Zinc (II)-vanillic acid complex: Synthesis, characterisation and evaluation of antidiabetic and antioxidative properties

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DECLARATION OF INDEPENDENT WORK

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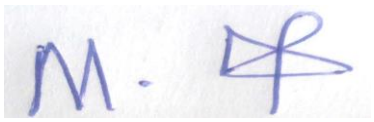

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DEDICATION

I dedicate this master's dissertation to Almighty Allah and my adorable kids Hameedat, Hassan, Huseeinat, and Haleem for their unending love and understanding.

ABSTRACT

Background: Diabetes is a major non-communicable disease that contributes to morbidity and mortality outcomes, globally. The morbidity and mortality outcomes of diabetes has been attributed to several vascular complications associated with the disease. Oxidative stress has been implicated in several mechanisms underlying the development and progression of diabetic complication. Blood glucose-lowering drugs are commonly used to manage diabetes and mitigate the development of vascular complication. However, many of these antidiabetic drugs have been associated with several unpleasant side effects, which have, somehow, discouraged their use. Moreover, some of these antidiabetic drugs are not affordable to most people in under-developed or developing countries, especially those in the middle- and low-income communities. Supplements and medicinal plants are, however, gaining attention in the prevention and management of many diseases, including diabetes and oxidative stress, perhaps due to their perceived holistic medicinal profile and minimal safety concern. In fact, studies have given credence to the antidiabetic and antioxidative pharmacological potentials of plant-derived phenolics. Vanillic acid is a natural phenolic acid with documented oxidative stress and diabetes related pharmacological properties. Also, zinc mineral has been reported to have insulin mimetic potentials. Data from clinical trials suggest that zinc may be useful in glycaemic control, as well as in diabetes prevention and management. Zn(II) has been complexed with many ligands, to develop potent antidiabetic agents. However, it has been mostly complexed with synthetic organic ligands that has potential toxic effects and little or no reported pharmacological property. In fact, antioxidant ligands such as natural phenolic acids have been scarcely studies as possible ligands of bioactive Zn(II) complexes, despite the minimal toxicity concerns of natural phenolic acids. Specifically, vanillic acid has not been studied as possible ligand to develop a bioactive Zn(II) complex. Therefore, the aim of this study was to synthesize and evaluate the antioxidative and antihyperglycaemic effects of a novel Zn(II)-vanillic acid complex.

Materials and methods: Zn(II)-vanillic acid complex was synthesised from zinc sulphate heptahydrate and vanillic acid precursors. After synthesis, the complex was characterised using Fourier-transform infrared (FT-IR) and proton nuclear magnetic resonance (^{1}H NMR) spectroscopic techniques. The effect of the complex on the viability of Chang liver cells and L-6 myotubes was evaluated. Then, different *in vitro*, cellular and *ex vivo* experimental models were used to measure the antihyperglycaemic and antioxidative activity of the complex, which was compared to the activity of the complex's precursors. The *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals scavenging and ferric reducing antioxidant activities of the complex and

precursors were measured. Also, the inhibitory activity of the test samples on α-glucosidase, α-amylase and glycation activities was measured *in vitro*. The effect of the test samples on glucose uptake was measured in L-6 myotubes and isolated rat psoas muscle tissue. Finally, the anti-lipid peroxidative effect of the test samples was measured in isolated rat liver tissues induced with oxidative stress.

Results and discussion: FT-IR and NMR data suggest that vanillic acid complexed with Zn(II) through a $Zn(O_6)$ coordination mode by using its carboxylic functional group. Thus, it is proposed that the complex has three moieties of vanillic acid. This structural property of the complex appears to influence its activity relative to vanillic acid. The DPPH (IC_{50} = 95.9 µM) and ABTS (IC_{50} = 12.2 µM) radicals scavenging and Fe^{3+} reducing (251 mmol/mol AAE at 40 µM) activities of the complex were, respectively, 2.3 ($p < 0.05$), 1.8 and 1.5 ($p < 0.05$) folds stronger than those of vanillic acid. Also, the anti-lipid peroxidative activity of the complex (IC_{50} = 667 µM) in rat liver tissue was 9.7 folds more potent ($p < 0.05$) than that of vanillic acid (IC_{50} = 6470 µM) and statistically comparable to that of ascorbic acid standard. Complexing Zn(II) with vanillic acid resulted in a complex with stronger α-glucosidase (IC_{50} = 48.3 µM; $p < 0.05$), amylase (IC_{50} = 5.86) and glycation (IC_{50} = 19.8 µM) inhibitory activities relative to those of vanillic acid. The potent activity of the complex may be partly attributed to its three vanillic acid moiety, which can collectively potentiate stronger activities compared to vanillic acid alone. Zn(II) conferred potent L-6 myotube (EC_{50} = 20.4 µM) and muscle tissue (EC_{50} = 612 µM) glucose uptake effects on vanillic acid. Cytotoxicity data showed that the complex did not reduced the viability of L-6 myotubes and Chang liver cells, suggesting it may not pose hepatotoxicity concerns.

Conclusion: Data of this study showed that complexing Zn(II) with vanillic acid resulted in a complex with improved antioxidant and antihyperglycaemic activity relative to vanillic acid. Zn(II) may be further studied as potential adjuvant for vanillic acid in developing bioactive antidiabetic and antioxidative nutraceutical for prevention and management of diabetes and oxidative complications.

Key words: Diabetes, oxidative stress, zinc mineral, vanillic acid, zinc-vanillic complex, antioxidative, antihyperglycaemic, muscle glucose uptake, NMR analysis and FT-IR analysis.

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LIST OF ABBREVIATIONS

^{1}H NMR	-	Proton nuclear magnetic resonance
ABTS	-	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AGEs	-	Advanced glycation end-products
Akt	-	Protein kinase B
AMPK	-	5' adenosine monophosphate-activated protein kinase
BSA	-	Bovine serum albumin
cAMP	-	Cyclic adenosine monophosphate
DMEM	-	Dulbecco's Modified Eagle Medium
DMSO	-	Dimethyl sulfoxide
DPP-4	-	Dipeptidyl peptidase 4
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
FRAP	-	Fe^{3+} reducing antioxidant power
FT-IR	-	Fourier transform infrared
GLUT-4	-	Glucose transporter type 4
GR	-	Glutathione reductase
GS	-	Glycogen synthase
GSH	-	Reduced glutathione
HbA1c	-	Glycated haemoglobin
IDF	-	International diabetes Federation
IGT	-	Impaired glucose tolerance
IRS-1	-	Insulin receptor substrate 1
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI-3K	-	Phosphatidylinositol 3-kinase

PPAR	-	Peroxisome proliferator-activated receptor
ROS	-	Reactive oxygen species
SGLT-2	-	Sodium-glucose co-transporter 2
SOD	-	Superoxide dismutase
STZ	-	Streptozotocin
T1D	-	Type 1 diabetes
T2D	-	Type 2 diabetes
WHO	-	World Health Organization

RESEARCH OUTPUTS

1. **Oke IM,** Ramorobi LM, Mashele SS, Bonnet SL, Makhafola TJ, Eze KC, Noreljaleel AEM, **Chukwuma CI*** (2021). Vanillic acid–Zn(II) complex: a novel complex with antihyperglycaemic and anti-oxidative activity Journal of Pharmacy and Pharmacology. https://doi.org/10.1093/jpp/rgab086**.** **Please see Appendix Section.**

Chapter 1
INTRODUCTION

Diabetes mellitus is a heterogenous metabolic disorder characterised by hyperglycaemia due to poor glycaemic control or glucose homeostasis (Punthakee et al., 2018). It occurs due to the inability of the pancreas to sufficiently produce insulin and/or as a result of failed response to insulin action in the target cells (Ndisang et al., 2017). Diabetes is classified into three main types, namely type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes, which occurs during pregnancy (IDF, 2019). The classical symptoms of diabetes include recurrent urination (polyuria), increased thirst (polydipsia) and increased hunger (polyphagia). However, persistent hyperglycemia without proper management can progress into diabetic ketoacidosis, heart disease, blindness, testicular dysfunction, kidney failure and other macro and microvascular complications (Wu et al., 2016).

Oxidative stress and inflammation are major mediators in the development of diabetic complications (Zhang et al., 2020). Oxidative stress occurs as a result of an imbalance between antioxidant status and free radical production in the body. Free radicals and other reactive oxygen species (ROS) cause oxidative damage to biological molecules, tissues and organs, which can lead to diabetic complications (Bazinet and Doyen, 2017). Elevated level of glycated haemoglobin (HbA1c) is a common diabetic biomarker linked to oxidative stress, which is a risk indicator of developing microvascular complications in diabetes (Zhang et al., 2020).

Despite available commercial anti-diabetic drugs, most of them cause side effects like gastrointestinal discomforts, dizziness, nausea and hypoglycaemia, which has discouraged their use (Rehani et al., 2019). Additionally, some of them are costly and unaffordable. However, phytochemicals such as plant-derived polyphenols are known antioxidants with diabetes-related medicinal properties and little or no side effects (Bazinet and Doyen, 2017). Phenolic compounds are known to be free radical scavengers and metal chelators, which help to mitigate oxidative injury in biological systems (Bazinet and Doyen, 2017).

Vanillic acid is a phenolic acid that is present in some edible plants and fruits. Early studies have documented its antimicrobial, antidiabetic and antioxidant properties (Tipparaju et al., 2004; Rupasinghe et al., 2006; Vinothiya et al., 2016; Dorevic et al., 2018; Mateos-Maces et al., 2020). Vanillic acid ameliorated diabetes by lowering blood glucose level and improving blood pressure and oxidative stress (Vinothiya et al., 2016).

Furthermore, supplements such as minerals and vitamins have been shown to be useful in diabetes management (Kimball et al., 2017). Zinc has been reported to play important roles in insulin secretion and functionality, as well as cellular processes involved in maintaining good health and preventing diseases (Chukwuma et al., 2020a). In human and mouse skeletal muscle cells, Zn(II) modulated insulin signalling, which increased glucose oxidation (Norouzi et al., 2018).

Considering the glycaemic control related properties of zinc mineral, it is safe to say that Zn(II) may be a promising adjuvant for vanillic acid in developing a complex with improved antihyperglycaemic and antioxidative properties. Therefore, in this study a novel zinc-vanillic acid complex was synthesised, characterised and the antidiabetic and antioxidative property of the complex was evaluated.

Chapter 2
LITERATURE REVIEW

2.1 Diabetes

Diabetes was first reported in an Egyptian manuscript 3000 years ago, which was associated with a sweet urine or blood (Ahmed, 2002). Advances in research has shown that it is a disease mainly characterised by abnormally high levels of blood glucose due to impairment of insulin secretion and/or action (Punthakee et al., 2018). The progression of diabetes can be devastating if not treated and could result in acute and chronic complications or even death.

Both genetic and environmental factors constitute the main cause of diabetes development. The environmental factors include the use of steroids and other drugs that can alter hormonal balance, thereby affecting insulin production. Also, sedentary lifestyle, bad eating habits, excessive intake of alcohol, smoking and some underlying disease conditions like obesity can be risk factors (Shrestha et al., 2019). Pancreatic infections by viruses and other microorganisms can, also, lead to the development of diabetes (Abu-Ashour et al., 2017).

The common symptoms of diabetes include polydipsia, weight loss, polyphagia and polyuria. Other associated non-specific symptoms include headache, fatigue, blurred vision, itchy skin and slow healing of cuts (IDF, 2019).

2.2 Prevalence of diabetes

In 2019, a total of 463 million people was estimated to be living with diabetes, representing 9.3% of adults (20 – 79 years), globally. This number is expected to increase to 578 and 700 million people by 2030 and 2045, respectively (IDF, 2019). The 2019 prevalence of diabetes in women and men was estimated to be 9.0% and 9.6%, respectively. It has, also, been shown that the prevalence of diabetes increases with age (Saeedi et al., 2019) (**Figure 2.1**).

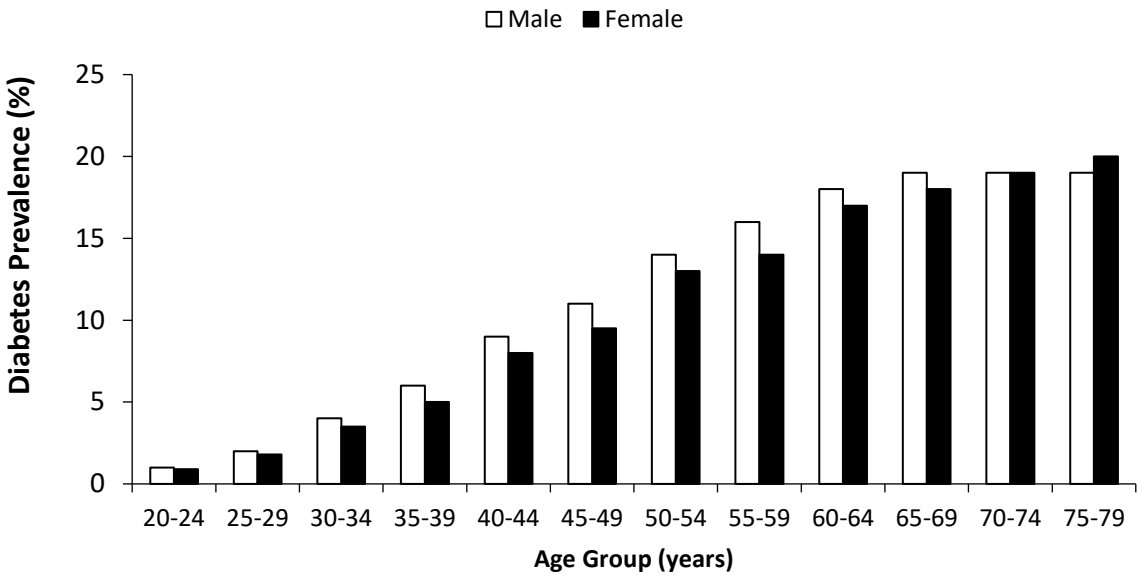


Figure 2.1 Diabetes prevalence by age and sex in 2019

Source: *Adapted without permission form Saeedi et al. (2019).*

Table 2.1 Top 10 countries in 2019 with the highest number people (age 20–79 years) with diabetes and the projection in 2030 and 2045

Rank	2019		2030		2045	
	Countries	**Number of people with diabetes (millions)**	**Countries**	**Number of people with diabetes (millions)**	**Countries**	**Number of people with diabetes (millions)**
1	China	116.4	China	140.5	China	147.2
2	India	77.0	India	101.0	India	134.2
3	USA	31.0	USA	34.4	USA	37.1
4	Pakistan	19.4	Pakistan	26.2	Pakistan	36.0
5	Brazil	16.8	Brazil	21.5	Brazil	26.0
6	Mexico	12.8	Mexico	17.2	Mexico	22.3
7	Indonesia	10.7	Indonesia	13.7	Egypt	16.9
8	Germany	9.5	Egypt	11.9	Indonesia	16.6
9	Egypt	8.9	Bangladesh	11.4	Bangladesh	15.0
10	Bangladesh	8.4	Germany	10.1	Germany	10.4

Source: *Adapted without permission form Saeedi et al. (2019).*

There is higher diabetes prevalence among high-income countries and middle-income countries compared to low-income countries. Also, a higher percentage of people living with diabetes reside in urban areas (Basit et al., 2018; IDF, 2019). This has been attributed to the sedentary lifestyle and unhealthy eating habits in urban life. Recent data and the 2030 and 2045 projections on the prevalence of diabetes in different countries show that China, India and USA are at the top of the list (**Table 2.1**), which could be due to the high population in these countries and their type of diet.

There are different types of diabetes. The notable ones are type 1 diabetes, type 2 diabetes and gestational diabetes. Type two diabetes is the most prevalent type of diabetes (IDF, 2019). Gestational diabetes occasionally occurs in pregnant women as high blood glucose due to glucose intolerance. To understand the etiology and pathophysiology of the different types of diabetes, it is rationale to first understand that normal metabolism of glucose.

2.3 Glucose metabolism and insulin signalling

Carbohydrate digestion, glucose synthesis from non-carbohydrate precursors (gluconeogenesis) and glycogen break down (glycogenolysis) are the major processes that contribute to the pool of circulating glucose (Ojha et al., 2019). The body signals the pancreatic beta cells (β-cells) to release insulin when the level of blood glucose is high and above normal range (Razzak et al., 2018). Insulin is a key hormone involved in regulating blood glucose level by signalling the uptake of circulating glucose into most cells of the body, especially the liver, fat and muscle cells. Concomitantly, insulin impedes the glycogenolytic action of glucagon (Röder et al., 2016) (**Figure 2.2**). Insulin helps to prevent the breaking down of fat in the adipose tissue by hindering intracellular lipase, an enzyme that hydrolysis triglycerides to release free fatty acids. Insulin is, also, indirectly involved in anabolic processes, including the accumulation of fat in adipose tissue (Aronoff et al., 2004).

In fed state insulin directly or indirectly signals cellular uptake of circulating glucose. At cellular level, glucose undergoes a catabolic process through the glycolytic pathway or an anabolic process to synthesise glycogen for storage in cells of tissues such as the liver and muscle (Aronoff et al., 2004). Glucose catabolism is facilitated by cellular respiration. During cellular respiration glucose is catabolised into pyruvate, which is used in the generation of adenosine triphosphate, an energy containing molecule (Röder et al., 2016). Glucose molecules can, also, be used for lipid synthesis (adipogenesis) in adipocyte. During fasting, there is a low level of circulating glucose, which signals a decrease in the release of insulin from the beta cells. Concomitantly, low level of circulating glucose signals an increase in glucagon secretion by the pancreatic α-cells, which facilitates the breakdown of stored glycogen and release of glucose into the blood (Aronoff et al., 2004; Röder et al., 2016) (**Figure 2.2**).

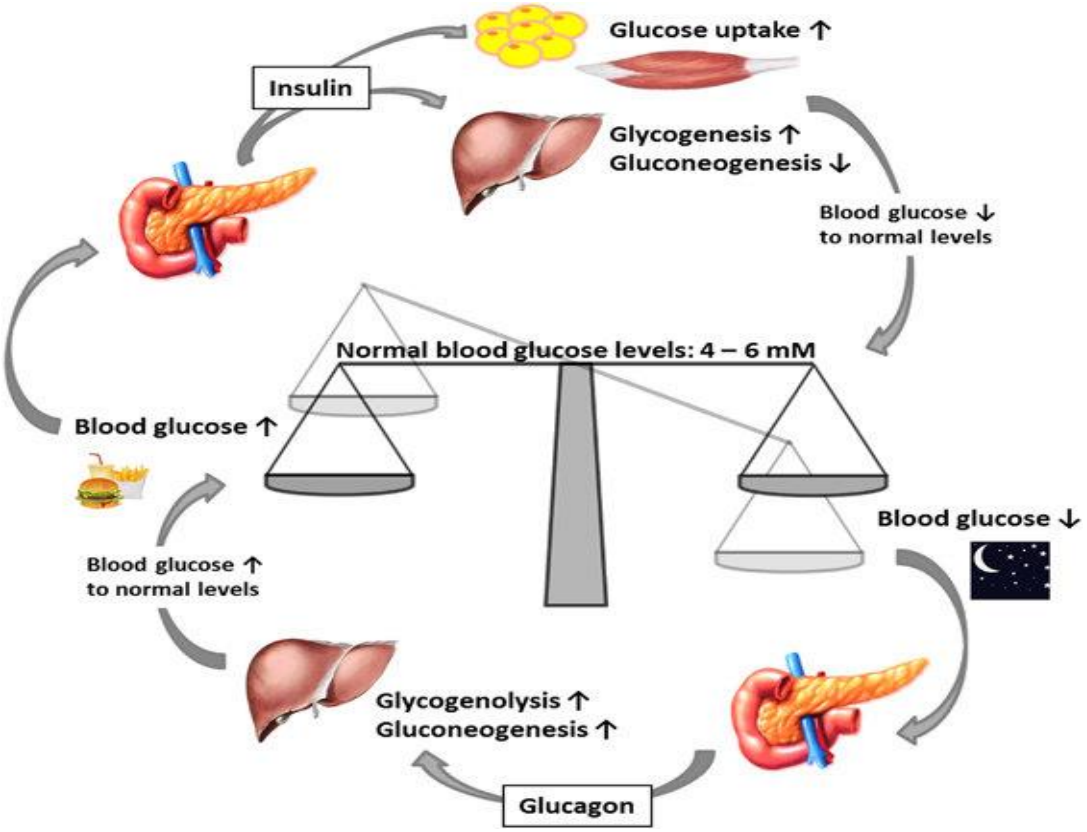


Figure 2.2 Glucose homeostasis regulation by insulin and glucagon

Source: *Adapted without permission from Röder et al. (2016).*

Insulin exerts blood glucose lowering effects by partly signalling the uptake of circulating in cells of different target tissues. The action of insulin in target tissues is mediated by a cascade of signalling events, largely regulated by phosphorylation and dephosphorylation in signalling protein targets (Yu and Chai, 2015) (**Figure 2.3**). Insulin released by the β-cell binds to the extracellular α-subunit of insulin receptor on the cell membrane of target cells. The binding of insulin activates the catalytic domain of the tyrosine kinases in the intracellular β-subunit of the insulin receptor through an autophosphorylation process. The activated kinases phosphorylate several substrate targets to activate them, with insulin receptor substrate 1 (IRS-1) being the important substrate with respect to insulin-mediated cellular glucose uptake (Khorami et al., 2015). Activated IRS-1 signals either the activation of the mitogen-activated protein kinase pathway or the activation or the activation of the phosphatidylinositol 3-kinase (PI-3K) pathway. The mitogen-activated protein kinase pathway is involved in the regulation of genes involved in cell growth and differentiation. Activation of PI-3K leads to subsequent activation/phosphorylation of protein kinase B (Akt or PKB), which mediates the metabolic action of insulin (Khorami et al., *2015*). Activated Akt, signals the translocation of glucose transporter type 4 (GLUT-4) form the cytosol to the plasma membrane, thereby allowing the influx of glucose from extracellular region into the cells (Yu and Chai, 2015; Khorami et al., 2015) (**Figure 2.3**). Activation

of Akt, also, leads to glycogen synthase kinase 3 activation, which activates gycogen sythase, an enzyme that catalyses the storage of excess cellular glucose as glycogen (Yu and Chai, 2015).

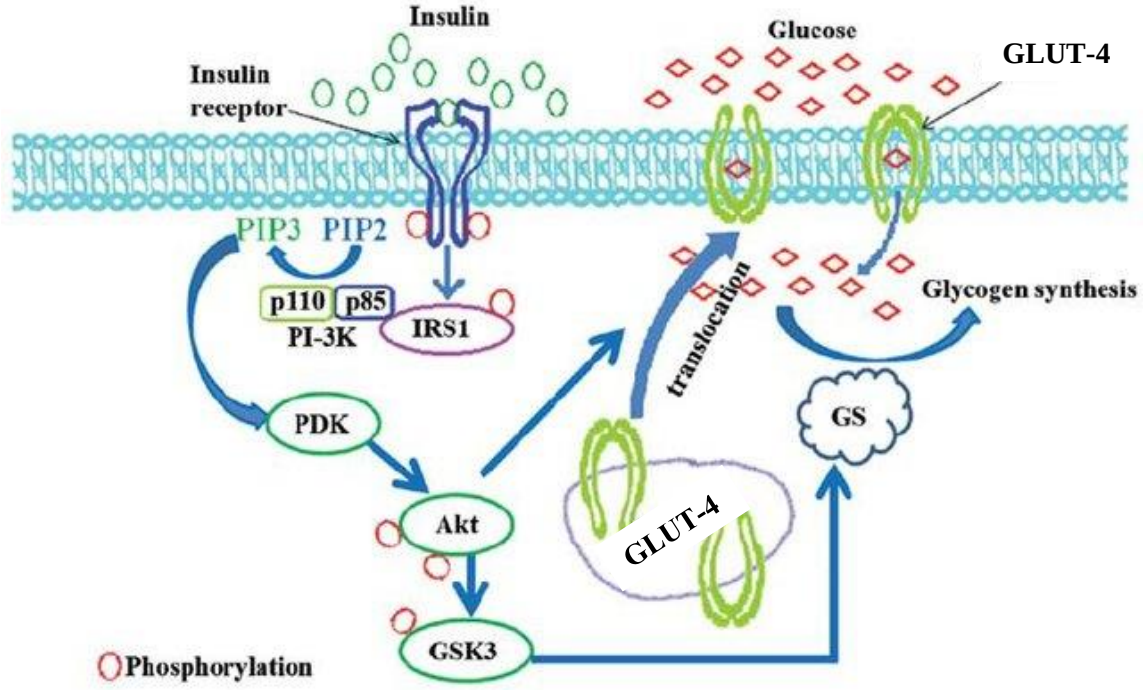


Figure 2.3 Schematic diagram showing the signalling of cellular glucose uptake by insulin

Source: *Adapted without permission from Yu and Chai (2015)*. **Abbreviations:** AGSK-3, glycogen synthase kinase 3; GS, glycogen synthase; IRS1, insulin receptor substrate 1; Akt, protein kinase B; GLUT-4, glucose transporter type 4; PI-3K, phosphatidylinositol 3-kinase; PDK, pyruvate dehydrogenase kinase.

2.4 Types of diabetes

Generally, T1D and T2D are the two most common types of diabetes. Gestational diabetes occurs sometimes during pregnancy as poor glycaemic control. Other special types of diabetes are caused by pancreatic exocrine infection or disease, anti-insulin hormones, xenobiotics, detrimental immune response and genetic factors (IDF, 2019). Also, non-diabetic individuals may show abnormally high levels of fasting blood glucose due to impaired glucose tolerance, which is termed “pre-diabetes” (IDF, 2019). The major types of diabetes are briefly discussed below.

2.4.1 Gestational diabetes

Gestational diabetes is a heterogeneous group of disorders that relate to glucose intolerance, which usually occur in the third trimester of pregnancies. Lee et al. (2008) reported that women who have a history of gestational diabetes are at risk of developing T2D later in their lives. Gestational diabetes may occur in pregnant women without a previous history of diabetes. Some may suddenly develop high blood sugar levels, which may cause detrimental outcomes during birth (Punthakee et al., 2018). Alterations in glucose metabolism can, also, occur in genetically predisposed women during perinatal

period, which can result in impaired secretion of insulin or insulin resistance (Whitelaw and Gayle, 2010). The condition may worsen as pregnancy progresses, and this could lead to the development of hyperglycaemia. However, in several cases, the condition improves or normalises following child delivery but may increase the child's risk of developing T2D in the future (Ashwal and Hod, 2015).

2.4.2 Type 1 diabetes

T1D, which is also known as insulin-dependent diabetes mellitus, occurs when the pancreas fails to produce enough insulin. It is primarily caused by a loss of β-cells function (Punthakee et al., 2018). β-cell damage in T1D is usually linked T-cell-mediated autoimmune attacks (Rother, 2007). T1D accounts for close to 10% of diabetes cases globally (Kharroubi and Darwish, 2015). It can be inherited from multiple genes having certain human leukocyte antigen genotypes that are known to influence the risk of developing diabetes (Kharroubi and Darwish, 2015). Environmental factors such as diet and viral infections are other risk factors in genetically susceptible individuals (Petzold et al., 2015).

2.4.3 Type 2 diabetes

Type 2 diabetes (T2D), which is also known as non-insulin-dependent diabetes mellitus or adult-onset diabetes, occurs when target cells fail to properly respond to insulin action or signalling (Punthakee et al., 2018). Poor dieting, detrimental weight gain, obesity and lack of adequate exercise are major risk factors of T2D (Punthakee et al., 2018). T2D is characterised by insulin resistance in target cells and progressive β-cell dysfunction (Kahn et al., 2014). It accounts for more than 90% of diabetes cases, globally (IDF, 2019), thus is our focus in this study.

2.5 Etiology and pathophysiology of type 2 diabetes

During the early stage of T2D, reduction in insulin sensitivity is the major defect. Target cells do not respond to insulin signalling, a defect referred to as insulin resistance (Yu and Chai, 2015). This results in impaired cellular glucose uptake and metabolism (**Figure 2.4**). Insulin resistance, also, leads to a defect in lipid and protein metabolism. Adipocytes do not properly differentiate into insulin responsive cells, thus leading to dyslipideamia and poor regulation of lipid metabolism (Hammarstedt et al., 2005; Goossens, 2008). These defects in lipid metabolism can increase the risk of metabolic syndrome and obesity (Hammarstedt et al., 2005; Goossens, 2008; Guilherme et al., 2008; Chatterjee et al., 2017).

