Evaluation of the potential benefits of L-ergothioneine on selected complications associated with type-2 diabetes in a rat model

By

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Submitted: June 2021.

PREFACE

The research study reported in this thesis was done by **Ayobami Dare** (**218084517**) in the Discipline of Physiology, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Westville Campus, Durban, South Africa, from March 2019 to June 2021. The study was supervised by Dr Anand Nadar and Professor Mahendra L. Channa.

This research study has not been submitted in any form for any degree to any other university, where use has been made of other works, it has been duly acknowledged in the text, the results reported in this study are original.

28/06/2021

Ayobami Dare — Date

28/06/2021

Dr Anand Nadar (Supervisor) — Date

28/6/2021

Prof. M.L. Channa (co-supervisor). — Date

DECLARATION 1- PLAGIARISM

I, Ayobami Dare, declared that:

1 The research reported in this thesis, except where otherwise stated or acknowledged, is my original work.

2 This thesis has not been submitted for any degree or examination at any other institution.

3 This thesis does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged as being sourced from other persons.

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5 This thesis does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the thesis and the reference sections.

28/06/2021

Signature

DECLARATION 2- PUBLICATIONS

Publication 1

Ayobami Dare, Mahendra L. Channa, and Anand Nadar: L-ergothioneine and metformin alleviate liver injury in experimental type-2 diabetic rats via reduction of oxidative stress, inflammation, and hypertriglyceridemia. Accepted for publication in Canadian Journal of Physiology and Pharmacology.

- Presented at the School of Laboratory Medicine and Medical Sciences research symposium September 2020 (poster presentation).
- Presented at the Annual conference on Pharmacology and Applied Toxicology, 09 July 2021 (oral presentation).

Publication 2

Ayobami Dare, Mahendra L. Channa, and Anand Nadar: L-ergothioneine and its combination with metformin attenuate renal dysfunction in type-2 diabetic rat model by activating Nrf2 antioxidant pathway. Submitted to the journal "Biomedicine & Pharmacotherapy," currently under consideration for publication (revision submitted).

- Presented at the Physiological Society of Southern Africa (PSSN)/ African Association of Physiological Sciences (AAPS) conference, September 2021 (Oral presentation).

Publication 3

Ayobami Dare, Ahmed Elrashedy, Mahendra L. Channa, and Anand Nadar: Cardioprotective effects and in-silico antioxidant mechanism of L-ergothioneine in experimental type-2 diabetic rats. Accepted for publication in "Cardiovascular and Hematological Agents in Medicinal Chemistry" journal.

Regarding the manuscripts above, I carried out the design, experimental procedures, analysis, and interpretation of results. The co-authors contributed to the in-silico analysis, editing, proof reading, and supervision of the study.

[redacted] 28/06/2021

Signature.

DEDICATION

To GOD - the source of my strength and wisdom.

To my parents, Mr. and Mrs. **Samson. O. Dare**.

To my supportive wife, **Oluwakemi,** and my handsome son, **Ireoluwa**.

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I return all praises to God for his lovingkindness, grace, strength, and wisdom that He has bestowed upon me throughout this journey. Indeed, I have come to say THANK YOU.

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LIST OF ABBREVIATIONS

%	- percentage
µ	- micro
AGE	- Advanced Glycated End-product
AGUI	- Auto Dock Graphical Interface
ALP	- Alkaline phosphatase
ALT	- Alanine aminotransferase
AMBER	- Assisted Model Building with Energy Refinement
AMPK	- Adenosine Monophosphate activated Protein Kinase
ANOVA	- Analysis of Variance
AR	- Aldose reductase
ARE	- Antioxidant Response Elements
AST	- Aspartate aminotransferase
ATP	- Adenosine Triphosphate
BMI	- Body Mass Index
BUN	- Blood Urea Nitrogen
CAT	- Catalase
CrCl	- Creatinine clearance
CKD	- Chronic Kidney Disease
CK-MB	- Creatine Kinase-MB
CVD	- Cardiovascular disease
DM	- Diabetes Mellitus
DN	- Diabetic nephropathy
DNA	- Deoxyribonucleic Acid

ECM	- Extracellular matrix
EFSA	- European Food and Safety Authority
ELISA	– Enzyme-Link Immunosorbent Assay
ESRD	- End stage renal damage
FAS	– Fatty Acid Synthase
FBG	- Fasting blood glucose
FDA	- Food and Drug Administration
FFA	- Free fatty acid
g	- gram
GAFF	- General Amber Force Field
GAPDH	- Glyceraldehyde-3-Phosphate dehydrogenase
GFR	- Glomerular Filtration Rate
GSH	- reduced glutathione
H&E	- Hematoxylin and Eosin
HbA1c	- Glycated hemoglobin
HO1	- Heme-Oxygenase 1
HOMA-IR	- Homeostasis Model Assessment of Insulin Resistance
hs-CRP	- High-sensitivity C-reactive protein
IL-10	- Interleukin-10
IL-6	-Interleukin-6
IR	– Insulin resistance
JAK/STAT	- Janus Kinase and Signal Transducer and Activator
Keap1	- Kelch-like ECH-associated protein 1
KIM-1	- Kidney Injury Molecule-1

LDH	- Lactate dehydrogenase
L-egt	– L-ergothioneine
LPL	- Lipoprotein lipase
mA	- milliampere
MAP	- Mean Arterial Pressure
MCP1	- Monocyte Chemotactic Protein-1
MD	- Molecular dynamic
mg/dl	- Milligram/deciliter
MGL	- Molecular Graphics Laboratory
MI	- Myocardial infarction
mins	- minutes
ml	- milliliter
MM/GBSA	- Molecular mechanics with generalized Born and surface area
MM/PBSA	- Molecular mechanics Poisson–Boltzmann surface area
mmHg	- millimeter of mercury
mmol/L	- millimole/liter
MRBP	- Mouse and Rat tail cuff method Blood Pressure Systems
mRNA	- Messenger Ribonucleic acid
NADH	- Nicotinamide Adenine Dinucleotide (NAD) + hydrogen (H)
NAFLD	– Non-Alcoholic Fatty Liver Disease
NFBG	- Non-Fasting Blood Glucose
NF-kB	– Nuclear Factor kappa-B
ng	- nanogram
nmol	nanomole

NQO1	- NAD(P)H: Quinone Oxidoreductase-1
Nrf2	– Nuclear factor erythroid 2- Related Factor 2
ns	- nanosecond
OCTN1	- Organic Cation Transporter Novel type 1
OGTT	- Oral Glucose Tolerance Test
p38 MAPK	- p38 Mitogen-Activated Protein Kinases
PAS	- Periodic Acid Schiff
pg	- Picogram
PKC	- Protein Kinase – C
PMEMD	- Particle Mesh Ewald Molecular Dynamics
PPARα	- Peroxisome Proliferator-Activated Receptor-α
RAAS	- Rennin Angiotensin-Aldosterone System
rAGE	- receptor of Advance Glycated End-product
RMSD	- Root-Mean-Square Deviation
RNA	- Ribonucleic Acid
RNS	- Reactive nitrogen species.
ROS	- Reactive Oxygen species
Rt-qPCR	- Reverse Transcriptase quantitative Polymerase Chain Reaction
SASA	- Solvent Accessible Surface Area
sCr	- Serum Creatinine
SLC2A44	- Solute Carrier Family 22 member 4
SOD	- Super Oxide Dismutase
SREBP-1c	– Sterol Regulatory Element-Binding protein-1c
STZ	- Streptozotocin

T1DM	- Type-1 diabetes mellitus
T2D	- Type-2 diabetes
TBA	- Thiobarbituric Acid
TG	- Triglycerides
TGF-β1	– Transforming Growth Factor-β1
TLR	- Toll-Like Receptor
TNF-α	- Tumor Necrotic Factor-α
U/mg	- Unit/milligram
Ualb	- Urine albumin
UCR	- Urine creatinine
Upro	- Urine protein
v	- volt
α	- Alpha
β	- Beta
γ	- gamma
ΔG	- Binding free energy

THESIS OUTLINE

This thesis is written and submitted in manuscript form, comprising of five chapters and appendices.

Chapter 1: This provides information on the introduction to the study, review of relevant literature, problem statement, justification of the study, aim, objectives, and novelty of this study.

Chapter 2: This chapter contains the first manuscript produced from this study, reporting that L-ergothioneine and its combination with metformin alleviate liver injury in experimental type-2 diabetic rats via reduction of oxidative stress, inflammation, and hypertriglyceridemia. This manuscript was authored by Ayobami Dare, Mahendra L. Channa, Anand Nadar and has been accepted for publication in the journal "**Canadian Journal of Physiology and Pharmacology**" (Appendix 3).

Chapter 3: This chapter presents the second manuscript produced from this study, reporting that L-ergothioneine and its combination with metformin attenuate renal dysfunction in type-2 diabetic rat model by activating the Nrf2 antioxidant pathway. This manuscript was authored by Ayobami Dare, Mahendra L. Channa, Anand Nadar and is published in the journal "**Biomedicine and Pharmacotherapy**" (Appendix 4).

Chapter 4: This chapter presents the third manuscript produced from this study, reporting the Cardioprotective effects and in-silico antioxidant mechanism of L-ergothioneine in experimental type-2 diabetic rats. This manuscript was authored by Ayobami Dare, Ahmed Elrashedy, Mahendra L. Channa, Anand Nadar and has been accepted for publication in the journal "**Cardiovascular and Hematological Agents in Medicinal Chemistry"** (Appendix 5).

Chapter 5: This chapter presents the synthesis/discussion, conclusion, and limitations that provide opportunities for further studies.

Appendices: This comprises of the ethical approval letter, certificates of presentation, and publications.

ABSTRACT

Several pathogenic factors promote type-2 diabetic complications in patients, including cardiomyopathy, nephropathy, and non-alcoholic fatty liver disease (NAFLD). Specific nutraceuticals from food may act as a medicinal adjuvant in managing diabetic complications. L-ergothioneine (L-egt), a bioactive compound obtained from medicinal mushrooms, beans and some meat products, has been shown to reduce lipid accumulation, provide cytoprotection in tissue injury and enhances therapeutic efficacy when used as adjuvant. This study investigated the effect of L-ergothioneine with or without metformin on pathogenic metabolic pathways and biomarkers associated with selected diabetic complications in a type-2 diabetic rat model.

Ninety (90) adult male Sprague-Dawley (175±20)g rats were divided into three study groups [study 1 (36), study 2 (30) and study 3 (24)]. A 10% fructose solution was provided *ad libitum* to adult male Sprague-Dawley (175±20)g rats for 14 days followed by a single intraperitoneal injection of low dose streptozotocin (STZ 40mg/kg bwt, *i.p*) to induce type-2 diabetes after which the animals were randomly divided into six, five, and four groups (n=6) in studies 1 (liver), 2 (kidney), and 3 (heart), respectively. The control groups were administered 1ml/100g distilled water, while L-egt (35mg/kg bwt), metformin (500mg/kg bwt), and losartan (20mg/kg bwt) were administered in the other groups. At the end of each study, animals were euthanized via decapitation, blood samples were collected, while the heart, kidney, and liver tissue were excised and used for biochemical, RT-qPCR, ELISA, western blotting, and histopathological analysis. An in-silico study was done to evaluate the molecular antioxidant mechanism of L-egt.

Administration of L-egt, with or without metformin, to diabetic animals positively altered selected biomarkers of hepatic, renal, and cardiac dysfunction and prevented structural damage in these tissues. This treatment regimen mitigated oxidative stress, inflammation, and fibrosis by downregulating ($p<0.05$) SREBP1c, FAS, NF-kB, fibronectin, TGFβ1, and Keap1 expression and upregulating ($p<0.05$) Nrf2, Sirt1, NQO1, and HO1 expression compared with the diabetic control animals. Interestingly, co-administration of L-egt and metformin improved glucose homeostasis and reduced HOMA-IR. The in-silico study showed that L-egt binds to the active site of Nrf2 and may serve as a ligand to activate this potent antioxidant molecule.

The overall result from this study showed the potential benefits of L-ergothioneine in the management of selected complications associated with type-2 diabetes. This bioactive compound may be an effective adjuvant to attenuate hypertriglyceridemia, oxidative stress, and inflammation, thereby protecting vital organs associated with diabetic complications against injury and improving glycemic control when co-administered with metformin to delay the onset of diabetic complications.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND

Diabetes mellitus (DM) is a chronic metabolic disorder affecting more than 451 million adults worldwide, and its incidence is increasing steadily with the high prevalence of obesity, aging population, and changing lifestyles (1, 2). DM is a multifactorial disease characterized by increased blood glucose level (hyperglycemia), together with biochemical alterations in protein, fat, and carbohydrate metabolism resulting from absolute deficiency in insulin production (type-1) or insulin resistance (type-2) by the target tissue (3). About 90–95% of diabetic patients have type-2 diabetes (T2D), and this chronic disorder is often associated with major complications (e.g., nephropathy, cardiomyopathy, and hepatic injury) if not properly managed (4). These complications are characterized by functional and structural changes in the kidney, heart, and liver caused by hyperglycemia, hyperlipidemia, and insulin resistance (IR) that stimulates oxidative stress, inflammation, apoptosis, and mitochondrial dysfunction (5, 6).

Clinical trials reveal that there is no overall effective treatment for diabetic complications, and this has become a serious medical challenge (7, 8). Despite close-fitting glycemic control by conventional treatments, patients remain at high risk of these associated complications even during intensive therapy. Thus, the need to provide more robust strategies to alleviate diabetic complications has become an active area of research, with the primary goal of preventing their onset or delay their progression. Experimental and clinical trials have shown that combination therapies exert better treatment outcomes than monotherapy in managing diabetic complications (9, 10). However, conventional treatment with the combination of dietary and pharmacological interventions has suffered significant setbacks due to non-compliance to dietary protocols and adverse side-effects of pharmaceutical agents (11, 12). To this end, there has been a paradigm shift towards combination therapy involving the synergistic administration of bioactive compounds with existing pharmacological agents that target various underlying mechanisms implicated in these complications, as well as improve efficacy and reduce the side effect of existing pharmacological regimen (13, 14).

Recently, studies have shown that natural products ameliorate some metabolic dysfunction associated with diabetes due to bioactive compounds that target different pathways (15). Evidence has shown that L-egt, a bioactive compound obtained from mushrooms, beans, and certain meat products, provides effective cyto-protection, especially to vital organs exposed to injury via its antioxidant and anti-inflammatory activity (16). Numerous experimental studies have documented the therapeutic benefits of this nutraceutical in the

management of different ailments, including neurodegenerative diseases, pre-eclampsia, metabolic disorders, cardiovascular disease, and diabetic embryopathy (17-20). However, the effects of L-egt on major complications associated with diabetes have not been reported. Furthermore, in vitro and in vivo studies have reported that the supplementation of L-egt with available treatment regimen (such as hispidin and melatonin) prevent memory deficit and cellular injury (21, 22). Therefore, this study seeks to evaluate the potential benefits of L-egt, alone and combined with pharmacological intervention, on selected complications associated with type-2 diabetes.

1.2 DIABETES

Globally, diabetes accounts for more than a million deaths per year, thereby putting diabetes among the top 10 leading causes of mortality worldwide (23). Although giant strides over the past decades increase our understanding of this health menace, the incidence of diabetes is increasing in many developed and developing countries of the world, with a projection of 693million patients by 2045 if proper interventions are not employed to curtail this incidence (1). Generally, type-1 and type-2 diabetes are the most widely recognized endocrine disorders contributing to the number of diseases globally, exerting a heavy burden on the national health care system and reducing the quality of life of each patient (2, 24). Several environmental (such overnutrition and physical inactivity) and genetic factors (e.g., Hepatocyte nuclear factor-1A gene (HNF-1A)) account for the progressive decline in β-cell function and downregulation of insulin receptors with clinical presentation of hyperglycemia, which predisposes diabetic patients to various complications. However, the onset and progression of such complications differ in both types of diabetes. Therefore, the provision of effective therapies for each type of diabetes will require adequate evaluation of the various pathways/factors that causes β-cell dysfunction or reduced sensitivity of body tissues to insulin as well as the attenuation of several biochemical changes implicated in diabetes (25).

The pathophysiology of type-1 is well understood, and accounts for proper diagnosis and adequate management of type-1 before complication. On the other hand, the pathway to β-cell dysfunction is poorly understood in type-2 with significant contributions from numerous other factors that promote insulin resistance, which accounts for its poor diagnosis and management. In fact, most patients present with complications of type-2 diabetes before diagnosis (26, 27).

1.2.1 TYPE-2 DIABETES MELLITUS

T2D is a complex metabolic disease characterized by insulin resistance that causes alterations in carbohydrate, fat, and protein metabolism (28). T2D usually develops due to abnormal lifestyle patterns such as unhealthy diet, poor physical activity, and sedentary lifestyle that promote fat accumulation and

obesity. In some cases, this could often be hereditary, but disease progression has been mostly correlated with poor lifestyle ethics, including diet and poor physical activities (29, 30). Significant advances in diabetes research have increased our understanding of the etiology and progression of this disease. T2D is usually diagnosed using a blood glucose test (i.e., both fasting blood glucose (FBG) and 2-hours post-prandial glucose (2-h PG) value) or glycated hemoglobin-A1c test (A1c) (31). According to the World Health Organization, a fasting blood glucose (FBG) value ≥ 7.0mmol/L (126mg/dL) obtained after 8hours of fasting or a 2-h PG ≥ 11.1mmol/L (200mg/dL) obtained after 2 hours oral glucose tolerance test (OGTT) as well as glycated hemoglobin level ≥ 48 mmol/mol (6.5%) may be used to confirm diabetes. These tests provide a reasonable and convenient diagnosis of diabetes, as shown in Figure 1 (32, 33). T2D, in particular, has already attained epidemic proportions due to its multiple pathogenic factors, late diagnosis, and inadequate management, with 90–95% of people suffering from type 2 diabetes. Type 1 diabetes (T1D) is increasing in incidence (34). The driving force behind the escalating prevalence of T2D is the global pandemic of obesity (approximately 13% of the world's adult population), with about 39% among men and 40% among women (35).

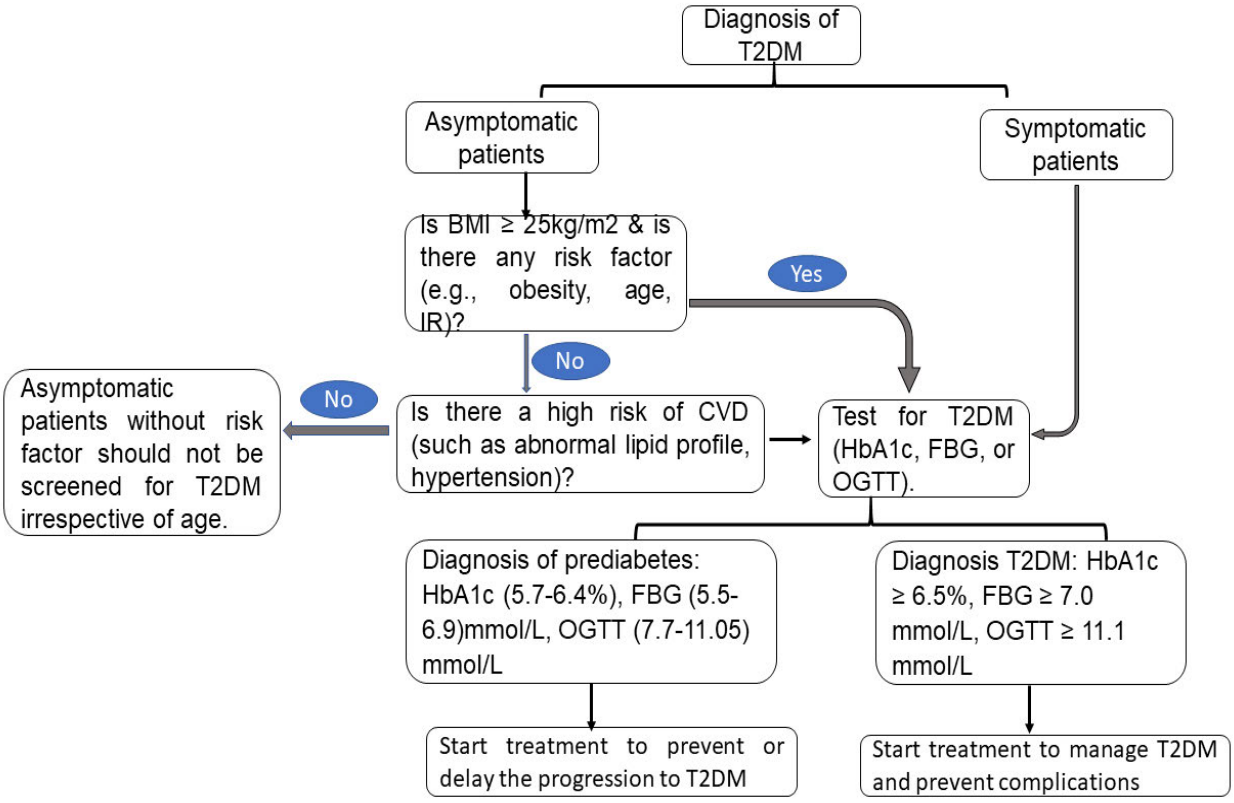

Figure 1: Flow chart for the diagnosis of type-2 diabetes. Adapted from Redmon et al., (36).

1.2.1.1 Epidemiology of type-2 diabetes mellitus

It has been reported that approximately 6.28% (about 462 million) of the global population (20–70 years) are afflicted with T2D, corresponding with an incidence rate of 6059 cases /100,000 in 2017. The incidence rate is increasing sporadically and has been projected to 7079 cases/100,000 by 2030 if proper interventions are not implemented to curtail the global prevalence (24). About 45 million of this population live in Africa, with a projected figure of 110 million by 2045, with South Africa having the highest incidence of type-2 in Africa (37, 38). This increasing prevalence is due to rapid economic development and urbanization associated with lifestyle changes, obesity, aging, and nutritional transition (39). Other factors like genetic factors and reduced physical activities can contribute to the accelerated increase in the T2D epidemic (40). A higher prevalence has been recorded in men than in women due to larger visceral fat (41, 42). Epidemiological reports have shown that lifestyle modifications, behavioral and biological factors promote the risk of T2D, while a higher BMI is a significant risk factor for T2D. The increased prevalence of T2D correlates with increasing incidence of obesity and increased plasma concentrations of inflammatory biomarkers like Tumor necrotic factor-α (TNF-α), C-reactive protein (CRP), while a higher adiponectin levels, anti-inflammatory biomarkers, have been shown to reduce T2D risk (43).

1.2.1.2 Pathophysiology of type-2 diabetes mellitus

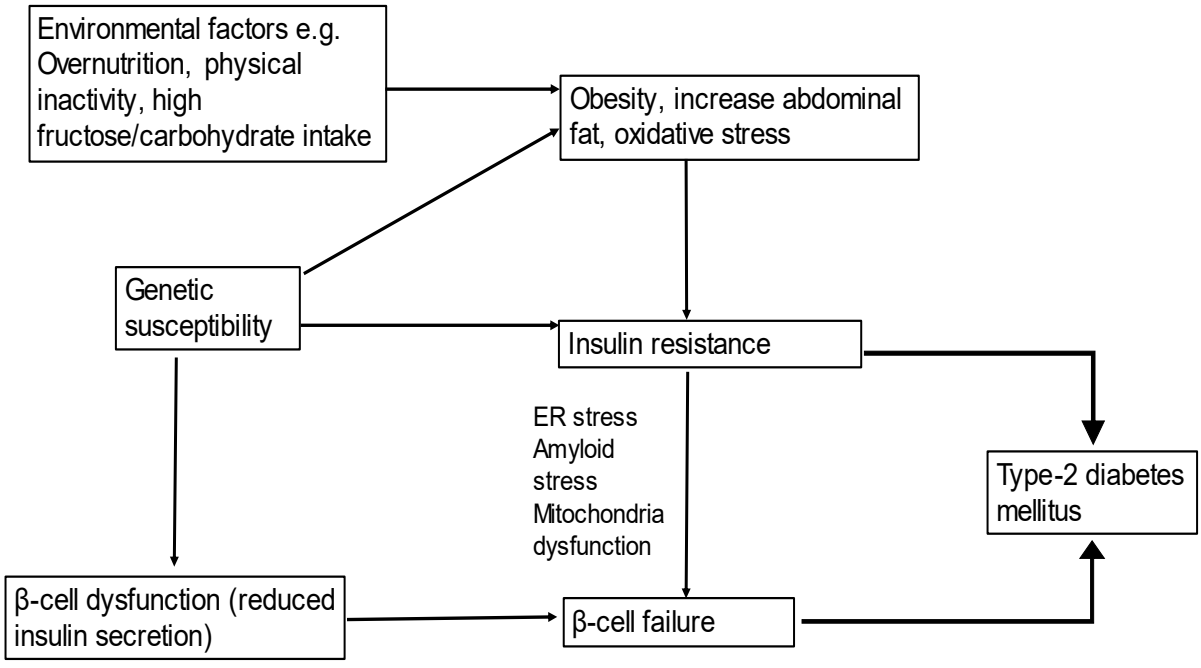

Figure 2: Schematic diagram summarizing the pathophysiology of type-2 diabetes mellitus. Adapted with modification from Tarngvarasittichai, (44).

Type-2 diabetes is a polygenic disorder with multiple factors implicated in its pathogenesis. Majorly, insulin resistance by the glucoregulatory tissues (e.g., skeletal muscle, liver, and adipose tissue) account for the hyperglycemia regarded as the primary clinical symptom of this disorder (45). The persistent increase in blood glucose promotes insulin secretion by the β-cells, which causes hyperinsulinemia that enhance the diagnosis of T2D (46). However, the interplay between the environmental and genetic factors (as shown in Figure 2) promotes metabolic overload, insulin resistance, and systemic inflammation that negatively affect the function of β-cells (47).

Environmental factors (such as overnutrition and physical inactivity) can promote metabolic overload (characterized by obesity and increased abdominal fat), oxidative stress, and systemic inflammation that cause IR in glucoregulatory tissues (48). IR by the skeletal muscle reduces glucose uptake, glycogenesis, and glycogen storage, while adipose tissue IR promotes glucose intolerance, increase plasma triglycerides and cellular infiltration, increase hepatic gluconeogenesis and compromise other insulin glucoregulatory effects that worsen hyperglycemia (49). Furthermore, liver IR alters lipid metabolism in addition to reduced glucose utilization by the hepatic cells and contribute to the pathogenesis of T2D by activating Sterol Regulatory Element-Binding Protein (SREBP) and Fatty Acid Synthase (FAS) that mediate hepatic lipid metabolism (50, 51). In an attempt to reduce blood glucose level, β-cell stimulates several compensatory mechanisms to increase insulin secretion. However, these mechanisms synergistically activate pathological processes (such as Endoplasmic Reticulum stress, mitochondria dysfunction, amyloid stress, loss of islet integrity) that reduce β-cell functions and, eventually, β-cell failure (52, 53). These pathologic processes progress with persistent hyperglycemia, in addition to increased glucagon synthesis, to compromise β-cell function that characterize the development of T2D.

In addition, studies have shown that genetic variation may contribute to the increased risk of diabetes, and this has been observed among Indian population with increased obesity and waist circumference, compared to other racial population (54, 55). Also, genome wide association studies have reported that several genetic defects, including mutation in Hepatocyte nuclear factor-1A gene (HNF-1A) may contribute to β-cell dysfunction, with a resultant decrease in insulin secretion to promote diabetes (56, 57). Altogether, it is evident that insulin resistance in glucoregulatory tissues and β-cell dysfunction are the two principal and interrelated abnormalities in the pathophysiology of T2D. However, other defects such as increased lipolysis, excessive glucose reabsorption by the kidney, dysfunction of the neurotransmitters (e.g., low dopamine and increased catecholamines), and excessive glucagon secretion may increase blood glucose, which is used in diagnosing this disorder (28, 58).

1.2.2 COMPLICATIONS OF TYPE-2 DIABETES

The development and progression of severe complications account for the increased mortality and morbidity in T2D patients. These complications (macro-and microvascular) results from poor glucose regulation and altered fatty acid metabolism that affect the functions and structures of vital tissues in the body (59). The microvascular complications (including nephropathy, liver disease, retinopathy) results from chronic injury to vital organs such as the kidneys and liver. In contrast, macrovascular complications (such as cardiovascular damage, cerebrovascular disease) predominantly result from long-term injury to the heart and blood vessels. A study including 28 countries across Europe, Asia, Africa, and America has shown that 50% of T2D patients have different microvascular complications, while 27% of these patients have macrovascular complications, with 10-20 times increased risk of developing complications compare to their non-diabetic counterparts (60).

1.2.2.1 Diabetic-related liver complications

The increasing prevalence of T2D has been associated with a high incidence of liver dysfunction, particularly nonalcoholic fatty liver disease (NAFLD), and hepatic steatosis. NAFLD is present in approximately 70-80% of T2D patients and approximately 30-40% of adults with T1DM (61, 62). The development of both diseases (liver disease and T2D) can be affected by each other because T2D can enhance the progression of steatosis to exacerbate liver damage, while liver dysfunction can increase the severity of diabetic complications in T2D patients (6). Urbanization, sedentary lifestyle, and over nutrition that cause T2D are major risk factors that promote obesity and resultant liver disease. At the same time, body mass index (BMI), triglycerides, and low-density lipoprotein correlate linearly with the severity of liver disease (63, 64). However, experimental evidence has shown that IR, altered lipid profile, and defective triglyceride metabolism promote triglyceride accumulation in the liver, linking T2D with liver complications (65, 66).

Abnormal lipid profiles can be caused by (1) increased lipolysis in the adipose tissue via impaired insulin lipogenic action; (2) enhanced *de novo* lipogenesis via sterol receptor element-binding protein1c (SREBP-1c); and (3) reduced oxidation of fatty acids. These processes enhanced the influx of free fatty acids (FFAs) into the hepatocytes, thereby increasing hepatic acetyl CoA with a resultant increase in glycogenesis and hepatic triglycerides in the liver (67, 68). Furthermore, the excess FFAs in the liver can increase free radical production to promote lipid peroxidation via excessive oxidative phosphorylation in the mitochondria (69, 70). This can eventually lead to liver fibrogenesis via stellate cell proliferation, while the excess free radicals produced may promote oxidative stress to increase cytokine production and macrophage infiltration into the hepatocytes (71, 72). In addition, the excess FFA may activate major transcription factors in the inflammatory pathway such as NF-kB and mitogen-activated protein kinase (MAPK), thereby promoting

the release of cytokines, chemokines, and adhesion molecules that alter hepatic function. (73, 74). Thus, effective therapeutic strategies (either monotherapy or combined treatment) to improve glucose homeostasis and lipid metabolism and alleviate oxidative stress and hepatic inflammation have shown significant benefits in managing diabetes-related liver injury (75, 76).

Improvement of lipid metabolism by natural products has been associated with reduced serum triglyceride and cellular infiltration, which has helped to alleviate the incidence of steatosis and NAFLD amongst T2D patients (77, 78). Recently, there have been increasing reports on the metabolic benefits of mushrooms, owing to the presence of numerous bioactive compounds in this food, including flavonoids, triterpenes, and L-ergothioneine (L-egt). Furthermore, L-egt has been reported to mitigate metabolic derangements, inhibit oxidative stress and inflammatory cytokines in the liver, and reduced lipid accumulation in the liver, with possible application in the treatment of NAFLD (17, 79, 80). There is however a paucity of reports on the potential benefits of L-egt on biomarkers of liver injury associated with type-2 diabetes. Therefore, Chapter 2 of this study investigated the hepatoprotective effect of L-ergothioneine (either as a stand-alone therapy or an adjuvant) in a type-2 diabetic animal model with a major focus on the role of this nutraceutical on liver triglyceride level, oxidative injury, hepatic inflammation, and other associated biomarkers of liver functions.

Literature has shown that hyperglycemia causes redox imbalance, and stimulate systemic and renal inflammation, which are implicated in the pathogenesis of diabetic nephropathy (81, 82). These biochemical changes damage vital cellular components (e.g., DNA, proteins, lipids) and further activate various transcription factors that promote structural and functional derangement in the kidney (83, 84). Thus, the next section highlights the pathogenesis of renal complications associated with type-2 diabetes.

1.2.2.2 Diabetes-related renal complications

Diabetes-induced renal complication, commonly referred to, as diabetic nephropathy (DN), is a major cause of chronic kidney disease (CKD) that eventually leads to End-Stage Renal Damage (ESRD). Globally, DN usually develops in approximately 20-40% of patients with T2D (85, 86). In Africa, renal complication is a major health threat for diabetic patients, with an incidence rate ranging from 11-84%. Estimation of this incidence suggests that approximately 95% of diabetic patients may develop proteinuria after 10 years of diabetes, while 35% may progress to ESRD after 5 years, and nephropathy may account for 18% mortality after 20 years of diabetes (87). However, the etiology of DN in type 2 diabetes is multifaceted, with variable grades of glomerulosclerosis, tubulointerstitial fibrosis, and vasculopathy (via hemodynamic and metabolic alterations). In addition, lipotoxicity resulting from lipid accumulation in the glomerular and tubular portion of the kidney may contribute to renal lesion during diabetes (88, 89).

Experimental studies to understand the pathogenesis of DN showed that the abnormal glucose metabolism via the hemodynamic and metabolic pathway mediates major structural and functional damages observed in the kidney during diabetes. These pathways stimulate oxidative stress, upregulate pro-inflammatory and pro-fibrotic factors that stimulate glomerular hypertension and hyperfiltration, albuminuria, and reduced glomerular filtration rate (GFR) (90, 91). In addition, the metabolic pathways can promote oxidative stress that increases the expression of NF-kB and TGFβ1 transcription cascades in the renal cells with resultant glomerulosclerosis, fibrosis, and cell death that alters renal structure and function. TGFβ1 upregulate matrix protein synthesis and inhibits matrix degradation, causing extracellular matrix accumulation and fibrosis (92, 93). Advanced Glycated End-products (AGEs) promote the accumulation of Extracellular Matrix (ECM) protein (e.g., collagen and fibronectin) in the glomerular mesangium and tubulo-interstitium marked by increased fibronectin expression, with resultant alteration of both structure and function of the kidney. Excessive ECM production is further complicated by increasing interstitial fibrosis, cytokine release and cellular infiltration (94, 95). Moreover, insulin resistance impairs lipid metabolism as occurring in T2D, to promote fatty acid influx in the renal cells, thereby causing lipotoxicity, which has been correlated with oxidative stress, fibrosis, and inflammation that mediate renal dysfunction (96, 97).

The use of natural products to delay the onset of DN and mitigate its progression has shown significant benefits. These products exert significant biological effects (including antidiabetic, antioxidant, and anti-inflammation) to mitigate renal damage (98, 99). Previous experimental studies have reported that L-egt exerts antioxidant and anti-inflammatory properties to protect kidney functions and improve treatment efficacy to provide cytoprotection (21, 100). However, the effect of L-egt on renal complications associated with diabetes is yet to be reported. Therefore, Chapter 3 of this study investigated the effect of L-ergothioneine on selected biomarkers (including serum creatinine, BUN, proteinuria, albuminuria, GFR, KIM-1, mesangial expansion, and fibrosis) associated with renal injury in type-2 diabetes and further evaluated the probable mechanism of action of this bioactive compound.

Studies have shown that type-2 diabetic patients have a higher risk of developing cardiovascular disease (CVD), ranging from mild endothelial dysfunction to severe conditions like stroke and heart failure. The incidence of CVD in these patients has been attributed to increased extracellular fluid volume and dyslipidemia that affect the vascular endothelium and the activation of oxidative injury and cardiac inflammation that compromise cardiac functions (101). Thus, the subsequent paragraphs highlight the pathogenesis of diabetic-related cardiovascular injury in type-2 diabetes.

1.2.2.3 Diabetes-related cardiovascular complications

Macrovascular conditions, such as stroke, hypertension, coronary artery disease, myocardial infarction (MI), and heart failure are the major cardiovascular complications associated with T2D (102). Cardiovascular complications account for significant mortality and morbidity among diabetic patients, with an incidence rate of 2 to 4-fold increase compared to non-diabetic subjects irrespective of age or underlying comorbidities (103, 104). Literature has shown that 30% of T2D patients have at least one cardiovascular complication with a 19% mortality rate over 8 years and 40% cardiovascular disease (CVD)-related death (105, 106). In addition, studies have shown that obesity, dyslipidemia, poor physical activity, smoking, and hypertension are strong predictors of CVD in diabetic patients (107, 108).

