Synthesis, characterisation and evaluation of the antidiabetic and antioxidative properties of caffeic acid and ferulic acid-zinc(II) complexes

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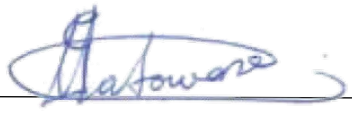
DECLARATION OF INDEPENDENT WORK

I **Retshedisitswe Godfrey Matowane**, 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 fulfillment (or partial fulfilment) of the requirements for the attainment of any qualification. As such, where external sources were utilized, due acknowledgement 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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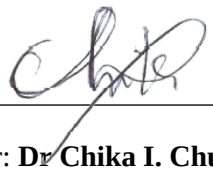

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DEDICATION

This thesis is dedicated to my wife,

Tshegofatso Matowane,

my siblings,

Thabo Matowane and Hlohonolofatso Matowane,

and my parents,

Sello Thomas Matowane and Joyce Matowane

ABSTRACT

Background: The morbidity and mortality outcomes of diabetes, as well as the associated expenditure on health care remain notable contributors to global socioeconomic burden. The close association to oxidative stress is a major factor aggravating the notoriousness of diabetes, which has been implicated in many complications, as well as the morbidity and mortality outcomes of the disease. Therapeutic approaches with holistic functional profiles and minimal side effects are being explored to manage the disease; approaches that could manage hyperglycaemia and mitigate or reduce the risk of oxidative complications. In this context, supplements and phytochemicals have gained popularity due to their safety profile and numerous biological benefits, including antioxidant functions and beneficial role in glucose and lipid metabolism. In recent years, zinc has been explored as a supplement for diabetes due to its function in insulin integrity and function. On the other hand, natural phenolic acids are known dietary antioxidants with diabetes-related pharmacological potentials. In this study, we took advantage of the insulin mimetic potential of zinc and the antioxidant and pharmacological potentials of ferulic and caffeic acid to develop novel zinc(II) complexes of ferulic and caffeic acid, which demonstrated improved antioxidant and antidiabetic effects.

Materials and methods: Zinc sulphate was complexed with ferulic acid, while zinc acetate was complexed with caffeic acid. Zn(II) was complexed with each phenolic acid in a 1:2 mole ratio, respectively. The synthesized complexes were spectroscopically characterized using NMR, FT-IR, high resolution-mass spectroscopy and HP-LC. The cellular toxicity of the complexes was assessed in Chang liver cells and L-myotubes. *In vitro*, cellular, and isolated tissue models were used to evaluate the antioxidant and antidiabetic properties of the complexes, relative to their precursors. Molecular docking was used to investigate the interaction between the complexes and molecular protein targets that are linked to diabetes. These include GLUT-4, protein kinase B (Akt/PKB), α-glucosidase and α-amylase. The zinc complex of caffeic acid was further subject to in *vivo* antidiabetic and antioxidant evaluation. Male SD rats were induced with diabetes using 10% fructose and 40 mg/kg bw streptozotocin. Thereafter, the diabetic rats were treated with the Zn(II)-caffeic acid complex and its precursors (caffeic acid and zinc acetate) for 4 weeks at predetermined doses. The effect of the treatments on diabetes and oxidative stress related parameters was measured.

Results: Complexation resulted in a bi-caffeic acid-zinc acetate complex and a Zn(II)-biferulate.$2H_2O$ complex, thus affording the complexes a moiety of Zn(II) and two moieties of their respective phenolic acids. The complexes showed *in vitro* radical scavenging, antiglycation, α-glucosidase, α-amylase inhibitory activity that were up to 2.6 folds stronger than that of their precursor phenolic acids. The ability of the complexes to inhibit lipid peroxidation and GSH depletion in hepatocytes was comparable to that of ascorbic acid and up to 3 folds more potent than their precursor phenolic acids. Complexation improved the glucose uptake activity of the phenolic acids in L-6 myotubes and isolated rat muscle tissues. Molecular docking showed the complexes had stronger interaction with the target proteins than their precursor phenolic acids. The complexes were not hepatotoxic and myotoxic. The bi-caffeic acid-zinc acetate complex ameliorated diabetic alterations in diabetic rats. It reduced polyphagia and polydipsia and appreciably recovered weight loss. It increased insulin secretion, insulin sensitivity, hepatic and muscle glycogen, muscle hexokinase activity and Akt phosphorylation, which resulted in improved glucose tolerance and reduced blood glucose in diabetic rats. The complex concomitantly reduced systemic and tissue lipid peroxidation and increased antioxidant enzymes activity in diabetic rats. Notably, the complex outperformed the antidiabetic and antioxidative action of its precursors and had a broader bioactivity profile. Complexing zinc acetate with caffeic acid improved their ameliorative effect on insulin resistance by ~24 and 42%, respectively, as well as their anti-hyperglycaemic action by ~24 – 36% and ~42 – 47%, respectively. In some instances, the antidiabetic action of the complex was comparable to metformin, while its antioxidant effect was better than that of metformin. It is plausible that the two moieties of the phenolic acids in each of the complexes potentiated their improved antioxidant action, while Zn(II) conferred a potent glycaemic control modulatory attribute on the complexes, which suggests a complexation-mediated synergistic potential.

Conclusion: Zinc(II) complexation with these phenolic acids may be an alternative approach to improving the efficacy of antidiabetic and antioxidative therapy with minimal adverse or side effects.

Keywords: *Oxidative stress, Diabetes, Zinc(II), Caffeic acid, Ferulic acid, Complexation, Bioactive synergism*.

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LIST OF ABBREVIATIONS AND ACRONYMS

^{1}H NMR	-	Proton nuclear magnetic resonance
ABTS	-	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ADP	-	Adenosine diphosphate
AGEs	-	Advanced glycation end-products
Akt/PKB	-	Protein kinase B
AMPK	-	5' adenosine monophosphate-activated protein kinase
ANOVA	-	Analysis of variance
ATP	-	Adenosine triphosphate
ATCC	-	American Type Culture Collection
BCA	-	Bicinchoninic acid
BSA	-	Bovine serum albumin
cAMP	-	Cyclic adenosine monophosphate
DMEM	-	Dulbecco's Modified Eagle Medium
DMSO	-	Dimethyl sulfoxide
DPP-4	-	Dipeptidyl peptidase 4
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
ELISA	-	Enzyme-linked immunosorbent assay
FAK	-	Focal adhesion kinase
FBG	-	Fasting blood glucose
FFA	-	Free fatty acids
FOXO1	-	Fork-head protein O1

FRAP	-	Fe^{3+} reducing antioxidant power
FSI	-	Fasting serum insulin
FT-IR	-	Fourier transform infrared
GDM	-	Gestational diabetes mellitus
GH	-	Growth hormone
GIP	-	Gastric-inhibiting polypeptides
GLP1	-	Glucagon-like peptide 1
GLUT-1	-	Glucose transporter type 1
GLUT-2	-	Glucose transporter type 2
GLUT-4	-	Glucose transporter type 4
GPDH	-	Glycerol-3-phosphate dehydrogenase
GR	-	Glutathione reductase
GS	-	Glycogen synthase
GSH	-	Reduced glutathione
GSK3	-	Glycogen synthase kinase 3
GT	-	Glucose tolerance
H_2O_2	-	Hydrogen peroxide
HbA1c	-	Glycated haemoglobin
HFD	-	High-fat diet
HOMA-IR	-	Homeostatic Model Assessment for Insulin Resistance
HPLC	-	High-performance lipid chromatography
HR-MS	-	High-resolution mass spectroscopy
IDF	-	International diabetes Federation
IGF-1R	-	Insulin-like growth factor-1 receptor

IGT	-	Impaired glucose tolerance
IR	-	Insulin receptor
IRS-1	-	Insulin receptor substrate 1
MAPK	-	Mitogen-activated protein kinase
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFBG	-	Non-fasting blood glucose
NMR	-	Nuclear magnetic resonance
OGTT	-	Oral glucose tolerance test
PEPCK	-	Phosphoenolpyruvate carboxykinase
PI3K	-	Phosphatidylinositol 3-kinase
pJAK2Tyr813	-	Phosphorylated Janus kinase-2 at tyrosine 813
PPAR	-	Peroxisome proliferator-activated receptor
QTOF	-	Quadrupole time-of-flight
RER	-	Rough endoplasmic reticulum
RNS	-	Reactive nitrogen species
ROS	-	Reactive oxygen species
RPMI	-	Roswell Park Memorial Institute
O^{2-}	-	Superoxide anion
SD	-	Sprague Dawley
SGLT2	-	Sodium-glucose co-transporter 2
SOD	-	Superoxide dismutase
STZ	-	Streptozotocin
T1D	-	Type 1 diabetes
T2D	-	Type 2 diabetes

TBARS	-	Thiobarbituric acid reactive substance
TCA	-	Trichloroacetic acid
TNF-α	-	Tumour necrosis factor alpha
TZD	-	Thiazolidinedione
UPLC	-	ultra-pressure liquid chromatography
ZnT8	-	Zinc transporter 8

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RESEARCH OUTPUTS

Publications and/or submitted manuscripts from this thesis

1. **Matowane, G. R.**, Ramorobi, L. M., Mashele, S. S., Bonnet, S. L., Noreljaleel, A., Swain, S. S., Makhafola, T. J., & Chukwuma, C. I. (2022). Complexation potentiated promising anti-diabetic and anti-oxidative synergism between Zn(II) and ferulic acid: A multimode study. ***Diabetic Medicine***, *39*(9), e14905. https://doi.org/10.1111/dme.14905
2. **Matowane, G. R.**, M Ramorobi, L., S Mashele, S., L Bonnet, S., E M Noreljaleel, A., S Swain, S., J Makhafola, T., & I Chukwuma, C. (2022). Novel Caffeic Acid - Zinc Acetate Complex: Studies on Promising Antidiabetic and Antioxidant Synergism Through Complexation. ***Medicinal Chemistry***, 19, 147 – 162. https://doi.org/10.2174/1573406418666220620144601
3. **Matowane, G.R.**, Mashele, S.S., Makhafola, T.J., & Chukwuma, C.I. Zinc(II) and caffeic acid synergistically improved muscle Akt phosphorylation, insulin action, glycaemic control and antioxidant status in diabetic rats. ***Biomedicine & Pharmacotherapy*** (Manuscript ID: **BIOPHA-D-22-06241**). Submitted for publication on **11 December 2022 (Under review).**

Other co-authored publications during doctoral programme

1. 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 glycemic control and antioxidative study. ***Journal of Food Biochemistry***, *46*(10), e14360. https://doi.org/10.1111/jfbc.14360
2. 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

3. Tshane, L., Mashele, S. S., **Matowane, G. R.**, Bonnet, S. L., Makhafola, T. J., Noreljaleel, A., Swain, S. S., Sekhoacha, M., & Chukwuma, C. I. (2021). Zinc(II) mineral increased the *in vitro*, cellular and *ex vivo* antihyperglycemic and antioxidative pharmacological profile of p-hydroxybenzoic acid upon complexation. ***Journal of Food Biochemistry***, *45*(2), e13609. https://doi.org/10.1111/jfbc.13609
4. Motloung, D. M., Mashele, S. S., **Matowane, G. R.**, Swain, S. S., Bonnet, S. L., Noreljaleel, A., Oyedemi, S. O., & Chukwuma, C. I. (2020). Synthesis, characterization, antidiabetic and antioxidative evaluation of a novel Zn(II)-gallic acid complex with multi-facet activity. ***The Journal of Pharmacy and Pharmacology***, *72*(10), 1412–1426. https://doi.org/10.1111/jphp.13322
5. Chukwuma, C. I., Mashele, S. S., Eze, K. C., **Matowane, G. R.**, Islam, S. M., Bonnet, S. L., Noreljaleel, A., & Ramorobi, L. M. (2020). A comprehensive review on zinc(II) complexes as anti-diabetic agents: The advances, scientific gaps and prospects. ***Pharmacological Research***, *155*, 104744. https://doi.org/10.1016/j.phrs.2020.104744
6. 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. ***Revista Brasileira de Farmacognosia*** (Manuscript ID: **RBFA-D-22-00747R1**). Accepted for publication on **16 January 2023**.

Conference presentation

1. **Matowane RG**, Ramorobi LM, Mashele SS, Bonnet SL , Noreljaleel AME, Makhafola TJ , Chukwuma CI. Zn(II)-syringic acid complex: A novel complex with improved bioactivity and promising antidiabetic and antioxidative potentials. Presentation at the ***54th SASBCP Annual Conference***, which took place virtually on ***22 October 2021***.

CHAPTER 1

INTRODUCTION

Diabetes mellitus is a set of metabolic conditions characterised by chronic hyperglycaemia initiated by variations in insulin secretion and/or ineffectiveness (Tesauro & Mazzotta, 2020). Hyperglycaemia is the major hallmark of the disease (Sadi *et al.*, 2018). The prevalence of diabetes and its complications poses a major global health risk (Zheng *et al.*, 2018). Since the beginning of the 21st century to date, diabetes has been amongst the fastest growing health emergencies worldwide (IDF, 2021). The International Diabetes Federation (IDF) estimates that there are currently 537 million people living with diabetes (IDF, 2021). This number is expected to increase to 643 million by the year 2030, and to reach 783 million before 2045 (IDF, 2021). There are several reasons behind this alarming escalation. The reasons include urbanisation, economic development, sedentary lifestyles, unhealthy eating habits and population ageing (Zheng *et al.*, 2018).

There are three main categories of diabetes, namely: (a) type 1 diabetes, which is triggered by autoimmune destruction of β-cells, leading to total insulin deficiency; (b) type 2 diabetes (T2D), which is caused by progressive loss of insulin sensitivity and insulin secretion from the β-cells; (c) gestational diabetes, which is often detected in the second or third trimester of pregnancy but was not seen before gestation (Tesauro & Mazzotta, 2020). Over 90% of diabetes mellitus cases are T2D (Zheng *et al.*, 2018).

Insulin has a key role to play in maintaining glucose homeostasis, thus preventing hyperglycaemia and its oxidative insult. It has been documented that hyperglycaemia results in the production of reactive oxygen species (ROS), which eventually leads to heightened oxidative stress in a range of tissues (Newsholme *et al.*, 2019). Moreover, oxidative stress is also known to play a pivotal role in the development and progression of diabetic complications (Bhattacharya & Sil, 2018). There are two main categories of diabetes complications: microvascular, which occur because of damage to small blood vessels, and macrovascular, which are a result of damage of larger blood vessels (Blair, 2016; IDF, 2021).

There are several commercial medications for the treatment of diabetes. These include glucagon-like peptide 1 (GLP-1) agonists, sodium-glucose cotransporter (SGLT2), biguanides (metformin), dipeptidyl peptidase 4 (DPP-4) inhibitors, sulfonylureas, α-glucosidase

inhibitors, meglitinide and thiazolidinedione (TZD) (Rines *et al.*, 2016; Chaudhury *et al.*, 2017). Even though the above-mentioned diabetes treatments have displayed appreciable success in reducing blood glucose levels, their impact is sometimes not sustained, and their continual use may lead to undesirable side effects (Rines *et al.*, 2016). To alleviate this epidemic, we urgently need new treatments that are safer and more potent as alternative and/or complementary therapeutic agents (Kleinert *et al.*, 2018).

Plants have been used for medical treatments since the beginning of civilization. Much research has gone into studying medicinal plants and in most cases, they show positive effects on diabetic patients (Bhattacharya & Sil, 2018). These effects are due to various phytoconstituents found in these plants. Dietary polyphenols have been proposed as effective supplements for managing and preventing T2D and associated oxidative insults. Polyphenols are seen as strong antioxidant phytochemicals owing to their unique structures and ability to form stable phenoxy radicals with radical scavenging or quenching attributes (Bhattacharya & Sil, 2018). Antioxidants have been extensively investigated because of their ability to suppress oxidative stress by inhibiting the formation of ROS, scavenging free radicals, or increasing the antioxidants defence enzyme capabilities in diabetes (Bhattacharya & Sil, 2018). There are four main categories of polyphenols: flavonoids, phenolic acids, stilbenes and tannins (Loureiro & Martel 2019). These polyphenols are found in plant-based foods, such as coffee, wine, tea, cocoa, berries, cereal grains, legumes and fruit (Ota & Ulrih, 2017). Simple phenolic acids are very common in many fruits and plant-based foods and are known to contribute to the antioxidant benefits of many such foods (Kumar & Goel, 2019). They have also been shown to potentiate both antioxidant and antidiabetic effects (Vinayagam *et al.*, 2016).

Ferulic acid and caffeic acid are the simple phenolic acids of focus in this study, due to their wide occurrence in fruits, cereals, teas, coffee, vegetables and herbs, as well as their promising antioxidant attributes (Kumar & Goel, 2019). Ferulic acid is a hydroxy-cinnamic acid derivative primarily found in grains and cereals (Mishra *et al.*, 2022). It is used as a food preservative and an antioxidant (Kumar & Goel, 2019). Ferulic acid possesses anticancer, antidiabetic, anti-inflammatory and cardioprotective properties. Ferulic acid is absorbed more easily into the body and remains in the blood longer than any other phenolic acid (Zdunska *et al.*, 2018).

Caffeic acid is also a hydroxy-cinnamic acid derivative found in beverages, including teas and coffee, as well as culinary herbs and some fruits (Mattila & Kumpulainen, 2002). Caffeic acid

exhibits antioxidant, antidiabetic and anticancer activities (Jung *et al.*, 2006). Its treatment on C57BL/KsJ-db/db mice resulted in increased expression of antioxidant enzymes and reduced lipid peroxidation (Jung *et al.*, 2006).

Other than phytochemicals, supplements and vitamins are also beneficial to our overall health, particularly oxidative and metabolic health (Liu *et al.*, 2018; Wong *et al.*, 2020). For the development and preservation of life and health, trace elements are necessary. The absence or insufficiency of these trace elements produces a functional impairment or can result in various diseases. Zinc (Zn(II)) is a functional trace element that is relevant in diabetes-related metabolic processes and is the second most abundant trace element in the human body (Jeddi & Torabi, 2019). However, Zn(II) levels are entirely dependent on dietary intake because Zn(II) cannot be stored by the human body (Jeddi & Torabi, 2019). Zn(II) has been intensively researched as a potential therapeutic option for diabetes in both animal models and human patients due to its antioxidative qualities and its specific involvement in insulin storage and production in the pancreatic β-cells (Gerber & Rutter, 2017). Physiological concentration of Zn(II) inhibits the production of ROS, which could explain the fact that Zn(II) deficiency has been reported to lead to the development of T2D (Olechnowicz *et al.*, 2018; Jeddi & Torabi, 2019). Zn(II) supplementation has demonstrated insulin-improving effects, lowering of blood glucose and helping to improve the functioning of β-cells in preclinical studies (Maret, 2017). To this end, Zn(II) was complexed with different kinds of ligands in an effort to develop insulin mimetic and antidiabetic complexes (Chukwuma *et al.*, 2020).

In both *in vitro* and *in vivo* studies, Zn(II) and its complexes demonstrated insulin-like activity (Chukwuma *et al.*, 2020). Zn(II) complexation with ligand is thought to increase bioavailability while having positive effects on glucose metabolism. The results of these developments confirm that Zn(II) complexes have antidiabetic properties (Chukwuma *et al.*, 2020).

Although Zn(II) complexes have shown encouraging therapeutic potentials, mainly synthetic ligands with negligible pharmacological credence and toxicity concerns have been explored as the ligands for therapeutic Zn(II) complexes (Chukwuma *et al.*, 2020). This suggests a scientific gap or flaw in the research trajectory. On the other hand, dietary supplements or phenolics with diabetes and oxidative stress related pharmacological credence and better safety profile are under-explored as promising ligands for therapeutic Zn(II) complexes (Chukwuma *et al.*, 2020). Studies show that complexes of Zn(II) with some supplements and plant-derived phenolics improved their efficacy and bioavailability, making them potential adjuvants for

antidiabetic phenolics (Chukwuma *et al.*, 2020). Considering the bioactivity and safety profile of caffeic acid and ferulic acid, it is rational to hypothesize that they may be promising ligands in developing potent therapeutic Zn(II) complexes. However, this study has not been undertaken in the present context. Hence the aim of this study is to develop novel Zn(II) complexes of ferulic and caffeic acid and evaluate their antidiabetic and antioxidative potentials.

CHAPTER 2

LITERATURE REVIEW

2.1. Diabetes

Diabetes mellitus is a set of complex metabolic conditions with diverse etiological factors. Diabetes is characterised by chronic hyperglycaemia (high circulating glucose levels), which is associated with an impairment of carbohydrate, fat, and protein metabolisms as a result of deficiencies in insulin secretion, action, or both (Chabosseau & Rutter, 2016; Sadi *et al.*, 2018; Tesauro & Mazzotta, 2020). Patients are at a high risk of developing long-term macro- and microvascular complications because of the ongoing metabolic imbalance associated with this illness, and eventual morbidity and mortality outcomes if they are not provided with high-quality treatment (Chaudhury *et al.*, 2017; Gupta *et al.*, 2017).

Glucose metabolism is predominantly affected by impaired insulin secretion and/or action in diabetes, which cause hyperglycaemia. The defects in glucose metabolism can include the following: (a) reduced glucose uptake into cells; (b) reduced storage and use of glucose by peripheral tissues; (c) increased glucose synthesis by the liver. Persistent hyperglycaemia can result in weight loss, frequent hunger, blurred vision, excessive urination, increased thirst and a compensatory increased fluid intake. These are the main clinical symptoms of diabetes. Also, hyperglycaemia is linked to increased rates of glucose auto-oxidation, non-enzymatic protein glycosylation, and increased influx to the polyol pathway, which are implicated in the development and progression of diabetic complications (Sadi *et al.*, 2018).

2.2. Prevalence of diabetes

One of the biggest international health crises of the twenty-first century is diabetes. In 2021, approximately 6.7 million people between the ages of 20 and 79 are estimated to have died from diabetes and its complications (IDF, 2021). This is equivalent to 12.2% of global deaths from all causes in this age group (IDF, 2021). Diabetes is found in an estimated 537 million adults between the ages of 20 and 79 worldwide and according to projections, 643 million adults aged 20-79 are expected to be living with diabetes by 2030 and 783 million by 2045 (**Figure 2.1**) (IDF, 2021).

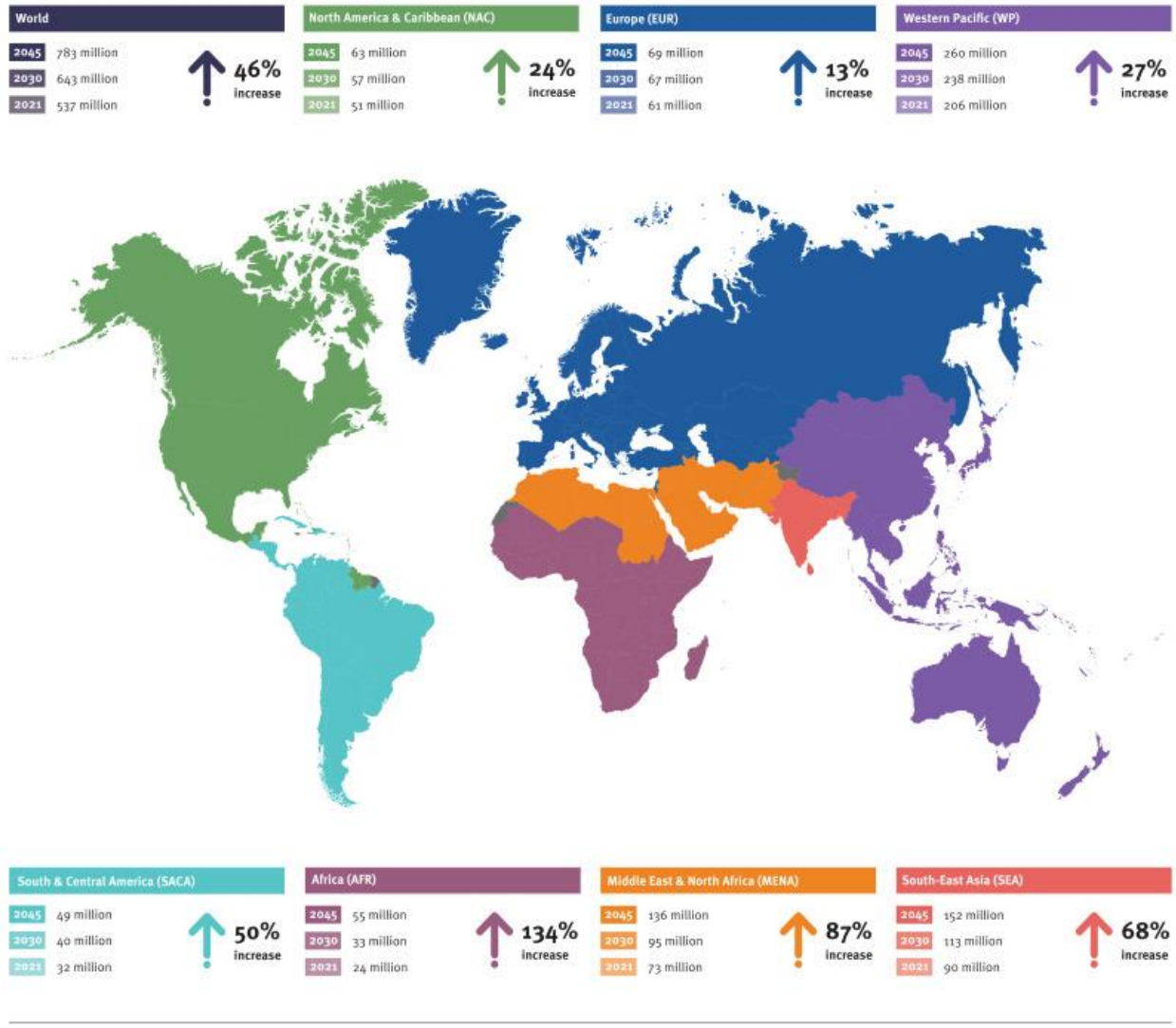

Figure 2.1: Number of people with diabetes worldwide in 2021–2045 (20–79 years) (Adopted without permission from IDF, 2021)

Long considered an illness of the wealthy Western countries of Europe and North America, type 2 diabetes (T2D) has now spread all over the world (**Figure 2.1**). More people living with diabetes now live in emerging countries than in the developed nations. The regions in Africa, the Middle East, and Northern Africa, as well as South-East Asia, are anticipated to experience the greatest increases of people living with diabetes during the next two decades. Compared to people from affluent countries, those from these regions get sicker quicker and die earlier (IDF, 2021).

Table 2.1: Global diabetes estimates and projections.

At a glance	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)			
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	-	-
Total health expenditure due to diabetes (USD)	$966 billion	$1,028 billion	$1,054 billion
Hyperglycaemia in pregnancy (20-49 years)			
Proportion of live births affected	16.7%	-	-
Number of live births affected	21.1 million	-	-
IGT (20-79 years)			
Prevalence	10.6%	11.0%	11.4%
Number of people with IGT	41.0 million	622.7 million	730.3 million
Impaired fasting glucose			
Prevalence	6.2%	6.5%	6.9%
Number of people with impaired glucose tolerance	319.0 million	369.7 million	440.8 million
Type 1 diabetes (0-19 years)			
Number of children and adolescents with T1D	1.2 million	-	-
Number of newly diagnosed cases each year	184,100	-	-

Adopted without permission from IDF, 2021; IGT, impaired glucose tolerance; T1D, type 1 diabetes.

Understandably, the expense of treating the illness and its repercussions has also considerably increased, placing a financial strain on healthcare systems (**Table 2.1**) (IDF, 2021). Clearly, greater action is required to enhance diabetes outcomes and lessen the burden of diabetes worldwide (IDF, 2021).

2.3. Glucose metabolism and insulin signalling

Plasma glucose concentration is determined by the amount of glucose entering the bloodstream and is balanced by the amount of glucose leaving circulation. The three major sources that contribute to plasma glucose are: intestinal absorption after food ingestion, glycogenolysis and gluconeogenesis. Endogenous glucose is mostly obtained from hepatic processes: glycogenolysis and gluconeogenesis during the fasting state (**Figure 2.2**) (Aronoff *et al.*, 2004; Rines *et al.*, 2016).

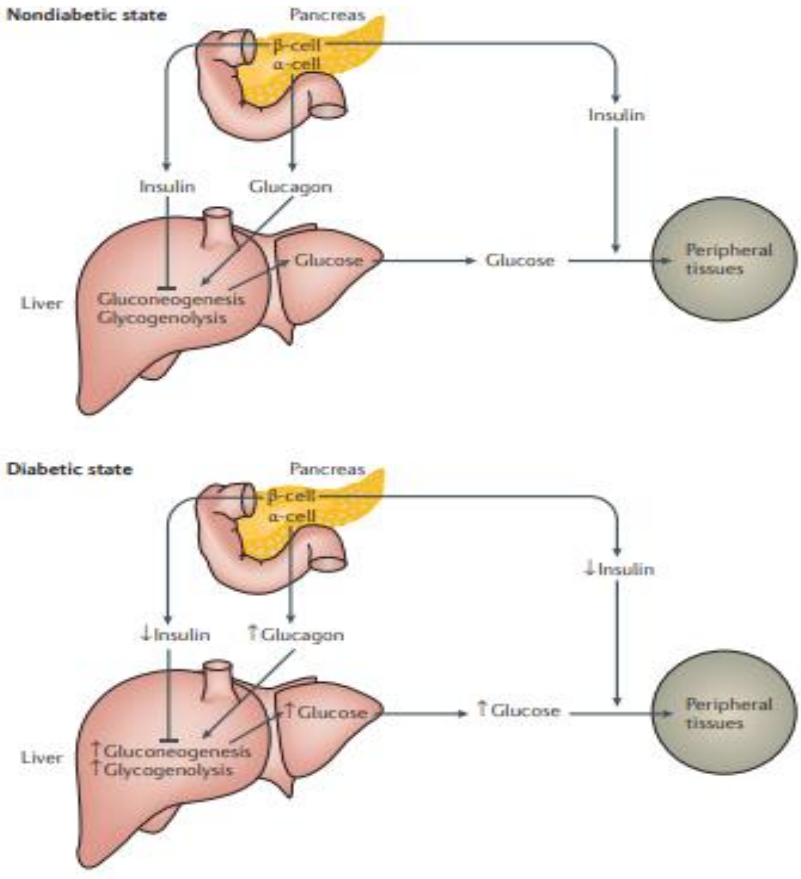

Figure 2.2: Schematic of glucose homeostasis in non-diabetic and diabetic states (Adopted without permission from Rines *et al.*, 2016)

The glucoregulatory hormones are programmed to regulate plasma glucose concentrations within a comparatively small range. Glucose exits the bloodstream at a steady pace in a fasting state and endogenic glucose development is required to keep pace with the disappearance of glucose. The liver is the primary source of endogenous glucose production for all practical purposes (**Figure 2.2**) (Aronoff *et al.*, 2004; Rines *et al.*, 2016). Renal gluconeogenesis adds greatly to the systemic glucose pool only during times of intense malnutrition. Even though numerous tissues are capable of hydrolyzing glycogen, only the liver and kidneys play a significant role in the release of glucose into the bloodstream through the action of glucose-6-phosphatase (Aronoff *et al.*, 2004; Rines *et al.*, 2016).

The pancreatic β-cells in the islets of Langerhans produce the peptide hormone known as active insulin (Rains & Jain, 2011; Ramachandran *et al.*, 2015). This hormone has a molecular weight of 5808 Da and is made up of 51 amino acids (Ramachandran *et al.*, 2015). Initially, pre-pro-insulin is produced as a precursor to insulin. Insulin first migrates to the rough endoplasmic reticulum where signal peptidases remove its signal peptide from the N-terminal, turning it into pro-insulin. The linking strand (C-peptide) from proinsulin is then removed, resulting in the formation of the double-chain insulin molecule. The two chains are joined by two disulfide linkages that attach to cysteine on both chains. One peptide chain comprises 21 amino acids, while the other has 30. Insulin and the C-peptide are both stored in membrane-bound storage granules and released into the portal circulation at the same concentration in response to stimulation. The C-peptide levels are a specific endogenous marker for insulin synthesis (Tesauro & Mazzotta, 2020).

When it comes to maintaining glucose homeostasis, insulin is crucial (Iliya *et al.*, 2016). When fasting, the liver's production of glucose via the pathways of glycogenolysis and gluconeogenesis is coordinated to regulate glucose homeostasis. In fed state, circulating glucose is mainly cleared by hepatic and muscle glycogen synthesis and cellular glucose metabolism/utilization. Insulin increases glucose absorption into muscle and adipose tissue while decreasing hepatic glucose output. The insulin-sensitive glucose transporter type 4 (GLUT-4) mediates glucose absorption in myocytes and adipocytes, whereas the glucose transporter type 2 (GLUT-2) releases glucose in the liver (Chadt & Al-Hasani, 2020).

The regulation of lipid, protein and carbohydrate metabolisms depends largely on insulin. Regarding carbohydrate metabolism, insulin aids in the transfer of glucose into cells, as well as its utilization in energy metabolism. Additionally, insulin promotes the synthesis of

glycogen in the muscles and liver from glucose and lactate sources (Iliya *et al.,* 2016). With respect to protein metabolism, insulin inhibits gluconeogenesis from protein sources in the liver, which is enhanced during diabetes, thus causing excessive production of glucose. Moreover, insulin increases the incorporation of amino acids into peptides to accelerate proteogenesis and growth. It modulates the actions of numerous intracellular structures involved in protein biosynthesis and improves the synthesis of mRNA (Iliya *et al..*, 2016). during fat metabolism, insulin exerts an antiketogenic effect in the liver. Also, it modulates lipogenesis from glucose in adipose tissue, while concomitantly decreasing cholesterolemia and lipemia. It prevents the liver from storing excessive amounts of lipid and inhibits lipolysis in the adipose tissue. Target tissues for insulin action include the liver, muscles, and adipose tissues. Insulin also facilitates the movement of glucose, amino acids, and electrolytes across cell membranes (Iliya *et al..,* 2016).

Insulin secretion follows a daily cycle, with higher release postprandial and lower amounts in the evening. Rapid, low-amplitude secretions alternate with slow, high-amplitude secretions in the hepatic portal vein, where insulin is secreted in an oscillatory pulsatile pattern (Tesauro & Mazzotta, 2020). Insulin secretion involves the transfer of glucose to the β-cell through the GLUT-2, which is eventually phosphorylated by glucokinase and then metabolized to further generate adenosine triphosphate (ATP). The resulting rise in the ATP:ADP (adenosine diphosphate) ratio closes the K^+ATP pathway, which induces depolarization as a result of intracellular K^+ entrapment. This opens the Ca^{2+} voltage-closed channel, which activates the release of insulin. Even a minor increase in glucose levels triggers insulin secretion (Tesauro & Mazzotta, 2020).

Postprandial glucose-induced insulin secretion is mediated through combined activation of many intestinal peptides, such as GLP-1 and gastric-inhibiting polypeptides (GIPs). This phenomenon is known as the incretin effect and is the basis for many of the current T2D treatments. Small intestine L cells release GLP-1 in response to food consumption, which activates insulin secretion. GLP-1 then binds to its receptor, thus stimulating adenyl cyclase. This enables the development of cyclic adenosine monophosphate (cAMP), which enhances glucose-mediated insulin secretion (Tesauro & Mazzotta, 2020). The insulinotropic activity of GLP-1 relies on the concentration of glucose, which explains why GLP-1 does not induce insulin production during fasting cycles (Tesauro & Mazzotta, 2020).

Secreted insulin signals glucose uptake in actively respiring muscle cells or adipocytes through a signalling cascade, also known as the insulin signalling pathway (**Figure 2.3**) (Gabbouj *et al.*, 2019). Insulin has a high affinity for the insulin receptor that is embedded in the cell membrane and a low affinity for the insulin-like growth factor-1 receptor (IGF-1R). The insulin receptor is a hetero-tetrameric structure made up of 2α and 2β subunits connected by disulfide linkages. The α subunits mediate mechanical insulin binding, whereas the β subunits are crucial for autophosphorylation to encourage tyrosine kinase activity, which is required for the initiation of the downstream signalling for the activation of the insulin receptor substrate, especially IRS-1 in the context of GLUT-4-mediated glucose uptake. Insulin works through two main pathways (Gabbouj *et al.*, 2019).

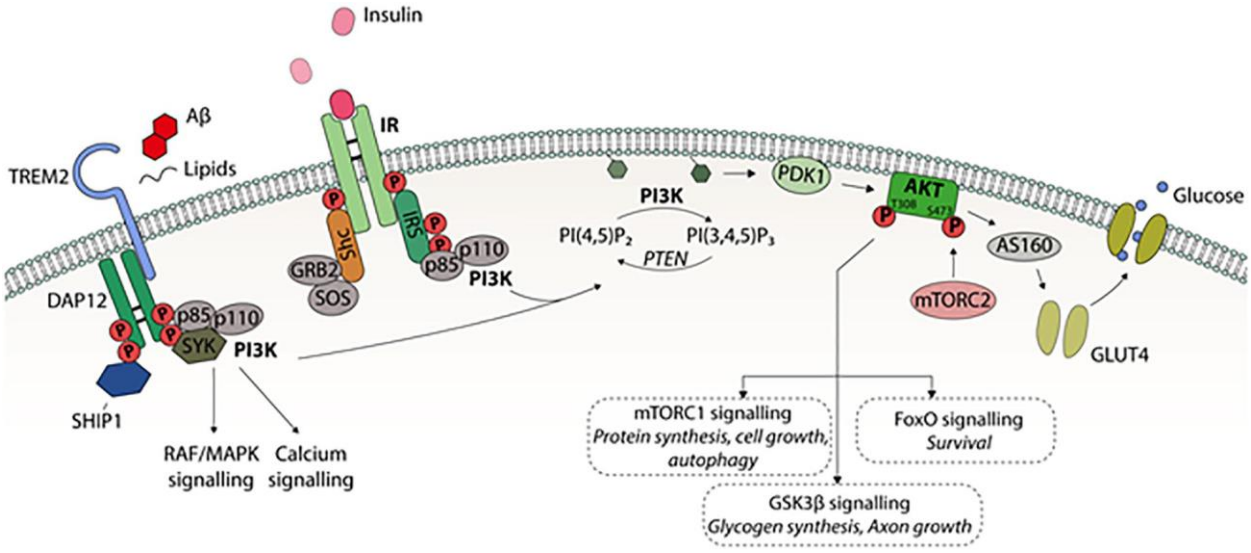

Figure 2.3: Insulin signalling (Adopted without permission from Gabbouj *et al.*, 2019)

The first pathway includes phosphoinositide-3-kinase (PI3K) working downstream by phosphorylation of multistep signalling intermediates to activate protein kinase B (AKT/PKB) (Tesauro & Mazzotta, 2020). Here, insulin induces protein anabolism through the mammalian target of rapamycin (mTOR), increases glycogen synthesis of glycogen synthase kinase 3 (GSK3), promotes cell survival through inhibition of antiapoptotic proteins, enhances gluconeogenesis by increasing the expression of phosphoenolpyruvate carboxykinase (PEPCK) by fork-head protein O1 (FOXO1) and increases GLUT-4 translocation to the plasma membrane. The other pathway includes the activation of two mitogen-activated protein kinases

(MAPKs) known as MEK1 and MEK2. Consequently, these phosphorylate the Elk-1 transcription factor, which promotes the expression of genes that possess a pro-mitogenic role (Tesauro & Mazzotta, 2020).

A key function of insulin is the stimulation of Na^+-independent GLUT channels to the plasma membrane, facilitating glucose absorption in the cells. In the context of cellular glucose uptake, predominantly translocated GLUT channels are GLUT-4. However, GLUT-1 is also translocated by the MAPK pathway in states of elevated insulin concentrations, this is despite GLUT-1 transporters remaining on the plasma membrane permanently (Gabbouj *et al.*, 2019).

Insulin also facilitates glucose absorption through upregulation of the glycolytic enzymes hexokinase and 6-phosphofructokinase to accelerate the rate of glycolysis, allowing for further glucose incorporation in the cell by restoring the concentration gradient through the GLUT transporters. Insulin also encourages glycogen synthesis by directly upregulating glycogen synthase enzyme (Tesauro and Mazzotta, 2020). Additionally, insulin lowers the activity of hormone-sensitive lipase. This results in a decrease in the blood's mobilization of free fatty acids (FFAs), which indirectly promotes the internalization of glucose. Insulin, also, facilitates the expression of malonyl-CoA in the skeletal muscle. Malonyl-CoA decreases the activity of the enzyme carnitine palmitoyltransferase-1, which reduces mitochondrial shuttling and, thus, reduces β oxidation (Tesauro and Mazzotta, 2020).

2.4. Types of diabetes

Type 1 diabetes (T1D), type 2 diabetes (T2D), and gestational diabetes are the three main types of diabetes.

2.4.1. Gestational diabetes

Gestational diabetes mellitus (GDM) is essentially hyperglycaemia that is first detected during pregnancy (Tesauro & Mazzotta, 2020). According to IDF (2021) estimates, there were some forms of hyperglycaemia in pregnancy in 21.1 million live births to women in 2021. 80.3% of these were brought on by GDM (IDF, 2021). GDM typically affects pregnant women in their second and third trimesters of pregnancy, but it may occur at any point of the pregnancy

(Gilbert *et al.*, 2019). GDM is presently the most common medical complication of pregnancy (McIntyre *et al.*, 2019, IDF, 2021).

The incidence of GDM is rising globally because of rising obesity rates and older mothers, placing a significant financial burden on the public health care system. In fact, GDM increases the risk of serious pregnancy problems that affect both mother and unborn child. These include caesarean birth, shoulder dystocia, macrosomia, and new-born hypoglycaemia. Additionally, women with GDM are significantly more likely to become obese, acquire hypertension, T2D, and cardiovascular disease after giving birth, while their children are more likely to grow up obese and develop T2D (Chiefari *et al.*, 2017).

Managing GDM generally requires frequent monitoring of blood glucose levels, ensuring it is within the normal range for pregnant women (Sandu *et al.*, 2021). Also, dietary alterations and exercise can help in managing GDM. Insulin or hypoglycaemic agents may be used when abnormally high blood glucose persists (Sandu *et al.*, 2021).

2.4.2. Type 1 diabetes

Type 1 diabetes is a multifactorial disorder in which genetic predisposition is linked to an initiating occurrence that stimulates autoimmune pathways within the pancreatic β-cells, causing a failure of insulin secretion (Tesauro & Mazzotta, 2020). It is essentially an autoimmune-mediated deterioration of insulin-producing pancreatic β-cells, which leads to loss of insulin secretion and later hyperglycaemia (Newsholme *et al.*, 2019). This means that very little or no insulin is produced by the body. Postprandial glucose concentrations increase due to insufficient insulin-stimulated glucose disappearance, poorly controlled hepatic glucose synthesis and accelerated or irregular stomach emptying after eating (Aronoff *et al.*, 2004).

Although type 1 diabetes can occur at any age, the disease tends to affect mostly children and adolescents (IDF, 2021). Nevertheless, its manifestation is frequently seen in adults (Jansen *et al.*, 2009; IDF, 2021). Daily exogenous insulin treatment is required for people with type 1 diabetes to maintain glucose homeostasis and prevent both early and late diabetic complications (Jansen *et al.*, 2009; IDF, 2021). The pathogenesis of T1D is proposed as a continuum separated into phases linked to the detection of autoantibodies and progress to β-cell death, dysglycaemia and, eventually, signs consistent with hyperglycaemia (Katsarou *et al.*, 2017).

2.4.3. Type 2 diabetes

The most common form of diabetes, T2D, poses a danger to both the general public's health and the socioeconomic standing of people in much of the world (IDF, 2021). About 90% of all instances of diabetes are T2D (IDF, 2021). It is a very common disease in many modern societies, and it is fast becoming a serious global public health threat.

T2D is characterised by diminished peripheral insulin sensitivity, loss of adequate glucose uptake in target tissues such as skeletal muscle, liver and adipose tissue, compromised hepatic glucose output and decreased pancreatic β-cell function (Galicia-Garcia *et al.,* 2020). These metabolic defects eventually lead to failure of the β-cells (Hudish *et al.,* 2019). Hyperinsulinemia occurs as insulin secretion cannot compensate for insulin resistance and this eventually exhausts and damages the β-cells, thus leading to impaired insulin secretion (Cerf, 2013). Elevated insulin resistance and progressive loss of insulin secretion are two of the key pathophysiological defects that contribute to hyperglycaemia in T2D (Chukwuma *et al.,* 2018; Galicia-Garcia *et al.*, 2020). T2D has a large genetic aspect that is influenced by environmental factors such as age, weight, nutrition and a lack of physical activity (Tesauro & Mazzotta, 2020). T2D is more prevalent in the elderly, however, it is being observed more and more in children, adolescents and younger adults as a result of increasing levels of obesity, physical inactivity and poor diet (IDF, 2021).

2.5. Etiology and pathophysiology of T2D

2.5.1. Insulin resistance

As mentioned above, T2D is characterised by insulin resistance accompanied by pancreatic β-cell dysfunction (Jung *et al.*, 2006). 'Insulin resistance' is the phrase used to describe a reduced sensitivity to the effects of insulin on target tissue (Tesauro & Mazzotta, 2020). Some risk factors include poor dieting, obesity and sedentary behaviour (Czech, 2017). It primarily affects the skeletal muscle, which predominantly internalizes and utilizes glucose, relative to other peripheral tissues. Insulin antagonists such as excess glucagon, adrenaline, growth hormone (GH), and catecholamines are present in some diabetes patients and contribute to insulin resistance by preventing insulin physiological actions (Tesauro & Mazzotta, 2020). Thus, by extension, insulin resistance is a deficiency in signal transduction.

The action of insulin in insulin-sensitive tissues including the liver, muscle, and adipose tissue and insulin production by pancreatic islet β-cells occur without synchrony when the feedback loops between insulin action and insulin secretion are not functioning properly, resulting in elevated blood glucose levels (Galicia-Garcia *et al.*, 2020). Insulin resistance in T2D triggers increased glucose synthesis in the liver and impaired glucose absorption in muscle and adipose tissue. In addition, β-cell dysfunction leads to diminished insulin release which becomes inadequate for maintaining normal glucose levels (Zheng *et al.*, 2018). Insulin resistance is already well established when impaired glucose tolerance is observed, and the rise in glucose, even within the normal range, is brought on by a persistent loss in β-cell activity (Wilcox, 2005; Cai *et al.*, 2019). Crosstalk between the β-cell and the insulin-sensitive tissues is necessary for this feedback loop to function. The insulin-sensitive tissues' ability to absorb glucose, amino acids, and fatty acids is mediated by insulin, which is released in response to β-cell stimulation (Fu *et al.*, 2013). In turn, these tissues communicate with the islet to indicate their need for insulin. The β-cell boosts its insulin production to maintain normal glucose tolerance when insulin resistance is present, which is most frequently encountered in obesity. However, when the β-cell is incapable of this task, the result is an increase in blood glucose (Kahn *et al.*, 2014). More gradual loss of β-cell activity is ultimately the result of the growing natural history of the condition, from reduced glucose tolerance to T2D (Kahn *et al.*, 2014; Alejandro *et al.*, 2015).

2.5.2. Hyperinsulinemia and partial pancreatic β-cell dysfunction

Hyperinsulinemia is linked to down-regulation of insulin receptors and metabolic stress, which contribute to the fatigue and damage of insulin producing β-cells (**Figure 2.4**) (Jansen *et al.*, 2009). It has been documented that functional pancreatic β-cell mass reduces over time and is aggravated in T2D (Saisho, 2019). The excessive compensatory production of insulin during this period of insulin resistance leads to a hyperinsulinemic condition (Saisho, 2019) and consequently exhausts the pancreatic β-cells, thus impeding their function and insulin secretion (Kahn *et al.*, 2014; Saisho, 2019). The clinical picture of hyperglycaemia in diabetes is characterised by peripheral insulin resistance, increasing β-cell failure, and decreased availability of insulin, amylin, and GLP-1 (Aronoff *et al.*, 2004).

Genetic predisposition and metabolic stress and inflammation are some of the major factors that cause β-cell failure damage in T2D (Saisho, 2019). Chronic hyperglycaemia leads to β-cell deficiencies through overproduction of glucose within the cells, resulting in endoplasmic

reticulum (ER) and mitochondrial stress (Galicia-Garcia *et al.*, 2020). The unfolded protein response is triggered by ER stress and results in cell death, while too much glucose substrate in the mitochondria results in too much ROS generation. With hyperglycaemia, the mitochondrial membrane's integrity gradually deteriorates over time, allowing cytochrome C to escape into the cytosol and ultimately causing apoptosis in the affected β-cells (**Figure 2.4**) (Saisho, 2019). B-cells are exposed to elevated glucose concentrations for an extended period when there is chronic hyperglycaemia. When the typical process of glycolysis is saturated, surplus glucose is diverted to other ROS-forming routes, including as the glucosamine pathway, glucose autoxidation, and glycosylation, all of which cause an accumulation of ROS and the development of oxidative stress (Wang and Wang, 2017).

2.5.3. Impaired insulin secretion, glucose intolerance and persistent hyperglycaemia

Normal pancreatic β-cells can counteract insulin resistance by secreting more insulin; however, prolonged exposure of pancreatic β-cells to high glucose levels results in β-cell dysfunction, which is linked to decreased insulin secretion and biosynthesis (Jung *et al.*, 2006).

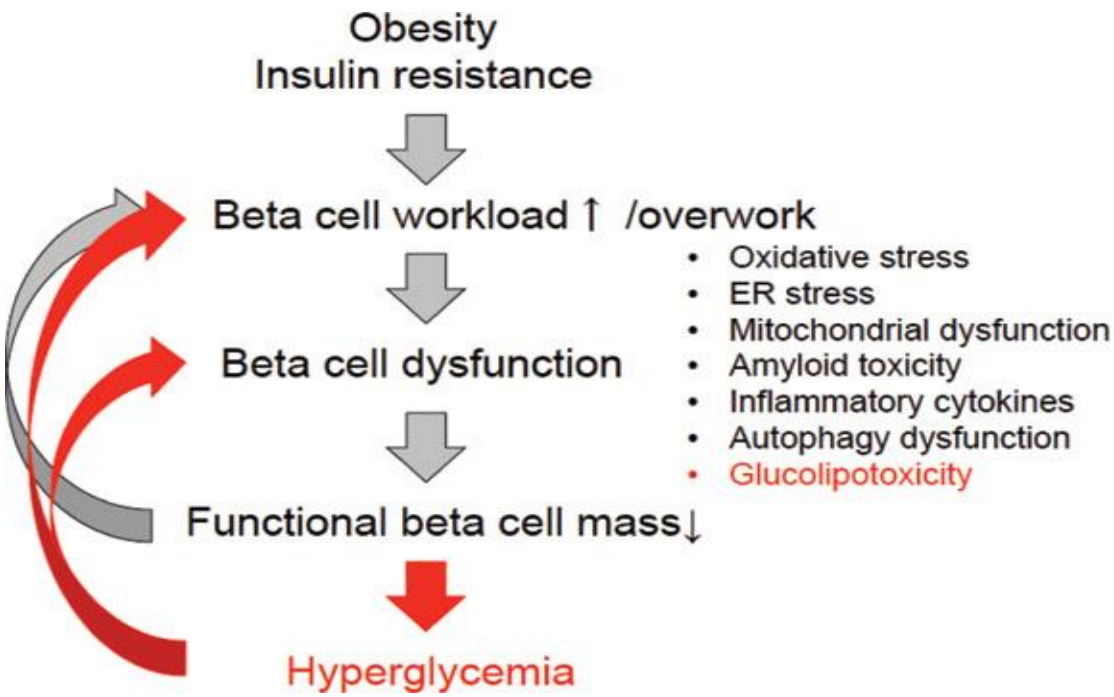

Figure 2.4: β-cell failure in T2D (Adopted without permission from Saisho, 2019)

These disorders in insulin action and glucose metabolism eventually lead to poor blood glucose homeostasis or glycaemic control and persistent hyperglycaemia (Pourghassem-Gargari *et al.*, 2011). Chronic hyperglycaemia encourages the oxidation of proteins, lipids, and glucose, which raises the physiological levels of free radicals and ROS and results in oxidative stress (Pourghassem-Gargari *et al.*, 2011; Bhattacharya & Sil, 2018).

2.6. Oxidative stress and diabetes

A free radical is any chemical species which comprises one or more unpaired electrons in its outer shell (Phaniendra *et al.*, 2015). These are made from molecules when a chemical connection is broken in a way that each piece of the bond keeps one electron. Free radicals are produced by mitochondria as a result of the production of ATP when cells use oxygen to fuel their energy production (Pharm-Huy *et al.*, 2008). Free radicals are also produced from external sources like pollution, cigarette smoke, radiation, and medications, among others (Lobo *et al.*, 2010). Some oxidants are produced as by-products that can easily lead to free radical reactions in living organisms. ROS and reactive nitrogen species (RNS) are more inclusive terms defining both radical and non-radical oxidants (Phaniendra *et al.*, 2015). Due to their potential for either injury or benefit to the body, ROS and RNS serve a dual purpose as both poisonous and helpful molecules (Pharm-Huy *et al.*, 2008). They have positive effects on cellular reactions and immunological activities at low concentrations, whilst on the other hand, high concentrations and where the abundance of free radicals produced cannot be sufficiently removed by the cells, normal tissue may be damaged, leading to a phenomenon called oxidative stress (Di Meo *et al.*, 2016; Bhattacharya & Sil, 2018). Therefore, oxidative stress is essentially an imbalance between the biological systems' antioxidant defences and ROS generation, leading to cellular injury (Betteridge, 2000; Balasubashini *et al.*, 2004; Sies *et al.*, 2017).

T2D is associated with increased oxidative stress and oxidative damage due to increased formation of free radicals (Pourghassem-Gargari *et al.*, 2011; Ramachandran *et al.*, 2015; Babiker & Dubayee, 2017). ROS causes oxidative damage to biological molecules like proteins and nucleic acids (Cruz *et al.*, 2015). Also, oxidative stress increases lipid peroxidation, which compromises cell membrane integrity, thus causing oxidative cellular injury (Cruz *et al.*, 2015).

It is important to note that β-cells are among the most metabolically active tissues and rely on oxidative phosphorylation for ATP synthesis due to the high demand for insulin (Wang & Wang, 2017). In addition, high oxygen consumption is a major factor for insulin secretion,

particularly when responding to high blood glucose levels, which increases the risk of ROS production and oxidative stress for β-cells (Galicia-Garcia *et al.*, 2020). Moreover, β-cells are extremely susceptible to oxidative stress, primarily due to their minimal of antioxidant enzymes content relative to other tissues, which therefore compromises the ability of β-cells to defend themselves against oxidative stress (Wang & Wang, 2017).

The cell, or even the tissue, is overrun by oxidative stress in the absence of an adequate compensatory response from oxidative stress, which activates intracellular stress-related pathways (Gerber & Rutter, 2017). Activation of these has been implicated in mediating insulin resistance, impaired insulin secretion and late diabetic complications.

Under normal conditions, the body is equipped with a network of antioxidant mechanisms to mitigate the insults caused by oxidative stress (Kurutas, 2015). This antioxidant system is, however, compromised in diabetic conditions (Singh *et al.*, 2022).

To sustain oxidative homeostasis, free radicals and antioxidant defence mechanisms must balance one another. Disequilibrium in favour of pro-oxidants would lead to pathological oxidative stress. The cellular antioxidant defence system counteracts oxidative stress by inhibiting the activity of free radicals through several mechanisms, including the destruction of free radicals or conversion into less harmful products, binding or chelating metals that stimulate the production of free radicals, inhibiting the formation of free radicals and acting as scavengers of free radicals (Malireddy *et al.*, 2012). Cellular antioxidants comprise both non-enzymatic molecules and enzymes (Rains & Jain, 2011; Malireddy, *et al.*, 2012).

Natural supplements like vitamins and glutathione that help to scavenge free radicals or unpaired electrons are non-enzymatic antioxidants (Pietta, 2000; Mirończuk-Chodakowska *et al.*, 2018). They consist of, amongst others, carotenoids (vitamin A), α-tocopherol (vitamin E) GSH, and L-ascorbic acid (vitamin C) (Evans & Halliwell, 2001; Mirończuk-Chodakowska *et al.*, 2018).

The enzymatic antioxidants consist of superoxide dismutase, catalase, glutathione peroxidase, glutathione S-transferase and glutathione reductase (Asmat *et al.*, 2016). When present in low quantities, they aid in delaying or inhibiting the oxidation of biological substrates or molecules by scavenging and neutralizing pro-oxidants or transforming them into non-toxic forms (Mirończuk-Chodakowska *et al.*, 2018). Superoxide dismutase (SOD) helps to reduce superoxide anion (O^{2-}) to oxygen and hydrogen peroxide (H_2O_2); catalase, which helps to decompose H_2O_2 into water and oxygen; glutathione peroxidase which helps reduce H_2O_2 to

water and oxygen or organic peroxides to alcohol and oxygen; glutathione reductase, which helps to replenish reduced glutathione in the antioxidant system (Mirończuk-Chodakowska *et al.*, 2018). Micronutrients like selenium, copper, and zinc, as well as other cofactors, support the functioning of these antioxidant enzymes (Mirończuk-Chodakowska *et al.*, 2018).

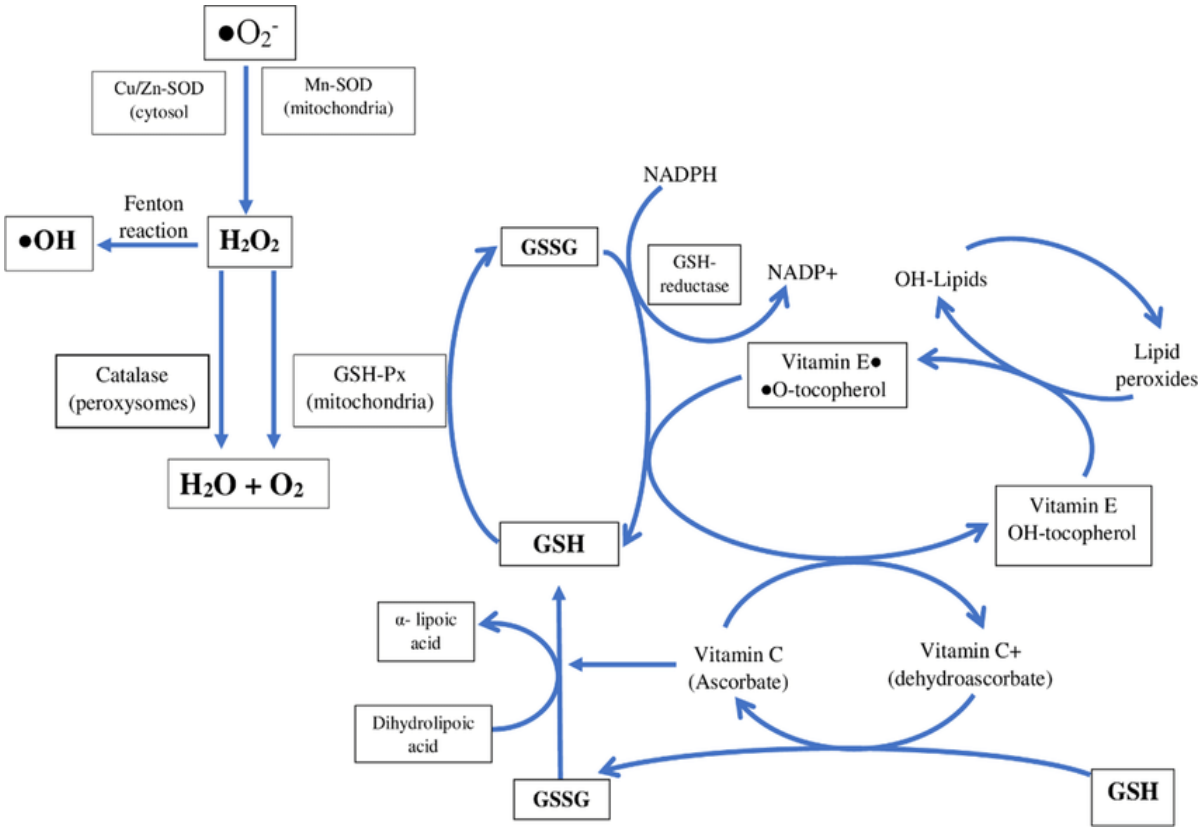

Figure 2.5: The antioxidant defence system (Adopted without permission from Mirończuk-Chodakowska *et al.* 2018)

The capacity of antioxidants to ward off the oxidative stress brought on by hyperglycaemia and increased amounts of free fatty acids raises the possibility that oxidative stress plays a causal role in the onset of diabetic complications (Rehman & Akash, 2017; Newsholme *et al.*, 2019). In fact, increased oxidative stress is a broadly accepted participant in the pathogenesis of diabetes complications and still represents the main culprit in diabetic morbidity and mortality (Balasubashini, 2004; Iacobini *et al.*, 2021).

2.7. Diabetic complications

Excessive ROS/RNS production and the resulting oxidative stress contributes to the development and progression of T2D and its complications (Newsholme *et al.*, 2016; Gerber & Rutter, 2017; Ekoru *et al.*, 2019; Papachristoforou *et al.*, 2020; Singh *et al.*, 2022). There are two types of diabetes complications: microvascular and macrovascular. Microvascular complications are due to the damage of small blood vessels, whilst macrovascular complications are caused by damage to larger blood vessels. Microvascular complications include kidney injury (nephropathy), leading to renal failure; retinal injury (retinopathy), leading to blindness; and the damage to peripheral nerves (neuropathy), causing impotence and diabetic foot disorders that can result in infections and amputations. Macrovascular complications comprise cardiovascular diseases, such as strokes, heart attacks, and atherosclerosis (Blair., 2016; Khamaisi *et al.*, 2019).

Retinopathies manifest in non-proliferative and proliferative forms. Non-proliferative retinopathy occurs as narrow blood vessels become slightly blocked, leading to microaneurysms and the leakage of capillary fluid (Curtis *et al.*, 2009). Proliferative retinopathy occurs when retinal capillaries become occluded and the body forms new capillaries as a result (Nentwich & Ulbig, 2015). Haemorrhage results from these aberrant and weak new vessels and tears may result from the developing vessels pulling the retina out of alignment. Consequently, diabetes patients are frequently prone to develop glaucoma and cataracts (Blair, 2016).

More than half of all diabetics acquire diabetic neuropathy (Feldman *et al.*, 2019). The nerves and blood vessels are likely damaged as a result of constant elevated blood glucose. Ageing, obesity, and having a related peripheral vascular disease are additional risk factors. Alterations in sensation and ischemia are symptoms of peripheral neuropathy, which often affects both the lower and upper body, initially in the lower limbs and then progressively migrating upward (Kazamel & Dyck, 2015). Diabetes patients can experience a complete lack of sensation, paresthesia, numbness, and loss of temperature sensation. Complications resulting from this include ulcers, amputations, muscular atrophy, and loss of fine movements. The "diabetes foot" is the most typical form of presentation, when an injury that the patient was unaware of because of loss of sensitivity precedes an infected ulcer (Blair, 2016).

Furthermore, diabetes patients have a twofold increased risk for cardiovascular disease. This is because diabetes exacerbates the mechanisms that cause atherosclerosis and heart failure (Abdul-Ghani *et al.*, 2017; Einarson *et al.*, 2018).

The detrimental macrovascular and microvascular consequences will continue to be a significant burden for decades to come if more coordinated efforts are not made to address the pathophysiology and management of this disease.

2.8. Management of diabetes

Although genetic makeup may contribute to how an individual responds to environmental changes, the key drivers of the global epidemic of T2D include the rise in obesity, sedentary lifestyles, energy-dense diets, and population ageing (Zheng *et al.*, 2018). Therefore, it is anticipated that diabetes will become an even bigger health issue in the future, meriting more attention.

2.8.1. Commercially available antidiabetic drugs

Current therapy for diabetes incorporates a change of lifestyle, such as diet and exercise, and the application of a range of pharmacological agents (Marín-Peñalver *et al.*, 2016). Non-pharmacological methods, involving dietary management, increased exercise, and patient education, are applied as first-line treatment of diabetes (Raveendran *et al.*, 2018). Pharmacological tools become necessary for the treatment of diabetes when there is a very high blood glucose level and nutritional and physical activity fail to improve the diabetic conditions (Rendell, 2004). Pharmacological therapy can be thought of as a tactic to prevent the development of T2D complications by managing hyperglycaemia (Zheng *et al.*, 2018). These medications aim to reduce hepatic glucose synthesis, boost insulin sensitivity, and improve insulin secretion (Balasubashini *et al.*, 2004).

Metformin, a biguanide, is the most utilized initial pharmacological treatment in the world. A variety of combination therapy alternatives are available if treatment with a single antidiabetic drug is insufficient. These therapy options include sulfonylureas, thiazolidinediones (TZD), dipeptidyl peptidase 4 (DPP-4) inhibitors, sodium-glucose cotransporter (SGLT2) inhibitors,

GLP-1 agonists, and acarbose. Insulin injections may be prescribed if oral hypoglycaemic drugs fail to manage hyperglycaemia to specified targets (IDF, 2021).

Biguanides (metformin)

The Middle Ages saw the beginning of the discovery of biguanide and its derivatives for the treatment of diabetes. It was discovered that the herbaceous plant *Galega officinalis* contains the compounds guanidine, galegine, and biguanide, which lower blood glucose levels (Chaudhury *et al.*, 2017). Biguanides act by stimulating glycolysis in tissues, increasing glucose removal from blood, reducing hepatic gluconeogenesis, slowing glucose absorption from gastrointestinal tract, and reducing plasma glucagon level (Di Magno *et al.*, 2022).

Through intricate interactions with the mitochondrial enzymes, metformin activates the liver's adenosine monophosphate-activated protein kinase, causing hepatic absorption of glucose and blocking gluconeogenesis (Rena *et al.*, 2017). By lowering hepatic glucose production and making peripheral tissues more responsive to insulin, metformin has been demonstrated to slow the progression of T2D, lower the risk of complications, and decrease mortality rates in patients (Chatterjee *et al.*, 2017). Additionally, it raises tyrosine kinase activity and activates insulin receptor expression to increase insulin sensitivity (Chatterjee *et al.*, 2017). Additionally, recent research reveals that metformin reduces cardiovascular illnesses by lowering plasma lipid levels via a peroxisome proliferator-activated receptor (PPAR)- route (Chaudhury *et al.*, 2017).

Metformin

Metformin, which has a half-life of around 5 hours, is absorbed by organic cation transporters after consumption. It is then broadly dispersed throughout the body's tissues, including the colon, liver, and kidney (Chaudhury *et al.*, 2017; Di Magno *et al.*, 2022). The kidney is the main organ of excretion. Patients with advanced stages of renal failure should not use metformin (Chaudhury *et al.*, 2017).

