TEA FLAVONOIDS AND THEIR EFFECT ON CHRONIC INFLAMMATION INDUCED IN *Trypanosoma brucei brucei* INFECTED MICE

By

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DECLARATION AND RECOMMENDATION

DECLARATION

I declare that this thesis is my original work and it has not been previously presented for the award of a degree in this or any other University.

Sign:…… Date: …….

Stephen Karori Mbuthia.

RECOMMENDATION

This thesis has been submitted for examination with our approval as University supervisors.

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DEDICATION

This work is dedicated 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.

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ABSTRACT

Emerging scientific data from pharmacological and physiological studies continue to show that tea has beneficial effects on human health by boosting immunity. *In vitro* studies have shown that flavonoids help immune response by acting as anti-cancer, anti-viral and anti-bacterial agents. In this study, different types of commercial tea samples were assayed for their phenolic composition, antioxidant activity and their effect on chronic inflammation induced by *Trypanosoma brucei brucei* in mice. High performance liquid chromatography was used for the identification and quantification of catechins. Subsequently, total phenolic content was determined spectrophotometrically using Folins-ciocalteus method. Total theaflavins and thearubigins were also determined. The radical scavenging behavior of the polyphenols on 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) was also studied spectrophotometrically. Total polyphenols, total catechins and antioxidant activity were significantly ($P<0.05$) different in the tea samples. Green tea had the highest levels of catechins, total polyphenols and total antioxidant activity. White tea was not significantly different from green tea. Epigallocatechin gallate (EGCG) was the most potent catechin and the most potent in antioxidant activity ($r=0.989$ ***). Black tea contained high levels of theaflavins and thearubigins, which accounted for most its antioxidant potential ($r=0.930$*** and $r=0.930$***, respectively). Gallic acid (GA) also showed significant($r=0.530$*) contribution to the antioxidant activity in black tea. Green, black and white tea products processed from Kenyan tea cultivars originally selected for black tea had significantly ($P<0.05$) higher antioxidant activity than green tea processed from tea cultivars from Japan and China. These seem to suggest that the cultivar type is critical in determining the antioxidant potency of tea product and that black teas processed from suitable cultivars could be potent in antioxidant activity when compared to green teas. *In vivo* study was carried out to determine the effect of tea on an animal model of Swiss albino mice infected with *Trypanosoma brucei brucei* isolate KETRI 2710. The purity and trueness to type of the isolate was screened using polymerase chain reaction (PCR). The isolate produced a similar clinical picture after a pre-patent period of 5 days post infection (DPI). The levels of parasitemia in the control infected mice and those given different teas developed exponentially at similar rates reaching a peak on 7 DPI. However, the decline on 9 to 13 DPI was significantly ($P<0.05$) different with that of treated mice decreasing more rapidly. This demonstrated that tea lowered parasitemia level, which can be attributed to the trypanolytic effect of tea flavonoids. A fall in erythrocyte packed cell volume (PCV) occurred within 4 DPI due to hemolysis of erythrocytes and consequent anaemia by trypanosomes. A significant difference ($P<0.05$) was observed on 11 DPI between the infected mice given tea and the infected untreated mice. Thus tea enhanced resistance to erythrocyte hemolysis signifying it could have a therapeutic role in cases of anaemia. The effect of tea on acute phase response and chronic inflammation was observed because tea produced a significant ($P<0.01$) elevation of parasite-induced hypoalbuminemia as compared to the infected untreated mice. The same effect was observed in the pathology of liver sections where tea reduced periportal and parenchymal infiltration and the resulting karyohexis and karyolysis compared to the infected untreated animals. Additionally tea showed a significant ($P<0.05$) effect on the survival rate between the infected untreated mice and those given tea. Although green and white teas were superior in most of these characteristics, black tea, which is the principle tea product from Kenya, displayed remarkable properties some even comparable to those of green tea. Tea was more efficacious than an anti-inflammatory drug (dexamethasone), demonstrating its potential as a therapeutic agent.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AA- Antioxidant activity

APP-Acute Phase Protein

APR- Acute Phase Response

BCG- Bromo Cresol Green

DM- Dry matter

DPPH- 2, 2-diphenyl-1-picrylhydrazyl radical

EC- Epicatechin

ECG- Epichatechin gallate

EGC- Epigallocatechin

EGCG- Epigallocatechin gallate

g/L- Grams per litre

GA- Gallic acid

GrTE- Green tea extract

HPLC-High Performance Liquid Chromatography

KTDA- Kenya Tea Development Authority

PCV- Packed Cell Volume

rpm- Revolution per minute

TF's- Theaflavins

TR's- Thearubigins

TRC - Trypanosomiasis Research Centre

CHAPTER ONE

INTRODUCTION

1.1 Background

Tea is one of the most popular beverages in the world, is manufactured from the young tender leaves of the plant *Camellia sinensis* (Cabrera *et al.,* 2003). Two types of teas are most widely consumed; green and black tea (Obanda *et al.,* 2001). Kenya is the fourth largest producer of tea globally, which proves her success in tea growing and manufacturing. The tea industry is the largest sub-sector in the agricultural sector, contributing significantly to the country's economy (Economic survey, 2005).

The tea plant biosynthesizes several polyphenols during growth. The polyphenols dominated by catechins (flavan-3-ols) have recently aroused considerable interest because of their potential effects on the human health (Lekh *et al.,* 2004). Increasingly, new research findings are demonstrating the value of tea as a pharmacological agent (Magoma *et al.,* 2001). In particular, tea may have protective effects against cardiac diseases, combating cancer and possesses some anti-viral, anti-bacterial and anti-mutagenic properties (Picard, 1996; Bukowoski, 2004). However, the effectiveness and mode of the anti-inflammatory action of tea flavonoids especially from black tea, the principle tea product produced and consumed in Kenya is yet to be elucidated.

Inflammation comprises a series of cellular changes, which ultimately facilitate phagocytosis, killing of pathogenic microorganisms and the digestion of cell debris. Plant flavonoids are thought to attenuate inflammation through inhibition of pro–inflammatory signaling molecules (Manthey, 2000). This effect of ameliorating inflammation is normally accompanied by the biosynthesis of protein cytokines (Whicher and Evans, 1992).

The investigation of the pathophysiological role of systemic cytokines in the host response to infection with *Trypanosome* in mice is hampered by low concentration of these cytokines in plasma and their short half-life (Ngure *et al.,* 1997). Cytokines mainly stimulate hepatocyte production of acute phase proteins (APPs) such as albumin, serum amyloid -P (SAP), haptoglobin (Hp) among others. These APPs are more stable in circulation than cytokines and can be measured as a means of assessing the systemic cytokine response in the *Trypanasoma* infected host (Eckersall, 2000; Murata and Yoshioka, 2004). Since experimental approaches to

study the role of cytokines in the acute phase response in Trypanosomosis is hampered by the instability and transient presence of these mediators in circulation, a more effective, though indirect process is used. The systemic cytokines activated during disease are quantified by measuring plasma concentration of APPs, which are recognized makers of inflammation (Ngure *et al.,* 1997; Eckersall, 2001).

Changes in mouse serum acute phase proteins in lipopolysaccharides (LPS) and apolipoprotein-induced inflammation have been observed (Wait *et al.,* 2005). Further more, major acute phase response of haptoglobin and serum amyloid protein following experimental infection of mice with *Trypanosoma brucei* has been observed (Ngure *et al.,* 1997). While the *in vivo* serum profile of APP namely albumin using experimental trypanosomes in mice model was monitored, the present research tried to establish the role of tea flavonoids in down-regulating inflammation.

Albumin concentration in plasma was measured to evaluate the status of the parasite and subsequent degree of inflammation in a well established animal model system, namely mice infected with *Trypanasoma brucei.* The effects of tea flavonoids were evaluated by oral administration of flavonoid extracts to the animal model and subsequent measurement of serum acute protein mainly albumin concentration.

Results obtained from this study on tea will be used as valuable marketing tool since it will demonstrate to the tea industry the potential health benefits associated with tea.

1.2 Statement of the problem

Emerging scientific data from pharmacological and physiological studies continue to show that tea has beneficial effects on human health by boosting immunity. A number of *in vitro* studies have shown that flavonoids help immune response by acting as anti-allergic, anti-cancer, anti-viral and anti-bacterial agents. While these activities of tea have been widely reported, little is known about the interaction of tea flavonoids with the immune regulatory functions namely, inflammation. Tea product development for medicinal use and other forms of drinks such as instant tea is receiving very active research in other tea producing countries. However, the state of research on tea in this area in Kenya is limited and majority of work has been conducted on green tea and little on black tea. It is therefore necessary to initiate research promptly in this area.

1.3 General objective

The aim of this study was to investigate levels of flavonoids and antioxidant capacity in various commercial teas, determine whether tea flavonoids could efficiently, and effectively down-regulate chronic inflammation in an experimental mouse model of *Trypanosoma brucei brucei* infected mice.

1.4 Specific objectives

a. To determine the flavonoid levels in different types of commercial Kenyan teas.
b. To evaluate the antioxidant capacity of different commercial Kenyan teas.
c. To determine the effect of tea flavonoids on the acute phase response during murine trypanosomiasis.
d. To compare effectiveness of green and black tea in the down- regulation of chronic inflammation using a mice model.

1.5 Hypotheses

To meet the objectives of this study, the following null hypotheses (H_o) were postulated.

i. There is no difference in the flavonoid levels of different commercial Kenyan teas.
ii. Different commercial Kenyan tea products have no difference in their antioxidant capacity.
iii. Tea flavonoids have no effect on the acute phase response.
iv. Tea has no effect on chronic inflammation during murine trypanosomiasis.

1.6 Justification of the project

Kenya is now the third largest producer of black tea in the world, which proves her success in tea growing and manufacturing (Economic survey, 2005). Despite this encouraging trend, there is declining tea consumption in the local market, which is a major concern to tea producers. The ongoing promotion campaign on “tea for health for life” is a defining moment to the local industry. Currently, tea’s potential health benefits continue to be a focus of many scientific studies, and new research is emerging regularly. While this emerging research is promising,

more studies on the role of tea must be completed before tangible conclusions about its contribution to health are reached. The efficiency of tea flavonoids to ameliorate inflammation is not fully yet studied and research in this area is still needed. Results from such work would contribute to promoting the “tea for health for life” message being passed across the world today. It is envisaged that this would contribute to an increase in the domestic consumption of tea and hence expand the current shrinking market for Kenyan tea.

CHAPTER TWO
LITERATURE REVIEW

2.1 Tea production in Kenya

Tea, a beverage from the tea plant (*Camellia sinensis* L.O Kuntze) is one of the most widely consumed beverages in the world. Green and black teas are the most widely consumed. Green tea is mainly consumed in China, Japan and the Middle East while black tea mostly consumed in India, Sri-lanka, European countries and regions of Africa (Figure 1). Both 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; Record and Lane, 2001; Takeda, 2004).

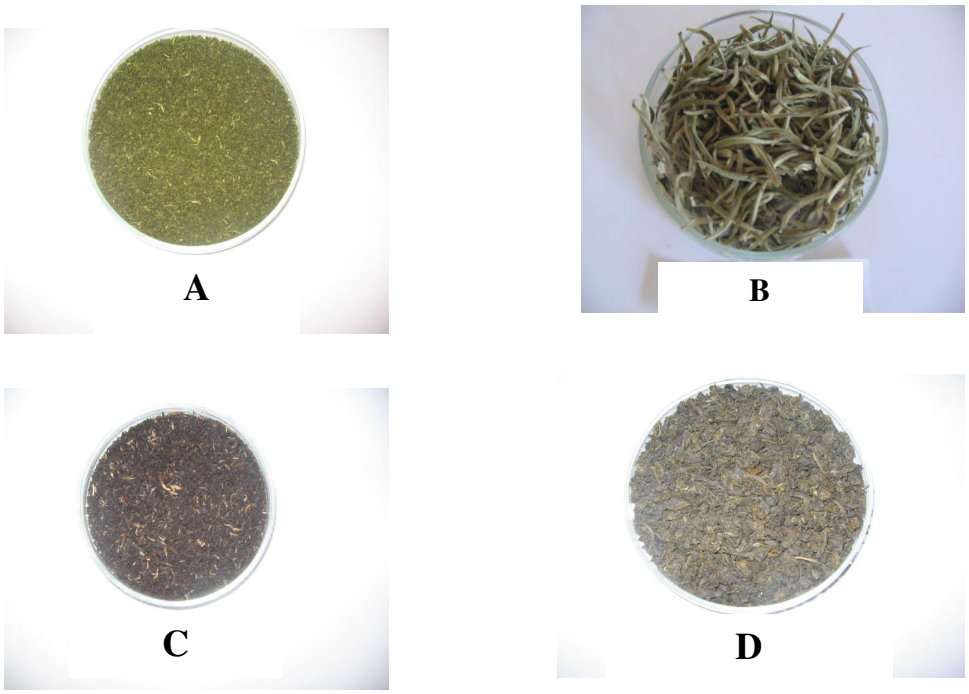
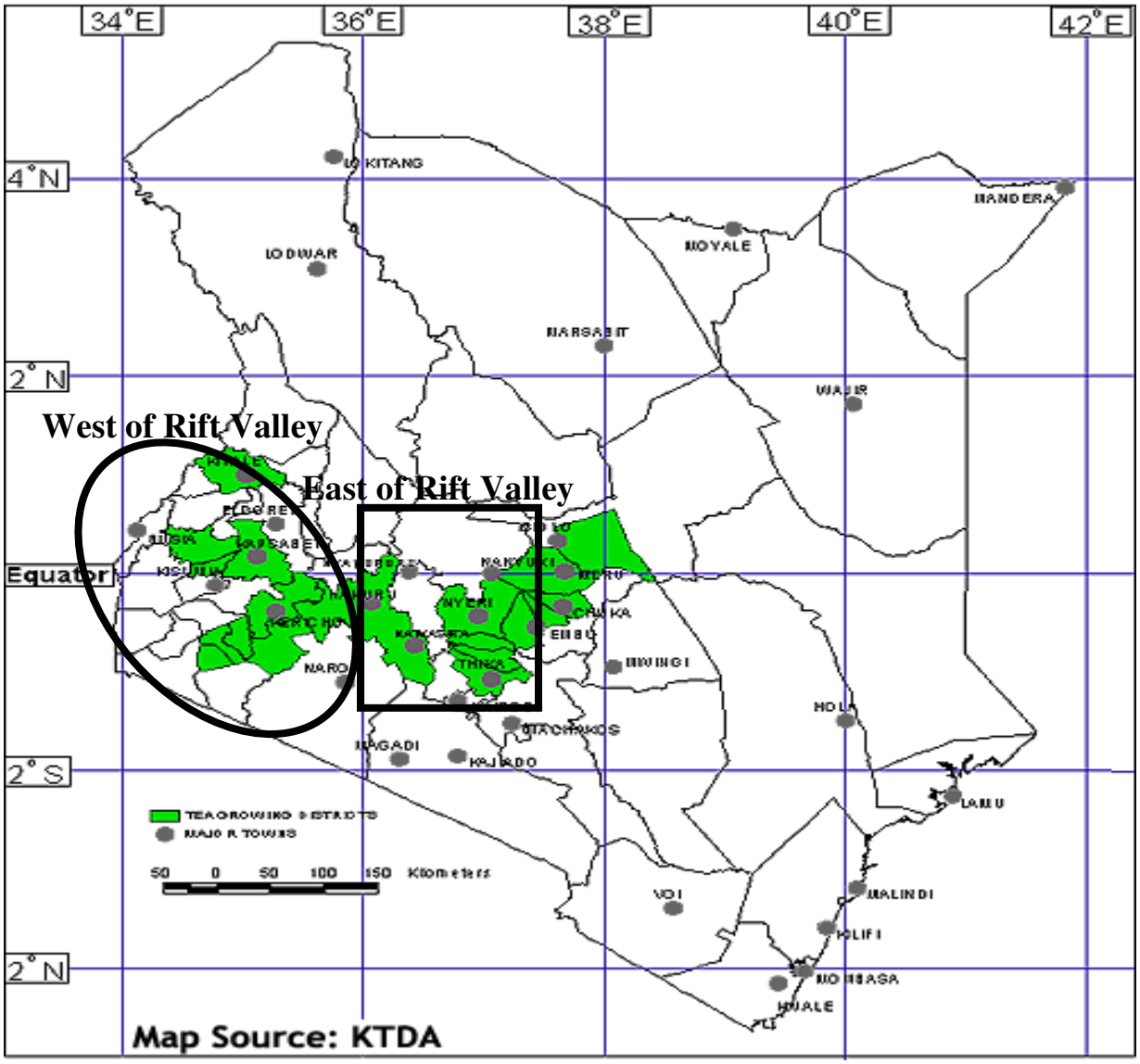


Figure 1: Different types of tea products namely: A. Green CTC (non-orthodox) tea, B. White tea (silvery tips), C. Black orthodox tea and; D. Green orthodox tea

The tea crop is widely grown in the highlands East and West of the Great Rift Valley in Kenya (Anon, 2000). In the East of the Rift are the cool Aberdare highlands, the home to the snow capped Mt. Kenya and the panoramic Nyambene hills. In the West of the Rift defined by the Mau escarpment are the Nandi Hills, highlands around Kericho, Mt. Elgon and the Kisii highlands as shown in Figure 2.



Map Source: Kenya Tea Development Agency (KTDA)

Figure 2: Tea growing areas, West and East of the Great Rift Valley in Kenya.

It is on the slopes of these highlands between the altitudes of between 1500 to 2700m above mean sea level that tea is grown. These regions are endowed with an ideal climate for tea growing manly adequate rain ranging between 1200mm and 2700 mm annually. Growing conditions of tea have an effect on the composition and quality of fresh green leaf. Rapid growth under high temperatures and humidity and at low altitude tends to give more stalks and coarse leaf while slow growth at high altitudes under cooler, drier conditions gives finer leaf with fewer stalks. This explains the variations in the quality of tea obtained from the West and East of the rift (Gesimba *et al.,* 2005).

Kenya is the world's fourth largest producer with a 20% share of the world export market. Production has expanded rapidly during the last 40 years and the country now commands 10% of the world production (Ng'etich and Stephens, 2001). Tea provides employment and a direct means of livelihood for more than 200,000 people, with over 3 million Kenyans, (10%) being directly and indirectly employed by the tea industry. Tea therefore is the largest sub-sector in the agricultural sector, contributing significantly to the Kenyan economy (Wachira and Ronno, 2004). Being the largest export commodity, tea is also a major foreign exchange earner. In 2003, about 270,000 tonnes of processed tea was exported earning the country about US$435 million. Annually, tea contributes about 26% of the total export earning and 4% of the gross domestic product (Economic survey, 2004; Gesimba *et al.,* 2005).

The smallholder growers process and market their crop through their own management agency, the Kenya Tea Development Agency (KTDA Ltd). This is the largest single producer of tea in the world, producing about 60% of the total crop in the country. The balance of 40% is produced by the large multinational estates as shown in figure Figure 3. A review of unit prices fetched by Kenya's tea at the local auction centre 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). 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).

Figure 3: A commercial tea plantation at Timbilil estate, Kericho.

Through this research continum, remarkable achievements on tea research have been made and for example, improved tea cultivars capable of exceptionally high yields of upto 8000 kgmt/ha under good management have been developed and commercialized (Wachira, 2004; Wachira and Kamunya, 2004; Wachira and Ronno, 2004; Gesimba *et al.,* 2005). 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 in Kenya (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.

