AN INVESTIGATION OF THE ANTI-HYPERGLYCAEMIC, BIOCHEMICAL AND MOLECULAR EFFECTS OF 4-HYDROXYISOLEUCINE AND FENUGREEK SEED EXTRACT IN COMPARISON TO METFORMIN IN VITRO AND IN VIVO

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DECLARATION

I, Nikita Naicker, declare that:

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Signed:                                          16th July 2018
Ms. Nikita Naicker                            Date
DEDICATION

To my grandfather, grandmother and aunt, the late Mr M. Naicker, Mrs D. Durgapersad and Mrs B. Naidoo. I am honoured to dedicate my accomplishment to the both of you. You both have imparted the desire to pursue sheer happiness, righteousness, brilliance, boundless knowledge and unconditional love, for which I am undoubtedly grateful.
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God
I am thankful for the wisdom, knowledge and strength to persevere through every trial and tribulation, and appreciate every victory.

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Published manuscripts:


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2. Nikita Naicker; Savania Nagiah; Pragalathan Naidoo; Sanil Singh; Linda Bester; Sooraj Baijnath; Anand Krishnan; Anil Chuturgoon (2017). 4-hydroxyisoleucine regulates PCSK9 and PPAR during dyslipidemia, in hyperglycaemic HepG2 cells and C57BL/6 male mice. European Journal of Pharmacology (In review) – Manuscript no. EJP-47425.

3. Nikita Naicker; Savania Nagiah; Pragalathan Naidoo; Sanil Singh; Linda Bester; Sooraj Baijnath; Anand Krishnan; Anil Chuturgoon (2017). 4-hydroxyisoleucine potentiates antioxidant regulators; Nrf2, LonP1 and SIRT3 during chronic hyperglycaemia in HepG2 cells and C57BL/6 male mice. Submitted to Biomedicine & Pharmacotherapy (In review) – Manuscript no. BIOPHA_2017_6359.
PRESENTATIONS


2. Poster presentations: International Conference on Targeting Diabetes and Novel Therapeutics September 14-16, 2015 Las Vegas, Nevada, USA. Trigonella foenum-graecum seed extract promotes cholesterol and lipid synthesis, in comparison to insulin, under a hyperglycaemic condition in HepG2 cells.

3. Poster presentations: International Conference on Targeting Diabetes and Novel Therapeutics September 14-16, 2015 Las Vegas, Nevada, USA. Trigonella foenum-graecum seed extract, 4-hydroxyisoleucine and metformin stimulate proximal insulin signaling and increases expression of glycogenic enzymes and GLUT2 in HepG2 cells.

4. Poster presentation: College of Health Science, School of Laboratory Medicine and Medical Sciences, annual research day 2016 - University of KwaZulu-Natal – Graduate School of Business and Leadership Auditorium, Westville Campus, Durban. 05 August 2016. Trigonella foenum-graecum seed extract promotes cholesterol and lipid synthesis, in comparison to insulin, under a hyperglycaemic condition in HepG2 cells.

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Contents

DECLARATION ................................................................................................................................. i
DEDICATION ............................................................................................................................... ii
ACKNOWLEDGEMENTS .................................................................................................................. iii
PUBLICATIONS ............................................................................................................................. iv
PRESENTATIONS .......................................................................................................................... v
LIST OF FIGURES ........................................................................................................................ x
LIST OF TABLES ........................................................................................................................... xii
ABBREVIATIONS .......................................................................................................................... xiii
ABSTRACT ...................................................................................................................................... xvi
CHAPTER ONE ............................................................................................................................. 1
INTRODUCTION ............................................................................................................................ 1

  2.8 References ............................................................................................................................ 4

CHAPTER TWO ............................................................................................................................. 9
LITERATURE REVIEW ................................................................................................................... 9

  2.1 Type 2 Diabetes Mellitus .................................................................................................... 9
    2.1.1 Epidemiology .................................................................................................................. 9
    2.1.2 Associated Risk Factors ................................................................................................. 10
    2.1.3 Screening and Diagnosis ............................................................................................... 11
      2.1.3.1 Diagnostic Tests for Type 2 Diabetes Mellitus ......................................................... 12
    2.1.4 Management .................................................................................................................. 13
      2.1.4.1 Pharmacological Agent: Metformin ......................................................................... 13
    2.1.5 Pathophysiology .......................................................................................................... 15
  2.2 Glucose Sensing and Insulin Receptor Signalling ............................................................... 16
    2.2.1 Insulin Signalling .......................................................................................................... 17
  2.3 Lipid Metabolism .................................................................................................................. 18
    2.3.1 PCSK9, an Inhibitory Regulator of LDLr ...................................................................... 20
    2.3.2 PPARG; A Positive Regulator of Lipid Metabolism ...................................................... 22
  2.4 Oxidative Stress and Mitochondrial Health ......................................................................... 25
    2.4.1 Nuclear factor erythroid 2-related factor 2 and the Antioxidant Response ................. 26
CHAPTER 7
SYNTHESIS, CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH

6.1 Synthesis ................................................................. 125
6.2 General conclusions ................................................... 126
6.3 Implications for further research ..................................... 127
6.4 References ............................................................... 127

CHAPTER 7 ........................................................................ 129
Appendix 1: Animal ethics approval ................................................................. 129
Appendix 2: Supplementary data for chapter 3 (Table numbers correlate with chapter 3) .... 130
Appendix 4: Supplementary data for chapter 4 (Table and figure numbers correlate with chapter 4) .................................................................................................................. 134
Appendix 4: Supplementary data for chapter 5 (Table numbers correlate with chapter 5) ...... 141
Appendix 5: Liquid chromatography–mass ........................................................................ 147
Appendix 6 .................................................................................................................... 151
Appendix 7 .................................................................................................................... 163
LIST OF FIGURES


Figure 2.2: Mechanism of action of metformin on hepatic and muscle glucose metabolism (Brufani et al. 2013). AMPK: Adenosine monophosphate-activated protein kinase, ACC; acetyl-coenzyme A carboxylase, SREBP-1; sterol regulatory element-binding protein-1, VLDL; very-low density lipoprotein. ............................................................................................................................................. 14

Figure 2.3: Pathophysiology of normal and abnormal glucose metabolism in type 2 diabetes mellitus (Shulman 2000). Normal glucose metabolism during a (A) fed state and (B) fasted state. “Abnormal” glucose metabolism during a (C) fed state and (D) fasted state.................................................................................................................. 16

Figure 2.4: Normal physiological process of insulin on genes and proteins involved in the proximal and distal signalling cascade (Prepared by author).................................................................................................................. 18

Figure 2.5: Regulation of plasma lipoproteins during elevated glucose levels by proprotein convertase subtilisin-like/kexin type (PCSK9) and peroxisome proliferator-activated receptor gamma (PPARG) (Prepared by author). .................................................................................................................. 20

Figure 2.6: Low density lipoprotein receptor mediated pathway (http://i0.wp.com/www.namrata.co/wp-content/uploads/2012/11/cholesterol-metabolism.jpg?resize=628%2C484) .................................................................................................................. 21

Figure 2.7: Cellular trafficking of PCSK9 (Poirier and Mayer 2013) .................................................................................................................. 22

Figure 2.8: Production and metabolism of high density lipoprotein via reverse cholesterol transport (https://www.nature.com/articles/nrneph.2015.180/figures/1) .................................................................................................................. 23

Figure 2.9: Protective functions of high density lipoproteins (https://www.nature.com/articles/nrneph.2015.180/figures/2) .................................................................................................................. 25

Figure 2.10: Schematic representation presenting the mechanism of cytoprotection via Nrf2 activators (Rashmi et al. 2017).................................................................................................................. 29

Figure 2.11 Regulation of mitochondrial dysfunction, during type 2 diabetes mellitus via Nrf2, LonP1, SIRT3 and PGC-1α (Prepared by Author).................................................................................................................. 31

Figure 2.12 Trigonella foenum-graecum (A) leaves, (B) pods and (C) seeds used for medicinal purposes (http://www.punmiris.com/himg/o.12961.jpg) .................................................................................................................. 33
Figure 2.13 Dark yellow, four-faced stone like structure of fenugreek seeds (http://qafexporters.com/wp-content/uploads/2015/08/1280px-Methi_by_49264526.jpg) ............................................................................................................................................. 34

Figure 2.14 Chemical structure of 4-hydroxyisoleucine
(http://www.sigmaaldrich.com/content/dam/sigmaaldrich/structure3/194/mfcd07357252.eps/_jcr_content/renditions/mfcd07357252-large.png) ............................................................................................................................................. 35
LIST OF TABLES

Table 1 Recommendations for the screening of type 2 diabetes mellitus

Table 2 Criteria for the diagnosis of type 2 diabetes mellitus
ABBREVIATIONS

AMPK  5’ adenosine monophosphate-activated protein kinase
ACC  Acetyl-CoA carboxylase
AGEs  Advanced glycation end-products
Akt  Protein Kinase B
ARE  Antioxidant response element
ATP  Adenosine triphosphate
AO  Antioxidant
Apo A1  Apolipoprotein A1
BSA  Bovine serum albumin
BW  Body weight
C  Control
CB  Citrate buffer
CCM  Complete culture medium
cDNA  Complementary deoxyribonucleic acid
CVD  Cardiovascular disease
CHO  Carbohydrate
DAG  Diacylglycerol
DBC  Diabetic control
DM  Diabetes mellitus
ETC  Electron transport chain
FA  Formic acid
Gck  Glucokinase
GIT  Gastrointestinal tract
GLUT  Glucose transporter
GPx  Glutathione Peroxidase
GSK3α/β  Glycogen synthase kinase 3α/β
GS  Glycogen synthase
GSH  Reduced glutathione
4-OH-Ile  4-hydroxyisoleucine
FA  Fatty acids
FFA  Free fatty acids
FPG  Fasting plasma glucose
FSE  Fenugreek seed extract
Hr    Hour
HDL  High density lipoprotein
HDLc High density lipoprotein cholesterol
HepG2 Human hepatoma cell line
HG   Hyperglycaemic
H2O2 Hydrogen peroxide
IDF  International diabetes federation
I.p  Intraperitoneal
IR   Insulin receptor
IRβ  Insulin receptor β
IRS  Insulin receptor substrate
Keap1 Kelch-like ECH-associated protein 1
LDL  Low density lipoprotein
LDLc Low density lipoprotein cholesterol
LDLr Low density lipoprotein receptor
LonP1 Lon protease 1
MF   Metformin
Min  Minute
NCD  Non-communicable disease
MDA  Malondialdehyde
ND   Non-diabetic
NFDM Non-fat dry milk
NG   Normoglycaemic
NSP  Non-starch polysaccharides
Nrf2 Nuclear factor E2-related factor 2
OGTT Oral glucose tolerance test
OS   Oxidative stress
p-   Phosphorylated-
PBS  Phosphate buffer solution
PKC  Protein kinase C
PPARG Peroxisome proliferator-activated receptor gamma
PCSK9 Proprotein convertase subtilisin-like/kexin type
PGC-1α Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ptd</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>p85 regulatory subunit of PI-3-kinase</td>
</tr>
<tr>
<td>PG</td>
<td>Plasma glucose</td>
</tr>
<tr>
<td>RBD</td>
<td>Relative band density</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>Sec</td>
<td>Second</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Sirtuin</td>
<td>SIRT</td>
</tr>
<tr>
<td>SREBP1c</td>
<td>Sterol regulatory binding element 1c</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TC</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween20-Tris-buffered saline</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
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<td>WHO</td>
<td>World health organization</td>
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Type two diabetes mellitus (T2D) is a significant cause of premature death and disability, accompanied with negative socio-economic impacts. This metabolic disorder is characterized by hyperglycaemia and defective insulin signalling. Long-term exposure to hyperglycaemia gives rise to altered fat metabolism and reactive oxygen species (ROS) generation. These precursors are central to the progression of dyslipidaemia and attenuated antioxidant (AO) response and detoxification system, respectively. Diabetic dyslipidaemia and oxidative stress (OS) are risk factors for the onset and progression of cardiovascular disease (CVD) and other diabetic complications. The treatment regimen for T2D comprises self-care and anti-diabetic drugs such as metformin. However, due to the lack of compliance to self-care recommendations and some undesirable side effects of metformin, there is the necessity for alternate therapy.

Natural products have been used for the treatment of many disorders, including T2D. Trigonella foenum-graecum commonly known as fenugreek is a plant that possesses anti-diabetic effects. These effects are attributed to its bioactive compound – 4-hydroxyisoleucine (4-OH-Ile), which constitutes approximately 80% of the bio-composition of the fenugreek seed. Despite these effects, biochemical and molecular effects of 4-OH-Ile on insulin signalling, lipid metabolism, and ROS production is not well-documented. This study investigated the effects of 4-OH-Ile in comparison to metformin and fenugreek seed extract (FSE) on hyperglycaemic human hepatoma (HepG2) cells and C57BL/6 male mice. Treatments were conducted under normoglycaemic and hyperglycaemic conditions as follows; control, 4-OH-Ile (in vitro: 100ng/ml; in vivo: 100mg/kg Body weight) metformin (in vitro: 20mM; in vivo: 20mg/kg Body weight) and FSE (in vitro: 100ng/ml; in vivo: 100mg/kg Body weight) treatment groups. The experiments included; blood glucose measurements, lipid profile analysis, spectrophotometric assays (in vitro), western blotting for protein expression and qPCR for mRNA expression.

First, to validate the effects on insulin signalling and glucose sensing, glucose levels were measured with completion of an oral glucose tolerance test. 4-OH-Ile treatment attenuated glucose levels, and elevated the mRNA levels of glycogen synthase (GS) and glucokinase (Gck). This was followed by the investigation of the protein and gene expression of insulin signalling regulators: insulin receptor β (IRβ), insulin receptor substrate 1 (IRS1), phosphorylated protein kinase B (pAkt), phosphorylated glycogen synthase kinase 3α/β (pGSK3α/β) and glucose transport 2 (GLUT2). In in vivo hyperglycaemia, 4-OH-Ile increased the expression of the investigated proteins and genes. The results showed that 4-OH-Ile was just as potent as MF, and FSE in stimulating the insulin signalling cascade.
Second, the effect of 4-OH-lle on dyslipidaemia was investigated by measuring mRNA levels of sterol regulatory binding element 1c (SREBP1c) and fatty acid synthase (FAS) – key factors in fatty acid metabolism. Both genes were up-regulated and correlated with the changes in triglyceride and cholesterol levels. Next the protein expression of proprotein convertase subtilisin-like/kexin type (PCSK9) - a regulator of low density lipoprotein cholesterol (LDLc) and peroxisome proliferator-activated receptor gamma (PPARG) – a regulator of high density lipoprotein (HDLc) was evaluated. The data showed that 4-OH-lle down-regulated protein and mRNA expression of PCSK9 and up-regulated protein expression of PPARG. The reduction in PCSK9 levels correlated with the changes observed in low density lipoprotein receptor (LDLr) and LDLc, whereas the increase in PPARG correlated with the elevated mRNA expression of apolipoprotein A1 (Apo A1) and HDLc. Together these results provide substantial evidence for the regulatory effect of 4-OH-lle, in comparison to metformin, and FSE on PCSK9, PPARG and related lipid factors.

Finally, the effect of 4-OH-lle on redox status and AO response was assessed by measuring nuclear factor E2-related factor 2 (Nrf2). In both models, there was an increase in the protein expression of phosphorylated Nrf2 accompanied by an increase in mRNA levels of superoxide dismutase 2 (SOD2) and glutathione peroxidase (GPx), and GSH levels. Mitochondria play a central role in contributing to elevated ROS levels. While nuclear responses like Nrf2 regulate ROS, mitochondria possess their own maintenance proteins. These include mitochondrial Lon protease 1 (LonP1), Sirtuin 3 (SIRT3) and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) which play an integral role in combatting OS and mitochondrial dysfunction. The results showed that 4-OH-lle displayed a potent effect in inducing the AO response and increasing mitochondrial regulatory proteins.

In conclusion, 4-OH-lle improved the compromised insulin signalling and the altered lipid profile as well as induced the AO response and mitochondrial maintenance proteins, in the presence of elevated glucose. Furthermore, the effect of 4-OH-lle was greater than the first-line drug; metformin and FSE, albeit in cultured human liver cells and a mouse model. Also, the crude seed extract displayed promising effects on all investigated parameters. Considering the active role of chronic hyperglycaemia in the onset and progression of CVD and diabetic complications, 4-OH-lle poses as a highly favourable alternate therapy in the treatment of T2D. Moreover, this has great importance in socio-economically challenged communities where T2D is a common disorder, access to healthcare facilities is limited, and plants serve as sources of easily accessible treatments.
CHAPTER ONE
INTRODUCTION

Type 2 diabetes mellitus (T2D) is a chronic, progressive disorder attributed to exacerbated levels of blood glucose. Chronic hyperglycaemia observed in affected individuals’ increases the risk of defective insulin signalling, resulting in insulin resistance. T2D poses a global healthcare problem and has been listed amongst the four priority non-communicable diseases (NCDs) (WHO 2016). Globally in 2014, approximately 422 million individuals were diagnosed with diabetes mellitus (DM), in comparison to 108 million individuals in 1980 (WHO 2016). Evidence shows that the incidence of DM has almost doubled since 1980, escalating from 4.7 to 8.5% in the mature population (WHO 2016). In 2015 the international diabetes federation (IDF) reported 2.28 million cases (7% of adults’ aged 21 to 79) of DM in South Africa, however, a significant number of these individuals remain undiagnosed (The International Diabetes Federation 2015). DM and its complications result in a considerable economic loss to affected individuals and their relatives, healthcare systems and national economies. The socio-economic impact of T2D has pushed the biochemical and molecular investigations of this disorder to the forefront of medical research.

The main contributors to the development of T2D are excessive calorie consumption and sedentary lifestyles. In addition, individuals who are genetically predisposed to this disorder are considered high risk (WHO 2016). Rapid urbanization has exposed South Africans to high caloric diets and processed foods which contribute to increased rates of T2D. Intense advertising food campaigns, non-conducive physical activity environments and lack of interest by the government further exacerbates the problem. The healthcare system provides comprehensive facilities for DM prevention and care but these facilities are not commonly implemented (WHO 2016). This could be the consequence of a lack of funds or maladministration, resulting in poor treatment. Furthermore, the public sector is known for shortages of drugs, and improved treatments with minimal side effects has yet to be discovered. In addition to drug shortages, high treatment costs in both public and private sectors are also contributing factors (WHO 2016). A key impediment to successful T2D therapy is the lack of inexpensive insulin, ultimately resulting in diabetic complications and premature deaths (WHO 2016). It has been reported that oral hypoglycaemic agents, as well as insulin, are only obtainable in a minority of low-income countries. Moreover, medicines that are critical to controlling DM as well as its complications, are often inaccessible in both low and middle-income nations (WHO 2016).
The liver is the metabolic hub of the body and central to glucose metabolism. Hepatic insulin signalling is fundamental for metabolic processes such as gluconeogenesis, glycogen storage and recruitment of glucose transporters (GLUT) (Leturque et al. 2009). Insulin signalling is regulated by insulin receptor (IR) which is responsible for the phosphorylation and stimulation of insulin receptor substrate (IRS). This activation is crucial for the integration of extracellular signals into intracellular responses (Copps et al. 2016). These intracellular responses include the stimulation of protein kinase B (Akt) which potentiates the phosphorylation of glycogen synthase kinase 3α/β (GSK3α/β), and recruitment of GLUT2. During T2D, compromised IR signalling and accompanied downstream events lead to insulin resistance, resulting in the disruption of the body’s physiological response to hyperglycaemia. Consequently, attenuating hyperglycaemia and targeting defective IR signalling is central in maintaining glucose homeostasis.

Following defective insulin signalling, hyperglycaemia induces the over-production of free fatty acids (FFA), resulting in an abnormal lipid profile; termed dyslipidaemia. Diabetic dyslipidaemia is characterised by reduced plasma levels of high density lipoprotein (HDL) and elevated low density lipoprotein (LDL), and triglycerides (TG) (Mullugeta et al. 2012). Restoration of plasma lipoproteins involve targeting abnormal LDL cholesterol (c) and HDL cholesterol (c) levels. New advancements in lipid-lowering agents has focused on regulators of proprotein convertase subtilisin/kexin type 9 (PCSK9) and agonists of peroxisome proliferator-activated receptor gamma (PPARG). PCSK9 functions as an LDL receptor (LDLr) inhibitor, targeting the receptor for lysosomal degradation (Burke et al. 2017). Therefore, a reduction in PCSK9, increases the LDLr pool ultimately reducing LDLc. PPARG agonists positively regulate PPARG expression, which in turn increases HDLc levels (Gervois et al. 2000). In T2D, diabetic dyslipidaemia poses a risk for the progression of atherosclerosis and subsequent cardiovascular disease (CVD). As a result, targeting both PCSK9 and PPARG could serve as prospective therapeutic interventions in T2D therapy.

The cytotoxic effects of hyperglycaemia are further exacerbated by excessive reactive oxygen species (ROS) production, causing a redox imbalance termed oxidative stress (OS). T2D individuals are negatively affected by OS, which is important in the onset and progression of CVD and diabetic complications (Baynes 1991). Uncontrolled ROS production impairs the antioxidant (AO) response and detoxification system, elevates protein glycosylation and promotes mitochondrial dysfunction (Baynes 1991). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a master regulator in controlling the expression of AO defence proteins (Allard et al. 2016). Activated Nrf2 directly impacts the expression of cytoprotective genes: mitochondrial superoxide dismutase 2 (SOD2) and glutathione peroxidase (GPx), and non-enzymatic AO: reduced glutathione (GSH) (Giralt and Villarroya 2012, Dinkova-Kostova and
Abramov 2015). In addition to hyperglycaemic-induced ROS, mitochondria are also accountable for elevating ROS production via oxidative phosphorylation. Mitochondria possess Lon protease 1 (LonP1) which proteolytically clears glycosylated proteins, that ultimately forming advanced glycation end-products (AGEs) (Pomatto et al. 2017). Another mitochondrial regulatory pathway involves NAD-dependent deacetylase Sirtuin 3 (SIRT3) that target proteins required for energy metabolism and the rate of ROS production (Buler et al. 2012, Giralt and Villarroya 2012). This deacetylase activates peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) which sequentially induces the ROS-detoxifying gene SOD2 and drives mitochondrial biogenesis (Kong et al. 2010). Accordingly, controlling defective insulin signalling and hyperglycaemia is central to regulating dyslipidaemia, and OS which is essential in managing CVD and diabetic complications.

The T2D regimen comprises of both self-care and anti-diabetic drugs. Self-care includes physical activity, no smoking, weight loss, diabetic diet and nutritional counselling. Following the failure of self-care treatment, the use of anti-diabetic drugs is the next line of treatment. Anti-diabetic drugs include multiple classes and their selection is dependent on the severity of DM and accompanying factors. T2D treatment include agents that raise insulin secretion in the pancreas, promote the sensitivity of specific organs to insulin and reduce glucose absorption rate in the gastrointestinal tract (GIT) (Haupt et al. 1991, Hermann et al. 1991, Owen et al. 2000). These agents are grouped into the following classes: alpha-glucosidase inhibitors, glycosurics, peptide analogs, sensitizers and secretagogues. The widely used first-line drug for treatment metformin (MF) belongs to the sensitizer group of drugs. This oral hypoglycaemic agent reduces hepatic glucose output and improves tissue sensitivity to insulin (Anisimov 2013). Although highly effective, MF is associated with some undesirable side effects such as dizziness, diarrhoea, and nausea and/or vomiting (Hermann 1979, Siavash et al. 2017). Consequently, poor self-care and the shortfalls of first-line therapy urges the requisite for complementary or alternate medicine for the treatment of T2D.

The National Centre for Complementary and Alternative Medicine propose natural products for the possible treatment of T2D. Several studies support the use of various plants for their glucose lowering effects such as: bitter melon, fenugreek, gymnema, ginseng, prickly pear cactus and tronadora (Shapiro and Gong 2002, Tundis et al. 2010, Rios et al. 2015). Fenugreek (Trigonella foenum-graecum) is a yearly legume belonging to the Fabaceae family (Rios et al. 2015). It is known in the culinary world as a spice, enhancing the taste of food, and the medicinal world for its anti-diabetic, anti-carcinogenic, hypcholesterolaemic, AO and immunological properties (Rios et al. 2015). These reported effects are attributed to a bioactive compound - 4-hydroxyisoleucine (4-OH-lle), a peculiar amino acid located within
the plant’s seed. Numerous reviews have reported the medicinal value of fenugreek and its active compound but there is insufficient evidence in supporting its biochemical and molecular effects in T2D (Basch et al. 2003, Fuller and Stephens 2015, Rios et al. 2015). With regards to T2D, chronic hyperglycaemia negatively affects insulin signalling, lipid metabolism, and OS. Consequently, the regulation of these integrated pathways will allow for the restoration of glucose, lipids and ROS levels. Studies provide evidence from cell culture and animal models which demonstrated the ability of 4-OH-lle and fenugreek seeds in stimulating the insulin signalling pathway and enhancing insulin sensitivity (Fowden et al. 1973, Sauvaire et al. 1998, Broca et al. 2000, Maurya et al. 2014, Naicker et al. 2016). Furthermore, studies also reported the glucose lowering effect of both 4-OH-lle and fenugreek seeds (Broca et al. 1999, Singh et al. 2010, Haeri et al. 2012). 4-OH-lle and fenugreek seeds also regulate plasma TGs, total cholesterol, FFAs, HDLc and LDLc, accompanied by the improvement of liver function (Narender et al. 2006, Haeri et al. 2009, Singh et al. 2010, Avalos-Soriano et al. 2016). Diabetic animal studies have reported fenugreek and 4-OH-lle to reduce abnormal ROS levels via the regulation of thiobarbituric acid reactive substances (TBARS), catalase, SOD2 and related liver enzymes comparable to controls as well as disrupt free radical metabolism (Ravikumar and Anuradha 1999, Mohamad et al. 2004, Dixit et al. 2005, Belguith-Hadriche et al. 2010, Dutta et al. 2014).

These studies provide compelling evidence on the anti-hyperglycaemic, anti-lipidemic and anti-oxidative effects of both fenugreek and its active compound. However, there is a lack of evidence focusing on the effect of 4-OH-lle in comparison to MF, and fenugreek seed on the specific biochemical and molecular responses within these integrating pathways. Understanding the interaction of this plant extract and its active compound will further enable its use as possible anti-diabetic agents. As a result, we hypothesize that 4-OH-lle and FSE regulate genes and proteins responsible for attenuating hyperglycaemia, dyslipidaemia and OS in hyperglycaemic HepG2 cells and C57BL/6 male mice.

2.8 References


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CHAPTER TWO
LITERATURE REVIEW

2.1 Type 2 Diabetes Mellitus

Diabetes, a disease initially described as sweet urine disease, has been affecting individuals for decades. In the early 1910’s, the American Diabetes Association reported that medical professionals initiated the first steps towards determining a cause and treatment regimen for DM (Kohen 2004). Insulin injections were the first effective treatment for this disorder, however, some cases were unresponsive to insulin injections (Kohen 2004). In 1963, Diabetic Medicine published that “sweet urine disease” could be defined as “insulin-sensitive” and “insulin-insensitive” (Kohen 2004, Zarshenas et al. 2014). The terms went on to be further categorised as type 1 diabetes mellitus (T1D) and T2D. T1D is regarded as an autoimmune disorder targeting pancreatic β-cells, rendering the individual incapable of producing insulin (Ashcroft and Rorsman 2012). T2D comprises of an array of hyperglycaemic-induced dysfunctions; accompanied with insufficient insulin secretion, resistance to insulin action, and disproportionate and/or inappropriate secretion of glucagon (Kohen 2004). With regards to T1D, there is no cure, however, treatment involves the infusion of insulin with a syringe, insulin pen or pump (Kohen 2004). As with T1D, there is no cure for T2D however, this condition can be managed in numerous ways such as: lifestyle modifications (control of carbohydrate (CHO) intake, physical activity, and weight loss), pharmacological agents and insulin. Therefore, it has become apparent that individuals diagnosed with T2D are efficiently able to reverse this disorder.

2.1.1 Epidemiology

In 2014, it was reported by the world health organization (WHO) that approximately 9% of the world’s inhabitants were burdened with DM, and more than 90% of these cases were T2D (WHO 2016). Among the list of NCDs, DM soars among the four main diseases, which include CVD, cancers and chronic lung diseases (WHO 2016). T2D is responsible for approximately 5 million deaths per year (WHO 2016). By the year 2030, it is expected that T2D will escalate to the 7th cause of death worldwide. T2D is associated with obesity, and the major burden arises in developing and middle-income nations due to urbanisation (WHO 2016). T2D is often undiagnosed and investigations to measure the number of newly occurring cases are complex, resulting in limited data on true incidence. Furthermore, in low- and middle-income countries there are limited statistics on the income gradient of DM (WHO 2016). However, existing statistics propose the prevalence of DM to be highest among the wealthy population, but this trend is reversing in a few middle-income countries (WHO 2016). For instance in sub-Saharan Africa, the number of individuals with DM is predicted to escalate from 19.8 million (2013) to 41.5 million (2035) (WHO
In 2015, the IDF reported 2.28 million cases of DM in South Africa, conversely, reports have revealed that there is inadequate statistics accessible on the prevalence of T2D in South Africa (Olokoba et al. 2012, WHO 2016). However, studies investigating data trends within Africa provide evidence of a dramatic rise in prevalence in rural and urban settings (Olokoba et al. 2012). In Africa, the majority of the DM burden is T2D, with less than 10% of cases being T1D (Olokoba et al. 2012). The prevalence of T2D in adults is predicted to increase in the next two decades.

2.1.2 Associated Risk Factors
The increasing prevalence of T2D has a massive impact on the healthcare system worldwide. Recent statistics from the IDF propose that DM is directly responsible for approximately 5 million deaths annually, exceeding the combined burden of HIV/AIDS, tuberculosis and malaria (Bailey et al. 2016). DM, if poorly controlled, may cause blindness, kidney failure, lower limb amputation and numerous long-term consequences that negatively impact the quality of life (Baquer et al. 2009). However, there is huge potential in attenuating and reversing the DM pandemic via the modification of risk factors through lifestyle changes. There are several risk factors which are associated with T2D (Fig 2.1). These include:

- **Age**, a driving force in the incidence of T2D. As an individual ages, both impaired fasting glucose and glucose intolerance are progressively prevalent (Pippitt et al. 2016). Evidence estimates a normal glucose metabolism of approximately 30% over the age of 80 years old (Bailey et al. 2016).

- **Obesity**, the main potentially modifiable risk factor for T2D. Visceral adiposity confers the highest risk accompanied with T2D (Wing 2010). Research has proven that obesity is accountable for 80-85% of the risk of developing T2D (Wing 2010).

- **Physical activity**, positively impacts glucose metabolism and attenuates the risk of obesity. However, the lack of high intensity physical activity is not as alarming as sedentary behaviour amongst vast populations (Wing 2010).