However, the pathogenic role of both hyperglycemia and IR in the development and progression of cardiovascular complications involves the activation of several molecular pathways and cellular processes. Experimental evidence has shown that hyperglycemia, via the electron transport chain in the mitochondria, increases the production of free radicals (such as RNS, ROS) and peroxynitrites that downregulates nitric oxide production with resultant endothelial dysfunction (109). In addition, chronic hyperglycemia causes a persistent glucose metabolism via the hexosamine and polyol pathway, thereby activating the mTOR signaling pathway that promotes cardiomyocyte expansion with resultant cardiac hypertrophy and cardiac failure. Also, overexpression of the aldose reductase enzyme via the activation of the polyol pathway contributes to cardiac failure by reducing glutathione levels with resultant induction of oxidative injury, DNA damage, and cell death in the cardiomyocytes (110, 111). AGEs and the activation of its receptor (rAGE) stimulate the release of inflammatory molecules (e.g., cytokines, adhesion molecules) via the NF-kB signaling pathway to enhance macrophage infiltration and subsequent vascular inflammation (112). AGEs can directly modify extracellular proteins and cause alteration in the structure and function of cardiac tissue or increase the production of free radicals to enhance oxidative stress as well as expansion of the extracellular matrix to promote plaque formation and cardiac fibrosis that damages the heart (113, 114).

IR also plays a significant role in the development of endothelial and cardiovascular dysfunction associated with diabetes (115). According to the insulin resistance atherosclerotic study (IRAS 1996), IR promotes dyslipidemia that contribute to atherosclerosis, and it is a significant risk factor for cardiovascular disease, while HOMA-IR (homeostatic model of assessment-insulin resistance) has been used as a diagnosis for endothelial dysfunction (116, 117). IR is associated with reduced bioavailability of NO which then results in vasoconstriction. However, the compensatory hyperinsulinemia may activate the MAPK pathway, promoting cardiac inflammation that damages the heart, with sodium and water reabsorption that increase extracellular fluid volume and blood pressure (118, 119). In addition, alteration in lipid metabolism causes fatty acid accumulation by activating peroxisome proliferator-activated receptor-α (PPARα) in the

cardiomyocytes with resultant lipotoxicity that promote oxidative injury in the cardiac cells (120, 121). At the molecular level, upregulation of the Nrf2 transcription-signaling cascade and its downstream antioxidant genes have been implicated in the management of diabetic cardiomyopathy (122, 123). Activation of this cascade alleviate oxidative injury and endothelial dysfunction. Thus, Nrf2 activators have been suggested as a therapeutic strategy to inhibit oxidative injury and alleviate cardiac inflammation to improve cardiac function in diabetes.

Studies have shown the potential of L-egt to protect vascular endothelium by preventing monocyte infiltration into the endothelial cells, while increased L-egt level has been correlated with a lower risk of cardio-metabolic diseases and mortality (17, 124, 125). Recent studies have reported that L-egt exerts a beneficial effect on cardiometabolic health, improves the clinical characteristics of pre-eclampsia, and modulates antioxidant and anti-inflammatory pathways to confer cyto-protection (126-128). However, the effects of L-ergothioneine on cardiovascular dysfunction associated with type-2 diabetes have not been reported in the literature. Thus, Chapter 4 of this study investigated the cardioprotective effect of L-ergothioneine in a type-2 diabetic animal model and further evaluated the probable mechanism of action of this bioactive compound.

1.2.3 PATHOGENESIS OF DIABETIC COMPLICATIONS

The persistent hyperglycemia increases glucose flux via the metabolic and hemodynamic pathway in susceptible individuals, causing significant alteration in the structural and functional integrity of vital tissues in the body, leading to diabetic complications. Insulin resistance also contributes to the severity of these complications by disrupting lipid metabolism with resultant infiltration of triglycerides into the cells, thus, promoting cellular dysfunction (129, 130). Glucose flux via the metabolic pathways activates protein kinase C, hexosamine, polyol pathways and increases the formation of advanced glycemic end products (AGEs) with the activation of its receptors (rAGEs). In addition, glucose flux through the hemodynamic pathway activates the renin-angiotensin-aldosterone system (RAAS) that increases water reabsorption and vasoconstriction, with subsequent increase in blood pressure. Thus, RAAS activation also plays a significant role in the development and progression of diabetic complications. Activation of these pathways upregulates several intracellular signals and transcription cascades that stimulate oxidative stress and cellular inflammation mediating tissue injuries.

1.2.3.1 Oxidative stress in type-2 diabetic complications

The activation of various metabolites in the glucose pathways increase the production of free radicals and compromise the activity of the antioxidant enzymes, which promotes oxidative stress that plays a central role in diabetic complications (131). Experimental and clinical studies that evaluate the underlying mechanisms of diabetic complications showed that the pro-oxidant nature of hyperglycemia contributes significantly to oxidative stress, as shown in Figure 3. However, altered lipid metabolism and obesity, act in synergy to aggravate the oxidative injury and each tissue-specific abnormality, which are clinically observed in diabetic patients (44, 132).

Excessive formation of advanced glycation end products (AGEs) has been reported as a major pathway through which hyperglycemia causes oxidative stress. The formation of these products increases ROS production via electron leakage from the mitochondria, and further compromises the antioxidant system's efficacy by downregulating the expression of major transcription factors (Nrf2, Sirt1) involved in the antioxidant pathway (133, 134). Increased activity of the polyol pathway contributes to oxidative stress by increasing NADPH consumption, which consequently reduces glutathione concentration; increased production of superoxide by sorbitol dehydrogenase when converting sorbitol to fructose of NADH/NAD^+ redox imbalance leading to oxidative stress (135, 136); increased production of AGEs and intense metabolic activities resulting from increased fructose production. Thus, the AGEs produced from this pathway result in free radical generation (137).

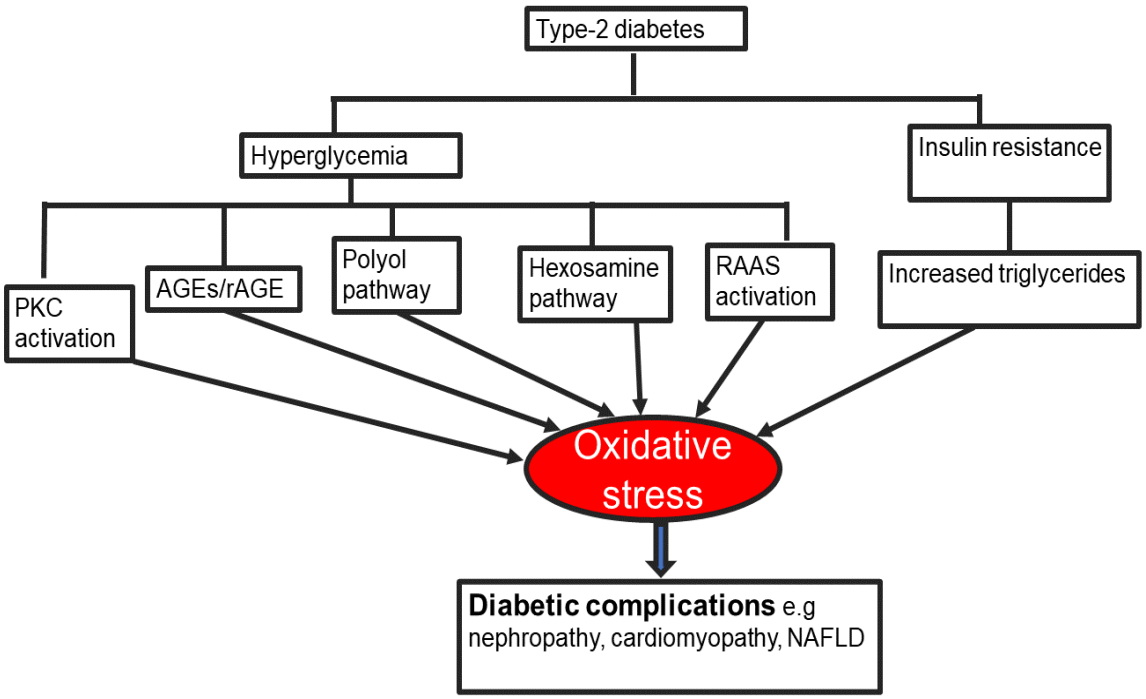

Figure 3: A hypothetical flow chart showing major cellular processes underlying the induction of oxidative stress that mediates complications in type-2 diabetes. Adapted with modification from Oguntibeju, (138).

Activation of the PKC pathway and its isoform due to persistent hyperglycemia is another mechanism contributing to diabetes-induced oxidative injury. Hyperglycemia modulates PKC activity by improving the production of diacylglycerol, a major co-factor necessary to activate both conventional and novel PKC isoforms (α, β1 and 2, γ), which are rapidly translocated to the cell. Activation of PKC isoforms increases the production of NADPH oxidase that generates superoxide. The inhibitory effect of glucosamine-6-phosphate in the hexosamine pathway decreased the production of reduced glutathione and inhibit catalase activity, thereby contributing to oxidative stress (139). Upregulation of the renin-angiotensin-aldosterone system (RAAS) increases angiotensin II and aldosterone concentration, thereby disrupting the insulin pathway, increasing the generation of free radicals and inflammation associated with vascular complications (140). Angiotensin-II acts as the major effector of the RAS pathway and has been reported to increase free radical production via NAD(P)H (141). In addition, IR enhances lipolysis that increases serum FFAs. Infiltration and accumulation of FFAs in the tissue cause lipotoxicity that contribute to oxidative injury and mitochondrial dysfunction (142). In addition to free radical production, the potency of the antioxidant defense system is reduced in diabetes. Studies have reported that plasma total antioxidant capacity is reduced, and the antioxidants may also undergo oxidation during diabetes (143). The scavenging abilities of the endogenous enzymes (glutathione peroxidase, catalase, superoxide dismutase) and the concentration of antioxidants such as glutathione are reduced during diabetes (144, 145). Furthermore, cellular oxidative injury can promote chronic inflammation in tissues contributing to diabetic complications by activating various inflammatory pathways in target organs (82, 146).

1.2.3.2 Inflammation in type-2 diabetic complication

Chronic Inflammation is another crucial physiological process that promotes the development and progression of diabetic complications resulting from the production of bioactive metabolites generated during glucose metabolism, as shown in Figure 4 (147). Alteration in glucose metabolism during chronic hyperglycemia increases the production of proinflammatory cytokines (TNF-α, TGF-β1), monocyte chemoattractant protein-1 (MCP1), intercellular adhesion molecules (ICAM) that infiltrate and inhibit cellular functions. In addition, hyperlipidemia, as it occurs during obesity, can reduce adiponectin level and activate the toll-like receptor (TLR) to recruit inflammatory cytokines and the activation of monocytes (148). At the molecular level, binding of AGEs to its receptor (rAGE) upregulates nuclear factor-kappa B (NF-kB) transcription factor and its downstream gene to promote inflammation, enhance monocyte infiltration, inhibit endothelial nitric oxide activity, and fibrosis (149, 150). Also, translocation of PKC

isoform into the cell membrane triggers several transcription factors, e.g., NF-kB and MAPK that inhibit nitric oxide synthesis and increase generation of ROS from the endothelium (151). Increased activity of aldose reductase in the polyol pathway increases angiotensin-II production from the hemodynamic pathway, and excessive ROS production during diabetes also activates the JAK/STAT signaling cascade that mediates cellular inflammation (152, 153).

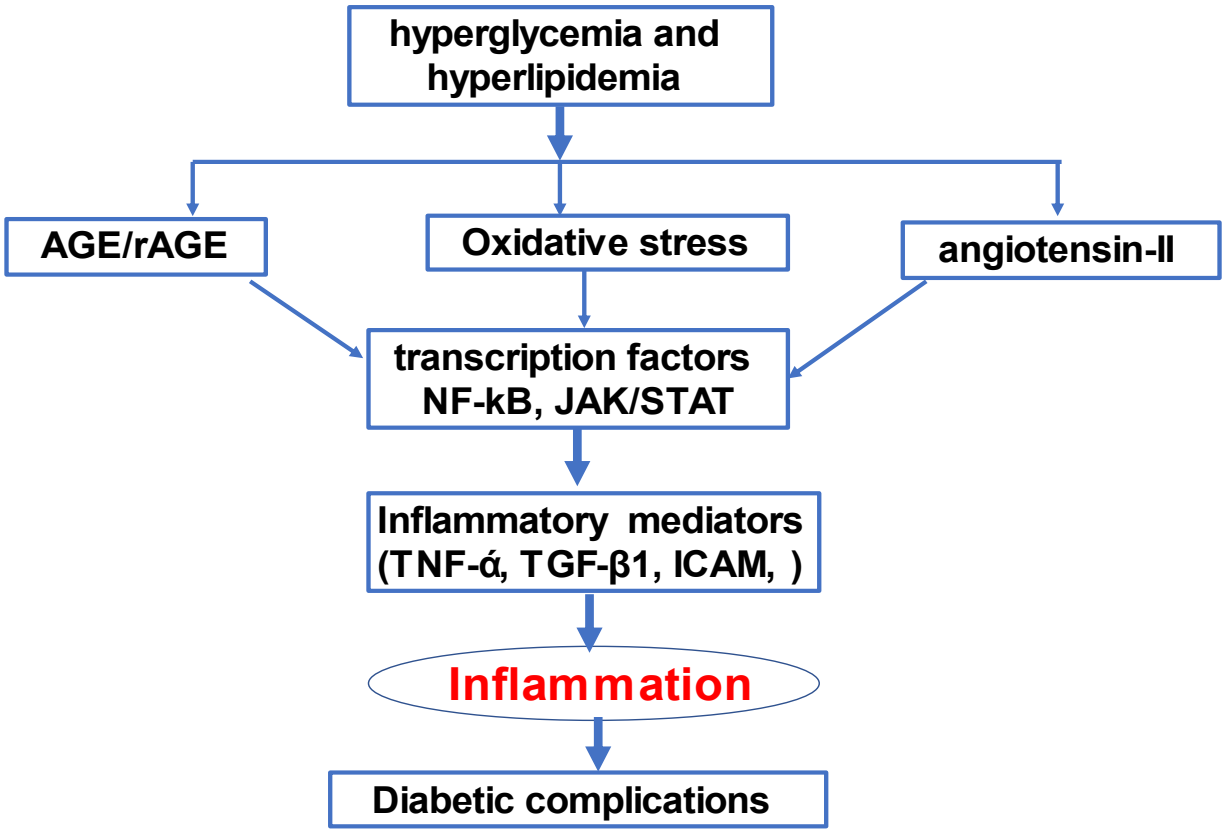

Figure 4: A hypothetical flow chart showing a major inflammatory pathway that mediates complications in type-2 diabetes. Adapted from Matoba et al., (154).

1.2.4 MANAGEMENT OF TYPE-2 DIABETIC COMPLICATIONS

Early diagnosis is required for the proper management of diabetes and its complications. However, due to late diagnosis of T2D, most patients often present with complications at the time of diagnosis, thereby contributing to the severity of its complications. After diagnosis, this disorder requires lifestyle intervention and pharmacological agent for adequate management to delay the onset or progression of complications

(155, 156). Giant strides over the past decades have increased our knowledge of each of these interventions, and the subsequent section highlights some of these effects.

1.2.4.1 Lifestyle intervention

T2D has been recognized as a lifestyle disease, which may be managed with dietary modifications according to the expert guidelines on a healthy diet and improved physical activity (157). American diabetes association recommends lifestyle modification as the foremost intervention to prevent the progression of T2D upon diagnosis before significant pharmacology treatment. Diabetes prevention studies have shown that lifestyle modifications, even at the prediabetes stage, can significantly decrease the occurrence of T2D (158, 159).

The management of T2D with lifestyle intervention relies on dietary modification and increased exercise (160, 161). These dietary regimens involve the consumption of a plant-based diet containing several bioactive compounds (such as polyphenols, sterol, amino acids, and triterpenes). These compounds can activate several molecular mechanisms to improve glucose homeostasis, reduce oxidative injury, and enhance lipid metabolism, thereby reducing body weight, and increasing well-being (162-164). Lifestyle interventions that combine dietary modification with adequate physical exercise is more effective than diet or exercise alone in managing T2D (161). Regular exercise has been reported to decrease HbA1c with or without dietary changes, causing a short-term increase in insulin activity via insulin-independent glucose uptake. In addition, vigorous exercise as short as one week in an adult with T2D caused an improvement in glycemic index, lipid metabolism, and cardiovascular functions (165-167). However, this treatment intervention is also compromised in the management of T2D by inadequate and poorly sustained physical exercise resulting in a short-lived efficacy of the exercise program. Dietary modification may be expensive, especially in developing countries with low economic status and the inability to adhere to a diet plan as advised by a dietitian. Taken together, non-compliance to lifestyle modification has accounted for the significant setback in the efficacy of this intervention in the management of T2D (168, 169).

1.2.4.2 Pharmacological intervention

Most patients solely rely on pharmacological agents to manage T2D due to the non-compliance to lifestyle intervention (170). However, the choice of pharmacological agents should be guided by glycemic control, safety profiles, comorbidities, contra-indications, patient preference, and cost. Also, the selected pharmacological agent should target multiple etiologies implicated in the development of the disease, such as reduced insulin production and sensitivity, reduced incretin effect, reduced lipolysis, reduced glucose

uptake and hepatic gluconeogenesis, increased glucagon release (171-173). However, consensus by the American diabetes association and American College of Endocrinologists has provided a strategic approach to manage T2D using effective pharmacotherapy (of different classes), with the primary aim of achieving HbA1c <6.5% (174).

Metformin, a class of biguanides, has been recommended as the first-line medication for newly diagnosed T2D due to its safety and efficacy, especially in patients without contraindication. Metformin reduces blood glucose by inhibiting hepatic gluconeogenesis and increase insulin sensitivity in skeletal muscles (175, 176). Besides its antihyperglycemic effect, metformin reduces cardiovascular risk, and mortality especially in obese patients (177); inhibit renal oxidative injury, inflammation, and fibrosis (178), and attenuates hepatic inflammation and lipid peroxidation to mitigate the onset of NAFLD (179). In addition, other classes of anti-diabetic drugs (including sulfonylureas, meglitinides, thiazolidinediones, dipeptidyl peptidase-IV inhibitors, and α-glucosidase inhibitors) that regulate the glycemic index via different pathways have been reported in experimental studies and clinical trials to exert various degrees of effectiveness in the management of T2D and its complications (180-182). However, none of these agents could provide overall efficacy, and the majority are associated with side effects that reduce their clinical application. Metformin and α-glucosidase inhibitors (e.g., colesevelam) have been reported to cause gastrointestinal disorders affecting over 50% of users, while chronic administration could cause deficiency of Vitamin B_{12} and aggravate neuropathy (183, 184). Also, the clinical use of dipeptidyl peptidase-IV inhibitors has been associated with nasopharyngitis, and severe hypersensitivity reaction, e.g., anaphylaxis and edema. Thiazolidinediones (rosiglitazone) have been restricted due to high risk of cardiovascular injury, and bone fracture while sulfonylureas have been reported to cause hypoglycemia and increase body weight (185, 186). In addition to the adverse event recorded, most pharmacological agents are expensive, not readily available, and there is an increased incidence of non-compliance with drugs during therapy (187), worsening T2D and causing early development severe progression of complications. Thus, the clinical efficacy of these pharmacological interventions may be limited in the management of T2D if appropriate lifestyle modifications or other adjuvants are not incorporated. Thus, research interest to develop alternative strategies with better efficacy has been on the increase.

1.2.4.3 Alternative treatment

The search for accessible, affordable, safe, and effective therapeutic strategies to manage diabetic complications has been an active research area. Emphasis has been laid on potent natural compounds with multiple bioactivities targeting the heterogeneous pathological mechanisms (such as oxidative stress, mitochondrial dysfunction, apoptosis, inflammation) mediating diabetic complications. The use of various

classes of natural compounds (such as triterpenes, flavonoids, and saponins) as a stand-alone therapy has been reported with significant improvements. These compounds can also be used as adjuvant to curtail diabetic-induced complications and improve treatment outcome, with some compounds (e.g., resveratrol, Bardoxolone-methyl) already undergoing clinical trials (188-190). The efficacy of these bioactive compounds has been attributed to their ability to improve glycemic control and insulin response; alleviate oxidative injury and cellular inflammation (191); prevent mitochondrial dysfunction and cell death (192) as well as improving therapeutic outcome with available pharmacological agents (193).

Recently, mushrooms have been identified as a vital part of the human diet used as food or medicine because of their ability to alleviate some ailments (including diabetes and its complications) and improve the general well-being of the body (194). This natural food contains several metabolites, including alkaloids, triterpenes, polysaccharides, and amino acids, that exert antioxidant, anti-inflammatory, antidiabetic functions and improve lipid metabolism (195, 196). Among the essential amino acids derived from mushroom and some other foods are L-ergothioneine and histidine. These amino acids have been reported in several studies to reduce blood glucose, alleviate oxidative injury, cellular inflammation, reduce obesity and metabolic syndrome, as well as augment the efficacy of existing therapies (80, 197-199). In addition to their biochemical activities, these metabolites are readily available, safe, with no reported adverse effects (200, 201). Histidine has been extensively studied for its antidiabetic potential, and it was established that histidine decreases blood glucose by downregulating the expression of hepatic glucogenic enzyme (such as glucose-6-phosphatase) and improve insulin response by suppressing inflammation, thereby reducing hepatic gluconeogenesis (198, 202). In addition, histidine prevents obesity and metabolic syndrome by reducing fat accumulation and cellular inflammation (197). Thus, the present study focuses on a histidine derivative, L-ergothioneine, with promising health benefits. This nutraceutical has been approved by the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) to be used as a supplement (203).

1.3 L-ERGOTHIONEINE

L-ergothioneine (L-egt) is a dietary water-soluble, sulfur-containing amino acid derivative of histidine obtained from mushrooms, black beans, red beans, and some meat products, like kidney (204, 205). It is not directly synthesized in humans, but it is widely present in human and animal tissues via uptake by its transporter (206). This compound was discovered in 1909 and was initially named after the ergot fungus, from where it was first purified (207). Since its discovery, there have been few studies, with a major focus on its synthesis, chemical properties, and possible physiological functions (208, 209); until two decades ago. Research interest on L-egt increased after the discovery of its specific transporter (OCTN1) widely

expressed in several tissues (plasma membrane, kidney, liver, eye) in the body by Grundemann et al. (210). Consequently, studies are reporting the potential benefits of L-egt in the body, thereby posing L-egt for a scientific breakthrough. In solution, L-egt exists as a tautomer between thiol and thione forms (Figure. 5), but at physiological pH, it exists predominantly as the thione, thus conferring greater stability under physiological conditions (206). Thus, L-egt does not readily undergo auto-oxidation as rapidly as other 'putative antioxidants' such as glutathione (GSH) which can generate free radicals in the process (211).
In animals (including humans), L-egt accumulates in various cells and tissue such as erythrocytes, liver, kidney, seminal vesicle, and bone marrow at high concentrations (212), via its specific transporter, suggesting the beneficial role of L-egt in the body. L-egt is well-tolerated, readily reabsorbed from the circulation, avidly retained in the body with no reported toxicity or adverse effect (200, 213). Recently, L-egt has attained European Food Safety Authority approval (EFSA) in Europe and is generally recognized as safe by the Food and Drug Administration (FDA) in the US to be used as a supplement (203).

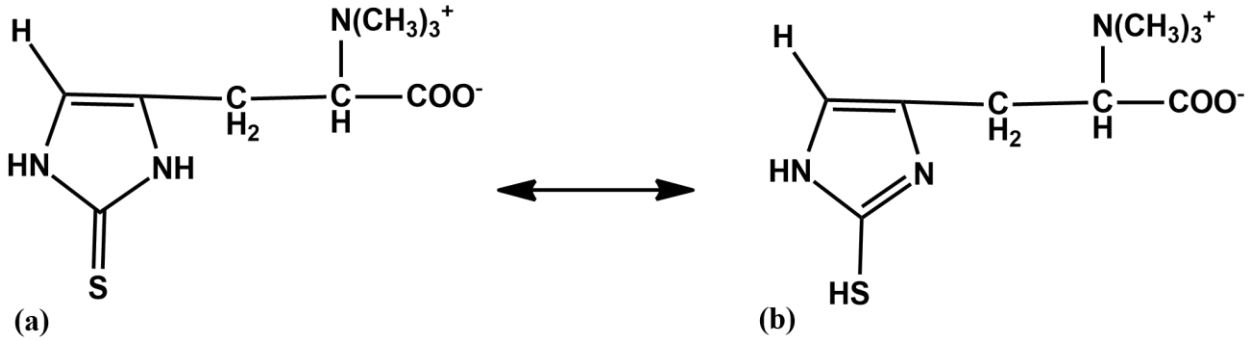

Figure. 5: Chemical structure of L-ergothioneine. In solution at physiological pH, L-egt exists predominantly in the thione (a) rather than the thiol (b). Adapted from Halliwell et al. (214).

1.3.1 Adaptive nature of L-ergothioneine

Experimental studies have shown the accumulation of L-egt at tissue injury sites via its transporter, Organic cation transporter-1 (OCTN-1) encoded by solute carrier (SLC22A4) gene that is ubiquitously expressed in the body (210). This increased accumulation has been hypothesized to be a deliberate mechanism of conferring cyto-protection to the tissues at risk of damage and the injured tissues (214, 215). An In vitro experiment using Human Embryonic Kidney (HEK-293) cells shows that L-egt was transported ~100 times faster by SLC22A4, a sodium dependent transporter, which shows a high affinity and relative specificity for L-egt (210). Cells lacking L-egt transporter are more susceptible to oxidative stress (16). Administering exogenous L-egt to animals (including humans) during tissue injuries such as in pre-eclampsia, NAFLD, pressure overload, and the infarcted heart have been proposed to be of therapeutic significance (18, 216).

L-egt have been demonstrated to exert limited antioxidant activity in healthy animals suggesting that it does not affect the normal function of ROS/RNS in healthy tissues but can be of great benefit when the free radical becomes excessive overwhelming the antioxidant defense system, thus causing oxidative damage to healthy tissues (217). Notably, some tissues (red blood cells, liver, lens of the eye) and seminal fluid have been shown to adapt to excessive and frequent exposure to oxidative stress by ensuring that the L-egt levels is constantly kept high (212). There have been established reports on the unique antioxidant, anti-inflammatory, and cytoprotective effect of L-egt compared to histidine using both in vivo and in vitro experimental models (16, 199, 218, 219). However, in vivo studies on its direct glucoregulatory effects and protective functions against diabetic complications have not been reported. Therefore, this study therefore investigated the potential benefits of L-egt on selected diabetes-induced complications, such as liver, kidney, and cardiovascular functions. The findings from this study may provide further evidence to support the therapeutic benefits of this compound either as a stand-alone or adjuvant in the effort to delay the onset or progression of these complications in type-2 diabetes.

1.4 EXPERIMENTAL ANIMAL MODEL OF TYPE-2 DIABETES

The significant contributions of diabetes mellitus to the global incidence of mortality and morbidity have prompted the need to have a proper understanding of the heterogeneous feature of this pathology, thereby making the use of appropriate animal model imperative. The search for a suitable animal model of diabetes that mimics the natural course of human diabetes has been an active area of research since the discovery of insulin using an in-vivo experiment carried out on dogs (220, 221). However, legal and ethical policies on the use of laboratory animals for experiments have prompted researchers to reduce the quantities of animals required for their studies, replace animals with another organism or model where necessary, or refine the experimental procedure for approval by the ethical committee. Despite the several restrictions from the Animal Ethics Committee, suitable animal models are highly required to understand the pathogenesis of diabetes and its associated complications and evaluate the efficacy of various therapeutic strategies formulated to mitigate the development and progression of diabetes.

Several diabetic animal models have been developed, which allows scientists to regulate factors (e.g., genetic, or environmental) implicated in the etiology of diabetes, thereby providing detailed information on the management of this disorder in humans. However, each of these animal models is associated with its own advantages and disadvantages. Thus, the researcher chooses a suitable model, usually influenced by the diabetes model, study duration, financial implication, and research goal. Based on these, rats are the most widely used in vivo model due to their accessibility, ease of breeding, and handling (222, 223). Most of the widely used animal models are chemically-induced diabetes (e.g., alloxan, streptozotocin),

spontaneously diabetic rats (e.g., Zucker diabetic fatty rat, non-obese diabetic mouse), diet-induced diabetic rats (high-fat diet, high caloric diet), or genetically knockout model (Got Kakizaki rat, Long Evans Tokushima lean rats). However, some scientists combine any two of the previously mentioned models (e.g., high fat/ streptozotocin or high fructose/streptozotocin) depending on the research goal (224, 225). Diet-induced animal models are commonly used in the experimental induction of type-2 diabetes as they promote IR and obesity (226). The primary characteristic of type-2 diabetes is the failure of β-cells to produce more insulin to compensate for insulin resistance during chronic hyperglycemia. Thus, an accurate animal model of type-2 diabetes should mimic this natural pathogenic history as occurring in humans. The high cost of obtaining and maintaining a genetic animal model and the setback in achieving both IR and β-cells dysfunction with either chemical or diet alone has reduced their use in developing this heterogeneous disorder (227). Thus, it has been hypothesized that combining diet (e.g., fructose, fat) and chemical (e.g., streptozotocin, alloxan) will produce a better and more cost-effective model of type-2 diabetes. This hypothesis has been validated using the high fat plus STZ model as well as nicotinamide and STZ model (228, 229).

Of interest in the present study is the combination of diet and chemical-induced model using excessive consumption of fructose solution plus STZ-treated animal model to induce type-2 diabetes. In this model, 10% fructose in drinking water is supplied *ad libitum* to the animal for 14 days and subsequent intraperitoneal injection of low dose (40mg/kg) of STZ (230). This experimental model has been shown to mimic the natural history of human type-2 diabetes where excessive fructose consumption promotes IR, and the low dosage of STZ causes partial loss of β-cell function to produce more insulin and compensate for the insulin resistance. It has been established that STZ at a lower dose ≤ 40mg/kg bwt could not induce T2D while doses greater than 50mg/kg is mostly used to mimic T1D (231, 232). In addition, excessive fructose consumption promotes hypertriglyceridemia, impaired glucose tolerance, and hyperinsulinemia. At the same time, several experimental studies and clinical trials have shown that fructose-feeding induces IR with compensatory hyperinsulinemia by reducing the expression of hepatic insulin receptor substrate-2, promoting de novo lipogenesis, and inhibit oxidation of fatty acid (233, 234). However, inducing T2D with fructose alone will require a longer duration and might cause nutritional intolerance with no clinical symptom of glucose intolerance (235). Therefore, a combination of short-term fructose feeding and low dose STZ injection has been used to induce T2D with clinical characteristics of its pathogenesis (236-238). In addition, the benefits of this animal model that showed major pathogenesis of T2D will assist in the formulation of better therapeutic strategies to manage type-2 diabetic complications.

1.5 PROBLEM STATEMENT

Type-2 diabetes (T2D) is recognized as a global issue of public health concern, with increasing prevalence in many regions of the world. Chronic hyperglycemia promotes oxidative injury, inflammation and apoptosis that compromise tissue function during diabetes. Thus, poorly managed T2D is often associated with renal, hepatic, and cardiovascular complications marked by structural and functional damage in the kidney, liver, and heart. Currently, dietary modification, pharmacological interventions, and their combination are used to manage diabetic complications. However, these therapeutic measures could not confer overall treatment due to non-compliance to dietary modification, while cost, side effects, and other setbacks limit the efficacy of the pharmacological interventions. Thus, there is the need for effective strategies that can improve glucose homeostasis, mitigate risk factors of diabetic complications, and improve the efficacy of available treatment options.

1.6 JUSTIFICATION OF THE STUDY

L-ergothioneine (L-egt), a bioactive compound obtained from mushroom and other food products, is rapidly absorbed, well-tolerated, and retained in tissues. L-egt has been granted full approval by the FDA and EFSA to be used as a supplement (203), and the identification of its specific transporter suggests its vital role in the body. Studies have reported the antioxidant, cytoprotective, and anti-inflammatory properties, while its accumulation in tissues has been hypothesized as an adaptive mechanism to protect against injury (199, 214). Experimental studies have shown that L-egt protects against hyperglycemia-induced cell toxicity, prevent embryo malformation in diabetic pregnant rats and protect tissues (such as liver, kidney, heart, and testes) exposed to injury as well as enhances the efficacy of existing therapies (17, 21, 79, 128). However, the role of this nutraceutical on glucose metabolism, insulin resistance, triglycerides, proteinuria, oxidative stress, inflammation, serum concentration of liver and cardiac enzymes that characterize structural and functional derangements in the kidney, liver, and heart in type-2 diabetes is yet to be reported. Thus, this study was designed to evaluate the effect of L-egt on renal, hepatic, and cardiovascular complications associated with type-2 diabetes.

1.7 RESEARCH QUESTIONS

The research questions in this study were:

- Can L-ergothioneine administration alleviate hepatic triglyceride levels, oxidative stress, and inflammation in type-2 diabetes?

- Does the combination of L-ergothioneine with metformin improve glucose homeostasis, insulin resistance, and treatment outcomes compared to monotherapy?

- Can L-ergothioneine alone, or in combination with metformin, alleviate structural and functional derangement to protect renal function in type-2 diabetes?

- Does the reno-protective effect of L-ergothioneine have any relation with the Nrf2 antioxidant pathway as observed in studies investigating the therapeutic effect of this nutraceutical?

- Can L-ergothioneine administration mitigate risk factors implicated in the pathogenesis of diabetic cardiomyopathy to delay the progression of cardiovascular injury associated with type-2 diabetes?

- What is the structure-function relationship between L-egt and the Nrf2-Keap1 protein complex that regulates the nuclear translocation of this import transcription factor?

1.8 AIM OF THE STUDY

This study aims to evaluate the potential benefits of L-ergothioneine on risk factors of hepatic, renal, and cardiovascular complications as stand-alone and in combination with metformin in a type-2 diabetic rat model.

1.9 OBJECTIVES

The objectives of this study include:

- To evaluate the effects of L-egt, alone or combined with metformin, against liver injury in type-2 diabetic rats, by analyzing blood glucose levels and IR, liver enzymes, liver triglycerides, oxidative stress and hepatic inflammation, mRNA expression of major transcription factors of lipid metabolism, antioxidants and inflammation, and histopathological analysis of the liver.

- To evaluate the reno-protective effects and probable mechanism of action of L-egt, alone or combined with metformin, in type-2 diabetic rats, by analyzing fluid intake and urine output, kidney hypertrophy, renal function biomarkers, KIM-1 concentration, oxidative stress, and renal inflammation, protein and mRNA expression of Nrf2, and mRNA expression of major transcription factors of antioxidants, inflammation, and fibrosis, as well as histopathological (H&E, PAS and Masson trichrome) analysis of the kidney.

- To evaluate the cardioprotective effect of L-egt in type-2 diabetic rats and its molecular antioxidant mechanism, by analyzing biomarkers of cardiac injury, mean arterial pressure, oxidative stress, and cardiac inflammation, mRNA expression of major transcription factors in the antioxidant pathway as well as evaluating the antioxidant mechanism of L-egt via molecular docking and molecular dynamic studies.

1.10 NOVELTY OF THIS STUDY

As part of the novelties of this study, (1) this is the first study to report that L-egt downregulates Sterol Element Binding Protein-1 (SREBP1c) and Fatty Acid Synthase (FAS) expression that alters fatty acid metabolism, thereby reducing liver triglyceride. (2) L-egt administration reduced mesangial matrix expansion and fibrosis by downregulating fibronectin and TGF-β1 gene expression. (3) this study provides structure-based evidence to support L-egt as a potent activator of the Nrf2 signaling cascade. (4) Altogether, this study as shown that this newly approved nutraceutical can be used as an adjuvant to improve the management of complications during diabetes, which is a life-long challenge.

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CHAPTER 2

PROLOGUE

Studies have shown that both hyperglycemia and IR associated with type-2 diabetes promotes liver complications (such as hepatic steatosis and NAFLD) via lipid accumulation, oxidative damage, and inflammation of the hepatocytes. Thus, therapeutic measures that can effectively inhibit oxidative injury, prevent inflammation, and reduce fatty infiltration into the hepatocytes may provide adequate cyto-protection against liver damage. Numerous studies over the past five decades have shown that the combination of pharmacological and dietary interventions may provide better results in the management of diabetic complications. However, non-compliance to dietary protocols and adverse effects associated with pharmacological regimens compromised the efficacies of these interventions. Therefore, there is the need for a refined strategy that could target the risk factors involved in the development and progression of liver complications in type-2 diabetes.

