INFLUENCE OF GALLIC ACID ON α-AMYLASE AND α-GLUCOSIDASE INHIBITORY AND ANTIOXIDANT PROPERTIES OF ACARBOSE

BY

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CERTIFICATION

This is to certify that this thesis was written as carried out by OGUNBADEJO MARIAM DAMILOLA, Matric no: BTH/11/0255 under the supervision of PROF. GANIYU OBOH and submitted to the department biochemistry, Federal University, Oye-Ekiti.

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DEDICATION

This thesis is dedicated to Almighty God, in whom I have grace, wisdom, knowledge and understanding.

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is a chronic progressive disease that has continued to be a global heath and economic burden. Acarbose is an antidiabetic drug, which acts by inhibiting alpha amylase and alpha glucosidase; while gallic acid is a simple phenolic acid that is widespread in plant foods and beverages such as tea and wine.This study therefore, sought to investigate the influence of gallic acid on α-amylase and α-glucosidase inhibitory and antioxidant properties of acarbose (*in vitro*). Aqueous solution of acarbose and gallic acid were prepared to a final concentration of 25µM each. Thereafter, mixtures of the samples (50% acarbose + 50% gallic acid; 75% acarbose + 25% gallic acid; 25% acarbose + 75% gallic acid) were prepared. The results showed that the combination of 50% acarbose and 50% gallic acid showed the highest α-glucosidase inhibitory effect, while 75% acarbose + 25% gallic acid showed highest α-amylase inhibitory effect. Furthermore, all the samples caused the inhibition of Fe^{2+}-induced lipid peroxidation (*in vitro)* in rat pancreatic tissue homogenate, with the combination of 50% acarbose and 50% gallic acid causing the highest reduction in the malondialdehyde content. In addition, all the samples showed antioxidant properties (ferric reducing property, 2, 2'-azino-bis (-3-ethylbenzthiazoline-6-sulphonate (ABTS*) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals scavenging abilities, and Fe^{2+} chelating ability). Therefore, the combinations of gallic acid with acarbose could be employed in the management of T2DM with the comparative advantage of possible reduction of the side effects of acarbose; nevertheless the combination of 50% acarbose and 50% gallic acid seems the best combinatory therapy for the management of type 2 diabetes mellitus.

CHAPTER ONE

1.0 INTRODUCTION

Diabetes Mellitus (DM) commonly referred to as diabetes is a group of metabolic diseases characterized by hyperglycemia (High blood sugar levels over a prolonged period), either because the pancreas does not produce enough insulin, or because the cells do not respond to the insulin that is produced (David and Gardner, 2011). The main symptoms of high blood sugar include polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger). If left untreated, this chronic disease can cause many complications (Cooke and Plotnick, 2008).

Type 2 diabetes is the most common form of diabetes (Shi and Hu 2014); it is characterized by insulin resistance, which may be combined with relatively reduced insulin secretion (David and Gardener, 2011), leading to hyperglycemia and ultimately malfunctioning of the pancreatic β-cells. Prolonged hyperglycemia results in increased generation of reactive oxygen species (ROS) and alteration of endogenous antioxidants (Ohkuwa *et al.,* 1995). Oxidative stress resulting from the hyperglycemic condition in Type 2 diabetes has been implicated in the impairment of the pancreatic β-cells and diabetes complications such as diabetes nephropathy (damage to the kidney) (Shukla *et al.,* 2003), diabetes retinopathy (damage to the eye) (Bearse *et al.,* 2004; Hove *et.,* 2004) and diabetes neuropathy (damage to the nerves of the body) (Seki *et al.,* 2004).

A practical approach to reducing the postprandial hyperglycemia is to retard the absorption of carbohydrates after food intake (Oboh and Ademiluyi, 2013). This could be achieved through the inhibition of α-amylase and α-glucosidase present in the gastrointestinal

tract (shim *et al.,* 2003). Inhibitors of these enzymes slow down carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Rhabasa- Lhoret and Chiasson, 2004). The dietary saccharides are first broken down to monosaccharides by certain gastrointestinal enzymes, since only monosaccharides can be absorbed from the intestinal lumen. Polysaccharides are hydrolyzed to oligosaccharides and disaccharides by α-amylase and intestinal α- glucosidase further hydrolyzes it to glucose before being absorbed into the intestinal epithelium entering the blood circulation (Oboh *et al.,* 2011).

Several reports have been published on established enzyme (α-glucosidase and α-amylase) inhibitors such as Acarbose, Miglitol, voglibose, nojirimycin and 1- deoxynojirimycin and their favorable effects on blood glucose levels after food uptake (Kim *et al.,* 2005). Enzyme inhibitors may also act as effective anti-obesity agents (Kotowaroo, *et al.,* 2006). This could be due to inhibition of saccharide assimilation, by inhibiting starch breakdown (Oboh *et al.,* 2010). The reduced amount of amylase available for the breakdown enables complex saccharides to have a better chance for travelling through the gastrointestinal tract (GIT) without being assimilated, which are eventually excreted from the body instead of being converted into storage fat (Oboh *et al.,* 2010).

Acarbose is an oral alpha-glucosidase and alpha amylase inhibitor for use in the management of Type 2 diabetes mellitus (Wang *et al.,* 2014). It is chemically known as O-4,6-dideoxy-4-[[(1S,4R,5S,6S)-4,5,6-trihydroxy-3-(hydroxymethyl)-2-cyclohexen-1-yl]amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose (Bayer Healthcare Pharmaceuticals, 2011). The antihyperglcemic action of acarbose results from a competitive, reversible inhibition of pancreatic alpha amylase and a membrane bound intestinal alpha

glucoside hydrolase enzyme. Acarbose is shown to reduce and slow down the intestinal absorption of glucose, which subsequently minimize the postprandial rise of blood glucose and insulin concentration (Wang *et al.,* 2014). It was first extracted from the culture broths of actinomycetes by Puls and his colleagues in the 1970s, and was applied in clinical studies for more than 10 years (Coniff and Krol, 1997; Scheen, 1998; Junger *et al.,* 2000). It reversibly inhibits alpha-glycosidases that exist in the brush-border of the small intestinal mucosa (Clissold and Edwards, 1988). Acarbose does not cause hypoglycemia and its minor gastrointestinal side effects can be prevented by gradual dosage increments (Wang *et al.,* 2014).

In recent years, there has been an increased interest in the application of antioxidants to medical treatment, as information is available linking the development of human diseases to oxidative stress (Giustarini *et al.,* 2009). Natural foods are known to contain natural antioxidants that can scavenge free radicals. Small molecule dietary antioxidants, such as vitamin C, vitamin E and carotenoids have procreated particular interest as defenses against degenerative diseases (Kohlmier and Hastings, 1995; Stampfer and Rimm, 1998). However, some studies have indicated that phenolic acids are considerably more potent antioxidants than vitamin C and vitamin E (Vinson *et al.,* 1995; Cao *et al.,* 1997). Phenolic compounds form a substantial part of plant foods, most of them have shown antioxidant properties both in *in vitro* and *in vivo* studies (Rice- Evans *et al.,* 1996).

Gallic acid is a ubiquitous natural product with various industrial applications including ink dyes, tanning products, and paper (Eslami *et al.,* 2010). Recent studies have documented that gallic acid and its esters [e.g., (-)-epi-gallocatechin-3-gallate] exert antioxidant, anticancer, antiviral, and many other biological effects (Sohi *et al.,* 2003; Tachibana *et al.,* 2004; Sameermahmood *et al.,* 2010).

1.1 JUSTIFICATION

Acarbose is an established antidiabetic drug that inhibits α- glucosidase and α- amylase (key enzymes relevant to type 2 diabetes) activities, but with well reported deleterious side effects. Gallic acid is a phenolic acid which is ubiquitous in many food/ natural sources; it can be classified as one of the dietary antioxidants. Consequently, this aims to investigate the effect of gallic acid on the enzyme (α- glucosidase and α- amylase) inhibitory and antioxidant properties of acarbose *in vitro*.

1.2 OBJECTIVES

The specific objectives of this project are to:

- Evaluate the effect of gallic acid on the *in vitro* inhibitory effect of acarbose on α-glucosidase and α- amylase
- Evaluate the effect of gallic acid on antioxidant properties of acarbose.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 DIABETES MELLLITUS

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Goldenberg and Punthakee, 2013). Several pathogenic processes are involved in the development of diabetes, these ranges from autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues (Diabetes care, 2002). Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently co-exist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia.

2.1.1 PATHOPHYSIOLOGY OF DIABETES MELLITUS

The uptake of glucose from the blood into most cells of the body, especially liver, muscle, and adipose tissue is regulated by insulin. Therefore, deficiency of insulin or the insensitivity of its receptors plays a central role in all forms of diabetes mellitus (American Diabetes Association, 1997).

The body obtains glucose from three main sources: the intestinal absorption of food, the breakdown of glycogen, the storage form of glucose found in the liver and gluconeogenesis (the generation of glucose from non-carbohydrate substrates in the body) (David and Gardner, 2011). Insulin functions in balancing glucose levels in the body. Insulin can inhibit the breakdown of glycogen or the process of gluconeogenesis, it can stimulate the transport of glucose into fat and muscle cells, and it can stimulate the storage of glucose in the form of glycogen (David and Gardner, 2011).

Insulin is released into the blood by beta cells (β-cells), found in the islets of Langerhans in the pancreas, in response to rising levels of blood glucose, typically after eating. Insulin is used by about two-thirds of the body's cells to absorb glucose from the blood for use as fuel, for conversion to other needed molecules, or for storage. Lower glucose levels result in decreased insulin release from the beta cells and in the breakdown of glycogen to glucose. This process is mainly controlled by the hormone glucagon, which acts in the opposite manner to insulin (Kim and Barrett, 2012). If the amount of insulin available is insufficient, if cells respond poorly to the effects of insulin (insulin insensitivity or insulin resistance), or if the insulin itself is defective, then glucose will not be absorbed properly by the body cells that require it, and it will not be stored appropriately in the liver and muscles. The net effect is persistently high levels of blood glucose, poor protein synthesis, and other metabolic derangements, such as acidosis (David and Gardner, 2011).

When the glucose concentration in the blood remains high over time, the kidneys will reach a threshold of reabsorption, and glucose will be excreted in the urine (glycosuria) (Robert and Murray, 2012). This increases the osmotic pressure of the urine and inhibits reabsorption of

water by the kidney, resulting in increased urine production (polyuria) and increased fluid loss. Lost blood volume will be replaced osmotically from water held in body cells and other body compartments, causing dehydration and increased thirst (polydipsia) (David and Gardner, 2011).

2.1.2 CLASSIFICATION OF DIABETES MELLITUS

Assigning a type of diabetes to an individual often depends on the circumstances present at the time of diagnosis, and many diabetic individuals do not easily fit into a single class. For example, a person with gestational diabetes mellitus (GDM) may continue to be hyperglycemic after delivery and may be determined to have, in fact, type 2 diabetes (William, 2008). Alternatively, a person who acquires diabetes because of large doses of exogenous steroids may become normoglycemic once the glucocorticoids are discontinued, but then may develop diabetes many years later after recurrent episodes of pancreatitis (Williams, 2008). Another example would be a person treated with thiazides who develops diabetes years later (Mayen *et al.,* 2002). Because thiazides in themselves seldom cause severe hyperglycemia, such individuals probably have type 2 diabetes that is exacerbated by the drug (Mayen *et al.,* 2002). Thus, for the clinician and patient, it is less important to label the particular type of diabetes than it is to understand the pathogenesis of the hyperglycemia and to treat it effectively (Diabetes care, 2002).

2.1.2.1 Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)

Type 1 diabetes is also frequently referred as insulin-dependent, juvenile or childhood diabetes characterized by inefficient or no insulin production by the pancreas. It is one of the most common types of diabetes in children and is divided into two types: Type 1A and Type 1B diabetes (Arora *et al.,* 2013). In Type 1A (immune mediated diabetes), the immune cells are

responsible for the destruction of the islet of Langerhans β cells (responsible for insulin production) present in pancreas while the type 1B diabetes is caused by the inefficient production of insulin by the islet of Langerhans β cells (Devendra *et al.*, 2004). The resulting deficiency in insulin also means a deficiency in the other co-secreted and co-located cell hormone, amylin (Kruger *et al.,* 1999). As a result, postprandial glucose concentrations rise due to lack of insulin-stimulated glucose disappearance, poorly regulated hepatic glucose production, and increased or abnormal gastric emptying following a meal. The cause for this diabetes is still unknown and is usually cured by administration of external insulin (Devendra *et al.*, 2004).