- **Diet**, numerous dietary factors are implicated in the onset of DM. Despite total caloric intake, certain dietary factors such as processed meat, unprocessed red meat, and sugar-sweetened beverages are associated with the risk of T2D (Wing 2010, Pippitt et al. 2016). Following the strong relationship between T2D and obesity, evidence for prevention of T2D arises from studies which couple dietary intervention with elevated physical activity (Wing 2010).

- **Socio-economic factors**, two out of three diabetic individuals reside in urban areas, however, individuals in the lower socio-economic classes are disproportionately affected (Foster et al. 2015). This occurrence is poorly understood, however unhealthier lifestyles serve as a mediating factor.
Globally, lower-middle income nations contribute to the prevalence of T2D, as these are the countries where urbanisation and economic progression has severely altered lifestyles (McIntyre et al. 2006).

- **Stress**, cortisol and adrenalin are associated with stress reactions. These hormones are recognized for their surging effect on glucose levels, in response to insulin (Pernicova and Korbonits 2014).


### 2.1.3 Screening and Diagnosis

T2D is a metabolic disorder characterized by raised glucose levels accompanied with disrupted CHO, lipid and protein metabolism due to deficiencies in the secretion and/or action of insulin (Fisher and Kahn 2003). Uncontrolled DM can result in blindness, limb amputations, kidney failure, vascular disease and heart disease (Alberti and Zimmet 1998). Hence screening patients prior to the development of signs and symptoms results in earlier diagnosis and treatment. It is imperative that diagnostic testing is performed in patients with a clinical past symptomatic of DM (Pippitt et al. 2016). Symptoms prompting consideration of DM include blurry vision, fatigue, numbness, polyuria, polydipsia, poor wound healing, tingling
sensations and weight loss (Pippitt et al. 2016). Screening for T2D is important for the following reasons (Pippitt et al. 2016):

- Global increase in prevalence of T2D
- Considerable proportion of people with T2D are undiagnosed
- Significant fraction of new cases of T2D present with evidence of microvascular complications
- A lengthy, latent and asymptomatic period in which T2D is detectable
- Importance of the direct effects and long-term complications of T2D
- Evidence in support of controlling blood-glucose, blood-pressure and blood-lipid levels in T2D
- Evidence that treatment of dyslipidaemia and hypertension can avoid CVD in individuals with T2D

The recommendations for the screening of T2D are listed below in table 1.

**Table 1** Recommendations for the screening of type 2 diabetes mellitus

<table>
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<tr>
<th>Screen asymptomatic individuals if the following risk factors are present:</th>
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<tr>
<td>Age ≥ 45 years</td>
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<td>CVD or family history of T2D</td>
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<tr>
<td>Chronic glucocorticoid exposure</td>
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<tr>
<td>HDLc level &lt; 0.91mmol/L and/or a TG level &gt; 2.8mmol/L</td>
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<tr>
<td>History of gestational DM or delivery of a baby &gt; 4.1kg</td>
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<tr>
<td>Hypertension (blood pressure &gt; 140/90 mmHg or prescribed medication for hypertension)</td>
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<tr>
<td>Glucose intolerance, impaired fasting glucose and/or metabolic syndrome</td>
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<tr>
<td>Nonalcoholic fatty liver disease</td>
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<tr>
<td>Overweight or obese</td>
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<td>Polycystic ovary syndrome</td>
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<tr>
<td>Sedentary lifestyle</td>
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<td>Sleep disorders in the presence of glucose intolerance</td>
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**Persons with two or more risk factors should be screened annually**

### 2.1.3.1 Diagnostic Tests for Type 2 Diabetes Mellitus

T2D is diagnosed based on the following: plasma glucose (PG) criteria, fasting plasma glucose (FPG), 2h PG value following a 75g oral glucose tolerance test (OGTT) and/or the A1C criteria (Table 2) (Association 2017).
Table 2 Criteria for the diagnosis of type 2 diabetes mellitus

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<td>1.</td>
<td>FPG ≥7.0 mmol/L (Fasting includes no caloric intake for at least 8h)</td>
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<tr>
<td>2.</td>
<td>2h PG ≥11.1 mmol/L during an OGTT (as per the WHO)</td>
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<tr>
<td>3.</td>
<td>A1C ≥6.5% (48 mmol/L)</td>
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<tr>
<td>4.</td>
<td>Patient with classic symptoms of hyperglycaemia or hyperglycaemic crisis, with a PG ≥11.1 mmol/L</td>
</tr>
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2.1.4 Management

The goals in managing T2D include the elimination of symptoms and reduced rate of development of complications. Both lifestyle and diet modifications are the most effective way to manage T2D (Olokoba et al. 2012). The incidence of T2D declined, in association with a body mass index of >25kg/m², a high fibre and low saturated fat diet, consistent exercise, abstinence from smoking and moderate alcohol consumption (Olokoba et al. 2012). This suggests that T2D can be prevented by lifestyle modification. In some instances lifestyle modifications may be insufficient in controlling blood glucose levels – since T2D is a progressive disease – other interventions are necessary. These include the administration of pharmacological agents such as MF.

2.1.4.1 Pharmacological Agent: Metformin

Globally, the first line drug of choice for T2D treatment is the oral biguanide antiglycaemic agent, MF (1,1-dimethylbiguanide hydrochloride) (Group 2002). The primary mechanism of action is increasing peripheral insulin sensitivity and inhibition of hepatic glucose production (Group 2002). 5' adenosine monophosphate-activated protein kinase (AMPK) is a crucial cellular regulator of both glucose and lipid metabolism, and is directly regulated by MF (Viollet et al. 2012). It is a multi-subunit enzyme regulating biosynthetic lipid pathways, owing to its role in the phosphorylation of significant enzymes such as acetyl-CoA carboxylase (ACC) (Fig 2.2) (Zhou et al. 2001). In the liver, MF activates AMPK resulting in reduced ACC activity, fatty acid (FA) oxidation, and suppressed expression of lipogenic enzymes (Fig 2.2) (Zhou et al. 2001). In addition, AMPK activation suppresses expression of sterol regulating binding element 1c (SREBP1c), a key lipogenic transcription factor (Fig 2.2) (Streicher et al. 1996). The suppression of SREBP1c down-regulates gene expression of essential lipogenic enzymes, causing reduced fatty liver, and elevated hepatic sensitivity of insulin. With regards to glucose metabolism MF is responsible for attenuating gluconeogenesis. Gluconeogenesis is an energy dependent process, which depends on mitochondrial function. Metformin accumulates within the mitochondria inhibiting mitochondrial complex 1, and suppressing the production of adenosine triphosphate (ATP). As a result, the attenuated ATP levels reduce gluconeogenesis (Fig 2.2) (Rena et al. 2017). Apart from its effect on
lipid and glucose metabolism, MF treatment is thought to have additional positive effects, including stabilization of weight gain and weight loss (Brufani et al. 2013). This occurs by suppression of hepatic glucose production, increase in insulin sensitivity, improving glucose uptake via phosphorylating GLUT-enhancer factor, increasing FA oxidation, and reducing glucose absorption from the GIT (Group 2002, Brufani et al. 2013). Although highly effective and safe, MF therapy is associated with side effects, such as acute infections of the nose and throat and irritations of the GIT (Hermann 1979, Siavash et al. 2017). The sustained effectiveness of this compound is controversial, resulting in a constant search for new T2D therapies.

**Figure 2.2**: Mechanism of action of metformin on hepatic and muscle glucose metabolism (Brufani et al. 2013). AMPK; Adenosine monophosphate-activated protein kinase, ACC; acetyl-coenzyme A carboxylase, SREBP-1; sterol regulatory element-binding protein-1, VLDL; very-low density lipoprotein.
2.1.5 Pathophysiology

The pathophysiology of T2D comprises: peripheral insulin resistance, compromised regulation of hepatic glucose production, and diminished β-cell function resulting in β-cell failure (Fig 2.3) (Mahler and Adler 1999). This results in reduced transport of glucose to the liver, adipose, and muscle cells (Olokoba et al. 2012). Insulin resistance arises when liver, adipose and muscle cells become non-responsive to insulin. Under these conditions, TGs in adipose cells are degraded to produce FFAs for energy (Mahler and Adler 1999). Muscles are disadvantaged of an energy source and liver cells fail to produce glycogen (Mahler and Adler 1999). The inability of cells to utilize glucose causes an overall rise in circulating glucose. Glycogen stores become markedly reduced, resulting in decreased availability of glucose accessible for essential cell functioning (Shulman 2000).

Following CHO intake, there is an increase in plasma glucose and insulin secretion (Fig 2.3A). In muscle cells, insulin elevates glucose transport allowing for both glucose entry and glycogen storage (Fig 2.3A). In the liver, glycogen synthesis and lipogenesis are promoted by insulin, whilst gluconeogenesis is inhibited (Shulman 2000). In adipose cells, insulin reduces lipolysis and stimulates lipogenesis (Fig 2.3A). This results in elevated hepatic gluconeogenesis and glycogenolysis, lipolysis and reduced hepatic lipid production (Fig 2.3B) (Khedoe et al. 2015). During T2D, ectopic lipid accumulation dysregulates lipid production (Fig 2.3C). Insulin-mediated glucose uptake in skeletal muscle is also impaired as a result of intramyocellular lipid accumulation, diverting glucose to the liver (Fig 2.3C). The capability of insulin to control gluconeogenesis and activate glycogen synthesis is impaired by hepatic lipid accumulation (Fig 2.3D) (Shulman 2000). In contrast, unaffected lipogenesis together with the elevated glucose leads to increased lipogenesis (Fig 2.3D). In adipose cells impaired insulin action permits increased lipolysis, promoting re-esterification of lipids in tissues (such as liver), further impairing insulin resistance (Fig 2.3D) (Khedoe et al. 2015). As a result, hyperglycaemia is coupled with a decline in pancreatic β-cell insulin secretion. As mentioned above increased FFA levels, inflammatory cytokines from adipose and oxidative factors are involved in the pathogenesis of T2D, and related CVD complications.
Figure 2.3: Pathophysiology of normal and abnormal glucose metabolism in type 2 diabetes mellitus (Shulman 2000). Normal glucose metabolism during a (A) fed state and (B) fasted state. “Abnormal” glucose metabolism during a (C) fed state and (D) fasted state.

2.2 Glucose Sensing and Insulin Receptor Signalling
Pancreatic β-cells release the anabolic hormone, insulin in response to raised levels of nutrients in the blood. Insulin initiates the uptake of amino acids, FAs and protein into muscle, adipose and liver; stimulating the storage of these nutrients as protein, lipids and glycogen, respectively (Fisher and Kahn 2003). However failure in the uptake and storage of these nutrients, due to the inability of insulin binding to its receptor and subsequent insulin resistance, results in T2D. The binding of insulin to insulin receptor-β (IRβ), initiates a cascade of tyrosine kinases which result in the uptake and storage of nutrients.
During T2D, this conventional insulin-receptor binding is defective, resulting in an imbalance in glucose homeostasis.

2.2.1 Insulin Signalling

The IR comprises two extracellular α subunits and two transmembrane β subunits linked via disulphide bonds. Binding of insulin to the α subunits, induces a conformational change followed by autophosphorylation of several tyrosine residues within the β subunit (Arner et al. 1987, Pirola et al. 2004). These residues are recognized by phospho-tyrosine-binding domains on adaptor proteins such as members of the IRS family (Saltiel and Kahn 2001, Lizcano and Alessi 2002). Receptor mediated-activation phosphorylates crucial tyrosine residues on IRS proteins, some of which are recognized through the Src homology 2 domain of phosphoinositide-3-kinase (PI3K) (Shulman 2000). The catalytic subunit of PI3K - p110, phosphorylates phosphatidylinositol (4, 5) bisphosphate (PtdIns (4, 5) P2) causing the formation of Ptd (3, 4, 5) P3, which is a significant downstream stimulant of Akt (Nolan et al. 1994).

Activation of Akt is governed by a dual regulatory mechanism; Akt is first recruited to the plasma membrane, and subsequent phosphorylation via phosphoinositide-dependent kinase-1 and mTOR Complex 2 (Lizcano and Alessi 2002, Gao et al. 2014). Once active, Akt enters the cytoplasm where it phosphorylates and inactivates GSK3α/β (Fig 2.4). This serine/threonine protein kinase facilitates addition of phosphate molecules onto serine and threonine amino acid residues (McManus et al. 2005). The main substrate of GSK3α/β is glycogen synthase (GS), an enzyme that catalyses the last step in glycogen synthesis (Fig 2.4). Phosphorylation of GS via GSK3α/β impedes glycogen synthesis, consequently the inactivation of GSK3α/β via Akt promotes glucose storage as glycogen (McManus et al. 2005). Inactivated GSK3α/β is a major regulatory step in the stimulation of hepatic GS via insulin. Furthermore, glucose-6-phosphate is a precursor for the formation of hepatic glycogen. Glucokinase (Gck) is a hepatic enzyme which facilitates the phosphorylation of glucose to glucose-6-phosphate, providing a substrate for the conversion to glycogen. In muscle and adipocytes, insulin promotes glucose uptake via translocation of GLUT4, and via GLUT2 in the liver (Fig 2.4). GLUT4 translocation involves the PI3K/Akt pathway (Carlson et al. 2003), where as GLUT2 translocation involves activation of SREBP1c via insulin (Fig 2.4) (Horton et al. 2002).

The transcription factor SREBP1c is responsible for regulating the transcription of genes involved in cholesterol and lipid synthesis (Sharawy et al. 2016). Raised glucose levels promote binding of SREBP1c to GLUT2 promoters, initiating GLUT2 transcription (Sharawy et al. 2016). GLUT2 recruitment
facilitates the uptake of extracellular glucose, ultimately, reducing circulating blood glucose and maintaining glucose homeostasis. Conversely, during T2D the above physiological processes are attenuated. Thus insulin resistance, compromised regulation of hepatic glucose production, and deteriorating β-cell function are attributable to a reduction in the normal physiology (Fig 2.4).

**Figure 2.4:** Normal physiological process of insulin on genes and proteins involved in the proximal and distal signalling cascade (Prepared by author)

### 2.3 Lipid Metabolism

Insulin signalling is central in regulating several steps of lipid metabolism. Following disrupted IR signalling and glucose sensing, T2D individuals present with abnormal lipid profiles (Mullugeta et al. 2012). Abnormal lipid profiles classified as diabetic dyslipidaemia are characterized by elevated TGs and LDL particles, and depleted HDLc. Uncontrolled glucose levels potentiates FA flux, which gives rise to diabetic dyslipidaemia promoting TG production in hepatocytes. In the presence of heightened glucose levels, hepatocytes increase in packaging of TGs, which are transported to the cells via very low density lipoproteins (VLDL) (Biesenbach 1989). TGs are stripped from VLDL particles, which facilitates the delivery and subsequent digestion of TGs in the cells (Ginsberg et al. 2005). VLDL particles become denser and undergo remodelling to form LDL particles, which function to transport cholesterol used within the membranes and/or for production of steroid hormones (Ginsberg et al. 2005). Cellular cholesterol uptake is mediated via receptor mediated endocytosis. The LDL particles containing
cholesterol bind to specific LDLr, where hydrolysis within endosomes release cholesterol for use in the cell; which is followed by the recycling of the receptor to the cell surface (Biesenbach 1989, Taskinen 2002).

In T2D, over-production of LDLc overwhelms the LDLr pool, resulting in insufficient removal of LDLc. As a result untreated diabetic dyslipidaemia increases the risk for the onset of CVD and subsequent microvascular and macrovascular complications (Lorber 2014). Therefore, it is imperative to target the modifiable risk factors, which lead to development of long-term complications. Statins are the most popular class of lipid-lowering drugs. These drugs function by inhibiting the enzyme HMG CoA reductase which decreases the production of cholesterol in the liver. Unfortunately, the use of statins is accompanied by a range of side effects, from muscle damage and pain to liver damage (Banach et al. 2015). New advancements, differing from the action of statins have emphasized the use of regulators of PCSK9 and agonists of PPARG in the treatment of dyslipidaemia (Gervois et al. 2000, Burke et al. 2017).

Studies have reported PCSK9 as a potent circulating regulator of LDLc through its ability to induce degradation of the LDLr in lysosomes of hepatocytes (Grefhorst et al. 2008, Bhat et al. 2015, Burke et al. 2017, Laugier-Robiolle et al. 2017). The transcription factor PPARG is responsible for mediating physiological effects on both glucose homeostasis and lipid metabolism (Memon et al. 2000, Kendall et al. 2006). The effects of PPARG in T2D has been well-documented, which include a direct impact on HDLc levels (Inoue et al. 2005, Kendall et al. 2006, Bermudez et al. 2010, Ahmadian et al. 2013).

Following the disparity in lipid homeostasis present in T2D, targeting PCSK9 and PPARG will contribute to the restoration of lipid homeostasis (Fig 2.5).
2.3.1 PCSK9, an Inhibitory Regulator of LDLr

Lipoprotein transfer of cholesterol within plasma plays a functional role in the cell membrane, energy production and hormone synthesis (Virella and Lopes-Virella 2012). Cells use cholesterol via the internalization of lipoprotein ligands comprising chylomicrons, LDL and VLDL particles mediated by LDLr, followed by an endocytic process (Fig 2.6) (Olofsson et al. 2000, Virella and Lopes-Virella 2012). LDLr-mediated endocytosis aids in understanding lipoprotein clearance and LDLr deficiency, as risk factors for developing CVD. Raised LDL is a predominant risk factor for coronary artery disease and other atherosclerotic diseases, which governs the largest cause of all morbidities and mortalities in T2D individuals.

LDLr is a cell membrane glycoprotein which functions by binding and internalizing circulating cholesterol containing lipoprotein particles (Virella and Lopes-Virella 2012). This receptor is ubiquitously expressed and central to the maintenance of cholesterol homeostasis in mammals. On the cell surface, clathrin-coated pits contain LDLr which bind to LDLc via adaptin (Virella and Lopes-Virella 2012). Following binding, the pits are pinched off to form clathrin-coated vesicles within the cell (Fig 2.6) (Virella and Lopes-Virella 2012). This allows LDLc to undergo endocytosis and avoids LDL diffusing around the membrane surface. This occurs in nucleated cells but predominantly within the liver, which is responsible for 70% of LDL removal from circulation. Once the coated vesicle is internalized, it sheds the clathrin-coat and fuses with an acidic endosome (Virella and Lopes-Virella 2012). Following this cycle, LDLr is either destroyed or recycled via endocytic cycle to the cell surface, to receive another LDL particle (Fig 2.6). The synthesis of LDLr is regulated by levels of free intracellular cholesterol, as well as, other molecules such as PCSK9.

PCSK9 regulates the degradation of the LDLr in response to cholesterol levels within the cell; by binding to an extracellular part of the LDLr (Fig 2.7) (Burke et al. 2017). PCSK9 inhibits LDLr from creating a closed conformation, causing the receptor to be susceptible to enzymatic degradation (Fig 2.7) (Horton et al. 2007). LDLr’s without PCSK9 bound to them are therefore more likely to be recycled to the cell surface. Increased function of PCSK9 causes hypercholesterolemia, while reduced function is accompanied by low LDL. Therefore, inhibiting PCSK9 allows more LDLr’s to be recycled to the cell surface, causing an elevation in the clearance of LDLc from circulation. Several approaches to the
pharmacological inhibition of PCSK9 has been investigated. Antisense oligonucleotides and small interfering RNAs are responsible for the formation of PCSK9 (Burke et al. 2017). Furthermore, small adnectin polypeptides and monoclonal antibodies bind to mature PCSK9 preventing interaction with LDLr (Burke et al. 2017). Consequently, attenuating the levels of PCSK9 can be achieved prior to/following formation of these molecules, resulting in therapeutic opportunities for targeting PCSK9.

Figure 2.6: Low density lipoprotein receptor mediated pathway
Figure 2.7: Cellular trafficking of PCSK9 (Poirier and Mayer 2013)

2.3.2 PPARG; A Positive Regulator of Lipid Metabolism

Lipoproteins are multifaceted particles responsible for the transport of lipids (Lund-Katz and Phillips 2010). The anti-atherogenic lipoprotein, HDL, facilitates the efflux of cellular cholesterol. HDL partakes in the reverse cholesterol transport process, whereby surplus cholesterol within peripheral cells are transported to the liver for excretion (Zhou et al. 2015). Apolipoprotein-facilitated interactions of HDL particles with lipid transporters and receptors on the cell surface are crucial for this process (Lund-Katz and Phillips 2010). The interchangeable apolipoproteins within HDL are A, C and E families, which are structurally similar (Lund-Katz and Phillips 2010, Zhou et al. 2015). Lipoprotein particles in circulation act as binding agents for cell surface receptors, and facilitate lipoprotein remodelling (Zhou et al. 2015). With regards to HDL, the apolipoproteins contribute to particle remodelling via the transfer of proteins and enzymes. These reactions are essential for HDL metabolism and reverse cholesterol transport (Lund-Katz and Phillips 2010, Zhou et al. 2015).

HDL particles protect against CVD, OS and systemic inflammation via its anti-thrombotic, anti-inflammatory, and AO activities, as well as, reverse cholesterol transport (Lund-Katz and Phillips 2010). Apolipoprotein A1 (Apo A1) is manufactured and released into circulation via the liver. The partial lipidation of ApoA1 by phospholipids and cholesterol form nascent HDL particles (Lund-Katz and Phillips 2010) (Fig 2.8). In vascular tissue, these particles initiate the release and transfer of free
cholesterol to the surface of HDL; free cholesterol is then re-esterified and stored within the HDL core (Lund-Katz and Phillips 2010, Zhou et al. 2015). This process converts lipid-deprived HDL3 to cholesterol ester-rich HDL2, which separate and release into circulation (Lund-Katz and Phillips 2010, Zhou et al. 2015). In circulation, part of the HDL cholesterol-ester is transferred to intermediate density lipoprotein (IDL) and LDL in exchange for TGs (Zhou et al. 2015). HDL2 then binds to HDL docking receptors allowing for the removal of HDL cholesterol-ester and hydrolysis of its TG, and phospholipid contents cellular uptake (Fig 2.8) (Zhou et al. 2015). Subsequently, the lipid-poor HDL separates and proceeds to circulation to repeat the cycle.

As previously mentioned, HDL confers important protective functions in addition to its role in mediating reverse cholesterol transport (Fig 2.9) (Lund-Katz and Phillips 2010). HDL is a protecting agent against OS via the actions of its constituent AO enzymes, GPx and paraoxonase (Fig 2.9). Systemic inflammation is mitigated by HDL via the removal of oxidized phospholipids and FAs from LDL, VLDL, and IDL (Fig 2.9) (Howard et al. 2000). This process limits the formation of oxidized phospholipids and their disposal in the liver. Anti-atherogenic HDL also stimulates proliferation and inhibits injury and apoptosis of
endothelial cells via attenuated caspase-3 activity and ROS generation (Giacco and Brownlee 2010).

Furthermore, HDL enables restoration, relocation, and growth of endothelial cells and elevates the quantity of circulating endothelial progenitor cells - which are vital for vascular repair and inhibition of plaque formation (Lorber 2014). Subsequently, HDL particles in circulation are responsible for regulating multiple factors, hence targeting this lipoprotein is imperative in maintaining cholesterol homeostasis.

A possible inducer of the anti-atherogenic lipoprotein is PPARG, which belongs to a subfamily of ligand-inducible transcription factors (Issemann and Green 1990). This transcription factor is central in controlling the expression of gene networks involved in lipid metabolism, glucose homeostasis, inflammation, and cell proliferation (Forman et al. 1997, Dussault and Forman 2000, Evans et al. 2004).

Ligand binding is followed by the formation of heterodimers with retinoid X receptor (RXR). PPARG-RXR heterodimers bind to peroxisome proliferator response element, on the promoter region of specific target genes (Gervois et al. 2000). Transcription is triggered upon recruitment of different transcriptional cofactors (Gervois et al. 2000). PPARG is responsible for inducing apolipoproteins which play an integral role in transporting excess cholesterol to its acceptor – HDL, initiating reverse cholesterol transport (Gervois et al. 2000). The application of PPARG agonists has been investigated in multiple disease conditions, the only approved use for PPARG ligands is the use of thiazolidinedione’s in T2D (Fruchart et al. 2001, Fitzgerald et al. 2002, Lalloyer and Staels 2010). These complete PPARG agonists first arose as a new class of therapy alleviating insulin resistance in individuals with T2D (Fruchart et al. 2001, Fitzgerald et al. 2002, Lalloyer and Staels 2010). However, drugs from the thiazolidinedione group were withdrawn from the market due to severe adverse effects. These effects were caused by full PPARG activation and opposing agonistic effects of endogenous PPARG ligands such as FAs and prostanoids (Dussault and Forman 2000, Balakumar and Kathuria 2012). The PPARG agonist pioglitazone is currently used to treat both diabetes and fatty liver disease (Amano et al. 2018; Chandra et al. 2017).

Consequently, research efforts have recently explored the prospective role of selective PPARG agonists - compounds that increase glucose homeostasis but reduce partial PPARG agonism side effects (Schupp et al. 2005, Balakumar and Kathuria 2012). Consequently, targeting PPARG poses potential benefit in potentiating the levels of HDL and its downstream effects in T2D.
2.4 Oxidative Stress and Mitochondrial Health

Excessive nourishment and sedentary lifestyle potentiates glucose and FA overload, resulting in the over-production of ROS (Baynes 1991). Disproportionate ROS causes OS, which is representative of the imbalance between ROS and the ability to detoxify their harmful effects (Baynes 1991). In T2D, hyperglycaemia induces ROS production via attenuation of the AO response and detoxification system, stimulation of protein kinase C (PKC) pathway, the formation of AGEs and reduced mitochondrial function. OS has been identified as a pathogenic mechanism in diabetic-related CVD and long-term complications. As a result, targeting factors responsible for mitigating OS levels and neutralizing the adverse effects of hyperglycaemia, are central to T2D treatment.

Studies have established the role of nuclear factor erythroid 2-related factor 2 (Nrf2) in alleviating the complications of DM. Nrf2 is a transcription factor that functions as the major regulator of the
endogenous AO and detoxification system. Nrf2 executes its function once released from Kelch-like
ECH-associated protein 1 (Keap1) repression, resulting in the stimulation of the antioxidant response
element (ARE). This response element is central to the activation of genes coding for a number of AOs.
Furthermore, protein kinase pathways such, as the diacylglycerol (DAG)-PKC pathway, has been
associated in transducing OS signals to genes mediated through the ARE. This pathway is known to be
activated by chronic DAG levels, resulting in elevated levels of PKC, promoting Nrf2 activation; whereas
the formation of cytotoxic AGEs are counteracted by Nrf2 and mitochondrial Lon protease.

An important source of ROS is the mitochondria, via oxidative phosphorylation. Interestingly, Nrf2
counterbalances mitochondrial produced ROS via the transcription of cytoprotective genes; SOD2, GPx
and GSH. Furthermore, the mitochondria possess regulatory proteins, enabling these organelles to combat
the over-production of ROS. These regulatory proteins include both SIRT3 and PGC-1α. SIRT3 is central
to regulating mitochondrial function since it is accountable for deacetylation of mitochondrial proteins,
and regulating mitochondrial biogenesis via the induction of PGC-1α. Mitochondria are essential for
bioenergetic and metabolic processes, including glucose and lipid metabolism (Kim et al. 2008).
However, when mitochondria are unable to perform their primary function, this results in mitochondrial
dysfunction (Dinkova-Kostova and Abramov 2015). Collectively, impaired AO defences and
mitochondrial dysfunction form a vicious cycle causing OS. Thus, therapeutic intervention using AO
supplements may be beneficial in interrupting this cycle.

2.4.1 Nuclear factor erythroid 2-related factor 2 and the Antioxidant Response
OS is central to the pathogenesis of T2D, and the onset of CVD and diabetic complications. As a result
improving the endogenous cellular AO response and detoxification system will enable cells to prevent
ROS induced damage (Allen and Tresini 2000, Ramachandran et al. 2011). Nrf2 regulates the
endogenous AO and detoxification system, providing cells the ability to adapt to OS, by mediating the
induction of cytoprotective genes (Fig 2.10) (Zhang 2006). Under non-stressed conditions, Nrf2 is
inhibited by a negative regulator Keap1 which is located in the cytoplasm (Zhang 2006, Taguchi et al.
2011). Keap1 is a repressor protein that binds to Nrf2 and stimulates its degradation via the ubiquitin
proteasome pathway (Kansanen et al. 2013). Exposure to OS allows Nrf2 to escape Keap1 repression,
translocating to the nucleus, which results in the phosphorylation and activation of Nrf2 (Fig 2.10). In the
nucleus pNrf2 binds to and initiates ARE-dependent gene expression to preserve cellular redox balance
(Zhang 2006). The ARE comprises a sequence involved in controlling the coordinated transcriptional
activation of genes coding for various AO enzymes (Fig 2.10). The AO function of Nrf2 is central to the
preservation of glucose metabolism in insulin-sensitive tissues, via insulin secretion and glucose
The cytoprotective genes induced by Nrf2 include the endogenous AOs; 
SOD2 and GPx, which are involved in synthesizing the non-enzymatic AO; GSH. Reduced GSH serves 
as a major cellular AO determinant between cellular protection and toxic damage (Pompella et al. 2003, 
Victor et al. 2011). When this endogenous AO fails to provide adequate compensatory response to restore 
the redox balance, GSH levels are reduced and OS ensures (Allen and Tresini 2000). Following elevated 
ROS, superoxide radicals are converted to hydrogen peroxide by SOD2 which is reduced by GPx to water 
(Vats et al. 2015). The reduction of hydrogen peroxide to water is complemented by the conversion of 
reduced GSH into oxidized GSH. Studies have reported decreased Nrf2 in diabetic mice and patients with 
T2D, which contribute to elevated OS, endothelial dysfunction, insulin resistance and elevated cardiac 
insult (Li et al. 2011, Tan et al. 2011, Cheng et al. 2012). Furthermore, studies have showed that Nrf2 
Studies also confirm the relationship between activated Nrf2 and its downstream effects on both SOD2 
and GPx, which are reduced in T2D (Dong et al. 2008, Ramachandran et al. 2011, Giralt and Villarroya 
2012). Nrf2 induction alters AO, energy intake, and gluconeogenesis related gene expression in metabolic 
tissues (Uruno et al. 2013). Subsequently, Nrf2 plays an invaluable role in modulating the metabolic 
aberrations present in T2D, by initiating the AO response and detoxification system.

