# STUDIES ON THE CYTOTOXICITY AND ANTIOXIDANT ACTIVITY OF TEA KOMBUCHA


**A RESEARCH PROJECT REPORT SUBMITTED**

**BY**

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**THIS DISSERTATION IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MSC. BIOCHEMISTRY DEGREE.**

**AUGUST, 2015**

## DECLARATION

I, Eugene Aidoo, do declare that the experimental work described in this project report was performed by me in the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon and the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Legon, under the supervision of Prof. L.K.N Okine, Rev. Dr. W.S.K. Gbewonyo and Dr. Regina Appiah-Opong.

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## DEDICATION

I dedicate this project to The Almighty God, My family, especially my wife, Gloria O. K. Aidoo, Mr. and Mrs. Adom-Oduro, my mum, Elizabeth S. Asiamah and dad, Gilbert K. Aidoo, as well as my wonderful siblings for their immense support and encouragement.

I also thank the Board and members of Emmanuel Assemblies of God Church, Bubiashie, for their prayers and encouragements.

**ACKNOWLEDGEMENT**

Thank God for the gift of life. I wish to express my profound gratitude to my supervisors Prof. L.K.N Okine, Rev. Dr. W.S.K Gbewonyo and Dr. Regina Appiah-Opong for their guidance which contributed to the success of this study.

My dear wife Gloria O.K. Aidoo's financial, spiritual, emotional and mechanical support was overwhelming. She has immensely contributed to the success of this study; as a result I wish to express my deepest gratitude to her.

I also wish to express my appreciation to all staff of the Department of Clinical Pathology of the Noguchi Memorial Institute for Medical Research, especially Mrs. Eunice Apenteng (Dotse), Ms. Abigail Aning, Mr. Ebenezer Ofori-Attah, Ms. Abena Kissi-Twum and Mr. Justice Kumi for their guidance and great support.

I would like to thank my friends, Ms. Dorcas Frimpong, Mr. Christian Kweku Arhin, Mr. Richmond Adzadogo and Mr. Fred Ayertey for their support and prayers.

May the good Lord bless all those who contributed to the success of this study.

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## ABSTRACT

Fermentation of sugared tea with a symbiotic culture of acetic acid bacteria and yeast (tea fungus) yields kombucha tea which is consumed worldwide for its refreshing taste and beneficial effects on human health. It is claimed to prevent various types of cancer and cardiovascular diseases, promote liver function, and stimulate the immune system. The anti-proliferative and antioxidant activities of tea kombucha and unfermented tea were, therefore, investigated in this study. The cytotoxic effect of the tea kombucha was studied using an MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium) assay while its antioxidant activity was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power (the ferric reducing/antioxidant power (FRAP)) assays. The total phenolic content of the tea kombucha was also studied using the modified Folin-Ciocalteau colorimetric assay. It was observed that the antioxidant activity of tea kombucha was higher compared to the unfermented tea and this reflected in the total phenolics contents. Tea kombucha had 2.4-fold and 7.3-fold significantly ($p<0.05$) higher phenolic content at concentrations of 2.5 and 5.0 mg/ml, respectively than the unfermented tea. The tea kombucha showed insignificantly low cytotoxicity against the Jurkat P9 leukemia cells many orders of magnitude below that of the standard drug, curcumin, which had an $IC_{50}$ = 7.22 mg/ml whilst the unfermented tea was without such effect. These results suggest that kombucha tea with its high antioxidant activity may help protect cells against oxidative damage and possibly cancer.

# CHAPTER ONE

## 1.0 INTRODUCTION AND LITERATURE REVIEW

### 1.1 INTRODUCTION

Cancer is a multifactorial disease that involves modulation of multiple pathways and targets. In India, breast cancer is the second leading cause of cancer deaths in women and the risk of its incidence is increasing every year. Several researches are going on to identify chemotherapeutic and chemopreventive agents that can act on multiple signaling targets (Aggarwal *et al*., 2004).

Evidence suggests that the plant kingdom is considered a good candidate for chemoprevention and cancer therapy due to the high concentration and wide variety of antioxidants such as resveratrol, genestein, beicalein, vitamin A, vitamin C, polyphenols, (–)–Epigallocatechin 3-gallate, flavonoids, polyphenols, gallic acid, glycosides, verbascoside, calceorioside, epicatechin, quercetin, curcumin, lovastatin, and many other types of compounds with the capability to inhibit the cell proliferation of different cancer cells *in vitro* and *in vivo*, such as colon cancer (HT-29, SW48, HCT116), breast (MCF7, MDA), cervix (HeLa, SiHa, Ca-Ski, C33-A), liver (Hep G2), skin (A 431), fibroblasts (3T3 SV40), and many other malignant cells (Grover *et al*., 2003; Cetojević-Simin *et al*., 2008). Studies have indicated that antioxidants can be employed efficiently as chemopreventives and as effective inhibitors of cell proliferation, promoting cell apoptosis, and increasing detoxification enzymes, and inhibiting gene expression and scavenger reactive oxygen species (ROS). Thus, many researchers are working with different types of natural antioxidants with the aim of finding those with the greatest capacity to inhibit the development of cancer both *in vitro* as well as *in vivo*. This is because these compounds have exhibited high potential for use not

only in the treatment of this disease, but they also act as good chemoprotective agents. Identification of pharmacologically safe phytochemicals that have multitargeting effect is a 'hotspot' in cancer research. One such phytochemical is curcumin, the principle ingredient from the yellow spice turmeric. It is a well-known plant derived chemopreventive agent, common in Southeast Asian countries and used as a folk medicine and traditional food for several centuries (Mohandas and Desai, 1999; Barclay *et al*., 2000).

It has been shown that dietary phytochemicals can interfere with each stage of the development of carcinogenesis (Manson, 2003). As in the case of direct antioxidant effects, dietary polyphenols are most likely to exert their chemopreventive effects on the gastrointestinal tract, where they are present at highest concentrations (Aggarwal *et al*., 2004). Indeed, studies have shown that various polyphenol-rich fruits and vegetables are particularly effective in protecting against several types of cancer development (Mouria, 2002). Dietary polyphenols may exert their anticancer effects through several possible mechanisms, such as removal of carcinogenic agents, modulation of cancer cell signaling and antioxidant enzymatic activities, and induction of apoptosis as well as of cell cycle arrest. Some of these effects may be related, at least partly, with their antioxidant activities (Greenwald, 1996).

At the cellular level, there is good evidence that polyphenols present in tea, red wine, cocoa, fruit juices, and olive oil; at some level, they are able to stimulate carcinogenesis and tumor development (Mouria, 2002). For example, they may interact with reactive intermediates and activated carcinogens and mutagens, they may modulate the activity of the key proteins involved in controlling cell cycle progression, and they may influence the expression of many cancer-associated genes. Perhaps most notably, the anticancer properties of green tea

flavanols have been reported in animal models and in human cell lines, as well as in human intervention studies. On the other hand, green tea consumption has been proposed as significantly reducing the risk of cancer of the biliary tract, bladder, breast, and colon (Patterson *et al*., 1997).

Tea kombucha is a traditional drink made from a particular fermentation of sugared-black tea and a symbiosis of yeast species (fungi) and acetic acid bacteria. It is commonly consumed throughout the world as a medicinal health-promoting beverage (Dutta and Gachhui, 2007).

Tea kombucha is prepared by placing the kombucha culture (tea fungus) in a sugared tea broth for fermentation (Chanda and Dave, 2009). If the kombucha culture is cultivated according to the standard recipe with black tea, sweetened with sucrose, it turns this substrate into a refreshing beverage called tea fungus beverage with high nutritive value and medicinal properties (Lončar *et al*., 2000). The popularity of kombucha expanded like many other traditional beverages due to its beneficial effects on human health and its ease in home preparation. The amounts of tea, sugar, and tea fungus differ in different places (Chen and Liu, 2000).

The taste of the kombucha changes during fermentation from a pleasantly fruity sour-like sparkling flavour after a few days to a mild vinegar-like taste after a long incubation period. Currently, tea kombucha is praised as "the ultimate health drink" or damned as "unsafe medicinal tea" (Blanc, 1996; Hartmann *et al*., 2000). There are many opinions regarding the health benefits and toxicity of kombucha beverage. Though it is claimed to be beneficial for several medical ailments, very little or no clinical evidence is available for that. Studies on tea kombucha were reviewed earlier (Dufresne and Farnworth, 2000; Ernst, 2003). Research on tea kombucha was highly boosted during the past decade, but there were no review reports

published during the period. Several investigations have been conducted under static conditions on the beverage (Kumar *et al*., 2008; Wang *et al*., 2010; Yang *et al*., 2010). Yeasts and bacteria in tea kombucha are involved in metabolic activities that utilize substrates by different and in complementary ways (Blanc, 1996).

Many *in vivo* and *in vitro* studies performed to evaluate the capability of antioxidants against cancer, such as chemopreventive or therapeutic agents, were conducted employing natural antioxidants from fruits and vegetables (Block *et al*., 1992; Helbock *et al*., 1998). Thus, humans are forced to consume antioxidants in a more direct manner, either in the form of a tablet, a pill, or any other form in order to supply the levels that the body requires of these compounds to protect it against cell damage caused by oxidation reactions, thus reducing the risk of certain cancer types, especially those of the epithelial surface and in the upper part of the body, such as breast, lung, kidney, liver, intestine, and many others that have been well documented (Patterson *et al*., 1997). However, further investigations are expected before a better understanding of the function of many antioxidants and their utilization in the prevention and treatment of cancer and other degenerative diseases could be obtained. Thus this study investigated whether tea kombucha has anti-proliferative and antioxidant activities with the view to establishing its potential for cancer prevention or treatment.

**Aim**

The main aim of the study was to evaluate the cytotoxicity and antioxidant activities of tea kombucha.

**Specific objectives**

- To determine the cytotoxic effect of tea kombucha on Jurkat P9 leukemia cell lines using the MTT assay.
- To evaluate the antioxidant activity of tea kombucha using DPPH and reducing power assays.
- To assess the total phenolic content of tea kombucha using Folin-Ciocalteau colorimetric assay.

## 1.2 LITERATURE REVIEW

### 1.2.1 Tea Kombucha

Tea kombucha is a slightly sweet, slightly acidic refreshing beverage consumed worldwide. It is obtained from infusion of tea leaves by the fermentation of a symbiotic association of bacteria and yeasts forming "tea fungus" (Chen and Liu, 2000; Goh *et al*., 2012). A floating cellulosic pellicle layer and the sour liquid broth are the 2 portions of tea kombucha. It tastes like sparkling apple cider. Though green tea can be used for kombucha preparation, black tea and white sugar are considered the finest substrates. Kombucha is the internationally used Germanized form of the Japanese name for this slightly fermented tea beverage. It was first used in East Asia for its healing benefits. Kombucha originated in northeast China (Manchuria) where it was prized during the Tsin Dynasty ("Ling Chi"), about 220 B.C., for its detoxifying and energizing properties. In 414 A.D., the physician Kombu brought the tea fungus to Japan and he used it to cure the digestive problems of the Emperor Inkyo. It is presently cultivated in at least 30 countries around the world (Stoner and Mukhtar, 1995).

