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ORIGINAL ARTICLE

Quercetin-3-*O*-rutinoside from *Moringa oleifera* Downregulates Adipogenesis and Lipid Accumulation and Improves Glucose Uptake by Activation of AMPK/Glut-4 in 3T3-L1 Cells

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Abstract
Natural product-based therapeutic alternatives have drawn immense interest to deal with growing incidence of metabolic disorders. Rutin (quercetin-3-*O*-rutinoside) is found in a variety of fruits, vegetables, and plant beverages. In the present study, rutin was isolated from *Moringa oleifera* Lam., leaves and its anti-lipidemic and anti-adipogenic activity was evaluated through inhibition of key digestive enzymes and *in vitro* cell culture experiments using 3T3-L1 adipocytes. Rutin treatment substantially reduced α-glucosidase and pancreatic lipase activities with IC_{50} values of 40 and 35 µg/ml, respectively. MTT assay with 3T3-L1 cells demonstrated the non-toxic effect of rutin up to 160 µg/ml. Oil Red O-stained images of rutin-treated 3T3-L1 cells depicted that rutin considerably reduced lipid content and adipogenesis (79.9%), and enhanced glycerol release in 3T3-L1 cells when compared to untreated cells. Rutin significantly ($p < 0.05$) enhanced glucose uptake in 3T3-L1 adipocytes and also led to reduced levels of leptin but enhanced levels of adiponectin. Western blot analysis of rutin-treated (40 µg/ml) adipocytes showed phosphorylation of AMPK, upregulated expression of Glut-4 (1.31-fold) and UCP-1 (1.47-fold), but downregulated expression of PPAR-γ by 0.73-fold. At transcriptional level, similar trends were observed in the mRNA expression of the above genes, except AMPK. Our results demonstrate that rutin isolated from *M. oleifera* significantly alleviates lipid content and adipogenesis, and improves glucose uptake through regulating PPAR-γ and AMPK signaling pathways; thus, rutin can be considered as a potential therapeutic agent against adiposity and glucose intolerance.

Keywords Adipogenesis · Adipokines · α-Glucosidase · Glucose uptake · Lipase · Rutin

Introduction

The prevalence of overweight and obesity has nearly tripled in the past two decades across the world and is now regarded as "global public health syndrome" because of the vast array of obesity-centered diseases including hypertension, diabetes mellitus, dyslipidaemia, cardiovascular diseases, and some types of cancers (Hruby and Hu 2015). If this scenario continues, it leads to a growing population of chronic ailing people that debilitate socioeconomic conditions and even reduce life span of the current and emerging generations. Moreover, recent reports by the World Obesity Federation have highlighted the potential link between obesity and high mortality rate in COVID-19-infected patients (Popkin et al. 2020). Multiple preventive and therapeutic methods have been tried; nevertheless, these efforts have not produced the expected results (Harms and Seale 2013; Crimmins 2015). Therefore, there is an imminent need to develop effective therapeutic alternatives to treat obesity ailments.

Different therapeutic strategies have been proposed like identifying molecules that act as appetite suppressants, lipase inhibitors, inhibitors of adipogenesis, and inducers of thermogenesis to contain obesity, insulin resistance, and co-morbidities (Azhar et al. 2016; Balaji et al. 2016). Emerging research evidence manifests the promising role of lipase inhibitors because they reduce lipid digestion, transport, and accumulation (Birari and Bhutani 2007; Liu et al. 2020). Similarly, inhibition of amylase and glucosidase could reduce carbohydrate digestion and dietary

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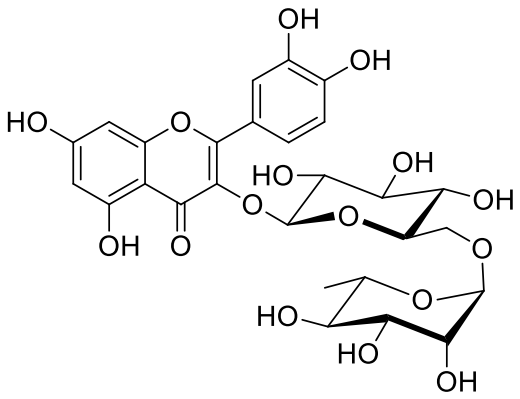
energy intake. The excess triacylglycerides are stored in the liver, making it fatty liver or in adipocytes causing adipose tissue growth (Les et al. 2018). Adipogenesis is the accumulation of fat in visceral, subcutaneous, and peritoneal tissues. Hypertrophy and hyperplasia are two primary events that cause adipose tissue to expand, resulting in overweight and obesity (Liu et al. 2020). At the molecular level, adipogenesis involves the regulation of several signaling molecules involved in adipocyte differentiation and lipogenesis, such as CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding proteins (SREBPs), and associated genes such as acyl Co-A carboxylase (ACC) and fatty acid synthase (FAS) (Shen et al. 2014). The AMP-activated protein kinase enzyme is important for maintaining energy balance. Glut-4 glucose absorption is facilitated by drugs that activate AMPK (Chow et al. 2017). AMPK and related signaling molecules have previously been identified as possible targets in the treatment of obesity and diabetes. Mitochondria, through its uncoupling protein 1 (UCP-1), play an important role in the thermogenesis process. UCP-1 is an important isoform of the UCP family that is found in BAT and is a well-studied marker of adipocyte beiging/browning. And UCP-1 appears in the WAT in times of previous stimulus such as cold and exercise. It has been postulated that activation of UCP-1 by stimuli such as cold, exercise, and diet uncouples respiration from energy production and increases non-shivering thermogenesis (NST), resulting in higher energy expenditure and obesity reduction (Li et al. 2018). As a result, finding molecule(s) that function on several targets could be more effective in combating obesity disorders.