2.4.2 Diacylglycerol-Protein kinase C Pathway

Hyperglycaemic-induced intracellular and extracellular changes alter signal transduction pathways which 
negatively impact gene expression and protein function. PKC is a serine/threonine-related protein kinase, 
fundamental in several cellular functions, and signal transduction pathways (Geraldes and King 2010). 
There are various isoforms of PKC that function in a multiplicity of biological systems, which are 
stimulated by DAG (Geraldes and King 2010). DAG is a glyceride consisting of two FA chains 
covalently bonded to a glycerol molecule (Geraldes and King 2010). The levels of DAG are chronically 
heightened in a hyperglycaemia, owing to an elevation in the glycolytic intermediate - dihydroxyacetone 
phosphate (Geraldes and King 2010). Dihydroxyacetone phosphate is reduced to glycerol-3-phosphate, 
which consequently raises the de novo synthesis of DAG. In DM, DAG levels are raised in vascular 
(heart, retina and kidney) and non-vascular tissues (liver and skeletal muscles). Studies by Kang et al, 
2000 and Kang et al, 2001 provided substantial evidence on the involvement of PKC in phosphorylating 
Nrf2, and initiating its nuclear translocation in response to OS (Kang et al. 2000, Kang et al. 2001). 
Accordingly, PKC phosphorylates Nrf2 at Serine-40, promoting its dissociation from Keap1 (Huang et al. 
2002). This action of PKC is a crucial signalling event resulting in the ARE-mediated cellular AO 
response.
2.4.3 Advanced Glycation End-Products Pathway

Diverse biochemical pathways have been suggested in linking the antagonistic effects of hyperglycaemia to vascular impediments. In addition to the DAG-PKC pathway, is an increase in the AGEs pathway. Glycation is the process whereby uncontrolled glucose forms covalent adducts with plasma proteins (Chevion et al. 2000). Glycation is a non-enzymatic process, central to the onset of diabetic complications, such as, nephropathy, neuropathy and retinopathy (Singh et al. 2014). Glucose-derived dicarbonyl precursors are responsible for accumulation of intracellular AGEs (Singh et al. 2014). Intracellular AGEs are important stimuli for triggering intracellular signalling pathways (Brownlee 2001).

Glycation hinders normal functioning of proteins, which disrupts conformation, enzymatic activity, degradation ability, as well as receptor recognition (Brownlee 1995, Hammes et al. 1999). Glycation modifies cellular functions via denaturation and functional failing of target proteins, initiation of receptor-mediated signalling and production of oxidative and carbonyl stress (Yonekura et al. 2005, Hsieh et al. 2007). As a result, reducing the formation of AGEs is imperative in attenuating its effects on protein structure and function, and subsequent vascular complications. In addition to, the role of Nrf2 in mitigating hyperglycaemic-induced OS, Nrf2 plays a functional role in reducing the levels of AGEs. Recent evidence for this interaction was provided by Sampath et al, 2016 (Sampath et al. 2017).

Following the multifaceted effects of Nrf2, the mitochondrial matrix possess an important protease, Lon protease 1 (LonP1) which is responsible for degrading oxidatively damaged proteins (Gumeni and Trougakos 2016, Pomatto et al. 2017). This protease degrades oxidized and damaged proteins, in association with chaperones which preserve the protein in an unfolded state until the initiation of the proteolytic reaction (Pomatto et al. 2017). Increased LonP1 will enable cells to efficiently reduce damaged proteins and attempt to restore cellular homeostasis. Studies compared diabetic and lean mice livers, and observed LonP1 protein levels to be significantly lower in diabetic mice (Lee et al. 2011). This suggests that LonP1 down-regulation is critical in mitochondrial dysfunction, and may be involved in the progression of insulin resistance and T2D (Lee et al. 2011).
2.4.4 Mitochondrial Regulatory Pathways

In mitochondria, ROS are generated at low levels as by-products of the electron transport chain (ETC) (Brownlee 2001). Mitochondria are double-membrane organelles with multiple critical cellular functions which include oxidative phosphorylation. Oxidative phosphorylation is the final stage of cellular respiration, consisting of two meticulously connected components; the ETC and chemiosmosis (Giacco and Brownlee 2010). The ETC comprises of proteins and organic molecules located in the inner mitochondrial membrane. In a sequence of redox reactions, electrons are transferred from one member of the ETC to the next (Liu et al. 2002). Energy is released during these reactions, which is captured as a proton gradient. This facilitates the production of ATP in a process called chemiosmosis (Montgomery and Turner 2015). During these processes, ROS production is necessary for the normal functioning of the cell, however excessive ROS production can be detrimental, making AO defences essential. In addition to the role of Nrf2 in the AO system, Nrf2 also influences mitochondrial function by protecting against mitochondrial toxins. The main small molecule AO - GSH is also a product of numerous downstream

Figure 2.10: Schematic representation presenting the mechanism of cytoprotection via Nrf2 activators (Rashmi et al. 2017)
target genes of Nrf2, which counterbalances the production of mitochondrial ROS (Holmström et al. 2016). Mitochondrial integrity is critical for overall functioning. Mitochondrial integrity is regulated via mitophagy, a process which removes damaged mitochondria. Autophagic adaptor protein sequestosome-1 (SQSTM1/p62) is a key protein responsible for mitophagy (Holmström et al. 2016). This protein competes with Nrf2 for its binding site on Keap1, as a result, raised p62 levels triggers the Nrf2 pathway, creating a positive feedback loop (Holmström et al. 2016).

In order to maintain proper mitochondrial function, lysine acetylation has become an important post-translational alteration of mitochondrial proteins (Nogueiras et al. 2012). In response to environmental stimuli, vital metabolic enzymes are acetylated within mitochondria. The SIRT family consists of seven members, three (SIRT 3, 4, 5) of which are located in the mitochondria (Nogueiras et al. 2012). SIRT3 is important in regulating mitochondrial function, as it is accountable for deacetylation of mitochondrial proteins. SIRT3 is responsible for targeting proteins involved in energy metabolism processes, which include the respiratory chain, tricarboxylic acid cycle and FA β-oxidation (Buler et al. 2012). Through these processes, SIRT3 regulates the flow of mitochondrial oxidative pathways and subsequently, the rate of ROS production (Giralt and Villarroya 2012). SIRT3-mediated deacetylation stimulates enzymes accountable for quenching ROS, thereby employing a profound protective action against OS-dependent pathologies, such as T2D (Nogueiras et al. 2012). In T2D, elevated OS contributes to the occurrence of mitochondrial dysfunction. As a result, elevated SIRT3 levels increase cellular respiration ultimately reducing ROS production (Fig 2.11). In addition, SIRT3 is essential for the induction of PGC-1α, and PGC-1α-dependent induction of ROS-detoxifying enzyme, SOD2 (Fig 2.11) (Kong et al. 2010). PGC-1α is a transcriptional coactivator which reacts with a wide range of transcription factors, involved in various biological responses such as mitochondrial biogenesis, glucose and FA metabolism (Moreno-Santos et al. 2016). This transcriptional coactivator is documented to be a master-regulator of mitochondrial biogenesis - interacting with a complement of transcription factors and nuclear hormone receptors associated with mitochondrial function (Fig 2.11) (Moreno-Santos et al. 2016). With regards to T2D, chronic hyperglycaemia is related to reduced expression of PGC-1α (Moreno-Santos et al. 2016). Therefore, stimulation of PGC-1α expression is a crucial regulatory event, leading to initiation of energy metabolic pathways, which elevate ATP production and exert homeostatic control (Moreno-Santos et al. 2016). Evidence has shown the significance of acetylation/deacetylation of mitochondrial proteins via SIRT3, and its possible role in the onset of insulin resistance. Thus, the development of new SIRT3-targeted drugs may aid in regaining regular cellular redox status in T2D patients.

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2.5 Natural Compounds for Medicinal Treatment

Globally in rural areas, traditional medicine is a common practice. Apart from its use by traditional healers, its demand and use has increased with limited knowledge of side effects and therapeutic efficacy (Ulbricht et al. 2007). It was estimated by the WHO that 80% of the world’s population is dependent on natural compounds for their primary healthcare (Khosla et al. 1995). Between 1983 and 1994 the United States Food and Drug Administration permitted approximately 78% of new drugs from unmodified natural products or semi-synthetic drugs acquired from natural sources (Suffredini et al. 2006). During a survey, the usage of natural compounds increased from 3% (1993) to 37% (1998) (Briskin 2000). This shift has been facilitated by the low cost of herbal drugs coupled with the developing world. The ‘green’ movement in the developed world campaigns the essential safety of natural products, and the individualistic philosophy of society that encourages self-medication - with countless people choosing to treat themselves with herbal remedies (Neelakantan et al. 2014).

Plants have been termed natural producers of medicinal compounds, which led to the discovery of new, inexpensive drugs, with high therapeutic potential. The exploitation of such medicinal compounds is
prehistoric. In the Middle Ages in Europe, internal bleeding and upper respiratory tract infections were treated by yarrow tea (Zarshenas et al. 2014). Salix leaves were prescribed by Hippocrates to reduce fever, (Zarshenas et al. 2014) and garlic and onions were thought to lower blood glucose, serum cholesterol and blood pressure, due to its proposed antibiotic properties (Bayan et al. 2014). Inflammation and pain were treated with salicin (white willow tree extract), which is now synthetically produced, and used as a staple over-the-counter drug (Huie 2002). Gymnema sylvestre is a woody plant that has been reported to be beneficial in diabetes therapy, via its ability to supress the desire for sugary foods (Leach 2007). Other potential sources of anti-diabetic properties include Momordica charantia (bitter melon) and Opuntia ficus-indica (prickly pear cactus), which has been documented to display glucose lowering effects (Leung et al. 2009, Joseph and Jini 2013). Another commonly used plant is Trigonella foenum-graecum (fenugreek). Its leaves and seeds are not only used as a nutritional source, but are also commonly prescribed in traditional medicine (Basch et al. 2003, Ulbricht et al. 2007).

Trigonella foenum-graecum frequently known as fenugreek is a promising medicinal plant from the Fabaceae family (Basch et al. 2003). Fenugreek is indigenous to Southern Europe and Western Asia but cultivated worldwide. Fenugreek seeds contain a rare amino acid, 4-OH-Ile – its biologically active compound (Ulbricht et al. 2007). Medical uses of fenugreek in Indian and Chinese medicine, include inducing labour, assisting digestion, and as a general tonic to increase metabolism and health (Basch et al. 2003, Ulbricht et al. 2007). In ancient times, Egyptians used fenugreek together with honey for the treatment of anaemia, constipation, DM, dyspepsia, rheumatism and rickets (Ulbricht et al. 2007). Preliminary studies have proposed potential hypoglycaemic and anti-hyperlipidaemic properties of fenugreek seed powder. However, at present, evidence is insufficient to endorse fenugreek for or against the treatment of hyperglycaemia or hyperlipidaemia.

2.5.1 Trigonella foenum-graecum (Fenugreek)
Fenugreek is an annual self-pollinating leguminous bean. The seeds are sown in well-prepared soil which sprouts in approximately three days. Seedlings grow semi-erect, erect or branched based on its variety and attains a height of approximately 30 to 60cm (Fig 2.12A) (Ulbricht et al. 2007). It has compound trifoliate pinnate leaves, axillary yellow to white flowers, and 3 to 15cm long thin pointed hoop-like beaked pods (Fig 2.12B) (Ulbricht et al. 2007). Every pod comprises 10 to 20 oblong brown-greenish seeds with unique hoop-like groves (Ulbricht et al. 2007). Seed shape-size, number of seeds in a pod, pods and plant height varies from one fenugreek species to another (Fig 2.12C) (Ulbricht et al. 2007).
Figure 2.12 *Trigonella foenum-graecum* (A) leaves, (B) pods and (C) seeds used for medicinal purposes (http://www.punmiris.com/himg/o.12961.jpg)

2.5.2 Fenugreek Seeds

Fenugreek seeds are known to be powerfully aromatic and pungent. These seeds are common spices widely used for both their culinary and medicinal properties. The seeds are the most important and useful part of the plant. These seeds are hard, dark yellow in colour, small in size and have a four-faced stone like structure (Fig 2.13) (Yoshikawa et al. 1997). Naturally the seed is 3-6mm long, 2-5mm wide and 2mm thick (Yoshikawa et al. 1997). The seeds are rich in phytonutrients, vitamins, minerals and soluble dietary fibre (Grant Reid and Derek Bewley 1979). Major fibre content in the plant comprises of non-starch polysaccharides (NSPs), such as, saponins, hemicellulose, mucilage, tannin, and pectin (Grant Reid and Derek Bewley 1979). These NSPs aid in reducing LDLc via inhibition of bile salt re-absorption; confers protection to the colon mucus membrane by binding to toxins present in food; as well as augments bowel movements (Grant Reid and Derek Bewley 1979, Fuller and Stephens 2015). The medicinal properties of the plant are attributed to the phytochemical compounds within the seed, such as, choline, diosgenin, gitogenin, neotigogens, trigonelline and yamogenin (Sauvaire et al. 1998). Likewise, this seed is an exceptional source of minerals like copper, calcium, iron, manganese, magnesium, potassium, selenium and zinc (Yoshikawa et al. 1997). Potassium is significant in controlling heart rate and blood pressure, and iron is essential for red blood cell production. Moreover the seed is rich in essential vitamins which are optimum for healthy living, such as, folic acid, niacin, pyridoxine, thiamin, riboflavin, vitamin A and C (Yoshikawa et al. 1997). In addition to these properties it has been recognised that 4-OH-Ile has facilitator action on insulin secretion (Sauvaire et al. 1998).
Figure 2.13 Dark yellow, four-faced stone like structure of fenugreek seeds

2.5.3 Biologically Active Compound: 4-hydroxyisoleucine

The composition of fenugreek consists of a large number of chemical components, such as, amino acids, proteins, lipids and alkaloids, which include trigonelline, vitamins, minerals, galactomannan, fibre, flavonoids, saponins and coumarin (Al-Jasass and Al-Jasser 2012). Active compounds of the plant include soluble fibre, saponins, trigonelline, diosgenin and 4-OH-lle. Moreover hypoglycaemic activity has been primarily attributed to dietary fibre, saponin and 4-OH-lle (Sauvaire et al. 1998). The natural non-proteinogenic amino acid, 4-OH-lle possesses biological insulinotropic activity (Khosla et al. 1995). 4-OH-lle is a branched-chain amino acid, extracted from fenugreek seeds, with an absolute stereo configuration of (2S, 3R, 4S) (Fig 2.14) (Fowden et al. 1973). 4-OH-lle has been reported to function by enhancing “glucose-induced release of insulin” (Broca et al. 1999). In comparison to other pharmacological drugs used for T2D therapy (e.g. sulfonylureas), the insulin response facilitated by 4-OH-lle is dependent on glucose concentration (Fowden et al. 1973, Khosla et al. 1995). This response is ineffective at 3-5mmol/l glucose, conversely 4-OH-lle potentiates insulin secretion induced by supranormal, 6.6 - 16.7mmol/l glucose (Broca et al. 2000). This property of 4-OH-lle permits the occurrence of undesirable side-effects such as, hypoglycaemia in T2D therapy (Korthikunta et al. 2015). Glucose-induced insulin release is increased by 4-OH-lle, in the range of 100μmol/l to 1mmol/l (Korthikunta et al. 2015). In both rats and humans, this is mediated via a direct effect on isolated islets of Langerhans. In addition, in isolated perfused rat pancreas treated with 4-OH-lle, it was shown i) that insulin secretion was biphasic, ii) this effect ensued in the absence of changes in pancreatic activity, and iii) greater the glucose concentration, greater the response of insulin (Korthikunta et al. 2015). Moreover,
4-OH-lle did not interact with other agonists of insulin secretion, such as leucine, arginine, tolbutamide, and glyceraldehyde (Korthikunta et al. 2015).

Figure 2.14 Chemical structure of 4-hydroxyisoleucine
(http://www.sigmaaldrich.com/content/dam/sigmaaldrich/structure3/194/mfcd07357252.eps/_jcr_content/renditions/mfcd07357252-large.png)

2.5.4 Pharmacological Action of Fenugreek Seeds and 4-hydroxyisoleucine

As previously mentioned, fenugreek possesses anti-diabetic and anti-lipidaemic effects. However, the particular mechanism of action remains unclear, as more work is required. The anti-diabetic property of fenugreek is owed to the formation of a colloidal-type suspension in the stomach and intestine (Yadav and Baquer 2014). Whereas the anti-lipidaemic effects of fenugreek is due to inhibited intestinal cholesterol absorption, improved loss of bile through faecal excretion, and effects of amino acid patterns on serum cholesterol (Yadav and Baquer 2014, Zarshenas et al. 2014). Fenugreek also comprises coumarins and additional constituents which affect platelet aggregation. Furthermore, this plant’s components have also shown evidence of cardiotonic, diuretic, anti-inflammatory, anti-hypertensive, and anti-viral properties (Zarshenas et al. 2014).

2.5.4.1 Evidence Based Studies on the Anti-diabetic Properties of Fenugreek

The action of fenugreek on lowering blood glucose levels was said to be almost analogous to that of insulin, mimicking its effects (Baquer 2011). Baquer et al, 2011 and Ardekani et al, 2009 reported the unusual amino acid, to display in vitro insulinotrophic activity and, anti-diabetic properties in animal models (Haeri et al. 2009). According to Ardekani et al, 2009, the amino acid is a useful and well-tolerated treatment for insulin resistance, as it serves as a hypoglycaemic and, a protective agent for hepatocytes. (Haeri et al. 2009). In addition, a meta-analysis on the effect of herbs on glucose homeostasis
in T2D patients, stated that HbA1c was significantly reduced in the group supplemented with fenugreek (Suksomboon et al. 2011). Furthermore, it was reported that ocular histopathological, and biochemical irregularities relevant to diabetic retinopathy, were controlled when using fenugreek, and sodium orthovandate independently, or in low dose combination (Prabhakar and Doble 2011). Another review article by Assad and Morse, 2013 included three separate studies that assessed the effect of fenugreek in patients with DM (Assad 2013). The first study assessed the effect of fenugreek use in patients with T1D, and showed a significant reduction of fasting blood glucose, TGs, total cholesterol, VLDL, and LDL levels (Assad 2013). The second study assessed the effect of fenugreek usage (25g fenugreek powder/day) in patients with T2D, and showed that fasting blood glucose levels were decreased, and glucose tolerance was improved (Assad 2013). The third study showed fenugreek to improve glycaemic control, insulin sensitivity, and hypertriglyceridemia in newly diagnosed T2D patients (Assad 2013).

2.5.4.2 Evidence Based Studies on the Anti-lipidemic Properties of Fenugreek

Anti-lipidemic properties were noted in the Hazra et al, 1996 trial on T1D; minor but statistically significant decreases were found in TG, and LDLc levels, but HDLc levels remained unchanged (Sharma et al. 1996). These results were based on a group of 15 non-obese, asymptomatic, hyperlipidaemic adults, who ingested 100g defatted fenugreek powder/day over 3 weeks (Sharma et al. 1996). In a later study, 60 patient diets were supplemented with 25g powered fenugreek seed/day for 24 weeks; lipid profiles were normalized by a decrease of 14-16% in LDLc and TG levels, and a 10% rise in HDLc (Sharma et al. 1996). Likewise, Sowmya and Rajyalakshmi, 1999 observed substantial decreases in TG and, LDLc levels in 20 adults with hypercholesterolemia, who received 12.5-18g powdered, germinated fenugreek seeds for a month - but no changes were observed in HDLc, VLDL, or TG levels (Sowmya and Rajyalakshmi 1999). In another study, Sharma et al, 1990 also described a reduction in total cholesterol levels in 5 diabetic patients treated with fenugreek seed powder (25g orally/day) over 21 days (Neelakantan et al. 2014). Also, Bordia et al, 1997 investigated the effects of fenugreek seed powder (2.5g administered twice daily for 3 months) in a group of 40 individuals with coronary artery disease and T2D, and observed reductions in TG levels but no changes in HDLc levels (Bordia et al. 1997).

2.5.4.3 Evidence Based Studies on the Anti-diabetic and Anti-lipidemic Properties of 4-hydroxyisoleucine

The molecular mechanism of action of 4-OH-Ile has been displayed in in vitro studies. For example in rat muscle cells, glucose uptake and GLUT4 translocation to the plasma membrane was elevated following a 16h exposure to 4-OH-Ile (Jaiswal et al. 2012). Another study treated L6 myotubes with 4-OH-Ile, resulted in reduced insulin resistance (Maurya et al. 2014). 4-OH-Ile displayed its inhibitory effect on
both the production of ROS, as well as, reduced activation of the JNK1/2 pathway. The anti-inflammatory potential of 4-OH-Ile on 3T3-L1 adipocytes was confirmed by an increase in glucose uptake accompanied with reduced TNF-α mRNA expression and secretion (Yu et al. 2013). As previously mentioned, the anti-diabetic properties of 4-OH-Ile are associated with its capability to stimulate insulin. This was observed in both diabetic dogs and rats which displayed an improvement in glucose and insulin tolerance, and reduced hyperglycaemia (Broca et al. 1999). The secretagogue potential of 4-OH-Ile is of particular interest, with regards to insulin resistance. In STZ-treated rats the stimulating effect of 4-OH-Ile resulted in an improved diabetic state (Broca et al. 2004). Also, in normal dogs and rats, 4-OH-Ile improved insulin secretion, and glucose tolerance (Broca et al. 1999). During a diabetic state, reversal of defective insulin secretion is highly desirable, as well as, improving insulin sensitivity in both peripheral and hepatic tissues. The insulin-sensitising efficacy of 4-OH-Ile was confirmed in two rat models. These models used the hyperinsulinaemic clamp method, which improved insulin sensitivity, and reduced hepatic glucose output in sucrose and lipid-fed rats (Broca et al. 2004). Alloxan-induced diabetic rats treated with fenugreek seed powder containing 28% 4-OH-Ile, displayed improvements in blood glucose level, and body weight, when compared to the diabetic control (Khosla et al. 1995). A study by Narender et al, 2006 observed reduced plasma TGs, total cholesterol (TC), and FFAs, complemented with a 39% rise in HDLc, in dyslipidaemic hamsters (Narender et al. 2006). Haeri et al, 2009 investigated the effect of 4-OH-Ile (50mg/kg for 8 weeks) on fructose-fed and STZ-induced diabetic rats (Haeri et al. 2009). These rats displayed improved liver function markers, decreased blood glucose and restoration of blood lipid levels (Haeri et al. 2009). The glucose-dependent and insulin-sensitising potential of 4-OH-Ile has been observed via its effects on pancreatic islets, liver, muscle and adipose tissue. These effects, culminated with the lack of acute toxicity, suggests that this compound, serves as a prospective natural therapeutic intervention in obesity and insulin resistance.

2.5.4.4 Evidence Based Studies on the Antioxidant Properties of Fenugreek Seed and 4-Hydroxyisoleucine

Studies have reported the AO effect of fenugreek in attenuating ROS levels. Mohamad et al, 2004 observed SOD2 and related liver enzyme levels, comparable to the diabetic group of fenugreek seed treated rats (Mohamad et al. 2004). Ravikumar and Anuradha, 1999 showed supplementation of diabetic animal diets with fenugreek seed, interrupted free radical metabolism (Ravikumar and Anuradha 1999). In a second study, they also showed that treatment with fenugreek seed powder improved AO status, via reduction in peroxidation (Anuradha and Ravikumar 2001). Another study, investigated the AO activities of various extracts of fenugreek seeds in cholesterol-fed rats, and observed a reduction in TBARS, and increase in catalase and SOD2 gene expression (Belguith-Hadriche et al. 2010). Additionally Dixit et al,
2005 revealed significant AO potential of germinated fenugreek seeds, which is due to the presence of flavonoids and polyphenols (Dixit et al. 2005). Kandhare et al, 2015 observed standardized fenugreek seed extract to execute anti-fibrotic efficacy, via stimulation of Nrf2, which in turn controls anti-inflammatory and fibrogenic molecules (Kandhare et al. 2015). The bioactive component of fenugreek seeds, 4-OH-llle, independently possesses AO activity as shown by Dutta et al, 2014. This study proved that 4-OH-llle scavenges hydroxyl, superoxide anion, hydrogen peroxide and DPPH radicals, decreased lipid peroxidation and protein carbonyl levels, and concomitantly increased GSH levels (Dutta et al. 2014). An important study, by Mayakrishnan et al, 2015 found both trigonelline and diosgenin to display protective effects via a substantial decrease in liver TGs, expression of liver ER stress marker proteins, and rise in liver glycogen content (Mayakrishnan et al. 2015). Other findings, based on other organ systems also validates the potent AO potential of fenugreek seeds (Kaviarasan et al. 2004, Gupta et al. 2010).

2.5.5 Adverse Effects and Safety

Fenugreek has been considered non-toxic and well-tolerated, with minimal side effects has been related with its usage (Wani and Kumar, Muraki et al. 2011). Fenugreek should not be taken during pregnancy and lactation and/or individuals with liver or kidney impairment should be avoided, due to inadequate data suggesting usage (Neelakantan et al. 2014). Other reported side effects include dizziness, flatulence, hypoglycaemia, transient diarrhoea and maple syrup urine, and decrease in blood urea (Vijayakumar et al. 2005, Neelakantan et al. 2014). Moreover, fenugreek has been documented to cause allergic reactions such as hoarseness, facial angioedema, nasal congestion, persistent coughing, shock and wheezing (Neelakantan et al. 2014). Continual chronic use of fenugreek should be avoided to prevent adverse reactions or toxicities, due to long-term data being unavailable (Vijayakumar et al. 2005, Neelakantan et al. 2014). Most importantly hypoglycaemia is an expected effect, thus care should be taken following fenugreek supplementation (Neelakantan et al. 2014).

Despite these documented adverse effects and warnings, there has been reports on the tolerability and outcome of fenugreek treatment. No clinical hepatic and/or renal toxicity and haematological irregularities were observed in a group of diabetic individuals, who were administered 25g/day of fenugreek for 24 weeks (Sharma et al. 1996). It was also shown in an animal study that fenugreek failed to induce signs of toxicity or mortality (Muralidhara et al. 1999). However, the data generated to date is minimal regarding the adverse effects and safety, and requires well-designed clinical trials to assess the outcome of fenugreek on insulin resistance and secretion, and cholesterol metabolism (Basch et al. 2003).
2.5.6 Drug Interactions

The administration of fenugreek in combination with other drugs could pose potential negative interactions, affecting their activity. Fenugreek powder is abundant in fibre and could hinder the absorption of oral medication (Wani and Kumar). Furthermore, simultaneous use of the plant with other hypoglycaemic agents, such as insulin and MF may result in hypoglycaemic episodes (Wani and Kumar, Neelakantan et al. 2014). Also, fenugreek can impede the activity of corticosteroids, warfarin, insulin and hormone therapy, due to the high content of mucilaginous fibre in the plant, and viscosity in the gut (Neelakantan et al. 2014) (Muralidhara et al. 1999). Coumarins found in fenugreek have anti-platelet effects (Neelakantan et al. 2014); thus simultaneous use of anti-platelet or anti-coagulant drugs (aspirin, clopidrogel, non-steroidal anti-inflammatory drugs, such as ibuprofen, diclofenac, naproxen, heparin, dalteparin and enoxaparin) may elevate the risk of bruising and bleeding (Neelakantan et al. 2014).

Therefore immense care should be taken upon administration of fenugreek in combination with other drugs.

2.6 Research Problem and Significance

T2D continues to negatively impact socio-economic development in multiple communities, despite efforts to control the disorder (WHO 2016). The incidence of T2D in different communities is a well-known phenomenon but under-diagnosed and poorly managed. Both self-care and anti-diabetic drugs have been widely employed as the priority therapy in treating T2D. However, the lack in compliance to self-care requirements, and unwanted side effects accompanying MF pose a potential problem (Rios et al. 2015). When strictly adhered to, self-care regimens efficiently maintain basal glucose levels. However, due to excessive calorie consumption, and sedentary lifestyle self-care regimens have become redundant. This failure in compliance, leads to the use of anti-diabetic drugs, such as MF which achieve basal glucose levels. Upon achieving this goal, unwanted side effects and complications make the use of this drug undesirable. Therefore, posing the requirement for new anti-diabetic therapy as T2D is a threat to public health especially in poor communities.

Long-term exposure to chronic hyperglycaemia due to insulin resistance plays an active role in elevating FFAs and ROS production. The elevated levels of FFAs contributes to an abnormal lipid profile, predisposing individuals to dyslipidaemia. Furthermore, an over-production in ROS surpasses the endogenous AO response and detoxification system, resulting in a highly toxic oxidative state. Both dyslipidaemia and elevated OS are major risk factors for the onset and progression of CVD, and microvascular and macrovascular complications. Therefore, uncontrolled glucose levels either due to lack of self-care or unwanted side effects of MF, pose severe health risks for T2D individuals.
Consequently, there is a need for developing high quality treatment alternatives for chronic hyperglycaemia, to improve the long-term outcomes of T2D. Over decades plants have been used for treatment of several disorders, and several bioactive compounds isolated from these plants produced effective drugs (Rios et al. 2015). Moreover, natural therapy is currently a significant source for the development of new treatments (Rios et al. 2015). Natural therapy possesses chemical diversity, which aids an array of biological functions, posing plants as an important source for new treatment opportunities (Rios et al. 2015). Fenugreek has been traditionally used to treat an array of illnesses and disease, many of which has been documented (Basch et al. 2003). In addition, studies have validated the proposed traditional usage and therapeutic outcomes of both 4-OH-lle and fenugreek seed (Neelakantan et al. 2014, Fuller and Stephens 2015, Gong et al. 2016). However, there is minimal evidence with regards to 4-OH-lle and the seed; and specific genes, proteins, and related factors in managing and controlling glucose and its long-term effects. This indicates the need for research on the molecular and biochemical aspects of 4-OH-lle and fenugreek seed in regulating hyperglycaemia and subsequent downstream effects.

Consequently, we hypothesized that 4-OH-lle in comparison to MF, and FSE increased biochemical and molecular responses following chronic exposure to hyperglycaemia in human hepatoma (HepG2) cells and C57BL/6 male mice. In order to test our hypothesis we measured anti-diabetic effects of 4-OH-lle in comparison to MF, and FSE relative to insulin signalling, lipid metabolism and OS, during normal and hyperglycaemic conditions in HepG2 cells and C57BL/6 male mice.

2.7 Research Aims, Objectives and Hypothesis

2.7.1 Research Aim

This study investigated the anti-hyperglycaemic, anti-lipidaemic and anti-oxidative effects of 4-OH-lle in hyperglycaemic HepG2 cells and C57BL/6 male mice.

2.7.2 Research Objectives

To determine the effect of 4-OH-lle in comparison to MF, and FSE in hyperglycaemic HepG2 cells and C57BL/6 male mice:

1. On a broad class of proteins and genes regulating insulin signalling and glucose sensing.
2. On PCSK9, PPARG, and the lipid profile relative to lipid metabolism.
3. On oxidative markers, AO response and mitochondrial proteins relative to OS.
2.7.3 Research Hypotheses

During hyperglycaemia, 4-OH-ile regulates insulin signalling and glucose sensing, dyslipidaemia and OS, in comparison to MF, and FSE.

