INVESTIGATION OF THE ANTI-PROLIFERATIVE AND ANTIOXIDANT ACTIVITIES OF COCOA KOMBUCHA

BY

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DECLARATION

I, Dorcas Akosua Frimpong, do hereby declare that the work presented in this dissertation is solely the results of my own investigations under the supervision of Prof. Laud K.N. Okine, Rev. Dr. W.S.K. Gbewonyo and Dr. Regina Appiah-Oppong, and that except previous research works which have been duly acknowledged, this work has never been submitted to this university or elsewhere in part or whole for the award of any degree.

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Dr. Regina Appiah-Oppong ……
(Supervisor) Date

DEDICATION

I dedicate this work to those who believed in my potential.

ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude and indebtedness to my supervisors Prof. Laud Okine, Rev. Dr. W. S. K. Gbewonyo and Dr. Regina Appiah-Oppong for their inspiring guidance, constructive and valuable suggestions throughout this work. Their abled knowledge and expert supervision with unswerving patience fathered/mothered my work at every stage, for without their warm affection and encouragement, the fulfillment of the task would have been very difficult.

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LIST OF ABBREVIATIONS

ROS	Reactive Oxygen Species
DPPH	2,2- Diphenyl-1- picryl-hydrazyl
BHT	Butylated Hydroxytoluene
CK	Cocoa Kombucha
CC	Unfermented Cocoa
MTT	(3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)

ABSTRACT

Cocoa kombucha (CK) is a new alcoholic beverage developed from cocoa which has many health benefits, so its antioxidant activity and anti-proliferative activity in Jurkat P9 cancer cells were determined and compared to unfermented cocoa and selected standards. The antioxidant activity of CK extract was determined by DPPH assay and total phenolic content, as well as its iron reducing power. The MTT assay was used to determine the anti-proliferative activity of cocoa kombucha. Results indicated that cocoa kombucha had higher antioxidant activity, represented by significantly ($p<0.05$) higher reducing power and free radical scavenging activity, compared to the unfermented cocoa. However, both cocoa extracts did not show any anti-proliferative activity in Jurkat P9 leukemia cells compared to the standard anti-cancer drug, curcumin. These findings suggest that cocoa kombucha, at the concentrations used in this study, though showed significant antioxidant activity was not cytotoxic to the Jurkat P9 cells.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

In today's society, human activities generate numerous forms of environmental oxidative stress. Oxidative stress has been implicated in many diseases such as cancer (Gibbs, 2003). Cancer is a widespread life-threatening disease that attacks people of all ages, especially those over 65 years. It is a disease related to lifestyle, environmental factors and less commonly genetic factors. Cancer is the third leading cause of death worldwide, being preceded by cardiovascular and infectious diseases. This presents major health concerns which need to be researched.

Kombucha, also named as tea fungus is consumed worldwide as a health drink for a very long time, especially in China, Russia and Germany (Dufresne and Farnworth, 2000). Kombucha is a symbiosis of *Acetobacter*, including *Acetobacter xylinum* as a characteristic species, and various yeasts such as the genera of *Brettanomyces*, *Zygosaccharomyces* and *Saccharomyces*, depending on the source (Mayser *et al*., 1995). The tea fungus broth is composed of two portions; a floating cellulosic pellicle layer and the sour liquid broth. Acetic acid, ethanol and gluconic acid are the major components of the liquid broth with other minor constituents such as lactic acid, glucuronic acid, phenolic acid, groups of vitamin B and enzymes (Blanc, 1996). This tea has been claimed to have antioxidant properties and other health benefits (Jayabalan *et al*., 2014) but with limited scientific evidence. The health benefits are largely attributed to the vitamins, amino acids and other micronutrients produced during the fermentation process. The organic acids produced during fermentation are believed to help the body in diverse ways,

including protection against cancer and cardiovascular diseases (Dufresne and Farnworth, 2000) but this remains to be proven scientifically.

Cocoa beans are derived from the fruit of the plant *Theobroma cacao L*. In Ghana, dry cocoa beans are mainly exported as a foreign exchange earner, while a small percentage of the cocoa beans serve as raw material for cocoa powder, cocoa butter and chocolate products (Adeyeye *et al.*, 2010). Cocoa as a food ingredient is fast becoming very popular in the food and confectionery industry worldwide. It is available in a wide variety of forms, colors and flavors, and used in numerous applications (Borchers *et al.*, 2000). A good quality cocoa powder should be relatively free flowing, stable and uniform in color and flavor, of good microbiological quality, and easy to handle by the user (Vu *et al.*, 2003). Moreover, a range of other characteristics such as pH, fineness and fat content define the powder and have an important impact on the end product for which the cocoa is used. Cocoa beans and its products are also a rich source of phytonutrients and polyphenols, particularly catechins and procyanidins (Lecumberri *et al.*, 2007).The nutritional quality of cocoa products are determined largely by the chemical composition of the cocoa powder, which is dependent on the quantum of proteins, carbohydrates, fats, minerals and phytochemicals in the cocoa products and the corresponding digestibility coefficient (Belscak *et al.*, 2009; Adeyeye *et al.*, 2010; Lettieri-Barbato *et al.*, 2012). These nutrients are similar to those found in tea which has been found to have antioxidant activities (Vina *et al.*, 2014).

1.1.1 Justification

Although, some research has been carried out on the antioxidant and anti-proliferative activities of tea kombucha, there is little information about cocoa kombucha although cocoa

has phytochemical constituents similar to tea. Thus, there is the need to investigate the free radical scavenging activity and anti-proliferative effect of cocoa kombucha.

1.1.2 Aim of Study

The study was aimed at determining the antioxidant and anti-proliferative activities of cocoa kombucha.

Specific objectives

The specific objectives were:

i. To determine the antioxidant activity of cocoa kombucha.

ii. To evaluate the effect of cocoa kombucha on the viability of Jurkat P9 leukemia cells

1.2 LITERATURE REVIEW

1.2.1 Kombucha Beverages

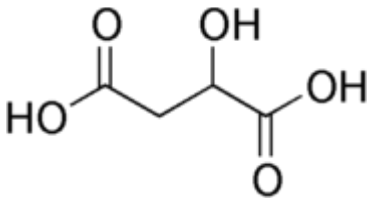
Kombucha is produced by the fermentation of sugared-tea by a symbiotic association of bacteria and yeasts forming a ‘‘tea fungus’’. It is also known as “Che” or “Kvass”. It originated in China where the ‘‘Divine Che’’ was prized 220 BC during the Tsin Dynasty for its detoxifying and energizing properties (Sievers *et al*., 1995). ‘‘Tea Kvass’’ was introduced into Russia by oriental merchants and then into Europe around the turn of this century. This refreshing beverage tasting like sparkling apple cider is often produced in the home by fermentation using a tea fungus passed from home to home. Kombucha is typically made by fermenting sugared-tea with a symbiosis of yeast species, fungi, and acetic acid bacteria at ambient temperature for about 7-14 days. This drink comprises a floating cellulose pellicle layer (mat) and a sour liquid broth (Liu *et al*., 1996). The mat can be used to ferment subsequent black tea to obtain the kombucha drink.

Tea leaves are infused in boiling water for about 5 minutes after which the leaves are removed. Sucrose is dissolved in the hot tea and the preparation is left to cool. The prepared tea is poured into a wide-mouthed clean vessel and is acidified by the addition of vinegar or already prepared kombucha. Tea fungus is laid on the surface, and the jar is carefully covered with a clean cloth and fastened properly. The preparation is incubated at room temperature. During fermentation, a daughter tea fungus is formed at the surface. The taste of the kombucha changes during fermentation; from a pleasant fruit sour-like lightly sparkling flavor after few days, to a mild vinegar-like taste with prolonged incubation (Sievers *et al*., 1995).

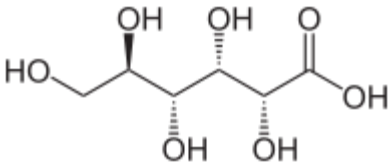
Bacteria and fungus present in kombucha form a powerful symbiosis able to inhibit the growth of potential contaminating bacteria (Liu *et al*., 1996; Balentine *et al*., 1997).The close

association between micro-organisms that make up the fungus and their interaction with the substrates supporting fermentation have been studied (Sievers *et al*., 1995; Balentine *et al*., 1997; Yurkevich and Kutyshenko, 1998). *Acetobacter xylinum* has the ability to synthesize a floating cellulose network which enhances the association formed between bacteria and fungi (Balentine *et al*., 1997). Some organic acids are also synthesized (Fig. 1.1).

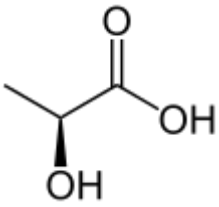
A large amount of information has been published concerning the effects of tea and its major constituents on human health (Jayabalan *et al*., 2010). This beverage has been consumed in many countries for a very long time and interest is still growing because scientific reports indicate that tea could bring benefits for health and may help prevent chronic diseases (Zheng *et al*., 2012). Tea was first introduced into European countries from China by Portuguese and Dutch explorers as a medicinal herb (Rams *et al*., 2000). Over the years, tea consumption has been associated with eating and living habits just like coffee or soft drinks, without regards to its benefits. The aging of the population and limitations of modern medicine have caused many people to look for new ways to improve their health. Doubts surrounding lifestyle and diet along with the growing interest in functional foods have contributed to this trend.



