# COMPLEXATION OF SYRINGIC AND P-COUMARIC ACIDS WITH ZINC(II) TO IMPROVE THEIR ANTIDIABETIC EFFICACY

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## DECLARATION OF INDEPENDENT WORK

I Limpho Martha Ramorobi, student number _____, hereby declare that this research project submitted to the Central University of Technology, Free State for the degree DOCTOR OF HEALTH SCIENCES IN BIOMEDICAL TECHNOLOGY is my independent work; and complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification. As such, where external sources were utilized, due acknowledgment was given by means of a comprehensive list of references in accordance with departmental requirements. I therefore give copyright of this dissertation in favour of the Central University of Technology.

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# DEDICATION

Thesis dedicated to my mother, Maleoatle Ramorobi, who have supported me throughout my education. Thank you for your prayers, words of encouragement and love.

# ABSTRACT

**Background:** The concept of Zn(II) complexation has long been explored as a possible scientific approach to developing potential therapeutic agents for managing impaired glucose tolerance in diabetes. This has been partly due to the safety profile of zinc mineral and its functional role in insulin preservation, secretion and function. Many types of ligands have been explored as suitable ligands to develop potent antidiabetic Zn(II) complexes. However, due to the critical role of oxidative stress in the development and progression of diabetes and vascular complications, the use of polyphenols as alternative ligands for antidiabetic Zn(II) complexes has been explored by scientists. It is perceived that the antioxidant attributes of natural polyphenols may afford a Zn(II) complex an antioxidant perspective in combating diabetic oxidative stress and reducing the risk of diabetic complications. Data from some studies that have complexed zinc with dietary flavonoids support this perception to an appreciable extent. However, natural phenolic acids, which are known dietary antioxidant have been scarcely explored as antioxidant ligands for developing antidiabetic Zn(II) complexes. Therefore, in the present study, p-coumaric acid and syringic acid were used as alternative ligands to develop Zn(II) complexes, due to their promising antioxidant attributes and some other diabetes-related pharmacological attributes.

**Methodology:** p-Coumaric acid and syringic acid were separately complexed with $ZnSO_4$ in a 1:2 mole ratio, respectively. The complexes were characterized by spectroscopically using FT-IR, proton NMR and high-resolution mass spectroscopy. The cytotoxic effect of the complexes was evaluated in Chang liver cells and L-6 myotubes. The antioxidant and antihyperglycaemic potential of the complex and precursors were evaluated with different experimental models. Molecular docking with target proteins linked to diabetes was performed. The antioxidant and antihyperglycaemic potential of the complexes, relative to their respective precursors were determined using *in vitro*, cellular and *ex vivo* experimental models. The complexes and their respective phenolic acids were comparatively docked against protein targets linked to diabetes. The Zn(II) complex of syringic acid was subjected to *in vivo* antidiabetic and antioxidant study in diabetic rats. Diabetes was induced in SD rats using 10% fructose and 40 mg/kg bw streptozotocin and thereafter subjected to a 4-week treatment with the complex and its precursors (Syringic acid

and zinc sulphate) for 4 weeks at predetermined doses. Then, the effect of the treatments on diabetes and oxidative stress related parameters was measured.

**Results:** Spectroscopic analysis showed the complexes were formed as a double hydrate Zn(II)-biphenolate product [Zn(II)-bicoumarate.2$H_2O$ and Zn(II)-bisyringate.2$H_2O$ complexes], which each had a moiety of Zn(II) and 2 moieties of their respective phenolic acids. The *in vitro* radical scavenging, α-glucosidase inhibitory, antiglycation and anti-lipid peroxidative activities of the complexes were several folds stronger than their respective phenolic acids. In Chang liver cells and rat liver tissues, the complexes inhibited lipid peroxidation and GSH depletion, which was notably stronger than their respective phenolic acids and comparable to ascorbic acid. Zn(II) and the phenolic acids synergistically modulated glucose uptake in L-6 myotubes and rat muscle tissue, which may be majorly influenced by to the observed complexation-mediated increase in muscle zinc uptake. Tissue glucose uptake activity of the complexes was accompanied by increased muscle hexokinase activity, suggesting increased glucose utilization. Moreover, treatment increased muscle phospho-Akt/pan-Akt ratio, while the complexes had stronger molecular docking interaction with insulin signalling protein targets (GLUT-4, Akt/PKB, insulin receptor tyrosine kinase and IRS-1) than their respective phenolic acid precursors. The Zn(II)-bisyringate.2$H_2O$ complex was more potent than the Zn(II)-bicoumarate.2$H_2O$, thus was subjected to *in vivo* antidiabetic and antioxidant study in diabetic rats. The complex improved diabetic polyphagia, polydipsia and weight loss. Complexing Zn(II) with syringic acid improved their anti-hyperglycaemic action by ~28 – 36% and ~37 - 40%, respectively, suggesting a complexation-mediated synergism. This may be attributed to the observed modulatory action of the complex on insulin secretion and sensitivity, tissue glycogen production, muscle hexokinase activity and Akt phosphorylation, thus improving glucose tolerance in diabetic rats, relative to its precursors. Concomitantly, the complex reduced systemic and tissue lipid peroxidation and increased antioxidant enzymes activity in the diabetic rats, while outperforming its precursors. In some cases, the antidiabetic action of the complex was comparable to metformin. The molecular properties of the complexes (i.e., the Zn(II) and biphenolate moieties) appears to be influential in the improved bioactivities of the complexes relative to their respective precursors, suggesting a complexation-mediated synergistic potential.

**Conclusion:** Complexing Zn(II) with these phenolic acids (p-coumaric and syringic acid) may be an underexplored therapeutic approach to improving the effectiveness of therapies for diabetes and oxidative stress management.

***Keywords:*** *Diabetes, Oxidative stress, Zn(II), Syringic acid, Coumaric acid, Complexation, Bioactive synergism*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $^{1}$H NMR | Proton nuclear magnetic resonance |
| AGEs | Advanced glycation end |
| AKT | Serine/threonine kinase (also known as protein kinase B) |
| cAMP | Cyclic adenosine monophosphate |
| DN | Diabetic nephropathy |
| DPP-4 | Dipeptidyl peptidase-4 |
| DPPH | 2,2-diphenyl-1-picrylhydrazy |
| DR | Diabetic retinopathy |
| FFAs | Free fatty acids |
| FT-IR | Fourier transform infrared |
| GDM | Gestational diabetes |
| GLP-1 | Glucagon-like peptide-1 |
| GLUT 2 | Glucose transporter 2 |
| GLUT 4 | Glucose transporter 4 |
| GSH | Reduced glutathione |
| HbA1c | Haemoglobin A1c |
| IGT | Impaired glucose tolerance |
| INRS | Insulin receptor |
| IRS-1 | Insulin receptor substrate 1 |
| MDA | Maldondialdehyde |
| PI3K | Phosphoinositide-3-kinase |
| PKB | Protein kinase B |
| PPARγ | Peroxisome proliferator-activated receptor γ |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SGLT2 | Sodium dependent glucose co-transporter 2 |
| SOD | Superoxide dismutase |

| | |
|---|---|
| TBARS | Thiobarbituric acid reactive substances |
| T1D | Type 1 diabetes |
| T2D | Type 2 diabetes |
| TZDs | Thiazolidinediones |
| WHO | World Health Organization |
| ZnT8 | Zinc transport family member 8 |

# RESEARCH OUTPUTS

**Published articles or submitted manuscript from this thesis:**

1. **Ramorobi LM**, Matowane GR, Mashele SS, Swain SS, Makhafola TJ, Mfengwana PH, Chukwuma CI (2022). Zinc(II) - Syringic acid complexation synergistically exerts antioxidant action and modulates glucose uptake and utilization in L-6 myotubes and rat muscle tissue. Biomedicine & Pharmacotherapy. doi: 10.1016/j.biopha.2022.113600.
2. **Ramorobi LM**, Matowane GR, Mashele SS, Bonnet SL, Noreljaleel EAEM, Swain SS, Makhafola TJ, Chukwuma CI (2022). Bioactive Synergism Between Zinc Mineral and p-Coumaric Acid: A multi-mode Glycaemic Control and Antioxidative Study. Journal of Food Biochemistry. doi: 10.1111/jfbc.14360.
3. **Ramorobi LM**, Matowane GR, Mashele SS, Erukainure OL, Makhafola TJ, Chukwuma CI. Therapeutic Antidiabetic and Antioxidative Synergism of Zn(II)-syringic acid complexation. Revista Brasileira de Farmacognosia (**Manuscript ID: RBFA-D-22-00747R1**). Accepted for publication on **16 January 2023**.

**Other published articles during doctoral program:**

1. Matowane GR, **Ramorobi LM**, Mashele SS, Bonnet SL, Noreljaleel EAEM, Swain SS, Makhafola TJ, Chukwuma CI (2022). Complexation Potentiated Promising Anti-diabetic and Anti-oxidative Synergism Between ZN(II) and Ferulic Acid: A Multimode Study. Diabetic Medicine. https://doi.org/10.1111/dme.14905.
2. Matowane GR, **Ramorobi LM**, Mashele SS, Bonnet SL, Noreljaleel EAEM, Makhafola TJ, Chukwuma CI (2022). Novel Caffeic Acid – Zinc Acetate Complex: Studies on Promising Antidiabetic and Antioxidative Synergism Through Complexation. Medicinal Chemistry. doi: 10.2174/1573406418666220620144601.
3. 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**.**

4. Chukwuma CI, Mashele SS, Eze KC, Matowane GR, Islam SMD, Bonnet SL, Noreljaleel AEM, **Ramorobi LM** (2020). A comprehensive review on zinc(II) complexes as antidiabetic agents: The advances, scientific gaps and prospects. Pharmacological Research, 155, available online 7 March 2020.

**Conference presentations:**

1. **Ramorobi LM**, Matowane GR, Mashele SS, Bonnet SL, Makhafola TJ, Chukwuma CI (2022). ANTIDIABETIC AND ANTIOXIDATIVE SYNERGISTIC POTENTIAL BETWEEN ZN(II) AND CAFFEIC ACID: IMPROVING THERAPEUTIC POTENTIAL THROUGH COMPLEXATION. Virtual presentation at the **3rd Molecules Medicinal Chemistry Symposium (MMCS): Shaping Medicinal Chemistry for the New Decade**, held at Sapienza University of Rome, Rome, Italy during 27 – 29 July 2022.

# CHAPTER 1

## INTRODUCTION

Diabetes mellitus is a metabolic disorder linked to disturbances of carbohydrate, fat and protein metabolism due to loss of insulin secretion or insulin action or both. The metabolic defects lead to elevated blood glucose. High blood glucose levels cause vascular damage that can affect the heart, eyes, kidneys and nerves resulting in various complications. Other characteristics of diabetes include polyuria, polydipsia and polyphagia (Mukhtar *et al*., 2020; Cho *et al*., 2018; Wu *et al*., 2016; Wu *et al*., 2014). Recently, the International Diabetes Federation (IDF) reported that in 2021, approximately 537 million people globally have diabetes, and this figure is expected to increase to 783 million by the year 2045. Also, about 6.7 million deaths caused by diabetes in adults aged 20-79 years are estimated (IDF, 2021). These estimates of diabetes prevalence and deaths pose socioeconomic burden across the world (Cho *et al*., 2018).

There are three main types of diabetes recognised by the World Health Organization (WHO); gestational diabetes mellitus (GDM), type 1 diabetes mellitus (T1D) and type 2 diabetes mellitus (T2D) (Nagaraj *et al*., 2021; Ramachandran *et al*., 2015). Type 1 and type 2 diabetes are the two major types (Dey *et al*., 2002). Type 1 diabetes being the major type of diabetes in childhood and type 2 diabetes the most prevalent, accounting for more than 90% of diabetes cases worldwide (IDF, 2021; *Wu et al*., 2014).

Gestational diabetes mellitus (GDM) is caused by glucose intolerance during pregnancy. It is characterised by insufficient levels of insulin caused by pancreatic β-cell dysfunction (Baz, *et al*., 2016). Type 1 diabetes, also known as insulin-dependent diabetes mellitus, is an autoimmune disorder that develops when the body's immune system destroys pancreatic β-cell, the body's insulin-producing cells (Remedi & Emfinger, 2016). Thus, type 1 diabetes patients do not adequately secrete insulin and require exogenous insulin to regulate blood glucose level (Dey *et al*., 2002; Sakurai, 2002).

Type 2 diabetes, which is also known as non-insulin dependent diabetes mellitus, results from impaired insulin action and it is characterised by derangements in lipid and glucose metabolism, as well as insulin resistance and/or impaired insulin secretion (Tripathi & Srivastava, 2006). Insulin resistance leads to β-cell dysfunction, impaired tissue glucose uptake and energy metabolism, which leads to persistent hyperglycaemia. (Fu *et al*., 2013; Kahn, 2003). The risk factors of type 2 diabetes include unhealthy dieting, obesity, lack of physical activity, aging, and stress (Kaku, 2010). Obesity has been shown to be one of the leading pathogenic factors linked to insulin resistance (Fu *et al*., 2013). Oxidative stress, also, plays a major role in the development and advancement of diabetes and its complications. It occurs due to an imbalance between free radical formation and the action of an organism's natural antioxidant mechanism. (Singh & Venkatesan, 2016). Free radicals and other reactive oxygen species (ROS) can oxidatively damage biological molecules, cells, tissues and organs, which can lead to diabetes complications (Bazinet & Doyen, 2017).

Therapeutic approach for diabetes management includes the use of synthetic commercial antidiabetic drugs and lifestyle and dietary adjustments (Blaslov *et al*., 2018). Synthetic antidiabetic drugs have, however, been linked to several unpleasant side effects and contraindications, which has discouraged their use to some degree (Meneses *et al*., 2015). Vitamins and minerals have shown promise as safer alternatives for diabetes management because of their role in several metabolic process linked to nutrient metabolism and antioxidant status metabolism (Liu *et al*., 2018; Laclaustra *et al*., 2009). Their deficiencies have been associated with diabetes-associated disorders (O'Connell, 2001; Mooradian *et al*., 1994). Particularly is zinc (Zn), which plays an important role in insulin storage and secretion (Kazi *et al*., 2008). Zinc has been shown to have antidiabetic and antioxidant properties, insulin mimetic activity and hypoglycaemic potential (Ranasinghe *et al*., 2015; Sakurai & Adachi, 2005; Fugono *et al*., 2002). Zinc is also an essential structural part of important antioxidant enzymes such as superoxide dismutase. The synthesis of these enzymes is impaired by deficiency of zinc leading to increased oxidative stress. Studies have shown that diet supplemented with zinc have beneficial effect in diabetic rats by stimulating pancreatic islet β-cells regeneration, reducing body weight gain and lowering blood glucose level (Anyakudo & Adewunmi, 2017).

Plant-derived polyphenols have, also, been reported to show several antidiabetic and antioxidant potentials. Syringic acid and p-coumaric acid are examples of phenolic compounds widely distributed in a variety of plants and certain fungal species (Ramachandran *et al*., 2015; Wei *et al*., 2012). Several studies have shown that syringic acid has useful antidiabetic and antioxidant properties, as well as the potential use in the management of diabetes in humans. A study conducted by Wei *et al*. (2012) showed that syringic acid prevented diabetic cataract in rat lenses by non-competitively inhibiting the activity and suppressing the gene expression of aldose reductase, a key enzyme involved in diabetic cataract development (Wei *et al*., 2012). Another study has shown syringic acid decreases the levels of plasma glucose while increasing the levels of plasma insulin and C-peptide when administered orally in alloxan induced diabetic rats (Muthukumaran *et al*., 2013), suggesting its glycaemic control potential. Furthermore, Srinivasan *et al*. (2014) demonstrated that similar administration of syringic acid in diabetic rats increased the levels of insulin, haemoglobin and glycogen content (Srinivasan *et al*., 2014). It has also been shown that syringic acid has antioxidant effects. In this study, it was demonstrated that the levels of total oxidant status, oxidative stress index and lipid hydroperoxide were increased and the total antioxidant status and sulfhydryl levels were decreased. These results suggests that syringic acid reduce the oxidative damage in pancreatic tissue through inhibition of lipid peroxidation (Cikman *et al*., 2015).

p-Coumaric acid has been shown to alleviate diabetes by reducing the intestinal absorption of dietary carbohydrate, regulating enzymes involved in glucose metabolism, stimulating insulin secretion and increasing antioxidant and anti-inflammatory responses (Pei *et al*., 2016). In a study conducted by Amalan *et al*. (2016), reduced blood glucose level, glucose-6-phosphatase and fructose-1,6-bisphosphatase activities, and increased insulin level were observed in diabetes induced rats treated with p-coumaric acid. In plasma and tissues, there was also reduced total cholesterol as well as triglycerides. A degreased expression of glucose transporter 2 (GLUT 2) mRNA in the pancreas was also observed, thus, demonstrating the ability of p-coumaric acid to modulate glucose and lipid metabolism through GLUT 2 activation in the pancreas (Amalan *et al*., 2016). Yoon *et al*. (2013) have also demonstrated the ability of p-coumaric acid to modulate glucose and lipid metabolism in skeletal muscle cells via 5´ adenosine monophosphate-activated protein kinase (AMPK) activation (Yoon *et al*., 2013). p-Coumaric acid has been shown to

demonstrate 2,2-diphenyl-1-picrylhydrazy (DPPH) radical scavenging and ferric ion reducing activity *in vitro* (Kilic & Yesiloglu, 2013), as well as anti-lipid peroxidative and antioxidant enzyme modulatory action in hyperlipidaemic and diabetic nephropathic rats (Mani *et al*., 2022; Shen *et al*., 2019).

The synergistic application of natural therapeutic agents has an appreciable reception in management of diseases. The concept can be beneficial in the following ways: limiting toxicity or side effects linked to high dose of drug and providing a platform for multi-mode therapeutic targets and effects (Lehár *et al*., 2009). Advances in research have shown possible diabetes-related therapeutic synergism through mineral-ligand complexation. Zinc mineral has been complexed with various types of ligands to develop zinc complexes with improved bioactivity (Chukwuma *et al*., 2020). A critical review on antidiabetic zinc complexes suggests they possess antidiabetic potential, which is linked to their insulin mimetic and insulin modulatory properties (Chukwuma *et al*., 2020). However, the research trajectory suggests most of the studied antidiabetic Zn(II) complexes have been synthesized using synthetic organic ligands with scarcely documented pharmacological evidence and safety concerns, while natural dietary phenolics with antioxidant and other pharmacological credence have been scarcely explored as alternative ligands for therapeutic Zn(II) complexes (Chukwuma *et al*., 2020). Considering the above-mentioned antioxidant and antidiabetic prospects of syringic and p-coumaric acids, it is fair to speculate that they may be promising targets for developing potent antidiabetic zinc complexes with broader scope of antidiabetic and antioxidant properties and, perhaps, lesser side effects compared to the synthetic ligands. Therefore, the aim of this study was to investigate the antidiabetic and antioxidant effect of novel syringic acid and p-coumaric acid Zn(II) complexes.

# CHAPTER 2

## LITERATURE REVIEW

### 2.1 Diabetes

The occurrence of diabetes dates to 3000 years ago. The ancient Egyptians described a condition with characteristics similar to diabetes. These included excessive urination, thirst and weight loss (Sazid *et al*., 2017; Ahmed, 2002). The term "diabetes" was first conceived by Araetus of Cappodocia (81-133 AD), who stated "no essential part of the drink is adsorbed by the body while great masses of the flesh are limbs (liquefied) into urine" when describing diabetes (Sazid *et al*., 2017).

Later in 1675, a British physician Thomas Willis added the term "mellitus" (of honey) due to the sweet taste of urine and blood of patients with diabetes. Matthew Dobson (in 1776) confirmed that the presence of sugar in urine and blood was the cause of the sweetness (Sazid *et al*., 2017; Ahmed, 2002). It was later hypothesized by Claude Bernard that excess secretion of sugar by the liver was the cause of diabetes (Gutteridge, 1999). Currently diabetes mellitus is defined as a group of metabolic diseases characterized by high levels of blood glucose (hyperglycaemia). Hyperglycaemia is caused by impaired insulin secretion and/or insulin action (Cho *et al*., 2018). The most common symptoms of diabetes mellitus include polyuria, polydipsia, polyphagia, hyperglycaemia, weight loss and fatigue (Ramachandran, 2014). If left unchecked over the long term, hyperglycaemia can lead to damage of body organs and life-threatening health complications such as cardiovascular diseases, nerve damage (neuropathy), kidney damage (nephropathy) and eye disease (leading to retinopathy, visual loss and even blindness) (IDF, 2021).

### 2.2 Prevalence of diabetes

Diabetes mellitus is a major public health concern, worldwide, that is affecting the health, quality of life and life expectancy of patients (Aldukhayel, 2017). In recent years, the global prevalence of diabetes and impaired glucose tolerance in adults has increased significantly. The recent edition of the IDF Diabetes Atlas estimated the prevalence of diabetes worldwide for adults aged 20-79

years and projected to the years 2030 and 2045. It has been estimated that in 2021, about 6.7 million deaths were caused by diabetes (IDF, 2021). This figure is high compared to what was reported in 2019 where the number of deaths due to diabetes was estimated at 4.2 million (IDF, 2019). The current IDF report estimated that 536.6 million adults globally have diabetes, and this is expected to increase to 642.7 million by 2030 and 783.2 million by the year 2045 (**Table 2.1**) (IDF, 2021).

**Table 2.1:** Estimated total number of adults (20–79 years) with diabetes in 2021, 2030 and 2045

| | **2021** | **2030** | **2045** |
|---|---|---|---|
| Total world population | 7.9 billion | 8.6 billion | 9.5 billion |
| Adult population (20-79 years) | 5.1 billion | 5.7 billion | 6.4 billion |
| **Diabetes (20-79 years)** | | | |
| Global prevalence | 10.5% | 11.3% | 12.2% |
| Number of people with diabetes | 536.6 million | 642.7 million | 783.2 million |
| Number of deaths due to diabetes | 6.7 million | - | - |

Adopted without permission from IDF Diabetes Atlas, 2021.

It has been estimated that 24 million adults aged 20-79 years living in Africa have diabetes and the numbers are expected to increase to 33 million in 2030 and 55 million in 2045 (**Table 2.2**). The number of deaths caused by diabetes in Africa was estimated to be 416000 in 2021 (IDF, 2021).

**Table 2.2:** Estimate number of adults in Africa (20-79 years) with diabetes in 2021, 2030 and 2045

| | **2021** | **2030** | **2045** |
|---|---|---|---|
| Adult population (20-79 years) | 527 million | 696 million | 1.05 billion |
| **Diabetes (20-79 years)** | | | |
| Regional prevalence | 4.5% | 4.8% | 5.2% |
| Age-adjusted comparative prevalence | 5.3% | 5.5% | 5.6% |
| Number of people with diabetes | 24 million | 33 million | 55 million |
| Number of deaths due to diabetes | 416000 | - | - |

Adopted without permission from IDF Diabetes Atlas, 2021.

In South Africa, the number of people (aged 30 years and older) with diabetes is increasing. About 2 million people had diabetes in 2009 (Pheiffer *et al*., 2018) and in 2021 the number has increased to 4.2 million for people aged 20-79, putting South Africa at number 1 of 5 top countries with highest numbers of diabetes cases in Africa (**Table 2.3**) (IDF, 2021).

**Table 2.3:** Top five countries in Africa for number of people with diabetes (20-79 years) in 2021

| **Countries** | **Number of people with diabetes (millions)** |
|---|---|
| South Africa | 4.2 |
| Nigeria | 3.6 |
| United Republic of Tanzania | 2.9 |
| Ethiopia | 1.9 |
| Democratic Republic of the Congo | 1.9 |

Adopted without permission from IDF Diabetes Atlas, 2021.

Worldwide, diabetes prevalence has been shown to be higher among high-income countries (11.1%) and middle-income countries (10.8%) compared to low-income countries (5.5%). It is, also, more prevalent among people residing in urban areas (12.1%) compared to those in the rural settlements (8.3%). The latter has been attributed to the sedentary lifestyle and unhealthy eating habits in urban areas (IDF, 2021).

There are three main types of diabetes, namely gestational diabetes mellitus, and other two which are the major categories: type 1 diabetes mellitus and type 2 diabetes mellitus. Type 2 diabetes is the most prevalent, as it accounts for more than 90% of diabetes cases worldwide (IDF, 2021; Nagaraj *et al*., 2021; Ramachandran *et al*., 2015; Wu *et al*., 2014). For better understanding the aetiology, pathogenesis and pathophysiology of the different types of diabetes, it is crucial to understand the metabolism and homeostasis of glucose.

## 2.3 Glucose metabolism and insulin signalling

Plasma glucose is controlled by the balance between glucose absorption from the intestines, production by the liver and uptake and metabolism by peripheral tissues (Saltiel & Kahn, 2001). Glucose homeostasis is maintained by rapid action of insulin. Insulin stimulates glucose uptake

and metabolism in peripheral tissues, the skeletal muscles being the primary site of glucose disposal in the insulin-stimulated state (Choi & Kim, 2010).

Insulin increases glucose uptake in muscle and fat and inhibits hepatic glucose production, hence, serving as the primary regulator of blood glucose concentration. It also stimulates cell growth and differentiation, promotes the storage of substrates in fat, liver and muscle by stimulating lipogenesis, glycogen and protein synthesis, and inhibits lipolysis, glycogenolysis as well as protein breakdown. The deficiency or resistance of insulin affects these processes and produce elevations in fasting and postprandial glucose and lipid levels (Saltiel and Kahn, 2001).

Insulin signals circulate glucose uptake via the signalling pathway, which involves a series of signalling events (**Figure 2.1**). These includes the phosphorylation of insulin receptor substrate (IRS) proteins, the activation of the phosphatidylinositol (PI) 3- kinase and Akt (also known as protein kinase B) (Saini, 2010). Insulin signalling is initiated by insulin binding to its cell surface receptor, followed by autophosphorylation of the receptor, then activation of receptor tyrosine kinases (Choi & Kim, 2010; Saini, 2010).

The insulin receptor tyrosine kinase in turn phosphorylates the IRS proteins. The phosphotyrosine residues on IRS proteins become good targets for the p85 regulatory subunit of PI3-kinase. The activated PI3-kinase generates 3′-phosphoinositides [phosphatidyl-inositol-3,4-bisphosphate (PIP2) and phosphatidyl-inositol-3,4,5-trisphosphate (PIP3)], which bind to the phosphoinositide-dependent kinase 1 (PDK1). Substrates of the PDKs includes the protein kinase B (PKB) and the protein kinase C (PKC). Following the activation of PI3-kinase, the serine/threonine kinase Akt (PKB), triggers insulin effects on the liver, such as glycogen synthesis and the suppression of hepatic glucose production.

Akt plays an important role by linking glucose transporter (GLUT4), the insulin-dependent glucose transporter protein, to the insulin signalling pathway (**Figure 2.1**). It signals the activation of GLUT-4, which moves to the cell surface to transport glucose into the cell (Petersen & Shulman, 2018; Saini, 2010; Choi & Kim, 2010; Morino *et al*., 2006). The insulin-sensitive GLUT-4 plays a major role in the insulin-stimulated glucose uptake in white adipose tissue and skeletal muscle,

contributing to glucose homeostasis. Adipose tissue and skeletal muscle specifically express GLUT-4 protein (Okamoto *et al*., 2004).

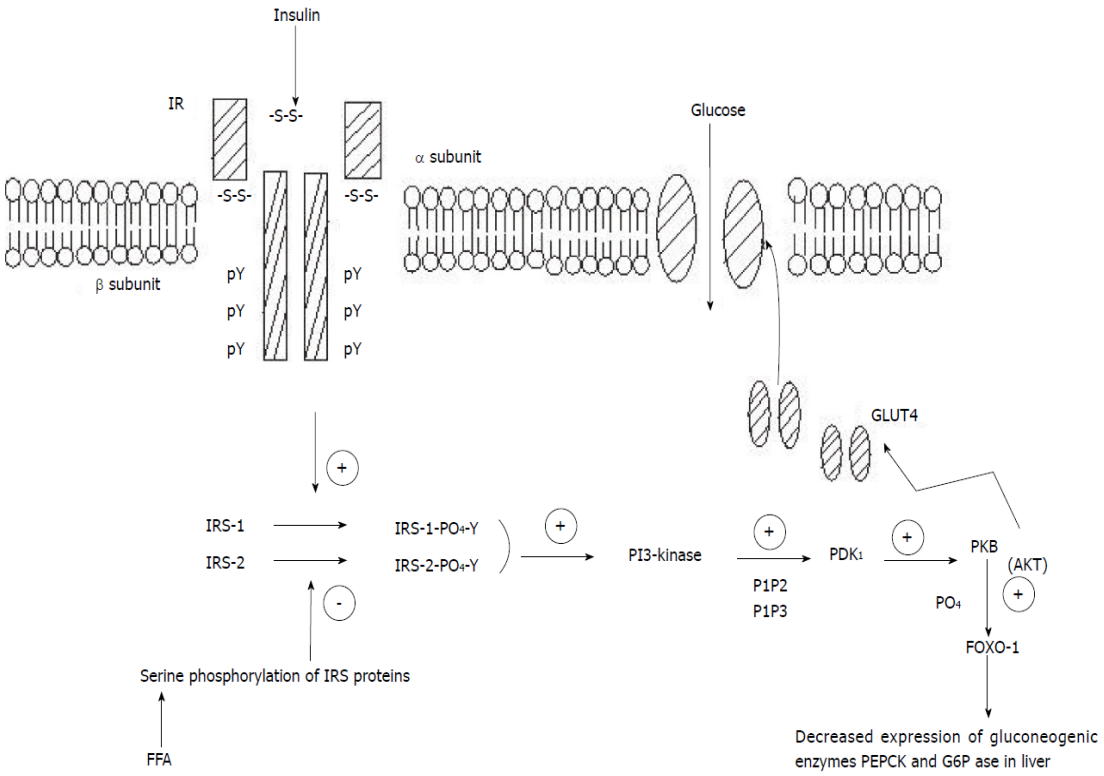



**Figure 2.1:** Insulin signalling pathway showing binding of insulin with the insulin receptor leading to the activation of glucose transporter 4 which imports glucose into the cell. (Adopted without permission from Saini, 2010).

## 2.4 Types of diabetes

There are three main forms of diabetes mellitus recognised by the world health organization: gestational diabetes mellitus (diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation), type 1 (due to autoimmune β-cell destruction, usually leading to absolute insulin deficiency) and type 2 diabetes mellitus (due to a progressive loss of β-cell insulin secretion frequently on the background of insulin resistance) (ADA, 2018; Ramachandran *et al*., 2015). Recent data reported by the World Health Organization (WHO) on the classification of diabetes mellitus also list a number of other specific types of diabetes (**Figure**

**2.2**). Newly diagnosed types of diabetes that do not fit into any of the categories are classified as unclassified diabetes (IDF, 2021; WHO, 2019).

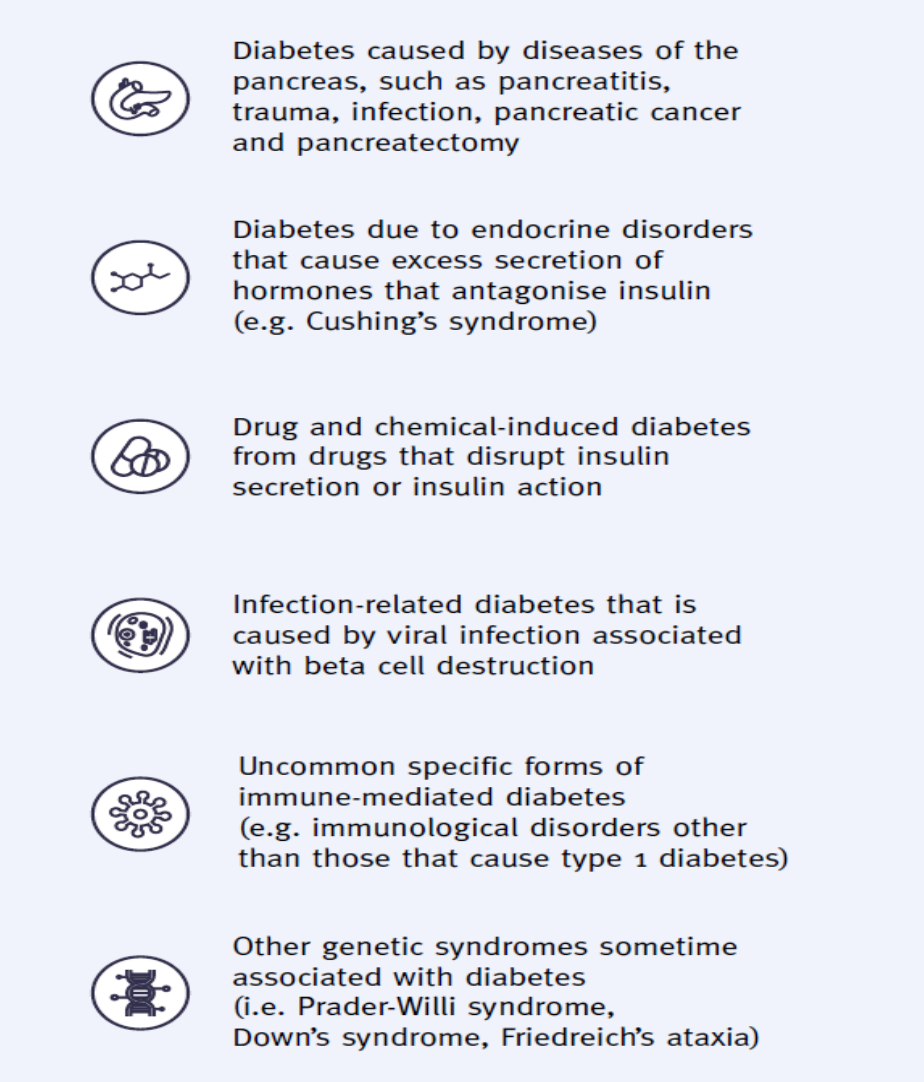



**Figure 2.2:** Other specific types of diabetes (Adopted without permission from IDF Diabetes Atlas, 2021).

### 2.4.1 Gestational diabetes

Gestational diabetes mellitus is a glucose intolerance first detected during pregnancy (Buchanan *et al*., 2012). It affects 3-8% of pregnant women and the risk of developing gestational diabetes

mellitus has been reported to be higher among obese and women older than 25 years (Zargar *et al*., 2004). The pathogenesis of gestational diabetes mellitus involves insulin resistance and insulin deficiency (Garvey *et al*., 1993). Insulin resistance during pregnancy is likely due to increased maternal adiposity and defects of hormonal products of the placenta (Buchanan and Xiang, 2005). Hormones present in high concentration during pregnancy such as progesterone, oestrogen, human placental lactogen and prolactin, have been shown to induce insulin resistance and cause β-cell dysfunction (Zargar *et al*., 2004; Sivan *et al*., 1998). β-cells normally secrete more insulin during pregnancy to compensate for a decrease in insulin sensitivity, therefore, their dysfunction is a cause of gestational diabetes (Saisho *et al*., 2010; Buchanan and Xiang, 2005).

During pregnancy, maternal glucose easily crosses the placenta and causes hyperglycaemia, which in turn results in intrauterine hyperglycaemia, foetal hyperinsulinemia, and possible modification of growth and development of the foetus, respiratory disorders, polycythaemia, cardiomyopathy and obesity in later life (Clausen *et al*., 2008; Zargar *et al*., 2004). Women with gestational diabetes are later in life, at high risk of developing impaired glucose tolerance and type 2 diabetes (Buchanan *et al*., 2012; Retnakaran *et al*., 2010).

### 2.4.2 Type 1 diabetes

Type 1 diabetes, also known as insulin-dependent diabetes mellitus, is an autoimmune disorder that develops when the body's immune system destroys pancreatic β-cell, the body's insulin-producing cells (Dey *et al*., 2002; Sakurai, 2002). Activated T cell and macrophages release several proinflammatory cytokines such as interleukin-1β, interferon-γ and tumour necrosis factor-α, which are mediators of β-cell destruction in type 1 diabetes. The cytokines damage β-cell through specific receptors by inducing several signal transduction pathways that lead to alterations in gene and protein expressions. The β-cell dysfunction leads to decreased insulin secretion, and eventually, hyperglycaemia (Alofi *et al*., 2016; Fu *et al*., 2013). Hyperglycaemia in turn induces overproduction of oxygen free radicals and as a result, increases protein and lipid oxidation (Singh and Venkatesan, 2016).

Type 1 diabetes development may also be influenced by dietary factors including early infant feeding status, vitamin D and omega 3 polyunsaturated fatty acid intakes, and duration of exposure to gluten (Alofi *et al*., 2016; Fu *et al*., 2013). Vitamins protect islets against oxidative stress. The key vitamins are vitamin A, vitamin D, biotin, nicotinamide, vitamin C and vitamin E (Singh and Venkatesan, 2016). Vitamin D for example regulate the pathogenesis of type 1 diabetes through its immunomodulatory and anti-inflammatory actions, reducing the inflammatory reaction in the pancreatic islets and reducing the autoimmune insulitis (Davi *et al*., 2010). β-cells are direct target of vitamin D. Impaired glucose stimulated insulin secretion is observed with vitamin D deficiency, which is improved following vitamin D supplementation (Al-Agha and Ahmad, 2015; Davi *et al*., 2010).

### 2.4.3 Type 2 diabetes

Type 2 diabetes is characterised by metabolic disturbances in nutrient metabolism, caused by insulin resistance and/or impaired insulin secretion (Fu *et al*., 2013; Tripathi and Srivastava, 2006). Obesity has been shown to be a leading pathogenic factor for developing insulin resistance. Insulin resistance result in β-cell dysfunction and it is associated with impairment in energy metabolism. This causes increase in intracellular fat content in skeletal muscle, liver and pancreatic islet (Fu *et al*., 2013; Kahn, 2003). Reduced β-cell mass and insulin secretory granules are responsible for hyperglycaemia.

Common symptoms of type 2 diabetes include:

- Polyuria, increased thirst and nocturia – due to hyperglycaemia
- Fatigue- due to the inability to use glucose as an energy source

These symptoms can sometimes be accompanied by the following:

- Weight loss (resulting from the breakdown of protein and fat as an alternative energy source)
- Blurred vision (caused by a change in lens refraction)

Urinary tract infections (due to increased serum glucose which provides growth medium

## 2.5 Aetiology and pathophysiology of type 2 diabetes

Type 2 diabetes is caused by defects in insulin action and insulin secretion, which is caused by the non-responsiveness of target cells to insulin action and progressive pancreatic β-cell dysfunction and loss of β-cell mass (Olokoba *et al*., 2012; Alonso-Magdalene *et al*., 2011; Kaku, 2010). In addition to the genetic factors, environmental factors, such as obesity, aging, lack of exercise and stress and other lifestyle habits, such as smoking and excessive alcohol consumption can contribute to the development of type 2 diabetes (Ozougwu *et al*., 2013; Olokoba *et al*., 2012; Kaku, 2010). The environmental factors affect β-cell function and could promote tissue insulin insensitivity (**Figure 2.3**) (Scheen, 2003).

Type 2 diabetes is characterised by abnormal high levels of glucose in the blood. The maintenance in the balance of plasma glucose levels in the blood is dependent on the production, secretion and action of insulin (Alonso-Magdalene *et al*., 2011). The insufficient action and secretion of insulin results in hyperglycaemia (Kaku, 2010). Disturbance in the transfer of signals between endocrine pancreas, liver, skeletal muscle, adipose tissue, gut and central nervous system may lead to the pathophysiology type 2 diabetes (Scheen, 2003).

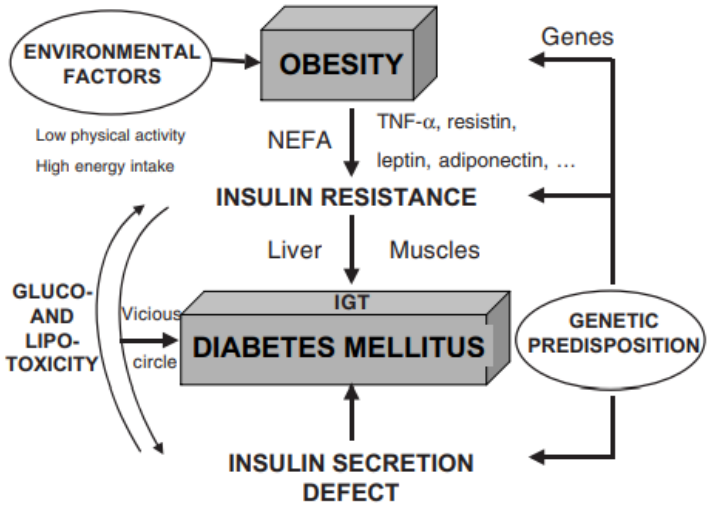



**Figure 2.3:** Contribution of genetic predisposition and environmental factors in the pathogenesis of type 2 diabetes and interplay between defective insulin resistance leading to a vicious circle explaining the progression from impaired glucose tolerance (IGT) to type 2 and the progressive aggravation of the disease (Adopted without permission from Scheen, 2003).

### 2.5.1 Insulin resistance

Insulin resistance occurs when insulin does not exert sufficient action proportional to blood glucose concentration (Kaku, 2010). Insulin resistance is central to the pathophysiology of type 2 diabetes (Le Marchand-Brustel *et al*., 2002). It develops and expands before type 2 diabetes onset (Taylor, 2013). Insulin resistance is a complex metabolic disorder that defies a single etiological pathway. Pathways such as formation of glycation end products, accumulation of lipid metabolites, activation of inflammatory pathway, and oxidative stress have been shown to be involved in the pathogenesis of insulin resistance (Lasram *et al*., 2014; Samuel and Shulman, 2012).

Studies have shown that skeletal insulin resistance is associated with defects at proximal levels of insulin signalling, including signalling at levels involving insulin receptor (INRS), insulin receptor substrate (IRS), phosphoinositide-3-kinase (PI3K) and serine/threonine kinase (AKT), also known as protein kinase B activities (Petersen and Shulman, 2018). A decrease in the IRS 1 tyrosine phosphorylation in response to insulin can cause insulin resistance. This have been observed in models of insulin resistance and in diabetic humans (Le Marchand-Brustel *et al*., 2002).

Insulin activates tyrosine kinase activity after binding to its receptor, leading to phosphorylation of various substrates including the IRS proteins. The tyrosine phosphorylated IRS serves as docking molecules for molecules, such as the p85 subunit of PI3K. This induces the activation of PI3K, which is in turn required for the translocation of vesicles containing glucose transporter 4 (GLUT 4) molecules, leading to the entry of glucose into the cell (Shulman, 2014; Moreno *et al*., 2010; Le Marchand-Brustel *et al*., 2002). It has been demonstrated that serine phosphorylation of IRS proteins can reduce activation of PI3K thereby causing impaired insulin signal transduction (Saini, 2010).

PI3K consists of a regulatory subunit p85, which is tightly associated with a catalytic subunit p110. Ordinarily, the p85 subunit exist in excess to the p110 subunit, hence there is usually a pool of free p85 monomers not associated with the p110 catalytic subunit. The p85-p110 heterodimer is responsible for the PI3K activity. The p85 monomer and p85-p110 heterodimer compete with tyrosine phosphorylated IRS proteins for the same binding sites.

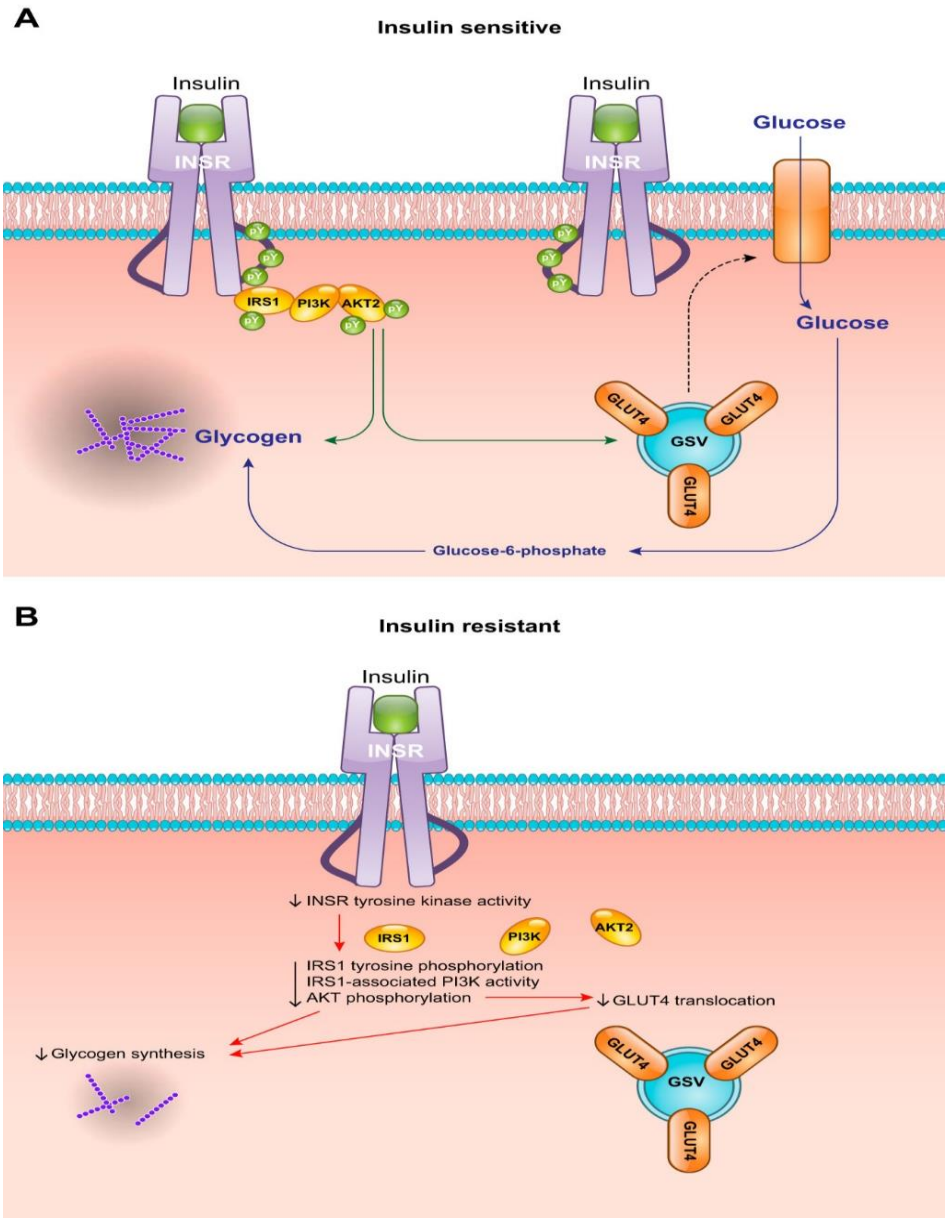



**Figure 2.4:** Functional consequences of skeletal muscle insulin resistance. A: an insulin-sensitive skeletal myocyte activates the metabolic insulin receptor substrate 1 (IRS 1)-phosphoinositide-3-kinase (PI3K)-AKT arm of the insulin signalling cascade to increase glucose uptake and glycogen synthesis. B: an insulin-resistant myocyte exhibits impairments in proximal insulin signalling events, blunting insulin's ability to stimulate GLUT 4 translocation and glycogen synthesis (adopted without permission from Petersen and Shulman, 2018).

Therefore, if there is as imbalance between the two, there could be a decrease or increase in the activity of PI3K. An increase in the expression of p85 subunit caused by human placental growth

hormone, for instance has been shown to affect the ability of insulin to stimulate the association of the p85-p110 heterodimer with IRS 1, which reduces the PI3K insulin signalling and the subsequent activation of the serine/threonine kinase AKT (also known as protein kinase B). This defect triggers a cascade of signalling event that limits GLUT-4 translocation and impairs cellular glucose uptake, thus causing insulin resistance (Saini, 2010). GLUT-4 translocation defects have, also, been shown to contribute to insulin resistance (Saini, 2010). The mechanism for insulin resistance to muscle glucose disposal is shown in **Figure 2.4**.

Adipose tissue also has a role in insulin resistance (**Figure 2.5**). Free fatty acids (FFAs) derived from adipocytes are increased in many insulin-resistant states and it has been suggested that they contribute to the insulin resistance in diabetes and obesity (Sears and Perry, 2015).

Elevated FFAs inhibit glucose uptake, glycogen synthesis, glucose oxidation, and increase hepatic glucose output. Increased levels of FFAs are, also, associated with a reduction in insulin-stimulated IRS-1 phosphorylation and IRS-1-associated PI(3)K activity (Sears and Perry, 2015). The high levels of circulating FFAs and insulin resistance might lead to accumulation of triglycerides and fatty acid-derived metabolites (diacylglycerol, fatty acyl-CoA and ceramides) in muscle and liver (Sears and Perry, 2015; Saltiel and Kahn, 2001).

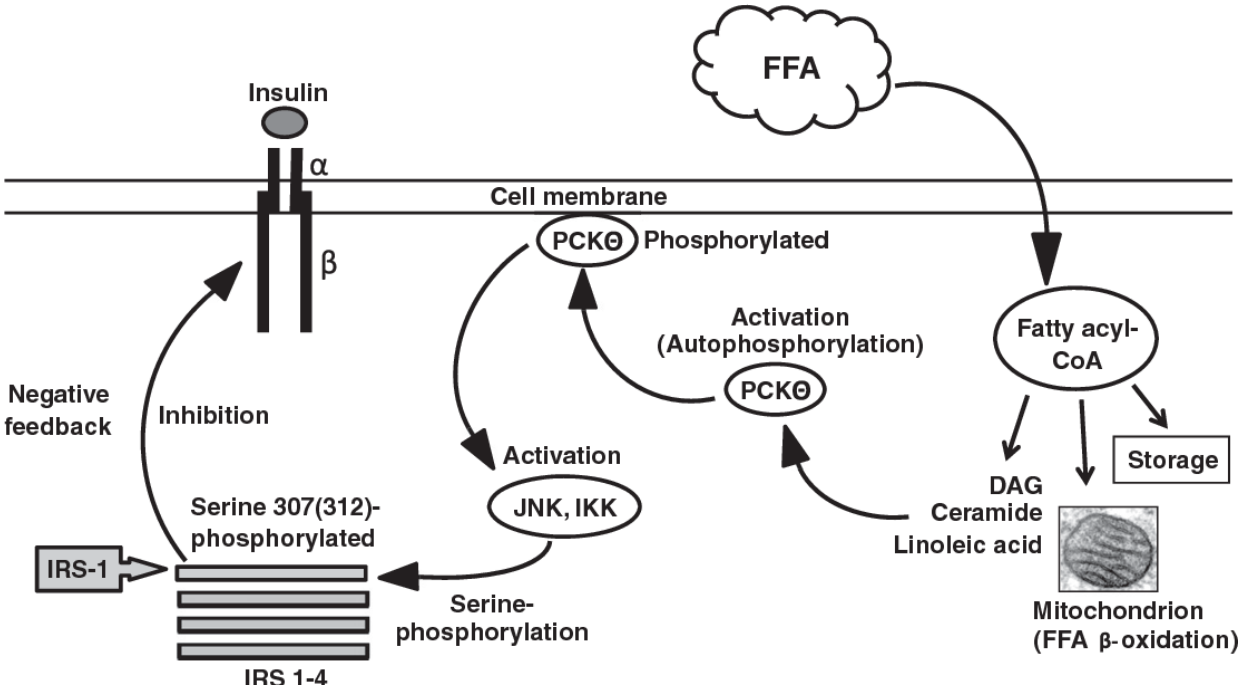



**Figure 2.5:** Adiposity and free fatty acid mediated tissue insulin resistance and impaired cellular glucose uptake (Adopted without permission from Capurso and Capurso, 2012).

### 2.5.2 Hyperinsulinemia and partial pancreatic β-cell dysfunction

Hyperinsulinemia is a compensation for systemic insulin resistance. Insulin resistance causes hyperinsulinemia as a result of the pancreatic β cells producing and releasing more insulin (Mehran *et al*., 2012). Pancreatic β cells sense changes in blood glucose and secrete insulin to maintain normal concentration of sugar in the blood (Lu *et al*., 2010). The increase in the plasma glucose results in increase in insulin secretion (Saisho, 2015; Choi *et al*., 2008; Abdul-Ghani *et al*., 2006). β cells function decline with persistently high plasma glucose levels. Insulin sensitivity also affects insulin secretion (Saisho, 2015; Mari *et al*., 2011). When insulin sensitivity decreases, insulin secretion increases to maintain normoglycaemia. Failure in increase of insulin secretion to compensate the decrease in insulin sensitivity results in abnormal glucose tolerance (Saisho, 2015). Insulin resistance and β-cell dysfunction lead to persistent hyperglycaemia which characterizes type 2 diabetes (Saisho, 2015; Cerf, 2013).

Free fatty acids (FFAs) have also been associated with insulin secretion and β-cells function. FFAs stimulate insulin secretion, however if β-cells are exposed for a long time to high FFA level, their glucose responsiveness could be reduced (Cnop *et al*., 2010). Short-term exposure to FFAs increases glucose-stimulated insulin secretion and enables storage of excess calories as fat, contributing to overweight and obesity. Long-term exposure to FFAs suppresses glucose-stimulated insulin secretion and leads to impaired glucose metabolism, reduced insulin biosynthesis, and beta cell loss. Thus, elevated FFAs may contribute to progressive β-cell dysfunction and loss in type 2 diabetes (Cerf, 2013; Cnop *et al*., 2010).

### 2.5.3 Impaired insulin secretion, glucose intolerance and persistent hyperglycaemia

Impaired insulin secretion caused by alterations and function of pancreatic of β-cell plays a crucial role in the progression from normal glucose tolerance to impaired glucose tolerance. The defect in insulin secretion leads to hyperglycaemia and contribute to the development of type 2 diabetes (Gastaldelli, 2011; Abdul-Ghani *et al*., 2008; De Rooij *et al*., 2006). Impaired insulin secretion is a decrease in glucose-mediated insulin release, which contributes to impaired glucose tolerance and sustained postprandial hyperglycaemia (Kaku, 2010). Hyperglycaemia itself causes further decreases in glucose-stimulated insulin secretion (Brownlee, 2003).

Impaired insulin secretion is generally progressive and involves glucose toxicity and lipo-toxicity. When untreated, these has been shown to cause a decrease in pancreatic β-cell mass in animal experiments (Kaku, 2010). The progression of the impairment of pancreatic β-cell function greatly affects the long-term control of blood glucose (Kaku, 2010). It has been reported that various mechanism associated with hyperglycaemia cause oxidative stress in the blood and tissue of diabetic patients (Kashiwagi, 2001).

## 2.6 Oxidative stress and diabetes

Oxidative stress is an imbalance between production of highly reactive molecular species, mainly oxygen and nitrogen, and biological antioxidant defences, leading to tissue damage. Oxidative stress results from increased content of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS). Examples of reactive oxygen species include charged species such as superoxide

and hydroxyl radicals, and uncharged species such as hydrogen peroxide (Araki and Nishikawa, 2010; Evans *et al*., 2003). Reactive oxygen species production occurs in patients with diabetes mellitus because of the impaired carbohydrate metabolism under hyperglycaemic conditions. Hyperglycaemia results in increased generation of active oxygen and antioxidant defence impairment, leading to the activation of redox-sensitive transcriptional factors including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Kashiwagi, 2001).

Antioxidants directly scavenge active oxygen. Endogenous antioxidants include ascorbate, vitamin E, reduced glutathione, β-carotene, various amino acids, proteins, uric acid, bilirubin, etc. Enzymes involved in the scavenging of active oxygen generated in cells include the following:

1. Superoxide dismutase (SOD), which converts endogenous $O_2^-$ into hydrogen peroxide (including Cu, Zn-SOD, Mn-SOD, and extracellular SOD)
2. Catalase and the glutathione redox cycle (GR cycle), which convert hydrogen peroxide ($H_2O_2$) into water (Kashiwagi, 2001).

The catalase and the glutathione redox cycle is dependent on the activity of glutathione peroxidase and glutathione reductase, intracellular contents of NADPH (reduced nicotinamide adenine dinucleotide phosphate), and the activity of the pentose phosphate pathway. **Figure 2.6** presents the biological antioxidant defence system.

Under hyperglycaemic conditions, the Superoxide dismutase is glycated, and the peptide chain is cut, resulting in a decrease in its activity. The activity of the glutathione redox cycle is also decreased due to the impaired activation of the pentose phosphate pathway (Fiorentino *et al*., 2013; Rolo and Palmeira, 2006; Kashiwagi, 2001). **Figure 2.7** shows pathways that contribute to oxidative stress in response to increased glucose flux.

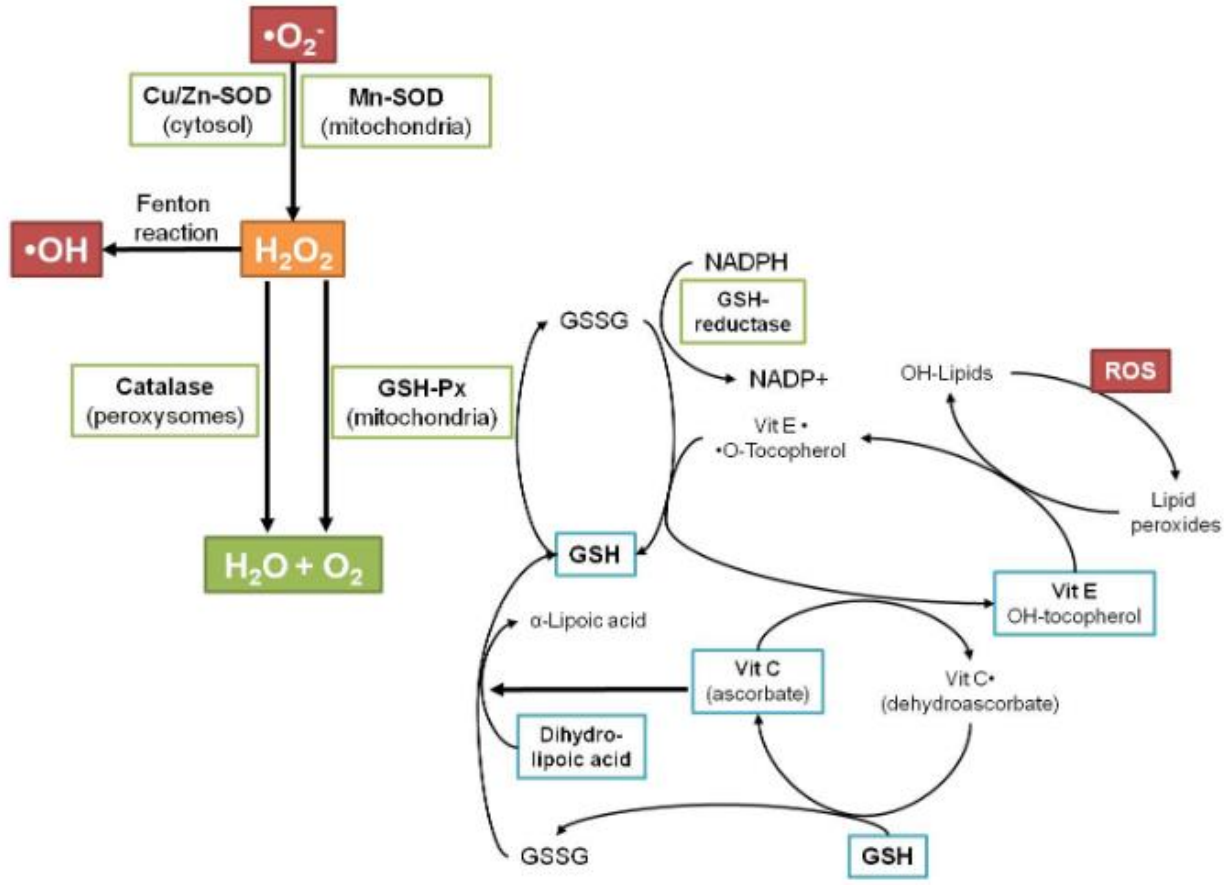



**Figure 2.6:** Antioxidant defenses in the organism (Adopted without permission from Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013).

It has also been reported that increased FFA levels may also result in ROS formation. Increased levels of FFA have adverse effects on mitochondrial function. These include the uncoupling of oxidative phosphorylation and the generation of ROS. FFA also impair endogenous antioxidant defences by reducing intracellular glutathione (Evans *et al*., 2003). ROS are believed to play a key direct role in the pathogenesis of late diabetic complications such as diabetic cardiomyopathy, microvascular and macrovascular diseases (Evans *et al*., 2003; Rolo and Palmeira, 2006).

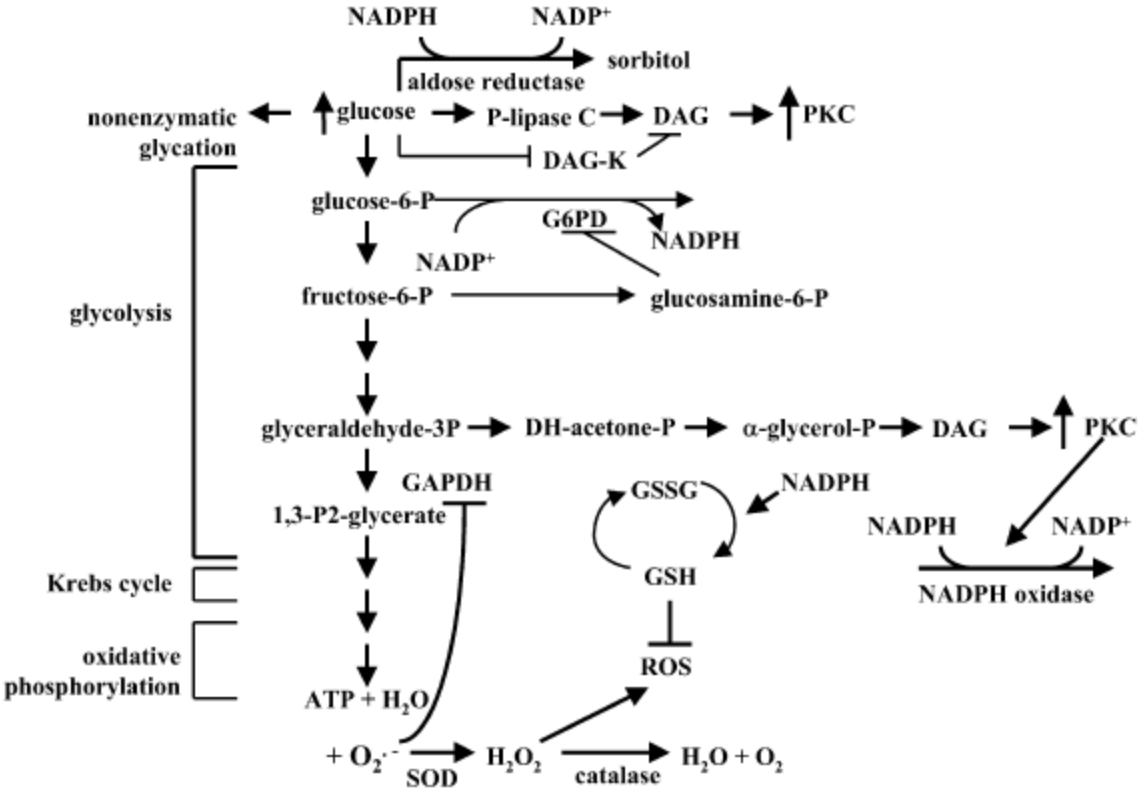



**Figure 2.7:** Schematic diagram of pathways that contribute to oxidative stress in response to increased glucose flux (Adopted without permission from King and Loeken, 2004).

The elevated production of superoxide radical and several other reactive oxygen species and depletion of antioxidants due to oxidative stress, can oxidatively damage cellular biomolecules and, eventually, tissues and organs. Persistent hyperglycaemia can also 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 (Oke *et al*., 2021; Chukwuma *et al*., 2018; Chukwuma and Islam, 2017).

## 2.7 Diabetic complications

Diabetes is associated with several complications, which are becoming the world's most significant cause of morbidity and mortality (Pickering *et al*., 2018; Singh *et al*., 2014). The biochemical mechanisms causing the pathogenesis of diabetes complications relate to genetic and

epigenetic modifications, nutritional factors, sedentary lifestyle and oxidative stress (Papatheodorou *et al*., 2016; Reddy *et al*., 2015). Hyperglycaemia is suggested to be the major cause of diabetic complications (Volpe *et al*., 2018; Nowotny *et al*., 2015). Hyperglycaemia triggers several metabolic signalling pathways that lead to inflammation, cytokines secretion, cell death and eventually diabetic complications (Volpe *et al*., 2018).

Oxidative stress and defects in insulin signalling have been implicated in the progression of diabetic complications (Papatheodorou *et al*., 2015). Oxidative stress plays a pivotal role in the development of diabetes complications, both at the microvascular and macrovascular levels (Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013). In addition, formation of advanced glycation end products (AGEs) is another contributing factor. AGEs increase the formation of reactive oxygen species and impair antioxidant systems, as a result contributing chronic stress conditions in diabetes (Nowotny *et al*., 2015; Volpe *et al*., 2018). The protein kinase C (PKC) pathway and the hexosome pathway, also, lead to insulin resistance, β-cell dysfunction and ultimately development of diabetic complications (Tan and de Haan, 2014). These complications are represented as inflammation in the vessels and/or in the nerves (Tan and de Haan, 2014; Volpe *et al*., 2018).

Diabetic complications occur in the majority of individuals with both type 1 and type 2 diabetes and they are grouped into microvascular disease, due to damage to small blood vessels, and macrovascular disease, due to damage to the arteries (Forbes and Cooper, 2013).

### 2.7.1 Microvascular complications

Microvascular complications include nephropathy (kidney disease), neuropathy (neural damage) and retinopathy (eye disease). These complications are induced by persistent hyperglycaemia and the underlying mechanisms may include production of AGEs, creation of proinflammatory microenvironment and the induction of oxidative stress (Papatheodorou *et al*., 2016; Papatheodorou *et al*., 2015; Nowotny *et al*., 2015; Tan and de Haan, 2014).

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease. Oxidative stress is the major cause of DN. The early stage of DN is characterised by glomerular hyperfiltration,

glomerular and tubular hypertrophy, and microalbuminuria. As it progresses, expansion of mesangial matrix and thickening of the glomerular basement membrane occur. Glomerular sclerosis, proteinuria and the detection of macro-albuminuria occur at the advanced stages of DN (Pickering *et al*., 2018).

The mechanisms for injury in DN involve the increased polyol pathway and AGE formation. AGE binding to its receptors plays a role in renal damage, fibrosis and inflammation associated with diabetic nephropathy. The actions of AGE potentiate oxidative stress, while synergizing with rennin-angiotensin system activation, and ultimately cause kidney failure (Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013).

Diabetic retinopathy (DR) creates a major health burden globally, affecting 93 million people worldwide (Pickering *et al*., 2018). It is the leading cause of blindness among adults aged 20–74 years. Diabetic retinopathy is characterized by a spectrum of lesions within the retina. These include changes in vascular permeability, capillary microaneurysms, capillary degeneration, and excessive formation of new blood vessels (neovascularization) (Pickering *et al*., 2018; Forbes and Cooper, 2013). The retina is particularly susceptible to oxidative damage due to a high metabolic rate and the rapid rate of oxygen consumption by the cells of the retina including the rod and cone photoreceptors (Pickering *et al*., 2018).

Diabetic retinopathy is clinically separated into non-proliferative and proliferative disease stages. In the initial non-proliferative stage, most people do not notice any visual impairment. However, there is intramural pericyte death and thickening of the basement membrane due to hyperglycaemia. This contributes to changes in the integrity of blood vessels within the retina, altering the blood-retinal barrier and vascular permeability. Degeneration of retinal capillaries progresses the disease into the proliferative phase, where neovascularization and accumulation of fluid within the retina, termed macula edema, contribute to visual impairment.

Severe cases of diabetic retinopathy can lead to bleeding and distortion of the retinal architecture, including development of a fibrovascular membrane. This can subsequently lead to retinal

detachment (Forbes and Cooper, 2013). AGEs and ROS are the causes of retina cell damage (Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013).

Diabetic neuropathy causes somatic and autonomic divisions of the peripheral nervous system. Development of vascular abnormalities, such as capillary basement membrane thickening and endothelial hyperplasia with subsequent diminishment in oxygen tension and hypoxia are the typical characteristics of disease progression in neuropathy. Hyperalgesia, paresthesias, and allodynia also occur in a proportion of patients, with pain evident in 40–50% of those with diabetic neuropathy (Forbes and Cooper, 2013).

