ANTIDIABETIC AND WOUND HEALING PROPERTIES OF SELECTED KENYAN TEA CULTIVARS

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DECLARATION AND RECOMMENDATIONS

DECLARATION

This thesis is my original work and has not been submitted or presented for examination in any institution.

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SM14/2735/10

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RECOMMENDATION

We wish to confirm that this research thesis has been prepared under our supervision and have our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

To my loving parents Mr. and Mrs. Wycliffe C. Tomno

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ABSTRACT

Diabetes mellitus is a metabolic disorder of multiple etiology, characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both. It is associated with significant morbidity and mortality due to cardiovascular complications as a result of imbalance in lipid metabolism. Diabetic foot ulcers (DFUs) are estimated to occur in 15% of all patients with diabetes and precede 84% of all diabetes-related lower leg amputations. Tea is rich in bioactive molecules that may play a role in modulating carbohydrate metabolism and wound healing. The study sought to determine if tea can prevent or reverse metabolic disturbances induced by diabetes, including occurrence of diabetic wounds. Green and black teas were processed from four different cultivars and assayed for their total monomeric anthocyanin content, total polyphenols content, catechin profiles and theaflavin profiles. Teas rich in anthocyanins, epigallocatechin gallate (EGCG), theaflavins (TF) and total polyphenols were chosen for assay in an animal model. An alloxan induced diabetic mouse model was used to study the anti diabetic potential of processed tea. Hyperglycaemia resulting from alloxan administration caused a significant ($p<0.05$) increase in blood glucose and cholesterol levels as well as low body weights and packed cell volume (PCV). Oral administration of tea helped alleviate these complications. Blood glucose and cholesterol levels were lowered in treated groups compared to controls. PCV levels increased while body weights declined in both diabetic and healthy mice on tea. Anthocyanin rich tea produced more beneficial effects compared to other teas.

Wound healing, angiogenesis and wound repair was augmented in anthocyanin treated group when compared to the placebo group, findings well documented by histological investigations. Results of this study demonstrate that hyperglycaemia and deranged carbohydrate metabolism plays a significant role in diabetic complications including artheriosclerosis and delayed wound healing. Moreover, these results demonstrate beneficial effects of tea in wound healing and diabetic complications.

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CETP	Cholesterol ester transfer proteins
CHD	Coronary heart diseases
CVD	Cardiovascular disease
DFUs	Diabetic foot ulcers
DM	Diabetes mellitus
EC	Epicatechin
ECG	Epicatechingallate
EGC	Epigallocatechin
EGCG	Epigallocatechingallate
EPCs	Endothelial progenitor cells
GC	Gallocatechin
HDL	High density lipoprotein
LDL	Low density lipoprotein
PCV	Packed cell volume
TF	Theaflavins
TFDG	Theaflavin di-gallate
TG	Triglycerides
TR	Thearubigins
TRFK	Tea Research Foundation of Kenya
VLDL	Very low density lipoprotein

CHAPTER ONE
INTRODUCTION

1.1. Background Information

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and/or increased cellular resistance to insulin. Chronic hyperglycemia and other metabolic disturbances of DM lead to long-term tissue and organ damage as well as dysfunction involving the eyes, kidneys, nervous and vascular systems (American Diabetes Association, 1998).

Type 2 diabetes mellitus is the most common form of DM worldwide and its prevalence is increasing (Smyth and Heron, 2005). Its underlying defects can vary from predominant insulin resistance with relative insulin deficiency to a predominant insulin secretory defect with insulin resistance (Erkelens, 2001). A great deal of heterogeneity exists and most patients with type 2 DM do not initially require insulin therapy (Pignone *et al*., 2010).

Because the onset is frequently insidious, many patients with type 2 DM are asymptomatic and remain undiagnosed for years. Upper body obesity is a recognized risk factor because it results in peripheral insulin resistance (Caballero *et al*, 2003). The beta cells compensate for this resistance by increasing insulin secretion and maintaining normal glucose tolerance (Griffin *et al*., 2000).

Type 2 DM is associated with significant morbidity and mortality due to cardiovascular complications (Turner *et al*., 1998). The incidence of cardiovascular disease (CVD) is more common in patients with type 2 diabetes than in the general population (Kannel and McGee, 1979). Dys-lipidemia, an established risk factor for CVD, is strikingly common in patients with type 2 diabetes, affecting almost 50% of this population (Saydah *et al*., 2004). In addition to hyperglycemia and hypertension, dys-lipidemia is a modifiable CVD risk factor that remains largely uncontrolled in patients with type 2 diabetes (Turner *et al*., 1998).

Hyperglycemia increases the risk of microvascular complications (Stratton *et al*., 2005) while dys-lipidemia is a major risk factor for macrovascular complications in patients with type 2 diabetes (Turner *et al*., 1998; Farmer, 2008,). Elevated low-density lipoprotein cholesterol (LDL-C) is a major risk factor for CVD. As such, management of LDL-C is the primary goal of therapy for diabetic dys-lipidemia (Goff *et al*., 2007; Brunzel *et al*., 2008). Furthermore, type 2 diabetes

increases the risk of CVD mortality independent of LDL-C levels, adding to the greater overall cardiovascular risk in this population (Stamler *et al*., 1993). Therefore, aggressive lipid treatment goals have been recommended for patients with type 2 diabetes (Grundy *et al.,* 2004).

The foot ulcer is a leading cause of hospital admissions for people with diabetes in the developing world (Reiber, 1999). It is a major morbidity associated with diabetes, often leading to pain, suffering and a poor quality of life for patients. Diabetic foot ulcers (DFUs) are estimated to occur in 15% of all patients with diabetes and precede 84% of all diabetes-related lower leg amputations (Reiber *et al*., 1995). Despite the existence of protocols to standardize care, the physiological impairments that can result in DFUs complicate the healing process. Currently, the only food and drug administration approved growth factor and cell therapies for DFUs are not routinely used during treatment, preventing professionals from implementing evidence-based protocols (Brem *et al*., 2006).

Small dense LDL particles become oxidized and form plaques in arteries causing arteriosclerosis (Haffner *et al*., 1999). In addition, diabetic foot ulcers mainly occur as a result of impaired angiogenesis (Jeffcoate and Harding, 2003). Endothelial progenitor cells, a major cell type in angiogenesis have impaired function in diabetes (Roncalli *et al*., 2008) in addition hyperglycemia increases generation of superoxide anions in skin tissue resulting in poor wound healing (Luo *et al*., 2004). Currently there are no medications for type 2 diabetes and its complications. However management of the condition is thus through exercise and diet.

In this study, the effect of tea flavonoids on cholesterol profiles and wound healing in diabetic mice models was evaluated. Tea flavonoids are antioxidants and it was hypothesised that they might help in lowering levels of oxidized LDL, as well as scavenging for free radicals. Recent studies have revealed that diabetes can increase oxidant stress in blood and treatment with antioxidants such as vitamin E and flavonoids may be used to lower oxidative stress and other diabetic complications (Crespy and Williamson, 2004). There is increasing evidence that as antioxidants, polyphenols may protect cell constituents against oxidative damage and therefore limit the risk of various degenerative diseases associated with oxidative stress (Luqman and Rizvi, 2006). Tea flavonoids have a positive effect against glucose metabolism disorders and diabetes-induced fat disorders hence decreasing the risk of diabetes complications (Crespy and Williamson, 2004).

1.2 Statement of the Problem

Diabetes mellitus has been on the increase over the years. Hyperglycemia increases the formation of oxidized LDL and glycated LDL cholesterol, which are important factors in the onset of atherosclerosis and increased risk of death from cardiovascular diseases. In addition, amputation as a result of impaired wound healing is a serious complication of diabetes. Green tea could help prevent oxidation of LDL as well as reactive oxygen species (ROS) accumulation, which are major causes of cardiovascular diseases and poor wound healing respectively. Current management strategies for diabetes mellitus involve insulin therapy which is costly while diet and exercise control methods are seldom practiced by diabetic patients. Therefore, there is need to develop a cheap and commonly available therapeutic intervention to stabilize sugar and cholesterol levels and to accelerate the rate of wound healing.

1.3 Objectives

1.3.1 General objective

To investigate the effect of tea flavonoids on blood biochemical parameters and wound healing in type 2 diabetes.

1.3.2 Specific objectives

i. To determine profiles of flavonoids and polyphenols in green and black tea products processed from different tea cultivars.
ii. To determine the effect of processed green and black teas on glucose and cholesterol levels in a diabetic mouse model.
iii. To evaluate effect of processed green and black teas on the rate of wound healing in a diabetic mouse model.

1.4 Hypotheses

i. Processed green and black teas from different cultivars do not have different flavonoid and polyphenol profiles.
ii. Processed green and black teas have no effect on blood glucose and cholesterol levels in diabetic mice
iii. Processed green and black teas have no significant effect on wound healing in diabetic mice.

1.5 Justification

Diabetes mellitus has been on the rise over the years. The two main features of this disease are insulin resistance and glucose intolerance which are also considered risk factors for cardiovascular diseases. Vascular complications associated with type 2 diabetes are the major clinical problems facing diabetic patients. In addition, amputations resulting from poor wound healing is a major morbidity associated with diabetes, often leading to pain, suffering and a poor quality of life for patients. Because diabetes is now affecting many in the workforce, it has a major and deleterious impact on both individual and national productivity. The socioeconomic consequences of diabetes and its complications could have a seriously negative impact on the economies of developed and developing nations. These complications need to be addressed in order for diabetic patients to live normal and productive lives. Management of type 2 diabetes focuses on lifestyle intervention, including diet and exercise. These management practices are poorly practiced among diabetic patients. Although insulin has become one of the most important therapeutic agents known to medicine, there is a continuous need to find substitutes, insulin stimulators from plant sources for diabetes management. It is probable that tea which is one of the most popular beverages can provide the needed therapy for these complications due to its antioxidant properties resulting from polyphenolic content.

1.3 Expected Outputs

i. Characterization of the flavonoid and polyphenol profiles of green and black teas processed from popular Kenyan tea cultivars.
ii. Identification of a possible management therapy for diabetes mellitus that involves tea.
iii. Research manuscript published in a peer refereed journal.

CHAPTER TWO

LITERATURE REVIEW

2.1 Diabetes Mellitus

Diabetes mellitus is a group of syndromes characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins and an increased risk of cardiovascular disorders (Pickup and Williams, 2003). The minimum deficiency characteristic feature to identify diabetes mellitus is a chronic and sustained hyperglycemia (Ziv *et al*., 1999).

Diabetes may present a sudden potentially lethal metabolic catastrophe. Furthermore, it can be associated with a few, if any, symptoms and many escape detection for many years (Zimmet *et al*., 2004). These extremes of clinical manifestations constitute a basis for subdividing DM into type 1 DM, previously known as insulin dependent DM and type 2 DM also referred to as non-insulin dependent DM (Beverley and Eschwège, 2003). Type 1 DM patients have beta cell destruction which is usually immune mediated with the majority of patients who develop absolute insulin deficiency becoming ketosis prone (American Diabetes Association, 2009). Type 2 DM encompasses the most prevalent form of the disease. Most patients exhibit insulin resistance and ultimately develop concomitant insulin secretion defects (Erkelens, 2001).

2.1.1 Classification and Aetiology of Diabetes Mellitus

Diabetes mellitus may be categorized into several types but the two major types are type 1 and type 2 (WHO, 1985; Zimmet *et al*., 2004). Type 1 diabetes results from severe absolute lack of insulin caused by reduction in beta cell mass. The three interlocking mechanisms responsible for islet destruction include genetic susceptibility, acute autoimmunity and environmental factors (Pickup and Williams, 2003). The two metabolic defects that are characteristic of type 2 DM include derangement of insulin secretion that is delayed or insufficient, relative to glucose load and inability of peripheral tissues to respond to insulin (Pignone *et al*., 2010).

Type 1 DM is characterized by the presence of islet cell antibody, anti-glutamic acid decarboxylate, or insulin antibodies that identify the autoimmune process with ß-cell destruction

(Zimmet *et al*., 2004). Autoimmune diseases such as Grave's disease, Hashimoto's thyroiditis and Addison's disease may be associated with type 1 DM (Atkinson and Maclaren, 1994). There is no known etiological basis for type 1 DM. Some of the type 1 diabetes patients have permanent insulinopaenia and are prone to ketoacidosis, but have no evidence of autoimmunity (McLarty *et al*., 1990). Generally, people with type 1 diabetes exhibit acute symptoms and have elevated blood glucose levels (Burtis *et al*., 2006). Type 1 DM usually occurs in children and people with this form need daily injections of insulin to control blood glucose levels (Kuzuya *et al*., 1994).

Type 2 DM, is the major form of diabetes caused by insulin resistance (DeFronzo *et al*., 1997). Therefore, type 2 DM has two hallmarks namely hyperglycemia and hyper-insulinemia due to the insulin resistance (Erkelens, 2001; Goldstein, 2002). Majority of patients with this form of diabetes are overweight and obesity itself aggravates insulin response (Campbell and Carlson, 1993). In Western countries the disease affects up to 7% of the population (WHO, 1994; Harris *et al*., 1998). Globally, it affects 5-7% of the world's population but this prevalence is underestimated because many cases, perhaps 50% in some population, remain undiagnosed (King *et al*., 1998). Traditionally, type 2 diabetes is common in individuals over the age of 40 years. The disease is usually controlled through dietary therapy, exercise and hypoglycemic agents (Miller *et al*., 2001).