In view of the considerable adverse effects associated with existing synthetic drugs, there is growing public inclination towards plant-based therapeutics, and dietary phytochemicals are gaining preference to treat/prevent diseases owing to their non-toxic and edible nature (Torres-Fuentes et al. 2015). Thus, many researchers are interested to explore potential natural components from foods, vegetables, and spices to combat obesity ailments. *Moringa oleifera* Lam, Moringaceae, also referred as drumstick tree or "miracle tree" has drawn enormous attention because its leaves are abundantly rich in many beneficial secondary metabolites, vitamins, and minerals and it is reported for multiple health benefits (Ghasi et al. 2000; Vergara-Jimenez et al. 2017). Flavonoids comprise an important group of bioactive polyphenolic compounds found in a variety of plants, vegetables, and fruits including *M. oleifera* leaves. Previously, we reported the bioactivity of *M. oleifera* leaf extract against oxidative stress and obesity, and in the present study, we isolated rutin (**1**, quercetin-3-*O*-rutinoside), a flavanol glycoside from *M. oleifera* leaves and carried out anti-obesity studies.



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Some research studies have shown that rutin has antioxidant, anti-inflammatory, anti-hyperglycemic, antihypertensive, and anti-hyperlipidemic properties (Niture et al. 2014; Yoo et al. 2014; Livingston Raja et al. 2021). However, there are no elaborate studies on how it attenuates obesity and improves glucose tolerance, and the mechanism of action is not totally understood. Therefore, the present study was carried out with 3T3-L1 cells, the well established *in vitro* cell culture model for adiposity studies.

Materials and Methods

LC–MS Analysis

Moringa oleifera leaves were collected from Tirupati market, Andhra Pradesh, India. Its identity was authenticated by Dr. Madhava Chetty, Department of Botany, at S.V. University, Tirupati, voucher number 136, and a specimen has been preserved at the departmental herbarium. Leaves were shad dried, pulverized, and soaked in maceration chambers with alcohol and water mixture (50:50) at room temperature. The collected hydro-alcoholic crude extract of *M. oleifera* was concentrated in a rota-evaporator. This extract was fractionated with different polarity-based solvent systems through column chromatography using silica gel as the column material. The active fractions were subjected to LC–MS analysis (UPLC H-class LC-MSD 3100 series) for identifying a series of compounds. To isolate and purify rutin (**1**), the fraction containing rutin was subjected to preparative reverse-phase HPLC using a C18 column. The mobile phase used was (A) 0.1% formic acid solution and (B) acetonitrile. The gradient was set up as 5% B for 5 min, 5 to 50% B for 10 min, 50 to 100% B for 20 min, and 100% B up to 30 min, at a flow rate of 15 ml/min. Rutin quantification was carried out in duplicates using a standard rutin absorption curve.

α-Glucosidase Activity Inhibition

The α-glucosidase inhibitory potential of rutin was determined based on a slightly modified version of a previously published method (Chipiti et al. 2017). Briefly, 250 µl of rutin (soluble in 0.01% DMSO) and/or acarbose (standard), both at varying concentrations (10–80 µg/ml), was incubated with 500 µl of α-glucosidase (1 U/ml) in phosphate buffer (with a concentration of 100 mM, pH 6.8) for 15 min at 37 °C. Then, 250 µl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) solution prepared in phosphate buffer was added to the reaction mixture and further incubated for 20 min at 37 °C. The *p*-NPG was used as substrate and the absorbance of the released *p*-nitrophenol was measured at 405 nm. The inhibitory activity was reported as the proportion of the control devoid of inhibitors using this equation.

$$Inhibition(\%) = 1 - \left(absorbance\ of\ \frac{sample}{control} \right) \times 100$$

Lipase Activity Inhibition

Pancreatic lipase inhibitory activity of rutin was determined using a slightly modified procedure as reported by Ganjayi et al. (2017). About 100 µl of rutin (soluble in 0.01% DMSO) and/or orlistat (standard drug), both at varying concentrations (10–80 µg/ml), was incubated with 880 µl enzyme buffer (2.5 mg/ml of lipase solution in 10 mM morpholine propane sulphonic acid (MOPS) and 1 mM EDTA, at pH 6.8) followed by addition of 20 µl of substrate solution (10 mM *p*-nitrophenyl butyrate (*p*-NPB) in dimethyl formamide) for 15 min at 37 °C. The lipase inhibitory activity was extrapolated by recording the hydrolysis of *p*-NPB to p-nitrophenol by measuring absorbance at a wavelength of 405 nm. The inhibition rate of lipase by rutin was calculated by using the following expression.