Reiss (1994) has suggested that 50 g sucrose/L provides the optimal concentrations of ethanol and lactic acid, and this sugar concentration has been used in traditional recipes for the preparation of "teakwass" (another name for kombucha) for a long time. An optimum fermentation time of eight days is required for the production of kombucha with pleasant flavour and taste. Longer fermentation periods produce high levels of acids (like mild vinegar) that may pose potential risks when consumed (Sreeramulu *et al*., 2000)

### 1.2.2 Chemical Composition of Tea Kombucha

Chemical analysis of tea kombucha showed the presence of various organic acids (Fig. 1.1), such as acetic, gluconic, glucuronic, citric, L-lactic, malic, tartaric, malonic, oxalic, succinic, pyruvic, usnic; also sugars, such as sucrose, glucose, and fructose; the vitamins $B_1$, $B_2$, $B_6$, $B_{12}$, and C; 14 amino acids, biogenic amines, purines, pigments, lipids, proteins, some hydrolytic enzymes, ethanol, antibiotically active matter, carbon dioxide, phenol, as well as some tea polyphenols, minerals, anions, as well as insufficiently known products of yeast and bacterial metabolites.

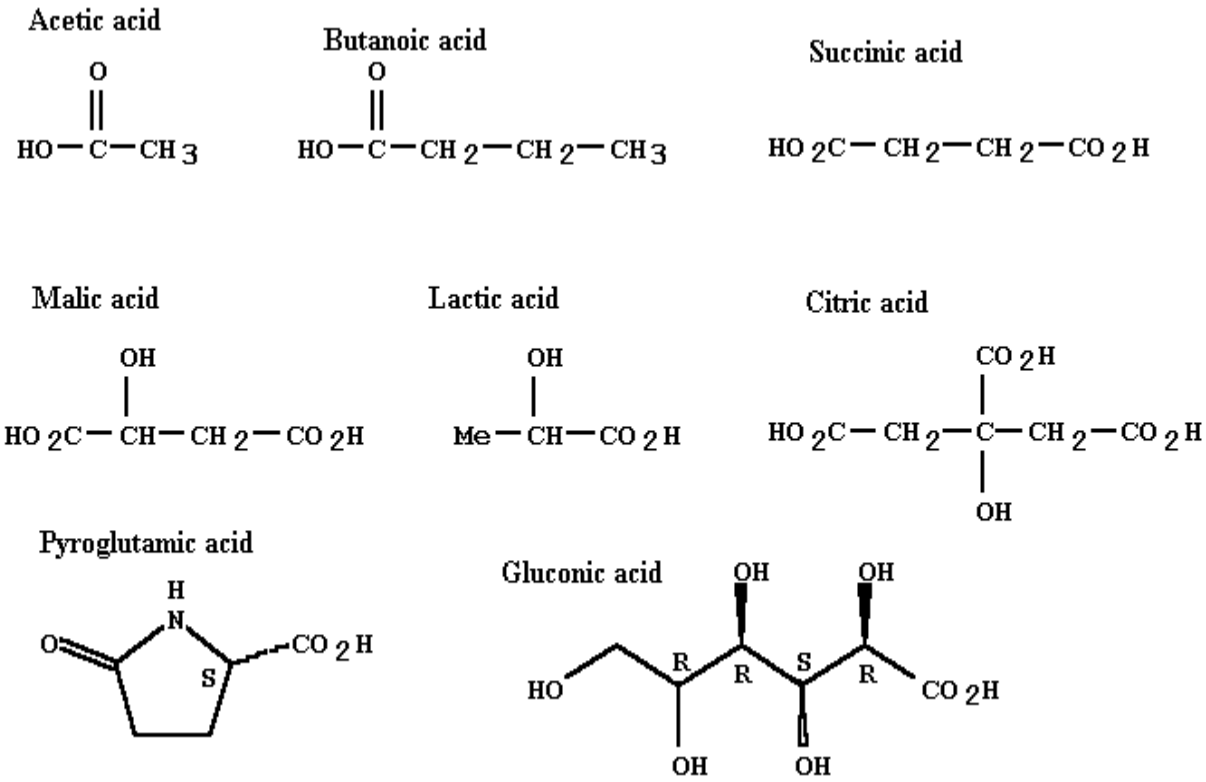
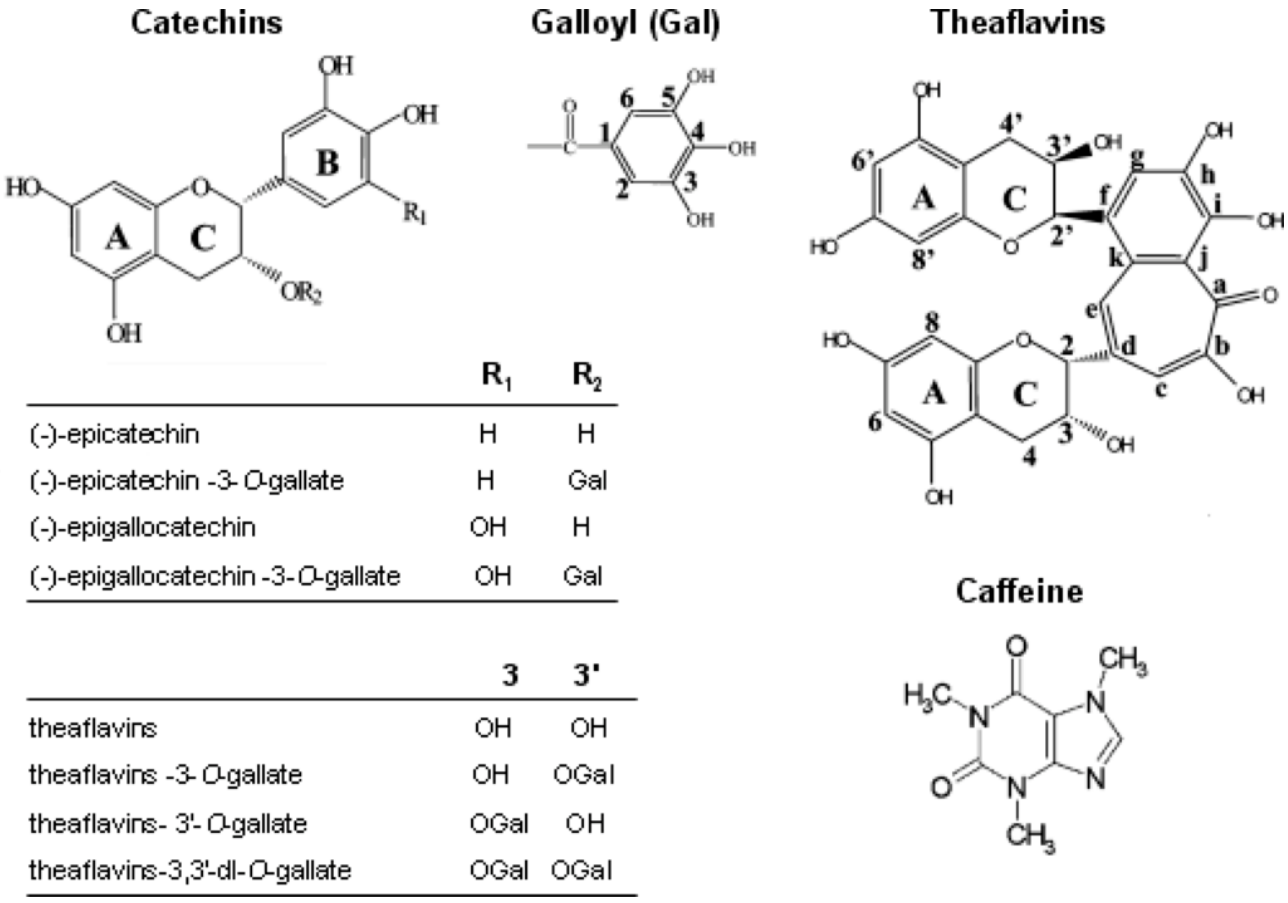



**Fig. 1.1: Structures of some organic acids present in tea kombucha.**

Yeasts hydrolyze sucrose into glucose and fructose by invertase activity and produce ethanol via glycolysis, with a preference for fructose as a substrate. Acetic acid bacteria make use of glucose to produce gluconic acid and ethanol to produce acetic acid. The pH value of tea

kombucha beverage decreases due to the production of organic acids during fermentation (Dufresne and Farnworth, 2000; Jayabalan *et al*., 2007).

Tea contains polyphenols and flavonoids (theaflavins and thearubigins), catechin, theobromine , caffeine, gallic acid, catechin gallates, tannins, gallotannin, small amounts of aminophylline (Fig. 1.2) and a yellow volatile oil that is solid at ordinary temperatures and has strong aromatic odour and taste (Jayabalan *et al*., 2008).




| | $R_1$ | $R_2$ |
|---|---|---|
| (-)-epicatechin | H | H |
| (-)-epicatechin -3- *O*-gallate | H | Gal |
| (-)-epigallocatechin | OH | H |
| (-)-epigallocatechin -3- *O*-gallate | OH | Gal |

| | 3 | 3' |
|---|---|---|
| theaflavins | OH | OH |
| theaflavins -3- *O*-gallate | OH | OGal |
| theaflavins- 3'- *O*-gallate | OGal | OH |
| theaflavins-3,3'-di- *O*-gallate | OGal | OGal |

**Fig. 1.2: Chemical structure of some tea constituents (Adapted from Lambert and Yang, 2003)**

The catechins and gallic acid complexes such as theaflavins, theaflavinic acids, thearubigins or theasinensis present in the kombucha prepared from the black tea, possess a significant

degree of bioavailability. The composition of kombucha beverage indicates the presence of numerous compounds and it depends on cultivation substrate, time and temperature of fermentation process, as well as the microorganisms present in the culture (Chen and Liu, 2000). It also depends on the applied method of analysis.

### 1.2.3 Beneficial Effects of Tea Kombucha

Tea kombucha has been claimed by kombucha drinkers all over the world to have many beneficial effects on human health. However, most of the benefits have been studied in animal models only and there is a lack of scientific evidence based on clinical trials. Non-human studies regarding antimicrobial, antioxidant, hepatoprotective, and anticancer properties of tea kombucha have been carried out and the biological activities have been reported (Table 1.1).