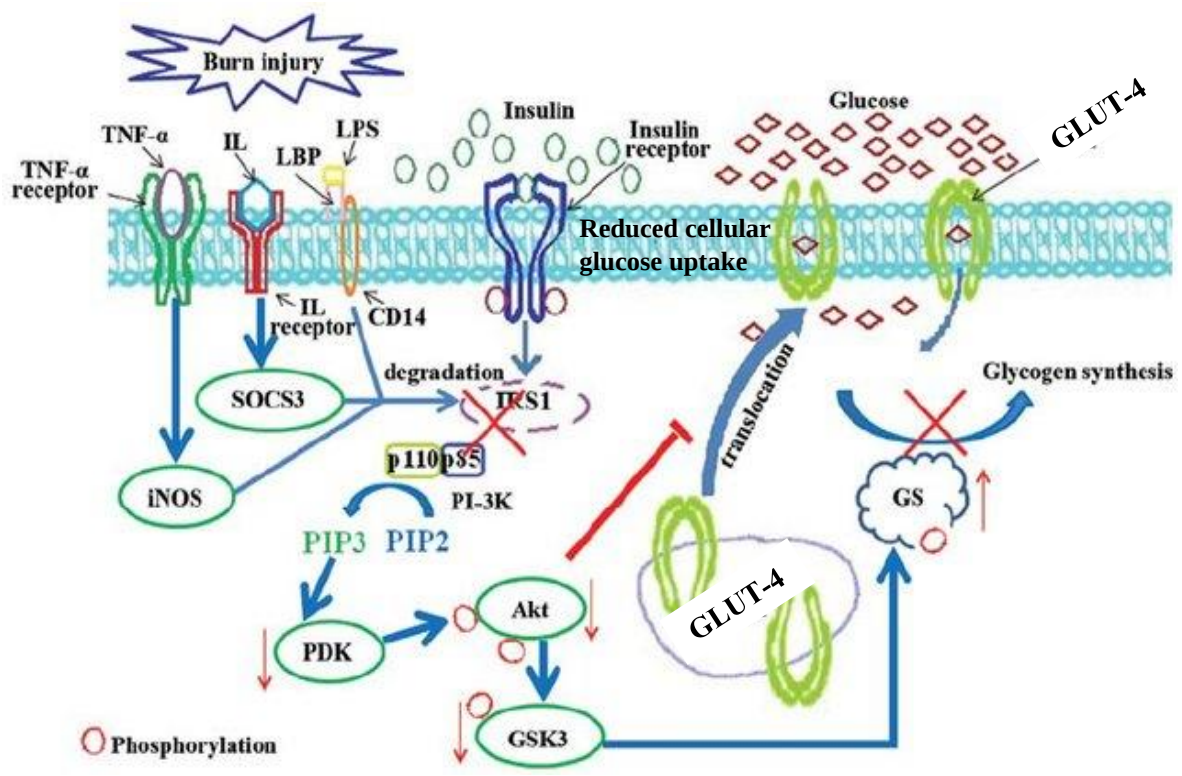


Figure 2.4 Schematic diagram showing cellular insulin resistance and impaired glucose uptake

Source: *Adapted without permission from Yu and Chai (2015).* **Abbreviations:** GSK-3, glycogen synthase kinase 3; GS, glycogen synthase; IRS1, insulin receptor substrate 1; Akt, protein kinase B; GLUT-4, glucose transporter type 4; PI-3K, phosphatidylinositol 3-kinase; PDK, pyruvate dehydrogenase kinase; SOCS3, suppressor of cytokine signaling 3; TNF, tumor necrosis factor; iNOS, inducible nitric oxide synthase; IL, interlukin; LPS, lipopolysaccharide; LBP, LPS binding protein.

Insulin resistance progressively leads to hyperinsulinaemia, due to continuous insulin secretion to compensate for insulin resistance in target cells. Eventually, this may lead to exhaustion of pancreatic β-cells, impaired glucose-induced insulin secretion and persistent hyperglycaemia (Kahn et al., 2014; Ojha et al., 2019). Additionally, hyperglycaemia may also cause oxidative stress-mediated glucotoxicity to pancreatic β-cells, which can worsen the condition of the β-cells. Beta cell dysfunction can, also, be influenced by other physiological defects such as obesity, cytokine-induced inflammation and high levels of saturated fat and free fatty acids (Ferrannini, 2010; Talchai et al., 2012).

Elevated blood glucose due to impaired insulin secretion and action can result in impaired glucose tolerance (IGT), which occurs during prediabetes. Abnormally blood glucose levels occur during IGT because the body cannot normalise postprandial blood glucose increase, which is caused by decrease in hepatic glucose uptake and glycogen synthesis (Punthakee et al., 2018, IDF, 2019). The World Health Organization (WHO) diagnostic criteria for differentiating between IGT and diabetes as follows: For IGT, it is fasting plasma glucose < 126 mg/dL (7.0 mmol/L) or plasma glucose ≥ 140 mg/dL (7.8 mmol/L) and < 200 mg/dL (11.1mmol/L) 2 hours post oral glucose administration, while for diabetes,

it is fasting plasma glucose ≥ 126 mg/dL (7.0 mmol/L) or plasma glucose ≥ 200 mg/dL (11.1 mmol/L) 2 hours post oral glucose administration (WHO, 2006).

The gastrointestinal tract is also implicated in glucose tolerance. When food is ingested, incretin hormones such as glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide are secreted, which are implicated in mitogenesis, differentiation and survival of beta cells, as well as stimulation of insulin secretion (Vella et al., 2015; Nauck and Meier, 2018). In T2D, impaired glucose tolerance is also linked to diminished stimulatory effect of incretin hormone on insulin secretion (Nauck and Meier, 2018).

Prolonged impaired glucose tolerance contributes to persistent hyperglycaemia. Persistent and uncontrolled high blood glucose promotes the development of several vascular complications and oxidative stress has been implicated as a major culprit (Pourghassem-Gargari et al., 2011; Chukwuma and Islam, 2017).

2.6 Oxidative stress in diabetes and antioxidant defence

Oxidative stress is caused by an imbalance between pro-oxidants and antioxidants in the body system. It is caused by both physiological and environmental stimulants. The major environmental stimulants include tobacco smoking, lifestyle (Youn et al., 1992), air pollutants (Boffetta and Nyberg, 2003) and UV radiation (Grattagliano et al., 2008). Endogenously, pro-oxidants such ROS, reactive nitrogen species and free radicals are produced by various physiological processes (**Figure 2.5**). Mitochondrial energy metabolism as wells as, xanthine oxidase, NADPH oxidase, cyclo-oxygenase, lipo-oxygenase, nitric oxide synthase and cytochrome P450 pathways are some of the processes that are associated with pro-oxidants production in the body (Cai and Harrison, 2000). Hydroxyl radical is produced in Fenton reaction and Haber–Weiss reaction pathway, while hypochlorous acid is produced from chloride ions and H_2O_2 through the myeloperoxidase reaction (Collin, 2019).

Oxidative stress causes damage to different vital cellular molecules such as proteins, lipids and DNA, thereby disrupting cellular function (Forni et al., 2019). Oxidative stress is implicated in insulin resistance, pancreatic islets dysfunction and tissue damage, which contributes to late pathological outcomes of diabetes (Ligouri et al., 2018). Overproduction of ROS such as superoxide radical and hydrogen peroxide has been reported in diabetes, which causes damage to the tissues and facilitate the development of diabetic complications (Cai and Harrison, 2000; Hath et al., 2017; Ligouri et al., 2018).

During diabetes, the alteration of the mitochondrial membrane leads to the leakage of electrons, which contribute to excessive superoxide radical formation (Nishikawa et al., 2000; Al Ghouleh et al., 2011). Also, NADPH oxidases reactions produce ROS, which are, also, implicated in the pathophysiology of

many cardiovascular diseases (Selemidis et al., 2008; Dal-Ros et al., 2009, 2010 and 2011). NADPH oxidases reactions are, also, sources of glucose-induced ROS production in the vasculature (Li and Shah, 2003; Dal et al., 2016), kidney (Li and Shah, 2003) and liver (Dal et al., 2015; 2016), indicating that some enzymes are mediators of diabetic complications.

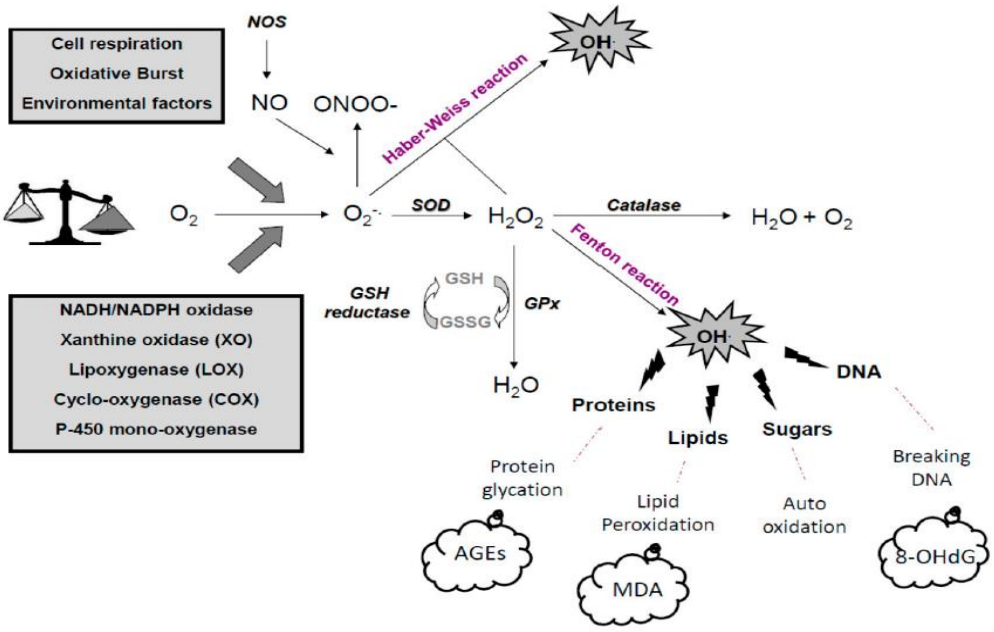


Figure 2.5 Endogenous pro-oxidant generation, oxidative stress and some antioxidant systems

Source: *Adapted without permission from Dal and Sigrist (2016).* **Abbreviations:** AGEs, advanced glycation end-products; SOD, superoxide dismutase; MDA, malondialdehyde; GSH, reduced glutathione; GPx, glutathione peroxide.

Furthermore, in diabetes, glucose and its metabolites react with hydrogen peroxide. In the presence of iron and copper ions, the reaction forms hydroxyl radical through an auto-oxidation process (Thomas et al., 2013). Lipid peroxidation, mitogen-activated protein kinase, hydrogen peroxide production by mesangial cells, activation of protein kinase C and cytokine production, also, play key roles in development of renal injury in diabetes (Anjaneyulu and Chopra, 2004). Retinopathy, a microvascular diabetic complication, has been reported to be linked to hyperglycaemia-induced glycation and accumulation of advanced glycation end-products (AGEs) (Kowluru et al., 2003; Yamagishi et al., 2012).

The equilibrium between free radicals and antioxidant defence systems is crucial for maintaining oxidative homeostasis. Disequilibrium in favour of pro-oxidant would lead to pathological oxidative stress (Thomas et al., 2013). Biologically, free radicals generated are usually quenched the body's antioxidant defence system, which comprises of enzymatic and non-enzymatic antioxidants (**Figure 2.6)**. They help to delay or inhibit the oxidation of biological substrates or molecules when present at

low concentrations by scavenging and neutralising the pro-oxidants or converting them to non-toxic forms (Mirończuk-Chodakowska et al., 2018).

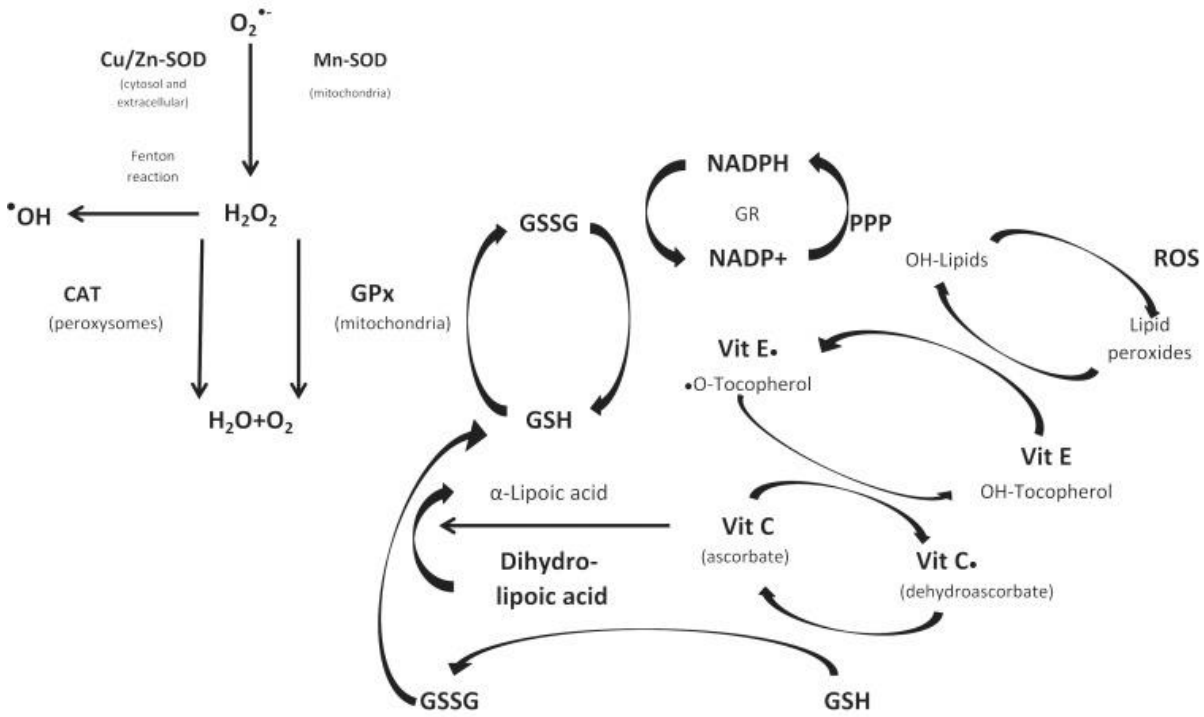


Figure 2.6 Biological enzymatic and non-enzymatic antioxidant defence system

Source: *Adapted without permission from Mirończuk-Chodakowska et al. (2018)*. **Abbreviations:** CAT, catalase; SOD, superoxide dismutase; GSH, reduced glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxide; GR, glutathione reductase; ROS, reactive oxygen species.

Some of the biological enzymatic antioxidants include superoxide dismutase (SOD), which helps to reduce superoxide anion (O_2^-) to oxygen and hydrogen peroxide (H_2O_2) (Ducrocq, et al., 2001); glutathione peroxidase, which helps reduce H_2O_2 to water and oxygen or organic peroxides to alcohol and oxygen; glutathione reductase, which helps to replenish reduced glutathione in the antioxidant system; catalase, which helps to decompose H_2O_2 into water and oxygen. The activity of these antioxidant enzymes is aided by micronutrients such as selenium, copper and zinc, as well as other cofactors (Evans and Halliwell, 2001; Mirończuk-Chodakowska et al., 2018).

Non-enzymatic antioxidants constitute natural supplements such as vitamins and glutathione, which help to scavenge radicals or unpaired electrons (Pietta, 2000; Mirończuk-Chodakowska et al., 2018) (**Figure 2.6**). They include α-tocopherol (vitamin E), carotenoids (vitamin A), GSH, L-ascorbic acid (vitamin C) etc. (Evans and Halliwell, 2001; Mirończuk-Chodakowska et al., 2018).

A depletion of these antioxidant is common in diabetic patients, which causes oxidative stress and increases the risk of diabetic complications, due to oxidative damage of biological molecule, cellular components, tissues and organs (Chukwuma and Islam, 2017; Mchunu et al., 2019).

2.7 Diabetic complications

The complications caused diabetes impose detrimental consequences on individuals, families, health systems and countries. It causes a reduction in life expectancy, morbidity and poor quality of life. Premature death appears to be twice as high among individuals with diabetes when compared with those without diabetes (Punthakee et al., 2018).

There are many complications associated with diabetes. These complications include both short- and long-term complications. They include nephropathy, neuropathy, peripheral vascular disease, stroke, chronic kidney disease, foot ulcers, retinopathy, diabetic dermadrome and cardiovascular diseases (Bourne et al., 2013). These are broadly classified as microvascular and macrovascular complications.

2.7.1 Microvascular complications

These are complications that affect small blood vessels for a long period. They include retinopathy, nephropathy, and neuropathy.

2.7.1.1 Diabetic retinopathy

This is the most common type of microvascular complication of diabetes. In the United States, it accounts for about 10 000 new cases of blindness every year (Fong et al., 2004). It is caused by prolonged and severe hyperglycaemic condition, which could develop several years before the diagnosis of diabetes in patients with T2D (Fong et al., 2004). Numerous pathological mechanisms have been proposed to be involved in the development of retinopathy. An example is through intracellular polyol pathway, which involves the conversion of excess glucose into sorbitol. High accumulation of sorbitol in cells causes osmotic stress, which consequently leads to thickening of basement membranes, microaneurysm formation and loss of pericytes (Duh et al., 2017). Additionally, some growth factors such as growth hormone, transforming growth factor β, and vascular endothelial growth factor play significant roles in the development of diabetic retinopathy (Duh et al., 2017; Wang and Lo, 2018).

Diabetic retinopathy is classified into two types, namely non-proliferative retinopathy and proliferative retinopathy. The non-proliferative retinopathy leads to the development of retinal haemorrhages, soft exudates, venous loops, hard exudates and microaneurysms. The proliferative retinopathy is seen as the advancement of non-proliferative retinopathy, which is characterised by the presence of new blood

vessels with or without vitreous haemorrhage (Wang and Lo, 2018). Diabetic patients with proliferative retinopathy need close monitoring to prevent visual loss (Watkins, 2003).

2.7.1.2 Nephropathy

Diabetic nephropathy is a major cause of renal failure globally (Menke et al., 2015). In the United States, approximately 30% and 40% of patients with T1D and T2D, respectively, develops nephropathy (Reutens, 2013; Saran et al., 2015). Diabetic nephropathy begins as microalbuminuria (urinary albumin excretion of 30–299 mg per 24 hours), which can develop to proteinuria (urinary protein level greater than 500 mg in 24 hours) and eventually, overt nephropathy (Fowler, 2008; Sulaiman, 2019). There is gradual decline in renal function, which eventually leads to severe renal disease. The mechanisms of injury could involve some of the mechanisms seen with diabetic retinopathy, particularly those involving the polyol pathway, vascular endothelial growth factor and AGEs. Diabetes-related pathological changes such as mesangial nodule formation, microaneurysm formation, increased glomerular basement membrane thickness, etc. promotes kidney damage in diabetic nephropathy. The screening for diabetic nephropathy or microalbuminuria can be carried out by using a 24-hour urine collection or a spot urine measurement of microalbumin (Micaheal and Fowler, 2008). Treatment with angiotensin-converting enzyme inhibitors has been shown to decrease the risk of developing nephropathy and cardiovascular events in patients with T2D (Didangelos et al., 2017).

2.7.1.3 Neuropathy

This is a heterogeneous condition that is linked to nerve pathology in individuals with diabetes, after ruling out other causes that are not related to diabetes. The risk of developing diabetic neuropathy is dependent on the duration and severity of hyperglycemia. Some genetic factors can, also, contribute to the development of neuropathy. Generally, neuropathy is primarily characterised by the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes (Inzucchi et al., 2016). Based on the affected nerve, diabetic neuropathy is usually categorised into different types, which include diffused, autonomic, sensory, focal, and motor neuropathy (Pop-Busui et al., 2017)

Peripheral neuropathy in diabetes is usually presented in many forms like autonomic, focal or multifocal and sensory neuropathies. The common type of neuropathy in diabetes is chronic sensorimotor distal symmetric polyneuropathy (Inzucchi et al., 2016). This is characterised by simple numbness, pains, burning, electrical pain, and tingling. In patients with simple numbness, painless foot ulceration can occur, which progressively leads to gangrene and diabetic foot. More than 80% of amputations in diabetic foot are linked to diabetic neuropathy (Boulton et al., 2005).

Diabetic mononeuropathy could manifest as diabetic amyotrophy, which is characterised by muscle weakness, severe pain and atrophy, mostly occurring in large thigh muscles (Boulton et al., 2005; Pop-Busui et al., 2017). Patient experience lack of sensation to light touch, temperature, and vibration.

2.7.2 Macrovascular complications of diabetes

Macrovascular diabetic complications include diseases that affects the peripheral, cerebrovascular and coronary arteries (Fowler, 2008). This type of complication is caused by prolonged hyperglycaemia, excess free fatty acid and insulin resistance and has been attributed to protein kinase activation, increased level of oxidative stress and activation of AGEs receptors. Early development of macrovascular diseases is linked to atherosclerotic plaque, which is usually formed in the vasculature that helps to carry blood to the brain, limbs, heart and other organs of the body. The late stage of macrovascular disease involves complete obstruction of these vessels, which can increase the risk of myocardial infarction, stroke, claudication and gangrene (Fowler, 2008; Chawla et al., 2016).

Development of macrovascular complications in diabetes may involve several physiological alterations, such as decrease in the level of nitric oxide and increase in the levels of endothelin and angiotensin II, which may lead to vasoconstriction and consequently, hypertension (Chawla et al., 2016). Also, decreased vascular nitric oxide level, increased nuclear factor-KB activation, increased angiotensin II levels and increased activation of activated protein-1 may increase inflammation due to the release of chemokines, cytokines and expression of cellular adhesion molecules (Rangel et al., 2019). Furthermore, elevated platelet tissue factor, reduced vascular nitric oxide level, elevated plasminogen activator inhibitor-1 and depleted prostacyclin may lead to thrombosis, hypercoagulation, platelet activation and decreased fibrinolysis. Consequently, these pathways may lead to the development of atherosclerosis, a major culprit of cardiovascular impairments and other macrovascular diabetic complications (Rangel et al., 2019).

Coronary heart disease has been reported as a prominent diabetic complication in several studies (Dokken, 2008; Leon and Maddox, 2015) and a primary cause of death in people with either T1D or T2D. Recent studies have documented coronary artery disease as a risk equivalent, rather than a risk factor due to the equivalent risk of myocardial infarction found in people with diabetes and nondiabetic patients with a history of previous myocardial infarction (Laing et al., 2003; Paterson et al., 2007). Premature cardiovascular events can be prevented or managed with antihypertensive and lipid-lowering agents. However, maintaining a normal range of blood glucose in diabetic patients require using hypoglycaemic or anti-hyperglycaemic agents, which is are, also, useful in preventing the development of diabetic complications.

2.8 Management of diabetes

Management approaches include exercise, healthy diet and weight loss (Dongowski et al., 2003). Fiber-rich foods are important in the diet because it assists in the control of the blood sugar level (Dongowski, et al., 2003). Also, low or no consumption of high glycaemic index foods and high fatty foods have been recommended (WHO, 2006) for people with diabetes and pre-diabetes. However, in persistent hyperglycaemic condition, management involves the use of antidiabetic drugs to maintain normal blood glucose levels.

2.8.1 Commercial antidiabetic drugs

Some of the major classes of known antidiabetic drugs include sulfonylureas, meglitinide, biguanides, thiazolidinedione, dipeptidyl peptidase 4 (DPP-4) inhibitors, sodium-glucose co-transporter 2 (SGLT-2) inhibitors, and α-glucosidase inhibitors (Chaudhury et al., 2017). These drugs have different modes of action and are used alone or combined.

2.8.1.1 Sulfonylureas

Sulfonylureas are used to lower the level of blood glucose in the body by increasing pancreatic insulin secretion (Chaudhury et al., 2017). They bind to the sulfonylurea receptor on the membrane of pancreatic β-cells, thus blocking the K_{ATP} channels or pumps, which causes membrane depolarisation (Bösenberg and Van Zyl, 2008; Chaudhury et al., 2017). Membrane depolarisation causes the influx of calcium into the β-cells, which stimulates insulin release. Sulfonylureas, also, help to limit hepatic gluconeogenesis and adipocyte lipolysis (Proks et al., 2002). They are divided into two groups, namely: The first-generation sulfonylureas, such as chlorpropamide, tolbutamide and tolazamide and the second-generation sulfonylureas, such as glimepiride, glyburide, and glipizide. The first-generation sulfonylureas have longer half-lives, higher risk of hypoglycemia and slower onset of action than the second-generation sulfonylureas (Bösenberg and Van Zyl, 2008). Hypoglycaemia and weight gain are some of the side effects of sulfonylureas (Chaudhury et al., 2017).

2.8.1.2 Biguanide

Currently, metformin is the most common biguanide that is used as a first line of oral drug for the treatment of T2D in all age-groups. The mechanism of action involves the activation of adenosine monophosphate-activated protein kinase in the liver, which causes hepatic uptake of glucose, inhibition of gluconeogenesis and suppression of hepatic glucose output (Viollet et al., 2012). Also, it improves insulin sensitivity by activating insulin receptor expression and upregulating tyrosine kinase activity (Hotta, 2019). Metformin has, also, been suggested to lower plasma lipid levels through the peroxisome

proliferator-activated receptor (PPAR)-α pathway, which helps to prevent the risk of cardiovascular diseases.

Low risk of hypoglycaemia, weight gain and diabetic complications have been linked to metformin treatment (Wulffelé et al., 2004; Lin et al., 2018). However, metformin is associated with side effects such as, diarrhoea, nausea, gastrointestinal discomforts, risk of hypoglycaemia and metallic after taste (McCreight et al., 2016). Also, reduction in drug's efficacy may occur with metformin as diabetes progresses (Rojas and Gomes, 2013).

2.8.1.3 Sodium-glucose cotransporter 2 (SGLT-2) Inhibitors

The SGLT-2 inhibitors are glucosuric agents, which include canagliflozin, dapagliflozin, and empagliflozin. They inhibit SGLT-2, thereby preventing glucose reabsorption in the proximal renal tubule and increasing urinary glucose excretion (Bösenberg and Van Zyl, 2008; Chaudhury et al., 2017). Due to the glucose-independent mechanism of action, SGLT-2 inhibitors are very useful in advanced stage of T2D, where there is a serious loss of pancreatic β-cells function. Also, they rarely lead to ketoacidosis and provides a modest weight loss and blood pressure reduction (Riser and Harris, 2013). However, they are associated with major side effects such as vomiting, nausea, diarrhoea and urinary tract infections (Burd et al., 2018; Chaudhury et al., 2017).

2.8.1.4 Thiazolidinediones

Thiazolidinediones help to improve insulin action or sensitivity. Rosiglitazone and pioglitazone are the major drugs in this class of antidiabetic drugs. They are agonists of PPAR-γ, which indirectly promotes glucose uptake in cells of several tissues such as adipose, muscle, and liver tissues. The mechanisms of action involve the diminution of free fatty acid accumulation, reduction in inflammatory cytokines, increasing levels of adiponectin levels, and preservation of β-cells integrity and function. However, they are not commonly used due to the major side effects which include heart attack, weight gain, bladder cancer and oedema (Chaudhury et al., 2017).

2.8.1.5 α-glucosidase inhibitors

The α-glucosidase inhibitors inhibit the activity of carbohydrate digestive enzymes including the glucosidase or disaccharidases, which are present in villi of the small intestine (Chaudhury et al., 2017). This class of drugs is usually taken before meal, thereby delaying carbohydrate digestion and decreasing intestinal glucose absorption and postprandial glucose level (Bösenberg and Van Zyl, 2008). The major side effects of α-glucosidase inhibitors are generally gastrointestinal discomforts, which has discouraged their recommendation in clinical practice. Particularly, α-glucosidase inhibitors are discouraged for patients with inflammatory bowel disease, colonic ulceration and intestinal obstruction

(Marín-Peñalver et al., 2016). Also, α-glucosidase inhibitors have been shown to promote weight gain. However, acarbose, a common α-glucosidase inhibitor, has been reported to sparingly increase body weight (Marín-Peñalver et al., 2016; Chaudhury et al., 2017).

2.8.1.6 Dipeptidyl peptidase 4 inhibitors

Dipeptidyl peptidase 4 (DPP-4) inhibitors are active inhibitors of DPP-4, an enzyme that deactivates incretin hormones, such as glucagon-like peptide-1 and glucose-dependent insulinotropic polypeptide (Tan et al., 2019). These incretin hormones enhance insulin secretion and function. Thus, inhibiting DPP-4, preserves their function, thereby enhancing insulin and function, as well as glycaemic control (Tan et al., 2019). The potential side effects of DPP-4 inhibitors are angioedema, pancreatitis and hypoglycaemia. DPP-4 inhibitors may not be used with insulin, sulfonylureas and thiazolidinediones or by patients with chronic kidney disease and high risk of hypoglycaemia (Bösenberg and Van Zyl, 2008; Chaudhury et al., 2017).

2.8.1.7 Insulin

Insulin is a polypeptide hormone that is produced in response to hyperglycaemia by the pancreatic β-cells (Aronoff et al., 2004). Once glucose enters the β-cells through the type 2 glucose transporters (GLUT 2), phosphorylation occurs. This increases metabolic flux through glycolysis and citric acid cycle, resulting in ATP generation (Fu et al., 2013). The elevated cellular ATP concentration causes a downregulation in the ATP-sensitive K^+ channel, which leads to depolarisation of the pancreatic β-cell membrane. Membrane depolarisation causes increase Ca^{2+} influx through the voltage-sensitive Ca^{2+} channels in the membrane, which stimulates insulin secretion (Fu et al., 2013). Randomised clinical trials have documented the use of insulin in the reduction of hyperglycaemia, as well as vascular complications of diabetes (Holman et al., 2008). However, insulin therapy can cause side effects such as hypoglycaemia and weight gain (Swinnen et al., 2009).

While synthetic anti-diabetic drugs may be useful in lowering blood glucose, the side effects that they pose remain a cause for concern among diabetic individuals. However, natural alternatives including supplements, fruits, vegetables and medicinal plants, as well as exercise, dieting and lifestyle adjustments are becoming popular in the prevention and management diabetes and oxidative complications in functional medicine. Moreover, they are considered as safe and present a holistic medicinal profile.