2.2 Tea consumption, processing and human health

There is declining tea consumption in the local Kenyan market, which continues to be a major concern to the producers in the country (Wachira, 2004). Out of a production of 328,498 metric tonnes of made tea in 2005, 96% of the produce was exported. While this makes Kenya the leading tea exporter in the world, it also reflects the lowest domestic consumption rate among producers with only 14,025 tonnes or 4% of its production being consumed locally. National per capita consumption has declined from 0.8 kg made tea/person/year in 1989 to 0.4 kg made tea

/person/year in 2004. During the same period the volume of tea consumed in Kenya declined from 17 million kg in 1989 to 14 million kg in 2004 (Economic survey, 2004; Wachira and Ronno, 2005). This calls for urgent interventions to diversify tea markets and more so, create a strong local demand in order to build the potential of increasing local consumption. In other countries where tea is produced, the beverage has been widely marketed as a health product. In addition, tea is increasingly being put to other uses in products 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 this assertion on the wide array of the beneficial uses of tea has been generated particularly using green tea, which is widely consumed in Asia (Picard, 1996; Lekh *et al.,* 2004). However, there is a dire paucity of information on the potential health benefits of black fermented tea, which is the principle type of tea product consumed in Kenya. Therefore, there is need to promptly initiate research on black tea to establish its beneficial effects on human health. This would not only be timely but also appropriate since few Kenyans know tea as food and are oblivious of its intrinsic health benefits.

Teas are used as beverages worldwide although consumers vary in their preferences for degree of fermentation, taste and color (Obanda *et al.,* 2001). Kenya prides itself as the producer of the best quality black tea in the world. This is because only the choicest of the upper two leaves and a bud are hand-plucked, followed by skillful manufacture under stringent conditions at source, to ensure maximum quality and cuppage. During its growth, the tea plant biosynthesizes several chemicals, which are responsible for the quality of its processed product (Obanda *et al.,* 2001).

The young shoots are plucked to make various kinds of tea beverages, which include black tea, green tea, and partially fermented teas such as oolong and pouching (Wilson and Clifford, 1992). Though most of the tea produced in the world can be classified as non-fermented or aerated green tea, semi-fermented (oolong) tea and fermented black tea (Reeves, *et al.,* 1987), processing has diversified to the production of speciality teas which may be defined as teas with distinctively different characteristics to those of regular tea and selling at enhanced prices. Such teas include white tea, flavored teas, organic teas, decaffeinated teas, herbal teas, scented teas and various other types of blends. The manufacturing techniques of the above types of tea products, which may be either orthodox or non-orthodox, vary considerably and have a

pronounced impact on the formative and degradative patterns of various cellular 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 (Mahanta and Hemanta, 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] from catalyzing oxidation of tea leaf catechins (Wilson and Clifford, 1992). In contrast, during black tea processing, tea shoots are macerated to initiate oxidation of catechins by PPO before firing and drying. This reaction enables the catechins to condense with the orthoquinones arising from the oxidation of the B ring di- 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. They act as oxidizing agents for substrates like gallic acid to form epitheaflavic acids. These acids combine with TFs to produce the chemically heterogeneous substances called thearubigins (TRs) that are responsible for the color, body and taste of tea. Molecular oxygen is essential in the formation of these compounds that are characterized by a benzotropolone ring structure (Obanda *et al.,* 2001)

The manufacturing process for semi-fermented oolong tea consists of solar withering, panning, rolling and drying. During this process, the characteristic floral aroma of oolong tea is produced. White tea is a rare speciality tea that gets its name from a specific hairy tea plant variety, as well as a particular post-harvest processing method that raises small silvery hairs on the dried buds. White tea contains a higher proportion of the buds that are covered with fine "silvery" hairs trichomes that impart a light white colour to the tea. The brew from white tea is pale yellow in colour with no "grassy" undertones sometimes associated with green tea. True white tea is lightly fermented, rapidly steamed and dried leaving the leaves "fresh". Unlike black, green and oolong teas, white tea is not rolled or crushed but it is steamed rapidly and air-dried to preserve most of the polyphenols. This unique processing produces a rare and expensive but highly refreshing drink. The various processes of manufacture result in differences in the types of tea products namely; black, green, oolong and white tea as shown in Figure 4.

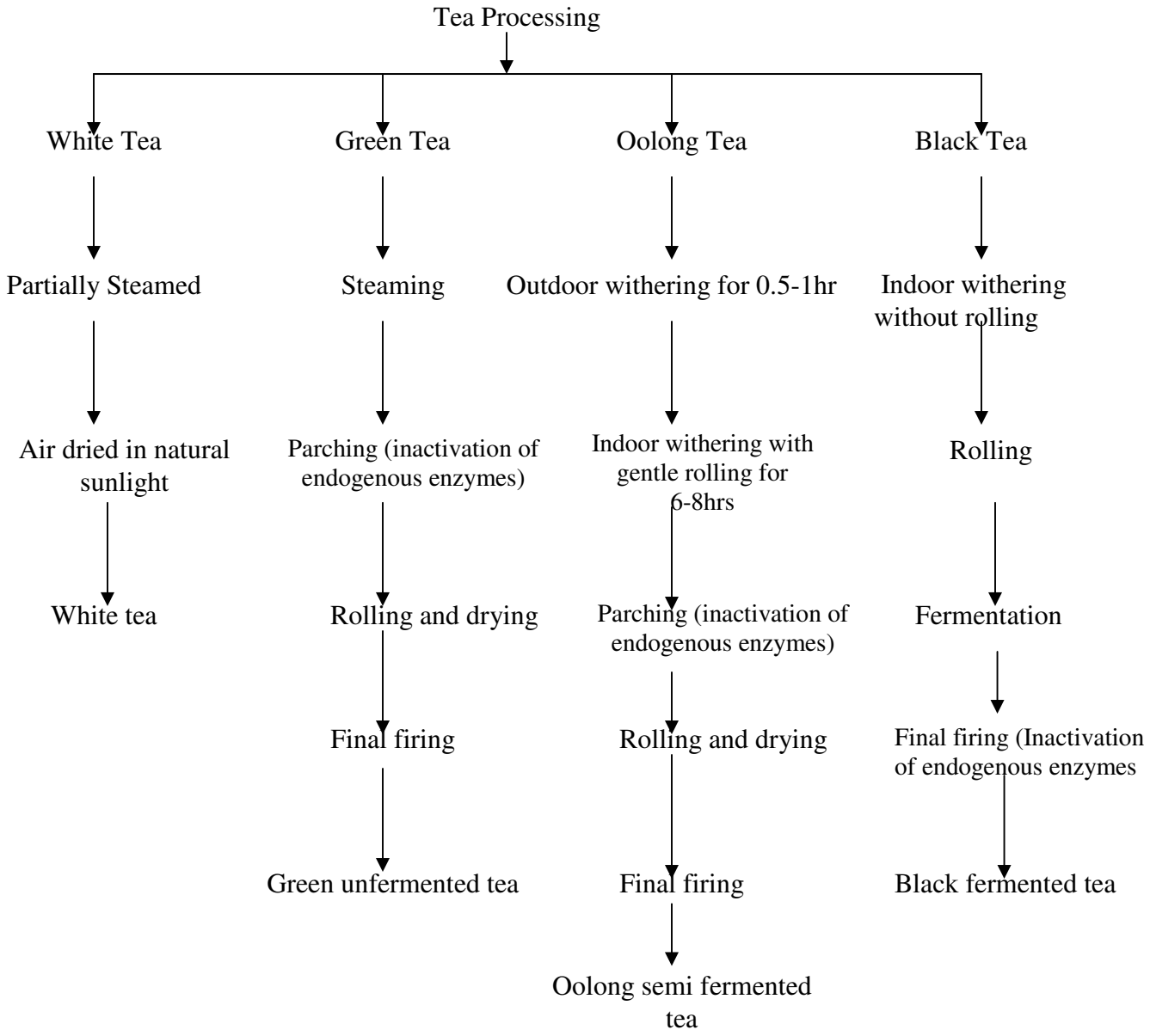


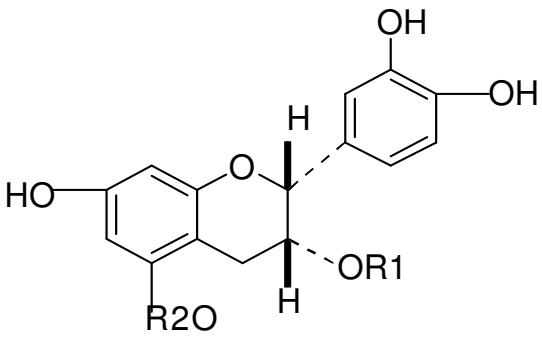
Figure 4: Schematic diagram of the conventional manufacturing process of green, black, oolong and white tea.

The chemical composition of tea is complex and includes polyphenols, alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, minerals, trace elements and other unidentified compounds. Among these, polyphenols constitute the most interesting group and are the main bioactive molecules in tea

(Cabrera *et al.,* 2003). The major polyphenolic compounds in tea are the flavan-3-ols called catechins which include: (-)-epicatechin (EC), (-)-Epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-Gallocatechins (GC) and (-)-gallocatechin gallate (GCG) (Figure 5). Catechins are present in large amounts in green tea (Peterson *et al.,* 2005). Based on their chemical structure, catechins that contain three hydroxyl groups in the B ring (positions 3', 4' and 5') are called gallocatechins while gallic acid substitution in position 3 of the ring is characteristic of catechin gallate (Pellilo *et al.,* 2002).

Catechins account for 6-16% of the dry weight of green tea leaves with EGCG constituting 10-50% of catechins and being the most bioactive due to its degree of gallation and hydroxylation (Stewert *et al.,* 2004). TFs and TRs are another group of polyphenolic compounds found in both black and oolong teas (Obanda *et al.,* 2001).

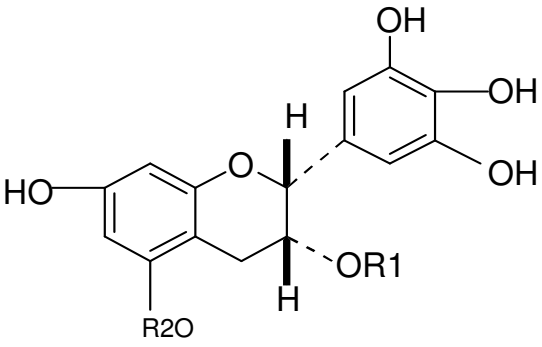
The tea beverage has continued to be considered a medicine since the ancient times because of its polyphenols. Research on the effects of tea on human health has been fuelled by the growing need to provide naturally healthy diets that include plant-derived polyphenols. In line with this, there has been a need to elucidate how known functional components in foods could expand the role of diet in disease prevention and treatment (Mandel *et al.,* 2005; Proestos *et al.,* 2006). There is already growing evidence that tea polyphenols reduce the risk of heart diseases and cancer in humans (Vanessa and Williamson, 2004). In some studies, tea has been associated with antiallergic action (Yamamoto, 2004), antimicrobial properties (Paola *et al.,* 2005), antidiarrhoeal properties (Besra *et al.,* 2003), antidiabetic activity (Sabu *et al.,* 2002) and also antihyperglycaemic activity (Gomes *et al.,* 1995). Further studies have demonstrated that the co-administration of drugs with catechins (EC and EGCG) inhibits glucoronidation and sulfonation of orally administered drugs thereby increasing the bioavailability of such drugs (Hang *et al.,* 2003). Moreover, some epidemiological studies have associated consumption of tea with a lower risk of several types of cancer including those of the stomach, oral cavity, oesophagus and lungs (Cabrera *et al.,* 2003; Hakim and Chow, 2004). Tea contains caffeine, which is known of stimulant effects. However, with mild addiction to some individuals, caffeine can cause nervousness, anxiety, insomnia and headache. Tea appears to be an effective prophylactic agent for toxic chemicals and carcinogens.



i (-)-epicatechin
R1=R2=H

ii(-)epicatechin-3-gallate
R1=3,4,5-trihydroxy-benzoyl
R2=H

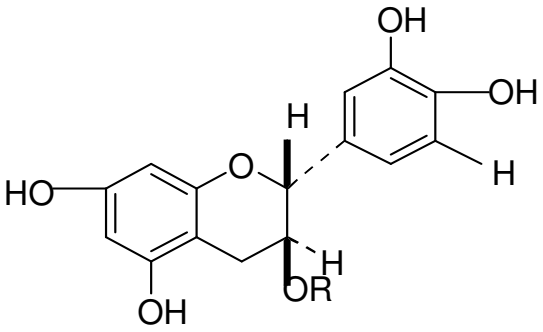
iii(-)-epicatechin-3-digallate
R1=R2=3,4,5-trihydroxybenzoyl



i (-)-epigallocatechin
R=R2=H

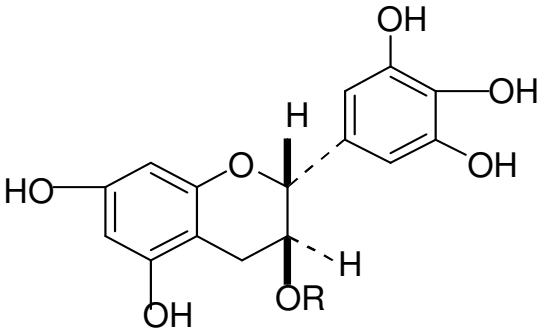
ii(-)-Epigallocatechin-3-gallate
R1=3,4,5-trihydroxybenzoyl
R2=H

iii(-)-Epigallocatechin-3,5-digallate
R1=R2=3,4,5-trihydroxybenzoyl



i(+)-epicatechin
R=H

ii(+)-catechin-3-gallate
R=3,4,5-Trihydroxybenzoyl



i(+)-Gallocatechin
R=H

ii(+)-Gallocatechin-3-gallate
R=3,4,5-trihydroxybenzoyl

Figure 5: The structure of catechins

The ability of tea polyphenols to scavenge for free radicals which act as potent oxidants is due to possession of a phenolic hydroxyl group attached to the flavan-3-ol structure that has been associated with teas' therapeutic action against free radical mediated diseases thereby attracting tremendous research interest (Amie *et al.,* 2003). Free radicals are known to contribute to numerous disorders in humans including cancer, atherosclerosis, arthritis, ischemia, central nervous system (CNS) injury, gastritis, dementia, renal disorders, Alzheimer's disease, Parkinson's disease, premature body ageing and Acquired Immune Deficiency Syndrome (AIDS) (Pourmorad *et al.,* 2006; Rao *et al.,* 2006). Free radicals are constantly generated *in vivo* due to environmental pollutants, radiation, chemicals, toxins, physical stress and the oxidation process of drugs and food. Many plant phenolics have been reputed to have antioxidant properties that are even much stronger than vitamins E and C. In addition, currently available synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT) and gallic acid esters have been suspected to cause or prompt negative health effects and hence the need to substitute them with naturally occurring antioxidants (Amie *et al.,* 2003; Aqil *et al.,* 2006; Pourmorad *et al.,* 2006). Free radicals are constantly generated due to environmental pollutants, radiation, chemicals, toxins, physical stress and the oxidation process of drugs and food. An imbalance between anti-oxidants and reactive oxygen species generated through the above factors results in oxidative stress leading to cellular damage (Wiseman, 1996) and ultimately inflammation, which is both a free-radical-producing process (Takabayashi *et al.,* 2004). Flavonoids which occur both in edible plants and foodstuffs derived from plants such as fruits, vegetables, wine and tea form a substantial constituent of human diet and are thought to have the capacity to attenuate inflammation and the immune response. This occurs by inhibiting important regulatory enzymes and also inhibiting the production of prostaglandins, which act on arachidonic acid in cell membranes, oxidizing arachidonic acid and forming potent pro-inflammatory metabolites, including prostaglandins, leukotrienes and throboxanes that are a group of powerful inflammatory signaling molecules (Asai *et al.,* 2004; Manthey, 2005).

2.3 Inflammation

Inflammation is a succession of changes that occurs in a living, vascularised tissue when it receives a sub-lethal injury. The process of inflammation is designed to dilute, destroy or otherwise inactivate the agent that caused the injury in the first place with an ultimate goal of

restoring the damaged or infected tissue to its original state (Whicher and Evans 1992). Anything that damages a tissue such as physical trauma, chemical toxins, thermal injury (heat or cold) and radiation, initiate the reaction of inflammatory response, which can either be acute or chronic. Acute inflammation is of short duration and is characterized by accumulation of neutrophils (Massimo *et al.,* 2003). If the acute response is not sufficient to deal with the problem at hand then chronic inflammation ensues. The principle cell types involved in the inflammation process include endothelial cells, mast cells, basophiles, platelets, and B and T lymphocytes. This is also accompanied by hyperemia, increased vascular permeability and migration of leucocytes from blood to the tissues (Whicher and Evans, 1992). A number of systemic changes accompany severe inflammation. These systemic changes are known as the acute phase response. The systemic changes in inflammation are mediated by cytokines, which are proteins produced by one cell and affect other cells. Macrophages are important sources of the major cytokines that produce acute phase response (Miller and Krangel, 1992). The major cytokines include Interleukin-1 (IL-1) and the tumor necrosis factor alpha (TNF-ά). During inflammation, macrophages are stimulated to produce IL-1. If the inflammation is severe, enough IL-1 and TNF will get into the blood stream and travel to distant parts of the body to produce systemic symptoms among which are the syntheses of acute phase proteins.

2.4 Acute phase proteins

Interleukin-1 and other cytokines travel to the liver, where they stimulate the cells of the liver hepatocytes to synthesize a variety of proteins that are needed for the inflammatory response and wound healing (Baumann and Gauldie, 1992). The proteins produced by hepatocytes can be divided into two groups;

Group I proteins: These are normally present in plasma, but their concentrations go up 2 to 3 fold in an acute phase response. These proteins include fibrinogen, complement proteins, and proteinase inhibitors.

Group II proteins: These are usually present in the plasma in only very small amounts. Their levels can increase 100- to 1000-fold during an acute phase response. These proteins include serum amyloid A protein (SAA), serum amyloid P (SAP), C-reactive protein (CRP). The serum amyloid A (SAA) and CRP can bind to certain bacteria and may serve to opsonize them. A

simple blood test to look for elevated levels of SAA and CRP can be used to diagnose a bacterial acute phase reaction (Pepys and Baltz, 1983).

Although several cytokines including IL-1 and TNF are implicated in the acute phase response, recent evidence indicates that the synthesis of the major APP such as CRP is induced by IL-6. The positive acute phase plasma proteins are useful non- specific indicators of tissue damage (Fleck, 1989; Akira and Kishimoto, 1992). The acute phase reaction is a normal response to tissue injury and is therefore a fundamental aspect in many diverse disease processes. It probably usually has a beneficial net function in limiting damage and promoting repair but in some circumstances it may have pathological consequences. One significant component of acute phase reaction is increased synthesis of hepatocyte of plasma acute phase proteins molecules important in limiting inflammation and, possibly in regulating immune responsiveness against a wide range of pathogens (Standynk and Gauldie, 1991). The principal pathway leading to the production of acute phase proteins involves initial release of pro-inflammatory cytokines by macrophages at the site of infection or inflammation, which results in a cascade of further release of cytokines. When this cytokines spill in circulation, they induce release of acute phase proteins by the liver. The circulating levels may be related to the severity of the response to an infection, and thus may provide valuable quantifiable biochemical indicators of an inflammatory response (Glass *et al.,* 2003). An example is albumin which is a negative APP since its level decreases during an acute phase reaction unlike in the case of positive APP like haptoglobin (HP), C-reactive protein (CRP) and serum amyloid-A (SAA) where the levels increases. The decrease in albumin levels during an acute phase response to trauma, inflammation or sepsis has been attributed to a decrease in the gene transcription rate of albumin mRNA. Previous studies using inflammation-induced rats showed a decrease in the rate of albumin synthesis (Nicholson *et al.,* 2000; Kaysen *et al.,* 2001). A sustained inflammatory response in critical illness may lead to prolonged inhibition of albumin synthesis indicating that a decline of its synthesis could be used as a prognostic marker of inflammation.