**Table 1.1: Various Biological Activities of Tea Kombucha.**

| **Biological activity** | **Experimental animal/cells** | **Treatment period/dose** | **Parameters studied** | **Reference** |
|---|---|---|---|---|
| Hypoglycaemic activity | Mice | 3 days and 1.71 mg/kg body weight | Blood sugar level | Shenoy, 2000 |
| Antioxidative stress against chromate | Rat | 30 days and 0.6 ml/200 g body weight | Plasma and tissue MDA levels, delayed type hypersensitivity response, GSH, peroxidise, catalase | Sai *et al.*, 2000 |
| Longevity | Mice | 3 years and free access | Longevity, general health, and open-field exploratory behavioural outcomes | Hartmann *et al.*, 2000 |
| Antistress activity against cold and hypoxia | Rat | 15 days and 1.6, 8.0 and 16 ml/kg body weight | plasma/blood MDA and reduced GSH, fecal output | Pauline *et al.*, 2001 |
| Antioxidative stress against lead | Rat | 45 days and 1 ml/kg body weight | Lipid peroxidation, creatine phosphokinase, GSH, DNA fragmentation in liver | Dipti *et al.*, 2003 |
| Cytogenic activity | Human peripheral blood lymphocytes | 1 hour and 40 µg/ml | Frequencies of sister chromatid exchange and micronuclei formation | Mrđanović *et al.*, 2007 |
| Antihyperglycaemic efficacy | Streptozotocin-induced rats | 45 days and 6 mg/kg body weight | Plasma insulin, haemoglobin, tissue glycogen | Srihari *et al.*, 2013b |

MDA= Malondialdehyde; GSH= reduced glutathione

Tea kombucha reduces cell damage induced by oxidative stress. It constitutes a potent therapeutic supplement that improves resistance against cancer, cardiovascular diseases, promotes digestive functions, stimulates the immune system and reduces inflammatory problems (Greenwalt *et al.*, 1998, Jayabalan *et al.*, 2008; Banerjee *et al*., 2011; El-Taher, 2011). The putative health benefits of tea kombucha have been largely attributed to the polyphenolic components of kombucha that have been transformed from the black tea (Bors *et al*., 1996). In addition, organic acids, vitamins, amino acids, antibiotics and a variety of micronutrients produced during fermentation of kombucha may also have a role in the health benefits to some extent.

### 1.2.4 Cancer

Cancer is the name given to a collection of related diseases. In all types of cancer, some of the body's cells begin to divide without stopping and spread into surrounding tissues. Cancer can start almost anywhere in the human body, which is made up of trillions of cells. Normally, human cells grow and divide to form new cells as the body needs them. When cells grow old or become damaged, they die, and new cells take their place.

When cancer develops, however, this orderly process breaks down. As cells become more and more abnormal, old or damaged cells survive when they should die, and new cells form when they are not needed. These extra cells can divide without stopping and may form growths called tumors. Many cancers form solid tumors, which are masses of tissue. Cancers of the blood, such as leukemias, generally do not form solid tumors.

Cancerous tumors are malignant, which means they can spread into, or invade nearby tissues. In addition, as these tumors grow, some cancer cells can break off and travel to distant places

in the body through the blood or the lymph system and form new tumors far from the original tumor. Unlike malignant tumors, benign tumors do not spread into, or invade, nearby tissues. Benign tumors can sometimes be quite large. However, when removed, they usually do not grow back, whereas malignant tumors sometimes do. Unlike most benign tumors elsewhere in the body, benign brain tumors can be life threatening.

Cancer, after cardiovascular disease, is the second leading cause of death (Turgay and Sar, 2005). Worldwide about 10 million people per year are diagnosed with cancer and more than 6 million die of the disease and over 22 million people in the world are cancer patients (Pinar, 1998). When cancer is diagnosed, therapists face a formidable range of challenges. Treatment usually consists of various combinations of surgery, radiation therapy, and chemotherapy but despite these therapeutic options, cancer remains associated with high mortality.

Cancer cells differ from normal cells in many ways that allow them to grow out of control and become invasive (Ertel *et al*., 2006). One important difference is that cancer cells are less specialized than normal cells. That is, whereas normal cells mature into very distinct cell types with specific functions, cancer cells do not. In addition, cancer cells are able to ignore signals that normally tell cells to stop dividing or that begin a process known as programmed cell death, or apoptosis, which the body uses to get rid of unneeded cells.

Cancer cells may be able to influence the normal cells, molecules, and blood vessels that surround and feed a tumor—an area known as the microenvironment. For instance, cancer cells can induce nearby normal cells to form blood vessels that supply tumors with oxygen and nutrients, which they need to grow. These blood vessels also remove waste products from tumors. Cancer cells are also often able to evade the immune system, a network of organs, tissues, and specialized cells that protects the body from infections and other conditions

(Parkin, 2006). Although the immune system normally removes damaged or abnormal cells from the body, some cancer cells are able to "hide" from the immune system. Tumors can also use the immune system to stay alive and grow. For example, with the help of certain immune system cells that normally prevent a runaway immune response, cancer cells can actually keep the immune system from killing cancer cells.

### 1.2.5 Causes of Cancer

Cancer is a genetic disease—that is, it is caused by changes to genes that control the way our cells function, especially how they grow and divide. Genetic changes that cause cancer can be inherited from our parents. They can also arise during a person's lifetime as a result of errors that occur as cells divide or because of damage to DNA caused by certain environmental exposures.

We all have a risk of developing cancer. Many cancers seem to develop for no apparent reason. However, certain risk factors are known to increase the chance that one or more of your cells will become abnormal and lead to cancer. Risk factors include the following:

#### 1.2.5.1 Chemical carcinogens

A carcinogen is something (chemical, radiation, etc) that can damage a cell and make it more likely to turn into a cancerous cell. As a general rule, the more the exposure to a carcinogen, the greater the risk. Well known examples of carcinogens include:

**Tobacco smoke**.

Smokers are more likely to develop lung cancer, mouth cancer, throat cancer, oesophageal cancer, bladder cancer and pancreas cancer. Smoking is thought to cause about a quarter of

all cancers. About 1 in 10 smokers die from lung cancer. The heavier you smoke, the greater the risk. If you stop smoking, your risk goes down considerably.

**Workplace chemicals**

This includes chemicals such as asbestos, benzene, formaldehyde, etc. If you have worked with these without protection you have an increased risk of developing certain cancers. For example, a cancer called mesothelioma is linked to past exposure to asbestos.

### 1.2.5.2 Effect of Age on cancer development

The older you become, the more likely you will develop a cancer. This is probably due to an accumulation of damage to cells in the body over time. Also, the body's defences against abnormal cells may become less good as you become older. For example, the ability to repair damaged cells, and the immune system which may destroy abnormal cells, may become less efficient with age. So, eventually one damaged cell may manage to survive and multiply out of control into a cancer. Most cancers develop in older people.

### 1.2.5.3 Lifestyle factors

Diet and other lifestyle factors can alter the risk of developing cancer. For example:

- If you eat a lot of fruit and vegetables you have a reduced risk of developing certain cancers. The exact way in which they protect against cancer is not fully understood. These foods are rich in vitamins and minerals, and also contain chemicals called antioxidants. They may protect against damaging chemicals that get into the body. We should all eat at least five portions of fruit and vegetables per day (some experts recommend even more).
- Eating too much fatty food possibly increases the risk of developing certain cancers.

- The risk of developing certain cancers is increased by obesity, lack of regular exercise (physical activity) and drinking a lot of alcohol.

In general, cancer cells have more genetic changes, such as mutations in DNA, than normal cells. Some of these changes may have nothing to do with the cancer; they may be the result of the cancer, rather than its cause (Harlin and Gajewski, 2008).

### 1.2.6 Types of Cancer

There are more than 100 types of cancer. Cancers are usually named after the organs or tissues where the cancers form. For example, lung cancer starts in cells of the lung, and brain cancer starts in cells of the brain. Cancers also may be described by the type of cell that formed them, such as an epithelial cell or a squamous cell (Worsham *et al* ., 2009). Here are some categories of cancers that begin in specific types of cells: carcinoma, sarcomas, leukemia, lymphoma etc.

#### 1.2.6.1 Blood cancers

Primarily, there are three basic types of blood cancer. Each of the variety may also include several variations, but in general this cancer is categorized into the following kinds

- ❖ Leukemia - With spurt in the multiplicity of cancerous cells affecting either the marrow or the blood; the ability of the circulatory system to produce blood is severely impaired.
- ❖ Lymphoma - The cancerous formation affecting the lymphocytes is referred to as the lymphoma. Lymphocytes are one of the varieties of white blood corpuscles.
- ❖ Myeloma – it is a cancer arising from plasma cells, a type of white blood cell which is made in the bone marrow. Bone marrow is the ‘spongy’ material found in the centre of the larger bones in the body. The bone marrow is where all blood cells are made.

### 1.2.6.2 Symptoms of blood cancer (leukemia).

Leukemia marked by an acute destruction of health sustaining red blood cells includes the symptoms of anemia, weakness and extreme fatigue. Consequently one affected by it is likely to sweat and come under bouts of breath shortness in course of performing day to day activities of the regular kind. Vulnerability to infection and swelling of the lymph nodes are some of the other fallouts of leukemia. Blood tests are likely to present higher counts of white blood corpuscles.

Leukemia can be chronic or acute. A person afflicted with the chronic type may not exhibit any of these symptoms. On the other hand, in leukemia of the acute type, the symptoms are likely to manifest with rapid intensity.

### 1.2.6.3 Treatment of Cancers

Treatment options vary, depending on the type of cancer and how far it has grown and spread. Briefly, the three most common treatments are:

- **Surgery**: It may be possible to cut out a cancerous (malignant) tumour.
- **Chemotherapy:** This is a treatment that uses anti-cancer medicines to kill cancer cells, or to stop them from multiplying. There are various different types of medicines used for chemotherapy. The medicine or combination of medicines selected depends on the type of cancer being treated.
- **Radiotherapy:** This is a treatment that uses high-energy beams of radiation which are focused on cancerous tissue. This kills cancer cells, or stops cancer cells from multiplying.

More recently, other treatments have been introduced which include:

- **Stem cell transplant:** High-dose chemotherapy may damage bone marrow cells and lead to blood problems. However, if you receive healthy bone marrow after the chemotherapy then this helps to overcome this problem.
- **Hormone therapy:** This is the use of medicines to block the effects of hormones. This treatment may be used for cancers that are hormone-sensitive such as some cancers of the breast, prostate and womb (uterus).
- **Immunotherapy:** Some treatments can boost the immune system to help to fight cancer. More specific immunotherapy involves injections of antibodies which aim to attack and destroy certain types of cancer cells. Research is underway to try to find vaccines that would stimulate your own immune system to make antibodies against cancer cells.
- **Gene therapy:** This is a new area of possible treatments. Research is underway to find ways of blocking, repairing or replacing abnormal genes in cancer cells.
- **Special techniques:** These can sometimes be used to cut off the blood supply to tumours. The tumour then dies.

For some cancers, a combination of two or more treatments may be used. A range of other treatments may also be used to ease cancer-related symptoms such as pain.

Certain herbs, at least two varieties of herbs known by the names of 'Garcinia Mangostana' and 'xanothenes' have been found to be effective with respect of leukemia. The herbs and compounds based on them have reflected intrinsic potential of growth inhibiting features (Mohandas and Desai, 1999).