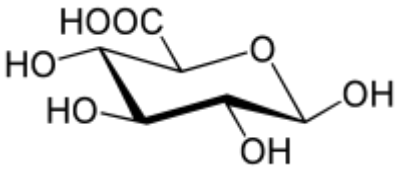
Malic acid



Gluconic acid



Lactic acid



Glucuronic acid

Fig. 1.1: Chemical structures of some organic acid constituents of kombucha.
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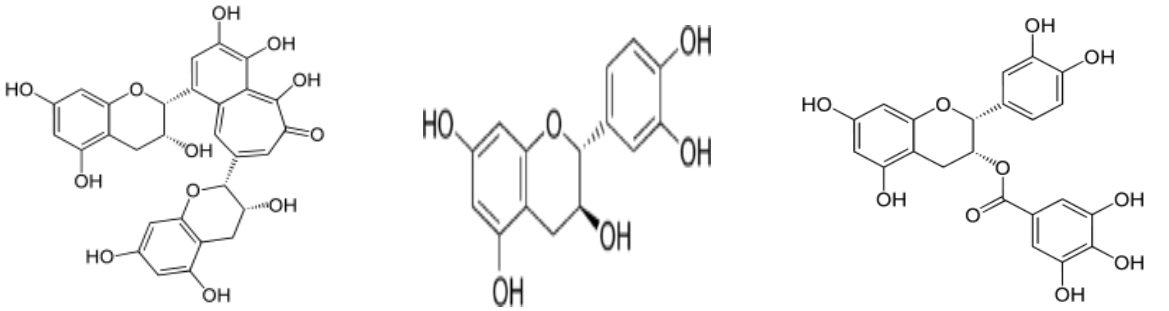
Substrate for the kombucha fermentation is black or green tea extract sweetened with 5% to 8% sucrose. Besides the traditional substrates, the possibility of use of alternative substrates has been established in various studies. Attempts in the use of non-traditional substrates such as Coca-Cola, red wine, white wine, vinegar, and extract of Jerusalem artichoke, milk, fresh sweet whey, reconstituted sweet whey, acid whey, Echinacea, and Mentha for kombucha fermentation has been shown (Leung and Foster, 1996). Studies have also revealed the possibility of using tea waste material for manufacturing kombucha beverage with satisfying quality (Jayabalan *et al*., 2007). Other studies of alternative cultivation medium have shown

that green tea and lemon balm tea have more stimulating effect on kombucha fermentation than black tea, thus providing the fermentation product in a shorter time (Pack *et al*., 2011), whilst some prepared kombucha beverage from mulberry tea, Japanese green tea, jasmine tea, and oolong tea. Some scientists attempted the kombucha fermentation on sweetened sour cherry juice (Velioglu *et al*., 1998; Heinonen *et al.,* 1998a).

Though green and oolong tea can be used for kombucha preparation, black tea and white sugar are considered the finest substrates. Gbewonyo (Personal communication) also reported that cocoa powder and white sugar can be used as a substrate for the preparation of kombucha.

1.2.2 Tea Kombucha

Tea belongs to the *Theaceae* family and come from two main varieties: *Camellia sinensis var. sinensis and Camellia sinensis var. assamica* (Hara *et al*., 1995a). The leaves are picked from the evergreen shrub and can be processed by different methods. Green tea is readily dried with or without a fixation step to inactivate enzymes (Hara *et al*., 1995b), whilst black tea is the result of the oxidation of leaf polyphenols through a multi-stage enzymatic process (Hara *et al*., 1995c). New polyphenol complexes are formed during the processing of black tea. Some of the polyphenols known are shown in Fig. 1.2.



Theaflavin Epicatechin Epicatechin-3-gallate

Fig. 1.2: Chemical structures of some tea polyphenols
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The composition and properties of tea have been scientifically proven, but not much scientific information is available concerning the composition and the effects of kombucha on health. Benefits of tea kombucha have been reported by testimony of users in different health conditions and with variable doses (Wang *et al.,* 2000). Many of the health benefits from kombucha have not been adequately researched. Initially tea kombucha was believed to be a detoxifying agent which aids in gastrointestinal health and also has energizing properties (Jayabalan *et al*., 2014). Consumers drink the kombucha beverage for the perceived benefits, including its supposed activity on immunity, obesity, atherosclerosis, hypertension, antioxidant capabilities and cancer prevention (Vina *et al*., 2014). These claimed benefits are being investigated to determine if there is a relationship between consumption of kombucha and improved health and what the mechanism(s) responsible may be (Jayabalan *et al.,* 2014). Research findings link majority of kombucha's alleged health benefits to kombucha's antioxidant properties and free radical scavenging ability (Rams *et al*., 2000; Banerjee *et al*., 2010). Also, it has been claimed to prolong life, lessen wrinkles, and promote wound healing, but these testimonies have not been validated scientifically (Dufresne and Farnworth, 2000). The beneficial effect of tea kombucha may be attributed to the presence of tea polyphenols and other components obtained after fermentation (Jayabalan *et al.*, 2014).

Although the consumption of kombucha generally presents no adverse side effects, a few cases of health complications have been reported. Upset stomach, some allergic reactions, particularly for those predisposed to acid sensitivities and kombucha associated renal disorders are usually improved by ceasing or lowering consumption (Pauline, 2001). Four cases of possible toxic reactions and two cases of unexplained severe metabolic acidosis have been reported apparently related to kombucha (Srini-vasan *et al*., 1997). One case of possible

hepatotoxicity (Perron *et al*., 1995) and one case of skin disease (Sadjadi, 1998) has also been reported. Some adverse health effects may be due to the acidity of the tea, which can cause acidosis, or may be a result of bacterial or fungal contamination during the brewing process. It is recommended that plenty of water is drunk to facilitate the elimination of toxins and to adjust consumption, when taking kombucha.

1.2.3 Cocoa

Seeds from *Theobroma cacao* L. (*Sterculiaceae*) are the base for the production of the most important and widespread functional food in human history. Cocoa powder a by-product of cocoa beans can serve as a substrate for kombucha (cocoa kombucha), but little or no literature is found on cocoa kombucha. Cocoa powder contains compounds such as purines, alkaloids, catechins, pro-anthocyanin, anthocyanin, and organic acids, which contribute to the taste of cocoa powder (Steinberg *et al*., 2003).

Researchers have focused attention on cocoa polyphenols, especially the flavonoids, and their function as potent antioxidants in human health. Report by Lee *et al.* (2003), suggests that cocoa is a richer source of dietary flavonoids than tea. Other phytochemicals present in cocoa includes phenolic acids, amides and stilbenoids (Kurosawa *et al*., 2005). Phenolic and flavonoid contents and total antioxidant capacities of cocoa are higher than that of other phytochemical-rich foods (Lee *et al*., 2003). Plants provide abundant minerals and phytochemicals when presented as food. Cocoa is a widely consumed food ingredient. Phytochemical profile in cocoa beans varies for different cultivars and among cocoa beans and cocoa-containing foods. In addition to polyphenols, cocoa contains methylxanthine compounds, predominantly theobromine and caffeine (Fig. 1.3).

events such as lipid peroxidation and oxidative DNA damage, but also causes physiologic adaptation phenomena and regulation of intracellular signal transduction (Guerra *et al.*, 2007).

Reactive oxygen species (ROS) can be produced from endogenous sources, such as mitochondria, peroxisomes and inflammatory cell activation (Klaunig and Kamendulis, 2004). When the mitochondrial electron transport chain is saturated with electrons, there is donation of single electrons to molecular oxygen, leading to superoxide generation. The exogenous sources of ROS may include environmental agents, pharmaceuticals, and industrial chemicals. The oxidative stress due to the ROS generated may cause DNA, proteins, and/or lipid damage (Fig. 1.4) leading to changes in chromosome instability, genetic mutations, and modulation of cell growth that may result in cancer (Kohen and Nyska, 2002).

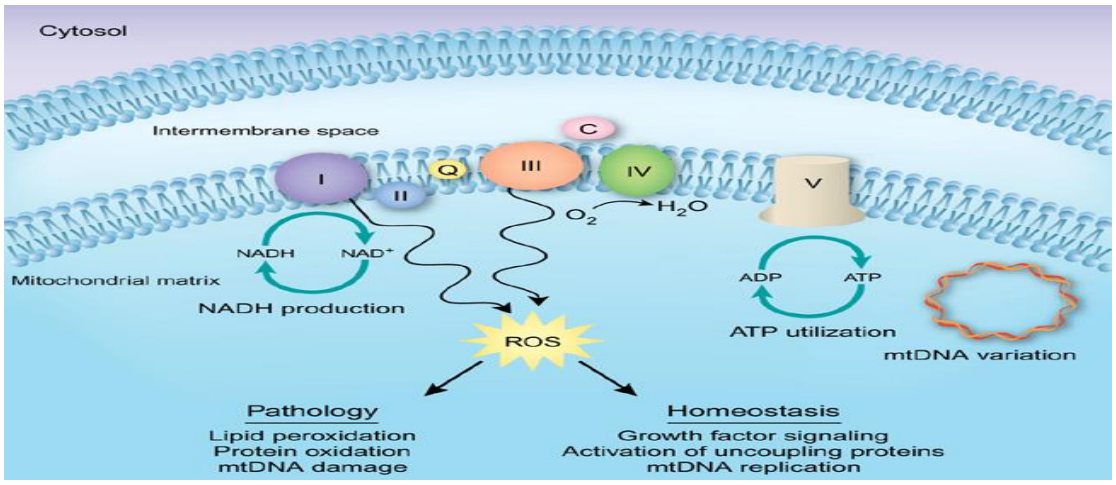

Fig 1.4: Overview of reactive oxygen species (ROS) generation in the mitochondrion.

