

**EFFECT OF TEA (*Camellia sinensis*) ON TUMOR PROPERTIES AND GENE
EXPRESSION PROFILES IN 4T1 METASTATIC BREAST CANCER MODEL**

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**A Thesis Submitted to the Graduate School in Partial Fulfillment for the Requirements of
the Degree of Doctor of Philosophy in Biochemistry of Egerton University**

EGERTON UNIVERSITY



March, 2015



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.

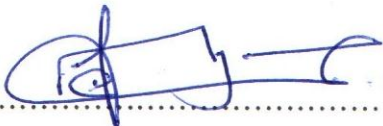
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DEDICATION

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.

ACKNOWLEDGEMENT

I would like to thank-the Almighty God for seeing me through the challenging times during this study. I wish to express my sincere appreciation to the following institutions for providing support in the course of my study: Egerton University (EU) for granting me an opportunity to undertake a doctorate study, the Director and Board of Directors and the entire team at Tea Research Foundation of Kenya (TRFK) for the financial support and allowing me to use their laboratory facilities, Tea Board of Kenya (TBK) for approving this project under the Tea and Health Theme, Kenya Tea Development Agency (KTDA) factories for providing tea samples for analysis, DAAD for financial support, Kenya Medical Research Institute (KEMRI) for providing facilities for cell culture assays and International Livestock Research Institute (ILRI-BeCA) for providing a platform for molecular assays and sequencing. I am greatly indebted to my supervisors, Prof. Francis N. Wachira, Prof. Raphael M. Ngure and Dr. Paul O. Mireji for their restless efforts, invaluable advice, guidance, criticism and patient supervision during the carrying out of this study and the preparation of this thesis. Prof. Wachira, I will remember the journey we have subjugated together in search of knowledge. I also extend thanks to the following for their unflagging support during the project; Dr. John Wanyoko of TRFK for assistance in sample collection and HPLC analysis, Dr. Sabina Wangui and Dr. Peter Kirira of KEMRI-Centre for Traditional Medicine and Drug Research (CTMDR) for the support during the importation of cell lines, Mr. Julius Muchiri for his effort in ensuring that I learnt and applied cell culture techniques during my studies. I wish to acknowledge the ILRI team led by Dr. Francesca Stomeo and Martina Kyalo for supervising me through molecular assays. Dr. Chalo Muoki, you have been very helpful during my research days. I owe a lot of gratitude to my lovely wife Eve and adorable kids Kita and Charles. Thanks for putting up with me guys and making me laugh my head off so often even when science took the better part of my conversation. And most importantly, I'd like to thank my Mum and Dad. Thank you for your love and support and for giving me so much over the years both emotionally and financially. I know you many times wondered if I'd ever finish; I owe you so much. I also extend my gratefulness to my reliable friends Moses Hardware Karuga, Anthony Junta Kariuki. Thanks for cheering me up even on the blackest of days. Jim, I will cherish our "collaboration" and the lonely *yoioto* nights.

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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 Folin 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 $IC_{50} = 13.12 \mu\text{g/ml}$. 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

TABLE OF CONTENTS

DECLARATION AND RECOMMENDATION	ii
DEDICATION	iii
ACKNOWLEDGEMENT	iv
COPYRIGHT.....	v
ABSTRACT	vi
TABLE OF CONTENTS	vii
LIST OF APPENDICES.....	x
LIST OF TABLES.....	xi
LIST OF FIGURES.....	xii
LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS	xiv
CHAPTER ONE.....	1
INTRODUCTION	1
1.1. Background Information.....	1
1.2. Statement of the Problem	4
1.3. Objectives	5
1.3.1. Main objective	5
1.3.2. Specific objectives.....	5
1.4 Hypotheses.....	5
1.5 Justification.....	6
CHAPTER TWO.....	7
LITERATURE REVIEW	7
2.1 Tea Plant.....	7
2.2 Tea Production in Kenya	7
2.3 Polyphenol Composition and Antioxidant Properties of Tea.....	12
2.5 Cancer Disease	17
2.6 Global Burden of Cancer.....	20
2.7 Cancer Situation in Kenya.....	21
2.8 The Role of Tea in Cancer Inhibition.....	22
2.9 Detection of Antimutagenicity by the Ames Test	24
2.10 Anti-mutagenic and Anti-carcinogenic Properties of Tea.....	27
2.13 Transcriptome Analysis of Genes.....	29
2.14 Candidate Gene Approaches by Northern Blot.....	30
2.15 Microarray Technology	30
2.16 Gene Profiling by Subtractive Hybridization	31
2.17 Sequence-Based Approaches to Studying Transcriptomes	31
2.18 Sanger Sequencing Technology	32
2.19 Expressed Sequence Tag	32
2.20 New-Generation Sequencing Methods.....	34
3.0 Cell line.....	41

