Effect of rutin on TNF-α-induced insulin resistance in 3T3-L1 cells

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DECLARATION

I Ndivhuwo Muvhulawa hereby declare that this thesis titled “Effect of rutin on TNF-α-induced insulin resistance in 3T3-L1 adipocytes” submitted for the MSc degree in Biochemistry to the North-West University is my work. I declare that no part of this work has been submitted for another degree to any other institution. The information obtained from literature has been acknowledged both in text and as a list of references.

DEDICATION

This thesis is dedicated to my father, Mr Ntanganedzeni Muvhulawa. Thank you for supporting me throughout my entire life. I am grateful for the love and encouragement you gave me.

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ABSTRACT

Obesity has become a growing epidemic globally and a burden to the health care system due to its association or contribution to the development of other chronic diseases. The excessive accumulation of fat in adipocytes or expansion of the adipocytes results in dysfunctional adipocytes which leads to the expression and secretion of cytokines, specifically pro-inflammatory cytokines such tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) which cause inflammation. Moreover, chronic inflammation has been linked with the development of insulin resistance which is a hallmark for type 2 diabetes. Rutin is a naturally occurring flavonoid found in various plants species with known therapeutic effects such as antioxidants, anti-diabetic and anti-inflammatory. This study aims to investigate whether rutin can ameliorate TNF-α-induced insulin resistance and inflammation in 3T3-L1 adipocytes.

In this study, 3T3-L1 preadipocytes were cultured, differentiated into mature adipocytes using cocktail media containing 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin, and rosiglitazone. Fully differentiated adipocytes were cultured in DMEM with or without 10 ng/mL TNF-α for 24h under tissue culture conditions. Thereafter, 3T3-L1 adipocytes were cultured with 1 µM insulin (added 15 minutes prior to termination), while 1 µM CL 316,243, and 10 µM rutin were kept for 6h. Cell viability was assessed using MTT assay. Lipid accumulation and lipolysis were evaluated using Oil Red O and glycerol release assay kit, respectively. Inflammation was evaluated using ELISA. Quantitative real-time polymerase chain reaction (qPCR) and Western blot were used to investigate mRNA and protein expression associated with inflammation, lipid metabolism, fat browning, and glucose metabolism.

MTT assay showed that apart from insulin which improved cell viability, culturing cells with TNF-α, CL 316,243, and rutin had no significant effect after 6h of treatment. A significant decrease in lipid accumulation by isoproterenol and TNF-α, and an increase by insulin, CL 316,243, and rutin after culturing with TNF-α for 24h was observed. There was an increase in lipolysis by CL 316,243, isoproterenol and TNF-α. Interestingly, all the treatments were able to supress TNF-α mediated lipolysis and rutin was most significant. TNF-α induced insulin resistance and inflammation after 24h, this was evident as pro-inflammatory markers TNF-α and IL-6 were increased and AKT phosphorylation was reduced. Also, positive controls insulin and CL 316,243, and rutin were able to abolish TNF-α- induced insulin resistance and inflammation. Furthermore, results showed that TNF-α affected glucose metabolism by blocking AKT phosphorylation. However, insulin and rutin reversed this effect on AKT. Subsequently, to elucidate the mechanism by which rutin could exert its beneficial effect, we investigated gene and protein markers associated with fat browning. Our results showed that fat browning markers PR-domain

containing 16 (PRDM16) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α) were decreased by TNF-α. All treatments were able to upregulate PGC1-α protein expression and only insulin and rutin restore *Prdm16* expression. In terms of lipid metabolism, culturing 3T3-L1 adipocytes with *Tnf-α* greatly reduced peroxisome proliferator-activated receptor (*Pparγ*), and carnitine palmitoyl transferase I (*Cpt1*) gene expression. Treating with insulin, CL 316,243, and rutin reversed the inhibition of *Pparγ*. Only CL316,243 and rutin were able to upregulate *Cpt1* gene expression. Lastly, rutin improved mitochondrial energy expenditure comparable to thepositive control CL 316,243 by increasing ATP production and mitochondrial spare capacity. Results demonstrated that rutin is a potential therapeutic compound for the treatment of TNF-α-induced inflammation and insulin resistance through inhibiting the expression of inflammatory cytokines and activating the insulin mediated insulin signaling pathway by activating AKT. Moreover, we observed that rutin upregulated *Prdm16* and PGC1-α which are markers found in brown fat, suggesting rutin induces white adipocyte browning and protected against TNF-α mitochondrial dysfunction. This could be a possible mechanism through which rutin ameliorates obesity-induced inflammation and associated disorders. Additional experiments, especially in vivo are required to confirm the role of AMPK in rutin response against inflammation.

LIST OF ABBREVIATIONS

AKT:	Protein kinase B
AMPK:	AMP-activated protein kinase
BAT:	Brown adipose tissue
CL 316,243:	5-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid
CPT1:	Carnitine palmitoyltransferase 1
C/EBP:	CCAAT-enhancer-binding proteins
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	Dimethyl sulfoxide
ERK:	Extracellular signal-regulated kinase
FBS:	Fetal bovine serum
GLUT4:	Glucose transporter type 4
IBMX:	3-isobutyl-1-methylxanthine
IFN-γ:	Interferon gamma
IL:	Interleukin-6
MAPK:	Mitogen-activated protein kinase
NF-κB:	Nuclear factor kappa B
ORO:	Oil Red O
PBS:	Phosphate-buffered saline
PGC1-α :	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPAR:	Peroxisome proliferator-activated receptor
PRDM16:	PR domain containing 16
SIRT1:	Sirtuin 1
T2D:	Type 2 diabetes
TBST:	Tris-buffered saline and tween20
TNF-α:	Tumor necrosis factor-alpha
UCP1:	Uncoupling protein 1

WAT:	White adipose tissue
WHO:	World health organization

OUTPUTS

Publications

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CHAPTER 1

This chapter gives a brief description of the current project including the hypothesis, aim and objectives of the study.

INTRODUCTION

The prevalence of obesity is on the rise has doubled in over 70 countries and steadily increased in most other countries since 1980 [1]. According to WHO,2016, it was estimated that 1.9 billion (39%) of the adult population are overweight of which more than 650 million adults were obese in 2016 [2]. In South Africa, 68% (41% obese and 27% overweight) of women and 31% (11% obese and 20% overweight) of men are overweight or obese, with severe obesity being common among the wealthier communities and mostly affecting women in urban areas than men [3]. Obesity is a growing health burden due to its role in the development of noncommunicable diseases such as cardiovascular disease, some cancers, insulin resistance, type 2 diabetes, non-alcoholic fatty liver disease, and dyslipidemia [4].

There's a global increase in the production and availability of non-nutritional processed foods. Overconsumption of energy-dense food coupled with a sedentary lifestyle result in excess energy in the body causing an imbalance between energy intake and expenditure which leads to increased growth of adipose tissue and this has been linked with insulin resistance, metabolic disorders, and obesity [5]. To manage excess energy, adipocytes either increase adipose tissue mass through hypertrophy (increase in cell mass) or hyperplasia (increase in cell number) [6]. The excess energy is stored in adipocytes as triglycerides and released during times of energy demand. These triglycerides are released as free fatty acids through a process known as lipolysis, however, the increased release of free fatty acids is linked with the development of metabolic complications such as insulin resistance [7]. Insulin resistance, a hallmark for type 2 diabetes (T2D) is said to develop in obesity mainly as a response to an increase in insulin stimulated excess energy storage in adipocytes [8]. Furthermore, elicitation of endoplasmic reticulum stress, aging of adipose tissue, hypoxia, genetic changes, oxidative stress, mitochondrial dysfunction, and inflammation are mechanisms involved in obesity-related insulin resistance [9].

Adipose tissue is an endocrine organ which releases hormones, chemokines, and cytokines that are important for regulating several functions in the body [7, 10]. The over expansion of adipocyte mass consequently results in dysfunctional adipocytes which is accompanied by the release of pro-inflammatory cytokines [8]. These pro-inflammatory cytokines such as MCP1, IL-1β, IL-6 and TNF-α initiate an inflammatory response. Interestingly, TNF-α has been implicated in the development of insulin resistance [11]. Moreover, in obese state, macrophages are recruited which exacerbate the release of pro-inflammatory cytokines and this imbalance among anti-

inflammatory and pro-inflammatory cytokines initiates low grade systemic inflammation, impairment of insulin sensitivity and dysregulation of lipid metabolism [7]. The link between obesity induced inflammation and various obesity-linked metabolic disorders makes targeting inflammation and inflammatory signaling pathways potential targets of treatment [8].

Polyphenols are secondary metabolites found abundantly in plants having several biological activities. Over 8000 varieties of polyphenols have been identified [12]. Flavonoids are the most common of the polyphenols and grouped into flavones, flavonols, flavanones, flavanols, isoflavones, anthocyanins, and proanthocyanidins [13]. These polyphenols have been shown to activate brown adipose tissue and induce the browning of white adipose tissue [14]. Brown adipose tissue plays a role in thermogenesis, making it a potential therapeutic target for combating human obesity and related metabolic complications. Polyphenols such as Resveratrol and Isoorientin were found to reduce lipid accumulation and promote insulin sensitivity through the activation of brown adipose tissue and by increasing the expression of thermogenic markers or markers associated with brown adipose tissue such as UCP1, PGC1-α , PPARγ, PPARα, and PRDM16 in white adipose tissue [14-16]. This activation or shift in physiology favours energy expenditure, offering a potential protective mechanism against obesity and obesity-related complications.

In the present study, rutin is of particular interest. Rutin is one such flavonoid with a growing body of research due to increased interest in its beneficial effects. Rutin is abundantly found in various food sources including blackberry, coriander, asparagus, buckwheat [17] and rooibos [18]. It has a wide range of pharmacological properties such as antioxidant, anti-diabetic, anti-obesity, and anti-inflammatory [19]. In a study conducted by Ma et al., in 2021 [20], it was shown that this flavonoid could activate brown fat and white adipose tissue browning both in vitro and in vivo in part through the activation of the CaMKKβ/AMPK pathway. A study by Yuan, 2017 [21] reported that rutin could mechanistically directly bind to SIRT1 resulting in the deacetylation of PGC1-α , activation of Tfam, subsequently leading to increased mitochondrial biogenesis and thermogenesis, ameliorating obesity and improving glucose homeostasis in both genetical and diet-induced obese mice. However, it is not known whether rutin can ameliorate insulin resistance, inflammation, and mitochondrial dysfunction mediated by TNF-α in obesity in vitro model.

Aim of the study

To investigate the ameliorative effects of rutin on TNF-α-induced insulin resistance and inflammation in 3T3-L1 adipocytes

Objectives

- To induce insulin resistance and inflammation in 3T3-L1 adipocytes
- To determine effect of rutin on glucose and lipid metabolism
- To investigate the molecular mechanism associated with fat browning
- To investigate the effect of rutin on mitochondrial activity

Basic hypothesis

Rutin can ameliorate TNF-α-induced insulin resistance, inflammation and induce fat browning in 3T3-L1 adipocytes

CHAPTER 2

This chapter reviews available scientific literature focused on rutin. It describes rutins pharmacokinetics and potential techniques to improve its solubility and bioavailability. Moreover, it summarises findings on rutin impact in metabolic diseases through targeting inflammation. This chapter also discusses some of the dietary plants in which rutin is found and their anti-inflammatory activity. The references for this review are included within the chapter and the references for the remaining chapters are compiled in chapter 6. This section of literature review has already been published and is available online: https://doi.org/10.1016/j.phrs.2022.106163

LITERATURE REVIEW

Literature review

Rutin ameliorates inflammation and improves metabolic function: A comprehensive analysis of scientific literature

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Abstract

Chronic inflammation remains an essential complication in the pathogenesis and aggravation of metabolic diseases. There is a growing interest in the use of medicinal plants or food-derived bioactive compounds for their antioxidant and anti-inflammatory properties to improve metabolic function. For example, rutin, a flavonol derivative of quercetin that is found in several medicinal plants and food sources has displayed therapeutic benefits against diverse metabolic diseases. Here, we searched the major electronic databases such as PubMed/MEDLINE, Scopus and Google Scholar to systematically extract and critically discuss evidence reporting on the impact of rutin against metabolic diseases by attenuating inflammation. In fact, available preclinical evidence suggests that rutin, through its strong antioxidant properties, can effectively ameliorate inflammation by reducing the levels of pro-inflammatory markers such as tumor necrosis factor-α, interleukin (IL)-6, cyclooxygenase-2, IL-1β, as well as blocking nuclear factor kappa B (NF-κB)/ mitogen-activated protein kinase (MAPK) activation to improve metabolic function. Notably, although clinical data on the impact of rutin on inflammation is limited, food-derived sources rich in this flavonol such as *Fagopyrum tataricum*, *Coffea arabica* and *Aspalathus linearis* (rooibos) have shown promise in improving metabolic function, in part by reducing markers of oxidative stress and inflammation. However, additional studies are still required to confirm the therapeutic properties of rutin in a clinical setting, including the enhancement of it low bioavailability profile.

Keywords:

Metabolic diseases; cardiovascular diseases; inflammation; therapeutic targets; flavonoids; rutin.

2.1 Introduction

Metabolic processes maintain life through the interconnection of various biochemical pathways that contribute towards balanced energy regulation [1]. Importantly, metabolic processes regulate optimal cellular functions and their dysregulation leads to metabolic dysfunction [2]. Some common metabolic disorders include visceral obesity, hypertension, dyslipidemia, insulin resistance, and an unregulated pro-inflammatory state [3]. These factors contribute to the development and exacerbation of metabolic diseases such as type 2 diabetes (T2D), nonalcoholic fatty liver disease (NAFLD), and cardiovascular diseases (CVDs) [4]. In fact, chronic inflammation is the apex of metabolic disease [5]. Accumulative research has proven this phenomenon can be a potential target in drug development to alleviate metabolic dysregulations and improve metabolic function [6-8]. Generally, inflammation is part of the innate and adaptive immune response, which is essential to fend-off infections and internal body injuries [9]. Currently, treatments used to dampen the pro-inflammatory response include non-steroidal anti-inflammatory drugs, glucocorticoids, hydroxychloroquine, immunosuppressants, and inflammatory cytokines-antagonists [10-12]. However, some of these medications have adverse effects and may lead to the progression of other diseases [13]. For example, a previous study revealed that the use of non-steroidal anti-inflammatory drugs may potentially increase the risk of kidney diseases in patients with T2D [14]. Most importantly, the increasing rate of metabolic disease-related deaths [15] warrants investigation into alternative effective therapies to prevent or protect against the development of such complications. Thus, a growing body of research is focusing on plant-based diet and medicinal plants, including their bioactive compounds to find an alternative treatment for inflammation and protect against metabolic diseases [16, 17]. Hence, the screening of bioactive compounds with strong anti-inflammatory properties is emerging as a vital area of research [17, 18].

Our group has previously focused in understanding the therapeutic properties of medicinal plants, including food-derived bioactive compounds for their ameliorative properties against metabolic complications. For instance, we have actively reviewed evidence informing on the molecular mechanisms related to the ameliorative properties of tea (*Camellia Sinensis*) or rooibos (*Aspalathus linearis*)-derived bioactive compounds like gallic acid and isoorientin against inflammation in metabolic diseases [19-21]. Furthermore, plant-derived bioactive compounds with robust antioxidant properties like quercetin or its derivative flavonol rutin are increasingly studied for their ameliorative effects against inflammation in various experimental models of metabolic diseases [22, 23]. In a study conducted by Khan et al. [24], rutin was shown to display hepatoprotective effects against carbon tetrachloride (CCl4)-induced hepatoxicity by enhancing endogenous liver antioxidant enzymes in rats. Such beneficial effects could be attributed to the capacity of rutin to enhance intracellular antioxidant levels of glutathione, glutathione peroxidase, glutathione S-transferase, glutathione reductase, superoxide dismutase and catalase [25, 26]. Furthermore, rutin has been studied and reviewed for its therapeutic effects in other biological settings, such as its beneficial effects against diabetes-related complications [27, 28], neurological diseases [29-31], cancer [32, 33], or

its broad pharmacological properties [22, 34]. However, none have critically discussed evidence on rutin and its effects on inflammation within a state of metabolic disease.

In the current systematic review, we identified relevant studies, reporting on the impact of rutin, the flavonol derivative of quercetin, on modulating inflammation in preclinical studies [35]. A conversation on the impact of plant-based foods containing this flavonoid is also included to underscore the therapeutic effects of rutin. Importantly, the current review also gives a brief summary on the bioavailability profile of rutin and mechanisms that are currently explored to enhance its solubility and bioavailability.

2.2 Methodology for literature search and study inclusion

The search for relevant articles to be included in the current review was conducted using major electronic databases such as PubMed/MEDLINE and Scopus, this was adapted to other relevant search engines including Google Scholar. The literature search was independently conducted by at least two authors, from inception until end of December 2021. The key search terms included “rutin”, “inflammation”, and “metabolic diseases”, and synonyms. There were no Language or date restrictions applied and EndNote version 10 (Clarivate Analytics, Philadelphia, PA, USA) was used to identify and remove duplicates. In this review, both in vitro and in vivo studies focusing on the impact of rutin on inflammation in metabolic disease were included. The presented evidence is discussed based on the experimental model of metabolic disease, effective doses, and duration of rutin, and its overall impact on inflammatory processes.

2.3 An overview of rutin, including its chemical and biological properties

Figure 1 displays a chemical structure of rutin (PubChem CID: 5280805), a flavanol glycoside with international union of pure and applied chemistry (IUPAC) name 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one, a molecular formula $C_{27}H_{30}O_{16}$, and a molecular weight of 610.51 g/mol [36, 37]. Briefly, rutin is widely distributed in plants that form most part of human diet such as *Coffea arabica*, *Schisandra chinensis*, and *Fagopyrum tataricum* (buckwheat) and rooibos. In fact, flavonoids like rutin have a great deal of attention in medical research due to their antioxidant [38], anti-inflammatory [39], antidiabetic [27], antibacterial [40], and antiviral properties [41]. Rutin is a hydrophobic compound having poor water solubility and bioavailability, hence the use of this compound in clinical settings is limited [42, 43]. Rutin is known to have low solubility in water of 0.125 g/L, while highly soluble in polar solvents such as methanol (55 g/L), ethanol (5.5 g/L), pyridine (37.3 g/L), and dimethyl sulfoxide (100 g/L) [44]. Other applicable solvents include dichloromethane, dimethylformamide, glycerine, and ethyl acetate [45]. In addition, rutin is normally dissolvable at room temperature, however, its solubility improves with increasing temperature [46].

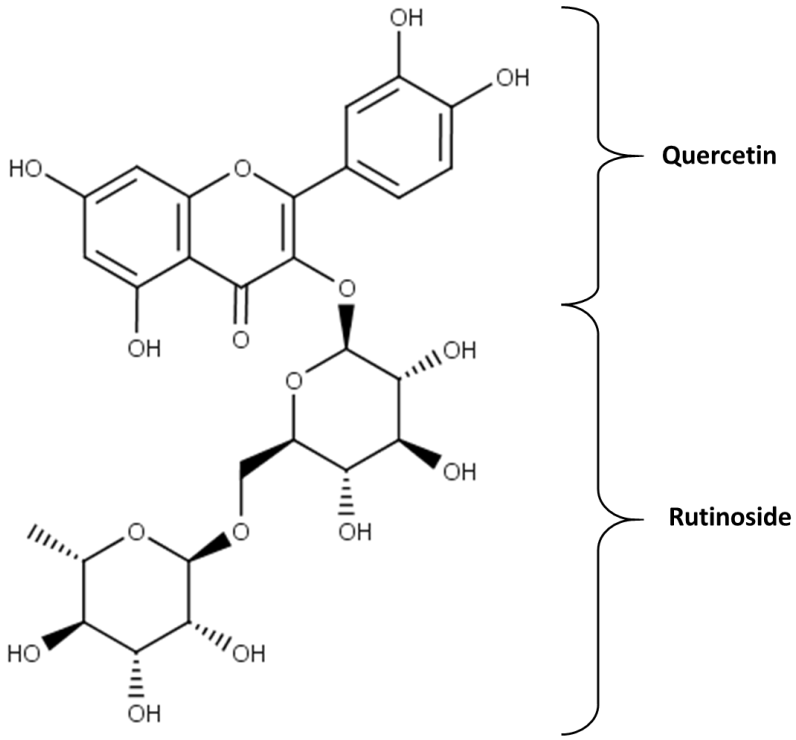

Figure 1: An overview of the chemical structure of rutin and its derivative, quercetin.

2.4 Pharmacokinetics profile of rutin

2.4.1. Absorption, bioavailability, and metabolism of rutin

Generally, the process of pharmacokinetics remains a critical component of determining the time course of drug absorption, distribution, metabolism, and elimination. This allows for proper prediction of the drug bioavailability, which is an amount of administered dose expected to enter the bloodstream and reach the site of action. The pharmacological profiling of bioactive compounds has a long application in preclinical and clinical research for the rational selection of dosing regimens [47]. The pharmacokinetics of rutin has long been studied in humans [48]. For instance, it was found that oral administration of rutin produces several metabolites including quercetin, isoquercetin and other phenol derivatives such as 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), 3-hydroxyphenylacetic acid (3-HPAA), and homovanillic acid (HVA) [48, 49]. As displayed in Figure 2, rutin can be hydrolyzed by intestinal enzymes like UDP-glucuronyl transferase (UGT) to form rutin sulfates and rutin glucuronides [50-52], whilst the conversion of rutin to quercetin is mostly done by acid hydrolysis catalyzed by α-rhamnosidase and β-glucosidase enzymes from gut microflora [53]. For this reason, rutin is absorbed more slowly than quercetin in the intestines.