2.8.2 Dietary and lifestyle adjustments in diabetes prevention and management

Nutrition and dieting are crucial components in both disease prevention and treatment. Usually, a healthy diet should comprise of low level of sugar, fruits, vegetables, whole grains, nuts, and foods with

polyunsaturated fats (Gray and Threlkeld, 2019). In the management of a diabetic patient, high glycaemic index food such as bread, rice, pasta etc. must be taken with great caution. Weight loss accomplished with low-calorie and high fibre diets and exercise have been linked to HbA1c reduction in diabetics (Wadden et al., 2006).

Furthermore, supplements, such as minerals, vitamins and plant-derived bioactive principles are known complementary and/or alternative agents with growing interests in diabetes prevention and treatment (Vinayagam et al., 2016; Kimball et al., 2017). In fact, plant-derived polyphenols have pharmacological prospects for the management of diabetes and related oxidative complications (Pandey and Rizvi, 2009; Vinayagam et al., 2016; Aryaeian et al., 2017).

2.8.3 Folk medicine and plant-derived polyphenols in diabetes and oxidative stress management

In recent years, the field of herbal medicine has increased tremendously. Many conventional medicines in use are obtained from medicinal plants and many orthodox drugs are gotten from prototypic molecules. The application of medicinal plants for remedial purposes is as ancient as human history. Several studies have documented the antidiabetic and antioxidative ethnobotanical uses of plants across different parts of the world (Dixit et al., 2008; Dodda and Ciddi, 2014; Chukwuma et al., 2019). The antidiabetic and antioxidative properties of medicinal plants have been largely attributed to their constituent chemicals, particularly the polyphenols (Pandey and Rizvi, 2009; Vinayagam et al., 2016; Aryaeian et al., 2017). These polyphenols are sub-categorised into different phytochemical classes as shown in **Figure 2.7**.

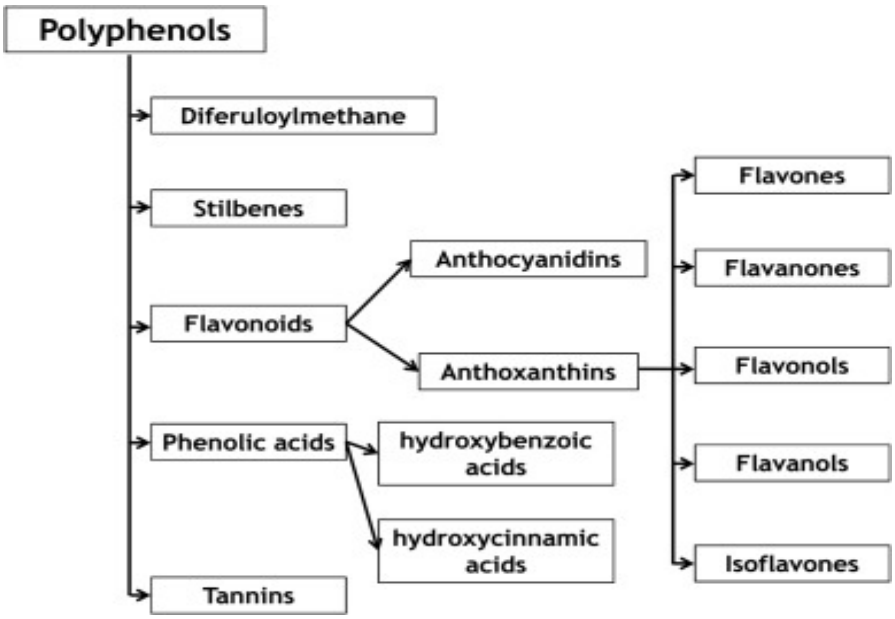


Figure 2.7 Classification of plant polyphenols

Source: *Adapted without permission from Pandareesh et al. (2015).*

Many formulations of plant-derived bioactive polyphenols are commercially available as supplements used for diabetes management. Nonetheless, the modes of action of these plant-derived formulations with respect to their antioxidative and antidiabetic potentials are yet to be fully elucidated. Some of the proposed mechanisms include inhibition of carbohydrate digestion and intestinal glucose absorption, insulinotropic effect, insulin-like action, stimulation of cellular glucose uptake, antioxidative effect, antiinflammatory effect, protective effect on pancreatic β-cells and hepatoprotective effect (Sun et al., 2020). **Table 2** shows the diabetes and oxidative stress-related pharmacologic properties of some plant-derived dietary polyphenols.

Table 2.2 Antidiabetic and antioxidative effects of plant-derived dietary polyphenols

Phenolic compounds	Foods	Effects promoted or increased	Effects inhibited or decreased
Flavonoids	Coffee, guava tea, Whortleberry, olive oil, Propolis, chocolate and cocoa	Intestinal microbiota	Glucose absorption; digestive enzymes; AGEs formation
Isoflavones	Soybean	Hypoglycaemic effects; insulin sensitivity; β-cells protection; anti-inflammation activity	Carbohydrate digestion; intestinal glucose uptake; oxidative stress; AGEs formation
Catechins	Tea leaves and red wine	Insulin sensitivity; AMPK activation; fecal excretion of bile acids and cholesterol	Elevated blood glucose and lipid; white fat deposition; α-glucosidase, α-amylase and maltase activity; ROS production
Hydroxycinnamic acids	Fruits and vegetables, especially the outer part of ripe fruits	Glucose intolerance and insulin resistance; glucokinase activity; β-cell function; AMPK activation; antioxidant and anti-inflammation effects	Hepatic glucose-6-phosphatase and phosphoenolpyruvate carboxykinase activities; cellular adipogenesis and gluconeogenesis
Caffeoylquinic acids	Potatoes, eggplants, peaches, prunes, and coffee beans	Insulin response	Human salivary and pancreatic α-amylase activity; hepatic glucose-6-phosphatase activity

Phenolic compounds	Foods	Effects promoted or increased	Effects inhibited or decreased
Anthocyanins, and anthocyanidins	Berries, eggplants, avocado, oranges, olives, red onion, fig, sweet potato, mango, and purple corn	Antioxidant and anti-inflammatory activity; blood glucose regulation	Oxidative stress; Elevation of blood cholesterol, triglycerides and LDL-cholesterol
Stilbenoids	Grapevine, berries, and peanuts	Metabolic control; insulin sensitivity; pancreatic β-cells and hepatocytes protection	Oxidative damage and inflammation; digestive enzymes activity
Procyanidins	Berries, red cabbage, apple, cocoa, cinnamon, wine and nuts	Insulin and AMPK signalling; cellular NAD^+ concentrations	Pro-inflammatory cytokine expression; digestive enzymes activity
Tannins	Coffee, tea, wine, grapes, berries, apples, apricots, barley, peaches, dry fruits, mint, basil, and rosemary	AMPK phosphorylation; glucose uptake in adipose tissue; IRS-1 phosphorylation	α-Amylase, α-glucosidase, maltase, sucrase and lactase activity; AGEs formation
Curcumin	Turmeric-flavoured foods and beverages	Pancreatic β-cells protection	Insulin resistance; diabetic cardiomyopathy; oxidative stress

Source: *Adapted without permission from Sun et al. (2020).* **Abbreviations:** AGEs, advanced glycation end products; AMPK, 5' adenosine monophosphate-activated protein kinase; IRS-1, insulin receptor substrate 1; LDL, low-density lipoprotein; NAD^+, oxidised nicotinamide adenine dinucleotide.

The radical quenching antioxidant property of polyphenols has been linked to their excellent hydrogen or electron-donating abilities. Polyphenols can form stable radical intermediates (phenoxy radical) and are not suitable targets for molecular oxygen (Chukwuma et al., 2018). Studies have shown that polyphenols from green tea, red cabbage, and red wine help to mitigate complications resulting from oxidative stress (Auberval et al., 2015; 2016). Also, epidemiological studies have documented an inverse link between plant derived dietary polyphenol consumption and the risk of T2D and oxidative complication (Middleton et al., 2000; Al-Awwadi et al., 2004; Vincent et al., 2007).

For the purpose of this study, we will be focusing on the class of polyphenols known as the phenolic acids, because they potent antioxidants that are abundant in fruits and vegetables.

2.8.4 Plant-derived phenolic acids and their antioxidative and antidiabetic potentials

Natural phenolic acids are polyphenols with a phenolic group and carboxylic acid functional group. There are two major classes of natural phenolic acids, namely the hydroxybenzoic acids and hydroxycinnamic acids (Heleno et al., 2015). The hydroxybenzoic acids include gallic acid, ellagic acid, vanillic acid, syringic acid, protocatechuic acid and salicylic acid, while the hydroxycinnamic acids include caffeic acid, caftaric acid, chlorogenic acid and cinnamic acid (Heleno et al., 2015). Many studies have given credence to antidiabetic and antioxidative pharmacological potentials of plant-derived phenolic acids (Vinayagam et al., 2016), which are discussed below.

Gallic acid is a natural trihydroxybenzoic acid present in tea leaves, grapes, several berries, vinegars and wine (Pandurangan et al., 2015). In streptozotocin (STZ)-induced diabetic rats, gallic acid treatments (20 mg/kg body weight) was shown to reduce blood glucose, increase plasma inulin and C-peptide concentrations and reverse diabetes-induced alterations of blood lipids and some biochemical makers of renal damage (Latha and Daisy, 2011). Also, the dose-dependent (25, 50 and 100 mg/kg bw) fasting blood glucose-lowering and cardioprotective effects of gallic acid have been reported in diabetic rats (Patel and Goyal, 2011). Gallic acid was, also, shown to reduce blood glucose by improving hepatic glucose metabolism in rats fed with high fructose diet (Huang et al., 2016). Additionally, diabetes-induced elevation in serum glucose, HbA1c, lipid peroxidation, serum tumor necrosis factor-α was reversed by gallic aicd treatment in rats, suggesting the antidiabetic and tissue protective potentials of gallic acid (Abdel-Moneim et al., 2017).

Studies have shown that p-coumaric acid may exert postprandial glycaemic control by inhibiting the activity of α-amylase and α-glucosidase activity (Pei et al., 2016). This natural hydroxycinnamic acid derivative is present in vegetables, fruits, mushrooms and cereals (Zabad et al., 2019). In T2D rats, p-coumaric acid potentiated antioxidant, anti-inflammatory and anti-apoptotic effects, thus suppressing hippocampal neurodegeneration (Abdel-Moneim et al., 2018). Also, p-coumaric acid ameliorated diabetic nephropathy in diabetic rats (Zabad et al., 2019). In rats, p-coumaric acid, upregulated the mRNA expression of PPARγ, thus ameliorating diabetic pathologies related to dyslipidaemia (Abdel-Moneim et al., 2018).

Syringic acid is present in different herbs including *Lentinula edode*s, *Herba dendrobii* and *Radix isatidis* (Sabahi et al., 2020) and has been reported to be an effective scavenger of free radicals (Rashedinia et al., 2020). Also, the protective effect of syringic acid against oxidative stress in the liver and kidney has been reported (Sabahi et al., 2020; Rashedinia et al., 2020). In STZ-induced diabetic rats, syringic acid ameliorated oxidative stress, reduced blood glucose and improved diabetes-induced alterations of enzyme markers of hepatic tissue damage (Sabahi et al., 2020). Syringic acid, also improved the renal antioxidant status of diabetic rats by increasing GSH concentration and suppressing

lipid peroxidation (Rashedinia, et al., 2020). Furthermore, diabetes-induced alterations in glucose and HbA1c levels, carbohydrate metabolic enzymes activities and hepatic and renal biochemical markers were restored in rats after treatment with syringic acid (Srinivasan et al., 2014). Data of the above studies suggests syringic acid may improve hyperglycaemic condition and mitigate oxidative stress-related pathologies in diabetic condition.

Ferulic acid an effective radical scavenger that is found in many cereals (Kikuzaki et al., 2002; Kumar and Goel, 2019). It enhances insulin secretion and regulates hepatic glucose production, thus improves glycaemic control (Son et al., 2010). Also, ferulic acid has been reported to reduce blood glucose levels and lipid peroxidation in STZ-induced diabetic mice and KK-A^y mice (Ohnishi et al., 2004). In the liver of high-fat diet and fructose-induced T2D rats, ferulic acid was shown to potentiate anti-diabetic effects by modulating the activity of insulin signalling molecules (Narasimhan et al., 2015). The above data suggests that ferulic acid may be useful in ameliorating hyperglycaemia and oxidative stress in individuals with diabetes.

Caffeic acid is a common natural hydroxycinnamic acids present in fruits, herbal spices, teas and coffee (Kumar and Goel, 2019). It decreased blood glucose in STZ and nicotinamide-induced diabetic rats (Mohammed et al., 2015). Concomitantly, it improved the antioxidant status of the diabetic rats by suppressing lipid peroxidation and increasing GSH level and the activities of catalase and SOD (Mohammed et al., 2015). In C57BL/KsJ-*db/db* mice, significant reduction in blood glucose and HbA1c levels and hepatic gluconeogenic enzymes activities, as well as appreciable increases in tissue glycogen content, hepatic GLUT-2 and adipocyte GLUT-4 expression and hepatic glucokinase activity were observed after caffeic acid treatment (Jung et al., 2006). Concomitantly, increased activity and expression of antioxidant enzymes, as well as reduced lipid peroxidation was observed in the caffeic acid-treated C57BL/KsJ-*db/db* mice (Jung et al., 2006), which suggests that caffeic acid may improve hyperglycaemic condition and mitigate oxidative stress-related pathologies in diabetic condition.

Cinnamic acid a natural phenolic acid found in the bark of *Cinnamomum* plant species and the nuts of African Shea tree. Data from several studies suggest that cinnamic acid may prevent diabetic complications (Adisakwattana, 2017). This phenolic acid demonstrated potent anti-glycation property, which suggests it may mitigate glycation process and associated oxidative damage in diabetics (Adisakwattana et al., 2012). In another study performed in rats, cinnamic acid reversed most diabetes-induced alterations in hepatic enzymes and lipid profiles and ameliorated oxidative stress in the kidney and liver (Anlar et al., 2018), suggesting that cinnamic acid may offer renal and hepatic protection and regulate blood lipid in diabetic conditions. Also, data from *ex vivo* and *in vivo* studies showed that cinnamic acid increased insulin secretion in isolated rat pancreatic islet and improved glucose tolerance in non-obese T2D Wistar rats (Hafizur et al., 2015), which suggests that cinnamic acid may improve the poor glycaemic control status of individuals with diabetes.

For the purpose of this study, we are focusing on vanillic acid, which is the phenolic acid that was used as the ligand for the synthesis of a novel zinc mineral complex.

2.8.5 Antidiabetic and antioxidative properties vanillic acid

Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a derivative of dihydroxybenzoic acid. Its chemical structure is shown in **Figure 2.8**. It is found in some herbs (e.g. *Angelica sinensis*, *Ocimum basilicum*, *Origanum vulgare*, *Salvia Rosmarinus* and *Thymus vulgaris*), some cereals (e.g. rice and maize), as well as some fruits and vegetables (e.g. *Euterpe oleracea*, *Phoenix dactylifera* and *Olea europaea fruits*), which contributes to the health benefits of some of the plants (Duke, 1992). It is, also, a metabolite of catechins from green tea, which contributes to the health benefits of green tea (Pietta et al., 1998).

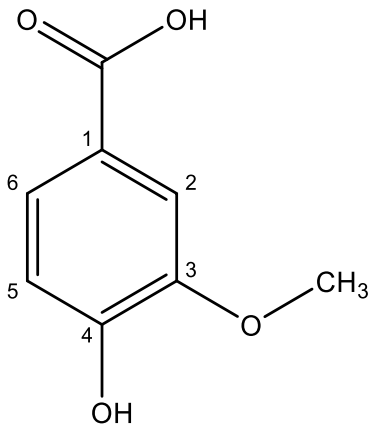


Figure 2.8 Chemical structure of vanillic acid

Vanillic acid is, also, derived from some edible plants and fruits that exhibit antimicrobial, antibacterial and antifilarial effects (Varma et al., 1993; Rai and Maurya, 1996). From previous studies, vanillic acid has been shown to have numerous bioactive properties, which include antioxidant, antidiabetic and antimicrobial activities (Tipparaju et al., 2004; Rupasinghe et al., 2006; Vinothiya et al., 2016). Vanillic acid isolated from the bark of *Rutaceae F. tessmannii* has been shown to demonstrate α-glucosidase inhibitory activity (Yin et al., 2014). Hamberg et al. (1974) reported that vanillic acid scavenges hydroxyl superoxide anion and lipid radicals *in vitro*. Wen-Fang et al. (2019) reported that vanillic acid alleviates palmitic acid-induced oxidative stress in human umbilical vein endothelial cells via the AMPK signalling pathway. Also, vanillic acid has been shown to increase glucose uptake activity in 3T3-L1 adipocytes and insulin-resistant FL83B mouse hepatocytes (Prabhakar & Doble, 2011; Chang et al., 2015). In both INS-1 cells and isolated rat pancreatic islets, vanillic acid modulated glucose-induced insulin secretion via cyclic AMP protein kinase A dependent activation of extracellular signal−regulated protein kinase (Mahendra et al., 2019).

In high-fat diet fed rats, 16-weeks oral administration of 30 mg/kg bw of vanillic acid reduced blood glucose and triglyceride levels and hyperinsulinemia and improved insulin resistance, glucose tolerance and insulin signalling (Chang et al., 2015). Additionally, 8-weeks oral administration of vanillic acid (50 mg/kg body weight) modulated the antioxidant status of diabetic hypertensive rats (Vinothiya & Ashokkumar, 2017).

Despite the potent medicinal relevance of plant phenolics in the management of diabetes, oxidative stress and related impairments, supplements such as minerals and vitamins, have also shown to be useful the prevention and management of diseases, including diabetes and oxidative stress.

2.8.6 Supplements in diabetes prevention and management

The advocacy to engage medicinal supplements and functional food in the management of metabolic, oxidative and vascular health is becoming popular because of the little to no side effects and holistic therapeutic profile of this approach (Tag et al., 2012). The antioxidant capacities of some vitamins and minerals in humans have been demonstrated in many studies. Vitamins, carotenoids, fiber, magnesium, and potassium have been shown to have synergistic beneficial effects (Stahl and Sies, 2005). **Table 3** summarises functional benefits of some supplements with respect to improving glycaemic control and ameliorating oxidative stress.

Previous studies have reported that vitamin C and vitamin E lowers oxidative stress markers and lipid peroxidation in diabetic condition (Balbi et al., 2018). Vitamin C helped to restore endothelial function in patients with T1D, thereby causing a reduction in the development of vascular complications (Timimi et al., 1998). Treatment with low or high dose of Vitamin E helped to decrease lipid peroxidation in patients with T1D (Engelen et al., 2000). In a clinical study, it was shown that vitamin E lowered the rate of cardiovascular events in individuals with diabetes and haptoglobin 2-2 genotype (Blum et al., 2010), which was corroborated by another meta-analysis study (Vardi et al., 2012).

Furthermore, the role of essential mineral such as zinc (Zn), chromium (Cr), Manganese (Mn), Copper (Cu), Iron (Fe) and Selenium (Se) in disease management has also been documented. Essential microminerals, zinc (Zn), chromium (Cr) and manganese (Mn) have biological functions that support good health and help in prevention and management of diseases, including diabetes. Hence, these minerals have been shown to be deficient in individuals with diabetes (Kazi et al., 2008). Zn, Cr and Mn play important roles in insulin secretion and functionality, oxidative health and metabolic processes involved in carbohydrate and lipid metabolism (Celafu and Hu, 2004; Chabosseau and Rutter, 2016; Li and Yang, 2018). Thus, have shown promising application as essential mineral that can improve glycaemic control and reduce the risk of developing insulin resistance and T2D in people with pre-diabetes and metabolic syndrome (Celafu and Hu, 2004; Ranasinghe et al., 2018; Li and Yang, 2018).

Table 2.3 Some supplements and their antidiabetic and antioxidative properties

Supplements	Experimental subject/model	Effects/outcomes	References
Vitamin D	Type 2 diabetic rats	Ameliorates nephropathy by activating the Keap1-Nrf2 antioxidant pathway	Nakai et al., 2014
β-carotene	Alloxan-induced diabetic rats	Improved antioxidant enzyme activity and oxidised glutathione and reduced lipid peroxidation in the heart, liver and kidney	Maritim et al., 2002
Vitamin C	Individuals with T1D	Restored endothelial function	Timimi et al., 1998
	Individuals with T1D	Reduced development vascular complications	Timimi et al., 1998
Vitamin E	Individuals with T1D	Decreased lipid peroxidation	Engelen et al., 2000
	Individuals with diabetes and haptoglobin 2-2 genotype	Lowered the rate of cardiovascular events	Blum et al., 2010; Vardi et al., 2012
Manganese	High-fat diet induced diabetic mice	Increased liver mitochondrial Mn-SOD activity and serum insulin level; improved glucose tolerance, islet insulin secretion and mitochondrial function; reduced islet lipid peroxidation levels	Lee et al., 2013
	Zucker diabetic fatty rats	reduces glucose-induced monocyte adhesion to endothelial cells and endothelial dysfunction	Burlet and Jain, 2013
Chromium	High-sucrose diet fed mice with insulin resistance	Modulated Akt phosphorylation and PI3-K activity	Dong et al., 2008
	High-fat diet fed mice with insulin resistance	Modulated Akt phosphorylation and PI3-K activity	Kandadi et al., 2011
	Adipocytes	Modulated GLUT-4 membrane translocation and glucose uptake	Chen *at al*., 2006
	Cultured monocytes and isolated human mononuclear cells	Suppressed oxidative stress in hyperglycaemic models	Jain and Kannan, 2001; Jain and Lim, 2006
	Individuals with T2D	Improved HbA1c and fasting serum glucose and insulin levels	Anderson et al., 1997
Selenium	Isolated rat adipocyte and skeletal muscle	Modulated glucose uptake by modulating plasma membrane GLUT-4 translocation and activating serine/threonine kinases.	Ezaki, 1990; Fürnsinn et al., 1996; Hei et al., 1998
	Individuals with T2D	Reduced blood glucose and HbA1c levels and improved blood cholesterol profile	Karalis, 2019

Abbreviations: Akt, protein kinase B; GLUT-4, glucose transporter type 4; HbA1c, glycated haemoglobin; PI-3K, phosphatidylinositol 3 kinase; SOD, superoxide dismutase; T1D, type 1 diabetes; T2D, type 2 diabetes.

Manganese is a known co-factor for mitochondrial superoxide dismutase, a notable physiological antioxidant enzyme that mops excess deleterious superoxide ion produced from mitochondrial energy metabolism (Lee et al., 2013). Its supplementation increases the activity of this enzyme and thus strengthens the antioxidant defence system and ameliorates oxidative stress (Lee et al., 2013; Li and Yang, 2018). Studies have, also, shown that Mn supplementation may protect against diet-induced diabetes (Lee et al., 2013) and hyperglycaemia-induced endothelial dysfunction (Burlet and Jain, 2013).

Chromium treatment restored the blunted Akt phosphorylation and the attenuated PI-3K activity in high-sucrose diet (Dong et al., 2008) and high-fat diet (Kandadi et al., 2011) models of insulin resistant mice. In adipocytes, treatment with chromium chloride and chromium picolinate modulated GLUT-4 translocation to the plasma membrane and insulin-mediated glucose transport across the plasma membrane (Chen et al., 2006). Also, the role of Cr in diabetic oxidative stress and inflammation has been documented. Chromium suppressed oxidative stress in hyperglycaemic models of cultured monocytes and isolated human mononuclear cells (Jain and Kannan, 2001; Jain and Lim, 2006). Individuals with T2D showed improvements in HbA1c, fasting plasma glucose and insulin levels, as well as 2-h fasting (i.e. levels 2 h after challenge) plasma glucose and insulin levels following supplementation of dietary Cr (Anderson et al., 1997). Clinical studies showed a strong correlation between reduced plasma Cr level and several diabetic pathologies such as high blood glucose, hyperinsulinemia, hypertension, impaired insulin sensitivity, high levels of inflammatory marker and risk of cardiovascular events (Ngala et al., 2018).

Studies have shown that dietary Se may reduce diabetes prevalence (Wei et al., 2015). Also, it has been proposed that Se may have insulin mimetic properties. Sodium selenite treatments dose-dependently modulated glucose uptake in isolated rat adipocytes (Ezaki, 1990, Hei et al., 1998) and skeletal muscle (Fürnsinn et al., 1996) by activating serine/threonine kinases and modulating the translocation of GLUT-4 to plasma membrane. In individuals with T2D, Se supplementation reduced blood glucose, HbA1c total and LDL cholesterol, but increased HDL cholesterol (Karalis, 2019). Furthermore, Selenium is a functional component of glutathione hyper-oxidase, an antioxidant enzyme that catalyses the removal of organic hyper-oxides and hydrogen peroxide from the tissues, thereby mitigating oxidative stress (Zoidis et al., 2018).

The present study focuses on zinc mineral, which was used in the synthesis of the mineral complex investigated in this study. Zinc mineral has gain notable attention in the management of diabetes because of its involvement in insulin secretion and functionality (Chukwuma et al., 2020a).

2.8.7 Cellular function of Zn(II) in insulin storage and secretion

Primarily, Zn^{2+} ion is transported in secretory granules of pancreatic β-cells, which helps insulin hexamerisation and storage (Steiner, 2011) (**Figure 2.9**). Insulin is produced in the ribosome of the pancreatic β-cells as inactive insulin, comprising of two polypeptide chains (A- and B-chains connected by a C-peptide) and a signal peptide attached to the N-terminal. In the lumen of the endoplasmic reticulum of β-cells the signal peptide is cleaved to form proinsulin, which is the direct precursor of insulin (Steiner, 1976). After the cleavage of the C-peptide moiety, proinsulin is transported into secretory granules of β-cells, where it is stored as Zn-insulin hexamer.

Insulin is stored initially as a monomer before a dimer formation, which occurs when there is an accumulation of insulin in the granules. During the insulin maturation process, Zn^{2+} lowers the solubility of the hexamer, thereby causing crystallisation within the granules. Thereafter, the matured insulin is packaged inside the granules or vesicles. Active insulin is released from the β-cell into the circulation through exocytosis when a stimulatory signal occurs (Yang, 2014). The secretory vesicles have an intrinsic proton pump that helps in lowering the pH of the granule. This acidic environment helps in the crystallisation the Zn^{2+}-insulin hexamer and the preservation of its integrity (Gold et al., 1984). For insulin to become active, the hexamer turns into a monomeric form. This conversion occurs rapidly when the inside the granule is exposed to the extracellular environment (Gold et al., 1984). Upon secretion of the monomeric active insulin, substantial amount of free Zn^{2+} is, also, released (**Figure 2.9**).

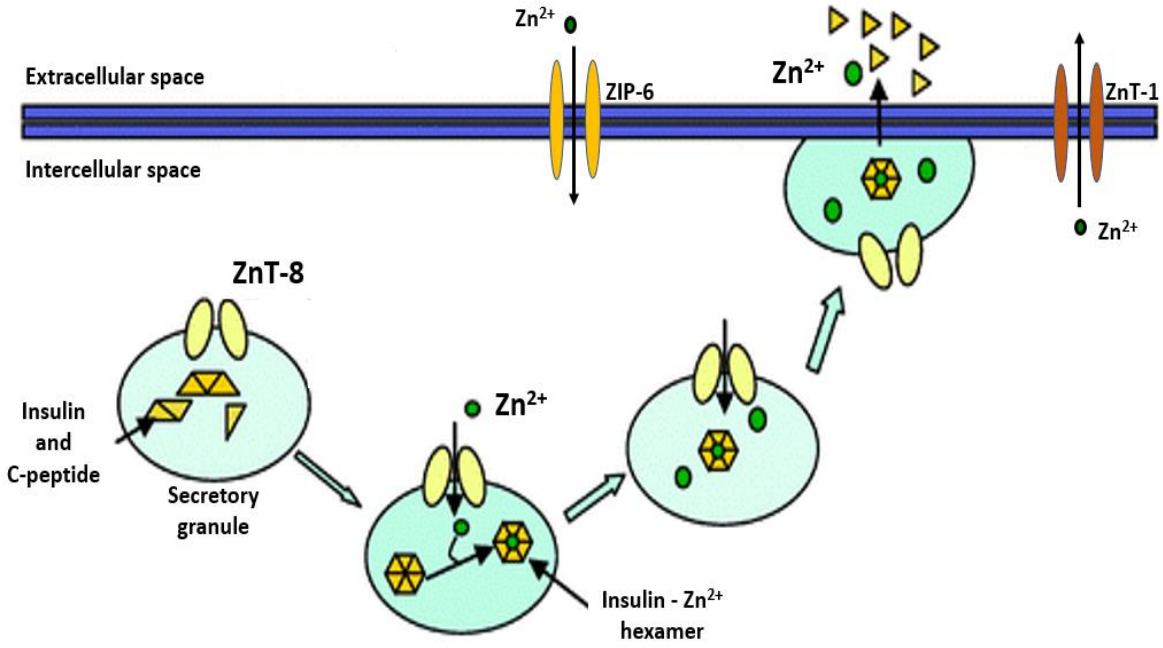


Figure 2.9 The role of zinc and zinc transporter in insulin storage and secretion

Source: *Adapted without permission from Meyer and Spence (2009).* **Abbreviations:** ZnT, Zn(II) transporter; ZIP, Zn(II) importer.