Previous studies have reported the potent antioxidant and anti-inflammatory properties of L-egt to protect against damage, especially in tissues exposed to injury. This compound was recently implicated in NAFLD treatment to reduce lipid accumulation in the liver and enhance therapeutic outcomes when combined with existing therapies. However, the therapeutic benefits of L-egt against liver complications associated with type-2 diabetes are yet to be reported. Thus, chapter 2 of this study evaluated the effects of L-egt, with or without metformin, on diabetic liver injury using an experimental type-2 diabetic rat model.

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L-ergothioneine and metformin alleviates liver injury in experimental type-2 diabetic rats via reduction of oxidative stress, inflammation, and hypertriglyceridemia

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ABSTRACT

Type-2 diabetes (T2D) is associated with liver toxicity. L-ergothioneine (L-egt) has been reported to reduce toxicity in tissues exposed to injury, while metformin is commonly prescribed to manage T2D. Hence, this study evaluates the hepato-protective role of L-egt, with or without metformin, in T2D male rats. Thirty-six (36) adult male Sprague-Dawley rats were randomly divided into non-diabetic (n=12) and diabetic (n=24) groups. After induction of diabetes, animals were divided into six groups (n=6) and treated either with de-ionized water (DW), L-egt (35 mg/kg bwt), metformin (500 mg/kg bwt) or a combination of L-egt and metformin orally for seven weeks. Bodyweight and blood glucose were monitored during the experiment. Thereafter, animals were euthanized, and liver tissue was excised for biochemical, ELISA, Rt-qPCR, and histopathological analysis. Results from this study showed that L-egt with or without metformin reduced liver hypertrophy, liver injury, triglycerides, oxidative stress, and inflammation. Also, L-egt normalizes mRNA expression of SREBP-1c, fatty acid synthase (FAS), NF-kB, TGF-β1, nuclear factor erythroid 2-related factor 2 (Nrf2) and Sirtuin-1 (Sirt1) in diabetic rats. Furthermore, co-administration of L-egt with metformin to diabetic rats reduced blood glucose and insulin resistance (IR). These results provide support to the therapeutic benefits of L-egt in the management of liver complications associated with T2D.

Keywords: Antioxidant, diabetes, L-ergothioneine, metformin, cytoprotection

INTRODUCTION

Type-2 diabetes (T2D), a chronic metabolic disorder of global prevalence, is drastically increasing in developing and industrialized countries due to excessive caloric intake, sedentary lifestyle, poor diagnosis and disease management. The etiology and progression of T2D are multifaceted and frequently associated with obesity, hypertriglyceridemia, insulin resistance and compromised insulin secretion by the pancreatic

islet with subsequent hyperglycemia Lozano et al. (2016); (Pandey et al. 2015). Liver complications are often seen in approximately 70% of people with T2D diabetes and account for about 2-4% mortality in T2D patients (Hazlehurst et al. 2016; Zoppini et al. 2014). These complications (including non-alcoholic fatty liver disease, steatosis) results from hyperglycemia-induced oxidative injury and low-grade inflammation that mediate liver damage, including fibrosis and apoptosis. Also, insulin resistance increases *de novo* lipogenesis under the influence of SREBP-1c and alters lipid metabolism by upregulating FAS, thereby promoting the influx of fatty acid into the liver cells (Gehrke and Schattenberg 2020). Excessive accumulation and infiltration of fats into the hepatocytes contribute to liver toxicity by increasing reactive oxygen species (ROS), stimulate inflammatory cytokines, mitochondria dysfunction, and endoplasmic reticular stress that mediate liver damage. Thus, therapeutic measures that can effectively inhibit oxidative injury, prevent inflammation, and reduce fatty infiltration into the hepatocytes may provide adequate cytoprotection against liver damage. However, attentions have been drawn towards the use of natural compounds with multiple bioactivities to provide adjuvant therapy in the management of diabetic liver complications.

The beneficial role of bioactive compounds (such as quercetin, resveratrol, hesperidin, curcumin, naringenin, and oleanolic acid) with antioxidants properties in the management of diabetic complications has been associated with their ability to increase antioxidant capacity, stimulate endogenous antioxidant enzymes and cytoprotective genes by activating various transcription factors such as Nrf2 and Sirt1 (Farghali et al. 2019; Jadeja et al. 2016). These compounds also exert anti-inflammatory activities by downregulating the Nuclear factor-kappa B signaling pathway to inhibit the production of cytokines, chemokines, and fibrosis in the liver (de Gregorio et al. 2020; McKinley and Willoughby 2014). Furthermore, natural compounds also help reduce serum cholesterol and triglycerides by downregulating the expression of lipogenic proteins, thereby reducing the influx of triglyceride into hepatocytes (Romano et al. 2021; Xu et al. 2020). Thus, there is a significant attention towards nutraceuticals that regulate glycemic control, upregulate the antioxidant pathway, inhibit cellular inflammation and improve lipid metabolism in the management of diabetic complications (Ma et al. 2019; Wang et al. 2018). Recently, the therapeutic benefits of mushroom in the management of diabetes and associated complications have received significant attention owing to the presence of various bioactive compounds, including flavonoids and L-ergothioneine (Azeem et al. 2021; Lindequist and Haertel 2020; Lo et al. 2020).

L-ergothioneine (L-egt), an adaptive antioxidant obtained from mushroom and some meat products (e.g., kidney and liver), was discovered over a century ago; however research interest on this biomolecule increased after the discovery of its specific transporter by (Ey et al. 2007; Gründemann et al. 2005) in 2005. Thus, there is paucity of information on the therapeutic benefits of L-egt. It has been reported that L-egt

exert antioxidant and anti-inflammatory activities as well as exhibit adaptive cytoprotective function by accumulating at the site of injury to protect against tissue damage (Halliwell et al. 2018; Salama and Omar 2021). L-egt protects against hyperglycemia-induced cell senescence, prevents embryo malformations in diabetic pregnant rats and enhances the therapeutic efficacies in vitro and in vivo (D'Onofrio et al. 2016; Guijarro et al. 2002; Song et al. 2017). Furthermore, administration of L-egt rich foods ameliorated dimethylnitrosamine-induced liver fibrosis and oxidative stress in mice (Tang et al. 2016) and has been shown in the treatment of non-alcoholic fatty liver disease (NAFLD) to downregulate SREBP1c and FAS expression, thereby inhibiting hepatic lipogenesis and lipid accumulation (Carbonero et al. 2019; Jeong and Park 2020). In addition, the hepatoprotective effect of biguanides, e.g., metformin, may result from its potency to regulate glycemic index by reducing hepatic gluconeogenesis and increased glucose uptake (Rena et al. 2017). However, metformin may be associated with side effects (such as diarrhea, weakness, and gastrointestinal discomfort) and patients still present with liver complications despite glycemic control, suggesting that metformin alone does not confer overall effective treatment. Studies have shown that combination therapies (e.g., vildagliptin, sulphonylurea) with metformin have better efficacy, allowing patients to reach their glycemic targets compared to continuing metformin monotherapy, without increasing the risk of hypoglycemia (Halimi et al. 2008; Madsen et al. 2019). In addition, literatures have reported the beneficial effect of combining natural compounds (like resveratrol, quercetin, curcumin) with metformin (Dludla et al. 2020; Roxo et al. 2019; Srivastava et al. 2013). These compounds improve the antihyperglycemic effect of metformin, reduced inflammation, and limit metformin toxicity by reducing dosage. Therefore, this study evaluated the role of L-egt and, in combination with metformin on liver injury in a rat model of type-2 diabetes.

MATERIAL AND METHODS

Drugs and chemicals

Pure L-egt was obtained from Tetrahedron limited, Paris, France. QPCR iTAQ SYBR Green and cDNA synthesis kits were purchased from Lasec (Cape Town, South Africa). Primers were synthesized by Inqaba Biotec (Pretoria, South Africa). Metformin was obtained from a local pharmacy (Pharmed, South Africa). All other chemicals, reagents and equipment were procured from standard commercial suppliers and of high analytical grade.

Experimental animals and ethical approval

Thirty-six (36) male Sprague-Dawley rats (175 ± 20 g) were obtained from the Biomedical Research Unit, Westville Campus, University of KwaZulu-Natal (UKZN), South-Africa and were housed in a room with standard laboratory conditions (12 hours light-dark cycles; temperature 23 ± 1°C, 40–60% humidity). The

animals were allowed access to rat feed and water *ad libitum* for an acclimatization period of one week before the experiment.

Ethics declaration: All animal and experimental procedures were approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, Durban, South Africa, with an approved ethic number: AREC/006/019D. All animal protocol was carried out as specified in the Guide for the Care and Use of Laboratory Animals (US National Research Council, Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Washington (DC), National Academies Press (US) 8th edition 2011).

Experimental design

After acclimatization, the animals were randomly divided into two major groups: the non-diabetic (n=12) and the diabetic (n=24) groups. All animals in the diabetic group were treated with fructose and streptozotocin (STZ) to induce type-2 diabetes using the established model described by (Wilson and Islam 2012). Briefly, the animals were supplied 10% fructose in drinking water ad-libitum for two weeks to induce insulin resistance and later injected *(i.p.)* 40mg/kg bwt STZ freshly prepared in 0.1M citrate buffer. The animals in the non-diabetic group were injected with the same volume of 0.1M citrate buffer. Animals with non-fasting blood glucose levels of >16.7mmol/L after one-week post-STZ injection were confirmed diabetic (Srinivasan et al. 2005) and included in the study. After successful diabetes induction, the non-diabetic animals were subdivided into two groups, while the diabetic animals were subdivided into four groups as follows.

Group 1(NC): non-diabetic plus de-ionized water (negative control).
Group-2 (NE): non-diabetic plus L-egt
Group-3 (DC): diabetic plus de-ionized water (positive control)
Group-4 (DE): diabetic plus L-egt
Goup-5 (DM): diabetic plus metformin
Group-6 (DEM): diabetic plus l-egt plus metformin.

Groups 1 and 3 were administered de-ionized water (1ml/100g), groups 2 and 4 were administered L-egt (35mg/kg bwt; concentration: 3.5mg/ml; volume: 1ml/100g), group 5 was administered metformin (500mg/kg bwt; concentration: 50mg/ml; volume: 1ml/100g), while group 6 was administered a combination of L-egt and metformin. The dosage of L-egt used in this study was based on previous in vivo studies using this nutraceutical (Tang et al. 2018; Williamson et al. 2020). All treatments were done daily by oral gavage and lasted for seven weeks. Fasting blood glucose was measured on day 1 and day 49. An oral glucose tolerance test (OGTT) was conducted on day 45 in overnight fasted animals administered (2g/kg bwt) oral glucose. Blood glucose was measured at 0, 30, 60, 120 minutes and the area under the curve was estimated using the trapezoid method.

Blood and tissue collection

After the seven-week treatment period, all animals were sacrificed by decapitation and blood was immediately collected into a serum vacutainer EDTA bottle and allowed to stand for 30 mins. The blood was then centrifuged at 3000 rpm for 10 mins at 4^0C to obtain serum. The serum samples obtained were stored in the bio-freezer (Snijers Scientific, Holland) at -80^0C until used for biochemical analysis. Afterward, incisions were made along the linea alba of the anterior abdominal wall to excise the liver. This organ weighed, rinsed with normal saline and snap-frozen in liquid nitrogen before been stored in the bio-freezer at -80^0C until used for analysis. Liver tissue was fixed in 10% neutral-buffered formalin for histological assessment.

Analysis of Bodyweight and liver index

Bodyweight was monitored weekly throughout the experimental period using a sensitive electronic weighing scale (Metler, Greifensee, Switzerland). The liver index (use to assess liver hypertrophy) was calculated as the ratio of liver weight to the body weight and expressed in percentage i.e.

Liver index = (Liver weight/ body weight) × 100.

Preparation of liver homogenates

The liver was thawed and homogenized in 10% phosphate buffer (0.1M, pH7.4, 1:9 w/v). The homogenates were centrifuged at 600g for 10mins to remove cell debris. The supernatant was subsequently centrifuged at 10,000g for 20mins to obtain the cytosolic fraction, which was used immediately for biochemical analyses.

Biochemical analysis

The serum concentration of liver enzymes, including Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), and Alkaline phosphatase (ALP) and triglyceride were analyzed at an accredited pathology laboratory (Global Clinic and Viral laboratories, Amazimtoti, South Africa). The concentration of triglyceride was also measured in the liver homogenate using an automatic biochemical analyzer. Blood glucose was measured at the end of the experiment using a glucometer (Accu-Chek Performa, USA). Serum insulin levels were measured by ELISA kits (Mercodia kit), and the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as previously described by Matthews et al. (1985) using the following formula:

HOMA-IR= fasting serum insulin (mU/L) X fasting blood glucose (mg/dl).

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Analysis of lipid peroxidation, antioxidant enzymes (SOD and CAT) and reduced glutathione level (GSH)

The liver homogenates were used to evaluate lipid peroxidation (using MDA as a biomarker), concentration of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) by spectrophotometric assay. Lipid peroxidation was evaluated by measuring the content of thiobarbituric acid (TBA) reactive product in the liver homogenates using the method of Mkhwanazi et al. (2014) and expressed as nmol MDA per milligram protein. GSH level and SOD activity were assessed using the method of Ellman (1959) and Marklund (1985), while CAT activity was assessed using the protocol of Aebi (1984).

Analysis of inflammatory biomarkers

The concentration of cytokine- tumor necrosis factor–α (TNF–α); chemokine, monocyte chemotactic protein-1 (MCP-1) and fibrotic cytokine, transforming growth factor-β (TGF-β) were quantified in the liver homogenates using Elabscience commercial ELISA kits according to the manufacturer's protocol.

Rt-PCR Analysis of SREBP1c, FAS, Nrf2, Sirt1, NF-kB and TGF-β1 mRNA expression

The relative mRNA expression of SREBP1c, FAS, Nrf2, Sirt1, NF-kB and TGF-β1 was quantified in the liver homogenates and analyzed using a light cycler. Total RNA was isolated in the liver using TRIzol reagent (40mg of tissue/mL, Trizol reagent). The isolated RNA quantity was determined by measuring absorbance at 260/280nm using nanodrop ND-1000 spectrophotometer (Thermo Scientific, Johannesburg, South Africa). The total RNA was converted into cDNA using iScript cDNA synthesis kit, Life Science research (Biorad, South Africa) following manufacturers' instruction. The complete reaction mixture was incubated on SimpliAmp^{TM} therma cycler, Applied Biosystems (Thermo Fischer Scientific), using the following reaction condition; priming 5mins at 25^0C, reverse transcription 20mins at 46^0C and RT inactivation 1min at 95^0C. Real-time polymerase chain reaction (RT-PCR) was done using iTaq Universal SYBR Green supermix (Biorad, CA, USA) as fluorescent dye on a light cycler 96 RT-PCR system (Roche, Mannheim, Germany). RT-qPCR was performed in a 10µL reaction volume containing 5µL SYBR Green Master Mix, 1µL of each primer, 1µL of nuclease-free water and 2µL of cDNA template. The primer sequences used are provided in Table-1. The purity and specificity of amplified PCR products were verified by melting curves generated at the end of each PCR. Relative mRNA expression was calculated using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001) and normalized in relation to the endogenous control expression, GAPDH. The primers sets were homology searched using an NCBI BLAST search to ensure that they were specific.

Table-1: Oligonucleotide sequence and accession number

Genes	Primer sequence	Genbank Accession number
GAPDH	F:5′-TGATGACATCAAGAAGGTGGTGGAG-3’ R: 5′-TCCTTGGAGGCCATGTAGGCCAT-3’	XM_017593963.1
Nrf_2	F: 5’-CAGCATGATGGACTTGGAATTG -3’ R: 5’-GCAAGCGACTCATGGTCATC -3’	NM_031789.2
Srt1	F: 5’-CCCAGATCCTCAAGCCATGTTC -3’ R: 5’-TGTGTGTGTGTTTTTCCCCC -3’	NM_001372090.1
TGF-β1	F: 5′-GGGCTACCATGCCAACTTCTG -3′ R: 5’-GAGGGCAAGGACCTTGCTGTA-3’	NM_021578.2
NF-kB	F: 5’-ACGATCTGTTTCCCCTCATCT-3’ R: 5’-TGCTTCTCTCCCCAGGAATA-3’	NM_199267.2
FAS	F: 5′-TGTGGGGTGGAAATCATCGG-3′ R: 5′-CATTGCTCCTTTGGGGTTGC-3′	NM_012820.1
SREBP-1c	F: 5’- GGAGCCATGGATTGCACATT-3’ R: 5’- AGGAAGGCTTCCAGAGAGGA-3’	NM_001276708.1

Histopathological analysis of Liver

Liver specimens were embedded in paraffin wax after dehydration in a graded series of ethanol and cleared in xylene. Serial sections were done using a rotary microtome; liver slices of 5-µm thick were fixed on a slide and stained with hematoxylin &eosin (H&E). The stained sections were visualized and captured using a nanozoomer S360 digital slide scanner (Hamamatsu Photonics, Japan) and nanozoomer digital pathology version 2.8 software for analysis by a pathologist.

Statistical analysis

Data were reported as mean ± SEM. GraphPad Prism Software version 7 (San Diego, CA) was used for statistical analysis. The differences between means were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test to determine the difference between groups. Statistical significance between groups was considered at $P < 0.05$.

RESULTS

Effect of L-egt with or without metformin administration on body weight, liver index, blood glucose, and HOMA-IR in T2D-rats

As presented in Table 2, the DC group had a significant reduction ($p<0.01$) in body weight vs. NC. L-egt, alone or with metformin in diabetic rats, caused no significant change in body weight vs. DC. There was a significant increase ($p<0.01$) in the liver index of DC rats vs. NC rats. However, both L-egt and its co-administration with metformin to diabetic rats significantly reduced ($p<0.05$) liver index-a measure of liver hypertrophy vs. DC. The blood glucose level of the DC group was significantly higher ($p<0.01$) than the NC group. Co-administration of L-egt with metformin significantly reduced ($p<0.05$) blood glucose level vs. DC, while L-egt treatment alone cause no significant change in blood glucose. There was a significant increase in HOMA-IR ($p<0.05$) in the DC group. However, L-egt with or without metformin significantly reduced HOMA-IR ($p<0.05$) vs. DC. Notably, L-egt administration to non-diabetic animals caused no significant change in body weight, liver index, blood glucose and IR.

Table-2: Effect of L-egt with or without metformin for seven weeks on body weight, liver index, blood glucose and HOMA-IR in T2D-rats.

Indices	NC	NE	DC	DE	DM	DEM
Body weight (g)	431.20±16.68	424.20±15.85	277.80±20.68**	321.40±8.44	338.60±15.79	358.60±12.97
Liver Weight (g)	13.42±0.65	13.47±0.65	11.77±0.56	11.26±0.78	11.39±0.76	11.70±0.24
Liver index (%)	3.11±0.08	3.17±0.08	4.29±0.24**	3.49±0.23#	3.35±0.08#	3.28±0.17#
Blood glucose (mg/dl)	98.64±2.97	91.08±553	465.48±19.63**	380.88±39.03	371.16±21.61#	340.2±13.81#
Insulin (mU/L)	7.90±0.74	8.10±0.73	5.2±1.02	5.4±1.08	5.46±0.79	5.7±0.78

HOMA -IR	1.91±0.16	1.82±0.12	5.97±1.27*	5.07±1.71#	5.00±0.68#	4.78±0.78#

L-ergothioneine; HOMA-IR, homeostasis model assessment of insulin resistance; T2D, type-2 diabetic; NC, negative control; DC, positive control; NE, non-diabetic + L-egt; DE, diabetic + L-egt; DM, diabetic + metformin group; DEM, diabetic + L-egt + metformin. **p < 0.01, *p < 0.05 vs. NC; #p < 0.05, ##p < 0.01 vs. DC. (n = 6).

Effect of L-ergothioneine with or without metformin on glucose tolerance

The effect of L-egt treatment alone or with metformin for seven weeks on oral glucose tolerance was presented in figure 1a, while the area under the curve was represented with the bar charts in figure 1b. The blood glucose level in the DC rats significantly increased ($p<0.01$) over the two hours experimental period when compared with the NC rats. However, treatment with metformin alone (DM) as well as co-administration of L-egt with metformin (DEM) significantly decreased ($p<0.05$) blood glucose level after 2hours of oral glucose administration while L-egt alone (DE) reduced blood glucose but not significant ($p>0.05$) when compared with the DC rats.

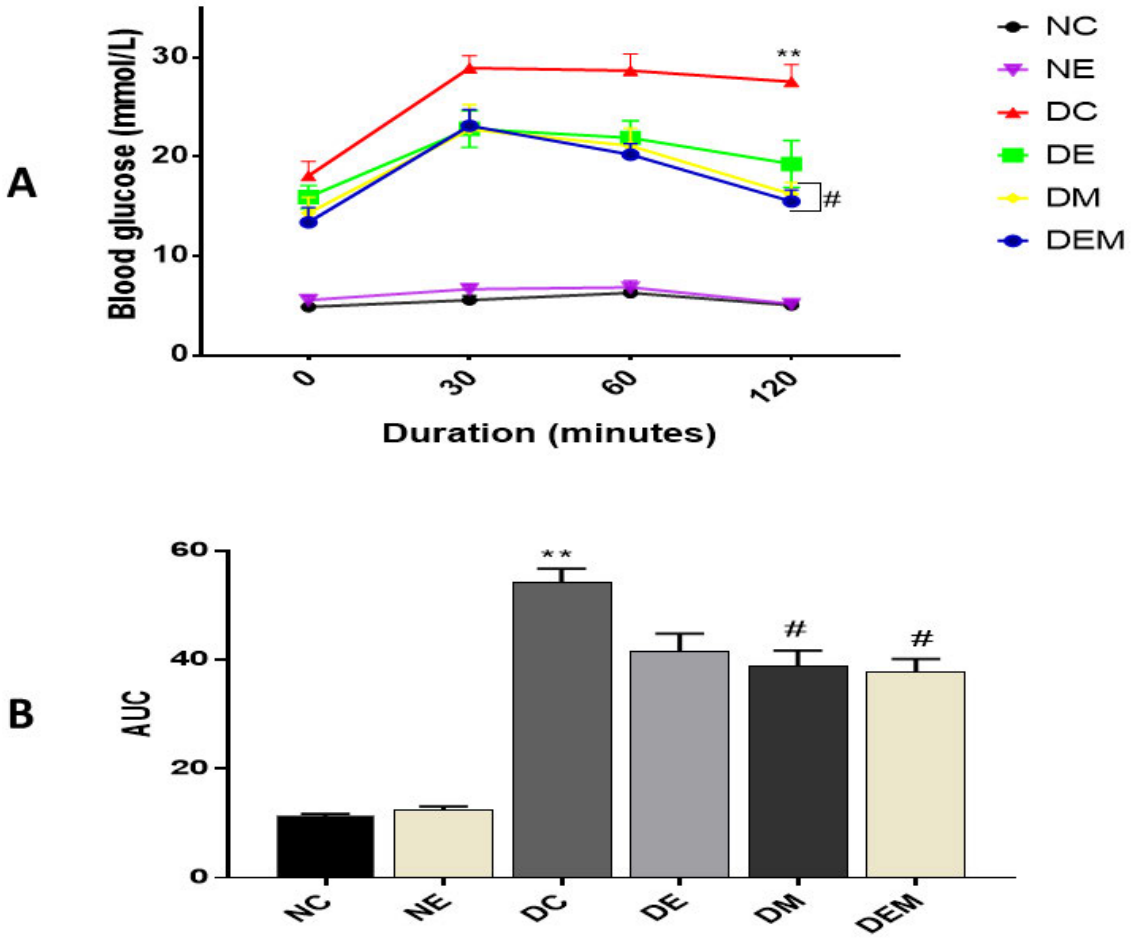

Figure 1a-b: Effect of L-egt, with or without metformin on (A) Oral glucose tolerance and (B) area under the curve in T2D rats. DM, diabetic + metformin group; NC, negative control; DC, positive control; NE, non-diabetic + L-egt; DE, diabetic + L-egt; DEM; diabetic + L-egt + metformin. $^{**}p<0.01$ vs NC; $^{\#}p<0.05$ vs DC. (n=6).

Effect of L-ergothioneine with or without metformin on liver injury in T2D rats.

The effects of L-egt treatment with or without metformin for seven weeks on biomarkers of liver injury (ALP, AST, and ALT) and liver triglyceride in type-2 diabetic rats were presented in Table 3. In the DC group, liver biomarkers (ALP, AST, and ALT) increased significantly ($p<0.05$; $p<0.01$ and $p<0.01$ respectively) vs. the NC group. However, L-egt, with or without metformin, significantly reduced ($p<0.05$) the concentration of liver biomarkers vs. DC. Furthermore, co-administration of L-egt with metformin significantly reduced ($p<0.05$) liver injury biomarkers vs. metformin treatment (DM) alone. This shows that L-egt enhances the efficacy of metformin treatment on liver dysfunctions. Liver triglyceride

significantly ($p<0.01$) increase in the DC group vs. NC group while L-egt (DE) and its co-administration with metformin cause a significant decrease ($p<0.05$) in hepatic triglyceride level vs. (DC). Serum TG increased significantly ($p<0.01$) in the DC rats vs. NC rats, while L-egt with or without metformin significantly reduced ($p<0.05$) serum TG vs DC rats. NE group showed no significant difference in liver injury biomarkers and hepatic triglyceride compared vs. the NC group.

Table-3: Effect of L-egt with or without metformin on liver enzymes, and triglycerides in T2D-rats.

Indices	NC	NE	DC	DE	DM	DEM
ALP (U/L)	129.60±15.58	124.20±24.00	239.60±29.04*	142.60±18.28#	141.60±10.26#	132.60±11.61#
AST (U/L)	78.80±6.15	89.20±14.18	184.20±14.51**	133.00±10.50#	134.20±7.41#	83.60±9.88##$
ALT (U/L)	87.20±9.28	86.80±14.97	184.20±7.84**	134.40±9.68#	132.20±9.35#	109.80±13.19##$
TG (mmol/g liver)	6.07±2.18	5.74±2.01	15.22±5.48**	12.44±3.27	9.50±4.26#	7.60±2.24#
TG (mmol/L)	1.07±0.18	0.93±0.18	3.29±0.48**	2.03±0.27#	1.96±0.26#	1.85±0.24#

ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TG, triglycerides; DM, diabetic + metformin group; NC, negative control; DC, positive control; NE, non-diabetic + L-egt; DE, diabetic + L-egt; DEM; diabetic + L-egt + metformin. $^{**}p < 0.01$, $^{*}p < 0.05$ vs NC; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. DC and $^{\$}p < 0.05$ vs DM (n = 6).

Effect of L-ergothioneine with or without metformin on lipid peroxidation and antioxidant enzymes

As presented in Figures 2a-d, liver MDA concentration in the DC group was significantly increased ($p<0.01$) vs. the NC group. Administration of L-egt alone and its co-treatment with metformin to diabetic rats caused a significant decrease in liver MDA concentration vs. the DC group ($p<0.05$ and $p<0.01$ resp.).

There was a significant decrease in SOD ($p<0.01$), GSH ($p<0.01$) and CAT ($p<0.05$) in the DC rats vs. NC. However, L-egt alone (DE) and its co-treatment with metformin (DEM) significantly increase ($p<0.05$) SOD and CAT activity and GSH in the diabetic rat vs. DC. Interestingly, a significant increase ($p<0.05$) in SOD was recorded in the group treated with L-egt and metformin vs. group-administered metformin only.

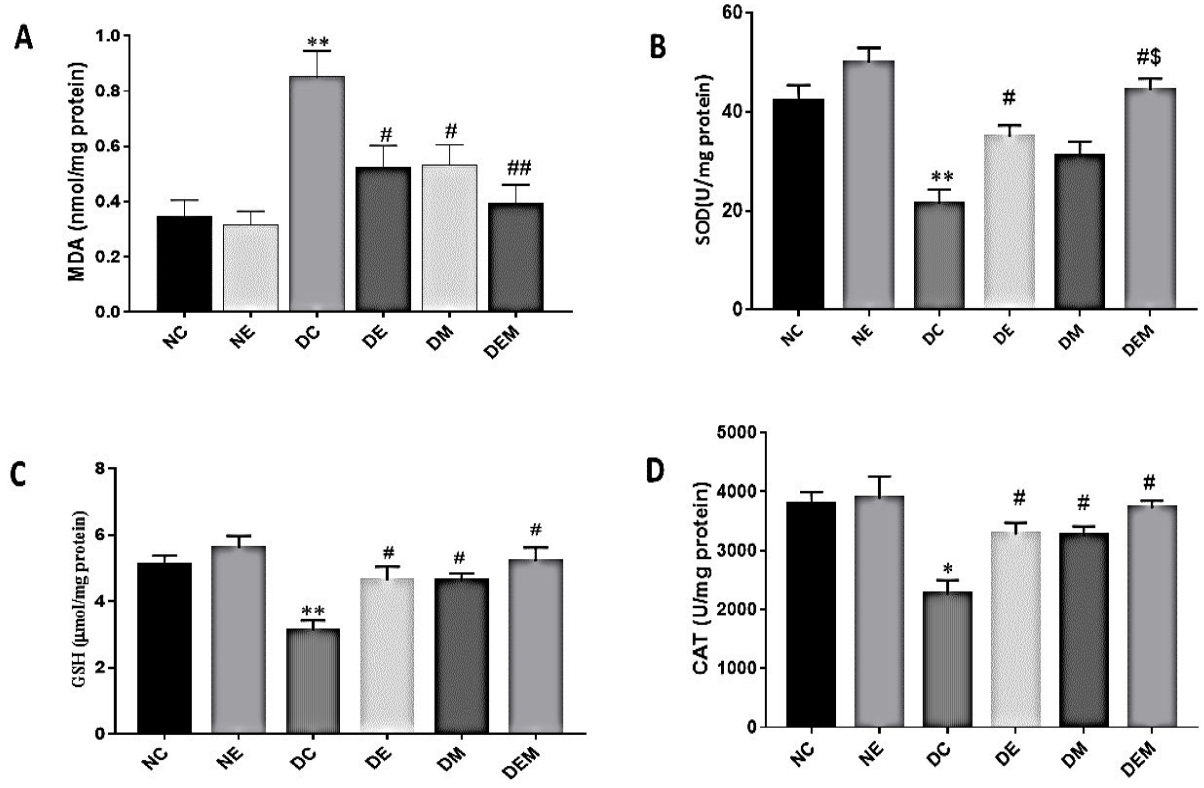

Figure 2a-d: Effect of L-egt, with or without metformin on Lipid peroxidation, antioxidant enzymes and glutathione in T2D-rats. (A) Malondialdehyde (MDA), (B) superoxide dismutase (SOD), (C) reduced Glutathione (GSH), and (D) Catalase (CAT). DM, diabetic + metformin group; NC, negative control; DC, positive control; NE, non-diabetic + L-egt; DE, diabetic + L-egt; DEM; diabetic + L-egt + metformin. **$p<0.01$, *$p<0.05$ vs NC; #$p<0.05$ vs DC, \$$p<0.05$ vs DM. (n=6).

Effects of L-egt with or without metformin on the levels of inflammatory biomarkers: TNF-α, MCP-1 and TGF-β1.

The concentration of TNF-α, MCP-1 and TGF-β1 in the liver homogenates after treatment with L-egt, with or without metformin, for seven weeks, was presented in figures 3a-c. DC group had a significant increase ($p<0.01$) in the concentration of TNF-α, MCP-1 and TGF-β1 vs. NC group. L-egt alone (DE) and its co-administration with metformin (DEM) to diabetic animals caused a significant reduction ($p<0.05$) in TNF-

α, MCP-1 and TGF-β1 vs. DC group. Also, DEM significantly reduced ($p<0.05$) TNF-α and TGF-β1 concentration with no change in MCP-1 when compared metformin alone (DM) group.

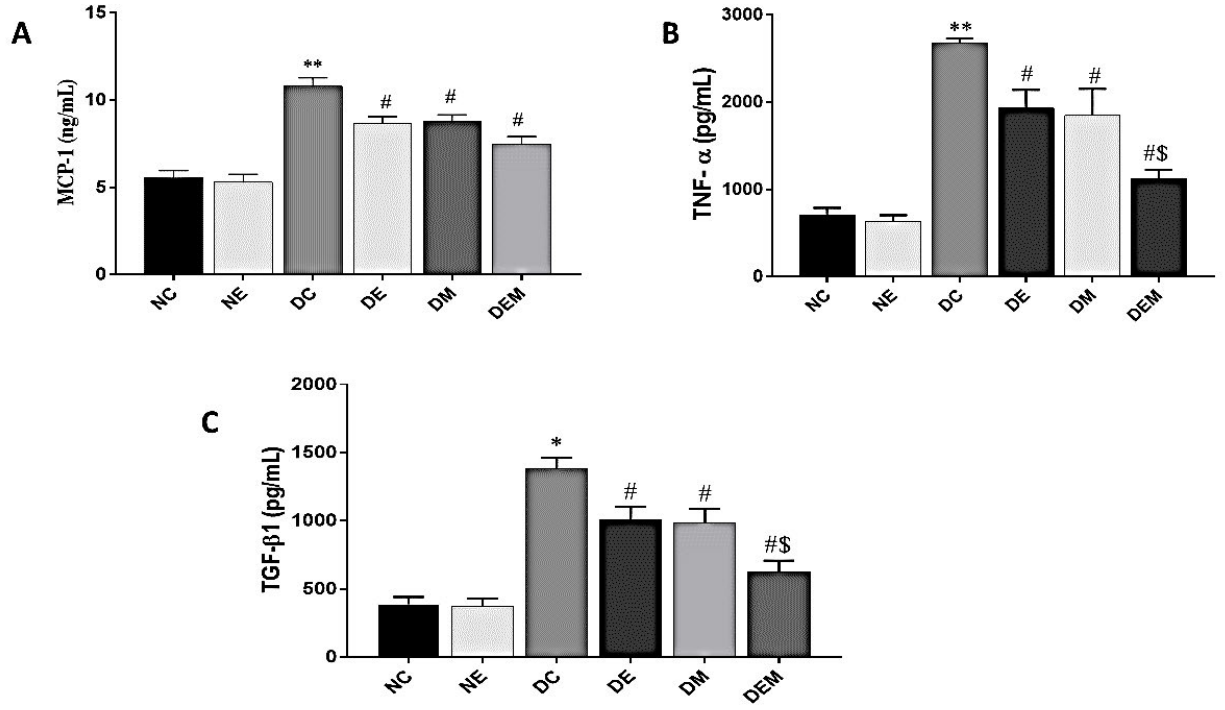

Figure 3a-c: Effect of L-egt, with or without metformin, on liver inflammatory biomarkers (A) Monocyte Chemotactic Protein 1 (MCP1), (B) Tumor Necrotic Factor- α (TNF-α), and (c) Transforming Growth Factor- β1 (TGF-β1) in T2D-rats. DM, diabetic + metformin group; NC, negative control; DC, positive control; NE, non-diabetic + L-egt; DE, diabetic + L-egt; DEM; diabetic + L-egt + metformin. **p<0.01, *p<0.05 vs NC; #p<0.05 vs DC, $p<0.05 vs DM. (n=6).

Effect of L-ergothioneine with or without metformin on liver mRNA relative expression of SREBP1c, FAS, NF-kB, TGFβ1, Nrf2, and Sirt1

The transcriptional levels of liver SREBP1c, FAS, NF-kB and TGF-β1 significantly increase ($p < 0.01$) in the DC group vs. NC group, while the administration of L-egt, with or without metformin, to diabetic rats, significantly reduced ($p<0.05$) the expression of these factors vs. DC rats (figures 4A and B). There was a significant decrease in mRNA expression of liver Sirt1 ($p < 0.05$) and Nrf2 ($p<0.01$) in the DC rats vs. NC rats (Fig. 4C). However, administration of L-egt, with or without metformin, to diabetic rats significantly increased ($p<0.05$) Nrf2 and Sirt1 mRNA levels vs. DC rats. Interestingly, co-administration of L-egt with metformin to diabetic rats (DEM group) significantly ($p < 0.05$) decreased SREBP1c and TGF-β1 but increases ($p<0.05$) Sirt1 mRNA expression vs. diabetic rats treated with metformin alone.

