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Inhibition of adipocyte differentiation in 3T3-L1 cell line by quercetin or isorhamnetin

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INHIBITION OF ADIPOCYTE DIFFERENTIATION IN 3T3-L1 CELL LINE BY QUERCETIN OR ISORHAMNETIN

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Dedicated to my beloved family
Carvajal-Aldaz and the most important person in my life Clara Vega, my grandmother

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ABBREVIATIONS

AMM	Adipocyte Maintenance Medium
ATCC	American Type Culture Collection
β-catenin	Beta-catenin
BSA	Bovine serum albumin
BMI	Body Mass Index
C/EBP-α	CCAAT/enhancer-binding protein-alpha
C/EBP-β	CCAAT/enhancer-binding protein-beta
C/EBP- δ	CCAAT/enhancer-binding protein-delta
CHD	Coronary heart disease
DM	Differentiation Medium
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
GRAS	General recognized as safe
hAMSCs	Human adipose tissue-derived stem cells
IBMX	3-isobutyl-1-methylxanthine
IL-6	Interleukin-6
IS	Isorhamnetin
LDL	Low-density lipoprotein
MCP-1	Macrophage chemoattractant protein -1
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PEM	Pre-adipocyte Expansion Medium
PPAR-γ	Peroxidase proliferator-activated regulator-gamma
PVDF	Polyvinylidene difluoride
Q	Quercetin
RIPA	Radioimminoprecipitation assay buffer
ROS	Reactive oxygen species

RXR	Retinoid X receptor
SREBP	Sterol regulatory element binding protein
TNF-α	Tumor necrosis factor-alpha
WAT	White adipose tissue
3T3-L1	Cell line which was originated from clonal expansion of murine Swiss 3T3 cells and contain only a single cell type

ABSTRACT

Obesity has become a major health problem worldwide. Obesity increases the risk of hypertension, diabetes, and certain types of cancer. Quercetin is a bioactive compound widely found in a variety of foods that are consumed daily. Isorhamnetin is a bioactive compound found in some foods and also is a quercetin metabolite. Many studies have reported the anti-obesity and anti-inflammation properties of quercetin and isorhamnetin. The objective of this study was to test the effect of quercetin or isorhamnetin at physiological and supraphysiological concentrations on the inhibition of the differentiation process of 3T3-L1 pre-adipocyte to adipocyte. Cell viability results demonstrated no significant difference ($P > 0.05$) between non differentiated cells, control and quercetin or isorhamnetin treated cells. During adipocyte differentiation for 8 days in the presence of quercetin or isorhamnetin, cell viability was above 94.84% and 97.63%, respectively. Red oil O staining assay was performed in order to evaluate the inhibitory effect of quercetin or isorhamnetin on cytoplasmic lipid droplet accumulation. Significant differences ($P < 0.05$) were reported. Isorhamnetin was more effective than quercetin in inhibiting cytoplasmic lipid droplet accumulation. Neither quercetin nor isorhamnetin had an effect on the expression of macrophage chemoattractant protein -1 (MCP-1). CCAAT/enhancer binding protein α (C/EBP-α) was down-regulated by quercetin or isorhamnetin. Compared to control quercetin decreased PPAR-γ 1 and 2 expressions by 45.03 ± 3.17% and 27.58 ± 12.39%, while isorhamnetin decreased PPAR-γ 1 and 2 expressions by 41.48 ± 9.51% and 2.01 ± 32.46%, respectively. β-catenin was not dose dependent either for quercetin or isorhamnetin and did not follow a specific trend. Taken together, our data indicate that isorhamnetin more than quercetin can exert potential anti-obesity effects by inhibiting differentiation of pre-adipocytes at physiological concentrations.

CHAPTER 1

INTRODUCTION

Obesity is a result of an imbalance between intake of energy and energy expenditure [1].Obesity is a serious health problem that is implicated in various diseases including hypertension, type II diabetes, coronary heart diseases, and cancer [2]. Obesity is characterized by increased adipose tissue mass that results from increased fat cell size (hypertrophy) or number (hyperplasia). Adipose tissue is a major energy reservoir in the body; it stores the excess energy as lipids and releases it on demand. Moreover, adipocytes constitute an endocrine system which secrete hormones known as adipokines [3]. Phytochemical treatments can regulate adipose tissue mass by the inhibition of adipogenesis from fibroblastic pre-adipocytes to mature adipocytes as demonstrated in recent studies [4].

Quercetin is one of the major flavonol in the western diet; the main sources are apples, onions, red wine and tea [5]. It is a bioactive compound widely studied due to its unique ability to act as an antioxidant (low doses) or a pro-oxidant (high doses) depending on its concentration [6]. Quercetin has been studied for its anti-inflammatory and lipid-regulating properties [7]. Since flavonoids are modified during absorption in the small intestine, several studies had been concentrated on plasma metabolites after quercetin intake. Three of the major metabolites of quercetin are quercetin as is, isorhamnetin and tamarixetin [8].

Isorhamnetin is a flavonoid found in seabuckthorn [9] or as a quercetin metabolite in plasma after quercetin intake [8]. Isorhamnetin has recently been reported to have antioxidant activity, the ability to increase the resistance of human low-density lipoprotein to oxidation, and antitumor activity [10].

Adipose tissue hyperplasia triggers the transformation of pre-adipocytes into adipocytes. Adipogenesis or adipocyte differentiation is a multifaceted process which includes changes in morphology, hormone sensitivity, and gene expression. In this process, members of CCAAT/enhancer-binding protein (C/EBP) - transcription factor family and peroxidase proliferator-activated regulator-γ (PPAR-γ) act together in order to regulate adipocyte differentiation [9]. During adipogenesis adipokines

as macrophage chemoattractant protein -1 (MCP-1) are released. MCP-1 is a novel adipokine which has an important role in the development of obesity-associated insulin resistance [11]. The expression of MCP-1 by adipose tissue induces inflammatory reactions and insulin resistance, suggesting the suppression of MCP-1 is important for the management of metabolic syndrome [12].

Several *in vitro* studies have shown that flavonoids at concentrations exceeding plasma levels have anti-inflammatory activity, decreased expression of PPAR-γ, C/EBP-α, and intracellular cytoplasmic lipid droplet accumulation; but the physiological significance of the results is questionable. Therefore, the main objective of this study was to evaluate and compare the effect of quercetin or isorhamnetin at physiological concentrations and supraphysiological dosages on 3T3-L1 cell line. Steps taken to achieve the objective included:

1. Determining cytotoxic concentrations of quercetin or isorhamnetin during adipocyte differentiation.
2. Demonstrating the lipid droplet accumulation after the adipocyte differentiation process and the inhibition of lipid droplet accumulation by quercetin or isorhamnetin treatments.
3. Investigating the secretion of MCP-1 during the adipocyte differentiation.
4. Studying the expression of β-catenin, C/EBP-α, and PPAR-γ in the presence or absence of quercetin or isorhamnetin.

CHAPTER 2

LITERATURE REVIEW

2.1. Definition of Obesity

Obesity is defined as abnormal or excessive fat accumulation, for women gluteofemoral or lower-body whereas for men abdominal or upper-body [2]. Obesity results from an excess in energy intake and low energy expenditure. There are different methods to measure body fat: (1) skin fold measurement, (2) waist circumference, or (3) body mass index. However, Body Mass Index (BMI) is commonly used. BMI is defined as the weight in kilograms divided by the square height in meters (kg/m^2), and the resulting index is classified as underweight, overweight, obese, or over-obese (Figure 2.1).

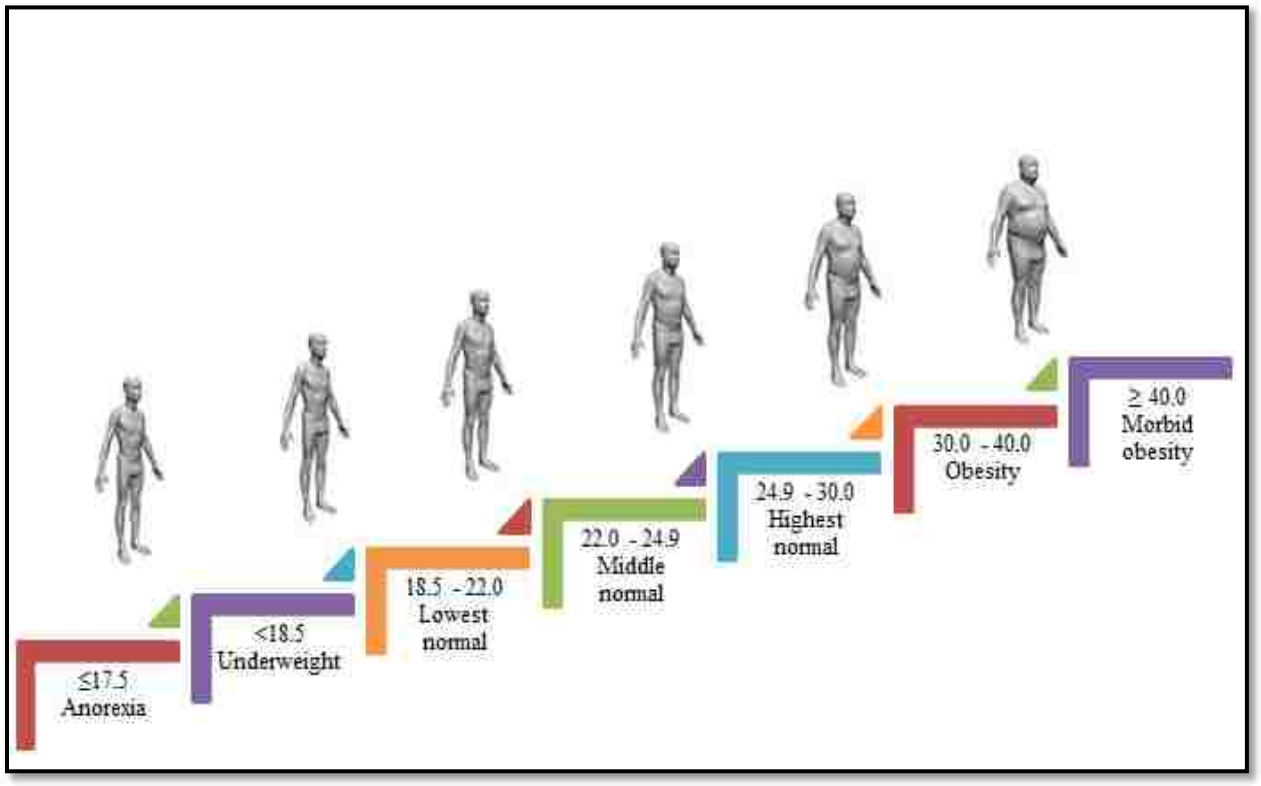


Figure 2.1. Body Mass Index classification. Adapted from:
http://www.scientificpsychic.com/fitness/diet.html.

2.2. Risk Factors for Obesity

Obesity is a product of the imbalance between energy intake and energy expenditure, which leads to the pathological growth of adipocytes [1]. The growth of the adipose tissue can be a result of hypertrophy and/or hyperplasia of the cells; which leads to increase in cell size and fat cell number, respectively [13]. Obesity is now recognized as a chronic and systematic inflammatory disease; the molecules secreted, known as adipokines, regulate carbohydrate and lipid metabolism, immune function and blood coagulability, and may serve as blood markers of cardiometabolic risk [14].

Obesity increases risk to acquire several disorders that are directly linked to high mortality and morbidity, including diabetes, hypertension, coronary heart disease (CHD), dyslipidemia, gallbladder disease, and certain kinds of cancer [2]. Obesity is a complex disorder with strong genetic basis and a multifactorial etiology [15].

2.3. Obesity and Adipogenesis

In periods of excess energy, the eukaryotes store excess energy as triacylglycerol and mobilize the stored lipids during deprivation periods; this energy reserve is known as white adipose tissue (WAT). A dramatic increase in the rate of obesity is a result of the excessive accumulation of WAT [16]. This phenomenon is called adipogenesis or adipocyte differentiation. During this, fibroblast-like pre-adipocytes differentiate into lipid-laden and insulin-responsive mature adipocytes [17]. Adipogenesis occurs under the regulation of several transcriptional factors, such as sterol regulatory element binding protein (SREBP), CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPAR- γ)[18].

The role of PPAR isoforms in adipogenesis is not completely understood. The PPARs form heterodimers with the retinoid X receptor (RXR) and regulate transcription through this binding. Peroxisome proliferator-activated receptor γ is the most adipose specific and has been shown to be enough to induce growth arrest or adipogenesis initiation besides playing a critical role in the regulation of adipocyte differentiation. Peroxisome proliferator-activated receptor α has been also reported to be

capable of inducing adipocyte differentiation, but is less adipogenic than PPAR- γ. Finally, peroxisome proliferator-activated δ is not adipocyte specific, however is highly expressed in adipose tissue [19].

C/EBP family was among the first transcription factors confirmed to perform an important role in adipocyte differentiation. Isoforms of C/EBP metabolize lipid and cholesterol-related compounds and are expressed in tissues such as liver [20]. Although C/EBP-α is not adipocyte specific, is expressed before the transcription of most adipocyte-specific genes has started, and in some instances is sufficient to induce differentiation. C/EBP-α, C/EBP-β, and C/EBP-δ are isoforms; each one has a distinct sequential and spatial expression during adipocyte differentiation. C/EBP-α express relatively late in differentiation, meanwhile β and δ are present in pre-adipocytes, and their levels increase during differentiation [21].

The expression of PPAR- γ is directly induced by the coexpression of C/EBP-β and δ; although C/EBP-α and PPAR- γ appearance rises intensely during adipocyte differentiation. This suggests that an increase in C/EBP-β above a certain level induces the expression of PPAR- γ. The ligand activation of PPAR- γ with C/EBP-α leads to full adipocyte differentiation [21]. An overview of stages in adipogenesis is represented in Figure 2.2.