2.1.2.1 Type 2 diabetes mellitus

This form of diabetes, previously referred to as non-insulin-dependent diabetes, type 2 diabetes, or adult-onset diabetes, is a term used for individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency (Reaven *et al.*, 1976; Olefsky *et al.*, 1982; DeFronzo *et al.,* 1979; Turner *et al.,* 1979). A marked postprandial increase in blood glucose is the hallmark of diabetes, but is also observed in pre-diabetic individuals with impaired glucose tolerance (DeFronzo *et al.,* 1979). Individuals with both conditions lack the ability to regulate the release of insulin, which prevents this postprandial increase in healthy people. Therefore, a significant aspect of the pathogenesis of type 2 diabetes is an increased blood glucose concentration, with maximum, but metabolically inadequate, stimulation of insulin secretion (Williams, 2008). One reason for this is the reduced number of insulin-producing beta cells in the pancreas (caused by an increased rate of apoptosis) which results in relative dominance of secretion of glucagon, the insulin antagonist. Inadequate insulin secretion reduces the glucose flow from blood into peripheral organs. Simultaneously, glucagon increases the stimulation of gluconeogenesis in the liver, both fasting and postprandially between meals.

Additionally, type 2 diabetics are insulin-resistant, which means that uptake of glucose into the cells of the peripheral organs is continuously deteriorating. In the long term, the continuously reducing number of beta cells and the decrease in peripheral glucose uptake causes an increase in glucose concentrations, and glucose toxicity intensifies. Hyperglycemia initiates a large number of regulatory processes which can exert harmful effects on the vascular system (Martinez *et al.,* 2005). Vessels are directly damaged, resulting in the increased morbidity and mortality associated with diabetes (Nishikawa *et al.,* 2000). The objective of any pharmacological and non-pharmacological therapeutic measures must therefore be a reduction in glucose toxicity, that is, in increased blood glucose concentrations (Scarlett *et al.,* 1982; Firth *et al.,* 1986). The algorithms of national and international guidelines for the treatment of diabetes are based on the principle that patients should be established on suitable oral therapies for the reduction of blood glucose concentrations and glucose toxicity depending on their glycosylated hemoglobin (HbA1c) concentrations (Samantha *et al.,* 2008). These therapies should be extended as required and ultimately be supplemented by insulin substitution to compensate for lack of adapted insulin release (Wang, 2008).

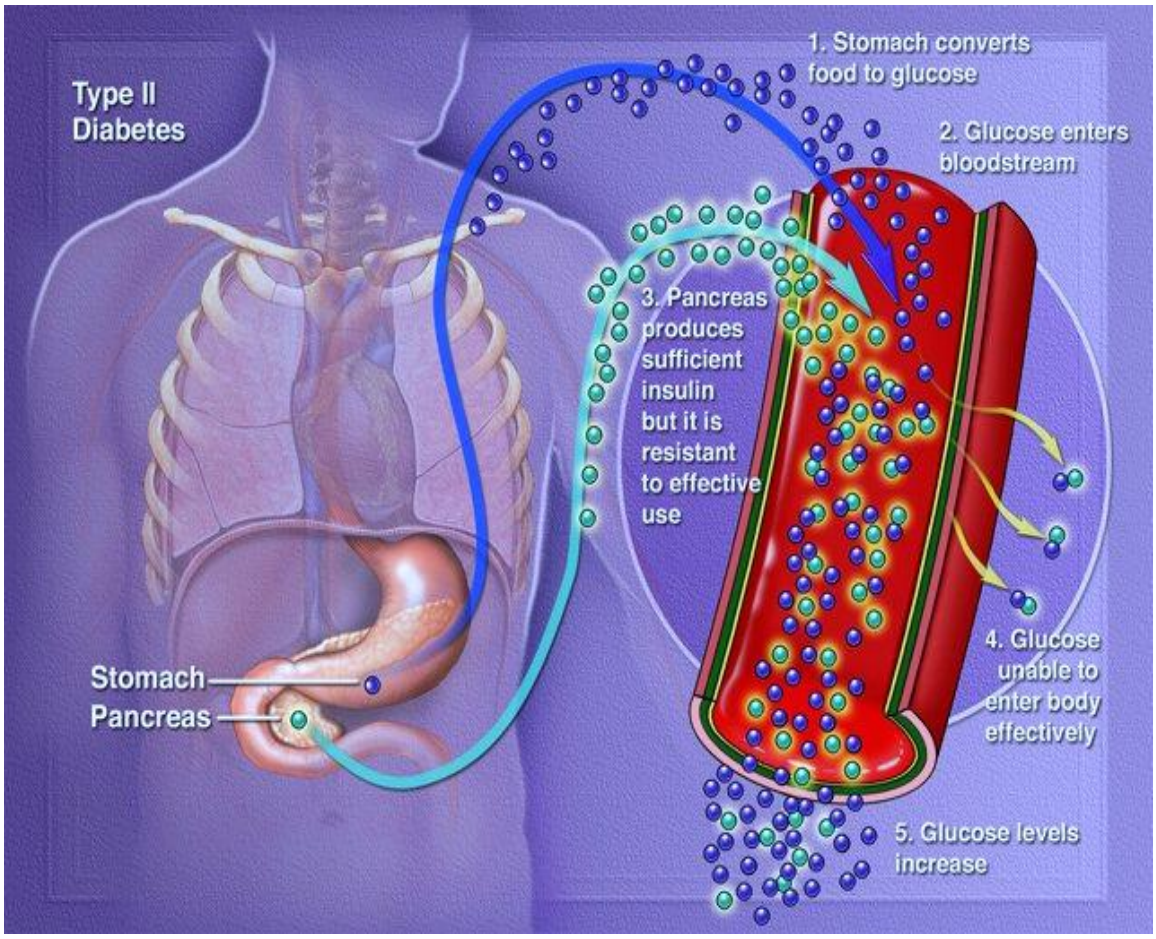

Figure 2.1: A diagram showing the pathogenesis of type 2 diabetes

Source: Wikipedia

2.1.3 PREVALENCE AND INCIDENCE OF DIABETES MELLITUS

Diabetes was found to be present in humans even in 200 A.D. as described by a Greek physician Arateus, who gave this disease its name (Arora *et al*, 2013). Although, it was present since so many years, still it is not considered a threat until the 20th century. This disease is now considered as a serious threat, because millions of people have died due to this. As stated by world health organization (WHO) in November 2014, 347 million people worldwide are reported to have different types of diabetes and there is a gradual increase in the number of patients. It was observed that 2.8% world population was suffering from diabetes in 2000, which increased to 6.8% in 2010 and is expected to further rise to 7.7% in 2030 (Wild *et al*, 2004; Shaw *et al*, 2010). WHO estimates that in 2012, approximately 1.5 million deaths were directly caused by diabetes, and more than 80 percent of these deaths occurred in low- and middle-income areas. In 2012, 29.1 million Americans, or 9.3% of the population, had diabetes. Approximately 1.25 million American children and adults have type 1 diabetes.

In April 2014, the National Institutes of Health (NIH) reported that the percentage of people with diabetes in the United States doubled since 1998. Nearly 1 in 10 adults have been diagnosed with the condition, and fewer people are thought to be undiagnosed—perhaps due to improved screening methods for diabetes. Globally, the five countries with the largest numbers of people with diabetes are China, India, US, Russia and Brazil, according to International Diabetes Federation estimates in 2011. The prevalence of type 2 diabetes mellitus among children in the UK is rising. In September 2014, the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health issued a report stating that factors that increase diabetes incidence may differ between the genders. According to the report, increased diabetes prevalence in women in the United States between 1976 and 2010 may be

attributed to higher body mass index (BMI) in women, an aging population, and changes in race and ethnicity. In men, increased diabetes prevalence may be associated with higher rates of overweight/obesity (indicated by higher BMI), improved survival times compared to women with the condition, and changes in physical activity, sleep, and other factors (World Health Organization, 2013).

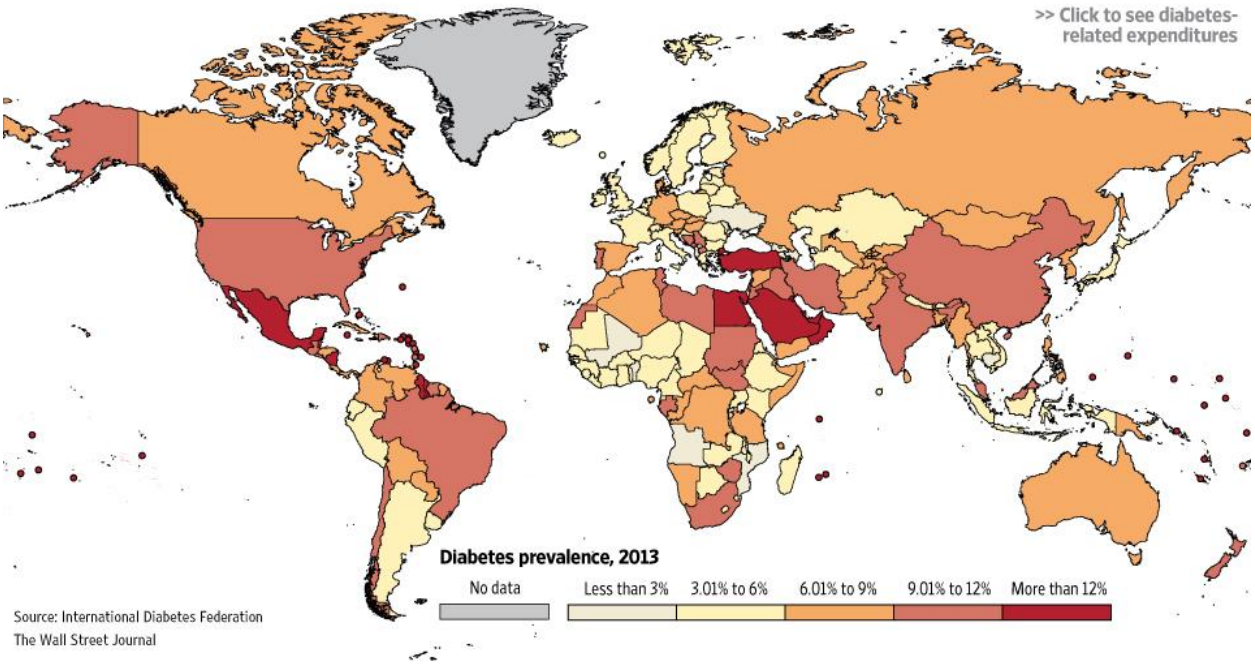

Figure 2.2: Map showing the prevalence of diabetes mellitus

Source: International Diabetes Federation, The wall Street Jouurnal (2014)

2.1.4 KEY ENZYMES LINKED TO TYPE-2 DIABETES MELLITUS

Starch is the major dietary carbohydrate source of glucose for the human, and the rate and extent of starch digestion is associated with glycemia-related problems such as diabetes and other metabolic syndrome conditions. To generate dietary glucose from starchy foods, salivary and pancreatic α-amylase and four small intestine mucosal α-glucosidase subunits are employed in the human body (Dhital *et al.,* 2013). Pancreatic alpha amylase is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of oligoglucans. These are then acted on by alpha glucosidase and further degraded to glucose which on absorption enters in to blood stream (Sabu and Kuttan 2009).