Metformin is generally well tolerated. Reduced food intake may result from incretin-like activities mediated by the hormone GLP-1. Thus, metformin may result in some weight loss in those at risk for diabetes who are overweight or obese (Chaudhury *et al.*, 2017; Chatterjee *et al.*, 2017).

Despite being regarded as safe, metformin has gastrointestinal side effects including nausea, anorexia, abdominal discomfort and diarrhoea (Nasri & Rafieian-Kopaei, 2014; Rines *et al.*, 2016; Marin-Penalver *et al.*, 2016). Also, metformin reduces intestinal absorption of vitamin B12 and is thus associated with vitamin B12 deficiency and folic acid deficiency (Marin-Penalver *et al.*, 2016; Chatterjee *et al.*, 2017). A less common side effect is lactic acidosis (Marin-Penalver *et al.*, 2016).

The fact that metformin loses effectiveness as diabetes worsens is another possible issue associated with its use. When there is sufficient insulin production, metformin is quite effective. However, metformin exhibits diminished efficacy when diabetes progresses to the point of β-cell failure, resulting in a type 1 diabetes phenotype (Chaudhury *et al.*, 2017). This necessitates a combination therapy with other medications (Rines *et al.*, 2016).

Thiazolidinedione (TZD)

The thiazolidinedione (TZD) class of medicines promote glucose absorption into peripheral tissues by acting as agonists for the peroxisome proliferator-activated receptor (PPAR). These tissues include adipose, muscle, and liver, thereby increasing insulin sensitivity. TZDs enhance insulin activity similarly to biguanides (Chaudhury *et al.*, 2017). TZDs also prevent the release of free fatty acids by preventing adipose tissue lipolysis (Rines *et al.*, 2016). Adiponectin levels are increased, free fatty acid build up is decreased, inflammatory cytokines are decreased, and β-cell integrity and function are preserved. These actions together enhance insulin resistance and β-cell function (Chaudhury *et al.*, 2017).

TZDs increase insulin sensitivity by increasing glucose utilization and reducing glucose production in the muscle, adipose tissue and liver. It binds to peroxisome proliferator-activated receptors (PPARs) (Liberato *et al.*, 2012). PPAR-gamma is mostly present in the brain, adipose tissue, macrophages, vascular endothelium, and pancreatic beta-cells. Obese and diabetic people have higher levels of PPAR-gamma in their skeletal muscle (Monsalve *et al.*, 2013). Weight gain caused by TZD is partially caused by the stimulation of PPAR-gamma in the central nervous system, which promotes increased feeding (Stump *et al.*, 2015).

TZDs include rosiglitazone and pioglitazone (Marin-Penalver *et al.*, 2016). Pioglitazone has some PPAR-alpha actions, whereas rosiglitazone is solely a PPAR-gamma agonist; as a result, they each have different effects on lipids. Pioglitazone produces a more beneficial lipid profile,

relative to Rosiglitazone (Lebovitz, 2019). LDL-cholesterol remains unaltered during treatment with Pioglitazone, while Rosiglitazone raises LDL-cholesterol (Marin-Penalver *et al.*, 2016). Also, Pioglitazone has a more reducing effect on triglyceride levels than Rosiglitazone (Marin-Penalver *et al.*, 2016).

TZDs are appreciably effective in combination therapy but are not preferred first-line or step-up therapy (Chaudhury *et al.*, 2017). Their use is associated with some adverse effects. They are, also, relatively more expensive than metformin, which has to some extent discouraged their use. It is recommended that TZDs should not be offered to diabetic patients with a history of heart failure or low bone mass (Marin-Penalver *et al.*, 2016). The major adverse effects of TZDs are edema, hypoglycaemia, weight gain/water retention, and heart failure (Erdmann *et al.*, 2009). Potential complications of this class of drugs include increased risks of myocardial infarction, skeletal fractures and bladder cancer (Rines *et al.*, 2016).

Sodium-glucose co-transporter 2 (SGLT2) inhibitors

Inhibiting SGLT2, which prevents the kidneys from reabsorbing glucose, is another strategy for reducing blood sugar levels and thus increasing urinary glucose excretion (Rines *et al.*, 2016; Chaudhury *et al.*, 2017). SGLT2 inhibitors are medications that reduce blood sugar without requiring insulin. By inhibiting SGLT2, they often prevent the proximal renal tubule from reabsorbing glucose (Chaudhury *et al.*, 2017).

Healthy individuals' kidneys filter 180 g of glucose each day through the renal glomerulus, where it is then reabsorbed in the proximal convoluted tubule (Provenzano *et al.*, 2021). This is made feasible by glucose transporter (GLUT) and SGLT conveyors, two types of passive and active co-carriers. There are two different forms of SGLT; SGLT1 is mostly found in the small intestine and proximal convoluted tubule, whereas SGLT2 is only found in segments 1 and 2 of the proximal tubule. Together, these two types of SGLT account for about 90% of glucose absorption. The other 10% of the glucose is reabsorbed by SGLT1 in segment 3 (Abdul Ghani *et al.*, 2015). SGLT2 inhibitors prevent the proximal tubule's SGLT2 transporter from carrying glucose, which reduces reabsorption and increases excretion in the urine (Abdul Ghani *et al.*, 2015). Since the glycaemic control mechanism of SGLT2 inhibitors is independent of insulin, there is low risk for hypoglycaemia, and none of exhaustion or overstimulation of the β-cells (Keller *et al.*, 2022). The SGLT2 efficiency of SGLT2 inhibitors is lower in patients

with renal failure since their mode of action requires a normal glomerular-tubular function (Marin-Penalver *et al.*, 2016).

Inhibitors of SGLT2, such as canagliflozin, dapagliflozin and empagliflozin, have been authorized as treatment for T2D (Ramachandran *et al.*, 2015; Chaudhury *et al.*, 2017). These inhibitors work well to lower hyperglycaemia and may lessen negative cardiovascular consequences in T2D patients (Rines *et al.*, 2016). Compared to placebo, these medications lower glycosylated haemoglobin (HbA1c) by 0.5–1.0% (5.5–11 mmol/mol) (Ramachandran *et al.*, 2015).

Increased glucosuria is associated with increased risk of urogenital tract infections. These are the most frequent side effects of SGLT2 inhibitors, particularly in females and men who have not undergone circumcision (Chaudhury *et al.*, 2017). Vulvovaginal candidiasis, vulvitis, vulvovaginitis, and vulvovaginal mycotic infection are examples of genital mycotic infections that may be found in female patients. Male patients on the other hand can be affected by balanitis and balanoposthitis. SGLT2 inhibitors are also associated with osmotic diuresis (Marin-Penalver *et al.*, 2016). Hypotension, vertigo, and deteriorating renal function are the less frequent negative effects (Ramachandran *et al.*, 2015; Rines *et al.*, 2016). Ketoacidosis is another rare side effect of SGLT2 inhibitors and patients are strongly advised to discontinue using SGLT2 inhibitors and seek medical help right away if they have ketoacidosis symptoms (Chaudhury *et al.*, 2017).

Sulfonylureas

The main oral drugs for treating hyperglycaemia in T2D are sulfonylureas (Rendell, 2004; Sola *et al.*, 2015). Since their introduction to clinical practice in the 1950s, sulfonylureas have been routinely used as a first- or second-line therapy for patients with T2D (Genuth, 2015). They have been used as a benchmark to contrast the effectiveness and safety of various hypoglycaemic medications, excluding insulin (Marin-Penalver *et al.*, 2016; Chaudhury *et al.*, 2017). Due to their long history of efficacy and safety, low price, and comprehensive clinical trial data confirming good glucose-lowering efficacy, sulfonylureas are the most affordable glucose-lowering medicines (Marin-Penalver *et al.*, 2016).

Sulfonylureas are frequently employed in the management of non-insulin-dependent diabetes mellitus to increase insulin secretion. They bind to the pancreatic β-cell protein Kir6.2/SUR1,

which closes the KATP channel and opens the voltage-dependent Ca^{2+} channel to increase insulin production (Rendell, 2004; Chaudhury *et al.*, 2017).

The mechanism of stimulation involves inhibiting the KATP potassium channel, an ATP-dependent potassium channel that establishes the resting membrane potential of the cell. β-cell depolarization and the activation of one or more L-type calcium channels result from a decrease in potassium outflow. This results in calcium influx, which triggers exocytosis and insulin release (Aguilar-Bryan *et al.*, 1995; Marin-Penalver *et al.*, 2016; Trexler & Taraska, 2017; Zhao *et al.*, 2021). Sulfonylureas like tolbutamide and glyburide inhibit the KATP channel, which depolarizes the cell and causes the release of insulin. Sulfonylureas also restrict gluconeogenesis in the liver, lower lipid breakdown to fatty acids, and lower insulin clearance in the liver (Chaudhury *et al.*, 2017).

First-generation sulfonylureas include chlorpropamide, tolazamide and tolbutamide, whilst second-generation sulfonylureas include glipizide, glimepiride, and glyburide. In comparison to second-generation sulfonylureas, first-generation sulfonylureas are known to have longer half-lives, a higher risk of hypoglycaemia, and a later initiation of action (Sola *et al.*, 2015). Second-generation sulfonylureas are currently given and favoured over first-generation drugs in clinical practice due to their higher potency, with glimepiride having the safest profile (Chaudhury *et al.*, 2017).

To prevent hypoglycaemia, it is important to use medications such as aspirin, allopurinol, sulfonamides, and fibrates cautiously because they can extend the effects of sulfonylureas. Sulfonylurea can be used with other oral diabetes medicines or insulin, however doing so may significantly raise the risk of hypoglycaemia (Chaudhury *et al.*, 2017).

The main issues associated with the use of sulfonylureas are loss of effectiveness, hypoglycaemia, and weight gain (Rendell, 2004; Sola *et al.*, 2015). These insulin secretagogues experience secondary failure over time as islet dysfunction and beta cell degeneration worsen. As a result, a smaller and smaller proportion of patients are able to maintain good glycaemic control. Sulfonylureas have demonstrated a higher rate of secondary failure than other medicines, even if this effect may possibly be connected to the course of the disease (Marin-Penalver *et al.*, 2016). Sulfonylureas, especially glyburide/glibenclamide, have been linked to an elevated risk of cardiovascular disease (Sola *et al.*, 2015; Marin-Penalver *et al.*, 2016). The most common adverse reaction to sulfonylureas is hypoglycaemia, while other less serious events like headache, nausea, and hypersensitivity reactions are also frequent. Due to the

potential for prolonged neonatal hypoglycaemia, sulfonylureas are contraindicated in individuals with hepatic and renal disorders, as well as in pregnant women (Chaudhury *et al.*, 2017).

Meglitinide

Meglitinides, including nateglinide and repaglinide, are non-sulfonylurea secretagogues that were authorized as a T2D treatment in 1997 (Chaudhury *et al.*, 2017). Meglitinides interact with the sulfonylurea receptor in the pancreatic β-cells via a similar mechanism to sulfonylureas. Meglitinide is a short-acting insulin secretagogue because its affinity to the receptor is lower than that of sulfonylurea, which allows for more flexibility in how it is administered. It is less efficient than sulfonylurea because a greater blood sugar level is required before it can activate β-cells to secrete insulin. In individuals with irregular mealtimes or experiencing late postprandial hypoglycaemia, rapid-acting secretagogues, such as meglitinides, may be used in place of sulfonylureas (Inzucchi *et al.*, 2015; Chaudhury *et al.*, 2017).

Glucagon-like peptide 1 (GLP-1) Receptor Agonists

The currently available GLP-1 receptor agonists are exenatide and liraglutide (Tran *et al.*, 2017). These drugs promote the function of incretin hormones and revive insulin secretion in type 2 diabetic patients (Chaudhury *et al.*, 2017). Young patients with a recent diagnosis of T2D, central obesity, and an aberrant metabolic profile may benefit from treatment with GLP-1 analogues, which would help with weight loss and ameliorate metabolic dysfunction (Chaudhury *et al.*, 2017; Tran *et al.*, 2017).

Human GLP-1 is secreted in response to meal consumption, which triggers the release of insulin (Hira *et al.*, 2021). GLP-1, which is generated and released mostly by L-cells in the distal ileum, and glucose-dependent insulinotropic polypeptide (GIP), which is secreted by enteroendocrine K-cells in the proximal gut, are the two incretins that have been identified (Boer & Holst, 2020). Treatment with GLP-1 in T2D patients enhances insulin secretion, decreases glucagon secretion, slows down gastric emptying, improves satiety and decreases food intake (Nauck *et al.*, 2021). GLP-1 guards against myocardial ischemia as well. Moreover, GLP-1 protects against endothelial dysfunction by promoting endothelium-independent arterial

relaxation in blood arteries. They also have an effect in protecting renal function by increasing diuresis and natriuresis. All of these activities help reduce blood pressure and have beneficial effects on cardiovascular risk indicators including brain natriuretic peptide and plasminogen activator inhibitor (Zhao, 2013).

The major side effects of GLP-1 are gastrointestinal, particularly nausea, vomiting and diarrhoea (Nauck & D'Alessio, 2022). When GLP-1 is used along with medications that elevate blood sugar, such as sulfonylureas and basal insulin, there is a slight risk of hypoglycaemic episodes. Reactions to injection sites are frequent and manifest as subcutaneous nodules, cellulitis, and abscess. Also, GLP-1 antibodies could appear (Marin-Penalver *et al.*, 2016). Lastly, GLP-1 analogues should not be used in renal failure (Chaudhury *et al.*, 2017).

DPP-4 Inhibitors

Dipeptidyl peptidase 4 inhibitors (DPP-4 inhibitors) include alogliptin, vidagliptin, linagliptin, saxagliptin and sitagliptin (Berger *et al.*, 2018). These drugs may be taken alone or in conjunction with TZD, metformin, or sulfonylurea (Chaudhury *et al.*, 2017).

DPP-4 inhibitors preserve the concentration and insulin secretion stimulatory function of endogenous incretins, including GLP-1. DPP-4 inhibitors, thus, clinically promote insulin secretion in a glucose-specific approach and subdue glucagon secretion (Makrilakis, 2019). DPP-4 inhibitors also affect postprandial lipid levels. Treatment with vidagliptin for four weeks was shown to decrease postprandial plasma triglyceride and the metabolism of apolipoprotein B-48-containing triglyceride-rich lipoprotein particle after a fat-rich meal in people living with T2D, who have never been exposed to the medication before. It was shown that sitagliptin medication enhanced coronary artery perfusion and cardiac performance in diabetic patients with coronary heart disease (Chaudhury *et al.*, 2017). DPP-4 inhibitors reduce blood sugar levels in the elderly, but they have little impact on calorie intake, which results in a reduced catabolic effect on muscle and total body protein mass (Chaudhury *et al.*, 2017).

Sitagliptin, which is widely used to treat T2D, can be used alone, in combination with sulfonylurea, metformin, or TZD, or as a third therapy (Shankar *et al.*, 2017). Sitagliptin is typically used once daily at a dose of 100 mg; patients with an eGFR of 30 to 50 mL/min receive half the usual dose, and those with an eGFR of less than 30 mL/min receive a quarter dose (Marin-Penalver *et al.*, 2016).

DPP-4 inhibitors are regarded as safe at lower doses in patients with moderate to severe renal failure (Chaudhury *et al.*, 2017). However, when combined with sulfonylureas or insulin, there is an elevated risk of hypoglycaemia. Hepatic dysfunction with vildagliptin and alogliptin has also been documented (Kaku *et al.*, 2019). Moreover, upper respiratory tract infection, nasopharyngitis, and headache are among the side effects of DPP-4 inhibitors that are frequently reported (Chaudhury *et al.*, 2017).

Alpha glucosidase inhibitors

Alpha glucosidase inhibitors reduce postprandial blood glucose increase through impeding the action of carbohydrate digesting enzymes, particularly the intestinal disaccharidases (Chaudhury *et al.*, 2017). There are a number of available agents of alpha glucosidase inhibitors, including miglitol, acarbose and voglibose. Alpha glucosidases are enzyme complexes situated in the brush border membrane of the small intestine and hydrolyse oligosaccharides into monosaccharides (Assefa *et al.*, 2019). Alpha glucosidase inhibitors cause a reversible inhibition of membrane-bound intestinal alpha-glucoside hydrolase enzymes. They are structurally identical to natural oligosaccharides with increased affinity for alpha-glucosidases (Marin-Penalver *et al.*, 2016).

Although alpha-glucosidase inhibitors lower postprandial triglycerides, they have uneven and negligible effects on fasting triglycerides, LDL cholesterol, and HDL cholesterol levels (Rosenblit, 2016). Alpha-glucosidase inhibitors seldom cause hypoglycaemia, because they hardly stimulate insulin release (Gong *et al.*, 2020). They also have no impact on body weight. Acarbose has been shown to reduce the risk of cardiovascular disease and delay the development of diabetes in patients with impaired glucose tolerance (Rosak & Mertes, 2012; Marin-Penalver *et al.*, 2016).

Side effects of alpha-glucosidase inhibitors are mainly gastrointestinal effects, including abdominal pain, flatulence and diarrhoea. Although these symptoms are usually mild, they are the most common reasons for discontinuing the use of alpha-glucosidase inhibitors. These symptoms are caused by undigested carbohydrates when they are fermented by colonic bacteria to release gases that cause abdominal discomforts. Patients with chronic intestinal diseases associated with poor digestion or absorption, as well as those who may have conditions that deteriorate with increased colonic gas production, should not take alpha-glucosidase inhibitors,

this includes conditions such as intestinal ulcers, intestinal obstruction and hernias (Derosa & Maffioli, 2012; Marin-Penalver *et al.*, 2016).

Lastly, alpha-glucosidase inhibitors are not endorsed for diabetes patients with creatinine clearance < 25 mL/min as they can produce asymptomatic rise of liver enzymes, thus it is necessary to monitor the levels of liver enzymes when the patients are on this class of drugs (Marin-Penalver *et al.*, 2016).

Insulin

A second oral drug, a GLP-1 receptor agonist, or basal insulin is added to the regimen if non-insulin monotherapy with metformin at the maximum tolerated dose fails to achieve or maintain the haemoglobin A1C target over three months, is not working, or if it does not work as well as it should. Insulin therapy ought to be introduced in individuals with recently identified T2D and frankly symptomatic and/or severely raised blood glucose levels (Donner & Munoz, 2012; Chaudhury *et al.*, 2017).

Several T2D patients will require insulin therapy at some point in the disease's progression. Insulin therapy for people with T2D and insufficient target glycaemic goals shouldn't be delayed. For any dose titration to accomplish target glycaemic goals and prevent hypoglycaemia, the patient must be closely and frequently monitored (Chaudhury *et al.*, 2017). To repair the damaging effects of high blood glucose levels on the pancreas, insulin therapy must be started, then once consistent glycaemic control is attained, oral medicines can take the role of insulin (Chaudhury *et al.*, 2017).

Basal insulin is the first course of insulin, starting at 10 U or 0.1–0.2 U/kg depending on the degree of hyperglycaemia. The use of a second agent is indicated when basal insulin use is greater than 0.5 U/kg (Mehta *et al.*, 2021). Basal insulin is usually added to oral metformin and possibly one additional non-insulin agent like a DPP-4 or SGLT2 inhibitor (Wolnik *et al.*, 2022). Although they are more expensive, newer, longer-acting basal insulin analogues have better pharmacodynamic profiles, a delayed onset and extended duration of action, and a reduced risk of hypoglycaemia (Chaudhury *et al.*, 2017).

Human insulin preparations (Neutral protamine Hagedorn insulin and regular insulin) do not mimic endogenous insulin secretion (basal and postprandial). Insulin analogues (aspart, lispro, glulisine, detemir, glargine, degludec and U-300) have been developed. They have improved

the adaptability and effectiveness of managing diabetes. While the long-acting insulin analogs have a longer duration of action permitting once-daily dosage and less day-to-day fluctuation, the very rapid-acting insulin analogues have a faster and shorter duration of action than conventional insulin for pre-meal coverage (Marin-Penalver *et al.*, 2016; Kramer *et al.*, 2021).

Instead of administering several injections, an insulin pump may be utilized. Patients and doctors frequently hesitate to increase therapy because of the risk of hypoglycaemia, the intricacy of the regimen, and the requirement for more frequent daily injections. To maintain specific treatment plans and glycaemic objectives, a flexible regimen is required. An ideal insulin regimen should replicate physiological insulin release and offer the best glycaemic control possible with the least amount of weight gain and daily injections possible (Chaudhury *et al.*, 2017; Seufert *et al.*, 2019).

Inhaled insulin is currently obtainable for use during mealtime. But the dosage range is constrained. Use of inhaled insulin entails pulmonary function testing before and after starting therapy. It is not recommended in patients with asthma or other lung conditions (Chaudhury *et al.*, 2017; Cunningham & Tanner, 2020).

Weight gain or loss may result from insulin injections. Additionally, insulin transports potassium into cells and may result in hypokalemia (Coregliano-Ring *et al.*, 2022). Exogenous insulin has the potential to cause allergies. Furthermore, insulin injections in combination with TZDs can cause cardiac failure (Chaudhury *et al.*, 2017).

The rising incidence of T2D in the world and the patients' gradual lack of metabolic control are stark indications that the present therapeutic approaches intended to protect the β-cells are mainly ineffective (Ranasinghe *et al.*, 2015). As seen in this section, the potential side effects of today's pharmacological treatment for diabetes include hypoglycaemia, weight gain, elevated liver enzymes, and heart failure. Therefore, the creation of the "ideal" antidiabetic agent—one with strong efficacy, a favourable safety profile, high clinical value, affordable price, and ease of use—has remained a crucial but elusive objective (Jansen *et al.*, 2009; Chaudhury *et al.*, 2017; Naveen & Baskaran, 2018). Therefore, there is a global search for therapies that are less expensive, more effective, and have no adverse effects (Ramachandran *et al.*, 2015).

In the context of safety and holistic functionality, and as a result of the unpleasant side effects connected with the majority of commercial antidiabetic medicines, dietary changes and the use

of nutraceuticals have become prominent treatment options for diabetes, particularly in functional medicine (Chaudhury *et al.*, 2017).

2.8.2. Dietary and lifestyle adjustments

In T2D, lifestyle interventions are essential in reaching the therapeutics goals (Marin-Penalver *et al.*, 2016). While there are many factors that can affect the development of T2D, it is clear that behaviours that are frequently connected to urbanisation and a modern lifestyle are the most significant. These include eating poorly, leading an inactive lifestyle, or engaging in sedentary activities (IDF, 2021). Therefore, the cornerstone of T2D treatment must be anchored on living a healthy lifestyle, which includes following a nutritious diet, getting more exercise, quitting smoking, and maintaining a healthy body weight (Gupta *et al.*, 2017; IDF, 2021).

Typically, diets high in whole grains, fruits, vegetables, nuts, and legumes and low in refined grains, red or processed meat, and sugar-sweetened beverages are recommended for preventing T2D (Zheng *et al.*, 2018). Hence, in the treatment of diabetes and its complications, agents that can inhibit and/or prevent oxidative stress at various levels are of special interest. According to conventional medical practices, the use of medicinal plants and their extracts may therefore be helpful in this (Naveen & Baskaran, 2018).

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

As long as there has been human history, there has been a relationship between man and plants. Man has studied plants from the dawn of time, especially as a source of food and a remedy for different health conditions. Herbal therapeutic agents are enlisted in naturopathic, ayurvedic, homeopathic, and other medicine practices acquired from natural sources (Ramachandran *et al.*, 2015). Numerous studies on medicinal plants have been conducted, and it has been shown that they have a distinct effect on the nervous, circulatory, respiratory, digestive, and urinary systems, as well as on the skin, sexual organs, vision, hearing, and taste. Thus, they have always been very good sources of therapeutic agents (Bhattacharya & Sil, 2018; Nimesh & Ashwlayan, 2018).

Diabetes is managed both preventatively and therapeutically using medicinal herbs. Their action is targeted on organs and their cellular tissue, particularly pancreatic β-cells, hepatic tissue, while preventing the activity on diabetic inducers. Medicinal plants may, also, show remedial action on affected tissue of pancreas, liver and organs related to diabetes. The presence of different phytoconstituents in these plants, particularly the polyphenols is responsible for the medicinal effects of plants. Polyphenols including, phenolic acids, flavonoids, terpenoids, alkaloids, anthraquinones, tannins and saponins are some vital components in medicinal plants that promote their antidiabetic potential (Yatoo *et al.*, 2017). These substances' direct effects on insulin secretion, stimulation of glycogenesis and hepatic glycolysis, adrenomimeticism, pancreatic β-cell potassium channel blocker activity, activation of cAMP, and modification of glucose absorption from the intestine are some of the proposed mechanisms of action for their antidiabetic effects (Gupta *et al.*, 2017).

Numerous studies have demonstrated that oxidative stress accelerates the disorder's course, but consuming foods high in antioxidants lowers the risk of developing diabetes (Ramachandran *et al.*, 2015). Antioxidants in diabetes management have become welcomed therapeutic because they minimize the actions of ROS (Gulcin, 2006). Plant polyphenols are recognized as potent antioxidant phytochemicals because of their unique structures and have been recognised to protect cells from oxidative stress in both plants and humans due to their antioxidant activity (Rasouli *et al.*, 2017; Bhattacharya & Sil, 2018). Plant polyphenols affect free radicals in various ways, including destroying free radicals, attaching to metals that promote the creation of free radicals, inhibiting the formation of free radicals and functioning as free radical scavengers. They are essential to preserving people's health and wellbeing (Bhattacharya & Sil, 2018).

This study will concentrate on the class of polyphenols known as the phenolic acids. Phenolic acids are strong antioxidants that are widely distributed in fruits and vegetables.

2.8.4. Natural phenolic acids as promising therapeutic agents for diabetes and oxidative stress

Polyphenols containing a phenolic group and a carboxylic acid functional group are natural phenolic acids. The hydroxybenzoic acids and the hydroxycinnamic acids are the two main groups of natural phenolic acids (Heleno *et al.*, 2015). Gallic acid, ellagic acid, vanillic acid, syringic acid, protocatechuic acid, and salicylic acid are hydroxybenzoic acids. Caffeic acid,

caftaric acid, chlorogenic acid, and cinnamic acid are hydroxycinnamic acids (Heleno *et al.*, 2015). Numerous studies have supported the pharmacological potential of phenolic acids derived from plants to be antidiabetic and antioxidative (Vinayagam *et al.*, 2016).

Cinnamic acid

Cinnamic acid and its derivatives occur naturally in fruits, vegetables, and whole grains. Studies have suggested that cinnamic acid may mitigate the progression of diabetic complications (Adisakwattana, 2017). The antiglycation potential of cinnamic acid and derivatives has been documented. Data suggest that cinnamic acid may suppress the development of glycation-mediated diabetic complications (Adisakwattana *et al.*, 2012). In diabetic rats, cinnamic acid treatment protected against deoxyribonucleic acid damage and hepatic and renal oxidative stress and reversed most diabetes-induced changes in hepatic enzymes levels and lipid profiles (Anlar *et al.*, 2018). In isolated rat pancreatic islet and non-obese type 2 diabetic Wistar rats, cinnamic acid increased insulin secretion and improved glucose tolerance, respectively (Hafizur *et al.*, 2015).

P-Coumaric acid

Vegetables, fruits, mushrooms, and grains all contain p-coumaric acid, a naturally occurring hydroxycinnamic acid derivative (Zabad *et al.*, 2019). The inhibition of α-amylase and α-glucosidase activity by p-coumaric acid raises the possibility of enhancing postprandial glycaemic management. P-coumaric acid may prevent hippocampus neurodegeneration by amplifying antioxidant, anti-inflammatory, and anti-apoptotic actions, according to findings from a prior study in T2D mice (Abdel-Moneim *et al.*, 2018). Experimental treatment with p-coumaric acid improved diabetic nephropathy in rats (Zabad *et al.*, 2019). Additionally, p-coumaric acid demonstrated antidiabetic benefits through increasing PPARγ mRNA expression (Abdel-Moneim *et al.*, 2018).

Syringic Acid

Syringic acid is a derivative of hydroxybenzoic acid. It is widely distributed in a variety of fruits and vegetables and certain fungal species (Sabahi *et al.*, 2020). It is a potent free radical

scavenger (Rashedinia *et al.*, 2020). Previous reports have documented the protective effect of syringic acid against hepatic and renal oxidative stress (Sabahi *et al.*, 2020; Rashedinia *et al.*, 2020). Syringic acid has been reported to show useful antidiabetic properties and potential use in the management of diabetes in humans. It ameliorated oxidative stress, improved liver mitochondrial biogenesis and reduced blood glucose and alkaline phosphatase levels in STZ-induced diabetic rats (Sabahi *et al.*, 2020). In diabetic rats, syringic acid increased kidney GSH level and reduced the renal TBARS level (Rashedinia *et al.*, 2020). Also, Srinivasan *et al.* (2014) showed that syringic acid restored diabetes-induced modifications in the levels of glucose and HbA1c, carbohydrate metabolic enzymes actions and hepatic and renal biochemical markers in diabetic rats. Wei *et al.* (2012) showed that syringic acid prevented diabetic cataract in rat lenses by non-competitively inhibiting the activity and gene expression of aldose reductase, a key enzyme involved in diabetic cataract development.

Ellagic acid

Ellagic acid is a natural antioxidant found in berries, nuts and vegetables. It is reported to mitigate hepatic oxidative stress and insulin resistance in T2D rats (Polce *et al.*, 2018). Studies have reported its antioxidative, anti-carcinogenic, anti-inflammatory and anti-mutagenic properties (Ahangarpour *et al.*, 2019). In addition, its cardioprotective role in STZ-induced diabetic mice has been reported (Chao *et al.*, 2005). Treatment with ellagic acid significantly improved the insulin secretion, which attenuated dyslipidaemia, improved glycaemic control and diminished cardiac oxidative stress and inflammation in the diabetic mice (Chao *et al.*, 2005). Oral ellagic acid administration reduced blood glucose and HbA1c levels in STZ-induced diabetic rats (Malini *et al.*, 2011). Oral administration of ellagic acid in STZ-induced diabetic rats decreased HbA1c level (Malini *et al.*, 2011). Ellagic acid has also been reported to have ROS scavenging activity (Priyadarsini *et al.*, 2002).

Gallic acid

Gallic acid (also known as 3,4,5-trihydroxybenzoic acid) is a phenolic acid commonly found in grapes, wine, several berries, vinegars and tea leaves. Studies have reported that gallic acid has antioxidant, anti-inflammatory, antimicrobial, anticancer, gastroprotective, cardioprotective and neuroprotective effects (Kahkeshani *et al.*, 2019). Gallic acid increased

the activity of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione-S-transferase and glutathione peroxidase in STZ-induced diabetic rats (Kade *et al.*, 2014). In STZ- and high-fat diet-induced diabetic rats, gallic acid improved insulin resistance and increased cellular glucose uptake by modulating PPAR-ϒ action and stimulating the PI3K/Akt signalling pathway and GLUT-4 translocation (Gandhi *et al.*, 2014). In rats fed with high fructose diet, gallic acid reduced blood glucose by improving hepatic glucose metabolism (Huang *et al.*, 2016).

Vanillic acid

Vanillic acid is found in herbs, cereals, fruits and vegetables. The antioxidant and antidiabetic properties of this phenolic acid have been reported. In insulin-resistant FL83B mouse hepatocytes and 3T3-L1 adipocytes, it improved glucose absorption activity (Prabhakar & Doble, 2011; Chang *et al.*, 2015). Vanillic acid, when given orally to high-fat diet-fed rats for 16 weeks, decreased blood sugar and triglyceride levels as well as hyperinsulinemia, but it also improved insulin resistance, glucose tolerance, and insulin signalling (Chang *et al.*, 2015). Furthermore, 8 weeks of oral vanillic acid therapy (50 mg/kg bw) altered the antioxidant state in diabetic hypertensive rats (Vinothiya & Ashokkumar, 2017), suggesting the potential role of vanillic acid in the prevention and management of diabetes and diabetic complications.

Caffeic acid

Caffeic acid [3,4-di(OH)-cinnamate] (**Figure 2.6**) is present in large amounts in several varieties of fruits, vegetables and coffee (Chao *et al.*, 2009; Khan *et al.*, 2016). It has demonstrated pharmacological antioxidant, anticancer and antimutagenic activity (Jung *et al.*, 2006; Okutan *et al.*, 2005). Caffeic acid prevented hydrogen peroxide formation, lipid peroxidation and the deleterious action of ROS by enhancing antioxidant enzyme activities in the erythrocyte and liver (Jung *et al.*, 2016). It has shown potentials to improve insulin secretion and suppress hepatic glucose production. Its antioxidant properties have shown promise in preventing or delaying the development of diabetes and its complications (Jung *et al.*, 2016; Oršolić *et al.*, 2021). In alloxan-induced diabetic mice, caffeic acid ameliorated diabetes-induced alterations in lipid and hematological parameters, as well as atherogenic outcomes, oxidative stress and hyperglycaemia and systemic and tissue DNA damage (Oršolić *et al.*,

2021). Caffeic acid was also shown to increase antioxidant enzymes activity in experimental diabetes (Xu *et al.*, 2020)

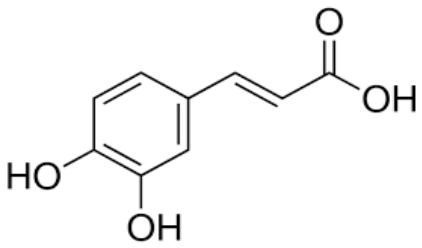

Figure 2.6: Structural formula of caffeic acid

Caffeic acid supplementation (0.02% of diet for 5 weeks) reduced blood sugar and increased insulin levels in C57BL/Ksj-db/db mice (Jung *et al.*, 2006). Concomitantly, the mRNA expression and activity of glucokinase and phosphoenolpyruvate carboxykinase, was increased and decreased, respectively, while hepatic glycogen content was increased following caffeic acid treatment. Additionally, caffeic acid treatment had a modulatory effect on the mRNA expression and activity of catalase, superoxide dismutase, and glutathione peroxidase, but suppressed hydrogen peroxide production and lipid peroxidation in the db/db mice (Jung *et al.*, 2006).

In another study, supplemental caffeic acid (2.5% and 5% were added to diabetic mice's diets during 12 weeks) dose-dependently decreased high blood levels of creatinine and urea nitrogen, suggesting renal protective effect of the treatment was accompanied by reduced , urinary glycated albumin, plasma HbA1c, renal carboxymethyllysine, and renal inflammatory makers, such as interleukin (IL)-6, IL-1b, and tumour necrosis factor alpha (TNF-α) (Chao *et al.*, 2009). This therefore suggests that caffeic acid exhibits a protective and/or ameliorative potential against diabetic nephropathy.

The insulin sensitivity modulatory potential of caffeic acid has also been documented. In hepatocytes with insulin resistance, caffeic acid was reported to promote glucose uptake (Huang *et al.*, 2009). *In vivo*, male rats fed a high-fat diet were treated with daily oral caffeic acid (30 mg/kg body weight) for 30 weeks. Caffeic acid treatment was shown to upregulate the expression of proteins related to cerebral insulin signalling, including phosphorylated Janus kinase-2 at tyrosine 813 (pJAK2Tyr813), PI3K, Akt, and GLUT-3 (Chang *et al.*, 2015).

Ferulic acid

Ferulic acid (**Figure 2.7**) is a hydroxy-cinnamic acid derivative. It is a naturally occurring antioxidant found in various cereal grains, such as maize (Kumar & Pruthi, 2014). It is, also, present in fruits and vegetables such as tomatoes, berries, as well as in coffee, beer and Chinese medicinal plants such as *Angelica sinensis*, *actaea racemosa*, and *Ligusticum chuangxiong* (Zdunska *et al.*, 2018; Alam, 2019).

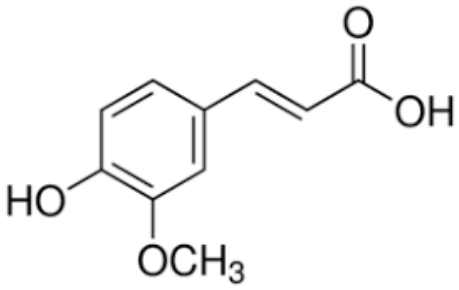

Figure 2.7: Chemical structure of ferulic acid

Ferulic acid intake at comparatively small doses enhances the production of antioxidant enzymes, thus neutralizing free radicals, which are the main source of increased tissue destruction in diabetic patients. Ferulic acid exhibits antioxidant activity by terminating free radical chain reaction (Balasubashini *et al.*, 2004; Ohnishi *et al.*, 2004; Ramar *et al.*, 2012;). Ferulic acid's complex antioxidant activity mechanism is based primarily on the prevention of the production of ROS or RNS (Di Meo *et al.*, 2016). The phenolic ring provides it with a high resonance stability, allowing it to readily accommodate the electron from a free radical. Ferulic acid is thus a direct scavenger of free radicals that are produced by hydrogen peroxide, superoxide, hydroxyl radicals, and nitrogen dioxide very effectively (Alam, 2019; Tao et al., 2019).

In addition to being a free radical scavenger, ferulic acid also inhibits enzymes that accelerate the production of free radicals and increases the activity of scavenging enzymes (Chen *et al.*, 2021). It has the ability to form stable phenoxy radicals, which can scavenge or neutralize a radical molecule, preventing a complex reaction cascade, such as lipid peroxidation that can lead to free radicals generation. Another function of ferulic acid is as a hydrogen donor, providing atoms directly to the radicals. This is crucial for preventing unwanted autooxidation

processes from occurring to the lipid acids in cell membranes. Ferulic acids and similar chemicals can bind transition metals like iron and copper as a secondary antioxidant. This avoids the production of dangerous hydroxyl radicals, which can result in the peroxidation of cell membranes (Zdunska *et al.,* 2018). These antioxidant attributes of ferulic acid largely influences it potent *in vivo* antioxidant potency in diabetes and related metabolic diseases.

In vitro, ferulic acid has been shown to inhibit alpha-glucosidase and alpha-amylase activity suggesting its potential to control postprandial glycemia (Zheng *et al.,* 2020). Ferulic acid improves glycaemic management by increasing insulin secretion and controlling hepatic glucose synthesis (Son *et al.,* 2011). Also, ferulic acid has been shown to reduce blood glucose levels and lipid peroxidation in STZ-induced insulin dependent diabetic mice and KK-A^y non-insulin dependent diabetic mice (Ohnishi *et al.,* 2004). In another study, ferulic acid potentiated antidiabetic effects in the liver of high-fat diet and fructose-induced T2D mice through regulating the activity of insulin signalling molecules (Narasimhan *et al.,* 2015). A previous report showed that ferulic acid increased the secretion of insulin from the cultured β cell and adiponectin from adipocyte, suggesting metabolic benefits (Adisakwattana *et al.,* 2008). Moreover, in diet-induced obese mice, ferulic acid administration reduced blood pressure, perhaps via enhancing blood cholesterol and glucose metabolism (Alam, 2019).

A previous study reported the ability of ferulic acid (10mg/kg bw) to potentiate antioxidant action in experimental diabetes, thus alleviating kidney, pancreas and liver and damage caused by alloxan-induced diabetes (Ramachandran *et al.,* 2015). Its protective effect was also attributed to inhibition of the proinflammatory factor nuclear factor-kappa B, an oxidative stress-responsive transcription factor identified as a key role player in cellular injury (Ramachandran *et al.,* 2015). Moreover, ferulic acid (25 and 20mg/kg BW for 6 weeks) decreased the hypertension caused by diabetes through a variety of mechanisms, including the prevention of inflammation and ROS generation and the enhancement of nitric oxide production and vascular contractility (Ramachandran *et al.,* 2015).

The protective effect of ferulic acid against diabetic nephropathy has been documented, with antioxidant and anti-inflammatory actions as the underlying mechanisms. Ferulic acid treatment in diabetic rats reduced blood lipids and improved biochemical and histological markers of renal insults. Concomitantly, the treatment downregulated the expression of inflammatory markers (p-NF-κB p65, TNF-α, TGF-β1), reduced lipid peroxidation and increased antioxidant enzymes activity in the renal tissues of the diabetic rats. In diabetic rats,

ferulic acid treatment resulted in substantial decline in the levels of thiobarbituric acid reactive substance (TBARS), hydroperoxides and FFA in liver ($p < 0.001$) (Balasubashini *et al.*, 2004). Concomitantly, the treatment significantly ($p < 0.001$) improved the antioxidant status of the diabetic rats (Balasubashini *et al.*, 2004). Also, diabetes-induced reduction of pancreatic islets was appreciably recovered by ferulic acid, with observed improvements in the islet histology (Balasubashini *et al.*, 2004). Overall, ferulic acid has been shown to have anti-inflammatory, antidiabetic, anti-cancer and cardio-protective effects (Zdunska *et al.*, 2018; Alam, 2019).

Antioxidants found in naturally occurring phytochemicals have assumed a significant role as powerful antioxidants for the prevention and/or treatment of a number of human illnesses and diseases. However, supplements and vitamins have also shown beneficial effects on human health, including oxidative and metabolic health (Liu *et al.*, 2018; Wong *et al.*, 2020).

2.8.5. Minerals and supplements in diabetes management

Vitamins (Vitamin A, Group B vitamins – Thiamine (B1), Pyridoxine (B6), Ascorbic acid (Vitamin C), Vitamin E, Vitamin D, Folic acid) and trace minerals, such as zinc (Zn(II)), copper, chromium, magnesium, selenium iron and manganese are recognized for their diabetes-associated health benefits (Mooradian *et al.*, 1994; Martini *et al.*, 2010). They serve as important coenzymes and cofactors for optimum glucose, lipid and protein metabolism (Mooradian *et al.*, 1994; Martini *et al.*, 2010). As a result, their inadequacies have been linked to diabetes and related diseases, while pharmacological research has supported their modulatory effects on glucose and lipid metabolism in conditions like diabetes, obesity, and other related metabolic disorders (Wu *et al.*, 2014).

A high degree of reciprocity characterizes the interaction between diabetes and several vitamins and minerals. Conversely, certain of these chemicals, especially those that have been classified as micronutrients, can directly influence glucose homeostasis in response to chronic uncontrolled hyperglycaemia (Mooradian *et al.*, 1993). While certain trace elements directly affect glucose metabolism, others act as antioxidants to stop membrane peroxidation.

Selenium is an essential trace element that can function as an antioxidant nutrient in various cell types by incorporating selenocysteine into selenoproteins via a genetic process that is expressed by the UGA codon. According to experimental research, antioxidant selenium

supplementation may prevent oxidative stress from contributing to the development of T2D (Wang & Wang, 2017).

When Schwartz & Mertz *et al*. (1959) discovered that Cr(III) appeared to be an essential ingredient in glucose metabolism, it was the first investigation on the beneficial influence of Chromium (Cr(III)) on the control of carbohydrate metabolism. Since then, various studies carried out on humans and animals have examined the part played by this element in the metabolism of carbohydrates and lipids. Studies on diabetic and animal-resistant models of the disease (mice and rats) revealed the therapeutic potential of a number of other Cr(III) compounds, which controlled the disturbed glucose and lipid metabolism in diabetes (Krol & Krejpcio, 2010; Ulas *et al.*, 2015).

Vincent (2015) showed in diabetic and insulin-resistant mice that Cr seems to improve insulin sensitivity. Its mode of action may entail the migration of Cr into tissues in an insulin-sensitive manner; as a result, Cr may serve as a second messenger for the transmission of signals from the receptor to the target cells (Vincent, 2015). Also, CrGly (Chromium Glycinate complex supplement) was shown to have hypoglycaemic potential. In insulin-resistant diabetic rats, it was shown that adding CrGly to water at doses of 40 and 80 g/kg b.w./day reduced the blood glucose/insulin ratio, restored depleted tissue Cr concentrations, and simplified the acquisition of memory (Sahin *et al.*, 2011). The complex displays considerable hypoglycaemic potential in insulin-resistant diabetic rats. It had more noticeable effects than chromium (III) picolinate. The balance of trace elements (Zn, Cu) was also restored in the diabetic rats, and CrGly did not accumulate in the internal organs (Krol *et al.*, 2019).

In recent years, zinc mineral has gained considerable attention as a promising supplement for glycaemic control in diabetics, which has been partially credited to the role of Zn(II) in insulin storage, integrity and function (Chabosseau & Rutter, 2016).

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

The trace metal Zn(II) is ubiquitous in the body, including the fluids and secretions, tissues, and organs (Olechnowicz *et al.*, 2018). Zn(II), which makes up about 2-4 g of the body's total trace metal content, is one of the most prevalent. The prostate has the highest tissue content of Zn(II) (about 200 µg/mL), followed by the pancreas (about 40 µg/mL), and muscle (about 50 µg/mL) (Norouzi *et al.*, 2017). Total Zn(II) in human plasma is delivered to cells and

subcellular organelles in amounts of 14–16 µM. In multicellular organisms, almost all Zn(II) is intracellular with the nucleus harbouring approximately 30–40%, the cytoplasm, organelles and specialised vesicles approximately 50%, and the cell membrane has about 10% (Norouzi *et al.*, 2017). Since there is no free Zn(II) in a healthy cell, compartmentalizing and controlling the distribution of cellular Zn(II) is crucial for maintaining Zn(II) homeostasis with the right cellular concentration and physiological range. A family of Zn(II) transporter proteins and metallothioneins accomplish this (Norouzi *et al.*, 2017).

After insulin was discovered and successfully used to treat diabetic patients, it was also realized that Zn(II) ions may be crucial for the crystallization of insulin. Insulin synthesis, storage, and release are all processes in which Zn(II) has an important role to play.

The mature insulin molecule is a 6 kDa protein comprising two polypeptide chains labelled A and B that are joined by two pairs of disulphide bonds with an extra intramolecular disulphide bond in the A chain. An inactive pre-proinsulin molecule composed of two chains joined by the C-peptide and a signal peptide attached to its N-terminus is produced when insulin mRNA is first translated. The rough endoplasmic reticulum (RER) lumen is the site of pre-proinsulin synthesis, before the signal peptide is cleaved there to produce proinsulin. Next, proinsulin is transferred to the trans-Golgi network and packaged into secretory granules where maturation takes place. The prohormone convertases cleave proinsulin to create C-peptide and a natural insulin molecule (Chabosseau & Rutter, 2016). Insulin is stored at first as a monomer and then forms dimer as it accumulates in the maturating granules. The dimers aggregate to form a hexamer around two Zn(II) ions when Zn(II) is present (Chabosseau & Rutter, 2016). Hexamerization increases the storage capacity of the insulin secreting vesicles by decreasing insulin solubility and promoting its crystallization. It, also, reduces insulin's susceptibility to enzymatic degradation (Chabosseau & Rutter, 2016).

Zinc transporter 8 (ZnT8) is a crucial transporter for the manufacture, storage, and action of insulin since it plays a significant role in Zn transfer from the cytoplasm into insulin secretory granules within islets (Chimienti *et al.*, 2006; Chabosseau & Rutter, 2016; Maret, 2017).

Considering the involvement of Zn(II) in insulin production and secretion by the pancreas, it may be relevant in diabetes management, and it may lead to the development of type 2 diabetic therapy (Jeddi & Torabi, 2019). Moreover, decreased concentration of Zn(II) status has been observed in people with diabetes, compared to healthy people (Olechnowicz *et al.*, 2018).

2.8.7. Potentials of Zn(II) in diabetes management

Recently, Zn(II) has attracted considerable interest as a supplement for the possible development of antidiabetic agents. Studies have shown that there is an important link between diabetes and Zn(II) (Chabosseau & Rutter, 2016). Studies have shown that reduced levels of Zn(II) are associated with low insulin secretion and high resistance of insulin by tissues, while supplementation of Zn(II) has been shown to reduce symptoms of diabetes (Chabosseau & Rutter, 2016; Gerber & Rutter, 2017).

It has been demonstrated that mice with a Zn(II) shortage may have ZnT8 depletion, which may contribute to the islets of Langerhans' dysfunction and an increase in insulin, increasing their chance of developing T2D mellitus (Maret, 2017). Additional studies have revealed that mice with the deleted ZnT8 gene showed reduced peripheral insulin levels, decreased glucose tolerance, and insulin production (Nicolson *et al.,* 2009; Wijesekara *et al.,* 2010). In another study, the abnormal morphology and noticeably less dense insulin cores were present in the insulin secretory granules of β-cells with reduced ZnT8 (Nicolson *et al.,* 2009; Wijesekara *et al.,* 2010). In a different study, increased ZnT8 expression in mice improved glucose tolerance and insulin sensitivity while decreasing pancreatic α-cells' ability to secrete glucagon (Lemaire *et al.,* 2009).

It has been shown that zinc(II) may possess insulin mimetic effects or modulate insulin action or sensitivity. Zn(II) has demonstrated the ability to enhance glucose transport and lipogenesis in adipocytes (Ezaki, 1989; Shisheva *et al.,* 1992). It could inhibit endogenous glycogen synthase kinase-3β, leading to increased glycogen synthesis (Ilouz *et al.,* 2002). Its modulatory action on cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase activity has also been documented (Percival *et al.,* 1997; Yoshikawa *et al.,* 2004), which can lead to a downregulatory effect on cAMP-mediated lipolytic and glycogenolytic signalling. Zinc treatment has, also, been shown to modulate glycolytic enzymes activity *in vitro* (Tamaki *et al.,* 1983).

Zn(II) treatment modulated the tyrosine phosphorylation of the β subunit of the insulin receptor, activation of PI3K and GLUT-4 translocation in adipocytes (Jansen *et al.,* 2009). It was proposed that Zn(II) activates PI3K without the involvement of IRS-1 (Jansen *et al.,* 2009). Possibility exists that Zn(II) stimulates the synthesis of H_2O_2 by epididymal cells, which in turn activates focal adhesion kinase (FAK), thereby activating the PI3K-Akt pathway (Jansen *et al.,* 2009). In fact, the modulatory effect of Zn(II) on the phosphorylation-mediated Akt activation

and GLUT-4 translocation was blocked by wortmannin, an inhibitor of PI3K, which underlines the significance of PI3K for the activation of Akt by Zn(II) (Jansen *et al.*, 2009).

Animal studies have demonstrated that Zn(II) has beneficial effects on diabetes. Earlier studies in ob/ob mice (Begin-Heick *et al.*, 1985) and db/db mice (Simon & Taylor, 2001) have shown that Zn(II) has the potential to ameliorate hyperinsulinemia and attenuate hyperglycaemia. Zn(II) supplementation in diabetic rats has been shown to improve the antioxidant status by reducing lipid peroxidation and increasing the activity of antioxidant enzymes (Duzguner & Kaya, 2007; Tang *et al.*, 2010). In diabetic rats, three months supplementation ameliorated diabetic nephropathy by reducing renal inflammatory makers, improving renal antioxidant status, reducing elevated urinary proteins and attenuating renal pathological alterations (Tang *et al.*, 2010). Moreover, Zn(II) plays a functional role in the antioxidant defence system by functioning as a co-factor for some antioxidant enzymes, in the synthesis of antioxidant metallothioneins, and in the downregulation of NADPH-Oxidase activity, thus preserving the integrity of cell membrane against oxidative damage and protecting against diabetic oxidative tissue damage (Marreiro *et al.*, 2017). In diabetic rats, Zn(II) recovered diabetes-induced morphological abnormalities in the pancreas and improved insulin resistance, hyperinsulinemia and glycaemic control (Barman & Srinivasan, 2016). In fact, a very recent systemic review on Zn(II) in experimental diabetes concludes that Zn(II) supplementation could potentiate glycemic control and protect against diabetic oxidative stress and insults (Martins *et al.*, 2022).

Some clinical data and meta-analyses have, also, supported the positive effects of Zn(II) supplementation on diabetes management. A previous meta-analysis demonstrated that Zn(II) supplementation has positive effects on glycaemia and blood lipids in T2D patients (Ranasinghe *et al.*, 2015). Notable reductions in fasting blood glucose, postprandial blood glucose and HbA1c was observed in T2D patients following supplementation of zinc over a period (Ranasinghe *et al.*, 2015). Also, the meta-analysis revealed that zinc supplementation causes systemic cholesterol, LDL-cholesterol and triglyceride reduction in T2D patients, with concomitant reduction in HDL-cholesterol level (Ranasinghe *et al.*, 2015). Results from an Australian longitudinal study on women's health (Vashum *et al.*, 2013), as well as a meta-analysis (Pompano & Boy, 2021), suggests that zinc supplementation could reduce the risk of developing T2D and cardiovascular events.

The ongoing and future trend of therapeutic agents comprise structural modification of biologically active agents or synergistic interaction to achieve a safer profile, a more selective

and bioavailable attribute and better efficacy. In this context, Zn(II) has been complexed with different types of ligands to develop potent antidiabetic and insulin mimetic complexes. It is probable that complexation increases bioavailability (Chukwuma *et al.*, 2020).

2.8.8. Zn(II) complexes and their antidiabetic potential

The insulin-mimetic activity of Zn(II) has been demonstrated through Zn(II) complexation. Complexes exhibited an inhibitory effect on epinephrine-induced release of free fatty acids, as well as a modulatory effect on glucose uptake in isolated adipocytes, suggesting insulin mimetic or modulatory actions (Chukwuma *et al.*, 2020). Zn(II) has been complexed with different types of ligands including synthetic and natural ligands, as well as supplements and antidiabetic drugs. The lipophilicity of the ligands was substantially correlated with the insulin mimetic action of these complexes, suggesting that the complex must enter intracellular space. The partition coefficient index and coordination modes of Zn(II) complexes are, also, factors that could influence the bioavailability, efficacy and bioactivity profile of Zn(II) complexes at intracellular levels (Chukwuma *et al.*, 2020).

In order to create complexes with different coordination modes and glycaemic control and insulin mimic potentials in isolated cells and experimental diabetes, Zn(II) has been complexed with numerous synthetic ligands.

Several Zn(II) complexes with synthetic organic ligands have been synthesized and studied for their antidiabetic effects (**Table 2.2)**. Consistent data suggest that the complexes possess insulin mimetic properties or the ability to modulate insulin signalling. Yoshikawa *et al*. (2002) reported that the zinc complex of 6-methylpiconilic acid inhibited lipolysis in isolated rat adipocytes, suggesting an insulin mimetic property of the complex. In T2D KK-A^y mice, 14 d i.p. treatment (3 mg Zn/kg) of the complex reduced blood glucose and HbA1c levels and improved glucose tolerance (Yoshikawa *et al.* 2002). The complex also reduced blood glucose and hyperinsulinemia in T2D GK rats. Bioavailability studies showed that more Zn(II) (1.5 fold) was found in the blood following treatment with the complex than with $ZnCl_2$, which may influence the more potent anti-lipolytic activity of the complex relative to $ZnCl_2$. Furthermore, Zn(II) complexes of 2-mercaptotropolone and 2-hydroxypyridine-N-oxide showed insulin mimetic activity by inhibiting lipolysis and enhancing glucose uptake in isolated rat adipocytes (Yoshikawa *et al.*, 2000). In T2D KK-A^y mice, 25 d oral treatment (10 mg Zn/kg) of 2-

mercaptotropolone-Zn(II) complex reduced blood glucose, HbA1c and hyperinsulinemia levels and improved glucose tolerance.

Other Zn(II) complexes of synthetic organic ligands, such as 1-oxy-2-pyridine-thiol, ethylmaltol, 3-hydroxy-4-pyrone, thioallixin-Nmethyl, 2-ethyl-3-hydroxy-4-pyrone and 3-hydroxy-2-methyl-4(H)-pyran-4-thione have also shown cellular glucose uptake and anti-lipolytic activities that were stronger than that of zinc mineral alone (Yoshikawa *et al*., 2000; Adachi *et al*., 2004; Adachi *et al*., 2006; Yoshikawa *et al*., 2011; Nishiguchi *et al*., 2018), suggesting that Zn(II) could be a promising adjuvant for ligands in developing potent insulin mimetic complexes. In different animal models, such as T2D GK rats, T2D KK-A^y mice and T2D ob/ob mice, some of the complexes, such as Zn(II) complexes of 3-hydroxy-2-methyl-4(H)-pyran-4-thione, 1-oxy-2-pyridine-thiol, 6-methylpicolinic acid, thioallixin-N-methyl reduced blood glucose and HbA1c levels but improved glucose tolerance (Adachi *et al*., 2006; Yoshikawa *et al*., 2011; Moroki *et al*., 2014; Nishiguchi *et al*., 2018). In fact, studies using 3T3-L1 adipocytes suggest that the insulin mimetic and antidiabetic properties of 1-oxy-2-pyridine-thiol-Zn(II) complex may be linked to the modulation Akt and glycogen synthase kinase 3β (GSK3β) phosphorylation and GLUT-4 translocation (Basuki *et al*., 2007).

Maltol, a naturally occurring flavour enhancer, is the most studied natural organic ligand of Zn(II) complexes. Consistent data of different studies showed that maltol-Zn(II) complex potentiated insulin mimetic effects by suppressing FFA release and modulating glucose uptake activity in rat adipocytes (Yoshikawa *et al.*, 2000; Adachi *et al.*, 2004; Nishiguchi *et al.*, 2018). The underlying insulin mimetic mechanisms of maltol-Zn(II) has been reported to include modulating the activity of cellular insulin receptor tyrosine kinase, PI3K, GLUT-4 and phosphodiesterase, as well as increasing Akt phosphorylation (Yoshikawa *et al.*, 2004; Naito *et al.*, 2019). *In vivo*, the complex normalised blood glucose and HbA1c levels and improved glucose tolerance in T2D KK-A^y mice (Adachi *et al.*, 2004). Some other studies have also reported the insulin mimetic and blood glucose lowering effects of the Zn(II) complexes of some other natural organic ligands, including picolinic acid, allixin, hinokitiol and quinic. The Zn(II) complexes of these ligands suppressed FFA release and increased glucose uptake in rat adipocytes (Yoshikawa *et al.*, 2002; Adachi *et al.*, 2004). In T2D KK-A^y mice, they reduced blood glucose and HbA1c levels and improved glucose tolerance (Kojima *et al.*, 2002; Adachi *et al.*, 2004; Naito *et al.*, 2017). Studies using adipocytes suggest that picolinic acid-Zn(II) complex may exert insulin mimetic effects by modulating the activity of cellular insulin receptor tyrosine kinase, PI3K and phosphodiesterase (Yoshikawa *et al.*, 2004).

Table 2.2: Antidiabetic properties of Zn(II) complexes

Class of complex	Ligand	Complex	CM	Antidiabetic activity	References
Zn(II) complexes with synthetic organic compounds as ligands	2-mercaptotropolone	Bis(2-mercaptotropolonato)-zinc (II)	$Zn(S_2O_2)$	Inhibited lipolysis (IC_{50} = 12 μM) and increased glucose uptake (EC50 = 0.7 μM) in isolated rat adipocytes. Oral treatment (10 mg Zn/kg; 25 d) reduced BG (≃50%) and HbA1c (≃58%,) levels and hyperinsulinemia (≃40%) and improved GT in T2D KK-A^y mice	Murakami *et al.*, 2012
	3-hydroxy-2-methyl-4(H)-pyran-4-thione	Bis(3-hydroxy-2-methyl-4(H)-pyran-4-thiono)zinc(II)	$Zn(S_2O_2)$	Inhibited lipolysis and increased glucose uptake in isolated rat adipocytes. Oral treatment (2.5 - 10 mg Zn/kg; 28 d) reduced blood glucose, HbA1c and hyperinsulinaemia, improved glucose tolerance and increased islet number and size in T2D ob/ob mice	Nishiguchi *et al.*, 2017
	1-oxy-2-pyridine-thiol	Bis(1-oxy-2-pyridine-thiolato)zinc(II)	$Zn(S_2O_2)$	Inhibited lipolysis in isolated rat adipocytes. Modulated Akt and glycogen synthase kinase 3β phosphorylation and GLUT-4 translocation in 3T3-L1 ad	Basuki *et al.*, 2007

Class of complex	Ligand	Complex	CM	Antidiabetic activity	References
	Ethylmaltol	Bis(ethylmaltolato) zinc(II)	$Zn(O_4)$	Inhibited lipolysis in isolated rat adipocyte	Adachi *et al.*, 2004
	3-hydroxy-4-pyrone	Bis(3-hydroxy-4-pyronato)zinc(II)	$Zn(O_4)$	Inhibited free fatty acid release in adrenalin treated rat adipocytes.	Adachi *et al.*, 2004
	Thioallixin-N-methyl	s(thioallixin-N-methyl)zinc(II)	$Zn(S_2O_2)$	Oral treatment (15 mg Zn/kg; 28 d) exerted blood glucose-lowering and glycaemic control effects in T2D KK-A^y mice	Adachi *et al.*, 2006
Zn(II) complexes with naturally occurring organic compounds as ligands	3-hydroxy-2-methyl-4-pyrone (Maltol)	Bis(maltolato)zinc(II)	$Zn(O_4)$	Inhibited FFA release and increased glucose uptake in isolated rat adipocytes. I.p. treatment (4.5 mg Zn/kg; 14 d) lowered blood glucose and HbA1c levels and improved glucose tolerance in T2D KK-A^y	Adachi *et al.*, 2004
				Modulated the activity of insulin receptor tyrosine kinase, PI3K, GLUT-4 and phosphodiesterase in adipocytes.	Yoshikawa *et al.*, 2004
				Single i.p. treatment (10 mg Zn/kg) modulated phosphorylation of Akt in the adipose and liver tissues of ICR mice	Naito *et al.*, 2019
	3-hydroxy-5-methoxy-6-	Bis(allixinato)zinc(II)	$Zn(O_4)$	Inhibited FFA release and increased glucose uptake in isolated rat adipocytes. I.p.	Adachi *et al.*, 2006

Class of complex	Ligand	Complex	CM	Antidiabetic activity	References
	methyl-2-pentyl-4-pyrone (allixin)			treatment (4.5 mg Zn/kg; 14 d) lowered blood glucose and HbA1c levels and improved glucose tolerance in T2D K	
	Pyridine-2 carboxylic acid (Picolinic acid)	Bis(picolinato)zinc(II)	$Zn(N_2O_2)$	I.p (3 mg Zn/kg; 14 d) treatment lowered blood glucose and HbA1c but improved glucose tolerance in T2D KK-A^y mice.	Kojima *et al.*, 2002
				Inhibited lipolysis in isolated rat adipocytes	Yoshikawa *et al.*, 2002
				Modulated the activity of insulin receptor tyrosine kinase, PI3K and phosphodiesterase in adipocytes.	Yoshikawa *et al.*, 2004
	D-(-)-quinic acid	Bis(quinate)zinc(II)	$Zn(O_4)$	Inhibited lipolysis in isolated rat adipocytes (IC_{50} = 0.98 mM); I.p. treatment (3 mg Zn/kg; 13 d) reduced BG (≃47%) and HbA1c (≃30%) levels in T2D KK-A^y mice	Kojima *et al.*, 2003
Zn(II) complexes with ligands	L-threonine	Bis(L-threoninato) zinc(II)	$Zn(N_2O_2)$	Inhibited lipolysis in rat adipocytes. I.p. treatment (3 mg Zn/kg; 14 d) reduced blood glucose and improved glucose tolerance in T2D KK-A^y mice	Yoshikawa *et al.*, 2001

Class of complex	Ligand	Complex	CM	Antidiabetic activity	References
used as medication and/or supplement				Modulated the activity of insulin receptor tyrosine kinase, PI3K and phosphodiesterase in adipocytes.	Yoshikawa *et al.*, 2004
	Ascorbic acid	Bis(ascorbate)zinc(II)	$Zn(O_4)$	Modulated adipogenesis and expression of GLUT-4, GPDH, C/EBPα and PPAR-ϒ in 3T3-L1 adipocytes	Ghosh *et al.*, 2013
				Suppressed FFA release in isolated rat adipocytes	Matsumoto *et al.*, 2011
	Pioglitazone hydrochloride	[Bis(Pioglitazone) zinc(II)]2Cl^- complex	$Zn(O_4)$	Single oral treatments (10 mg/kg bw) reduced blood glucose in alloxan-induced diabetic Wistar rats after 8 h	Prakash & Iqbal, 2014
	Glibenclamide	Bis(Glibenclamide) zinc(II) complex	$Zn(N_2O_2)$	Single oral treatments reduced blood glucose level in alloxan-induced diabetic Wistar rats after 8 h	Rasheed *et al.*, 2008
	L-carnitine	Zn(L-carnitine)$_2Cl_2$ complex	$Zn(O_4)$	Inhibited lipolysis in isolated rat adipocytes (IC_{50} = 0.8 mM). Oral treatment (20 mg Zn/kg; 16 d) reduced BG (≃32%) level, and improved GT in T2D KK-A^y mice.	Yoshikawa *et al.*, 2003

Abbreviations: Akt, protein kinase B; BG, blood glucose; C/EBPα, CCAAT enhancer-binding protein alpha; FFA, free fatty acid; GLUT-4, glucose transporter type 4; GPDH, glycerol-3-phosphate dehydrogenase; GT, glucose tolerance; HbA1c, glycated hemoglobin; IC_{50}, concentration of test sample that can cause 50% inhibition; PI3K, phosphatidylinositol 3-kinases; PPAR-ϒ, Peroxisome Proliferator-Activated Receptor-ϒ; T2D, type 2 diabetes.

Zn(II) complexes of vitamins (vitamin C and U), amino acids (threonine, asparagine, proline, valine, glycine, alanine, aspartic acid and glutamine) and antidiabetic drugs (glibenclamide and pioglitazone) are some of the Zn(II) complexes of ligands used as supplements and/or drugs that have been studied as potent antidiabetic nutraceuticals.

Although most of the Zn(II) complexes of amino acids exhibited anti-lipolytic activity in rat adipocytes (Yoshikawa *et al*., 2001), Zn(II) complex of threonine, further, exhibited glucose uptake activity and antidiabetic effects in rat adipocytes and T2D T2D KK-A^y, respectively (Yoshikawa et al., 2001). Studies using adipocytes suggest that threonine-Zn(II) complex potentiates insulin mimetic effects by modulating the activity of cellular insulin receptor tyrosine kinase, PI3K and phosphodiesterase (Yoshikawa *et al*., 2004).

