EFFECT OF TEA (*Camellia sinensis*) ON TUMOR PROPERTIES AND GENE EXPRESSION PROFILES IN *4TI* METASTATIC BREAST CANCER MODEL

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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DEDICATION

To my dear parents Charles and Mary Mbuthia who set an example of love, discipline and hard work that has stayed with me to this day. To my dear wife Evelyne Kiruri Karori, my cherished son Charles Arthur Karori and my lovely daughters Kita Wangui Karori and Collete Wanjiru Karori-you are such an amazing family.

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ABSTRACT

Polyphenolic fractions in tea are potent bioactive molecules with health benefits including potential anticancer properties. However, the mechanism of action of tea as an anticancer or chemo-preventive agent is poorly understood, with no information on black tea and purple tea. The polyphenolic composition of 25 different types of Kenyan tea was determined using the High Performance Liquid Chromatography (HPLC) and the Folins Ciocalteus methods. Total polyphenols (TP), Total catechins (TC), individual catechins and antioxidant activity (AA) were significantly ($P<0.05$) different among tea varieties, with green tea having the highest levels of TP. *In vitro* bioassay carried out using 2, 2'-diphenyl picryl hydrazyl radical (DPPH) showed that Epigallocatechin gallate (EGCG) was the most potent catechin in antioxidant activity ($r=0.968^{***}$). Black tea contained high levels of theaflavins (TFs) and thearubigins (TRs) (2.072% to 17.12%), respectively which accounted for most of the antioxidant potential in this type of tea product ($r=0.803^{***}$ and $r=0.859^{***}$). These results suggest that conversion of catechins during black tea processing did not affect the free-radical potency of black tea.

Results on Ames test based on the *Salmonella typhimurium* tester strain obtained showed that tea had no toxicity or mutagenic activity at a concentration of 20% (w/v), unlike the mutagen sodium azide. Tea extracts had a significant ($P<0.05$) antimutagenic activity with percent inhibition of 65%, 38% and 19.17% for green, purple and black tea, respectively. Further, *in vitro* assays were carried out on *4TI* cancer cell line to determine the effect of tea on cancer cell growth and proliferation. Results obtained revealed that green tea had the highest inhibition on *4TI* cells proliferation at a concentration of IC_{50} =13.12µg/ml. Further analysis of the *4TI* cancer cell line treated with green, black and purple teas using 454 pyrosequencing generated 425,696 reads with an input mean length of 286.54. Trimmed sequences were imported on a CLC genomic workbench v7.03 and annotated on a reference mouse genome (*Mus musculus* strain *C57BL/6J*). Results revealed a differential expression of apoptosis related genes in the transcriptome. *Casp8, Casp9, Casp3, Casp6, Casp8AP2, Aifm1, Aifm2* and *Apopt1* genes were significantly up regulated indicating the process of apoptosis was initiated and executed. These findings on caspases offer valuable information on the mechanism of tea as an anticancer agent. This demonstration of *4TI* cancer cell growth inhibition induced by tea flavonoids will

[illegible] treatments.

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

AMV-	Avian myeloblastosis virus
ATCC-	American type culture collection
BLAST -	Basic local alignment search tool
DMA-	Davis minimal agar
DPPH -	2, 2-Diphenyl picryl hydrazyl radical
DMRT-	Duncans Multiple Range Test
EGCg-	Epigallocatechingallate
EST-	Expressed sequence tags
FCS-	Fecal calf serum
His^--	Histidine deficient strain of *S. typhimurium*
His^+-	Prototrophic (wild type) strain of *S. typhimurium*
HPLC-	High performance liquid chromatography
IBMK-	Isobutyl methylketone(4-methylpentan-2-one)
ILRI-	International Livestock Research Institute
IREC-	Institutional Research Ethic Committee
KEMRI-	Kenya Medical Research Institute
KTDA-	Kenya Tea Development Agency
MEM-	Minimal essential media
MID-	Mutiplex Identifier
MTT-	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
NCD-	Non communicable diseases
ncRNA-	Non coding messenger ribonucleic acid
NGS-	Next generation sequencer
PBS-	Phosphate buffered saline
SMPs-	Strepatavidin magnetic coated particles
TFs-	Theaflavins
TRFK-	Tea Research Foundation of Kenya
TRs-	Thearubigins