2.1.5 ALPHA- GLUCOSIDASE INHIBITORS

Alpha-glucosidase (EC3.2.1.20, maltase, glucoinvertase, glucosidosucrase, maltase-glucoamylase, alpha-glucopyranosidase, glucosidoinvertase, alpha-D-glucosidase, alpha-glucoside hydrolase, alpha-1,4-glucosidase, alpha-D-glucoside glucohydrolase) is a glucosidase located in the brush border of the small intestine that acts upon 1,4-alpha bonds (Bruni *et al,* 1970; Flanagan and Forstner, 1978; Larner, 1960; Sivikami and Radhakrishnan, 1973; Sorensen, 1982). The membrane-bound intestinal alpha-glucosidases hydrolyze oligosaccharides, trisaccharides, and disaccharides to glucose and other monosaccharides in the small intestine.

Alpha-glucosidase inhibitors are saccharides that act as competitive inhibitors of enzymes (especially alpha glucosidase) needed to digest carbohydrates. Currently, three (3) drugs are therapeutically used as anti-glucosidases: acarbose, miglitol and voglibose (DeFronzo, 1999). These drugs have greater glycemic control over hyperglycemia in diabetes mellitus type 2, particularly with regard to postprandial hyperglycemia, by interfering with the rate of digestion

of dietary carbohydrate. Therefore, less glucose is absorbed because the carbohydrates are not broken down into glucose molecules. In diabetic patients, the short-term effect of these drugs therapies is to decrease current blood glucose levels: the long-term effect is a small reduction in glycosylated hemoglobin (glycohemoglobin or hemoglobin A1C) level (Samantha *et al.,* 2008).

2.1.6 ALPHA AMYLASE INHIBITORS

α-Amylase (α- 1,4- glucan-4-glucanohydrolase) is an endo-acting enzyme EC3.2.1.1 that hydrolyses alpha bonds of α-(1,4) glycosidic linkages of polysaccharides, such as starch and glycogen, yielding glucose and maltose (Maureen, 2000). It is the major form of amylase found in humans and other mammals (Voet and Voet, 2005). It is also present in seeds containing starch as a food reserve, and is secreted by many fungi. α- amylase inhibitors such as acarbose, miglitol, voglibose, nojirimycin and 1- deoxynojirimycin, also known as carbo- blockers (Kim *et al.,* 2000) prevent degradation of complex dietary carbohydrates to oligosaccharides and dissacharides. Theses inhibitors are indirectly helpful in weight loss due to ability to prevent sugar assimilation, through inhibition of starch hydrolysis (Kim *et al.,* 2000).

2.2 FREE RADICALS AND OXIDATIVE STRESS

Free radicals are continuously produced by the body's normal use of oxygen (Tiwari, 2004). Hence, free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Halliwell and Gutteridge, 1999). Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are produced by the mitochondria. These by-products are generally reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) that result from the cellular redox

process. The free radicals have a special affinity for lipids, proteins, carbohydrates and nucleic acids (Velavan, 2011).

Reactive oxygen species can be classified into oxygen-centered radicals and oxygen-centered non radicals. Oxygen-centered radicals are superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), alkoxyl radical ($RO\cdot$), and peroxyl radical ($ROO\cdot$). Other reactive species are nitrogen species such as nitric oxide ($NO\cdot$), nitric dioxide ($NO_2\cdot$), and peroxynitrite ($OONO^-$). Oxygen-centered non-radicals are hydrogen peroxide (H_2O_2) and singlet oxygen (O_2), hypochlorous acid and ozone (Halliwell *et al*, 1995; Simon *et al*, 2005).

It has been established that ROS can be both harmful and beneficial in biological systems depending on the environment and concentration (Glade, 2003; Lopaczynski and Zeisel, 2011). Beneficial effects of ROS involve, for example, the physiological roles in cellular responses to noxia such as defense against infectious agents, and in the function of a number of cellular signaling systems and gene expression. In contrast, at high concentrations, ROS can mediate damage to cell structures, including lipids and membranes, proteins and nucleic acids; this damage is often referred as "oxidative stress" (Poli *et al.*, 2004). Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants or reactive oxygen species (Rahman *et al.*, 2012). This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism (Durackova, 2010).

2.3 HYPERGLYCAEMIA AND OXIDATIVE STRESS

Increased oxidative stress, which contributes to the pathogenesis of diabetes and its complications, is the consequence of either enhanced ROS production or attenuated ROS scavenging capacity (Maria *et al.*, 2007). Several mechanisms, including auto-oxidative

glycosylation, formation of advanced glycosylated end products (AGEs), and increased polyol pathway activity contribute to increased oxidative stress (Kaneto *et al.,* 1996). Free radicals generated in glucose oxidation, is believed to be the main source of free radicals. Glucose, in its enediol from, is oxidatized in a transition-metal-dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals (Araki and Nishikawa, 2010). The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transitional metals, can lead to production of extremely reactive hydroxyl radicals (Jiang *et al.,*, 1990). Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals. Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway to generate free radicals (Tsai *et al.*, 1994).

Another important source of free radicals in diabetes is interaction of glucose with proteins to lead to protein glycation. Glycation involves the condensation of glucose with the ε-amino group of lysine, the α-amino group of an N-terminal amino acid or the amines of nucleic acids, which will result in the formation of advanced glycosylated end products (AGEs) (Uribarri *et al.*, 2010). The increased availability of glucose in diabetes mellitus induces enhanced production of AGEs. This process has been described as glucosylation, and is probably the major source of increased generation of ROS in diabetes patients (Wolff, 1993). AGEs are believed to be involved in the genesis of many of the irreversible complications of diabetes, including expanded extracellular matrix, cellular hypertrophy, hyperplasia, and vascular complication (Munch *et al.*, 1997). The formation of glycoxidation products is not only the result of glucose-induced oxidative stress. Fructose, which is increased as a consequence of activation of the polyol pathway, leads to the formation of AGE precursors: methylglyoxal and 3-deoxyglucosone

(Takagi *et al.,* 1995). These AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions, promote free radicals formation, and quench and block anti-proliferative effects of nitric oxide. By increasing intracellular oxidative stress, AGEs activate the transcription factor NF-κB, thus promoting up-regulation of various NF-κB controlled target genes (Mohamed *et al.,* 1999). NF-κB enhances production of nitric oxide, which is believed to be a mediator of islet β-cell damage.

In addition, hyperglycaemia leads to glycation of antioxidant enzymes, which could alter the structure and function of antioxidant enzymes such that they are unable to detoxify free radicals, exacerbating oxidative stress in diabetes. Therefore, the process of glucose oxidation might be responsible not only for increased ROS products but also for decrease availability of antioxidant enzymes (Li *et al.,* 2008).

2.4 LIPID PEROXIDATION

Lipid peroxide, a compound formed by chain reaction, involves the non-radical lipids being converted to radicals by species such as $O^{\cdot}_2$, $OH^{\cdot}$, $NO^{\cdot}$ and other reactive oxygen species (ROS). The hydrogen groups attached to the lipids have a proton and an electron, and once the hydrogen atoms are removed by free radicals, it leaves behind an unpaired electron in the lipids (Niki, 2009). This in turn leads to a chain reaction, thus reacting with other biomolecules (Krajcovicová-Kudlácková *et al.,* 2004; Niki, 2009). The reaction is shown below:

$$L\text{-}H + OH^{\cdot} \longrightarrow H_2O + L^{\cdot}$$

This lipid peroxidation results in the chain reaction and damages the various other molecules, finally leading to the cell damage (Gutteridge, 1995). Upon lipid peroxidation, a variety of products are formed depending on the type of lipids and the location of the electron.

These products are malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), lipid hydroperoxide (LOOH), isoprostanes, conjugated dienes, lipid-DNA adduct, lipid-protein adduct, lipofuscin pigments, exhaled gases (Devasagayam *et al.*, 2003).

The lipid peroxidation during diabetes is generally caused by both the enzymatic methods as well as the non- enzymatic methods. Diabetes is linked with the high blood glucose level and the high lipid content of the adipose tissues during obesity. This leads to the increase in the size of adipocytes and thus leading to the generation of phospholipase A2. The activation of phospholipase A2 finally leads to the process of the lipid peroxidation (Spiteller, 2003). The non-enzymatic process is usually caused by the mitochondrion. It is well known that mitochondrion is responsible for the transformation of oxygen into water. This involves the conversion of oxygen into superoxide anion by the transfer of an electron. These superoxide radicals are sometimes able to escape and react with water, thus generating $HO_2^{\cdot}$, which is converted to H_2O_2 and O_2. The H_2O_2 thus formed is a radical and reacts with Fe^{2+} ion to form an $OH^{\cdot}$ radical. The $OH^{\cdot}$ radical removes a hydrogen atom from CH_2 group and initiates the chain reaction leading to the process of lipid peroxidation (Spiteller, 2003). The lipid peroxidation is generally linked with the reduced tolerance among the T cells for the self-molecules due to the general processes, infections and diseases like diabetes (Wuttge, 1999).

The chain reaction which continuously supplies free radicals that intimate further peroxidation occurs in three steps: initiation, propagation and termination (Marnette, 1999).

Initiation

This step involves the production of fatty acid radicals. The initiators in living cells are most notably reactive oxygen species (ROS), such as $OH\cdot$ and $HOO\cdot$, which combines with a

hydrogen atom to make water and a fatty acid radical or lipid peroxide radical $ROO^{\bullet}$. This peroxidation process is inhibited by tocopherols, mannitol and formate.

$RH \rightarrow R^{*} + H_2O$

$ROOH \rightarrow ROO^{*} + H^{+}$

Propagation

Since the fatty acid radical generated is not a very stable molecule, it reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This radical is also an unstable species that reacts with another free fatty acid, producing a different fatty acid radical and a lipid peroxide, or a cyclic peroxide if it had reacted with itself. This cycle continues, as the new fatty acid radical reacts in the same way.

$R^{*} + O_2 \rightarrow ROO^{*}$

$ROO^{*} + RH \rightarrow ROOH + R$

Termination

The term "chain reaction mechanism" simply refers to a process whereby, a radical reacts with a non-radical to produce another radical. The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough thereby causing collision of two radicals, enabling lipid peroxidation.

$ROO + ROO \rightarrow ROOR + O_2$

$R + R \rightarrow R{-}R$

$ROO + R \rightarrow ROOR$

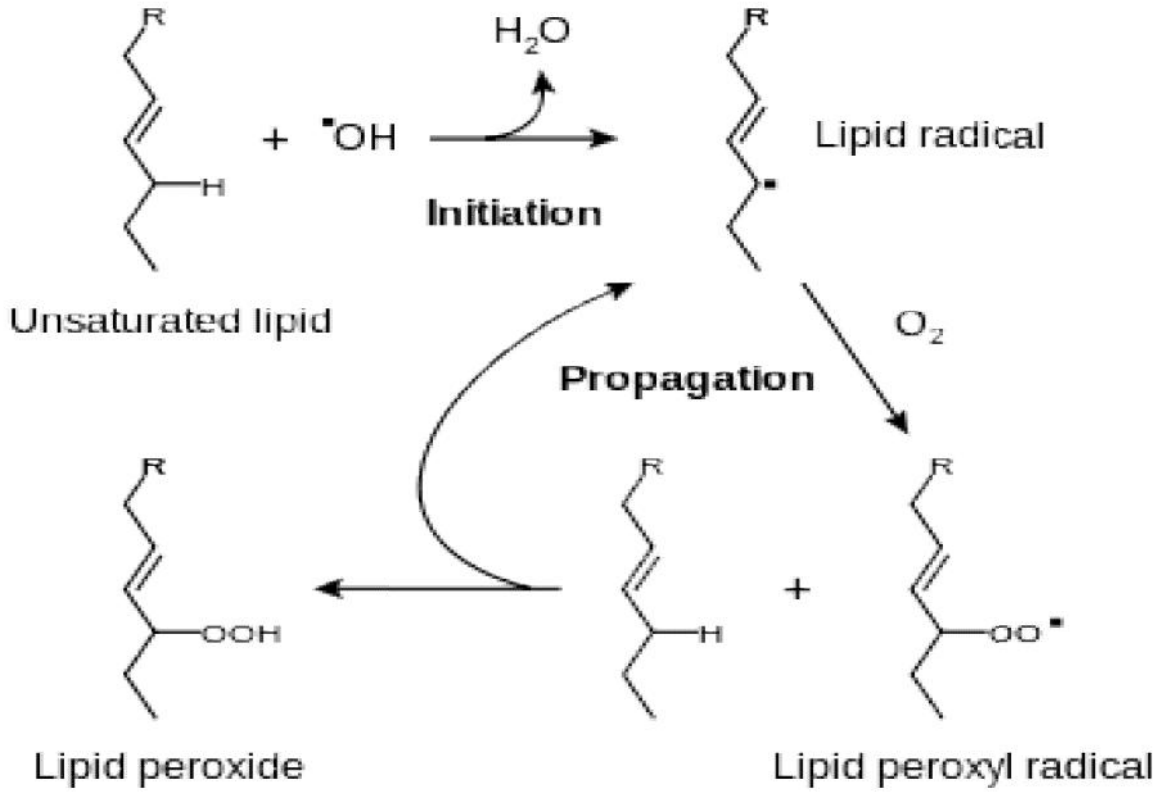

Figure 2.3: Mechanism of lipid peroxidation

Source: Wikipedia

2.5 ANTIOXIDANTS

Antioxidant refers to any molecule capable of stabilizing or deactivating free radicals before they attack cells (Eboh, 2014). Humans have evolved highly complex antioxidant systems (enzymatic and nonenzymatic), which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage. The antioxidants can be endogenous or obtained exogenously e.g., as a part of a diet or as dietary supplements. Some dietary compounds that do not neutralize free radicals, but enhance endogenous activity may also be classified as antioxidants.