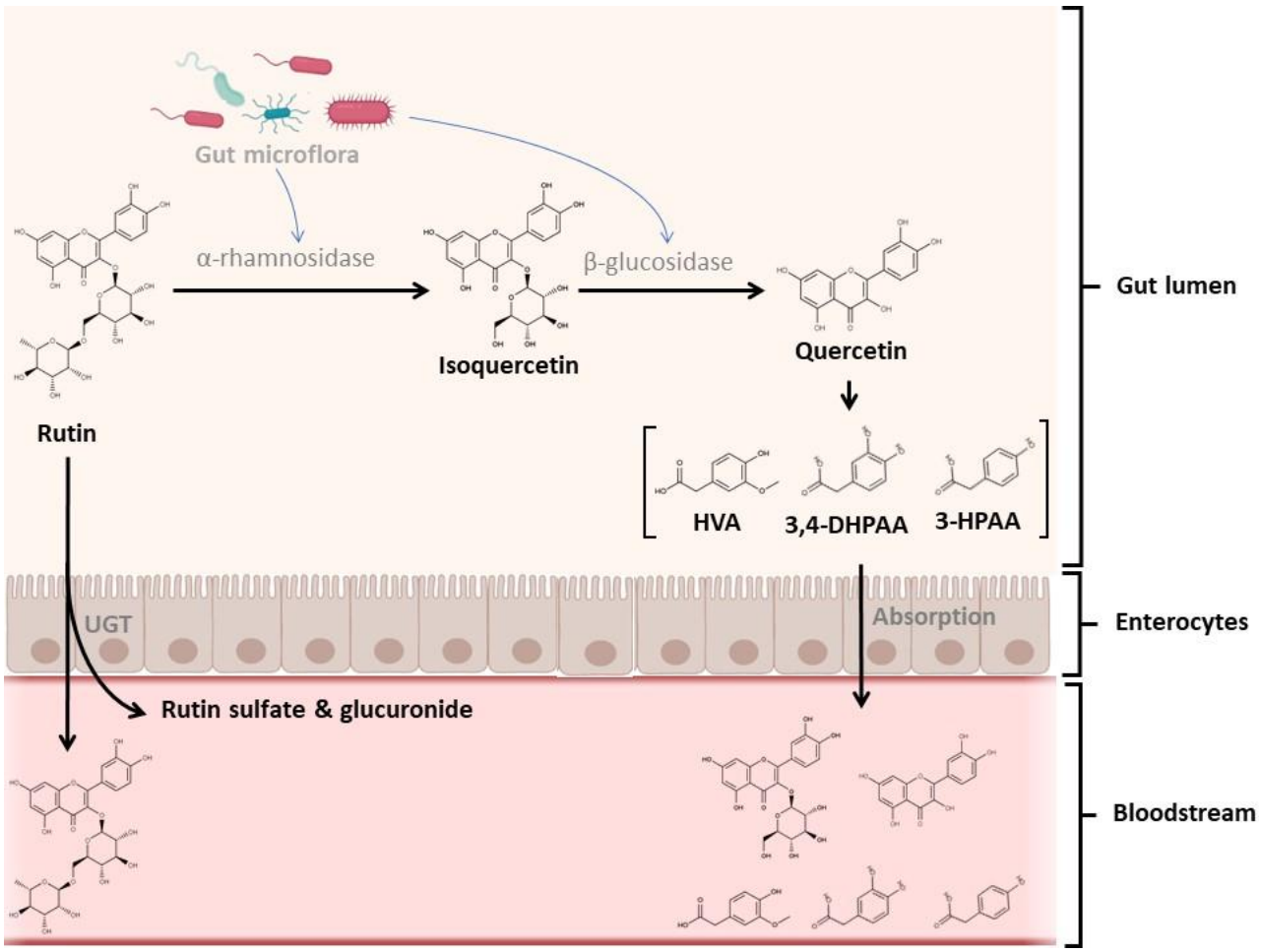

Figure 2: A brief overview of mechanisms affecting the absorption and metabolism of rutin in small intestines. Briefly, rutin can be hydrolyzed by intestinal enzymes like UDP-glucuronyl transferase (UGT) to form rutin sulfate and rutin glucuronide, and the conversion of rutin to quercetin is mostly done by acid hydrolysis catalyzed by α-rhamnosidase and β-glucosidase enzymes from gut microflora. For this reason, rutin is absorbed more slowly than quercetin in the intestines. Abbreviations: 3,4-DHPAA: 3,4-dihydroxyphenylacetic acid; 3-HPAA: 3-hydroxyphenylacetic acid; HVA: homovanillic acid.

In fact, some factors are associated with poor absorption of flavonoids and these include molecular weight, metabolic conversion, glycosylation, and the interaction with gut microflora [54]. An in vitro study showed that rutin was slowly absorbed and hydrolyzed by cecal microbiota to its derivative, quercetin [55]. In another study, it was found that rutin was absorbed in the small intestines of rats at a very slow rate [56]. Furthermore, rutin can bind to and pass through the small intestines which was suggested as a possible explanation for the slow absorption or limitation to its absorption [56]. Though the low water solubility of the flavonoids usually prevents toxicity, this translates to their limited use in medicine [57]. Moreover, the structure of the flavonoid determines the site of absorption (small intestines or colon), with aglycones being readily absorbed in the intestines and glycosides being converted to aglycones [58]. Interestingly, the

solubility of rutin is different to that of its aglycone quercetin due to the presence of the sugar moiety [59], which may also limit its absorption in the small intestines [60].

Besides its low absorption rate, rutin has low bioavailability. A clinical study showed that rutin, at a dose of 500 mg was hydrolyzed to quercetin maximally at 6-7 hours, however, it was completely cleared within 24 hours [61]. Whereas varying quercetin plasma concentrations among individuals may range from 40-220 ng/ml [61]. The low bioavailability is also attributed to the poor stability and limited membrane permeability of rutin which then potentially restricts its use as an oral drug in clinical settings [62]. Since humans and animals cannot synthesize flavonoids like rutin, their dietary intake and absorption has become an important aspect to determine and promote, especially due to their envisaged health properties [63]. Due to various factors, it remains difficult to determine the daily flavonoid intake on a global scale, however, it was estimated to be 400 mg/day [64]. With regards to rutin, there is no specific recommended daily intake, however, studies have used rutin at doses of 500 mg/day. For example, in one study, healthy individuals were given rutin daily for 6 weeks, at a dose of 500 mg in order to assess its bioavailability and antioxidant activity [61]. This dose when combined with other supplements did not exhibit any toxic effects [65]. Hence indicating that improving the bioavailability could yield better therapeutic effects. However, this aspect remains to be evaluated and confirmed in future clinical trials.

2.4.2. Strategies to improve bioavailability of rutin

There are several strategies that have been explored to improve the solubility or the bioavailability profile of rutin. The implicated techniques have been well documented [44]. Briefly, different formulations, including cyclodextrin complex, enzymatic oligomerization, and nanoparticulate systems have been used to enhance the solubility and bioavailability of rutin [66-68]. This evidence suggests that application of nanocrystal formulations or encapsulations for oral delivery, along with reducing drug particle size, can enhance the solubility and dissolution rate, which allows the capsules to be easily absorbed by the small intestines resulting in increased bioavailability at the site of action. Notably, it has been shown that nanocrystal-loaded tablets can potentially enhance the solubility and bioavailability profile of rutin [69]. Similarly, a non-aqueous self-double emulsifying carrier system can enhance the stability and dissociation rate of rutin in both gastric and intestinal fluid [70]. Also, it was evident that cyclodextrin complex formulations of rutin, had greater solubility and permeated through the gut membrane 2-fold to that of normal rutin [71]. Apparently, literature has explored the use of natural deep eutectic solvents, through their advantages of biodegradability and biocompatibility, which could potential be used to enhance the bioavailability profile of natural products with low solubility like rutin [72, 73]. In fact, a combination of proline and glutamic acid could solubilize rutin more than methanol and ethanol solvents [74]. Proline-glutamic acid solvent drastically increased rutin solubility from 120.0 µg/ml in water to 2938.4 µg/ml in the solvent, interestingly, oral administration showed great improvement in bioavailability evident from increased plasma concentration compared to rutin suspension in water [74]. Previously, Ravi et al., [25] showed that a nano-lipid complex of rutin had greater aqueous

solubility, and increased serum concentration compared to pure rutin. Notably, not only do these formulations potentially increase the solubility and bioavailability of rutin but may also enhance its therapeutic potential [67]. For example, it has been previously reported that a rutin-phospholipid complex could enhance its permeability through the membrane of the skin, leading to its protection against carrageenan paw edema inflammation in rats [75]. Nonetheless, emerging research has indicated that food derived compounds like rutin may target prominent mechanisms involved in disease development, like ameliorating an undesired pro-inflammatory response to improve metabolic function [76, 77].

2.5 The impact of rutin on inflammation in experimental models of metabolic disease

Through the use of various experimental models, it has increasingly apparent that augmented secretion and circulation of inflammatory markers such as interleukin (IL)-6, tumor necrosis factor alpha (TNF-α), and IL-1β are associated with the development and aggravation of metabolic complications [78, 79]. This includes the toll-like receptor 4 (TLR4), as well as the myeloid differentiation factor 88 (MyD88)/ nuclear factor kappa B (NF-κB) signaling pathway that can promote the activation of cytokines such as TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1) to aggravate inflammation, subsequently causing damage to metabolically active tissues [79, 80]. Literature already indicates that elevated levels of TNF-α, IL-6, and MCP-1, mainly mediated by the actions of NF-κB, are the major pro-inflammatory markers increasingly investigated for their role in the development of insulin resistance and the progression of T2D [81-84]. Moreover, TNF-α concentrations have been found to be high in the serum of patients with T2D, consistent with the development of diabetes-related complications like nephropathy [85]. In addition, in vitro evidence suggests that pro-inflammatory cytokines like TNF-α and IL-6 are implicated in the development of coronary endothelial dysfunction through the upregulation of reactive oxygen species [86]. Thus, there is a general interest in understanding how different therapeutic interventions affect the levels of pro-inflammatory markers in relation to improved metabolic function [87]. As such, Figure 3 gives an overview of various inflammatory pathways implicated in the pathogenesis of metabolic diseases. While Table 1 gives an overview of in vitro studies reporting on the effects of rutin on modulating different pro-inflammatory markers in experimental models of metabolic disease. Here, evidence supports the ameliorative effects of rutin against inflammation by suppressing the levels of TNF-α, nitric oxide, interferon gamma (IFNγ), IL-1β, IL-6, and MCP-1 in RAW 264.7 and murine macrophage cells, exposed to palmitate or lipopolysaccharides (LPS) [88-91].

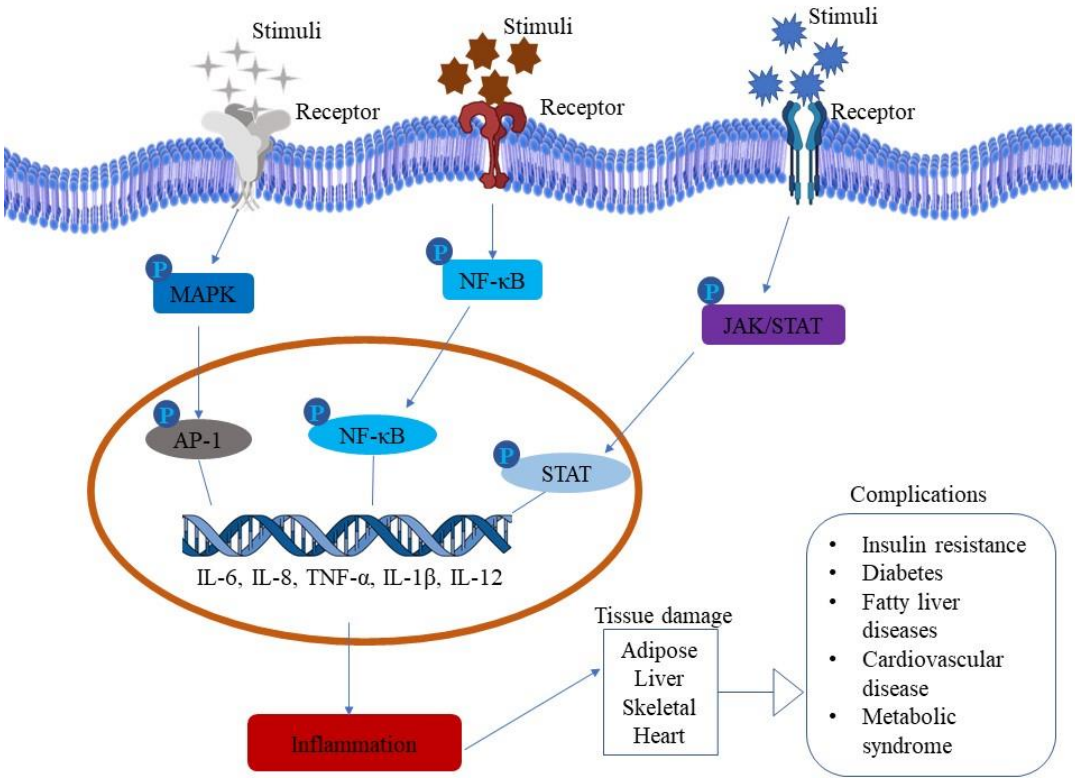

Figure 3: Implication of inflammation in the pathogenesis of metabolic diseases. Different stimuli bind to their respective receptors and initiate a signaling cascade leading to phosphorylation and activation of different inflammatory pathways that eventually lead to the transcription of inflammatory cytokines in the nucleus which then cause inflammation. The distribution of pro-inflammatory cytokines in different tissues results in tissue damage and dysfunctional organs, consequently progressing to the development of metabolic diseases. Abbreviations: AP-1: activator protein-1; JAK/STAT: Janus kinase-signal transducer activator of transcription; IL: interleukin; MAPK: mitogen activated protein kinase; NF-κB: nuclear factor kappa B; TNF-α: tumor necrosis factor alpha.

The biological properties of rutin in ameliorating an undesired pro-inflammatory response have been detected in other cellular models, besides the RAW 264.7 cells. Much of this evidence indicates that this flavonoid can reduce the levels of IL-6, TNF-α, inducible nitric oxide synthase (iNOS) and downregulate NF-κB in LPS-exposed skeletal muscle (C2C12) cells [92]. This is consistent with attenuating inflammation by reducing the expression levels of purinergic P2X7 ionotropic receptor (P2X7r), NLR family pyrin domain containing 3 (NLRP3), IL-1α, and protecting against fibrosis in HSC-T6 cells or primary rat hepatocytes [93]. These results are of interest since it has already been established that NLRP3 is involved in acute inflammation or inflammasomes-related responses, which are subject to enhanced therapeutic targeting to protect against inflammation-mediated conditions [94]. These findings further indicate that perhaps rutin may have a major role in protecting against NAFLD, through the modulation of inflammatory response, as

reported in in vitro models of liver disease [95]. Nonetheless, summarized in vitro evidence support that rutin can modulate diverse mechanisms of inflammation, ranging from reducing the levels of inhibitory kappa B kinase (IKK)α/β, I-κBα, TNF-α, IL-6, and NF-κB in experimental models mimicking metabolic disease.

To complement in vitro evidence, Table 2 summarizes the in vivo information on the therapeutic potential of rutin against inflammation in a state of metabolic disease. Of these experiments, it is evident that most models are focused on diabetes, obesity, and liver disease, which may be induced through common stressors such as with streptozotocin (STZ), high fat diet (HFD), CCL4, and ethanol (EtOH) [96-98]. Interestingly, these conditions are associated with an increased inflammatory state [99, 100]. From the current review, it was evident that indeed inflammation was prominent in a state of metabolic disease (Table 2). However, supplementation with rutin at a dose, ranging from 5 mg/kg to 200 mg/kg, from a period as little as 4 h to 24 weeks, could reverse the effects caused by these stressors and ameliorate inflammation (Table 2). Mechanistically, this flavonoid showed an enhanced capacity to block the expression of pro-inflammatory markers in adipose tissue or reduce their levels in serum, including TNF-α, MCP-1, and IFNγ, IL-6, IL-2, NF-κB, MyD88, and lipopolysaccharide binding protein (LBP), while enhancing antioxidant responses through the upregulation of nuclear factor erythroid 2-related factor 2 (NRF2) and heme oxygenase-1 (HO-1) [89, 101-106]. Beyond these mechanisms, it is evident that rutin can exert its ameliorative effects against inflammation in vivo through blocking various pathways such as Janus kinase (JAK), signal transducer and activator of transcription (STAT)3, and mitogen-activated protein kinase (MAPK) (Table 2).

2.6 Anti-inflammatory properties of the major dietary plant source of rutin

Rutin, the flavonol derivative of quercetin, is highly abundant in natural sources, particularly plant species that are widely used in traditional medicine for their anti-inflammatory properties. For example, in a study conducted by [107], on commercially available fresh produce, rutin was found in a number of dietary fruits and vegetables, including apples, tangerines, watermelon, bean sprout, mustard, and broccoli [107]. Hence, it is an important dietary constituent of food and plant-based beverages. Moreover, rutin has been identified in many foods derived plant-sources, such as buckwheat, and herbal teas like rooibos with strong anti-inflammatory properties [108]. Relevant to the current review, the below sections briefly give an overview of these rutin containing plant sources, and also their potential properties in ameliorating inflammation in conditions of metabolic disease.

Table 1: An overview of *in vitro* studies reporting on the effect of rutin on inflammation.

Author, year	Study design		Main findings
	Experimental model	**Rutin dosage and duration**	
Wu et al., 2009 [164]	High glucose-induced pro-inflammatory cytokines secretion in THP-1 cells	20 μM for 72 h	Attenuated high glucose (hyperglycemia)-induced inflammation by modulating the expression of TNF-α, IL-6, COX-2, IkBα, NF-κB and ERK1/2
Xu et al., 2012 [165]	LPS-treated RAW 264.7 cells	10 and 50 μM for 24 h	Counteracted LPS induced inflammatory response by lowering COX-2 expression and reducing PGE_2 levels in a dose dependent manner
Gao et al., 2013 [89]	Palmitic acid-treated RAW 264.7	100, 200, and 400 μM for 16h	Blocked the expression of inflammatory markers IFNγ, IL-1β, IL-6, MCP-1 and reduced concentration of TNF-α
Agbo et al., 2014 [88]	LPS-induced RAW 264.7 cells	5, 25, 100 μM for 2 h	Rutin pretreatment exhibited anti-inflammatory activity by suppressing the production of nitric oxide (NO) and inhibiting the expression of pro-inflammatory cytokine TNF-α
Choi et al., 2015 [166]	LPS-IFNγ -treated RAW 264.7 cells	2.5, 5, 10 μg/ml for 24 h	Pretreatment with rutin attenuated inflammatory response by reducing NO production and cytokine expression of IL-6, NF-κB, COX-2, and iNOS
Saraphanchotiwitthaya and Sripalakit, 2015 [167]	LPS-stimulated RAW 264.7 macrophages	12.21, 24.42, 48.84 μg/ml	Attenuated LPS associated inflammation by reducing the concentration levels of IL-1β and reduced NO
Liu et al., 2019 [92]	LPS-C2C12 cell line	10, 25,50 and100 μM for 6 h	Reduced LPS-induced inflammation in muscle cells by downregulating expression of IL-6, TNF-α and iNOS possibly through downregulating the inflammatory pathway of NF-κB
Liu et al., 2019 [90]	LPS-treated RAW 246.7	20 and 40 μM for 2 h	Attenuated fat induced inflammation and liver autophagy significantly through decreasing TNF-α and IL-1β concentrations
	Oleic acid treated HepG2		Rutin protected against nonalcoholic liver damage by reducing COX-2 generation caused by hyperlipidemia due to oleic acid overload
Ganeshpurkar and Saluja, 2020[168]	LPS-treated macrophages from Wistar rats	1, 10, 100 μM for 6 h	Hindered macrophage function by lowering TNF-α and IL-1β in a dose dependent manner, moreover, it significantly reduced IL-6 and NO levels
Lee et al., 2020 [169]	LPS-induced RAW 264.7	50 μM for 24 h	Rutin inhibited the generation of NO induced by LPS and downregulated the protein expression of iNOS and COX-2 while also downregulating IKKα/β, IκBα, and NF-κB p65
Hou et al., 2020 [93]	HSC-T6 cells	10, 20, 30, 40,50 μM for 6 h	Protected against hepatic fibrosis and reduced inflammation through inhibiting caspase-1, IL-1β, IL-1α, IL-18, IL-6, and TNF-α expression, potentially through P2X7r
	Primary hepatocytes from Sprague-Dawley rats	40 and 50 μM for 6 h	Rutin reduced the expressions of P2X7r, NLRP3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain, and IL-1α protecting against fibrosis and reduced TLR4, CD14, MyD88, IRAK1, IRAK4, TRAF-1, and IRF-3

Abbreviations: CD14: cluster of differentiation 1; COX-2: cyclooxygenase; ERK1/2: extracellular signal-regulated kinase; IkBα: nuclear factor-kappa-B inhibitor alpha; IFNγ: interferon gamma; IL: interleukin; iNOS: nitric oxide synthase; IRAK: interleukin-1 receptor-associated kinase; IRF-3: interferon regulatory transcription factor-3; LPS: lipopolysaccharides; MCP-1: monocyte chemoattractant protein; MYD88: myeloid differentiation primary response 88; NF-κB: nuclear factor kappa B; NLRP3: NLR Family Pyrin Domain Containing 3; NO: nitric oxide; P2X7r: P2X purinoceptor 7; PGE_2: prostaglandin E_2; TLR4: toll-like receptor 4; TNF-α: tumor necrosis factor alpha; TRAF-1: TNF receptor-associated factor 1.