The mechanisms leading to neuronal injury in diabetic neuropathy remain unclear. Several mechanisms including the polyol pathway, AGEs formation and production of ROS have been associated with the pathogenesis of diabetic neuropathy (Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013). Protein kinase c activation may also contribute to diabetic neuropathy by a neurovascular mechanism, such as blood flow and conduction velocity (Geraldes and King, 2010).

### 2.7.2 Macrovascular complications

The major macrovascular complications include accelerated cardiovascular disease which results in myocardial infarction and cerebrovascular disease manifesting as strokes (Reddy *et al*, 2015; Forbes and Copper, 2013). Diabetic macrovasculopathy is associated with structural and functional changes in large vessels that lead to blood flow obstruction, hypertension, myocardial infarction and possibly death (Geraldes and King, 2010).

Atherosclerosis is the most common macrovascular complication in people with diabetes mellitus (Papatheodorou *et al*., 2016). It is characterized by endothelial dysfunction, cytokine expression, monocyte infiltration, smooth muscle cells proliferation, impaired fibrinolysis combined with increased thrombosis, and chronic inflammation, causing ischemic heart diseases, myocardial infarction, stroke, and peripheral arterial diseases (Geraldes and King, 2010).

Atherosclerosis occurs as a result of chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system. These damages cause accumulation of oxidized lipids from low-density lipoprotein particles in the endothelial wall of arteries, whose rupture leads to acute vascular infarction. It has also been proposed that increased superoxide production is involved in the pathogenesis of complications, as it is the central and major mediator of endothelial tissue damage and activation of oxidative stress pathways. Endothelial cells also contain high amounts of aldo-keto reductase and are thus, prone to increased polyol pathway activation (Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013).

Hyperglycaemia is believed to participate in atherosclerotic plaque formation. Activation of protein kinase c isoforms, especially the β-isoform, has also been associated with Atherosclerosis (Geraldes and King, 2010; Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013).

## 2.8 Management of diabetes

Several studies have shown that type 2 diabetes can be prevented by lifestyle and diet modification including, maintenance of healthy body weight (body mass index of 25 $kg/m^2$), consuming a healthy diet (eating high fibre, unsaturated fat, diet low in saturated and trans-fats and glycaemic index), regular exercise, abstinence from smoking and moderate consumption of alcohol (Zheng *et al*., 2018; Olokoba *et al*., 2012).

The goal of lifestyle management is to manage blood glucose and cardiovascular risk factors to reduce risk for diabetes-related complications (Davies *et al*., 2018; Chaudhury *et al*., 2017). Pharmacological therapy can also be used in diabetes management (Chaudhury *et al*., 2017). Usually, lifestyle management combined with pharmacological therapy is recommended to patients with diabetes to lower blood glucose levels or improve other outcomes (Davies *et al*., 2018; Reusch and Manson, 2017).

### 2.8.1 Commercially available antidiabetic drugs

Several antidiabetic medications that can lower blood glucose or improve other outcomes in patients with Type 2 diabetes are available commercially. These include α-glucosidase inhibitors,

biguanides, sulfonylureas, meglitinides, glucagon-like peptide-1 (GLP-1) receptor agonists, thiazolidinedione (TZD), dipeptidyl peptidase 4 (DPP-4) inhibitors, sodium-glucose cotransporter (SGLT2) inhibitors and insulins. This section summarises the mechanism of action, advantages and disadvantages of the antidiabetic medications.

**α-Glucosidase inhibitors**

Following a meal containing complex carbohydrates, enzymatic action is required to breakdown the carbohydrates to monosaccharides to ensure absorption in the intestines. The degradation is by the action of enzymes such β-galactosidases, α-amylases and α-glucosidases. Absorption of monosaccharides results in the increase of glucose levels in the blood. α-glucosidase inhibitors regulate the rate of digestion of complex carbohydrates by inhibiting the action of α-glucosidases. The increase in blood glucose levels is delayed, giving pancreatic β-cells time to secrete insulin in response to plasma glucose levels. It is therefore recommended that α-Glucosidase inhibitors be taken at the beginning of meals. Examples of α-Glucosidase inhibitors used as antidiabetic drugs are acarbose, miglitol and voglibose. However, there are several side effects associated with these drugs including, flatulence, diarrhoea, and abdominal discomfort caused by altered metabolism of disaccharides in the colon (Meneses *et al*., 2015).

**Biguanides**

Metformin is a biguanide that is the main first-line oral drug of choice in the management of type 2 diabetes across all age groups (Chaudhury *et al*., 2017; Thrasher, 2017). It reduces plasma glucose through several mechanisms (Davies *et al*., 2018). It acts by blocking liver gluconeogenesis through regulation of the gluconeogenic flux. Recent studies have shown that metformin activates adenosine monophosphate-activated protein kinase in the liver, resulting in glucose uptake, fatty acid oxidation in liver and muscle, inhibition of gluconeogenesis, cholesterol and triglyceride synthesis and lipogenesis (Chaudhury *et al*., 2017; Meneses *et al*., 2015).

It has also been suggested recently that metformin lowers plasma lipid levels through a peroxisome proliferator-activated receptor (PPAR)-α pathway, which prevents cardiovascular diseases. Metformin is highly efficient when there is enough insulin production; however, when diabetes

reaches the state of failure of β-cells and resulting in a type 1 phenotype, metformin loses its efficacy (Chaudhury *et al*., 2017). In addition, metformin improves endothelial dysfunction, haemostasis, oxidative stress, insulin resistance, lipid profiles, and fat redistribution. Furthermore, some of the advantages of metformin include but not limited to, mild side effects, minimal risk of hypoglycaemia, low chances of cardiovascular disease events and weight gain. The disadvantages of this drug include rare cases of lactic acidosis, vitamin B12 and folic acid deficiency and gastrointestinal side effects (Thrasher, 2017).

**Sulfonylureas**

Sulfonylureas were the first pharmacological option to treat non-insulin-dependent diabetes mellitus, besides insulin injections. Examples sulfonylureas are glimepiride, glipizide, glicazide and glibenclamide (glyburide). Researchers consider glimepiride as a second generation of sulfonylureas, while others classify it as third-generation agent. Sulfonylureas are inexpensive, widely available, and have high glucose lowering efficacy. They lower glucose by stimulating insulin secretion from pancreatic β-cells (Davies *et al*., 2018).

Sulfonylureas act on β-cells' sulfonylurea receptor 1, which closes the ATP-dependent potassium channels. This stops the potassium flow across the plasma membrane leading to depolarization and opening of calcium channels. The uptake of extracellular calcium activates cytoskeletal system, which causes translocation of secretory granules to the cell surface and extrusion of insulin through exocytosis. This implies that sulfonylureas action can lead to hypoglycaemia and hyperinsulinemia (Chaudhury *et al*., 2017; Meneses *et al*., 2015). In addition, they are associated with weight gain, lack of durable effect on glucose lowering and risk of cardiovascular diseases. (Davies *et al*., 2018).

**Meglitinides**

Nateglinide, repaglinide and mitiglinide are examples of meglitinides (Davies *et al*., 2018). Meglitinides are insulin secretagogues with a similar mechanism of action to sulfonylureas. They act on ATP-dependent potassium channels, promoting insulin secretion by closing the channels in the membrane of pancreatic β-cells. They offer an alternative to sulfonylurea treatment but have

almost the same disadvantages and a more complex dosing schedule. Repaglinide binds to a nearby location of the receptor site for sulfonylureas drugs and generates a more rapid reaction. (Chaudhury *et al*., 2017; Meneses *et al*., 2015). Repaglinide is rapidly absorbed in the intestine. It is metabolized in the liver to inactive metabolites and is predominantly excreted via the bile into the faeces. This makes it a good option for patients with renal failure. It can be used in combination with metformin or thiazolidinediones (Meneses *et al*., 2015).

Nateglinide is a D-phenylalanine derivative that quickly and reversibly binds to the sulfonylurea receptor 1 like sulfonylureas. It has binding specificity for pancreatic sulfonylurea receptor 1, hence its binding site differs from that of repaglinide. It also inhibits the ATP-dependent potassium channels faster than repaglinide and effective in combination with metformin or thiazolidinediones. Nateglinide is rapidly absorbed in a dose-dependent manner and is mostly excreted in urine (Meneses *et al*., 2015).

**Glucagon-like peptide-1 (GLP-1) receptor agonists**

GLP-1 receptor agonists (GLP-1 RAs) available are albiglutide, dulaglutide, exenatide, liraglutide and lixisenatide. GLP-1 is an incretin hormone released from the gut after a meal. It enhances insulin release, decreases glucagon secretion from the pancreas in a glucose-dependent manner, improves satiety, promotes weight loss and delays gastric emptying (Thrasher, 2017). They are currently delivered by subcutaneous injection (Davies *et al*., 2018; Thrasher, 2017). GLP-1 exerts direct effects in several organs, due to the interaction with its receptor located in organs such as pancreas, brain, heart, lung, stomach, intestine, and kidney. On pancreatic β-cells, this interaction leads to activation of adenylate cyclase and production of cAMP, which mediates its stimulatory effect on insulin secretion via protein kinase A. Also, GLP-1 stimulates insulin secretion by inhibiting ATP-dependent potassium channels (Meneses *et al*., 2015). The GLP-1 receptor agonists are associated with a low risk of hypoglycaemia, except when used with insulin or agents that stimulate insulin secretion (sulfonylureas). Gastrointestinal disorders, thyroid C-cell tumours, pancreatitis and acute kidney injury have been linked to these drugs (Chaudhury *et al*., 2017; Thrasher, 2017).

**Thiazolidinedione (TZD)**

Thiazolidinedione like biguanides improve insulin action. Rosiglitazone and pioglitazone are representative agents. TZDs are synthetic activators of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), which is abundantly expressed in key target tissues for insulin action, such as adipose tissue, but is also present in muscle, liver, endothelium and pancreatic β-cells (Chaudhury *et al*., 2017; Meneses *et al*., 2015). The mechanisms of action of TDZs include diminution of free fatty acid accumulation, reduction of inflammatory cytokines, elevation adiponectin levels, and preservation of β-cell integrity and function, all leading to improvement of insulin resistance and β-cell exhaustion **(**Chaudhury *et al*., 2017).

TZDs increase high-density lipoprotein cholesterol, and pioglitazone has been shown to reduce cardiovascular endpoints and hepatic steatohepatitis. However, TDZs may be associated with heart failure, weight gain, bone fracture and bladder cancer (Davies *et al*., 2018).

**Dipeptidyl peptidase 4 (DPP-4) inhibitors**

Dipeptidyl peptidase 4 (DPP-4) inhibitors include alogliptin, linagliptin, sitagliptin, saxagliptin and vildagliptin. These medications may be used as a single therapy, or with metformin, sulfonylurea, or TZD (Chaudhury *et al*., 2017). DPP-4 inhibitors are well tolerated, have a neutral effect on weight and have minimal risk of hypoglycaemia when used as monotherapy. When added to sulfonylurea therapy, however, the risk for hypoglycaemia is increased 50% compared with sulfonylurea therapy alone (Davies *et al*., 2018; Thrasher, 2017).

DPP-4 inhibitors reduce the enzymatic degradation of the incretin hormones, GLP-1, and glucose-dependent insulinotropic polypeptide by reducing the activity of serum DPP-4. This increases the availability of endogenous incretins, thus stimulating insulin secretion from β-cells and inhibiting glucagon release from pancreatic α-cells (Davies *et al*., 2018; Thrasher, 2017). Side effects that have been reported are rare pancreatitis, musculoskeletal side effects, nasopharyngitis, upper respiratory tract infection, and headaches (Davies *et al*., 2018; Chaudhury *et al*., 2017).

**Sodium-glucose cotransporter (SGLT2) inhibitors**

Sodium-glucose cotransporter inhibitors are new classes of glucosuric agents. Some examples include canagliflozin, dapagliflozin, ertugliflozin and empagliflozin. SGLT2 inhibitors provide glucose lowering by blocking glucose reabsorption in the proximal renal tubule through the inhibition of SGLT2. These drugs may be effective in advanced stages of type 2 diabetes when pancreatic β-cell reserves are permanently lost because of their glucose-independent mechanism of action. It is suggested that SGLT2 expression is increased in patients with type 2 diabetes, resulting in increased glucose reabsorption and preservation of elevated blood glucose levels.

SGLT2 inhibitors provide modest weight loss, blood pressure reduction, are generally well tolerated, and associated with a low risk of hypoglycaemia. Urinary tract infections leading to urosepsis and pyelonephritis, as well as genital mycosis, may occur with the use of SGLT2 inhibitors. They may cause ketoacidosis, although it is rare (Davies *et al*., 2018; Chaudhury *et al*., 2017; Thrasher, 2017).

**Insulins**

Insulin remains the most potent glucose-lowering agent, particularly for patients with high HbA1c levels. Insulin is available in rapid-acting/prandial (lispro, aspart, glulisine), short-acting (human regular), intermediate-acting (human isophane [neutral protamine Hagedorn]), and premixed formulations (Thrasher, 2017). The main advantage of insulin over other glucose-lowering medications is that insulin lowers glucose in a dose-dependent manner over a wide range, to almost any glycaemic target as limited by hypoglycaemia.

Older formulations of insulin have demonstrated reduction in microvascular complications (Davies *et al*., 2018). The disadvantages of insulin include weight gain and the need for injection, frequent titration for optimal efficacy and glucose monitoring. The effectiveness of insulin depends highly on its appropriate use; patient selection and training; adjustment of dose for changes in diet, activity or weight; and titration to acceptable and safe glucose targets (Davies *et al*., 2018).

### 2.8.2 Dietary and lifestyle adjustments in the management of diabetes

Lifestyle management is a fundamental aspect of diabetes care and includes diabetes self-management education, diabetes self-management support, nutrition therapy, physical activity, smoking cessation, counselling and psychosocial care. Clinical trials have shown that lifestyle modifications are cost effective and safe in preventing or delaying the onset of diabetes (ADA, 2019; Chaudhury *et al*., 2017).

Moderate weight loss (≈7% of body weight) is important in the prevention and treatment of diabetes, as it can improve blood glucose levels, and can also positively impact blood pressure and cholesterol levels (Chaudhury *et al*., 2017). Weight loss can be achieved through a healthy balanced diet, with control of total calories and free carbohydrates. Studies have suggested that consumption of complex dietary fibre and whole grains also improve glycaemic control (Reynolds *et al*., 2020).

Other studies have shown that exercise can improve glycaemic control by lowering HbA1C level by 0.66%, with or without significant decrease in body weight, and improve the total well-being of patients (Chaudhury *et al*., 2017; van Dijk and van Loon, 2015). The combination of dietary changes for weight reduction and physical exercise improves hyperglycaemia and reduces cardiovascular risk factors more than dietary interventions or physical activity alone (Davies *et al*., 2018).

Epidemiological evidence indicates that excessive time spent in sedentary behaviours (too much sitting) is associated with an increased risk of type 2 diabetes. Observational and experimental studies have shown consistent evidence that reducing and frequently breaking up prolonged sitting with light-intensity physical activities and standing may be practical strategies for improving type 2 diabetes prevention and management (Dempsey *et al*., 2016).

Other lifestyle measures considered in the diabetes treatment are moderate alcohol consumption (≤1 drink for women, ≤2 drinks for men). Consumption of alcohol, especially in a fasted state, can precipitate life-threatening hypoglycaemia and coma (Chaudhury *et al*., 2017). Binge drinking increases the risk of hypoglycaemia especially in sulfonylureas treated type 2 diabetes

patients (Pietraszek *et al*., 2010). Alcohol is particularly known to heighten insulin and sulfonylurea induced hypoglycaemia. Intake of alcohol stimulates glycogenolysis that could contribute to hyperglycaemia (Kalaria *et al*., 2021). Reduction in sodium intake especially in patients with comorbidities such as hypertension, habitual tobacco use, and lacking immunizations (influenza, diphtheria, pertussis, tetanus, pneumococcal, and hepatitis B) is also very important (Chaudhury *et al*., 2017). Hypertension is associated with a range of adverse outcomes in patients with type 2 diabetes. Dietary sodium intake is correlated with blood pressure levels therefore, it is assumed that any blood pressure lowering associated with reduced dietary salt intake may be translated into protection from end-organ damage in the context of diabetes (Ekinci *et al*., 2011). Patients with diabetes mellitus are at increased risk for hepatitis B virus (HBV) infection hence, HBV vaccination is recommended for adults with diabetes (Van Der Meeren *et al*., 2016). Cigarette smoking is associated with high triglyceride levels and the development of cardiovascular disease in type 1 diabetes patients (Reynolds *et al*., 2011).

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

The use of herbal products is not limited to dietary uses; they are also used in the treatment and prevention of several diseases including diabetes (**Figure 2.8**). The use of herbal medicines (also known as phytomedicine) as alternative medicinal therapy have a long history compared to conventional medicine. It dates back in Mesopotamia in 2600 B.C. and the oldest record on practice of medicinal plants for drug preparation was engraved on a Sumerian clay slab, created over 5,000 years ago. To date, medicinal plants are used as complementary and alternative therapy. Compared to modern medicines, phytomedicine, have the advantages of having few side effects and low cost (Choudhury *et al*., 2018; Rahimi-Madiseh *et al*., 2016).

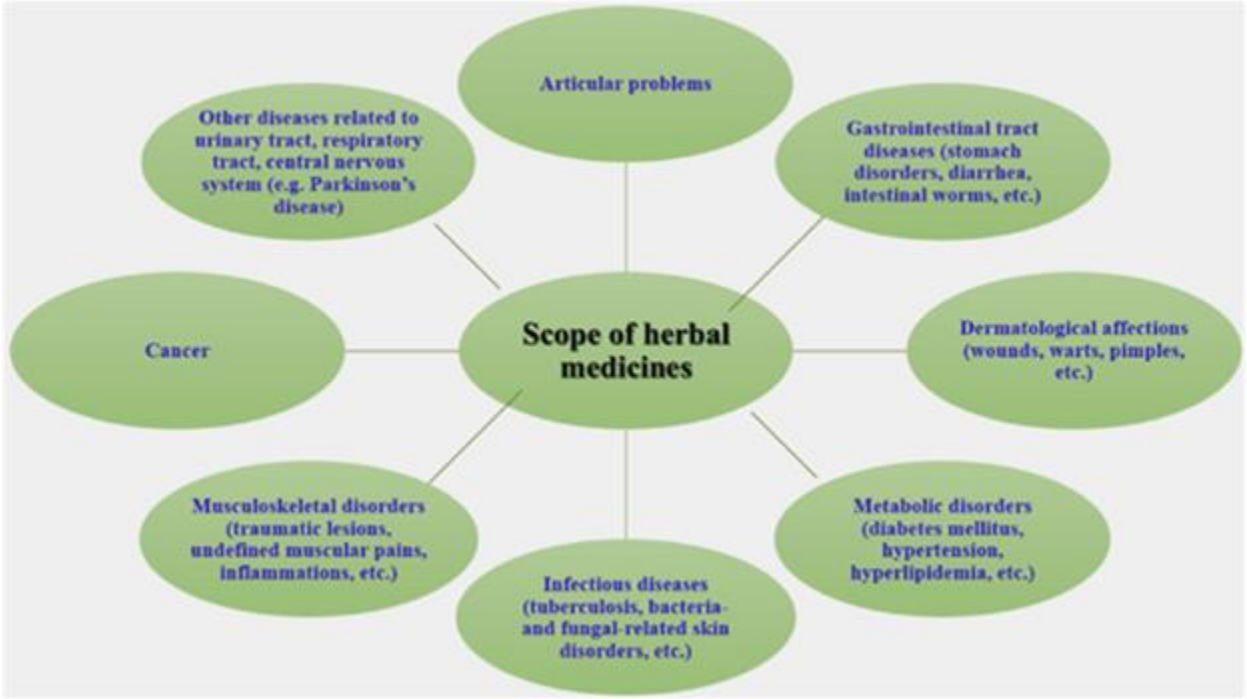



**Figure 2.8:** The traditional utilization of herbal medicines in different field of medical field (adopted without permission from Choudhury *et al*., 2018).

There has been a growing interest in herbal agents because of the side effects associated with oral hypoglycaemic agents used in the treatment of diabetes (El-Refaei *et al*., 2015). Several medicinal plants and their extracts have been reported have potential relevance in the treatment of diabetes as they are rich sources of flavonoids, gallotannins, amino acids and other related polyphenols which have antidiabetic, antihyperlipedaemic and antioxidant potentials (Hasan and Mohieldein, 2016; Vinayagam *et al*., 2016).

Polyphenols are a group of phytochemicals found in plant-based foods. They are classified into several categories such as flavonoids, phenolic acid, and stilbenes, based on the number of phenol rings and structural elements that bind the rings (Aryaeian *et al*., 2017). Polyphenols have been shown to prevent and manage Type 2 diabetes by protecting pancreatic islet β-cell, promoting β-cell proliferation, attenuating oxidative stress, activating insulin signalling, and secretion. They have also been shown to inhibit glucose absorption and formation of advanced glycation end products as well as ameliorating diabetic complications (Sun *et al*., 2020).

Among the polyphenols, the phenolic acids are known natural and dietary antioxidants present in fruits, vegetables and culinary herbs (Kumar and Goel, 2019). They are potent radical scavenging agents due to their electron donating attribute, forming stable phenoxy radicals that have the capacity to scavenge deleterious radicals and mitigate oxidative stress (Kumar and Goel, 2019). Also, their antidiabetic potentials have been documented (Vinayagam *et al*., 2016).

### 2.8.4 Plant-derived phenolic acids and their anti-diabetic and anti-oxidative properties

Phenolic acids belong to a class of plant polyphenols mostly found in vegetables, fruits and nuts (Oke *et al*., 2021; Vinayagam *et al*., 2016). Phenolic acids are aromatic carboxylic acids with hydroxyl derivatives that have only one phenolic ring in their structure derivatives (Sun *et al*., 2020). Phenolic acids are classified as hydroxybenzoic acid and hydroxycinnamic acid derivatives (Sun *et al*., 2020; Pandareesh *et al*., 2015). Hydroxybenzoic acid derivatives include gallic acid, protocatechuic acid, and *p*-hydroxybenzoic acid and the hydroxycinnamic acid derivatives include caffeic, p-coumaric, ferulic, and sinapic acids (Sun *et al*., 2020). Phenolic acids have been shown to have anti-diabetic and anti-oxidative effects such as improved insulin signalling, glucose uptake and insulin secretion as well as reduced blood glucose and glycated haemoglobin (Gandhi *et al*., 2014; Jung *et al*., 2006).

Caffeic acid is a natural phenolic compound widely distributed in plant-derived materials such as tea, wine and olive oil. It is also found in many types of fruit and in coffee in high concentrations (Adisakwattana *et al*., 2009). Caffeic acid has pharmacological properties such as antioxidant, anticancer and ant-mutagenic activities (Adisakwattana *et al*., 2009). A study by Chao *et al*. (2009), has demonstrated that caffeic acid is an agent against diabetes-associated cardiac inflammation. In the study, it was shown that dietary supplementation of caffeic acid improved glycaemic control and lipid metabolism in diabetic mice. Caffeic acid provided triglyceride lowering, as well as anti-coagulatory, antioxidant and anti-inflammatory protections for cardiac tissue. These findings show that caffeic acid might be helpful for the prevention of diabetic cardiomyopathy (Chao *et al*., 2009). Caffeic acid has also been shown to have therapeutic properties against diabetic kidney disease (Matboli *et al*., 2017).

Gallic acid is another phenolic acid found in tea, several fruits and wine (Ramachandran *et al*., 2015). Gallic has been known to have anti-diabetic, antioxidant and hypolipidemic effects. It decreases serum glucose, increases insulin levels and decreases total cholesterol, triglyceride and low-density lipoprotein cholesterol. Therefore, patients with diabetes may benefit from the anti-hyperlipidaemic effect of gallic acid (Ramachandran *et al*., 2015; Seo *et al*., 2009). Gallic acid has, also, been shown to exhibit radical scavenging and anti-lipid peroxidative activity, modulatory action on antioxidant enzymes in experimental diabetes (Ramkumar *et al*., 2014; Lee *et al*., 2009).

*p*-Hydroxybenzoic acid has been isolated from many sources including carrots, oil palm, grapes and from several other plants (Khadem and Marles, 2010). *p*-Hydroxybenzoic acid has been shown to have antioxidant activity (Dekdouk *et al*., 2015; Manuja *et al*., 2013). One study has shown that oral administration of *p*-Hydroxybenzoic acid caused a decrease in plasma glucose levels in diabetic rats. The results suggested that *p*-Hydroxybenzoic acid has insulin-like activities, resulting in hypoglycaemic effect mediated by an increase in peripheral glucose consumption (Peungvicha *et al*., 1998). A recent study also demonstrated the modulatory action of *p*-hydroxybenzoic acid on insulin secretion, PPAR-γ activity and hepatic glycogen storage as possible underlying antidiabetic mechanisms using *in silico*, *in vitro* and *in vivo* experimental models (Rosiles-Alanis *et al*., 2022).

For the purpose of this study, p-coumaric and syringic acids will be the phenolic acid of focus. p-coumaric acid (**Figure 2.9**) is found in plants and mushrooms in free or bound form. It is also widely distributed in fruits, vegetables and cereals (Ramachandran *et al*., 2015). p-Coumaric acid has antioxidant, anti-inflammatory, anti-mutagenic, anti-ulcer, anti-platelet and anti-cancer activities (Shairibha *et al*., 2014). p-Coumaric acid alleviates several conditions including, atherosclerosis, oxidative cardiac damage and diabetes (Pei *et al*., 2016s). It can alleviate diabetes by reducing the intestinal absorption of dietary carbohydrate, regulating enzymes involved in glucose metabolism, stimulating insulin secretion and increasing antioxidant and anti-inflammatory properties (Bahadoran *et al*., 2013). p-Coumaric acid inhibits α-amylase, α-glucosidase and β-glucosidase (Aleixandre *et al*., 2022; Jeong *et al*., 2012;), hence may mitigate postprandial hyperglycaemia in diabetic state. p-Coumaric acid increases insulin sensitivity and inhibits adipogenesis (Pei *et al*., 2016). p-Coumaric acid have been shown to suppress

hippocampal neurodegeneration in type 2 diabetic rats by potentiating antioxidant, anti-inflammatory and anti-apoptotic effects. It has, also, been shown that p-coumaric acid exhibited an antidiabetic effect by upregulating the mRNA expression of PPARγ in diabetic rats (Abdel-Moneim *et al*., 2018). Another study demonstrated that p-coumaric acid ameliorated diabetic nephropathy in diabetic rats (Zabad *et al*., 2019).

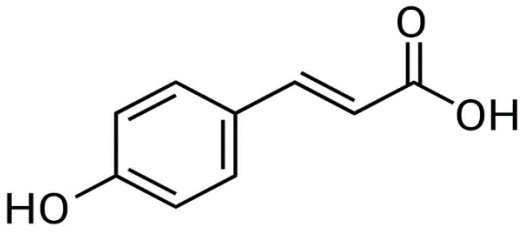



**Figure 2.9:** Chemical structure of p-coumaric acid.

Syringic acid (**Figure 2.10**) is one of the phenolic compounds that are widely distributed in a variety of fruits 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 (Rashedinia *et al*., 2020; Sabahi *et al*., 2020).

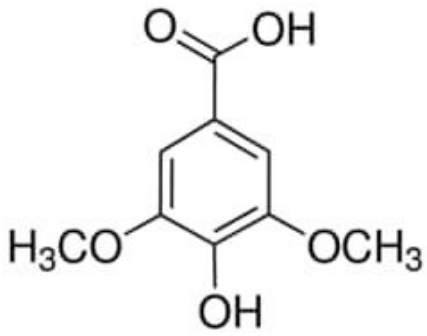



**Figure 2.10:** Chemical structure of syringic acid.

Syringic acid have been shown to ameliorate oxidative stress, reduce blood glucose and improve diabetes-induced alterations of enzyme markers of hepatic tissue damage in streptozotocin induced diabetic rats (Sabahi *et al*., 2020). It has also been shown to improve the renal antioxidant status of diabetic rats by increasing glutathione 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). Syringic acid has been shown to inhibit *in vitro* protein glycation and formation of advanced glycation end products (Bhattacherjee and Datta, 2015; Wu *et al*., 2010), suggesting its potential to mitigate diabetic oxidative vascular complications

While natural and dietary phenolics have shown promise as therapeutic agent to manage diabetes and oxidative complications, mineral supplements and vitamins play key role in metabolic processes, which are beneficial to human health, including oxidative and metabolic health (Wong *et al*., 2020; Liu *et al*., 2018).

### 2.8.5 Minerals and supplements in diabetes management

Vitamins and minerals play a major role in glucose metabolism and are, therefore, recommended to diabetes mellitus patients (Laclaustra *et al*., 2009). Vitamins have antidiabetic properties mainly because they protect islets against oxidative stress. The key vitamins are vitamin A, vitamin C, vitamin D, vitamin E biotin and nicotinamide (Singh and Venkatesan, 2016).

Vitamin D supplementation improves glycaemic control in patients with type 2 diabetes, impaired fasting glucose or normal glucose tolerance, as well as elevated HbA1C levels (Hu *et al*., 2019). Vitamin D is known to have immuno-modulatory and anti-inflammatory effects, which could improve peripheral insulin resistance and increase insulin secretion in type 2 diabetes by altering the balance between intracellular and extracellular calcium in $\beta$ cells (Krul-Poel, *et al*., 2017; Davi *et al*., 2010). Pancreatic beta-cells are direct target of vitamin D because they contain the vitamin D receptor. Studies have suggested that vitamin D and calcium may promote $\beta$-cell function and insulin sensitivity (Davi *et al*., 2010). The mechanism of vitamin D action may be through; nuclear factor kappa B (NF-κB) inhibition or translocation; chemokine, cytokine and major histocompatibility complex class 1 reduction; non-genomic interaction of vitamin D with putative plasma membrane vitamin D receptor; reduction of Nitric oxide; and prevention of apoptosis (Singh and Venkatesan, 2016).

Vitamin C is essential for optimum beta cell function as it modulates calcium channel of beta-cell. Deficiency of vitamin C leads to decreased ATP generation with glucose stimulation (Singh and Venkatesan, 2016). Vitamin C reduces fasting and postprandial oxidative stress (Chambial *et al*., 2013). It reacts directly with oxygen before oxidation, preventing reactions that can lead to protein glycation (Davi *et al*, 2010; Martini *et al*., 2010). In a study conducted by Ceriello *et al*. (2013), it was shown that vitamin C reduces the generation of oxidative stress and inflammation and improve endothelial dysfunction. This was observed during induced acute hypoglycaemia (Ceriello *et al*., 2013).

Vitamin A deficiency causes increased alpha cell/beta cell ratio, hyperglycaemia, and hyperglucagonemia and the re-introduction of vitamin A restores this effect (Trasino *et al*., 2015). Vitamin A also prevents beta-cell apoptosis. Vitamin E protects islet by conferring resistance against nitric oxide induced toxicity. Biotin increases beta cell function, and nicotinamide is known to protect human beta-cells against free-radical induced necrosis (Singh and Venkatesan, 2016).

Micronutrients such as zinc, chromium, copper, magnesium, iron, manganese and selenium are known for their health benefits. Trace elements function as essential coenzymes and cofactors for metabolic processes required in the maintenance of normal glucose, lipid and protein metabolism. Their deficiencies have been associated with diabetes (O'Connell, 2001; Mooradian *et al*., 1994).

Chromium is an essential trace element involved in glucose, insulin and lipid metabolism. It functions as a cofactor in insulin regulating activities. Chromium facilitates insulin binding and subsequent uptake of glucose in the cell. It decreases fasting glucose levels, improves glucose tolerance, lowers insulin levels, decreases total cholesterol and triglycerides and increases high-density lipoprotein cholesterol in type 2 diabetes patients (Mooradian *et al*., 1994). Chromium deficiency has been implicated in emergence of glucose intolerance and chromium supplementation reverse glucose intolerance (O'Connell, 2001).

Copper (Cu) is an essential heavy metal since it is a cofactor of several enzymes that are important in many metabolic processes but also highly toxic to the cell as it can enhance reactive oxygen

species formation (Lowe *et al.*, 2017). In diabetes, impaired copper regulation can manifest in defective antioxidant defences, damage to the blood vessels, heart, kidneys, retina and nerves (Cooper, 2012). It is, therefore, essential to manage Cu homeostasis for the treatment of diabetes. Metal chelation drugs could be considered as a promising alternative especially for Cu ions. These can decrease reactive oxygen species, thus ameliorate diabetes complications (Lowe *et al*., 2017).

Magnesium (Mg) regulates transport of glucose across cell membranes. It is a cofactor in various enzymatic pathways involved in glucose oxidation. Mg deficiency is common in type 2 diabetes and has been associated with complications of diabetes, especially retinopathy (Mooradian *et al*., 1994; O'Connell, 2001). Mg deficiency affects the post- receptor signalling of insulin (Lopez-Ridaura *et al*., 2004). Studies have shown that Mg supplementation improves insulin-mediated glucose disposal and insulin secretion as well as elevation of high-density lipoprotein cholesterol level in patients with type 2 diabetes (Song *et al*., 2006; Song *et al*., 2004; de Valk, 1999).

Manganese (Mn) is required for normal insulin synthesis and secretion (Lee *et al*., 2013). Mn serves as a cofactor for many enzymes, including arginase, pyruvate carboxylase and manganese superoxide dismutase (MnSOD) (Burlet and Jain, 2013). MnSOD is a major mitochondrial antioxidant, and it protects the cell from reactive oxygen species. MnSOD catalyse the conversion of superoxide radicals to hydrogen peroxide, which is further metabolised to water (Burlet and Jain, 2013). Mitochondrial dysfunction is both a contribution mechanism and complication of diabetes and oxidative stress contributes to the dysfunction (Lee *et al*., 2013). It has been shown that changes in dietary $Mn^{2+}$ induce modulatory changes in MnSOD activity. Supplementation of $Mn^{2+}$ has been reported to have a beneficial effect on lipid metabolism, by decreasing serum cholesterol, aorta cholesterol and reducing the risk of atherosclerosis. *In vitro* and *in vivo* studies have demonstrated that $Mn^{2+}$ supplementation can lead to a decrease in monocyte adhesion to endothelial cells and lower the risk of endothelial dysfunction in diabetes. Endothelial cells play a role in vascular maintenance and angiogenesis and contribute to wound healing in type 1 and type 2 diabetes patients (Burlet and Jain, 2013). A Study conducted by Madsen-Bouterse *et al*. (2010) have demonstrated that overexpression of MnSOD has a potential of preventing the development of retinopathy in diabetic patients (Madsen-Bouterse *et al*., 2010).

Selenium is a key component of a number of selenoproteins involved in the antioxidant defence systems, which protect against oxidative damages caused by free radicals and oxygen species. Increased free radical level impairs glucose stimulated insulin secretion (Park *et al*., 2012: Stranges *et al*., 2010; Bleys *et al*., 2007). Selenium is involved in a complex system of defence against oxidative stress. Because of its antioxidant properties, selenium might prevent the development of diabetes and oxidative complications (Bleys *et al*., 2007). Studies have shown that selenium deficiency impaired islet function and free radical scavenging systems in rats, resulting in decreased insulin secretory reserve (Zhou *et al*., 2013). Human trials have shown that selenium consumption in type 2 diabetic patients could improve glycaemic control and lipid profile (Karalis, 2019).

In recent years zinc has gained considerable attention as a therapeutic supplement for diabetes, which is attributed to its role in insulin secretion and action (Chabosseau and Rutter, 2016). Also, clinical research has suggested that deficiency of zinc can increase the risk of glucose intolerance and diabetic complications (Kazi *et al*., 2008; Al-Maroof and Al-Sharbatti, 2006). Zinc is required as a cofactor in protein, lipid, and glucose metabolism. It is involved in synthesis, storage, secretion, and conformational integrity of insulin monomers. Zinc supplementation has been reported to improve insulin sensitivity in patients with T2D (Zargar *et al*., 1998). Zinc supplementation in T2D increased serum zinc level, improved glycaemic control and promotes healthy lipid parameters (Al-Maroof and Al-Sharbatti, 2006).

### 2.8.6 Role of Zn(II) in insulin storage and secretion

Zinc is present in all human tissues with pancreas and bone having a particularly high content, which shows its importance for human health. In mammalian pancreas, $Zn^{2+}$ is essential for the correct processing, storage, secretion and action insulin in β-cells (Nygaard *et al*., 2014; Li, 2014; Slepchenko and Li, 2012). Inactive insulin is formed in the cellular ribosomes, and it is composed of 2 polypeptide chains (A and B chains) connected by 2 pairs of di-sulfide bonds and an additional intramolecular di-sulfide bond in the A chain (Chabosseau and Rutter, 2016). It is stored in β-cells of the pancreas as a hexamer containing two Zinc ions (Zhao, 2019). Part of the zinc contained in the β-cells and concentrated within the dense core of insulin secreting granules helps in insulin preservation (Chabosseau and Rutter, 2016). β-cells are rich in zinc ($Zn^{2+}$) because it is required

for zinc-insulin crystallization within secretory vesicles and also for its exocrine and endocrine functions (Nygaard *et al*., 2014; Chimienti *et al*., 2006).

As insulin accumulates in the maturing granules, it is initially stored as a monomer, then forms a dimer. In the presence of zinc, the dimers aggregate to form a hexamer around two zinc ions. The process of hexamerization reduces insulin solubility and triggers its crystallization, increasing the storage capacity of the insulin secreting vesicles. When insulin is secreted, the hexamers are converted to active monomers. During this process, significant concentration of zinc ions is released (Chabosseau and Rutter, 2016). Insulin secretion is correlated with zinc release by the β-cells in response to glucose stimulation. This contributes to the paracrine communication in the pancreatic islets. In the presence of zinc within the cell, insulin assembles to a hexamer with two zinc ions and one calcium ions in the centre. This form of insulin is stored and secreted as insulin-zinc crystal (Davidson *et al*., 2014; Nygaard *et al*., 2014; Slepchenko and Li, 2012; Chimienti *et al*., 2005). $Zn^{2+}$ regulates the activity of ATP-sensitive potassium channels and calcium ($Ca^{2+}$) channels, which are involved in glucose induced insulin secretion (Nygaard *et al*., 2014).

Studies have shown that zinc-transporting proteins (zinc-regulated transporter, iron-regulated transporter-like proteins [ZIPs] and zinc transporters [ZnTs]) and metal-buffering proteins (metallothioneins, MTs) regulate intracellular zinc homeostasis (Nygaard *et al*., 2014). Zinc transporter ZnT-8 has been suggested to be a key protein for both zinc accumulation and regulation of insulin secretion in pancreatic β-cells (Chimienti, 2013; Chimienti *et al*., 2006). Zinc transporter ZnT-8 is localized in secretion vesicles membrane and facilitates the import of cytosolic $Zn^{2+}$ into secretory granules. This transporter enables the accumulation of zinc from the cytoplasm into intracellular insulin-containing vesicles and it is a major component providing zinc for insulin maturation and/or storage in insulin-secreting pancreatic beta cells (Rutter *et al*., 2016; Chimienti *et al*., 2005). $Zn^{2+}$ is not only essential for synthesis, storage and structural stability of insulin, but also to protect against oxidative stress in type 1 and type 2 diabetes and their associated pathologies (Rutter *et al*., 2016).

### 2.8.7 Zn(II) in diabetes management

Zinc (Zn) can act as an intracellular signalling molecule and regulate cellular metabolism and functional activities (Slepchenko and Li, 2012). Zinc is important in insulin action and carbohydrate metabolism. Zinc is a structural part of key antioxidant enzymes such as superoxide dismutase, and its deficiency impairs their synthesis, leading to increased oxidative stress (Marreiro *et al*., 2017; Ruz *et al*., 2013).

In adipocytes isolated from rats, zinc has been shown to exert modulatory and suppressive effects on glucose uptake and lipolysis, respectively, while treatment in type 2 diabetes KK-A$^{y}$ mice exerted glycaemic control (Adachi *et al*., 2004). Zn(II) promotes endogenous glycogen synthesis by inhibiting the action of endogenous glycogen synthase kinase-3β (Ilouz *et al*., 2002). The modulatory action of Zn(II) on cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase activity has been documented (Percival *et al*., 1997; Yoshikawa *et al*., 2004), which potentiates downregulation of cAMP-mediated lipolytic and glycogenolytic signalling. *In vitro*, the activity of glycolytic enzymes has been shown to be modulated by Zn(II) (Tamaki *et al*., 1983).

A study conducted on *db/db* showed that Zn supplementation attenuates hyperglycaemia and hyperinsulinemia (Simon and Taylor, 2001). A study on streptozotocin-induced diabetic rats revealed that zinc supplementation improves polydipsia and increased high-density lipoprotein cholesterol levels. The level of maldondialdehyde (MDA), which is an oxidative stress marker, was reduced in the liver of high fat-fed rats, suggesting a hepatoprotective potential. An increase in the expression of metallothioneins in the liver of rats was also observed (Wang *et al*., 2012). Treatment with zinc in type 2 diabetes KK-A$^{y}$ mice exerted glycaemic control (Adachi *et al*., 2004). Deleting Zinc transport family member 8 (ZnT8) genes that are involved in pancreatic Zn(II) transport was shown to cause impaired glucose tolerance in mice (Nicolson *et al*., 2009). Zinc supplementation in streptozotocin-induced diabetic rats exerted significant control on hyperglycaemia and hypoinsulinemia (Barman and Srinivasan, 2016). Reduction in protein glycosylation, glucosuria, and urinary excretion of proteins and urea in the diabetic animals maintained on a zinc supplemented diet was also observed. The diabetic rats also showed higher plasma albumin and lower plasma urea and creatinine levels (Barman and Srinivasan, 2016),

suggesting the renal protective potential of zinc. Zinc-supplemented diet has also been shown to have an antidiabetic effect in diabetic rats by stimulating pancreatic islet β-cells regeneration, reducing body weight gain and lowering of blood glucose level (Anyakudo and Adewunmi, 2017).

Human studies have also shown that zinc supplementation has beneficial effects on glycaemic control and lipid profile in type-2 diabetes (Rutter *et al*., 2016; Ranasinghe *et al*., 2015; Jayawardena *et al*., 2012). In different studies, zinc supplementation in the form of $ZnSO_4$, $Zn_3(PO_4)_2$, zinc acetate and zinc gluconate had several beneficial metabolic and clinical effects in type diabetic patients. These benefits included improved glycaemic control and lipid parameters. Zinc supplementation caused significant reduction in fasting blood glucose, post prandial blood glucose, HbA1c, blood glucagon, total cholesterol, low-density lipoprotein cholesterol and triglyceride levels, while increasing serum insulin and high-density lipoprotein cholesterol (Afkhami-Ardekani *et al*., 2008; Al-Maroof *et al*., 2006; Kadhim *et al*., 2006; Partida-Hernndez *et al*., 2006; Hayee *et al*., 2005; Gupta *et al*., 1998; Hegazi *et al*., 1992).

In both type 1 and type 2 diabetic patients, zinc gluconate exerted anti-lipid peroxidative effects suggesting its potential to mitigate oxidative insults in diabetes (Anderson *et al*., 2001; Faure *et al*., 1995). In another study, adult subjects with HbA1c >7.5% were supplemented for six months with 30 mg/day of zinc as zinc gluconate or placebo. The effect of zinc supplementation on plasma zinc (Zn), plasma thiobarbituric acid reactive substances (TBARS), Cu-Zn superoxide dismutase (SOD) and glutathione peroxidase activities (GPX) in red blood cells, blood lipids and lipoproteins, HbA1c and fasting glucose were measured at the beginning of the study and after three and six months. The results showed a decrease of plasma TBARS, no alteration of HbA1c and glucose homeostasis hence potential antioxidant effects of Zn supplementation in persons with type 2 diabetes (Roussel *et al*., 2003).

The diabetes-related therapeutic potential of zinc has led to its use as a precursor in synthesizing Zn(II) complexes with glycaemic control effects. Zinc has been complexed with different types of ligands, including natural organic ligands, synthetic organic ligands, and supplements to synthesize Zn(II) complexes with insulin mimetic and modulatory effects (Chukwuma *et al*., 2020).

### 2.8.8 Antidiabetic effects of Zn(II) complexes

In the recent past years, new concept for treating diabetes mellitus has been investigated. This involves the use of several metal complexes such as vanadium, manganese, cobalt, selenium and zinc in place of insulin injection and synthetic therapeutics for type 1 and type 2 diabetes (Fugono *et al*., 2002). Zinc, however, has gained notable attention as a promising metal for synthesizing potent insulin mimetic complexes (**Table 2.4**).

Adachi *et al*. (2004) reported the modulatory and suppressive effects of Zn(II)-maltol and Zn(II)-allixin complexes on rat adipocytes' glucose uptake and lipolysis, respectively. Also, the complexes ameliorated hyperglycaemia in T2D KK-Ay mice (Adachi *et al*., 2004). Zinc complexes of picolinic acid and threonine has been shown to reduce blood glucose and HbA1c in T2D KK-Ay (Kojima *et al*., 2002). In adipocytes the complexes potentiated insulin mimetic effects by modulating the action of insulin signalling proteins, including insulin receptor tyrosine kinase, phosphodiesterase, phosphoinositide 3-kinase and glucose transporter type 4 (Yoshikawa *et al*., 2004).

In a study conducted by Yoshikawa *et al*. (2003), the insulin mimetic activity of bis(L-carnitinato)Zn(II) complex, $Zn(car)_2Cl_2$, using isolated rat adipocytes treated with epinephrine was evaluated. In this study, the complex inhibited the release of free fatty acid. In addition, the complex was found to have *in vitro* insulin mimetic activity. The insulin mimetic activity of the complex was evaluated by using isolated rat adipocytes treated with epinephrine in terms of the inhibition of free fatty acid release. The inhibitory effect of $Zn(car)_2Cl_2$ was compared with those of $VOSO_4$ and $ZnSO_4$ as positive controls. The inhibitory concentration of the free fatty acid released in $Zn(car)_2Cl_2$ was found to be more active ($IC_{50}$ = 0.80±0.05mM) than $VOSO_4$ ($IC_{50}$ = 1.00±0.08mM) and almost the same activity as $ZnSO_4$ ($IC_{50}$ = 0.81±0.10mM). The complex was also found to have *in vivo* blood glucose lowering effect. It lowered the blood glucose levels KK-$A^y$ mice with type 2 diabetes mellitus when administered orally and improved glucose tolerance (Yoshikawa *et al*., 2003).

In a study by Kojima *et al*. (2003), Zn(II) complexed with L-lactic acid (lac), and D-(2)-quinic acid (qui) ($[Zn(lac)_2]_2$ and $Zn(qui)_2$) have been reported to lower blood glucose in KK-$A^y$ mice

with type 2 diabetes, when injected intraperitoneally (Kojima *et al*., 2003). $HbA1_c$ and serum parameters results suggested that the blood glucose lowering effect of $Zn(qui)_2$ complex was long-term compared to that of $[Zn(lac)_2]_2$. $Zn(qui)_2$, $[Zn(lac)_2]_2$ and $Zn(bet)_3(ClO_4)_2$ were found to have insulin mimetic activities *in vitro.* These was determined by inhibition of the release of FFAs from isolated rat adipocytes treated with epinephrine (Kojima *et al*., 2003). *bis*(3-hydroxy-2-methyl-4(H)-pyran-4-thiono)Zn, [Zn(hmpt)2] and *bis*(3-hydroxy-2-methyl-4(H)-pyran-4-seleno)Zn, [Zn(hmps)2] complexes has also been shown to have potent blood glucose-lowering effects and result in improved HbA1c values in KK-$A^y$ mice with type 2 diabetes (Nishiguchi *et al*., 2017).

Some other Zn(II) complexes that have *in vitro* insulin-mimetic activity include Bis(aspirinato)zinc(II) Complex and di(2-mercaptotropolonato)zinc(II) (ZT2) (Kolesa-Dobravc *et al*., 2018; Murakami *et al*., 2012; Yoshikawa *et al*., 2011). The inhibitory effects on free fatty acid release from the isolated rat adipocytes treated with epinephrine were measured on selected vanadium and zinc complexes. All the studied complexes showed the insulin-mimetic activity by inhibition of free fatty acid release from isolated rat adipocytes (Kolesa-Dobravc *et al*., 2018).

Zn-3,4-heptanedione-bis($N^4$-methylthiosemicarbazonato) (Zn-HTSM) and bis(1,2-dimethyl-3-hydroxy-4(1H)-pyridinonate)zinc(II), $Zn(dmpp)_2$, have, also, demonstrated antidiabetic activity *in vivo* (Kadowaki *et al*., 2013; Moniz *et al*., 2011). Antidiabetic activity of Zn-3,4-heptanedione-bis($N^4$-methylthiosemicarbazonato) (Zn-HTSM) complex was examined in KK-$A^y$ type 2 diabetes mice. It was shown that the complex had potent hypoglycaemic activity, improved glucose intolerance and had lipophilicity for cellular permeability (Kadowaki *et al*., 2013).

**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 (IC50 = 0.54 mM) and increased glucose uptake (EC50 = 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 (IC50 = 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 (IC50 = 145µM) and increased glucose uptake (EC50 = 19µM) in isolated rat adipocytes | Murakami *et al*., 2012 |

| Class of complex | Ligands | Complex | $C_M$ | Antidiabetic activity | Reference |
|---|---|---|---|---|---|
| | α-furonic acid | Bis(α-furonate)zinc(II) | $Zn(O_4)$ | Inhibited lipolysis (IC50 = 0.31mM) ad increased glucose uptake (EC50 = 0.23mM) in isolated rat adipocytes | Nishide *et al*., 2008 |
| 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 IC50 =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 (IC50 = 0.63mM) | Yoshikawa *et al*., 2001 |

Adopted 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.

The above-mentioned studies suggest zinc complexes have potential for clinical application and development of new and more effective nutraceuticals for diabetes. However, for the development of metallopharmaceutics, intense research of zinc complexes on long term toxicity and side effects is deemed essential. Advances in research have started exploring natural phenolics as alternative ligands for Zn(II), which is perceived to result in a complex with better safety profile due to the natural attributes of phenolics. Also, considering the diabetes and oxidative stress related pharmacological potential of natural polyphenols (Umeno *et al*., 2016), it is most probable that Zn(II) complexes of natural polyphenols will possess potent antidiabetic and antioxidative profile, mediated through several mechanisms. A few studies on some Zn(II) complexes of some plant flavonoids support the medicinal potentials of Zn(II) complexes of plant derived polyphenols.

### 2.8.9 Antidiabetic and antioxidative potentials of Zn (II) complexes of plant-derived polyphenols

Several zinc complexes have been formulated using plant secondary metabolites as ligands, and their antidiabetic properties evaluated. Gopalakrishnan *et al*. (2015) conducted a study to evaluate hypoglycaemic properties of zinc-diosmin complex. Diosmin (diosmetin-7-O-rutinoside) is a naturally occurring bioflavon and it has been reported to have a wide range of pharmacological properties including antioxidant and antidiabetic properties. In their study, zinc-diosmin complex was orally administrated in high fat diet fed-low dose streptozotocin (STZ) induced experimental type 2 diabetic rats. The diabetic rats were treated with the complex at a concentration of 20mg/kg bw/rat/day for 30 days. The results showed that treatment with zinc-diosmin complex improved insulin sensitivity by enhancing protein metabolism and alteration in the levels of muscle and liver glycogen. Treatment with zinc-diosmin complex also reduced blood glucose and glycated haemoglobin (HbA1c) levels. Furthermore, it improved glucose tolerance, insulin resistance in high-fat diet and streptozotocin-induced type 2 diabetes Wistar rat (Gopalakrishnan *et al*., 2015).

In another study, a Zn–morin complex also demonstrated antidiabetic and antioxidant potentials in high fat diet (HFD)-fed streptozotocin (STZ) induced diabetic rats (Sendrayaperumal *et al*., 2014). After oral administration of the complex to diabetic rats (5 mg/kg body weight/day) for 30 days, there was a decrease in blood glucose and HbA1c levels. The complex also improved hyperglycaemia, glucose intolerance, and insulin resistance (Sendrayaperumal *et al*., 2014).

**Table 2.5:** Antidiabetic effects of Zn(II) complexes with plant-derived polyphenols as ligands

| Ligands | Complex | $C_M$ | Antidiabetic activity | Reference |
|---|---|---|---|---|
| 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 levels 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 |
| Silibinin | Bis(silibinin)zinc(II) complex | $Zn(O_4)$ | 30 d oral treatment (5 mg/kg bw) reduced BG (≃56%) and HbA1c (≃43%) levels; increased insulin concentration (≃122%); and improved pancreas histology and antioxidant status in HFD and STZ-induced T2D Wistar rats | Umamaheswari and Subramanian, 2015 |

Adopted without permission from Chukwuma *et al*., 2020. Abbreviations: $C_M$, coordination mode; BG, blood glucose; HFD, high-fat diet; HbA1c, glycated haemoglobin; GT, glucose tolerance; IR, insulin resistance; T2D, type 2 diabetes; STZ, streptozotocin.

Treatment of STZ-induced diabetic rats with zinc-flavonol complex have, also, been reported to improve antioxidant level and suppress inflammatory and oxidative stress markers NF-κB p65, NO, TNF-α, IL-1β and IL-6 levels. Histological observations showed that the complex protected pancreatic β-cells against oxidative damage (Vijayaraghavan *et al*., 2013).

Ali-Ali *et al*. (2016) reported the antidiabetic properties of curcumin-zinc complex in STZ-induced diabetic rats. Curcumin (diferuloylmethane) is the most active component of turmeric, and it has been reported to have a potential in the treatment of diabetes. Treatment of STZ-induced diabetic rats with the curcumin-zinc complex resulted in reduced blood glucose, glycosylated haemoglobin (Hb)A1c, and lipid profile parameters levels, as well as an improvement in plasma insulin levels **(**Ali-Ali *et al*, 2016). **Table 2.5** shows antidiabetic effects of some Zn(II) complexes with plant-derived polyphenols as ligands.

## 2.9 Problem statement

The tendency to explore natural polyphenols as ligands in the synthesis of antidiabetic Zn(II) complexes is fuelled by not only their potential health benefits, but their natural origin as well, which may potentially afford their Zn(II) complexes improved pharmacological and safety profiles. It is, however, unfortunate that the previous research trajectory on Zn(II) complexes have favoured the use of synthetic organic ligands, which have scarce information on their pharmacological effects and elusive safety profile as compared to the natural counterparts. In fact, a 2020 review done on reported Zn(II) complexes revealed that Zn(II) complexes of synthetic organic ligands comprised about 72% of the studied Zn(II) complexes (Chukwuma *et al*., 2020), which depicts an unwanted paradigm shift towards synthetic therapy as opposed to natural and functional medicine. Natural polyphenols have been scarcely studied as ligands for antidiabetic Zn(II) complexes, making up only 5% of the ligands used in synthesizing the studied complexes (Chukwuma *et al*., 2020). In fact, simple phenolic acids, which are known dietary antioxidants with antidiabetic potentials (Vinayagam *et al*., 2016) were not explored as ligands for antidiabetic Zn(II) complexes (Chukwuma *et al*., 2020). Syringic acid and p-coumaric acid are natural phenolic acids with potent antioxidant and glycaemic control potentials (Shen *et al*., 2019; Srinivasulu *et al*., 2018; Amalan *et al*., 2016) and may be promising alternative ligands for synthesizing Zn(II) complexes with improved and broader antidiabetic and antioxidative profiles, as well as

appreciable safety profiles. However, this hypothetical reasoning has not been investigated or studied.

## 2.10 Aim and objectives

The aim and objectives of this study are given below.

### 2.10.1 Aim

The aim of this study was to investigate the antidiabetic and antioxidant effect of novel syringic acid and p-coumaric acid zinc complexes.

### 2.10.2 Objectives

The following were the objectives of this study:

- Synthesis of Zn(II) complexes of syringic acid and p-coumaric acid.
- Spectrophotometric characterization of the synthesized complexes
- Evaluation of the cytotoxicity of the complexes
- *In vitro*, *in silico* and *ex vivo* antidiabetic and antioxidative evaluation of the complexes
- *In vivo* antidiabetic and antioxidative investigation of the most potent complex
- Elucidation of the possible mechanism(s) of actions of the complexes

# CHAPTER 3

## MATERIALS AND METHODS

Unless otherwise mentioned, all reagents and chemicals used in this section were purchased from Sigma Aldrich, Johannesburg, South Africa.

### 3.1 Synthesis of the complexes

#### 3.1.1 Zn(II) complex of p-coumaric acid

Zn(II) was complexed with p-coumaric acid in a 1:2 mole ratio using a previous method (Kalinowska *et al*., 2011) with some modifications. First, 287.56 mg of Zn(II) sulphate heptahydrate (Mr = 287.56 g/mol) and 396.34 mg of p-coumaric acid (Mr = 164,08 g/mol) were separately dissolved in 5 mL of distilled water and 5 mL of methanol, respectively. A solution (168.02 mg in 5 mL of distilled water) of sodium hydrogen carbonate (Mr 84.01 g/mol) was then added to the p-coumaric acid solution. The mixture was stirred until no effervescence was observed. Thereafter, the Zn(II) sulphate heptahydrate solution was gradually added to the mixture, while stirring. Stirring continued until complete precipitation of a white precipitate. The mixture was filtered using a filter paper to recover the precipitate. The precipitate was washed thrice with 50% methanol and freeze dried (Alpha 1-2 LDplus Freeze Dryer, Martin Christ, Osterode am Harz, Germany).

#### 3.1.2 Zn(II) complex of syringic acid

The complex was formed from Zn(II) mineral and syringic by using a mole ratio of 1:2, respectively. A previous method (Kalinowska *et al*., 2011) with some modifications was used for the synthesis. Initially, 287.56 mg of $ZnSO_4.7H_2O$ (molar mass = 287.56 g/mol) and 396.34 mg of syringic acid (molar mass = 198.17 g/mol) were respectively dissolved in distilled water and methanol. The volume of the solvents was 5 mL. Next, 5 mL of a 0.4 M $NaHCO_3$ aqueous solution (molar mass = 84.01 g/mol) was mixed with the syringic acid solution. The mixture was stirred until no effervescence was observed. While stirring, the $ZnSO_4.7H_2O$ solution was gradually added. The mixture was stirred continuously until the complete precipitation of a white substance.

A filter paper was used to recover the precipitate. A 50% methanol solution was used to wash the precipitate three times. The precipitate was freeze-dried (Alpha 1-2 LDplus Freeze Dryer, Martin Christ, Osterode am Harz, Germany).

## 3.2 Spectrophotometric characterization

### 3.2.1 Fourier-transform infrared spectroscopy (FT-IR)

For FT-IR analysis, the instrument used was a Perkin Elmer Spectrum 100 FT-IR Spectrometer (MA, USA). An ART accessory was attached to the instrument. Onto to the sample holder, ≈ 2 mg of the complexes or their respective phenolic acids was loaded. The sample was scanned from 4000 $cm^{-1}$ to 380 $cm^{-1}$. A scan rate of 40 $s^{-1}$ was applied. Data acquisition was done using a Spectrum Software V 6.3.4, which recorded the FT-IR spectra.

### 3.2.2 Proton nuclear magnetic resonance spectroscopy ($^1$H NMR)

For $^1$H NMR, experiment was done using a 400 MHz Bruker Avance spectrometer (Bruker Corporation, MA, USA) and an MNOVA software was used for the data acquisition, visualization, and analysis. The sample was dissolved in deuterated DMSO (DMSO-d6; δH = 2.50) and a tetramethylsilane internal standard was used in the experiment. The chemical shifts were presented in parts per million after recording. Recording was done in the delta ($\delta$) scale. The coupling constants ($J$) were all set correctly to 0.01 Hz.

### 3.2.3 High-resolution mass spectroscopy (HR-MS)

The Central Analytical Facility, which is located at Stellenbosch University, Cape Town performed the high-resolution mass spectroscopic (HR-MS) analysis. A Waters Synapt G2 (Waters Corporation, MA, USA), coupled to an ESI probe and ESI Pos was the instrument used for analysis. The instrument was supplied with a cone voltage of 15 V. To perform the HR-MS, 1 mg of sample was dissolved in 1 mL of methanol (Romil), followed by a further 10-fold dilution into methanol. Then, 2 µL of the sample was injected into a stream of methanol flowing at 0.3 mL/min, using a Waters ultra-pressure liquid chromatography (UPLC) system (Waters, Mitford, USA), which conveyed the sample to a Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer used for high-resolution accurate mass analysis. Data was acquired in scan mode.

The mass spectrometer was optimized for best sensitivity. The cone voltage was 15 V. The desolvation gas was nitrogen at 650 L/h and desolvation temperature 275°C. The instrument was operated with an electrospray ionization probe in the positive mode. Sodium formate was used for calibration and leucine encephalin was infused in the background as lock mass for accurate mass determination.

### 3.3 Measurement of possible cytotoxic effect of the complex on Chang liver cells

American Type Culture Collection (ATCC), Virginia, USA (Chang live cells; ATCC® CCL-13™) supplied the Chang liver cells and cell viability assay was performed according to the MTT cell viability assay. First, cells were grown in EMEM media. Ten percent FBS was contained in the culturing media. A NÜVE EC 160 $CO_2$ incubator (NÜVE, Ankara, Turkey) was used to culture the cells. The running conditions of the incubator was 95% oxygen, 5% $CO_2$ and 37°C temperature. When the cell confluence reached 80%, the cells were plated into a 96-well plate with 200 µL aliquot of the culturing medium. The cell density in each well of the plate was 50,000 cells/mL. The plate was incubated for 48 h, during which the cells attached to the plate. After incubation, the medium in each well was removed by aspiration. Next, the complex was dissolved in fresh culturing medium, which was added to designated wells. The complexes were added to the wells at three different concentrations (7.17, 71.7, and 717 µM of the Zn(II) syringic acid complex and 8.17, 81.7 and 817 µM of the Zn(II) complex of p-coumaric acid in the incubation medium). Some wells were assigned as the control, which contained the vehicle of the complex (0.5% DMSO). The plate was, then, incubated for 36 h. After incubation, a solution (0.5 mg/mL) of MTT (Sigma Aldrich, Johannesburg, South Africa) was aliquoted into each well of the plate at a volume of 100 µL and incubation continued for additional 3 h. The liquid in the wells was removed by aspiration after incubation and phosphate-buffered saline was used to wash the cells in the wells. Immediately, an MTT solubilisation solution (Sigma Aldrich, Johannesburg, South Africa) was aliquoted into each well of the plate at a volume of 100 µL. The absorbance of each well was, then, measured at 570 nm. Absorbance measurement was done with a Multiskan Go plate reader (Thermo Fischer Scientific, Waltham, MA, USA). The viability (%) of the complex-treated cells was calculated using the cells treated with the vehicle (0.5% DMSO) as reference. The assay was done in three biological repeats.

## 3.4 Measurement of antioxidant and antidiabetic effects *in vitro*

### 3.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

A previous method (Oke *et al*., 2021) was adopted for this assay. In a 96-well clear plate, 75 µL of the tested samples (3.75 – 60 µM in reaction mixture) or their solvents (control) was added. Next, 37.5 µL of a 0.5 mM DPPH solution was added. The mixture was kept in the dark for 30 min under room temperature. Absorbance was measured at 517 nm (SpectraMax M2 microplate reader, Molecular Devices, California, USA) after the 30 min incubation. Sample blanks or sample solvents were used as the blank. The DPPH radical scavenging activity (%) was computed using the formula below:

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

### 3.4.2 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\bullet+}$) scavenging assay

The method reported by Oke *et al*. (2021) was used for this assay. Equal volumes of 7 mM ABTS and 2.4 mM potassium persulphate solutions were mixed and incubated at room temperature in the dark for 12 – 16 h to generate the radicals ($ABTS^{\bullet+}$). The mixture was, then, diluted 5 times with methanol. The assay was done in a 96-well clear plate. The assay mixture contained 50 µL of the tested samples (3.75 – 60 µM in reaction mixture) or their solvents (control) and 125 µL of the diluted radicals ($ABTS^{\bullet+}$) solution. After 15 min incubation in the dark, absorbance was measured at 734 nm. Sample blanks or sample solvents were used as the blank. The radical scavenging activity (%) was computed relative to the control as follows:

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

### 3.4.3 $Fe^{3+}$ reducing antioxidant power (FRAP) assay

A previous method (Oke *et al*., 2021) was slightly modified to perform this assay. The assay was done in a 96-well clear plate. First, 25 µL of the tested samples (40 µM in reaction mixture) or increasing concentrations (3.75 – 60 µM in reaction mixture) of ascorbic acid, 25 µL 0.2 M phosphate buffer (pH 6.6) and 25 µL of 1% potassium ferricyanide were mixed in the wells of the plate. The mixture was incubated for 20 min at 50 ˚C, before successively adding, 25 µL of 10% TCA, 100 µL of distilled water and 50 µL of 0.1% $FeCl_3$ solution. Thereafter, absorbance was then measured at 700 nm and blanked with the sample blank. The FRAP was determined from ascorbic acid standard curve and computed as mmol/mol equivalent of the ascorbic acid (mmol/mol AAE) using the following formula:

$$FRAP\ (mmol/mol\ AAE) = \frac{C \text{ x } SV}{M}$$

Where, "C" is the concentration (mmol/mL) extrapolated from gallic acid standard curve; "SV" is the sample volume (mL), and "M" is the amount (mole) of the sample in SV (mL) of the sample solution; "AAE" means "ascorbic acid equivalent".

### 3.4.4 Measurement of the inhibitory effect on Linoleic acid peroxidation

The protocol reported in a previous article (Choi *et al*., 2002) was slightly modified to measure the inhibitory effect of the complex on linoleic acid peroxidation. Thirty microlitres of the tested samples or the reference standards (ascorbic acid and Trolox) was aliquoted into 2 mL vials. The concentrations of the samples and reference standards ranged from 5 µM to 80 µM in the assay volume. Some vials were assigned as the control and negative control, which contained an equivalent volume of the solvent used in dissolving the tested samples. Next, 30 µL of 50 mM linoleic acid and 20 µL of 100 mM Tris-HCl buffer (pH = 7.5) were, successively added into the vials. Thereafter, 20 µL of a 2 mM $FeSO_4.7H_2O$ solution was aliquoted into the vials containing the samples or reference standards and the vials that were assigned as the negative control to initiate linoleic acid peroxidation. An equivalent volume of distilled water was aliquoted into the vials that were assigned as the control. Immediately, all the vials were put in an incubator set at

37℃. After the 30 min of incubation, 80 µL of 5.5% trichloroacetic acid solution and 50 µL of 0.25% thiobarbituric acid solution (dissolved in 50 mM NaOH solution) were successively aliquoted into each vial. A boiling water bath was, then, used to heat all the vials for 30 min. After heating, the vials were cooled under room temperature and centrifuged for 10 min at 3500 *g* and ambient temperature. One hundred and fifty microlitres of the supernatant in each vial was aliquoted into a 96-well plate and absorbance was read at 532 nm using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA). The inhibitory activity (%) of the tested samples on linoleic acid peroxidation was calculated as follows:

$$\text{Inhibition}(\%) = \frac{\left(A_{Negative\ control} - A_{Normal\ control}\right) - \left(A_{Test} - A_{Normal\ control}\right)}{\left(A_{Negative\ control} - A_{Normal\ control}\right)} \times \frac{100}{1}$$

Where, "A" means "Absorbance".