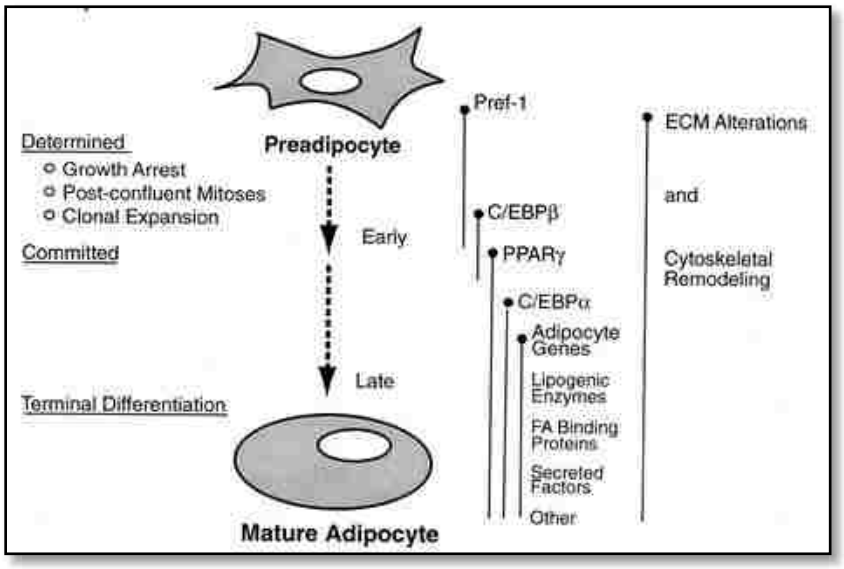


Figure 2.2. Stages in adipogenesis [19]

2.4 Obesity and Inflammation

Adipocyte cells have an active role as endocrine cells releasing various bioactive substances called “adipocytokines” or “ adipokines”, which impact directly the regulation of food intake, insulin sensitivity, energy metabolism, and the vascular microenviroment [12]. Recently, research has focused on the concept that obesity as a chronic low-grade systematic inflammation stimulates insulin resistance and the production of inflammatory biomarkers [22]. Adipocytes release adipokines and bioactive peptides, including leptin, adiponectin, tumor necrosis factor-alpha (TNF-α), resistin, interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1), macrophage chemoattractant protein-1 (MCP-1) [23].The situation worsens when adipocytes release excessive amounts of cytokines/ adipokines as TNF-α, resistin, IL-6, PAI-1, MCP-1 and others; which circulate via the vascular system to insulin target tissues such as liver, muscle and islet cells inducing insulin resistance [24]. Inflammation produced by obesity is partially mediated by multiple cellular stresses, such as oxidative stress, endoplasmic reticulum, and hypoxia [25].

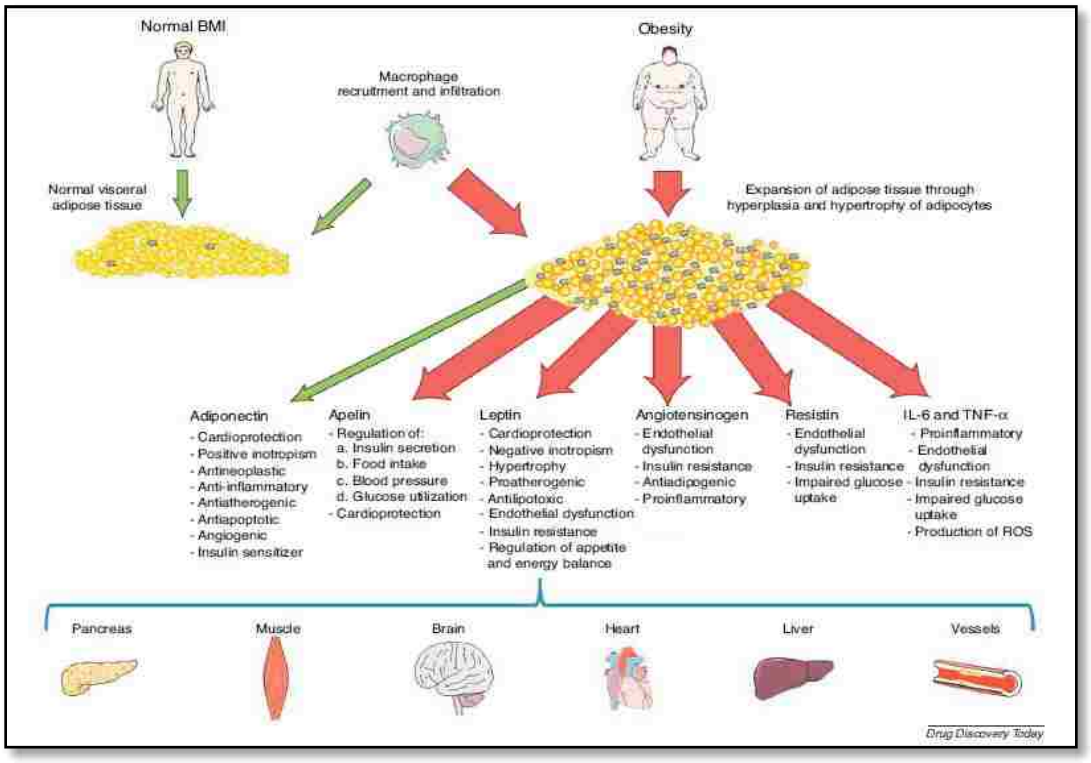


Figure 2.3. Contribution of adipokines to obesity and metabolic syndrome abnormalities [26].

2.4.1. Adipokines

Adiponectin is the most abundant and studied adipokine; it is exclusively released in adipocytes [14]. Full-length adiponectin is constituted of 244 amino acids, found in plasma at levels of 3-30 mg/ml, and forms three major oligomeric complexes with biological functions as trimer, hexamer, and high-molecular-mass form (Figure 2.4) [27]. Adiponectin is also found in a smaller form that consists of globular domain existing in plasma in very small amounts [28]. Adiponectin increases fatty acid oxidation while decreases glucose production, and also plays a role as anti-inflammatory [29].

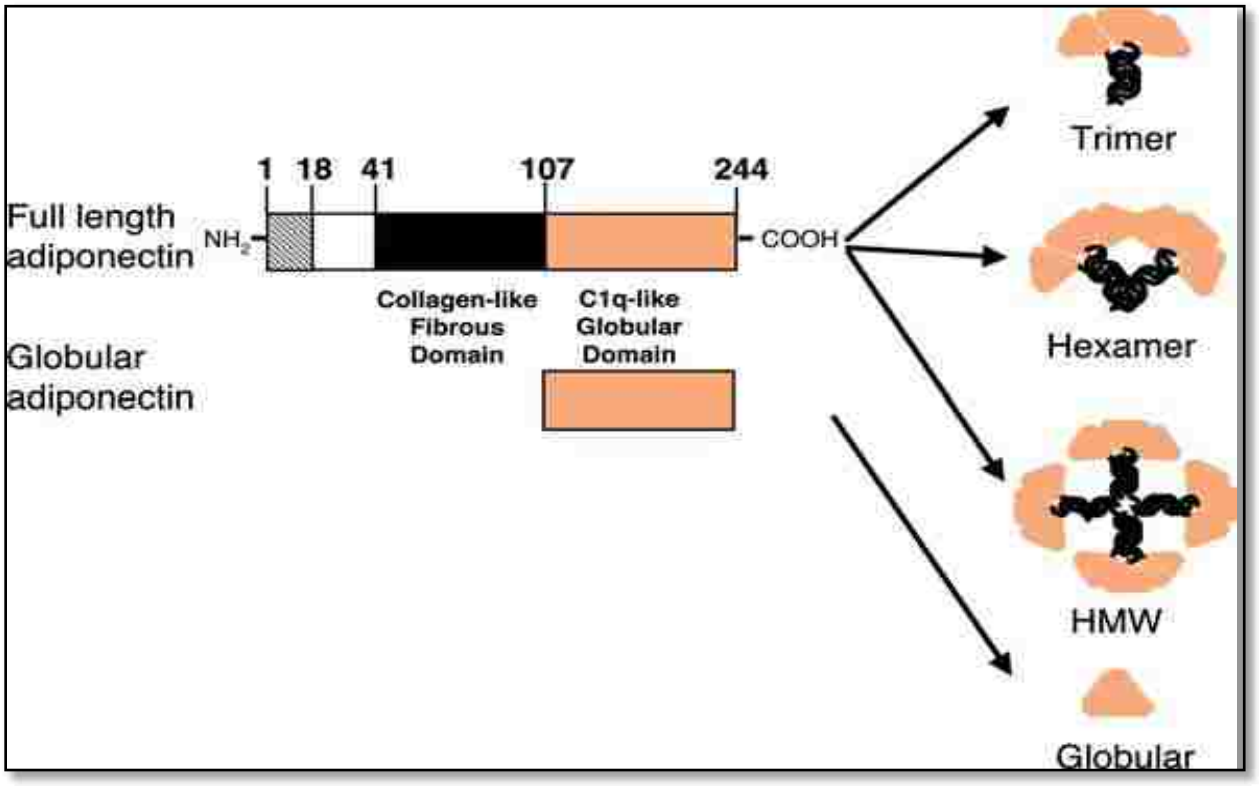


Figure 2.4. Domains and structure of adiponectin [27]

Numerous studies reveal the negative correlation of plasma adiponectin and obese subjects depicting a higher concentration of adiponectin in non-obese subjects [30]. Arita et al. [30] found a negative correlation between plasma concentration of adiponectin and BMI in men and women (r = -0.71, P < 0.0001; and r = -0.51, P < 0.0001 respectivily). Individuals with low concentrations of plasma adiponectin have a reduced insulin sensitivity, and tend to develop Type II diabetes [31]. Elevated plasma

adiponectin levels were found in low BMI diabetic and non-diabetic subjects [32]. The physiological role of adiponectin in humans is not yet clearly elucidated [30].

Leptin is another adipocytokine product of the obese (*ob*) gene, that reduces appetite by sending a satiety signal to the central nervous system [27]. It is a 16 kDa hormone and mainly released by adipocytes in order to control body weight [14]. Concentration of leptin circulation in plasma is proportional to total body adiposity and direct nutritional state [33]. It is present in human serum in ranges of 1-15 ng/ml in non-obese individuals, and levels more than 30 ng/ml in individuals with BMI $\geq$30 kg/m^2[34]. Leptin also stimulates thyroid-mediated thermogenesis and fatty acid oxidation. Deficiency of leptin is associated with increased appetite and manifest obesity in mice and humans [35].

Interleukin-6 (IL-6) is an inflammatory cytokine that is correlated with hyperglycemia, insulin resistance, and type 2 diabetes mellitus [36]. Mice chronically exposed to IL-6 develop hepatic insulin resistance [37]. About 25% of circulating IL-6 in humans is released by subcutaneous adipose tissue [38].

Tumor necrosis factor-alpha (TNF-α) is a cytokine whose expression is increased in adipose tissue and highly found in circulation of obese and insulin resistant individuals [39]. TNF-α has an important effect on whole body lipid and glucose metabolism [40], it inhibits tyrosine phosphorylation of IRS-1 which decreases insulin signaling [41]. Moreover, TNF- α is directly involved in the activation of pro-inflammatory subcellular pathways and induces the production of reactive oxygen species (ROS) [42].

Plasminogen activator inhibitor-1 (PAI-1) is secreted in high concentration by adipose tissue. Plasma and adipocyte concentrations of PAI-1 correlate with the levels of visceral fat and triglycerides [43]. It has been suggested that PAI-1 may probably destroy adipocytes causing hypertrophy [26].

Macrophage chemoattractant protein -1 (MCP-1) is a novel adipokine which has an important role in the development of obesity-associated insulin resistance [11]. The expression of MCP-1 by adipose tissue induces inflammatory reactions and insulin resistance, suggesting their suppression of MCP-1 is important for the management of metabolic syndrome [12].

2.5. Dietary Approach to Reduce Obesity

Consumption of vegetables, legumes and fruits have been associated with a large number of health benefits, some of them are prevention of obesity, type 2 diabetes mellitus and cardiovascular disease, because of their flavonoid and polyphenolic content [44]. Polyphenols, including flavonoids, are bioactive compounds with anti-oxidative and anti-inflammatory properties [45]. Flavonoids are polyphenolic compounds that are widely found in fruits and vegetables in glycosylated forms. Quercetin is one of the major flavonol in the western diet; the main sources are apples, onions, red wine and tea [5]. The estimated average intake of flavones and flavonols per day is approximated 25 mg [46]. An extensive literature demonstrates that phenolic compounds including quercetin have important anti-inflammatory and antiobesity properties [7, 22, 24, 45, 47].

2.5.1. Quercetin

Quercetin (3, 3', 4', 5-7-pentahydroxyflavone) is a polyphenol extensively found in a variety of foods that are consumed daily. Quercetin has the basic structure of one or more hydroxylated benzene rings, and also contains several hydroxyl groups attached to the diphenyl propane (C6-C3-C6) backbone [48].This bioactive compound has been studied because of its unique ability to act as an antioxidant (low doses) or a pro-oxidant (high doses) depending on its concentration [6]. Quercetin has also been studied for its anti-inflammatory and lipid-regulating properties [7].

Quercetin dosage of up to 100 mg/day is general recognized as safe (GRAS). Sixty percent of orally ingested quercetin is absorbed. The daily intake is only 6 to 31 mg. Quercetin can be absorbed as glycoside or as an aglycone. Few studies have been done on the evaluation of absorption of quercetin, most studies were performed with low doses that could be achieved by normal diet [6]. Egert et al. established that quercetin could be absorbed without difficulty. They found quercetin in blood plasma after two weeks of administration, in averages of 145 nM, 217 nM, and 380 nM when subjects received supplements of 50 mg/day, 100 mg/day, and 150 mg/day respectively. The metabolites, isorhamnetin and tamarixetin, were between 9 and 23 nM [8].

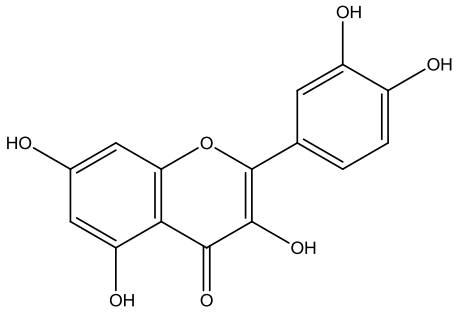


Figure 2.5. Quercetin structure

2.5.2. Isorhamnetin

Isorhamnetin (3,4',5,7-tetrahydroxy-3'-methoxyflavone) is an active flavonol aglycone. It is one of the primary metabolites of quercetin. It is recognized for its antioxidant activity, cytoprotective capacity, anti-adipogenesis and anti-inflammatory effects [49]. Its antioxidant activity had been attributed to its ability to increase the resistance of human low-density lipoprotein (LDL) to oxidation by Cu^{2+} [10]. Isorhamnetin activate the p38MAPK pathway preventing endothelial cell injuries caused by oxidized LPL [50]. Anti-adipogenesis properties had been reported by the down regulation of the expression of C/EBP-α and PPAR-γ in 3T3-L1 murine cells [9], and also by the stabilization of the β-catenin protein in human adipose tissue-derived stem cells (hAMSCs) [51].

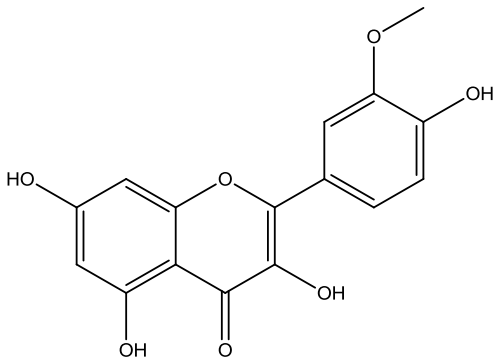


Figure 2.6. Isorhamnetin structure

2.6. 3T3-L1 Cell Line

Many models and techniques are being used in order to evaluate and understand adipocyte biology [52]. 3T3-L1 is a pre-adipose cell line which was originated from clonal expansion of murine

Swiss 3T3 cells and contain only a single cell type [53]. This cell line has been using widely in more than 5000 published articles on adipogenesis and the biochemistry of adipocytes for the last 30 years, because of its potential to differentiate from fibroblast to complete adipocytes [54]. Several investigations use 3T3-L1 cells because it helps in identifying key molecular markers including transcription factors and various pathways during pre-adipocyte differentiation [52]. Numerous protocols can be used to induce differentiation from pre-adipocytes to adipocytes, but the most commonly used agents are insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) [55] at concentrations of 1 μg/ml, 0.25 μM, and 0.5 μM, respectively. Pre-adipocytes contain less amount of lipid droplets accumulated, but four days after induction they start to accumulate lipids that grow in size and number over the differentiation time [54].