CHAPTER ONE

INTRODUCTION

1.1. Background Information

Tea, from *Camellia sinensis* L.O Kuntze is one of the most widely consumed beverages in the world. Tea was first introduced in Kenya in1904 by the British settlers. Its cultivation has since expanded to cover an area of around 157,720 ha in the highlands East and West of the Great Rift Valley in Kenya (Wachira, 2004). Young leaves of tea are processed into different types of products, the predominant ones being black, green, white and oolong tea. Green tea is mainly consumed in China, Japan and the Middle East, while black tea is mostly consumed in India, Sri-lanka, European countries and regions of Africa. Popularity of tea is due to its aroma, pleasant taste and medicinal benefits (Lin *et al.,* 2003). Tea from Kenya is better perceived since it is grown free of agrochemicals in an ideal environment that naturally deters pests and attack by plant diseases. This pleasant natural condition guarantees the consumer the safest and most refreshing sought after health drink in the world. The tea plant is an evergreen bush that grows to 15m high in the wild, and 60–100 cm under cultivation. Cultivated tea is usually pruned to form a table from which the young leaves are harvested and a cyclic pruning is carried out after every 3 to 4 years. Commercial harvesting is carried out either by hand or machines (Vo, 2006).

Kenya is a leading exporter of black tea in the world market and contributes 26% of the total foreign exchange earnings annually; 4% of the Gross Domestic Product (Wachira and Ronno, 2004). Over 560,000 small holder farmers who own KTDA account for 60% of the tea that Kenya exports annually that is more than 200 million kilograms of made tea. In 2011 Kenya earned Kshs 109 billion from the sale of 377.9 million kilogram of tea, which shot up to Kshs 112.2 billion from the sale of 369.2 million kilogram of tea in 2012 contributing about 22% of the manufactured product (TBK, 2012). The crop offers a direct source of livelihood to about 8% of the total rural population (Mbadi and Owuor, 2008) and contributes to environmental conservation through enhanced water infiltration, reduced surface erosion and mitigation of global warming through carbon sequestration (Cheserek, 2011). Despite this, the share of tea that is consumed locally has continued to decrease (TBK 2012). For example, national per capita consumption in Kenya has

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in 1989 to 14 million kg in 2004 (Wachira and Ronno, 2005). It is estimated that on average each

person in Kenya drinks an average of 400g of tea per year compared to the 1.5 kg, 2kgs and 2.26 kgs of tea consumed during the same period in Pakistan, UK and Ireland, respectively. There is a need to double the figure to 800g per person or 10 percent of the tea produced in the next few years (Anon, 2009). This calls for urgent interventions to diversify black tea markets and more so, create a strong local demand in order to build the potential of increasing local consumption. Additionally, tea prices have continued to dip especially in 2014 and this was occasioned by political instability in key markets of Egypt, Pakistan, Sudan, Afghanistan etc. This demonstrates the weakness of Kenya's marketing strategy which focuses on a few traditional markets and lack of product diversification to capture the emerging markets.

In other countries where tea is produced, the beverage has been widely marketed as a health product. In addition, tea has increasingly been put to other uses other than in food and drinks. Indeed, numerous environmentally friendly industrial cleaning agents, deodorizers and anti-microbial agents have been formulated using tea (Magoma *et al.,* 2001; Wachira, 2004). Data to support the view that tea is pharmacologically active has been generated particularly using green tea, which is widely consumed in Asia (Picard, 1996; Lekh and Okubo, 2004). However, there is dearth of information on the health benefits of black aerated or fermented tea the principle type of tea product consumed in Kenya and much of the world. There is therefore need to conduct systematic research on the pharmacological properties of Kenyan black tea in order to generate requisite data to drive the ''tea and health'' market promotion.

Phytochemicals and functional components in tea are receiving a lot of attention due to their potential benefits in health when consumed as part of a varied diet on a regular basis and at effective levels. Many nutraceuticals, functional foods and naturally occurring polyphenols have physiological and pharmacological activities including their well characterized antioxidant properties (Wachira and Kamunya 2005; Karori *et al.,* 2007). Since the scientific community and food industry communities share a common goal of extending the quality of human life, through development of viable options for the management of chronic diseases through the use of nutraceuticals, functional foods have become a potential start point. This is because functional foods are fairly affordable, readily available and extremely active, have profound effect on cell metabolism and often demonstrate few side effects (Mandel *et al.,* 2005). It is evident that their usefulness to human health.