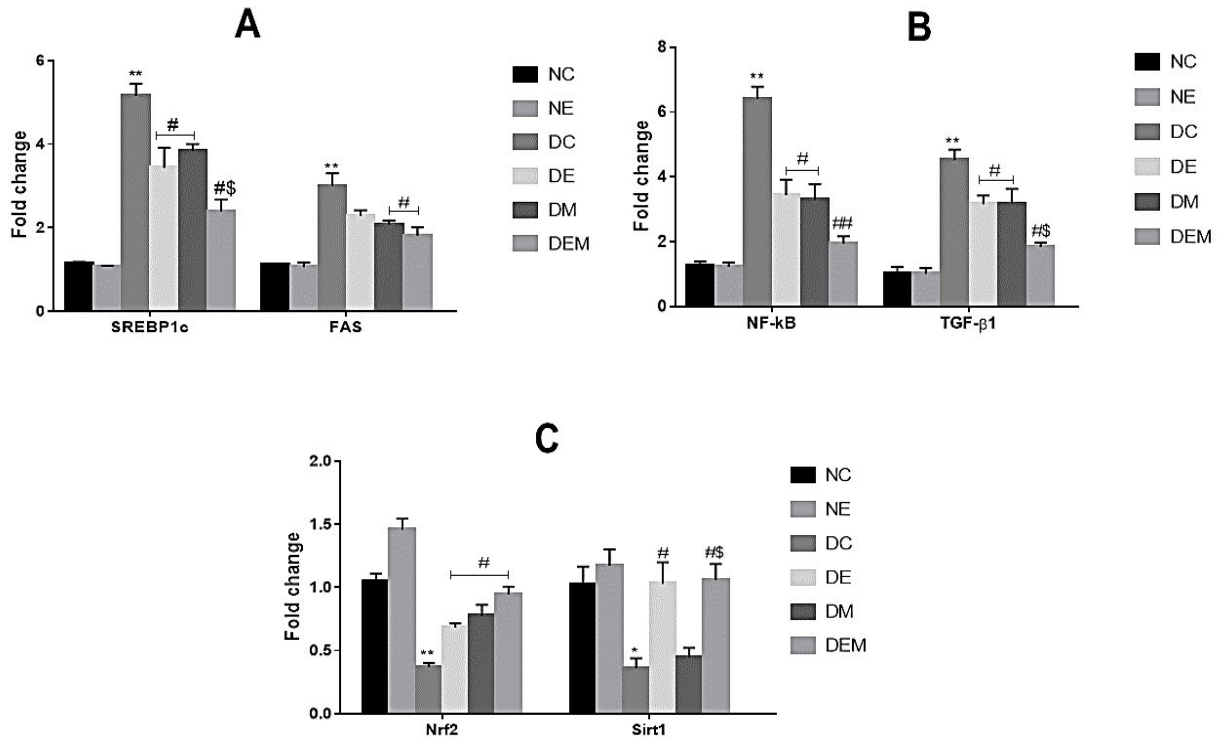

Figure 4a-c: Real time-PCR analysis of (A) Sterol Regulatory Element Binding Protein 1c (SREBP1c) and Fatty Acid Synthase (FAS), (B) Nuclear Factor- kappa B (NF-$_k$B) and transforming Growth Factor- β1 (TGF-β1), (C) Nuclear Factor Erythroid 2 related factor 2 (Nrf2) and Situin-1 (Sirt1), relative gene expression in liver tissues of diabetic animals treated with L-egt with or without metformin for 7 weeks. **p<0.01, *p<0.05 vs NC, #p<0.05, ##p<0.01 vs DC and $ p<0.05 vs DM.

Effect of L-egt with or without metformin on liver histopathological changes.

Morphological evaluation of the liver sections in the experimental animals after seven weeks of treatment showed that L-egt, with or without metformin, alleviates hepatic injury in T2D animals, as shown in fig 5A-F. The photomicrograph of liver sections in NC (Fig 5A) and NE (Fig 5B) groups showed normal liver histoarchitecture with normal morphology of the central vein and hepatic sinusoids. The liver section in the DC animal (fig 5C) showed liver injury characterized by disrupted hepatic sinusoids, congested central vein with mild hepatocyte degeneration compared to NC. Administration of L-egt (DE) or metformin (DM) only reduced sinusoid disruption and congestion of the central vein (fig 5D and 5E resp). In contrast, the liver section in DEM animals shows similar histoarchitecture with NC animals (fig 5F).

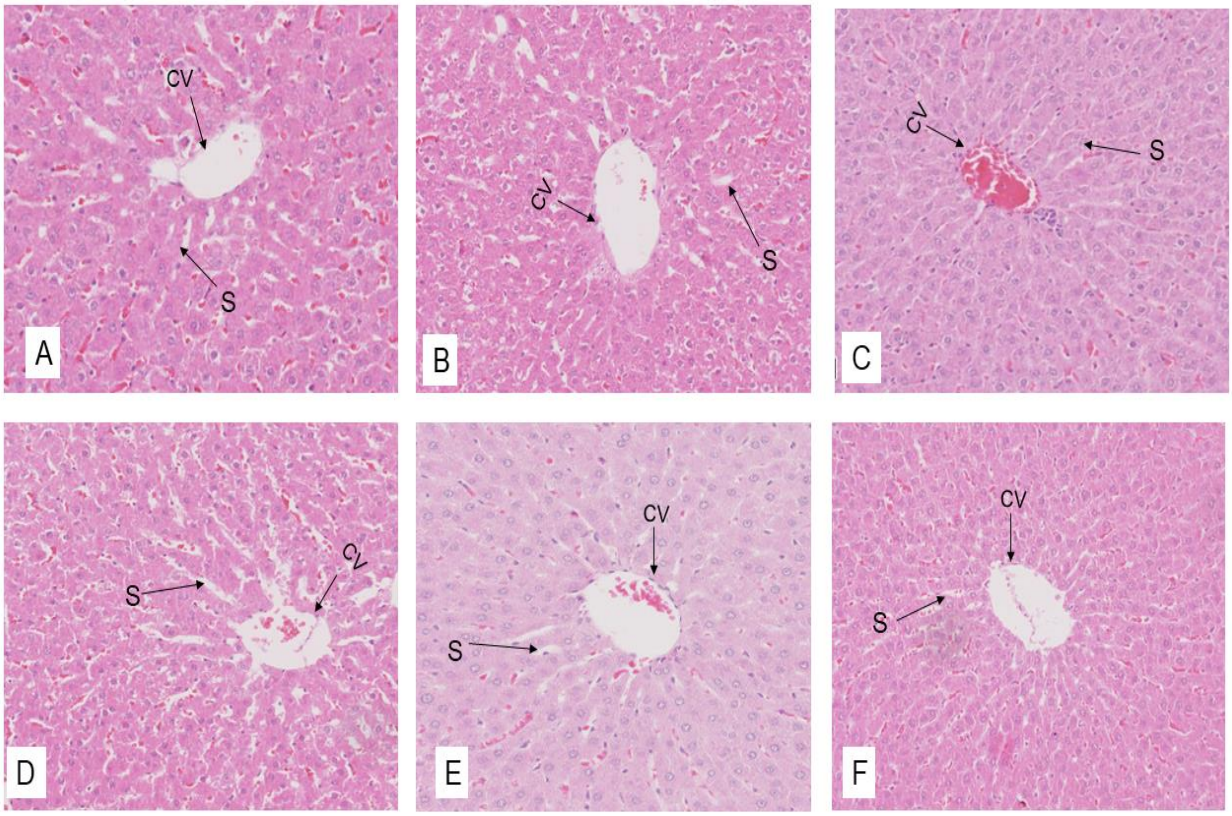

Figure 5A-F: Histoarchitecture of liver sections in T2D-rats administered L-egt with or without metformin (H&E 400X; 250µm). CV=central vein. S=sinusoid. (A) Negative control (NC), (B) Non-diabetic + L-egt (NE), (C) positive control (DC), (D) Diabetic + L-egt (DE), (E) Diabetic + Metformin (DM) and (F) Diabetic + L-egt + Metformin (DEM).

DISCUSSION

The aim of this study was to examine the role of L-ergothioneine, with or without metformin, on liver injury in a type-2 diabetic rat model. Both hyperglycemia and IR increase the production of reactive oxygen species (ROS) that alter the structure and functions of vital organs (including the liver) with the resultant pathogenesis of diabetic complications. In this regard, the use of natural compounds with significant bioactive potential have attracted greater interest due to their reduced side effect, increased accessibility and efficacy against the molecular and cellular triggers involved in diabetic complications (Choudhury et al. 2017; Gothai et al. 2016).

A significant reduction in body weight has been reported in poorly managed diabetes (Magalhães et al. 2019). A similar observation was reported in this study where the DC rats showed substantial weight

reduction compared to NC. This reduction could be correlated with metabolic derangements associated with poor glucose utilization. This metabolic derangement promotes excessive catabolism of adipose tissue and breakdown of structural proteins and reduced protein synthesis in all tissues, that caused muscle wasting. In this study, L-egt administration to diabetic rats, alone or in combination with metformin, improves body weight but not significant. This suggests that L-egt treatment (35mg/kg/day) for seven weeks could not fully compensate for the significant loss of adipose tissues and proteolysis, causing muscle wasting during diabetes. A previous study by (Marone et al. 2016) also reported no significant change in body weight over a 90-day administration course of L-egt.

In this study, liver hypertrophy was seen in the DC rats compared to the NC rats, and this was in accordance with other studies where increased liver weight was reported in diabetic rats (Ayepola et al. 2013; Zhang et al. 2019). The increased liver weight may result from IR and hypertriglyceridemia associated with liver steatosis and NAFLD with significant influx and accumulation of fatty acids into the liver cells (Mohamed et al. 2016; Zhang and Lu 2015). Administration of L-egt alone or with metformin to diabetic rats significantly reduced liver hypertrophy, suggesting that L-egt may reduce the influx of fatty acids into the liver or promote β-oxidation of fatty acids in the hepatic mitochondria. Thus, reducing liver hypertrophy by preventing excessive accumulation of fatty acids and triacylglycerol in the liver.

Effective regulation of fasting and postprandial blood glucose level plays a significant role in reducing diabetes-induced organ damage. In this study, co-administration of L-egt with metformin to diabetic rats enhanced the antihyperglycemic efficacy of metformin (a conventional drug used in the management of type-2 diabetes). This could be responsible for the reduced blood glucose observed in the DEM rat. Also, the reduced insulin resistance assessed by HOMA-IR in the DEM group indicates improved insulin sensitivity and glycemic control. Metformin reduces blood glucose by increasing insulin sensitivity and reducing hepatic gluconeogenesis via AMP-activated protein kinase (AMPK). These mechanisms promote glucose uptake into the cells and inhibit glycogenolysis in the liver (Foretz et al. 2019; Kim et al. 2008). Thus, L-egt supplementation during metformin therapy may upregulate this pathway to prevent hyperglycemia-induced liver damage. Also, the cytoprotective effect of L-egt on the liver may improve the antihyperglycemic efficacy of metformin because the liver is a major site of metformin activity where it reduces hepatic lipogenesis and improves fatty acid oxidation (Zheng et al. 2015).

Liver enzymes, including Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and alkaline phosphatase (ALP), are common biomarkers used to evaluate liver injury. These enzymes help catalyze critical chemical reactions (e.g., amino transfer) and their serum concentration increases during liver damage (Fu et al. 2020; Kwo et al. 2017). An increase in serum aminotransferase is mostly reported in type-2 diabetes due to membrane damage in the hepatocytes, while increased ALT level may also indicate fatty

liver resulting from reduced insulin sensitivity by the hepatocytes (Islam et al. 2020; Mandal et al. 2018). The increased serum level of ALP, AST and ALT recorded in this study indicates significant liver injury in the DC rats. Interestingly, L-egt with or without metformin attenuated liver damage, as evidenced by reduced serum level of liver enzymes. This result suggests hepatoprotective function, which may result from the ability of L-egt to enhance membrane integrity in the hepatocytes to reduce leakage of liver enzymes into circulation. Notably, there was no significant change in the serum level of liver enzymes in the NE rats when compared with NC rats suggesting that L-egt does not alter liver functions in the normal rats and provides further credence to its safety evaluation.

In this study, hypertriglyceridemia observed in DC rats indicates a substantial alteration in fatty acid metabolism, which is another risk factor for diabetic complications. This result is in accordance with similar studies that reported a significant association between increased serum triglycerides (TG) and type-2 diabetes (Rašković et al. 2019; Thambiah et al. 2016). The altered metabolism increased serum TG, which promotes the influx of free fatty acids into the systemic circulation with subsequent infiltration into the hepatocytes, as shown by the increased TG in the liver homogenates (Table 3). This may promote the influx of free fatty acids into the systemic circulation and infiltration into the hepatocytes with resultant steatosis or NAFLD. Besides, IR can inhibit lipoprotein lipase (LPL) that enhances the mobilization of TG to skeletal muscles and adipose tissue, thereby reducing the TG clearance from circulation (Arguello et al. 2015; Jiang et al. 2013). From this study, the significant reduction in serum and liver TG in diabetic animals treated with L-egt alone or in combination with metformin may result from the inhibition of *de novo* biosynthesis as well as enhance lipoprotein lipase activity to increase the TG clearance from the circulation thereby reducing the risk of fatty liver and related injuries (Alves-Bezerra and Cohen 2017; Kawano and Cohen 2013). Furthermore, the reduced TG infiltration into the hepatocyte in the L-egt treated groups may result from the downregulation of SREBP1c and FAS genes that promote TG synthesis, as shown in Fig 4a.

Chronic hyperglycemia has been reported to increase free radical production by the neutrophils and activated kupffer cells. Elevated free radicals (e.g., O_2^-, NO, OH^-) compromises the integrity of the cells via lipid peroxidation in the cell membrane, upregulate inflammatory signals and induce hepatic apoptosis as well as irreversible damage to other biomolecules in the body (e.g., protein, RNA). These biochemical events may result in significant structural and functional damage in the liver (Lucchesi et al. 2015; Masarone et al. 2018). In the present study, the reduced lipid peroxidation in the L-egt treated groups suggests that L-egt may inhibit oxidative degradation of the lipid bilayers in the cell membrane to prevent cell damage thereby improving cellular integrity and the viability of membrane proteins that help in cellular communication. The increased SOD, and CAT levels and glutathione level work synergistically to reduce the deleterious effect of free radicals in the tissues. An increased SOD level in the liver homogenates

facilitates the detoxification of free radicals by enhancing the conversion of superoxide into hydrogen peroxide and oxygen, while CAT helps to neutralize the free radicals by degrading hydrogen peroxide into water and oxygen molecule. Increased GSH reduces hydrogen peroxides to water and other lipid peroxides to alcohol, usually in the mitochondria and cytosol (Ighodaro and Akinloye 2018). In this study, L-egt with or without metformin enhanced the efficacy of these antioxidant enzymes in diabetic rats. Notably, L-egt has been hypothesized to exert potent antioxidant potential compared to other 'putative' compounds (such as ascorbic acid, α-Tocopherol) by penetrating the mitochondria via its specific transporter known as organic cation transporter (OCTN1), with reported evidence of its accumulation in the hepatic mitochondria to reduce free radical production from the mitochondria (Apostolova and Victor 2015; Kawano et al. 1982). The increased GSH concentration observed in non-diabetic and diabetic rats treated with L-egt gives further credence to this hypothesis. These results indicate that L-egt with or without metformin protects against oxidative damage by maintaining cell integrity and complement the efficacy of antioxidant enzymes against ROS. The improved antioxidant defense system observed in the L-egt treated group may result from the upregulation of Sirt1 and Nrf2 genes (Fig 4c), which are the major transcription factors that activates the antioxidant signaling pathways. Nrf2 is a master regulator of cellular antioxidant response that stimulates the production of phase II cytoprotective antioxidant genes (e.g., heme-oxygenase-1 and NAD(P)H: quinone oxidoreductase-1) and ROS-detoxifying enzyme (e.g., SOD, CAT, and GPx) that mediate redox balance (Ma 2013; Tonelli et al. 2017). Also, Sirt1 exerts a wide variety of biological functions, acting as a metabolic sensor that regulates lipid metabolism, inhibit free radical production from the respiratory chain, increase the detoxification of ROS by activating antioxidant enzymes and downregulate the NF-kB signaling pathway (Li 2014; Singh et al. 2018).

The suppression of inflammatory molecules in the liver cells in response to tissue injury has attracted significant interest in managing liver disease. Low-grade chronic inflammation in metabolic diseases, IR and their deleterious effect on liver function have been reported in previous studies (Hou et al. 2018; Ning et al. 2015). TNF-α is a proinflammatory cytokine implicated in the development of NAFLD and hepatic steatosis by mediating the inflammatory response to ROS-induced injury, promotes necrosis of the hepatocytes, and the concentration of TNF-α varies directly with the severity of the liver disease (Seo et al. 2013; Yang and Seki 2015). MCP-1 is a chemokine that promotes inflammatory reaction in the liver by recruiting activated monocytes during injury as well as increase proinflammatory cytokines and adhesion molecules (Mandrekar et al. 2011). TGF-β1, on its part, is a pleiotropic peptide that may upregulate the Smad-dependent signaling pathway in the liver to enhance the accumulation of fatty acids and induced cell death via ROS production (Nair and Nath 2020). Taken together, the reduced inflammatory and fibrotic markers in the liver homogenates of diabetic animals treated with L-egt and metformin shows that this

regimen may prevent hepatic inflammation and fibrosis, immune cell activation and apoptosis, as well as macrophage infiltration into the hepatocytes to promote structural and functional damage (Del Campo et al. 2018; Gehrke and Schattenberg 2020). This result is further supported by the downregulation of NF-kB-p65 and TGF-β1 gene expression in the L-egt treated groups. Thus, L-egt may protect against liver injury by reducing hepatic inflammation and fibrosis (Fabregat et al. 2016).

The histopathological examination of liver tissue provides essential information on the structural integrity of the liver. Disruption of the hepatic sinusoids and congested central vein with mild degeneration of the hepatocytes observed in the DC rats suggests significant liver damage. This result is consistent with other studies (Ogar et al. 2019; Rodríguez et al. 2018). However, improvement in the structural integrity of the diabetic liver treated with L-egt and metformin can be associated with the hepato-protective function of this treatment regimen. The regular appearance of hepatic sinusoids and central veins in the DEM rats may enhance adequate blood supply from the hepatic artery and portal vein towards the central veins, thus preventing ischemic cell death.

In conclusion, this study showed that L-egt may protect liver function during diabetes by alleviating oxidative damage by upregulating Sirt1/Nrf2 expression and its downstream antioxidant molecules, downregulate NF-kB and TGF-β1 mRNA expression to reduce hepatic inflammation and fibrosis as well as reduce SREBP1c and FAS expression to lower hypertriglyceridemia and attenuating hepatocyte fatty acid accumulation in diabetic rats. Thus, supplementation of L-egt could be used as an adjuvant regimen with metformin therapy in the early stage of diabetes to prevent the development or progression of liver complications associated with diabetes. However, in vitro analysis and evaluation of L-egt transporters were not done due to limited funding. Thus, further studies to evaluate the status of L-egt transporters, protein expression of various transcription factors and in vitro study are required to provide the detailed mechanism of action of L-egt.

AUTHOR STATEMENT

AD: conceptualization, methodology, formal analysis, writing original draft, funding acquisition.
MC: Writing-editing, supervision, and funding acquisition.

AN: Methods, writing-editing, supervision, and funding acquisition.

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COMPETING INTEREST

The authors declare that there is no competing interest.

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CHAPTER 3

PROLOGUE

It has been reported that most patients with type-2 diabetes already have renal complications (characterized by microalbuminuria and reduced glomerular filtration rate) at the time of diagnosis, contributing to the increasing prevalence of chronic kidney failure (CKD). Despite the available treatment options, patients remain at high risk of this complication even during intensive therapy. Therefore, developing a therapeutic regimen that can effectively alleviate renal complications associated with diabetes is an active area of research. Recently, combination therapies have been reported to exert potent therapeutic effects when compared to monotherapy. Thus, attention is drawn towards using bioactive compounds with numerous therapeutic potentials to complement the efficacy of available pharmacological interventions. L-egt has been approved by FDA and EFSA to be used as a supplement. At the same time, studies have shown that L-egt possesses biological potentials to mitigate the risk factors (such as oxidative stress, inflammation, hypertriglyceridemia) that are implicated in the pathogenesis and progression of diabetes-induced renal damage. Therefore, Chapter 3 of this study evaluates the effects of L-egt, alone or in combination with metformin, on renal complications associated with type-2 diabetes using an experimental rat model.

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Manuscript-2

L-ergothioneine and its combination with metformin attenuates renal dysfunction in type-2 diabetic rat model by activating Nrf2 antioxidant pathway.

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ABSTRACT

L-ergothioneine (L-egt) is a bioactive compound recently approved by the food and drug administration as a supplement. L-egt exerts potent cyto-protective, antioxidant and anti-inflammatory properties in tissues exposed to injury, while metformin is a first-line prescription in type-2 diabetes to regulate blood glucose. Therefore, the present study investigated the protective effect of L-egt alone, or combined with metformin, on renal damage in a type-2 diabetic (T2D) rat model. T2D was induced in male Sprague-Dawley rats using the fructose-streptozotocin rat model. L-egt administration, alone or combined with metformin, began after confirming diabetes, and was administered orally for seven weeks. After the experiment, all animals were sacrificed by decapitation, blood samples were collected, and both kidneys were harvested. Biochemical analysis, Enzyme-link Immunoassay (ELISA), Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), western blotting, and histological analyses were done to evaluate various biomarkers and structural changes associated with renal damage. Untreated diabetic rats showed loss of kidney functions characterized by increased serum creatinine, blood urea nitrogen, proteinuria, triglycerides, lipid peroxidation, inflammation, and decreased antioxidant enzymes. Histological evaluation showed evidence of fibrosis, mesangial expansion, and damaged basement membrane in the nephrons. However, L-egt alleviates these functional and structural derangements in the kidney, while co-administration with metformin reduced hyperglycemia and improves therapeutic outcomes. Furthermore, L-egt treatment significantly increased the expression of major antioxidant transcription factors, cytoprotective genes and decreased the expression of inflammatory genes in the kidney. Thus, combining L-egt and metformin may improve therapeutic efficacy and be used as an adjuvant therapy to alleviate renal damage in type-2 diabetes.

Keywords: Diabetes, L-ergothioneine, metformin, kidney, antioxidants, cytoprotection.

1.0 INTRODUCTION

Type-2 diabetes (T2D) is a metabolic disorder exerting a heavy toll on both the individual and society with several complications, including nephropathy (1). Diabetic nephropathy (DN) is a major microvascular complication that causes chronic kidney disease (CKD) and eventually leading to end-stage renal disease (ESRD) requiring renal hemodialysis therapy or kidney transplant (2, 3). DN develops in approximately 30-40% of patients with diabetes and progresses with time, characterized by increased urinary albumin excretion, decreased glomerular filtration rate (GFR), and increased peripheral arterial blood pressure (4, 5). The pathogenesis of DN in T2D involves a complex and multifactorial process resulting from hyperglycemia and insulin resistance (IR). The persistent hyperglycemia generates excess free radicals (e.g., ROS) that overwhelms the antioxidant defense system, resulting in oxidative injury that promotes renal inflammation and mitochondrial dysfunction (6, 7). Insulin resistance also causes dyslipidemia associated with progressive loss of renal functions via transforming growth factor- β (TGF-β) signaling pathway. (8, 9). These biochemical processes stimulate different cellular signals that damage vital biomolecules and cellular components of the kidney (including DNA, proteins, podocyte, mesangial and tubular cells), exerting significant abnormalities on renal structure and function with subsequent ESRD (10). Therefore, the prevention and management of DN should be multi-targeted, focusing on cellular and molecular switches involved in the pathogenesis of DN.

The significant role of increased ROS activity in DN calls for a refined approach to antioxidant therapy, with several reports supporting the hypothesis that bioactive compounds with potent antioxidant activities can delay the development and halt the progression of DN (11, 12). L-ergothioneine (2-mercaptohistidinetrimethylbetaine) is a bioactive compound obtained solely from diets (such as mushroom, black bean, red beans, and certain meat products) and recently approved by the Foods and Drugs Administration (FDA) and European Food Safety Authority (EFSA) to be used as supplements (13, 14). L-egt possesses antioxidant and anti-inflammatory properties, while its accumulation at the sites of tissue injury has been hypothesized as an adaptive mechanism of protecting tissues at risk of damage and regenerating injured tissues (15, 16). Also, L-egt has been reported to activate the Nrf2 (nuclear factor erythroid-2 related factor-2) antioxidant signaling pathway to protect against cellular injury, enhance glutathione level and reduce oxidative damage in the kidney as well as activates sirt1 and 6 to protect against high glucose-induced cell senescence (17-19). Furthermore, coadministration of L- egt with existing therapy (such as melatonin and hispidin) significantly increases treatment benefits both in vitro and in vivo (20, 21), suggesting that L-egt may improve the efficacy of available treatment options. In addition, it has

been shown that the reno-protective effect of metformin goes beyond its antihyperglycemic effect; metformin alleviates oxidative stress, suppresses TGF-β1 inflammatory pathway, and attenuates apoptosis (22, 23). However, the effect of L-egt and its co-administration with metformin against renal complications associated with diabetes is yet to be established. Thus, this study was designed to investigate the effect of L-egt, with or without metformin, on renal dysfunctions associated with diabetes in a rat model of type-2 diabetes.

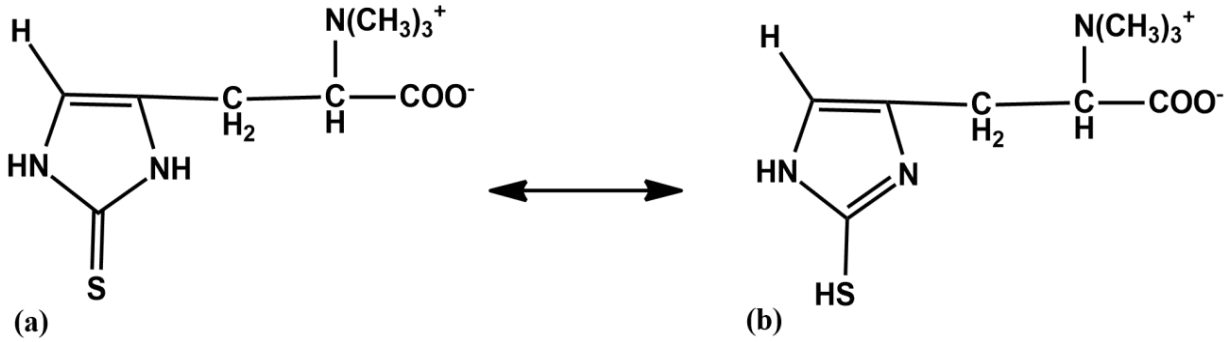


Fig 1: Chemical structure of thione-thiol tautomers of L-ergothioneine. In solution at physiological pH, Egt exists predominantly in the thione (a) rather than the thiol (b) (15).

2.0 MATERIALS AND METHODS

2.1 Drugs and Chemicals

Pure L-ergothioneine was sponsored by Tetrahedron (Paris, France www.tetrahedron.fr). Streptozotocin (STZ) was obtained from Sigma-Aldrich (St., Louis, MO, USA). Acucheck glucose strips and metformin (Austell Laboratories Pvt. Ltd.) were purchased from Pharmed Ltd., (Durban, South Africa. Fructose (Radchem (PTY) Ltd) was purchased from Laboquip (Durban, South Africa). All chemicals and reagents used in this study were analytical grades and available commercially.

2.2 Animals

Thirty (30) adult male Sprague-Dawley rats weighing (175±20) g were used in this study. The animals were obtained from the Biomedical Research Unit (BRU), Westville Campus, University of KwaZulu-Natal, Durban, South Africa. The animals were allowed to acclimatize to standard laboratory conditions (temperature 23 ± 1°C, 40–60% humidity) and 12 h light-dark cycles with free access to standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* for one week before the experiment. All animal protocol was done according to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the University of KwaZulu-Natal Animal Research Ethics Committee, Durban, South Africa, with ethical clearance (AREC/006/019D).

2.2.1 Induction of experimental diabetes

Type-2 diabetes rat model was induced using fructose plus a low dose of streptozotocin model reported previously by (24). Briefly, after acclimatization, the animals were randomly divided into two major groups: non-diabetic (n=6) and diabetic groups (n=24). The diabetic group was supplied with 10% fructose solution *ad libitum* in drinking water for 14 days to induce insulin resistance, while the non-diabetic groups were supplied with distilled water. After 14 days, all animals were fasted for 8-10 hours before intraperitoneal injections. The diabetic groups were injected a single low dose of streptozotocin (STZ) (40mg/kg bwt) dissolved in freshly prepared 0.1M citrate buffer (pH 4.5), while the non-diabetic groups were injected with the same volume of citrate-buffer only. One week after STZ injection, non-fasting blood glucose (NFBG) levels were measured in all animals using a portable glucometer Accu-Chek Active (Roche Diagnostics GnbHD-68298 Mannheim, Germany) in the blood collected from the tail vein. Animals with NFBG levels> 16.7 mmol/L were considered diabetic (25) and included in the study.

2.3 Experimental protocol

After the induction of diabetes, the diabetic rats were randomly divided into four groups (n=6) and treated as follows.

- Non-diabetic control (NC) - 1ml/100g bwt of distilled water.
- Diabetes control (DC) - 1ml/kg bwt of distilled water.
- Diabetes + L-egt (DE) - 35mg/kg bwt of L-egt.
- Diabetes + metformin (DM) - 500mg/kg bwt of metformin.
- Diabetes + L-egt + metformin (DEM).

Distilled water, L-egt, and metformin were administered orally for seven weeks after confirmation of diabetes. In addition, body weight was recorded every week. Dosage of L-egt was selected based on distribution and accumulation of dietary ergothioneine in mouse tissues and in accordance with previous studies that used this nutraceutical (26, 27).

2.4 Collection of samples

After seven weeks of treatment, the rats were placed individually in a metabolic cage for 24 hours to measure water intake and collect urine samples using a sterile container. Urine volume was recorded, and the collected samples were centrifuged at 2000rpm, 10mins at 4^0C to remove any suspended particles. The urine samples were then aliquoted in Eppendorf tubes and stored at -20^0C until analysis. Rats were sacrificed by decapitation, and blood samples were collected into a serum vacutainer bottle; centrifuged at 3000rpm for 10 mins to obtain serum and stored in the bio-freezer at -80^0C until used for analysis. The kidneys were rapidly excised, cleaned of adhering tissues, weighed, and rinsed in normal saline. The right

kidney was rapidly frozen in liquid nitrogen and stored at –80°C until used for analysis, while the left kidney was fixed in 10% neutral-buffered formalin histopathological analysis.

2.4.1 Preparation of kidney homogenates

The kidney samples were thawed and homogenized in 10% phosphate buffer (0.1M, pH7.4). The homogenates were vortexed and centrifuged at 600g for 10min to remove cell debris. The supernatant was subsequently centrifuged at 10,000g for 20mins to obtain the cytosolic fraction and used for biochemical analyses.

2.5 Assessment of body weight, kidney weight, and blood glucose

The bodyweight of all animals was recorded weekly. The kidney index was assessed by calculating the kidney-to-bodyweight ratio. Non-fasting blood glucose levels were recorded before and after the experiment using a glucometer (Accu-Chek Performa, USA).

2.6 Biochemical analysis

Serum and urine samples were analyzed at Global Clinical and Viral Laboratories (Amanzimtoti, South Africa) for quantification of serum creatinine (sCr), Blood Urea Nitrogen (BUN), and Urinary albumin (Ualb), Urine creatinine (UCR), and triglyceride (TG) using a biochemical analyzer (BCA-733 plus Semi-auto Biochemical Analyzer). Urine protein was quantified by Bradford assay (Sigma Aldrich Chemical Company, Missouri, and St Louis, USA). The renal index was estimated by calculating the ratio of kidney weight to body weight. Creatinine clearance was used to estimate glomerular filtration rate and was calculated using the formula:

$$\text{CrCl (ml/min)} = \frac{\text{Urine creatinine (mg/dl) x urine volume (ml/24hrs)}}{\text{Serum creatinine (mg/dl)} \times 60\text{mins} \times 24\text{hrs}}$$ (28).

2.6.1 Evaluation of lipid peroxidation and antioxidant enzymes

The concentration of malondialdehyde (MDA), a marker of lipid peroxidation, was evaluated by measuring the content of thiobarbituric acid (TBA) reactive product in the kidney homogenates using the protocol previously described by Mkhwanazi et al. (29). The concentration of antioxidant enzymes (SOD, GSH, and CAT) was also measured in the kidney homogenates by spectrophotometric assay. Reduced-GSH and SOD levels were assessed using the method Ellman, (30) and Marklund, (31). CAT activity was analyzed following the method reported by Aebi, (32).

2.6.2 Evaluation of renal inflammation and fibrosis biomarkers

Pro-inflammatory cytokine, tumor necrosis factor –α (TNF–α); chemokine, monocyte chemotactic protein-1 (MCP-1) and fibrotic cytokine, tumor growth factor-β (TGF-β) were quantified in the kidney homogenates using their specific ELISA kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) according to the manufacturer's protocol. Absorbance was measured using the microplate reader, SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenburg, LGBW, Germany).

2.6.3 Histopathological examination of the kidney

Kidney samples were fixed in 10% neutral-buffered formalin. Specimens were then dehydrated in a graded series of ethanol cleared in xylene and embedded in paraffin wax. The samples were then cut into slices (5-µm thick) using a micron rotary microtome and stained with hematoxylin and eosin (H&E). Kidney sections were also sent to an accredited laboratory (Lancet laboratory, South Africa) for Periodic Acid Schiff (PAS) and Masson's trichome special stains. The stained sections were visualized using a nanozoomer S360 digital slide scanner (Hamamatsu Photonics, Japan) and nanozoomer digital pathology version 2.8 software for analysis by a pathologist. H&E-stained sections were used to determine the degree of damage in the kidney tissue; PAS and Masson trichome stained were used to evaluate hyperplasia in the glomerular mesangial area and renal fibrosis, respectively. The percentage of collagen deposit (used as a biomarker of renal fibrosis) was quantified using ImageJ 1.53e software (NIH, USA). Mesangial matrix index was calculated and expressed in percentage.

$$\text{Mesangial matrix index (\%)} = \frac{\text{Mesangial matrix area}}{\text{Total glomerular area}} \times 100$$

Collagen fibers were stained green. Twenty glomeruli were randomly selected from each section, and the green stained collagen area in each glomerulus was scored by a pathologist blinded to the study using the method (33): 0 = <25% stained collagen area; 1 = 25- 50% stained collagen area; 2 = 50-75% stained collagen area; and 3 = >75% stained collagen area. Ten to twelve fields of images were selected in the H&E-stained section at x200 magnification to measure the tubular diameter. ImageJ analysis software was used to measure the diameters of the renal tubules.

2.6.4 Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol reagent (40mg of tissue/mL, Trizol reagent) was used to extract total RNA from the kidney. The quality and quantity of isolated RNA were evaluated by measuring absorbance at 260/280nm with a nanodrop ND-1000 spectrophotometer (Thermo scientific, Johannesburg, South Africa). After that, iScript

cDNA synthesis kit, Life Science research (Biorad, South Africa), was used to convert the RNA into cDNA according to the manufacturers' instructions.

Real-time qPCR of samples was used to determine the mRNA expression level of Nrf2, Ho-1, NQO-1, sirt1, NF-kB, TGF-β1, fibronectin, and GAPDH using iTaq Universal SYBR Green PCR master mix analysis (Biorad, CA, USA) on a light cycler 96 RT-PCR system (Roche, Mannheim, Germany). RT-qPCR was performed in a 10-µL reaction volume containing 5-µL SYBR Green Master Mix, 1-µL of each primer, 1-µL of nuclease-free water, and 2-µL of cDNA template. The primer sequences are shown in Table-1. The purity and specificity of amplified PCR products were verified by melting curves generated at the end of each PCR. Data from the real-time analysis were calculated using the v1.7 sequence detection software from PE Biosystems (Foster City, CA). Relative mRNA expression of the genes of interest was calculated using the $2^{-\Delta\Delta ct}$ method (34) and normalized in relation to the expression of the endogenous control, GAPDH. The primers sets were homology searched using an NCBI BLAST search to ensure that they were specific.