Table 2: A summary of *in vivo* studies on the effect of rutin on inflammation.

Author, year	Study design		Main findings
	Experimental model	**Rutin dosage and duration**	
Alsaif et al., 2009 [170]	STZ-induced diabetic Wistar rats	100 mg/kg for 5 weeks	Decreased pro-inflammatory cytokines TNF-α and IL-6 in serum of diabetic rats
Challa et al., 2009 [105]	STZ-induced type 1 diabetes Albino Wistar rats with ischemia reperfusion injury	5 and 10 mg/kg for 4 h	Ameliorated the inflammatory response by reducing myeloperoxidase and serum IL-12 levels in myocardial ischemia reperfusion injury and diabetic subject
Domitrović et al., 2012 [171]	Carbon tetrachloride-induced hepatotoxicity BALB/c mice	10, 50, and 150 mg/kg for 5 days	Displayed a strong hepatoprotective effect by reducing plasma transaminases and improving acute liver damage and attenuating the inflammation through down-regulation of NF-κB, TNF-α and COX-2 and increasing HO-1, reduced iNOS
Hu et al., 2012 [172]	Fructose-fed Sprague-Dawley rats	50 and 100 mg/kg for 4 weeks	Decreased the concentration and gene expression of inflammatory markers IL-1β, IL-6, IL-18 and TNF-α possibly through the down regulation of NLRP3 and caspase-1
Gao et al., 2013 [89]	HFD-induced obesity and fatty liver in C57BL/6 mice	50 mg/kg twice weekly	Protected against high fat diet induced obesity and ameliorated inflammation in white adipose tissue through lowering the expression of genes associated activated macrophages F4/80, Cd11c, and Cd68, Cd206, increased Arg1 and reduced inflammatory cytokines TNF-α, MCP-1, and IFNγ. Protected liver against fat induced inflammation by reducing expression of TNF-α, and IFNγ
Lee et al., 2013 [173]	Ethanol-induced liver damage in C57BL/6 mice	11.5 mg/kg for 4 weeks	Protected against liver damage by downregulated IL-1β and IL-6
	CCL4-induced liver damage in C57BL/6 mice	11.5 mg/kg for 8 weeks	Protected liver from inflammatory injury through downregulating the expression of TNF-α, IL-1β, and IL-6
Aruna et al., 2014a [174]	Ethanol and HFD-induced pancreatitis in albino Wistar rats	100 mg/kg for 60 days	Protected against tissue injury through inhibiting ASC complex via upregulating its CARD and downregulating PYD domain while also inhibiting the expression of caspase-1, TNF-α, MPO, IL-18 and IL-6
Aruna et al., 2014b [175]	Ethanol–Cerulein-treated albino Wistar rats	100 mg/kg for 3 weeks	Downregulated the plasma concentration and mRNA expression of IL-1β, IL-18, caspase-1, pancreatic MPO and TNF-α
Sikder et al., 2014 [176]	High cholesterol diet-induced hepatoxicity in Swiss albino mice	100 mg/kg for 8 weeks	Attenuated liver injury and downregulated iNOS2, CRP, TNF-α, and IL-6 inflammatory signaling by hindering p65 activation
Wang et al., 2015 [177]	STZ- induced diabetes in Wistar rats	8 mg/kg for 1 week	Rutin protected against diabetic cardiomyopathy by reducing TNF-α and IL-6 levels, alleviating cardiac inflammation
Nafees et al., 2015 [178]	Cyclophosphamide-induced oxidative stress and inflammation in Wistar rats	50 and 100 mg/kg for 20 days	Attenuated oxidative stress and blocked inflammation by lowering serum TNF-α, IL-6, iNOS, COX-2, p38-MAPK, and NF-κB
Hafez et al., 2015 [179]	CCl4-induced hepatotoxicity in male Wistar rats	70 mg/kg twice a week for 4 weeks	Rutin protected against liver damage through hindering the expression of IL-6, JAK and STAT3 levels
Abreu et al., 2016 [180]	L-arginine-induced pancreatitis in swiss mice	75 mg/kg once every 24, 36, 48, and 60 h	Reduced serum IL-6 and CRP significantly

Li et al., 2016 [181]	Age-related metabolic dysfunction in Sprague Dawley rats	25 and 50 mg/kg for 6 weeks	Attenuated metabolic dysfunction associated with age and ameliorated inflammation by reducing serum levels of IL-1β and TNF-α in a dose dependent manner
Saklani et al., 2016 [102]	STZ-induced diabetes in Wistar albino rats	50 mg/kg for 24 weeks	Showed cardioprotective effects through downregulation of TNF-α and CRP
Tian et al., 2016 [104]	STZ-induced diabetic neuropathy in Sprague–Dawley rats	5, 25, 50 mg/kg for 2 weeks.	Attenuated neuroinflammation by decreasing IL-6 and TNF-α concentrations and NF-κB, IκBα and p-IκBα in a dose dependent manner
Liu et al., 2017 [90]	HFD-induced non-alcoholic fatty liver disease C57BL/6 mice	200 mg/kg for 4 weeks	Presented hepatoprotective effects and reduced protein expression of Pro-inflammatory TNF-α and IL-1β significantly
Al-Roujeaie et al., 2017 [101]	STZ-induced diabetic erectile dysfunction in Wistar albino rat	50 and 100 mg/kg for 5 weeks	attenuated inflammation by decreasing levels of IL-6, IL-1β, and tumor TNF-α in serum diabetic rats Increased expression of NRF2 and HO-1
AlDrak et al., 2018 [182]	Lead acetate induced hepatotoxicity in albino Wistar rats	50 mg/kg for 7 days	Pre-administration of rutin reduced serum levels of TNF-α, IL-1B, and IL-6 protecting against liver toxicity
Xianchu et al., 2018 [183]	LPS-induced heart injury in BALB/c mice	100 mg/kg for 8 days	Pretreatment significantly protected against heart injury inflammation by reducing TNF-α and IL-6 mRNA expression and levels
Yu et al., 2018 [184]	HFD-induced insulin resistance in C57BL/6J	5, 10, 20 mg/kg for 18 weeks	Attenuated liver insulin resistance through reduced expression of TNF-α, IL-1, and IL-6 and reduced protein expression of JNK, p38, ERK in a dose dependent manner
Mittal et al., 2018 [103]	STZ-induced diabetic neuropathy in male wistar rats	100 and 200 mg/kg for 8 weeks	reduced serum levels of TNF-α, caspase-3, and NF-κB activity and activated the NRF-2 and HO-1 pathway
Guo et al., 2018 [185]	HFD-induced obesity inC57BL/6J mice	6.4 mg/g for 20 weeks	Ameliorated inflammation by reducing inflammatory cytokines including IFN-γ, IL-4, IL-6, IL-2, and TNF-α and mediators NF-κB, MyD88, TNF-α and lipopolysaccharide binding protein
Caglayan et al., 2019 [186]	Mercuric chloride-induced hepatotoxicity in Sprague Dawley rats	50 and 100 mg/kg for 7 days	Protected against hepatotoxicity by inactivating p38α MAPK, p53, Bcl-3, NF-κB and reducing TNF-α and IL-1B levels
Manzoni et al., 2019 [187]	Poloxamer-407-induced hyperlipidemia in Wistar rats	50 mg/kg for 30 days	Treatment with rutin protected against hyperlipidemia inflammation by increasing lymphocyte generation while decreasing neutrophils and controlled inflammation through reducing MPO, adenosine deaminase in serum and prevented E-ADA activity
Hou et al., 2020 [93]	Thioacetamide-induced hepatic fibrosis in BALB/C mice	10, 25, and 50 mg/kg for 28 days	Protected against fibrosis and inflammation by inhibiting mRNA and protein expression of P2X7r, NLRP3, caspase-1, IL-1β, IL-18, IL-6, IL-1α and TNF-α while also downregulating TLR2, TLR3, TLR4, CD14, MyD88, IRAK1, IRAK4, TRAF-1, and IRF-3
Küçükler et al., 2021 [188]	Deltamethrin-induced hepatotoxicity in Sprague Dawley rats	25 and 50 mg/kg for 30 days	Showed hepatoprotective effects through decreasing the production of inflammatory mediators, TNF-α, IL-1β, NF-κB, iNOS, COX-2, and p38α MAPK

Abbreviations: Arg1: arginase 1; BCL-3: B-cell lymphoma 3-encoded protein; CD14: cluster of differentiation 1; COX-2: cyclooxygenase; CRP: C-reactive protein; ERK1/2: extracellular signal-regulated kinase; HFD: high fat diet; HO-1: heme oxygenase-1; JAK/STAT: janus kinase-signal transducer activator of transcription; IkBα: nuclear factor-kappa-B inhibitor alpha; IFNγ: interferon gamma; IL: interleukin; iNOS: nitric oxide synthase; IRAK: interleukin-1 receptor-associated kinase; IRF-3: interferon regulatory transcription factor-3; LPS: lipopolysaccharides; MCP-1: monocyte chemoattractant protein; MPO: myeloperoxidase; MYD88: myeloid differentiation primary response 88; NF-κB: nuclear factor kappa B; NLRP3: NLR Family Pyrin Domain Containing 3; NO: nitric oxide; P2X7r: P2X purinoceptor 7; NRF2: nuclear factor erythroid 2-related factor 2; p38α MAPK: p38α mitogen-activated protein kinase; PGE_2: p53: tumor protein P53; prostaglandin E_2; STZ: streptozotocin; TLR: toll-like receptor; TNF-α: tumor necrosis factor alpha; TRAF-1: TNF receptor-associated factor 1

2.6.1. An overview on *Fagopyrum tataricum* (Tartary buckwheat) and its potential anti-inflammatory properties

Fagopyrum tataricum commonly known as Tartary buckwheat, is a widely cultivated and consumed pseudo cereal crop from the family Polygonaceae [109]. Tartary buckwheat is native to western China, and is grown in mountainous areas of China, Bhutan, Northern India, and Nepal [110]. It is famous for its high nutritional and health benefits. In fact, the foods made from the tartary buckwheat grain have shown protective effects against several chronic diseases, including obesity, hypertension and CVDs, such effects are mainly attributed to high phenolic content, particularly flavonoids such as rutin [109-111]. Of interest, the anti-inflammatory properties of this food source have been uncovered both in vitro and in vivo. Indeed, a study by Lee et al. [112] showed that 100 µg/ml buckwheat extracts inhibited adipogenesis and downregulated genes associated with adipocyte inflammation, including TNFα, IL-6, MCP-1, and iNOS in 3T3-L1 cells. Consistently, Kim et al. 2019 [113] showed that administration of 5 or 10 g/kg buckwheat in HFD-induced obese Sprague Dawley rats improved serum lipid profile, while also reducing the mRNA expression of major genes involved in adipogenesis such as peroxisome proliferator-activated receptor (PPAR)γ and transcription factor CCAAT/enhancer binding protein (CEBP)α, as well as downregulating well-known pro-inflammatory markers like TNF-α, IL-6, and MCP-1. In fact, although these beneficial effects are identified, very limited information is available on the clinical impact of tartary buckwheat. Available evidence suggests that daily replacement of a portion of the staple food with tartary buckwheat can ameliorate insulin resistance and improve lipid profiles in patients with T2D [114]. Indeed, a study by Wieslander et al. [115] did indicate that daily intake of buckwheat cookies containing relatively high levels of rutin (approximately 16.5-359.7 mg) for two weeks could lower serum cholesterol and reduce the levels of myeloperoxidase (MPO), a known marker of inflammation.

2.6.2. An overview on *Crataegus spp.* And its potential anti-inflammatory properties

Briefly, *Crataegus spp*, also known as Hawthorn/thornapple, is a wild edible fruit that largely grows in northern temperate zones in East Asia, Europe, and eastern North America and has long been used in traditional medicine for the treatment of CVDs [116]. Hawthorn extract was found to have protective effects against atherosclerosis in HFD-fed mice [117]. Hawthorn contains bioactive compounds namely, chlorogenic acid, vitexin, vitexin 2”-O-rhamnoside, hyperoside, quercetin, and isoquercetin, with rutin being the most abundant in the plant flowers with varying 19oncentrateions from 0.02 mg/g in Crataegus monogyna to 3.64 mg/g in Crataegus pseudomelanocarpa [118]. The hawthorn fruit extracts have shown ferric-reducing antioxidant power activity [119], to scavenge free radicals, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), and oxygen radical absorbance capacity [120]. In traditional Chinese medicine, it is used to aid with digestion and can be eaten raw or processed into flakes, juice, jellies, or jams [120]. Of recent, Hamza et al., reported on the protective effect of hawthorn against CCl4-induced liver damage in rats [121]. Here, hawthorn at a dose of 350 mg/kg

could improve the antioxidant status and ameliorate liver inflammation by downregulating the expression levels of IL-6, TNF-α, MPO, cyclooxygenase-2 (COX-2), and NF-κB [121]. Moreover, it could protect against doxorubicin-induced chronic heart failure in Wistar rats at doses of 100 or 200 mg/kg for 6 weeks, while also reducing serum markers of inflammation such as IL-6, IL-8, IL-1β, and TNF-α [122]. Moreover, it reduced levels of TNF-α, IL-1β, and MCP-1 in trimethylamine-N-oxide-exacerbated atherogenesis in apolipoprotein E knock-out mice fed a HFD [123]. While also proving protective effect against complications of dyslipidemia in rats [124]. Clinical data suggests that hawthorn extract (standardized to 50 mg oligomeric procyanidin per 250 mg extract), has the potential to lower blood pressure by reducing flow mediated dilation (a marker of endothelial function) in hypertensive patients [125]. However, this hawthorn extract (given at 450 mg twice daily for 6 months) provides no symptomatic or functional benefit to patients with heart failure.

2.6.3. An overview on *Coffea arabica* and its potential anti-inflammatory properties

Native to Ethiopia [126], *Coffea arabica*, commonly referred to as Arabian coffee, is one of the important commercially traded Coffea species that is highly recognized for the production of high-quality coffees [127]. The plant contains several phenolic compounds, including chlorogenic acid as the highest content [128]. Others have indicated that besides chlorogenic acids and mangiferin, the flavonol rutin and anthocyanins have been notably identified and quantified from the pulp and peels of Arabica coffee [129, 130]. This plant has been traditionally used to treat pain, headaches, cough, liver diseases, diarrhea, and amoebiasis [131]. Scientific research indicates that individuals consuming 121.2 mg of green coffee extract per day present with reduced blood pressure, as well as low levels of leptin (a pro-inflammatory marker), glucose, and insulin in individuals with metabolic syndrome [132]. Recently, Al-Megrin et al., found that the green *Coffea arabica* extract, at a dose of 50 and 100 mg/kg, was able to reverse testicular dysfunction in a rat model of T2D, and this was done by mainly increasing the antioxidant status while decreasing the pro-inflammatory markers like TNF-α and IL-1β [133]. Interestingly, Roshan et al. conducted a clinical study and found that green coffee extract at a dose of 800 mg per day for 8 weeks could attenuate metabolic features such as blood pressure, and insulin resistance in individuals with metabolic syndrome [134]. Such findings are also confirmed by a recent meta-analysis showing that a green coffee extract supplementation could improve fasting plasma glucose and serum levels of insulin and total cholesterol in patients with metabolic syndrome [135].

2.6.4. An overview on *Schisandra chinensis* and its potential anti-inflammatory properties

Native to Japan, Korea, China, and Eastern Russia, *Schisandra chinensis* is used in traditional medicine as a dietary supplement to treat cough, amnesia, dyspnea, insomnia, diaphoresis, and nocturnal dysentery

[136]. This plant is known to contain abundant antioxidant and antimicrobial activities [137], anticancer [138], and hepatoprotective effects [139]. Research indicates that three flavonoid glycosides (rutin, quercitrin, and isoquercitrin) and two flavonoid aglycones (myricetin and quercetin) are among the major bioactive compounds found in different extracts of *Schisandra chinensis* [140-142]. Park et al. showed that 500 mg/kg of the extract could decrease excessive adiposity, in part by modulating thermogenesis and increasing energy expenditure, while also ameliorating insulin resistance in HFD-obese mice [143]. Using RAW 264.7 cells exposed to LPS, Kang et al. showed that 500 µg/ml fruit extract of this plant could ameliorate inflammation by downregulating the expression of IL-1β and iNOS [144]. Furthermore, Dilshara et al. consistently showed that this extract could block inflammation by inhibiting iNOS and NF-κB activation, while increasing insulin signaling through the phosphorylation of protein kinase B (AKT) in RAW 264.7 macrophages [145]. On the other hand, Jang et al. affirmed that *Schisandra chinensis* extract can attenuate endoplasmic reticulum stress to protect against liver injury in HFD-fed obese mice and palmitate-treated HepG2 cells, in part by reducing the expression of pro-inflammatory markers such as IL-6, TNF-α, and MCP-1 [146]. Interestingly, a randomized controlled trial did indicate that tongmai yangxin pill, containing *Schisandra chinensis*, along with other plant-compositions, can alleviate complications linked with coronary artery disease, in particular by ameliorating macrophage foam cell formation and exhibited NF-κB signaling pathway activity [147].

2.6.5. An overview on *Aspalathus linearis* (rooibos) and its potential anti-inflammatory properties

Rooibos is a popular medicinal herb indigenous in the Western Cape of South Africa [148]. It is commercially used as a tea product (rooibos tea), and the traditional use of rooibos dates back to about a century ago [149-151]. Although known to contain abundant dihydrochalcones, that are increasingly studied like aspalathin [152, 153], rooibos also comprises of *O*-glycosyl derivatives of quercetin, such as quercetin-3-*O*-robinobioside and rutin, which seemingly contribute to the enhanced therapeutic potential of rooibos [154, 155]. In fact, beyond its well-known antidiabetic and cardioprotective effects [108, 156-158], rooibos can exhibit anti-inflammatory activity in LPS-induced liver injury in rats through decreasing TNF-α and IL-6 levels [159]. Furthermore, a study by Lawal et al., [160] showed that an aqueous extract of rooibos (50 mg/kg) can protect against diesel exhaust particles induced inflammation by ameliorating the levels of IL-8, TNFα, IL-1β in the aorta and hearts of Wistar rats. In a clinical study conducted by Marnewick and colleagues, it was evident that regular consumption of six cups of rooibos tea can markedly improve the lipid profile as well as redox status in individuals at increased risk of CVD [161]. Interestingly, other clinical studies have indicated that consumption of rooibos tea indeed displays some potential to improve cardiovascular outcomes in patients at risk of CVD [162, 163]. However, there is limited studies reporting on its effect on inflammatory markers in a clinical setting.

2.7 Summary and future perspective

Cumulative evidence from this review suggests that the supplementation with rutin in preclinical models of metabolic disease can effectively ameliorates inflammation through the downregulation of inflammatory cytokines such as TNF-α, IL-6, COX-2, IL-1β (Table 1 and 2). This includes blocking the associated inflammatory pathways such as NF-κB, MAPK, and JAK/STAT. The activation of NRF2, enhances intracellular antioxidant responses and alleviates inflammation. This may be a key therapeutic mechanism of rutin in these preclinical settings. Interestingly, available clinical data suggests that plant based-food and medicinal plants containing relatively high levels of rutin, such as *Fagopyrum tataricum* [115], *Crataegus spp.* [125], *Coffea arabica* [134], *Schisandra chinensis* [147], and rooibos [162, 163], can reduce blood pressure and cholesterol levels, in part by reducing oxidative stress and mediators of inflammation such as MPO, lipid-laden macrophages and NF-κB signaling activity (Figure 4). While the potential therapeutic benefits of rutin or its enriched plants are acknowledged, available evidence still suggests that additional studies are still required to clarify its bioavailability profile. This will lay an important foundation for future studies reporting on the clinical uses of rutin as a pure compound in ameliorating metabolic complications, including oxidative stress and inflammation to enhance the quality of life of the general population.