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ROS molecules participate in multi-faceted activities within cells, acting as secondary signaling molecules for inflammation and immune responses, in addition to directly harming

microbes that invade tissues (Lau *et al*., 2008). Typically, low concentration of ROS is essential for normal physiological functions like gene expression, cellular growth and defense against infection. Sometimes they also act as the stimulating agents for biochemical processes within the cell.

One of the early responses of host innate immunity is the ROS production in reaction to microbial invaders. Free oxygen radicals are highly toxic to pathogens and are utilized as a tool to prevent colonization of tissues by microorganisms. ROS are also a key part of the intracellular redox profile influencing a wide variety of signaling networks (Circu and Aw, 2010). Uncontrolled generation of ROS can lead to their accumulation causing oxidative stress in the cells. However, due to excessive generation of ROS which may lead to oxidative stress, cells have evolved defense mechanisms for protection against ROS mediated oxidative damage. These include antioxidant defenses to keep a check on the generation of ROS.

The body has natural antioxidants to counteract damages caused by ROS. Antioxidants are effective because they can donate their own electrons to ROS and thereby neutralizing the adverse effects of the latter. More often, the defense mechanisms are overwhelmed by the high production of ROS, thus the need for exogenous free radicals scavengers. The exogenous antioxidants include vitamins C and E and polyphenols found in diets. The prophylactic properties of dietary plants have been attributed to the antioxidants and polyphenols present in them.

Polyphenols are secondary metabolites of plants and represent a huge gamut of substances having aromatic ring(s) bearing one or more hydroxyl moieties (Bors *et al*., 1996). Polyphenols are effective ROS scavengers and metal chelators due to the presence of multiple hydroxyl groups. Polyphenols are synthesized by fruits, vegetables, teas, cocoa and other plants.

Examples of polyphenolic natural antioxidants derived from plant sources include vitamin E, flavonoids, cinnamic acid derivatives, curcumin, caffeine, catechins, gallic acid derivatives, anthocyanins and tannins (Bors *et al*., 1996). All polyphenols, including flavonoids, offer numerous health benefits. The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity (Heim *et al*., 2002).

Vitamin C, the water soluble natural vitamin, plays a crucial role in regenerating lipid soluble antioxidants like vitamin E. Both vitamins E and C are used as standards for evaluating the antioxidant capacity of new molecules (Kohen and Nyska, 2002). Healthy people on good diet have sufficient ascorbic acid to exert maximum antioxidant effects, whilst low levels are found in unhealthy individuals and thus predisposed to ROS-mediated diseases. Studies have shown that kombucha contains both vitamins and polyphenols (Blanc, 1996) and can serve as a source of exogenous antioxidants. A study has demonstrated that kombucha has *in vivo* antioxidant activities (Hertog *et al*., 2000).

1.2.5 Cancer

Cancer is a complex genetic disease that is caused primarily by environmental factors. The cancer-causing agents (carcinogens) can be present in food and water, in the air, and in chemicals and sunlight that people are exposed to. Factors that contribute to regional differences in the types of cancer include regional variations in the prevalence of major risk factors, availability and use of medical practices such as cancer screening, availability and quality of treatment, and age. In 2008, two of the four leading cancers in men (stomach and liver) and women (cervix and stomach) in developing countries were related to infection (Worsham *et al*., 2009).

1.2.5.1 Causes of cancer

The incidence, geographic distribution, and behavior of specific types of cancers are related to multiple factors, including sex, age, race, genetic predisposition, and exposure to environmental carcinogens. Of these factors, environmental exposure is probably the most important. Chemical carcinogens (particularly those in tobacco smoke) as well as azo dyes, aflatoxins, asbestos, and benzene have been clearly implicated in cancer induction in humans and animals (Ziech *et al*., 2010).

Over the past few decades, a number of researchers have attempted to estimate the proportion of cancer cases or deaths due to environmental and occupational exposures. Tobacco use is the cause of about 22% of cancer deaths. Another 10% is due to obesity, a poor diet, lack of physical activity, and consumption of alcohol (Worsham *et al*., 2009). Other factors include certain infections, exposure to ionizing radiation, and other environmental pollutants. Approximately 15% of all incident cancers worldwide are attributable to infections (Parkin, 2006). This percentage is about three times higher in developing countries (26%) than in developed countries (8%). In the developing world nearly 20% of cancers are due to infections such as hepatitis B (Newcomb *et al*., 1994).

1.2.5.2 Cancer treatment

Cancer management and treatment involves the use of surgery and/or chemotherapy. It usually depends on the type of cancer and whether it is malignant or not. Surgery involves the use of surgical instruments to remove the tumor, thus, prevent its spreading to other cells. In chemotherapy, strong anticancer agents are given either as an injection or tablet. These treatment regimens do not completely eliminate the tumors but control the rate of metastasis (rate of proliferation).

Curcumin is an example of an anticancer drug. It is the product obtained by solvent extraction of turmeric i.e., the ground rhizomes of *Curcuma longa L*. (*Curcuma domestica Valeton*) and purification of the extract by crystallization. The product consists essentially of coloring principles 1,7-bis-(4-hydroxy-3-methoxy-phenyl)-hepta-1,6-diene-3,5-dione (curcumin) and its desmethoxy- and bis-desmethoxy-derivatives (Fig. 1.5) in varying proportions. Curcumin is an oil soluble pigment, practically insoluble in water at acidic and neutral pH, soluble in alkali. It is stable at high temperatures and in acids, but unstable in alkaline conditions and in the presence of light (Barclay *et al*., 2000).

Curcumin's potent anti-oxidant and free-radical quenching properties play an important role in the inhibitory effects of the compound on the initial stages of carcinogenesis (Oda, 1995). It has been shown that curcumin has the ability to suppress UV irradiation-induced DNA mutagenesis (Ellatar and Virgi, 2000). In addition to the inhibitory effects on the production of nitric oxide (NO) and the ability to scavenge DNA damaging superoxide radicals, curcumin also affects both the Phase I and Phase II enzymes of the hepatic cytochrome P450 enzyme system involved in the oxidation and detoxification of toxic substances (Mohandas and Desai, 1999).

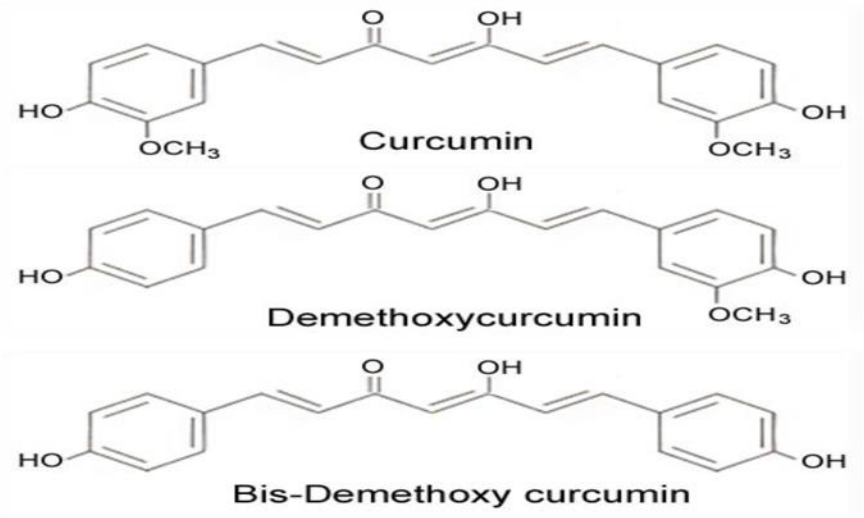


Fig. 1.5: Structures of the curcuminoids; curcumin, demethoxycurcumin and bis-demethoxycurcumin

Curcumin has been studied in multiple human carcinomas including melanoma, breast, colon, and ovarian cancers (Aggarwal *et al*., 2004; Ziech *et al*., 2010). Epidemiological studies attribute the low incidence of colon cancer in India to the chemo-preventive and antioxidant properties of diets rich in curcumin (Mohandas and Desai, 1999). The mechanisms by which curcumin exerts its anti-cancer effects are comprehensive and diverse, targeting many levels of regulation in the processes of cellular growth and apoptosis.

A wide array of compounds in fruits and vegetables in addition to antioxidants may contribute to the reduction of cancer. Foliate deficiency, one of the most common vitamin deficiencies, causes extensive chromosome breaks in human genes (MacGregor *et al*., 1990). Also, iron depletion through chelation has been explored as a possible therapeutic intervention in a variety of cancers.