Several regulatory proteins, such as the Zn(II) importers and Zn(II) transporters are involved in the regulation of Zn(II) homeostasis in the cytosol of pancreatic β-cells (**Figure 2.9**). Studies have shown that these cellular transporters of Zn(II) may play a role in the zinc's involvement in insulin secretion, glycemic control and diabetic disorders (Chabosseau and Rutter, 2016). Zn(II) transporter 8, which is encoded by the SLC30A8 gene, appears to be the most influential Zn(II) transporter on insulin secretion and functionality (Chimienti et al., 2006; Sladek et al., 2007). It transports cytosolic Zn(II) into insulin secretory granules for hexamerisation and storage of insulin (Chabosseau and Rutter, 2016). According to the genome-wide association studies, SLC30A8 gene has been identified as a genetic risk factor for diabetes, since possession of a variant allele (rs13266634) of this gene may increase the risk of diabetes by 17 % (Chimienti et al., 2006; Sladek et al., 2007).

2.8.8 Zinc mineral in diabetes management

Several epidemiological studies have documented the relationship between physiological status of zinc mineral and diabetes. zinc deficiency has been associated with poor glycaemic control (Farooq, 2019). Hence, its supplementation may be associated with diabetes-related beneficial effects. Zn(II) has been shown to stimulate lipogenesis and glucose transport in adipocytes (Ezaki, 1989; Shisheva et al., 1992). Studies in human and mouse skeletal muscle cells suggest that Zn(II) enhances glucose oxidation and glycaemic control by modulating insulin signalling (Norouzi et al., 2018). Other studies suggest that Zn(II) downregulates cAMP-mediated lipolytic and glycogenolytic signalling by modulating the cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase activity; an enzyme that inactivates cAMP (Percival et al., 1997; Yoshikawa et al., 2004; Adachi et al., 2004). The above data suggest that Zn(II) may possess insulin mimetic effects and modulatory effects on insulin signalling.

In streptozotocin-induced diabetic rats, zinc supplementation helps to reduced polydipsia and increased HDL cholesterol levels (Wang et al., 2012), which suggests its ameliorative effect on diabetes and diabetes alteration in blood lipid profile. In obese mice zinc supplementation reduced fasting hyperglycaemia and hyperinsulinemia (Begin-Heick et al., 1985). Also, zinc mineral supplementation has been shown to improve glycaemic and lipid profiles in diabetic subjects (Jayawardena et al., 2012).

Advances in research reveal that researchers are taking advantage of the above-mentioned modulatory effects of Zn(II) on glucose and lipid metabolism to develop potent antidiabetic zinc complexes. Zn(II) complexation with ligand appears to improve bioavailability, while affording beneficial effects on glucose metabolism (Chukwuma et al., 2020a).

2.8.9 Antidiabetic effects of Zn(II) complexes

Research and reviews on pharmacologically active Zn(II) complexes have been carried out in many studies (Yoshikawa and Yasui, 2012; Ranasinghe et al., 2018; Chukwuma et al., 2020a). While there

are several proposed mechanisms underlying the glycaemic control properties of Zn(II) complexes, insulin mimetic and insulin signalling modulatory effects appear as the most prominent antihyperglycaemic modes of action (Chukwuma et al., 2020a). These Zn(II) has been shown to coordinate with its ligands through different coordination modes depending on the type of ligand. For the purpose of this study, the antihyperglycaemc studies on Zn(II) complexes will be discussed according to the class of ligand in the Zn(II) complexes. **Table 4** summarises the antihyperglycaemic and antioxidative properties of Zn(II) complexes of different classes of ligands.

Table 2.4 Antidiabetic effects of Zn(II) complexes

Class of complex	Ligands	Complex	C_M	Antidiabetic activity	Reference
Zn(II) complexes with naturally occurring organic compounds as ligands	Hinokitiol	Bis(hinokitiolato)zinc(II)	$Zn(O_4)$	120 d oral treatment (10-30mg Zn/kg) reduced HbA1c level, hyperinsulinemia and improved IR and GT in T2D KK-A^y mice	Naito et al., 2017
	3-hydroxy-2-methyl-4-pyrone (Maltol)	Bis(maltolato)zinc(II)	$Zn(O_4)$	Inhibited lipolysis (IC_{50} = 0.54 mM) and increased glucose uptake (EC_{50} = 1 mM) in isolated rat adipocytes; 14 d i.p. treatments reduced BG and HbA1c levels and improved GT in T2D GK rats	Adachi et al., 2004
	Pyridine-2 carboxylic acid (Picolinic acid)	Bis(picolinato)zinc(II)	$Zn(N_2O_2)$	14 d i.p. (3mg Zn/kg) treatment reduced BG and HbA1c levels and improved GT in T2D KK-A^y mice	Kojima et al., 2002
	3-hydroxy-5-methoxy-6- methyl-2-pentyl-4-pyrone (allixin)	Bis(allixinato)zinc(II)	$Zn(O_4)$	Showed glucose uptake and anti-lipolytic effects in isolated rat adipocytes; 14 d i.p. treatment (4.5 mg Zn/kg) reduced BG and HbA1c levels and hyperinsulinemia, and improved GT T2D KK-A^y mice	Adachi et al., 2004
Zn(II) complexes with synthetic organic compounds as ligands	3-methylpicolinic acid	Bis(3-methylpicolinato) zinc(II)	$Zn(N_2O_2)$	Inhibited lipolysis in isolated rat adipocytes (IC_{50} = 0.4mM)	Yoshikawa et al., 2002
	3-hydroxy-2-methyl-4(H)-pyran-4-thione	Bis(3-hydroxy-2-methyl-4(H)-pyran-4-thiono)zinc(II)	$Zn(S_2O_2)$	28 d oral treatment (2.5 – 10 mg Zn/kg) reduced BG and HbA1c levels and hyperinsulinemia; improved GT and increased islet number and size in T2D *ob/ob* mice	Nishiguchi et al., 2017
	2-mercapto-thiotropolone	Bis(2-mercapto-thiotropolonato)zinc(II)	$Zn(S_4)$	Inhibited lipolysis (IC_{50} = 145µM) and increased glucose uptake (EC_{50} = 19µM) in isolated rat adipocytes	Murakami et al., 2012
	α-furonic acid	Bis(α-furonate)zinc(II)	$Zn(O_4)$	Inhibited lipolysis (IC_{50} = 0.31mM) ad increased glucose uptake (EC_{50} = 0.23mM) in isolated rat adipocytes	Nishide et al., 2008

Class of complex	Ligands	Complex	C_M	Antidiabetic activity	Reference
Zn(II) complexes with ligands used as medication and/or supplement	L-threonine	Bis(L-threoninato) zinc(II) OR Zn(L-Thr)2	$Zn(N_2O_2)$	14 d i.p. treatment (3 mg Zn/kg) reduced BG and HbA1c levels and improved GT in T2D KK-A^y mice	Kojima et al., 2002
	L-carnitine	Zn(L-carnitine)2Cl2 complex	$Zn(O_4)$	Inhibited lipolysis in isolated rat adipocytes (Relative IC_{50} =0.8mM). 16 d oral treatment (20 mg Zn/kg) reduced BG (≈32%) level, and improved GT in T2D KK-A^y mice	Yoshikawa et al., 2003
	Ascorbic acid	Bis(ascorbate)zinc(II)	$Zn(O_4)$	Dose-dependently modulates adipogenesis and expression of GLUT-4, GPDH, C/EBPα and PPAR-ϒ in 3T3-L1 adipocytes	Gosh et al., 2013
	Glycine	Zn(Gly)2	$Zn(N_2O_2)$	Inhibited lipolysis in isolated rat adipocytes (IC_{50} = 0.63mM)	Yoshikawa et al., 2001
Zn(II) complexes of plant-derived polyphenol ligands	Morin	Bis(morin)zinc(II) complex	$Zn(O_4)$	30 d oral treatment (5 mg/kg bw) reduced BG and HbA1c levels and improved GT	Sendrayaperumal et al., 2014
	Curcumin	Curcumin-zinc(II) complex	$Zn(O_2)$	45 d oral treatment (150 mg/kg bw) reduced plasma glucose and HbA1c levels and increased plasma insulin level in STZ-induced diabetic SD rats	Al-Ali et al., 2016
	Flavonol	Flavonol-zinc(II) complex		30 d oral treatment (5 mg/kg bw) reduced BG and HbA1c levels and improved GT	Vijayaraghavan et al., 2013
	Diosmin	Bis(diosmin)zinc(II) complex	$Zn(O_4)$	30 d oral treatment (20 mg/kg bw) reduced BG and HbA1c levels and improved GT, IR in HFD and STZ-induced T2D Wistar rat	Gopalakrishnan et al., 2015
	3-hydroxyflavone	3-Hydroxyflavone-zinc(II) complex	$Zn(O_2)$	30 d oral treatment (5 mg/kg bw) reduced BG and HbA1c levels and improved GT in STZ-induced diabetic Wistar rat	Vijayaraghavan et al., 2013

Source: *Adapted without permission from Chukwuma et al., 2020*. **Abbreviations:** C_M, coordination mode; BG, blood glucose; HFD, high-fat diet; HbA1c, glycated haemoglobin; GLUT-4, glucose transporter type 4; GT, glucose tolerance; IT, insulin tolerance; IR, insulin resistance; i.p., intraperitoneal injection; T2D, type 2 diabetes, C/EBP-α, CCAAT/enhancer binding protein alpha; PPAR-ϒ, peroxisome proliferator activated receptor gamma; GDPH, glycerol-3-phosphate dehydrogenase; STZ, streptozotocin.

2.8.9.1 Zinc complexes with synthetic organic ligands

Many of the anti-diabetic Zn(II) complexes are known to be synthesised from synthetic organic compounds. The antilipolytic and glucose uptake activities of Zn(II) with complexes of thiol-or mercapto-containing tropolone, pyridine and pyrone, as well as their derivatives have been reported in isolated rat adipocytes (Chaves et al., 2010; Murakami et al., 2012; Nishiguchi et al., 2017; Naito et al., 2019). Zn(II) complex of 2-mercapto-thiotropolone [bis(2-mercapto-thiotropolonato)-Zn(II) complex] showed potent inhibitory and modulatory effects on lipolysis (IC_{50} = 145µM) and glucose uptake (EC_{50} = 19µM), respectively, in isolated rat adipocytes (Murakami et al., 2012).

Furthermore, the insulin mimetic and antidiabetic activities of Zn(II) complexes of piconilic acid derivatives have been reported. In isolated rat adipocytes, bis (3-methylpicolinic acid)-Zn(II) complex inhibited lipolysis and increased glucose uptake (Yoshikawa et al., 2002). In type 2 diabetic KK-A^y mice, bis(6-methylpicolinic acid)-Zn(II) complex reduced blood glucose and HbA1c levels and improved glucose tolerance (Fugong et al., 2002).

2.8.9.2 Zn(II) complexes with naturally occurring organic ligands

Naturally occurring organic compounds like maltol, hinokitiol, betaine, tropolone, D-(-)-quinic acid, etc. are some compounds that have been studied as natural ligands of antidiabetic Zn(II) complex, with maltol being the most studied. Adachi et al. (2004) reported the antilipolytic (IC_{50} = 0.54 mM) and glucose uptake (EC_{50} = 1 mM) effects of bis(maltolato)-Zn(II) complex in isolated rat adipocytes. In type 2 diabetic KK-A^y mice, it reduced blood glucose and HbA1c levels by approximately 52% and 19%, respectively, and improved glucose tolerance after a 14-days i.p. treatment of 4.5 mg Zn/kg bw (Adachi et al., 2004). Zn(II) complex of hinokitiol, a naturally occurring ligand, has, also, been reported to increase glucose uptake and inhibit lipolysis in adipocytes (Murakami et al., 2012). In islet cells bis(hinokitiol)-Zn(II) complex increased glucose-induced Akt phosphorylation. In T2D KK-A^y, mice the antihyperglycaemic effect of bis(hinokitiol)-Zn(II) complex was, also, reported (Naito et al., 2019).

2.8.9.3 Zn(II) complexes with antidiabetic drugs and supplements as ligands

Several antidiabetic drugs such as metformin, pioglitazone, chlorpropamide and glibenclamide and supplements like L-carnitine, vitamins and amino acids have been studied as potent ligands of antidiabetic Zn(II) complexes. Zn(II) complexes of pioglitazone and glibenclamide showed blood glucose-lowering effects in alloxan-induced diabetic Wistar rats, 8 h after oral administration (Rasheed et al., 2008; Prakash and Iqbal, 2014). Treatment with glibenclamide-Zn(II) complex showed stronger hypoglycaemic effect than treatment with glibenclamide alone (Rasheed et al., 2008), which suggests that Zn(II) may be a promising adjuvant for antidiabetic drugs.

Furthermore, Zn(II) complex of L-carnitine inhibited lipolysis in adipocytes (Yoshikawa et al., 2003; Matsumoto et al., 2011). The blood glucose-lowering and glucose tolerance-improving effects of L-carnitine-Zn(II) complex in type 2 diabetic KK-A^y mice has, also, been reported (Yoshikawa et al., 2003). Zn(II) complexes of vitamin U and C are the studied Zn(II)-vitamin complexes with antilipolytic activity (Matsumoto et al., 2011). Data from previous studies suggest that the insulin mimetic effect of bis(ascorbate)-Zn(II) complex is through modulation of the GLUT-4 activity and lipid metabolising proteins (Ghosh et al., 2013). Most of the studied Zn(II) complexes amino acids showed antilipolytic activity in adipocytes (Yoshikawa et al., 2001). Zn(II) complex of L-threonine, also, showed anti-hyperglycaemic effects in type 2 diabetic KK-A^y mice (Yoshikawa et al., 2001; Kojima et al., 2002). Mechanistic studies, further, showed that Zn(II) complex of L-threonine exerts insulin mimetic effects by modulating the activities PI3-K, GLUT-4 and phosphodiesterase in adipocytes (Yoshikawa et al., 2004).

2.8.9.4 Zn(II) complexes with plant polyphenols ligands

Many plant-derived polyphenols are known for their antioxidative and antidiabetic properties. (Upadhyay and Dixit, 2015). As a result, they have been explored as possible ligands for Zn(II) complexes with antidiabetic and antioxidative properties. Bis(morin)-Zn(II) complex was shown to reduce blood glucose and HbA1c levels, improve glucose and insulin tolerance and increase insulin concentration and tissue glycogen contents in T2D rats (Sendrayaperumal et al., 2014). In two other studies, the antidiabetic and antioxidative properties of bis(silibinin)-Zn(II) and bis(diosmin)-Zn(II) complexes were shown in high-fat diet and STZ-induced T2D Wistar rats (Gopalakrishnan et al., 2015; Umamaheswari and Subramanian, 2015). These complexes reduced blood glucose and HbA1c levels and improved *in vivo* antioxidant status (Gopalakrishnan et al., 2015; Umamaheswari and Subramanian, 2015). They, also, increased glycogen synthesis (Gopalakrishnan et al., 2015) and improved pancreatic histology (Umamaheswari and Subramanian, 2015) in the diabetic rats.

A 30-days oral treatment (5 mg/kg bw) of Zn(II) complex of 3-hydroxyflavone reduced plasma glucose and HbA1c levels, increased plasma insulin and C-peptide concentrations and improved glucose tolerance in STZ-induced diabetic rats (Vijayaraghavan et al., 2012). Zn(II) complex of curcumin, also, showed related effects in STZ-induced diabetic rats by reducing blood glucose and HbA1c levels and increasing insulin concentrations (Al-Ali et al., 2016). The anti-diabetic activities of the curcumin-zinc complex were 1.4 to 3.5 folds more potent than that of curcumin alone (Al-Ali et al., 2016), which suggests that Zn(II) may be a promising adjuvant for plant-derived polyphenols in developing potent antidiabetic and antioxidative Zn(II) complexes.

2.9 Problem statement

As indicated previously, studies have shown that the complexation of Zn(II) with several ligands, including synthetic and natural ligands, may improve their bioactivity and bioavailability. Few studies have, also, demonstrated the potency of Zn(II) complexes of some plant flavonoids. However, A critical analysis of antidiabetic Zn(II) complexes revealed that majority (about 72 %) of the Zn(II) complexes that have been synthesised and studied for possible antidiabetic activities are Zn(II) complexes of synthetic organic ligands that have little or no diabetes-related pharmacological history, while plant-derived polyphenols with antidiabetic and antioxidative pharmacological credence remain the least studied (5%) (Chukwuma et al., 2020b). In fact, studies on the antidiabetic and antioxidative effects of Zn(II) complexes of plant-derived phenolic acids, including vanillic acid have not been undertaken.

2.10 Aim and objectives

The aim and objectives of this study are given below.

2.10.1 Aim

The aim of this study is to investigate the antihyperglycaemic and antioxidative properties of Zn(II)-vanillic acid complex.

2.10.2 Objectives

1. To synthesise a novel zinc(II)-vanillic acid complex.
2. To characterise the complex using Fourier transform infrared (FT-IR) and proton nuclear magnetic resonance (^{1}H NMR) spectroscopies.
3. To evaluate the cytotoxicity of the complex.
4. To evaluate the antihyperglycaemic and antioxidative properties of the complex using *in vitro*, cellular and *ex vivo* experimental models.

Chapter 3
MATERIALS AND METHODS

3.1 Chemicals and reagents

Vanillic acid monohydrate, zinc sulphate heptahydrate, potassium ferricyanide, dimethyl sulfoxide (DMSO), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), etramethylsilane, starch, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), fetal calf serum, methanol, malondialdehyde (MDA), phosphate buffered saline, Trolox, potassium persulfate, para-nitrophenyl β-D-glucopyranoside, trichloroacetic acid, distilled water, iron(III) chloride, bovine serum albumin (BSA), acarbose, bovine serum albumin (BSA), porcine pancreatic amylase, ascorbic acid, Eagle's Minimum Essential Media, dinitrosalicylate colour reagent, 2,2-diphenyle-1-picrylhydrazyl (DPPH), α-glucosidase from rice, sodium carbonate, Dulbecco's Modified Eagle Medium (DMEM), aminoguanidine, RPMI medium, glucose (GO) assay kit and insulin were purchased from Sigma Aldrich, South Africa. Insulin used for *ex vivo* muscle glucose uptake was purchased form a local store (Clicks Pharmacy).

3.2 Synthesis of Zn(II)-vanillic acid complex

The complex was synthesised from Zn(II) sulphate heptahydrate and vanillic acid in a 1:2 mole ratio using a previous method (Kozlevčar et al., 2006) with some modifications. Briefly, 336.3 mg of vanillic acid (Mr = 168.15 g/mol) and 287.56 mg Zn(II) sulphate heptahydrate (Mr = 287.56 g/mol) were each separately dissolved in 3 ml of methanol and distilled water, respectively. Both solutions were, then slowly mixed. Thereafter, 3 mL of $NaHCO_3$ solution (168.02 mg in 3 mL of distilled water; Mr = 84.01 g/mol) was added until no CO_2 effervescence was observed. The complex was formed as a white precipitate, which was recovered by filtration. The precipitate was washed 3 times with 50% (v/v) methanol, dried and stored at room temperature in air-tight glass vials.

3.3 Characterisation of synthesised complex using proton nuclear magnetic resonance (^{1}H NMR) and Fourier-transform infrared (FT-IR) spectroscopies

For the ^{1}H NMR analysis the 600 MHz Bruker Avance™ spectrometer (Bruker Corporation, Massachusetts, United States) was used. The complex was dissolved in DMSO-d6 (δH = 2.50). Tetramethylsilane was used as the internal standard. The chemical shifts were recorded using the delta

(δ) scale and presented in parts per million (ppm). The coupling constants (J) were all set correctly to 0.01 Hz.

For the infrared spectrum analysis of the complex, an FT-IR spectrometer having an ART accessory (Perkin Elmer Spectrum 100 FTIR Spectrometer, Massachusetts, United States) was used. About 1mg of the complex or vanillic acid was loaded to cover the equipment's crystal sample holder. The sample was, then, scanned and the infra-red spectra were recorded using a Spectrum Software V 6.3.4. The infra-red analysis was scanned at a scan rate of 40 per second between 4000 – 380 cm^{-1}.

3.4 Cytotoxicity evaluation of complex using Chang liver cells

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure the cytotoxic effect of the complex on human Chang liver cells (ATCC® CCL-13™, American Type Culture Collection, Virginia, USA). The Chang liver cells were maintained in Eagle's Minimum Essential Media. The cell lines were supplemented with 10% heat-inactivated fetal bovine serum and grown in a humidified CO_2 incubator (NÜVE EC 160, Ankara, Turkey) set at 95% oxygen, 5% CO_2, and 37°C. At about 80% confluence, the cells were sub-cultured with 0.25% (w/v) trypsin.

To analyse the effect of the complex on the cell viability, cells were, first, seeded in 100 µl medium in 96-well plates at a concentration of 1×105 cells/mL and incubated for 24 h in the CO_2 incubator to allow cells to attach to the bottom of the wells. Thereafter, the medium was replaced with 100 µL of fresh culture medium containing varying concentrations (6, 60, and 600 µM in final assay volume) of the complex. The control cells included vehicle-treated cells exposed to growth medium containing 0.5% DMSO. After a 36-hours treatment period, the cells were exposed to MTT reagent (0.5 mg/mL in incubation medium) and incubated for 3 h. The wells were, then, aspirated and the cells were washed with phosphate buffered saline. Thereafter, 100 µL of MTT de-staining or solubilisation solution was, added into wells and absorbance was measured at 570 nm wavelength using a Multiskan Go plate reader (Thermofischer Scientific, Waltham, MA, USA). Sample blank was included and used to normalise the results. The samples were evaluated in three independent experimental repeats and each sample was evaluated in triplicate for each experimental repeat. The results given are representative of the average percentage inhibition of all the experimental repeat. The viability of cells was computed as the percentage of the sample blank with the vehicle control treated cells regarded as 100% cell viability.

3.5 *In vitro* antioxidant evaluation of the complex and precursors

Three antioxidant assays were used to measure the *in vitro* antioxidant capacity of the complex and its precursors. These include 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2′-Azino-bis (3-

ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging and Ferric (Fe^{3+}) reducing antioxidant assays.

3.5.1 Measurement of DPPH radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical characterised by a delocalisation of the spare electron over the molecule, so that the molecule does not dimerise, like most radicals. The delocalisation also gives rise to a deep violet colour, with an absorption in ethanol solution at around 520 nm. On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet colour. The ability of the complex or precursors to neutralise a dark blue/purple DPPH radical was measured using a previous method (Chukwuma et al., 2020b). To perform this assay, 75 µL of different concentrations (3.75 – 60 µM in final reaction mixture) of samples or standards (ascorbic acid and Trolox) or their solvents (control) were mixed with 35.7 µL of DPPH solution (0.3 mM in final assay volume) to make a final volume of 112.5 µL. Absorbance was, then, measured at 517 nm (SpectraMax M2 microplate reader, Molecular Devices, San Jose, CA, USA) after a 30 min incubation in the dark. The radical scavenging activity of test samples was computed as follow:

$$Scavenging\ activity(\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

3.5.2 Measurement of ABTS radical scavenging activity

The ABTS radical was prepared by mixing 7 mM ABTS and 2.4 mM potassium persulphate solutions at equal volumes and incubating for 12 to 16 hours in the dark to generate the dark blue/green ABTS radical solution. The ABTS radical solution was, then, diluted 5 time with methanol and used for the assay. To measure the neutralising effect of the complex or precursors on ABTS radical, a previous method (Oyedemi et al., 2017) was used with slight modifications. In a 96-well plate, 50 µL of different concentrations of samples, positive controls (Trolox and ascorbic acid) or their solvents together with 125 µL of the diluted ABTS solution was mixed. After a 15-minutes incubation in the dark, absorbance was measured at 734 nm wavelength. The radical scavenging activity of the samples was calculated as follows:

$$Scavenging\ activity(\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

3.5.3 Measurement of the Fe^{3+} reducing antioxidant power (FRAP)

The ability of the complex or precursors to reduce Fe^{3+} to Fe^{2+} was measured by using a ferricyanide chromogenic indicator according to a slightly modified method of a previous study (Chukwuma et al., 2020b). First, 25 µL each of 0.2 M phosphate buffer (pH of 6.6) and 1% of potassium ferricyanide was

added to a 96-well plate containing 25 µL of a certain concentration (40 µM in assay volume) of samples or positive control (Trolox) or 25 µL of standard concentrations (4 – 80 µM in reaction mixture) of ascorbic standard. The mixture was, then, incubated for 20 min at a temperature of 50 ˚C. Next, 25 µL of 10% TCA, 100 µL of distilled water and 50 µL of 0.1% $FeCl_3$ solution was added into each well of the plate in the order the reagents are mentioned. The plate was swirled gently to mix properly and absorbance was immediately measured at 700 nm. To compute the FRAP of samples, an ascorbic acid standard curve was, first, computed. Then, the FRAP of the samples and positive control was extrapolated from the standard curve, computed using the formula below and expressed as mmol/mol equivalent of the ascorbic acid standard.

$$FRAP\ (mmol/molAAE) = \frac{C \times SV}{M}$$

Where "C" = extrapolated concentration in mmol/mL
"SV" = sample volume (mL)
"M" = amount in mole of the sample in SV (mL) of the sample solution.
"AAE" = ascorbic acid equivalent.

3.6 *In vitro* antihyperglycaemic and antiglycation evaluation of the complex and precursors

The following assays were used to measure the *in vitro* antihyperglycaemic and antiglycation capacity of the complex and its precursors. These include rice α-glucosidase, porcine pancreatic α-amylase and bovine serum albumin (BSA) glycation inhibitory assays.

3.6.1 Measurement of α-glucosidase inhibitory activity

Alpha glucosidase is an intestinal digesting enzyme that hydrolyses disaccharides to monosaccharides like glucose, which is absorbed into circulation. Inhibiting the activity of this enzyme been a mode of action can reduce glucose absorption postprandial blood glucose level. The inhibitory effect of the complex and precursors on α-glucosidase activity was measured by monitoring the enzymatic hydrolysis of para-Nitrophenyl-β-D-glucopyranoside at 405 nm in the presence of the compounds. To perform this assay a previous method (Sanni et al., 2019) was used with slight modification. First, 25 µL of different concentrations (3.75 - 60 µM in assay volume) of samples or standard (acarbose) or their solvents (control) was mixed with 50 µL of a solution of α-glucosidase from rice (2 U/mL; dissolved in100mM phosphate buffer, pH 6.8) in a 96-well plate and incubated for 10 min at a temperature of 37˚C. Then, 25 µL of a solution of para-Nitrophenyl-β-D-glucopyranoside (5 mM; dissolved in100 mM phosphate buffer, pH 6.8) was added and incubation continued for 25 min. After incubation, 100 µL of

100 mM Na_2CO_3 solution was added to stop the reaction and absorbance was measured at a wavelength of 405 nm. The enzyme inhibition was calculated as follows:

$$Enzyme\ activity\ inhibition\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

3.6.2 Measurement of α-amylase inhibitory activity

Alpha amylase is a digesting enzyme that hydrolyses starch to release reducing sugar moieties, predominantly short oligosaccharides and disaccharides. Inhibiting the action of this enzyme can reduce postprandial hyperglycemia. Reducing sugars resulting from alpha amylase action can reduce yellow dinitrosalicylic acid to a reddish-brown 3-amino-5-nitrosalicylic acid. This reaction can be monitored at 540 nm. Based on this principle, the inhibitory effect of the complex and precursors on α-amylase activity was measured. To perform this assay, the method reported by Chukwuma et al. (2020b) was used with slight modifications. In a 2 mL vial, 50 µL of different concentrations (5 - 80 µM in assay volume) of samples or standard (acarbose) or their solvents (control) was mixed with 50 µL of a solution of porcine pancreatic amylase (3 U/mL; dissolved in100 mM phosphate buffer, pH 6.8) and incubated at for 15 min at 37 ˚C. Then, 50 µL of a solution starch (1% w/v; dissolved in100 mM phosphate buffer, pH 6.8) was added and incubation continued for 30 min. Thereafter, 50 µL of dinitrosalicylate colour reagent was added and the vials were put in a boiling water bath set a 110 ˚C for 10 min. The vials were, then, allowed to cool and, thereafter, centrifuged (5000 rpm for 5 min) in a Hettich Mikro 200 microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany). A 200 µL aliquot of supernatant in each vial was transferred into respective wells of a 96-well plate and absorbance was measured at a wavelength of 540nm. The enzyme inhibition of the samples or standard was computed as follows:

$$Enzyme\ activity\ inhibition\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

3.6.3 Measurement of inhibitory activity on glucose-induced BSA glycation

The effect of the complex and precursors on *in vitro* glucose-induced BSA glycation was measured with a method described by Chukwuma et al. (2020b) with a slight modification. Into a black 96-well plate, 50 µL volume of varying concentrations (5 – 80 µM in assay volume) of the samples or standard (aminoguanidine) or their solvents (control) was mixed with 50 µL of glucose (90 mg/mL) and 50 µL of BSA (10 mg/mL). The glucose and BSA solutions were prepared in 50 mM sodium phosphate buffer containing 0.02% sodium azide (pH 6.8). The mixture was incubated in the dark for 3 weeks at 37 ˚C. The fluorescence was, then, measured at excitation and emission wavelengths of 360 and 420 nm, respectively. The glycation inhibition of the samples or standard was calculated as follows:

$$Glycation\ Inhibition(\%) = \frac{Fluorescence\ of\ control - Fluorescence\ of\ test}{Fluorescence\ of\ control} \times 100$$

3.7 Measurement of the effect of the complex and precursors on glucose uptake in L6-myotubes

The method used to evaluate the effect of the complex and precursors on cellular glucose uptake was adapted from the method described by Van Huyssteen et al. (2011) and Oyedemi et al. (2013) with some modifications. L-6 myoblast cells from rat muscle (ATCC® CRL-1458™, American Type Culture Collection, Virginia, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing low glucose. The medium was supplemented with 10% fetal calf serum. The cells were sub-cultured by trypsinisation. To seed the cells, a volume of 200 µL of medium containing 5000 cells was put into each well of a 96-well plate and incubated in a humidified CO_2 incubator set at 5% CO_2, 95% oxygen at 37°C. When the cells were 80% confluence, the incubation medium was aspirated and a fresh differentiation medium (DMEM with 2% horse serum) was added to the cells. Then, incubation continued for 5 days so that the myoblast can differentiate into myotubes. After differentiation, the L-6 myotubes were incubated in 200 µL of fresh medium that contained varying concentrations (5, 50, and 100 µM in incubation volume) of samples or their solvent (control). Some wells contained only the glucose medium without cells, which was used as the blank control. After 48 h incubation, the incubation medium was aspirated and the differentiated cells were washed with phosphate buffered saline. Thereafter, 100 µL of RPMI medium containing 8 mM glucose and 0.1% of BSA was added to the wells and incubation continued for 2 h. A 1 µM insulin was used as a positive control group. After incubation, glucose concentration (µM) was measured in aliquots of incubation medium collected from each well. Glucose concentration was measured using the Glucose (GO) Assay Kit (Sigma Aldrich, South Africa). The viability of the myotubes was, also, measured after treatment with test samples using MTT assay protocol. Two biological repeats of three technical replicates were performed in the glucose uptake assay. The glucose uptake of the test samples and control wells was calculated relative to the blank control as follows:

$$Glucose\ uptake(\%) = \frac{\Delta GC\ of\ test\ or\ control - \Delta GC\ of\ blank\ control}{\Delta GC\ of\ blank\ control} \times 100$$

Where "ΔGC" is the difference between the initial and final concentration of glucose the in incubation medium. The EC_{50} of the tests, which is the effective concentration (µM) of sample needed to increase glucose uptake by 50% relative to the control was, also, computed.