CHAPTER 3.

MATERIALS AND METHODS

3.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Life Technologies, Parsley, PA), calf serum and fetal bovine serum (FBS) were obtained from ATLANTA® biological (Lawrenceville, GA). Dexamethasone, insulin, isorhamnetin, and oil red O were purchased from Sigma-Aldrich (St. Louis, MO). Methylisobutylxanthine (IBMX) was obtained from Cayman Chemical Company (Ann Arbor, MI). Quercetin was purchased from LKT Labs (St. Paul, MN). Anti-β-catenin, β-actin, C/EBP-α, and PPAR-γ monocolonal were obtained from Cell Signaling (Danvers, MA). Alkaline phosphate-conjugated secondary antibody was from Santa Cruz Biotechnology® (Santa Cruz, CA). SuperSignal® West Pico Chemiluminescent Substrate was from Thermo Scientific (Rockford, IL).

3.2. Cell Culture

In this study, 3T3-L1 cell line was used to model the inhibitory effects of quercetin or isorhamnetin against pre-adipocyte differentiation to adipocyte cells. The cell line was obtained from American Type Culture Collection (ATCC) (Rockville, MD). 3T3-L1 is a continuous substrain of 3T3 (Swiss albino) developed through clonal isolation [54].

3T3-L1 pre-adipocyte differentiation was performed following the ATCC protocol, illustrated in Figure 3.1. Cells were seeded and maintained with Pre-adipocyte Expansion Medium (PEM) for 48 h by incubating at 37°C and 5% CO_2, or after reaching 100% confluence. Next, the identical volume of medium was replaced with Differentiation Medium (DM) (Day 0) and incubated for 48 h at 37°C in humidified atmosphere containing 5% CO_2. After that, the Differentiation Medium was replaced with Adipocyte Maintenance Medium (AMM); AMM was changed every 72h. Finally, the cells were fully differentiated at day 10 after induction. Pre-adipocyte cells could be fully differentiated between 8 to 12 days after DM application. To examine the effect of quercetin or isorhamnetin on adipocyte differentiation, 3T3-L1 cells were treated with PEM until day 10 in the absence or presence of

concentrations from 20 nM to 25 µM of quercetin or isorhamnetin. Also, a flask of cells containing PEM (non-differentiated cells) was maintained until day 10.

Media formulations are described below in Table 3.1.

Table 3.1. Description of media formulations

MEDIUM	FORMULATION
Pre-adipocyte Expansion	90% Dubelcco's Modified Eagle's Medium (DMEM), and 10% Bovine Calf Serum
Differentiation	90% DMEM, 10% Fetal Bovine Serum (FBS), 1.0 µM Dexamethasone, 0.5 mM Isobutylmethylxanthine (IBMX), and 1.0 µg/ml Insulin
Adipocyte Maintenance	90% DMEM, 10% FBS, and 1.0 µg/ml Insulin

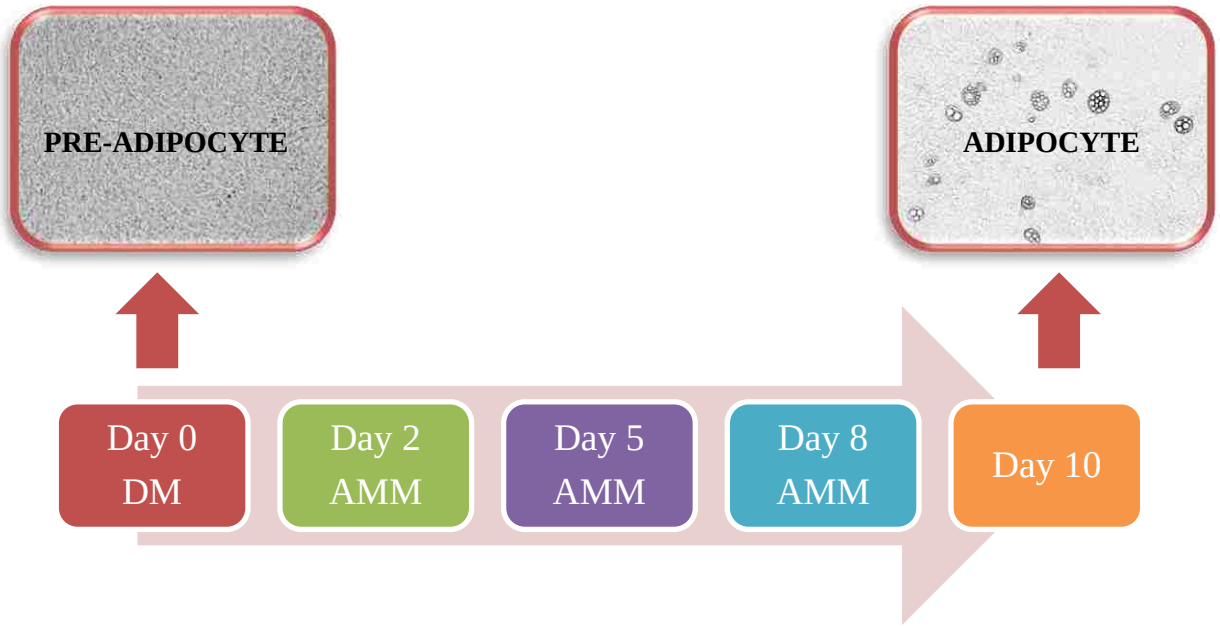


Figure 3.1. Protocol for chemical-induction of differentiation. Differentiation Medium (DM), Adipocyte Maintenance Medium (AMM).

3.3. MTS Cell Viability Assay

3T3-L1 cells were seeded in 96-well plates at a density of $1.0x10^4$ cells per well. The cells were initiated for differentiation as previously described. Stock solutions of 100 µM and 10 µM of pure quercetin or isorhamnetin were prepared with PEM, DM, and AMM. Different concentrations of quercetin (25 nM to 25 µM), and isorhamnetin (25 nM to 25 µM) of the total volume of the media used were tested during the differentiation period of eight days. The cells were incubated for 1, 3, 5, 7, and 8 days at 37°C in an incubator with 5% CO_2. After incubation, cell viability was determined according to

the protocol provided by the supplier of CellTiter 96 AQ_{ueous} One solution (Promega, Madison, WI). Twenty microliters of MTS reagent was added to every well and incubated under the same conditions for 4h. Absorbance of the plates was read at 490 nm in a BioRad Model 680 micro plate reader (Hercules, CA). The number of viable cells was directly proportional to the absorbance of formazan formed due to the reduction of MTS. Cell viability was expressed as the percentage of control cells. Treatments were tested in triplicates.

3.4. Oil Red O Staining Intracellular Triglycerides

3T3-L1 cells were seeded in 6-well plates at a density of $8x10^4$ cells per well. Cells were subjected to differentiation following the protocol described before including non-differentiated, control, and different concentrations of quercetin (25 nM to 25 μM) or isorhamnetin (25 nM to 25 μM) in triplicates. At day 10 of differentiation, 3T3-L1 adipocytes were washed with PBS and fixed with 10% formalin for 30 min. Then they were washed with distilled water twice, the cells were stained for 45 minutes in a 37°C incubator with diluted oil red O solution. Treatments were photographed with a microscope OLYMPUS Model DP72 (Center Valley, PA). Finally, the dye retained in 3T3-L1 cells was eluted with isopropanol and pipetted in a 96 well-plate to measure the absorbance by a microplate spectrophotometer BIORAD Benchmark Plus™ (Philadelphia, PA) at 510 nm. Briefly, the oil red O solutions was prepared from 0.05 g of oil red O dissolved in 100 ml of propanol (stock solution), then six parts of stock solution was mixed with four parts of distilled water, and this mixture was filtered by vacuum.

3.5 Biomarkers during 3T3-L1 Differentiation

In order to study the inhibitory effects of quercetin, or isorhamnetin on differentiation; the prevention approach was tested. Cells were treated in presence or absence of quercetin or isorhamnetin before and during the adipocyte differentiation process. The 3T3-L1 cells were seeded in T25 flasks at a density of $200x10^3$ cells per flask and subjected to differentiation following the protocol described before. Cells were treated with quercetin or isorhamnetin at concentrations of 0 to 25 μM from the expansion

period until the completion of adipocyte differentiation on day 10. Finally, the supernatant and the cell lysates were collected for further analysis.

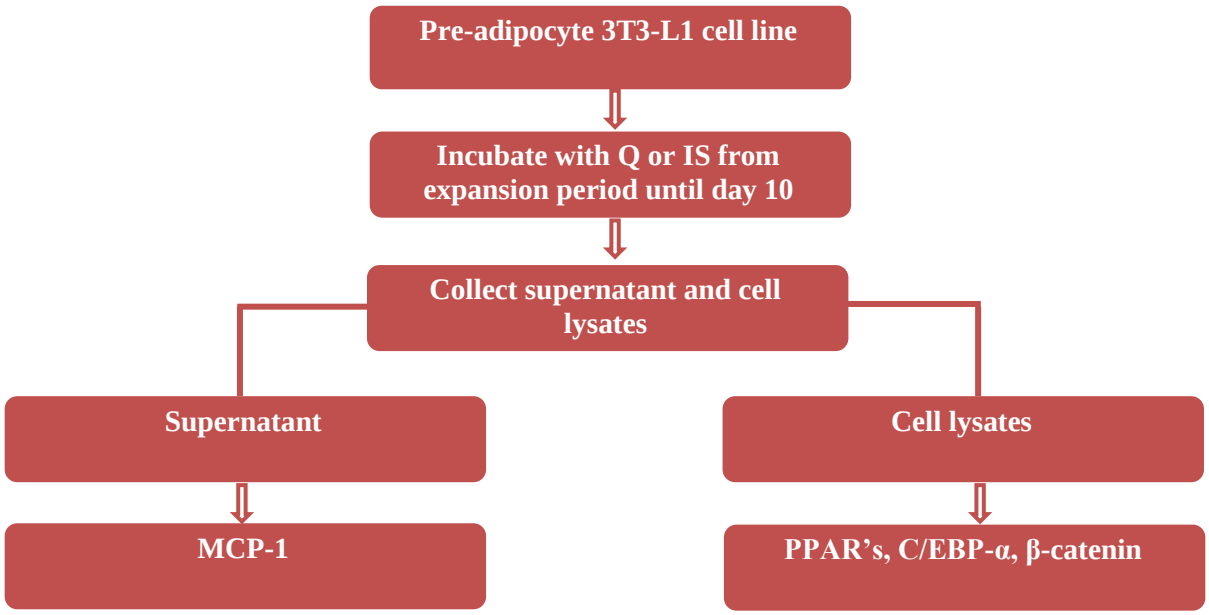


Figure 3.3. Interaction of bioactive compounds with 3T3-L1 cell line.

3.6. Enzyme Linked Immunosorbent Assay for Pro-inflammatory Cytokines

To determine the inhibitory effect of quercetin or isorhamnetin on differentiation, the supernatants were collected on day 0, 2, 5, 7, and 10 and MCP-1 levels were determined by ELISA using commercial kits from eBioscience® (San Diego, CA). All analyzes were performed in triplicates.

3.7. Western Blot Analysis

In order to determine the anti-adipogenesis properties of quercetin or isorhamnetin during 3T3-L1 differentiation, PPAR-γ, C/EBP-α, and β-catenin were identified on day 10 after differentiation. 3T3-L1 cells were rinsed once with ice-cold phosphate buffered saline (1X PBS), then lysed in 240 µl of radioimminoprecipitation assay buffer (RIPA) supplemented with protease inhibitor, sodium orthovanadate and PMSF for 30 min at 4°C. Cells were harvested by scraping and transferred into an eppendorf, incubated on ice for 20 min, centrifuged at 12,000 rpm for 30 min, and the supernatant was saved in a fresh eppendorf. Protein concentration in the supernatant was determined by the BCA method

using BCA protein Assay Reagents (Pierce). Extracts containing 20 µg protein from control, quercetin or isorhamnetin treated 3T3-L1 cells were mixed with LDS sample buffer (Invitrogen, Carlsbad, CA), water, boiled for 3 minutes for protein denaturation, and 20 µl of samples were loaded onto a 12 well 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA), ran for 35 min at 200 V and 125 mA. After protein separation, they were transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA) for 1h 10 min at 30 V and 170 mA. Five percent bovine serum albumin (BSA) in 1X PBS containing 0.05% Tween 20 (PBS-Tween) was used to block the membrane for 1h at room temperature. Then the membrane was incubated with primary antibody (dissolved in PBS-Tween containing 5% BSA as manufacturer's instructions) overnight at 4°C with gentle agitation. The next day, the membrane was washed 3 times for 10 min each with PBST, secondary antibody (dissolved in PBS-Tween containing 5% BSA as manufacturer's instructions) was incubated for 1h at room temperature, and washed again 3 times for 10 min each with PBST. Finally the blots were developed by enhanced chemiluminescence with SuperSignal® West Pico Chemiluminescent substrate and exposed to X-ray film (Kodak X-omat 1000A processor).

3.8. Statistical Analysis

Data were expressed as means ± standard deviation from triplicates of each experiment. Statistical analysis was performed using the Statistical Analysis Software (SAS) (version 9.2). Differences between control and treatments were determined by analysis of variance (ANOVA) and followed by Tukey analysis. A *P*-value of < 0.05 was considered statistically significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Cell Viability

To determine the effects of quercetin or isorhamnetin on cell viability during adipocyte differentiation period of 8 days, MTS cell viability assay was performed on 3T3-L1 cells treated with 0 to 25 µM quercetin or 0 to 10 µM isorhamnetin, and non-differentiated cells maintained only on PEM. Data were collected on days 1, 3, 5, 7, and 8. The cell viability results are expressed as the percentage test cells surviving compared to control cells. Treatments were performed in triplicates.

The cell viability results for quercetin are shown in Figure 4.1. There was no significant difference ($P = 0.3405$; $P > 0.05$) between non differentiated cells, control, and 25 nM to 10 µM quercetin treatments. The cell viability results for isorhamnetin are shown in Figure 4.2. Similarly to quercetin, there was no significant difference ($P = 0.8349$; $P > 0.05$) between non differentiated cells, control, and 25 nM to 10 µM isorhamnetin treated cells. During adipocyte differentiation in the presence of quercetin or isorhamnetin, average cell viability was 94.84 and 97.63%, respectively. Based on the results, quercetin or isorhamnetin at the concentrations used were not cytotoxic to 3T3-L1 during the differentiation from pre-adipocytes to adipocytes.