1.2.5.3 Cancer prevention

The purpose of primary prevention is to limit the incidence of cancer by controlling exposure to recognized risk factors in susceptible populations to prevent the disease. Consumption of adequate fruits and vegetables is associated with a lowered risk of degenerative diseases such as cancer, cardiovascular disease, and immune dysfunction (Helbock *et al*., 1998). Nearly 200 studies in the epidemiological literature have been reviewed and relate, with great consistency, the lack of adequate consumption of fruits and vegetables to cancer incidence (Block *et al*., 1992). Improvements in hygiene have reduced cancers that are related to infections (Neukam *et al.*, 2007). Reduction in the exposure to environmental risk factors such as chemicals could greatly reduce the incidence of cancer, and also healthy life style and eating habits could minimize the risk of cancer.

1.2.6 *In vitro* Evaluation of Antioxidant Activity

Scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) in the DPPH assay is the simplest and most widely reported method for screening antioxidant activity in foods and many plant-based drugs (Brand-Williams *et al.*, 1995). In this assay, the purple chromogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The procedure involves measurement of decrease in absorbance of DPPH at its absorption maxima of 518 nm, which is proportional to concentration of free radical scavenger added to DPPH reagent solution. The activity is expressed as inhibitory concentration IC_{50}; that is the amount of antioxidant necessary to decrease by 50% the initial DPPH• concentration. Thus the lower the IC_{50}, of a chemical entity, the higher its "anti-radical efficiency".

1.2.7 Evaluation of Cytotoxicity

Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenases, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometry. The MTT Cell Proliferation Assay measures the cell proliferation rate, and conversely, when metabolic events lead to apoptosis or necrosis, there is reduction in cell viability (Plumb, 2004).

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 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical reagents were purchased from Wako Pure Chemical Industries Ltd (Tokyo, Japan). All other chemicals were obtained from commercially available sources in their pure forms.

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

2.2 METHODS

2.2.1 Preparation of Cocoa Kombucha/ Unfermented Cocoa Extract

Cocoa kombucha was prepared according to the method of Gbewonyo (Personal communication). Unfermented cocoa extract was prepared without starter culture, and served as control for cocoa kombucha.

2.2.2 Freeze-Drying of Cocoa Kombucha/ Unfermented Cocoa Extract

About 600 ml each of cocoa kombucha and unfermented cocoa were freeze-dried. The beverage was transferred into small containers 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 the dark at 4°C. They were reconstituted in appropriate solvent before use.

2.2.3 Test for Cell Viability and Proliferation

Cell viability was determined according to the method of Plumb (2004). This assay measures the reduction of yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) by succinate dehydrogenase to an insoluble purple formazan product. Aliquots of suspended Jurkat P9 cells containing 1 x 10^5 cells/mL were added to each well of a 96-well flat-microtiter plate and incubated with various concentrations of two-fold dilution of both the cocoa kombucha and unfermented cocoa extract (highest concentration of 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.

Acidified isopropanol (150 µl) was added to stop the reaction and absorbance at 570 nm was measured using a plate reader. The inhibition rates were calculated as follows:

$$\% \text{ inhibition} = \frac{[\text{mean absorbance of control - mean absorbance of extract}]}{\text{Blank (control)}} \times 100$$

2.2.4 *In vitro* Antioxidant Activity of Cocoa Kombucha

The antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay described by Brand-Williams *et al.* (1995). The cocoa kombucha or unfermented cocoa was constituted into solution with distilled water to a final concentration of 0.5 mg/ml. Methanolic DPPH (100 µl of 0.5 mM) was added to 100 µl of the extract and the reaction mixture incubated in the dark for 20 minutes. The absorbance was measured at 517 nm using a spectrophotometer. Butylated hydroxyltoluene (BHT) served as a positive standard with absolute methanol as the blank. The assay was done in triplicate. Percentage antioxidant activity was determined as:

$$\% \text{ Antioxidant Activity} = \frac{\text{Blank- treated}}{\text{Blank (control)}} \times 100$$

2.2.5 Iron Reducing Power

This is an assay that measures the reduction of Fe^{3+} to Fe^{2+} (Ferreira *et al.*, 2007). Various concentrations (0-5 mg/ml) of both cocoa kombucha and unfermented cocoa extracts (200 µl each) in triplicates were mixed with phosphate buffer (2.5 ml, 0.2 M) and potassium ferricyanide (200 µl, 0.5 M). The mixture was kept at 50°C in a water bath for 20 minutes. After cooling, trichloroacetic acid (200 µl at 10% (w/v)) 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 0.1 M ferric chloride solution (40 µl) and incubated for 30 minutes. The absorbance was measured at 700 nm. Ascorbic acid and BHT at various concentrations were used as standards.

2.2.6 Total Phenolic Assay

The phenolic content was determined using the Folin- Ciocalteau method. Both the cocoa kombucha and unfermented cocoa extracts (2.5 mg/ml and 5 mg/ml) were aliquoted (10 µl each) separately into Eppendorf tubes in triplicates. Distilled water (0.79 ml) was added, followed by 50 µl of Folin-Ciocalteau reagent and then mixed thoroughly. The mixture was incubated at room temperature for 8 minutes, after which 2.0 M Na_2CO_3 (150 µl) solution was added, mixed, and incubated at room temperature for 2 hours. The mixtures were aliquoted into a 96-well plate and absorbance measured at 750 nm with a plate reader. The total phenolic content of the extract was extrapolated from a calibration curve of varying concentrations of gallic acid (0-25 µg/ml) as standard.

2.2.7 Statistical Analysis

Statistical analysis was carried out using SPSS 16.0. Data were expressed as means ± SD of triplicate determinations and analyzed using one-way analysis of variance followed by student's t-test within and between extracts. P values of <0.05 were considered as statistically significant.

CHAPTER THREE

3.0 RESULTS

3.1 CELL VIABILITY STUDY

The effect of cocoa kombucha and unfermented cocoa extracts on the percentage cell viability was tested on Jurkat P9 leukemia cells (Fig 3.1). Unfermented cocoa and cocoa kombucha had no cytotoxic effect on Jurkat P9 cells. The anti-cancer standard drug, curcumin caused a concentration-dependent cytotoxicity in the cells with an IC_{50} = 17.42 µg/ml.

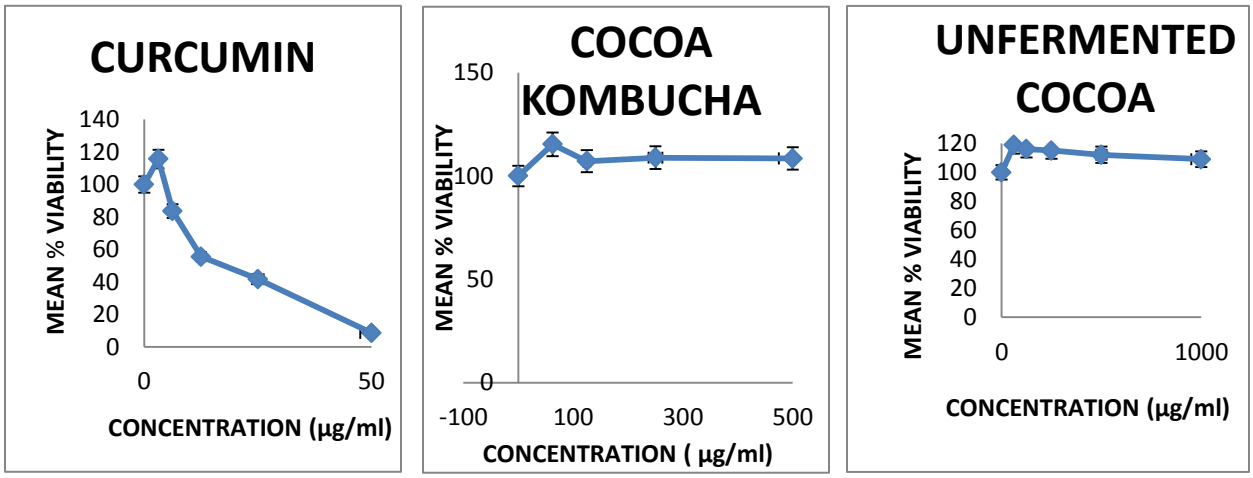


Fig 3.1: Effect of curcumin, cocoa kombucha and unfermented cocoa concentrations on percentage viability of Jurkat P9 cells. Results are means of ± SD of n=3.

3.2 TOTAL PHENOLIC CONTENT

The total phenolic content of cocoa kombucha (CK) and unfermented cocoa (CC) with Gallic acid as standard were determined (Fig. 3.2). Cocoa kombucha had total phenolic content of 3.7 and 5.2 times that of the unfermented cocoa at 2.5 mg/ml and 5.0 mg/ml, respectively. These differences were statistically significant ($p<0.05$).