$$Inhibition(\%) = 1 - \left(absorbance\ of\ \frac{sample}{control} \right) \times 100$$

Adipogenesis Induction

The murine 3T3L1 pre-adipocytes from American Type Culture Collection (ATCC) were cultured in DMEM with 10% FBS and 1% penicillin and streptomycin, at 37 °C in humidified atmosphere with 5% CO_2, and for adipogenesis studies, 3T3-L1 cells were grown to confluence; after that, cells were stimulated with adipocytes' differentiation medium of induction (DMI) consisting of DMEM, 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone (DEX), and insulin (10 µg/ml), incubated for 2 days followed by treatment of the cells with differentiation medium, DM (DMEM with 10% FBS and 10 µg/ml of insulin) for additional 8 days. Rutin was solubilized in 0.01% of DMSO and used for further *in vitro* assays at different concentrations (10, 20, and 40 µg/ml) and DMSO was used as vehicle control.

Cell Viability

A colorimetric assay to determine the toxicity of the test compound on the cells based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals by the lactate dehydrogenase present in live cells was used. The 3T3L1 pre-adipocytes were cultured in DMEM, and cell viability assay was conducted as previously described (Ganjayi et al. 2020). Cells were plated at a density of 1.5×10^4 cells/ml in a 96-well plate, treated with rutin at varying concentrations (10–160 µg/ml), and incubated with MTT solution for 3 h at 37 °C. The supernatants were aspirated, DMSO was added to each well, and the plates were agitated to dissolve the crystal product. Absorbance was measured after 24 and 48 h at 570 nm using a micro-well plate reader (Bio-Rad). The cytotoxicity was compared using the absorbance value of untreated control cells as the baseline.

Lipid Content

The 3T3-L1 pre-adipocytes were seeded in a 6-well plate, allowed to reach 70–80% confluency. After that, cells were provided with differentiation medium as mentioned above to undergo differentiation. On the 8th day of cell differentiation, the control and rutin-treated (10, 20, and 40 µg/ml) adipocytes were washed with phosphate-buffered saline (PBS) (pH 7) and then fixed with 10% buffered formalin for 1 h at room temperature. The cells were then stained with 0.5% ORO staining solution for 30 min followed by 3 washes with wash buffer and the images were visualized under a bright-field microscope at 40 × magnification. The lipid content (triacylglycerides) was quantified by extracting the stain from adipocytes in 60% isopropanol (to extract intracellular Oil Red O stain). The absorbance was measured at 540 nm by a microplate reader.

Lipolysis Assay

Glycerol levels released into the culture medium were measured using a commercial kit (Lipolysis assay kit, ab185433, Abcam, Shanghai) according to the manufacturer's instructions. The differentiated 3T3-L1 adipocytes were treated for 24 h with rutin. At room temperature, 50 µl of medium was incubated with 50 µl of reaction mixture for 30 min. The

glycerol content was determined using a microplate reader by measuring absorbance at 570 nm. The measurements were made in triplicates, and the results are given in nmol/well.

Glucose Uptake Assay

Differentiated 3T3-L1 adipocytes were used to evaluate the role of rutin in glucose uptake. Rutin was added to differentiated adipocytes in a 96-well plate on day 8. Insulin stimulation and glucose uptake were conducted on day 12. The treated/untreated cells were starved in DMEM for 16 h, washed with Krebs–Ringer HEPES buffer (pH 7.4), and incubated with 0.1% BSA for about 30 min at 37 °C. The uptake of glucose was terminated by removal of glucose solution from wells followed by a wash with ice-cold KRP buffer. Further, the cells were lysed with 0.1 M NaOH, and using a glucose assay kit, glucose levels were measured in aliquots of cell lysates. Three independent experiments were performed, and the data was tabulated to determine the percentage enhancement of glucose uptake over the controls. Measurements were done using the Bio-Rad microplate reader at an absorbance of 420 nm in a kinetic program with a read every 1 min for 30 min. Glucose uptake values were then normalized to the protein value of the cell lysates as determined by the BSA Protein Assay method described elsewhere.

Quantitative RT-PCR

After differentiation, the total RNA was isolated from 3T3-L1 cells by using TRIzol reagent according to the manufacturer's protocol. The RNA pellet was dissolved and quantified using a Nanodrop. Complementary DNA (cDNA) was synthesized from 2 µg of total RNA by reverse transcription using cDNA synthesis kit (Applied Bio Systems, Foster City, USA). Two nanograms of cDNA was used for qRT-PCR. PCR amplification was performed with SYBR green PCR kit and transcript-specific primers are shown in Table 1. β-Actin was used as the housekeeping gene for normalization. The relative expressed levels of the selected genes were normalized by using the $2-\Delta\Delta Ct$ method.