The changes in lipid accumulation and morphology of the cells were clearly observed on day 4, after the differentiation media was added. The complete adipocyte differentiation could be achieved between 8 to 10 days [54]. Because the viability of control, quercetin or isorhamnetin treated, and non-differentiated cells was higher than 90 %; 3T3-L1 cells were maintained for 10 days for further observations in terms of lipid accumulation, morphology and biomarker measurements. Yang et al. performed a cell proliferation assay where the cell viability was measured on the third day after the drug addition (quercetin-3-O-(6”-Feruloyl)-β-D-Galactopyranoside or quercetin) and their results showed no significant difference in proliferation [13].

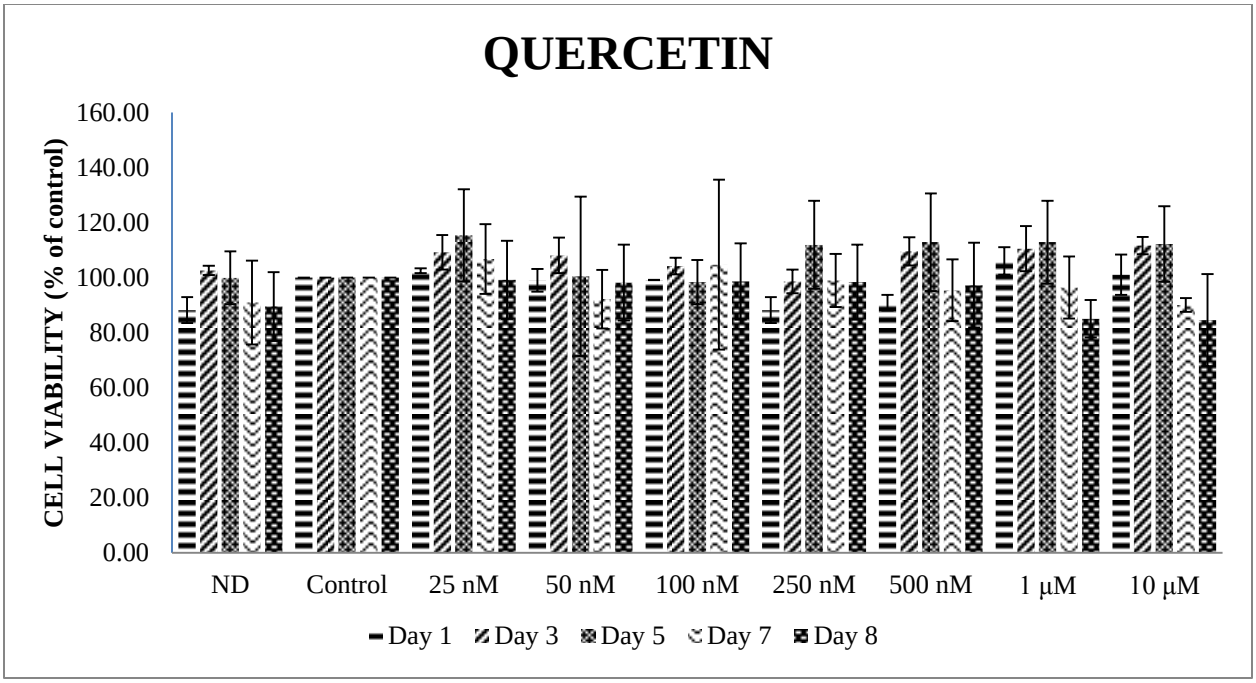


Figure 4.1. Effects of quercetin on 3T3-L1 cell viability. Non-differentiated 3T3-L1 cells were treated with quercetin (0 to 25 μM) for 0 to 8 days. The values are expressed as percentage of control cells.

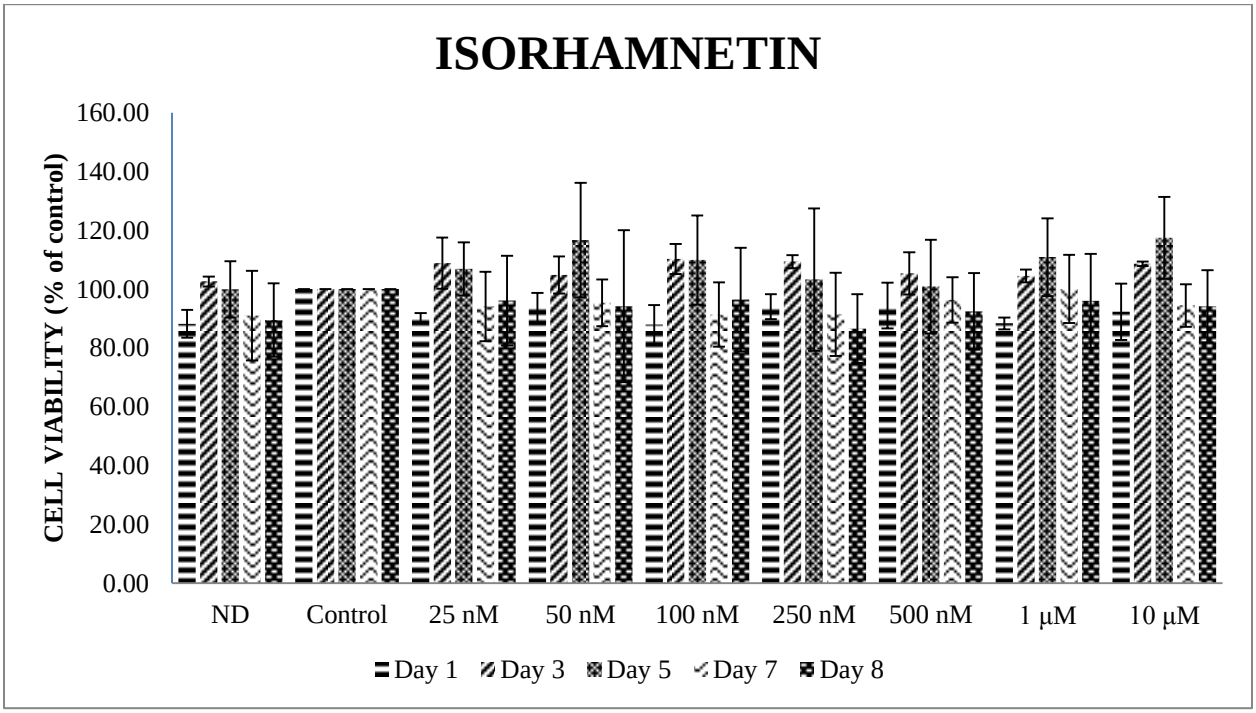


Figure 4.2. Effects of isorhamnetin on 3T3-L1 cell viability. Non-differentiated 3T3-L1 cells were treated with isorhamnetin (0 to 10 μM) for 0 to 8 days. The values are expressed as percentage of control cells.

4.2. Oil Red O Staining

The inhibitory effect of quercetin or isorhamnetin on lipid accumulation was evaluated on day 10 of treatment, after the differentiation was induced by adding the differentiation media containing various concentrations of quercetin or isorhamnetin. The retained dye by the intracellular lipids was eluted with isopropanol and measured at 510 nm. Treatments were compared to control or non-differentiated cells. There were significant statistical differences ($P < 0.0001$; $P < 0.05$) between non differentiated cells, control and quercetin or isorhamnetin treated cells. Statistical and Tukey's indicated that there were differences between control – isorhamnetin treated, quercetin treated – non-differentiated, quercetin treated – isorhamnetin treated, and non-differentiated – control. The results are expressed as percentage of control. Non-differentiated cells showed a lipid accumulation of 41.71 ± 9.35% less than control.

4.2.1. Effect of Quercetin on Lipid Accumulation

The oil red O staining results for quercetin are shown in Figure 4.3. There was significant difference ($P = 0.0002$; $P < 0.05$) between non differentiated cells, control, and 25 nM to 25 μM quercetin treated cells. Tukey's test stated that cells treated with 25 μM quercetin were not significantly different from non-differentiated cells. Cells treated with 25 μM quercetin down regulated the lipid droplet accumulation by 8.64 ± 9.54% compared to control. However, the difference was significant between non-differentiated cells and 25 nM to 10 μM quercetin.

Similarly, Yang et al. stated in their investigation that concentration of 25 μM decreased lipid accumulation around 15.9 ± 2.5%, and concentration of 12.5 μM was not significantly different from control [4]. Furthermore, they found that combinations of quercetin with resveratrol down regulated the lipid accumulation by 68.7 ± 0.7%. On the other hand, Yang et al. reported that concentrations of quercetin from 0.1 to 20 μM have potential in demonstrating concentration-dependent inhibition of adipocyte differentiation [13]. Our results suggest that quercetin alone might not be effective in inhibiting lipid accumulation as a combination of quercetin and resveratrol.

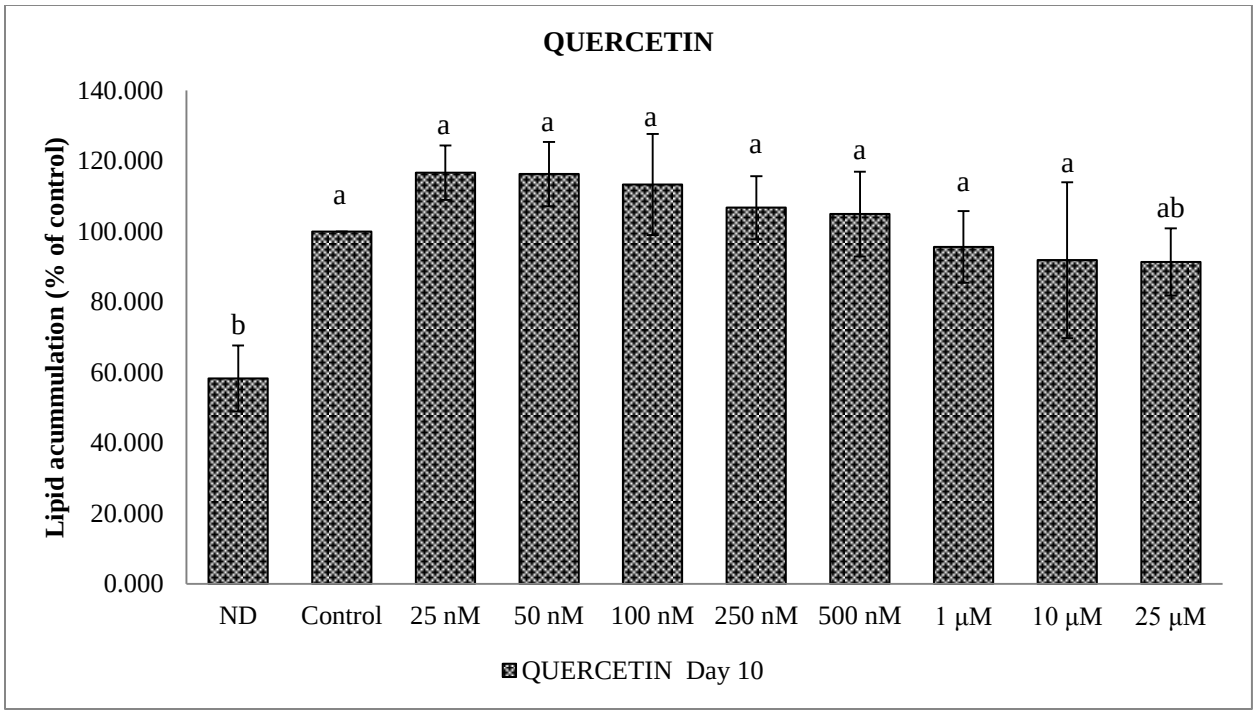
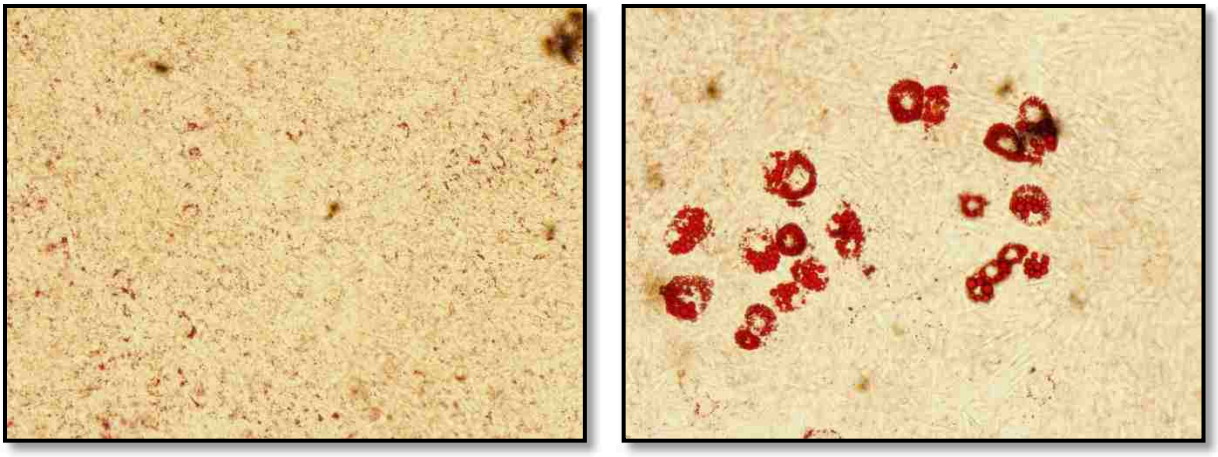


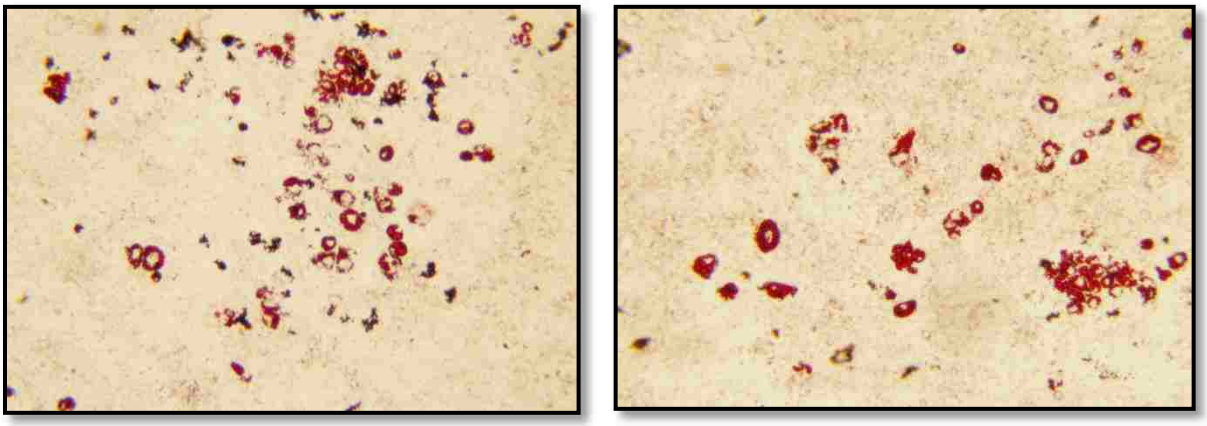
Figure 4.3. Effect of quercetin on lipid accumulation in 3T3-L1 cells. The values were expressed as percentage of control cells. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different ($P < 0.05$) among groups.