Gestational DM refers to the onset or initial recognition of glucose intolerance during pregnancy, usually in the second or third trimester (American Diabetes Association, 2001). It occurs in about 4% of all pregnancies. Patients with GD have a 30% to 50% chance of developing DM and usually of type 2. Classical symptoms including polydipsia, polyuria, and rapid weight loss associated with an overall and clear elevation of blood glucose of ≥200mg/dl at diagnosis. A fasting plasma glucose level of ≥126mg/dl on two occasions is diagnostic of diabetes (Borch-Johnsen, 2001).

2.2 Complications of Diabetes Mellitus

Most of the consequences of diabetes result from its macrovascular effects including coronary heart diseases and stroke, and the accompanying micro vascular complications such as retinopathy, neuropathy and nephropathy (Bearse *et al*., 2004). The age-adjusted mortality, mostly due to coronary heart disease (CHD) in many but not all populations, is 2-4 times higher than in the non-diabetic population (Morrish *et al*., 2001).

Diabetes mellitus is one of the leading causes of renal failure and has become the commonest cause of end stage renal disease that requires dialysis or kidney transplant (USRDS, 2002). Diabetes nephropathy occurs as a clinical complication with albuminuria which steadily lowers the rate of glomerular filtration and higher risk of CVD (Young *et al*., 2003).

Neuropathy occurs when DM damages nerves all over the body. Diabetic sensory neuropathy is characterized by peripheral damage of nerves of the lower extremities and eventual loss of sensation leading to numbness (Mohan *et al*., 2007).

People with diabetes have a two-fold increased risk of developing stroke (Bell, 1994). Increased oxidative stress plays a major role in both development and progression of diabetes (Maritim *et al.*, 2003). Hyperglycemia leads to production of reactive oxygen species and ultimately major diabetic complications and endothelial dysfunctions (Figure 1). Increased glucose as well as free fatty acids have been shown to induce oxidative stress which then plays a major role in causing insulin resistance and cell dysfunction (Manish *et al*., 2008). The same time extremity amputations are at least 10 times more common in people with diabetes than in non-diabetic individuals in developed countries with more than half of all non-traumatic lower limb amputations resulting from diabetes (Siitonen *et al*., 1993). In addition within the developed countries, diabetes is one of the leading causes of visual impairment and blindness (Kuzuya *et al*., 1994).

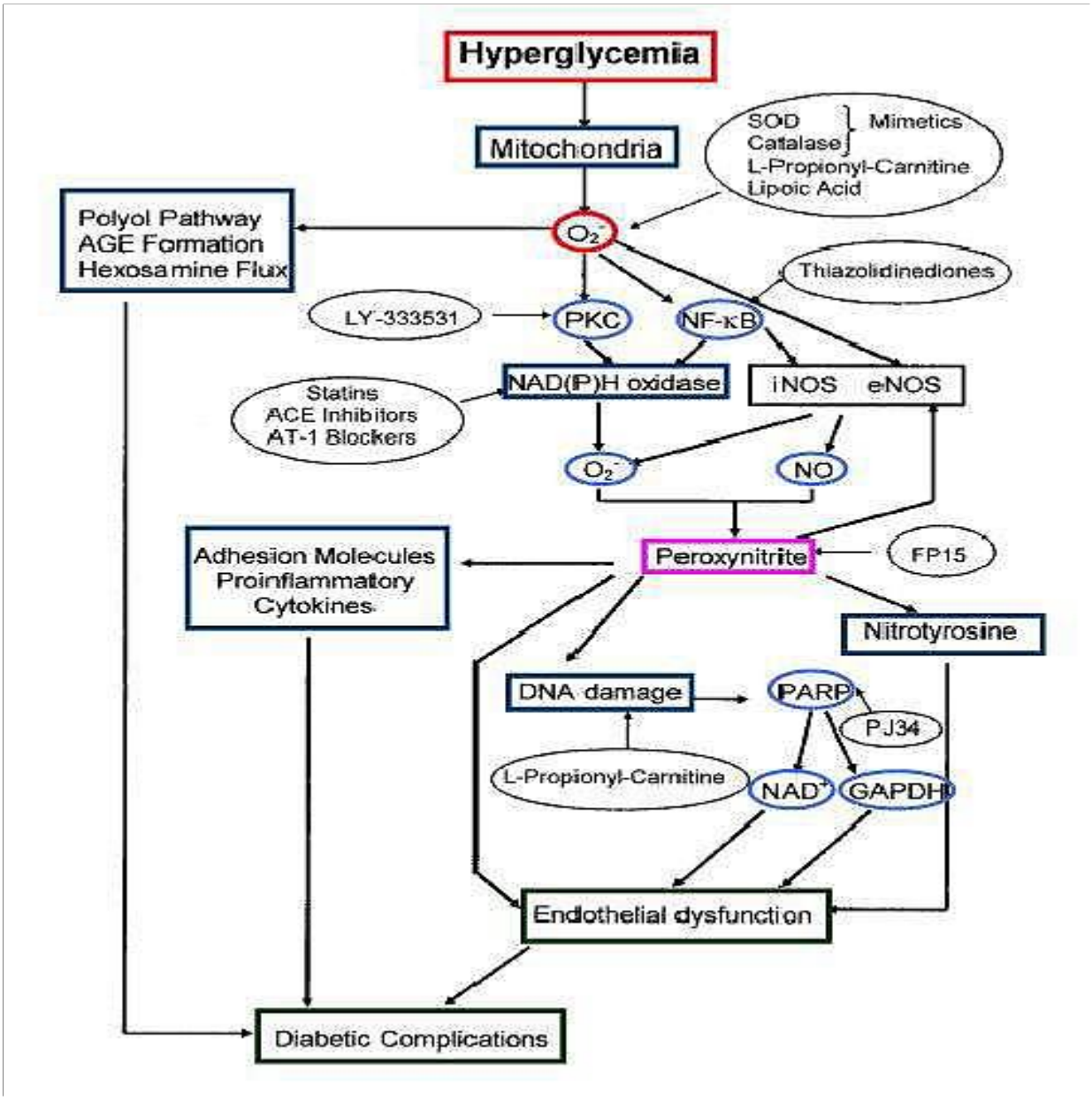


Figure 1: Hyperglycemia causes oxidant stress which in turn results into endothelial dysfunction and diabetic complications (source: Ishii *et al*., 2001).

2.2.1 Dyslipidemia in type 2 diabetes

The hallmarks of type 2 diabetes include hyperglycemia, insulin resistance and insulin deficiency. It is evident that insulin resistance is the major contributor of dys-lipidemia associated with type 2 diabetes (Adiels *et al.*, 2006).

The disturbance in lipid metabolism is an early event towards development of type 2 DM and has the potential of preceding the disease for several years (Figure 2) (Adiels *et al*., 2006). Moreover, plasma lipid and lipoprotein abnormalities are believed to be metabolically linked (Taskinen, 2005). The dys-lipidemia that is linked with insulin resistance is characterized by an elevation of very low density lipoprotein (VLDL) and triglyceride levels, a reduction of high density lipoprotein (HDL) cholesterol levels and the presence of small triglyceride-enriched low density lipoproteins (LDL) (Eckel *et al*., 2002; Ginsberg and Huang, 2007).

In addition, triglyceride- rich lipoproteins, remnant lipoproteins, Apo lipoprotein B 100 (Apo B) and small dense HDL particles have been shown to be elevated in type 2 diabetes patients (Haffner *et al.,* 1999).

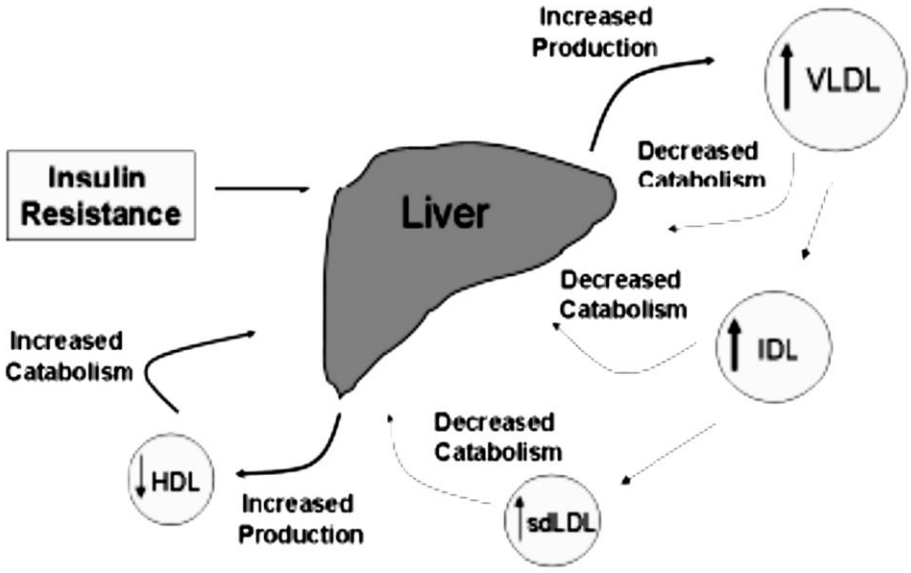


Figure 2: Atherogenic dys-lipidemia and changes in lipoprotein metabolism associated with type 2 DM (source: Adiels *et al.*, 2006).

Dys-lipidemia results from impaired regulation of circulating lipoprotein and glucose levels (Adiels *et al*., 2006; Farmer, 2008). Research suggests that impairment in the ability of insulin to suppress hepatic production of large tryglyceride (TG) rich VLDL (VLDL-TGs) in diabetic patients, increases plasma TG levels (Taskinen, 2005).

Impaired action of insulin in adipocytes results in impaired suppression of intracellular hydrolysis of TGs. This is followed by the release of non-esterified free fatty acids (NEFAs) into

circulation (Krentz, 2003). The massive entry of NEFAs into the liver promotes synthesis of TG and assembly of large VLDL characterized by high plasma VLDL levels (Jacobs *et al*., 2005). Elevated VLDL triglycerides reduce levels of cardio-protective HDL cholesterol (HDL-C) accompanied by low anti-oxidant and anti-atherogenic activities (Solano and Goldberg, 2006). When LDL particles become small and dense, they become susceptible to oxidation and subsequently adhere to and invade arterial walls leading to atherosclerosis (Krentz, 2003). Cholesterol ester transfer protein (CETP) also plays a pathological role in diabetic dys-lipidemia, that is, reduction of HDL-C levels as a result of increased catabolism (de Vries *et al*., 2003).

Activation of rennin- angiotensin aldosterone system (RAAS) can interfere with signaling of insulin (Wang *et al.,* 2007). This promotes and aggravates insulin resistance in type 2 diabetes patients. Activation of RAAS increases oxidative stress decreasing nitric oxide (NO) production and activates protein kinase signaling pathways. This leads to excessive generation of angiotensin II, vascular complications and endothelial damage to type 2 diabetes therefore, resulting in increased risks of CVD (Coccheri, 2007). Indeed CVD is a major cause of illness, disability and death in patients with type 2 diabetes (Gu *et al*., 1998). Macrovascular complications of diabetes including CVD, cerebrovascular disease and peripheral disease contribute for more than 70% of all deaths in diabetic patients (Gu *et al*., 1998).

2.3 Cellular and Molecular Basis of Wound Healing in Diabetes

Impaired wound healing is a common complication of DM (LoGerfo and Coffman, 1984). Amputation as a result of poor wound healing is a serious complication in diabetes (Marrotte *et al.,* 2010). Inadequate angiogenesis contribute to poor blood flow at the site of the wound and this leads to impaired endogenous regenerative response (Jeffcoate and Harding, 2003). Endothelial progenitor cells (EPCs) are major cell types in angiogenesis which migrate to the ischemia site and contribute to cellular maintenance and angiogenesis (Roncalli *et al*., 2008; Barcelos L. S, 2009). However studies show that EPCs are impaired in function in diabetic patients. The studies also show that the number of circulating EPCs is greatly reduced in type 1 and type 2 diabetes (Fadini, 2005; Loomans, 2005). Consequently, EPC dysfunction represents a mechanism for impaired angiogenesis and subsequent poor wound healing in diabetes (Caballero, 2007). Healing in patients with DM is characterized by reduced tensile strength of wounds when compared with controls, suggesting either defective matrix production or

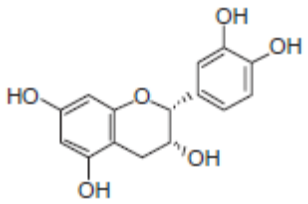
deposition (Vlassara *et al*., 1995; Ferguson *et al*., 1996). It is presumed that diabetic complications result from periods of poor glycemic control. However, aberrant growth factor expression or factors secondary to diabetes, such as advanced glycation and cross-linking of matrix protein, may also be involved (Walker *et al*., 1988). Growth factor 52 involvement has been implicated not only in diabetic wounds but also in other diabetic complications, such as diabetic retinopathy and nephropathy (Yamamoto *et al*., 1993). In addition, hyperglycemia and glycosidation products also contribute to poor wound healing in diabetes (Peppa *et al*., 2009). Hyperglycemia is the main feature of diabetes and it increases ROS and cellular damage (Brownlee, 2001; Rolo and Palmeira, 2006). Hyperglycemia increases generation of superoxide anions in skin tissue by activating nicotinamide adenosine diphosphate reduced (NADPH) oxidase and protein kinase C, resulting in poor wound healing in diabetic mice (Luo *et al.*, 2004). Increased production of ROS in the case of hyperglycemia leads to ROS-mediated mitochondrial release of cytochrome C followed by activation of caspase-3, leading to hyperglycemia induced myocardial cell apoptosis (Baynes and Thorpe, 1999). ROS accumulation leads to cellular damage and poor wound vascularization (Tie *et al.*, 2009). Normal EPCs have been demonstrated to posses' high levels of manganese superoxide dismutase (MnSoD). This enzyme plays a major role in EPCs resistance to oxidative stress by scavenging mitochondrial ROS (mt ROS) (Chen and Chen, 2006).