The cascade of events and systemic responses of the acute phase response includes triggering factors, primary cellular reaction, mediators and secondary systemic reactions as demonstrated in Figure 6.

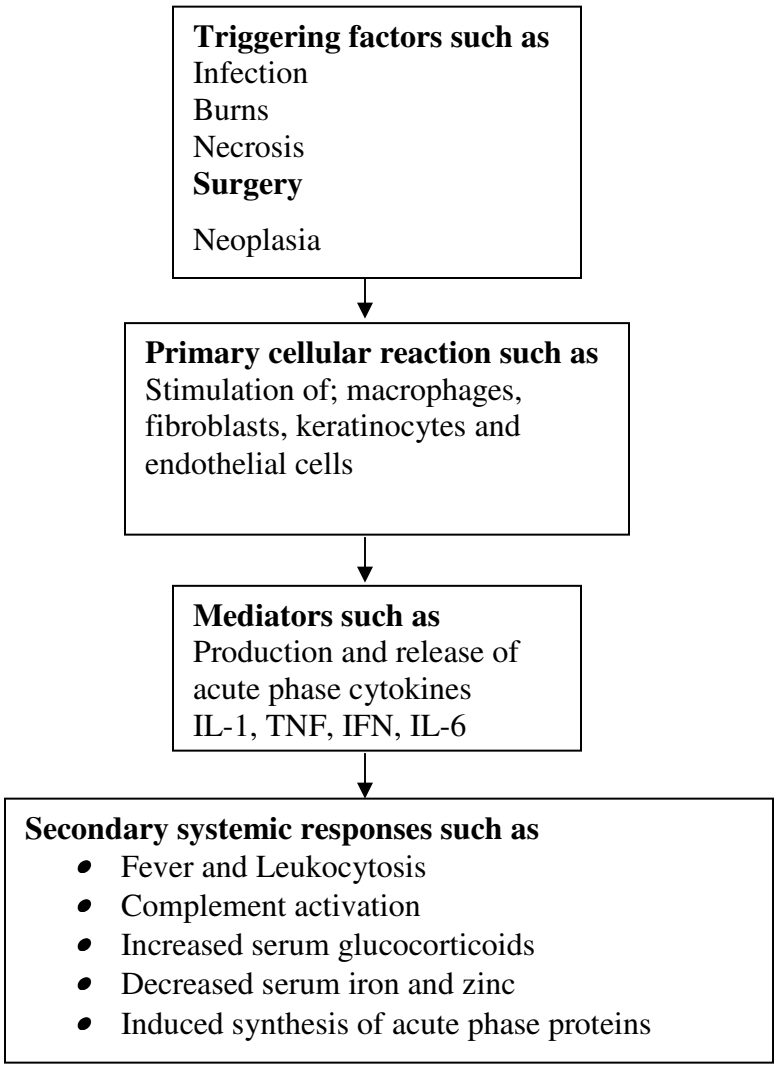


Figure 6: A flow chart showing the cascade of events during inflammation

2.5 Trypanosomosis

Trypanosomosis caused by tissue invasive species (*Trypanasoma brucei)* leads to a severe inflammatory response, extensive tissue damage and untimely death when left untreated (Murray *et al.,* 1974). In response to bacterial and parasitic infections the host mounts an acute phase response, which leads to many systemic effects such as fever, cachexia and stimulation of hepatocytes-derived acute phase proteins (Ngure *et al.,* 1997; Eckersall, 2000: Murata *et al.,* 2004). This response is mediated by the release of cytokines such as IL-1, IL-2, IL-6 and TNF-ά. The experimental approaches to study the role of cytokines in acute phase response in

Trypanosomosis are, however, hampered by their instability and transient presence in circulation (Ngure *et al.,* 1997; Eckersall, 2001).

A more effective, although, indirect means of demonstrating that cytokines have been activated during disease is quantification of their activity by measuring the plasma concentration of acute phase proteins (APP). These proteins are recognized markers of inflammation with their plasma concentration increasing 2 and 1000 times for several days following infection (Murata *et al.,* 2004). The APP consists of "negative" and "positive" proteins that show a decrease and an increase in levels, respectively, in response to a challenge (Murata *et al.,* 2004). The negative APP includes albumin, the most abundant and constitutive plasma protein in healthy individuals. The positive APP are glycoprotein's synthesized mainly by hepatocytes upon stimulation by pro-inflammatory cytokines and released into the blood stream. The positive APP includes haptoglobin, C-reactive protein, serum amyloid-A and alpha-1-acid glycoprotein (Murata *et al.,* 2004).

In the present study, albumin concentration in plasma was measured to evaluate the status of the protein as a marker in a well established animal model, namely mouse infected with *Trypanasoma brucei* which is a tissue invasive parasite that causes the mice to develop extensive tissue damage similar to that which occurs in man (Ngure *et al.,* 1997).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Determination of flavonoid levels in various commercial tea products

3.1.1 Tea samples

A set of twelve tea samples; eight commercial Kenyan teas that included fermented (black), semi fermented (oolong), non-fermented (green) and white tea from different tea factories in Kenya and two of each Japanese and Chinese green teas were analyzed. The samples had been manufactured in commercial factories using standard manufacturing conditions. Black teas had been manufactured using physical withering up to 50-65% moisture content for 18 hours; fermentation at 24 ^{0}C for 1-2 hours and a final firing in a fluid bed drier at 120 ^{0}C for 20-25 minutes. The oolong teas had been processed using outdoor withering under sunlight for 30-60 minutes; indoor withering at room temperature with turned over treatment for 6-8 hours and then rolling and final firing at 100 ^{0}C for about 30 minutes. The green teas had been manufactured using standard green tea manufacturing procedures of steaming for 1 hour and then final firing in a fluid bed drier at 120 ^{0}C for 20-25 minutes. White tea had been processed from the hairy tip buds of clone AHP S15/10 only by partial steaming and air drying in natural sunlight. Preliminary assay were carried out to establish the appropriate amount of sample for analysis and to ensure that the samples had not been damaged or destroyed during transportation. All biochemical analysis was carried out in duplicate.

3.1.2 Sample treatment for polyphenol and catechin analysis

Tea samples of a coarse granular structure were minced and ground to a fine powder. Two grams of the sample was placed on a pre-weighed moisture dish and left for 16 hours at 103 ^{0}C in the oven to dry for the determination of dry matter. Of these, 0.2 ± 0.001g was weighed into an extraction tube. Five milliliter of hot 70 % v/v methanol/water was dispensed into the sample as an extraction mixture and vortexed. Heating of the extraction tube continued in the water bath for 10 minutes with mixing in the vortex mixer after every 5 minutes. The extraction tubes were then removed from the water bath and allowed to cool. The tubes were then placed in a centrifuge at 3500 rpm for 10 minutes. The supernatant was decanted into a graduated tube and

the extraction procedure repeated. The extracts were combined and made up to 10 ml with cold methanol/water mixture. One milliliter of the sample extract was transferred into a graduated tube and diluted to 5 ml with a stabilizing solution (10 % v/v acetonitrile with 500 µg/ml EDTA and ascorbic acid). The solution was further filtered through a 0.45 µm nylon membrane filter. A 20 µl aliquot of this solution was injected into HPLC machine for analysis.

3.1.3 Catechin analysis using HPLC

A modified method of Zuo *et al,* (2002) was used. A Shimadzu LC 20 AT HPLC machine fitted with a SIL 20A auto sampler and a SPD-20 UV-Visible detector with a class LC 10 chromatography workstation was used for the analysis of the prepared samples. A Luna ™ 5 µM C18, 25 cm x 4.6 i.d (Phenomenex, Torrance, CA, USA) column with a Reodyne precolumn filter 7335 model was used. All solvents were filtered through a 0.45 µm millipore membrane filter disk and degassed before injection into the HPLC system. A gradient elution was carried out using the following solvent systems: Mobile phase A (acetonitrile/acetic acid/ double distilled water- 9/2/89 v/v/v), Mobile phase B (acetonitrile/acetic acid/double distilled water - 80/2/18 v/v/v). The mobile phase composition for a binary gradient condition started at 100% solvent A for 10 minutes then over 15 minutes a linear gradient to 60 % mobile phase A, 32 % mobile phase B and held at this composition for 10 minutes. The condition was reset to 100 % mobile phase A and allowed to equilibrate for 10 minutes before the next injection. The flow rate of the mobile phase was 1 ml/ min and the temperature of the column was performed at 35 ± 0.5 ^{0}C. The identification of individual catechins was carried-out by comparing the retention times and UV- absorbance of unknown peaks with peaks obtained from the mixed known catechins standards under the same conditions. The quantification of catechins was performed at 278 nm and was achieved using a caffeine external standard with a calibration curve R^2 = 0.9984 in conjunction with the consensus individual catechin relative response (RRF) values with respect to caffeine calculated on dry matter basis. Total catechin as percentage by mass on a sample dry matter basis was given on the summation of individual catechins.

%Total catechin = [%ECG + (%+C) + (%EC) + %EGCG + %ECG] content.

3.1.4 Total polyphenols determination

The Folin-ciocalteu phenol reagent method was used to determine total polyphenols as described by Pourmorad *et al.,* (2006). The reagent was used since it contains phospho-tungstic acid as oxidants. Reduction of these oxidants by the readily oxidized phenolic hydroxyl yields a blue colour with a broad maximum absorption at 765 nm. This is due to the formation of tungsten and molybdenum blue complexes. One milliliter of the sample extract was transferred to a one-mark volumetric flask, diluted to the mark with water and mixed. One milliliter of the diluted sample extract was transferred in duplicate into separate tubes. Ten percent (10 % v/v) of dilute Folins-ciocalteus was pipetted into each tube and mixed. Within 3-8 minutes after the addition of the Folins-ciocalteus phenol reagent, 4 ml of 7.5 % w/v sodium carbonate solution was added to each tube, stoppered and mixed well. The mixture was allowed to stand at room temperature for 60 minutes and then optical densities (OD) measured using a CE 393 Cecil digital grating spectrophotometer set at 765 nm. A calibration curve was obtained for gallic acid over a concentration range from 10 µg/ml to 60 µg/ml. The OD readings of the test samples were referenced to the calibration curve to determine the total polyphenols content of the samples.

3.1.5 Total theaflavins (TF's) content analysis/flavognost

Total TF were determined by the flavognost method of Hilton (1973). A tea infusion was made with 375 ml of boiling water, added from an overhead boiler into a tared flask. The flask was agitated in a mechanical shaker for 10 minutes; the infusion filtered through a rough cotton wool and allowed to cool to room temperature. Ten milliliter (10 ml) of the infusion was pipetted into 10 ml isobutylmethylketone (4-methylpentan-2-one, IBMK). The mixture was shaken for 10 minutes and allowed to stand until the layers separated. Two milliliters (10 ml) of the upper layer were pipetted into a test tube followed by 4 ml ethanol and 2 ml flavognost (2 g diphenyl boric acid – 2- aminoethyl ester dissolved in 100 ml ethanol). The contents were mixed and color allowed to develop. The absorbance at 625 nm was read against an IBMK/ethanol (1:1 v/v) blank. The total theaflavins were calculated by the formulae;

TF (µmol/g) = A 625 x 47.9 x 100/DM

Where A 625 is the absorbance at 625 nm and DM is the dry matter of the sample.

3.1.6 Spectrophotometric measurement of total Thearubigins (TR's)

Total thearubigins were determined using the method of Robert and Smith, (1961). Six milliliters of 1% v/v aqueous solution of anhydrous disodium hydrogen orthophosphate was added to six milliliters of cooled tea infusion. The resulting mixture was extracted with 10 ml ethyl acetate by vigorous shaking for one minute. The mixture was allowed to settle and the aqueous layer drained off. Five milliliters of ethylacetate was added to the ethylacetate extract containing the TF fraction in the separating funnel. Ten milliliters of the ethylacetate extract was diluted to 25 ml with methanol in 25 ml volumetric flask (solution E_1). One milliliter of the tea infusion was mixed with 9 ml of distilled water and made to 25 ml in a volumetric flask with methanol (solution E_2). One milliliter of saturated aqueous 10 % oxalic acid was added to 1ml of infusion and 8 ml of water and made to 25 ml with methanol (solution E_3). The absorbencies of solution E_1, E_2 and E_3 were obtained at 380nm and 460 nm respectively using CE 393 Cecil digital grating spectrophotometer with distilled water as the blank. Each sample was extracted in duplicate for determination of TF and TR fractions. TF and TR values were then calculated using the formulae's;

$$TF\% = 2.25 \times E_1 \times DM\ \%$$

$$TR\% = 7.06 \times (4\ E_3 - E_1) \times DM\%$$

Where DM is the dry matter of the sample.

3.2 Determination of antioxidant activity of tea

The stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was used for the determination of free radical scavenging of the tea extracts using a modified method of Brand-Wiliams *et al.* (1995). The assay is based on the measurement of the scavenging ability of antioxidants towards the stable DPPH radical. From the methodological point of view, the DPPH is recommended as easy and accurate with regard to measuring the antioxidant activity of fruits, vegetable juices and plant extracts (Moreno, 2002). Five grams of tea was infused in 100 ml of boiling double-

distilled water followed by stirring with a magnetic stirrer and additional steeping for 30 minutes at room temperature. The extracts were strained through a nylon mesh (120 µm) followed by a filter paper (Whatman No. 54). Aliquots of the extracts were kept frozen at -18 ^{0}C until further use. The soluble solid extract was standardized to give stock solutions of 50 mg soluble solids per 100 ml of 50 % methanol. A 50 µl methanolic solution of the antioxidant was placed in a cuvette and 2 ml of 6.0 x 10^{-5} M methanolic solution of DPPH was added (DPPH solution was made using 80 % methanol). The decrease in absorbance at 517 nm was determined using a CE 393 digital grating spectrophotometer until the absorbance stabilized. Reading was done between 15 and 30 minutes interval before the reaction reached a plateau phase. The DPPH solution was prepared afresh and kept in the dark to minimize the loss of free radical stock solution. All determinations were performed in duplicate. The (%) inhibition of the DPPH radical was calculated from the absorbance data according to Yen and Duh, (1994).

% Inhibition against DPPH = [(AB -AA)/AB] x100

Where AB is the absorbance of the blank sample (50 µl double distilled water and 2ml DPPH) and AA is the absorbance of the tested sample after 15 minutes.

3.3 Determination of the effects of tea flavonoids on acute phase response

3.3.1. Preliminary assay for appropriate tea dosage determination

3.3.1.1 Animals

Male Swiss albino mice 6 – 8 weeks old and weighing between 24-30 g were obtained from the Trypanosomosis Research Centre (TRC) laboratory. The animals were housed in standard mice cages in a controlled environment and provided *ad libitum* with food and water. The mice animal model was chosen for this study because trypanosomiasis caused by *Trypanosoma brucei brucei* mimics human trypanosomiasis disease caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense.* The mice model therefore reflects all stages of human sleeping sickness and is now widely utilized as a model to study the disease and evaluate the efficacy of chemotherapeutic agents. Animal care protocols and procedures used in the current study were reviewed and approved by the institutional animal care and use committee.

3.3.1.2 Consumption of green tea extracts in water

Initially, it was tested whether the Swiss Albino mice would voluntarily drink water supplemented with 10 g/L sucrose and various concentrations of green tea extract (GrTE) (0 – 20 g/L. For each concentration, the tea infusion was prepared by adding 1 litre of boiling water to the weighed leaves of *Camellia sinensis* and extracted for 1 minute. After cooling, the aqueous extract of the mixture was filtered and given to the mice.

The mice were acclimatized for 2 weeks during which the animals were treated once using 1% Ivermectin (Ivomec®) at a dose of 0.01 mls per Kg/body weight during the first week in order to exclude any helminthes infestation. The animals were then randomly allocated into 5 groups each of six mice per group and each group of mice was housed separately. Over a period of 10 days each group was subjected to either: (a). Water with 10 g/L sucrose (control); (b). Water supplemented with 10 g/L sucrose + 5 g/L GrTE; (c). Water supplemented with 10 g/L sucrose + 10 g/L GrTE; (d). Water supplemented with 10 g/L sucrose + 15 g/L GrTE; (e). Water supplemented with 10 g/L sucrose + 20 g/L GrTE. Daily consumption of water was monitored and packed cell volume (PCV) determined using the standard micro-haematocrit method. The animals were also weighed and monitored for any sign of disease.

3.3.1.3 Statistical analyses

Statistical analysis on the determination of flavonoid levels in various commercial tea products was carried out in duplicates and data was subjected to analysis of variance using SPSS version 11.5 software. Percent data from antioxidant activity was arcsine transformed before statistical analysis. The Duncan's Multiple Range Test (DMRT) at a P value of < 0.05 was used to separate the means.

Statistical analysis on determination of the effects of tea on inflammation was done. Data from each group were expressed as means ± standard deviation (SD) of the mean of *n* observations, which represents the number of animals studied. Statistical comparison between groups was done using Statsview Statistical (SAS) software programme release 9.1 followed by post-hoc test for group comparison. $P < 0.05$ was considered to be statistically significant. Univariate survival analysis using Kaplan-meir method was used to analyze the effect of tea on the survival rate of infected animals.

3.3.2 Effect of tea flavonoids on acute phase response in *Trypanosoma brucei brucei* infected mice

3.3.2.1 Trypanosomes

Stabilate made from *Trypanasoma brucei brucei* (KETRI 2710), that had been cryopreserved in liquid nitrogen at -196 ^{0}C at TRC trypanosome bank was used. The parasite was reactivated and maintained in clean Swiss albino mice few days before the commencement of the research. The purity and trueness to type of the trypanosome strain used was confirmed using the Polymerase Chain Reaction (PCR) analysis as described in section 3.3.2.2.

3.3.2.2 Template preparation, PCR amplification of trypanosome DNA, electrophoresis and filming

The blood sample was collected from the tail of infected donor mice and the contents expelled into single 1.5 mL micro-centrifuge tubes containing 0.5 mL lysis buffer (1% v/v Triton -100 in 10 mM Tris-HCL, pH 7.5) in which they were vortexed briefly. The material was pelleted by centrifugation at 13000 rpm for 10 minutes and the supernatant discarded. This step was repeated until haemoglobin was removed. The final pellet was resuspendend in 100 µL of PCR buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCL, 1.5 mM $MgCL_2$) with 60 µgmL $^{-1}$ proteinase K. The mixture was incubated at 55 ^{o}C for one hour and then heated to 95 ^{o}C to denature the proteinase K. Two microlitres of this extract was used as template for amplification.