Western Blotting Analysis

Protein was isolated from treated and untreated 3T3-L1 cells using ice-cold RIPA lysis buffer by centrifugation at $13{,}000 \times g$ for 10 min and protein quantification was done using BCA method. Equal amount of protein was loaded on 10% SDS-PAGE gel and transferred onto a PVDF membrane. To block non-specific binding sites, membrane was incubated with 5% skimmed milk for 1 h at room temperature followed by overnight incubation with selected primary antibodies of rabbit anti-Glut-4, anti-UCP-1, anti-PPAR-γ, and anti-AMPK (1:1000 dilution) and mouse anti-β-actin (Abclonal Technology, USA) (1:10,000 dilution) at 4 °C. The immune reactive antigen was then recognized by incubation with HRP-conjugated secondary antibody (1:20,000 dilution, Abclonal Technology, USA). After washing with TBST, the membrane was treated with chemiluminescent ECL detection reagent (Bio-Rad) followed by exposure to Chemiluminescence detection system and the specific protein bands appeared were recorded and quantified.

Adipokines' Levels

Using enzyme-linked immunosorbent assay kits (Crystal Chem, Downers Grove, IL, USA), the secreted levels of major adipokines such as leptin and adiponectin were measured in 3T3-L1 adipocytes according to the manufacturer's instructions. The assays were done in triplicate and the adipokines' levels were expressed in ng/ml.

Statistical Analysis

The data are expressed as mean ± standard deviation (SD), and comparison was made by using one-way ANOVA program (SPSS version 17.0, SPSS Inc. Chicago, IL, USA),

Table 1 Primers used for quantitative RT-PCR analysis

Gene	Accession number	Primer sequence	BP length
UCP-1	NM_009463.3	F: 5′-CACGGGGACCTACAATGCTT-3′	20
		R: 5′-ACAGTAAATGGCAGGGGACG-3′	
PPAR-γ	NM_001308354.1	F: 5′-GGCTGCAGCGCTAAATTCTT-3′	20
		R: 5′-GGCATTGTGAGACATCCCCA-3′	
Glut-4	NM_001359114.1	F: 5′-GGCTCTGACGTAAGGATGGG-3′	20
		R: 5′-CAGCTCCTATGGTGGCGTAG-3′	
AMPK	NM_001355640.1	F: 5′-TCATCAAACTTTGGACGAAAAGGA-3′	24
		R: 5′-TTCGGCAACCAAGAACGGTA-3′	
β-Actin	NM_007393.5	F: 5′-TAGGCGGACTGTTACTGAGC-3′	20
		R: 5′-CTCAGACCTGGGCCATTCAG-3′	

followed by Tukey's post hoc tests to study the significance level ($p < 0.05$ is considered significant).

Results and Discussion

Isolation and Purification

From *Moringa oleifera* dried leaf powder (100 g), the yield obtained for hydro-alcoholic extract was 31% from which we could get 57% yield of active methanolic fraction. About 15 major compounds were identified in *M. oleifera* leaf active methanolic fraction along with rutin (Ganjayi et al. 2020). By using reverse-phase preparative HPLC and UPLC-MS, rutin was purified, confirmed, and quantified. The final rutin content obtained was 2.12 g. This compound was identified by comparison with an authentic sample and literature data (Atawodi et al. 2010).

Effect on Digestive Enzymes

To elucidate the anti-adipogenesis effects of rutin, we attempted to examine the effect of rutin on digestive enzyme inhibitory activities through *in vitro* study. The inhibition of α-glucosidase and pancreatic lipase activities by rutin was estimated with acarbose and orlistat as positive controls respectively. With increasing concentration of rutin, the activities of both the enzymes were substantially decreased (Fig. 1). The maximum inhibition of α-glucosidase and pancreatic lipase was 83 and 98% and their IC_{50} values were 40 and 35 µg/ml, respectively.

Cytotoxic Effect

MTT assay was performed on 3T3-L1 cells to measure the effect of rutin on cell viability during 24- and 48-h intervals. The results showed that there was no cytotoxicity in 24 h treatment, but, in 48 h, some cytotoxicity was observed with above 100 µg/ml of rutin, but it was not significant (Fig. 2A). Based on these results, 12.5, 25, and 50 µg/ml were chosen for the succeeding experiments.

Effect on Lipid Accumulation

To demonstrate the effect of rutin on lipid accumulation, 3T3-L1 pre-adipocytes were induced to differentiate with differentiation medium of induction (DMI). Oil Red O-stained images depict that rutin-treated 3T3-L1 adipocytes had remarkably reduced intracellular lipid droplets/lipid content and adipogenesis in a concentration-dependent manner (Fig. 2B). We also quantified the lipid content in terms of extracted Oil Red O stain into the culture media from 3T3-L1 adipocytes and the results showed a fivefold decrease in lipid content in rutin-treated adipocytes when compared to untreated adipocytes (Fig. 2C).

Effect on Free Glycerol Release

The lipolytic capability of rutin was demonstrated by quantifying the glycerol content released from adipocytes into the medium. Maximum glycerol release was observed in rutin-treated (40 µg/ml) adipocytes, which is five- to eightfold higher than untreated and other treated cells confirming its lipolytic activity (Fig. 2D).

Effect on Glucose Uptake

To assess the effect of rutin on glucose uptake in matured 3T3-L1 adipocytes, a colorimetric assay was performed. The basal half-time of GLUT-4 translocation to the plasma membrane is approximately 3 h, whereas insulin reduces this half-time to about 9 min and rutin administration further enhances this efficiency. Our data revealed that rutin treatment markedly increased glucose uptake ($p < 0.01$) in comparison to untreated cells and higher than insulin-treated cells (Fig. 3).