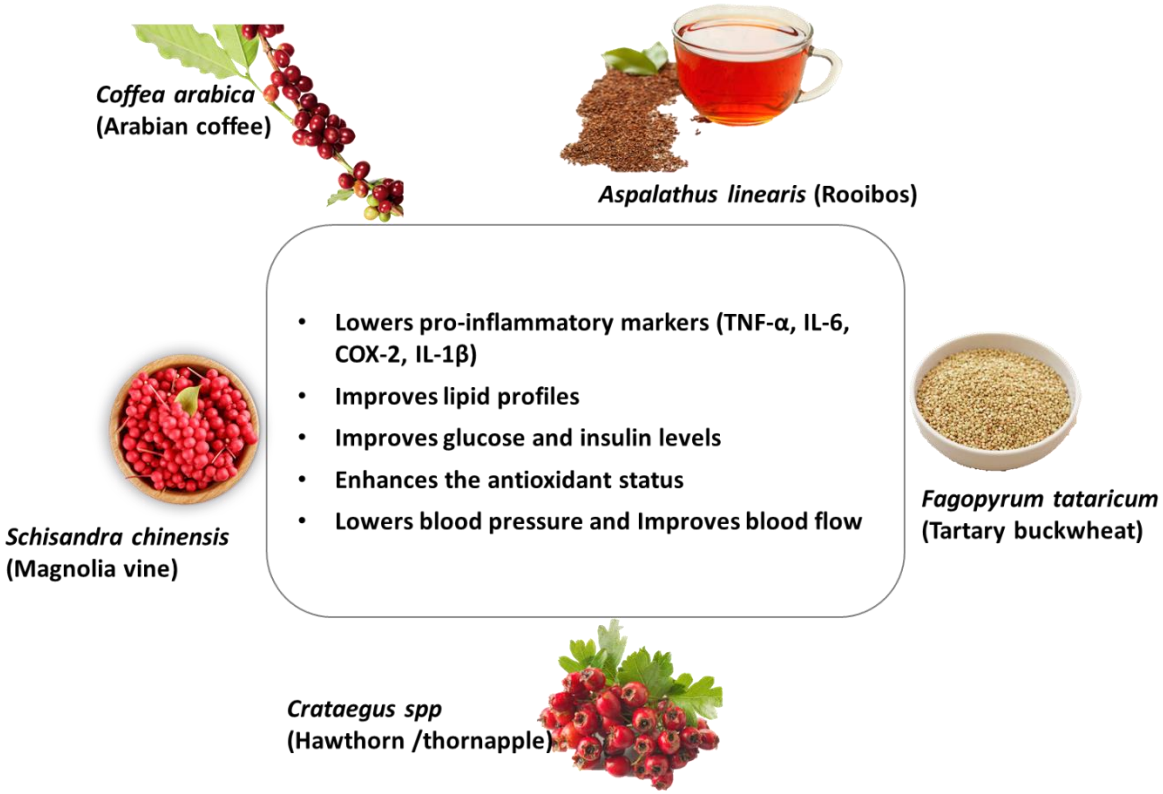

Figure 4: An overview of the plants/food sources that are known to contain relatively high levels of rutin, and these include Fgopyrum tataricum, Crataegus spp., Coffea arabica, Schisandra chinensis, and rooibos. An overview of the plants/food sources that are known to contain relatively high levels of rutin, and these include Agopyrum tataricum, Crataegus spp., Coffea arabica, Schisandra chinensis, and rooibos. Summarized evidence, though preclinical and clinical models, suggests these sources of rutin can ameliorate inflammation and alleviate some complications linked by metabolic disease, such as serum lipid profiles, blood pressure and lower cardiovascular disease (CVD)-risk, in part by enhancing the intracellular antioxidant status. Some of the predominant markers of inflammation that are targeted include, tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, cyclooxygenase 2 (COX-2), and IL-1β.

Abbreviations

ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); AP-1, activator protein-1; ARE, antioxidant response element; ASC, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain; BCL, B-cell lymphoma; CARD, caspase activation and recruitment domain; CCL4, carbon tetrachloride; COX, cyclooxygenase; CRP, C-reactive protein; CVDs, cardiovascular diseases; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ERK, extracellular signal-regulated kinase; INFγ, interferon gamma; IKKα/β, inhibitory kappa B kinase; IκBα, NF-κB inhibitor alpha; IL, interleukin; iNOS, Inducible nitric oxide synthase; LPS. Lipopolysaccharide; JAK/STAT, janus kinase-signal transducer activator of transcription; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinase; MDA, malondialdehyde; MCP-1, monocyte chemoattractant protein 1; MPO. Myeloperoxidase; MyD88, myeloid differentiation factor 88; NAFLD, non-alcoholic fatty liver disease; NO. nitric oxide; NRF-2, nuclear factor erythroid 2-related factor 2; NF-κB, nuclear factor kappa B; NLRP3, NLR Family Pyrin Domain Containing 3; OH-1, heme oxygenase-1; P2X7r, P2X purinoceptor 7; PGE_2, prostaglandin E_2; STAT3, Signal transducer and activator of transcription 3; STZ, streptozotocin; T2D, type 2 diabetes; TLR, toll-like receptor; TNF-α, tumour necrosis factor alpha.

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Declaration of competing interest

The authors declare no conflict of interest.

Authors' contributions

N. Muvhulawa carried out the literature search, study selection, and data extraction and manuscript writing; all other co-authors and supervisors were involved in editing and approval of the final draft.

Data availability statement

Data related to search strategy, study selection and extraction items will be made available upon request after the manuscript is published.

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CHAPTER 3

This chapter entails methods used in this study.

MATERIALS AND METHODS

3.1 Cell culture

3.1.1 Thawing of 3T3-L1 adipocytes cells

The 3T3-L1 cells (ATTC Cat No. CL-173) cryopreserved at -80°C were thawed in a water bath at 37°C. The cells were thawed until a little ice remained and were immediately removed from the water bath. The cryotube was sprayed with 70% ethanol before being placed in the hood. Using a 3 mL pasteur pipette the cells were transferred into a 15 mL tube containing 3 mL pre-warmed growth media (DMEM supplemented with 10% FBS and 1% pen/strep) (Lonza, Walkersville, MD, USA). The cryotube was rinsed with 1 mL pre-warmed growth media to ensure collection of all the cells. The tube was then centrifuged using a Nuve NF 200 benchtop centrifuge (Nüve, Saracalar, Akyurt, Turkey) tube at 112 RCF for 5 minutes to pellet the cells. Thereafter, the supernatant was aspirated leaving only the pellet. Cells were then separated by flicking the bottom of the tube and resuspended in 6 mL of growth media. Thereafter the 1 mL of cells were transferred into a 25 cm^2 flask. The cells were distributed by slightly shaking the flask. The cells were then visualized under a light microscope to insure proper distribution. Thereafter, cells were placed in an incubator with the conditions 37°C and 5% $C0_2$ in humidified air. Subculturing between experiments was kept below passage 15 to eliminate inconsistencies and reduction of specific cell phenotypes. The growth media was replaced with fresh media after 24 hours.

3.1.2 Cell maintenance and sub-culturing (splitting)

After reaching a confluency of 70-80%, cells were sub-cultured into a 75 cm^2 flasks at a density of 2.0×10^4 cells/mL. Briefly, the old media was aspirated out and cells were washed with 4 mL phosphate buffer saline (PBS) for roughly 10-15 seconds to remove all unwanted substances. Thereafter the PBS was aspirated out and 2 mL of trypsin was added to the flask and incubated for 5 minutes at 37°C and 5% $C0_2$ in humidified air to allow the cells to detach from the flask. The cells were viewed under a light microscope to confirm detachment from the flask. Thereafter, 4 mL of growth media was added the to stop trypsin reaction. The cells were collected into a 15 mL tube and centrifuged at 112 RCF for 5 minutes. After centrifugation, media was aspirated, and the pellet was then broken for single cells by gently flicking the tube. Thereafter, the cells were resuspended in 5 mL of pre-warmed growth media and mixed by gentle pipetting. Cells were sub-cultured into 75 cm^2 flasks, adding 1 mL of cells with 11 mL pre-warmed growth media, making a

total volume of 12 mL. Thereafter, cells were incubated at 37°C and 5% CO_2 in humidified air, media was refreshed every 2 days until cells reached 70-80% confluency.

3.1.3 Freezing cells and counting of cells

Upon confluency, the cells were cryopreserved between subcultures to prepare stock. Cells were dislodged from the flask as previously described in section 3.1.2 (sub-culturing). Briefly after trypsinization, 100 µL media was transferred into a microtube for counting and other cells were spun down at 800 rpm for 5 min. For counting, 10 µL of cells was mixed with 10 µL of 0.4% (W/V) trypan blue. Thereafter, 10 µL of the mixture was placed on one the cell counting slide and cells were counted using the countess cell counter and number of cells to be frozen (cells/mL) were determined. For freezing media (7% DMSO in DMEM containing 10% FBS) was freshly prepared each day. After counting, the cells were re-suspended in freezing media at a concentration of $1x10^6$ cells/mL., then aliquoted into well labelled cryotubes and frozen immediately at -80 degrees.

3.1.4 Seeding of 3T3-L1 adipocytes into plates

When cells reached 70-80% confluency, media from the flask was aspirated out and cells were washed with 8 mL pre-warmed PBS. The PBS was aspirated out and 2 mL of trypsin was added. The cells were incubated for 5 minutes at 37°C and 5% CO_2 in humidified air. Cells were then visualized under a light microscope to see if they had detached. Thereafter, trypsinization was stopped with 8 mL of pre-warmed media. Cells were then counted as previously described in sections 3.1.3. Cells were seeded into various multi-well plates (table 3) at a density of 2.0×10^4 cells/mL. Thereafter, cells were incubated at 37°C and 5% CO_2 in humidified air for 3 days until the cells were 90-100% confluent.

Table 3: Cell seeding density

Plate	Concentration (cells/mL)	Volume (µL)
96 well	2.0×10^4	200
24 well	2.0×10^4	1000
6 well	2.0×10^4	3000

3.1.5 The differentiation of 3T3-L1 adipocytes

On day 3, cells had reached 100% confluency and were ready for differentiation. To induce differentiation, growth media was aspirated out and replaced with differentiating media (Day 0) containing 10% FBS, 1% pen/strep, 0.5 mM IBMX, 1 µM dexamethasone, 5 µg/mL insulin, and 1 µM rosiglitazone. Cells were further cultured in differentiating media for 3 days and media was replaced with adipocytes maintenance media (Day 3) containing DMEM supplemented with 10% FBS, 1% pen/strep, and 5 µg/mL insulin and cultured for further 2 days (Day 5). Thereafter, on day 5, adipocytes maintenance media was replaced with DMEM media containing 10% FBS and 1% pen/strep only for an extra 2 days (Day 7). On the 8^{th} day, inflammation was induced in fully differentiated adipocytes by culture with 10 ng/mL TNF-α containing media for 24 h. thereafter, cells were treated with positive controls insulin, CL 316,243 and rutin for 6 h. After treatment, relevant biochemicals assays could be performed.

3.2 Biochemical assay

3.2.1 The 3- [4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide assay (MTT assay)

MTT assay described by Mosmann [22] was used to assess cell viability in 3T3-L1. Briefly, 3T3-L1 adipocytes were cultured in a 96-well plate at a density of 4×10^3 cells/well and were treated with or without TNF-α 10 ng/mL for 24 hours, after 24 hours, cells were treated with rutin (10 µM), with or without positive controls insulin (1 µM), CL 316,243 (1 µM) for 6 hours. After 6 hours of treatment of cells, media was removed. Thereafter, 50 µl of DMEM (without phenol red) and 50 µl of MTT solution (5 mg/mL prepared in tissue culture-treated water) was added with to each well of the 96 well plate and incubated at 37°C under standard cell culture conditions for 30 minutes. Thereafter, MTT solution was removed and 100 µl of DMSO was added into each well followed by 50 µL of Sorenson's glycine buffer (0.1 M Glycine and 0.1 M Sodium chloride, pH 10.5) to dissolve formazan crystals and halt the MTT reaction, respectively. The plate was covered with foil and allowed to shake on an orbital shaker for 15 minutes. The absorbance was read at 570 nm using the Thermo Scientific Multiskan GO (Thermo Fisher Scientific, Finland).

3.2.2 Oil Red O (ORO)

After exposure to TNF-α for 24 hrs and treatment with rutin for 6 hrs, ORO was performed to assess the accumulation of lipids in adipocytes. Cells were washed with 500 µL of pre-warmed PBS and 200 µL of 10% neutral buffered formalin was added to each well and incubated for 30 minutes at room temperature. After incubation, cells were washed and stained with 200 µL of 0.7% ORO working solution and incubated for 30 minutes at room temperature. Thereafter, the stain was removed, and cells were washed 3 times with 400 µL of dH20 this step was repeated three times. After the last wash, 200 µL of isopropanol was added into each well and swirled gently to aid in extraction of the stain. 100 µL of the extract was transferred to a 96-well plate and absorbance was read at 510 nm in a Thermo Scientific Multiskan GO (Thermo Fisher Scientific, Finland) plate reader. The remaining volume was discarded, and the cells were washed with 200 µL of 70% ethanol. Ethanol was removed to near dryness and 400 µL of crystal violet stain was added and incubated at room temperature for 5 minutes. The stain was removed, and cells washed 3 times with PBS. After the third wash, 70% ethanol was added to each well to extract the stain. A 100 µL of the extract was transferred to a 96-well plate and read at 570 nm in a Thermo Scientific Multiskan GO (Thermo Fisher Scientific, Finland) plate reader. Results were calculated by normalizing lipid content (measured at 510 nm) to cell density (measured at 570 nm).

3.2.3 Glycerol assay

Glycerol release was performed using glycerol colorimetric assay according to the manufacturer instructions. Briefly, a master mix and standards were prepared of appropriate volume. Then in a 96-well plate, 10 µL of the standards and samples were aliquoted into different wells. Thereafter, 100 µL of the master mix was added into each well and mixed by pipetting. The reaction was incubated for 20 minutes at room temperature aways from light. The absorbance was read at 570 nm with a Thermo Scientific Multiskan GO (Thermo Fisher Scientific, Finland) plate reader.

3.2.4 Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed to assess inflammation by detecting the production of pro-inflammatory marker IL-6 according to the manufacturers protocol (R&D Systems, Minneapolis,MN, USA). Briefly, a 96-well plate was coated with 100 µL of capture antibody, the plate was sealed and incubated overnight at room temperature. Thereafter, the capture antibody was aspirated out and the plate was washed 3 times with 400 µL of wash buffer. The wells were then blocked with 300 µL of reagent diluent for an hour at room temperature. The plate was washed again, and wash

buffer was removed till near dryness. 100 µL of the standards and samples were added, covered with an adhesive strip and incubated at room temperature for 2 hours. After, wells were washed and 100 µL of the detection antibody was added, the plates were incubated at room temperature for another 2 hours. Thereafter, wash step was repeated and 100 µL of streptavidin-HRP was added to each well and incubated for 20 minutes away from light. The plate was washed again, and 100 µL of substrate solution was added before the plate was incubated for another 20 minutes away from light. After incubation, 50 µL of stop solution was added directly into each well and the plate was gently tapped to insure thorough mixing. The optical density was read at 450 nm and 540 for wavelength correction using Thermo Scientific Multiskan GO (Thermo Fisher Scientific, Finland) plate reader.

3.2.5 Quantitative real-time polymerase reaction

3.2.5.1 RNA extraction

Total RNA extraction of good quality and quantity is a crucial step which is required for many biological experiments such as qPCR for a good gene expression analysis outcome. Total RNA was isolated using Trizol. After treatment, 3T3-L1 cells were lysed with 1 mL Trizol. A cell scraper was used to detach cells from the plates and then transferred into a 2 mL tube. The cell lysate was allowed to stand at room temperature for 10 minutes and then centrifuged at 12 000 × g for 10 minutes at 4°C. Thereafter, 200 µL of chloroform was added and allowed to incubate at room temperature for 10 minutes (samples were vortexed 4 times in between the 10 minutes to allow thorough mixing for phase separation). The samples were then centrifuged at 18 400 x g for 20 minutes at 4°C to get phase separation. The aqueous phase (upper, colourless phase) was transferred into a new 1.5 mL tube. The RNA was precipitated by mixing with 0.5 mL of cold isopropanol and incubating on ice for 10 minutes. After incubation, the solution was centrifuged at 18 400 x g for 40 minutes at 4°C and the supernatant was removed leaving only the pellet. The pellet was washed twice by adding 1 mL (75%) ethanol and centrifuging at 12 000 x g for 10 minutes at 4°C. The ethanol was discarded, and pellet allowed to air dry for 15 minutes. The pellet was dissolved in RNase-free water depending on the size of the pellet and stored at -80°C freezer until use.

3.2.5.2 RNA concentration and integrity

RNA concentration was measured using the Nanodrop one/one^{c} (Thermo Fisher Scientific, Waltham, MA, USA) according to manufactures recommendations. Briefly, RNase-free water (1 µL) was first used to blank the machine then 1 µL of the sample was placed on the centre of the

lower read head and the absorbance was read at a wavelength of 260 nm in duplicates. Thereafter the RNA integrity was analysed using 1% agarose gel electrophoresis. Briefly, in a 500 mL beaker, 1 g of agarose was mixed with 100 mL Tris-acetate-EDTA (TAE) buffer (1X). The solution was swirled and heated in a microwave at high heat to dissolve the agarose for 2-3 minutes and shaking at 30 second intervals until solution was clear. The solution was left to cool for about 5 minutes and 1.5 µL of ethidium bromide was added. The solution was poured into the gel tray and a rubber comb was placed. The gel was left to rest at room temperature for 20-30 minutes until the gel solidified. After the gel solidified, the comb was carefully removed, and gel was placed in the electrophoresis chamber, and it was filled with TAE buffer until it covered the gel. Samples mixed with a gel loading dye were then loaded into the wells and electrophoresis was ran at 100 volts for 50 minutes. Thereafter, bands were visualized using a Gel Doc™ XR+ machine with image lab™ software (Bio-Rad, Hercules, CA, USA).

3.2.5.3 cDNA synthesis

Total RNA was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit. Briefly, total RNA (12 µL) was incubated with 2 µL of gDNA for 2 minutes at 42 degrees then immediately placed on ice. Thereafter, a master mix prepared by adding Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and a RT Primer Mix (Table 4) was added into each tube at 6 µL to make a final volume of 20 µL. A non-template control was prepared by mixing the reaction components without total RNA. The contents were gently mixed, and the tubes were incubated at 42°C for 15 minutes using the T100 thermal cycler (Bio-Rad Laboratories, CA, USA). Tubes were again incubated at 95°C for 3 minutes to terminate the reaction. The cDNA was diluted by adding 180 µL of RNase-free water making a total of 200 µL. The cDNA was stored at -20°C until use.

Table 4: Reaction components used for cDNA synthesis

Components	Quantity
Quantiscript Reverse Transcriptase	1 µL
Quantiscript RT Buffer	4 µL
RT Primer Mix	1 µL
Entire genomic DNA elimination reaction	14 µL

3.2.5.4 quantitative polymerase chain reaction (qPCR)

The qPCR was performed by adding 4 µL of cDNA samples into individual wells according to the plate layout. A master mix was prepared by adding 5 µL taqman master mix, 1 µL probe, and 1 µL RNase-free water (table 5). 6 µL of master mix was added to each well making a total reaction volume of 10 µL (table 5). The plate was sealed and placed on a shaker for 5 minutes and placed in the PCR machine. Genes that were investigated are listed in table 6. Act B and B2M were used as a house keeping gene The plate was ran at the following cycling conditions 50°C (2 min), 95°C (2 min), 95°C (15 sec) and 60°C (1 min).

Table 5: Reaction components used in qPCR

Components	Quantity
Taqman master mix	5 µL
Probe	1 µL
H_2O	1 µL
cDNA	4 µL

Table 6: List of probes used

Name	Number
B-actin	Mm02619580_g1
Cpt1a	Mm01231183_m1
Pparγ	Mm00440940_m1
Prdm16	Mm01266507_g1
Tnf-α	Mm00443258_m1
Ucp1	Mm01244861_m1
B2m	Mm00437762_m1

3.2.6 Western blot

3.2.6.1 Protein extraction

In a 1.5 mL tube, 1 mL Lysis buffer (1X) was mixed with 10 µL PMSF (1%) on the day of extraction and stored on ice. In a 6 well plate containing 3T3-L1 adipocytes, 200 µL of the lysis buffer with PMSF was added into each well and a cell scraper was used to scrape the cells off the plate. The lysates were transferred to fresh 1.5 mL tubes and incubated on ice for 30 minutes. The samples

were centrifuged at maximum speed for 20 minutes at 4°C. The supernatant was transferred into a new 1.5 mL tube and centrifuged again. After centrifugation, the sample was transferred into a new tube and stored at -20°C until use in western blot analysis.

3.2.6.2 Protein assay

A Bradford assay was used to determine the protein concentration of thesamples. Samples were first diluted 1:5 with molecular grade water. A Standard was prepared using BSA of different concentrations from a 50 mg/mL stock. The protein dye assay (5x) was first diluted with water to make a 50 mL (1x) working solution. The working solution was filter sterilized and covered in foil. Briefly, in 96 well plate, 10 µL of samples or standard were used in each well and 200 µL of the 1X Bradford solution was added into each well. After adding the Bradford solution, the plate was incubated by covering with foil and mixed using a shaker for 5 minutes. After incubation, the plate was read at 540 nm using a Thermo Scientific Multiskan GO (Thermo Fisher Scientific, Finland) plate reader and the absorbance was recorded.

Table 7: BSA protein standard used to determine unknown protein concentration.

Tubes	Standard (µL)	Buffer (µL)	Final concentration (mg/mL)	Final volume (µL)
1	1000	0	2	625
2	375	125	1.5	167
3	333	167	1	125
4	375	125	0.75	167
5	333	167	0.5	250
6	250	250	0.25	250
7	250	250	0.125	500
8	0	500	0	500

From a 50 mg/mL BSA stock, a 1mL (2mg/mL) working solution was prepared and was used to prepare serial dilutions of different standard concentrations.