2.8 References


Chapters 3, 4 and 5 comprise the three manuscripts which have been submitted to journals. Each manuscript has been formatted as per the specific journal requirements. However for thesis consistency the margins, font, line spacing, numbering of sections and figures were adjusted.
CHAPTER 3

4-hydroxyisoleucine enhances glucose sensing and insulin receptor signaling, in streptozotocin-induced diabetic C57BL/6 male mice

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Abstract

Type two diabetes mellitus (T2D) is a metabolic disorder characterized by chronic hyperglycaemia and compromised insulin receptor (IR) signaling ultimately contributing to insulin resistance. Previous studies showed beneficial glucose lowering effects of a novel amino acid, 4-hydroxyisoleucine (4-OH-lle) found abundantly in fenugreek seeds, displaying potent anti-diabetic activity. Based on our previous in vitro study, we investigated the effect of 4-OH-lle and metformin (MF), on hepatic IR signaling in normoglycaemic (NG) and streptozotocin-induced diabetic (hyperglycaemic –HG) C57BL/6 male mice. Mice (both normoglycaemic and hyperglycaemic) were treated via oral administration with 4-OH-lle and MF for 15 days. The oral glucose tolerance test was performed to evaluate glucose sensing ability. 4-OH-lle enhanced glucose utilization, exceeding the effects of MF, under both conditions. We investigated protein and gene expression of IRβ, glucose transporter2 (GLUT2), phosphorylated - IR substrate1 (pIRS1); protein kinase B (pAkt) and glycogen synthase kinase3α/β (pGSK3α/β). 4-OH-lle induced the most significant effect on the insulin signaling cascade with increased protein and gene expression of IRβ, pIRS1, pAkt, pGSK3α/β, and GLUT2, under both conditions. The glucose-lowering effect of 4-OH-lle exceeds that of MF by stimulation of the hepatic insulin signaling pathway in mice.

Keywords: Hyperglycaemia/ insulin signaling/ insulin resistance/ C57BL/6 male mice
Introduction

Hepatic insulin signalling is central to metabolic processes including glycogen synthesis, glycogen storage, recruitment of glucose transporters and lipid synthesis [1]. Autophosphorylation of the insulin receptor (IR) by insulin recruits and activates intracellular down-stream signalling molecules, leading to glucose uptake and subsequent biological effects. Conversely, defective insulin signalling, defined as insulin resistance results in post-prandial hyperglycaemia, dysregulated glucose utilization and lipid metabolism [1]. During insulin resistance, there is conventional binding of insulin to the IR but signal transduction is impaired [2]. The integral role of this signalling cascade in glucose homeostasis leads to a disorder classified by chronic hyperglycaemia - type 2 diabetes mellitus (T2D).

Presently, the treatment of T2D principally involves a sustained reduction in hyperglycaemia using agents that increase insulin secretion in the pancreas, stimulate the sensitivity of the liver to insulin and decrease glucose absorption rate in the gastrointestinal tract (GIT) [1, 2, 3]. The commonly used first-line drug for treatment of T2D is metformin (MF) which belongs to the sensitizer group of drugs. This oral hypoglycaemic agent decreases hepatic glucose output and improves tissue sensitivity to insulin [4]. Although highly effective, some minor side effects have been recorded with chronic use; there is still an increasing demand for new compounds or therapeutic alternatives, with less or no side effects for the treatment of T2D.

Alternative therapeutic interventions have become a focus for the treatment for T2D. Alternatives such as herbal medicines have gained attention for anti-diabetic potential [15, 16]. Trigonella foenum-graecum, commonly known as fenugreek, has been investigated as a therapeutic intervention in diabetes [16-18]. Cell culture, animal model and meta-analysis studies by Khosla et al, 1995, Vijayakumar et al, 2005 and Neelakantan et al, 2014 established the glucose lowering properties of fenugreek seeds [18, 19, 20]. The anti-diabetic effect of fenugreek seeds is largely attributed to a high content of the branched-chain amino acid derivative, 4-OH-lle, which comprises about 80% free amino acid content within the seed [21]. Animal studies demonstrated 4-OH-lle to possess an insulinotrophic effect; functions as an insulin secretagogue only at elevated blood glucose levels and stimulates the insulin signalling pathway [17, 21, 22], making 4-OH-lle a strong candidate as an antidiabetic compound. Most importantly, Vijayakumar and his group observed the hypoglycaemic effect of FSE in vivo, and concluded the effect to be mediated by the activation of an insulin signalling pathway in adipocytes and liver cells [19]. Our previous in vitro study using HepG2 human liver cells as a model was the first to show that FSE and its biologically active compound 4-OH-lle, in comparison to MF, significantly influenced the insulin signalling pathway and subsequently increased hepatic glucose uptake [13]. The objective of the present in vivo study was
therefore to investigate the effect of 4-OH-Ile in comparison to MF, on glucose sensing, IRβ-IRS1 and Akt signalling in normal and streptozotocin (STZ)-induced diabetic C57Bl/6 male mice.

Materials and methods

Materials

4-OH-Ile (50118) and STZ (S0130) were purchased from Sigma Aldrich (St Louis, MO, USA). Whole fenugreek seeds were purchased from Agricol Niche Brands, a South African seed company. A herbarium voucher of flowering material was lodged at the Ward Herbarium (UDW-UKZN; N.Naicker 1). All other consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

Animals

Six-week-old male C57BL/6 mice (n=40) were procured from the Biomedical Resource Unit at the Westville Campus of the University of KwaZulu-Natal (UKZN), Durban, South Africa. Mice with a mean body weight (BW) of 20 ± 2.99g were randomly divided into 2 groups: non-diabetic (normoglycaemic) and diabetic (hyperglycaemic-HG). Each group were further subdivided into 4 groups of 5 mice each as follows: Control (C), MF, FSE and 4-OH-Ile. Mice were housed in polycarbonated cages in a humidity and temperature controlled room (40-60% humidity, 23 ± 1°C) with a 12 hour (hr) light dark cycle. The mice were fed a commercially available pellet diet and normal drinking water ad libitum throughout the 15 day experimental period. The mice were maintained according to the rules and regulations of the Experimental Animal Ethics Committee of the UKZN (Ethical approval number: AREC/057/016).

Induction of diabetes

Diabetes mellitus is a disease characterized by a relative or absolute lack of insulin resulting in hyperglycaemia. Type 2 diabetes mellitus is associated with insulin resistance, as well as a lack of appropriate compensation by beta cells, causes insulin deficiency [63-66]. Type 2 diabetes mellitus is established in both non-obese and obese animal models with varying degrees of insulin resistance and beta cell failure. The mice model should possess characteristics which emulate the pathophysiology and complications of T2D comparable to the human condition [63-66]. Streptozotocin is a chemical that is principally toxic to the insulin-producing β cells of the pancreas in mammals [23, 61]. Streptozotocin offers the additional benefit of being able to select specific traits of interest with regards to disease specific conditions [24]. In relation to T2D, STZ allows for the replication of the metabolic characteristics and disturbances observed in this disease [25]. Administration of multiple low doses or a low single dose of STZ damages pancreatic β cells through alkylation of DNA - by causing partial destruction (and not total knockout) of these cells, resulting in hyperglycaemia [23, 26]. Therefore displaying the
pathophysiological characteristics of T2D which include inadequate β-cell mass and β-cell dysfunction. As a result T2D was induced in all mice in the diabetic group (HG group) by an intraperitoneal (i.p.) administration of STZ (50mg/kg BW) dissolved in 0.1M citrate buffer (pH 4.4) following an overnight fast (12hr) [27, 28]. The optimal dosage of 50mg/kg was determined by a pilot study which included a range of STZ concentrations (50mg/kg, 100mg/kg and 150mg/kg BW). The blood was collected from the tail vein, using a glucometer (Accu-Chek) to monitor and measure the fasting blood glucose (FBG) over a 10 day period prior to administration of treatment. Once a blood glucose of >7mmol/L and <16mmol/L was achieved and stable, the treatment period was inducted.

Treatment preparation of stock solutions
Whole fenugreek seeds were crushed using a mortar and pestle, suspended in deionized water (1000 mg/mL), and placed on a stirrer at room temperature (RT) for 3Hr. The aqueous solution was transferred to a sterile conical tube and centrifuged (3600xg for 10min) at RT. Supernatant (FSE) was removed, freeze-dried, and stored at -20oC. Metformin tablets were also crushed with a mortar and pestle and suspended in 0.1M phosphate-buffered saline (PBS) (50mg/mL). The solution was then filter sterilized (0.45-mm filter). 4-hydroxyisoleucine was obtained in a liquid form, and treatments were prepared from a stock solution (as per manufacture instructions). Subsequent treatments were prepared in 0.1M phosphate buffer solution (PBS) solution for in vivo.

Treatment preparations
Preparation of treatments were guided by the protocol Naicker et al, 2016 [13]. The concentration of 4-OH-Ile (100mg/kg BW), MF (20mg/kg BW), FSE (100mg/kg BW) and were based on previous animal studies which evaluated a range of concentrations and reported the outcomes of the range which we based our optimal concentration [17, 29-32]. Mice were treated once daily for the 15 day treatment period via oral gavage.

Oral glucose tolerance test (OGTT)
Glucose tolerance ability was measured by the OGTT performed on day 15 (last experimental day) of the experimental period. After an overnight fast (12hr), mice in NG and HG groups were orally dosed with a D-glucose solution (2.0g/kg BW). The blood glucose concentrations were subsequently measured at 0 (prior to oral glucose dosing), 30, 60, 90, and 120 min after the oral dosing of glucose. In order to give a clear quantitative indication of glucose intolerance in the different animal groups, area under the curve (AUC) values were calculated using the formula below (Supplementary data, table 1, 2) [33]:

\[
AUC = \frac{(B2 + B1)}{2} \times (A2 - A1)
\]
B1 and B2 = Initial and final blood glucose values (mg/dl) at a given time period respectively

A1 and A2 = Initial and final time periods (min) respectively

Where: \((A2 - A1) = (30 - 0), (60 - 30), (90 - 60)\) and \((120 - 90)\)

\((B2 + B1) = \text{Blood glucose values at the above mentioned time periods}\)

**Animals post treatment**

At the end of the treatment period, the mice were sacrificed using isoflurane. Fasting plasma samples were obtained from the body of the mice; blood samples were collected using anticoagulant EDTA tubes. The blood glucose levels were measured at an accredited laboratory (AMPATH). Liver samples were harvested, rinse twice in saline, dissected and stored in cytobuster and triazol at -80°C until analysis.

**Western blotting**

Western blots were performed to quantify expression of phosphorylated (p-) and total proteins in the insulin signaling pathway. These include phosphorylated and total; IRS1 (pIRS1/IRS1), Akt (pAkt/Akt), GSK3α/β (pGSK 3α/β/pGSK 3α/β) and total insulin receptor β (IRβ) and GLUT2. Crude protein was isolated using Cytobuster (Novagen) supplemented with protease and phosphate inhibitors (Roche: 04693124001 and 04906837001). Liver samples were homogenized and incubated in cell lysis buffer for 10 min and then centrifuged (4°C, 12,000g). Crude protein was quantified by the bicinchoninic acid (BCA) assay and standardized to 1mg/mL. Samples were boiled in Laemmli buffer [dH₂O, 0.5M Tris–HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, and 1% bromophenol blue] for 5 min. Samples were electrophoresed on a sodium dodecyl sulphate polyacrylamide gel (4% stacking and 10% resolving) for 1hr at 150V and transferred on to nitrocellulose using the TransBlot Turbo Blotting System (BioRad) using a preinstalled Standard SD program. All membranes were blocked for 2hr in 3% BSA in Tween20-Tris-buffered saline (TTBS - 0.15M NaCl, 2.68M KCl, 24. 86M Tris, 500µl Tween20, pH 7.4) at RT on a shaker. Thereafter, the membranes were incubated with primary antibody [pIRS1 (CS2381), anti-IRβ (CS3050), anti-GLUT2 (AB54460), pAkt (CS9271) and pGSK3α/β (CS9331); 1:5000] at 4°C overnight. The membranes were then equilibrated to RT on a shaker for 1hr, followed by 5 washes (10min) with TTBS. Membranes were then probed with horseradish peroxidase-conjugated-secondary antibody [anti-rabbit 1:10000 (CS7074)] for 1hr, followed by 5 washes (10min) with TTBS. Chemiluminescent signal was detected using ECL Clarity Western detection reagent (BioRad) and captured on the BioRad ChemiDoc Viewing System. Data were expressed as relative band density (RBD) and expression of proteins was analyzed with the BioRad ChemiDoc MP Imaging System with Image Lab software. Membranes were quenched (5% H2O2 at 37°C for 30min) and proteins were normalized against anti-IRS1 (CS2382), anti-Akt (CS9331), anti-GSK3α/β (cs9315) and β-actin (A5316), 1:1000.
Quantitative PCR

The RNA expression of genes of interest was determined by qPCR. These included IRβ, IRS1, Akt, GSK3α/β, GS, GK, GLUT2 and SREBP1c. Total RNA was isolated using an in-house protocol [34]. RNA was quantified using a spectrophotometer (Nanodrop2000) and standardized to 1,000ng/µl. Standardized RNA was reverse transcribed to complementary DNA (cDNA) using the iScript™ cDNA Synthesis kit (Bio-Rad; 107-8890) as per the manufacturer’s instruction. A reaction volume of 10µl was prepared consisting of 5X IQ™ SYBR® green supermix (Bio-Rad; 170-880), nuclease free water, 1µl cDNA template (1,000ng/ml), and 1µl sense and anti-sense primer (25µM primer stock, Inqaba Biotec™). The mRNA expression was normalized against a housekeeping gene (18S). Thermocycler conditions were carried out using the CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA) as follows: initial denaturation (95°C, 10min), 40 cycles of denaturation [95°C, 15seconds (s)], annealing and extension (72°C, 30s). mRNA expression was determined using the method described by Livak and Schmittgen, 2001 to calculate relative fold change [35]. Primer sequences and annealing temperatures are shown supplementary data, table 3.

Measurement of glucose concentration

Measurement of glucose concentration was performed by an accredited pathology laboratory AMPATH Laboratories, Durban, South Africa (Incorporated Pathology Laboratory practices of: Drs Du Buisson, Kramer, Swart, Bouwer Inc).

Liquid chromatography–mass spectrometry (LC-MS)

Liquid chromatography–mass spectrometry (LC-MS) is an analytic chemistry technique which combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). 1mg of crude FSE was weighed and dissolved in 10ml MeOH. Each sample was vortexed and sonicated to allow for complete dissolution. Then the extract was subjected to solid phase extraction (SPE), using a Supelco C18 100mg solid phase cartridge. The extract was eluted with MeOH. A 1:100 dilution of the sample was prepared using MeOH and injected into the LC-MS. The instrumentation used was the Shimadzu 202 UFLC-MS, mobile A: 0.1% FA in H2O and mobile phase B: 0.1% FA in CAN. Separation was achieved using a YMC Triart C18 analytical column (4.6mm x 150mm), using a gradient elution method from 5% B to 95 B over 25min. Data was collected at 265 nm and analyzed using the Shimadzu Lab Solutions software (Supplementary data Fig 1, 2, 3 and table 4).
Statistical analyses

Statistical analyses was performed using GraphPad Prism v5.0 software (GraphPad Software, Inc.). The data was expressed as relative fold change to respective controls in the qPCR and western blotting experiments. With regards to western blotting, the phosphorylated proteins were normalized against the total protein. Followed by the total protein against actin. The data was expressed as the mean + SD for the OGTT and glucose measurements. Following each experiment in vitro and in vivo, 4-OH-lle was compared to MF and the untreated control. We then analyzed FSE in comparison to the untreated control. Statistical comparisons for 4-OH-lle, MF and the untreated control were made using a one-way analysis of variance (ANOVA), nonparametric test (Kruskal Wallis test) and a Dunn’s post-test; and a non-parametric Mann-Whitney test was used for FSE and the untreated control. The data were considered statistically significant with a value of p < 0.05.

Results

4-hydroxyisoleucine reduces excess blood glucose levels

Glucose utilization of male C57BL/6 mice were assessed by an OGTT and quantification of circulating glucose levels. Treated mice under both NG (p=0.0154; Fig 1 a) and HG (p=0.0245; Fig 1 b) conditions efficiently utilized glucose, compared to their respective control groups. Two hours after the glucose administration, the blood glucose levels of the HG group were significantly higher than the NG group (p<0.005). More importantly, under a HG state, the excess blood glucose was reduced following treatment with 4-OH-lle (p=0.0021; Fig.1 d). Under NG conditions (p=0.00231) the blood glucose levels of MF and 4-OH-lle were significantly lower than the NC (p<0.005; Fig.1 c).

Table 1 Area under the curve (AUC) values for the OGTT at day 15 of experimental period for normoglycaemic group

<table>
<thead>
<tr>
<th>Area under curve (AUC)</th>
<th>Control</th>
<th>FSE</th>
<th>MET</th>
<th>4-OH-lle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC 1 (30-0 min)</td>
<td>217.5 ± 50*</td>
<td>199.5 ± 21#</td>
<td>192 ± 47*</td>
<td>183 ± 24*</td>
</tr>
<tr>
<td>AUC 2 (60-30 min)</td>
<td>222 ± 34*</td>
<td>202.5 ± 72#</td>
<td>190.5 ± 24*</td>
<td>187.5 ± 48+</td>
</tr>
<tr>
<td>AUC 3 (90-30 min)</td>
<td>223.5 ± 61*</td>
<td>201 ± 45#</td>
<td>192.45 ± 13+</td>
<td>185.25 ± 23+</td>
</tr>
<tr>
<td>AUC 4 (120-90 min)</td>
<td>221.25 ± 19*</td>
<td>207 ± 35#</td>
<td>190.65 ± 175+</td>
<td>184.5 ± 25+</td>
</tr>
<tr>
<td>Total AUC</td>
<td>884.25 ± 124</td>
<td>810 ± 264</td>
<td>765.6 ± 119</td>
<td>740.25 ± 249</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD of 5 animals; *, #, + Values with different superscript letters within a row are significantly different from each group of animals (Tukey’s multiple range posthoc test, p < 0.05). FSE: fenugreek seed extract, MET: Metformin, 4-OH-lle: 4-hydroxyisoleucine
Table 2  Area under the curve (AUC) values for the OGTT at day 15 of experimental period for hyperglycaemic group

<table>
<thead>
<tr>
<th>Area under curve (AUC)</th>
<th>Control</th>
<th>FSE</th>
<th>MET</th>
<th>4-OH-lle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC 1 (30-0 min)</td>
<td>498 ± 29*</td>
<td>484.5 ± 17#</td>
<td>447 ± 33*</td>
<td>423 ± 17*</td>
</tr>
<tr>
<td>AUC 2 (60-30 min)</td>
<td>508.5 ± 54*</td>
<td>465 ± 89#</td>
<td>438 ± 55*</td>
<td>453 ± 18*</td>
</tr>
<tr>
<td>AUC 3 (90-30 min)</td>
<td>519 ± 45*</td>
<td>448.5 ± 16#</td>
<td>433.5 ± 12*</td>
<td>445.5 ± 19*</td>
</tr>
<tr>
<td>AUC 4 (120-90 min)</td>
<td>519 ± 34*</td>
<td>448.5 ± 10#</td>
<td>435.75 ± 11*</td>
<td>445.5 ± 15*</td>
</tr>
<tr>
<td>Total AUC</td>
<td>2044.5 ± 163*</td>
<td>1846.5 ± 257#</td>
<td>1754.25 ± 175*</td>
<td>1767 ± 138</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD of 5 animals; *, #, + Values with different superscript letters within a row are significantly different from each group of animals (Tukey’s multiple range posthoc test, p < 0.05). FSE: fenugreek seed extract, MET: Metformin, 4-OH-lle: 4-hydroxyisoleucine

Fig.1  Average concentrations (mean±SD) of blood glucose levels in the serum of mice, and oral glucose tolerance test at day 15 of experimental period; blood glucose levels were measured at 0, 30, 60, 90 and 120 min time intervals following an overnight fast, following treatment with metformin (MF), fenugreek seed extract (FSE) and 4-hydroxyisoleucine (4-OH-lle), compared to the relative control (C). Average
blood glucose (A) normoglycaemic ($p=0.00231$) and (B) hyperglycaemic ($p=0.0245$). Oral glucose tolerance test (C) normoglycaemic ($p=0.0154$) and hyperglycaemic ($p=0.0021$).

4-hydroxyisoleucine positively regulates the total protein expression of IRS1, IRβ, Akt, GSK3α/β and GLUT2 and phosphorylated protein expression of pIRS1, pAkt and pGSK3α/β

The effects of 4-OH-Ile and MF on phosphorylated total expression (IRS1, IRβ, GSK3α/β and GLUT2) and (pAkt and pGSK3α/β) of proteins regulating the insulin signaling cascade were assessed by western blotting. Under NG conditions, 4-OH-Ile significantly up-regulated the total protein expression of IRβ 2.2-fold (1.08±0.02RBD), (Fig.2 a). 4-OH-Ile (2.1-fold; 0.98±0.01RBD) and MF (2.5-fold; 1.28±0.03RBD) upregulated the phosphorylated expression of IRS1 ($p=0.00279$) greater than the total protein expression (Fig.2 a). However, 4-OH-Ile increased the total protein expression of IRS1 ($p=0.00279$) by 1.6-fold (0.98±0.01RBD) (Fig.2 a). More importantly, under a HG condition, MF and 4-OH-Ile also increased total protein expression of IRβ ($p=0.0121$) by 2.8 (0.96±0.01RBD) - and 1.6 (0.89±0.01RBD) -fold (Fig.2 b). The phosphorylated expression of IRS1 ($p=0.00279$) was increased under a HG condition (Fig.2 b) by 4-OH-Ile (1.6-fold; 0.89±0.01RBD) as well as the total protein expression by 1.5-fold (0.96±0.03RBD). Our data shows that the novel amino acid 4-OH-Ile has the potential to work as efficiently as the first line of drug MF in stimulating the IR signal. Above all, in a HG state 4-OH-Ile stimulated a strong receptor signal as seen by the 2.4-fold change in IRβ total protein expression. This shows that 4-OH-Ile can effectively increase the total protein expression of IRβ and translate this activation into the phosphorylation of pIRS1. Also, under a normal condition 4-OH-Ile significantly upregulated the total protein expression of GLUT2 ($p=0.0011$) by 2.8-fold (1.08±0.03RBD), (Fig.2 a). Under a HG state 4-OH-Ile elicited the most significant response by increasing GLUT2 ($p=0.0022$) total protein expression by 2.9 (1.28±0.03RBD) (Fig.2 b). Again, 4-OH-Ile consistently maintained an increase in GLUT2 total protein expression under both conditions. Following the increase in total and phosphorylated protein expression of the IR’s – 4-OH-Ile significantly increased the total and phosphorylated protein expression of Akt and GSK3α/β under both conditions. 4-OH-Ile upregulated pAkt by 2-fold (1.08±0.03RBD) and Akt by 2.2-fold (1.10±0.02RBD) under a normal condition ($p=0.0121$) (Fig.3 a). The expression of pAkt and Akt was increased by 2.3-fold (1.08±0.03RBD) and 1.9-fold (1.20±0.03RBD) under a HG condition ($p=0.0351$) (Fig.3 b). Phosphorylated protein expression of GSK3α/β was also up-regulated by 4-OH-Ile 1.8- and 1.5-fold, under a NG ($p=0.0468$) and HG ($p=0.0357$) condition, respectively (Fig.3 a, b). Lastly the total protein expression of GSK3α/β was increased by 1.9- and 2.1-fold under a NG and HG condition (Fig.3 a, b).
Fig. 2 Metformin (MF) and 4-hydroxisoleucine (4-OH-Ile) treatments relative to the untreated control (C) on phosphorylation of insulin receptor β (IRβ), insulin receptor substrate 1 (IRS1) and glucose transporter 2 (GLUT2) on mice liver under normoglycaemic (A) and hyperglycaemic (B) conditions. *P < 0.05; **P < 0.005 relative to control.
4-hydroxyisoleucine increases the gene expression of IRS1, IRβ, GLUT2 and SREBP1c

The effects of MF and 4-OH-Ile on IRS1, IRβ and GLUT2 gene expression was investigated using qPCR. The gene expression of IRS1 ($p=0.0049$), IRβ ($p=0.0100$) and GLUT2 ($p=0.0110$) was increased by MF and 4-OH-Ile under a NG condition (Fig.5). 4-OH-Ile (2.5- and 3.9 fold) displayed a greater increase in gene expression of IRS1 compared to MF under both conditions (Fig.6 a). 4-OH-Ile preceded the effects of MF on IRβ (2.3- and 3.1-fold) and GLUT2 (1.6- and 2.8-fold) gene expression (Fig.5 b, c). MF (1.5-fold) and 4-OH-Ile (1.6-fold) also elevated the gene expression of SREBP1c under a NG state (Fig.5 d). Similar trends were displayed under a hyperglycaemic condition. Most importantly, 4-OH-Ile displayed the greatest increase in IRS1, IRβ, GLUT2 and SREBP1c gene expression compared to MF (Fig.5).

**Fig.3** Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control
(C) on phosphorylation of protein kinase b (Akt) and glycogen synthase kinase 3α/β on mice liver under normoglycaemic (A) and hyperglycaemic (B) conditions. *P< 0.05; **P< 0.005 relative to control.

4-hydroxyisoleucine increases the gene expression of AKT, GSK3α/β, GS and Gck

The effects of MF and 4-OH-lle on AKT, GSK3α/β, GS and Gck gene expression was investigated using qPCR. The gene expression of AKT (p=0.0031) was increased by MF (1.4- and 1.6-fold) and 4-OH-lle (3.6- and 3-fold) by both conditions, respectively (Fig.6 a). This increase in AKT gene expression correlates with the increased gene expression of GSK3α/β (p=0.0072) by MF (1.1- and 1.4-fold) and 4-OH-lle (1.1- and 2.5-fold), under a NG and HG condition (Fig.6 b). GSK3α/β is important for the activation of GS. The gene expression of GS (p=0.0081) was profoundly increased by 4-OH-lle (3.2-fold) under a HG condition (Fig.6 c). Gck (p=0.0436) displayed a steady increased in gene expression by MF (1.5- and 1.9-fold) and 4-OH-lle (1.6- and 1.7-fold) (Fig.6 d) under both condition, respectively.

Fig.4 Fenugreek seed extract (FSE) treatment relative to the untreated control (C) on phosphorylation of insulin receptor β (IRβ), insulin receptor substrate 1 (IRS1), glucose transporter 2 (GLUT2), protein kinase b (Akt) and glycogen synthase kinase 3α/β on mice liver under normoglycaemic (A and C) and hyperglycaemic conditions (B and D). *P< 0.05; **P< 0.005 relative to control.
Effect of FSE on protein expression of IRS1, IRβ, Akt, GSK3α/β and GLUT2 and phosphorylated protein expression of pIRS1, pAkt and pGSK3α/β and gene expression of IRS1, IRβ, GLUT2, SREBP1c, AKT, GSK3α/β, GS and Gck

Following the preceding data on 4-OH-Ile, we further investigated the effect of FSE on the protein expression of IRS1, IRβ, Akt, GSK3α/β and GLUT2 and phosphorylated protein expression of pIRS1, pAkt and pGSK3α/β. Interestingly under NG conditions, FSE significantly up-regulated the total protein expression of IRβ (p=0.0729) by 2.5-fold (1.28±0.03RBD) (Fig. 4a). FSE (2.2-fold; 1.08±0.02RBD) upregulated the phosphorylated expression of IRS1 (p=0.00279) greater than the total protein expression (Fig. 4a). FSE, under HG conditions, significantly increased the total protein expressions of IRβ (p=0.0121) by 2.5-fold (1.98±0.03RBD) (Fig. 4b) and IRS1 by (2.1-fold; 1.06±0.03RBD). Under NG conditions, FSE significantly upregulated the total protein expression of GLUT2 (p=0.0011) by 2.3 (1.12±0.03RBD). However, under a HG state FSE increased GLUT2 (p=0.0022) total protein expression by 2.5 (1.28±0.03RBD) (Fig. 4a). Following the increase in total and phosphorylated protein expression of the IR’s – FSE significantly increased the total and phosphorylated protein expression of Akt and GSK3α/β under both conditions. FSE upregulated pAkt by 1.9-fold (1.08±0.03RBD) and Akt by 2-fold (1.10±0.02RBD) under NG conditions (p=0.0121) (Fig. 5). The expression of pAkt and Akt was increased by 2.1-fold (1.08±0.03RBI) and 2-fold (1.20±0.03RBD) under HG conditions (p=0.0351) (Fig. 6). Phosphorylated protein expression of GSK3α/β was also up-regulated by FSE 2- and 2.5-fold, under a NG (p=0.0468) and HG (p=0.0357) condition, respectively (Fig.5, 6). Lastly the total protein expression of GSK3α/β was increased by 2.1- and 2-fold under a NG and HG condition.

Further, we investigated the gene expression of IRS1, IRβ, GLUT2, SREBP1c, AKT, GSK3α/β, GS and Gck by FSE. The gene expression of IRS1 (p=0.0049), IRβ (p=0.0100) and GLUT2 (p=0.0110) was increased by FSE under a NG condition (Fig.7). FSE (2.1-fold) showed an increase in the gene expression of IRS1 (Fig.7 a). Also FSE increased IRβ and GLUT2 (3.8- and 2.6-fold) gene expression, respectively (Fig.7 b, c). FSE (2.2-fold) elevated the gene expression of SREBP1c under a NG state. The gene expression of AKT (p=0.0031) was increased by FSE (2.2- and 3.2-fold), under a NG and HG condition, respectively (Fig.8 a). This increase in AKT gene expression correlates with the increased gene expression of GSK3α/β (p=0.0072) by FSE (2.2- and 3.5-fold) under both conditions (Fig.8 b). The gene expression of GS (p=0.0081) was increased by FSE (2.6-fold) under a HG condition (Fig.8 c). Gck (p=0.0436) displayed a steady increased in gene expression by FSE (2.6- and 3.5-fold) (Fig.8 d) under both condition, respectively.
Fig. 5 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of insulin receptor substrate 1 (A), insulin receptor β (B), glucose transporter 2 (C) and sterol regulatory binding protein 1c on mice liver under normo- and hyperglycaemic conditions. *$P<0.05$; **$P<0.005$ relative to control.
Fig. 6 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of protein kinase B (A), glycogen synthase kinase 3α/β (B), glycogen synthase (C) and glucokinase (D) on mice liver under normo- and hyperglycaemic conditions. *P < 0.05; **P < 0.005 relative to control.
Fig. 7 Fenugreek seed extract (FSE) treatment relative to the untreated control on the gene expression of insulin receptor substrate 1 (A), insulin receptor β (B), glucose transporter 2 (C) and sterol regulatory binding protein 1c on mice liver under normo- and hyperglycaemic conditions. *$P < 0.05$; **$P < 0.005$ relative to control.
Fig. 8 Fenugreek seed extract (FSE) treatments relative to the untreated control on the gene expression of protein kinase B (A), glycogen synthase kinase 3α/β (B), glycogen synthase (C) and glucokinase (D) on mice liver under normo- and hyperglycaemic conditions. *P < 0.05; **P < 0.005 relative to control.