Adipogenesis and Glucose Uptake Genes

To investigate the effect of rutin on mRNA expression of key genes linked to adipogenesis and/or glucose uptake (PPAR-γ, UCP-1, GLUT-4, and AMPK), we did qRT-PCR in control and rutin-treated 3T3-L1 cells using specific

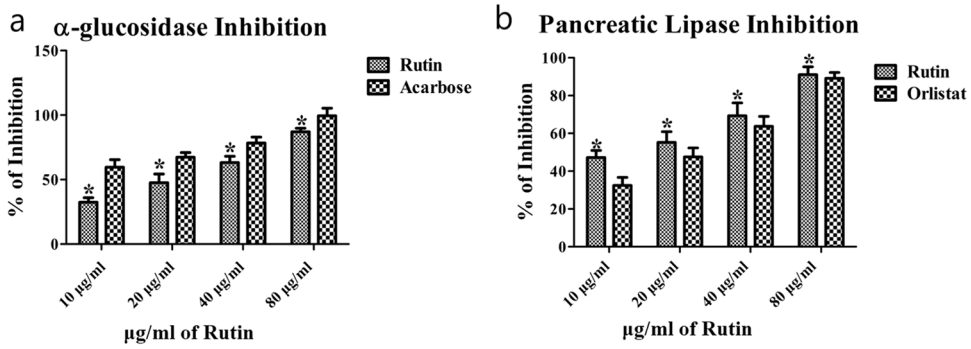

Fig. 1 Effect on key digestive enzymes. **a** α-Glucosidase inhibition. **b** Pancreatic lipase inhibition (*$p < 0.01$). Acarbose and orlistat were the standards used for glucosidase and lipase respectively

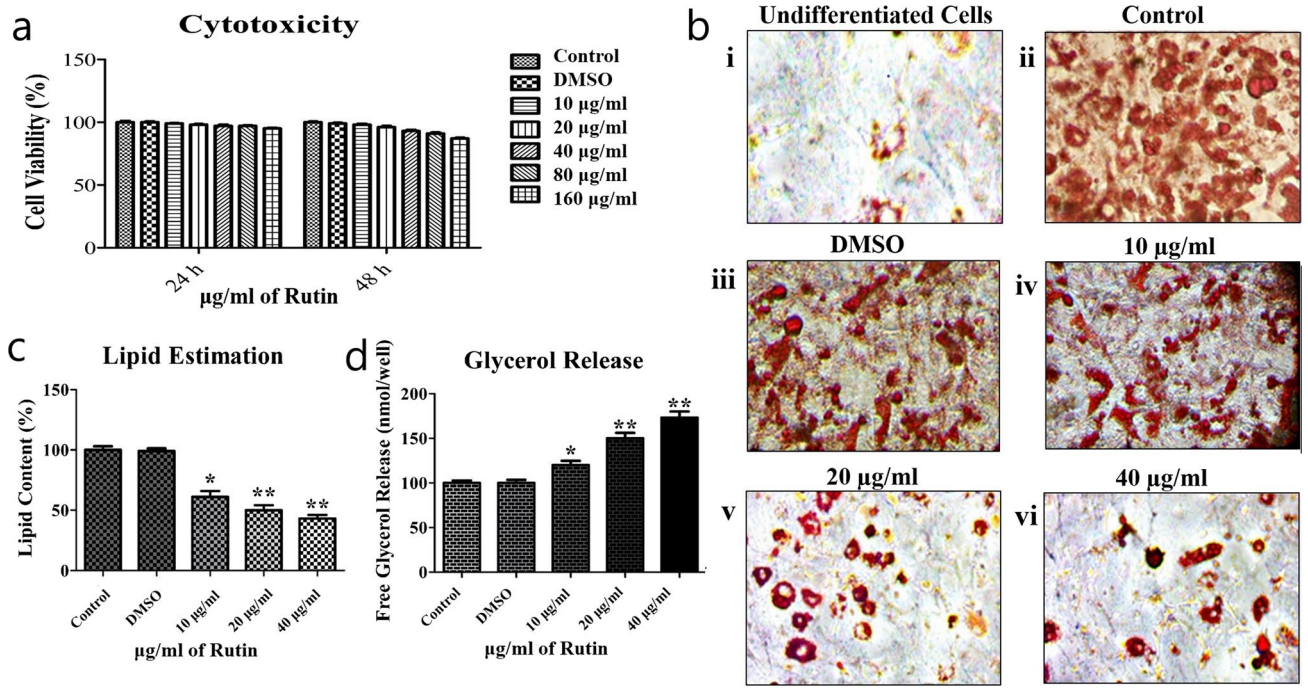

Fig. 2 Effect on 3T3-L1 adipocytes' differentiation. **a** Percentage of cell viability at 24 and 48 h by using MTT assay. **b** Total lipid content estimated from the lysate of ORO-stained 3T3-L1 cells. **c** ORO-stained adipocytes' images illustrating reduced lipid droplets in a concentration-dependent manner (magnification 40×). **d** Estimated glycerol released into the culture media by lipolysis assay. *$p < 0.05$ and **$p < 0.01$ significant difference between control and treated adipocytes, respectively

primers. Treatment with rutin substantially enhanced the mRNA expression levels of UCP-1 and GLUT-4 and reduced that of PPAR-γ and AMPK remains unchanged in a concentration-dependent manner when compared to untreated adipocytes as depicted in Fig. 4.