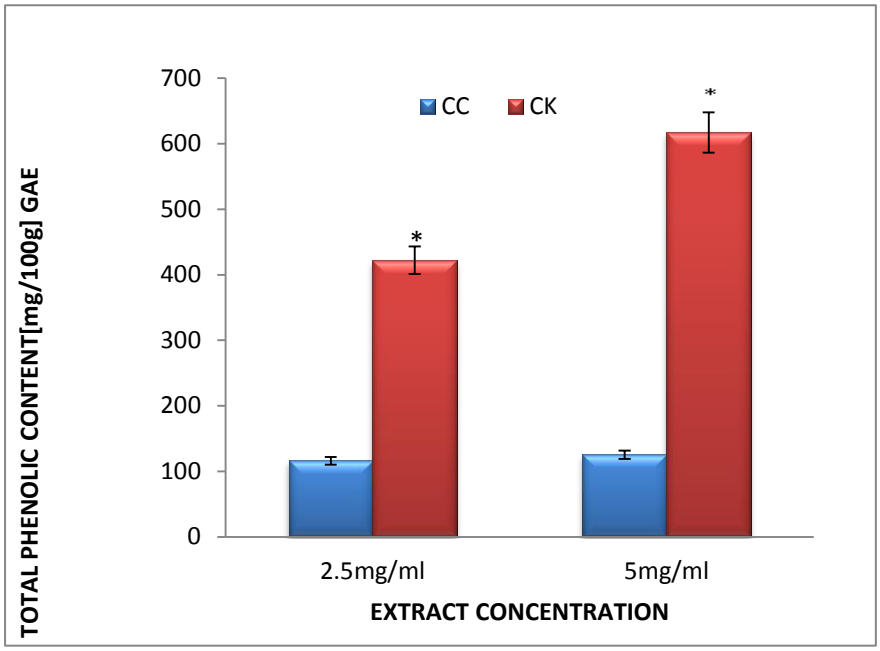


Fig. 3.2: Total phenolic content of unfermented cocoa (CC) and cocoa kombucha (CK) extracts at 2.5 and 5.0 mg/ml. Results are means ± SD of n=3. * Value significantly different from CC; $p<0.05$. GAE= Gallic acid equivalents.

3.3. DPPH SCAVENGING ACTIVITY

The antioxidant activity of cocoa kombucha and the unfermented cocoa was investigated with BHT as standard control (Fig. 3.3). The antioxidant activity of cocoa kombucha was more than twice that of the unfermented cocoa with an EC_{50} of 1.21 mg/ml and 2.43 mg/ml, respectively which represented 3.1 and 6.2 percent of the antioxidant activity, respectively of the standard antioxidant BHT (EC_{50}= 0.0376 mg/ml).

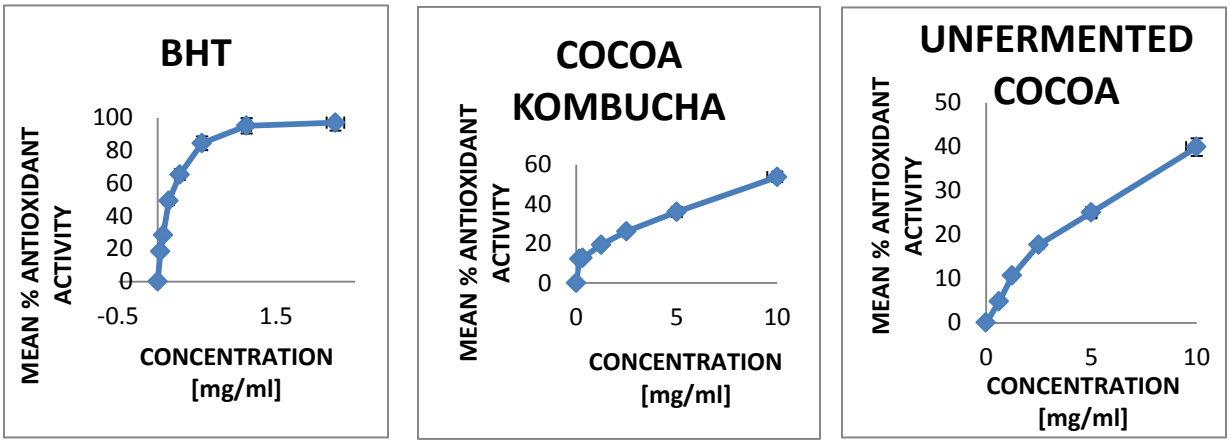


Fig. 3.3: Mean percentage antioxidant activities of BHT, cocoa kombucha and unfermented cocoa at varying concentrations. Results are means ± SD of n=3.

3.4 IRON REDUCING POWER

The iron reducing power of cocoa kombucha and unfermented cocoa compared to BHT and ascorbic acid as standard reducing agents are shown in Fig 3.4. Reducing power of BHT and ascorbic acid increased with increasing concentration reaching a plateau at concentration of 0.75 mg/ml and 0.2 mg/ml, with EC_{50} of 0.11 mg/ml and 0.07 mg/ml respectively. Cocoa kombucha had a steeper slope than that of the unfermented cocoa. The reducing power of cocoa kombucha was, therefore, about 1.5 times that of the unfermented cocoa with $EC_{50} > 40$ mg/ml.

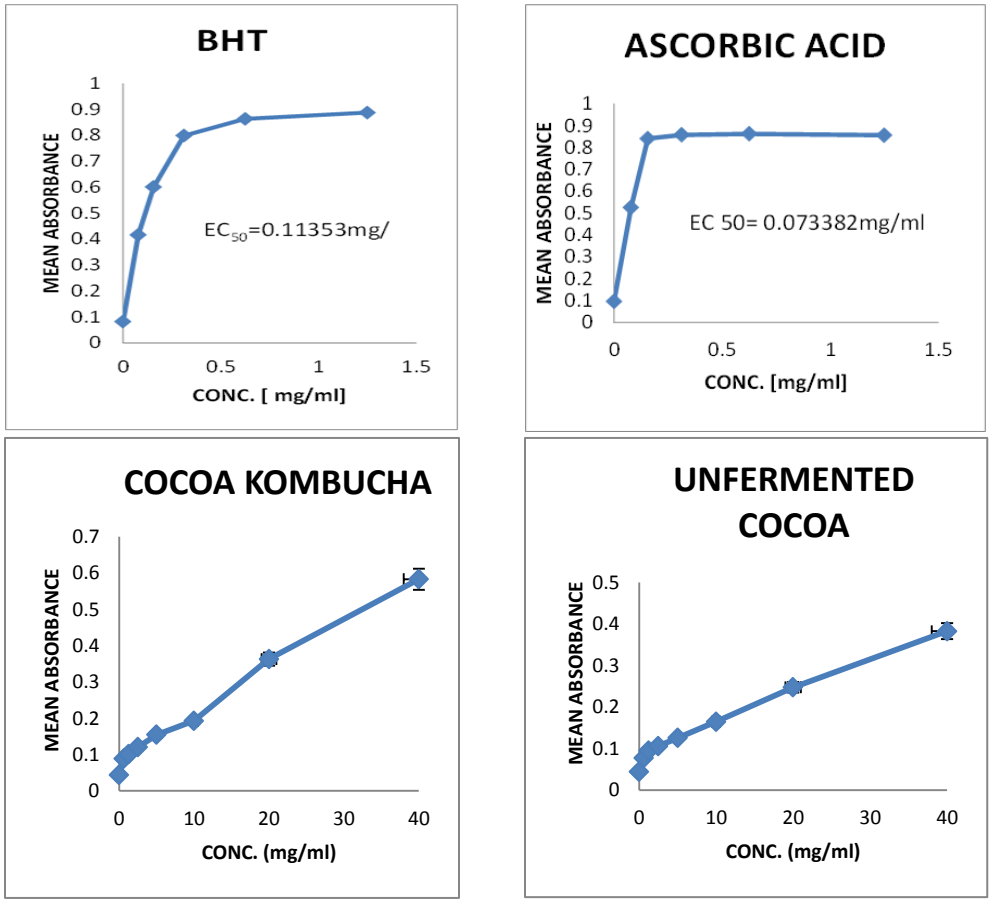


Fig. 3.4: Reducing power of BHT, ascorbic acid, cocoa kombucha and unfermented cocoa at varying concentrations. Results are means ± SD of n=3.

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 DISCUSSION

Cancer is the third leading cause of death worldwide, preceded by cardiovascular and infectious diseases. It is a generic term for a group of more than 100 diseases that can affect any part of the body. Various plant parts are extracted for the treatment of cancers. Although there are many therapeutic strategies including chemotherapy to treat cancer, high systemic toxicity and drug resistance limit the successful outcomes in most cases. Accordingly, several new strategies are being developed to control and treat cancer (Parkin *et al.,* 2005). One such approach is a combination of an effective natural product with chemotherapeutic agents, which when combined, would enhance efficacy while reducing toxicity to normal tissues.

Viability test is used to determine the efficacy of an extract or drug as a cytotoxic agent and its safety for human consumption. MTT assay is used to investigate anticancer properties of compounds. In this study, the cytotoxicity effect of cocoa kombucha (CK) extract on Jurkat P9 leukemia cells was investigated. The effect of CK was compared with both unfermented cocoa extract and the anticancer agent, curcumin. From the results, curcumin exhibited cytotoxicity on the Jurkat P9 cells, while Cocoa kombucha had no cytotoxic effect on the cells because it showed a non-concentration dependent increase in percentage cell viability. A similar observation was made with the unfermented cocoa extract (Fig. 3.1). However, laboratory research agrees with epidemiologic studies that tea can reduce the incidence and the multiplicity of esophageal and gastrointestinal cancers (Gao *et al*., 1994, Weisburger *et al*., 1998). These observations may suggest that changes occurring during fermentation of cocoa

either did not produce cytotoxic constituents or the concentrations produced may be too low to exert cytotoxic effects on Jurkat P9 cells.