CHAPTER THREE	42
MATERIALS AND METHODS	42
3.1 Tea Products and Determination of Flavonoid Levels.....	42
3.1.1 Tea samples.....	42
3.1.2 Processing of the tea leaves	42
3.1.3 Treatment of tea sample for polyphenol and catechin analysis	42
3.1.4 Determination of catechins in tea extracts	43
3.1.5 Determination of total polyphenols in the tea extracts	44
3.1.6 Anthocyanin extraction and purification.....	46
3.1.7 Determination of total monomeric anthocyanin content.....	46
3.1.8 Fractionation of anthocyanins by HPLC.....	47
3.1.9 Determination of antioxidant activity of tea	47
3.1.10 Analysis of the content of total theaflavins and individual theaflavin fractions content in the tea samples	48
3.1.11 Spectrophotometric determination of total thearubigins in the tea samples	49
3.2 Antimutagenicity Assays	49
3.2.1 Cell line	49
3.2.2 Preparation of tea extracts.....	50
3.2.3 Preparation of culture medium.....	50
3.2.4 Mutagenicity assay.....	50
3.2.5 Antimutagenicity assays	51
3.2.6 Statistical analysis	51
3.3 <i>4TI</i> Cell Culture	52
3.3.1 Study site.....	52
3.3.2 Cell culture.....	52
3.3.3 Measurement of cancer cell survival and growth	52
3.3.3.1 Trypan blue assay.....	52
3.3.3.2 Cell growth and proliferation assay.	53
3.3.4 Statistical analysis	54
3.3.5 Cell harvesting for mRNA isolation	55
3.4 Extraction and Quantification of mRNA	55
3.5 Preparation of cDNA Library for Transcriptome Sequencing.....	56
3.6 Emulsion PCR Amplification	57
3.7 454 Sequencing.....	58
3.8 Sequence Analysis	58
3.8.1 Quality control and <i>de novo</i> assembly of sequencing reads	58
CHAPTER FOUR	60
RESULTS AND DISCUSSION	60
4.1 Chromatographic and Spectrophotometric Analysis of Black, Green and White Tea	60
4.1.1 Tea polyphenols	60
4.1.2 Total catechin content	62
4.1.3 Catechin fractions	67
4.1.4 Gallic acid	72
4.1.6 Theaflavin fractions	77
4.1.6 Antioxidant activity of green, black, purple and white tea products	78

4.1.7 Caffeine.....	83
4.2 Antimutagenicity of tea.....	85
4.3 <i>4TI</i> Cell Culture Assay.....	94
4.4 Molecular Analysis	101
4.4.1 Quantification of extracted mRNAs	101
4.4.2 Assessment of mRNA quality.....	102
4.4.3 cDNA synthesis.....	102
4.4.4 <i>De novo</i> sequencing of the <i>4TI</i> coding transcriptome.....	105
CHAPTER FIVE.....	111
CONCLUSION AND RECOMMENDATIONS	111
5.1 Conclusion	111
5.2 Recommendations	112
6.0 REFERENCES.....	113

LIST OF APPENDICES

APPENDIX 1: KEMRI Ethical Review Committee.....	136
APPENDIX 2: NIH CERTIFICATE OF COMPLETION.....	137
APPENDIX 3: ILRI IREC.....	138
APPENDIX 4a: ANOVA from the Thaflavin fraction assay	139
APPENDIX 4: ANOVA from the Thaflavin fraction assay	140
APPENDIX 5: ANOVA from the Antimutagenicity Assay	141
APPENDIX 6: ANOVA FROM THE CYTOTOXICITY ASSAY	142
APPENDIX 7: REPRESENTATIVE CURVE OF FROM A NANODROP ASSAY	143
APPENDIX 8: READS DISTRIBUTION GENERATED AFTER TRIMMING OF THE 454 PYROSEQUENCE SEQUENCES.....	144
APPENDIX 9: A SNAPSHOT OF THE TRANSCRIPTOME ANALYSIS INDICATING SOME OF THE GENES AFFECTED BY GREEN, BLACK AND PURPLE TEAS.	145