It has been reported that ascorbic acid-Zn(II) complex dose-dependently modulated adipogenesis and expression of GLUT-4, glycerol-3-phosphate dehydrogenase, CCAAT/enhancer binding protein alpha and PPAR-ϒ in 3T3-L1 adipocytes (Ghosh *et al*., 2013), which suggests the insulin mimetic potential of the ascorbic acid-Zn(II) complex. Studies on the Zn(II) complexes of antidiabetic drugs, glibenclamide and pioglitazone, showed that the complexes had hypoglycaemic properties in diabetic animal models. Single oral treatment (10 mg/kg bw) of pioglitazone-Zn(II) and glibenclamide-Zn(II) complexes reduced blood glucose level in alloxan-induced diabetic Wistar rats 8 hours post-treatment (Prakash & Iqbal, 2015). The hypoglycaemic effect of glibenclamide-Zn(II) was 1.2-fold that of glibenclamide alone (Prakash & Iqbal, 2015), which suggests that Zn(II) may be a promising adjuvant for antidiabetic drugs. Matsumoto *et al*. (2007) investigated the anti-lipolytic effects of L-carnitine-Zn(II) complex in 3T3-L1 adipocytes. A dose-dependent suppression of glycerol and free fatty acid release was observed, suggesting an antilipolytic and/or insulin mimetic effect. Additionally, carnitine-Zn(II) complex reduced blood glucose level and improved glucose tolerance in T2D KK-A^y mice (Yoshikawa *et al*., 2003). In fact, the glucose-lowering effect of the complex was 3.6 folds that of carnitine alone (Yoshikawa *et al*., 2003) indicating the potent antidiabetic property of the complex.

According to Chukwuma *et al*. (2020), Zn(II) complexes of organic synthetic ligands remain the most studied Zn(II) complexes. This raises serious concerns in the quest for potent bioactive and safe nutraceuticals for diabetes and oxidative mediated complication because the majority of these synthetic ligands have little or no pharmacological credence and are toxic. Plant-derived dietary phenolics, on the other hand, have been shown to be safe and possess promising

antioxidant properties. Thus, they could be preferred ligands for Zn(II) in developing potent and safe nutraceutical complexes. Some plant flavonoids have been previously complexed with Zn(II) to form complexes that show potent antidiabetic, antioxidant and anti-dyslipidaemic properties, which supports the promising lead of Zn(II)-polyphenol complex in the prevention and management of diabetes and oxidative complications.

Although minimally, the use of natural polyphenols as ligands for Zn(II) complexation has been explored. The rationale for this is understandable considering that dietary polyphenols are potent antioxidants with minimal toxicity concerns, thus may improve the medicinal potential of their respective Zn(II) complexes. In this context, few flavonoids have been complexed with zinc(II) to afford complexes with potent antioxidant and antidiabetic potentials (Chukwuma *et al.,* 2020).

2.8.9. Zn(II) complexes of plant-derived polyphenols with antioxidative and antidiabetic potentials

Globally, there is still a need for antidiabetic therapeutics with enhanced pharmacological qualities and fewer side effects. Studies have shown that Zn(II) enhanced the antidiabetic pharmacological properties of some plant flavones and flavonols (Vijayaraghavan *et al.,* 2012; Sendrayaperumal *et al.,* 2014; Gopalakrishnan *et al.,* 2015; Al-Ali *et al.,* 2016), making it a promising adjuvant for plant-derived phenolics.

In a previous study, Al-Ali *et al*. (2016) used safe, reproducible, and efficient methods to design, synthesize, and characterize a curcumin-Zn complex. Also investigated were the antidiabetic potentials of curcumin, Zn(II), and curcumin-Zn complex (Al-Ali *et al.,* 2016). Their research demonstrated that utilizing STZ to induce diabetes resulted in considerable increases in plasma glucose and glycosylated haemoglobin and significant decreases in baseline plasma insulin levels. The curcumin administration lowered blood glucose levels by 45 mg/dL in the STZ-induced diabetic rats after 45 days treatment, while Zn(II) did not notably alter the plasma glucose levels of the diabetic rats . The effect of the curcumin–Zn complex on plasma glucose level in the diabetic rats was, however, stronger than that of curcumin, as the complex reduced plasma glucose by 77.7 mg/dL (Al-Ali *et al.,* 2016). Moreover, plasma insulin level showed a marked elevation in the diabetic rats administered with the complex, relative to the untreated diabetic rats, regardless of the weak effect of curcumin and Zn(II) on

the plasma insulin of the untreated diabetic rats (Al-Ali *et al.*, 2016), suggesting the complexation mediated synergism between Zn(II) and curcumin.

The STZ-induced untreated diabetic rats showed an increase in HbA1c levels, an important marker for assessing the progression and risk of diabetic complications The curcumin–Zn treatment reduced the diabetes-induced elevation of HbA1c to a greater extent than those of the curcumin or Zn treatments (Al-Ali *et al.*, 2016). In the diabetic rats, the complex showed approximately 2 folds lowering effect on serum HDL-cholesterol compared to curcumin and Zn(II), while it concomitantly outperformed curcumin in lowering serum triglyceride and increasing HDL-cholesterol. The data above suggested a synergistic potential between curcumin and Zn(II) in mitigating against diabetic vascular and metabolic complications (Al-Ali *et al.*, 2016). Moreover, blood biochemical parameters suggest diabetes induction led to hepatic and renal dysfunction, which was ameliorated more effectively by the complex, relative to its precursors, suggesting a tissue protective effect. The improved bioactivity of the complex may be attributed to improvements of curcumin bioavailability by Zn(II) complexation (Al-Ali *et al.*, 2016). Moreover, the *in vivo* antidiabetic, antioxidative, anti-inflammatory and tissue protective effects of curcumin have been documented (Den Hartogh *et al.*, 2019).

In another study, the antidiabetic and anti-oxidative properties of zinc-silibinin complex were evaluated in diabetic rats induced with high-fat diet and low dose of STZ (Umamaheswari & Subramanian, 2015). The complex reduced blood glucose and HbA1c levels but increased plasma insulin level. In fact, its antidiabetic effect was comparable to that of metformin, suggesting Zn(II) improved the bioactivity of the complex. The complex also mitigated oxidative stress caused by hyperglycaemia by improving the levels of both enzymatic and non-enzymatic physiological antioxidants, which suggest a potent antioxidant property. Moreover, the increased expression of pro-inflammatory markers in the diabetic rats was, also, decreased, which further supports the antioxidant and anti-inflammatory properties of the zinc-silibinin complex (Umamaheswari & Subramanian, 2015). In addition to the antidiabetic and anti-oxidative properties of Zn(II) complexes of plant-derived polyphenols, Sendrayaperumal *et al.* (2014) reported anti-dyslipidaemic potentials of Zn(II)-morin complex in high-fat diet- and STZ-induced diabetic rats. Thirty days administration significantly improved hyperglycaemia, glucose intolerance, lipid profile and insulin resistance (Sendrayaperumal *et al.*, 2014). Also, the elevated levels of lipid peroxides and tumour necrosis factor alpha (TNFα) were reduced, and the antioxidant status of the diabetic was improved (Sendrayaperumal *et al.*, 2014).

In other studies, on STZ-induced diabetic Wistar rats, 30 days' oral treatment (5 mg/kg bw) of Zn(II) complexes of 3-Hydroxyflavone (Vijayaraghavan *et al.*, 2012) and flavonol (Vijayaraghavan *et al.*, 2013) lowered blood glucose, HbA1c and some biochemical indices of diabetes-induced tissue inflammation or damage. Further histological observation showed that the Zn(II) complex of flavonol protected the pancreas from oxidative damage, by improving the pancreatic antioxidant status, which preserved the insulin secreting ability of the pancreas (Vijayaraghavan *et al.*, 2013). Other studied Zn(II) complexes of plant flavonoids include Zn(II) complexes of diosmin. This complex showed promising blood glucose and HbA1c lowering effects (≃ 44 to 61%) and increased insulin secretion in type 2 diabetic rats induced with high-fat diet and STZ (Sendrayaperumal *et al.*, 2014; Umamaheswari & Subramanian, 2015; Gopalakrishnan *et al.*, 2015). Diosmin-Zn(II) complexes also increased muscle and liver glycogen contents and improved glucose tolerance (Sendrayaperumal *et al.*, 2014; Gopalakrishnan *et al.*, 2015) in the diabetic rats.

Table 2.3: Zn(II) complexes of plant-derived polyphenols and their antidiabetic properties

Ligand	**Complex**	**CM**	**Antidiabetic activity**	**References**
Curcumin	Curcumin-zinc(II) complex	$Zn(O_2)$	Oral treatment (150 mg/kg bw; 45 d) reduced PG (≃24%) and HbA1c (≃36%) levels and increased PI levels (≃108%) in STZ-induced diabetic SD rats	Al-Ali *et al.*, 2016
3-hydroxyflavone	3-Hydroxyflavone–zinc(II) complex	$Zn(O_2)$	Oral treatment (5 mg/kg bw; 30 d) reduced BG (≃52%) and HbA1c (≃46%) levels; increased insulin (≃122%) and C-peptide (≃46%) concentrations; and improved GT in	Vijayaraghavan *et al.*, 2012

Ligand	Complex	CM	Antidiabetic activity	References
			STZ-induced diabetic Wistar rats	
Silibinin	Bis(silibinin)-zinc(II) complex	$Zn(O_4)$	Oral treatment (5 mg/kg bw; 30 d) 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
Morin	Bis(morin)zinc(II) complex	$Zn(O_4)$	Oral treatment (5 mg/kg bw; 30 d) reduced BG (≃61%) and HbA1c (≃44%) levels; increased insulin concentration (≃30%) and muscle (≃109%) and liver (≃51%) glycogen contents; and improved GT, IT and antioxidant status in HFD and STZ-induced T2D Wistar rats	Sendrayaperum al *et al*., 2014
Diosmin	Bis(diosmin)-zinc(II) complex	$Zn(O_4)$	Oral treatment (20 mg/kg bw; 30 d) reduced BG (≃56 %) and HbA1c (≃43 %) levels and liver	Gopalakrishnan *et al*., 2015

Ligand	Complex	CM	Antidiabetic activity	References
			glycogen phosphorylase activity (≃16 %); increased insulin (≃27 %) and C-peptide (≃55 %) concentrations, glycogen synthase activity (≃52 %) and muscle (≃84 %) and liver (≃69 %) glycogen contents; and improved GT and IR (≃44 %) in HFD and STZ-induced T2D Wistar rats	
Flavonol	Flavonol-zinc(II) complex	$Zn(O_2)$	Oral treatment (5 mg/kg bw; 30 d) reduced BG (≃58 %) and HbA1c (≃35 %) levels; increased insulin (≃122 %) concentration; and improved GT, antioxidant status and pancreatic histology in STZ induced diabetic Wistar rats	Vijayaraghavan *et al.*, 2013

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

2.9. Problem statement

The medicinal and antioxidant credence of polyphenols and their presumed safety profile are likely to be some of the influencing factors driving exploring them as promising alternative

ligands for therapeutic Zn(II) complexes. Supporting data showing their contribution to the bioactivity profiles of their respective complexes further validates this trajectory of research. It is, however, unfortunate that the research trajectory on therapeutic Zn(II) complexes has favoured the use of synthetic organic compounds, with little or no pharmacological credence and poor safety profile as ligands for synthesizing Zn(II) complexes. By contrast, the use of polyphenols as promising ligands has not been explored extensively, despite their promising medicinal/pharmacological credence and better safety profiles (Chukwuma *et al.*, 2020). This depicts the unwanted and detrimental paradigm shift towards synthetic therapy as opposed to natural and functional medicine. A number of natural phenolic acids, which are known dietary antioxidants have not been explored as alternative ligands for developing promising Zn(II) complexes with improved therapeutic potential and appreciable safety profile. Considering the antidiabetic and antioxidative potential of caffeic acid and ferulic acid, which are two common medicinal dietary phenolic acids, it is reasonable to speculate that they may be promising ligands for Zn(II) in developing therapeutic Zn(II) complexes with broad and improved pharmacologic profile that will be relevant in managing diabetes and oxidative complication with minimal toxicity concerns. However, this hypothesis has not been investigated.

2.10 Aims and Objectives

Aim

To investigate the antidiabetic and anti-oxidative effects of novel Zn(II) complexes of caffeic acid and ferulic acid.

Objectives

- Synthesis of caffeic acid and ferulic acid zinc (II) complexes
- Characterisation of the complexes using spectroscopic techniques
- *In vitro, in silico* and *ex vivo* antidiabetic and antioxidative screening of the complexes
- *In vivo* antidiabetic and antioxidative investigation of the most potent complex
- Elucidation of the mechanism(s) of actions of the complexes

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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. Complexing ferulic acid with zinc sulphate

Zinc(II) was complexed with ferulic acid in a 1:2 mole ratio using a previous method (Kalinowska *et al.*, 2011) with some modifications. First, 287.56 mg zinc(II) sulphate heptahydrate (Mr = 287.56 g/mol) and 396.34 mg ferulic acid (Mr = 194.18 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 ferulic acid solution. The solution was stirred until no effervescence was observed. Thereafter, the zinc(II) sulphate heptahydrate solution was gradually added to the mixture while stirring. Stirring continued until complete precipitation of a light yellowish precipitate. The mixture was filtered using a filter paper to recover the precipitate. The precipitate was washed thrice with 50% methanol and freeze dried using a freeze dryer (Alpha 1–2 LDplus Freeze Dryer, Martin Christ).

3.2. Complexing caffeic acid with zinc acetate

Zinc(II) was complexed with caffeic acid in a 1:2 molar ratio. A previous article was consulted (Motloung *et al.*, 2020). First, 219.51 mg of $Zn(CH_3COO)_2 \cdot 2H_2O$ (molecular mass = 219.51 g/mol) and 360.32 mg of caffeic acid (molecular mass = 180.16 g/mol) were dissolved individually in glass vials containing 5 mL of methanol. Both solutions were then gradually mixed in another glass vial while stirring. Stirring continued until complete precipitation of a dark yellow precipitate. The precipitate was recovered by filtration, washed twice with 50% methanol, and lyophilized in an Alpha 1-2 LDplus Freeze Dryer, Martin Christ, Osterode am Harz, Germany).

3.3. Complex characterization using spectroscopic methods

The complex was structurally characterized using Fourier-transform infrared spectroscopy (FT-IR), proton nuclear magnetic resonance spectroscopy (^{1}H NMR), high-resolution mass spectroscopy (HR-MS), and high-performance liquid chromatography (HPLC).

3.3.1. Fourier-transform infrared spectroscopy (FT-IR) characterization

For FT-IR, a Perkin Elmer Spectrum 100 FT-IR Spectrometer (MA, USA) was used. The instrument was fitted with an ART accessory. For analysis, roughly 1 mg of either the complexes or their respective phenolic acids was put on the crystal sample holder. Then scanning was done at a wavelength range of 4000 – 380 cm^{-1}, using a 40 s^{-1} scan rate. A V 6.3.4 Spectrum Software was used to record the spectra.

3.3.2. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) characterization

For ^{1}H NMR, a Bruker Avance spectrometer (400MHz; Bruker Corporation) was used, which recorded the ^{1}H NMR data. DMSO-d6 (δH = 2.50) was used as the solvent with a tetramethylsilane 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 0.01Hz.

3.3.3. High-resolution mass spectroscopy (HR-MS) characterization

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 operated at a cone voltage of 15 V.

To perform the HR-MS analysis, 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, Midford, 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 desolvation gas was nitrogen at 650 L/h and desolvation temperature 275 ºC. The instrument was operated with an electrospray ionization probe in positive mode. Sodium formate was used for calibration and leucine encephalin was infused in the background as lock mass for accurate mass determination.

3.3.4. High-performance liquid chromatography (HPLC) characterization

HPLC was further used to verify the purity of the complex. The HP-LC was performed using a HPLC-DAD (Agilent 1100 series, Agilent, Waldbronn, Germany) instrument and a Phenomenex Luna C_{18} column (Separations, Johannesburg) having a dimension of 150 x 4.6 mm and particle size of 5 µm. The mobile phase was HPLC grade H_2O containing 0.1% methanoic acid (solvent A) and acetonitrile (solvent B). The running condition and detection mode was according to the previously reported method (Akuru *et al.*, 2021). A 20 µL injection volume of complex (1mg/mL) was applied. Absorption at 280 nm was used to capture the signal data of the chromatography.

3.4. Cytotoxicity evaluation of complex in Chang liver cells

Chang liver cells were procured from 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. Cell culture was carried out in a NÜVE EC 160 CO_2 incubator (NÜVE) with oxygen, CO_2 supply, and temperature maintained at 95%, 5% and 37°C, respectively. At about 80% confluence, 100µl of cell suspension was seeded (80,000cells/ml) into each well of a 96-well sterile plate. The plate was incubated for 48h to allow the cells attach to the plate. Thereafter, the medium in the wells was aspirated and replaced with new medium containing 7.27, 72.7 and 727µM of the Zn(II)-biferulate.$2H_2O$ complex or 7.36, 73.6, and 736 µM of the bi-caffeic acid-zinc acetate complex or 0.5% DMSO (vehicle control) and incubation continued for 36h. Thereafter, 100µl 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 content in the wells were removed, and the wells were washed with phosphate-buffered saline. One hundred microliters of MTT de-staining or solubilization solution (Sigma Aldrich) was quickly added into the wells, and absorbance was captured at 570nm using the Multiskan Go plate

reader (Thermo Fischer Scientific, Waltham, MA, USA). The viability (%) of sample-treated cells was calculated using the control as a reference. Calculation was done with triplicate data of three biological repeats.

3.5. *In vitro* antioxidant and antidiabetic measurements

3.5.1. 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:

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

3.5.2. 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:

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

3.5.3. 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 measured at 700 nm against 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".

3.5.4. Measurement of linoleic acid peroxidation inhibition

The effect of the complex on linoleic acid peroxidation was measured by consulting a previous method (Choi *et al.*, 2002) and adding some modifications. Into vials containing 30µl of varying concentrations (5 – 80 µM in assay volume) of samples or positive controls (ascorbic acid and Trolox) or the solvents (normal controls and negative control), 30µl of 50mM linoleic acid and 20µl of 100mM Tris–HCl buffer (pH = 7.5) were sequentially added. Next, 20µL of 2mM $FeSO_4.7H_2O$ solution was aliquoted into the vials. The mixtures were kept for 30min in an incubator set at 37 °C. The normal control vial contained 20µL of distilled water instead of $FeSO_4.7H_2O$, while the negative control vial contained 20µl of the $FeSO_4.7H_2O$ solution. After incubation, the mixtures were acidified with 80 µl of 5.5% TCA before addition of 50µL of 0.25% thiobarbituric acid (dissolved in 50 mM NaOH) solution. A boiling water bath was used to heat the vials for 30 min before cooling under room temperature. The vials were then centrifuged at 3500 x g for 10 min under ambient temperature. In total, 150 µL of the

supernatants was pipetted into a 96-well plate and the absorbance was measured at 532 nm using a SpectraMax M2 microplate reader. The percentage inhibition of linoleic acid peroxidation was calculated as follows:

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

where, 'A' means 'absorbance'

3.5.5. Measurement of α-glucosidase inhibition

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 in100 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 in100 mM phosphate buffer, pH 6.8) was added and incubation continued for an additional 20 min under the same incubation conditions. 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:

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

3.5.6. Measurement of α-amylase inhibition

To perform this assay, the method reported by Oke *et al*. (2021) was used with slight modifications. In a 2 mL vial, 50 µL of different concentrations (5 - 80 µM in assay volume) of samples or standard (acarbose) or their solvents (control) was mixed with 50 µL of a solution

of porcine pancreatic amylase (3 U/mL; dissolved in100 mM phosphate buffer, pH 6.8) and incubated for 10 min at 37 ˚C. Then, 50 µL of starch solution (1% w/v; dissolved in100 mM phosphate buffer, pH 6.8) was added and incubation continued for 30 min. Thereafter, 50 µL of dinitrosalicylate colour reagent was added and the vials were heated in a boiling water bath for 10 min. The vials were then allowed to cool and, thereafter, centrifuged (4280 g for 5 min) in a Hettich Mikro 200 microcentrifuge (Hettich Lab Technology, Tuttlingen, Germany). A 200 µL aliquot of supernatant in each vial was transferred into respective wells of a 96-well plate and the absorbance was measured at a wavelength of 540 nm. The enzyme inhibition of the samples or standard was computed as follows:

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

3.5.7. Measurement of the inhibition of glucose-induced protein glycation

The effect of the tested samples on *in vitro* glucose-induced protein glycation was measured with a method described previously (Oke *et al*., 2021) with slight modification. Into a black 96-well plate, 50 µL volume of varying concentrations (5 – 80 µM in assay volume) of the samples or standard (aminoguanidine) or their solvents (control) was mixed with 50 µL of glucose (90 mg/mL) and 50 µL of bovine serum albumin (BSA) (10 mg/mL) solutions. The glucose and BSA solutions were prepared in 50 mM sodium phosphate buffer containing 0.02% sodium azide (pH 6.8). The mixture was incubated in the dark for 2 weeks at 37 ˚C. The fluorescence was then measured at excitation and emission wavelengths of 360 and 420 nm, respectively. The glycation inhibition of the samples or standard was calculated as follows:

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

3.6. Measurement of cellular antioxidant and antidiabetic properties

3.6.1. Evaluating the effect of complex on lipid peroxidation and reduced glutathione (GSH) concentration in Chang liver cells induced with oxidative stress

A recent article (Akuru *et al.*, 2021) with minor modifications was adopted to assess the complex's cellular antioxidant effect and that of its precursors. Chang liver cells (ATCC® CCL-13™, ATCC, Virginia, USA) were cultured in a culture flask as described above (section 3.4.). At about 85% confluence, the cells were harvested by trypsinization and 200 µL of the cell suspension was plated with the culture medium (EMEM media containing 10% FBS) in a 96 well plate at a concentration of 50000 cells/mL. Plates were incubated in a CO_2 incubator for 48 hours allow the cells attach. Thereafter, the spent medium was discarded and 200 µL 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 $FeSO_4{\cdot}7H_2O$ solution (5 mM in assay volume) to induce oxidative stress. While the negative control contained the $FeSO_4{\cdot}7H_2O$ solution, the normal control contained the equivalent volume of distilled water. The plate was incubated for another 1 hour. Thereafter the incubation medium was aspirated, and the cells were lysed with 250 µL of cold 50 mM sodium phosphate buffer (pH = 7.5), containing 0.5% v/v Triton X-100. Lysing was done under cold conditions by gently agitating the plate. The plate was then centrifuged, and the supernatant was used to estimate lipid peroxidation and reduced glutathione (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), 500 µL of 0.25% w/v thiobarbituric acid and 200 µL each of 20% v/v acetic acid and distilled water heated in a boiling water bath for 50 min. The vials were allowed to cool and then centrifuged for 10 min at 3500 x *g* and ambient temperature. Aliquots (200 µL) were plated, and absorbance was measured at 532 nm. The lipid peroxidation was estimated as using the malondialdehyde standard linear plot.

To estimate the GSH concentration, the protein was precipitated from the supernatant by mixing with an equal volume of 10% trichloroacetic acid and centrifuging for 5 min at 3250 g (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany). Fifty microlitres of the supernatant or GSH standards (0.002, 0.02, 0.2, 2 and 20 µM in assay volume) were plated. 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 allowed to stand for 5 min under ambient temperature and absorbance was measured at 412 nm. The GSH concentration was computed from a linear graph plotted using the GSH standard concentrations.

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

3.6.2 Evaluating the effect on glucose uptake in L-6 myotubes

To measure the effectiveness of the complex on glucose uptake, previous methods (Odeyemi *et al.*, 2013; van Huyssteen *et al.*, 2020) were consulted. L-6 myoblast cells from rat muscle (ATCC CRL-1458, ATCC, VA, USA) were used for the assay. The cells were cultured in low glucose containing Dulbecco's Modified Eagle Medium (DMEM), which contained 10% fetal calf serum. Culturing was done in a CO_2 incubator. After culturing, the cells were harvested by trysinization and seeded into a 96 well plate with fresh medium at a density 4000 cells/well. At 80% confluence, DMEM containing 2% horse serum was used to differentiate the cells for 4 – 5 days by replacing the used medium in the wells. The myoblasts differentiated into myotubes during the 4 – 5 days incubation period. Thereafter, differentiation medium was removed and 200 µL of the fresh medium that contained the samples at different concentrations (5, 50 and 100 µm in incubation volume) was pipetted into wells. The sample solvent was used as the vehicle control. Some wells, which contained only the medium without cells were used as the blank control. The culture medium was removed after 48 hours of incubation, and the cells were washed with PBS. Then, 100 µL of RPMI medium, which contained 8 mM glucose and 0.1% of BSA, was pipetted into the wells and the plate was incubated for another 2 hours. Insulin (1µM) was used as a 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 MTT viability assay, as described above, was used to measure the viability of the myotubes following the treatment with samples under investigation. The experiment was done in two biological repeats and each biological repeat had three technical replicates. The blank was used to normalize glucose uptake of the test samples and control as shown below:

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

"ΔGC" denotes glucose concentration change (*i.e.*, the difference between initial and final glucose concentration in incubation solutions). The EC_{50} of samples, which stands for sample concentrations (M) needed to cause a 50% increase in glucose absorption, was also computed.

3.7. Measurement of *ex vivo* antioxidant and antidiabetic properties

3.7.1. Animals

This study was conducted as reported previously (Chukwuma & Islam, 2015; Akuru *et al.*, 2021) with slight modifications. 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 regulation of the ethics committee. The university animal facility supplied Sprague Dawley (SD) rats that were 8 weeks old. The animals were fasted overnight. Thereafter, 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.

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

A previous method (Akuru *et al.*, 2021) was adopted with minor changes to perform this experiment. Into 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) for 25 min in 900 µL of Kreb's buffer (118 mM NaCl, 4.7mM 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 different sample or standard (ascorbic acid) 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. For the controls (control and negative control), the tissue was incubated in buffer containing the equivalent volume of solvents used in dissolving the test samples. After

pre-incubation, 100 µL of $FeSO_4.7H_20$ solution (1 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. The plate was incubated for 90 min. After incubation time elapsed, a pair of sterile forceps was used to remove the tissues from the incubation solution. The tissues were cleaned with Krebs buffer. A D1000 Handheld Homogenizer (Merck) was, then, used to homogenize the tissues in 1 ml of 50mM sodium phosphate buffer (contained 0.5% v/v Triton X-100, pH, 7.5) under ice cold condition. The tissue homogenate was centrifuged at 9600 g for 10 min to recover the supernatants. Lipid peroxidation and GHS concentration were estimated in the supernatants using similar methods described above in **subsection 3.6.1**. The inhibitory effect (%) of samples or standard on lipid peroxidation and GHS depletion was also calculated, which was used to compute the IC_{50} values of samples or standard. Experiment was performed as triplicate biological repeats.

3.7.3. Evaluating the effect of complex on glucose uptake isolated rat psoas muscle tissue

Previous studies (Chukwuma & Islam, 2015 and Oke *et al*., 2021) were consulted, and the experiment was performed with some changes. Into 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 sample concentrations (10, 20, 40 and 80 µM in total incubation volume) or 50 mU insulin positive control (NovoRapid® FlexPen®, Novo Nordisk Limited). For the control, the tissue was incubated in buffer containing the equivalent volume of solvents used in dissolving the test samples. One hundred microlitres of glucose solution (11.1 mM in total incubation volume) was added after the pre-incubation. Thereafter, additional 90 min incubation was done. After the 90 min incubation, aliquots (30 µL) from the incubation medium in each well was then used for glucose concentration measurement using the Glucose (GO) Assay Kit (Sigma Aldrich). The absorbance value of the control was used as the reference value for computing the glucose uptake increase (%) of test samples and positive control as shown below. Experiment was performed as triplicate biological repeats.

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

In addition, the EC_{50}, which denotes the sample concentrations (µM) needed to effect 50% glucose uptake increase was computed.

3.7.4. Measurement of muscle tissue zinc concentration, hexokinase activity and Akt phosphorylation

Immediately after the 90 min incubation, zinc concentration and hexokinase activity, as well as pan-Akt and phosphor-Akt were measured in the muscle tissues without treatment (control), as well as the tissues that were treated with the highest concentration of the test samples and 50 mU insulin. First, a pair of sterile forceps was used to remove the tissues from the incubation solution. The tissues were washed with Krebs buffer, homogenised in 1 mL of ice cold 50mM sodium phosphate buffer (contained 0.5% v/v Triton X-100 and 1 mM EDTA, pH, 7.5). The tissue homogenates were centrifuged at 10,400 g for 10 min at 4°C to recover the supernatants. Protein concentration was measured in the supernatants using the bicinchoninic acid method.

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

Zinc concentration was measured in the tissue supernatant using a zinc Assay Kit (catalogue number MAK032, Sigma Aldrich) and expressed in ng/µL.

An ELISA method was adopted to measure panAkt and phosphor-Akt using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (catalogue number RAB0012, Sigma Aldrich). The phospho-Akt/pan-Akt ratio was computed.

3.8. Bioactivity fold change determination

The activity of the complex was compared to the precursors by fold ratio using the effective concentrations (IC_{50} and EC_{50} values). The following equation was used to calculate the fold ratio:

$$\textbf{Fold ratio} = \left(\frac{IC_{50}\ of\ complex}{IC_{50}\ of\ precursor}\right)^{-1}\ or\ \left(\frac{EC_{50}\ of\ complex}{EC_{50}\ of\ precursor}\right)^{-1}$$

3.9. Molecular docking analysis against enzyme targets linked to carbohydrate metabolism and diabetes

The antidiabetic potential of complexes and their respective phenolic acids was investigated by computationally docking them as ligands against putative target proteins linked to diabetes using previous methods (Swain *et al*., 2020; Motloung *et al*., 2020). The target enzymes include alpha-amylase (AmyL), α-glucosidase (GAA), GLUT-4, and protein kinase B (PKB). Briefly, the 3-D structures of each enzyme were retrieved from the protein data bank, while GLUT-4 was theoretically generated by a homology modelling approach due to unavailability. Molecular docking was carried out using AutoDock Vina 4.2.20. Five docking poses were generated for each ligand against a particular target enzyme and based on the minimum docking score (kcal/mol), the best docking pose was selected. Then, the BIOVIA-DSV software was used to generate visuals of the protein-ligand molecular interaction.

3.10. *In vivo* antidiabetic and antioxidant evaluation

3.10.1. Animals

Ethical approval for the use of animals was obtained from the University of the Free State (UFS) Animal Ethics Committee (**protocol approval reference: UFS-AED2019/0152/2020, approved on 16 November 2020**) and all animal procedures were in accordance with 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**). Experiments were conducted at the UFS animal research facility. Forty-one (41) 7 weeks-old male Sprague Dawley (SD) rats were procured by the UFS animal research facility and allowed to acclimatize for 5 days with free access to 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 ^{O}C; set point at 22 ^{O}C) and humidity (40-70%; set point 60%) controlled room with a 12 h

light-dark cycle. Six (6) of the rats were used as normal control group animals, while diabetes was induced in the remaining thirty-five (35) rats.

3.10.2. Induction of diabetes

To induce diabetes, 10% fructose and low dose (40 mg/kg bw) streptozotocin were used to induce insulin resistance and partial pancreatic β-cell damage, respectively, in rats. A method reported previously (Wilson and Islam, 2012) was used with slight modifications. First, the 35 rats were given 10% fructose solution, *ad libitum*, in place of drinking water for 2 weeks. On the other hand, the six (6) rats in the normal control group continued with normal drinking water. The fructose solution was removed after the 2 weeks and replaced with normal drinking water. Immediately after that, the 35 rats were administered a single intraperitoneal injection of streptozotocin (40 mg/kg body weight, dissolved in citrate buffer, pH 4.5), at a dosing volume of 0.5 mL/200 g body weight, using a 1 mL syringe and 25 G needle. On the other hand, the six (6) rats in the normal control group were injected with only citrate buffer at a volume of 0.5 mL/200 g body weight. Confirmation of diabetes induction was done on the 5th day after streptozotocin injection 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). Blood glucose measurement was obtained in mmol/L and was converted to mg/dL using a multiplication factor of 18.018. Rats with NFBG between 250 and 600 mg/dL were considered diabetic and were used in the study. Of the 35 rats induced with diabetes, 5 rats had extreme adverse conditions and were considered not suitable for the study. Thus, the rats were excluded from the study. The remaining 30 rats were considered suitable for the study and were used in the study. This implies an 86% success rate of diabetes induction.

3.10.3. Animal treatment

The 6 normal control group rats were in the first group. The 30 diabetic rats selected for the study were randomly divided into 5 groups with 6 animals each. The 5 groups of diabetic animals had average blood glucose levels within similar ranges. A total of six groups with six rats each as shown below were used in the study. The six groups of rats were treated five times in a week for 4 weeks as shown below.

- **Normal control:** Nondiabetic rats treated with the vehicle (water) of test substances
- **Diabetic control:** Diabetic rats treated with the vehicle (water) of test substances
- **Caffeic acid:** Diabetic animals treated with 45 mg/kg bw of caffeic acid
- **Zinc acetate:** Diabetic animals treated with 15 mg/kg bw of zinc acetate
- **Complex:** Diabetic animals treated with the complex at a dose of 45 mg/kg bw
- **Metformin:** Diabetic animals treated with 250 mg/kg bw Metformin

Food was removed during administration of the treatments and returned thereafter. The test substances were dissolved in drinking water (vehicle) and administered orally. The test substances were administered once a day, for 20 days (i.e., 5 times in a week for 4 weeks) at a dosing volume of 0.5 mL/kg body weight using an oral dosing needle. Oral dosing was done in the first 5 days of each week. In the last two days of each week, the animals were allowed to recover from any stress caused by oral dosing. All the rats had free access to commercial rat chow and drinking water during the intervention period, unless mentioned otherwise.

3.10.4. Measurement of body weight, food intake and water intake

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

3.10.5. Blood glucose measurements

The tail-tip blood glucose method was employed for blood glucose measurements using a glucometer. NFBG level was measured in the 1st, 2nd and 4th week of the intervention period. It was measured on the 6th day of each of the above-mentioned weeks.

The oral glucose tolerance test (OGTT) was done on the 6th day of the 3rd week of the intervention period. The rats were fasted for 7 hours on the day of the OGTT, and the blood glucose levels of the rats were immediately measured (i.e., blood glucose at time 0 minutes). The rats were then orally administered with 2 g/kg bw glucose at a dosing volume of 0.5 mL/kg body weight. Thereafter, the blood glucose levels of the rats were consecutively measured four more times at 30 minutes intervals (i.e., at 30, 60, 90 and 120 minutes). Food was returned after OGTT. The area under the curve (AUC) for OGTT was computed using a previously reported formula (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 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 measured on the day of euthanization. The rats were fasted for 7 hours, before they were administered with the treatments. After 1 hour 30 minutes, the FBG was measured before the rats were euthanized.

3.10.6. Animal euthanization and sample collection

The animals were euthanized using isoflurane. Once animals were confirmed dead, exsanguination was done to collect blood through cardiac puncture. The blood was collected into non-heparinized tubes using a 5 mL syringe and a 22 G needle. The liver, pancreas, and muscle tissues of the rats were harvested and immediately snap frozen in liquid nitrogen and stored at -80 ^{O}C. To recover the serum, the collected blood was centrifuged (Labnet Prism™ R Refrigerated Microcentrifuge, Labnet International Inc, New Jersey, USA) at 3500 g for 10 min at 4 ^{O}C and the supernatant (serum) was stored at -80 ^{O}C.

3.10.7. Fasting serum insulin and homeostatic model assessment for insulin resistance (HOMA-IR)

An enzyme-linked immunosorbent assay (ELISA) method was adopted to measure fasting serum insulin (FSI) concentration. A Rat Ins1 Insulin ELISA Kit (Sigma Aldrich, Johannesburg, South Africa; product number: RAB0904) was used.

The HOMA-IR was computed using the FBG measured on the day of euthanization and the FSI concentration according to a previously reported formula (Vilela *et al*., 2016):

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

3.10.8. Measurement of muscle and liver tissue glycogen content

The phenol–sulfuric acid method (Lo *et al.,* 1970) was adopted for this assay with slight modifications. Approximately 0.5 g of tissue was put into glass vials containing 1.5 mL of 30% KOH solution. The vials were boiled for 40 minutes until the tissue was digested and a homogenous solution was formed. The vials were allowed to cool. Then, 2 mL of 95% ethanol was added to the vials and sugars/glycogen were precipitated at 2 – 8 ^{O}C for 30 minutes. The vials were centrifuged (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany) at 1200 g for 30 minutes at ambient temperature. The supernatant was decanted, and the precipitate or residue was washed twice with 2 mL of 95% ethanol before dissolving in 3 mL of distilled water. A 200 µL volumes of the dissolved residue or glycogen standards (0.005 – 0.1 mg/200 µL) was transferred into glass vials. Next, 1 mL of 5% phenol and 5 mL of 98% H_2SO_4 were added, successively. The content was gently mixed before aliquoting 250 µL volume into a 96-well plate. Absorbance was, then, measured at 490 nm (SpectraMax M2 microplate reader, Molecular Devices, San Jose, CA, USA). The tissue glycogen content was computed from the glycogen standard curve using 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.

3.10.9. Tissue sample preparation and protein concentration determination

A 100 mg portion of muscle, liver or pancreatic tissue 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)

and centrifuged at 10 400 x g for 10 min at 4 ^{O}C to recover the supernatant. The concentration of protein was then estimated in the supernatant using the bicinchoninic acid (BCA) method.

3.10.10. Measurement of hexokinase activity, Akt phosphorylation and zinc concentration

Hexokinase activity was measured in the muscle tissue 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.

The pan-Akt and phosphor-Akt levels in the muscle tissue supernatant were estimated colorimetrically by an ELISA method using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (catalogue number RAB0012, Sigma Aldrich, Johannesburg, South Africa). The phospho-Akt/pan-Akt ratio was determined as an index for Akt phosphorylation.

Zinc concentration was measured in the serum, as well as the muscle and pancreatic tissue supernatants using a Zinc Assay Kit (catalogue number MAK032, Sigma Aldrich, Johannesburg, South Africa) and expressed in ng/µL.

3.10.11. Measurement of antioxidant parameters

Antioxidant parameters were measured in the serum, as well as the liver and pancreatic tissue supernatants. Lipid peroxidation and reduced glutathione concentration (GSH) were measured using methods reported above in **subsection 3.7.2**.

Catalase activity was measured using a Catalase Assay Kit (Sigma Aldrich, Johannesburg, South Africa; catalogue number: CAT100) by measuring the rate of H_2O_2 decomposition at room temperature and was expressed in mmol/mL/min. Enzyme activity was measured at 1 mg/mL sample protein concentration.

The Superoxide dismutase (SOD) activity was measured using an SOD Assay Kit (Sigma Aldrich, Johannesburg, South Africa; product number: 19160). 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] and was expressed as a percentage.

3.11. Data and statistical analysis

Data were analysed using the 2016 version of MS Excel program. The analysed data were reported as mean ± standard deviation of replicates (n = 3 for the *in vitro*, cellular and *ex vivo* studies and n = 6 for the *in vivo* study). Statistical analysis was done on the Windows 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.

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CHAPTER 4

COMPLEXATION POTENTIATED PROMISING ANTIDIABETIC AND ANTI-OXIDATIVE SYNERGISM BETWEEN Zn(II) AND FERULIC ACID: A MULTIMODE STUDY

Prescript

The *in vitro*, cellular and *ex vivo* antidiabetic and antioxidant potential of a Zn(II) complex of ferulic acid was investigated in this chapter. The content of this chapter has been published as follows: ***Matowane, G. R.***, *Ramorobi, L. M., Mashele, S. S., Bonnet, S. L., Noreljaleel, A., Swain, S. S., Makhafola, T. J., & Chukwuma, C. I. (2022). Complexation potentiated promising anti-diabetic and anti-oxidative synergism between Zn(II) and ferulic acid: A multimode study.* ***Diabetic Medicine****; 39(9), e14905.* https://doi.org/10.1111/dme.14905. The content of this chapter was adopted from the above-mentioned publication.

4.1. Abstract

This study was undertaken to investigate the antidiabetic and antioxidative synergism between zinc(II) and ferulic acid through complexation. Zinc sulphate was complexed with ferulic acid in a 1:2 molar ratio. The complex was characterized using Fourier-transform infrared spectroscopy, proton NMR and high-resolution mass spectrometry techniques and evaluated for cellular toxicity. *In silico, in vitro,* cell-based and tissue experimental models were used to test the antidiabetic and antioxidant activities of the complex relative to its precursors. A zinc(II)-biferulate.$2H_2O$ complex was formed. The *in vitro* radical scavenging, anti-lipid peroxidative and α-glucosidase and α-amylase inhibitory activity of the complex was 1.7–2.1 folds more potent than ferulic acid. Zn(II) complexation increased the anti-glycation activity of ferulic acid by 1.5 folds. The complex suppressed lipid peroxidation (IC_{50} = 48.6 and 331μM) and GHS depletion (IC_{50} = 33.9 and 33.5 μM) in both Chang liver cells and isolated rat liver tissue. Its activity was 2.3–3.3 folds more potent than ferulic acid and statistically comparable to ascorbic acid. Zn(II) complexation afforded ferulic acid improved glucose uptake activity in L-6 myotube (EC_{50} = 11.7 vs. 45.7 μM) and isolated rat muscle tissue (EC_{50} = 501 and 1510μM). Complexation increased muscle tissue zinc(II) uptake and hexokinase activity. Docking scores of the complex (−7.24 to −8.25 kcal/mol) and ferulic acid (−5.75 to 6.43kcal/mol) suggest the complex had stronger interaction with protein targets related to diabetes, which may be attributed to the 2 ferulic acid moieties and Zn(II) in the complex. Moreover, muscle tissue showed increased phospho-Akt/pan-Akt ratio upon treatment with complex. The complex was not hepatotoxic and myotoxic at in vitro cellular level. Zn(II) complexation may be promising therapeutic approach for improving the glycaemic control and antioxidative potential of natural phenolic acids.

4.2. Introduction

Recent data from the International Diabetes Federation (IDF) depicts a sharp increase in global diabetes prevalence (IDF, 2021), which is imposing more socio-economic and health burden on affected countries. Since 2019, the global prevalence has increased by 74 million people (IDF 2019; IDF, 2021). Type 2 diabetes (T2D) has consistently remained the most prevalent type of diabetes (IDF 2019; IDF, 2021). This is partly attributed to its association with obesity, sedentary lifestyle, poor dieting and lack of exercise (Al-Goblan *et al.*, 2014; Sami *et al.*, 2017). The aforementioned are risk factors of insulin resistance and impaired glucose tolerance, which

can lead to persistent hyperglycemia and eventually T2D if not properly managed (Al-Goblan *et al.*, 2014; Sami *et al.*, 2017; Galicia-Garcia *et al.*, 2020). Insulin resistance is characterized by poor insulin signalling to cells, particularly myocytes and adipocytes (Saini, 2010). There is poor insulin-mediated phosphorylative activation of key signalling proteins, such as insulin receptor, insulin receptor substrate-1, phosphatidylinositol 3-kinase, serine/threonine kinase Akt, etc. at cellular level, which results in impaired cellular glucose transporter type 4 (GLUT-4) translocation, as well as impaired glucose uptake and utilization (Saini, 2010). Thus, glucose persists in the blood and increases the risk of diabetic complications.

Oxidate stress is crucial in the development and progression of diabetic complications, due to oxidative damage caused by free radicals and reactive oxygen species on cellular biomolecules. Persistent hyperglycaemia drives excess production of superoxide ion through carbohydrate and energy metabolism pathways, which can cause oxidative stress and oxidative damage in the absence of adequate antioxidant defence mechanisms (Giacco & Brownlee, 2010; Fakhruddin *et al.*, 2017). Additionally, persistent hyperglycaemia drives biomolecule glycation, as well as the formation of advanced glycation end-products, which are biomarkers of degenerative complications associated with diabetes (Giacco & Brownlee, 2010).

The concept of synergism has a wide reception and application in the therapeutic management of diseases. The concept affords the benefits of overcoming toxicity or side effects linked to high dose of drug and potentiating multi-mode therapeutic targets and effects, thus improving therapeutic effects (Lehar *et al.*, 2009). Advances in research have reported the complexation of Zn(II) with various types of ligands with the aim of developing potent antidiabetic zinc complexes; the premise being that zinc possess insulin mimetic action and may improve the bioavailability and bioactivity of its ligands through various mechanisms (Chukwuma *et al.*, 2020). Zn(II)–threonine complex was shown to demonstrate insulin-like effects by modulating the activity of phosphatidylinositol 3-kinase, phosphodiesterase, insulin receptor tyrosine kinase and GLUT-4 in adipocytes (Yoshikawa *et al.*, 2004). The complex also suppressed lipolysis in rat adipocytes and potentiated glycaemic control in T2D KK-A^y mice (Yoshikawa *et al.*, 2001). Zn(II) complexes of two natural ligands (maltol and allixin), also, showed suppressive and modulatory effects on lipolysis and glucose uptake, respectively, in rat adipocytes (Adachi *et al.*, 2004). Intraperitoneal administration of both complexes to T2D KK-A^y mice reduced blood glucose, HbA1c and hyperinsulinemia and improved glucose tolerance (Adachi *et al.*, 2004).

Despite the therapeutic potential of zinc–ligand synergism, there are some pitfalls associated with the research trajectory in this area of research. In a recent review, it was reported that most of the studied antidiabetic Zn(II) complexes are those of synthetic ligands with little documented pharmacological credence and toxicity concerns, while plant phenolics with documented antioxidant and glycaemic control potential remain understudied (Chukwuma *et al.*, 2020). Several phenolic acids, including ferulic acid have not been explored as possible ligands of therapeutic Zn(II) complexes. Ferulic acid is a natural antioxidant present in some cereal grains, including maize (Kumar & Pruthi, 2014). It is an effective radical scavenger (Kikuzaki *et al.*, 2002). It enhances insulin secretion and regulates hepatic glucose production, thus improving glycaemic control (Son *et al.*, 2011). In addition, ferulic acid has been shown to reduce blood glucose levels and lipid peroxidation in STZ-induced insulin-dependent diabetic mice and KK-A^y non-insulin-dependent diabetic mice (Ohnisi *et al.*, 2004). In the liver of high-fat diet and fructose-induced T2D rats, ferulic acid potentiated antidiabetic effects by modulating the activity of insulin signalling molecules (Narasimhan *et al.*, 2015).

The above data suggest that ferulic acid may be a promising ligand for Zn(II) in developing a Zn(II) complex with potent antidiabetic and antioxidative synergistic property. However, this has not yet been studied. Therefore, the aim of this study was to investigate the antidiabetic and antioxidative properties of a novel Zn(II) complex of ferulic acid.

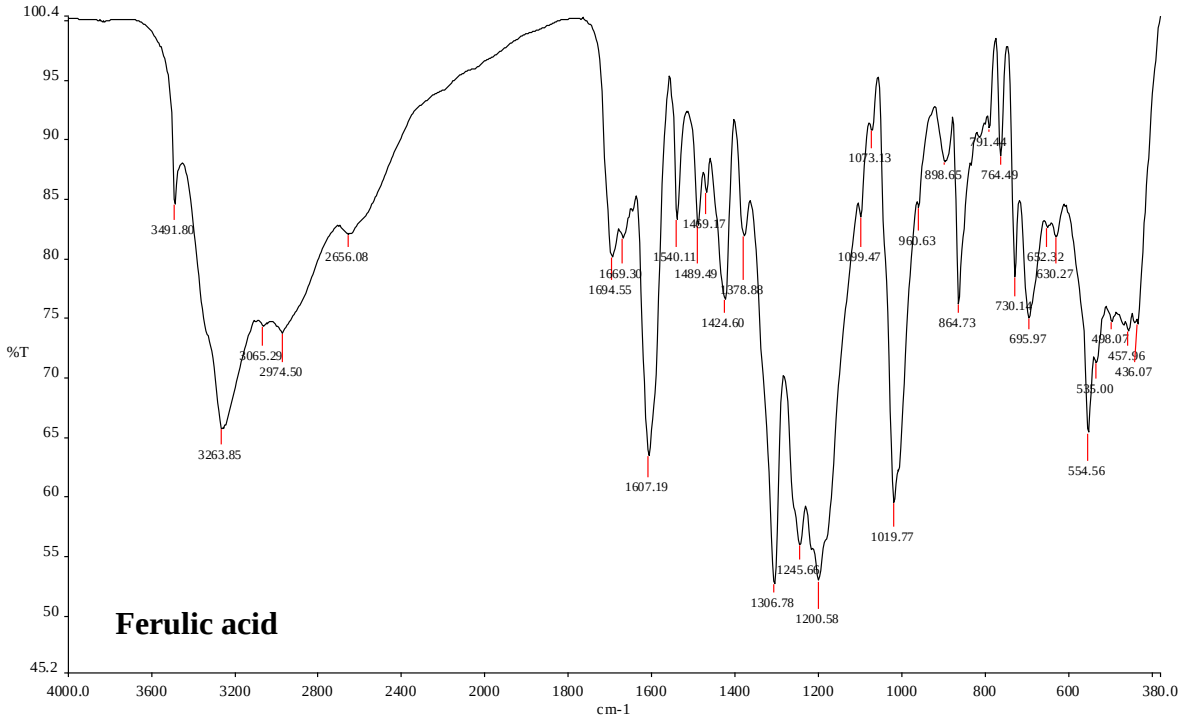
4.3. Materials and methods

Please refer to **sections 3.1, 3.3, 3.4, 3.5, 3.6, 3.7 and 3.9 of chapter 3** for the materials and methods used in this chapter.

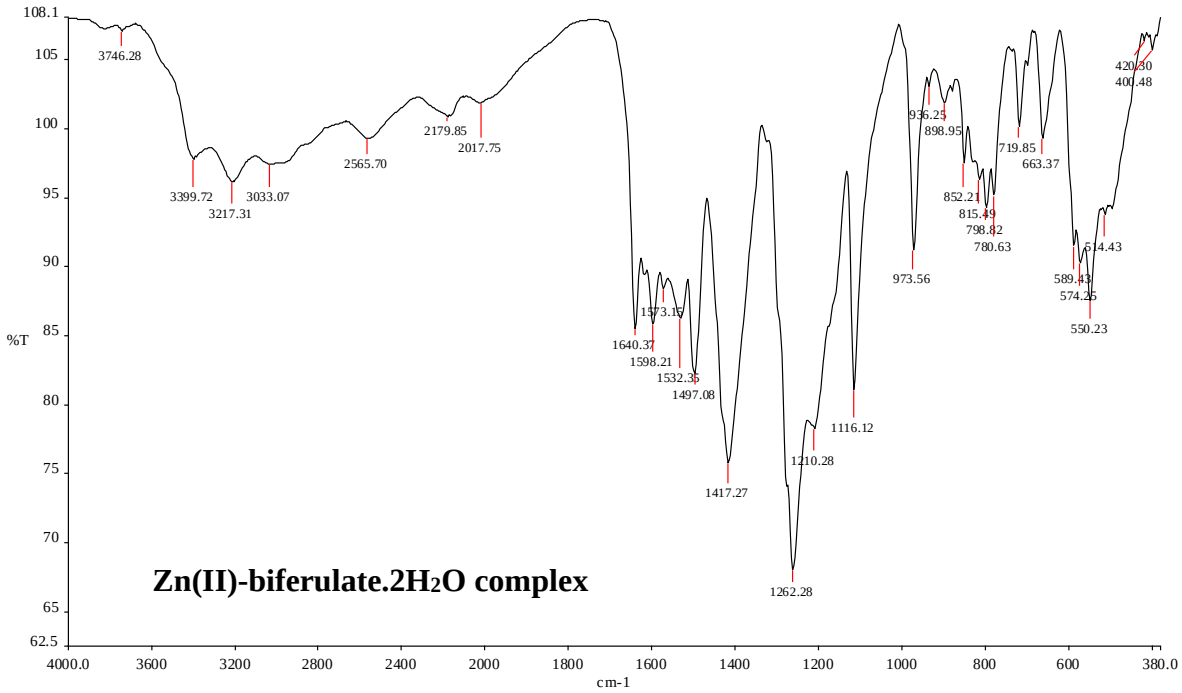
4.4. Results

4.4.1. Spectroscopic characterization

Figure 4.1 shows the FT-IR spectra of ferulic acid and its complex. The spectrum of ferulic acid showed a broad peak centred at 3491 cm^{-1}, which is indicative of a carboxylic O-H stretch in the compound (**Figure 4.1a**). This region of carboxylic O-H stretching, also, appeared in the FT-IR spectrum of the complex in the region centred at 3399 cm^{-1} (**Figure 4.1b**).



(a)



(b)

Figure 4.1: (a) Ferulic acid **(b)** complex FT-IR spectra

The lower carboxylic O-H stretch wave number value of the complex suggests an interaction of Zn(II) with the carboxylic O-H bond. Probably zinc ion donated electrons to the carboxylic O-H bond, thus weakening the bond and resulting in a lower wave number. This suggests that ferulic acid interacted with Zn(II) using its carboxylic O-H group as proposed in **Figure 4.2**.

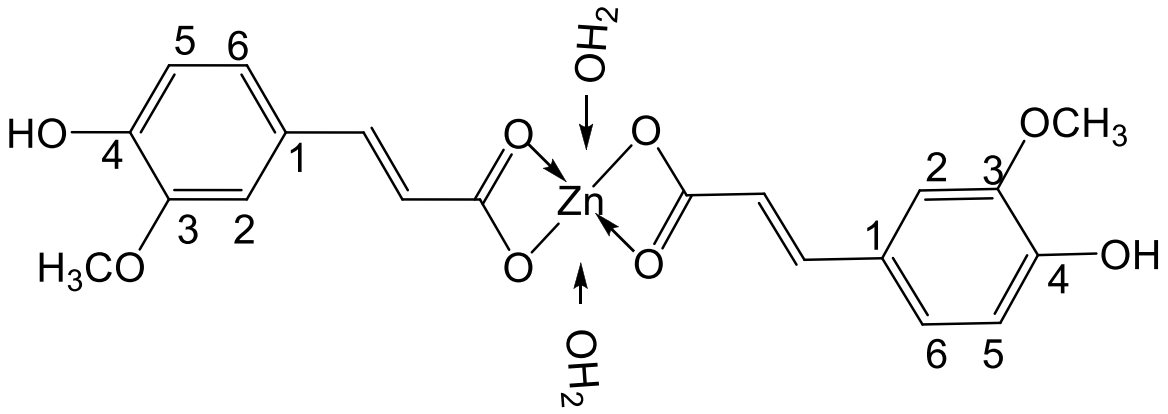
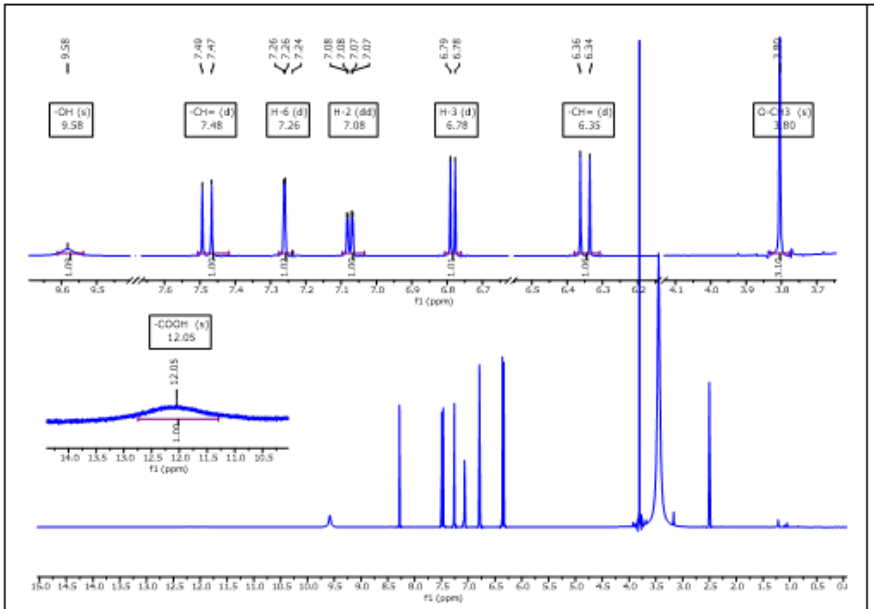
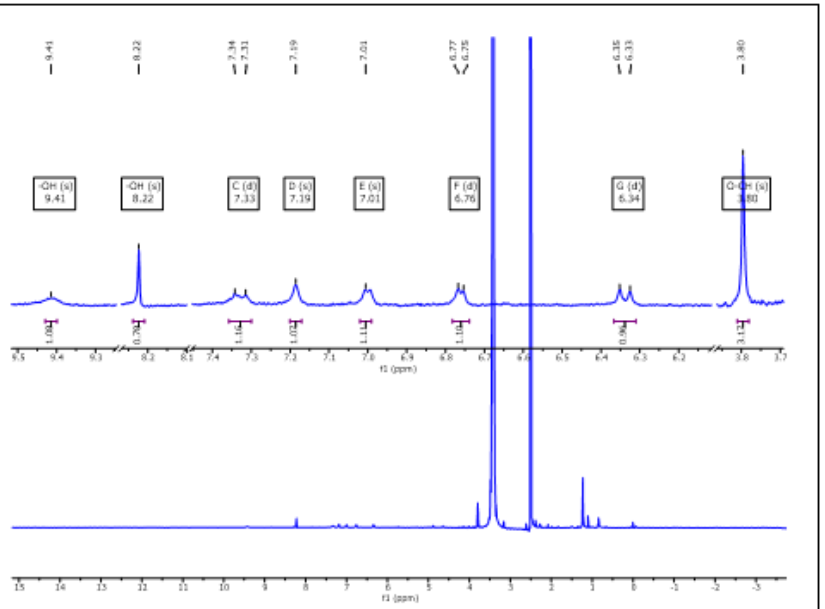


Figure 4.2: Proposed Zn(II)-biferulate.2H_2O complex

In addition, the sharp peaks at 1694 and 1640 cm−1 indicate carboxylic C=O stretching in the phenolic acid (**Figure 4.1a**) and the complex (**Figure 4.1b**), respectively. The lower wave number value of the complex suggests a C=O bond weakening, probably due to electrons donated by zinc ion. This suggests that zinc(II) coordinated with the carboxylic carbonyl group of the phenolic acid. The alteration of the carboxylic O-H and carbonyl IR signals suggests zinc(II) complexed with ferulic acid through the carboxylic functional group.

A comparative proton NMR analysis between the starting material (ferulic acid) and the complex is presented in (**Figure 4.3**). The ^{1}H-NMR spectrum of the complex showed ABX system of ferulic acid moiety. Doublet of doublet peak at 7.01 corresponding to H-2, while H-6 and H-3 is observed as a doublet at δ 7.19 and 6.76 respectively. The values at 6.34 and 7.33 represent the double bond. The singlet peak at δ 9.41 is characteristic of phenolic hydroxyl group. The carboxylic proton observed as singlet in the ferulic acid spectra at δ 12.05 was not picked up in the spectra of the complex (**Figure 4.3**), possibly due to involvement in the complexation with zinc(II) ions. The double bond proton closed to the carboxylic group of ferulic acid was observed at δ 7.48. For the complex, this was observed at δ 7.33. The change from δ 7.48 to δ 7.33 may be due to the coordination of the carboxylic group with zinc(II) ions.





Group	Chemical Shift and *J*-Coupling Hz for ferulic acid	Chemical Shift and *J*-Coupling Hz for complex
-OCH_3	3.80 (3H, s)	3.80 (3H, s)
-CH=	6.34(1H, d, *J*=15.96 Hz)	6.34(1H, d, *J*=15.90 Hz)
H-3	6.78 (1H, d, *J*=8.20 Hz)	6.76 (1H, d, *J*=8.20 Hz)
H-2	7.08 (1H, dd)	7.01(1H, dd)
H-6	7. 26 (1H, d)	7.19(1H, d)
-CH=	7.48 (1H, d, *J*=15.96 Hz)	7.33(1H, d, *J*=15.90 Hz)
OH	9.58 (1H, s)	9.41(1H, s)
-COOH	12.05 (IH, s)	-

Figure 4.3: Comparative ^{1}H NMR spectral analysis between ferulic acid and complex**.**

The high-resolution mass spectroscopy (HR-MS) data of the complex are presented in **Figure 4.4**. For the negative mode, the signal at m/z 485.0015 on the mass spectrum showing a difference of 0.0411 from calculated m/z 485.0426 represents the two ferulic acid and two water molecules coordinating/interacting with zinc ion, which indicates the complex was successfully formed. Fragment was observed at m/z 293.1785 (calculated; 293.0004), signifying a coordination/interaction between one ferulic acid and two water molecules with zinc ion. The fragment observed at m/z 264.9455 is attributed to the loss of a methyl group from one molecule of ferulic acid coordinating with zinc ion. The fragment with 100% abundance at m/z 193.0501 (calculated m/z 193.0501) represents ferulic acid moiety.

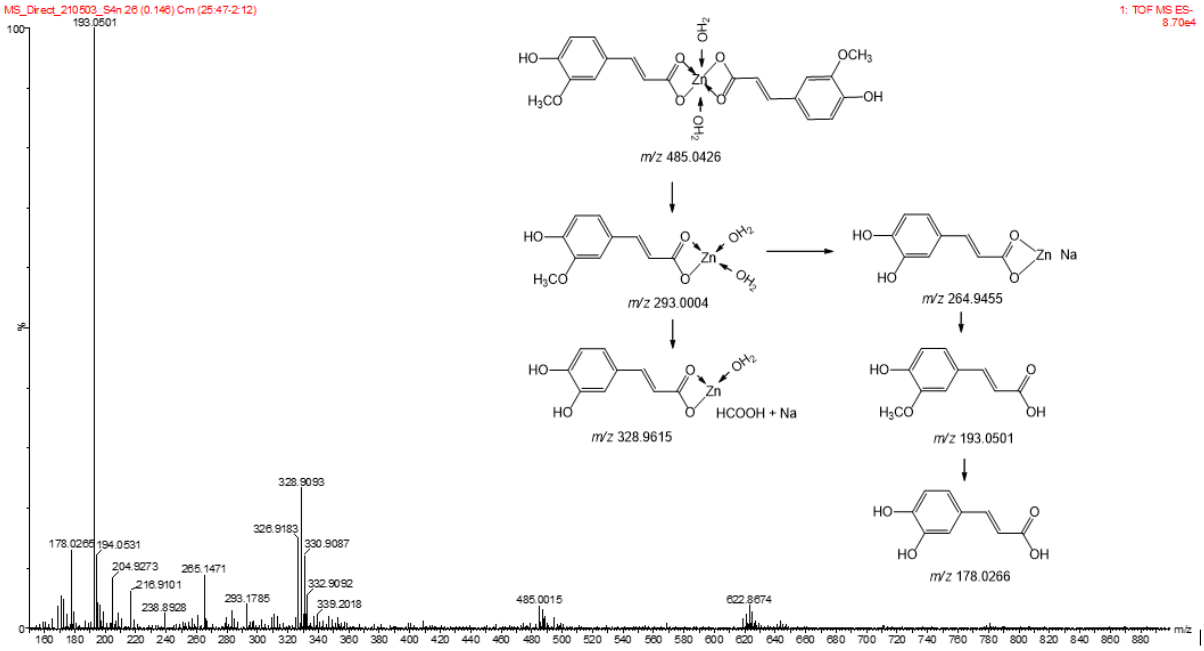
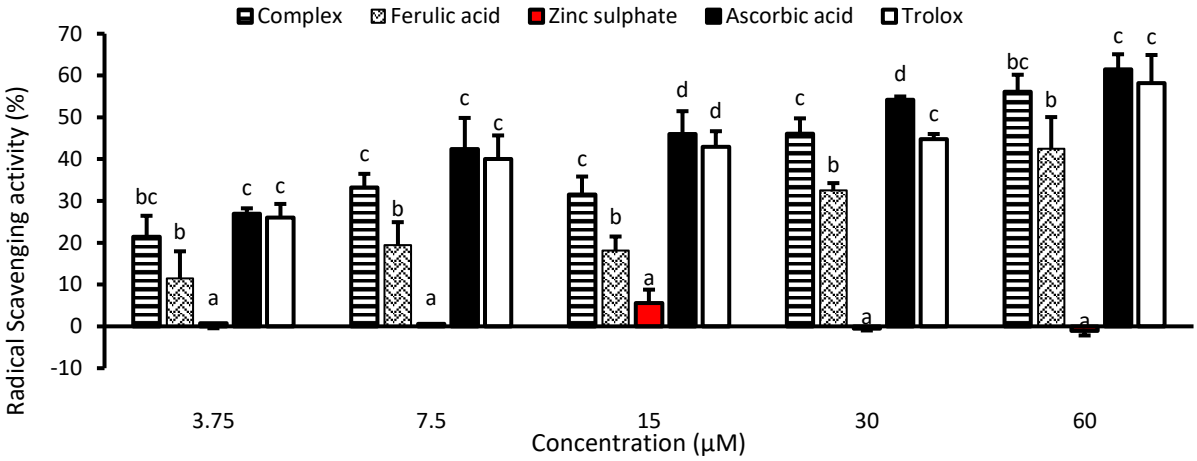


Figure 4.4: High resolution-mass spectroscopic analysis of complex

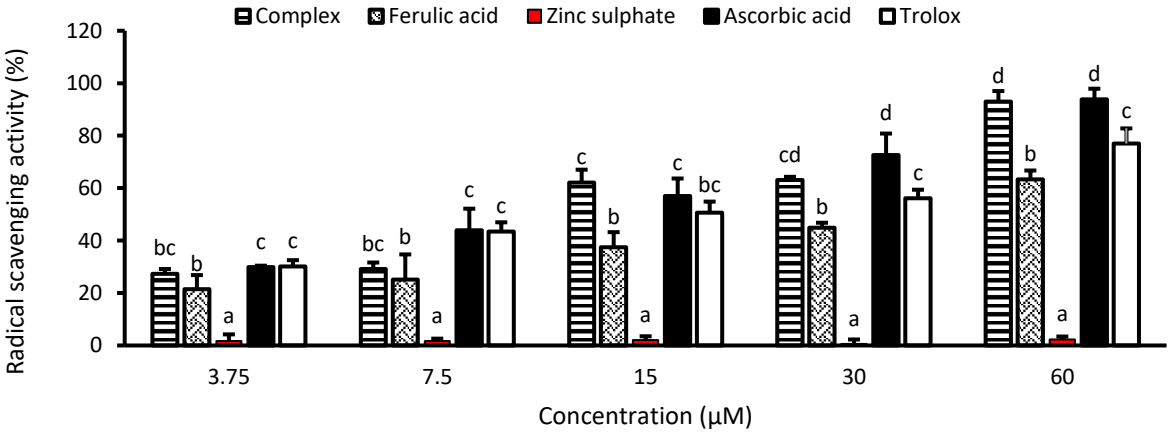
The above spectroscopic characterization suggests that Zn(II) complexed with two moieties of ferulic acid to form a Zn(II)-biferulate.2H_2O product as shown in **Figure 4.2**. Based on the above spectroscopic data and analysis, it is most probable that the carboxylate group coordinated to Zn(II) ions in a bidentate or bridging mode.