### 1.2.7 Phytochemicals

Phytochemicals and their synthetic derivatives have, over the decades, attracted huge attention and made significant contribution in modern drug discovery programs for their relevance in leveraging the severity or cure of several human diseases, including cancer. These natural products and their derivatives thereof have demonstrated immense pharmacological and biological properties. Although the molecular mechanisms of action of a majority of these phytochemicals are yet to be elucidated, cumulative evidence and the continued generation of new scientific data on their health benefits in disease prevention and cure have accrued over the years. Recent advancement in molecular biology, high throughput screening, biomarker identifications, target selection and genomic approaches have enabled researchers to understand salient interactions of natural products or their derivatives with cancer cells. Most phytochemicals exhibit their pharmacologic effects in nature through a multi-targeted approach; a property that is highly desirable since therapy for carcinomas invariably involves dysregulation of multiple genes and associated cell-signalling pathways at various stages of initiation, progression and metastasis. On the other hand, in cancer initiation and progression, acquired genetic alterations, microenvironment-mediated epigenetic (heritable changes in gene activity and expression that occur without alteration in DNA sequences and are sufficiently powerful to regulate the dynamics of gene expression) perturbations have primarily been considered to play an important role in neoplastic development (Ashendel, 1995). One of the most widely studied phytochemical with anticancer properties is curcumin. Indeed, curcumin together with a number of related chemically-defined derivatives have been used extensively in the treatment of a number of malignant growths, such as breast cancer.

### 1.2.7.1Curcumin

The rhizome of the plant *Curcuma longa* L., commonly known as turmeric, has been used for centuries as a spice and colouring agent. The dry rhizome of turmeric contains curcumin, the main bioactive component. Curcumin displays a diverse range of molecular targets, supporting the concept that it acts upon numerous biochemical and molecular cascades (Fig. 1.3).

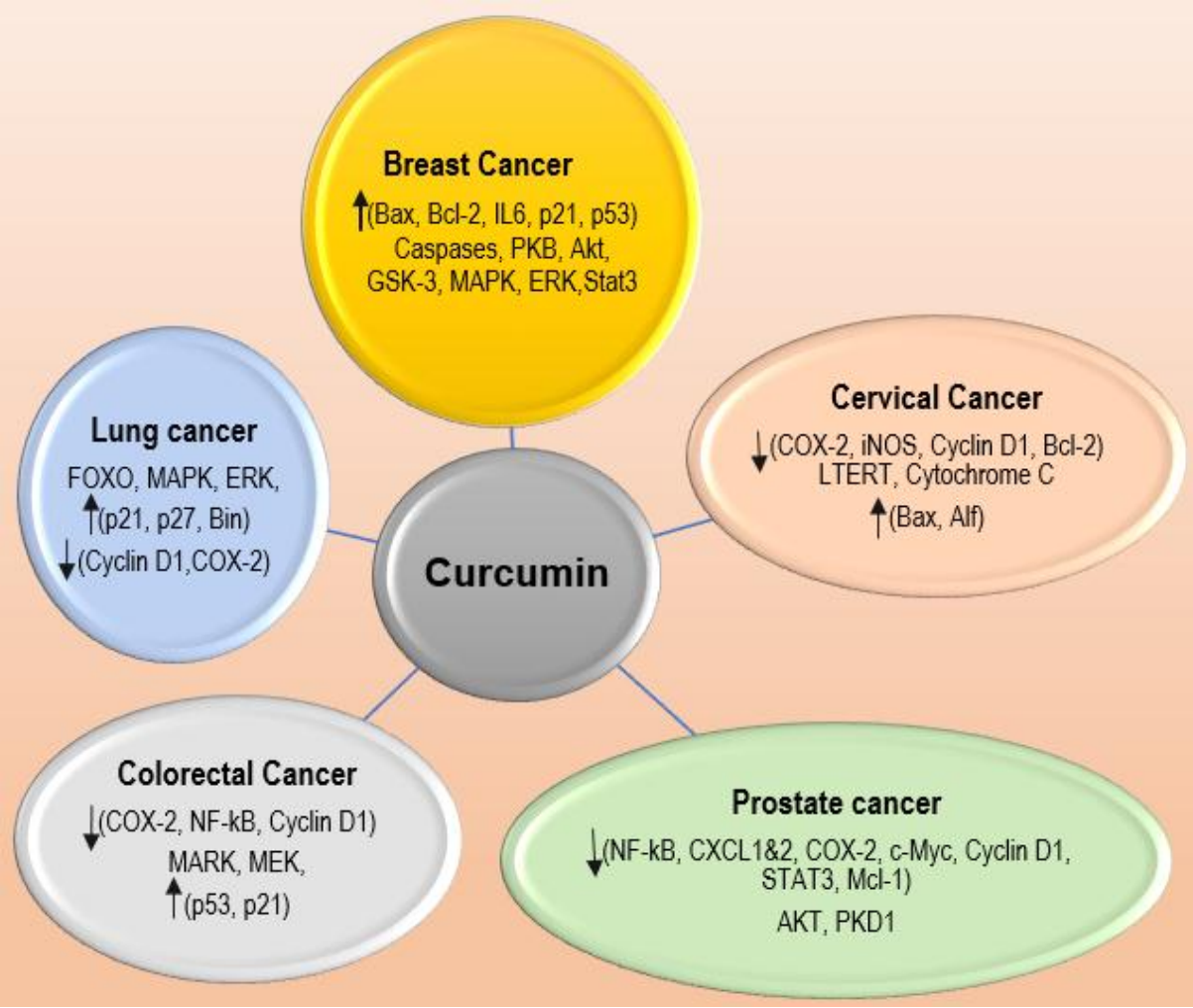


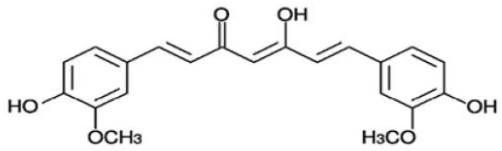

Fig. 1.3: Molecular targets of curcumin and/or its chemically-related analogues and possible mechanisms of action in various types of malignant growths (Huang *et al*., 1997).

Although the precise mode of action of this compound is yet to be fully elucidated, studies have shown that the chemopreventive action of curcumin might be due to its ability to induce apoptosis by several pathways (Kuo *et al*., 1996). This is a pleiotropic molecule which has many pharmacological properties and popularly known for its anticarcinogenic, antioxidant and anti inflammatory activities (Aggarwal *et al*., 2004; Ziech *et al*., 2010). Since phytochemicals exhibit their therapeutic effect through multi-mechanism of action, research into the mechanism of action of curcumin in cancer has demonstrated its relevance in various biochemical pathways. The modulation of anti-apoptotic and survival pathways by curcumin as a strategy to induce apoptosis in cancer cells is well documented as well as its ability to inhibit proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell-signalling proteins (Khar *et al*., 2001). A list of some of apoptotic and growth inhibitory pathways activated by curcumin in tumour cells has been well documented (Piwocka *et al*., 2001).

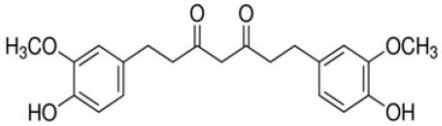
#### 1.2.7.2 Medicinal plants

Natural products, especially plants, have been used for the treatment of various diseases for thousands of years. Terrestrial plants have been used as medicines in Egypt, China, India and Greece from ancient times and an impressive number of modern drugs have been developed from them. The first written records on the medicinal uses of plants appeared in about 2600 BC from the Sumerians and Akkaidians (Samuelson, 1999). The "Ebers Papyrus", the best known Egyptian pharmaceutical record, which documented over 700 drugs, represents the history of Egyptian medicine dated from 1500 BC. The Chinese *Materia Medica*, which

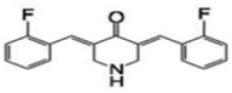
describes more than 600 medicinal plants, has been well documented with the first record dating from about 1100 BC (Cragg *et al*., 1997). Documentation of the Ayurvedic system recorded in Susruta and Charaka dates from about 1000 BC (Kappor, 1990). The Greeks also contributed substantially to the rational development of the herbal drugs. Dioscorides, the Greek physician (100 A.D.), described in his work "*De Materia Medica*" more than 600 medicinal plants. Phytochemicals have been proposed to offer protection against a variety of chronic ailments including cardiovascular diseases, obesity, diabetes, and cancer. As for cancer protection, it has been estimated that diets rich in phytochemicals can reduce cancer risk by 20%.The compounds that are responsible for medicinal property of the drug are usually secondary metabolites.



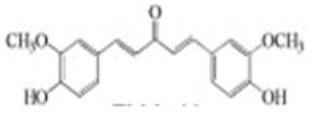
Curcumin



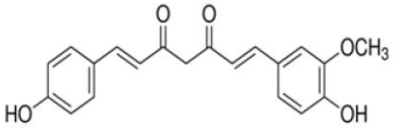
Tetrahydrocurcumin (THC)



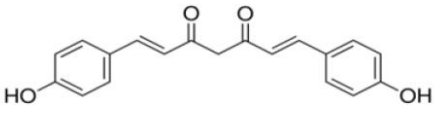
3,5-*bis*(2-flurobenzylidene)piperidin-4-one



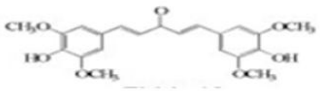
FLLL-11



Demethoxycurcumin



*bis*demethoxycurcumin



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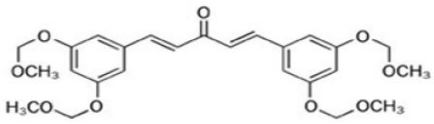
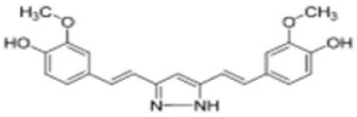
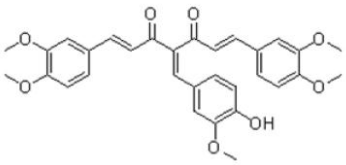


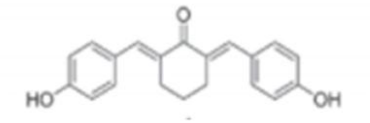
GO-YO30



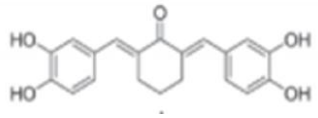
Hydrazinocurcumin



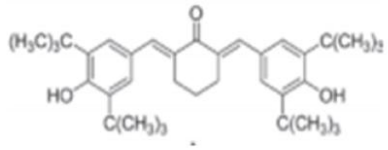
4-arylidene curcumin (T63)



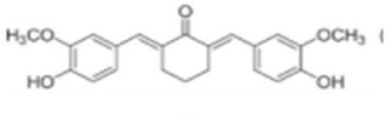
2,6-*bis*(4-hydroxybenzylidene)-cyclohexanone



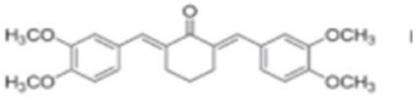
2,6-*bis*(3,4-dihydroxybenzylidene)-cyclohexanone



2,6-*bis*(3,5-di-tert-butyle-4-hydroxylbenzylidene)-cyclohexanone



2,6-*bis*(4-hydroxy-3-methoxybenzylidene)-cyclohexanone



2,6-*bis*(3,4-dimethoxybenzylidene)-cyclohexanone

**Fig. 1.4: Chemical structure of curcumin and related derivatives/analogues widely used in the treatment of various forms of cancers (Fang *et al*., 2013)**

Plants have a long history of use in the treatment of cancer. Hartwell, in his review of plants used against cancer, lists more than 3000 plant species that have reportedly been used in the treatment of cancer. It is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms. Indeed, molecules derived from natural sources (so called natural products)**,** including plants, marine organisms and micro-organisms have played and continue to play, a dominant role in the discovery of leads for the development of conventional drugs for the treatment of most human diseases. The search for anti-cancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins. These discoveries prompted the United States National Cancer Institute (NCI) to initiate an extensive plant collection program in 1960. This led to the discovery of many novel chemotypes showing a range of cytotoxic activities, including the taxanes and camptothecins (Cragg and Newmann, 2005).