Hyperglycemia elevates oxidative stress and inhibits cell proliferation, nitrogen oxide production, matrix metallopeptidase 9 (MMP9) activities and migration, hence decreased EPC survival (Krankel, 2005; Balestrieri, 2008). These observations elucidate that loss of tolerance to oxidative stress in EPC may lead to their dysfunction in diabetes and that a powerful antioxidant might be helpful.

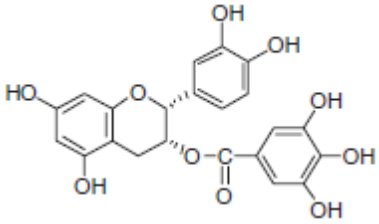
2.4 Tea Flavonoids

Tea consists of over 200 components, the best known being the catechins also called flavonoids or polyphenolic compounds, in addition to polysaccharide conjugates (TPCs), amino acids, caffeine and vitamins (Fujihara *et al.*, 2007; Monobe *et al.*, 2008; Chen *et al.*, 2009). Among these, polyphenols constitutes the most interesting group and are the main bioactive molecules in tea (Cabrera *et al*., 2003). Flavonoids are abundant in plants and almost all plant

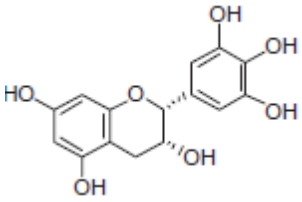
tissues can manufacture flavonoids (Hollman *et al*., 1997). The levels of flavonoids in various beverages like tea and wine is very high (Hertog *et al*., 1993). The flavinol polyphenolic compounds are the most abundant accounting for nearly 30% of the dry weight of the green tea leaves (Mukhtar and Ahmad, 1999). Polyphenolic compounds of green tea include epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG), catechin (C) and gallocatechin (GC). The most abundant catechin which accounts for about 65% of the total catechin content is EGCG (Chu and Juneya, 1997).



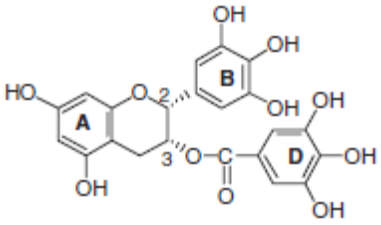
epicatechin (EC)



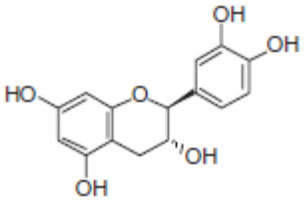
epicatechin gallate (ECG)



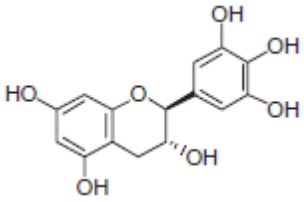
epigallocatechin (EGC)



epigallocatechin gallate (EGCG)



Catechin (C)



gallocatechin (GC)

Figure 3: Structures of the major polyphenolic catechins present in green tea.

The fermentation process used to produce black tea results in the conversion of catechins to theaflavins including theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate, and theaflavin-3, 3'- digallate and thearubigin polymers (Miura *et al*., 2000; Kris-Etherton and Keen, 2002). The major fraction of black tea polyphenols is composed of thearubigins, which account for 20% of the solids and 47% of the total flavonoids (Yang and Landau, 2000). It is known that theaflavins, which account for 2–6% of the dry weight of solids in brewed black tea, contribute greatly to the quality of tea in terms of color, 'mouthfeel' and extent of tea cream formation (Powell *et al*., 1993). Recently, theaflavins have attracted considerable interest because of their potential benefits for human health, including antimutagenicity, suppression of cytochrome P450 1A1 in cell culture, anticlastogenic effects in bone marrow cells of mice, suppression of extracellular signals and cell proliferation, and anti-inflammatory and cancer chemo preventive action (Feng *et al*., 2002).

Anthocyanins are polyphenolic compounds that occur naturally and gives red, blue and purple pigments to fruits, vegetables and some grains (Kong *et al*., 2003). More than 600 different anthocyanins have been identified but only six are predominant found in plants and include cyanidin, peonidin, petunidin, pelargonidin, delphinidin and malvidin (Kong *et al.,* 2003). Anthocyanins have been found to possess superior antioxidant activities compared to other polyphenols. Just like the catechins in the tea plant, they have been reported to have a wide range of benefits which including antioxidant potential (Bae and Suh, 2007). Indeed they have also been reported to have antimicrobial, anti-carcinogenic and anti-inflammatory properties (Youdim and Joseph, 2001; Viskelis *et al.,* 2009).

2.5 Tea and Health

Tea, a product made from the leaf and bud of the plant *Camellia sinensis*, is the second most consumed beverage in the world, well ahead of coffee, beer, wine and carbonated soft drinks (Costa *et al*., 2002). The economic and social interest of tea is clear and its consumption is part of many people's daily routine, as an everyday drink and as a therapeutic aid in many illnesses (Rietveld and Wiseman, 2003). The ability to scavenge for free radicals by tea polyphenols due to possession of a phenolic hydroxyl group attached to the flavan-3-ol structure has been associated with teas' therapeutic action against free radical mediated diseases thereby attracting tremendous research interest (Amie *et al*., 2003).

On anticancer actions, several studies have demonstrated the ability of tea in prevention of tumor initiation, promotion and progression (Huang *et al*., 1992). Several studies have shown that green tea polyphenol is a free radical scavenger (Zhao *et al*., 1985; Guo *et al*., 1996). In addition, green tea polyphenols protect against oxidative stress on deoxyribo-nucleic acids (DNA) molecules (Leanderson *et al*., 1997). Tea components possess antioxidant, antimutagenic, and anticarcinogenic effects and could protect humans against the risk of cancer by environmental agents (Mukhtar *et al*., 1992). Tea has been shown to posses anticarcinogenic effects against breast cancer in experimental studies (Zhang *et al*., 2008).

Several epidemiological studies have established a converse relationship between tea consumption and cholesterol (Zhu *et al*., 1999). Green tea and its polyphenols have been shown to be hypocholesterolemic (Yang and Koo, 2000) and prevent development of atherosclerotic plaques (Chyu *et al*., 2004). Moreover, oral administration of green tea reduces aortic lesion formation and prolonged delay of LDL oxidation (Tijburg *et al*., 1997). Some studies indicated that green tea has an anti-proliferative action on hepatoma cells and a hypolipidemic activity in hepatoma-treated rats, as well as the prevention of hepatoxicity (Vanessa and Gary, 2004). Polyphenols can prevent oxidation of LDL *in vitro* which is considered a key mechanism in atherosclerosis which results in the diminished oxidation of LDL lipids and of α-tocopherol (Zhu *et al*., 1999). Other animal studies have demonstrated that tea catechins reduce hepatic cholesterol (Muramatsu *et al*., 1986).

The anti-diabetic potential of polyphenols has been reported from various studies. Tea catechins have also been studied for their anti-diabetic potential (Rizvi *et al*, 2005). Polyphenols can affect glucose balance by either hindering glucose absorption from the gut into the intestines or its uptake by peripheral tissues (Matsui *et al*., 2002). Inhibition of glucose transporters and intestinal glycosidases by polyphenols has also been studied (Matsui *et al*., 2002). Polyphenols such as catechin, epicatechin, EGC, ECG decreases S-Glut-1 mediated transport of glucose in intestines. Animal experiments have shown that black and green tea extracts and EGCG increases glucose uptake by rat epididymal adipocytes, both in the presence or absence of insulin (Anderson *et al*., 2002). Polyphenols may exert different actions on peripheral tissues that would diminish glycaemia including the inhibition of gluconeogenesis (Arion *et al*., 1997; Waltner *et al*., 2002), adrenergic stimulation of glucose uptake (Cheng and Liu, 2000) or the stimulation of insulin release by pancreatic β-cells (Ohno *et al*., 1993). Recent studies indicate that tea has a

wide range of preventive effects on diabetes and obesity for animal and human health (Kao *et al*., 2006; Wolfram *et al*., 2006). Green tea consumed orally may protect against disorders related to obesity including diabetes, hypertension, and atherosclerosis (Kao *et al*., 2000). *In vivo* effects of EGCG also inhibit lipid oxidation and can also modulate glucose levels (Tsuneki *et al*., 2004). Green tea also possesses anti-diabetic effects in animal models that show resistance to insulin (Wu *et al*., 2004). It is well known that dietary antioxidants, including anthocyanins, protect pancreatic β-cells from glucose induced oxidative stress (Al-Awwadi *et al*., 2005). Jayaprakasam *et al*. (2005) demonstrated glucose-induced insulin release from pancreatic β-cells by anthocyanins and anthocyanidins.

Moreover, green tea polyphenols has been shown to inhibit free radical induced lyses' of red blood cells (Zhang *et al*., 1997). Recent studies suggested that green tea polyphenols might protect against Parkinson's and Alzheimer's diseases and other neurodegenerative diseases (Weinreb *et al*., 2004). Studies have also demonstrated tea's activity of neuro-protection in cell cultures and animal models, such as the prevention of neurotoxin-induced cell injury (Pan *et al*., 2003). Besides, purple tea anthocyanins have been demonstrated to improve treatment outcomes of human African trypanosomiasis in experimental mice. (Rashid *et al*., 2014). Green tea is considered to be useful for insect stings attributed to its anti-inflammatory effects and its capacity to stop bleeding (Sagesaka-Mitane *et al*., 1998; Dvorakova *et al*., 1999). Some studies have suggested a relationship between green tea consumption and lowered risk of kidney stone formation (McKay and Blumberg 2002; Ishizuk *et al*., 2003). Green tea also prevents cataracts by preserving the antioxidant defense system of the lens (Gupta *et al*., 2002).

2.6 Alloxan Monohydrate

Alloxan (2, 4, 5, 6-tetraoxypyrimidine; 2, 4, 5, 6-pyrimidinetetrone) is an oxygenated pyrimidine derivative which is present as alloxan hydrate in aqueous solution (Lenzen, 2008). Alloxan is a urea derivative which causes selective necrosis of the pancreatic islet β-cells. It is used to induce diabetes in animals such as rabbits, rats, mice and dogs (Huralikuppi, 1991). The drug has been noted to exert its diabetogenic action when administered parenterally namely intravenously, intraperitoneally or subcutaneously. Furthermore, the dose of alloxan required for inducing diabetes depends on the animal species, route of administration and nutritional status (Federiuk *et al*., 2004). Moreover, alloxan has been demonstrated to be non-toxic to the human

beta-cells, even in very high doses, the reason of which may be attributed to the differing glucose uptake mechanisms in humans and rodents (Eizirik *et al*., 1994; Tyrberg *et al*., 2004).

The mechanism of action of alloxan has been widely studied and several experimental studies have demonstrated that alloxan causes a sudden rise in insulin secretion in the presence or absence of glucose appearing just after alloxan treatment (Lachin and Reza, 2012). The alloxan-induced insulin release occurs for short time followed by the complete suppression of the islet response to glucose even when high concentrations of glucose were used (Kliber *et al*., 1996). Further, the alloxan action in the pancreas is preceded by its rapid uptake by pancreatic beta cells that have been proposed to be one of the important features determining alloxan diabetogenicity. Moreover, in pancreatic beta cells, the reduction process occurs in the presence of different reducing agents like reduced glutathione (GSH), cysteine, ascorbate and protein-bound sulfhydryl (-SH) groups (Lenzen and Munday, 1991). Alloxan reacts with two -SH groups in the sugar binding site of glucokinase resulting in the formation of the disulfide bond and inactivation of the enzyme. As a result of alloxan reduction, dialuric acid is formed which is then re-oxidized back to alloxan establishing a redox cycle for the generation of ROS and superoxide radicals (Das *et al*., 2012). The superoxide radicals liberate ferric ions from ferritin and reduce them to ferrous and ferric ions (Sakurai and Ogiso, 1994). In addition, superoxide radicals undergo dismutation to yield hydrogen peroxide (H_2O_2) in the presence of superoxide dismutase. As a result, highly reactive hydroxyl radicals are formed according to the Fenton reaction in the presence of ferrous and H_2O_2.