4.4.2. Antioxidant activities

In vitro, ferulic acid showed scavenging activities on DPPH (IC_{50} = 75.4 µM) and ABTS (IC_{50} = 66.9 µM) radicals, reduced Fe^{3+} ion (504 mmol/mol AAE) and inhibited linoleic acid peroxidation (IC_{50} = 66.7 µM) (**Figures 4.5 and 4.6; Table 4.1**).

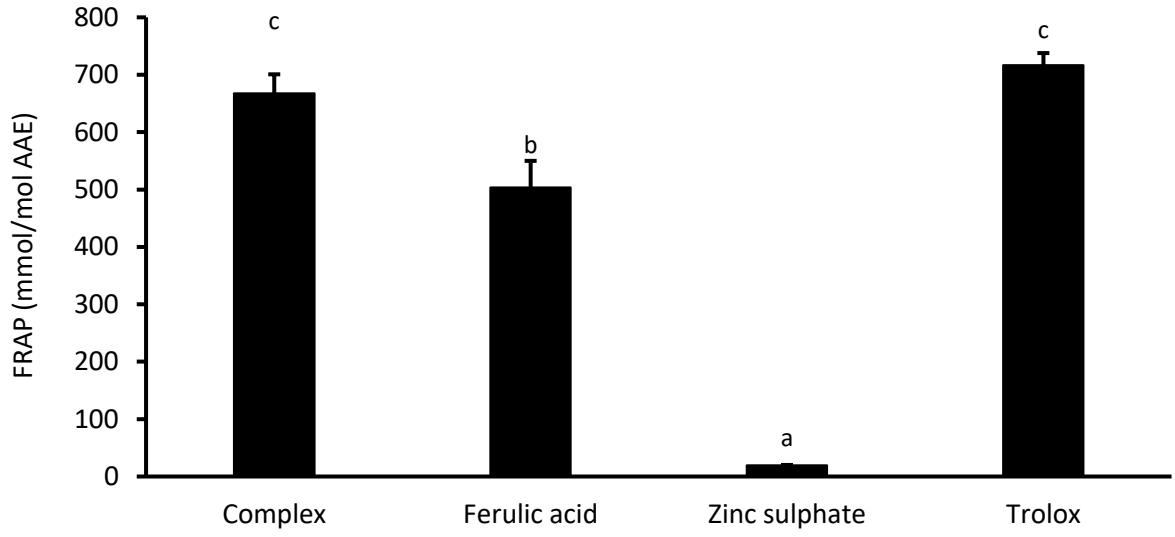


(a)

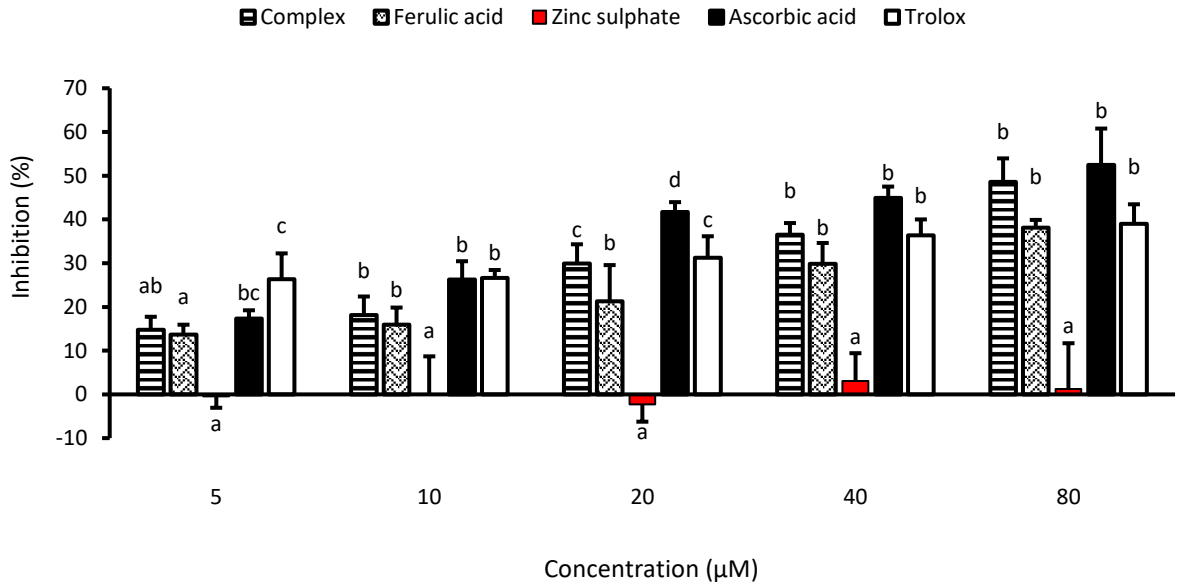


(b)

Figure 4.5: The *In vitro* **(a)** DPPH radical scavenging **(b)** $ABTS^{\bullet+}$ radical scavenging activities of the tested samples. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.



(a)



(b)

Figure 4.6: The *In vitro* **(a)** Fe^{3+} reducing and **(b)** linoleic acid lipid peroxidation inhibitory activities of the tested samples. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

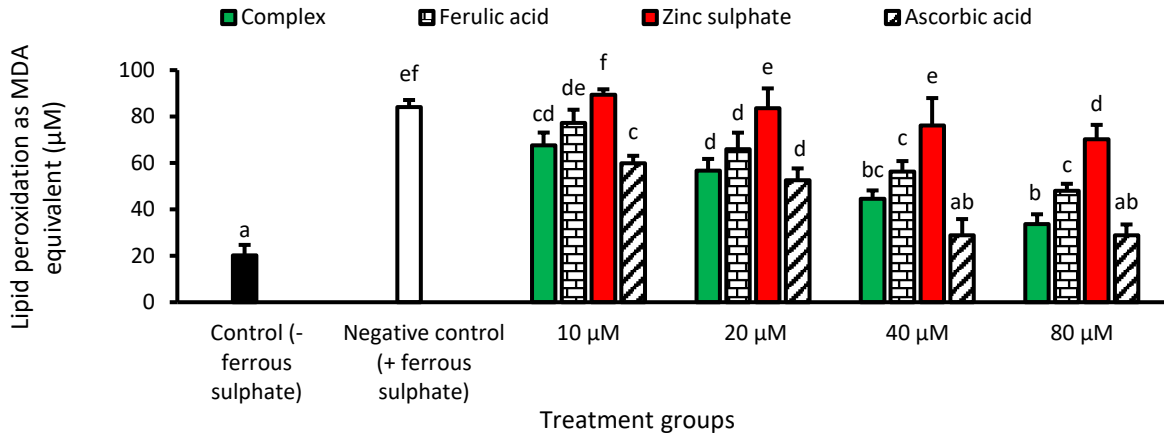
Table 4.1: The IC_{50} and EC_{50} values of ferulic acid, its Zn(II)-biferulate.$2H_2O$ complex and standards

Parameters or activity	Zn(II)-biferulate.$2H_2O$ complex	Ferulic acid	Zn(II)	Ascorbic acid	Trolox	Acarbose	Amg
	IC_{50} or EC_{50} values (µM)						
ABTS radical scavenging activity (IC_{50})	32.0 ± 7.27^{b}	66.9 ± 17.3^{a}	ND	26.3 ± 10.9^{b}	49.3 ± 5.58^{ab}	NA	NA
DPPH radical scavenging activity (IC_{50})	43.8 ± 6.23^{b}	75.4 ± 5.48^{a}	ND	14.3 ± 1.46^{c}	12.7 ± 4.62^{c}	NA	NA
Inhibition of *in vitro* linoleic acid peroxidation	40.1 ± 6.41^{bc}	66.7 ± 11.3^{a}	ND	13.9 ± 4.38^{c}	37.8 ± 11.1^{ab}	NA	NA
Antiglycation activity (IC_{50})	64.5 ± 11.2^{ab}	97.8 ± 17.7^{a}	60.8 ± 16.5^{b}	NA	NA	NA	8.70 ± 2.04^{c}
α-glucosidase inhibition (IC_{50})	22.1 ± 2.88^{b}	45.2 ± 2.54^{b}	208 ± 40.8^{a}	NA	NA	6.90 ± 2.75^{b}	NA
α-Amylase inhibition (IC_{50})	20.1 ± 4.45^{b}	40.6 ± 10.7^{a}	ND	NA	NA	17.2 ± 2.89^{b}	NA
Glucose uptake increase in L-6 myotubes (EC_{50})	11.7	45.7	34.7	NA	NA	NA	NA
Glucose uptakes increase in isolated rat psoas muscle (EC_{50})	501	1510	603	NA	NA	NA	NA

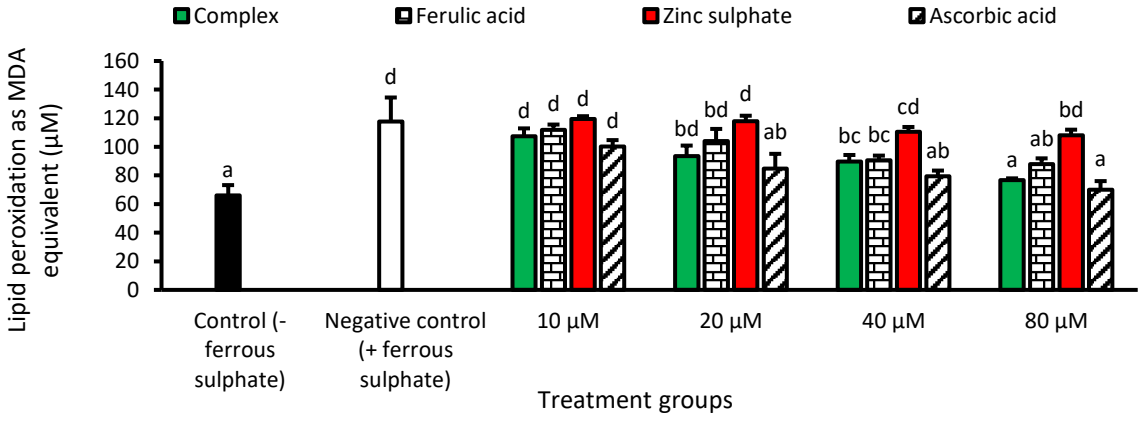
Parameters or activity	Zn(II)-biferulate.2H$_2$O complex	Ferulic acid	Zn(II)	Ascorbic acid	Trolox	Acarbose	Amg
	IC_{50} or EC_{50} values (µM)						
Inhibition of oxidative stress-induced lipid peroxidation in Chang liver cells (IC_{50})	48.6 ± 6.96^{c}	116 ± 24.8^{b}	743 ± 51.3^{b}	30.3 ± 8.52^{a}	NA	NA	NA
Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver (IC_{50})	331 ± 116^{b}	758 ± 3069^{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})	35.5 ± 5.57^{b}	117 ± 25.8^{b}	2150 ± 437^{a}	27.0 ± 2.48^{b}	NA	NA	NA

"Amg" 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 (n = 3). For each parameter, statistical comparison was done between treatment groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

Zinc sulphate showed no observable *in vitro* antioxidant activity (**Figures 4.5 and 4.6; Table 4.1**). However, the DPPH (IC_{50} = 43.8 µM) and ABTS (IC_{50} = 32.0 µM) radical scavenging and *in vitro* anti-lipid peroxidative (IC_{50} = 40.1 µM) activities of the complex were 2.1, 1.7 and 1.7 folds more potent than that of ferulic acid according to computations from the IC_{50} values (**Table 4.1**). In addition, some antioxidant activities of the complex were statistically comparable ($p > 0.05$) and/or more potent relative to Trolox and ascorbic acid (**Table 4.1**).



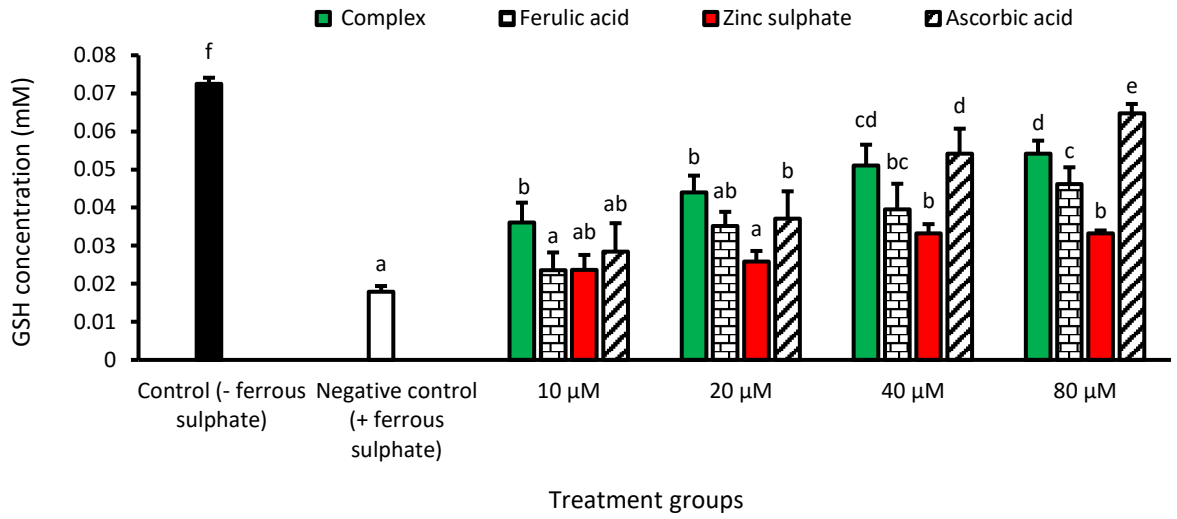
(a)



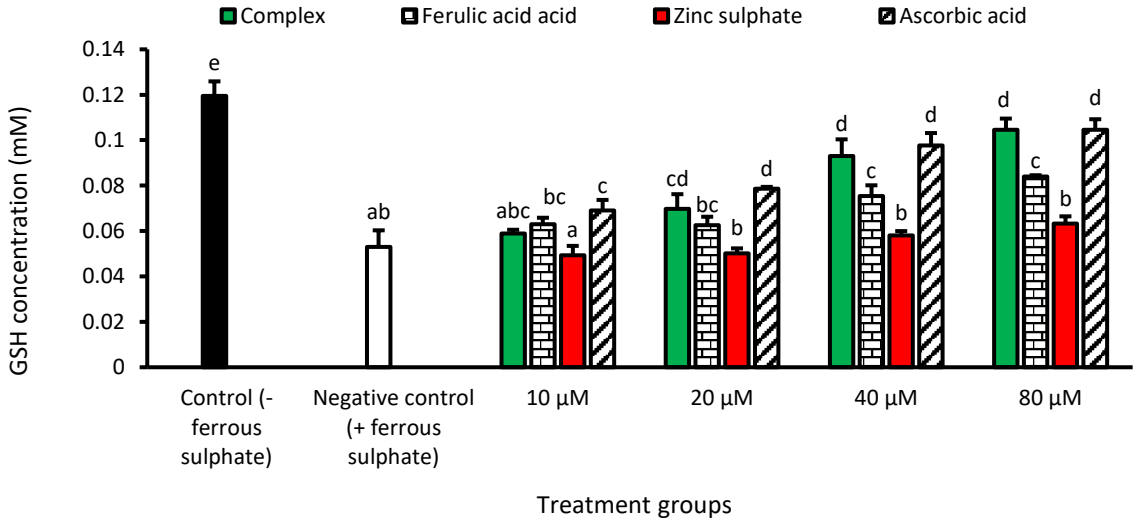
(b)

Figure 4.7: The anti-lipid peroxidative effects of the tested samples in **(a)** Chang liver cells and **(b)** isolated rat liver tissue induced with oxidative stress. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups, as well as between treatment groups and the controls (normal

control and negative control). There is significant difference ($p<0.05$) when the compared groups have no similar letter s.



(a)



(b)

Figure 4.8: The effects of the tested samples on the GSH levels of **(a)** Chang liver cells and **(b)** isolated rat liver tissue induced with oxidative stress. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups, as well as between treatment groups and the controls (normal control and negative control). There is significant difference ($p<0.05$) when the compared groups have no similar letter.

In both Chang liver cells and isolated rat liver tissues, ferulic acid dose-dependently inhibited oxidative stress-induced lipid peroxidation (IC_{50} = 116 and 758 µM, respectively) and reduced GSH depletion (IC_{50} = 85.4 and 117 µM, respectively) (**Figures 4.7 and 4.8; Table 4.1**). The cellular and tissue antioxidant activity of zinc sulphate was not as potent as ferulic acid. The complex showed significantly ($p > 0.05$) stronger cellular (IC_{50} = 48.6 µM) and tissue (IC_{50} = 331µM) anti-lipid peroxidative activity than ferulic acid (**Table 4.1**). The inhibitory effect of the complex on GSH depletion in Chang liver cell (IC_{50} = 33.9 µM) and isolated rat liver tissue (IC_{50} = 35.5 µM) was 2.5 and 3.3 folds more potent than ferulic acid based on computations from the IC_{50} values (**Table 4.1**). In most instances, the activity of the complex did not differ significantly ($p > 0.05$) from ascorbic acid.

4.4.3. Antidiabetic activities

Although not as potent as aminoguanidine (IC_{50} = 8.70 µM), zinc sulphate exhibited appreciable *in vitro* dose dependent anti-glycation effect (IC_{50} = 60.8 µM) (**Figure 4.9 and Table 4.1**). It, also, increased the anti-glycation effect of ferulic acid (IC_{50} = 97.8 µM) by 1.5-folds upon complexation (IC_{50} = 64.5 µM) (**Figure 4.9 and Table 4.1**).

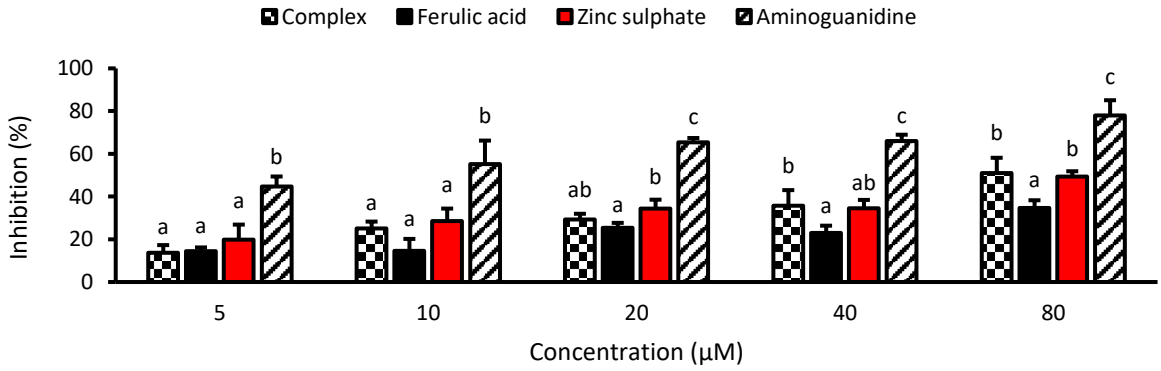
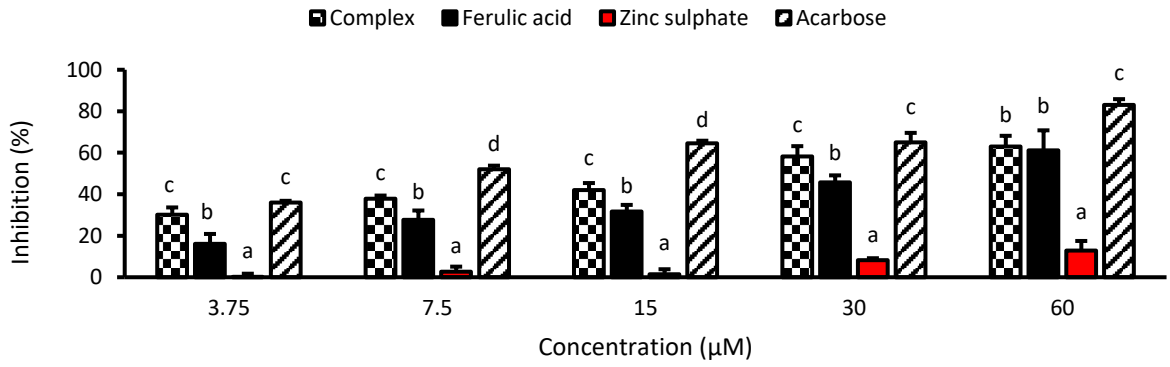
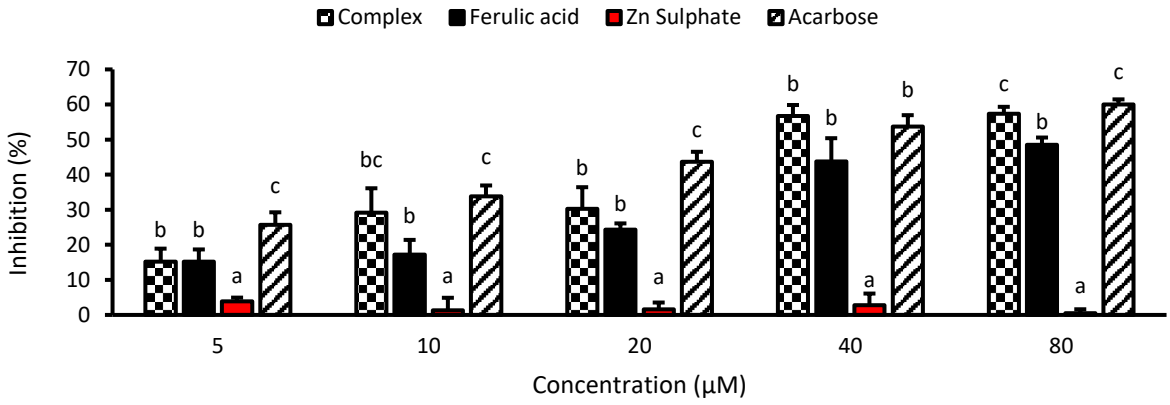


Figure 4.9: The *in vitro* inhibitory effect of the tested samples on glucose-induced protein glycation. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

Both ferulic acid and the complex dose dependently inhibited α-glucosidase (IC_{50} = 45.2 and 22.1 µM, respectively) and α-amylase (IC_{50} = 40.6 and 20.1 µM, respectively) activity *in vitro*, with the complex having stronger enzyme inhibitory activities than ferulic acid (**Figure 4.10 and Table 4.1**).



(a)

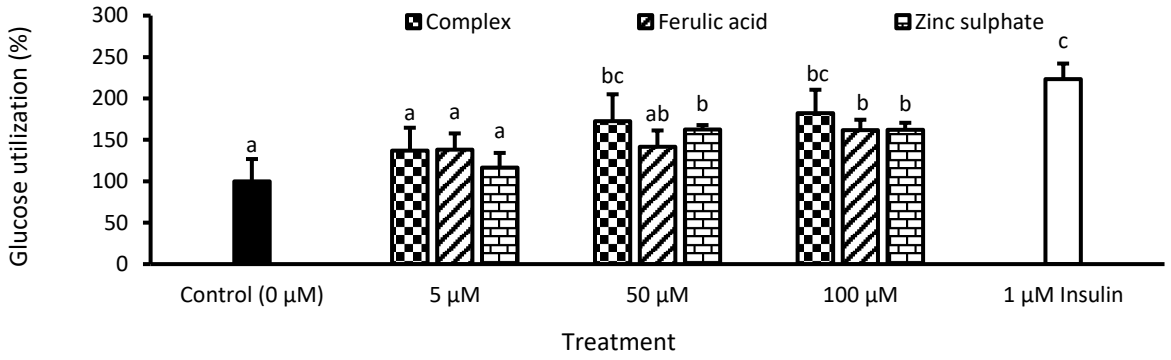


(b)

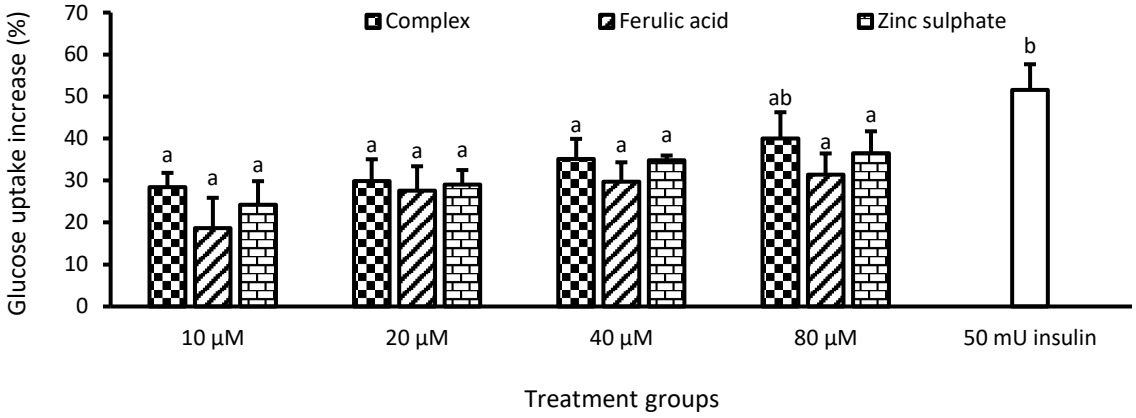
Figure 4.10: The *in vitro* inhibitory effect of the tested samples on **(a)** α-glucosidase activity **(b)** α-amylase activity. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups. Significant difference ($p<0.05$) means a difference in letters. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

The potency of the complex was statistically comparable ($p > 0.05$) with that of acarbose (IC_{50} = 6.90 and 17.2 µM) (**Table 4.1**). Zinc sulphate showed a relatively lower α-glucosidase inhibitory activity (IC_{50} = 208 µM) without observable inhibition on α-amylase (**Figure 4.10 and Table 4.1**).

In both L-6 myotubes and isolated rat psoas muscle tissue, zinc sulphate dose dependently increased glucose uptake (IC_{50} = 34.7 and 603 µM, respectively) (**Figure 4.11 and Table 4.1**).



(a)

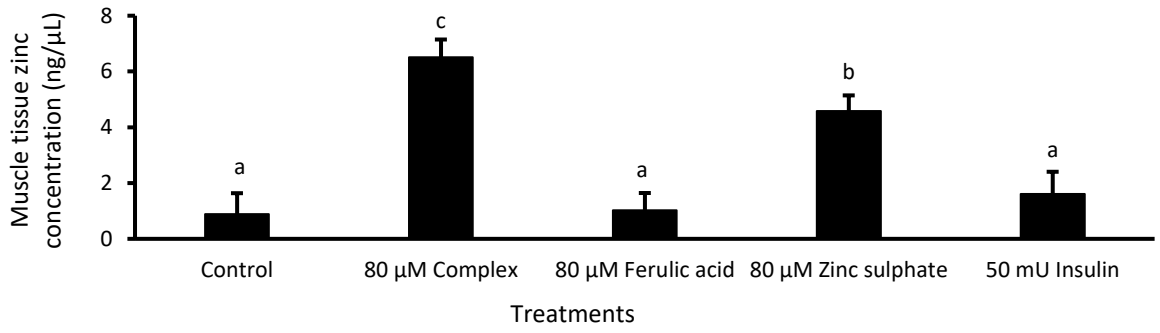


(b)

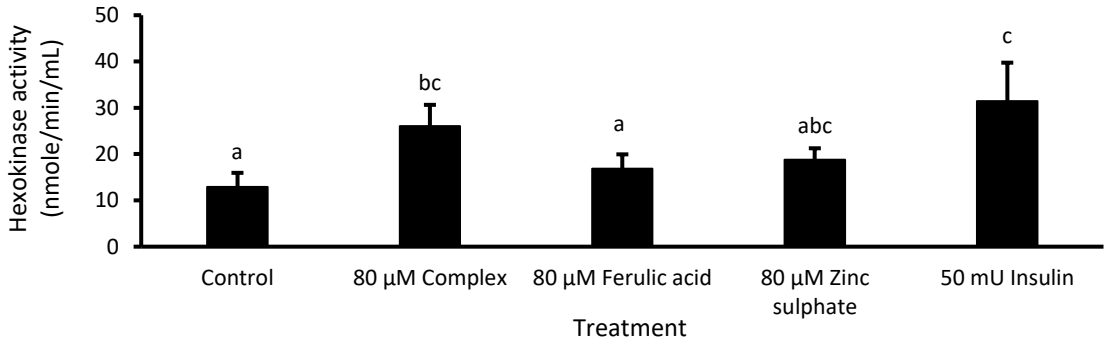
Figure 4.11: The effect of the tested samples on glucose uptake in **(a)** L6-myotubes and **(b)** isolated rat psoas muscle. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups, as well as between treatment groups and the controls (control and insulin). There is significant difference ($p<0.05$) when the compared groups have no similar letter.

Although not as potent as zinc sulphate, ferulic acid, also, dose-dependently increased glucose uptake in the myotubes (IC_{50} = 45.7 µM) and muscle tissues (IC_{50} = 1510 µM) (**Table 4.1**). However, Zn(II) complexation increased the cellular and tissue glucose uptake effect of ferulic acid by 3.9 and 3.0 folds, respectively (**Table 4.1**). At highest concentrations tested the cellular and tissue glucose uptake effect of the complex was statistically comparable ($p > 0.05$) to that of insulin (**Figure 4.11**).

Zinc(II) concentration in the complex-treated muscle tissue was higher than that of the ferulic acid ($p < 0.05$) and zinc sulphate–treated tissues (**Figure 4.12a**).



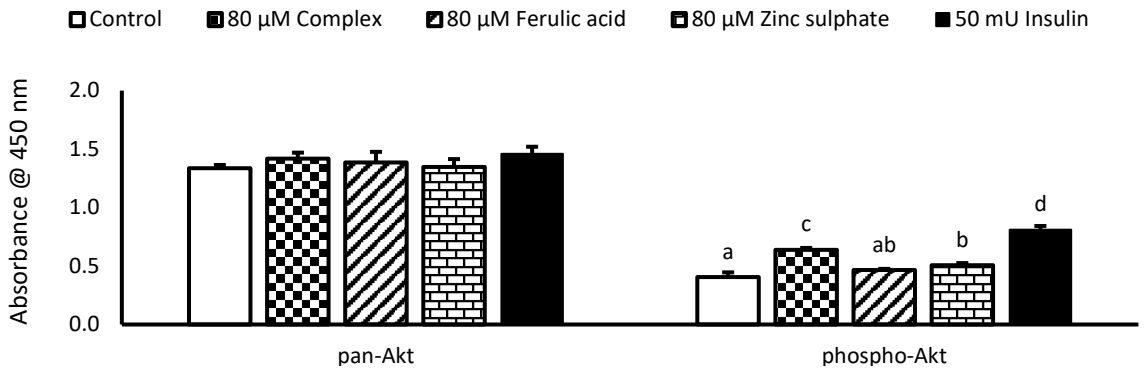
(a)



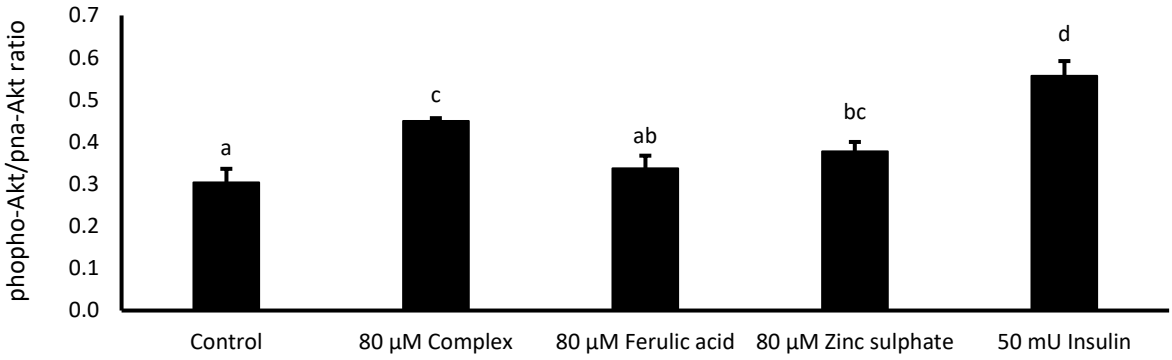
(b)

Figure 4.12: The effect of the highest concentration of 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). There is significant difference ($p<0.05$) when the compared groups have no similar letter.

Hexokinase activity in the complex-treated muscle tissue was significantly higher ($p < 0.05$) than that of the ferulic acid and zinc sulphate–treated tissues (**Figure 4.12b**). Phospho-Akt and phospho-Akt/pan-Akt ratio in the complex-treated muscle tissue was higher than that of the ferulic acid ($p < 0.05$) and zinc sulphate–treated tissues (**Figure 4.13**).



(a)



(b)

Figure 4.13: The effect of the highest concentration of tested samples on **(a)** phospho-Akt and pan-Akt levels and **(b)** phospho-Akt/pan-Akt ratio in the isolated rat psoas muscle tissues used for glucose uptake study. Data are shown as mean ± SD of triplicate analysis (n = 3). For each parameter, statistical comparison was done between treatment groups, as well as between treatment groups and the controls (control and insulin). There is significant difference ($p<0.05$) when the compared groups have no similar letter.

The docking analysis of ferulic acid and its Zn complex against α-glucosidase (GAA), amylase (AmyL), GLUT-4, and protein kinase B (PKB) protein targets is presented in **Figure 4.14**, while the minimum docking scores (kcal/mol) are shown in **Table 4.2**.

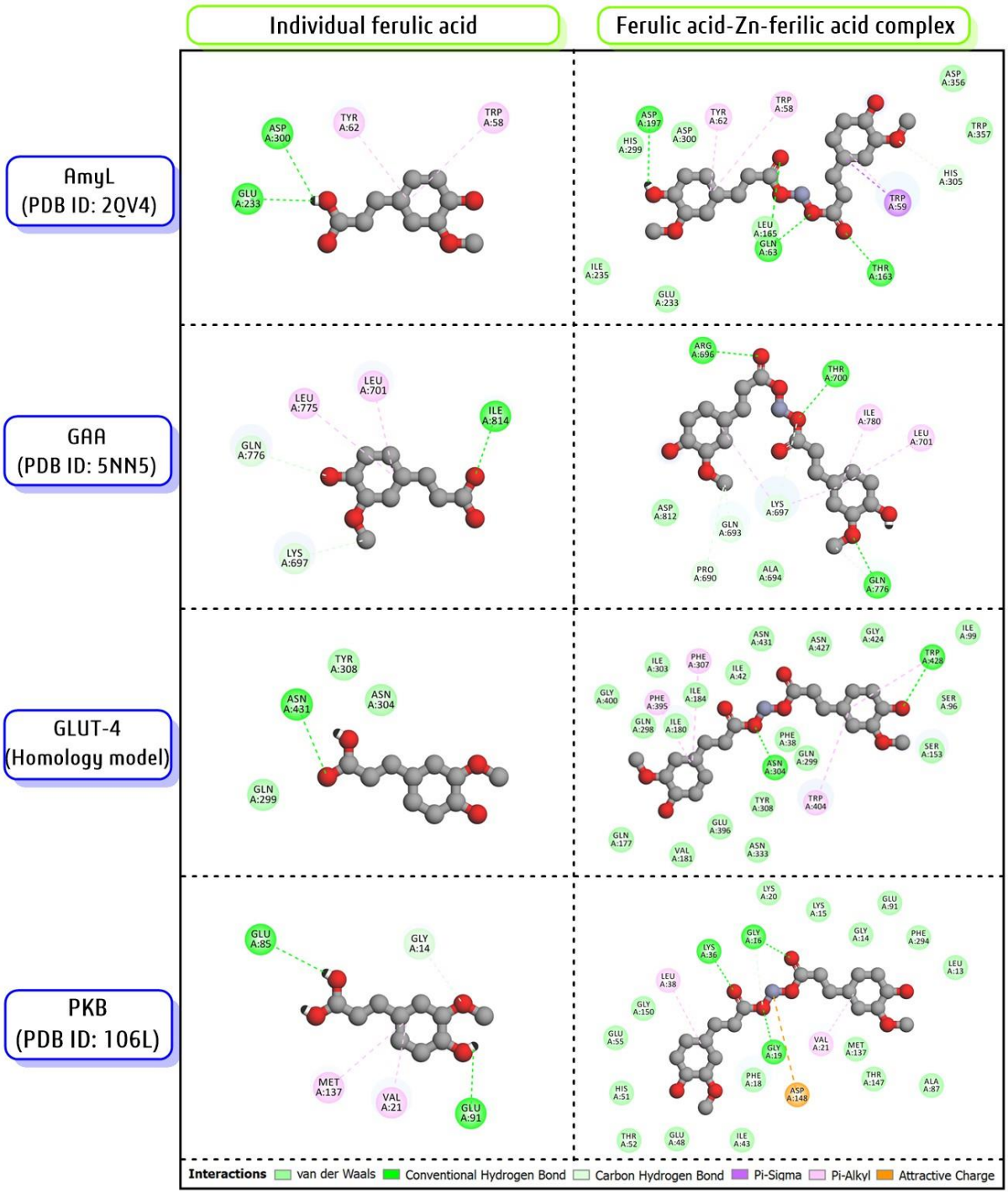


Figure 4.14: Molecular docking interaction of ferulic acid and its complex with four enzyme targets (α-glucosidase, α-amylase, GLUT-4 and PKB) linked to diabetes.

For protein targets, the docking scores of the complex (−7.24 to −8.25 kcal/mol) were higher than those of ferulic acid (−5.75 to −6.43 kcal/ mol) (**Table 4.2**).

Table 4.2: Molecular docking scores of ferulic acid and its Zn(II) complex against four enzyme targets (α-glucosidase, α-amylase, GLUT-4 and PKB) linked to diabetes

Target enzymes	**Docking scores (kcal/mol)**	
	Ferulic acid	**Zn(II)-biferulate.2H$_2$O complex**
AmyL (PDB ID: 2QV4)	-6.15	-7.74
GAA (PDB ID: 5NN5)	-5.75	-6.98
GLUT-4 (Newly modelled)	-6.16	-8.83
PKB (PDB ID: 106L)	-6.43	-8.25

AmyL, alpha-amylase; GAA, *α-glucosidase;* GLUT-4, glucose transporter type 4; PKB, protein kinase B

In both Chang liver cells and L-6 myotubes, the complex did not adversely affect viability (**Figure 4.15**).

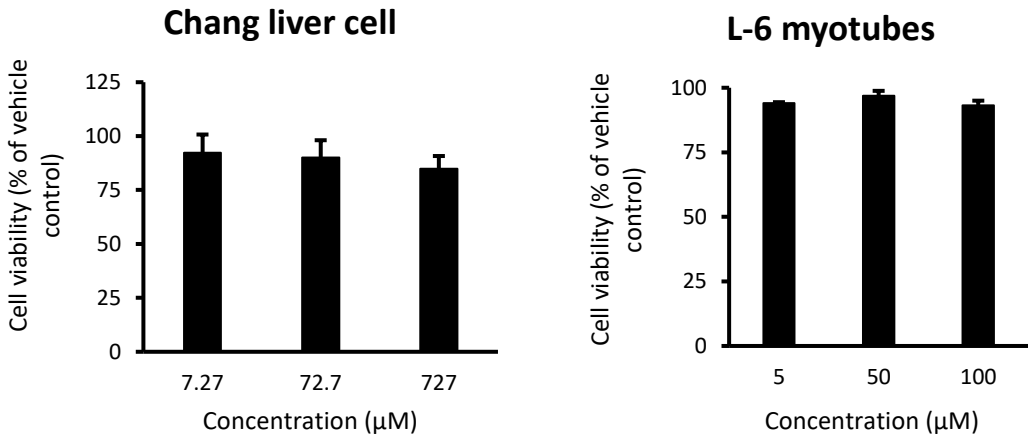


Figure 4.15: The effects of the tested samples on the viability of Chang liver cells and L-6 myotubes using MTT cell viability assay. Data are shown as mean ± SD of triplicate analysis (n = 3).

4.5. Discussion

The search for more effective approach in preventing or managing diabetes and oxidative complication remains a major strategy for combating the disease. In some cases, a combination of several drugs is used to achieve a targeted effect but poses the threat of drug toxicity and detrimental side effects. In this regard, natural medicines are often encouraged as supplemental therapy to minimize toxicity concerns. In this study, we took advantage of the insulin-related functions of zinc mineral and the oxidative stress related pharmacological credence of ferulic acid to develop a potent anti-hyperglycaemic and antioxidative complex from these natural precursors. Spectroscopic characterization suggests a Zn(II)-biferulate.$2H_2O$ product was formed (**Figure 4.2**). *In silico* molecular docking studies suggest that the complex is more favourably accommodated by putative enzyme targets and may therefore exhibit more promising bioactivity than ferulic acid.

It is an established fact that oxidative stress is implicated in the development and progression several diabetic complications (Giacco & Brownlee, 2010). Prooxidants, including free radicals, oxidatively damage biological molecules and facilitate processes like lipid peroxidation in diabetic conditions. Natural antioxidants are important in mitigating the detrimental effects of prooxidants in diabetes due to their ability to quench radical reactions and oxidative processes (Giacco & Brownlee, 2010). Ferulic acid is a natural antioxidant with radical quenching properties (Kikuzaki *et al.,* 2002), which could be attributed to its electron or hydrogen donating characteristic and ability to form a stable phenoxy radical (Chen *et al.,* 2020). In this study ferulic acid was shown to scavenge ABTS and DPPH and inhibit peroxidation of linoleic acid *in vitro* (**Figures 4.5 and 4.6b; Table 4.1**). However, when complexed with zinc sulphate its activities increased by 1.7, 2.1 and 1.7 fold, respectively, and was comparable to ascorbic acid and or Trolox (**Table 4.1**). Considering zinc sulphate had no noticeable *in vitro* radical scavenging or anti-lipid peroxidative activity (**Figures 4.5 and 4.6b; Table 4.1**), the improved activity of the complex may be attributed to the 2 moieties of ferulic acid in the complex (**Figure 4.2**). In liver cells and liver tissues, similar antioxidant trend was observed (**Figures 4.7 and 4.8; Table 4.1**). The cellular and tissue inhibitory activity of the complex on oxidative stress induced lipid peroxidation and GSH depletion was stronger than that of ferulic and zinc by several folds and statistically comparable ($p > 0.05$) to the activity of ascorbic acid (**Table 4.1**), which could also be linked to the two moieties of ferulic acid afforded by complexation (**Figure 4.2**). The data suggest that complexation could be a

therapeutic approach to improve the antioxidant potency of phenolic acids and their ability to mitigate oxidative stress.

Furthermore, the data of this study suggest that the Zn(II)-biferulate.2H2O complex may also possess promising diabetes-related therapeutic benefits. *In vitro*, the complex exhibited inhibitory activities on α-glucosidase and α-amylase (**Figure 4.10 and Table 4.1**). Inhibition of these carbohydrate digesting enzymes could translate into reduced glucose absorption and postprandial blood glucose level, which is the mechanism of action for antidiabetic drugs known as α-glucosidase inhibitors. The α-glucosidase and α-amylase inhibitory activity of the complex was 2 folds stronger than ferulic acid and comparable to acarbose (**Table 4.1**), a known α-glucosidase inhibitor. Considering that the inhibitory activity of zinc sulphate was not as potent as ferulic acid (**Table 4.1**), it is rational to hypothesize that the greater potent inhibitory activity of the complex could be attributed to the two moieties of ferulic that were acquired by the complex during complexation (**Figure 4.2**). Moreover, ferulic acid had appreciable α-glucosidase and α-amylase inhibitory activities (**Table 4.1**), which has also been previously documented (Zheng *et al.*, 2020). Additionally, docking data support this structure–function interaction of the complex (**Figure 4.14**). The docking scores or binding energies (**Table 4.2**) showed the complex had stronger *in silico* molecular interaction with α-glucosidase and α-amylase enzyme/protein targets than its precursor phenolic acid. The two ferulic acid moieties in the complex potentiated more hydrogen bond interactions with target enzymes, resulting in stronger binding energies and interaction (**Figures 4.2 and 4.14**). The data suggest that zinc mineral complexation with ferulic acid could be a therapeutic approach to improving postprandial glycaemic control of ferulic acid, and therefore warrants future antidiabetic studies in appropriate diabetic animal models.

In both L-6 myotubes and isolated rat psoas muscle tissue the complex showed promising glucose uptake modulatory effect and was more potent than its precursors (**Figure 4.11 and Table 4.1**). The uptake of circulating glucose into cells of peripheral tissues is a known mechanism for ensuring physiological glucose homeostasis, which is stimulated by insulin (Aronoff *et al.*, 2004; Saini, 2010). Thus, the modulatory effect of the complex on cellular and tissue glucose uptake suggests it may potentiate glycaemic control *in vivo*.

Furthermore, a complex with improved *in vitro* inhibitory activity on glucose induced BSA glycation (**Figure 4.9 and Table 4.1**), relative to its precursor phenolic acid was developed in the present study. Hyperglycaemia-induced glycation causes abnormally high glycation end

products, which is an important biomarker of severe diabetes and risk index of diabetic complications (Singh et al., 2014). Thus, the potent antiglycation effect of the complex suggests it may be further studied as a nutraceutical with possible mitigatory potential against glycation-mediated diabetic complications. Although the *in vitro* antiglycation activity of ferulic acid has been previously documented (Sompong et al., 2013), it appears zinc(II) further improved its antiglycation activity upon complexation (Table 1). Zinc sulphate has been shown to inhibit protein glycation in vitro (Tupe et al., 2015) and reduce protein glycation and protein carbonyl formation in rats with diabetes (Sacan et al., 2016). Thus, Zn(II) could be a promising adjuvant in improving the antiglycation property of ferulic acid through complexation.

Furthermore, glucose uptake activity in the complex-treated muscle tissues was accompanied by higher tissue zinc concentration relative to the ferulic acid and zinc sulphate–treated tissues (**Figure 4.12a**). This suggests complexation increased muscle tissue zinc uptake, which possibly influenced tissue glucose uptake. Documented evidence has shown that, through complexation, non-bioactive 1-oxy-2-pyridinethiol ligands potentiated insulin mimetic effects in adipocytes by enhancing cellular zinc uptake (Basuki *et al.*, 2007; Yoshikawa *et al.*, 2011). In the treated muscle tissues, hexokinase activity followed the same trend as glucose uptake activity (**Figure 4.12b**). Hexokinase activity in the complex treated muscle tissue was higher than that of ferulic acid and zinc sulphate–treated muscle tissues (**Figure 4.12b**), suggesting that the glucose uptake activity of the complex may be linked to cellular glucose utilization. This is evidenced by the higher phopho-Akt/pan-Akt ratio in the complex-treated muscle tissue relative to the ferulic acid and zinc sulphate–treated muscle tissues (**Figure 4.13**). Phosphorylative activation of Akt is known to be involved in the downstream signalling of glucose uptake in peripheral tissues, such as adipose and muscle tissues (Saini, 2010; Beg *et al.*, 2017). This suggest that the modulatory effect of the complex on glucose uptake and utilization may be linked to possible modulatory action on Akt activation.

From a synergistic point of view, the potent glucose uptake effect of the complex may be attributed to complexation-mediated synergism between its zinc(II) and ferulic acid moieties. Documented evidence has reported the insulin signalling modulatory effects of zinc sulphate, including modulatory effects on cellular glucose uptake (Chukwuma *et al.*, 2020), while ferulic acid has been shown to enhance glucose uptake in isolated rat muscle tissues (Salau *et al.*, 2021). The present study, also, showed that zinc sulphate and ferulic acid had notable glucose uptake effects in L-6 myotubes and isolated rat muscle tissues (**Figure 4.11 and Table 4.1**). Thus, both the zinc(II) and the two ferulic acid moieties in the complex (**Figure 4.2**) may work

synergistically to potentiate improved cellular and tissue glucose uptake and utilization. Further docking studies (**Figure 4.14 and Table 4.2**) showed insulin signalling proteins, GLUT-4 and PKB/Akt, may be implicated in the potent glucose uptake activity of the complex. Docking scores of the complex against GLUT-4 and PKB/Akt target proteins were higher than those of ferulic acid (**Table 4.2**), suggesting a stronger binding affinity with the protein targets. This could be attributed to the two ferulic acid moieties and Zn(II) components complex (**Figure 4.2**). Technically, the presence of two ferulic acid moieties and Zn(II) potentiated solid-binding affinity. The molecular interaction showed that the Zn-metal ion produced a minimum of two and maximum of four hydrogen bonds with van der Waals, P-alkyl and P-sigma covalent bonds (**Figure 4.14**). Moreover, isolated rat muscle tissues showed an increased phospho-Akt/pan Akt ratio upon treatment with the complex, suggesting modulated Akt activation.

Toxicity evaluation showed that the complex was not myotoxic or hepatotoxic, at least at *in vitro* cellular level (**Figure 4.15**). The data suggest that Zn(II) complexation may be a promising therapeutic approach for improving the glycaemic control potential of ferulic acid with minimal toxicity concerns.

4.6. Conclusion

Natural medicine, including supplements and plant derived phenolic compounds have become popular in functional medicine, perhaps due to their holistic functional and medicinal properties and minimal safety concerns. In this study, natural moieties, zinc mineral and ferulic acid, demonstrated promising antihyperglycaemic and antioxidative synergism on complexation. Zn(II) complexation improved the bioactivities of ferulic acid and conferred a broader scope of therapeutic potentials, which may be attributed to the molecular characteristics of the Zn(II)-biferulate.$2H_2O$ complex. Zn(II) complexation may be a promising therapeutic approach for improving the glycaemic control and antioxidative potential of natural phenolic acids. Thus, developing potent nutraceuticals with improved and broader antihyperglycaemic and antioxidative pharmacological profile, as well as minimal toxicity concerns. Further *in vivo* antidiabetic and antioxidative studies in appropriate animal models are recommended for additional evidenced-based data.

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CHAPTER 5

NOVEL CAFFEIC ACID – ZINC ACETATE COMPLEX: STUDIES ON PROMISING ANTIDIABETIC AND ANTIOXIDATIVE SYNERGISM THROUGH COMPLEXATION

Prescript

The *in vitro*, cellular and *ex vivo* antidiabetic and antioxidant potential of a novel zinc acetate-caffeic acid complex was investigated in this chapter. The content of this chapter has been published as follows: ***Matowane, G.R.***, *Ramorobi, L.M., Mashele, S.S., Bonnet, S.L., Noreljaleel, A.E.M., Swain, S.S., Makhafola, T.J., & Chukwuma, C.I. (**2022**). Novel Caffeic Acid - Zinc Acetate Complex: Studies on Promising Antidiabetic and Antioxidant Synergism Through Complexation.* ***Medicinal Chemistry***; 19, 147 – 162. https://doi.org/10.2174/1573406418666220620144601. The content of this chapter was adopted from the above-mentioned publication.

5.1. Abstract

The role of Zn(II) in insulin storage, secretion and function has been documented. Plant phenolics, on the other hand, have antioxidant benefits credence. The study aimed at synthesizing a novel medicinal Zn(II) complex. The medicinal properties of zinc(II) and caffeic acid were considered in synthesizing a novel complex with promising and improved antioxidant and antihyperglycaemic attributes. The complex synthesis was done using a 1:2 mole ratio of zinc acetate to caffeic acid and the product was characterized using NMR, FT-IR, high resolution-mass spectroscopy and HPLC. Its cellular toxicity was assessed in Chang Liver cells and L-myotubes. *In vitro*, cellular, and isolated tissue models were used to evaluate the antioxidant and antihyperglycaemic properties of the complex relative to its precursors. Molecular docking was used to investigate the interaction with insulin signalling target proteins: GLUT-4 and protein kinase B (Akt/PKB). Zinc(II) and caffeic acid interacted *via* $Zn:O_4$ coordination, with the complex having one moiety of Zn(II) and 2 moieties of caffeic acid. The complex showed *in vitro* radical scavenging, α-glucosidase and α-amylase inhibitory activity up to 2.6 folds stronger than caffeic acid. Its ability to inhibit lipid peroxidation (IC_{50} = 24.5 and 29.2 µM) was about 2 folds stronger than caffeic acid. Complexation improved glucose uptake activity of caffeic acid in L-6 myotubes (EC_{50} = 23.4 versus 169 µM) and isolated rat muscle tissues (EC_{50} = 339 versus 603 µM). Molecular docking showed better interaction with insulin signalling targeting proteins (GLUT-4 and Akt/PKB) than caffeic acid. The complex was not hepatotoxic or myotoxic. Data suggests a synergistic antioxidant and antihyperglycaemic potential between zinc and caffeic acid, which could be attributed to the $Zn:O_4$ coordination. Thus, it may be of medicinal relevance.

5.2. Introduction

The prevalence of diabetes and oxidative impairments is still increasing globally, with oxidative stress being a major culprit of diabetic complications. Recent data from the International Diabetes Federation (IDF) revealed that the global diabetes population has increased by 74 million since 2019, with type 2 diabetes being the most dominant diabetes (IDF, 2019). The high prevalence of type 2 diabetes is largely attributed to its strong association with poor dieting, sedentary lifestyle, and obesity or weight gain (Al-Goblan *et al.*, 2014; Sami *et al.*, 2017). These factors increase the risk of insulin resistance and poor glucose and lipid metabolism, which could lead to impaired glucose tolerance, impaired circulating glucose

uptake and utilization, hyperinsulinemia, progressive decline in pancreatic β-cell function, and persistent hyperglycaemia (Al-Goblan *et al.*, 2014; Sami *et al.*, 2017; Galicia-Garcia *et al.*, 2020).

Prolonged elevated blood glucose increases the risk of developing diabetic vascular complications, and oxidative stress has been implicated in the development of diabetic vascular complications (Giacco & Brownlee, 2010). Glycation and advanced glycation end production are notably increased when high blood glucose persists (Giacco & Brownlee, 2010). This leads to increased production of reactive oxygen species and reactive radicals, causing oxidative damage to biomolecules and increasing the risk of diabetic vascular complications (Giacco & Brownlee, 2010).

Functional foods and nutraceuticals are increasingly being used to support metabolic and oxidative health because they are perceived to be affordable, safe, and possess a holistic medicinal profile. Moreover, commercial drugs are not readily affordable among the low-income population. However, minerals and vitamins in diabetes management have been documented (Martini *et al.*, 2010). For instance, zinc plays a major role in metabolic health and insulin secretion and has been shown to modulate insulin action (Chabosseau & Rutter, 2016; Chukwuma *et al.*, 2020). In fact, zinc has been complexed with different types of ligands to develop zinc-ligand complexes with modulatory potential on glycaemic control and lipid homeostasis (Chukwuma *et al.*, 2020).

Zinc(II) complex of threonine was shown to inhibit lipolysis in adipocytes from rats and modulate glycaemic control in T2D KK-A^y mice following 14 days of intraperitoneal administration at a dose of 3mg Zn/kg body weight (Yoshikawa *et al.*, 2001). It also mimicked the effect of insulin by modulating the activity of phosphoinositide 3-kinase, phosphodiesterase, insulin receptor tyrosine kinase, and glucose transporter type 4 (GLUT-4) in adipocytes (Yoshikawa *et al.*, 2004). Zinc(II)-maltol and zinc(II)-allixin complexes inhibited lipolysis and increased uptake of glucose in adipocytes from rats (Adachi *et al.*, 2004). In T2D KK-A^y, 14 days intraperitoneal injection (4.5 mg Zn/kg) of the complexes reduced blood glucose, HbA1, and hyperinsulinemia and improved glucose tolerance (Adachi *et al.*, 2004). Naito *et al.* (2017) reported that zinc(II) complex of hinokitiol increased glucose-induced Akt phosphorylation, indicating insulin mimetic and glycaemic control potential.

Although zinc-ligand complexes show promising prospects in type 2 diabetes management, a recent review suggests that most of these complexes had been obtained from synthetic and non-

bioactive ligands that are potentially toxic, while natural bioactive phenolics remain underexplored as ligands (Chukwuma *et al.*, 2020). In fact, natural antioxidant phenolic acids, including caffeic acid, have not been studied as ligands for antidiabetic zinc complexes. Caffeic acids is a natural antioxidant found in coffee and teas (Espindola *et al.*, 2019). The potent *in vitro* radical scavenging and anti-lipid peroxidative activity of caffeic acid has been reported (Gülcin, 2006). After caffeic acid treatment, C57BL/KsJ-db/db mice showed significantly lower blood glucose and HbA1c levels, hepatic gluconeogenic enzyme activities, as well as noticeably higher tissue glycogen content, hepatic GLUT-2 and adipocyte GLUT-4 expression, and hepatic glucokinase activity (Jung *et al.*, 2006). Concomitantly, C57BL/KsJ-db/db mice treated with caffeic acid showed improved activity and expression of antioxidant enzymes decreased lipid peroxidation (Jung *et al.*, 2006).

Considering the antidiabetic and antioxidant properties of caffeic acid, it is safe to say that it is a promising ligand for developing a bioactive zinc(II) complex. However, the study has not been undertaken. Therefore, this study was done to investigate the antihyperglycaemic and antioxidative qualities of a novel zinc(II) complex of caffeic acid.

5.3. Materials and methods

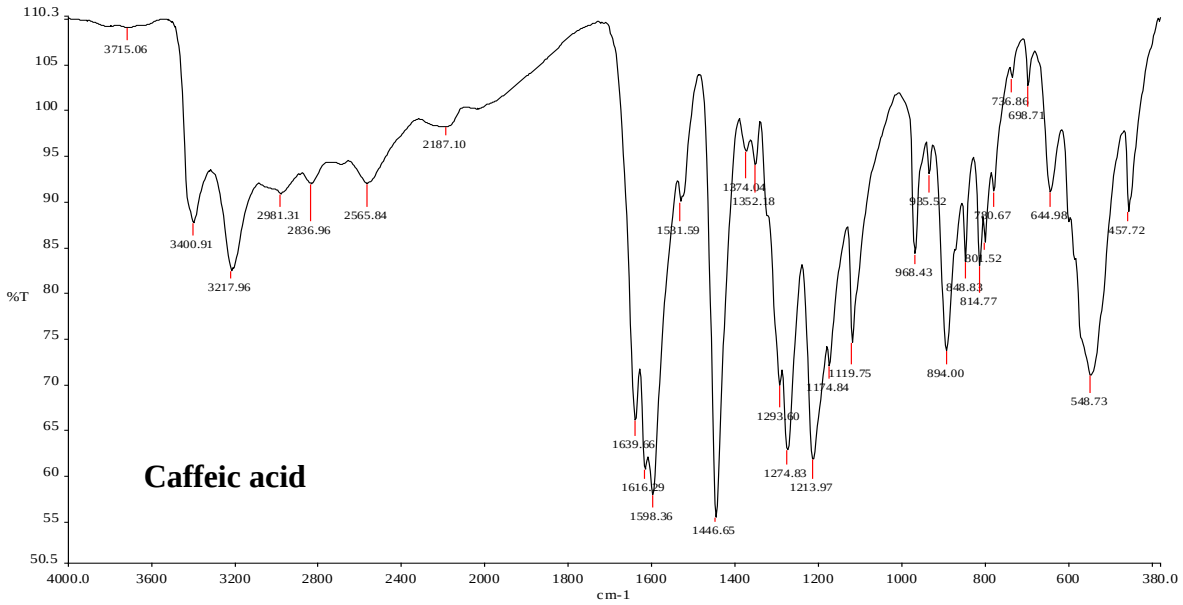
Please refer to **sections 3.1, 3.3, 3.4, 3.5, 3.6, 3.7 and 3.9 of chapter 3** for the materials and methods used in this chapter.

5.4. Results

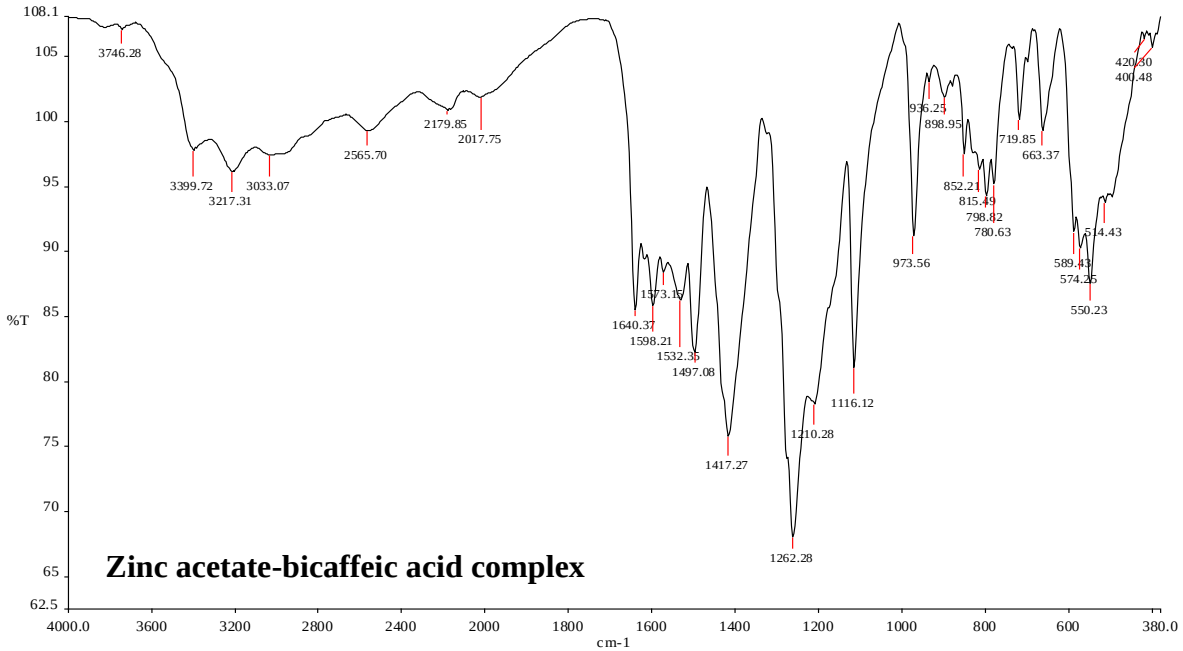
5.4.1. Structural and spectroscopic characteristics of the complex

The signal of the broad peaks (3400 – 2565 cm^{-1}) in the FTIR spectrum of caffeic acid (**Figure 5.1a**) was notably lessened in the FTIR spectrum of the complex (**Figure 5.1b**). The peaks represent a stretch in the carboxylic O-H functional group of the caffeic acid moiety. The alteration of the signals suggests that zinc (II) – caffeic acid complexation occurred through the carboxylic end of caffeic acid, as shown in (**Figure 5.2a**). Even the signal of the sharp peaks between 1700 and 1600 cm^{-1} also lessened in the FT-IR spectrum of the complex (**Figure 5.1b**) relative to that of the caffeic acid spectrum (**Figure 5.1a**). The sharp peaks are the result of carboxylic C=O stretching. Thus, the alteration of the signal further suggests the

involvement of caffeic acid's carboxylic end in caffeic acid – Zn(II) complexation (**Figure 5.2a**).

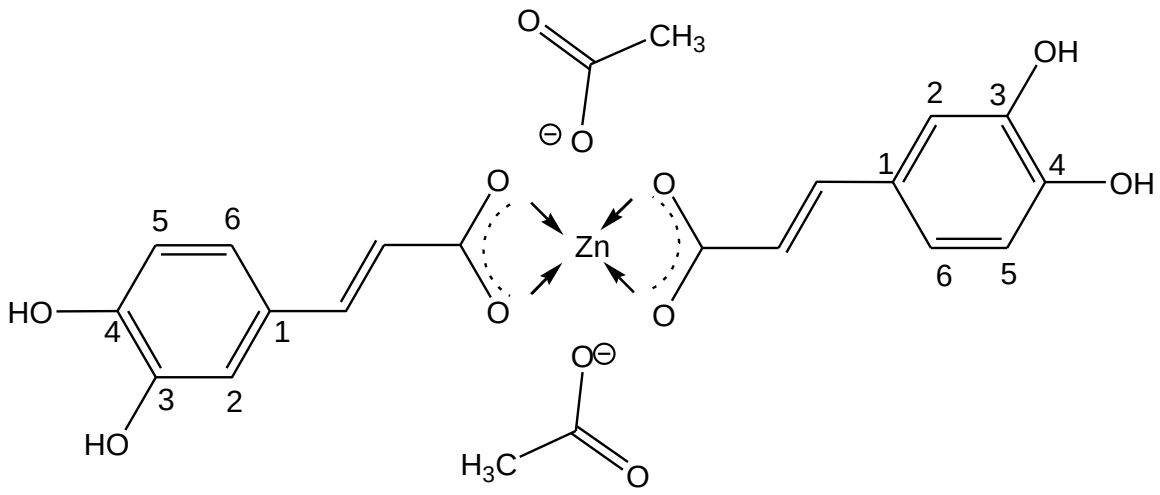


(a)

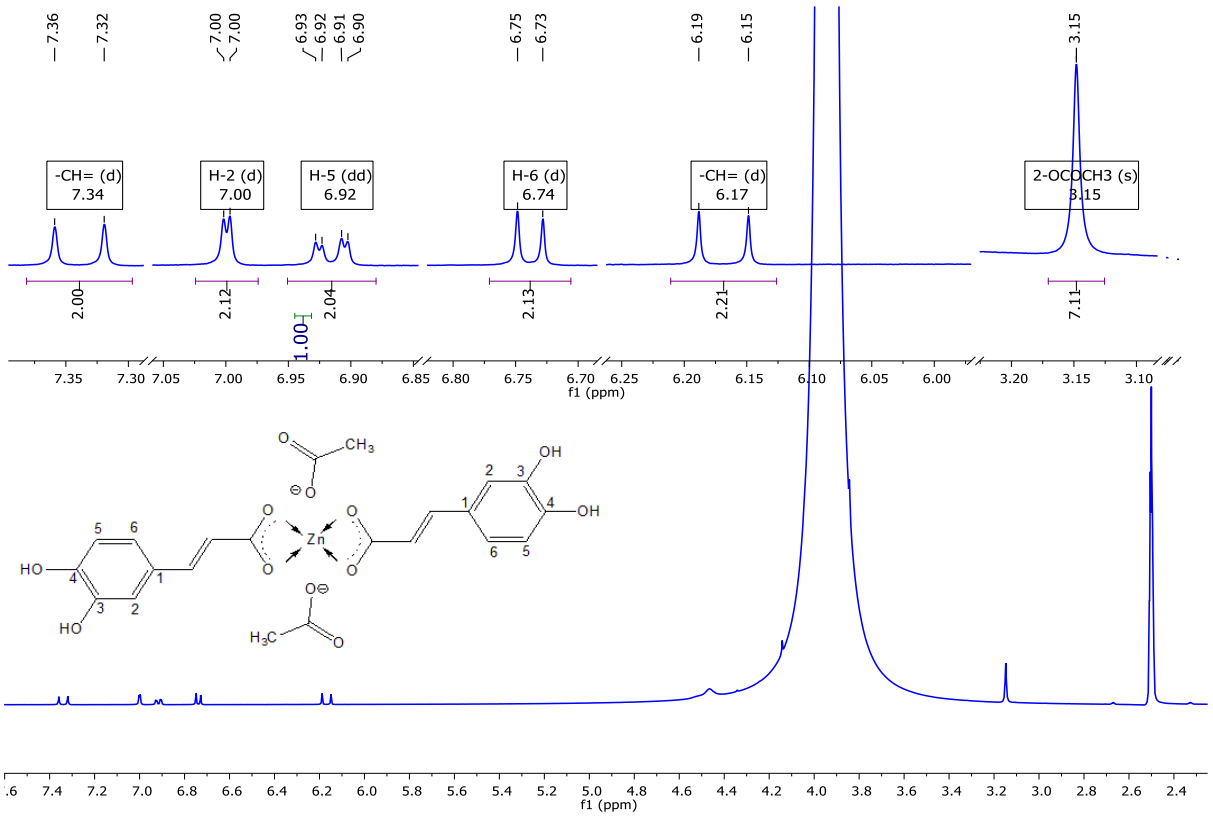


(b)

Figure 5.1: FT-IR of spectra of **(a)** caffeic acid **(b)** zinc acetate-bicaffeic acid complex.