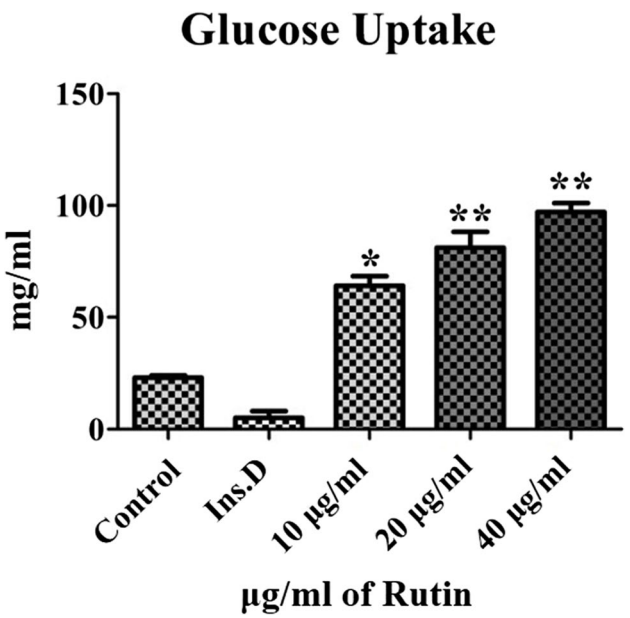

Fig. 3 Effect on glucose uptake. Ins. D, insulin deficient. *$p < 0.05$ and **$p < 0.01$ significant difference between control and treated adipocytes, respectively

Adipogenesis and Glucose Uptake Proteins

To elucidate the potential effect of rutin on expression of key proteins associated with adipogenesis and glucose homeostasis, we performed western blotting analysis in 3T3-L1 adipocytes. A concentration-dependent downregulation of PPAR-γ and upregulation of UCP-1, *p*-AMPK, and GLUT-4 were observed with rutin treatment, the maximum effect being exhibited at 40 µg/ml of rutin when compared to control cells (Fig. 5).

Adipokines Levels

To further understand the effect of rutin on adipokines, we measured leptin and adiponectin levels in 3T3-L1 adipocytes. As shown in Fig. 6, when compared to the control group, we found significantly higher levels of leptin but lower levels of adiponectin in adipocytes. However, administration of rutin led to reduced levels of leptin and increased levels of adiponectin ($p < 0.01$).

Considering the complexity associated with obesity biology and its therapy, different therapeutic models have been proposed to contain obesity (Balaji et al. 2016). One