Discussion

4-hydroxyisoleucine has been proposed as an anti-diabetic alternative therapy, being linked to improved insulin action, reduced post-prandial glucose levels and stimulation of the insulin signalling pathway [29, 36-38, 40]. We previously showed, using an in vitro (HepG2 liver cells) model, the reduction of extracellular glucose levels by 4-OH-lle, MF and FSE via induction of the insulin signalling pathway [13]. Furthermore, this study showed that 4-OH-lle stimulated the proximal and distal insulin signalling pathways, and subsequently GLUT2 recruitment. This study was validated by investigating the effect of 4-OH-lle in comparison to MF on glucose sensing, insulin signalling, and consequently GLUT2 recruitment in C57BL/6 male mice. 4-OH-lle significantly improved glucose homeostasis, IRβ-IRS1 and Akt signalling in normal and STZ-induced diabetic mice by effectively lowering blood glucose levels and enhancing receptor signalling, respectively. Chronic hyperglycaemia is the hallmark of T2D, and primary
treatment involves managing the high glucose levels. A recent study on skeletal muscle cells showed that 4-OH-lle inhibited inflammation-stimulated IRS1 serine phosphorylation and restored insulin-stimulated IRS1 tyrosine phosphorylation in the presence of palmitate, leading to enhanced insulin sensitivity [41]. A previous in vitro study by Broca et al, 1999 showed that 4-OH-lle (200 μM) potentiated glucose (16.7 mM)-induced insulin release from rat-isolated islets [42]. Haeri et al, 2009 examined the effect of 4-OH-lle on liver function and blood glucose and concluded that 4-OH-lle is a well-tolerated treatment for insulin resistance, both directly as a hypoglycaemic and as a protective agent for the liver [43]. More importantly Singh et al, 2010 confirmed the anti-hyperglycaemic property of 4-OH-lle where they showed that 4-OH-lle suppressed progression of T2D by enhancement of insulin sensitivity and glucose uptake in peripheral tissue [30]. In our in vivo study, we compared the effect of 4-OH-lle and MF on proximal and distal signalling and recruitment of GLUT2 under both normal and hyperglycaemic conditions.

Glucose uptake by the target tissues of insulin is enabled mostly by translocation of glucose transporters from an intracellular site to the plasma membrane. It was reported that IR’s and GLUT2 form a receptor transporter complex in liver cells [46-47]. This results in a mechanism of insulin facilitated hepatic glucose regulation. Using C57BL/6 male mice livers (both normo- and hyperglycaemic) we investigated the total protein expression of GLUT2. Under both conditions 4-OH-lle significantly increased the protein expression of GLUT2 (Fig.2 a, b). Furthermore, these findings correlated well with the increased gene expression of GLUT2 (Fig.5 b). The sterol regulatory binding protein 1c (SREBP1c) plays a key role in the translocation of GLUT2 to the cell surface (Ono et al. 2003). We investigated the gene expression of SREBP1c; 4-OH-lle increased the gene expression of SREBP1c under both conditions (Fig.5d).

The action of insulin is initiated by its binding to the IR. This leads to autophosphorylation of the IR and subsequent increase in tyrosine phosphorylation of several proteins including pIRS1 and pAkt. In this study the effects of 4-OH-lle on tyrosine phosphorylation of the IRβ and the downstream signalling molecules in the primary cellular targets of liver cells were investigated. The results revealed that under both conditions 4-OH-lle activated the tyrosine phosphorylation of IRβ (Fig.2 a, b), subsequently enhancing tyrosine phosphorylation of IRS1 (Fig.2 a, b) and Akt (Fig.2 a, b). These results correlate well with the increased gene expressions of IRβ, IRS1 and Akt. Activated Akt phosphorylates substrates that control insulin-mediated glucose transport, protein and glycogen synthesis [49]. An important response to Akt activation is the phosphorylation and inactivation of GSK3α/β [54]. A major substrate of GSK3α/β is GS, an enzyme that catalyses the final step in glycogen synthesis [50]. Phosphorylation of GS by GSK3α/β inhibits glycogen synthesis; conversely, inactivation of GSK3α/β by pAkt promotes glucose storage as glycogen [7]. Thereafter, the effect of 4-OH-lle on the total and phosphorylated protein
expression of GSK3α/β and the gene expression of GSK3α/β, GS and Gck was investigated. The results showed a consistent amplification by 4-OH-Ile in comparison to MF in the protein expression of GSK3α/β (Fig.3 a, b). This increased protein expression correlated with increases in GSK3α/β gene expression (Fig.5, 6). Furthermore, 4-OH-Ile significantly stimulated the gene expression of GS and Gck. This strongly suggests that the liver could be target site for 4-OH-Ile and activating the insulin signalling pathway.

Based on our previous in vitro data and literature, we further investigated FSE using the same parameters as 4-OH-Ile and MF. FSE significantly increased the total and phosphorylated protein expressions of IRS1 (pIRS1/IRS1), Akt (pAkt/Akt), GSK3α/β (pGSK 3α/β), total insulin receptor β (IRβ) and GLUT2. Also, FSE elevated the gene expressions of IRβ, IRS1, Akt, GSK3α/β, GS, GK, GLUT2 and SREBP1c. It is well established that 4-OH-Ile is the most abundant unusual free amino acid in fenugreek seeds and accounts for the seeds antidiabetic effects. Interestingly, this study shows that 4-OH-Ile exerts a potent effect on receptor signalling and glucose sensing as opposed to that of FSE alone. Studies have also reported the presence of diosgenin – a biologically active steroid sapogenin as a possible mediator in the seeds effect to maintain glucose homeostasis and insulin signalling [55]. Diabetic animal models provide evidence for the role of diosgenin in glycaemic control by decreasing proteins involved in gluconeogenesis and glucose export [56]. Furthermore, fenugreek seeds constitute a large amount of soluble fibers such as galactomannans [36]. Soluble fibers have been reported to enhance glycaemic control by inhibiting lipid and carbohydrate proteins in the digestive system [57-58].

In conclusion, this set of data confirms the induction of total and phosphorylated protein and gene expressions of GLUT2, IRβ, IRS1, Akt and GSK3α/β by 4-OH-Ile under both conditions. The effect of 4-OH-Ile is greater than MF with discernible relevance to the effect displayed during a chronic HG state. Following an insulin resistant state and the insufficiency of insulin signalling components in the liver, this effect of 4-OH-Ile is significant. Hence, understanding the effect of 4-OH-Ile relative to glucose sensing and insulin signalling provides evidence for the use of 4-OH-Ile in T2DM treatment.

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Conflict of interest
Authors declare no conflicts of interest.
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References


CHAPTER 4

4-hydroxyisoleucine regulates PCSK9 and PPARG during dyslipidemia in HepG2 cells and diabetic C57BL/6 male mice

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Abstract

Scope: In T2DM; defective insulin action and hyperglycaemia, lead to diabetic dyslipidemia.

Characteristic features include high plasma levels of triglycerides, LDLc and low HDLc. Diabetic dyslipidemia is a risk factor for CVD. Our previous study demonstrated anti-diabetic effects of 4-hydroxyisoleucine - bioactive component of fenugreek seeds, in comparison to metformin. We evaluated 4-hydroxyisoleucine compared to metformin on the lipid profile and hepatic expression of PCSK9 and PPARG in HepG2 cells and C57BL/6 mice.

Methods and results: Treatments were conducted over 72-hours (HepG2 cells) and 15-days (C57BL/6 mice) under normoglycaemic and hyperglycaemic conditions. Lipid profile analysis was conducted. Protein expressions of PCSK9 and PPARG were determined by western blotting. mRNA levels of PCSK9, SREBP1c, FAS, LDLr and Apo A1 were evaluated by qPCR. In both models, 4-OH-Ile increased SREBP1c and FAS expressions, PPARG protein expression and decreased PCSK9.

Conclusion: 4-OH-Ile improved lipid homeostasis by regulating SREBP1c processing, PCSK9 and PPARG expression.
Introduction

Type two diabetes mellitus (T2D) results from defective insulin signaling/resistance and leads to increased blood glucose levels. T2D individuals present with dyslipidemia, characterized by elevated triglycerides (TG), low high-density lipoprotein cholesterol (HDLc) and a predominance of small-dense low-density lipoprotein (LDL) particles. These changes in the lipid profile are attributed to increased free fatty acid (FFA) flux due to elevated blood glucose levels. Diabetic dyslipidemia is a risk factor for the development of cardiovascular disease (CVD); this increases the risk of microvascular and macrovascular complications, accompanied by morbidity and mortality (1). It is important to manage CVD risk factors in T2D (1).

The first-line drug therapy for the treatment of T2D is metformin (MF), which possesses anti-lipidemic properties that include improved LDL, HDL and TG levels (2). Also, statins produce favourable changes in altered lipid profiles via inhibition of HMGCoA reductase (3). However an optimum individualistic regimen remains to be defined as intolerance and insufficiency is a common occurrence. Advancements in lipid-lowering agents have focused on regulators of proprotein convertase subtilisin/kexin type 9 (PCSK9) and agonists of peroxisome proliferator-activated receptor gamma (PPARG). Studies have reported PCSK9 as a circulating regulator of LDLc through its ability to induce degradation of the LDL receptor (LDLr) in the lysosome of hepatocytes (4, 5). In T2D, elevated glucose levels cause a concomitant rise in LDLc, whilst PCSK9 regulators shift LDLr traffic from degradation to recycling in hepatic cells. In addition to LDLc clearance, there is the requirement for an associated rise in HDLc. PPARG is responsible for mediating physiological effects on both glucose homeostasis and lipid metabolism (6), and directly impacts on HDLc level (6, 7). Also, individuals with a dominant-negative mutation in the PPARG gene showed severe hyperglycaemia, providing a genetic link between PPARG and T2D (8). The disparity in lipid homeostasis in T2D, regulators of PCSK9 and agonists of PPARG may contribute to its restoration.

Nutritional supplements provide an opportunity for T2D individuals to normalize lipid levels. Plant extracts are a potential source in reducing elevated lipid levels. Amongst these plants is Trigonella foenum-graecum (an aromatic plant), commonly known as fenugreek (9), and the seeds possess anti-diabetic effects (10). The main biological content of fenugreek seeds is a branched-chain amino acid derivative - 4-hydroxyisoleucine (4-OH-Ile) which itself displays anti-diabetic effects. Among its beneficial effects are regulation of blood glucose, plasma TGs, total cholesterol and FFA levels, and improvement of liver function (11). A diabetic rat model showed that 4-OH-Ile was well-tolerated in control animals and improved HDLc levels (12). A dyslipidemic hamster model showed that 4-OH-Ile
significantly decreased plasma TG and increased HDLc levels (13). Similarly in C57BL/6 male mice 4-
OH-Ile reduced blood glucose, plasma insulin, TGs, total cholesterol and LDLc levels and raised plasma
HDLc levels (14). Previously, we showed that 4-OH-Ile, MF and fenugreek seed extract (FSE) effectively
regulated insulin signaling both in vitro (15) and in vivo. The objective of this study was to investigate the
regulatory effect of 4-OH-Ile in comparison to MF – on PCSK9 and PPARG signaling and the plasma
lipid profile, in hyperglycaemic induced HepG2 cells and C57BL/6 male mice.

Materials and methods
Materials
4-hydroxyisoleucine (50118), MF (PHR1084) and streptozotocin (STZ) (S0130) were purchased from
Sigma Aldrich (St Louis, MO, USA). Whole fenugreek seeds were purchased from Agricol Niche Brands,
a South African seed company. A herbarium voucher of flowering material was lodged at the Ward
Herbarium (UDW-UKZN; N. Naicker 1). All other consumables were purchased from Merck (Darmstadt,
Germany), unless otherwise stated.

Treatment preparation of stock solutions
Whole fenugreek seeds were crushed using a mortar and pestle, suspended in deionized water (1000
mg/mL), and placed on a stirrer at room temperature (RT) for 3 hour (hr). The aqueous solution was
transferred to a sterile conical tube and centrifuged (3600xg for 10min) at RT. Supernatant (FSE) was
removed, freeze-dried, and stored at -20°C. Metformin tablets were also crushed with a mortar and pestle
and suspended in 0.1M phosphate-buffered saline (PBS) (50mg/mL). The solution was then filter
sterilized (0.45mm filter). 4-hydroxyisoleucine was obtained in a liquid form, and treatments were
prepared from a stock solution (as per manufacture instructions). Subsequent treatments were prepared in
complete culture medium (CCM) for in vitro and in 0.1M PBS solution for in vivo.

Cell culture and treatment preparation
HepG2 cells were cultured (37°C, 5% CO2) in 25cm3 flasks in complete CCM comprising Eagles
minimum essential medium, 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone (Lonza
Biowhittaker; Basel, Switzerland). Cells were grown to 90% confluency prior to treatment for 72hr. Cells
were subject to normoglycaemic (NG; 5mM glucose) and hyperglycaemic (HG; 25mM glucose)
conditions. Treatments were replenished every 24hr. The methods used for the preparation of 4-OH-Ile,
MF and FSE was as per the protocol followed by Naicker et al, 2016 (15). The optimized treatment
concentrations by Naicker et al, 2016 were used in this study which include; 4-OH-Ile (100ng/ml), MF
(2mM) and FSE (100ng/ml) (15). All experiments were conducted in triplicate and repeated independently 3 times.

Animals

Six-week-old male C57BL/6 mice (n=40) were procured from the Biomedical Resource Unit at the Westville Campus of the University of KwaZulu-Natal (UKZN), Durban, South Africa. Mice with a mean body weight (BW) of 20 ± 2.99g were randomly divided into 2 groups: non-diabetic (NG) and diabetic (HG). Each group were further subdivided into 4 groups of 5 mice each as follows: Control (C), 4-OH-Ile, MF and FSE. Mice were housed in polycarbonated cages in a humidity and temperature controlled room (40-60% humidity, 23 ± 1°C) with a 12hr light dark cycle. The mice were fed a commercially available pellet diet and normal drinking water ad libitum throughout the 15 day experimental period. The mice were maintained according to the rules and regulations of the Experimental Animal Ethics Committee of the UKZN (Ethical approval number: AREC/057/016).

Induction of diabetes

Administration of a low single dose of STZ damages pancreatic β cells through alkylation of DNA by causing partial destruction of these cells, resulting in hyperglycaemia. This process displays the pathophysiological characteristics of T2D which include inadequate β-cell mass and β-cell dysfunction. Type two diabetes was induced in all mice in the diabetic group (HG) by an intraperitoneal administration of STZ (50mg/kg BW) dissolved in 0.1M citrate buffer (pH 4.4) following an overnight fast (12hr). The optimal dosage of 50mg/kg was determined by preliminary investigation which included a range of STZ concentrations (50mg/kg, 100mg/kg and 150mg/kg BW). Blood was collected from the tail vein, using a glucometer (Accu-Chek®) to monitor and measure the fasting blood glucose over a 10 day period prior to administration of treatment. Once a blood glucose of >7mmol/L and <16mmol/L was achieved and stable, the treatment period was inducted.

Treatment preparations

Preparation of treatments were guided by the protocol followed by Naicker et al, 2016 (15). The concentration of 4-OH-Ile (100mg/kg BW), MF (20mg/kg BW) and FSE (100mg/kg BW) were based on previous animal studies which evaluated a range of concentrations and reported the outcomes of the range which we based our optimal concentration (14, 16). Mice were treated once daily for the 15 day treatment period via oral gavage.
Animals post treatment
At the end of the treatment period, the mice were sacrificed using isoflurane. Blood samples were collected using anticoagulant EDTA tubes, in order to measure blood glucose levels. The blood glucose levels were measured at an accredited laboratory (AMPATH, Amanzimtoti, South Africa). All mice livers were harvested, rinsed twice in saline, dissected and then stored in Cytobuster (Novagen, Darmstadt, Germany) and Qiazol (Qiagen; Hildenburg, Germany) at -80°C until analysis.

Western blotting
Western blots were performed to quantify relative protein expression of PCSK9 and PPARG. Crude protein was isolated using Cytobuster (Novagen, San Diego, CA, USA) supplemented with protease and phosphate inhibitors (Roche: 04693124001 and 04906837001). HepG2 cells and mice liver samples (homogenized) were incubated in Cytobuster for 10min on ice and then centrifuged (4°C, 12,000g). Crude protein samples was quantified by the bicinchoninic acid assay and standardized to 1mg/mL. Samples were boiled in Laemmli buffer [dH2O, 0.5M Tris–HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol, and 1% bromophenol blue] for 5min. Samples were electrophoresed on a sodium dodecyl sulphate polyacrylamide gel (4% stacking and 10% resolving) for 1Hr at 150V and transferred on to nitrocellulose using the TransBlot Turbo Blotting System (Bio-Rad; Hercules, CA) using a preinstalled Standard SD program. All membranes were blocked for 2hr in 3% BSA in Tween20-Tris-buffered saline (TTBS - 0.15M NaCl, 2.68M KCl, 24. 86M Tris, 500µl Tween20, pH 7.4) at RT on a shaker. Thereafter, the membranes were incubated with primary antibody PCSK9 (ab125251, 1:5,000) and PPARG (ab, 1:5,000) at 4oC overnight. The membranes were then equilibrated to RT on a shaker for 1hr, followed by 5 washes (10min) with TTBS. Membranes were then probed with horseradish peroxidase conjugated-secondary antibody [anti-rabbit 1:10,000 (CS7074)] for 1hr, followed by 5 washes (10min) with TTBS. Chemiluminescent signal was detected using ECL Clarity Western detection reagent (Bio-Rad) and captured on the Bio-Rad ChemiDoc Viewing System. Data were expressed as relative band density (RBD) and expression of proteins was analyzed with the Bio-Rad ChemiDoc MP Imaging System with Image Lab software. Membranes were quenched (5% H2O2 at 37°C for 30min) and proteins were normalized against β-actin (A5316), 1:1,000.

Quantitative PCR
The mRNA levels of genes regulating lipid homeostasis: PCSK9, LDLr, SREBP1c, FAS and ApoA1, was determined by qPCR. Total RNA was isolated using extraction buffer (Qiazol) and an in-house protocol (17). RNA was quantified using a spectrophotometer (Nanodrop2000, Biotech) and standardized to 1000ng/µl. Standardized RNA was reverse transcribed to complementary DNA (cDNA) using the
iScript™ cDNA Synthesis kit (Bio-Rad; 107-8890) as per the manufacturer’s instruction. A reaction volume of 10μl was prepared consisting of 5X IQ™ SYBR® ssoAdvanced SYBR Green (Bio-Rad; 170-880), nuclease free water, 1μl cDNA template (1000ng/ml), and 1μl sense and anti-sense primer (Inqaba Biotec™). The mRNA expression was normalized against a housekeeping gene (18S). Thermocycler conditions were carried out using the CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA) as follows: initial denaturation (95°C, 10min), 40 cycles of denaturation [95°C, 15seconds (s)], annealing and extension (72°C, 30s). Relative fold change was calculated using the method described by Livak and Schmittgen, 2001 to calculate relative fold change (18). Primer sequences and annealing temperatures are shown in Table 1 (supplementary data).

Lipid profile and glucose analysis
Lipid profile analysis and measurement of glucose concentration was performed by an accredited pathology laboratory (AMPATH laboratories, Amanzimtoti, South Africa). The supernatant from each cell culture sample was lyophilized and reconstituted in 500μl of 0.1M phosphate buffer solution and sent for analysis. And the plasma from each mouse sample was analyzed. Glucose and oral glucose tolerance test analysis are shown in supplementary data (Appendix 3).

Liquid chromatography–mass spectrometry
Liquid chromatography–mass spectrometry (LC-MS) is an analytic chemistry technique which combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS). 1mg of crude FSE was weighed and dissolved in 10ml methanol (MeOH). Each sample was vortexed and sonicated to allow for complete dissolution. The extract was then subjected to solid phase extraction, using a Supelco C18 100mg solid phase cartridge. The extract was eluted with MeOH. A 1:100 dilution of the sample was prepared using MeOH and injected into the LC-MS. The instrumentation used was the Shimadzu 202 UFLC-MS, mobile A: 0.1% formic acid (FA) in water and mobile phase B: 0.1% FA in acetonitrile. Separation was achieved using a YMC Triart C18 analytical column (4.6mm x 150mm), using a gradient elution method from 5% B to 95 B over 25min. Data was collected at 265nm and analyzed using the Shimadzu Lab Solutions software (Supplementary data Fig 2, 3, 4 and Table 2).

Statistical analyses
Statistical analyses was performed using GraphPad Prism v5.0 software (GraphPad Software, Inc.). The data was expressed as relative fold change to respective controls in the qPCR and western blotting experiments. With regards to western blotting, the phosphorylated proteins were normalized against the
total protein. Followed by the total protein against actin. Following each experiment in vitro and in vivo, 4-OH-Ile was compared to MF and the untreated control. We then analyzed FSE in comparison to the untreated control. Statistical comparisons for 4-OH-Ile, MF and the untreated control were made using a one-way analysis of variance (ANOVA), nonparametric test (Kruskal Wallis test) and a Dunn’s post-test; and a non-parametric Mann-Whitney test was used for FSE and the untreated control. The data were considered statistically significant with a value of p < 0.05.

Results

4-OH-Ile regulates protein expression of PCSK9 and gene expression of PCSK9 and LDLr under NG and HG conditions, in vitro and in vivo

PCSK9 is an inhibitory enzyme which regulates the LDLr pool. The relative protein expression of PCSK9 in HepG2 cells was maintained by both treatments (Fig 1A). However, under HG conditions, 4-OH-Ile reduced the expression of PCSK9 0.8-fold (0.99±0.03RBD) compared to MF (Fig 1A). Similarly, in the liver cells of C57BL/6 mice, the protein expression of PCSK9 was maintained by all treatments, under a NG state (Fig 1B). However, under a HG state, 4-OH-Ile (0.5-fold; 0.89±0.03RBD) and MF (0.8-fold; 0.99±0.03RBD) reduced its expression (Fig 1 C; D). These reductions in protein expression by 4-OH-Ile correlate with the decrease in mRNA levels of PCSK9 in both models (Fig 2). Under normal conditions in vitro and in vivo (Fig 1A, C), metformin treatment increased the protein expression of PCSK9. Under a hyperglycaemic condition in vitro (Fig 1B) metformin treatment had no effect compared to the untreated control. However, under a hyperglycaemic condition in vivo, metformin treatment significantly reduced the protein expression of PCSK9. The changes observed in protein expression correlated with the changes in gene expression, under both conditions and models. Therefore, we can conclude that elevated glucose levels could have an impact on the action of metformin on PCSK9. 4-OH-Ile decreases the expression of PCSK9, and thereby reduces its inhibitory effect on the LDLr. This will increase the LDLr pool and consequently reduces the extracellular levels of LDLc. We then investigated the changes in mRNA expression of LDLr. In vitro, 4-OH-Ile significantly increased LDLr mRNA levels 1.8-fold compared to MF 1.5-fold, under a HG state (Fig 2C). Whereas in vivo, 4-OH-Ile elevated the gene expression under both conditions (Fig 2E). To correlate these results, a lipid profile was analyzed on the supernatant of each treatment (Fig 2D) and on the serum of treated mice (Fig 2F), to measure the extracellular levels of LDLc. Following the reported changes in both protein and mRNA levels of PCSK9, these changes translated into the reduced levels of LDLc (Fig 2 D, F).
4-OH-Ile regulates protein expression of PPARG and gene expression of Apo A1 under NG and HG conditions, *in vitro* and *in vivo*

We evaluated the protein expression of PPARG by western blotting. *In vitro*, both treatments significantly increased the protein expression of PPARG under both conditions (Fig 1). *In vivo*, 4-OH-Ile (2.6-fold; 1.90±0.03RBD) elicited the highest response in elevating the expression of PPARG compared to MF (Fig 1). As previously mentioned, PPARG is responsible for regulating HDLc. Apolipoprotein A1 (Apo AI) is a major constituent of HDLc that removes cholesterol from peripheral cells and transports it to the liver for their ultimate removal. Therefore the effect of 4-OH-Ile and MF on *Apo A1* mRNA levels was investigated by qPCR. *In vitro* and *in vivo*, 4-OH-Ile (2.6-fold; 1.7-fold) and MF (2.5-fold; 1.8-fold) increased the gene expression of *Apo A1* under HG conditions (Fig 3 A, C). This increase in mRNA content correlates with the increase in extracellular HDLc in both HepG2 cells (Fig 3 B) and C57BL/6 mice (Fig 3 D), under similar conditions. Subsequently, both the increase in protein expression of PPARG and gene expression of *Apo A1*, positively contribute to the elevated levels of HDLc.

**Figure 1.** Protein expression analysis of pro-protein convertase subtilisin/kexin type 9 (PCSK9) and peroxisome proliferator-activated receptor gamma (PPARG) in 4-hydroxyisoleucine (4-OH-Ile) and metformin (MF) treated HepG2 cells (normoglycaemic – A, p=0.0272; hyperglycaemic – B, p=0.0313) and mouse liver (normoglycaemic – C, p=0.0296; hyperglycaemic – D, p=0.0040). *P*< 0.05 relative to control
**Figure 2** 4-hydroxyisoleucine (4-OH-Ile) and metformin (MF) treatments increase mRNA levels of PCSK9 in HepG2 cells (A, \( p=0.0207 \)) and mice liver samples (B, \( p=0.0335 \)). LDLr mRNA was significantly elevated in hyperglycaemic HepG2 samples treated with MF and 4-OH-Ile (C, \( p=0.0478 \)) with a concomitant decrease in extracellular LDLc concentration (D, \( p=0.0293 \)). The *in vivo* data substantiated this with significantly elevated LDLr transcripts (E, \( p=0.0255 \)) and significantly reduced plasma LDLc levels (F, \( p=0.0112 \)) in both MF and 4-OH-Ile treatments under both conditions. *\( P<0.05 \); **\( P<0.005 \) relative to control.
Figure 3: Quantitative PCR results show 4-hydroxyisoleucine (4-OH-Ile) and metformin (MF) increased mRNA levels of SREBP1c (A; \( p = 0.0143 \)) and FAS (B; \( p = 0.00513 \)) in HepG2 cells. Extracellular cholesterol levels were significantly reduced relative to the control under hyperglycaemic conditions (C, \( p = 0.0478 \)) and TGs were significantly reduced by MF and 4-OH-Ile under both conditions (D, \( p = 0.0441 \)). mRNA quantification in substantiated increased SREBP1c (E, \( p = 0.0043 \)) and FAS (F, \( p = 0.0102 \)) in the presence of MET and 4-OH-Ile. Lipid profile analysis showed reduced plasma levels of cholesterol (G, \( p = 0.0121 \)) and TGs (H, \( p = 0.0231 \)) of treated mice. *\( P < 0.05 \), **\( P < 0.005 \) relative to control.
Figure 4 4-hydroxyisoleucine (4-OH-Ile) and metformin (MF) treatments relative to the untreated control on the gene expression Apo A1 on HepG2 cells (A, \( p=0.0036 \)) and mice liver (C, \( p=0.0272 \)) under normoglycaemic and hyperglycaemic conditions. A lipid profile analysis was performed on the supernatant of the treated HepG2 cells (B, \( p=0.0206 \)) and serum (D, \( p=0.0336 \)) of treated mice to assess the extracellular HDLc levels, under normoglycaemic and hyperglycaemic conditions. *\( P < 0.05 \) relative to control.

4-OH-Ile regulates gene expression of SREBP1c and FAS under NG and HG conditions, *in vitro* and *in vivo*

SREBP1c is a major transcription factor regulating the expression of LDLr. SREBP1c regulates FAS which is responsible for TG production. We investigated the effect of 4-OH-Ile and MF on the gene expression of SREBP1c and FAS (*in vitro* and *in vivo*) using qPCR. *In vitro*, under both conditions, 4-OH-Ile up-regulated the mRNA levels of SREBP1c (2.1 and 3.8-fold; Fig 4 A). Gene expression of FAS was increased by 4-OH-Ile under both conditions (NG: 2.5 and HG: 2.8-fold; Fig 4 B). *In vivo*, both 4-OH-Ile (1.7-fold) and MF (1.8-fold) elevated mRNA levels of SREBP1c (Fig 4 E). Similarly, 4-OH-Ile (NG: 1.6-
fold; HG: 1.7-fold) elevated the expression of FAS under both conditions (Fig 4 F). A lipid profile measuring the extracellular levels of cholesterol (Fig 4 C, G) and TG (Fig 4 D, H) in the cell culture supernatant (Fig 4 C, D) of each treatment and in the serum of treated mice (Fig 4 G, H) was determined. 4-OH-lle up-regulated SREBP1c which is responsible for cholesterol synthesis. This was confirmed by the change in extracellular cholesterol levels. Increased mRNA levels of SREBP1c, which regulates FAS, resulted in an increased FAS mRNA and extracellular TG levels. Triglyceride’s form a part of the VLDL complex which in turn form LDL particles. These LDL particles form LDLc which is endocytosed by LDLr.