Tea is rich in polyphenols, associated with numerous pharmacological properties which include such anti-diabetic, antimicrobial (Koech *et al.,* 2013), anti-inflammatory (Karori *et al.,* 2008), anti-aging properties (Khan and Mukhtar, 2007, Mbuthia *et al.,* 2011), antimalarial properties (Sannella *et al.,* 2006) and antioxidant activity (Karori *et al.,* 2007; Kerio *et al.,* 2013, Wachira *et al.,* 2013). Cancer, accelerated aging and other chronic disease associated with free radical induced oxidative damage (Mandel *et al.,* 2005) can be ameliorated by phytochemicals in tea since antioxidants in green tea have been shown to protect DNA from damage induced by free radicals and slow or halt initiation and progression of cancerous tumor growth (Amie *et al.,* 2005). Epicatechingallate (EGCG) a polyphenol in tea, inhibit growth of cancer cells as well as play an important role in stimulating apoptosis or programmed cell death both of which are crucial aspects of cancer prevention (Liu *et al.,* 1998; Hafeez *et al.,* 2006). Biochemical actions of green tea and mainly the catechin EGCG in chemoprevention and anticancer effect have been widely studied (Ahmad *et al.,* 1997, August *et al.,* 1999, Gupta *et al.,* 2001, Hakim *et al.,* 2004, Fujiki *et al.,* 2005 and Chan *et al.,* 2006). Further, there is also increasing evidence that tea provides significant immuno-protective qualities to cancer patients undergoing radiation and chemotherapy (Fujiki *et al.,* 2005). However, mechanisms of action of anti-cancer properties are poorly understood. Additionally, published data have primarily focused on green tea yet black tea is the most widely consumed tea product worldwide.

This study conducted to establish anti-mutagenic properties Kenyan teas using the *Salmonella typhimurium* (TA 1538) AMES test. Further, the study set out to establish possible mechanisms of cancer prevention by Kenyan tea using the *4TI* metastatic breast cancer model. The Gene expression profiles of the *4TI* cancer cell line were analyzed using the 454 pyrosequencer technique since it is a fast, simple, and cost effective method of determining candidate genes involved in major metabolic pathways. The technology also provides a more comprehensive and efficient way to measure transcriptome composition and obtain RNA expression profiles (Shi *et al.,* 2011). In addition, whole-genome sequencing and analysis of cancer and matched normal genomes with next-generation sequencing platforms can illuminate commonly mutated genes and transcript-level events that contribute to the underlying cancer biology.

1.2. Statement of the Problem

Humans are exposed to a wide range of carcinogenic agents which include endogenous and man-made chemicals, radiation, physical agents and viruses. Constant exposure to these carcinogens has led to a significant increase in the cases of cancer and other chronic diseases. World Health Organization (WHO) in 2012 reported that cancer accounted for 7.9 million deaths which is about 13% of all deaths worldwide making cancer a leading cause of death worldwide. The annual mortality rates attributed to main types of cancer includes lung cancer (1.3 million deaths), stomach cancer (803,000 deaths), colorectal cancer (639,000 deaths), liver cancer (610,000 deaths), breast cancer (519,000 deaths) cervical cancer (450,000) and oesophageal cancer (380,000). Statistics in Kenya has shown that about 50 Kenyans die daily from various forms of cancers. However, despite this worrying figures limited advances have been made in managing cancer, due to inadequate resources, infrastructure and trained personnel. Others include advanced stage of majority of cancers at time of presentation and limited access to chemotherapy drugs. The strategies of prevention and intervention vary, but use of natural and synthetic agents to prevent progression of premalignant lesions to invasive cancers is a feasible way. An ideal chemo-preventive agent should be non-toxic, easily available, economical, and should be aimed at multiple targets. Diet and lifestyle have been demonstrated to be critical interventions that can be successfully applied to minimize the likelihood of development of cancer. Indeed, dietary interventions can be used to control this chronic disease, either in the general population or in the susceptible subpopulations. The evaluation of mechanisms involved in cancer may reveal targets amenable to tea nutraceuticals based prevention strategies since tea is cheap, readily available and non-toxic.

1.3.1. Main objective

To determine the effect exerted by different types of Kenyan teas in ameliorating cancer and postulate possible a molecular mechanism of inhibition of cancer by tea.

1.3.2. Specific objectives

1. To characterize green, purple and black teas processed from Kenyan tea cultivars for their total polyphenols, total catechins, catechin fractions, anthocyanin profiles, theaflavins and thearubigins content.
2. To determine the antioxidant activity of green, purple and black teas processed from Kenyan tea cultivars using 2, 2'-diphenyl picryl hydrazyl radical (DPPH).
3. To determine the antimutagenic activity of selected Kenyan green, purple and black teas using the *Salmonella typhimurium* (TA 1538) Ames test.
4. To determine the effect of selected Kenyan teas on growth and proliferation of the *4TI* metastatic breast cancer model.
5. To determine the gene expression profiles in *4TI* metastatic breast cancer model treated with tea using 454 next generation pryosequencer.
6. To determine the underlying molecular mechanism responsible for teas anticancer effect.