Differentiation was induced in 3T3-L1 cells for 10 days with or without quercetin or isorhamnetin. Oil red O stained adipocytes treated with quercetin (0 – 25 µM) were photographed at day 10. Microscopic observation was conducted with an OLYMPUS Model DP72 (Center Valley, PA) at 5X magnification. Figure 4.4 shows the pictures obtained for quercetin treatments in comparison with non-differentiated cells or control.

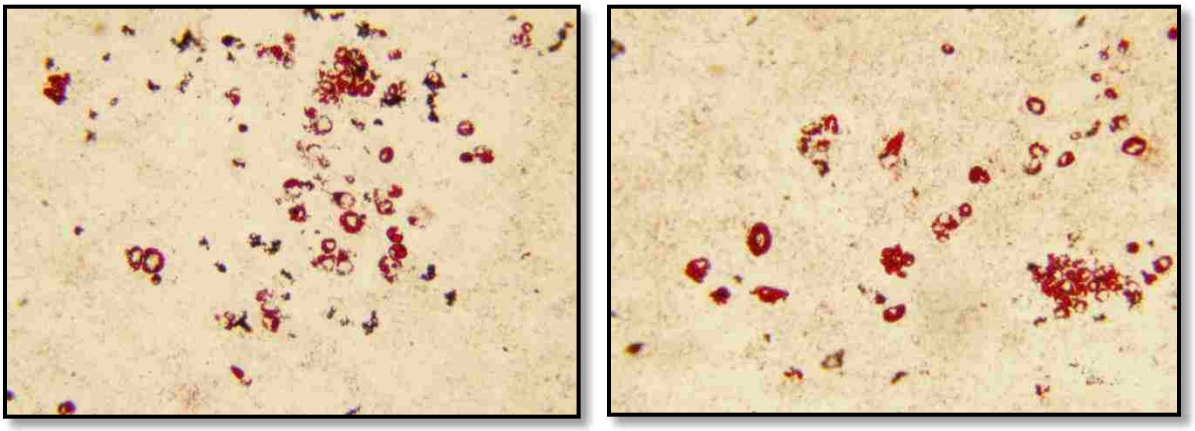
The pictures clearly show the difference between non-differentiated cells and control at day 10. Non-differentiated cells (Fig. 4.4. A) maintained the initial fibroblast morphology and show no lipid accumulation except for control (Fig. 4.4. B), where the intracellular lipid accumulation is noticeable by oil red O staining. As shown in Figure 4.4. (C-J), the concentration of quercetin from 25 nM to 25 µM did not highly attenuate the lipid accumulation in differentiated adipocytes as demonstrated by oil red O staining.



A. Non Differentiated cells day 10 (5X) B. Control day 10 (5X)



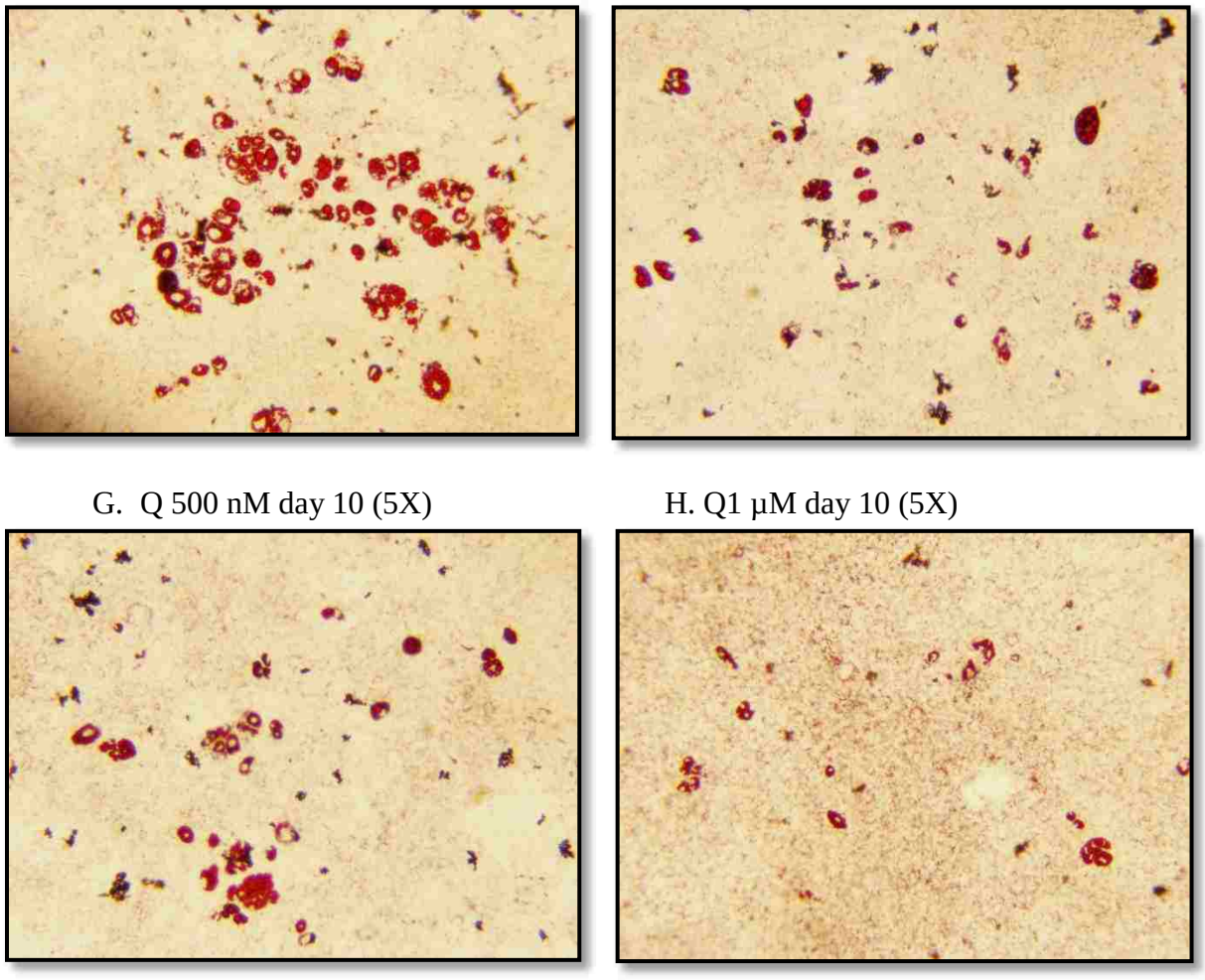
C. Q 25 nM day 10 (5X) D. Q 50 nM day 10 (5X)



E. Q 100 nM day 10 (5X) F. Q 250 nM day 10 (5X)

Figure 4.4. Effect of quercetin on 3T3-L1 cells. (A) non-differentiated cells, (B) control, and from (C) to (J) 25 nM to 25 µM quercetin treated, respectively. Pictures were obtained with a microscope OLYMPUS Model DP72 and taken at 5X magnification.

(Figure 4.4. continued)



G. Q 500 nM day 10 (5X)

H. Q1 µM day 10 (5X)

I. Q 10 µM day 10 (5X)

J. Q 25 µM day 10 (5X)

Figure 4.4. Effect of quercetin on 3T3-L1 cells. (A) non-differentiated cells, (B) control, and from (C) to (J) 25 nM to 25 µM quercetin treated, respectively. Pictures were obtained with a microscope OLYMPUS Model DP72 and taken at 5X magnification.

4.2.2 Effect of Isorhamnetin on Lipid Accumulation

The oil red O staining results for isorhamnetin are shown in Figure 4.5. There was significant difference ($P = 0.001$; $P < 0.05$) between non differentiated cells, control and 25 nM to 25 µM isorhamnetin treated cells. Tukey's test results showed that cells treated from 50 nM to 25 µM isorhamnetin were not significantly different from non-differentiated cells. However, control cells were significantly different from all other treatments. Even 25 nM isorhamnetin was significantly different

from control with a difference of 22.86 ± 12.62% compared to the control. In contrast Lee, et al. reported that concentrations of 25 and 50 µM isorhamnetin significantly lower the lipid accumulation [9].

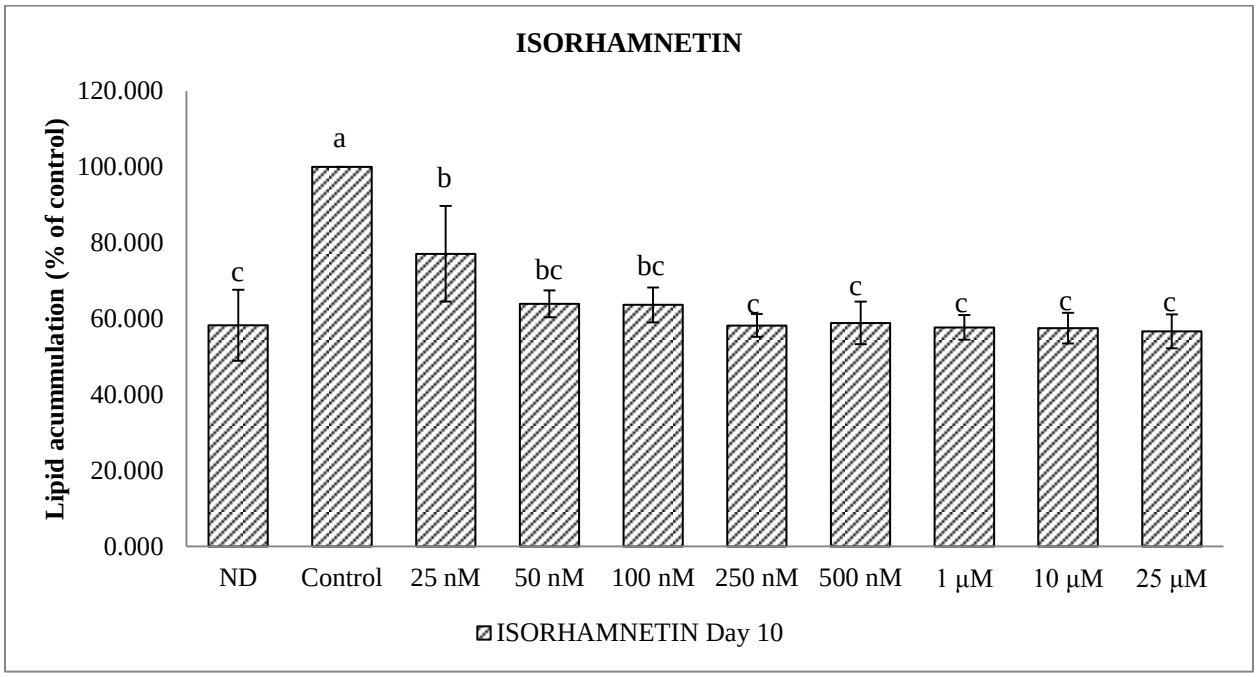
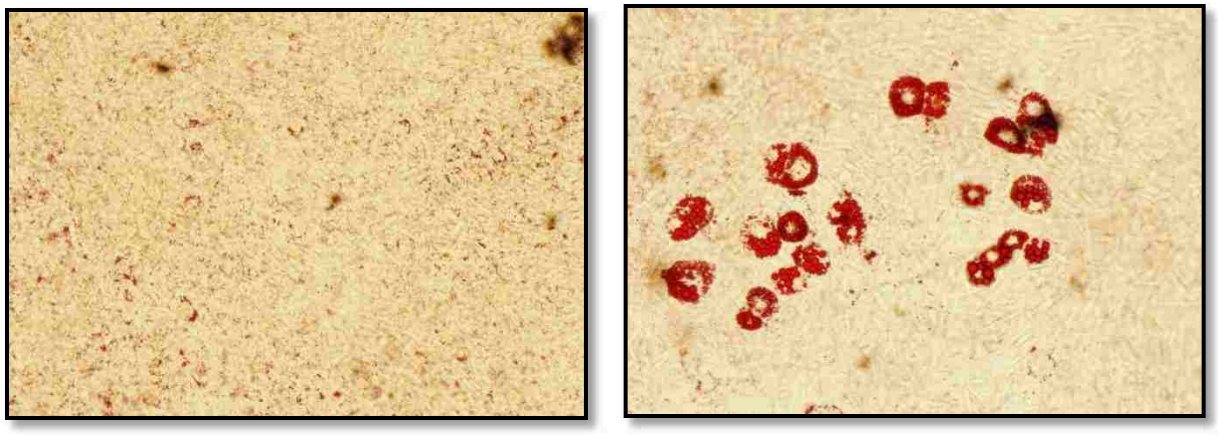


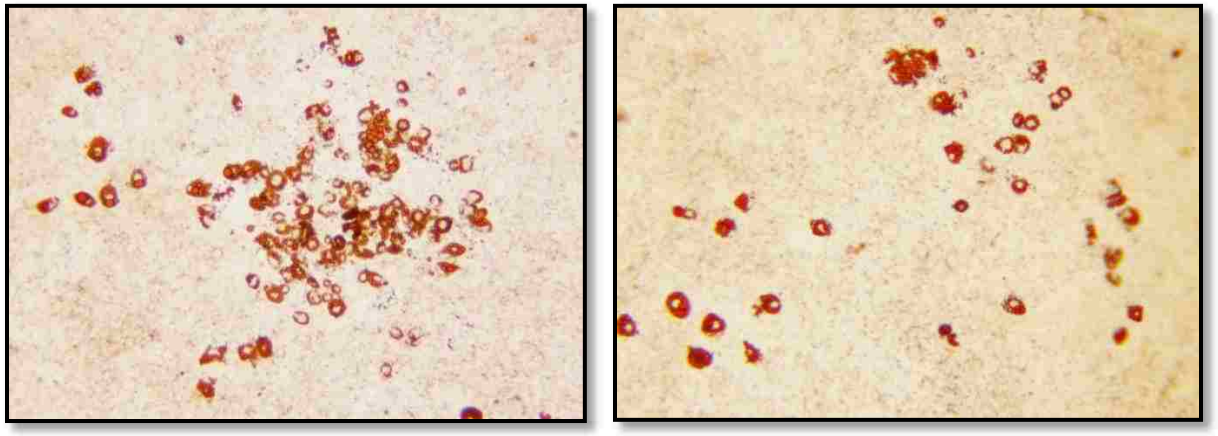
Figure 4.5. Effect of isorhamnetin on lipid accumulation on 3T3-L1 cells. The values are expressed as percentage of control cells. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different (P < 0.05) among groups.