Table-1: Nucleotide sequence, accession number, and product size

Genes	Primer sequence	GenBank Accession number	Product size (bp)
GAPDH	F: 5′-TTCAACGGCACAGTCAAGG-3' R: 5′-CGGCATGTCAGATCCACAA-3'	NM_017008.4	578
HO-1	F: 5′-CGACAGCATGTCCCAGGATT-3′ R: 5'-TCGCTCTATCTCCTCTTCCAGG-3.'	NM_012580.2	184
NQO-1	F: 5'-CATTCTGAAAGGCTGGTTTGA-3' R: 5'-CTAGCTTTGATCTGGTTGTCG-3'	NM_017000.3	486
Nrf_2	F: 5'-GCCAGCTGAACTCCTTAGAC-3' R: 5'-GATTCGTGCACAGCAGCA -3'	NM_031789.2	466
Sirt1	F: 5'- CCCAGATCCTCAAGCCATGTTC-3' R: 5'- TGTGTGTGTGTTTTTCCCCC-3'	NM_001372090.1	119
Fibronectin	F: 5'-GTGGCTGCCTTCAACTTCTC-3' R: 5'-AGTCCTTTAGGGCGGTCAAT-3'	XM_006245158.4	231
TGFβ1	F: 5'-GGGCTACCATGCCAACTTCTG-3' R- 5'- GAGGGCAAGGACCTTGCTGTA-3'	NM_021578.2	82
NF-kB	F: 5'-ACGATCTGTTTCCCCTCATCT-3' R: 5'- TGCTTCTCTCCCCAGGAATA-3'	NM_199267.2	150

2.6.5 Western blot analysis of Nrf2 protein expression

Total protein was extracted from the kidney tissues using RIPA cell lysis buffer. Nrf2 protein expression was evaluated by western blotting technique. Beta-actin was used as the housekeeping protein. The amount of protein in the lysate was quantified using an RC DC protein assay kit (Biorad, CA, USA). An equal amount of protein was separated by electrophoresis using 8-16% SDS-PAGE (Bio-Rad, California, USA) at 100V, 25mA for 90mins, and transferred to a PVDF membrane (Amersham Biosciences). After transfer, the membrane was blocked with intercept (PBS) blocking buffer (Celtic Molecular diagnostic, South Africa) for 2 hours at room temperature with gentle shaking. Then, the membrane was washed with PBST and incubated overnight at 4^0C with mouse anti-Nrf2 antibody (ab89443, 1:500 dilution) and β-actin (1:2000). Next, the membrane was washed three times with PBST and later incubated with a secondary antibody, IRDye 680, 1:15,000 dilution (Li-Cor Bioscience, Lincoln, USA) in a blocking buffer containing 0.2% Tween-20 for one hour at room temperature in the dark. The protein bands were detected using an Odyssey CLx imaging system (Li-Cor Bioscience, Lincoln, United States), and the intensities were quantified using Odyssey image studio v2.1. Results were expressed as the ratio of Nrf2/ β-actin.

2.7 Statistical analysis

Data were presented as the mean ± SEM and analyzed by GraphPad Prism version 7 (GraphPad, San Diego, CA) using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison tests to determine differences between groups. $P < 0.05$ was considered statistically significant.

3.0. RESULTS

3.1 Effect on kidney hypertrophy, Fasting blood glucose, water intake, and triglyceride level

The effect of L-egt, with or without metformin, on kidney hypertrophy (KW/BW), blood glucose, water intake, and TG after seven weeks of treatment is presented in Figure 2a-d. Kidney hypertrophy was expressed as a percentage ratio of kidney weight to body weight. DC animals showed a significant increase ($p<0.05$) in kidney hypertrophy vs. NC animals, while the administration of L-egt with or without metformin to diabetic rats significantly reduced ($p<0.05$) kidney hypertrophy vs. DC rats. There was a significant increase ($p<0.001$) in blood glucose in the DC animal vs. NC animals. While the administration of L-egt alone (DE) showed a non-significant decrease ($p>0.05$) in blood glucose, coadministration with metformin (DEM group) caused a significant decrease in blood glucose level vs. DC rats. There was a significant increase ($p<0.01$) in serum TG in DC animals vs. NC animals, while L-egt administration with or without metformin caused a significant reduction ($p<0.05$) in TG level vs. DC animals.

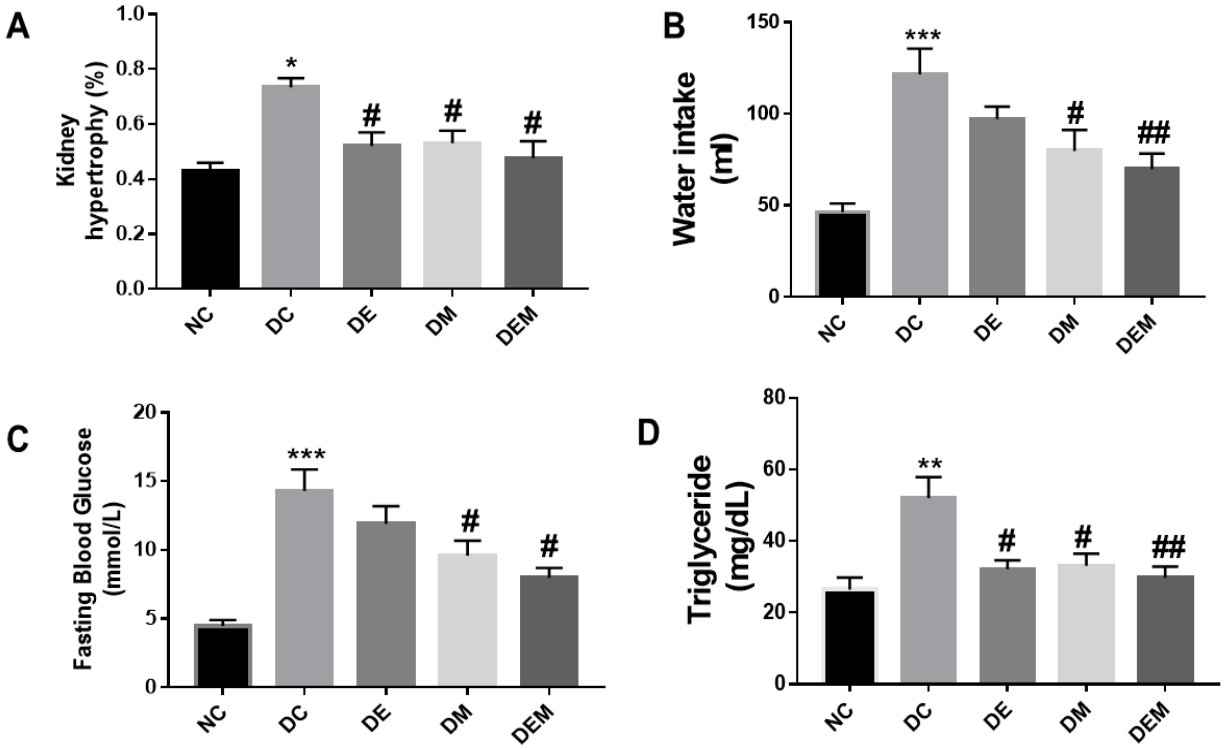
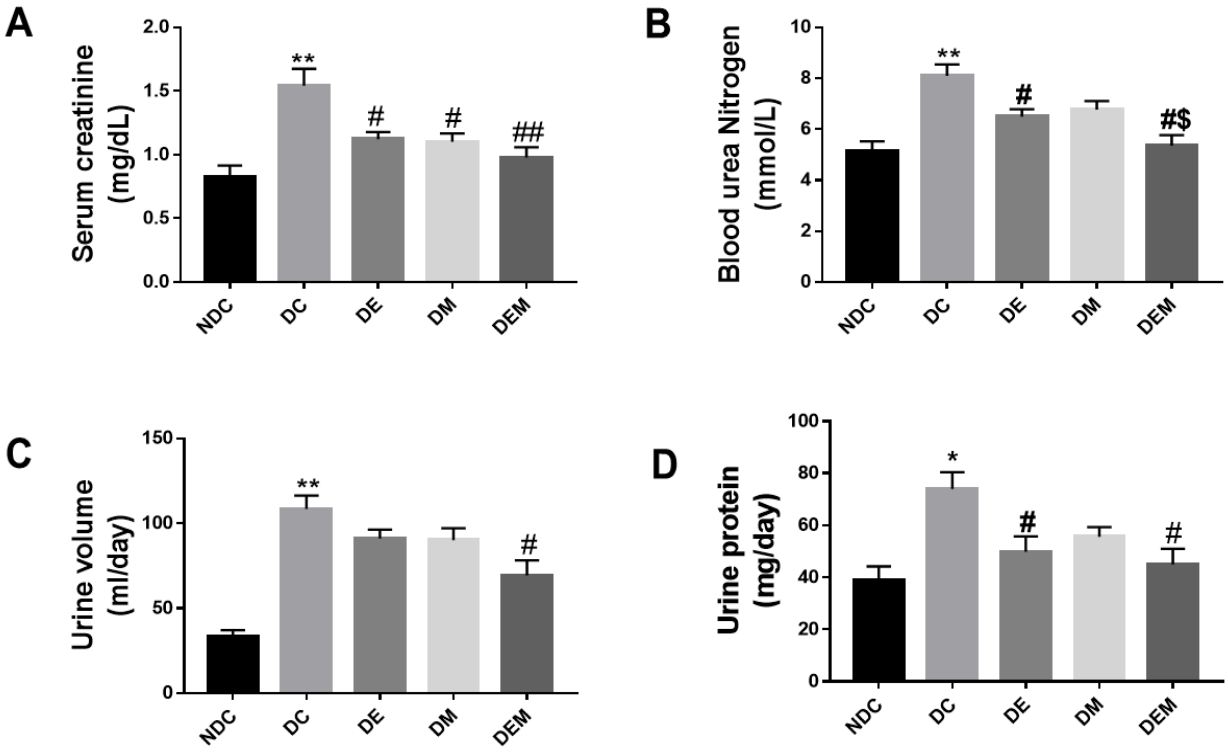


Figure 2a-d: Effect on kidney hypertrophy, blood glucose, water intake and triglyceride level. *P<0.05, **p<0.01, ***p<0.001 vs NC while $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs DC and $^{\$}$p<0.05 vs DM. (n=6). KW/BW= Kidney weight: body weight, FBG= fasting blood glucose, TG- triglycerides. Non-diabetic control (NDC), Diabetic control (DC), Diabetic treated plus L-egt only (DE), Diabetic plus metformin only (DM), Diabetic plus L-egt and metformin (DEM).

3.2 Effect on biomarkers of renal function

The effects of L-egt treatment on biomarkers of renal function after seven weeks of administration are presented in Table 3. Serum creatinine (sCr) and blood urea nitrogen (BUN) in the DC animals significantly increased (p<0.01) vs. NC animals. However, there was a significant reduction (p<0.05) in these serum biomarkers after seven weeks of administering L-egt vs DC animals. Interestingly, coadministration of L-egt with metformin (DEM) caused a significant reduction (p<0.05) in BUN vs diabetic rats treated with metformin only (DM). Furthermore, DC animals showed a significant increase in urinary protein (p<0.05), urine albumin (p<0.05), and urine volume (p<0.01) vs NC animals. However, L-egt treatment, with or without metformin, to diabetic animals significantly reduced (p<0.05) urine protein and albumin vs. DC,

while urine volume decreased significantly ($p<0.05$) in the DEM group vs DC group. Creatinine clearance (CrCl) decreased significantly ($p<0.01$) in the DC vs. NC animals. Treatment with L-egt alone caused a non-significant ($p>0.05$) increase in CrCl similar to metformin vs. rats, while coadministration of both drugs caused a significant ($p<0.05$) increase in Ccr. Kidney injury molecule-1 (KIM-1) concentration in the kidney homogenates of DC animals significantly increased ($p<0.05$) vs. NC animals. However, administration of L-egt with or without metformin to diabetic rats caused a significant decrease ($p<0.05$) in renal KIM-1 concentration compared with DC rats.



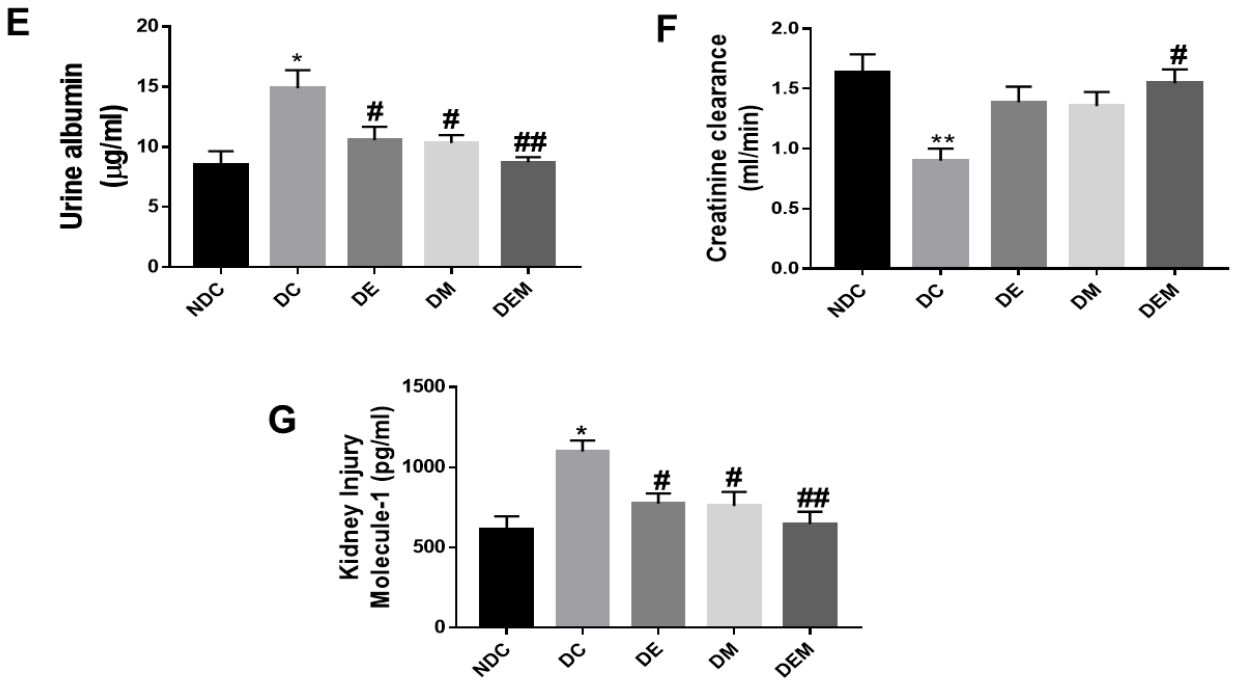


Figure 3a-g: Effect of L-egt alone with or without metformin on biomarkers of renal functions $^{*}p<0.05$, $^{**}p<0.01$ vs NC while $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs DC and $^{\$}p<0.05$ vs DM. (n=6). (A) Serum creatinine, (B) Blood urea nitrogen, (C) urine volume, (D) urine protein, (E) urine albumin, (F) creatinine clearance, and (G) Kidney injury molecule 1. Non-diabetic control (NDC), Diabetic control (DC), Diabetic treated plus L-egt only (DE), Diabetic plus metformin (DM), Diabetic plus L-egt, and metformin (DEM).

3.3 Effect on lipid peroxidation and antioxidant biomarkers

Figure 4a-d shows the effect of L-egt on kidney malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), and catalase (CAT) level in the kidney homogenates after seven weeks of treatment. There was a significant increase ($p<0.01$) in MDA level in the DC group vs. NC group, while L-egt administration, with or without metformin, to diabetic rats, significantly reduced MDA levels (DE: $p<0.05$; DEM: $p<0.01$) vs. DC group. In addition, antioxidant enzymes (SOD, GSH, and CAT) were significantly reduced ($p<0.05$) in the DC group vs. NC group. However, administration of L-egt with or without metformin to diabetic rats significantly increased ($p<0.05$) SOD and GSH level vs. DC group, while CAT level significantly increased ($p<0.05$) in the DEM group vs DC group. Interestingly, coadministration of L-egt with metformin to diabetic rats (DEM group) significantly increased ($p<0.05$) GSH level vs. DM group.

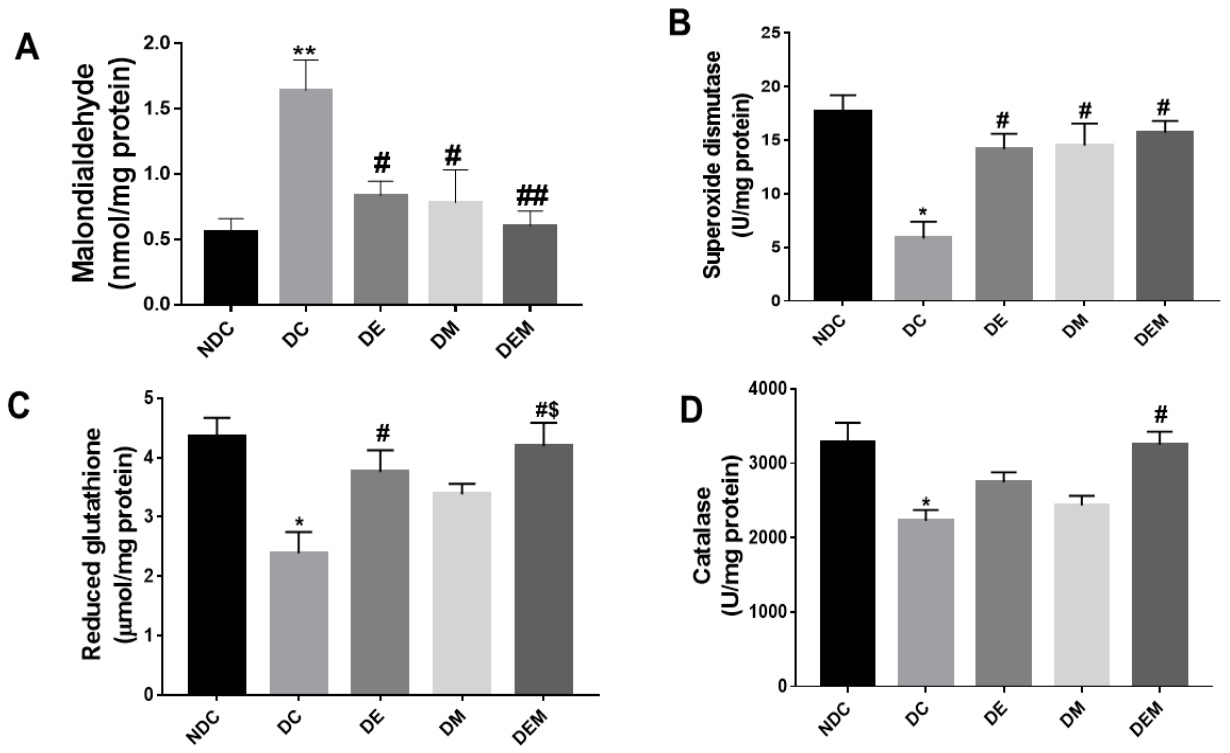


Figure 4a-d: Effect of L-egt alone with or without metformin on lipid peroxidation and antioxidant biomarkers. $^{*}p<0.05$, $^{**}p<0.01$ vs NC while $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs DC and $^{\$}p<0.05$ vs DM. (n=6). Non-diabetic control (NC), diabetic control (DC), Diabetic treated plus L-egt only (DE), Diabetic plus metformin (DM), Diabetic plus L-egt, and metformin (DEM). (A) Malondialdehyde (MDA), (B) Superoxide dismutase (SOD), (C) Reduced glutathione (GSH), and (D) Catalase (CAT).

3.4. Effect on Inflammatory biomarkers

Effects of L-egt, with or without metformin, on tumor necrotic factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and Transforming growth factor (TGF-β1) level in the kidney homogenates after seven weeks of treatment is presented in Figure 5. There was a significant increase in TNF-α, MCP-1, and TGF-β1 ($p<0.05$) in the DC rats vs. NC rats. Conversely, L-egt administration to diabetic rats (DE) significantly reduced TNF-α and TGF-β1 levels ($p<0.05$) with a non-significant decrease in MCP-1 level ($p>0.05$) vs. DC rats. Interestingly, coadministration of L-egt with metformin (DEM) to diabetic animals significantly reduced inflammatory biomarkers (TNF- α: $p<0.05$, MCP-1: $p<0.05$; TGF- β1: $p<0.01$) vs. DC rats.

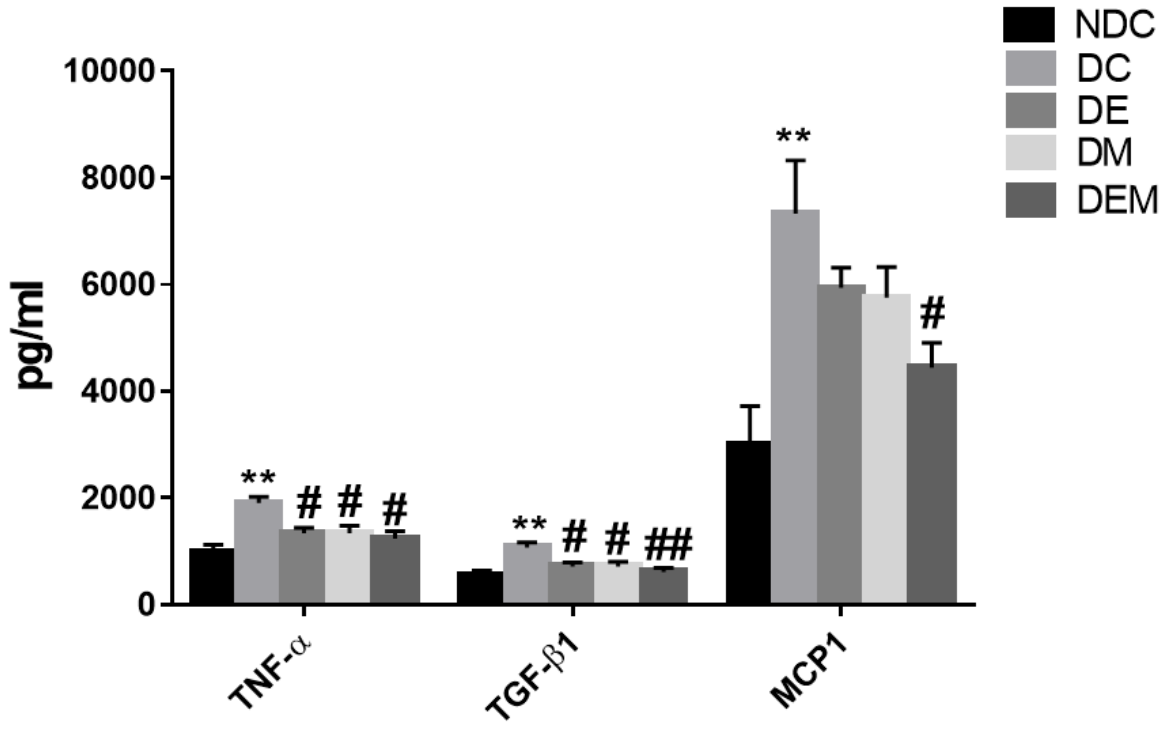


Fig 5: Effect of L-egt with or without metformin on inflammatory biomarkers. $^{**}p<0.01$, vs NDC while $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs DC. (n=6). Non-diabetic control (NC), diabetic control (DC), Diabetic treated plus L-egt only (DE), Diabetic plus metformin (DM), Diabetic plus L-egt and metformin (DEM). TNF-α: tumor necrotic factor- α; MCP-1: monocyte chemoattractant protein-1 and TGF-β1: Transforming growth factor-β1.

3.5 Effect on mRNA expression of antioxidant and inflammatory transcription factors.

The mRNA level of major transcription factors (e.g., Sirt1, Nrf2, HO-1, NQO-1, NF-kB, TGF- β1, and fibronectin mRNA expression) mediating the antioxidant and inflammatory signaling pathway is presented in Figure 6a-b. The result showed that the transcriptional level of Sirt1, Nrf2, and NQO-1 reduced significantly ($p<0.05$) in the DC group vs. NC group, while L-egt, with or without metformin, significantly increased ($p<05$) mRNA expression of these transcription factors vs. DC. Interestingly, Coadministration of L-egt with metformin to diabetic rats (DEM group) significantly increased ($p<0.05$) Nrf2 and HO-1 mRNA expression vs. DM group (Figure-6a). However, the DC group showed a significant increase ($p<0.01$) in NF-kB, TGF- β1, and fibronectin mRNA expression vs. the NC group (Figure-6b). Administration of L-egt, with or without metformin, to diabetic rats significantly reduced (DE: $p<0.05$; DEM: $p<0.01$) mRNA expression of these inflammatory biomarkers vs. DC group.

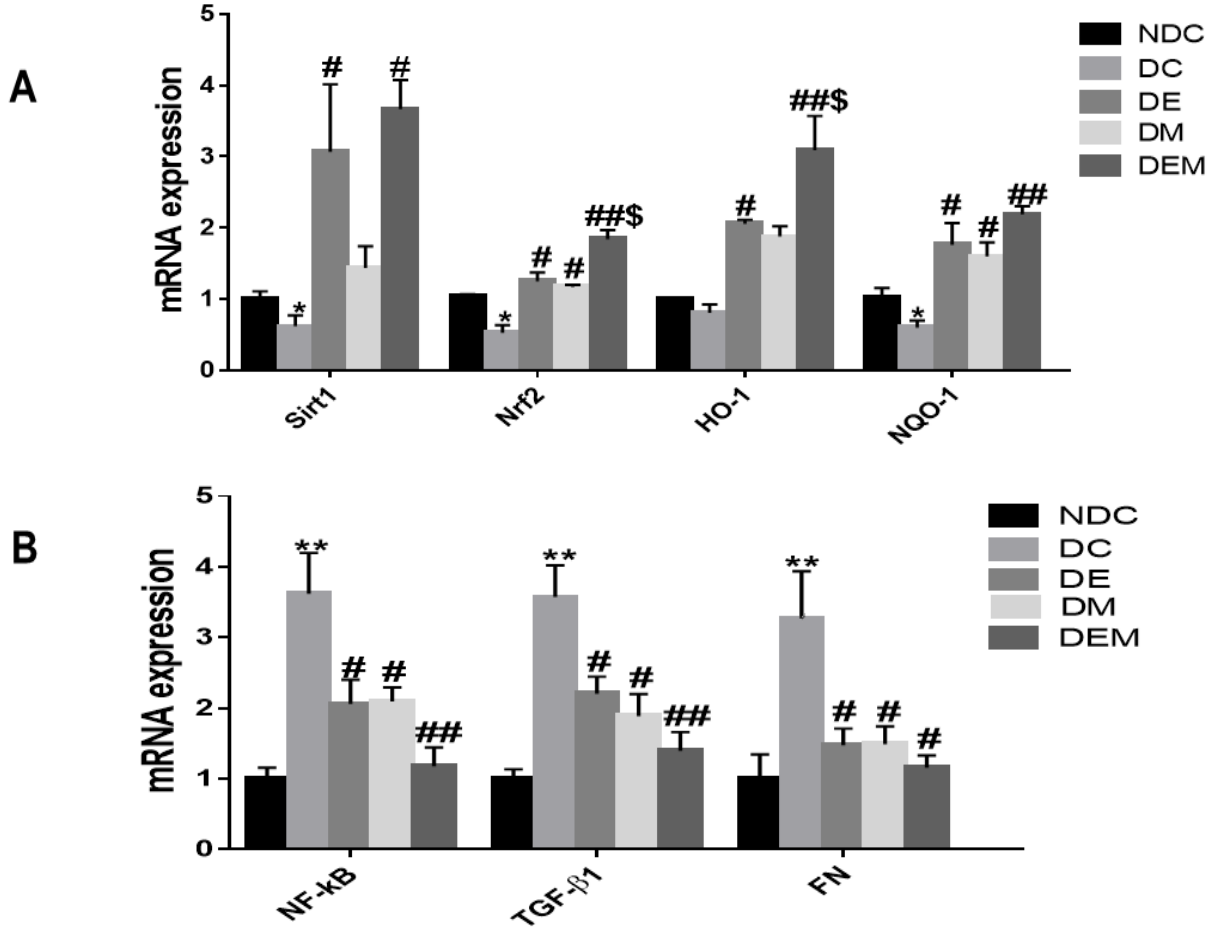


Figure 6a-b: Effect of L-egt with or without metformin on relative mRNA expression. $^{**}p<0.01$, $^{*}p<0.05$ vs NC while $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs DC. $^{\$}p<0.05$ vs DM. Non-diabetic control (NC), Diabetic control (DC), Diabetic treated plus L-egt only (DE), Diabetic plus metformin only (DM), Diabetic plus L-egt and metformin (DEM). Nrf2= Nuclear factor erythroid-2 related factor-2, Sirt1= sirtuin-1, HO1= hemeoxygenase-1, NQO1= NAD(P)H quinone oxidase-1, NF-kB= Nuclear factor kappa-B, TGF-β1: Transformin growth factor- β1 and FN= fibronectin.

3.6. Effect on Nrf2 protein expression

The relative protein expression of Nrf2 in the kidney is presented in Figure 7a-b. The result showed that Nrf2 protein expression significantly decreases in the DC group Vs. NC group. L-egt, with or without metformin, significantly increased Nrf2 protein expression Vs. DC group.

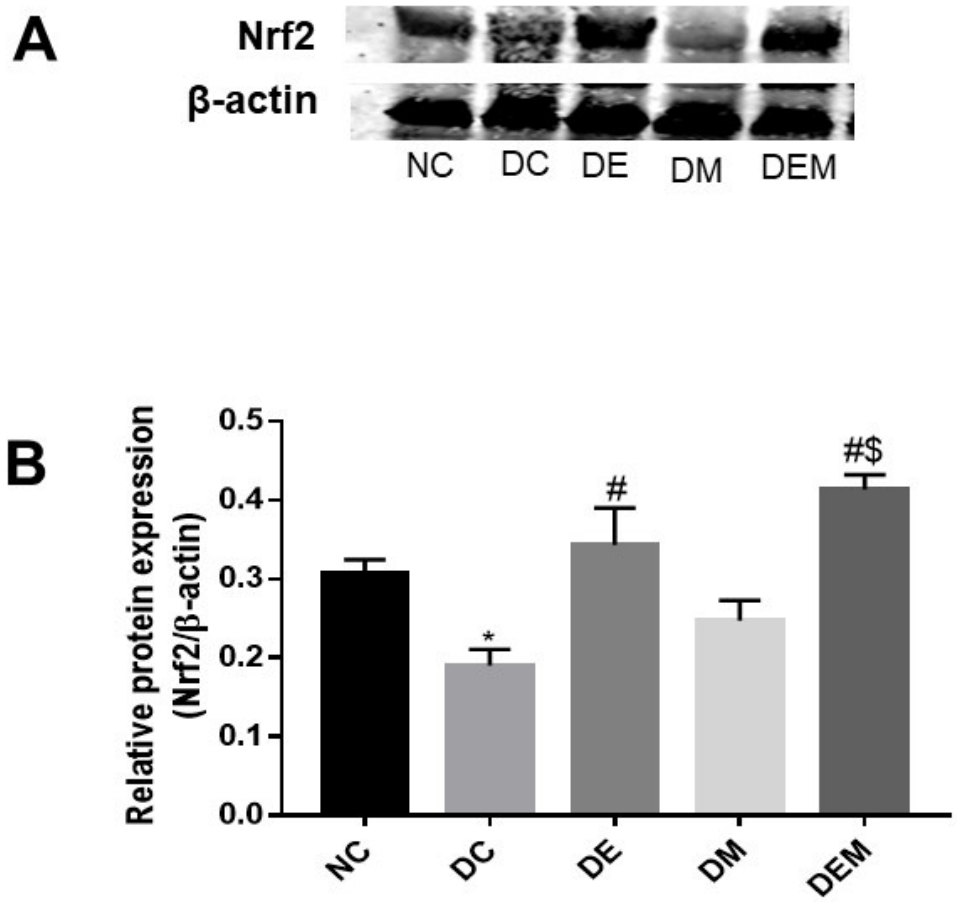


Figure 7a-b: Effect of L-egt with or without metformin on relative Nrf2 protein expression. $^{*}p<0.05$ vs NC while $^{\#}p<0.05$, vs DC. $^{\$}p<0.05$ vs DM. Non-diabetic control (NC), diabetic control (DC), diabetic treated plus L-egt (DE), diabetic plus metformin (DM), diabetic plus L-egt, and metformin (DEM). (A) immunoblot of Nrf2 and β-actin. (B) Relative Nrf2 protein expression

3.7 Effect on histological changes in the kidney

Histological evaluation of the kidney sections after seven weeks of administering L-egt, with or without metformin, to T2D animals is shown in Figure 8a-c.

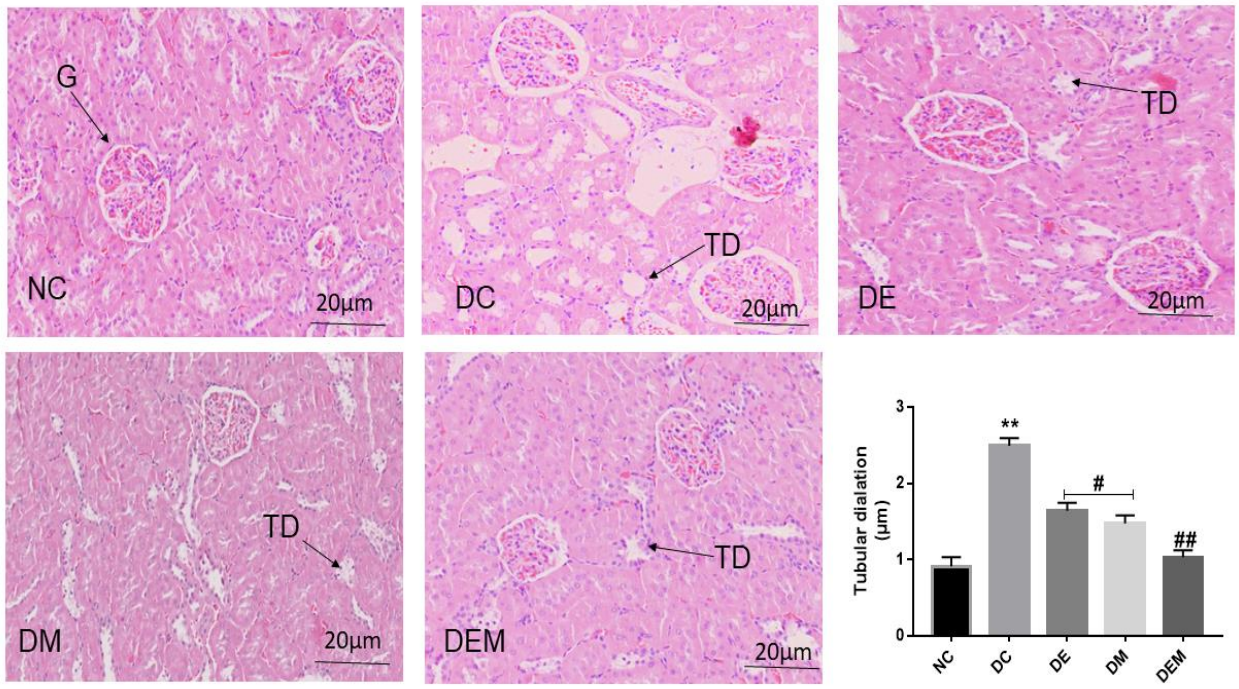


Figure 8a. Photomicrographs of kidney sections stained with hematoxylin and eosin (x200; scale bar: 20µm). NC rats showed normal renal histoarchitecture with normal size and morphology of the glomerulus and renal tubules. Compared to NC, the renal section in the DC rats showed dilation of the bowman's capsule (DB) and renal tubules (TD) with a slightly enlarged renal corpuscle. Kidney histology in the DE and DM rats showed mild tubular dilation, while coadministration of both L-egt and metformin to diabetic rats (DEM) prevent renal damage with a morphological structure similar to the NC rats.