(a)



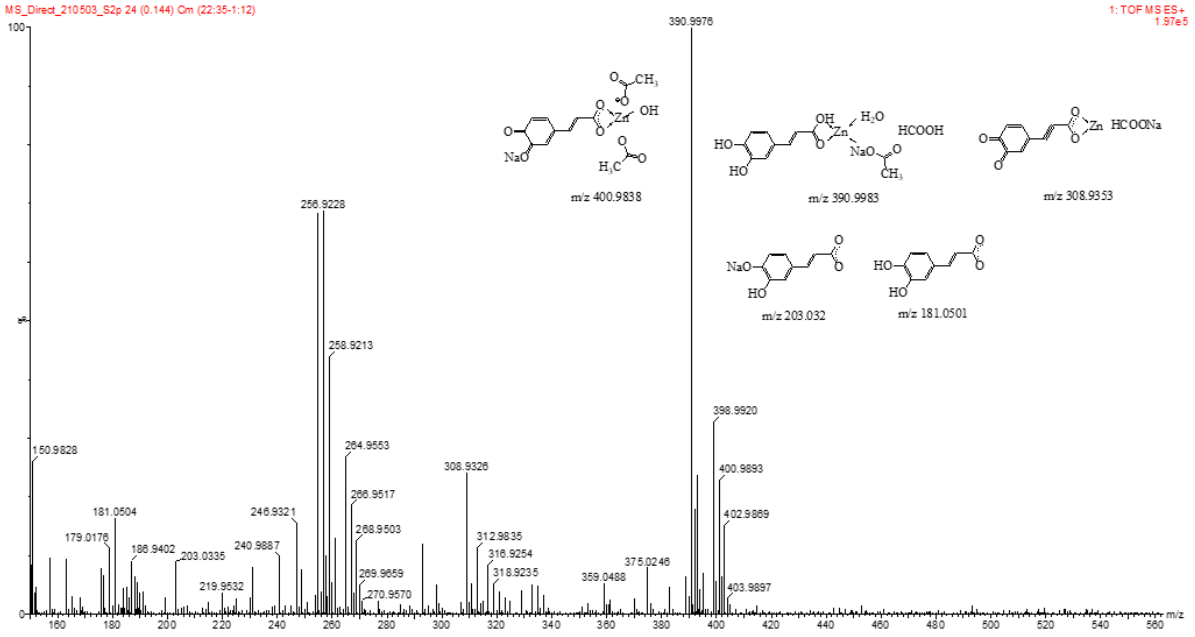
(b)

Figure 5.2: (a) proposed structure and **(b)** proton NMR spectrum of the caffeic acid – zinc acetate complex**.** ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 (d, J = 15.9 Hz, 1H, H-β), 7.00 (d, J = 2.1 Hz, 1H, H-2), 6.92 (dd, J = 8.2, 2.1 Hz, 1H, H-5), 6.74 (d, J = 8.1 Hz, 1H, H-6), 6.17 (d, J = 15.9 Hz, 1H, H-α), 3.15 (s, 3H, -OCO**CH**$_3$).

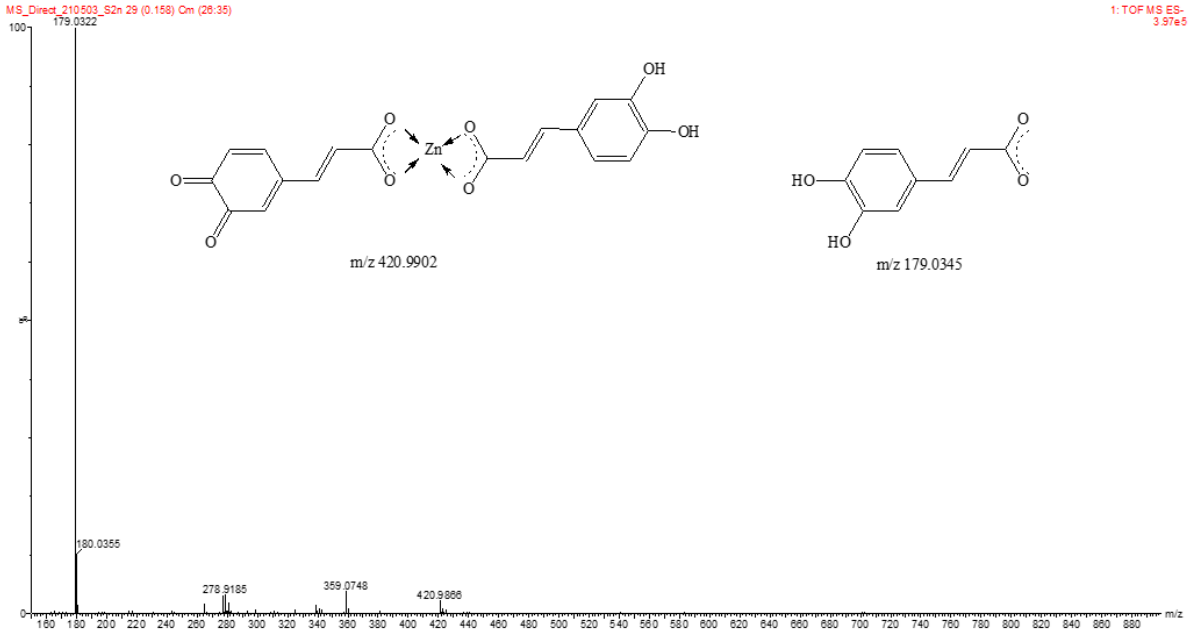
Proton NMR analysis (**Figure 5.2b**) also supports the proposed structure of the complex (**Figure 5.2a**). The ^{1}H-NMR spectrum of the complex showed an ABX system of the aromatic ring. The proton at position 2 was noticed as a doublet peak at δ 7.00, while the proton at position 5 was assigned as a doublet at δ 7.32. The proton at position 5 was observed as a doublet at δ 7.24. The doublet peak at δ 7.34 with J = 15.9 Hz was characteristic of the double bond β-proton, while the doublet peak at δ 6.35 with the same J-coupling = 15.9 Hz represents the α-proton of the double bond. The two methyl groups of the zinc acetate moiety were observed at δ 3.15. The ratio between the integral of the different protons of caffeic acid and acetate group (1:3, respectively) indicated that the caffeic acid–zinc acetate complex was synthesized successfully.

The High-Resolution Mass Spectroscopy (HR-MS) data showed the positive (**Figure 5.3a**) and negative (**Figure 5.3b**) modes of the complex's mass spectra, which also support the proposed structure of the complex (**Figure 5.2a**). The positive mode and negative mode showed fragments containing caffeic acid, zinc ion, and the acetate group, providing evidence that there was a successful formation of the caffeic zinc – acetate complex. The HR-MS of the complex in positive mode (**Figure 5.3a**) showed the main peak with an intensity of 100% at *m/z* = 390.9976 with a calculated mass of 390.9983. This denotes a difference of 7 units, possibly due to the complex accepting sodium formulate during the mass spectrometry process. The value *m/z* = 400.9893 with a calculated mass *m/z* of 400.9838 has a difference of 55 units, representing the caffeic zinc diacetate complex. The fragment of one molecule of caffeic acid in the zinc(II) complex was observed at a value of *m/z* = 308.9353, which differed by 27 units relative to the calculated mass of 308.9326. The fragmentations *m/z* = 181.0504 differed by 3 units from the calculated mass (*m/z* = 180.0501), representing the loss of one caffeic acid moiety. The fragment with *m/z* = 203.0335 differed by 15 units relative to the calculated mass of 203.0320, suggesting the sodium adduct of caffeic acid moiety. The HR-MS of caffeic acid Zinc acetate in negative mode (**Figure 5.3b**) showed a value at *m/z* = 420.9866 and differed from the calculated mass (420.9902) by 36 units, which denotes caffeic zinc di-acetate. The fragment of caffeic acid in negative mode is assigned *m/z* = 179.0345 and differed by 23 units relative to the calculated mass of *m/z* = 179.0322.

The HPLC chromatogram of the synthesized complex is presented in **Figure 5.4**. The chromatogram showed a single predominant peak (% peak area = 90.5%) at a retention time of 10.839 min, suggesting the complex was formed as a single unit.



(a)



(b)

Figure 5.3: (a) positive **(b)** negative modes for High–resolution mass spectroscopy (HR-MS) spectra of the caffeic acid-zinc acetate complex.

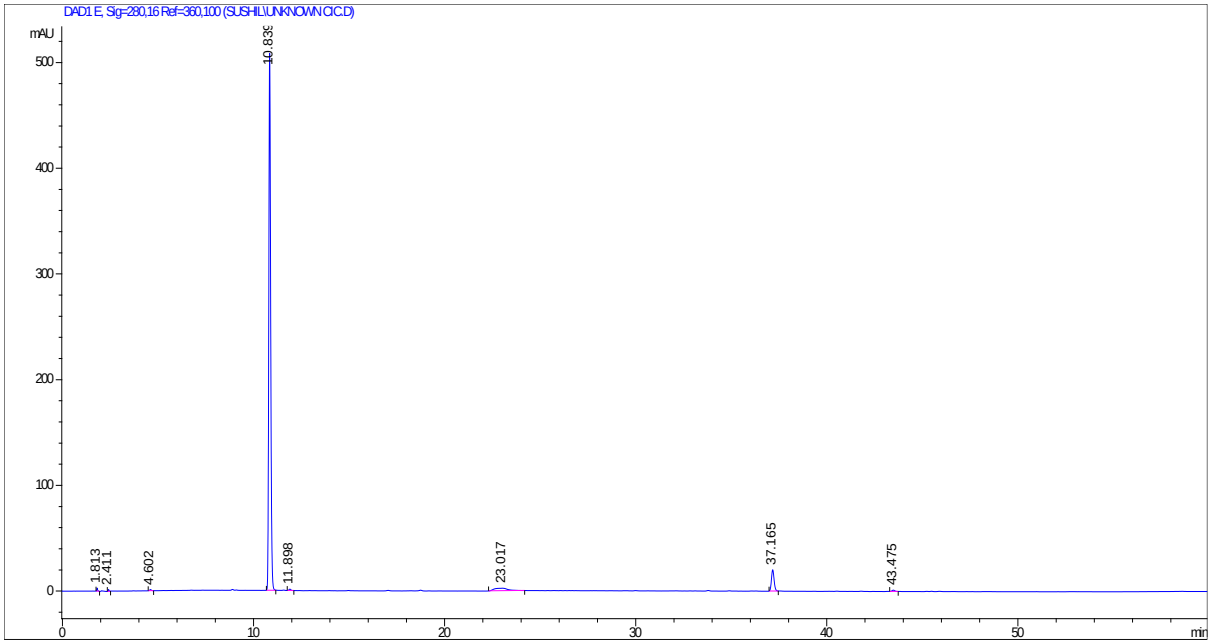


Figure 5.4: HP-LC chromatogram of caffeic acid – zinc acetate complex**.**

5.4.2. Antioxidant properties of the zinc acetate-caffeic acid complex and precursors

Both caffeic acid and its Zn(II) complex showed significantly ($p < 0.05$) more potent and/or statistically similar *in vitro* Fe^{3+} reducing, ABTS radical scavenging, and anti-lipid peroxidative activity relative to ascorbic acid and Trolox (**Figures 5.5 and 5.6 and Table 5.1**).

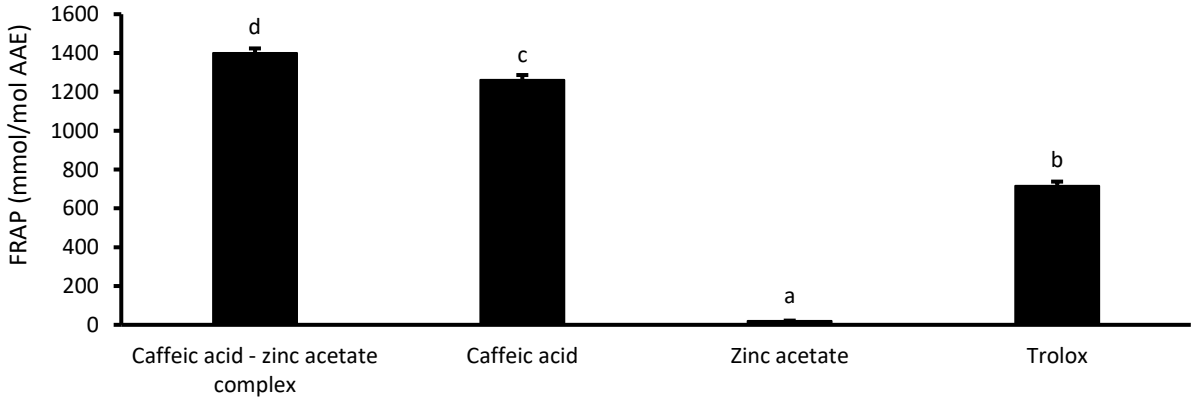
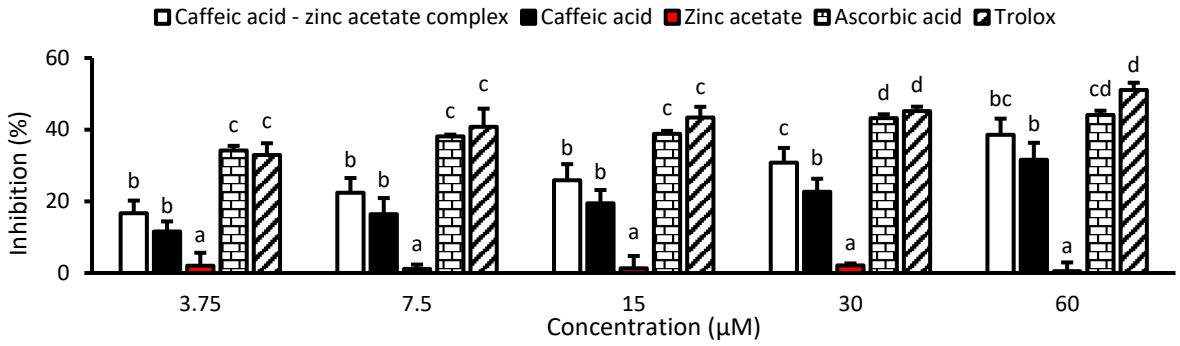
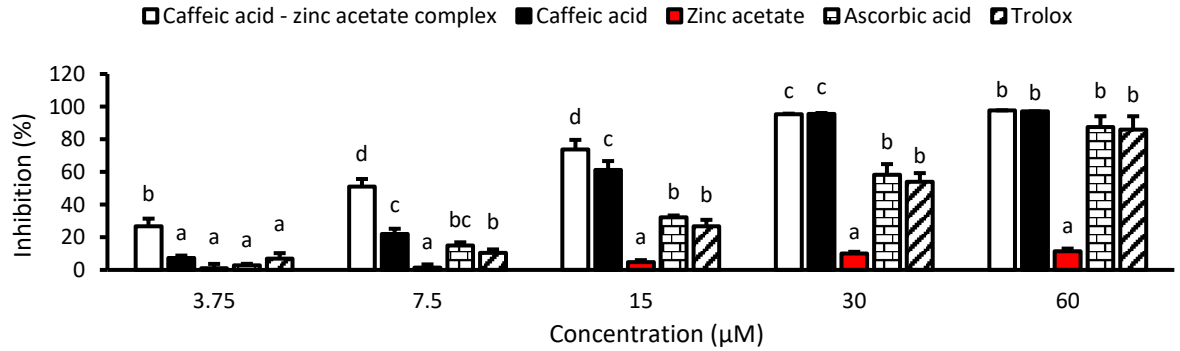


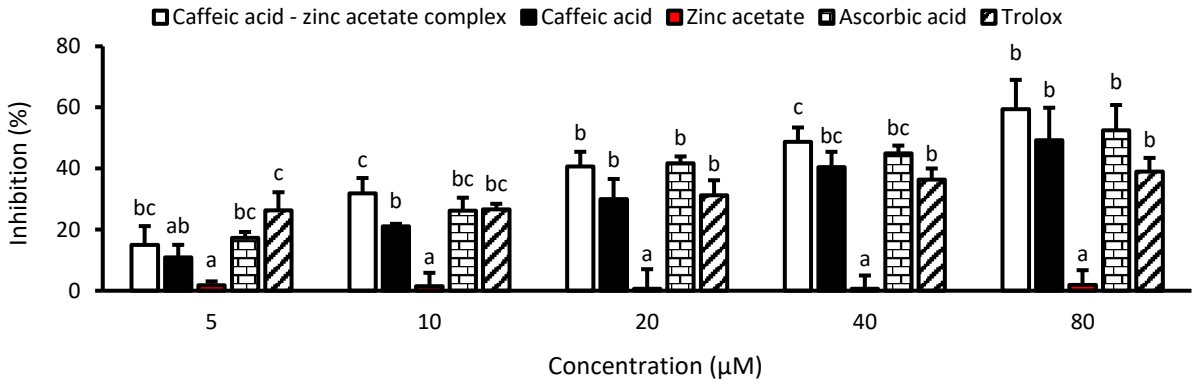
Figure 5.5: Fe^{3+} reducing activity of the complex, precursors and standard. Data are shown as mean ± SD of triplicate analysis (n = 3). Statistical comparison was done between treatment groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.



(a)



(b)



(c)

Figure 5.6: *In vitro* **(a)** DPPH radical scavenging **(b)** $ABTS^{\bullet+}$ radical scavenging and **(c)** linoleic acid lipid peroxidation inhibitory activities of the complex, precursors and standards. Data are shown as mean ± SD of triplicate analysis. Within each tested concentration, statistical comparison was done between treatment groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

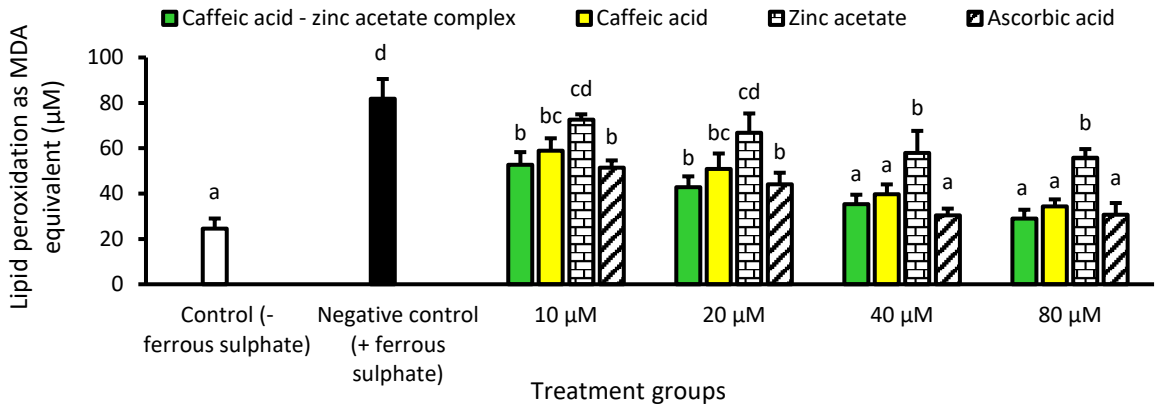
Table 5.1: The IC_{50} and EC_{50} values of caffeic acid, its complex with Zn(II) and other tested substances.

Parameters or activity	Caffeic acid - zinc acetate complex	Caffeic acid	Zinc acetate	Ascorbic acid	Trolox	Acarbose
	IC_{50} or EC_{50} values (µM)					
ABTS radical scavenging activity (IC_{50})	5.07 ± 1.84^{c}	9.51 ± 1.25^{c}	ND	46.2 ± 10.0^{b}	92.9 ± 6.73^{a}	NA
DPPH radical scavenging activity (IC_{50})	32.0 ± 6.45^{b}	68.2 ± 6.39^{a}	ND	9.98 ± 2.04^{c}	6.20 ± 3.18^{c}	NA
Inhibition of *in vitro* linoleic acid peroxidation	8.80 ± 0.86^{b}	20.8 ± 5.86^{b}	ND	13.9 ± 4.38^{b}	37.8 ± 11.1^{a}	NA
α-glucosidase inhibition (IC_{50})	17.3 ± 3.68^{b}	23.2 ± 2.15^{b}	90.4 ± 17.7^{a}	NA	NA	9.02 ± 3.18^{b}
α-amylase inhibition (IC_{50})	44.7 ± 5.55^{a}	53.2 ± 10.4^{a}	ND	NA	NA	6.36 ± 1.08^{b}
Inhibition of oxidative stress-induced lipid peroxidation in Chang liver cells (IC_{50})	26.4 ± 2.43^{b}	43.4 ± 11.2^{b}	393 ± 41.0^{a}	24.5 ± 6.21^{b}	NA	NA
Inhibition of oxidative stress-induced GSH depletion in Chang liver cells (IC_{50})	16.8 ± 5.71^{b}	35.3 ± 10.6^{b}	744 ± 310^{a}	29.2 ± 5.51^{b}	NA	NA

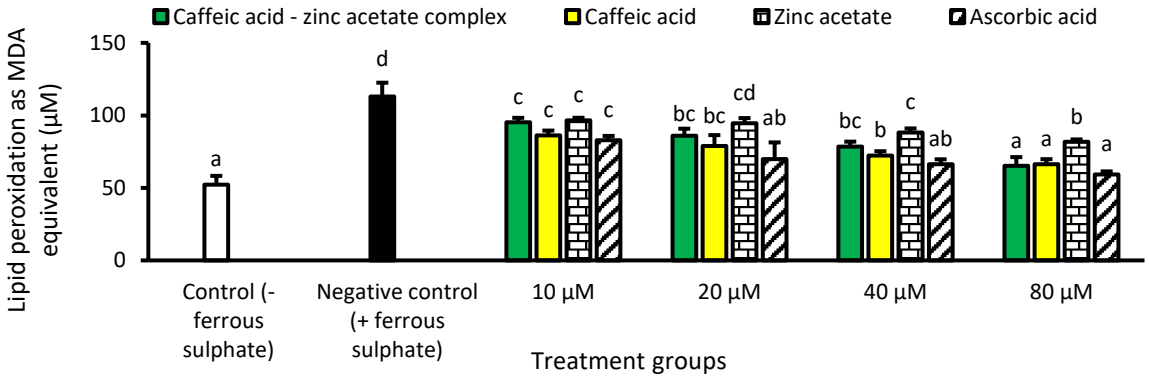
Parameters or activity	Caffeic acid - zinc acetate complex	Caffeic acid	Zinc acetate	Ascorbic acid	Trolox	Acarbose
	IC_{50} or EC_{50} values (µM)					
Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver (IC_{50})	172 ± 52.3^{b}	223 ± 20.9^{b}	3000 ± 910^{a}	100 ± 36.3^{b}	NA	NA
Inhibition of oxidative stress-induced GSH depletion in isolated rat liver (IC_{50})	26.4 ± 3.05^{b}	69.2 ± 10.4^{b}	1120 ± 420^{a}	23.8 ± 3.41^{b}	NA	NA
Glucose uptake increase in L-6 myotubes (EC_{50})	23.4	169	93.3	NA	NA	NA
Glucose uptakes increase in isolated rat psoas muscle (EC_{50})	339	603	398	NA	NA	NA

"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, 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 (n = 3). For each parameter, statistical comparison was done between treatment groups. Different letters 'a', 'b' and 'c' represent significant difference ($p<0.05$) between treatment groups.

However, the *in vitro* DPPH radical scavenging activity of caffeic acid (IC_{50} = 68.2 µM) and its zinc acetate complex (IC_{50} = 32.0 µM) was significantly less potent than that of ascorbic acid and Trolox (**Table 5.1**). Zinc acetate did not show any appreciable *in vitro* antioxidant activity (**Figures 5.5 and 5.6**). However, when complexed with caffeic acid, the resulting complex showed *in vitro* antioxidant activity that was up to 2.4 folds more potent ($p < 0.05$) than its precursor caffeic acid as computed using IC_{50} values (**Table 5.1**).



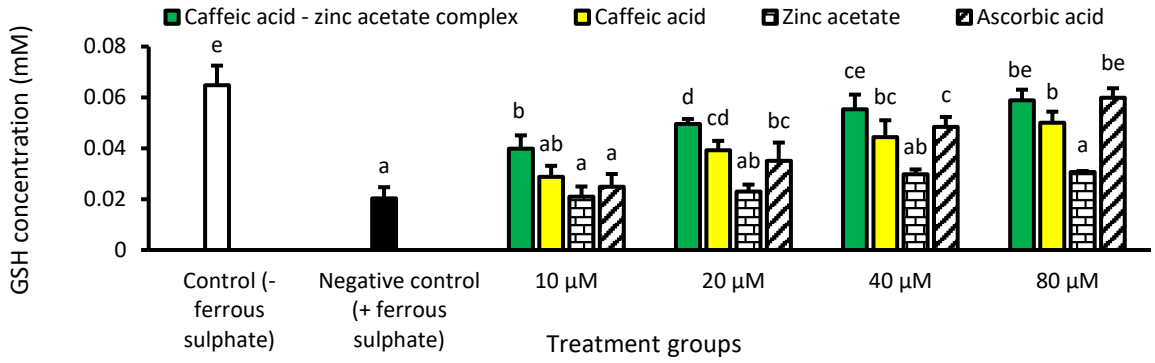
(a)



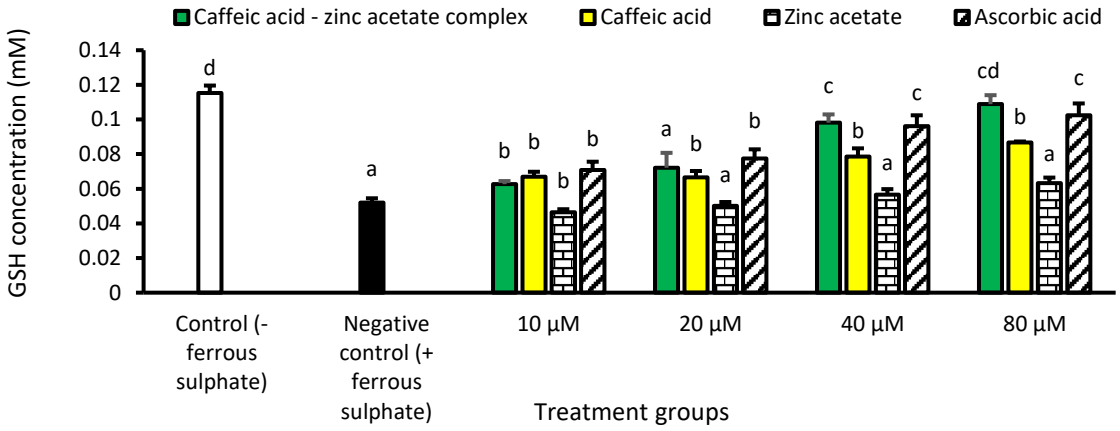
(b)

Figure 5.7: Oxidative stress-induced lipid peroxidation levels in **(a)** Chang liver cells and **(b)** isolated rat liver tissues dose-dependently treated with the tested samples. Within each tested concentration, statistical comparison was done between treatment groups, as well as between treatment groups and the controls (normal control and negative control). Data are shown as mean ± SD of replicate analysis. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

In Chang liver cells and isolated rat liver tissue, zinc acetate remained significantly ($p < 0.05$) least potent with respect to inhibiting oxidative stress-mediated lipid peroxidation and GSH depletion (**Figures 5.7 and 5.8; Table 5.1**). With respect to the IC_{50} values in **Table 5.1**, the cellular and tissue antioxidant activity of both caffeic acid and the complex was statistically not different ($p > 0.05$) compared to that of ascorbic acid. However, the activity of the complex was 1.3 to 2.6 folds more potent than its precursor caffeic acid.



(a)

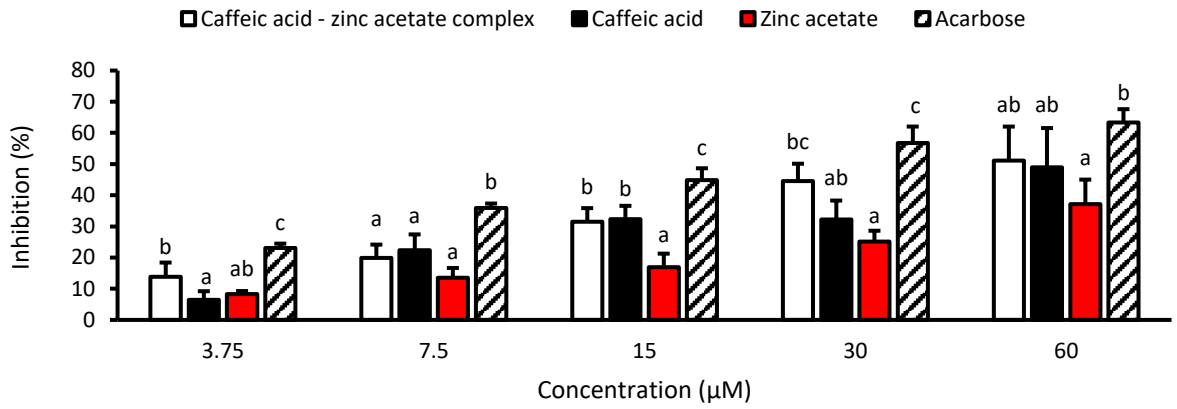


(b)

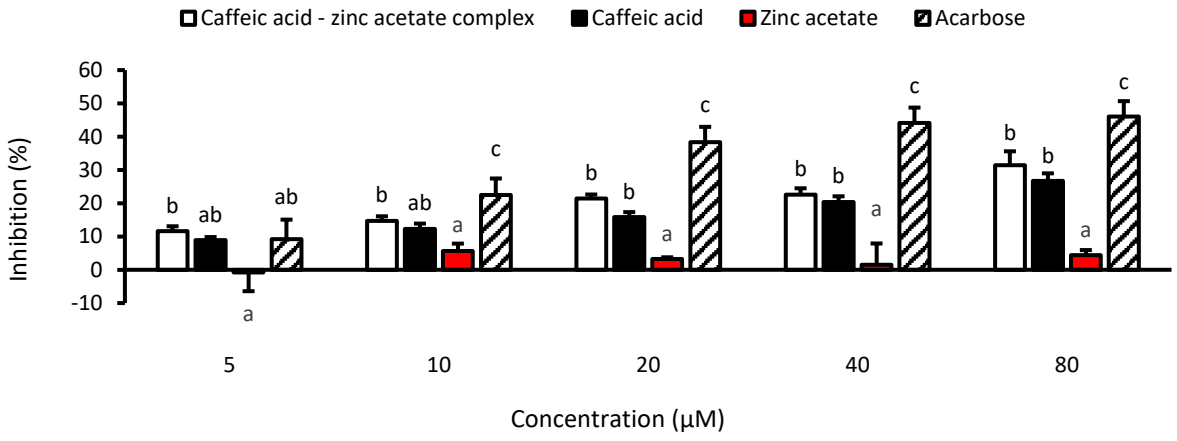
Figure 5.8: The effects of the tested samples on glutathione reduced (GSH) concentrations in **(a)** Chang liver cells and **(b)** isolated rat liver tissues induced with oxidative stress. Within each tested concentration, statistical comparison was done between treatment groups, as well as between treatment groups and the controls (normal control and negative control). Data are shown as mean ± SD of replicate analysis. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

5.4.3. Antihyperglycaemic activities of the zinc acetate-caffeic complex and precursors

In vitro, zinc acetate showed lower α-glucosidase inhibitory activity (IC_{50} = 90.4 µM) than caffeic acid (IC_{50} = 23.2 µM) and the complex (IC_{50} = 17.3 µM), without observable inhibition on α-amylase (**Figure 5.9 and Table 5.1**).



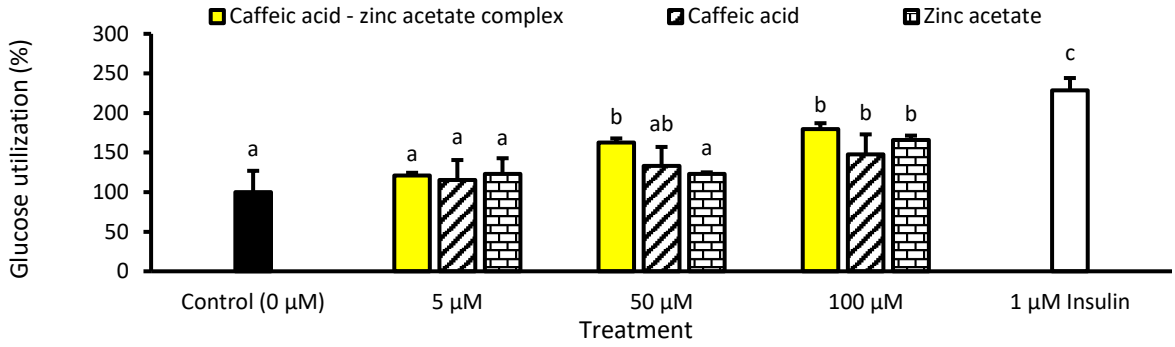
(a)



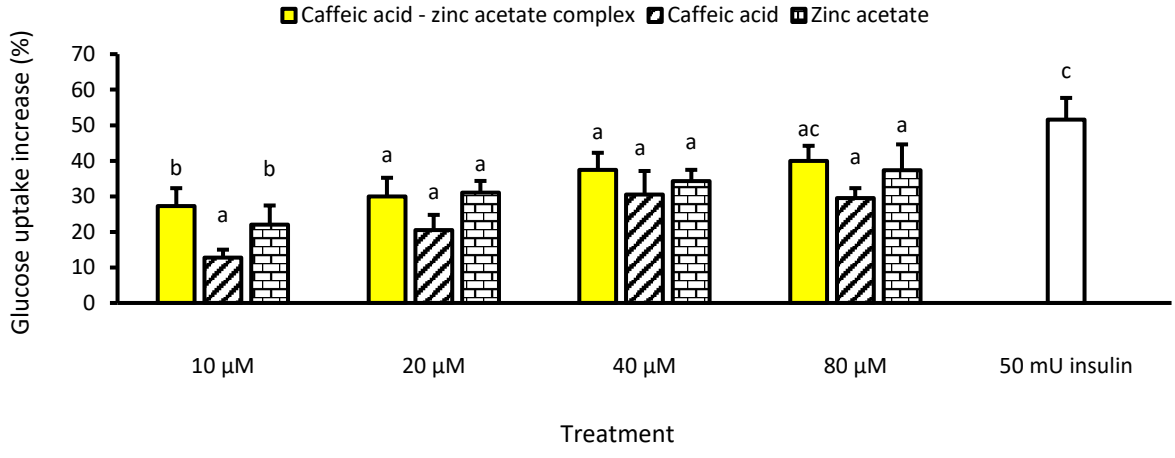
(b)

Figure 5.9: *In vitro* **(a)** α-glucosidase and **(b)** α-amylase inhibitory activities of the zinc acetate-caffeic acid complex and precursor. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

The α-glucosidase inhibitory activity of caffeic acid and the complex was statistically not different ($p > 0.05$) compared to that of acarbose, while acarbose significantly ($p < 0.05$) outperformed caffeic acid and the complex in inhibiting α-amylase (IC_{50} = 6.36 µM versus 53.2 µM and 44.7 µM, respectively). Although no significant difference ($p < 0.05$) was observed between the activity of caffeic acid and the complex, the α-amylase and α-glucosidase inhibitory activity of the complex was more potent than that of caffeic acid (**Table 5.1**).



(a)



(b)

Figure 5.10: the effects of the zinc acetate-caffeic acid and precursors on glucose uptake in **(a)** L6-myotubes and **(b)** isolated rat psoas muscle. Data are shown as mean ± SD of triplicate analysis (n = 3). Within each tested concentration, statistical comparison was done between treatment groups, as well as between treatment groups and the controls (control and insulin). There is significant difference ($p<0.05$) when the compared groups have no similar letter.

In both L6-myotubes and psoas muscle tissues isolated from rats, the complex was more effective in increasing glucose uptake/utilization compared to caffeic acid and zinc acetate (**Figure 5.10 and Table 5.1**), although, it was not as potent as insulin. Based on the EC_{50} value (23.4 µM) it outperformed caffeic acid and zinc acetate by 7 and 4 folds, respectively, at the cellular experimental level. At concentrations ranging from 7.36 to 736 µM and 5 to 100 µM, the complex did not notable compromise the viability of Chang liver cells and L6-myotubes, respectively (**Figure 5.11**).

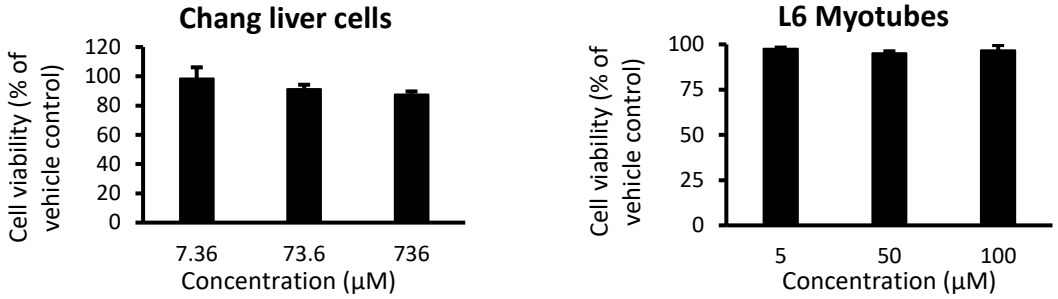


Figure 5.11: Figures showing the effects of the tested samples on the viability of Chang liver cells and L-6 myotubes using MTT cell viability assay. Data are shown as mean ± SD of triplicate analysis (n = 3).

Computational docking data of complex and caffeic acid against two insulin signalling target enzymes (GLUT-4 and PKB) are shown in Figure **5.12 and Table 5.2**. Caffeic acid showed docking scores of -6.78 and -6.34 kcal/mol, while the complex showed docking scores of -9.91 and -9.72 kcal/mol against GLUT-4 and PKB, respectively. The docking scores were higher against GLUT-4 than PKB for both caffeic acid and the complex. The data suggests that the complex had stronger *in silico* interaction with the target enzymes than caffeic acid.

Table 5.2. Molecular docking score (kcal/mol) of caffeic acid and the zinc acetate-caffeic acid complex against GLUT-4 and PKB.

Target enzymes	Caffeic acid	Zinc acetate-caffeic acid complex
GLUT-4	-6.78	-9.91
PKB	-6.34	-9.72

GLUT-4, glucose transporter type 4; PKB, protein kinase B.

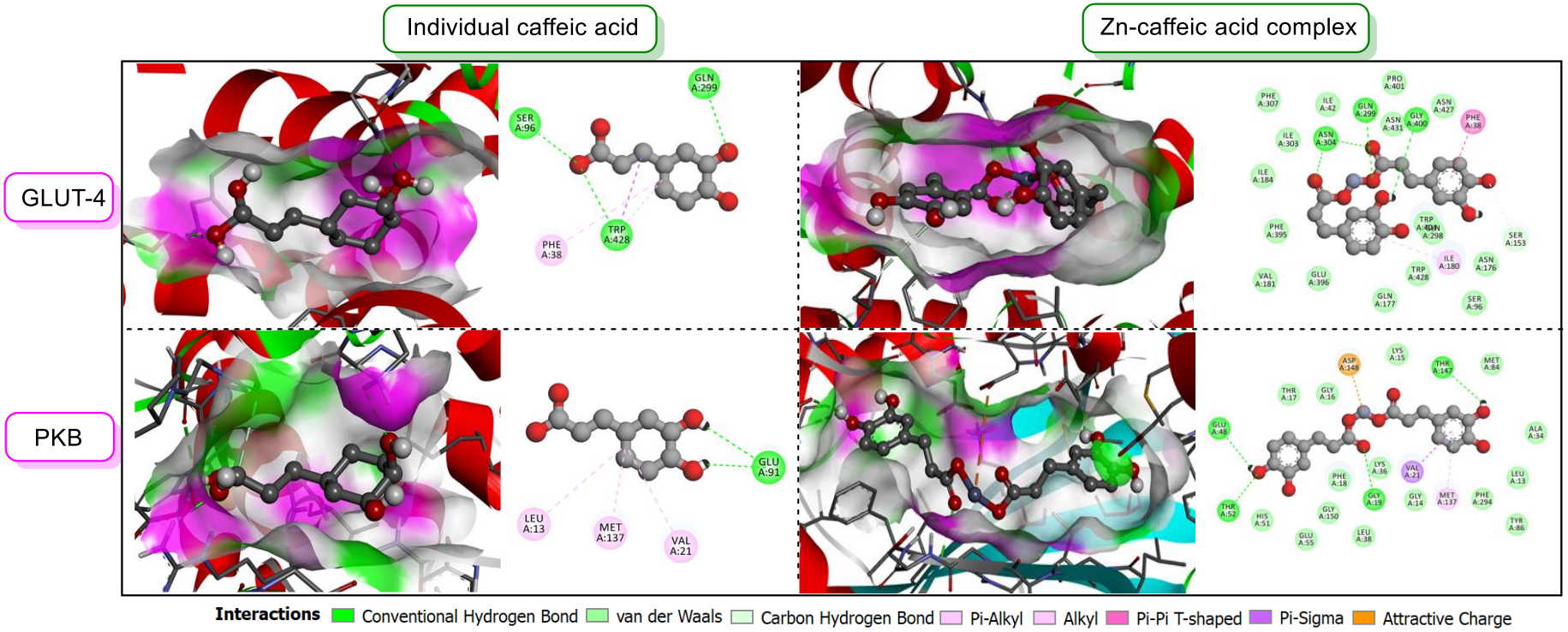


Figure 5.12: Docking analysis of complex and caffeic acid against GLUT-4 and PKB/Akt**.**

5.5. Discussion

The detrimental role of oxidative injuries in the progression and complications of several diseases, including diabetes, cannot be over emphasized. Caffeic acid is a known natural oxidant (Espindola *et al.*, 2019), ameliorating oxidative stress in diabetic conditions (Jung *et al.*, 2006). Zinc mineral (Zn^{2+}), on the other hand, plays an important role in insulin storage and secretion and has been shown to possess insulin mimetic potentials (Norouzi *et al.*, 2018; Chukwuma *et al.*, 2020).

In this study, we took advantage of these attributes to develop a novel zinc(II) complex of caffeic acid, which had structural properties that potentiated promising antidiabetic and antioxidant activities relative to its precursors.

Reactive oxygen species and free radical production have been shown to increase during diabetes, which are major culprits of diabetic oxidative tissue damage (Giacco & Brownlee, 2010). Processes like glycation (Giacco & Brownlee, 2010), electron transport chain energy metabolism (Fakhruddin *et al.*, 2017), and lipid peroxidation (Fatani *et al.*, 2016) are some of the key intiators and promoters of pro-oxidant and radical production during diabetes. The detrimental action of these radicals is strongly linked to their highly reactive nature and thus could oxidatively alter biological macromolecules and processes (Di Meo & Venditti, 2020). Mopping these biological radicals could limit or delay the progression of oxidative complications in diabetes.

Consistent with previous documentation (Gülcin, 2006), caffeic acid appreciably scavenged DPPH and ABTS radicals and inhibited linoleic acid peroxidation *in vitro* (**Figure 5.6 and Table 5.1**), which can be attributed to its stable phenoxy radical forming, electron donating and hydrogen atom transferring abilities (Chukwuma *et al.*, 2018; Chen *et al.*, 2020). However, when complexed with zinc acetate, the resulting complex showed DPPH and ABTS radical scavenging and anti-lipid peroxidative activity that was, respectively, 2.1, 1.9 and 2.4 fold more potent than caffeic acid, based on computed IC_{50} values (**Table 5.1**). The *in vitro* antioxidant effect of the complex was statistically comparable and/or more potent than ascorbic acid and Trolox, demonstrating its potential relevance in mitigating against lipid peroxidation-mediated radical production relative to caffeic acid. Considering that no notable *in vitro* antioxidant activity was shown by zinc, it may be hypothesized that the two moieties of caffeic acid in the complex (**Figures 5.2a**) influenced the observed 2 fold increase in activity relative to its caffeic acid precursor. Additionally, the phenolic acid's meta- and para- O-H group, which are crucial

to its antioxidant activities (Chen *et al.*, 2020), were unaltered and did not involve complexation (**Figures 5.1b and 5.2a**), thus preserving this antioxidant attribute in the complex.

In Chang liver cells and isolated rat liver tissue induced with oxidative stress, zinc acetate inhibited lipid peroxidation and GSH depletion to some degree (**Figures 5.7 and 5.8; Table 5.1**). The cellular and tissue antioxidant effect of zinc acetate may be attributed to the involvement of molecular zinc(II) in the functioning of some antioxidant enzymes (as a co-factor), synthesis of antioxidant metallothioneins, suppression of NADPH-Oxidase activity, and stabilization of cell membrane against oxidative damage (Marreiro *et al.*, 2017). Nevertheless, zinc acetate's cellular and tissue antioxidant effect was not as potent as that of caffeic acid (**Table 5.1**). The promising dose-dependent anti-lipid peroxidase property of caffeic acid has been previously demonstrated to show 68.2 and 75.8% efficacy at 10 and 30 µg/mL, respectively (Gülcin, 2006). In C57BL/KsJ-db/db mice, caffeic acid was shown to reduce hepatic lipid peroxidation by 49% and concomitantly increased hepatic GSH concentration significantly ($p < 0.001$), which was attributed to its modulatory effect on hepatic antioxidant enzymes (Jung *et al.*, 2006). However, complexation of caffeic acid with Zn(II) resulted in stronger (2.6 and 1.3 folds, respectively) anti-lipid peroxidative activity compared to caffeic acid in Chang liver cells and rat liver tissues (**Table 5.1**). Similarly, it was shown that the complex's ability to inhibit oxidative stress-induced cellular and tissue GSH depletion was, respectively, 1.6 and 2.1 folds more potent than caffeic acid precursor, which may be attributed to two caffeic moieties in the complex. The data suggests that Zn(II) may be a promising adjuvant for improving the efficacy of caffeic acid with regard to managing tissue oxidative stress and associated injuries. This may be largely influenced by the $Zn:O_4$ coordination structure afforded by complexation (**Figure 5.2a**).

Other than antioxidant effects, caffeic acid also showed potential for postprandial glycaemic control. It showed an *in vitro* inhibitory effect on α-glucosidase and α-amylase (**Figure 5.9 and Table 5.1**), which is also consistent with previous documentations (Oboh *et al.*, 2015). Inhibition of these enzymes is a known therapeutic approach for diabetes (Moelands *et al.*, 2018). Thus, if mimicked *in vivo*, the *in vitro* inhibitory activity of caffeic acid (**Table 5.1**) can translate into reduced glucose absorption and subsequently postprandial glycaemic control. On the other hand, zinc acetate showed moderate α-glucosidase inhibitory action relative to caffeic acid (**Figure 5.9a and Table 5.1**) without observable α-amylase inhibitory action (**Figure 5.9b**). However, upon complexation with caffeic acid, a more potent α-glucosidase (1.4 folds)

and α-amylase inhibitory activity was observed (**Table 5.1**), which suggest that zinc(II) complexation with caffeic acid may potentiate appreciable postprandial glucose control.

Furthermore, postprandial glycaemia is controlled by the clearance of circulating glucose to promote homeostasis. Insulin signals the uptake of circulating glucose into peripheral tissues when blood glucose is above normal (Aronoff *et al.,* 2004). Some antidiabetic drugs act as insulin sensitizers. They stimulate and modulate insulin signalling, thus facilitating insulin-induced lowering of blood glucose (Chaudhury *et al.,* 2017). Evidence that zinc could promote insulin signalling or exert insulin mimetic effects has been documented and may be useful in diabetes management. In adipocytes, zinc exerted stimulatory effects on adipogenesis and glucose transport (Shisheva *et al.,* 1992). Studies in skeletal muscle cells from humans and mice suggest that Zn(II) enhances glucose oxidation and glycaemic control by modulating insulin signalling (Norouzi *et al.,* 2018). Clinical documentation reported that supplementation of zinc minerals could cause improvements in glycaemic and lipid profiles of diabetic subjects (Jayawardena *et al.,* 2012). In the present study, zinc acetate increased glucose uptake in both L-6 myotubes and psoas muscle tissues isolated from rats (**Figure 5.10 and Table 5.1**). Also, zinc(II) afforded the complex cellular and tissue glucose uptake activity (**Figure 5.10 and Table 5.1**). In fact, computations using the EC_{50} values showed that the cellular glucose uptake activity of the complex was, respectively, 4.0 and 7.3 folds more potent than that of zinc acetate and caffeic acid, while the muscle tissue glucose uptake of the complex was, respectively, 1.2 and 1.8 folds more potent than zinc acetate and caffeic acid. The fold increase may be attributed to a synergistic action of both zinc acetate and caffeic acid moieties in the complex (**Figure 5.2a**). Moreover, caffeic acid showed a modulatory effect on cellular (EC_{50} = 169 µM) and tissue (EC_{50} = 603 µM) glucose uptake (**Table 5.1**), while studies have shown that it increases glucose transport in rat skeletal muscle independent of insulin by modulating the activity of 5'AMP-activated protein kinase (Tsuda *et al.,* 2012). Data suggest that caffeic acid–zinc acetate complex may be further studied as a potent nutraceutical with insulin modulatory and glycaemic control functions.

Further molecular docking data (**Figure 5.12 and Table 5.2**) showed that the complex had stronger interaction with insulin signalling protein targets (GLU-4 and PKB) compared to caffeic acid. In the current era of drug discovery, bioinformatic tools, including molecular docking simulation are widely used by academic and industrial researchers towards exploring or developing potential candidates targeting desired target enzymes. The presence of double mole of caffeic acid in the complex potentiated stronger binding affinity with target enzyme

(**Figure 5.12**). Additionally, due to the presence of hydroxy group (-OH), the two caffeic acid moieties in the complex formed double hydrogen bonds with the target enzymes. PHE38, GLN299, SER96, and TRP299 in GLUT-4, as well as LEU13, VAL21, and MET137 in PKB, are some common and conserved interactions found in docking against the target enzymes (**Figure 5.12**). GLUT-4 and PKB are important proteins involved in insulin signalling (Chang *et al.*, 2004), which suggests possible insulin signalling modulatory action of the complex.

The present study showed some linear correlation in the structure-activity relationship. For example, the *in vitro* radical scavenging and anti-lipid peroxidase activity of the complex were approximately 2 fold (2.1, 1.9 and 2.4) more potent than its caffeic acid precursor. Thus, it was logical to attribute the double fold increase to the two moieties of caffeic acid in the complex since zinc acetate did not show *in vitro* antioxidant activity. Similarly, the inhibitory activity of the complex cellular and tissue GSH depletion was two fold more potent than its caffeic acid precursor. Since the activity of zinc acetate was far less than that of caffeic acid (**Table 5.1**), we attributed the two fold activity of the complex to the combined action of its two caffeic acid moieties. In some other instances, the non-linear correlation was observed. For example, in L6-myotubes and rat muscle tissue, the complex's glucose uptake activity showed a fold increase that varied from 1.2 to 7.3 folds relative to that of its precursors (caffeic acid and zinc acetate). Both zinc acetate and caffeic acid also increased glucose uptake. Hence, the activity of the complex may be attributed to possible synergism between its zinc(II) and caffeic moieties.

5.6. Conclusion

The role of supplements and natural medicine remains pertinent in functional therapy, possibly due to their holistic functional effects and minimal toxicity concerns. In this study, it was shown that complexing zinc(II) with caffeic acid resulted in a non-cytotoxic complex with improved glycaemic control and antioxidant potentials relative to its precursors, which may be attributed to the $Zn:O_4$ structural property of the complex. The *in vitro*, cellular, *ex vivo* and *in silico* data suggests a synergistic antioxidant and glycaemic interactive potential between zinc(II) and caffeic acid, which can be further studied in appropriate animal model to see if it will be mimicked. The study suggests that the complex may be a potential candidate to control diabetes and related oxidative assaults and could be medicinally relevant following more pharmacological validations.

5.7. Postscript

Based on the EC_{50} values (**Tables 4.1 and 5.1**) the muscle tissue glucose uptake activity of the zinc acetate-caffeic acid complex (EC_{50} = 339 μM) was more potent than that of the Zn(II)-biferulate.$2H_2O$ complex (EC_{50} = 501 μM). Together with the stronger antioxidant activity of the former relative to the latter (**Tables 4.1 and 5.1**), it was rationale to further subject the zinc acetate-caffeic acid complex to *in vivo* antidiabetic and antioxidant evaluation using a fructose and low dose STZ rat model of diabetes to see if its promising complexation mediated antidiabetic and antioxidant potential will be mimicked or reproduced *in vivo*. The complex was subjected to *in vivo* antidiabetic and antioxidant evaluation and its effects were compared to that of its precursors (caffeic acid and zinc acetate). This study and the outcome are reported in the **chapter 6** of this thesis.

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CHAPTER 6

ZINC(II) AND CAFFEIC ACID SYNERGISTICALLY IMPROVED MUSCLE AKT PHOSPHORYLATION, INSULIN ACTION, GLYCAEMIC CONTROL AND ANTIOXIDANT STATUS IN DIABETIC RATS

Prescript

The *in vivo* antidiabetic and antioxidant potential of a novel zinc acetate-caffeic acid complex was investigated in this chapter. The content of this chapter has been submitted for publication on **11 December 2022** and is currently under review as follows: ***Matowane, G.R.***, *Mashele, S.S., Makhafola, T.J., & Chukwuma, Zinc(II) and caffeic acid synergistically improved muscle Akt phosphorylation, insulin action, glycaemic control and antioxidant status in diabetic rats.* ***Biomedicine & Pharmacotherapy*** *(Manuscript ID:* ***BIOPHA-D-22-06241****).* The content of this chapter was adopted from the above-mentioned manuscript.

6.1. Abstract

Zinc(II) and caffeic acid are natural supplements with insulin mimetic and antioxidant attributes, respectively. Recently, we demonstrated their antioxidative and glycaemic control synergism *in vitro*, cellular and *ex vivo* experimental models by complexing them. This study was done to evaluate the complexation-mediated antidiabetic and antioxidative synergism between zinc(II) and caffeic acid in diabetic and the possible underlying mechanisms. Male SD rats were induced with diabetes using 10% fructose and 40 mg/kg bw streptozotocin. The diabetic rats were treated with the Zn(II)-caffeic acid complex and its precursors (caffeic acid and zinc acetate) for 4 weeks at predetermined doses. The effect of the treatments on diabetes and oxidative stress related parameters was measured. The complex ameliorated diabetic alterations. It reduced polyphagia and polydipsia and appreciably recovered weight loss. It increased insulin secretion, insulin sensitivity, hepatic and muscle glycogen, muscle hexokinase activity and Akt phosphorylation, which resulted in improved glucose tolerance and reduced blood glucose in diabetic rats. The complex concomitantly reduced systemic and tissue lipid peroxidation and increased antioxidant enzymes activity in diabetic rats. Notably, the complex outperformed the antidiabetic and antioxidative action of its precursors and had a broader bioactivity profile. Complexing zinc acetate with caffeic acid improved their ameliorative effect on insulin resistance by ~24 and 42%, respectively, as well as their anti-hyperglycaemic action by ~24 – 36% and ~42 – 47%, respectively, suggesting a complexation-mediated synergism. In some instances, the antidiabetic action of the complex was comparable to metformin, while its antioxidant effect was better than that of metformin. Zinc(II)-caffeic acid complexation may be an alternative approach to improving the efficacy of antidiabetic and antioxidative therapy with minimal adverse or side effects.

6.2. Introduction

The global prevalence of diabetes is increasing at an alarming rate. Between 2019 and 2021, the global number of adults with diabetes increased by approximately 74 million (IDF, 2021; 2022). Diabetes is associated with severe morbidity and mortality outcomes, as well as the enormous health care expenditures, which is causing economic strain in many countries. Classically, the disease is characterized by abnormally high blood glucose levels, which is caused by impaired insulin secretion or action depending on the type of diabetes (Trikkalinou *et al*., 2017). Type 2 diabetes (T2D) has been the most common type of diabetes (IDF, 2021;

2022). Risk factors include some lifestyle related factors, such as unhealthy eating and inactivity, as well as obesity (Al-Goblan *et al*., 2014; Sami *et al*., 2017). Insulin resistance and impaired glucose tolerance are the main causes of hyperglycaemia in T2D. Cells, particularly those of peripheral tissues become non-responsive to the action of insulin in normalizing blood glucose, which results to poor circulating glucose uptake and glucose/lipid metabolism, impaired glucose storage and hormonal control of glycogen breakdown, hyperinsulinaemia, progressive loss of pancreatic β-cell function, etc. (Galicia-Garcia *et al*., 2020). These together contribute to impaired glucose tolerance and persistent hyperglyceamia (Galicia-Garcia *et al*., 2020).

While there are several known antidiabetic drugs for managing diabetes, there have been documented incidences of many unpleasant side effects (Chaturvedi *et al*., 2018), perhaps because they are synthetic and exogenous. Natural medicine and supplements are becoming popular, perhaps due to their holistic functional/medicinal profile and minimal adverse concerns. Zinc mineral is an endogenous substance that is linked to insulin storage and function (Chabosseau and Rutter, 2016), thus considerable attention has been given to it as a potential therapeutic supplement for diabetes. Zinc has been shown to modulate glucose uptake and inhibit lipolysis in rat adipocytes (Adachi *et al*., 2004). Also, it has been shown to reduce HbA1c and improve hyperglycaemia in T2D KK-A^y mice (Adachi *et al*., 2004). Mice with deleted ZnT8 genes responsible for pancreatic zinc transport were reported to display impaired glucose tolerance (Nicolson *et al*., 2009). In diabetic subjects, zinc supplementation has been shown to improve blood lipid and glycemic profiles (Jayawardena et al., 2012).

Antioxidant agents are also becoming more relevant in managing diabetes and its complications by mitigating oxidative insults (Bajaj and Khan, 2012; Kanwugu *et al*., 2022). This is partly because oxidative stress has been implicated in many complications of diabetes (Giacco and Brownlee, 2010). Processes such as hyperglycaemia-induced glycation and glucose energy metabolism can lead to the generation of reactive oxygen species (ROS) during diabetes, which can cause oxidative injuries to cellular biomolecules and organelles (Giacco and Brownlee, 2010). Some ROS compromise cellular membrane integrity through lipid peroxidation, while releasing more deleterious ROS in the process (Giacco and Brownlee, 2010).

Some natural antioxidants, including plant polyphenols have been reported to potentiate antioxidative action in experimental diabetes, which ameliorated diabetic alterations

(Vinayagam *et al*., 2016). Caffeic acid, for example, is a natural phenolic acid with potent radical scavenging properties and anti-lipid peroxidative activity (Gülçin, 2006). Its treatment in C57BL/KsJ-*db/db* mice resulted in increased expression of antioxidant enzymes and reduced lipid peroxidation (Jung *et al*., 2006).

Therapeutic synergism is a growing approach in disease management because it affords multi-mode effects, which can improve therapeutic outcomes (Lehár *et al*., 2009). Research advances have shown complexation-mediated synergism between zinc and some natural ligands, which resulted in improved insulin mimetic effects (Chukwuma *et al*., 2020). In adipocytes, Zn(II)–threonine complex demonstrated effects that resemble that of insulin by increasing the activity of phosphatidylinositol 3-kinase, phosphodiesterase, insulin receptor tyrosine kinase and GLUT-4 (Yoshikawa *et al*., 2004). In rat adipocytes, the complex reduced lipolysis (Yoshikawa *et al*., 2001). It, also, showed glycaemic control effects in T2D KK-A^y mice by reducing blood glucose and increasing glucose tolerance (Yoshikawa *et al*., 2001). In adipocytes from rat Zn(II) complexes of two natural ligands (maltol and allixin) were shown to modulate and suppress glucose uptake and lipolysis, respectively (Adachi *et al*., 2004).

Recently, we complexed zinc(II) to caffeic acid to form a potent anti-hyperglycaemic and antioxidant complex. Spectroscopic characterization showed a $Zn:O_4$ coordination between zinc and caffeic acid (Matowane *et al*., 2022), which afforded the complex two moieties of caffeic acid. The complex showed a promising glucose uptake activity in both L-6 myotubes and isolated rat muscle tissue, while its two caffeic acid moieties afforded it improved radical scavenging activity and anti-lipid peroxidative activity that was comparable and/or more potent than ascorbic acid or Trolox (Matowane *et al*., 2022). The activity of the complex was several fold more potent than its precursors, suggesting a potent complexation-mediated synergism, which may be mimicked *in vivo*. However, the *in vivo* antidiabetic and antioxidative potential of the complex but has not been studied. The present study was, therefore, undertaken to investigate the *in vivo* antidiabetic and antioxidative effects of the Zn(II)-caffeic acid complex in fructose/streptozotocin diabetic rat model.

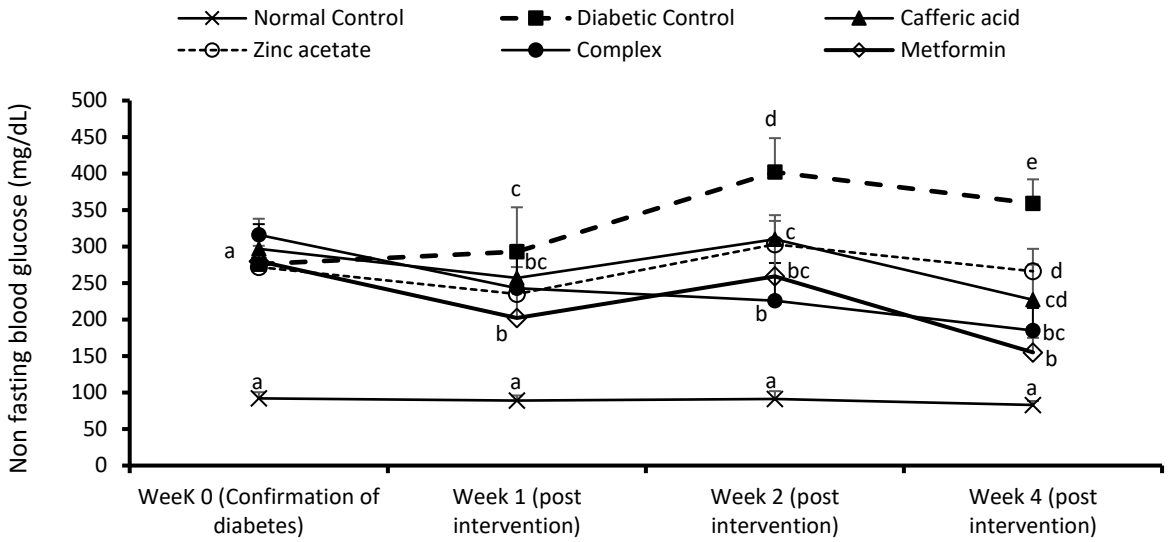
6.3. Materials and methods

Please refer to **sections 3.8 and 3.9 of chapter 3** for the materials and methods used in this chapter.

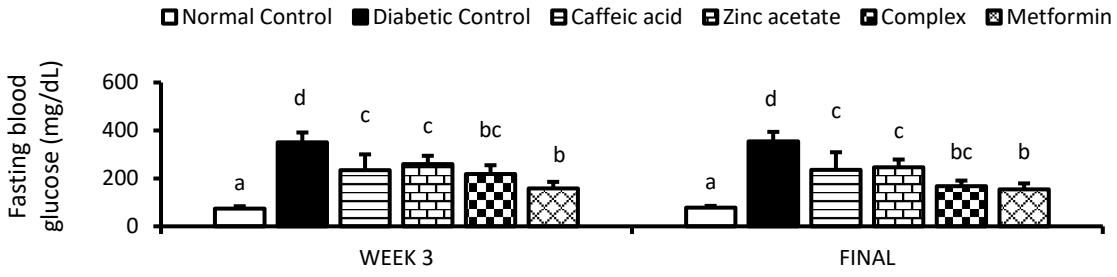
6.4 Results

6.4.1. Diabetes related biological effects

The NFBG differed significantly ($p < 0.05$) between the normal and diabetic rats at the start of intervention, with the diabetic rats showing higher NFBG (**Figure 6.1a**).



(a)



(b)

Figure 6.1: (a) non-fasting blood glucose (NFBG) at diabetes confirmation, as well as in the 1st, 2nd and 4th week of the intervention period and **(b)** 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 animals per group. For each period in the experiment, statistical comparison was done between animal groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

Table 6.1: Oral glucose tolerance and area under the curve (AUC) during the experimental period

Groups	Blood glucose (mg/dL)					AUC (mmol.h/L)
	0 min	30 min	60 min	90 min	120 min	
Normal control	74.2 ± 9.83^{a}	118 ± 22.5^{a}	124 ± 5.05^{a}	94.9 ± 9.65^{a}	88.0 ± 6.18^{a}	214 ± 18.4^{a}
Diabetic control	350 ± 41.5^{c}	547 ± 17.5^{d}	549 ± 9.56^{d}	551 ± 8.25^{d}	526 ± 25.4^{e}	1040 ± 20^{d}
Caffeic acid	234 ± 66.1^{c}	494 ± 62.4^{cd}	496 ± 71.8^{cd}	432 ± 66.6^{c}	371 ± 47.9^{d}	863 ± 106^{c}
Zinc acetate	272 ± 10.9^{c}	439 ± 62.8^{c}	440 ± 21.6^{c}	396 ± 41.7^{c}	383 ± 31.6^{d}	809 ± 43.2^{c}
Complex	218 ± 36.9^{bc}	479 ± 33.7^{cd}	459 ± 67.3^{c}	353 ± 47.5^{c}	276 ± 55.4^{c}	776 ± 80.2^{c}
Metformin	158 ± 27.5^{b}	329 ± 43.6^{b}	313 ± 42.4^{b}	212 ± 27.5^{b}	182 ± 35.5^{b}	530 ± 65.7^{b}

Data are shown as mean ± SD of six animals 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. Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp, Armonk, NY, USA). Tukey multiple range post hoc tests and the one-way analysis of variance (ANOVA) was adopted for multiple comparison of the data averages.