Stained adipocytes treated with isorhamnetin (0 – 25 µM) were photographed on day 10 after oil red O staining by a microscope OLYMPUS Model DP72 (Center Valley, PA) at 5X magnification. Figure 4.6 shows the pictures obtained for isorhamnetin treatments in comparison with non-differentiated cells and control. The pictures clearly exhibit the difference between non-differentiated cells and control at day 10. Non-differentiated cells (Fig. 4.6. A) on day 10 maintained the fibroblast morphology and show no lipid accumulation compared to the control (Fig. 4.6. B), where the morphological alterations were evident as accumulation of lipid droplets in the cytoplasm. As shown in Figure 4.6. (C-J), the concentration of isorhamnetin from 25 nM to 25 µM attenuated the lipid accumulation in differentiated adipocytes as demonstrated by oil red O staining. These observations match with the results obtained in the dye quantification at 510 nm by spectrophotometry.



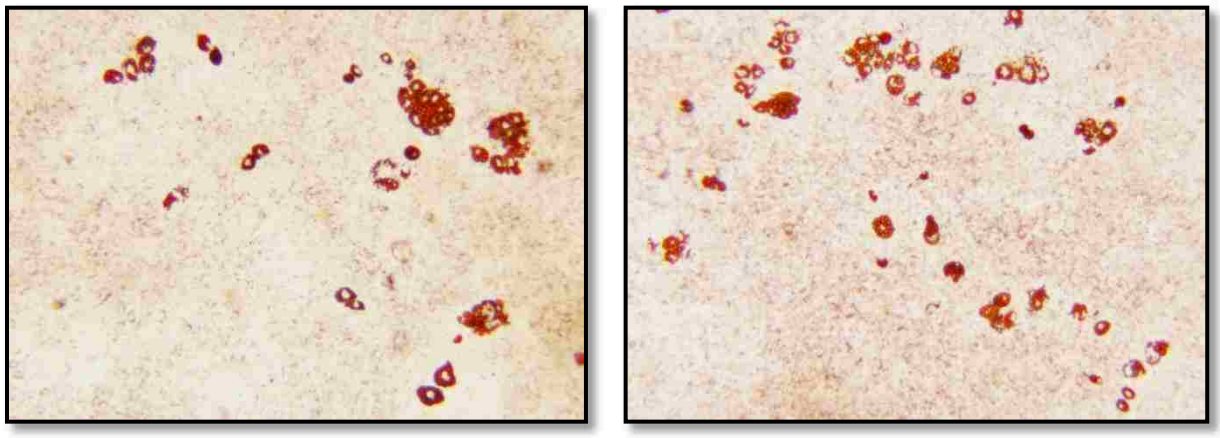
A. Non Differentiated cells day 10 (5X)

B. Control day 10 (5X)



C. IS 25 nM day 10 (5X)

D. IS 50 nM day 10 (5X)

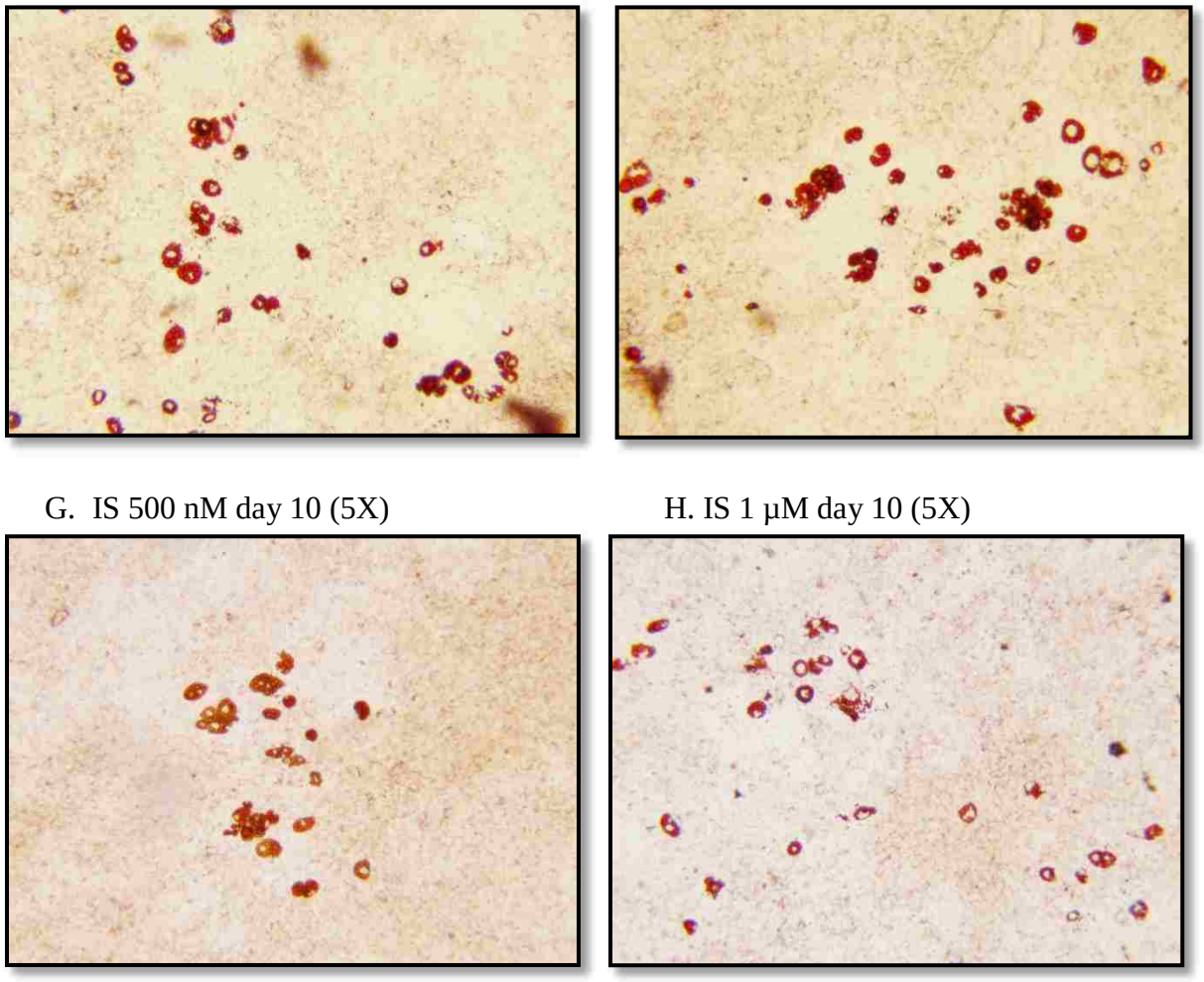


E. IS 100 nM day 10 (5X)

F. IS 250 nM day 10 (5X)

Figure 4.6. Effect of isorhamnetin on 3T3-L1 cells. (A) non-differentiated cells, (B) control, and from (C) to (J) 25 nM to 25 µM quercetin treated, respectively. Pictures were obtained with a microscope OLYMPUS Model DP72 and taken at 5X magnification.

(Figure 4.6. continued)



G. IS 500 nM day 10 (5X)

H. IS 1 µM day 10 (5X)

I. IS 10 µM day 10 (5X)

J. IS 25 µM day 10 (5X)

Figure 4.6. Effect of isorhamnetin on 3T3-L1 cells. (A) non-differentiated cells, (B) control, and from (C) to (J) 25 nM to 25 µM quercetin treated, respectively. Pictures were obtained with a microscope OLYMPUS Model DP72 and taken at 5X magnification.

4.3. Effects of Quercetin or Isorhamnetin on the Secretion of MCP-1 during Cell Differentiation

To determine the effect of quercetin or isorhamnetin on secretion of cytokine MCP-1, the supernatants were collected on day 0, 2, 5, 7, and 10. Only samples from days 0, 2, and 10 were measured by ELISA using commercial kits from eBioscience® (San Diego, CA). Treatments were compared against the control and non-differentiated cells. There were significant statistical differences ($P < 0.0001$; $P < 0.05$) between non differentiated cells, control and quercetin or isorhamnetin treatments. The means separation by Tukey's suggested that there are differences between non-differentiated – control, control –

isorhamnetin treated, and quercetin treated – non-differentiated. Between quercetin treated cells and isorhamnetin treated cells significant difference was not evident. The results were expressed per the protein concentration of the supernatant obtained by BCA protein assay.

4.3.1. Effects of Quercetin on the Secretion of MCP-1 during Cell Differentiation

The MCP-1 results for secretion over the adipocyte differentiation of quercetin are shown in Figure 4.7. There was no significant difference ($P = 0.1511$; $P > 0.05$) between non differentiated cells, control and 25 nM to 25 µM quercetin treated cells. These data suggest that quercetin does not affect the MCP-1 levels per day during the adipocyte differentiation. Extracts rich in *p*-Coumaric acid, quercetin, and resveratrol change levels of MCP-1 [56] suggesting that quercetin alone might not have inhibition properties against MCP-1. Studies on 3T3-L1 cells with auraptene at concentrations from 0 to 100 µM had reported results that suggest a decrease in the levels of MCP-1. Additionally, the effects of levels of auraptene on MCP-1 were dose dependent [12].

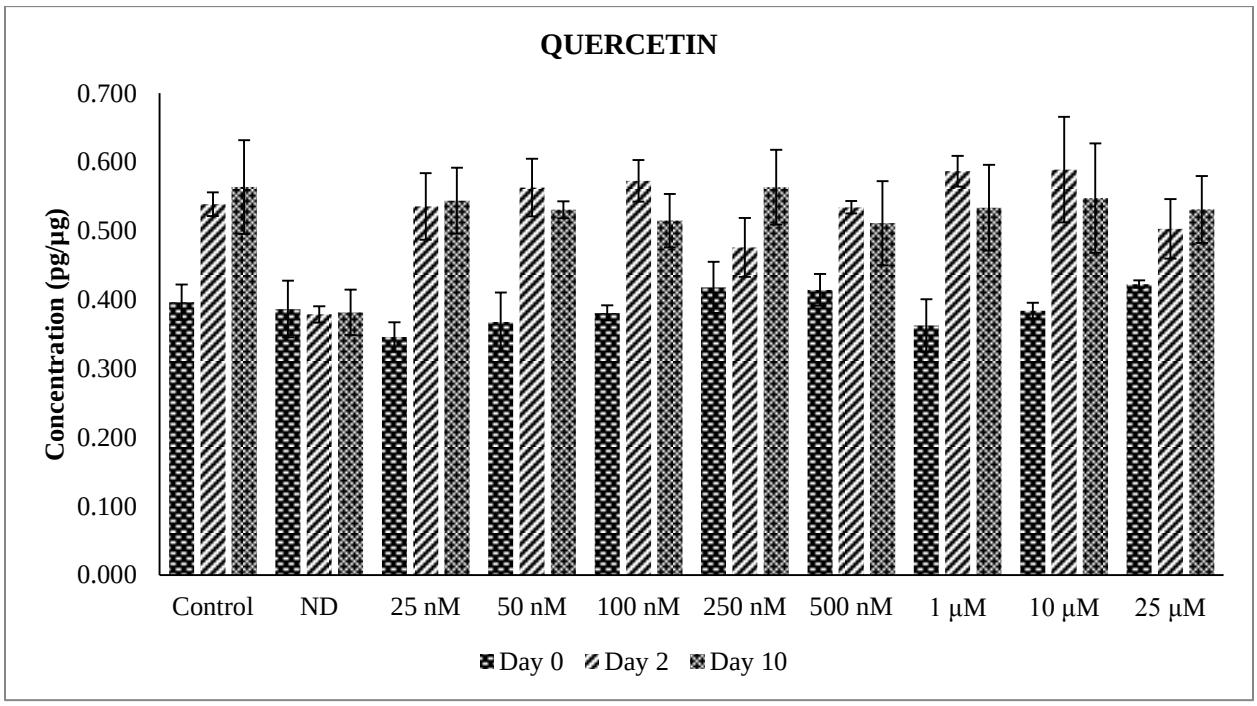


Figure 4.7. Effect of quercetin on MCP-1 levels (pg/µg protein) during 3T3-L1 adipocyte differentiation. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different ($P < 0.05$).

4.3.2. Effects of Isorhamnetin on the Secretion of MCP-1 during Cell Differentiation

The levels of MCP-1 in the supernatants of 3T3-L1cells treated with vehicle, undifferentiated, or isorhamnetin are shown in Figure 4.8. There was no significant difference ($P = 0.1275$; $P > 0.05$) between non differentiated cells, control and 25 nM to 25 µM isorhamnetin treatments. These data suggest that isorhamnetin does not affect the MCP-1 levels during adipocyte differentiation. Other studies in human umbilical artery smooth muscle cells stimulated with TNF-α for 24 h in order to arouse the MCP-1 secretion showed that quercetin at 2 µM to 10 µM decreased the secretion of MCP-1 to 18% and 67%, respectively. However, isorhamnetin which is a metabolite of quercetin did not affect MCP-1 secretion [57].

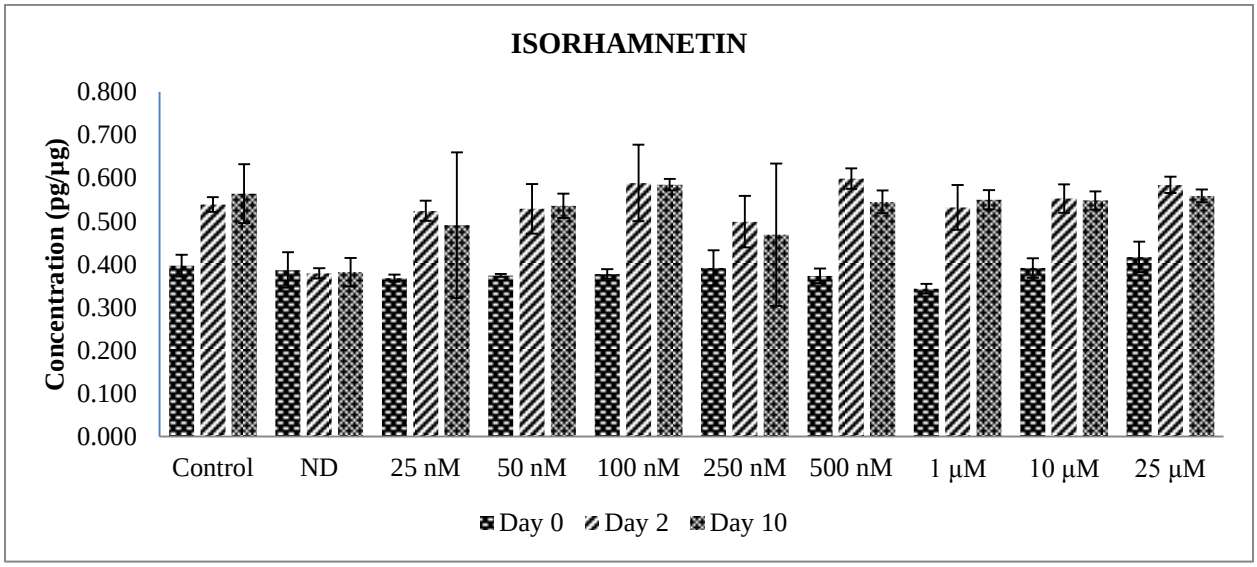


Figure 4.8. Effect of isorhamnetin on MCP-1 levels (pg/µg protein) during 3T3-L1 adipocyte differentiation. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different ($P < 0.05$).