An ideal antioxidant should be readily absorbed and quench free radicals, and chelate redox metals at physiologically relevant levels. It should also work in both aqueous and/or membrane domains and effect gene expression in a positive way. Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus systemic health and well- being. However, under conditions which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary antioxidants may be required to maintain optimal cellular functions.

The most efficient enzymatic antioxidants involve glutathione peroxidase, catalase and superoxide dismutase (Mates *et al.,* 1999). Non-enzymatic antioxidants include Vitamin E and C, thiol antioxidants (glutathione, thioredoxin and lipoic acid), melatonin, carotenoids, natural flavonoids, and other compounds (McCall, 2000). Some antioxidants can interact with other antioxidants regenerating their original properties; this mechanism is often referred to as the "antioxidant network" (Sies, 2005). There is growing evidence to support a link between increased levels of ROS and disturbed activities of enzymatic and non-enzymatic antioxidants in diseases associated with aging.

2.5.1 ENDOGENUOS ANTIOXIDANTS

Endogenous antioxidants include various antioxidant defenses in aerobic cells. They vary according to organelles and cell function; they help in maintaining the normal redox balance of a cell ensuring that ROS generation is transitory. The most important of these systems are glutathione, catalase and superoxide dismutase (SODs). Reducing glutathione (GSH), a tripeptide with a free thiol group, is a major antioxidant in human tissues. GSH is involved in important cell functions including vitamin C metabolism, chelation of copper ions, and the bio transformation of foreign substances and intermediate oxygen metabolite. An adequate intra-cellular supply is essential for cell survival: reduction of cell concentration usually indicate a pathological state preceding apoptosis in viral infections (Sen, 1999; Sen, 2000). GSH is synthesized primarily in the liver and it is the main intra cellular defense against ROS, free radicals, and electrophilic xenobiotics in the hepatocytes.

Availability of cysteine, the unstable precursor of GSH synthesis in most cells, is a critical determinant of cellular GSH levels (Sen, 1999; Sen, 2000). GSH provides reducing equivalents for the glutathione peroxidase (GP_X) catalyzed reduction of hydrogen peroxide and lipid hydro peroxides to water and the respective alcohol. During this process, GSH becomes oxidized glutathione (GSSG). GSSG is then recycled to GSH through interaction with the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), catalyzed by glutathione reductase (GR);

$$H_2O_2 + GSH \rightarrow GSSG + H_2O$$

$$GSSG + NADPH + H \rightarrow 2GSH + NADPH^{+}$$

Catalase presents in the cytosol and mitochondrial matrix promotes conversion of hydrogen peroxides to water and molecular oxygen:

$2H_2O_2 \rightarrow 2H_2O + O_2$

Catalase also uses hydrogen peroxide to oxidize toxins including phenols, formic acid, formaldehyde and alcohols:

$H_2O_2 + RH_2 \rightarrow 2H_2O + R$

SODs catalyze transformation of superoxide anions to oxygen and hydrogen peroxide, protecting cells against the toxic effect of oxygen metabolism.

2.5.2 DIETARY ANTIOXIDANTS

The term dietary antioxidants comprise a variety of structurally distinct compounds that have reported to act as scavengers of either ROS or RNS (Hallliwell and Gutteridge, 2007). Diets, such as fruits, vegetables, nuts and seeds provide a rich source of antioxidant vitamins, and other phytochemicals with antioxidant characteristics, which are important exogenous sources of compounds able to augment cellular responses to oxidative stress. The water soluble antioxidant vitamin C has a high reducing power and able to quench a variety of ROS. Ascorbic acid (Vitamin C), dietary antioxidant has 4 −OH groups that can donate hydrogen to an oxidizing system. Due to the −OH groups on adjacent carbon atoms, vitamin C is able to chelate metal ions (Fe^{++}). It also scavenges free radicals, quenches O_2^-, and acts as a reducing agent. At high levels (>1000 mg/kg), vitamin C shifts the balance between ferrous (Fe^{2+}) and ferric iron (Fe^{3+}), acts as an oxygen scavenger, and inhibits oxidation. However, at low levels (<100 mg/kg), it can catalyze oxidation (in muscle tissue; Ahn *et al.*, 2007; Yetella and Min 2008).

α-Tocopherol (vitamin E), a fat-soluble carotenoid is the major vitamin E compound in plant leaves where it is located in the chloroplast envelope and thylakoid membranes in proximity to phospholipids (Onibi *et al.,* 2000). It deactivates photosynthesis-derived reactive oxygen species (especially O_2^-) and prevents the propagation of lipid peroxidation by scavenging lipid peroxyl radicals in thylakoid membranes (Munn´e-Bosch, 2005). Trolox is a water-soluble derivative of vitamin E. Structurally related lipid-soluble antioxidants that differ in the number of methyl groups (δ-tocopherol compared with α-tocopherol) have different free radical-scavenging activities and different surface activities (Chaiyasit *et al.,* 2005).

Experimental dietary studies are supportive as to the beneficial effects of dietary plants rich in antioxidants. Yeum *et al.,* (2009) reported synergistic effects between ascorbic acid and α-tocopherol in protecting an *in vitro* biological model system. It may be that ascorbic acid regenerates α-tocopherol after α-tocopherol donates Hydrogen to an oxidizing lipid (Brewer, 2011). α-Tocopherol can also inhibit oxidation of protein. According to Est´evez and Heinonen (2010), α-tocopherol reduced formation of α-aminoadipic and γ-glutamic semialdehydes from oxidized myofibrillar proteins. Dietary supplementation of α-tocopherol increases incorporation of the antioxidant into the phospholipid membrane region where the polyunsaturated fatty acids are located. Including α-tocopherol in livestock diets has been shown to have significant effects on the antioxidative activities of their tissues and the stability of meat derived from them (Boler *et al.,* 2009; Lahucky *et al.,* 2010).

Protein and amino acids are responsible for the synthesis of antioxidant enzymes. GSH and Carnosine are the small peptides, nitrogenous metabolites like creatine and uric acid are the direct scavengers of reactive metabolites (Rassaf *et al.,* 2002). The oxidative stress is due to increase level of tissue iron in protein deficient patient, in which iron-binding protein are

deficient including transferrin, lactoferrin and ferritin. This iron overload exhibits the cardiovascular injury. Similarly, high protein diet exhibit oxidative stress. Homocysteine elevation exhibits endothelial superoxide anion in vasculature, increase inducible and constitutive NOS synthesis, and stimulate ROS generation in polymorphonuclear leukocytes and monocytic cells (Garcion *et al.*, 1997; Gurujeyalakshmi *et al.*, 2000).

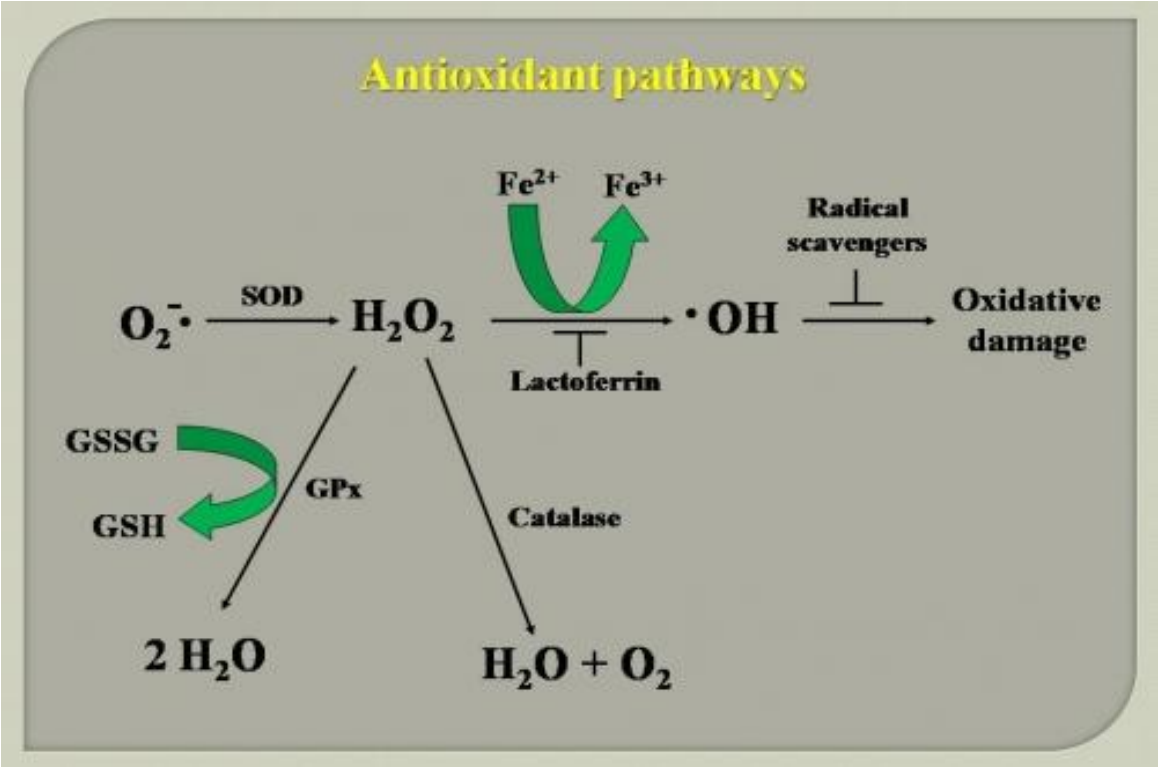

Figure 2.4: The antioxidant pathway

Source: Wikipedia

2.6 POLYPHENOLS

Polyphenols are secondary metabolites that plants produce in response to biotic and abiotic stress (Cesoniene *et al.*, 2009; Cushnie and Lamb, 2011). Dietary polyphenols have been shown to play important roles in human health. High intake of fruits, vegetables and whole grains, which are rich in polyphenols, has been linked to lowered risks of many chronic diseases including cancer, cardiovascular disease, chronic inflammation and many degenerative diseases (Milner, 1994; Duthie and Brown, 1994). Studies have revealed that many of these diseases are related to oxidative stress from reactive oxygen and nitrogen species. Phytochemicals, especially polyphenols, are the predominant contributor to the total antioxidant activities of fruits, rather than vitamin C (Wang *et al.*, 1996). Polyphenols have been found to be strong antioxidants that can neutralize free radicals by donating an electron or hydrogen atom. Polyphenols suppress the generation of free radicals, thus reducing the rate of oxidation by inhibiting the formation of or deactivating the active species and precursors of free radicals. More frequently, they act as direct radical scavengers of the lipid peroxidation chain reactions (chain breakers). Chain-breakers donate an electron to the free radical, neutralizing the radicals and themselves becoming stable (less reactive) radicals, thus stopping the chain reactions (Rice-Evans *et al.*, 1996; Pietta, 2000; Guo, 2009). In addition to radical scavenging, polyphenols are also known as metal chelators. Polyphenols can induce antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase that decompose hydroperoxides, hydrogen peroxide and superoxide anions, respectively, and inhibit the expression of enzymes such as xanthine oxidase (Du *et al.*, 2007).