1.4 Hypotheses

1. There are no significant differences in the polyphenolic composition green, purple and black teas processed from Kenyan tea cultivars.
2. Green, purple and black teas processed from Kenyan tea cultivars are not significantly different in their total antioxidant activity.
3. Kenyan teas do not have any antimutagenic activity as measured using the *Salmonella typhimurium* (TA 1538) designed for Ames test and are not significantly different in their antimutagenic activity.
4. Kenyan teas do not inhibit growth and proliferation in the *4TI* metastatic breast cancer model.

are not different.

6. There are no underlying mechanism responsible for teas anticancer effect.

1.5 Justification

Cancer is a growing problem in most parts of the world and is particularly increasing in prevalence in Africa. Potential role of tea in protection against cancer has been supported by evidence from studies in cell culture and animal studies. However, some epidemiological studies have also generated inconsistent results, with some associating tea with reduced risk of cancer, and others indicating that tea lacks protective activity against certain cancers. The inconsistency is largely ascribed to the chemical composition of tea samples used and therefore raises questions about the actual role of tea in cancer prevention. The problem is aggravated further by the fact that many studies have only been carried out using green tea, with very few studies or none conducted using black and white tea, yet black tea is the principle tea product consumed the world over. In addition, purple tea which is new in the market has received a lot of attention due to its unique biochemical make up, but data on its health associated properties is scanty. This study therefore investigated whether Kenyan black, purple and green tea can ameliorate cancer and also provide a better understanding on the underlying molecular mechanisms effected by the beverage.

CHAPTER TWO
LITERATURE REVIEW

2.1 Tea Plant

The tea plant was first described taxonomically in 1753 by Linnaeus in *species Plantarum*. Linnaeus referred to the tea plant as *Thea* but later refined the species into black tea (*Thea bohea*) and green tea (*Thea viridis*). By the early 1900s, taxonomists recognized that green and black tea were both from the same species, *Camellia sinensis* (L.) O. Kuntze which is an evergreen tree or shrub that has yellow-white flowers and long, serrated leaves (Banerjee, 1991; Banerjee 1992; Selena and Steep, 2012). The cultivated plant species *Camellia sinensis* ((L.) O. Kuntze) is the source of the raw material from which the popular tea beverage is processed. Although the crop is cultivated in many countries, there are several different types of tea plant, each with its own identifiable character and potential for unique cup quality. Green and black teas are the most widely consumed the world all over although production has diversified to other specialty types of teas. Green tea is mainly consumed in China, Japan and the Middle East while black tea mostly consumed in India, Sri-lanka, European countries, Kenya and other regions of Africa. The teas are prepared from the leaf of *Camellia sinensis* var. *assamica* (Assam variety), var. *sinensis* (China variety) or var. *cambodiensis* (Cambod variety). Tea plants belonging to the var. *sinensis* are characterized by a bush type with small leaves, resistance to cold, are largely used for making green and semi-fermented tea while var. *assamica* are tall trees with large leaves, less resistance to cold, and are suitable for making black tea (Wilson and Clifford, 1992; Takeda, 2004; Wachira *et al.*, 2012).

2.2 Tea Production in Kenya

The species is now cultivated commercially in Asia, Africa and South America. Major producers of the crop include China, India, Kenya, Sri Lanka and Indonesia (Table 1). Kenya is currently the largest single exporter of tea (Table 2). Being the largest export commodity from Kenya, tea is also a major foreign exchange earner for the country. The tea crop is widely grown in the highlands East and West of the Great Rift Valley in Kenya (Anon, 2000) as shown in the figure 1. In 2003, about 270,000 tonnes of processed tea was exported, earning the country about US$435 million. It is projected by the Food and Agriculture Organization (FAO) that the black tea export share of the global tea export (FAO, 2012). Annually, tea contributes about 26% of the total export

earnings and 4% of the gross domestic product (Economic survey, 2004; Gesimba *et al.,* 2005). However, a review of unit prices fetched by Kenya's tea at the local auction center in Mombasa reveals that this has declined over the last decade contributing to decreased returns for tea farmers (Wachira *et al.,* 2004; Wachira and Ronno, 2005; FAO, 2012). To respond to the changing circumstances in the tea business, research interventions have therefore continued to be formulated with the aim of developing technologies for cost effective, efficient and sustainable tea production in Kenya. The current tea research and development activities in Kenya include the following broad objectives of germplasm improvement; development of appropriate tea processing methods; pest and disease management; plant environment and ecosystem management; product diversification and value addition (Wachira *et al.,* 2004).

