UNIVERSITY *of the*
WESTERN CAPE

Proteome Profiling as Tool to Determine the Effectiveness of Traditional Africa Therapies for Type 2 Diabetes Mellitus

By

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Submitted in fulfillment of the requirements for the degree

Doctor of Philosophy

UNIVERSITY *of the*
WESTERN CAPE

In the

Department of Medical Bioscience

Faculty of Nature Science

University of the Western Cape

Supervisor

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December 2022

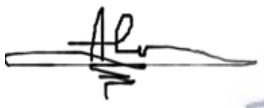
Declaration

I declare that:

The study Proteome Profiling as Tool to Determine the Effectiveness of Traditional Africa Therapies for Type 2 Diabetes Mellitus is my own work—it has not been submitted for any degree or examination at any other university and all the sources I have used or quoted have been indicated and acknowledged by complete references.

Ali M B Nouh

Signature:



Date: December 2022

Abstract

Type 2 diabetes mellitus (T2DM) involves multiple organ dysfunction accompanied by a chronic inflammatory state and oxidative stress resulting from a combination of factors such as hyperglycaemia and dyslipidaemia. Although medication to treat T2DM is currently available, several limitations and harmful effects of anti-hyperglycaemic diabetes synthetic drugs make it necessary to investigate novel drug therapies that are safer and more efficient. The biggest challenges remain in the management of T2DM and its complications. Therefore, it is necessary to prevent the early onset of diabetes and the progression of the disease individually by using pharmacological and traditional therapies.

Three traditional African herbs and a standard Western medicine for treating diabetes mellitus were selected for consideration in this study. The herbs selected were *Trigonella foenum-graecum* (*T. foenum-graecum*), *Cinnamon verum* (*C. verum*) and *Artemisia afra* (*A. afra*). The standard Western medicine chosen was metformin. These treatments were selected to assess potential effects on regulating the pro-inflammatory marker response and metabolism disorder bio-actives of mimic diabetic cells using *in vitro* assays.

The murine macrophage cell line (RAW 264.7) cultures were selected to assess the effects of plant extracts and metformin on the inflammatory response and metabolic disorder biomarkers regulation. The effects on RAW 264.7 cells were monitored in the absence or presence of high glucose and lipopolysaccharide.

The objectives of this study were to extract and analyse the chemical components of the plants *T. foenum-graecum*, *C. verum* and *A. afra* to verify their main bio-active compound concentrations; demonstrate cells expressing cytokines; assess the effects of plant extracts on pro-inflammatory (cytokines and chemokines) and biomarker activities in the presence or absence of high-glucose and lipopolysaccharide stimulation; evaluate the effects of herb extracts in comparison to metformin.

In conclusion, this study presents parameters of the pro-inflammatory cytokines, chemokines, and growth factors of mimic diabetes cell expression. These protein molecules can be biomarkers and may be used for reducing diabetic complications because of low-grade/chronic inflammation, oxidation and lipid metabolism. They may also describe defects in the pathophysiology of T2DM and might offer an advantage in drug screening.

Furthermore, evaluation of the effectiveness of traditional plants has revealed that the best herbal agent is *A. afra* – it may have better anti-inflammatory action and the potential to regulate metabolism disorders, in the diabetic condition, than *C. verum* and *T. foenum-graecum*. These natural herbal extracts are more effective than metformin in inhibiting the pro-inflammatory response and regulation of oxidant activity in high glucose.

This finding may be significant in contributing to increasing evidence that natural herbal therapies have potent anti-oxidant and anti-inflammatory properties that may result in the better regulation of dyslipidaemia. It may also open the way forward to novel therapeutic strategies for managing diabetic complications with minimal

side effects. Patients with T2DM should be monitored and supplemented with anti-inflammation and anti-oxidant therapies, combined with Western medicine, to avoid the progression of diabetes complications and achieve the most beneficial treatment.

Keywords: Type 2 diabetes mellitus, High glucose, *Artemisia afra*, *Cinnamon verum*, *Trigonella foenum-graecum*, Metformin, Macrophage cell, Inflammation, Cytokines, Chemokines, Metabolic syndrome

Acknowledgments

I would like to convey my sincere gratitude to my supervisor, Prof. Edmund John Pool, for his unwavering support, encouragement, supervision, guidance and inspiration throughout this research. Without his guidance, the completion of this research project would not have been possible.

I thank Mr Lucky Mokwena from the Central Analytical Facilities (CAF) at Stellenbosch University for assistance in performing analyses of the bio-active compounds of traditional Africa plants.

I also express appreciation to my colleagues and friends, in and outside of our laboratory—they were sincere in helping me.

I extend my heartfelt gratitude to my household for their unconditional love and support during this PhD study—to my wife, for her loving support and encouragement, continually encouraging me to progress in my career and scientific research.

I acknowledge and thank the Ministry of Higher Education of Libya for their financial support during this research project.

List of acronyms and abbreviations

A. afra	Artemisia afra
AGIs	Alpha-glucosidase inhibitors
ANOVA	Analysis of variance
β-cells	Pancreatic islet beta cells
C. verum	Cinnamon verum
CO_2	Carbon dioxide
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
DPP-4	Dipeptidyl peptidase-4
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DR	Diabetic retinopathy
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FBS	Fetal-bovine serum
FFA	Free fatty acids
G	grams
GC-MS	Gas chromatography-mass spectrometry
G-CSF	Granulocyte colony-stimulating factor
GLUT-4	Glucose transporter-4
HbA1c	Haemoglobin A1c
HG	High glucose

IC_{50}	Inhibitory concentration
ICAM-1	Intercellular adhesion molecule-1
IL-1ra	Interleukin-1 receptor antagonist
IL-1α	Interleukin-1alpha
IL-1β	Interleukin-1beta
IL-27	Interleukin-27
IL-6	Interleukin-6
IL-16	Interleukin-16
iNOS	Inducible nitric oxide synthase
IP-10	Interferon gamma-induced protein-10
IR	Insulin resistance
IRs	Insulin receptor/s
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2
I-TAC	Interferon–inducible T cell alpha chemo-attractant
LDL	Low-density lipoprotein
LG	Low glucose
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MEM	Minimum essential medium
MIP-1α	Macrophage inflammatory protein-1alpha

MIP-1β	Macrophage inflammatory protein-1beta
MIP-2	Macrophage inflammatory protein-2
ml	milliliter
mm	millimeter
mM	millimolar
NF-Kb	Nuclear factor kappa-B
Nm	nanometer
OS	Oxidative stress
Ox-LDL	Oxidative low-density lipoprotein
PBS	Phosphate-buffered saline
PI	Protease inhibitor
PI3k	Phosphatidylinositol 3-kinase
PIP-3	Phosphatidylinositols-3, 4, 5-trisphophate
PKC	Protein kinase C
RANTES	Regulated on activation, normal T cell expressed and secreted
RAW 264.7	Murine macrophage cells
ROS	Reactive oxidative species
SD	Standard deviation
SEM	Standard error mean
SFU	Sulfonylureas
SOD-2	Superoxide dismutase
IRs	Surface insulin receptors

T. foenum-graecum	Trigonella foenum-graecum
T2DM	Type 2 diabetes mellitus
TAT	Traditional African therapies
TG	Triglycerides
TNF-α	Tumour necrosis factor-alpha
TZDs	Thiazolidinediones
WHO	World Health Organization
XTT	Labelling reagent for cytotoxicity [2,3-Bis-(2 methoxy-4- nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide]
3T3-L1	Mouse pre-adipocytes

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Chapter 1

Introduction and objectives

1.1. Type 2 diabetes mellitus

Type 2 Diabetes Mellitus (T2DM) is the most common type of diabetic condition. It is a long-term metabolic disorder characterised by the inability to maintain glucose homeostasis, associated with metabolic syndrome. Metabolic disorders are the result of an excess or a deficiency of substances, such as glucose or lipids, produced by the body, resulting in a variety of complications (Piero et al., 2015). Obesity, oxidative stress, and chronic inflammation are often associated with T2DM, which affects carbohydrate, protein, and lipid metabolism (Baynes, 2015; Asmat et al., 2016). This multi-factorial abnormal metabolism may lead to defects in insulin sensitivity, pancreatic islet beta cells (β-cells) dysfunction, hepatic glucose dysfunction and glycogen storage.

Dysfunction of the β-cells accompanied by insulin resistance (IR) leads to failure in controlling blood glucose levels (Kaur & Valecha, 2014). These disorders may then cause hyperglycaemia, hyperlipidaemia and the development of overt hyperinsulinemia (Hayden, 2020). Several multi-organ complications of T2DM reduce life expectancy, including neuropathy, nephropathy, retinopathy, and cardiomyopathy (Rao Kondapally et al., 2011). Therefore, these are significant causes of rising morbidity and mortality among individuals living with diabetes resulting in severe economic concerns/challenges for the health-care system.

It is estimated that 90–95% of patients with diabetes are diagnosed with T2DM (Graves, 2007). Epidemiological studies have indicated that the prevalence of T2DM among females (19.0%) is significantly higher than among males (9.8%) (Bani, 2015). The

International Diabetes Foundation has reported that the prevalence of diabetes will increase worldwide from 400 million in the year 2030 to more than 640 million in 2040 (Marin-Penalver, 2016).

In a worldwide epidemic of diabetes, in 2021, one in two (239,7 million aged 20-79) people with diabetes were not diagnosed (Sun et al., 2022). According to estimates, two-thirds of T2DM cases will occur in developing countries by 2045, with 47 million Africans expected to have the condition (Godman et al., 2020). This increase over time in the prevalence of diagnosed diabetes mellitus (DM) indicates the complexity of the pathogenesis of diabetes and its complications. Therefore, these complications need to be prevented or lowered by reducing inflammation and OS, among other things.

1.2. Onset of diabetes mellitus

The onset of diabetes is indicated by pre-diabetes in healthy subjects, a condition where the blood sugar level is slightly higher than normal levels. At this stage, an individual's blood sugar is still low enough to be assigned the diagnosis of diabetes, and the person usually has no symptoms (Khan et al., 2019). This rise in blood sugar levels in pre-diabetes commences when the body begins to show signs of IR. An IR occurs when the β-cells fail to secrete sufficient amounts of insulin to activate the uptake of glucose. The resulting IR is the main factor marking the progression from pre-diabetes to diabetes.

Insulin is an essential hormone released by the pancreas to process glucose metabolism in the body's cells. This process disorder leads to a high level of blood sugar, which is referred to as impaired glucose tolerance. Uncontrolled and untreated impaired glucose tolerance can lead to hyperglycaemia and dyslipidaemia, which may result in the development of the pathophysiology of T2DM (Padberg et al., 2014) (see Figure 1.1).

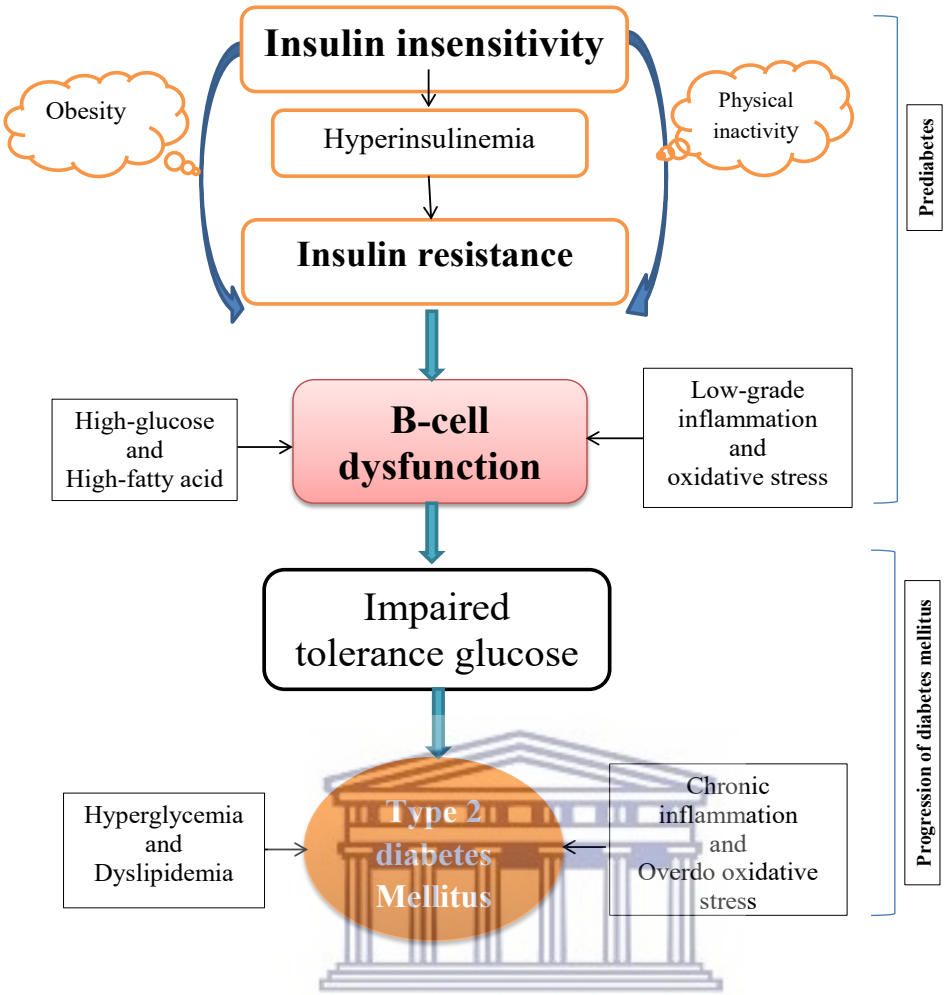


Figure 1.1. Diagram showing some common pathophysiology of T2DM, such as obesity and physical inactivity causes high fatty acid and insulin resistance. (The author's design)

1.3. Peripheral tissues and hepatic insulin resistance

1.3.1 Physiological function of insulin

Skeletal muscles are the primary location of glucose uptake in the human body where 80% of glucose uptake occurs (Hong et al., 2015). In response to increased blood glucose, the glucose transporter-2 transports glucose into the β-cells and the liver. Then, it stimulates insulin production, releasing it into the bloodstream, where the insulin binds to the cell surface insulin receptors (IRs) of the muscle tissue. This mechanism of insulin action stimulates glucose transport into the cell by promoting the translocation of glucose

transporter-4 (GLUT-4) to the cell surface (Thorens & Mueckler, 2010).Phosphorylation of intracellular substrate proteins is the result of insulin binding to IR_S, known as insulin receptor substrate-1 (IRS-1), more prominent in the skeletal muscle, and insulin receptor substrate-2 (IRS-2), which is more prominent in the liver (Eckstein et al., 2017). The IRS-1 and IRS-2 proteins attract phosphoinositide 3-kinases, thus leading to the production of phosphatidylinositol 3,4,5-triphosphate (PIP-3) and activation of a serine-threonine kinase. This action promotes the translocation of GLUT-4 to the cell membrane, thus promoting glucose transport into the cell (Leto & Saltiel, 2012).

Upon glucose entry into the peripheral tissue (skeletal muscle, adipocytes) and liver cells, glucose is converted to glucose-6-phosphate by the enzyme glucokinase to metabolise by glycolysis or stored as glycogen. Phosphorylated IRS-1 and IRS-2 bind to phosphatidylinositol 3-kinase (PI3k), which converts phosphatidylinositols-4,5-bisphosphate to PIP-3. These actions lead to the activation of the PI3k protein into the cellular surface, then transferring signals inside cells and regulating the nuclear factor kappa-B (NF-kB) translocation factor to the binding gene (Swiderska et al., 2018; Tokarz et al., 2018). Glucose-6-phosphate plays a critical role in glucose-induced insulin secretion – approximately two-thirds is converted to glycogen, and the remainder enters the glycolysis and oxidative cycles (Hong et al., 2015) (see Figure 1.2).

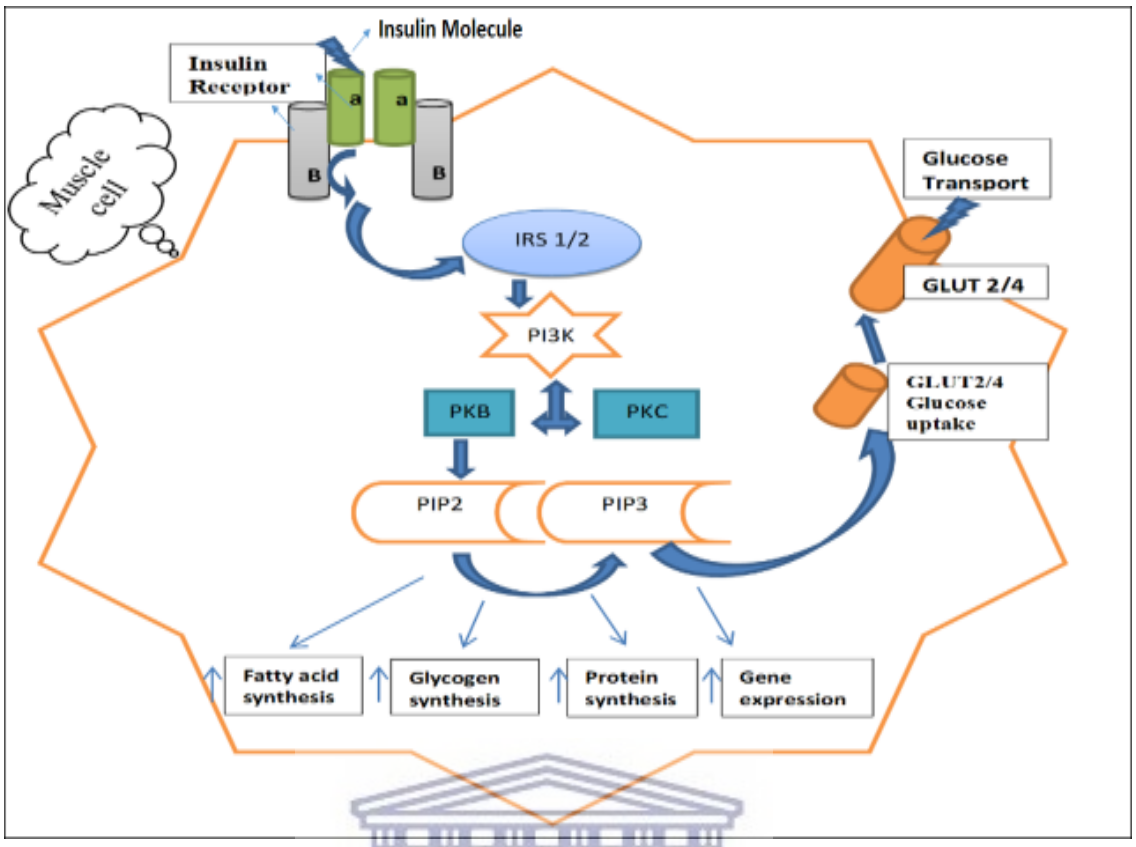


Figure 1.1. Schematic diagram showing how insulin receptors and glucose transport are involved in metabolism (The author's design)

1.3.2. Peripheral tissue insulin resistance

Insulin resistance plays an essential role in the pathogenesis of T2DM, resulting in impaired glucose uptake by the peripheral tissue, which leads to hyperglycaemia (Patel et al., 2016). Furthermore, sequential signalling transduction cascades involved in the insulin action of DM causes impaired cells in the skeletal muscle (Choi & Kim, 2010). A dysfunctional insulin-induced glucose transfer and translocation of GLUT-4 occur in diabetes when impaired insulin-induced glucose uptake is present (Richter & Hargreaves, 2013).

In a study by Cerf (2013), it was suggested that this is the result of inflammation in the peripheral tissues, in addition to insensitivity to insulin and because of pancreas β-cell

dysfunction. In other studies, researchers suggested that IR may be attributable to post-receptor defects caused by an impaired insulin-stimulated PI3k (Semple et al., 2009; Swiderska et al., 2019). Conditions of OS could be caused leading to the activation of various stress pathways, such as NF-κB and mitogen-activated protein kinase (MAPK) signalling pathway. There is an increase in reactive oxygen species (ROS) because of multiple disruptions in the cell functions (Rains & Jain, 2011).

Several reports suggest that T2DM impairs endothelial nitric oxide synthase (eNOS) activity as well as enhancing the production of ROS, thus resulting in reduced nitric oxide (NO) bio-availability (Tousoulis et al., 2013). Insulin is a normal regulator of eNOS activation and NO production through sequential phosphorylation. However, in T2DM, insulin reduces this pathophysiological process and suppresses bioavailability of NO, leading to atherogenic alteration (Capellini et al., 2010). Inducible nitric oxide synthases (iNOS) also play an essential role in inflammation. Activation of iNOS expression produces NO to mediate the cytotoxic effect involved in the pathogenesis of cell damage and IR (Soskic et al., 2011). All these disorders are major risk factors in the early onset and progression of DM.

1.3.3. Hepatic insulin resistance

The liver is usually exposed to the highest insulin concentrations compared to the other insulin-sensitive organs. Insulin can immediately reach the liver to suppress glucagon secretion and gluconeogenesis (Rojas & Schwartz, 2014), which is a result of the feedback loop between blood glucose levels and insulin secretion because of an increase in blood glucose (Van der Meulen et al., 2015; Guemes et al., 2016). The insulin action also stimulates the liver cells to take up and store glucose. This mechanism occurs

because of the translocation of GLUT-4 glucose transporters from cytoplasmic vesicles to the plasma membrane. When the glucose and insulin blood levels decrease, the GLUT-4 transporters are reprocessed and returned to the cytoplasm (Wang et al., 2020).

In the liver, insulin mediates the transfer of triglycerides (TG) to apolipoprotein-B and regulates lipoprotein lipase (LPL) activity. In T2DM, the combination of hyperinsulinaemia with hyperglycaemia and high free fatty acids (FFA) leads to hepatic uptake of TG-rich lipoproteins, resulting in hepatic IR, which is crucial in metabolic syndrome and the mitochondrial oxidation system (Mathur et al., 2016). IR in hepatocytes directly alters glucose metabolism, which may lead to increased levels of FFA and their metabolites, OS and altered expression in adipocytokines (Leclercq et al., 2007). Subsequently, suppressed LPL activity leads to overproduction of very-low-density lipoprotein cholesterol, then resulting in the formation of arteriosclerosis (Low et al., 2016).

1.4. Obesity related to the development of diabetes mellitus

The mechanisms that induce IR related to obesity have not yet been fully addressed (Kalinkovich & Livshits, 2017). Several studies suggest that weight loss in obese subjects may play a crucial role in preventing T2DM (Grams & Garvey, 2015; Magkos et al., 2020). Not only is obesity a high risk factor for the progression of T2DM and its complications, but it also has enormous socio-economic consequences, such as family history, low socioeconomic status, and certain unhealthy lifestyle behaviours (Wu et al., 2014; Montesi & Marchesini, 2016).

Obesity is characterised by a high body mass index (≥ 40 kg/m^2) and excess body fat in adipose tissue. A risk factor for developing IR resulting in a decrease in glucose uptake

by cells, is an increased adipose tissue mass (Qatanani & Lazar, 2007). Impaired muscle insulin sensitivity may occur because of the decreased number of IR_S on the cell surface (Petersen & Shulman, 2006).

In obese patients with diabetes, insulin insensitivity related to increased fat mass in adipose tissue leads to enhanced OS and elevated expression of pro-inflammatory cytokines known as adipocytokines (Fernandez-Sanchez et al., 2011) (see Figure 1.3).

Oxidative stress is also induced by obesity because of an increase in free radicals and FFA, resulting in increased lipid peroxidation (Morita et al., 2012). This disorder leads to decreased adiponectin levels and reduced adipocyte-derived anti-inflammatory markers (Ohashi et al., 2014). Furthermore, there is a mechanism that may lead to the production of oxidants that overload antioxidant activity in the body, resulting in damage to the cellular machinery that could trigger increased IR (Shum et al., 2021).

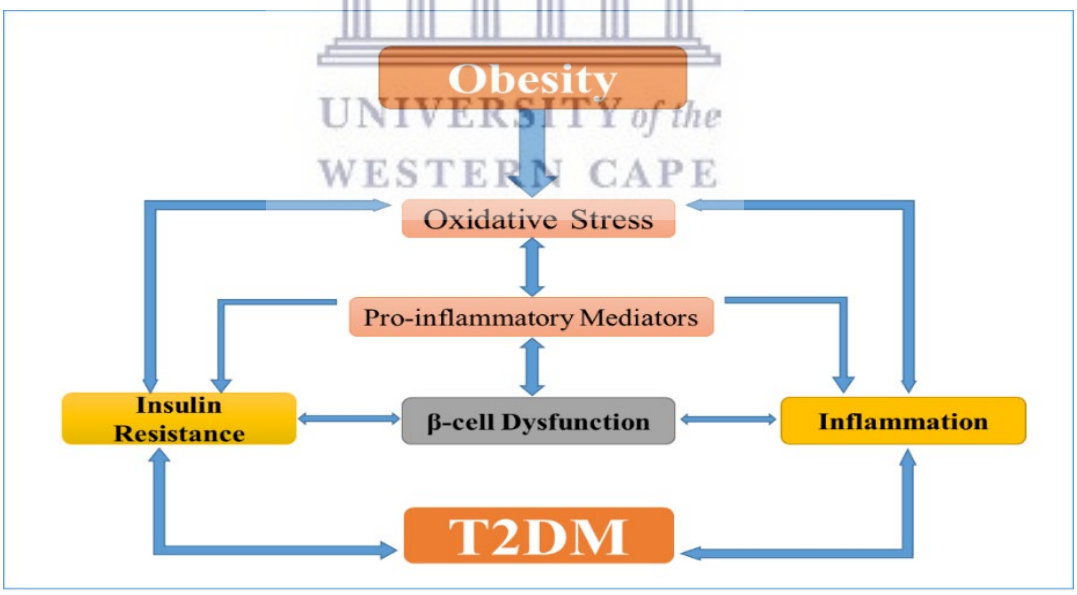


Figure 1.2. Mechanism of obesity: oxidative stress enhances inflammation, resulting in the impairment of the insulin signalling pathways, which lead to the development of T2DM. (The author's design)

1.5. Inflammation related to the development of diabetes

The biological mechanism underlying the association of chronic inflammation with diabetes remains poorly understood (Chen et al., 2015). One of the main pathways that regulate cytokine expression by subsequent binding to deoxyribonucleic acid is NF-κB activation (Laveti et al., 2013; Christian et al., 2016). The inhibition of the transcription factor NF-κB with treatment reduces the cytokine production of a pro-inflammatory response (Ferraz et al., 2015). Other mechanisms of action trigger the MAPK pathway that controls gene expression. Cytokines also induce secondary responses by increasing the inflammatory mediated responses such as tumour necrosis factor-alpha (TNF-α) (Kim et al., 2012; Sabio & Davis, 2014).

In general, inflammation is characterised by an enhanced systemic cytokine production along with an increase in the migration and infiltration of the immune cells into the injury sites (Chen et al., 2019). Dysregulation of the inflammatory system can result in uncontrollable inflammation, leading to infectious conditions such as tissue lesions. These dysregulations also play a critical role in the pathogenesis of several illnesses and conditions such as atherosclerosis, DM and cardiovascular diseases (Meshkani & Sanaz, 2016).

These pro-inflammatory mediators, such as interleukin-6 (IL-6), decrease tyrosine phosphorylation of IRS-1 and reduce the regulatory subunit of PI3k linked to IRS-1 (Patel et al., 2016). The PI3k pathway is responsible for the action of insulin on glucose transportation into cells and inhibition of gluconeogenesis in liver cells (Rodgers et al., 2010; McCracken et al., 2018). These impaired responses to insulin, including the

dysfunction of β-cells, may lead to hyperglycemia, hyperinsulinemia and hyperlipidaemia (Lin & Sun, 2010).

Hyperglycaemia also results in OS by high-glucose glycation end products, which promotes the pathogenesis of T2DM (Rains & Jain, 2011). The mechanism proceeds through activating the protein kinase pathway that induces IR by serine phosphorylation of IRS proteins and activation of transcription NF-κB (Hua et al., 2012). As a result, pro-inflammatory signalling is involved in endothelial dysfunction and microvascular complications. The inflammatory mediators can make the tissue cells less sensitive to insulin, and cells do not respond well to insulin activity. Because of disruptive functions, such as mitochondrial oxidative phosphorylation, triggering to release of pro-inflammatory modulators occurs (Lopez-Armada et al., 2013; Hafiz et al., 2015).

These pro-inflammatory modulator proteins bind their matching cell-surface receptors and trigger intracellular signalling pathways, which, in turn, alter cellular functions (Burkholder et al., 2014; Turner et al., 2014). This action results in an excessive number of cell-surface receptors and the production of other cytokine molecules (Jamaluddin et al., 2012; Arango & Descoteaux, 2014). These pathogenesis mechanisms are linked with high pro-inflammatory mediator levels, such as IL-6, TNF-α and monocyte chemoattractant protein-1 (MCP-1). These cytokines can impair insulin signalling pathways and lead to β-cell dysfunction. These molecules affect the behaviour of other cells as mediators of cell communication, resulting in the cell's response to the signal, which induces a physiological or pathophysiological effect on cell function (Moro et al., 2007).

The chronic inflammatory state is thus associated with the pathogenesis of many diseases, such as metabolic disorders and impaired microvascular and macrovascular in diabetes mellitus (Guarner & Rubio-Ruiz, 2015). The role of OS and insulin resistance-induced inflammation in the progression of T2DM is widely acknowledged (see Figure 1.4).

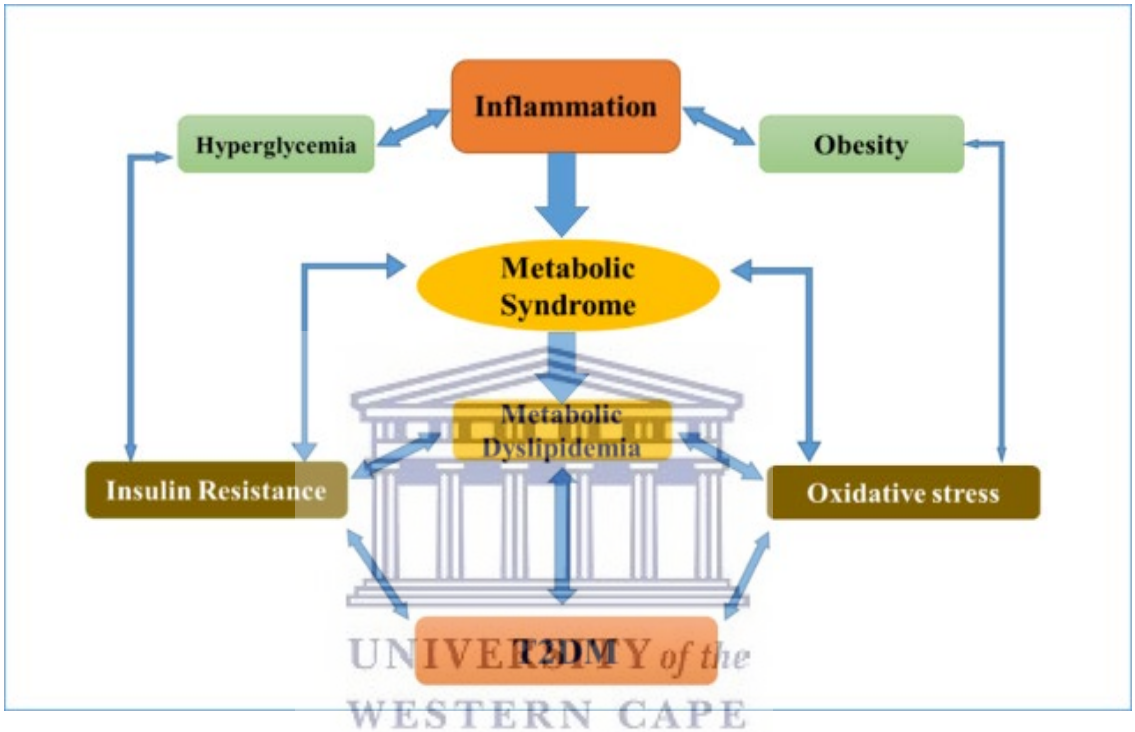


Figure 1.3. Schematic showing the mechanisms of inflammation associated with obesity and hyperglycaemia that enhance the metabolic syndrome, followed by an increase in oxidative stress and insulin resistance, which lead to the progression of T2DM. (The author's design)

1.5.1. Pro-inflammatory cytokine and chemokine markers

Cytokines are produced by several body cells, such as T-lymphocytes, B-lymphocytes, macrophages and mast cells (Turner et al., 2014). They are also released by various connective tissue cells (stromal cells), such as fibroblasts, endothelial cells, pericytes and adipose tissue (Correa-Gallegos et al., 2021). Primary pro-inflammatory mediators such

as IL-6 or TNF-α are strongly associated with T2DM, which is play a critical role in obesity pathophysiology. In obesity, pro-inflammatory molecules are overexpressed, suppressing adiponectin and leptin levels (Mirza et al., 2012; Liu et al., 2016).

Cytokines are cell-signalling molecules that mediate the inflammatory response by binding to cell-surface receptors. Their binding promotes intracellular signalling pathways resulting in an alteration of cell functions (Turner et al., 2014). An increase in pro-inflammatory cytokines, such as TNF-α, MCP-1 and IL-6, that inhibit insulin-mediated tyrosine phosphorylation of the IR_S and IRS-1. This alternative leads to defective activation of downstream insulin signalling to PI3k and translocation of GLUT-4 to the cell surface (Monroy et al., 2009; Daniele et al., 2014). These variations of the molecular activation mechanism are significantly increased in long-term diabetes conditions, but they remain unsolved.

Macrophages are strong factors implicated in the pathogenesis of IR and inflammation. Macrophages play a significant role in the immune system (innate and adaptive) and metabolic dysfunction (McLaughlin et al., 2017). They are a source of pro-inflammatory cytokines, chemo-attraction markers and adipocyte dysfunction, associated with IR (McArdle et al., 2013). Macrophages and T cells stimulate cytokine release, which activates the inflammatory state in adipocytes. The result of this process is the elevation of monocyte chemoattractant protein-1 (MCP-1) cytokine, which may increase the migration of monocytes/macrophages in obese people and ultimately lead to obesity-induced IR (Goncalves & Martel, 2015; Wang & Wu, 2018a).

Pro-inflammatory chemokines such as MCP-1 and their receptor expression are increased in visceral and subcutaneous adipose tissue in obesity (Mraz et al., 2011). This

expression leads to a pro-inflammatory state in fatty tissue – large amounts of MCP-1, macrophage inflammatory protein-1beta (MIP-1β) and other chemokines are secreted by macrophages (Jung & Choi, 2014). Elevated levels of MCP-1 and MIP-1β have also been reported in diabetic subjects with retinopathy, which suggests they play a role in activating and attracting leukocytes in an inflammation response (Rangasamy et al., 2012; Shiraya et al., 2017).

A review article provides evidence that even a slight elevation in MCP-1 levels could induce IR in skeletal muscle cells with T2DM (Sell & Eckel, 2009). However, others suggested that inhibition of the C-C motif chemokine receptor-2 signalling pathway could enhance the inflammatory responses and the progression of diabetes (Wada & Makino, 2013). Moreover, a study observed that elevated levels of MCP-1, MIP-1β, Granulocyte Colony-Stimulating Factor (G-CSF), Regulated on Activation-Normal T Cell Expressed and Secreted (RANTES), and TNF-α in T2DM with micro-albuminuria, compared to in normal albuminuria subjects (Liu et al., 2010).

RANTES, known as the C-C ligand 5, plays a critical role in the regulation of inflammatory cells involved in atherogenesis (Madani et al., 2009; Mikolajczyk et al., 2016). Increased levels of the RANTES chemokine are associated with early-onset diabetes and cardiovascular health risks (Koh et al., 2009; Dworacka et al., 2014). In atherosclerotic plaques and early atherosclerotic endothelium, RANTES triggers the detention of circulating monocytes (Braunersreuther et al., 2007). Therefore, it is considered imperative to use these cytokines as markers, specifically related to inflammation, which may be an efficacious goal for future considerations of herbal therapeutic approaches in preventing the development of DM.

1.6. Lipid abnormalities and diabetes mellitus

Lipid metabolism dysfunction is a common metabolic disorder related to obesity and pro-inflammation, resulting in an increased risk for diabetes. T2DM is characterised by a metabolic disorder that contributes to the development of diabetic complications (Fowler, 2011; Cooper et al., 2015). The metabolic abnormalities in T2DM include glucose intolerance, IR, hyperglycaemia, dyslipidaemia and hypertension. With diabetes, obesity-induced systemic low-grade inflammation appears to be a central factor underlying the pathophysiology of IR and metabolic disorders (Ford & Sattar, 2008). Evidence shows that lipid metabolism dysfunction begins with excess in adipose tissue (Cameron et al., 2008; Donath & Shoelson, 2011).

Generally, insulin promotes TG synthesis (lipogenesis) and adipocyte storage, inducing LPL (Gathercole et al., 2011; Czech et al., 2013). LPL is mainly expressed in vascular smooth muscle cells, endothelial cells and macrophages (He et al., 2018). LPL's primary function is the hydrolysis of TG in chylomicrons to release FFA and transport lipids (Xie et al., 2010). The concern is the macrophage-derived LPL related to the formation of foam cells, atherosclerotic plaques and the progression of T2DM. In this diabetes condition, because of low-density lipoprotein (LDL) uptake by macrophages, there is a rapid accumulation of intracellular cholesterol, leading to foam cell formation (Takahashi et al., 2013; Volobueva et al., 2018). Inflammation and ROS increase macrophage LPL expression macrophage LPL modulates response and may promote atherosclerosis (Zhu et al., 2015).

It has been reported that macrophage LPL inhibition, through the transplantation of LPL-deficient bone marrow, decreases atherosclerotic plaque formation (Evans, 2005),

suggesting that macrophage-derived LPL plays a critical role in the inflammatory response in macrophages (Xie et al., 2016). Down-regulation of LPL secreted by skeletal muscle and adipose tissue is related to metabolic disorders, such as obesity and diabetes. Over-expression of macrophage LPL is also related to the pathogenesis of foam formation in diabetes patients (Christou & Kiortsis, 2013; Li et al., 2020).

Fatty acids are taken up by the main metabolic tissues, adipocytes and liver cells, and then used for lipogenesis (Ameer et al., 2014). But high levels of circulating FFA can also cause peripheral tissue IR and stimulate cytokine expression of macrophages (Suganami et al., 2012). Increased accumulation of macrophages in pericardial adipose tissue mass that may be linked to the inflammatory intermediaries such as TNF-α and IL-6 leads to metabolic complications (Zhou et al., 2007). While the accumulation of esterified cholesterol in macrophages initiates the formation of foam cells, it progresses to atherosclerotic lesions in the arterial wall (Narasimhulu et al., 2016). Up/down-regulated expression of LPL is associated with high TG, which contributes to an increased risk for cardiovascular disease (Geldenhuys et al., 2017).

Earlier reports have suggested that long-term exposure of macrophage cells to HG induces ROS, resulting in an accumulation of lipoproteins which impairs LPL activity (Marseglia et al., 2015; Giri et al., 2018). This mechanism promotes OS and vascular inflammation to stimulate the proliferation and migration of endothelial cells. Thus, long-term HG induces increases in vascular endothelial permeability, indicative of increased ROS, and then impairment of insulin action through IRS (Monteiro & Azevedo, 2010). Increased levels of ROS cause defects in OS and inflammatory cytokines (Wang et al., 2015; Zilaee & Shirali, 2016; Alkhatib, 2019).

Oxidative stress plays an essential role in the progress of metabolic disorders, mainly IR, through the inhibition of protein-tyrosine phosphatases and phosphorylation of proteins, which leads to impaired insulin action (Le Lay et al., 2014). Fitness and exercise are known to be important in diabetes: intense aerobic exercise, over the long term, improves glycaemic control and reduces the OS of T2DM (Nojima et al., 2008). Therefore, the regulation of LPL can also decrease the expression of lipid composition in macrophages, which, in turn, may inhibit OS and the inflammatory response, which may reduce or prevent the progression of atherosclerosis (Xie et al., 2016). (see Figure 1.5).