Antioxidants are essential for the mopping-up of excess radicals generated during mitochondrial oxidation. They help reduce the risk of exposure to diseases such as cancer and diabetes. Compounds or extracts with good source of antioxidants are potential therapeutic candidates. The antioxidant activity of both cocoa kombucha and unfermented cocoa extracts was investigated and compared with BHT as positive control. Compared with the results of BHT, both cocoa kombucha and the unfermented cocoa had appreciable free radical scavenging abilities (Fig. 3.3). This may be due to the vitamins B and C found in cocoa and its products. This is supported by the observation that cocoa and its products exhibit a wide range of physiological properties including antioxidant activity (Benavente-Garcia *et al*., 1997; Parr and Bolwell, 2000). Furthermore, this result supports the claim that fermented products (such as wine and alcoholic beverages) are a good source of vitamins and minerals, thus good source of antioxidants (Chen and Liu, 2000). The higher antioxidant activity expressed by cocoa kombucha compared with the unfermented cocoa may be due to the production of various constituents including vitamins during the fermentation process. Similar reports suggested that tea kombucha is a good source of antioxidants than the unfermented tea (Conney *et al*., 1999).

To support the antioxidant activity of the cocoa kombucha, total phenolic content was assayed (Fig. 3.2). Cocoa kombucha recorded a significantly higher phenolic content than unfermented cocoa, an indication that more phenolic were reduced during the fermentation process. Chen and Liu (2000) suggested that fermented products exhibit potent biological activities including antioxidant properties than their unfermented counterparts, which appear to support the higher antioxidant activity of cocoa kombucha compared to the unfermented cocoa. Cocoa is known

to be rich in polyphenols (Belscak *et al*., 2009) and it is possible that the concentration of these polyphenols is greatly increased during fermentation. Similar observation has been reported with tea kombucha (Pauline, 2001).

Reducing power is associated with antioxidant activity and may serve as a significant measure of the antioxidant activity (Oktay *et al*., 2003). Compounds with reducing power are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Chanda and Dave, 2009). Presence of reducers causes the conversion of $Fe^{3+/}$ferricyanide complex to the ferrous form. Any compound or extract that is capable of reducing Fe^{3+} to Fe^{2+} in tumor cells can be a potent anticancer or antioxidant agent. Both unfermented cocoa and cocoa kombucha exhibited a concentration-dependent reducing power, and the effective concentrations (EC_{50}) of both extracts were above 40 mg/ml, compared with BHT and ascorbic acid which had EC_{50} of 0.11 mg/ml and 0.07 mg/ml respectively. The use of iron reducers as a possible adjunct to chemotherapy has been studied extensively (Dang, 1999). Cancer cells require much iron (Fe^{3+}) for cellular metabolism and proliferation than required by normal functioning cells. Hence, ability to reduce the bioavailability of these essential ions would deprive the cancer cells of nutrients, thus, inhibiting proliferation. Although this was not the case with the Jurkat P9 leukemia cells, it suggests that the reducing power of the two cocoa extracts was not high enough to prevent the proliferation of the cancer cells.

4.2 CONCLUSION

Cocoa kombucha has higher antioxidant activity, measured by its Fe^{3+} reducing power and radical scavenging activity, than the unfermented cocoa which may be attributed to its higher phenolic content. These degrees of antioxidant activity and iron reducing power offered by CK may not be sufficient to reduce the proliferation of the Jurkat P9 leukemia cells *in vitro* as seen with the standard anticancer drug, curcumin a strong antioxidant.

4.3 RECOMMENDATIONS

- The reducing power of cocoa kombucha should be extensively investigated by varying the concentration and the time of exposure.
- Further studies should be carried out on the potential anticancer property of cocoa kombucha using different cancer cell lines.
- The various phenols present in cocoa kombucha and their antioxidant activities should be investigated.
- Lipid peroxidation activity of cocoa kombucha should be studied.

REFERENCES

Adeyeye, I.E., Akinyeye, O.R., Ogunlade, I., Olaofe, O., Boluwade O. J. (2010). Effect of farm and industrial processing on the amino acid profile of cocoa beans. *Food Chem.* **118**: 357-363.

Aggarwal, S., Takada, Y., Singh, S., Myers, J.N., Aggarwal, B.B. (2004). Inhibition of growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor-kB signaling. *Int J. Cancer*. **111**:679-692.

Balentine, D., Wiserman, A., Bouwens, L. (1997). The chemistry of tea flavonoids. *Sci Nutr*. **137**: 693–704.

Banerjee, D., Hassarajani, S., Maity, B., Narayan, G., Bandyopadhyay, S., Chattopadhyay, S. (2010). Comparative healing property of kombucha tea and black tea against indomethacin-induced gastric ulceration in mice: possible mechanism of action. *Food Funct*. **1**(**3**):284–293.

Barclay, L., Vinqvist, M., Mukai, K., Goto, H., Hashimoto, Y., Tokunaga, A., Uno, H. **(**2000). The antioxidant mechanism of curcumin: classical methods are needed to determine antioxidant mechanism and activity. *Org. Lett*. **2(18)**: 2841-2843.

Belscak, A., Komes, D., Horzic, D., Ganic, K., Damir, K.D. (2009). Comparative study of commercially available cocoa products in terms of their bioactive composition. *Food Research Inter*. **42**: 707-716.

Benavente-Garcia, O., Castillo, J., Ortuno, A., Del Rio, J. (1997). Uses and properties of citrus flavonoids. *J. Agric. Food Chem*. **45**: 4505–4515.

Blanc, PJ. (1996). Characterization of the tea fungus metabolites. *Biotechnol Lett.* **18**: 139–142.

Block, G., Patterson, B., Subar, A. (1992). Fruit, vegetables and cancer prevention: A review of the epidemiologic evidence. *Nutr. Cancer*. **18**: 1–29.

Borchers, A.T., Keen, C.L., Hannum, S.M., Gershwin, M.E. (2000). Cocoa and chocolate: composition, bioavailability, and health implications. *J. Med.Foods*, **3**: 77-103.

Bors, W., Heller, W., Michel, C. (1996). Flavonoids and Polyphenols: chemistry and biology. In Handbook of Antioxidants. **409:** 36-40.

Brand-Williams, W., Cuvelier, M., Berset, C. (1995). *Lebensm. Wiss. Technol.* **28**: 25-30.

Chanda, S., Dave, R. (2009). *In vitro* models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. *African J. Microbiol. Res.* **3(13):** 981-996.

Chen, C., Liu, B. (2000). Changes in major components of tea fungus metabolites during prolonged fermentation. *J. Appl. Microbiol*. **89**:834-839.

Circu, M., Aw, T. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic. Biol. Med.* **48 (6)**: 749–762.

Conney, A., Lu, Y., Lou, R., Xie, J., Huang, M. (1999). Inhibitory effect of green and black tea on tumor growth. Proceedings of the Society for Experimental Biology and Medicine. *Biosci. Biotechnol. Biochem*. **59(9)**: 1609-1612.

Dang, C. (1999). C-myc target genes involved in cell growth, apoptosis, and metabolism. *Mol. Cell. Biol.* **19:**1–11.

Dufresne, C., Farnworth, E. (2000). Tea, kombucha, and health: a review. *Food Res. Int.* **33**: 409-421.

Elattar ,T., Virji, A. (2000). The inhibitory effect of curcumin, genistein, quercetin and cisplatin on the growth of oral cancer cells in-vitro. *Anticancer Res*. **20**:1733-1738.

Ellam, S., Williamson, G. (2013). Cocoa and human health. *Annu. Rev. Nutr*. **33**: 105-128.

Ferreira, I., Baptista, M., Vilas- Boas, L. (2007). Free radical scavenging capacity and reducing power of wild edible mushrooms from north east Portugal. Food Chem. **100**: 1511-1516.

Gao, Y., McLaughlin, J., Blot, W., Ji, B., Dai, Q., Fraumeni, J. (1994). Reduced risk of esophageal cancer associated with green tea consumption. *J. Natl. Cancer Inst.* **86**: 855-858.

Gbewornyo, W.S.K.(2013). Personal Communication.

Gibbs, W. (2003). Untangling the roots of cancer. New evidence challenges old theories of how cancer develops. *Scientific American*. **3**: 57-65.

Guerra, A., Gavala, M., Chung, H. (2007). Nucleotide receptor signalling and the generation of reactive oxygen species. *Purinergic Signal*. **3***:* 39–51.

Hara, Y., Luo, S.J., Wickremashinghe, R.L., Yamanishi, T. (1995a). Botany of tea. *Food Rev. Inter*. **11**: 371-374.

Hara, Y., Luo, S.J., Wickremashinghe, R.L., Yamanishi, T. (1995b). IV. Processing tea. *Food Rev. Inter*. **11**: 409-434.

Hara, Y., Luo, S.J., Wickremashinghe, R.L., Yamanishi, T. (1995c). V. Chemical composition of tea. *Food Rev. Inter*. **11**: 435-456.

Heim, K., Tagliaferro, A., Bobilya, D. (2002). Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. *J. Nutr. Biochem*. **13**: 572–584.