Natural and some synthetic compounds can prevent, suppress, or reverse the progression of cancer. Although tumors have traditionally been treated with chemotherapeutic agents, the advents of compounds which prevent malignancies represent an emerging field and offer new options. The first agents to advance into clinical use were the isolation of the vinca alkaloids, vinblastine and vincristine (Fig. 1.5) from the Madagascar periwinkle, *Catharanthus roseus* (Apo-cynaceae) introduced a new era of the use of plant material as anticancer agents. They were the first agents to advance into clinical use for the treatment of cancer. Vinblastine and vincristine are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular

cancer, breast and lung cancers, and Kaposi's sarcoma. The discovery of paclitaxel from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. (Taxaceae), is another evidence of the success in natural product drug discovery. Various parts of *Taxus brevifolia* and other *Taxus* species (e.g., *Taxus Canadensis*, *Taxus baccata* ) have been used by several Native American Tribes for the treatment of some noncancerous cases (Cragg and Newmann, 2005). Paclitaxel is significantly active against ovarian cancer, advanced breast cancer, small and non-small cell lung cancer. Camptothecin, isolated from the Chinese ornamental tree *Camptotheca acuminate* (Nyssaceae), was advanced to clinical trials by NCI in the 1970s but was dropped because of severe bladder toxicity. Topotecan and irinotecan are semi-synthetic derivatives of camptothecin and are used for the treatment of ovarian and small cell lung cancers, and colorectal cancers, respectively. Epipodophyllotoxin is an isomer of podophyllotoxin which was isolated as the active antitumor agent from the roots of *Podophyllum* species, *Podophyllum peltatum* and *Podophyllum emodi* (Berberidaceae). Etoposide and teniposide are two semi-synthetic derivatives of epipodophyllotoxin and are used in the treatment of lymphomas and bronchial and testicular cancers.

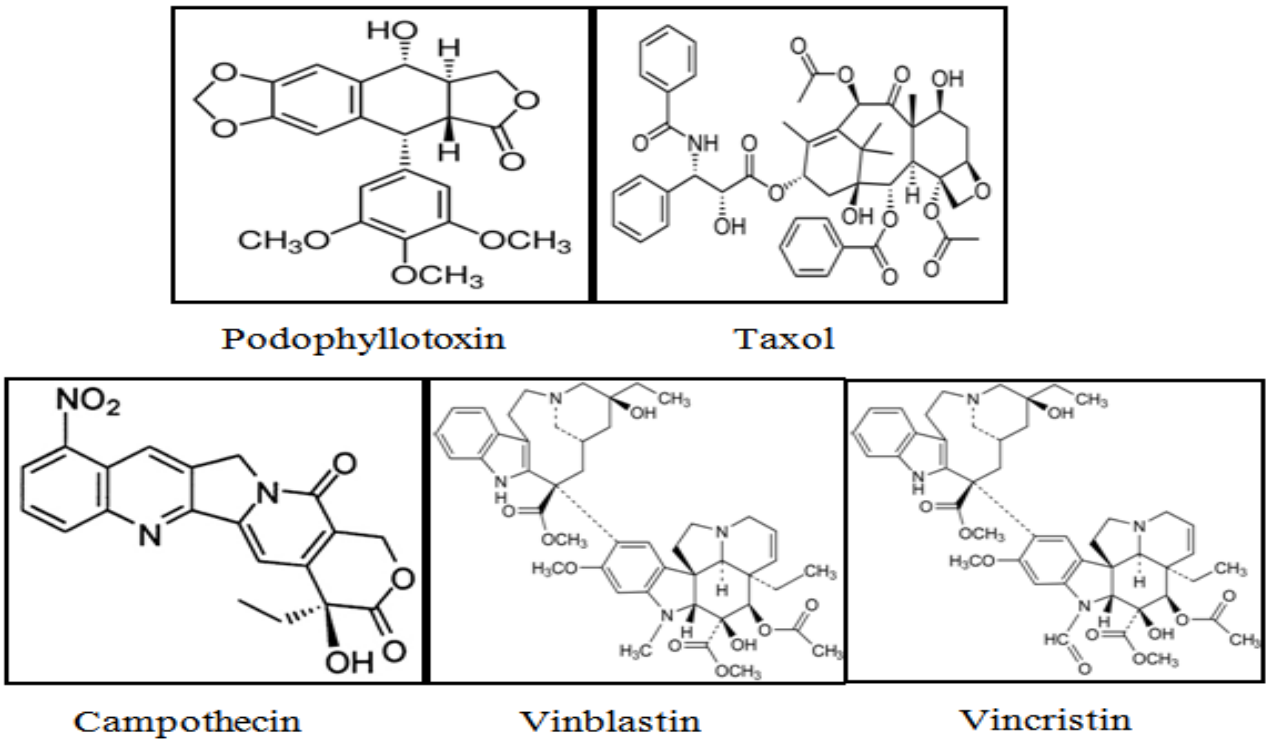



**Fig. 1.5: Chemical structures of some anti-cancer agents (Gordon and David, 2005).**

### 1.2.8 Importance of Antioxidants.

It has commonly been observed that people, particularly kids undergoing treatment for blood cancer, respond better to curative measures if there is no drastic cut in the supply of antioxidants. So, even with allopathic remedial options, it is important to go for a diet rich in greens and antioxidants, in order to add to the supportive base of the treatment (Block *et al* ., 1992; Helbock *et al* ., 1998). Children oriented to a balanced diet with an adequate supply of greens and raw fruits are less likely to develop blood cancer/leukemia.

### 1.2.9 Tea Kombucha as Antioxidant Source

There has been a global trend toward the use of phytochemicals as antioxidants and functional foods. Bioactive molecules of natural sources are being utilized in the food industry, and there is evidence that these molecules can act as anti-oxidants within the human body. Antioxidant activity of tea kombucha may be correlated with its many claimed beneficial effects like cancer prevention, immunity enhancement, and alleviation of inflammation and arthritis. Jayabalan *et al.* (2008) reported on the free radical scavenging ability of tea kombucha prepared from green tea, black tea, and tea waste material. They have shown that total phenolic compounds, scavenging activity on DPPH radical, superoxide radical, and inhibitory activity against hydroxyl radical-mediated linoleic acid were increased with an increase in fermentation time, whereas reducing power, hydroxyl radical scavenging ability and anti-lipid peroxidation ability were decreased.

Malbaša *et al.* (2011) studied the influence of 3 starter cultures (mixed culture of acetic acid) bacteria and *Zygosaccharomyces* sp., mixed culture of acetic acid bacteria and *S. cerevisiae*, and native local kombucha) on the antioxidant activities of green tea and black tea kombucha beverage. They observed the highest antioxidant activity with native kombucha on green tea beverage and acetic acid bacteria with *Zygosaccharomyces* sp. culture on black tea beverage. The antioxidant property of kombucha tea was tested against tertiary butyl hydroperoxide (TBHP)-induced cytotoxicity using murine hepatocytes. The results obtained showed that tea kombucha neutralized the TBHP-induced changes and prevented cell death. These counter effects were also shown by the unfermented black tea, but the tea kombucha was found to be more efficient (Malbaša, 2004; Bhattacharya *et al*., 2011). Kombucha exhibited increased free radical scavenging activities during fermentation. The extent of the activity depended

upon the fermentation time, type of tea material, and the normal microbiota of the kombucha culture, which in turn determined the nature of their metabolites. Although free radical scavenging properties of kombucha showed time-dependent profiles, prolonged fermentation is not recommended because of accumulation of organic acids, which might reach harmful levels for direct consumption. The identification of extracellular key enzymes responsible for the structural modification of components during kombucha fermentation and potent metabolites responsible for the free radical scavenging abilities are necessary to elucidate the metabolic pathway leading to the products of kombucha fermentation. Metabolic manipulations may be one of the effective methods to enhance the antioxidant activities and fermentation efficiency of kombucha.

The possible anticancer mechanisms of tea polyphenols accepted by most researchers now are as follows:

- inhibition of gene mutation;
- inhibition of cancer-cell proliferation;
- induction of cancer-cell apoptosis and
- termination of metastasis (Conney *et al*., 2002).

The anticancer properties of tea kombucha might be due to the presence of tea polyphenols and their degradation products formed during fermentation.

### 1.2.10 Nontoxic Nature of Tea Kombucha

The U.S. Food and Drug Administration and Kappa Laboratories, Miami, Florida, U.S.A. (1995), have carried out microbiological and biochemical tests and reported that tea kombucha is safe for human consumption. Vijayaraghavan *et al*., (2000) studied the subacute

(90 days) oral toxicity potency of tea kombucha using rats by recording body weight, feed intake, water intake, general behaviour, and histological examinations. They concluded that kombucha feeding for 90 days to rats did not show any toxic signs. Hematological and biochemical variables of rats studied were within clinical limits. Their study indicated that rats fed tea kombucha for 90 days did not show any toxic effects. Pauline *et al*. (2001) studied the toxicity of tea kombucha by feeding the rats orally for 15 days using 3 different doses of tea kombucha (normal dose and 5 and 10 times that dose) and by measuring various biochemical and histopathological parameters. They observed that tea kombucha displayed no significant toxicity.

### 1.2.11 Anti-proliferative Activity (Viability of Cells)

Cytotoxicity assays are widely used in *in vitro* toxicology studies. The LDH leakage assay, a protein assay, the neutral red and the MTT assays are the most commonly employed for the detection of cytotoxicity or cell viability, following exposure to toxic substances (Conney *et al*., 2002, Jayabalan *et al*., 2011)

#### 1.2.11.1 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium (MTT) Assay

MTT is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan (Fig. 1.6) by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan produced is impermeable to the cell membranes and therefore accumulates in healthy cells (Barltrop and Owen, 1991).

The MTT reduction assay was the first homogeneous cell viability assay developed for a 96-well plate format that was suitable for high throughput screening (HTS) (Mosmann, 1983).

The MTT tetrazolium assay technology has been widely adopted and remains popular in academic laboratories as evidenced by thousands of published articles. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT (Marshall *et al.*, 1995). Speculation in the early literature involving specific mitochondrial enzymes has led to the assumption in numerous publications that MTT measures mitochondrial activity (Berridge *et al*., 1993; Berridge *et al.*, 1996).