3.2.6.3 Profiling and western blot

Samples for profiling were prepared according to the calculated sample protein concentrations. Samples were added together with 2x-laemmli buffer containing β-mercaptoethanol. Thereafter samples were heated at 95°C for 5 minutes and then allowed to cool. Mini-PROTEAN TGX gels

were placed in the gel electrophoresis chamber, and it was filled with the appropriate amount of 1x running buffer. The wells were rinsed with the buffer then the samples were loaded into the gels. All blue marker was used for profiling and western c for western blot. The samples were run at 200 V for 40 minutes or until the blue colour ran out. For profiling, the gel was stained with Coomassie overnight. The stain was removed, and the gel washed with destaining solution for 30 minutes on a shaker 3 times. The gel was viewed to check separation of proteins using a gel doc. For western blot, the gel was placed on a wet filter paper with nitrocellulose membrane on top of the filter paper and another wet filter paper placed on top of the gel creating a sandwich. Making sure to roll out any air bubbles that may have formed. Protein transfer was done using the Transblot machine. The runs were set according to the proteins of interest. After the run the membranes were placed in ponceau stain for 1 min to check if the protein transfer was successful. After, the stain was washed off with water once then 3 times with TBST for 5 minutes on shaker or until stain was completely removed.

3.2.6.4 Blocking and Antibody incubation

The membranes were blocked with 5% milk for 2 hours on a shaker at room temperature. Thereafter, it was washed 3 times with TBST for 5 minutes each wash. The membranes were then incubated in primary antibodies prepared in 5% BSA overnight on a shaker in a cold room (4 degrees). The membrane was then washed and incubated with secondary antibody with HRP-conjugate prepared in 5% milk for 90 minutes. The membrane was washed again and stained with ECL PRIME (1:1) and incubated for 2 minutes. The membrane was then viewed using a gel doc. The antibodies used in this study are on table 8 and the catalogue numbers and suppliers are in the index.

Table 8: List of antibodies

Antibody	Dilution	% Gel
pAKT	1:1000	10
tAKT	1:1000	10
pAMPK	1:800	10
tAMPK	1:1000	10
B-actin	1:1000	10
PGC1-α	1:1000	10

3.2.7 Seahorse

The 3T3-L1 adipocytes were seeded and cultures as previously described by Ziqubu et al., 2020 [23] with slight modifications, briefly cells were seeded at 5 000 cells/well in 80 µL of growth media containing 10 % FBS and 1%P/S in an Agilent seahorse XF-96 cell culture microplate and incubated at 37°C, 5% CO_2 with humidified air. After 48h cells reached confluency and were differentiated into mature adipocytes. For differentiation, growth media was supplemented with 0.5 mM IBMX, 1 µM dexamethasone, 5 µg/mL insulin, and 1 µM rosiglitazone for 3 days. On day 3 the differentiation media was replaced with maintenance media containing growth media supplemented with 5 µg/mL insulin for further 2 days. On day 5, cells were incubated with or without 10 ng/mL TNF-α for 24h. Thereafter, cells were treated with CL 316,243 and rutin for 6h, insulin treatment was added 15 minutes before termination.

In preparation for Seahorse assay, a day prior to assay, 20 ml seahorse XF calibrant was aliquoted into a 50 mL tube and placed in a non-CO_2 incubator overnight. From the XFe96 Extracellular Flux Assay Kit, the utility plate was filled with 200 µL of sterile water in each well and the sensor cartridge was submerged into the water making sure to avoid bubble formation (Figure 5). The cartridge and utility plate were then placed in the non-CO_2 incubated overnight at 37°C sealed with parafilm to avoid evaporation of water. On the day of assay, the utility plate with cartridge were removed from the incubator and the water was replaced with 200 µL of the calibrant. The sensor cartridge was submerged into the calibrant. The utility plate was then incubated in a non-CO_2 incubator at 37°C for an hour. After, the modulators were added into the cartridge ports as shown in Table 9. The utility plate and cartridge were placed in the XFe96 analyser and allowed to calibrate for 15-30 minutes. Cells were washed once with 180 µL seahorse media, DMEM supplemented with 2 mM L-glutamate, 8 mM glucose, and 1 mM sodium pyruvate. After washing, 180 µL of seahorse media was added to each well. The plate was incubated at 37°C in a non-CO_2 incubator for 10 minutes. Thereafter, the plate was ran using the XFe96 analyser (Figure 5).

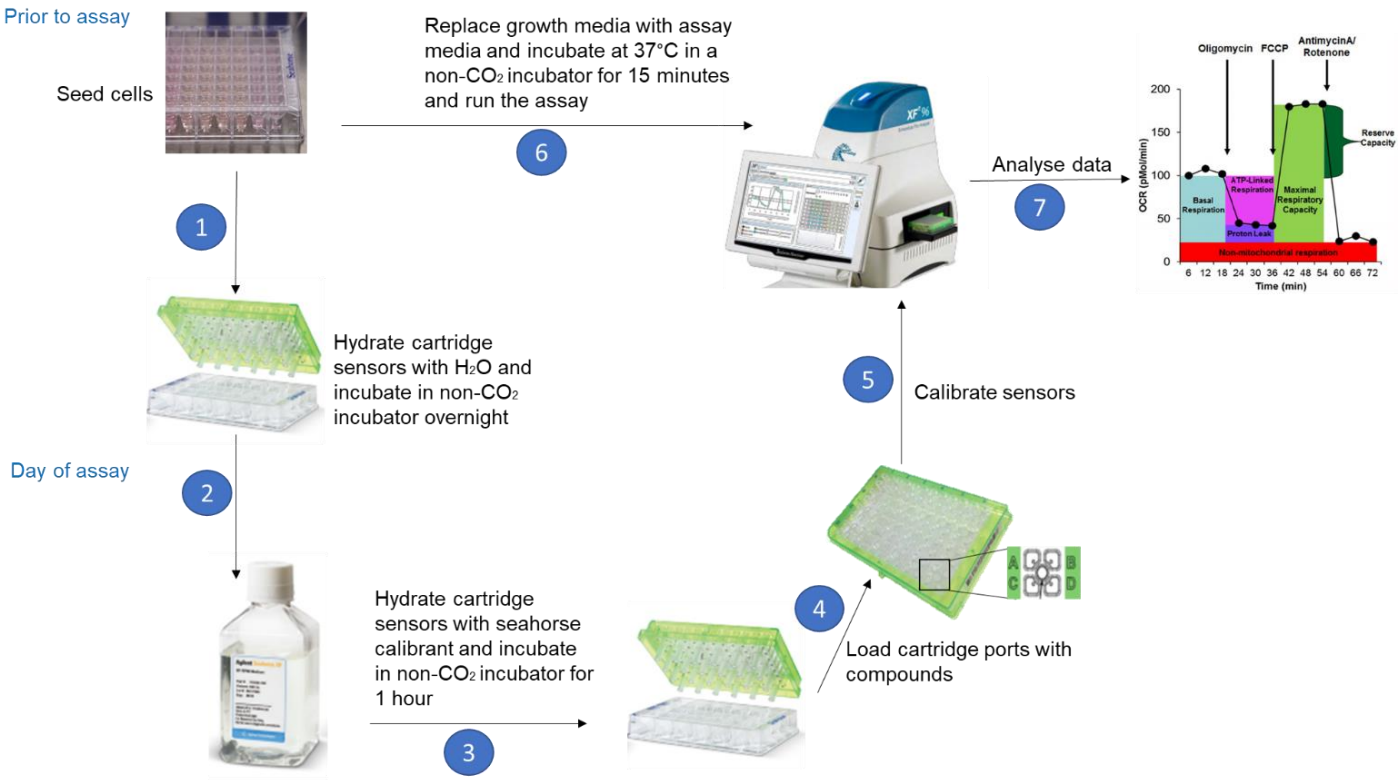

Figure 5: The cell Mito Stress Test work flow diagram

Table 9: Different modulators used to assess mitochondrial respiration

Modulators	Port	Volume (µL)	Concentration (µM)
Oligomycin	A	20	20
FCCP	B	22	5
Rotenone/Antimycin A	C	25	5
2-DG	D	27	500

3.3 Statistical analysis

Statistical analysis was done using Graphpad Prism software. The significance of difference was determined using one-way analysis of variance (ANOVA), followed by the Tukey post-hoc test or Student t test where appropriate. A value of $p < 0.05$ was considered significant.

CHAPTER 4

This chapter reports on the findings from this study after conducting the assays in chapter 3.

RESULTS

4.1 Rutin treatment did not affect the cell viability of 3T3-L1 adipocytes exposed to TNF-α

The MTT assay remains one of the most versatile and widely used assay for the analysis of cell viability and metabolic activity through the action of mitochondrial reductase [24]. In this study the MTT assay was conducted to determine the effect of rutin on the viability of 3T3-L1 adipocytes prior and after exposure to TNF-α. In normal cells, insulin a positive control, increased the cell viability by 15.57% ($p < 0.05$) relative to normal control while CL 316, 243, a β3-adrenergic receptor agonist developed as an antiobesity and antidiabetic drug and the test compound rutin showed no significant change on cell viability. Alternatively, culturing 3T3-L1 adipocytes with TNF-α for 24 hrs reduced cell viability by 10.37%, albeit not significant. Moreover, treatment with insulin increased cell viability from 89.63 ± 7.53% to 112.4 ± 14.17% ($p < 0.001$) when compared to TNF-α. Notably, rutin treatment slightly improved cell viability at a level equivalent to that of positive control CL 316, 243 and normal control, although it was not significant (Figure 6).

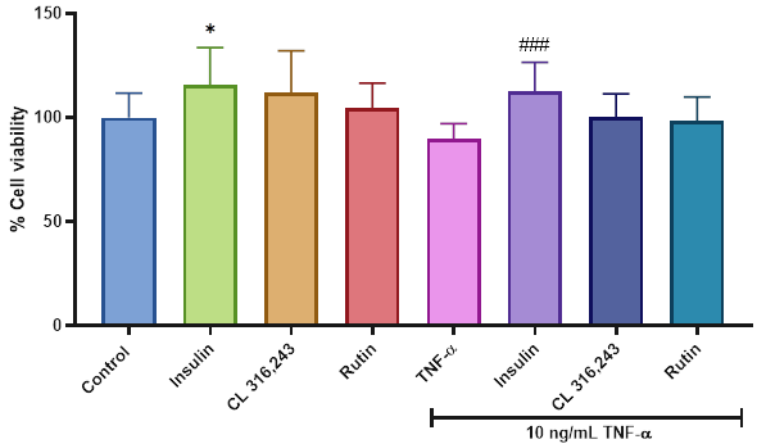

Figure 6: Effect of rutin on the viability of 3T3-L1 adipocytes before and after exposure to TNF-α.

The 3T3-L1 adipocytes were cultured in DMEM containing 8 mM glucose in the absence and presence of 10 ng/mL TNF-α for 24 h, followed by treatment with positive controls insulin (1 µM) for 15 min, CL 316, 243 (1 µM) for 6hrs and compound of interest rutin (10 µM) for 6 h. Mitochondrial activity was measured using the MTT assay. Results are reported as the mean ± SD of 3 independent experiments at 100%, $^{*}p < 0.05$ versus control; $^{###}$ $p < 0.001$ versus TNF-α.

4.2 Rutin suppresses TNF-α- induced pro-inflammatory response in 3T3-L1 adipocytes

Excessive storage of fatty acid and lipid accumulation in adipose tissue has been strongly linked with inflammation and secretion of several pro-inflammatory adipokines, such as IL-6, IL-8, IFN-γ, and TNF-α. To induce inflammation differentiated 3T3-L1 adipocytes were treated with 10 ng/mL of TNF-α for 24 hrs, *Tnf-α* mRNA expression and IL-6 protein expression were evaluated. Results demonstrated a significant increase in *Tnf-α* gene expression from 100 ± 0% to 5173 ± 1584% ($p<0.001$). Interestingly, treatment with rutin for 6 hrs significantly decreased this expression to 1.82 ± 0.66%. Similarly, positive controls insulin and CL 316,243 also showed a significant decrease to 5.05 ± 0.79% ($p<0.001$) and 1.55 ± 0.30% ($p<0.001$), when compared to TNF-α treated cells respectively (Figure 7A). Similar results were observed, inflammatory cytokine IL-6, was increased from 100± 46.57% to 2438 ± 123.8 ($p<0.001$). In the TNF-α treated group compared to the control. Interestingly, rutin was able to reverse this increase to 11.93 ± 2.42% ($p<0.001$). Moreover, rutin was able to decrease IL-6 protein expression values the most compared to the positive controls insulin (41.83 ± 13.19%, $p<0.001$) and CL 316,243 (13.11 ± 4.66%, $p<0.001$) (Figure 7B).

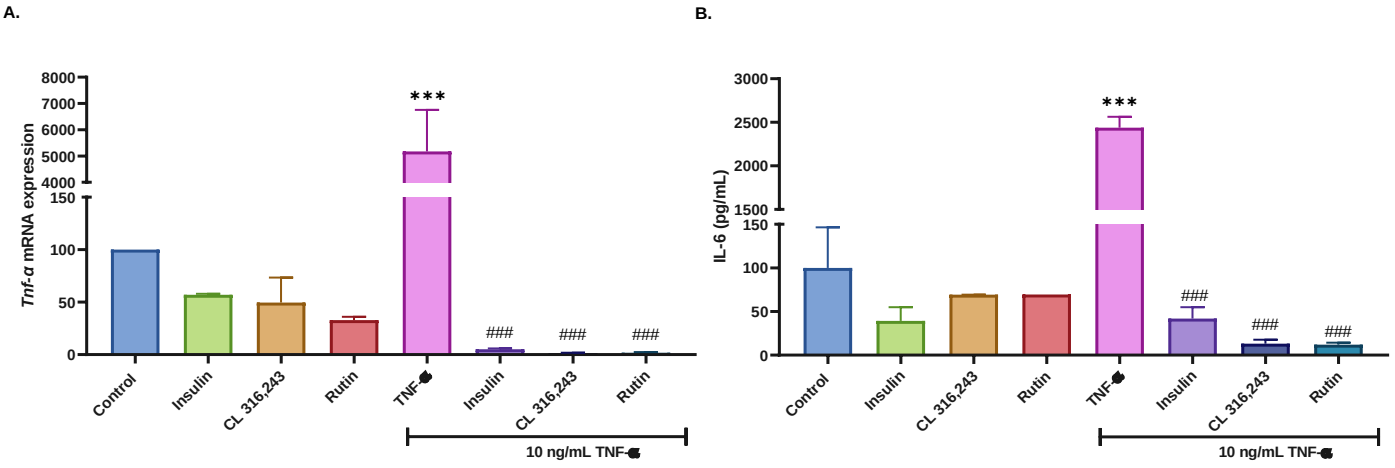

Figure 7: Rutin suppresses TNF-α- induced pro-inflammatory response in 3T3-L1 adipocytes

3T3-L1 adipocytes were cultured in DMEM with 8 mM glucose in the absence and presence of 10 ng/mL TNF-α for 24 h, and then treated with insulin (1 μM) for 15 min, CL 316, 243 (1 μM), and rutin (10 μM) for 6 h. Cells were lysed, and gene expression was quantified using qPCR **(A)** and protein expression was analysed using ELISA **(B)**. qPCR results **(A)** were normalised to housekeeping genes β-actin and B2M. Results are given as the mean ± SD of 3 independent experiments. Significance is shown as *** $p < 0.001$ versus control; $^{###}$ $p < 0.001$ versus TNF-α treated cells.

4.3 Effect of rutin on lipid accumulation and lipolysis in TNF-α induced inflammation and insulin resistance in 3T3-L1 adipocytes

After induction of inflammation and insulin resistance with TNF-α and culturing with or without rutin for 6 hrs lipid droplet accumulation using oil-red-o staining was evaluated. Results showed that isoproterenol, a nonspecific β-adrenergic receptor agonist and potent lipolysis stimulator decreased lipid accumulation by 40.82% ($p < 0.001$). Culturing the 3T3-L1 cells with TNF-α decreased lipid accumulation by 30.9% ($p < 0.01$). Interestingly, treatment with insulin, CL 316,243, and rutin increased lipid accumulation from 59.18 ± 1.21% to 112.7 ± 18.46% (45.57%, $p < 0.001$), 106.1 ± 17.35% (37.0%, $p < 0.001$), and 107.0 ± 16.88%) (37.85%, $p < 0.001$) (Figure 8A). In terms of lipolysis as measured with glycerol release assay, culturing 3T3-L1 adipocytes with positive control CL 316,243, a β3-adrenergic receptor agonist increased lipolysis by 151.6% ($p < 0.001$) and isoproterenol drastically increased by 403.2% $p < 0.001$ compared to the control. Incubating the 3T3-L1 adipocytes in media containing TNF-α for 24h resulted in a significant increase in lipolysis compared to the control by 113.8% ($p < 0.01$). Treatment with rutin was able to reverse this effect. Resulting in the decrease in lipolysis from 213.8 ± 17.6% ($p < 0.01$) to 81.23 ± 12.02% ($p < 0.001$) compared to TNF-α. Interestingly, this decrease in lipolysis was greater compared to the positive controls of which insulin, CL 316,243, and isoproterenol decreased lipolysis to 103.2 ± 27.07% ($p < 0.01$), 119.8 ± 10.57% ($p < 0.01$), and 125.9 ± 8.05% ($p < 0.05$), respectively (Figure 8B).

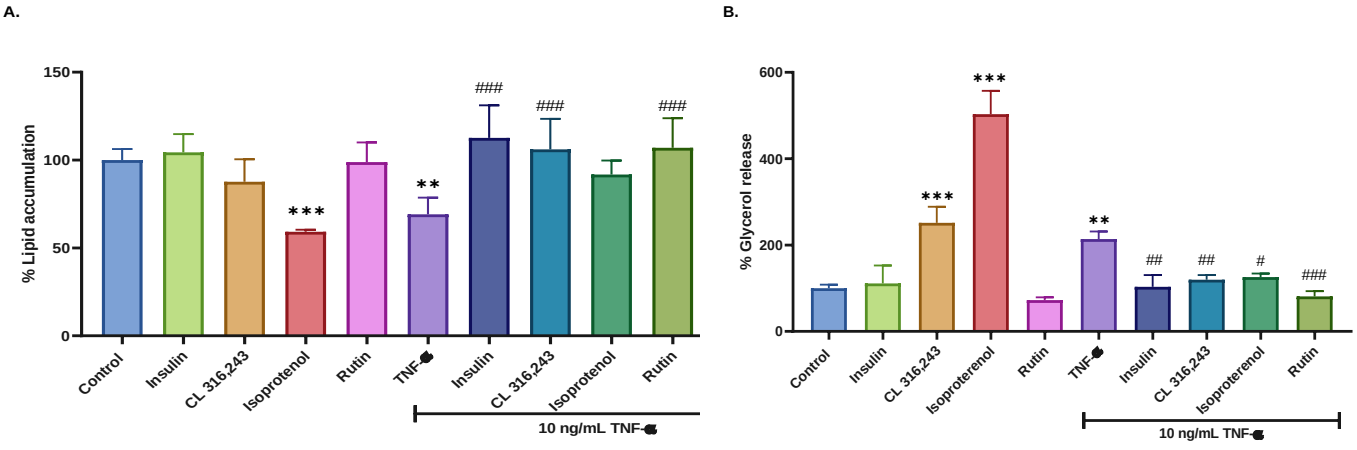

Figure 8: Effect of rutin on lipid accumulation and lipolysis in TNF-α-induced inflammation and insulin resistance in 3T3-L1 adipocytes.

3T3-L1 adipocytes were cultured in DMEM with 8 mM glucose in the absence and presence of 10 ng/mL TNF-α for 24 h, and then treated with insulin (1 μM) for 15 min, CL 316, 243 (1 μM), isoproterenol (10 μM) and rutin (10 μM) for 6 h. Lipid accumulation was measured using Oil-Red-O staining (A) and lipolysis was measured using a glycerol assay kit (B). A one-way analysis of variance (ANOVA) with Tukey post-hoc test

was used for statistical analysis. A value of $p < 0.05$ was considered statistically significant. Results are given as the mean ± SD of 3 independent experiments. Significance is shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ versus TNF-α.

4.4 Effect of rutin on lipid metabolism in 3T3-L1 adipocytes

As a key transporter of fatty acids to mitochondria for beta-oxidation, carnitine palmitoyltransferase I (CPT1) mRNA expression was measured in 3T3-L1 adipocytes exposed to TNF-α. TNF-α significantly reduced mRNA expression of *Cpt1* by 49.19% ($p<0.05$) relative to normal control. Insulin treatment in TNF-α cells showed no effect. Rutin treatment reversed this effect from 50.81 ± 24.48% to 187.4 ± 19.42% ($p<0.001$). CL 316,243 showed a slight increase in *Cpt1* gene expression compared to normal control, however, not significant. In TNF-α cells, CL 316,243 greatly improved *Cpt1* mRNA expression by 169.0% (219.8 ± 18.50%, $p<0.001$) (Figure 9A). Peroxisome proliferator- activated receptor gamma (PPARγ) is a key adipogenic transcriptional factor involved in the differentiation of both brown and white adipocytes [25]. TNF-α greatly abolished the *Pparγ* mRNA expression by reducing expression by 92.22% ($p<0.001$). The addition of insulin increased this expression from 7.78 ± 1.83% ($p<0.001$) to 193.5 ± 11.76% ($p<0.001$). CL 316,243 and rutin increased *Pparγ* mRNA expression in TNF-α cells to 231,2 ± 39,36 ($p<001$) and 258,6 ± 21,41 ($p<0.001$), respectively (Figure 9B).