Heinonen, I., Lehtonen, P., Hopia, A. (1998a). Antioxidant activity of berry and fruit wines and liquors. *J. Agric. Food Chem*. **46**: 25-31.

Helbock, H., Beckman, K., Shigenaga, M., Walter, P., Woodall, A., Yeo, H., Ames, B. (1998). DNA oxidation matters: The HPLC-electrochemical detection assay of 8-oxodeoxy-guanosine and 8-oxo-guanine. *Proc. Natl. Acad. Sci*. **95**: 288-293.

Hertog, M., Hollman, P., Katan, M., Kromhout, D. (2000). Intake of potentially anti-carcinogenic flavonoids and their determinants in adults in Netherlands. *Nutr. Cancer*. **20**: 21-29.

Jayabalan, R., Marimuthu, S., Swaminathan, K (2007). Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. *Food Chem*. **102**:392-398.

Jayabalan, R., Malini, K., Athishkumar, M., Swaminathan, K., Yun, S. (2010). Biochemical characteristics of tea fungus produced during kombucha fermentation. *Food Sci. Biotech.* **19:** 843-847.

Jayabalan, R., Malbas, R., Loncar, E., Vitas, J., Sathishkumar, M. (2014). A review on kombucha tea microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. *Comp. Rev. Food Sci*. **13**: 538-550.

Klaunig, J., Kamendilus, L. (2004). The role of stress in carcinogenesis. *Ann. Rev. Pharmacol. Toxicol*. **.44**: 239-267.

Kohen, R., Nyska, A. (2002). Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol. Pathol.* **30**:620-630.

Kurosawa, T., Itoh, F., Nozaki, A., Nakano, Y., Katsuda, S., Osakabe, N., Tubone, H., Kondo, K., Itakura, H. (2005). Suppressive effect of cocoa powder on atherosclerosis in Kurosawa and Kusanagi- hypercholesterolemic rabbits. *J. Atheroscler. Thromb*. **12**: 20-28.

Lau, A., Wang, Y., Chiu, J. (2008). Reactive oxygen species: current knowledge and applications in cancer research and therapeutic. *J. Cell Biochem*.**104 (2):** 657-667.

Lecumberri, E., Mateos, R., Izquierdo-Pulido, M., Ruperez, P., Goya, L., La Bravo, L. (2007). Dietary fibre composition, antioxidant capacity and physico-chemical properties of a fibre-rich product from cocoa (Theobroma cacao L.). *Food Chem*. **104**: 948-954.

Lee, K., Kim, Y., Lee, H., Lee, C. (2003). Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *J. Agric. Food Chem*., **51**: 7292-7295.

Lettieri-Barbato, D., Villano, D., Beheydt, B., Guadagni, F., Trogh, I., Serafini, M. (2012). Effect of ingestion of dark chocolates with similar lipid composition and different

cocoa content on antioxidant and lipid status in healthy humans. *Food Chem*. **132**: 1305-1310.

Leung, A., Foster, S. (1996). Encyclopedia of common natural ingredients used in food, drugs and cosmetics, 2nd ed., John Wiley and Sons, New York, p. 44.

Liu, C., Hsu, W., Lee, F., Liao, C. (1996). The isolation and identification of microbes from a fermented tea beverage, Haipao, and their interactions during Haipao fermentation. *Food Microbiol*. **13**: 407-415.

MacGregor, J., Schlegel, R., Wehr, C., Alperin , P., Ames, B. (1990). Cytogenetic damage induced by folate deficiency in mice is enhanced by caffeine. *Proc. Natl. Acad. Sci.* **87**: 9962-9965.

Mayser, P., Fromme, S., Leitzmann, C., Grunder, K. (1995). The yeast spectrum of "tea fungus Kombucha". *Mycodes.* **38**: 289-295.

Mohandas, K., Desai, D. (1999). Epidemiology of digestive tract cancers in India. V. Large and small bowel. *Indian J. Gastroenterol*. **18**: 118-121.

Neukam, K., Stahl, W., Tronnier, H., Sies, H., Heinric, U. (2007). Consumption of flavonol-rich cocoa acutely increases microcirculation in human skin. *Eur. J. Nutr*. **46**: 53-56.

Newcomb, P., Storer, B., Longnecker, M., Mittendorf, R., Greenberg, E., Clapp, R., Burke, K., Willett, W., MacMahon, B. (1994). Lactation and a reduced risk of premenopausal breast cancer. *N. Engl. J. Med*. **330**: 81-87.

Oda, Y. (1995). Inhibitory effect of curcumin on SOS functions induced by UV irradiation. *Mutat. Res*. **348**:67-73.

Oktay, M., Gulci, I., Kufrevioglu, O. (2003). Determination of *in vitro* antioxidant activity of fennel *(Foeniculum vulgare)* seed extracts. *Lebensum. Wiss. U. Technol*. **3**: 11-14.

Pack, S., Jung, C., Lee, W. (2011). A combination of Green Tea Extract and L- Theanine Improves Memory and Attention in Subjects with Mild Cognitive Impairment: A Double-Blind Placebo-Controlled Study. *J. Med. Food*. **14(4)**: 334-343.

Parkin, D., Bray, F., Ferlay, J., Pisani, P. (2005). Global cancer statistics. *Cancer J. Clin.* **55**: 74-108.

Parkin, D. (2006) .The global health burden of infection-associated cancers in the year 2002. *Int. J. Cancer*. **118**: 3030-3044.

Parr, A., Bolwell, G. (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sc. Food Agric*. **80**: 985-995.

Pauline, T. (2001). Studies on toxicity, anti-stress and hepato-protective properties of kombucha tea. *Biomed. Environ. Sc*. **14**: 207-213.

Perron, A., Patterson, J., Yanofsky, N. (1995). Kombucha `mushroom hepatotoxicity. *Annals Emergency Med*. **26**: 660-661.

Plumb, J.A. (2004). Cell sensitivity assays: the MTT assay. *Methods Mol. Med*. **88**:165-169.

Rams, M., Prasad, D., Kain, A., Mongia, S., Sharma, S., Singh, B. (2000). Effect of kombucha tea on chromate (VI)-induced oxidative stress in albino rats. *J. Ethnopharmacol*. **71**: 235-240.

Sadjadi, J. (1998). Cutaneous anthrax associated with the Kombucha mushroom in Iran (letter). *J. Amer. Med. Assoc*. **280**:1567-1568.

Sievers, M., Lanini, C., Weber, A., Schuler-Schmid, U., Teuber, M. (1995). Microbiology and fermentation balance in kombucha beverage obtained from a tea fungus fermentation. *System App. Microbiol*. **18**: 590-594.

Solà, R., Valls, R., Godàs, G., Perez-Busquets, G., Ribalta, J., Girona, J., Heras, M. Srinivasan, R., Smolinske, S., Greenbaum, D. (1997). Probable gastrointestinal toxicity of kombucha tea. *J. Gen. Intern. Med*. **12**(10): 643-645.

Srini-vasan, R., Smolinske, S., Greenbaum, D. (1997). Probable gastrointestinal toxicity of kombucha tea. *J. Gen. Intern. Med*. **12**: 643-644.

Steinberg, F., Bearden, M., Keen, C. (2003). Cocoa and chocolate flavonoids: Implications for cardiovascular health. *J. Am. Diet. Assoc*. **103**: 215-223.

Tomaru, M., Takano, H., Osakabe, N., Yasuda, A., Inouse, K., Yangisa,W., Ohwatari, T., Uematsu, H. (2007). Dietary supplementation with cacao liquor proanthocyanidins prevents elevation of blood glucose levels in diabetic obese mice. *Nutrition* **23**: 351-355.

Velioglu, Y., Mazza, G., Gao, L., Oomah, B. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J. Agric. Food Chem*. **46**: 4113-4117

Vina, I., Semjonovs, P., Linde, R., Denina, I. (2014). Current evidence on physiological activity and expected health effects of kombucha fermented beverage. *J. Med. Food* **17**(2):179-188.

Vu, T.O., Galet, L., Fages, J. Oulahna, D. (2003). Improving the dispersion kinetics of a cocoa powder by size enlargement. *Powder Technol.* **130(1-3)**: 400-406.

Wang, H., Provan, G., Helliwell, K. (2000). Tea flavonoids, their functions, utilization and analysis. *Food Sci. Tech*. **11**: 152-160.

Weisburger, J., Rivenson, A., Reinhardt, J., Aliaga, C., Braley, J., Pittman, B., Zang, E. (1998). Effect of black tea on azox- ymethane-induced colon cancer. *Carcinogenesis*. **19**: 229-232.

Worsham, M., Raju, U., Lu, M. (2009). Risk factors for breast cancer from benign breast disease in a diverse population. *Breast Cancer Res. Treat*. **118(1)**:1-7.