### 3.4.5 Measuring the inhibitory effect of the tested samples on α-glucosidase activity

To perform the α-glucosidase inhibition assay, a previous method (Oke *et al*., 2021) was adopted. It was done in a 96-well clear plate. First, 25 µL of the tested samples or acarbose (at 3.75 – 60 µM in reaction mixture) or their solvents (control) and 25 µL of a 4 U/mL of α-glucosidase solution (dissolved in 100 mM phosphate buffer, pH 6.8) were incubated for 10 min at 37 $^{O}$C. Next, 50 µL of 10 mM 4-nitrophenyl-β-D-glucopyranoside substrate solution (dissolved in 100 mM phosphate buffer, pH 6.8) was added and incubation continued for additional 20 min at the same incubation condition. After incubation, the enzyme-substrate reaction was stopped by adding 100 µL of 0.1 M $Na_2CO_3$ solution and absorbance was measured at 405 nm. The absorbance was blanked using the sample and solvent blanks. The enzyme inhibition activity of the samples was computed using the formula below:

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

## 3.5 Cellular antioxidant and antidiabetic experiments

### 3.5.1 Measuring the effect of the complex on oxidative stress-induced alteration of lipid peroxidation and reduced glutathione (GSH) concentration in hepatocytes

The protocol reported in a previous article (Akuru *et al*., 2021) was slightly modified to perform this assay. First, Chang liver cells (ATCC® CCL-13™, ATCC, Virginia, USA) were grown in an EMEM media, which contained 10% FBS. When the cell confluence reached 85%, the cells were trypsinized and plated into a 96-well plate with 200 µL aliquot of the culturing medium. The cell density in each well of the plate was 15,000 cells/mL. The plate was incubated for 36 h in a $CO_2$ incubator, during which the cells attached to the plate. After the incubation, the medium in the wells was removed by aspiration. Thereafter, the tested samples were dissolved in fresh culturing medium, which was added to designated wells of the plate. The tested samples were added to wells at five different concentrations (5, 10, 20, 40 and 80 µM in incubation medium). Some wells were assigned as the control and negative control, which contained the solvent used in dissolving the tested samples. The plate was incubated for 30 min. Thereafter, 50 µL of $FeSO_4.7H_2O$ solution (1 mM in incubation medium) was aliquoted into the wells containing the tested samples and the wells that were assigned as the negative control to initiate oxidative stress. An equivalent volume of distilled water was aliquoted into the wells that were assigned as the control. Immediately, the plate was put into a $CO_2$ incubator and kept there for 1 h. Thereafter, the plate was taken out of the $CO_2$ incubator. The medium in the wells was removed by aspiration and replaced with 250 µL of cold lysis buffer (50 mM sodium phosphate buffer with 0.5% v/v Triton X-100, pH 7.5). A gentle agitation was applied to lyse the cells in the wells. Next, the plate was immediately centrifuged for 1 min at 5000 *g* and 4°C to pellet the cell debris. The supernatant was quickly collected and kept on ice. Immediately, lipid peroxidation and GSH concentration were measured in the supernatant using slightly modified protocols of a previous article (Akuru *et al*., 2021).

To estimate lipid peroxidation, 100 µL of the supernatant or malondialdehyde standards (0, 7.5, 15, 22.5, 30, and 45 µM in the assay volume) was put into designated vials. Next, 0.25% w/v thiobarbituric acid, 20% v/v acetic acid, and distilled water were aliquoted into the vials at a volume ratio of 500 µL:200 µL, respectively. A boiling water bath was, then, used to heat all the vials for 50 min. After heating, the vials were cooled under room temperature and centrifuged for

10 min at 3500 *g* and ambient temperature. Two hundred microlitres of the supernatant in each vial was aliquoted into a 96-well plate and absorbance was read at 532 nm. A malondialdehyde standard curve was used to compute the lipid peroxidation, which was estimated as thiobarbituric acid reactive substances.

To estimate the GSH concentration, the supernatant was, first, mixed with an equal volume of 10% TCA. This was done to precipitate the proteins in the cell lysates. At an angular speed of 4000 *g* and for a duration of 5 min (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany), the supernatant without proteins was recovered. Into a 96-well plate, 50 µL of the supernatant or GSH standards (0.002, 0.02, 0.2, 2, 20, 200 µM in assay volume) were aliquoted into designated wells. Next, 200 µL of Ellman's reagent [0.1 mM of 5,5′ -dithio-bis-(2-nitrobenzoic acid) dissolved in 0.1 M Tris-HCl buffer, pH = 7.4)] was aliquoted into each well. Thereafter, the plate was put in an incubator set at 25 ℃ for 5 min. The absorbance of each well was measured at 412 nm. A GHS standard curve was used to extrapolate the GHS concentration of the supernatant contained in each well.

The percentage inhibition of lipid peroxidation and GSH depletion was calculated and used to compute the $IC_{50}$ values (the concentration required to inhibit lipid peroxidation and GSH depletion by 50%) of the tested samples.

### 3.5.2 Measuring the glucose uptake effect of the complex in L-6 myotubes

The protocols reported in previous articles (Oyedemi *et al*., 2013; van Huyssteen *et al*., 2011) were slightly modified to perform this assay. The cells used in the experiment were L-6 myoblast (ATCC CRL-1458, ATCC, VA, USA). The cells were cultured in a $CO_2$ incubator. A low glucose Dulbecco's Modified Eagle Medium (DMEM) was used as the culturing medium. The medium, also, contained 10% fetal calf serum. The cells were trypsinized after an 85% confluence was attained and seeded into a 96-well plate with fresh media. Each well had approximately 4000 cells. The plate was incubated until an 80% cell confluence was reached. The myoblasts were, then, differentiated into myotubes by replacing the used medium with DMEM medium containing 2% horse serum and incubating the for 4–5 days. After differentiating the myoblasts, the medium in each well of the plate was removed by aspirating and 200 µL of fresh medium containing different

concentrations (5, 50, and 100 µL in the incubation volume) of the tested samples was aliquoted into the plate. The sample solvent was used in the vehicle control (control) wells. Some wells were assigned as the blank control, which contained only the medium without cells. Incubation continued for 48 h. After incubation, the used medium in each well was removed and PBS was used to wash the cells. The cells were, then, treated with 8 mM glucose solution for 2 h. The glucose solution was dissolved in an RPMI medium containing 0.1% BSA. Insulin (1 µM) was used as the positive control group. After the treatment period elapsed, glucose concentration was measured in the medium of each well using a Glucose-GO Assay Kit (Sigma Aldrich, South Africa). The MTT assay was, also, employed to measure the effect of the treatments on the viability of the myotubes. The experiment was conducted in three biological repeats. The formula below was used to compute the glucose uptake of the test samples and control (vehicle control) relative to the blank control.

$$\text{Glucose}(\%) = \frac{\Delta\text{GC of test or control} - \Delta\text{GC of blank control}}{\Delta\text{GC of blank control}} \times \frac{100}{1}$$

"ΔGC" denotes change in glucose concentration (i.e., initial–final glucose concentration in incubation solutions). Also, the $EC_{50}$ of samples, which denotes the concentration (µM) of samples needed to effect 50% glucose uptake increase was computed.

### 3.6 *Ex vivo* antioxidant and antidiabetic experiments

The protocol reported in this section was adopted from previous reports (Oke *et al*., 2021; Akuru *et al*., 2022) and some modifications were added.

#### 3.6.1 Animals

The Animal Research Ethics Committee of the University of the Free State, Bloemfontein, South Africa ethically approved the protocol (**protocol approval reference: UFS-AED2019/0152/2020, approved on 16 November 2020**). The protocol was executed in accordance with the guidelines of the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). The University of the Free State Animal Unit supplied the male Sprague Dawley rats used for the study. The rats were 8 weeks old. First, the rats were subjected to an

overnight fast. Thereafter, isoflurane was used to euthanize the rats. Immediately, the liver and psoas muscle tissues of the rats were collected, which were quickly used for measuring the *ex vivo* antioxidant and antidiabetic properties of the complex.

### 3.6.2 Measuring the effect of the complex on oxidative stress-induced alteration of lipid peroxidation and reduced glutathione (GSH) concentration in the liver tissues

A protocol reported in a previous article (Akuru *et al*., 2022) was slightly modified to perform this experiment. About 200 ± 5 mg portions of the freshly harvested liver tissues were put into a 48-well plate containing 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) and different concentrations (10, 20, 40 and 80 µM in the total assay volume) of the tested samples or reference standard (ascorbic acid). Some wells were assigned as the control and negative control, which contained the solvent used in dissolving the tested samples. The plate was placed in a $CO_2$ incubator (NAPCO series, 5400 $CO_2$ incubator, Thermo Scientific, South Africa) set at 37°C for 25 min. After the pre-incubation period, 100 µL of $FeSO_4.7H_2O$ solution (2 mM in incubation medium) was aliquoted into the wells containing the tested samples or reference standard and the wells that were assigned as the negative control to initiate oxidative stress. An equivalent volume of distilled water was aliquoted into the wells that were assigned as the control. Immediately, the plate was put into the $CO_2$ incubator and kept there for 90 min. At the end of the incubation period, the tissue in each well was removed with a pair sterile forceps and rinsed in Kreb's buffer. The tissues were homogenized in 1 mL of 50 mM sodium phosphate buffer (containing 0.5% v/v Triton X-100; pH 7.5). The supernatant was obtained with an angular speed of 10,400 g for a duration of 10 min. The methods previously described above were adopted to estimate lipid peroxidation and GHS concentration in the supernatant of each tissue homogenate. The inhibitory percentage of the tested samples or reference standard on lipid peroxidation and GHS depletion was calculated, which was used to compute the $IC_{50}$ values of the samples.

### 3.6.3 Measuring the glucose uptake effect of the complex using the muscle tissues isolated from rats

A protocol reported in a previous article (Oke *et al*., 2021) was slightly modified to evaluate the tissue glucose uptake effect of complex. About 300 ± 10 mg portions of the freshly harvested psoas muscle tissues were put into a 48-well plate containing 900 µL of Kreb's buffer and different concentrations (10, 20, 40 and 80 µM in the total assay volume) of the samples or reference standard (50 mU insulin; NovoRapid® FlexPen®, Novo Nordisk Limited, West Sussex, UK). Some wells were assigned as the control, which contained the solvent used in dissolving the tested samples. The plate was placed in a $CO_2$ incubator (NAPCO series, 5400 $CO_2$ incubator, Thermo Scientific, South Africa) set at 37°C for 25 min. After the pre-incubation period, 100 µL of glucose solution (11.1 mM in total incubation volume) was aliquoted into the wells of the plate. Immediately, the plate was put into the $CO_2$ incubator and kept there for 90 min. At the end of the incubation period, a portion of the incubation medium in each was aliquoted into a new 96-well plate and glucose concentration was spectrophotometrically estimated in the medium using a Glucose (GO) Assay Kit (Sigma Aldrich, South Africa). The absorbance value of the control was used as a reference to compute the glucose uptake increase (%) of the tested samples and insulin according to the formula below:

$$\text{Glucose}(\%) = \frac{\Delta\text{GC of test or control} - \Delta\text{GC of blank control}}{\Delta\text{GC of blank control}} \times \frac{100}{1}$$

The $EC_{50}$ of the samples, which represents the concentration (µM) of the sample required to increase tissue glucose uptake by 50% was computed.

### 3.6.4 Measurement of muscle tissue hexokinase activity, zinc concentration and phospho-Akt levels

Immediately after the 90 min incubation, zinc concentration and hexokinase activity were measured in the muscle tissues that were treated with the highest concentration of the test samples. First, the tissues were washed with Kreb's buffer, homogenized in 1 mL of ice-cold 50 mM sodium phosphate buffer (containing 0.5% v/v Triton X-100 and 1 mM EDTA; pH, 7.5) and centrifuged

at 10 400 *g* for 10 min at 4°C to recover the supernatant. The concentration of protein was estimated in the supernatant using the bicinchoninic acid (BCA) method.

Hexokinase activity was measured in the supernatant at a protein concentration of 15 mg/mL using a Hexokinase Colorimetric Assay Kit (catalog number MAK091, Sigma Aldrich, Johannesburg, South Africa). The hexokinase activity was expressed in nmol/min/mL, which is equivalent to milliunit/mL. One unit of hexokinase represents the amount of enzyme that will generate 1.0 µmol of NADH per minute at pH 8.0 at room temperature.

Zinc concentration was measured in the tissue supernatant using a Zinc Assay Kit (catalog number MAK032, Sigma Aldrich, Johannesburg, South Africa) and expressed in ng/µL. The pan- Akt and phosphor-Akt in the tissue supernatants were estimated by an ELISA method using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (catalog number RAB0012, Sigma Aldrich, Johannesburg, South Africa). The phospho-Akt/pan-Akt ratio was determined.

### 3.7 Molecular docking analysis

The complexes and their respective phenolic acids were docked to five different types of enzymes associated with diabetic development and progression (Motloung *et al*., 2020; Swain *et al*., 2020). They include glucose transporter type 4 (GLUT4), human insulin receptor tyrosine kinase domain (HIR-TKD), insulin receptor substrate-1 (IRS-1), human maltase–glucoamylase (MGAM) and protein kinase B (PKB). HIR-TKD (PDB ID: 1IRK), IRS-1 (PDB ID: 1IRS), MGAM (PDB ID: 3L4U), and PKB (PDB ID: 106L) were retrieved from protein data bank. GLUT-4 was theoretically generated followed by the homology modelling platform. All the attached heteroatoms from each retrieved protein structure were removed and saved in.pdb file format for the docking studies. The 3D structures of the ligands were optimized using Avogadro software to get the reliable docking score. The structures were also saved in.pdb file format. Molecular docking study was done using AutoDock Vina 4.2. The docking of the proposed ligands that had the best pose was selected based on the minimum docking score (kcal/mol). To visualize the molecular interaction between the proteins and ligands, the BIOVIA-DSV software was used.

## 3.8 *In vivo* antidiabetic and antioxidant evaluation

### 3.8.1 Experimental animals

Animal experiment was done in the University of the Free State (UFS) animal facility. Approval was obtained from the UFS Animal Ethics Committee to use animals for experimental purposes (**protocol approval reference: UFS-AED2019/0152/2020, approved on 16 November 2020**). The rules and regulations of the Ethics Committee, which followed the guidelines of the South African National Standard for the Care and Use of Animals for Scientific Purposes (**SANS 10386:2008**) were adhered to in the experiment. The UFS animal facility supplied forty-one (41) male Sprague Dawley (SD) rats that were 7 weeks old. The rats acclimatized for 5 days and were fed ad libitum with commercial rat chow and drinking water. The rats were housed two to three animals per cage in large-sized cages with the following dimensions: 598mm (L) x 380mm (W) x 200mm (H) with Grid top hatch access of 220mm (L) x 225mm (W) x 70mm (H). The animals were housed in a temperature (20 – 24°C; set point at 22°C) and humidity (40-70%; set point 60%) controlled room with a 12 h light-dark cycle. Of the 41 rats 6 were put into the normal control group (NMC), while the remaining 35 were induced with diabetes.

### 3.8.2 Diabetes induction

Diabetes induction was done using 10% fructose and low dose (40 mg/kg bw) streptozotocin (STZ). The fructose induced insulin resistance, while STZ induced partial pancreatic β cell damage as described previously (Wilson and Islam, 2012). For 2 weeks, the 35 rats received 10% fructose as drinking water. Thereafter, they were administered a single intraperitoneal injection of streptozotocin (40 mg/kg body weight, dissolved in citrate buffer, pH 4.5), while the 6 rats in the NMC were injected with only citrate buffer at an equivalent dosing volume. After a week, diabetes was confirmed by measuring the tail tip non fasting blood glucose (NFBG) levels of the rats using a Contour Plus Blood Glucose Monitoring System (Ascensia Diabetes Care Holdings AG, Basel, Switzerland. NFBG between 250 and 600 mg/dL was the inclusion criterium for experimental diabetes in the study. Diabetes induction was 86% success rate. The selected diabetic rats used in the study were grouped into 5 diabetic groups (DBC, SYA, SYZ, ZNS and MET) with 6 animals each.

### 3.8.3 Treatments

There were six groups of experimental animals in total with six animals each. They include one normal control or non-diabetic group and five diabetic groups. The groups and the treatments are given below

- **NMC:** Non-diabetic rats treated with the vehicle (water) of test substances
- **DBC:** Diabetic rats treated with the vehicle (water) of test substances
- **SYA:** Diabetic animals treated with 45 mg/kg bw of syringic acid
- **SYZ:** Diabetic animals treated with 45 mg/kg bw of the complex
- **ZNS:** Diabetic animals treated with 15 mg/kg bw of zinc sulphate heptahydrate
- **MET:** Diabetic animals treated with 250 mg/kg bw Metformin

Administration was done orally once a day, for 20 days (i.e., 5 times in a week for 4 weeks) using an oral dosing needle. The rats freely ate food and drank water, unless mentioned otherwise.

### 3.8.4 Body weight, food intake and water intake measurements

Body weight and food intake were measured on the $6^{th}$ day of each week during the four weeks intervention period. Water intake was measured daily throughout the study.

### 3.8.5 Blood glucose measurements

All blood glucose measurements were done using the tail-tip method. In the $1^{st}$, $2^{nd}$ and $4^{th}$ week of the intervention period, NFBG was measured. In the $3^{rd}$ week of the intervention period oral glucose tolerance test (OGTT) was measured. First, the rats were fasted for 7 hours. Immediately the blood glucose of rats was immediately measured, which represented the OGTT at time 0 minutes. After that, glucose was orally administered to the rats at 2 g/kg bw. Blood glucose was, then, measured at 30 minutes intervals (i.e., at 30, 60, 90 and 120 minutes). A previously reported formula was used to compute the area under curve (AUC) for OGTT (Sakaguchi *et al*., 2015).

$$AUC = \frac{BG0 + (BG30\ x\ 2) + (BG60\ x\ 3) + (BG120\ x\ 2)}{4}$$

Where BG0, BG30, BG60 and BG120 mean blood glucose at 0, 30, 60 and 120 minutes, respectively.

The OGTT at time 0 minutes was, also, considered as the fasting blood glucose (FBG) of the rats at the 3rd week of the intervention period. FBG was also measure on the day of euthanization. The rats were fasted for 7 hours. Next, they were administered with the treatments. After 1 hour 30 minutes, the FBG was measured before the rats were euthanized.

### 3.8.6 Euthanization of rats and collection of biological samples

Euthanization was done using isoflurane. Cardiac puncture was employed for exsanguination immediately animal death was confirmed. Non-heparinized tubes were used to collect blood and a 5 mL syringe, and a 22 G needle was used. Immediately, the liver, pancreas, and muscle tissues of the rats were removed and snap frozen in liquid nitrogen. The tissues were stored at -80°C. To serum was recovered from the blood by centrifuging (Labnet Prism™ R Refrigerated Microcentrifuge, Labnet International Inc, New Jersey, USA) at 3500 g for 10 min at 4°C. The serum was stored at -80°C.

### 3.8.7 Measurement of fasting serum insulin and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) computation

A method involving enzyme-linked immunosorbent assay (ELISA) method was used in fasting serum insulin (FSI) concentration measurement. This was done using A Rat Ins1 Insulin ELISA Kit (Sigma Aldrich, Johannesburg, South Africa; product number: RAB0904).The FBG that was measured just before euthanization and the FSI of the animals were used to compute the HOMA-IR based on a previously reported formula (Vilela *et al*., 2016):

$$HOMA - IR = \frac{Fasting\ blood\ glucose(mmol/L)\ x\ Fasting\ serum\ insulin\ (mU/L)}{22.5}$$

### 3.8.8 Muscle and liver tissue glycogen content measurement

The method reported previously (Lo *et al.*, 1970) was used with some modifications in this assay. Into vials about 0.5 g of tissue was put. Next, 1.5 mL of 30% KOH solution was added. The vials were placed in a boiling water bath for 40 minutes to digest and make the content a homogenous solution. After cooling the vials, 2 mL of 95% ethanol was added, and the vials were allowed to stand for 30 min at 2 – 8°C to precipitate sugars/glycogen. The precipitated glycogen was recovered and washed twice in 2 mL 95% ethanol by centrifuging (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany) at 1200 g for 30 minutes at ambient temperature. Next, the glycogen residue was dissolved in 3 mL of distilled water. A 200 µL volume of the dissolved residue or glycogen standards (0.005 – 0.1 mg/200 µL) was aliquoted into glass test tubes and 1 mL of 5% phenol and 5 mL of 98% $H_2SO_4$ were added, successively. The mixture was agitated and 250 µL volume was aliquoted into a 96-well plate. Absorbance was, then, measured at 490 nm (SpectraMax M2 microplate reader, Molecular Devices, San Jose, CA, USA). A glycogen standard curve was used to derive the tissue glycogen content according to the following formula:

$$Tissue\ glycogen\ content\ (mg/g\ tissue) = \frac{C\ x\ D}{M}$$

Where, C is the extrapolated concentration (mg/200 µL); D is the dilution factor = 31, which is the dilution of 200 µL of the dissolved residue in 6.2 mL of assay volume; M = 0.5 g, which is the mass (g) of tissue used in the assay.

### 3.8.9 Preparation of tissue samples and determination of protein concentration

Approximately 100 mg portion of the harvested tissues was homogenised in 1 mL of ice-cold 50 mM sodium phosphate buffer (containing 0.5% v/v Triton X-100 and 1 mM EDTA; pH 7.5). The supernatant was recovered by centrifuging at 10 400 g for 10 min at 4°C. Protein concentration was measured in the supernatant using the bicinchoninic acid (BCA) method.

### 3.8.10 Assays to measure hexokinase activity, Akt phosphorylation and zinc concentrations

At sample protein concentration of 15 mg/mL hexokinase activity was measured in the muscle tissue supernatant at a protein concentration of 15 mg/mL. A Hexokinase Colorimetric Assay Kit (catalogue number MAK091, Sigma Aldrich, Johannesburg, South Africa) was used. The hexokinase activity was expressed in nmol/min/mL, which is equivalent to milliunit/mL. One unit of hexokinase denotes the enzyme activity that will generate 1.0 µmol of NADH per minute at pH 8.0 at room temperature. In the muscle tissue supernatant, the pan-Akt and phosphor-Akt levels were measured calorimetrically by an ELISA method using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (catalogue number RAB0012, Sigma Aldrich, Johannesburg, South Africa). The index for Akt phosphorylation was determined by computing the ratio between phospho-Akt and pan-Akt ratio. In the serum, as well as the muscle and pancreatic tissue supernatants zinc concentration was measured using a Zinc Assay Kit (catalogue number MAK032, Sigma Aldrich, Johannesburg, South Africa) and expressed in ng/µL.

### 3.8.11 Measurement of antioxidant indices

In the serum, as well as the liver and pancreatic tissue supernatants antioxidant parameters were measured. Lipid peroxidation and reduced glutathione concentration (GSH) were measured using methods reported above in **sub-section 3.5.1**.

A Catalase Assay Kit (Sigma Aldrich, Johannesburg, South Africa; catalogue number: CAT100) was used to measure catalase activity at 1 mg/mL sample protein concentration. It is done by measuring the rate of $H_2O_2$ decomposition at room temperature, which was expressed in mmol/mL/min.

A superoxide dismutase (SOD) Assay Kit (Sigma Aldrich, Johannesburg, South Africa; product number: 19160) was used to measure the SOD activity in the samples, which was measured a 15 mg/mL sample protein concentration. SOD catalyses the reduction of superoxide ion to $H_2O_2$ and $O_2$. Thus, SOD activity in the samples was determined by measuring the inhibitory action on superoxide ion-mediated colorimetric reduction of a water-soluble tetrazolium salt [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], which was expressed as a percentage.

# CHAPTER 4

## BIOACTIVE SYNERGISM BETWEEN ZINC MINERAL AND P-COUMARIC ACID: A MULTI-MODE GLYCEMIC CONTROL AND ANTIOXIDATIVE STUDY

### Prescript

This chapter investigated the *in vitro,* cellular and *ex vivo* antidiabetic and antioxidant potential of a novel Zn(II) complex of p-coumaric acid. The content of this chapter has been published as follows: ***Ramorobi, L. M.***, *Matowane, G. R., Mashele, S. S., Bonnet, S. L., Noreljaleel, A., Swain, S. S., Makhafola, T. J., & Chukwuma, C. I. (**2022**). Bioactive synergism between zinc mineral and p-coumaric acid: A multi-mode glycaemic control and antioxidative study.* ***Journal of Food Biochemistry****, 46(10), e14360. https://doi.org/10.1111/jfbc.14360*. The above-mentioned publication was adopted in writing the content of this chapter.

## 4.1 Abstract

Natural supplements are important in diabetes and oxidative stress management. A complexation-mediated antihyperglycemic and antioxidant synergism between Zn(II) and p-coumaric acid was investigated. p-Coumaric acid was complexed with $ZnSO_4$ and characterized by FT-IR, $^1H$ NMR, and mass spectroscopy. The antioxidant and antihyperglycemic potential of the complex and precursors were evaluated with different experimental models. Molecular docking with target proteins linked to diabetes was performed. A Zn(II)-bicoumarate.$2H_2O$ complex was formed. The *in vitro* radical scavenging, α-glucosidase inhibitory, antiglycation, and anti-lipid peroxidative activities of the complex were several folds stronger than p-coumaric acid. In Chang liver cells and rat liver tissues, the complex inhibited lipid peroxidation ($IC_{50}$ = 56.2 and 398 μM) and GSH depletion ($IC_{50}$ = 33.9 and 38.7 μM), which was significantly stronger (2.3–5.4-folds) than p-coumaric acid and comparable to ascorbic acid. Zn(II) and p-coumaric synergistically modulated (1.7 and 2.8-folds than p-coumaric acid) glucose uptake in L-6 myotubes ($EC_{50}$ = 10.7 μM) and rat muscle tissue ($EC_{50}$ = 428 μM), which may be linked to the observed complexation-mediated increase in tissue zinc uptake. Glucose uptake activity was accompanied by increased hexokinase activity, suggesting increased glucose utilization. Docking scores α-glucosidase, GLUT-4, and PKB/Akt showed stronger interaction with the complex (−6.31 to −6.41 kcal/mol) compared to p-coumaric acid (−7.18 to −7.74 kcal/mol), which was influenced by the Zn(II) and bi-coumarate moieties of the complex. *In vitro*, the complex was not hepatotoxic or myotoxic. Zn(II) complexation may be a therapeutic approach for improving the antioxidative and glycaemic control potentials of p-coumaric acid.

## 4.2 Introduction

The search for better and safe therapeutic agents for diabetes and associated complications remains an ongoing trend, due to the limitations of existing therapeutic agents (MacLean *et al*., 2006) and the increasing prevalence and detrimental consequences of the disease (IDF, 2021, 2019). Type 2 diabetes (T2D), which is the most prevalent type of diabetes, has posed serious socioeconomic and health burdens on most countries of the world (IDF, 2021). Its high prevalence has been attributed to its association with obesity and lifestyle-related factors (Sami *et al*., 2017; Al-Goblan *et al*., 2014). These factors promote metabolic disorders, such as insulin resistance, glucose intolerance,

and hyperinsulinemia, which are metabolic indices and risk factors for developing T2D (Galicia-Garcia *et al*., 2020; Sami *et al*., 2017; Al-Goblan *et al*., 2014). Impaired insulin signalling and poor circulating glucose uptake and utilization in peripheral tissues, as well as progressive pancreatic β-cell damage are some of the derangements that facilitate poor glucose homeostasis and persistent hyperglycaemia in T2D (Galicia-Garcia *et al*., 2020).

Persistent hyperglycaemia is the key instigator of most complications associated with diabetes. Oxidative stress has been implicated as a major mediator and culprit of the progression and outcomes of some of the diabetic complications (Giacco & Brownlee, 2010). This is largely attributed to increased generation of reactive oxygen species (ROS) and free radicals, which can oxidatively damage important biological molecules, resulting in degenerative conditions in the absence of adequate and effective antioxidant defense mechanisms (Di Meo & Venditti, 2020). Persistent hyperglycaemia drives the incomplete reduction of molecular oxygen in cellular electron transport chain and energy metabolism, thus increasing the production of superoxide ion, a deleterious biological ROS (Fakhruddin *et al*., 2017). Also, hyperglycaemia promotes glycation processes and formation of advanced glycation end products, which are biomarkers of degenerative conditions and complications of diabetes (Giacco & Brownlee, 2010). The increased radical production associated with diabetes, also, initiates lipid peroxidation processes, which releases more deleterious free radicals, while comprising the integrity of functional cellular lipids (Fatani *et al*., 2016).

The synergistic application of natural therapeutic agents has an appreciable reception in management of diseases. The concept can be beneficial in the following ways: Limiting toxicity or side effects linked to high dose of drug and providing a platform for multi-mode therapeutic targets and effects (Lehár *et al*., 2009). Advances in research have shown possible diabetes-related therapeutic synergism through mineral-ligand complexation. Zinc mineral has been complexed with various types of ligands to develop zinc complexes with improved bioactivity (Chukwuma *et al*., 2020). It has been reported that zinc possesses insulin-mimetic action and complexation may afford improved bioavailability and multi-facet bioactivity (Chukwuma *et al*., 2020). Zn(II) complex of maltol inhibited lipolysis and increased glucose uptake in adipocytes isolated from rats (Adachi *et al*., 2004). A mechanistic study suggests its effect may be through modulatory action

on adipocyte insulin receptor tyrosine kinase, phosphoinositide 3-kinase, GLUT-4, and phosphodiesterase (Yoshikawa *et al*., 2004). Studies on T2D KK-Ay mice showed the complex reduced blood glucose and HbA1c levels, and improved glucose tolerance (Adachi *et al*., 2004). Zn(II) complexes of several amino acids (asparagine, proline, valine, glycine, alanine, glutamine, aspartic acid, and threonine) (Yoshikawa *et al*., 2001) and L-carnitine (Yoshikawa *et al*., 2003) suppressed lipolysis in isolated rat adipocytes, while Zn(II) complex of threonine and L-carnitine potentiated antihyperglycemic action in T2D KK-Ay mice (Yoshikawa *et al*., 2001, 2003). In fact, the blood glucose-lowering effect of the Zn(II) complex of L-carnitine was approximately 3.6 times more potent than that of L-carnitine (Yoshikawa *et al*., 2003), which suggest the improving action of Zn(II) on its ligands.

Although studies on antidiabetic Zn(II) complexes are potential therapeutic leads to the discovery of antidiabetic nutraceuticals, the research trajectory appears to have a major flaw. A broad perspective into the reported studies on antidiabetic Zn(II) complexes reveals that most of the studied antidiabetic Zn(II) complexes are synthesized using synthetic ligands that have little pharmacological credence and toxicity concerns (Chukwuma *et al*., 2020). Plant phenolics with documented antioxidant and glycaemic control potential remain the least explored ligands of antidiabetic Zn(II) complexes (Chukwuma *et al*., 2020), which suggests a research trajectory that favours synthetic rather than natural medicines. Several phenolic acids, including p-coumaric acid, have not been studied as possible ligands of Zn(II) complexes with promising bioactivities. p-Coumaric acid is a natural hydroxycinnamic acid derivative that is present in vegetables, fruits, mushrooms, and cereals (Zabad *et al*., 2019) and has been reported to possess radical scavenging antioxidant properties (Kiliç & Yeşiloğlu, 2013; Kadoma & Fujisawa, 2008). p-Coumaric acid inhibits α-glucosidase activity, which suggests potential of improving postprandial glycaemic control (Pei *et al*., 2016). Results from a previous study in T2D rats suggest that p-coumaric acid may inhibit hippocampal neurodegeneration by potentiating antioxidant, anti-inflammatory, and anti-apoptotic effects (Abdel-Moneim *et al*., 2018). In rats, p-coumaric acid alleviated experimentally induced diabetic nephropathy (Zabad *et al*., 2019), while in T2D rats, it exhibited antidiabetic effects by upregulating the mRNA expression of PPARγ (Abdel-Moneim *et al*., 2018).

The above studies suggest that p-coumaric acid may be a promising ligand for Zn(II) in developing promising a Zn(II) complex with improved and broader scope of bioactive action. However, this hypothesis has not been studied. Therefore, this study was done to investigate the possible complexation-mediated glycaemic control and antioxidant synergism between Zn(II) and p-coumaric acid.

## 4.3 Materials and methods

For the materials and methods of this chapter, please consult **sub-sections 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7 and 3.9 of chapter 3**.

## 4.4 Results

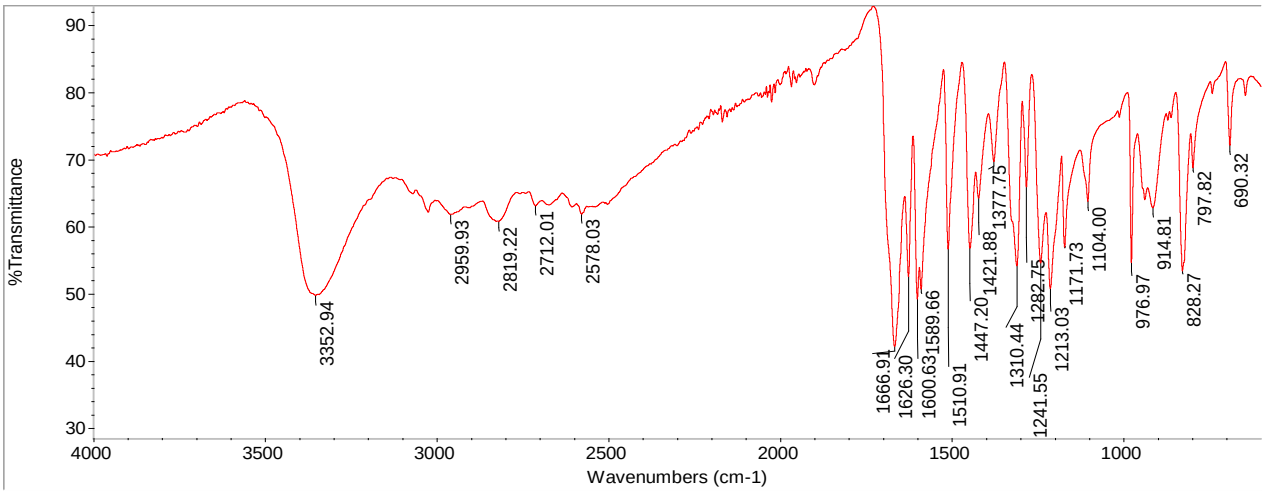
### 4.4.1 Spectroscopic characteristics of the complex

**Figure 4.1** presents the FT-IR data. Both the IR spectra of p-coumaric acid **Figure 4.1a**) and the complex (**Figure 4.1b**) showed broad picks centred at 3352.94 and 3351.73 $cm^{-1}$, respectively. These signals are caused by the phenolic O-H stretching. This shows the phenolic OH groups were not involved in the complexation, and thus are intact. The sharp signal observed in the IR spectrum of p-coumaric acid at 1666.91 $cm^{-1}$ (**Figure 4.1a**) is caused by the carboxylic carbonyl (C=O) stretching. Carboxylic C=O stretching signal was, also, observed in the IR spectrum of the complex at 1636.41 $cm^{-1}$ (**Figure 4.1b**). However, in the IR spectrum of the complex, the signal was reduced, and the wavenumber was lesser relative to the signal in the IR spectrum of p-coumaric acid. The reduced signal and wavenumber suggest zinc donated electrons to the carboxylic group of the phenolic acid, which indicates Zn(II) complexation occurred at the carboxylic end of the phenolic acids as shown in **Figure 4.2a**.

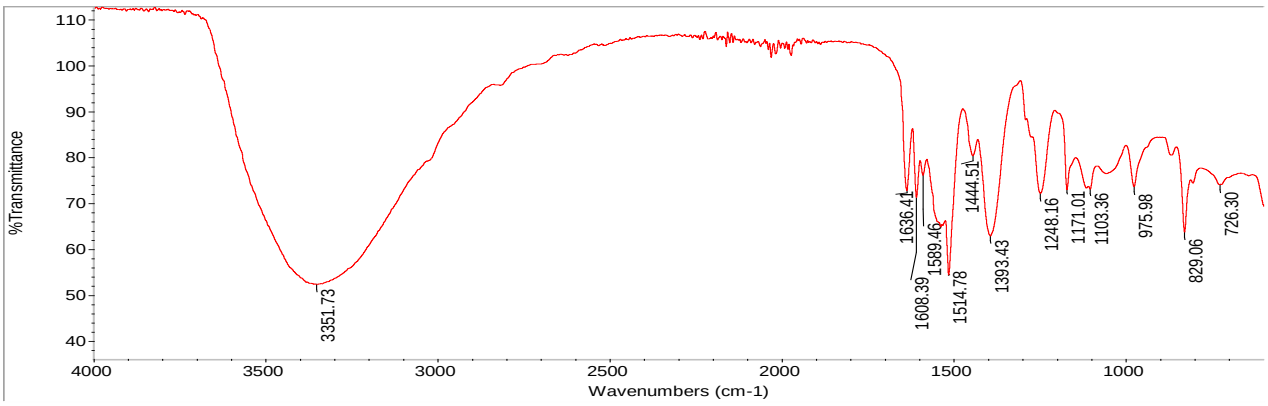
Proton NMR analysis is presented in Figure 2b. The 1H NMR represent all required protons at the different environments, proving that p-coumaric acid zinc sulphate complex was successfully achieved (Figure 2b). The protons H-2 and H-6; H-3 and H-5 observed at δ 6.76 (d, $J$ = 8.2 Hz, 2H) and δ 7.42 (d, $J$ = 8.1 Hz, 2H), respectively, indicated a doublet. The important signal: the hydroxyl group observed at δ 9.78 ppm showed a singlet signifying that the OH group is not involved in the complex formation (**Figure 4.2b**). The two protons of the double bond (H-α, and

H-β) appearing at positions δ 6.28 (d, *J* = 15.8 Hz, 1H) and δ 7.31 (d, *J* = 15.8 Hz, 1H), respectively, also showed a doublet (**Figure 4.2b**).The high-resolution mass spectroscopy of the complex is presented in **Figure 4.3**. The negative mode spectrum showed zinc associated with two molecules of p-coumaric acid and two molecules of water, which had a mass of *m/z* = 224.9784 and differed from the calculated mass by 0.0431 unit. The fragment of coumaric acid had a mass of *m/z* = 163.0388 and differed from the calculated mass by 0.0007 unit.

The above data show that a double hydrated Zn(II)-bicoumarate complex was formed.

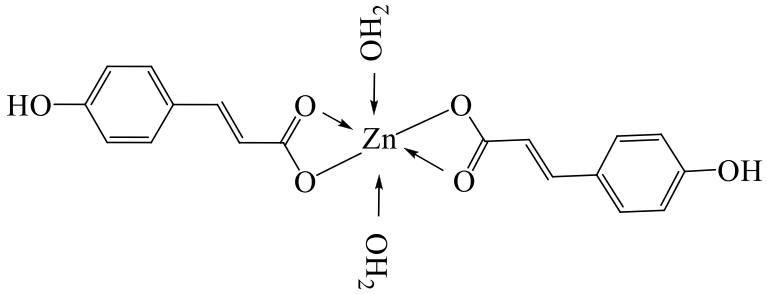



**(a)**

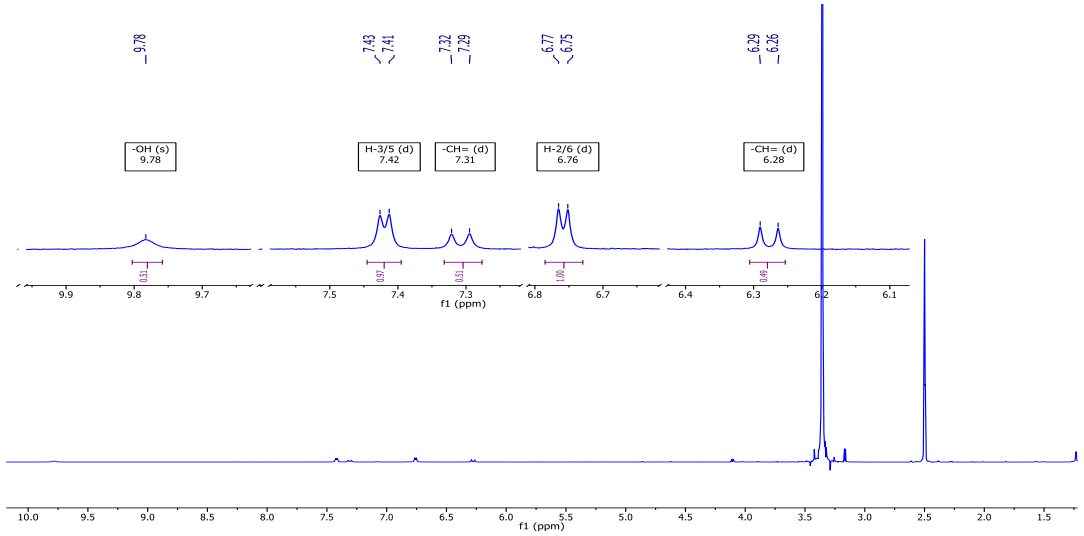



**(b)**

**Figure 4.1:** Spectra for FT-IR of **(a)** p-coumaric acid and **(b)** the Zn(II)-bicoumarate.2$H_2O$ complex.




**(a)**




| Position | $^1$H NMR |
|---|---|
| 1 | - |
| 2 | 6.76 (d, $J$ = 8.2 Hz, 1H) |
| 3 | 7.42 (d, $J$ = 8.1 Hz, 1H) |
| 4 | - |
| 5 | 7.42 (d, $J$ = 8.1 Hz, 1H) |
| 6 | 6.76 (d, $J$ = 8.2 Hz, 1H) |
| α | 7.31 (d, $J$ = 15.8 Hz, 1H) |
| β | 6.28 (d, $J$ = 15.8 Hz, 1H) |
| -OH | 9.78 (s, 1H) |

**(b)**

**Figure 4.2:** Diagrams showing the **(a)** proposed structure and **(b)** $^1$H NMR spectrum of the Zn(II)-bicoumarate.2$H_2O$ complex.

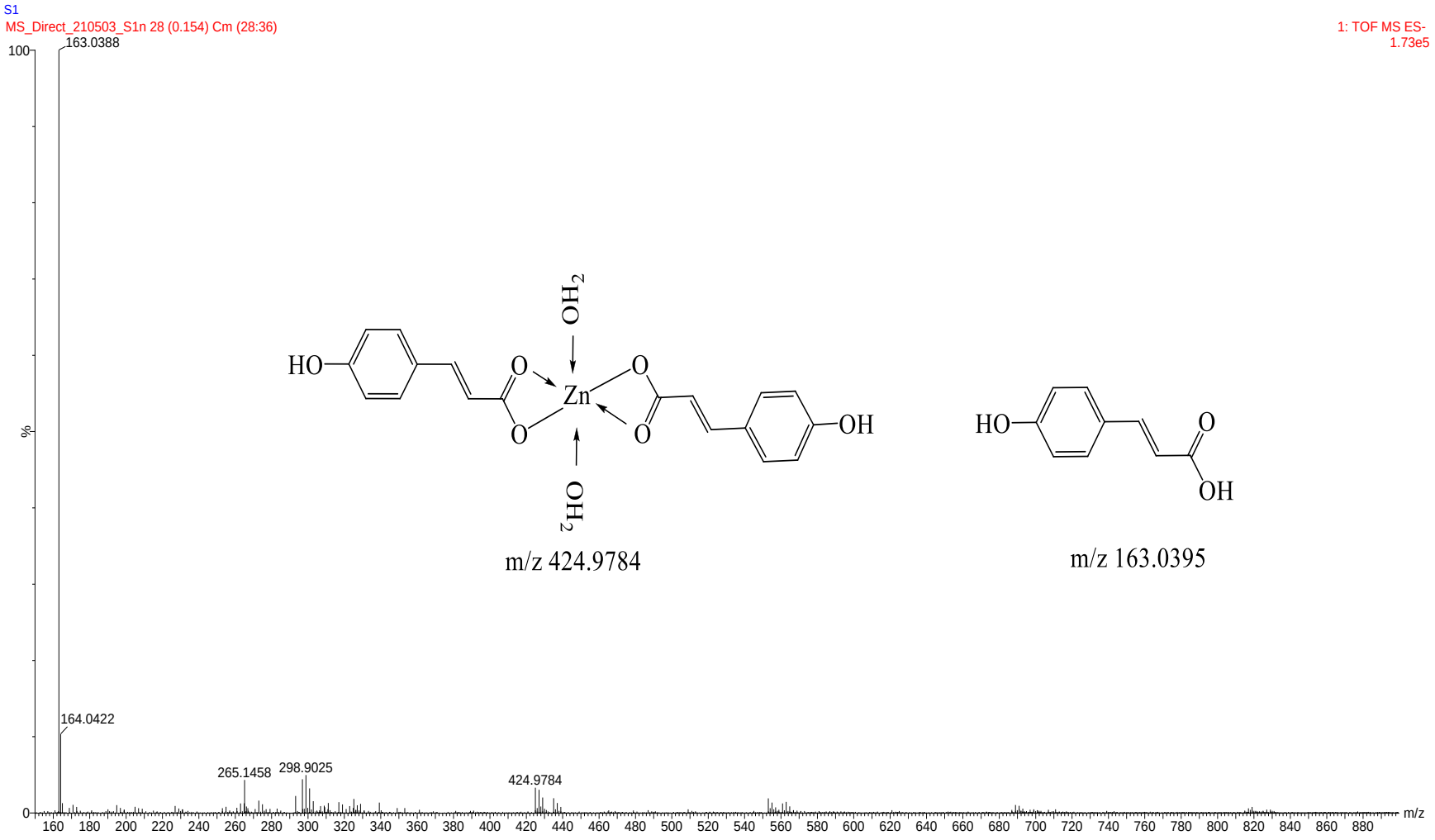
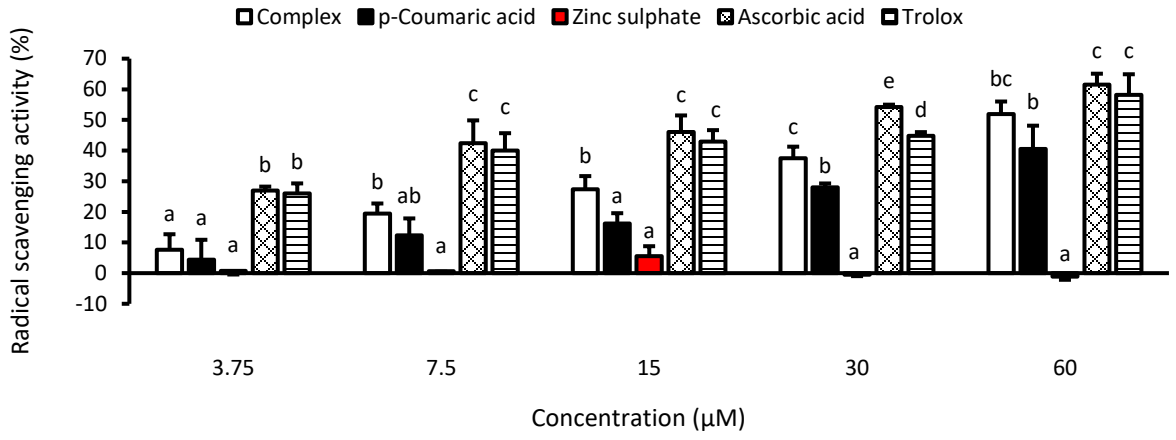


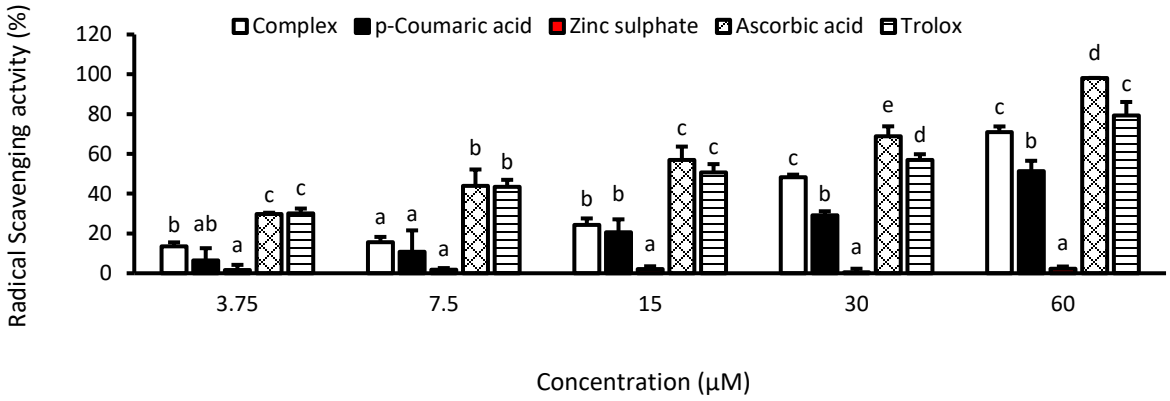

**Figure 4.3:** High-resolution mass spectroscopy data of the Zn(II)-bicoumarate.2$H_2O$ complex.

### 4.4.2 Oxidative stress-related bioactivities of complex and precursors

p-Coumaric acid showed DPPH ($IC_{50}$ = 50.8 µM) and ABTS ($IC_{50}$ = 169 µM) radical scavenging, $Fe^{3+}$ reducing, and anti-linoleic acid peroxidative ($IC_{50}$ = 75.8 µM) activities *in vitro*, while zinc sulphate did no show *in vitro* radical scavenging, $Fe^{3+}$ reducing and anti-lipid peroxidative activities (**Figures 4.4 and 4.5; Table 4.1**).



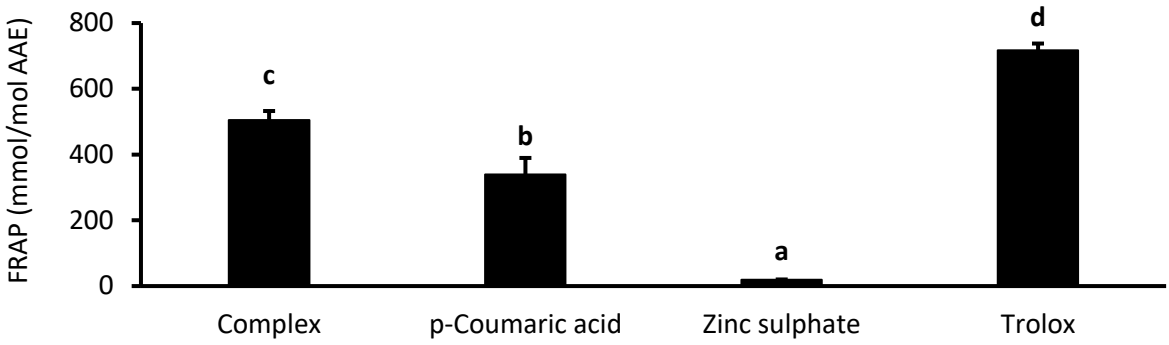

**(a)**



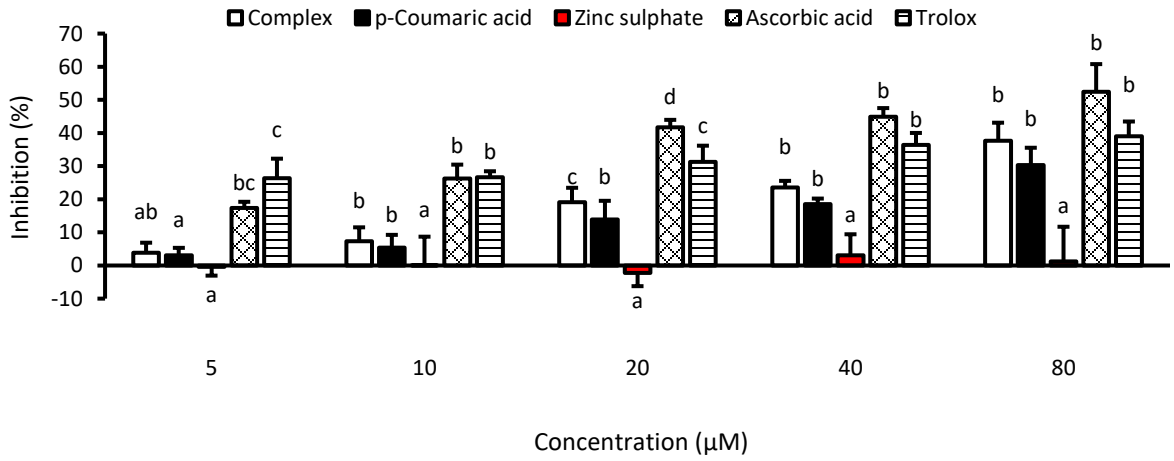

**(b)**

**Figure 4.4:** *In vitro* **(a)** DPPH radical scavenging and **(b)** $ABTS^{\bullet+}$ radical scavenging activities of the tested samples. Data are 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.

However, complexation of the aforementioned resulted in a complex with significantly ($p < .05$) stronger DPPH ($IC_{50}$ = 24.6 µM) and ABTS ($IC_{50}$ = 115 µM) radical scavenging, $Fe^{3+}$ reducing and anti-linoleic acid peroxidative ($IC_{50}$ = 51.2 µM) activities (**Table 4.1**). The *in vitro* anti-lipid peroxidative effect of the complex was statistically comparable ($p > .05$) to that of Trolox ($IC_{50}$ = 37.8 µM) and ascorbic acid ($IC_{50}$ = 13.9 µM) (**Table 4.1**).




**(a)**




**(b)**

**Figure 4.5:** *In vitro* **(a)** $Fe^{3+}$ reducing and **(b)** lipid peroxidation inhibitory activities of the tested samples. Data are 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.

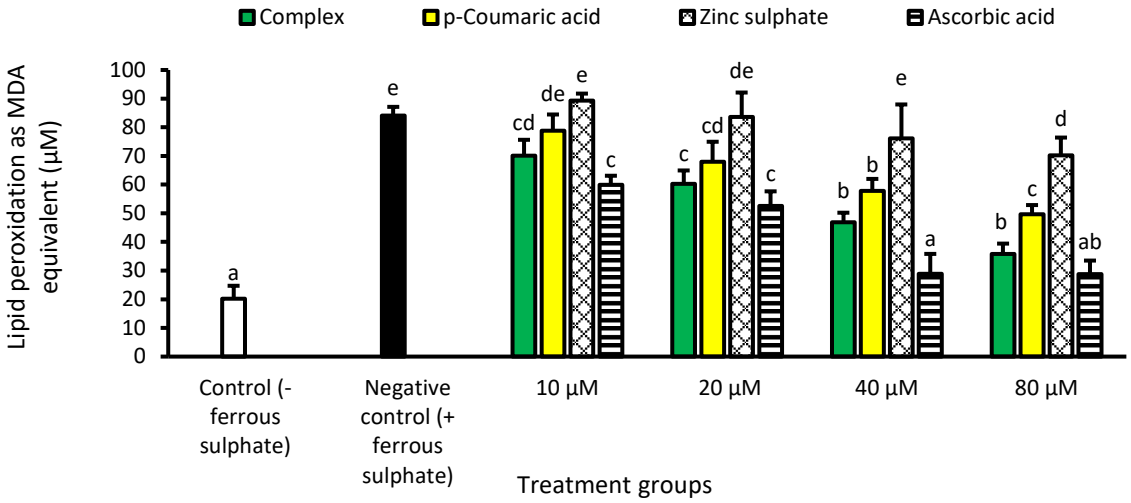
**Table 4.1:** $IC_{50}$ and $EC_{50}$ values of the Zn(II)-bicoumarate.$2H_2O$ c[illegible]ors and standards for the different experimental parameters.

| Experimental parameters | Complex | p-Coumaric acid | Zn(II) | Ascorbic acid | Trolox | Acarbose | Amgd |
|---|---|---|---|---|---|---|---|
| | $IC_{50}$ or $EC_{50}$ values (µM) | | | | | | |
| ABTS radical scavenging activity ($IC_{50}$) | $115 \pm 5.52^{b}$ | $169 \pm 15.2^{a}$ | ND | $53.0 \pm 10.1^{c}$ | $62.1 \pm 16.7^{c}$ | NA | NA |
| DPPH radical scavenging activity ($IC_{50}$) | $24.6 \pm 7.16^{b}$ | $50.8 \pm 3.93^{a}$ | ND | $14.3 \pm 1.46^{bc}$ | $12.7 \pm 4.62^{c}$ | NA | NA |
| Inhibition of *in vitro* linoleic acid peroxidation | $51.2 \pm 8.50^{bc}$ | $75.8 \pm 16.8^{a}$ | ND | $13.9 \pm 4.38^{c}$ | $37.8 \pm 11.1^{ab}$ | NA | NA |
| Antiglycation activity ($IC_{50}$) | $39.9 \pm 11.0^{a}$ | ND | $34.6 \pm 5.84^{a}$ | NA | NA | NA | $8.70 \pm 2.04^{b}$ |
| α-glucosidase inhibition ($IC_{50}$) | $19.6 \pm 8.66^{b}$ | $46.3 \pm 2.99^{b}$ | $207 \pm 40.8^{a}$ | NA | NA | $14.0 \pm 4.71^{b}$ | NA |
| Glucose uptake increase in L-6 myotubes ($EC_{50}$) | 10.7 | 18.6 | 34.7 | NA | NA | NA | NA |
| Glucose uptakes increase in isolated rat psoas muscle ($EC_{50}$) | $428 \pm 45.9^{b}$ | $1180 \pm 164^{a}$ | $450 \pm 87.0^{b}$ | NA | NA | NA | NA |

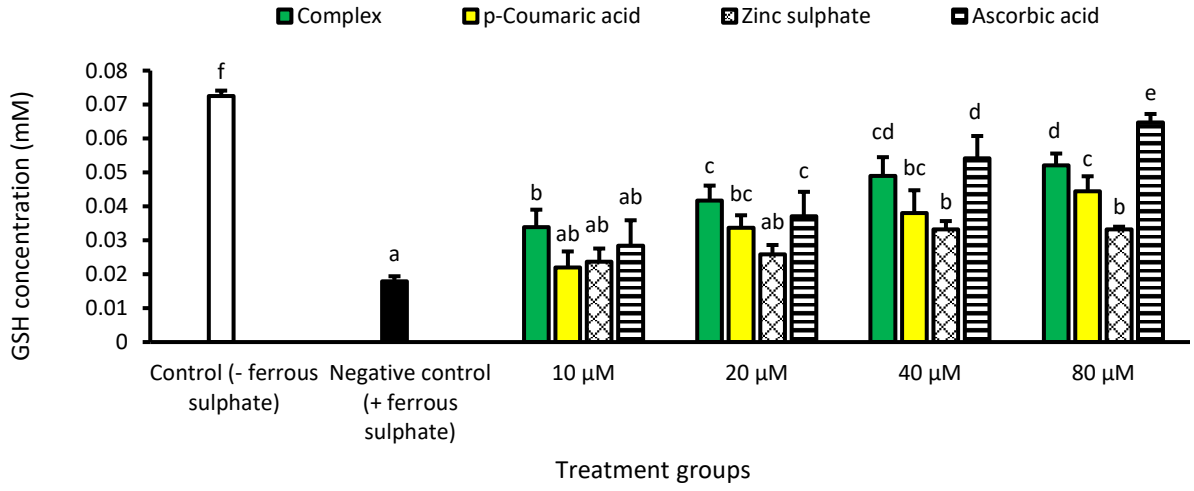
| Experimental parameters | Complex | p-Coumaric acid | | Ascorbic acid | Trolox | Acarbose | Amgd |
|---|---|---|---|---|---|---|---|
| | $IC_{50}$ or $EC_{50}$ values (µM) | | | | | | |
| Inhibition of oxidative stress-induced lipid peroxidation in Chang liver cells ($IC_{50}$) | 56.2 ± 8.35$^{c}$ | 131 ± 26.6$^{b}$ | 743 ± 51.3$^{a}$ | 30.3 ± 8.52$^{c}$ | NA | NA | NA |
| Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver ($IC_{50}$) | 398 ± 189$^{b}$ | 2160 ± 129$^{a}$ | ND | 189 ± 71.5$^{b}$ | NA | NA | NA |
| Inhibition of oxidative stress-induced GSH depletion in Chang liver cells ($IC_{50}$) | 33.9 ± 13.1$^{b}$ | 85.4 ± 31.9.0$^{b}$ | 709 ± 265$^{a}$ | 27.3 ± 5.51$^{b}$ | NA | NA | NA |
| Inhibition of oxidative stress-induced GSH depletion in isolated rat liver ($IC_{50}$) | 38.7 ± 7.15$^{c}$ | 147 ± 18.0$^{b}$ | 2150 ± 437$^{a}$ | 27.0 ± 2.48$^{c}$ | NA | NA | NA |

"Amgd" means "aminoguanidine"; "ND" means "not determined; "NA" means "not applicable"; "GSH" means "reduced glutathione"; $IC_{50}$ is concentration needed to inhibit the activity of carbohydrate digesting enzymes, bovine serum albumin glycation, lipid peroxidation and reduced glutathione depletion 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%. Data are shown as mean ± SD of triplicate analysis. Different letters 'a', 'b' and 'c' represent significant difference ($p<0.05$) between treatment groups.

At cellular (Chang liver cells) and tissue (isolated rat liver tissue) hepatic levels, the complex, also, exhibited significantly ($p < .05$) stronger antioxidant actions compared to its precursors (zinc sulphate and p-coumaric acid) (**Figures 4.6 and 4.7; Table 4.1**).



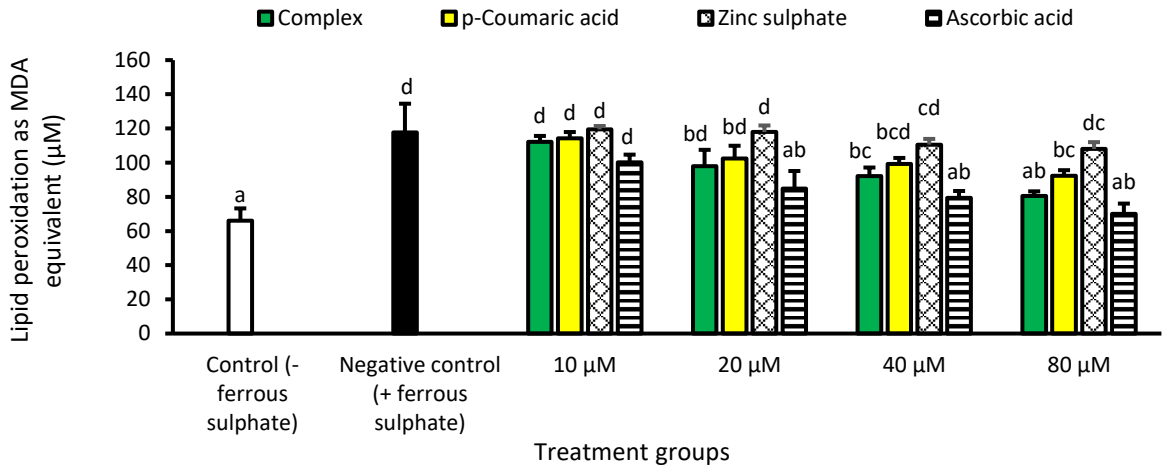

**(a)**



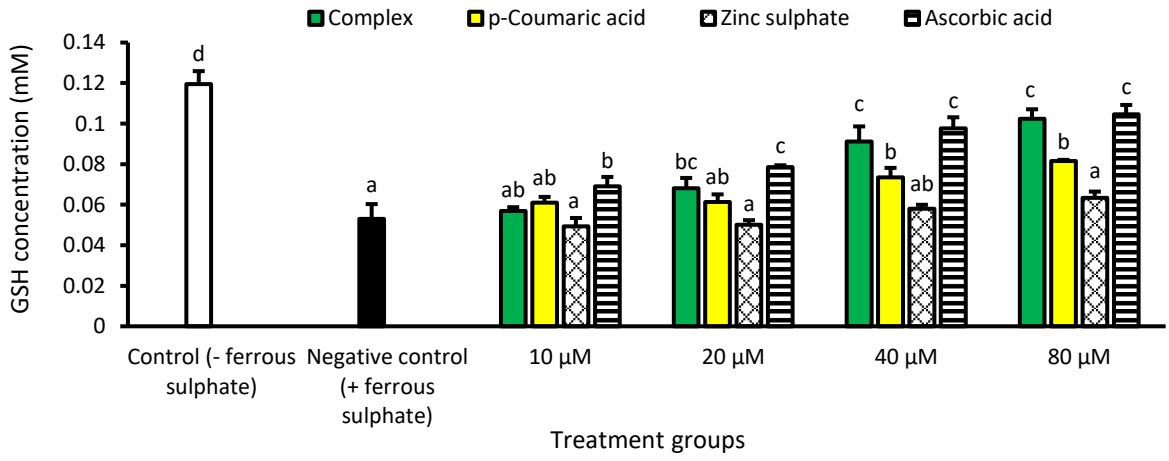

**(b)**

**Figure 4.6:** Graphs showing how the complex, precursors and standards inhibit **(a)** lipid peroxidation and **(b)** GSH depletion in Chang liver cells. Data are shown as mean ± SD of replicate 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.

The cellular and tissue inhibitory action of the complex on oxidative stress-induced lipid peroxidation ($IC_{50}$ = 56.2 and 398 µM) and GSH depletion ($IC_{50}$ = 33.9 and 38.7 µM) was more potent than that of p-coumaric acid and did not differ significantly ($p > .05$) from that of ascorbic and Trolox (**Table 4.1**).




**(a)**

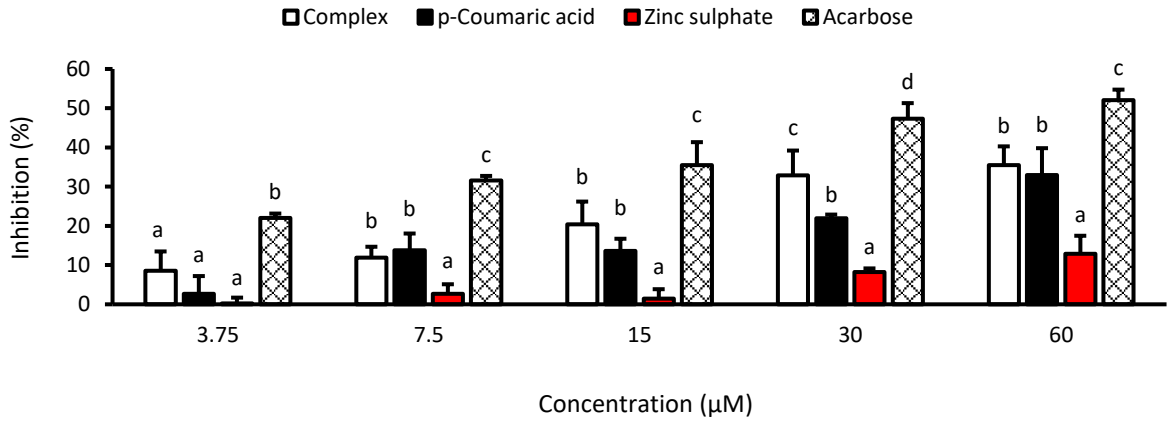



**(b)**

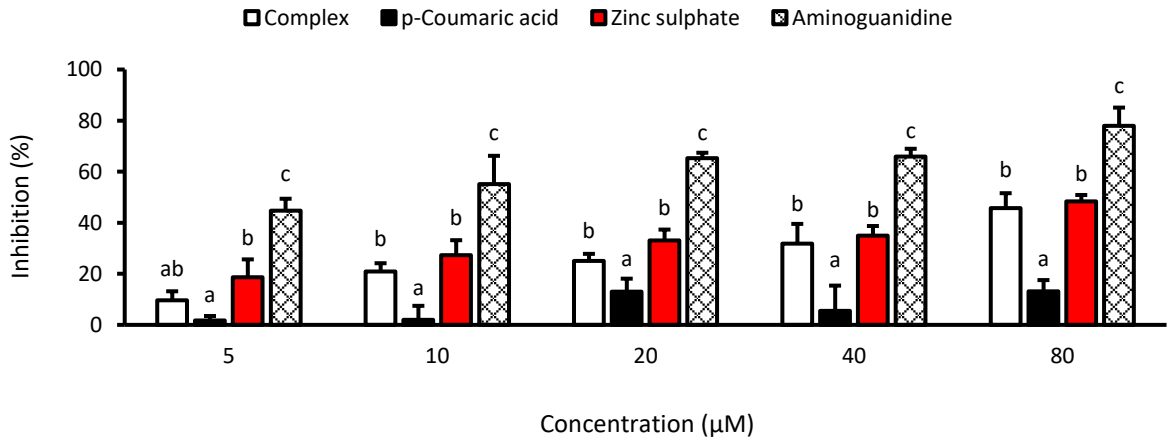
**Figure 4.7:** Graphs showing how the complex, precursors and standards inhibit **(a)** lipid peroxidation and **(b)** GSH depletion in isolated rat liver tissues. Data are shown as mean ± SD of replicate analysis. Different letters 'a', 'b', 'c' and 'd' represent significant difference ($p<0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

### 4.4.3. Diabetes-related bioactivities of complex and precursors

p-Coumaric acid had a dose-dependent inhibitory effect ($IC_{50}$ = 46.3 µM) on α-glucosidase enzyme *in vitro* (**Figure 4.8a and Table 4.1**). Relative to p-coumaric acid ($p < .05$), zinc sulphate showed a mild α-glucosidase inhibitory activity ($IC_{50}$ = 207 µM) (**Table 4.1**). The complex had a more potent inhibitory activity ($IC_{50}$ = 19.6 µM) compared to its precursors and was statistically comparable ($p > .05$) to acarbose ($IC_{50}$ = 14.0 µM) (**Table 4.1**).