Amplification of trypanosome DNA was performed as a 20µL –reaction mix in a micro-centrifuge tube. The reaction mixture consisted of 10mM Tris HCL, pH 8.3, 1.5 mM $MgCL_2$, 50 mM KCL, 150 µM of each deoxynucleotide triphosphate (dNTPs), 0.8 µM of each pair of trypanozoon specific primers (SRA A: 5'-GACAACAAGTACCTTGGCGC- 3' and SRA B: 5'-CAGCAACCATATTCAGAGCC- 3' for *Trypanosoma rhodesiense*; TBR_1: 5'-CGAATGAAT AATAAACAATGCGCAGT- 3' and TBR_2: 5'-AGAACCATTTATTAGCTTTGTTGC- 3') for *Trypanosoma brucei* (PE Applied Biosystems ,USA), 2µL of template DNA extract and 0.5 units of Taq DNA polymerase (Fischer ,USA). A negative control, which only contained sterile distilled water instead of trypanosome DNA was used in the assay. The reaction mixture was placed in a thermocycler and incubated at 94^{o}C for 1 minute followed by 30 cycles of denaturation at 92 ^{o}C for 30 seconds, annealing at 60 ^{o}C for 45 seconds, extension at 72 ^{o}C for 45 seconds and final extension at 72 ^{o}C for 4 minutes. An aliquot of 15 µL of each PCR product was analyzed by gel electrophoresis through 1.5% (w/v) Agarose gel in Tris-borate-EDTA buffer

(89 mM Tris HCL,pH 8.3, 89 mM boric acid and 2.5 mM EDTA) stained with 0.5 µg/mL ethidium bromide. The agarose gel was viewed on a UV transilluminator at 312nm and photographed using a Polaroid camera.

3.3.2.3 Infection and treatment

A total of 105, eight weeks old male adult healthy Swiss albino mice weighing 20-30 g were used in all experiments. All control animals were age matched with experimental animals. The mice were randomly divided into seven equal groups (n =15 per group) and subjected to one of the following treatments: green tea, black tea, oolong tea, white tea, anti-inflammatory drug (dexamethasone), water only (infected) and water only (non-infected/placebo). Except for the placebo group, animals in other groups were infected with *Trypanosoma brucei brucei* isolate KETRI 2710 after one mouse passage from stabilate. Inoculation was by intraperitoneal injection (*i.p*) with approximately 10^4 trypanosome stabilate in a 0.2 ml phosphate buffered saline (PBS), pH 8.0 containing 1.5 % (w/v) glucose. Although *Trypanosoma brucei brucei* is not infective to man, it belongs to the same sub-genus (trypanozoon) as the human infective parasites species. The similarities include tissue invasiveness and causing chronic infection in domestic and laboratory animals with similar characteristics such as, the involvement of the central nervous system (Jenings and Gray, 1983).

3.3.2.4 Parasitemia

To estimate the circulating parasite numbers in infected mice, two methods were used. In the first instance, a drop of blood from each infected mouse was taken by tail snip daily and a wet smear prepared for examination at x400 magnification by phase-contrast microscopy. Parasitemia was most often estimated by counting the number of parasites present in at least 10 fields, but unusually heavy parasite loads were quantified according to the rapid "matching" method Herbert and Lumsden (1976) as illustrated in Figure 7 and Table 1. Negative samples were further examined by the more sensitive buffy coat technique as described by Murray *et al.* (1977). Briefly, infected blood was collected in a heparanised haematocrit tube and centrifuged at 15,000 rpm for 5 minutes. The buffy coat was collected onto a glass slide and observed under phase contrast microscopy at x400 magnification.

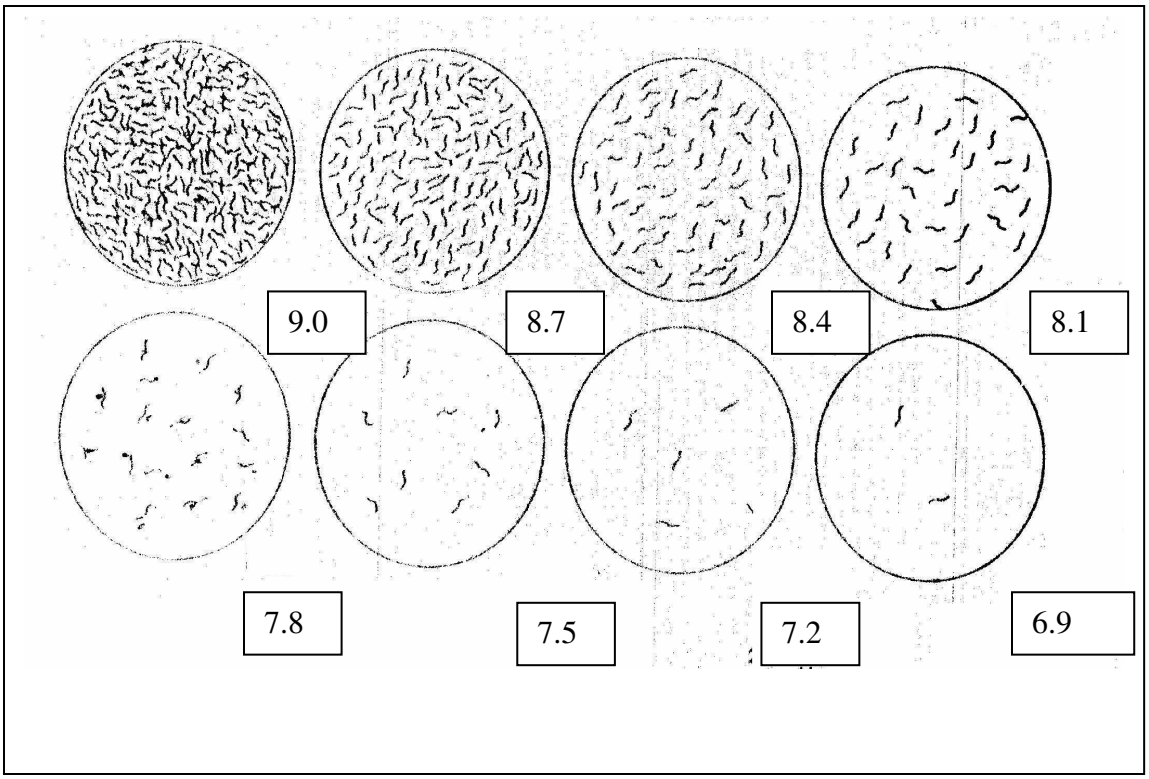


Figure 7: Chart for estimating trypanosome parasitemia. The circles are used for matching when more than one organism per microscope field is present. The values in the boxes in the charts indicate the logarithm of the number of trypanosomes per milliliter as computed for *Trypanosoma brucei* infections in mouse blood inspected under x400 magnification. For viewing at 25 cm, the circles are drawn with a diameter of 6.5 cm. they contain representations of trypanosomes (6mm) that decrease in number by twofold steps.

Table 1: Tables used for estimating lower trypanosome parasitemia. The values indicate the logarithm of the number of trypanosomes per milliliter as computed for *Trypanosoma brucei* infections in mouse blood examined at x400 magnification.

5 fields	10 fields	20 fields
4-5 trypanosomes 6.6	2-3 trypanosomes 6.0	2-3 trypanosomes 5.7
7 trypanosomes 6.3		4 trypanosomes 5.4
		0 trypanosomes <5.4

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3.3.2.5 Blood sampling and determination of packed cell volume

Blood samples were obtained from three healthy animals prior to infection on day 0 and analyzed for baseline data. Subsequent data was obtained by serial sacrificing of 3 mice per group at each sampling time after every seven days. At each point, blood was taken by tail snip and gently squeezing the tail. Blood was collected by capillary action in 100µl microhaematocrit tubes coated with heparin-sodium and centrifuged at 10,000 rpm for 5 minutes using a Hawksley-microhaematocrit centrifuge. Packed Cell Volume (PCV) was read using a Hawksley-microhaematocrit reader. The mice were anaesthetised using carbon dioxide (CO_2) and immediately blood collected from the heart by cardiac puncture using a 21 G needle and a 1ml syringe into plain bottles void of anticoagulant. The syringe was inserted in the left ventricle of the heart and blood was drawn in small jerks. After the collapse of the left ventricle, blood was again drawn from the right ventricle. The blood was allowed to clot and later centrifuged at 10,000 rpm for 10 minutes. Serum was collected in sterile cryovials and stored at -20 ^{0}C until use.

3.3.2.6 Albumin assay

Serum albumin levels was measured using the BCG® photometric colorimetric method as described by Tsirpanlis *et al.* (2005). This test uses bovine albumin as a standard. It is based on the formation of a colored complex by albumin in citrate buffer and bromocresol green. The absorbance of this complex is proportional to the albumin concentration in the sample. Ten µL (10µL) of the sample and the standard was pipetted into separate cuvettes. Into each of these, 1 mL of the reagent, consisting of 30mM/L of citrate buffer (pH 4.2) and 260 µM/L of bromocresol green was added. The contents of the cuvettes were mixed and incubated for 5 minutes at 25 ^{0}C. The absorbance of the sample and standard was measured using a LM-7211 colorimeter at 578 nm against a blank of the reagent within 30 minutes. Albumin concentration (C) was calculated using the formula below;

$$C = \frac{40 \times A\ Sample}{A\ Standard}\ g/dL$$

Where A is the absorbance at 578nm

3.3.2.7 Tissue collection and histopathological analysis

The whole liver was removed from the mice after sacrifice. Fresh liver blocks were trimmed and immediately fixed in 10 % phosphate–buffered formalin for 2 weeks before histochemical examination. The samples were then dehydrated in graded alcohols, infiltrated in hot paraffin and embedded in paraffin wax (Sherwood Medical co USA). Samples were sectioned serially at 4 µm thickness using a rotary microtome, rehydrated and stained with hematoxylin and eosin dyes. Stained sections were observed under light microscopy to determine the degree of inflammation and liver damage.

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CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Flavonoid levels in different types of tea products

To obtain an adequate resolution of the peaks within a reasonable time of analysis, a gradient elution program was developed. The best results were obtained with double distilled water –acetonitrile-acetic acid at a flow rate of 1 ml /min which allowed the separation of catechins within 18 minutes. To avoid interaction of the free hydroxyl groups of catechins and the stationery phase, all solutions were prepared in an acidic media because catechins are stable in acidic media (Pellilo *et al.,* 2002). The Luna ™ Phenomenex column was chosen because of its high stationery phase surface and a constant support dimension that permitted a complete separation of catechins within a short time. The column technique employed was exclusively RP because of its high resolution for separation and quantification of phenolic substances. In addition, 70 % aqueous methanol solution was used for extraction because of its protective role on phenolic substances from being oxidized (Proestos *et al.,* 2006). The major catechins were identified by a comparison of their retention times with those of authentic standards at UV absorption spectra of 278 nm. Under these operating conditions, the retention times in minutes for the studied compounds were as follows: EGC (8.1), +C (10.2), caffeine (13.5), EC (14.8), EGCG (16.4), ECG (21.7), GC (6.1) and GA (4.3) as shown in Figure 7. Previously, results for green tea (Ferruzzi and Green, 2005) and for clonal fresh leaves (Ender *et al.,* 2004) were comparable to the observed results. Separation chromatograms of the major catechins by reverse phase (RP) 18 HPLC is as shown in Figure 8.

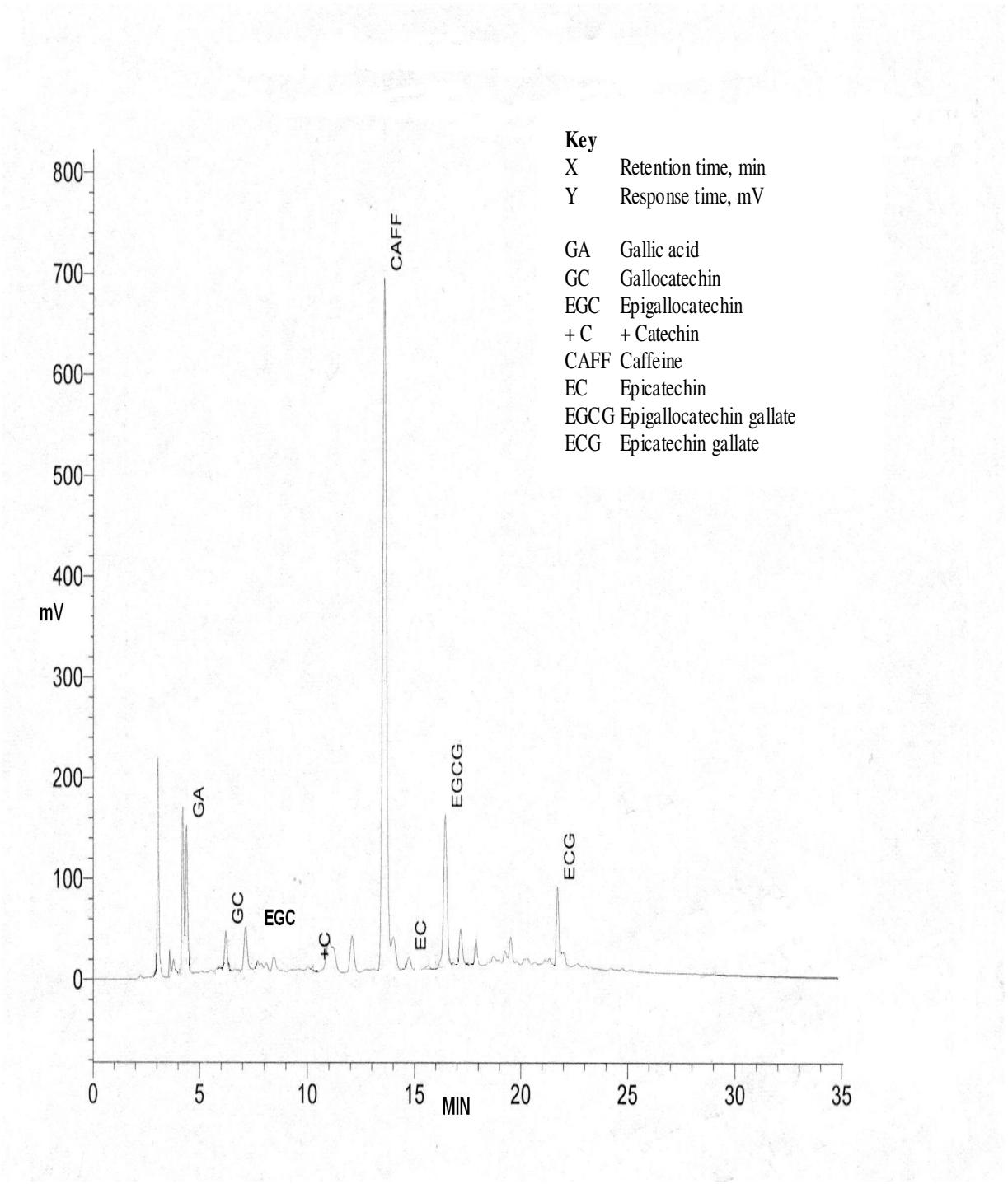

Figure 8: A high performance liquid chromatogram of green BP CTC tea sample.

The total catechin content was significantly higher ($P<0.05$) in green tea than oolong and black tea as indicated in Table 2. This demonstrated clearly that the degree of fermentation

during the manufacturing process had an influence on the catechin content of the final product. Black tea is obtained by a post-harvest fermentation which is an auto-oxidation reaction catalyzed by the enzyme polyphenol oxidase, whereas green teas are steamed to inactivate oxidation. Oolong tea is obtained by a partial oxidation of the leaf, an intermediate process between green and black tea manufacture (Peterson *et al.,* 2005). White tea is a product of partial steaming and air-drying of the hairy tips. This unique processing preserves most of the catechins in white tea (Table 2).

Individual catechins varied significantly ($P<0.05$) among the teas with EGCG, GC and EGC levels being the highest and +C, ECG and EC being less abundant. These results are similar to those of Ender *et al,* (2004). White tea which is predominantly manufactured from the young apical hairy bud only, showed high levels of EGCG and ECG that are present in higher amounts in fresh young leaves (Table 2). This latter result corroborates the result by Saijo *et al.,* (2004) who determined the chemical constituents of young tea leaves and the change occurring during leaf development. The decrease in the gallic acid esters of catechin such as EGCG and ECG during leaf development means that there is a slow biosynthesis of gallic acid moiety in each catechin gallate compared with dry matter production. Since catechin biosynthesis is slower than dry matter production from young leaves to the less young leaves, it is apparent that there is no weight increase in the less young and mature leaves and as a result catechin moves to other young leaves or are metabolized to other products. This accounts for the change in catechin levels in various leaf developmental stages and hence the levels of residual catechins in tea manufactured from different ages as exemplified in the differences in catechin levels between white tea and other types of teas in this study (Table 2).

Table 2: Phenolic composition and antioxidant activity of various teas.

Sample	Total polyphenols	Total catechins	Individual catechins						TFs	TRs	AA+
			EGC	EGCG	GC	ECG	+C	EC			
Kenyan teas											
Black PD (CTC)	20.65 cd,7	5.91 e,9	1.12 de,9	2.82 de, 9	4.88 a, 1	0.99 b, 3	0.37 b,6	0.62 g,9	11.61 c, 3	10.30 d,4	73.31 d, 6
Black BP (CTC)	17.45 f,11	3.07 f,14	0.63 e,11	1.43 ef,11	3.60 cd,, 4	0.57 cde,9	0.20 de,11	0.25 h,11	18.75 a, 1	15.65 a, 1	71.19 fg,10
Green PF (CTC)	26.85 a, 2	14.93 a, 1	5.47 a, 1	6.78 a, 1	3.26 cd 5,	0.75 bc,4	0.43 b, 2	1.56 ab,2	0.46 fg,8	5.59 h,11	77.22 a, 1
Green BP (CTC)	25.70 a,4	10.04 d,7	5.17 ab, 2	3.11 cd,8	2.45 ef,9	0.28 e,12	0.30 b,4	1.21 cde,5	1.63 e, 6	8.38 f,8	73.31d, 5
Black orthodox	22.25 b,5	4.62 ef,10	1.20de,10	1.87 def,10	3.00 de,7	0.62 cd,8	0.33 bc,8	0.79 fg,9	13.80 b, 2	11.51 c,3	71.52 ef,9
Green orthodox	27.10 a, 1	11.06 cd,4	4.91 ab,4	4.72 b,5	3.84 bc,3	0.35 de,11	0.38 b,5	0.98 ef,7	0.41 fg,10	5.77 h,10	75.64 b,3
Oolong orthodox	26.15 a,3	9.49 d,8	2.31 c, 7	4.37 bc,7	3.01 de,6	1.03 b, 2	0.57 a, 1	1.30 bcd,4	6.81 d, 5	8.75 ef,7	74.78 c, 4
White tea/ Silvery tip	21.30 c,6	10.20 cd,6	1.73 cd,8	5.53 ab,2	4.46 ab,2	1.505 a, 1	0.41 b, 3	1.00 def,6	0.85 f, 7	1.75 i,12	76.01 b, 2
Other ungraded teas											
Japanese teas											
Green CTC cultivar Yabukita	19.36 de,9	12.69 ab,2	4.75 b,5	5.05 b,3	1.83 fg,10	0.70 bc,7	0.35bc,7	1.85 a,1	0.25g,12	9.06 e,6	71.76 ef,8
Green CTC cultivar Yutakamidori	19.78 de,8	12.24 bc,3	4.97 ab,3	4.18 b,4	1.79 fg,11	0.74 bc,5	0.23cde,10	1.50 bc,3	0.45fg,9	7.69 g,9	70.54 g,11
Chinese teas											
Green CTC cultivar Hanlu	18.82 ef,10	11.19 cd,5	4.65 b,6	4.64 b,6	2.48 ef,8	0.71 bc,6	0.31bcd,9	0.90 fg,8	0.31fg,11	9.30 e,5	69.21 h,13
Green CTC cultivar Yinghong	11.42 g,12	3.31 f,12	0.60 e,12	0.78 f,12	1.20 g,12	0.36de,10	0.14 e,12	0.22 h,12	11.57 c,4	13.55 b,2	67.62 i,14
Fresh unprocessed vegetables											
Spinach	-	-	-	-	-	-	-	-	-	-	69.34 h,12
Onion	-	-	-	-	-	-	-	-	-	-	72.35 e,7
Grand mean	21.48	9.95	3.12	3.82	2.98	0.72	0.34	1.01	5.62	8.88	72.41
LSD (P≤0.05)	1.423	1.856	0.6995	1.423	0.7431	0.3265	0.1206	0.2953	0.5697	0.5947	0.9021
C.V (%)	3.01	8.30	10.20	16.92	11.34	20.58	4.59	13.26	16.93	3.04	0.55

DMRT ranking - Means within a column followed by the same letter are not significantly different at P<0.05 according to Duncan's Multiple Range Test –DMRT. Numerical ranking is from the highest value of the parameter to the lowest. +- Data has been arcsine transformed. Tea grades: PD, pekoe dust; BP, broken pekoe; PF, pekoe fanning's; CTC, curl tear crush (non-orthodox). EGC, Epigallocatechin; EGCG, Epigallocatechin gallate; GC, Gallocatechin; ECG, Epicatechin gallate; +C, Catechin; EC, Epicatechin; TFs, Theaflavins; TRs, Thearubigins; AA, Antioxidant Activity.