FSE regulates the protein expression of PSCK9 and PPARG and the gene expression of PCSK9, Apo A1, SREBP1c and FAS, in vitro and in vivo
We also investigated the effect of FSE on the protein expression of PCSK9 and PPARG, gene expression of PCSK9, LDLr, SREBP1c, FAS and Apo A1 and lipid profiles, both in vitro and in vivo. During both conditions in vitro, FSE maintained the protein expression of PCSK9 (Fig 5A, B). However in vivo, FSE increased the protein expression of PCSK9 (Fig 5C, D). Interestingly, FSE increased the gene expression of PCSK9 under both conditions. However the gene expression of LDLr and the LDLc levels were also increased. A potential explanation for this effect of FSE could be the cause of a contribution of other compounds within the seed, as opposed to 4-OH-lle (an isolated compound). This reduction in PCSK9 expression correlated with the change in LDLr expression (Fig 6 C, E), followed by the concomitant reduction in LDLc levels, both in vivo and in vitro (Fig 6 D, F). This effect of FSE (in vitro; 4.1-fold; in vivo; 3.2-fold) was much greater in comparison to 4-OH-lle (in vitro; 1.8-fold; in vivo; 2.5-fold) under a HG state. FSE also positively affected the protein expression of PPARG. In vitro FSE (2.5-fold; 1.90±0.03RBD) up-regulated the expression of PPARG under a HG state (Fig 5 A, B). The similar pattern of expression was displayed in vivo, FSE increased PPARG expression by 1.9-fold (1.10±0.03RBD) (Fig 5 C, D). In vitro and in vivo, FSE increased the gene expression of Apo A1 2.2-fold and 3.9-fold respectively, under a HG state (supplementary data, Fig1). This rise in expression translated to the elevated HDLc levels measured in the HepG2 (supplementary data, Fig 1) and mice liver cells (supplementary data, Fig 2). In comparison to 4-OH-lle, these responses were significant but not as great as 4-OH-lle. With regards to the gene expression of SREBP1c and FAS, FSE again displayed its stimulating affects as an increase in both genes are observed under both conditions, in both models (supplementary data, Fig 2). Moreover, these changes in gene expression translated into the changes observed in the cholesterol and TG levels, in vitro and in vivo (supplementary data). Similarly, FSE elicited a significant response but not as great as 4-OH-lle.
Figure 5 Protein expression analysis of pro-protein convertase subtilisin/kexin type 9 (PCSK9) and peroxisome proliferator-activated receptor gamma (PPARG) in fenugreek seed extract (FSE) treated HepG2 cells (normoglycaemic – A, \( p=0.0142 \); hyperglycaemic – B, \( p=0.0021 \)) and mouse liver (normoglycaemic – C, \( p=0.0352 \); hyperglycaemic – D, \( p=0.0141 \)). *\( P < 0.05 \) relative to control.
Figure 6 Fenugreek seed extract treatment increase mRNA levels of PCSK9 in HepG2 cells (A, $p=0.0363$) and mice liver samples (B, $p=0.00412$). LDLr mRNA was significantly elevated in hyperglycaemic HepG2 samples treated with FSE (C, $p=0.0223$) with a concomitant decrease in extracellular LDLc concentration (D, $p=0.0360$). The in vivo data substantiated this with significantly elevated LDLr transcripts (E, $p=0.0142$) and significantly reduced plasma LDLc levels (F, $p=0.0133$) in both MF and 4-OH-Ile treatments under both conditions. *$P<0.05$ relative to control.
Figure 7 Quantitative PCR results show fenugreek seed extract (FSE) increased mRNA levels of 
SREBP1c (A; \( p=0.0412 \)); and FAS (B; \( p=0.0021 \)) in HepG2 cells. Extracellular cholesterol levels were 
significantly reduced relative to the control under hyperglycaemic conditions (C, \( p=0.0312 \)) and TGs 
were significantly reduced by MF and 4-OH-Ile under both conditions (D, \( p=0.0122 \)). mRNA 
quantification in substantiated increased SREBP1c (E, \( p=0.0043 \)) and FAS (F, \( p=0.0102 \)) in the presence
of MET and 4-OH-Ile. Lipid profile analysis showed reduced plasma levels of cholesterol (G, \( p=0.0121 \)) and TGs (H, \( p=0.0231 \)) of treated mice. \(^*P<0.05\) relative to control.

\[\text{Figure 8}\] Fenugreek seed extract treatment relative to the untreated control on the gene expression \textit{Apo A1} on HepG2 cells (A, \( p=0.0122 \)) and mice liver (C, \( p=0.0272 \)) under normoglycaemic and hyperglycaemic conditions. A lipid profile analysis was performed on the supernatant of the treated HepG2 cells (B, \( p=0.0233 \)) and serum (D, \( p=0.0332 \)) of treated mice to assess the extracellular HDLc levels, under normoglycaemic and hyperglycaemic conditions. \(^*P<0.05\) relative to control

\[\text{Discussion}\]
The role of 4-OH-Ile as an anti-lipidemic alternative has been supported by improved lipid profiles (19). We previously showed in an in vitro liver model, the reduction of extracellular glucose levels by 4-OH-Ile, MF and FSE via induction of the insulin signaling pathway and subsequent GLUT2 recruitment. (15). We also validated the above study in an in vivo mice model. The present study consolidated our previous
findings on 4-OH-Ile, by investigating the effect of 4-OH-Ile in comparison to MF and FSE on PCSK9
and PPARG signaling and related plasma lipid profiles in hyperglycaemic HepG2 cells and C57BL/6
male mice. 4-hydroxyisoleucine significantly improved the altered lipid profile, SREBP1c-FAS, PCSK9
and PPARG signaling in both models by effectively regulating altered lipid levels, enhancing SREBP1c-
FAS and PPARG signaling and reducing PCSK9.

An abnormal lipid profile is the hallmark abnormality of diabetic dyslipidemia. Studies have reported the
l lipid lowering effect of fenugreek in reducing abnormal lipid levels. In human studies by Sharma et al,
1990 and Kassaian et al, 2009, both researchers concluded that the lipid lowering effect of fenugreek
seeds was attributed to 4-OH-Ile (19, 29). In dyslipidemic hamsters’ 4-OH-Ile decreased plasma TGs,
total cholesterol, and FFAs, accompanied with an elevation by 39% of the HDLc: TC ratio. Another study
by Haeri et al, 2009 (12) displayed improved liver function markers, decreased blood glucose and
restoration of blood lipid and uric acid levels following 4-OH-Ile treatment (12). An important study by
Vijayakumar et al, 2010 (10) investigated the hypolipidemic effect of fenugreek seeds in 3T3-L1 and
HepG2 cells. They showed that the inhibition of fat accumulation and upregulation of LDLr, decreased
both TGs and LDLc (10). However no studies have reported the lipid lowering effects of 4-OH-Ile on
both PCSK9 and PPARG in regulating lipid homeostasis. In our study, we compare the effect of 4-OH-Ile
to MF and FSE on plasma lipoproteins and regulation of PCSK9 and PPARG under both normal and HG
conditions, in HepG2 cells and C57BL/6 male mice.

In this study, we showed the potential of 4-OH-Ile in activating SREBP1c gene expression.
Interestingly, 4-OH-Ile elicited a more potent effect than MF under NG and HG conditions, in both
models. The activation of SREBP1c is largely regulated by insulin which plays a profound role in
insulin’s effect on the transcription of the hepatic gene FAS. The significance of this direct
communication of insulin with SREBP1c was supported by Azzout-Marniche et al, 2000 (20). This
interaction between insulin and SREBP1c is crucial for the genomic actions of insulin on both
carbohydrate and lipid metabolism (20). Furthermore, the results of Dif et al, 2006 strongly suggest that
SREBP1c transcription factors are the main mediators of insulin action on SREBP1c expression in human
tissues. Other studies are also in agreement of the imperative role of insulin on SREBP1c (21). Therefore,
we investigated the effect of 4-OH-Ile in comparison to MF on SREBP1c mRNA expression. In both
models, we observed 4-OH-Ile significantly increase SREBP1c with a profound effect during
hyperglycaemia (Fig 4). In addition, the transcript levels of FAS also increased, with 4-OH-Ile inducing a
greater increase as compared to MF (Fig 4). During FA synthesis, both SREBP1c and subsequent FAS
activation is central to the formation of TGs (22, 23). Triglycerides form a major component of very-low
density lipoprotein (VLDL) which serve as both energy sources and transporters of dietary fat (2). Again, our results showed that 4-OH-Ile preceded the effect of MF in regulating TG levels (Fig 4). Collectively these findings validate the potential of 4-OH-Ile in regulating SREBP1c and FAS activation during chronic hyperglycaemia.

The impact of SREBP1c activation is fundamental for the formation of lipoproteins (23). Following the formation of TGs, their uptake into the cell stimulates VLDL assembly which is an attempt of the liver to maintain lipid homeostasis (24). This is followed by the removal of TG remnants from VLDL particles, resulting in particles with a higher cholesterol content – forming LDLc (25, 26). Several studies have evaluated the role of LDLc in lipid homeostasis (27, 28). A study by Mohan et al, 2005 concluded that LDL is associated with diabetes and a TG/HDL ratio ≥ 3.0 could serve as a marker of LDL (27). A patient study supported recommendations for aggressive control of LDLc in diabetic individuals, with a target level of <5.5mmol/L (29). In addition, studies have reported the use of PCSK9 regulators in controlling LDLc levels (4). Seidah, 2009 reported Annexin A2 to specifically bind and inhibit PCSK9 (30). Another study raised the possibility that pharmacologic inhibition of PCSK9 might lower LDLc levels in patients with hypercholesterolemia (31). In the destructive hyperglycaemic environment 4-OH-Ile down-regulated the total protein expression of PCSK9 (Fig 1B, D). This effect translated into the observed reduction in PCSK9 gene expression (Fig 1, 2). More importantly, we observed a concomitant rise in the transcript levels of LDLr (Fig 1; 2). Our results infer the potential of 4-OH-Ile in restoring lipid homeostasis which is on par or even better than MF.

Despite lowering LDLc levels, it is of utmost importance that lowered LDLc is accompanied by a concomitant rise in HDLc. The term ‘good’ cholesterol is given to HDLc which is formed following the removal of cholesterol from LDLc. The formation of HDLc is necessary for reverse cholesterol transport which is accomplished by Apo A1, a major constituent of HDLc (32, 33). Apolipoprotein A1 functions to remove cholesterol from peripheral cells and transports it to the liver for its removal from circulation (34). The well-known transcription factor PPARG is responsible for positively contributing to the cholesterol pool by elevating HDLc levels. Studies have suggested PPARG agonists might have therapeutic potential in the treatment of diabetic dyslipidemia (35-37). Our results are in agreement with these studies, as 4-OH-Ile displayed a significant ability in increasing the protein expression of PPARG in both chronic hyperglycaemic models (Fig 1). Furthermore, 4-OH-Ile exhibited stronger potential than MF in increasing the gene expression of Apo A1 (Fig 4). The outcome of this increase was validated by the elevated HDLc levels, in both HG models (Fig 4). The increase in HDLc further exemplifies the potency of 4-OH-Ile in restoring an abnormal lipid profile.
Following our promising results and the supporting literature, we further analyzed FSE under the same parameters, in comparison to 4-OH-lle and MF. We observed FSE significantly decrease total protein expression of PCSK9 and increase the total protein expression of PPARα (Fig 5). In addition, FSE elevated the gene expression levels of PCSK9, LDLr, SREBP1c, FAS and Apo A1. These results preceded the effect of MF but were similar to 4-OH-lle. Interestingly, 4-OH-lle could account for the potency of FSE, as 4-OH-lle has been documented to be abundant in fenugreek seeds. Furthermore, 4-OH-lle is postulated to account for the seeds anti-diabetic and anti-lipidemic effects. Interestingly, our study shows that 4-OH-lle is potent in its effect on both PCSK9 and PPARα expression and related lipid factors as opposed to the independent effect of FSE. Studies have also reported diosgenin – a biologically active steroid sapogenin as a possible mediator in the seeds effect to maintain glucose and lipid homeostasis (38). Animal models have recently supported the role of diosgenin in reducing glycaemia in a diabetic state by reducing the proteins involved in hepatic gluconeogenesis and glucose export. Also, soluble fibers such as galactomannans largely constitutes the fiber content of fenugreek seeds (39). Studies reported that these fibers enhance glycaemic control by inhibiting lipid and carbohydrate proteins in the digestive system (40).

4-hydroxyisoelucine potentiates an anti-lipidemic response in hyperglycaemic HepG2 cells and C57BL/6 male mice. Collectively, the results show that the liver response to 4-OH-lle exposure augments PCSK9 and PPARα expression and the abnormal lipid profile. These results were particularly profound in a hyperglycaemic state. Furthermore, the results provide substantial evidence for the use of FSE as a possible lipid-lowering agent. This data may help develop a better understanding of the molecular and biochemical interactions of both 4-OH-lle and FSE, associated with risks of diabetic dyslipidemia. This has great importance in socio-economically challenged communities where T2D individuals are unable to access healthcare facilities and natural products are first-line treatment.

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**Conflict of interest**

Authors declare no conflict of interest.
Author contributions
Experiment design: Nikita Naicker, Savania Nagiah, Pragalathan Naidoo and Anil A. Chuturgoon.
Execution of experiments, data analysis and research article: Nikita Naicker, Savania Nagiah, Anand Krishnan and Anil A Chuturgoon
Review of research article: Savania Nagiah and Anil A. Chuturgoon.

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CHAPTER 5

4-hydroxyisolecuine potentiates hepatic Nrf2-antioxidant response and mitochondrial maintenance proteins during chronic hyperglycaemia in vitro and in vivo

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Abstract

During type 2 diabetes mellitus (T2D), defective insulin action and hyperglycaemia, initiate over-production of reactive oxygen species (ROS) and compromise mitochondrial integrity. This imbalance causes oxidative stress (OS) contributing to diabetic complications. 4-hydroxyisoleucine (4-OH-Ile) - the bioactive component of fenugreek seeds, possesses anti-diabetic and anti-lipidemic effects. Our study evaluated the effects of 4-OH-Ile on hepatic Nrf2-mediated antioxidant response and mitochondrial maintenance proteins (LonP1, SIRT3, PGC-1α), in vitro (HepG2 cells) and in vivo (C57BL/6 male mice). In addition, the effects of metformin and fenugreek seed extract (FSE) were also investigated. Treatments included 4-OH-Ile, metformin and FSE and were conducted over 72 hours (in vitro) and 15 days (in vivo) under normoglycaemic and hyperglycaemic (in vitro: 25mM glucose; in vivo: 50mg/kg body weight streptozotocin) conditions. In vitro GSH (GSH assay), MDA (TBARS) and protein carbonyl (protein carbonyl assay) levels were measured. Protein expression of pNrf2/Nrf2, LonP1, SIRT3 and PGC-1α were measured by western blotting. And mRNA levels of SIRT3, PGC-1α, SOD2 and GPx were evaluated by qPCR. Thereafter, these results were validated using an in vivo diabetic mouse model. In vitro, 4-OH-Ile increased GSH concentrations and mitigated OS markers (MDA, protein carbonyl), greater than metformin. Since 4-OH-Ile enhanced the antioxidant status we validated the above regulatory proteins both in vitro and in vivo. In both models, 4-OH-Ile significantly increased mRNA levels of Nrf2 targets and mitochondrial maintenance genes and proteins, exceeding that of metformin. However, the overall effect of 4-OH-Ile was similar to FSE. In conclusion, 4-OH-Ile improves the OS status in T2D by regulating pNrf2, LonP1 and SIRT3 expression. This provides a possible novel therapeutic intervention for the treatment of OS and associated diabetic complications.

Keywords: type 2 diabetes mellitus, 4-hydroxyisoleucine, oxidative stress, hyperglycaemia, Nrf2, mitochondrial proteins
Introduction

During type two diabetes mellitus (T2D) compromised insulin signaling potentiates insulin resistance, causing chronic hyperglycaemia [1]. Long term elevation in blood glucose levels initiates the over-production of reactive oxygen species (ROS) via attenuating endogenous antioxidant (AO) enzymes, mitochondrial dysfunction, stimulation of protein kinase C (PKC) and generation of advanced glycation end-products (AGEs) [2, 3]. The accumulation of ROS results in oxidative stress (OS) - a pathological condition linked to the development and progression of cardiovascular disease (CVD) and diabetic complications. Uncontrolled ROS production in T2D patients is central to the pathogenesis of micro- and macro-vascular complications, and associated morbidity and mortality [1]. It is therefore critically important in treating modifiable CVD risk factors in T2D individuals.

Improving endogenous cellular AO responses and detoxification systems would be beneficial in attenuating CVD and diabetic complications. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) functions as the principal regulator of the endogenous AO system, with secondary functions in detoxification and mitochondrial homeostasis. This transcription factor is the first line of defence against OS by mediating the transcription of cytoprotective and ROS detoxification genes. Reduced Nrf2 expression has been reported in diabetic mice and humans - contributing to elevated OS, endothelial dysfunction and insulin resistance [4, 5]. Acute hyperglycaemia has been associated with increased Nrf2 function whereas chronic hyperglycaemia has resulted in reduced function of Nrf2 [4-6]. This transcription factor is regulated through multi-signaling processes which involve cytosolic regulation, nuclear translocation and export, and DNA binding [4, 7]. Studies have confirmed that the diabetic milieu dysregulates several aspects of the Nrf2 signaling pathway [4, 7-9]. Therefore, targeting Nrf2 may prove beneficial in counteracting the pathological effects of T2D.

The relationship between diabetes and mitochondrial dysfunction is well-established [7, 10]. Mitochondria generate low levels of superoxide anion radicals as by-products of the electron transport chain during respiration [11]. At basal levels, ROS play a role in redox signaling; however, over-production of ROS leads to free radical interaction with cellular macromolecules, often resulting in deleterious effects [11]. It is well-documented that mitochondria are one of the main endogenous producers of ROS [7, 10]. In an attempt to counteract this ROS production, Nrf2 further extends its AO effect to the mitochondria [7, 10]. It has been reported that Nrf2 affects availability of substrates for mitochondrial respiration, leading to its effect on mitochondrial ROS production [7, 10]. Furthermore, mitochondria possess its own maintenance pathways which include sirtuin 3 (SIRT3), peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) and mitochondrial lon protease 1.
(LonP1). A well-characterized marker of oxidative damage includes lipid peroxidation which yields lipid peroxy-radicals [12]. The metabolic alterations associated with T2D increases susceptibility to lipid peroxidation, and is considered a driver of atherosclerotic progression [12]. Additionally, ROS interaction with amino acid side chains, produces protein carbonyls and dysfunctional oxidatively-modified proteins [12]. Hyperglycaemia elevates protein damage via protein glycosylation, resulting in the production of AGEs. LonP1 is a mitochondrial protease, which proteolytically clears damaged proteins, which preserves proteostasis under normal metabolic conditions, and avoids proteotoxicity during environmental and cellular stress [13]. SIRT3 is a NAD-dependent deactylase, targeting proteins involved in energy metabolism and the rate of ROS production [14]. SIRT3 serves to elevate cellular respiration and attenuate ROS levels. More importantly, SIRT3 is essential for the induction of PGC-1α - a regulator of mitochondrial ROS scavenging enzyme; superoxide dismutase 2 (SOD2) and transcriptional coactivator driving mitochondrial biogenesis [15]. Initiation of PGC-1α expression in liver is a regulatory event causing the activation of energy metabolic pathways which exert homeostatic control. However, elevated glucose levels compromise the expression of PGC-1α. Consequently, in T2D attenuated LonP1, SIRT3 and PGC-1α levels further exacerbate mitochondrial dysfunction and OS.

Metformin (MF) is the first-line drug therapy for treating T2D and demonstrates some AO activity [16-18]. Alternative therapeutic interventions, however, provide a cost effective and easily accessible means of treating T2D. Trigonella foenum-graecum, frequently known as fenugreek, has been investigated as a therapeutic intervention in diabetes [19, 20]. Documented effects of fenugreek have been attributed to the seed of the plant which has a high content of a branched-chain amino acid derivative - 4-hydroxyisoleucine (4-OH-lle) [20, 21]. A study by Mohamad et al, 2004 observed increased SOD2 and liver enzymes in rats treated with 4-OH-lle [22]. In cholesterol-fed rats, extracts of fenugreek seeds reduced thiobarbituric acid-reactive substances (TBARS) and increased catalase and SOD2 in the liver [23]. More importantly a study by Dutta et al, 2014 revealed that the 4-OH-lle rich fraction possesses AO characteristics which is evident from its ability to scavenge toxic radicals in a chemically defined in vitro system [24]. In our previous study, we investigated the effects of 4-OH-lle, MF and fenugreek seed extract (FSE) on proteins and genes involved in regulating insulin signaling and dyslipidemia in vitro [25] and in vivo (PHYMED-D-17-01253). These studies supported the use of 4-OH-lle and fenugreek seed as a possible alternate therapy for reducing hyperglycaemia and improving an abnormal lipid profile. The objective of our current study was to investigate the regulatory effect of 4-OH-lle in comparison to MF on hepatic OS and mitochondrial maintenance proteins in hyperglycaemic HepG2 cells and C57BL/6 male mice.
Materials and methods

Materials

4-OH-Ile (50118), MF (PHR1084) and streptozotocin (STZ) (S0130) were purchased from Sigma Aldrich (St Louis, MO, USA). Whole fenugreek seeds were purchased from Agricol Niche Brands, a South African seed company. A herbarium voucher of flowering material was lodged at the Ward Herbarium (UDW-UKZN; N. Naicker 1). All other consumables were purchased from Merck (Darmstadt, Germany), unless otherwise stated.

Liquid chromatography–mass spectrometry (LC-MS)

LC-MS was used to separate multiple components and structurally identify individual components with high molecular specificity, within a crude extract. 1mg of crude FSE was weighed and dissolved in 10ml methanol (MeOH). Each sample was vortexed and sonicated to allow for complete dissolution. The extract was then subjected to solid phase extraction, using a Supelco C18 100mg solid phase cartridge. The extract was eluted with MeOH. A 1:100 dilution of the sample was prepared using MeOH and injected into the LC-MS. The instrumentation used was the Shimadzu 202 UFLC-MS, mobile A: 0.1% formic acid (FA) in water and mobile phase B: 0.1% FA in acetonitrile. Separation was achieved using a YMC Triart C18 analytical column (4.6mm x 150mm), using a gradient elution method from 5% B to 95 B over 25min. Data was collected at 265nm and analyzed using the Shimadzu Lab Solutions software (Graphs 2, 3, 4 and Table 2).

Treatment preparation of stock solutions

Whole fenugreek seeds were crushed using a pestle and mortar, suspended in deionized water (1,000 mg/mL) and placed on a stirrer at room temperature (RT) for 3 hour (hr). The aqueous solution was transferred to a sterile conical tube and centrifuged (3,600xg; 10min; 24°C). The aqueous phase was removed, freeze-dried and stored at -20°C. Metformin tablets were crushed with a pestle and mortar, suspended in 0.1M phosphate-buffered saline (PBS) (50mg/mL) and filter sterilized (0.45-mm filter). 4-hydroxyisoleucine was obtained in a liquid form and treatments were prepared from a stock solution (as per manufacturer’s instructions). Subsequent treatments were prepared in complete culture medium (CCM) for in vitro and in 0.1M PBS solution for in vivo administration.

Cell culture and treatment preparation

HepG2 cells were cultured (37°C, 5% CO2) in 25cm3 flasks in complete CCM comprising Eagles minimum essential medium, 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone (Lonza Biowhittaker; Basel, Switzerland). Cells were grown to 90% confluency prior to treatment for 72hr. Cells
were subject to normoglycaemic (NG; 5mM glucose) and hyperglycaemic (HG; 25mM glucose) conditions and treatments were replenished every 24hr. The methods used for the preparation of 4-OH-Ile, MF and FSE were as per the protocol followed by Naicker et al, 2016 [25]. The optimized treatment concentrations by Naicker et al, 2016 were used in this study which include; 4-OH-Ile (100ng/ml), MF (2mM) and FSE (100ng/ml) [25]. All experiments were conducted in triplicate and repeated independently 3 times. The following spectrophotometric assay: TBARS, GSH and protein carbonyl assay were only performed in vitro, these results prompted us to further investigate the protein and gene expression in vitro and in vivo.

TBARS

The TBARS assay determined extracellular levels of MDA – an end-product of lipid peroxidation in HepG2 cells. The assay was conducted as the method previously described by Phulukdaree et al, 2010 [26]. The absorbance was measured at 532nm with a reference wavelength of 600nm using a Bio-Tek µQuant spectrophotometer. The average of 3 replicates were calculated and divided by the absorption coefficient, 156 mM-1 to determine the average concentration of MDA (µM).

GSH Assay

The GSH-Glo™ Glutathione Assay (Promega, Madison, USA) was used to detect reduced GSH content in HepG2 cells. For the detection of GSSG, a stronger thiol reductant, tris (2-carboxyethyl) phosphine hydrochloride (TCEP), was used to release GSH bound to proteins, providing a reading for GSSG+GSH. GSSG was calculated by subtracting the GSH quantification from GSSG+GSH. The assay was conducted as per the method previously described by Nagiah et al, 2015 [27]. The plates were read on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, CA) and GSH concentrations (µM) were determined by extrapolation from the standard curve.

Protein carbonyl assay

The protein carbonyl assay was used as a measure of oxidative damage to proteins in HepG2 cells. Following treatment, cells were rinsed twice with PBS; 200µl of cell lysis buffer was added for 10min on ice and then centrifuged (4oC, 12,000g). Crude protein samples were quantified by the bicinchoninic acid assay and standardized to 2mg/mL in 200µl. For each sample, 200µl of protein was transferred to a 15ml conical centrifuge tube. 800µl of 10mM DNPH in 2,5M HCl was added to 200µl of protein of each sample, except the blank (800µl of 2.5M HCl). Samples were left for 1hr to incubate at room temperature and vortexed every 15min. 1ml of 20% TCA was added to each sample, and left on ice for 10min. This was followed by centrifugation for 10min at 4000xg for the collection of protein precipitates. Another
wash was performed using 1ml of 10% TCA. Samples were then centrifuged at 2000g for 10min at RT. To remove the free DNPH, the pellets were washed twice with 1ml of ethanol-ethyl acetate (1:1, v/v). The final precipitates were dissolved in 500μl of 6M guanidine hydrochloride and left for 10min at 37°C with general vortex mixing. Any insoluble materials were removed by additional centrifugation (2,000g, 10min, RT). Samples were plated at 100μl per well in triplicate and protein carbonyl concentration was determined at an absorbance of 370nm.

**Animals**

Six-week-old male C57BL/6 mice (n=40) were procured from the Biomedical Resource Unit at the Westville Campus of the University of KwaZulu-Natal (UKZN), Durban, South Africa. Mice with a mean body weight (BW) of 20 ± 2.99g were randomly divided into 2 groups: non-diabetic (NG) and diabetic (HG). Each group were further subdivided into 4 groups of 5 mice each as follows: Control (C), 4-OH-Ile, MF and FSE. Mice were housed in polycarbonated cages in a humidity and temperature controlled room (40-60% humidity, 23 ± 1°C) with a 12hr light dark cycle. The mice were fed a commercially available pellet diet and normal drinking water ad libitum throughout the 15 day experimental period. The mice were maintained according to the rules and regulations of the Experimental Animal Ethics Committee of the UKZN (Ethical approval number: AREC/057/016).

**Induction of diabetes**

Administration of a low single dose of STZ damages pancreatic β-cells through alkylation of DNA by causing partial destruction of these cells, resulting in hyperglycaemia [28, 29]. This process displays the pathophysiological characteristics of T2D which include inadequate β-cell mass and β-cell dysfunction. Type two diabetes was induced in all mice in the diabetic group via intraperitoneal administration of STZ (50mg/kg BW) dissolved in 0.1M citrate buffer (pH 4.4) following an overnight fast (12hr). The optimal dosage of 50mg/kg was determined by preliminary investigation which included a range of STZ concentrations (50mg/kg, 100mg/kg and 150mg/kg BW). Blood was collected from the tail vein, using a glucometer (Accu-Chek®) to monitor and measure the fasting blood glucose over a 10 day period prior to administration of treatment. Once a blood glucose of >7mmol/L and <16mmol/L was achieved and stable, the treatment period was inducted.

**Treatment preparations**

Preparation of treatments were guided by the protocol followed by Naicker et al, 2016 [25]. The concentration of 4-OH-Ile (100mg/kg BW), MF (20mg/kg BW) and FSE (100mg/kg BW) were based on previous animal studies which evaluated a range of concentrations and reported the outcomes of the range
which we based our optimal concentration [19, 30-33]. Mice were treated once daily for the 15 day
treatment period via oral gavage.

Animals post treatment
At the end of the treatment period, mice were sacrificed using isoflurane. Blood samples were collected
using anticoagulant EDTA tubes, in order to measure blood glucose levels. The blood glucose levels were
measured at an accredited laboratory (AMPATH, Amanzimtoti, South Africa). All mice livers were
harvested, rinsed twice in saline, dissected and stored in Cytobuster (Novagen, Darmstadt, Germany) and
Qiazol (Qiagen; Hildenburg, Germany) at -80°C until analysis.

Glucose analysis
Glucose analysis was performed by an accredited pathology laboratory (AMPATH laboratories,
Amanzimtoti, South Africa). The supernatant from each cell culture sample was lyophilized and
reconstituted in 500µl of 0.1M PBS before analysis. Plasma isolated from each mouse was also analyzed
for glucose levels. Glucose and oral glucose tolerance test analysis are shown in Figure 1 (supplementary
data).

Western blotting
Western blots were performed to quantify relative protein expression of pNrf2/Nrf2, SIRT3, PGC-1α and
LonP1. Crude protein was isolated using Cytobuster supplemented with protease and phosphate inhibitors
(Roche: 04693124001 and 04906837001). HepG2 cells and mice liver samples (homogenized) were
incubated in Cytobuster for 10min on ice and then centrifuged (4°C, 12,000g). Crude protein samples
were quantified by the bicinchoninic acid assay and standardized to 1mg/mL. Samples were boiled in
Laemml buffer [dH2O, 0.5M Tris–HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol and 1%
bromophenol blue] for 5min. Samples were electrophoresed on a sodium dodecyl sulphate
polyacrylamide gel (4% stacking and 10% resolving) for 1hr at 150V and transferred on to nitrocellulose
using the TransBlot Turbo Blotting System (Bio-Rad; Hercules, CA) using a preinstalled Standard SD
program. All membranes were blocked for 2hr in 3% BSA in Tween20-Tris-buffered saline (TTBS -
0.15M NaCl, 2.68M KCl, 24.86M Tris, 500µl Tween20, pH 7.4) at RT on a shaker. Thereafter, the
membranes were incubated with primary antibody pNrf2 (CS8882, 1:5,000), SIRT3 (CSC73E7, 1:5,000),
PGC-1α (CS2178, 1:5,000) and LonP1 (Sigma HPA002192, 1:5,000) at 4°C overnight. The membranes
were then equilibrated to RT on a shaker for 1hr, followed by 5 washes (10min) with TTBS. Membranes
were then probed with horseradish peroxidase conjugated-secondary antibody [anti-rabbit 1:10,000
(CS7074)] for 1hr, followed by 5 washes (10min) with TTBS. Chemiluminescent signal was detected
using ECL Clarity Western detection reagent (Bio-Rad) and captured on the Bio-Rad ChemiDoc Viewing System. Data were expressed as relative band density (RBD) and expression of proteins was analyzed with the Bio-Rad ChemiDoc MP Imaging System with Image Lab software. Membranes were quenched (5% H2O2 at 37oC for 30min) and pNrf2 was normalized against total Nrf2 (ab31163, 1:5,000) and other proteins were normalized against β-actin (A5316, 1:5,000).

**Quantitative PCR**

The mRNA expression of SIRT3, PGC-1α, GPx and SOD2 was determined by qPCR. Total RNA was isolated using Qiazol extraction buffer (Qiagen, Hilden, Germany) and an in-house protocol [34]. RNA was quantified using a spectrophotometer (Nanodrop2000, Biotech) and standardized to 1,000ng/µl. Standardized RNA was reverse transcribed to complementary DNA (cDNA) using the iScript™ cDNA Synthesis kit (Bio-Rad; 107-8890) as per the manufacturer’s instruction. A reaction volume of 10µl was prepared consisting of 5X IQ™ SYBR® ssoAdvanced SYBR Green (Bio-Rad; 170-880), nuclease free water, 1µl cDNA template (1,000ng/ml), and 1µl sense and anti-sense primer (Inqaba Biotec™). The mRNA expression was normalized against a housekeeping gene (18S). Thermocycler conditions were carried out using the CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA) as follows: initial denaturation (95°C, 10min), 40 cycles of denaturation (95°C, 15sec), annealing and extension (72°C, 30sec). Relative fold change was calculated using the method described by Livak and Schmittgen, 2001 to calculate relative fold change [35]. Primer sequences and annealing temperatures are shown in (Supplementary data, table 1).

**Statistical analyses**

Following each experiment in vitro and in vivo, 4-OH-Ile was compared to MF and the untreated control. We then analyzed FSE in comparison to the untreated control. Statistical analyses were performed using GraphPad Prism v5.0 software (GraphPad Software, Inc.). Statistical comparisons for 4-OH-Ile, MF and the untreated control were made using a one-way analysis of variance (ANOVA), nonparametric test (Kruskal Wallis test) and a Dunn’s post-test; and a non-parametric Mann-Whitney test was used for FSE and the untreated control. The data were considered statistically significant with a value of p < 0.05.