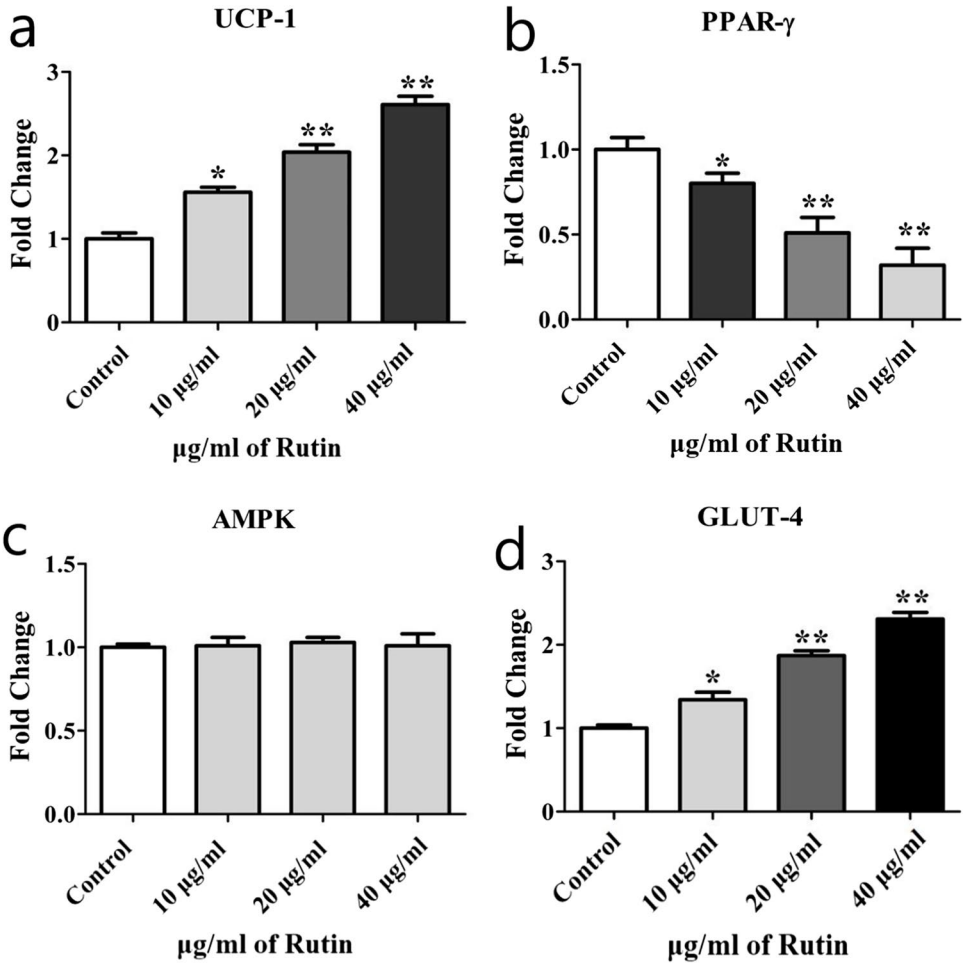

Fig. 4 Effect on mRNA expression of **a** UCP-1, **b** PPAR-γ, **c** AMPK, and **d** GLUT-4. $*p < 0.05$ and $**p < 0.01$ significant difference between control and treated adipocytes, respectively

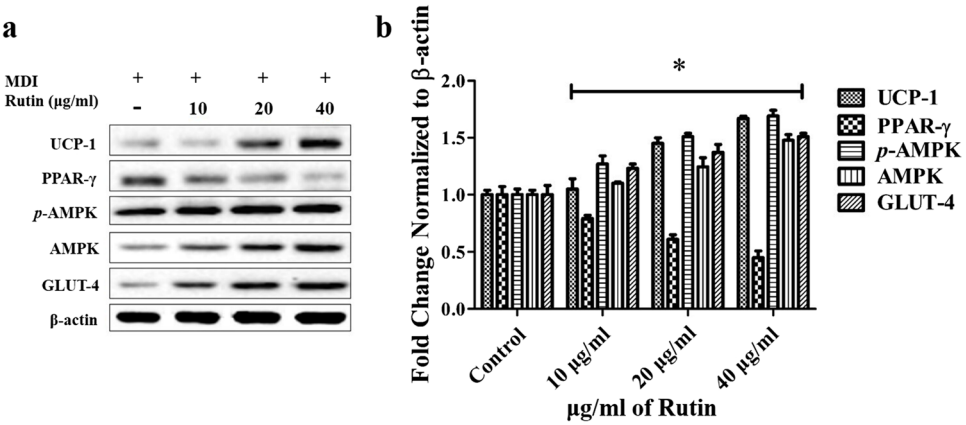

Fig. 5 Effect on protein expression. **a** Protein expression analysis of UCP-1, PPAR-γ, *p*-AMPK, AMPK, and GLUT-4. **b** Graphical representation of their protein expression levels. $*p < 0.05$ and $**p < 0.01$ significant difference between control and treated adipocytes, respectively

of the key strategies in the treatment of obesity and glucose homeostasis is to develop inhibitors to slow down nutrient digestion and absorption of lipids and carbohydrates. Pancreatic lipase is a principal enzyme that digests dietary lipids into monoglycerides and fatty acids. Similarly, pancreatic amylase and glucosidase bring about digestion of dietary starch into simple sugars. Therefore, inhibiting such digestive enzymes is an important means for reduced digestion of dietary fat, starch, and energy intake which leads to prevention/controlling of obesity and hyperglycemia (Les et al. 2018). Orlistat has been widely used in clinical practice as a lipase inhibitor to treat obesity. Many studies with synthetic and natural products are underway to develop effective lipase inhibitors (Birari and Bhutani 2007). In the present

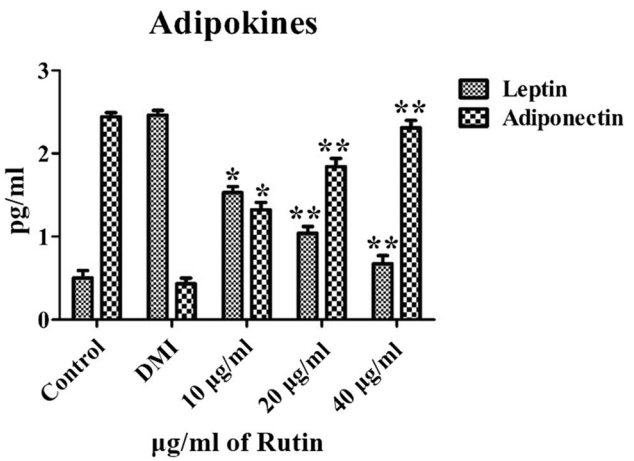

Fig. 6 Effect on adipokines. DMI, differentiation medium of induction. *$p < 0.05$ and **$p < 0.01$ indicate significant difference between DMI and rutin-treated adipocytes, respectively

study, rutin had effectively inhibited the activities of lipase (IC_{50} 35 µg/ml) as well as glucosidase (IC_{50} 40 µg/ml), indicating the promising role of *M. oleifera* leaves in decreasing energy intake (Fig. 1).