The variation in the polyphenolic composition of the different tea products resulted from the leaf maceration during manufacturing. The rolling and cutting of the tea shoots in non-orthodox manufacture causes a release of polyphenol oxidase which interacts with phenolic compounds, one simple catechin and one gallocatechin, to produce theaflavins and thearubigins that posses a benzotropolone skeleton (Reeves *et al.,* 1987; Mahanta and Hemanta, 1992). Owuor and Obanda (2006) investigated the use of green tea flavan-3-ols in predicting black tea quality potential and revealed that a correct balance of the trihydroxylated flavan-3-ols and dihydroxylated flavan-3-ols was necessary to ensure maximum formation of the theaflavins. The trihydroxylflavan-3-ols are oxidized faster during the fermentation phase of black tea processing explaining the high levels of EGCG and EGC in green tea and the subsequent reduction in black tea. Theaflavins are further oxidized to form thearubigins that are heterogeneous in nature and contribute significantly towards taste, color and body of tea (Obanda *et al.,* 2004; Li *et al.,* 2005). Black tea therefore has high levels of TFs and TRs that are the main fermentation products as evident in Table 2.

Results from the present study however clearly showed that TRs were present in green tea (Table 2). Further observation revealed that in green tea, TRs were formed in the presence of low levels of TFs unlike in black tea where the levels were almost similar (Table 2). This may suggest that theaflavins are not the only source of thearubigins. Wilson and Clifford (1992) explained the factors affecting the formation and degradation of theaflavins and thearubigins in black tea and observed that maximum synthesis of theaflavins occurs when oxygen is in excess to support benzotropolone ring formation. However, under a limiting oxygen concentration, polyphenol oxidase, which has a high affinity for the substrate, has a preferential demand for oxygen and theaflavins formation is suppressed at the expense of catechin quinone formation. This competition for oxygen is particularly noticeable during the early stages of fermentation when the concentration of the catechins is at its highest and enzyme turnover is unimpeded by substrate availability. This occurs during green tea manufacture since the enzyme is active before deactivation through steaming. For this reason, high enzyme activity in an already low oxygen concentration creates almost total anaerobiosis, which suppresses benzotropolone ring formation. Consequently as a result of this, thearubigins are formed, mainly from gallocatechins since the simple catechins are unable to react in benzotropolone ring formation. Moreover, it might be possible to minimize thearubigins formation by deactivating the enzyme immediately after

plucking through a steaming procedure although this is hardly achievable during commercial tea processing. Further research is desirable to explain in details the existence of this thearubigins in green tea and the importance of steaming during tea processing.

4.2 Antioxidant capacity of tea

The polyphenolic composition of tea and especially its catechins has aroused interest in their potential as radical scavenging compounds. Data on antioxidant capacity is presented in Table 2. Overall, green and white teas' had significantly ($p< 0.05$) higher antioxidant activity compared to black tea. There was no significant difference in the antioxidant capacity of black tea manufactured using orthodox and non-orthodox methods. Table 3 presents data on the correlation between tea polyphenols contents and the antioxidant activity of different types of tea products. Total catechins significantly ($p<0.001$) correlated with antioxidant activity ($r=0.959$). EGCG was identified as the most potent antioxidant ($r=0.989$, $P<0.001$). EC, EGC, +C and ECG contents also showed significant influence on the antioxidant activity. Therefore, the antioxidant activity was higher in tea extracts containing high levels of EGCG, EC, EGC, +C and ECG. These results are similar to those of Gramza *et al.* (2006). This antioxidative effect of polyphenols has been attributed to the phenolic hydroxyl groups in their structures that make them potent free radical scavengers (Amie *et al.,* 2003). On the basis of these results, it appears that the most effective radical scavengers are catechins with a 3', 4' and 5'-trihydroxylated substitution pattern on the B ring and/or hydroxyl group at C-3 position of the catechin structure (Figure 4). This hydroxylation confers a higher degree of stability on the catechin phenoxyl radical by participating in electron delocalisation that is an important feature of the antiradical potential. This explains why radical scavenging is high in the gallocatechins namely EGCG and EGC that are potent antioxidants (Zhu *et al.,* 2001; Amie *et al.,* 2003; Rao *et al.,* 2006).

Table 3: Correlation coefficient matrix analyses between various tea chemical parameters

TP	TFs	TRs	EGC	EGCG	ECG	+C	EC	GC	GA	AA	TC	
1.00	0.881***	0.734***	0.919***	0.791***	0.530*	0.832***	0.920***	0.675**	0.637**	0.807***	0.899***	TP
	1.00	0.952***	0.894***	0.908***	0.262	0.766***	0.885***	0.308	0.584*	0.920***	0.955***	TFs
		1.00	0.706**	0.922***	0.658**	0.738***	0.803***	0.450	0.378	0.930***	0.892***	TRs
			1.00	0.782***	0.674**	0.624**	0.860***	0.644**	0.815**	0.787***	0.919***	EGC
				1.00	0.597*	0.769***	0.873***	0.333	0.505*	0.989***	0.965***	EGCG
					1.00	0.578*	0.261	0.757***	0.771***	0.579*	0.255	ECG
						1.00	0.887***	0.401	0.442	0.766***	0.794***	+C
							1.00	0.627**	0.449	0.860***	0.937***	EC
								1.00	0.184	0.400	0.369	GC
									1.00	0.530*	0.645**	GA
										1.00	0.959***	AA
											1.00	TC

** - Correlation significant at the P≤ 0.05 level*
*** - Correlation significant at the P≤ 0.01 level*
**** - Correlation significant at the P≤ 0.001 level*

There is much discussion in literature about the mechanism of the antioxidative action of flavonoids, but until now, these mechanism and structural requirements have not been fully understood. However, flavonoids as polyphenolic compounds have the ability to act as antioxidants by a free radical scavenging mechanism with the formation of less reactive flavonoid compounds. The high potential of flavonoid compounds (FL-OH) to scavenge free radical ($R^{\bullet}$) in reactive oxygen species (ROS) may be explained by their ability to donate a hydrogen atom from their hydroxyl group for scavenging free radicals and therefore the higher the degree of hydroxylation, the greater the realized effect.

$$FL\text{-}OH + R^{\bullet} \rightarrow FLO^{\bullet} + RH$$

Scavenging reaction

This reaction gives the flavonoid phenoxyl radical ($FLO^{\bullet}$) and a stable molecule (RH). $FLO^{\bullet}$ subsequently undergoes a change in resonance structure by redistributing the unpaired electron on the aromatic core. Thus, flavonoid phenoxyl radicals exhibit a much lower reaction compared to $R^{\bullet}$. $FLO^{\bullet}$ would react further to form unreactive compounds probably by radical-radical termination.

$$FLO^{\bullet} + R^{\bullet} \rightarrow FLO\text{-}R$$

Radical-radical coupling reaction

$$FLO^{\bullet} + FLO^{\bullet} \rightarrow FLO\text{-}OFL$$

Radical-radical coupling reaction

This is in agreement with literature whereby in the case of a flavonoid quartecin, abstraction of 3-OH hydrogen leads to a more stable phenoxyl radical tautomer which undergoes resonance stabilization (Amie *et al.,* 2003).

Black teas analyzed in this study exhibited some antioxidant activity with a high DPPH radical scavenging activity though less than that of green, white and oolong tea. During black tea manufacture, the gallocatechins are first to be oxidized and dimerised to TFs and TRs because of their high oxidation potential and high concentration in the leaves. These major phenolic compounds in black tea also contributed significantly to the radical scavenging activity namely TFs ($r=0.920$, $P<0.001$), TRs (0.807, $P<0.001$) and GA ($r=0.530$, $P<0.05$). Interestingly, TFs, which are the major phenolic products in black tea, had a higher radical scavenging activity compared to some of its precursors ECG, EGC and EC (Table 3). This confirms that conversion of catechins to TFs during black tea processing did not affect the radical scavenging potency.

These observations are consistent with those of Leung *et al.* (2001) who showed that black tea posses more or less the same antioxidant potency as catechins present in green tea. EGCG and EGC contribute significantly to the formation of TFs. These are B ring trihydroxylated catechins, which are oxidized at a much faster rate than the B ring dihydroxylated catechins including EC, ECG and +C due to their lower oxidation potential (Owuor and Obanda, 2006). TFs formed from this reaction have hydroxyl groups (OH) considered necessary for free radical scavenging activity. These additional groups increase the total number of phenyl hydroxyl groups and make the gallate containing catechins and TFs more able to donate protons due to resonance delocalization thereby expressing the observed antioxidant activity of black tea. Similarly, gallic acid contributed significantly to the radical scavenging activity in black tea because it is a potent hydrogen donator to DPPH. Additionally, the present study provided evidence of the contribution of TRs towards the antioxidant activity of black tea ($r=0.807$, $P<0.001$). The antioxidant activity of TRs can be explained by the presence of 3-OH groups, which are more or less esterified by gallic acid in the TRs structure. However, this is a highly speculative hypothesis since to date there is no definite data on TRs structures (Li *et al.,* 2005). Oolong a semi-fermented tea which was intermediate between green and black tea did not contain high levels of the major anti-oxidative gallocatechins and also did not contain a great amount of theaflavins and thearubigins which are found in fully fermented black tea. Consequently, this type of tea had an antioxidant activity that was intermediate of that of green and black tea (Table 2).

A comparison of the antioxidant activity of the Kenyan commercial teas derived from the Kenyan varieties with those of green teas from Japanese and Chinese cultivars was carried out to determine the effect of the variety from which the tea products were processed on antioxidant activity. This study revealed that Kenyan tea products both green and black were rich in total polyphenols as shown in Table 2. The high polyphenol content of Kenyan tea products is not unexpected since the tea-breeding programme in Kenya has indirectly and consistently selected germplasm for high total phenol content, which produce black teas with high levels of TFs and TRs. A previous study had confirmed the superiority of Kenyan tea germplasm in its total polyphenol content (Wachira and Kamunya, 2005). Tea germplasm from Japan and China that is traditionally used for green tea manufacture is selected to be low in astringency and bitterness and consequently low in total polyphenols. A comparison of the antioxidant activity of tea and

popularly consumed vegetables such as spinach and onion showed that the antioxidant activity of tea was significantly ($P<0.05$) higher than that of the fresh unprocessed vegetable (Table 2) which demonstrated the potency of tea as a potentially health enhancing food.

Despite the growing number of studies on the antioxidant properties of green, black and oolong tea, limited information is available on white tea. In the present study, the antioxidant capacity of white tea was shown to be similar to that of green tea (Table 2). This can be attributed to the high levels of EGCG, which is the most potent antioxidant, and ECG that is present in large amounts in the young fresh leaves or the hairy bud traditionally used in the manufacture of this rare speciality type of tea (Stewart *et al.,* 2004; Takeda*,* 2004).

4.3 Effect of tea on inflammation induced in *Trypanosoma brucei brucei* infected mice

The compelling evidence in favour of antioxidant activity of different types of commercial tea products prompted a further investigation on the effect of tea on chronic inflammation in *Trypanosoma brucei brucei* infected mice. Mice were reared in standard cages (Figure 9).

Figure 9: Male Swiss albino mice used in the experiment as seen in an open standard mice cage.

Results on appropriate tea dosage determination using green tea extract on healthy mice indicated a significant difference (P< 0.05) between the various dosages on daily water intake but no significant difference on packed cell volume (PCV). This showed that tea extract did not have a toxic effect on the mice and therefore recommended for the main study. Twenty (20 g/L), the most consumed and tolerated concentration, was not significantly different from the control as shown in Figure 10. This concentration was thus selected as a standard dosage for the main experiment since it had the best chance to show activity when administered orally and still ensures absence of toxicity.

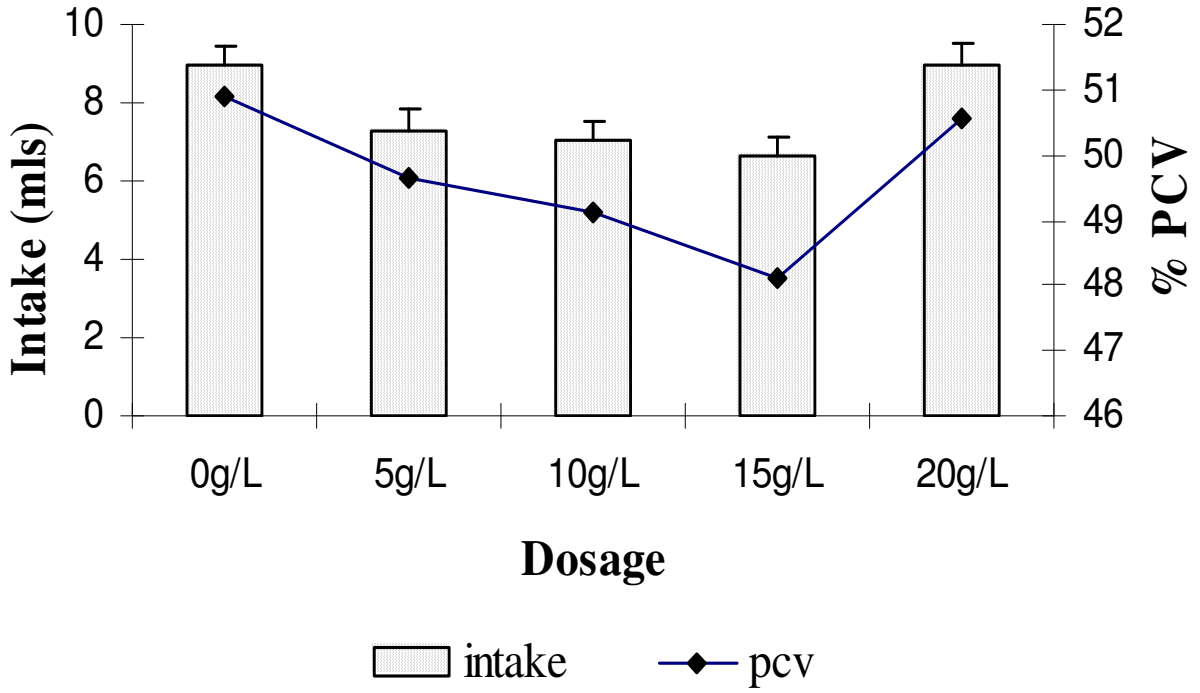

Figure 10: Effect of oral administration of green tea extract on water intake and PCV on male Swiss albino mice. Data are means ± standard error of the means (SEM), *n* =30, *P*<0.05.

4.4 Template preparation and Trypanosome DNA amplification

Trypanosoma brucei brucei stabilate 2710, which was used in this study, was screened for its purity and trueness to type before inoculation in experimental animals. DNA assay based

on PCR analysis on whole blood samples of infected donor mice was used to provide improved levels of accuracy and sensitivity. PCR amplification using TBR_1, TBR_2 primers designed for identification of *Trypanosoma brucei* gave positive amplicons for lanes M2, M3, and M4 which represented *Trypanosoma brucei* KETRI 2710, *Trypanosoma brucei* 3738, *Trypanosoma brucei* 42 respectively. No amplification was elicited using *T. brucei* specific primers for the *Trypanosoma congolense* (lane M5) and the negative control (lane M7). However, no amplification products were present using the SRA A and SRA E primers, which were specific for *Trypanosoma rhodisiense* (Figure 11).

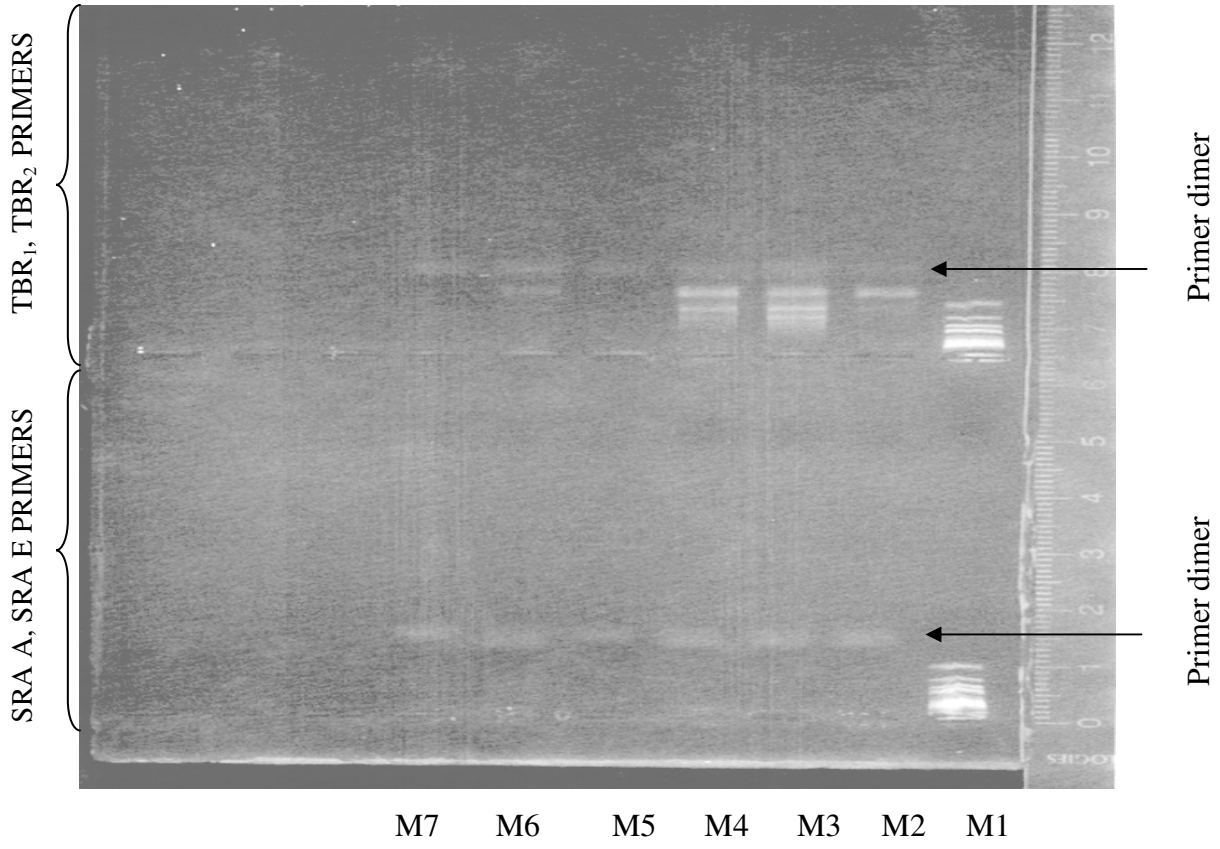

Figure 11: Composite figure of PCR products amplified from trypanosome-infected mice using stabilate KETRI 2710 electrophoresed through 1.5 % Agarose gel. Samples from right to left: 1). M1- 100 bp ladder marker, 2). M2- KETRI 2710 (sample), 3). M3- *T. brucei* 3738, 4). M4- *T. brucei* 42, 5). M5- *T. congolense*, 6. M6- (unknown field sample of trypanosoma species). 7).