LIST OF TABLES

Table 1: World production of tea (Metric tons) and percent share	8
Table 2: World export of tea (Metric tons) and percent share.....	8
Table 3: List of <i>Salmonella typhimurium</i> strains used in Ames test	26
Table 4: Criteria of evaluation as the inhibition of mutagenicity.....	52
Table 5: Total polyphenols (TP) (%) levels of different tea products used in this study	62
Table 6: Total catechins (TC) (%) levels of different tea products analyzed.....	64
Table 7: Individual anthocyanidins and their retention times.	66
Table 8: Mean of anthocyanin profile from the selected clone 306/1	67
Table 9: Retention times for individual catechins	68
Table 10: Individual catechin (%) levels of different green tea products analyzed	70
Table 11: Individual catechin (%) levels of different black tea products analyzed	71
Table 12: Gallic acid (GA) (%) levels of different tea products analyzed	73
Table 13: Total theaflavins and total thearubigins (%) levels of black, green, purple and white tea products processed from different cultivars	76
Table 14: Correlation coefficient matrix analyses between various theaflavin fractions.....	78
Table 15: Percent antioxidant capacity (AA) of green, black and white tea products analyzed	79
Table 16: Correlation coefficient matrix analyses between various tea chemical parameters.....	82
Table 17: Caffeine levels of different tea products analyzed	85
Table 18: Inhibitory effect of water extracts of green, TRFK-360 purple and black teas on the mutagenicity of sodium azide towards <i>Salmonella typhimurium</i>	89
Table 19: Cytotoxicity of various tea extract	95
Table 21: Reads Distribution Statistics	107
Table 22: Transcriptome analysis of genes affected by green, black and purples teas.	109

LIST OF FIGURES

Figure 1: Tea growing areas, West and East of the Great Rift Valley in Kenya [Map Source: Kenya Tea Development Agency (KTDA)-2009].....	9
Figure 2: Schematic diagram of the conventional manufacturing process of green, black, oolong and white tea.	11
Figure 3: Structures of green tea catechin derivatives (1-6) and structures of formal dimers of catechins found in black tea (7-10).....	14
Figure 4. Schematic representation of the main molecular pathways leading to apoptosis.	20
Figure 5: An overview of how ESTs are constructed.....	33
Figure 6: Diagram showing pyrosequencing process.....	36
Figure 7: a). Illumina cluster generation, b) Extension and c) Illumina reading.....	38
Figure 8: a) Beads on a slide, b) Components of SOLiD, c) Methods of SOLiD and d) Illustration of SOLiD mechanism.	40
Figure 9: A representative plate used in the MTT cell proliferation assay	54
Figure 10: A representative HPLC chromatogram of processed black tea sample from purple colored cultivar TRFK 306/1	65
Figure 11: A representative high performance liquid chromatogram of green tea cultivar	68
Figure 12: Molecular structure of theaflavins. theaflavin (TF): R' = R'' = OH, theaflavin-3- gallate (TF-3-G): R' = H, R'' = galloyl; theaflavin-3'-gallate (TF-3'-G): R' galloyl, R'' H; theaflavin-3, 3'-digallate (TF-dG): R' = R'' = galloyl.....	77
Figure 13: Representative of Ames spot test showing no spontaneous His ⁺ revertants where tea was added in the absence of sodium azide.	86
Figure 14: Representative of Ames spot test showing numerous spontaneous His ⁺ revertants where tea was added in the absence of sodium azide.	87
Figure 15: Representative of Ames spot test showing spontaneous His ⁺ revertants in the presence of sodium azide. The mean number of revertants observed from the open CFU was 88.67 colonies.....	88
Figure 16: Representative of Ames spot test showing reduced spontaneous His ⁺ revertants where green tea was added in the presence of sodium azide. The mean number of revertants observed from the open CFU was 30.67 colonies.....	88
Figure 17: Representative of Ames spot test showing reduced spontaneous His ⁺ revertants where purple tea was added in the presence of sodium azide. The mean number of revertants observed from the open CFU was 54.33 colonies.	89
Figure 18: Representative of Ames spot test showing reduced spontaneous His ⁺ revertants where black tea was added in the presence of sodium azide. The mean number of revertants observed from the open CFU was 71.67 colonies.....	90
Figure 19: Quantification of the inhibitory concentration (IC ₅₀ µg/ml) of purple, black and green teas on 4T1 cancer cells.....	94
Figure 20: A monolayer of 4T1 control cells after 48 hours in DMEM media.....	95

Figure 21: Sensitivity of <i>4TI</i> cancer cells to black tea at IC ₅₀ of 44.04µg/ml showing disruption of monolayer.	96
Figure 22: Sensitivity of <i>4TI</i> cancer cells to purple tea at IC ₅₀ of 29.27µg/ml showing disruption of monolayer.	97
Figure 23: Sensitivity of <i>4TI</i> cancer cells to green tea at IC ₅₀ of 13.12µg/ml showing disruption of monolayer.	97
Figure 24: Gel electrophoresis image of mRNA from cells treated with the representative tea samples.	102
Figure 25: Representative images indicating the size distribution and quality of cDNA library assessed on an Agilent bioanalyzer high sensitivity DNA chip. The cDNA produced a peak between 600 and 1200bp as shown by the arrow.	104
Figure 26: Gel-like image of the cDNA library samples as run on an Agilent bioanalyzer high sensitivity DNA chip. The samples produced a smear that ranged from 0.2kb to 7kb as shown in the arrow.	105
Figure 27: Box plot showing quality scores of trimmed sequences.	106