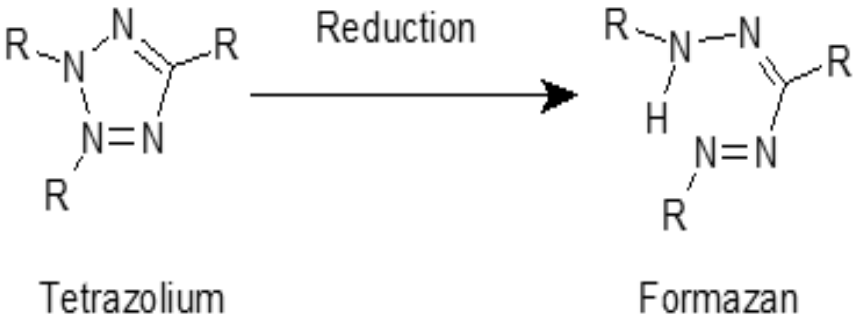



**Fig. 1.6: The reduction of MTT to Formazan**

Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. It has been used in hepatocarcinoma, HepG2 cell lines and in rat lung epithelial cells after exposure to cadmium chloride as well as in oligodendrocytes to assess cell viability.

### 1.2.12 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Assay

Determination of antioxidant activity of various types of foods using DPPH is comparable to other methods. It is very difficult to select a suitable antioxidant assay method since anti-oxidants act by several mechanisms and no one assay can capture the different modes of action of antioxidant. Conventional cuvette assay of radical scavenging activity has been replaced by 96-well plate microtitre assay for the past couple of years. Cuvette assay method uses UV-Visible spectrophotometer to obtain the absorbance, where as 96-well plate method uses ELISA plate reader for absorbance. The first method (cuvette assay) is very tedious, time consuming and allows only one sample to be read at a time and requires high quantity of reagent; whereas the second method (96 – well plate micro litre) is time saving and reads about 96 samples at a time, with small amounts of reagent.

There are several advantages of the use of DPPH assay over other methods. These include

- DPPH is allowed to react with the whole sample and sufficient time is given to allow DPPH to react slowly even with weak anti-oxidants.
- DPPH method may be utilized in aqueous and non-polar organic solvents and can be used to examine both hydrophilic and lipophilic antioxidants.
- DPPH assay is considered a valid accurate, easy and economic method for evaluation of radical scavenging activity of antioxidants, since the radical compound is stable and need not be generated.

### 1.2.13 Reducing Power

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Denizot and Lang, 1986). Compounds with reducing

power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Plumb *et al.,* 1989). In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Presence of reducers causes the conversion of the $Fe^{3+}$/ferricyanide complex used in this method to the ferrous form. By measuring the formation of Pearl's Prussian blue at 700 nm, it is possible to determine the concentration of $Fe^{3+}$ ion.

# CHAPTER TWO

## 2.0 MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 Chemicals and Reagents

RPMI-1640 culture medium, absolute isopropanol, and trypan blue solutions were obtained from Sigma Aldrich Chemicals Co., Ltd. (St. Louis, MO, USA). Butylated hydroxytoluene (BHT) and 1, 1-diphenyl-2-picryl hydrazyl (DPPH) free radical reagents were purchased from Wako Pure Chemical Industries Ltd (Japan).

### 2.1.2 Kombucha Starter Culture

Kombucha starter culture was provided by Rev. Dr. W.S.K. Gbewonyo of the department of biochemistry, cell and molecular biology, University of Ghana, Legon.

### 2.1.3 Leukemia cell line

Jurkat P9 cells were obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shangai, China).

## 2.2 METHODS

### 2.2.1 Preparation of Tea Kombucha

Tea kombucha was prepared according to the method by Malbaša *et al.* (2011).

Sugared-water was prepared with 40 grams sugar (sucrose) per litre of boiled drinkable water. The boiled sugared-water was then infused with one bag of Lipton tea (thread hanging outside) and boiled again for 2 minutes after which the tea bag was removed. The boiled

sugared-tea was transferred into a clean transparent plastic container and allowed to cool to room temperature. It was inoculated with 3% (w/v) of freshly grown tea fungus that had been cultured in the same medium for 14 days and 10% (v/v) of previously fermented liquid tea broth aseptically. The fermentation container was then covered, leaving a little opening for aeration, which is required for proper fermentation of the tea. The set up was then kept in a clean environment and left to grow in the dark at room temperature over a period of 14 days.

### 2.2.2 Freeze-Drying of Tea Kombucha

About 600 ml each of tea kombucha and unfermented tea were freeze-dried. The beverage was transferred into small containers (150 ml) and placed in a Super Modulyo freeze-drying machine (Thermo Scientific, USA) for 7 days to obtain dried extracts. The extracts were weighed and stored in a cool dry place in the dark. They were reconstituted in distilled water before use.

### 2.2.3 MTT Assay for Cell Viability

Cell viability was determined according to the method of Ayisi *et al.*, (1991). This assay measures the reduction of yellow MTT by succinate dehydrogenase to an insoluble purple formazan product. Aliquots (100 µl) of Jurkat P9 leukemia cells containing 1 x $10^5$ cells/ml were added to each well of a 96-well flat-microtiter plate and incubated (24 hours at room temperature) with various concentrations of two-fold dilution of both the tea kombucha and unfermented tea extract (highest concentration:1000 µg/ml). Three replicates were used for each concentration in the experiment. After 24 hour incubation, 20 µl of MTT was added to each well, mixed and then incubated for 4 hours at room temperature. A 150 µl of acidified

isopropanol was added. After an overnight incubation at 37ºC, absorbance was measured at 570 nm using a spectrophotometer. The inhibition rates were calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Mean absorbance of treated sample- Mean absorbance of } \mathbf{c.c}}{\text{Mean absorbance of untreated sample –Mean absorbance of } \mathbf{c.c}} \times 100\%$$

**c.c** means colour control

### 2.2.4 Antioxidant Determination using DPPH Assay

The antioxidant activity was determined using the DPPH free radical scavenging assay described by Brand-Williams *et al*. (1995). The tea kombucha or unfermented tea was diluted in distilled water to a final concentration of 0.5 mg/ml. A 100 µl of 0.5 mM methanolic DPPH was added to 100 µl of extract. This was done in triplicate and the reaction mixture incubated in the dark for 20 minutes. The absorbance was measured at 517 nm using spectrophotometer. Butylated hydroxyltuolene (BHT) served as a positive standard control, and absolute methanol as the blank. Percentage antioxidant activity was determined as:

$$\% \text{ Antioxidant Activity} = \frac{\text{(Absorbance of blank well-(}\mathbf{Abs.}\text{ of sample - }\mathbf{Abs.}\text{ of }\mathbf{c.c}\text{))}}{\text{Absorbance of Blank}} \times 100\%$$

**c.c** means colour control and **Abs.** means Absorbance

### 2.2.5 Reducing Power

This is an assay that measures the reduction of $Fe^{3+}$ to $Fe^{2+}$ as described by Plumb *et al.* (1989). Various concentrations (0-5 mg/ml) of both tea kombucha and unfermented tea extracts (200 µl each) were mixed with 0.2 M phosphate buffer (2.5 ml) and 0.5 M potassium ferricyanide (200 µl). The mixture was kept at 50°C in a water bath for 20 minutes. After

cooling, 200 µl of 10% (w/v) trichloroacetic acid was added and centrifuged at 3000 rpm for 10 minutes. The upper layer of solution (200 µl) was mixed with distilled water (200 µl) and a freshly prepared 0.1M ferric chloride solution (40 µl) and incubated for 30 minutes at room temperature. The absorbance was measured at 700 nm. Ascorbic acid at various concentrations (1-5 mg/ml) was used as standard.

### 2.2.6 Total Phenolic Content

It was evaluated using a modified colorimetric method described previously by Singleton and Rossi (1965). An Aliquot of 10 µl of tea kombucha/unfermented tea or gallic acid (as calibration standard) was placed into an Eppendorf tube. Distilled water (0.79 ml) was then added. After that 50 µl of Folin-Ciocalteau reagent was added and mixed thoroughly. The mixture was then incubated at room temperature for 8 min. Sodium carbonate (20 g/100ml) solution (150 µl) was added, mixed and incubated at room temperature for 2 hours. An aliquot of 100 µl was placed into a 96 – well plate. The absorbance was measured at 750 nm using a plate reader.

### 2.2.7 Statistical Analysis:

The statistical analysis was carried out using SPSS for Windows version 10.0 statistical software (SPSS Inc, Chicago, USA). Statistically significant differences between the groups were compared using one-way analysis of variance (ANOVA). The data are displayed as means ± standard deviation (SD) and p-values less than 0.05 are considered “statistically significant”.

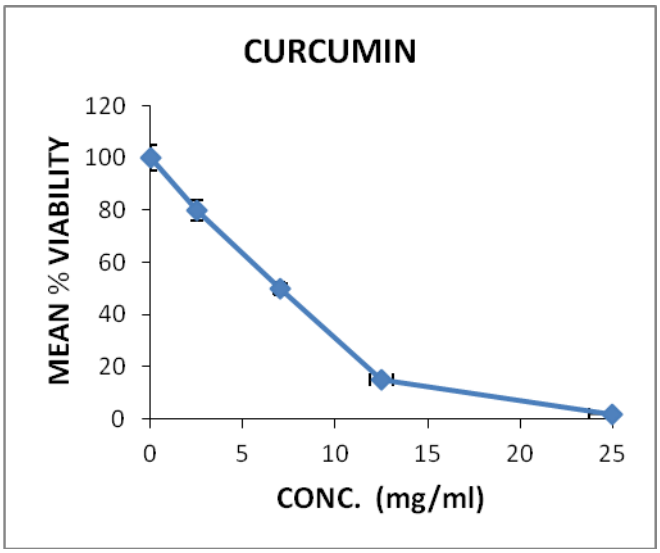
# CHAPTER THREE

## 3.0 RESULTS

### 3.1 Cell Viability

Curcumin, the standard positive control, showed decreasing % cell viability with increasing concentration (Fig. 3.1A). However, the cell viability of the tea kombucha and the unfermented tea used as normal control, increased slightly at lower concentration to about 120 % but slightly decreased upon increasing the concentration albeit to a higher degree in the tea kombucha. Curcumin had an $IC_{50}$ value of 7.22 mg/ml while that of the tea kombucha and unfermented tea could not be determined within the concentration range tested (since the % cell viability was above 70% at the highest concentration of 1000 µM). Curcumin showed cytotoxic effect but the tea kombucha and the unfermented tea showed no cytotoxic effects on Jurkat cells (Fig. 3.1).