After intake, some polyphenols are directly absorbed through the stomach and the small intestine (Brown *et al.*, 2004; Eckel *et al.*, 2005). After absorption, polyphenols undergo

extensive biotransformation by enterocytes and liver, a necessary step aiming to increase hydrophilicity therefore favoring urinary excretion. Sulfation, glucuronidation, methylation and glycine-conjugation are the most common biotransformation reactions (Moco *et al.*, 2012). Importantly, liver may play a larger role in the metabolism of flavonoids absorbed in the small intestine compared to metabolism of compounds taken up by colon (Heim *et al.,* 2002). Polyphenols are not able to saturate metabolic pathways similarly to drugs, thus hindering the establishment of high plasma levels (Scalbert and Williamson, 2000). Absorption of flavonoids may be influenced by dosage, vehicle of administration, prior diet, food matrix, gender and differences in the gut microbial populations (Erlund *et al.,* 2001; Heim *et al.,* 2002). Recent research strongly supports the concept that the consumption of fruits and plant-derived foods is inversely correlated with type 2 diabetes prevalence and the occurrence of cardio metabolic complications (Morimoto *et al.,* 2012; Bauer *et al.,* 2013; Eshak *et al.,* 2013; Sabin *et al.,* 2012 and Chan *et al.,* 2012). It has been suggested that 90% of type 2 diabetes cases could be potentially prevented by lifestyle modifications (Willett, 2002), including increased physical activity, weight loss and consuming a diet rich in plant-derived foods (*e.g*. whole grains, fruits and vegetables) (Lindström *et al.*, 2006).

Examples of polyphenolic natural antioxidants derived from plant sources include vitamin E, flavonoids, cinnamic acid derivatives, curcumin, caffeine, catechins, gallic acid derivatives, salicylic acid derivatives, chlorogenic acid, resveratrol, folate, anthocyanins and tannins (Bors *et al.,* 1996).

2.7 GALLIC ACID

Gallic acid (GA), 3, 4, 5-trihydroxybenzoic acid, and its derivatives are biologically active compounds which are widely present in plants (Kahkonen *et al.,* 1999; Lee *et al.,* 2000). Gallic acid is a strong natural antioxidant (Aruoma *et al.,* 1993; Heinonen *et al.,* 1998; Khan *et al.,* 2001). It is able to scavenge hypochlorous acid at a rate sufficient to protect α – 1 — antiproteinase against inactivation by this molecule. Gallic acid decreases the peroxidation of ox brain phospholipids (Milic *et al.,* 1998). Free radicals have been implicated in the etiology and pathogenesis of numerous disease states including cardiovascular disease, cancer and diabetes (Inoue *et al.,* 1995; Sakagami *et al.,* 1997; Aoki *et al.,* 2001). Free radicals occur as a natural consequence of cell metabolism. They are also produced as results of oxidative stress (Schmidt *et al.,* 1995; Koga *et al.,* 1999; Terasaka *et al.,* 2000). Antioxidant capacity of gallate esters against hydroxyl, azide, and superoxide radicals has also been reported (Masaki *et al.,* 1995; Satoh *et al.,* 1998; Bors and Michel, 1999; Pulido *et al.,* 2000; Metelitza *et al.,* 2001). Gallic acid is widespread in plant foods and beverages such as tea and wine and was proven to be one of the anticarcinogenic polyphenols present in green tea (Ho *et al.,* 1992; Kerry and Abbey, 1997; Abu-Amsha *et al.,* 2001; Landrault *et al.,* 2001). Antioxidants present in red wine have been shown to have a protective role against oxidation of LDL *in vitro* (Arce *et al.,* 1998). Gallic acid is a strong chelating agent and forms complexes of high stability with iron (III) (Sroka *et al.,* 1994; Li *et al.,* 2000). It has shown phytotoxity and antifungal activity against *Fusarium semitectum, F. fusiformis* and *Alternaria altternata* (Dowd *et al.,* 1997). Gallic acid is of great interest in arteriosclerosis prevention (Abella and Chalas, 1977)

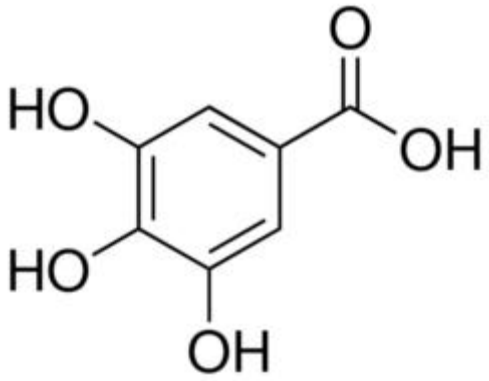

Figure 2.5: Molecular structure of Gallic acid

Source: Mämmelä et al., 2000; Wang et al., 2003

2.8 ACARBOSE

Acarbose belongs to the group of noninsulinotropic oral antidiabetic agents. Because of its unique mode of action, acarbose not only plays an essential and direct role in carbohydrate uptake from food into the blood, but also has an indirect role in the optimization of glucose metabolism over the whole day, as it contributes to the adaptation of insulin secretion (Rosak and Mertes, 2012). To enable glucose uptake and absorption by the body and availability as an energy source, intestinal cleavage of starch and oligosaccharides is necessary, because only monosaccharides can be taken up into the blood. Oligosaccharides are cleaved into monosaccharides by enzyme complexes called alpha- glucosidases, which are present in the brush border membrane of the small intestine (Elsenhans and Caspary, 1987). Acarbose is structurally similar to natural oligosaccharides, but has a 10^4 to 10^5 times higher affinity for alpha-glucosidases (Rosak and Mertes, 2012). This means that these enzyme complexes are competitively inhibited and that their availability to the oligosaccharides from dietary starch is reduced. Thus, monosaccharide formation decreases and less insulin is required for further

metabolisation, leading to a reduction of food-induced postprandial increases in blood glucose and insulin (Bischoff, 1991; Puls, 1996). Therefore, the effect is not a classic reduction in blood glucose by increased insulin secretion as a reaction to an increase in blood glucose, but a reduction in blood glucose rise as an antihyperglycemic effect. Because reduced blood glucose concentrations result in markedly lower stimulation of insulin synthesis and insulin secretion, the hyperinsulinemia induced by insulin resistance is also decreased (Bischoff *et al.,* 1995). Given that acarbose acts in the intestine, it can be combined in long-term treatment with all other antidiabetic agents to enhance its effect without potentiating adverse events.

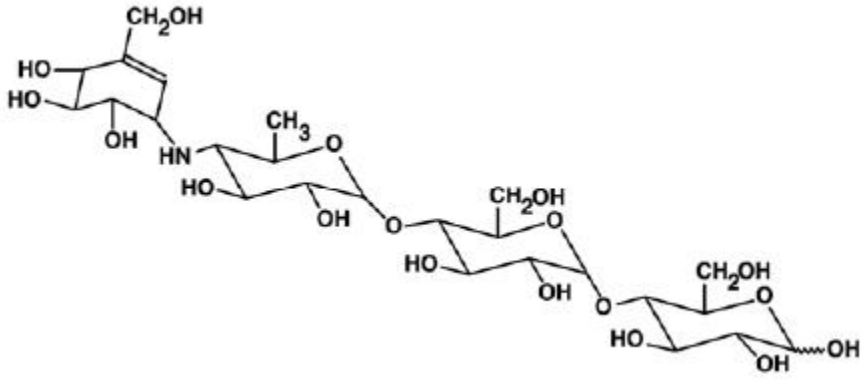

Figure 2.6: Molecular structure of Acarbose

Source: Bischoff, 1991.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sample collection

The Acarbose was purchased from Glenmark Generics (Europe) pharmaceutical limited. Gallic acid used was purchased from Sigma Al-drich Co. (St Louis, Missouri, USA).

3.1.2 Sample preparation

Acarbose and Gallic acid were dissolved in distilled water to a final concentration of 25µM. Thereafter, sample mixtures were prepared thus:

S1= 100% Acarbose (25µM)

S2= 100% Gallic acid (25µM)

S3 = 50% Acarbose + 50% Gallic acid

S4 = 75% Acarbose + 25% Gallic acid

S5 = 25% Acarbose + 75% Gallic acid

All samples were kept in the refrigerator at 4^0C for subsequent analysis.

3.1.3 Chemicals and Reagents

Chemical reagents such as Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH (2, 2-diphenyl-1picrylhydrazyl), thiobarbituric acid (TBA), gallic acid, porcine pancreatic

α-amylase and 1,10-phenanthroline were procured from Sigma Al-drich Co. (St Louis, Missouri, USA). Trichloroacetic acid (TCA) was sourced from Sigma Al-drich, Chemie GmbH (Steinheim, Germany), hydrogen peroxide, methanol, acetic acid, hydrochloric acid, aluminium chloride, potassium acetate, sodium dodecyl sulphate,Iron (II) sulphate, potassium ferrycyanide and ferric chloride were sourced from BDH Chemicals Ltd., (Poole, England). Ascorbic acid and starch were products of Merck (Darmstadt, Germany). Except stated otherwise, all other chemicals and reagents were of analytical grades and the water was glass distilled.

3.2 METHODS

3.2.1 Alpha glucosidase activity assay

Appropriate dilution of the sample (50 µl) and 100µl of α-glucosidase solution (EC 3.2.1.20; 1.0 U/ml) in 0.1M phosphate buffer (pH 6.9) was incubated at 25°C for 10 min. Thereafter, 50µl of 5 mM pnitrophenyl- α-D-glucopyranoside solution in 0.1M phosphate buffer (pH 6.9) was added. The mixtures were incubated at 25°C for 5 min the absorbance read at 405 nm in the spectrophotometer. The α-glucosidase inhibitory activity was expressed as percentage inhibition (Apostolidis *et al.*, 2007).

3.2.2 Alpha amylase activity assay

The aqueous sample dilution (500µl) and 500µl of 0.02M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) containing 0.5 mg/ml Hog pancreatic α-amylase (EC 3.2.1.1) were incubated at 25°C for 10 min. Thereafter, 500µl of 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9 with 0.006M NaCl) was added to each reaction mixture. The reaction mixtures was incubated at 25°C for 10 min and stopped with 1.0ml of dinitrosalicylic acid (DNSA) color reagent. Thereafter, the mixture was incubated in a boiling water bath for 5 min,

and cooled to room temperature. The reaction mixture was then diluted by adding 10 ml of distilled water, and absorbance measured at 540 nm. The reference samples included all other reagents and the enzyme with the exception of the test sample. The percentage enzyme inhibitory activity of the extract was subsequently calculated (Worthington, 1993).

3.2.3 Fe^{2+} chelation assay

The Fe^{2+} chelating ability of the samples was determined using the method of Minotti and Aust (1987) as modification by Puntel et al. (2005). Freshly prepared 500µmol/l $FeSO_4$ (150µl) was added to a reaction mixture containing 168µl of 0.1mol/l Tris-HCl (pH 7.4), 218µl saline and the extract (0 – 100µl). The reaction mixture was incubated for 5 min before the addition of 13µl of 0.25% 1: 10-phenanthroline (w/v).The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe^{2+} chelating ability of the extract was subsequently calculated as percentage of the control.

3.2.4 Inhibition of lipid peroxidation and thiobarbituric acid reactions

Albino rats were immobilized by cervical dislocation and the pancreas was rapidly isolated and placed on ice and weighed. This tissue was subsequently homogenized in cold saline (1/10 w/v) with about 10-up-and –down strokes at approximately 1200 rev/min in a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 3000 × *g* to yield a pellet that was discarded, and the low-speed supernatant (S1) was kept for lipid peroxidation assay (Belle *et al*. 2004). The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al*., (1979). Briefly 100µl S1 fraction was mixed with a reaction mixture containing 30µl of 0.1M pH 7.4 Tris – HCl buffer, sample (0 – 100µl) and 30µl of 250µM freshly prepared $FeSO_4$. The volume was made up to 300µl by water before incubation at 37 ^oC for 1hr. The color reaction was developed by adding 300µl 8.1% SDS (Sodium dodecyl sulphate) to the reaction mixture

containing S1, this was subsequently followed by the addition of 600µl of acetic acid/HCl (pH 3.4) mixture and 600µl 0.8% TBA (Thiobarbituric acid). This mixture was incubated at 100^{o}C for 1hr. Thiobarbituric acid reactive species (TBARS) produced was measured at 532 nm and expressed using MDA (Malondialdehyde) equivalent.