Another mechanism that has been reported is the effect of ROS on the deoxyribonucleic acid (DNA) of pancreatic islets. The fragmentation of DNA takes place in the beta cells exposed to alloxan that causes DNA damage, which stimulates poly adenosine diposphate-ribosylation, a process participating in DNA repair (Ebelt *et al*., 2000).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Tea Sampling and Processing for Analysis

Based on existing literature for various teas and their biochemical contents (Koech *et al.*, 2012), four tea cultivars were selected for processing and analyses namely TRFK 306, TRFK 6/8, TRFK 303/577 and AHP S15/10. All the tea leaves for processing were obtained from the Tea Research Foundation of Kenya (TRFK), Kericho. The young tender shoots comprising of two leaves and a bud were harvested by hand. In the preparation of green tea, the withered leaves were steamed and then dried relatively rapidly after plucking to minimize chemical and enzymatic reactions. The black teas were dried and macerated in the curl, tear and cut (CTC) machine, fermented for 90 minutes at ambient temperature followed by firing at 121^0C in a fluid bed drier (Luo *et al*., 2008).

3.2 Tea Sample Treatment for Polyphenol and Catechin Analysis

Three processed samples of each tea cultivar were obtained to assay for total polyphenols and green tea catechins. Tea samples of a coarse granular structure were 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 g 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. The samples were heated in a 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 centrifuged 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 a high performance liquid chromatography (HPLC) machine for analysis.

3.2.1 Catechin analysis using HPLC

A modified method of Zuo *et al.,* (2002) was used to assay for the major tea flavonoids; catechins. A Shimadzu LC 20 AT HPLC machine fitted with a SIL 20A auto sampler and a SPD-20 ultra violet-visible detector with a class LC 10 chromatography workstation was used for the analysis of the prepared samples. A Luna ^{TM} 5µM C18, 25cm 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 0.45µm millipore membrane filter disk and degassed before injection into the HPLC system. A gradient elution was carried out using solvent systems made of 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 the binary gradient conditions was 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 then 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 1ml/min and the temperature of the column was kept 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 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.

3.2.2 Total polyphenols determination

The Folin-ciocalteu phenol reagent method was used to determine total polyphenols as described by Pourmorad *et al.* (2006). One milliliter of the sample extract was transferred to a one-mark volumetric flask, diluted to the mark with water then 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 then 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 then 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 10µg/ml to 60µg/ml. The optical density readings of the test samples were referenced to the calibration curve to determine the total polyphenols content of the samples.

3.3 Extraction of Anthocyanins

Five grams (5g) of ground tea sample was weighed into 250 ml conical flasks, covered with foil and mixed with 50 ml MeOH/HCL (99:1 v/v) and magnetically stirred at 900rpm for four hours at room temperature. The resultant solution was filtered and evaporated to dryness using a rotary evaporator (Buchi Rotavapour R-300, Switzerland) under reduced pressure at 35^{o}C. The extract was then dissolved in 10 mls distilled water and passed through a membrane filter 0.45 µM and stored at 4^{o}C until required for analysis.

3.3.1 Purification of anthocyanin fraction

The tea extracts were passed through a C-18 reverse phase octadecylsilane (ODS; Phenomenex Inc. Torrance CA, USA) cartridge previously activated with acidified methanol (10% HCl/methanol v/v). Anthocyanins were adsorbed onto the column while sugars, acids and other water soluble compounds were washed out using 0.01% HCl in distilled water. The anthocyanins were recovered using acidified methanol (10% formic acid/methanol v/v). The catridges were then washed with ethyl acetate (Fischer Scientific, UK) to remove phenolic compounds other than anthocyanins.

3.3.2 Determination of total monomeric anthocyanin content

The analysis was carried out in triplicate using the pH differential method as described by Kerio *et al.,* (2012) using two buffer systems; 0.025 potassium chloride (KCL) buffer at pH 1.0 and 0.4M sodium acetate ($NaC_2H_3O_2$) buffer at pH 4.5. Two hundred microliters of the anthocyanin sample was mixed separately with 1.8ml of potassium chloride and sodium acetate buffer and the absorbance at 520 nm and 700 nm determined. The difference in absorbance of the sample was determined as follows:

A= (A520 nm-A700 nm) pH 1.0- (A520 nm-A700 nm) pH 4.5

The monomeric anthocyanin pigment concentration (mg/dl) in the original sample was determined using the formula;

Monomeric anthocyanin pigment (mg/dl) = (A x MW x DF x 1000)/ (ε x 1)

Where;

A- Absorbance (Absorbance difference in the two pH ranges)

ε- cyaniding-3-glucoside molar absorbance (26 900)

MW- molecular weight of anthocyanin (449.2g)

DF- Dilution factor

3.4 Analysis of Total Theaflavin Content in the Tea Sample

Both green and black teas were assayed for total theaflavins (TF) using the flavognost method of Hilton and Palmer Jones (1973). A tea infusion was made with 375 ml of boiling water, added from an overhead boiler into a tared flask. The flask was then agitated in a mechanical shaker for 10 minutes; the infusion was then filtered through rough cotton wool and allowed to cool to room temperature. Ten milliliter of the tea infusion was pipetted into 10ml isobutylmethylketone (4-methylpenta-2-one, IBMK). The mixture was shaken for 10 minutes and allowed to stand until layers got separated. Two milliliters of the upper layer was 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 of ethanol). The contents were mixed and colour were allowed to develop. The absorbance at 625 nm was then read against an IBMK/ethanol (1:1 v/v) blank. 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.

3.4.1 Spectrophotometric determination of total thearubigin in tea samples

Total thearubigins (TR) was determined in the tea samples using the method of Roberts and Smith (1964). Six milliliters of 1% v/v aqueous solution of anhydrous disodium hydrogen orthophosphate was added to 6ml of cooled tea infusion. The resulting mixtures were extracted with 10 ml ethyl acetate by vigorous shaking for 1 minute. The mixture was then allowed to settle and the aqueous layer drained off. Five milliliters of ethyl acetate was added to the ethyl acetate extract containing the TF fraction in the separating funnel. Ten milliliters of the ethyl acetate extract was diluted to 25 ml with methanol in 25 ml volumetric (solution E_1). One

milliliter of the tea infusion was mixed with 9ml of distilled water and made to 25ml in a volumetric flask with methanol (solution E_2). One milliliter of saturated aqueous 10% oxalic acid was added to 1ml of tea infusion and 8ml of water and made to 25 ml with methanol (solution E_3). The absorbencies of solution E_1, E_2 and E_3were obtained at 380nm and 460 nm, respectively, using a 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;

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

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

Where DM is the dry matter of the sample.

3.5 Experimental Animals

70 adult male Swiss albino mice of approximately 12 weeks of age, weighing 20-30 g were obtained from the Veterinary Investigation Laboratories Kabete, Kenya. Mice were housed in standard mice cages at room temperatures on a 12 hour light: 12 hour dark cycle and provided with water and feed *ad libitum*. The mice pellet feeds was obtained from Unga Feeds Ltd Kenya. The beddings consisted of wood chippings changed regularly. De-worming of all the mice was done using ivermectin with a single dose of 0.2 ml equivalent to 10 mg/ml ivermectin which was injected subcutaneously before commencement of experiment. Skin marking of the animals was done at their backs using picric acid for easy identification. Following eight week acclimatization period, animals were divided into two broad groups of 25 mice each. Group I comprised of diabetic mice and group II healthy mice.

3.5.1 Diabetic animals

Hyperglycaemia was induced in 27 overnight fasted mice by a single intra-peritoneal injection of freshly prepared alloxan monohydrate (Szudelski, 2001) at a dose of 150 mg/kg body weight. The Blood glucose level from each mouse was randomly measured for a period of three days. Animals with sustained blood glucose levels of over 200 mg/dl were considered diabetic and selected for use in the study.

The 25 diabetic animals were further divided into five groups of 5 mice each as follows: group I on anthocyanin rich tea, group II on EGCG rich tea, group III on theaflavin rich tea, group IV on total polyphenol rich tea and group IV serving as control receiving only water.

3.5.2 Healthy animals

The healthy mice were similarly grouped into five groups of 5 mice each as follows: group I on anthocyanin rich tea, group II on EGCG rich tea, group III on theaflavin rich tea, group IV on total polyphenol rich tea and group V serving as a negative control on water only. All the freshly prepared tea solutions were administered orally at a concentration of 20 g/l.

3.6 Packed Cell Volume and Body Weight

The packed cell volume of all the experimental mice was done at intervals of 7 days. Blood was collected by tail snip into 100 µl microhematrocrit tube and centrifuged at 10,000 revolutions per minute for five minutes. The hematocrit was determined using a micro-haematocrit reader and expressed as a percentage (%) of the total blood volume (Bull and Rittenbach, 1990).

Live body weights (grams) of all the mice were also done at intervals of one week using the analytical electronic balance.

3.7 Blood Glucose and Cholesterol Levels

Blood samples for glucose and cholesterol analysis was aseptically collected from the tail tip from all animals. Blood glucose levels (mg/dl) was measured at 72 hour intervals using Extracare^{TM} blood glucose monitor for a period of 21 days. Cholesterol levels (mg/dl) were measured using Easy Touch® GCHb monitoring systems at intervals of 21 days for a period of 45 days. Both the glucose and cholesterol monitors are rapid kits and uses test strips. A drop of blood was applied at the tip of the test strip and readings obtained in 60 seconds.

3.8 Wound Healing Experiments

A total of 20 mice were used for this experiment. Diabetes was induced in ten of these mice as described in 3.5.1. The backs of all the mice were shaved and skin wounds prepared using the modified methods of Whitby and Ferguson (1991). Wound incisions were done down to the panniculus carnosus muscle on the dorsum of the experimental mice. All the wound areas were left open and monitored for infection. The mice were then grouped into four groups of five mice each as follows: diabetic mice on anthocyanin rich tea, healthy mice on anthocyanin rich tea, diabetic mice on water and healthy mice on water.

The experiment was run for ten days with daily monitoring of wound diameters and wound closure by digital imaging. Wound areas were measured on day 0, 3, 7 and day 10.using a

transparent sheet and a marker. Wound area recordings were then measured on graph paper. The period of epithelialization was determined by observing the day of scab falling without any residual raw wound. On the last day, the mice were sacrificed and the entire wound area, including the skin and tissues near the wound, were dissected and fixed using 10% paraformaldehyde for histological studies.

3.8.1 Histological studies

The skin samples were processed for paraffin embedding procedures. Paraffin embedded sections (6μm thick) were cut perpendicular to the wound surface, mounted onto slides, and stained with hematoxilin and eosin (H & E) for light microscopy evaluation.

3.9 Data Analysis and Presentation

The cholesterol levels, blood glucose levels, body weights PCV and wound areas were expressed as the mean ± standard error of the mean (mean±SEM) and analyzed using Graph pad Prism version 5.0. Significance of difference between means for PCV, live body weights, blood glucose and cholesterol levels was determined by one way ANOVA and Tukey post hoc test was performed to determine differences among group means. The p values of <0.05 were considered to be statistically significant. Graphs were plotted to show the trend of the various response variables.

CHAPTER FOUR
RESULTS

4.1 Results of Tea Analysis

4.1.1 Levels of total polyphenols

The levels of the total polyphenols in different tea clones is presented in figure 4. Total polyphenol levels varied in the different tea products with the green teas having higher total polyphenolic content than the black teas. Processed green tea from cultivar TRFK 6/8 had the highest total polyphenols at 25.0 ± 0.31% with green teas from cultivars TRFK 303/577, AHP S15/10 and TRFK 306 having levels of 24.03 ± 0.75%, 24.07 ± 0.07% and 21.03 ± 0.82%, respectively. Black teas had lower total polyphenols with TRFK 6/8, TRFK 303/577, AHP S15/10 and TRFK 306 having 18.4 ± 0.12%, 19.13 ± 2.78%, 19.8 ± 0.19% and 17.93 ± 0.37%, respectively. Owing to its high total polyphenolic levels, green tea of cultivar TRFK 6/8 was chosen for use in the animal model study.

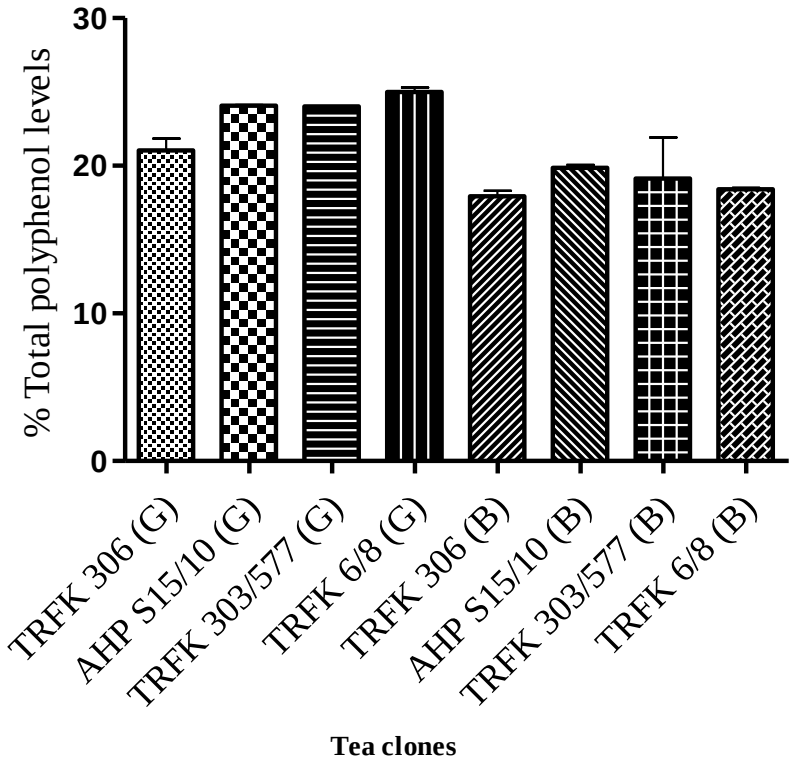


Figure 4: Percentage total polyphenol concentration in processed green and black teas from four cultivars

4.1.2 Epigallocatechin gallate levels

Catechin profiling of the different tea products using HPLC showed presence of catechins; epigallocatechin galate, epigallocatechin, epicatechin gallate, epicatechin. Epigallocatechin gallate was the most abundant catechin (Table 1).