**(A)**




$IC_{50}$ = 7.22 mg/ml

**(B)**

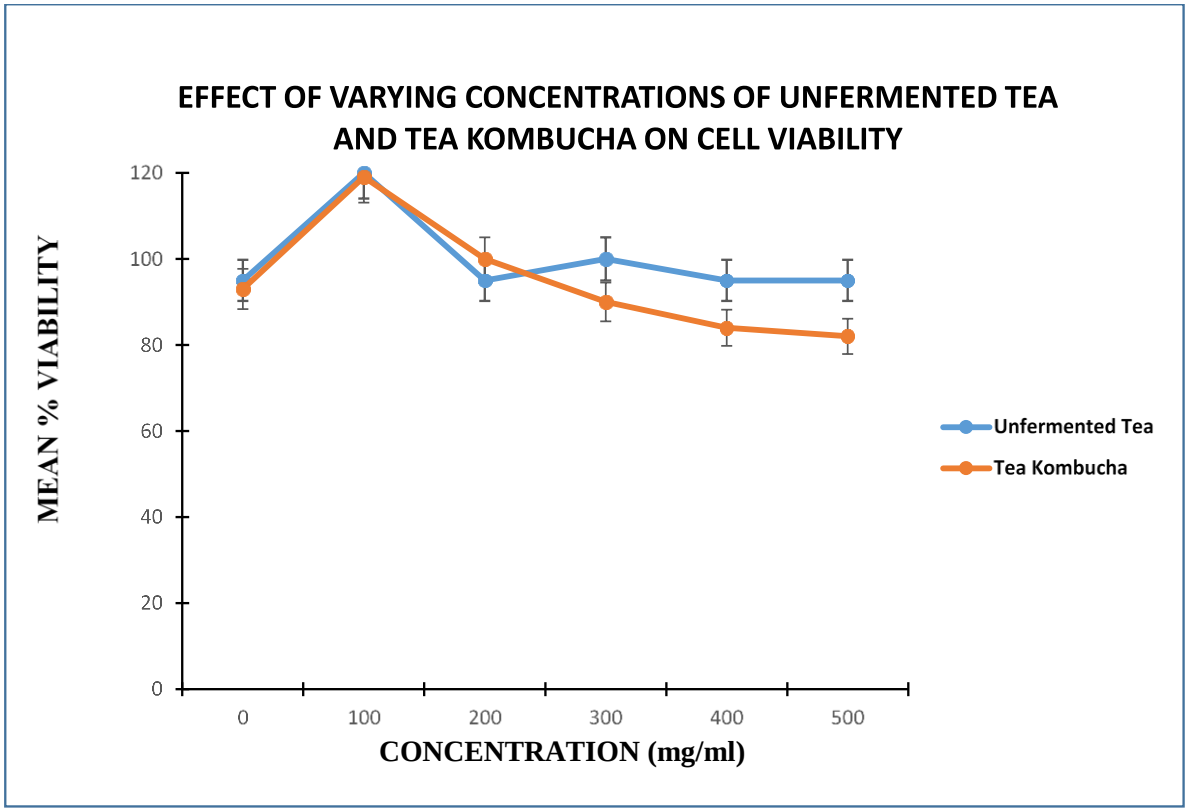
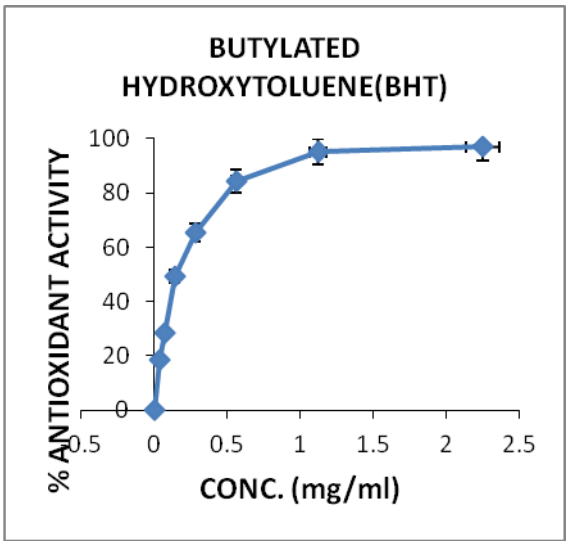



**Fig. 3. 1: Effect of varying concentrations of curcumin (A), tea kombucha and unfermented tea (B) on viability of Jurkat P9 leukemia cells. Results are means of ± SEM of n= 3**

### 3.2 DPPH Antioxidant Activity

DPPH free radical-scavenging ability of the tea kombucha (TK) was measured together with butylated hydroxytoluene (BHT) was used as a standard positive control and unfermented tea (BT) as a normal control (Fig. 3.2). The plot of concentration against % antioxidant activity gave sigmoid curves from which $EC_{50}$ values were obtained.

**(A)**




$EC_{50}$ = 0.148 mg/ml

**(B)**

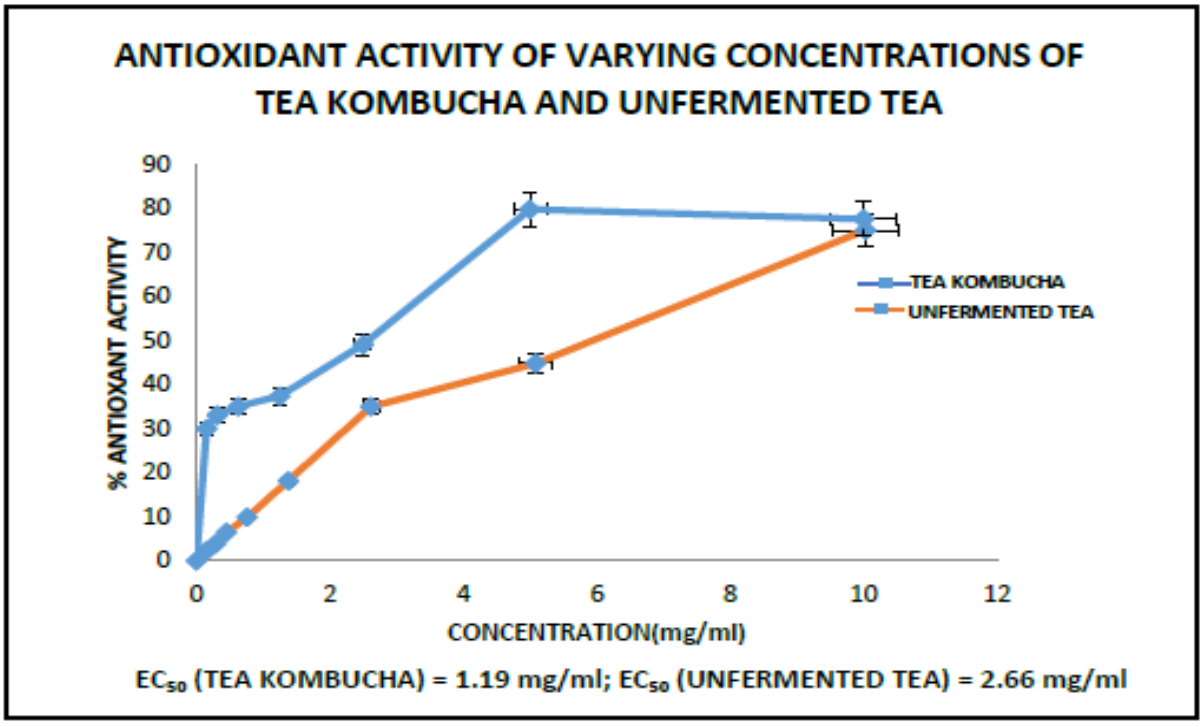
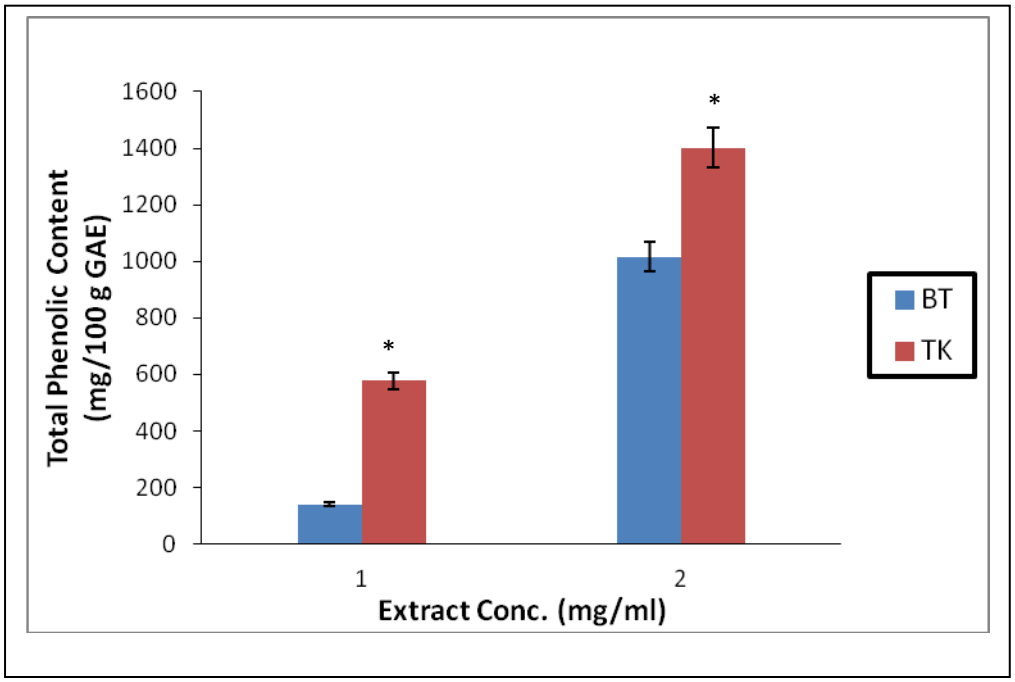



**Fig. 3.2: Antioxidant activity of varying concentrations of BHT (A), tea kombucha and unfermented tea (B). Results are means of ± SEM of n= 3**

### 3.3 Total Phenolic Content

The total phenolic content of the tea kombucha (TK) and the unfermented tea (BT) increased with increasing concentration (Fig. 3.3). At a concentration of 2.5 mg/ml the total phenolic content expressed as mg/100 mg gallic acid equivalent (GAE) was 1016.0 and 139.2 for TK and BT, respectively; and at 5 mg/ml the total phenolic content was 1401.1 and 577.6 for TK and BT, respectively. Thus at extract concentration of 2.5 mg/ml, the total phenolic content of TK was 7-fold higher than the BT, whilst at 5.0 mg/ml total phenolic content of the TK was 2.5-fold higher than the BT.