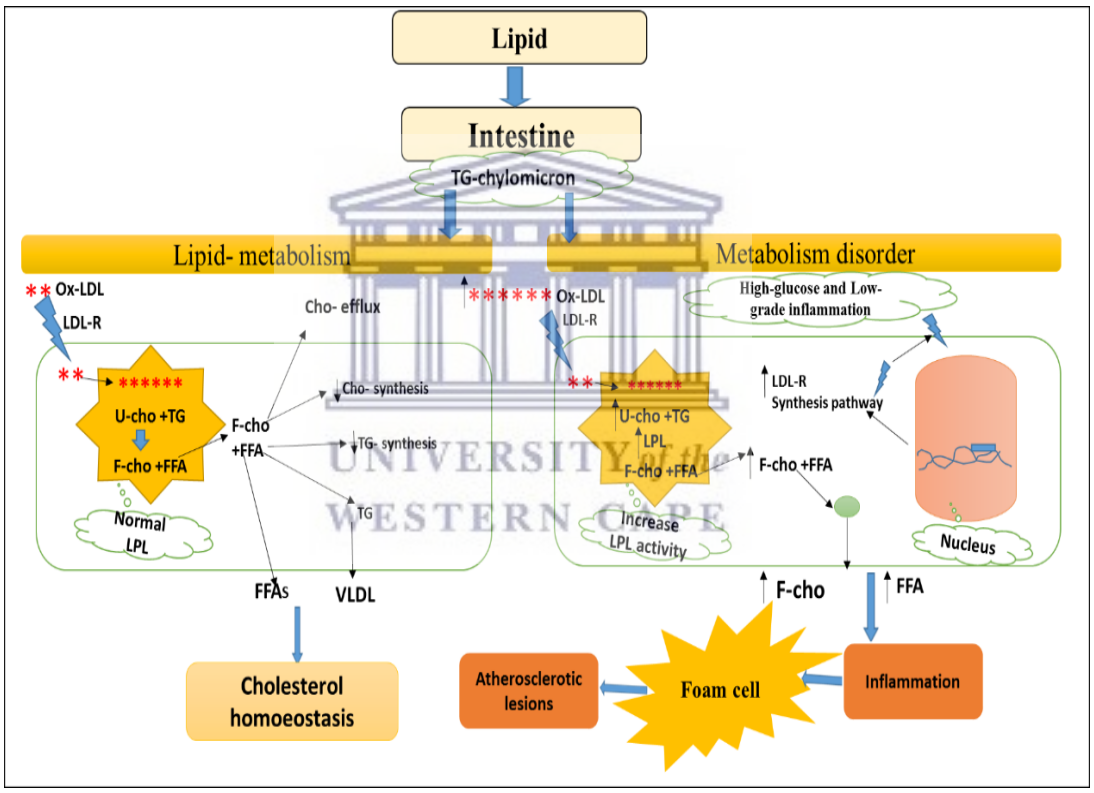


Figure 1.4. Schematic diagram showing of cellular cholesterol homoeostasis in (lipid metabolism) healthy subjects and patients with diabetes with metabolic disorder. LDL, low-density lipoprotein cholesterol; LDL-R, low-density lipoprotein cholesterol receptor; U-cho, unesterified cholesterol; TG, triglycerides; LPL, lipoprotein lipase; F-cho, free cholesterol; FFA, free fatty acids; VLDL, very-low-density lipoprotein. (The author's design)

1.7. Diabetic complications

1.7.1. Cardiovascular diseases

Common cardiovascular disorders associated with DM are early atherosclerosis and myocardial infarction. These disorders are a primary cause of morbidity and mortality among patients with diabetes (Das et al., 2018). Atherosclerosis is a complex process involving numerous types of cell interactions that lead to the formation of atherosclerotic plaques. These result in myocardial infarction, unstable angina or strokes in patients with diabetes (Badimon et al., 2012; Forbes & Cooper, 2013). Atherosclerosis is a chronic inflammatory disorder involving macrophage activity and lipoprotein modification in T2DM (Ley et al., 2011; Buckley & Ramji, 2015).

Hyperlipidaemia and local inflammation appear to be the main processes responsible for atherogenesis, characterised by a long-term inflammation state affecting the arterial intima (Fenyo & Gafencu, 2013). Hyperglycaemia may also induce diabetic angiogenesis through the generation of OS, leading to NO pathways (Vanessa et al., 2013). In addition, the disorder of lipid and long-term HG induces endothelial cells to produce adhesion molecules leading to the recruitment and differentiation of monocytes to macrophages. The macrophages then attract modified lipoproteins, forming foam cells. These foam cells are a primary source of pro-inflammatory mediators MCP-1, TNF-α and IL-6 (Viola & Soehnlein, 2015).

1.7.2. Diabetic retinopathy

Diabetes causes several serious complications such as peripheral vascular disease, renal disorders, neuropathy, hypertension, retinopathy and inflammation (Forbes & Cooper, 2013). Diabetic retinopathy (DR) refers to damage to the eye's retina and visual

impairment occurring with long-term diabetes. It is a microvascular complication susceptible to chronic inflammation and oxidation caused by toxicity to the microvascular walls, which can cause diabetic tissue damage (Tang & Kern, 2011; Urbancic et al., 2013). Considering the biomarkers of inflammation appear impairment in the neurovascular system. But still some knowledge of the role mechanisms underlying the inflammatory pathways associated with DR (Rubsam et al., 2018). A significant factor in the retinal inflammation associated with long-term hyperglycaemia is the up-regulation of MCP-1, an inflammatory chemokine (Semeraro et al., 2015).

1.7.3. Diabetic nephropathy

Diabetic nephropathy is a chronic disease characterised by proteinuria, decreased glomerular filtration, and hypertrophy, ending with renal failure (Sun et al., 2013). Hyperglycaemia and chronic inflammation may relate to the development of renal failure (Lim & Tesch, 2012). Up-regulation of MCP-1 chemokine levels reveals kidney dysfunction and plays a significant role in the development of diabetic renal injury (Kanamori et al., 2007; Gupta et al., 2013). Several studies have shown that macrophages accumulate in the glomeruli of patients and animals with diabetes. This condition is characterised by prolonged hyperglycaemia, increased chemokine production, and progressive kidney fibrosis (Hickey & Martin, 2013).

1.7.4. Diabetic neuropathy

Neuropathy is a common diabetes complication (affecting about 50% of patients with diabetes), associated with loss of peripheral nerve fibres (Zenker et al., 2013; Shi et al., 2013). Diabetic neuropathy is also a common cause of foot ulcers, the leading cause of non-traumatic limb amputation (Farmer et al., 2012). Hyperglycaemia, dyslipidaemia

and insulin deficiency are suggested to play a critical role in the progress of diabetic neuropathy. They result in an increased expression of pro-inflammatory cytokines because of peripheral nerve injury, which leads to the recruitment of macrophages and the pathogenesis of nephropathy (Pop-Busui et al., 2016). Macrophages exacerbate diabetic neuropathy through a variety of mechanisms, such as excess production of cytokines, ROS and cellular oxidative damage (Hosseini & Abdollahi, 2013). In addition, diabetic neuropathy results in increased levels of TNF-α, and inhibition of this cytokine may be an ideal approach to treat the condition (Yamakawa et al., 2011; Shi et al., 2013).

Diabetes is characterised by chronic hyperglycaemia, with the nerve cells more susceptible to ischaemia-reperfusion injury cells, resulting in wide-ranging infiltration of macrophages in diabetic peripheral nerves (Meshkani & Vakili, 2016). Therefore, for most people, reducing or preventing these complications will require managing their high glucose (HG) and lipids levels, including regulating inflammation and OS.

1.8. A proteomic approach to diabetes

Increasing the efficiency and number of protein biomarkers that can be measured in relation to diabetes has enhanced the efficiency of protein biomarkers techniques (Chen & Gerszten, 2020). More research is required in this area, using *in vitro* models to evaluate its effectiveness and the mechanism of action, using a proteomic approach/proteomic studies. The application of proteomic techniques may offer new insights into the mechanism of diabetes by allowing researchers to compare physiological and pathophysiological pathways. Proteomic techniques cover an entire set of proteins expressed in various tissues, cells or organelles (Chandramouli & Qian, 2009; Giordano et al., 2020), enabling proteins in cells or tissues to be identified by high-throughput

methods. A comprehensive view of the proteomic changes associated with the progression of a disease is thus provided (Ohlendieck, 2011).

Different techniques have been used to identify protein profiles to evaluate biomarker discovery and validation and the development of novel drug therapies. These techniques allow for reliable and reproducible biomarkers that require identical protocols for collecting, handling, processing and storing samples (Rai & Vitzthum, 2006). A variety of techniques exists that analyse protein profiles in biological samples. These different techniques could be used to identify predictors of disease pathogenesis and be helpful in designing specific drugs (Nandal & Burt, 2017).

Techniques included are:

i) The utilization of isobaric tags for the relative and absolute quantification of protein abundance (Karp et al., 2010);

ii) The two-dimensional gel electrophoresis-mass spectrometry, which allows a systematic and comparative analysis of proteomic changes and mass spectrometry peptide identification of a large number of proteins (Gygi & Aebersold, 2000);

iii) A combination of liquid chromatography-mass spectrometry, allowing the detection of protein markers by their dynamic range and with greater depth and enhanced accuracy of quantification (Peck, 2009).

iv) The utilisation of antibody-based immune-detection assays, such as Western blotting and enzyme-linked immunosorbent assay (ELISA), which use specific antibodies to identify potential biomarkers.

These methods are routinely used for protein measurements because they are convenient, rapid, sensitive and provide high sample throughput results (Mahmood & Yang, 2012).

1.9. Aims

The overall aim of this study was to determine the efficacy of herbal therapies commonly used in African countries for the management of T2DM using *in vitro* assays. The anti-inflammatory and antioxidant activity of the three plants *Artemisia afra (A. afra), Cinnamon verum (C. verum) and Trigonella foenum-graecum (T. foenum-graecum)* were compared with that of the western pharmaceutical, metformin.

Specific aims were to:

1. Use proteome profiling to compare the protein expression of cells cultured under normal glucose and HG (diabetes) with and without stimulation conditions.
2. Set up quantitative assays for proteins showing the main differences between normal and diabetic conditions with and without stimulation (diabetes biomarkers).
3. Compare the effectiveness of traditional African herbal treatments with metformin at normalising diabetes biomarkers of cells grown under diabetic and inflammatory conditions to levels present under normal conditions.

1.10. Objectives

The objectives of this study were, first, to extract and analyse the chemical components of the plants *T. foenum-graecum*, *C. verum* and *A. afra* to verify the main bio-active compound concentrations. The bio-active compounds in these plants may have anti-inflammatory and antioxidant properties.

In the second objective, the effects of HG on RAW 264.7 cells cytokine expression were evaluated in the presence or absence of lipopolysaccharides (LPS), which were used in this study to evaluate the effects of herbal extracts. These parameters included cytotoxicity and inflammatory biomarkers.

The third objective was to assess the effects of plant extracts compared to metformin on pro-inflammatory cytokines and chemokines in the presence or absence of HG and LPS stimulation.

The fourth objective of this study was to evaluate the effects of herbal extracts, compared to metformin, on biomarker activity enzymes (lipoprotein lipase and superoxide dismutase) in the presence or absence of HG and LPS stimulated cells.

1.11. Expected results and outcomes

1. Obtaining data on the toxicity of commonly used traditional African herbs.
2. Identifying biomarkers that can be used to diagnose and assess treatment for chronic inflammation related to T2DM.
3. Obtaining data on the effectiveness of commonly used African herbal remedies for treating inflammation and metabolic disorders associated with diabetes.

1.12. Layout of thesis

This dissertation comprises seven chapters.

Bio-active compound analysis of ethanolic extracts of plants and the effects of selected African herbs on the pro-inflammatory response and metabolic disorders linked to DM are addressed in Chapters 3, 5 and 6.

In efforts to evaluate the various inflammatory biomarkers that might induce inflammation linked with HG, the effect of HG on macrophage cell expression pro-inflammatory markers was assessed, as described in Chapter 4.

Further details of these chapters, with titles, follow:

Chapter 1: *Introduction and objectives*

This chapter provides an introduction to T2DM, inflammation and metabolic disorders. The outline of the thesis is also described in this chapter.

Chapter 2: *Overview of western medicine and traditional herbal remedies for T2DM*

In this second chapter, an overall view is given of the standard (Western) diabetes medicines and their effects and side effects on the management of DM. The traditional herbs and their effects on and safety in regulating inflammation and metabolic disorders related to DM are also considered.

Chapter 3: *Chemical analysis of African medicinal plants with anti-inflammatory and anti-diabetic properties*

The preparation of plant extracts for use in chemical analysis is discussed in this chapter. Using gas chromatography-mass spectrometry (GC-MS), analysis of bio-active compounds in ethanolic extracts was done to determine the fraction molecules of plants.

Gas chromatography-mass spectrometry (GC-MS) analysis of bio-active compounds in ethanolic extracts was carried out to determine the fraction molecules of plants.

Chapter 4: *The effect of high glucose, related to diabetes mellitus, on macrophage cell expression of pro-inflammatory markers*

The evaluation of cell viability and inflammatory biomarker expressions are addressed in this chapter. A proteome profile analysis of cytokines and chemokines is used after exposing RAW 264.7 cells to HG and LPS.

Chapter 5: *Evaluation of the anti-inflammatory effects of Artemisia afra, Cinnamon verum and Trigonella foenum-graecum in high glucose-induced macrophage cells*

In this chapter, an evaluation of the cell viability and the ability of African medicinal plants to regulate the pro-inflammatory biomarkers related to DM produced by the RAW 264.7 cell line after exposure to HG and LPS is addressed.

Chapter 6: *Evaluation of the effects of Artemisia afra, Cinnamon verum and Trigonella foenum-graecum on macrophage metabolic markers in high glucose-induced lipopolysaccharides*

The determination of macrophage cells' metabolic bio-active marker expressions to determine whether the African medicinal plants regulate lipid dysfunction and OS, then possible inhibition of the inflammatory response is described in this chapter.

Chapter 7: *Conclusions and recommendations*

In this final chapter, the main findings of this research are highlighted, and recommendations for future work are provided.

Chapter 2

Overview of Western medicine and traditional herbal remedies for T2DM

2.1. The treatment of T2DM using Western medicine

The focus of T2DM treatment has been on regulating insulin levels and actions by directly improving tissue sensitivity to insulin, which is achieved by inducing insulin secretion or reducing the rate of carbohydrate absorption from the gastrointestinal tract. This action causes a reduction in blood glucose levels after a meal (Drucker, 2007). Some therapies prevent the development and the progression of T2DM by preventing dysfunction of the β-cells and avoiding a series of complications in T2DM patients, whereas other treatments act by reducing hepatic glucose production through a reduction in glycogenolysis and gluconeogenesis (Horton, 2008; Marin-Penalver et al., 2016).

The most recently recommended monitoring system for effective diabetes treatment and reduction of complications, as suggested by the American Diabetes Association, is to control haemoglobin A1c (HbA1c) levels (Nathan et al., 2009). Unfortunately, these target goals are not appropriate therapeutic strategies as they focus only on optimal glycaemic control to achieve and prevent the progression of diabetes complications. Chronic inflammation and dyslipidaemia are associated with many diseases and long-term T2DM, which causes microvascular and macrovascular complications. T2DM is also a chronic disease that requires long-term medication; therefore, side effects of these pharmaceuticals could become more dominant because of continuous use (Nyenwe et al., 2011; Low et al., 2016). For monitoring and controlling diabetes, treatment should focus on reducing the chronic inflammatory state and metabolic disorders (Yaribeygi et al.,

2019a). The management of diabetes is still considered a global problem, and there is, as yet, no successful confident therapy.

Various current available oral anti-diabetic medicines, their mechanisms of action, advantages and side effects are now described.

2.1.1. Biguanides (metformin)

Metformin, also known as Glucophage, is one of the most widely used medications for treating T2DM. Metformin acts on the body by inhibiting intestinal absorption of glucose and decreasing the hepatic production of glucose by inhibiting hepatic lactate uptake (Meneses et al., 2015; Bailey, 2017). Metformin also increases insulin sensitivity of cells and promotes insulin-stimulated glucose uptake in muscle cells (Turban et al., 2012). Suggestions are that metformin inhibits low-grade inflammation in obese patients and regulates macrophage response through the activation of 5-adenosine monophosphate-activated protein kinase (Jing et al., 2018), which may enhance insulin sensitivity and glucose uptake into the cell.

Beneficial effects of metformin include weight reduction, decreased hyper-insulinaemia and improved lipid profiles associated with cardiovascular risk (Fruzzetti et al., 2017). Metformin side effects include gastrointestinal complaints such as nausea, diarrhoea and/or abdominal cramping, occurring in up to 50% of treated patients (Florez et al., 2010). Long-term metformin therapy results in mild vitamin B_{12} malabsorption in up to 10–30% of patients (de Jager et al., 2010). According to Acharya et al. (2013), a new combination of metformin and sulfonylurea anti-diabetic therapy controls glucose levels without the associated weight gain.

2.1.2. Sulfonylureas

Sulfonylureas (SFU) are among the most widely used drugs for treating T2DM and are effective in lowering plasma glucose by stimulating pancreatic β-cell insulin secretion (insulin secretagogues) and slightly improving IR in the liver and peripheral tissues (Gangji et al., 2007; Seino et al., 2011). Earlier studies reported that SFU agents appear to reduce glycosylated HbA1c levels in patients with diabetes (Uemura et al., 2021). A recent study compared SFU with vildagliptin, an oral anti-diabetic agent with the dipeptidyl peptidase-4 (DPP-4) group, and showed that vildagliptin is more beneficial in reducing HbA1c than SFU (Shete et al., 2013).

Delayed wound healing is a common complication in patients with diabetes, often resulting in limb amputation (Icli et al., 2016). One of the diabetic complications is a permanent inflammatory condition characterised by an increase in macrophage cytokines. However, no studies have yet found any effects of SFU on wound healing in diabetic individuals (Salazar et al., 2016). Side effects of SFU include permanent neurological disability, weight gain, hypoglycaemia and cardiovascular risk (Luna & Feinglos, 2001; Blahova et al., 2021). A relationship between SFU and the risk of death, possibly because of low blood glucose, has been reported (Bodmer et al., 2008).

2.1.3. Dipeptidyl peptidase-4 inhibitors

DPP-4 is an enzyme belonging to a family of cell membrane proteins that are expressed in many tissues. DPP-4 inhibitors enhance the effects of glucose-dependent insulinotropic peptides (neuropeptides) and glucagon-like peptide-1. These neuropeptides are the main insulinotropic peptides of intestinal origin (incretins) and are rapidly broken down by DPP-4 (Nathan et al., 2009; Kabade et al., 2016). DPP-4

inhibitors act by increasing glucose-mediated insulin secretion from pancreatic β-cells and suppressing glucagon secretion from pancreatic α-cells, which significantly reduce HbA1c (Boada & Martinez-Moreno, 2013).

DPP-4 inhibitors have been reported to reduce inflammatory markers and platelet aggregation while improving endothelial function (Kodera et al., 2014). In a study on the treatment of T2DM patients, DPP-4 inhibitors were shown to reduce pro-inflammatory cytokines such as IL-6, Interleukin-18 and TNF-α compared with sitagliptin and vildagliptin (Rizzo et al., 2012). DPP-4 inhibitors carry a low risk of hypoglycaemia, transient nausea and vomiting and cause moderate weight loss (Boada & Martinez-Moreno, 2013).

2.1.4. Meglitinides

Meglitinides are insulin secretagogue agents. Their mechanism of action closely resembles that of SFU. They act by stimulating the release of insulin from the pancreatic β-cells to lower blood sugar levels after meals (Bailey, 2011; He et al., 2015). This drug is suitable for combination use with metformin or thiazolidinedione, effectively targeting IR (Nyenwe et al., 2011). No studies have reported that meglitinide medication has anti-inflammatory activity (Yaribeygi et al., 2019b). They are used less commonly because of their short duration of action. Risk factors of meglitinides include hypoglycaemia (because of the drug's prompt action on the pancreas to secrete insulin), weight gain, gastrointestinal upset, upper respiratory tract infection and back pain (Nathan et al., 2009).

2.1.5. Thiazolidinediones (glitazones)

Thiazolidinediones (TZDs) are commonly used to enhance insulin-mediated glucose uptake and decrease peripheral IR. TZDs achieve this by binding to peroxisome proliferator-activated receptors in adipocytes and reducing the secretion of adiponectin, which, in turn, leads to regulating glucose and lipid metabolism (Du, 2014). TZDs improve insulin sensitivity, but their mechanism of action is poorly understood. TZDs also improve β-cell function (Kabade et al., 2016). TZDs prevent inflammation by preventing macrophage infiltration and inhibition of NF-κB activation, leading to the reduced expression of pro-inflammatory mediators, as revealed in a study (Chawla, 2010). Therefore, TZDs inhibit inflammation and possibly improve the healing of chronic wounds in patients with diabetes (Mirza et al., 2015). Adverse effects of TZDs include liver failure (hepatotoxic), peripheral oedema, weight gain and increased adipose tissue mass (Colhoun et al., 2012).

2.1.6. Alpha-glucosidase inhibitors

Alpha-glucosidase inhibitors (AGIs) lower glucose levels without causing hypoglycaemia, specifically in people who have raised post-prandial blood glucose (Nathan et al., 2009). They inhibit intestinal alpha-glucosidase enzymes, which are responsible for the hydrolysis of dietary carbohydrates into sugars (Du, 2014). AGIs are less effective than other oral drugs in reducing plasma glucose in T2DM and thus used in combination with other drugs (He et al., 2015). AGIs are also known to have anti-inflammatory effects through reduced angiogenesis marker expression levels and improved inflammation of endothelial cells, which may lead to the prevention of developing atherosclerosis in patients with diabetes (Shimazu et al., 2009; Derosa et al.,

2011). Side effects observed with these agents include dyspepsia, flatulence and diarrhoea (He et al., 2014). AGIs are not recommended for patients with renal dysfunction, inflammatory bowel disease, or a history of bowel obstruction (Brunetti & Kalabalik, 2012).

2.2. Costs of anti-diabetic drugs

Medication economics play a significant role in medical practice. Medical economics looks at the manufacturing cost and efficacy of drugs used for treatment. In developing countries, the cost of medication is a primary concern for physicians, patients, prescribers and health care providers (Abidi et al., 2016). A report from Zion Market Research Group (2017) estimated the global oral anti-diabetes drug market (biguanides, SFU, meglitinides and AGIs) at US$ 19.94 billion in 2016 – this figure is expected to reach $ 35.91 billion by 2022 (see Figure 2.1).

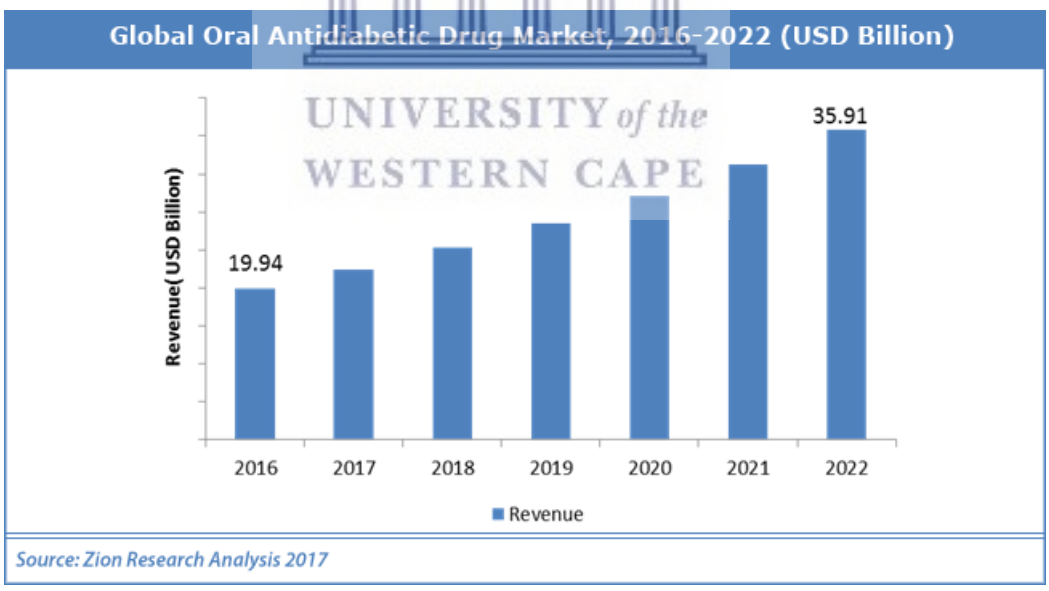


Figure 2.1. Global rising costs in the core oral anti-diabetic drug market, include biguanides, sulfonylureas, meglitinides, and alpha-glucosidase inhibitors (Zion Market Research Group, 2017)

Differential pricing could be a practical strategy to improve access to essential drugs in low- and middle-income states where most patients pay for out-of-pocket drugs (Lu et al., 2011). For example, in single-drug therapy, the price variation between the SFU group of medicines is as follows: for Glimepiride (1 mg), the minimum to maximum price variation is 562%, while metformin shows a difference of 492% (Mehani & Sharma, 2018). The percentage variation demonstrates that the cost of oral antidiabetic drugs between individual drugs in an SFU group is above 100%. These wide-ranging variations in the prices of the different drugs of the same class have severe economic inferences (Abidi et al., 2016; Mehani & Sharma, 2018). Despite considerable progress in the treatment of T2DM, there remains a significant challenge of achieving a cost-effective treatment and administration regimen coupled with few associated side effects (Philippe & Raccah, 2009; Pontarolo et al., 2015).

As T2DM is a chronic disease that requires long-term medication, side effects could become more dominant because of continuous use (Nyenwe et al., 2011). Several limitations and harmful effects of diabetes synthetic drugs make it necessary to research novel drug therapies that are safer and more efficient (Akhtar et al., 2007).

2.3. The traditional herbal remedies for T2DM available in Africa

A strategy to obtain successful therapy for T2DM includes the challenge of minimising or preventing the progression of diabetes complications. Current therapies minimize the adverse effects of hyperglycaemia and weight gain (Davies et al., 2018). DM represents a group of metabolic diseases characterised by persistent hyperglycaemia, OS and inflammation, resulting in various chronic complications. Therefore, the goal of therapy

for diabetes is to normalise blood glucose levels, regulate dyslipidaemia, and inhibit chronic inflammation in the prevention of diabetes complications (Testa et al., 2017).

Herbal medicines may have beneficial effects in combination with other herbs or synthetic drugs. There are instances where the combination of Western drugs with traditional therapies has increased the range of options available for the treatment of T2DM (Ezuruike & Prieto, 2014). Several studies have focused on the combination of herbal therapy and western drugs in treating diabetes. These results seem to suggest that herbal medicines have a better efficacy when used in conjunction with synthetic drugs than using the latter alone (Satyanarayana et al., 2007; Kaur et al., 2013).

In 2013, the World Health Organization (WHO) Traditional Medicine Strategy reported that the strategic approach for the next decade (2014–2023) would be to support the member states' use of traditional medicine for health care. For optimal treatment, research should be encouraged and combined with the production of traditional herbal medicines to ensure the safety of products and enhance the efficacy of treatment (Ezuruike & Prieto, 2014; Chege et al., 2015).

In many regions of Africa, traditional plant therapies are the most readily available health resources accessible to the public. It has been reported in several studies that 80% of the population in Africa depends on traditional remedies for the treatment of DM (Matheka & Demaio, 2013). Hence, there is an increasing scientific interest in herbs that have traditionally been used to treat DM in Africa (Gemeda et al., 2015). For example, in Morocco, 11% of the local population were using traditional medicine, while 67% used traditional and Western medicine; interestingly, 75% of women used herbal medicines compared to 25% of men (Bousta et al., 2014; Bouyahya et al., 2017). (Ziyyat et al.,

1997; Bousta et al., 2014). Information on the percentage of people who used traditional medicinal plants for their primary health care in the following African countries was Ethiopia, 80% (Gemeda et al., 2015), South Africa, 80% (Street & Prinsloo, 2013), Uganda, 60%, and Tanzania, 60% (Payyappallimana, 2010).

Across the African region, there are various types of herbs used as traditional medicine for the management of DM as well as for their anti-inflammatory activity – commonly used herbs are *T. foenum-graecum, C. verum* and *A. afra* (Islam & Choi, 2008; Sunmonu & Afolayan, 2013).

Traditional herbal treatments have also been observed as safe, inexpensive, readily usable and culturally acceptable compared to Western medicines. Many studies have been done on these plants – the leaf part has been the most widely investigated (see Figure 2.2).

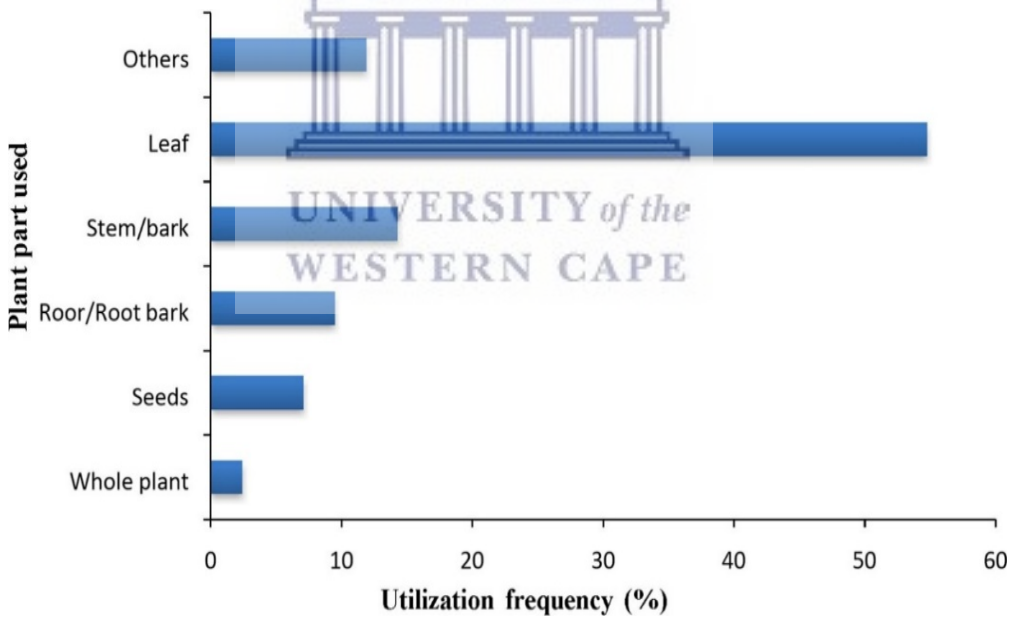


Figure 2.2. Frequency of plant parts used in anti-diabetic studies in southern African countries (Sabiu et al., 2019)

2.3.1. Trigonella foenum-graecum

T. foenum-graecum, commonly known as *fenugreek*, is a plant belonging to the family Fabaceae. It is an annual plant native to North Africa, the Middle East and India, and it has a long history of usage in cooking and traditional herbal medicine (Srinivasan, 2006; Zandi et al., 2017). Medicinal properties of its seeds and leaves have been found to be anti-inflammatory, antioxidant, and anti-diabetic (Xue et al., 2007; Sheikhlar, 2013).

Observations indicate its effectiveness in the inhibition of carbohydrate digestive enzymes because of the slow absorption rate, causing glucose-induced insulin to be released (Chauhan et al., 2010). A possible explanation may be because *T. foenum-graecum* seeds contain 50% fibre slowing the rate of postprandial glucose absorption (Hannan, 2007). These effects, confirmed by available reports in the literature, suggest that *T. foenum-graecum* is efficacious in stimulating and regenerating pancreatic β-cells that result in lowering blood glucose levels in people with diabetes (Shashikumar et al., 2019).

T. foenum-graecum, through different mechanisms of action, is known to have various pharmacological effects, such as hypoglycaemia and anti-hyperlipidaemia, through different mechanisms of action (Ramesh et al., 2010; Yadav et al., 2011). In several studies, supplementing *T. foenum-graecum* seed extracts to patients with T2DM has shown beneficial effects on lipid profiles. *T. foenum-graecum* can be combined with Western medicine metformin in managing dyslipidaemia related to diabetes (Geberemeskel et al., 2019; Kaur & Singh, 2019). *T. foenum-graecum* contains polysaccharides, saponins, gelatin and tannins. This mixture of components helps to

minimise the LDL cholesterol in the blood by obstructing the re-absorption of bile salts in the colon (Wani & Kumar, 2018).

2.3.1.1. Efficacy

2.3.1.1.1. Evaluation of anti-inflammatory and antioxidant activity

Reactive oxygen species are formed in the body because of biological oxidation, promoting inflammation in people with diseases such as diabetes. Increasing these species can lead to NF-κB activation in β-cells and inflammatory response, thus driving β-cell interleukin-1beta (IL-1β) secretion (Menu et al. 2012). The flavonoid content of *T. foenum-graecum* seeds has been found to have anti-inflammatory effects (Mandegary et al., 2012). Free radicals have been implicated in the pathogenesis of DM; oxidative stress plays a key role in complications (Fernandez-Sanchez et al., 2011; Rains & Jain, 2011). Because of the overproduction of free radicals such as hydroxyl radicals, superoxide anion radicals and hydrogen peroxide, which can cause damage to the body organs and alter gene expression resulting from OS (Pham-Huy et al., 2008; Lobo et al., 2010).

T. foenum-graecum seeds have been evaluated for their antioxidant activity, hydroxyl radical scavenging activity and the inhibition of hydrogen peroxide-induced lipid peroxidation in rat liver cells (Kaviarasan et al., 2007). *T. foenum-graecum* was found to be effective against free radical-mediated diseases (Subhashini et al., 2011). Therefore, *T. foenum-graecum* may offer benefits regarding physiological antioxidant activities that include anti-diabetic properties (Bhanger et al., 2008; Gautama & Kalia, 2013; Neelakantan et al., 2014), which may help to prevent or slow the progression of diabetes and its complications.

2.3.1.1.2. Evaluation of T. foenum-graecum activity on glucose uptake

An *in vitro* study revealed that ethanol extracts of *T. foenum-graecum* (0.33–3.3 mg/ml) reduced and inhibited glucose uptake of intestinal sodium-dependent glucose transport (Al-Habori et al., 2001). In another study, the anti-diabetic properties of *T. foenum-graecum* extract were indicated, showing a significant increase in GLUT-4 translocation on an L6 muscle cell (Kadan et al., 2013). In human hepatoma cells and mouse pre-adipocytes (3T3-L1), *T. foenum-graecum* promotes the tyrosine phosphorylation of the IR_S and improves glucose uptake into cells (Vijaykumar, 2005).

T. foenum-graecum also inhibits the α-amylase activity of glucosidase activity in liver cells. Furthermore, it affects the suppression of hepatic gluconeogenesis, the enhancement of hepatic glycogen storage, and the acceleration of glucose metabolism through the hexose monophosphate pathway (Gad et al., 2006). In a human study, *T. foenum-graecum* seeds exerted a hypoglycaemic effect by stimulating glucose-dependent insulin secretion from β-cells of the pancreas, also inhibiting the activities of α-amylase and sucrose (Ganeshpurkar et al., 2013).

The administration of *T. foenum-graecum* seed powder to diabetic animals lowers blood glucose levels efficiently, which may be linked to the inhibition of intestinal glucose uptake by the higher fibre content and stimulation of insulin secretion (Srinivasan, 2019). Furthermore, *T. foenum-graecum* seed extracts improve glucose utilisation in peripheral tissues and stimulate insulin secretion (Mullaicharam et al., 2013).

2.3.1.2. Safety

In a study investigating the change in liver cells by measuring the ability of live cells to induce cytotoxicity when exposed to *T. foenum-graecum* seed extract for 24 hours,

findings suggested that *T. foenum-graecum* can be considered cytoprotective (Kaviarasan et al., 2007). Acute doses of the herb showed no change in all morphological, haematological, biochemical or physiological functions in vital organs. However, chronic doses caused highly significant spermatotoxic effects in male mice that subsequently developed inflammation during 30–60 days of treatment (Al-Ashban et al., 2010).

Although *T. foenum-graecum* has been considered reasonably safe and well-tolerated, further research is still required to clarify the safety of *T. foenum-graecum* in diabetes patients who are allergic to *T. foenum-graecum. T. foenum-graecum* may cause neurobehavioral and neurodevelopmental side effects (Faeste et al., 2009; Ouzir et al., 2016).

Indications are that the antioxidant, anti-inflammatory and anti-diabetic potential of *A. afra, C. verum and T. foenum-graecum*, as determined in *in vitro* or *in vivo* trials, can reduce oxidative stress and inflammatory response. Therefore, it is recommended that the safety and efficacy of these traditional plants be evaluated in more detail and then compared to Western drugs through the regulation of protein expression related to inflammation and metabolic disorders in the diabetes condition.

2.3.2. *Cinnamon verum*

C. verum, known as *Cinnamomum zeylanicum*, belongs to the family Lauraceae and genus *Cinnamomum*. This plant is native to Sri Lanka, southern India, Bangladesh and Myanmar (Ranasinghe et al., 2013; Hussain et al., 2019). Different cultures in African countries, India and Brazil, commonly use the *C. verum* bark as an ingredient in the food chain (Farias et al., 2020). It has been found that *C. verum* has several pharmacological

activities, including anti-inflammatory, antioxidant and anti-diabetic activities (Mazimba et al., 2015).

2.3.2.1. Efficacy

2.3.2.1.1. Anti-inflammatory activity

Adipokines have been implicated in obesity and inflammation in adipose tissue, which ultimately leads to increased local and various pro-inflammatory cytokine levels. These cytokines include TNF-α, IL-6 and IL-1β. They may result in IR and the development of T2DM (Kim et al., 2011; Leiherer et al., 2013). Therefore, this suggests that inflammation plays a critical role in the progression of T2DM complications (Donath & Shoelson, 2011).

C. verum bark is one of the oldest herbal medicines used for inflammation. In several scientific studies, it has been shown that the bark produces anti-inflammatory activity (Vetal et al., 2013). In one study, ethanol extracts of the bark were found to inhibit TNF-α gene expression in human peripheral blood mononuclear cells (Joshi et al., 2010). In another study of macrophages exposed to *C. verum* extract, a decrease in NO and TNF-α production was shown (Gunawardena et al., 2015).

2.3.2.1.2. Antioxidant activity

Plant extracts are natural antioxidants that have shown inhibitory effects on free radicals in cells. The equilibrium between the generation of free radicals and antioxidant defence in the body has significant health implications (Ismail et al., 2017). *C. verum* has noticeable antioxidant potential and may be beneficial in moderating complications related to OS in people with diabetes (Ranjbar et al., 2006; Sontakke et al., 2019).

An *in vitro* study of Wistar rat livers exposed to the essential oil of *C. verum* showed that *C. verum* decreases 3-nitrotyrosine formation and inhibits the peroxynitrite-induced lipid peroxidation (Chericoni et al., 2005). In another *in vitro* study, using the extract of *C. verum* bark, it was found that it has free radical scavenging capacity of ROS, including superoxide anions and hydroxyl radicals (Mathew & Abraham, 2006). Methanol extracts of *C. verum* used on human cancer cell lines showed activity against 2,2-diphenyl-1-picryl-hydroxyl-hydrate (DPPH) radical scavenging and chelation power of ferrous ions (Sudan et al., 2013). A more recent study used mouse 3T3-L1cells and normal liver cells to investigate the potential activity of *C. verum* antioxidant. *C. verum* bark significantly inhibited the formation of DPPH and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) free radicals (Ismail et al., 2017).

Consequently, there is a hypothesis that *C. verum* may be beneficial as an antioxidant, which may prevent the onset and development of diabetic complications (Perez-Matute et al., 2009).

2.3.2.1.3. Anti-insulin resistance activity

The first desirable treatment goals for patients with T2DM are directed at increasing insulin sensitivity and insulin action in peripheral tissues (Shen et al., 2015). It has been suggested that *C. verum* can be used effectively to lower blood glucose and cholesterol levels in T2DM (Neto et al., 2020).

It has been shown that aqueous extracts of *C. verum* may potentiate insulin action (Anderson et al., 2004). An *in vitro* study showed that cinnamon extracts enhance insulin action by activating insulin receptor kinase activity and autophosphorylation of IRs in 3T3-L1 cells (Jarvill-Taylor et al., 2001). In another *in vitro* study, in which methanolic

extracts of *C. verum* bark were used, decreases in α-amylase and α-glucosidase enzymes involved in the digestion of carbohydrates, were shown to achieve hyperglycaemia control in T2DM (Nair et al., 2013). The above findings suggest that *C. verum* may be valuable in treating IR and beneficial in controlling glucose intolerance (Mollazadeh & Hosseinzadeh, 2016).

2.3.2.2. Safety

In a WHO survey, it was reported that about 70–80% of the world's population mainly use traditional herbal medicine in their primary health care (Payyappallimana, 2010). Toxicology studies are therefore necessary before using an integrating agent in health management. In their study, Ranasinghe et al. (2012) suggested that *C. verum* is more efficient and safe than *C. verum*-related species such as *Cinnamon cassia*. In another study evaluating the cytotoxicity of the essential oil activity from *C. verum* in rat fibroblasts, results showed essential oil and significant cytotoxicity in cell lines at concentrations > 15 µg/ml (Unlu et al., 2010).

Cytotoxic analysis of 100 µg/ml of *C. verum* bark on four human cancer cell lines (breast, colon, hepatocellular and lung) showed no cytotoxicity of the *C. verum* bark but only weak toxicity against the colon carcinoma (Moustafa et al., 2014). The study also included cytotoxicity assays of a 50% ethanolic *C. verum* extract (500 g/ml) on the hepatocellular liver carcinoma cell and rat L6 muscle cell line. ELISA was used to evaluate GLUT-4 translocation to the plasma membrane. Results indicated a significant increase in GLUT-4 activity on the plasma membranes at non-cytotoxic concentrations (Kadan et al., 2013). However, indications are that the observed anti-diabetic properties of this plant may be through the regulation of GLUT-4 translocation (Kadan et al., 2013).

2.3.3. *Artemisia afra*

A. afra (Jacq. Ex. Willd), 'African Wormwood', is a diverse genus of the family Asteraceae, of which the roots, leaves and stems are used in many different therapeutic ways (Van Wyk et al., 2008; Ntutela et al., 2009; Arendse, 2013). *A. afra* is a common species of the genus *Artemisia* and is widely used in traditional medicine (Patil et al., 2011).

2.3.3.1. Efficacy

In vitro use of *A. afra* shows antioxidant potential and reduced risk of chronic diseases (Burits et al., 2001). An *in vivo* study confirmed that *A. afra* has antioxidant and anti-diabetic properties; blood glucose levels were significantly reduced by decreasing OS, which may regenerate pancreatic β-cells (Afolayan & Sunmonu, 2011). In another *in vivo* study, an ethanolic extract of *A. afra* significantly lowered blood glucose by 49.8% at a dose of 1000 mg/kg – 500–750 mg/kg doses significantly decreased blood glucose levels in alloxan-induced diabetic mice (Issa & Hussen, 2015). Thus, from the limited available studies, *A. afra* appears to have anti-diabetic properties. A recent study evaluated the anti-inflammatory response of aqueous extracts of *A. afra* and found significantly decreased pain in Wistar rats (Gondwe et al., 2018).