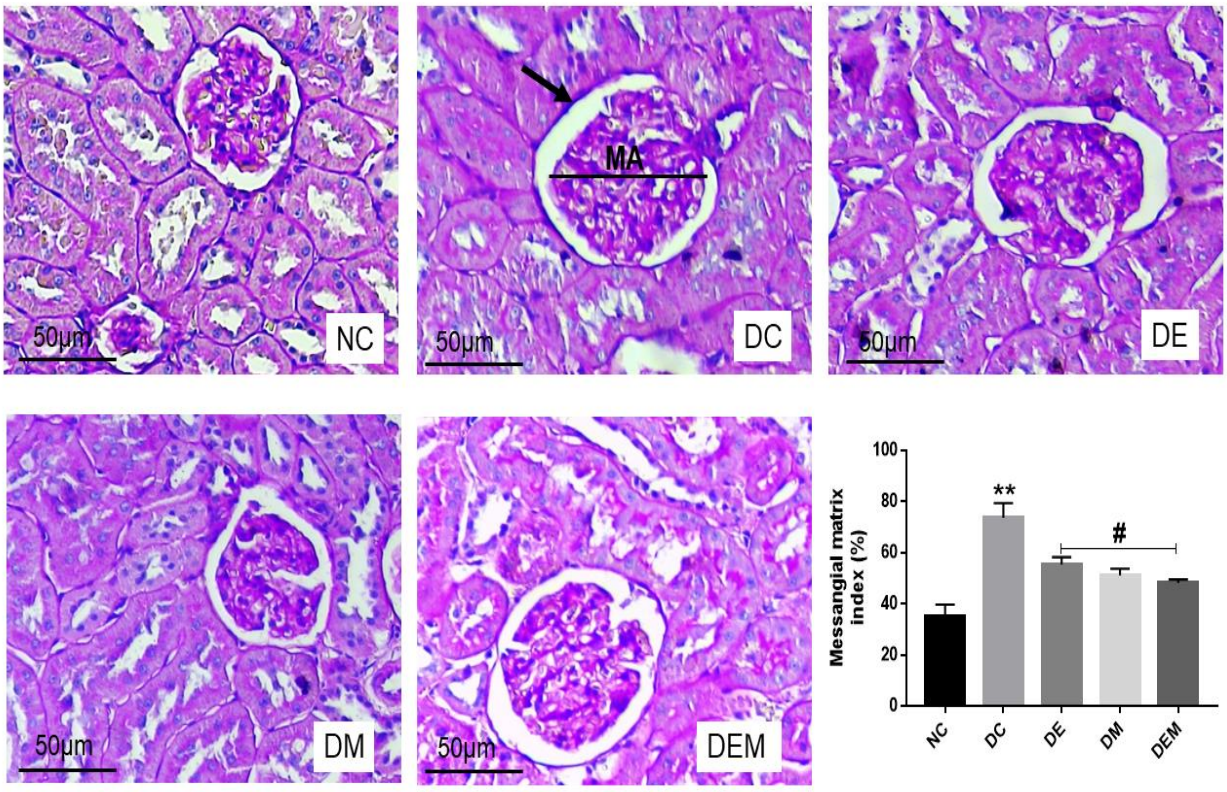


Figure 8b. Photomicrographs of kidney sections stained with PAS (x400; scale bar: 50µm) and mesangial matrix index. NC rats showed kidney histology with a normal glomerular tuft and tubules. The histology of the DC rats showed glomerular hypertrophy with mesangial area (MA) expansion and diffuse thickening of the basement membrane (arrow). There is a reduction in the mesangial area with a normal glomerular tuft in all the treated diabetic groups compared to the DC group.

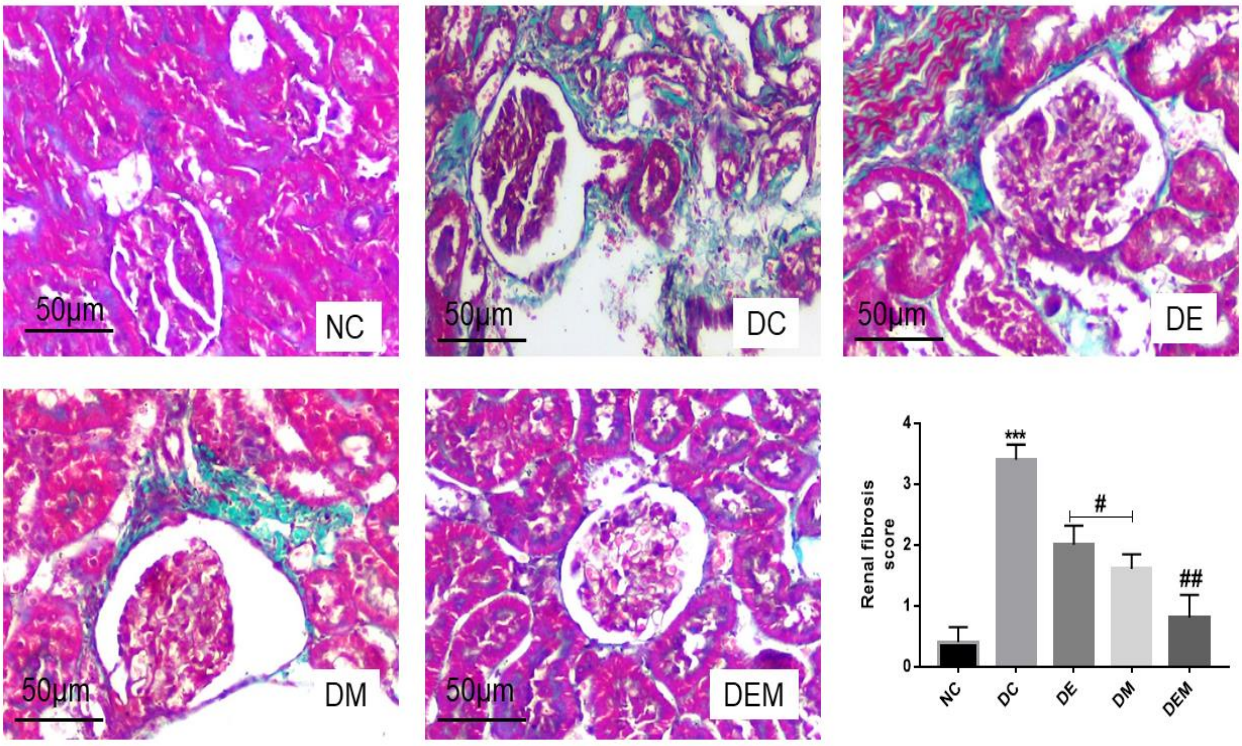


Figure 8c. Photomicrographs of kidney sections stained with Masson trichrome stain (x400; scale bar: 50µm) and renal fibrosis. The NC group showed normal renal interstitium. The DC rats showed renal fibrosis characterized by excessive collagen deposit (green stain) in the renal interstitium, staining both the glomerular and tubular membrane. The DE and DM group showed mild fibrosis with a diffuse deposit of collagen in the renal interstitium. The kidney in the DEM group showed renal histology like the negative control (NC) with less collagen deposit in the renal interstitium.

4.0 DISCUSSION

Recently, attention has been drawn to the use of drug adjuvants to reduce the dosage of administered drugs, minimize adverse effects, improve the efficacy of therapeutic interventions, and delay the onset or halt the progression of diabetic complications (35, 36). Thus, the present study evaluated the benefits of L-ergothioneine alone and combined with metformin in renal dysfunctions associated with T2D in a fructose-Streptozotocin rat model. Significantly, this study shows that L-egt exerts reno-protective effects and improves therapeutic outcomes when administered in combination with metformin, thus, providing further support to the use of combination therapies to manage diabetic nephropathy. Also, previous studies have reported that L-egt activates Nrf2 and inhibits NF-kB in several animal models (37, 38). To this end, we evaluated the beneficial role of L-egt-induced Nrf2 activation in diabetes-induced kidney disease.

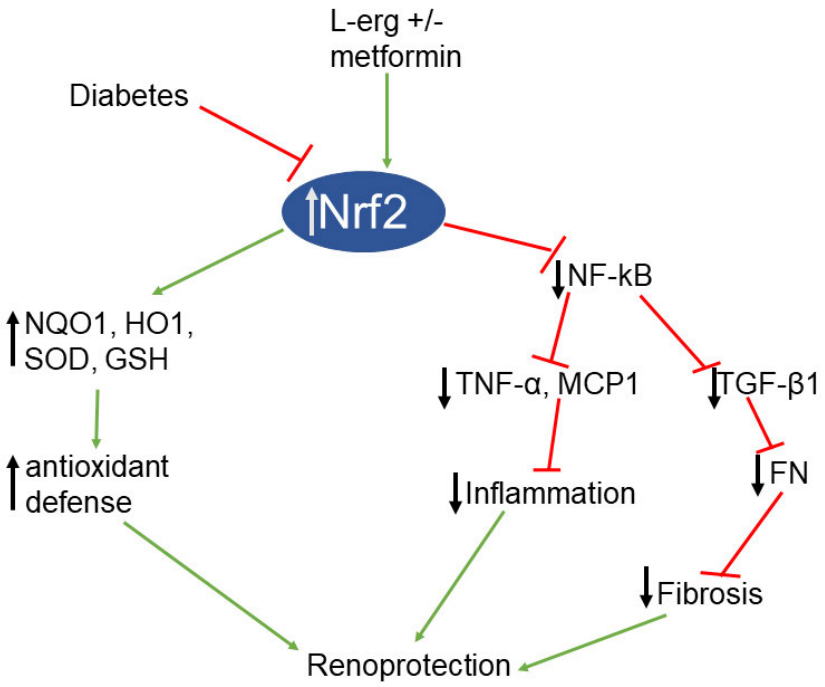


Figure 9: Probable reno-protective pathway of L-egt-induced Nrf2 activation. Nrf2 activation exerts two effects. 1) it upregulates the cytoprotective genes and antioxidant enzymes to enhance the antioxidant defense system. 2) it inhibits the NF-kB inflammatory gene to reduce inflammatory cytokines and downregulate TGF-β1 gene and its downstream fibrotic protein in the extracellular matrix. The improved antioxidant defense and reduced renal inflammation alleviate diabetes-induced renal damage. Red= inhibition while green= activation.

Liver is the primary target for metformin activity where it increases hepatic glucose uptake and decrease hepatic gluconeogenesis (39). L-egt has been reported to protect against liver damage during diabetes, thereby promoting antihyperglycemic effect of metformin. This may account for the improve glucose homeostasis observed in the diabetic rats co-administered with L-egt and metformin. Increased serum triglycerides have also been reported as an independent risk factor contributing to the development of renal damage in diabetes (40, 41). Previous studies in both human and animal models have reported the lipid-lowering effect of mushrooms, attributed to the bioactive content in this food (42, 43). Thus, the reduced serum triglycerides in diabetic animals treated with L-egt suggests that this nutraceutical can alleviate risk factors associated with renal dysfunction. This result is in accordance with a previous study done by (44), where ergothioneine-rich mushroom extract reduced intracellular triglyceride level by downregulating sterol regulatory binding protein-1c (SREBP1c), and hepatic lipogenesis

Kidney hypertrophy is well correlated with chronic hyperglycemia, resulting from excessive proliferation of mesangial cells and substantial accumulation of extracellular matrix. Kidney hypertrophy is accompanied by tubular basement membrane thickening and has been identified as a marker of diabetic nephropathy (45, 46). The potency of L-egt to reduce kidney hypertrophy in diabetic animals suggests that this compound may inhibit mesangial cell proliferation and excessive growth of the tubular epithelial cells that contribute significantly to kidney hypertrophy, thereby preventing structural damage and loss of kidney function. The proximal tubular cells and the mesangial cells are the primary targets of hyperglycemia due to their inability to downregulate glucose uptake (47). Therefore, chronic exposure to hyperglycemia and other risk factors promotes cellular injury in the kidney, resulting in compromised renal function. In this study, renal function was significantly reduced in the diabetic control animals, as shown in Table 3, which is similar to previous reports (48, 49). The reduced sCr, BUN, uPro, uAlb, KIM-1, and increased CrCl in diabetic rats treated with L-egt indicates that L-egt improves glomerular and tubular functions, which may be attributed to the potency of this nutraceutical to protect against cell injury and enhance the integrity of the glomerular filtration barrier. Studies have shown that metformin improves glycemic control, alleviates oxidative stress, and inhibits apoptosis in diabetic nephropathy (22, 50). The combination regimen improves renal function compared to either L-egt or metformin administration alone.

Oxidative stress plays a crucial role in the development and progression of diabetic kidney disease. The altered redox balance causes oxidative injury in the renal cells, promoting fibrosis and inflammation that mediate glomerulosclerosis and tubulointerstitial injury (51-53). Previous studies have reported that the activation of the Nrf2 signaling pathway protects the kidney against oxidative injury by enhancing the antioxidant defense system (54-56). In this study, L-egt-induced Nrf2 activation in non-diabetic and diabetic animals was confirmed by the upregulation of Nrf2 protein and gene expression. This promotes the upregulation of Nrf2 downstream cytoprotective genes, including HO-1 and NQO-1 that act as the central effector of the L-egt-induced Nrf2 activation. Consequently, the increased antioxidant defense system caused a reduction in lipid peroxidation and increased SOD, CAT, and GSH that works synergistically to reduce the harmful effect of free radicals in the renal cells. These results suggest that L-egt can activate the Nrf2 antioxidant pathway to reduce renal injury during diabetes. Furthermore, literature has shown that metformin alleviates renal oxidative damage in addition to its antihyperglycemic effect (22, 57). Therefore, the complementary effect of L-egt with metformin may account for the improved biological response compared to the administration of either treatment alone. Notably, clinical trial with Nrf2 activator such bardoxolone methyl has been terminated due to its potential contribution to cardiac injury (58). However, not all Nrf2 activators have been associated with cardiac failure, and studies are yet to report the adverse effect of ergothioneine. Therefore, further studies are still needed to translate the experimental data

from the bench to the bedside, focusing on evaluating the adverse effect of ergothioneine-induced Nrf2 activation in clinical studies.

The significant role of inflammation in the pathogenesis of DN has been reported (59, 60). Both IR and hyperglycemia stimulate low-grade inflammation characterized by an increased circulating level of inflammatory cytokines (TNF-α), chemokines (MCP-1), and fibrotic factors (TGF-β1), with significant loss of renal function (60, 61). In this study, the L-egt-induced Nrf2 activation is accompanied by a reduction in renal inflammation in diabetic rats, which was associated with the downregulation of NF-kB gene expression and reduced concentration of its downstream cytokines TNF-α and MCP-1 in the diabetic kidney. This result is similar to previous studies reporting a significant reduction in NF-kB activity due to Nrf2 activation (62, 63). During diabetes, monocytes infiltrate the renal cells to promote matrix expansion and mesangial proliferation (64). Interestingly, monocyte infiltration significantly decreased in the L-egt-treated animals as evidenced by the reduced MCP-1 in the kidney homogenates; suggesting the anti-inflammatory activity of L-egt to reduce renal inflammation. Besides, studies have shown that the reno-protective effect of metformin goes beyond its antihyperglycemic efficacy; metformin protects against renal inflammation by inhibiting the NF-kB transcription pathway, thereby preventing the release of inflammatory cytokines (65, 66). Therefore, combined administration of L-egt and metformin may exert a potent anti-inflammatory effect in renal tissues more than the administration of either L-egt or metformin alone. The protective effect of L-egt against diabetes-induced renal injury can also be attributed to the inhibition of TGF-β1, a potent profibrotic cytokine, via Nrf2 activation. In this study, L-egt administration decreased the concentration and gene expression of TGF-β1 in the kidney as well as downregulate fibronectin expression in the extracellular matrix of diabetic animals.

Histological examination of the kidney provides detailed insight into the structural architecture of the renal corpuscle and tubules. In this study, the glomerular hypertrophy, mesangial expansion, thickened basement membrane, tubular degeneration, and excessive collagen deposit (a biomarker of renal fibrosis) observed in the diabetic control group are characteristic features of early kidney injury associate with T2D, and these have also been reported in similar studies (22, 67, 68). However, most of these structural derangements are reduced in the L-egt treated group, while synergistic treatment with L-egt and metformin prevents histological aberrations in the kidney. These results suggest that L-egt may reduce cellular injury to stabilize the structural derangements evident in the T2D kidney. These observations are further supported by other indices of renal function, including the reduction in albuminuria, KIM-1, TGF-β1, and oxidative injury evaluated in this study.

5.0 CONCLUSION

This study showed that the combination of L-egt and metformin to diabetic animals improves therapeutic management of renal dysfunctions compared to either treatment alone. This therapeutic outcome is associated with the activation Nrf2 antioxidant signal to improve renal structure and function. L-egt-induced Nrf2 activation upregulates antioxidant cytoprotective genes (HO-1 and NQO1) to enhance the antioxidant defense system. Also, Nrf2 can downregulate NF-kB, TGF-β1, and fibronectin expression to inhibit renal inflammation. Furthermore, the combination of L-egt with metformin in diabetic animals reduced hypertriglyceridemia and improves glucose homeostasis. Thus, these findings highlight the therapeutic benefits of L-egt, which can be explored to alleviate metabolic disorders and use as an adjuvant regimen in the management of renal dysfunctions associated with T2D. However, the in-vitro analysis and analysis of specific L-egt transporters could not be done due to limited funding. Thus, further studies may be required to evaluate the detailed mechanism of action of L-egt fully.

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Conflict of interest

The authors declared that there is no conflict of interest

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CHAPTER 4

PROLOGUE

Renal injury has been reported to compromise cardiac function via alteration in cardiac hemodynamics. Also, hyperglycemia and dyslipidemia observed in type-2 diabetes promote oxidative damage, endothelial injury, and low-grade inflammation in the cardiac cells. Thus, it has been proposed that treatment options that improve renal functions and target major risk factors (such as dyslipidemia, oxidative stress, and inflammation) may play a significant role in delaying the onset or halt the progression of diabetic cardiomyopathies. Studies have shown that increased plasma L-egt level reduce mortality and cardiometabolic disease risk in human. In addition, we reported in chapters 2 and 3 of this study that L-egt may improve renal function, reduce triglyceride accumulation in tissue, reduce oxidative damage and cellular inflammation. However, its effect on cardiovascular functions during diabetes is yet to be established. Therefore, the cardio-protective effect of L-egt in a type-2 diabetic rat model and its in-silico antioxidant mechanism was investigated and reported in chapter 4 of this study.

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Cardioprotective effects and in-silico antioxidant mechanism of L-ergothioneine in experimental type-2 diabetic rats.

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ABSTRACT

Introduction/background: Diabetic cardiotoxicity is commonly associated with oxidative injury, inflammation, and endothelial dysfunction. L-ergothioneine (L-egt), a diet-derived amino acid, has been reported to decrease mortality and risk of cardiovascular injury, provides cytoprotection to tissues exposed to oxidative damage, and prevents diabetes-induced perturbation. This study investigated the cardioprotective effects of L-egt on diabetes-induced cardiovascular injuries and its probable mechanism of action.

Methods: Twenty-four male Sprague-Dawley rats were divided into non-diabetic (n=6) and diabetic groups (n=18). Six weeks after the induction of diabetes, the diabetic rats were divided into three groups (n=6) and administered distilled water, L-egt (35mg/kg), and losartan (20mg/kg) by oral gavage for six weeks. Blood glucose and mean arterial pressure (MAP) were recorded before and after L-egt or metformin treatment. After the completion of experiment, all animals were euthanized. Then, biochemical, ELISA, and Rt-PCR analyses were conducted to determine oxidative stress and inflammatory biomarkers in cardiac tissue as well as cardiac injury and serum triglycerides levels. In addition, an in-silico study, including docking and molecular dynamic simulations of L-egt toward the Keap1-Nrf2 protein complex, was done to provide a basis for the molecular antioxidant mechanism of L-egt.

Results: Administration of L-egt to diabetic animals reduced serum triglyceride, water intake, MAP, biomarkers of cardiac injury (CK-MB, LDH), lipid peroxidation, and inflammation. Also, L-egt increased

body weight, antioxidant enzymes, upregulated Nrf2, HO-1, NQO1 expression, and decreased Keap1 expression. The in-silico study showed that L-egt inhibits Keap1-Nrf2 complex by binding to the active site of Nrf2 protein, thereby preventing its degradation.

Conclusion: L-egt protects against diabetes-induced cardiovascular injury via the upregulation of Keap1-Nrf2 pathway and its downstream cytoprotective antioxidants.

Keywords: cardio-protection, diabetes, L-ergothioneine, molecular docking, molecular dynamics.

1.0 INTRODUCTION

Diabetes exerts a significant economic and social burden as well as reduced quality of life due to associated micro- and macro-vascular complications leading to coronary heart disease, cardiomyopathy, cerebrovascular disease and arrhythmia (1-3). Globally, cardiovascular disease (CVD) is well correlated with significant mortality and morbidity in type-2 diabetes patients due to an accelerated decline in cardiac function (4, 5), which often result from risk factors such as hyperglycemia, insulin resistance (IR), obesity, and dyslipidemia (6, 7). These factors cause excessive generation of free radicals and a defective antioxidant pathway that promotes oxidative injury, endothelial dysfunction, and low-grade chronic inflammation identified as the main pathogenic mechanisms connecting CVD and diabetes (8). Therefore, effective strategies that enhance the antioxidant pathway to reduce damage by free radicals and cardiac inflammation have been proposed to manage cardiovascular related complications in diabetes (9, 10). Recently, attention has been drawn to the beneficial role of the Keap1-Nrf2 antioxidant signaling pathway in managing diabetes and its related complications, including cardiac injury (11-13).

Keap1-Nrf2-ARE (Kelch-like ECH-associated protein-1/nuclear factor erythroid-2-related factor-2, antioxidant response elements) protein complex is a major antioxidant-signaling pathway that provide cyto-protection to tissues exposed to oxidative injury. Activation of this pathway enhances the antioxidant defense system in various disease conditions, including diabetes, becoming a drug target in the treatment of diabetes and its associated complications (14, 15). During conditions of oxidative stress or the presence of activators (e.g., bardoxolone), the interaction between the Keap1-Nrf2 protein complex is disturbed, thus reducing the degradation of Nrf2 by its repressor protein Keap1. This stabilizes Nrf2 in the cytoplasm and its translocation into the nucleus to activate the antioxidant response element (ARE), thereby stimulating the transcription of various cytoprotective genes with resultant upregulation of the antioxidant cyto-protective enzymes and inhibition of cellular inflammation (14, 16). Thus, the administration of specific

ligands that inhibit the Keap1-Nrf2 protein complex from promoting nuclear translocation of Nrf2 may prevent oxidative injury in the cardiomyocytes.

L-ergothioneine (L-egt), a naturally occurring amino acid, is found abundantly in mushrooms, beans, and certain meat products (17). It has been recognized as a dietary supplement with full approval by the European Food and Safety Authority as well as Food and Drug Administration (18, 19). L-egt is a physiological cryoprotectant and antioxidant nutraceutical with significant biological activities (20-22). Previous studies have shown that L-egt increases SIRT 1 expression to activate various transcription factors that modulate antioxidant and anti-inflammatory pathways (23). It exerts significant cardio-protective functions by preventing monocyte infiltration into the endothelium (24), protects against infarction during ischemic reperfusion (25), inhibits high glucose-induced impairment of rat aorta, and promotes maximum response of the coronary artery to acetylcholine in STZ-induced diabetic rats (26, 27). In humans, increased plasma L-egt has been linked with reduced mortality and well-correlated with a lower risk of cardio-metabolic diseases (28). Thus, this study was designed to evaluate the antioxidant mechanism and cardioprotective effects of L-egt in an experimental type-2 diabetic rat model.

2.0 MATERIALS AND METHODS

2.1 Chemicals and Reagents

Pure L-egt was provided by Tetrahedron (Paris, France www.tetrahedron.fr). Streptozotocin (STZ), reduced glutathione (GSH), and thiobarbituric acid were purchased from Sigma-Aldrich (USA), iTAQ SYBR green master mix, and cDNA kits were obtained from Bio-Rad (Johannesburg, South Africa), PCR primers were produced by Inqaba Biotec (Pretoria, South Africa). Losartan (LOS) was purchased from a local pharmacy (Aurobindo Pharma, Johannesburg, South Africa). Other analytical grade chemicals and reagents were obtained from local suppliers.

2.2 Experimental animals

Twenty-four adult male Sprague-Dawley rats weighing (150-190)g were obtained from the Biomedical Research Unit (BRU), Westville Campus, University of KwaZulu-Natal, Durban, South Africa. The animals were allowed to acclimatize under standard laboratory conditions (temperature 23 ± 1°C, 40–60% humidity) with free access to rat feed and water *ad libitum* for one week before the commencement of the experiment. This study was approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, Durban, South Africa (Ethic number: AREC/006/019D), and animal protocols were

according to the guidelines published by the US National Institute of Health for the proper care and use of laboratory animals (NIH publication N0. 85-23, 1996).

2.3 Experimental protocol and treatment

Type-2 diabetes was induced using 10% fructose solution plus a low dosage of streptozotocin to induce insulin resistance and partial damage to β-cells with resultant hyperglycemia (29). After acclimatization, the experimental rats were randomly divided into non-diabetic (ND) and diabetic (DB) groups. The diabetic groups (DB) were provided 10% fructose solution *ad libitum* in drinking water for two weeks, while the non-diabetic group (ND) was supplied with distilled water. After that, all animals were fasted for 8-10 hours before intraperitoneal injections; the diabetic groups were injected a single low dose of freshly prepared STZ (40mg/kg bwt) in 0.1M citrate buffer (pH 4.5), while the non-diabetic group was injected with the same volume of 0.1M citrate-buffer only. Diabetes was confirmed after one week of STZ injection by measuring non-fasting blood glucose (NFBG) level using a portable glucometer Accu-Chek Active (Roche Diagnostics GnbHD-68298 Mannheim, Germany) in the blood collected from the tail vein. Animals with NFBG levels > 16.7 mmol/L were considered diabetic (30) and included in the study. After six weeks of diabetes, the diabetic animals were subdivided into three groups (n=6) and treated orally for six weeks as follows: non-diabetic + distilled water (ND+DW), diabetic + distilled water (DB+DW), diabetic + L-egt (DB+EGT), and diabetic + losartan (DB+LOS). Distill water- 1ml/100g, L-egt- 35 mg/kg bwt (20, 31) and losartan- 20 mg/ kg bwt (32, 33).

2.4 Evaluation of body weight, blood glucose, and blood pressure measurement

Bodyweight and non-fasting blood glucose of all experimental animals were measured using standard laboratory weighing balance (Metler, Switzerland) and Accu-check glucometer. Mean arterial pressure of all the experimental animal was recorded at week 6 (pre-treatment) and week 12 (post-treatment) by a non-invasive tail-cuff method using the non-invasive MRBP IITC Model 31, multichannel tail-cuff blood pressure system (Life Sciences, Woodland Hills, CA) following the established protocol described by Mkhwanazi et al., 2014. Briefly, the animals were placed in a rat restrainer (3" ID (75mm)-12" length) enclosed in a warming chamber (IITC Model 303sc animal Test Chamber, Woodland Hills, CA) at 32^0C. The rat tail was then attached to the cuff; blood pressure was then measured by occluding blood flow in the tail artery. The mean of 3 readings was taken for each measurement and statistically analyzed.

2.5. Sample collection and preparation

At the end of the experiment, rats were weighed and sacrificed by decapitation; blood was collected into a serum vacutainer (BD Vacutainer, SST^{TM} Advance, Plymouth, UK) and centrifuged for 10 minutes at 3000rpm. The serum obtained was stored in the bio-freezer at -80^0C until used for analysis (triglycerides and cardiac biomarkers). The hearts were harvested, cleared of adhering tissues, washed in normal saline,

blotted, weighed, and immediately snap-frozen in liquid nitrogen, and later stored in a bio-freezer (Snijers Scientific, Tilburg, The Netherlands). The heart-to-body weight ratio (heart index) was used to assess possible hypertrophy. Heart homogenates were prepared in phosphate-buffered saline (pH 7.5; 10%w/v) and used to quantify malondialdehyde (MDA), antioxidant and inflammatory biomarkers.

2.6 Biochemical assays

Triglycerides (TG), LDH, hs-CRP, CK-MB, antioxidants enzymes (SOD, GSH, and CAT), Lipid peroxidation (MDA), and inflammatory markers (TNF-α, MCP-1, and TGF-β1) were analyzed.

2.6.1 Evaluation of serum triglycerides and biomarkers of cardiac injury

Serum triglyceride (TG) and lactate dehydrogenase (LDH) were analyzed at an accredited pathology laboratory (Global Clinical and Viral laboratories, Amanzimtoti, South Africa) using a biochemical analyzer. Serum high-sensitivity C-reactive protein (hs-CRP) and CK-MB were done immediately after euthanasia using an ELISA kit (Elabscience Biotechnology Co., Ltd) according to the manufacturer's protocol, and the absorbance was measured at 450nm using the microplate reader, Spectrostar Nano Spectrophotometer (BMG Labtech, Ortenburg, LGBW, Germany).

2.6.2 Evaluation of cardiac inflammatory biomarkers

Level of tumor necrotic factor- α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor-1 (TGF-β1) were measured in the heart homogenates using specific rat ELISA kits ((Elabscience Biotechnology Co., Ltd) according to the manufacturer's protocol and absorbance was measured at 450nm using the microplate reader.

2.6.3 Evaluation of lipid peroxidation, antioxidant biomarkers, and nitric oxide in cardiac homogenates

The heart homogenates were used to measure the concentration of MDA, SOD, GSH, and CAT by spectrophotometric assay. GSH and SOD levels were assessed using Ellman's method (34) and Marklund (35). CAT activity was analyzed following the protocol of Aebi (36). MDA, a marker of lipid peroxidation, was assayed using the previously described method (37) and expressed as nmol/mg protein.

2.7 Real time-quantitative Polymerase Chain Reaction (Rt-qPCR) analysis in cardiac tissue

PCR analysis of the relative mRNA expression level of Kelch-like ECH-associated protein 1 (Keap1), Nuclear factor erythroid 2–related factor 2 (Nrf2), Heme oxygenase-1 (HO-1), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were done using SYBR Green PCR master mix analysis (Biorad, CA, USA) on a light cycler 96 PCR system (Roche, Mannheim, Germany). TRIzol reagent (Life Technologies, Inc., MD, USA) was used for the extraction of total RNA from the heart and converted to cDNA using iScript cDNA synthesis kit (Biorad, Sandton, South Africa) and incubated on SimpliAmp™ thermal cycler, Applied Biosystems (Thermo Fischer Scientific) according to the manufacturer's protocol. RT-qPCR was

performed in a 10-µL reaction volume containing 5µL SYBER Green PCR master mix, 1µL of each primer, 1µL of nuclease water, and 2µL of cDNA. The primer sequence is listed in Table 1. The relative expression of the gene of interest was analyzed with reference to the housekeeping gene, GAPDH, and calculated using RQ= $2^{-\Delta\Delta Ct}$ (38).

Table 1: List of primers

Gene	**Primer sequence**	**Product size (bp)**	**Gene accession number**
GAPDH	F: 5′- TGATGACATCAAGAAGGTGGTGGAG -3’ R: 5′- TCCTTGGAGGCCATGTAGGCCAT -3’	578	XM_017593963.1
Nrf2	F: 5’-CAGCATGATGGACTTGGAATTG-3’ R: 5’-GCAAGCGACTCATGGTCATC -3’	466	NM_031789.2
HO-1	F: 5′-AGAGGCTAAGACCGCCTTCC -3′ R: 5’-ATTTTCCTCGGGGCGTCTCT-3’	167	NM_012580.2
Keap1	F:5’-AACTCGGCAGAATGTTACTACCC-3’ R:5’-CTACGAAAGTCCAGGTCTCTGTCTC-3’	190	NM_057152.2

2.8 Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). All statistical comparisons were carried out using a one-way analysis of variance (ANOVA) test followed by Tukey's test post hoc analysis using GraphPad Prism version 7 (GraphPad Software, CA, USA). A value of $p<0.05$ was considered statistically significant.

2.9. Molecular modeling: docking study

The in-silico study further evaluates the beneficial effects of bioactive compounds via computer simulations to decipher and understand their molecular mechanisms of action with reference to biological experiments. Thus, a molecular modeling study was done using Autodock Vina software to evaluate the binding free energies of the L-(+)-ergothioneine toward the Nrf2-Keap1 complex, thereby providing a basis for the antioxidant mechanism of this nutraceutical.

2.9.1 System preparation

The X-ray crystal structures of the Nrf2-Keap1 complex were obtained from the RCSB Protein Data Bank with code 4L7B (39). The missing residues were modeled using MODELLER 9.19 (40) integrated with Chimera software (41).

2.9.2 Molecular docking study of the experiment L-(+) - ergothioneine

Docking of the L-(+)-ergothioneine was conducted using the Autodock Vina software (42). The Gasteiger partial charges were used to assign the atomic charges of both protein and ligand, and the nonpolar hydrogen atoms were merged to carbon atoms (43). The Auto dock graphical interface (AGUI) provided by MGL tools illustrates the Auto dock atom type (44). L-(+) -ergothioneine compound was then docked using rigid docking into the binding pocket of the Nrf2-Keap1 receptor (by defining the grid box with a spacing of 1 Å and size of 9.97 × 7.04 × 10.14 pointing in x, y, and z directions), with eight exhaustiveness. The Lamarckian genetic algorithm in Autodock vina was used to produce different docked conformation and dynamic ligand positions within the protein conformational space (43).

2.9.3 Validation of the docking method

Previous studies have confirmed that docking will lead to the best geometric configuration of the docked complex, but short molecular dynamics (MD) simulations may not preserve the complex's stability and lead to molecule distortion. Thus, the most suitable way to verify the stability of the docked complex is via MD simulation. In this study, NRF- L-(+) – ergothioneine complex was subjected to further MDs studies (60 ns) as described below.

2.10 Molecular dynamics simulations

In the study of biological systems, the integration of molecular dynamic (MD) simulations enables the analysis of the physical motion of atoms and molecules that cannot be easily obtained by any other means. (45). The knowledge gained from this kind of simulation offers a detailed view of the complex evolution of biological systems, such as conformation changes and molecule association (45). MD simulation studies were performed on all systems using the GPU version of the PMEMD engine in the AMBER 18 package. (46).

The partial atomic charge of each compound was generated using the General Amber Force Field (GAFF) protocol in an ANTECHAMBER package (47). Each system was implicitly solvated within an orthorhombic box of TIP3P water molecules within 10Å of any box edge, performed by the Leap module of the AMBER 18 package. Via the addition of Na+ and Cl- counter ions integrated with the Leap module, neutralization of each system was further implemented. The systems were minimized with applied restraint of 100 kcal/mol Å for 2500 steps, followed by 1000 steps of full minimization steps. The systems were then gradually heated for 50 ps from 0 K to 300 K such that a fixed number of atoms and a fixed volume (NVT) are maintained with a potential harmonic restriction of 10 kcal/mol Å and a collision frequency of 1.0 ps^{-1}. The systems were then equilibrated at a temperature of 300 K without constraints at a constant pressure of 1 bar using the Berendsen barostat (48). This was followed by the production of MD for 250 ns per system, in which the SHAKE algorithm was used to constrict hydrogen atom bonds.

2.10.1 Post-MD Analysis

The trajectories generated after MD simulations were each saved every 1 ps, followed by analysis using the CPPTRAJ (49) module implemented in the AMBER18 suite. All plots and visualizations were completed utilizing the Origin (50) data analysis tool and Chimera (41).

2.10.2 Binding free energy calculation

The relative binding free energy (ΔG_{bind}) of L-(+) - ergothioneine toward NRF2 was computed using molecular mechanics integrated with the Poisson-Boltzmann or generalized Born and surface area continuum solvation (MM/PBSA and MM/GBSA) method (51). MM/GB-SA and MM/PB-SA rely on molecular simulations of the ligand-protein complex to compute rigorous statistical-mechanical binding free energy within a specified force field (52, 53).

Binding free energy averaged over 600 snapshots extracted from the entire 60 ns trajectory. The estimation of the change in binding free energy (ΔG) for each molecular species (complex, ligand, and receptor) can be represented as follows (54):

$$\Delta G_{bind} = G_{complex} - G_{receptor} - G_{ligand} \quad (1)$$

$$\Delta G_{bind} = E_{gas} + G_{sol} - TS \quad (2)$$

$$E_{gas} = E_{int} + E_{vdw} + E_{ele} \quad (3)$$

$$G_{sol} = G_{GB} + G_{SA} \quad (4)$$

$$G_{SA} = \gamma SASA \quad (5)$$

The term E_{gas}, E_{int}, E_{ele}, and E_{vdw} represent gas-phase energy, internal energy, Coulomb energy, and van der Waals energy, respectively. The E_{gas} was directly assessed from the FF14SB force field terms. Solvation-free energy (G_{sol}) was evaluated from the energy involvement from the polar states (G_{GB}) and nonpolar states (G). The nonpolar solvation free energy (G_{SA}) was determined from the Solvent Accessible Surface Area (SASA) (55) using a water probe radius of 1.4 Å. In contrast, the polar solvation (G_{GB}) contribution was assessed by solving the GB equation. Items S and T symbolize the total entropy of the solute and temperature, respectively.