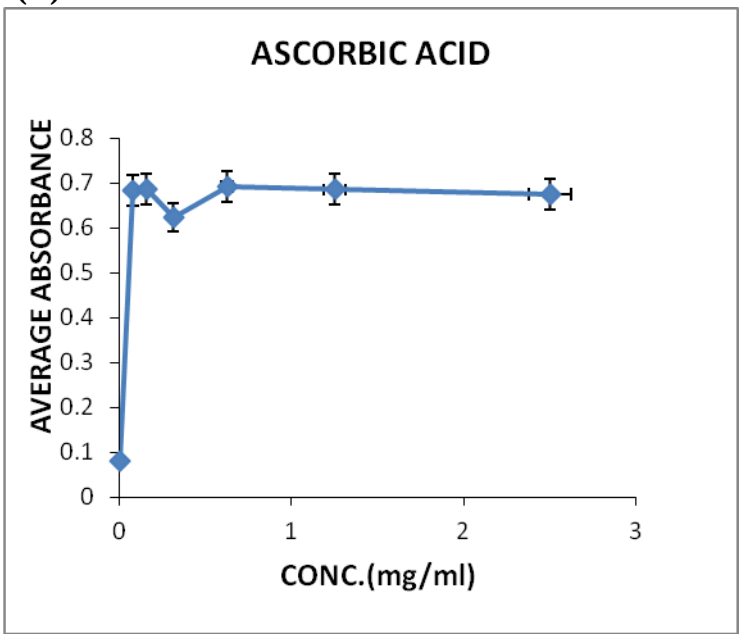

Extract conc. 1= 2.5 mg/ml and 2= 5.0 mg/ml GAE = gallic acid equivalent

**Fig. 3.3: Total phenolic content of tea kombucha (TK) and unfermented tea (BT) (A) and calibration curve for gallic acid (B). Results are means ± SEM of n= 3 for (A) and means of n=2 for (B). *value significantly different from BT control; $p < 0.05$; # value significantly different from 2.5 mg/ml extract; $p < 0.05$**

### 3.4 Reducing Power

The $Fe^{3+}$ reducing power of the standard ascorbic acid reached a maximum at 1.0 mg/ml at a mean absorbance of 0.70 while that of the tea kombucha reached a maximum at 20 mg/ml with a mean absorbance of 0.70. The reducing power of the unfermented tea, however, showed an absorbance of 0.5 at 20 mg/ml which continued to increase to about 0.70 at 40 mg/ml. Thus the tea kombucha has about 2-fold higher reducing power than the unfermented tea (Fig. 3.4).

**(A)**




B)

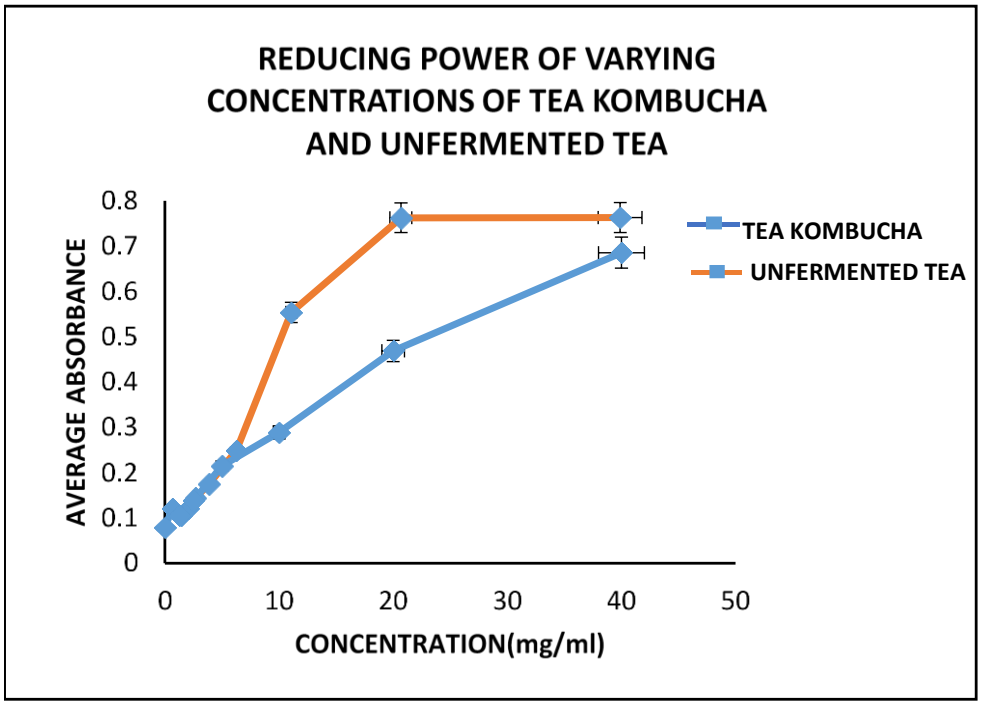



**Fig. 3.4: Reducing power of varying concentrations of ascorbic acid (A), tea kombucha and unfermented tea (B). Results are means ± SEM of n=3**

# CHAPTER FOUR

## 4.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

### 4.1 DISCUSSION

Cell-based assays are often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death. Regardless of the type of cell-based assay being used, it is important to know how many viable cells are remaining at the end of the experiment. There are a variety of assay methods that can be used to estimate the number of viable eukaryotic cells. In this study MTT assay was used.

The half maximal inhibitory concentration, $IC_{50}$, for the tea samples compared to the standard, curcumin, were very high. Thus, the tea samples showed very low inhibitory activities against the growth of the Jurkat P9 leukemia cells. It is possible that much higher concentrations of the samples may be required for inhibition. According to the U.S. Food and Drug Administration and Kappa Laboratories, Miami, Florida, U.S.A. (1995) strong cytotoxic agents must have $IC_{50}$ values less than 30 µg/ml. The percentage cell viability at the highest concentration tested for the tea kombucha was lower than that of the unfermented tea (Fig. 3.1). This suggests that some cytotoxic substances may be produced as a result of the fermentation process. The weak anti-proliferative activity of the tea samples against the Jurkat P9 leukemia cells as compared to the standard curcumin, which showed strong inhibitory effects, suggests that the samples are less likely to inhibit cell growth. This finding supports the observations made by Vijayaraghavan et al. (2000) and Pauline et al. (2001) in which tea kombucha displayed no significant toxicity.

Total antioxidant capacity of tea kombucha and unfermented tea were tested using the DPPH free radical scavenging activity. The $EC_{50}$ values of 1.19 mg/ml and 2.66 mg/ml for tea kombucha and unfermented tea, respectively indicates that tea kombucha has a 2-fold stronger antioxidant activity than the unfermented tea. This suggests that the tea kombucha contained more antioxidants than the unfermented tea probably as a result of the fermentation process. This corresponds to work done by Chu and Chen (2006) that examined tea kombucha and established that total phenol content of all kombucha samples showed a linear increase with fermentation time. And that its antioxidant activity correlates with its total phenolic content. Antioxidants, especially polyphenols, have been found to be promising agents against cervical cancer, including induction of apoptosis, growth arrest, inhibition of DNA synthesis, and modulation of signal transduction pathway; additionally, polyphenols can interfere with each stage of carcinogenesis initiation, promotion, and progression for the prevention of cancer development (Aggarwal *et al*., 2004). Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity of a compound (Denizot and Lang, 1986). Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Plumb *et al*., 1989). In this assay, there were more $Fe^{3+}$ reducers in the tea kombucha than the unfermented tea (Fig. 3.3) suggesting that the fermentation process caused the production of substances with reducing power thus increasing antioxidant activity. This corresponds to findings by Jayabalan *et al*. (2008) in which they reported on the free radical scavenging abilities of tea kombucha prepared from green tea, black tea and tea waste.

The total phenolic content expressed as mg/ 100 g GAE at 2.5 mg/ml and 5.0 mg/ml of tea extracts were 7.3 – fold and 2.4 – fold, respectively higher in tea kombucha than unfermented tea (Fig. 3.3). This suggests that at either concentration of extracts the total phenolic content of the tea kombucha was significantly higher than the unfermented tea. This corroborates with the higher reducing power or radical scavenging activity of the tea kombucha vis-à-vis the unfermented tea indicating that the total phenolic content may be responsible for antioxidant activity expressed by both extracts (Vissoto *et al*., 2013). This was established by Jayabalan *et al*. (2007) in which a highly pronounced increase of total phenol content in all kombucha samples was recorded. Also Chu and Chen (2006) proved that the content was up to 7.8 mM gallic acid equivalent (GAE; 15$^{th}$ day of fermentation) and only around 4 mM GAE for black tea.

Tea kombucha showed a higher antioxidant activity compared to the unfermented tea due to compounds such as glucuronic acids, polyphenols and other organic acids produced during the fermentation process, hence its ability to shield the Jurkat P9 leukemia cells from oxidative damage. The antioxidants present in the tea kombucha may mop up the oxyradicals and neutralize their effects thereby protecting the cells from damage. This may explain why the tea kombucha showed no cytotoxic effect on the Jurkat P9 leukemia cells (Jayabalan *et al.*, 2008).

## 4.2 CONCLUSION

Tea kombucha was observed to have higher antioxidant activity than unfermented tea and it slightly reduced Jurkat P9 leukemia cell viability many orders of magnitude below that of the standard drug, curcumin, a potent antioxidant, whilst the unfermented tea was without effect.

## 4.3 RECOMMENDATION

- Tea kombucha extracts of different concentrations should be investigated compared to unfermented tea extract.
- The tea extracts concentrations exhibiting cytotoxicity should be tested in normal and other cancer cell lines to establish selectivity in cytotoxicity.
- The effects of the tea extracts on lipid peroxidation should be determined.
- The apoptotic activity of the tea extracts should also be investigated.

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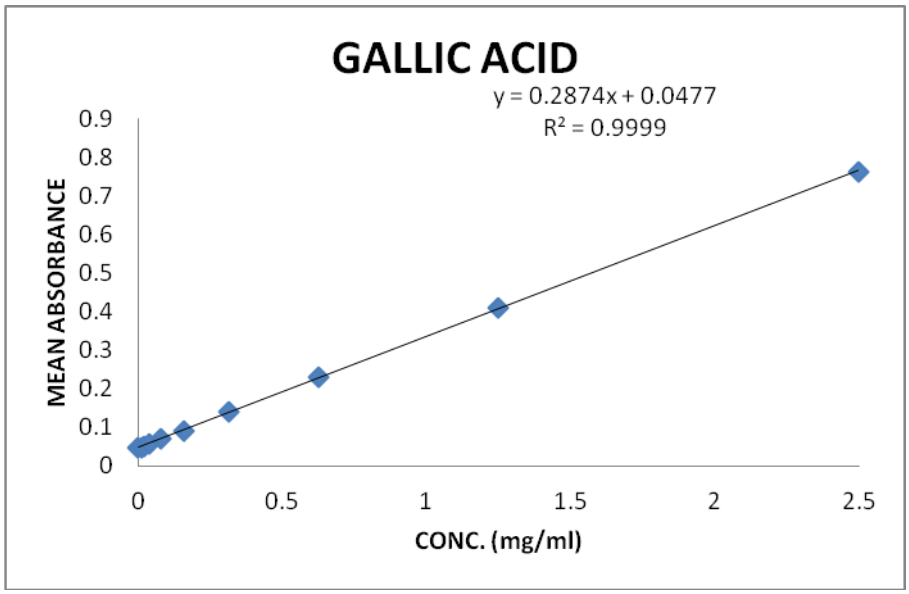
## APPENDIX

### CALIBRATION CURVE FOR GALLIC ACID




**Gallic acid (calibration standards)**

(a) Stock [5 mg/ml]

Gallic acid (50 mg) was dissolved in 1 ml of absolute ethanol. Distilled water was then added to dilute it to 10ml. Solution was then stored at 4 $^{o}$C.

(b) Dilutions

(c) Serial dilutions of stock using distilled water were done to obtain concentrations for calibration curve.

0.5, 0.25, 0.125