At the end of the intervention period (i.e., week 4 post treatment), the rats in the treatment groups had significantly ($p < 0.05$) lower NFGB than the untreated diabetic rats (diabetic control). Among the treatments, the complex and metformin had the lowest NFBG, which was significantly ($p < 0.05$) lower than that of caffeic acid and zinc acetate (**Figure 6.1a**). The FBG of the rats at the end of intervention followed a similar trend as the NFBG for the different treatment groups (**Figure 6.1b**). Glucose tolerance was significantly improved by the different treatments relative to the untreated diabetic rats, as shown by the AUC values with metformin significantly ($p < 0.05$) outperforming other treatments (**Table 6.1**).

Diabetes induction reduced FSI concentration, although not significantly (**Figure 6.2**). Both zinc acetate and the complex caused appreciable increase in the FSI concentration of the diabetic rats with the effect of the complex being significant ($p < 0.05$) relative to the untreated diabetic rats (**Figure 6.2**).

The Elevated HOMA-IR index of the untreated diabetic rats relative to the normal control group rats suggests diabetes induction caused significant insulin resistance in the rats (**Figure 6.3**). However, HOMA-IR was appreciably reduced by the treatments relative to the untreated diabetic rats. The complex and metformin treatment caused the most reducing effect on HOMA-IR, with the effect of metformin being significant ($p < 0.05$).

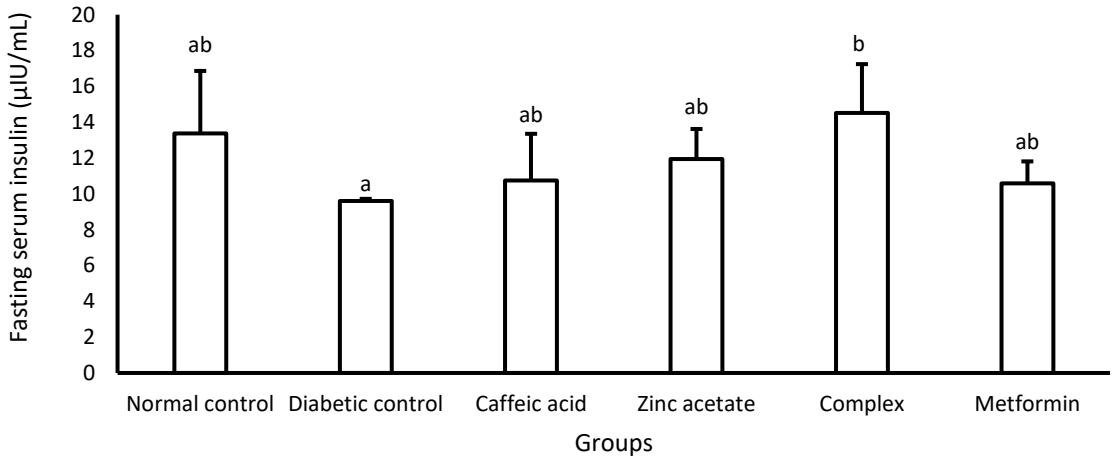


Figure 6.2: Fasting serum insulin (FSI) concentration and at the end of the experiment. Data are shown as mean ± SD of six animals per group. Statistical comparison was done between animal groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

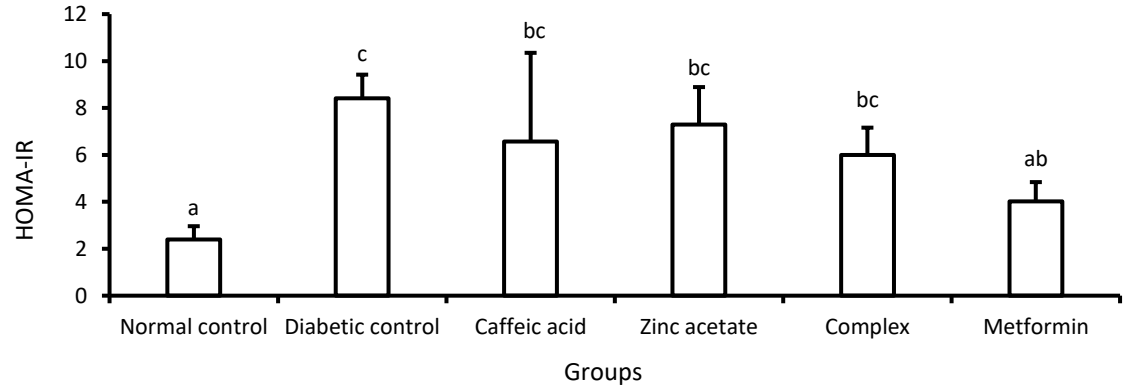


Figure 6.3: HOMA-IR score at the end of the experiment. Data are shown as mean ± SD of six animals per group. Statistical comparison was done between animal groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

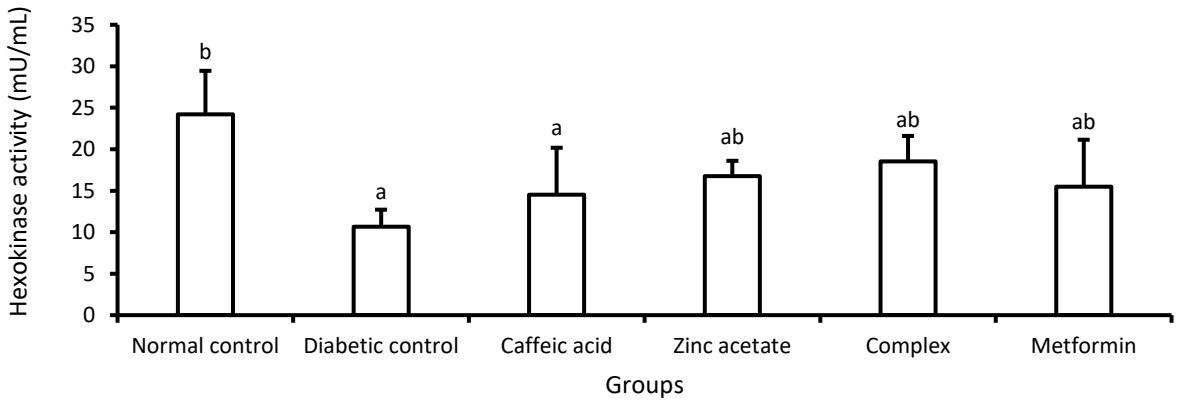
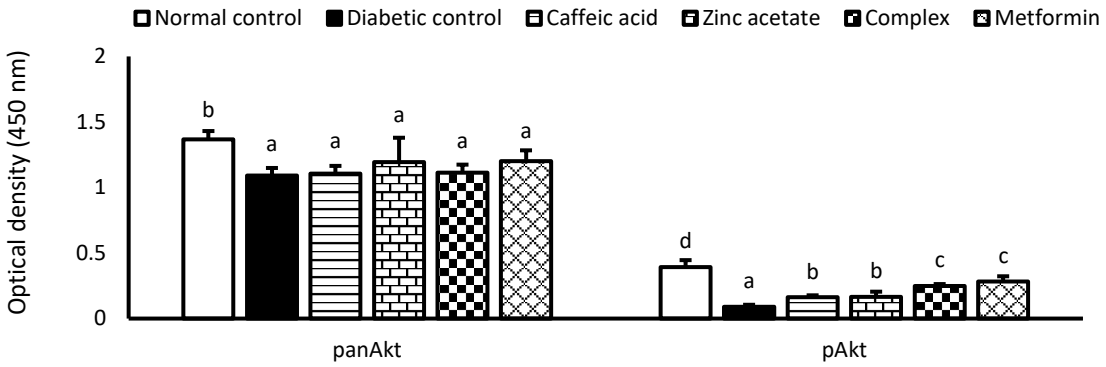


Figure 6.4: Muscle tissue hexokinase activity at the end of the experiment. Data are shown as mean ± SD of six animals per group. Statistical comparison was done between animal groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

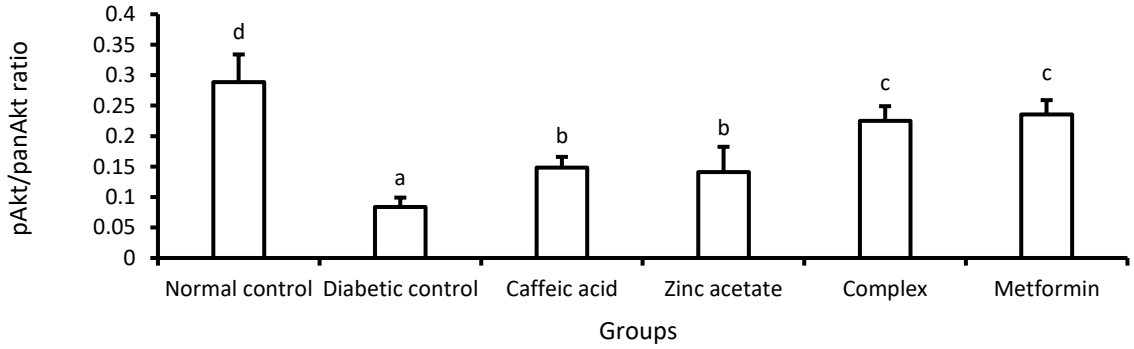
Muscle hexokinase activity was significantly ($p < 0.05$) reduced by diabetes induction (**Figure 6.4**). Although not significantly, the treatments increased the muscle tissue hexokinase activity relative to the untreated diabetic rats with the complex causing the highest increase.

The level of phosphorylated Akt in the muscle tissue reduced significantly ($p < 0.05$) in the untreated diabetic rats compared to the non-diabetic rats (**Figure 6.5**). All the treatments significantly ($p < 0.05$) increased phosphorylated Akt level in the muscle tissue of the diabetic rats. The effect of the complex was significantly ($p < 0.05$) stronger than that of zinc acetate

and caffeic acid, while the effect of metformin was significantly ($p < 0.05$) stronger than that of the complex (**Figure 6.5**).



(a)



(b)

Figure 6.5: 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. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

The liver and muscle glycogen contents of the rats were significantly ($p < 0.05$) reduced by diabetes induction (**Table 6.2**). The diabetes-induced reduction of liver glycogen content was significantly recovered by zinc acetate, the complex and metformin treatments. Although the muscle glycogen content followed similar trend among the groups, the complex was the only treatment with significant ($p < 0.05$) recovery effect (**Table 6.2**).

Table 6.2: Serum and tissue zinc concentration and tissue glycogen content at the end of the animal study

Groups	Zinc concentration (ng/µL)			Glycogen content (mg per gram tissue)	
	Serum	Muscle	Pancreas	Liver	Muscle
Normal control	5.72 ± 0.55^{ab}	1.07 ± 0.30^{a}	1.65 ± 0.32^{ab}	30.4 ± 1.74^{c}	2.05 ± 0.44^{c}
Diabetic control	4.05 ± 0.67^{a}	0.93 ± 0.18^{a}	1.47 ± 0.20^{a}	11.5 ± 2.95^{a}	0.54 ± 0.24^{a}
Caffeic acid	4.73 ± 1.03^{a}	1.34 ± 0.12^{a}	1.56 ± 0.39^{ab}	19.4 ± 6.78^{ab}	0.76 ± 0.48^{ab}
Zinc acetate	6.91 ± 1.96^{ab}	1.27 ± 0.44^{a}	2.05 ± 0.12^{ab}	21.5 ± 4.37^{bc}	1.37 ± 0.41^{ac}
Complex	8.46 ± 3.44^{d}	2.16 ± 0.40^{b}	2.13 ± 0.45^{b}	25.3 ± 5.49^{bc}	1.43 ± 0.70^{bc}
Metformin	4.09 ± 0.60^{a}	0.97 ± 0.28^{a}	1.52 ± 0.41^{ab}	26.5 ± 4.74^{bc}	1.36 ± 0.46^{ac}

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. Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp, Armonk, NY, USA). Tukey multiple range post hoc tests and the one-way analysis of variance (ANOVA) was adopted for multiple comparison of the data averages.

Table 6.3: Body weight, food intake and water intake during experimental period.

Groups	Body weight (g)						Food intake (g/day/rat)	Water intake (mL/day/rat)
	Pre-diabetes induction	At diabetes confirmation	1 week post intervention	2 weeks post intervention	3 weeks post intervention	4 weeks post intervention		
Normal control	213 ± 21.0	251 ± 18.9	269 ± 18.8^{b}	289 ± 19.0^{b}	300 ± 16.7^{b}	315 ± 15.3^{b}	40.8 ± 5.37^{a}	55 ± 5.00^{a}
Diabetic control	220 ± 25.6	252 ± 25.7	247 ± 19.0^{ab}	235 ± 16.7^{a}	244 ± 17.4^{a}	248 ± 22.4^{a}	63.4 ± 1.97^{c}	174 ± 12.5^{b}
Caffeic acid	235 ± 11.4	264 ± 16.4	253 ± 16.4^{ab}	260 ± 16.2^{ab}	249 ± 17.3^{a}	266 ± 19.1^{a}	59.2 ± 2.50^{bc}	157 ± 25.0^{b}
Zinc acetate	204 ± 14.0	236 ± 12.5	227 ± 11.1^{a}	249 ± 11.2^{a}	248 ± 14.8^{a}	266 ± 15.2^{a}	57.4 ± 3.80^{bc}	169 ± 14.2^{b}
Complex	231 ± 14.9	263 ± 17.4	255 ± 15.8^{ab}	260 ± 14.3^{ab}	259 ± 19.8^{a}	279 ± 27.8^{ab}	53.5 ± 3.27^{b}	149 ± 19.2^{b}
Metformin	220 ± 18.1	250 ± 22.0	260 ± 11.3^{b}	265 ± 16.4^{ab}	268 ± 21.0^{a}	279 ± 20.3^{ab}	55.6 ± 2.23^{bc}	143 ± 16.7^{b}

Data are shown as mean ± SD of six animals per group. For each experimental period and/or each measured parameter, statistical comparison was done between animal groups. Significant difference ($p<0.05$) means a difference in letters. Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp, Armonk, NY, USA). Tukey multiple range post hoc tests and the one-way analysis of variance (ANOVA) was adopted for multiple comparison of the data averages.

Diabetes induction did not significantly alter the concentration of zinc in the serum, muscle and pancreas (**Table 6.2**). Zinc acetate treatment in diabetic rats caused an insignificant elevation of zinc concentration in the serum and pancreas, while the complex treatment significantly ($p < 0.05$) increased zinc concentration in the serum, muscle and pancreas relative to the untreated diabetic rats (**Table 6.2**).

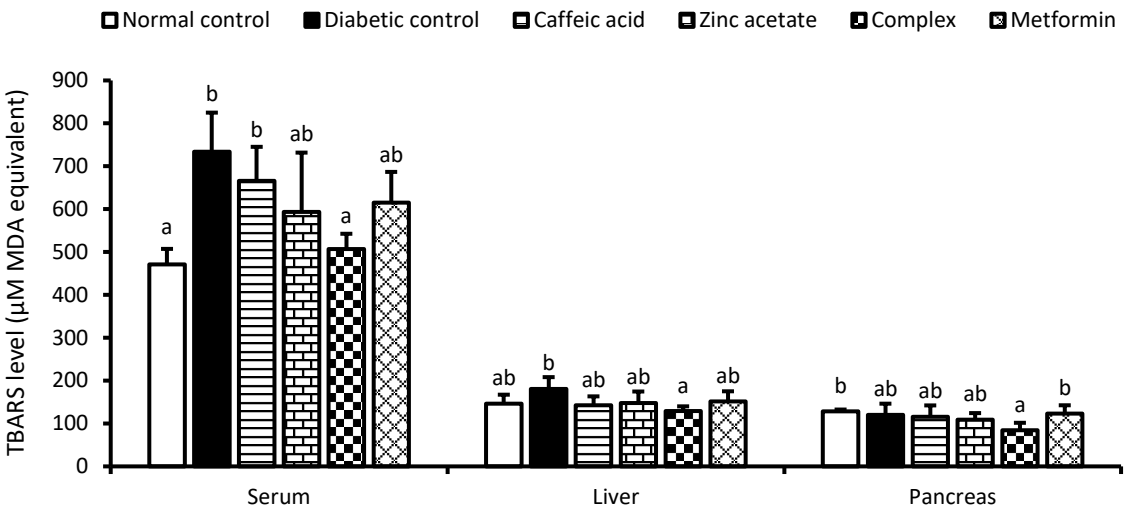
Food and water intake of rats significantly ($p < 0.05$) increased following diabetes induction (**Table 6.3**). However, the treatments reduced food and fluid consumption with the complex and metformin showing the strongest effects in the diabetic rats. Diabetes induction also caused significant ($p < 0.05$) weight loss in the rats, but was gradually recovered after treatment with the complex and metformin treatment causing the recovery effect (**Table 6.3**).

6.4.2. Oxidative stress related effects

Diabetes induction caused significant ($p < 0.05$) and non-significant ($p > 0.05$) increases in systemic and hepatic lipid peroxidation, respectively, without notably altering pancreatic lipid peroxidation (**Figure 6.6a**). Of all the treatments, only the complex had significant ($p < 0.05$) lowering effect on systemic and hepatic lipid peroxidation relative to the untreated diabetic rats. Although not significant, the complex also appreciably reduced diabetes-induced pancreatic lipid peroxidation (**Figure 6.6a**).

Diabetes induction significantly ($p < 0.05$) reduced systemic glutathione (GSH) concentrations without significantly altering hepatic and pancreatic GSH concentration (**Figure 6.6b**). Hepatic GSH concentration of diabetic rats was marked by significant decrease ($p < 0.05$) following the treatments with zinc acetate and the complex. None of the treatments caused significant alteration in the systemic and pancreatic GSH concentration of the diabetic rats (**Figure 6.6b**).

Diabetes induction exerted significant lowering effects on systemic and hepatic catalase activity ($p < 0.05$) (**Figure 6.7a**). The pancreatic catalase activity of rats was also reduced following diabetes induction but insignificantly ($p > 0.05$). The diabetes-induced reduction in systemic, hepatic and pancreatic catalase activity was increased by the different treatments. The effect of the treatments on systemic catalase activity was significant ($p < 0.05$) for caffeic acid and the complex, while only the complex treatment exerted significant ($p < 0.05$) recovery effect on the hepatic and pancreatic catalase activity of the diabetic rats (**Figure 6.7a**).



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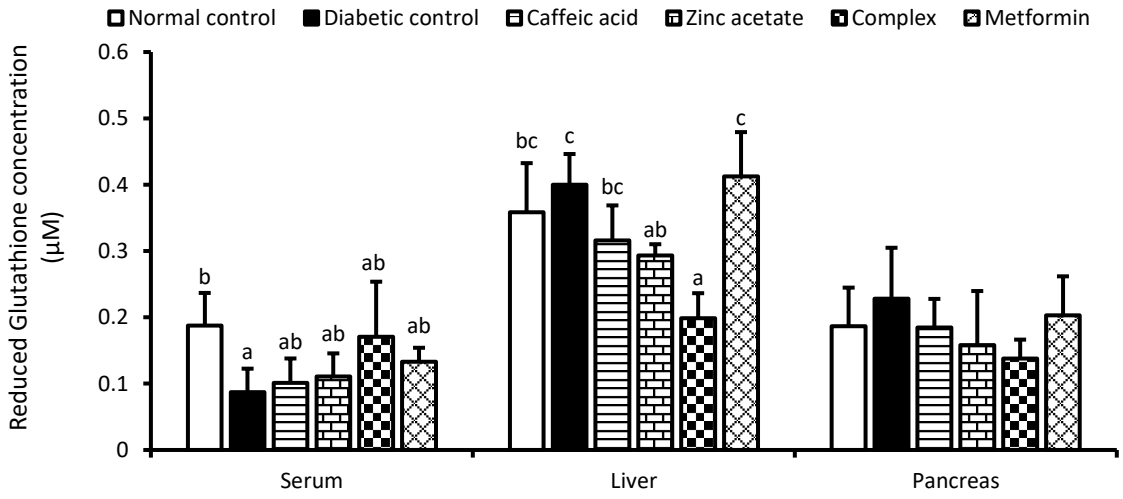
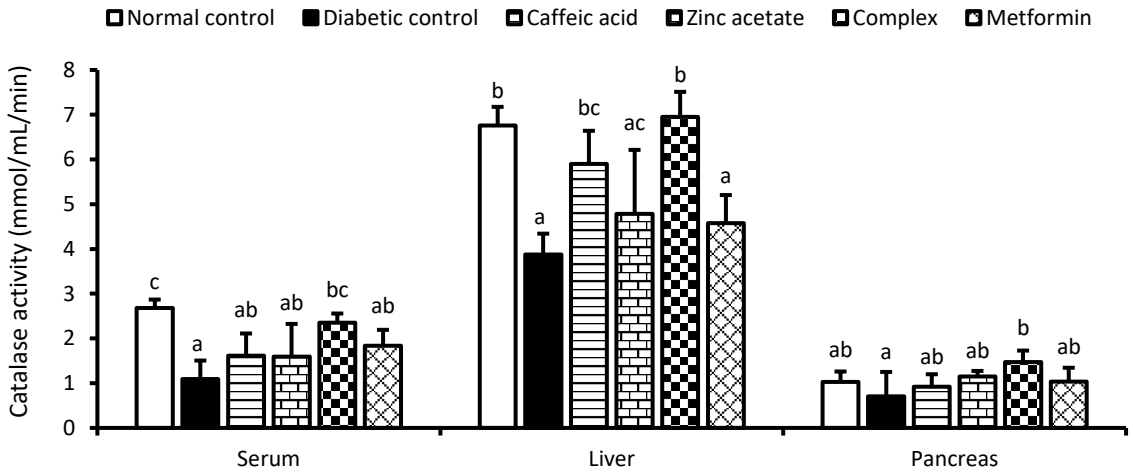


(b)

Figure 6.6: Serum, liver and pancreatic **(a)** Lipid peroxidation and **(b)** Reduced glutathione (GSH) concentration at the end of the animal study. For each biological sample, statistical comparison was done between animal groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter

SOD activity followed similar trend with significant ($p < 0.05$) decreases in the serum, liver and pancreas of untreated diabetic rats compared to the rats in the normal control group (**Figure**

6.7b). The treatments caused observable increase in the systemic, hepatic and pancreatic SOD activity of the diabetic rats with caffeic acid and the complex potentiating significant ($p < 0.05$) ameliorative effect on the hepatic SOD activity of the diabetic rats (**Figure 6.7b**).



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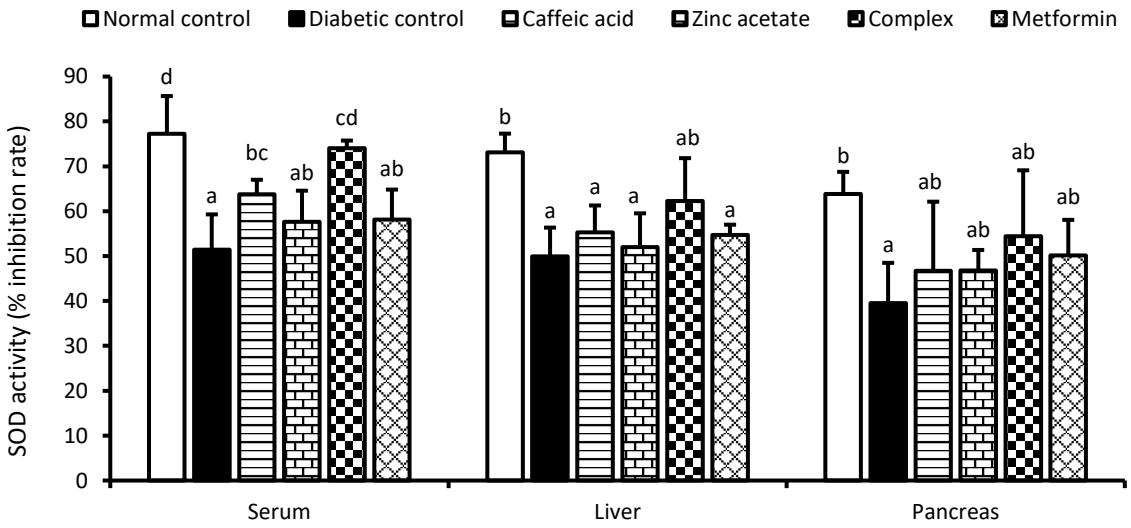

(b)

Figure 6.7: Serum, liver and pancreatic **(a)** Catalase and **(b)** Superoxide dismutase (SOD) activity at the end of the animal study. For each biological sample, statistical comparison was done between animal groups. There is significant difference ($p<0.05$) when the compared groups have no similar letter.

6.5. Discussion

There has been a paradigm shift from natural therapeutic agents towards their synthetic counterparts in functional medicine due to the functional properties and minimal toxicity of natural therapeutics. To this end, supplements and natural antioxidants are becoming popular in managing several diseases, including diabetes and oxidative stress. Zinc mineral, for example, has shown promise in diabetes management due to its role in insulin secretion and function (Jayawardena *et al*., 2012; Chabosseau and Rutter, 2016), while natural phenolics have been shown to have pharmacological credence that is relevant to diabetes and oxidative (Vinayagam *et al*., 2016). In a recent study, the innate bioactive potentials of zinc mineral and a natural phenolic acid (caffeic acid) were considered in developing a zinc(II)-caffeic acid complex. *In vitro*, cell-based and *ex vivo* experimental models showed the glycaemic control and antioxidant effect of the complex was several fold stronger than its precursors and comparable to some positive control standards, suggesting a complexation-mediated synergism. In the present study the promising glycaemic control and antioxidant properties of the complex were demonstrated in a fructose/streptozotocin model of diabetic rats.

Significant ($p < 0.05$) body weight loss and increased food and water intake was caused by diabetes induction in the rats (**Table 6.3**), which are classical symptoms of diabetes resulting from deranged nutrient metabolism by insulins. Although not significant, the treatments caused a reducing trend on food intake and an increasing trend on body weight, which suggest possible ameliorative effects. The effect of the complex and metformin was most potent (**Table 6.3**), which showed potentials of improving in long-term treatment.

Persistent high blood glucose remains the hall mark of a diabetic state, which is accompanied by insulin resistance and partial pancreatic malfunction in T2D (Galicia-Garcia *et al*., 2020). Previous studies have shown the possible modulatory effect of zinc(II) on insulin action, including promoting glucose uptake and glycogenesis and inhibiting lipolysis and glycogenolysis (Ezaki, 1989; Shisheva *et al*., 1992; Percival *et al*., 1997; Ilouz *et al*., 2002; Adachi *et al*., 2004), which could potentiate glycaemic control. On the other hand, caffeic acid has been shown to have anti-hyperglycaemic action in diabetic animals (Jung *et al*., 2006; Oršolić *et al*., 2021). In the present study, both caffeic acid and zinc acetate significantly ($p < 0.05$) reduced diabetes-induced elevation of NFBG and FBG in rats after 4 weeks treatment (**Figures 6.1**). However, complexing zinc acetate with caffeic acid improved the anti-hyperglycaemic action of caffeic acid by ~24 – 36% and zinc acetate by ~42 – 47%, which

was comparable to the anti-hyperglycaemic action of metformin. The treatments also ameliorated diabetes-induced alterations of oral glucose tolerance in a fairly similar pattern with the complex having a stronger ameliorative effect than caffeic acid and zinc acetate (**Table 6.1**). The data suggest a complexation-mediated glycaemic control potential between caffeic acid and zinc acetate.

The significantly ($p < 0.05$) higher HOMA-IR score of the untreated diabetic rats relative to normal control rats (**Figure 6.3**) suggests a case of insulin resistance in the untreated diabetic rats, which may be attributed to the 2 weeks *ad libitum* supply of 10% fructose. Documented evidence has exposed the association between fructose and insulin resistance in peripheral tissues (Basciano *et al*., 2005; Softic *et al*., 2020). Induction of diabetes also caused a notable but insignificant reduction in FSI concentration (**Figure 6.2**), possibly potentiated by the low dose STZ-induced pancreatic insult, suggesting that hyperglycaemia in the diabetic rats was possibly influenced by a decline in insulin action and secretion. The zinc acetate and caffeic acid treatments appreciably recovered FSI (**Figure 6.2**). However, the complex recovered FSI significantly ($p < 0.05$), which may be attributed to its composite moieties, and further suggests the possible synergistic antidiabetic interaction between caffeic acid and zinc acetate. Moreover, the role of zinc(II) in insulin storage and secretion has been documented (Chabosseau and Rutter, 2016; Chukwuma *et al*., 2020), while caffeic acid has been reported to enhance insulin secretion in INS-1E cells (Bhattacharya *et al*., 2014).

Both caffeic acid and zinc acetate reduced HOMA-IR score in the diabetic rats, although not significantly (**Figure 6.3**). Considering that fasting serum insulin is a multiplication factor for computing HOMA-IR, it is likely that the increased FSI in the zinc acetate treated rats influenced the observed relatively higher HOMA-IR score in the rats compared to the caffeic acid treated rats. Nevertheless, the effects of caffeic acid and zinc acetate on HOMA-IR in the diabetic rats was increased by ~24 and 42%, respectively, following complexation. Although not as potent as metformin, the effect of the complex was statistically comparable to that of metformin, which suggests a complexation-mediated synergistic potential in ameliorating insulin resistance in experimental diabetes.

While caffeic acid has been reported to promote glucose uptake in hepatocytes with insulin resistance (Huang *et al*., 2009) and hepatic glycolysis in *db/db* mice (Jung *et al*., 2006), several mechanisms have been put forward to explain the modulatory potential of zinc(II) on insulin sensitivity and action. Zn(II) has been shown to enhance glucose transport and lipogenesis in

adipocytes (Ezaki, 1989; Shisheva *et al*., 1992). It could inhibit endogenous glycogen synthase kinase-3β, leading to increased glycogen synthesis (Ilouz *et al*., 2002). Its modulatory action on cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase activity has been documented (Percival *et al*., 1997; Yoshikawa *et al*., 2004), which can lead to a downregulatory effect on cAMP-mediated lipolytic and glycogenolytic signaling. Zinc treatment has also been shown to modulate glycolytic enzymes activity *in vitro* (Tamaki *et al*., 1983). In the present study both caffeic acid and zinc acetate increased diabetes-induced depleted hepatic and muscle glycogen, which became significant ($p < 0.05$) and comparable to the effect of metformin following treatment with the complex (**Table 6.2**). Muscle hexokinase activity was also appreciably increased in the diabetic rats following treatment with the complex. The complex was more effective than caffeic acid and zinc acetate in modulating muscle hexokinase activity (**Figure 6.4**). The data suggest a complexation-mediated synergistic modulatory action on insulin signalling, thus improving insulin resistance and promoting glucose utilization and glycaemic control. Moreover, reported preliminary studies on complex showed that it increased glucose uptake and utilization in L-6 myotubes and isolated rat psoas muscle tissues by several fold relative to its precursors (Matowane *et al*., 2022).

Muscle phospho-Akt data suggest diabetes impaired Akt phosphorylation, which was significantly ($p < 0.05$) enhanced by caffeic acid and zinc treatments (**Figure 6.5**). Phosphorylation-mediated Akt activation is a notable downstream signalling action through which insulin signals cellular glucose uptake and utilization (Beg *et al*., 2017), which suggests caffeic acid and zinc acetate may exert modulatory action on insulin signaling and glucose uptake in peripheral tissues. However, complexation significantly ($p < 0.05$) increased Akt phosphorylation by 54 and 59% relative to caffeic acid and zinc acetate, respectively (**Figure 6.5**), which further supports the complexation-mediated glycaemic control potential between zinc(II) and caffeic acid.

In the serum, zinc concentration was higher in the complex and zinc acetate treated diabetic rats, relative to the other treatments (**Table 6.2**), suggesting increased zinc bioavailability. A similar pattern of zinc concentration among the treatment groups was observed in the pancreas (**Table 6.2**). Considering the role of zinc in insulin storage and secretion (Chabosseau and Rutter, 2016) it is rational to speculate that the higher pancreatic zinc concentration of the complex and zinc acetate treated diabetic rats, relative to the other treatments (**Table 6.2**) may have influenced the observed higher FSI of the complex and zinc acetate treated diabetic rats relative to the other treatments (**Figure 6.2**). Muscle zinc concentration of the complex treated

diabetic rats was, however, significantly ($p < 0.05$) higher than that of the zinc acetate treated diabetic rats (**Table 6.2**), suggesting that complexation modulated zinc transport in the muscle, which may have in synergy with caffeic acid potentiated the observed promising insulin signaling modulatory effects of the complex. Moreover, documented evidence has demonstrated that non-pharmacologically active 1-oxy-2-pyridinethiol ligands have the potential of increasing cellular zinc uptake via complexation, which resulted in insulin mimetic effects (Basuki *et al*., 2007; Yoshikawa *et al*., 2011).

Oxidative stress remains pivotal in the progression and development of complications of diabetes (Giacco and Brownlee, 2010). Hence, it was reasonable to measure the antioxidant capacity of the complex. Systemic oxidative stress was notable altered by diabetes with significant ($p < 0.05$) elevation of lipid peroxidation in the serum of the untreated diabetic rats relative to the normal control rats (**Figure 6.6a**). Hepatic lipid peroxidation was also elevated by diabetes induction (**Figure 6.6a**). However, the diabetes-induced elevations in systemic and hepatic lipid peroxidation were significantly ($p < 0.05$) reduced by the complex, which outperformed other treatments. Similarly in the pancreas, the lowest lipid peroxidation level was observed in the complex treated rats (**Figure 6.6a**), which suggests a potent antioxidant action of the complex. The stronger anti-lipid peroxidative effect of the complex relative to its precursors has been previously demonstrated *in vitro*, as well as in Chang liver cells and isolated rat liver tissue, which was majorly attributed to the innate antioxidant properties (Gülçin, 2006; Jung *et al*., 2006; Xu *et al*., 2020) of the two moieties of caffeic acid in the complex (Matowane *et al*., 2022)

Furthermore, diabetes-induced depletion of systemic GHS, a known biological molecule involved in the antioxidant defense system (Birben *et al*., 2012), was recovered by the complex treatment (**Figure 6.6b**), which supports promising antioxidant potential of the complex. However, in the liver and pancreas, the GHS level was notably lowest in the complex treated rats. This could be attributed to reduced tissue lipid peroxidation or oxidative stress of the rats (**Figure 6.6a**), which adapted the tissues to a reduced tissue GSH level (**Figure 6.6b**).

The trend in the systemic and tissue activities of catalase (**Figure 6.7a**) and SOD (**Figure 6.7b**), also suggests that the diabetes induction and treatment intervention caused notable alterations in the antioxidant status of the diabetic rats. Catalase and SOD forms part of the antioxidant defense system that helps to convert superoxide ion, a deleterious ROS, to a less harmful water (Birben *et al*., 2012). Diabetes induction significantly ($p < 0.05$) and/or notably reduced

systemic, hepatic and pancreatic catalase and SOD activity, suggesting a suppressed antioxidant defense system and risk of oxidative stress. Both zinc acetate and caffeic acid increased SOD activity in the diabetic rats. The modulatory action of zinc acetate on antioxidant enzymes may be attributed to the involvement of molecular zinc(II) as co-factor and structural component in the functioning of important antioxidant enzymes (Marreiro *et al*., 2017). Caffeic acid, on the other hand, has been shown to increase antioxidant enzymes activity in experimental diabetes (Xu *et al*., 2020). It was therefore not surprising that complexation of zinc acetate and caffeic acid resulted in an improved modulatory effect on the catalase and SOD activity relative zinc acetate and caffeic acid (**Figure 6.7**), which suggests a complexation-mediated antioxidant synergism between zinc(II) and caffeic acid.

6.6. Conclusion

There has been an ongoing search for agents with more effective therapeutic outcomes in managing diabetes and associated oxidative complications with minimal adverse effects. In this study, the *in vivo* antidiabetic and antioxidant potentials of a Zn(II)-caffeic acid complex that was developed from natural precursors, zinc(II) and caffeic acid, were evaluated. Previous preliminary data has shown that the complex outperformed its precursors in exerting in vitro, cellular and *ex vivo* antioxidant and glycaemic control potential. Data of the present study showed that the complex appreciably replicated a closely related effect in diabetic rats. It significantly ameliorated diabetic derangements and symptoms by lowering blood glucose, improving glycaemic control, increasing glycogen storage and modulating the activity of enzymes and/or proteins involved in insulin signalling and glucose utilization. Concomitantly it ameliorated oxidative stress by supressing systemic and tissue lipid peroxidation and increasing the activity of key antioxidant enzymes. Importantly, the complex outperformed its precursors, suggesting a complexation-mediated antioxidant and glycaemic control synergism between the zinc(II) and caffeic acid. Zinc(II)-caffeic acid complexation may, therefore, be an alternative approach to improving the efficacy of antidiabetic and antioxidative therapy with minimal adverse or side effects.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

7.1. Discussion

The morbidity and mortality outcomes of diabetes, as well as the associated expenditure on health care remain notable contributors to global socioeconomic burden (IDF, 2021). The close association with oxidative stress is a major factor aggravating the notoriousness of diabetes, which has been implicated in many complications, as well as the morbidity and mortality outcomes of the disease (Giacco and Brownlee, 2010). In spite of available commercial medications, stakeholders are still searching for new therapeutic approaches for diabetes to compensate for the issues relating to efficacy and adverse effects that are associated with the existing conventional medications (Chaudhury *et al*., 2017; Chaturvedi *et al*., 2018). Therapeutic approaches with holistic functional profiles and minimal side effects are being explored; approaches that could manage hyperglycaemia and mitigate or reduce the risk of oxidative complications. In this context, supplements and phytochemicals have gained popularity due to their safety profile and numerous biological benefits, including antioxidant functions and beneficial role in glucose and lipid metabolism (Martini *et al*., 2010; Vinayagam *et al*., 2016; Liu *et al*., 2018; Sun *et al*., 2020; Cross and Thomas, 2021). In recent years, zinc mineral has been explored as a supplement for diabetes due to its function in insulin integrity and function (Chabosseau and Rutter, 2016). On the other hand, natural phenolic acids are known dietary antioxidants with diabetes-related pharmacological potentials (Vinayagam et al., 2016; Kumar and Goel, 2019).

In this study, we took advantage of the insulin mimetic potential of zinc (Chabosseau and Rutter, 2016) and the antioxidant and pharmacological potentials of ferulic (Srinivasan *et al*., 2007; Narasimhan *et al*., 2015; Zduńska *et al*., 2018; Li *et al*., 2022) and caffeic acid (Gülçin, 2006; Jung *et al*., 2006; Genaro-Mattos *et al*., 2015) to develop novel zinc(II) complexes of ferulic and caffeic acid, which demonstrated improved antioxidant and antidiabetic effects relative to their precursor, suggesting a complexation mediated synergism. Spectroscopic characterization showed that the complexes were formed as bi-caffeic acid-Zinc acetate complex (**Figure 5.2a**) and Zn(II)-biferulate.$2H_2O$ complex (**Figure 4.2**). Zn(II) was afforded two moieties of the phenolic acids upon complexation, which influenced the bioactive

properties of the complexes relative to their precursors in different experimental models, without causing observable cytotoxicity in liver and kidney cells (**Figures 4.15 and 5.11**)

Phenolic acids, including ferulic acid and caffeic acid have been shown to be potent scavengers of radicals and chelators of metals (Kadoma and Fujisawa, 2008; Genaro-Mattos *et al*., 2015; Zduńska *et al*., 2018), which has been largely attributed to their electron donating, hydrogen transferring and stable phenoxy radical forming abilities (Chen *et al*., 2020). In the present study, caffeic acid and ferulic acid showed dose dependent *in vitro* radical scavenging and Fe^{3+} reducing activities without observable effects shown by Zn(II) (zinc acetate and zinc sulphate) (**Figures 4.5, 4.6a, 5.5, 5.6a and b; Tables 4.1 and 5.1**). *In vitro*, caffeic acid and ferulic acid, also, showed anti-linoleic acid peroxidative effects (**Figures 4.6a and 5.6c; Tables 4.1 and 5.1**), which reflects their previously reported *in vivo* anti-lipid peroxidative effects (Balasubashini *et al.*, 2004; Jayanthi and Subash, 2010). The phenolic acid further inhibited lipid peroxidation and reduced glutathione (GSH) depletion in both Chang liver cells and isolated rat live tissues (**Figures 4.7, 4.8, 5.7 and 5.8; Tables 4.1 and 5.1**), suggesting their protective potential against hepatic oxidative insults. However, when complexed with Zn(II) the *in vitro*, cellular and *ex vivo* antioxidant profile of the phenolic acids was improved (**Tables 4.1 and 5.1**). The activity of the complexes outperformed their respective precursors and was comparable to ascorbic acid and/or Trolox (**Tables 4.1 and 5.1**), which may be attributed to the two moieties of caffeic acid or ferulic acid that were acquired by their respective complexes (**Figures 4.2 and 5.2a**), thus increasing antioxidant activity by several folds. In both Chang liver cells and isolated rat liver tissues, Zn(II) (zinc acetate or zinc sulphate) potentiated appreciable antioxidant effects, which may be attributed to the functional role of molecular zinc in cellular antioxidant status (Marreiro *et al*., 2017). This may influence the promising cellular and tissue antioxidant activity of the complexes relative their precursors, suggesting an antioxidant synergism between Zn(II) and the phenolic acids.

It is important to note that based on the IC_{50} values, the antioxidant activity of caffeic acid was stronger than that of ferulic acid, which was translated into a similar trend of efficacy in their respective Zn(II) complexes (**Tables 4.1 and 5.1**). This suggests that caffeic acid may be a better alternative than ferulic acid in developing Zn(II) complex with improved antioxidant profile.

In vitro, the antidiabetic potency of the complexes was assessed by measuring their inhibitory action on digestive enzymes (α-glucosidase and α-amylase), as well as glucose-induced protein

glycation. Inhibition of the enzymes suggests potential to suppress postprandial glycaemia in diabetic individuals (Moelands *et al*., 2018), while antiglycation action could mitigate hyperglycaemia induced glycation and AGEs production and reduce the risk of associated diabetic complications (Rhee and Kim, 2018). Zinc acetate-bi-caffeic acid complex inhibited α-glucosidase and α-amylase and outperformed its precursors (**Figure 5.9 and Table 5.1**), suggesting a postprandial glycaemic control potency. On the other hand, Zn(II)-biferulate.2H_2O complex inhibited α-glucosidase, α-amylase and glucose-induced protein glycation and outperformed it precursors by 1.5 to 2 fold (**Figures 4.9 and 4.10; Table 4.1**), suggesting an improved potency to control postprandial glycaemia and mitigate glycation-induced diabetic complications. It is plausible that the two moieties of caffeic acid or ferulic acid in the respective complexes (**Figures 4.2 and 5.2a**) potentiated the stronger enzyme inhibitory action of complexes, considering zinc sulphate and zinc acetate had low to mild enzyme inhibition effects, relative to the phenolic acids (**Tables 4.1 and 5.1)**. Moreover, the enzyme inhibitory effects of the phenolic acids have also been documented previously (Oboh *et al*., 2015; Zheng *et al*., 2020). On the other hand, it is plausible that Zn(II) afforded the Zn(II)-biferulate.2H_2O complex a potent antiglycation effect relative to ferulic acid, considering the observed antiglycation effect of zinc sulphate (**Figure 4.9 and Table 4.1**), which has been previously demonstrated *in vitro* (Tupe *et al*., 2015) and in diabetic rats (Sacan *et al*., 2016). The data suggest that Zn(II) could a promising adjuvant in improving the glycaemic control and antiglycation property of the phenolic acids through complexation.

The complexes further demonstrated antidiabetic potential by modulating glucose uptake in both L6-myotubes and isolated rat psoas muscle (**Figure 4.11 and 5.10; Tables 4.1 and 5.1**). Glucose uptake in peripheral tissues, including muscle and adipose tissue is one of the mechanisms by which the body maintains glucose homeostasis (Aronoff *et al*., 2004), suggesting the glycaemic control potential of the complexes. Consistent with the other results, the glucose uptake activity of the complexes was several fold more potent than that of their respective phenolic acid and Zn(II) precursors, which may have been afforded by a synergistic interaction between the respective phenolic acid and the Zn(II) components of the complexes. Moreover, both phenolic acids (caffeic acid and ferulic) and Zn (II) (zinc sulphate and zinc acetate) displayed appreciable cellular and tissue glucose uptake activities (**Figure 4.11 and 5.10; Tables 4.1 and 5.1**), while documented evidence has shown the ability of caffeic acid (Tsuda *et al*., 2012) and ferulic acid (Salau *et al*., 2022), as well as Zn(II) (Chukwuma *et al*., 2020) to modulate glucose transport and glucose uptake in rat skeletal muscle. However, the

stronger activity of Zn (II) (zinc sulphate and zinc acetate) relative to the phenolic acids (**Tables 4.1 and 5.1**), further suggests that the Zn(II) component of the complexes may be the predominant component influencing the potent glucose uptake activity of the complexes. Moreover, the insulin mimetic and insulin action modulatory potential of Zn(II) have been well documented (Chukwuma et al., 2020).

Zinc treatment has been shown to modulate glycolytic enzymes activity *in vitro* (Tamaki *et al*., 1983). Other studies suggest the modulatory action of zinc on insulin signalling molecular targets, including insulin receptor, GLUT-4, and Akt, which facilitates cellular glucose transport and glycaemic control (Ezaki, 1989; Tang and Shay, 2001; Norouzi *et al*., 2018). In the present study, Zn(II) conferred on the Zn(II)-biferulate.$2H_2O$ complex the ability to modulate hexokinase activity in muscle tissues (**Figure 4.12b**), which suggests a potential to enhance cellular glucose utilization. Also, Zn(II) conferred on the Zn(II)-biferulate.$2H_2O$ complex the ability to modulate Akt phosphorylation (**Figure 4.13**), an important signalling step in the insulin-mediated signalling of glucose uptake in skeletal muscles (Beg *et al*., 2017), suggesting a complexation-mediated insulin signalling modulatory action. Furthermore, computational studies showed interactions between the complexes [zinc acetate-caffeic acid and Zn(II)-biferulate.$2H_2O$] and some insulin signalling molecular targets (GLUT-4 and protein kinase B (PKB)/Akt) (**Figure 4.14**). The computed docking scores suggest that the complexes had stronger interactions with the molecular targets than their respective phenolic acid precursors (**Tables 4.2 and 5.2**), which supports the notion that the complexes may potentiate glycaemic control by modulating the action of insulin signalling molecular targets, thus enhancing muscle glucose uptake.

It is important to note that based on the EC_{50} values (**Tables 4.1 and 5.1**) the muscle tissue glucose uptake activity of the zinc acetate-bicaffeic acid complex (EC_{50} = 339 µM) was more potent than that of the Zn(II)-biferulate.$2H_2O$ complex (EC_{50} = 501). Together with the stronger antioxidant activity of the former relative to the latter (**Tables 4.1 and 5.1**), it was rational to further subject the zinc acetate-bicaffeic acid complex to *in vivo* antidiabetic and antioxidant evaluation using a fructose and low dose STZ rat model of diabetes. The complex was subjected to *in vivo* antidiabetic and antioxidant evaluation and its effects were compared to that of its precursors (caffeic acid and zinc acetate).

Diabetes-induced polyphagia, polydipsia and weight loss in the rats were gradually recovered by the complex (**Table 6.3**), suggesting an ameliorative potential of the complex. The blood

glucose profile (**Figure 6.1 and Table 6.1**), which is the hallmark of diabetes also suggested that the complex had ameliorative potential on diabetes. After 20 days treatment, the complex significantly ($p < 0.05$) reduced NFBG in diabetic rats, appreciably outperformed its precursors and was statistically comparable ($p > 0.05$) to metformin treatment (**Figure 6.1a**). Post-treatment, a similar trend of data was observed for FBG in the diabetic rats (**Figure 6.1b**). Glucose tolerance in the diabetic rats was also significantly ($p < 0.05$) improved by the complex and its precursors (**Table 6.1**), with the complex outperforming its precursors, suggesting a complexation-mediated synergistic glycaemic control potential between zinc acetate and caffeic acid in diabetic condition.

Further analyses provide insight into the possible anti-hyperglycaemic modes of action of the complex. HOMA-IR indices suggest the complex notably improved diabetic insulin resistance (**Figure 6.3**), thus improving glucose intolerance (**Table 6.1**). Diabetes-induced depletion in FSI level was also significantly ($p < 0.05$) recovered by the complex (**Figure 6.2**), which may be mainly attributed to its Zn(II) moiety, considering the functional role of zinc in insulin preservation, integrity and secretion (Chabosseau and Rutter, 2016). Supporting data showed the complex potentiated elevated pancreatic zinc concentration (**Table 6.2**), which may have influenced the observed increase in FSI (**Figure 6.2**). Diabetes-induced reduction of hepatic and muscle glycogen was notably ($p < 0.05$) increased by the complex (**Table 6.2**). Hepatic and muscle glycogen synthesis is a major glycaemic control mechanism of insulin (Aronoff *et al*., 2004), and has been shown to be downregulated in diabetic condition (Nikoulina *et al*., 2001), which suggests an anti-hyperglycaemic potential of the complex in ameliorating diabetes. Zinc acetate also showed a potent stimulatory action to increase the depleted hepatic and muscle glycogen in diabetic rats (**Table 6.2**) and may, together with the two caffeic acid moieties influence the potent increasing effect of the complex on hepatic and muscle glycogen. Moreover, studies have shown that Zn(II) could modulate glycogen synthesis by inhibiting endogenous glycogen synthase kinase-3β (Ilouz *et al*., 2002), while caffeic acid has been shown to modulate glycogen synthesis by upregulating the phospholipase C–protein kinase C pathway (Huang and Shen, 2012), as well as insulin receptor tyrosyl phosphorylation and the downstream insulin signalling (Huang *et al*., 2009). The combined effect of both Zn(II) and caffeic acid potentiated the promising effect of the complex, which further reflects a complexation mediated synergism with Zn(II) and caffeic acid.

Furthermore, the complex increased Akt phosphorylation in muscle tissues, which was previously downregulated by diabetes induction (**Figure 6.5**). The modulatory action of the

complex on Akt phosphorylation was more effective than caffeic acid, which correlates with the *in silico* docking data (**Figure 5.12 and Table 5.2**), where the complex had stronger interaction with Akt/PKB molecular target than caffeic acid. The modulatory action of the complex on Akt phosphorylation was also as potent as that of metformin. Considering the signalling role of Akt phosphorylation in insulin-mediated muscle glucose uptake (Beg *et al*., 2017), the data suggest the remarkable potential of the complex to promote glucose uptake and utilization. Consistent muscle hexokinase data further support the modulatory potential of the complex on tissue glucose utilization, as the complex, relative to caffeic acid appreciably recovered the significantly depleted muscle hexokinase activity in diabetic rats (**Figure 6.4**), which suggests a modulation of glucose utilization via glycolysis. In fact, the complex was stronger than that of metformin, suggesting the remarkable glycaemic control potential of the complex. Zinc acetate also had appreciable modulatory action on muscle hexokinase activity (**Figure 6.4**) and may be the predominant component of the complex influencing its modulatory effect on muscle tissue hexokinase activity. This is partly because complexation increased muscle tissue zinc bioavailability relative to zinc acetate treatment (**Table 6.2**), which may contribute to the improved modulatory effect of the complex on muscle hexokinase activity compared to its precursors. Moreover, zinc treatment has been shown to modulate glycolytic enzymes activity *in vitro* (Tamaki et al., 1983). The data further reflect a complexation-mediated glycaemic control improvement in diabetic rats.

Further analysis showed that the complex diplays another pharmacological property that reflects its antioxidant prospects in diabetic rats. Caffeic acid is a known natural antioxidant with radical scavenging, anti-lipid peroxidative and antioxidant enzyme modulatory attributes (Jayanthi and Subash, 2010; Khan *et al*., 2016). Molecular zinc, on the other hand is a known co-factor involved in the functioning of some antioxidant enzymes and has been shown to improve antioxidant status (Jarosz *et al*., 2017). In the present study, the antioxidant attributes of caffeic acid and zinc(II) were acquired by the complex to potentiate a stronger antioxidant effect in diabetic rats. This was further influenced by the two caffeic acid moieties in the complex (**Figure 5.2a**). The complex significantly reduced lipid peroxidation and increased catalase and SOD activities in the serum, liver and pancreas of diabetic rats (Figure and Table). Its antioxidant action in the diabetic rats was more potent than that of its precursors and metformin, suggesting its potential to improve diabetic oxidative stress and mitigate associated complications.

7.2. Conclusion

The use of supplements and natural therapeutics in the management of diabetes and oxidative stress has long been in existence and in recent years becoming popular. Also, the concept of synergism in functional medicine has become a welcomed approach because it affords the benefits of potentiating multimode therapeutic targets and effects, thus improving therapeutic effects. Zinc is a supplement that has insulin related functions, while caffeic acid and ferulic acid are natural dietary antioxidants. In the present study both phenolic acids were individually complexed with zinc(II) to develop Zn(II) complexes of caffeic acid and ferulic with the bioactivity attributes of their respective precursors. The complexes demonstrated *in vitro* radical scavenging, anti-lipid peroxidative and digestive enzyme inhibitory effects. They, also, inhibited oxidative stress-induced lipid peroxidation and GSH depletion in Chang liver cells and isolated rat liver tissues and modulated glucose uptake in L-6 myotubes and isolated rat muscle tissues. The antioxidant effect of the complexes was several fold more potent than their respective precursors, possibly due to the two moieties of caffeic acid or ferulic acid in the respective complexes, which was afforded by complexation. On the other hand, it appears the complexes acquired the insulin mimetic and glucose uptake potential of Zn(II), which in synergy with the phenolic acids potentiated improved glucose uptake and utilization activity. The *in vitro*, cell-based and *ex vivo* data suggest caffeic acid may be a more potent alternative for Zn(II) complexation relative to ferulic acid and thus its Zn(II) complex was subjected to *in vivo* antidiabetic and antioxidative evaluation. The complex improved glycaemic control, insulin sensitivity and signalling, and glycolytic action in diabetic rats. Concomitantly, it reduced systemic and tissue lipid peroxidation and improved antioxidant enzymes activity in the diabetic rats. Consistent with the *in vitro*, cellular and *ex vivo* studies, the *in vivo* antidiabetic and antioxidant potential of the Zn(II)-caffeic acid complex was more potent than that of its precursors, suggesting a complexation-mediated antidiabetic and antioxidative synergism between Zn(II) and the phenolic acids. Zinc(II) complexation with these phenolic acids may be an alternative approach to improving the efficacy of antidiabetic and antioxidative therapy with minimal adverse or side effects.

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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:** Matowane, G. R., Ramorobi, L. M., Mashele, S. S., Bonnet, S. L., Noreljaleel, A., Swain, S. S., Makhafola, T. J., & Chukwuma, C. I. (2022). Complexation potentiated promising anti-diabetic and anti-oxidative synergism between Zn(II) and ferulic acid: A multimode study. **Diabetic Medicine**; 39(9), e14905. https://doi.org/10.1111/dme.14905.
3. **Publication 2 of thesis:** Matowane, G.R., Ramorobi, L.M., Mashele, S.S., Bonnet, S.L., Noreljaleel, A.E.M., Swain, S.S., Makhafola, T.J., & Chukwuma, C.I. (**2022**). Novel Caffeic Acid - Zinc Acetate Complex: Studies on Promising Antidiabetic and Antioxidant Synergism Through Complexation. **Medicinal Chemistry**; 19, 147 – 162. https://doi.org/10.2174/1573406418666220620144601.
4. **Publication 3 of thesis (proof of manuscript submission):** Matowane, G.R., Mashele, S.S., Makhafola, T.J., & Chukwuma, C.I. Zinc(II) and caffeic acid synergistically improved muscle Akt phosphorylation, insulin action, glycaemic control and antioxidant status in diabetic rats. **Biomedicine & Pharmacotherapy** (manuscript under review; manuscript ID: **BIOPHA-D-22-06241**).
5. **Proof of scientific conference presentation:** Matowane RG*, Ramorobi LM, Mashele SS, Bonnet SL , Noreljaleel AME, Makhafola TJ , Chukwuma CI. Zn(II)-syringic acid complex: A novel complex with improved bioactivity and promising antidiabetic and antioxidative potentials. **Presentation at the 54th SASBCP Annual Conference, which took place virtually on 22 October 2021**.

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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DIABETIC Medicine

RESEARCH: BASIC SCIENCE

Complexation potentiated promising anti-diabetic and anti-oxidative synergism between ZN(ii) and ferulic acid: A multimode study

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Funding information
National Research Foundation South Africa

Abstract

Aim: This study was done to investigate the anti-diabetic and anti-oxidative synergism between zinc(II) and ferulic acid through complexation.

Methods: Zinc sulphate was complexed with ferulic acid in a 1:2 molar ratio. The complex was characterized using Fourier-transform infrared spectroscopy, proton NMR and high-resolution mass spectroscopy techniques and evaluated for cellular toxicity. In silico, in vitro, cell-based and tissue experimental models were used to test the anti-diabetic and anti-oxidant activities of the complex relative to its precursors.

Results: A zinc(II)-biferulate.$2H_2O$ complex was formed. The in vitro radical scavenging, anti-lipid peroxidative and α-glucosidase and α-amylase inhibitory activity of the complex was 1.7–2.1 folds more potent than ferulic acid. Zn(II) complexation increased the anti-glycation activity of ferulic acid by 1.5 folds. The complex suppressed lipid peroxidation (IC_{50} = 48.6 and 331 μM) and GHS depletion (IC_{50} = 33.9 and 33.5 μM) in both Chang liver cells and isolated rat liver tissue. Its activity was 2.3–3.3 folds more potent than ferulic acid and statistically comparable to ascorbic acid. Zn(II) complexation afforded ferulic acid improved glucose uptake activity in L-6 myotube (EC_{50} = 11.7 vs. 45.7 μM) and isolated rat muscle tissue (EC_{50} = 501 and 1510 μM). Complexation increased muscle tissue zinc(II) uptake and hexokinase activity. Docking scores of the complex (−7.24 to −8.25 kcal/mol) and ferulic acid (−5.75 to 6.43 kcal/mol) suggest the complex had stronger interaction with protein targets related to diabetes, which may be attributed to the 2 ferulic acid moieties and Zn(II) in the complex. Moreover, muscle tissue showed increased phospho-Akt/pan-Akt ratio upon treatment with complex. The complex was not hepatotoxic and myotoxic at in vitro cellular level.

Conclusion: Zn(II) complexation may be promising therapeutic approach for improving the glycaemic control and anti-oxidative potential of natural phenolic acids.

KEYWORDS

diabetes, experimental pharmacology, oxidative stress, therapeutics, zinc(II)-ferulic acid complexation

1 | INTRODUCTION

Recent data from the International Diabetes Federation (IDF) depicts a sharp increase in global diabetes prevalence,[1] which is imposing more socio-economic and health burden on affected countries. Since 2019, the global prevalence has increased by 74 million people.[1,2] Type 2 diabetes (T2D) has consistently remained the most prevalent of the type of diabetes,[1,2] which is partly attributed to its association with obesity, sedentary lifestyle, poor dieting and lack of exercise.[3,4] The aforementioned are risk factors of insulin resistance and impaired glucose tolerance, which can lead to persistent hyperglycemia and eventually T2D if not properly managed.[3–5] Insulin resistance is characterized by poor insulin signaling to cells, particularly myocytes and adipocytes.[6] There is poor insulin-mediated phosphorylative activation of key signaling proteins, such as insulin receptor, insulin receptor substrate-1, phosphatidylinositol 3-kinase, serine/threonine kinase Akt, etc. at cellular level, which results in impaired cellular glucose transporter type 4 (GLUT-4) translocation, as well as impaired glucose uptake and utilization.[6] Thus, glucose persists in the blood and increases the risk of diabetic complications.

Oxidate stress is crucial in the development and progression of diabetic complications, due to oxidative damage caused by free radicals and reactive oxygen species on cellular biomolecules.[6] Persistent hyperglycemia drives excess production of superoxide ion through carbohydrate and energy metabolism pathways, which can cause oxidative stress and oxidative damage in the absence of adequate anti-oxidant defense mechanisms.[7,8] Additionally, persistent hyperglycemia drives biomolecules glycation, as well as, the formation of advanced glycation end-products, which are biomarkers of degenerative complications associated with diabetes.[7]

The concept of synergism has a wide reception and application in the therapeutic management of diseases. The concept affords the benefits of overcoming toxicity or side effects linked to high dose of drug and potentiating multimode therapeutic targets and effects, thus improving therapeutic effects.[9] Advances in research have reported the complexation of Zn(II) with various types of ligands with the aim of developing potent anti-diabetic zinc complexes; the premise being that zinc possess insulin mimetic action and may improve the bioavailability and bioactivity of its ligands through multifacet mechanisms.[10] Zn(II)–threonine complex was shown to demonstrate insulin-like effects by modulating the activity of phosphatidylinositol 3-kinase, phosphodiesterase, insulin receptor tyrosine kinase and GLUT-4 in adipocytes.[11] The complex also suppressed lipolysis in rat adipocytes and potentiated glycaemic control in T2D KK-Ay mice.[12] Zn(II) complexes of two natural ligands (maltol and allixin), also, showed suppressive and modulatory effects on lipolysis and glucose uptake, respectively, in rat adipocytes.[13] Intraperitoneal administration of both complexes to T2D KK-Ay mice reduced blood glucose, HbA1c and hyperinsulinemia and improved glucose tolerance.[13]

Despite the therapeutic potential of zinc–ligand synergism, there are some pitfalls associated with the research trajectory in this area of research. In a recent review, it was reported that most of the studied anti-diabetic Zn(II) complexes are those of synthetic ligands with little documented pharmacological credence and toxicity concerns, while plant phenolics with documented anti-oxidant and glycemic

Highlights

- Zinc mineral is known to be involved in insulin secretion and function and thus have been complexed with various types of ligands with the aim of developing potent anti-diabetic zinc complexaes.
- Majority of the studied Zn(II) complexes are those of synthetic ligands with little documented pharmacological credence and toxicity concerns, while plant phenolics with documented anti-oxidant and glycemic control potential remain understudied.
- We took advantage of the insulin-related functions of zinc mineral and the oxidative stress related pharmacological credence of ferulic acid to develop a potent anti-hyperglycaemic and anti-oxidative complex.
- Zn(II) complexation increased the radical scavenging, enzyme inhibitory, anti-glycation, hepatic anti-lipid peroxidative and muscle glucose uptake effects of ferulic acid by several folds.
- Zn(II) complexation may be promising therapeutic approach for improving the glycaemic control and anti-oxidative potential of natural phenolic acids.

control potential remain understudied.[10] Several phenolic acids, including ferulic acid have not been explored as possible ligands of therapeutic Zn(II) complexes. Ferulic acid is a natural anti-oxidant present in some cereal grains, including maize.[14] It is an effective radical scavenger.[15] It enhances insulin secretion and regulates hepatic glucose production, thus improving glycaemic control.[16] In addition, ferulic acid has been shown to reduce blood glucose levels and lipid peroxidation in STZ-induced insulin-dependent diabetic mice and KK-Ay non-insulin-dependent diabetic mice.[17] In the liver of high-fat diet and fructose-induced T2D rats, ferulic acid potentiated anti-diabetic effects by modulating the activity of insulin signaling molecules.[18]

The above data suggest that ferulic acid may be a promising ligand for Zn(II) in developing a Zn(II) complex with potent anti-diabetic and anti-oxidative synergistic property. However, this has not yet been studied. Therefore, this study was done to investigate the anti-diabetic and anti-oxidative properties of a novel Zn(II) complex of ferulic acid.

2 | MATERIALS AND METHODS

2.1 | Complexing ferulic acid with zinc sulphate

Zinc(II) was complexed with ferulic acid in a 1:2 mole ratio using a previous method[19] with some modifications. First, 287.56 mg zinc(II) sulphate heptahydrate (Mr = 287.56 g/mol) and 396.34 mg ferulic acid (Mr = 194.18 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 ferulic acid solution. The was stirred until no effervescence was observed. Thereafter, the zinc(II) sulphate heptahydrate solution was gradually added to the mixture while stirring. Stirring continued until complete precipitation of a light yellowish precipitate. The mixture was filtered using a filter paper to recover the precipitate. The precipitate was washed thrice with 50% methanol and freeze dried using a freeze dryer (Alpha 1–2 LDplus Freeze Dryer, Martin Christ).

2.2 | Spectroscopic characterization

The complex was characterized using Fourier-transform infrared spectroscopy (FT-IR), proton nuclear magnetic resonance spectroscopy (^{1}H NMR) and high-resolution mass spectroscopy.

For FT-IR, a Perkin Elmer Spectrum 100 FT-IR Spectrometer was used. The instrument had an ART accessory. Roughly 2 mg of either the complex or ferulic acid was put on the crystal sample holder. Then, scanning was done from a wavelength range of 4000–380 cm^{-1} at 40 s^{-1} scan rate. A V 6.3.4 Spectrum Software was used to record the spectra.

For ^{1}H NMR, a Bruker Avance spectrometer (400 MHz; Bruker Corporation) was used, which recorded the ^{1}H NMR data. DMSO-d6 (δH = 2.50) was used as the solvent with a tetramethylsilane 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.

The HR-MS analysis was done by the Central Analytical Facility, Stellenbosch University, Cape town. Instrument used was Waters Synapt G2 (Waters Corporation), ESI probe, ESI Pos, Cone Voltage 15 V.

2.3 | Cytotoxicity evaluation of complex in Chang liver cells

Chang liver cells were procured from 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 media containing 10% FBS was used to culture the cells. Cell culture was carried out in a NÜVE EC 160 CO_2 incubator (NÜVE) with oxygen, CO_2 supply, and temperature maintained at 95%, 5% and 37°C, respectively. At about 80% confluent, 100 µl of cells were seeded (30,000 cells/ml) in a 96-well sterile plate. The plate was incubated for 48 h to allow the cells attach to the plate. Thereafter, the medium in the wells was aspirated and replaced with new medium containing 7.27, 72.7 and 727 µM of complex or 0.5% DMSO (vehicle control) and incubation continued for 36 h. Thereafter, 100 µl of 0.5 mg/ml MTT solution (Sigma Aldrich) was added to the plate and the plate was further incubated for 3 h. After incubation, the content in the wells were removed, and the wells were washed with phosphate-buffered saline. One hundred microliters of MTT de-staining or solubilization solution (Sigma Aldrich) was quickly added into the wells, and absorbance was captured at 570 nm using the Multiskan Go plate reader (Thermo Fischer Scientific). Viability (%) of sample-treated cells was calculated using the control as a reference. Calculation was done with triplicate data of three biological repeats.