Table 1: Percentage catechins in various black and green tea cultivars.

	TEA CULTIVARS							
Catechins	TRFK 306/3 (G)	AHP S15/10 (G)	TRFK 6/8 (G)	TRFK 303/577 (G)	TRFK 306/3 (B)	AHP S15/10 (B)	TRFK 6/8 (B)	TRFK 303/577 (B)
GA	0.93	0.79	0.59	0.69	0.27	0.47	0.24	0.20
EGC	2.3	6.62	7.58	6.35	0.29	2.89	1.80	3.19
C	0.08	0.45	0.58	0.32	0.00	0.23	0.17	0.20
CAFF	2.34	3.94	3.15	3.35	2.47	4.61	3.86	3.17
EC	1.55	1.54	2.11	2.05	0.09	0.67	0.57	0.61
EGCG	3.86	9.69	8.85	9.38	0.19	1.41	0.56	0.98
ECG	2.67	3.08	3.05	3.13	0.25	1.57	0.83	1.16

Key: GA- Gallic acid, EGC- Epigallocatechin, C- Catechin, CAFF- Caffeine, EC- Epicatechin, EGCG- Epigallocatechin gallate, ECG- Epicatechin gallate.

The epigalocatechin levels varied in the different teas with black teas having means of 0.19 ± 0.04%, 0.56 ± 0.18%, 0.98 ± 0.05% and 1.41 ± 0.67% in cultivars TRFK 306, TRFK 6/8, TRFK 303/577 and AHP S15/10, respectively. Green teas however had higher EGCG levels with AHP S15/10 containing higher EGCG levels at 9.70 ± 0.28% as compared to TRFK 306/3, TRFK 6/8 and TRFK 303/577 which had 3.83 ± 0.11%, 8.85 ± 0.41% and 9.38 ± 0.14% respectively (Figure 5).

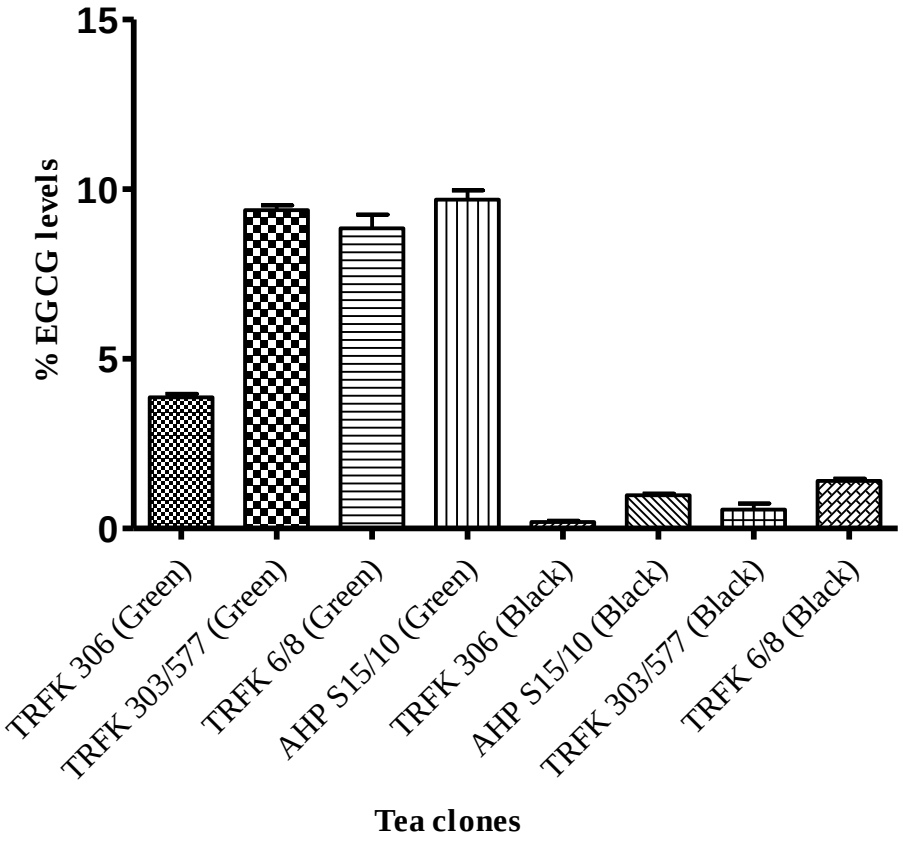


Figure 5: Epigallocatechin gallate concentration in processed green and black teas from four cultivars.

4.1.3. Anthocyanin levels

Processed green tea from cultivar TRFK 306 had the highest total monomeric anthocyanin levels (9.71 mg/dl) when compared to other the tea samples (Figure 6). Some teas had amounts as little as 0.2 mg/dl (black teas from cultivars TRFK 303/577 and AHP S15/10), 0.05 mg/dl (green teas from cultivars AHP S15/10 and TRFK 303/577 and black tea TRFK 6/8), 0.6 mg/dl (green tea TRFK 6/8) and 1.07 mg/dl (black tea from TRFK 306).

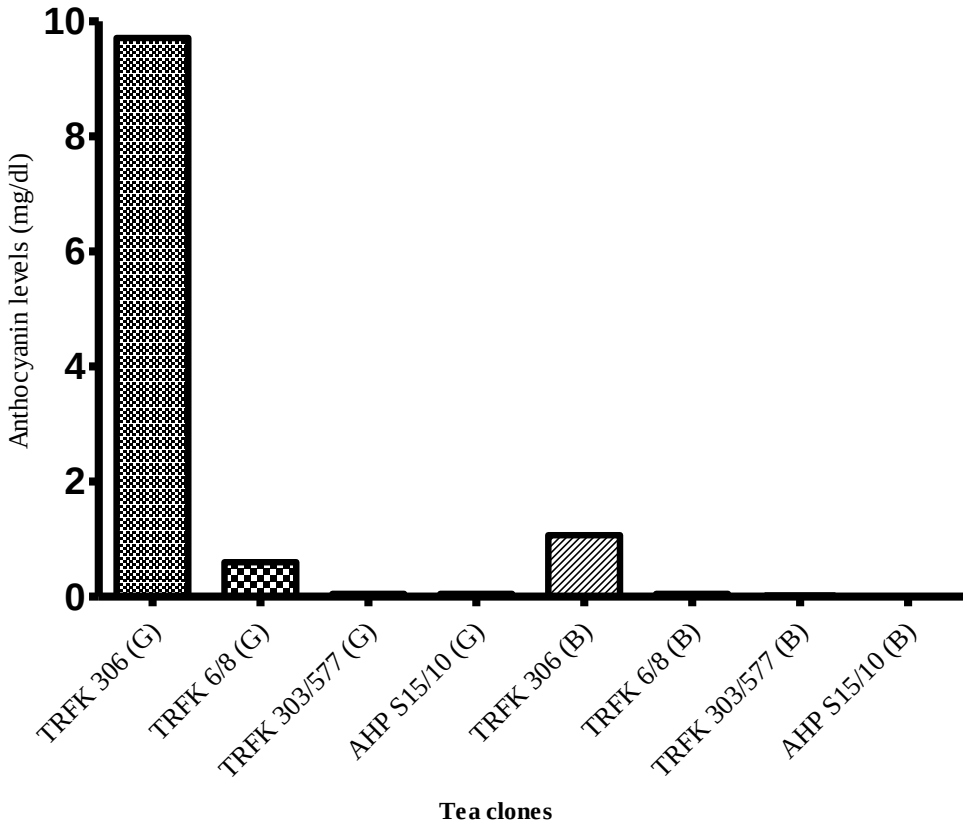


Figure 6: Total monomeric anthocyanin concentration in processed green and black teas from four cultivars

4.1.4 Theaflavin levels

The levels of theaflavins in the different cultivars of tea are presented in figure 7. The black teas from all the assayed cultivars had higher theaflavin levels compared to the green teas. Black tea from cultivar TRFK 6/8 had the highest theaflavin levels at 1.65 ± 0.27%. Black teas from cultivars TRFK 303/577, AHP S15/10 and TRFK 306 had theaflavin levels of 1.38 ± 0.03%, 1.43 ± 0.07% and 0.70 ± 0.03%, respectively. The green teas had significantly lower amounts of theaflavins which ranged from as low as 0.28 ± 0.01% to 0.38 ± 0.01%. Black tea from cultivar TRFK 6/8 was selected to be used in the mice experiments due to its high theaflavin levels.

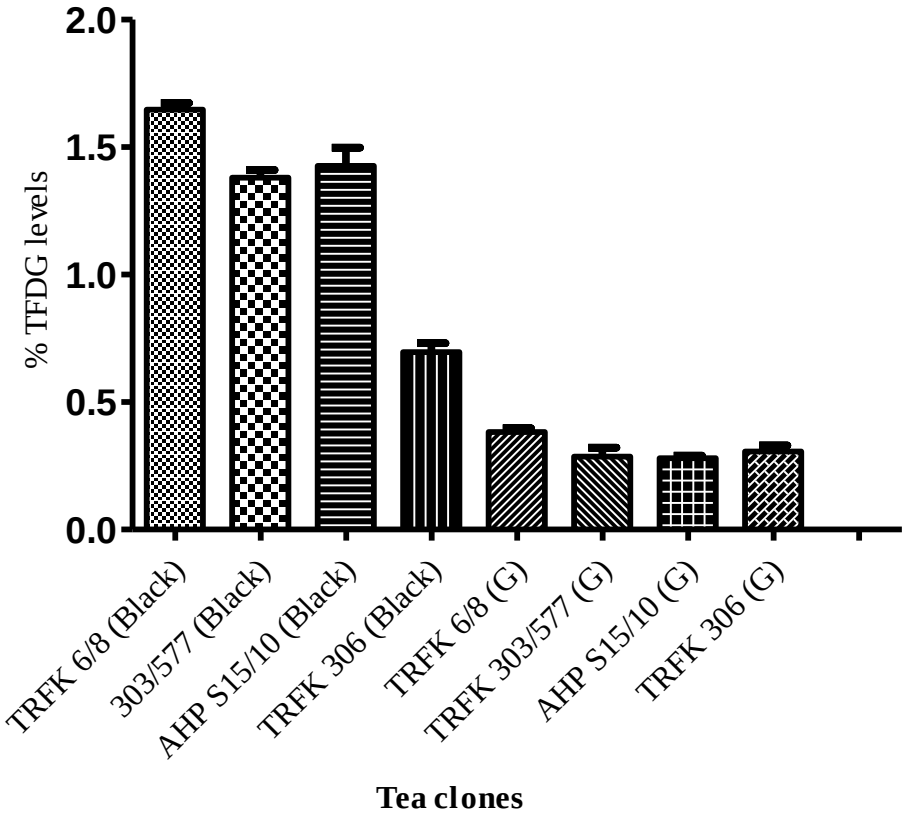


Figure 7: Percentage theaflavin concentration in processed green and black teas from four cultivars

4.2 The Effect of Tea on Blood Biochemical Parameters

4.2.1 Cholesterol profile

Table 2 shows the cholesterol levels of both treated and untreated control mice over time. Healthy mice supplemented with tea exhibited insignificant changes as healthy untreated mice throughout the experimental period.

Table 2: Change in cholesterol levels of healthy mice supplemented with different teas over time. All values are expressed as mean ± SEM.

Cholesterol levels in mg/dl

Days post tea administration	Untreated control	Anthocyanin rich tea	EGCG rich tea	Theaflavin rich tea	Total polyphenol rich tea
-4	176.24±2.36	171.52±2.67	173.25±2.70	175.61±2.59	174.16±2.10
21	176.10±2.54	170.18±3.11	173.21±3.23	174.81±2.31	173.87±3.01
42	177.34±3.15	170.01±2.98	172.87±2.63	174.20±2.63	173.80±2.27

The changes in the cholesterol levels in diabetic mice fed with or without different tea over time are presented in table 3. Three weeks post diabetes induction; there was a slight increase in mean cholesterol levels across the mice groups. Following tea administration, there was a marked drop in blood cholesterol levels in the experimental groups declining from 175.72 ± 3.51 mg/dl, 173.96 ± 2.66 mg/dl, 176.08 ± 2.43 mg/dl, 174.06 ± 3.43 mg/dl to 168.26 ± 3.45 mg/dl, 171.24 ± 2.10 mg/dl, 174.42 ± 2.35 mg/dl and 173.32 ± 3.72 mg/dl for animals on anthocyanin (ACN) rich tea, TF rich tea, total polyphenol rich tea and EGCG rich tea, respectively. The control animals registered a marginal increase in cholesterol levels which reached 187.33 ± 0.83 mg/dl from 179.24 ± 2.90 mg/dl.