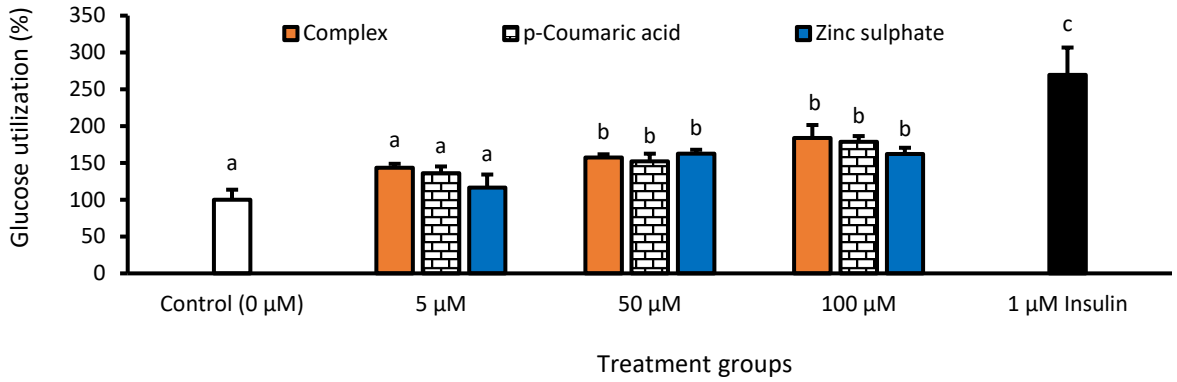

**(a)**



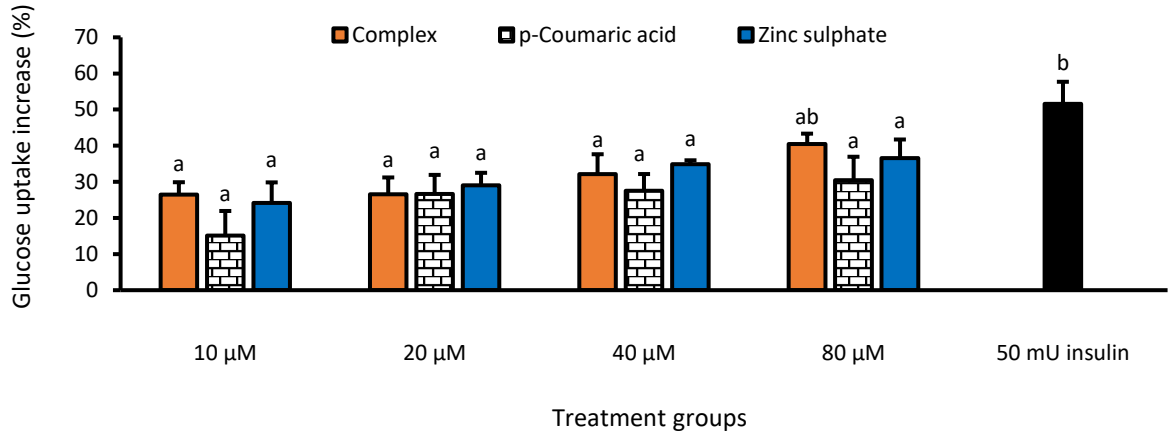

**(b)**

**Figure 4.8:** *In vitro* **(a)** α-glucosidase and **(b)** BSA glycation inhibitory effects of complex, precursors and standards. Data are shown as mean ± SD of triplicate analysis. Different letters 'a', 'b', 'c' and 'd' represent significant difference ($p<0.05$) between treatment groups.

Zinc sulphate dose-dependently inhibited ($IC_{50}$ = 34.6 µM) glucose-mediated glycation of BSA protein in vitro, while p-coumaric acid showed no observable antiglycation effect (**Figure 4.8b and Table 4.1**). Although not as potent as the antiglycation effect of aminoguanidine ($IC_{50}$ = 8.70 µM), zinc sulphate conferred a complexation-mediated antiglycation property on p-coumaric acid ($IC_{50}$ = 39.9 µM) (**Figure 4.8b and Table 4.1**).




**(a)**

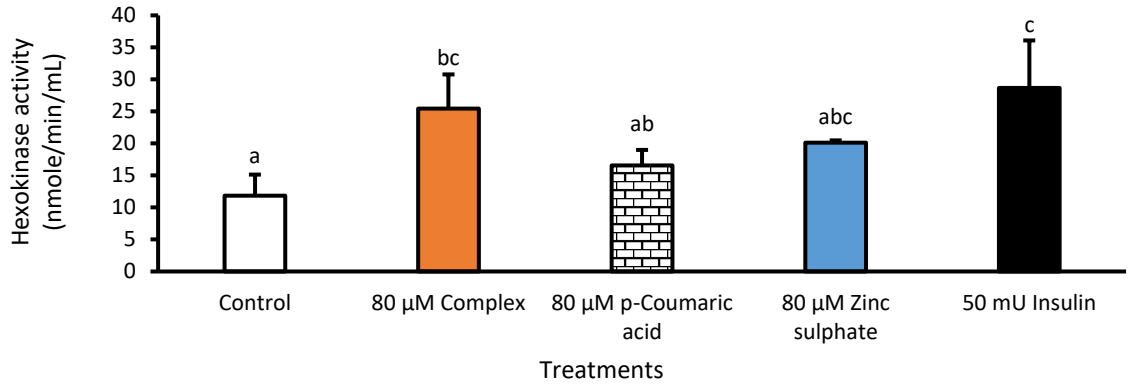



**(b)**

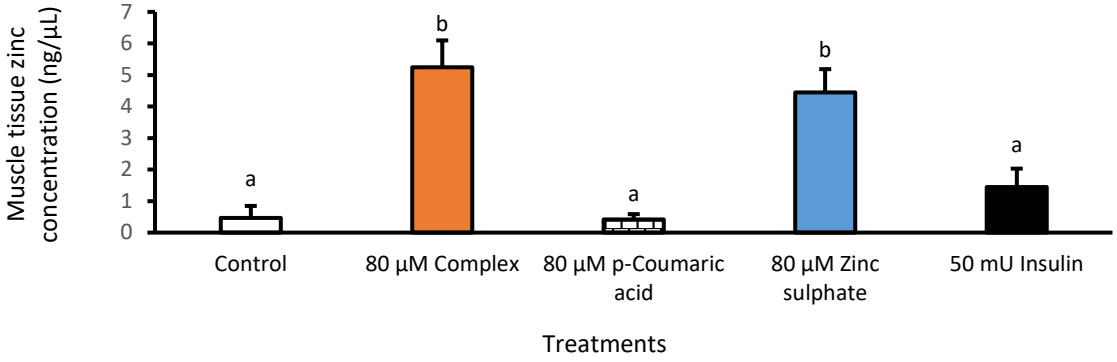
**Figure 4.9:** Effect of complex, precursors and standards on glucose uptake in (**a**) L6-myotubes, (**b**) isolated rat psoas muscle tissues. Data are shown as mean ± SD of replicate analysis (n = 3). Different letters 'a', 'b' and 'c' represent significant difference ($p<0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

In L-6 myotubes and isolated rat muscle tissues, p-coumaric acid, zinc sulphate, and the complex showed a dose-dependent modulatory effect on glucose uptake (**Figure 4.9 and Table 4.1**). The complex had the most potent effect at cellular ($EC_{50}$ = 10.7 µM) and tissue levels

($EC_{50}$ = 428 µM) based on $EC_{50}$ values. Its glucose uptake effect was 2.8-folds more potent ($p < .05$) than that p-coumaric acid ($EC_{50}$ = 1180 µM) in the rat muscle tissue (**Table 4.1**).




**(a)**




**(b)**

**Figure 4.10:** Effect of the highest concentration of the tested samples on **(a)** zinc concentration and **(b)** hexokinase activity in the isolated rat psoas muscle tissues used for glucose uptake study. Data are shown as mean ± SD of triplicate analysis (n = 3). Statistical comparison was done between treatment groups, as well as between treatment groups and the controls (control and insulin). Significant difference ($p<0.05$) means a difference in letters.

Elevated hexokinase activity was also, observed in treated isolated rat psoas muscle tissues showing modulated glucose uptake (**Figure 4.10a**). Muscle tissue zinc concentration was significantly higher ($p < .05$) in the complex and zinc sulphate-treated muscle tissues than the tissues treated with p-coumaric acid and insulin (**Figure 4.10b**)

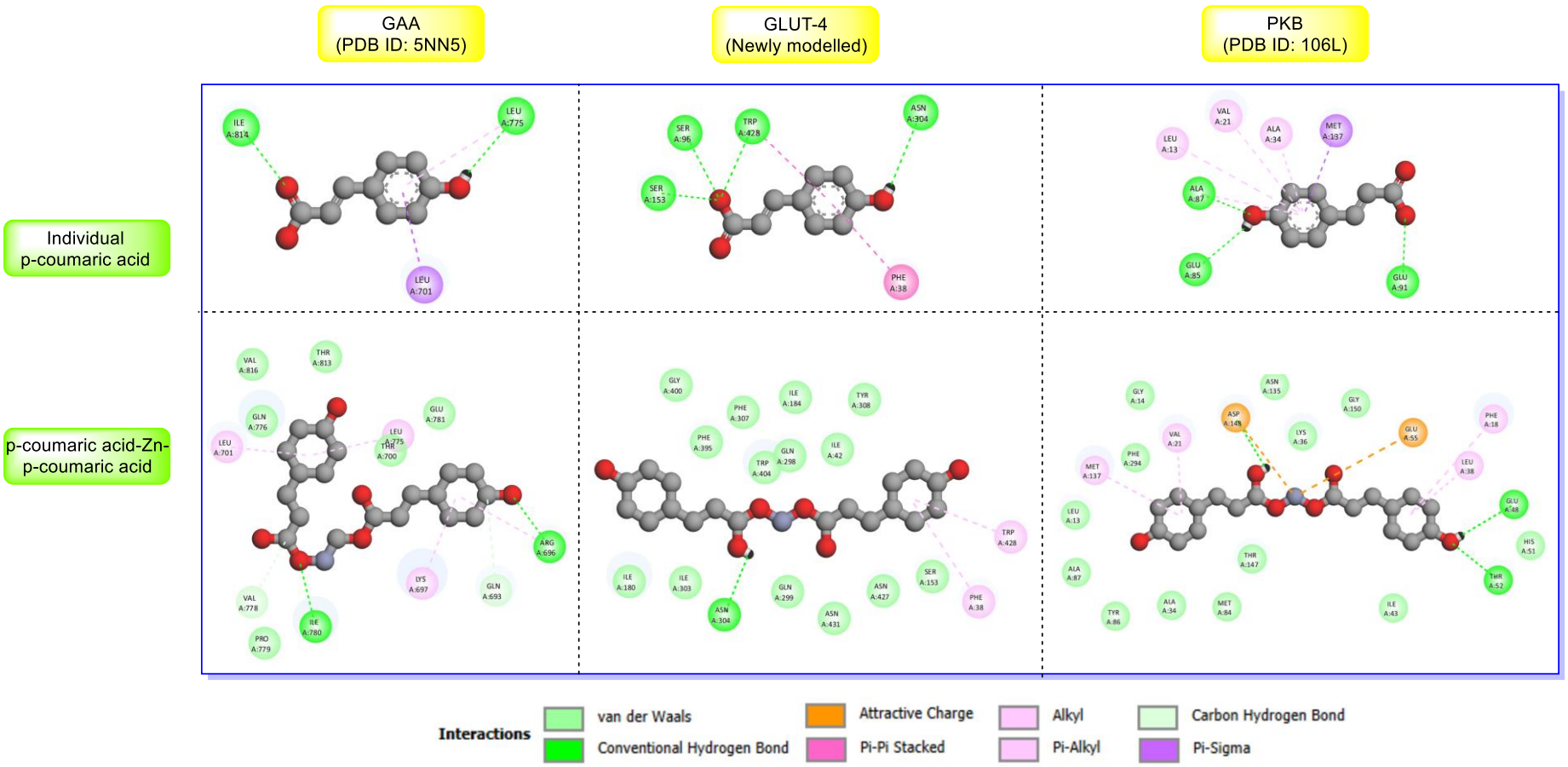



**Figure 4.11:** Molecular docking interaction of ferulic acid and its complex with four enzyme targets (α-glucosidase, GLUT-4 and PKB) linked to diabetes. GAA, α-glucosidase; GLUT4, glucose transporter type 4; PKB, protein kinase B.

**Table 4.2**. Molecular docking score (kcal/mol) of p-coumaric acid and its Zn(II) complex with target enzymes linked to diabetes.

| Target enzymes | p-coumaric acid | Complex |
|---|---|---|
| GAA (PDB ID: 5NN5) | -6.31 | -7.18 |
| GLUT-4 (Newly modelled) | -6.35 | -7.58 |
| PKB (PDB ID: 106L) | -6.41 | -7.74 |

GAA, *α-glucosidase;* GLUT4, glucose transporter type 4; PKB, protein kinase B.

The visualized docking interaction between p-coumaric acid or its Zn(II) complex and the protein targets (α-glucosidase, GLUT-4, and PKB) is shown in **Figure 4.11**, while **Table 4.2** depicts the minimum docking scores (kcal/mol). The docking scores of the complex (−7.18 to −7.74 kcal/mol) against the enzyme targets were higher than those of p-coumaric acid (−6.31 to −6.41 kcal/mol) (**Table 4.2**).

At *in vitro* cellular level, the complex was not myotoxic or hepatotoxic at tested concentrations ranging from 5 to 817 µM (**Figure 4.12**).

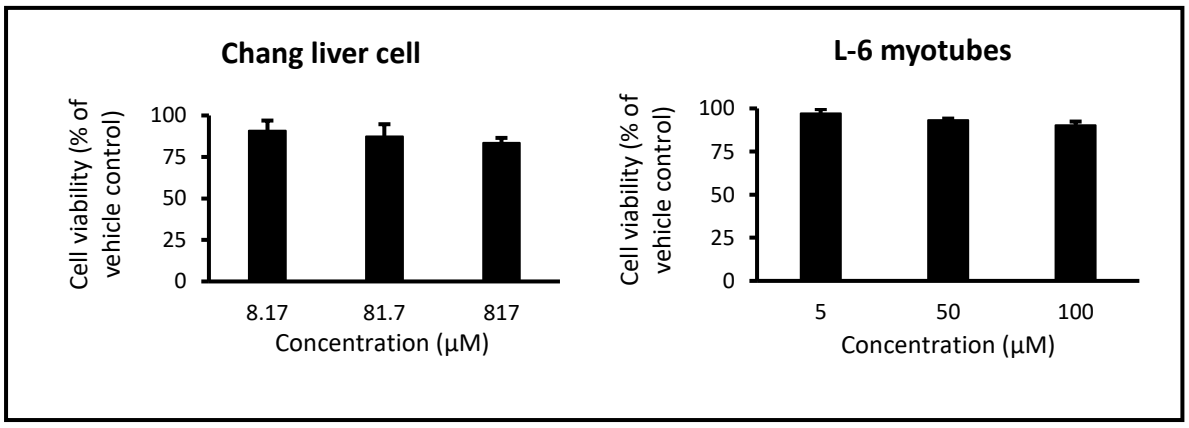



**Figure 4.12:** Effect of the complex on the viability of Chang liver cells and L6-myotubes. Data are shown as mean ± SD of replicate analysis.

## 4.5 Discussion

In the past two decades, considerable attention has been drawn to zinc mineral as a possible therapeutic supplement for diabetes due to its role in insulin secretion and reported insulin mimetic potentials (Chukwuma *et al*., 2020). Although many researchers have explored zinc as a precursor for developing metal–ligand complexes with possible diabetes-related bioactivity, they have concentrated on synthetic ligands with toxicity concerns and no documented pharmacological credence (Chukwuma *et al*., 2020). In this study, we considered a natural phenolic acid (p-coumaric acid) as an alternative ligand to develop a Zn(II)-p-coumaric acid complex with improved diabetes and oxidative stress-related bioactivity. The potent bioactivity of the complex relative to its precursor suggests a possible complexation-mediated synergism between zinc sulphate and p-coumaric, which is partly attributed to Zn(II)-bicoumarate structure of the complex (**Figure 4.2a**).

p-Coumaric acid is a natural antioxidant with radical scavenging activity (Kiliç & Yeşiloğlu, 2013; Kadoma & Fujisawa, 2008), which could be attributed to its ability to donate electron or transfer hydrogen, thus forms a stable phenoxy radical intermediate (Chen *et al*., 2020). Also, its anti-lipid peroxidative effect has been shown in high-fat diet mice model (Shen *et al.*, 2019), which may be linked to its quenching capacity of radicals associated with lipid peroxidation process. In the present study, consistent antioxidant effects in the form of *in vitro* radical scavenging and anti-linoleic acid peroxidative activity were shown by p-coumaric acid (**Figures 4.4 and 4.5; Table 4.1**). Also, in Chang liver cells and isolated rat liver tissues, p-coumaric acid demonstrated antioxidant effects by inhibiting oxidative stress-induced lipid peroxidation and GSH depletion (**Figures 4.6 and 4.7; Table 4.1**). However, upon complexation with zinc sulphate, the resulting complex acquired two moieties of p-coumaric acid, which afforded it the ability to potentiate stronger antioxidant activities relative to its precursors. Based on computations using $IC_{50}$ values (**Table 4.1**), the complex was 1.5-to 5.4-folds more potent than p-coumaric acid and was statistically comparable ($p > .05$) to Trolox and ascorbic acid. The data suggest that Zn(II) complexation may be a therapeutic approach for improving the antioxidant capacity of p-coumaric acid, which may be relevant in mitigating oxidative stress and its insults on different tissues.

Elevated oxidative stress in diabetic condition has been shown to be fuelled by persistent hyperglycaemia and processes like glycation and increased mitochondrial superoxide ion

production are known to be key culprits (Fakhruddin *et al*., 2017; Giacco & Brownlee, 2010). Elevated glycation is a major risk factor of diabetic complications. Improving glycaemic control is important in limiting ROS prooxidant production processes and mitigating diabetic oxidative damage. Postprandial blood glucose has been shown to be controlled by inhibiting α-glucosidase, a key carbohydrate hydrolysing enzyme in the brush borders of the small intestine (Lebovitz, 1997). In this study, p-coumaric acid dose dependently inhibited α-glucosidase *in vitro* (**Figure 4.8a and Table 4.1**), which has been previously (Jeong *et al*., 2012) and recently (Aleixandre *et al*., 2022) documented. Zinc sulphate showed a relatively lower *in vitro* α-glucosidase inhibition (**Table 4.1**). However, it acquired two moieties of p-coumaric acid through complexation (**Figure 4.2a**) to potentiate a stronger (2.4-folds) α-glucosidase inhibition compared to p-coumaric acid alone (**Table 4.1**). Further, in silico docking analysis also suggests a structure–function relationship (**Figure 4.11 and Table 4.2**). More hydrogen bonding between complex and enzyme was observed due to the presence of two moieties of p-coumaric acid (**Figure 4.11**). This influenced its higher docking scores against α-glucosidase protein target compared to p-coumaric acid (**Table 4.2**).

On the other hand, it appears zinc sulphate conferred an *in vitro* antiglycation property on p-coumaric acid through complexation (**Figure 4.8b and Table 4.1**). Moreover, zinc sulphate has been shown to inhibit glucose-induced protein glycation *in vitro* (Tupe *et al*., 2015). Additionally, in diabetic rats, zinc sulphate treatment was shown to lower glycation and protein carbonyl interaction (Sacan *et al*., 2016). The above data suggest that Zn(II) complexation may be a therapeutic approach for improving the postprandial glycaemic control of p-coumaric acid, while affording other benefits that could mitigate diabetic complications. *In vivo* studies on the complex using appropriate animal models are, however, recommended moving forward.

Impaired insulin signalling is a major factor that drives persistent hyperglycaemia, particularly in T2D. There is impaired circulating glucose uptake and utilization, particularly in peripheral tissues, such as muscle and adipose tissues, which leads to poor glycaemic control and hyperglycaemia (Galicia-Garcia *et al*., 2020). The insulin-mimetic potential of Zn(II) and its modulatory effect on insulin signalling have been documented. Akt phosphorylation was induced in mouse and human skeletal cells after Zn(II) treatment, which modulated insulin signalling (Norouzi *et al*., 2018). In 3 T3-L1 pre-adipocytes and adipocytes, Zn(II) treatment potentiated increased glucose uptake through insulin receptor and Akt activation (Tang & Shay, 2001). In the present study, complexing p-coumaric and Zn(II) lead to a modulatory effect on

glucose uptake in L-6 myotubes and muscle tissues isolated from rats (**Figure 4.9; Table 4.1**). The complex outperformed its precursors (**Table 4.1**). In the complex and zinc-treated tissues, glucose uptake activity was accompanied by an increase in the concentration of zinc relative to the non-treated tissues (**Figure 4.10b**). Also, zinc concentration was higher in the complex-treated muscle tissues compared to the zinc sulphate-treated muscle tissues (**Figure 4.10b**), suggesting that the complex increased tissue or cellular Zn(II) uptake. This effect may be relevant in increasing in glucose uptake. Documented evidence has shown that non-pharmacologically active 1-oxy-2-pyridinethiol ligands potentiated insulin-mimetic effects in adipocytes by enhancing cellular zinc uptake upon complexation (Yoshikawa *et al*., 2011; Basuki *et al*., 2007).

The present study, however, suggests a synergistic interaction between the Zn(II) and p-coumaric acid components of the complex in potentiating a stronger glucose uptake. p-Coumaric acid has been shown to modulate glucose and lipid metabolism in L-6 skeletal muscle cells through an AMP-activated protein kinase-dependent mechanism (Yoon *et al*., 2013). In the present study, p-coumaric acid was shown to moderately increase cellular and tissue glucose uptake (**Figure 4.9; Table 4.1**). Hence, it is hypothesized that while complexing p-coumaric acid with Zn(II) enhanced cellular bioavailability of Zn(II), it concomitantly contributed to the glucose uptake effect potentiated by Zn(II). Hexokinase activity followed the same trend as tissue glucose uptake activity (**Figure 4.10a**), suggesting that the observed glucose uptake activity may be linked to increase in cellular glucose utilization.

Molecular docking data (**Figure 4.11 and Table 4.2**) suggest that the glucose uptake effect of the complex may be associated with some insulin signalling enzyme targets (GLUT-4 and PKB). These enzymes play key roles in the signalling of glucose uptake by insulin (Chang *et al*., 2004). Data showed that the docking score or binding energy against these enzyme targets was higher for the complex compared to p-coumaric acid (**Table 4.2**), which suggests more interaction with the enzymes.

Additional toxicity data revealed the complex was not toxic to hepatocytes and myocytes, at least under *in vitro* conditions (**Figure 4.12**). The study suggests that Zn(II)-p-coumaric acid complexation may be a therapeutic approach for synergistic glycaemic control with minimal toxicity, thus warrants future *in vivo* studies in type 2 diabetic animal model.

## 4.6 Conclusion

Synergistic therapy remains an important therapeutic approach for several diseases, including diabetes and associated oxidative insults. Also, natural medicines including supplements and phytochemicals have gained wide reception as supplemental therapies in functional medicine. Through complexation, we took advantage of the antioxidative and/or glycaemic control potentials of zinc mineral and p-coumaric acid to form a complex with an improved and multifaceted antioxidative and/or glycaemic control potential, as well as minimal cellular toxicity. The complex had a Zn(II)-bicoumarate molecular attribute that afforded a structure–function relationship. Zn(II) complexation may be a therapeutic approach for improving the antioxidative and glycaemic control potentials of p-coumaric acid, which may be medicinally relevant in combating diabetes and associated oxidative complications. It is important to note that the present study was limited to *in vitro*, cellular, and *ex vivo* experimental models. *In vivo* antioxidant, antidiabetic, and toxicity studies using appropriate animal models are recommended in further future studies.

# CHAPTER 5

## ZINC(II) – SYRINGIC ACID COMPLEXATION SYNERGISTICALLY EXERTS ANTIOXIDANT ACTION AND MODULATES GLUCOSE UPTAKE AND UTILIZATION IN L-6 MYOTUBES AND RAT MUSCLE TISSUE

### Prescript

This chapter investigated the *in vitro*, cellular and *ex vivo* antidiabetic and antioxidant potential of a novel Zn(II) complex of syringic acid. The content of this chapter has been published as follows: ***Ramorobi, L. M.***, *Matowane, G. R., Mashele, S. S., Swain, S. S., Makhafola, T. J., Mfengwana, P. H., & Chukwuma, C. I. (**2022**). Zinc(II) - Syringic acid complexation synergistically exerts antioxidant action and modulates glucose uptake and utilization in L-6 myotubes and rat muscle tissue. Biomedicine & Pharmacotherapy; 154, 113600. https://doi.org/10.1016/j.biopha.2022.113600*. The above-mentioned publication was adopted in writing the content of this chapter.

## 5.1 Abstract

Zinc and syringic acid have metabolic and antioxidant medicinal potentials. A novel Zn(II)–syringic acid complex with improved anti-hyperglycaemic and antioxidant potential was developed. Zn(II) was complexed with syringic acid in a 1:2 molar ratio and characterized using FT-IR, $^{1}$H NMR and LC-MS. Different experimental models were used to compare the anti-hyperglycaemic and antioxidant properties between the complex and precursors. A Zn(II)-bisyringate.2$H_2O$ complex was formed. The *in vitro* radical scavenging and $Fe^{3+}$ reducing antioxidant, antiglycation, and α-glucosidase inhibitory activities of the complex were 1.8–5.2 folds stronger than those of the syringic acid precursor and comparable to those of the positive controls. The complex possessed an increased ability to inhibit lipid peroxidation (by 1.6–1.7 folds) and glutathione depletion (2.8–3 folds) relative to syringic acid in Chang liver cells and liver tissues isolated from rats. The complex exhibited a higher glucose uptake effect ($EC_{50}$ = 20.4 and 386 µM) than its precursors ($EC_{50}$ = 71.1 and 6460 µM) in L6-myotubes and psoas muscle tissues isolated from rats, respectively, which may be linked to the observed increased cellular zinc uptake potentiated by complexation. Tissue glucose uptake activity was accompanied by increased hexokinase activity, suggesting increased glucose utilization. Moreover, treatment increased tissue phospho-Akt/pan-Akt ratio. The complex had strong molecular docking scores than syringic acid with target proteins linked to diabetes. The presence of two syringic acid moieties and Zn(II) in the complex influenced its potency. The complex was not hepatotoxic and myotoxic in vitro. Zinc-syringic acid complexation may be a novel promising therapeutic approach for diabetes and oxidative complications.

## 5.2 Introduction

With the recent global diabetes population standing at 537 million, the disease is increasingly affecting people of different age groups (IDF, 2021). Diabetes presents severe hyperglycaemic conditions due to poor nutrient metabolism, of which poor insulin secretion and action are the most influential culprits (Galicia-Garcia *et al*., 2020; Atkinson *et al.*, 2014). Type 2 diabetes (T2D) is the most prevalent diabetes, accounting for about 95% of incidences, which is largely fuelled by its association with poor nutrition, weight gain, obesity, and lack of exercise (Sami *et al*., 2017; Al-Goblan *et al*., 2014). Typically, these factors promote insulin resistance, poor functioning of pancreatic β-cells, and associated metabolic disorders, which leads to poor glucose and lipid metabolism, impaired circulating glucose clearance, impaired glucose

tolerance, hyperinsulinemia and, eventually persistent hyperglycaemia (Galicia-Garcia *et al*., 2020; Sami *et al*., 2017).

Oxidative stress plays a critical part in the progression of diabetes and the development of associated vascular complications (Giacco *et al*., 2010). Persistent hyperglycaemia increases glycation and accumulation of advanced glycation end products (AGEs), processes that increase the production of prooxidants and free radicals (Giacco *et al*., 2010). Prooxidants and free radicals are major catalysts of oxidative biomolecular inflammation and damage, which are implicated in diabetic vascular complications (Giacco *et al*., 2010).

While there are several synthetic diabetic drugs used for lowering blood glucose and mitigating diabetic complications, advances in research have shown that natural products such as phenolics can be safe alternatives for managing diabetes and oxidative complications (Sun *et al*., 2020). Polyphenols, including natural phenolic acids, possess innate radical quenching and antioxidant properties. The antioxidant properties of polyphenols are partly influenced by their ability to form stable phenoxy radical intermediates by donating an electron or transferring a hydrogen atom (Chen *et al*., 2020; Chukwuma *et al*., 2020). Additionally, phenolic acids have been shown to potentiate glucose lowering effects via various mechanisms (Vinayagam *et al*., 2016). Syringic acid is a phenolic acid that is naturally found in *Schumannianthus dichotomus* (Roxb.) Gagnep (Rob *et al*., 2020), as well as some fruits, such as grapes and berries (Pezzuto, 2008). It is an effective scavenger of free radicals (Rashedinia *et al*., 2021) and has been shown to ameliorate hepatic and renal oxidative stress (Rashedinia *et al*., 2021; Sabahi *et al*., 2020). In streptozotocin-induced diabetic rats, syringic acid ameliorated oxidative stress, reduced blood glucose, and improved diabetes-induced alterations of enzyme markers that are linked to hepatic tissue damage (Sabahi *et al*., 2020). Syringic acid also improved the renal and neuronal antioxidant status of diabetic rats by suppressing lipid peroxidation and/or increasing glutathione (GSH) concentration (Rashedinia *et al*., 2021; Rashedinia *et al*., 2020).

Furthermore, the role of nutrition in diabetes management has been documented (Bantle *et al*., 2006). Minerals and vitamins have shown promising outcomes in the prevention and management of T2D (Martini *et al*., 2010). Zinc mineral is known for its role in insulin secretion and has been reported to possess insulin mimetic potential (Chukwuma *et al*., 2020). Numerous zinc-ligand complexes with insulin mimetic potential have been synthesized using different types of ligands and Zn(II). Adachi *et al*. (Adachi *et al*., 2004) reported the

antilipolytic and glucose uptake effects of Zn(II)-maltol complex in adipocytes isolated from rat. In T2D KK-$A^y$ mice, it reduced the levels of blood glucose and glycated haemoglobin (HbA1c) by approximately 52% and 19%, respectively, and improved glucose tolerance after 14-days i.p. treatment (4.5 mg Zn/kg bw) (Adachi *et al*., 2004). Zn(II) complex of hinokitiol, a naturally occurring ligand, has been reported to increase glucose uptake and inhibit lipolysis in adipocytes (Murakami *et al*., 2012). In islet cells, it increased glucose-induced Akt phosphorylation, while in T2D KK-$A^y$ mice it showed anti-hyperglycaemic action (Naito *et al*., 2017).

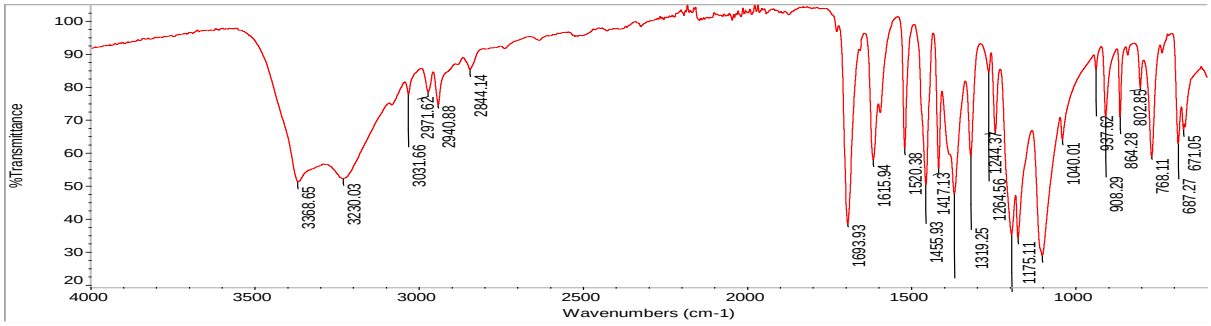
The concept of zinc-ligand complex as potential antidiabetic and insulin mimetic agents has been extensively studied (Chukwuma *et al*., 2020). However, majority (≈ 72%) of the studied zinc complexes are those with synthetic ligands that have no/limited known innate medicinal biological effects (Chukwuma *et al*., 2020). Many of these ligands pose toxicity concerns. Hence, a ligand that has known medicinal properties and minimal toxicity concern was explored in this study, which will better complement the glycaemic potential of zinc without posing toxicity concerns. In this context, syringic acid, a natural phenolic acid with antioxidant properties and several documented diabetes-related bioactivities was selected or considered. Also, syringic acid, as well as several phenolic acids have not been studied as promising pharmacological ligands in synthesizing zinc complexes that have potential medicinal relevance for diabetes and oxidative stress and do not present toxicity issues. The observed ongoing research trend negates the trajectory towards natural medicine. Therefore, this study was undertaken to investigate the glycaemic control, antioxidant and toxicity profiles of a novel Zn(II)-syringic acid complex.

## 5.3 Materials and methods

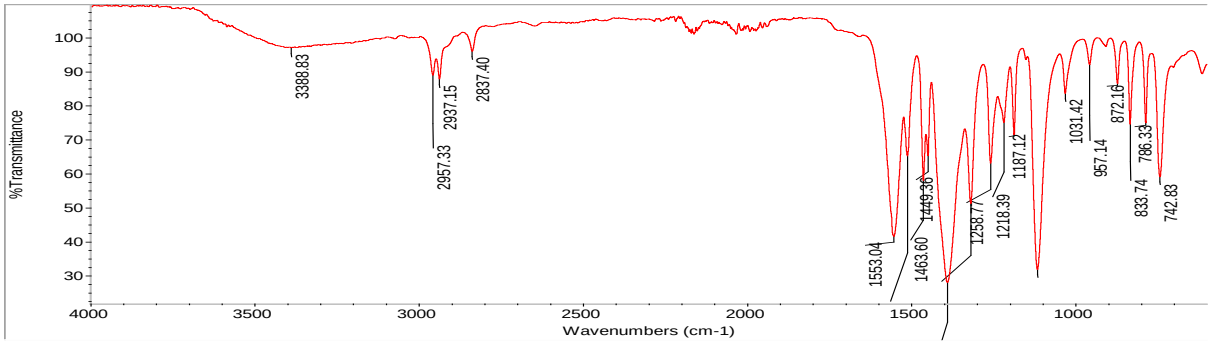
For the materials and methods of this chapter, please consult **sub-sections 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7 and 3.9 of chapter 3**.

## 5.4 Results

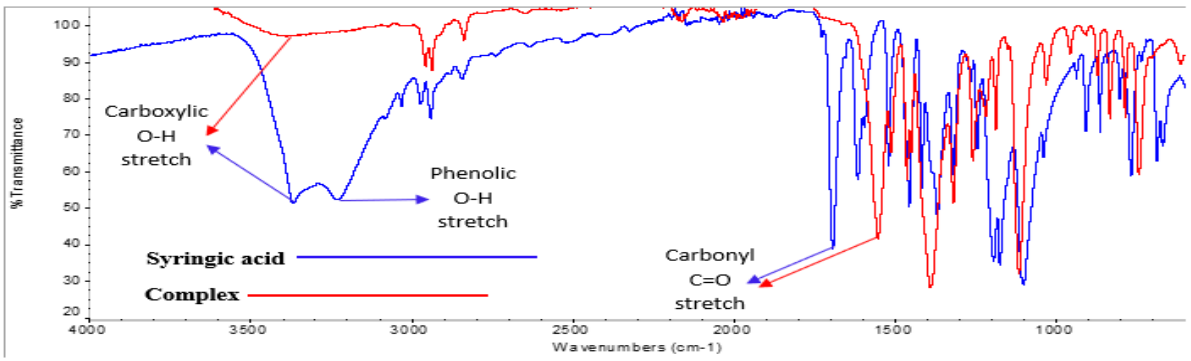
### 5.4.1 Characterization data




**(a)**




**(b)**




**(c)**

**Figure 5.1: (a)** syringic acid, **(b)** zinc(II)-bisyringate.2$H_2O$ complex and (**c**) superimposed FT-IR spectra.

The FT-IR data are presented in **Figure 5.1**. The FT-IR spectrum of syringic acid (**Figures 5.1a and 5.1c**) depicts a broad peak centred at 3368 $cm^{-1}$, which represents a signal caused by the carboxylic O-H stretch. The broad peak centred at 3230 $cm^{-1}$ is typical of a phenolic O-H stretch (**Figures 5.1a and 5.1c**). In the FT-IR of the complex (**Figures 5.1b and 5.1c**) the intensity of the carboxylic O-H stretch peak (3388 $cm^{-1}$) diminished, suggesting that the carboxylic end of the phenolic acid was involved in the Zn(II) complexation. The disappearance of the phenolic O-H stretch peak may be due to intermolecular hydrogen bond interaction of the phenolic OH group during synthesis, which may also influence its concentration in the molecule. The strong sharp peak (1693 $cm^{-1}$) caused by carbonyl C=O stretching in syringic acid (**Figures 5.1a and 5.1c**) notably shifted to a lower wave number (1553 $cm^{-1}$) in the FT-IR of the complex (**Figures 5.1b and 5.1c**). This shift is due to the donating of electrons from zinc to the C=O group of syringic acid, thus weakening the C=O bond, which strongly suggests that the complexation between Zn(II) and syringic acid occurred through its carboxylic end as depicted in **Figure 5.2**.

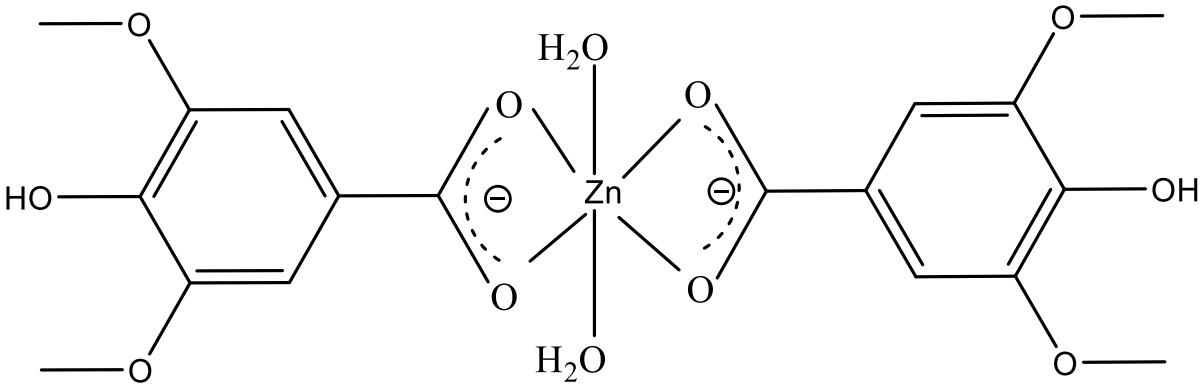



**Figure 5.2:** Proposed structure of the Zn(II)-bisyringate.$2H_2O$ complex.

**Figure 5.3** shows the high-resolution mass spectroscopic analysis of the complex. The value of 463.0602 (*m/z*) with calculated mass of 463.0582 (*m/z*) had a difference of 20 units, which represents the complex after losing two methyl groups and sulphate group. When the complex lost three of its methyl groups and a sulphate group, the mass of 418.9987 (*m/z*) was observed, which differed from the calculated mass (*m/z* = 418.9956) by 31 units. When the complex lost all of its methyl groups and a sulphate group the mass of 409.0078 (*m/z*) was observed, which differed from the calculated mass (*m/z* = 409.0118) by 40 units.

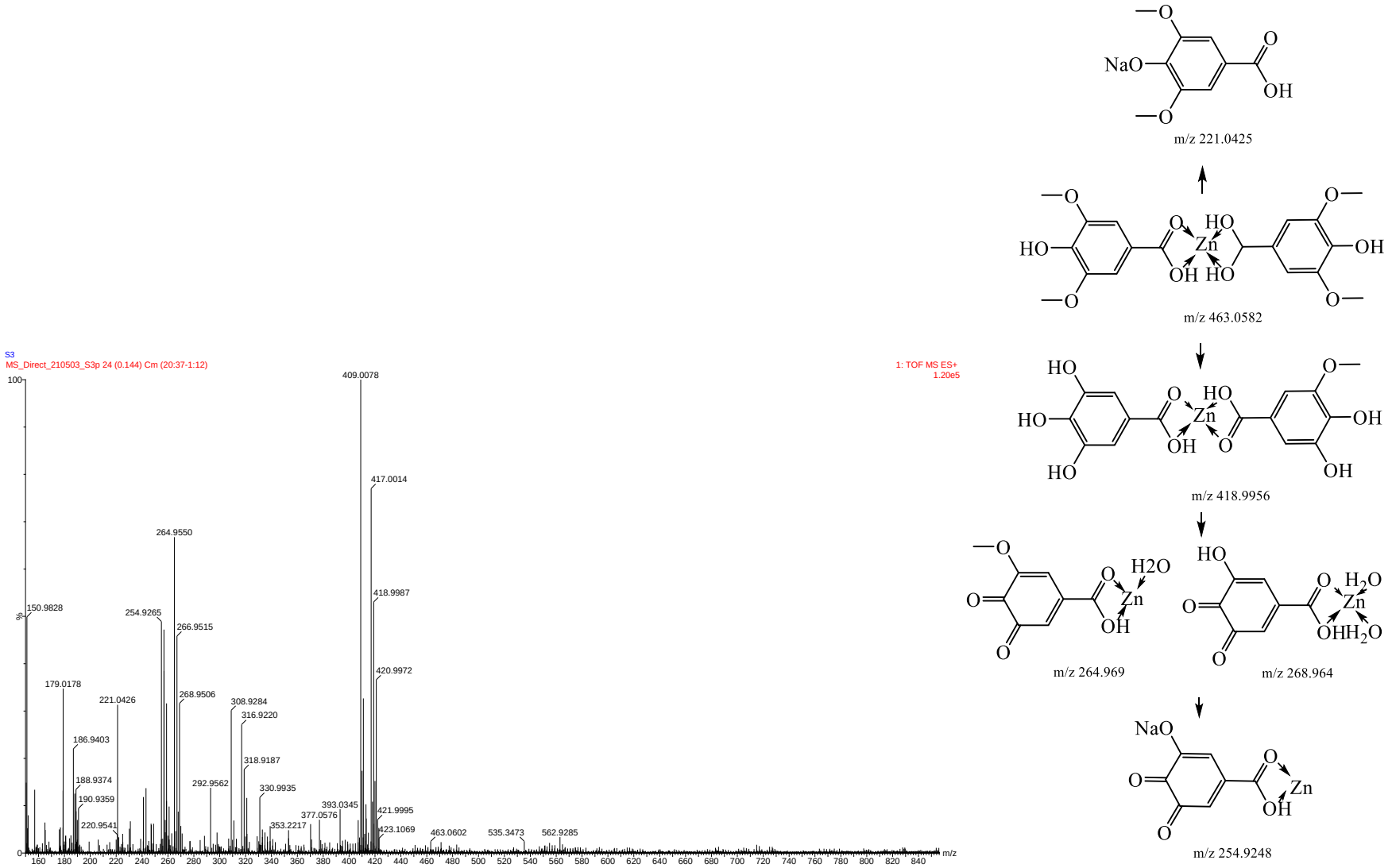



**Figure 5.3:** High resolution-mass spectroscopic (HR-MS) analysis of the Zn(II)-bisyringate.2$H_2O$ complex

The fragments *m/z* 264.9550 (calculated mass *m/z* m/z 264.969), *m/z* 268.9506 (calculated mass *m/z* 268.964) and *m/z* 254.9265 (calculated mass *m/z m/z* 254.9248) represent the loss of one syringic acid moiety. The fragment with *m/z* = 221.0426 is a sodium adduct of syringic zinc, which differed from the calculated mass (*m/z* = 221.0425) by 1 unit.

Proton NMR analysis (**Figure 5.4**) also supports the proposed structure of the complex shown in **Figure 5.2**. The $^{1}$H NMR showed the four methoxy groups of the syringic acid moieties resonated at $\delta$ 3.79, while the equivalent protons on the aromatic ring are located at $\delta$ 7.19 (**Figure 5.4**). The hydroxyl group of the phenolic moiety was observed at $\delta$ 9.24, which provides evidence that this group was not involved in complexation.

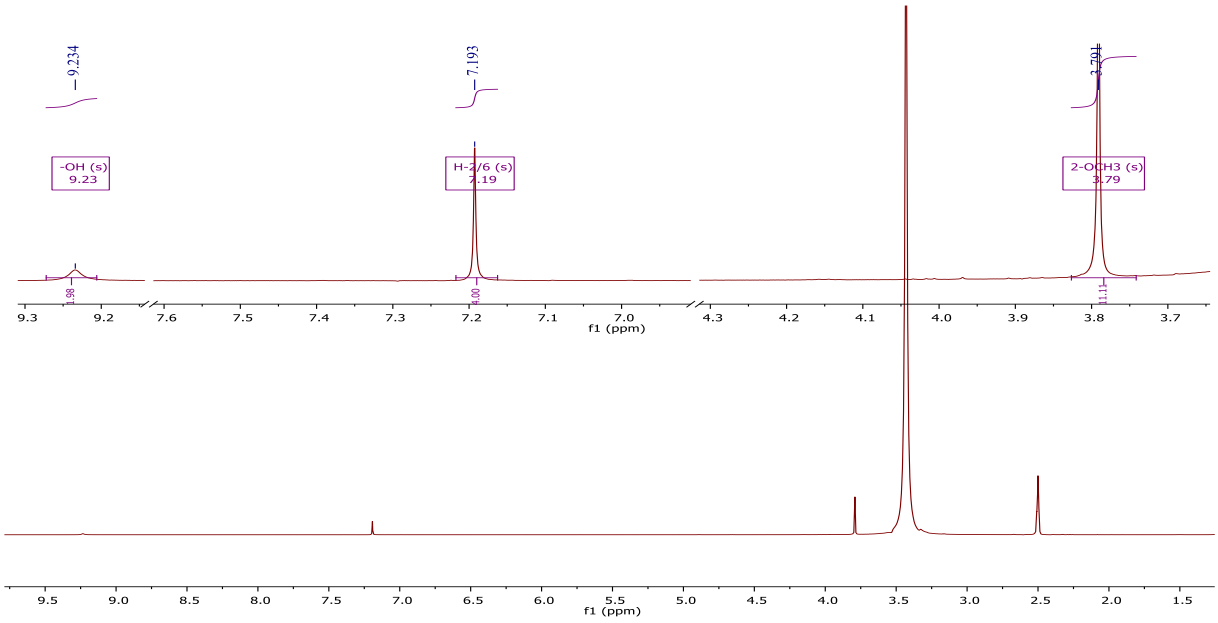
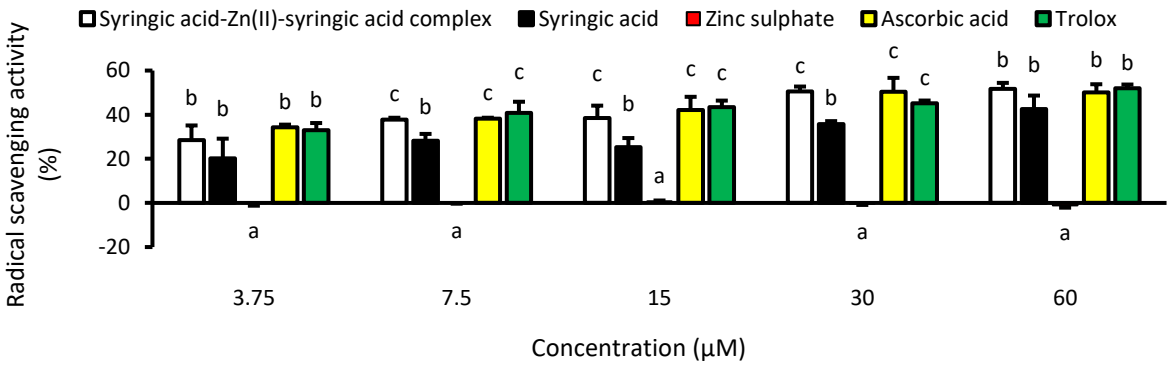



**Figure 5.4:** $^{1}$H NMR spectrum of the zinc(II)-bisyringate.2$H_2O$ complex. $^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.23 (s, 2-ArOH), 7.19 (s, 4H, H-2/6), 3.79 (s, 12H, 4-$OCH_3$)

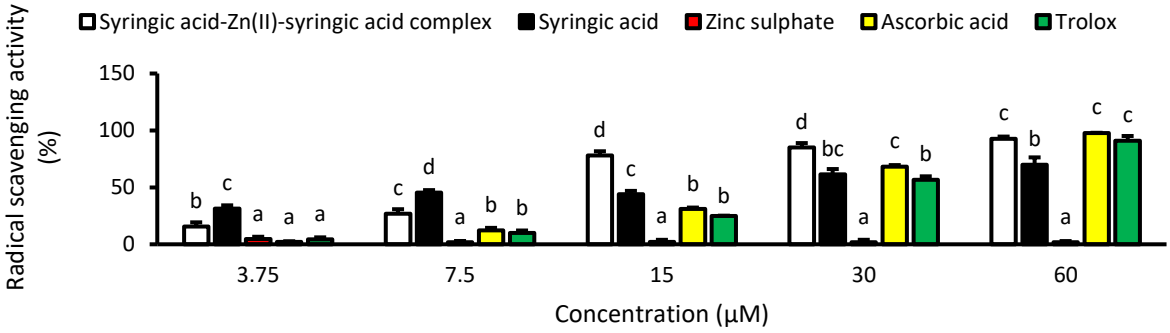
Summarily, the above characterization suggests that zinc sulphate complexed with two moieties of syringic acid through the carboxylic functional group of the two phenolic acid moieties (**Figure 5.2**). In the present study we consider syringic acid to be a chelating agent for Zn(II), which was mediated through the carboxylate group. This was influenced by using the sodium salt of syringic acid (sodium syringate) in the complex synthesis. A previous study (Kalinowska *et al*., 2011) has, also, reported the Zn(II) chelating properties of the carboxylate group of cinnamic acid by using the sodium salt of cinnamic acid (sodium cinnamate) in the complex synthesis, which supports our study.

### 5.4.2 Antioxidant properties

Syringic acid inhibited DPPH ($IC_{50}$ = 29.8 µM) and ABTS ($IC_{50}$ = 29.7 µM) radicals, reduced $Fe^{3+}$ ion (529 µmol/mol AAE) and inhibited linoleic acid peroxidation ($IC_{50}$ = 40.5 µM) *in vitro* (**Figures 5.5 and 5.6; Table 5.1**).




**(a)**




**(b)**

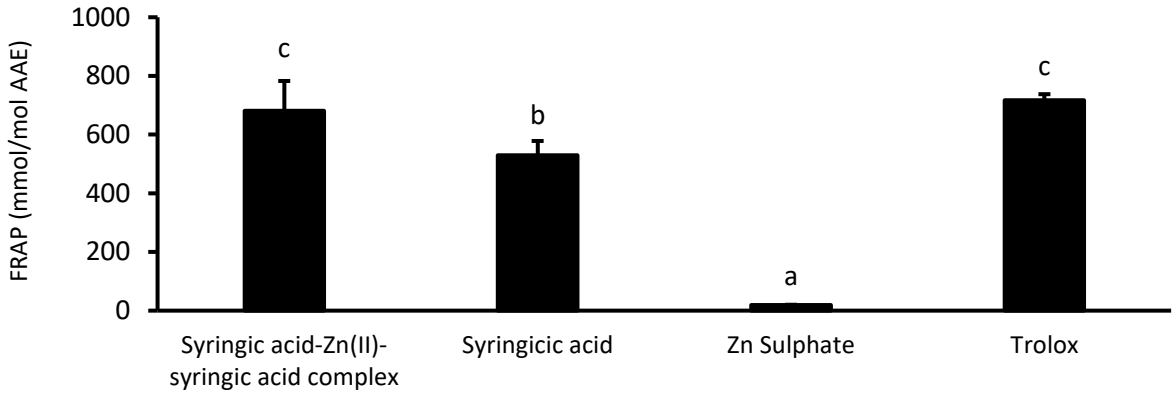
**Figure 5.5:** *In vitro* **(a)** DPPH radical scavenging and **(b)** $ABTS^{\bullet+}$ radical scavenging activities of complex, precursors and standards. The data are shown as mean ± SD of the triplicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups. Significant difference ($p<0.05$) is presented as a difference in the letter annotations.

**Table 5.1:** $IC_{50}$ and $EC_{50}$ values of syringic acid, its complex with Zn(II)

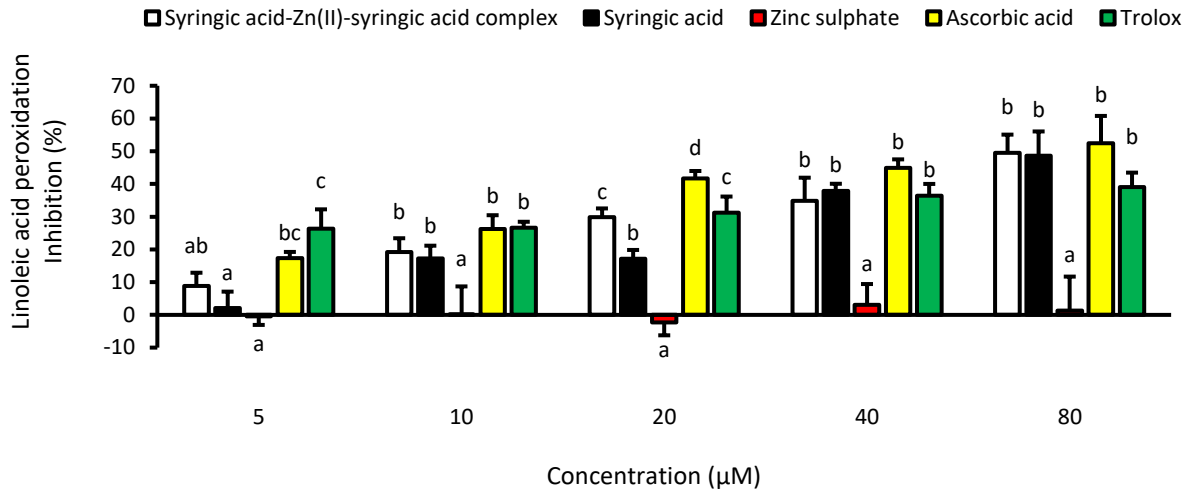
| Parameters or activity | Complex | Syringic acid | Zinc sulphate | Ascorbic acid | Trolox | Acarbose | Amgdn |
|---|---|---|---|---|---|---|---|
| | $IC_{50}$ or $EC_{50}$ values (µM) | | | | | | |
| ABTS radical scavenging activity ($IC_{50}$) | 5.76 ± 1.82$^{c}$ | 29.7 ± 5.92$^{b}$ | ND | 47.1 ± 4.47$^{b}$ | 100 ± 34.3$^{a}$ | NA | NA |
| DPPH radical scavenging activity ($IC_{50}$) | 10.7 ± 4.36$^{b}$ | 29.8 ± 5.83$^{a}$ | ND | 13.1 ± 6.11$^{b}$ | 10.0 ± 3.75$^{b}$ | NA | NA |
| Inhibition of *in vitro* linoleic acid peroxidation | 22.2 ± 2.78$^{bc}$ | 40.5 ± 4.90$^{a}$ | ND | 13.9 ± 4.38$^{c}$ | 37.8 ± 11.1$^{ab}$ | NA | NA |
| Antiglycation activity ($IC_{50}$) | 13.6 ± 6.27$^{b}$ | 30.8 ± 9.54$^{a}$ | 46.2 ± 7.57$^{a}$ | NA | NA | NA | 6.52 ± 0.93$^{b}$ |
| α-glucosidase inhibition ($IC_{50}$) | 16.8 ± 6.04$^{c}$ | 40.0 ± 4.08$^{b}$ | 78.7 ± 15.6$^{a}$ | NA | NA | 9.02 ± 3.18$^{c}$ | NA |
| Glucose uptake increase in L-6 myotubes ($EC_{50}$) | 20.4 | 6460 | 71.1 | NA | NA | NA | NA |
| Glucose uptakes increase in isolated rat psoas muscle ($EC_{50}$) | 386 ± 13.5$^{b}$ | 693 ± 31.9$^{a}$ | 450 ± 87.0$^{b}$ | NA | NA | NA | NA |

| Parameters or activity | Complex | Syringic acid | sulphate | orbic acid | Trolox | Acarbose | Amgdn |
|---|---|---|---|---|---|---|---|
| | $IC_{50}$ or $EC_{50}$ values (µM) | | | | | | |
| Inhibition of oxidative stress-induced lipid peroxidation in Chang liver cells ($IC_{50}$) | 25.7 ± 12.2[b] | 40.2 ± 12.2[b] | 538 ± 37.1[a] | 24.5 ± 6.21[b] | NA | NA | NA |
| Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver ($IC_{50}$) | 376 ± 123[b] | 650 ± 129[a] | ND | 244 ± 94.0[b] | NA | NA | NA |
| Inhibition of oxidative stress-induced GSH depletion in Chang liver cells ($IC_{50}$) | 18.1 ± 4.83[b] | 49.8 ± 17.0[b] | 449 ± 144[a] | 29.2 ± 5.51[b] | NA | NA | NA |
| Inhibition of oxidative stress-induced GSH depletion in isolated rat liver ($IC_{50}$) | 29.4 ± 2.62[b] | 89.7 ± 18.0[b] | 885 ± 190[a] | 23.8 ± 3.41[b] | NA | NA | NA |

"Amgdn" means "aminoguanidine"; "ND" means "not determined; "NA" means "not applicable; "GSH" means "reduced glutathione"; $IC_{50}$ is concentration needed to inhibit the activity of carbohydrate digesting enzymes, bovine serum albumin glycation, lipid peroxidation and reduced glutathione depletion 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' and 'c' represent significant difference ($p<0.05$) between treatment groups.




**(a)**

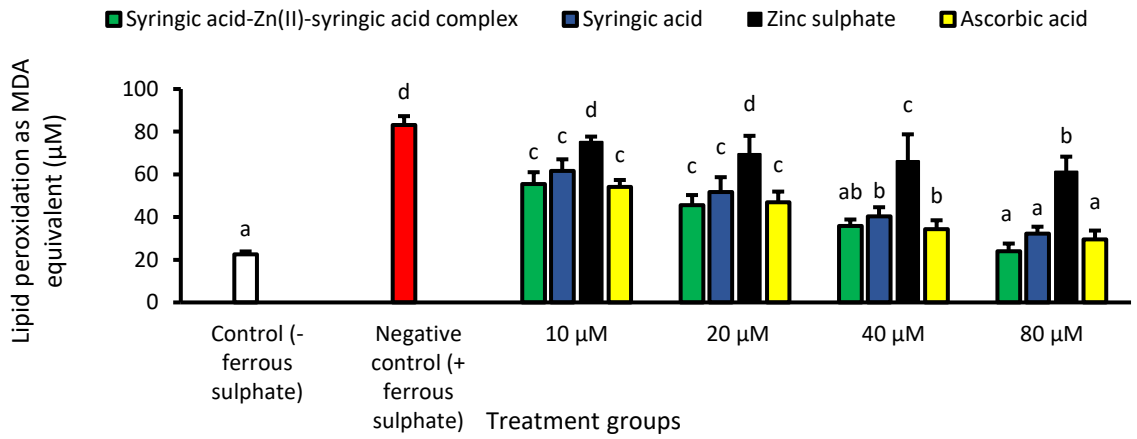



**(b)**

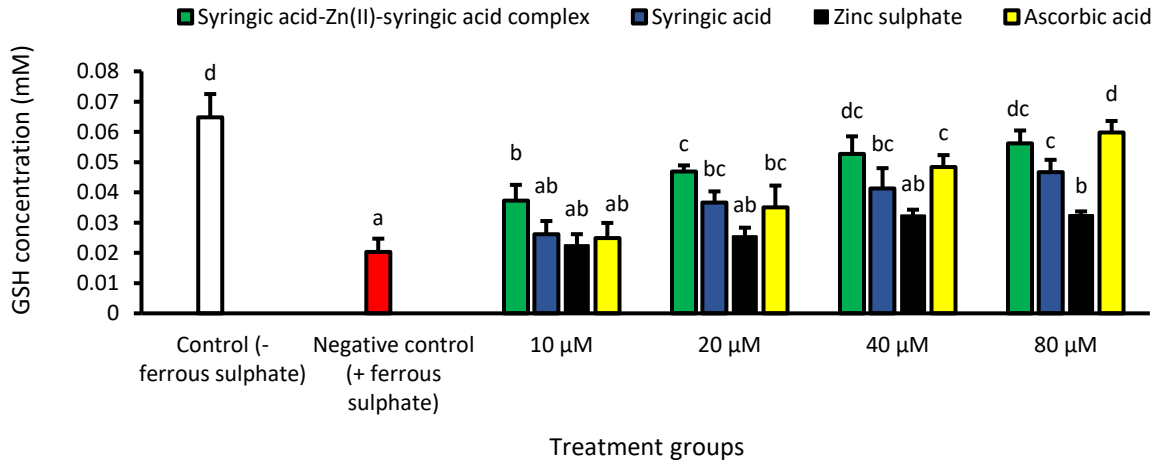
**Figure 5.6:** *In vitro* **(a)** $Fe^{3+}$ reducing and **(b)** linoleic acid lipid peroxidation inhibitory activities of complex, precursors and standards. The data are shown as mean ± SD of the triplicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups. Significant difference ($p<0.05$) is presented as a difference in the letter annotations.

The DPPH scavenging, $Fe^{3+}$ reducing and anti-lipid peroxidative activity of syringic acid was significantly ($p < 0.05$) less potent than that of ascorbic acid ($IC_{50}$ = 13.1 and13.9 µM). However, the complex showed DPPH ($IC_{50}$ = 10.7 µM) and ABTS ($IC_{50}$ = 5.76 µM) radical scavenging, $Fe^{3+}$ reducing (681 mmol/mol AAE) and anti-lipid peroxidative ($IC_{50}$ = 22.2 µM)

activities that were, respectively, 2.8, 5.2, 1.3 and 1.8 folds more potent than the activities of syringic acid and statistically comparable and/or more potent than that ascorbic acid and Trolox based on $IC_{50}$ values. Zinc sulphate showed no notable *in vitro* antioxidant effect (**Figures 5.5 and 5.6; Table 5.1**).

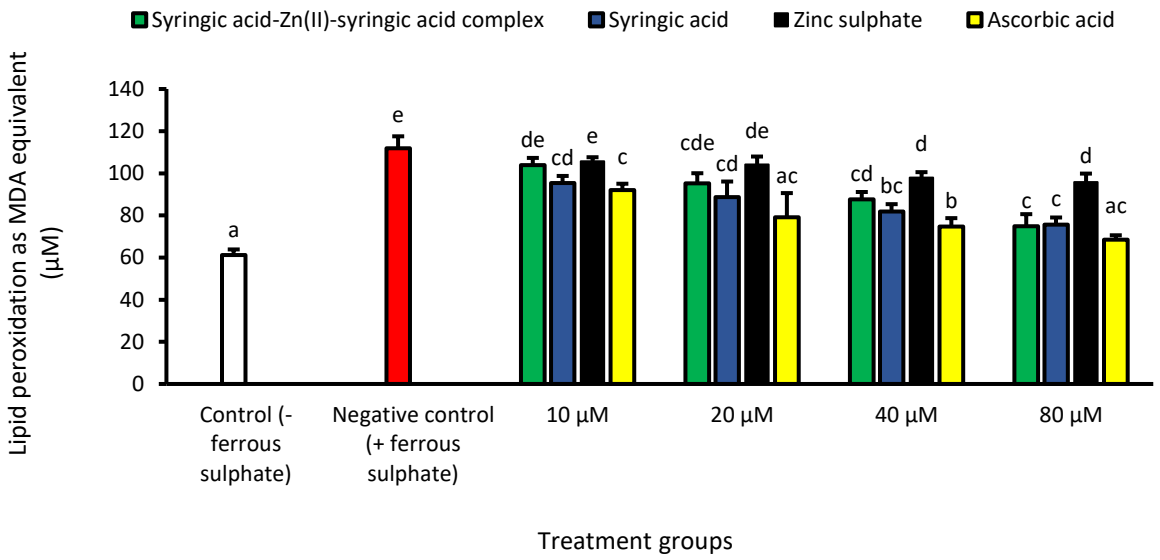



**(a)**

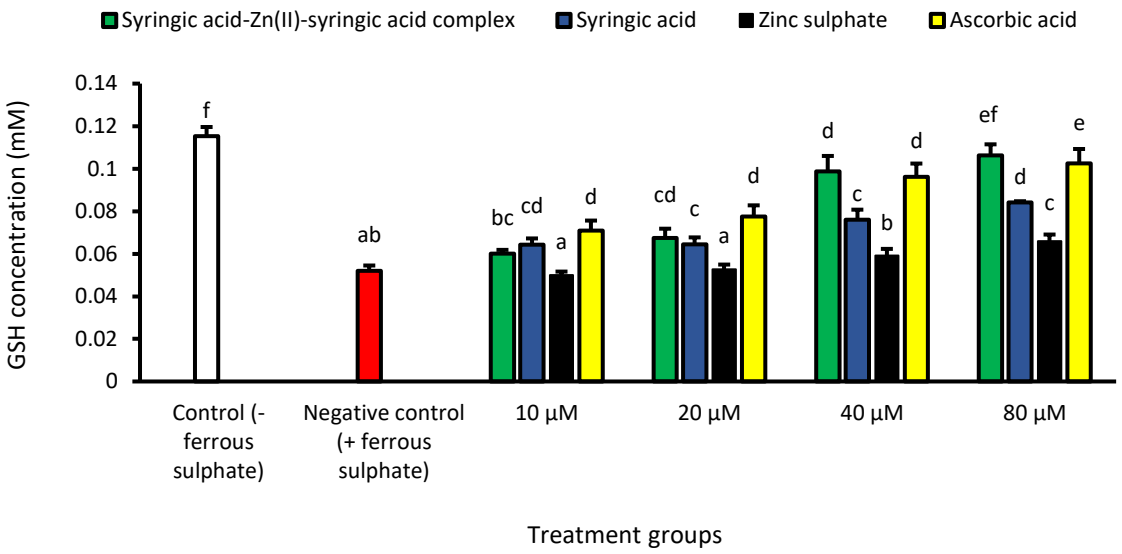



**(b)**

**Figure 5.7:** Graphs showing how the complex, precursors and standards inhibit **(a)** lipid peroxidation and **(b)** GSH depletion in Chang liver cells. Data are shown as mean ± SD of replicate analysis. Different letters at the top of each bar represent significant difference ($p<0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls.




**(a)**




**(b)**

**Figure 5.8:** Graphs showing how the complex, precursors and standards inhibit **(a)** lipid peroxidation and **(b)** GSH depletion in isolated rat liver tissues. Data are shown as mean ± SD of replicate analysis. Different letters at the top of each bar represent significant difference ($p<0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

In both Chang liver cells (**Figure 5.6 and Table 5.1**) and isolated rat liver tissue (**Figure 5.7 and Table 5.1**), $ZnSO_4$ minimally inhibited lipid peroxidation and GSH depletion ($IC_{50}$ = 449–885 µM) induced by oxidative stress. Syringic acid showed a significantly ($p < 0.05$) stronger cellular and tissue antioxidant activity ($IC_{50}$ = 40.2–650 µM) compared to $ZnSO_4$ but was not as potent as ascorbic acid (**Figures 5.6 and 5.7; Table 5.1**). However, the antioxidant activity of the complex showed a 36–67% increment compared to syringic according to the $IC_{50}$ values in **Table 5.1**. Moreover, the cellular and tissue antioxidant activity of the complex was statistically comparable ($p < 0.05$) to that of ascorbic acid (**Table 5.1**).

### 5.4.3 Antihyperglycemic properties

Data showed $ZnSO_4$ ($IC_{50}$ = 78.7 and 46.2 µM, respectively) and syringic acid ($IC_{50}$ = 40.0 and 30.8 µM, respectively) moderately inhibited α-glucosidase and BSA glycation in vitro, which was not as potent ($p < 0.05$) as the activity of acarbose ($IC_{50}$ = 9.02 µM) and aminoguanidine ($IC_{50}$ = 6.52 µM) (**Figures 5.9 and 5.10; Table 5.1**).