2.3.3.2. Safety

Traditionally, *Artemisia* is widely used, although there is little evidence to validate its safety. In the 1970s, the WHO declared the plant unsafe for consumption, but it remains extensively used in traditional therapy (Patil et al., 2011). Several *in vivo* studies have investigated the safety of *A. afra* by determining its toxicity after acute and chronic administration in rodents. One such study showed no significant changes in organ

weights or histopathological results after using *Artemisia* (Mukinda & Syce, 2007). Another study suggested that *A. afra* may protect the liver and reduce the blood disorders caused by HG levels (Sunmonu and Afolayan 2013). In yet another study, aqueous extracts of *A. afra* (5500 mg/kg (1 hour) and 5000 mg/kg (24 hours)) were found to be non-toxic in mice (Issa & Hussen, 2015). However, there seems to be no data nor scientific literature that justifies its usage in *in vitro* studies that use proteomic biomarkers to show the mechanism of action and efficacy of herbs.

Chapter 3

Chemical analysis of extracts from African medicinal plants with anti-inflammatory and anti-diabetic properties

Abstract

Traditional plants have been an important source in the discovery of new drugs. Plant extracts are commonly used for medicinal purposes. There is a lack of sufficient scientific data supporting herbal medicine used for specific diseases. A particular concern is which components of a herbal remedy are pharmaceutically effective in treatment, with little or no side effects. Among the objectives of this study was to generate data about the anti-inflammatory and antioxidant properties of traditional African herbs. The chemical composition of plant extracts of *T. foenum-graecum*, *C. verum* and *A. afra* were also analysed to determine the bio-active compounds that could be involved in anti-inflammatory and antioxidant activity. Compound analysis of ethanolic extracts of plants was performed using GC-MS.

The results showed new chemical compounds and chemical compounds previously detected in studies on *C. verum* and *A. afra* extract analysis. Analysis of the following three plant extracts revealed the following primary bio-actives: *T. foenum-graecum,* hexadecanoic acid (31.06%); *C. verum,* (E)-cinnamaldehyde (35.43%); *A. afra,* artemisia ketone (36.05%).

3.1. Introduction

Traditional plants have been an important source in the discovery of new drugs. More than 400 herbal remedies are used for diabetes treatment, but only a few have been scientifically and clinically tested to determine their efficacy (Singh et al., 2011; Chang et al., 2013). The safety and efficacy of various plant extracts have been tested in several *in vivo* and *in vitro* trials. In most studies thus far, plant extracts have been studied on animals to determine whether they can treat diabetes complications (Ilhan et al., 2016; Pandey et al., 2011). Indications are that some plants have been scientifically studied as remedies for T2DM and can undeniably decrease blood sugar levels (Chege et al., 2015; Kibiti & Afolayan, 2015).

Plant extracts are commonly used for medicinal purposes (Beavers, 2011). There is a lack of sufficient scientific data supporting herbal medicine used for specific diseases. A particular concern is which components of a herbal remedy are pharmaceutically effective in treatment, with little or no side effects.

T. foenum-graecum is a plant that has been extensively used as an anti-diabetic medicine (Adedapo et al., 2014; Olaiya & Soetan, 2014). The leaves and seeds of the plant show anti-inflammatory, antioxidant and anti-diabetic properties (Haber & Keonavong, 2013; Yadav & Baquer, 2014). According to earlier studies, 4-hydroxyisoleucine-extracted and purified from *T. foenum-graecum* seed has insulinotropic activity and is valuable in treating DM (Baquer et al., 2011; Kumar et al., 2012).

C. verum, also known as *C. Zeylanicum*, can be used for diabetes treatment because of its potent anti-inflammatory activity (Gunawardena et al., 2014). Several studies revealed

that *C. verum* reduces chronic inflammation (Ranasinghe et al., 2012; Rathi et al., 2013). The most important chemical compounds extracted from *C. verum* are cinnamaldehyde, cinnamyl alcohol and eugenol (Winias, 2015; De La Torre et al., 2017). The primary *C. verum* compounds responsible for anti-inflammatory activity are (E)-cinnamaldehyde and *o*-methoxy cinnamaldehyde (Gunawardena et al., 2015). *C. verum* affects immune responses by regulating anti- and pro-inflammatory genes and glucose transporter gene expression (Cao et al., 2008; Cao et al., 2010).

A. afra is one of the oldest and best known indigenous medicines in South Africa (Patil et al., 2011). The active chemical compound from *A. afra* is an essential oil that appears pale in colour (Issa & Hussen 2015). A few laboratory studies have shown that *A. afra* extracts have antioxidant potential and anti-diabetic activities (Afolayan & Sunmonu, 2011). *In vitro* studies report that *A. afra* has anti-inflammatory activity. Studies indicate that *A. afra* down-regulates the synthesis of NO, IL-6, macrophage inflammatory proteins, TNF-α, MCP-1 and cyclooxygenase-2 (Meng et al., 2013; Gunawardena et al., 2014; Palencia et al., 2015).

The objective of this part of the study is to analyse the chemical composition of *T. foenum-graecum, C. verum* and *A. afra* plant extracts and determine the bio-active compounds that could be involved in anti-inflammatory and antioxidant activity.

3.2. Materials and methods

3.2.1. Plant collection

T. foenum-graecum seeds (200 g), *C. verum* bark powder (100 g) and *A. afra* leaves (200 g) were obtained from a local supplier, Health Connection Wholefoods (Pty) Ltd (Cape Town, South Africa). Equal amounts of plant seeds and leaves of the three plants

were washed thoroughly with distilled water and allowed to dry at room temperature under shade. The dry *T. foenum-graecum* seeds and *A. afra* leaves were crushed to fine powder and stored in airtight containers until required for use.

3.2.2. Plant extraction procedure

Ethanol extracts of the plants were prepared at elevated temperature at 45°C, the most common method used for extracting organic constituents from dried plant material (Pandey et al., 2011; Pandey & Tripathi, 2014). Ethanol is a suitable solvent for extracting polar and non-polar compounds (Altemimi et al., 2017). *T. foenum-graecum* and *C. verum* (20 g each) were mixed with 100 ml ethanol at 45°C for 24 hours.

The mixture was then centrifuged at 4000 rpm for 10 min, after which the supernatant was filtered through a sterile syringe filter (0.2 µM). *A. afra* leaves were first allowed to air dry, then ground into a fine powder. The ground *A. afra* was mixed with 1:5 m/v ethanol. The mixture was centrifuged at 4000 rpm for 10 min and then filtered. Images of the extraction procedure are shown in Figure 3.1. The final concentrations of *T. foenum-graecum, C. verum* and *A. afra* extracts obtained were 16.6 g/100 ml, 16.6 g/100 ml and 20 g/100 ml, respectively.

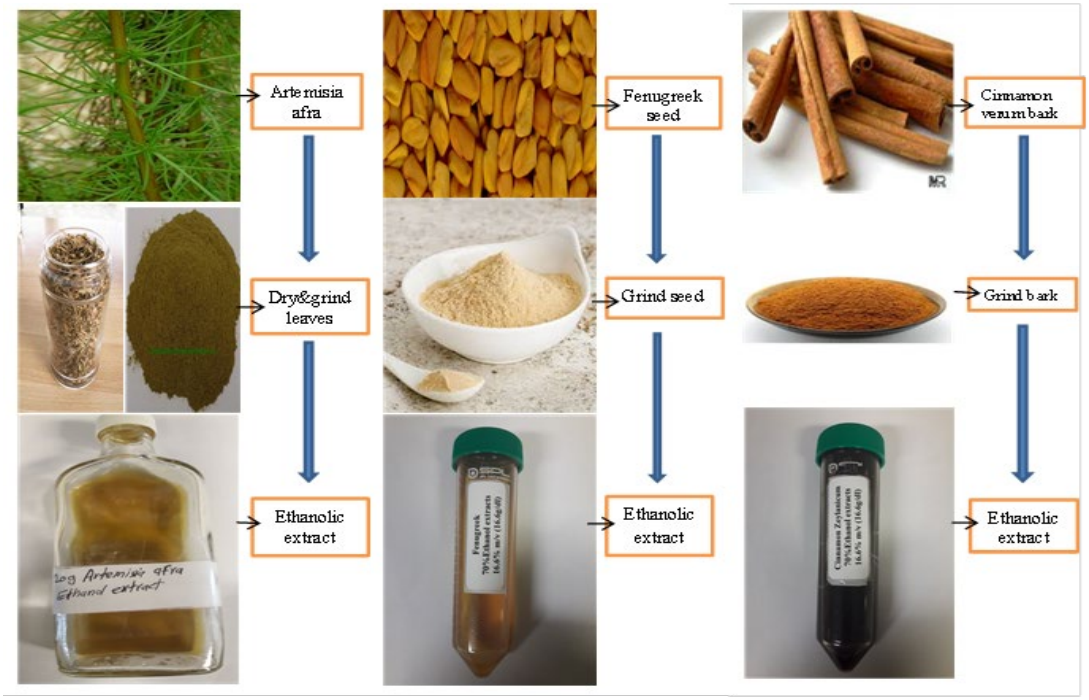


Figure 3.1. Extraction procedure used for *A. afra* leaves, *T. foenum-graecum* (fenugreek) seed and *C. verum* bark: 45°C, 24 hours

Analysis of bioactive compounds of the ethanolic extracts of *T. foenum-graecum*, *C. verum* and *A. afra* was performed by GC-MS analysis (Central Analytical Facilities, Stellenbosch University). GC-MS analysis was coupled with electron impact (EI) or chemical ionization (CI) mass spectrometry (Agilent Technologies inert XL mass selective detector). GC is combined with the high sensitivity and specificity of mass spectrometry (MS). In mass spectrometry, compounds are identified based on their molecule mass. Using the gas chromatograph, the sample is effectively vaporized into the gas phase and separated into its various components using a capillary column coated with a stationary liquid phase. As a result of vaporizing the sample in the source, molecules have practical limitations.

Comparisons between system detector sensitivities of plant compound analysis are complex because of the changing effects resulting from various samples (Abidi, 2001).

However, the detection system's sensitivity can be attributed to the various plant constituents of leaves, seeds and bark, extraction procedure, solvents used and the technique for analysis (Altemimi et al., 2017).

3.3. Results

Phytochemical analysis of the ethanolic extracts of *T. foenum-graecum, C. verum* and *A. afra* were carried out. The chemical compositions of *T. foenum-graecum*, *C. verum* and *A. afra* are tabulated in Table 3.1, Table 3.3 and Table 3.5, respectively, with the corresponding percentage abundance in the extract as depicted in Figure 3.1 (starting peak at time 39.74 min and the highest peak at 42.21 min) obtained from ethanolic seed extract; Figure 3.2, (starting peak at time 29.25 min and the highest peak at 40.61 min) obtained from ethanolic bark extract; Figure 3.3, (starting peak at time 2.43 min and the highest peak at 4.23 min) obtained from ethanolic leaf extract, respectively. Tables 3.2, 3.4 and 3.6 list the compounds with known anti-diabetic, anti-inflammation and antioxidant properties found in the extracts of *T. foenum-graecum, C. verum* and *A. afra*, respectively.

Table 3.1. Chemical composition of the ethanolic extract of *T. foenum-graecum* seeds

No	Retention Time (min)	Compound Name	(%) in the extract	Chemical Formula	Molecular weight (g/mol)
1	11.8687	2-Hexanol	0.57	$C_6H_{14}O$	102.1748
2	13.6149	Isopentanol	0.61	$C_5H_{12}O$	88.1482
3	16.8602	4,7-Diazaindole	0.30	$C_6H_5N_3$	119.1240
4	24.1784	3-Octanol	0.92	$C_8H_{18}O$	130.2280
5	38.1624	Benzene acetic acid	0.10	$C_8H_8O_2$	136.1479
6	39.0478	1,2,3,6-Tetrahydropyridine	0.28	C_5H_9N	83.1317
7	39.1391	1-Chloro-2-methylcyclopropane	0.25	C_4H_7Cl	90.5514
8	**39.5045**	**2- Phenyle ethanol**	**0.22**	**$C_8H_{10}O$**	**122.1644**
9	39.739	Phenol, 2,6-bis-(1,1-dimethylethyl)-4-methyl-(CAS)	1.42	$C_{16}H_{26}O_2$	250.3764
10	39.8882	3-Methyl-3a,4,5,6,7,7a-hexahydro-inden-1-one	0.40	$C_{10}H_{14}O$	150.22
11	40.1419	2-Methyl-1-octene	0.15	C_9H_{18}	126.2392
12	40.2762	Phenol	0.04	C_6H_6O	94.1112
13	40.485	2-Isopropyl-5-methyl-1, 4-benzoquinone	0.16	$C_{10}H_{12}O_2$	164.2011
14	40.5754	Trans-cinnamaldehyde	0.11	C_9H_8O	132.16
15	40.6967	Cyclohexanone, O-methyloxime	0.49	$C_7H_{13}NO$	127.1842
16	40.7876	Ethyl 5-methoxy-1,2-dimethylindole-3-carboxylate	0.09	$C_{14}H_{17}NO_3$	247.2900
17	40.8331	Isobutyramide	0.12	C_4H_9NO	87.1204
18	40.9695	2-(1-methylethyl)-5-methyl-phenol	0.13	$C_{10}H_{14}O$	150.22
19	41.0604	Ethyl N-benzylcarbamate	1.04	$C_{10}H_{13}NO_2$	179.219
20	**41.3597**	**2-Methoxy-4-prop-2-enylphenol**	**8.29**	**$C_{10}H_{12}O_2$**	**164.2011**
21	41.431	Butylphenol	6.98	$C_{10}H_{14}O$	150.2180
22	**41.5951**	**Benzoic acid**	**6.50**	**$C_7H_6O_2$**	**122.1213**
23	41.8795	3-Phenyl-5H-1,2,4-triazolo[3,4-b][1,3]-benzothiazin-5-one	3.90	$C_{15}H_9N_3OS$	279.3165
24	42.0613	11-Methoxy-[5]-metacyclophane	0.75	$C_{12}H_{16}O$	176.26
25	**42.1976**	**Hexadecanoic acid* (Palmitic acid)**	**31.06**	**$C_{16}H_{32}O_2$**	**256.4241**
26	42.6217	para-tert-Amylphenol	6.03	$C_{11}H_{16}O$	164.2441
27	42.8187	1,3,5-tri-tert-Butylbenzene	1.86	$C_{18}H_{30}$	246.4308
28	42.8944	1,13-Dihydroxy-herbertene	1.84	$C_{15}H_{22}O_2$	234.339
29	**42.9702**	**2,4-Bis-(2-methyl-2-butanyl) phenol**	**1.40**	**$C_{16}H_{26}O$**	**234.3770**
30	**43.2428**	**Furan-2-ylmethyl acetate**	**1.10**	**$C_7H_8O_3$**	**140.1366**
31	43.4398	Pyrrolo-[3,2,1-jk]-carbazole	2.66	$C_{29}H_{30}N_2O_3$	454.57
32	**43.5307**	**(2E,4E)-Octadeca-2,4-dienoic acid (Linoleic acid)**	**11.31**	**$C_{18}H_{32}O_2$**	**280.452**
33	43.7428	2-Cyclohexylidene ethanol	1.00	$C_8H_{14}O$	126.1962
34	43.8185	Dimethyl-Bisphenol-A	1.06	$C_{17}H_{20}O_2$	256.34
35	**43.9245**	**(E,7R,11R)-3,7,11,15-Tetramethylhexadec-2-en-1-ol**	**2.09**	**$C_{20}H_{40}O$**	**296.53**
36	44.3336	Allyldimethyl (vinyl) silane	1.27	$C_7H_{14}Si$	126.274
37	**44.485**	**Tetradecanoic acid**	**0.31**	**$C_{14}H_{28}O_2$**	**228.376**
38	44.5305	Butyl phthalate	0.35	$C_{16}H_{22}O_4$	278.348
39	44.6971	Muscopyridine	0.73	$C_{16}H_{25}N$	231.3764

- **Main constituent of T. foenum-graecum extracts.*
- *Compounds in bold were found in previous studies.*

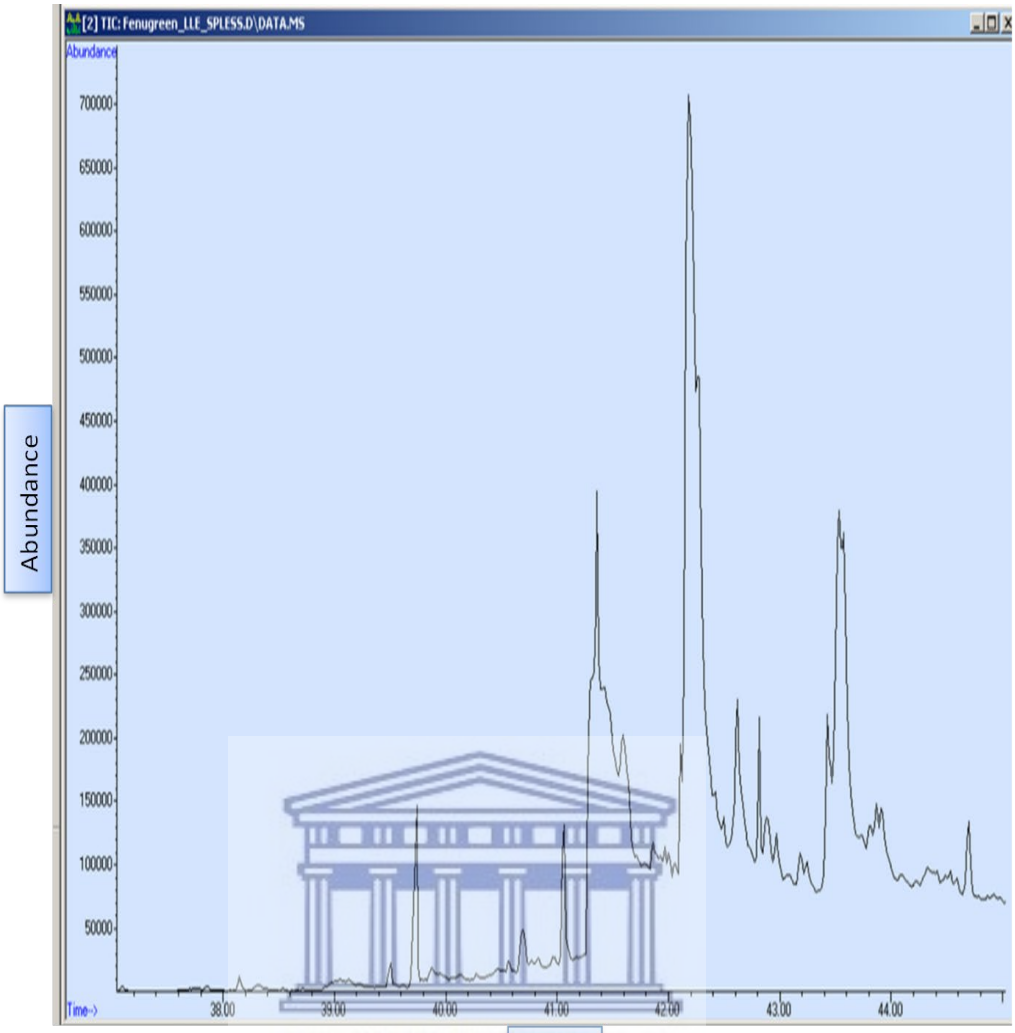


Figure 3.2. Gas chromatogram of *T. foenum-graecum* components

Table 3.2. Chemical compounds in *T. foenum-graecum* (indicated in bold in Table 3.1) with reported biological activities

No	Compound Name	(%)	Formula	Molecular weight g/mol	Biological activity (Diabetes)	References
1	2-Phenyl ethanol	0.22	$C_8H_{10}O$	122.164	Anti-oxidant and anti-inflammatory	*Mebazaa et al., 2011; Pittala et al., 2018*
2	2-Methoxy-4-prop-2-enyl phenol	8.29	$C_{10}H_{12}O_2$	164.201	Inhibiting α-glucosidase	*Mebazaa et al., 2011*
3	Benzoic acid	6.50	$C_7H_6O_2$	122.12	Reduces free radial factors and lipid peroxidation	*Mebazaa et al., 2011; Gayathri & Kannabiran, 2012*
4	Hexadecanoic acid (Palmitic acid)	31.06	$C_{16}H_{32}O_2$	256.424	Anti-inflammatory, stimulates glucose uptake and anti-oxidant	*Mebazaa et al., 2011; Gu et al., 2017; Akbari et al., 2019*
5	2,4-Bis-(2-methyl butan-2-yl)-phenol	1.40	$C_{16}H_{26}O$	234.377	Regulate fat metabolism	*Mebazaa et al., 2011; Kim et al., 2015*
6	Furan-2-ylmethyl acetate	1.10	$C_7H_8O_3$	140.137	Increased glucose uptake	*Mebazaa et al., 2011*
7	(2E,4E)-Octadeca 2,4-dienoic acid (Linoleic acid)	11.31	$C_{18}H_{32}O_2$	280.452	alpha-Gucosidase inhibitors	*Naidu et al., 2011; Gu et al., 2017; Akbari et al., 2019*
8	(E,7R,11R)-3,7,11,15-tetramethyl hexadec-2-en-1-ol	2.09	$C_{20}H_{40}O$	296.53	Anti-inflammatory and anti-oxidant	*Silva et al., 2014; Lee et al., 2016*
9	Tetradecanoic acid (myristic acid)	0.31	$C_{14}H_{28}O_2$	228.376	Anti-diabetes, lower body weight and anti-oxidant	*Takato et al., 2017; Gu et al., 2017; Mujeeb et al., 2014*

Table 3.3. Chemical composition of the ethanolic extract of *C. verum* bark

No	Retention Time (min)	Compound Name	(%)	Chemical Formula	Molecular weight (g/mol)
1	11.779	2-Hexanol	0.10	$C_6H_{14}O$	102.1748
2	13.5459	Isopentanol	0.10	$C_5H_{12}O$	88.1482
3	29.2525	alpha-Cubebene	3.99	$C_{15}H_{24}$	204.351
4	**29.516**	**Benzaldehyde-(CAS)**	**0.45**	**C_7H_6O**	**106.1219**
5	33.1309	1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)-(CAS)	0.44	$C_{15}H_{24}$	204.357
6	36.4557	alpha-Amorphene, (-)-	0.78	$C_{15}H_{24}$	204.357
7	37.1197	alpha-Elemene	0.36	$C_{15}H_{24}$	204.357
8	37.3806	alpha-Muurolene	1.17	$C_{15}H_{24}$	204.351
9	37.5125	beta-Bisabolene	0.49	$C_{15}H_{24}$	204.357
10	37.9678	delta-Cadinene	3.12	$C_{15}H_{24}$	204.357
11	38.2108	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	0.34	$C_{15}H_{22}$	202.3352
12	38.3081	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-(CAS)	0.12	$C_{15}H_{24}$	204.357
13	38.4539	alpha-Cadinene	0.07	$C_{15}H_{24}$	204.357
14	38.9199	Naphthalene,1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-(1S-cis)-(CAS)	2.11	$C_{15}H_{22}$	202.341
15	39.0659	8-Oxo-neoisolongifolene	0.23	$C_{15}H_{22}O$	218.3346
16	39.5043	Benzene ethanol-(CAS)	0.05	$C_8H_{12}O$	122.1644
17	39.5775	4-(1-Hydroxyethyl)-benzaldehyde	2.09	$C_9H_{10}O_2$	150.1745
18	**39.8881**	**Tetradecanol-(CAS)**	**0.53**	**$C_{14}H_{30}O$**	**214.3874**
19	40.0224	Ethyl thieno[2,3-b]pyridine-6-carboximidate	0.07	$C_{10}H_{10}N_2OS$	206.2640
20	40.1418	Benzene, (1,1-dimethyl-2-propenyl)-(CAS)	0.21	$C_{11}H_{14}$	146.2289
21	40.2313	Ethyl cinnamate	0.20	$C_{11}H_{12}O_2$	176.215
22	40.3058	4-Bromo-5-methyl-2(5H)-Furanone	0.03	$C_5H_5BrO_2$	176.997
23	**40.6056**	**2-Propenal, 3-phenyl-**	**32.6**	**C_9H_8O**	**132.1592**
24	**40.7723**	**1,4,4,7-tetra-Methyltricyclo[5.3.1.02,6]undecan-11-ol**	**0.18**	**$C_{15}H_{26}O$**	**222.372**
25	40.8632	(5R)-2-Methyl-5-[(2S)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene	1.55	$C_{15}H_{24}$	204.357
26	41.2467	delta-Cadinene, (+)-	0.10	$C_{15}H_{24}$	204.3511
27	**41.3595**	**(E)-3-Phenylprop-2-enoic acid (Cinnamic acid)**	**2.83**	**$C_9H_8O_2$**	**148.161**
28	41.4905	Epi-biocyclosesquipheilandrene	1.48	$C_{15}H_{24}$	204.3511
29	41.58	T-Cadinol	3.19	$C_{15}H_{26}O$	222.3663
30	41.7292	alpha.-Bisabolol	0.78	$C_{15}H_{26}O$	222.372
31	41.8339	1,4-Dimethyl-7-(1-methylethyl)-azulene	0.90	$C_{15}H_{18}$	198.3034
32	42.0157	Hexadecanoic acid, ethyl ester (CAS)	2.34	$C_{18}H_{36}O_2$	284.484
33	**42.1671**	**n-Hexadecanoic acid**	**7.38**	**$C_{16}H_{32}O_2$**	**256.42**
34	**42.5307**	**1-Methyl-4-propan-2-ylcyclohexa-1,4-diene**	**0.93**	**$C_{10}H_{16}$**	**136.23**
35	42.6216	3-Methyl-benzo-(b)-thiophene-1-oxide	1.79	C_9H_8OS	164.224
36	42.8488	para-Methoxycinnamaldehyde	5.82	$C_{10}H_{10}O2$	162.1850
37	42.9852	Chromen-2-one	12.50	$C_9H_6O_2$	146.1427
38	43.1821	9-Octadecenoic acid-(Z)-, ethyl ester (CAS)	1.10	$C_{20}H_{38}O_2$	310.5145
39	43.4548	Ethyl linoleate	1.34	$C_{20}H_{36}O_2$	308.4986

Table 3.3, cont.

40	**43.6063**	**Dibromoacetic acid**	**0.13**	**$C_2H_2Br_2O_2$**	**217.844**
41	43.7123	Exo-7-ethyl-5-methyl-6,8-dioxabicyclo-[3.2.1]-oct-3-ene	0.45	$C_9H_{14}O_2$	154.2060
42	44.1062	Dehydroabietal	0.17	$C_{20}H_{28}O$	284.4357
43	44.2728	5H-Thieno-[2,3-c]-pyran-5-one, 7-methyl-	0.39	$C_8H_6O_2S$	166.194
44	44.4849	n-Tetradecanoic acid	0.73	$C_{14}H_{28}O_2$	228.376
45	11.7346	2-Pentanol, 4-methyl-	0.14	$C_6H_{14}O$	102.1748
46	13.4772	1-Butanol, 3-methyl-	0.14	$C_5H_{12}O$	88.1482
47	24.0613	3-Octanol	0.21	$C_8H_{18}O$	130.2279
48	**25.5996**	**Decanaldehyde**	**0.44**	**$C_{10}H_{20}O$**	**156.27**
49	29.2673	alpha-Cubebene	5.29	$C_{15}H_{24}$	204.357
50	29.443	Benzaldehyde	0.78	C_7H_6O	106.124
51	32.4392	Bicyclo-[3.1.1]-hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	0.22	$C_{15}H_{24}$	204.357
52	33.1165	1S,2S,5R-1,4,4-Trimethyltricyclo-[6.3.1.0(2,5)]-dodec-8(9)-ene	0.75	$C_{15}H_{24}$	204.3511
53	33.5412	2-Decenal, (E)-	0.18	$C_{10}H_{18}O$	154.2493
54	34.5663	Benzofuran, 2-methyl-	0.17	C_9H_8O	132.1592
55	34.8068	Cycloheptane	0.11	C_7H_{14}	98.1890
56	35.5953	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	0.21	$C_{15}H_{24}$	204.3511
57	36.4559	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	1.15	$C_{15}H_{24}$	204.357
58	36.7547	Cyclodecane	0.13	$C_{10}H_{20}$	140.270
59	37.2635	2-Isopropenyl-4a,8-dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	0.20	$C_{15}H_{24}$	204.3511
60	37.3955	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)-	1.63	$C_{15}H_{24}$	204.3511
61	37.5128	1,6,10-Dodecatriene, 7,11-dimethyl-3-methylene-, (E)-	0.93	$C_{15}H_{24}$	204.357
62	37.7327	2H-Pyran-2-one, tetrahydro-	0.12	$C_5H_8O_2$	100.1158
63	37.968	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	4.25	$C_{15}H_{24}$	204.3511
64	38.2111	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	0.49	$C_{15}H_{22}$	202.3352
65	38.3245	Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)-	0.23	$C_{15}H_{24}$	204.3511
66	38.4703	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,[1R-(1.alpha.,4a.alpha.,8a.alpha.)]-	0.15	$C_{15}H_{24}$	204.3511
67	38.7295	11-Hexadecen-1-ol, (Z)-	0.08	$C_{16}H_{32}O$	240.4247
68	38.9201	Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-	2.89	$C_{15}H_{22}$	202.3352
69	39.0662	Benzyl Alcohol	0.13	C_7H_8O	108.1378
70	39.1392	Butanoic acid, butyl ester	0.09	$C_8H_{16}O_2$	144.2114
71	39.194	Propanoic acid, 2-methyl-, 2-methylpropyl ester	0.09	$C_8H_{16}O_2$	144.2114
72	39.5045	Phenylethyl Alcohol	0.09	$C_8H_{10}O$	122.1644
73	39.5777	1,5,6,7-Tetrahydro-4-indolone	2.57	C_8H_9NO	135.1632
74	39.739	Butylated Hydroxytoluene	2.25	$C_{15}H_{24}O$	220.35
75	39.8883	1,13-Tetradecadiene	0.52	$C_{14}H_{26}$	194.3562
76	40.0375	2,4,5-Trichlorophenyl cinnamate	0.58	$C_{15}H_9Cl_3O_2$	327.585
77	40.1569	Alpha-Ethyltryptamine	0.37	$C_{12}H_{16}N_2$	188.274
78	40.2315	trans-1,2-Dibromocyclohexane	0.08	$C_6H_{10}Br_2$	241.954
79	40.321	3-Methylbenzoylhydrazide	0.23	$C_8H_{10}N_2O$	150.181
80	**40.621**	**(2E)-3-Phenylacrylaldehyd (Cinnamaldehyde, (E)-)***	**35.43**	**C_9H_8O**	**132.1592**

Table 3.3, cont.

81	40.7725	Thiourea, 1-(4-fluorophenyl)-3-(3-hydroxypropyl)-	0.43	$C_{10}H_{13}FN_2OS$	228.29
82	40.8634	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,7.beta.,8a.alpha.)]-	1.94	$C_{15}H_{24}$	204.357
83	41.0605	Benzoic acid, 4-nitroso-, ethyl ester	0.37	$C_9H_9NO_3$	179.1730
84	**41.1905**	**2-Propenoic acid, 3-phenyl-, ethyl ester**	**0.24**	**$C_{11}H_{12}O_2$**	**176.2118**
85	41.2469	Naphthalene, 1,2,4a,5,8,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1S-(1.alpha.,4a.beta.,8a.alpha.)]-	0.14	$C_{15}H_{24}$	204.3511
86	41.3597	1H-Cycloprop[e]azulene, decahydro-1,1,7-trimethyl-4-methylene-, [1aR-(1a.alpha., 4a.alpha.,7.alpha.,7a.beta.,7b.alpha.)]-	1.28	$C_{15}H_{24}$	204.357
87	41.4907	(+)-Epi-bicyclosesquiphellandrene	0.73	$C_{15}H_{24}$	204.3511
88	41.5802	1,5,6,7-Tetramethylbicyclo-[3.2.0]-hept-6-en-3-one	1.12	$C_{11}H_{16}O$	164.248
89	41.7294	alpha-Bisabolol	0.53	$C_{15}H_{26}O$	222.37
90	41.8492	Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl-	0.69	$C_{13}H_{18}$	174.2820
91	42.1219	Oxirane, [[4-(1,1-dimethylethyl)-phenoxy]methyl]-	0.71	$C_{13}H_{18}O_2$	206.283
92	42.2128	7-Isopropenyl-1,4a-dimethyl-4,4a,5,6,7,8-hexahydro-3H-naphthalen-2-one	0.21	$C_{15}H_{22}O$	218.3346
93	42.334	2-(2-Hydroxycyclohexylmethyl)pyridine oxide	0.25	$C_{12}H_{17}NO_2$	207.273
94	42.4552	Diethyl Phthalate	0.88	$C_{12}H_{14}O_4$	222.2372
95	**42.5309**	**2,6,6Trimethylbicyclo[3.1.1]hept-2-ene**	**0.65**	**$C_{10}H_{16}$**	**136.23**
96	42.6218	Phenol, 4-(1,1-dimethylpropyl)-	0.32	$C_{11}H_{16}O$	164.2441
97	42.849	2-Propenal, 3-(2-methoxyphenyl)-	5.49	$C_{10}H_{10}O_2$	162.1852
98	42.9854	2H-1-Benzopyran-2-one	10.53	$C_9H_6O_2$	146.145
99	43.0914	1-Naphthalenol, 5,6,7,8-tetrahydro-2,5-dimethyl-8-(1-methylethyl)-	0.32	$C_{15}H_{22}O$	218.3346
100	43.1823	Ethyl Oleate	1.06	$C_{20}H_{38}O_2$	310.5145
101	43.3338	4,6-di-tert-Butyl-m-cresol	0.39	$C_{15}H_{24}O$	220.3505
102	43.455	Linoleic acid ethyl ester	1.41	$C_{20}H_{36}O_2$	308.506
103	43.6065	1H-Pyrazol-5-amine, 3-methyl-1-phenyl-	0.40	$C_{10}H_{11}N_3$	173.2144
104	43.7428	Cyclohexadecane	0.29	$C_{16}H_{32}$	224.4253
105	43.8792	9,12,15-Octadecatrienoic acid, ethyl ester, (Z,Z,Z)-	0.81	$C_{20}H_{34}O_2$	306.49
106	**44.1064**	**Benzyl Benzoate**	**0.18**	**$C_{14}H_{12}O_2$**	**212.248**
107	44.273	2-Methoxybenzyl alcohol	0.20	$C_8H_{10}O_2$	138.1638
108	44.4699	Tetradecanoic acid	0.68	$C_{14}H_{28}O_2$	228.3709
109	44.6063	Pyridine, 2,6-dimethyl-	0.05	C_7H_9N	107.1531
110	44.6972	Ethyl 1-methyl-2-oxo-1,2-dihydroquinoline-4 carboxylate.	0.23	$C_{13}H_{13}NO_3$	231.2470

- **Main constituent of C. verum bark extract.*
- *Compounds in bold were found in previous studies.*

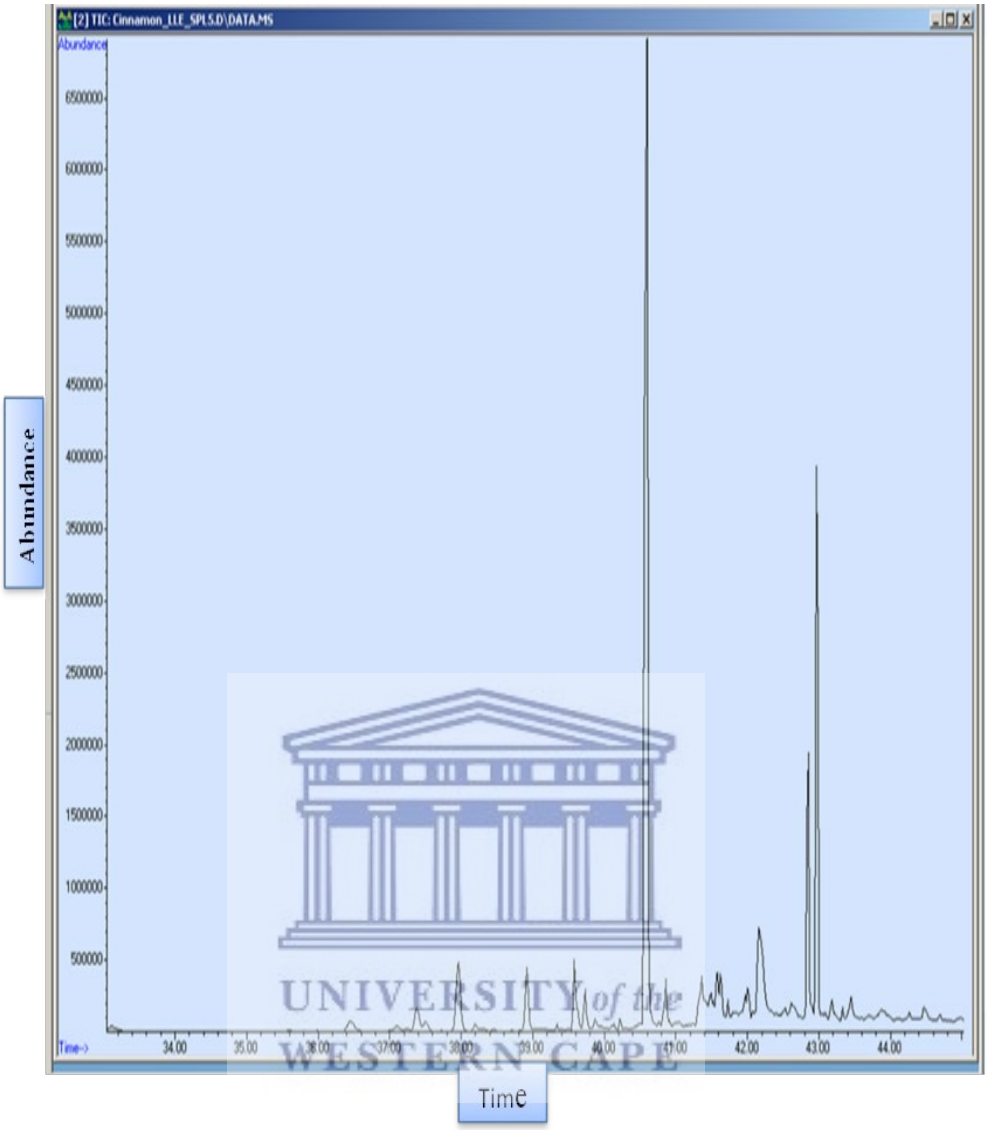


Figure 3.3. Gas chromatogram of *C. verum* components

Table 3.4. Chemical compounds in *C. verum* (bold in Table 3.1) with reported biological activities

No	Compound Name	(%)	Formula	Molecular weight g/mol	Biological activity (Diabetes)	References
1	Benzaldehyde - (CAS)	0.45	C_7H_6O	106.123	Inhibit formation of non-enzymatic glycosylation products.	*Muhammad & Dewettinck, 2017; Jayaprakasha & Rao, 2011*
2	Tetradecan-1-ol- (CAS)	0.53	$C_{14}H_{30}O$	214.387	Anti-inflammatory	*Marongiu et al., 2007; Mujeeb et al., 2014*
3	2-Propenal, 3-phenyl-	32.6	C_9H_8O	132.159	Decreased HbA1C, LDL and TG levels.	*Liyanage et al., 2017; Marongiu et al., 2007; Babu et al., 2007*
4	1,4,4,7-Tetramethyltricyclo-[5.3.1.02,6]-undecan-11-ol	0.18	$C_{15}H_{26}O$	222.372	Anti-inflammatory and anti-oxidant.	*Marongiu et al., 2007*
5	(E)-3-Phenylprop-2-enoic acid	2.83	$C_9H_8O_2$	148.161	alpha-Glucosidase inhibitors and stimulating insulin secretion.	*Farag et al., 2018; Jayaprakasha & Rao, 2011*
6	n-Hexadecanoic acid	7.38	$C_{16}H_{32}O_2$	256.42	Anti-inflammatory and anti-diabetic activities.	*Marongiu et al., 2007; Aparna et al., 2012*
7	1-Methyl-4-propan-2-ylcyclohexa-1,4-diene	0.93	$C_{10}H_{16}$	136.23	None	*Marongiu et al., 2007*
8	Dibromoacetic acid	0.13	$C_2H_2Br_2O_2$	217.844	Improved various diabetic metabolism.	*Farag et al., 2018; Sakakibara et al., 2006*
9	Decanaldehyde	0.44	$C_{10}H_{20}O$	156.27	Anti-oxidant	*Muhammad & Dewettinck, 2017; Liu et al., 2012*
10	(2E)-3-Phenylacrylaldehyd (Cinnamaldehyde, (E)-)	35.43	C_9H_8O	132.159	Hypoglycemic and hypolipidemic effects	*Farag et al., 2018; Babu et al., 2007; Zhu et al., 2017; Sontakke et al., 2019*
11	2,6,6-Trimethylbicyclo-[3.1.1]-hept-2-ene	0.65	$C_{10}H_{16}$	136.23	Anti-oxidant	*Liyanage et al., 2017; Sontakke et al., 2019*
12	Benzyl benzoate	0.18	$C_{14}H_{12}O_2$	212.248	Anti-inflammatory	*Marongiu et al., 2007; Gunawardena et al., 2015*
13	2-Propenoic acid, 3-phenyl-, ethyl ester	0.16	$C_{11}H_{12}O_2$	176.21	None	*Sontakke et al., 2019*

Table 3.5. Chemical composition of the ethanolic extract of *A. afra* leaves

No	Retention Time (min)	Compound Name	(%)	Chemical Formula	Molecular weight (g/mol)
1	2.4254	Tricyclene-(1, 7, 7-Trimethy ltricyclo-[2. 2. 1. 02, 6]-heptane)	0.35	$C_{10}H_{16}$	136.238
2	**2.7712**	**Camphene-(3,3-dimethyl-2-methylidenebicyclo-[2.2.1]-heptane)**	**7.53**	**$C_{10}H_{16}$**	**136.238**
3	3.3356	Mesitylene-(1,3,5-trimethylbenzene)	0.25	C_9H_{12}	120.195
4	3.4132	Yomogi alcohol-(2,5,5-trimethylhepta-3,6-dien-2-ol)	0.36	$C_{10}H_{18}O$	154.253
5	**3.6178**	**alpha-Terpinene-(1-methyl-4-propan-2-ylcyclohexa-1,3-diene)**	**0.47**	**$C_{10}H_{16}$**	**136.238**
6	**3.7236**	**p-Cymene-(1-methyl-4-propan-2-ylbenzene)**	**3.97**	**$C_{10}H_{14}$**	**134.222**
7	**3.8224**	**1,8-Cineole-(2,2,4-trimethyl-3-oxabicyclo-[2.2.2]-octane)**	**18.15**	**$C_{10}H_{18}O$**	**154.253**
8	**3.8859**	**cis-Ocimene-((3Z)-3,7-dimethylocta-1,3,6-triene)**	**0.17**	**$C_{10}H_{16}$**	**136.238**
9	**4.2316**	**Artemisia ketone-(3,3,6-trimethylhepta-1,5-dien-4-one)***	**36.05**	**$C_{10}H_{16}O$**	**152.237**
10	**4.4362**	**Artemisia alcohol-(3,3,6-trimethylhepta-1,5-dien-4-ol)**	**0.15**	**$C_{10}H_{18}O$**	**154.253**
11	**4.4926**	**alpha-Terpinolene-(1-methyl-4-propan-2-ylidenecyclohexene)**	**0.20**	**$C_{10}H_{16}$**	**136.238**
12	4.6196	Isoamyl 2-methylbutyrate-(3-methylbutyl 2-methylbutanoate)	0.53	$C_{10}H_{20}O_2$	172.268
13	4.662	2-Methylbutyl 2-methylbutyrate	1.01	$C_{10}H_{20}O_2$	172.268
14	**4.9583**	**cis-Sabinenehydrate-(4-methyl-1-propan-2-ylbicyclo[3.1.0]hexan-4-ol)**	**0.17**	**$C_{10}H_{18}O$**	**154.253**
15	**5.1417**	**Camphor (4,7,7-trimethylbicyclo-[2.2.1]-heptan-3-one)**	**6.78**	**$C_{10}H_{16}O$**	**152.237**
16	5.2476	Verbenyl ethyl ether-(2-ethoxy-4,6,6-trimethylbicyclo-[3.1.1]-hept-3-ene)	0.15	$C_{12}H_{20}O$	180.291
17	5.3605	Endo-Borneol-((1S,3R,4S)-4,7,7-trimethylbicyclo-[2.2.1]-heptan-3-ol)	0.83	$C_{10}H_{18}O$	154.253
18	**5.4733**	**4-Terpineol-(1-isopropyl-4-methyl-cyclohex-3-enol)**	**0.64**	**$C_{10}H_{18}O$**	**154.25**
19	5.7555	2-(1-Methylpropyl)-5-methylcyclohexanone	0.18	$C_{11}H_{20}O$	168.28
20	5.8261	6-Methylene-6,7-dihydro-4H-thiazolo-[2,3-c]-[1,2,4]-triazin-4-one	0.15	$C_6H_5N_3OS$	167.191
21	5.8755	1,5-Dimethyl-2-pyridithione	0.45	C_7H_9NS	139.22
22	6.1154	Cuminic aldehyde-(4-propan-2-ylbenzaldehyde	0.13	$C_{10}H_{12}O$	148.205

Table 3.5, cont.