Table 3: Change in cholesterol levels of diabetic mice supplemented with different teas over time. All values are expressed as mean ± SEM. Treatments denoted with the same letter across the groups are not significantly different at $p>0.05$

Days post tea administration	Cholesterol levels in mg/dl				
	Untreated control	Anthocyanin rich tea	EGCG rich tea	Theaflavin rich tea	Total polyphenol rich tea
-4	179.24±2.90[a]	173.32±2.94[b]	174.08±3.29[b]	171.58±2.49[b]	175.66±2.48[b]
21	183.06±2.76[a]	175.72±3.51[b]	174.26±3.43[b]	173.96±2.63[b]	176.08±2.42[b]
42	187.33±0.83[a]	168.26±3.45[b]	173.32±3.72[b]	171.24±2.10[b]	174.42±2.35[b]

4.2.2 Blood glucose levels

Figure 8 shows the changes in blood glucose levels of both treated and untreated control mice over time. Healthy animals on tea supplements showed a marginal drop in blood glucose levels throughout the experimental period. However, tea rich in anthocyanins exerted the highest and significant drop ($P < 0.05$) from 112.2 ± 5.99 mg/dl to 97.4 ± 4.81 mg/dl as compared to mice fed with tea rich in EGCG, TF and total polyphenols which dropped from 122.4 ± 8.02 mg/dl, 118.8 ± 9.30 mg/dl, 110.2 ± 5.25 mg/dl to 115.1 ± 8.98 mg/dl, 114.0 ± 4.31mg/dl and 106.8 ± 3.61 mg/dl respectively. However, the control group did not show any significant difference in mean blood glucose levels.

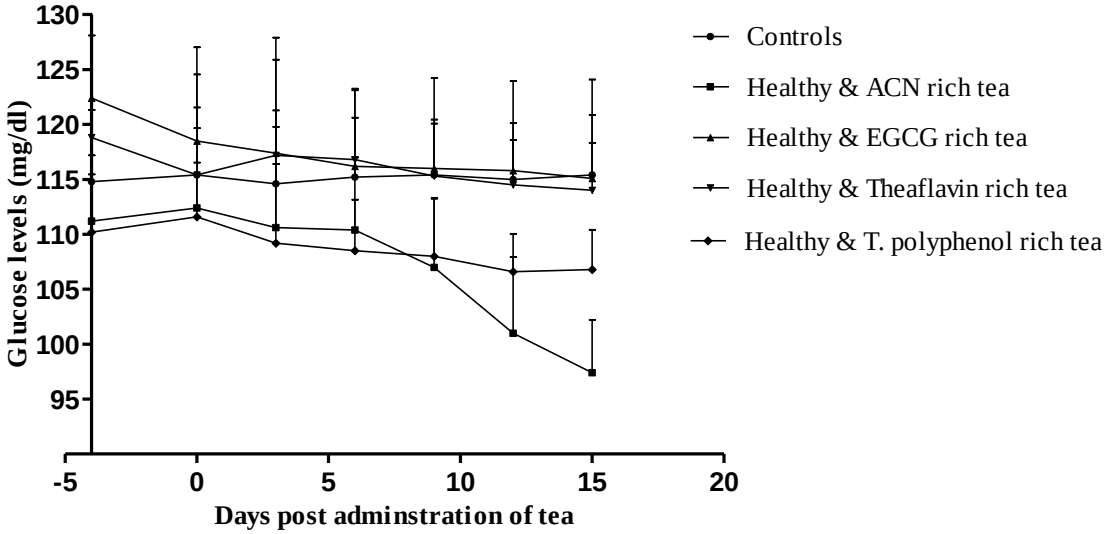


Figure 8: Change in glucose levels of tea treated and control healthy mice

Figure 9 shows the changes in blood glucose levels in treated and untreated diabetic mice over time. Hyperglycaemia, observed by the elevated blood glucose levels was evident immediately following alloxan injection and continued consistently for the next three days. Following oral tea administration, animals recorded a sharp decrease in glucose levels in both diabetic and healthy mice. This was followed by a steady decrease throughout the experimental period. The drop in blood glucose levels was significantly higher in animals on anthocyanin rich tea reaching 248.6 ± 42.96 mg/dl from 468 ± 80.66 mg/dl. Animals on EGCG, TF and total polyphenol rich tea also had consistently lower blood glucose levels until the last day of experiment dropping from 497.0±61.61mg/dl, 437.8 ± 62.25 mg/dl and 514.0 ± 31.73 mg/dl to 347.0 ± 67.31 mg/dl, 312.6 ± 39.87 mg/dl and 363.6 ± 23.77 mg/dl, respectively. Diabetic untreated mice exhibited a steady increase in blood glucose levels throughout the experimental period rising from 457.4 ± 77.03 mg/dl to 490.4 ± 69.12 mg/dl.

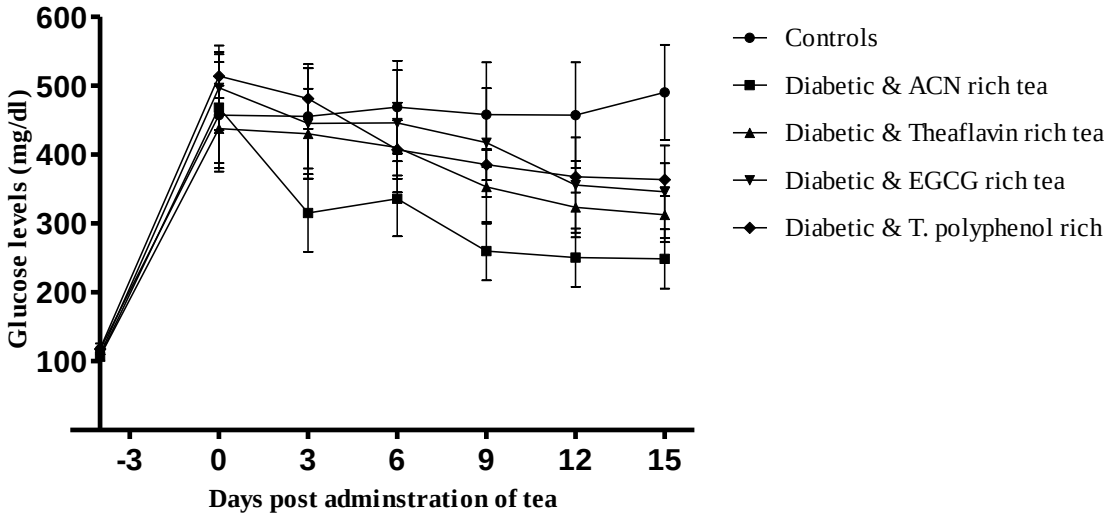


Figure 9: Change in glucose levels of tea treated and control untreated diabetic mice.

4.2.3 Changes in Mice Body weights

Healthy mice on tea also recorded a drop in their mean body weights with anthocyanin rich tea supplemented mice having the highest body weight decline from 31.86 ± 0.83 g to 27.64 ± 0.74 g as compared to TF, EGCG and total polyphenol rich tea which dropped from 30.14 ± 0.78 g, 28.26 ± 1.35 g and 29.40 ± 0.28 g to. 27.60 ± 0.74 g, 24.98 ± 0.97 g and 27.70 ± 0.15 g

respectively. Healthy untreated control mice however, recorded a steady increase in mean body weights rising from 26.42 ± 0.45 g to 31.82 ± 0.52 g (Figure 10).

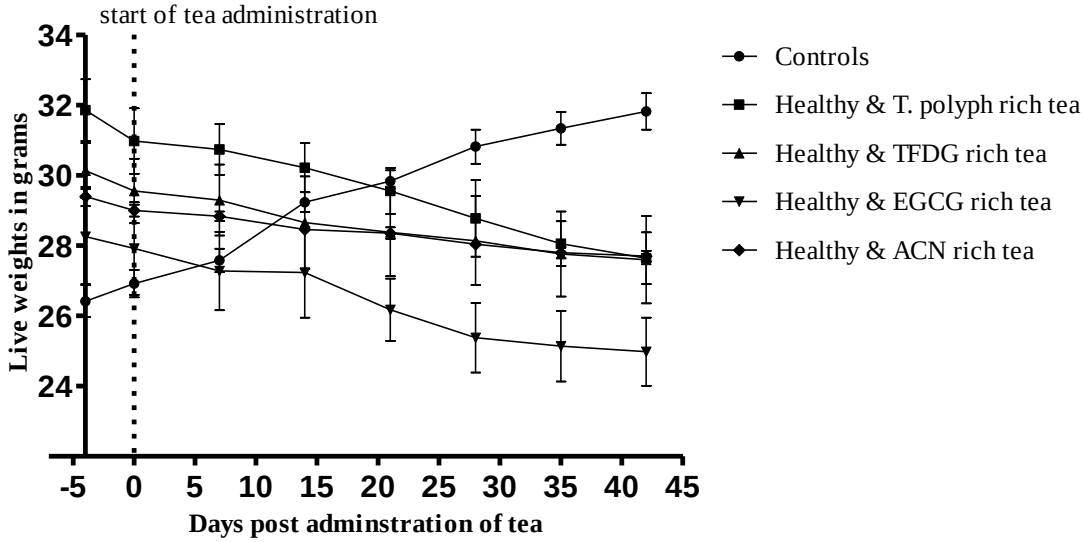


Figure10: Changes in live body weights of tea treated and untreated control healthy mice.

Figure 11 shows the changes in live body weights of diabetic mice over time. Diabetic animals recorded a sharp decrease in body weights following alloxan injection. All the animals on tea supplements including the diabetic control group lost weight consistently throughout the experimental period. Diabetic mice on anthocyanin rich tea recorded the greatest decline in body weights dropping from 29.98 ± 1.21 g to 26.02 ± 1.36 g as compared to TF, EGCG, total polyphenol rich tea and water, which dropped from 29.32 ± 1.44 g, 29.86 ± 1.42g, 27.32 ± 1.83 g and 26.62 ± 0.63 g to 26.8 ± 2.48g, 26.34 ± 1.23 g, 24.8 ± 1.20 g and 22.7 ± 0.35 g, respectively.

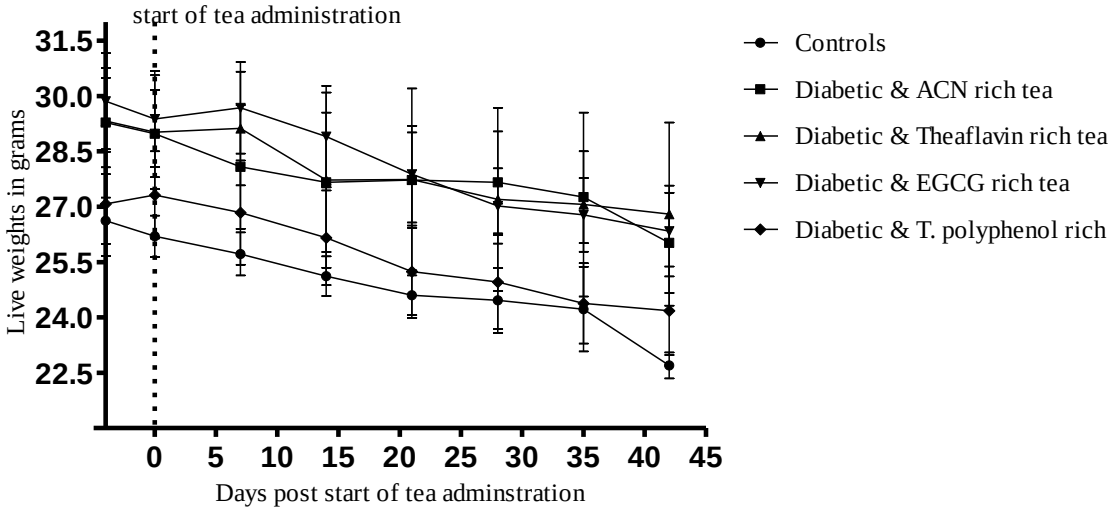


Figure 11: Changes in live body weights of tea treated and untreated control diabetic mice.

4.2.4 Packed cell volume

The changes in the packed cell volume in the healthy mice are presented in figure 12. There was a slight increase in mean PCV levels in healthy mice on tea supplements with anthocyanin rich tea showing the highest marginal increase at 4.37% compared to EGCG, TF and total polyphenol rich teas which had 3.57%, 4.30% and 4.27%, respectively. Healthy control mice also recorded a slight increase in PCV levels at 1.19% rising from 51.0 ± 0.78 to 51.6 ± 1.03%.