3.2.5 Determination of Ferric Reducing Antioxidant property

The reducing property of the samples was determined by assessing its ability to reduce $FeCl_3$ solution as described by Pulido et al. (2000). 2.5ml aliquot of the extract was mixed with 2.5ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and thereafter, 2.5ml of 10% trichloroacetic acid was added. This mixture was centrifuged at 805g for 10 min; 5ml of the supernatant was mixed with an equal volume of water and 1ml of 0.1% ferric chloride. The absorbance was measured at 700 nm in the spectrophotometer after allowing the solution to stand for 30 min. A graph of absorbance against concentration of extract was plotted to observe the reducing property where higher absorbance values indicated a higher reducing property. The reducing property was subsequently calculated using ascorbic acid equivalent.

3.2.6 2, 2'-azino-bis (-3-ethylbenzthiazoline-6-sulphonate ($ABTS^{\cdot+}$) scavenging ability

Trolox equivalent antioxidant capacity (TEAC) of the samples was determined by their 2, 2'-azino-bis(-3-ethylbenzthiazoline-6-sulphonate ($ABTS^{\cdot+}$) scavenging ability according to the method described by Re *et. al*.(1999). The $ABTS^{\cdot+}$ was generated by reacting (7 mmol/l) $ABTS^{\cdot+}$aqueous solution with $K_2S_2O_8$ (2.45 mmol/l, final concentration) in the dark for 16 h and adjusting the absorbance at 734 nm to 0.700 in a spectrophotometer with ethanol. 0.2ml of appropriate dilution of the extract was added to 2.0ml $ABTS^{\cdot+}$ solution and the absorbance was

measured at 734 nm in a spectrophotometer after 15 min. The trolox equivalent antioxidant capacity was subsequently calculated using trolox as the standard.

3.2.7 1, 1-diphenyl–2-picrylhydrazyl (DPPH*) free radical scavenging ability

The scavenging ability of the extracts against DPPH* (1, 1-diphenyl–2-picrylhydrazyl) free radical was evaluated as described by Gyamfi et al. (1999) with slight modifications. 1ml of 0.4mM DPPH* in methanol was mixed with 0.05ml of the extract. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm in the spectrophotometer. The DPPH* free radical scavenging ability was subsequently calculated as percentage of the control.

3.3 Data Analysis

The results of replicates were pooled and expressed as mean standard error and the least significance difference (Zar, 1984) and one- way analysis of variance (ANOVA) will be determined.

CHAPTER FOUR

4.0 RESULT

The effect of gallic acid on α-glucosidase inhibitory property of acarbose (Figure 4.1) revealed that 100% acarbose (S1) had significantly higher ($P<0.05$) inhibitory effect (66.2±0.7%) than 100% gallic acid (S2; 43.9±0.7%). However, considering the combinations, a combination of 50% acarbose and 50% gallic acid (S3) showed the highest significant ($P<0.05$) inhibitory effect (65.7±1.4%), which was not significantly different ($P>0.05$) from the inhibitory effect of 100% acarbose.

The effect of gallic acid on α-amylase inhibitory activity of acarbose is presented in figure 4.2. The result reveals that 100% acarbose (S1) had significantly higher ($P<0.05$) enzyme inhibitory effect (82.8±0.7%) than 100% gallic acid (S2; 49.0±1.4%). It also showed that a combination of 75% acarbose and 25% gallic acid (S4) had the highest significant ($P<0.05$) inhibitory effect (82.2±1.6%) of the various combinations; there was however, no significant difference ($P>0.05$) in the inhibitory effect of S4 and S1.

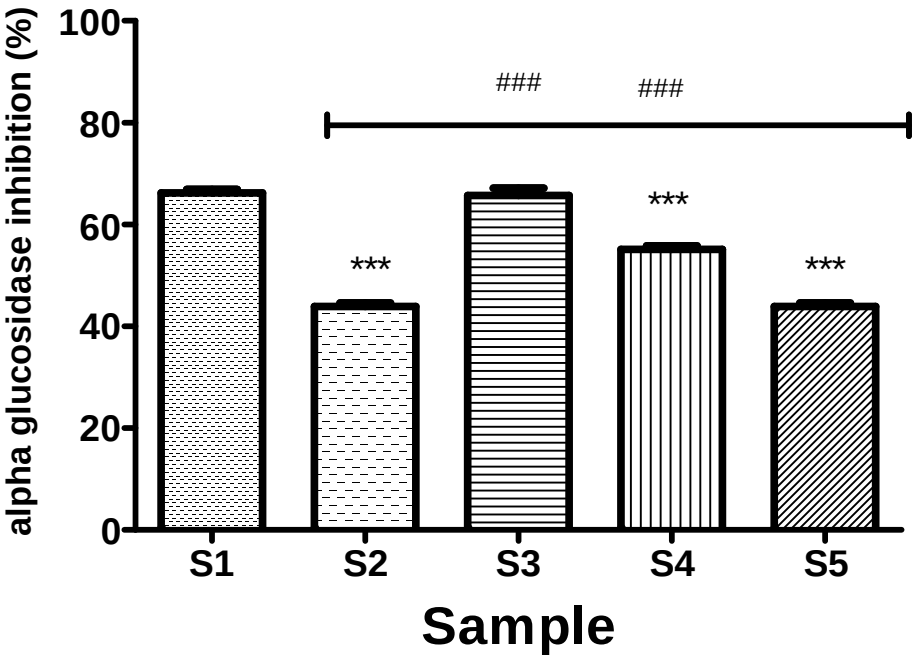

Figure 4.1: Effect of Gallic acid on α- Glucosidase inhibitory ability of Acarbose (AC) *in vitro.*

* mean values are significantly different (P<0.05) compared to S1

mean values are significantly different (P<0.05) compared to S2

S1= 100% Acarbose (25µM)
S2= 100% Gallic acid (25µM)
S3 = 50% Acarbose + 50% Gallic acid
S4 = 75% Acarbose + 25% Gallic acid
S5 = 25% Acarbose + 75% Gallic acid

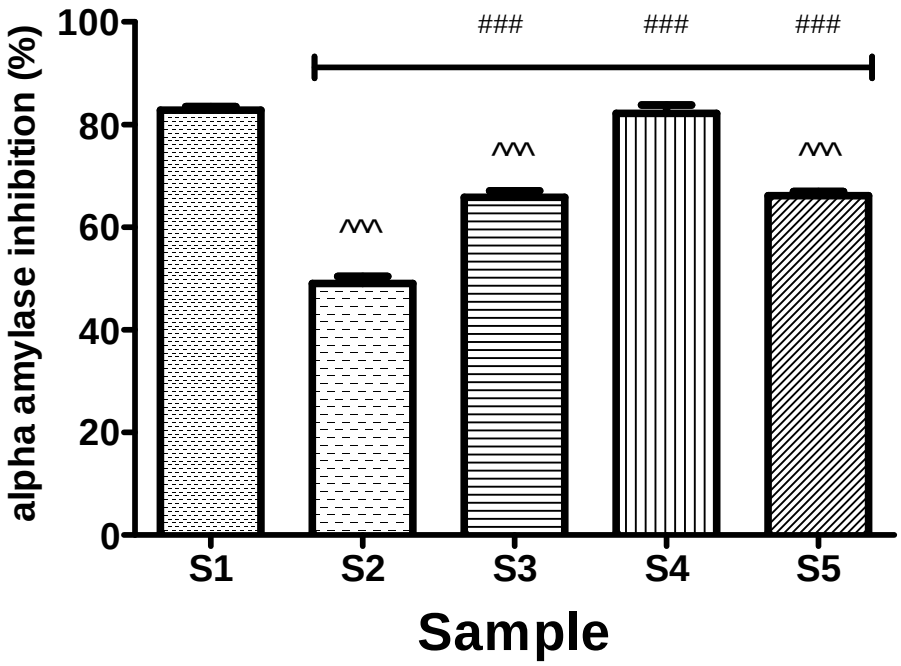

Figure 4.2: Effect of Gallic acid on α- Amylase inhibitory ability of Acarbose *in vitro.*

^ mean values are significantly different (P<0.05) compared to S1

mean values are significantly different (P<0.05) compared to S2

S1= 100% Acarbose (25µM)
S2= 100% Gallic acid (25µM)
S3 = 50% Acarbose + 50% Gallic acid
S4 = 75% Acarbose + 25% Gallic acid
S5 = 25% Acarbose + 75% Gallic acid

The result of the Fe^{2+} chelating ability as shown in figure 4.3, revealed that 100% acarbose (S1) had significantly higher ($P<0.05$) chelating ability (80.9±1.3%) compared to 100% gallic acid (S2; 52.3±3.2%). However, considering the combinations, a combination of 75% acarbose and 25% gallic acid (S4) had the highest chelating ability (90.5±0.6%) compared to other combinations, but was not significantly different ($P>0.05$) from the chelating ability (80.9±1.3%) of the combination of .25% acarbose and 75% gallic acid (S5).

Incubation of rat's pancreas homogenates in the presence of Fe^{2+} induced a significant ($P<0.05$) increase (152.6±0.7%) in the malodialdehyde (MDA) content (Figure 4.4). However, introduction of all the samples (S1-S5) inhibited lipid peroxidation in thee pancreatic tissue homogenate by causing a significant ($P<0.05$) reduction in the MDA content; nevertheless, there was no significant difference ($P>0.05$) in the inhibitory effects of 100% acarbose (S1; 61.7±2.2%) and 100% gallic acid (S2; 64.8±0.7%). Considering the combinations, a combination of 50% acarbose and 50% gallic acid (S3) caused the highest significant ($P<0.05$) reduction in the MDA content (56.6±0.7%).

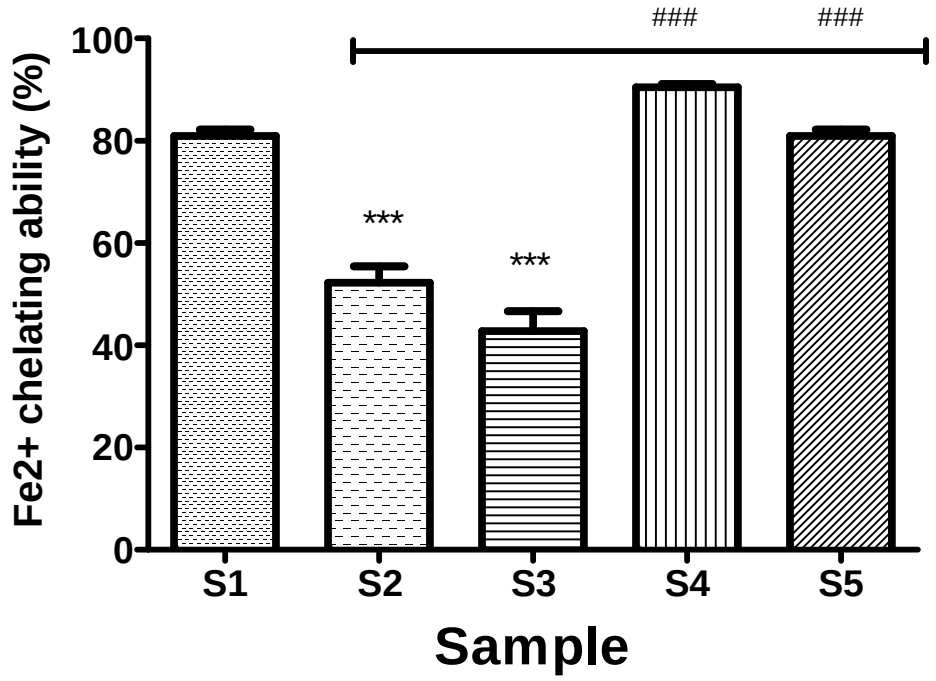

Figure 4.3 Effect of Gallic acid on Fe^{2+} chelating ability of Acarbose *in vitro*