23	**6.32**	**Chrysanthenyl acetate-((4,6,6-trimethyl-7-bicycle-[3.1.1]-hept-3-enyl) acetate)**	**0.58**	**$C_{12}H_{18}O_2$**	**194.274**
24	6.5669	iso-Bornyl acetate-(4,7,7-trimethyl-3-bicycle-[2.2.1] -heptanyl) acetate)	0.28	$C_{12}H_{20}O_2$	196.290
25	6.6375	Ethyl nonanoate	0.30	$C_{11}H_{22}O_2$	186.295
26	7.4065	alpha-Copaene	0.23	$C_{15}H_{24}$	204.357
27	7.4276	Nonanal diethyl acetal-(1,1-diethoxynonane)	0.35	$C_{13}H_{28}O_2$	216.365
28	7.4912	Benzyl Isovalerate-(benzyl 3-methylbutanoate)	0.24	$C_{12}H_{16}O_2$	192.258
29	7.5405	Ethyl caprate-(ethyl decanoate)	0.16	$C_{12}H_{24}O_2$	200.322
30	7.5617	N-Tetradecane-(tetradecane)	0.19	$C_{14}H_{30}$	198.394
31	7.8016	beta-Caryophyllene-([(1R,4E,9S)-11,11-dimethyl-8-methylidene-4-bicycle-[7.2.0]-undec-4-enyl]methanol)	0.43	$C_{15}H_{24}O$	220.356
32	7.9568	beta-Phenylethyl butyrate-(2-phenylethyl butanoate)	0.12	$C_{12}H_{16}O_2$	192.258
34	8.1614	Alloaromadendrene-(1,1,7-trimethyl-4-methylidene-2,3,4a,5,6,7,7a,7b-octahydro-1aH-cyclopropa-[e]-azulene)	0.56	$C_{15}H_{24}$	204.357
35	8.3096	Ar-Curcumene-(1-methyl-4-(6-methylhept-5-en-2-yl) benzene)	0.82	$C_{15}H_{22}$	202.341
36	8.3801	beta-Selinene-((3R,4aS,8aR)-8a-methyl-5-methylidene-3-prop-1-en-2-yl-1,2,3,4,4a,6,7,8-octahydronaphthalene)	5.46	$C_{15}H_{24}$	204.357
37	8.5142	beta-Bisabolene-((4S)-1-methyl-4-(6-methylhepta-1,5-dien-2-yl)cyclohexene)	0.14	$C_{15}H_{24}$	204.357
38	9.1139	(+)- Spathulenol-((1aS,4aS,7R,7aS,7bS)-1,1,7-trimethyl-4-methylidene-1a,2,3,4a,5,6,7a,7b-octahydrocyclopropa[h]azulen-7-ol)	0.61	$C_{15}H_{24}O$	220.356
39	9.1632	Caryophyllene oxide	1.24	$C_{15}H_{24}O$	220.356
40	9.2761	n-Butyl-beta-phenylpropionate	0.44	$C_{13}H_{18}O_2$	206.285
41	9.5654	Tetracyclo-[6.3.2.0(2,5).0(1,8)]-tridecan-9-ol, 4,4-dimethyl-	0.20	$C_{15}H_{24}O$	220.356
42	9.8123	alpha-Cedrene oxide	0.25	$C_{15}H_{24}O$	220.356
43	9.9323	Nootkatone-((4R,4aS,6R)-4,4a-dimethyl-6-prop-1-en-2-yl-3,4,5,6,7,8-hexahydronaphthalen-2-one)	0.54	$C_{15}H_{22}O$	218.34
44	10.9765	6,10,14-trimethyl-2-Pentadecanone	0.15	$C_{18}H_{36}O$	268.485
45	11.9501	Ethyl palmitate-(ethyl hexadecanoate)	0.46	$C_{18}H_{36}O_2$	284.484
46	15.0544	beta-Amyrin-(beta.-Amyrenol)-(4,4,6a, 6b, 8a,11,11,14b-octamethyl-1,2,3,4a, 5,6,7,8,9,10,12,12a,14,14a-tetradecahydropicen-3-ol)	1.54	$C_{30}H_{50}O$	426.729

- ***Main constituent of A. afra leaf extract.***
- *Compounds in bold were found in previous studies.*

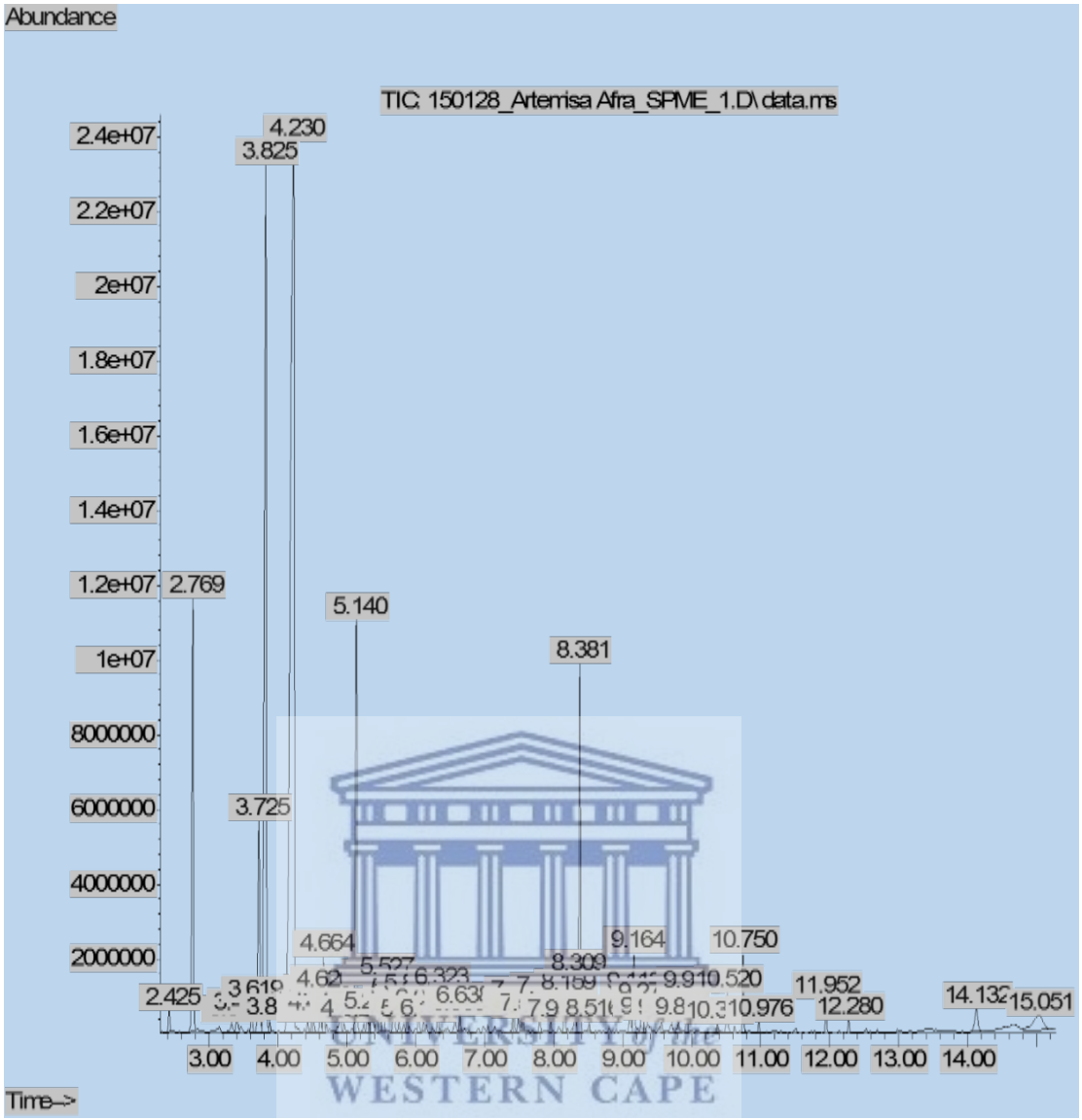


Figure 3.4. Gas chromatogram of *A. afra* components

Table 3.6. Chemical compounds found in the current *A. afra* ethanolic extract (indicated in bold in Table 3.5) previously reported in the literature

No	Compound Name	%	Chemical Formula	Molecular weight (g/mol)	Biological activity	References
1	Camphene (3,3-dimethyl-2-methylidenebicyclo-[2.2.1]-heptane)	7.53	$C_{10}H_{16}$	136.238	No Literature review reports	Mbokane & Moyo, 2020
2	alpha-Terpinene (1-methyl-4-propan-2-ylcyclohexa-1,3-diene)	0.47	$C_{10}H_{16}$	136.238		
3	p-Cymene (1-methyl-4-propan-2-ylbenzene)	3.97	$C_{10}H_{14}$	134.222		
4	1,8-Cineole (2,2,4-trimethyl-3-oxabicyclo-[2.2.2]-octane)	18.15	$C_{10}H_{18}O$	154.253		
5	Cis-Ocimene ((3Z)-3,7-dimethylocta-1,3,6-triene)	0.17	$C_{10}H_{16}$	136.238		
6	Artemisia ketone (3,3,6-trimethylhepta-1,5-dien-4-one)	36.05	$C_{10}H_{16}O$	152.237		
7	Artemisia alcohol (3,3,6-trimethylhepta-1,5-dien-4ol)	0.15	$C_{10}H_{18}O$	154.253		
8	alpha-Terpinolene (1-methyl-4-propan-2-ylidenecyclohexene)	0.20	$C_{10}H_{16}$	136.238		
9	Cis-Sabinenehydrate (4-methyl-1-propan-2-ylbicyclo-[3.1.0]-hexan-4-ol)	0.17	$C_{10}H_{18}O$	154.253		
10	Camphor (4,7,7-trimethylbicyclo-[2.2.1]-heptan-3-one)	6.78	$C_{10}H_{16}O$	152.237		
11	4-Terpineol (1-isopropyl-4-methyl-cyclohex-3-enol)	0.64	$C_{10}H_{18}O$	154.25		
12	Chrysanthenyl acetate ((4,6,6-trimethyl-7-bicycle-[3.1.1]-hept-3-enyl) acetate)	0.58	$C_{12}H_{18}O_2$	194.274		

3.4. Discussion

GC-MS is a suitable analytical tool to analyse and accurately quantify some bioactive and low molecular weight compounds in herb extracts used in pharmaceutical industries for drug production, as established in earlier studies (Iordache et al., 2009; Capriotti et al., 2015). In the present study, the chemical constituents of the plant extracts were also analysed using GC-MS. However, more compounds were discovered in the extracts than those reported in previous studies.

T. foenum-graecum

In this study, 39 chemical compounds were detected in the extracts of *T. foenum-graecum*. Of these, 30 were new compounds, i.e., not previously reported in T. *foenum-graecum* extracts (Table 3.1). Nine bioactive compounds were identified in the *T. foenum-graecum* extracts in this study that were also found in previous studies and affected inflammation, oxidation, and diabetes (Table 3.2). Chemical analysis of *T. foenum-graecum* seeds showed that hexadecanoic acid is the main component in the extract (31.06%). Earlier studies report that hexadecanoic acid has potential anti-inflammatory and anti-diabetes activities (Mebazaa et al., 2011; Kadan et al., 2013*)*. Moreover, findings indicate that the *T. foenum-graecum* extract may inhibit inflammation associated with T2DM (Uemura et al., 2010; Xie & Du, 2011; Gu et al., 2017).

C. verum

A total of 110 chemical compounds were detected in extracts of *C. verum.* Of these, 97 were novel compounds not previously reported in extracts of C. *verum* (Table 3.3). We also identified 13 bioactive compounds in the *C. verum* extracts in this study that were

found in previous studies. These 13 bioactive compounds affected inflammation, oxidation, and diabetes (Table 3.4). (E)-Cinnamaldehyde was the main compound obtained from *C. verum* (35.43%). In a recent study on the chemical constituents of *C. verum*, essential oil extract also indicated cinnamaldehyde as the main bioactive (Ainane et al., 2019). Several studies have shown that the (E)-cinnamaldehyde lowers LDL and TG levels in the blood (Liyanage et al., 2017; Farag et al., 2018) and suggest that *C. verum* may have anti-inflammatory and anti-diabetes properties.

A. afra

Fourty-six chemical compounds were detected in the *A. afra* extracts, of which 34 were new compounds not previously reported in *A. afra* extracts (Table 3.5). Twelve compounds identified in the *A. afra* extract in this study, were also found in previous studies (Table 3.6). The main compound in the *A. afra* extract was artemisia ketone (36.05%). In a recent study high ketone levels in *A. afra* essential oil (5.5%) were also reported (Mbokane & Moyo, 2020). Investigators found that the phytochemical substances in *A. afra*, in earlier studies, showed potential anti-inflammatory, antioxidant and anti-diabetic activity (Mukinda & Syce, 2007; Issa & Hussen, 2015; Hoosen & Pool, 2019). However, no earlier studies used a specific chemical compound of *A. afra* to evaluate its potent anti-inflammation and antioxidative activity (Table 3.6).

3.5. Conclusions and recommendations

In this part of the study, the objective is to analyse the chemical composition of *T. foenum-graecum, C. verum*, and *A. afra* plant extracts to identify the particular chemical compounds. The main compounds in the *T. foenum-graecum* extract showed that (hexadecanoic acid), *C. verum* extract ((E)-Cinnamaldehyde) and *A. afra* extract

(artemisia ketone). Several compounds detected were compared to those previously reported in the literature and may be involved in anti-inflammatory and antioxidant activity. These previous studies used similar current study ethanolic extracts for *T. foenum-graecum seeds, C. verum bark*, and *A. afra leaves.*

GC-MS was successfully used to identify new compounds and compounds previously reported in extracts of *T. foenum-graecum*, *C. verum*, and *A. afra*. Some of the chemical constituents in the extracts are known to have anti-inflammatory, antioxidant and anti-diabetic activity. However, no previous studies reported particular activities of specific constituents in *A. afra* extracts on anti-inflammatory antioxidants associated with DM.

Earlier studies indicated that these compounds have the potential to inhibit chronic inflammation and OS related to diabetes. Further studies should be directed at evaluating the effects of these plants concerning regulating the levels of inflammatory mediators in metabolic disorders such as T2DM, which is often associated with low-grade inflammation.

Chapter 4:

The effect of high glucose, related to diabetes mellitus, on macrophage cell expression of pro-inflammatory markers

Abstract

Macrophage activation plays a significant role in the inflammatory response induced by long-term hyperglycaemia, leading to progression complications in diabetes. In this part of the study, various cellular biomarkers that indicated pro-inflammatory in response to long-term exposure to HG and determined its toxicity were examined. A significant decrease in cell viability above 25 mM glucose concentration was present. The cell cultures were treated to different glucose concentrations, incubated for 24 hours at 37°C, and then exposed to LPS for 24 hours. Differences in the protein expression of RAW 264.7 cell cytokines ICAM-1, MIP-1α, MIP-1β, MIP-2, IL-16, IL-1ra and IL-27 in LG without LPS stimulation were found in this study. While in HG without LPS stimulation, the cells expressed ICAM-1, MIP-1α and IL-16, with the down-regulation level of MIP-1β without LPS, compared to LG without LPS incubated for 48 hours.

The RAW 264.7 cell expressions of cytokines and chemokines in LG with LPS stimulation were IP-10, I-TAC, TNF-α, G-CSF, IL-6, MCP-1, MIG, ICAM-1, MIP-1α, MIP-1β, IL-16, IL-1α, MIP-2, RANTES, IL-1ra and IL-27. While in HG with LPS stimulation, it expressed IP-10, I-TAC, TNF-α, G-CSF, IL-6, MCP-1, MIG, ICAM-1, MIP-1α, MIP-1β, IL-16, MIP-2, RANTES, IL-1ra and IL-27, with the down-regulation level of MCP-1, IL-16, IL-1ra and IL-27, compared to LG with LPS incubated for 48 hours.

Results showed that the levels of cytokines and chemokines were up-regulated in LG and HG with LPS stimulation compared with LG and HG without LPS stimulation. The cytokine proteins obtained may be used to assess the state of inflammation as well as to characterise the pathophysiology of T2DM.

4.1. Introduction

Symptoms of T2DM include progressive dysfunction of the β-cells and an impaired insulin signalling pathway (Fuentes et al., 2013). Almost 90% of all individuals with diabetes show IR. It has been suggested that chronic inflammation plays a crucial role in the pathogenesis of IR in T2DM reports (Calle & Fernandez, 2012; Cruz et al., 2013; Rehman & Akash, 2016). The molecular mechanisms linking HG levels and diabetic-inflammatory cytokines are not well-characterised. Evidence suggests that various inflammatory mediator pathways exist in early-onset diabetes and the development of its complications (King, 2008; Dobrian et al., 2019). Thus, there is a concern about whether diabetes causes inflammation or vice versa.

Hyperglycaemia can initiate pathways that can result in the production of reactive oxygen intermediates and advanced glycation end products. These products can induce and promote inflammation in diabetes patients (Hua et al., 2012). As a result of ROS production, OS plays a critical role in the pathogenesis of T2DM, as stated in several studies (Nolan et al., 2011; Volpe et al., 2018). This progression is because of free radicals generated by HG levels and glycated molecules (Perez-Matute et al., 2009; Zheng et al., 2016). These can disrupt insulin signalling pathways, with the pathway inducing pro-inflammatory cytokines (Meng et al., 2013; Asmat & Ismail, 2016).

Therefore, pro-inflammatory mediators are associated with the development and pathogenesis of diabetes complications (Giacco & Brownlee, 2010).

Obesity is associated with chronic low-grade inflammation (Capurso & Capurso, 2012) and is a well-known risk factor for the progression of T2DM. An increase in the mass of adipocytes releases lipids, FAs and various molecule adipocytokines, which heighten the inflammation triggering the development of IR (Leiherer et al., 2013). The glucose uptake in T2DM is decreased as a result of IR.

The innate immune system is regulated in normal inflammatory responses, but T2DM impairs the regulation of inflammation. Pro-inflammatory cytokines such as IL-6, TNF-α, MCP-1, MIP-1α, MIP-2 and ICAM-1 can increase the induction of iNOS and production of NO Wellen and Hotamisligil 2005; Tang & Kern, 2011). The iNOS activity results in reduced activity of the core mediator of IRs, which inhibits the activity of the insulin signalling pathway (Zeyda & Stulnig, 2009). To a greater extent, NO is a highly reactive nitrogen radical involved in multiple biological processes such as the inflammatory state (Xiang et al., 2015). It has been reported that NO generation is associated with altered vascular homeostasis contributing to atherosclerosis developing in T2DM (Elahi et al., 2007).

Additionally, inflammatory activity associated with diabetic complications is influenced by the production of different chemoattractant factors (Forbes & Cooper, 2013). This activity or process develops in increased ICAM-1 levels through the activation and proliferation of leukocytes and endothelial cells in diabetes, resulting in diabetic vascular complications and contributing to cellular dysfunction (van den Oever et al., 2010). Therefore, if chronic inflammation-associated diabetes remains untreated, it may lead to

deleterious micro- and macro blood vessels (Manabe, 2011). Consequently, it is necessary to understand the effects of HG on the inflammatory mediator expression during diabetic disease.

The aim of this part of the study was to identify pro-inflammatory biomarkers that might induce inflammation associated with long-term hyperglycemia, which could lead to early diagnosis and novel treatments for diabetes.

4.2. Materials and methods

4.2.1. Material

Dulbecco's modified Eagle's medium (DMEM) was obtained from Lanza (Belgium), while antibodies and D-glucose were purchased from Sigma-Aldrich (Germany). All the primary antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). Mouse macrophage cells, RAW 264.7, were obtained from American Type Culture Collection (USA) and 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT reagent) from Roche Diagnostic GmbH (Germany).

4.2.2. Cell viability assay

Raw 264.7 cells were cultured in DMEM media supplemented with 10% heat-inactivated fetal-bovine serum (FBS) (Hyclone), 1% L-glutamine (Gibco) (v/v), 1% antibiotic–antimycotic mix (Sigma) (v/v) and 0.5% gentamycin (Sigma) (v/v). The cells were grown in 75-cm^2 tissue culture flasks (Sigma) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. After reaching 70–80% confluence, the cells were suspended and centrifuged. Then, 200 µl cell suspension/well plates were seeded in seven tissue

cultures treated 96-well microtiter plates (Nunc) at a density of 6×10^4 cells per well. The cells were incubated in 5% CO_2 at 37°C until 70–80% confluence was reached.

Three plates of cell cultures were exposed to different concentrations of D-glucose (5, 11.3, 17.5, 30 and 55 mM) and incubated for 48 hours in 5% CO_2 at 37°C. Another three plates were exposed to varying concentrations of D-glucose (5, 15, 25 and 45 mM) and incubated for 24 hours, after which LPS (0.2 µg/ml) was added and the plates incubated for a further 24 hours. After exposure for 48 hours, the supernatant was collected and stored at –80°C, until required for further analysis. The monolayer cell cultures were washed with phosphate-buffered saline (PBS) and used for the cytotoxicity assay, XTT.

4.2.2.2. Determination of nitric oxide production

After the 48-hour incubation of the RAW 264.7 cells at LG (5 mM), HG (25 mM), and LPL stimulation, the amount of nitrite produced by the cells was measured in the supernatant. NO production was assayed by quantifying the stable end product of NO oxidation, nitrite (NO^-_2) and reacted as per the Griess Reagent System protocol (Shultis 2018). The amount of NO produced was measured against a solution of nitrite standard at an initial concentration of 100 µM (Sigma-Aldrich). A 100 µl volume of the supernatant collected or nitrite standard was mixed with 100 µl of Griess reagent (1:1 of 1% sulphanilamide and 0.1% naphtylethlemidimine-dihydrochloride in 2.5% phosphoric acid) (Sigma-Aldrich). The plate was subsequently incubated at room temperature for 15 min. The absorbance was read at 540 nm on a microplate reader (Multiskan Ex, Thermo Electron Corporation), and the amount of NO produced by the RAW 264.7 cells was quantified.

4.2.2.3. Extraction of cell proteins and determination of protein concentration

Cells (6×10^5 in 4 ml medium) were seeded in 60-mm-diameter petri dishes (Greiner Bio-One, GmbH company). After the RAW 264.7 cells reached approximately 70–80% confluence, the medium was replaced with 5 mM LG and 25 mM HG DMEM media, and incubated for a further 24 hours. Supernatants were removed and stored at –80°C for cytokine proteome profile array assay. After removing the supernatants, cells were washed four times with Dulbecco's phosphate-buffered saline (DPBS) (Lonza).

The protein extraction solution was prepared by diluting protease inhibitor (PI) (Sigma-Aldrich). The protein extraction solution was added to all the petri dishes (500 µl/dish). The cell extracts were scraped, stored in Eppendorf tubes (Greiner Bio-One) and placed on ice before sonication. The cells in the Eppendorf tubes were then sonicated (QSonica, LLC. Misonix sonicators, XL-200 Series). The sonicated samples were centrifuged (Supermini centrifuge, MiniStar Plus) at relative centrifugal force 12100 for 1 min, after which the protein concentration was determined.

Samples and standards were diluted in buffer solution (PI, PBS/Tween) to determine the protein concentration to yield a final volume of 10 µl. An initial standard concentration of 1 000 µg/ml of human serum albumin was used to determine the protein concentration of unknown samples. Then, double dilutions were performed. The assay reagent was diluted from 1:5 with distilled water. One hundred microliters of Bradford reagent were added to each Eppendorf tube, and the absorbance read at 620 nm (Fluo-star Omega, BMG Labtech). The quantitative protein abundance based on normalised spectral counts was used to perform qualitative and statistical analysis for every single protein biomarker.

4.2.2.4. Determination of MIP-1α production

Mouse MIP-1α was determined using 96-well ELISA plates (Nunc-Immuno plates, Sigma-Aldrich, Denmark) and an ELISA kit (Affymetrix, ebioscience, USB). The detailed experimental procedures were performed according to the manufacturer's instructions. Absorbance was measured at 450 nm, and the concentration calculated by regression analysis of a standard curve. Experiments were conducted in triplicate.

4.2.2.5. Determination of the protein expression

A comprehensive cytokine proteome profile approach was used to identify changes in protein expression in the culture medium. With glucose concentrations of 5, 15, 25 and 45 mM, LPS-stimulated and unstimulated RAW 264.7 cells were induced. The human proteome profiler array kit (cytokine and chemokine) was used according to the manufacturer's protocol (R&D Systems, USA). In brief, membranes were blocked and incubated for 1 hour. Protein samples with antibodies (detection antibody cocktail) mixed were incubated with each array at 2–8°C overnight on a rocking platform shaker. The arrays were washed four times with washing buffer (1 part ELISA wash buffer to 9 parts deionized water). Arrays were incubated with the conjugated peroxidase (Streptavidin-HRP) for 30 minutes on the rocking platform shaker at room temperature, and subsequently washed four times with washing buffer. Substrate solution Ultra TMB (Thermo Scientific, USA) was then added to the arrays on the rocking platform shaker. The arrays were washed with distilled water to terminate the reaction when protein spots were visualised.

4.3. Statistics and protein array analysis

Using the mean, ± standard deviations (SD) values and P-value, one-way ANOVA was used to evaluate HG and LPS effects on RAW 264.7cell. Statistical significance was a probability value of $P < 0.01$. Analysis of proteins (duplicated array spots) by transparency and stamped identification supplied in the Array Kits.

4.4. Results

4.4.1. The effects of glucose concentration on RAW 264.7 cells

4.4.1.1. Cell viability

Results obtained in this study showed that glucose affects macrophage cell viability ≥ 55 mM significantly reduced ($P < 0.001$) (indicated by **), compared to 5 mM normal glucose, as shown in Figure 4.1. However, glucose concentrations of 30 mM had no effect on cell viability.

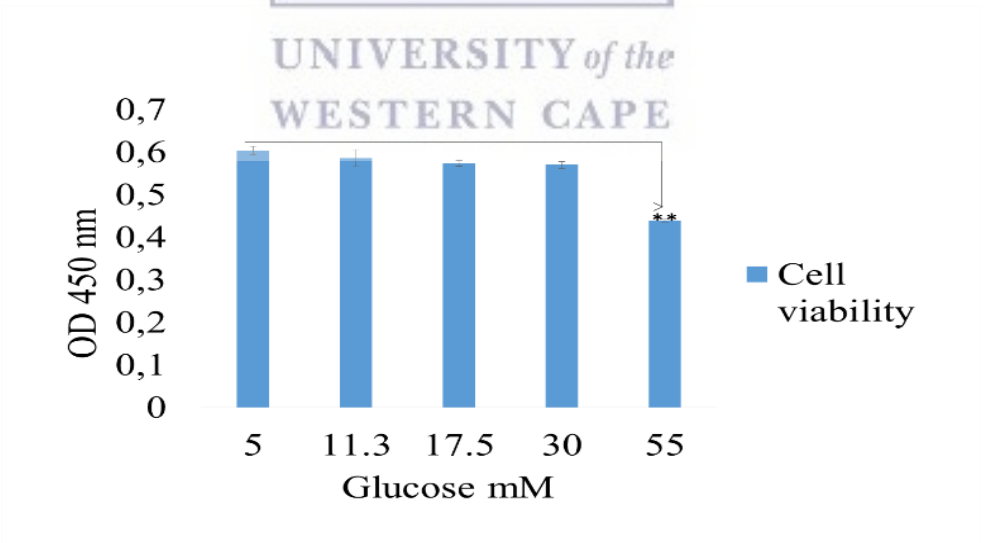


Figure 4.1. Effects of glucose concentration on the viability of RAW 264.7 cells at increasing glucose concentrations

4.4.1.2. The effects of glucose on nitric oxide production by RAW 264.7 macrophages

The NO production is affected by glucose, with a significant increase at 55 mM ($P < 0.001$). At 30 mM glucose concentrations, the cells show no NO response, as shown in Figure 4.2.

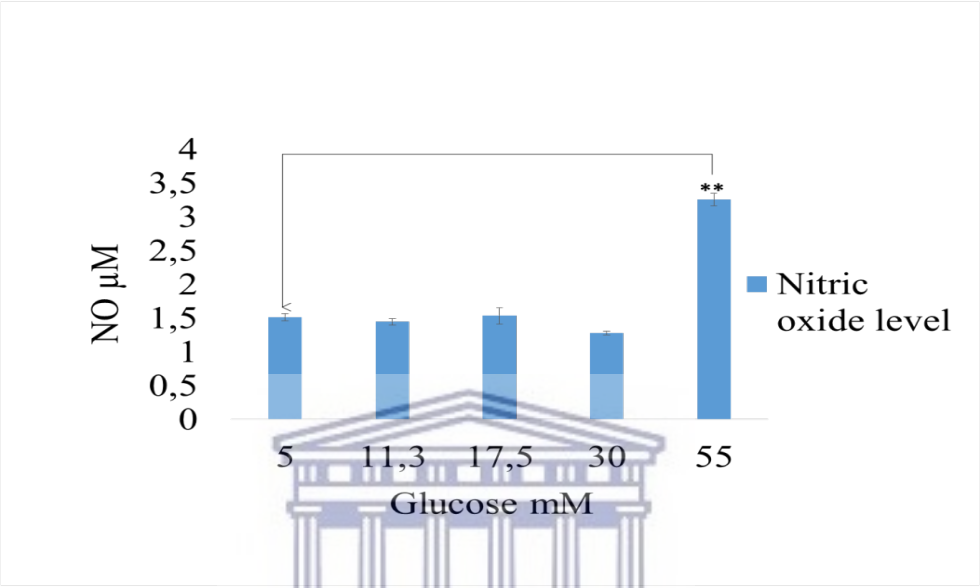


Figure 4.2. Nitric oxide levels of RAW 264.7

4.4.2. The effects of glucose and lipopolysaccharides on RAW 264.7 cells

4.4.2.1. Cell viability

Results obtained in this section of the study showed that glucose, with and without LPS, significantly decreases cell viability at 45 mM glucose concentration ($P < 0.001$) compared to 5 mM. See Figure 4.3. There were no effects of glucose with and without LPS at a glucose concentration of 25 mM.

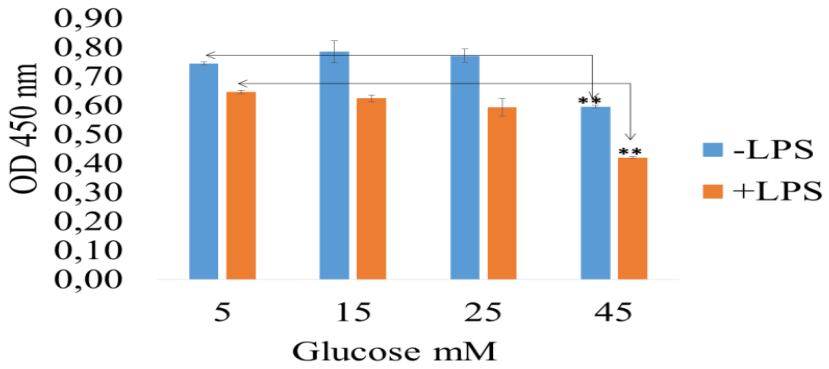


Figure 4.3. Effects of glucose with LPS on the viability of RAW 264.7 cells cultures exposed to different glucose concentrations

4.4.2.2. Inflammatory response of nitric oxide and macrophage inflammatory protein-1α production

At all glucose concentrations considered here, there was no change in NO and MIP-1α production without LPS stimulation. While the cell products, the higher level of NO and MIP1α with LPS stimulation in different glucose concentrations. See Figure 4.4 (i and ii, respectively).

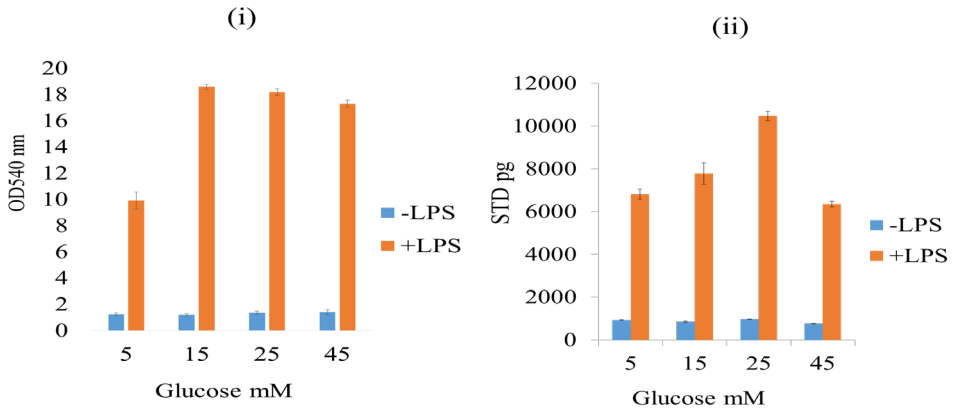
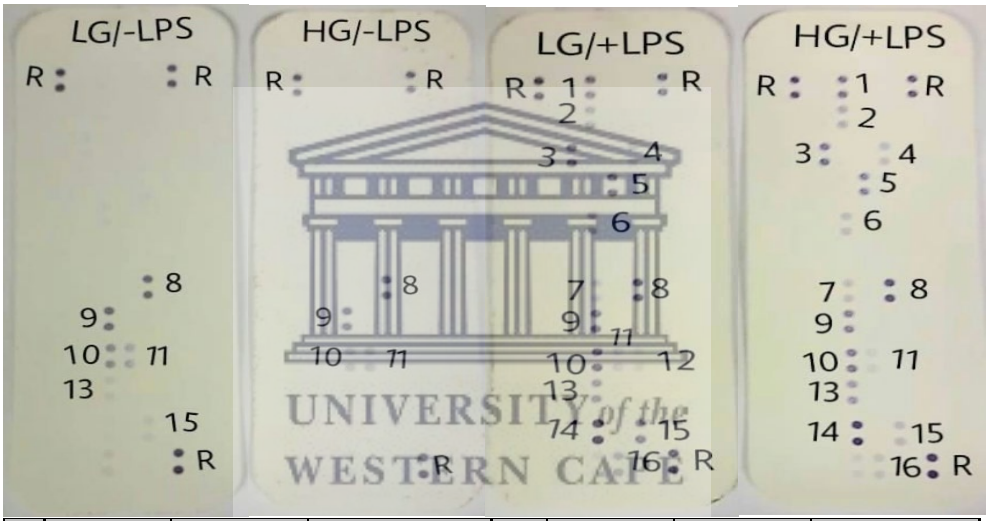


Figure 4.4. Effects of HG, with and without LPS on the inflammatory response of RAW 264.7 cells

4.4.3. Effects of HG on Raw 264.7 cell expression of pro-inflammatory cytokines

Conventional differential analysis was performed by pairwise comparisons between nitrocellulose membrane spots (LG and HG "unstimulated" vs LG and HG "stimulated"). The RAW 264.7 cell expression of 16 cytokine biomarkers, inducible protein-10 (IP-10), interferon-inducible T-cell alpha chemoattractant (I-TAC), TNF-α, G-CSF, IL-6, MCP-1, monokine induced by gamma (MIG), ICAM-1, MIP-1α, MIP-1β, interleukin-16 (IL-16), interleukin-1α (IL-1α), MIP-2, RANTES, IL-1ra and interleukin-27 (IL-27) are shown in Figure (4.5).



No	Markers	LG vs HG (-LPS)	LG vs HG (+LPS)	No	Markers	LG vs HG (-LPS)	LG vs HG (+LPS)
1	IP-10	-/-	+++/+++	9	MIP-1α	+++/+++	+++/+++
2	I-TAC	-/-	++/++	10	MIP-1ß	++/+	+++/+++
3	TNF-α	-/-	+++/+++	11	IL-16	++/++	++/+
4	G-CSF	-/-	++/++	12	IL-1α	-/-	+/-
5	IL-6	-/-	+++/+++	13	MIP-2	+/-	++/++
6	MCP-L	-/-	++/+	14	RANTES	-/-	+++/+++
7	MIG	-/-	+/+	15	IL-1ra	+/-	++/+
8	ICAM-1	+++/+++	++++/++++	16	IL-27	+/-	++/+

Figure 4.6. Nitrocellulose membrane spots showing the LG and HG effects on RAW 264.7 cell expression of cytokine biomarkers that were incubated in LG and HG for 24 hours and then with LPS for 24 hours.

Cell expression of seven cytokines in LG without LPS was added to the culture medium, and four cytokines in HG without LPS stimulation. Up-regulation levels of cell expression of 16 cytokines in LG in the presence of LPS stimulation were compared to 15 cytokines cell expression in HG with LPS stimulation. Differences in the protein expressions are shown in Table (4.1). In a variety of conditions, the expression of cytokines were either up-regulated or down-regulated.