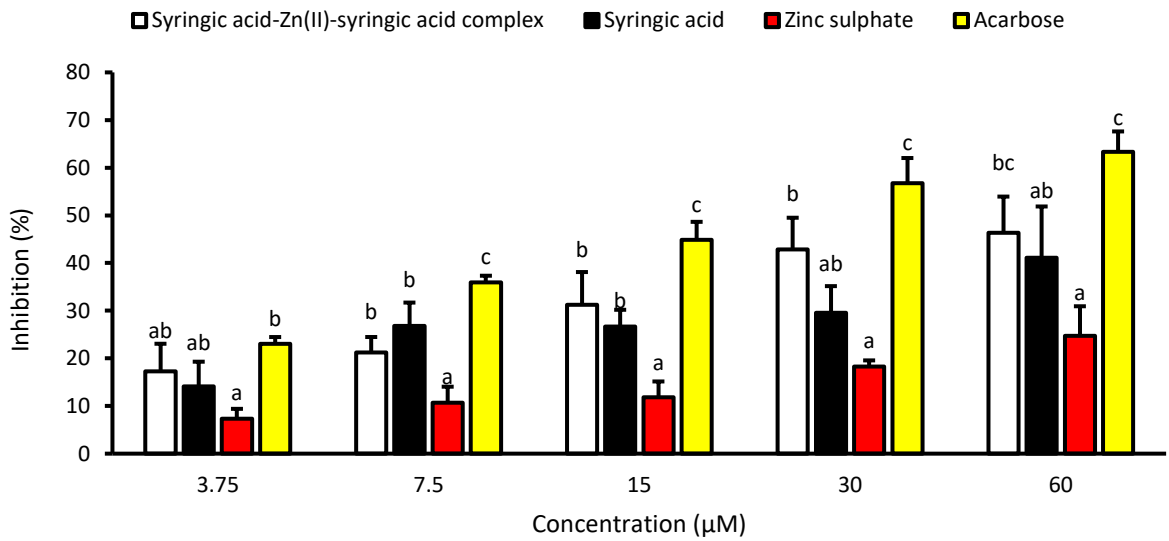



**Figure 5.9:** Effect of the complex, precursors and standards on *in vitro* α-glucosidase activity. The data are shown as mean ± SD of the triplicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups. Significant difference ($p<0.05$) is presented as a difference in the letter annotations.

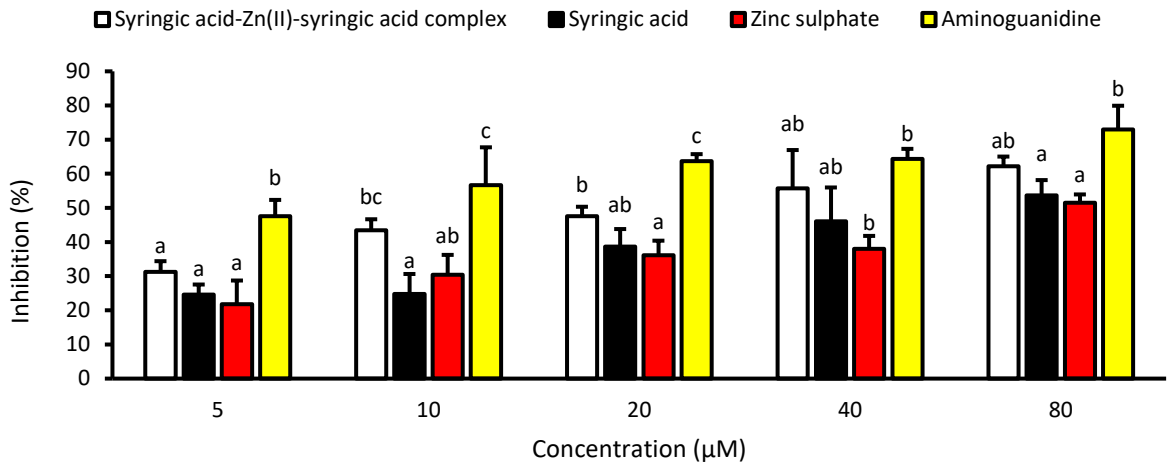
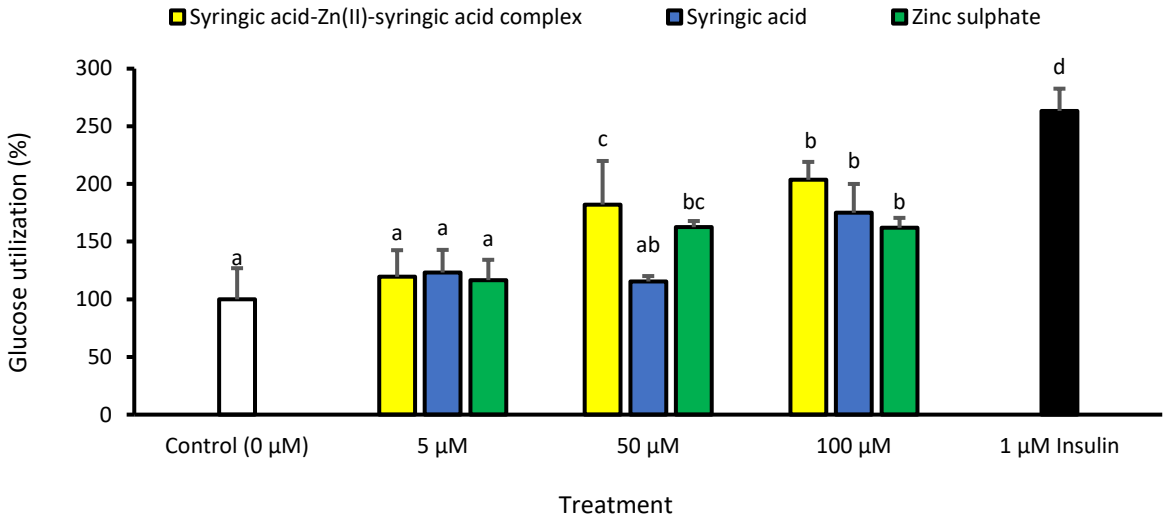



**Figure 5.10:** Effect of the complex, precursors and standards on *in vitro* protein (bovine serum albumin) glucose-induced glycation. The data are shown as mean ± SD of the triplicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups. Significant difference ($p<0.05$) is presented as a difference in the letter annotations.

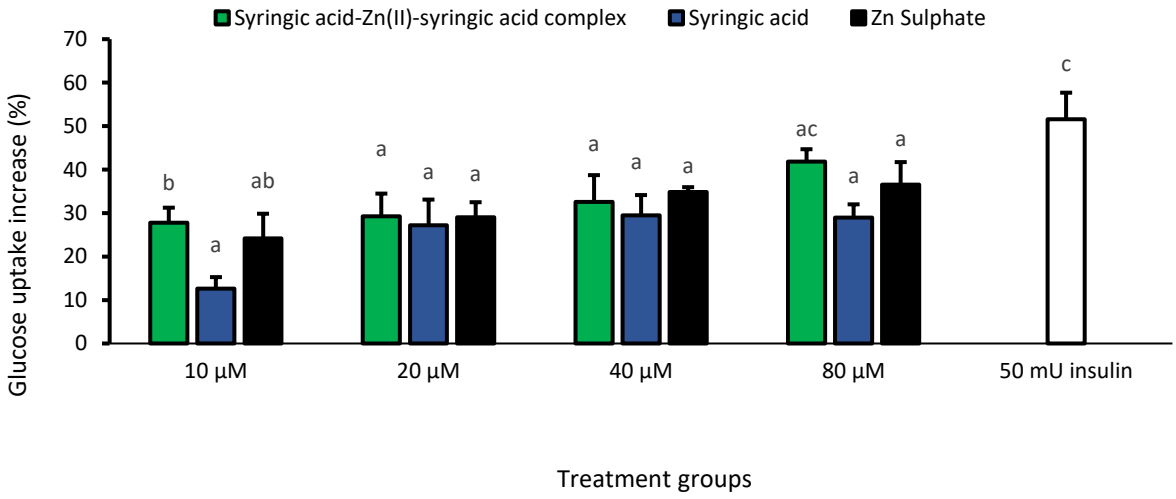
However, the complex had α-glucosidase inhibitory ($IC_{50}$ = 9.02 µM) and anti-glycation ($IC_{50}$ = 9.02 µM) activity that was statistically more potent ($p < 0.05$) than syringic acid (2.4 and 2.3 folds, respectively) and $ZnSO_4$ (4.7 and 3.4 folds, respectively) and statistically comparable ($p < 0.05$) to the activity of the positive control or standard drugs (acarbose and aminoguanidine).

In both L6-myotubes (**Figure 5.11a**) and isolated rat psoas muscle tissue (**Figure 5.11b**), the complex ($EC_{50}$ = 20.4 and 386 µM, respectively), syringic acid ($EC_{50}$ = 6460 and 693 µM, respectively) and $ZnSO_4$ ($EC_{50}$ = 71.1 and 450 µM, respectively) showed a dose-dependent increases in glucose uptake/utilization (**Table 5.1**), although they were not as potent as insulin at the different tested concentrations. The glucose uptake effect of syringic acid was significantly ($p < 0.05$) lower compared to $ZnSO_4$, However, both formed a complex with a more potent glucose uptake effect (**Table 5.1**). In isolated rat psoas muscle tissues, glucose uptake was accompanied by elevated hexokinase activity (**Figure 5.12a**). Muscle tissue zinc concentration was significantly higher ($p < 0.05$) in the complex and zinc sulphate treated tissue samples than the tissue samples

treated with syringic acid and insulin (**Figure 5.12b**). Similarly, the complex-treated tissues showed higher phospho-Akt/pan-Akt ratio than its precursors (**Figure 5.12c**).

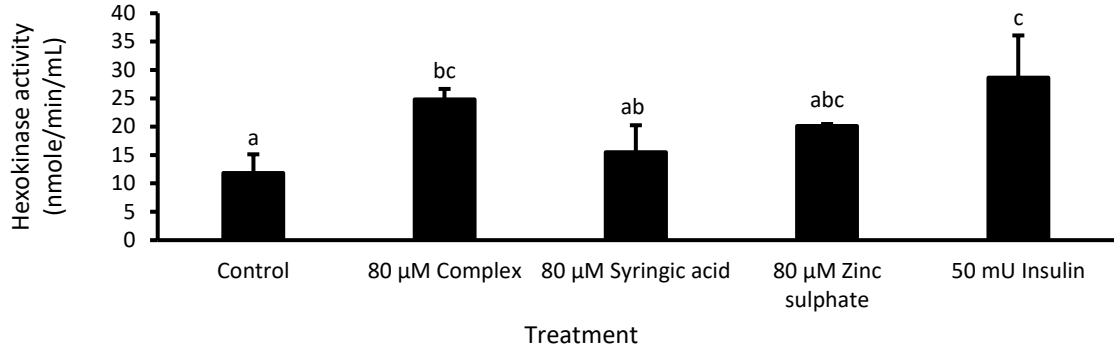



**(a)**

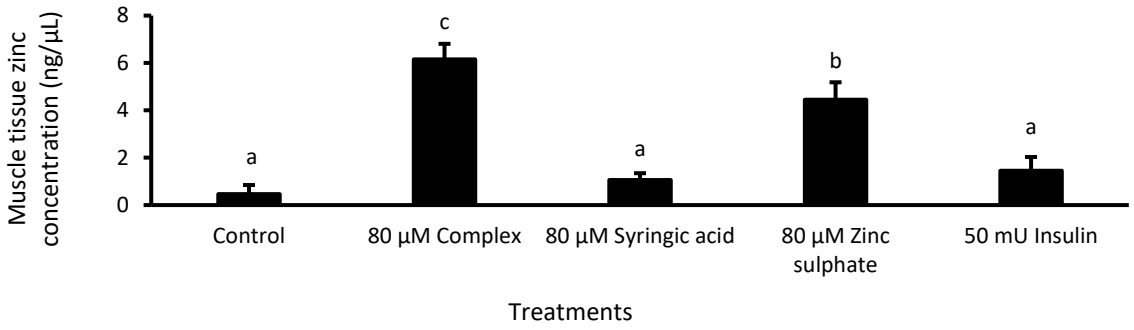



**(b)**

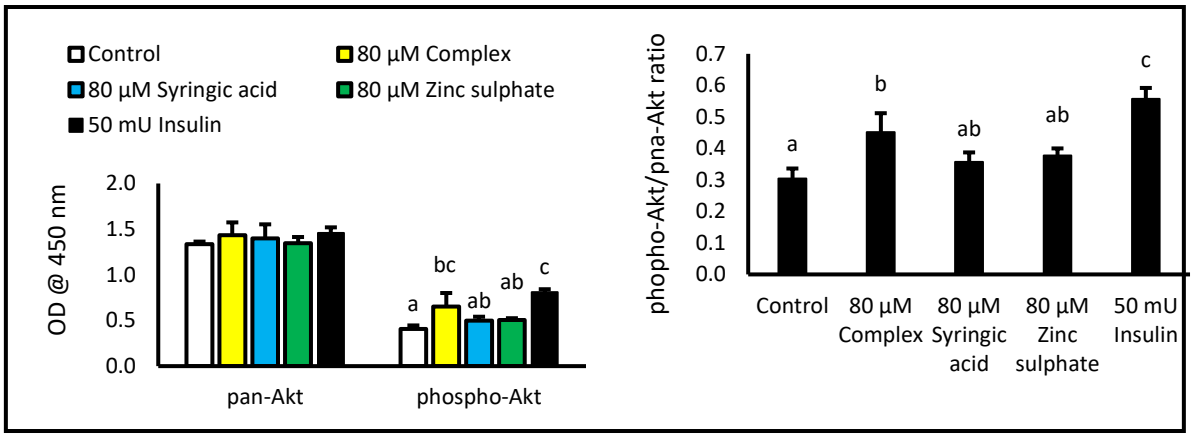
**Figure 5.11:** Effect of complex, precursors and standards on glucose uptake in (**a**) L6-myotubes, (**b**) isolated rat psoas muscle tissues. The data are shown as mean ± SD of the replicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups, as well as between the treatment groups and the controls (normal control and positive control). Significant difference ($p<0.05$) is presented as a difference in the letter annotations.




**(a)**




**(b)**




**(c)**

**Figure 5.12:** Effect of the highest concentration of the tested samples on **(a)** zinc concentration, **(b)** hexokinase activity and **(c)** Akt phosphorylation in the isolated rat psoas muscle tissues used for glucose uptake study. Statistical comparison was done between the treatment groups, as well as between the treatment groups and the controls (normal control and positive control). Significant difference ($p<0.05$) is presented as a difference in the letter annotations.

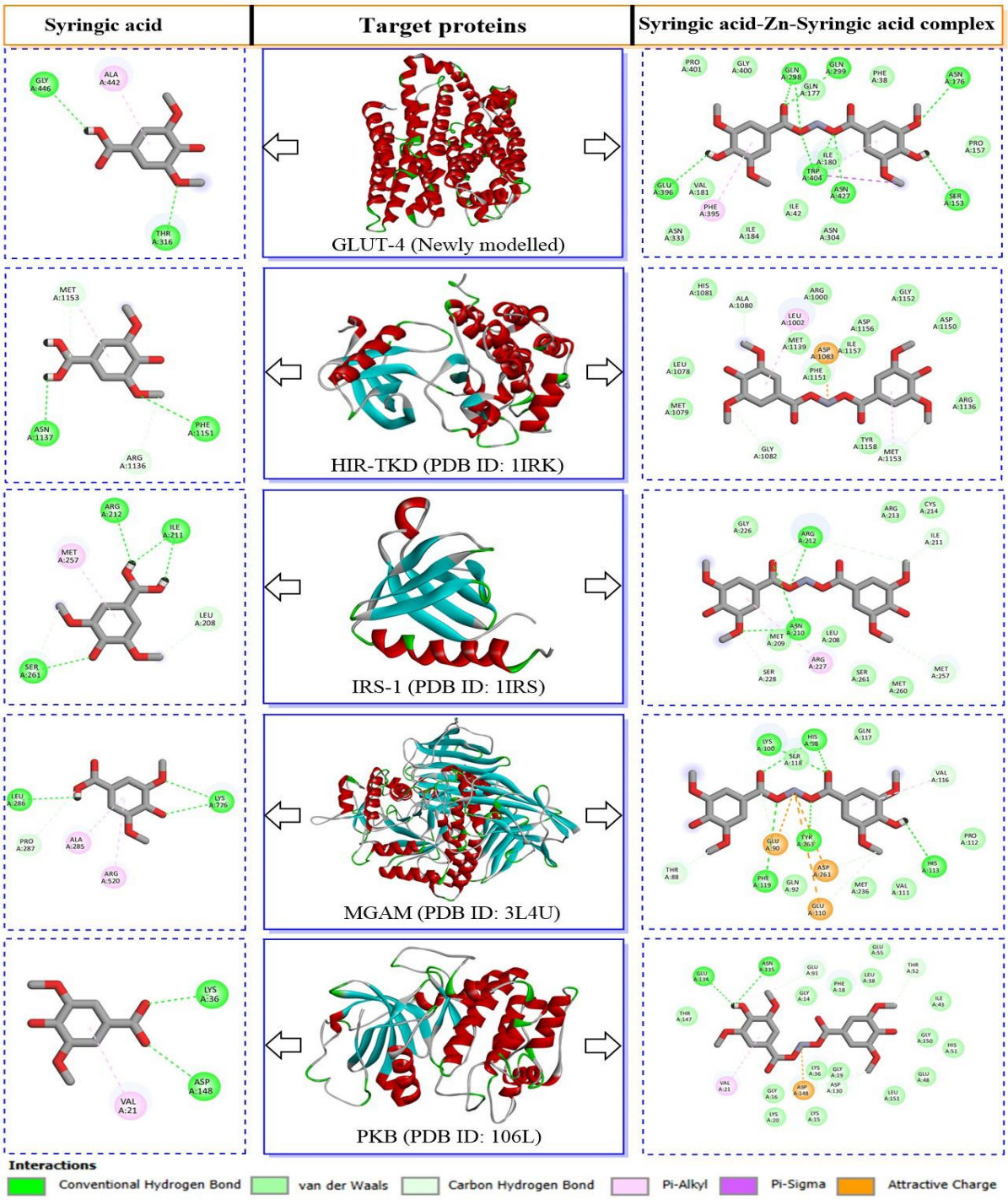



**Figure 5.13:** Visualized molecular docking interaction of syringic acid and the complex with five target enzymes (GLUT-4, HIR-TKD, IRS-1, MGAM and PKB). GLUT-4, glucose transporter type 4; HIR-TKD, human insulin receptor-tyrosine kinase domain; IRS-1, insulin receptor substrate-1; MGAM, *Human maltase–glucoamylase;* PKB, protein kinase B.

The molecular docking analysis is shown in **Figure 5.13**. The complex and syringic acid were docked to 5 target enzymes (glucose transporter type 4, human insulin receptor tyrosine kinase, insulin receptor substrate-1, human α-glucosidase and protein kinase B) that are linked to insulin signalling and diabetes. The docking scores of the complex (- 7.31, - 6.38, - 5.78, - 6.03 and - 6.39 kcal/mol, respectively) were higher than the docking scores of syringic acid (- 5.95, - 5.81, - 4.53, - 5.32 and - 5.50 kcal/mol) as shown in **Table 5.2**.

**Table 5.2**. Docking scores for molecular docking study of syringic acid and its Zn(II) complex with five target enzymes.

| **Target enzymes** | **PDB ID** | **Syringic acid** | **Zn(II) complex** |
|---|---|---|---|
| | | **Docking scores (kcal/mol)** | |
| GLUT-4 | Newly modelled | -5.95 | -7.31 |
| HIR-TKD | 1IRK | -5.81 | -6.38 |
| IRS-1 | 1IRS | -4.53 | -5.78 |
| MGAM | 3L4U | -5.32 | -6.03 |
| PKB | 106L | -5.50 | -6.39 |

GLUT-4, glucose transporter type 4; HIR-TKD, human insulin receptor-tyrosine kinase domain; IRS-1, insulin receptor substrate-1; MGAM, *Human maltase–glucoamylase;* PKB, protein kinase B

Cytotoxicity evaluation showed that the complex had no notable adverse effect on the viability of Chang liver cells and L6-myotubes (**Figure 5.14**).

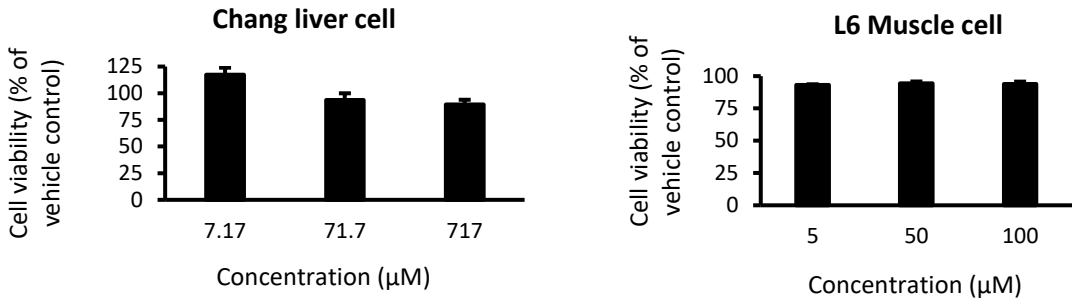



**Figure 5.14:** Effect of the complex on the viability of Chang liver cell and L-6 myotubes.

## 5.5 Discussion

In the present study, we considered syringic acid, a natural phenolic acid, as a suitable ligand for developing an antihyperglycaemic and antioxidant zinc complex with improved and multi-facet bioactivity profile. Emanating data showed the developed complex had improved and multifaceted bioactivity profile, suggesting a complexation mediated glycaemic control and antioxidant synergistic potential. Spectroscopic characterization suggests a double hydrated bisyringate-Zn(II) complex was formed (**Figures 5.1 – 5.4)**, which influenced the biological properties of the complex.

Oxidative stress remains pivotal in degenerative diseases, including diabetes, due to cellular and tissue oxidative injuries caused by reactive oxygen species and radicals (Giacco & Brownlee, 2010). These prooxidants initiate the oxidative degradation of cellular lipids (lipid peroxidation), thus damaging cellular components, with concomitant production of free radicals (Giacco & Brownlee, 2010). Syringic acid is considered a natural antioxidant (Rob *et al*., 2020). It possesses free radical quenching ability (Rashedinia *et al.*, 2021), which can be attributed to its electron donating ability and stable phenoxy radical intermediate (Chen *et al*., 2020; Chukwuma *et al*., 2020). This radical quenching ability of syringic acid has been implicated in its inhibitory effects on lipid peroxidation and GSH depletion in hepatic, renal, and neuronal tissues of diabetic rats (Rashedinia *et al*., 2021; Rashedinia *et al*., 2020). In this study, syringic acid scavenged DPPH and ABTS radicals, reduced $Fe^{3+}$ ion and inhibited the peroxidation of linoleic acid *in vitro* (**Figures 5.5 and 5.6; Table 5.1**). In both Chang liver cells, and rat liver tissues induced with oxidative stress, it mitigated lipid peroxidation and the depletion of GSH (**Figures 5.7 and 5.8; Table 5.1**).

However, when complexed with zinc sulphate, its *in vitro*, cellular and tissue antioxidant activity appreciable and/or significantly ($p < 0.05$) increased by various percentages (36.1–80.6%). In most cases, the antioxidant effect was comparable and/or more potent than that of ascorbic acid and Trolox (**Table 5.1**). Considering that zinc sulphate did not exhibit *in vitro* antioxidant effects and mildly potentiated cellular and tissue antioxidant effects relative to syringic acid (**Figures 5.5 - 5.8; Table 5.1**), it is fair to say that the stronger antioxidant activity of the complex compared to syringic acid was as a result of the two moieties of syringic acid in the complex (**Figure 5.2**). It is proposed that this molecular property of the complex potentiated a stronger antioxidant activity relative to its precursor phenolic acid. These data suggest that zinc may be an adjuvant for improving the antioxidant efficacy of syringic acid in disease conditions including diabetes.

The progression of oxidative stress-related diabetic complications is largely fuelled by persistent hyperglycaemia. Glycation of proteins and lipids and formation of AGEs are the most notable culprits linking persistent hyperglycaemia with elevated prooxidant production and diabetic oxidative stress (Giacco & Brownlee, 2010). Additionally, hyperglycaemia drives the production of superoxide radical, a deleterious prooxidant, via glucose metabolism and mitochondrial electron transport and energy generation, thus increasing diabetic oxidative stress (Fakhruddin *et al*., 2017). Improving glycaemic control is thus important in limiting diabetic oxidative stress. One mechanism is by reducing postprandial blood glucose increase through inhibiting carbohydrate digesting enzymes such as α-glucosidase and α-amylase (Moelands *et al*., 2018). A computational study has speculated that some natural phenolic acids, including syringic acid, can notably inhibit α-glucosidase activity (Rasouli, 2017) and glycation (Purnamasari *et al*., 2021). Moreover, syringic acid has been shown to inhibit *in vitro* protein glycation and formation of AGEs (Bhattacherjee & Datta, 2015; Wu *et al*., 2010).

In this study, syringic acid inhibited both *in vitro* α-glucosidase and glucose-induced BSA glycation (**Figures 5.9 and 5.10; Table 5.1**). Zinc sulphate moderately inhibited α-glucosidase relative to syringic acid but showed a comparable *in vitro* BSA anti-glycation activity relative to syringic acid (**Figures 5.9 and 5.10; Table 5.1**), which correlates with the previously documented *in vitro* anti-glycation properties of zinc sulphate (Tupe *et al*., 2015), as well as its ability to mitigate the glycation and carbonyl formation with proteins in diabetic rats (Sacan *et al*., 2016)

and rats suffering from protein malnourishment (Adebayo *et al*., 2014). However, complexing syringic acid with zinc sulphate resulted in a complex with stronger α-glucosidase and glycation inhibitory activity relative to syringic acid (2.4 and 2.3 folds, respectively) and zinc sulphate (4.7 and 3.4 folds, respectively) as shown in **Table 5.1**, suggesting a synergistic glycaemic control and anti-glycation potential between syringic acid and zinc sulphate through complexation. In fact, the activity of the complex was comparable to acarbose and aminoguanidine (**Table 5.1**), which may be influenced by the two syringic acid moieties and the zinc sulphate components of the complex (**Figure 5.2**). Docking study further showed the complex had higher docking scores against human α-glucosidase target enzyme than syringic acid (**Table 5.2**). Due to the presence of more -OH groups and Zn(II) in the complex, it is able to interact better with the target enzyme through hydrogen bond and ionic interactions (**Figure 5.13**).

Furthermore, Zn(II) has been shown to exhibit glycaemic control effects through insulin mimetic actions by modulating insulin signalling and glucose uptake. In mouse and human skeletal cells, Zn(II) treatment was shown to induce Akt phosphorylation, thus modulating insulin signalling (Norouzi *et al*., 2018). In 3T3-L1 pre-adipocytes and adipocytes, the modulatory effect of Zn(II) on insulin receptor and Akt activation potentiated increased glucose uptake (Tang & Shay, 2001). In the present study, zinc sulphate increased glucose uptake in both L-6 myotubes and isolated rat muscle tissues and complexing it with syringic acid formed a complex that outperformed both syringic acid and zinc sulphate (**Figure 5.11 and Table 5.1**). In the complex-treated muscle tissues, glucose uptake activity was accompanied by higher tissue/cellular zinc concentrations relative to the nontreated muscle tissues (**Figure 5.12b**). In fact, intracellular zinc concentration was higher in the complex-treated tissues compared to the zinc sulphate-treated tissues (**Figure 5.12b**), suggesting that Zn(II) complexation with syringic acid increased tissue/cellular Zn(II) uptake, which potentiated a stronger glucose uptake effect. Documented evidence has shown that non-pharmacologically active 1-oxy-2-pyridinethiol ligands could potentiate cellular insulin mimetic effects by enhancing cellular zinc uptake through complexation (Yoshikawa *et al*., 2011; Basuki *et al*., 2007).

Our study, however, suggests a synergistic interaction between the Zn(II) and syringic acid components of the complex in potentiating glucose uptake. Syringic acid was shown to

moderately increase cellular and tissue glucose uptake (**Figure 5.11 and Table 5.1**) and the complex was afforded 2 moieties of syringic acid (**Figure 5.2**). Hence, it is hypothesized that while complexing syringic acid with Zn(II) enhanced the cellular bioavailability of Zn(II), it concomitantly contributed to the glucose uptake potentiated by zinc(II). Hexokinase activity followed the same trend as tissue glucose uptake activity (**Figure 5.12a**), suggesting that the glucose uptake activity may be linked to an increase in cellular/tissue glucose utilization. Docking data further showed the complex had higher docking scores (**Table 5.2**) and stronger interaction (**Figure 5.13**) with four target enzymes (GLUT-4, IRS-1, PKB/Akt and insulin receptor tyrosine kinase) linked to insulin signalling and cellular glucose uptake, which suggests the glucose uptake potency of the complex may be linked to a modulatory action on associated signalling proteins. Moreover, isolated rat muscle tissues treated with the complex showed significantly ($p < 0.05$) elevated tissue phospho-Akt/pan-Akt ratio (**Figure 5.12c**). Akt activation is an important downstream signalling step to facilitate insulin-mediated cellular glucose uptake and utilization (Beg *et al*., 2017), which suggests Zn(II)-syringic acid complexation may potentiate glycaemic control by modulating insulin signalling and cellular glucose uptake. It is noteworthy that the complex may not pose toxicity concerns, as it did not adversely alter the viability of Chang liver cells and L-6 myotubes.

## 5.6 Conclusion

This study revealed that complexing syringic acid with zinc sulphate resulted in a synergistic interaction between both. The outcome was a non-cytotoxic complex with a multimode and improved antioxidant and glycaemic control potential. The interaction between the two moieties of syringic acid and Zn(II) of the complex appears to be influential in the improved and multi-facet bioactivity profile of the complex. Through complexation, Zn(II) may be a promising adjuvant for syringic acid in developing novel nutraceuticals with improved medicinal relevance against diabetes and oxidative complications.

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## 5.7 Postscript

Based on the stronger antioxidant, antiglycation and enzyme inhibitory effect of the Zn(II)-bisyringate.$2H_2O$ complex compared to the Zn(II)-bicoumarate.$2H_2O$ complex, as well as its

remarkable complexation-mediated improvement in cellular and tissue glucose uptake, its rational to subject the Zn(II)-bisyringate.$2H_2O$ complex to *in vivo* antidiabetic and antioxidative evaluation to see whether the synergistic effects are mimicked or replicated *in vivo*. The antidiabetic and antioxidant potential of the Zn(II)-bisyringate.$2H_2O$ complex was investigated in fructose and low-dose STZ induced diabetic rats and compared to the effect of its precursors (syringic acid and zinc sulphate), which is reported in the chapter 6 of this thesis.

# CHAPTER 6

## ZN(II) AND SYRINGIC ACID EXERT SYNERGISTIC MODULATORY ACTION ON AKT PHOSPHORYLATION, INSULIN SENSITIVITY, GLYCOLYTIC ACTION, GLYCAEMIC CONTROL, AND ANTIOXIDANT STATUS IN DIABETIC RATS

### Prescript

This chapter investigated the *in vivo* antidiabetic and antioxidant potential of a novel Zn(II) complex of syringic acid. The content of this chapter has been accepted in **Revista Brasileira de Farmacognosia** on **16 January 2023** as follows: ***Ramorobi, L. M.***, *Matowane, G. R., Mashele, S. S., Erukainure, O. L., Makhafola, T. J., & Chukwuma, C. I. Therapeutic Antidiabetic and Antioxidative Synergism of Zn(II)-syringic acid complexation (**Manuscript ID: RBFA-D-22-00747R1**)*. The above-mentioned publication was adopted in writing the content of this chapter.

## 6.1 Abstract

Zn(II) and syringic are natural supplements with medicinal attributes. We recently showed their complexation-mediated muscle glucose uptake and hepatic anti-lipid peroxidative synergism in vitro. This study was done to investigate the antidiabetic and antioxidative synergism between Zn(II) and syringic acid in diabetic rats by complexing both. Diabetes was induced in SD rats using 10% fructose and 40 mg/kg bw streptozotocin and thereafter subjected to a 4-week treatment with the complex and its precursors (Syringic acid and zinc sulphate) for 4 weeks at predetermined doses. Then, the effect of the treatments on diabetes and oxidative stress related parameters was measured. Diabetic polyphagia, polydipsia and weight loss were improved by the complex. Complexing Zn(II) with syringic acid improved their anti-hyperglycaemic action by ~28 – 36% and ~37 - 40%, respectively, suggesting a complexation-mediated synergism. This is attributed to the modulatory action on insulin secretion and sensitivity, tissue glycogen production, muscle hexokinase activity and Akt phosphorylation, thus improving glucose tolerance in diabetic rats, relative to its precursors. Concomitantly, the complex reduced systemic and tissue lipid peroxidation and increased antioxidant enzymes activity in the diabetic rats, while outperforming its precursors. In some cases, the antidiabetic action of the complex was comparable to metformin. Complexing Zn(II) and syringic acid may be an underexplored therapeutic approach to improving the effectiveness for diabetes and oxidative stress management.

## 6.2 Introduction

Reports have consistently mentioned that the growing prevalence of type 2 diabetes (T2D) is fuelled by poor eating habits, sedentary lifestyle, detrimental weight gain and obesity (Al-Goblan *et al*., 2014; Sami *et al*., 2017). The most recent data of the International Diabetes Federation has shown that that about 537 million adults globally have diabetes and T2D accounts for more than 90% of this prevalence (IDF, 2021). Impaired insulin action and glucose intolerance are the main pathophysiologic defects of T2D. Cells of peripheral tissues, including muscle, liver and adipose tissues do not respond to the blood glucose normalization action of insulin, which adversely affects circulating glucose uptake/utilization and glucose/lipid metabolism (Galicia-Garcia *et al*., 2020). Hormonal control of glucose storage and glycogen breakdown is impaired, resulting in hyperinsulinemia and progressive loss of pancreatic β-cell function. (Galicia-Garcia *et al*., 2020),

which to together with the above defects influence impaired glucose tolerance and sustained hyperglycaemia (Galicia-Garcia *et al*., 2020). Although there are different commercials drugs for managing diabetes, they are mostly synthetic, which partly contributes to the documented side effects associated with their use (Chaturvedi *et al*., 2018). On the other hand, natural medicine and supplements are becoming more receptive in diabetes management, perhaps due to their safety profile and functional effects (Cross and Thomas, 2021). Zinc mineral is one of the mineral supplements that has shown some promise in diabetes management. It plays an important role in insulin secretion and function (Chabosseau and Rutter, 2016). In adipocytes isolated from rats, zinc has been shown to exert modulatory and suppressive effects on glucose uptake and lipolysis, respectively, while treatment in T2D KK-$A^y$ mice exerted glycaemic control (Adachi *et al*., 2004). Deleting ZnT8 genes that are involved in pancreatic Zn(II) transport was shown to cause impaired glucose tolerance in mice (Nicolson *et al*., 2009). Clinically, studies have shown that zinc supplementation could improve glycaemic control and blood lipids profile (Jayawardena *et al*., 2012).

Documented evidence has supported the relevance of natural antioxidants in managing diabetes and complications (Kanwugu *et al*., 2022), which may be partly because of the critical role oxidative stress plays in the development and progression of diabetic complications (Giacco and Brownlee, 2010). Persistent hyperglycaemia has been shown to induce glycation processes and production of reactive oxygen species (ROS). Energy metabolism through the electron transport chain and lipid peroxidation are, also, known to initiate and promote ROS production during diabetes (Giacco and Brownlee, 2010). ROS, including free radicals cause oxidative damage to biomolecules and organelles in cells, which results in several complications (Giacco and Brownlee, 2010). Also, the integrity of cellular membrane lipids can be compromised by lipid peroxidation (Giacco and Brownlee, 2010).

Plant polyphenols are some of the natural antioxidants that have be explored in mitigating oxidative stress and insults in diabetes. Dietary polyphenols, including phenolic acids have shown some promise in concomitantly potentiating glycaemic control and antioxidant action (Vinayagam *et al*., 2016; Sun *et al*., 2020). Syringic acid is natural phenolic acid found in berries and grapes

(Pezzuto, 2008). It is an effective radical scavenger and has been shown to ameliorate diabetic oxidative stress (Sabahi *et al*., 2020; Rashedinia *et al*., 2020; 2021).

Synergistic therapy has been a useful therapeutic approach because it affords multi-mode effects, which can improve therapeutic outcomes (Lehár *et al*., 2009). In this context, metal-ligand complexation has been researched to develop products with therapeutic advantages. Zinc metal has been complexed with some natural ligands to synthesize complexes with insulin mimetic and antidiabetic potentials (Chukwuma *et al*., 2020). Adachi *et al*. reported the modulatory and suppressive effects of Zn(II)-maltol and Zn(II)-allixin complexes on rat adipocytes' glucose uptake and lipolysis, respectively (Adachi *et al*., 2004). Also, the complexes ameliorated hyperglycaemia in T2D KK-Ay mice (Adachi *et al*., 2004). Zinc complexes of picolinic acid and threonine has been shown to reduce blood glucose and HbA1c in T2D KK-Ay (Kojima *et al*., 2002). In adipocytes the complexes potentiated insulin mimetic effects by modulating the action of insulin signalling proteins, including insulin receptor tyrosine kinase, phosphodiesterase, phosphoinositide 3-kinase and glucose transporter type 4 (Yoshikawa *et al*., 2004).

In a recent study we took advantage of the insulin mimetic potential of zinc and the antioxidant properties of syringic acid to develop a Zn(II)-syringic acid complex with improved and broader scope of *in vitro* glycaemic control and antioxidant profile (Ramorobi *et al*., 2022). Spectroscopic characterization showed the complex had two moieties of syringic acid and a moiety of Zn(II), which influenced the observed complexation mediated synergism (Ramorobi *et al*., 2022). The complex showed *in vitro* radical scavenging, α-glucosidase inhibitory and antiglycation activities. In both L-6 myotubes and isolated rat psoas tissues it increased glucose uptake. It further reduced oxidative stress in Chang liver cells and isolated rat liver tissues by suppressing lipid peroxidation and reduced glutathione depletion (Ramorobi *et al*., 2022). In many instances, the bioactivity of the complex was several folds more potent that of its precursors (syringic acid and zinc sulphate) and comparable and/or more effective than tested standard drugs. The potency of the complex suggests a synergistic interaction between its constituents, which warranted further *in vivo* studies to see if the bioactive properties of the complex will be mimicked *in vivo*. Hence, the present study was done to investigate the antioxidant and antidiabetic potential of the Zn(II)-syringic acid complex in a diabetic rat model induced using fructose and low-dose streptozotocin.

## 6.3 Materials and methods

For the materials and methods of this chapter, please consult **sub-sections 3.8 and 3.9 of chapter 3**.

## 6.4 Results

### 6.4.1 *In vivo* effects associated with the diabetes

At the end of the study, body weight was significantly ($p < 0.05$) lower in the untreated diabetic rats (DBC) compared to rats in the normal control group (NMC) (**Figure 6.1**). The food and water intake were significantly ($p < 0.05$) higher comparing the DBC group to the NMC group (Figure 6.2). Although not normalized, the complex (SYZ) and metformin (MET) appreciably recovered the diabetes-induced alteration of body weight, food intake and intake (**Figures 6.2**).

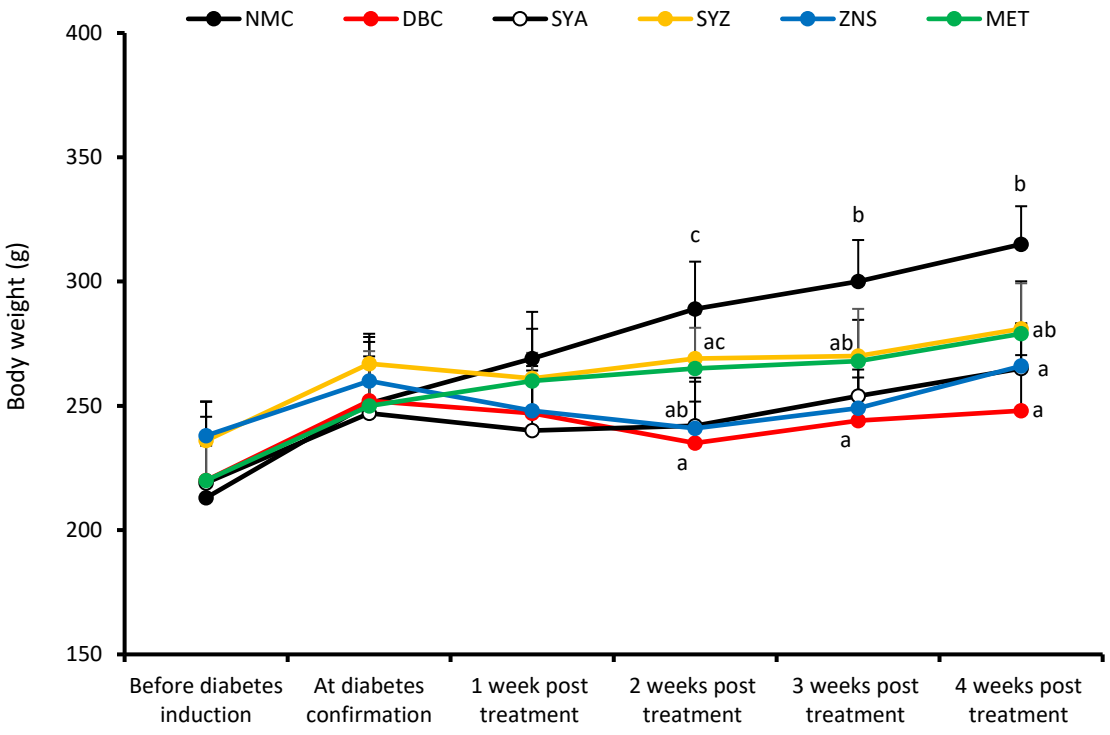



**Figure 6.1:** Figures showing the weekly body weight of rats during the experimental period. Data are shown as mean ± SD of six rats per group. For each experimental period, statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

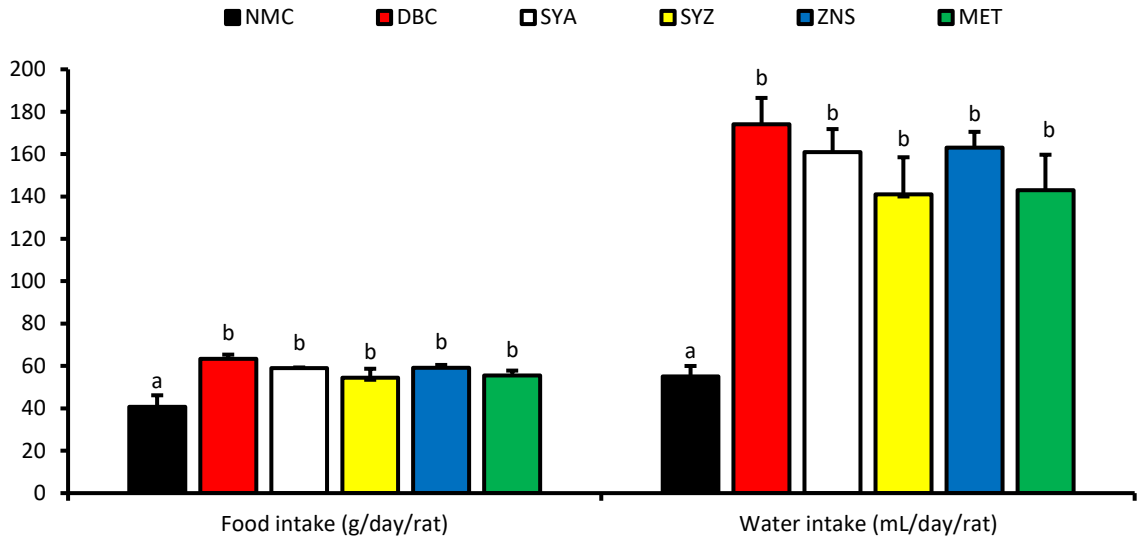
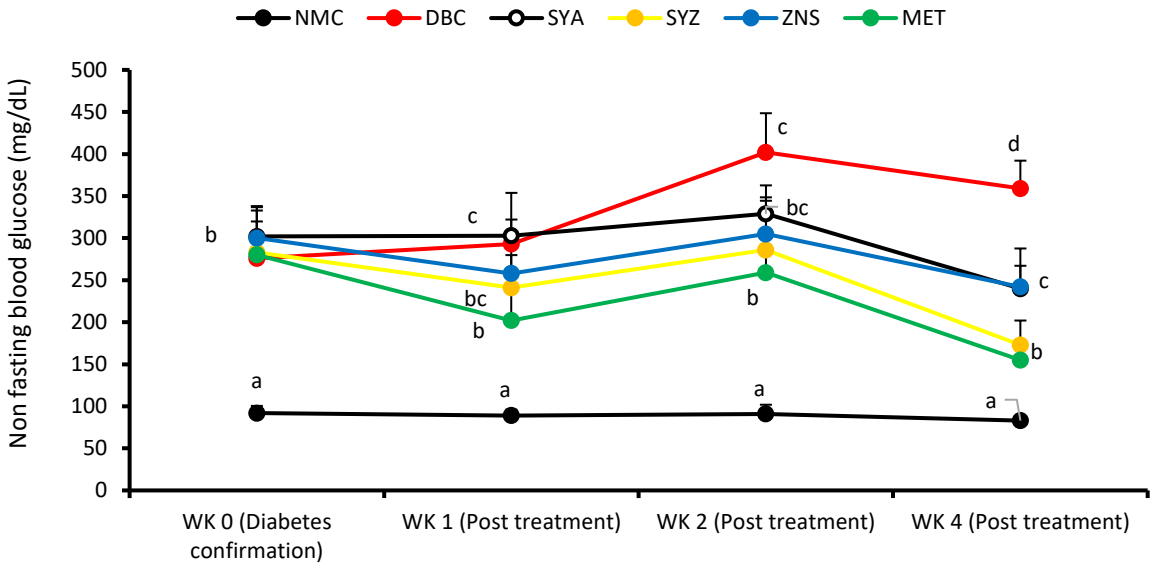



**Figure 6.2:** Figures showing the food and fluid intake of rats during the experimental period. Data are shown as mean ± SD of six rats per group. For each measured parameter, statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

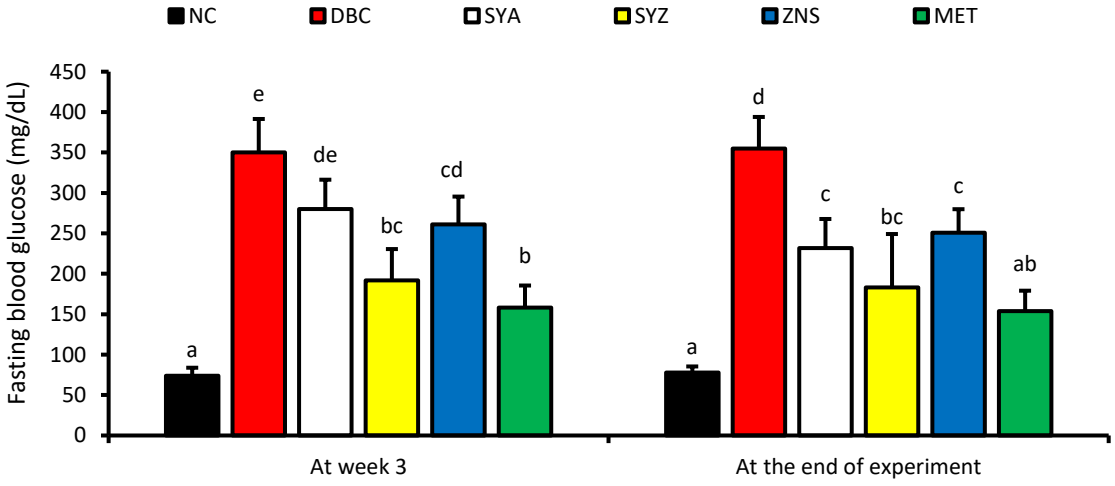
At diabetes confirmation, it was observed that the rats in all the diabetic groups (DBC, SYA, SYZ, ZNS and MET) had relatively similar NFBG level, which was significantly ($p < 0.05$) higher than that of the rats in the normal control group (**Figure 6.3a**). As treatment progressed, the NFBG of the rats in the different treatment groups differed from the NFBG of the rats in the DBC group. At the end of 4 weeks, the different treatments significantly ($p < 0.05$) reduced the NFBG elevation caused by diabetes without normalizing it. The complex exerted the most significant ($p < 0.05$) reducing effect on NFBG, which was comparable to the effect of metformin treatment (Figure 2a). The treatments, also, affected the FBG of the animals in a fashion similar their effect on the NFBG at the end of the experiment (**Figure 6.3b**).

Oral glucose tolerance test (OGTT) data showed that diabetes induction significantly ($p < 0.05$) altered glucose tolerance in rats by impairing glycaemic control (**Figure 6.4a**), which was illustrated by the significantly ($p < 0.05$) higher AUC of the rats in the DBC group relative to the rats in the NMC group (**Figure 6.4b**). Although not normalized, treatment with syringic acid, the complex and metformin significantly ($p < 0.05$) improved the impaired glycaemic control caused

by diabetes induction. Metformin and the complex were significantly ($p < 0.05$) more effective than syringic acid and zinc sulphate treatment (**Figure 6.4b**).



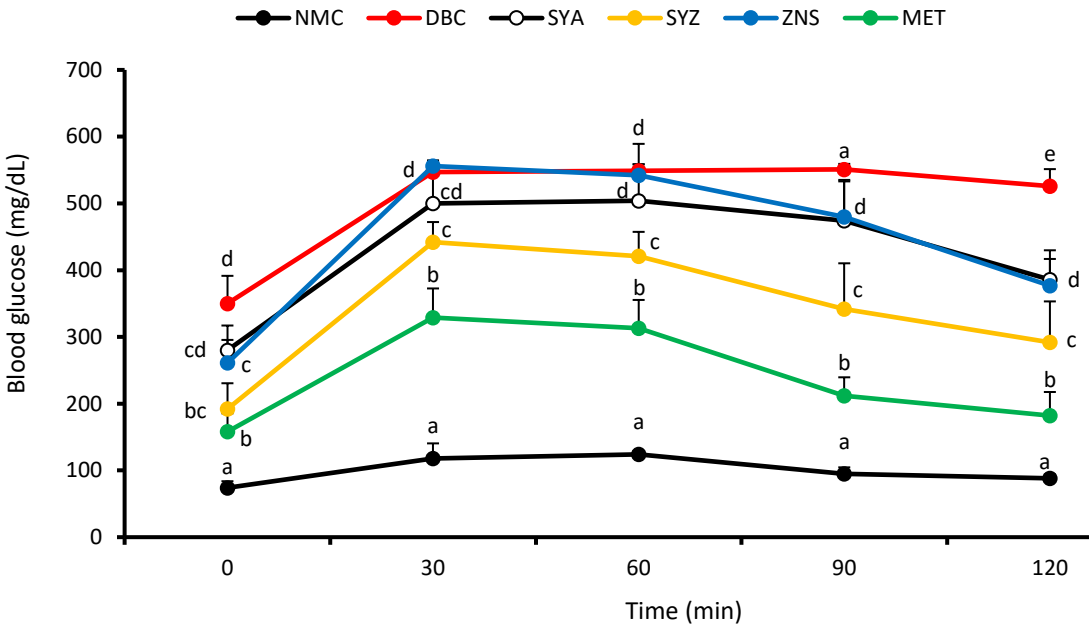

**(a)**



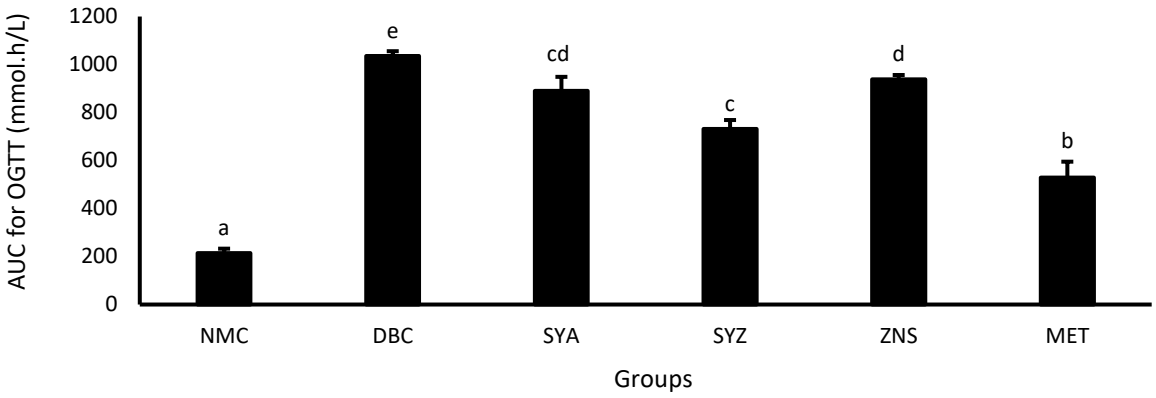

**(b)**

**Figure 6.3:** Figures showing the **(a)** weekly non fasting blood glucose (NFBG) during the experimental period and **(b)** the fasting blood glucose (FBG) at the 3rd week of the intervention period and at the end of the animal study. Data are shown as mean ± SD of six rats per group. For

each period in the experiment, statistical comparison was done between animal groups in each measured parameter. Significant difference ($p<0.05$) means a difference in letters.



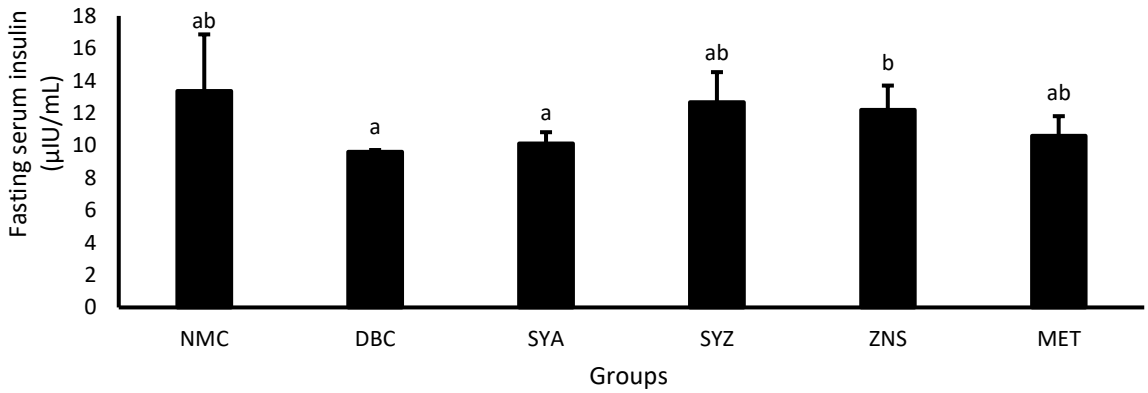

**(a)**



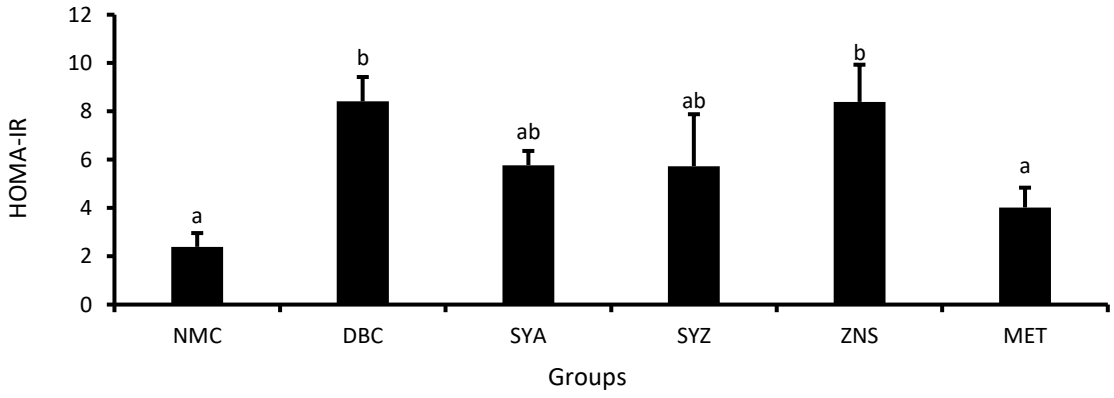

**(b)**

**Figure 6.4:** Figures showing the **(a)** oral glucose tolerance and **(b)** area under the curve (AUC) at the 3rd week of the intervention period. Data are shown as mean ± SD of six rats per group. For each time interval and/or each measured parameter, statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

Diabetes induction notably decreased fasting serum insulin (FSI) but not significantly (**Figure 6.5a**). The complex and zinc sulphate treatments notably increased the depleted fasting serum insulin in the diabetic rats with the effect of zinc sulphate being significant ($p < 0.05$). The significantly ($p < 0.05$) higher HOMA-IR value of the DBC group compared to the NMC group, suggests significant insulin resistance in the untreated diabetic rats (DBC) relative to the rats in the NMC group (**Figure 6.5b**). Syringic acid, metformin and the complex notable reduced the elevated HOMA-IR in the diabetic rats with the effect metformin being significant ($p < 0.05$). The effect the complex and syringic acid was however statistically comparable to that of metformin (**Figure 6.5b**).



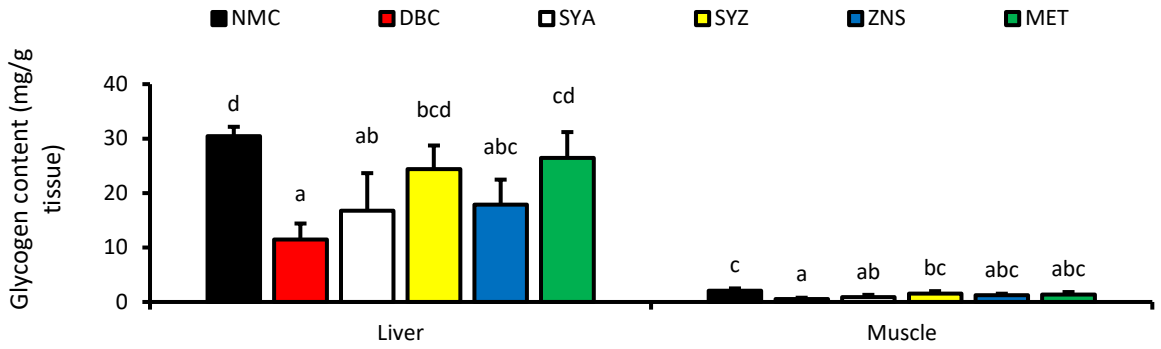

**(a)**



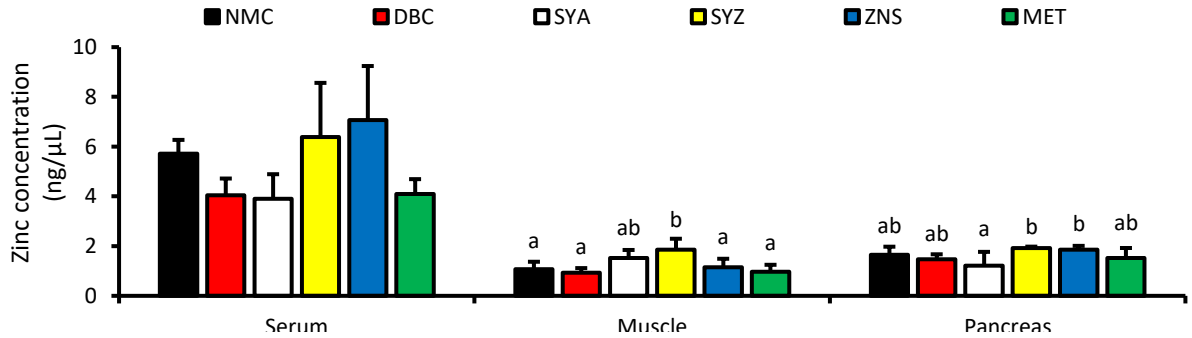

**(b)**

**Figure 6.5:** Figures showing the **(a)** fasting serum insulin and **(b)** HOMA-IR score at the end of the experiment. Data are shown as mean ± SD of six animals per group. Statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

Both liver and muscle tissue glycogen contents were significantly ($p < 0.05$) reduced in the untreated diabetic rats relative to the rats in the NMC group at the end of the experiment (**Figure 6.6a**). Elevated liver and muscle tissue glycogen content was observed in the rats of treatment groups (SYA, SYZ, ZNS and MET) compared to the rats of the DBC group with significant ($p < 0.05$) elevation observed in the SYZ and MET groups (**Figure 6.6a**).




**(a)**




**(b)**

**Figure 6.6:** Figures showing the **(a)** tissue glycogen content and **(b) s**erum and tissue zinc concentration at the end of the animal study. Data are shown as mean ± SD of six animals per group. For each biological sample in each measured parameter, statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

Diabetes induction notably reduced serum zinc concentration without significantly altering muscle and pancreatic zinc concentration (**Figure 6.6b**). The complex and zinc sulphate treatments potentiated notable increases in the serum zinc concentration of the diabetic rats. In the muscle, significant ($p < 0.05$) increase in zinc concentration was observed in the diabetic rats following treatment with the complex. In the pancreas, zinc concentration was slightly higher in the SYZ and ZNS groups relative to the NMC, DBC and MET groups and significantly ($p < 0.05$) higher relative to the SYA group (**Figure 6.6b**).

Induction of diabetes caused significant ($p < 0.05$) decrease in muscle tissue hexokinase activity (**Figure 6.7**). Although not significant, all the treatments increased the diabetes-induced reduction in muscle hexokinase activity.

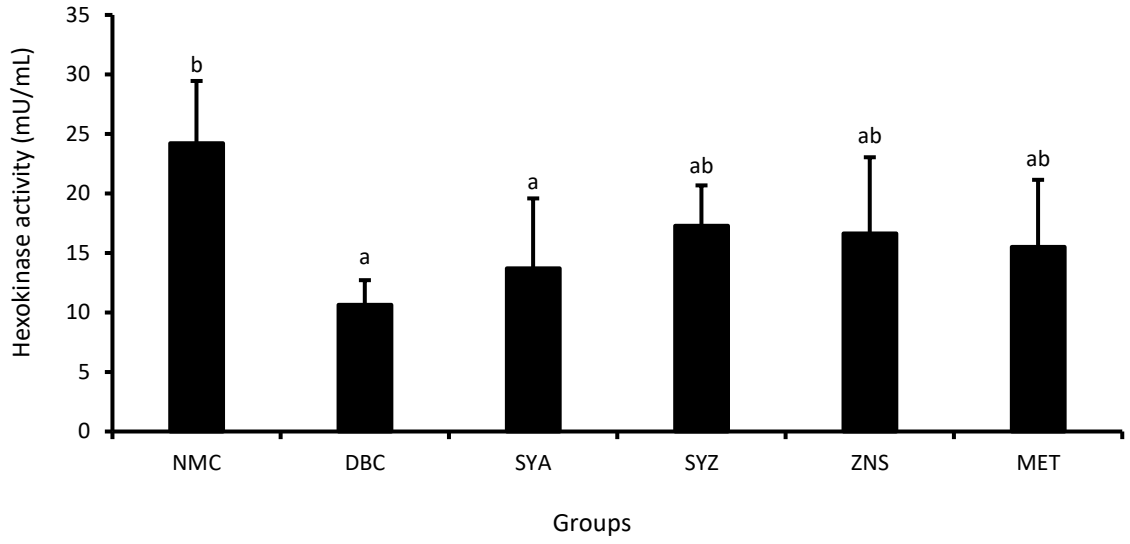
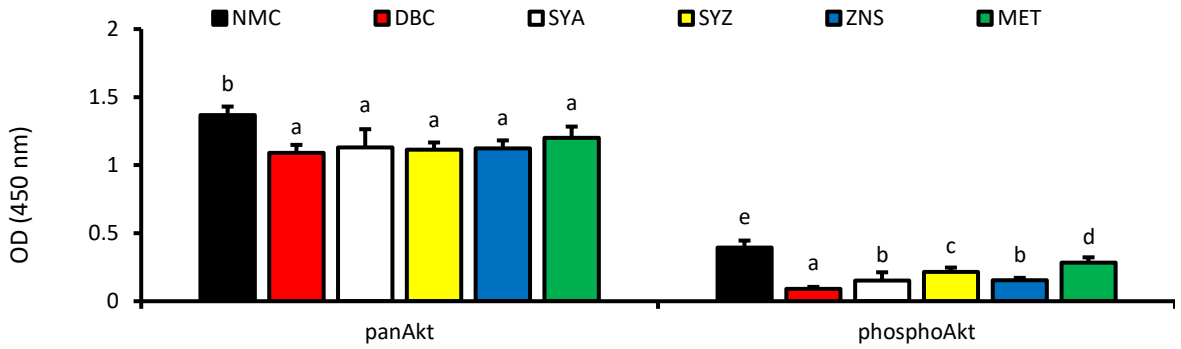


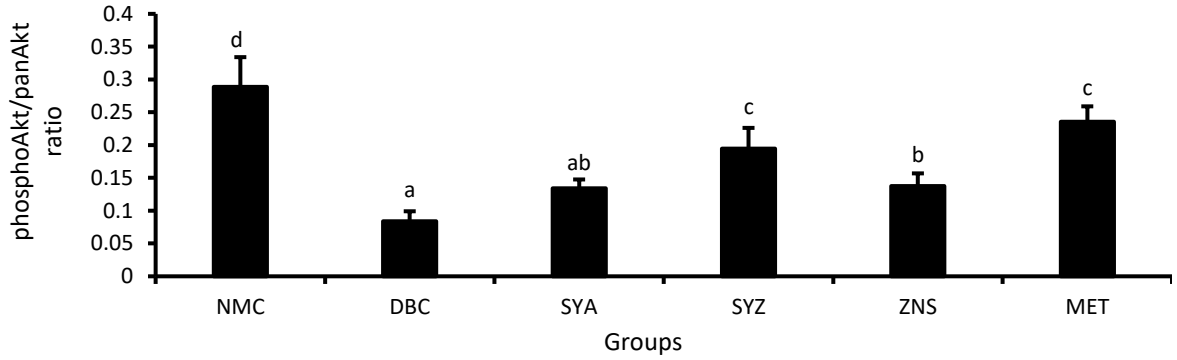

**Figure 6.7:** Figures showing the muscle tissue hexokinase activity at the end of the animal study. Data are shown as mean ± SD of six animals per group. Statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

At the end of the study, significant ($p < 0.05$) reduction in muscle phospho-Akt level was observed in the rats of the DBC group relative to the rats of the NMC group (**Figure 6.8a**). Also, the phopho-Akt/pan-Akt ratio data suggest diabetes induction significantly ($p < 0.05$) suppressed Akt phosphorylation (**Figure 6.8b**). Although not normalized, all treatments notably and/or significantly ($p < 0.05$) increased muscle Akt phosphorylation and phospho-Akt level (**Figures 6.8a and b)**. The complex and metformin treatments had the most potent effects in the diabetic

rats, which was significantly ($p < 0.05$) more effective than the effect syringic acid and zinc sulphate (**Figures 6.8a and b**).




**(a)**




**(b)**

**Figure 6.8:** Figures showing the muscle tissue **(a)** phospho-Akt and pan-Akt levels and **(b)** phospho-Akt/pan-Akt ratio in the isolated rat psoas at the end of the experiment. Data are shown as mean ± SD of six animals per group. Statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

### 6.4.2. *In vivo* effects associated with the oxidative stress

Diabetes induction caused marked and significant ($p < 0.05$) elevation of lipid peroxidation in the liver and serum, respectively, without notably altering pancreatic lipid peroxidation (**Table 6.1**). Relative to the untreated diabetic rats (DBC group), a level of decrease in hepatic and systemic

lipid peroxidation was observed in the rats of the different treatment groups (SYA, SYZ, ZNS and MET). The complex caused the most notable decrease among the treatments, which was significant ($p < 0.05$) in the liver and serum (**Table 6.1**).