M7- Negative control (sterile distilled water). Group samples were amplified and run together in-order to compensate for differences in PCR conditions and gel staining / image analysis.

Trypanosoma brucei rhodisiense co-exists in domestic animals and are morphologically identical to *Trypanosoma brucei brucei*, which is not pathogenic to humans. However, there are no safe means of differentiating these parasites morphologically (Picozzi *et al.,* 2002). PCR analysis unequivocally differentiated *Trypanosoma brucei rhodesiense* from *Trypanosoma brucei brucei.* This can be attributed to the fact that strains of *Trypanosoma brucei rhodesiense* have a serum resistant associated gene (SRA), which has proven to be a reliable molecular marker for distinguishing it from *Trypanosoma brucei brucei* and other trypanosomes of subgenus trypanozoon (Gibson *et al.,* 2002). This SRA gene is not present in samples designated as *Trypanosoma brucei brucei* and therefore no amplification product was observed in our study. Comparison of our samples with other *Trypanosoma brucei* stabilates confirmed its purity since only a single band (lane M2) was observed as opposed to the multiple bands of samples in lanes M3 and M4 (*T. brucei* 3738 and *T. brucei* 42), respectively, which indicate multiplicity of genotypes.

4.5 Clinical symptoms

The pure *Trypanosoma brucei brucei* isolate 2710 stabilate was inoculated into experimental animals following this confirmation. The stabilate produced similar clinical effect in all infected animals. After a pre-patent period of five days the mice developed clinical signs of the disease. This was at first characterized by reduced food and water intake coinciding with the first wave of parasitemia. During the second week, the animals appeared sluggish, and had a starring coat. This was followed by late signs of the disease which included weight loss, oedema, hypoproteinemia, reduced activity and gasping, reduced co-ordination of the hind limbs during movement and enlarged abdomen due to splenomegaly. Ultimately the animals developed anaemia, sleepiness and a severe illness leading to death. However the infected mice given water only had died on day 11 post infection.

4.6 Parasitemia and PCV

Parasites were observed by microscopic examination of peripheral blood smears from infected mice on day five post infection which is in line with the parasites known incubation period of 5-10 days (Mare, 2000). Figure 12 shows a fixed smear of trypanosomes in their long slender form as seen by microscopic examination on day 8 post infection.

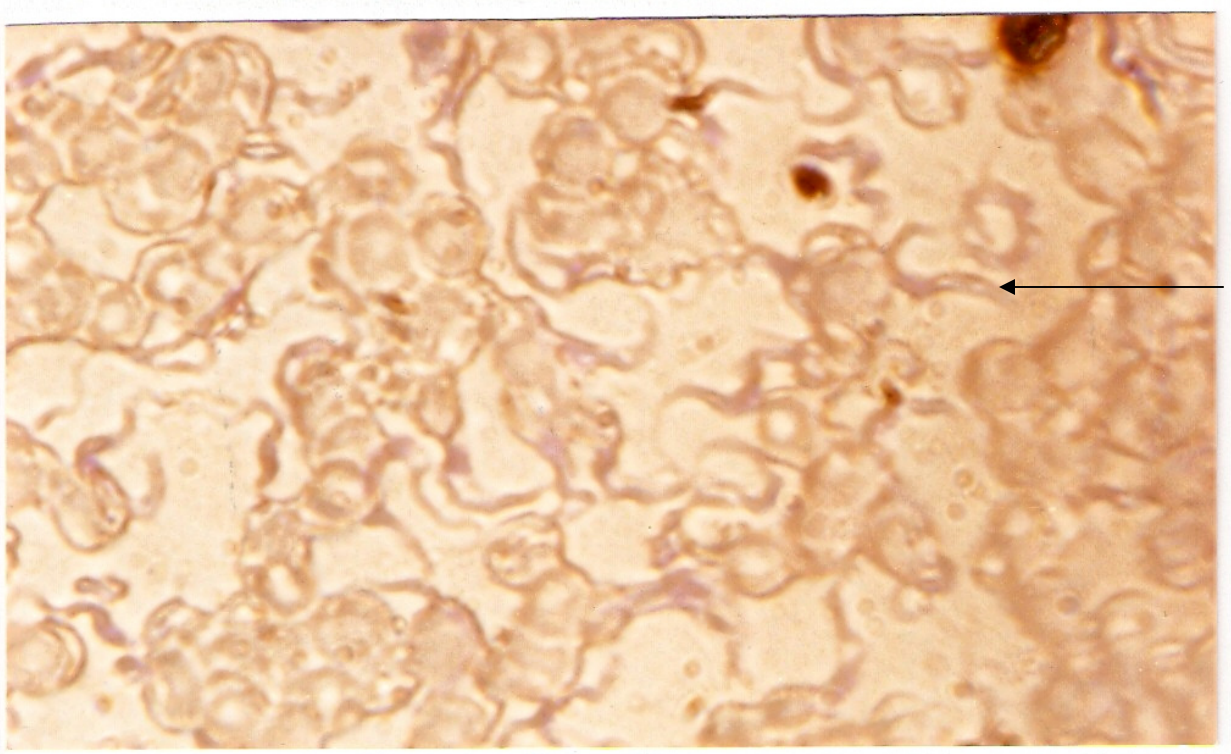

Figure 12: A fixed smear of trypanosomes (indicated by the pointer) in their long slender form as seen by microscopic examination on day 8 post infection (magnification x 3000).

Levels of parasitemia in control mice and experimental mice developed exponentially at similar rates and reached similar densities at the peak of parasitemia on the same day, namely day 7 post infection (Figure 13).

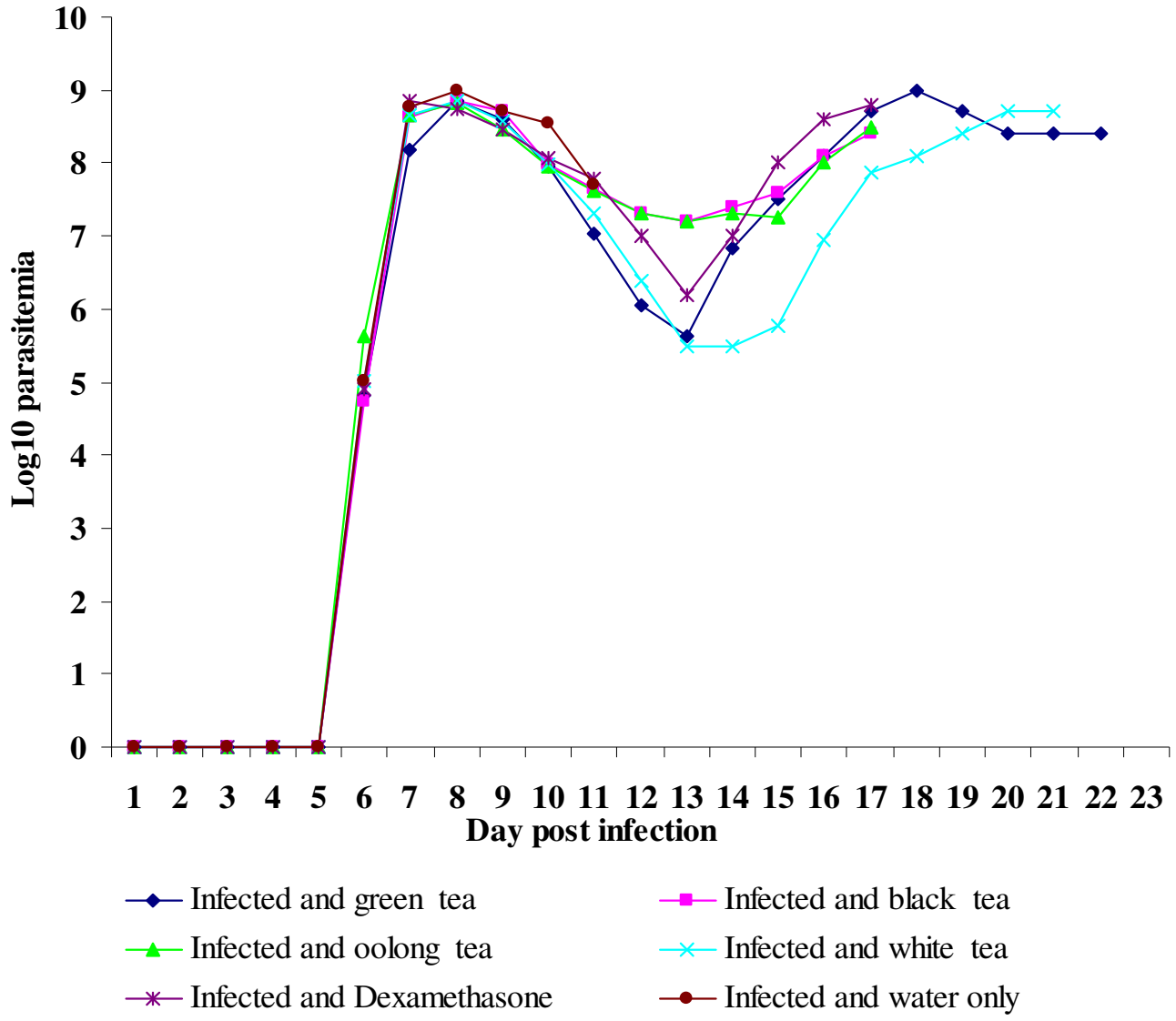

Figure 13: Time course of *Trypanosoma brucei brucei* stabilate KETRI 2710 for different treatments plotted as Log_{10} of parasites per milliliter of blood; the scale is linear and ranges from 0-10.

Transient parasitemia decline after parasitemic peak on day 9 to 13 post infection was significantly different ($P<0.05$) between the various groups. Parasitemia in the treated mice,

however, decreased more rapidly. The decline in parasitemia was analyzed at day 11 and 13 post infection to evaluate its levels and the effects of tea extracts on the parasites (Table 4). Day 11 post infection was chosen for analysis of the infected mice and given water since this was the last obtainable sample. However, the other treatments survived beyond this particular day.

Table 4: Values (Means ± SEM) of Log_{10} Parasitemia in mice infected with *Trypanosoma brucei brucei*.

Treatment	Day 11	Day 13
Infected and green tea	8.225 ±0.062 [a]	7.545 ±0.078 [a]
Infected and white tea	8.220 ±0.064 [a]	7.414 ±0.086 [a]
Infected and dexamethasone	8.371 ±0.069 [a]	7.865 ±0.120 [b]
Infected and oolong tea	8.293 ±0.077 [a]	7.995 ±0.010 [b]
Infected and black tea	8.358 ±0.069 [a]	8.041 ±0.096 [b]
Infected and water only	8.532 ±0.078 [b]	D
Non infected and water only	ND	ND
	C.V 7.44, P<0.05	C.V 5.38, P<0.01

Treatments marked with the same letters are not significantly different at P<0.05.
ND- not done since the animals were not infected with the parasite
D-indicates the animals had died on 11 DPI

On day 11 post infection it was evident that mice given tea had a significant reduction in parasitemia level from the infected and water only but no significant difference (P>0.05) was observed between tea treatments (Table 4). Though tea was not able to eradicate the parasites, it significantly reduced the level of parasitemia enabling the mice to relapse and thus extending the mean survival rate. At day 13 post infection, a significant parasitemia reduction was evident, with green and white teas having the highest reduction and significantly different (P<0.05) from other treatments including the drug (Table 4). The ability of tea to lower the level of parasitemia can be attributed to the toxic activity of polyphenols present in it. These compounds have the ability to complex with extracellular and soluble proteins and also parasite cell wall thereby disrupting the parasite cell membrane. The site and number of hydroxyl groups are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation

results in increased toxicity (Cowan, 1999). This explains why green and white teas were more effective in parasite reduction, since they contain catechins that are highly hydoxylated compared to black tea and oolong tea that have oxidized polyphenols. Recent studies using trypanosomes established that flavonoids possess *in vitro* trypanocidal activity against *Trypanosoma brucei rhodisiense* and *Trypanosoma cruzi* (Paveto *et al.,* 2004; Tasmedir *et al.,* 2006). These previous findings could help suggest that the *in vivo* efficacy of tea against *Trypanosoma brucei brucei* observed in this study is as a result of the phenolic compounds especially the flavan-3-ol group. We can therefore speculate that without the host immunological assistance, high concentration of tea flavonoids would be necessary to reduce *Trypanosoma brucei brucei* in the host. This indicates the need for detailed mechanistic studies together with the development of parasite-specific drug formulations, since the current treatment regimens, based on chemotherapy for these parasites are limited and not ideal since they are associated with severe side effects and development of drug resistance.

Accompanying these events, severe loss of total packed cell volume and tissue damage developed. *Trypanosoma brucei brucei* is a tissue invasive parasite, which induced a reduction in PCV in infected mice as in other susceptible animals such as rabbits, rats, cattle and dogs (Jenkins *et al.,* 1980; Erah *et al.,* 2003). The fall in PCV occurred within 4 days and this remained below the normal values until the terminal stages of the disease (Figure 14).

The reduction in PCV within 4 days post infection as evident in this study resulted to the rapid onset of the observed anaemia, which was associated with the parasitemia crises. Jennings *et al*. (1974) have reported similar results on studies in mice infected with *Trypanosoma brucei.* To study the progressive reduction of PCV and the effect of various treatments over time, the mean change in PCV was analyzed on day 11 and 17. The change in PCV was obtained by taking the difference from day 0 to day 11 and 17 post infection indicating therefore that the larger the PCV difference, the more there was a decline in PCV values as shown in Table 5. On day 11 there was a significant PCV difference ($P<0.05$) between animals treated using different teas and those infected and given water only. However no significant difference was observed between the tea treatments even on day 17. These observations suggest that tea could have a therapeutic role in cases of total packed erythrocyte volume reduction and the resulting anaemia.

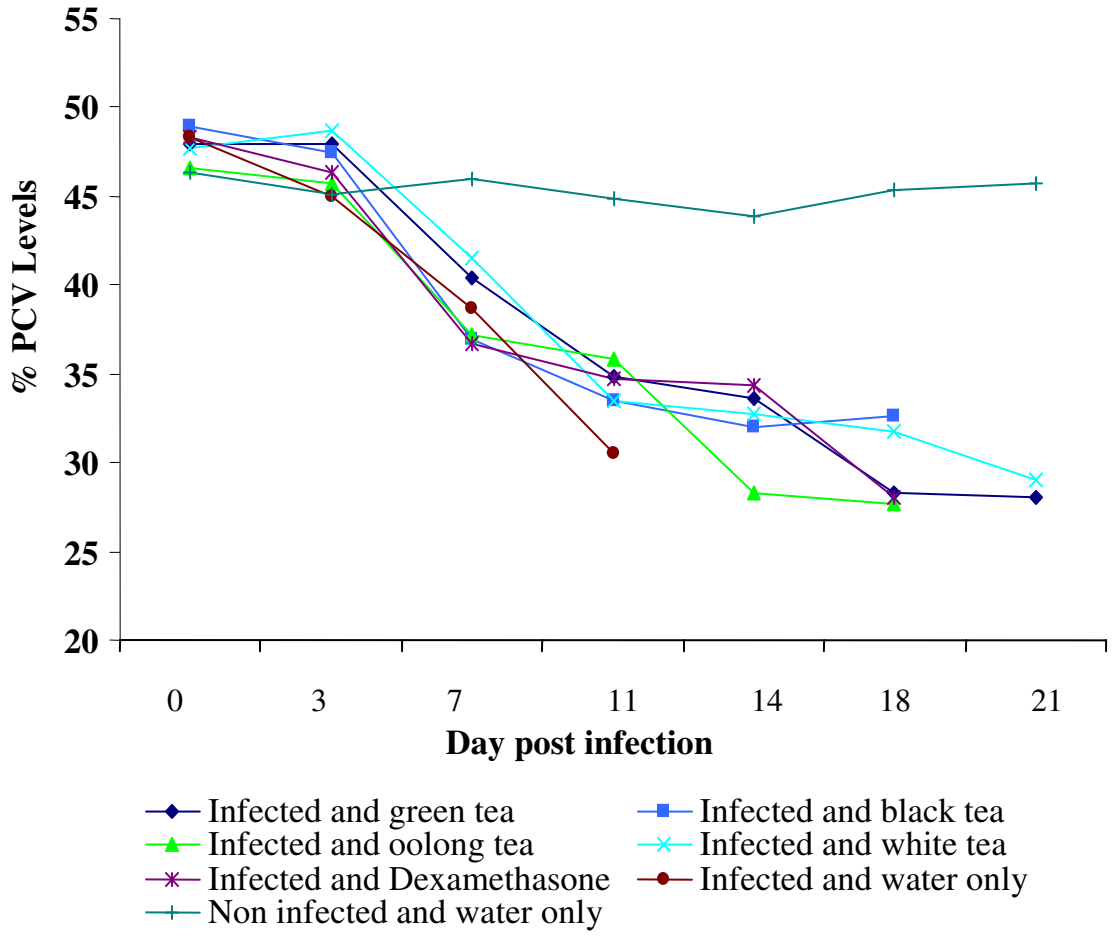

Figure 14: Changes in PCV during the period of study.

Table 5: Mean change (Means ± SEM) in PCV % of the treated animals and the control group from day 0 to day 11 and 17 post infection.

Treatment	Day 11	Day 17
Infected and green tea	13.300 ± 1.263 [a]	21.750 ± 2.412
Infected and white tea	13.714 ± 1.510 [a]	14.000 ± 2.413
Infected and dexamethasone	15.333 ± 1.631 [a]	22.333 ± 2.786
Infected and oolong tea	13.000 ± 1.787 [a]	21.000 ± 2.876
Infected and black tea	15.333 ± 1.631 [a]	13.000 ± 2.786
Infected and water only	21.250 ± 1.988 [b]	D
Non infected and water only	ND	ND
	C.V 6.97, P<0.037	C.V 6.29, NS

Treatments marked with the same letters are not significantly different at P<0.05.

ND- not done since the animals were not infected with the parasite

D-indicates the animals had died on 11 DPI

PCV reduction and anaemia are common and critical features in the pathogenesis of African trypanosomiasis contributing to the morbidity and mortality and thus curtailing the disease longetivity (Jennings *et al.,* 1974; Kagira *et al.,* 2006). The loss of total packed erythrocyte volume early in the infection may be due to haemolysis, which plays an important role in the generation of anaemia. This results from the direct binding of the trypanosomes antigens with specific receptors on the red blood cells giving rise to complexes which elicits the production of antibodies mainly IgM and endotoxins with a consequent lysis of red blood cells (Turay, 2005). The sensitized erythrocytes fix the complement and are then recognized by the mononuclear phagocytic cells of the spleen and liver. These phenomena facilitate phagocytosis of IgM and complement-sensitized erythrocytes via the C_3 receptor resulting to erythrocytes destruction (Amole *et al.,* 1982). This accounts for the invariable enlargement of the spleen (splenomegaly) in our infected mice. Blood-borne protozoal infections are frequently characterized by splenomegaly and subsequent sensitization of erythrocytes with immunoglobulins and this has been advanced as a contributory or exclusive cause of anaemia associated with *Plasmodium*

falcipirum which leads to malaria in humans (Jennings *et al.,* 1974). This may be analogous to the erythrophagocytosis seen in trypanosomiasis.