3.8 *Ex vivo* antihyperglycaemic and antioxidative evaluation of complex and precursors

The *ex vivo* antihyperglycaemic effect of the complex and precursors was evaluated by measuring their effect on glucose uptake in isolated rat psoas muscle, while the *ex vivo* antioxidative effect of the complex and precursors was evaluated by measuring their anti-lipid peroxidative effect in isolated rat liver tissue induced with oxidative stress.

3.8.1 Animals

Ten weeks old Sprague Dawley rats were used in this study. The rats were procured from the animal research facility of the University of the Free State, Bloemfontein campus. First, the animals were fasted for 16 h (overnight) and, then, they were euthanised using isoflurane and carbon(iv) oxide. The psoas muscle and liver were harvested and immediately used to perform the above-mentioned *ex vivo* experiments. All the animal protocols were done according to the rules and regulations of the Animal Research Ethics Committee of the University of the Free State (Protocol approval reference: UFS-AED2019/0152; please Appendix Section).

3.8.2 Measurement of glucose uptake in isolated rat psoas muscle tissue

The method used to evaluate the *ex vivo* muscle glucose uptake effect of the complex and precursors was adapted from the method described by Chukwuma and Islam (2015) with some modifications. In a clear 48-well plate, 300 ± 10 mg portions of the harvested muscle tissues were, first, incubated in 900 µL of Kreb's buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 0.5 mM K_2HPO_4; pH 7.4 ± 2 at 25 ^{O}C), which contained different concentrations (10, 20, 40 and 80 µM in final incubation volume) of the test samples or insulin (50 mU in final 1 mL incubation volume; NovoRapid® FlexPen®, Novo Nordisk Limited, West Sussex, UK) as positive control. Incubation was done for 25 min in a CO_2 incubator (NAPCO series 5400 CO_2 incubator, Thermo Scientific, South Africa) that was set at 5% CO_2, 95% oxygen, and 37°C. For the control wells the muscle tissue was incubated in 900 µL of Kreb's buffer only. After pre-incubation, 100 µL of Kreb's buffer containing glucose (11.1 mM in incubation volume) was added to the wells. The plate was further incubated for 90 min. Thereafter, glucose concentration (µM) was spectrophotometrically measured in aliquots of incubation medium collected from each well using the Glucose (GO) Assay Kit (Sigma Aldrich, South Africa). The glucose uptake increase (%) of tests and positive control was calculated relatively to the control as follows:

$$Glucose\ uptake\ increase\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control}\ \ x\ 100$$

The EC_{50} was, also, computed.

3.8.3 Measurement of anti-lipid peroxidative effect in isolated rat liver tissue

The method used to evaluate the *ex vivo* anti-lipid peroxidative effect of the complex and precursors was adapted from the method described by Chukwuma et al. (2020b) with some modifications. In a clear 48-well plate, 200 ± 5 mg portions of the harvested liver tissue were incubated in 900 µL of Kreb's buffer, which contained different concentrations (10, 20, 40 and 80 µM in final incubation volume) of the test samples or ascorbic acid as positive control. Incubation was done in a CO_2 incubator that was set at 5% CO_2, 95% oxygen, and 37°C. The control and negative control wells contained 900 µL of Kreb's buffer. After pre-incubation, 100 µL of $FeSO_4.7H_20$ solution (0.7 mM in final incubation volume) was added to the test, positive control and negative control wells to induce oxidative stress, while an equivalent volume of Kreb's buffer was added to the control well. The plate was further incubated for 90 min. After incubation, the tissues were removed from the incubation solution and washed in Kreb's buffer. The tissues were, then, homogenised in 1 mL of 50 mM sodium phosphate buffer (with 0.5% v/v Triton X-100; pH, 7.5) and the supernatant was recovered by centrifuging for 10 min at 10000 rpm (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany). The level of lipid peroxidation or thiobarbituric acid reactive substances (TBARS) were, then, measured in the supernatants using a previous method (Mchunu et al., 2019) with slight modifications. First, 100 µL of the supernatants or increasing concentrations (0, 7.5, 15, 22.5, 30, 45, and 60 µM in assay volume) of malondialdehide (MDA), 500 µL of 0.25% w/v thiobarbituric acid, 200 µL of 20% v/v acetic acid and 200 µL of distilled water were mixed in vials. The mixture was, then, incubated for 50 min in a boiling water bath set at 110 °C. The vials were cooled after boiling. A 200 µL aliquot of the content was, then, transferred into a 96-well plate and the absorbance was measured at a wavelength of 532 nm. The level of lipid peroxidation or TBARS was extrapolated from the MDA standard plot and expressed as an equivalent of MDA concentration. The anti-lipid peroxidative effect of the test samples was calculated as follows:

$$Lipid\ peroxidation\ inhibition\ (\%) = \frac{TBARS\ of\ negative\ control - TBARS\ of\ test}{Absorbance\ of\ negative\ control}\ \ x\ 100$$

The IC_{50}, which is the concentration (µM) of sample required to cause 50% anti-lipid peroxidative effect was, also, computed.

3.9 Data and statistical analysis

Data analysis was done with Microsoft (2016) Excel and GraphPad Prism 5. The results were represented as mean ± standard deviation of replicates. The IBM SPSS version 23.0 (IBM Corp, Armonk, NY, USA) was used to do the statistical analysis of the data. The Tukey's multiple range post

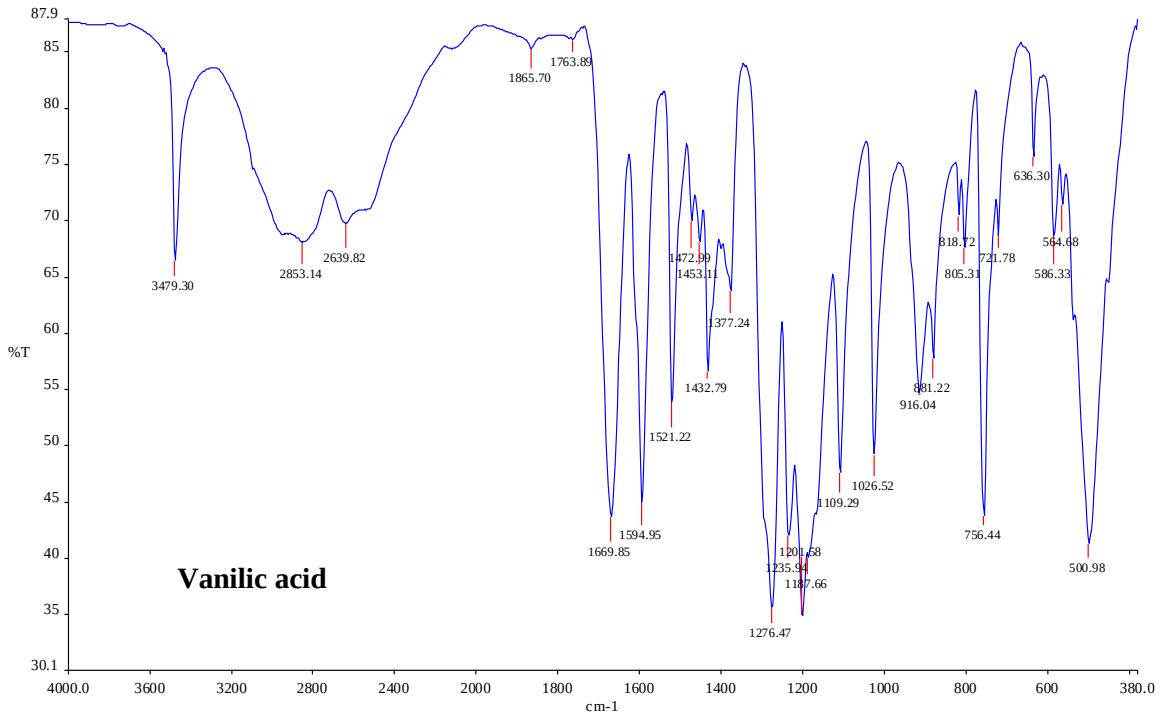
hoc and the one-way analysis of variance (ANOVA) were used for comparing the data of multiple groups. The significant difference was set at $p < 0.05$ when comparing the data between two treatment groups.

Chapter 4
RESULTS

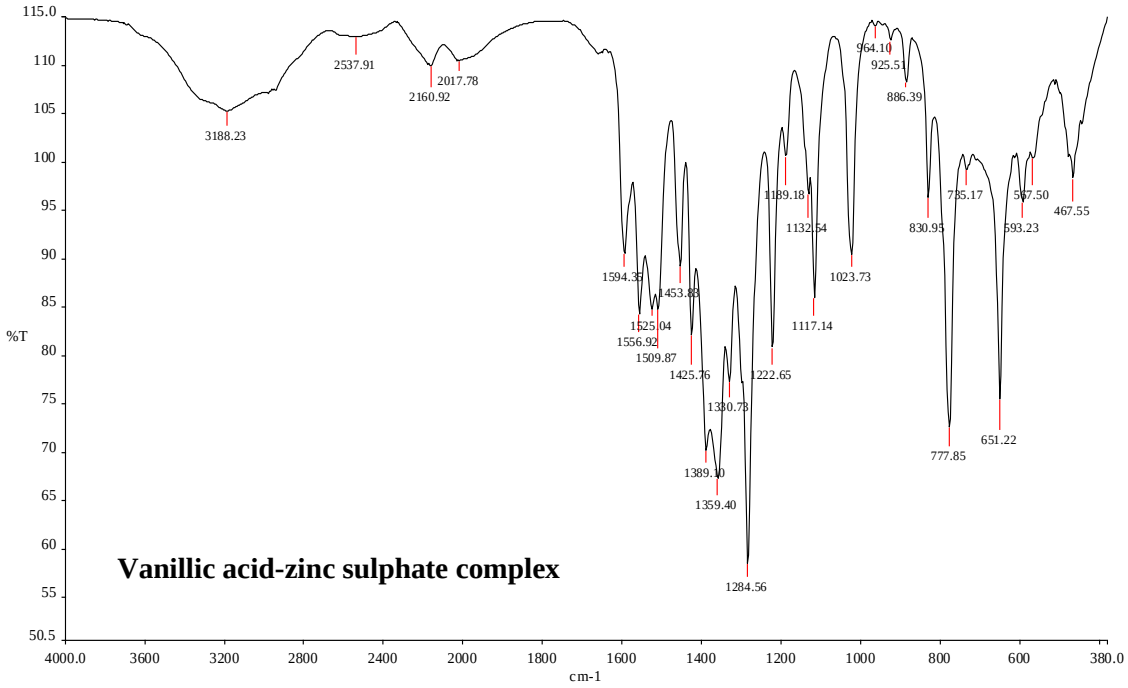
4.1 FT-IR and ^{1}H NMR structural characteristics of Zn(II)-vanillic acid complex

The synthesised Zn(II)-vanillic acid complex appeared as a white powder, which is slightly soluble in DMSO. FTIR analysis, showed some comparable features between the complex and its precursor phenolic acid (vanillic acid). The broad peak around 3500 to 3200 cm^{-1} in the IR spectra of vanillic acid (**Figure 4.1a**) and its complex (**Figure 4.1b**) is due to intermolecular O-H stretching (Oliveira et al., 2016; Merck, 2020). The strong sharp peaks at 1276 cm^{-1} (**Figure 4.1a**) and 1285 cm^{-1} (**Figure 4.1b**) is due to C-O stretching of an alkyl aryl ether (Merck, 2020), which is indicative of the methoxy group in vanillic acid (**Figure 4.2a**) and its complex (**Figure 4.2b**). The broad peak shown in the vanillic acid IR spectrum around 3300 – 2400 cm^{-1} (**Figure 4.1a** and **10b**) is due to carboxylic O-H stretching (Soichi and Noriyuki, 1966; Oliveira et al., 2016; Merck 2020). This peak was greatly diminished in the IR spectrum of the complex (**Figure 4.1b**), which suggests that the carboxylic acid group of the vanillic acid moiety was involved in Zn(II)-vanillic acid coordination as shown in **Figure 4.2b**. Also, the carboxylic C=O stretching (1670 cm^{-1}) (Soichi and Noriyuki 1966; Oliveira et al., 2016; Merck, 2020) shown in the IR spectrum of vanillic acid (**Figure 4.1a**) was absent in that of the complex (**Figure 4.1b**), which, further, supports the involvement the vanillic acid's carboxylic acid group in Zn(II)-vanillic acid coordination (**Figure 4.2b**).

The NMR analysis of the complex is presented in **Figure 4.3**. The ^{1}H NMR spectra of the ABX system of ring A was observed as doublet of doublet of H-5A at δ 7.56 and doublet of H-5A at δ 7.24, while H-2A was observed at δ 7.90 (**Figure 4.3a**). The ABX system of ring C was observed as doublet of doublet of H-5C at δ 7.46 and doublet of H-5C at δ 7.12, while H-2C was observed at δ 7.05 (**Figure 4.3a**). The ABX system of ring B in complex was observed as doublet of doublet of H-2B at δ 7.44, doublet of H-6B at δ 7.24 and doublet peak of H-5B at δ 6.7 (**Figure 4.3a**). The methoxy protons were observed at ranged between 3.15-3.41. Observation of broad peak at δ 9.74 with 3H integral indicated that the three hydroxyl groups of vanillic acid were not involve in complex formation. However, disappearance of carboxylic group indicated its involvement in the complex formation. Cosey experiments showed three ABX system of the three vanillic acid, which correlates with the AB of all three vanillin acid molecules (**Figure 4.3b**). Based on the FTIR and ^{1}H NMR data it was proposed that Zn(II) complexed with 3 moieties vanillic acid through a ZnO_6 coordination as shown in **Figure 4.2b**.

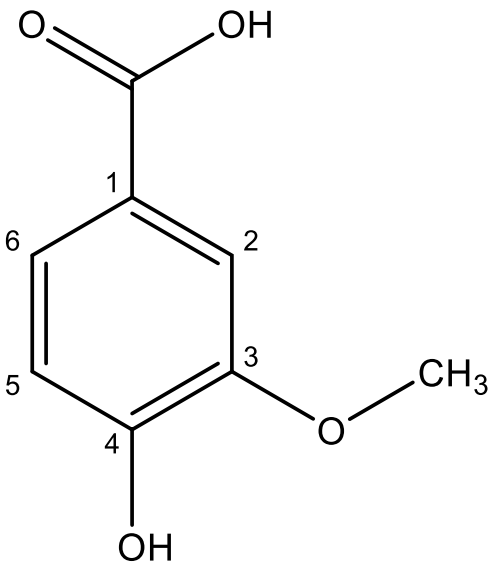


(a)

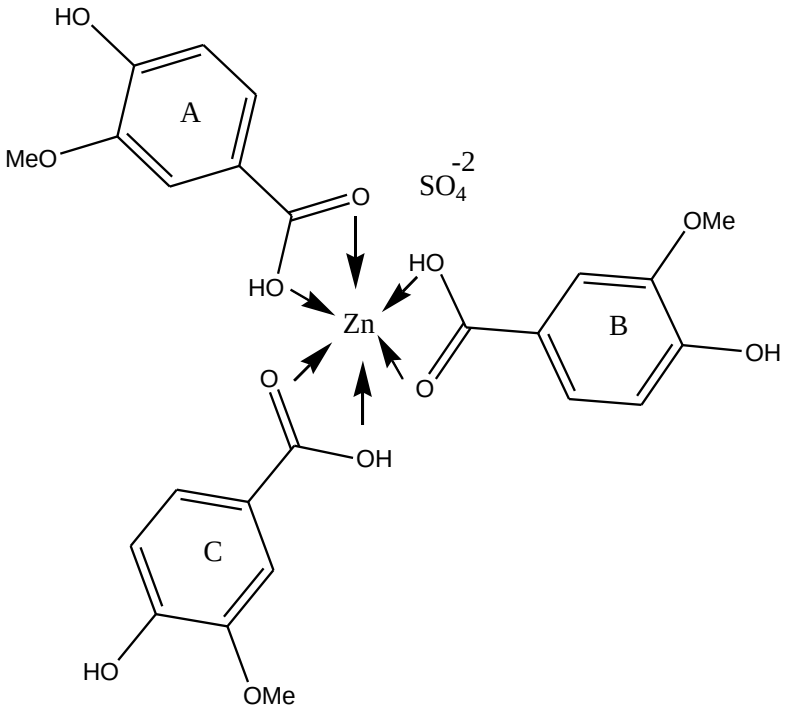


(b)

Figure 4.1 FT-IR spectra of (a) vanillic acid and (b) Zn(II)-vanillic acid complex

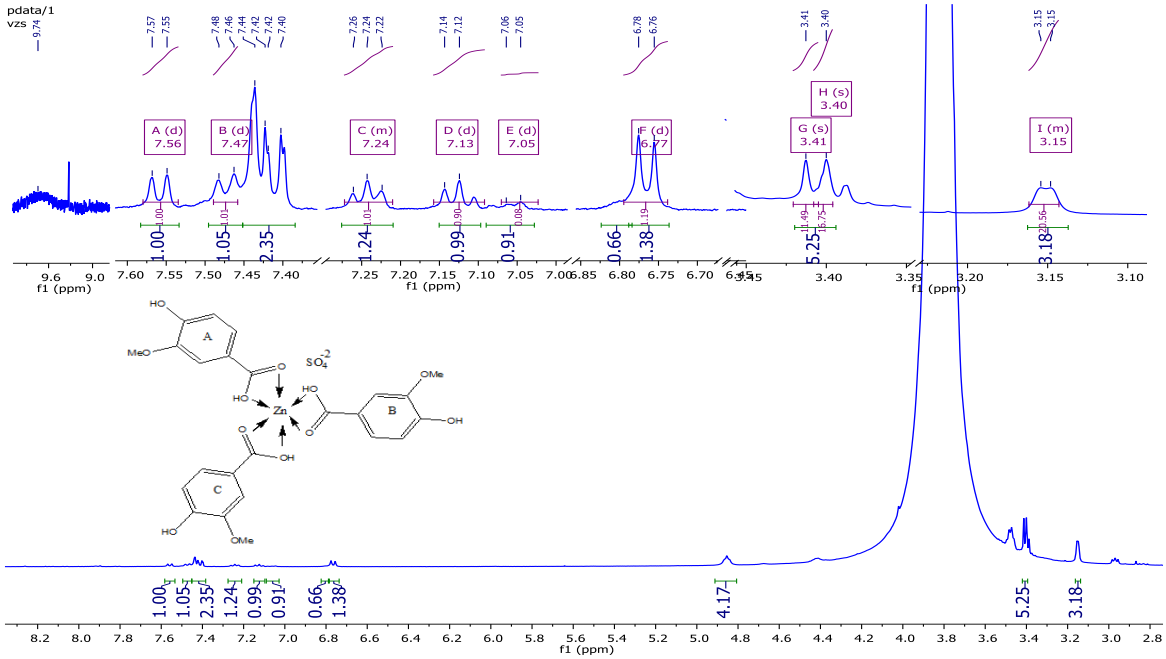


(a)

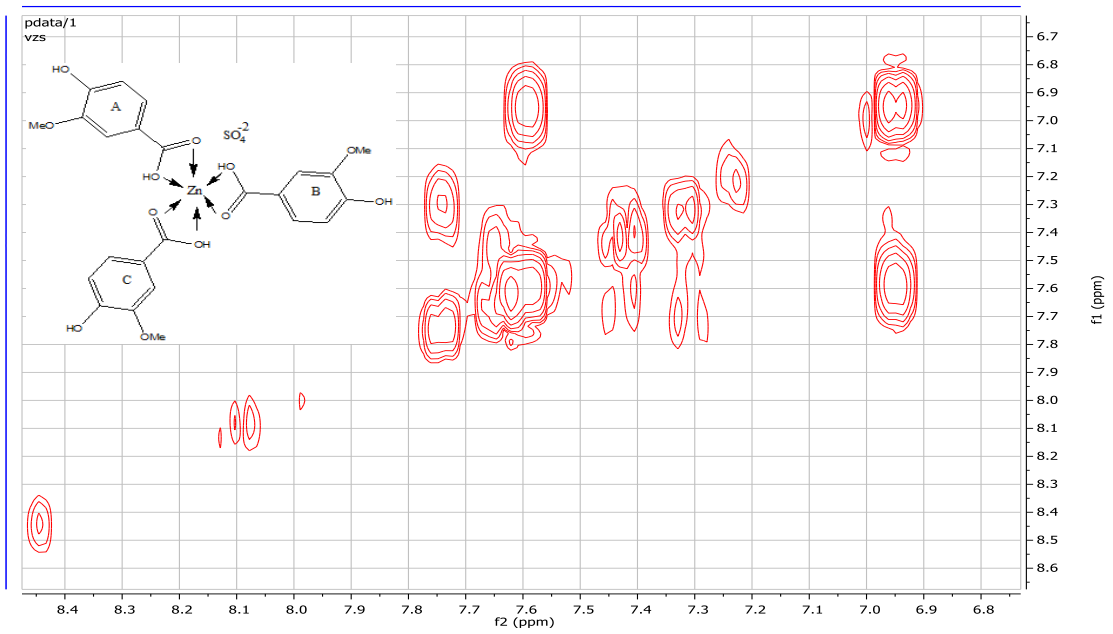


(b)

Figure 4.2 (a) structure of vanillic acid and (b) proposed structure of Zn(II)-vanillic acid complex



(a)



(b)

Figure 4.3 (a) ^{1}H-NMR spectrum and (b) Cosey experiments of Zn(II)-vanillic acid

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.56 (d, J = 7.6 Hz, 1H, H-5A), 7.47 (d, J = 7.8 Hz, 1H, H-5C), 7.44 (d, 1H, H-2A), 7.42 (dd, J = 7.4 Hz, 1H, H-6B), 7.24 (d, J = 7.6 Hz, 1H, H-2C), 7.10 (d, J = 7.4 Hz, 1H, H-2A), 7.05 (m, H, H-2A) 6.79 (m, H, H-2C), 6.77 (d, J = 8.1 Hz, 1H, H-5B), 3.41 (s, 3H, -CH3), 3.40 (s, 3H, -CH3), 3.15 (d, J = 3.1 Hz, 3H, -CH3).

4.2 Effect of Zn(II)-vanillic acid complex on the viability of Chang liver cells and L-6 myotubes

The cytotoxic evaluation the complex is presented in **Figure 4.4**. The complex did not notably reduce the viability of Chang liver cells and L-6 myotubes at tested concentrations (5 to 600 µM).

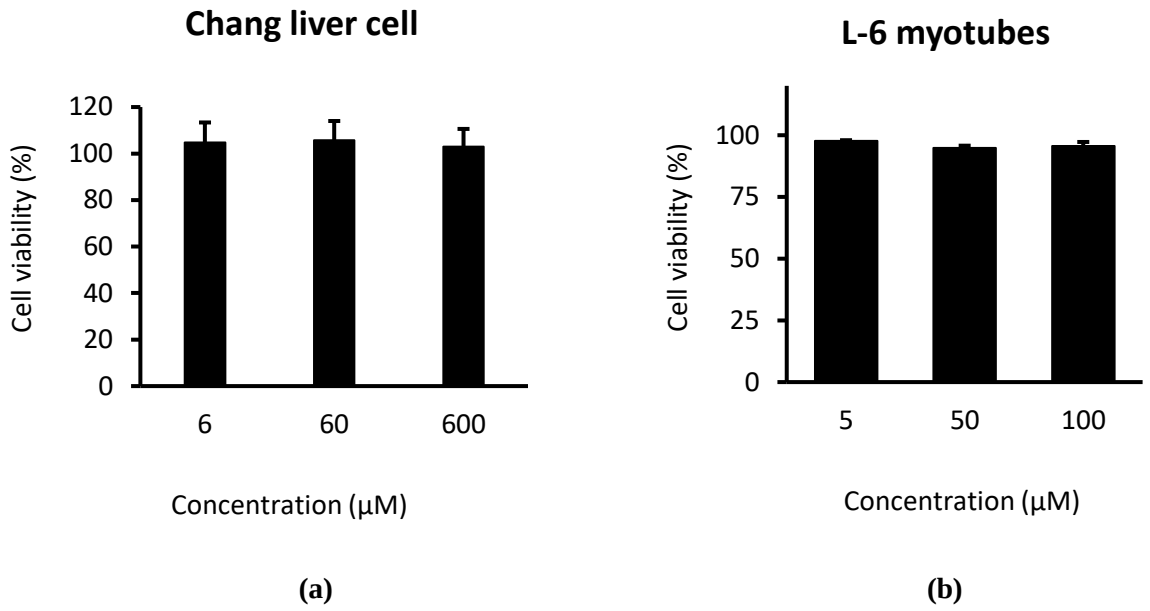
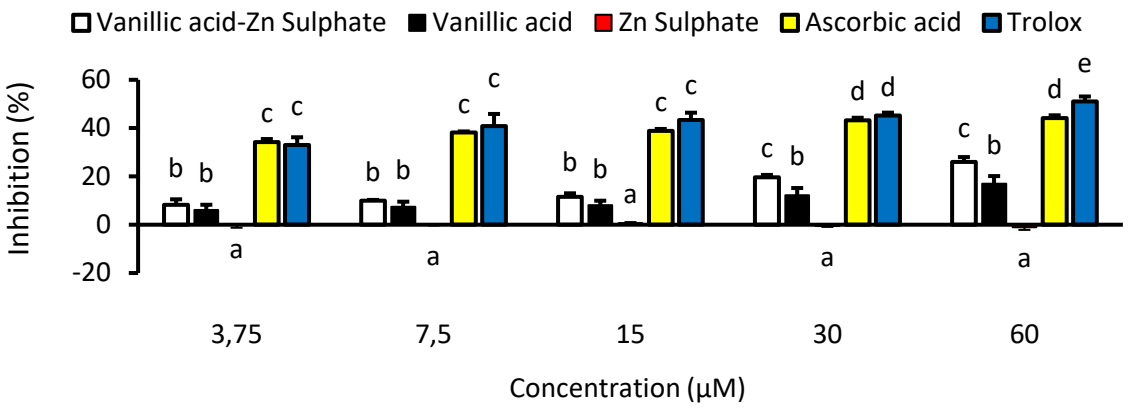


Figure 4.4 Figures showing the effect of Zn(II)-vanillic acid complex on the viability of (a) Chang liver cell and (b) L-6 myotubes

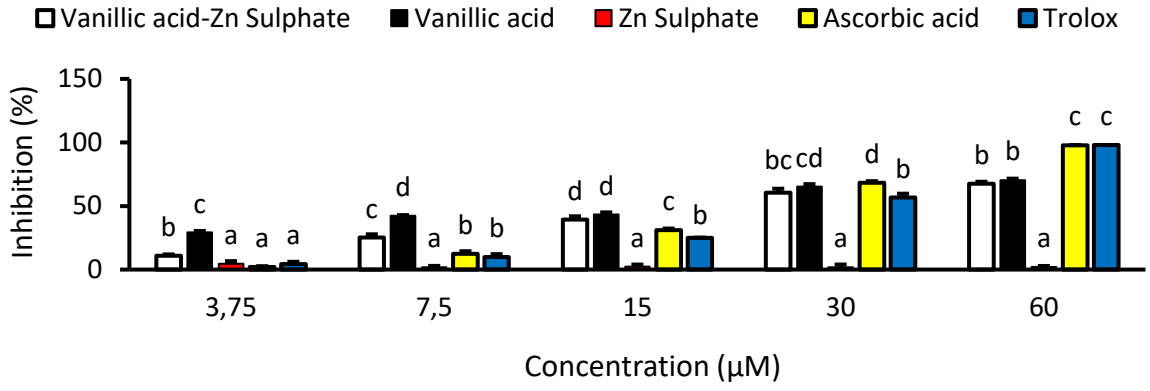
Data is shown as mean ± SD of replicate analysis.