Serum GSH concentration was significantly ($p < 0.05$) decreased in the untreated diabetic rats (DBC group) relative to the normal rats (NMC group), while hepatic and pancreatic GSH concentration was not significantly altered by diabetes induction (**Table 6.1**). Although not significantly, the treatments slightly increased serum GSH concentration relative to untreated diabetic rats with the complex having the most potent effect. On the other hand, pancreatic and hepatic GSH concentrations were lower in the treated rats (SYA, SYZ, ZNS and MET groups) relative to the untreated diabetic rats (DBC group). The lower GSH concentration in the complex-treated rats (SYZ group) relative to the untreated diabetic rats (DBC group) was significant ($p < 0.05$) (**Table 6.1**).

**Table 6.1:** Serum and tissue lipid peroxidation and reduced glutathione (GSH) at the end of the animal study.

| Groups | Lipid peroxidation presented as TBARS (µM MDA equivalent) | | | GSH concentration (µM) | | |
|---|---|---|---|---|---|---|
| | Liver | Pancreas | Serum | Liver | Pancreas | Serum |
| NMC | $146 \pm 20.8^{ab}$ | $128 \pm 4.57$ | $471 \pm 36.4a$ | $0.36 \pm 0.07^{bc}$ | $0.19 \pm 0.06^{ab}$ | $0.19 \pm 0.05^{b}$ |
| DBC | $181 \pm 91.1^{b}$ | $120 \pm 26.0$ | $734 \pm 91.1^{c}$ | $0.40 \pm 0.05^{bc}$ | $0.23 \pm 0.08^{b}$ | $0.09 \pm 0.04^{a}$ |
| SYA | $150 \pm 22.2^{ab}$ | $116 \pm 18.2$ | $621 \pm 87.4^{ac}$ | $0.31 \pm 0.03^{ab}$ | $0.18 \pm 0.02^{ab}$ | $0.10 \pm 0.03^{ab}$ |
| SYZ | $135 \pm 22.8^{a}$ | $101 \pm 13.9$ | $574 \pm 94.1^{ab}$ | $0.21 \pm 0.07^{a}$ | $0.11 \pm 0.03^{a}$ | $0.15 \pm 0.07^{ab}$ |
| ZNS | $147 \pm 35.6^{ab}$ | $113 \pm 24.3$ | $700 \pm 61.7^{bc}$ | $0.33 \pm 0.06^{bc}$ | $0.17 \pm 0.06^{ab}$ | $0.13 \pm 0.06^{ab}$ |
| MET | $151 \pm 23.7^{ab}$ | $123 \pm 19.1$ | $615 \pm 72.0^{abc}$ | $0.41 \pm 0.07^{c}$ | $0.20 \pm 0.06^{ab}$ | $0.13 \pm 0.02^{ab}$ |

Data are shown as mean ± SD of six animals per group. For each biological sample, statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

Catalase and SOD activities were markedly and/or significantly reduced by diabetes induction in the liver, pancreas and serum of rats (**Table 6.2**). In the serum and tissue, the diabetes-induced reduction in catalase and SOD activities were increased by the different treatments. Syringic acid and the complex had the most potent ameliorative effect on the depleted enzyme activities of the diabetic rats. The complex, however, outperformed syringic acid, and almost statistically normalized the activity of the antioxidant enzymes (**Table 6.2**).

**Table 6.2:** Serum and tissue catalase and superoxide dismutase (SOD) activity at the end of the animal study

| Groups | Superoxide dismutase activity (% inhibition rate) | | | Catalase (mmol/mL/min) | | |
|---|---|---|---|---|---|---|
| | **Liver** | **Pancreas** | **Serum** | **Liver** | **Pancreas** | **Serum** |
| NMC | $73.1 \pm 4.19^{c}$ | $63.9 \pm 4.92^{b}$ | $77.3 \pm 8.38^{c}$ | $6.76 \pm 0.42^{b}$ | $1.03 \pm 0.24$ | $2.68 \pm 0.18^{c}$ |
| DBC | $50.0 \pm 6.39^{a}$ | $39.5 \pm 8.95^{a}$ | $51.4 \pm 7.88^{a}$ | $3.88 \pm 0.46^{a}$ | $0.71 \pm 0.54$ | $1.10 \pm 0.41^{a}$ |
| SYA | $57.4 \pm 11.3^{ab}$ | $50.5 \pm 11.2^{ab}$ | $61.0 \pm 5.28^{ab}$ | $5.57 \pm 0.61^{bc}$ | $1.11 \pm 0.41$ | $1.50 \pm 0.30^{ab}$ |
| SYZ | $65.4 \pm 2.33^{bc}$ | $56.6 \pm 10.6^{ab}$ | $71.4 \pm 6.80^{bc}$ | $6.33 \pm 0.59^{b}$ | $1.27 \pm 0.52$ | $2.09 \pm 0.61^{bc}$ |
| ZNS | $50.4 \pm 10.2^{a}$ | $49.2 \pm 6.17^{ab}$ | $60.7 \pm 2.10^{ab}$ | $4.20 \pm 0.26^{a}$ | $1.09 \pm 0.40$ | $1.30 \pm 0.40^{ab}$ |
| MET | $54.7 \pm 2.32^{ab}$ | $50.1 \pm 7.93^{ab}$ | $58.2 \pm 6.65^{a}$ | $4.58 \pm 0.63^{ac}$ | $1.04 \pm 0.31$ | $1.83 \pm 0.36^{ab}$ |

Data are shown as mean ± SD of six animals per group. For each biological sample, statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters.

## 6.5 Discussion

Advances in research have shown that zinc complexation may be a promising therapeutic approach for diabetes management (Chukwuma *et al*., 2020). However, the synthetic origin and the little or no pharmacological credence of the ligands in most of the studied antidiabetic zinc complexes has raised toxicity and efficacy concerns (Chukwuma *et al*., 2020). To address this concern, we considered natural phenolic acids as an alternative ligand for therapeutic zinc complex, due to their

natural origin and antioxidant credibility. In the present study, syringic acid was chosen due to its ameliorative potential in diabetic oxidative stress (Sabahi *et al*., 2020; Rashedinia *et al*., 2020; 2021). Our previous study showed that complexing Zn(II) with syringic acid resulted in a double hydrated Zn(II)-bisyringate complex (Ramorobi *et al*., 2022). Preliminary in vitro, cell-based and *ex vivo* studies showed the complex possessed potent antioxidant and muscle glucose uptake activity and was several folds more potent than its precursors (Ramorobi *et al*., 2022), suggesting a complexation-mediated antioxidant and glycaemic control synergistic effect. It the present study we investigated whether the synergistic effects will be mimicked in fructose/streptozotocin-induced diabetic rats and the observed outcome was promising.

Classical diabetes symptoms, polyphagia and polydipsia, were observed in untreated diabetic rats as elevated ($p < 0.05$) food and fluid intake (**Figure 6.1**). Diabetes induction, also, caused significant ($p < 0.05$) body weight loss (**Figure 6.2**) due to impaired nutrient metabolism by insulin. Although not normalized, the treatments improved the diabetes-induced alteration in the rats' body weight, food intake and fluid intake, which suggests an ameliorative effect. The complex outperformed syringic acid and zinc sulphate and was comparable to metformin (**Figures 6.1 and 6.2**), suggesting a promising ameliorative effect, possibly potentiated by synergism.

The loss of insulin action and pancreatic β-cell function are the main critical factors causing persistent hyperglycaemia in T2D (Galicia-Garcia *et al*., 2020). In the present study, elevated blood glucose in the untreated diabetic rats (**Figures 6.3**) was accompanied by reduced ($p < 0.05$) FSI (**Figure 6.5a**), increased ($p < 0.05$) HOMA-IR (**Figure 6.5b**) and impaired ($p < 0.05$) oral glucose tolerance (**Figure 6.4**), which suggest a type 2 diabetic pathophysiology (Galicia-Garcia *et al*., 2020). Syringic acid and zinc sulphate significantly ($p < 0.05$) reduced the elevated NFBG (**Figure 6.3a**) and FBG (**Figure 6.3b**) of the diabetic animals at the end of the study, suggesting promising antidiabetic action. However, when complexed, the blood glucose lowering action of syringic acid and zinc sulphate in the diabetic rats increased ($p < 0.05$) by ≈28 – 36% and ≈37 - 40%, respectively, suggesting a potent antidiabetic synergism. The AUC of OGTT, also, showed that the modulatory effect of the complex on the glucose tolerance of the diabetic rats was better than that of syringic acid and zinc sulphate (**Figure 6.4b**), suggesting a complexation-mediated synergistic glycaemic control. The diabetes-induced depletion of FSI was appreciably recovered

by the complex and zinc sulphate (**Figure 6.5a**). The effect of the complex on FSI may be influenced by its Zn(II) moiety, which has been documented to play an important role in insulin storage and secretion (Chabosseau and Rutter, 2016).

The complex reduced HOMA-IR in the diabetic rats (**Figure 6.5b**), suggesting an ameliorative effect on diabetic insulin resistance. The data, also, showed that syringic acid reduced HOMA-IR in the diabetic rats, and may be an influencing component of the complex in improving diabetic insulin resistance. Moreover, it has been shown that syringic acid could promote insulin sensitivity in experimental diabetes (Muthukumaran *et al*., 2013). Nevertheless, it is, also, plausible that the Zn(II) moiety worked in synergy with the syringic acid moiety in potentiating the observed ameliorative effect of the complex on diabetic insulin resistance, despite zinc sulphate did not reduce HOMA-IR. This is because, the modulatory action of Zn(II) on insulin action has been documented (Chukwuma *et al*., 2020). Considering that FSI is a multiplication factor for computing HOMA-IR, it is reasonable to assume that the increased FSI caused by zinc sulphate (**Figure 6.5a**) may have, also, influenced the relatively higher HOMA-IR value in the rats of the ZNS group relative to the rats of the other treatment groups (**Figure 6.5b**).

Several mechanisms underlying the insulin mimetic action of Zn(II) have been documented. Zn(II) promotes adipocyte glucose transport and lipogenesis (Ezaki, 1989; Shisheva *et al*., 1992). Zinc(II) promotes endogenous glycogen synthesis by inhibiting the action of endogenous glycogen synthase kinase-3β (Ilouz *et al*., 2002). The modulatory action of Zn(II) on cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase activity has been documented (Percival *et al*., 1997; Yoshikawa *et al*., 2004), which potentiates downregulation of cAMP-mediated lipolytic and glycogenolytic signalling. In vitro, the activity of glycolytic enzymes has been shown to be modulated by Zn(II) (Tamaki *et al*., 1983). Syringic acid treatment, one the other hand, has been shown to increase glycogen synthesis in experimental diabetes (Srinivasan *et al*., 2014). In the present study, complexing syringic acid and zinc sulphate increased liver glycogen content (by ≈59 and 50%, respectively) and muscle glycogen content (by ≈63 and 28%) of both precursors (**Figure 6.6a**), suggesting a complexation mediated synergistic modulation of glycogen storage in experimental diabetes.

Furthermore, the complex had a potent modulatory effect on muscle hexokinase activity (**Figure 7**), which can be attributed to its Zn(II) moiety. The modulatory action of the complex on glycogen synthesis and muscle hexokinase activity was comparable to that of metformin, suggesting its potential to promote glucose uptake and utilization and improve glycaemic control in experimental diabetes. Moreover, the complex, relative to its precursors significantly ($p < 0.05$) increased Akt phosphorylation (**Figure 6.8**), a key activation step involved in the insulin-mediated signalling of glucose uptake and utilization in peripheral tissues (Beg *et al*., 2017).

The higher muscle zinc concentration in the complex treated diabetic animals relative to the other animals (Figure 5b) suggests that complexation modulated muscle Zn(II) transport, which may partly influence the insulin mimetic and glycaemic control potential of the complex. Moreover, it has been shown that through complexation non-pharmacologically active 1-oxy-2-pyridinethiol ligands has the potential of increasing cellular zinc uptake, thus causing insulin mimetic effects (Basuki *et al*., 2007; Yoshikawa *et al*., 2011). Considering the role of Zn(II) in insulin storage, integrity and secretion, it is reasonable to speculate that the higher pancreatic zinc concentration of the rats in the complex and zinc sulphate treated groups compared to the rats in the syringic acid and metformin treated groups (**Figure 6.6b**) influenced the observed higher FSI in the former relative to the later (**Figure 6.5a**). Thus, supporting the potential therapeutic benefits of Zn(II) complexation in diabetes management.

From antioxidant perspective, it was observed that that the complex, also, ameliorated diabetic oxidative stress. It reduced systemic, hepatic and pancreatic lipid peroxidation in experimental diabetes (**Table 6.1**), suggesting its mitigatory potential against radical production and tissue oxidative insults in diabetes. The depleted serum GHS in the diabetic rats was, also, recovered by the complex treatment. GSH is a known biological antioxidant molecule that forms part of a network of antioxidant defence system in the body (Birben *et al*., 2012), suggesting the complex's potential to improve systemic antioxidant capacity. Concomitantly, the complex appreciably recovered catalase and SOD activity in diabetic rats (**Table 6.2**). Catalase and SOD catalyse the reduction of superoxide ion and detoxification of $H_2O_2$, respectively, to form water, thus averting the deleterious oxidative injury that can be caused by superoxide ion and $H_2O_2$ (Birben *et al*., 2012). Therefore, the recovery of these enzymes in the diabetic rats further suggests the complex's

potential to improve systemic antioxidant capacity and mitigate oxidative insults in diabetes. It is, however, noteworthy to mention that the systemic and tissue antioxidant effect of the complex was notably stronger than its precursors and metformin (**Table 6.2**), suggesting that Zn(II) – syringic acid complexation has the capacity to improve the antioxidant potency of its precursors, and thus may be a therapeutic alternative for diabetic oxidative stress.

## 6.6 Conclusion

The preference for natural therapeutic agents over the synthetic counterparts has been an ongoing trend due the perceived holistic functional advantage and minimal adverse effects of the former relative to the later. In the present study the antidiabetic and antioxidant potential of a novel complex developed from a natural antioxidant (syringic acid) and insulin mimetic zinc mineral was investigated using fructose/streptozotocin-induced diabetic rats. It was observed that the complex acquired the bioactive properties of its precursors, thus exhibiting a broader and improved bioactivity profile (**Figure 6.9)**.

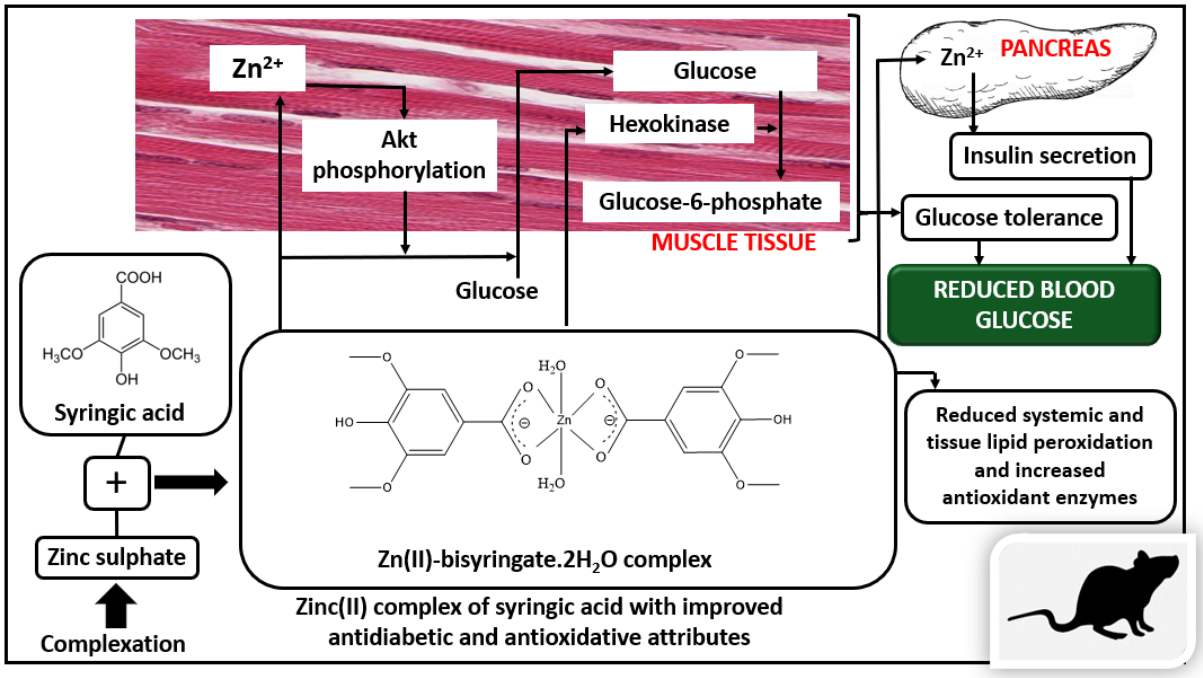



**Figure 6.9:** A summary figure depicting the complexation mediated antioxidant and antidiabetic synergistic potential between zinc (II) and syringic acid.

It potentiated glycaemic control and ameliorated diabetes induced biochemical alteration. It concomitantly suppressed lipid peroxidation and recovered key antioxidant enzymes in diabetic rats. Zinc-syringic acid complexation may, therefore, be an alternative approach to improving the efficacy of antidiabetic and antioxidative therapy with minimal adverse or side effects.

# CHAPTER 7

## GENERAL DISCUSSION AND CONCLUSION

### 7.1 Discussion

There has been an ongoing search for better and more effective therapeutic approaches to combat diabetes and related complications due to issues relating to efficacy and detrimental side effects observed with the use of most of the current commercial synthetic antidiabetic drugs (Chaudhury *et al*., 2017; Chaturvedi *et al*., 2018). The increasing prevalence of diabetes and its associated morbidity and mortality outcomes has, also, prompted the search for effective therapy to manage diabetes and mitigate the associated oxidative complications. Therapeutic synergism has been a welcomed approach for diabetes and oxidative stress management because it affords a multi-target/multi-facet therapeutic benefit, which improves therapeutic outcomes (Lehár *et al*., 2009). Many herbal products containing pharmacologically active phenolics potentiate their multi-mode pharmacological benefits through synergistic mechanisms (Roh and Choi, 2011; Moukette *et al*., 2017). Still in the context of synergism, zinc, an insulin related functional element, has been complexed with many types of ligands, including natural organic ligands, synthetic organic ligands, supplements, antidiabetic drugs and flavonoids to develop complexes with improved glycaemic control potential (Chukwuma *et al*., 2020). The zinc complexes of some flavonoids potentiated improved antidiabetic and antioxidant actions that were comparable to some known antidiabetic drugs (Vijayaraghavan *et al*., 2012; 2013; Sendrayaperumal *et al*., 2014; Umamaheswari and Subramanian, 2015; Al-Ali *et al*., 2016). In the present study, two natural phenolic acids (p-coumaric acid and syringic acid), which have not been previously considered, where explored as promising ligands in synthesizing Zn(II) complexes with potent antidiabetic and antioxidant activities; the premise being that they are natural antioxidants with diabetes related pharmacological potentials (Abdel-Moneim *et al*., 2018; Zabad *et al*., 2019; Rashedinia *et al*., 2020; 2021). Thus, may be promising ligands in developing Zn(II) complexes with multi-facet antidiabetic and antioxidant therapeutic effects.

Zinc sulphate was individually complexed with p-coumaric acid and syringic acid in a 1:2 mole ratio of zinc sulphate to phenolic acid, respectively. Spectroscopic characterization (**Figures 4.1,**

**4.2, 4.3, 5.1, 5.3 and 5.4)** showed that both complexes were formed as a double hydrated Zn(II)-biphenolate complex: Zn(II)-bicoumarate.$2H_2O$ (**Figure 4.2a**) and Zn(II)-bisyringate.$2H_2O$ (**Figure 5.2**) complexes. Complexation afforded the synthesized complexes a moiety of Zn(II) and two moieties of their respective phenolic acids, which influenced improved antioxidant and antidiabetic potentials of the complexes relative to their precursors, suggesting a complexation mediated synergism.

The complexes demonstrated *in vitro* radical scavenging and anti-linoleic acid peroxidative activities that were stronger than those of their respective precursors and comparable to those of ascorbic acid and/or Trolox (**Figures 4.4, 4.5, 5.5 and 5.6; Tables 4.1 and 5.1**). The two phenolic acid moieties in the complexes (**Figures 4.2a and 5.2**) may be responsible for to the complexes' stronger *in vitro* antioxidant activities considering zinc sulphate had no observable *in vitro* antioxidant effect, while the phenolic acids showed appreciable *in vitro* radical scavenging, Fe3+ reducing and anti-lipid peroxidative effects (**Figures 4.4, 4.5, 5.5 and 5.6; Tables 4.1 and 5.1**). The antioxidant effects of the phenolic acids may be attributed to their ability to form stable phenoxy radicals with radical scavenging and metal chelating property (Chen *et al*., 2020). Thus, there was a combined effect from the two moieties in the complex as compared to a single molecule.

In Chang liver cells and isolated rat liver tissue, the complexes also outperformed their respective precursors in inhibiting oxidative stress-induced lipid peroxidation and reduced glutathione (GSH) (**Figures 4.6, 4.7, 5.7 and 5.8 and Tables 4.1 and 5.1**), which may, also, be attributed to the combined effect of the two phenolic acid moieties in the complexes (**Figures 4.2a and 5.2**). Moreover, p-coumaric acid (Shen *et al*., 2019) and syringic acid (Rashedinia *et al*., 2020; 2021) have been shown to have *in vivo* anti-lipid peroxidative properties. The combined anti-lipid peroxidative effect of the two phenolic acid moieties in each complex was as potent as that of ascorbic acid (**Figures 4.6, 4.7, 5.7 and 5.8 and Tables 4.1 and 5.1**), suggesting Zn(II) complexation may be an alternative novel approach to improving the antioxidant properties of these phenolic acids. It is noteworthy that the antioxidant effects of the Zn(II)-bisyringate.$2H_2O$ complex was stronger than the antioxidant effects of the Zn(II)-bicoumarate.$2H_2O$ complex

(**Tables 4.1 and 5.1**), suggesting that in the context of modulating antioxidant action through Zn(II) complexation, syringic acid may be more favourable than p-coumaric acid.

In an antidiabetic context the complexes demonstrated potentials to improve postprandial hyperglycaemia and mitigate hyperglycaemia-induced complications. The complexes inhibited α-glucosidase enzyme *in vitro* (**Figures 4.8a and 5.9; Tables 4.1 and 5.1**). Inhibition of this enzyme is a pharmacological mode of action to reduce glucose absorption and postprandial elevation of blood glucose in diabetic patients (Moelands *et al*., 2018), which suggests the potential of the complexes to modulate postprandial glycaemic control. Also, the complexes inhibited glucose-induced protein glycation *in vitro* (**Figures 4.8b and 5.10; Tables 4.1 and 5.1**), suggesting a potential to mitigate glycation and AGEs mediated diabetic complications (Singh *et al*., 2014). For the enzyme inhibition data, it appeared there was a synergistic action between Zn(II) and the phenolic acids, which potentiated improved α-glucosidase inhibitory effect (**Tables 4.1 and 5.1**). The synergistic α-glucosidase inhibitory action of the complexes was stronger for the complex with syringic acid than that with p-coumaric acid as evident in the $IC_{50}$ values of their respective complexes (16.8 µM versus 19.6 µM, respectively) (**Tables 4.1 and 5.1**). For the antiglycation data, it appeared Zn(II) conferred a potent antiglycation action on the Zn(II)-bicoumarate.$2H_2O$ complex but functioned in synergy with syringic acid to potentiate an improved antiglycation effect in the Zn(II)-bisyringate.$2H_2O$ complex, which was 2.9 folds stronger than that of the Zn(II)-bicoumarate.$2H_2O$ complex and statistically comparable to that of aminoguanidine (**Tables 4.1 and 5.1**), a known antiglycation standard. The data suggest syringic acid may be more promising than p-coumaric acid in developing a Zn(II) complex with mitigatory potential against hyperglycaemia-induced glycation and associated vascular impairments. Moreover, syringic acid has been shown to inhibit *in vitro* protein glycation and formation of AGEs (Wu *et al*., 2010; Bhattacherjee and Datta, 2015), while zinc sulphate has been shown to exert *in vitro* antiglycation action and suppress protein-carbonyl interaction in diabetic rats (Sacan *et al*., 2016).

The complexes, further, showed antidiabetic potential by increasing glucose uptake in L-6 myotubes and isolated rat psoas muscle tissues (**Figures 4.9 and 5.11; Tables 4.1 and 5.1**). Insulin-induced glucose uptake and utilization in peripheral tissues, such as muscle and adipose tissues is a known glycaemic control mechanism of insulin (Aronoff *et al*., 2004), which suggests

that the complexes may improve glycaemic control. In rat muscle tissues, increased hexokinase activity was caused by the complexes (**Figures 4.10a and 5.12a**), suggesting a modulation in a glycolytic glucose utilization. The Zn(II)-bisyringate.$2H_2O$ further modulated Akt phosphorylation (**Figure 4.12c**), a key signalling step in insulin signalling and cellular glucose uptake (Beg *et al*., 2017). The data suggest a modulatory action of the complexes on insulin signalling and glucose utilization, thus facilitating tissue glucose uptake. The complexes had stronger modulatory effect on glucose uptake and utilization compared zinc sulphate and their respective phenolic acids.

In silico docking also showed molecular targets (GLUT-4, Akt/PKB, IRS-1 and insulin receptors) involved in insulin signalling and cellular glucose uptake had stronger interaction with the complexes than with their respective phenolic acids (**Figures 4.11 and 5.13; Tables 4.2 and 5.2**), which may also account for the more potent modulatory effect of the complexes on muscle Akt phosphorylation, glucose uptake and glucose utilization relative their respective phenolic acids. It appeared Zn(II) conferred promising cellular and tissue glucose uptake and utilization activities on the complexes and further acted in synergy with the phenolic acid moieties to potentiate the observed improved activities in the complexes relative to their phenolic acid precursors, suggesting a complexation-mediated synergistic glycaemic control potential.

Based on the stronger antioxidant, antiglycation and enzyme inhibitory effect of the Zn(II)-bisyringate.$2H_2O$ complex compared to the Zn(II)-bicoumarate.$2H_2O$ complex, as well as its remarkable complexation-mediated improvement in cellular and tissue glucose uptake, it is rational to subject the Zn(II)-bisyringate.$2H_2O$ complex to *in vivo* antidiabetic and antioxidative evaluation to see whether the synergistic effects are mimicked or replicated *in vivo*. The antidiabetic and antioxidant potential of the Zn(II)-bisyringate.$2H_2O$ complex was investigated in fructose and low-dose STZ induced diabetic rats and compared to the effect of its precursors (syringic acid and zinc sulphate).

Diabetes induction resulted in polyphagia and polydipsia (**Figure 6.2**), which are classic symptoms of diabetic condition (Fournier, 2000). Diabetes induction, further, caused significant ($p < 0.05$) weight loss (**Figure 6.1**), which may be attributed to a defect in insulin function and nutrient

metabolism. Although not significantly, the complex and its precursors reversed diabetic polyphagia, polydipsia and weight loss (**Figures 6.1 and 6.2**), suggesting an ameliorative effect on diabetes. The effect of the complex was stronger than its precursors and statistically ($p > 0.05$) as potent as metformin. The data showed potential of normalizing these diabetic alterations with longer-term treatment, suggesting that the complex has antidiabetic potential.

Blood glucose profile, which is a critical index for assessing the severity of diabetes suggests the promising antihyperglycaemic action of the complex. Both zinc sulphate and syringic acid exerted significant ($p < 0.05$) reduction effect on the NFBG and FBG of diabetic rats after 20 days treatment (**Figure 6.3**). However, upon complexation, their blood glucose lowering capacity further improved significantly ($p < 0.05$) and became comparable with the blood glucose lowering capacity of metformin. Even the modulatory effect of the complex on glucose tolerance in diabetic rats was better than its precursors (**Figure 6.4**), suggesting a complexation-mediated glycaemic control synergism between Zn(II) and syringic acid in diabetic rats.

Diabetes-induced depletion of FSI was recovered by the complex (**Figure 6.5a**), which may have, also, influenced its glycaemic control potential. Moreover, endogenous insulin release is the most potent mechanism for systemic blood glucose homeostasis (Aronoff *et al*., 2004). The elevated FSI potentiated by the complex may be majorly attributed to its Zn(II) moiety (**Figure 5.2**), considering the functional role of molecular zinc in pancreatic insulin preservation and secretion (Chabosseau and Rutter, 2016). Moreover, studies in mice have shown that deleting the gene encoding the proteins responsible for pancreatic β-cell Zn(II) transport (ZnT8) resulted in the impairment of insulin secretion, reduction of peripheral insulin concentration and impairment of glucose tolerance (Nicolson *et al*., 2009; Wijesekara *et al*., 2010). Supporting biochemical data in the present study showed elevated pancreatic zinc concentration in the complex and zinc sulphate treated diabetic rats (**Figure 6.6b**), which is consistent with and may account for the elevated FSI in these groups of rats (**Figure 6.5a**).

Furthermore, syringic acid-Zn(II) complexation increased muscle tissue zinc concentration and/or bioavailability after treatment in the diabetic rats. Elevated zinc concentration in the muscle may have partly acted in synergy with syringic acid potentiated the observed elevated insulin mimetic

or modulatory action shown by the complex at cellular and tissue levels. Zn(II) has been shown to promote endogenous glycogen synthesis by inhibiting the action of endogenous glycogen synthase kinase-3β (Ilouz *et al*., 2002). *In vitro*, the activity of glycolytic enzymes has been shown to be modulated by Zn(II) (Tamaki *et al*., 1983). Other studies suggest Zn(II) may have modulatory action insulin signalling, which may be mediated by a modulatory activation of insulin receptor, IRS-1, Akt signalling and GLUT-4 translocation (May and Contoreggi, 1982; Ezaki, 1989; Tang and Shay, 2001; Miranda and Dey, 2004; Yoshikawa *et al*., 2004; Wu *et al*., 2016; Norouzi *et al*., 2018). On the other hand, syringic acid has been shown to increase glycogen synthesis in experimental diabetes (Srinivasan *et al*., 2014).

In the present study, the complex showed modulatory effect on hepatic and muscle glycogen production (**Figure 6.6a**), as well as muscle hexokinase enzyme activity (**Figure 6.7**) and Akt phosphorylation (**Figure 6.8**), which suggests a modulatory effect on insulin signalling, glucose uptake and utilization, and thus potentiating glycaemic control action in diabetic rats as presented in **Figure 5.15**. The ability to the complex to reduce diabetic HOMA-IR score (**Figure 6.5b**) suggests its ability to improve diabetic insulin resistance, which further reflects the ability to improve insulin action and signalling, thus potentiating glycaemic control in experiment diabetes. The data suggest possible complexation mediated therapeutic approach for improving glycaemic control and reducing hyperglycaemia in diabetes.

Systemic, pancreatic and hepatic data suggest the complex may exert promising protective effect against diabetic oxidative stress and insults in the liver and pancreas. Syringic acid has been previously shown to reduce lipid peroxidation and restore antioxidant enzymes activity in experimental diabetes, thus improving diabetic oxidative stress (Sabahi *et al*., 2020; Rashedinia *et al*., 2020; 2021). Consistent data was observed in the present study were syringic acid reduced diabetes-induced elevation of serum, hepatic and pancreatic lipid peroxidation (**Table 6.1**), a key ROS generating processing that causes oxidative stress and tissue damage (Ayala *et al*., 2014). Concomitantly, syringic acid notably recovered key antioxidant enzymes (SOD and catalase), which were notable depleted in the serum, liver and pancreas by diabetes induction (**Table 6.1**). These antioxidant enzymes catalyse the reduction and detoxification of highly oxidant and

deleterious superoxide ion and $H_2O_2$ to less harmful water (Birben *et al*., 2012), which suggests the mitigatory potential of syringic acid against diabetic oxidative stress and insult.

However, complexation-mediated acquisition of a bi-syringate structure (**Figure 5.2**) further improved the antioxidant action of syringic acid, and in some instances appreciably normalizing the diabetes-induced elevated lipid peroxidation and depleted antioxidant enzyme activity. Although the systemic and tissue antioxidant effect zinc sulphate may not be as potent as syringic acid (**Tables 6.1 and 6.2**), it is important not to "downplay" or neglect the possible influence of the Zn(II) moiety in the improved antioxidant action of the complex. Moreover, the importance of molecular zinc as a functional co-factor in the action of some key antioxidant enzymes has been well documented (Jarosz *et al*., 2017). Thus, it is most probable that there was a synergistic interaction between the Zn(II) and the bi-syringate moieties of the complex, which potentiated the observed stronger *in vivo* antioxidant effect of the complex in diabetic rats relative to its precursors.

## 7.2 Conclusion

The concept of Zn(II) complexation has long been explored as a possible scientific approach to developing insulin mimetic complexes with glycaemic control potential, which can potentially be used to manage impaired glucose tolerance in diabetes. This has been partly due to the safety profile of zinc mineral and its functional role in insulin preservation, secretion and function. Many types of ligands including supplements and antidiabetic drugs have been explored as suitable ligands to develop potent therapeutic Zn(II) complexes for managing diabetes. However, due to the critical role of oxidative stress in the development and progression of diabetes and vascular complications, the use of polyphenols as alternative ligands for antidiabetic Zn(II) complexes has been explored by scientists. It is perceived that the antioxidant attributes of natural polyphenols may afford a Zn(II) complex an antioxidant perspective in combating diabetic oxidative stress and reducing the risk of diabetic complications. Data from some studies that have complexed zinc with dietary flavonoids support this perception to an appreciable extent. However, natural phenolic acids, which are known dietary antioxidant have been scarcely explored as antioxidant ligands for developing antidiabetic Zn(II) complexes. In the present study, p-coumaric acid and syringic acid were used as alternative ligands to develop Zn(II) complexes, due to their promising antioxidant

attributes and some other diabetes-related pharmacological attributes. Zn(II) was separately complexed with two molecules of each phenolic acid to form a Zn(II)-bicoumarate.$2H_2O$ complex and a Zn(II)-bisyringate.$2H_2O$ complex, which each had a Zn(II) moiety and two moieties of their respective phenolic acids. This structural characteristic of the complexes appeared to have influenced their bioactivity relative to their precursors.

The complexes exhibited *in vitro* radical scavenging and anti-lipid peroxidative effects and concomitantly inhibited oxidative stress-induced lipid peroxidation and GSH depletion in Chang liver cells and isolated rat liver tissues, suggesting their potential to suppress oxidative tissue insults. Also, the complexes inhibited α-glucosidase and glucose-induced protein glycation, suggesting a potential to suppress postprandial glycaemia and mitigate hyperglycaemia-induce glycation and associated complications. In both L-6 myotubes and isolated rat muscle tissues, the complexes increased glucose uptake and utilization with causing cytotoxicity. Modulation in insulin signalling and glycolysis appeared to be the possible underlying mechanisms. The Zn(II)-bisyringate.$2H_2O$ complex outperformed the Zn(II)-bicoumarate.$2H_2O$, and thus was subjected to further studies in fructose/STZ-induced diabetic rats. The complex appreciably recovered diabetic polydipsia, polyphagia and weight loss. It also increased insulin secretion, tissue glycogen and glycolysis and improved insulin resistance, thus potentiating glycaemic control in the diabetic rats. Concomitantly, the complex suppressed systemic and tissue lipid peroxidation and modulated the activity of key antioxidant enzymes in the diabetic rats. The complexes had antioxidant and antidiabetic effects that were stronger than their respective precursors. Perhaps, with some contribution from Zn(II) two moieties of the phenolic acid present in their respective Zn(II) complexes appear to be the major factor influencing the improved antioxidant properties of the complexes. On the other hand, it appears that Zn(II) functioned synergistically with the phenolic acid counterparts as the more influencing moiety in potentiating glycaemic control. Possibly, by modulating glucose uptake and utilization. The data of this study suggests a complexation-mediated antioxidative and antidiabetic synergism between Zn(II) and the phenolic acids. Therefore, complexing Zn(II) with these phenolic acids (p-coumaric and syringic acid) may be an underexplored therapeutic approach to improving the effectiveness of therapies for diabetes and oxidative stress management.

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# APPENDICES

The following below are the contents of the appendix:

1. **Animal ethics approval document for conducting studies involving the use of animals:** Ethical clearance was gotten from the Animal Research Ethics Committee of the University of the Free State, Bloemfontein, South Africa (**protocol approval reference: UFS-AED2019/0152/2020; approved on 16 November 2020**)
2. **Publication 1 of thesis:** Ramorobi LM, Matowane GR, Mashele SS, Swain SS, Makhafola TJ, Mfengwana PH, Chukwuma CI (2022). Zinc(II) - Syringic acid complexation synergistically exerts antioxidant action and modulates glucose uptake and utilization in L-6 myotubes and rat muscle tissue. Biomedicine & Pharmacotherapy. doi: 10.1016/j.biopha.2022.113600
3. **Publication 2 of thesis:** Ramorobi LM, Matowane GR, Mashele SS, Bonnet SL, Noreljaleel EAEM, Swain SS, Makhafola TJ, Chukwuma CI (2022). Bioactive Synergism Between Zinc Mineral and p-Coumaric Acid: A multi-mode Glycaemic Control and Antioxidative Study. Journal of Food Biochemistry. doi: 10.1111/jfbc.14360.
4. **Publication 3 of thesis (proof of accepted manuscript):** Ramorobi LM**,** Matowane GR, Mashele SS, Erukainure OL, Makhafola TJ, Chukwuma, CI. Therapeutic Antidiabetic and Antioxidative Synergism of Zn(II)-syringic acid complexation. Revista Brasileira de Farmacognosia (**Manuscript ID: RBFA-D-22-00747R1**). Accepted for publication on **16 January 2023**.
5. **Proof of scientific conference presentation:** Ramorobi LM*, Matowane GR, Mashele SS, Bonnet SL, Makhafola TJ, Chukwuma CI (2022). ANTIDIABETIC AND ANTIOXIDATIVE SYNERGISTIC POTENTIAL BETWEEN ZN(II) AND CAFFEIC ACID: IMPROVING THERAPEUTIC POTENTIAL THROUGH COMPLEXATION. Virtual presentation at the **3rd Molecules Medicinal Chemistry Symposium (MMCS): Shaping Medicinal Chemistry for the New Decade**, held at Sapienza University of Rome, Rome, Italy during 27 – 29 July 2022.

**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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# Bioactive synergism between zinc mineral and p-coumaric acid: A multi-mode glycemic control and antioxidative study

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## Abstract

Natural supplements are important in diabetes and oxidative stress management. A complexation-mediated antihyperglycemic and antioxidant synergism between zinc(II) and p-coumaric acid was investigated. p-Coumaric acid was complexed with $ZnSO_4$ and characterized by FT-IR, $^1$H NMR, and mass spectroscopy. The antioxidant and antihyperglycemic potential of the complex and precursors were evaluated with different experimental models. Molecular docking with target proteins linked to diabetes was performed. A Zn(II)-bicoumarate.$2H_2O$ complex was formed. The in vitro radical scavenging, α-glucosidase inhibitory, antiglycation, and anti-lipid peroxidative activities of the complex were several folds stronger than p-coumaric acid. In Chang liver cells and rat liver tissues, the complex inhibited lipid peroxidation ($IC_{50}$ = 56.2 and 398 μM) and GSH depletion ($IC_{50}$ = 33.9 and 38.7 μM), which was significantly stronger (2.3–5.4-folds) than p-coumaric acid and comparable to ascorbic acid. Zn(II) and p-coumaric synergistically modulated (1.7- and 2.8-folds than p-coumaric acid) glucose uptake in L-6 myotubes ($EC_{50}$ = 10.7 μM) and rat muscle tissue ($EC_{50}$ = 428 μM), which may be linked to the observed complexation-mediated increase in tissue zinc uptake. Glucose uptake activity was accompanied by increased hexokinase activity, suggesting increased glucose utilization. Docking scores α-glucosidase, GLUT-4, and PKB/Akt showed stronger interaction with the complex (−6.31 to −6.41 kcal/mol) compared to p-coumaric acid (−7.18 to −7.74 kcal/mol), which was influenced by the Zn(II) and bicoumarate moieties of the complex. In vitro, the complex was not hepatotoxic or myotoxic. Zn(II) complexation may be a therapeutic approach for improving the antioxidative and glycemic control potentials of p-coumaric acid.

## Practical applications

In functional medicine, natural supplements, plant-derived phenolics, and nutraceuticals are becoming popular in the management of diseases, including diabetes and oxidative stress. This has been largely attributed to their perceived holistic medicinal profile and the absence of notable toxicity concerns. In the past two decades, considerable attention has been drawn toward zinc mineral as a possible therapeutic supplement for diabetes due to its role in insulin secretion and reported insulin mimetic potentials. p-Coumaric acid is a known natural antioxidant with reported

diabetes-related pharmacological effects. In this study, we took advantage of these properties and complexed both natural supplements, which resulted in a more potent nutraceutical with improved glycemic control and antioxidant potential. The complexation-mediated synergistic interaction between zinc and p-coumaric acid could be an important therapeutic approach in improving the use of these natural supplements or nutraceuticals in managing diabetes and associated oxidative complications.

**KEYWORDS**
antioxidant, complexation, glycemic control, p-coumaric acid, synergism, zinc mineral

## 1 | INTRODUCTION

The search for better and safe therapeutic agents for diabetes and associated complications remains an ongoing trend, due to the limitations of existing therapeutic agents (MacLean et al., 2006) and the increasing prevalence and detrimental consequences of the disease (IDF, 2019, 2021). Type 2 diabetes (T2D), which is the most prevalent type of diabetes, has posed serious socioeconomic and health burdens on most countries of the world (IDF, 2021). Its high prevalence has been attributed to its association with obesity and lifestyle-related factors (Al-Goblan et al., 2014; Sami et al., 2017). These factors promote metabolic disorders, such as insulin resistance, glucose intolerance, and hyperinsulinemia, which are metabolic indices and risk factors for developing T2D (Al-Goblan et al., 2014; Galicia-Garcia et al., 2020; Sami et al., 2017). Impaired insulin signaling and poor circulating glucose uptake and utilization in peripheral tissues, as well as progressive pancreatic β-cell damage are some of the derangements that facilitate poor glucose homeostasis and persistent hyperglycemia in T2D (Galicia-Garcia et al., 2020).

Persistent hyperglycemia is the key instigator of most complications associated with diabetes. Oxidative stress has been implicated as a major mediator and culprit of the progression and outcomes of some of the diabetic complications (Giacco & Brownlee, 2010). This is largely attributed to increased generation of reactive oxygen species (ROS) and free radicals, which can oxidatively damage important biological molecules, resulting in degenerative conditions in the absence of adequate and effective antioxidant defense mechanisms (Di Meo & Venditti, 2020). Persistent hyperglycemia drives the incomplete reduction of molecular oxygen in cellular electron transport chain and energy metabolism, thus increasing the production of superoxide ion, a deleterious biological ROS (Fakhruddin et al., 2017). Also, hyperglycemia promotes glycation processes and formation of advanced glycation end products, which are biomarkers of degenerative conditions and complications of diabetes (Giacco & Brownlee, 2010). The increased radical production associated with diabetes, also, initiates lipid peroxidation processes, which releases more deleterious free radicals, while comprising the integrity of functional cellular lipids (Fatani et al., 2016).

The synergistic application of natural therapeutic agents has an appreciable reception in management of diseases. The concept can be beneficial in the following ways: Limiting toxicity or side effects linked to high dose of drug and providing a platform for multi-mode therapeutic targets and effects (Lehár et al., 2009). Advances in research have shown possible diabetes-related therapeutic synergism through mineral-ligand complexation. Zinc mineral has been complexed with various types of ligands to develop zinc complexes with improved bioactivity (Chukwuma et al., 2020). It has been reported that zinc possesses insulin-mimetic action and complexation may afford improved bioavailability and multi-facet bioactivity (Chukwuma et al., 2020). Zinc(II) complex of maltol inhibited lipolysis and increased glucose uptake in adipocytes isolated from rats (Adachi et al., 2004). A mechanistic study suggests its effect may be through modulatory action on adipocyte insulin receptor tyrosine kinase, phosphoinositide 3-kinase, GLUT-4, and phosphodiesterase (Yoshikawa et al., 2004). Studies on T2D KK-Ay mice showed the complex reduced blood glucose and HbA1c levels, and improved glucose tolerance (Adachi et al., 2004). Zn(II) complexes of several amino acids (asparagine, proline, valine, glycine, alanine, glutamine, aspartic acid, and threonine) (Yoshikawa et al., 2001) and L-carnitine (Yoshikawa et al., 2003) suppressed lipolysis in isolated rat adipocytes, while Zn(II) complex of threonine and L-carnitine potentiated antihyperglycemic action in T2D KK-Ay mice (Yoshikawa et al., 2001, 2003). In fact, the blood glucose-lowering effect of the Zn(II) complex of L-carnitine was approximately 3.6 times more potent than that of L-carnitine (Yoshikawa et al., 2003), which suggest the improving action of Zn(II) on its ligands.

Although studies on antidiabetic Zn(II) complexes are potential therapeutic leads to the discovery of antidiabetic nutraceuticals, the research trajectory appears to have a major flaw. A broad perspective into the reported studies on antidiabetic Zn(II) complexes reveals that most of the studied antidiabetic Zn(II) complexes are synthesized using synthetic ligands that have little pharmacological credence and toxicity concerns (Chukwuma et al., 2020). Plant phenolics with documented antioxidant and glycemic control potential remain the least explored ligands of antidiabetic Zn(II) complexes (Chukwuma et al., 2020), which suggests a research trajectory that favors synthetic rather than natural medicines. Several phenolic acids, including p-coumaric acid, have not been studied as possible ligands of Zn(II) complexes with promising bioactivities. p-Coumaric acid is a natural hydroxycinnamic acid

derivative that is present in vegetables, fruits, mushrooms, and cereals (Zabad et al., 2019) and has been reported to possess radical scavenging antioxidant properties (Kadoma & Fujisawa, 2008; Kiliç & Yeşiloğlu, 2013). p-Coumaric acid inhibits α-glucosidase activity, which suggests potential of improving postprandial glycemic control (Pei et al., 2016). Results from a previous study in T2D rats suggest that p-coumaric acid may inhibit hippocampal neurodegeneration by potentiating antioxidant, anti-inflammatory, and anti-apoptotic effects (Abdel-Moneim et al., 2018). In rats, p-coumaric acid alleviated experimentally induced diabetic nephropathy (Zabad et al., 2019), while in T2D rats, it exhibited antidiabetic effects by upregulating the mRNA expression of PPARγ (Abdel-Moneim et al., 2018).

The above studies suggest that p-coumaric acid may be a promising ligand for Zn(II) in developing promising a Zn(II) complex with improved and broader scope of bioactive action. However, this hypothesis has not been studied. Therefore, this study was done to investigate the possible complexation-mediated glycemic control and antioxidant synergism between zinc(II) and p-coumaric acid.

## 2 | MATERIALS AND METHODS

### 2.1 | Zinc sulfate complexed with p-coumaric

Zinc(II) was complexed with p-coumaric acid in a 1:2 mole ratio using a previous method (Kalinowska et al., 2011) with some modifications. First, 287.56 mg of zinc(II) sulfate heptahydrate (Mr = 287.56 g/mol) and 396.34 mg of p-coumaric acid (Mr = 164,08 g/mol) were separately dissolved in 5 ml of distilled water and 5 ml of methanol, respectively. A solution (168.02 mg in 5 ml of distilled water) of sodium hydrogen carbonate (Mr 84.01 g/mol) was then added to the p-coumaric acid solution. The mixture was stirred until no effervescence was observed. Thereafter, the zinc(II) sulfate heptahydrate solution was gradually added to the mixture, while stirring. Stirring continued until complete precipitation of a white precipitate. The mixture was filtered using a filter paper to recover the precipitate. The precipitate was washed thrice with 50% methanol and freeze-dried (Alpha 1–2 LDplus Freeze Dryer, Martin Christ, Osterode am Harz, Germany).

### 2.2 | Spectroscopic characterization of complex

For spectroscopic characterization, Fourier-transform infrared spectroscopy (FT-IR), proton nuclear magnetic resonance ($^1$H NMR), and high-resolution mass spectroscopy (HR-MS) were employed.

For the IR analysis, a Thermo Scientific Nicolet iS50 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used. To collect the spectrum of samples (p-coumaric acid and the complex), a reference (or background) spectrum without sample was obtained. About 2 mg of the finely ground sample was loaded into the transmission holder and locked. A scan of 40 $s^{-1}$ rate was done from 4000 to 400 $cm^{-1}$. The background data were subtracted to obtain the signals from the samples.

For $^1$H NMR, a 400 MHz Bruker Avance spectrometer (Bruker Corporation, MA, USA) was used, which recorded the $^1$H NMR data. DMSO-d6 (δH = 2.50) was used as the solvent, while tetramethylsilane was used as the internal standard. The chemical shifts were recorded in the delta (δ) scale and presented in parts per million (ppm). The coupling constants (*J*) were all set correctly to 0.01 Hz.

For HR-MS, the experiment was performed by the Central Analytical Facility, which is located at Stellenbosch University, Cape Town. Instrumentation included A Waters Synapt G2 (Waters Corporation, MA, USA), which was coupled to an ESI probe and ESI Pos. The instrument was operated with a cone voltage supply of 15 V.

### 2.3 | Cytotoxicity evaluation of complex in Chang liver cells

Chang liver cells were procured from the American Type Culture Collection (ATCC), Virginia, USA (Chang live cells; ATCC® CCL-13™). Cytotoxicity evaluation was done using the MTT cell viability protocol. An EMEM medium containing 10% FBS was used to culture the cells. Culturing was done in a NÜVE EC 160 $CO_2$ incubator (NÜVE, Ankara, Turkey) at 95% oxygen and 5% $CO_2$ supply and 37°C temperature. At 80% confluent, 100 µl of the medium containing cells was seeded (30000 cells/ml) in a 96-well sterile plate and incubated for 48 h to allow the cells attach to the plate. Thereafter, the used medium was aspirated and replaced with fresh medium containing 8.17, 81.7, and 817 µM of complex or 0.5% DMSO (vehicle control). Incubation continued for 36 h. One hundred microliters of 0.5 mg/ml MTT solution (Sigma-Aldrich, Johannesburg, South Africa) was added to the plate and the plate was further incubated for 3 h. After incubation, the wells were aspirated and washed with phosphate-buffered saline. One hundred microliters of MTT de-staining solution (Sigma-Aldrich, Johannesburg, South Africa) was quickly added into the wells, and absorbance was measured at 570 nm using the Multiskan Go plate reader (Thermo Fischer Scientific, Waltham, MA, USA). Viability (%) of sample-treated cells was computed relative to the control using the triplicate data of three biological repeats.

### 2.4 | In vitro antioxidant and antidiabetic measurements

#### 2.4.1 | Radical scavenging and $Fe^{3+}$ reducing antioxidant assays

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging

assays, as well as the $Fe^{3+}$ reducing antioxidant assay were performed according to a previous study (Chukwuma et al., 2021). For DPPH and ABTS, the samples were tested at concentrations 3.75–60 μM in final assay volume and expressed as scavenging activity (%). Ascorbic acid and Trolox were used as positive controls. For $Fe^{3+}$ reducing antioxidant assay, the samples were tested at 40 μM. The $Fe^{3+}$ reducing antioxidant activity was computed from an ascorbic acid standard curve (3.75–60 μM in final assay volume) and expressed as mmol/mol ascorbic acid equivalent. Trolox was used as the positive control.

### 2.4.2 | Linoleic acid peroxidation inhibition assay

The inhibitory effect of the complex on linoleic acid peroxidation was measured using a previous method (Choi et al., 2002) with some modifications. Into vials containing 30 μl of different concentrations (5–80 μM in assay volume) of samples or standard (ascorbic acid and Trolox) or the solvents (normal controls and negative control), 30 μl of 50 mM linoleic acid and 20 μl of 100 mM Tris–HCl buffer (pH = 7.5) were added. Next, 20 μl of 2 mM $FeSO_4.7H_2O$ was added and the mixture was incubated for 30 min at 37°C. The normal control vial contained 20 μl of distilled water instead of $FeSO_4.7H_2O$ solution, while the negative control contained 20 μl of the $FeSO_4.7H_2O$ solution. After incubation, the mixtures were acidified with 80 μl of 5.5% TCA, before addition of 50 μl of 0.25% thiobarbituric acid (dissolved in 50 mM NaOH solution). The vials were placed in a boiling water bath for 20 min and allowed to cool at room temperature. The vials were then centrifuged at 3500*g* for 10 min at ambient temperature. A 150 μl aliquot of the supernatants was transferred into a 96-well plate and absorbance was measured at 532 nm using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA). The percentage inhibition of linoleic acid peroxidation was calculated as follows:

$$\text{Inhibition}\,(\%) = \frac{\left(A_{\text{Negative control}} - A_{\text{Normal control}}\right) - \left(A_{\text{Test}} - A_{\text{Normal control}}\right)}{\left(A_{\text{Negative control}} - A_{\text{Normal control}}\right)} \times \frac{100}{1} \quad (1)$$

where, "A" means "Absorbance".

### 2.4.3 | α-Glucosidase and bovine serum albumin (BSA) glycation inhibition assays

The in vitro inhibitory effect on α-glucosidase activity and BSA glycation was measured using methods reported previously (Chukwuma et al., 2021). For α-glucosidase inhibition assay, the samples and positive control were tested at concentrations ranging from 3.75 to 60 μM and acarbose was used as the positive control. For BSA glycation inhibition, the samples and positive control were tested at concentrations ranging from 5 to 80 μM and aminoguanidine was used as the positive control.

## 2.5 | Measurement of cellular antioxidant and antidiabetic properties

### 2.5.1 | Evaluating the effect of the complex on lipid peroxidation and GSH concentration in Chang liver cells induced with oxidative stress

A previous method (Akuru et al., 2021) with slight modifications was adopted to measure the cellular antioxidant effect of the complex and its precursors. Chang liver cells (ATCC® CCL-13™, ATCC, Virginia, USA) were cultured in a culture flask as mentioned above. At about 85% confluence, the cells were harvested by trypsinization and 200 μl of the medium (EMEM media containing 10% FBS) with cells was plated in a 96-well plate at a concentration of 15,000 cells/ml. The plate was incubated in a $CO_2$ incubator for 36 h to allow cells attach. Thereafter, the spent medium was discarded and 200 μl of fresh medium containing different concentrations (10–80 μM in assay volume) of test samples or standard (ascorbic acid) or equivalent volume of solvents (normal control and negative control) was added to the wells. Incubation continued for 30 min, before the addition of 50 μl of $FeSO_4.7H_2O$ solution (1 mM in assay volume) to induce oxidative stress. While the negative control contained the $FeSO_4.7H_2O$ solution, the normal control contained the equivalent volume of distilled water. The plate was incubated for another 1 h. Thereafter, the incubation medium was aspirated, and the cells were lysed with 250 μl of cold lysis buffer (50 mM sodium phosphate buffer with 0.5% v/v Triton X-100, pH 7.5) and gentle agitation. The plate was then centrifuged at 3500*g* and the supernatant was used to estimate lipid peroxidation and GSH concentration according to the methods described below (Akuru et al., 2021).

To estimate lipid peroxidation, 100 μl of supernatant or malondialdehyde standards (0, 7.5, 15, 22.5, 30, and 45 μM in assay volume) was put into vials. Five hundred microliters of 0.25% w/v thiobarbituric acid, 200 μl of 20% v/v acetic acid (200 μl), and 200 μl of distilled water were added, successively. The vials were placed in a boiling water bath for 50 min and then allowed to cool. Aliquot of 200 μl was plated and absorbance was measured at 532 nm. The lipid peroxidation was estimated as thiobarbituric acid reactive substances, which was extrapolated from a malondialdehyde standard curve.

To estimate the GSH concentration, protein was precipitated from the supernatant by mixing with equal volume of 10% trichloroacetic acid and centrifuging for 5 min at 4280*g* (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany). Fifty microliters of the supernatant or GSH standards (0.002, 0.02, 0.2, 2, 20, 200 μM in assay volume) were put into a 96-well plate. Next, 200 μl of Ellman.s reagent [0.1 mM of 5,5′-dithio-bis-(2-nitrobenzoic acid) dissolved in 0.1 M (Tris–HCl buffer, pH 7.4)] was added. The mixture was incubated for 5 min at 25°C and absorbance was measured at 412 nm. The GSH concentration was extrapolated from a GSH standard curve.

The percentage inhibition of lipid peroxidation and GSH depletion were calculated and used to compute the $IC_{50}$ values (concentration of sample causing 50% inhibition) of the tested samples.

### 2.5.2 | Evaluating the effect on glucose uptake in L-6 myotubes

To measure the effect of the complex on glucose uptake, the methods previously reported (Oyedemi et al., 2013) were consulted. L-6 myoblasts from rat muscle (ATCC CRL-1458, ATCC, VA, USA) were used for the assay. The cells were cultured in a low glucose containing Dulbecco's Modified Eagle medium (DMEM), which had 10% fetal calf serum. Culturing was done in a $CO_2$ incubator. After culturing, the cells were harvested by trypsinization and seeded with fresh medium into a 96-well plate at a density of 3000 cells/well. At 80% confluence, a differentiation medium (DMEM) containing 2% horse serum was used to replace the old medium and incubation continued for 4–5 days. The myoblasts differentiated into myotubes during the 4–5 days incubation period. The spent medium was replaced with 200 µl of the fresh medium that contained the samples at different concentrations (5, 50, and 100 µm in incubation volume). The sample solvent was used as the vehicle control. Some wells were used as the blank control, which contained only the medium without cells. The culture medium was removed after 48 h of incubation, and the cells were washed with PBS. Then, 100 µl of RPMI medium containing 8 mm glucose and 0.1% of BSA was added to the wells and the plate was incubated for another 2 h. Insulin (1 µM) was used in the positive control group. Aliquots from the incubation medium of each well were then used for glucose concentration measurement (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. Three biological repeats were adopted for the assay. The glucose uptake effect of the test samples and control wells was calculated relative to the blank control as follows:

$$\text{Glucose uptake}(\%) = \frac{\Delta \text{GC of test or control} - \Delta \text{GC of blank control}}{\Delta \text{GC of blank control}} \times 100 \quad (2)$$

"ΔGC" denotes change in glucose concentration (i.e., initial – final glucose concentration in incubation solutions). Also, the $EC_{50}$ of samples, which denotes the concentration (µM) of samples needed to effect 50% glucose uptake increase was computed.

## 2.6 | Measurement of ex vivo antioxidant and antidiabetic properties

### 2.6.1 | Animals

First ethical clearance was obtained from the Animal Research Ethics Committee of the University of the Free State, Bloemfontein, South Africa (protocol approval reference: UFS-AED2019/0152/2020, approved on 16 November 2020). Animal handling was done according to the rules and regulations of the ethics committee. Eight weeks old Sprague Dawley rats were procured from the animal facility of the university. The study was conducted as reported previously (Oke et al., 2021) with slight modifications. The animals were fasted overnight. They were euthanized using isoflurane and the liver and psoas muscle tissues were immediately harvested and used for the ex vivo antioxidant and antidiabetic experiments.

### 2.6.2 | Evaluating the effect of the complex on lipid peroxidation and GSH concentration in isolated rat liver tissue induced with oxidative stress

A method previously reported (Oke et al., 2021) was adopted for this assay with slight modification. In a 48-well plate, approximately equal portions (200 ± 5 mg) of the harvested liver tissues were pre-incubated (NAPCO series 5400 $CO_2$ incubator, Thermo Scientific, South Africa) for 25 min in 900 µl of Krebs 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) that contained the samples or standard (ascorbic acid) at different concentrations (10, 20, 40, and 80 µM in total incubation volume). Incubation conditions were 5% $CO_2$ and 95% oxygen supply and 37°C temperature. The controls (normal control and negative control) contained the tissue, the equivalent volume of the buffer, and the solvent of the test samples. After pre-incubation, 100 µl of $FeSO_4.7H_20$ solution (2 mM in total incubation volume) was used to induce oxidative stress. While the negative control contained the $FeSO_4.7H_2O$ solution, the normal control contained the equivalent volume of distilled water. Additional 90 min incubation was done. After the 90 min incubation, the tissues were taken out from the incubation solution and cleaned with Krebs buffer. The tissues were, then, homogenized in 1 ml of 50 mM sodium phosphate buffer (containing 0.5% v/v Triton X-100; pH, 7.5) and centrifuged at 10400 g for 10 min to recover the supernatants. Lipid peroxidation and GHS concentrations were estimated in the supernatants using similar methods described above. The inhibitory percentage of the samples on lipid peroxidation and GHS depletion was computed, which was used to determine the $IC_{50}$ values of the tested samples.

### 2.6.3 | Evaluating the effect of the complex on glucose uptake isolated rat psoas muscle tissue

A method recently reported (Oke et al., 2021) was adopted for this assay with slight modification. In a 48-well plate, approximately equal portions (300 ± 10 mg) of the harvested muscle tissues were pre-incubated for 25 min in 900 µl of Krebs buffer that contained different concentrations (10, 20, 40, and 80 µM in total incubation volume) the tested samples or 50 mU insulin (NovoRapid® FlexPen®, Novo Nordisk Limited, West Sussex, UK) as the positive

control. The control contained the tissue, the equivalent volume of the buffer, and the solvent of the tested samples. One hundred microliters of glucose solution (11.1 mM in total incubation volume) was added after pre-incubation. Additional 90 min incubation was done. After the 90 min incubation, an aliquot from the incubation medium in each well was used for glucose concentration measurement using the Glucose (GO) Assay Kit (Sigma-Aldrich, South Africa). The glucose uptake increase (%) of the tested samples and positive control was calculated using the absorbance values of the control as a reference according to the formula below:

$$\text{Glucose uptake increase}\,(\%) = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100 \quad (3)$$

Also, the $EC_{50}$, which denotes the concentration (μM) of the samples needed to effect 50% glucose uptake increase was computed.

Immediately after the 90 min incubation, zinc concentration and hexokinase activity were measured in the muscle tissues that were treated with the highest concentration of the tested samples. First, the tissues were washed with Krebs buffer, homogenized in 1 ml of ice-cold 50 mM sodium phosphate buffer (containing 0.5% v/v Triton X-100 and 1 mM EDTA; pH 7.5), and centrifuged at 10400 g for 10 min at 4°C to recover the supernatants. Protein concentration was measured in the supernatants using the bicinchoninic acid (BCA) method. Hexokinase activity was measured in the supernatant at a protein concentration of 15 mg/ml using Hexokinase Colorimetric Assay Kit (catalog number MAK091, Sigma-Aldrich, Johannesburg, South Africa) and expressed in nmole/min/ml, which is equivalent to milliunit/ml. One unit of hexokinase represented the amount of enzyme that will generate 1.0 mmole of NADH per minute at pH 8.0 under room temperature. Also, zinc concentration was measured in the tissue supernatants using a Zinc Assay Kit (catalog number MAK032, Sigma-Aldrich, Johannesburg, South Africa) and expressed in ng/μl.

## 2.7 | Molecular docking study

The molecular docking study was carried out using p-coumaric acid (4-hydroxycinnamic acid) and its Zn(II) complex against three target enzymes that are linked to diabetes. The in silico interaction between compounds and the enzyme targets was compared. The target enzymes include α-glucosidase (GAA), glucose transporter type 4 (GLUT-4), and protein kinase B (PKB). The 3-D structures of each of the enzymes were retrieved from the protein data bank. Due to unavailability of the crystallographic structure of GLUT-4, it was theoretically constructed by template-based homology modeling approach. Following previous methods, the molecular docking study was carried out using the AutoDock Vina 4.2 software (Motloung et al., 2020; Swain et al., 2020). Five docking poses were generated for each ligand against a particular target enzyme and the minimum binding energy (kcal/mol) was selected as the best docking score. The BIOVIA-DSV software was employed for visualizing the protein-ligand molecular interaction.

## 2.8 | Statistical analysis

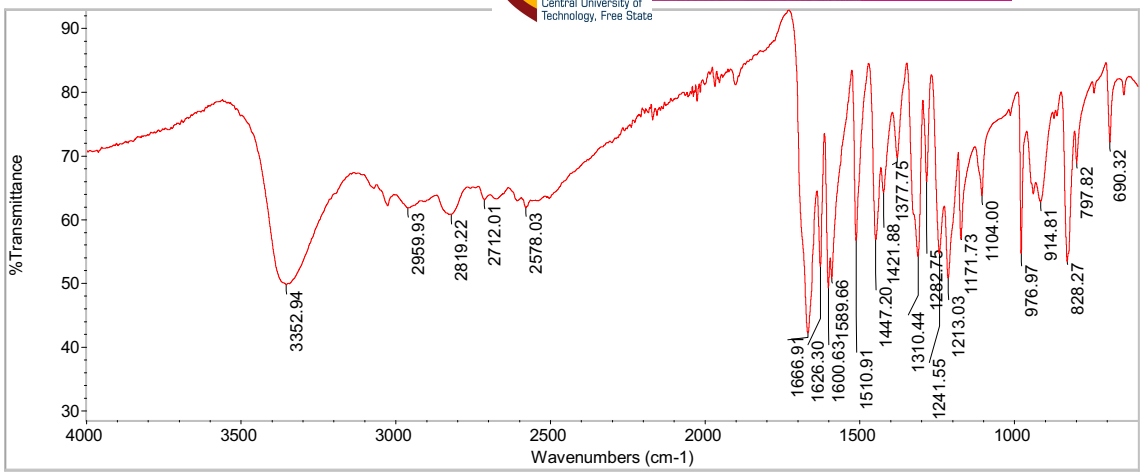
For analyzing the data of this study, the 2016 version of MS Excel and GraphPad Prism 5 were used. $IC_{50}$ and $EC_{50}$ values were computed as a linear and/or non-linear fit of transformed (log10) x-axis (sample concentration) versus activity (y-axis). The analyzed data were reported in triplicates using the average ± standard deviation format. Statistical analysis was done on the Windows version 27.0 of IBM SPSS (IBM Corp, Armonk, NY, USA). Tukey multiple range post hoc test and the one-way analysis of variance (ANOVA) were adopted for multiple comparison of the data averages and statistically significant difference (p) of comparison was set at $p < .05$.

# 3 | RESULTS

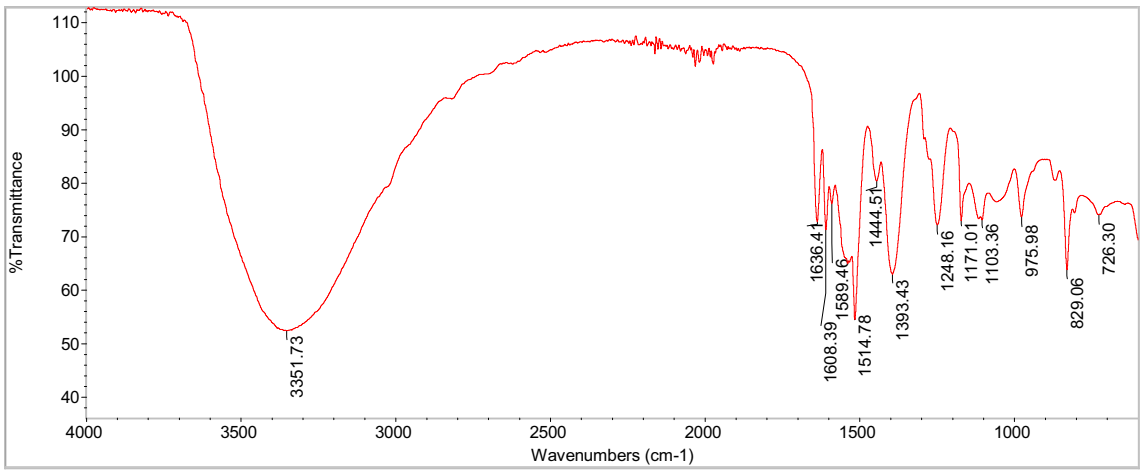
## 3.1 | Spectroscopic characteristics of the complex

Figure 1 presents the FT-IR data. Both the IR spectra of p-coumaric acid (Figure 1a) and the complex (Figure 1b) showed broad picks centered at 3352.94 and 3351.73 $cm^{-1}$, respectively. These signals are caused by the phenolic O-H stretching. This shows the phenolic OH groups were not involved in the complexation, and thus are intact. The sharp signal observed in the IR spectrum of p-coumaric acid at 1666.91 $cm^{-1}$ (Figure 1a) is caused by the carboxylic carbonyl (C=O) stretching. Carboxylic C=O stretching signal was, also, observed in the IR spectrum of the complex at 1636.41 $cm^{-1}$ (Figure 2b). However, in the IR spectrum of the complex, the signal was reduced, and the wavenumber was lesser relative to the signal in the IR spectrum of p-coumaric acid. The reduced signal and wavenumber suggest zinc donated electrons to the carboxylic group of the phenolic acid, which indicates Zn(II) complexation occurred at the carboxylic end of the phenolic acids as shown in Figure 2a.