Table 1: World production of tea (Metric tons) and percent share

Country	2006		2007		2008		2009	
	Production	% Volume	Productio n	% Volume	Productio n	% Volume	Productio n	% Volume
China	1,028,064	28.7	1,140,000	30.0	1,200,000	32.5	1,358,642	34.5
India	981,805	27.4	944,678	25.9	980, 818	25.4	978,999	24.9
Kenya	310,578	8.7	369,606	9.7	345,817	8.9	314,198	8.0
Sri lanka	310,822	8.7	304,613	8.0	318,697	8.2	289,774	7.4
Indonesia	146,847	4.1	137,248	3.6	137,499	3.6	136,481	3.5
Others-Africa	172,052	4.8	189,845	5.1	172,022	4.5	201,767	5.1
Others	629,481	17.6	664,904	17.7	649,337	16.9	656,235	16.6
World Totals	3,579,649	100	3,750,894	100	3, 804, 190	100.00	3, 936, 096	100.00

Table 2: World export of tea (Metric tons) and percent share

Country	2006		2007		2008		2009	
	Production.	% Volume	Production	% Volume	Production	% Volume	Production	% Volume
China	1,028,064	28.7	1,140,000	30.0	296,935	18.1	302, 949	19.2
India	981,805	27.4	944,678	25.9	193,000	11.8	193,000	12.2
Kenya	310,578	8.7	369,606	9.7	383,444	23.4	342,482	21.7
Sri lanka	310,822	8.7	304,613	8.0	297,469	18.2	279, 839	17.7
Indonesia	146,847	4.1	137,248	3.6	96,210	5.9	92,304	5.9
Others-	172,052	4.8	189,845	5.1	144,317	8.8	162,886	10.3
Totals								