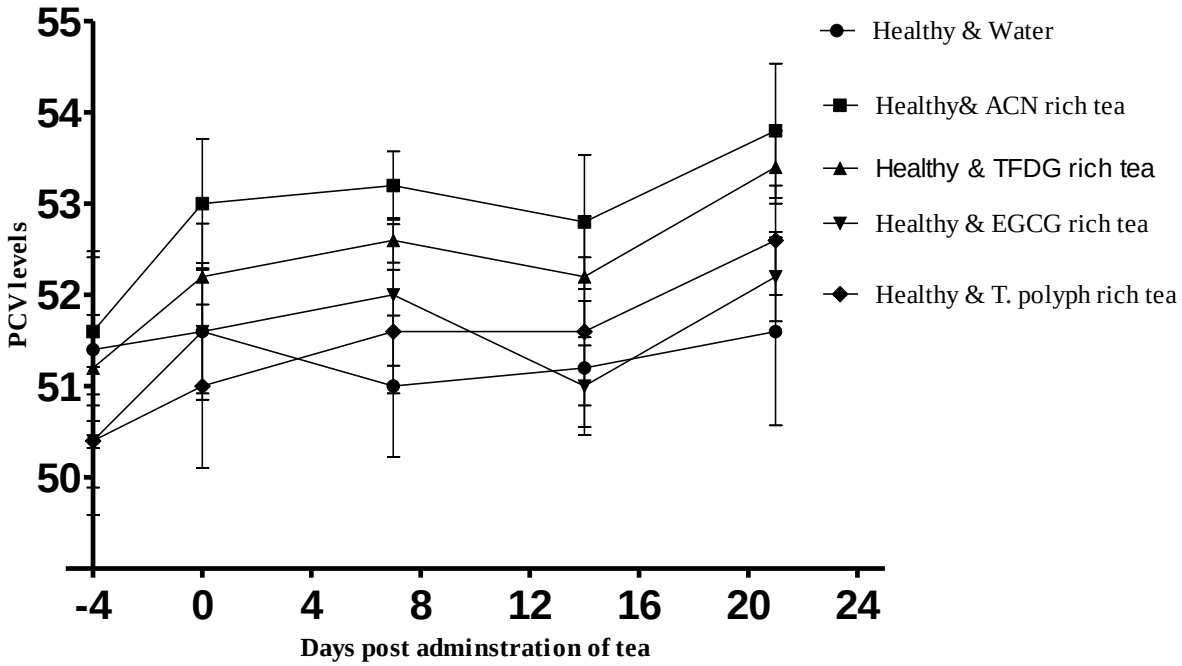


Figure 12: Changes in PCV levels in tea treated and control untreated healthy mice

Figure 13 shows changes in PCV levels in diabetic mice over time. Packed cell volume increased significantly in all groups following tea administration. However, the percentage drop depended on the type of tea administered. Anthocyanin rich tea had the highest percentage gain in PCV at 11.93% rising from 48.6±1.22% to 54.4 ± 0.81% as compared to EGCG, TFDG and total polyphenol rich teas which increased from 48.4 ± 0.93%, 48.8 ± 0.73 and 48.8 ± 0.73% to 51.2 ± 0.58%, 52.0 ± 0.63% and 52.0 ± 0.55% respectively. The percentage increase for EGCG, TF and total polyphenol teas were 5.79%, 6.56% and 6.56%, respectively. The diabetic control group of animals showed declining PCV levels up to 3.24%, reaching 47.8 ± 1.07% from 49.4 ± 0.68%.

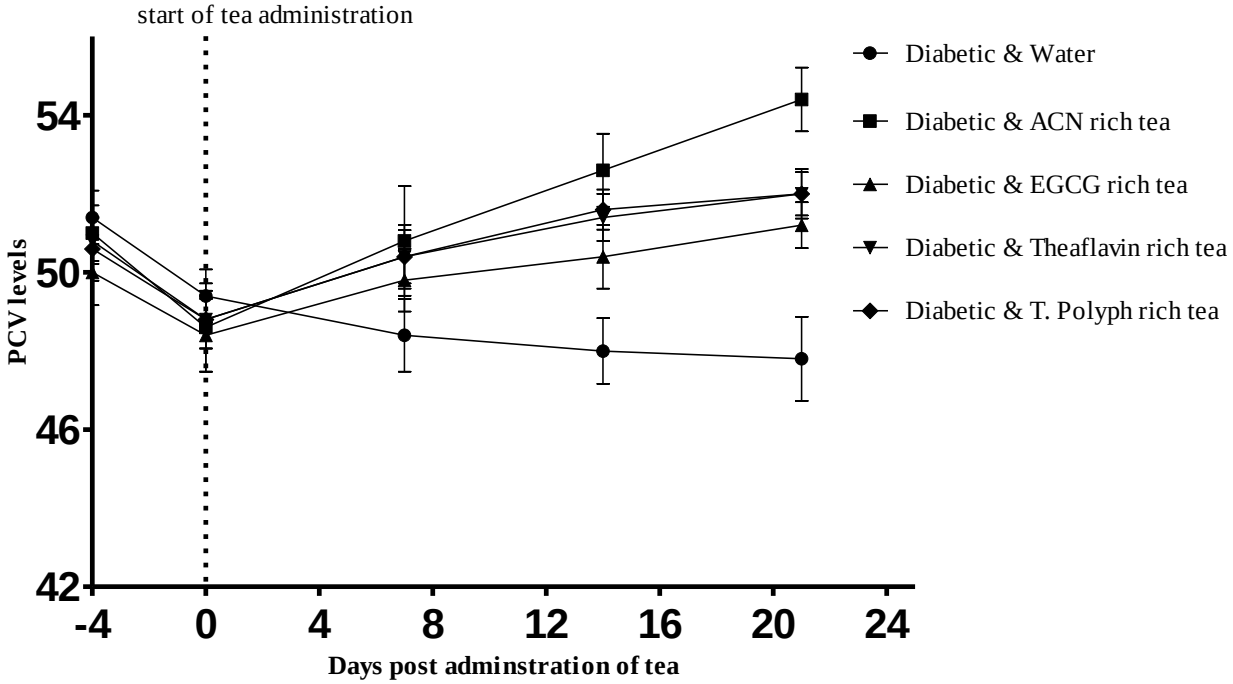


Figure 13: Changes in PCV levels over time in diabetic mice

4.3 Wound Healing Experiments

4.3.1 Wound closure

The changes in skin excision sites were evaluated in both tea untreated controls and mice supplemented with anthocyanin rich tea. Data from 5 mice in each group is expressed as the mean percentage of wound closure ± SEM at each time point (table 4). Wound closure was more accelerated in mice supplemented with anthocyanin rich tea with the wound size decreasing from 26.6 ± 2.38 mm^2 to 15.7 ± 2.31 mm^2 as compared to the diabetic controls which dropped from 28.6 ± 4.18 mm^2 to 23.6 ± 2.54 mm^2. The percentage decrease in wound areas were 40.98%, 17.48%, 33.70% and 21.07% for diabetic mice on tea, diabetic mice on water, healthy mice on tea and healthy mice on water, respectively. These results demonstrate that wound healing was accelerated in the diabetic mice on anthocyanin rich tea as compared to the tea untreated control mice.

Table 4: Mean wound areas in mm^2. All values are expressed as mean ± SEM. The difference between the four groups was not statistically significant at $p>0.05$.

Days post wounding	Wound areas in mm^2			
	Diabetic water	Diabetic tea	Healthy tea	Healthy water
0	28.6±4.18	26.6±2.38	27.6±1.84	29.9±2.82
3	26.1±3.24	23.3±2.71	25.1±1.68	27.3±2.64
7	23.9±3.20	18.6±2.18	21.9±1.59	25.2±2.47
10	23.6±2.54	15.7±2.31	18.3±1.10	23.6±2.51

Digital images of wound sites were taken at day 0 immediately after the injury. On day 10 post-injury, the wounds in the control and diabetic mice on anthocyanin rich tea were diverse with diabetic control mice exhibiting delayed wound closure (figure 14). Significant increase in wound healing was observed in the activity of wound healing in mice on tea supplements as compared to the control. Both healthy and diabetic animals on anthocyanin rich tea showed a decrease in epithelialization period and an increased percentage in wound contraction compared to the controls.

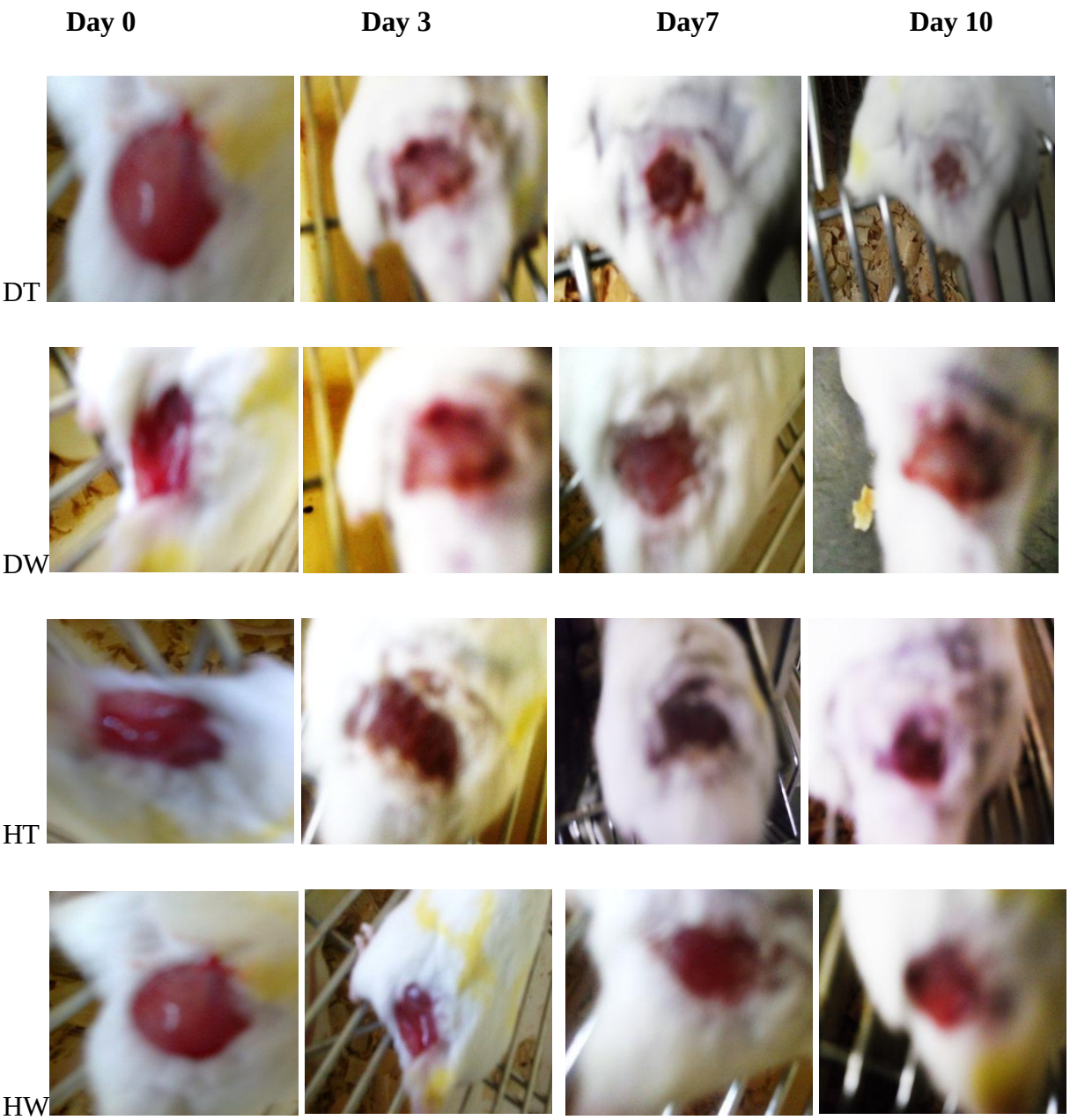


Plate 1: Wound images on day 0, 3, 7, 10 post wounding. Key: (DT-mice on tea supplement, DW- diabetic mice on water, HT- healthy mice on tea, HW- healthy mice on water)

4.3.2 Histopathological studies

Wounds were excised and processed for histology on day 10 post-wounding. Histological examinations of wounds for mice on tea supplements had more collagen fibres and proliferating blood capillaries indicating a more advanced stage of wound healing. Collagen fibres in the granulation tissue are a characteristic of the proliferative stage of wound healing. Animals in the control groups on the other hand exhibited more inflammatory cells an indicator of incomplete epithelialization. Inflammatory cells are mainly observed in wounds that are undergoing inflammation; the first stage in wound healing.