4.3 *In vitro* antioxidant property of Zn(II)-vanillic acid complex and precursors

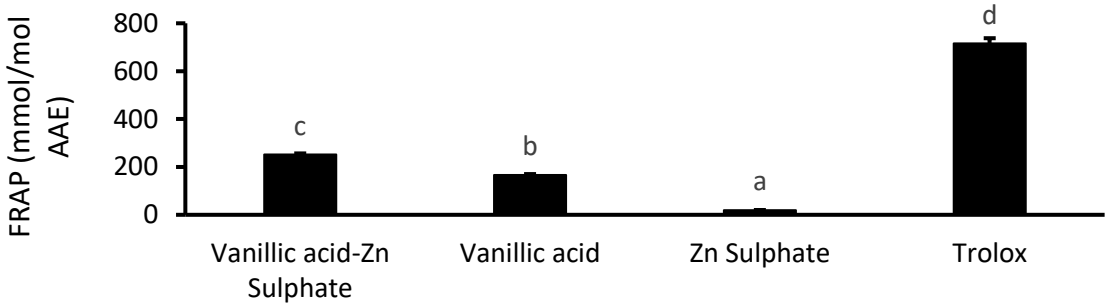
Vanillic acid dose-dependently scavenged DPPH (IC_{50} = 216 µM; **Figure 4.5a** and **Table 4.1**) and ABTS (IC_{50} = 22.0 µM; **Figure 4.5b** and **Table 4.1**) radicals and showed Fe^{3+} reducing activity (166 mmol/mol AAE; **Figure 4.5c**). $ZnSO_4$ did not show radical scavenging and Fe^{3+} reducing activities. The DPPH radical (IC_{50} = 95.9 µM) scavenging and Fe^{3+} reducing (251 mmol/mol AAE) activities of Zn(II)-vanillic acid complex was significantly ($p < 0.05$) more potent than that of vanillic acid. The ABTS radical scavenging activity of the complex (IC_{50} = 12.2 µM) was more potent than that of vanillic acid and ascorbic acid (IC_{50} = 47.1 µM) and significantly ($p < 0.05$) more potent than that of Trolox (IC_{50} = 153 µM).



(a)



(b)



(c)

Figure 4.5 Figures showing the (a) DPPH radical scavenging, (b) ABTS radical scavenging and (c) Fe^{3+} ion reducing effects of Zn(II)-vanillic acid complex and precursors

Data is shown as mean ± SD of triplicate analysis. Different letters 'a', 'b', 'c', 'd' and 'e' represent significant difference ($p < 0.05$) between treatment groups at the different tested concentrations.

Table 4.1 IC_{50} and EC_{50} values of vanillic acid, its complex with Zn(II) and other studied agents

Parameters or activity	Zn(II) complex	Phenolic acid	Zn(II)	Ascorbic acid	Trolox	Acarbose	Aminoguanidine	Insulin
	IC_{50} or EC_{50} values (µM)							
ABTS radical scavenging activity (IC_{50})	12.2 ± 3.16[a]	22.0 ± 8.54[a]	ND	47.1 ± 4.47[a]	153 ± 32.8[b]	NA	NA	NA
DPPH radical scavenging activity (IC_{50})	95.9 ± 9.97[b]	216 ± 23.7[a]	ND	9.98 ± 2.04[c]	6.20 ± 3.18[c]	NA	NA	NA
Antiglycation activity (IC_{50})	19.8 ± 7.38[bc]	52.1 ± 4.54[a]	23.3 ± 6.51[b]	NA	NA	NA	6.52 ± 0.93[c]	NA
α-amylase inhibition (IC_{50})	5.86 ± 1.08	7.91 ± 2.40	ND	NA	NA	6.36 ± 1.08	NA	NA
α-glucosidase inhibition (IC_{50})	48.3	147	46.7	NA	NA	9.08	NA	NA
Glucose uptake increase in L-6 myotubes (EC_{50})	20.4	6460	71.1	NA	NA	NA	NA	ND
Glucose uptake increase in isolated rat psoas muscle (EC_{50})	612 ± 125[b]	1190 ± 169[a]	872 ± 138[ab]	NA	NA	NA	NA	ND
Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver (IC_{50})	667 ± 184[b]	6470 ± 596[a]	ND	270 ± 93.2[b]	NA	NA	NA	NA

"ND" means "not determined; "NA" means "not applicable; IC_{50} is concentration needed to inhibit the activity of carbohydrate digesting enzymes and bovine serum albumin glycation or scavenge DPPH and ABTS radicals by 50%; EC_{50} is the effective concentration needed to increase glucose uptake in L-6 myotubes and isolated rat psoas muscle by 50%. Different letters 'a', 'b', 'c' and 'd' represent significant difference ($p<0.05$) between treatment groups.

4.4 Anti-lipid peroxidative effect of Zn(II)-vanillic acid complex and precursors in isolated rat liver tissue

The lipid peroxidation or TBARS level in the liver tissues treated with only $FeSO_4$ (negative control) was significantly ($p < 0.05$) higher than the level in the tissues without $FeSO_4$ treatment (normal control), suggesting that $FeSO_4$ induced oxidative stress in the liver tissue (**Figure 4.6**). Treatment with vanillic acid and Zn(II)-vanillic acid complex dose-dependently inhibited (IC_{50} = 6470 and 667 µM, respectively) lipid peroxidation in the liver tissues treated with $FeSO_4$, while $ZnSO_4$ treatment did not result in anti-lipid peroxidative effect (**Figure 4.6** and **Table 4.1**). The anti-lipid peroxidative effect of Zn(II)-vanillic acid complex was significantly ($p < 0.05$) stronger than that of vanillic acid and was not significantly different from that of ascorbic acid (IC_{50} = 270 µM).

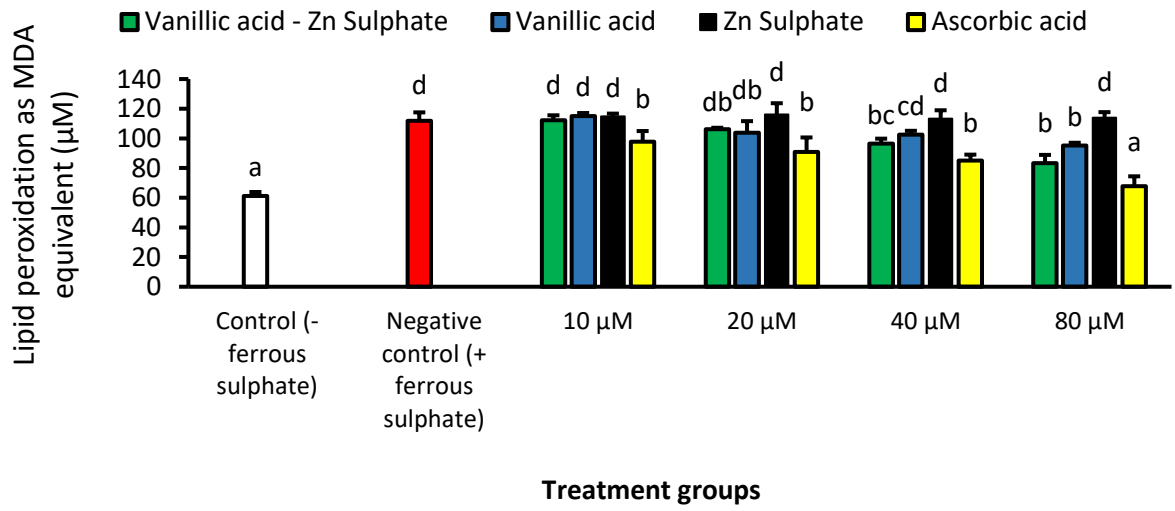
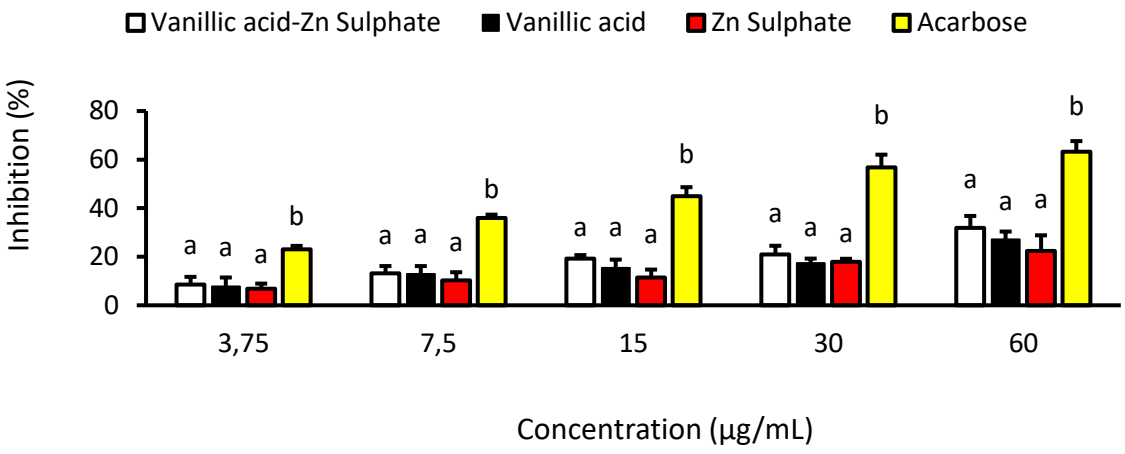


Figure 4.6 Figure showing the hepatic anti-lipid peroxidative effects of Zn(II)-vanillic acid complex and precursors

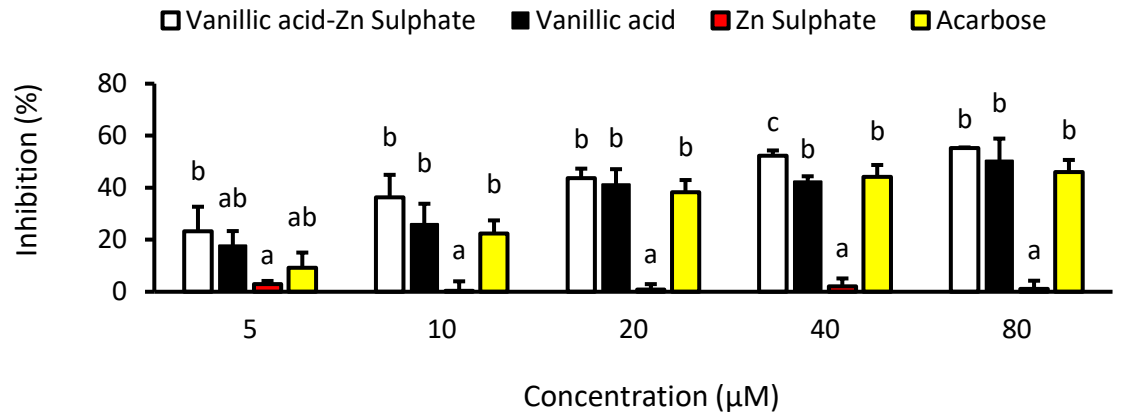
Data is shown as mean ± SD of triplicate analysis. Different letters 'a', 'b', 'c' and 'd' represent significant difference ($p < 0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

4.5 *In vitro* inhibitory effect of Zn(II)-vanillic acid and precursors on α-glucosidase, α-amylase and BSA glycation activities

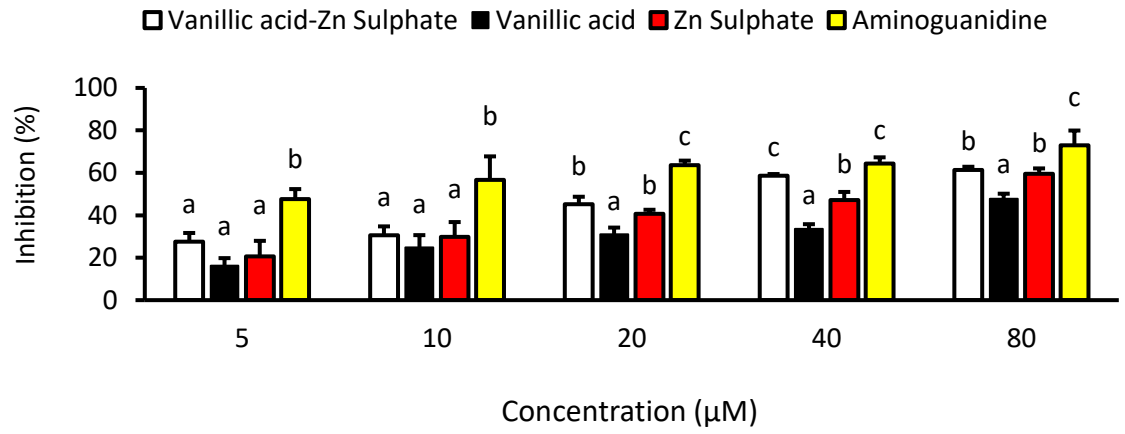
Both vanillic acid and Zn(II)-vanillic acid complex dose-dependently inhibited α-glucosidase (IC_{50} = 147 and 48.3 µM, respectively), α-amylase (IC_{50} = 7.91 and 5.86 µM, respectively) and BSA glycation (IC_{50} = 52.1 and 19.8 µM, respectively) activities, while $ZnSO_4$ only inhibited α-glucosidase (IC_{50} = 46.7 µM) and BSA glycation (IC_{50} = 23.3 µM) activities (**Figure 4.7 and Table 4.1**).



(a)



(b)



(c)

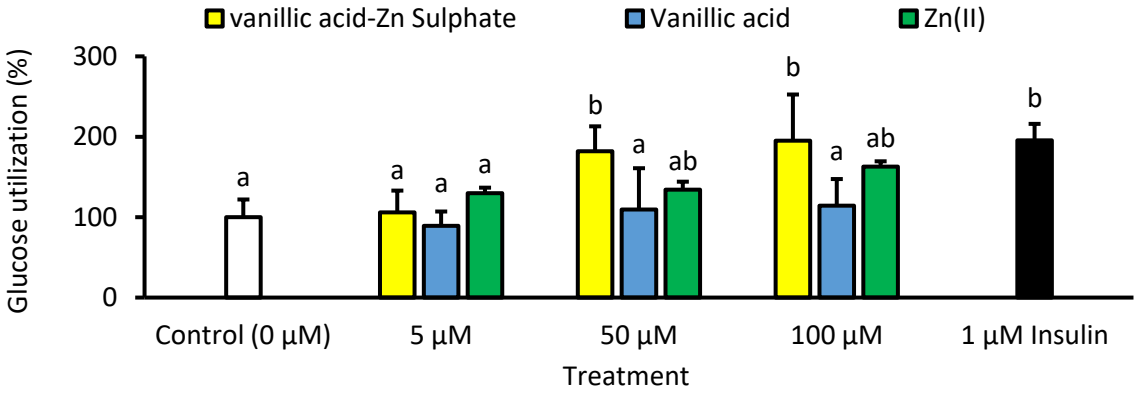
Figure 4.7 Figures showing the (a) α-glucosidase, (b) α-amylase and (c) BSA glycation inhibitory effects of the complex and precursors

Data is shown as mean ± SD of triplicate values. Different letters 'a', 'b', and 'c' represent significant difference ($p<0.05$) between treatment groups at the different tested concentrations.

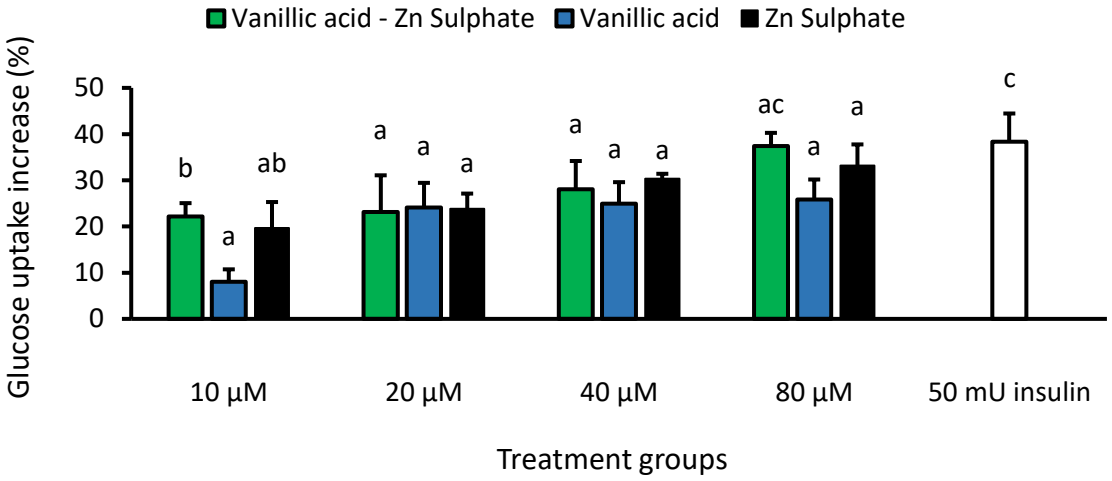
The α-glucosidase inhibitory activity of the complex and $ZnSO_4$ was stronger than that of vanillic acid, but was not as potent as that of acarbose (IC_{50} = 9.08 µM) (**Figure 4.7a and Table 4.1**). Similarly, the anti-glycation activity of the complex and $ZnSO_4$ was significantly ($p < 0.05$) stronger than that of vanillic acid, while the anti-glycation activity of the complex was comparable to that of aminoguanidine (IC_{50} = 6.52 µM) (**Figure 4.7c and Table 4.1**). However, there was no significant difference between the α-amylase inhibitory activity of vanillic acid (IC_{50} = 7.91 µM), the complex (IC_{50} = 5.86 µM) and acarbose (IC_{50} = 6.36 µM) (**Figure 4.7b and Table 4.1**).

4.6 The effect of Zn(II) vanillic acid complex and precursors on glucose uptake in L-6 myotubes and isolated rat psoas muscle tissue

Both Zn(II) and the Zn(II)-vanillic acid complex dose-dependently increased glucose uptake in L-6 myotube (EC_{50} = 20.4 and 71.1 µM, respectively) and isolated rat psoas muscle tissue (EC_{50} = 612 and 872 µM, respectively) (**Figure 4.8 and Table 4.1**). Their glucose uptake activities were more potent than that of vanillic acid (**Table 4.1**). At the highest tested concentrations, the glucose uptake activity of Zn(II)-vanillic acid complex was comparable to that of a 1 µM insulin in L-6 myotube (**Figure 4.8a**) and a 50 mU insulin in isolated rat psoas tissue (**Figure 4.8b**).



(a)



(b)

Figure 4.8 Figures showing the effect of the complex and precursors on glucose uptake in (a) L-6 myotubes and (b) isolated rat psoas muscle

Data is shown as mean ± SD of replicate analysis. Different letters 'a', 'b', and 'c' represent significant difference ($p < 0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

Chapter 5
DISCUSSION AND CONCLUSION

5.1 Discussion

Dietary polyphenols and supplements such essential minerals and vitamins are increasingly becoming popular in disease prevention and management, particularly in functional medicine. Zn(II) has been shown to be involved in insulin secretion and functionality and thus, has been useful in diabetes prevention and management (Chukwuma et al., 2020a). On the other hand, phenolic acids belong to a class of dietary polyphenol with antidiabetic and antioxidative pharmacological credence. In this study, we showed that complexing Zn(II) with vanillic acid, a known bioactive phenolic acid, resulted in a complex with improved antidiabetic and antioxidative profile, which suggests that Zn(II) may be a promising adjuvant for phenolic acids in the management of diabetes and oxidative stress-related disorders. FT-IR (**Figure 4.1**) and NMR (**Figure 4.3**) data analyses suggest that Zn(II) complexed with vanillic acid at the carboxylic functional group through a $Zn(O_6)$ coordination mode, thus the resulting Zn(II)-vanillic acid complex was proposed to have three moieties of vanilic acid as shown in **Figure 4.2b**. This structural property of the complex appears to influence the bioactivity of the complex relative to its precursors as discussed below.

The *in vitro* radical scavenging capacity of vanillic acid (Tai et al., 2012) and its ability to improve antioxidant status, as well as impede oxidative stress and lipid peroxidation in rats have been documented (Stanely et al., 2011; Vinothiya and Ashokkumar, 2017). This has been linked to its ability to form a stable phenoxy radicals by donating hydrogen atom (Shahidi and Wanasundara, 1992), which can scavenge pro-oxidant radicals associated with biological oxidative damage (Pourghassem-Gargari et al., 2011). In this study, vanillic acid demonstrated dose-dependent DPPH (IC_{50} = 216 µM; **Figure 4.5a** and **Table 4.1**) and ABTS (IC_{50} = 22.0 µM; **Figure 4.5b** and **Table 4.1**) radicals scavenging activity and Fe^{3+} reducing capacity (166 mmol/mol AAE at 40 µM; **Figure 4.5c**). However, complexation of vanillic acid with Zn(II) increased its DPPH and ABTS radicals scavenging activity and Fe^{3+} reducing capacity by 2.3 ($p < 0.05$), 1.8 and 1.5 ($p < 0.05$) folds, respectively (**Figure 4a–c** and **Table 4.1**), which suggests that Zn(II) may be a promising adjuvant to improve the antioxidative property of phenolic acids.

In isolated rat liver Zn(II), also, increased the antioxidative property of vanillic acid. The anti-lipid peroxidative activity of Zn(II)-vanillic acid complex (IC_{50} = 667 µM) in rat liver tissue was 9.7 folds more potent ($p < 0.05$) than that of vanillic acid (IC_{50} = 6470 µM) and statistically comparable ($p > 0.05$) to that of ascorbic acid standard (**Figure 4.6** and **Table 4.1**). The increased antioxidant capacity

of the complex relative to vanillic acid may be attributed to the three moieties of vaniilic acid in the complex (**Figure 4.2b**), which collectively potentiated a stronger antioxidant activity.

Zn(II), also, influenced some diabetes-related properties of vanillic acid. For instance, is the inhibitory property of vanillic on carbohydrate digesting enzymes, α-amylase and α-glucosidase. The inhibition of these enzymes is a known mechanism of controlling postprandial hyperglycemia in diabetics (Sanni et al., 2019). Vanillic acid has been shown to inhibit (IC_{50} = 69.4 µM) α-glucosidase *in vitro* (Yin et al., 2014), which was also observed in this study (IC_{50} = 147 µM; **Figure 4.7a** and **Table 4.1**). It was, also, observed that vanillic acid showed a remarkable dose-dependent α-amylase inhibitory activity (IC_{50} = 7.91 µM), which was comparable that of acarbose (IC_{50} = 6.36 µM) (**Figure 4.7b** and **Table 4.1**). Zn(II), on the other hand, showed a moderate α-glucosidase inhibition (IC_{50} = 46.7 µM) relative to acarbose (IC_{50} = 9.08 µM), without any observable amylase inhibition (**Figure 4.7a** and **b**; **Table 4.1**). However, complexing Zn(II) with vanillic acid resulted in a complex with stronger α-glucosidase (IC_{50} = 48.3 µM; $p < 0.05$) and amylase (IC_{50} = 5.86) inhibitory activity relative to that of vanillic acid (**Figure 4.7a** and **b**; **Table 4.1**). These results suggest that while the Zn(II) and vanillic acid components of the complex may synergistically contribute to its more potent α-glucosidase inhibitory activity relative to vanillic acid, the three moieties of vanillic acid in the complex (**Figure 4.2b**) appears to be the major component influencing the stronger amylase inhibitory activity of the complex relative to vanillic acid. Thus, Zn(II) may be a potential adjuvant to increase the postprandial glycemic control profile of phenolic acids. Further *in vivo* studies using diabetic rat models are, however, need to investigate the effects of the complex on digestive enzymes and postprandial glucose.

It was, also, shown that Zn(II) increased the *in vitro* inhibitory activity of vanillic acid on glycation and advanced glycation end product (AGE) formation process - a process that has been implicated in hyperglycemia-induced complications of diabetes due to oxidative damage in organs and tissues (Cooper, 2004). Previous studies have shown that vanillic acid moderately inhibits methylglyoxal-mediated glycation in apoptotic Neuro-2A cells (Huang et al., 2008a; 2008b), suggesting it may impede AGE formation. In the present study, vanillic acid dose dependently inhibited *in vitro* glucose-induced BSA glycation and AGE formation (IC_{50} = 52.1 µM) (**Figure 4.7c** and **Table 4.1**), although not as potent as ($p < 0.05$) that of aminoguanidine standard (IC_{50} = 6.52 µM). The anti-glycation effect of vanillic acid, however, increased ($p < 0.05$) by 2.6 folds and became statistically comparable with that of aminoguanidine standard when vanillic acid was complexed with Zn(II) (**Table 4.1**). The more potent ($p < 0.05$) anti-glycation effect of Zn(II)-vanillic acid complex (IC_{50} = 19.8 µM) compared to vanillic acid may be attributed to the collective effects of the three moieties of vanillic acid in the complex (**Figure 4.2b**), as well as the potent activity (IC_{50} = 23.3 µM) of Zn(II) (**Figure 4.7c** and **Table 4.1**). Moreover, evidences that $ZnSO_4$ exerts *in vitro* inhibitory effect on BSA glycation has been documented (Tupe et al., 2015), while $ZnSO_4$ supplementation has been shown to reduce protein glycation and

protein carbonyl formation in diabetic (Sacan et al., 2016) and protein malnourished (Adebayo et al., 2014) rats, suggesting that Zn(II)-vanillic acid complex may be further studied as an anti-glycation supplement for preventing glycation-induced oxidative complications in diabetes.

In both L-6 myotubes (20.4 μM) and isolated rat psoas muscle tissue (612 μM) the Zn(II)-vanallic acid complex, also, increased glucose uptake (**Figure 4.8a** and **b**; **Table 4.1**), which could be mostly attributed the Zn(II) moiety of the complex. Moreover, the involvement of Zn(II) in insulin secretion and signalling, as well as the insulin mimetic effect of Zn(II) and its complexes have been well documented (Chukwuma et al., 2020a). In isolated rat adipocytes, Zn(II) treatment modulated the activation of insulin receptor kinases and increased lipogenesis, glycolysis and glucose oxidation (Ezaki, 1989; Shisheva et al., 1992). In 3T3-L1 pre-adipocytes and adipocytes, Zn(II) increased glucose transport into cells by modulating phosphorylation-mediated activation of insulin receptor β-subunit and Akt (Tang and Shay, 2001). It is, therefore, not surprising that despite documented evidences on the modulatory effect of vanillic acid on hepatic insulin resistance (Chang et al., 2015), β-cell (INS-1 cells) insulin secretion (Mahendra et al., 2019) and adipocyte (3T3-L1 cells) glucose uptake (Prabhakar and Doble, 2011), its glucose uptake activity in L-6 myotubes and isolated rat psoas muscle tissue was increased significantly ($p < 0.05$) when complexed with Zn(II) (**Figure 4.8a** and **b**; **Table 4.1**). In fact, the glucose uptake activity of the complex at the highest tested concentration was comparable to that of insulin (**Figure 6a and b**). Removal of excess circulating glucose through insulin-mediated signalling of glucose uptake and utilisation in cells of peripheral tissues, including muscle tissue is crucial for maintaining blood glucose homeostasis, suggesting that Zn(II)-vanallic acid complex may be further studied as a promising glycemic control nutraceutical. Also, the complex did not adversely affect the viability of Chang liver cells (**Figure 4.4a**) and the L-6 myotubes (**Figure 4.4b**), suggesting it may not pose hepatotoxicity concerns.

5.2 Conclusion

Data of this study suggest that Zn(II) complexed with vanillic acid through a $Zn(O_6)$ coordination, thus the resulting complex acquired three moieties of vanillic acid. The three moieties of vanillic acid in the complex may have influenced the *in vitro* anti-oxidative, anti-glycation and α-glucosidase and α-amylase inhibitory activities of the complex by collectively potentiating stronger effects. The stronger hepatic anti-lipid peroxidative activity of the complex relative to vanillic acid may, also, be attributed to this structural property of the complex. The data of this study, also, suggest that Zn(II) conferred both cellular and tissue glucose uptake properties on the Zn(II)-vanillic acid complex. Cytotoxicity data, further, showed that the Zn(II)-vanillic acid complex did not reduce the viability of L-6 myotubes and Chang liver cells. Zn(II) may be further studied as potential adjuvant for vanillic acid and other phenolic

acids in developing bioactive antidiabetic and antioxidative nutraceutical for prevention and management of diabetes and oxidative complications.

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APPENDIX SECTION

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS UV

Animal Research Ethics

02-Dec-2019

Dear Chukwuma, Chika Ifeanyi CI

Student Project Number: UFS-AED2019/0152

Project Title: Improving the anti-diabetic efficacy of plant-derived anti-diabetic phenolic acids through zinc II mineral complexation

The above-mentioned protocol has been **CONDITIONALLY APPROVED** by the Ethics Committee with the following remarks:

Phase 1 of the study (ex-vivo studies): APPROVED

(See the two uploaded documents in the section “DOCUMENTS” of the application form.)

Phase 2 of the study (in-vivo studies): CONDITIONAL APPROVAL

Provision: Letter from ARC that they can supply the animals for phase 2 and are able to provide the necessary infrastructure and resources to perform Phase 2 of the study.

Studies on live animals will be performed at the ARC of the UFS

Awaiting section 20 approval.