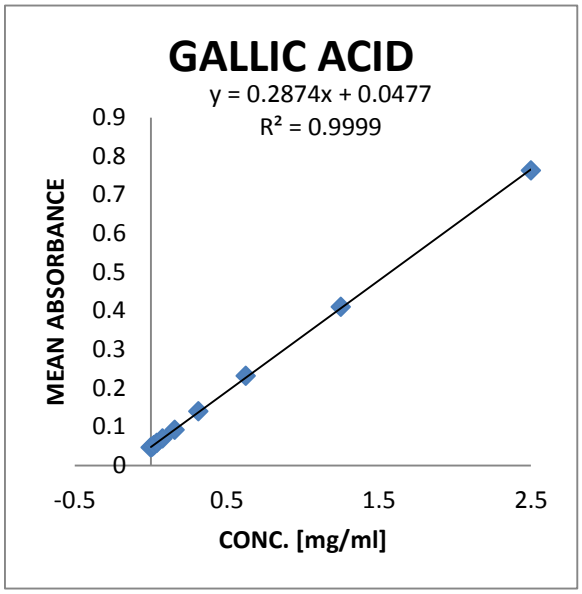
Yurkevich, D., Kutyshenko, V. (1998). Study of glucose utilization during the growth of tea fungus by 1H NMR spectroscopy. *Biofizika*. **43**: 319-322.

Zheng, F., Shi, L., Yang, J., Deng, X., Wu, Y., Yan, X., Huang, N. (2012). Effect of tea polyphenols on the adhesion of highly metastatic human lung carcinoma cell lines to endothelial cells *in vitro*. *Asian Pacific J. Cancer Prev*. **13(8):** 3751-3755.

Ziech, D., Franco, R., Georgkalikas, A., Georgakila, S., Malamou-Mitsi, V., Schoneveld, O. (2010). The role of reactive oxygen species and oxidative stress in environmental carcinogenesis and biomarker development. *Chem. Biol. Interact*. **188**: 334-339.

APPENDICES

APPENDIX I: STANDARD CALIBRATION CURVE OF GALLIC ACID



APPENDIX II: PREPARATION OF REAGENTS

I) Na_2CO_3 SOLUTION

Anhydrous Na_2CO_3 (20 g) was dissolved in 80 ml of distilled water and the mixtures bring to boil. The solution was allowed to cool and a few crystals of Na_2CO_3 were added. Solution was kept at room temperature for 24 hours. After, solution was filtered and 20 ml of distilled water was added to obtain 100 ml Na_2CO_3 solution.

II) GALLIC ACID CALIBRATION STANDARDS

a) Stock (5 mg/ml)

Gallic acid (50 mg) was dissolved in 1 ml of absolute ethanol. The solution was diluted with 9 ml of distilled water and stored at 4^oC until further use.

b) Dilution

Serial dilution was performed using the stock to obtain calibration concentrations of 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml.

APPENDIX III: PLATE SHEET TEMPLATE FOR *IN VITRO* VIABILITY TEST

Sample ID	Initial Conc.	Final Conc	Log Conc.	ABS 1	ABS 2	ABS3	DC	% Viab 1	% Viab 2	% Viab 3	Avrg % Viab	% Inhibitn	STDEV
	0	0	0	1.197961	1.197961	1.197961	0.455419	100	100	100	100	0	0
CUR[uM]	31.25	3.125	0.49485	1.118	1.1072	1.1321	0.2606	115.4682	114.0137	117.367	115.6163	-15.6163	1.677215
	62.5	6.25	0.79588	1.0532	1.0539	1.0767	0.4408	82.47341	82.56768	85.6382	83.55976	16.44024	1.566966
	125	12.5	1.09691	0.9779	0.9727	0.6687	0.4615	69.54486	68.84456	27.90413	55.43118	44.56882	20.87173
	250	25	1.39794	0.5093	0.9111	0.8448	0.4459	8.538233	62.64962	53.72084	41.63623	58.36377	10.54612
	500	50	1.69897	0.5793	0.5646	0.5482	0.5002	10.65259	8.672908	6.464283	8.596594	91.40341	1.253701
	0	0	0	1.268013	1.268013	1.268013	0.455419	100	100	100	100	0	0
CK1[ug/ml]	625	62.5	1.79588	1.5662	1.311	1.294	0.4533	136.9564	105.5508	103.4587	115.322	-15.322	6.332309
	1250	125	2.09691	1.4654	1.2866	1.2565	0.4655	123.0503	101.0467	97.34251	107.1465	-7.1465	4.950533
	2500	250	2.39794	1.4752	1.2759	1.2975	0.4649	124.3301	99.80377	102.4619	108.8653	-8.86528	4.657964
	5000	500	2.69897	1.4413	1.2605	1.3031	0.4534	121.5735	99.32382	104.5663	108.4879	-8.48788	4.597871
	10000	1000	3	1.513	1.533	1.2317	0.4482	131.0371	133.4983	96.41954	120.3183	-20.3183	18.79582
C3	0	0	0	1.268013	1.268013	1.268013	0.455419	100	100	100	100	0	0
	625	62.5	1.79588	1.5022	1.3314	1.3063	0.45	129.4865	108.4674	105.3785	118.9769	-18.9769	14.86274
	1250	125	2.09691	1.5084	1.296	1.2521	0.4584	129.2157	103.0772	97.67478	116.1465	-16.1465	18.4827
	2500	250	2.39794	1.496	1.2955	1.2591	0.4607	127.4067	102.7327	98.25317	115.0697	-15.0697	17.44719
	5000	500	2.69897	1.4323	1.2949	1.5369	0.4521	120.626	103.7171	133.4983	112.1715	-12.1715	11.95633
	10000	1000	3	1.5045	1.2713	1.2609	0.4589	128.6743	99.97605	98.6962	109.1155	-9.1155	5.682273

APPENDIX IV: PLATE SHEET TEMPLATE FOR *IN VITRO* ANTIOXIDANT ACTIVITY

SAMPLE ID[mg/ml]	INI. CONC	FIN, CONC.	LOG CONC.	ABS1	ABS2	ABS3	DC	% AOA 1	% AOA 2	% AOA 3	AVERAGE	STDEV
	0	**0**	0	1.568083	1.568083	1.568083	0.0419	0	0	0	**0**	0
BHT	0.070313	**0.03515625**	-1.45399746	1.261	1.3068	1.2902	0.0422	20.14065	17.1397	18.22739	**18.50258**	1.519283
	0.140625	**0.0703125**	-1.15296746	1.1234	1.1542	1.1335	0.0435	29.24179	27.22369	28.58001	**28.3485**	1.028779
	0.28125	**0.140625**	-0.85193746	0.7511	0.8649	0.8358	0.0416	53.51148	46.05497	47.96169	**49.17605**	3.873742
	0.5625	**0.28125**	-0.55090747	0.5627	0.5807	0.5689	0.0421	65.88877	64.70935	65.48252	**65.36021**	0.599144
	1.125	**0.5625**	-0.24987747	0.2736	0.2853	0.278	0.0411	84.76592	83.9993	84.47762	**84.41428**	0.387213
	2.25	**1.125**	0.051152522	0.1156	0.1188	0.1176	0.0421	95.18406	94.97439	95.05302	**95.07049**	0.105923
	4.5	**2.25**	0.352182518	0.0874	0.088	0.0876	0.0413	96.97939	96.94008	96.96629	**96.96192**	0.020018
	0	**0**	0	1.358467	1.358467	1.358467	0.04075	0	0	0	**0**	0
CC	0.3125	**0.15625**	-0.80617997	1.211	1.2119	1.2273	0.0444	11.46807	11.39977	10.23108	**11.03297**	0.695301
	0.625	**0.3125**	-0.50514998	1.265	1.2675	1.2568	0.0444	7.370073	7.180346	7.992356	**7.514258**	0.424773
	1.25	**0.625**	-0.20411998	1.2862	1.3126	1.2993	0.045	5.806758	3.803295	4.812621	**4.807558**	1.001741
	2.5	**1.25**	0.096910013	1.2165	1.2417	1.2106	0.0466	11.21763	9.305233	11.66538	**10.72942**	1.253531
	5	**2.5**	0.397940009	1.0884	1.1679	1.1525	0.0516	21.31844	15.28528	16.45396	**17.6859**	3.199687
	10	**5**	0.698970004	1.0295	1.0751	1.05	0.0636	26.69896	23.23843	25.14324	**25.02688**	1.733196
	20	**10**	1	0.854	0.9178	0.8787	0.0914	42.12717	37.28545	40.25271	**39.88844**	2.441323
	0	**0**	0	1.358467	1.358467	1.358467	0.04075	0	0	0	**0**	0
CKK	0.3125	**0.15625**	-0.80617997	1.204	1.1953	1.1732	0.0435	11.93099	12.59123	14.26837	**12.26111**	1.204992
	0.625	**0.3125**	-0.50514998	1.2145	1.1874	1.2005	0.0429	11.08863	13.14521	12.15107	**12.64814**	1.028482
	1.25	**0.625**	-0.20411998	1.2533	1.2383	1.2204	0.0434	8.182086	9.320418	10.67883	**9.393777**	1.249986
	2.5	**1.25**	0.096910013	1.128	1.1038	1.0883	0.0444	17.76684	19.60335	20.77963	**19.38327**	1.518404
	5	**2.5**	0.397940009	1.0111	1.0308	1.0171	0.0461	26.76726	25.27225	26.31193	**26.11715**	0.7663
	10	**5**	0.698970004	0.8862	0.9242	0.8869	0.0557	36.97431	34.09054	36.92119	**35.99534**	1.649827

20	**10**	1	0.696	0.6643	0.6696	0.067	52.26592	54.67159	54.26938	**53.73563**	1.288598