**Results**

**4-OH-Ile regulates MDA, GSH and protein carbonyl levels under NG and HG conditions, in vitro**

We first determined the effect of 4-OH-Ile on OS markers (MDA and protein carbonyls) and cellular AO content (GSH/GSSG). Figure 1A demonstrates OS induction as indicated by increased extracellular MDA levels, under a HG state (0.38 ± 0.004), 4-OH-Ile (0.13 ± 0.004) and MF (0.15 ± 0.004) significantly
reduced the levels of MDA (Fig 1A). During hyperglycaemia, GSH levels are attenuated which is observed in the hyperglycaemic control (4.0 ± 0.002) (Fig 1B). Again, both 4-OH-Ile (NG: 30.0 ± 0.002; HG: 18.0 ± 0.002) and MF (NG: 32.0 ± 0.001; HG: 27 ± 0.004) significantly increased GSH levels compared to the untreated controls (Fig 1B). In addition, the levels of GSSG were reduced under both conditions by 4-OH-Ile (NG: 0.2 ± 0.006; HG: 1.3 ± 0.005) and MF (NG: 0.9 ± 0.005; HG: 1.5 ± 0.006) compared to the untreated controls (NG: 2.9 ± 0.001, HG: 1.8 ± 0.017) (Fig 1C). Hyperglycaemia causes the oxidation of proteins, forming protein carbonyls. We observed the rise in protein carbonyls in the hyperglycaemic state (0.0000015 ± 0.0005) (Fig 1D). However, as seen in figure 1D 4-OH-Ile (NG: 0.0000008 ± 0.003; HG: 0.0000009 ± 0.002) and MF (NG: 0.0000006 ± 0.001; HG: 0.0000007 ± 0.004) attenuated the protein carbonyl levels.

**Figure 1.** Intracellular reactive oxygen species measured as malondialdehyde (MDA) (A–normoglycaemic, p=0.0024; hyperglycaemic, p=0.0012), GSH (B– normoglycaemic, p=0.0424; hyperglycaemic, p=0.0232), GSSG (C– normoglycaemic, p=0.0121; hyperglycaemic, p=0.0242), and protein carbonyl (D– normoglycaemic, p=0.0420; hyperglycaemic, p=0.0112) levels in HepG2 cells.
treated with metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) at 72h, *$P<0.05$; **$P<0.05$ relative to control.

**4-OH-Ile regulates protein expression of pNrf2/Nrf2 and gene expression of SOD2 and GPx under NG and HG conditions, in vitro and in vivo**

The changes in MDA and GSH levels observed *in vitro* prompted further investigation of the transcriptional regulation of the cellular AO response. *In vitro*, 4-OH-Ile elevated the expression of pNrf2 (NG: 2.2-fold, 1.40±0.03RBD; HG: 2.5-fold, 1.38±0.02RBD) (Fig 2A, B). This was validated *in vivo*; where we observed 4-OH-Ile increase the expression of pNrf2 (NG: 2-fold, 1.40±0.02RBD; HG: 2.3-fold, 1.38±0.01RBD) (Fig 2C, D). Increased pNrf2 is indicative of nuclear translocation and activation of Nrf2, followed by binding to the ARE; inducing transcription of various antioxidant genes. This was evidenced by increased SOD2 (Table 1) and GPx (Table 1) transcript levels in 4-OH-Ile treatments. In both HG *in vitro* and *in vivo* models 4-OH-Ile increased SOD2 gene expression 2.4-fold (Table 1) and 2.2-fold (Table 1), respectively. Likewise, 4-OH-Ile increased GPx gene expression 2.5-fold (Table 1) and 2.6-fold (Table 1).

**Figure 2.** Phosphorylated (p) Nuclear factor erythroid 2-related factor 2 (Nrf2) and total Nrf2 expression in 4-hydroxyisoleucine (4-OH-Ile) and metformin (MF) treated HepG2 cells (normoglycaemic –A, $p=0.0112$; hyperglycaemic –B, $p=0.0113$) and mouse liver (normoglycaemic –C, $p=0.0164$; hyperglycaemic –D, $p=0.0011$). *$P< 0.05$ relative to control.*
Table 1. Gene expression of SIRT3, PGC-1α, SOD2 and GPx following 4-OH-Ile and MF treatment in vitro and in vivo, under NG and HG conditions

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<th>Gene</th>
<th>Normoglycaemic</th>
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<tr>
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<td>SIRT3</td>
<td>PGC-1α</td>
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<td>Control</td>
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<tr>
<td>4-OH-Ile</td>
<td>1.2*</td>
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<td>MF</td>
<td>1.3*</td>
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<tr>
<td>4-OH-Ile</td>
<td>1.2*</td>
<td>1.45*</td>
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<td>MF</td>
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4-OH-Ile and MF treatments increase mRNA levels of SIRT3, PGC-1α, SOD2 and GPx in HepG2 cells and mice liver samples. SIRT3 (p=0.00132), PGC-1α (p=0.00133), SOD2 (p=0.00331) and GPx (p=0.0012) mRNA was significantly elevated in hyperglycaemic HepG2 samples treated with MF and 4-OH-Ile. The in vivo data substantiated this with significantly elevated SIRT3 (p=0.00112), PGC-1α (p=0.00211), SOD2 (p=0.00221) and GPx (p=0.00122) in both MF and 4-OH-Ile treatments under both conditions. *P< 0.05 relative to control.

4-OH-Ile regulates protein expression of LonP1, SIRT3 and PGC-1α and mRNA expression of SIRT3 and PGC-1α under NG and HG conditions, in vitro and in vivo

We evaluated the effects of 4-OH-Ile and MF on protein expression of LonP1, SIRT3 and PGC-1α as an assessment of mitochondrial stress. We proposed oxidized protein levels diminished following the elevation of LonP1 in vitro; (NG: 2.3-fold, 1.28±0.02RBD; HG: 2.8-fold, 1.58±0.01RBD) (Fig 3A, B) and in vivo; 1.7-fold (NG: 1.40±0.03RBD) (Fig 3C) and 2.2-fold (HG: 1.27±0.02RBD) (Fig 3D). This effect was quantified in vitro as seen via the reduction in protein carbonyl levels (Fig 1D). Next, the activation of mitochondrial regulatory proteins were confirmed in vitro, via the increased protein expression of both SIRT3 (NG: 2-fold, 1.38±0.02RBD; HG: 2.8-fold, 1.58±0.02RBD) and PGC-1α (NG: 1.9-fold, 1.08±0.02RBD; HG: 3-fold, 1.70±0.02RBD) (Fig 3A, B). These results were validated by the observed increases in vivo, (Fig 3C, D). SIRT3 increased by 1.6-fold (NG: 0.90±0.02RBD) (Fig 3C) and 1.9-fold (HG: 1.00±0.02RBD) (Fig 3D) whilst PGC-1α expression increased by 2.4-fold (NG: 2.40±0.02RBD) (Fig 3C) and 2.8-fold (HG: 1.57±0.02RBD) (Fig 3D). Furthermore, these observed
changes in protein expression were accompanied by a concomitant rise in mRNA expression of both 
*SIRT3* and *PGC-1α* (Table 1). In both models, the expression of these genes were more prominent in a 
HG condition. *In vitro* 4-OH-Ile increased *SIRT3* by 1.7-fold and *PGC-1α* by 1.55-fold (Table 1); and *in 
vivo* 4-OH-Ile elevated *SIRT3* by 1.7-fold and *PGC-1α* by 1.5-fold (Table 1). Again, the anti-diabetic drug 
MF failed to exceed the effects of 4-OH-Ile on both *SIRT3* and *PGC-1α*.

**Figure 3.** Protein expression analysis of Sirtuin 3 (SIRT3), peroxisome proliferator-activated receptor 
gamma coactivator 1-alpha (PGC-1α) and mitochondrial lon protease 1 (LonP1) in 4-hydroxyisoleucine 
(4-OH-Ile) and metformin (MF) treated HepG2 cells (normoglycaemic –A, *p*=0.0242; hyperglycaemic –B, *p*=0.0323) and mouse liver (normoglycaemic – C, *p*=0.0269; hyperglycaemic – D, *p*=0.0030). *P*<
0.05 relative to control

**FSE regulates protein expression of pNrf2/Nrf2, LonP1, SIRT3 and PGC-1α and gene expression of 
*SIRT3*, *PGC-1α*, *SOD2* and *GPx* under NG and HG conditions, *in vitro* and *in vivo***

We also investigated the effect of FSE on GSH, MDA and protein carbonyl levels *in vitro*. We observed 
that FSE possesses the ability to regulate markers of OS as efficiently as 4-OH-Ile (Fig 4). Consequently, 
*in vitro* and *in vivo* we investigated the phosphorylated and total protein expression Nrf2. pNrf2 increased 
1.3-fold (NG: 1.01±0.03RBD) (Fig 5A) and 2.3-fold (HG: 1.50±0.02RBD) (Fig 5B) *in vitro* and 2.2-fold 
NG: 1.70±0.04RBD) (Fig 5C) and 1.4-fold (HG: 0.90±0.03RBD) (Fig 5D) *in vivo*. *In vitro*, 4-OH-Ile 
elevated total protein expression of Nrf2 (NG: 1.4-fold, 1.40±0.01RBD; HG: 1.5-fold, 1.38±0.02RBD) 
and *in vivo* (NG: 1.5-fold, 1.40±0.04RBD; HG: 1.2-fold, 1.38±0.02RBD) expression of Nrf2 (Fig 5).
Following the effects of 4-OH-lle, we can conclude that FSE is also a substantial regulator of this major transcription factor.

LonP1 displayed a 1.4-fold (NG: 1.01±0.02RBD) (Fig 6A) and 1.9-fold (HG: 1.50±0.02RBD) (Fig 6B) increase in vitro and 2.1-fold (NG: 1.01±0.02RBD) (Fig 6C) and 1.2-fold (HG: 0.90±0.02RBD) (Fig 6D) rise in vivo. However, in both models these effects did not exceed 4-OH-lle but were potent in eliciting a response to the extract. In vitro, FSE significantly up-regulated the total protein expression of SIRT3 by 2.2-fold (NG: 1.18±0.03RBD) (Fig 6A) and 2.5-fold (HG: 1.28±0.02RBD) (Fig 6B). Also, under a HG condition, FSE significantly increased the total protein expression of PGC-1α 2.6-fold (2.00±0.02RBD) (Fig 6B) which exceeded the effects of 4-OH-lle. In vivo, FSE displayed the similar changes in protein expression; NG: 1.8-fold (1.90±0.02RBD) (Fig 6C) and HG: 1.65-fold (1.60±0.02RBD) (Fig 6D). Interestingly, FSE continued to display its efficacy via the marked increase in SIRT3 and PGC-1α.

The gene expression of SOD2, GPx, SIRT3 and PGC-1α was increased by FSE under both conditions (Table 2). In vitro, FSE (2.1-fold) displayed an increase in gene expression of SIRT3 (Table 2) which surpassed the effect of 4-OH-lle. Also FSE (1.8- and 1.75-fold) increased PGC-1α and SOD2 gene expression, respectively (Table 2). In vivo, the similar increases were observed following treatment with FSE - SIRT3 increased 1.9-fold, PGC-1α; 2.1-fold and SOD2; 2.6-fold. In vitro, exceeding the effects of 4-OH-lle, FSE increased GPx gene expression 2.4-fold (in vitro; HG) (Table 2) and 2-fold (HG; in vivo) (Table 2).
Figure 4. Intracellular reactive oxygen species measured as GSH (A – normoglycaemic, \( p=0.0124 \); hyperglycaemic, \( p=0.0132 \)), GSSG (B – normoglycaemic, \( p=0.0221 \); hyperglycaemic, \( p=0.0142 \)), malondialdehyde (C – normoglycaemic, \( p=0.0034 \); hyperglycaemic, \( p=0.0022 \)) and protein carbonyl (D – normoglycaemic, \( p=0.0320 \); hyperglycaemic, \( p=0.0212 \)) levels in HepG2 cells treated with fenugreek seed extract (FSE) at 72h, \(*P<0.05\); **\( p<0.05 \) relative to control.
Figure 5. Phosphorylated (p) Nuclear factor erythroid 2-related factor 2 (Nrf2) and total Nrf2 expression in fenugreek seed extract (FSE) treated HepG2 cells (normoglycaemic – A, \( p=0.0212 \); hyperglycaemic – B, \( p=0.0213 \)) and mouse liver (normoglycaemic – C, \( p=0.0164 \); hyperglycaemic – D, \( p=0.0011 \)). *\( P < 0.05 \) relative to control.

Figure 6. Protein expression analysis of Sirtuin 3 (SIRT3), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1\( \alpha \)) and mitochondrial lon protease 1 (LonP1) in fenugreek seed extract.
(FSE) treated HepG2 cells (normoglycaemic – A, \( p=0.0231 \); hyperglycaemic – B, \( p=0.0233 \)) and mouse liver (normoglycaemic – C, \( p=0.0269 \); hyperglycaemic – D, \( p=0.0030 \)). *\( P < 0.05 \) relative to control

**Table 2.** Gene expression of SIRT3, PGC-1\( \alpha \), SOD2 and GPx following FSE treatment *in vitro* and *in vivo*, under NG and HG conditions

<table>
<thead>
<tr>
<th>Normoglycaemic</th>
<th>Hyperglycaemic</th>
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</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
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</tr>
<tr>
<td><strong>In vitro</strong></td>
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<tr>
<td>Control</td>
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</tr>
<tr>
<td>FSE</td>
<td>1.9*</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
</tr>
<tr>
<td>FSE</td>
<td>2.2*</td>
</tr>
</tbody>
</table>

FSE treatment increased mRNA levels of SIRT3, PGC-1\( \alpha \), SOD2 and GPx in HepG2 cells (A-D) and mice liver samples (E-H). SIRT3 (\( p=0.00121 \)), PGC-1\( \alpha \) (\( p=0.00132 \)), SOD2 (\( p=0.00231 \)) and GPx (\( p=0.00112 \)) mRNA was significantly elevated in hyperglycaemic HepG2 samples treated with FSE. The *in vivo* data substantiated this with significantly elevated SIRT3 (\( p=0.00312 \)), PGC-1\( \alpha \) (\( p=0.00311 \)), SOD2 (\( p=0.0321 \)) and GPx (\( p=0.00322 \)) in both MF and 4-OH-Ile treatments under both conditions. *\( P < 0.05 \) relative to control

**Discussion**

In our previous investigations we proved the salient role of 4-OH-Ile as a promising therapeutic intervention for T2D therapy, via the regulation of insulin signaling and dyslipidemia. Following these favourable effects on two critical pathways related to diabetic complications, prompted us to evaluate OS and mitochondrial health. Compelling evidence proposes that prolonged OS plays a causal role in the pathogenesis of T2D; and improving endogenous cellular AO responses and detoxification will reduce OS and attenuate diabetic complications. The major regulator of cellular redox status – Nrf2 has been implicated in mitigating the features of diabetic milieu. Therefore, our study investigated the regulatory effect of 4-OH-Ile in comparison to MF on hepatic OS and mitochondrial maintenance pathways in hyperglycaemic human liver (HepG2) cells and C57BL/6 male mice. We observed the protective effects of 4-OH-Ile via Nrf2 induction and mitochondrial maintenance proteins; LonP1, SIRT3 and PGC-1\( \alpha \).
Hyperglycaemic-induced ROS production is the main risk factor contributing to OS and subsequent CVD and diabetic complications. Studies have reported the AO effect of fenugreek in reducing ROS levels. A study by Mohamad et al, 2004 observed SOD2 and liver enzyme levels similar to the normoglycaemic control group in fenugreek seed treated rats [22]. Ravikumar and Anuradha, 1999 displayed that interrupted free radical metabolism in diabetic animals was regulated by dietary fenugreek seed supplementation [36]. Another study investigated the AO activities of fenugreek seeds extracts in cholesterol-fed rats and observed a decrease in TBARS and rise in catalase and SOD2 expression [23]. Additionally Dixit et al, 2005 revealed substantial AO potential of germinated fenugreek seeds which is due to the presence of flavonoids and polyphenols [37]. The bioactive component of fenugreek seeds, 4-OH-Ile, independently possesses AO activity as shown by Dutta et al, 2014. This study evidenced that 4-OH-Ile scavenges hydroxyl, superoxide anion, hydrogen peroxide and DPPH radicals, reduced lipid peroxidation and protein carbonyl levels and concomitantly amplified GSH levels, in a chemically defined in vitro system [24]. Other substituents of fenugreek seeds, trigonelline and diosgenin, display similar effects and are identified as potent activators of the AO transcriptional regulator, Nrf2 [38]. This central role player in endogenous AO response has yet to be investigated in relation to 4-OH-Ile.

Nrf2 is deemed the master regulator of the endogenous AO response; it enables cells to adapt to an oxidative environment via the induction of cytoprotective genes. Nrf2 is positioned in the cytoplasm where it is linked to a negative regulator Kelch-like ECH-associated protein 1 (Keap1) [39]. Following exposure to OS, Nrf2 gains protein stability (via phosphorylation) and escapes Keap1-mediated repression; translocating into the nucleus. This dissociation from Keap1 causes the phosphorylation and activation of Nrf2. Within the nucleus, pNrf2 initiates the antioxidant response element (ARE); a regulatory sequence involved responsible for transcriptional activation of genes coding for AO enzymes. These include cytoprotective genes SOD2 and GPx which are involved in the synthesis of GSH. Cell culture studies demonstrated the activation of Nrf2 in response to hyperglycaemic induced-oxidative and chemical stress [40, 41]. Other studies also reported the decrease of Nrf2 in both diabetic mice and T2D individuals; contributing to increased OS, endothelial dysfunction, insulin resistance and microvascular complications [4-6]. Our study was the first to demonstrate the AO response of 4-OH-Ile via the induction of pNrf2 in both hyperglycaemic Hepg2 cells and the liver of C57BL/6 male mice. Furthermore, the induction of pNrf2 by 4-OH-Ile exceeded the effects of MF and FSE. This result was validated by the increase in transcript levels of SOD2 and GPx, with 4-OH-Ile inducing a greater response as compared to MF and FSE (Fig 4). During OS, both SOD2 and GPx activation is central to the synthesis of GSH [42, 43]. Glutathione forms a major component of the AO response which functions to alleviate OS by directly
To further confirm the above response, our results showed that 4-OH-Ile preceded the effect of MF and FSE in regulating GSH (Fig 1).

Protein kinase pathways are implicated in transducing OS signals to gene expression facilitated through the ARE. Hyperglycaemia causes chronic elevation of diacylglycerol (DAG); a secondary messenger that activates proteins involved in a multiplicity of signaling cascades [2]. In T2D, DAG levels are elevated in non-vascular tissues, such as the liver which is responsible for activating protein kinase C (PKC) [2]. The initiation of PKC is of great value as evidence proves the involvement of PKC in phosphorylation of Nrf2 on Ser40, promoting its dissociation from Keap1 [2]. Furthermore, phosphatidylinositol 3-kinase and its downstream target protein kinase B (Akt) have been associated with activation of the ARE in hepatoma cells [44]. Our previous study on insulin signaling evaluated Akt signaling and provided evidence on the elevating effect of 4-OH-Ile on hepatic Akt (Submitted for publication). Both PKC and Akt activation comprise the group of biochemical pathways proposed in linking the adverse effects of hyperglycaemia with diabetic complications. Another cellular mechanism includes the activation of AGE pathway. Elevated ROS causes the formation of oxidatively damaged proteins, forming protein carbonyls [45]. Protein carbonyls are by-products of glycosylated proteins which ultimately form the toxic compounds - AGEs. Previous studies support the worsening effect of AGEs on metabolic control in T2D [3, 46, 47]. An important study by Sampath et al, 2017 confirmed the reduction of AGEs via Nrf2 [48]. In addition to Nrf2-regulated AO response, mitochondria possess LonP1 which enable cells to effectively remove damaged proteins. We showed 4-OH-Ile significantly potentiated the protein expression of LonP1 in both chronic hyperglycaemic models (Fig 3). To further support this finding in vitro, we showed the ability of 4-OH-Ile to attenuate the levels of protein carbonyls (Fig 1).

Hyperglycaemia induced OS coupled with insulin resistance causes a decline in mitochondrial function. Mitochondria produce ATP via oxidative phosphorylation. However, this process is linked to the production of ROS. Mitochondria possess their own maintenance proteins - SIRT3 and PGC-1α, enabling these organelles to alleviate the effects of OS. In addition to these proteins, Nrf2 also plays a functional role in counterbalancing mitochondrial produced ROS via SOD2, GPx and GSH. SIRT3 is a NAD+-dependent protein deacetylase which is located and exerts its function in the mitochondria. Among the SIRTs located in the mitochondria, SIRT3 is responsible for regulating mitochondrial function via deacetylation of mitochondrial proteins [49]. SIRT3 induction is regulated by caloric restriction and stress which is central to the effect of SIRT3 on the transcription of PGC-1α [14]. Studies provide substantial evidence for the communication between SIRT3 and PGC-1α, which is imperative for the AO response and regulation of mitochondrial biogenesis [50]. Our results clearly displayed the interaction between...
SIRT3 and PGC-1α. More importantly, we further exemplify the AO potency of 4-OH-Ile by displaying its ability in significantly up-regulating the protein and gene expression of both SIRT3 and PGC-1α (Fig 3). It is of greater significance that we observed these effects in a chronic glucose condition which surpassed the effects of MF and FSE.

Following our favourable results and the supporting literature, we evaluated FSE under the same parameters, in comparison to 4-OH-Ile and MF. We observed FSE significantly increase the phosphorylated and total protein expression of Nrf2 as well as the total protein expression of LonP1, SIRT3 and PGC-1α (Fig 6, 7). In addition, FSE elevated the transcript levels of SOD2, GPx, SIRT3 and PGC-1α. These results surpassed the effect of MF but were similar to 4-OH-Ile. 4-OH-Ile could account for the potency of FSE, as 4-OH-Ile has been documented to be abundant in fenugreek seeds, and suggested to account for the seeds anti-diabetic and anti-lipidemic effects. An important study by Mayakrishnan et al, 2015 found both trigonelline and diosgenin to exhibit protective effects via a substantial decrease in serum enzymes, liver TGs, expression of liver ER stress marker proteins and elevated liver glycogen content and AOs [38]. Therefore, the effects observed by FSE may also be due to the culminated effect of these active compounds in the seed. Interestingly, our study was the first to show that 4-OH-Ile is potent in stimulating the Nrf2-AO response and detoxification system.

Oxidative stress has been implicated as a contributor to the onset and progression of CVD and associated diabetic complications. The consequence of an oxidative environment is the development of compromised AO defence mechanisms and mitochondrial dysfunction, which ultimately leads to a diabetic disease state. 4-hydroxyisoeelucine potentiates an AO response in hyperglycaemic HepG2 cells and C57BL/6 male mice. Collectively, the results show that 4-OH-Ile exposure augments the expression of Nrf2, LonP1, SIRT3 and PGC-1α, particularly in a hyperglycaemic state. Furthermore, the results provide significant evidence for the use of FSE as a possible AO agent. This data may help develop a better understanding of the molecular and biochemical interactions of both 4-OH-Ile and FSE, associated with risks of elevated ROS production and diabetic complications. This is importance in socio-economically challenged communities where T2D individuals are diagnosed with CVD, unable to access healthcare facilities and natural products serve as first-line therapy.

Acknowledgements

The authors would like to acknowledge the NRF for the Scarce Skills Doctoral Scholarship (grant no. 94953) and the College of Health Sciences (UKZN) for financial support. Linda A. Bester and the BRU at the Westville Campus of UKZN for their support and resources throughout the study.
Funding
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Conflict of interest
Authors declare no conflict of interest.

References


CHAPTER SIX
SYNTHESIS, CONCLUSIONS AND IMPLICATIONS FOR FURTHER RESEARCH
6.1 Synthesis

Diabetes mellitus significantly contributes to the morbidity and mortality rates, resulting in negative socio-economic impacts. Type 2 diabetes mellitus, a subtype of DM, is progressively observed among children, teenagers and younger adults (WHO, 2016). The epidemic of T2D is attributed to a multifaceted collection of genetic and molecular systems interacting within a complex social framework that controls behaviour and environmental influences (WHO, 2016). T2D is characterized by hyperglycaemia, insulin resistance and relative lack of insulin production. These disturbances negatively impact the normal physiological, molecular and biochemical activities of the body (Ashcroft & Rorsman, 2012). The treatment approach for T2D comprises both self-care and anti-diabetic drugs. Unfortunately, the lack of complying with self-care recommendations and the associated unwanted side effects of anti-diabetic drugs such as MF, demands the need for new therapeutic interventions. This study investigated the anti-hyperglycaemic, anti-lipidaemic and anti-oxidative effect of 4-OH-Ile in comparison to MF, and FSE in a hyperglycaemic in vitro and in vivo model. It was observed that 4-OH-Ile and FSE improved compromised insulin signalling and glucose sensing, the altered lipid profile, and an imbalance in OS.

4-OH-Ile up-regulated the proteins and genes pertinent to insulin signalling and glucose sensing. IRβ and IRS1 are important proteins in initiating the insulin signalling cascade (Guo, 2014). 4-OH-Ile displayed the greatest effect in elevating the phosphorylated and total protein and mRNA expression of IRβ and IRS1, in both models. This elevation translated to the phosphorylation and activation of Akt and subsequent increase in GSK3α/β activity. The dominant effect of 4-OH-Ile was further observed in the increased mRNA levels of GS and GK; which in turn regulate the conversion of excess glucose into glycogen. The entry of glucose into the cell is essential for regulating excess glucose levels (Cho, Thorvaldsen, Chu, Feng, & Birnbaum, 2001). We showed that 4-OH-Ile possesses the strongest potential in regulating glucose entry into the cell via the up-regulation of the protein and mRNA expression of GLUT2. This was further supported by the reduction in glucose levels. Therefore, this study provides evidence that 4-OH-Ile elicited a stronger response than both MF and FSE in regulating the specific proteins and genes compromised during a hyperglycaemic state.

Insulin signalling plays a principal role in regulating lipid metabolism (Mullugeta, Chawla, Kebede, & Worku, 2012). In T2D, defective insulin signaling alters the lipid profile resulting in diabetic dyslipidaemia (Tangvasittichai, 2015). The levels of TGs are controlled by SREBP1c and FAS (Horton, Goldstein, & Brown, 2002), which were elevated by 4-OH-Ile. This change in gene expression correlated with the regulation of both TG and cholesterol levels. Furthermore, 4-OH-Ile down-regulated protein and gene expression of PCSK9 and up-regulated the protein expression of PPARG, which are crucial in...
restoring LDLc and HDLc levels, respectively (Amy C. Burke, 2017; Gervois, Torra, Fruchart, & Staels, 2000). The effect of 4-OH-Ile on PCSK9 was validated by the increase in LDLr and reduction in LDLc, whereas the effect of 4-OH-Ile on PPARG was validated via the elevation in gene expression of ApoA1 and HDLc levels. Together, these results show that 4-OH-Ile in comparison to MF, and FSE efficiently restores the lipid profile via the regulation of PCSK9 and PPARG; which were posed as possible targets for the treatment of diabetic dyslipidaemia (Gervois et al., 2000; Horton, Cohen, & Hobbs, 2007).

Therefore, in regulating both insulin signalling and dyslipidaemia, 4-OH-Ile possesses the ability to control the progression of CVD that may present in T2D.

In addition, hyperglycaemic-induced OS is a major risk factor for the onset and progression of CVD and diabetic complications (John W Baynes, 1991, J W Baynes & Thorpe, 1999). 4-OH-Ile induced the AO response via pNrf2 and subsequent increase in SOD2, GPx and GSH levels. In T2D over and above hyperglycaemic-induced ROS, mitochondria are also responsible for an increase in ROS production (Brownlee, 2001). Mitochondrial ROS is reduced by the activity of pNrf2 as well as its own maintenance proteins (Dinkova-Kostova & Abramov, 2015; Giralt & Villarroya, 2012; Pomatto, Raynes, & Davies, 2017). Further, 4-OH-Ile increased the activities of these proteins which include LonP1, SIRT3, and PGC-1α. The elevation of these proteins is essential in attenuating mitochondrial dysfunction present in T2D (Dinkova-Kostova & Abramov, 2015; Giralt & Villarroya, 2012; Pomatto et al., 2017). The results further exemplify the potency of 4-OH-Ile in comparison to MF, and FSE, by inducing the Nrf2-AO response in a chronic hyperglycaemic state, attenuating OS and the accompanying risk of developing CVD and diabetic complications.

6.2 General conclusions

During T2D, insulin signalling, dyslipidaemia, and OS have been implicated as major contributors to the onset and development of CVD and diabetic complications. The consequences of defective insulin signalling promote dyslipidaemia and an oxidative environment. This gives rise to the development of an abnormal lipid profile and compromised AO defence mechanisms and mitochondrial dysfunction, ultimately causing a diabetic disease state. The data provides evidence on the potency of 4-OH-Ile in regulating insulin signalling, lipid metabolism, and OS, in both hyperglycaemic HepG2 cells and C57BL/6 male mice. Collectively, the results show that the hepatic response to 4-OH-Ile augments the expression of specific proteins, genes and, related factors, altered in T2D. Furthermore, the results provide substantial evidence for the use of FSE as a possible therapeutic intervention, as the seed extract also elicited potent responses, although not as potent as 4-OH-Ile.
The data assists in developing a better understanding of the molecular and biochemical interactions of both 4-OH lle and FSE. This has a great impact on socio-economically challenged communities where T2D individuals are diagnosed with CVD and/or other diabetic complications but are unable to access healthcare facilities. In addition, these affected individuals have access to natural products which possess invaluable medicinal properties.

6.3 Implications for further research
Future studies to extend on the current findings would be to investigate the effects of 4-OH lle and FSE in other organ systems such as the pancreas and skeletal muscle since these organs play a central role in regulating insulin production and glucose utilization. Furthermore, the pathways investigated in this study coupled with existing literature, could provide substantial evidence for the possible initiation of a clinical trial for the use of 4-OH lle and FSE as possible anti-diabetic therapy.

6.4 References


Horton, Jay D., Goldstein, Joseph L., & Brown, Michael S. (2002). SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. The Journal of Clinical Investigation, 109(9), 1125-1131. doi:10.1172/JCI15593


Appendix 1: Animal ethics approval

03 October 2016

Professor Anil Chuturgoon (34866)
School of Laboratory Medicine & Medical Sciences
Westville Campus

Dear Professor Chuturgoon,

Protocol reference number: AREC/057/016
Project title: Effect of fenugreek seed extract, 4-OH-ile and metformin on insulin signalling, lipid metabolism and epigenetic regulation of type 2 diabetic C57Bl/6 black male mice

Full Approval – Research Application

With regards to your revised application received on 15 September 2016 and 31 August 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and FULL APPROVAL for the protocol has been granted.