Modifications requied

The following statement is made throughout the protocol: "This will be done by an authorized veterinarian or personnel at the UFS animal facility". It must be noted that all controlled substances must be handled by a veterinarian. The PI must ensure that duly registered or authorized personnel perform the specific procedures.

Some of the procedures must be performed by a veterinarian only. These procedures must be identified,

and corrected in the protocol to state when the personnel of the ARC are not allowed to perform these procedures.

Please respond via RIMS and do the necessary changes. Submit again.

Contact the Ethics office if you need any help or clarification: Amanda Smith Tel. 051 4439011 / smitham@ufs.ac or RIMS related questions: Maricel van Rooyen Tel. 051 4019451 / vanrooyenm2@ufs.ac.za

Yours sincerely

Mr. Gerhard Johannes van Zyl
Chair: Animal Research Ethics Committee

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA
UFS UV

Animal Research Ethics

16-Nov-2020

Dear Dr Chika Ifeanyi Chukwuma

Student Project Number: UFS-AED2019/0152/2020

Project Title: Improving the anti-diabetic efficacy of plant-derived anti-diabetic phenolic acids through zinc II mineral complexation

Department: Environmental Health Sciences - CUT

You are hereby kindly informed that, at the meeting held on , the Interfaculty Animal Ethics Committee approved the above project.

Kindly take note of the following:

1.
A progress report with regard to the above study has to be submitted Annually and on completion of the project. Reports are submitted by logging in to RIMS and completing the report as described in SOP AEC007: Submission of Protocols, Modifications, Amendments, Reports and Reporting of Adverse Events which is available on the UFS intranet.

2.
Researchers that plan to make use of the Animal Experimentation Unit must ensure to request and receive a quotation from the Head, Mr. Seb Lamprecht.

3.
Fifty (50%) of the quoted amount is payable when you receive the letter of approval.

Yours Sincerely

Mr. Gerhard Johannes van Zyl
Chair: Animal Research Ethics Committee

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Research Paper

Vanillic acid–Zn(II) complex: a novel complex with antihyperglycaemic and anti-oxidative activity

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Abstract

Objectives Our aim was to synthesize, characterize and evaluate the antihyperglycaemic and anti-oxidative properties of a new Zn(II) complex of vanillic acid.

Methods The complex was synthesized using $ZnSO_4.7H_2O$ and vanillic acid as precursors. NMR and FTIR techniques were used to characterize the synthesized complex. The cytotoxicity of the complex was measured. The antihyperglycemic and anti-oxidative properties of the complex were evaluated using in vitro, cell-based and ex vivo models and compared with those of its precursors.

Key findings Zn(II) coordinated with vanillic acid via a $Zn(O_6)$ coordination, with the complex having three moieties of vanillic acid. The radical scavenging, Fe^{3+} reducing and hepatic antilipid peroxidative activity of the complex were, respectively, 2.3-, 1.8- and 9.7-folds more potent than vanillic acid. Complexation increased the α-glucosidase and glycation inhibitory activity of vanillic acid by 3- and 2.6-folds, respectively. Zn(II) conferred potent L-6 myotube (EC50 = 20.4 μm) and muscle tissue (EC50 = 612 μm) glucose uptake effects on vanillic acid. Cytotoxicity evaluation showed that the complex did not reduce the viability of L-6 myotubes and Chang liver cells.

Conclusions The data suggest that Zn(II)–vanillic acid complex had improved bioactivity relative to vanillic acid. Thus, Zn(II) may be further studied as an antihyperglycaemic and anti-oxidative adjuvant for bioactive phenolic acids.

Keywords: zinc mineral; vanillic acid; Zn(II)–vanillic acid complex; antioxidants; diabetes; glucose uptake

Introduction

Diabetes remains a global health problem. Despite the alarming global prevalence of diabetes (463 million people), it has been predicted to increase by 50% by 2045, which will worsen the socioeconomic- and health-related burdens already caused by this disease.[1] Type 2 diabetes (T2D) is the most prevalent type of diabetes, partly because it is largely influenced by the lifestyle and dietary habits of people, as well as several disease-related risk factors, including, obesity.[1] It is characterized by disorders in energy and nutrient metabolism, with insulin resistance and progressive pancreatic β-cell dysfunction being the most predominant disorders.[2] In T2D, cells of peripheral tissues, particularly adipocytes, muscle cells and hepatocytes do not adequately respond to insulin signaling. Thus, insulin does not effectively regulate circulating glucose uptake, nutrient metabolism, hepatic glucose release and general blood glucose homeostasis.[2, 3] These disorders, eventually, contribute to persistent hyperglycemia, progressive pancreatic β-cell dysfunction and impaired insulin secretion.

Persistent uncontrolled high blood glucose contributes to the development of several vascular diabetic complications and oxidative stress has been implicated as a major mediator.[4, 5] Persistent hyperglycaemia in a diabetic state can lead to elevated production of superoxide radical and several other reactive oxygen species, which can oxidatively damage cellular biomolecules and, eventually, tissues and organs.[4, 5] Also, persistent hyperglycemia can lead to glycation processes, production of advance glycation end-products (AGEs) and release of pro-oxidants, which can mediate physiological oxidative damage and progressive vascular complications.[6]

Despite available commercial antidiabetic drugs, supplements, such as minerals and vitamins, are known complementary or alternative agents with growing interests for diabetes management in functional medicine, perhaps due to their perceived minimal safety concern and holistic medicinal profile.[7, 8] Zn(II) mineral, a focus of this study, plays important roles in insulin secretion and functionality, as well as metabolic processes involved in carbohydrate and lipid metabolism.[9, 10] Thus, its deficiency has been associated with poor glycemic control.[11] Zn(II)-stimulated insulin binding to insulin receptors in rat adipocytes and hepatocytes, as well as human lymphocytes and placenta tissue membranes has been previously reported.[12] Also, dietary zinc (50 mg $ZnSO_4.7H_2O$ per kg diet) reduced fasting blood glucose levels, increased islet β-cells regeneration and improved glucose tolerance in alloxan-induced diabetic rats after 8 week's supplementation.[13] Zinc supplementation has, also, been shown to improve glucose handling in individuals with prediabetes.[14] Thus, Zn(II) could be a promising mineral to develop nutraceuticals for preventing and managing diabetes.

On the other hand, plant-derived phenolic acids have been shown to be potent antioxidants with diabetes-related bioactivity.[15] Vanillic acid is a phenolic acid found in herbs (e.g. *Angelica sinensis*, *Ocimum basilicum*, *Origanum vulgare*, *Salvia Rosmarinus* and *Thymus vulgaris*), cereals (e.g. rice and maize), as well as some fruits and vegetables (e.g. *Euterpe oleracea*, *Phoenix dactylifera* and *Olea europaea* fruits) and contributes to the health benefits of some of these plants.[16] It is, also, a metabolite of catechins from green tea, which contributes to the health benefits of green tea.[17] Studies have shown the antidiabetic and antioxidant properties of vanillic acid. It increased glucose uptake activity in 3T3-L1 adipocytes and insulin-resistant FL83B mouse hepatocytes.[18, 19] In both INS-1 cells and isolated rat pancreatic islets, vanillic acid modulated glucose-induced insulin secretion via cAMP-PKA dependent ERK1/2 activation.[20] In high-fat diet fed rats, 16 week oral administration of 30 mg/kg bw reduced blood glucose and triglyceride levels and hyperinsulinaemia and improved insulin resistance, glucose tolerance and insulin signaling.[18] Additionally, 8 week oral administration of vanillic acid (50 mg/kg bw) modulated the antioxidant status of diabetic hypertensive rats.[21]

Studies have shown that the complexation of Zn(II) with several ligands, including synthetic and natural ligands improved their bioactivity and bioavailability.[10] However, a critical analysis of antidiabetic Zn(II) complexes revealed that majority (about 72%) of the Zn(II) complexes that have been synthesized and studied for possible antidiabetic activity are Zn(II) complexes of synthetic organic ligands that have little or no diabetes-related pharmacological history, while plant-derived polyphenols with antidiabetic and anti-oxidative pharmacological credence remain the least studied (5%).[10] In fact, studies on the antidiabetic and anti-oxidative effects of Zn(II) complex of vanillic acid, a known bioactive phenolic acid have not been undertaken. This study takes advantage of the insulin mimetic properties of Zn(II), as well as the diabetes and oxidative stress-related pharmacological properties of vanillic acid, with the aim of synthesizing a novel Zn(II)–vanillic acid complex with improved and broader scope of pharmacological activity.

Methods

Synthesis of Zn(II)–vanillic acid complex

The synthesis of the complex was done using Zn(II) sulphate heptahydrate and vanillic acid as precursors. A previous method[22] was adapted with some modifications. First, 336.3 mg of vanillic acid (Mr = 168.15 g/mol) was dissolved in 3 mL of methanol, while 287.56 mg Zn(II) sulphate heptahydrate (Mr = 287.56 g/mol) was dissolved in 3 ml of distilled water. Next, the vanillic acid and $ZnSO_4.7H_2O$ solutions gradually mixed together. Then, 3 mL of $NaHCO_3$ solution (168.02 mg in 3 mL of distilled water; Mr = 84.01 g/mol) was added to the mixture, while the mixture was stirred. The reaction between Zn(II) and vanillic acid was monitored by CO_2 effervescence. Slowly, a white precipitate was formed, which was the complex. The mixture was, then, filtered to recover the precipitate. Thereafter, a 50% (v/v) methanol solution was used to wash the precipitate. The washing was done trice. After washing, the precipitate was dried and stored in glass vials that are air-tight.

Fourier Transform Infrared analysis of complex

For the infrared spectrum analysis of the complex, a Fourier Transform Infrared (FT-IR) spectrometer having an ART accessory (Perkin Elmer Spectrum 100 FTIR Spectrometer, MA, USA) was used. About 1 mg of the complex or vanillic acid was loaded to cover the equipment's crystal sample holder. The sample was, then, scanned and the infra-red spectra were recorded using a Spectrum Software V 6.3.4. The infra-red spectral scanning was done at a scan rate of 40 s^{-1} between 4000 and 380 cm^{-1}.

Proton NMR (^{1}H NMR) analysis of complex

For the ^{1}H NMR analysis, the 600 MHz Bruker Avance spectrometer (Bruker Corporation, MA, USA) was used. It recorded the ^{1}H NMR of the complex in DMSO-d6 (δH = 2.50). Tetramethylsilane was used as the internal standard. The chemical shifts were recorded

using the delta (δ) scale and presented in parts per million (ppm). The coupling constants (J) were all set correctly to 0.01 Hz.

Cytotoxicity evaluation of complex

The complex was evaluated for any possible cytotoxicity in Chang liver cell lines (ATCC CCL-13, American Type Culture Collection, VA, USA). This was done by measuring the viability of the cells using standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to a method reported previously.[23]

In vitro antioxidant assays

The DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt] radical scavenging, as well as the Fe^{3+} reducing assays were used as in vitro models to measure the antioxidant properties of the test samples of samples. The DPPH radical scavenging and Fe^{3+} reducing antioxidant activity of the samples were measured using the methods reported by Ak and Gülçin [24] according to the modified version reported by Motloung *et al.*[23] The ABTS radical scavenging activity of the samples was measured using a method reported by Re *et al.*[25] according to the modified version reported by Motloung *et al.*[23].

Measurement of *in vitro* glycation inhibition

The effect of the complex and precursors on *in vitro* glucose-induced BSA glycation was measured according to the method reported by Motloung *et al.*[23], which was modified from a previously reported method.[26] Briefly, 50 µL volume of increasing concentrations (5 – 80 µm in final assay volume) of the samples or aminoguanidine standard or their solvents (control) was mixed with equal volumes of glucose (90 mg/mL) and BSA (10 mg/mL) solutions in a black 96-well plates. The plate was, then, incubated for three weeks in the dark at 37°C. After incubation, the fluorescence was measured at excitation and emission wavelengths of 360 and 420 nm, respectively, using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA). The glycation inhibition was computed as follows:

$$Glycation\ inhibition\ (\%) = \frac{Fluorescence\ of\ control - Fluorescence\ of\ test}{Fluorescence\ of\ control} \times 100$$

In vitro carbohydrate digestive enzyme inhibition assay

The inhibitory effect of the complex and other test samples on α-amylase and α-glucosidase activity was measured and the inhibition percentage was evaluated using the methods reported by Ademiluyi and Oboh[27] according to the modified version reported by Motloung *et al.*[23]

Measurement of the effect complex and precursors on glucose uptake in L-6 myotubes

The method used to evaluate the cellular glucose uptake effect of the complex and precursors was adapted from the method described by Motloung *et al.*[23] with some modifications. L-6 myoblast cells from rat muscle (ATCC CRL-1458, American Type Culture Collection, VA, USA) were grown in Dulbecco's Modified Eagle Medium (DMEM) containing low glucose. The medium was supplemented with 10% fetal calf serum (FCS). The cells were sub-cultured by trypsinization. To seed the cells a volume of 200 µL of medium containing 5000 cells was put into each well of a 96-plate well and incubated in a humidified CO_2 incubator set at 5% CO_2, 95% oxygen at 37°C. At 80% confluence, the spent medium was replaced with a differentiation medium (DMEM) containing 2% horse serum and incubation continued for 5 days so that the myoblast can differentiate into myotubes. After differentiation, the L-6 myotubes were incubated in 200 µL of the fresh medium that contained varying concentrations (5, 50 and 100 µm in incubation volume) of samples or their solvent (control). Some wells contained only the glucose medium without cells, which was used as the blank control. After 48 h incubation, the culture medium was removed and the myotubes were washed with PBS. Thereafter, 100 µL of RPMI medium containing 8 mm glucose and 0.1% of BSA was added to the wells and incubation continued for 2 h. An 1 µg/mL insulin was used as a positive control group. After incubation, glucose concentration (µm) was measured in aliquots of incubation medium collected from each well. Glucose concentration was measured using the Glucose (GO) Assay Kit (Sigma Aldrich, South Africa). The viability of the myotubes was, also, measured after treatment with test samples using MTT assay protocol. Two biological repeats of three technical replicates were performed in the glucose uptake assay. The glucose uptake of the test samples and control wells was calculated relative to the blank control as follows:

$$Glucose\ uptake\ (\%) = \frac{\Delta GC\ of\ testor\ control - \Delta GC\ of\ blank\ control}{\Delta GC\ of\ \ blank\ control} \times 100$$

Where 'ΔGC' stands for change in glucose concentration (i.e. initial – final glucose concentration in incubation solutions). The EC50, which is the concentration (µm) of sample needed to increase glucose uptake by 50% relative to the control was, also, computed.

Ex vivo antidiabetic and anti-oxidative evaluation of complex and precursor

Animals

Ten weeks old Sprague Dawley rats were procured from the animal research facility of the University of the Free State, Bloemfontein campus. The animals were fasted for 16 h (overnights), and thereafter, the animals were euthanized using isoflurane and carbon(iv) oxide. Immediately, the psoas muscle and liver were harvested. The psoas muscle was immediately used to evaluate the effect of the complex and precursors on glucose uptake, while the liver was immediately used to evaluate the effect of the complex and precursors on oxidative stress-induced lipid peroxidation. All animal procedures were done in accordance with the rules and regulations of the Animal Research Ethics Committee (AREC) of the University of the Free State (UFS). The protocol was approved by the UFS's AREC on 02 December 2019 under the protocol approval reference: UFS-AED2019/0152.

Measurement of glucose uptake in isolated rat psoas muscle tissue

The method previously reported by Chukwuma and Islam[28] was used in this assay with slight modifications. First, 300 ± 10 mg portions of muscle tissue were pre-incubated in 900 µL of Kreb's buffer (118 mm NaCl, 4.7 mm KCl, 1.2 mm $MgSO_4$, 1.25 mm $CaCl_2$, 1.2 mm KH_2PO_4, 25 mm $NaHCO_3$ and 0.5 mm K_2HPO_4; pH 7.4 ± 2 at 25°C) containing different concentrations (10, 20, 40 and 80 µm in final 1 mL incubation volume) of the test samples or a 50 mU insulin (NovoRapid FlexPen, Novo Nordisk Limited, West Sussex, UK) as the positive control. The incubation was done in a 48-well clear

plate. Blank control wells contained an equivalent volume of Kreb's buffer with tissue. In the control wells, a portion of muscle tissue was incubated in 900 µL of Kreb's buffer only. Pre-incubation was done for 25 min in a CO_2 incubator (NAPCO series 5400 CO_2 incubator, Thermo Scientific, South Africa) set at 5% CO_2, 95% oxygen and 37°C. After pre-incubation, 100 µL of glucose solution (11.1 mm in final 1 mL incubation volume) was added to the wells and incubation continued for an additional 90 min. Thereafter, glucose concentration was measured spectrophotometrically using the glucose oxidase/peroxidase kit [Glucose (GO) assay kit, Sigma Aldrich, South Africa). The glucose uptake increase (%) of tests and positive control was calculated using the absorbance values of the control as a reference according to the formula below:

$$Glucose\ uptake\ increase\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ test}{Absorbance\ of\ control} \times 100$$

The concentration in µm needed to increase glucose uptake by 50% relative to the control (EC50) was, also, computed.

Measurement of oxidative stress-induced lipid peroxidation in isolated liver tissue

The method previously reported by Chukwuma *et al.*[29] was used for this assay with slight modification. First, 200 ± 5 mg portions of liver tissue were pre-incubated in 900 µL of Kreb's buffer only (normal control and negative control) or containing different concentrations (10, 20, 40 and 80 µm in final 1 mL incubation volume) of the test samples or ascorbic acid (positive control) using a 48-well clear plate. Pre-incubation was done for 25 min in a CO_2 incubator set at 5% CO_2, 95% oxygen and 37°C. After pre-incubation, 100 µL of $FeSO_4.7H_20$ (0.7 mm in final 1 mL incubation volume) was added to the negative control, test and positive control wells to induce oxidative stress in the liver tissue samples, while the equivalent volume of the buffer was added to the normal control wells. Incubation continued for an additional 90 min. Thereafter, the tissues were removed from the incubation solution, washed in Kreb's buffer, homogenized in 1 mL of 50 mm sodium phosphate buffer (containing 0.5% v/v Triton X-100; pH, 7.5) and the supernatant was recovered at 2370 g for 10 min (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany). Lipid peroxidation or thiobarbituric acid reactive substances expressed as an equivalent of malondialdehyde (MDA) concentration were measured in the supernatant using methods previously described by Mchunu *et al.*[30] with slight modifications. Briefly, 100 µL of supernatant, 500 µL of 0.25% w/v thiobarbituric acid, 200 µL of 20% v/v acetic acid and 200 µL of distilled water were mixed in vials and the mixture was incubated in a boiling water bath set at 110°C for 50 min. After cooling, 200 µL aliquot was transferred to 96-well plates and absorbance was measured at a wavelength of 532 nm. The MDA concentrations in supernatants were extrapolated from an MDA standard plot (0, 7.5, 15, 22.5, 30, 45 and 60 µm in assay volume) and presented as lipid peroxidation or thiobarbituric acid reactive substances (TBARS).

Percentage inhibition of lipid peroxidation used was computed as follows:

$$Lipid\ peroxidation\ inhibition\ (\%) = \frac{TBARS\ of\ negative\ control - TBARS\ of\ test}{Absorbance\ of\ negative\ control} \times 100$$

The percentage inhibition of lipid peroxidation was used to compute the IC50 (concentration in µm required to cause 50% inhibition) of test samples.

Statistical analysis

Microsoft Excel (2016 version) and GraphPad Prism 5 were used to analyse the data of this study. The analysed data were reported as mean ± standard deviation of replicates. The Window's version 23.0 of IBM SPSS (IBM Corp, Armonk, NY, USA) was used to statistically analyse the data of this study. Mean multiple comparison was done using Tukey's multiple range post hoc tests and the one-way analysis of variance (ANOVA). $P < 0.05$ was the set point for statistical significant difference when comparing the mean values of the different groups.

Results and Discussion

Dietary polyphenols and supplements such as essential minerals and vitamins are increasingly becoming popular in disease prevention and management, particularly in functional medicine. Zn(II) has been shown to be involved in insulin secretion and functionality and thus, has been useful in diabetes prevention and management.[10] On the other hand, phenolic acids belong to a class of dietary polyphenol with antidiabetic and anti-oxidative pharmacological credence. In this study, we showed that complexing Zn(II) with vanillic acid, a known bioactive phenolic acid, resulted in a complex with improved antidiabetic and anti-oxidative profile, which suggests that Zn(II) may be a promising adjuvant for phenolic acids in the management of diabetes and oxidative stress-related disorders.

FTIR and ^{1}H NMR characterization of complex

The synthesized Zn(II)–vanillic acid complex appeared as a white powder, which is slightly soluble in DMSO. FTIR analysis showed some comparable features between the complex and its precursor phenolic acid (vanillic acid). The broad peak around 3500 to 3200 cm^{-1} in the IR spectra of vanillic acid (Figure 1a) and its complex (Figure 1b) is due to intermolecular O–H stretching.[31, 32] The strong sharp peaks at 1276 cm^{-1} (Figure 1a) and 1285 cm^{-1} (Figure 1b) are due to C–O stretching of an alkyl aryl ether,[31] which is indicative of the methoxy group in vanillic acid (Figure 2a) and its complex (Figure 2b). The broad peak shown in the vanillic acid IR spectrum around 3300 – 2400 cm^{-1} (Figure 1a) is due to carboxylic O–H stretching.[31–33] This peak was greatly diminished in the IR spectrum of the complex (Figure 1b), which suggests that the carboxylic acid group of the vanillic acid moiety was involved in Zn(II)–vanillic acid coordination as shown in Figure 2b. Also, the carboxylic C=O stretching (1670 cm^{-1})[31–33] shown in the IR spectrum of vanillic acid (Figure 1a) was absent in that of the complex (Figure 1b), which, further, supports the involvement the vanillic acid's carboxylic acid group in Zn(II)–vanillic acid coordination (Figure 2b).

First, comparing the ^{1}H NMR of the complex with that of the starting material (vanillic acid; Supplementary Data) confirms the formation of a complex. The NMR analysis of the complex is presented in Figure 3. The ^{1}H NMR spectra of the ABX system of ring A were observed as doublet of doublet of H-5A at δ 7.56 and doublet of H-5A at δ 7.24, while H-2A was observed at δ 7.90 (Figure 3a). The ABX system of ring C was observed as doublet of H-5C at δ 7.46 and doublet of H-5C at δ 7.12, while H-2C was observed at δ 7.05 (Figure 3a). The ABX system of ring B in the complex was observed as doublet of H-2B at δ 7.44, doublet of H-6B at δ 7.24 and doublet peak of H-5B at δ 6.7 (Figure 3a). The methoxy protons were observed at ranged between 3.15 and 3.41. Observation of broad peak at δ 9.74 with 3H integral indicated that the three

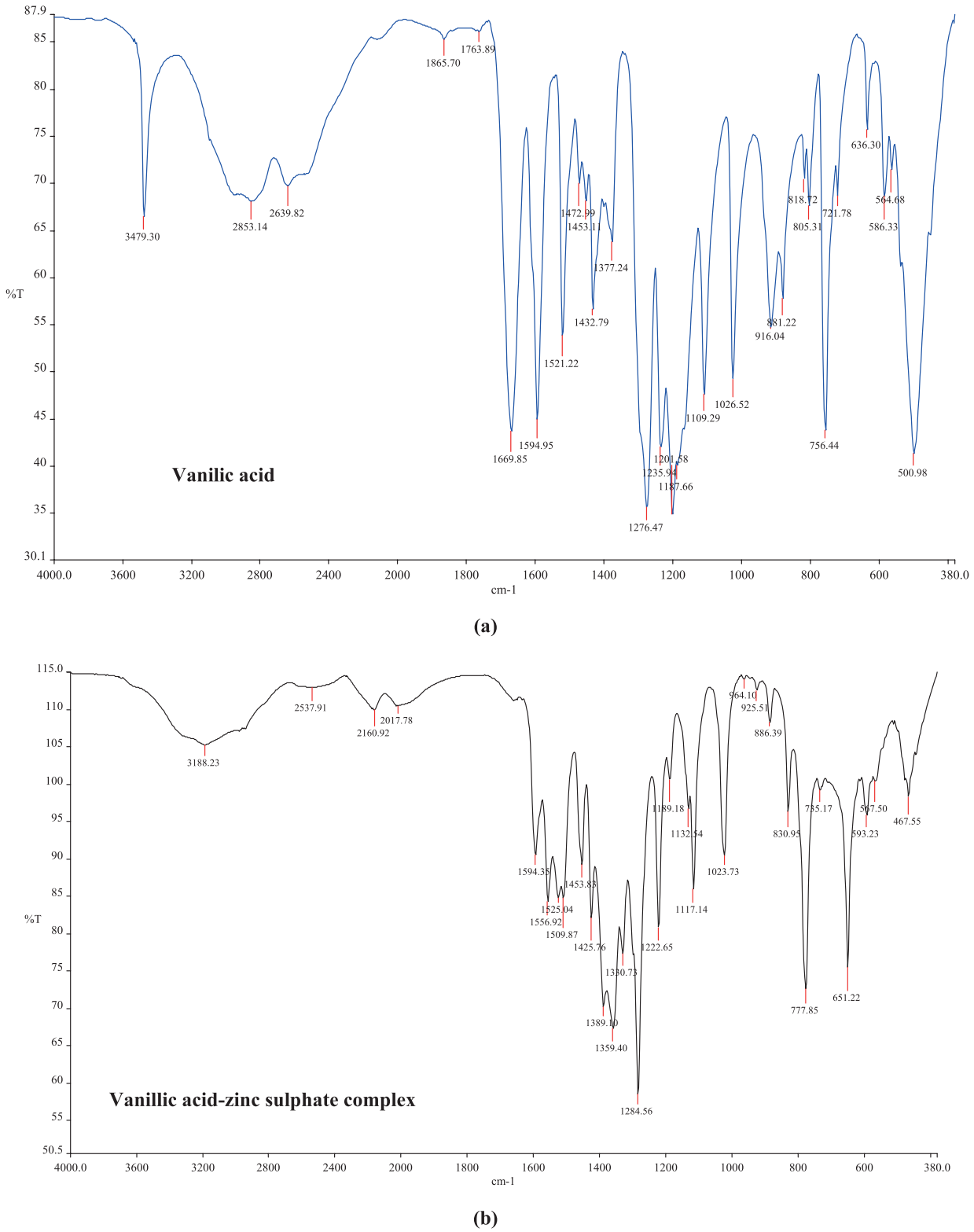


Figure 1 FT-IR spectra of **(a)** vanillic acid and **(b)** Zn(II)–vanillic acid complex.

hydroxyl groups of vanillic acid were not involved in the complex formation. However, the disappearance of carboxylic group indicated its involvement in the complex formation. Cosey experiments showed three ABX system of the three vanillic acid, which correlates with the AB of all three vanillin acid molecules (Figure 3b). Based on the FTIR and ^{1}H NMR data it was proposed that Zn(II) complexed with three moieties vanillic acid through a Zn(O_6) coordination as shown in Figure 2b.

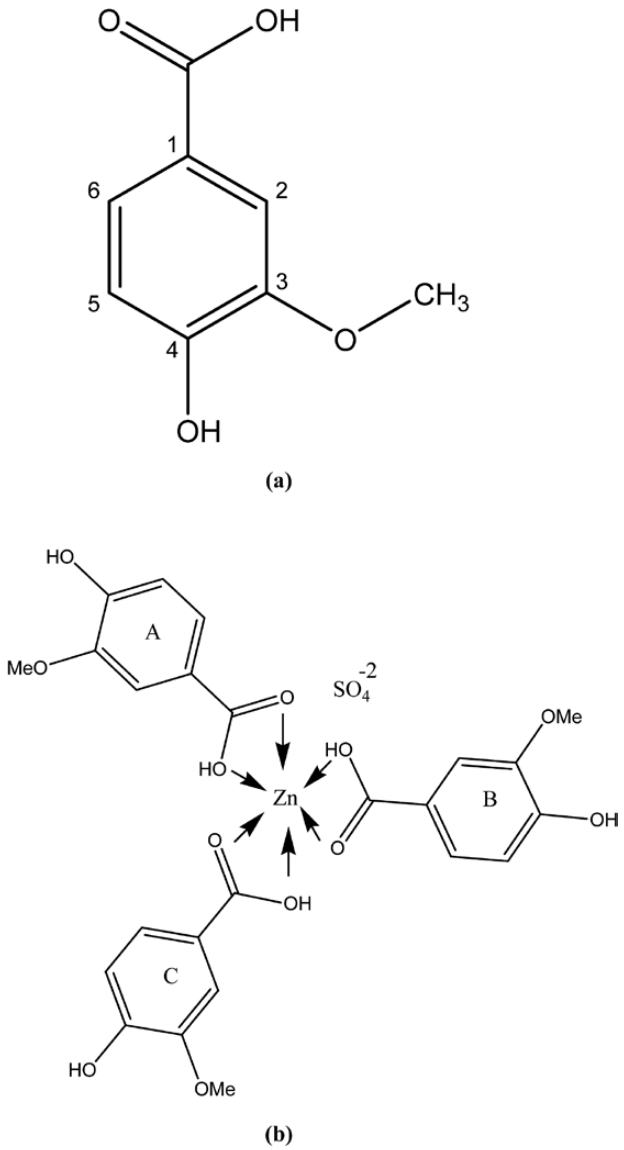


Figure 2 **(a)** Structure of vanillic acid and **(b)** proposed structure of Zn(II)–vanillic acid complex.