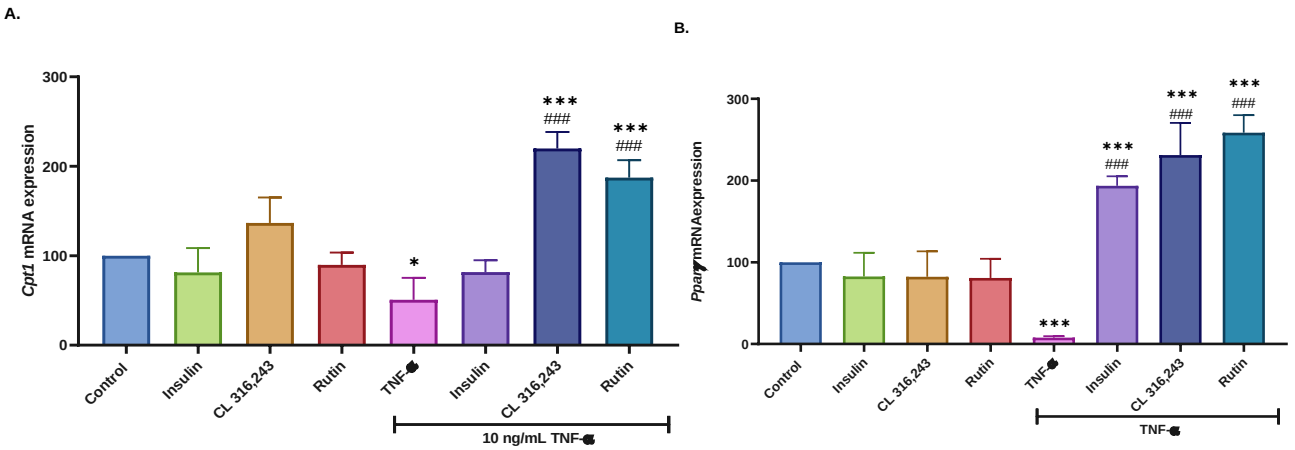

Figure 9: Effect of rutin on lipid metabolism in 3T3-L1 adipocytes

3T3-L1 adipocytes were cultured in DMEM with 8 mM glucose in the absence and presence of 10 ng/mL TNF-α for 24 h, followed by treated with insulin (1 µM) for 15 min, CL 316, 243 (1 µM), and rutin (10 µM) for 6 h. cells were lysed, and mRNA extracted was quantified using qPCR. Results were normalised to housekeeping genes β-actin and B2M. Results are given as the mean ± SD of 3 independent experiments. Significance is shown as * $p < 0.05$, *** $p < 0.001$ versus control; $^{\#\#\#}$ $p < 0.001$ versus TNF-α.

4.5 Rutin counteracts the impact of TNF-α in 3T3-L1 adipocytes by promoting UCP1-independent thermogenic program and mitochondrial biogenesis

The uncoupling protein 1 (UCP1) is an anion carrier protein, which uncouple substrate oxidation from ATP synthesis, to dissipating excess energy as heat and promote thermogenesis [26]. *Ucp1* mRNA expression was elevated by CL 316,243 to 3904 ± 42.59% ($p<0.001$) compared to the normal control. Insulin and rutin showed no effect. Culturing cells with TNF-α slightly downregulated Ucp1 mRNA expression, however, was not significant. Treatment with CL 316,243 drastically increased *Ucp1* mRNA levels from 72.23 ± 14.21 to 1370 ± 277.9% ($p<0.001$) (Figure 10A). The effect of rutin on fat browning was investigated, insulin unexpectedly reduced PR domain containing 16 (PRMD16) expression by 55.74% ($p<0.05$). Interestingly, CL316, 243 enhanced *Prdm16* mRNA expression albeit not significant, moreover, rutin enhanced *Prdm16* mRNA expression by 81,12% ($p<0.001$), when compared to normal control respectively (Figure 10B). Culturing cells with TNF-α resulted in a reduction of *Prdm16* mRNA expression by to 65.70% compared to the normal control. However, treatment with rutin appeared to have reversed this effect and increased levels to 170.6 ± 26.17% ($p<0.001$) compared to TNF-α (34.30±19.64, $p<0.01$) (Figure 10B). Insulin also reduced Peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC1-α) protein expression by 43.52% ($p<0.001$) while rutin increased PGC1-α protein expression by 33.81% compared to normal control TNF-α decreased the protein expression of PGC1-α by 87.93% ($p < 0.001$) compared to the normal control However, in the TNF-α treated cells, CL and rutin increased PGC1-α protein expression from 12.07 ± 0.97% ($p <0.001$) to 105.1 ± 6.67% ($p<0.001$) and 145.4 ± 14.36% ($p<0.001$), respectively. Insulin was the most significant with a 189.3% increase (201.5 ± 12.61%, $p < 0.001$) (Figure 10C).

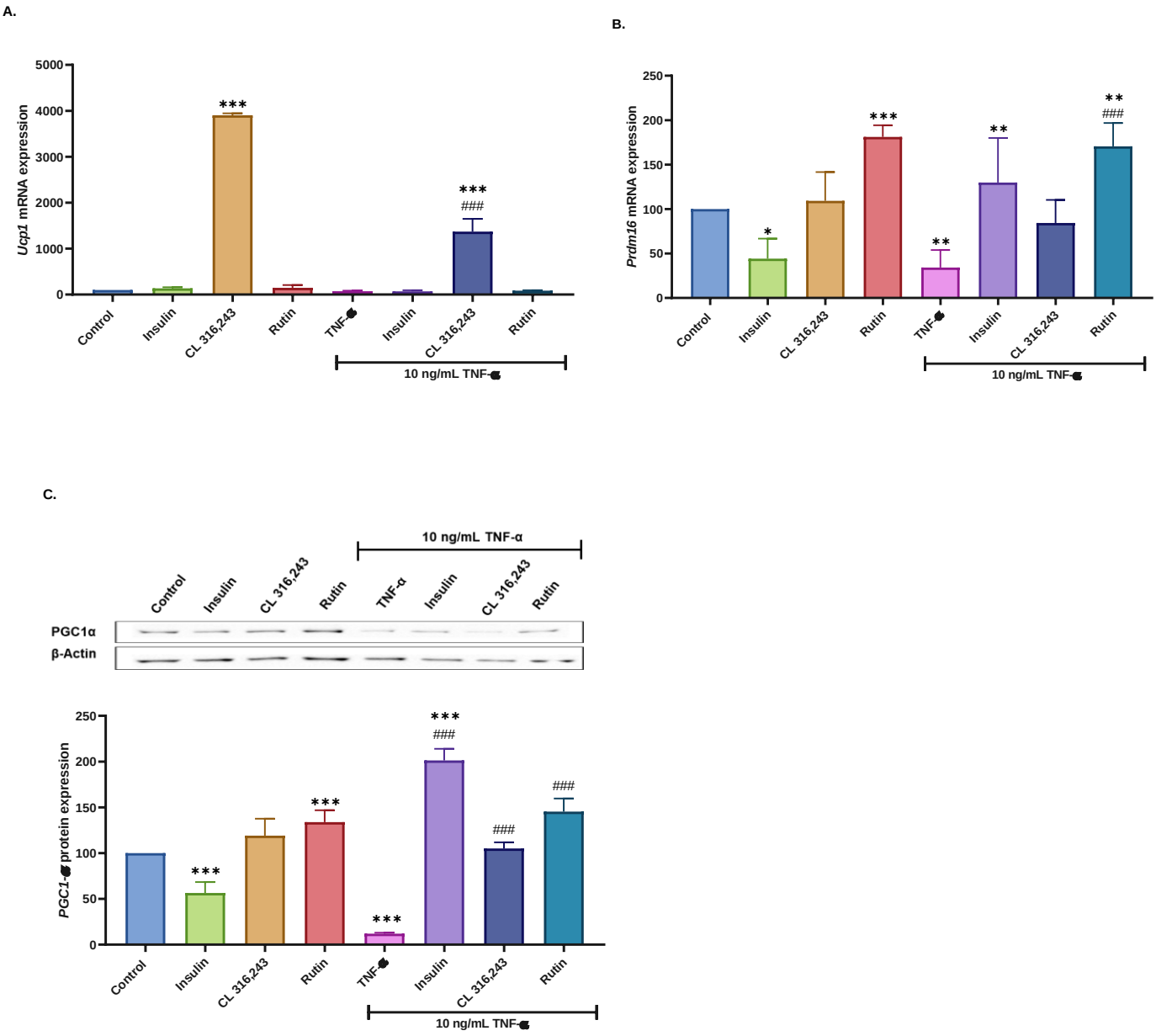

Figure 10: Effect of rutin on thermogenic program and mitochondrial biogenesis in 3T3-L1 adipocytes exposed to TNF-α.

3T3-L1 adipocytes were cultured in DMEM with 8 mM glucose in the absence and presence of 10 ng/mL TNF-α for 24 h, and then treated with insulin (1 µM) for 15 min, CL 316, 243 (1 µM), and rutin (10 µM) for 6 h. Cells were lysed and subjected to qPCR **(A, B)** and western blot **(C)** analysis. Results are given as the mean ± SD of 3 independent experiments. Significance is shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control; $^{###}$ $p < 0.001$ versus TNF-α.

4.6 Effect of rutin on glucose metabolism in TNF-α induced inflammation and insulin resistance 3T3-L1 adipocytes

To assess the effect of rutin on glucose metabolism, phosphorylation of protein kinase B (pAKT) and AMP-activated protein kinase (pAMPK) were investigated. CL 316,243 and rutin increased the protein expression of pAKT compared to normal control by 91.16% ($p<0.001$) and 130.9% ($p<0.001$). insulin showed no significant effect. Exposure of 3T3-L1 adipocytes to TNF-α reduced pAKT protein expression by 28.15% ($p<0.05$). rutin and insulin increased AKT phosphorylation from 71.8 ±16.11% ($p<0.05$) to 114.7 ± 22.89% (0.001) and 246.2 ± 9.53% ($p<0.001$), respectively (Figure 11A). AMPK is a master regulator of cellular energy metabolism [27]. Insulin, CL 316,243, and rutin resulted in a significant increase in pAMPK protein expression by 32.35% (132.4 ± 18.10%, $p<0.05$), 50.01% (150.0 ± 18.98%, $p<0.001$), and 65.75% (165.8 ± 10.33%, $p<0.001$) compared to normal control. Culturing 3T3-L1 adipocytes with TNF-α showed no effect in pAMPK protein expression and treatment with either insulin, CL 316,243, or rutin had a significant effect (Figure 11B).

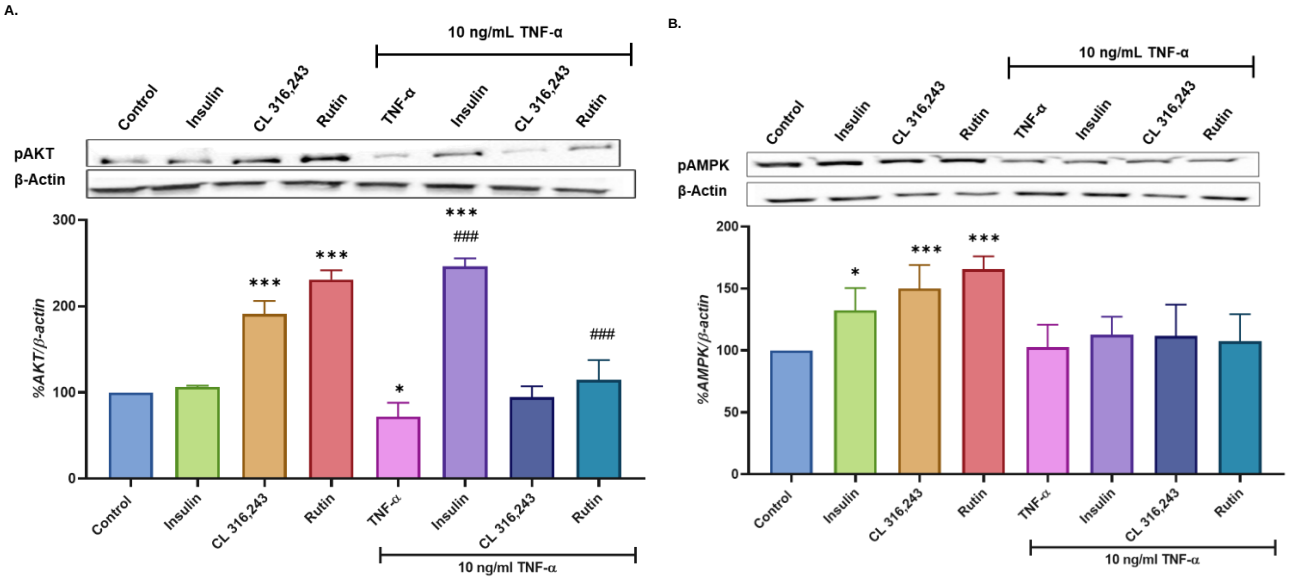

Figure 11: Effect of rutin on glucose metabolism in TNF-α induced inflammation and insulin resistance 3T3-L1 adipocytes

3T3-L1 adipocytes were cultured in DMEM with 8 mM glucose in the absence and presence of 10 ng/mL TNF-α for 24 h, and then treated with insulin (1 μM) for 15 min, CL 316, 243 (1 μM), and rutin (10 μM) for 6 h. Cells were lysed and subjected to western blot analysis. Results were normalised to β-actin. A one-way analysis of variance (ANOVA) with Tukey post-hoc test was used for statistical analysis. A value of $p < 0.05$ was considered statistically significant. Results are given as the mean ± SD of 3 independent experiments. Significance is shown as $^{*}p < 0.05$, $^{***}p < 0.001$ versus control; $^{###}p < 0.001$ versus TNF-α.

4.7 TNF-α reduced mitochondrial function in 3T3-L1 adipocytes

This study investigated the effect of rutin on real-time mitochondrial respiration in TNF-α challenged 3T3-L1 cells using Mito stress assay. Representative graphs of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) are presented in (Figure 12 A and B).

In terms of basal respiration (Figure 12C), the positive control CL 326,243 which is a potent β-3 adrenergic agonist known to increase thermogenesis and metabolic rate resulted in a decrease in basal OCR from 152.0 ± 8.27% to 135.2 ± 14.59% (16.8%, $p < 0.05$) compared to normal control. Incubation with TNF-α showed no significant change, however, when cells were treated with CL 316,243, an increase in basal OCR from 150.2 ± 18.77% to 175.8 ± 15.44% (25.60%, $p < 0.001$) compared to TNF-α and increased by 23.79% ($p < 0.01$) compared to the normal control was observed.

Furthermore, ATP production after injection with the mitochondrial ATP synthase inhibitor oligomycin (2μM) was investigated. As with basal respiration, The results show ATP production was not affected by TNF-α. Interestingly treatment with positive control CL 316,243, and compound of interest rutin increased ATP production from 92.08 ± 13.19% to 122.9 ± 14.25% (30.85%, $p < 0.001$), and 116.9 ± 12.09% (24.85%, $p < 0.001$) compared to TNF-α, respectively (Figure 12 D). Furthermore, this increase was greater compared to that of the normal control with CL 326,243 increased by 26.87% and rutin increased by 20.87%.

After injection with FCCP an uncoupler of mitochondrial membrane potential, maximal respiration was measured (Figure 12E). The results showed that TNF-α reduced maximal respiration from 285.6 ± 51.44 to 245.3 ± 26.53% (40.29%, $p < 0.05$). The addition of CL 316,243 significantly increased maximal respiration from 245.3 ± 26.53% to 315.8 ± 27.17 (70.47%, $p < 0.001$). No significant change was observed in Rutin treated cells compared to TNF-α group, however, it was significantly decreased by 34.63% ($p < 0.05$) compared to the normal control. The spare capacity of the mitochondria which measures the differences between basal OCR and maximal respiration used by cells during periods of increased energy demand and implying mitochondrial health was measured after injecting Rotenone and Antimycin A (Figure 12F). TNF-α incubation slightly decreased space capacity, however, it was not significant. Treating these cells with CL 316,243 and rutin however significantly increased spare capacity from 45.88 ± 6.72% to 55.73 ± 6.50% (9.86%, $p < 0.001$) and 52.2 ± 3.8% (6.33%, $p < 0.05$).

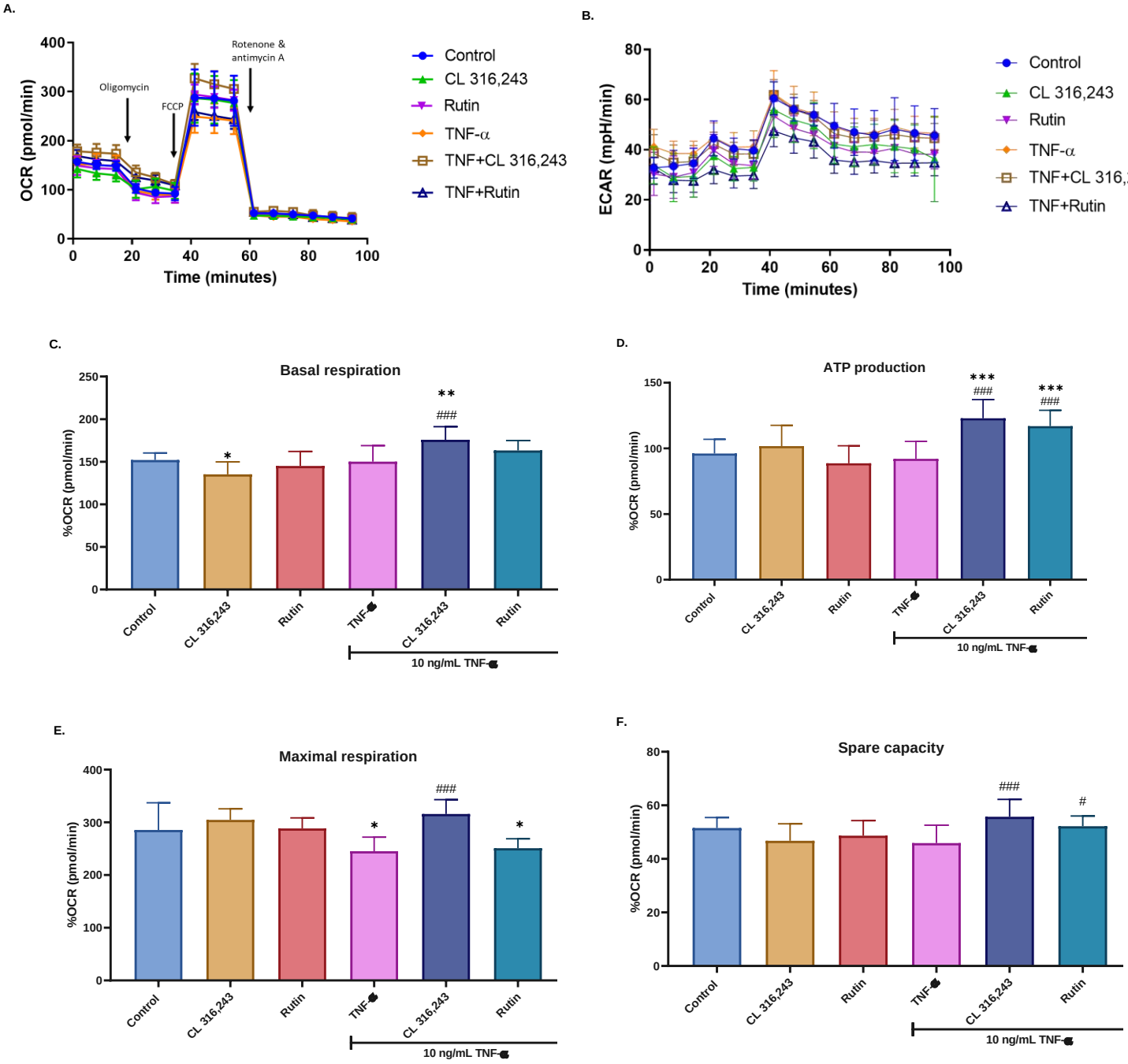

Figure 12: TNF-α reduced mitochondrial function in 3T3-L1 adipocytes.

The graphs represent the oxygen consumption rate (A), extracellular acidification rate (B) for all treatments, basal OCR (C), ATP production (D), maximal respiration (E), and spare capacity (F) of individual treatments. 3T3-L1 adipocytes were cultured in DMEM with 8 mM glucose in the absence or presence of 10 ng/mL TNF-α for 24 h, and then treated with insulin (1 µM) for 15 min, CL 316, 243 (1 µM), and rutin (10 µM) for 6 h. The OCR was measured under basal conditions and after the addition of 2 µM oligomycin, 0.5 µM FCCP, 0.5 µM rotenone and 0.5 µM antimycin A. A one-way analysis of variance (ANOVA) with Tukey post-hoc test was used for statistical analysis. A value of $p < 0.05$ was considered statistically significant.

Results are given as the mean ± SD of 2 independent experiment. Significance is shown as *p < 0.05, **p < 0.01, ***p < 0.001 versus control; # p < 0.05, ### p < 0.001 versus TNF-α.

CHAPTER 5

This chapter provides the overall discussion of the study.

DISCUSSION

This study aimed to investigate the effect of rutin on TNF-α-induced insulin resistance and inflammation in 3T3-L1 adipocytes. The mitochondrial activity, various genes and proteins associated with lipid metabolism, inflammation, and glucose metabolism were investigated. furthermore, the effect of rutin on browning of fat by assessing the gene and protein expression of various markers associated with fat browning was explored. In terms of metabolic activity MTT assay, a measure of metabolically active cells in a group or population of cells which gives an indication of the number of living cells and dead cells within that population was used [28]. This study reports the effect of rutin, positive controls insulin and CL 316, 243 on mitochondrial activity in normal and in TNF-α induced insulin resistance and inflammation cells. Treating with 10 ng/mL TNF-α for 24h had no significant effect on mitochondrial activity. These results are similar to those reported by Jack et al., 2022 [29], in 3T3-L1 adipocytes containing different concentrations of TNF-α (5, 10, 25 and 50 ng/mL) for 24h had no significant effect of cell viability [29]. Interestingly, Xia et al., 2017 [30] reported a reduction in cell viability at 100 ng/mL. Treatment with rutin showed to have significant effect in normal cells after 6h of treatment, while in TNF-α induced insulin resistance and inflammation cells, rutin enhanced mitochondrial activity although not significantly. Similar to the findings from this study, Ma et al., 2021 [20] showed that different concentrations (1, 5, 10, 50, 100 μM) of rutin did not affect mitochondrial activity and measured using the cell counting kit-8 even after a 48h incubation period. Moreover, the positive controls, CL 316, 243 had no effect on mitochondrial activity, however, insulin favoured metabolic activity of the cells.