4.4. Expression of PPAR-γ, C/EBP-α, and β-catenin during 3T3-L1 Cell Differentiation

In order to understand the differentiation process of 3T3-L1 cells during the cell culture the expression of β-catenin, PPAR-γ, and C/EBP-α was monitored. Cell lysates were collected after pre-adipocyte expansion medium (PEM) was replaced with differentiation medium (DM). That was considered day 0 (D0); next samples were collected after 5, 16, and 24 hours. Subsequently after 48 h,

DM was replaced by adipocyte maintenance medium (AMM) every 72 h, samples were saved at day 2 (D2), day 5 (D5), day 7 (D7), and day 10 (D10). Finally, a sample from non-differentiated cells was collected on day 10. The total cell lysates were analyzed by western blot analysis as described under the materials and methods. Results are shown in Figure 4.9.

Figure 4.9, demonstrated that the β-catenin level decreased until day 5, and increased slightly at day 7 and 10. Non-differentiated cells had a similar level of β-catenin as D0. Peroxisome proliferator-activated receptor-γ (PPAR-γ) levels increased by day 10 (D10). CCAAT/enhancer binding protein α (C/EBP) was visible on day 10 (D10) and β-catenin level decreased. Lee et al. showed that β-catenin level decreased at early period after DM was added, while differentiation was critical as revealed by the significant up-regulation of PPAR-γ and C/EBP-α [58].

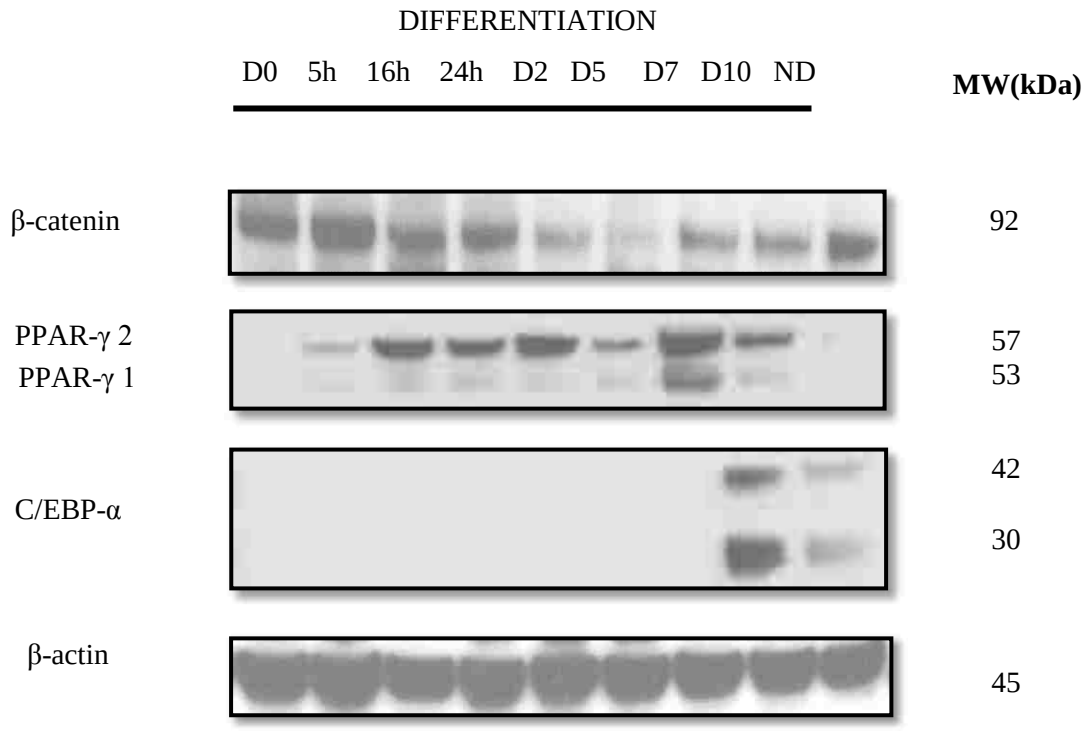


Figure 4.9. Western blot analysis results for control cells during the adipocyte differentiation period of 10 days.

Western blots developed by enhanced chemiluminescence were quantitatively analyzed by Quantity One software. Figure 4.10. shows the results obtained for β-catenin expression during adipocyte differentiation. β-catenin level was decreased slowly until D5, and increased in day 7, and 10. There was significant difference for β-catenin expression ($P < 0.0001$; $P < 0.05$) during adipocyte differentiation. These data show that β-catenin expression decreased to 41.90 ± 2.43% of control during adipocyte differentiation.

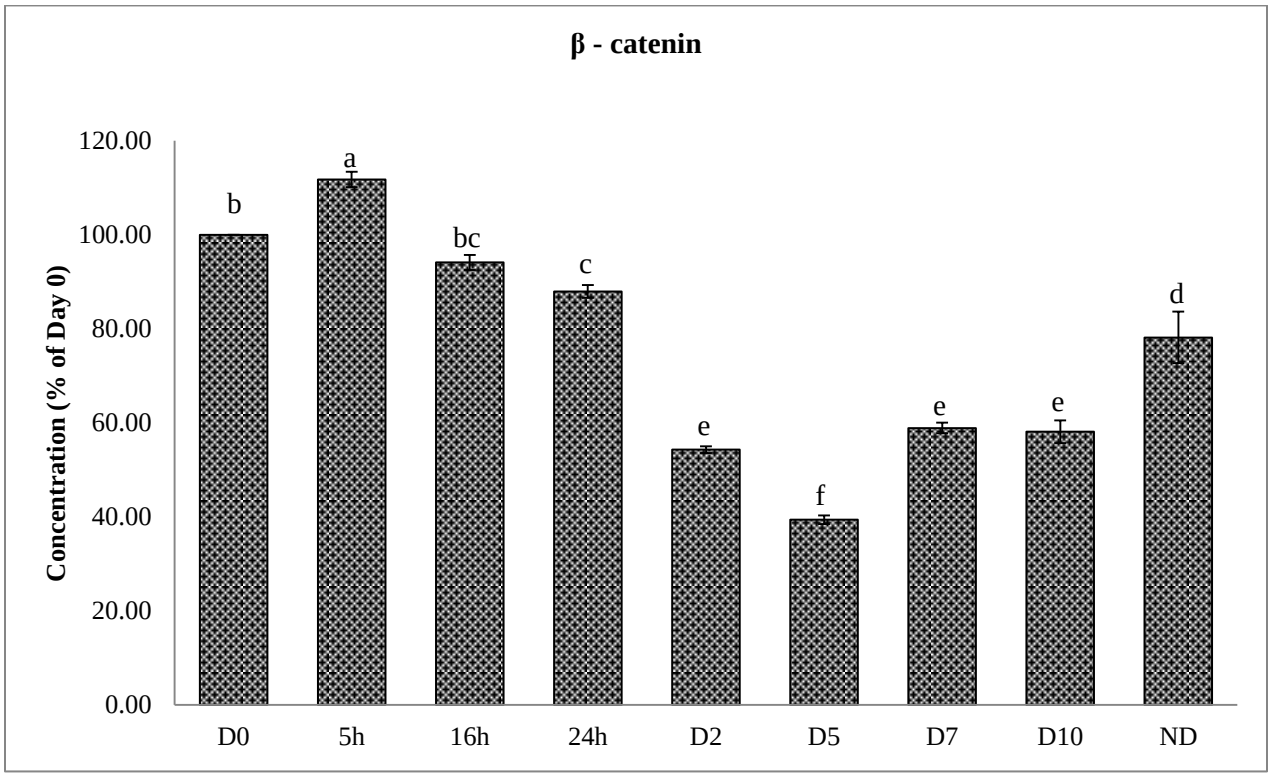


Figure 4.10. β-catenin expression in control cells during adipocyte differentiation. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different ($P < 0.05$).

C/EBP-α expression through adipocyte differentiation is shown in Figure 4.11.; there was significant difference ($P < 0.0001$; $P < 0.05$). C/EBP-α p30 and p42 were maintained the same from D0 to D7, and increased significantly in 69.53 ± 0.40% and 65.61 ± 1.24% respectively from D7 to D10. Also, C/EBP-α p30 and p42 level in non-differentiated cells was slightly higher than the level maintained until D7.

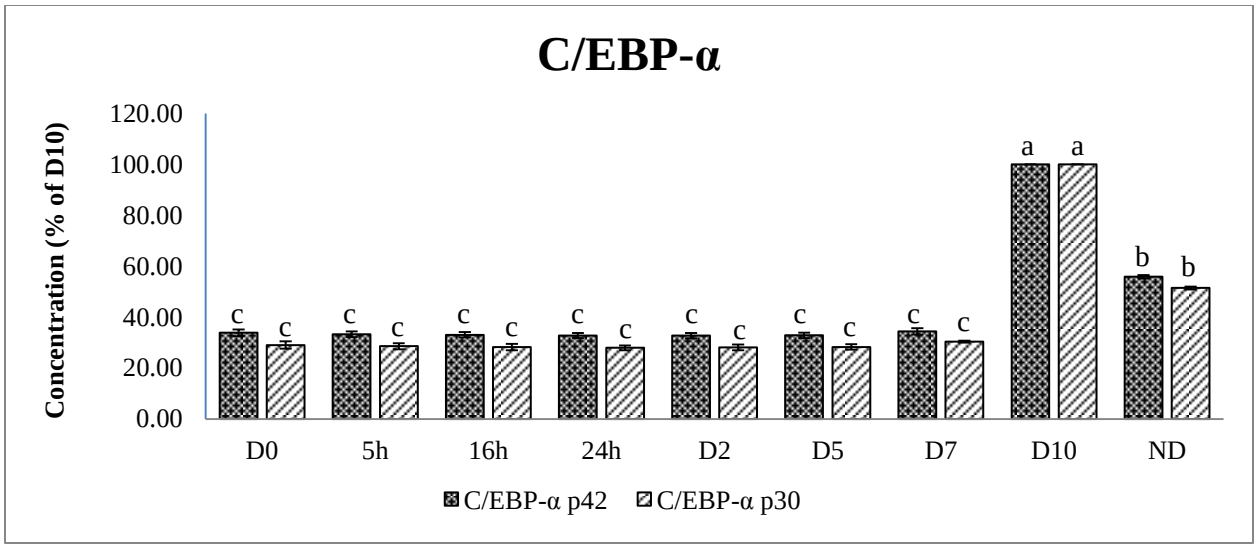


Figure 4.11. C/EBP-α expression during adipocyte differentiation. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different (P < 0.05).

PPAR-γ expression through adipocyte differentiation is shown in Figure 4.12.; there was significant difference (P < 0.0001; P < 0.05). Highest levels of PPAR-γ 1 and 2 were at D7 and decreased by 113.69 ± 1.34% and 44.09 ± 4.06% respectively from D7 to D10. Also, PPAR-γ 1 and 2 levels of non-differentiated cells on D10 were not significantly different from D0.Other important observation was that PPAR-γ 1 and 2 levels were normally increasing until D2 and decreased by 3.27 ± 0.91% and 47.65 ± 0.34% respectively at D5.

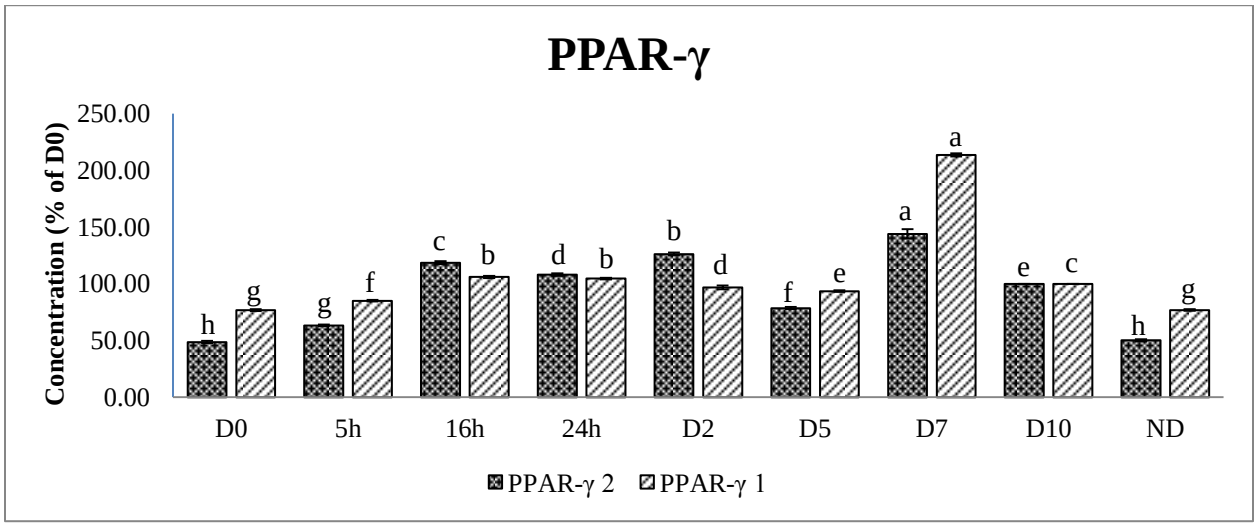


Figure 4.12. PPAR-γ expression during adipocyte differentiation. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different (P < 0.05).

4.5. Effects of Quercetin on PPAR-γ, C/EBP-α, and β-catenin during 3T3-L1 Cell Differentiation

To quantify the effect of quercetin - adipocyte differentiation by quercetin, we determined the expression of β-catenin, PPAR-γ, and C/EBP-α in lysates of quercetin treated cells at day 10, and compared with control and non-differentiated cells as described under the material and methods. The results shown in Figure 4.13 clearly show that β-catenin level is more intense in non-differentiated cells; and decreased after quercetin treatment. PPAR-γ decreased with increasing concentration of quercetin; at 25 µM quercetin the PPAR-γ 1 and 2 were at the lowest levels and lowers even than non-differentiated cells. C/EBP p30 and p42 levels behaved similarly to PPAR-γ 1 and 2. They decreased critically at 25 µM quercetin and were even lowers than non-differentiated cells. These results suggest that PPAR-γ 1 and 2 and C/EBP-α p30 and p42 expressions were concentration dependent.