Table 4.1. The RAW 264.7 cell expression of cytokines and chemokines in LG and HG in the absence or presence of LPS stimulation

No	Markers	LG vs HG (-LPS)*	LG vs HG (+LPS)*	Physiological Actions	Pathological Processes	References
1	**IP-10**	-/-	+++/+++	Regulation of immune responses and activation.	Promotes inflammation state.	*Reza Vazirineja et al., 2016*
2	**I-TAC**	-/-	++/++	Chemo-active for T cells	Inflammatory response.	*Kohli et al., 2021*
3	**TNF-α**	-/-	+++/+++	Pro-inflammatory cytokines.	Promoting IR in peripheral tissues.	*Castro et al., 2017.*
4	**G-CSF**	-/-	++/++	Promotes macrophage activation.	Pathogenesis of diabetic complications.	*Emmerson et al., 2012.*
5	**IL-6**	-/-	+++/+++	Regulation of metabolic, regenerative process.	Promoting inflammation and IR.	*Castro et al., 2017& Scheller et al., 2011.*
6	**MCP-1**	-/-	++/+	Activate immunity cells to the site of inflammation.	Induce adipose inflammation and IR	*Daniele et al., 2014.*
7	**MIG**	-/-	+/+	Activated immunity cells.	Induce Inflammatory by CXCR3-binding.	*Kohli et al., 2021*
8	**ICAM-1**	+++/+++	++++/++++	Activates leukocyte to adhesion to the endothelium.	Endothelial cell inflammatory and apoptosis.	*Jing et al. 2014*
9	**MIP-1α**	+++/+++	+++/+++	-Monocyte/ macrophage infiltration and activation.	Induced adipose inflammation.	*Ahmad et al., 2020*
10	**MIP-1ß**	++/+	+++/+++	Attracting leukocytes in inflammatory state.	High level induce inflammation and hepatocellular carcinoma.	Sadeghi *et al., 2015*
11	**IL-16**	++/++	++/+	Promotes migration of lymphocytes.	Role in the inflammatory process	*Schernthaner et al., 2017*
12	**IL-1α**	-/-	+/-	Anti-inflammatory cytokine.	Regulate the inflammatory factors.	*Di Paolo & Shayakhmetov et al., 2016*
13	**MIP-2**	+/-	++/++	Homoeostatic chemokine , tumour metastasis and tissue regeneration.	Induce Inflammatory response.	*Qin et al., 2017*
14	**RANTES**	-/-	+++/+++	Adherence and transmigration of monocytes.	Induce atherosclerosis, Dyslipidaemia and IR.	*Lin et al., 2018*
15	**IL-1ra**	+/-	++/+	Anti-inflammatory factors and inhibit by HG.	Up-regulation of the inflammatory response.	Lindblad et al., 2019
16	**IL-27**	+/-	++/+	- Anti-inflammatory factors. Inhibit T-helper cells differentiation.	-Up-regulation in inflammatory.	*Wang & Liu 2016*
* Spot density		++++ 		+++ 	++ 	+ 

4.5. Discussion

The inflammatory response is a pathway that has pathogenic effects in hyperglycaemia. Previous studies have shown that HG concentrations *in vitro* induced the expression of pro-inflammatory cytokines (Gonzalez et al., 2012; Kumar et al., 2014). Therefore, this study evaluated the cell viability and pro-inflammatory markers of macrophages in HG environments that mimic hyperglycaemia. It emerged that HG enhances the toxicity of the cells and stimulates inflammatory responses, leading to macrophage activation of NO production. The increased production of the free radical NO led to inhibited mitochondrial function (Zalewska et al., 2019). In this study, it was also revealed that higher glucose concentrations of (45 mM) decreased cell viability, in agreement with other reported findings that HG and LPS affect cell viability.

The RAW 264.7 cell is often used as a model of the physiological and pathological interaction process. These cells are highly differentiated and express a variety of protein markers (Jia et al., 2015). A number of diabetic complications are linked to HG, such as micro- and macrovascular disorders and damage to multiple organs (Kapoor & Kakkar, 2012). Thus, the purpose of this study was to determine which cytokine and chemokine biomarker(s) indicate the effect of HG regulation on macrophage cell protein production in the presence of LPS.

In this part of the study, differences in the expression of RAW 264.7 cell cytokines, ICAM-1, MIP-1α, MIP-1β, IL-16, MIP-2, IL-1ra and IL-27 in LG without LPS stimulation were found. While in HG without LPS stimulation, it expressed ICAM-1, MIP-1α, MIP-1β and IL-16, with the down-regulation level of MIP-1β in HG without LPS, compared to LG without LPS incubated for 48 hours.

The RAW 264.7 cell expressions of cytokine and chemokine in LG with LPS stimulation were IP-10, I-TAC, TNF-α, G-CSF, IL-6, MCP-1, MIG, ICAM-1, MIP-1α, MIP-1ß, IL-16, IL-1α, MIP-2, RANTES, IL-1ra and IL-27. While in HG with LPS stimulation, it expressed IP-10, I-TAC, TNF-α, G-CSF, IL-6, MCP-1, MIG, ICAM-1, MIP-1α, MIP-1ß, IL-16, MIP-2, RANTES, IL-1ra and IL-27, with the down-regulation level of MCP-1, IL-16, IL-1ra and IL-27, compared to LG with LPS incubated for 48 hours.

Pro-inflammatory cytokines of TNF-α and IL-6 inhibit insulin-mediated tyrosine phosphorylation of the IR_S and IRS-1 (Monroy et al., 2009; Daniele et al., 2014). Earlier studies suggest that HG down-regulates CD33 expression and enhances mitochondrial overproduction of the superoxide anion ($O2^-$), which promotes the secretion of TNF-α by peripheral monocytes and macrophages (Gonzalez et al., 2012).

These mechanisms transpire to induce the activation of PKC and NF-κB, which triggers the release of TNF-α, IL-6 and other pro-inflammatory factors (Nishikawa et al., 2000; Hua et al., 2012). In this study, TNF-α and IL-6 secretion in LG and HG with LPS-stimulation compared to LG and HG without LPS-stimulation of the macrophage cells (Figure 4.5 (3 and 5)). In a recent study, it was suggested that the activation of a pro-inflammatory response contributes to the increase of intracellular ROS because of chronic hyperglycaemia and not with HG alone in diabetes patients (Cantuaria et al., 2018). However, TNF-α and IL-6 production play a significant role in generating microvascular complications such as renal disease and chronic eye inflammation in diabetes patients (Devaraj et al., 2007; Elmarakby & Sullivan, 2012).

HG up-regulates endothelial ICAM-1 expression, and has a direct association with OS and inflammation state (Elmarakby & Sullivan, 2012). This current study showed

enhanced macrophage cell secretion of ICAM-1. Hence, up-regulation of ICAM-1 expression plays an essential role in inducing neuro-inflammation and the risk of inflammation in organs such as blood vessels, the heart, kidneys and retina in T2DM patients (Jing et al., 2014).

MIP-1α is an endotoxin-inducible chemokine that plays an essential role in the recruitment of macrophages (Noh et al., 2014). Previous studies have shown that MIP-1α proteins are elevated in the adipose tissue, causing systemic inflammation, and are positively associated with elevated insulin levels in the blood (Huber et al., 2008; Jiao et al., 2009; Sell & Eckel, 2009). MIP-1α may be a suitable target for modulating hyperglycaemia, adipose inflammation, OS-inflammation and IR (Noh et al., 2014).

In the present study, we showed that HG can increase in MIP-1α expression by the macrophage cells compared to the LG concentration level ($P < 0.01$) (Figure 4.4 (ii)). This result showed that the MIP-1α appears to release RAW 264.7 macrophage cells in qualitative evaluation with LG and HG with or without LPS stimulation (Figure. 4.5 (9)), suggesting that MIP-1α may be a suitable target for modulating obesity-induced adipose inflammation and IR (Noh et al., 2014).

MIP-1β is a chemokine macrophage inflammatory that enhances cell migration from the bloodstream into tissues through set lesions (Charo & Ransohoff, 2006). The present study has shown that HG without LPS stimulation inhibits the macrophage cell expression of MIP-1β, compared to LG without LPS stimulation (Figure 4.5 (10)). RAW 264.7 cells appear to up-regulate MIP-1β production in LG and HG with LPS stimulation compared to LG and HG without LPS stimulation (Figure 4.5 (10)). A recent study suggested that MIP-1β and its receptor, C-C chemokine receptor-5 play various roles in

the inflammatory actions underlying diabetes and cardiovascular diseases (Chang & Chen, 2016).

Effects of hyperglycaemia are a disturbance of anti-inflammatory cytokine levels, such as IL-1ra, IL-1α and IL-27, which play critical roles in the chronic inflammation in T2DM (Banerjee & Saxena, 2012). According to previous studies, IL-1ra regulation improves pancreatic B-cell function and prevents atherosclerosis vascular disease (Sauter et al., 2008; Banerjee & Saxena, 2012). IL-27 is a cytokine chiefly produced by antigen-presenting cells of macrophages and immunoregulatory functions (Aparicio-Siegmund & Garbers, 2015; Meka et al., 2015). This cytokine might have anti-inflammatory activity that down-regulates extreme immune responses (Ringkowski et al., 2016).

In this study, our results revealed that IL-1ra and IL-27 expression by RAW 264.7 cells in LG without LPS-stimulation compares to results with HG without LPS, while IL-1ra down-regulation of expression exposed to HG with LPS compares to results with LG with LPS-stimulation (Figure 4.5 (15 and 16), respectively). However, IL-1α stimulates expression in LG with LPS-stimulation (Figure 4.5 (12)). LPS stimulates the production of IL-1ra, IL-1α and IL-27 in LG compared to HG with LPS stimulation (Figure 4.5 (12, 15 and 16)).

Present results support the finding by Boni-Schnetzler and Meier (2019) that HG inhibits the IL-1ra production because of exposure of cells to HG, which reduces IL-1ra expression in T2DM patients. The current result finding also confirmed the down-regulation of inflammatory cytokine by IL-27 activation, indicating an early marker of potential therapeutic use in managing diabetic complications (Meka et al., 2015).

G-CSF is a haematopoietic growth factor required for the proliferation and differentiation of cells promoting angiogenesis and inhibiting apoptosis. G-CSF cells were identified as cell surface antigens, and a progenitor cell marker increased in non-diabetic individuals and impaired mobilisation in patients with diabetes (Golbidi et al., 2011). In the present study, we showed a low expression of G-CSF growth factor in LG and HG with LPS stimulation (Figure 4.5 (4)). The finding confirms that low expression of G-CSF cells in DM patients is associated with poor mobilisation in response to G-CSF (Ferraro et al., 2011). Treatment with G-CSF increases sympathetic activity in the bone marrow, leading to suppressed LPS-induced secretion of TNF-α and monocyte chemoattractant protein-5 genes (Méndez-Ferrer et al., 2008).

MCP-1 plays a role in recruiting macrophages to the site of tissue inflammation (Deshmane et al., 2009). Several studies suggest that suppression of the MCP-1 signalling pathway improved inflammatory responses and progression of IR (Wada & Makino, 2013; Daniele et al., 2014). Results of the present study show that HG stimulated by LPS induced MCP-1 expression, compared to LG and HG without LPS-stimulation. Results also show down-regulation of MCP-1 expression in HG stimulated by LPS compared to LG with LPS stimulation (Figure 4.5 (6)). In contrast, a recent study reported no difference in chemokine production in different glucose concentrations (Cantuaria et al., 2018).

MIP-2 cytokine activates neutrophils to adherent endothelial cells, triggering and preserving inflammation in obese diabetics (Rouault et al., 2013). Results of the present study show that MIP-2 is also increasingly secreted into LG and HG with an LPS stimulated medium by macrophage cells (Figure 4.5 (13)). It is suggested that the increased level of MIP-2 promotes inflammation in diabetes, which is a primary cause of

the interval in the healing of diabetic foot abscesses (Pettersson et al., 2011; DeClue & Shornick, 2015).

RANTES is a chemokine marker associated with obesity in adipose tissue and the liver. T-cells and macrophages are expressed, and secreted – expression negatively correlates with adiponectin. The secretion is correlated positively with T-cell and macrophage accumulation, a primary event in adipose tissue inflammation (Harford et al., 2011). Several studies suggested that elevated RANTES levels may be associated with metabolic syndrome and atherosclerosis (Veillard et al., 2004; Ueba et al., 2014). Up-regulation of RANTES is associated with increased systemic inflammation in obese subjects (Matter & Handschin, 2007). According to the present study, LG and HG stimulated by LPS up-regulated RANTES levels (Figure 4.5 (14)).

IP-10, MIG and I-TAC are chemokines that may modulate the inflammatory response (Hardison et al., 2006). These three chemokines have binding affinity in a unique receptor known as chemokine (C-X-C motif) receptor-3 (CXCR-3) in the recruitment of activated T cells to sites of infection (Rotondi et al., 2007). IP-10 acts on angiogenesis by activating CXCR3-A to a proliferation of various cell types. It also has anti-apoptosis activity by signalling the CXCR3-B receptor inhibiting the migration and proliferation of cells (Ahmadi et al., 2013). In particular, MIG is a chemokine-induced by IFN-γ and could provide a prolonged function and bio-activity in the IFN-γ signalling pathway (Berthoud et al., 2009). Neuropathy is a common disorder occurring in nearly 50% of patients with T2DM (Darivemula et al., 2019). I-TAC is a crucial effector for T-cell chemotaxis in the pathophysiology of neuro-inflammatory disorders (Guo et al., 2017).

In this study, we have shown unregulated IP-10, MIG and I-TAC chemokine expression in LG and HG with LPS-stimulation (Figure 4.5 (1, 2 and 7)), suggesting that the cytokines act as anti-inflammatory markers induced by inflammatory stimuli (Salmaggi et al., 2002).

The IL-16 cytokine plays a crucial role in the inflammatory process; it acts as a chemoattractant for peripheral immune cells to promote the migration of T-cell lymphocytes and induce the expression of pro-inflammatory proteins (Schernthaner et al., 2017). Increased production of IL-16 contributes to MIP-1α and β, and IP-10, which has been shown in the regulation of DM and modulation of neuro-inflammation (Skundric et al., 2015). The present study shows un-regulated IL-16 expression in LG and HG with LPS stimulation (Figure 4.5 (11)). In patients with diabetes, approximately 50% are affected by chronic heart failure; there is evidence that IL-16 is a mediator of myocardial fibrosis and apoptosis (Tamaki et al., 2013). Therefore, it is suggested that the regulation of IL-16 may represent a novel therapy for the prevention of myocardial fraction (Schernthaner et al., 2017).

4.6. Conclusion

Macrophages can produce pro- and anti-inflammatory cytokines, chemokines and growth factors in an HG environment with an induced-stimulating factor. Effects of HG are a disturbance of anti-inflammatory cytokine levels and enhancement of the toxicity of the cells and stimulation of inflammatory responses which play critical roles in the chronic inflammation in T2DM. These protein molecules play a critical role in the early onset of diabetes and the development of complications. This RAW 264.7 macrophage cell may also describe defects in the pathophysiology of T2DM and might offer an advantage in

drug screening. In the present study, the parameters (cytokines and chemokines) that could be used are presented for evaluating the effectiveness of herbal treatments for chronic inflammation associated with diabetes. Evaluation of herbal cytotoxicity was also performed. It may offer new approaches for early diagnosis and novel treatments for diabetes and its complications.

Chapter 5:

Evaluation of the anti-inflammatory effects of *Artemisia afra, Cinnamon verum* and *Trigonella foenum-graecum* in high glucose-induced macrophage cells

Abstract

Some traditional African therapies (TAT) have shown much promise because of their safety, low cost and general acceptability of the therapies compared to standard Western medicine. The purpose of this part of the study was to examine the anti-inflammatory properties of *A. afra, C. verum* and *T. foenum-graecum*, commonly used in African countries for treating diabetes. The results were compared with those of pharmaceutical-grade metformin. The present study revealed that *A. afra, C. verum* and *T. foenum-graecum* extracts significantly inhibited the expression of several mediators of inflammation by the cell, including MIP-1β, TNF-α, IL6, G-CSF, MCP-1, RANTES and NO. Metformin had no effect on MIP-1β, RANTES or NO levels. Results indicated that different herbs inhibited inflammatory mediators at different concentrations. *A. afra* and metformin had the lowest cytotoxicity compared to *C. verum* and *T. foenum-graecum*. Furthermore, *A. afra* was found to exhibit a higher anti-inflammatory effect than the other herbs tested. These findings suggest that *A. afra* plant extracts may be used to regulate low-grade/chronic inflammation related to diabetes and prevent its complications.

5.1. Introduction

T2DM is a common pathological disorder associated with metabolic syndrome – carbohydrate, lipid, and protein metabolism changes are often related to obesity, chronic

inflammation, and OS (de Carvalho et al., 2012; Matsuda & Shimomura, 2013). Alterations in hyperglycaemia and hyperlipidaemia are linked to metabolic disorders, such as elevated FFA and OS (Galicia-Garcia et al., 2020). Chronic inflammation and lipid accumulation in skeletal muscle cells also contribute to OS activation, impairing insulin signalling pathways (Liu et al., 2014; Rani et al., 2016). The effects of hyperglycaemia on the body's OS have been studied, enhancing the pro-inflammatory modulator responses leading to IR and resulting in diabetic complications (Xiu et al., 2014). Several studies have shown that inflammation plays an essential role in the development of diabetes and the progression of complications associated with this disease (Wada & Makino, 2013; Meshkani & Sanaz, 2016).

Metformin (biguanides) is generally used to treat T2DM in males with more positive results than in females. Higher hospitalisation and mortality rates caused by cardiovascular disease were found in females than males using metformin (Arnetz et al., 2014). Therapies that reduce and control glucose levels are ineffective in preventing the development of diabetes complications (Kahn et al., 2014). In a prospective diabetes study, Nathan et al. (2009) evaluated three glucose-lowering drugs (sulfonylurea, metformin and insulin) but could not establish which drug was superior regarding diabetes complications.

Many antihyperglycaemic drugs (biguanides, meglitinides, SFU, AGIs, TZDs and DPP-4 inhibitors) are associated with small decreases in inflammatory markers (Oguntibeju 2019). The use of herbal medicines in the treatment of diabetes is safe, inexpensive, widely available, and acceptable compared with standard Western medicines (Pandey et al., 2011; Ilhan et al., 2016). Recently, several traditional therapies have been formally introduced in medical treatments worldwide. The efficacy of other traditional medicines

to treat diabetes is currently being evaluated scientifically and medically (Mustafa et al., 2017; Chege et al., 2015). Earlier studies suggest that *A. afra*, *C. verum* and *T. foenum-graecum* can be used for treating diabetes (Ku & Lin, 2013; Sunmonu & Afolayan, 2013).

The *A. afra* plant's roots, leaves and stems have a variety of therapeutic uses (Ntutela et al., 2009). Yet it is still necessary to conduct *in vitro* research to clarify specific biochemical pathways in *A. afra* to prevent diabetic complications. In diabetic rats, *A. afra* has been shown to significantly lower glucose levels and enable the regrowth of pancreatic β-cells that have been inhibited by OS (Afolayan & Sunmonu, 2011; Sunmonu & Afolayan, 2013). *C. verum* is reported to have pharmacological activities with anti-inflammatory, antioxidant and anti-diabetic potential (Mazimba et al., 2015). The herb *T. foenum-graecum* also has antioxidant, anti-inflammatory, and anti-diabetic properties (Rajan & Dharman, 2014; Goyal et al., 2016).

Macrophage cells contribute to diabetes complications through immune cells interacting with inflammatory mediators to cause cardiovascular and neuropathy, nephropathy, and retinopathy diseases, as suggested in some studies (Meshkani & Sanaz, 2016). Therefore, activated macrophages are considered a potential therapeutic target for treating chronic complications associated with DM (Pop-Busui et al., 2016).

The aim was to determine the *in vitro* effectiveness and safety of plant remedies commonly used in African countries to regulate the pro-inflammatory markers associated with chronically high glucose levels in mimic-diabetic cells. A macrophage cell line, RAW 264.7, was exposed to HG levels, followed by exposure to *A. afra*, *C. verum* and *T. foenum-graecum*. The results obtained were compared with those for the antidiabetic activity of the commonly used Western pharmaceutical, metformin. This

study may offer a new strategy for using traditional inhibitor agents to synthesise pro-inflammatory mediators as potential novel treatments for diabetes and its complications.

5.2. Materials and Methods

5.2.1. Preparation of plant extracts

In stock extracts of *A. afra* leaves (20 g/100 ml), *C. verum* bark (16.6 g/100 ml) and *T. foenum-graecum* seeds (16.6 g/100 ml) were prepared in ethanol solutions 70%. Plants were obtained from a local manufacturer (Health Connection Wholefoods (Pty) Ltd, Diep River, Cape Town). The plant extracts were prepared using approximately 1 g plant mass per 5 ml ethanol at 45°C for 24 hours (mixing the extraction material and solvent every 6 hours). After centrifuging for 10 min at 4000 rpm, the extract was filter-sterilised using a 0.2 µM sterile syringe filter. Extracts were stored at 4°C, until required for use.

5.2.2. Cell cultures

Cells (RAW 264.7) were cultured in DMEM. DMEM was supplemented with 10% heat-inactivated FBS (HyClone) (v/v), 1% L-glutamine (v/v) (Gibco), 1% antibiotic-antimycotic mix (v/v) (Danvers, MA, USA) and 0.5% gentamycin (v/v) (Danvers, MA, USA). Cells were cultured in 75 cm^2 flasks (Sigma-Aldrich, Germany) at 37°C in a humidified atmosphere containing 5% carbon dioxide. Upon reaching 70–80% confluence, the cells were suspended in the medium and centrifuged (10 minutes at 2000 rpm). RAW 264.7 cells were seeded in four treated 96-well microtiter plates (Nunc) at 6×10^5 cells per well, and the cells were grown in a humidified atmosphere of 5% CO_2 at 37°C for approximately 48 hours until they reached 80–90% confluence.

The cells were then pre-exposed to low (5 mM) and high D-glucose (25 mM) concentrations (LG and HG) (v/v), which were purchased from Sigma-Aldrich and incubated for 24 hours. The cell plate medium was replaced with a fresh exposure medium containing LG and HG plant extracts. Varying concentrations of *A. afra, C. verum* and metformin solution (Sigma-Aldrich) (0–1000 µg/ml) and exposure medium were supplemented with *T. foenum-graecum* (0–5000 g/ml). The cells were left unstimulated or stimulated with LPS (0.2 µg/ml) (v/v) that was purchased from Sigma-Aldrich to induce inflammation. The plates were incubated for 24 or 48 hours under standard tissue culture conditions. Then, 100 µl of culture supernatant was collected from each culture well after 24 hours and used for NO oxide and cytokine biomarker determination.

5.2.3. Cytotoxicity assay

After exposure for 48 hours and removal of the cell culture supernatants, the monolayer cell cultures were washed with PBS. Cytotoxicity was measured by adding 50 µl of a 1/10 dilution of XTT reagent to each well according to the manufacturer's instructions (Roche Diagnostic GmbH, Germany). The absorbance of each well was measured immediately after XTT addition at a wavelength of 450 nm using a Multiskan Ex microplate reader (Thermo, Electron Corporation). Subsequently, the plate was incubated at 37°C, and readings were taken every 10 min. After exposure for 48 hours and removal of the cell culture supernatants, the monolayer cell cultures were washed with PBS. To determine mitochondrial activity and viability, formazan formation at 450 nm was measured. The procedure was performed in triplicate.

5.2.4. Nitric oxide production measurement

After 48 hours incubation of the RAW 264.7 cells in LG (5 mM) and HG (25 mM) containing LPL-stimulation, supernatants were measured for nitrite production. Quantification of nitrite (NO^-_2), the stable end product of NO oxidation, was used to assess NO production. Using the Griess assay, we determined NO^-_2 levels (Shultis, 2018). The amount of NO produced was measured against a standard curve prepared by a 2-fold dilution range of 100 µM nitrite standard (Sigma-Aldrich). One hundred microliters of the supernatant collected or nitrite standard were mixed with 100 µl of Griess reagent (1:1 of 1% sulfanilamide and 0.1% naphtylethlemidimine-dihydrochloride in 2.5% phosphoric acid) (Sigma-Aldrich). After 15 min incubation at room temperature, the plate was examined. After 15 min incubation at room temperature, the plate was examined and the absorbance in each well measured at 540 nm on a a microplate reader (Multiskan Ex, Thermo Electron Corporation). The NO production by the RAW 264.7 cells was quantified by determining the amount of NO produced.

5.2.5. Determination of pro-inflammatory markers in culture supernatant

Pro-inflammatory biomarkers were assayed using commercially available ELISA kits. Mouse ELISA kits were purchased from R&D SYSTEMS, Inc. (Minneapolis, MN, USA) and eBioscience, Inc. (San Diego, CA, USA). In the supernatant of RAW 264.7 cells stored at –80°C, cytokine and chemokine concentrations were determined with ELISA kits. The following biomarkers were quantified, MIP-1β (cat. No, DY451); TNF-α (cat. No, 410); IL-6 (cat. 88-7064-88); G-CSF (cat. No, DY414); RANTES (cat. No, DY478) and MCP-1 (cat. No, DY479), R&D Systems, Bio-Techne Brands, (Minneapolis, Minnesota).

Culture supernatants were diluted with sample diluent for each specific biomarker quantitation as follows, MIP-1β production in the absence of LPS (1/10 v/v); production of MIP-1β in the presence of LPS (1/500 v/v); TNF-α production in the presence of LPS (1/200 v/v); IL-6 production in the presence of LPS (1/200 v/v); and G-CSF, RANTES and MCP-1 production in the presence of LPS (1/500 v/v). The detailed experimental procedures were carried out according to the manufacturer's instructions (manufacturer's reference mentioned above). Microplate readers (Multiskan Ex, Thermo Electron Corporation) were used to measure absorbance at 540 nm. The concentration of the biomarkers was calculated by regression analysis of a standard curve. A triplicate experiment was performed.

5.2.6. Statistical analysis

Data were analysed using averages and ± SDs. The graph data and concentration parameters for each treatment were compared with the controls (no treatment) for each experiment. SigmaPlot 12.0 was used to assess significant differences using a one-way analysis of variance (ANOVA). Significant differences compared to the controls were considered for samples with a $P < 0.01$.

5.3. Results

In this study, we compared the effects of three TAT with standard Western medicine on RAW 264.7 cell cytokine secretion profiles exposed to HG and evaluated the inflammatory response to TAT and possible cytotoxicity. The results showed that HG treatment of mouse macrophage cells stimulated with LPS markedly induced the expression of the protein markers in cell culture supernatants.

We found high levels of pro-inflammatory cytokines, including MIP-1β, TNF-α, IL6, G-CSF, MCP-1 and RANTES, in the supernatants of cultures treated with HG and LPS in this study. We also found that high glucose levels without LPS resulted in no elevated RAW 264.7 cell secretion of the inflammatory cytokines/chemokines TNF-α, IL-6, G-CSF, MCP-1 and RANTES, while low levels of MIP-1β secretion were observed (data not shown).

5.3.1. Effects of *A. afra* extracts on cell viability

5.3.1.1. Cytotoxicity assay of A. afra extract

The XTT assay was used to assess the toxicity of the *A. afra* extract on RAW 264.7 cells. Cells of mouse macrophages were exposed to HG for 24 hours, and then treated with increasing concentrations of *A. afra* extract (up to 1000 µg/ml) for 24 hours. The viability of cells was determined by cell proliferation assays, under different conditions, such as LG vs HG and in the presence or absence of LPS stimulation.

Cell proliferation was observed in LG and HG media in the presence of LPS (Figure 5.1 (i)). A reduction in cell viability was also observed at *A. afra* concentrations > 125 µg/ml compared to the control at 0 µg/ml (Figure 5.1 (i)). The viability of RAW 264.7 cells declined significantly at an *A. afra* concentration of ≥ 250 µg/ml ($P < 0.001$). Thus, the cell viability in LG vs HG, with and without LPS stimulation, is clearly dependent on the concentration of *A. afra* (Figure 5.1 (ii, iii, iv and v)). The results were compared with those of an untreated control for statistical analysis. In Table 5.1, we present half-maximal IC_{50} of *A. afra* extract for the various biomarkers.

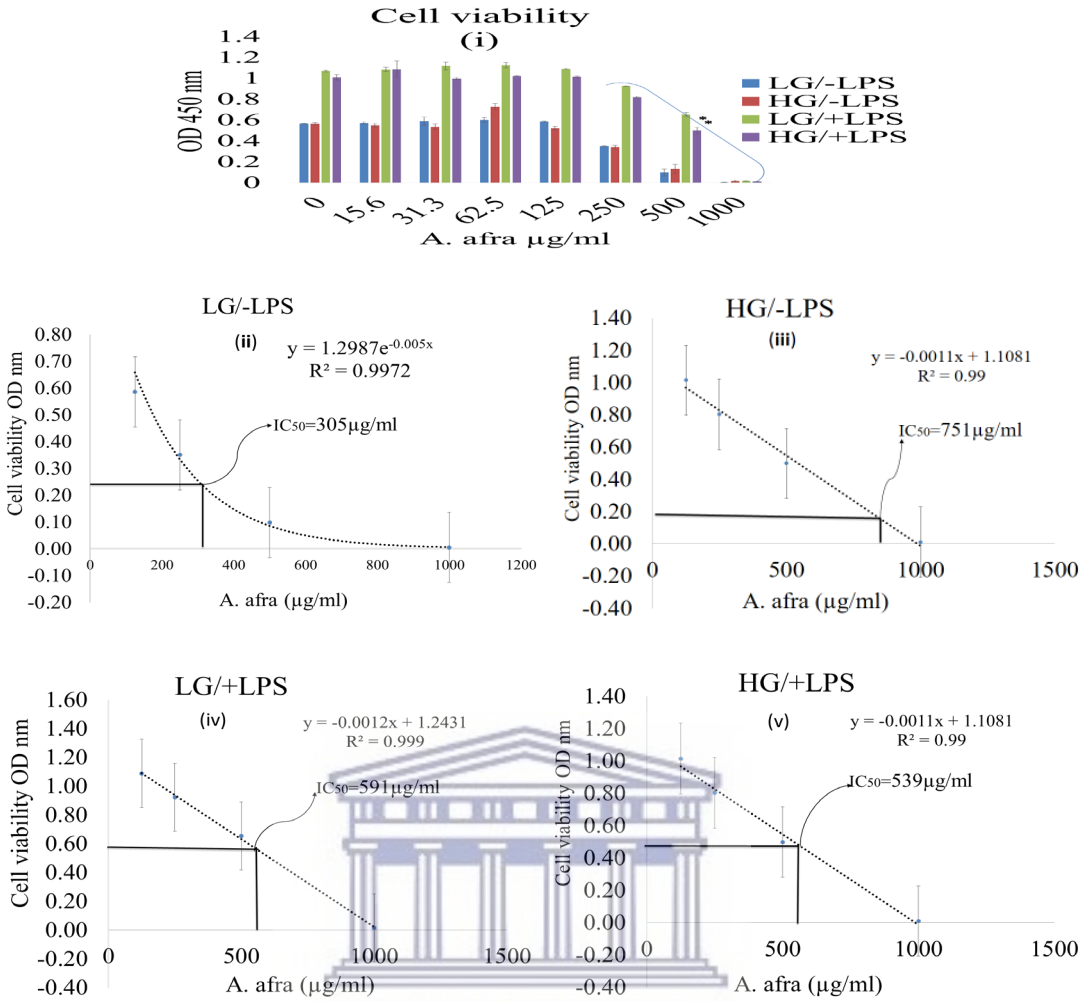


Figure 5.1. Cell viability of RAW 264.7 cells treated with various concentrations of *A. afra* under LG and HG with and without LPS-stimulation (i). Bars marked with ** indicate a statistically significant difference ($P < 0.001$) compared to 0 µg/ml *A. afra* extract. The IC_{50} curves for RAW 264.6 cells under various incubation conditions are indicated in legends (ii, iii, iv and v).

Table 5.1. IC_{50} of the *A. afra* extract for the secretion of various inflammatory and pro-inflammatory biomarkers by RAW 264.7 cells

Markers		MIP1β	TNF α	IL6	G-CSF	MCP-1	RANTES	NO	Cell viability
A. afra IC_{50} µg/ml	LG/-LPS	75							305
	HG/-LPS	56							751
	LG/+LPS	147	242	76	187	331	83	88	591
	HG/+LPS	166	207	50	207	540	59	75	539

5.3.1.2. Effects of A. afra on nitric oxide secretion

The Griess assay was used to determine NO in the supernatant of RAW 264.7 cell culture supernatants after treatment with various *A. afra* concentrations under LG and HG conditions in the presence of LPS. Results showed that NO secretion was significantly inhibited ($P < 0.001$) by increasing the concentration of *A. afra* (Figure 5.2 (i)). The IC_{50} of *A. afra* on NO production under LG with LPS was 88 µg/ml, while under HG with LPS conditions, it was 75 µg/ml. *A. afra* inhibition of NO was concentration-dependent (Figure 5.2 (ii and iii)). For statistical analyses, the results were compared with the untreated control.

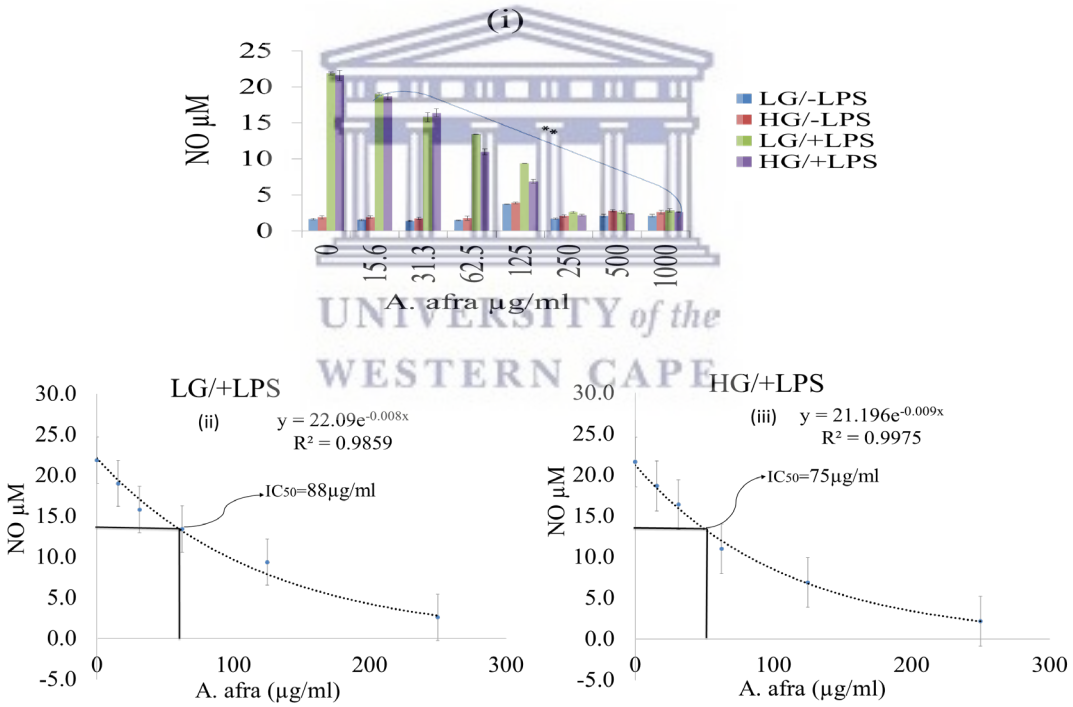


Figure 5.2. Effects of *A. afra* extract on NO production in LG and HG with and without LPS-stimulated RAW 264.7 cells (i). Data represent the mean ± SEM. Statistically significant difference** ($P < 0.001$) compared to the 0 µg/ml *A. afra* concentration. IC_{50} determination of *A. afra* extract inhibition on the RAW 264.7 cell production of NO in LG and HG with LPS-stimulated cells is compared to the 0 µg/ml *A. afra* concentration (ii and iii).

5.3.1.3. Effects of A. afra on cytokine and chemokine secretions

5.3.1.3.1. Effects of A. afra on MIP-1β expression levels

RAW 264.7 cells produce low levels of MIP-1β in the presence of LG and HG when no LPS is added to the culture medium. However, when the cells were cultured with LG and HG in the presence of LPS, much higher concentrations of MIP-1β were secreted into the culture medium (Figure 5.3 (i and ii)). *A. afra* extract at concentrations ≥ 31.3 µg/ml significantly inhibited MIP-1β secretion ($p < 0.001$ and $P < 0.01$) into the culture supernatant in a concentration-dependent manner in LG and HG media without and in the presence of LPS-stimulation (Figure 5.3 (i and ii)).

The IC_{50} values for *A. afra* inhibition of MIP-1β secretion in LG and HG with and without LPS-stimulation are shown in (Figure 5.3 (iii, iv, v and vi)).

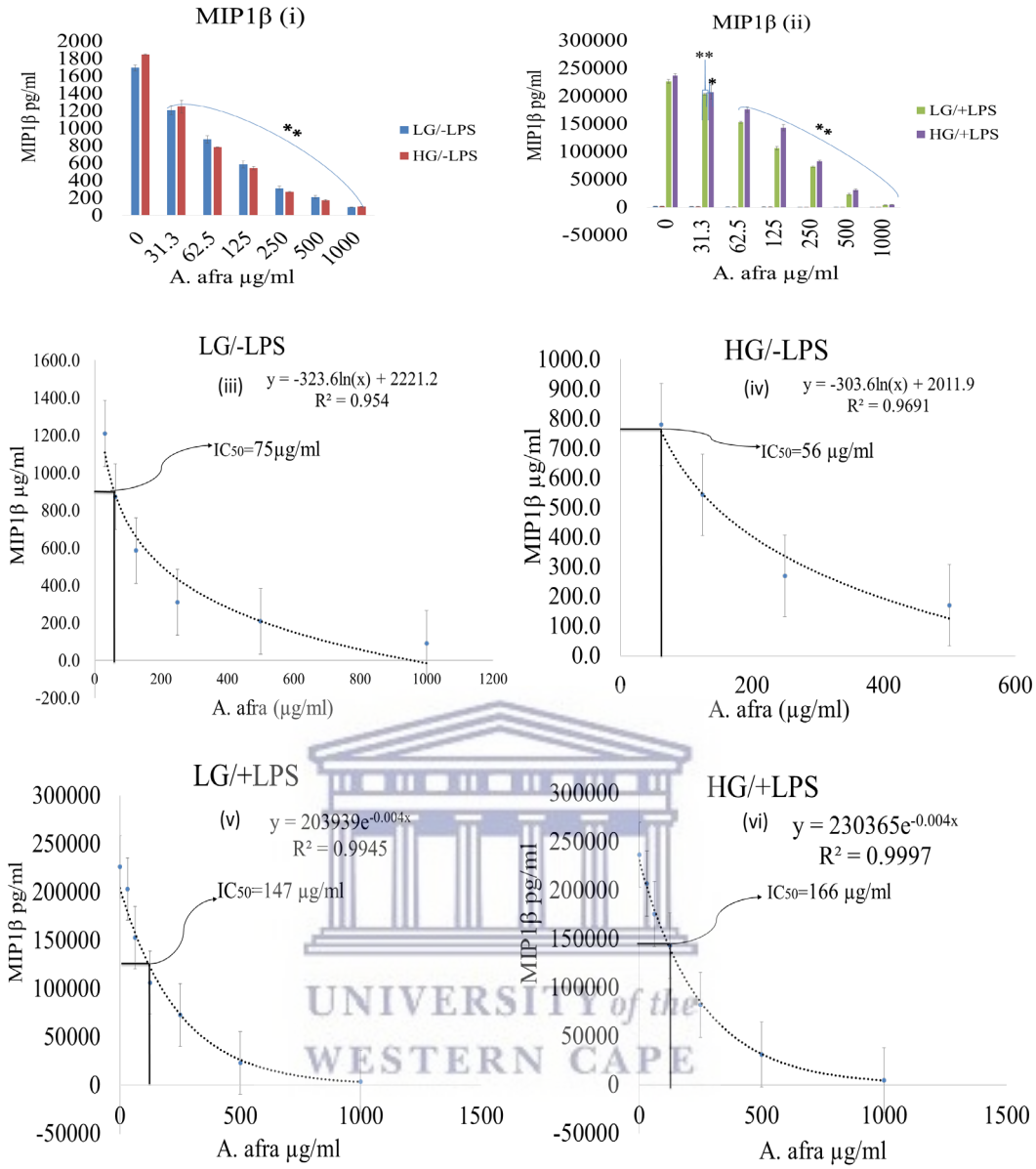


Figure 5.3. Effect of *A. afra* extract on MIP-1β secretion by RAW 264.7 cells cultured in LG and HG media in the absence (i) or presence ii) of LPS. IC_{50} determination of *A. afra* inhibition of MIP-1β in LG and HG with and without LPS-stimulated cells are shown in (iii, iv, v and vi).

5.3.1.3.2. Effects of A. afra on TNF-α and IL6 expression levels

Results further showed that *A. afra* extracts significantly decreased TNF-α levels at 62.5 µg/ml (statistical significance,* $P < 0.01$) and at ≥ 125 µg/ml (** $P < 0.001$) in LG and HG media under LPS-stimulated conditions (Figure 5.4 (i)). *A. afra* at 13.3 µg/ml

significantly decreased IL6 secretion by RAW 264.7 cells when incubated in LG with LPS ($P < 0.01$) and HG with LPS ($P < 0.001$). *A. afra* at ≥ 62.5 µg/ml significantly decreased IL6 secretion ($P < 0.001$) in LG and HG media supplemented with LPS (Figure 5.4 (ii)). The IC_{50} concentrations of the *A. afra* extracts were calculated for TNF-α and IL6, as shown in (Figure 5.4 (iii, iv, v and vi)).