Infected mice given tea extracts in this study showed a significant increase in PCV compared to the infected mice given water only which can be ascribed to an enhanced resistance to erythrocyte haemolysis. This demonstrates clearly that tea flavonoids possess *in vivo* ability to protect erythrocytes from haemolysis, which can be attributed to the following factors. Firstly, tea flavonoids have been shown earlier in this study to lower parasitemia levels in infected mice and thereby decreasing the direct binding of trypanosomes antigens on the red cells and consequent lysis of the cells. Secondly, erythrocytes have membranes with a high content of polyunsaturated lipids and a rich oxygen supply making them vulnerable to lipid peroxidation. Reactive oxygen species generated during infections like trypanosomiasis can attack erythrocytes membrane, induce its oxidation and trigger haemolysis. However the antioxidant activity of tea reported earlier, might have elicited a rise in plasma antioxidant capacity stirring up a reduction in the susceptibility of erythrocyte membrane destruction. This is consistent with Zhu *et al.* (2002) observation that consumption of flavonol rich- cocoa and chocolate can result to an increased plasma antioxidant activity in healthy human adults. With these findings, we can hypothesize that ingestion of tea would reduce the risk of free radical induced oxidative damage to the erythrocytes though further work involving characterization of membrane binding and action of tea flavonoids is clearly warranted. Similar work might further explain if tea can be used as a treatment in cases of anaemia resulting from erythrocyte destruction. During the late stages of infection, when the infected mice were immunosuppressed, haemodilution and splenomegaly contributed to the progressive decline in PCV (Murray *et al.,* 1974; Amole *et al.,* 1982). This led to the low metabolic rate, general weakness and death of the animals.

4.7 Effect of Tea on Albumin levels

The changes in serum albumin levels are shown in Figure 15. Treatment with various teas resulted in a significant amelioration ($P<0.01$) of parasite-induced hypoalbuminemia.

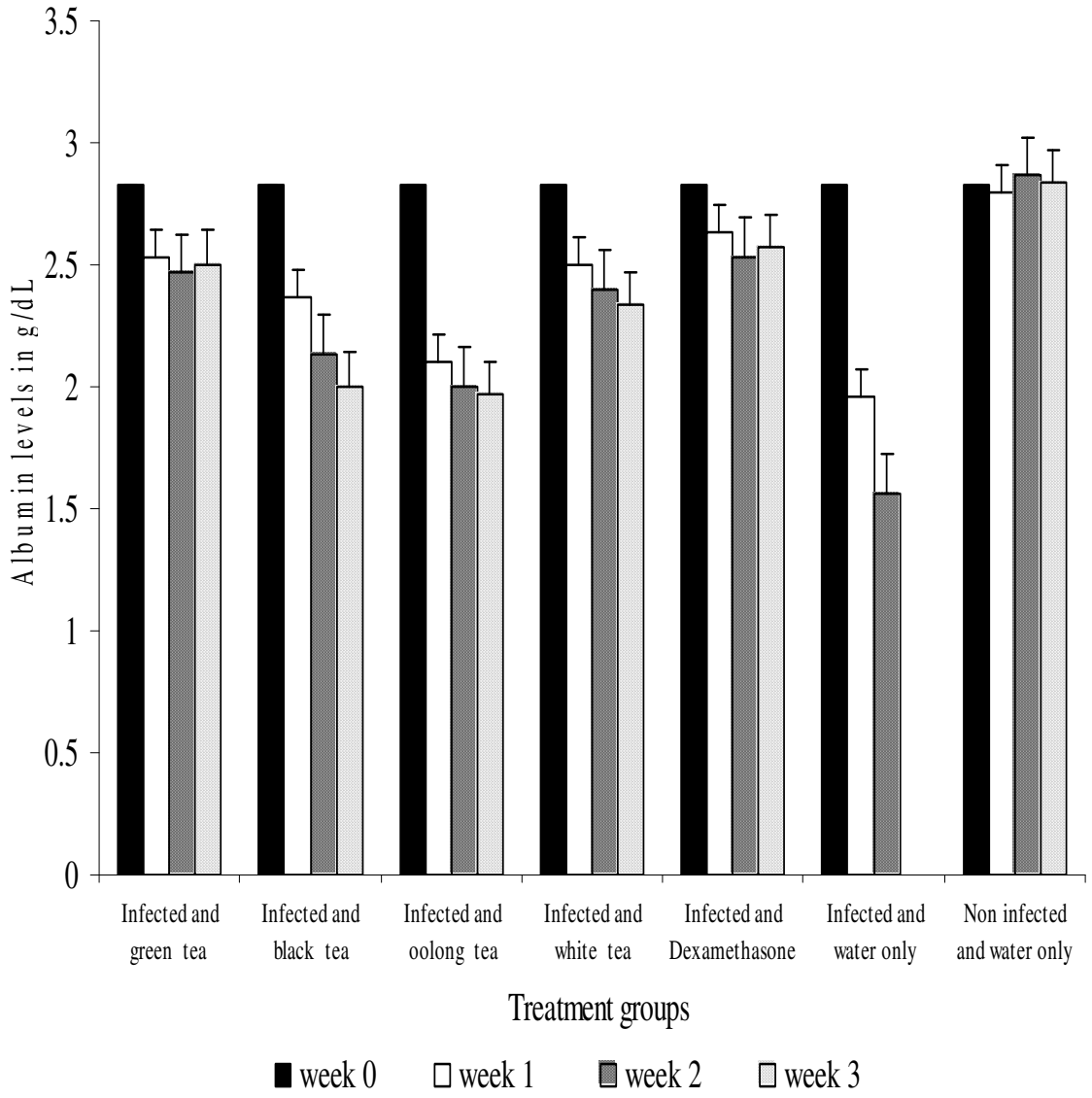

Figure 15: Means (± SEM) in albumin levels post infection in various treatments.

The change in albumin levels was analyzed on day 11 and 17 post infection to evaluate the effect of various treatments over time as shown in Table 6.

Table 6: Albumin levels g/dL (Means ± SEM) of the treated animals and the infected and non-treated animals between day 11 and 17.

Treatment	Day 11	Day 17
Infected and green tea	2.611 ± 0.048 [a]	2.583 ± 0.053 [a]
Infected and white tea	2.578 ± 0.048 [a]	2.517 ± 0.053 [a]
Infected and dexamethasone	2.670 ± 0.048 [a]	2.641 ± 0.053 [a]
Infected and oolong tea	2.311 ± 0.052 [a]	2.250 ± 0.056 [b]
Infected and black tea	2.433 ± 0.048 [a]	2.325 ± 0.053 [b]
Infected and water only	2.110 ± 0.048 [b]	D
Non infected and water only	2.876	2.876
	C.V 5.88, P<0.01	C.V 7.47, P<0.01

Treatments marked with the same letters are not significantly different at P<0.05.

D-indicates the animals had died on 11 DPI

The non-infected (placebo) group had albumin levels within the normal range throughout the duration of the experiment. On day 11, mice treated with dexamethozone, an anti-inflammatory drug produced the highest albumin but it was not significantly different P<0.01) from other treatments. Dexamethasone is a catabolic glucocorticoid and a potent ant-inflammatory drug with broad spectrum of mechanism of action. The observed increase in albumin levels could have resulted from the ability of dexamethasone to increase albumin mRNA content by inducing the transcription of the albumin gene (Douglas *et al.,* 2005). Black and oolong teas had marginally lower albumin levels than those of green and white teas though not significantly different (P>0.05). Infected untreated mice had the lowest levels compared to other groups. However on day 17, black and oolong teas had a lower and significantly different P<0.01 albumin level compared to other treated animals.

Albumin is produced entirely by the liver as a principle target of systemic inflammatory mediators. Its production helps in supplying the necessary components for immediate defense at the site of tissue damage, clearing harmful agents and ultimately aiding in tissue repair

(Baumann and Gauldie, 1994). In regulating immune responsiveness, the liver cells influence the synthesis of acute phase proteins, which mount a systemic acute phase response in the mammalian host against infection, inflammation or trauma (Stadnyk and Gauldie, 1991; Baumann and Gauldie, 1994; Kitani *et al.,* 2004). *Trypanosoma brucei brucei* infection leads to the stimulation of an acute phase response that causes a multi-factorial non-specific host defense mechanism, which is triggered by a systemic release of pro-inflammatory cytokines. These cytokines activate receptors on different target cells leading to a systemic reaction that is characterized by fever and changes in the concentration of serum proteins (Gruys *et al.,* 1994; Eckersall *et al.,* 2001 and Maina *et al.,* 2004).

These latter proteins include albumin, which is a negative acute phase protein since it shows a decrease (hypoalbuminemia) in levels upon specific stimulation by cytokines (Gruys *et al.,* 1994; Kitani *et al.,* 2004; Orhue *et al.,* 2005; Ramiro *et al.,* 2005). The rate of albumin synthesis in the liver has been shown to vary with disease states and its concentration decreases, often dramatically, from early in the course of a critical illness without an expected increase until the recovery phase of the illness (Nicholson *et al.,* 2000). Sustained inflammatory response in critical illnesses like in this case of *Trypanosoma brucei brucei,* causes a prolonged reduction of albumin synthesis. The levels of albumin in such a case can therefore be used as a prognostic indicator in the progress of an inflammatory response. Studies have previously demonstrated that serum albumin is a late-acting acute phase protein clinically evident during inflammation (Tsirpanlis *et al.,* 2005). In rats with turpentine-induced inflammation, serum albumin concentration was markedly decreased emphasizing on its important role as a valuable predictor of inflammatory conditions (Moshage *et al.,* 1987). Kaysen *et al.* (2001) observed that chronic inflammation is associated with hypoalbuminemia and used serum albumin levels as markers of inflammation in hemodialysis patients. Besides this, the reduction in albumin levels observed in this study may have contributed to the facial and scrotal oedema observed in the infected mice. This is because it is of great importance in regulating flow of water between the plasma and tissue fluid by its effect on colloid osmotic pressure (Kaysen *et al.,* 2001; Orhue *et al.,* 2005).

Oral administration of tea extracts in this study produced a significant ($P<0.01$) elevation in albumin levels in *Trypanosoma brucei brucei* infected mice thereby indicating a decreased effect on inflammation induced by the trypanosome parasite. This effect can be ascribed to the presence of flavonoids. Tea flavonoids and evidence for their role in the prevention of many

degenerative diseases is emerging (Tasmedir *et al.,* 2006). The ability of tea flavonoids to elevate albumin levels and the resultant putative anti-inflammatory effects can be accredited to various properties. These ubiquitous compounds have the ability to exert strong antioxidant effects based in part on their structural characteristic especially the 3', 4', -dihydroxylation of the B-ring in the catechol moiety. These structural features of flavonoids represent the molecular basis for their hydrogen donating (radical-scavenging) and reduction of reactive oxygen species, which have been implicated in the pathogenesis of inflammatory diseases (Hansely *et al.,* 2000). Green tea contains flavan-3-ols or catechins, which include EGCG, ECG and EC with EGCG being the major constituent and also the component with the highest antioxidant property. Catechins undergo major enzymatic biotransformation to form theaflavins and thearubigins which are the characteristic constituents in black tea but which have less antioxidant capacity as described earlier in this study. During inflammation, toxic oxidants, including oxygen species are generated. The phenolic hydroxyl substitutions present mainly in EGCG act as potent radical scavengers, increasing the capacity of endogenous antioxidant defenses and thereby modulating the cellular redox state (Amie *et al.,* 2003; Mandel *et al.,* 2005). This ability to strengthen the physiological antioxidant defense system helps improve the chronic inflammatory condition as observed in this study.

Teas flavonoids have the potential to augment an individual's antioxidant system and therefore the need to market tea as a radical scavenger/antioxidative drink. There are other cellular mechanisms through which tea flavonoids can affect inflammation besides the scavenging of free radicals of toxic oxidants and these include stimulation of suppression of inflammatory cells during infections thus lowering nitric oxide (NO)-mediated endothelial changes (Tedeshi *et al.,* 2004). Millar *et al.* (1999) infected mice with *Trypanosoma brucei brucei* and observed an increase in NO production resulting to a slow decline in parasitemia after the first parasitemia peak. Nitric oxide is synthesized by the enzyme NO-synthase (NOS) and may have a protective role as an antiparasite. However an aberrant expression of NOS enzyme leads to inappropriate NO production and thus has been implicated in human inflammatory diseases (Chen *et al.,* 2004; Tedeshi *et al.,* 2004). To attenuate inflammation, tea is thought to block the cytokine-induced nuclear factor-κB, which controls a wide variety of genes active in inflammation including those for the enzyme NO-synthase (Varilek *et al.,* 2001). Since NF-κB plays an important role in the pathogenesis of chronic inflammation, its inhibitors have been

shown to inhibit inflammatory diseases (Varilek *et al.,* 2001; Tedeshi *et al.,* 2004). Previous studies using concentrated forms of green tea polyphenols on mice model of chronic inflammatory bowel disease (IBD) showed a decrease in inflammation, which was postulated to increased NF-κB inactivation by the polyphenols (Varilek *et al.,* 2001).

Therefore, the elevation of serum albumin levels which was used as a marker of inflammation induced in *Trypanosoma brucei brucei* infected mice clearly indicates that ECGG, the main polyphenol in green and white teas and TFs present in black tea have the ability to inhibit oxidative stress activation and this may be responsible for the anti-inflammatory effects of tea. It is evident from this study that tea flavonoids elevated albumin levels and this may be promising at least for tea as an auxiliary anti-inflammatory in chronic inflammatory diseases. The potential for the consumption of tea or tea polyphenols to prevent or ameliorate chronic disease is currently the subject of considerable scientific investigation. Although a number of mechanisms have been proposed for the beneficial effects of tea in different models of chronic disease, the radical scavenging and antioxidant properties of tea polyphenols are frequently cited as important contributors. Much of the evidence supporting an antioxidant function for tea polyphenols is derived from assays of their antioxidant activity *in vitro*. However, evidence that tea polyphenols are acting directly or indirectly as antioxidants *in vivo* is more limited. Animal studies offer a unique opportunity to assess the contribution of the antioxidant properties of tea polyphenols to the physiological effects during oxidative stress

4.8 Histopathological findings

To further validate the anti-oxidative and anti- inflammatory properties of tea extracts on the mice, the effect of tea on liver injury was determined. Following infection using *Trypanosoma brucei brucei* there was an infiltration of lymphocytes mainly at the periportal regions. However, as time progressed during the infection period, the lymphocytes were replaced by macrophages. In addition to the periportal infiltration, late stage of the infection was characterized by cells infiltration into the liver parenchymal tissue. This was accompanied by the liver cells undergoing karyohexis and later karyolysis.

Comparison of the pathology between the liver of animals infected and treated using various tea extracts and infected untreated animals indicated a reduction in the pathology of tea treated animals. The effect was observed as a reduction in the cellular infiltration both at the

periportal region and in the liver parenchyma. However, for green tea especially at the early stages (day 7 post infection) there was a marked increase in periportal infiltration despite the effective reduction in parenchymal infiltration (Figure 16). The order of increased reduction after green tea was black tea followed by white tea.

At day 11 post infection, white tea showed an improvement in the reduction of periportal and parenchymal infiltration followed by green tea, oolong tea and black tea respectively (Figure 17). At day 21 post infection, the same trend was observed with the reduction more marked in the liver of mice treated with white tea followed by green tea, black tea and oolong tea, respectively (Figure 18). In general, green tea showed a reduced pathology throughout the infection period whereas white tea showed improved reduction in later stages of infection namely day 11 and 21. Black tea performed well at day 7 but showed reduced effect on day 11 and 21. Oolong tea showed intermediate effect at mid infection (day 11 post infection) but had little effect by day 21.

Specific organ damage during trypanosomiasis is one of the major contributing factors to the disease progress. Infection of mammalian host with this pathogenic intracellular protozoan parasite results in chronic persistence of the parasite, which is associated with a progressive inflammatory destruction of the target tissues including the liver (Murray *et al.,* 1974; Muller *et al.,* 2001). In this study, tea reduced the severity of liver damage as observed in the minimal degree of cellular infiltration into the periportal and parenchymal regions and ultimately reducing cellular death (karyohexis and karyolysis) as compared to the untreated mice. The minimal infiltration is an indication that tea could have aided the host immune resistance during the experimentally induced trypanosomiasis. A previous study has shown that tea enhances innate immunity during experimentally induced trypanosomiasis (Bukowski, 2004). Elsewhere tea polyphenols have been shown to prevent toxin-induced hepatotoxicity in mice (Chen *et al.,* 2004) though the mechanism underlying this protective effect on liver damage is not known. It can be speculated that oxidative stress during trypanosomiasis infection caused liver damage. Therefore tea acted as a potent radical scavenger, reducing any free radical metabolites thus preventing liver hepatotoxicity and ultimate inflammation. This explains why white and green teas performed better in reducing liver damage because of the presence of unoxidised catechins especially EGCG which previously have been shown to be potent radical scavengers compared to oolong and black teas whose catechins have been oxidized to TFs and TRs. Because tea can

be consumed over long periods of time without any obviously known side effects, its possible role as adjunct therapeutic agent in human inflammatory liver disease deserves consideration.