In obesity, there is an increased level of free fatty acids a result of the hypertrophic dysfunctional adipocytes which secrete these free fatty acid. Moreover, elevated levels of these free fatty acids in circulation have been implicated in the development of insulin resistance and activation of the nuclear factor kappa B (NF-κB) pathway, which induces the secretion of proinflammatory cytokines and inflammation [31]. Chronic low-grade inflammation is associated with adipose tissue in obesity. Inflammation is mainly regulated by cytokines. In obesity there is a dysfunction of adipose tissue which results in the dysregulation of these cytokines promoting increased secretion of pro-inflammatory cytokines over anti-inflammatory. In obesity, the expanding adipose tissue undergoes phenotypic changes along with surrounding immune cells that promote inflammation and hindering the production of anti-inflammatory cytokines and this response of accumulated inflammatory cytokines affects insulin signaling leading to insulin resistance and

other obesity-linked disorders [32]. Pre-clinical and clinical studies have shown that there is increased circulation of pro-inflammatory cytokines in obese animals and humans compared to lean animals and humans. TNF-α is a known cytokine secreted by adipose tissue and is an important key regulator/mediator of inflammation or inflammatory responses. Studies have confirmed that TNF-α is able to facilitates inflammation [33, 34]. This cytokine promotes the expression and generation of other inflammatory cytokines such as IL-8, IL-6, MCP-1, IL-1β and TNF-α itself [29]. In the present study, incubation with TNF-α drastically augmented the mRNA gene expression of TNF-α. Suggesting that TNF-α can induce its transcription at the gene level. Moreover, TNF-α greatly increased the protein levels of IL-6. The findings are corroborated by those of Jack et al.[29]. IL-6 plays a role in various physiological process through its plasticity/ability to switch between pro-inflammatory and anti-inflammatory activity [35]. Clinical studies established that IL6 increased glucose disposal, lipolysis, and fatty acid oxidation [36, 37]. However, Han et al., 2020 [38] suggested that the source of IL-6 expression regulates its inflammatory action. They found that when adipocytes expressed IL-6 in obese state, this resulted in increased adipose tissue macrophages recruitment thereby promoting inflammation, however, when expressed by myeloid cells, this resulted in suppressed adipose tissue macrophage accumulation [38]. The findings from this study indicate an increased inflammatory state by TNF-α in adipocytes. Rutin reduced gene expression of TNF-α and protein levels IL-6 in lipopolysaccharide -induced inflammation in C2C12 cells, moreover, rutin attenuated the activation of NF-κB [39]. Yoo et al., 2014 [40] showed similar effects in HMGB1-induced inflammatory responses in HUVECs cells. *In vivo* studies also show that rutin is able to ameliorate acute liver inflammation induced by carbon tetrachloride in mice by downregulating TNF-α, COX-2, HO-1 and NF-κB [41]. In this study, treatment with rutin stopped inflammation induced by TNF-α by downregulating the levels of TNF-α and IL-6 to normal levels. Pro-inflammatory cytokines, especially IL-6 and TNFα, are present and highly expressed in almost all inflammatory states, making them a potential therapeutic target. The results from this study showed that rutin exerts anti-inflammatory effects against TNF-α mediated inflammation, making rutin a potential therapeutic to treat obesity associated inflammation.

It is well documented that inflamed-insulin resistant adipose tissue is distinguished by loss of lipogenic capacity and increased lipolysis favouring increased secretion of free fatty acids [42]. In this study, the effect TNF-α could have on intracellular lipid content was evaluated. The results showed a significant reduction in lipid accumulation compared to the normal control. These results are in agreement with those in literature in terms of lipid metabolism. Previous studies have reported that TNF-α blocks adipocyte differentiation and lipid accumulation in 3T3-L1 adipocytes

[43-45]. A recent study conducted by Jack and colleagues [29] showed that TNF-α at a dose of 10 ng/mL decreased lipid accumulation after 24h. While Jin et al., 2014 [46] showed that 10 ng/mL TNF-α not only significantly reduced lipid droplet size but also decreased lipid droplet number. Administration of rutin displayed no change in lipid accumulation in normal cells. These results are consistent with those of Jean et al., 2014 [47]. However, Naowaboot [48] reported that rutin was able to activate adipogenesis by enhancing adipogenic transcription factor genes CCAAT/Enhancer-binding Protein (C/EBP) α, peroxisome proliferator-activated receptor gamma (PPARγ), and adipocyte fatty acid-binding protein (aP2) resulting in increased lipid accumulation in a concentration dependent manner (3-100 μM). whereas Han [49] and Ma [20] reported a decrease in lipid accumulation by rutin, with a downregulation of PPARγ, C/EBPα, aP2, FAS, and LPL. These contrasting results could be because of differences in the concentration of rutin used, suggesting that different concentrations of rutin initiate different effects. In this study, to investigate the mechanism through which rutin restores lipid accumulation, The transcription of PPARγ was assessed. It was found that no significant changes in PPARγ expression in normal cells which correlates with the results found in this study in terms of lipid accumulation. when administered as a treatment in TNF-α cells, rutin showed a reversible effect and increased lipid accumulation to near normal control levels. Also an increase in the expression of PPARγ which was inhibited by TNF-α was observed. These results show that rutin is able to restore adipocyte lipid storage capacity abolished by TNF-α.

Reduction in lipid accumulation is associated with increased lipolysis. In this study, an increase in lipolysis after incubation with TNF-α was observed. Therefore, these results supports what is in the literature that TNF-α induces insulin resistance by increasing the rate of adipose tissue lipolysis and elevated levels of free fatty acids [50, 51]. TNF-α induces lipolysis through the regulation of lipid droplet proteins and lipases. TNF-α increases adipose triglyceride lipase activity mainly by upregulating the expression of its co-activator CGI-58 and also by downregulating its inhibitor GOS2 [52]. This enzyme is responsible for the release of fatty acids from triglycerides and catalyzes the first step in lipolysis. Moreover, it decreases hormone sensitive lipase (HSL) which is an enzyme that is crucial to the hydrolysis of triglycerides, diacylglycerols, and monoacylglycerols [52]. Lipolysis by TNF-α is mediated through the activation of MAP kinases such as the extracellular signal-regulated protein kinase (ERK) signal transduction pathway [53]. The activation of ERK1/2 by TNF-α downregulates perilipin [51, 54] which is a protein found on the surface of lipid droplets and plays a role in the adipose lipolysis [55]. Moreover, Green et al., 2004 [51] reported that elevated levels of glucose (hyperglycaemia) exacerbated lipolysis. This study showed that TNF-α significantly increased lipolysis and these results are consistent with

those of Jack et al., 2022 [29], who reported on the increased TNF-α induced lipolysis and also found a decrease in mRNA expression of the HSL, suggesting TNF-α lipolysis could be attributed to the inhibition of HSL [29]. *In vivo* study conducted by Chen et al., 2015 [56] showed that rutin administered at 100 mg in high fat diet-induced obese mice decreased the phosphorylation of HSL and perilipin in epididymal adipose tissue, however, in subcutaneous adipose tissue, rutin had an opposite effect and increased the protein expression of perilipin. In this study, treatment with rutin inhibited TNF-α mediated lipolysis. This result show that rutin has antilipolytic activity. Rutin could improve insulin sensitivity through its antilipolytic activity.

Carnitine palmitoyl transferase 1 (CPT1) is the rate-limiting step of fatty acid oxidation. It is attached to the outer mitochondrial membrane and catalyses long-chain fatty acids into long-chain acyl-carnitine facilitating their transfer in the mitochondria for β-oxidation [57, 58]. Fatty acid oxidation in white adipose tissue is minimal compared to metabolic active tissues such as liver, heart and brown adipose tissue since CPT1 is rarely expressed in white adipose tissue [57]. Expression of CPT1 in adipocytes protects from fatty acid induced insulin resistance and inflammation [58]. Thereby, increased expression of CPT1 in adipocytes could hold potential therapeutic effects. CPT-1 was significantly increased by rutin in skeletal muscle of high fat diet-induced obese rats [59]. This effect was also observed in liver of broiler chickens in a dose dependant manner [60]. Liu et al., 2017 [61] found that treatment with rutin was able to restore CPT1 expression which was downregulated by Oleic Acid. In this study, rutin treatment greatly increased the expression of *Cpt1* in 3T3-L1 adipocytes which was downregulated by TNFα. PPARγ, a master regulator of adipocyte differentiation, plays an important role as a regulator of lipid metabolism and insulin sensitivity [62]. Fatty acids bind to and initiate PPARγ activity, thereby resulting in increased fatty acid uptake by adipose tissue. In vivo studies showed that the selective removal of PPARγ in mature mouse adipocytes, resulted in adipocytes death and hypertrophy of the remaining cells [63, 64]. Whereas the selective activation of PPARγ results in improved insulin sensitivity devoid of weight gain [65]. This suggest that PPARγ is vital for proper functioning of mature adipocytes. Inflammatory cytokines can inhibit or interfere with the function of PPARγ, thus contributing to the development of numerous diseases including atherosclerosis, cancer cachexia, insulin resistance, and inflammation [66]. Ye [66] suggested three mechanisms by which TNF-α inhibits PPARγ activity. One through the obstruction of PPARγ gene expression and the remaining two through suppression of PPARγ ligand-dependent transcription activity. In this study, rutin was able to reverse the downregulation of PPARγ in 3T3-L1 mediated by TNF-α. These results suggest that rutin can initiate a transcriptional program to restore functionality of dysfunctional white adipocytes in states of obesity with chronic inflammation.

Browning of fat, a process of transforming white adipose tissue into beige adipose which holds the same characteristics as brown adipose tissue has become a research interest due to its potential of burning fat, thus making it a potential anti-obesity agent. This browning effect can be induced by β-adrenergic agonist [67], exercise [68], cold exposure [69], and bariatric surgery [70]. Research has shown that plant polyphenols possess this browning capabilities [71]. Making polyphenols a safe and inexpensive promising therapeutic for obesity and obesity related disorders. Hu et al., 2017 [72] showed that rutin could ameliorate polycystic ovary syndrome in rats by brown fat activation, increased thermogenesis, and also improved insulin sensitivity. Ma et al., 2021 [20] found increased gene and protein expression levels of thermogenic genes uncoupling protein 1 (UCP1) and peroxisome proliferator-activated receptor co-activator-1α (PGC1-α) in 3T3-L1 adipocytes and in white adipose tissue of mice fed a high fat diet after treatment with rutin. Rutin induced browning of subcutaneous white adipose tissue by expressing beige specific markers and brown thermogenic genes in both diet-induced obese and genetically obese mice [21]. Moreover, they found that rutin could activate brown adipose tissue by directly binding to Sirtuin 1 (SIRT1) resulting in the deacetylation of PGC1-α thereby stimulating activation of mitochondrial transcription factor and eventually increasing the number of mitochondria and UCP1 activity [21].

The results showed that rutin in normal cells increased BAT specific mRNA gene PR-domain containing 16 (PRDM16). PRDM16 is involved in physiological processes in adipose such as glucose and lipid metabolism and energy homeostasis [73]. It is a key transcriptional regulator of thermogenesis, brown fat development and function [74]. PRDM16 is able to induce the browning program by binding to C/EBPβ, forming a transcriptional complex which then induces the browning of fat and which results in increased expression of PGC1-α and PPARγ which are vital for maturation of brown fat [73]. Also, PRDM16 can directly bind to PPARγ and PGC1-α thereby increasing browning through increased adipogenesis and thermogenesis [73, 75]. PPARγ is deacetylated by SIRT1 at Lys268 and Lys293, which then recruits PRDM16 [76]. Moreover the PPARγ/PRDM16 complex determines adipocytes identity whereas binding of PGC1-α to this complex regulates adipocyte function [62]. Loss of PRDM16 in brown fat result in loss of brown fat characteristics [75]. Similar to Ma et al., 2021 [20], this study found that rutin increased protein levels of PGC1-α . PGC1-α is a key transcriptional coactivator which stimulates mitochondrial biogenesis and regulates energy metabolism [77]. The expression of PGC1-α in white adipocytes is associated with increased mitochondrial biogenesis and increased expression UCP1 and thermogenesis [77]. UCP1 also known as thermogenin is a marker for brown fat or thermogenic fat expressed highly in the mitochondria. Its role in brown fat is to uncouple oxidative

phosphorylation from ATP synthesis and producing heat as a product. However, this data showed no significant changes in UCP1 expression. Moreover, Ma et al., 2021 [20] suggested rutin may induce browning of white adipocytes through the activation of the AMPK pathway in part through regulation by the calmodulin-dependent protein kinase kinase β (CaMKKβ). AMPK was not affected by TNF-α. Moreover, treatment with rutin had no effect although there was a significant increase in normal cells. These findings indicating that rutin can induce brown fat development and increase mitochondrial biogenesis,

TNF-α secretion is increased in obesity by dysfunctional adipocytes. Numerous studies have reported on TNF-α role in inducing insulin resistance and inflammation [34, 78, 79]. TNF-α has been reported to induce insulin resistance in skeletal muscle, adipose tissue and peripheral tissues [79]. TNF-α induces insulin resistance in human adipocytes by impairing Insulin stimulated glucose uptake, blocking GLUT4 expression through inhibiting protein kinase B (AKT/PKB) phosphorylation [34]. Méndez-García et al., 2018 [80] reported that TNF-α at a dose of 5 ng/mL mediated insulin resistance through reduced insulin stimulated glucose uptake in 3T3-L1 adipocytes, reduced insulin receptor substrate-2 and AKT phosphorylation, while simultaneously increasing protein-tyrosine phosphatase 1B phosphorylation. In this study, TNF-α at a dose of 10 ng/mL for 24h induced insulin resistance by blocking the phosphorylation of AKT in fully differentiated 3T3-L1 adipocytes. This study further investigated its effect on AMP-activated protein kinase (AMPK), however, there was no alteration in phosphorylated AMPK production by TNF-α. This suggests that TNF-α mediated insulin resistance is mainly through the AKT pathway.

TNF-α increases inflammation by increasing the expression and secretion of pro-inflammatory cytokines. Jack et al., 2022 [29] showed that at a dose of 10 ng/mL, TNF-α induced inflammatory response in 3T3-L1 adipocytes by increasing both the expression and secretion of inflammatory cytokines MCP-1, IL-6, and IL-1β. In human adipocytes challenged with TNF-α, it was found that inflammation was achieved by the upregulation of genes ICAM-1, CXCL-10, MCP-1, and downregulating ADIPOQ [34]. This study confirmed inflammation by evaluating mRNA gene expression of TNF-α and protein secretion of IL-6. It was found that exposure of 3T3-L1 adipocytes to TNF-α greatly exacerbated the expression of TNF-α gene and production of IL-6 proteins in the medium. This study shows that exposure to TNF-α aggravates the expression and secretion of pro-inflammatory cytokines resulting in chronic inflammation.

To evaluate rutins role in insulin signaling, the effect of rutin on insulin signaling pathways was assessed, focusing on the AKT and AMPK pathways which are insulin dependent and insulin-independent signaling pathways, respectively. AKT also referred to as protein kinase B is a

serine/threonine kinase vital to insulin-stimulated glucose uptake by various insulin-responsive tissues. The activation of AKT pathway ameliorates insulin resistance via translocating glucose transporters to the cell membrane resulting in increased uptake of glucose by the cells [81]. TNF-α has been implicated in mediating insulin resistance [82]. TNF-α induced insulin resistance in 3T3-L1 adipocytes by increasing phosphorylation of IRS-1 thereby suppressing IRS-1 activity and decreased the insulin-stimulated phosphorylation of AKT [83]. This study found a decreased expression of pAKT by TNF-α. Similar to these findings, Quarta et al., 2021 and Medina et al., 2005 [34, 84] showed that TNF-α reduce AKT protein levels in 3T3-L1 adipocytes, moreover the impairment of AKT was in a dose and time dependent manner due to caspase-dependent degradation of AKT via the cleavage and ubiquitination of Akt [84]. Lim et al., 2021 [85] showed that rutin activated insulin-stimulated glucose uptake by activating the phosphorylation of AKT at a concentration of 10 µM. Similarly, the findings from this study demonstrated that rutin was able to upregulate the protein expression of pAKT in both normal cells and TNF- α treated cells indicating that rutin can reverse the TNF-α mediated insulin resistance by activating the AKT pathway.

Various factors such as exercise, increased AMP/ATP ratio, and diabetic drug metformin are able to activate the AMPK pathway and activation of this pathway results in increased free fatty acid oxidation and glucose uptake [81]. In the present study, rutin elevated the protein levels of phosphorylated AMPK in normal cells. These results are in agreement with those of Lim et al., 2021 and Ha et al., 2010 [85, 86], they reported that rutin at a concentration of 10 µM activated AMPK suggesting a role of rutin in insulin-independent glucose uptake. On the other hand, TNF-α did not elicit any difference in phosphorylated AMPK levels. Different to these results, Cheng et al. 2019 [45] showed a decreased levels of phosphorylated AMPK after incubation with TNF-α of the same concentration and time period in same cell line. However, Nagahara [87] found that at a dose of 50 ng/mL, TNF-α had no effect of AMPK activity. When rutin was added as a treatment in these cells, there was no significant changes observed. These results suggest that rutin can ameliorate TNF-α mediated insulin resistance mainly through the activation of the insulin-dependent AKT pathway without affecting insulin-independent AMPK pathway.

Mitochondria are the main site of a greater amount of energy production in the form of ATP which is needed for the proper functioning of the cell through the electron transport chain and mitochondrial ATP synthase [88]. Any irregularities in mitochondrial activities can cause mitochondrial dysfunction. TNF-α has been shown to cause mitochondrial dysfunction through reduced mitochondrial biogenesis, changing mitochondrial morphology, increasing the generation of reactive oxygen species, decreased mitochondrial electron respiratory complex expressions,

diminishing the mitochondrial membrane potential, and reducing ATP synthesis [89-91]. Measuring changes in mitochondrial respiration parameters such as basal respiration, ATP production, proton leak, maximal respiration, spare capacity, and non-mitochondrial respiration is a useful tool to assess mitochondrial function [92]. Hahn et al., 2014 [93] previously reported that after prolonged exposure (96h) of the 3T3-L1 adipocytes to 1 nM of the inflammatory cytokine TNF-α, basal OCR, ATP production, and maximal respiration were decreased. The results of this study showed TNF-α-mediated mitochondrial dysfunction through decreased mitochondrial maximal respiration after 24h incubation. Interestingly, rutin was able to increase ATP production and spare capacity in TNF-α challenged cells comparable to the positive control CL 316,243. Although literature on rutin's effect on mitochondrial energetics is limited, Trumbeckaite et al., 2006 [94] found that rutin at a dose of 44.4 ng/ml in isolated heart mitochondria uncoupled oxidative phosphorylation and the same dose showed no significant effect was detected on ATP synthesis by mitochondria. They suggested that partial mitochondrial uncoupling without affecting ATP synthesis could be the mechanism which rutin uses to exert its beneficial effects. Results from this study indicate that rutin can increase the mitochondrial capacity when exposed to increased energy demand and is, therefore, able to generate more ATP to sustain cellular functions under stress [95] and therefore could be a potential player in mitochondrial energetics.

Conclusion

- TNF-α is a cytokine secreted by macrophages and adipocytes, can induce inflammation and obesity-related insulin resistance.
- This study showed that rutin has antilipolytic activity, which could be one way rutin can protect against TNF-α insulin resistance since free fatty acids are released during lipolysis and are known to mediators of insulin resistance.
- Rutin showed improved adipocyte function by increasing Pparγ and Cpt1.
- Rutin showed to attenuate TNF-α-induced inflammation by suppressing the gene and protein expression of pro-inflammatory cytokines.
- Resulted in the activation of AKT which is responsible for insulin mediated glucose uptake, suggesting that rutin ameliorates insulin resistance through activation of the AKT pathway.
- Rutin initiated fat browning program and increased mitochondrial biogenesis by increasing Prdm16 and PGC1-α.
- These findings provide evidence which supports rutin as a potential therapeutic in combating TNF-α-induced inflammation and insulin resistance in obesity.

Study limitations and considerations for future studies

- Due to a limited budget, we were unable to investigate NF-kB pathway and GLUT4.
- For future studies it would be interesting to investigate the expression of GLUT4 for insulin signalling and exploring its effect on inflammatory pathways such as the NF-kB pathway.
- The effect of rutin on TNF-α-induced insulin resistance and inflammation should further be investigated *in vivo* to get a better understanding of its effects and to establish the underlying mechanisms.
- Further experiments needed to confirm the role of AMPK in rutin response against inflammation.