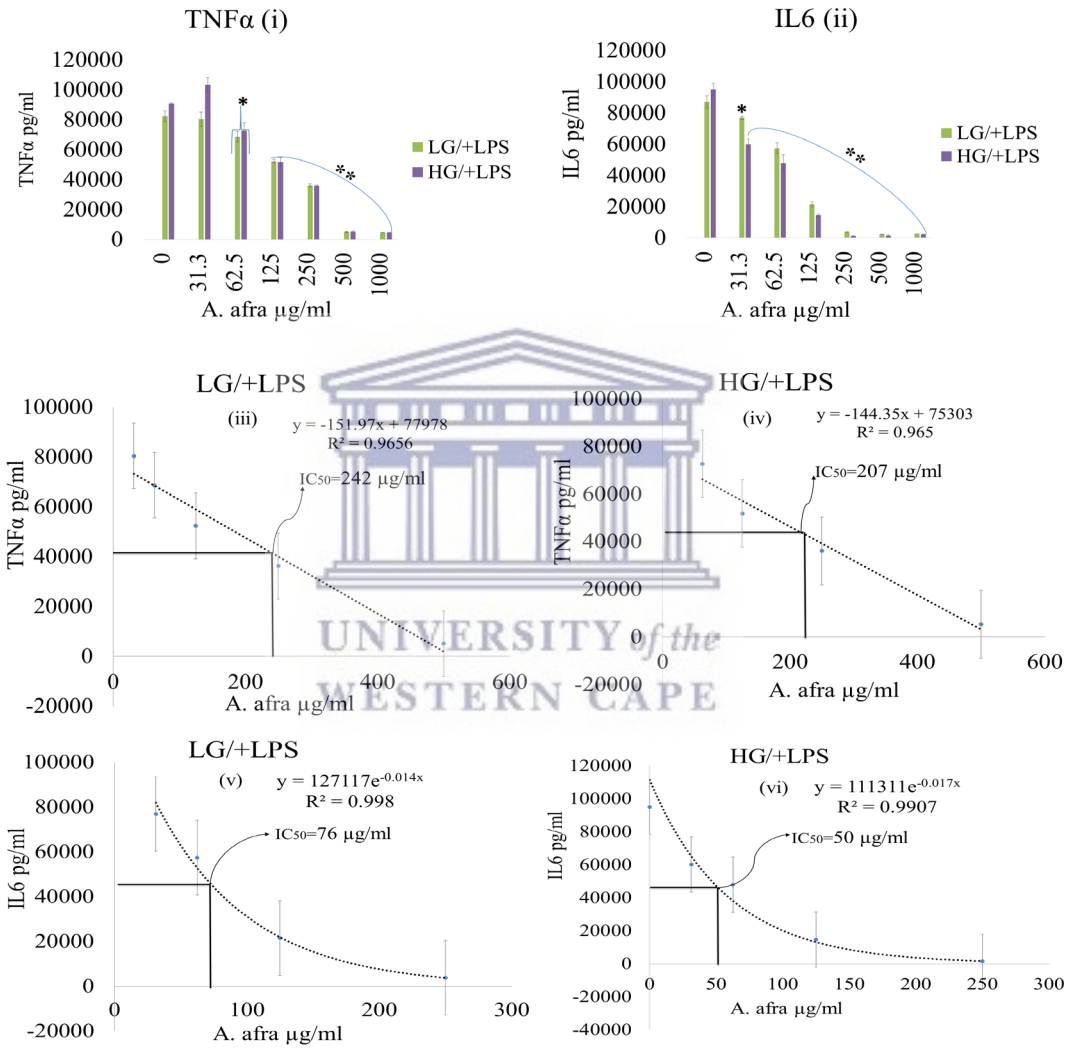


Figure 5.4. Effects of the *A. afra* extract on TNF-α and IL-6 secretion by RAW 264.7 cells incubated with LG and HG media containing LPS-stimulation (i and ii). Statistically significant difference levels are indicated as * ($P < 0.01$) and ** ($P < 0.001$). The data generated were also used to calculate the IC_{50} for *A. afra* extract on TNF-α and IL-6 synthesis (iii, iv, v and vi).

5.3.1.3.3. Effects of A. afra on G-CSF and MCP-1 expression levels

Exposure to *A. afra* significantly decreased ($P < 0.001$) the synthesis of G-CSF at a concentration of 13.3 µg/ml in LG medium containing LPS and by $P < 0.01$ in HG medium containing LPS-stimulation. At ≥ 62.5 µg/ml, *A. afra* significantly decreased G-CSF ($P < 0.001$) in LG and HG media containing LPS-stimulation (Figure 5.5 (i)). MCP-1 production was significantly reduced ($P < 0.001$) by *A. afra* in a concentration-dependent manner in LG and HG media containing LPS-stimulation (Figure 5.5 (ii)). The IC_{50} values for *A. afra* inhibition of G-CSF and MCP-1 expression are presented in (Figure 5.5 (iii, iv, v and vi)).

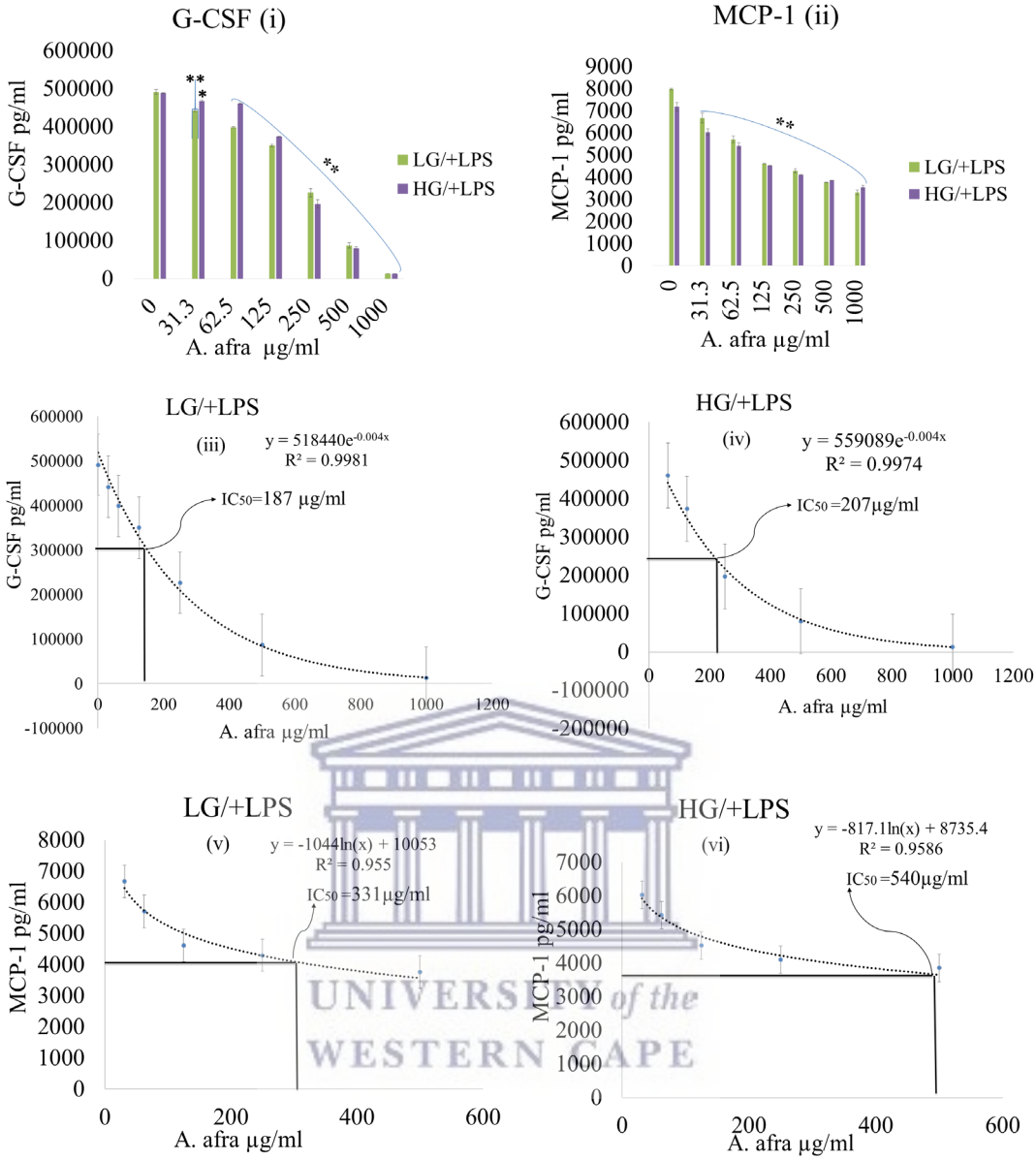


Figure 5.5. Effect of the *A. afra* extract on G-CSF secretion in LG and HG media containing LPS-stimulation (i). Effects of the *A. afra* extract on MCP-1 secretion in LG and HG media containing LPS-stimulation (ii). The IC_{50} for *A. afra* extract inhibition of G-CSF and MCP-1 in LG and HG media containing LPS-stimulation (iii, iv, v and vi).

5.3.1.3.4. Effects of A. afra on RANTES expression levels

Exposure of LPS-stimulated RAW 264.7 cells to *A. afra* significantly decreased ($P < 0.001$) RANTES secretion levels at ≥ 31.25 µg/ml. RANTES secretion was inversely proportional to the *A. afra* concentration (Figure 5.6 (i)). The IC_{50} of *A. afra* for RANTES

secretion was 83 µg/ml in LG medium containing LPS and 59 µg/ml in HG medium containing LPS-stimulation (Figure 5.6 (ii and iii)).

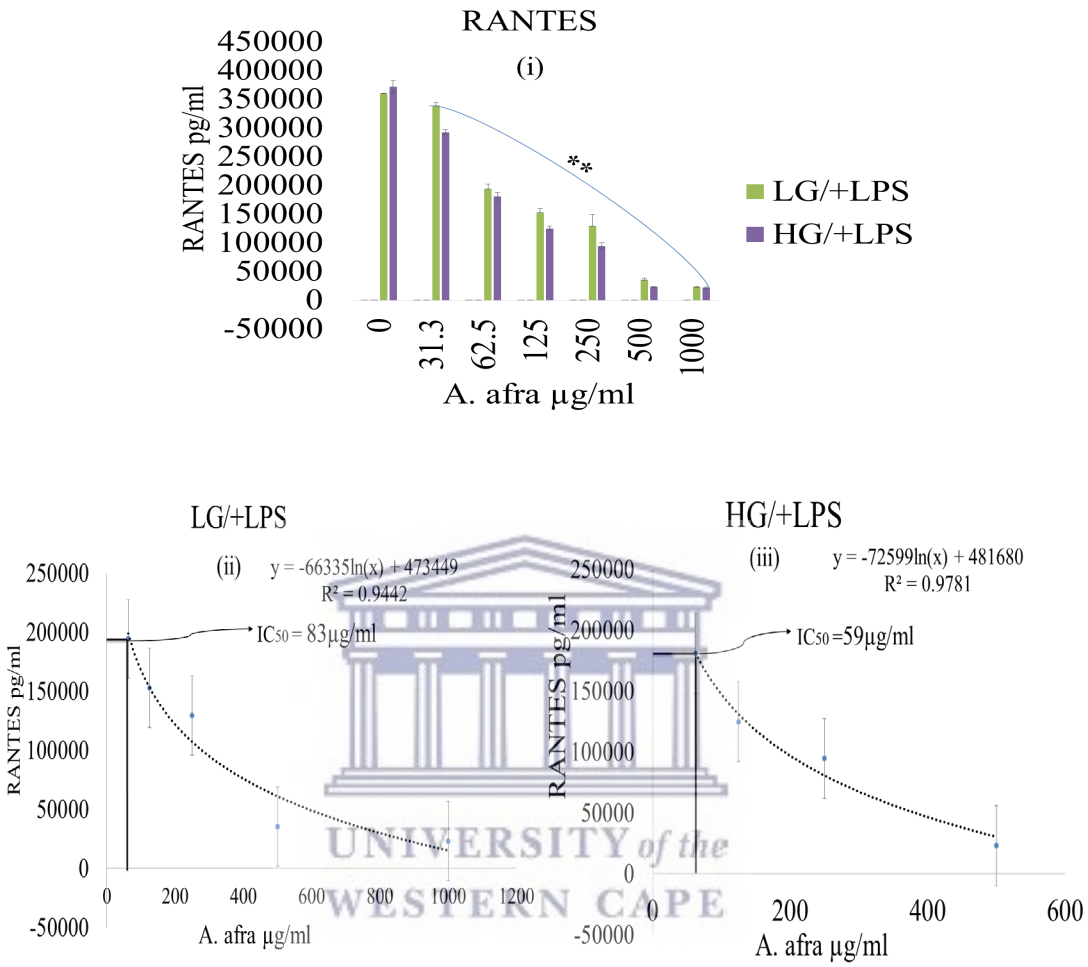


Figure 5.6. Effect of the *A, afra* extract on RANTES secretion in LG and HG media containing LPS compared to the control 0 µg/ml (i). IC_{50} determination of *A. afra* inhibition of RANTES in LG vs HG medium stimulated with LPS (ii and iii).

5.3.2. Effects of *C. verum* extracts on RAW 264.7 macrophages

5.3.2.1. Cytotoxicity assay of C. verum extract

The XTT assay was used to assess the cytotoxicity of *C. verum* extract on RAW 264.7 cells. Mouse macrophage cells were exposed to HG for 24 hours, and then treated with

increasing concentrations of *C. verum* extract (up to 1000 µg/dL) for 24 hours. The XTT cell proliferation assay was used to determine the cell viability after incubation with various concentrations of *C. verum* in LG and HG media with and without LPS-stimulation. *C. verum* decreased cell viability at concentrations > 250 µg/ml, compared to the control at 0 µg/ml. The effects of *C. verum* were only observed at concentrations ≥ 500 µg/ml, and cell viability was significantly reduced ($P < 0.001$) (Figure 5.7 (i)). The IC_{50} values of the *C. verum* extract for the various biomarkers analysed are summarised in Table 5.2.

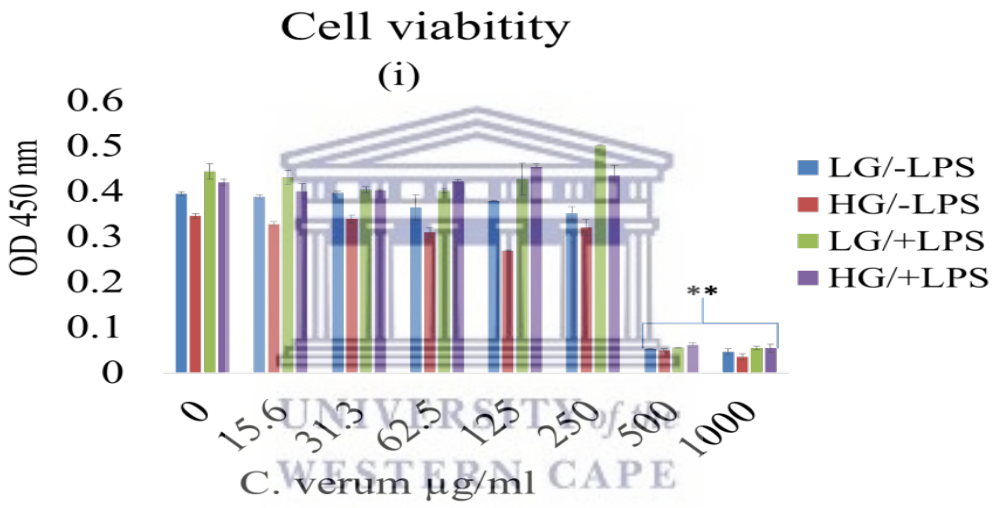


Figure 5.7. Cell viability of RAW 264.7 cells treated with *C. verum*. Statistically significant difference** ($P < 0.001$) is shown at the higher concentrations compared to 0 µg/ml *C. verum*.

Table 5.2. IC_{50} of the *C. verum* extract for the secretion of various inflammatory biomarkers by RAW 264.7 cells

Markers		MIP1β	TNF α	IL6	MCP-1	RANTES
C. verum IC_{50} µg/ml	LG/-LPS	293				
	HG/-LPS	293				
	LG/+LPS	321	183	60	285	274
	HG/+LPS	327	172	54	312	287

5.3.2.2. Effects of C. verum on nitric oxide secretion

C. verum concentrations showed a low inhibition of NO in HG and LG media-containing LPS (Figure 5.8 (i)). The *C. verum* effects reduced the NO secretion-dependent concentration and at higher concentrations because of a decrease in cell proliferation. All data were compared to the results obtained from cultures incubated in the absence of *C. verum* (Figure 5.8 (i)).

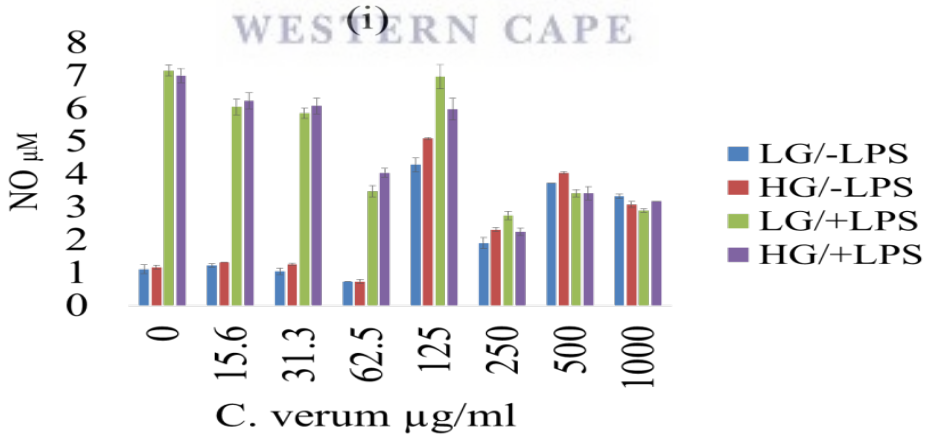


Figure 5.8. Effects of the *C. verum* extract on NO production in LG vs HG with and without LPS-stimulated RAW 264.7 cells compared to 0 µg/ml of *C. verum* concentration.

5.3.2.3. Effects of C. verum on cytokine and chemokine secretions

5.3.2.3.1. Effects of C. verum on MIP-1β expression levels

C. verum extract significantly decreased MIP-1β secretion ($P < 0.01$) at 31.3 µg/ml and ($P < 0.001$) at concentrations ≥ 62.5 µg/ml in LG medium without LPS. In HG medium without LPS, the *C. verum* extract significantly decreased MIP-1β secretion ($P < 0.01$) at 62.5 µg/ml and ≥ 125 µg/ml ($P < 0.001$) (Figure 5.9 (i)). *C. verum* extracts at ≥ 125 µg/dl in LG and HG media containing LPS significantly decreased MIP-1β secretion ($P < 0.001$) (Figure 5.9 (ii)). The IC_{50} of *C. verum* extract on MIP-1β expression was calculated by generating a polynomial curve, as shown in (Figure 5.9 (iii, iv, v and vi)).

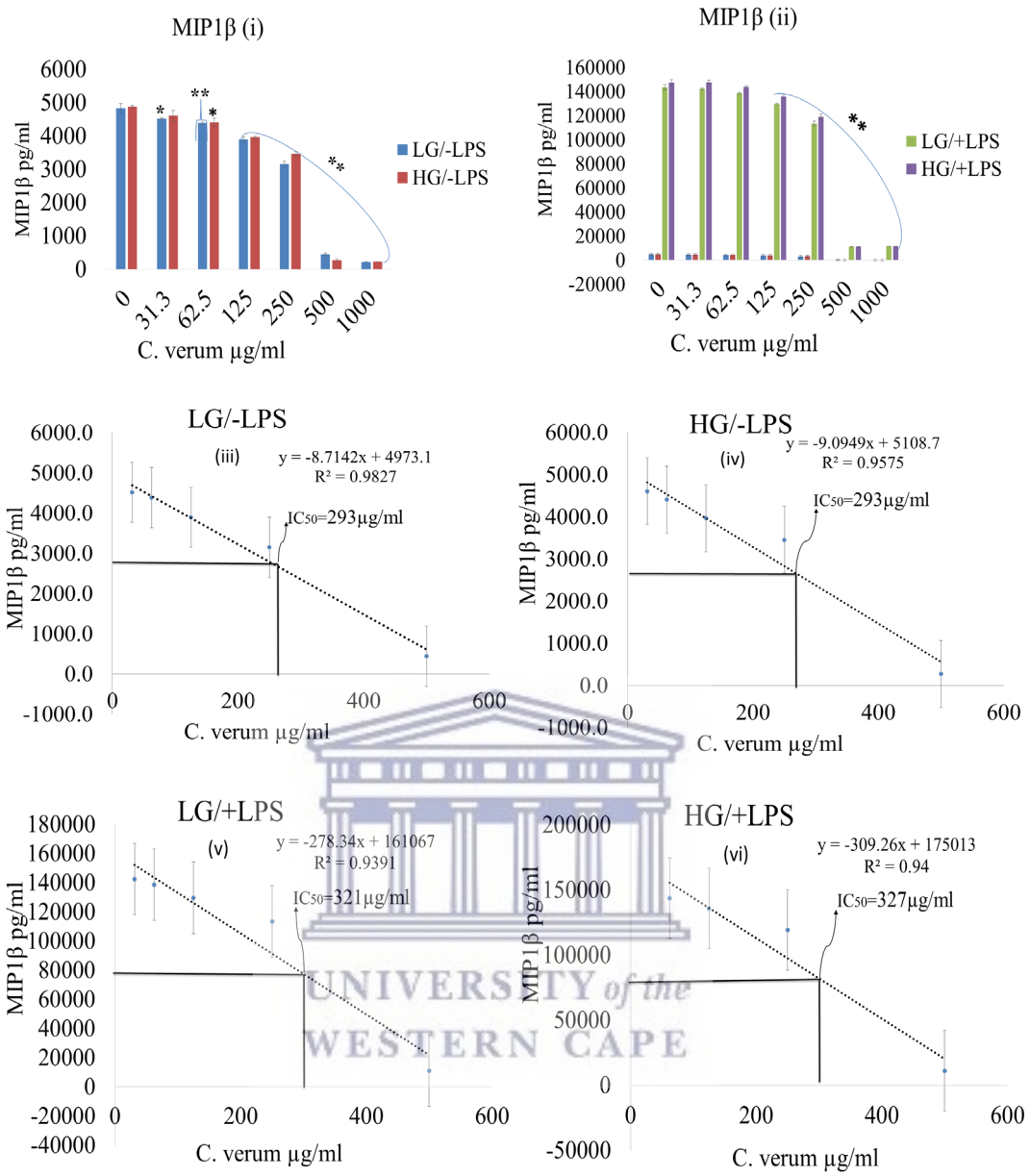


Figure 5.9. Effect of the *C. verum* extract on MIP-1β secretion levels by RAW 264.7 cells in LG and HG media without LPS-stimulation (i) and LG and HG media with LPS stimulation (ii). Secretion levels significantly different from those with the 0 µg/ml *C. verum* extract are indicated as * for $P < 0.01$ and ** for $P < 0.001$. IC_{50} determination of *C. verum* inhibition of MIP-1β in LG vs HG with and without LPS-stimulated cells (iii, iv, v and vi).

5.3.2.3.2. Effects of C. verum on TNF-α and IL-6 expression levels

C. verum extract exposure of RAW 264.7 cells at 62.5 µg/ml in HG medium containing LPS significantly decreased TNF-α secretion levels ($P < 0.01$) and by $P < 0.001$ at ≥ 125 µg/ml extract in LG and HG media containing LPS-stimulation (Figure 5.10 (i)). *C. verum* extract exposure of RAW 264.7 cells in LG and HG media containing LPS significantly decreased IL-6 secretion ($P < 0.001$) (Figure 5.10 (ii)). The IC_{50} values of the *C. verum* extracts were 183 µg/ml for TNF-α and 60 µg/ml for IL-6 in LG medium containing LPS-stimulation, while the IC_{50} values of the *C. verum* extracts were 172 µg/ml for TNF-α and 54 µg/ml for IL-6 in HG medium containing LPS (Figure 5.10 (iii, iv, v and vi)).

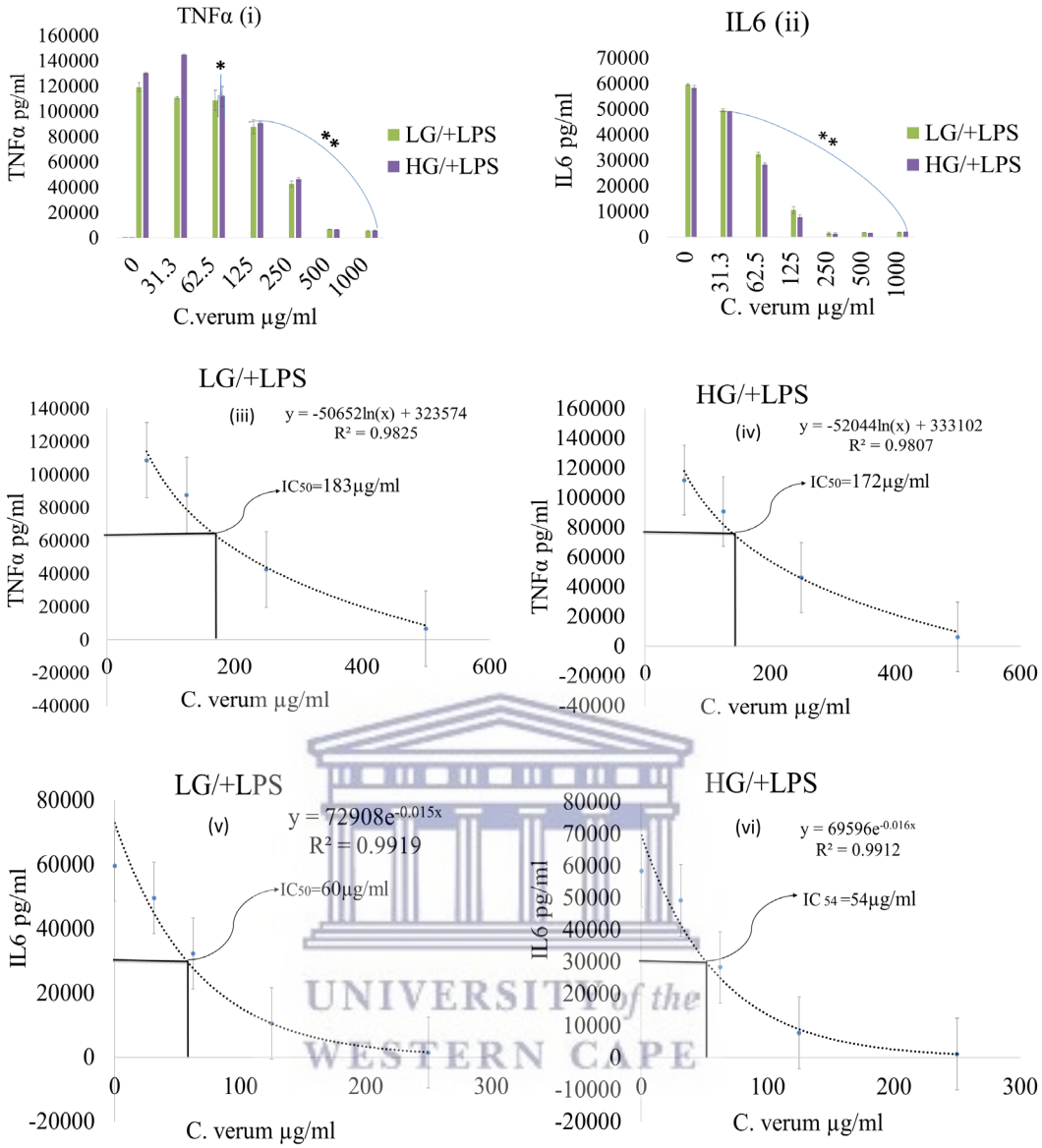


Figure 5.10. Effect of the *C. verum* extract on TNF-α and IL-6 secretion levels in LG vs HG media containing LPS-stimulation (i and ii). IC_{50} determination of the *C. verum* extract on TNF-α and IL-6 secretion in LG and HG media containing LPS-stimulation (iii, iv, v and vi).

5.3.2.3.3. Effects of C. verum on G-CSF and MCP-1 expression levels

C. verum exposure had no effects on G-CSF secretion by RAW 264.7 cells cultured in LG and HG media containing LPS, while it inhibited G-CSF secretion because the cell

proliferation decreases at concentrations of 500 and 1000 µg/ml (Figure 5.11 (i)). However, *C. verum* exposure at concentrations ≥ 125 µg/ml in LG and HG media containing LPS significantly decreased MCP-1 expression ($P < 0.001$) (Figure 5.11 (ii)). The results, reproducing the half-maximal inhibitory concentration of *C. verum* extracts, were measured (IC_{50}) to be for MCP-1 expression, as shown in (Figure 5.11 (iii and iv)).

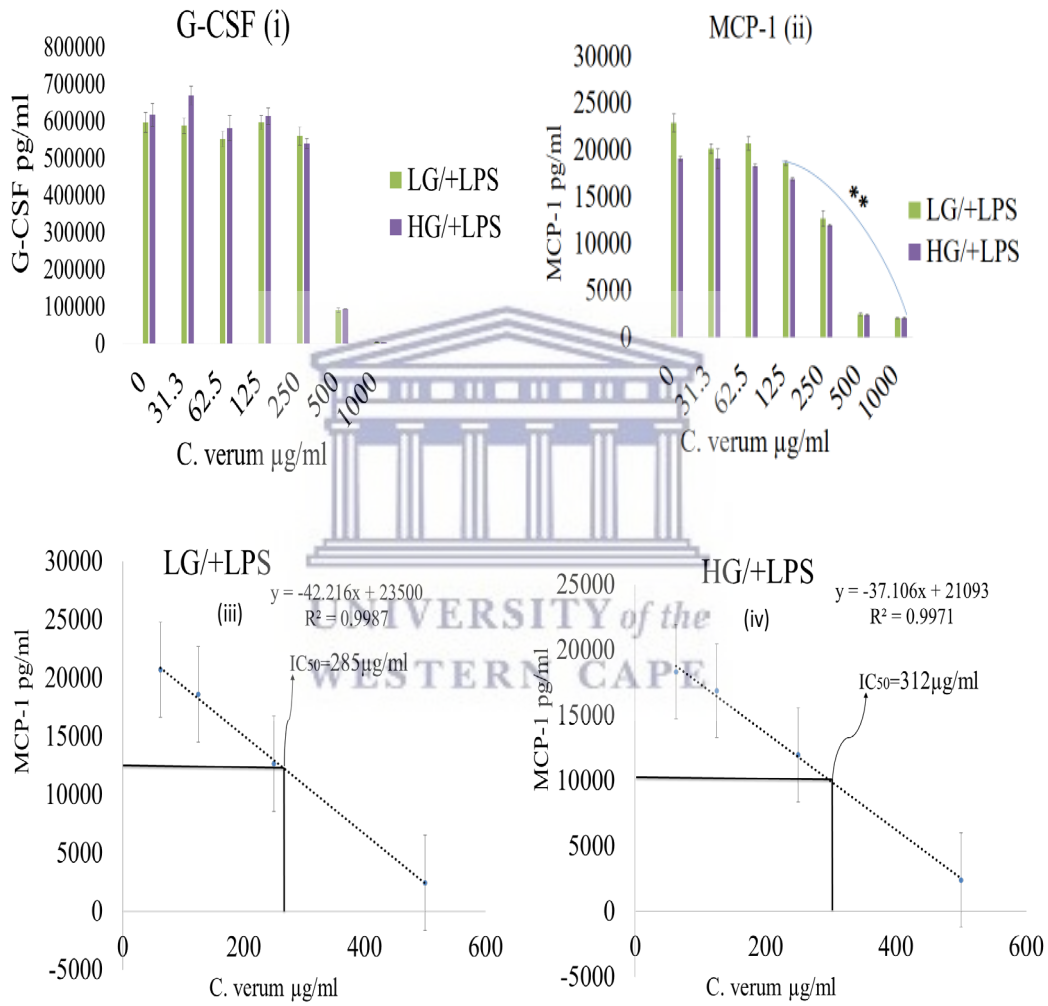


Figure 5.11. Effect of the *C. verum* extract on MCP-1 and G-CSF secretion in LG and HG media containing LPS-stimulation (i and ii). IC_{50} determination of the *C. verum* extract on MCP-1 secretion by RAW 264.7 cells cultured in LG and HG media containing LPS-stimulation (iii and iv).

5.3.2.3.4. Effects of C. verum on RANTES expression levels

Exposure to *C. verum* significantly decreased RANTES secretion levels by RAW 264.7 cells at concentrations ≥ 62.5 µg/ml by $P < 0.001$ in LG and HG media containing LPS-stimulation (Figure 5.12 (i)). The IC_{50} values of *C. verum* for RANTES secretion by RAW 264.7 cells cultured in LG and HG media containing LPS were 274 and 287 µg/ml, respectively (Figure 5.12 (ii and iii)).

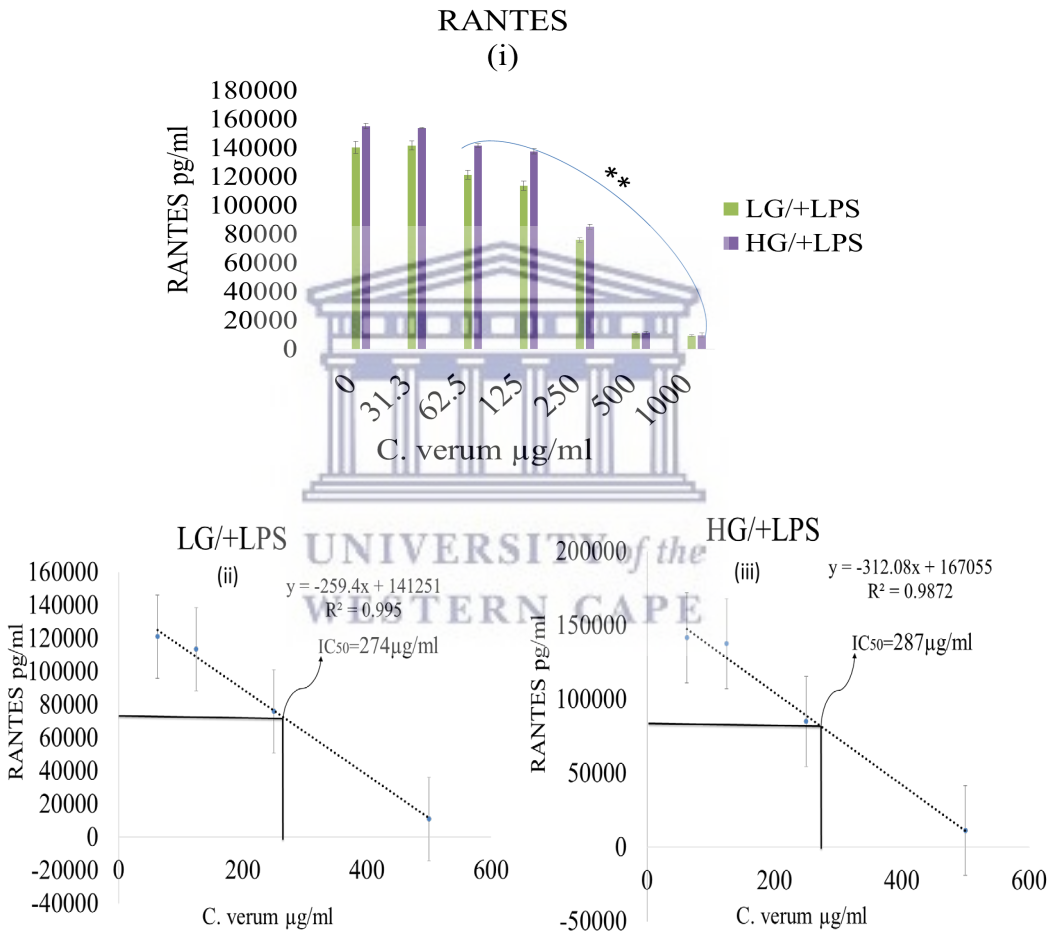


Figure 5.12. Effects of the *C. verum* extract on RANTES secretion by RAW 264.7 cells cultured in LG and HG media containing LPS-stimulation (i). The IC_{50} determination for *C. verum* on RANTES secretions by RAW 264.7 cells cultured in LG and HG media containing LPS stimulation (ii and iii).

5.3.3. Effects of *T. foenum-graecum* extracts on RAW 264.7 macrophages

5.3.3.1. Cytotoxicity assay T. foenum-graecum extract

The toxicity of *T. foenum-graecum* extracts on RAW 264.7 cells cultured in LG and HG media containing LPS was assessed using the XTT assay. Mouse macrophage cells were exposed to HG for 24 hours, and then treated with increasing concentrations of *T. foenum-graecum* extracts (up to 5000 µg/ml) for 24 hours. The results showed that LPS increased cell proliferation in LG and HG media (Figure 5.13 (i)). The results also showed that *T. foenum-graecum* had no effect at levels ≤ 2500 µg/ml but reduced cell viability at concentrations higher than 2500 µg/ml compared to the control 0 µg/ml *T. foenum-graecum* control (Figure 5.13 (i)). Cell viability decreased significantly ($P < 0.001$) at the higher concentration of 5000 µg/ml *T. foenum-graecum* (Figure 5.13 (i)). In Table 5.3, the IC_{50} values of the *T. foenum-graecum* extract for the various biomarkers analysed.

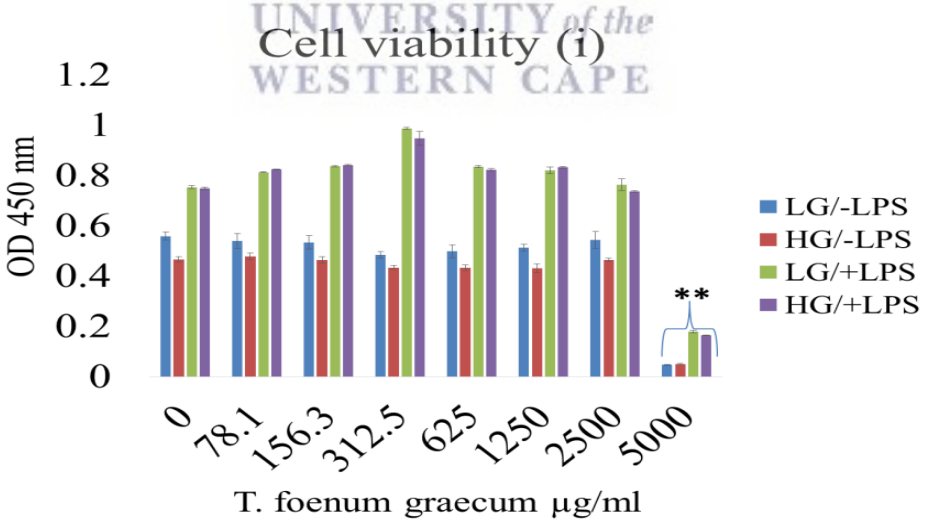


Figure 5.13. Effect of the *T. foenum-graecum extract* on RAW 264.7 cells viability. Statistical significance is indicated by ** ($P < 0.001$) at the higher concentration compared with 0 µg/ml *T. foenum-graecum* extract.

Table 5.3. IC_{50} of *T. foenum-graecum* extract for the secretion of various inflammatory biomarkers by RAW 264.7 cells

Markers		MIP1β	TNF α	IL6	G-CSF	MCP-1
T. foenum-graecum IC_{50} µg/ml	LG/-LPS					
	HG/-LPS					
	LG/+LPS	1258	1357	481	640	2709
	HG/+LPS	1068	1155	475	504	3955

5.3.3.2. Effects of T. foenum-graecum on nitric oxide secretion

The NO production assay was performed on the cell culture medium supernatant of RAW 264.7 cells exposed to *T. foenum-graecum* in LG and HG media containing LPS. The *T. foenum-graecum* significantly inhibited ($P < 0.001$) the production of NO at the higher concentration (2500 µg/ml) and inhibited NO secretion because the cell proliferation decreases at a concentration of 5000 µg/ml, compared to 0 µg/ml in LG and HG media containing LPS-stimulation (Figure 5.14 (i)).

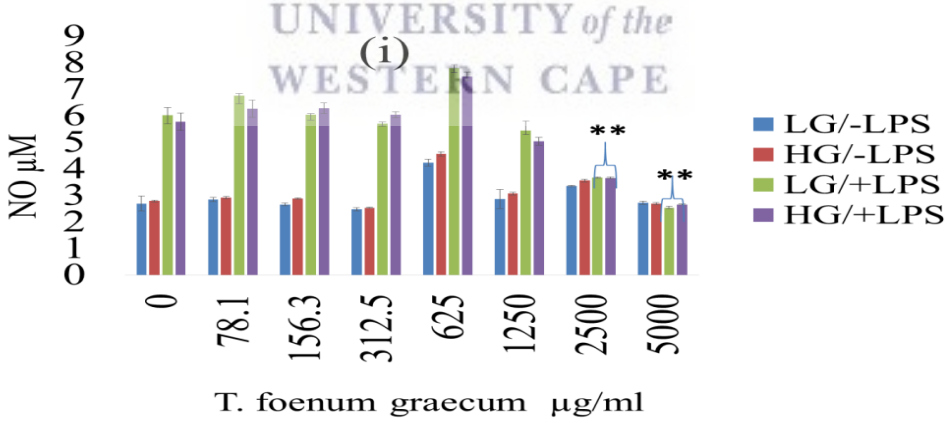


Figure 5.14. Effects of *T. foenum-graecum* extract on NO production by RAW 264.7 cells cultured in LG and HG media with and without LPS-stimulation (i). Data represent the mean ± SEM. Differential significance was denoted by $P < 0.01$ and ($P < 0.001$) (indicated by **) at the higher concentration in LG and HG media containing LPS compared to 0 µg/ml *T. foenum-graecum*.