2.4 | In vitro anti-oxidant and anti-diabetic measurements

2.4.1 | Radical scavenging and Fe^{3+} reducing anti-oxidant assays

The DPPH and ABTS radical scavenging assays, as well as the Fe^{3+} reducing anti-oxidant assay was performed

according to a previous study.[20] For DPPH and ABTS, the samples were tested at different concentration (3.75–60 μM in final assay volume) and expressed as % scavenging activity. Ascorbic acid and Trolox were the positive controls in these assays. For Fe^{3+} reducing anti-oxidant assay, the samples were tested at 40 μM. The Fe^{3+} reducing anti-oxidant activity was computed from an ascorbic acid standard curve (3.75–60 μM in total assay volume) and expressed as mmol/mol ascorbic acid equivalent (AAE). Trolox was used as the positive control.

2.4.2 | Linoleic acid peroxidation inhibition assay

The effect of the complex on linoleic acid peroxidation was measured by consulting a previous method[21] and adding some modifications. Into vial containing 30 μl of varying concentrations (5–80 μM in assay volume) of samples or positive controls (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 sequentially added. Next, 20 μl of 2 mM $FeSO_4.7H_2O$ was aliquoted into the vial. The mixtures were kept for 30 min in an incubator set at 37°C. The normal control vial contained 20 μl of distilled water instead of $FeSO_4.7H_2O$, 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). A boiling water bath was used to heat the vials for 30 min and before cooling under room temperature. The vials were then centrifuged at 3500*g* for 10 min under ambient temperature. In total, 150 μl of the supernatants was pipetted into a 96-well plate and absorbance was measured at 532 nm using a SpectraMax M2 microplate reader (Molecular Devices). The percentage inhibition of linoleic acid peroxidation was calculated as follows:

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

where, 'A' means 'absorbance'.

2.4.3 | α-Glucosidase and α-amylase inhibition assays

The in vitro inhibitory effect on α-glucosidase and α-amylase activity was done using methods reported in a previous study.[20] For α-glucosidase inhibition assay, the samples and positive control were tested at concentrations ranging from 3.75 to 60 μM. For α-amylase inhibition, samples and positive control were tested at concentrations ranging from 5 to 80 μM. Acarbose was used to positively control the experiments.

2.5 | Measurement of cellular anti-oxidant and anti-diabetic properties

2.5.1 | Evaluating the effect complex on lipid peroxidation and reduced glutathione concentration in Chang liver cells induced with oxidative stress

A recent study[22] with minor modifications was adopted to assess the cellular anti-oxidant effect of the complex and its precursors. Chang liver cells (ATCC® CCL-13™, ATCC) were cultured in a culture flask as mentioned above. At about 85% confluence the cells were harvested by trypsinization and 200 μl of culture medium (EMEM media containing 10% FBS) containing cells was aliquoted into 96-well plate at a concentration of 15,000 cells/ml. The plates was incubated in a CO_2 incubator for 36 hours so that the cells can attach to the bottom of the plate wells. Thereafter, spent medium was discarded and 200 μl of fresh medium containing different concentration (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 induced 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 containing 0.5% v/v Triton X-100, pH 7.5) by gently agitating under cold condition. The plate was then centrifuged at 2000*g* for 2 min. Lipid peroxidation and reduced glutathione (GSH) concentration were measured in the supernatant of each well using methods described below.[22] Experiment was performed as triplicate biological repeats.

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 a vial. Five hundred microlitres of 0.25% w/v thiobarbituric acid, 200 μl of 20% v/v acetic acid and 200 μl of distilled water were added, successively. A boiling water bath was used to heat the vials for 50 min before cooling at ambient temperature. An aliquot of 200 μl was transferred into a 96-well plate and absorbance was measured at 532 nm. The lipid peroxidation was estimated as thiobarbituric acid reactive substances,

which was computed from the malondialdehyde linear graph.

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 4200*g* (Hettich Mikro 200 microcentrifuge, Hettich Lab Technology). Fifty microlitres of the supernatant or GSH standards (0.002, 0.02, 0.2, 2, 20, 200 μM in assay volume) was transferred 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 allowed to stand for 5 min under ambient temperature, and absorbance was measured at 412 nm. The GSH concentration was computed from the GSH linear graph.

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

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

To measure the effect of the complex on glucose uptake a previous method[23] was consulted. L-6 myoblast cells from rat muscle (ATCC CRL-1458, ATCC) 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 trysinization and seeded with fresh medium into a 96-well plate at a density of 4000 cells/well. At about 80% confluence, DMEM containing 2% horse serum was used to replace spent medium in for 4–5 days. This was done to differentiate the cells. The myoblasts differentiated into myotubes during the 4–5 days incubation period. Thereafter, the differentiation medium was removed and 200 μl of fresh medium that contained the samples at different concentrations (5, 50 and 100 μm in incubation volume) was pipetted into wells. The sample solvent was used as the vehicle control. Some wells were used as the blank control, in which only the medium was pipetted and had no cells. The culture medium was removed after 48 hours incubation, and the cells were washed with PBS. Then, 100 μl of RPMI medium, which contained 8 mm glucose and 0.1% of BSA was pipetted into the wells and the plate was incubated for another 2 h. Insulin (1 μM) was used as a positive control group. Aliquot from the incubation medium of each well was then used for glucose concentration measurement (Glucose-GO Assay Kit, Sigma Aldrich). The MTT viability assay, as described above, was used to measure the viability of the myotubes following the treatment with samples. Experiment was done in three biological repeats and each biological repeat had three technical replicates. The blank was used to normalize glucose uptake of the test samples and control as shown below:

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

'ΔGC' denotes glucose concentration change (i.e., difference between initial and final glucose concentration in incubation solutions). In addition, the EC_{50} of samples, which denotes sample concentrations (μM) needed to effect 50% glucose uptake increase was computed.

2.6 | Measurement of ex vivo antioxidant and anti-diabetic properties

2.6.1 | Animals

This study was conducted as reported previously[22,24] with slight modifications. First 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). Animal handling was done according to the rules and regulation of the ethics committee. The university animal facility supplied Sprague Dawley rats that were 8 weeks old. The animals were fasted overnight. Thereafter, they were euthanized using isoflurane and the liver and psoas muscle tissues were immediately harvested and used for the ex vivo antioxidant and anti-diabetic experiments.

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

A previous method[22] was adopted with minor changes to perform this experiment. Into 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) for 25 min in 900 μl of Kreb's buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 0.5 mM K_2HPO_4; pH 7.4 ± 2 at 25°C) that contained different sample or standard (ascorbic acid) 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. For the controls (control and negative control), the tissue was incubated in buffer containing the equivalent volume of solvents used in dissolving the test

samples. After pre-incubation, 100 µl of $FeSO_4.7H_20$ solution (1 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. The plate was incubated for 90 min. After incubation time elapsed, a pair of sterile forceps was used to remove the tissues from the incubation solution. The tissues were cleaned with Kreb's buffer. A D1000 Handheld Homogenizer (Merck) was, then, used to homogenize the tissues in 1 ml of 50 mM sodium phosphate buffer (contained 0.5% v/v Triton X-100; pH, 7.5) under ice cold condition. The tissue homogenate was centrifuged at 9600 g for 10 min to recover the supernatants. Lipid peroxidation and GHS concentration were estimated in the supernatants using similar methods described above. The inhibitory effect (%) of samples or standard on lipid peroxidation and GHS depletion was also calculated, which was used to compute the IC_{50} values of samples or standard. Experiment was performed as triplicate biological repeats.

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

A previous study[24] was consulted and experiment was performed with some changes. Into 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 Kreb's buffer that contained different sample concentrations (10, 20, 40 and 80 µM in total incubation volume) or 50 mU insulin positive control (NovoRapid® FlexPen®, Novo Nordisk Limited). For the control, the tissue was incubated in buffer containing the equivalent volume of solvents used in dissolving the test samples. One hundred microlitres of glucose solution (11.1 mM in total incubation volume) was added after pre-incubation. Thereafter, additional 90 min incubation was done. After the 90 min incubation, aliquot from the incubation medium in each well was then used for glucose concentration measurement using the Glucose (GO) Assay Kit (Sigma Aldrich). The absorbance value of the control was used as the reference values for computing the glucose uptake increase (%) of test samples and positive control as shown below. Experiment was performed as triplicate biological repeats.

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

In addition, the EC_{50}, which denotes the sample concentrations (µM) 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 test samples and 50 mU insulin. First, a pair of sterile forceps was used to remove the tissues from the incubation solution. The tissues were washed with Krebs buffer, homogenised in 1 ml of ice cold 50 mM sodium phosphate buffer (contained 0.5% v/v Triton X-100 and 1 mM EDTA; pH, 7.5). The tissue homogenates were centrifuged at 10,400 g for 10 min at 4°C to recover the supernatants. Protein concentration was measured in the supernatants using the bicinchoninic acid method. Hexokinase activity was measured in the supernatant at protein concentration of 15 mg/ml using a 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 represents the amount of enzyme that will generate 1.0 mmole of NADH per minute at pH 8.0 at room temperature. In addition, zinc concentration was measured in the tissue supernatant using a zinc Assay Kit (catalog number MAK032, Sigma Aldrich) and expressed in ng/µl. An ELISA method was adopted to measure pan-Akt and phosphor-Akt using a Phospho-Akt (pSer473)/pan-Akt ELISA Kit (catalog number RAB0012, Sigma Aldrich). The phospho-Akt/pan-Akt ratio was computed.

2.7 | Molecular docking analysis against enzyme targets linked to carbohydrate metabolism and diabetes

The in silico anti-diabetic potential of complex and ferulic acid were investigated by computationally docking them as ligands against four putative target proteins linked to diabetes. The target enzymes include alpha-amylase (AmyL), α-glucosidase (GAA), GLUT-4, and protein kinase B (PKB) potency. Briefly, the 3-D structures of each enzyme were retrieved from the protein data bank, while GLUT-4 was theoretically generated by homology modeling approach due to unavailability. Molecular docking was carried out using AutoDock Vina 4.2.[20] Five docking poses were generated for each ligand against a particular target enzyme and based on the minimum docking score (kcal/mol), the best docking pose was selected. Then, the BIOVIA-DSV software was used to generate visuals of the protein-ligand molecular interaction.

2.8 | Data and statistical analysis

For analysing the data of this study, 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 fit of transformed (log10) *x*-axis (sample concentration). The analysed data were reported in triplicates using the average ± standard deviation format. Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp). Tukey multiple range post-hoc tests and the one-way analysis of variance (ANOVA) was adopted for multiple comparison of the data averages and statistically significant difference (p) was set at $p < 0.05$ when comparing the mean values of the different groups.

3 | RESULTS

3.1 | Spectroscopic characterization

Figure 1 shows the FT-IR spectra of ferulic acid and its complex. The spectrum of ferulic acid showed a broad peak centered at 3491 cm^{-1}, which is indicative of a carboxylic O-H stretch in the compound (Figure 1a). This region of carboxylic O-H stretching, also, appeared in the FT-IR spectrum of the complex in the region centered at 3399 cm^{-1} (Figure 1b). The lower carboxylic O-H stretch wave number value of the complex suggests an interaction of Zn(II) with the carboxylic O-H bond. Probably zinc ion donated electrons to the carboxylic O-H bond, thus weakening the bond and causing a lower wave number. This suggests that ferulic acid interacted with Zn(II) using its carboxylic O-H group as proposed in Figure 2b. In addition, the sharp peaks at 1694 and 1640 cm^{-1} indicate carboxylic C=O stretching in the phenolic acid (Figure 1a) and the complex (Figure 1b), respectively. The lower wave number value of the complex suggests a C=O bond weakening, probably due to electrons donated by zinc ion. This suggests that zinc(II) coordinated with the carboxylic carbonyl group of the phenolic acid. The alteration of the carboxylic O-H and carbonyl IR signals suggests zinc(II) complexed with ferulic acid through the carboxylic functional group.

A comparative proton NMR analysis between the starting material (ferulic acid) and the complex is presented in (Figure 2b and Appendix S1). The ^{1}H-NMR spectrum of the complex showed ABX system of ferulic acid moiety. Doublet of doublet peak at 7.01 corresponding to H-2, while H-6 and H-3 is observed as a doublet at δ 7.19 and 6.76 respectively. The values at 6.34 and 7.33 represent the double bond. The singlet peak at δ 9.41 is characteristic of phenolic hydroxyl group. The carboxylic proton observed as singlet in the ferulic acid spectra at δ 12.05 was not picked up in the spectra of the complex (Figure 2b and Appendix S1), possibly due to involvement in the complexation with zinc(II) ions. The double bond proton closed to the carboxylic group of ferulic acid was observed at δ 7.48. For the complex, this was observed at δ 7.33. The change from δ 7.38 to δ 7.33 may be due to the coordination of the carboxylic group with zinc(II) ions.

The high-resolution mass spectroscopy (HR-MS) data of the complex are presented in Figure 3. For the negative mode, the signal at m/z 485.0015 on the mass spectrum showing a difference of 0.0411 from calculated m/z 485.0426 represents the two ferulic acid and two water molecules coordinating/interacting with zinc ion, which indicates the complex was successfully formed. Fragment was observed at m/z 293.1785 (calculated; 293.0004) signifying a coordination/interaction between one ferulic acid and two water molecules with zinc ion. The fragment observed at m/z 264.9455 is attributed to the loss of a methyl group from one molecule of ferulic acid coordinating with zinc ion. The fragment with 100% abundance at m/z 193.0501 (calculated m/z 193.0501) represents ferulic acid moiety.

The above spectroscopic characterization suggest that Zn(II) complexed with two moieties of ferulic acid to form a Zn(II)-biferulate.2H_2O product as shown in Figure 2a. Based on the above spectroscopic data and analysis, it is most probable that the carboxylate group coordinated to Zn(II) ions in a bidentate or bridging mode.

3.2 | Anti-oxidant activities

In vitro, ferulic acid showed scavenging activities on DPPH (IC_{50} = 75.4 μM) and ABTS (IC_{50} = 66.9 μM) radicals, reduced Fe^{3+} ion (504 mmol/mol AAE) and inhibited linoleic acid peroxidation (IC_{50} = 66.7 μM) (Figure 4a–d and Table 1). Zinc sulphate showed no observable in vitro anti-oxidant activity (Figure 4a–d and Table 1). However, the DPPH (IC_{50} = 43.8 μM) and ABTS (IC_{50} = 32.0 μM) radical scavenging and in vitro anti-lipid peroxidative (IC_{50} = 40.1 μM) activities of the complex were 2.1, 1.7 and 1.7 folds more potent than that of ferulic acid according to computations from the IC_{50} values (Table 1). In addition, some anti-oxidant activities of the complex were statistically comparable ($p > 0.05$) and/or more potent relative to Trolox and ascorbic acid (Table 1).

In both Chang liver cells and isolated rat liver tissues, ferulic acid dose-dependently inhibited oxidative stress-induced lipid peroxidation (IC_{50} = 116 and 758 μM, respectively) and reduced GSH depletion (IC_{50} = 85.4 and 117 μM, respectively) (Figure 5a,b; Table 1). The cellular and tissue anti-oxidant activity of zinc sulphate was not as potent as ferulic acid. The complex showed significantly ($p > 0.05$) stronger cellular (IC_{50} = 48.6 μM) and tissue (IC_{50} = 331 μM) anti-lipid peroxidative activity than ferulic acid (Table 1). The inhibitory effect of the complex on GSH depletion in Chang liver cell (IC_{50} = 33.9 μM) and

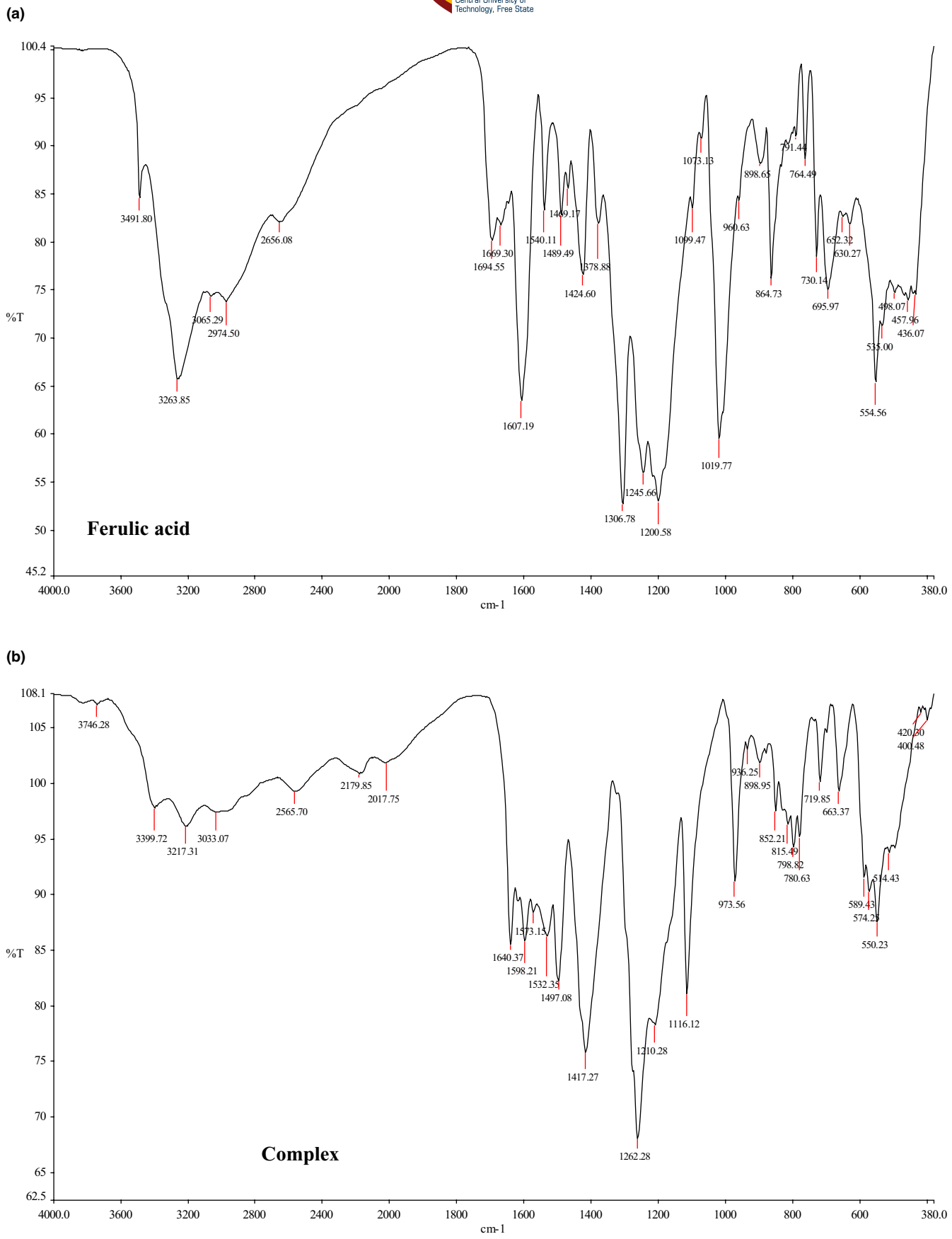
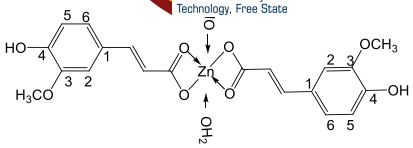
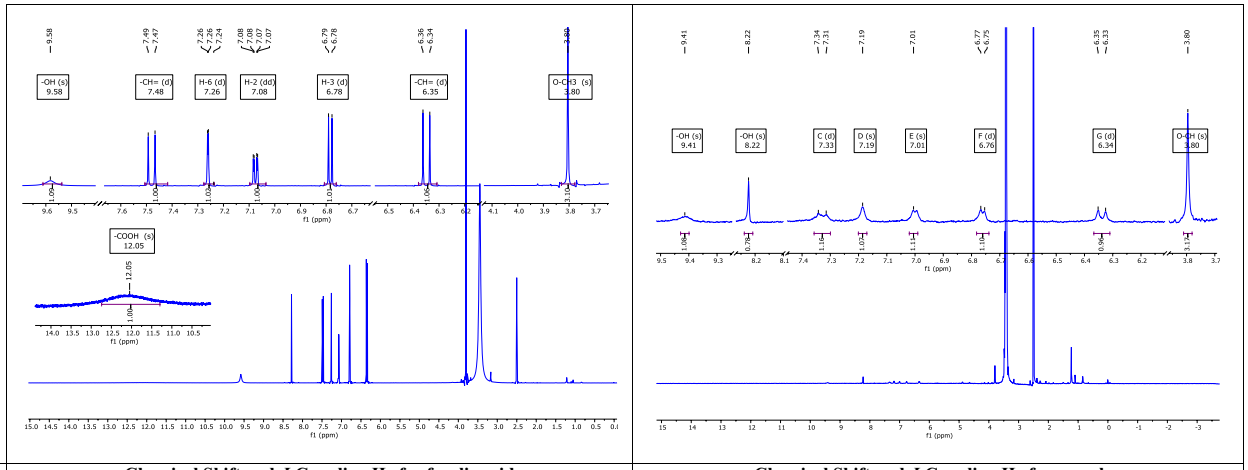


FIGURE 1 (a) Ferulic acid (b) complex Fourier-tranform infrared spectra.

(a)



(b)



Group	Chemical Shift and *J*-Coupling Hz for ferulic acid	Chemical Shift and *J*-Coupling Hz for complex
-OCH_3	3.80 (3H, s)	3.80 (3H, s)
-CH=	6.34(1H, d, *J*=15.96 Hz)	6.34(1H, d, *J* =15.90 Hz)
H-3	6.78 (1H, d, *J*=8.20 Hz)	6.76 (1H, d, *J* =8.20 Hz)
H-2	7.08 (1H, dd)	7.01(1H, dd)
H-6	7. 26 (1H, d)	7.19(1H, d)
-CH=	7.48 (1H, d, *J* =15.96 Hz)	7.33(1H, d, *J* =15.90 Hz)
OH	9.58 (1H, s)	9.41(1H, s)
-COOH	12.05 (IH, s)	-

FIGURE 2 (a) Proposed Zn(II)-biferulate.$2H_2O$ complex and (b) comparative ^{1}H NMR spectral analysis between ferulic acid and complex.

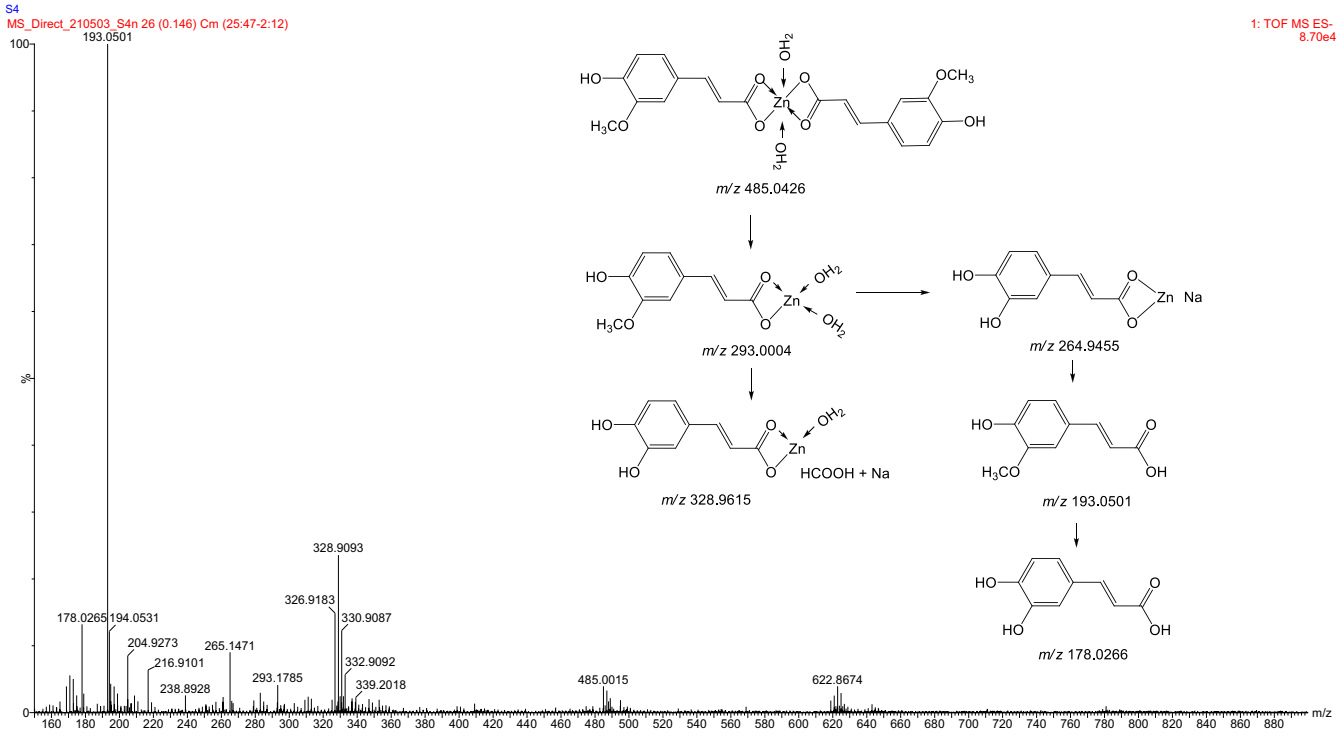


FIGURE 3 High-resolution mass spectroscopic analysis of complex.

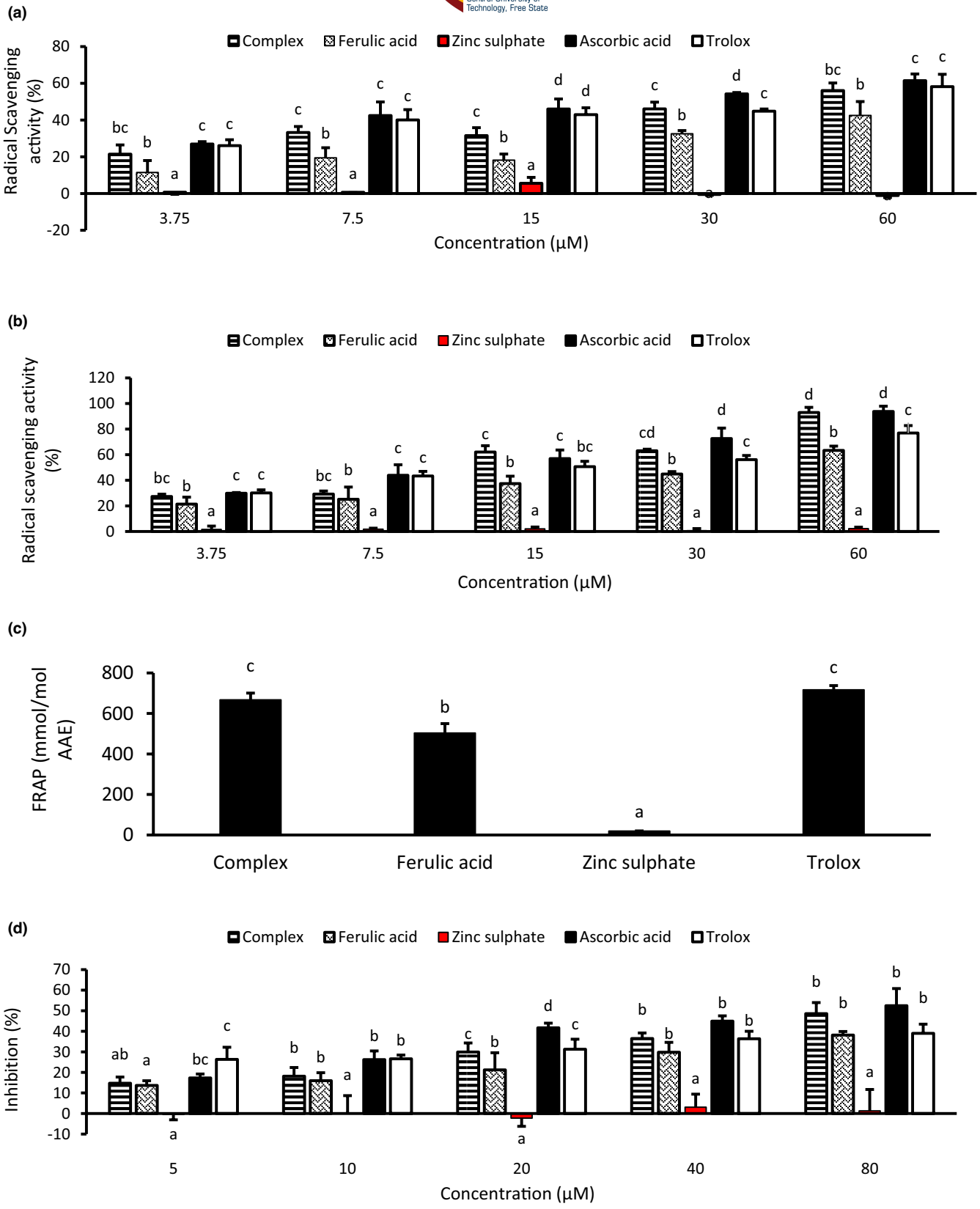


FIGURE 4 (a) DPPH radical scavenging (b) $ABTS^{\bullet+}$ radical scavenging (c) Fe^{3+} reducing and (d) in vitro linoleic acid lipid peroxidation inhibitory activities of complex, precursors and standards, in vitro. Data are shown as mean ± SD of triplicate analysis ($n = 3$). Within each tested concentration, statistical comparison was done between treatment groups. Significant difference ($p < 0.05$) means a difference in letters. Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp). Tukey multiple range post-hoc tests and the one-way analysis of variance (ANOVA) was adopted for multiple comparison of the data averages.

TABLE 1 IC_{50} and EC_{50} values of ferulic acid, its complex with Zn(II) and standards

Parameters or activity	Zn(II) – Ferulic acid complex	Ferulic acid	Zn(II)	Ascorbic acid	Trolox	Acarbose	Aminoguanidine	Insulin
	IC_{50} or EC_{50} values (μM)							
ABTS radical scavenging activity (IC_{50})	32.0 ± 7.27^{b}	66.9 ± 17.3^{a}	ND	26.3 ± 10.9^{b}	49.3 ± 5.58^{ab}	NA	NA	NA
DPPH radical scavenging activity (IC_{50})	43.8 ± 6.23^{b}	75.4 ± 5.48^{a}	ND	14.3 ± 1.46^{c}	12.7 ± 4.62^{c}	NA	NA	NA
Inhibition of in vitro linoleic acid peroxidation	40.1 ± 6.41^{bc}	66.7 ± 11.3^{a}	ND	13.9 ± 4.38^{c}	37.8 ± 11.1^{ab}	NA	NA	NA
Anti-glycation activity (IC_{50})	64.5 ± 11.2^{ab}	97.8 ± 17.7^{a}	60.8 ± 16.5^{b}	NA	NA	NA	8.70 ± 2.04^{c}	NA
α-glucosidase inhibition (IC_{50})	22.1 ± 2.88^{b}	45.2 ± 2.54^{b}	208 ± 40.8^{a}	NA	NA	6.90 ± 2.75^{b}	NA	NA
α-Amylase inhibition (IC_{50})	20.1 ± 4.45^{b}	40.6 ± 10.7^{a}	ND	NA	NA	17.2 ± 2.89^{b}	NA	NA
Glucose uptake increase in L-6 myotubes (EC_{50})	11.7	45.7	34.7	NA	NA	NA	NA	ND
Glucose uptakes increase in isolated rat psoas muscle (EC_{50})	501	1510	603	NA	NA	NA	NA	ND
Inhibition of oxidative stress-induced lipid peroxidation in Chang liver cells (IC_{50})	48.6 ± 6.96^{c}	116 ± 24.8^{b}	743 ± 51.3^{b}	30.3 ± 8.52^{a}	NA	NA	NA	NA
Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver (IC_{50})	331 ± 116^{b}	758 ± 3069^{a}	ND	189 ± 71.5^{b}	NA	NA	NA	NA
Inhibition of oxidative stress-induced GSH depletion in Chang liver cells (IC_{50})	33.9 ± 13.1^{b}	$85.4 \pm 31.9.0^{b}$	709 ± 265^{a}	27.3 ± 5.51^{b}	NA	NA	NA	NA
Inhibition of oxidative stress-induced GSH depletion in isolated rat liver (IC_{50})	35.5 ± 5.57^{b}	117 ± 25.8^{b}	2150 ± 437^{a}	27.0 ± 2.48^{b}	NA	NA	NA	NA

Note: 'GSH' means 'reduced glutathione'; 'ND' means 'not determined'; 'NA' means 'not applicable'; IC_{50} is concentration needed to inhibit the activity of carbohydrate digesting enzymes, bovine serum albumin glycation, lipid peroxidation and GSH 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.

isolated rat liver tissue (IC_{50} = 35.5 μM) was 2.5 and 3.3 folds more potent than ferulic acid based on computations from the IC_{50} values (Table 1). In most instances, the activity of the complex did not differ significantly ($p > 0.05$) from ascorbic acid.

3.3 | Anti-diabetic activities

Both ferulic acid and the complex dose dependently inhibited α-glucosidase (IC_{50} = 45.2 and 22.1 μM, respectively) and α-amylase (IC_{50} = 40.6 and 20.1 μM, respectively) activity in vitro, with the complex having stronger enzyme inhibitory activities than ferulic acid (Figure 6a,b; Table 1). The potency of the complex was statistically comparable ($p > 0.05$) with that of acarbose (IC_{50} = 6.90 and 17.2 μM) (Table 1). Zinc sulphate showed a relatively lower α-glucosidase inhibitory activity (IC_{50} = 208 μM) without observable inhibition on α-amylase (Figure 6a,b; Table 1).

Although not as potent as aminoguanidine (IC_{50} = 8.70 μM), zinc sulphate exhibited appreciable in vitro dose dependent anti-glycation effect (IC_{50} = 60.8 μM) (Figure 6c and Table 1). It, also, increased the anti-glycation effect of ferulic acid (IC_{50} = 97.8 μM) by 1.5-folds upon complexation (IC_{50} = 64.5 μM) (Figure 6c and Table 1).

In both L-6 myotubes and isolated rat psoas muscle tissue, zinc sulphate dose dependently increased glucose uptake (IC_{50} = 34.7 and 603 μM, respectively) (Figure 7a,b; Table 1). Although not as potent as zinc sulphate, ferulic acid, also, dose-dependently increased glucose uptake in the myotubes (IC_{50} = 45.7 μM) and muscle tissues (IC_{50} = 1510 μM) (Table 1). However, Zn(II) complexation increased the cellular and tissue glucose uptake effect of ferulic acid by 3.9 and 3.0 folds, respectively (Table 1). At highest concentrations tested the cellular and tissue glucose uptake effect of the complex was statistically comparable ($p > 0.05$) to that of insulin (Figure 7a,b). Zinc(II) concentration in the complex-treated muscle tissue was higher than that of the ferulic acid ($p < 0.05$) and zinc sulphate–treated tissues (Figure 7c). Hexokinase activity in the complex-treated muscle tissue was significantly higher ($p < 0.05$) than that of the ferulic acid and zinc sulphate–treated tissues (Figure 7d). Phospho-Akt/pan-Akt ratio in the complex-treated muscle tissue was higher than that of the ferulic acid ($p < 0.05$) and zinc sulphate–treated tissues (Figure 7e).

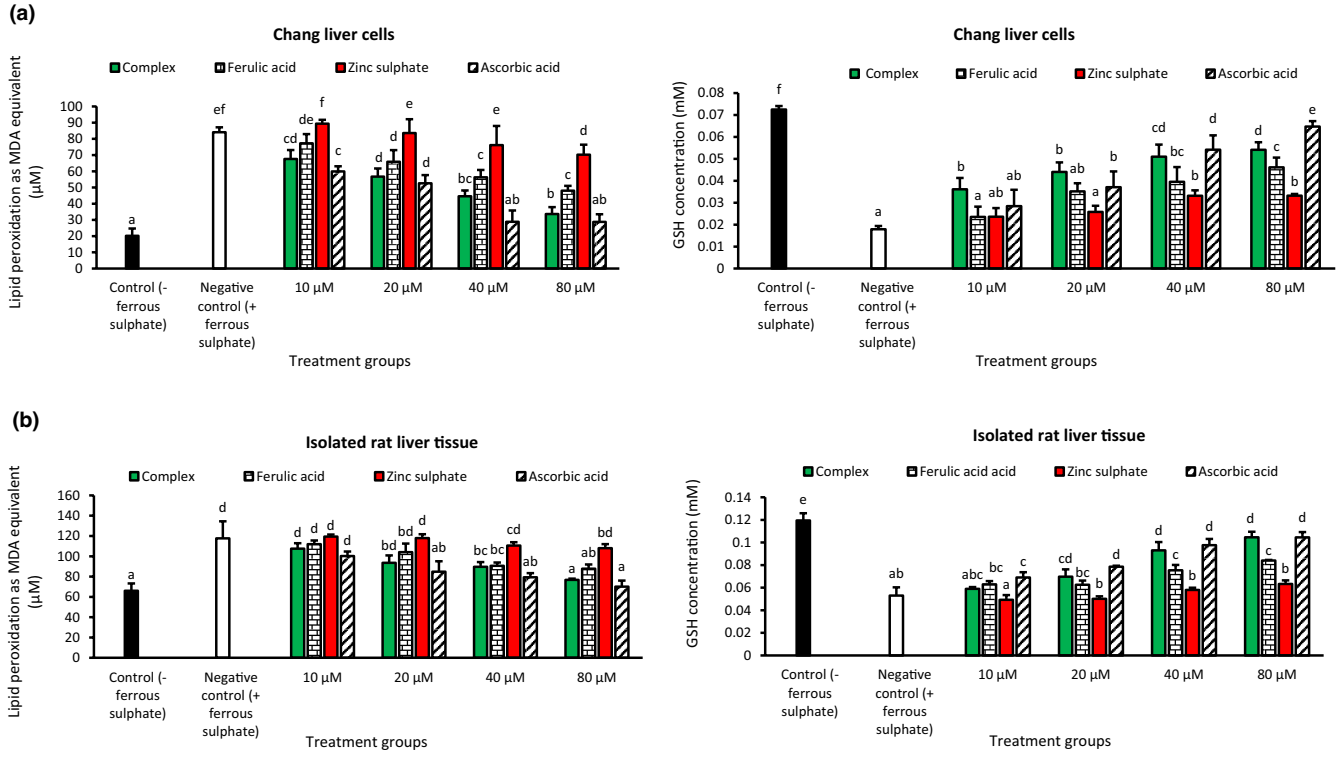


FIGURE 5 Figure showing how the complex, precursors and standards affects lipid peroxidation and GSH concentration in (a) Chang liver cells and (b) isolated rat liver tissue induced with oxidative stress. Data are shown as mean ± SD of triplicate analysis ($n = 3$). Within each tested concentration, statistical comparison was done between treatment groups, as well as between treatment groups and the controls (normal control and negative control). Significant difference ($p < 0.05$) means a difference in letters. Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp). Tukey multiple range post hoc tests and the one-way analysis of variance (ANOVA) was adopted for multiple comparison of the data averages.

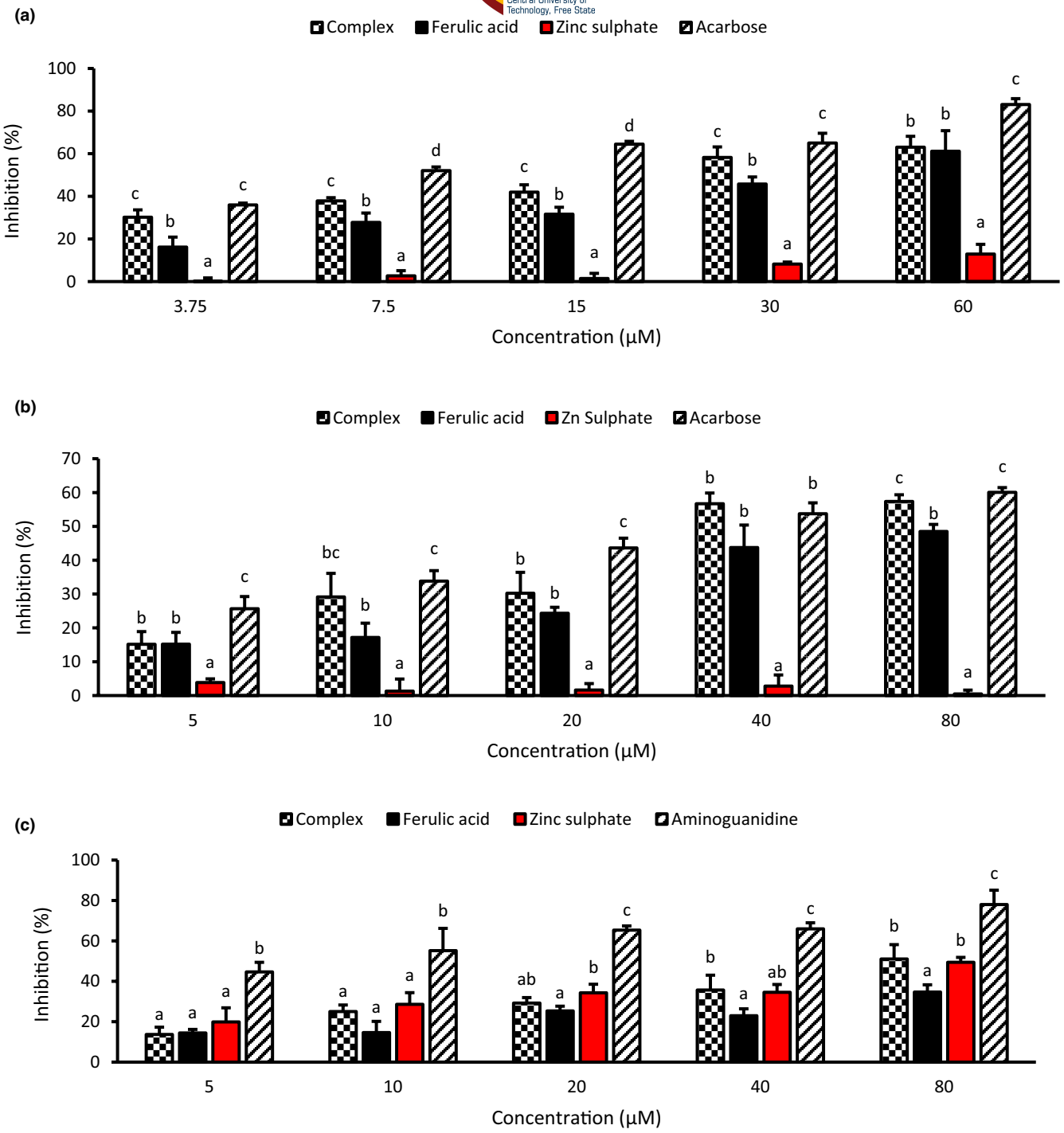


FIGURE 6 Figure showing the in vitro inhibitory effect of test samples on (a) α-glucosidase (b) α-amylase and (b) BSA glycation. Data are shown as mean ± SD of triplicate analysis ($n = 3$). Within each tested concentration, statistical comparison was done between treatment groups. Significant difference ($p < 0.05$) means a difference in letters. Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp). Tukey multiple range post hoc tests and the one-way analysis of variance (ANOVA) was adopted for multiple comparison of the data averages.

The docking analysis of ferulic and its complex against α-glucosidase (GAA), amylase (AmyL), GLUT-4, and protein kinase B (PKB) protein targets is presented in Figure 8, while the minimum docking scores (kcal/mol) are shown in Table 2. For protein targets, the docking scores of the complex (−7.24 to −8.25 kcal/mol) were higher than those of ferulic acid (−5.75 to −6.43 kcal/mol) (Table 2).

In both Chang liver cells and L-6 myotubes, the complex did not adversely affect viability (Figure 7f).

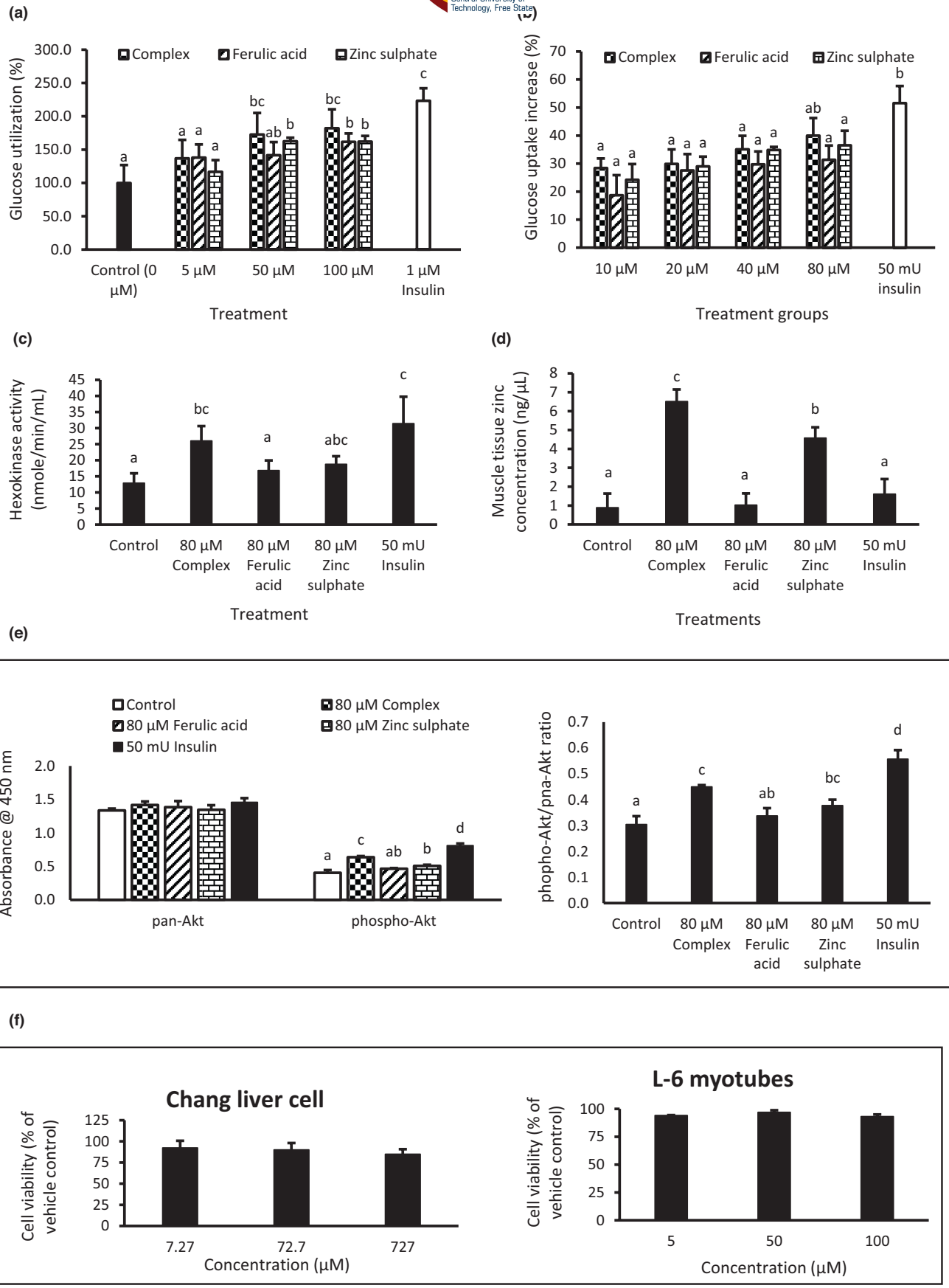


FIGURE 7 Figure showing the effect of test samples on (a) glucose uptake in L6-myotubes, (b) glucose uptake in isolated rat psoas muscle, glucose uptake-associated (c) hexokinase activity, (d) Zn(II) concentration and (e) Akt phosphorylation in isolated rat psoas muscle tissues and (f) viability of Chang liver cell and L6-myotubes. Data are shown as mean ± SD of triplicate analysis ($n = 3$). Within each tested concentration, 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. Statistical analysis was done on the Window's version 27.0 of IBM SPSS (IBM Corp). Tukey multiple range post-hoc tests and the one-way analysis of variance (ANOVA) was adopted for multiple comparison of the data averages.

4 | DISCUSSION

The search for more effective approach in preventing or managing diabetes and oxidative complication remains a major strategy for combating the disease. In some cases, a combination of several drugs is used to achieve a targeted effect but poses the treat of drug toxicity and detrimental side effects. In this regard, natural medicines are often encouraged as supplemental therapy to minimize toxicity concerns. In this study, we took advantage of the insulin-related functions of zinc mineral and the oxidative stress related pharmacological credence of ferulic acid to develop a potent anti-hyperglyceamic and anti-oxidative complex from these natural precursors. Spectroscopic characterization suggests a Zn(II)-biferulate.$2H_2O$ product was formed. In silico analysis suggests that the molecular property of the complex may influence its promising bioactivity relative to its precursors, indicating a possible structure–function relationship of the complex.

It is an established fact that oxidative stress is implicated in the development and progression several diabetic complications.[7] Prooxidants, including free radicals oxidatively damage biological molecules and facilitate processes like lipid peroxidation in diabetic condition. Natural antioxidants are important in mitigating the detrimental effects of prooxidants in diabetes due to their ability to quench radical reactions and oxidative processes.[7] Ferulic acid a natural anti-oxidant with radical quenching properties,[15] which could be attributed to its electron or hydrogen donating characteristic and ability to form a stable phenoxy radical.[25] In this study ferulic acid was shown to scavenge ABTS and DPPH and inhibit peroxidation of linoleic acid in vitro (Figure 4a,b,d; Table 1). However, when complexed with zinc sulphate its activities increased by 1.7, 2.1 and 1.7, respectively, and was comparable to ascorbic acid and or Trolox (Table 1). Considering zinc sulphate had no noticeable in vitro radical scavenging or anti-lipid peroxidative activity (Figure 4a,b,d; Table 1), the improved activity of the complex may be attributed to the 2 moieties of ferulic acid in the complex (Figure 2a). In liver cells and liver tissues, similar anti-oxidant trend was observed (Figure 5a,b; Table 1). The cellular and tissue inhibitory activity of the complex on oxidative stress induced lipid peroxidation and GSH depletion was stronger than that ferulic and zinc by several folds and statistically comparable ($p > 0.05$) to the activity of ascorbic acid (Table 1), which could, also, be linked to the two moieties of ferulic acid afforded by complexation (Figure 2a). The data suggest that complexation could be a therapeutic approach to improving the anti-oxidant potency of phenolic acids and their ability to mitigate oxidative stress.

Furthermore, the data of this study suggest that Zn(II)-biferulate.$2H_2O$ complex may also possess promising diabetes-related therapeutic benefits. In vitro, the complex exhibited inhibitory activities on α-glucosidase and α-amylase (Figure 6a,b; Table 1). Inhibition of these carbohydrate digesting enzymes could translate into reduced glucose absorption and postprandial blood glucose level, which is the mechanism of action for anti-diabetic drugs known as α-glucosidase inhibitors. The α-glucosidase and α-amylase inhibitory activity of the complex was 2 folds stronger than ferulic acid and comparable to acarbose (Table 1), a known α-glucosidase inhibitor. Considering that the inhibitory activity of zinc sulphate was not as potent as ferulic acid (Table 1), it is rational to hypothesize that the potent inhibitory activity of the complex could be attributed to the two moieties of ferulic that were acquired by the complex during complexation (Figure 2a). Moreover, ferulic acid had appreciable α-glucosidase and α-amylase inhibitory activities (Table 1), which has, also, been previously documented.[26] Additionally, docking data support this structure–function interaction of the complex (Figure 7). The docking scores or binding energies (Table 2) showed the complex had stronger in silico molecular interaction with α-glucosidase and α-amylase enzyme/protein targets than its precursor phenolic acid. The two ferulic acid moieties in the complex potentiated more hydrogen bond interactions with target enzymes, causing stronger binding energies and interaction (Figures 2a and 7). The data suggest that zinc mineral complexation could be a therapeutic approach to improving postprandial glycemic control of ferulic acid, which warrants future anti-diabetic studies in appropriate diabetic animal models.

Furthermore, a complex with improved in vitro inhibitory activity on glucose induced BSA glycation (Figure 6c and Table 1), relative to its precursor phenolic acid was developed in the present study. Hyperglycaemia-induced glycation causes abnormally high glycation end

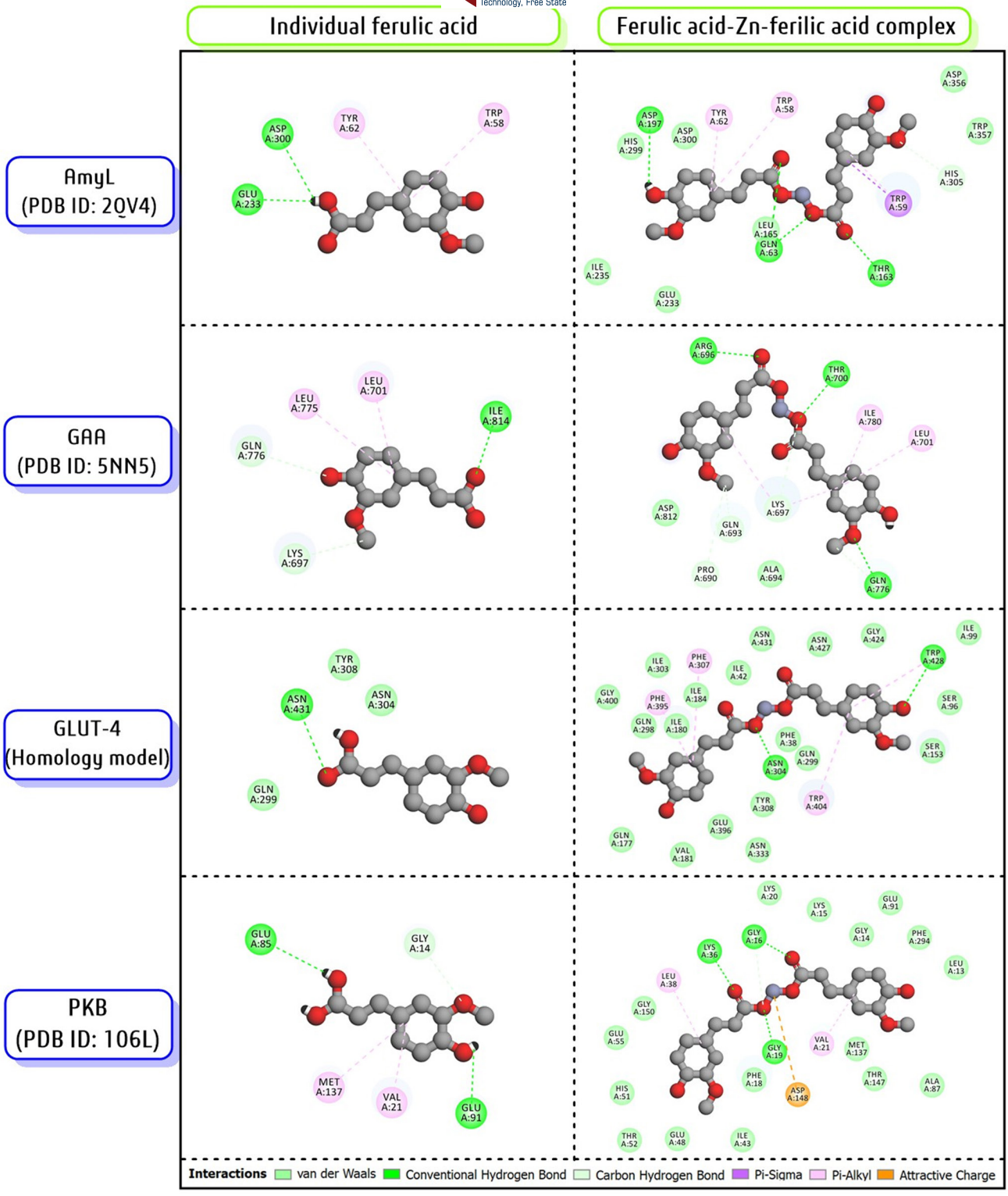


FIGURE 8 Molecular docking interaction of ferulic acid and its complex with four enzyme targets (α-glucosidase, α-amylase, GLUT-4 and PKB) linked to diabetes.

products, which is an important biomarker of severe diabetes and risk index of diabetic complications.[27] Thus, the potent anti-glycation effect of the complex suggests it may be further studied as a nutraceutical with possible mitigatory potential against glycation-mediated diabetic complications. Although the in vitro anti-glycation activity of ferulic acid has been previously documented,[28] it appears zinc(II) further improved its anti-glycation activity on complexation (Table 1). Zinc sulphate has been shown to inhibit protein glycation in vitro[29] and

TABLE 2 Molecular docking scores of ferulic acid and ferulic acid–zinc sulphate complex against four enzyme targets (α-glucosidase, α-amylase, GLUT-4 and PKB) linked to diabetes

Target enzymes	Docking scores (kcal/mol)	
	Ferulic acid	Ferulic acid-zinc sulphate complex
AmyL (PDB ID: 2QV4)	−6.15	−7.74
GAA (PDB ID: 5NN5)	−5.75	−6.98
GLUT-4 (Newly modelled)	−6.16	−8.83
PKB (PDB ID: 106L)	−6.43	−8.25

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

reduce protein glycation and protein carbonyl formation in rats with diabetes.[30] Thus, Zn(II) could a promising adjuvant in improving the anti-glycation property of ferulic acid through complexation.

In both L-6 myotubes and isolated rat psoas muscle tissue the complex showed promising glucose uptake modulatory effect and was more potent than its precursors (Figure 7a,b; Table 1). The uptake of circulating glucose in to cells of peripheral tissue is a known mechanism for ensuring physiological glucose homeostasis, which is stimulated by insulin.[6,31] Thus, the modulatory effect of the complex on cellular and tissue glucose uptake suggests it may potentiate glycaemic control in vivo.

Furthermore, glucose uptake activity in the complex-treated muscle tissues was accompanied by higher tissue zinc concentration relative to the ferulic acid and zinc sulphate–treated tissues (Figure 7d). This suggests complexation increased muscle tissue zinc uptake, which possibly influenced tissue glucose uptake. Documented evidence has shown that, through complexation, non-bioactive 1-oxy-2-pyridinethiol ligands potentiated insulin mimetic effects in adipocytes by enhancing cellular zinc uptake.[32,33] In the treated muscle tissues, hexokinase activity followed the same trend as glucose uptake activity (Figure 7c). Hexokinase activity in the complex treated muscle tissue was higher than that of ferulic acid and zinc sulphate–treated muscle tissues (Figure 7c), suggesting that the glucose uptake activity of the complex may be linked to cellular glucose utilization. This is evident by the higher phopho-Akt/pan-Akt ratio in the complex-treated muscle tissue relative to the ferulic acid and zinc sulphate–treated muscle tissues (Figure 7e). Phosphorylative activation of Akt is known to be involved in the downstream signaling of glucose uptake in peripheral tissues, such as adipose and muscle tissues.[6,34] This suggest that the modulatory effect of the complex on glucose uptake and utilization may be linked to possible modulatory action on Akt activation.

From a synergistic point of view, the potent glucose uptake effect of the complex may be attributed to complexation-mediated synergism between its zinc(II) and ferulic acid moieties. Documented evidence has reported the insulin signaling modulatory effects of zinc sulphate, including modulatory effects on cellular glucose uptake,[10] while ferulic acid has been shown to enhance glucose uptake in isolated rat muscle tissues.[35] The present study, also, showed that zinc sulphate and ferulic acid had notable glucose uptake effects in L-6 myotubes and isolated rat muscle tissues (Figure 7a,b; Table 1). Thus, both the zinc(II) and the two ferulic acid moieties in the complex (Figure 2a) may work synergistically to potentiate improved cellular and tissue glucose uptake and utilization. Further docking studies (Figure 8 and Table 2) showed insulin signaling proteins, GLUT-4 and PKB/Akt, may be implicated in the potent glucose uptake activity of the complex. Docking scores of the complex against GLUT-4 and PKB/Akt target proteins were higher than those of ferulic acid (Table 2), suggesting a stronger binding affinity with the protein targets. This could be attributed to the two ferulic acid moieties and Zn(II) components complex (Figure 2a). Technically, the presence of two ferulic acid moieties and Zn(II) potentiated solid-binding affinity. The molecular interaction showed that the Zn-metal ion produced a minimum of two and maximum of four hydrogen bonds with van der Waals, P-alkyl and P-sigma covalent bonds (Figure 8). Moreover, isolated rat muscle tissues showed increased phospho-Akt/pan-Akt ratio upon treatment with the complex, suggesting modulated Akt activation.

Toxicity evaluation showed that the complex was not myotoxic or hepatotoxic, at least at in vitro cellular level (Figure 7f). The data suggest that Zn(II) complexation may be promising therapeutic approach for improving the glycaemic control potential of ferulic acid with minimal toxicity concerns.

5 | CONCLUSION

Natural medicine, including supplements and plant derived the phenolic have become popular in functional medicine, perhaps due to their holistic functional and medicinal properties and minimal safety concerns. In this study, natural moieties, zinc mineral and ferulic acid, demonstrated promising anti-hyperglycemic and anti-oxidative synergism on complexation. Zn(II) complexation improved the bioactivities of ferulic acid and conferred a broader scope of therapeutic potentials, which may be attributed to the molecular characteristics of the Zn(II)-biferulate.$2H_20$ complex. Zn(II) complexation may be promising therapeutic approach for

improving the glycaemic control and anti-oxidative potential of natural phenolic acids. Thus, developing potent nutraceuticals with improved and broader anti-hyperglycemic and anti-oxidative pharmacological profile, as well as minimal toxicity concerns. Further in vivo anti-diabetic and anti-oxidative studies in appropriate animal models are recommended for additional evidenced-based data.

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CONFLICT OF INTEREST

No conflict of interest in this study was declared.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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RESEARCH ARTICLE

Novel Caffeic Acid - Zinc Acetate Complex: Studies on Promising Anti-diabetic and Antioxidative Synergism Through Complexation

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Abstract: ***Background*:** The role of Zn(II) in storage, insulin secretion and function has been documented, while plant phenolics have antioxidant and other pharmacological credence.

***Objective*:** The study aimed at synthesizing a novel medicinal Zn(II) complex. The medicinal properties of zinc(II) and caffeic acid were considered in synthesizing a novel complex with promising and improved antioxidant and anti-hyperglycaemic attributes.

***Methods*:** Complex synthesis was done using a 1:2 molar ratio of zinc acetate and caffeic acid and structurally characterized using NMR, FT-IR, high resolution-mass spectroscopy and HPLC. Its cellular toxicity was assessed in Chang liver cells and L-myotubes. *In vitro*, cellular, and isolated tissue models were used to evaluate the antioxidant and anti-hyperglycaemic properties of the complex relative to its precursors. Molecular docking was used to investigate the interaction with insulin signalling target proteins: GLUT-4 and protein kinase B (Akt/PKB).

***Results*:** Zinc(II) and caffeic acid interacted *via* Zn:O_4 coordination, with the complex having one moiety of Zn(II) and 2 moieties of caffeic acid. The complex showed *in vitro* radical scavenging, α-glucosidase and α-amylase inhibitory activity up to 2.6 folds stronger than caffeic acid. The ability to inhibit lipid peroxidation (IC_{50} = 26.4 µM) and GSH depletion (IC_{50} = 16.8 µM) in hepatocytes was comparable to that of ascorbic acid (IC_{50} = 24.5 and 29.2 µM) and about 2 folds stronger than caffeic acid. Complexation improved glucose uptake activity of caffeic acid in L-6 myotubes (EC_{50} = 23.4 versus 169 µM) and isolated rat muscle tissues (EC_{50} = 339 versus 603 µM). Molecular docking showed better interaction with insulin signalling target proteins (GLUT-4 and Akt/PKB) than caffeic acid. The complex was not hepatotoxic or myotoxic.

***Conclusion*:** Data suggest a synergistic antioxidant and anti-hyperglycaemic potential between zinc and caffeic acid, which could be attributed to the Zn:O_4 coordination. Thus, it may be of medicinal relevance.

Keywords: Zinc(II), caffeic acid, complexation, structure-function relationship, diabetes, oxidative stress.

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1. INTRODUCTION

The prevalence of diabetes and oxidative impairments is still increasing globally, with oxidative stress being a major culprit of diabetic complications. Recent data from the International Diabetes Federation (IDF) revealed that the global diabetes population has increased by 74 million since 2019, with type 2 diabetes being the most dominant diabetes [1]. The high prevalence of type 2 diabetes is largely attributed to its strong association with poor dieting, sedentary lifestyle, and obesity or weight gain [2, 3]. These factors increase the risk of insulin resistance and poor glucose and lipid metabolism, which could lead to impaired glucose tolerance, impaired circulating glucose uptake and utilization, hyperinsulinnaemia, progressive decline in pancreatic β-cell function, and persistent hyperglyceamia [2-4].

Prolonged elevated blood glucose increases the risk of developing diabetic vascular complications, and oxidative stress has been implicated in the development of diabetic

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vascular complications [5]. Glycation and advanced glycation end product production are notably increased when high blood glucose persists [5]. This leads to increased production of reactive oxygen species and reactive radicals, causing oxidative damage to biomolecules and increasing the risk of diabetic vascular complications [5].

Functional foods and nutraceuticals are increasingly being used to support metabolic and oxidative health because they are perceived to be affordable, safe, and possess a holistic medicinal profile. Moreover, commercial drugs are not readily affordable among the low-income population. However, minerals and vitamins in diabetes management have been documented [6]. For instance, zinc plays a major role in metabolic health and insulin secretion and has been shown to modulate insulin action [7,8]. In fact, zinc has been complexed with different types of ligands to develop zinc-ligand complexes with modulatory potential on glycaemic control and lipid homeostasis [8].

Zinc(II) complex of threonine was shown to inhibit lipolysis in adipocytes from rats and modulate glycaemic control in T2D KK-A^{y} mice following 14 days of intraperitoneal administration at a dose of 3 mg Zn/kg body weight [9]. It also mimicked the effect of insulin by modulating the activity of phosphoinositide 3-kinase, phosphodiesterase, insulin receptor tyrosine kinase, and glucose transporter type 4 (GLUT-4) in adipocytes [10]. Zinc(II)-maltol and zinc(II)-allixin complexes inhibited lipolysis and increased uptake of glucose in adipocytes from rats [11]. In T2D KK-Ay, 14 days intraperitoneal injection (4.5 mg Zn/kg) of the complexes reduced blood glucose, HbA1, and hyperinsulinemia and improved glucose tolerance [11]. Naito *et al.* [12] reported that zinc(II) complex of hinokitiol increased glucose-induced Akt phosphorylation, indicating insulin mimetic and glycaemic control potential.