2.10.3 Per-residue free energy decomposition analysis

Per-residue energy decomposition was carried out to estimate the individual binding free energy contribution of ATP binding site residues to the stabilization and affinity of studied compounds. This provides insight into the basis of Nrf2-Keap1 inhibition exhibited by L-(+)–ergothioneine. It has been noted that high residual energy contributions could depict crucial residues necessary for inhibitor binding (56).

3.0 RESULTS

3.1 L-egt prevented a drastic reduction in body weight, reduced water intake and triglycerides in diabetic rats.

As presented in Table-2, the result showed that the diabetic control (DB+DW) rats exhibited a significant ($P<0.01$) decrease in body weight when compared to the non-diabetic control rats (ND+DW) while L-egt (DB+EGT) and LOS (DB+LOS) administration significantly ($P<0.05$) increased body weight when compared to untreated diabetic rats (DB+DW). Food and water intake increased significantly ($p<0.05$ and $p<0.01$ respectively) in the diabetic control group compared with the non-diabetic group, while L-egt and losartan administration significantly ($p<0.05$) reduced water intake with no significant change in food intake when compared to the diabetic control group. The blood glucose level in the diabetic control group (DB+DW) was significantly ($p<0.01$) higher compared to the non-diabetic group (ND+DW), while treatment with either L-egt or Los shows no significant difference in blood glucose level. In this study, there was no significant difference in the heart index of all the experimental rats. Also, the diabetic control group showed a significant increase ($p<0.05$) in serum TG when compared to the non-diabetic group (ND+DW). However, treatment with L-egt (DB+EGT) significantly reduce ($P<0.05$) TG while the losartan treated group showed no difference in TG when compared with the diabetic control group.

Table-2: Effect of L-egt on body weight, blood glucose, heart index food, and water intake after a treatment period of 6 weeks.

Indices	**Groups**			
	ND+DW	DB+DW	DB+EGT	DB+LOS
Body weight (g/rat)	455.20 ± 17.67	271.20 ± 15.14**	337.60 ± 6.96$^{\#}$	352.80 ± 21.72$^{\#}$
Food consumption (g)	13.79 ± 2.53	29.50 ± 2.04*	25.45 ± 2.36	23.54 ± 3.54
Water intake (ml)	19.83 ± 3.66	139.67 ± 12.60**	91.67 ± 11.95$^{\#}$	90.00 ±8.94$^{\#}$
Blood glucose (mmol/L)	5.52 ± 0.14	25.55 ± 2.05**	23.97 ± 1.65	24.80 ± 2.06
Heart index (%)	0.33 ± 0.02	0.45 ± 0.03	0.3 ± 0.01	0.33 ± 0.02
TG (mg/dL)	17.04 ± 1.95	29.13 ± 2.89*	19.05 ± 2.89$^{\#}$	25.89 ± 2.19

ND+DW = non-diabetic control; DB+DW = Diabetic control; DB+L-egt = Diabetic rats treated with L-egt and DB+LOS = diabetic rats treated with losartan. $^{**}P<0.01$, $^{*}P<0.05$ compared to ND+DW; $^{\#}p<0.05$ compared to DB+DW.

3.2 L-egt prevents increase in mean arterial pressure

Figure-1 shows the effect of L-egt on mean arterial pressure (MAP) after six weeks of treatment. Before treatment, there was no significant difference in the MAP of all the diabetic animals compared to non-diabetic animals. However, after six weeks of untreated diabetes, MAP significantly increases ($P<0.05$) in the diabetic control group (DB+DW) compared with the non-diabetic group (ND+DW). On the other hand, the administration of L-egt (DB+L-egt) and LOS (DB+ LOS) for six weeks to diabetic animals significantly reduced ($p<0.05$) MAP compared to the diabetic control group (DB+DW).

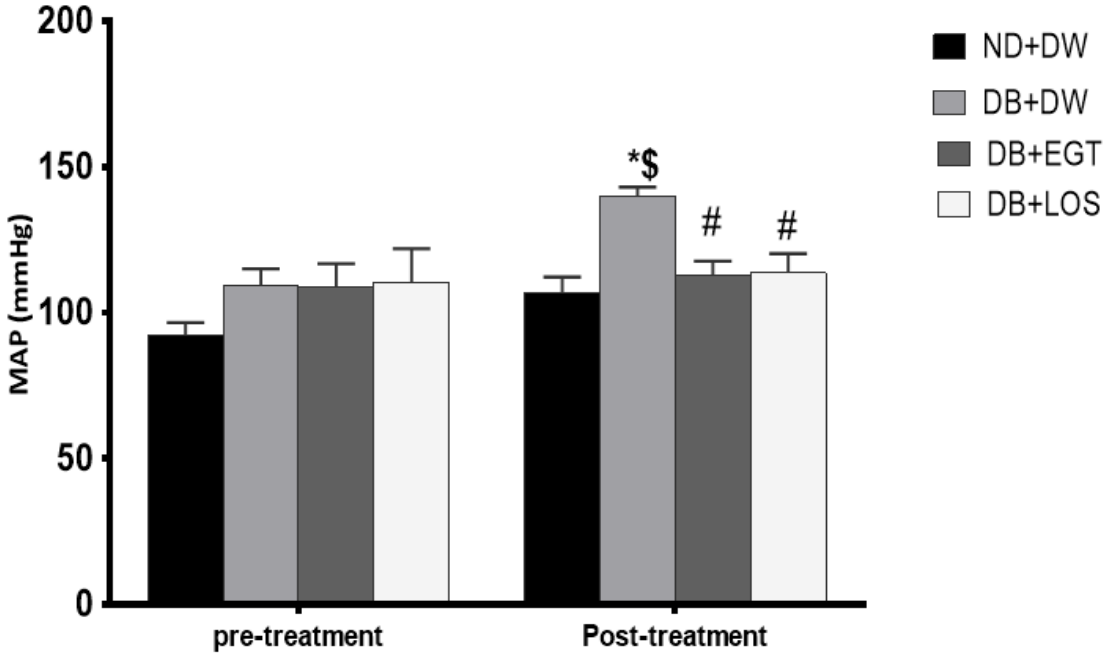


Figure-1: Effect of L-egt on mean arterial pressure before and after treatment for 6 weeks. ND+DW = non-diabetic control; DB+DW = Diabetic control; DB+L-egt = Diabetic rats treated with L-egt and DB+LOS = diabetic rats treated with losartan. $^{*}P<0.05$, compared to ND+DW; $^{\#}P<0.05$ compared to DB+DW; $^{\$}p<0.05$ compared to pretreatment.

3.3 L-egt alleviates biomarkers of cardiac injury

Serum creatinine kinase-MB (CK-MB), C-reactive protein (CRP), and lactate dehydrogenase (LDH) concentrations were measured at the end of the experiment. There was a significant ($p<0.05$) increase in serum CK-MB (Figure 2a), CRP concentration (Figure 2b), and LDH (Figure 2c) in the diabetic control group (DB+DW) compared with the non-diabetic (ND+DW) group. Administration of L-egt and LOS to diabetic rats significantly ($p<0.05$) reduced serum CK-MB and CRP with no significant difference in LDH

concentration in the diabetic group treated with either L-egt (DB+EGT) or Los (DB+LOS) when compared with the diabetic control group (DB+DW).

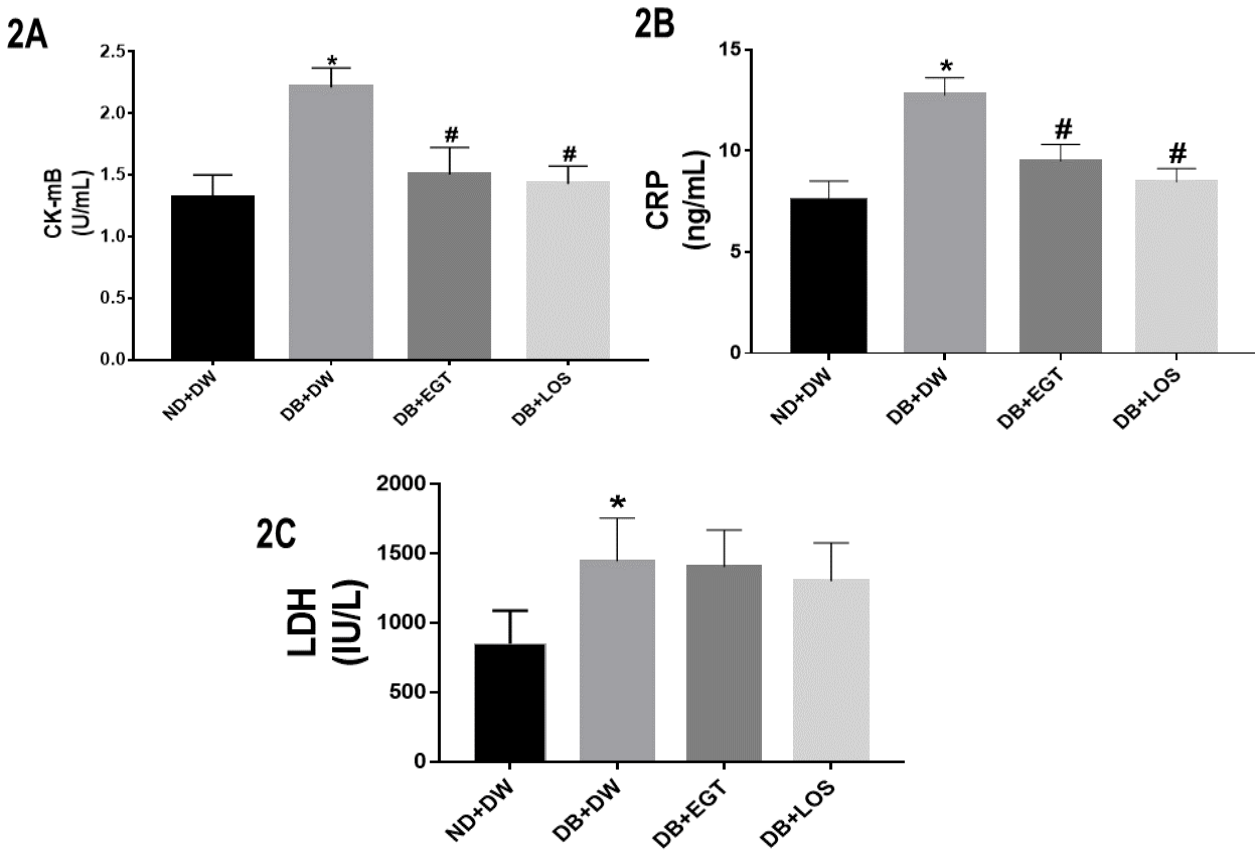


Figure 2a-c: Effect of L-egt on serum (a) CK-MB, (b) CRP and (c) LDH after a treatment period of 6 weeks. *p<0.05 vs ND+DW. #p<0.05 vs DB+DW. ND+DW = non-diabetic control; DB+DW = Diabetic control; DB+L-egt = Diabetic rats treated with L-egt and DB+LOS = diabetic rats treated with losartan.

3.4 L-egt reduced lipid peroxidation, increases antioxidant enzymes, and reduced glutathione in the cardiac homogenates

The result of L-egt treatment on cardiac lipid peroxidation (MDA), antioxidant enzymes (SOD, CAT), and GSH is presented in Figures 3a-d. There is a significant increase ($p<0.01$) in MDA in the diabetic control group (DB+DW) compared with the non-diabetic group (ND+DW) while treatment with L-egt (DB+L-egt) significantly ($p<0.05$) decreased cardiac MDA concentration compared with the diabetic group. On the other hand, SOD, GSH, and CAT significantly decrease ($p<0.05$) in the diabetic control group compared to the non-diabetic group, while L-egt significantly increases ($p<0.05$) GSH, SOD, and CAT compared

with the diabetic control group. A similar result was observed in the diabetic animals treated with losartan. However, there was no significant difference in CAT level compared with DB+DW.

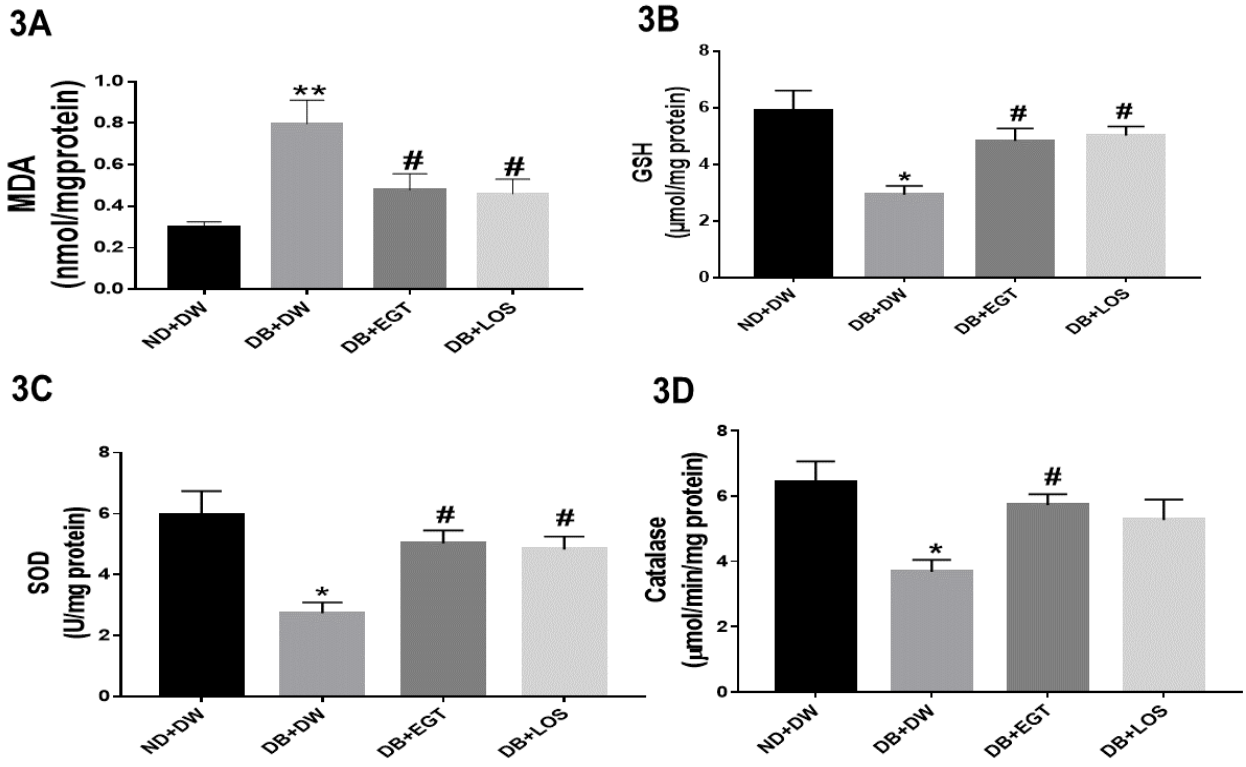


Figure-3a-d: Effect of L-egt on cardiac (a) malondialdehyde (b) GSH, (c) SOD and (d) CAT after a treatment period of 6 weeks. $^{**}p<0.01$, $^{*}p<0.05$ vs ND+DW. $^{\#}p<0.05$ vs DB+DW. ND+DW = non-diabetic control; DB+DW = Diabetic control; DB+L-egt = Diabetic rats treated with L-egt and DB+LOS = diabetic rats treated with losartan.

3.5 L-egt reduced inflammatory cytokine, chemokine, and fibrotic biomarkers in the cardiac homogenate.

The result of L-egt treatment on cardiac inflammatory cytokine (TNF-α), chemokine (MCP-1), and fibrotic factor (TGF-β1) after six weeks is presented in Figures 4a-b. TNF-α, MCP-1, and TGF-β1 significantly increased ($p<0.05$) in the diabetic control group (DB+DW) compared with the non-diabetic group (ND+DW) while treatment with L-egt significantly ($p<0.05$) reduced cardiac TNF-α and MCP-1 with no significant decrease in TGF-β1 compared with diabetic control. A similar result was observed in the LOS-treated group.

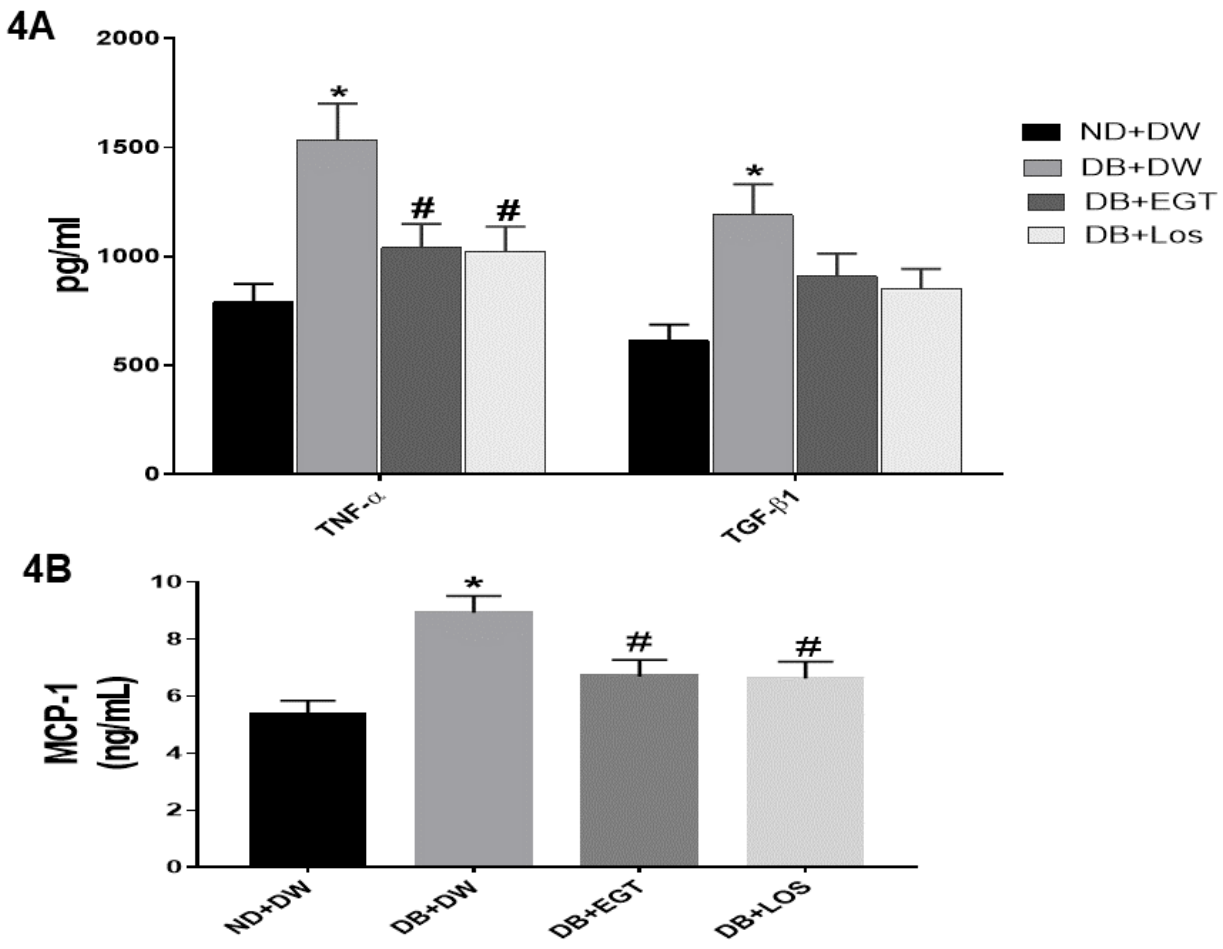


Figure-4a-b: Effect of L-egt on cardiac (a) TNF-α and TGF-β1 (b) MCP-1 after a treatment period of 6 weeks. $^{*}p<0.05$ vs ND+DW. $^{\#}p<0.05$ vs DB+DW. ND+DW = non-diabetic control; DB+DW = Diabetic control; DB+L-egt = Diabetic rats treated with L-egt and DB+LOS = diabetic rats treated with losartan.

3.6 L-egt upregulates total mRNA expression of Nrf2, HO1, and NQO1 and downregulates keap1 expression in cardiac tissue.

Figure 5 shows the relative mRNA level of keap1, Nrf2, HO-1, and NQO1 in the cardiac tissue of diabetic and non-diabetic animals. Keap1 mRNA level significantly increased ($p<0.05$), Nrf2 and NQO1 level significantly ($p<0.05$) decreased, while there was no significant difference in HO1 level in the diabetic control group vs non-diabetic group. Interestingly, the administration of L-egt to diabetic animals significantly reduced ($p<0.05$) Keap1 level with a significant increase in Nrf2, HO1, and NQO1 mRNA level in the cardiac tissue vs non-diabetic and diabetic control animals.

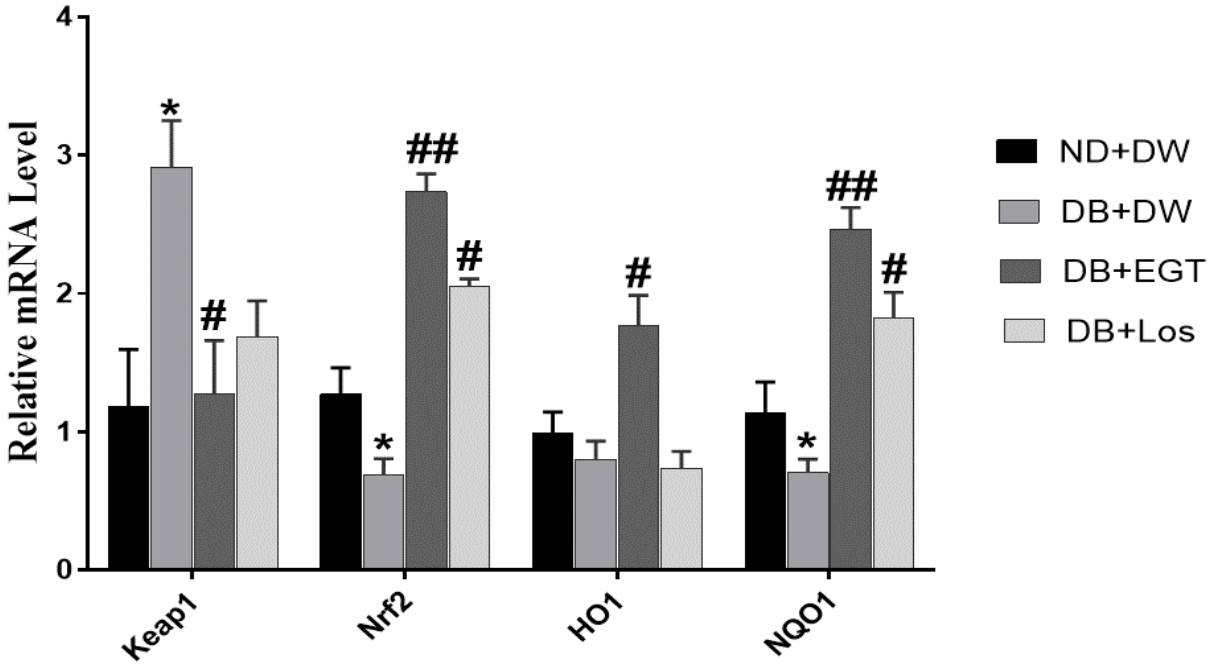


Figure-5: Effect of L-egt on relative mRNA expression of Keap1, Nrf2, HO-1 and NQO1 after a treatment period of 6 weeks. $^{*}p<0.05$ compared to ND+DW. $^{\#\#}p<0.01$, $^{\#}p<0.05$ compared to DB+DW. ND+DW = non-diabetic control; DB+DW = Diabetic control; DB+L-egt = Diabetic rats treated with L-egt and DB+LOS = diabetic rats treated with losartan.

3.7. Molecular docking

A docking study was carried out to determine the interaction between L-(+) – ergothioneine and Nrf2 peptide binding sites and compare affinities to the native co-crystallized ligand. The protein structure (PDB code: 4L7B) was first separated from the inhibitor and refined using molecular minimization with added hydrogen for the docking calculations. Docking calculation was conducted using the Autodock Vina software (42). L-(+) – ergothioneine compound was docked into the same groove of the binding site of the native co-crystallized 1VV-ligand (Table 3). The NRF- L-(+) – ergothioneine complex showed a relatively stable complex during the simulation (figure 6c). L-(+)–ergothioneine's thermodynamic contribution to the complex's total binding free energy surmounts the stability of L-(+) – ergothioneine in the Nrf2 peptide-binding pocket and thus the complex's stability throughout the simulation. The free binding energy of the system was summarized in Table 4.

Table 3: Representation of the native co-crystallized ligand pose (IVV) and L-(+) – ergothioneine bound to Nrf2 peptide binding site

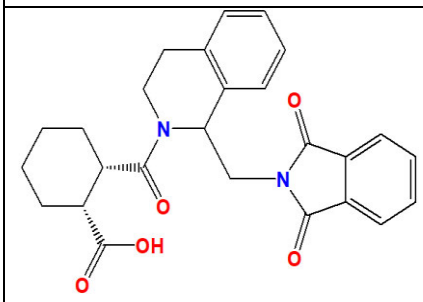
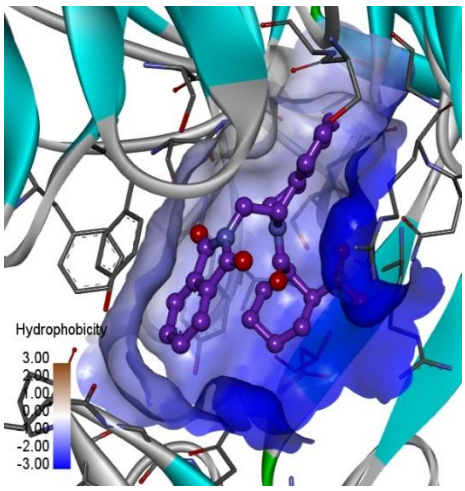
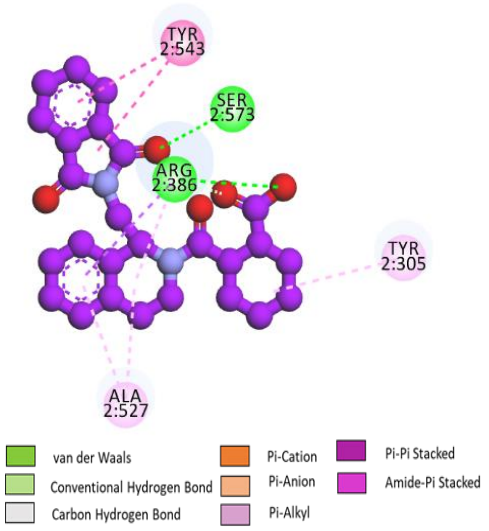
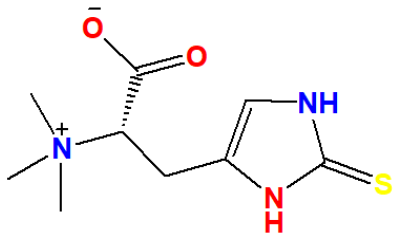
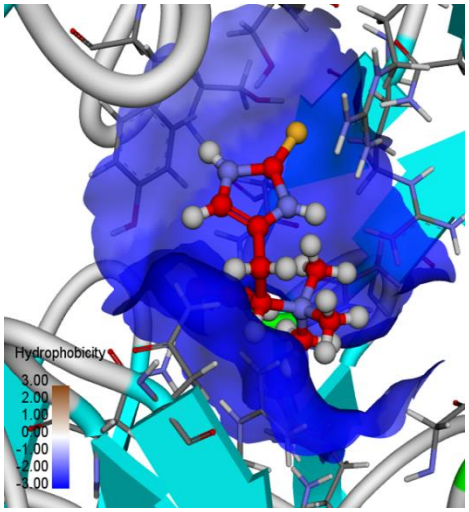
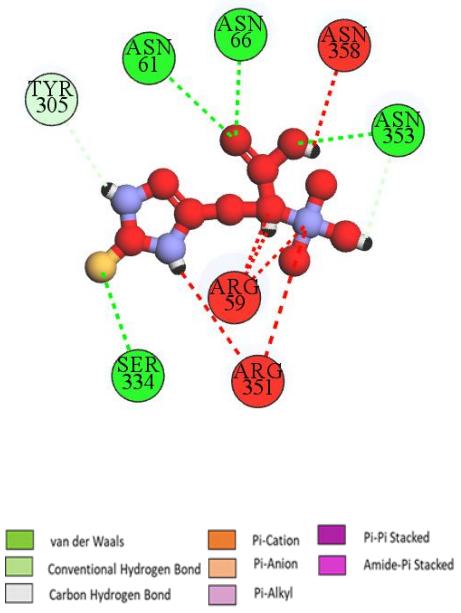
Compounds	Docked structure	Ligand interaction
 (1S,2R)-2-{[(1S)-1-[(1,3-dioxo-1,3-dioxo-1,3-dihydro-2H-isoindol-2 yl) methyl]ccn-3,4-dihydroisoquinolin 2(1H)yl]carbonyl}cyclohexane carboxylic acid) (**IVV**)	 Δ G = -11.01	
 **L-(+) – ergothioneine**	 Δ G = -11.54	

Table4: The MM-GBSA free binding energy calculations of NRF- L-(+) – ergothioneine complex.

NRF- L-(+) – ergothioneine complex					
Complex	**ΔE_{vdW}**	**ΔE_{elec}**	**ΔG_{gas}**	**ΔG_{solv}**	**ΔG_{bind}**
L-(+)– ergothioneine	-11.21 ± 0.11	-135.62 ± 0.62	-146.83 ± 0.63	135.11 ± 0.61	-11.71 ± 0.12

3.8 Molecular Dynamic Simulations and Binding Free Energy Analysis

A molecular dynamic simulation was conducted to investigate the inhibition performance and interactions of L- (+) – ergothioneine with the NRF binding site. Validation of system stability is significant for monitoring disruptive movement and avoiding artifacts during the simulation process. In this study, Root-Mean-Square Deviation (RMSD) was calculated to measure the systems' stability during the 60 ns simulations, and the result was presented in Figure 6c.

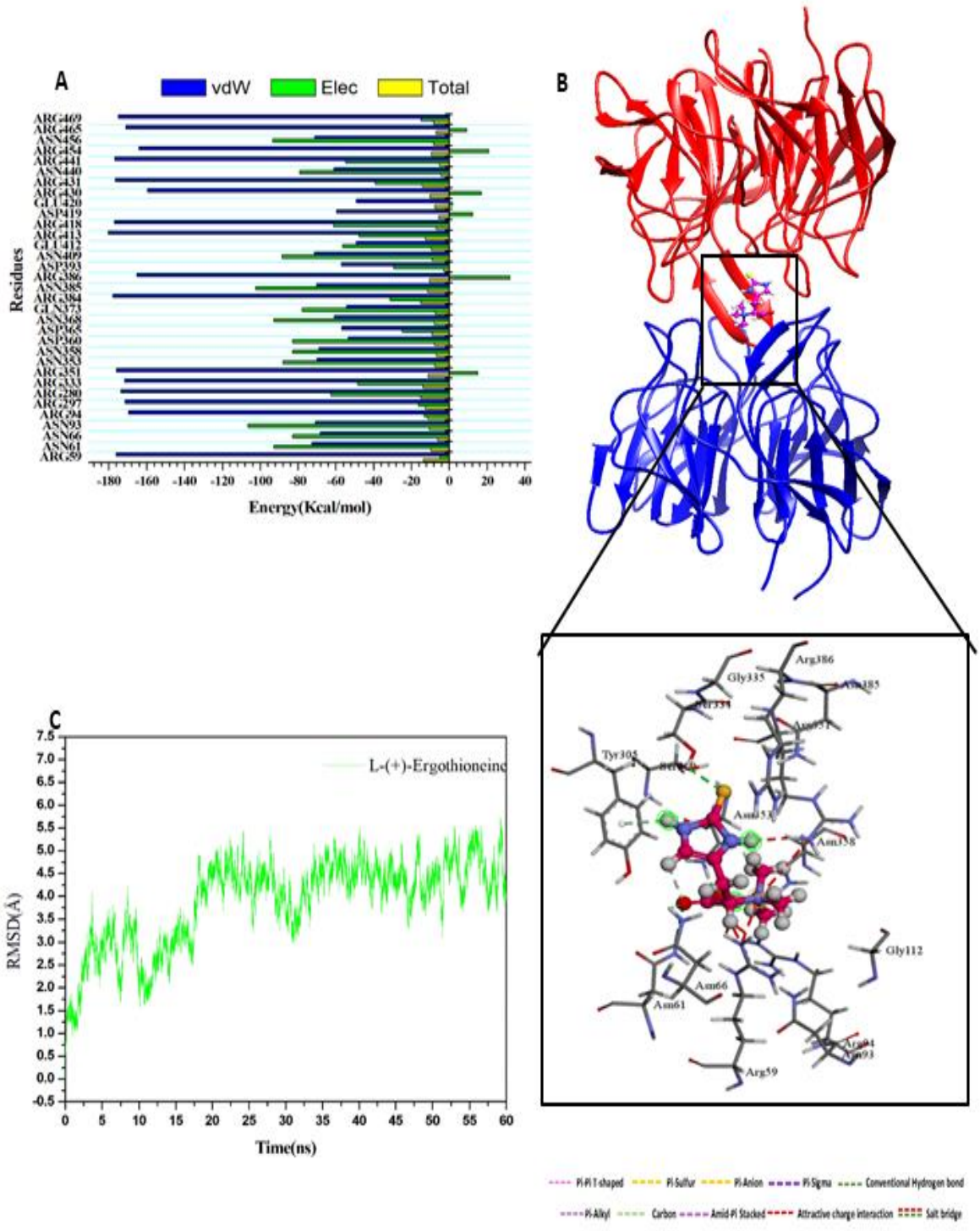


Figure 6: NRF- L-(+) – ergothioneine complex interactions (A) Per-residue decomposition analysis showing ARG59 and ASN353 to have the most significant bond fluctuations (B) Inter-molecular interactions between L-(+) – ergothioneine with NRF catalytic site residues, which was validated by (C) The time evolution of RMSD of the C-alpha atom backbone of the NRF- L-(+) – ergothioneine complex.

4.0 DISCUSSION

The present study aimed at evaluating the protective effect and antioxidant mechanism of L-egt against cardiovascular injury associated with type-2 diabetes. It has been reported that L-egt primarily accumulates in the liver (basal level of L-egt in the liver was 80.65ng/mg tissue, which increased to 202.60 ng/mg after oral dosing for 28 days), and 35 mg/kg/day L-egt for seven days is sufficient to cause a significant elevation in L-egt levels in most tissues (31, 57). This increased tissue L-egt content has been identified as an adaptive mechanism by which L-egt suppresses oxidative damage and delay further tissue injury (58, 59). However, the cardioprotective effect of L-egt was compared to the reference drug losartan, an angiotensin-II receptor blocker that reduces cardiac fibrosis and inflammation (60, 61).

The drastic weight loss observed in diabetic control animals has been attributed to the loss of adipose tissue, excessive catabolism of tissue protein, and muscle wasting resulting from abnormal energy metabolism during diabetes (62, 63). This reduction in the body weight of diabetic rats was inhibited with L-egt treatment suggesting that L-egt improved energy balance and prevented muscle wasting. Increased water intake observed in diabetic control animals confirms the successful induction of diabetes. However, the reduced water intake in L-egt-treated diabetic animals indicates the osmoregulatory effect of L-egt because hyperglycemia can increase the osmotic concentration of extracellular fluids, thereby stimulating the osmoreceptors in the hypothalamus to increase water intake (64). The increased blood glucose recorded in the diabetic rats was reduced when treated with L-egt, although this was not significant, which may be due to the delay in starting treatment. This observation is in line with the study (65), which reported that a delay in starting treatment might take longer to normalize blood glucose levels. However, previous studies have shown the potential of nutraceuticals (such as naringenin and L-egt) to alleviate diabetic complications (including vascular dysfunction) without affecting blood glucose levels (22, 66). This suggests that L-egt may target other risk factors (such as hypertriglyceridemia, inflammation, and oxidative injury) implicated in the pathogenesis of diabetic cardiomyopathy. Hypertriglyceridemia reported in diabetic control rats may result from altered lipid profile in type-2 diabetes and is recognized as a significant risk factor for CVD (67, 68). The reduction in serum triglycerides in the L-egt treated rats indicates the efficacy of this compound to inhibit de novo lipogenesis or enhanced oxidation of fatty acid in the liver during fructose consumption, thereby controlling hyperlipidemia (69, 70). This beneficial effect could prevent the risk of developing cardiovascular complications that are often associated with diabetes.