* mean values are significantly different (P<0.05) compared to S1

mean values are significantly different (P<0.05) compared to S2

S1= 100% Acarbose (25µM)
S2= 100% Gallic acid (25µM)
S3 = 50% Acarbose + 50% Gallic acid
S4 = 75% Acarbose + 25% Gallic acid
S5 = 25% Acarbose + 75% Gallic acid

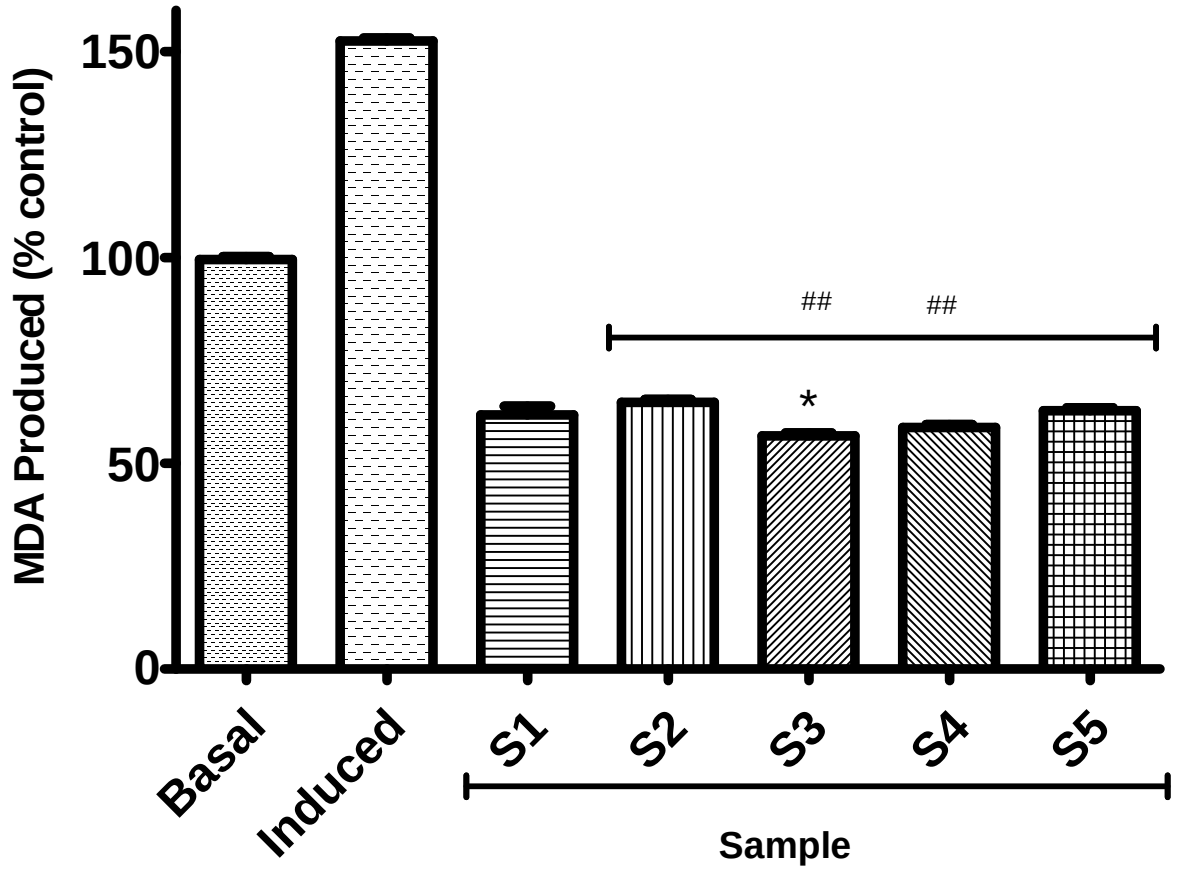

Figure 4.4 Effect of Gallic acid on Inhibition of Fe^{2+} induced lipid peroxidation in Rat's pancreas (*in vitro)* by Acarbose

* mean values are significantly different (P<0.05) compared to S1

mean values aresignificantly different (P<0.05) compared to S2

S1= 100% Acarbose (25µM)
S2= 100% Gallic acid (25µM)
S3 = 50% Acarbose + 50% Gallic acid
S4 = 75% Acarbose + 25% Gallic acid
S5 = 25% Acarbose + 75% Gallic acid

The ferric reducing antioxidant properties of the samples (S1-S4) were presented as ascorbic acid equivalent (AAE) figure 4.5. The result revealed that 100% acarbose had the highest significant ($P<0.05$) reducing property (319.7±26.9 mgAAE/g) compared to 100% gallic acid (162.1±22.5 mgAAE/g). However, a combination of 25% acarbose and 75% gallic acid (S5) had the highest significant ($P<0.05$) reducing property (403.2±11.4 mgAAE/g) compared to the other combinations, while the reducing property of the combination of 50% acarbose and 50% gallic acid (S3; 321.9±10.1 mgAAE/g) showed no significant difference ($P>0.05$) to that of 100% acarbose.

The result of the DPPH free radical scavenging ability (Figure 4.6) revealed that 100% gallic acid (S2) had significantly higher ($P<0.05$) scavenging ability (69.2±0.5%) compared to 100% acarbose (S1; 42.5±0.3%). However, a combination of 50% acarbose and 50% gallic acid (S3) has the highest significant ($P<0.05$) scavenging ability (73.2±0.1%), but not significantly different ($P>0.05$) from the scavenging ability (72.1±0.1%) of the combination of 25% acarbose and 75% gallic acid (S5).

$ABTS^{+}$ scavenging ability presented as trolox equivalent antioxidatant capacity (Figure 4.7) revealed that there was no significant difference ($P>0.05$) in the ABTS* scavenging ability of all the samples (S1= 25.67±3.55 mmolTEAC/g; S2= 26.61±3.55 mmolTEAC/g; S3=25.69±3.55 mmoLTEAC/g; S4= 26.63±3.55 mmoLTEAC/g; S5= 26.67±3.55 mmoLTEAC/g).

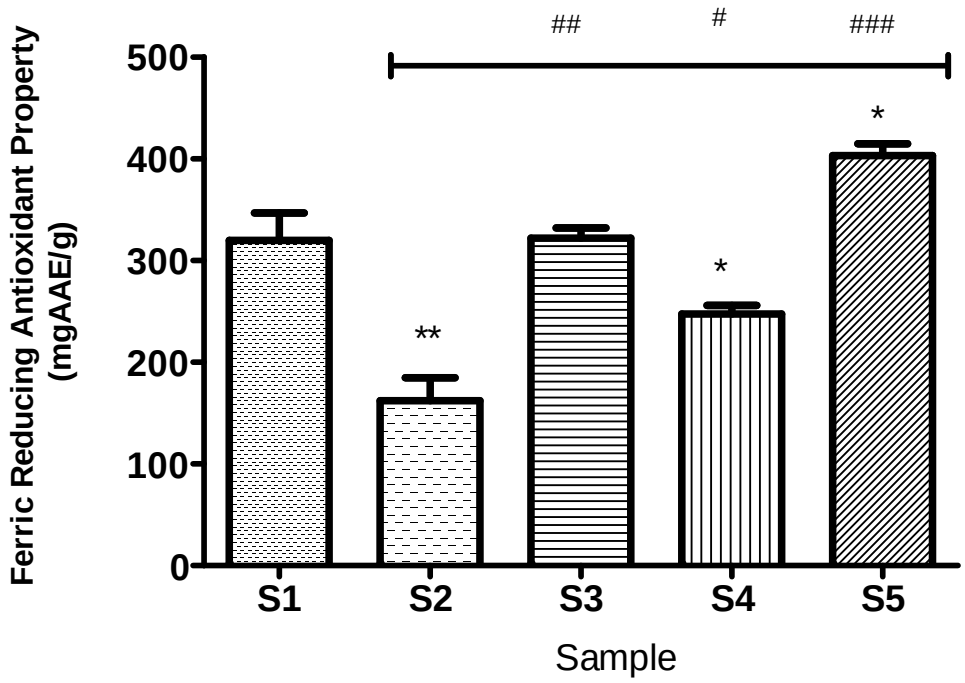

Figure 4.5 The effect of Gallic acid on Ferric Reducing Antioxidant Property of Acarbose *in vitro*.

* mean values are significantly different (P<0.05) compared to S1

mean values are significantly different (P<0.05) compared to S2

S1= 100% Acarbose (25µM)
S2= 100% Gallic acid (25µM)
S3 = 50% Acarbose + 50% Gallic acid
S4 = 75% Acarbose + 25% Gallic acid
S5 = 25% Acarbose + 75% Gallic acid

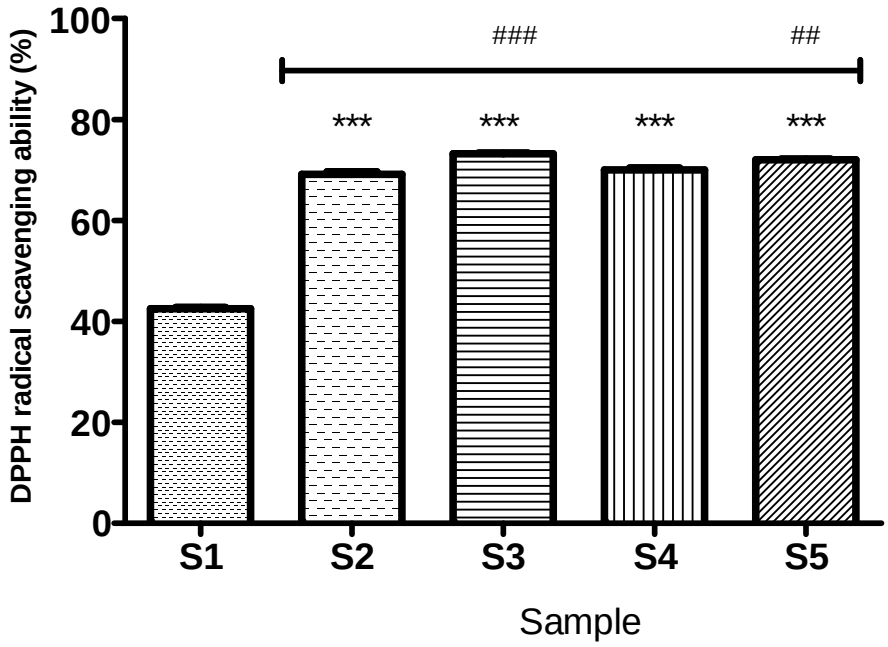

Figure 4.6 Effect of Gallic acid on DPPH Radical Scavenging ability of Acarbose *in vitro.*

* mean values are significantly different (P<0.05) compared to S1

mean values are significantly different (P<0.05) compared to S2

S1= 100% Acarbose (25µM)
S2= 100% Gallic acid (25µM)
S3 = 50% Acarbose + 50% Gallic acid
S4 = 75% Acarbose + 25% Gallic acid
S5 = 25% Acarbose + 75% Gallic acid