5.3.3.3. Effects of T. foenum-graecum extract on macrophage cytokine and chemokine secretions

5.3.3.3.1. Effects of T. foenum-graecum on MIP-1β expression levels

The *T. foenum-graecum* extract at concentrations ≥ 625 µg/ml significantly decreased MIP-1β secretion levels ($P < 0.001$) by RAW 264.7 cells cultured in LG and HG media containing LPS (Figure 5.15 (i)) and compared to the untreated control. The IC_{50} of *T. foenum-graecum* extract for MIP-1β expression by RAW 264.7 cells cultured in LG and HG media with LPS was calculated (Figure 5.15 (ii and iii)).

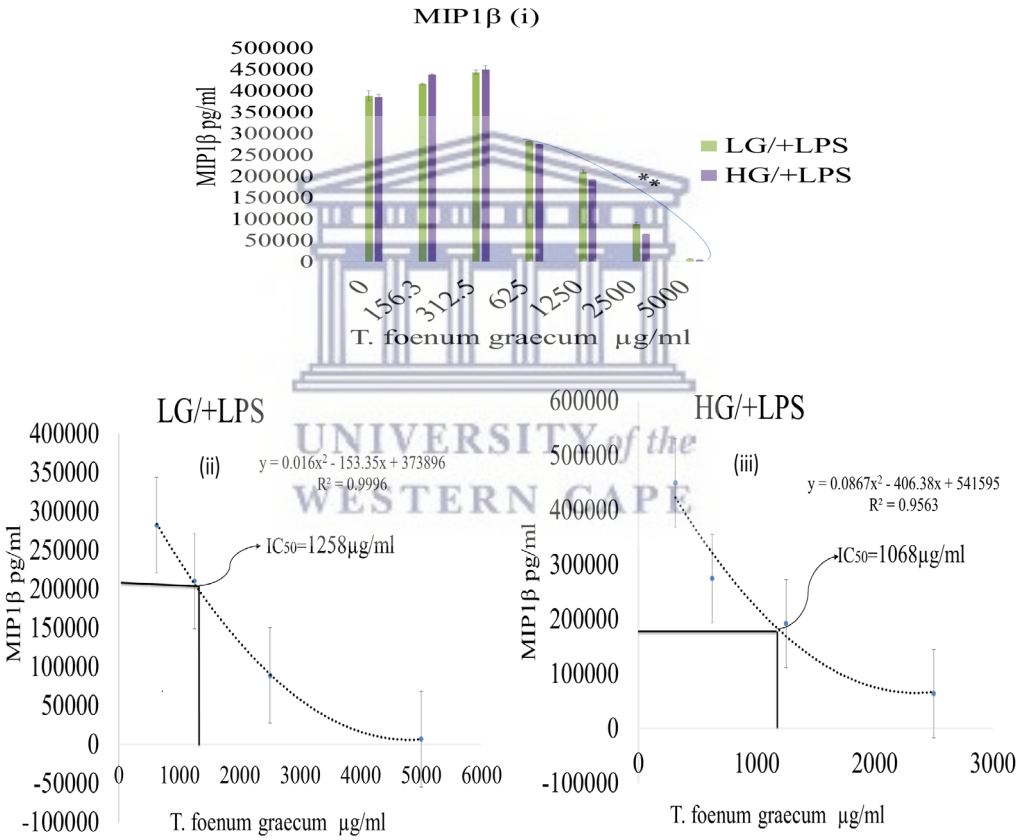


Figure 5.15. Effect of *T. foenum-graecum* extracts on MIP-1β secretion levels by RAW 264.7 cells at LG and HG in the absence and presence of LPS. (i) At concentrations ≥ 625 µg/ml in LG and HG with LPS-stimulated cells, the extract significantly inhibited MIP-1β secretion $P < 0.001$ (**).(ii and iii) IC_{50} determination of *T. foenum-graecum* inhibition of MIP-1β in LG and HG media with LPS-stimulated cells.

5.3.3.3.2. Effects of T. foenum-graecum on TNF-α and IL-6 expression levels

The *T. foenum-graecum* extract significantly decreased secretion of TNF-α ($P < 0.001$) by RAW 264.7 cells cultured in LG and HG media containing LPS at concentrations ≥ 625 µg/ml (Figure 5.16 (i)). *T. foenum-graecum* significantly decreased IL-6 secretion ($P < 0.001$) by RAW 264.7 cells cultured in LG and HG media containing LPS at concentrations ≥ 156.3 µg/ml (Figure 5.16 (ii)). The IC_{50} values of *T. foenum-graecum* for the secretion of TNF-α and IL-6 were calculated (Figure 5.16 (iii, iv, v and vi)).

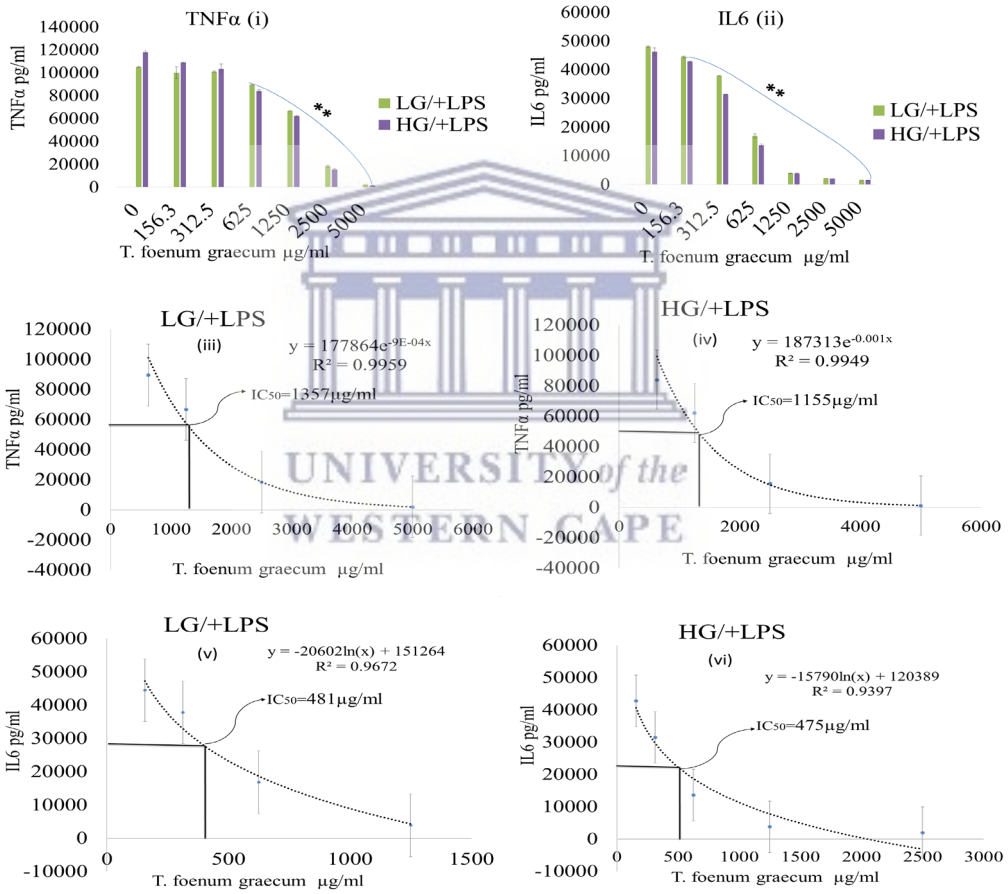


Figure 5.16. Effects of *T. foenum-graecum* extract on TNF-α and IL-6 secretion levels by RAW 264.7 cells cultured in LG and HG media containing LPS (i and ii). IC_{50} determination of *T. foenum-graecum* on the production of TNF-α and IL-6 by RAW 264.7 cells cultured in LG and HG media containing LPS-stimulation (iii, iv, v and vi), respectively.

5.3.3.3.3. Effects of T. foenum-graecum on G-CSF and MCP-1 expression levels

Exposure to *T. foenum-graecum* extracts significantly decreased the synthesis of G-CSF and MCP-1 ($P < 0.001$) by RAW 264.7 cells cultured in LG and HG media-containing LPS at concentrations ≥ 625 µg/ml compared to the 0 µg/ml *T. foenum-graecum* control (Figure 5.17 (i and ii)). The IC_{50} for G-CSF and MCP-1 expression was calculated (Figure 5.17 (iii, iv, v and vi)).

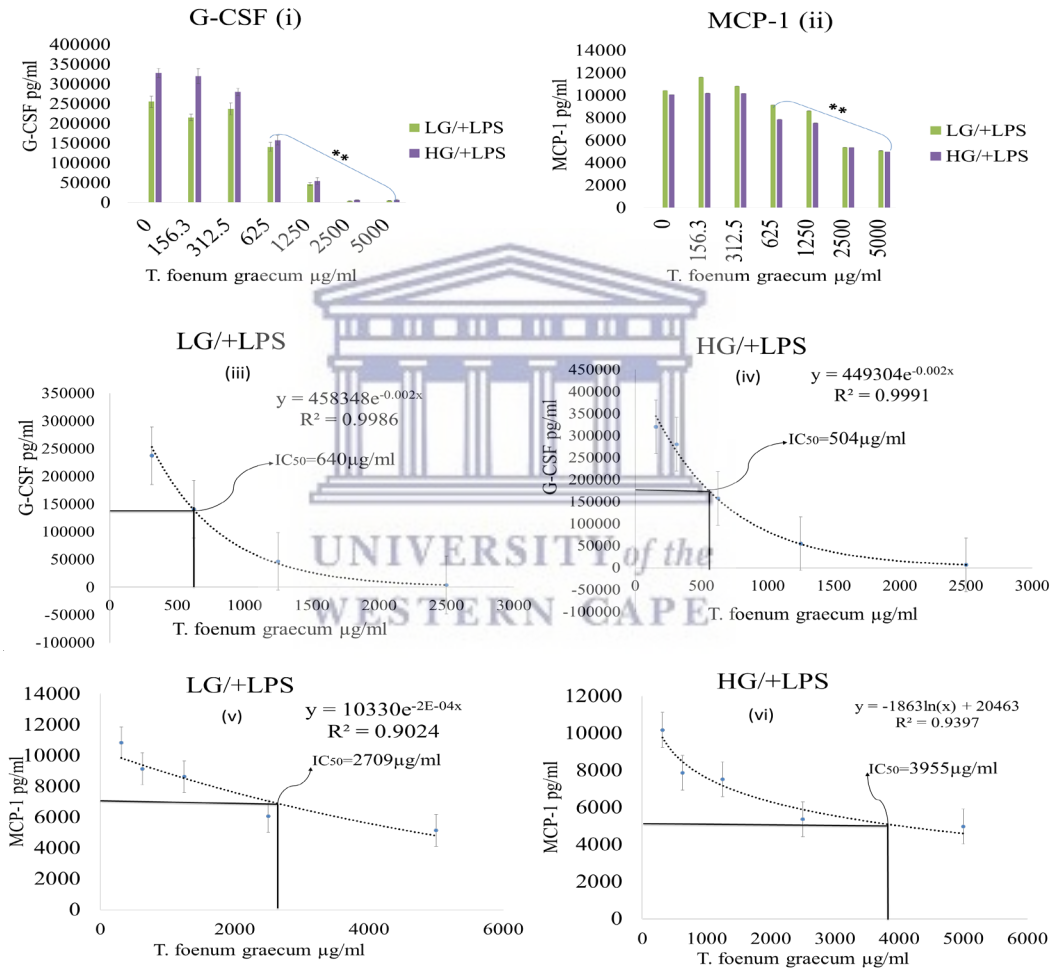


Figure 5.17. Effects of *T. foenum-graecum* extract on G-CSF and MCP-1 secretion levels by RAW 264.7 cells cultured in LG and HG media containing LPS (i and ii). IC_{50} determination of *T. foenum-graecum* on G-CSF and MCP-1 secretion by RAW 264.7 cells cultured in LG and HG media with LPS-stimulation (iii, iv, v and vi). ** $P < 0.001$.

5.3.3.3.4. Effects of T. foenum-graecum on RANTES expression levels

The *T. foenum-graecum* extract down-regulated RANTES significantly at a concentration of 1250 µg/ml ($P < 0.01$) and less so (at a concentration ≥ 2500 µg/ml $P < 0.001$) in LG medium containing LPS stimulation. A significant decrease ($P < 0.001$) was observed at concentrations ≥ 1250 µg/ml in HG medium containing LPS stimulation (Figure 5.18 (i)).

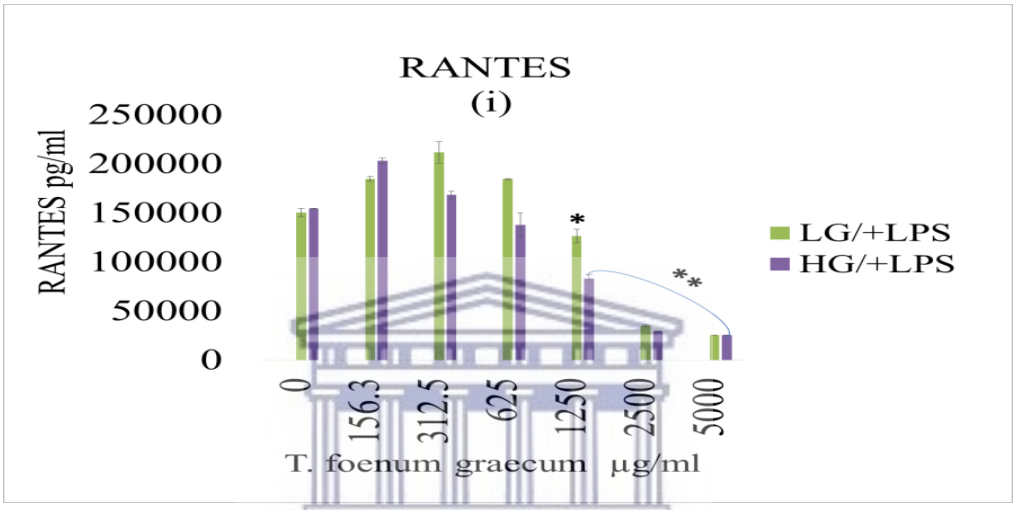


Figure 5.18. Effects of *T. foenum-graecum* extract on RANTES synthesis by RAW 264.7 cells cultured in LG and HG media containing LPS stimulation.

5.3.4. Effects of metformin solution on RAW 264.7 cells

5.3.4.1. Cytotoxicity assay of metformin solution

The XTT assay was used to assess the toxicity of metformin solution on RAW 264.7 cells. Mouse macrophage cells were exposed to HG for 24 hours, then treated with increasing concentrations of metformin solution (up to 1000 µg/ml) for 24 hours. The cell proliferation assay was used to determine the toxic activity concentration in LG and HG media with and without LPS stimulation.

Metformin significantly decreased cell proliferation at concentrations ≥ 250 µg/ml ($P < 0.001$) (Figure 5.19 (i)). The IC_{50} of metformin in LG medium without LPS was 260 µg/ml; in HG medium without LPS, it was 237 µg/ml; in LG medium containing LPS, it was 396 µg/ml; and in HG medium with LPS, it was 345 µg/ml (see Figure 3.19 (ii, iii, iv and v)). For statistical analyses, the results were compared with the untreated control. The IC_{50} values of metformin extract for the various biomarkers analysed are presented in Table 5.4.

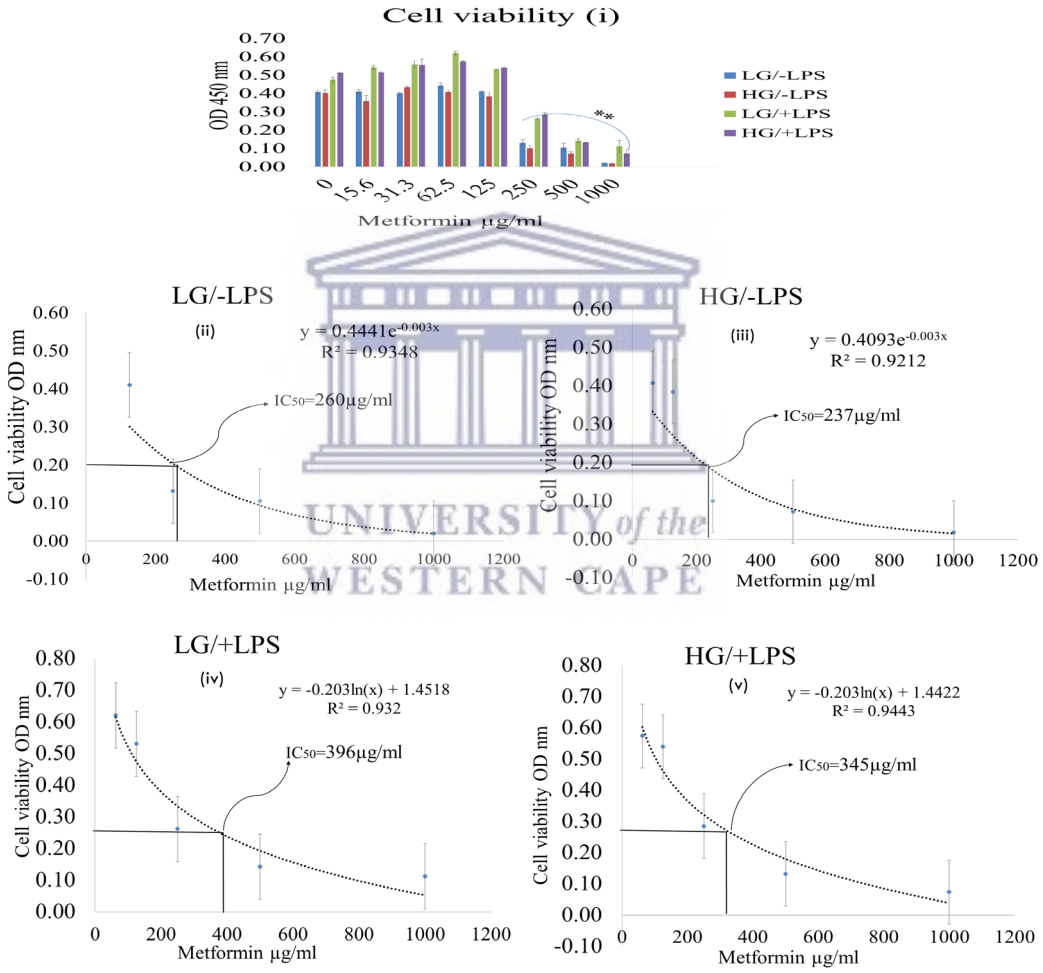


Figure 5.19. Cell viability of RAW 264.7 cells treated with metformin solution (i). The effect of metformin on cell proliferation was significant ($P < 0.001$) compared to 0 µg/ml metformin solution (i). A concentration-effect curve of metformin showing inhibition of cell viability in LG and HG with and without LPS-stimulated cells (ii, iii, iv and v), respectively.

Table 5.4. IC_{50} of the metformin extract for the secretion of various inflammatory factors by RAW 264.7 cells

Markers		MIP1β	TNF α	G-CSF	Cell viability
Metformin IC_{50} µg/ml	LG/-LPS	244			260
	HG/-LPS	181			237
	LG/+LPS		212	207	396
	HG/+LPS		268	246	345

5.3.4.2. Effects of metformin on nitric oxide secretion

Nitric oxide was measured in RAW 264.7 cell culture medium after exposure to metformin. Metformin at ≤ 125 µg/ml had no effect on NO secretion by RAW 264.7 cells incubated with LG and HG media containing LPS. Higher metformin concentrations inhibited NO secretion because of cell proliferation that decreased at concentrations ≥ 250 µg/ml (Figure 5.20 (i)). Data also show that metformin had no affect NO secretion by cells incubated in LG and HG media without LPS. All statistical analyses were compared with the untreated control.

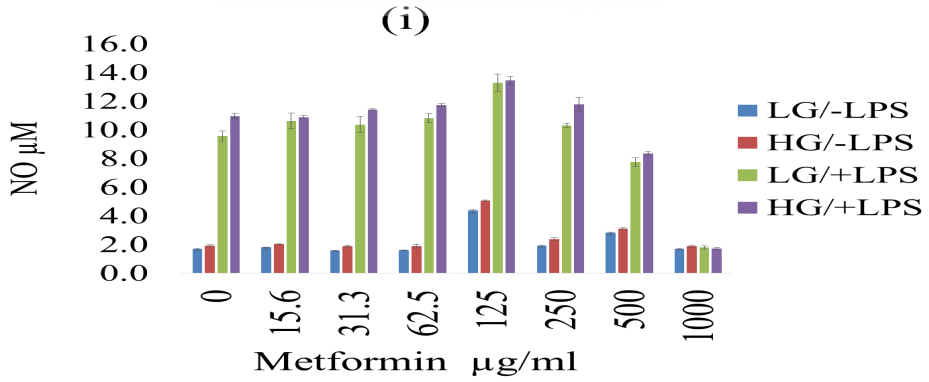


Figure 5.20. No effects of metformin solution on RAW 264.7 cell in LG and HG with and without LPS-induced NO production.

5.3.4.3. Effects of metformin solution on RAW 264.7 cell cytokine and chemokine secretions

5.3.4.3.1. Effects of metformin on MIP-1β expression levels

Metformin significantly decreased RAW 264.7 cell secretion of MIP-1β in LG medium without LPS at ≥ 125 µg/ml ($P < 0.001$). However, in HG medium in the absence of LPS at 31.3 µg/ml ($P < 0.01$) and ≥ 62.5 µg/ml ($P < 0.001$) (Figure 5.21 (i)). Metformin in LG and HG media containing LPS had no significant effect on MIP-1β secretion levels by RAW 264.7 cells (Figure 5.21 (ii)). An LPS-containing medium decreased MIP-1β levels that appeared at 500 µg/ml metformin because of a decrease in cell proliferation at a concentration of ≥ 250 µg/ml. A concentration-effect curve of metformin showed inhibition of MIP-1β in LG and HG media in the absence of LPS (Figure 5.21 (iii and iv)). For statistical analyses, the results were compared with the untreated control.

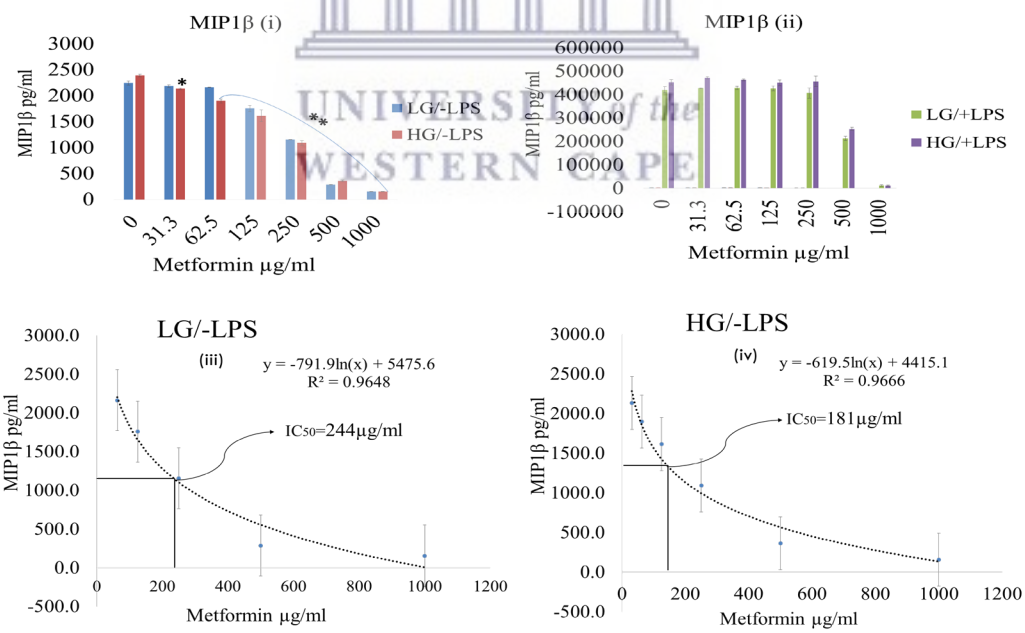


Figure 5.21. Effects of metformin on RAW 264.7 cell expression in LG and HG without LPS stimulation, significant levels by $P < 0.01$ (*) and $P < 0.001$ (**) decrease MIP-1β secretion levels (i). In LG and HG with LPS-stimulation, there was no significant

decrease in MIP-1β secretion levels (ii). IC_{50} determination of metformin inhibition of MIP-1β in LG and HG without LPS-stimulated cells (iii and iv) is shown.

5.3.4.3.2. Effects of metformin on TNF-α and IL-6 expression levels

Exposure of RAW 264.7 cells to metformin in an LG medium containing LPS inhibited TNF-α secretion significantly at ≥ 31.3 µg/ml ($P < 0.001$). RAW 264.7 cells cultured in an HG medium containing LPS showed a significant decrease in TNF-α secretion ($P < 0.001$) at a metformin concentration of ≥ 125 µg/ml (Figure 5.22 (i)). There was no significant decrease in IL-6 secretion levels at ≥ 125 µg/ml metformin (Figure 5.22 (ii)). The IC_{50} of metformin on TNF-α is shown in LG and HG media containing LPS-stimulation (Figure 5.22 (iii and iv)).

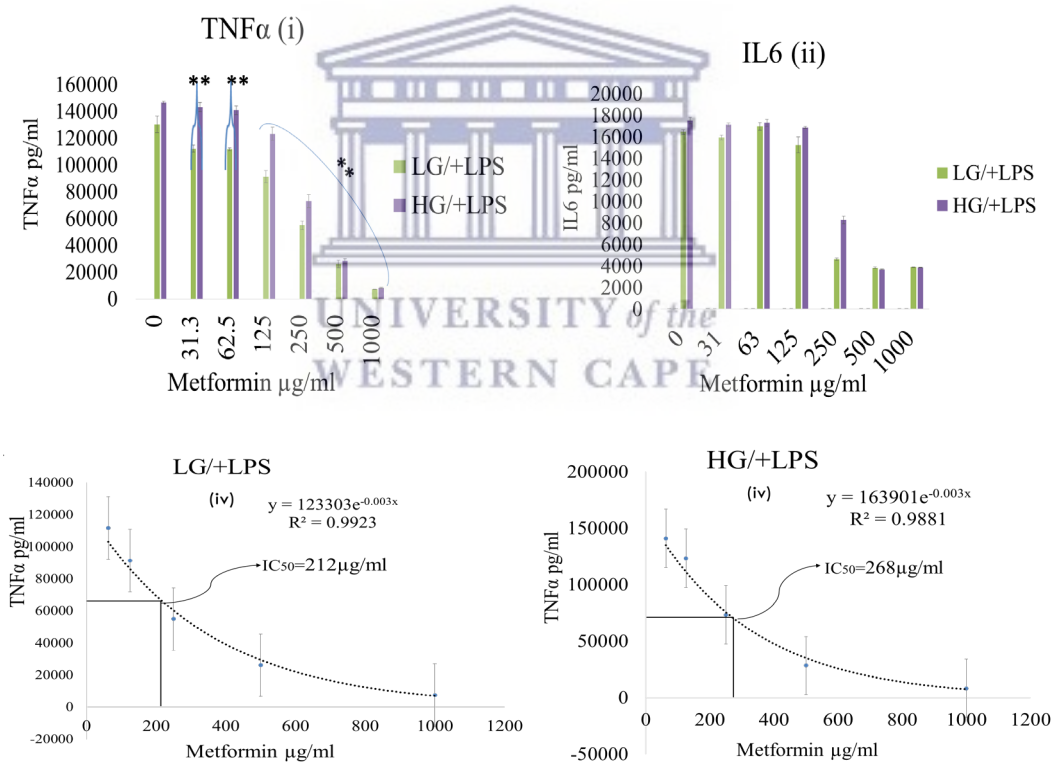


Figure 5.22. LPS-stimulated LG with metformin showed significant reductions in TNF-1 expression ($P < 0.001$)(i). A significant decrease in IL-6 secretion was not observed after LG- or HG-treatment of LPS-stimulated cells (ii). The inhibitory concentration (IC_{50}) of metformin solution for TNF-α expression is shown in Figures (iii and iv).

5.3.4.3.3. Effects of metformin on G-CSF and MCP-1 expression levels

Increasing the concentration of metformin decreased the expression level of G-CSF ($P < 0.001$) in LG medium containing LPS-stimulated cells. A concentration of 125 µg/ml and above significantly decreased G-CSF expression ($P < 0.001$) in an HG medium containing LPS-stimulated cells (Figure 5.23 (i)). Metformin down-regulated the MCP-1 secretion level, with significant differences of $P < 0.001$ at concentrations of 62.5 and 125 µg/ml in LG and HG media-containing LPS-stimulated cells, whereas higher metformin concentrations inhibited MCP-1 secretion because of the cell proliferation that decreased at concentrations ≥ 500 µg/ml (Figure 5.23 (ii)). A concentration-effect curve of metformin showed inhibition of G-CSF (Figure 5.23 (iii and iv)).

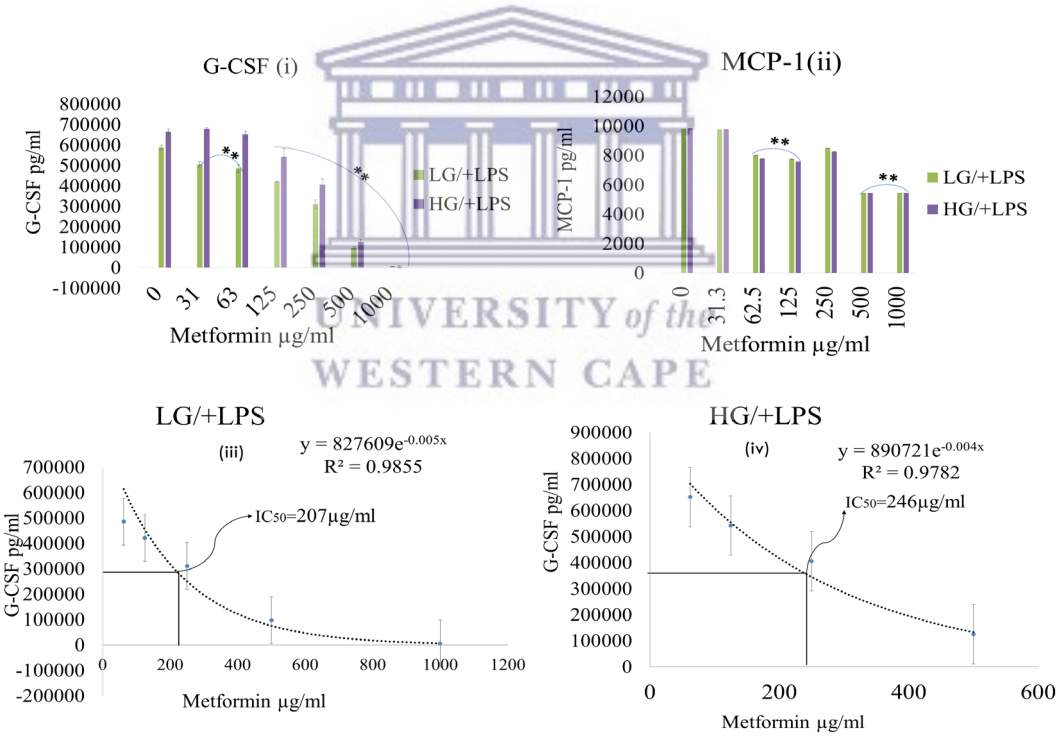


Figure 5.23. Metformin significantly decreased G-CSF secretion levels ($P < 0.001$ $^{(**)}$) in LG and HG with LPS-stimulated cells (i). MCP-1 secretion levels decreased significantly ($P < 0.001$) in LG and HG with LPS-stimulated cells (ii). IC_{50} determination of metformin production of G-CSF in LG and HG with LPS-stimulated cells is shown (iii and iv), respectively.

5.3.4.3.4. Effects of metformin on RANTES expression levels

Metformin caused no significant decrease in RANTES secretion levels in LG and HG media treated with LPS-stimulated cells, as shown in (Figure 3.24 (i)). Reductions appeared at ≥ 500 µg/ml metformin because of the cell proliferation that decreased in LPS-containing media.

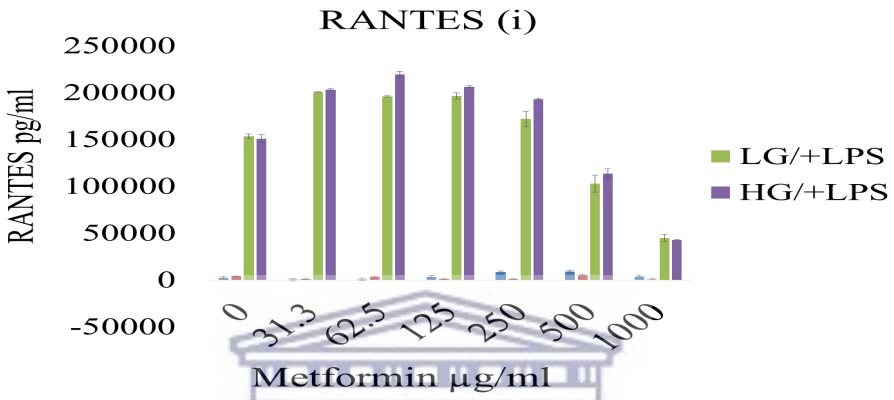


Figure 5.24. Metformin had no effect on RANTES secretion levels at increasing concentrations in LG- and HG-treated cells.

5.4. Discussion

Pharmaceutical agents such as metformin, which lower glucose levels and enhance glucose uptake, are insufficient to maintain or prevent the development of DM (Kahn et al., 2014; Hostalek et al., 2015). Therefore, it is necessary to handle early diabetes individually and prevent its progression using pharmacological and traditional therapies.

It has been suggested that the use of proteome profiles is a convenient approach for evaluating mechanisms involved in the anti-inflammatory role in diabetes complications (Lopez-Villar et al., 2015; Wang et al., 2018b). In the present study, biomarker proteins were used – findings indicated that plants used as traditional medicines inhibit pro-inflammatory cytokines such as MIP-1β, TNF-α, IL-6, G-CSF, MCP-1 and RANTES associated with HG.

In this study, the IC_{50} effects of TAT and standard Western medicine on RAW 264.7 cell viability were evaluated. *A, afra, C. verum, T. foenum-graecum* extracts and metformin reduced cell proliferation at different concentrations. The results indicated increased cell proliferation in LG and HG media containing LPS compared to cell proliferation in cells cultured in LG and HG media without LPS. The effects of *C. verum* and *T. foenum-graecum* on cell viability were observed at higher concentrations. The findings in the current study showed that *A. afra* and metformin were significantly more cytotoxic than *C. verum* and *T. foenum-graecum.*

The different herbs were found to have different IC_{50} values on the inflammatory marker expression. These results showed that the effects of *A. afra* extracts significantly inhibited MIP-1β, TNF-α, IL-6, G-CSF, MCP-1 and RANTES and NO. *C. verum* extract significantly inhibited MIP-1β, TNF-α, IL-6, MCP-1 and RANTES, and *T. foenum-*

graecum extract significantly inhibited MIP-1β, TNF-α, IL-6, G-CSF, MCP-1, RANTES and NO at higher concentrations and metformin solution significantly inhibited TNF-α, G-CSF, and MCP-1.

Results also indicated that *A. afra* had the highest anti-inflammatory activity compared to the other herbs in the study. The effects of *A. afra* may be a novel therapeutic target for the treatment of progression diabetes complications with minimal side effects.

The pro-inflammatory mediators related to the pathogenesis of T2DM have been identified in several studies. TNF-α and IL-6 play an essential role in activating other inflammatory cytokines, such as interferon-gamma and IL-1β, at the onset of diabetes-related obesity (Jamil et al., 2017; Garvey et al., 2017). TNF-α is the first pro-inflammatory cytokine associated with the pathogenesis of obesity-related impaired insulin signalling, as suggested by Esser et al. (2014). Increased TNF-α levels are also associated with renal complications in patients with T2DM (Chen et al., 2017; Umapathy et al., 2018).

Severe complications of inflammatory and metabolic disorders such as T2DM include chronic wounds (Pop-Busui et al., 2016). Several autoimmune diseases are associated with the dysregulation of the IL-6 signalling pathway, including T2DM (Kamimura et al., 2003). Therefore, the regulation of IL-6 levels may be used in the treatment and maintenance of chronic wounds derived from diabetes, as suggested in a study by van de Vyver et al. (2016).

The present study showed that *A. afra, C. verum* and *T. foenum-graecum* decreased TNF-α and IL-6 levels in a concentration-dependent manner in an HG medium containing LPS. Conversely, metformin showed a minimal effect on the TNF-α level and no effect

on the IL-6 concentration. In a recent study, the benefits of *metformin* combined with atorvastatin were reported to inhibit inflammatory markers, such as TNF-α and IL-6, in diabetic rats (Van Stee et al., 2018). Other studies reported that in LPS-treated cells *C. verum* extracts inhibited TNF-α and IL-6 production (Ho et al., 2013; Pathak & Sharma, 2021). It was also confirmed that trans-cinnamaldehyde and *p*-cymene from *C. verum* contributed to the inflammatory state (Schink et al., 2018). Furthermore, polysaccharides extracted from *T. foenum-graecum* seeds have an effective wound healing potential through their antioxidant activities, a finding that may improve insulin secretion/sensitivity and wound healing (Ktari et al., 2017).

Chronic inflammation in T2DM upregulates macrophage production of iNOS, causing tissue damage and impaired wound healing (Venneri et al., 2015). iNOS has an essential role in the inflammatory response, as activated iNOS produces NO that mediates the cytotoxic effect involved in the pathogenesis of cell damage and IR (Shreshtha et al., 2018). In earlier studies, it was demonstrated that the inhibition of NO production is essential in preventing the inflammatory response and improving endothelial dysfunction and chronic inflammation (Joo et al., 2014; Zhao et al., 2015). Metabolic syndrome is associated with low-grade inflammation, as evidenced by increased levels of MIP-1β (Loughrey et al., 2013; Ota, 2013), suggesting that MIP-1β in DM is involved in the pathogenesis of atherosclerosis and increased risk of stroke and cardiovascular disease (Chang & Chen, 2016).

In the present study, *A. afra, C. verum* and *T. foenum-graecum* decreased the MIP-1β concentration in LG and HG with and without LPS-stimulation of RAW 264.7 cells. The results also showed that *A. afra* decreased the NO concentration, while *C. verum* and *T. foenum-graecum* had effects at higher concentrations on NO in LG and HG with and

without LPS stimulation of cells. Metformin did not affect NO production in LG and HG with or without LPS but decreased MIP-1β levels in LG and HG without LPS. This finding shows that the above plants may be used as therapies for preventing or managing impaired wound healing in patients with diabetes.

The hematopoietic cytokine G-CSF enhances the proliferation and differentiation of neutrophil progenitor cells (Katakura et al., 2019). Therefore, G-CSF is crucial for neutrophil survival and the induction of inflammation to attack pathogen invasion (Kumar & Sharma, 2010). The G-CSF levels are increased in T2DM patients with FFA-induced inflammation, IR and atherosclerosis (Ordelheide et al., 2016). Therefore, regulating G-CSF levels may be an effective strategy for preventing diabetic cardiomyopathy and the progression of diastolic dysfunction (Lim et al., 2011). Our findings in the present study showed that *A. afra* and *T. foenum-graecum* effectively inhibit G-CSF expression through LPS-stimulated macrophages cultured in an HG medium. Metformin inhibits macrophage expression of G-CSF at higher concentrations (LG with LPS), while *C. verum* did not affect G-CSF synthesis.

RANTES is a chemokine expressed by many cell types, such as endothelial cells, macrophages, smooth muscle cells and platelets, in chronic inflammation. It activates T-cell adhesion and transmigration through the endothelial wall (Suffee et al., 2011). RANTES has been associated with pro- and anti-inflammatory effects and potentially regulates angiogenesis mediators. It may contribute to chronic inflammation and the process of inflammatory angiogenesis (Zernecke et al., 2008; Suffee et al., 2011). During angiogenesis, a new blood vessel is formed from an existing one which plays a critical role in inflammation and wound healing (Hall & Ran, 2010). Therefore, inhibiting this inflammatory mediator may be used to target and stimulate endothelial cells for wound

healing in DM (Elewa et al., 2010). In this study, *A. afra, C. verum* and *T. foenum-graecum* extracts effectively inhibited RANTES expression, while *metformin* did not affect RANTES levels in HG with LPS-stimulated cells.

In an earlier study that evaluated the effects of metformin on trophoblast cells (Sw.71), metformin was shown not to affect the HG modulation of G-CSF and RANTES secretion (Han et al., 2015). These plants may be potential therapeutic drugs to treat microvascular complications associated with DM by inhibiting angiogenic modulating effects.

High glucose was found to stimulate and increase macrophage infiltration into adipose tissue, facilitated by cell adhesion molecules and MCP-1, formed by the tubular and vascular cells (Fang et al., 2015). MCP-1 and its receptor, C-C motif chemokine receptor-2, have an essential role in promoting macrophage cell recruitment to kidney tubular cells (Awad et al., 2011; Meshkani & Vakili, 2016). MCP-1 plays a vital role in the progression of diabetes and as a biomarker for detecting renal failure (Lestarini et al., 2020).

In a recent study, metformin was shown to markedly reduce MCP-1 and thus could potentially be used to treat DR (Han et al., 2018). Previous studies did not show that *A. afra* reduced MCP-1 expression, while *T. foenum-graecum* and *C. verum* reduced the high expression of MCP-1 (Nurudeen, 2016; Han & Parker, 2017). However, in the present study, we found that *A. afra*, *C. verum*, *T. foenum-graecum* and metformin significantly reduced macrophage expression of MCP-1 and thus, may be used in managing and treating diabetes complications related to long-term inflammation.