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

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

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 03 October 2017.

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

Yours faithfully

Prof S Ismail, PhD
Chair: Animal Research Ethics Committee

Cc: Acting Academic Leader Research: Dr Michelle Gordon
Cc: Registrar: Mr Simon Msokona
Cc: NSPCA: Ms Jessica Light
Cc: BRU – Dr Sandi Singh

Animal Research Ethics Committee (AREC)
Ms Maritzia Smyrniot, (Administrator)
Westville Campus, Geovan Mbedi Building
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Telephone: +27 (0)31 260 8230 or 8231
Facsimile: +27 (0)31 260 4655
Email: arec@ukzn.ac.za
Website: http://ropriate.ukzn.ac.za/research-ethics/animal-ethics.aspx
The term control refers to the group of mice which were untreated during the experimental period. These mice were maintained under the same conditions but were not treated with metformin, 4-hydroxyisoleucine or fenugreek seed extract. Following the methods of our study there were two experimental arms (normoglycaemic and hyperglycaemic) – each arm had an untreated group of mice.

**Table 1** Absolute oral glucose tolerance test values (mmol) following treatment of mice with fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), and metformin (MF)

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Normoglycaemic state (Glucose in mmol/L)</th>
<th>Hyperglycaemic state (Glucose in mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (untreated)</td>
<td>FSE (100mg/kg BW)</td>
</tr>
<tr>
<td>0 min</td>
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<tr>
<td>30 min</td>
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<td>90 min</td>
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<tr>
<td>120 min</td>
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</table>
Table 2 Blood glucose measurements (mmol/L) at day 0, 3, and 10 of the ten day induction period of the hyperglycaemic group of mice

<table>
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<tr>
<th>Mice</th>
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<th>Day 3 (glucose in mmol/L)</th>
<th>Day 10 (glucose in mmol/L)</th>
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<td>4.3</td>
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</table>

All mice were labeled 1 to 20 by an ear piecing, to ensure the same mice were treated within the same group.
Table 3 Glucose measurements of each mouse per a treatment group (control, fenugreek seed extract metformin and 4-hydroxyisoleucine) under both conditions on day 0, 3, 6, 9, 12 and 15

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day 0 (glucose in mmol/L)</th>
<th>Day 5 (glucose in mmol/L)</th>
<th>Day 10 (glucose in mmol/L)</th>
<th>Day 15 (glucose in mmol/L)</th>
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<td>5.0</td>
<td>5.1</td>
<td>5.0*</td>
</tr>
<tr>
<td></td>
<td>2 4.8</td>
<td>4.2</td>
<td>4.2</td>
<td>4.8*</td>
</tr>
<tr>
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<td>40 13.9</td>
<td>10.0</td>
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<td>8.7*</td>
</tr>
</tbody>
</table>

All mice were labelled 1 to 40 by an ear piercing, to ensure the same mice were treated within the same group. The glucose was measured with a glucometer on day 0, 5, and 10. On day 15, the glucose levels were measured by the accredited laboratory.
mentioned in the methods and materials section. *Indicates these mice were chosen for the qPCR and western blot validation (based on blood glucose values).

Table 4 Mouse primer sequences and annealing temperatures (Ta) for qPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense</th>
<th>Anti-sense</th>
<th>Ta</th>
</tr>
</thead>
<tbody>
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<td>5'CCTCATCTTGGGTTGAACCT3'</td>
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<td>5'GGTGATGTTGAAACAGCTCCT3'</td>
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</tr>
<tr>
<td>GLUT2</td>
<td>5'GGCTAATTTCAGGACTGTT3'</td>
<td>5'TTTCTTTGCCCTGACCTCCT3'</td>
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<tr>
<td>Akt</td>
<td>5'ATCCCTCAACAACTCTCTCCT3'</td>
<td>5'CTTCCGTCACCTCTCTCTCTC'</td>
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<tr>
<td>GSK-3α/β</td>
<td>5'GCATTATCATTACCTAGCACC3'</td>
<td>5'ATTTTTACACTTAAAACATGACC3'</td>
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</tr>
<tr>
<td>GS</td>
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<td>5'CCAGCTGACAGGTTGACA3'</td>
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<td>Gck</td>
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<td>5'AAAAGGACTGGAGTAAGAC3'</td>
<td>59</td>
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</table>
Appendix 3: Supplementary data for chapter 4 (Table and figure numbers correlate with chapter 4)

Figure 1 Average concentrations (mean±SD) of blood glucose levels in the serum of mice, and oral glucose tolerance test at day 15 of experimental period; blood glucose levels were measured at 0, 30, 60, 90 and 120min time intervals following an overnight fast, following treatment with metformin (MF), fenugreek seed extract (FSE) and 4-hydroxyisoleucine (4-OH-Ile), compared to the relative control (C). Average blood glucose (A) normoglycaemic (p=0.00231) and (B) hyperglycaemic (p=0.0245). Oral glucose tolerance test (C) normoglycaemic (p=0.0154) and (D) hyperglycaemic (p=0.0021).
Table 1 Absolute oral glucose tolerance test values (mmol) following treatment of mice with fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), and metformin (MF)

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Normoglycaemic state (Glucose in mmol/L)</th>
<th>Control (untreated)</th>
<th>FSE (100mg/kg BW)</th>
<th>MF (20mg/kg BW)</th>
<th>4-OH-lle (100mg/kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td></td>
<td>4.80</td>
<td>4.40</td>
<td>4.30</td>
<td>4.00</td>
</tr>
<tr>
<td>30 min</td>
<td></td>
<td>4.90</td>
<td>4.50</td>
<td>4.20</td>
<td>4.20</td>
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<tr>
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<td></td>
<td>5.00</td>
<td>4.50</td>
<td>4.30</td>
<td>4.10</td>
</tr>
<tr>
<td>90 min</td>
<td></td>
<td>4.90</td>
<td>4.40</td>
<td>4.23</td>
<td>4.15</td>
</tr>
<tr>
<td>120 min</td>
<td></td>
<td>4.95</td>
<td>5.00</td>
<td>4.25</td>
<td>4.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Hyperglycaemic state (Glucose in mmol/L)</th>
<th>Control (untreated)</th>
<th>FSE (100mg/kg BW)</th>
<th>MF (20mg/kg BW)</th>
<th>4-OH-lle (100mg/kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td></td>
<td>13.9</td>
<td>10.90</td>
<td>10.00</td>
<td>9.00</td>
</tr>
<tr>
<td>30 min</td>
<td></td>
<td>14.8</td>
<td>10.50</td>
<td>9.80</td>
<td>10.20</td>
</tr>
<tr>
<td>60 min</td>
<td></td>
<td>14.8</td>
<td>10.00</td>
<td>9.60</td>
<td>9.80</td>
</tr>
<tr>
<td>90 min</td>
<td></td>
<td>14.8</td>
<td>9.90</td>
<td>9.70</td>
<td>10.10</td>
</tr>
<tr>
<td>120 min</td>
<td></td>
<td>15.1</td>
<td>10.10</td>
<td>9.65</td>
<td>9.50</td>
</tr>
</tbody>
</table>

Table 2 Area under the curve (AUC) values for the OGTT at day 15 of experimental period for normoglycaemic group

<table>
<thead>
<tr>
<th>Area under curve (AUC)</th>
<th>Control</th>
<th>FSE</th>
<th>MET</th>
<th>4-OH-lle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC 1 (30-0 min)</td>
<td>217.5 ± 50⁺</td>
<td>199.5 ± 21#</td>
<td>192 ± 47⁺</td>
<td>183 ± 24⁺</td>
</tr>
<tr>
<td>AUC 2 (60-30 min)</td>
<td>222 ± 34⁺</td>
<td>202.5 ± 72#</td>
<td>190.5 ± 24⁺</td>
<td>187.5 ± 48⁺</td>
</tr>
<tr>
<td>AUC 3 (90-60 min)</td>
<td>223.5 ± 61⁺</td>
<td>201 ± 45#</td>
<td>192.45 ± 13⁺</td>
<td>185.25 ± 23⁺</td>
</tr>
<tr>
<td>AUC 4 (120-90 min)</td>
<td>221.25 ± 19⁺</td>
<td>207 ± 35⁺</td>
<td>190.65 ± 175⁺</td>
<td>184.5 ± 25⁺</td>
</tr>
<tr>
<td>Total AUC</td>
<td>884.25 ± 124</td>
<td>810 ± 264</td>
<td>765.6 ± 119</td>
<td>740.25 ± 249</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD of 5 animals; *, #, + Values with different superscript letters within a row are significantly different from each group of animals (Tukey’s multiple range posthoc test, p < 0.05). FSE: fenugreek seed extract, MET: Metformin, 4-OH-lle: 4-hydroxyisoleucine
Table 3 Area under the curve (AUC) values for the OGTT at day 15 of experimental period for hyperglycaemic group

<table>
<thead>
<tr>
<th>Area under curve (AUC)</th>
<th>Control</th>
<th>FSE</th>
<th>MET</th>
<th>4-OH-lle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC 1 (30-0 min)</td>
<td>639 ± 29(^*)</td>
<td>484.5 ± 17(^#)</td>
<td>447 ± 33(^*)</td>
<td>423 ± 17(^*)</td>
</tr>
<tr>
<td>AUC 2 (60-30 min)</td>
<td>666 ± 54(^*)</td>
<td>465 ± 89(^#)</td>
<td>438 ± 55(^*)</td>
<td>453 ± 18(^*)</td>
</tr>
<tr>
<td>AUC 3 (90-60 min)</td>
<td>666 ± 45(^*)</td>
<td>448.5 ± 16(^#)</td>
<td>433.5 ± 12(^*)</td>
<td>445.5 ± 19(^*)</td>
</tr>
<tr>
<td>AUC 4 (120-90 min)</td>
<td>670.5 ± 34(^*)</td>
<td>448.5 ± 10(^#)</td>
<td>435.75 ± 11(^*)</td>
<td>445.5 ± 15(^*)</td>
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<tr>
<td>Total AUC</td>
<td>2641.5 ± 163(^*)</td>
<td>1846.5 ± 257(^#)</td>
<td>1754.25 ± 175(^*)</td>
<td>1767 ± 138</td>
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</tbody>
</table>

Data are shown as mean ± SD of 5 animals; \(^*\), \(^#\), \(^+\) Values with different superscript letters within a row are significantly different from each group of animals (Tukey’s multiple range posthoc test, p < 0.05). FSE: fenugreek seed extract, MET: Metformin, 4-OH-lle: 4-hydroxyisoleucine
Table 4 Blood glucose measurements (mmol/L) at day 0, 3, and 10 of the ten day induction period of the hyperglycaemic group of mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day 0 (glucose in mmol/L)</th>
<th>Day 3 (glucose in mmol/L)</th>
<th>Day 10 (glucose in mmol/L)</th>
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<td>13.5</td>
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</tr>
<tr>
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<td>4.3</td>
<td>6.5</td>
<td>14.4</td>
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<td>3</td>
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</tr>
<tr>
<td>5</td>
<td>5.5</td>
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<td>13.9</td>
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</tr>
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<td>4.4</td>
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<td>14.9</td>
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<td>5.0</td>
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</table>

All mice were labeled 1 to 20 by an ear piercing, to ensure the same mice were treated within the same group.
Table 5  Glucose measurements of each mouse per a treatment group (control, fenugreek seed extract metformin and 4-hydroxyisoleucine) under both conditions on day 0, 3, 6, 9, 12 and 15

<table>
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<th>Mice</th>
<th>Day 0 (glucose in mmol/L)</th>
<th>Day 5 (glucose in mmol/L)</th>
<th>Day 10 (glucose in mmol/L)</th>
<th>Day 15 (glucose in mmol/L)</th>
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<td>9.9*</td>
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<td>9.9</td>
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<td>9.0</td>
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</tr>
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<td></td>
<td>35 15.2</td>
<td>12.9</td>
<td>11.5</td>
<td>10.4*</td>
</tr>
<tr>
<td>4-OH-Ile</td>
<td>36 15.3</td>
<td>9.9</td>
<td>8.7</td>
<td>8.0</td>
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<td>37 14.5</td>
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<td>8.9*</td>
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<td>8.7*</td>
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<tr>
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<td>40 13.9</td>
<td>10.0</td>
<td>8.9</td>
<td>8.7*</td>
</tr>
</tbody>
</table>

All mice were labelled 1 to 40 by an ear piecing, to ensure the same mice were treated within the same group. The glucose was measured with a glucometer on day 0, 5, and 10. On day 15, the glucose levels were measured by the accredited laboratory.
mentioned in the methods and materials section. *Indicates these mice were chosen for the qPCR and western blot validation (based on blood glucose values).

**Table 6.** Primer sequences and annealing temperatures for qPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Type</th>
<th>Ta (°C)</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>SREBP1c</td>
<td>Sense</td>
<td>58</td>
<td>5'-GTGGCGCGCTGCATTGAGAGTGAAG-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-AGGTACCCGAGGGCATCCGAGAAT-3’</td>
</tr>
<tr>
<td>FAS</td>
<td>Sense</td>
<td>58</td>
<td>5'-CAAGAACTGCACGAGGTGT-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-AGCTGCCAGAGTCGGAGAAG-3’</td>
</tr>
<tr>
<td>LDLR</td>
<td>Sense</td>
<td>58</td>
<td>5'-CCCCGCAGATCAAACCCACC-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-AGACCCCCAGGCGAAAGGACAGCS-3’</td>
</tr>
<tr>
<td>ApoA1</td>
<td>Sense</td>
<td>58</td>
<td>5'-AGACAGCGCGAGACTATGTGTC-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-ACCTTCTGGCGGTAGAGCTC-3’</td>
</tr>
<tr>
<td>PCSK9</td>
<td>Sense</td>
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<td>5'-CCAAGATCTGCTGTCTTCC-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-ACATCAGCTCCACCAAACCTAA-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Sense</td>
<td></td>
<td>5'-TGACGGGTCACCCACTGTGCCCAT-3’</td>
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<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-CTAGAAGCATTTTGCGGTGGAGATGAGGG-3’</td>
</tr>
<tr>
<td>18S</td>
<td>Sense</td>
<td></td>
<td>5'-ACAGGGAGATGCTTGAGAAG-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-CAAATCGCTCCACCAAACCTAA-3’</td>
</tr>
<tr>
<td><strong>Mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SREBP1c</td>
<td>Sense</td>
<td>58</td>
<td>5'-ATGGGCAGCGGTGAGGTAGCTCGGC-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-ACTGGCTTTGATGGTTGAGCTGGAGCAT-3’</td>
</tr>
<tr>
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<td>Sense</td>
<td>55</td>
<td>5’ ATGGGCAGCGGTGAGGTAGCTCGGC-3’</td>
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<td></td>
<td>Antisense</td>
<td></td>
<td>5'-AGAGACGCTGCTCCCTGGACCTT-3’</td>
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<tr>
<td>LDLr</td>
<td>Sense</td>
<td>61</td>
<td>5'-GAAGTCGACACTGTGACCACC-3’</td>
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<td>Antisense</td>
<td></td>
<td>5'-CTTCCACTCCCTTCCTGACCAGC-3’</td>
</tr>
<tr>
<td>PCSK9</td>
<td>Sense</td>
<td>58</td>
<td>5'-TGCTCCAGAGGTGACCATCAG-3’</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td></td>
<td>5'-GTCCCAGCTCTGGAGTCCAG-3’</td>
</tr>
</tbody>
</table>

Ta - annealing temperature
Table 7 Absolute lipid profile values (mmol/L) for each experimental group, in vitro and in vivo at day 0 and day 15 of the experimental period

<table>
<thead>
<tr>
<th>In vitro</th>
<th>Control (untreated) Glucose in mmol/L</th>
<th>FSE (100mg/kg BW)</th>
<th>MF (20mg/kg BW)</th>
<th>4-OH-lle (100mg/kg BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoglycaemic</td>
<td>Day 15</td>
<td>Day 15</td>
<td>Day 15</td>
<td>Day 15</td>
</tr>
<tr>
<td>LDLc</td>
<td>2.6</td>
<td>2.13</td>
<td>2.17</td>
<td>2.07</td>
</tr>
<tr>
<td>HDLc</td>
<td>1.23</td>
<td>1.17</td>
<td>1.21</td>
<td>1.18</td>
</tr>
<tr>
<td>TC</td>
<td>4.12</td>
<td>4.23</td>
<td>4.4</td>
<td>4.33</td>
</tr>
<tr>
<td>TG</td>
<td>0.90</td>
<td>0.37</td>
<td>0.5</td>
<td>0.43</td>
</tr>
<tr>
<td>Hyperglycaemic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDLc</td>
<td>3.10</td>
<td>2.09</td>
<td>2.08</td>
<td>2.08</td>
</tr>
<tr>
<td>HDLc</td>
<td>0.87</td>
<td>1.08</td>
<td>1.4</td>
<td>1.18</td>
</tr>
<tr>
<td>TC</td>
<td>5.61</td>
<td>4.57</td>
<td>4.57</td>
<td>4.25</td>
</tr>
<tr>
<td>TG</td>
<td>1.43</td>
<td>0.8</td>
<td>0.7</td>
<td>0.83</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>In vivo</th>
<th>Normoglycaemic</th>
<th>Hyperglycaemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLc</td>
<td>0.58</td>
<td>0.70</td>
</tr>
<tr>
<td>HDLc</td>
<td>1.40</td>
<td>1.33</td>
</tr>
<tr>
<td>TC</td>
<td>3.50</td>
<td>4.93</td>
</tr>
<tr>
<td>TG</td>
<td>0.21</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Table 1 Concentration of glucose in the cell supernatant following incubation with fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), insulin and metformin for 72 hours.

<table>
<thead>
<tr>
<th>Normoglycaemic state</th>
<th>Hyperglycaemic state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (ng/ml)</td>
<td>Glucose mmol/L</td>
</tr>
<tr>
<td>Control (0)</td>
<td>5.0***</td>
</tr>
<tr>
<td>Metformin (100)</td>
<td>2.9***</td>
</tr>
<tr>
<td>FSE (100)</td>
<td>&lt; 1.7 (read too low)***</td>
</tr>
<tr>
<td>4-OH-lle (100)</td>
<td>2.4***</td>
</tr>
</tbody>
</table>
Figure 1 Average concentrations (mean±SD) of blood glucose levels in the serum of mice, and oral glucose tolerance test at day 15 of experimental period; blood glucose levels were measured at 0, 30, 60, 90 and 120min time intervals following an overnight fast, following treatment with metformin (MF), fenugreek seed extract (FSE) and 4-hydroxyisoleucine (4-OH-lle), compared to the relative control (C).

Table 1 Concentration of glucose in the cell supernatant following incubation with fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), insulin and metformin for 72 hours.

<table>
<thead>
<tr>
<th></th>
<th>Normoglycaemic state</th>
<th>Hyperglycaemic state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (ng/ml)</td>
<td>Glucose mmol/L</td>
<td>Treatment (ng/ml)</td>
</tr>
<tr>
<td>Control (0)</td>
<td>5.0***</td>
<td>Control (0)</td>
</tr>
<tr>
<td>Metformin (100)</td>
<td>2.9***</td>
<td>Metformin (100)</td>
</tr>
<tr>
<td>FSE (100)</td>
<td>&lt; 1.7 (read too low)***</td>
<td>FSE (100)</td>
</tr>
<tr>
<td>4-OH-lle (100)</td>
<td>2.4***</td>
<td>4-OH-lle (100)</td>
</tr>
</tbody>
</table>
Table 2 Absolute oral glucose tolerance test values (mmol) following treatment of mice with fenugreek seed extract (FSE), 4-hydroxyisoleucine (4-OH-lle), and metformin (MF)

<table>
<thead>
<tr>
<th>Time of measurement</th>
<th>Normoglycaemic state (Glucose in mmol/L)</th>
<th>Hyperglycaemic state (Glucose in mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (untreated)</td>
<td>FSE (100mg/kg BW)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>4.80</td>
<td>4.40</td>
</tr>
<tr>
<td>30 min</td>
<td>4.90</td>
<td>4.50</td>
</tr>
<tr>
<td>60 min</td>
<td>5.00</td>
<td>4.50</td>
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<tr>
<td>90 min</td>
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<td>4.40</td>
</tr>
<tr>
<td>120 min</td>
<td>4.95</td>
<td>5.00</td>
</tr>
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</table>

Table 3 Area under the curve (AUC) values for the OGTT at day 15 of experimental period for normoglycaemic group

<table>
<thead>
<tr>
<th>Area under curve (AUC)</th>
<th>Control</th>
<th>FSE</th>
<th>MET</th>
<th>4-OH-lle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC 1 (30-0 min)</td>
<td>217.5 ± 50</td>
<td>199.5 ± 21</td>
<td>192 ± 47</td>
<td>183 ± 24</td>
</tr>
<tr>
<td>AUC 2 (60-30 min)</td>
<td>222 ± 34</td>
<td>202.5 ± 72</td>
<td>190.5 ± 24</td>
<td>187.5 ± 48</td>
</tr>
<tr>
<td>AUC 3 (90-60 min)</td>
<td>223.5 ± 61</td>
<td>201 ± 45</td>
<td>192.45 ± 13</td>
<td>185.25 ± 23</td>
</tr>
<tr>
<td>AUC 4 (120-90 min)</td>
<td>221.25 ± 19</td>
<td>207 ± 35</td>
<td>190.65 ± 175</td>
<td>184.5 ± 25</td>
</tr>
<tr>
<td>Total AUC</td>
<td>884.25 ± 124</td>
<td>810 ± 264</td>
<td>765.6 ± 119</td>
<td>740.25 ± 249</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD of 5 animals; *, #, + Values with different superscript letters within a row are significantly different from each group of animals (Tukey’s multiple range posthoc test, p < 0.05). FSE: fenugreek seed extract, MET: Metformin, 4-OH-lle: 4-hydroxyisoleucine
Table 4 Area under the curve (AUC) values for the OGTT at day 15 of experimental period for hyperglycaemic group

<table>
<thead>
<tr>
<th>Area under curve (AUC)</th>
<th>Control</th>
<th>FSE</th>
<th>MET</th>
<th>4-OH-lle</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC 1 (30-0 min)</td>
<td>639 ± 29*</td>
<td>484.5 ± 17#</td>
<td>447 ± 33*</td>
<td>423 ± 17*</td>
</tr>
<tr>
<td>AUC 2 (60-30 min)</td>
<td>666 ± 54*</td>
<td>465 ± 89#</td>
<td>438 ± 55*</td>
<td>453 ± 18*</td>
</tr>
<tr>
<td>AUC 3 (90-60 min)</td>
<td>666 ± 45*</td>
<td>448.5 ± 16#</td>
<td>433.5 ± 12+</td>
<td>445.5 ± 19+</td>
</tr>
<tr>
<td>AUC 4 (120-90 min)</td>
<td>670.5 ± 34*</td>
<td>448.5 ± 10#</td>
<td>435.75 ± 11+</td>
<td>445.5 ± 15+</td>
</tr>
<tr>
<td>Total AUC</td>
<td>2641.5 ± 163*</td>
<td>1846.5 ± 257#</td>
<td>1754.25 ± 175+</td>
<td>1767 ± 138</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD of 5 animals; *,#,+ Values with different superscript letters within a row are significantly different from each group of animals (Tukey’s multiple range posthoc test, p < 0.05). FSE: fenugreek seed extract, MET: Metformin, 4-OH-lle: 4-hydroxyisoleucine
Table 5 Blood glucose measurements (mmol/L) at day 0, 3, and 10 of the ten day induction period of the hyperglycaemic group of mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day 0 (glucose in mmol/L)</th>
<th>Day 3 (glucose in mmol/L)</th>
<th>Day 10 (glucose in mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>5.6</td>
<td>7.8</td>
<td>13.5</td>
</tr>
<tr>
<td>2</td>
<td>4.3</td>
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<td>14.4</td>
</tr>
<tr>
<td>3</td>
<td>4.2</td>
<td>6.5</td>
<td>13.9</td>
</tr>
<tr>
<td>4</td>
<td>4.1</td>
<td>6.3</td>
<td>14.0</td>
</tr>
<tr>
<td>5</td>
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<td>7.7</td>
<td>13.9</td>
</tr>
<tr>
<td>FSE</td>
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</tr>
<tr>
<td>6</td>
<td>4.3</td>
<td>6.5</td>
<td>14.2</td>
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<td>7.3</td>
<td>14.9</td>
</tr>
<tr>
<td>MF</td>
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<td>4.3</td>
<td>7.9</td>
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<td>12</td>
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<td>15.2</td>
</tr>
<tr>
<td>4-OH-Ile</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>5.1</td>
<td>9.0</td>
<td>15.3</td>
</tr>
<tr>
<td>17</td>
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<td>19</td>
<td>4.8</td>
<td>8.5</td>
<td>15.7</td>
</tr>
<tr>
<td>20</td>
<td>4.3</td>
<td>8.4</td>
<td>13.9</td>
</tr>
</tbody>
</table>

All mice were labeled 1 to 20 by an ear piecing, to ensure the same mice were treated within the same group.
Table 6 Glucose measurements of each mouse per a treatment group (control, fenugreek seed extract metformin and 4-hydroxyisoleucine) under both conditions on day 0, 3, 6, 9, 12 and 15

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day 0 (glucose in mmol/L)</th>
<th>Day 5 (glucose in mmol/L)</th>
<th>Day 10 (glucose in mmol/L)</th>
<th>Day 15 (glucose in mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoglycaemic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>5.1</td>
<td>5.0</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.8</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5.0</td>
<td>4.9</td>
<td>4.9</td>
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All mice were labelled 1 to 40 by an ear piecing, to ensure the same mice were treated within the same group. The glucose was measured with a glucometer on day 0, 5, and 10. On day 15, the glucose levels were measured by the accredited laboratory.
mentioned in the methods and materials section. *Indicates these mice were chosen for the qPCR and western blot validation (based on blood glucose values).

### Table 7 Primer sequences and annealing temperatures for qPCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Type</th>
<th>Ta (°C)</th>
<th>Primer Sequence</th>
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<tr>
<td></td>
<td>Antisense</td>
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<td>5’-AGGTACCCGAGGGCATCCGAGAAT-3’</td>
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<tr>
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<td>5’-CAAGAACTGCACGGAGGTG-3’</td>
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<td>Antisense</td>
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<td>5’-AGGTGCCAGAGTGGAGAC-3’</td>
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<td>Sense</td>
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<td>5’-CCCCGCAGATCAAACCACCACC-3’</td>
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<td>5’-AGACCCCGAGGAAGCAGACG-3’</td>
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Ta - annealing temperature
Appendix 5: Liquid chromatography–mass

Figure 1 LCMS displaying molecular weight more than 200g/mol of compounds present in the fenugreek seed
Figure 2 LCMS displaying molecular weight less than 200g/mol of compounds present in the fenugreek seed.
Figure 3 Duplication of LCMS displaying molecular weight less than 200g/mol of compounds present in the fenugreek seed
Table 1 Molecular weight per LCMS of compounds present in fenugreek seed extract

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<th>Compound present in fenugreek seed</th>
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<td>Diosgenin</td>
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<td>Inositol</td>
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<td>Vitexin</td>
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Validation of qPCR data

Chapter 3: Insulin signalling

**IRβ**

Figure 1 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of insulin receptor β (P=0.0051) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **P< 0.005 relative to control.

**IRS**

Figure 2 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of insulin receptor substrate 1 (P=0.0051) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **P< 0.005 relative to control.
Figure 3 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of glucose transporter 2 (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **P < 0.005 relative to control.

Figure 4 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of sterol regulatory element binding protein 1c (P= 0.0078) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **P < 0.005 relative to control.
Figure 5 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of protein kinase B (P=0.0051) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **P<0.005 relative to control.

GSK-3αβ

Figure 6 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of glycogen synthase kinase 3α/β (P=0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **P<0.005 relative to control.
Figure 7 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of glycogen synthase ($P=0.0059$) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *$P<0.005$ relative to control.

Figure 8 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of glucokinase ($P=0.0059$) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **$P<0.005$ relative to control.
Figure 9 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of insulin receptor β (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P< 0.005 relative to control.

Figure 10 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of insulin receptor substrate (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P< 0.005 relative to control.
Figure 11 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of glucose transporter 2 (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P< 0.005 relative to control.

SREBP1c

Figure 12 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of sterol regulatory element binding protein 1c (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P< 0.005 relative to control.

Akt
Figure 13 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of protein kinase B (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P< 0.005 relative to control.

Figure 14 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of glycogen synthase kinase-3α/β (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P< 0.005 relative to control.
Figure 15 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of glycogen synthase (P = 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P < 0.005 relative to control.

Figure 16 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of glucokinase (P = 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P < 0.005 relative to control.

Chapter 4: Lipid metabolism
Figure 17 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of proprotein convertase subtilisin/kexin type 9 \( (P=0.0059) \) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **\( P<0.005 \) relative to control.

Figure 18 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of low density lipoprotein receptor \( (P=0.0059) \) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **\( P<0.005 \) relative to control.
Figure 19 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of fatty acid synthase ($P=0.0059$) on mice liver under normo-(white) and hyperglycaemic (grey) conditions. **$P<0.005$ relative to control.

APO A1

Figure 20 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of apolipoprotein A1 ($P=0.0059$) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **$P<0.005$ relative to control.

PCSK9
Figure 21 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of proprotein convertase subtilisin/kexin type 9 (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. *P< 0.005 relative to control.

Figure 22 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of low density lipoprotein receptor (P= 0.0059) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **P< 0.005 relative to control.
Figure 23 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of fatty acid synthase \( (P=0.0059) \) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **\( P<0.005 \) relative to control.

APO A1

Figure 24 Metformin (MF) and 4-hydroxyisoleucine (4-OH-Ile) treatments relative to the untreated control on the gene expression of apolipoprotein A1 \( (P=0.0059) \) on mice liver under normo- (white) and hyperglycaemic (grey) conditions. **\( P<0.005 \) relative to control.
Appendix 7
Validation of western blot data
Chapter 3: Insulin signalling

**Figure 1** Protein expression analysis of glucose transporter 2 in metformin, 4-hydroxyisoleucine (4-OH-Ile) and fenugreek seed extract (FSE) treated mouse liver (*p*=0.0067), under normal and hyperglycaemic conditions. *P* < 0.05 relative to control, **P** < 0.05 relative to control
Figure 2 Protein expression analysis of proprotein convertase subtilisin/kexin type 9 in metformin, 4-hydroxyisoleucine (4-OH-Ile) and fenugreek seed extract (FSE) treated mouse liver \((p=0.0010)\), under normal and hyperglycaemic conditions. \(^*P<0.05\) relative to control, \(^{**}P<0.05\) relative to control.

Figure 3 Protein expression analysis of peroxisome proliferator-activated receptor gamma in metformin, 4-hydroxyisoleucine (4-OH-Ile) and fenugreek seed extract (FSE) treated mouse liver \((p=0.0048)\), under normal and hyperglycaemic conditions. \(^*P<0.05\) relative to control, \(^{**}P<0.05\) relative to control.