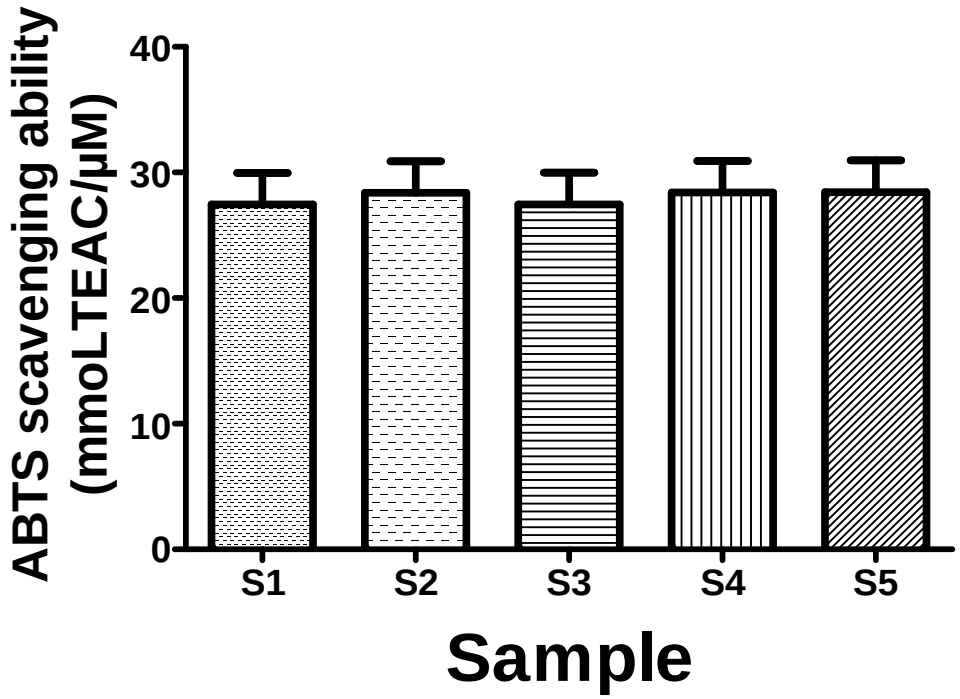

Figure 4.7 Effect of Gallic acid on ABTS Radical Scavenging ability of Acarbose *in vitro.*

S1= 100% Acarbose (25µM)
S2= 100% Gallic acid (25µM)
S3 = 50% Acarbose + 50% Gallic acid
S4 = 75% Acarbose + 25% Gallic acid
S5 = 25% Acarbose + 75% Gallic acid

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 DISCUSSION

Hyperglycemia is a condition of abnormal rise in blood glucose level and is an etiology of type-2 diabetes; a disease caused by insulin resistance (Oritz- Andrade *et al*., 2007; Jaspinder, 2014). As hyperglycemia plays an important role in the progression of diabetes and diabetic complications, control of postprandial hyperglycemia has been shown to be a practical way in the management of diabetes and its complications arising from oxidative stress (Oritz- Andrade *et al.,* 2007). Pancreatic α- amylase is involved in the breakdown of starch into disaccharides and oligosaccharides before intestinal α-glucosidase catalyzes the breakdown of disaccharides to liberate glucose which is later absorbed into the blood circulation. Inhibition of the α- amylase and α-glucosidase has been suggested to slow down the breakdown of starch in the gastrointestinal tract, which reduces the amount of glucose absorbed into the circulation (Oboh *et al.,* 2010). Various synthetic drugs such as acarbose are being used to reduce the postprandial hyperglycemia but several side effects are observed during long term use (Chakrabarti and Rajagopalan, 2002; Kimmel and Inzucchi, 2005). Therefore combinations of these drugs with diets rich antidiabetic and antioxidant phytochemicals such as phenolics, could help produce synergistic effects at reducing postprandial rise in blood glucose level while at the same time, offering possible reduction in the attendant side effects of these synthetic drugs. This is more so because previous studies have reported the antidiabetic and antioxidant properties of phenolic rich food sources (Ogunmodele *et al.,* 2012; Mhbele *et al.,* 2015).

The study revealed that acarbose, gallic acid and their various combinations caused the inhibition of both α- amylase and α-glucosidase activities *in vitro* with the 100% acarbose

exhibiting stronger inhibitory activities than the 100% gallic acid. A previous report has shown that acarbose serves as a strong inhibitor of enzymes associated with carbohydrate hydrolysis compared to phenolic compounds (Ademiluyi and Oboh, 2013). Specifically, as presented in figure 4.1, the combination of acarbose and gallic acid in equal proportions had a higher α-glucosidase inhibitory effect compared to 100% acarbose, a drug known to be a strong inhibitor of α-glucosidase. Therefore, this combination could be said to be synergistic, and could hence, serve as possible combinatory therapy in preference to 100% Acarbose, this is to possibly reduce the several side effects associated with the use of acarbose.

Furthermore, the inhibitory effect of acarbose, gallic acid and their various combinations on the α-amylase (Figure 4.2), shows that acarbose inhibits α-amylase more significantly stronger than it inhibits α-glucosidase. Nevertheless, it exhibits a stronger inhibiting property compared to gallic acid, which showed mild inhibition of α-amylase. This observations could be linked to the deleterious side effects of acarbose and also in agreement with previous reports which indicated that excessive inhibition of pancreatic α-amylase could result in the abnormal bacterial fermentation of undigested carbohydrates in the colon and therefore mild α-amylase inhibition activity is desirable (Shai *et al*., 2010). The result then therefore suggests that since the combination of acarbose and gallic acid at equal proportion serves as mild inhibitor of α-amylase and strong inhibitor of α- glucosidase, it could be of great therapeutic importance in addressing the side effects associated with acarbose in the management of type 2 diabetes which is linked with excess inhibition of α- amylase. Also, the combination of acarbose and gallic acid at the ratio 75:25 shows a synergistic inhibition of α- amylase. The inhibition of the enzymes using the various combinations could be very effective in reducing the postprandial increase in

the glucose level, hence reduces oxidative stress, and eventually prevents diseases associated with oxidative stress such as diabetes.

Also, as presented in figure 4.3, the samples were able to chelate Fe^{2+}, with the combinations of acarbose and gallic acid in the ratio of 75:25 and 25:75 being more potent in the chelation of Fe^{2+}. Nevertheless, 100% acarbose also shows a significantly higher chelating ability compared to 100% gallic acid. Fe^{2+} catalyzes one electron transfer reactions that generates reactive oxygen species (ROS), such as the OH, which is formed from H_2O_2 through the fenton reaction. Iron causes lipid peroxidation and also decomposes the lipid peroxides, which leads to the generation of peroxyl and alkoxyl radicals which favours the propagation of lipid peroxidation (Ademiluyi and Oboh, 2013). Furthermore, the incubation of isolated rat's pancreatic homogenates in the presence of Fe^{2+} caused a significant ($P<0.05$) increase in the malondialdehyde (MDA) content. Previous studies have shown that incubation of rat tissues in the presence of 25μm $FeSO_4$ solution caused a significant increase in their MDA content (Oboh *et al.,* 2007; Ademiluyi and Oboh, 2013). However, the introduction of acarbose, gallic acid and their various combinations caused significant ($P> 0.05$) decrease in the MDA content of the incubated pancreatic tissue homogenate as shown in figure 4.4. The possible mechanisms through which the sample protects against lipid peroxidation could be by Fe^{2+} chelation (Oboh *et al.,* 2007). Therefore, the decrease in the pancreatic MDA content by acarbose, gallic acid and their various contributions could be attributed to the Fe^{2+} chelating properties. Moreover, this study reveals that acarbose, gallic acid and their various combinations were able to protect the pancreas against Fe^{2+}- induced lipid peroxidation. However, a combination of 50% acarbose and 50% gallic acid had the highest inhibitory effect on MDA production.

According to Li *et al.* (*2*008), insulin secreting organ (pancreatic β- cells) is vulnerable to oxidative damage caused by the ROS due to their limited antioxidant defense systems. Antioxidants as diets, supplements or as nutraceuticals could help protect against the oxidative damage and thus prevents diabetes and its complications. Persistence hyperglycemia may induce free radical production via glycation and autoxidation. This free radical production contributes to β- cells destruction in type 2 diabetes (Maria *et al.,* 2007).

The ability of phytochemicals possessing antioxidant property to reduce oxidative species has been shown to be one of their antioxidative mechanisms (Islam, 2013). Reducing property of the samples was assessed based on their ability to reduce Fe^{3+} to Fe^{2+}. The result reveals that the 100% Acarbose exhibits a higher ferric reducing ability than 100% gallic acid. Of the various combinations, a combination of 25% acarbose and 75% gallic acid shows the highest reducing property and hence, a synergistic effect. This observations could possibly be attributed to synergistic chemical reaction between the phenolic acid (gallic acid) and acarbose.

Furthermore, this study also revealed that the samples scavenged DPPH free radicals. 100% gallic acid exhibited a higher radical scavenging ability than 100% acarbose, nevertheless, a combination of 50% acarbose and 50% gallic acid showed the highest scavenging ability, hence portraying a possible synergy in the DPPH free radical scavenging abilities of acarbose and gallic acid. The antioxidant property of gallic acid has being reported (Adefegha *et al*., 2015); hence, the presence of phenolic compound (gallic acid) in the various combinations could have enhanced their DPPH scavenging abilities. The ability of gallic acids to act as free radical scavengers is due to the presence of multiple hydroxyl groups in each compound which are able to donate their protons to finally break the chain reaction of the free radicals (Van Acker *et al.,* 1996).

ABTS is a water soluble free radical initiator that in the presence of reactive oxygen can be oxidized to form a stable green radical $ABTS^+$ (Zhang *et al.,* 2012). Antioxidants have being studied to react with $ABTS^+$, thereby fading the reaction colour system (Zhu and Jiao, 2005). The ABTS radical scavenging ability result (figure 4.7) reveals that there was no significant difference in the ABTS* scavenging ability of all the samples (acarbose, gallic acid and their various combiations). Nevertheless, the samples were able to scavenge the $ABTS^+$ radical.

The $ABTS^+$ and DPPH radicals scavenging abilities by gallic acid, acarbose and their various combinations could be attributed to structure-function relationship. Both compounds possess numerous hydroxyl groups, which have been well reported to contribute significantly their free radicals scavenging ability. Notably, the various combinations showed synergistic effect in the free radicals scavenging abilities and this could be as a result of the reaction between their OH groups.

5.2 CONCLUSION

Inhibition of key enzymes associated with the hydrolysis of carbohydrate has been reported to be involved in the management of type 2 diabetes. Several diabetic complications, which have been noted to result from oxidative stress can be controlled by antioxidants. Therefore, the combinations of gallic acid with acarbose could be employed in the management of T2DM with the comparative advantage of possible reduction of the side effects of acarbose; nevertheless the combination of 50% acarbose and 50% gallic acid seems the best combinatory therapy for the management of type 2 diabetes mellitus.

5.3 RECOMMENDATION

In view of the findings of this study, diets rich in gallic acid could serve as means of managing diabetes. In cases of chronic diabetes where drugs are to be taken, diets rich in gallic acid could be recommended with acarbose to reduce the side associated with the drug, and hence, means of management of type 2 diabetes.

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APPENDIX

CALCULATIONS

DPPH radical scavenging ability (%)

% scavenging ability = (Abs_{ref}- Abs_{sam}) / Abs_{ref}* 100

Where, Abs_{ref}= Absorbance of Reference

Abs_{sam}= Absorbance of Sample

Fe^{2+} chelating ability (%)

% Fe^{2+} chelating ability = (Abs_{ref}- Abs_{sam}) / Abs_{ref} * 100

Where, Abs_{ref} = Absorbance of Reference

Abs_{sam} = Absorbance of Sample

Ferric reducing antioxidant property (FRAP)

FRAP content = (Abssam Conc. Std)/(Absstd Conc.sam)

Where, Absstd = Absorbance of standard (Vitamin C)

Abssam = Absorbance of Sample

Conc.std = stockconcentration of Standard in mg/ml

Conc.sam = Stock Concentration of Sample in g/ml

ABTS* scavenging ability (mmol. TEAC/g)

% scavenging ability ((Abs_{ref}–Abs_{sam}) x 100

Where, Abs_{ref}= Absorbance of Reference

Abs_{sam} = Absorbance of sample/ standard

Therefore, ABTS scavenging ability (mmol/g) = (% sam x $Conc._{std}$) / (%stdx $Conc._{sam}$x TMW)

where, % std = percentage scavenging ability of standard (Trolox)

Enzyme Inhibition (%)

% Inhibition = $(Abs_{ref} - Abs_{sam}) / Abs_{ref} * 100$

Where, Abs_{ref} = Absorbance of Reference

Abs_{sam} = Absorbance of Sample

Lipid peroxidation

% sample = (ABSsamplel/ABSbasal)*100

% induced (ABS induceed/ABSbasal)*100