Chronic hyperglycemia is commonly associated with a sustained increase in blood pressure that usually promotes other microvascular complications. In this study, L-egt administration to diabetic rats prevented the increase in arterial pressure, and this is comparable with the previous studies where L-egt treatment reduced mean arterial pressure, alleviated symptoms of pre-eclampsia, and prevents endothelial dysfunction

(20, 71). This therapeutic benefit may be related to the potency of L-egt to reduce endothelial inflammation, promote nitric oxide bioavailability, and enhances the relaxation of vascular smooth muscles (including aorta and arterioles) with a resultant decrease in blood pressure (26, 71, 72). Besides, losartan regulates blood pressure by reducing vasoconstriction, aldosterone secretion, oxidative stress, and increasing nitric oxide and endothelial function (73, 74). Thus, L-egt may share some similar mechanism with losartan in reducing MAP. CRP, an important biomarker of cardiac inflammation, can be found in injured tissues and infarcted myocardium and serve as another risk factor to predict CVD, while cardiac enzymes (CK-MB and LDH) are major biomarkers associated with cardiac injury. A progressive increase in creatinine kinase and LDH varies with the severity of damage to the myocardial cells (75, 76). L-egt has been reported to reduce cellular inflammation in vital tissues via the inhibition of mitogen-activated protein kinase (p38-MAPK) and prevent apoptotic damage (77-79). These could account for the reduced cardiac CRP and CK-MB recorded in the diabetic rats treated with L-egt. Serum CK-MB is considerably more specific for myocardial damage, while peak rise in LDH is proportional to the extent of myocardial injury (80). Type-2 diabetes is associated with moderate myocardial injury that becomes severe after a long duration of the disease condition. This may account for the differences observed in the effect of L-egt on CK-MB and LDH.

Oxidative stress is a significant event implicated in the pathogenesis of diabetic cardiomyopathy (DCM). Experimental evidence has shown that hyperglycemia and insulin resistance stimulates excessive production of free radicals that promote inflammatory damage to vital biomolecules (e.g., protein and DNA) and apoptosis in the myocardium. In addition, chronic hyperglycemia can downregulate the efficacy of the antioxidant system (81-83). Thus, the excess free radical produced may overwhelm the antioxidant defense system to exert oxidative injury in the myocytes and endothelial cells, resulting in a decline in cardiac function. In the present study, we investigated the effect of L-egt on oxidative injury in type-2 diabetic rats by analyzing MDA, a marker of oxidative stress, and some antioxidant enzymes (SOD and CAT), and reduced-glutathione. The reduced MDA and increased SOD, CAT, and GSH observed in the L-egt treated diabetic rats indicates that L-egt was able to reduce lipid peroxidation and improve antioxidant activity providing further credibility to the potent antioxidant property of L-egt, which have been reported in different in vitro and in vivo studies (84-86). These antioxidant properties may provide cyto-protection against oxidative injury in the myocardium by scavenging the free radicals produced and converting them into less toxic molecules. Also, tissue distribution of L-egt has shown that this nutraceutical can accumulate in the mitochondria via active transport by its specific transporter- Organic cation transporter-1 (OCT-1) localized on the mitochondria membrane (87, 88). Thus, L-egt may penetrate the mitochondria to reduce the production of free radicals in the electron transport chain. Collectively, L-egt may protect against oxidative injury in the cardiac cells by reducing the production of free radicals, inhibit their activity, and

upregulate the antioxidant defense system in the body. Studies have shown that losartan also exerts antioxidant properties associated with increased glutathione and reduces MDA level (89), which have been reported as a major mechanism in which losartan improves cardiovascular functions. Thus, L-egt and losartan may protect against diabetes-induced oxidative to improve cardiovascular functions during diabetes.

L-egt has been shown to activate the Nrf2/ARE pathway, a signaling pathway for activating detoxifying enzymes and cytoprotective antioxidant gene, thereby improving the antioxidant defense system (90, 91). This report was also validated in this study, where the in-vivo mechanism of action of L-egt showed the upregulation of Nrf2, HO-1, and NQO1 expression while down-regulating Keap-1 expression in the heart. This result indicates that L-egt could mediate its antioxidant activities by inhibiting the degradation of Nrf2 from its repressor protein (Keap1) and promoting its influx into the nucleus to increase the production of its downstream antioxidant genes, including HO-1 and NQO1 (12, 92).

Chronic inflammation occurs due to tissue response to oxidative injury and contributes significantly to the development of CVD even before hyperglycemia (93, 94). The increased levels of TNF-α, MCP-1, and TGF-β1 in the cardiac homogenates of the diabetic control rats are characteristic feature of inflammation. Treatment of diabetic rats with L-egt reduced the level of these inflammatory molecules suggesting that L-egt exert anti-inflammatory activities in the cardiac tissue. Previous studies have also reported that L-egt reduced lung inflammation in cytokine-insufflated rats and inhibited inflammation-induced DNA damage in rats (95, 96). The anti-inflammatory activity can be attributed to the efficacy of L-egt to suppress the NF-kB and p38MAPK cellular transcription pathway, thereby reducing the release of pro-inflammatory molecules and macrophage activity (97, 98). Furthermore, losartan has been shown to attenuate coronary perivasculitis via its anti-inflammatory properties, associated with reduced serum levels of IL-6, TNF-α, IL-10, MCP-1, and expression of TGF-β1 expression (60, 99). Thus, L-egt may share similar anti-inflammatory properties with losartan to alleviate cardiac injury.

Previous experiments have reported that Nrf2 is a vital transcription protein in the cellular antioxidant signaling pathway, and its activation has shown therapeutic benefits in managing cardiovascular injuries, including diabetic cardiomyopathy, heart failure, and diabetic kidney disease (12, 100). The increased HO-1, NQO1, and Nrf2 mRNA expression with reduced Keap1 expression observed in the in-vivo study indicate that L-egt may activate Nrf2 by disrupting the Nrf2-Keap1 protein complex. This result was further confirmed by molecular docking, where L-egt binds effectively to the active site of Nrf2 more than the native ligand, thereby promoting Nrf2 dissociation from its repressor protein (Keap1). Thus, the data from this study support that L-egt may inhibit the ubiquitination of Nrf2, with subsequent activation of its downstream antioxidant molecules.

The molecular docking and simulations study further evaluates the relationship between L-egt and the Nrf2-Keap1 protein complex. The docked NRF- L-(+) – ergothioneine complex showed ionic interaction with fifteen residues (Tyr 305, ser 334, Gly 335, Arg 386, Asn 385, Arg 386, Arg 351, Asn 353, Asn 358, Gly 112, Asn 94, Asn 93, Arg 59, Asn 61, Asn 66, and Ser 334). In contrast to the co-crystallized ligand, it only showed ionic interaction with only five amino acid residues. Interestingly, however, a hydrogen bond acceptor was noted between Asn 353's OD1 and L-(+)-ergothioneine C4 at a distance of 2.67 Å, and a hydrogen bond donor was noted between Asn 353's ND2 and L-(+)-ergothioneine O1 at a distance of 2.56 Å; this was peculiar since Asn 353 was not engaged in any hydrogen interaction of the IVV – NRF complex. The pharmacophoric hot spot Tyr 305 has formed Pi-Pi interaction with IVV and L-(+)–ergothioneine. These ionic bond deviations between the systems might be because of the size of L-(+)–ergothioneine in contrast to IVV. L-(+)– ergothioneine was essentially diminished in size, containing the heterocyclic rings from the pharmacophore model. Because of the size of L-(+) – ergothioneine, the sulfur atom of the imidazole-thione was allowed to form a hydrogen bond with Ser 334 further into the Nrf2 hydrophobic binding site.

The L-(+) – ergothioneine contain imidazole-thione with several heteroatoms (N, S) suggest that they act as a polydentate ligand (101). A keen look into the individual energy contribution shows that L-(+)–ergothioneine interactions with the enzymes are driven by the more negative Electrostatic energy components resulting in the observed binding free energies, thus increasing gas-phase energy validating the free energy analysis.

Conclusion

This study showed that L-egt complements the antioxidant system by upregulating the Nrf2 signaling pathway and activating its downstream cyto-protective genes to alleviate cardiac inflammation and injury in type-2 diabetic rats. Furthermore, the molecular dynamic investigation showed higher binding energy and selectivity of L-(+) – ergothioneine toward the Nrf2-Keap1 complex, thereby reducing the degradation of Nrf2 by its repressor protein (Keap1). This insight highlights the potential mechanism of action of L-egt and provides the basis for further structure-based design hypotheses of novel analogs with higher efficacy. Therefore, supplementation of L-egt with the existing therapeutic regimen could provide additional benefits in the management of diabetic cardiomyopathy.

Ethics Approval and Consent to Participate

This study was approved by the Animal Research Ethics Committee (AREC) of the University of KwaZulu-Natal, Durban, South Africa, with a reference number: AREC/006/019D.

HUMAN AND ANIMAL RIGHT

No human were used in this study. All snimal protocols were according to the guidelines published by the US National Institute of Health for the proper care and use of laboratory animals (NIH publication N0. 85-23, 1996).

List of Abbreviations

ELISA = Enzyme-Link Immuno-Sorbent Assay

Rt-PCR = Reverse Transcriptase-quantitative Polymerase Chain Reaction

Keap1 = Kelch-like ECH-Associated Protein-1

Nrf2 = Nuclear factor erythroid-2-Related Factor-2

ARE = Antioxidant Response Elements

MAP = Mean Arterial Pressure

STZ = Streptozotocin

L-egt = L-ergothioneine

CK-mB = Creatinine Kinase-MB

LDH = Lactate Dehydrogenase

CRP = C-Reactive Protein

HO-1 = Heme Oxygenase-1

NQO1 = NADP(H) Quinone Oxidoreductase-1

GAPDH = Glyceraldehyde-3-phosphate Dehydrogenase

MRBP = Mouse and Rat tail cuff Blood pressure system

GSH = Glutathione

MCP-1 = Monocyte Chemoattractant Protin-1

TGF-β1 = Transforming Growth Factor- β1

TNF-α = Tumor Necrotic Factor- α

SOD = Super Oxide Dismutase

CAT = Catalase

MD = Molecular Dynamics

ns = nanoseconds

Consent for Publication

Not applicable.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article

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Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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CHAPTER 5

5.0 SYNTHESIS AND CONCLUSION

5.1 SYNTHESIS

Type-2 diabetes (T2D) has already attained epidemic levels, with 90–95% of diabetic patients suffering from T2D (1). This metabolic disease can cause or worsen the progression of renal, hepatic, and cardiovascular complications by promoting dyslipidemia, oxidative injuries, inflammation, and apoptosis, which damage vital cellular components, such as protein, lipids, and DNA (2). The use of combination therapies has been reported to improve treatment outcomes compared to monotherapy. Recently, attention has been drawn to the therapeutic benefits of medicinal foods (such as mushrooms) with potent bioactive compounds as an adjuvant in the management of diabetes complications (3, 4). L-ergothioneine, obtained from mushrooms and other food products, has been approved to be used as supplements (5). Studies have reported its adaptive cytoprotective function in tissues exposed to injuries and improved therapeutic efficacy in synergy with existing treatment (6, 7). Previous studies have shown that L-egt lowers the risk of cardiometabolic diseases and inhibits high-glucose induced endothelial dysfunction (8); reduces lipid accumulation, improves liver function in nonalcoholic liver disease (9), and modulate oxidative and inflammatory damage in the kidney (10). Therefore, this study was designed to evaluate the effects of L-ergothioneine alone or combined with metformin on selected biomarkers of renal, hepatic, and cardiovascular function in an experimental type-2 diabetic rat model. The probable mechanism of action of this compound was further highlighted using an in-silico model.

During diabetes, an increased *de novo* lipogenesis, decreased fatty acids oxidation, and triglyceride clearance from the circulation promotes the influx of fatty acid into the liver and compromises liver function (11, 12). In this study, the induction of type-2 diabetes using the fructose-STZ model promotes insulin resistance and hyperglycemia in diabetic rats with an increased hepatic triglyceride concentration. Insulin resistance increases hepatic triglyceride accumulation under the influence of SREBP1c that activates lipogenic enzymes such as FAS (13). Thus, reducing lipid accumulation in the liver is a vital strategy to alleviate liver injury, including hepatic steatosis and NAFLD. L-egt has been previously demonstrated to mitigate postprandial triglyceride response, and has been implicated in NAFLD treatment by reducing lipid accumulation in the liver (14). In this study, administration of L-egt alone or in combination with metformin decreased liver triglyceride accumulation. This observation was further supported by the downregulation of SREBP1c and FAS mRNA expression that activates lipogenic enzymes. As part of the novelty of this study, our study is the first to report that L-egt downregulates SREBP1c and FAS mRNA expression to reduce hepatic triglyceride accumulation.

Excessive lipid accumulation increases oxidative stress and stimulates the inflammatory response that causes structural and functional damage in the liver (15, 16). Oxidative injury and inflammation have been well-correlated with increased serum concentration of liver enzymes such as AST, ALT, and ALP which have been used as biomarkers to evaluate liver function thereby assisting in the diagnosis and management of liver injury (17). The results from this study showed that L-egt alone, and its combination with metformin reduced liver injury biomarkers, alleviated oxidative damage and hepatic inflammation. Interestingly, the coadministration of L-egt with metformin improves the antihyperglycemic effect of this biguanide by reducing IR and hepatic lipogenesis. Furthermore, the liver has been recognized as the primary site for metformin activity where it suppresses hepatic gluconeogenesis (18). This biological activity may be enhanced by the protective effect of L-egt on the liver. Furthermore, the liver histology showed that L-egt and metformin prevent congestion of the central vein and disruption of the hepatic sinusoids, thereby improving intrahepatic blood supply. Collectively, the results from this study indicate that administration of L-egt improves glycemic control, insulin sensitivity, reduce triglyceride accumulation, prevent hepatic oxidative injury and inflammation, and structural damage, to attenuate liver complication associated with type-2 diabetes.

The antioxidant and anti-inflammatory efficacy of L-egt was further evaluated by the expression of major transcription factors in these signaling pathways. It was observed that L-egt upregulates Nrf2 and Sirt1 mRNA expression. The activation of these transcription factors has been reported to stimulate the production of cytoprotective antioxidant gene (such as NQO-1 and HO1), improve lipid metabolism, detoxify ROS, and suppresses the NF-kB signaling cascade (19, 20). In addition, L-egt downregulates NF-kB and TGF-β1 expression to reduce hepatic inflammation implicated in the pathogenesis of diabetic liver injury. Thus, in chapter 2 of this study, we reported that L-egt and metformin alleviate liver injury in type-2 diabetic rats via the reduction of oxidative stress, inflammation, and hypertriglyceridemia.

Renal complication (diabetic nephropathy) is another microvascular complication associated with type-2 diabetes and is often diagnosed with proteinuria and decreased GFR (21). The persistent hyperglycemia increases free radical production, causing oxidative injury in the glomerular basement membrane. This compromises the membrane integrity and reduces the efficacy of the filtration barrier, promoting the leakage of proteins into the renal tubules (22, 23). Thus, strategies to attenuate hyperglycemia and enhance the antioxidant defense system have been hypothesized to be a robust approach to delaying the onset of kidney injury in diabetes. This study demonstrated that the administration of L-egt improved antioxidant efficacy, and its co-administration with metformin effectively regulates glucose homeostasis in type-2 diabetic rats. Indeed, L-egt, alone or in combination with metformin, attenuates renal dysfunction as shown

by the reduced serum creatinine, blood urea nitrogen, albuminuria, proteinuria, and KIM-1 concentration, as well as improved creatinine clearance (a biomarker of GFR).

Low-grade inflammation commonly associated with T2D can elicit structural and functional damage in the kidney via the NF-kB, JAK/STAT, and TGF-β1 transcription signaling. These pathways increase the secretion of inflammatory cytokines (TNF-α), chemokines (MCP-1), and growth factors (TGF-β1) that mediate apoptosis, immune response, and fibrosis of the renal cells (24, 25). Furthermore, the altered metabolic and hemodynamic pathway associated with diabetes upregulates fibronectin expression in the extracellular matrix, promoting fibrosis and mesangial matrix expansion, with resultant glomerulosclerosis and tubulointerstitial fibrosis. Therefore, increased fibronectin expression as identified in the renal cells, is a significant risk factor in diabetic nephropathy (26). Among the novelty of this study, L-egt administration reduced mesangial matrix expansion and renal fibrosis by downregulating fibronectin and TGF-β1 expression. In addition, L-egt, in the presence and absence of metformin, reduced TNF-α, MCP1, and TGF-β1 concentration in the kidney by downregulating NF-kB mRNA expression. The improved antioxidant effect of L-egt was further supported by the increase in protein and mRNA expression of the Nrf2 transcription factor, with subsequent upregulation of the cytoprotective antioxidant gene, including NQO1 and HO1. Altogether, the results from this study showed that L-egt, with or without metformin administration, mitigates oxidative stress and inflammation to reduce biomarkers of renal injury in diabetic animals. Thus, in chapter 3 of this study, we reported that L-egt might attenuate renal dysfunction in type-2 diabetic rats by activating the Nrf2 antioxidant pathway.

Studies have reported that cardiovascular dysfunctions are also associated with type-2 diabetes (27, 28), which are often characterized by increased arterial pressure, endothelial dysfunction, and increased serum levels of cardiac enzymes (Ck-MB, CRP, and LDH), resulting from altered lipid metabolism, oxidative stress, and cardiac inflammation (29, 30). These features were indeed observed in the untreated diabetic animals in this study, which was in line with the report of other studies on diabetic cardiomyopathy (31, 32). Excess fructose consumption promotes hypertriglyceridemia, contributing to atherogenesis and increased arterial pressure (33, 34). Also, elevated arterial pressure may result from increased peripheral resistance due to vascular remodeling and high extracellular fluid volume caused by IR and hyperglycemia (35). In addition to the mild increase in arterial pressure, cardiac injury characterized by increased serum concentration of cardiac enzymes (CK-MB, CRP, and LDH) was also observed in the diabetic control animals. This may be attributed to hyperglycemia-induced free radical production that promotes lipid peroxidation in the cardiac cells, thereby compromising membrane integrity, with subsequent leakage of cardiac enzymes into the circulation.

Furthermore, the increased oxidative stress promoted cardiac inflammation by activating the NF-kB and TGF-β1 transcription pathway, enhancing monocyte activation and fibrosis with resultant inflammation in the cardiomyocytes (35-37). Our results showed that the administration of L-egt mitigates cardiac dysfunction as observed by the reduction in arterial pressure, cardiac CK-MB, CRP, and hypertriglyceridemia in type-2 diabetic rats. This cardioprotective effect may be related to the potential of L-egt to improve lipid metabolism, as well as its potent antioxidant and anti-inflammatory properties (9, 38). As observed in the L-egt treated groups, the increased SOD, CAT, and GSH work synergistically to detoxify the free radicals produced by hyperglycemia, while the reduced cardiac concentration of TNF-α, TGF-β1, and MCP1 suggest the inhibition of cardiac inflammation. In addition, we carried out a molecular docking and dynamic study to evaluate the probable antioxidant mechanism of L-egt. Our result showed that L-egt binds effectively to the Nrf2 active site. This structural relationship may inhibit the Nrf2-Keap1 protein bond, releasing Nrf2 from its suppressor protein (Keap1). This result provides structure-based evidence that L-egt may be a potent activator of the Nrf2 antioxidant signaling cascade. The in-silico study was further supported by the upregulation of Nrf2 and its downstream cytoprotective genes (NQO1 and HO1) in the cardiac homogenate. These observations showed that L-egt might exert its potent antioxidant effect by activating the Nrf2 antioxidant signaling pathway. Thus, in chapter 4 of this study, we reported that L-egt administration might alleviate risk factors associated with cardiovascular injury in type-2 diabetic rats, with the evidence that L-egt might be a potent ligand that activates Nrf2 signaling.

5.2 Conclusion

The overall result from this study showed the potential benefits of L-ergothioneine in the management of selected complications associated with type-2 diabetes. L-egt may be an effective adjuvant to alleviate hypertriglyceridemia, oxidative stress, and inflammation, thereby protecting vital tissues (including kidney, liver, and heart) against injury and improving glycemic control when co-administered with metformin to delay the onset of diabetic complications. As reported in Chapter 2, L-ergothioneine and metformin administration alleviates liver injury in type-2 diabetic rats by reducing oxidative stress, inflammation, and hypertriglyceridemia; Chapter 3 showed that L-ergothioneine might activate the Nrf2 antioxidant pathway and its downstream cytoprotective genes to attenuate renal dysfunction in type-2 diabetic rats, while Chapter 4 showed the cardioprotective effects of L-ergothioneine in type-2 diabetic rats and in-silico antioxidant mechanism. Most importantly, the present study showed that supplementation of metformin therapy with L-egt has better treatment outcomes than monotherapy. Treatment regimen with L-egt will provide an affordable and cost-effective strategy, especially in low-middle income countries, as this compound can be obtained from dietary sources. In addition, the increase tissue uptake and accumulation

of L-egt at injury site as well its extended half-life in the body suggest its effectiveness to attenuate cellular injury. Clinically, the result from this study suggest that L-egt may be supplemented with conventional therapies, like metformin, to enable diabetes patients achieved glycemic control compared to prolonged use of metformin only. This may also help to reduce adverse effect of metformin by reducing the dosage of drug administered. Therefore, dietary sources of L-egt, like mushrooms and beans, may be incorporated as a simple dietary modification to manage T2D and its complications.

5.3 Scope for further studies

The limited funding available for this study provides the opportunity for further studies, which are required to evaluate the detailed preventative and therapeutic mechanisms of L-egt in diabetes.

1) In vitro studies using renal and hepatic cells are required to provide more evidence on the specific effect of L-egt.
2) L-egt transporter (OCTN-1) expression needs to be evaluated to provide detailed information on the distribution and accumulation of L-egt in these vital tissues (heart, kidney, and liver).
3) The immunohistochemical analysis of major signaling cascades (like Nrf2, Sirt1, TGF-β1, JAK/STAT) and apoptotic pathways involved in the pathogenesis of diabetic complications should be evaluated.
4) Also, electrocardiogram analysis (to support cardiovascular parameters) and biomarkers of endothelial dysfunction (nitric oxide and endothelin 1) should be evaluated.

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CHAPTER 6- APPENDICES

APPENDIX 1: ETHICAL CLEARANCE

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

20 June 2019

Mr Ayobami Dare (218084517)
School of Laboratory Medicine and Medical Sciences
Westville Campus

Dear Mr Dare,

Protocol reference number: AREC/006/019D
Project title: Role of L-ergothioneine on kidney functions of streptozotocin-induced diabetic male rats

Full Approval – Research Application

With regard to your revised application received on 28 February 2019. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following conditions:

RECOMMENDATIONS:

1. Use 'euthanise' instead of 'sacrifice' in future submissions, dissertations and articles.
2. Confirm the number of animals being used.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 12 April 2020.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

[redacted]

Dr Sanil D Singh, PhD
Deputy Chair: Animal Research Ethics Committee

/kr
cc Supervisor: Dr Anand Nadar
Cc Registrar: Mr Simon Mokoena

Animal Research Ethics Committee (AREC)
Ms Mariette Snyman (Administrator)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
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Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

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APPENDIX-2: SLMMS RESEARCH SYMPOSIUM CERTIFICATE

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

LMMS RESEARCH SYMPOSIUM 2020

CERTIFICATE OF PRESENTATION

Awarded to

Ayobami Dare

University of KwaZulu-Natal

for presenting the poster:

L-ergothioneine alleviates liver injury by docking oxidative stress, inflammation and hypertriglyceridemia in type-2 diabetic male Sprague-Dawley rats

At the

Annual Laboratory Medicine and Medical Sciences Research Symposium 2020 Which took place via Zoom, Durban, South Africa

On the 18th of September 2020

Yours sincerely,

[redacted]

Dr De Gama

Academic Leader Research SLMMS

Appendix 3: Galley Proof of manuscript 1

J_ID: CJPP Art No.: cjpp-2021-0247 Date: 4-September-21 Page: 1 Total Pages: 12 4/Color Figure(s): 1-5

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Canadian Science Publishing

ARTICLE

AQ1

L-ergothioneine and metformin alleviates liver injury in experimental type-2 diabetic rats via reduction of oxidative stress, inflammation, and hypertriglyceridemia

AQ2

Ayobami Dare, Mahendra L. Channa, and Anand Nadar

Abstract: Type-2 diabetes (T2D) is associated with liver toxicity. L-ergothioneine (L-egt) has been reported to reduce toxicity in tissues exposed to injury, while metformin is commonly prescribed to manage T2D. Hence, this study evaluates the hepatoprotective role of L-egt, with or without metformin, in T2D male rats. A total of 36 adult male Sprague–Dawley rats were randomly divided into non-diabetic (n = 12) and diabetic (n = 24) groups. After induction of diabetes, animals were divided into six groups (n = 6) and treated orally either with deionized water, L-egt (35 mg/kg bodyweight (bwt)), metformin (500 mg/kg bwt), or a combination of L-egt and metformin for 7 weeks. Bwt, and blood glucose were monitored during the experiment. Thereafter, animals were euthanized and liver tissue was excised for biochemical, ELISA, real-time quantitative PCR, and histopathological analysis. L-egt with or without metformin reduced liver hypertrophy, liver injury, triglycerides, oxidative stress, and inflammation. Also, L-egt normalized mRNA expression of SREBP-1c, fatty acid synthase, nuclear factor kappa B, transforming growth factor β1, nuclear factor erythroid 2–related factor 2, and sirtuin-1 in diabetic rats. Furthermore, co-administration of L-egt with metformin to diabetic rats reduced blood glucose and insulin resistance. These results provide support to the therapeutic benefits of L-egt in the management of liver complications associated with T2D.

Keywords: antioxidant, diabetes, L-ergothioneine, metformin, cytoprotection.

Résumé : Le diabète de type 2 (DT2) est associé avec la toxicité hépatique. On a rapporté que la L-ergothionéine (L-egt) permet d'abaisser la toxicité dans des tissus exposés à des lésions, tandis que la metformine est prescrite couramment dans la prise en charge du DT2. Par conséquent, la présente étude portait sur le rôle hépatoprotecteur de la L-egt, avec et sans metformine, chez des rats mâles atteints de DT2. Nous avons réparti aléatoirement 36 rats Sprague–Dawley mâles adultes dans des groupes non-diabétique (n = 12) et diabétique (n = 24). Après la production du diabète, nous avons réparti les animaux dans six groupes (n = 6) et leur avons administré de l'eau désionisée, de la L-egt (à 35 mg/kg de poids corporel), de la metformine (à 500 mg/kg de poids corporel) ou une association de L-egt avec de la metformine par voie orale pendant sept semaines. Nous avons surveillé le poids corporel et la glycémie au cours de l'expérience. Par la suite, les animaux ont été euthanasiés et nous avons prélevé du tissu hépatique en vue d'analyses biochimiques, d'ÉLISA, de PCR quantitatif en temps réel et d'histopathologie. Avec ou sans metformine, la L-egt permettait d'atténuer l'hypertrophie du foie, les lésions hépatiques, le stress oxydatif et l'inflammation de même que de faire diminuer les taux de triglycérides. De plus, la L-egt permettait de normaliser l'expression en ARNm de SREBP-1c, de FAS (« fatty acid synthase »), de NF-κB (« nuclear factor kappa B »), de TGF-β1 (« transforming growth factor β1 »), de Nrf2 (pour « nuclear factor erythroid 2–related factor 2 ») et de sirtuine-1 (Sirt1) chez les rats diabétiques. En outre, l'administration concomitante de L-egt avec de la metformine permettait de faire diminuer la glycémie et la résistance à l'insuline chez les rats diabétiques. Ces résultats viennent appuyer les bienfaits thérapeutiques de la L-egt dans la prise en charge des complications hépatiques associées avec le DT2. [Traduit par la Rédaction]

Mots-clés : antioxydant, diabète, L-ergothionéine, metformine, cytoprotection.

Introduction

Type-2 diabetes (T2D), a chronic metabolic disorder of global prevalence, is drastically increasing in developing and industrialized countries due to excessive caloric intake, sedentary lifestyle, and poor diagnosis and disease management. The etiology and progression of T2D are multifaceted and frequently associated with obesity, hypertriglyceridemia, insulin resistance (IR), and compromised insulin secretion by the pancreatic islet with subsequent hyperglycemia (Lozano et al. 2016; Pandey et al. 2015). Liver complications are often seen in approximately 70% of people with diabetes and account for about 2%–4% mortality in T2D patients (Hazlehurst et al. 2016; Zoppini et al. 2014). These complications (including non-alcoholic fatty liver disease (NAFLD), steatosis) result from hyperglycemia-induced oxidative injury and low-grade inflammation that mediate liver damage, including fibrosis and apoptosis. Also, IR increases de novo lipogenesis under the influence of SREBP-1c and alters lipid metabolism by upregulating fatty acid synthase (FAS), thereby promoting the influx of fatty acid into the liver cells (Perry et al. 2015; Gehrke and Schattenberg 2020; Arrese et al. 2019). Excessive accumulation and infiltration of fats into the hepatocytes contribute to liver toxicity by increasing

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Appendix-4: Manuscript 2

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L-ergothioneine and its combination with metformin attenuates renal dysfunction in type-2 diabetic rat model by activating Nrf2 antioxidant pathway

Ayobami Dare *, Mahendra L. Channa, Anand Nadar

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ARTICLE INFO

Keywords:
Diabetes
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Kidney
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ABSTRACT

L-ergothioneine (L-egt) is a bioactive compound recently approved by the food and drug administration as a supplement. L-egt exerts potent cyto-protective, antioxidant and anti-inflammatory properties in tissues exposed to injury, while metformin is a first-line prescription in type-2 diabetes. Therefore, the present study investigated the protective effect of L-egt alone, or combined with metformin, on renal damage in a type-2 diabetic (T2D) rat model. T2D was induced in male Sprague-Dawley rats using the fructose-streptozotocin rat model. L-egt administration, alone or combined with metformin, began after confirming diabetes and was administered orally for seven weeks. After the experiment, all animals were euthanized by decapitation, blood samples were collected, and both kidneys were excised. Biochemical analysis, Enzyme-link Immunoassay (ELISA), Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR), western blotting, and histological analyses were done to evaluate various biomarkers and structural changes associated with renal damage. Untreated diabetic rats showed loss of kidney functions characterized by increased serum creatinine, blood urea nitrogen, proteinuria, triglycerides, lipid peroxidation, inflammation, and decreased antioxidant enzymes. Histological evaluation showed evidence of fibrosis, mesangial expansion, and damaged basement membrane in the nephrons. However, L-egt alleviates these functional and structural derangements in the kidney, while co-administration with metformin reduced hyperglycemia and improves therapeutic outcomes. Furthermore, L-egt treatment significantly increased the expression of major antioxidant transcription factors, cytoprotective genes and decreased the expression of inflammatory genes in the kidney. Thus, combining L-egt and metformin may improve therapeutic efficacy and be used as an adjuvant therapy to alleviate renal damage in type-2 diabetes.

1. Introduction

Type-2 diabetes (T2D) is a metabolic disorder exerting a heavy toll on both the individual and society with several complications, including nephropathy [39]. Diabetic nephropathy (DN) is a major microvascular complication that causes chronic kidney disease (CKD) and eventually leading to end-stage renal disease (ESRD) requiring renal hemodialysis therapy or kidney transplant [50,70]. DN develops in approximately 30–40% of patients with diabetes and progresses with time, characterized by increased urinary albumin excretion, decreased glomerular filtration rate (GFR), and increased peripheral arterial blood pressure [11,63]. The pathogenesis of DN in T2D involves a complex and multifactorial process resulting from hyperglycemia and insulin resistance (IR). The persistent hyperglycemia generates excess free radicals (e.g., ROS) that overwhelms the antioxidant defense system, resulting in oxidative injury that promotes renal inflammation and mitochondrial dysfunction [6,30]. Insulin resistance also causes dyslipidemia associated with progressive loss of renal functions via transforming growth factor- β (TGF-β) signaling pathway [27,41]. These biochemical processes stimulate different cellular signals that damage vital biomolecules and cellular components of the kidney (including DNA, proteins, podocyte, mesangial and tubular cells), exerting significant abnormalities on renal structure and function with subsequent ESRD [65]. Therefore, the prevention and management of DN should be

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Appendix 5: Galley proof of manuscript 3

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Cardiovascular & Hematological Agents in Medicinal Chemistry, xxxx, xx, 1-15

RESEARCH ARTICLE

Cardioprotective Effects and In-Silico Antioxidant Mechanism of L-Ergothioneine In Experimental Type-2 Diabetic Rats.

Ayobami Dare[1,*], Ahmed A Elrashedy[2], Mahendra L. Channa[1] and Anand Nadar[1]

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Abstract: ***Background*:** Diabetic cardiotoxicity is commonly associated with oxidative injury, inflammation, and endothelial dysfunction. L-ergothioneine (L-egt), a diet-derived amino acid, has been reported to decrease mortality and risk of cardiovascular injury, provides cytoprotection to tissues exposed to oxidative damage, and prevents diabetes-induced perturbation.

***Objective*:** This study investigated the cardioprotective effects of L-egt on diabetes-induced cardiovascular injuries and its probable mechanism of action.

***Methods*:** Twenty-four male Sprague-Dawley rats were divided into non-diabetic (n=6) and diabetic groups (n=18). Six weeks after the induction of diabetes, the diabetic rats were divided into three groups (n=6) and administered distilled water, L-egt (35mg/kg), and losartan (20mg/kg) by oral gavage for six weeks. Blood glucose and mean arterial pressure (MAP) were recorded pre-and post-treatment, while biochemical, ELISA, and RT-qPCR analyses were conducted to determine inflammatory, injury-related and antioxidant biomarkers in cardiac tissue after euthanasia. Also, an in-silico study, including docking and molecular dynamic simulations of L-egt toward the Keap1-Nrf2 protein complex, was done to provide a basis for the molecular antioxidant mechanism of L-egt.

***Results*:** Administration of L-egt to diabetic animals reduced serum triglyceride, water intake, MAP, biomarkers of cardiac injury (CK-MB, CRP), lipid peroxidation, and inflammation. Also, L-egt increased body weight, antioxidant enzymes, upregulated Nrf2, HO-1, NQO1 expression, and decreased Keap1 expression. The in-silico study showed that L-egt inhibits the Keap1-Nrf2 complex by binding to the active site of Nrf2 protein, thereby preventing its degradation.

***Conclusion*:** L-egt protects against diabetes-induced cardiovascular injury via the upregulation of the Keap1-Nrf2 pathway and its downstream cytoprotective antioxidants.

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Keywords: Cardio-protection, diabetes, L-ergothioneine, molecular docking, molecular dynamics, Cardiovascular Disease (CVD).

1. INTRODUCTION

Diabetes exerts a significant economic and social burden as well as reduced quality of life due to associated micro and macro-vascular complications leading to coronary heart disease, cardiomyopathy, cerebrovascular disease, arrhythmia [1-3]. Globally, Cardiovascular Disease (CVD) is well-correlated with significant mortality and morbidity in type-2 diabetes patients due to an accelerated decline in cardiac function [4, 5], which often result from risk factors such as hyperglycemia, insulin resistance, obesity, and dyslipidemia [6, 7]. These factors cause excessive generation of free radicals and a defective antioxidant pathway that promotes oxidative injury, endothelial dysfunction, and low-grade chronic inflammation identified as the main culprits connecting CVD and diabetes [8]. Therefore, effective strategies that enhance the antioxidant pathway to reduce damage by free radicals and cardiac inflammation have been proposed to manage diabetic complications [9, 10]. Recently, attention has been drawn to the beneficial role of the Keap1-Nrf2 antioxidant signaling pathway in managing diabetes and its related complications, including cardiac injury [11-13].

Keap1-Nrf2-ARE (Kelch-like ECH-associated protein-1/nuclear factor erythroid-2-related factor-2, antioxidant response elements) protein complex is a master antioxidant signaling pathway that confers cytoprotection to tissues exposed to oxidative injury. Activation of this pathway enhances the antioxidant defense system in various disease con-

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APPENDIX 6: Conference presentation

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University of Kwa-Zulu Natal, South Africa

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"L-ergothioneine and metformin alleviate liver injury in experimental type-2 diabetic rats via reduction of oxidative stress, inflammation and hypertriglyceridemia"

at the "Annual Conference on Pharmacology and Applied Toxicology" held during July 09, 2021 | Webinar

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