CHAPTER 6

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APPENDIX 1

Table 10: List of reagents and kits used in this study

Product name	Catalogue number	Supplier
B-actin	sc-47778	Santa Cruz Biotechnology, Santa Cruz, CA, USA
2-mercaptoethanol	M3148	Sigma-Aldrich, St Louis, MO, USA
2X Laemmli sample buffer	161-0747	Bio-Rad, Hercules, CA, USA
3-Isobutyl-1-methylxanthine (IBMX)	I5879	Sigma-Aldrich, St Louis, MO, USA
3T3-L1 pre-adipocytes	CL-173	American Type Culture Collection (ATCC), Manassas, VA, USA
5' adenosine monophosphate-activated protein kinase (AMPK) Total AMPKα antibody	2532	Cell Signaling Technology, Danvers, MA, USA
5' adenosine monophosphate-activated protein kinase (AMPK) Phosphor-AMPKα (Thr172) antibody	2535	Cell Signaling Technology, Danvers, MA, USA
Acetic acid	695092	Sigma-Aldrich, St Louis, MO, USA
All blue	1610393	Bio-Rad, Hercules, CA, USA
Ambion nuclease-free water	AM9938	Ambion, Austin, TX, USA /invitrogen
Agarose	A9539	Sigma-Aldrich, St Louis, MO, USA
Bovine serum albumin (BSA)	15561-020	Invitrogen, Carlsbad, CA, USA
Calcium chloride	C4901	Sigma-Aldrich, St Louis, MO, USA

Cell extraction lysis Buffer	FNN0011	Life Technologies Corporation, Carlsbad, CA, USA
Chloroform	0757	AMRESCO, Solon, OH, USA
CL 316,243 hydrate	C5976	Sigma-Aldrich, St Louis, MO, USA
Clarity western ECL substrate	170-5060S	Bio-Rad, Hercules, CA, USA
Coomasie blue	161-0437	Bio-Rad, Hercules, CA, USA
Crystal violet	C0775	Sigma-Aldrich, St Louis, MO, USA
dexamethasone	D4902	Sigma-Aldrich, St Louis, MO, USA
Dimethyl sulfoxide (DMSO)	D8418	Sigma-Aldrich, St Louis, MO, USA
Dulbecco`s modified Eagle`s medium (DMEM)	12-604F	Lonza, Walkersville, MD, USA
Duoset ancillary reagent kit	DY008	Whitehead scientific, CPT, SA
Ethanol	34923	Sigma-Aldrich, St Louis, MO, USA
Ethidium bromide	15585-011	Invitrogen, Carlsbad, CA, USA
Extra thick blot paper filter paper	1703965	Bio-Rad, Hercules, CA, USA
Fetal Bovine Serum (FBS)	10270-106	GIBCO by Life technologies
Formalin solution, neutral buffered	HT501128	Sigma-Aldrich, St Louis, MO, USA
Gel loading dye purple (6X)	B7025S	Biolabs, UK
glucose	SAAR2676020EM	MERCK MILLIPORE
Glycerol assay kit	MAK117-1KT	Sigma-Aldrich, St Louis, MO, USA
Glycine	G7126	Sigma-Aldrich, St Louis, MO, USA
HEPES buffer solution (1M)	01955	Gibco life tecnologies
Hydrochloric acid (37%)	320331	Sigma-Aldrich, St Louis, MO, USA
Immune-Blot LP PVDF filter paper	162-0263	Bio-Rad, Hercules, CA, USA

Instant skimmed milk powder		Spar, SA
Insulin	I9278	Sigma-Aldrich, St Louis, MO, USA
Isopropanol (70%)	563935	Sigma-Aldrich, St Louis, MO, USA
Isoproterenol hydrochloride	I6604	Sigma-Aldrich, St Louis, MO, USA
Loading dye	B7025S	Biolabs
Lysis buffer	9803	Cell signaling technology, Danvers, MA, USA
Magnesium chloride	M8266	Sigma-Aldrich, St Louis, MO, USA
Methanol	322415	Sigma-Aldrich, St Louis, MO, USA
Mini-PROTEAN TGX gels	4561044	Bio-Rad, Hercules, CA, USA
Molecular biology grade water	51200	Lonza, Walkersville, MD, USA
Mouse duoset ELISA	DY406-05	Whitehead scientific, CPT, SA
Nitrocellulose membrane	162-0146	Bio-Rad, Hercules, CA, USA
Oil red o	O0625	Sigma-Aldrich, St Louis, MO, USA
Pen/Strep Amphotericin B (100X)	17-745E	Lonza, Walkersville, MD, USA
Peroxisome proliferator-activated receptor-gamma coactivator 1alpha (PGC1-α)	2178	Cell Signaling Technology, Danvers, MA, USA
Phosphate buffered saline (10X)	17-517Q	Lonza, Walkersville, MD, USA
Ponceau S	P3504	Sigma-Aldrich, St Louis, MO, USA
Potassium chloride	P4504	Sigma-Aldrich, St Louis, MO, USA
Protease Inhibitors	11697498001	Roche, Basel, Switzerland
Protein assay dye reagent concentrate	500-0006	Bio-Rad, Hercules, CA, USA

Protein kinase B (AKT) Phospho-AKT (Ser473) antibody	9271	Cell Signaling Technology, Danvers, MA, USA
Protein kinase B (AKT) Total AKT antibody	9272	Cell Signaling Technology, Danvers, MA, USA
QuantiTect reverse transcription kit	205313	Qiagen, Hilden, Germany
Quick start bovine serum albumin (BSA) standard	5000206	Bio-Rad, Hercules, CA, USA
Quick start Bradford 1X dye reagent	5000205	Bio-Rad, Hercules, CA, USA
Rosiglitazone	R2408	Sigma-Aldrich, St Louis, MO, USA
Running/ buffer 10x tris/glycine.SDS buffer	1610732	Bio-Rad, Hercules, CA, USA
rutin	PHL89270	Sigma-Aldrich, St Louis, MO, USA
Sodium bicarbonate	S8875	Sigma-Aldrich, St Louis, MO, USA
Sodium chloride	31434	Sigma-Aldrich, St Louis, MO, USA
Sodium dodecyl sulfate	1610302	Bio-Rad, Hercules, CA, USA
TAE buffer (50x)	K915	AMRESCO, Solon, OH, USA
Taqman fast advance master mix	4444557	Thermo Fisher Scientific, Waltham, MA, USA
Thiazolyl blue tetrazolium bromide (MTT)	M5655	Sigma-Aldrich, St Louis, MO, USA
Trizma base	93352	Sigma-Aldrich, St Louis, MO, USA
TRIzol reagent	15596018	Invitrogen, Carlsbad, CA, USA
Trypsin-EDTA (1X)		GIBCO

Tumor necrosis factor-α from mouse (TNF-α)	T7539	Sigma-Aldrich, St Louis, MO, USA
Tween20	P1379	Sigma-Aldrich, St Louis, MO, USA
Water for cell culture applications	17-724F	Lonza, Walkersville, MD, USA
Western c	1610398	Bio-Rad, Hercules, CA, USA

Table 11: List of equipment and software

Equipment	Catalogue number	Supplier
Centrifuge	15227589	Thermo Fisher Scientific, Waltham, MA, USA
Gel Doc Image lab software	Version 6.1	Bio-Rad, Hercules, CA, USA
Gel Doc XR	170-8199	Bio-Rad, Hercules, CA, USA
Graphpad Prism ®	Version 8.0.0	Graphpad Software Inc, CA, USA
Microsoft office	Excel / Word / PowerPoint	Microsoft Corporation, WA, USA
Multiskan GO plate reader	51119250	Thermo Fisher Scientific, Waltham, MA, USA
Nanodrop OneC Microvolume	AZY2123199	Thermo Fisher Scientific, Waltham, MA, USA
Nuve NF 200 benchtop centrifuge		NÜVE, Saracalar, Akyurt, Turkey
StepOnePlus Real-Time PCR system machine	4376600	Applied biosystems
T100 thermal cycler	186-1096	Bio-Rad, Hercules, CA, USA
Trans-Blot Turbo transfer system	1704150	Bio-Rad, Hercules, CA, USA
Vortex	CLS6776	Corning, MA, USA

Disposables	Catalogue number	Supplier
Bio-pure pipetting reservoir	Z679968	Sigma-Aldrich, St Louis, MO, USA
Microplates, 96-well	655101	Greiner bio-one, Frickenhausen, Germany
T25 flask	C6356	Greiner bio-one, Frickenhausen, Germany
T75 flask	C7231	Greiner bio-one, Frickenhausen, Germany
CELLBIND-96 well plates	3300	Corning, MA, USA
CELLBIND-24 well plates	3337	Corning, MA, USA
CELLBIND-6 well plates	3335	Corning, MA, USA
MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL	4346907	Thermo Fisher Scientific, Waltham, MA, USA
MicroAmp optical adhesive film	4311971	Thermo Fisher Scientific, Waltham, MA, USA
MicroAmp™ Reaction Tube with Cap, 0.2 mL	N8011540	Thermo Fisher Scientific, Waltham, MA, USA
Cell scrappers	710011	Whitehead scientific, CPT, SA

APPENDIX 2

Preparation of medium and buffers

- DMEM used in this study consisted of 10% FBS and 1% pen/strep
- Freezing media for cryopreservation of 3T3-L1 cells consisted of DMEM containing 10% FBS and 7% DMSO

Preparation of 0.1 mg/mL TNF-α stock

10 µg TNF-α was dissolved in 100 µL tissue culture treated water.

Table 12: Krebs ringer bicarbonate HEPES buffer (500 mL)

Reagent	Molecular weight	Final concentration	Weight
NaCl	58.44	115 mM	3.36 g
NaHCO3	84.01	24 mM	1.008 g
KCL	74.55	5 mM	0.186 g
MgCL2	95.21	1 mM	0.048 g
CaCl2	110.98	2.5 mM	0.139 g
BSA		0.1% (w/v)	0.5 g
HEPES	238.3	10 mM	5 mL

Sorenson's buffer

Sorenson's buffer was prepared by dissolving 0.751 g glycine and 0.584 g NaCl in 100 mL distilled water. The pH of the buffer was adjusted to 10.5 with 0.1 mM NaOH.

Table 13: Chemicals used in preparation of sorenson’s buffer

Chemical	Final concentration	Amount for 100 mL
Glycine	0.1 M	0.751 g

NaCl	0.1 M	0.584 g

RIPA buffer

NaCl and Tris were weighed and added into a 100 mL bottle together with 500 Triton X-100 and mixed with 40 mL dH2O. using a magnetic stirrer, the solution was mixed until the solvents had completely dissolved. The pH of the solution was adjusted to 8.0 using 37% HCL. Thereafter, 10% SDS was added to the solution and the volume was adjusted to 50 mL with dH2O. the buffer was aliquoted to 1.5 mL tubes and stored at 4°C and -20°C for short term and long term storage, respectively.

Table 14: Chemicals used in preparation of RIPA buffer

Chemical	Final concentration	Amount for 50 mL
NaCl (58.44 g/Mol)	150 mM	0.4383 g
Triton X-100	1%	500 µL
SDS	0.1%	500 µL of 10% SDS
Tris (121.14 g/Mol)	50 mM	0.30285 g

10X Running buffer

Tris (30.285 g) and glycine (145.34 g) were weighed and added into a 1 L bottle, thereafter, 750 mL of dH_2O was added. Using a magnetic stirrer, the solution was mixed until the solvents had completely dissolved. The pH of the solution was adjusted to 8.3 using 37% HCL. Thereafter, 10 % SDS (100 mL) was added to the solution and the volume was adjusted to 1 L using dH_2O. The buffer was stored at 4°C until use.

Table 15: Chemicals used in preparation of 10X Running buffer

Chemical	Final concentration	Amount for 1 L
Tris (121.14 g/Mol)	250 mM	30.285 g
Glycine (75.7 g/Mol)	1.92 M	145.34 g

SDS	1%	100 mL of 10% SDS

10X TBS

NaCl (87.66 g) and Tris (30.285 g) were weighed and added in a 1 L bottle and 850 dH_2O was added. Using a magnetic stirrer, the solution was mixed until all solvents had dissolved. The pH was adjusted to 7.5 with 37% HCL. Thereafter, dH_2O was used to adjust the final volume to 1 L and the buffer was stored at 4°C until use.

Table 16: Chemicals used in preparation of 10X TBS

Chemical	Final concentration	Amount for 1 L
NaCl (58.44 g/Mol)	1.5 M	87.66 g
Tris (121.14 g/Mol)	250 mM	30.285 g

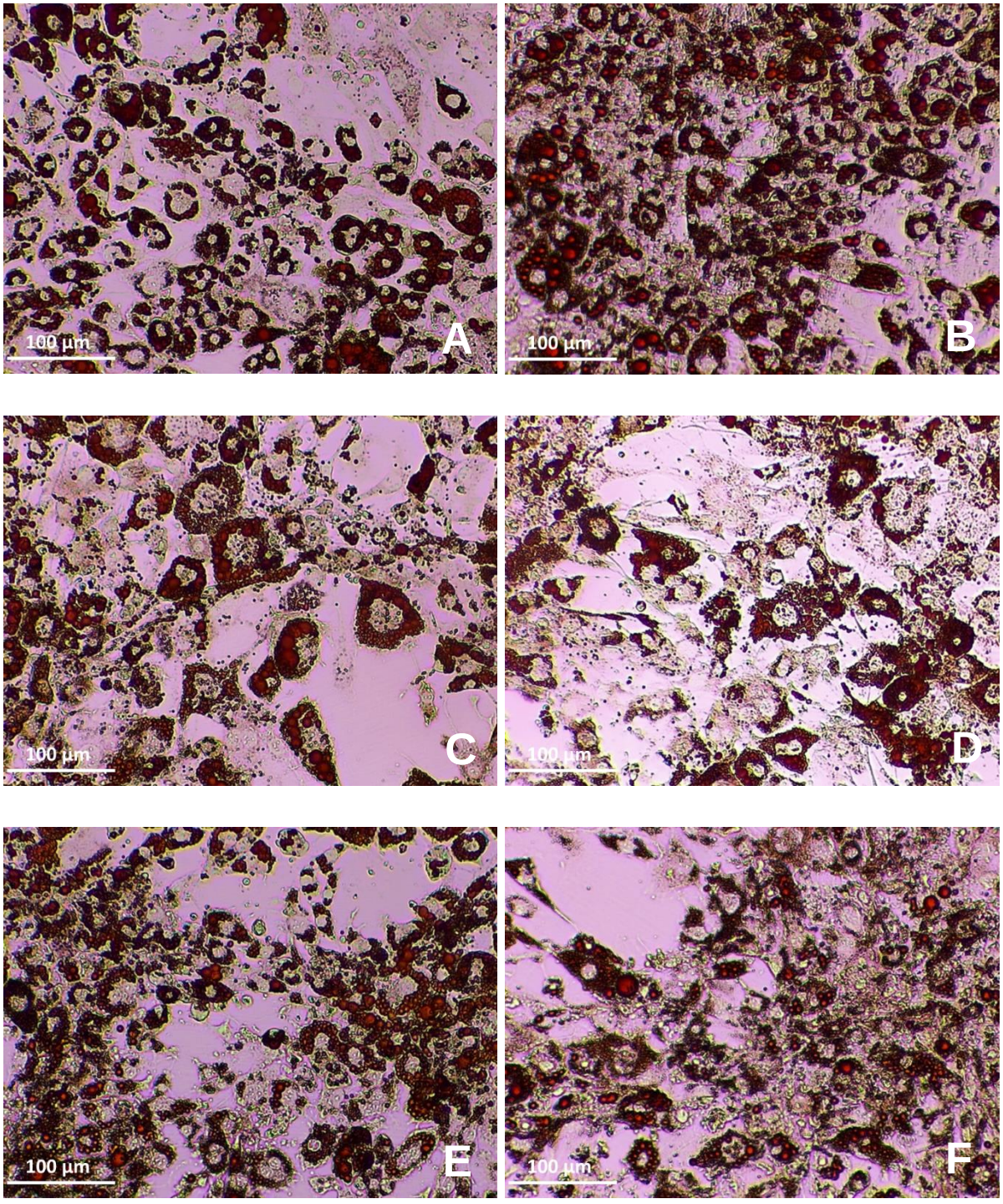
1x Tris-buffered saline and tween20 (1x TBST)

Working concentration of TBST was prepared by diluting 100 mL of 10x TBS with 900 mL of dH2O and 1 mL of tween20 was added to the buffer. The buffer was stored at 4 degrees until use.

APPENDIX 3

Oil red o staining

Lipid droplet accumulation in differentiated 3T3-L1 adipocytes cultured with and without TNF-α was assessed using oil-red-o staining.



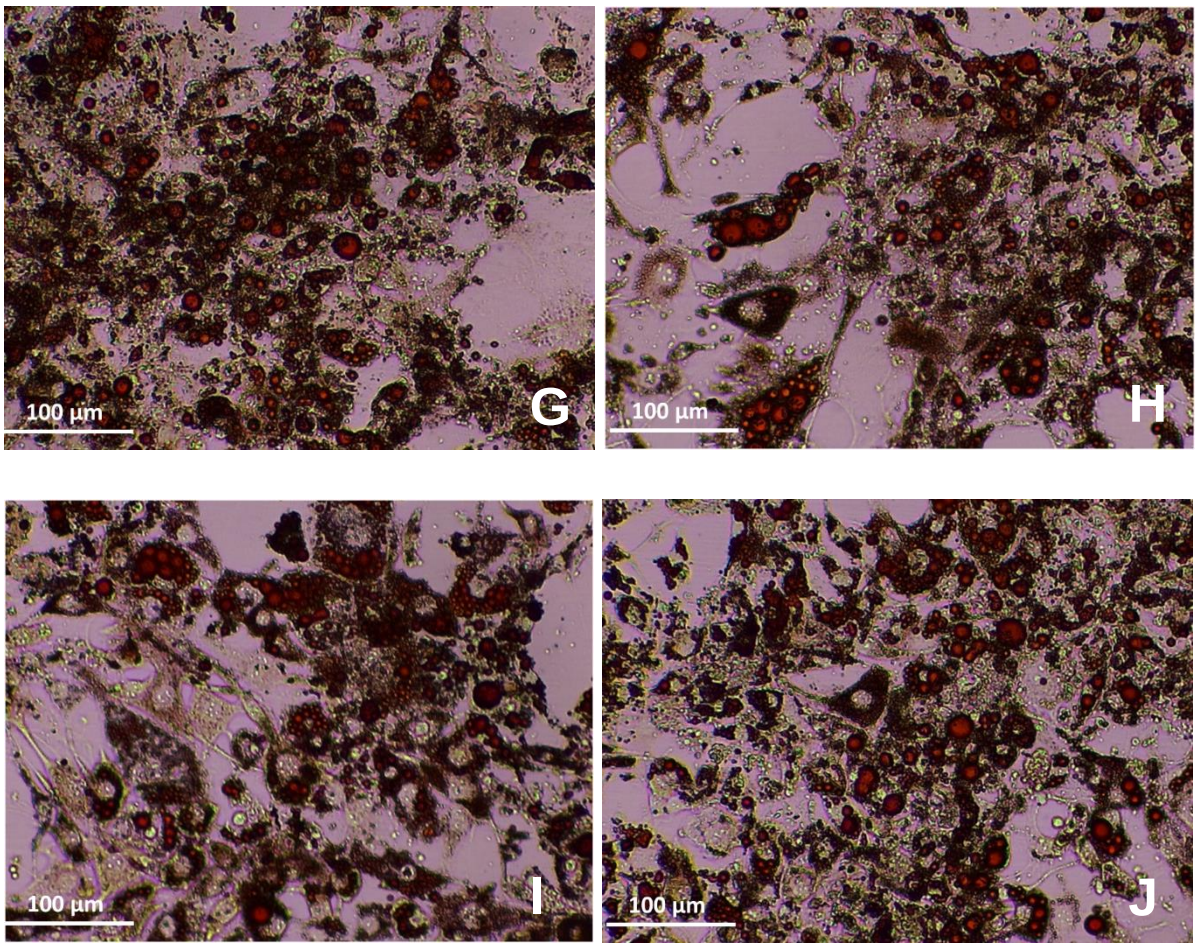


Figure 13. Effect of rutin on lipid accumulation in 3T3-L1 adipocytes. 3T3-L1 adipocytes were cultured in DMEM with 8 mM glucose in the absence and presence of 10 ng/mL TNF-α for 24 h, and then treated with insulin (1 µM) for 15 min, CL 316, 243 (1 µM), isoproterenol (10 µM) and rutin (10 µM) for 6 h. Images depict cells in the absence (**A**: Control, **B**: Insulin, **C**: CL 316,243, **D**: Isoproterenol, **E**: Rutin) and presence of TNF-α (**F**: TNF-α, **G**: insulin, **H**: CL 316,243, **I**: Isoproterenol, **J**: Rutin).

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North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

6 November 2021

RESEARCH ETHICS COMMITTEE LETTER OF DECISION: NO RISK

Based on the review by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 06/11/2021, the NWU-AnimCareREC hereby clears your study as a no risk study. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met, the study may be initiated, using the ethics number below.

Study title: Effect of rutin on TNF-α-induced insulin resistance in 3T3- L1 adipocytes
Principal Investigator/Study Supervisor/Researcher: Prof SE Mazibuko-Mbeje
Student: N Muvhulawa - 32917392

Ethics number:

N	W	U	-	0	0	4	4	4	-	2	1	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: No Risk
Commencement date: 06/05/2021

Risk: No Risk

General conditions:

The following general terms and conditions will apply:

- *The commencement date indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research;*
  - *withdraw or postpone clearance if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*