Proton NMR analysis is presented in Figure 2b. The $^1H$ NMR represent all required protons at the different environments, proving that p-coumaric acid zinc sulfate complex was successfully achieved (Figure 2b). The protons H-2 and H-6; H-3 and H-5 observed at δ 6.76 (d, $J$ = 8.2 Hz, 2H) and δ 7.42 (d, $J$ = 8.1 Hz, 2H), respectively, indicated a doublet. The important signal: the hydroxyl group observed at $\delta$ 9.78 ppm showed a singlet signifying that the OH group is not involved in the complex formation (Figure 2b). The two protons of the double bond (H-α, and H-β) appearing at positions $\delta$ 6.28 (d, $J$ = 15.8 Hz, 1H) and $\delta$ 7.31 (d, $J$ = 15.8 Hz, 1H), respectively, also showed a doublet (Figure 2b).




(a)




(b)

FIGURE 1 Spectra for FT-IR of (a) p-coumaric acid and (b) the complex.

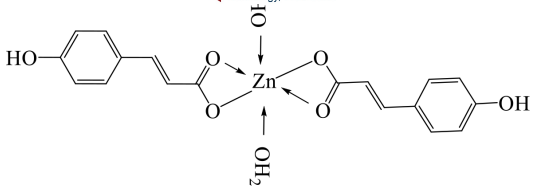
The high-resolution mass spectroscopy of the complex is presented in Figure 3. The negative mode spectrum showed zinc associated with two molecules of p-coumaric acid and two molecules of water, which had a mass of $m/z$ = 224.9784 and differed from the calculated mass by 0.0431 unit. The fragment of coumaric acid had a mass of $m/z$ = 163.0388 and differed from the calculated mass by 0.0007 unit.

The above data show that a double hydrated Zn(II)-bicoumarate complex was formed.

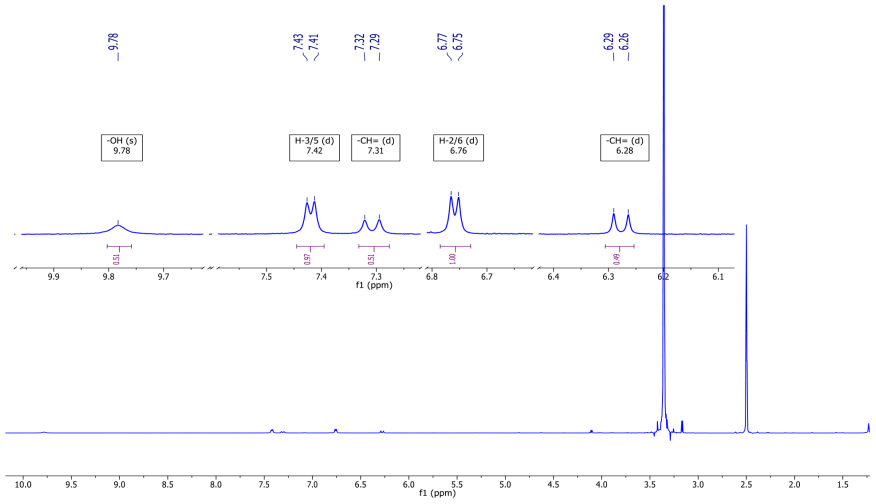
## 3.2 | Oxidative stress-related bioactivities of complex

p-Coumaric acid showed DPPH ($IC_{50}$ = 50.8 μM) and ABTS ($IC_{50}$ = 169 μM) radical scavenging, $Fe^{3+}$ reducing, and anti-linoleic acid peroxidative ($IC_{50}$ = 75.8 μM) activities in vitro, while zinc sulfate did no show in vitro radical scavenging, $Fe^{3+}$ reducing and anti-lipid peroxidative activities (Figure 4a–d and Table 1). However, complexation of the aforementioned resulted in a complex with significantly ($p < .05$) stronger DPPH ($IC_{50}$ = 24.6 μM) and ABTS ($IC_{50}$ = 115 μM) radical scavenging, $Fe^{3+}$ reducing and anti-linoleic acid peroxidative ($IC_{50}$ = 51.2 μM) activities (Table 1). The in vitro anti-lipid peroxidative effect of the complex was statistically comparable ($p > .05$) to that of Trolox ($IC_{50}$ = 37.8 μM) and ascorbic acid ($IC_{50}$ = 13.9 μM) (Table 1).

At cellular (Chang liver cells) and tissue (isolated rat liver tissue) hepatic levels, the complex, also, exhibited significantly ($p < .05$) stronger antioxidant actions compared to its precursors (zinc sulfate and p-coumaric acid) (Figure 5a,b and Table 1). The cellular and tissue inhibitory action of the complex on oxidative stress-induced lipid peroxidation ($IC_{50}$ = 56.2 and 398 μM) and GSH depletion ($IC_{50}$ = 33.9 and 38.7 μM) was more potent than that of p-coumaric acid and did not differ significantly ($p > .05$) from that of ascorbic and Trolox (Table 1).




(a)




| Position | $^1$H NMR |
|---|---|
| 1 | - |
| 2 | 6.76 (d, $J$ = 8.2 Hz, 1H) |
| 3 | 7.42 (d, $J$ = 8.1 Hz, 1H) |
| 4 | - |
| 5 | 7.42 (d, $J$ = 8.1 Hz, 1H) |
| 6 | 6.76 (d, $J$ = 8.2 Hz, 1H) |
| α | 7.31 (d, $J$ = 15.8 Hz, 1H) |
| β | 6.28 (d, $J$ = 15.8 Hz, 1H) |
| -OH | 9.78 (s, 1H) |

(b)

FIGURE 2 Diagrams showing the (a) proposed structure and (b) $^1$H NMR spectrum of the Zn(II) complex of p-coumaric acid.

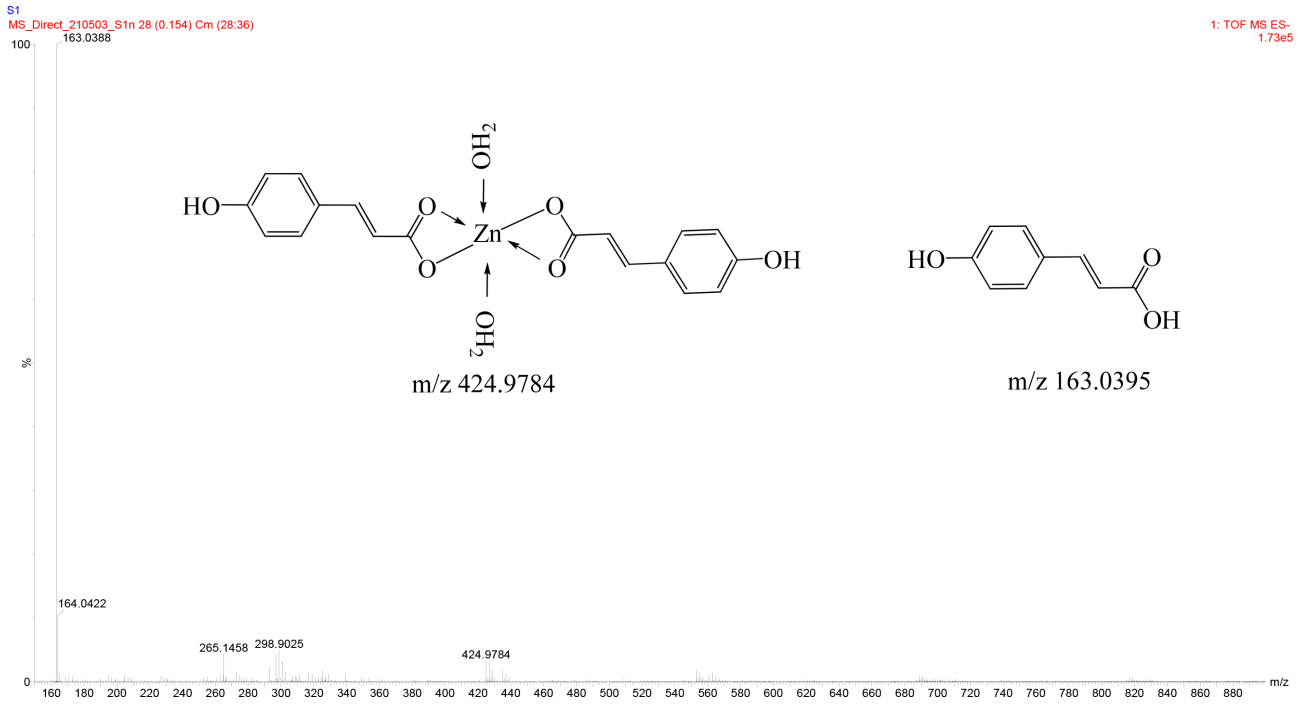



FIGURE 3 High-resolution mass spectroscopy data of the complex.

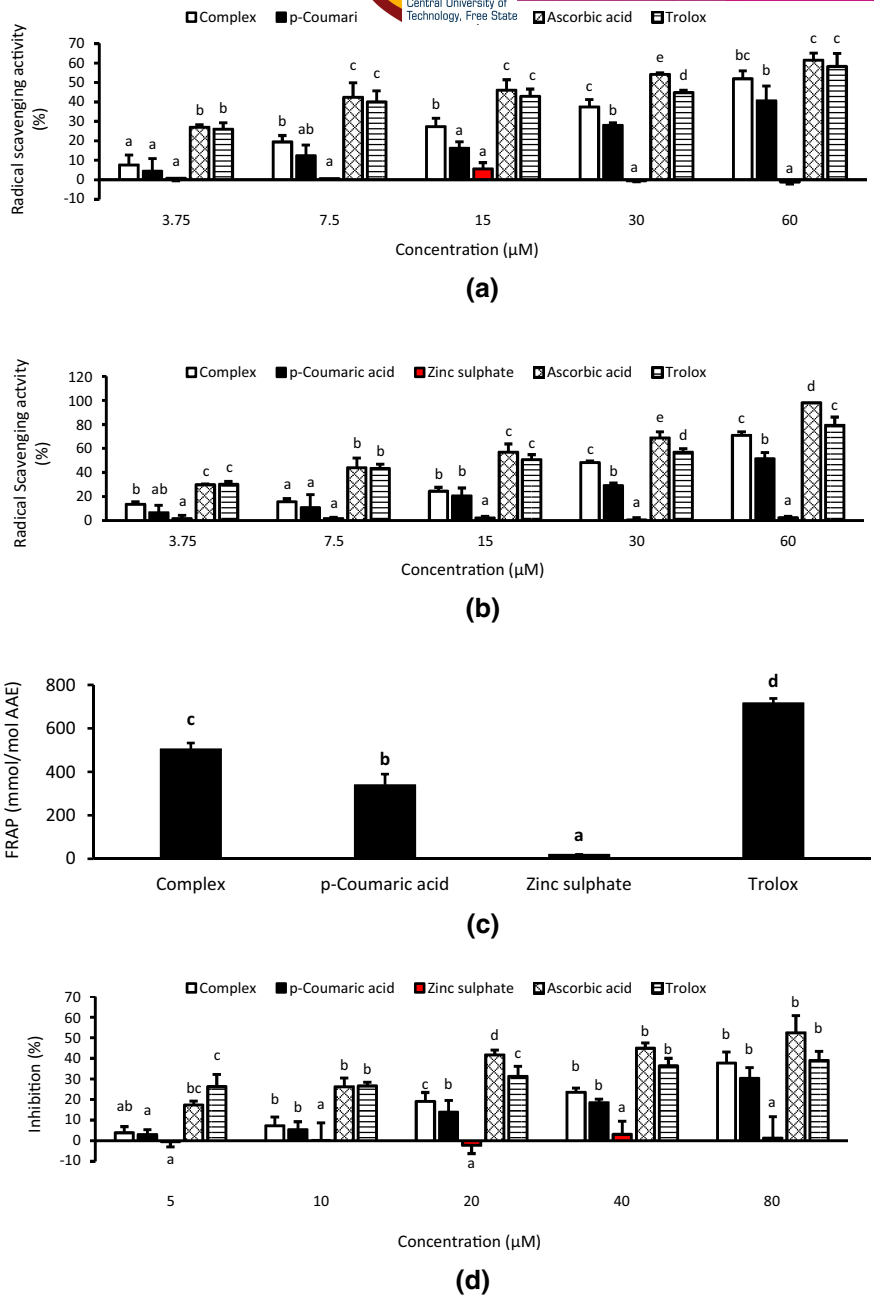



FIGURE 4 (a) DPPH radical scavenging (b) ABTS$^{\bullet+}$ radical scavenging (c) $Fe^{3+}$ reducing and (d) in vitro linoleic acid lipid peroxidation inhibitory activities of complex, precursors, and standards. Data are shown as mean ± SD of triplicate analysis. Different letters "a", "b", "c", and "d" represent significant difference ($p < .05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

### 3.3 | Diabetes-related bioactivities of complex

p-Coumaric acid had a dose-dependent inhibitory effect ($IC_{50}$ = 46.3 μM) on α-glucosidase enzyme in vitro (Figure 6a and Table 1). Relative to p-coumaric acid ($p < .05$), zinc sulfate showed a mild α-glucosidase inhibitory activity ($IC_{50}$ = 207 μM) (Table 1). The complex had a more potent inhibitory activity ($IC_{50}$ = 19.6 μM) compared to its precursors and was statistically comparable ($p > .05$) to acarbose ($IC_{50}$ = 14.0 μM) (Table 1).

Zinc sulfate dose-dependently inhibited ($IC_{50}$ = 34.6 μM) glucose-mediated glycation of BSA protein in vitro, while p-coumaric acid showed no observable antiglycation effect (Figure 6b and Table 1). Although not as potent as the antiglycation effect of aminoguanidine ($IC_{50}$ = 8.70 μM), zinc sulfate conferred a complexation-mediated antiglycation property on p-coumaric acid ($IC_{50}$ = 39.9 μM) (Figure 6b and Table 1).

In L-6 myotubes and isolated rat muscle tissues, p-coumaric acid, zinc sulfate, and the complex showed a dose-dependent

**TABLE 1** $IC_{50}$ and $EC_{50}$ values of complex, precursors, and standards for the different experimental parameters

| Experimental parameters | Complex | p-coumaric acid | Zn(II) | Ascorbic acid | Trolox | Acarbose | Aminoguanidine | Insulin |
|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ or $EC_{50}$ values (μM) | | | | | | | |
| ABTS radical scavenging activity ($IC_{50}$) | $115 \pm 5.52^{b}$ | $169 \pm 15.2^{a}$ | ND | $53.0 \pm 10.1^{c}$ | $62.1 \pm 16.7^{c}$ | NA | NA | NA |
| DPPH radical scavenging activity ($IC_{50}$) | $24.6 \pm 7.16^{b}$ | $50.8 \pm 3.93^{a}$ | ND | $14.3 \pm 1.46^{bc}$ | $12.7 \pm 4.62^{c}$ | NA | NA | NA |
| Inhibition of in vitro linoleic acid peroxidation | $51.2 \pm 8.50^{bc}$ | $75.8 \pm 16.8^{a}$ | ND | $13.9 \pm 4.38^{c}$ | $37.8 \pm 11.1^{ab}$ | NA | NA | NA |
| Antiglycation activity ($IC_{50}$) | $39.9 \pm 11.0^{a}$ | ND | $34.6 \pm 5.84^{a}$ | NA | NA | NA | $8.70 \pm 2.04^{b}$ | NA |
| α-glucosidase inhibition ($IC_{50}$) | $19.6 \pm 8.66^{b}$ | $46.3 \pm 2.99^{b}$ | $207 \pm 40.8^{a}$ | NA | NA | $14.0 \pm 4.71^{b}$ | NA | NA |
| Glucose uptake increase in L-6 myotubes ($EC_{50}$) | 10.7 | 18.6 | 34.7 | NA | NA | NA | NA | ND |
| Glucose uptakes increase in isolated rat psoas muscle ($EC_{50}$) | $428 \pm 45.9^{b}$ | $1180 \pm 164^{a}$ | $450 \pm 87.0^{b}$ | NA | NA | NA | NA | ND |
| Inhibition of oxidative stress-induced lipid peroxidation in Chang liver cells ($IC_{50}$) | $56.2 \pm 8.35^{c}$ | $131 \pm 26.6^{b}$ | $743 \pm 51.3^{a}$ | $30.3 \pm 8.52^{c}$ | NA | NA | NA | NA |
| Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver ($IC_{50}$) | $398 \pm 189^{b}$ | $2160 \pm 129^{a}$ | ND | $189 \pm 71.5^{b}$ | NA | NA | NA | NA |
| Inhibition of oxidative stress-induced GSH depletion in Chang liver cells ($IC_{50}$) | $33.9 \pm 13.1^{b}$ | $85.4 \pm 31.9.0^{b}$ | $709 \pm 265^{a}$ | $27.3 \pm 5.51^{b}$ | NA | NA | NA | NA |
| Inhibition of oxidative stress-induced GSH depletion in isolated rat liver ($IC_{50}$) | $38.7 \pm 7.15^{c}$ | $147 \pm 18.0^{b}$ | $2150 \pm 437^{a}$ | $27.0 \pm 2.48^{c}$ | NA | NA | NA | NA |

*Notes*: "ND" means "not determined; "NA" means "not applicable"; "GSH" means "reduced glutathione"; $IC_{50}$ is concentration needed to inhibit the activity of carbohydrate digesting enzymes, bovine serum albumin glycation, lipid peroxidation, and reduced glutathione depletion 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", and "c" represent significant difference ($p < .05$) between treatment groups.

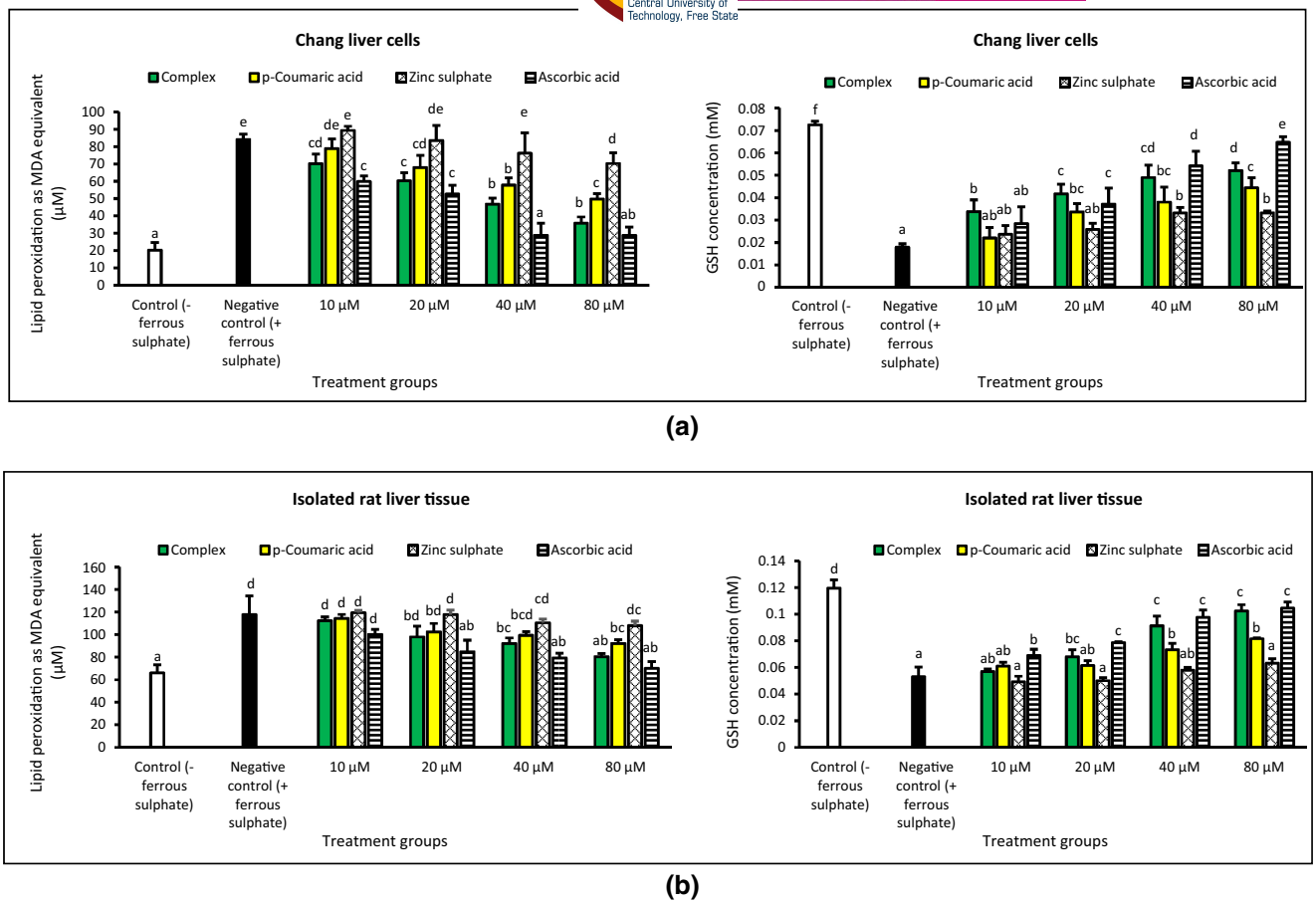



FIGURE 5 Graphs showing how the complex, precursors, and standards inhibit lipid peroxidation and GSH depletion in (a) Chang liver cells and (b) isolated rat liver tissue. Data are shown as mean ± SD of replicate analysis. Different letters "a", "b", "c", "d", and "e" represent significant difference ($p < .05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

modulatory effect on glucose uptake (Figure 7a,b and Table 1). The complex had the most potent effect at cellular ($EC_{50}$ = 10.7 μM) and tissue levels ($EC_{50}$ = 428 μM) based on $EC_{50}$ values. Its glucose uptake effect was 2.8-folds more potent ($p < .05$) than that p-coumaric acid ($EC_{50}$ = 1180 μM) in the rat muscle tissue (Table 1).

Elevated hexokinase activity was, also, observed in treated isolated rat psoas muscle tissues showing modulated glucose uptake (Figure 7c). Muscle tissue zinc concentration was significantly higher ($p < .05$) in the complex and zinc sulfate-treated muscle tissues than the tissues treated with p-coumaric acid and insulin (Figure 7d).

The visualized docking interaction between p-coumaric acid or its Zn(II) complex and the protein targets (α-glucosidase, GLUT-4, and PKB) is shown in Figure 8, while Table 2 depicts the minimum docking scores (kcal/mol). The docking scores of the complex (−7.18 to −7.74 kcal/mol) against the enzyme targets were higher than those of p-coumaric acid (−6.31 to −6.41 kcal/mol) (Table 2).

At in vitro cellular level, the complex was not myotoxic or hepatotoxic at tested concentrations ranging from 5 to 817 μM (Figure 7e).

## 4 | DISCUSSION

In the past two decades, considerable attention has been drawn to zinc mineral as a possible therapeutic supplement for diabetes due to its role in insulin secretion and reported insulin mimetic potentials (Chukwuma et al., 2020). Although many researchers have explored zinc as a precursor for developing metal–ligand complexes with possible diabetes-related bioactivity, they have concentrated on synthetic ligands with toxicity concerns and no documented pharmacological credence (Chukwuma et al., 2020). In this study, we considered a natural phenolic acid (p-coumaric acid) as an alternative ligand to develop a Zn(II)-p-coumaric acid complex with improved diabetes and oxidative stress-related bioactivity. The potent bioactivity of the complex relative to its precursor suggests a possible complexation-mediated synergism between zinc sulfate and p-coumaric, which is partly attributed to Zn(II)-bicoumarate structure of the complex (Figure 2a).

p-Coumaric acid is a natural antioxidant with radical scavenging activity (Kadoma & Fujisawa, 2008; Kiliç & Yeşiloğlu, 2013), which could be attributed to its ability to donate electron or transfer hydrogen, thus forms a stable phenoxy radical intermediate

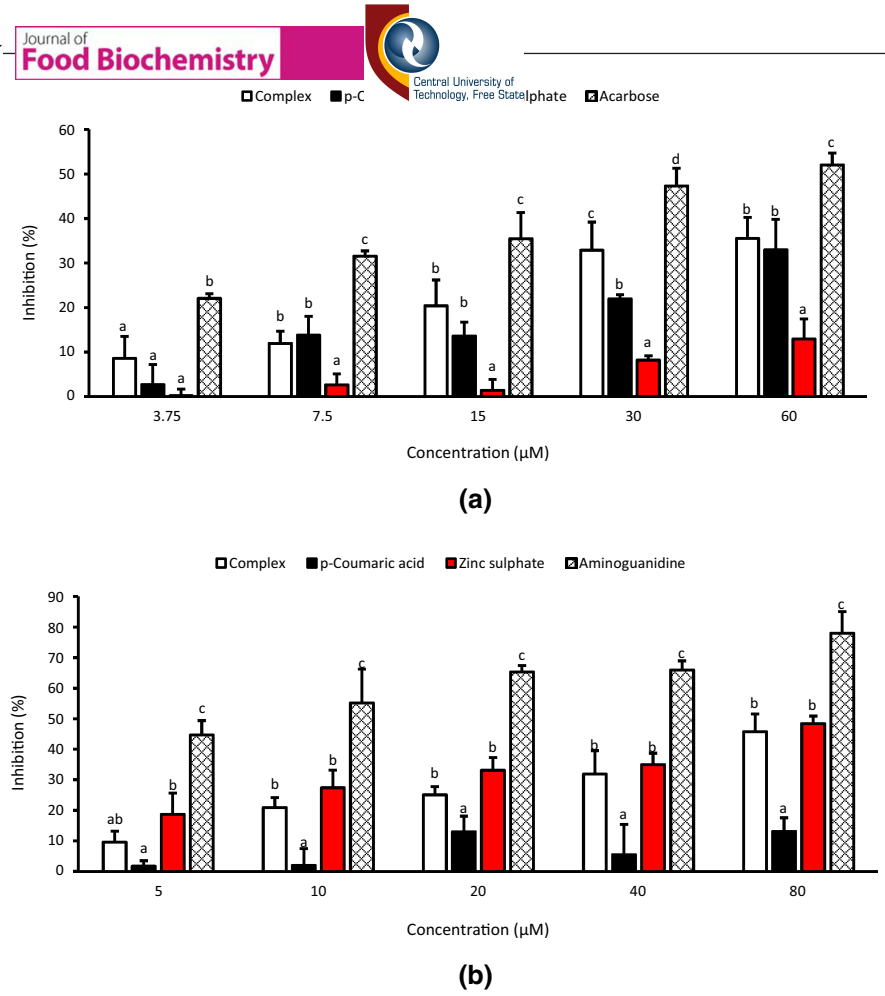



FIGURE 6 In vitro (a) α-glucosidase and (b) BSA glycation inhibitory effects of complex, precursors, and standards. Data are shown as mean ± SD of triplicate analysis. Different letters "a", "b", "c", and "d" represent significant difference ($p < .05$) between treatment groups.

(Chen et al., 2020). Also, its anti-lipid peroxidative effect has been shown in high-fat diet mice model (Shen et al., 2019), which may be linked to its quenching capacity of radicals associated with lipid peroxidation process. In the present study, consistent antioxidant effects in the form of in vitro radical scavenging and anti-linoleic acid peroxidative activity were shown by p-coumaric acid (Figure 4a,b and d; Table 1). Also, in Chang liver cells and isolated rat liver tissues, p-coumaric acid demonstrated antioxidant effects by inhibiting oxidative stress-induced lipid peroxidation and GSH depletion (Figure 5a,b; Table 1). However, upon complexation with zinc sulfate, the resulting complex acquired two moieties of p-coumaric acid, which afforded it the ability to potentiate stronger antioxidant activities relative to its precursors. Based on computations using $IC_{50}$ values (Table 1), the complex was 1.5- to 5.4-folds more potent than p-coumaric acid and was statistically comparable ($p > .05$) to Trolox and ascorbic acid. The data suggest that Zn(II) complexation may be a therapeutic approach for improving the antioxidant capacity of p-coumaric acid, which may be relevant in mitigating oxidative stress and its insults on different tissues.

Elevated oxidative stress in diabetic condition has been shown to be fueled by persistent hyperglycemia and processes like glycation and increased mitochondrial superoxide ion production are known to be key culprits (Fakhruddin et al., 2017; Giacco & Brownlee, 2010). Elevated glycation is a major risk factor of diabetic complications. Improving glycemic control is important in limiting ROS prooxidant production processes and mitigating diabetic oxidative damage. Postprandial blood glucose has been shown to be controlled by inhibiting α-glucosidase, a key carbohydrate hydrolyzing enzyme in the brush borders of the small intestine (Lebovitz, 1997). In this study, p-coumaric acid dose dependently inhibited α-glucosidase in vitro (Figure 6a and Table 1), which has been previously (Jeong et al., 2012) and recently (Aleixandre et al., 2022) documented. Zinc sulfate showed a relatively lower in vitro α-glucosidase inhibition (Table). However, it acquired two moieties of p-coumaric acid through complexation (Figure 2a) to potentiate a stronger (2.4-folds) α-glucosidase inhibition compared to p-coumaric acid alone (Table 1). Further, in silico docking analysis also suggests a structure–function relationship (Figure 8 and Table 2). More hydrogen bonding between complex and enzyme was observed due to the presence of two moieties of p-coumaric acid (Figure 8). This influenced its higher docking scores against α-glucosidase protein target compared to p-coumaric acid (Table 2).

On the other hand, it appears zinc sulfate conferred an in vitro antiglycation property on p-coumaric acid through complexation (Figure 6b and Table 1). Moreover, zinc sulfate has been shown to inhibit glucose-induced protein glycation in vitro (Tupe et al., 2015).

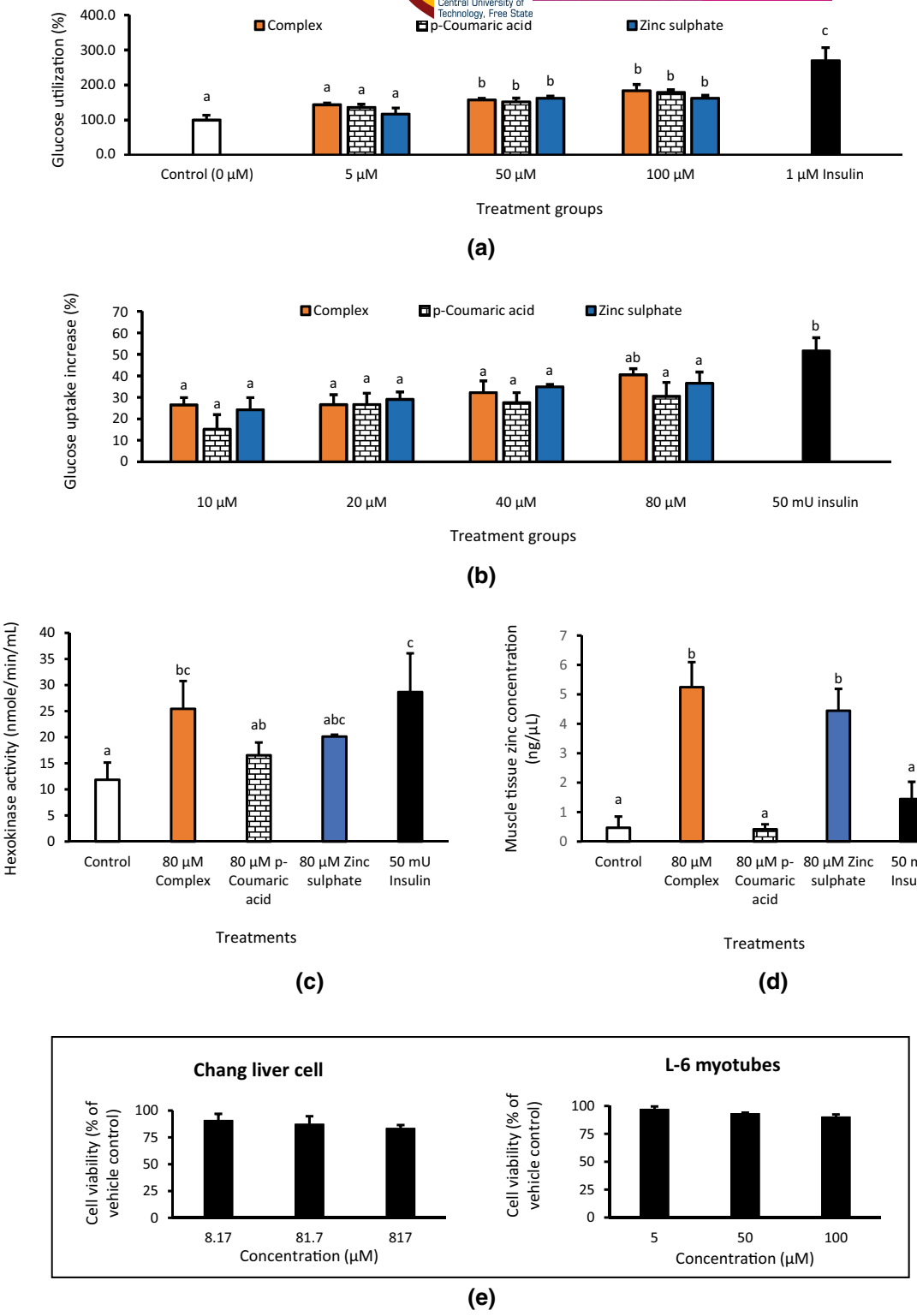



FIGURE 7 Effect of complex, precursors, and standards on (a) glucose uptake in L6-myotubes, (b) glucose uptake, (c) hexokinase activity, and (d) zinc concentration in isolated rat psoas muscle, and (e) viability of Chang liver cell and L6-myotubes. Data are shown as mean ± SD of replicate analysis. Different letters "a", "b", and "c" represent significant difference ($p < .05$) between treatment groups at the different tested concentrations or between treatment groups and controls.

Additionally, in diabetic rats, zinc sulfate treatment was shown to lower glycation and protein carbonyl interaction (Sacan et al., 2016). The above data suggest that Zn(II) complexation may be a therapeutic approach for improving the postprandial glycemic control of p-coumaric acid, while affording other benefits that could mitigate diabetic complications. In vivo studies on the complex using

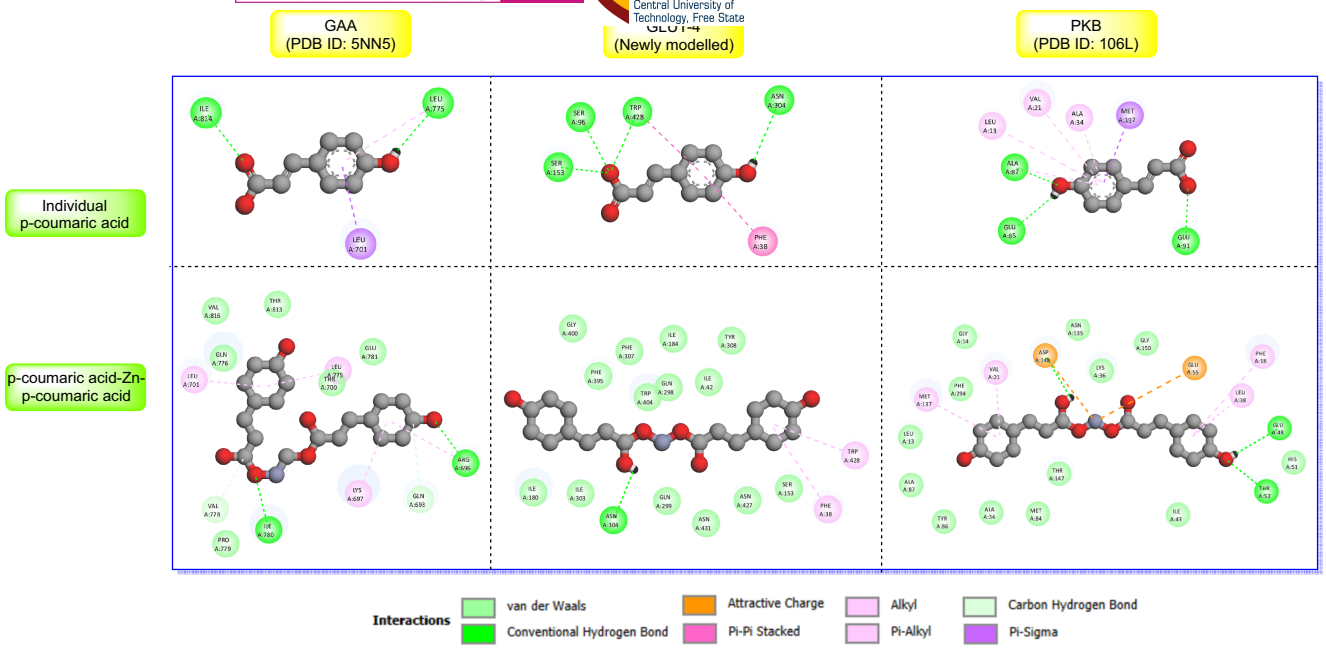



FIGURE 8 Molecular docking interaction of ferulic acid and its complex with four enzyme targets (α-glucosidase, GLUT-4, and PKB) linked to diabetes. GAA, α-glucosidase; GLUT4, glucose transporter type 4; PKB, protein kinase B.

TABLE 2 Molecular docking score (kcal/Mol) of p-coumaric acid and its Zn(II) complex with target enzymes linked to diabetes

| Target enzymes | p-coumaric acid | Complex |
|---|---|---|
| GAA (PDB ID: 5NN5) | −6.31 | −7.18 |
| GLUT-4 (Newly modeled) | −6.35 | −7.58 |
| PKB (PDB ID: 106 L) | −6.41 | −7.74 |

Abbreviations: GAA, α-glucosidase; GLUT4, glucose transporter type 4; PKB, protein kinase B.

appropriate animal models are, however, recommended moving forward.

Impaired insulin signaling is a major factor that drives persistent hyperglycemia, particularly in T2D. There is impaired circulating glucose uptake and utilization, particularly in peripheral tissues, such as muscle and adipose tissues, which leads to poor glycemic control and hyperglycemia (Galicia-Garcia et al., 2020). The insulin-mimetic potential of Zn(II) and its modulatory effect on insulin signaling have been documented. Akt phosphorylation was induced in mouse and human skeletal cells after Zn(II) treatment, which modulated insulin signaling (Norouzi et al., 2018). In 3 T3-L1 pre-adipocytes and adipocytes, Zn(II) treatment potentiated increased glucose uptake through insulin receptor and Akt activation (Tang & Shay, 2001). In the present study, complexing p-coumaric and Zn(II) lead to a modulatory effect on glucose uptake in L-6 myotubes and muscle tissues isolated from rats (Figure 7a,b; Table 1). The complex outperformed its precursors (Table 1). In the complex and zinc-treated tissues, glucose uptake activity was accompanied by an increase in the concentration of zinc relative to the non-treated tissues (Figure 7d). Also, zinc concentration was higher in the complex-treated muscle tissues compared to the zinc sulfate-treated muscle tissues (Figure 7d), suggesting that the complex increased tissue or cellular Zn(II) uptake. This effect may be relevant in increasing in glucose uptake. Documented evidence has shown that non-pharmacologically active 1-oxy-2-pyridinethiol ligands potentiated insulin-mimetic effects in adipocytes by enhancing cellular zinc uptake upon complexation (Basuki et al., 2007; Yoshikawa et al., 2011).

The present study, however, suggests a synergistic interaction between the Zn(II) and p-coumaric acid components of the complex in potentiating a stronger glucose uptake. p-Coumaric acid has been shown to modulate glucose and lipid metabolism in L-6 skeletal muscle cells through an AMP-activated protein kinase-dependent mechanism (Yoon et al., 2013). In the present study, p-coumaric acid was shown to moderately increase cellular and tissue glucose uptake (Figure 7a,b; Table 1) Hence, it is hypothesized that while complexing p-coumaric acid with zinc(II) enhanced cellular bioavailability of Zn(II), it concomitantly contributed to the glucose uptake effect potentiated by zinc(II). Hexokinase activity followed the same trend as tissue glucose uptake activity (Figure 7c), suggesting that the observed glucose uptake activity may be linked to increase in cellular glucose utilization.

Molecular docking data (Figure 8 and Table 2) suggest that the glucose uptake effect of the complex may be associated with some insulin signaling enzyme targets (GLUT-4 and PKB). These enzymes play key roles in the signaling of glucose uptake by insulin (Chang et al., 2004). Data showed that the docking score or binding energy against these enzyme targets was higher for the complex compared to p-coumaric acid (Table 2), which suggests more interaction with the enzymes.

Additional toxicity data revealed the complex was not toxic to hepatocytes and myocytes, at least under in vitro conditions

(Figure 7e). The study suggests that Zn(II)-p-coumaric acid complexation may be a therapeutic approach for synergistic glycemic control with minimal toxicity, thus warrants future in vivo studies in type 2 diabetic animal model.

## 5 | CONCLUSION

Synergistic therapy remains an important therapeutic approach for several diseases, including diabetes and associated oxidative insults. Also, natural medicines including supplements and phytochemicals have gained wide reception as supplemental therapies in functional medicine. Through complexation, we took advantage of the antioxidative and/or glycemic control potentials of zinc mineral and p-coumaric acid to form a complex with an improved and multifaceted antioxidative and/or glycemic control potential, as well as minimal cellular toxicity. The complex had a Zn(II)-bicoumarate molecular attribute that afforded a structure–function relationship. Zn(II) complexation may be a therapeutic approach for improving the antioxidative and glycemic control potentials of p-coumaric acid, which may be medicinally relevant in combating diabetes and associated oxidative complications. It is important to note that the present study was limited to in vitro, cellular, and ex vivo experimental models. In vivo antioxidant, antidiabetic, and toxicity studies using appropriate animal models are recommended in further future studies.

### AUTHOR CONTRIBUTIONS

**Limpho M. Ramorobi**: Investigation; Writing - original draft; Methodology; Software; Formal analysis. **Godfrey R. Matowane**: Investigation; Funding acquisition; Writing - review & editing; Methodology; Software; Formal analysis. **Samson S. Mashele**: Investigation; Funding acquisition; Writing - review & editing; Formal analysis; Supervision. **Susanna L. Bonnet**: Methodology; Writing - review & editing; Software; Formal analysis. **Anwar E.M. Noreljaleel**: Methodology; Writing - review & editing; Software; Formal analysis. **Shasank S. Swain**: Software; Formal analysis; Writing - review & editing; Methodology. **Tshepiso J. Makhafola**: Methodology; Writing - review & editing. **Chika I. Chukwuma**: Conceptualization; Funding acquisition; Writing - review & editing; Supervision; Software; Formal analysis.

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### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest or competing interest with this work.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### ETHICS APPROVAL

Ethical clearance was obtained from the Animal Research Ethics Committee of the University of the Free State, Bloemfontein, South Africa (protocol approval reference: UFS-AED2019/0152/2020, approved on 16 November 2020).

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# Zinc(II) – Syringic acid complexation synergistically exerts antioxidant action and modulates glucose uptake and utilization in L-6 myotubes and rat muscle tissue

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ABSTRACT

Zinc and syringic acid have metabolic and antioxidant medicinal potentials. A novel zinc(II)–syringic acid complex with improved anti-hyperglycaemic and antioxidant potential was developed. Zinc(II) was complexed with syringic acid in a 1:2 molar ratio and characterized using FT-IR, $^{1}$H NMR and LC-MS. Different experimental models were used to compare the anti-hyperglycaemic and antioxidant properties between the complex and precursors. A Zn(II)-bisyringate.2$H_2O$ complex was formed. The in vitro radical scavenging and $Fe^{3+}$ reducing antioxidant, antiglycation, and α-glucosidase inhibitory activities of the complex were 1.8–5.2 folds stronger than those of the syringic acid precursor and comparable to those of the positive controls. The complex possessed an increased ability to inhibit lipid peroxidation (by 1.6–1.7 folds) and glutathione depletion (2.8–3 folds) relative to syringic acid in Chang liver cells and liver tissues isolated from rats. The complex exhibited a higher glucose uptake effect ($EC_{50}$ = 20.4 and 386 μM) than its precursors ($EC_{50}$ = 71.1 and 6460 μM) in L6-myotubes and psoas muscle tissues isolated from rats, respectively, which may be linked to the observed increased cellular zinc uptake potentiated by complexation. Tissue glucose uptake activity was accompanied by increased hexokinase activity, suggesting increased glucose utilization. Moreover, treatment increased tissue phospho-Akt/pan-Akt ratio. The complex had strong molecular docking scores than syringic acid with target proteins linked to diabetes. The presence of two syringic acid moieties and Zn(II) in the complex influenced its potency. The complex was not hepatotoxic and myotoxic in vitro. Zinc-syringic acid complexation may be a novel promising therapeutic approach for diabetes and oxidative complications.

## 1. Introduction

With the recent global diabetes population standing at 537 million, the disease is increasingly affecting people of different age groups [1]. Diabetes presents severe hyperglycemic conditions due to poor nutrient metabolism, of which poor insulin secretion and action are the most influential culprits [2,3]. Type 2 diabetes (T2D) is the most prevalent diabetes, accounting for about 95 % of incidences, which is largely fueled by its association with poor nutrition, weight gain, obesity, and lack of exercise [4,5]. Typically, these factors promote insulin resistance, poor functioning of pancreatic β-cells, and associated metabolic disorders, which leads to poor glucose and lipid metabolism, impaired circulating glucose clearance, impaired glucose tolerance, hyperinsulinemia and, eventually persistent hyperglycemia [3–5].

Oxidative stress plays a critical part in the progression of diabetes and the development of associated vascular complications [6]. Persistent hyperglycemia increases glycation and accumulation of advanced glycation end products (AGEs), processes that increase the production of prooxidants and free radicals [6]. Prooxidants and free radicals are major catalysts of oxidative biomolecular inflammation and damage,

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which are implicated in diabetic vascular complications [6].

While there are several synthetic diabetic drugs used for lowering blood glucose and mitigating diabetic complications, advances in research have shown that natural products such as phenolics can be safe alternatives for managing diabetes and oxidative complications [7]. Polyphenols, including natural phenolic acids, possess innate radical quenching and antioxidant properties. The antioxidant properties of polyphenols are partly influenced by their ability to form stable phenoxy radical intermediates by donating an electron or transferring a hydrogen atom [8,9]. Additionally, phenolic acids have been shown to potentiate glucose lowering effects via various mechanisms [10]. Syringic acid is a phenolic acid that is naturally found in *Schumannianthus dichotomus* (Roxb.) Gagnep [11], as well as some fruits, such as grapes and berries [12]. It is an effective scavenger of free radicals [13] and has been shown to ameliorate hepatic and renal oxidative stress [13,14]. In streptozotocin-induced diabetic rats, syringic acid ameliorated oxidative stress, reduced blood glucose, and improved diabetes-induced alterations of enzyme markers that are linked to hepatic tissue damage [14]. Syringic acid also improved the renal and neuronal antioxidant status of diabetic rats by suppressing lipid peroxidation and/or increasing glutathione (GSH) concentration [13,15].

Furthermore, the role of nutrition in diabetes management has been documented [16]. Minerals and vitamins have shown promising outcomes in the prevention and management of T2D [17]. Zinc mineral is known for its role in insulin secretion and has been reported to possess insulin mimetic potential [18]. Numerous zinc-ligand complexes with insulin mimetic potential have been synthesized using different types of ligands and Zn(II). Adachi et al. [19] reported the antilipolytic and glucose uptake effects of Zn(II)-maltol complex in adipocytes isolated from rat. In T2D KK-$A^y$ mice, it reduced the levels of blood glucose and glycated hemoglobin (HbA1c) by approximately 52 % and 19 %, respectively, and improved glucose tolerance after 14-days i.p. treatment (4.5 mg Zn/kg bw) [19]. Zn(II) complex of hinokitiol, a naturally occurring ligand, has been reported to increase glucose uptake and inhibit lipolysis in adipocytes [20]. In islet cells, it increased glucose-induced Akt phosphorylation, while in T2D KK-$A^y$ mice it showed anti-hyperglycaemic action [21].

The concept of zinc-ligand complex as potential antidiabetic and insulin mimetic agents has been extensively studied [18]. However, majority (≈ 72 %) of the studied zinc complexes are those with synthetic ligands that have no/limited known innate medicinal biological effects [18]. Many of these ligands pose toxicity concerns. Hence, a ligand that has known medicinal properties and minimal toxicity concern was explored in this study, which will better complement the glycaemic potential of zinc without posing toxicity concerns. In this context, syringic acid, a natural phenolic acid with antioxidant properties and several documented diabetes-related bioactivities was selected or considered. Also, syringic acid, as well as several phenolic acids have not been studied as promising pharmacological ligands in synthesizing zinc complexes that have potential medicinal relevance for diabetes and oxidative stress and do not present toxicity issues. The observed ongoing research trend negates the trajectory towards natural medicine. Therefore, this study was undertaken to investigate the glycaemic control, antioxidant and toxicity profiles of a novel zinc(II)-syringic acid complex.

## 2. Experimental

### 2.1. Synthesis of the complex

The complex was formed from zinc(II) mineral and syringic by using a mole ratio of 1:2, respectively. A previous method [22] with some modifications was used for the synthesis. Initially, 287.56 mg of $ZnSO_4.7H_2O$ (molar mass = 287.56 g/mol) and 396.34 mg of syringic acid (molar mass = 198.17 g/mol) were respectively dissolved in distilled water and methanol. The volume of the solvents was 5 mL. Next, 5 mL of a 0.4 M $NaHCO_3$ aqueous solution (molar mass = 84.01 g/mol) was mixed with the syringic acid solution. The mixture was stirred until no effervescence was observed. While stirring, the $ZnSO_4.7H_2O$ solution was gradually added. The mixture was stirred continuously until the complete precipitation of a white substance. A filter paper was used to recover the precipitate. A 50 % methanol solution was used to wash the precipitate three times. The precipitate was freeze-dried (Alpha 1-2 LDplus Freeze Dryer, Martin Christ, Osterode am Harz, Germany).

### 2.2. Spectrophotometric characterization

For spectrophotometric characterization of the complex, the following techniques, including Fourier-transform infrared spectroscopy (FT-IR), proton nuclear magnetic resonance spectroscopy ($^1$H NMR) and high-resolution mass spectroscopy (HR-MS) were employed.

For FT-IR analysis, the instrument used was a Perkin Elmer Spectrum 100 FT-IR Spectrometer (MA, USA). An ART accessory was attached to the instrument. Onto to the sample holder, ≈ 2 mg of the complex or syringic acid was loaded. The sample was scanned from 4000 $cm^{-1}$ to 380 $cm^{-1}$. A scan rate of 40 $s^{-1}$ was applied. Data acquisition was done using a Spectrum Software V 6.3.4, which recorded the FT-IR spectra.

For $^1$H NMR, experiment was done using a 400 MHz Bruker Avance spectrometer (Bruker Corporation, MA, USA) and an MNOVA software was used for the data acquisition, visualization, and analysis. The sample was dissolved in deuterated DMSO (DMSO-d6; δH = 2.50) and a tetramethylsilane internal standard was used in the experiment. The chemical shifts were presented in parts per million after recording. Recording was done in the delta ($\delta$) scale. The coupling constants ($J$) were all set correctly to 0.01 Hz.

The Central Analytical Facility, which is located at Stellenbosch university, Cape town performed the high-resolution mass spectroscopic (HR-MS) analysis. A Waters Synapt G2 (Waters Corporation, MA, USA), coupled to an ESI probe and ESI Pos was the instrument used for analysis. The instrument was supplied with a cone voltage of 15 V.

To perform the HR-MS, 1 mg of sample was dissolved in 1 mL of methanol (Romil), followed by a further 10-fold dilution into methanol. Then, 2 μL of the sample was injected into a stream of methanol flowing at 0.3 mL/min, using a Waters ultra-pressure liquid chromatography (UPLC) system (Waters, Mitford, USA), which conveyed the sample to a Waters Synapt G2 quadrupole time-of-flight (QTOF) mass spectrometer used for high-resolution accurate mass analysis. Data was acquired in scan mode. The mass spectrometer was optimized for best sensitivity. The cone voltage was 15 V. The desolvation gas was nitrogen at 650 L/h and desolvation temperature 275 °C. The instrument was operated with an electrospray ionization probe in the positive mode. Sodium formate was used for calibration and leucine encephalin was infused in the background as lock mass for accurate mass determination.

### 2.3. Measurement of possible cytotoxic effect of the complex on Chang liver cells

American Type Culture Collection (ATCC), Virginia, USA (Chang live cells; ATCC® CCL-13™) supplied the Chang liver cells and cell viability assay was performed according to the MTT cell viability assay. First, cells were grown in EMEM media. Ten percent FBS was contained in the culturing media. A NÜVE EC 160 $CO_2$ incubator (NÜVE, Ankara, Turkey) was used to culture the cells. The running conditions of the incubator was 95 % oxygen, 5 % $CO_2$ and 37 °C temperature. When the cell confluence reached 80 %, the cells were plated into a 96-well plate with 200 μL aliquot of the culturing medium. The cell density in each well of the plate was 15,000 cells/mL. The plate was incubated for 48 h, during which the cells attached to the plate. After incubation, the medium in each well was removed by aspiration. Next, the complex was dissolved in fresh culturing medium, which was added to designated wells. The complex was added to the wells at three different

concentrations (7.17, 71.7, and 717 μM in incubation medium). Some wells were assigned as the control, which contained the vehicle of the complex (0.5 % DMSO). The plate was, then, incubated for 36 h. After incubation, a solution (0.5 mg/mL) of MTT (Sigma Aldrich, Johannesburg, South Africa) was aliquoted into each well of the plate at a volume of 100 μL and incubation continued for additional 3 h. The liquid in the wells was removed by aspiration after incubation and phosphate-buffered saline was used to wash the cells in the wells. Immediately, an MTT solubilization solution (Sigma Aldrich, Johannesburg, South Africa) was aliquoted into each well of the plate at a volume of 100 μL. The absorbance of each well was, then, measured at 570 nm. Absorbance measurement was done with a Multiskan Go plate reader (Thermo Fischer Scientific, Waltham, MA, USA). The viability (%) of the complex-treated cells was calculated using the cells treated with the vehicle (0.5 % DMSO) as reference. The assay was done in three biological repeats.

### 2.4. Measurement of antioxidant and antidiabetic effects in vitro

#### 2.4.1. Radical scavenging and $Fe^{3+}$ reducing antioxidant experiments

The ability to scavenge DPPH and ABTS radicals or reduce $Fe^{3+}$ ion was measured using the protocols reported in a previous study [23]. Different sample concentrations (3.75–60 μM in the final assay volume) were tested in the radical scavenging assays. The results were presented as radical scavenging activity (%). Ascorbic acid and Trolox were the reference standards used in the assay. A chosen sample concentration (40 μM in the final assay volume) was tested in the $Fe^{3+}$ reducing antioxidant assay. The result was computed from an ascorbic acid standard curve (3.75–60 μM in the final assay volume) and expressed as mmol/mol ascorbic acid equivalent (AAE). Trolox was the reference standard used in the $Fe^{3+}$ reducing antioxidant assay.

#### 2.4.2. Measurement of the inhibitory effect on Linoleic acid peroxidation

The protocol reported in a previous article [24] was slightly modified to measure the inhibitory effect of the complex on linoleic acid peroxidation. Thirty microlitres of the tested samples or the reference standards (ascorbic acid and Trolox) was aliquoted into 2 mL vials. The concentrations of the samples and reference standards ranged from 5 μM to 80 μM in the assay volume. Some vials were assigned as the control and negative control, which contained an equivalent volume of the solvent used in dissolving the tested samples. Next, 30 μL of 50 mM linoleic acid and 20 μL of 100 mM Tris-HCl buffer (pH = 7.5) were, successively added into the vials. Thereafter, 20 μL of a 2 mM $FeSO_4.7H_2O$ solution was aliquoted into the vials containing the samples or reference standards and the vials that were assigned as the negative control to initiate linoleic acid peroxidation. An equivalent volume of distilled water was aliquoted into the vials that were assigned as the control. Immediately, all the vials were put in an incubator set at 37 °C. After the 30 min of incubation, 80 μL of 5.5 % trichloroacetic acid solution and 50 μL of 0.25 % thiobarbituric acid solution (dissolved in 50 mM NaOH solution) were successively aliquoted into each vial. A boiling water bath was, then, used to heat all the vials for 30 min. After heating, the vials were cooled under room temperature and centrifuged for 10 min at 3500 *g* and ambient temperature. One hundred and fifty microlitres of the supernatant in each vial was aliquoted into a 96-well plate and absorbance was read at 532 nm using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA). The inhibitory activity (%) of the tested samples on linoleic acid peroxidation was calculated as follows:

$$\text{Inhibition } (\%) = \frac{(A_{\text{Negative control}} - A_{\text{Normal control}}) - (A_{\text{Test}} - A_{\text{Normal control}})}{(A_{\text{Negative control}} - A_{\text{Normal control}})} \text{ X } \frac{100}{1}$$

where, "A" means "Absorbance".

#### 2.4.3. Measuring the inhibitory effect of the complex on α-glucosidase activity and bovine serum albumin (BSA) glycation

The ability to inhibit α-glucosidase activity and BSA glycation in vitro was measured using the protocols reported in a previous study [23]. Different sample concentrations were tested in the assays. For the α-glucosidase inhibition assay, the tested concentrations ranged from 3.75 to 60 μM. For the antiglycation assay, the tested concentrations ranged from 5 to 80 μM. Acarbose and aminoguanidine were the reference standards used in the enzyme inhibition and antiglycation assays, respectively.

### 2.5. Cellular antioxidant and antidiabetic experiments

#### 2.5.1. Measuring the effect of the complex on oxidative stress-induced alteration of lipid peroxidation and reduced glutathione (GSH) concentration in hepatocytes

The protocol reported in a previous article [25] was slightly modified to perform this assay. First, Chang liver cells (ATCC® CCL-13™, ATCC, Virginia, USA) were grown in an EMEM media, which contained 10 % FBS. When the cell confluence reached 85 %, the cells were trypsinized and plated into a 96-well plate with 200 μL aliquot of the culturing medium. The cell density in each well of the plate was 15,000 cells/mL. The plate was incubated for 36 h in a $CO_2$ incubator, during which the cells attached to the plate. After the incubation, the medium in the wells was removed by aspiration. Thereafter, the tested samples were dissolved in fresh culturing medium, which was added to designated wells of the plate. The tested samples were added to wells at five different concentrations (5, 10, 20, 40 and 80 μM in incubation medium). Some wells were assigned as the control and negative control, which contained the solvent used in dissolving the tested samples. The plate was incubated for 30 min. Thereafter, 50 μL of $FeSO_4.7H_2O$ solution (1 mM in incubation medium) was aliquoted into the wells containing the tested samples and the wells that were assigned as the negative control to initiate oxidative stress. An equivalent volume of distilled water was aliquoted into the wells that were assigned as the control. Immediately, the plate was put into a $CO_2$ incubator and kept there for 1 h. Thereafter, the plate was taken out of the $CO_2$ incubator. The medium in the wells was removed by aspiration and replaced with 250 μL of cold lysis buffer (50 mM sodium phosphate buffer with 0.5 % v/v Triton X-100, pH 7.5). A gentle agitation was applied to lyse the cells in the wells. Next, the plate was immediately centrifuged for 1 min at 5000 *g* and 4 °C to pellet the cell debris. The supernatant was quickly collected and kept on ice. Immediately, lipid peroxidation and GSH concentration were measured in the supernatant using slightly modified protocols of a previous article [25].

To estimate lipid peroxidation, 100 μL of the supernatant or malondialdehyde standards (0, 7.5, 15, 22.5, 30, and 45 μM in the assay volume) was put into designated vials. Next, 0.25 % w/v thiobarbituric acid, 20 % v/v acetic acid, and distilled water were aliquoted into the vials at a volume ratio of 500 μL:200 μL:200 μL, respectively. A boiling water bath was, then, used to heat all the vials for 50 min. After heating, the vials were cooled under room temperature and centrifuged for 10 min at 3500 *g* and ambient temperature. Two hundred microlitres of the supernatant in each vial was aliquoted into a 96-well plate and absorbance was read at 532 nm. A malondialdehyde standard curve was used to compute the lipid peroxidation, which was estimated as thiobarbituric acid reactive substances.

To estimate the GSH concentration, the supernatant was, first, mixed with an equal volume of 10 % TCA. This was done to precipitate the proteins in the cell lysates. At an angular speed of 4000 *g* and for a duration of 5 min (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany), the supernatant without proteins was recovered. Into a 96-well plate, 50 μL of the supernatant or GSH standards (0.002, 0.02, 0.2, 2, 20, 200 μM in assay volume) were aliquoted into designated wells. Next, 200 μL of Ellman's reagent [0.1 mM of 5,5′-dithio-bis-(2-nitrobenzoic acid) dissolved in 0.1 M Tris-HCl

buffer, pH = 7.4)] was aliquoted into each well. Thereafter, the plate was put in an incubator set at 25 °C for 5 min. The absorbance of each well was measured at 412 nm. A GHS standard curve was used to extrapolate the GHS concentration of the supernatant contained in each well.

The percentage inhibition of lipid peroxidation and GSH depletion was calculated and used to compute the $IC_{50}$ values (the concentration required to inhibit lipid peroxidation and GSH depletion by 50 %) of the tested samples.

### 2.5.2. Measuring the glucose uptake effect of the complex in L-6 myotubes

The protocols reported in previous articles [26,27] were slightly modified to perform this assay. The cells used in the experiment were L-6 myoblast (ATCC CRL-1458, ATCC, VA, USA). The cells were cultured in a $CO_2$ incubator. A low glucose Dulbecco's Modified Eagle Medium (DMEM) was used as the culturing medium. The medium, also, contained 10 % fetal calf serum. The cells were trypsinized after an 85 % confluence was attained and seeded into a 96-well plate with fresh media. Each well had approximately 4000 cells. The plate was incubated until an 80 % cell confluence was reached. The myoblasts were, then, differentiated into myotubes by replacing the used medium with DMEM medium containing 2 % horse serum and incubating the for 4–5 days. After differentiating the myoblasts, the medium in each well of the plate was removed by aspirating and 200 μL of fresh medium containing different concentrations (5, 50, and 100 μm in the incubation volume) of the tested samples was aliquoted into the plate. The sample solvent was used in the vehicle control (control) wells. Some wells were assigned as the blank control, which contained only the medium without cells. Incubation continued for 48 h. After incubation, the used medium in each well was removed and PBS was used to wash the cells. The cells were, then, treated with 8 mM glucose solution for 2 h. The glucose solution was dissolved in an RPMI medium containing 0.1 % BSA. Insulin (1 μM) was used as the positive control group. After the treatment period elapsed, glucose concentration was measured in the medium of each well using a Glucose-GO Assay Kit (Sigma Aldrich, South Africa). The MTT assay was, also, employed to measure the effect of the treatments on the viability of the myotubes. The experiment was conducted in three biological repeats. The formula below was used to compute the glucose uptake of the test samples and control (vehicle control) relative to the blank control.

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

"ΔGC" implies initial glucose concentration minus final glucose concentration. The $EC_{50}$ (concentration of the sample that will increase glucose uptake by 50 %) of the tested samples was computed.

## 2.6. Ex vivo antioxidant and antidiabetic experiments

The protocol reported in this section was adopted from a previous report [28] and some modifications were added.

### 2.6.1. Animals

The Animal Research Ethics Committee of the University of the Free State, Bloemfontein, South Africa ethically approved the protocol (**protocol approval reference: UFS-AED2019/0152/2020, approved on 16 November 2020**). The protocol was executed in accordance with the guidelines of the South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008). The University of the Free State Animal Unit supplied the male Sprague Dawley rats used for the study. The rats were 8 weeks old. First, the rats were subjected to an overnight fast. Thereafter, isoflurane was used to euthanize the rats. Immediately, the liver and psoas muscle tissues of the rats were collected, which were quickly used for measuring the ex vivo antioxidant and antidiabetic properties of the complex.

### 2.6.2. Measuring the effect of the complex on oxidative stress-induced alteration of lipid peroxidation and reduced glutathione (GSH) concentration in the liver tissues

A protocol reported in a previous article [25] was slightly modified to perform this experiment. About 200 ± 5 mg portions of the freshly harvested liver tissues were put into a 48-well plate containing 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) and different concentrations (10, 20, 40 and 80 μM in the total assay volume) of the tested samples or reference standard (ascorbic acid). Some wells were assigned as the control and negative control, which contained the solvent used in dissolving the tested samples. The plate was placed in a $CO_2$ incubator (NAPCO series, 5400 $CO_2$ incubator, Thermo Scientific, South Africa) set at 37 °C for 25 min. After the pre-incubation period, 100 μL of $FeSO_4.7H_2O$ solution (2 mM in incubation medium) was aliquoted into the wells containing the tested samples or reference standard and the wells that were assigned as the negative control to initiate oxidative stress. An equivalent volume of distilled water was aliquoted into the wells that were assigned as the control. Immediately, the plate was put into the $CO_2$ incubator and kept there for 90 min. At the end of the incubation period, the tissue in each well was removed with a pair sterile forceps and rinsed in Kreb's buffer. The tissues were homogenized in 1 mL of 50 mM sodium phosphate buffer (containing 0.5 % v/v Triton X-100; pH 7.5). The supernatant was obtained with an angular speed of 10,400 g for a duration of 10 min. The methods previously described above were adopted to estimate lipid peroxidation and GHS concentration in the supernatant of each tissue homogenate. The inhibitory percentage of the tested samples or reference standard on lipid peroxidation and GHS depletion was calculated, which was used to compute the $IC_{50}$ values of the samples.

### 2.6.3. Measuring the glucose uptake effect of the complex using the muscle tissues isolated from rats

A protocol reported in a previous article [28] was slightly modified to evaluate the tissue glucose uptake effect of complex. About 300 ± 10 mg portions of the freshly harvested psoas muscle tissues were put into a 48-well plate containing 900 μL of Kreb's buffer and different concentrations (10, 20, 40 and 80 μM in the total assay volume) of the samples or reference standard (50 mU insulin; NovoRapid® FlexPen®, Novo Nordisk Limited, West Sussex, UK). Some wells were assigned as the control, which contained the solvent used in dissolving the tested samples. The plate was placed in a $CO_2$ incubator (NAPCO series, 5400 $CO_2$ incubator, Thermo Scientific, South Africa) set at 37 °C for 25 min. After the pre-incubation period, 100 μL of glucose solution (11.1 mM in total incubation volume) was aliquoted into the wells of the plate. Immediately, the plate was put into the $CO_2$ incubator and kept there for

90 min. At the end of the incubation period, a portion of the incubation medium in each was aliquoted into a new 96-well plate and glucose concentration was spectrophotometrically estimated in the medium using a Glucose (GO) Assay Kit (Sigma Aldrich, South Africa). The absorbance value of the control was used as a reference to compute the glucose uptake increase (%) of the tested samples and insulin according to the formula below:

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

The $EC_{50}$ of the samples, which represents the concentration (μM) of the sample required to increase tissue glucose uptake by 50 % was computed.

Immediately after the 90 min incubation, zinc concentration and hexokinase activity were measured in the muscle tissues that were treated with the highest concentration of the test samples. First, the tissues were washed with Krebs buffer, homogenized in 1 mL of ice-cold 50 mM sodium phosphate buffer (containing 0.5 % v/v Triton X-100 and 1 mM EDTA; pH, 7.5) and centrifuged at 10 400 *g* for 10 min at 4 °C to recover the supernatant. The concentration of protein was estimated in the supernatant using the bicinchoninic acid (BCA) method. Hexokinase activity was measured in the supernatant at a protein concentration of 15 mg/mL using a Hexokinase Colorimetric Assay Kit (catalog number MAK091, Sigma Aldrich, Johannesburg, South Africa). The hexokinase activity was expressed in nmol/min/mL, which is equivalent to milliunit/mL. One unit of hexokinase represents the amount of enzyme that will generate 1.0 μmol of NADH per minute at pH 8.0 at room temperature. Zinc concentration was measured in the tissue supernatant using a Zinc Assay Kit (catalog number MAK032, Sigma Aldrich, Johannesburg, South Africa) and expressed in ng/μL. The pan-Akt and phosphor-Akt in the tissue supernatants were estimated by an ELISA method using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (catalog number RAB0012, Sigma Aldrich, Johannesburg, South Africa). The phospho-Akt/pan-Akt ratio was determined.