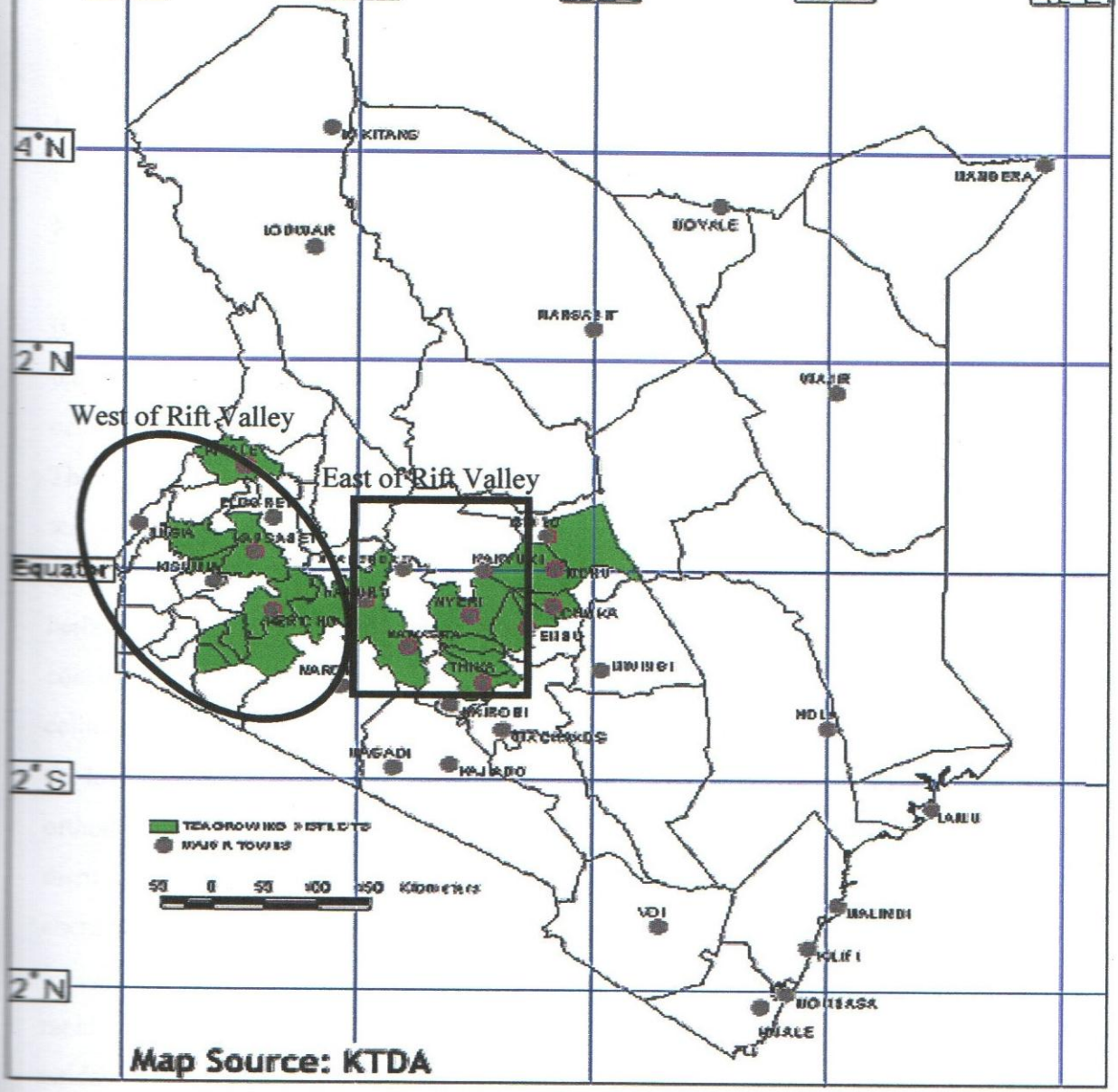

Figure 1: Tea growing areas, West and East of the Great Rift Valley in Kenya [Map Source: Kenya Tea Development Agency (KTDA)-2009]

Through this research interventions, remarkable achievements on tea research have been made, for example the Tea Research Foundation of Kenya (TRFK) has developed over 1,000 improved clones, out of which 50 have been selected for consistent superiority in yield and quality

Fourteen of these clones are capable of yielding between 5,000kg and 8,000kg of made tea per

hectare per year. These yield levels are some of the highest in the world and are in the magnitude of three times the average yields of unimproved tea (Wachira, 2004; Wachira and Kamunya, 2004; Wachira and Ronno, 2004; Gesimba *et al.,* 2005; Wachira and Ronno, 2005). Despite achieving the mentioned commendable increases in tea production, there are numerous challenges that continue to beset the tea industry in Kenya, the major one being the shrinking market outlets both locally and internationally.

Tea beverage is processed from the young tender leaves of the plant *Camellia sinensis* (Cabrera *et al.,* 2003). Two types of tea products are most widely consumed namely; green and black tea. In both cases, it is the chemical composition of the tea shoots and the reactions that occur during processing that determine the nature of the finished product and its quality (Figure 2). Though most of the tea produced in the world can be classified as non-fermented/un-aerated green tea, semi-fermented (oolong) tea and fermented black tea (Reeves *et al.,* 1987), processing has diversified to the production of specialty teas such as white, flavored, organic, decaffeinated, herbal and scented teas. The processing techniques of the above types of tea products vary considerably and have a pronounced impact on the formative and degradative patterns of various cellular and biochemical components. The conventional orthodox method which consists of rolling the leaf on a rolling bed, stretching and tearing the leaf has in some cases been replaced with non-orthodox methods or curl, tear and crush (CTC) which have a quicker and more severe leaf disruption leading to production of smaller fragments and consequently more oxidation of the leaf chemicals (Mahanta and Hemanta, 1992; Wilson and Clifford, 1992).

In the preparation of green tea, the withered leaves are steamed and then dried relatively rapidly after plucking to minimize chemical and enzymatic reactions. This stops the polyphenol oxidase [PPO] enzyme [EC 1.10.31] catalyzed oxidation of tea leaf catechins (Wilson and Clifford, 1992). In contrast, during black tea processing, tea shoots are macerated to initiate oxidation by polyphenol oxidase (PPO) before firing (Barnajee, 1991). This reaction enables the catechins to condense with the orthoquinones arising from the oxidation of ring B di-hydroxylated and trihydroxylated catechins to form theaflavins (TFs). TFs are homogenous substances, which give a yellow red coloration in fermented black tea and contribute to the briskness and brightness of tea liquor (Obanda *et al.,* 2001). TFs act as oxidizing agents for substrates like gallic acid to

[redacted]

substances called thearubigins (TRs) that are responsible for the color, body and taste of tea.