5.5. Conclusion

There is strong evidence that chronic inflammation is involved in the pathogenesis of the onset and progression of diabetes complications. This part of the study focused on the benefits of plant-based therapies for inflammation and DM. The findings of this study indicated the effects of *A. afra*, *C. Verum* and *T. foenum-graecum* on pro-inflammatory cytokines related to the progression of T2DM compared to metformin. *A. afra* and metformin were more cytotoxic than *C. Verum* and *T. foenum-graecum*. The results suggest that the plants could be used as anti-inflammatory agents to prevent or delay the onset of DM and its complications. *A. afra* may be a novel therapeutic target for treating progressive diabetes complications with minimal side effects. The prevention and management of chronic inflammatory conditions linked to diabetes may indeed benefit from plant-based therapies. Further studies are required to look for the bioactive molecules of *A. afra*, *C. Verum* and *T. foenum-graecum* as diabetes-related pro-inflammatory response inhibitors.

Chapter 6:

Evaluation of the effects of *Artemisia afra, Cinnamon verum* and *Trigonella foenum-graecum* on macrophage metabolic markers, linked with high glucose-induced lipopolysaccharides

Abstract

Dyslipidaemia and OS, a pathophysiological reaction to chronic inflammation and diabetes, play a significant part in metabolic syndrome. The aim of this study was to use three herbal extracts as therapies to evaluate the macrophage bio-active marker expression levels in metabolic disorders and compare these with standard Western medicine. In this study, *A. afra* (IC_{50} = 200 µg/ml), *C. verum* (IC_{50} = 250 µg/ml), *T. foenum-graecum* (IC_{50} = 2500 µg/ml) and metformin (IC_{50} = 200 µg/ml), were used to treat LPS stimulated cell cultures. LPL is overexpressed and SOD-2 inhibited in the presence of HG with and without LPS-stimulated RAW 264.7 cells. Assessments showed conclusively that *A. afra* could suppress LPL expression and NO production, besides up-regulating SOD-2 enzyme in HG with LPS-stimulated RAW 264.7 cells. Extracts of *C. verum* could suppress LPL and NO, and the *T. foenum-graecum* extract can suppress NO and up-regulate the SOD-2 enzyme in HG with LPS-stimulated cells. In comparison, metformin could suppress LPL activity in HG with LPS-stimulated cells. In conclusion, the *A. afra* herb could play an essential role in treating metabolic disorder(s) by reducing inflammation and OS and possibly preventing the progression of the diabetes disease.

6.1. Introduction

Metabolic disorders are characterised by hyperglycaemia, hyperlipidaemia, hypertension and osteoporosis (Tabatabaei-Malazy et al., 2015; Kina-Tanada et al., 2017). Dyslipidaemia and hyperglycaemia are part of a common pathologic condition of metabolic syndrome in DM (Hartman & Kilianska, 2012). The dysfunctioning of adipose tissue surrounding all ingested fat leads to fat storage in other organs, with noticeable significance on IR. IR, a pre-diabetes condition of T2DM, is commonly associated with metabolic dyslipidaemia (Kang et al., 2016).

Cholesterol and TG are transported together with proteins, which are then cleared from circulation by LPL. This enzyme plays an integral role in the transportation of lipids from the liver to peripheral tissues and reverse cholesterol transport (Vaziri et al., 2016; Lan et al., 2016). T2DM is often associated with metabolic stress caused by a metabolic overload of lipoproteins and free radical species (Gonzalez et al., 2018).

There is evidence that the accumulated lipids in the macrophages are the precursors of foam cells in the sub-endothelial layer, which results in the development of atherosclerotic lesions (McLaren et al., 2011; Narasimhulu et al., 2016). LPL in macrophages contributes to a significantly higher risk of atherosclerosis, where it acts as a molecular bridge between lipoproteins and receptors on the cell surface (Kersten, 2014; Li et al., 2014). The resulting increase in lipid accumulation and oxidation leads to an inflammatory response in the artery wall and then foam cell formation (Makoveichuk et al., 2012; Lillis et al., 2015). Regulation of LPL can suppress the inflammatory cytokine expression and composition of lipid and foam plague in macrophages (Zhang et al., 2017).

Oxidative low-density lipoprotein (Ox-LDL), particles derived from excess circulating LDL that may have generated in the body of diabetes subjects, inducing adipocyte proliferation by increasing the infiltration of macrophages (Parthasarathy et al., 2010). These mechanisms promoting the macrophage expression of LPL result in the accumulation of FFA in adipocyte and non-adipocyte organs (Aouadi et al., 2014). Furthermore, alteration of Ox-LDL production causes a decrease of adipokine levels, such as the release of anti-oxidant mediators, which inhibit the ROS synthesis (Gaens et al., 2013). Reports have suggested that the raised Ox-LDL in subjects with hyperlipidaemia may be due to the down-regulation of anti-oxidant capacity, such as the decreased activity of superoxide dismutase (SOD-2) enzyme (Wang & Trayhurn, 2006).

Free radical species are bio-active substances that occur under physiological conditions and increase in many pathophysiological situations such as obesity, diabetes and cardiovascular disease. They cause increases in the uptake of lipid species (native-LDL, oxidized-LDL particles or free fatty acids) and lipolysis in macrophages and mitochondria (Fernandez-Sánchez et al., 2011). Increasing ROS levels causes an increase in OS, due to the inability of antioxidants to neutralize ROS, including catalase and SOD-2 (Alkhatib, 2019). Hyperglycaemia also results in induced-OS, pro-inflammatory markers and decreased expression of the SOD-2 enzyme (Wang et al., 2015; Zilaee & Shirali, 2016).

In T2DM, mitochondrial dysfunction causes increased FFA oxidation. However, SOD-2 acts as an antioxidative enzyme, neutralising oxidant damage to mitochondria and maintaining the cell environment (Kasahara et al., 2005). In an earlier study, Sharma et al. (2010) suggested that increased OS linked to hyperglycaemia reduces the level of SOD-2 activity. Thus, regulation of LPL and SOD-2 may be a likely target for treating

pathological processes associated with microvascular complications in T2DM (Mollsten et al., 2007; Jakhotia et al., 2018) (as shown in Figure 6.1).

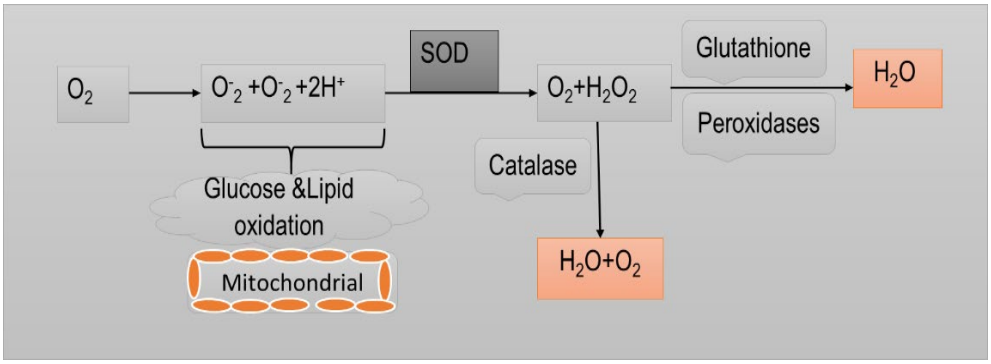


Figure 6.1. Schematic of antioxidants acting through enzymatic mechanisms (Chaudhary, 2018).

Great emphasis is placed on one or more marker proteins or enzymes that indicate a decrease in OS in skeletal muscle and the prevention of inflammation and IR (Schrauwen & Hesselink, 2004). In addition to controlling hyperglycaemia, the main issue in managing T2DM and its complications is minimising hyperlipidaemia and OS-induced chronic inflammation.

Therefore, the aim of this study is to determine macrophage bio-active marker expression levels regarding metabolic disorders and evaluate the herbs' therapeutic potential to regulate their activity. It is suggested that suppression of OS and inflammation may be an approach used to investigate the therapeutic target in managing atherosclerotic lesions and microvascular complications observed in DM.

6.2. Materials and Methods

6.2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) (Lanza, Belgium), Eagle's minimum essential medium, high D-glucose, 10% BSA, and metformin hydrochloride were purchased from Sigma-Aldrich (Germany). The mouse macrophage cell line was obtained from (Thermo Fisher Scientific Cell Line (USA)). XTT labelling reagent (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) was purchased from Roche Diagnostic GmbH (Germany). Ethanolic *A. afra*, *C. verum* and *T. foenum-graecum (fenugreek)* extracts were collected from a local manufacturer, Health Connection Wholefoods (Pty) Ltd (Cape Town, South Africa). LPS from *Escherichia coli* 0111:B4 was obtained from Amersham Biosciences (South Africa). Human/Mouse SOD-2-ELISA kits were purchased from R&D Systems (South Africa). *p*-Nitro phenyl-palmitate was purchased from Sigma-Aldrich (South Africa).

6.2.2. Methods

6.2.2.1. Cell viability assay

Raw 264.7 cells were cultured in a DMEM medium. The cells were grown in tissue culture flasks (T-25cm^2); 6 ml medium was supplemented with 10% heat-inactivated FBS, 0.2 mM/L L-glutamine, 1% antibiotic-antimycotic mix and 1% gentamycin (m/v). The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C. After reaching 70–80% confluence, the cells were seeded in a DMEM medium in a 96-well plate at a density of 1×10^5 cells per well. The cells were incubated in 5% CO_2 at 37°C until reaching 50–60% confluence. The supernatant DMEM medium was discarded and

replaced with a MEM medium supplement with a 10% FBS, amino acids 0.2 mM/L L-glutamine, 1% antibiotic-antimycotic mix and 1% gentamycin.

Raw 264.7 cells were divided into two groups (–LPS and +LPS). Each group was divided into normal glucose (5 mM) and HG (25 mM). The cells were incubated in 5% CO_2 at 37°C for 24 hours. The supernatant was discarded, cells were then exposed to effective concentrations of *A. afra*, metformin, *C. verum*, and *T. foenum-graecum* extracts in a 0.5% FBS-MEM medium. The cells were incubated in 5% CO_2 at 37°C for 24 hours. Then, 50 µl of the XTT labelling reagent was added to each well according to the manufacturer's instructions. The plate was read immediately (zero point) and then at 10-minute intervals for the next 40 minutes, using an ELISA plate reader at a wavelength of 450 nm – serving as a measure of cell viability.

6.2.2.2. Extraction of cell proteins

Cells (1×10^5 in 2 ml medium) were seeded in 6-well plates. After the RAW 264.7 cells reached approximately 50–60% confluence in each well, the medium was replaced with LG and HG in 10% FBS-MEM media. The cells were incubated in 5% CO_2 at 37°C for 24 hours. Following exposure to 1% LPS, the cells were treated with an effects concentration of *A. afra,* metformin*, C. verum*, and *T. foenum-graecum* in LG and HG 0.5% FBS-MEM media, then incubated in 5% CO_2 at 37°C for 24 hours.

After removing the supernatants, cells were washed with DPBS (Lonza). The protein extract was prepared by diluting phosphatase and PI buffer (Sigma-Aldrich) (250 µg/ml phosphatase +250 µg/ml PI + 250 µg/ml 10% Tween + 24.25 ml of PBS buffer). The protein extract was added to each well (150 µl/well). The cell extracts were scraped, stored in Eppendorf tubes (Greiner Bio-One) and placed on ice before sonication. The

cells in the Eppendorf tubes were then sonicated (Misonix sonicator, XL-200 Series; Q Sonica, LLC, Newton, CT, USA). The sonicated samples were centrifuged (Super mini centrifuge, Mini-Star Plus) at relative RCF 12100 for 1 minute, whereafter the protein concentration was determined.

6.2.2.3. Protein concentration assay

For determining the protein concentration, samples were diluted in a 1:10 buffer solution (PI, PBS/Tween). For the standard curve, human serum albumin (Sigma-Aldrich) was prepared in 2-fold dilutions from 1000 µg/ml to 0 µg/ml and used to determine the unknown sample of protein concentration. Bradford reagent was diluted 1:5 with distilled water. Then 100 µL of Bradford reagent was added to each well (10 µl sample). Absorbance was read at a wavelength of 620 nm (Fluo-star Omega BMG Lab-tech). For all the protein biomarkers, quantitative protein abundance was used to perform qualitative and statistical analysis based on normalised spectral counts.

6.2.2.4. Lipoprotein lipase assay

The LPL of macrophage cell expression associated with HG and LPS stimulation was investigated. The colorimetric method was used, and selective measurement of LPL was based on the activation of LPL by *p*-nitrophenyl-palmitate (p-NP palmitate) was prepared as follows, i. Substrate stock: 30 mM p-NP palmitate in 10 ml 2-isopropanol. ii. Buffer: 602 mg (2-amino-2-(methylamine) propane-1,3-diol, ($NH_2C(CH_2OH)_2$) + 100 mg arabic gum + 400 µl Triton×-100 (iso-octyl phenoxy polyethoxy ethanol) then adjusted to 100 ml, pH 8 with HCl. The reaction mixture consisted of 135 µL of a 1:10 dilution of the substrate stock solution in buffer pH 8 and 15 µL of cell extract. This mixture was

incubated at 37°C, and lipase activity determined the release of p-nitrophenol by measuring the absorbance at 410 nm.

6.2.2.5. Superoxide dismutase enzyme activities assay

To investigate whether the macrophage SOD-2 expression is regulated, the macrophage cell line (RAW 264.7) was treated and exposed to HG in the presence of LPS stimulation. Four different measurements of macrophage cell lysate associated with HG and LPS were treated with ethanol extracts of herbal agents and analysed according to the manufacturer's instructions.

6.2.3. Statistical analysis

Statistical analysis was determined by ANOVA using the Sigma Plot software. All the data are presented as means ± standard error of the mean (SEM). The statistical mean difference was considered significant at $P < 0.01$. Most experiments were performed in triplicate.

6.3. Results

6.3.1. Effects of *A. afra* and metformin on RAW 264.7 cells

6.3.1.1. Cell viability

Cytotoxicity of *A. afra* and metformin against RAW 264.7 cells was determined using an XTT assay. The results show that no effects on RAW 264.7 cell viability were detected when ethanol extracts of *A. afra* and metformin solution were added to the 200 µg/mL concentration. *A. afra* exhibited an increase in cell growth in 5 and 25 mM glucose concentration with or without LPS stimulation in comparison with metformin (Figure 6.2 (i and ii)).

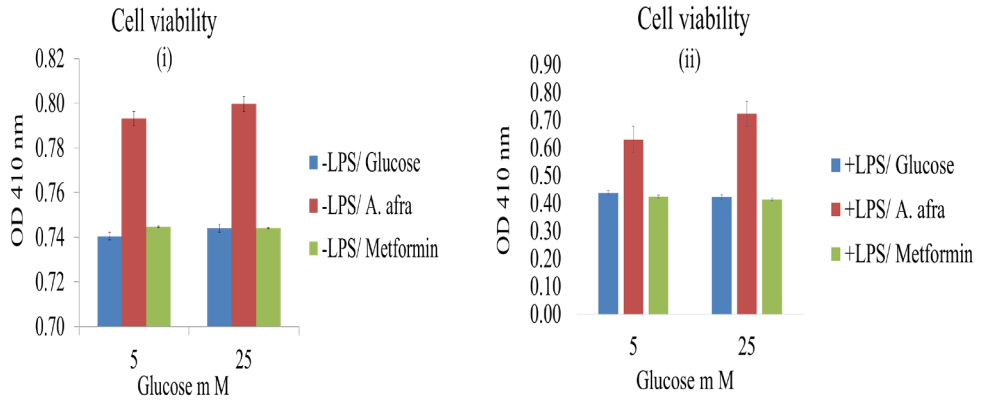


Figure 6.2. Results showing that *A. afra* increases cell growth in 5 and 25 mM glucose with or without LPS stimulation while metformin does not affect RAW 264.7 cell viability (i and ii).

6.3.2. *A. afra* and metformin regulation of RAW 264.7 cell lipase activity

LPL enzyme activity is an essential factor for lipid metabolism. It was measured to evaluate the effects of HG in LPS-induced RAW 264.7 cell expression and further assess whether *A. afra* or metformin regulated the LPL, which is crucial for initial foam cell formation. The results showed that the HG with and without LPS significantly increased expression of LPL compared to LG with and without LPS ($P < 0.001$), as shown in Figure 6.3 (i and ii). The results have shown that at concentrations of *A. afra* extract and metformin solution (200 µg/ml), down-regulation of LPL activity is observed, as shown in Figure 6.3 (i and ii).

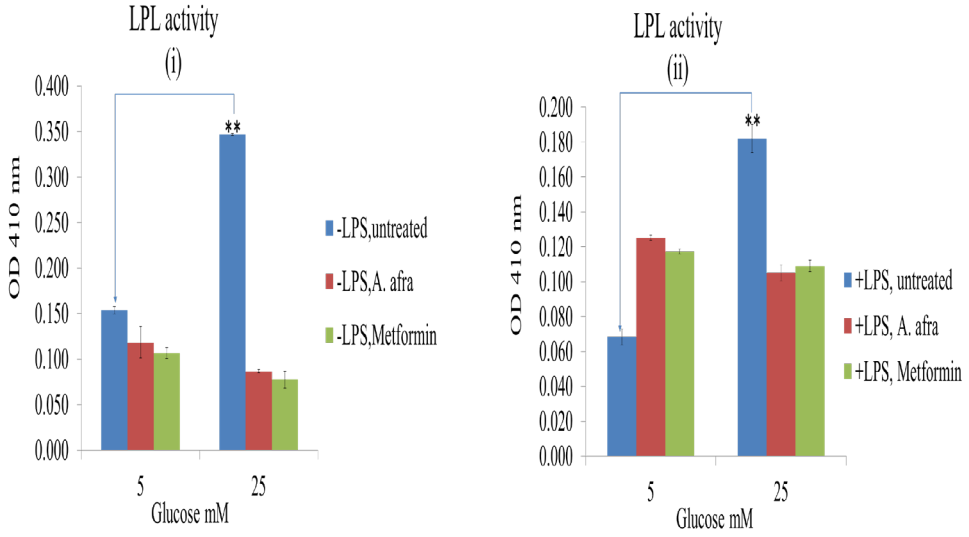


Figure 6.3. Effects of *A. afra* and metformin extracts on RAW 264.7 cell expression of LPL in LG and HG with and without LPS stimulation (i and ii). Cells were treated with *A. afra* and metformin in the presence of LPS. LPL activity values indicate a significant increase in HG with and without LPS stimulation ($P < .001$). LPL activity normalised with both treatments (*A. afra* and metformin) compared to the LG-stimulated and -unstimulated cells (i and ii).

6.3.3. The effects of *A. afra* and metformin on RAW 264.7 cells released by superoxide dismutase enzyme

SOD-2, one of the primary defence systems against OS, was measured to evaluate the antioxidative potential of *A. afra* and metformin to regulate oxidation factors. The results show that SOD-2 was statistically significantly decreased by HG with and without LPS stimulation ($P < 0.001$). SOD-2 was up-regulated by *A. afra* and metformin in HG without LPS with a statistically significant difference ($P < 0.001$) in Figure 6.4 (i). However, no effect of *A. afra* and metformin was observed on RAW 264.7 cell expression of SOD-2 in the HG with LPS stimulation, Figure 6.4 (ii).

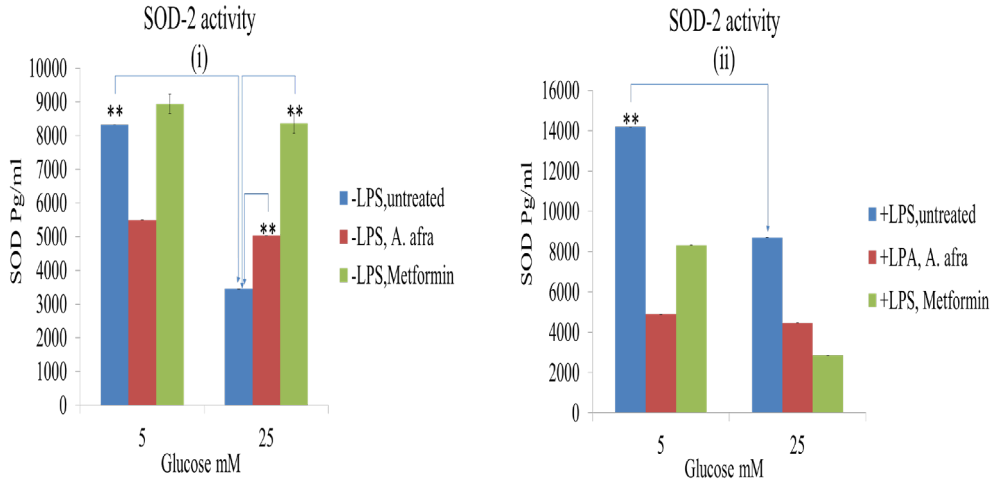


Figure 6.4. Effects of *A. afra* and metformin extract on RAW 264.7 cells released a superoxide dismutase enzyme (i and ii). SOD-2 was up-regulated by *A. afra* and metformin in HG without LPS-stimulated RAW 264.7 cells with a statistically significant difference ($P < 0.001$) (i). But no effect of *A. afra* and metformin on RAW 264.7 cell expression of SOD-2 in the HG with LPS stimulation is seen (ii).

6.3.4. Effects of *C. verum* and *T. foenum-graecum* on RAW 264.7 cell viability

First, we assessed the effects of treatments on the cell viability of RAW 264.7 cells. Cells were exposed to LG and HG and incubated at 37°C for 24 hours, then treated with 250 and 2500 µg/ml of *C. verum* and *T. foenum-graecum* extracts, respectively, for 24 hours. The results show increased cell growth of *C. verum* in LG and HG with and without LPS stimulation (Figure 6.5 (i and ii)). The *T. foenum-graecum* extract likewise displays increased cell growth in HG with LPS stimulation (Figure 6.5 (ii)).

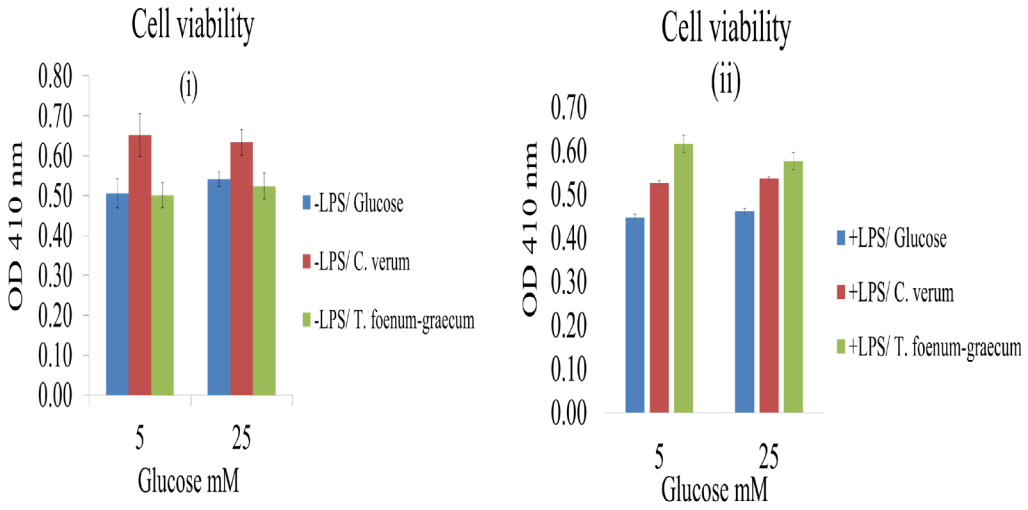


Figure 6.5. *C. verum* and *T. foenum-graecum* increased cell growth of RAW 264.7 cell viability at effective concentrations, respectively, in HG with LPS stimulation, while *C. verum* increased cell growth of RAW 264.7 cells in LG and HG without LPS stimulation (i and ii).

6.3.5. *C. verum* and *T. foenum-graecum* regulation of RAW 264.7 cell lipase activity

In the present study, we also investigated the effects of *C. verum* and *T. foenum-graecum* extracts on macrophage LPL activity. The results showed that the HG with and without LPS statistically significantly increased the expression of LPL compared to the LG with and without LPS ($P < 0.001$), as shown in Figure 6.6 (i and ii). Concentrations of 250 and 2500 µg/ml were used; *C. verum* and *T. foenum-graecum* extracts showed down-regulation of LPL activity in HG with and without LPS-stimulated RAW 264.7 cells, statistically significant differentiation ($P < 0.001$) (Figure 6.6 (i and ii)).

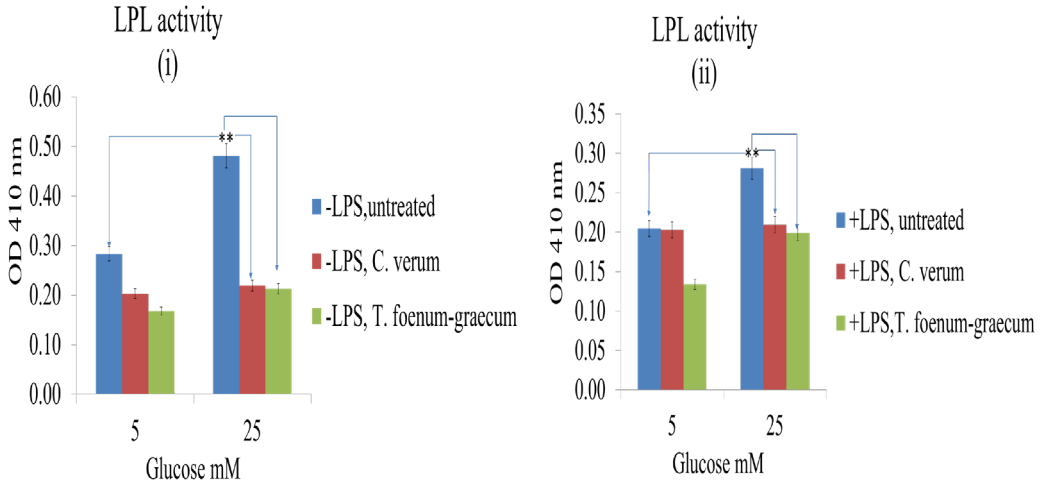


Figure 6.6. Increased expression of LPL compared to LG with and without LPS-stimulated cells. *C. verum* and *T. foenum-graecum* extracts show down-regulation of LPL activity in HG with and without LPS-stimulated cells (i and ii).

6.3.6. Effects of on RAW 264.7 cell released of the superoxide dismutase enzyme

Furthermore, in this study, we investigated the SOD-2 secretion in RAW 264.7 cells to evaluate the potential of the *C. verum* and *T. foenum-graecum* extracts to regulate antioxidant factors. Our results showed that SOD-2 was significantly decreased by HG with and without LPS stimulation ($P < 0.001$) (Figure 6.7 (i and ii)). Up-regulation by the *C. verum* extract in a concentration dependent manner significantly differed in SOD-2 activity in the LG with and without LPS stimulation ($P < 0.001$) (Figure 6.7 (i and ii)). No effect of *T. foenum-graecum* on the RAW 264.7 cell expression of SOD-2 in HG with LPS stimulation was observed (Figure 6.7 (ii)).

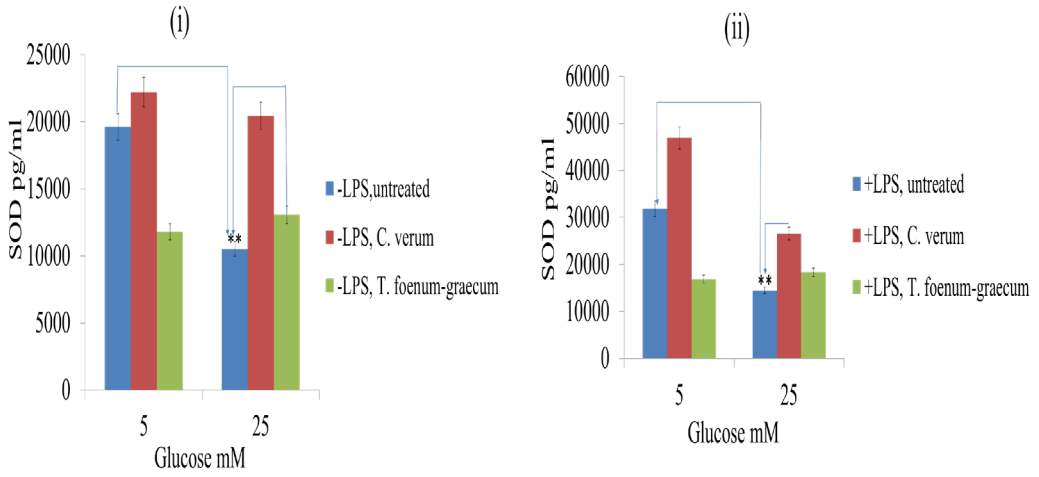


Figure 6.7. Effects of *C. verum* and *T. foenum-graecum* extracts on cell release of a superoxide dismutase enzyme. SOD-2 statistically significantly decreased in HG with and without LPS-stimulation (i and ii). Up-regulation by *C. verum* extract differed statistically significantly (i and ii). No effect of *T. foenum-graecum* on cell expression of SOD-2 in HG with LPS-stimulation (ii).

In this study, our results show comparisons between the *A. afra*, *C. verum*, and *T. foenum-graecum* extracts and the Western medicine (metformin) used for diabetes treated on RAW 264.7 cell to evaluate cell viability, LPL and SOD-2 secretion.. See Table 6.1.

Table 6.1. Comparison of the effects of *A. afra*, *C. verum*, and *T. foenum-graecum* extracts with metformin medicine on RAW 264.7 cell expression of metabolic biomolecule factors

Ethanol Extracts Concentration *(µg/ml)*	Cell Growth	LPL activity	SOD-2 activity
	LG and HG (-/+ LPS)		
A. afra *(200 µg/ml)*	Increase	Down regulation	Up-regulation HG (- LPS)
C. verum *(250 µg/ml)*	Increase	Down regulation	Up-regulation
T. foeum graecum *(2500 µg/ml)*	Increase HG (+LPS)	Down Regulation	Up-regulation HG (- LPS)
Metformin *(200 µg/ml)*	……	Down regulation	Up-regulation HG (- LPS)

6.4. Discussion

Several diseases accompany T2DM, causing metabolic disorders and promoting OS and inflammation (Park et al., 2009; Patel et al., 2016). Developing novel regulators that suppress inflammatory reactions and OS is a significant way to prevent diabetes complications (Chen et al., 2019). Thus, in this study, we evaluated the effectiveness of some herbal therapies compared to standard Western medicine to determine whether they may be beneficial as a therapeutic strategy to prevent the progress of diabetes complications.

The present study was designed to examine the effects of *A. afra*, *C. verum*, and *T. foenum-graecum* extracts and metformin on RAW 264.7 cells exposed to HG-induced LPS stimulation. It is considered that the LPL activity is overexpressed in the presence of HG with and without LPS-stimulated RAW 264.7 cells, in agreement with the reports of Sartippour and Renier (2000) and He et al. (2018).

In T2DM, antioxidant factors, such as SOD-2 enzyme levels, are altered, contributing to diabetes complications (Pourvali et al., 2016). This study revealed that HG inhibited SOD-2 in RAW 264.7 macrophages with and without LPS stimulation. This current result is in accord with findings of several studies that have reported that increased ROS release leads to decreased SOD-2 in dyslipidaemia (Hopps et al., 2010).

A. afra, C. verum and metformin inhibited LPL activity in LG and HG with and without LPS stimulation. The *T. foenum-graecum* extract inhibited LPL expression in HG without LPL-induced RAW264 cell stimulation. Previous reports similarly indicated that metformin suppressed lipid synthesis by inducing mitogen-activated protein kinases (Viollet et al., 2012). The *A. afra* extract seems to regulate the antioxidant defence

systems and improve lipid peroxidation in diabetic rats (Sunmonu & Afolayan, 2013). However, no studies describing the evaluation of *A. afra* and metformin effects on regulating LPL activity regarding metabolic disorders in diabetes exist.

Results of the present study also revealed that *C. verum* up-regulates the antioxidant SOD-2 enzyme in HG with or without stimulation of the RAW264 cell. In comparison, *A. afra, T. foenum-graecum* and metformin up-regulate in HG without LPL-induced stimulation. Previous studies have demonstrated that *A. afra* normalised levels of isoproterenol, which decreased levels of SOD-2 in rats (Sunmonu & Afolayan, 2010). Devasena and Menon (2007) reported that *T. foenum-graecum* seed increased SOD-2 hepatic OS during colon cancer.

In this part of the study, results indicate that *A. afra* and *T. foenum-graecum* extracts increase SOD-2, which improves the inflammatory state and has beneficial effects on glucose and lipid metabolism (Giacco & Brownlee, 2010; de Assis et al., 2012). However, the result shows a possibility of regulating inflammation factors by generating SOD-2 with decreased LPL-induced OS (Xie et al., 2016). Furthermore, our findings also confirm that the inhibition of macrophages-derived LPL activity has beneficial effects on lipid accumulation and inflammatory response in DM (Tian et al., 2012; Tian et al., 2014).

The pathogenesis of T2DM is associated with OS and chronic inflammation, implicated in dyslipidaemia and microvascular diabetes complications. For that reason, it is suggested to evaluate the potential effects of antioxidants and regulators of dyslipidaemia to prevent the development of DM (Abdollahi et al., 2013; Nasri & Rafieian-Kopaei, 2013). Current data indicate that these herbal therapies have the potential to inhibit LPL

activity and antioxidant properties. Thus, traditional therapies in Africa appear to be a novel therapeutic strategy in diabetes for patients with metabolic disorders.

6.5. Conclusion

There is no doubt that T2DM is one of the most prevalent complex disorders with prolonged hyperglycaemia and dyslipidaemia in the global healthcare system. Furthermore, it has been linked with conditions of OS and chronic inflammation. Currently, researchers still evaluate medication to prevent or treat the progress of diabetes complications. Results of the current study strongly suggest that *A. afra* and *C. verum* have higher antioxidant activity and regulate the LPL activity more efficiently than *T. foenum-graecum* and metformin. This finding may open the way to novel therapeutic strategies in managing metabolic disorders in diabetes. Thus, it appears that future studies will be required to address the bio-active molecules of these herbal therapies involved in this protocol. In addition, the mechanisms by which these herbal therapies regulate the suppression of the LPL activity and up-regulate the SOD-2 antioxidant factor in the macrophage need interpreting.

Chapter 7:

Conclusions and recommendations

7.1. General conclusions

In this study, the aim was to determine the efficacy of some herbal therapies commonly used in African countries and compare these to Western medicines, usually prescribed for managing T2DM using *in vitro* assays. For this study, we also intended to identify potential biomarkers that could be monitored for proteome profiling and enzyme bio-activity to compare the protein expression of cells cultured under normal glucose and HG (diabetes) conditions.

The murine macrophage RAW 264.7 cell line culture was exposed to the herbal therapies under normal glucose and HG concentrations. These conditions included basal levels and simulated the pro-inflammatory response and metabolic disorder. The research focused on using pharmacological and traditional therapies to prevent the progression of diabetes.

For the treatment of T2DM, various ways are currently in practice to regulate and lower blood sugar levels. One of the biggest challenges in managing T2DM is its complications besides minimising the side effects of prescribed medication. Thus, researchers are investigating possible therapeutic strategies that can reduce chronic inflammation and regulate metabolic disorders, including preventing the progression of this condition.

7.2. Specific conclusions

Two chapters, the introduction and an overview of the literature, for this study dealt with the following topics: T2DM, inflammation and metabolic disorders, including Western diabetes medicine and traditional herbs;. Then there are four chapters in which plant

extracts are subjected to chemical analysis, and the anti-inflammatory and antioxidant properties of these extracts are evaluated. We also assessed cell viability and expression of inflammation biomarkers through experiments as reported in these chapters. Specific conclusions of the present study are presented in the chapters that follow.

Chapter 3 – in this chapter, the chemical composition of plant extracts was prepared and analysed. The results show that 39, 110 and 46 chemical compounds were found in extracts of the *T. foenum-graecum, C. verum* and *A. afra* analysis, respectively. Furthermore, 30, 97 and 34 novel chemical compounds were separated from the ethanolic extracts of *T. foenum-graecum*, *C. verum* and *A. afra,* respectively, and nine, 13 and 12 chemical compounds in *T. foenum-graecum*, *C. verum* and *A. afra* respectively, were found parallel to previous studies.

The chemical composition of *T. foenum-graecum, C. verum* and *A. afra* showed the highest concentrations of Hexadecanoic acid (31.06%), Cinnamaldehyde-(E) (35.43%) and Artemisia ketone (36.05%), respectively. Previous studies demonstrated that some chemical compounds of *T. foenum-graecum* and *C. verum* and phytochemical compounds in *A. afra* have the potential for anti-inflammation and antioxidant properties. These findings might be a promising therapeutic strategy for the control of type 2 diabetes mellitus.

Chapter 4 –cell viability and inflammatory cytokines and chemokines were evaluated after exposing RAW 264.7 cells to different glucose concentrations with and without lipopolysaccharide. Our results indicated that the effects of different glucose concentrations with and without lipopolysaccharide on the viability of RAW264.7 cells

decreased in cell viability at 45 mM, but with no effects on glucose at 25 mM glucose concentration compared to 5 mM glucose.

These results show that the macrophage cells expressed the cytokines ICAM-1, MIP-1α, MIP-1β, MIP-2**,** IL-16, IL-1ra and IL-27 in LG (5 mM) unstimulated; while in HG (25 mM) unstimulated, it expressed ICAM-1, MIP-1α, MIP-1β and IL-16 after incubation of 48 hours. The cells not expressing cytokines MIP-2, IL-1ra and IL-27, and also lower-regulating MIP-1β expression levels in high-stimulated glucose compared to low-stimulated glucose.

The results showed that cytokines and chemokines were expressed at higher levels in LG and HG after lipopolysaccharide stimulation with the expression of IP-10, I-TAC, TNF-α, G-CSF, IL-6, MCP-1, MIG, ICAM-1, MIP-1α, MIP-1 β, IL-16, IL-1α, MIP-2, RANTES, IL-1ra and IL-27. Comparing cell expression to low and high-glucose unstimulated results has shown down-regulation of cytokines IL-16, IL-1ra and IL-27. Comparing cell expression to low and high-glucose unstimulated results has shown down-regulation of cytokines MCP-1, IL-16, IL-1ra and IL-27. Cell no expression of cytokine IL-1α in HG with LPS, compared to LG stimulated. These proteins can be biomarkers and may be used to regulate inflammation, IR and angiogenesis. It may also describe defects in the pathophysiology of T2DM and offer an advantage in drug screening.

Chapter 5 – we evaluate cell viability and effects of African medicinal plants to regulate the pro-inflammatory biomarkers related to diabetes mellitus in this chapter. These biomarkers were produced by RAW 264.7 cell line after being exposed to HG and lipopolysaccharide. Results have shown that *A, afra, C. verum, T. foenum-graecum*

extracts and the metformin solution reduce cell proliferation at different concentrations, *A afra* (≥ 250 µg/ml); *C. verum* (≥ 500 µg/ml); *T. foenum-graecum* (> 2500 µg/ml); and metformin (≥ 250 µg/ml).

We also found that the different herbs have a differential IC_{50} on cytokine and chemokine markers. This current finding showed that these natural herbal extracts have a higher effect than metformin in inhibiting pro-inflammatory responsee and regulating the oxidant activity in HG. Also, the study has shown that the best herbal agent, *A. afra*, has a higher anti-inflammatory activity than the other herbs. This result can be a target for treating the progression of diabetes complications with minimal side effects. Moreover, results confirm that DM is strongly implicated with the inflammatory state that may cause the progression of diabetes complications.

Chapter 6 – we determined macrophage metabolic bio-active marker expression. In addition, we assessed if the African medicinal plants regulate lipid dysfunction and OS in LG and HG with stimulation. Macrophage was treated at concentrations of *A. afra* (200 µg/ml), *C. verum* (250 µg/ml), *T. foenum-graecum* (2500 µg/ml) and metformin (250 µg/ml) with stimulation of RAW 264.7 cells (In the current study, it was measured by the concentration of plant extracts and metformin that affected cell viability). The following desired effects were obtained, *A. afra* can suppress lipoprotein lipase and NO and up-regulate superoxide dismutase enzyme in HG with stimulation. Compared with extracts of *C. verum*, lipoprotein lipase and nitric oxide can be suppressed, while the *T. foenum-graecum* extract can suppress NO and up-regulate the superoxide dismutase enzyme in HG with stimulation. These plants, when compared to metformin, can suppress lipoprotein lipase activity in HG with stimulation. Hence, it can be concluded, that *A. afra* could play a vital role in the treatment of metabolic disorders by reducing

inflammation and OS, supported by the marker evaluation, and may prevent the progression of complications of diabetes.

7.3. Future perspectives and recommendations

Diabetes patients should be monitored and supplemented with anti-inflammation and antioxidant therapies combined with Western medicine to achieve beneficial treatment to avoid the progression of diabetes complications.

The herbal therapies which were evaluated using extract forms should in future be used in the substance molecules in a similar condition in this research.

Interpreting the mechanisms by which these herbal therapies regulate the suppression of the pro-inflammatory modulators and metabolism biomarkers in the RAW 264.7 cells needs to be investigated.

In this study, only extracellular cytokines and chemokines and intracellular protein for metabolism biomarkers were monitored. A proteome profile of intracellular proteins and extracellular bioactive markers should be considered for future studies as this would give an overall picture of how the herbal therapies interact with the cells and the potential pathways that they may affect or interfere.

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