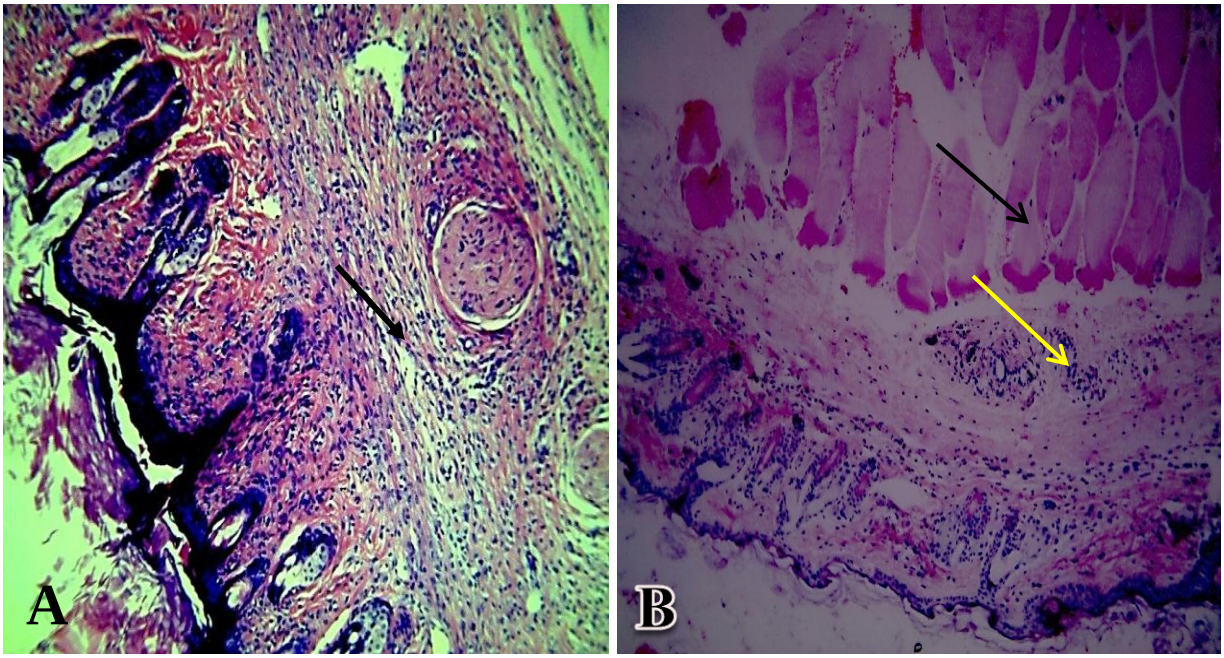


Plate 2: A) Organized collagen fibers with less inflammation (arrows). In diabetic treated mouse wound. B) Incomplete epithelialization in diabetic untreated control with presence of inflammatory cells as shown by arrows (H&E X40)

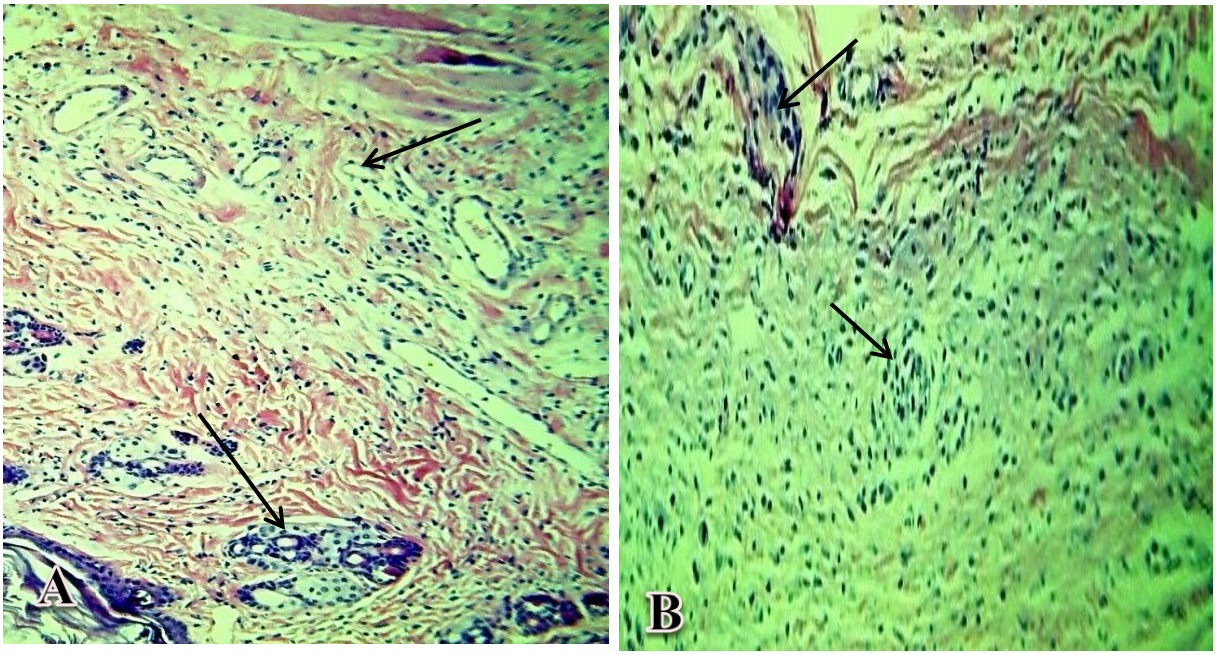


Plate 3: (A) Histological examination of healed wound of healthy mouse on tea. Collagen fibers are more organized and blood vessels observed (arrows) (B) wound of healthy untreated mouse. Collagen fibers are randomly distributed and the connective tissue is infiltrated by inflammatory cells (arrows). (H&E X40).

CHPTER 5
DISCUSSION

5.1 Tea Profiling and Variances in Flavonoid Content

The total polyphenol and total catechin content were significantly higher ($p<0.05$) in green teas than in black teas. This indicates that fermentation process during tea processing had an impact on the final product. Black tea is processed through fermentation, an oxidation reaction catalyzed by polyphenol oxidase enzyme. Green teas on the other hand are steamed to inactivate the process of oxidation. Individual catechins also varied among different teas with EGCG being the highest catechin. Green AHP S15/10 clone tea had the highest level of EGCG levels at 9.7%. Although total catechin content varies widely depending on species, clonal variation, growing location, season, light variation, and altitude, they are present in nearly all teas made from *Camellia sinensis*, including white tea, green tea, black tea and oolong tea (Peterson, 2005).

Generally black teas had higher thearubigin and theaflavin content compared to the green tea samples. The fermentation process used to produce black tea results in the conversion of catechins to theaflavins including theaflavin, theaflavin-3-gallate, theaflavin-3-gallate, and theaflavin-3, 3-digallate and thearubigin polymers (Dreosti, 2000; Kris-Etherton and Keen, 2002). The major fraction of black tea polyphenols is composed of thearubigins, which account for 20% of the solids (Yang and Landau, 2000) and 47% of the total flavonoids (Tijburg *et al*., 1997).

5.2 The Effect of Tea on Blood Glucose and Cholesterol Levels

This study clearly elucidates the continuous and consistent hypoglycemic effect of tea. Animals supplemented with tea recorded a steady reduction in blood glucose level throughout the experimental period as compared to the controls. Anthocyanin rich tea had the most potent effect in lowering glucose levels up to 248.6±42.96 mg/dl from 468±80.66 mg/dl. Diabetes mellitus is by far the world's most ubiquitous endocrine disorder which is characterized by chronic hyperglycemia that causes long term complications of diabetes (Brown *et al*., 2010); therefore, new methods to regulate blood sugar levels are highly valuable. Botanicals in general and especially polyphenols have been reported as potential effective mediators to control hyperglycemia and insulin resistance (Al-Awwadi *et al*., 2004; Stull *et al*., 2010). The ability of

tea flavonoids in inhibition of elevating blood glucose levels and improvement of insulin sensitivity has been attributed to enhanced activity of residual insulin in the alloxan diabetes induced animals or promotion of glucose uptake by peripheral tissues (Twaij and Al-Badr, 1988; Gupta, 1994). It is also possible that the tea supplement may have slowed down glucose absorption in the gastrointestinal tract or regulated glucose metabolism in the liver.

Hypercholesterolemia, is one of the major complications of diabetes and occurs in about 40% of diabetes cases (Wolfe *et al*., 2003; Kim *et al*., 2006), and is directly linked to insulin deficiency (Ramarathnam *et al*., 1997). Increase in total cholesterol levels was recorded in diabetic untreated animals over the entire experimental period. Similar consistent results have been reported (Mironova *et al*., 2000; Barnett and O' Gara, 2003). Diabetic treated animals recorded a significant reduction in total cholesterol levels with anthocyanin rich tea supplemented group recording the highest drop. Dyslipidemia occurs as a result of the inability of peripheral tissues to access blood glucose; therefore any attempt to control hyperglycemia will help in regulating cholesterol levels as well. Artheriosclerosis and ischemic condition can be prevented by effective lowering of total and LDL cholesterol and raising HDL (Fuller *et al*., 1980).

5.3 Packed Cell Volume and Body Weights

There was a significant drop in PCV levels of diabetic untreated animals as compared to the healthy controls. The presence of anemia in diabetes mellitus is due to the increased non-enzymatic glycosylation of red blood cell (RBC) membrane proteins, which is associated with hyperglycemia (Szudelski, 2001). When these glycosylated membrane proteins are oxidized coupled to hyperglycemia in DM, there is an increase in lipid peroxidase production resulting in hemolysis of RBC through many pathological consequences (Kolanjiappan *et al.*, 2002). Diabetic animals on tea exhibited a significant higher levels of PCV compared to the controls. This can be attributed to the lowered hemolysis of RBC in diabetic animals. Tea contains various flavonoids that are antioxidants in nature and have the ability to protect RBC from hemolysis. RBC membranes contain high amounts of polyunsaturated lipids and a rich supply of oxygen hence their susceptibility to lipid peroxidation. Free radicals are generated in hyperglycemia which then attacks the erythrocytes causing hemolysis (Karori *et al*., 2007). Tea flavonoids on the other hand are potent antioxidants that can help ameliorate hemolysis of RBC hence boosting their levels.

Alloxan induced diabetic animals recorded a sharp decrease in mean body weights and continued throughout the experimental period reaching 22.7±0.35g from 24.8±1.20g. Poorly controlled diabetes causes weight loss through muscle wasting (Elia M., 1981). In addition, having an insulin deficiency causes a decrease in muscle synthesis and increases its breakdown (Charlton and Nair, 1998).

All the animals on tea supplements similarly recorded a significant decline in body weight as compared to healthy controls which gained weight consistently throughout the experimental period. Flavonoids have been shown to inhibit the action of catechol-o-methyl transferase (COMT) the enzyme that degrades norepinephrine resulting in the prolonged action of catechol amines (Auvichayapat *et al*., 2008). Caffeine inhibits the phosphodiesterase-induced degradation of intracellular cyclic AMP leading to an increase in norepinephrine release. It is thought that these two actions combined can stimulate energy expenditure and promote fat oxidation resulting in the reduction of body weight. It had also been suggested that catechol amines might possibly play a role in satiety. Given the important role of the sympathetic nervous system and its neurotransmitter norepinephrine in the control of thermogenesis and fat oxidation, it is apparent that flavonoids, by inhibiting COMT, result in an increase in or a more prolonged effect of norepinephrine on thermogenesis and fat metabolism or both (Dulloo *et al*., 1996).

5.4 Wound Healing

Wound healing is a complicated process involving restoration of cell structures in a damaged tissue. Wound contraction is a process that occurs throughout the healing process, starting from the fibroblastic stage where the wound area shrinks (Ghasemi *et al*., 2012). Complications and associated problems may occur and eventually cause a delay in wound healing hence increase in the possibility of infection, delayed recovery and development of ugly scars (Tredget *et al*., 1997). There are three stages of wound healing depending on the type and extent of damage, the general state of the host's health, and the ability of the tissue to repair (Stadelmann *et al*., 1998). The inflammatory phase is characterized by hemostasis and inflammation, followed by epithelialization, angiogenesis, and collagen deposition in the proliferative phase (Ghasemi *et al*., 2012). In the maturational phase, the final phase of wound healing, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue (Shukla *et al*., 1999).

In the present study, wound healing analysis was divided into several stages with characteristic parameters: At wound infliction, 1 to 3 days post wounding: Morphologically, this stage included blood-clot formation (primary clot), 4 to 7 days post-wounding: Morphologically, this stage is marked by scab formation 8 to 10 days post wounding: Morphologically, scab detachment was observed. Histological results exhibit the formation of new epidermis that becomes differentiated by day 10. In addition, dermal closure is initiated, concomitant to granulation-tissue formation. Anthocyanin rich tea significantly accelerated the rate of wound healing. Oral administration of the purple tea sample significantly accelerated the rate of wound healing, and collagen, fibroblasts, and blood capillaries were contained in granulation tissue but without inflammatory cells. The wound healing effects may be attributed to regulation of the expression of collagen I (Bonte *et al*., 1994) and an increased wound tensile strength (Suguna *et al*., 1996). Accelerated healing process has been ascribed to an increase in collagen formation and angiogenesis (Trabucchi *et al*., 1986; Shukla *et al*., 1999). Angiogenesis in granulation tissues improves blood flow to the wound site, therefore providing nutrients and oxygen necessary for the healing (Szabo *et al*., 1995). There are many studies in green tea healing aspects in diseases, like anti-oxidant, anti-cancer, anti-aging, anti-inflammatory effects and preventive effects of collagen production and accumulation (Frei and Higdon, 2003). The majority of these properties could be attributed to the plant's polyphenolic compounds (Katiyar and Elmets, 2001).

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the results of this study, conclusions made include:

i. Teas high in flavonoids have beneficial effects in regulating blood glucose and cholesterol levels in diabetic mice.
ii. Oral administration of tea rich in flavonoids improves disturbances of hematological factors induced by diabetes and protects erythrocytes from oxidative stress. Tea also causes weight loss in healthy and diabetic mice models.
iii. Tea administered orally helps accelerate the process of wound closure and healing in mice models and may be a promising medication for wound management.

6.2 Recommendations

i. Further biochemical and pharmacological investigations need to be carried out to clearly elucidate the mechanism of action through which tea regulates blood glucose which will be useful in development of a diabetes therapy.
ii. Tea flavonoids should be individually extracted and tested for their anti-diabetic potential and wound healing properties. Further studies need to be done to identify and separate the active constituents responsible for anti-diabetic and wound healing activity.
iii. Purple tea should be considered a therapeutic agent and more plant should be grown and processed to be availed as a dietary supplement.

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