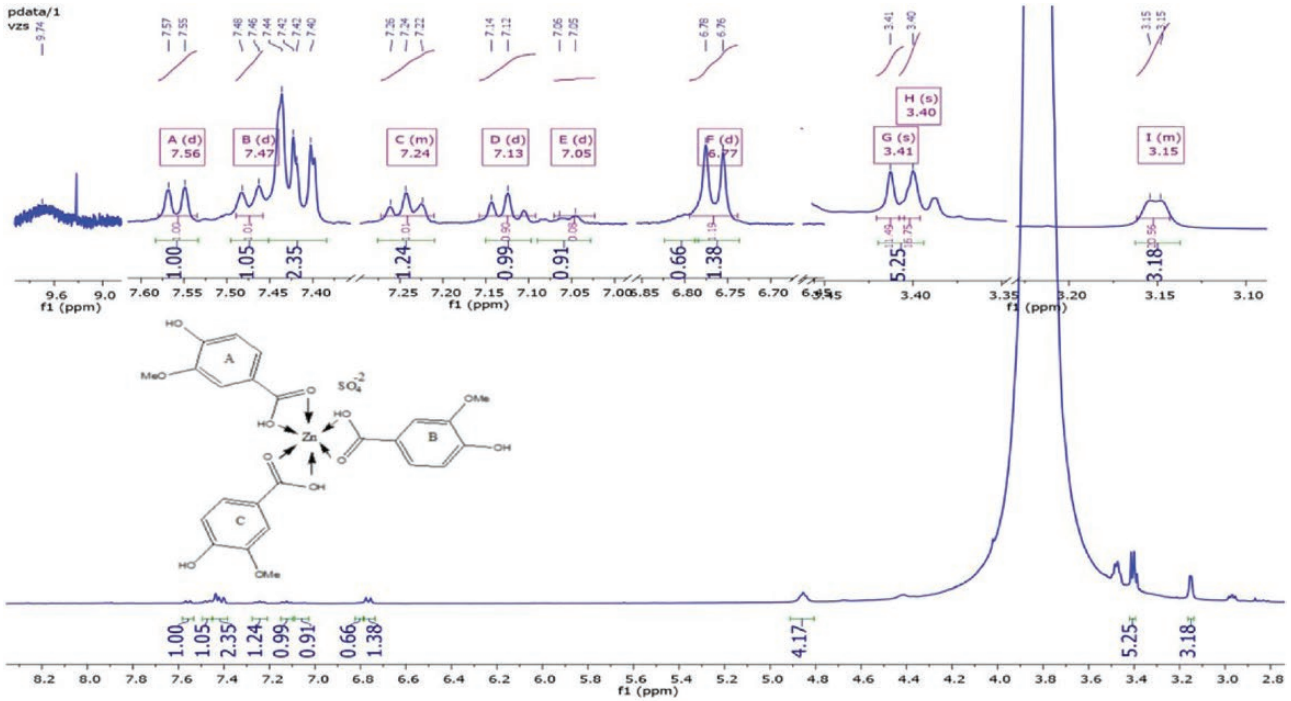
In vitro, cellular and ***ex vivo*** antihyperglycaemic and anti-oxidative properties of the complex and its precursors

The *in vitro* radical scavenging capacity of vanillic acid[34] and its ability to improve antioxidant status, as well as impede oxidative stress and lipid peroxidation in rats have been documented.[21, 35] This has been linked to its ability to form a stable phenoxy radical by donating hydrogen atom,[36] which can scavenge pro-oxidant radicals linked to biological oxidative damage.[5] In this study, vanillic acid demonstrated dose-dependent DPPH (IC50 = 216 µm; Figure 4a and Table 1) and ABTS (IC50 = 22.0 µm; Figure 4b and Table 1) radicals scavenging activity and Fe^{3+} reducing capacity (166 mmol/mol AAE at 40 µm; Figure 4c). However, complexation of vanillic acid with Zn(II) increased its DPPH and ABTS radicals scavenging activity and Fe^{3+} reducing capacity by 2.3 ($P < 0.05$), 1.8 and 1.5 ($P < 0.05$) folds, respectively (Figure 4a–c and Table 1), which suggests that Zn(II) may be a promising adjuvant to improve the anti-oxidative property of phenolic acids.

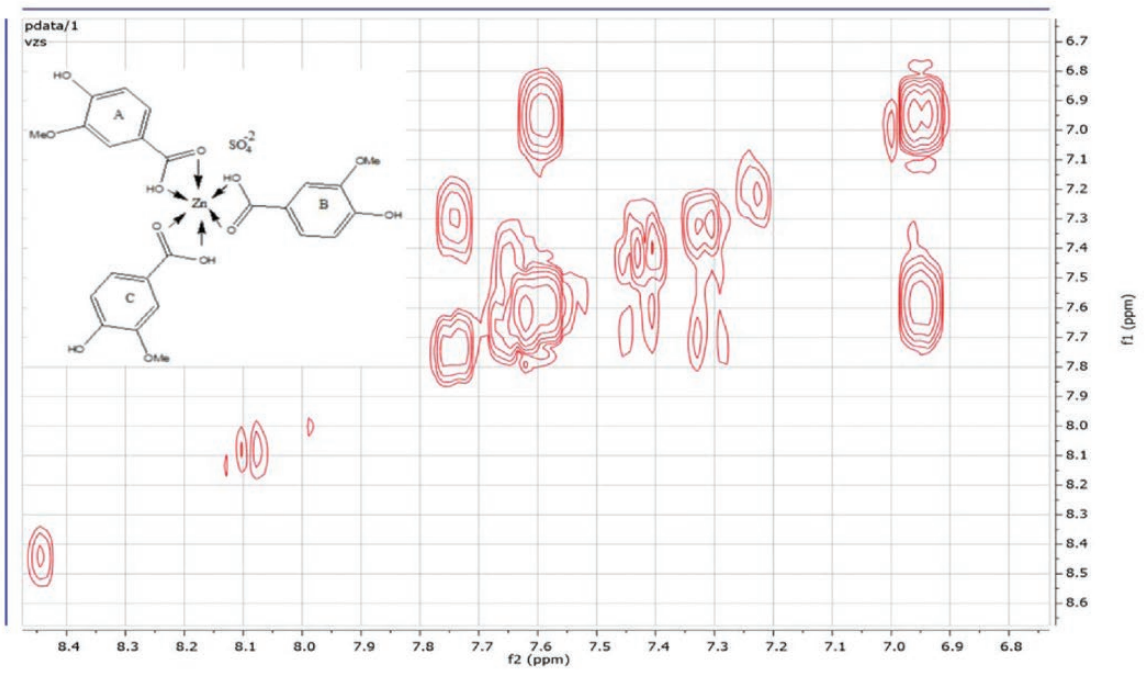
In isolated rat liver Zn(II), also, increased the anti-oxidative property of vanillic acid. The antilipid peroxidative activity of Zn(II)–vanillic acid complex (IC50 = 667 µm) in rat liver tissue was 9.7-folds more potent ($P < 0.05$) than vanillic acid (IC50 = 6470 µm) and statistically comparable ($P > 0.05$) to that of ascorbic acid standard (Figure 4d and Table 1). The increased antioxidant capacity of the complex relative to vanillic acid may be attributed to the three moieties of vaniilic acid acquired in the complex (Figure 2b), which collectively potentiated a stronger activity.

Zn(II), also, influenced some diabetes-related properties of vanillic acid. For instance, is the inhibitory property of vanillic on carbohydrate digesting enzymes, α-amylase and α-glucosidase. The inhibition of these enzymes is a known mechanism of controlling postprandial hyperglycemia in diabetics.[37] Vanillic acid has been shown to inhibit (IC50 = 69.4 µm) α-glucosidase *in vitro*,[38] which was observed in this study (IC50 = 147 µm; Figure 5a and Table 1). It was, also, observed that vanillic acid showed a remarkable dose-dependent α-amylase inhibitory activity (IC50 = 7.91 µm), which was comparable to that of acarbose (IC50 = 6.36 µm) (Figure 5b and Table 1). Zn(II), on the other hand, showed a moderate α-glucosidase inhibition (IC50 = 46.7 µm) relative to acarbose (IC50 = 9.08 µm), without any observable amylase inhibition (Figure 5a and b and Table 1). However, complexing Zn(II) with vanillic acid resulted in a complex with stronger α-glucosidase (IC50 = 48.3 µm; $P < 0.05$) and amylase (IC50 = 5.86) inhibitory activity relative to that of vanillic acid (Figure 5a and b and Table 1). These results suggest that while the Zn(II) and vanillic acid components of the complex may contribute to its more potent α-glucosidase inhibitory activity relative to vanillic acid, the three moieties of vanillic acid in the complex (Figure 2b) appears to be the major component influencing its stronger amylase inhibitory activity of the complex relative to vanillic acid. Thus, Zn(II) may be a potential adjuvant to increase the postprandial glycemic control profile of phenolic acids. Further in vivo studies using diabetic rat models are however need to investigate the effects of the complex on digestive enzymes and postprandial glucose.

It was, also, shown that Zn(II) increased the in vitro inhibitory activity of vanillic acid on glycation and AGE formation process – a process that has been implicated in hyperglycemia-induced complications in diabetics due to oxidative damage in organs and tissues.[6] Previous studies have shown that vanillic acid moderately inhibits methylglyoxal-mediated glycation in apoptotic Neuro-2A cells,[39, 40] suggesting it may impede AGE formation. In this study, vanillic acid dose dependently inhibited *in vitro* glucose-induced BSA glycation and AGE formation (IC50 = 52.1 µm) (Figure 5c and Table 1), although not as potent as ($P < 0.05$) that of aminoguanidine standard (IC50 = 6.52 µm). The antiglycation effect of vanillic acid, however, increased ($P < 0.05$) by 2.6-folds and became statistically comparable with the effect of aminoguanidine standard when vanillic acid was complexed with Zn(II) (Table 1). The more potent ($P < 0.05$) antiglycation effect of Zn(II)–vanillic acid complex (IC50 = 19.8 µm) compared to vanillic acid may be attributed to the collective effects of the three moieties of vanillic acid in the complex (Figure 2b), as well as the potent activity (IC50 = 23.3 µm) of Zn(II) (Figure 5c and Table 1). Moreover, evidences that $ZnSO_4$ exerts *in vitro* inhibitory effect on BSA glycation has been documented,[41] while its supplementation has been shown to reduce protein glycation and protein carbonyl formation in diabetic[42] and protein malnourished[43] rats, which suggest that Zn(II)–vanillic acid complex may be further studied as an antiglycation supplement for preventing glycation-induced oxidative complications in diabetes.



(a)



(b)

Figure 3 **(a)** ^{1}H-NMR spectrum and **(b)** COSY experiments of vanillic acid–zinc sulphate complex. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.56 (d, J = 7.6 Hz, 1H, H-5A), 7.47 (d, J = 7.8 Hz, 1H, H-5C), 7.44 (d, 1H, H-2A), 7.42 (dd, J = 7.4 Hz, 1H, H-6B), 7.24 (d, J = 7.6 Hz, 1H, H-2C), 7.10 (d, J = 7.4 Hz, 1H, H-2A), 7.05 (m, H, H-2A) 6.79 (m, H, H-2C), 6.77 (d, J = 8.1 Hz, 1H, H-5B), 3.41 (s, 3H, –CH3), 3.40 (s, 3H, –CH3), 3.15 (d, J = 3.1 Hz, 3H, –CH3).

In both L-6 myotubes (20.4 μm) and isolated rat psoas muscle tissue (612 μm), the Zn(II)-vanallic acid complex, also, increased glucose uptake (Figure 6a and b and Table 1), which could be mostly attributed to the Zn(II) moiety of the complex. Moreover, the involvement of Zn(II) in insulin secretion and its signaling, as well as the insulin mimetic effect of Zn(II) and its complexes has been well documented.[10] In isolated rat adipocytes, Zn(II) treatment modulated the activation of insulin receptor kinases and

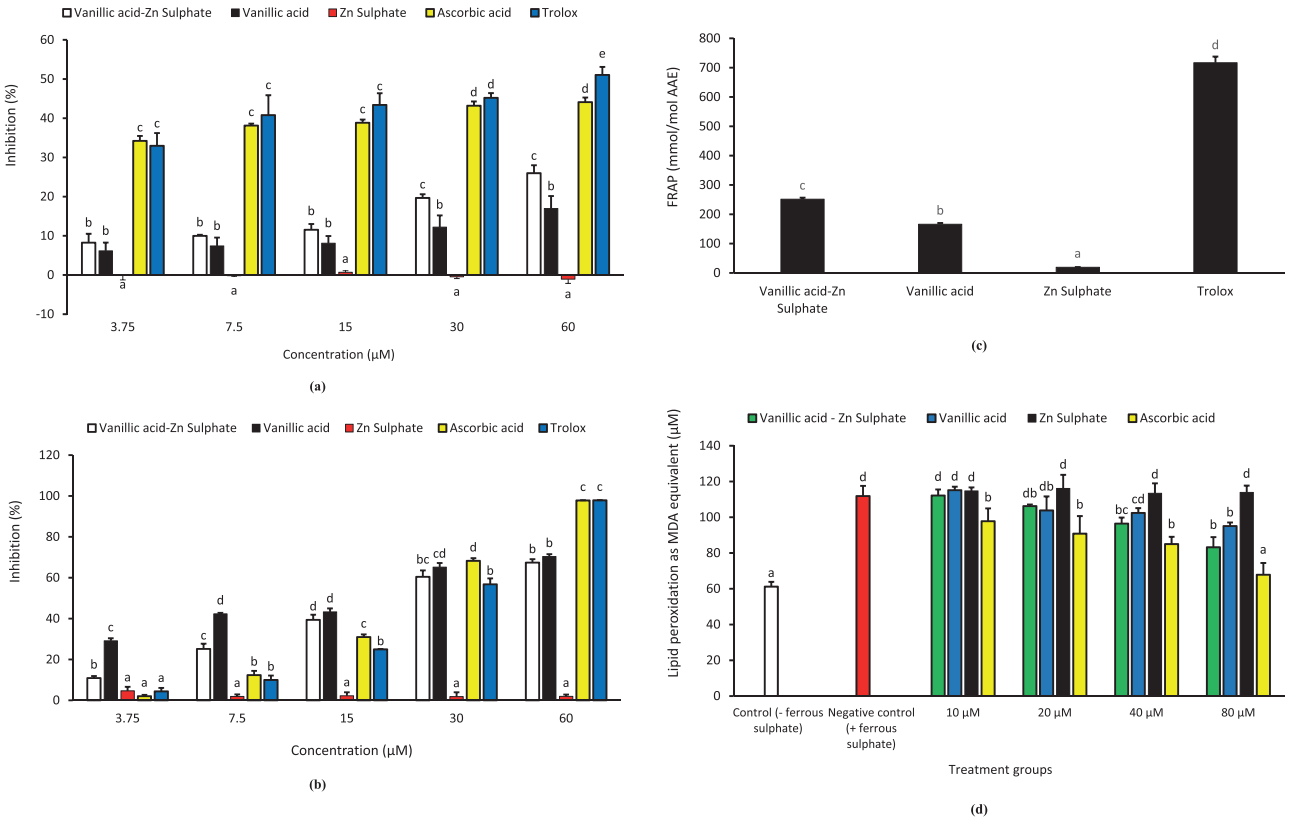


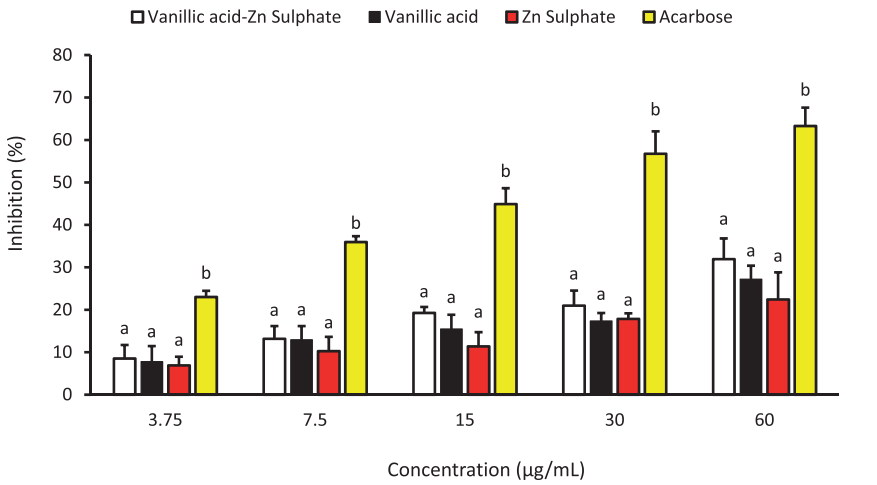
Figure 4 Figures showing the **(a)** DPPH radical scavenging, **(b)** ABTS radical scavenging, **(c)** Fe^{3+} ion reducing and **(d)** hepatic antilipid peroxidative effects of the complex and precursor. Data are shown as mean ± SD of triplicate analysis. Different letters 'a', 'b', 'c', 'd' and 'e' represent significant difference ($P < 0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

Table 1 IC50 and EC50 values of vanillic acid, its complex with Zn(II) and other studied agents

Parameters or activity	Zn(II) complex	Vanillic acid	Zn(II)	Ascorbic acid	Trolox	Acarbose	Aminoguanidine	Insulin
	IC50 or EC50 values (μm)							
ABTS radical scavenging activity (IC50)	12.2 ± 3.16[a]	22.0 ± 8.54[a]	ND	47.1 ± 4.47[a]	153 ± 32.8[b]	NA	NA	NA
DPPH radical scavenging activity (IC50)	95.9 ± 9.97[b]	216 ± 23.7[a]	ND	9.98 ± 2.04[c]	6.20 ± 3.18[c]	NA	NA	NA
Antiglycation activity (IC50)	19.8 ± 7.38[bc]	52.1 ± 4.54[a]	23.3 ± 6.51[b]	NA	NA	NA	6.52 ± 0.93[c]	NA
α-Amylase inhibition (IC50)	5.86 ± 1.08	7.91 ± 2.40	ND	NA	NA	6.36 ± 1.08	NA	NA
α-Glucosidase inhibition (IC50)	48.3	147	46.7	NA	NA	9.08	NA	NA
Glucose uptake increase in L-6 myotubes (EC50)	20.4	6460	71.1	NA	NA	NA	NA	ND
Glucose uptake increase in isolated rat psoas muscle (EC50)	612 ± 125[b]	1190 ± 169[a]	872 ± 138[ab]	NA	NA	NA	NA	ND
Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver (IC50)	667 ± 184[b]	6470 ± 596[a]	ND	270 ± 93.2[b]	NA	NA	NA	NA

'ND' means 'not determined'; 'NA' means 'not applicable'; IC50 is the concentration needed to inhibit the activity of carbohydrate digesting enzymes and bovine serum albumin glycation or scavenge DPPH and ABTS radicals by 50%; EC50 is the effective concentration needed to increase glucose uptake in L-6 myotubes and isolated rat psoas muscle by 50%. Different letters 'a', 'b', 'c' and 'd' represent significant difference ($P < 0.05$) between treatment groups.

increased lipogenesis, glycolysis and glucose oxidation.[44, 45] In 3T3-L1 preadipocytes and adipocytes, Zn(II) increased glucose transport into cells by modulating phosphorylation-mediated activation of insulin receptor β-subunit and Akt or protein kinase B.[46] It is, therefore, not surprising that despite documented evidences on the modulatory effect of vanillic acid on hepatic insulin resistance,[18] β-cell (INS-1 cells) insulin secretion[20] and adipocyte (3T3-L1 cells) glucose uptake,[19] its glucose uptake activity in L-6 myotubes and isolated rat psoas muscle tissue was increased significantly ($P < 0.05$) when complexed with Zn(II) (Figure 6a and



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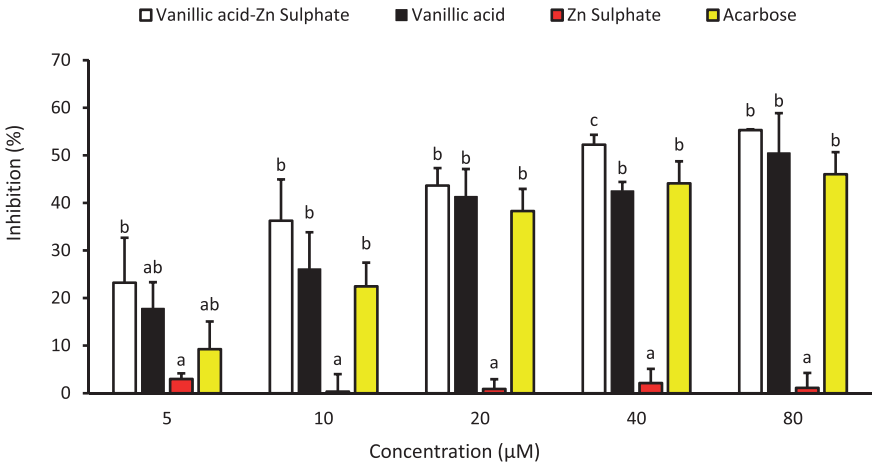

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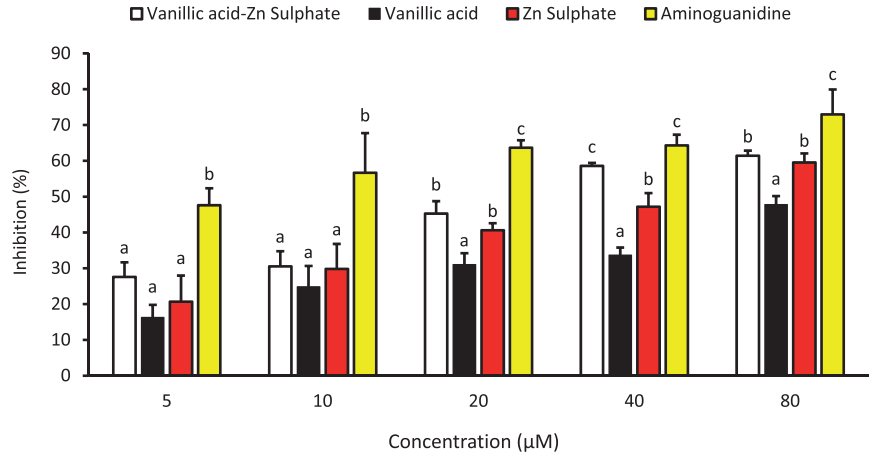

(c)

Figure 5 Figures showing the **(a)** α-glucosidase, **(b)** α-amylase and **(c)** BSA glycation inhibitory effects of the complex and precursors. Data are shown as mean ± SD of triplicate values. Different letters 'a', 'b' and 'c' represent significant difference ($P < 0.05$) between treatment groups at the different tested concentrations.

b and Table 1). In fact, the glucose uptake activity of the complex at the highest tested concentration was comparable to that of insulin (Figure 6a and b). Removal of excess circulating glucose through insulin-mediated signaling of glucose uptake and utilization in cells of peripheral tissues, including muscle tissue is crucial for maintaining blood glucose homeostasis, suggesting that

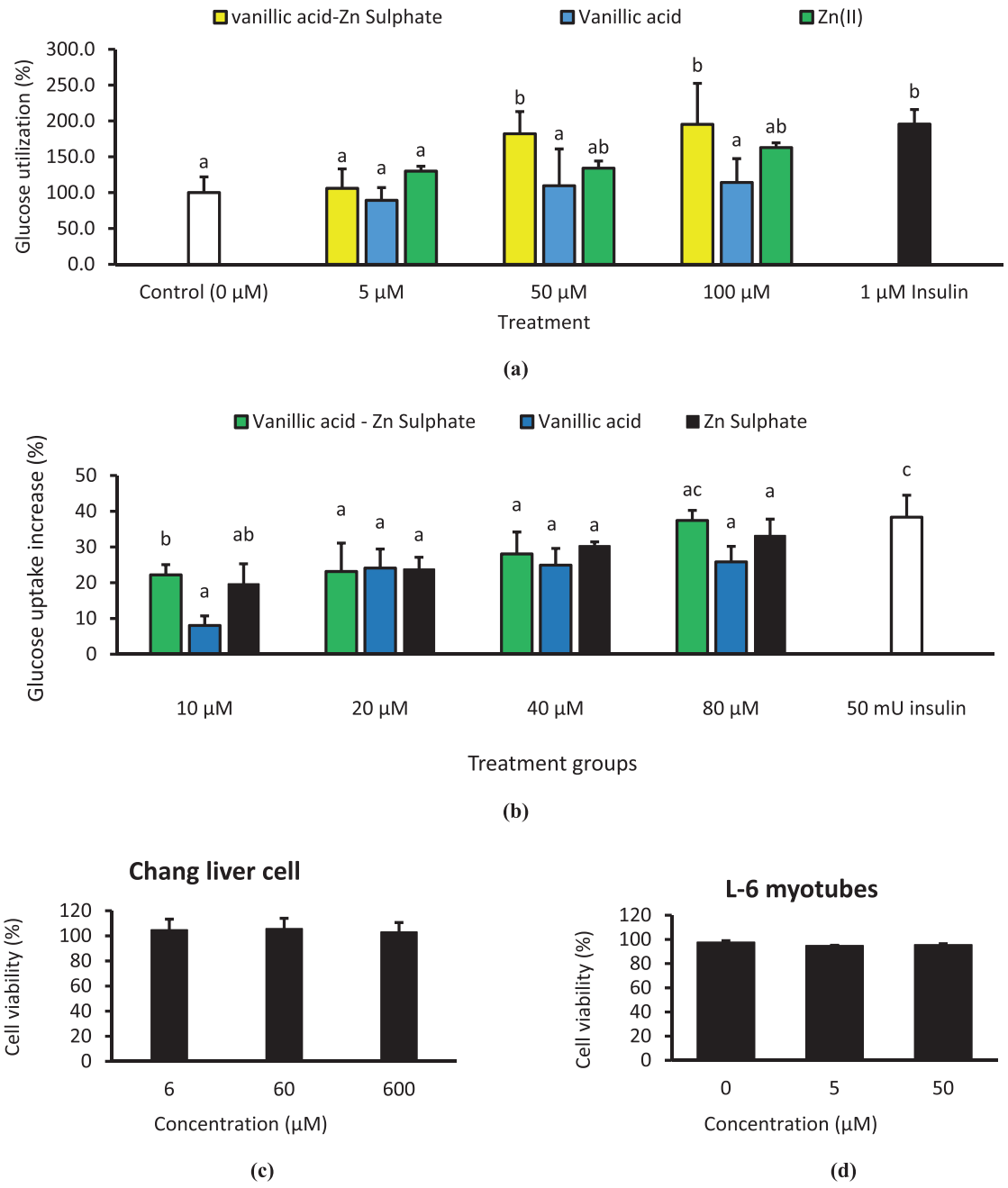

Figure 6 Figures showing the effect of the complex and precursors on **(a)** glucose uptake in L-6 myotubes, **(b)** glucose uptake in isolated rat psoas muscle, **(c)** Chang liver cell viability and **(d)** L-6 myotubes viability. Data are shown as mean ± SD of replicate analysis. Different letters 'a', 'b' and 'c' represent significant difference ($P < 0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

Zn(II)-vanallic acid complex may be used further studied as a promising glycemic control nutraceutical. Also, the complex did not adversely affect the viability of Chang liver cells and the L-6 myotubes (Figure 6c and d), suggesting it may not pose hepatotoxicity concerns.

Conclusion

Data of this study suggest that Zn(II) complexed with vanillic acid through a $Zn(O_6)$ coordination, thus the resulting complex acquired three moieties of vanillic acid. The three moieties of vanillic acid in the complex may have influenced the in vitro anti-oxidative, antiglycation and α-glucosidase and α-amylase inhibitory activity of the complex by collectively potentiating stronger effects. The stronger hepatic antilipid peroxidative activity of the complex relative to vanillic acid may, also, be attributed to this structural property of the complex. The data of this study, also, suggest that Zn(II) conferred both cellular and tissue glucose uptake on the Zn(II)–vanillic acid complex. Cytotoxicity data, further, showed that the Zn(II)–vanillic acid complex did not reduce the viability of L-6 myotubes and Chang liver cells. Zn(II) may be further studied as a potential adjuvant for vanillic acid in developing bioactive antidiabetic and anti-oxidative nutraceutical for the prevention and management of diabetes and oxidative complications.

Supplementary Material

Supplementary data are available at *Journal of Pharmacy and Pharmacology* online.

Author's Contribution

Chika I. Chukwuma conceptualized and designed the study. Ifedolapo M. Oke and Limpho M. Ramorobi carried out the synthesis and in vitro antidiabetic and anti-oxdative evaluation of the complex under the main supervision and co-supervision of Chika I. Chukwuma and Samson S. Mashele, respectively. Ifedolapo M. Oke prepared the initial draft of manuscript, while Chika I. Chukwuma and Samson S. Mashele made revision on the final version. Chika I. Chukwuma and Samson S. Mashele did the FITR, glucose uptake and cytotoxicity study and the data interpretation. Tshepiso J. Makhafola was partly involved in cytotoxicity and glucose uptake study, as well as editing the manuscript. Susanna L. Bonnet and Anwar E.M. Noreljaleel did the NMR study and the data interpretation, as well as revising the initial draft of manuscript. Kenneth C. Eze was partly involved in the FITR and glucose uptake study and revising initial draft of manuscript. All authors proof-read the final version of manuscript and made necessary inputs.

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Conflict of Interest

None declared.

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