Adipogenesis is the crucial process by which fibroblasts like pre-adipocytes differentiate into lipid-laden mature adipocytes leading to adiposity. In the present study on 3T3-L1 cells, rutin did not show any significant cytotoxicity until a concentration of 160 µg/ml either at 24 or 48 h (Fig. 2). Decreased adipogenesis was observed in 3T3-L1 adipocytes as evident from Oil Red O staining and glycerol estimation assays (Fig. 2). The proliferation and differentiation of pre-adipocytes play pivotal role in the process of adipogenesis, and it is regulated by a set of genes at transcriptional level such as members of PPARs, CEBPs, and SREBPs families (Moseti et al. 2016).

PPAR-γ is a transcription factor of nuclear receptor superfamily that plays a decisive role in adipocytes' differentiation and maturation (Choi et al. 2014). In the present study, the mRNA and protein expression profile of PPAR-γ was downregulated with increase in rutin concentration in 3T3-L1 cells (Figs. 4 and 5). PPAR-γ is predominantly found in adipose tissue and is considered as a master regulator as it regulates the expression of certain genes (SREBP-1c, FAS, ACC, etc.) and corresponding proteins involved in glucose and lipid metabolism (White and Stephens 2010). Downregulated expression of PPAR-γ at gene and protein levels by rutin indicates its interference at both transcriptional and translational levels, as well as through PPAR-γ signaling pathway it might influence the genes involved in lipogenesis and glucose homeostasis in favor of reducing adiposity and improving glucose tolerance. Our results are consistent with previous studies on 14-deoxy-11,12-didehydroandrographolide and astragalin, which inhibited adipogenesis through PPAR-γ signaling (Li et al. 2018; Ganjayi et al. 2020). Therefore, repression of PPAR-γ expression could inhibit adipocytes' differentiation and lipogenesis; thus, it is considered as a potential target in the treatment of obesity.

AMP-activated protein kinase (AMPK) is a master energy sensor of adipocyte membranes. Upon phosphorylation, AMPK turns the metabolic switch from anabolic mode to catabolic mode by regulating downstream target proteins. Its activation enhances insulin sensitivity in tissues, promotes glucose transport through glucose transporter-4 (GLUT-4), and inhibits adipocyte differentiation (Chow et al. 2017). In the present study, AMPK was phosphorylated and Glut-4 was upregulated with rutin treatment (Figs. 4 and 5).

When stimulated by insulin, GLUT-4 translocates from its intracellular compartment to the plasma membrane and, therefore, is responsible for insulin-stimulated glucose uptake which is augmented by rutin in the present context (Fig. 3). Previous reports with cinnamon extract showed AMPK-mediated enhanced glucose uptake in 3T3-L1 cells (Shen et al. 2014; Xie et al. 2018). Another study by Chow et al. (2017) had demonstrated that AMPK activators might block adipocyte development by downregulating the expression of PPAR-γ, C/EBP-α, and FAS. For instance, dihydrosanguinarine increased glucose uptake in mouse 3T3-L1 cells by activating Glut-4. Hence, AMPK signaling pathway could be a potential molecular target in the development of drugs for the treatment of type 2 diabetes and obesity (Shen et al. 2014).

Thermogenesis is an important means to inhibit adipogenesis by fat burning. This could be achieved by activating UCP-1, an inner mitochondrial membrane protein whose higher expression levels favor heat generation and enhance cellular catabolism and energy expenditure. Usually, UCP-1 is highly expressed in brown adipose tissue (BAT), but not in white adipose tissue (WAT); however, molecules that upregulate UCP-1 in WAT can result in heat dissipation. In agreement with above hypothesis and several previous studies, rutin in the present study has upregulated the mRNA and protein expression of UCP-1 in a concentration-dependent manner (Figs. 4 and 5). A few reports implicated PPAR-γ's role in regulation of UCP-1, but it is not clearly established (Shen et al. 2014; Zhang et al. 2019). Previous studies using β3-adrenergic receptor (β3-AR) activators confirmed that the sympathetic nervous system was the main trigger of UCP-1 activation, but the actual mechanism is not understood (Zhang et al. 2014, 2019).

Adipokines such as adiponectin and leptin are key adipokines secreted by adipose tissue and are involved in regulation of metabolic functions linked to glucose homeostasis, insulin resistance, and adipogenesis. In this study, treatment with rutin significantly decreased leptin level but increased adiponectin level in a concentration-dependant manner (Fig. 6) which might result in enhanced lipolysis and fatty

acid oxidation through AMPK activation. In addition, the present study also demonstrated that rutin had substantially inhibited lipase and glucosidase activities; it improved glucose uptake and reduced lipid content and adipogenesis in 3T3-L1 cells through working on different targets.

Conclusion

This study emphasizes the nutraceutical potential of *M. oleifera* leaves and recommends rutin as a potential pharmacological agent in drug development to alleviate obesity and glucose intolerance. However, further *in vivo* experiments are underway to confirm the therapeutic potential of rutin to combat obesity and diabetes.

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Author Contribution MB conceived and developed the idea. GMS and RSK did isolation and purification of rutin. GMS, RSK, and GS conducted cell culture experiments, analyzed the data, and performed statistical analysis. MB, GMS, and RSK interpreted the results and edited the manuscript. Final corrections were done by MB and GMS. All authors have read the final manuscript and agreed the submission.

Data Availability All data in this study have been included in this article.

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