Although zinc-ligand complexes show promising prospects in type 2 diabetes management, a recent review revealed that most of these complexes had been obtained from synthetic and non-bioactive ligands that are potentially toxic, while natural bioactive phenolics remain underexplored as ligands [8]. In fact, natural antioxidant phenolic acids, including caffeic acid, have not been studied as ligands for antidiabetic zinc complexes. Caffeic acid is a natural antioxidant found in coffee and teas [13]. The potent *in vitro* radical scavenging and anti-lipid peroxidative activity of caffeic acid has been reported [14]. After caffeic acid treatment, C57BL/KsJ-db/db mice showed significantly lower blood glucose and HbA1c levels, hepatic gluconeogenic enzyme activities, as well as noticeably higher tissue glycogen content, hepatic GLUT-2 and adipocyte GLUT-4 expression, and hepatic glucokinase activity [15]. Concomitantly, C57BL/KsJ-db/db mice treated with caffeic acid showed improved activity and expression of antioxidant enzymes and decreased lipid peroxidation [15].

Considering the antidiabetic and antioxidant properties of caffeic acid, it is safe to say that it is a promising ligand for developing a bioactive zinc(II) complex. However, the study has not been undertaken. Therefore, this study was done to investigate the anti-hyperglycaemic and antioxidative qualities of a novel zinc(II) complex of caffeic acid.

2. MATERIALS AND METHODS

2.1. Complexation of Zinc(II) with Caffeic Acid

Zinc(II) was complexed with caffeic acid in a 1:2 molar ratio. A previous article was consulted [16]. First, 219.51 mg of $Zn(CH_3COO)_2{\cdot}2H_2O$ (molecular mass = 219.51 g/mol) and 360.32 mg of caffeic acid (molecular mass = 180.16 g/mol) were dissolved individually in glass vials containing 5 mL of methanol. Both solutions were then gradually mixed in another glass vial while stirring. Stirring continued until complete precipitation of a dark yellow precipitate. Precipitate was recovered by filtration, washed twice with 50% methanol, and lyophilized in an Alpha 1-2 LDplus Freeze Dryer, Martin Christ, Osterode am Harz, Germany).

2.2. Complex Characterization

The complex was structurally characterized using Fourier-Transform Infrared Spectroscopy (FT-IR), proton nuclear magnetic resonance spectroscopy (^{1}H NMR), High-Resolution Mass Spectroscopy (HR-MS), and High-Performance Lipid Chromatography (HPLC).

For FT-IR, a Perkin Elmer Spectrum 100 FT-IR Spectrometer (MA, USA) was used. The instrument had an ART accessory. For analysis, roughly 1 mg of either the complex or caffeic acid was put on the crystal sample holder. Then, scanning was done at a wavelength range of 4000 – 380 cm^{-1}, using a 40 s^{-1} scan rate. A V 6.3.4 Spectrum Software was used to record the spectra.

For ^{1}H NMR, a Bruker Avance spectrometer (400 MHz; Bruker Corporation, MA, USA) was used, which recorded the ^{1}H NMR data. DMSO-d6 (δH = 2.50) was used as the solvent with a tetramethylsilane 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.

The HR-MS analysis was done by the Central Analytical Facility, Stellenbosch University, Cape Town. The instruments used were Waters Synapt G2 (Waters Corporation, MA, USA), ESI probe, ESI Pos, Cone Voltage 15 V.

HPLC was further used to verify the purity of the complex. The HP-LC was done using a HPLC-DAD (Agilent 1100 series, Agilent, Waldbronn, Germany) equipment and a Phenomenex Luna C_{18} column (Separations, Johannesburg) having a dimension of 150 × 4.6 mm and particle size of 5 µm. The mobile phase was HPLC grade H_2O with 0.1% methanoic acid (solvent A) and acetonitrile (solvent B). The running condition and detection mode was done according to the previously reported method [17]. A 20 µL injection volume of complex (1mg/mL) was applied. A UV wavelength of 280 nm was used to capture the signal data of the chromatography.

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 media con-

taining 10% FBS was used to culture the cells. Cell culture was carried out in a NÜVE EC 160 CO_2 incubator (NÜVE, Ankara, Turkey) with oxygen, CO_2 supply, and temperature maintained at 95%, 5%, and 37 °C, respectively. At 80% confluence, 100 µL of cells were seeded (30000 cells/mL) in a 96 well sterile plate for 48 hours to allow the cells to attach to the plate. Thereafter, the medium in the wells was aspirated and replaced with a new medium containing 7.36, 73.6, and 736 µM of complex or 0.5% DMSO (vehicle control) and incubation continued for 36 hours. 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 hours. After incubation, the content in the wells was removed and the wells were washed with phosphate-buffered saline. One hundred microliters of MTT de-staining or solubilization solution (Sigma Aldrich, Johannesburg, South Africa) was quickly added into the wells, and absorbance was captured at 570 nm using a Multiskan Go plate reader (Thermo Fischer Scientific, Waltham, MA, USA). Viability (%) of sample-treated cells was calculated using the control as a reference. The calculation was done with triplicate data of two 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, was performed according to a previous study [18]. For DPPH and ABTS, the samples were tested at different concentrations (3.75 – 60 µM in final assay volume) and expressed as % scavenging activity. Ascorbic acid and Trolox were the positive controls in these assays. For the Fe^{3+} reducing antioxidant assay, the samples were tested at 40 µM. The Fe^{3+} reducing antioxidant activity was computed from a ascorbic acid standard curve (3.75 – 60 µM in total assay volume) and expressed as mmol/mol ascorbic acid equivalent (AAE). Trolox was used as the positive control.

2.4.2. Linoleic Acid Peroxidation Inhibition Assay

The effect of the complex on linoleic acid peroxidation was measured by consulting a previous method [19] and adding some modifications. Into vial containing 30 µL of varying concentrations (5 - 80 µM in assay volume) of samples or positive controls (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 sequentially added. Next, 20 µL of 2 mM $FeSO_4.7H_2O$ was aliquoted into the vial. The mixture was kept for 30 min in an incubator set at 37 ^OC. The normal control vial contained 20 µL of distilled water instead of $FeSO_4.7H_2O$, 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 the addition of 50 µL of 0.25% thiobarbituric acid (dissolved in 50 mM NaOH solution). A boiling water bath was used to heat the vials for 30 minutes before cooling at room temperature. The vials were then centrifuged at 3500 g for 10 min under ambient temperature. One hundred and fifty microlitres of the supernatants were pipetted 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:

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

Were, “A” means “Absorbance”

2.4.3. α-Glucosidase and α-Amylase Inhibition Assays

The *in vitro* inhibitory effect on α-glucosidase activity and α-amylase was done using methods reported in a recent study [18]. For the α-glucosidase inhibition assay, the experiment was done on samples and positive control at concentrations ranging from 3.75 to 60 µM. For α-amylase inhibition, the experiment was done on samples and positive control at concentrations ranging from 5 to 80 µM. Acarbose was used to positively control the experiments.

2.5. Measurement of Cellular Antioxidant and Antidiabetic Properties

2.5.1. Evaluating the Effect Complex on Lipid Peroxidation and Reduced Glutathione (GSH) Concentration in Chang Liver Cells Induced with Oxidative Stress

A recent article [17] with minor modifications was adopted to assess the complex's cellular antioxidant effect 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 cells were plated with the culture medium (EMEM media containing 10% FBS) in a 96 well plate at a concentration of 30000 cells/mL. Plates were incubated in a CO_2 incubator for 48 hours so that the cells can attach to the bottom of the plate wells. Thereafter, the spent medium was discarded and 200 µL 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 (5 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 hour. Thereafter the incubation medium was aspirated, and the cells were lysed with 250 µL of cold 50 mM sodium phosphate buffer (pH = 7.5), containing 0.5% v/v Triton X-100. Lysing was done under cold conditions by gently agitating the plate. The plate was then centrifuged, and the supernatant was used to estimate lipid peroxidation and reduced glutathione (GSH) concentration according to the methods described below [17].

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 a vial. Five hundred microlitres of 0.25% w/v thiobarbituric acid, 200 µL of 20% v/v acetic acid, and 200 µL of distilled water were added successively. A boiling water bath was used to heat the vials for 50 min before cooling at ambient temperature. 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 computed from a linear graph plotted using the malondialdehyde standard concentrations.

To estimate the GSH concentration, the protein was precipitated from the supernatant by mixing with an equal volume of 10% trichloroacetic acid and centrifuging for 5 min at 3250 g ((Hettich Mikro 200 microcentrifuge, Hettich Lab Technology, Tuttlingen, Germany). Fifty microlitres of the supernatant or GSH standards (0.002, 0.02, 0.2, 2 and 20 µM in assay volume) were plated. 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 allowed to stand for 5 min under ambient temperature and absorbance was measured at 412 nm. The GSH concentration was computed from a linear graph plotted using the GSH standard concentrations.

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

2.5.2. Evaluating the Effect on Glucose Uptake in L-6 Myotubes

To measure the effectiveness of the complex on glucose uptake, few methods [20-22] were consulted. L-6 myoblast cells from rat muscle (ATCC CRL-1458, ATCC, VA, USA) were used for the assay. The cells were cultured in 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 trysinization and seeded into a 96 well plate with fresh medium at a density of 4000 cells/well. At 80% confluence, DMEM containing 2% horse serum was used to differentiate the cells for 4 - 5 days by replacing the used medium in the wells. The myoblasts differentiated into myotubes during the 4 - 5 days incubation period. Thereafter, the differentiation medium was removed and 200 µL of the fresh medium that contained the samples at different concentrations (5, 50 and 100 µm in incubation volume) was pipetted into wells. The sample solvent was used as the vehicle control. Some wells were used as the blank control, in which only the medium without cells was pipetted. The culture medium was removed after 48 hours of incubation, and the cells were washed with PBS. Then, 100 µL of RPMI medium, which contained 8 mm glucose and 0.1% of BSA, was pipetted into the wells and the plate was incubated for another 2 hours. Insulin (1µM) was used as a positive control group. Aliquot from the incubation medium of each well was then used for glucose concentration measurement (Glucose-GO Assay Kit, Sigma Aldrich, South Africa). The MTT viability assay, as described above, was used to measure the viability of the myotubes following the treatment with samples under investigation. The experiment was done in two biological repeats and each biological repeat had three technical replicates. The blank was used to normalize glucose uptake of the test samples and control as shown below:

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

"ΔGC" denotes glucose concentration change (*i.e.*, the difference between initial and final glucose concentration in incubation solutions). The EC_{50} of samples, which stands for sample concentrations (M) needed to cause a 50% increase in glucose absorption, was also computed.

2.6. Measurement of *Ex Vivo* Antioxidant and Antidiabetic Properties

2.6.1. Animals

This study was conducted as reported previously [23] with slight modifications. First 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). Animal handling was done according to the rules and regulations of the ethics committee. The university animal facility supplied Sprague Dawley rats that were 8 weeks old. The animals fasted overnight. Thereafter, 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 Reduced Glutathione (GSH) Concentration in Isolated Rat Liver Tissue Induced with Oxidative Stress

A method recently reported [23] was adopted with minor changes to perform this experiment. In a 48 well plate, approximately equal portions (200 ± 5 mg) of the harvested liver tissues were incubated (NAPCO series 5400 CO_2 incubator, Thermo Scientific, South Africa) for 25 min in 900 µL of Kreb's buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 0.5 mM K_2HPO_4; pH 7.4 ± 2 at 25 ^{O}C) that contained different sample or standard (ascorbic acid) concentrations (10, 20, 40 and 80 µM in total incubation volume). Incubation conditions were 5% CO_2, 95% oxygen supply, and 37°C temperature. The controls (normal control and negative control) contained only the tissue, as well as the buffer and the solvent of the test sample in equivalent amounts. After preincubation, 100 µL of $FeSO_4.7H_20$ solution (5 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 incubation time elapsed, a pair of clean and sterile forceps were used to remove the tissues from the incubation solution and the tissue was cleaned in Kreb's buffer. A D1000 Handheld Homogenizer (Merck, South Africa) was used to homogenize the tissue in 1 mL of 50 mM sodium phosphate buffer (containing 0.5% v/v Triton X-100; pH, 7.5) under ice cold conditions. The tissue homogenate was centrifuged at 8900 g for 10 min to recover the supernatants. Lipid peroxidation and GHS concentrations were estimated in the supernatants using similar methods described above [17]. The inhibitory percentage of samples on lipid peroxidation and GHS depletion was also calculated, which was used to compute the IC_{50} values of samples.

2.6.3. Evaluating the Effect of the Complex on Glucose Uptake Isolated Rat Psoas Muscle Tissue

A recent article [23] was consulted and an experiment was performed with minor changes. In a 48 well plate, approximately equal portions (300 ± 10 mg) of the harvested muscle tissues were incubated for 25 min in 900 µL of

Kreb's buffer that contained different sample concentrations (10, 20, 40 and 80 µM in total incubation volume) or 50 mU insulin positive control (NovoRapid® FlexPen®, Novo Nordisk Limited, West Sussex, UK). The control contained only the tissue, as well as the buffer and the solvent of the test sample in equivalent amounts. One hundred microlitres of glucose solution (11.1 mM in total incubation volume) were 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 then used for glucose concentration measurement using the Glucose (GO) Assay Kit (Sigma Aldrich, South Africa). The absorbance value of the control was used as the reference value for computing the glucose uptake increase (%) of tests and positive control, as shown below:

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

Also, the EC_{50}, which denotes the sample concentrations (µM) needed to effect a 50% glucose uptake increase, was computed.

2.7. Molecular Docking Analysis with GLUT-4 and Protein Kinase B (Akt/PKB)

The *in silico* glycaemic control potential of caffeic acid and the synthesized Zn(II)-caffeic acid complex were studied by docking against two target enzymes, Glucose Transporter Type 4 (GLUT-4) and Protein Kinase B (PKB). Molecular docking study was done using AutoDock Vina 4.2 [16, 24]. Based on the minimum docking score (kcal/mol), the best docking scores of the proposed ligands were selected. Then, the BIOVIA-DSV software was employed for the visualization of the protein-ligand molecular interaction [24].

2.8. Data and Statistical Analysis

The 2016 version of MS Excel and GraphPad Prism 5 were used for data analysis. IC_{50} and EC_{50} values were computed as a linear and/or non-linear fit of transformed (log10) x-axis (sample concentration). The analysed data were reported in replicates using the average ± standard deviation format. Statistical analysis was done on Window's 27.0 version of IBM SPSS (IBM Corp, Armonk, NY, USA) software. Tukey multiple range post hoc tests and the one-way analysis of variance (ANOVA) were adopted for multiple comparisons of the data averages. A statistically significant difference (p) was set at $p < 0.05$ for comparing the mean values of the different groups.

The activity of the complex was compared to its precursors by fold ratio using the effective concentrations (IC_{50} and EC_{50} values). The following equation was used to calculate the fold ratio:

$$\textbf{\textit{Fold ratio}} = \left(\frac{IC_{50}\ of\ complex}{IC_{50}\ of\ precursor}\right)^{-1}\ or\ \left(\frac{EC_{50}\ of\ complex}{EC_{50}\ of\ precursor}\right)^{-1}$$

3. RESULTS AND DISCUSSION

3.1. Structural Characteristics of Complex

The signal of the broad peaks (3400 - 2565 cm^{-1}) in the FTIR spectrum of caffeic acid (Fig. **1a**) was notably lessened in the FTIR spectrum of the complex (Fig. **1b**). The peaks represent a stretch in the carboxylic O-H functional group of the caffeic acid moiety. The alteration of the signals suggests that zinc(II) - caffeic acid complexation occurred through the carboxylic end of caffeic acid, as shown in (Fig. **2a**). Even the signal of the sharp peaks between 1700 and 1600 cm^{-1} also lessened in the FT-IR spectrum of the complex (Fig. **1b**) relative to that of the caffeic acid spectrum (Fig. **1a**). The sharp peaks are the result of carboxylic C=O stretching. Thus, the alteration of the signal further suggests the involvement of caffeic acid's carboxylic end in caffeic acid - Zn(II) complexation (Fig. **2a**).

Proton NMR analysis (Fig. **2b**) also supports the proposed structure of the complex (Fig. **2a**). The ^{1}H-NMR spectrum of the complex showed an ABX system of the aromatic ring. The proton at position 2 was noticed as a doublet peak at δ 7.00, while the proton at position 5 was assigned as a doublet at δ 7.32. The proton at position 5 was observed as a doublet at δ 7.24. The doublet peak at δ 7.34 with J = 15.9 Hz was characteristic of the double bond β-proton, while the doublet peak at δ 6.35 with the same J-coupling = 15.9 Hz represents the α-proton of the double bond. The two methyl groups of the zinc acetate moiety were observed at δ 3.15. The ratio between the integral of the different protons of caffeic acid and acetate group (1:3, respectively) indicated that the caffeic acid - zinc acetate complex was synthesized successfully.

The High-Resolution Mass Spectroscopy (HR-MS) data showed the positive (Fig. **3a**) and negative (Fig. **3b**) modes of the complex's mass spectra, which also support the proposed structure of the complex (Fig. **2a**). The positive mode and negative mode showed fragments containing caffeic acid, zinc ion, and the acetate group, providing evidence that there was a successful formation of the caffeic zinc - acetate complex. The HR-MS of the complex in positive mode F(ig. **3a**) showed the basic peak with an intensity of 100% at *m/z* = 390.9976 with a calculated mass of 390.9983. This denotes a difference of 7 units, possibly due to the complex accepting sodium formate during the mass spectrometry process. The value *m/z* = 400.9893 with a calculated mass *m/z* of 400.9838 has a difference of 55 units, representing the caffeic zinc diacetate complex. The fragment of one molecule of caffeic acid in the zinc(II) complex was observed at a value of *m/z* = 308.9353, which differed by 27 units relative to the calculated mass of 308.9326. The fragmentations *m/z* = 181.0504 differed by 3 units from the calculated mass (*m/z* m/z = 180.0501), representing the loss of one caffeic acid moiety. The fragment with *m/z* = 203.0335 differed by 15 units relative to the calculated mass of 203.0320, suggesting the sodium adduct of caffeic acid moiety. The HR-MS of caffeic acid Zinc acetate in negative mode Fig. (**3b**) showed a value at *m/z* = 420.9866 and differed from the calculated

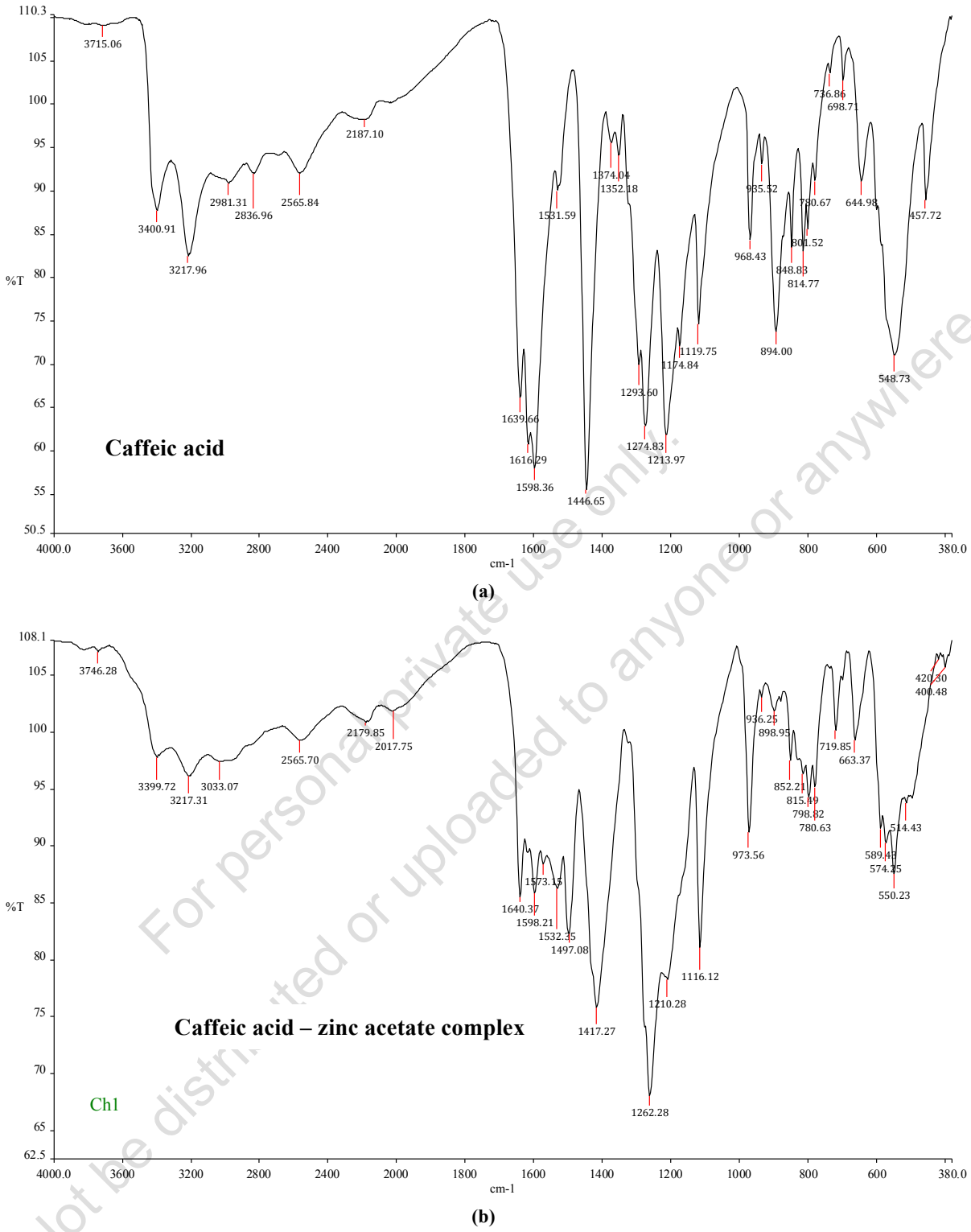
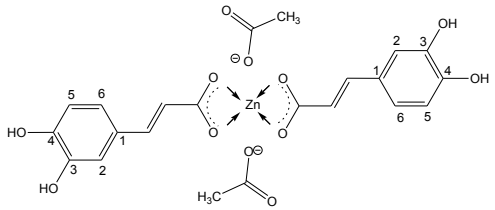
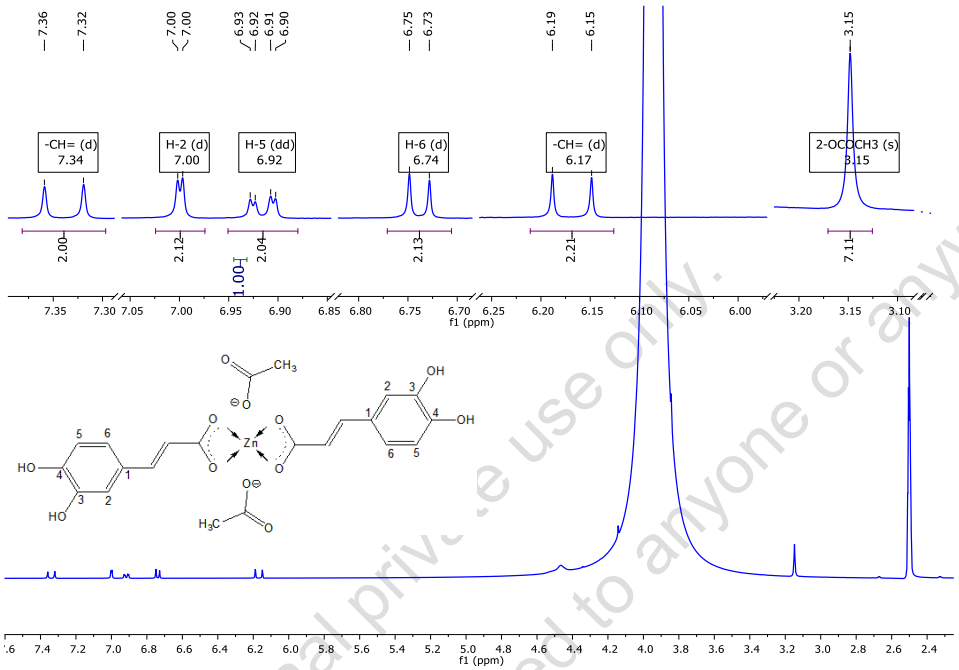


Fig. (1). FT-IR of spectra of **(a)** caffeic acid **(b)** caffeic acid - zinc acetate complex. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).



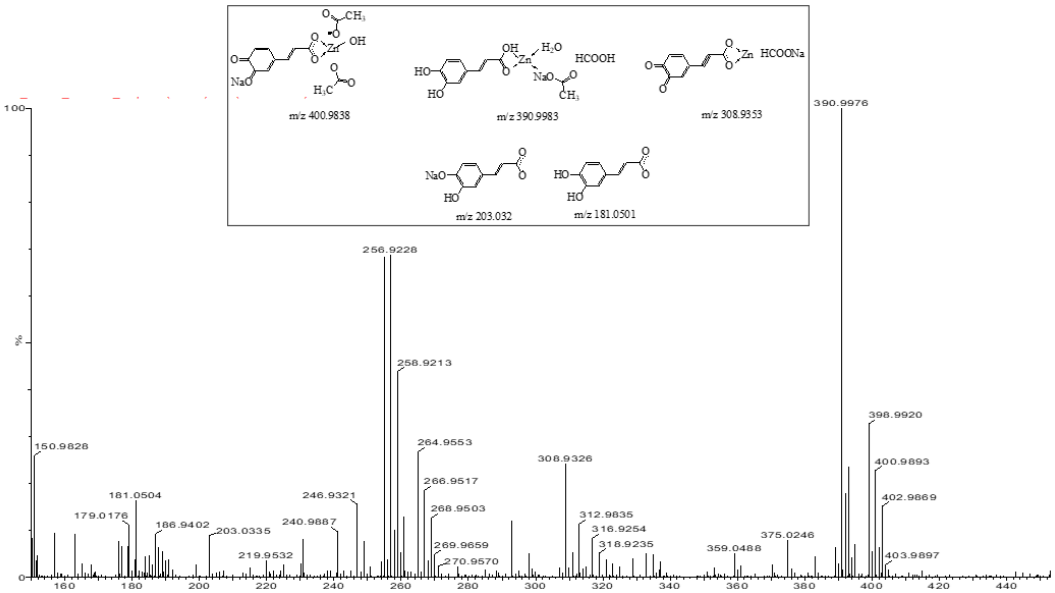
(a)



(b)

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.34 (d, J = 15.9 Hz, 1H, H-β), 7.00 (d, J = 2.1 Hz, 1H, H-2), 6.92 (dd, J = 8.2, 2.1 Hz, 1H, H-5), 6.74 (d, J = 8.1 Hz, 1H, H-5), 6.17 (d, J = 15.9 Hz, 1H, H-α), 3.15 (s, 3H, -OCO**CH$_3$**).

Fig. (2). (a) proposed structure and **(b)** ^{1}H NMR spectrum of caffeic acid - zinc acetate complex. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).



(a)

Fig. (3) Contd….

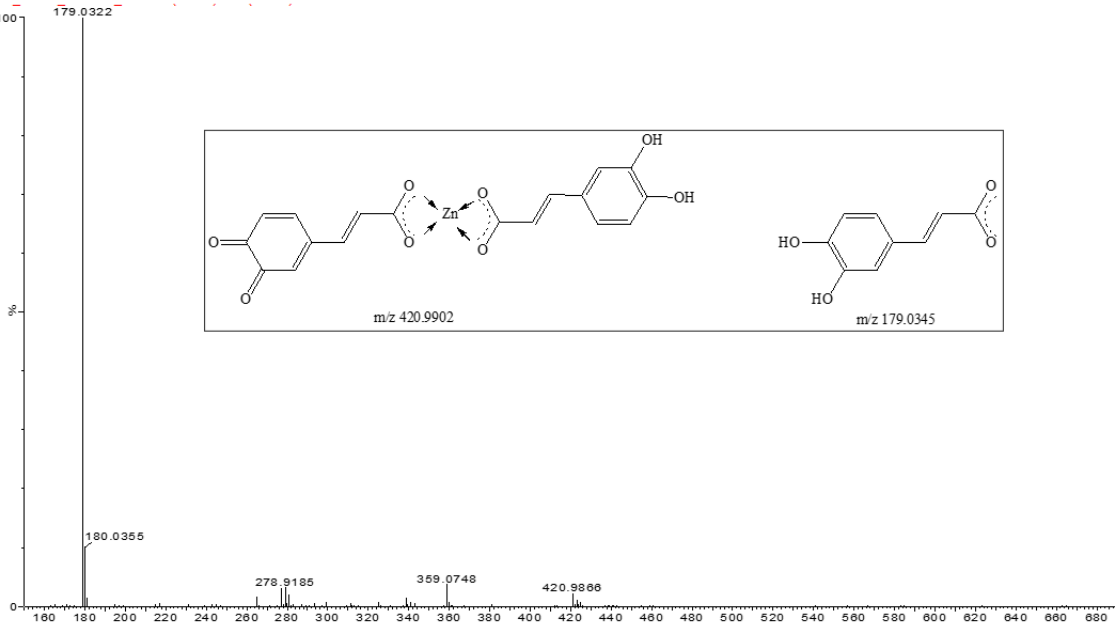


Fig. (3). (a) positive **(b)** negative modes for High – resolution mass spectroscopy (HR-MS) spectra of caffeic acid - zinc acetate complex.

mass (420.9902) by 36 units, which denotes caffeic zinc diacetate. The fragment of caffeic acid in negative mode is assigned *m/z* = 179.0345 and differed by 23 units relative to the calculated mass of *m/z* = 179.0322.

The HPLC chromatogram of the synthesized complex is presented in supplementary 1. The chromatogram showed a single predominant peak (% peak area = 90.5%) at a retention time of 10.839 min, suggesting the complex was formed as a single unit.

The above structural characterization of the complex indicates two caffeic acid moieties complexed with zinc acetate through a Zn:O_4 coordination model using the carboxylic functional group (Fig. **2a**).

3.2. Antioxidant Properties of Complex and Precursors

Both caffeic acid and its Zn(II) complex showed significantly ($p < 0.05$) more potent and/or statistically similar *in vitro* ABTS radical scavenging, Fe^{3+} reducing and anti-lipid peroxidative activity relative to ascorbic acid and Trolox (Fig. **4b** and **c** and Table **1**). However, the *in vitro* DPPH radical scavenging activity of caffeic acid (IC_{50} = 68.2 µM) and its zinc acetate complex (IC_{50} = 32.0 µM) was significantly less potent than that of ascorbic acid and Trolox (Table **1**). Zinc acetate did not show any appreciable *in vitro* antioxidant activity (Figs. **4a-d**). However, when complexed with caffeic acid, the resulting complex showed *in vitro* antioxidant activity that was up to 2.4 folds more potent ($p < 0.05$) than its precursor caffeic acid as computed using IC_{50} values (Table **1**).

In Chang liver cells and isolated rat liver tissue, zinc acetate remained significantly ($p < 0.05$) least potent with respect to inhibiting oxidative stress-mediated lipid peroxidation and GSH depletion (Figs. **5a** and **b** and Table **1**). With respect to the IC_{50} values in Table **1**, the cellular and tissue antioxidant activity of both caffeic acid and the complex was statistically not different ($p > 0.05$) compared to that of ascorbic acid. However, the activity of the complex was 1.3 to 2.6 folds more potent than its precursor caffeic acid.

3.3. Anti-Hyperglycaemic Activity of Complex and Precursors

In vitro, zinc acetate showed lower α-glucosidase inhibitory activity (IC_{50} = 90.4 µM) than caffeic acid (IC_{50} = 23.2 µM) and the complex (IC_{50} = 17.3 µM), without observable inhibition on α-amylase (Figs. **6a** and **b**; Table **1**). The α-glucosidase inhibitory activity of caffeic acid and the complex was statistically not different ($p > 0.05$) compared to that of acarbose, while acarbose significantly ($p < 0.05$) outperformed caffeic acid and the complex in inhibiting α-amylase (IC_{50} = 6.36 µM versus 53.2 µM and 44.7 µM, respectively). Although no significant difference ($p < 0.05$) was observed between the activity of caffeic acid and the complex, the α-amylase and α-glucosidase inhibitory activity of the complex was more potent than that of caffeic acid (Table **1**).

In both L6-myotubes and psoas muscle tissues isolated from rats, the complex was more effective in increasing glucose uptake/utilization compared to caffeic acid and zinc acetate (Figs. **7a** and **b**; Table **1**). Although, it was not as potent as insulin. Based on the EC_{50} values in Table **1**, the complex (EC_{50} = 6.36 µM) outperformed caffeic acid and zinc acetate by 7 and 4 folds, respectively, at the cellular experimental level.

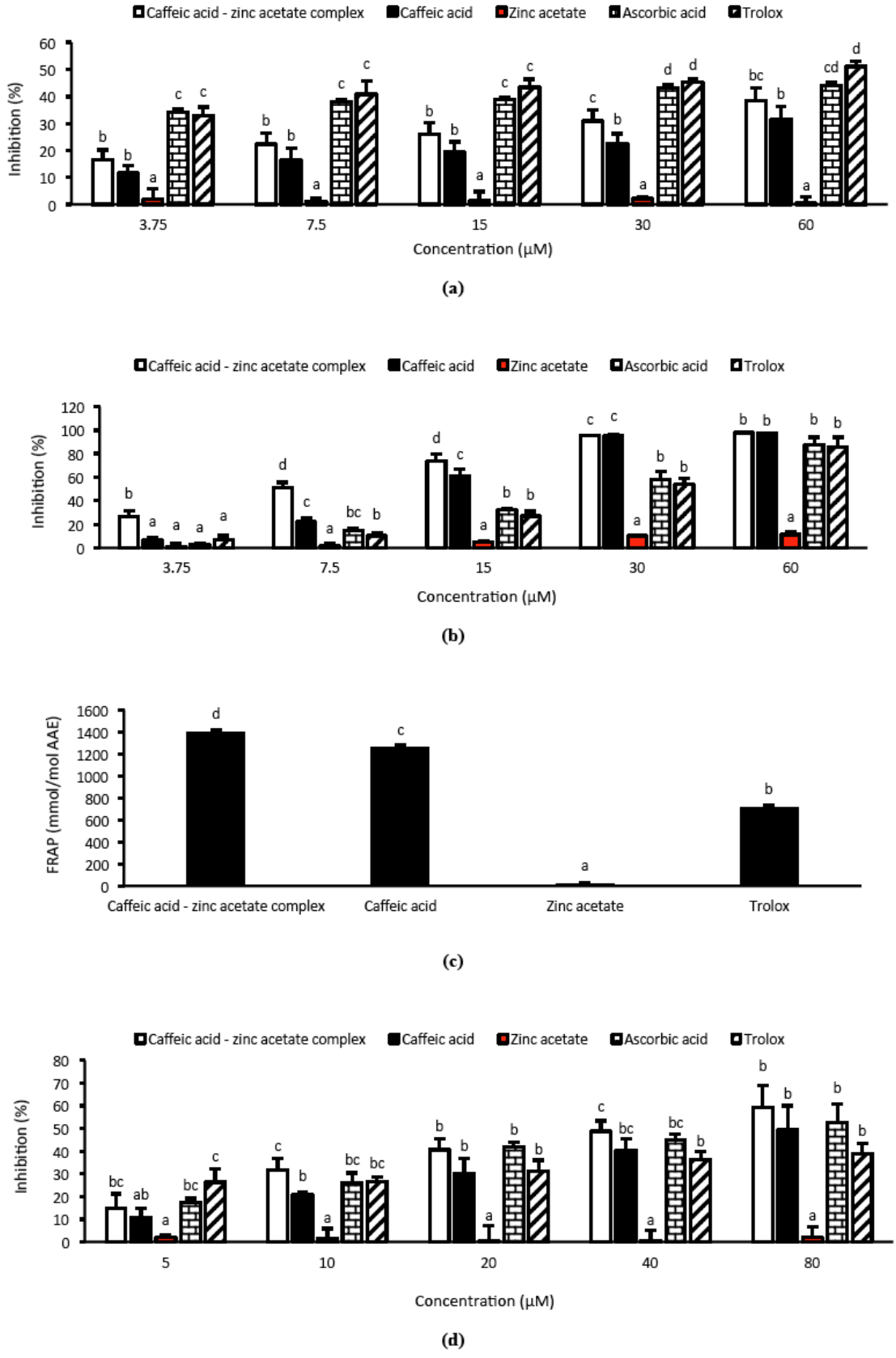


Fig. (4). (a) DPPH radical scavenging **(b)** $ABTS^{\bullet+}$ radical scavenging **(c)** Fe^{3+} reducing and **(d)** *in vitro* linoleic acid lipid peroxidation inhibitory activities of complex, precursors and standards. Data are shown as mean ± SD of triplicate analysis. Different letters 'a', 'b', 'c' and 'd' represent significant difference ($p<0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. IC_{50} and EC_{50} values of caffeic acid, its complex with Zn(II) and other studied agents.

Parameters or Activity	Caffeic Acid - Zinc Acetate Complex	Caffeic Acid	Zinc Acetate	Ascorbic Acid	Trolox	Acarbose	Insulin
	IC_{50} or EC_{50} Values (µM)						
ABTS radical scavenging activity (IC_{50})	5.07 ± 1.84^{c}	9.51 ± 1.25^{c}	ND	46.2 ± 10.0^{b}	92.9 ± 6.73^{a}	NA	NA
DPPH radical scavenging activity (IC_{50})	32.0 ± 6.45^{b}	68.2 ± 6.39^{a}	ND	9.98 ± 2.04^{c}	6.20 ± 3.18^{c}	NA	NA
Inhibition of *in vitro* linoleic acid peroxidation	8.80 ± 0.86^{b}	20.8 ± 5.86^{b}	ND	13.9 ± 4.38^{b}	37.8 ± 11.1^{a}	NA	NA
α-glucosidase inhibition (IC_{50})	17.3 ± 3.68^{b}	23.2 ± 2.15^{b}	90.4 ± 17.7^{a}	NA	NA	9.02 ± 3.18^{b}	NA
α-amylase inhibition (IC_{50})	44.7 ± 5.55^{a}	53.2 ± 10.4^{a}	ND	NA	NA	6.36 ± 1.08^{b}	NA
Inhibition of oxidative stress-induced lipid peroxidation in Chang liver cells (IC_{50})	26.4 ± 2.43^{b}	43.4 ± 11.2^{b}	393 ± 41.0^{a}	24.5 ± 6.21^{b}	NA	NA	NA
Inhibition of oxidative stress-induced GSH depletion in Chang liver cells (IC_{50})	16.8 ± 5.71^{b}	35.3 ± 10.6^{b}	744 ± 310^{a}	29.2 ± 5.51^{b}	NA	NA	NA
Inhibition of oxidative stress-induced lipid peroxidation in isolated rat liver (IC_{50})	172 ± 52.3^{b}	223 ± 20.9^{b}	3000 ± 910^{a}	100 ± 36.3^{b}	NA	NA	NA
Inhibition of oxidative stress-induced GSH depletion in isolated rat liver (IC_{50})	26.4 ± 3.05^{b}	69.2 ± 10.4^{b}	1120 ± 420^{a}	23.8 ± 3.41^{b}	NA	NA	NA
Glucose uptake increase in L-6 myotubes (EC_{50})	23.4	169	93.3	NA	NA	NA	ND
Glucose uptakes increase in isolated rat psoas muscle (EC_{50})	339	603	398	NA	NA	NA	ND

"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

Computational docking data of complex and caffeic acid against two insulin signalling target enzymes (GLUT-4 and PKB) are shown in Fig. (**8**) and Table **2**. Caffeic acid showed docking scores of -6.78 and -6.34 kcal/mol, while the complex showed docking scores of -9.91 and -9.72 kcal/mol against GLUT-4 and PKB, respectively. The docking scores were higher against GLUT4 than PKB for both caffeic acid and the complex. The data suggest that the complex had stronger *in silico* interaction with the target enzymes than caffeic acid.

At concentrations ranging from 7.36 to 736 µM and 5 to 100 µM, the complex did not notably compromise the viability of Chang liver cells and L6-myotubes, respectively Fig. (**7c**).

4. DISCUSSION

The detrimental role of oxidative injuries in the progression and complications of several diseases, including diabetes, cannot be over emphasized. Caffeic acid is a known natural antioxidant [13], ameliorating oxidative stress in diabetic conditions [15]. Zinc mineral (Zn^{2+}), on the other hand, plays an important role in insulin storage and secretion and has been shown to possess insulin mimetic potentials [8, 25]. In this study, we took advantage of these attributes to develop a novel zinc(II) complex of caffeic acid, which had structural properties that potentiated promising antidiabetic and antioxidant activities relative to its precursors.

Reactive oxygen species and free radical production have been shown to increase during diabetes, which are major culprits of diabetic oxidative tissue damage [5]. Processes like glycation [5], electron transport chain energy metabolism [26], and lipid peroxidation [27] are some of the key initiators and promoters of pro-oxidant and radical production during diabetes. The detrimental action of these radicals is strongly linked to their highly reactive nature and thus could oxidatively alter biological macromolecules and processes [28]. Mopping these biological radicals could limit or delay the progression of oxidative complications in diabetes.

Consistent with previous documentation [14], caffeic acid appreciably scavenged DPPH and ABTS radicals and inhibited linoleic acid peroxidation *in vitro* (Fig. **4** and Table **1**), which can be attributed to its stable phenoxy radical forming, electron donating and hydrogen atom transferring abilities [29, 30]. However, when complexed with zinc acetate, the resulting complex showed DPPH and ABTS radical scavenging and anti-lipid peroxidative activity that was, re-

spectively, 2.1, 1.9. and 2.4 folds more potent than caffeic acid based on computed IC_{50} values (Table **1**). The *in vitro* antioxidant effect of the complex was statistically comparable and/or more potent than ascorbic acid and Trolox, demonstrating its potential relevance in mitigating against lipid peroxidation-mediated radical production relative to caffeic acid. Considering that no notable *in vitro* antioxidant activity was shown by zinc, it may be hypothesized that the two moieties of caffeic acid in the complex Fig. (**2a**) influenced the observed double fold activity relative to its caffeic acid precursor. Additionally, the phenolic acid's meta- and para- O-H group, which is crucial to its antioxidant activities [30], was unaltered and did not involve complexation Figs. (**1** and **2**). Thus, preserving this antioxidant attribute in the complex.

In Chang liver cells and isolated rat liver tissue induced with oxidative stress, zinc acetate inhibited lipid peroxidation and GSH depletion to some degree (Fig. **5** and Table **1**). The cellular and tissue antioxidant effect of zinc acetate may be attributed to the involvement of molecular zinc(II) in the functioning of some antioxidant enzymes (as a co-factor), synthesis of antioxidant metallothioneins, suppression of NADPH-Oxidase activity, and stabilization of cell membrane against oxidative damage [31]. Nevertheless, zinc acetate's cellular and tissue antioxidant effect was not as potent as that of caffeic acid (Table **1**). The promising dose-dependent anti-lipid peroxidative property of caffeic acid has been previously demonstrated with 68.2 and 75.8% efficacy at 10 and 30 µg/mL, respectively [14]. In C57BL/KsJ-db/db mice, caffeic acid was shown to reduce hepatic lipid peroxidation by 49% and concomitantly increased hepatic GSH concentration significantly ($p < 0.001$), which was attributed to its modulatory effect on hepatic antioxidant enzymes [15]. However, complexation of caffeic acid with Zn(II) resulted in stronger (2.6 and 1.3 folds, respectively) anti-lipid peroxidative activity compared to caffeic acid in Chang liver cells and rat liver tissues (Table **1**). Similarly, it was shown that the complex's ability to inhibit oxidative stress-induced cellular and tissue GSH depletion was, respectively, 1.6 and 2.1 folds more potent than its caffeic acid precursor, which may be attributed to two caffeic acid moieties in the complex. The data suggest that Zn(II) may be a promising adjuvant for improving the potency of caffeic acid with regard to managing tissue oxidative stress and associated injuries. This may be largely influenced by the $Zn:O_4$ coordination structure afforded by complexation (Fig. **2a**).

Other than antioxidant effects, caffeic acid also showed potential for postprandial glycaemic control. It showed *in vitro* inhibitory effect on α-glucosidase and α-amylase (Fig. **6** and Table **1**), which is also consistent with previous documentations [32]. Inhibition of these enzymes is a known therapeutic approach for diabetes [33]. Thus, if mimicked *in vivo*, the *in vitro* inhibitory activity of caffeic acid (Table **1**) can translate into reduced glucose absorption and subsequently postprandial glycaemic control. On the other hand, zinc acetate showed moderate α-glucosidase inhibitory ac-

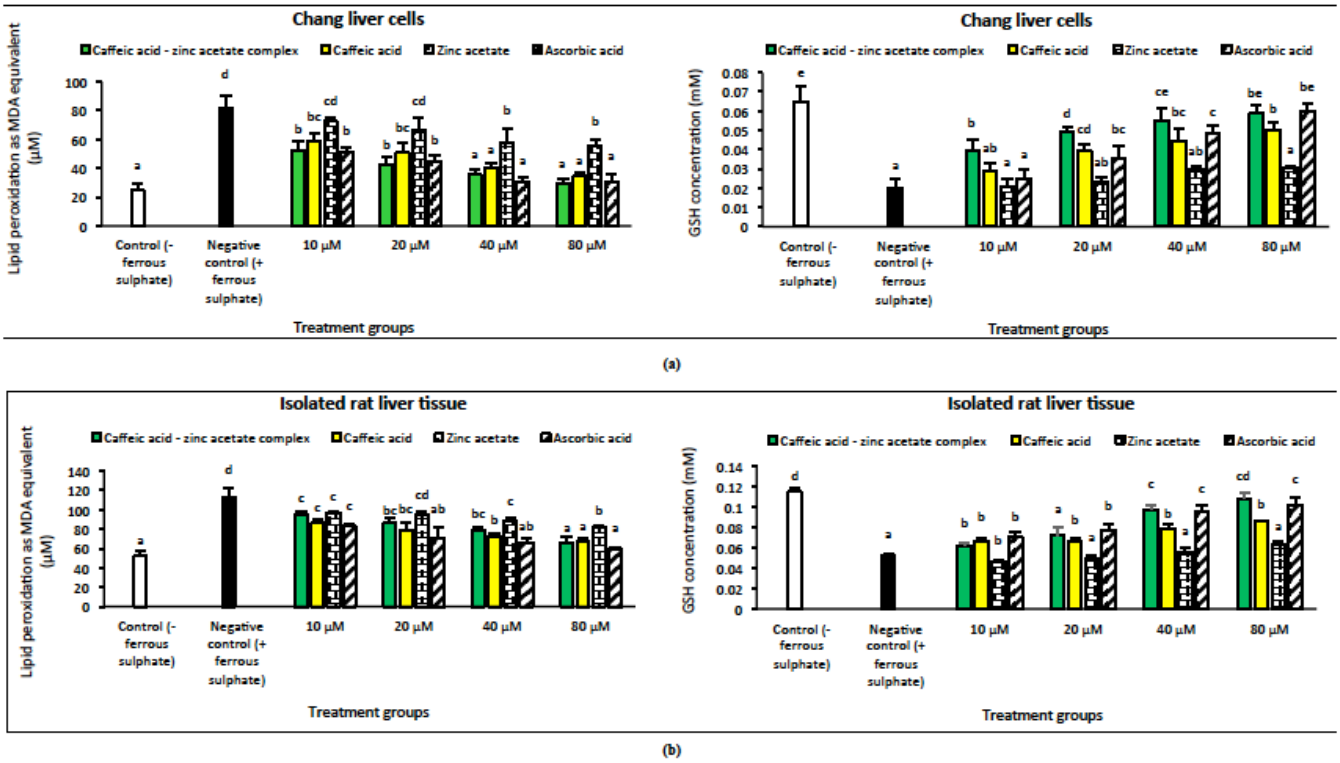
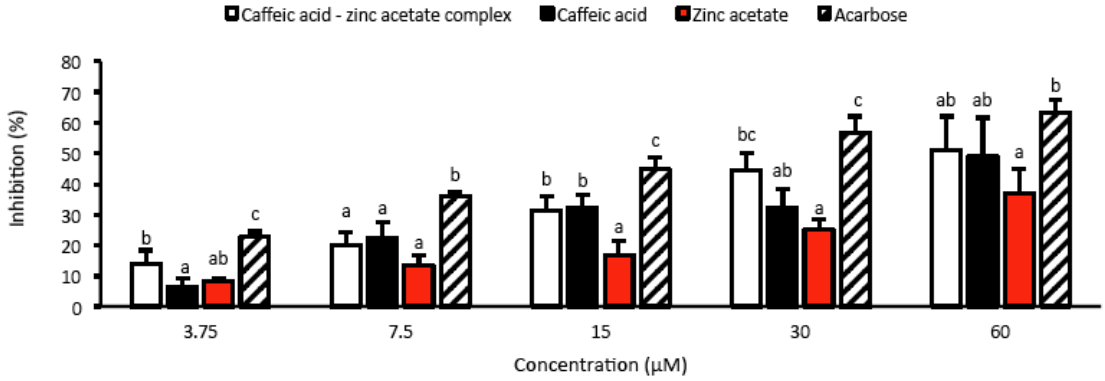
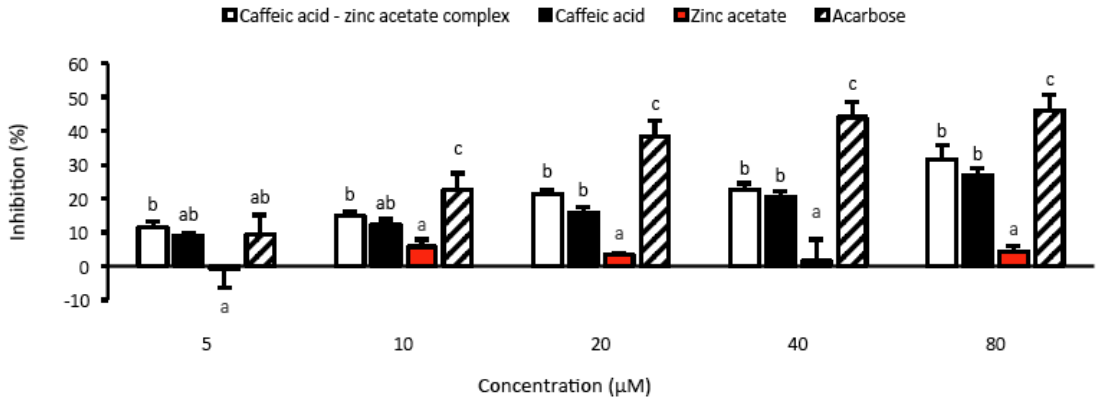


Fig. (5). Effect of complex, precursors and standards on lipid peroxidation and GSH concentration in **(a)** Chang liver cells and **(b)** isolated rat liver tissue. Data are shown as mean ± SD of replicate analysis. Different letters 'a', 'b', 'c', 'd' and 'e' represent significant difference ($p<0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).



(a)



(b)

Fig. (6). Effect of complex, precursors and standards on *in vitro* **(a)** α-glucosidase and **(b)** α-amylase activity. Data are shown as mean ± SD of triplicate analysis. Different letters 'a', 'b' and 'c' represent significant difference ($p<0.05$) between treatment groups. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

tion relative to caffeic acid (Fig. **6a** and Table **1**) without observable α-amylase inhibitory action (Fig. **6b**). However, upon complexation with caffeic acid, a more potent α-glucosidase (1.4 fold) and α-amylase inhibitory activity was observed (Table **1**), which suggests that zinc(II) complexation with caffeic acid may potentiate appreciable postprandial glucose control.

Furthermore, postprandial glycaemia is controlled by the clearance of circulating glucose to promote homeostasis. Insulin signals the uptake of circulating glucose into peripheral tissues when blood glucose is above normal [34]. Some antidiabetic drugs act as insulin sensitizers. They stimulate and modulate insulin signalling, thus facilitating insulin-induced lowering of blood glucose [35]. Evidence that zinc could promote insulin signalling or exert insulin mimetic effects has been documented and may be useful in diabetes management. In adipocytes, zinc exerted stimulatory effects on adipogenesis and glucose transport [36]. Studies in skeletal muscle cells from humans and mice suggest that Zn(II) enhances glucose oxidation and glycaemic control by modulating insulin signalling [25]. Clinical documentation reported that supplementation of zinc minerals could cause improvements in glycaemic and lipid profiles of diabetic subjects [37]. In the present study, zinc acetate increased glucose uptake in both L-6 myotubes and psoas muscle tissues isolated from rats (Figs. **7a** and **b**; Table **1**). Also, zinc(II) afforded the complex cellular and tissue glucose uptake activity (Figs. **7a** and **b**; Table **1**). In fact, computations using the EC_{50} (Table **1**) values showed that the cellular glucose uptake activity of the complex was, respectively, 4.0 and 7.3 folds more potent than that of zinc acetate and caffeic acid, while the muscle tissue glucose uptake of the complex was, respectively, 1.2 and 1.8 folds more potent than zinc acetate and caffeic acid. The fold increase may be attributed to a synergistic action of both zinc acetate and caffeic acid moieties in the complex (Fig. **2a**). Moreover, caffeic acid showed a modulatory effect on cellular (EC_{50} = 169 µM) and tissue (EC_{50} = 603 µM) glucose uptake (Table **1**), while studies have shown that it increases glucose transport in rat skeletal muscle independent of insulin by modulating the activity of 5'AMP-activated protein kinase [38]. Data suggest that caffeic acid - zinc acetate complex may be further studied as a potent nutraceutical with insulin modulatory and glycaemic control functions.

Further molecular docking data (Fig. **8** and Table **2**) showed that the complex had stronger interaction with insulin signalling protein targets (GLUT-4 and PKB) compared to caffeic acid. In the current era of drug discovery, bioin-

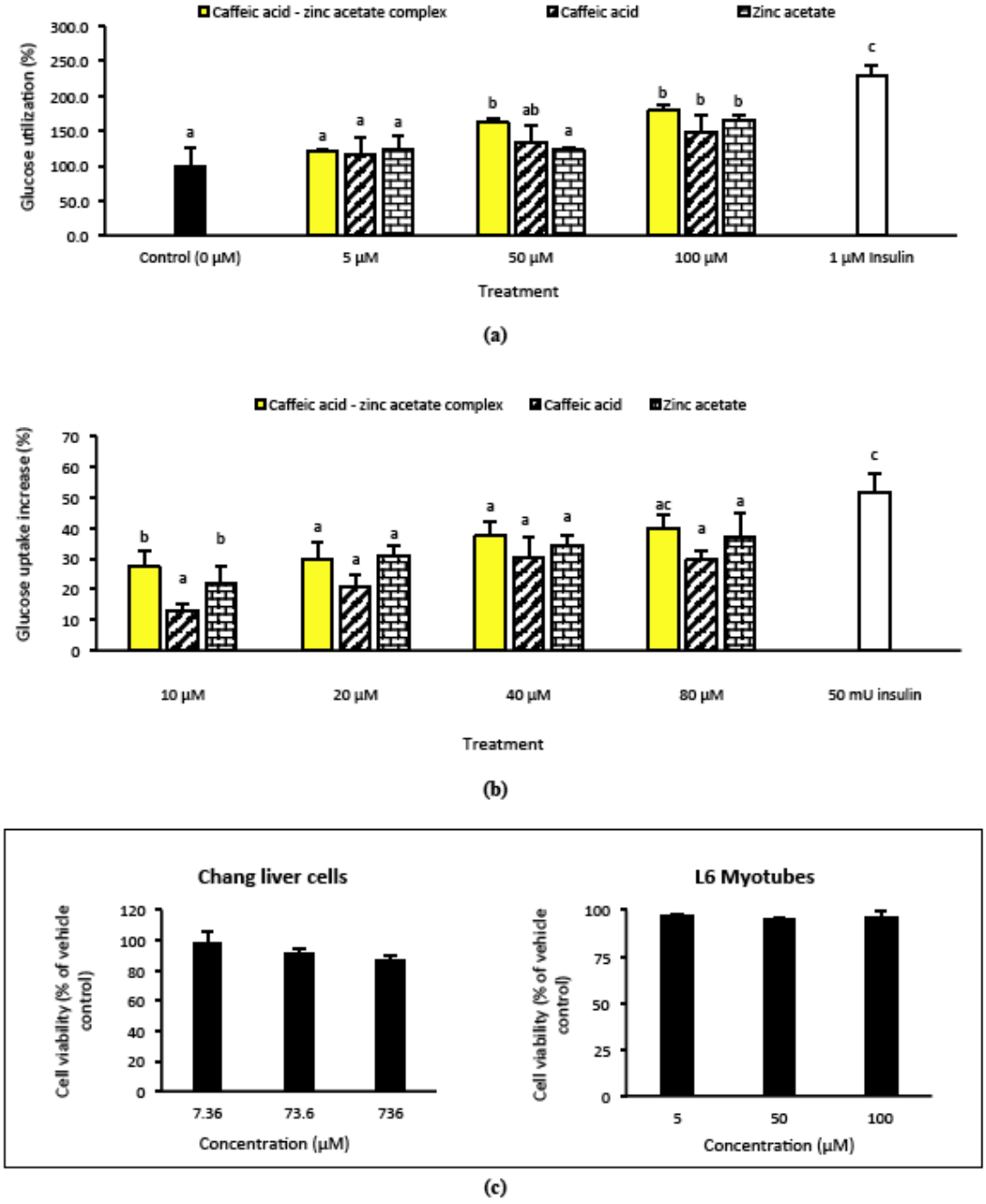


Fig. (7). Effect of complex, precursors and standards on glucose uptake in **(a)** L6-myotubes, **(b)** isolated rat psoas muscle and **(c)** viability of Chang liver cell and L6-myotubes. Data are shown as mean ± SD of replicate analysis. Different letters 'a', 'b' and 'c' represent significant difference ($p<0.05$) between treatment groups at the different tested concentrations or between treatment groups and controls. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

formatics tools, including molecular docking-simulation are widely used by academic and industrial researchers towards exploring or developing potential candidates targeting desired target enzymes. The presence of double mole molecular mass in the complex potentiated stronger binding affinity with the target enzyme (Fig. **8**). Additionally, due to the presence of hydroxy group (-OH), the two caffeic acid moieties in the complex formed double hydrogen bonds with the target enzymes. PHE38, GLN299, SER96, and TRP299 in GLUT-4, as well as LEU13, VAL21, and MET137 in PKB, are some common and conserved interactions found in docking against the target enzymes (Fig. **8**). GLUT-4 and PKB are important proteins involved in insulin signalling [39], which suggest possible insulin signalling modulatory action of the complex.

The present study showed some linear correlation in the structure-activity relationship. For example, the *in vitro* radical scavenging and anti-lipid peroxidative activity of the

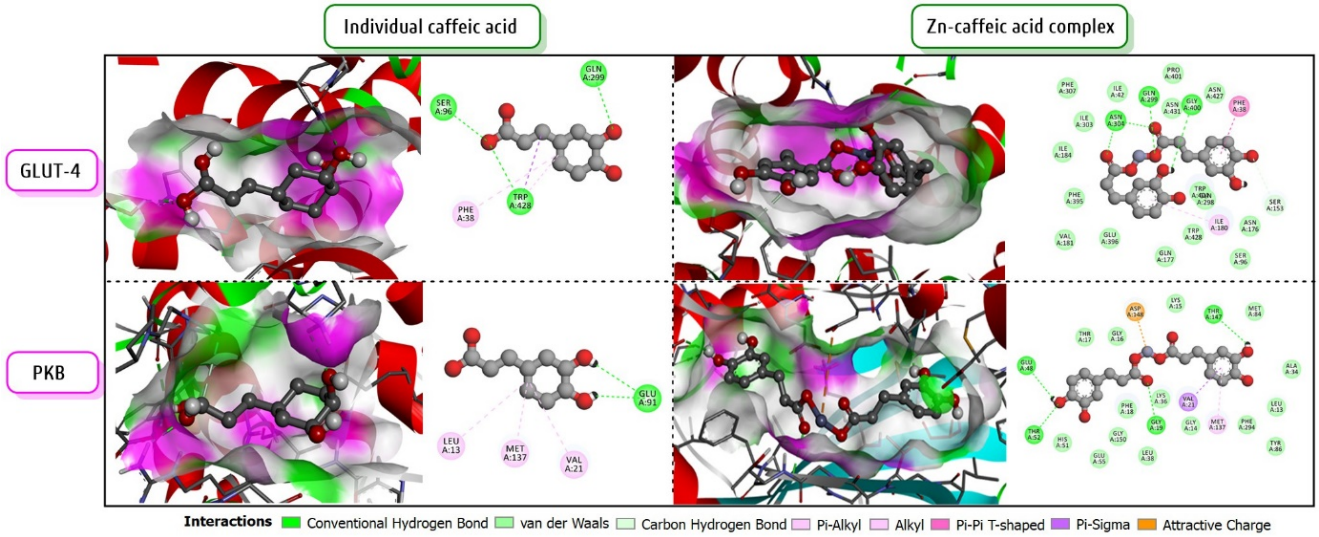


Fig. (8). Docking analysis of complex and caffeic acid against GLUT-4 and PKB/Akt. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 2. Molecular docking score (kcal/mol) of caffeic acid and the Zn(II)-caffeic acid complex against GLUT4 and PKB.

Target Enzymes	Caffeic Acid	Zn-Caffeic Acid Complex
GLUT-4	-6.78	-9.91
PKB	-6.34	-9.72

GLUT4, glucose transporter type 4; PKB, protein kinase B.

complex were approximately 2 folds (2.1, 1.9 and 2.4) more potent than its caffeic acid precursor. Thus, it was logical to attribute the double fold increase to the two moieties of caffeic acid in the complex since zinc acetate did not show *in vitro* antioxidant activity. Similarly, the inhibitory activity of the complex cellular and tissue GSH depletion was double fold more potent than its caffeic acid precursor. Since the activity of zinc acetate was far less than that of caffeic acid (Table **1**), we attributed the double fold activity of the complex to the combined action of its two caffeic acid moieties. In some other instances, the non-linear correlation was observed. For example, in L6-myotubes and rat muscle tissue, the complex's glucose uptake activity showed a fold increase that varied from 1.2 to 7.3 folds relative to that of its precursors (caffeic acid and zinc acetate). Both zinc acetate and caffeic acid also increased glucose uptake. Hence, the activity of the complex may be attributed to possible synergism between its zinc(II) and caffeic acid moieties.

CONCLUSION

The role of supplements and natural medicine remains pertinent in functional therapy, possibly due to their holistic functional effects and minimal toxicity concerns. This study showed that complexing zinc(II) with caffeic acid resulted in a non-cytotoxic complex with improved glycaemic control and antioxidant potentials relative to its precursors, which may be attributed to the $Zn:O_4$ structural property of the complex. The *in vitro*, cellular, *ex vivo* and *in silico* data suggest a synergistic antioxidant and glycaemic interactive potential between zinc(II) and caffeic acid, which can be further studied in an appropriate animal model to see if it will be mimicked. The study suggests that the complex may be a potential candidate for controlling diabetes and related oxidative assaults and could be medicinally relevant following more pharmacological validations.

LIST OF ABBREVIATIONS

IDF	=	International Diabetes Federation
FT-IR	=	Fourier-Transform Infrared Spectroscopy
^{1}H NMR	=	Proton nuclear magnetic resonance spectroscopy
HR-MS	=	High-Resolution Mass Spectroscopy
HPLC	=	High-Performance Lipid Chromatography

AUTHORS' CONTRIBUTIONS

Godfrey R. Matowane: Formal analysis, Investigation, Methodology, Software, Writing-original draft; Limpho M. Ramorobi: Formal analysis, Investigation, Methodology, Software, Writing-original draft; Samson S. Mashele: Funding acquisition, Investigation, Methodology, Software, Supervision, Writing-review & editing; Anwar E.M. Norel-jaleel: Formal analysis, Methodology, Resources, Software, Writing-review & editing; Susanna L. Bonnet: Formal analysis, Methodology, Resources, Software, Writing-review & editing; Shasank S. Swain: Methodology, Software, Writing-review & editing; Tshepiso J. Makhafola: Methodology,

Resources, Writing-review & editing; Chika I. Chukwuma: Conceptualization, Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Writing-review & editing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

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).

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. The reported experiments on animals were performed in accordance with the "South African National Standard for the Care and Use of Animals for Scientific Purposes (SANS 10386:2008)".

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

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

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CONFLICT OF INTEREST

The author(s) declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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Page: 1 of 1 (1 total submissions)

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Godfrey R.

Matowane

Dear Godfrey R. Matowane

Thank you for submitting your abstract entitled "***Zn(II)-syringic acid complex: A novel complex with improved bioactivity and promising antidiabetic and antioxidative potentials***" for consideration for presentation at the 54th SASBCP Annual Conference which will take place on the **22nd October 2021**. It is my great pleasure to inform you that your abstract has been accepted for **poster presentation** in the **basic pharmacology** category. Congratulations!

Please be advised that this year's conference will be a **fully virtual event** managed/administered by University of Cape Town (UCT)'s Conference Management Centre (CMC). For this reason, Roxanne Adams and her team at CMC require that your final presentation be sent to them by no later than Monday**, 18 October 2021**. Detailed instructions on how to record your presentation are outlined at the end of this letter.

Each poster presenter is required to do a graphical poster (1 page in pdf) and with audio recording up to 10 minutes. These will be uploaded to a cloud platform 2 days before the meeting for viewing by other delegates. Separate zoom links for themed live Q&A sessions will made available on the day of the conference 30 minutes before the start of the conference. Poster presenters are expected to be logged in at that time to participate in the discussions. Furthermore, we'd like to invite you to stay on to participate in the half-day event.

We are looking forward to your participation in the 54th SASBCP Annual Conference. Do not hesitate to contact us further if you have any questions.

Yours sincerely,

Phumla Sinxadi, on behalf of the 54th SASBCP Annual Conference Committee

Instructions for pre-recording presentations

CMC have requested that pre-recorded presentations done in PowerPoint are saved as an MP4 version.

- How to create and save your PowerPoint presentation with video and audio. https://www.youtube.com/watch?v=2m60HT3OMOI
- Save this to directly to the CMC drive, http://tiny.cc/62wjuz or via WeTransfer to roxanne.adams@uct.ac.za
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Central University of Technology, Free State

South African Society for Basic and Clinical Pharmacology

This certificate is awarded to

Godfrey R. Matowane

For attendance on 22 October 2021 at The 54th SASBCP Annual Conference

R van Wyk

Roxanne van Wyk, Conference Organiser

18 February 2022

DATE