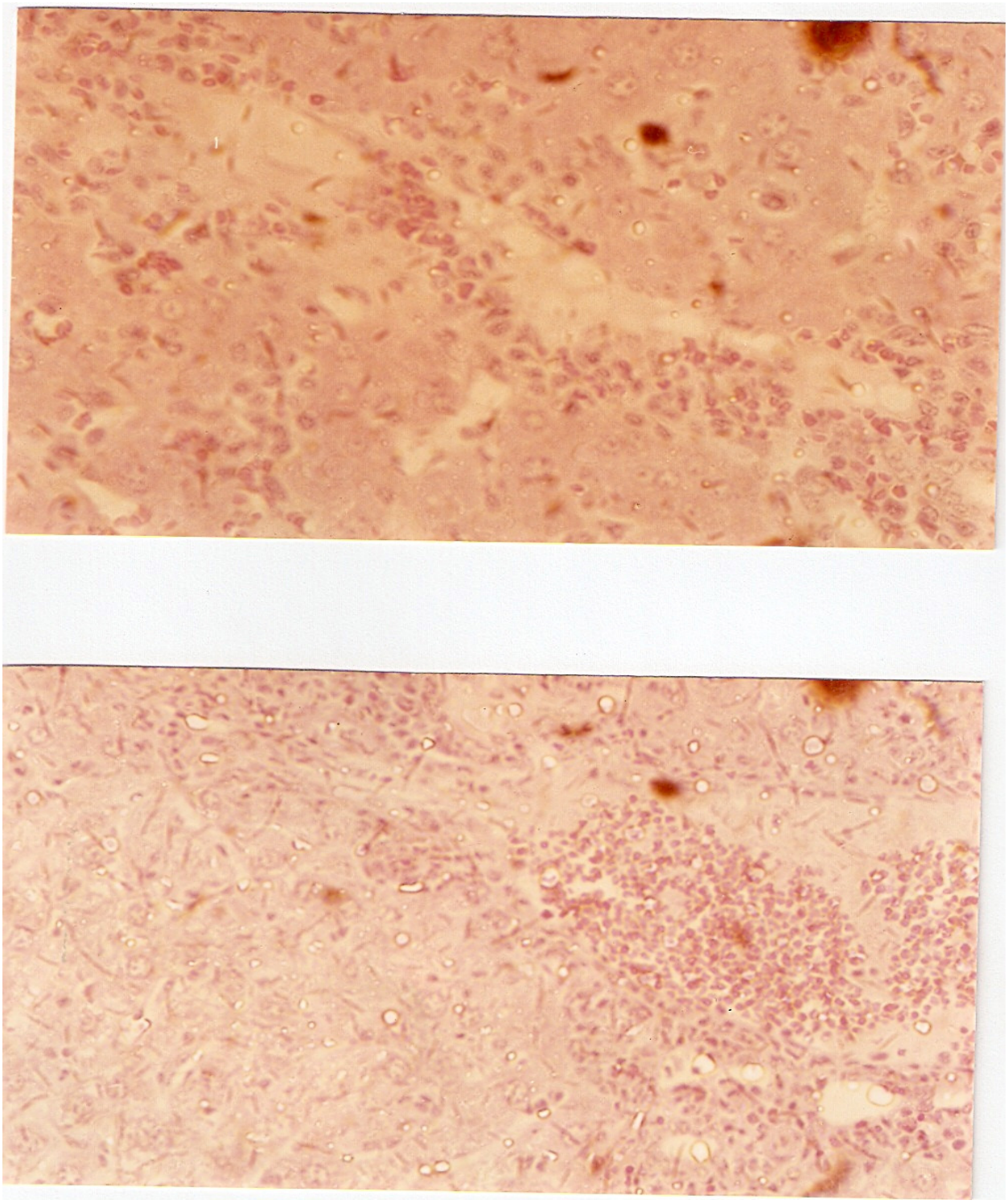

Figure 16: Representative liver sections showing histopathological profiles of mice infected using *Trypanosoma brucei brucei* and given water only (top) and green tea (bottom) as seen on day 7 post infection. The liver sections were stained with hematoxylin and eosin dyes and magnified at x3000 in group A and x1875 in group B.

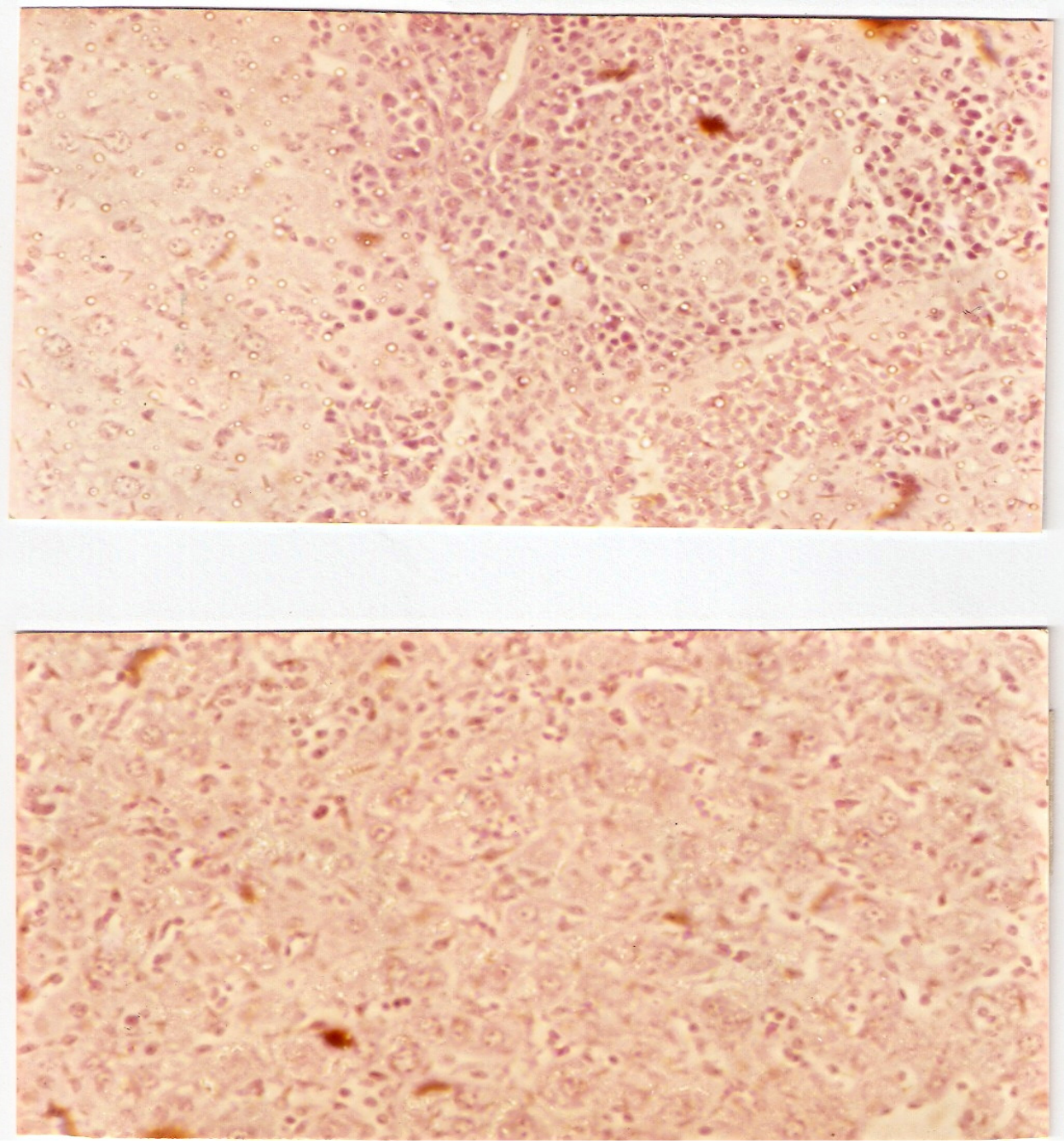

Figure 17: Representative liver sections showing histopathological profiles of mice infected using *Trypanosoma brucei brucei* and given water only (top) and white tea (bottom) as seen on day 11 post infection. The liver sections were stained with hematoxylin and eosin dyes and magnified at x1875.

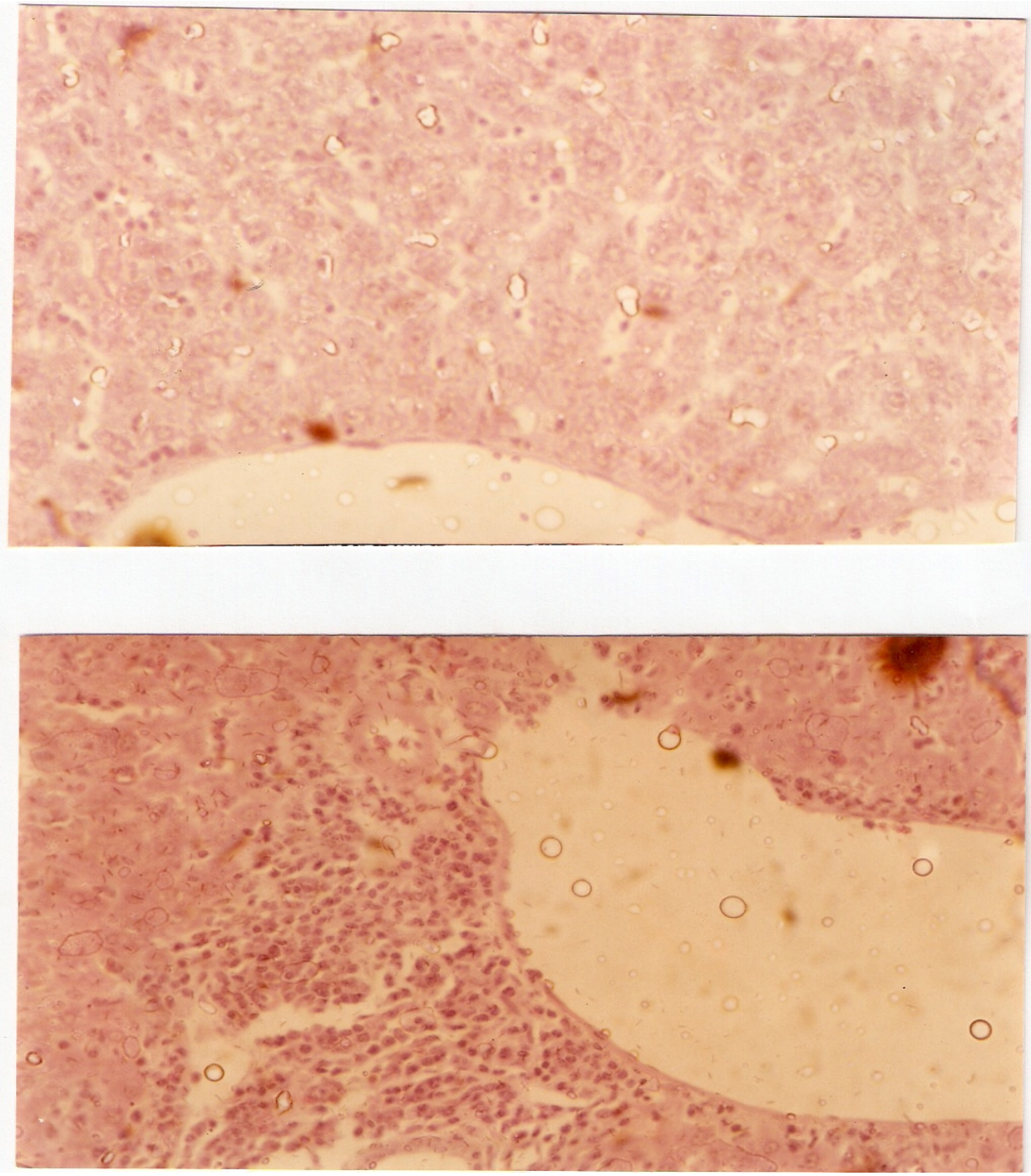

Figure 18: Representative liver sections showing histopathological profiles of mice infected using *Trypanosoma brucei brucei* and given white tea (top) and black tea (bottom) as seen on day 21 post infection. The liver sections were stained with hematoxylin and eosin dyes and magnified at x1875.

4.9 Effect of tea extracts on survival rate

The evidence emanating from this study on the antioxidant capacity and anti-inflammatory properties of tea flavonoids led us to investigate the effect of tea on the survival rate of infected treated mice. Univariate survival analysis using Kaplan-meir method showed that infected mice given tea extracts or dexamethasone had a significant difference $P<0.042$ (log-rank test) in the larger survival rates compared to the infected untreated group as shown in Figure 19. All mice were dead on the 22^{nd} day with the mice in the infected untreated group dead as at day 11^{th}. In contrast, the survival rates in the treated groups tended to improve with green and white teas showing the longest survival rates though marginally lower for black and oolong teas.

Thus, it was evident that tea flavonoids significantly prolonged the survival period of the infected animals even better than the known anti-inflammatory drug (dexamethasone). The ability of tea flavonoids to prolong life can be attributed to their ability to aid total antioxidant defense system. Tea has been shown to reduce oxidative stress by protecting the defense system against the damaging effects of reactive oxygen species such as singlet oxygen, peroxyl radicals, nitric oxide and peroxynitrite (Vanessa and Williamson, 2004). These antioxidative properties of flavonoids led to a down-regulation of inflammation as observed in this study and ultimately it can be hypothesized that they produced a profound effect on survival rate.

However, despite the observed effect on the survival rate, there is a scarcity of information on the mechanism through which tea is thought to prolong life. By mopping free radicals from the body, antioxidants are known to protect DNA from nucleotide oxidation and subsequent mutation whose accumulation among others is responsible for ageing. The capacity of tea to prolong life can however be resolved if extensive studies are performed to establish the mechanisms.

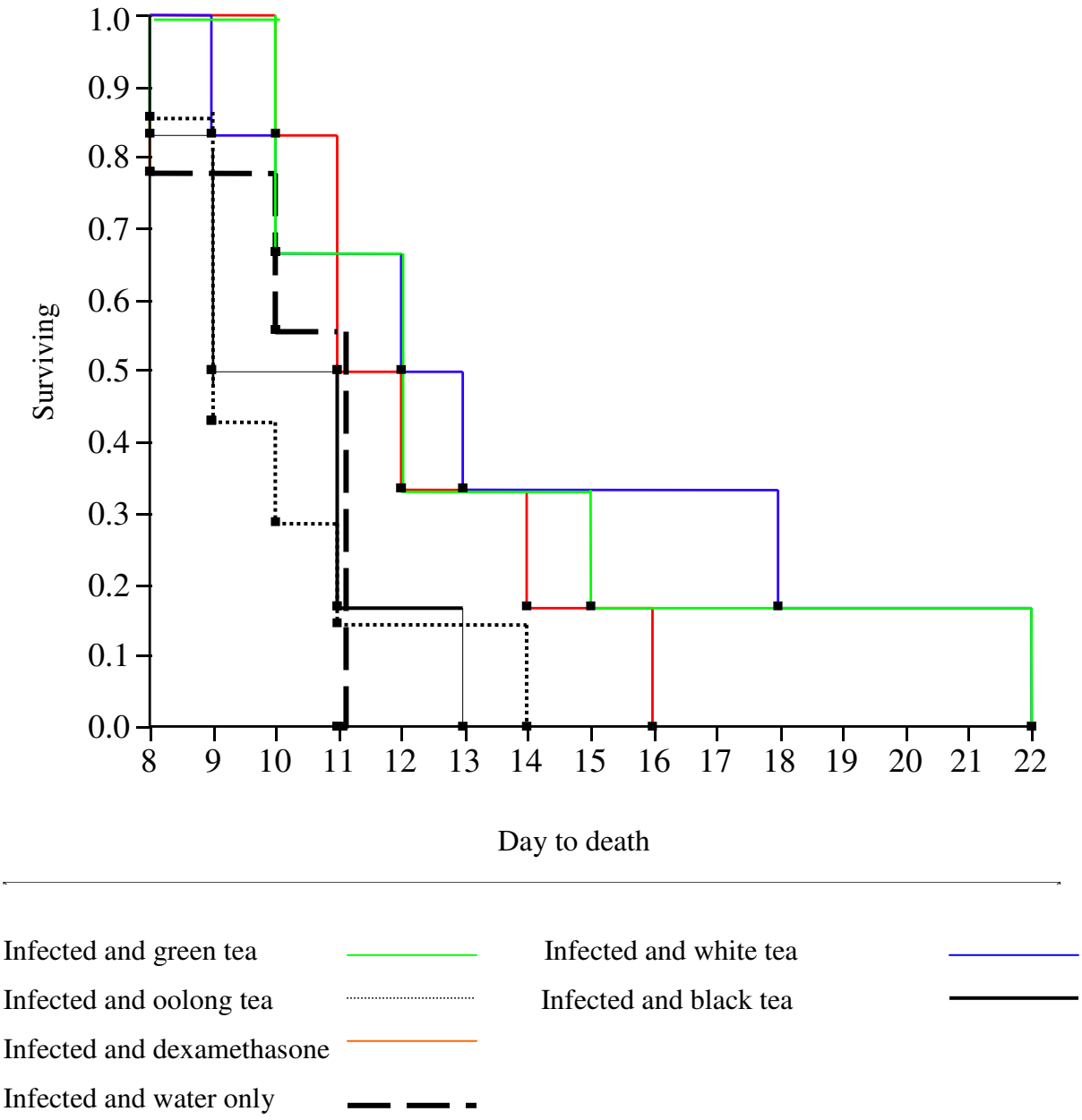

Figure 19: Kaplan-Meir survival curves to compare survival rate in mice treated with tea and dexamethasone with the infected control given water only. The survival rates are significantly different from the control group, $P<0.05$ (Logrank test).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The results obtained in this study clearly established that tea had a significant effect as an antioxidant, anti-inflammatory and a life prolonging compound. Inflammation and several diseases often result from the effects of free radicals the most important ones being superoxide, hydroxyl, singlet oxygen and nitrites. The efficient radical scavenging property of tea extracts is due to the presence of polyphenols a property that is of great importance in the treatment of degenerative diseases.

Tea, a widely consumed polyphenolic beverage plays a significant role as a naturally occurring health enhancing drink and hence contributes to human health. This can mainly be attributed to the presence of bioactive micronutrient molecules, which belong to a family of polyphenols. These functional components in tea are extremely active, have a profound effect on cell metabolism, often demonstrate few side effects, are water soluble and thus excrated by the body unlike fat based synthetic substances that are absorbed and retained even at potentially harmful levels (Mandel *et al.,* 2005; Aqil *et al.,* 2006).

It is these novel pharmacological activities of polyphenols that are arousing interest in their possible clinical use for prevention and therapeutic use in several diseases. Unfortunately, there are too few controlled studies with most of these micronutrients and considerable skepticism about their efficacy still exists, largely because the molecular mechanisms responsible for their beneficial health enhancing effects remain unknown. To try and resolve this impasse, a well controlled study was preformed both *in vitro* and *in vivo* to investigate the potential of tea polyphenols as health enhancing agents.

Additionally, this study compared and ranked the activity of theaflavins (TFs) and thearubigins (TRs) in black tea and catechins in green tea to be not significantly different. Under the same *in vitro* experimental conditions, TFs and TRs possessed antioxidant potency comparable to that of catechins on equal concentration basis. Although green and white teas were superior in most other characteristics, black tea, which is the principle tea product from Kenya, displayed remarkable properties some even comparable to those of green teas. This observation confirms that black tea processed from polyphenol rich germplasm (varieties) such

as those from Kenya can be pharmacologically efficacious as green teas. This information provides an appropriate marketing tool to the Kenyan tea industry.

5.2 RECOMMENDATIONS

From results obtained in this and other previous studies, tea polyphenols could serve as models for the rationale design of synthetic analogues with higher *in vitro* and *in vivo* activities and more favorable chemical properties. It is necessary to give serious considerations to this issue and support the fact that tea can be used as a support treatment to various degenerative diseases.

Few studies have in the past been carried out to ascertain the health properties of black tea, which is currently facing a glut in the market. To achieve an increase in the unit prices fetched from exported tea, there is a dire need to ensure concomitant growth of the black tea market and as well as product diversification. This will entail the value addition and branding of raw tea and the development of diversified tea products such as ready to drink (RTD) teas with high functional components for sale as health products and raw materials for fast moving consumer products and environmentally friendly home and industrial cleaning and microbial agents. Similarly, tea can be used as a pharmacological source of the anti-inflammatory compounds namely polyphenols which can be extracted and marketed as rich sources of the compounds thus expanding its use from the traditional beverage. However, to achieve the above, it will be necessary to fully index and describe black tea from Kenya so that it is no longer marketed as a generic product for blending teas of lesser quality from other traditional tea producing countries like is the case currently. If brought to bear, these efforts would enhance the returns from tea farming enterprise and also contribute to the creation of the many needed jobs.

It is important and critical for the Kenyan tea industry to continue enhancing the knowledge base on its tea products, which will enable the industry to promote “tea for health for life” message globally. This is because nutraceuticals and naturally bioactive compounds like tea will continue to be a focus to the scientific community and food consumers who are motivated to enhance their quality of life, and in particular, to manage chronic and associated aging diseases.

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