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 <i>S. typhimurium</i>
His ⁺ -	Prototrophic (wild type) strain of <i>S. typhimurium</i>
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-	Multiplex 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-	Streptavidin 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 in 1904 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

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 (Mandal *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, Mbutia *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 chemopreventive 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 pyrosequencer.
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

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	Production	% Volume	Production	% Volume	Production	% 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	3,579,649	100	3,750,894	100	1,857,655	100.00	1,574,428	100.00

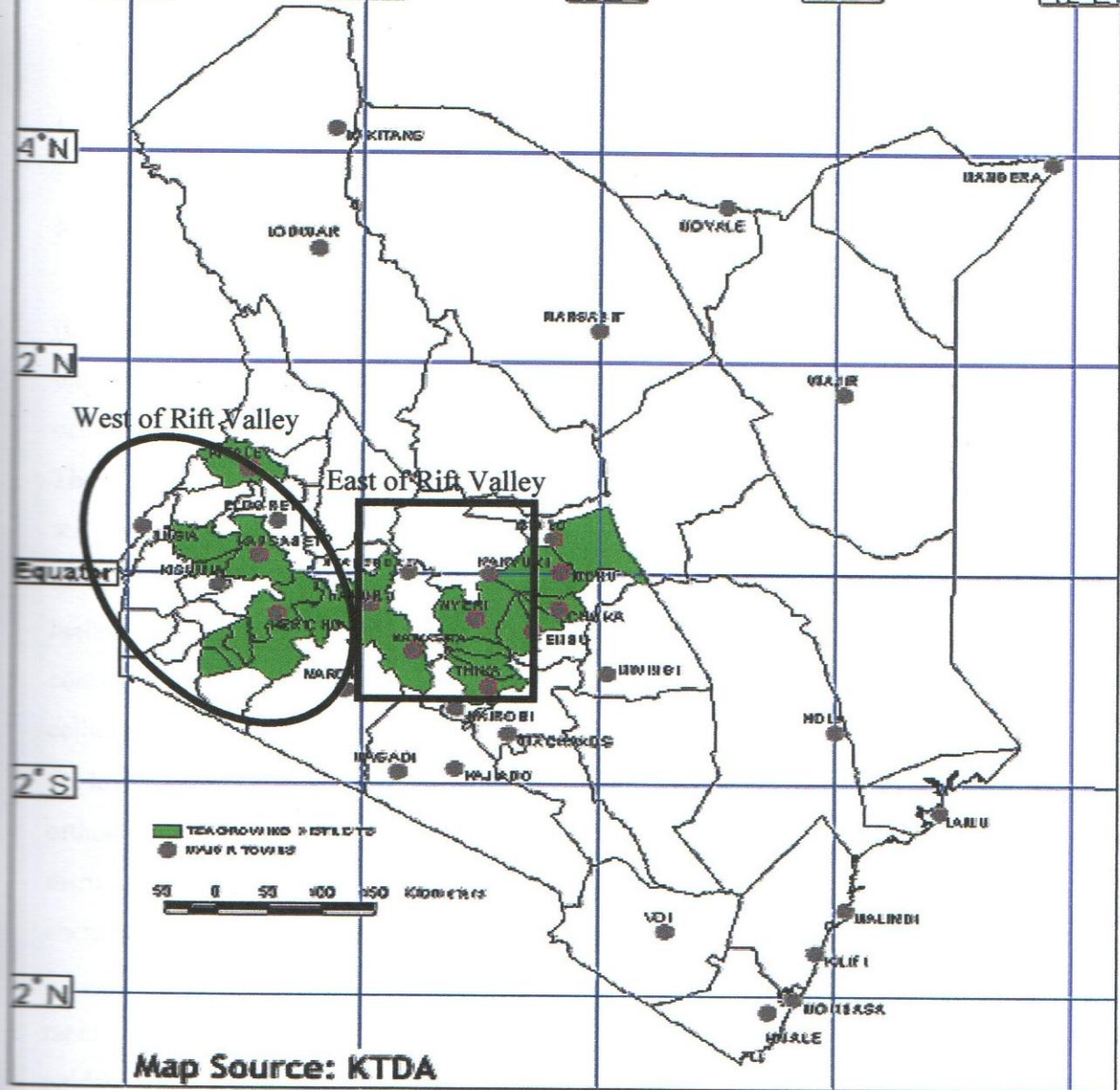


Figure 1: Tea growing areas, West and East of the Great Rift Valley in Kenya [Map Source: Kenya Tea Development Agency (KTDA)-2009]

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