### 2.7. Molecular docking

The complex and syringic acid were docked to five different types of enzymes associated with diabetic development and progression. They include glucose transporter type 4 (GLUT4), human insulin receptor tyrosine kinase domain (HIR-TKD), insulin receptor substrate-1 (IRS-1), human maltase–glucoamylase (MGAM) and protein kinase B (PKB). HIR-TKD (PDB ID: 1IRK), IRS-1 (PDB ID: 1IRS), MGAM (PDB ID: 3L4U), and PKB (PDB ID: 106L) were retrieved from protein data bank. GLUT-4 was theoretically generated followed by the homology modeling platform. All the attached heteroatoms from each retrieved protein structure were removed and saved in.pdb file format for the docking studies. The 3D structures of the ligands (syringic acid and its complex) were optimized using Avogadro software to get the reliable docking score. The structures were also saved in.pdb file format. Molecular docking study was done using AutoDock Vina 4.2. The docking of the proposed ligands that had the best pose was selected based on the minimum docking score (kcal/mol). To visualize the molecular interaction between the proteins and ligands, the BIOVIA-DSV software was used.

### 2.8. Data and statistical analysis

Data emanating from the study was analyzed using the 2016 version of MS Excel and GraphPad Prism 5 were. $IC_{50}$ and $EC_{50}$ values were computed as a linear and/or non-linear plot between the transformed (log10) sample concentration (x-axis) and bioactivity. The analysed data were reported as mean ± standard deviation of replicates (n = 3). Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp, Armonk, NY, USA). Multiple comparison of data averages among the groups was done using the Tukey multiple post hoc test and one-way analysis of variance (ANOVA) and statistically significant difference (p) was set at $p < 0.05$ when comparing the mean values of the different groups.

## 3. Results

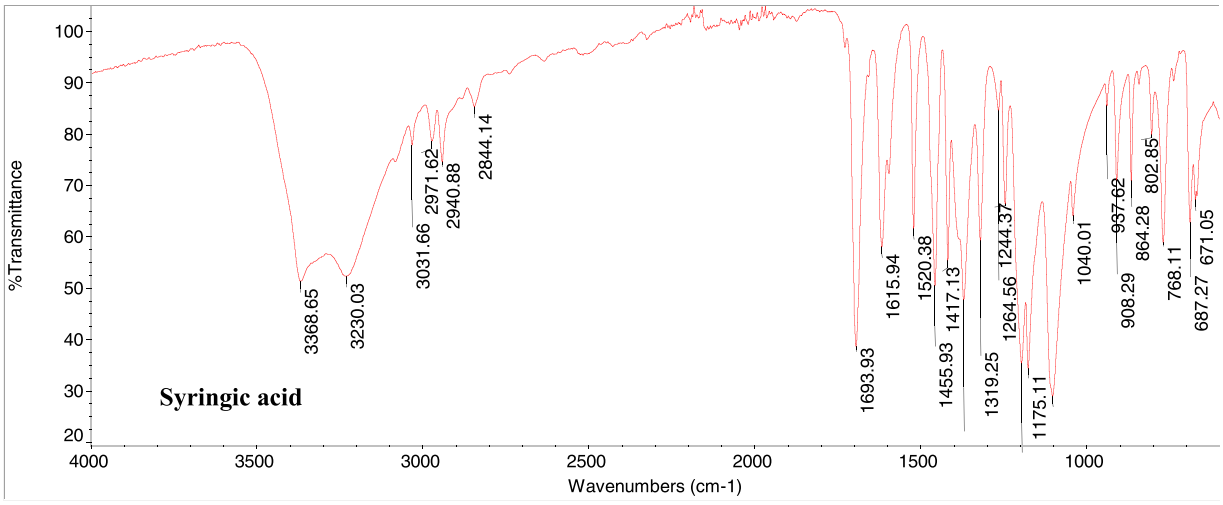
### 3.1. Characterization

The FT-IR data are presented in Fig. 1a–c. The FT-IR spectrum of syringic acid (Fig. 1a and c) depicts a broad peak centered at 3368 $cm^{-1}$, which represents a signal caused by the carboxylic O-H stretch. The broad peak centered at 3230 $cm^{-1}$ is typical of a phenolic O-H stretch (Fig. 1a and c). In the FT-IR of the complex (Fig. 1b and c) the intensity of the carboxylic O-H stretch peak (3388 $cm^{-1}$) diminished, suggesting that the carboxylic end of the phenolic acid was involved in the Zn(II) complexation. The disappearance of the phenolic O-H stretch peak may be due to intermolecular hydrogen bond interaction of the phenolic OH group during synthesis, which may also influence its concentration in the molecule. The strong sharp peak (1693 $cm^{-1}$) caused by carbonyl C═O stretching in syringic acid (Fig. 1a and c) notably shifted to a lower wave number (1553 $cm^{-1}$) in the FT-IR of the complex (Fig. 1b and c). This shift is due to the donating of electrons from zinc to the C═O group of syringic acid, thus weakening the C═O bond, which strongly suggests that the complexation between Zn(II) and syringic acid occurred through its carboxylic end as depicted in Fig. 2a.

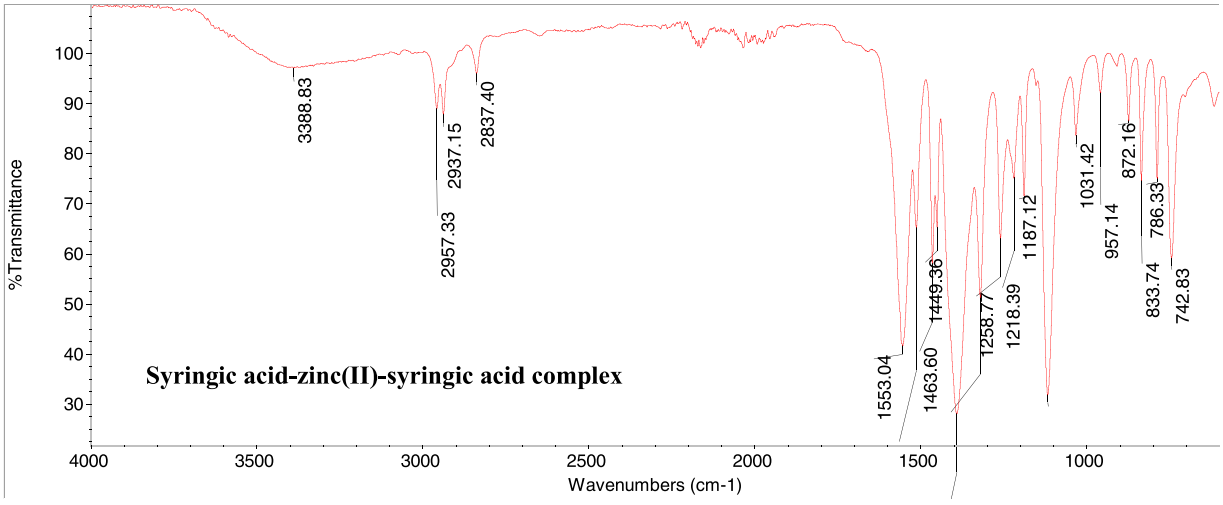
Proton NMR analysis (Fig. 2b) also supports the proposed structure of the complex shown in Fig. 2a. The $^1H$ NMR showed the four methoxy groups of the syringic acid moieties resonated at $\delta$ 3.79, while the equivalent protons on the aromatic ring are located at $\delta$ 7.19 (Fig. 2b). The hydroxyl group of the phenolic moiety was observed at $\delta$ 9.24, which provides evidence that this group was not involved in complexation.

Fig. 3 shows the high-resolution mass spectroscopic analysis of the complex. The value of 463.0602 (*m/z*) with calculated mass of 463.0582 (*m/z*) had a difference 20 units, which represents the complex after losing two methyl groups and sulfate group. When the complex lost three of its methyl groups and a sulfate group, the mass of 418.9987 (*m/z*) was observed, which differed from the calculated mass (*m/z* = 418.9956) by 31 units. When the complex lost all of its methyl groups and a sulfate group the mass of 409.0078 (*m/z*) was observed, which differed from the calculated mass (*m/z* = 409.0118) by 40 units. The fragments *m/z* 264.9550 (calculated mass *m/z* m/z264.969), *m/z* 268.9506 (calculated mass *m/z* 268.964) and *m/z* 254.9265 (calculated mass *m/z m/z* 254.9248) represent the loss of one syringic acid moiety. The fragment with *m/z* = 221.0426 is a sodium adduct of syringic zinc, which differed from the calculated mass (*m/z* = 221.0425) by 1 unit.

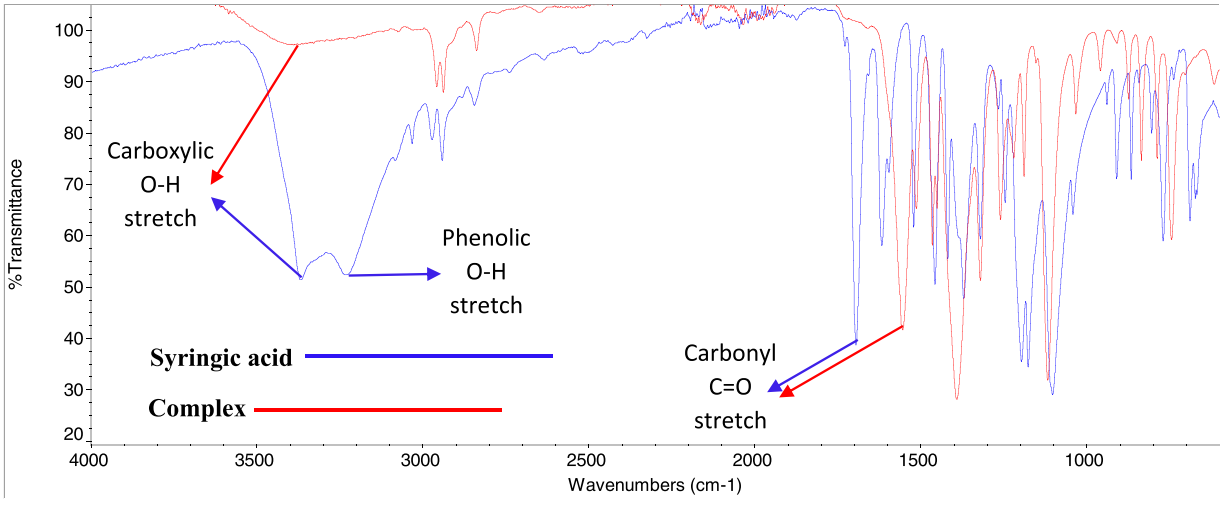
Summarily, the above characterization suggests that zinc sulfate complexed with two moieties of syringic acid through the carboxylic functional group of the two phenolic acid moieties (Fig. 2a). In the present study we consider syringic acid to be a chelating agent for Zn(II), which was mediated through the carboxylate group. This was influenced




(a)

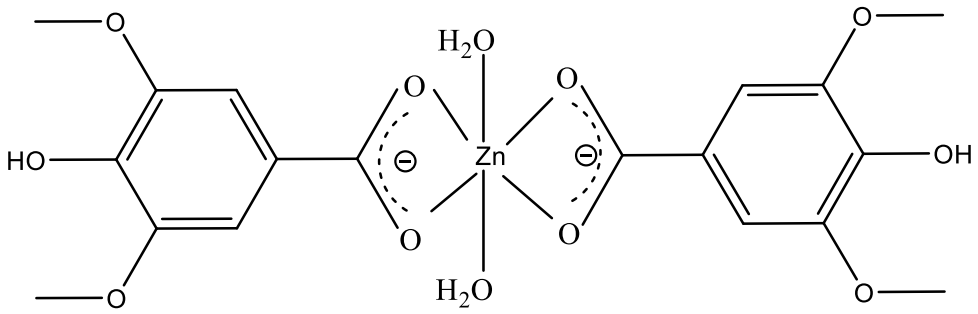



(b)

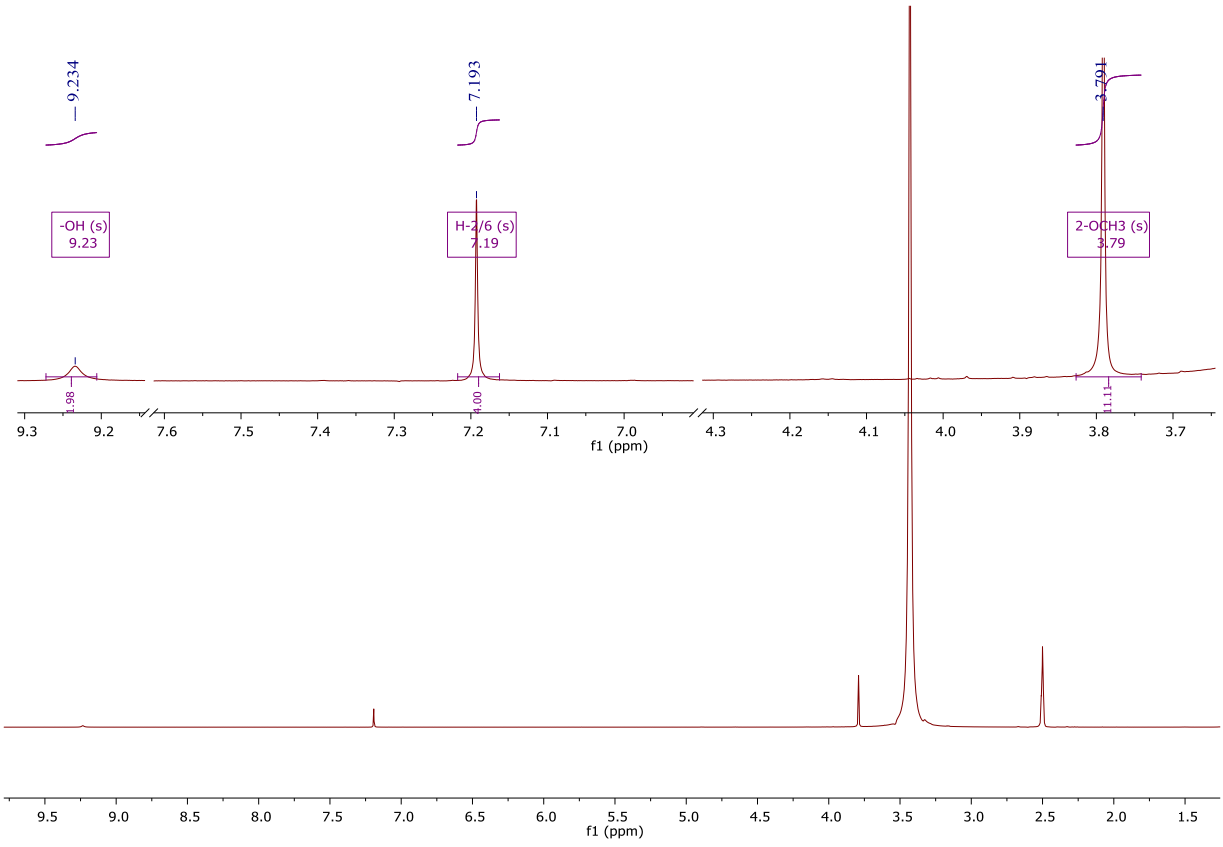



(c)

**Fig. 1.** FT-IR of spectra of **(a)** syringic acid and **(b)** zinc(II)-bisyringate.2$H_2O$ complex and (**c**) superimposed FT-IR spectra.




**(a)**




$^{1}$H NMR (400 MHz, DMSO-$d_6$) δ 9.23 (s, 2-ArOH), 7.19 (s, 4H, H-2/6), 3.79 (s, 12H, 4-OCH$_3$)

**(b)**

**Fig. 2.** **(a)** Proposed structure and **(b)** $^{1}$H NMR spectrum of the complex.

by using the sodium salt of syringic acid (sodium syringate) in the complex synthesis. A previous study [22] has, also, reported the Zn(II) chelating properties of the carboxylate group of cinnamic acid by using the sodium salt of cinnamic acid (sodium cinnamate) in the complex synthesis, which supports our study.

### 3.2. Antioxidant properties

Syringic acid inhibited DPPH ($IC_{50}$ = 29.8 μM) and ABTS ($IC_{50}$ = 29.7 μM) radicals, reduced $Fe^{3+}$ ion (529 μmol/mol AAE) and inhibited linoleic acid peroxidation ($IC_{50}$ = 40.5 μM) in vitro (Fig. 4a–d and Table 1). The DPPH scavenging, $Fe^{3+}$ reducing and anti-lipid peroxidative activity of syringic acid was significantly ($p < 0.05$) less potent than that of ascorbic acid ($IC_{50}$ = 13.1 and 13.9 μM). However, the complex showed DPPH ($IC_{50}$ = 10.7 μM) and ABTS ($IC_{50}$ = 5.76 μM) radical scavenging, $Fe^{3+}$ reducing (681 μmol/mol AAE) and anti-lipid peroxidative ($IC_{50}$ = 22.2 μM) activities that were, respectively, 2.8, 5.2, 1.3 and 1.8 folds more potent than the activities of syringic acid and statistically comparable and/or more potent than that ascorbic acid and Trolox based on $IC_{50}$ values. Zinc sulfate showed no notable in vitro antioxidant effect (Fig. 4a–d and Table 1).

In both Chang liver cells (Fig. 5a and Table 1) and isolated rat liver tissue (Fig. 5b and Table 1), $ZnSO_4$ minimally inhibited lipid peroxidation and GSH depletion ($IC_{50}$ = 449–885 μM) induced by oxidative stress. Syringic acid showed a significantly ($p < 0.05$) stronger cellular and tissue antioxidant activity ($IC_{50}$ = 40.2–650 μM) compared to

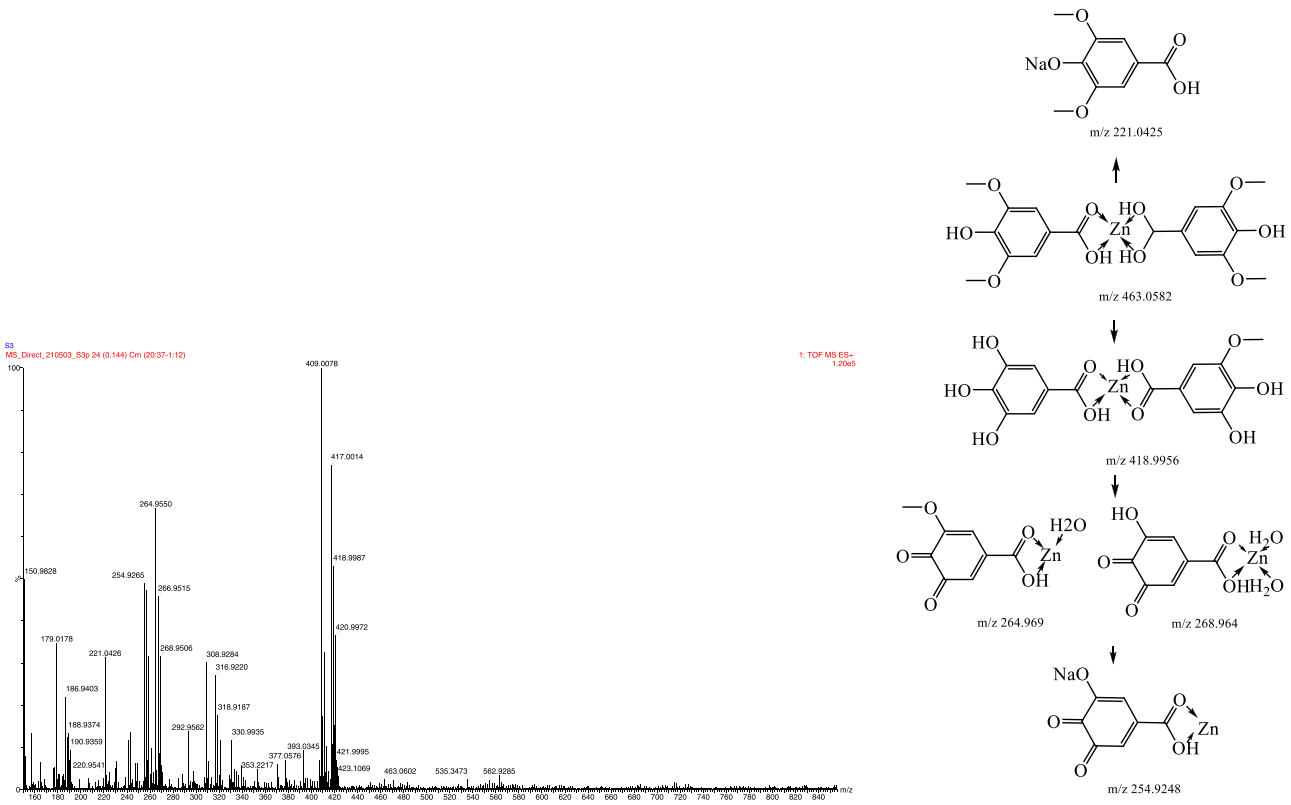



**Fig. 3.** High resolution-mass spectroscopic (HR-MS) analysis of the complex.

$ZnSO_4$ but was not as potent as ascorbic acid (Fig. 6a and b; Table 1). However, the antioxidant activity of the complex showed a 36–67 % increment compared to syringic according to the $IC_{50}$ values in Table 1. Moreover, the cellular and tissue antioxidant activity of the complex was statistically comparable ($p < 0.05$) to that of ascorbic acid (Table 1).

### 3.3. Antihyperglycemic properties

Data showed $ZnSO_4$ ($IC_{50}$ = 78.7 and 46.2 μM, respectively) and syringic acid ($IC_{50}$ = 40.0 and 30.8 μM, respectively) moderately inhibited α-glucosidase and BSA glycation in vitro, which was not as potent ($p < 0.05$) as the activity of acarbose ($IC_{50}$ = 9.02 μM) and aminoguanidine ($IC_{50}$ = 6.52 μM) (Fig. 6a and b; Table 1). However, the complex had α-glucosidase inhibitory ($IC_{50}$ = 9.02 μM) and anti-glycation ($IC_{50}$ = 9.02 μM) activity that was statistically more potent ($p < 0.05$) than syringic acid (2.4 and 2.3 folds, respectively) and $ZnSO_4$ (4.7 and 3.4 folds, respectively) and statistically comparable ($p < 0.05$) to the activity of the positive control or standard drugs (acarbose and aminoguanidine).

In both L6-myotubes (Fig. 7a) and isolated rat psoas muscle tissue (Fig. 7b), the complex ($EC_{50}$ = 20.4 and 386 μM, respectively), syringic acid ($EC_{50}$ = 6460 and 693 μM, respectively) and $ZnSO_4$ ($EC_{50}$ = 71.1 and 450 μM, respectively) showed a dose-dependent increases in glucose uptake/utilization (Table 1), although they were not as potent as insulin at the different tested concentrations. The glucose uptake effect of syringic acid was significantly ($p < 0.05$) lower compared to $ZnSO_4$, However, both formed a complex with a more potent glucose uptake effect (Table 1). In isolated rat psoas muscle tissues, glucose uptake was accompanied by elevated hexokinase activity (Fig. 7c). Muscle tissue zinc concentration was significantly higher ($p < 0.05$) in the complex and zinc sulfate treated tissue samples than the tissue samples treated with syringic acid and insulin (Fig. 7d). Similarly, the complex-treated tissues showed higher phospho-Akt/pan-Akt ratio than its precursors (Fig. 7e).

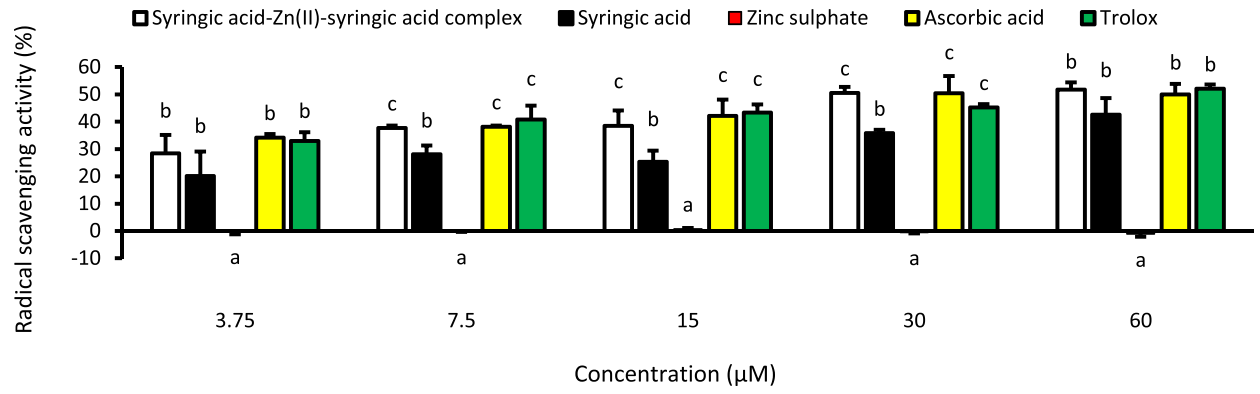
The molecular docking analysis is shown in Fig. 8. The complex and syringic acid were docked to 5 target enzymes (glucose transporter type 4, human insulin receptor tyrosine kinase, insulin receptor substrate-1, human α-glucosidase and protein kinase B) that are linked to insulin signaling and diabetes. The docking scores of the complex (− 7.31, − 6.38, − 5.78, − 6.03 and − 6.39 kcal/mol, respectively) were higher than the docking scores of syringic acid (− 5.95, − 5.81, − 4.53, − 5.32 and − 0.5.50 kcal/mol) as shown in Table 2.

Cytotoxicity evaluation showed that the complex had no notable adverse effect on the viability of Chang liver cells and L6-myotubes (Fig. 7f).

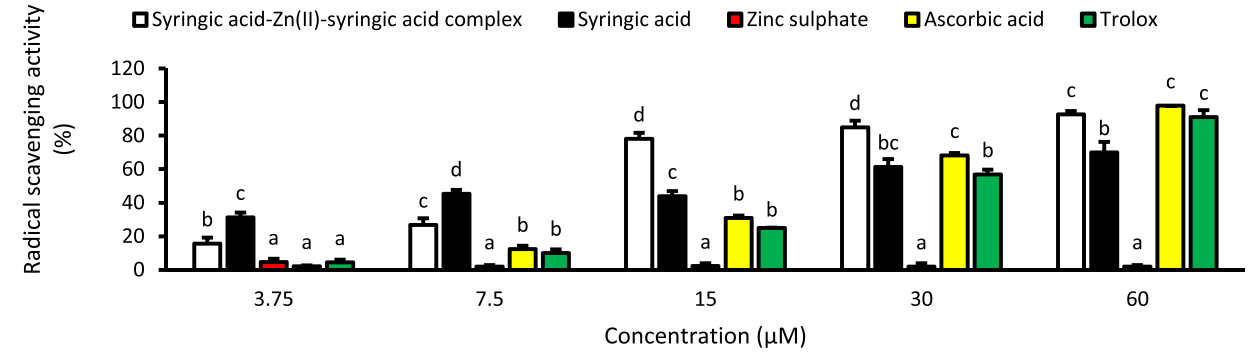
## 4. Discussion

In the present study, we considered syringic acid, a natural phenolic acid, as a suitable ligand for developing an anti-hyperglycaemic and antioxidant zinc complex with improved and multi-facet bioactivity profile. Emanating data showed the developed complex had improved and multifaceted bioactivity profile, suggesting a complexation mediated glycaemic control and antioxidant synergistic potential. Spectroscopic characterization suggests a double hydrated bisyringate-Zn(II) complex was formed, which influenced the biological properties of the complex.

Oxidative stress remains pivotal in degenerative diseases, including diabetes, due to cellular and tissue oxidative injuries caused by reactive oxygen species and radicals [6]. These prooxidants initiate the oxidative degradation of cellular lipids (lipid peroxidation), thus damaging cellular components, with concomitant production of free radicals [6]. Syringic acid is considered a natural antioxidant [11]. It possesses free radical quenching ability [13], which can be attributed to its electron donating ability and stable phenoxy radical intermediate [8,9]. This radical quenching ability of syringic acid has been implicated in its inhibitory effects on lipid peroxidation and GSH depletion in hepatic, renal, and neuronal tissues of diabetic rats [13–15]. In this study,




**(a)**

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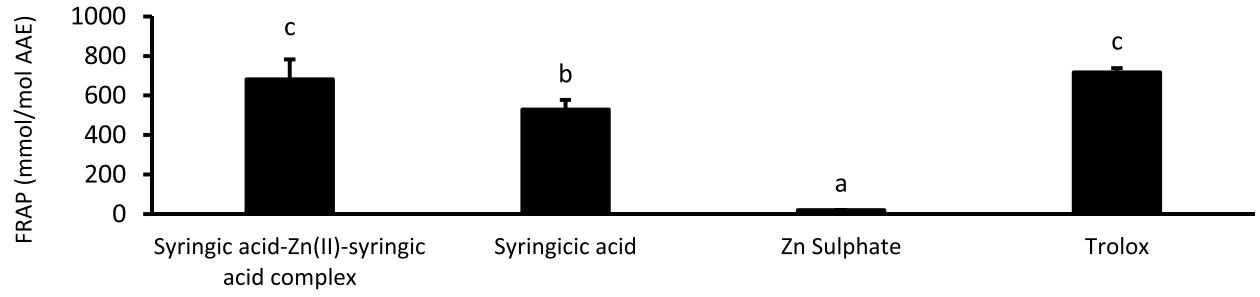
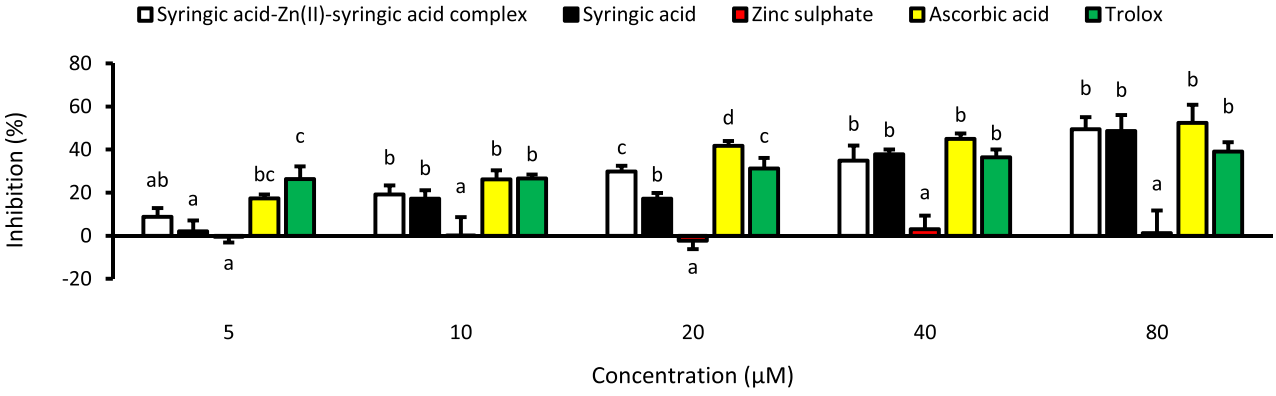



**(c)**




**(d)**

**Fig. 4.** **(a)** DPPH radical scavenging **(b)** ABTS$^{\bullet+}$ radical scavenging **(c)** $Fe^{3+}$ reducing and **(d)** in vitro linoleic acid lipid peroxidation inhibitory activities of complex, precursors and standards. The data are shown as mean ± SD of the triplicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups. Significant difference ($p < 0.05$) is presented as a difference in the letter annotations.

**Table 1**
$IC_{50}$ and $EC_{50}$ values of syringic acid, its complex with Zn(II) and standards.

| Parameters or activity | Complex | Syringic acid | Zinc sulfate | Ascorbic acid | Trolox | Acarbose | Aminoguanidine | Insulin |
|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ or $EC_{50}$ values (μM) | | | | | | | |
| ABTS radical scavenging activity ($IC_{50}$) | 5.76 ± 1.82$^{c}$ | 29.7 ± 5.92$^{b}$ | ND | 47.1 ± 4.47$^{b}$ | 100 ± 34.3$^{a}$ | NA | NA | NA |
| DPPH radical scavenging activity ($IC_{50}$) | 10.7 ± 4.36$^{b}$ | 29.8 ± 5.83$^{a}$ | ND | 13.1 ± 6.11$^{b}$ | 10.0 ± 3.75$^{b}$ | NA | NA | NA |
| Inhibition of in vitro linoleic acid peroxidation | 22.2 ± 2.78$^{bc}$ | 40.5 ± 4.90$^{a}$ | ND | 13.9 ± 4.38$^{c}$ | 37.8 ± 11.1$^{ab}$ | NA | NA | NA |
| Antiglycation activity ($IC_{50}$) | 13.6 ± 6.27$^{b}$ | 30.8 ± 9.54$^{a}$ | 46.2 ± 7.57$^{a}$ | NA | NA | NA | 6.52 ± 0.93$^{b}$ | NA |
| α-glucosidase inhibition ($IC_{50}$) | 16.8 ± 6.04$^{c}$ | 40.0 ± 4.08$^{b}$ | 78.7 ± 15.6$^{a}$ | NA | NA | 9.02 ± 3.18$^{c}$ | NA | NA |
| Glucose uptake increase in L-6 myotubes ($EC_{50}$) | 20.4 | 6460 | 71.1 | NA | NA | NA | NA | ND |
| Glucose uptakes increase in isolated rat psoas muscle ($EC_{50}$) | 386 ± 13.5$^{b}$ | 693 ± 31.9$^{a}$ | 450 ± 87.0$^{b}$ | NA | NA | NA | NA | ND |
| Inhibition of oxidative stress-induced lipid peroxidation in Chang liver cells ($IC_{50}$) | 25.7 ± 12.2$^{b}$ | 40.2 ± 12.2$^{b}$ | 538 ± 37.1$^{a}$ | 24.5 ± 6.21$^{b}$ | NA | NA | NA | NA |
| Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver ($IC_{50}$) | 376 ± 123$^{b}$ | 650 ± 129$^{a}$ | ND | 244 ± 94.0$^{b}$ | NA | NA | NA | NA |
| Inhibition of oxidative stress-induced GSH depletion in Chang liver cells ($IC_{50}$) | 18.1 ± 4.83$^{b}$ | 49.8 ± 17.0$^{b}$ | 449 ± 144$^{a}$ | 29.2 ± 5.51$^{b}$ | NA | NA | NA | NA |
| Inhibition of oxidative stress-induced GSH depletion in isolated rat liver ($IC_{50}$) | 29.4 ± 2.62$^{b}$ | 89.7 ± 18.0$^{b}$ | 885 ± 190$^{a}$ | 23.8 ± 3.41$^{b}$ | NA | NA | NA | NA |

"ND" means "not determined; "NA" means "not applicable; "GSH" means "reduced glutathione"; IC50 is concentration needed to inhibit the activity of carbohydrate digesting enzymes, bovine serum albumin glycation, lipid peroxidation and reduced glutathione depletion 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' and 'c' represent significant difference ($p < 0.05$) between treatment groups.

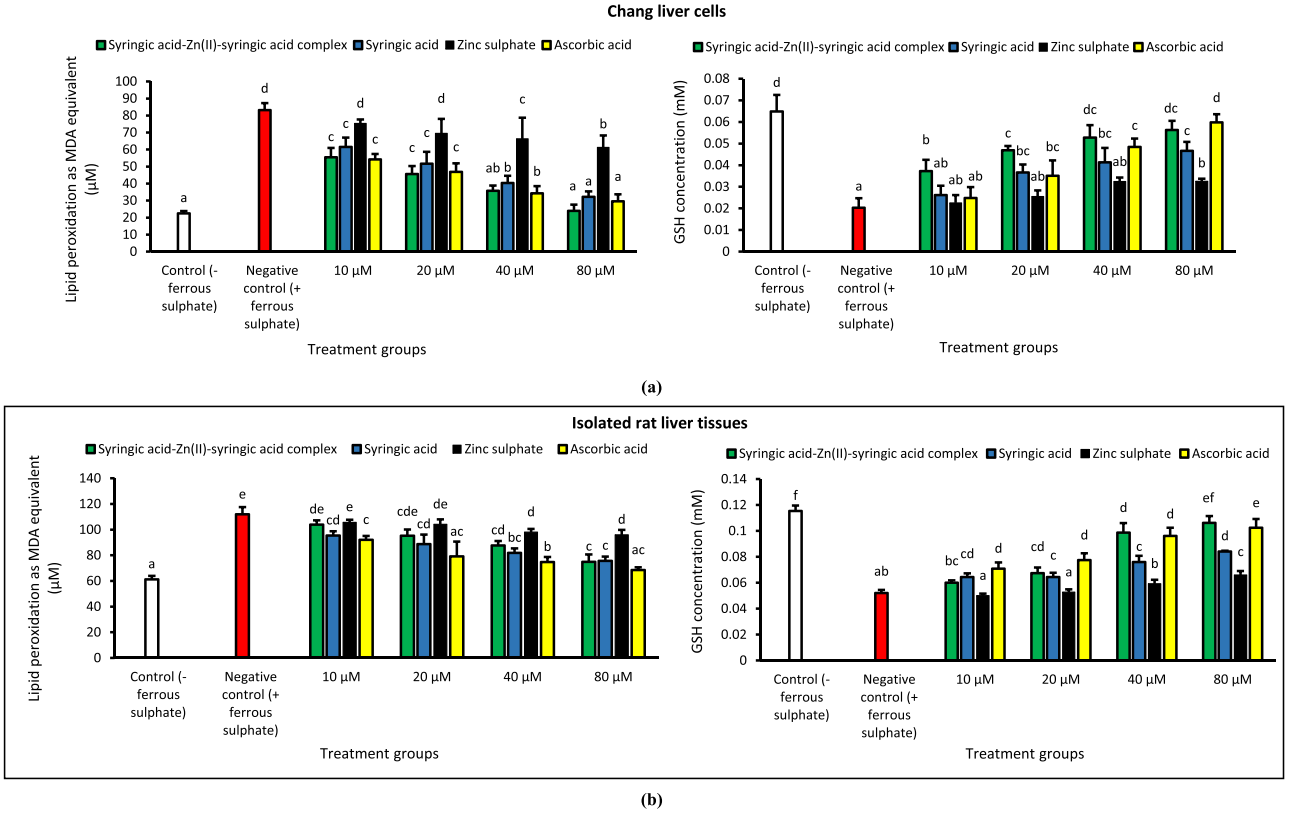



**Fig. 5.** Effect of the complex, precursors and standards on lipid peroxidation and GSH concentration in **(a)** Chang liver cells and **(b)** isolated rat liver tissue. The data are shown as mean ± SD of the replicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups, as well as between the treatment groups and the controls (normal control and negative control). Significant difference ($p < 0.05$) is presented as a difference in the letter annotations.

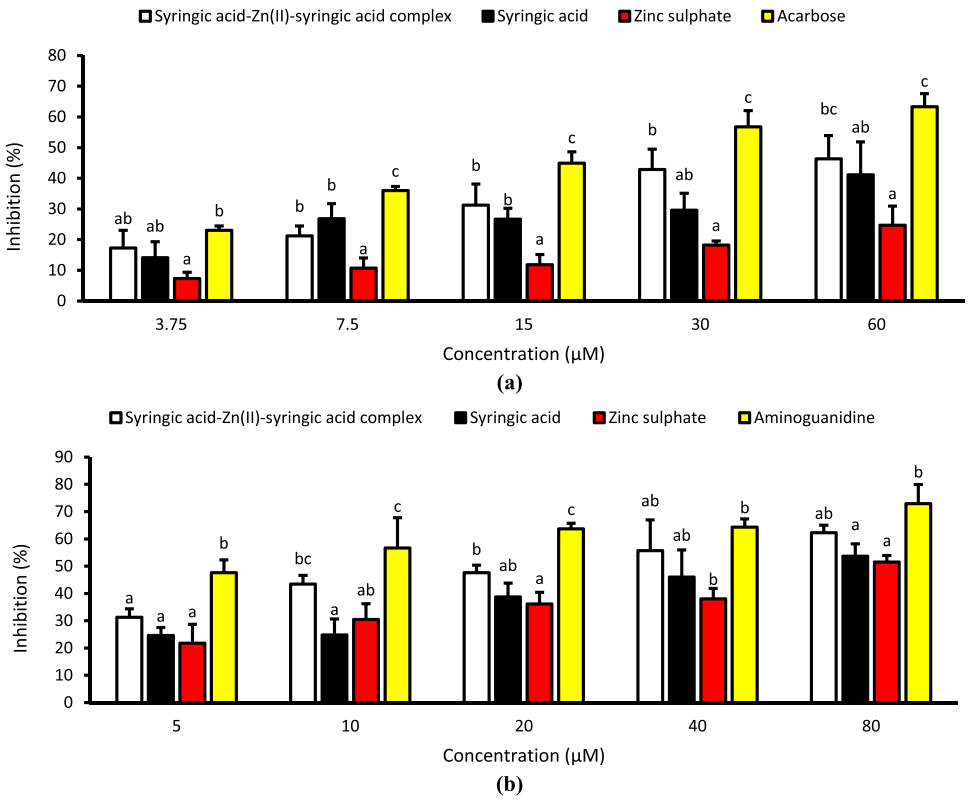



**Fig. 6.** Effect of the complex, precursors and standards on in vitro **(a)** α-glucosidase activity and **(b)** BSA glycation. The data are shown as mean ± SD of the triplicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups. Significant difference ($p < 0.05$) is presented as a difference in the letter annotations.

syringic acid scavenged DPPH and ABTS radicals, reduced $Fe^{3+}$ ion and inhibited the peroxidation of linoleic acid in vitro (Fig. 4a–d and Table 1). In both Chang liver cells, and rat liver tissues induced with oxidative stress, it mitigated lipid peroxidation and the depletion of GSH (Fig. 5a and b; Table 1). However, when complexed with zinc sulfate, its in vitro, cellular and tissue antioxidant activity appreciable and/or significantly ($p < 0.05$) increased by various percentages (36.1–80.6 %). In most cases, the antioxidant effect was comparable and/or more potent than that of ascorbic acid and Trolox (Table 1). Considering that zinc sulfate did not exhibit in vitro antioxidant effects and mildly potentiated cellular and tissue antioxidant effects relative to syringic acid (Fig. 4a–d; Fig. 5a and b; Table 1), it is fair to say that the stronger antioxidant activity of the complex compared to syringic acid was as a result of the two moieties of syringic acid in the complex (Fig. 2a). It is proposed that this molecular property of the complex potentiated a stronger antioxidant activity relative to its precursor phenolic acid. These data suggest that zinc may be an adjuvant for improving the antioxidant efficacy of syringic acid in disease conditions including diabetes.

The progression of oxidative stress-related diabetic complications is largely fueled by persistent hyperglycemia. Glycation of proteins and lipids and formation of AGEs are the most notable culprits linking persistent hyperglycemia with elevated prooxidant production and diabetic oxidative stress [6]. Additionally, hyperglycemia drives the production of superoxide radical, a deleterious prooxidant, via glucose metabolism and mitochondrial electron transport and energy generation, thus increasing diabetic oxidative stress [29]. Improving glycemic control is thus important in limiting diabetic oxidative stress. One mechanism is by reducing postprandial blood glucose increase through inhibiting carbohydrate digesting enzymes such as α-glucosidase and α-amylase [30]. A computational study has speculated that some natural phenolic acids, including syringic acid, can notably inhibit α-glucosidase activity [31] and glycation [32]. Moreover, syringic acid has been shown to inhibit in vitro protein glycation and formation of AGEs [33, 34].

In this study, syringic acid inhibited both in vitro α-glucosidase and glucose-induced BSA glycation (Fig. 6a and b; Table 1). Zinc sulfate moderately inhibited α-glucosidase relative to syringic acid but showed a comparable in vitro BSA anti-glycation activity relative to syringic acid (Fig. 6a and b; Table 1), which correlates with the previously documented in vitro anti-glycation properties of zinc sulfate [35], as well as its ability to mitigate the glycation and carbonyl formation with proteins in diabetic rats [36] and rats suffering from protein malnourishment [37]. However, complexing syringic acid with zinc sulfate resulted in a complex with stronger α-glucosidase and glycation inhibitory activity relative to syringic acid (2.4 and 2.3 folds, respectively) and zinc sulfate (4.7 and 3.4 folds, respectively) as shown in Table 1, suggesting a synergistic glycemic control and anti-glycation potential between syringic acid and zinc sulfate through complexation. In fact, the activity of the complex was comparable to acarbose and aminoguanidine (Table 1), which may be influenced by the two syringic acid moieties and the zinc sulfate components of the complex (Fig. 2a). Docking study further showed the complex had higher docking scores against human α-glucosidase target enzyme than syringic acid (Table 2). Due to the presence of more -OH groups and Zn(II) in the complex, it is able to interact better with the target enzyme through hydrogen bond and ionic interactions (Fig. 8).

Furthermore, zinc(II) has been shown to exhibit glycaemic control effects through insulin mimetic actions by modulating insulin signaling and glucose uptake. In mouse and human skeletal cells, Zn(II) treatment was shown to induce Akt phosphorylation, thus modulating insulin signaling [38]. In 3T3-L1 pre-adipocytes and adipocytes, the modulatory effect of Zn(II) on insulin receptor and Akt activation potentiated increased glucose uptake [39]. In the present study, zinc sulfate

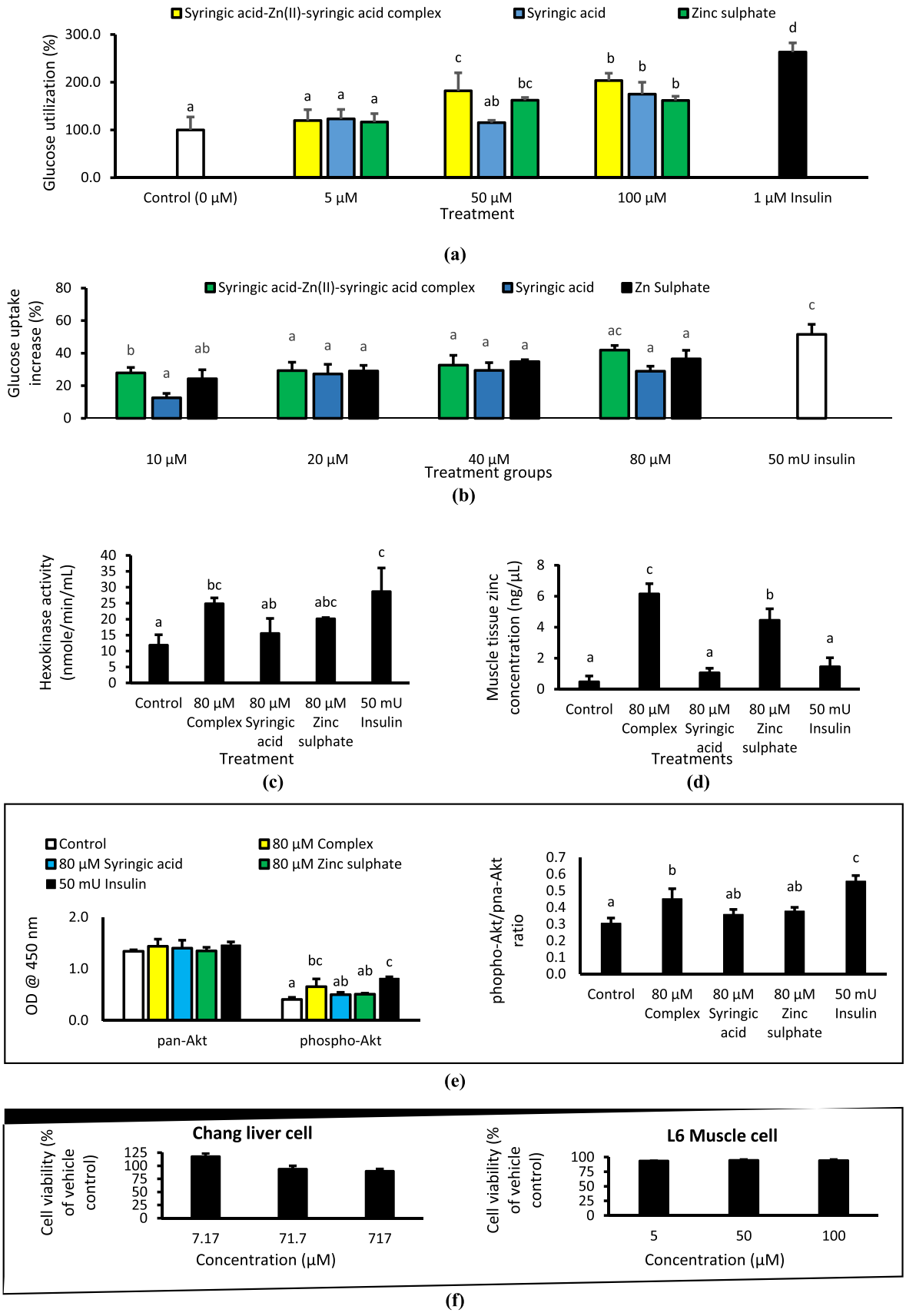



**Fig. 7.** Effect of the treatments on (**a**) glucose uptake in L6-myotubes, (**b**) glucose uptake, (**c**) hexokinase activity, (**d**) zinc concentration and (**e**) Akt phosphorylation in isolated rat psoas muscle and (**f**) the viability of Chang liver cell and L6-myotubes. The data are shown as mean ± SD of the replicate analysis. Within each tested concentration, statistical comparison was done between the treatment groups, as well as between the treatment groups and the controls (normal control and negative control). Significant difference ($p < 0.05$) is presented as a difference in the letter annotations.

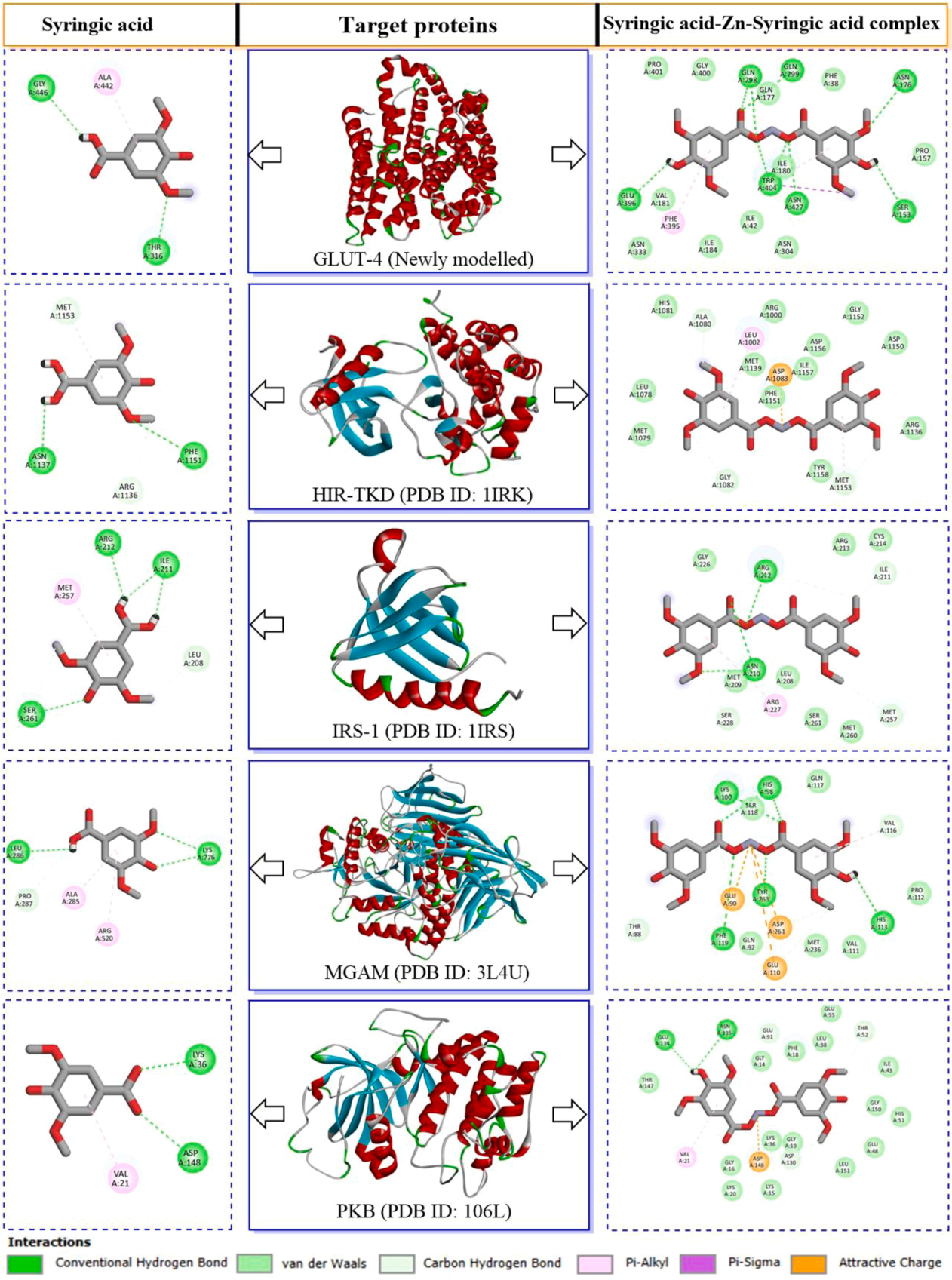



**Fig. 8.** Visualized molecular docking interaction of syringic acid and the complex with five target enzymes (GLUT-4, HIR-TKD, IRS-1, MGAM and PKB). GLUT-4, glucose transporter type 4; HIR-TKD, human insulin receptor-tyrosine kinase domain; IRS-1, insulin receptor substrate-1; MGAM, Human maltase–glucoamylase; PKB, protein kinase B.

**Table 2**
Docking scores for molecular docking study of syringic acid and syringic acid-–zinc(II)-syringic acid complex with five target enzymes.

| Target enzymes | PDB ID | Syringic acid | syringic acid–zinc (II)-syringic acid complex |
|---|---|---|---|
| | | Docking scores (kcal/mol) | |
| GLUT-4 | Newly modeled | -5.95 | -7.31 |
| HIR-TKD | 1IRK | -5.81 | -6.38 |
| IRS-1 | 1IRS | -4.53 | -5.78 |
| MGAM | 3L4U | -5.32 | -6.03 |
| PKB | 106L | -5.50 | -6.39 |

GLUT-4, glucose transporter type 4; HIR-TKD, human insulin receptor-tyrosine kinase domain; IRS-1, insulin receptor substrate-1; MGAM, Human maltase–-glucoamylase; PKB, protein kinase B.

increased glucose uptake in both L-6 myotubes and isolated rat muscle tissues and complexing it with syringic acid formed a complex that outperformed both syringic acid and zinc sulfate (Fig. 7a and b, Table 1). In the complex-treated muscle tissues, glucose uptake activity was accompanied by higher tissue/cellular zinc concentrations relative to the nontreated muscle tissues (Fig. 7d). In fact, intracellular zinc concentration was higher in the complex-treated tissues compared to the zinc sulfate-treated tissues (Fig. 7d), suggesting that Zn(II) complexation with syringic acid increased tissue/cellular Zn(II) uptake, which potentiated a stronger glucose uptake effect. Documented evidence has shown that non-pharmacologically active 1-oxy-2-pyridinethiol ligands could potentiate cellular insulin mimetic effects by enhancing cellular zinc uptake through complexation [40,41].

Our study, however, suggests a synergistic interaction between the Zn(II) and syringic acid components of the complex in potentiating glucose uptake. Syringic acid was shown to moderately increase cellular and tissue glucose uptake (Fig. 7a and b; Table 1) and the complex was afforded 2 moieties of syringic acid (Fig. 2a). Hence, it is hypothesized that while complexing syringic acid with zinc(II) enhanced the cellular bioavailability of Zn(II), it concomitantly contributed to the glucose uptake potentiated by zinc(II). Hexokinase activity followed the same trend as tissue glucose uptake activity (Fig. 7c), suggesting that the glucose uptake activity may be linked to an increase in cellular/tissue glucose utilization. Docking data further showed the complex had higher docking scores (Table 2) and stronger interaction (Fig. 8) with four target enzymes (GLUT-4, IRS-1, PKB/Akt and insulin receptor tyrosine kinase) linked to insulin signaling and cellular glucose uptake, which suggests the glucose uptake potency of the complex may be linked to a modulatory action on associated signaling proteins. Moreover, isolated rat muscle tissues treated with the complex showed significantly ($p < 0.05$) elevated tissue phospho-Akt/pan-Akt ratio (Fig. 7e). Akt activation is an important downstream signaling step to facilitate insulin-mediated cellular glucose uptake and utilization [42], which suggests Zn(II)-syringic acid complexation may potentiate glycaemic control by modulating insulin signaling and cellular glucose uptake. It is noteworthy that the complex may not pose toxicity concerns, as it did not adversely alter the viability of Chang liver cells and L-6 myotubes.

## 5. Conclusion

This study revealed that complexing syringic acid with zinc sulfate resulted in a synergistic interaction between both. The outcome was a non-cytotoxic complex with a multimode and improved antioxidant and glycaemic control potential. The interaction between the two moieties of syringic acid and Zn(II) of the complex appears to be influential in the improved and multi-facet bioactivity profile of the complex. Through complexation, zinc(II) may be a promising adjuvant for syringic acid in developing novel nutraceuticals with improved medicinal relevance against diabetes and oxidative complications.

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## CRediT authorship contribution statement

**Conceptualization:** Chika I. Chukwuma; **Data curation:** Limpho M. Ramorobi and Godfrey R. Matowane; **Funding acquisition:** Chika I. Chukwuma; **Methodology:** Limpho M. Ramorobi, Godfrey R. Matowane, Samson S. Mashele, Shasank S. Swain, Tshepiso J. Makhafola and Chika I. Chukwuma; **Resources:** Samson S. Mashele, Tshepiso J. Makhafola, Polo-Ma-Abiele H. Mfengwana and Chika I. Chukwuma; **Software:** Shasank S. Swain and Chika I. Chukwuma; **Supervision:** Samson S. Mashele and Chika I. Chukwuma; **Writing – original draft:** Limpho M. Ramorobi; **Writing – review & editing:** Limpho M. Ramorobi, Godfrey R. Matowane, Samson S. Mashele, Shasank S. Swain, Tshepiso J. Makhafola and Chika I. Chukwuma.

## Conflict of interest

The authors declare that there is no conflict of interest with this work.

## Data Availability

Data will be made available on request.

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# Sciforum.net abstract approved [sciforum-057892]

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Date: Thursday, 12 May 2022, 04:03 pm GMT+2

Dear Dr. Chukwuma,

We are pleased to inform you that your abstract has been approved by our editorial team.

Please note that, following the Chair´s assessment, we will let you know of the specific type of your contribution (oral presentation or poster) by the notification deadline.

To attend the event, please visit the event website at //MMCS2022.sciforum.net#registration and register, if you have not yet done so.

**Submission ID:** sciforum-057892
**Title:** ANTIDIABETIC AND ANTIOXIDATIVE SYNERGISTIC POTENTIAL BETWEEN ZN(II) AND CAFFEIC ACID: IMPROVING THERAPEUTIC POTENTIAL THROUGH COMPLEXATION
**Authors:** Limpho Martha Ramorobi, Godfrey R Matowane, Samson S. Mashele, Susanna L Bonnet, Tshepiso J Makhafola, Chika Ifeanyi Chukwuma *
**Event:** 3rd MMCS: Shaping Medicinal Chemistry for the New Decade
**Section:** Medicinal Chemistry Tales

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**Editor decision:** Approve
**Editor comments:** Please note that you will be informed early next week whether your abstract has been accepted as Oral or Poster Presentation for MMCS2022.

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Kind regards,
Your MMCS2022 Organizing Team
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# MMCS2022 List of Posters

## Poster Session A – Thursday, 28 July 2022; 13:45-14:45h

| Poster Session | Poster Number | Author Name | Title |
|---|---|---|---|
| A | 1 | Simeon Dimitrov | *In vivo* toxicity, redox-modulating capacity and intestinal permeability of novel aroylhydrazone derivatives with high *in vitro* antimycobacterial activity |
| A | 2 | Federico Appetecchia | Novel pyrazole- and pyrrole- based compounds active against multiple stages of P.falciparum |
| A | 3 | Harry Wilders | A high-throughput-chemistry, direct-to-biology approach discovers novel reactive fragments targeting SARS-CoV-2 |
| A | 4 | Maria Emília Sousa | Chiral xanthones reversing antimicrobial resistance: enantioselectivity in efflux pump inhibition |
| A | 5 | David Alam-Escamilla | Fucoidan as a protective agent against SARS-CoV-2 in Vero cells |
| A | 6 | Simona Sestito | High content Screening identification of new molecular tools against SARS-CoV-2 |
| A | 7 | Bahne Stechmann | Identifying molecules against SARS-CoV-2 and future pandemics collaboratively within an open-access research infrastructure initiative |
| A | 8 | Amgad Albohy | Molecular Dynamics simulation of SARS-CoV-2 main protease reveals role of Q189 loop flexibility in recognition of novel inhibitors |
| A | 9 | Sveva Pelliccia | The PADAM oxidation route for the synthesis of SARS-CoV-2 Main Protease inhibitors |
| A | 10 | Dariia Samofalova | Identification of novel potential inhibitors of dpre1 from mycobacterium tuberculosis and mycobacterium bovis |
| A | 11 | Olga Bobileva | 3-(Adenosylthio)methyl benzoic acid with modified adenosine as SARS-CoV-2 methyltransferases inhibitors |
| A | 12 | Alessandra Salerno | Targeted Degradation of Trypanothione Reductase as potential new approach to treat leishmaniasis |
| A | 13 | Federica Blua | Design and synthesis of encorafenib-based BRAF-V600E degraders |

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|---|---|---|---|
| A | 14 | Giuseppe Lamanna | DeLA-Drug: A Deep Learning Algorithm for Automated Design of Drug-like Analogues |
| A | 15 | Lorenzo Antonini | Leveraging information from essential oils biological assays through machine learning: PDIA3 as a case study |
| A | 16 | Mauro Nascimben | Quantized computational QSAR framework for molecular toxicity virtual screening |
| A | 17 | Michela Proietti | Self-explainable Graph Neural Network for Molecular Property Prediction using Concept Whitening |
| A | 18 | Salvatore Galati | VenomPred: A Machine Learning Based Platform for Molecular Toxicity Predictions |
| A | 19 | Marta Barral-Martínez | Evaluation of antimicrobial and antitumor activities from *Chamaemelum nobile* (L) All |
| A | 20 | Dominika Pindjakova | Antimalarial Activity of Novel Substituted *N*-Arylcinnamamides |
| A | 21 | Antia G. Pereira | Antimicrobial activity screening for drug development of Camellia japonica flowers (var. Conde de la Torre) |
| A | 22 | Fátima Vela | Ingol derivatives as PKC-modulating compounds to promote neurogenesis. |
| A | 23 | Paola Carta | Prenylated derivatives of *trans*-cinnamic acids effective against clinical Fusarium spp. responsible of onychomycosis infection |
| A | 24 | Marília O. F. Goulart | Therapeutic potential of natural products and thiols in maternal and child diseases |
| A | 25 | Zaynab Abdulghany | Total oligomeric flavonoids (TOF) of the herb tubers Cyperus rotundus induce growth inhibition, antioxidant activity and apoptosis in different cancer cell lines. |
| A | 26 | Manuel Schriefer | Synthesis of the lower binding arm of kibdelomycin, a novel gyr B- and topo IV-inhibitory antibiotic |
| A | 27 | Marc Panosetti | Design, synthesis and biological evaluation of new RNA ligands targeting miR-210: modulation of the circadian clock for cancer chemotherapy. |

## Poster Session B – Friday, 29 July 2022; 13:30-14:30h

| Poster Session | Poster Number | Author Name | Title |
|---|---|---|---|
| B | 1 | Miguel Gallardo | New bioorganometallic-heterocylic compounds based on ferrocene as potential antitumor agents |
| B | 2 | Yuri Mackeyev | [60]Fullerene to Alleviate the Consequences of Cosmic Radiation |
| B | 3 | Raid Abdel-Jalil | Design, synthesis, and bioactivity evaluation of novel cinnoline derivatives as antitumor agents |
| B | 4 | Beata Morak-Młodawska | Novel bis-dipyridothiazines - synthesis and *in silico* analysis |
| B | 5 | Anna Pasieka | Novel, potent and selective drug-like butyrylcholinesterase inhibitors with antiagregating properties |
| B | 6 | Amelia Bou Puerto | Selective inhibition of cancer cell proliferation by the action of styrylcarbamates |
| B | 7 | Alicja Gawalska | Structure-based molecular modeling approach in search for multi-target-directed ligands blocking phosphodiesterases 4B, 8A and TRPA1 ion channel with a potential application in the treatment of asthma and COPD |
| B | 8 | Alberto Pla-López | Synthesis and biological evaluation of triazole derivatives as inhibitory agents of cancer multitargets |
| B | 9 | Beatrice Macchi | Triorganotin derivatives act as metabolic inhibitors towards oral squamous cell carcinoma (OSCC) cells through suppression of glucose uptake. |
| B | 10 | Marek Jamrozik | *In silico* and *in vitro* evaluation of ASP9521 – a compound supporting anticancer activity of daunorubicin through the inhibition of aldo-keto reductase 1C3 and carbonyl reductase 1. |
| B | 11 | Lamis Yahia Mohamed Elkheir | Design and synthesis of a novel series of imidazo[1,2-b]pyridazines as antifungals against *Madurella mycetomatis*, the prime causative agent of Eumycetoma |
| B | 12 | Aminesena Başer | Development of activatable fluorophore for use in Super-resolution Fluorescence Microscopy |
| B | 13 | Manuela Sabatino | The Py-ComBinE Web App as a Tool to build Structure-Based Quantitative Models. Application to the BCL-2 Protein family inhibitors. |

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| B | 14 | Limpho Martha Ramorobi | Antidiabetic and antioxidative synergistic potential between zn(ii) and caffeic acid: improving therapeutic potential through complexation |
| B | 15 | Maurizio Pasi | Design and preliminary biological evaluation of dihydro-benzazepine tricyclic derivatives as new NEDD4 covalent inhibitors |
| B | 16 | Sanja Tomić | Dipeptidyl-Peptidase III Cancer Mutations |
| B | 17 | Krystof Skach | Discovery of Human Constitutive Androstane Receptor (CAR) Agonists with Imidazo[1,2-a]pyridine Structure |
| B | 18 | Kristina Puls | Dynamics matter: Deciphering Opioid Receptor Subtype Specificity of the Peripheral Analgesic HS-731 |
| B | 19 | Anna Kieronska-Rudek | Endothelium-targeted delivery of vitamin $K_1$ using hyaluronan-based capsules |
| B | 20 | Anna Szymczyk | New compositions of electrochemical DNA biosensors receptor layers for fast and sensitive detection of SARS-CoV-2 biomarkers |
| B | 21 | Karolina Lejwoda | Photostability and phototoxicity of ocular drugs, azelastine, ketotifen and timolol |
| B | 22 | Mbilo Misehe | Structure-based design and modular synthesis of novel PI4K class II inhibitors with 4-aminoquinazoline scaffold |
| B | 23 | Dioni Arrieche | Study of the cytotoxic activity of ethanolic extracts from four native plants of Northern Chile |
| B | 24 | Kamil Łątka | Virtual screening as a tool for the discovery of new BGT-1 transporter inhibitors |
| B | 25 | Arianna Colcerosa | Synthesis and biological evaluation of Schistosoma mansoni Sirtuin2 (SmSirt2) inhibitors |
| B | 26 | Noemi Villella | Targeting Plasmodium falciparum dihydroorotate dehydrogenase: design, synthesis, co-crystallization and biological evaluation of new 3-hydroxypyrazole scaffold-based inhibitors. |
| B | 27 | Jorge González Garcia | Study of the interaction of far-red fluorescence ligands with G-quadruplex structures |
| B | 28 | Alessandra Salerno | A fragment-based approach for the development of trypanothione reductase inhibitors as antileishmanial agents |

MMCS 2022

3rd Molecules Medicinal Chem osium

Shaping Medicinal Chemistry for the New Decade

27–29 JULY 2022, ROME, ITALY

Central University of Technology, Free State

# Certificate of Attendance

This certificate is given to

## Limpho Ramorobi

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**3rd Molecules Medicinal Chemistry Symposium: Shaping Medicinal Chemistry for the New Decade**

27–29 July 2022

Sapienza University of Rome, Rome, Italy

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