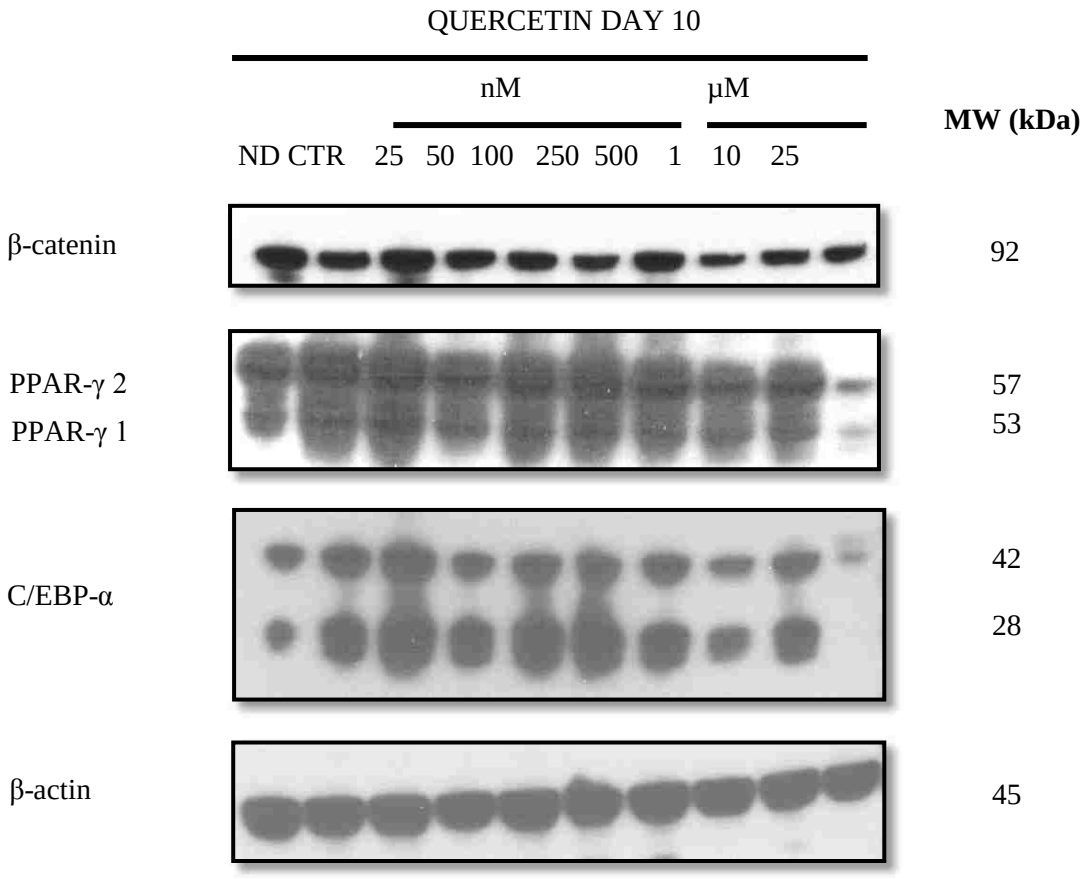


Figure 4.13. Western blot of β-catenin, PPAR-γ, and C/EBP-α on day 10 in response to quercetin concentration from 25 nM to 25 µM.

The results of β-catenin expression in cells treated with vehicle, non-differentiated or 25 nM to 25 μM quercetin are shown in Figure 4.14. The highest expression of β-catenin level was in non-differentiated cells, and the lowest at 25 μM quercetin. Level of β-catenin did not follow a trend, these suggest it expression was not dose dependent. There was significant difference in β-catenin expression ($P < 0.0001$; $P < 0.05$) among increasing concentrations of quercetin from 25 nM to 25 μM. Non-differentiated cells had a β-catenin level 36.79 ± 1.59% higher than control. However, these data suggest that quercetin concentrations can affect β-catenin expression.

C/EBP-α levels in quercetin treated cells are shown in Figure 4.15.; there was significant difference ($P < 0.0001$; $P < 0.05$). C/EBP-α level did not have a specific trend in response to quercetin concentrations. However, at increasing concentrations of quercetin the levels of C/EBP-α p30 and p42 decreased gradually. Overall, C/EBP- α p30 and p 42 levels decreased by 68.73 ± 2.66% and 54.48 ± 2.60%, respectively compared to control. Recent studies have shown that quercetin at 25 μM did not have any effect on the expression of C/EBP-α p30 and p42, whereas quercetin and resveratrol significantly decreased the expression by 60.2 ± 1.2% and 45.4 ± 1.2% respectively [4]

PPAR-γ levels in cells treated with 25 nM to 25 μM quercetin are shown in Figure 4.16.; there was significant difference ($P < 0.0001$; $P < 0.05$). PPAR-γ level had a decreased as quercetin concentration increased. We also observed that level of PPAR-γ decreased gradually, this suggest a dependent dosage relation between PPAR-γ and quercetin. PPAR-γ 1 and 2 lowest levels were observed at 25 μM quercetin.

Overall the PPAR-γ 1 and 2 levels decreased by 45.03 ± 3.17% and 27.58 ± 12.39% respectively compared to control. Similar study had reported a no significant effect on PPAR-γ 1 and 2 expression at concentrations of 12.5 or 25 μM quercetin [4]. Other flavonoid compounds as nobiletin at concentration from 0 to 100 μM had reported similar results about down-regulation of PPAR-γ expression as quercetin [3].

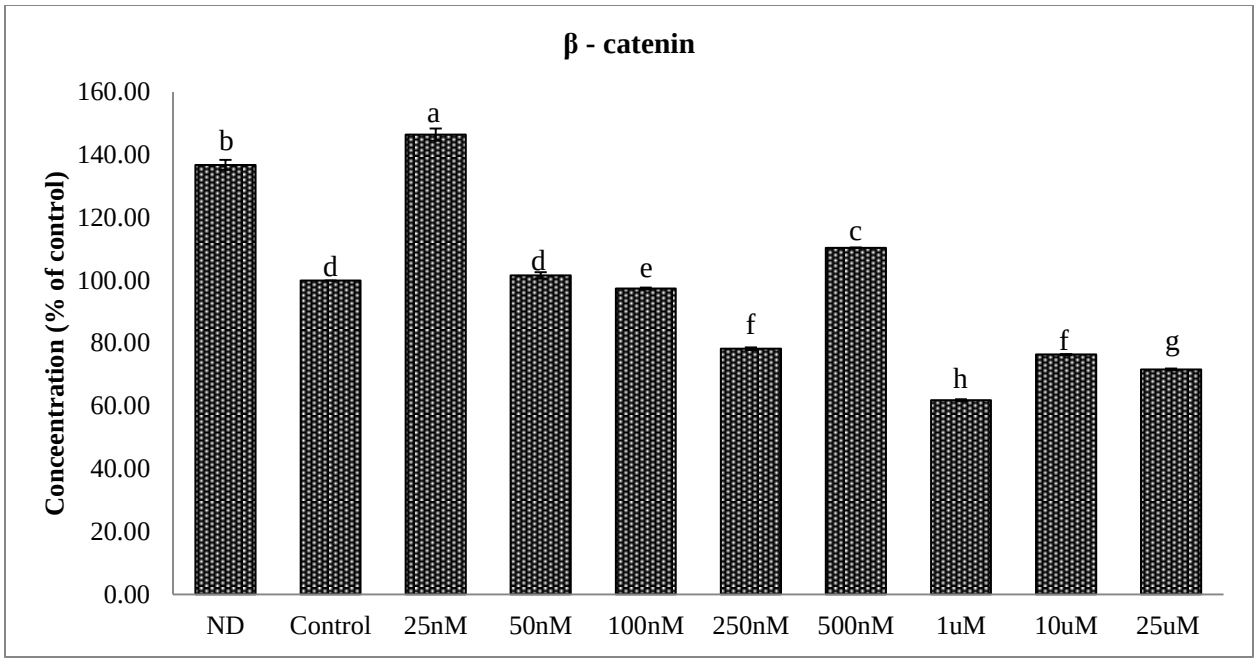


Figure 4.14. β-catenin expression in quercetin (25 nM to 25 µM) treated cells. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different (P < 0.05).

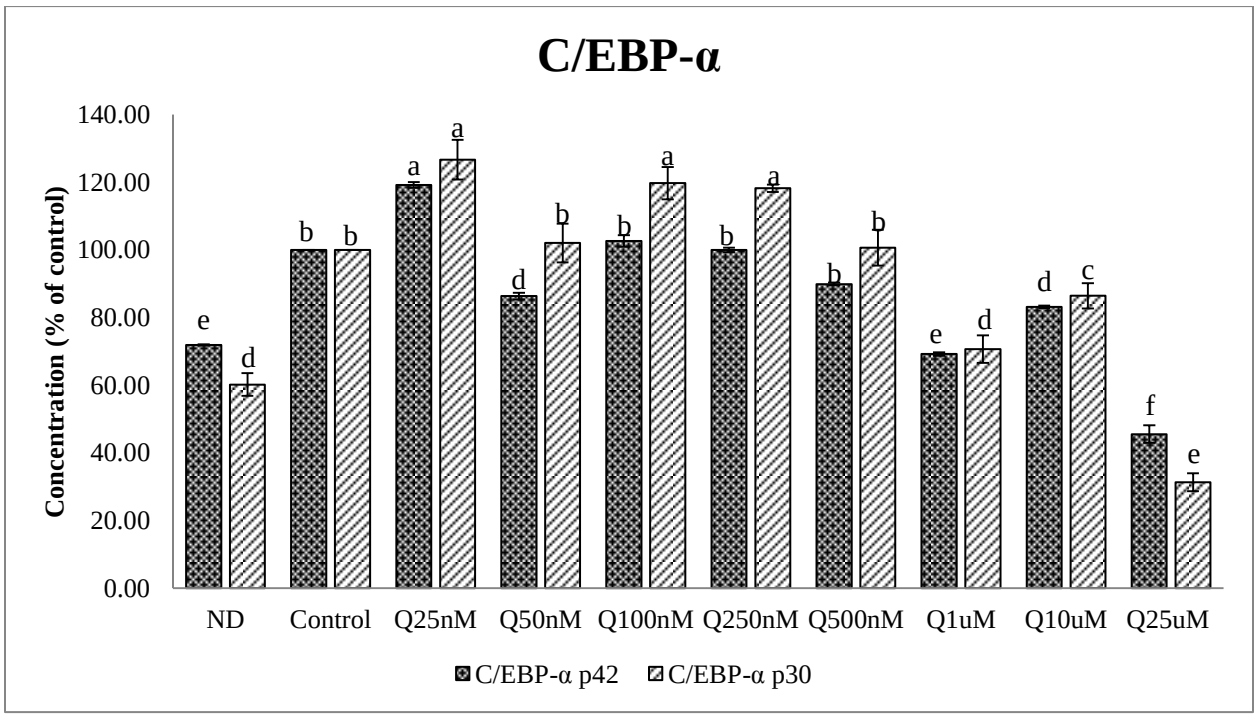


Figure 4.15. C/EBP-α expression in quercetin (25 nM to 25 µM) treated cells. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different (P < 0.05).

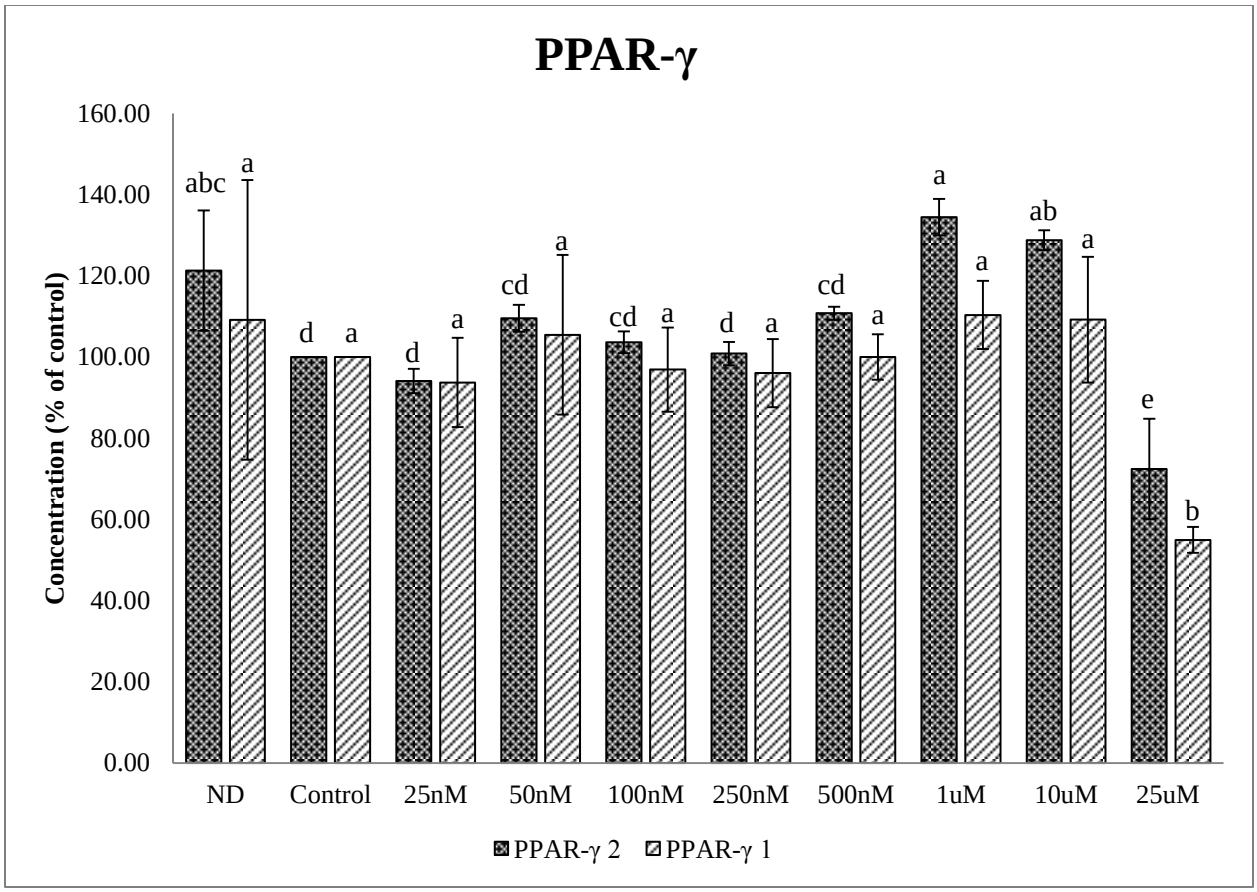


Figure 4.16. PPAR-γ expression in quercetin (25 nM to 25 µM) treated cells. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different ($P < 0.05$).

4.6 Effects of Isorhamnetin on PPAR-γ, C/EBP-α, and β-catenin during 3T3-L1 Cell Differentiation

To quantify inhibition of adipocyte differentiation by isorhamnetin, the expression of β-catenin, PPAR-γ, and C/EBP-α in cells treated with 25 nM to 25 µM was examined on day 10, and compared with control and non-differentiated cells as described in the material and methods. The expression of β-catenin, PPAR-γ and C/EBP-α on D10 is shown in Figure 4.17.

The β-catenin level is highly expressed in non-differentiated cells; also β-catenin is expressed among isorhamnetin treatments. PPAR-γ 1 and 2 expressions decreased sensitively with increasing concentrations of isorhamnetin PPAR-γ 1 and 2 levels were lowest even in comparison with non-differentiated cells. C/EBP-α p30 and p42 levels also decreased. These results suggest that PPAR-γ and C/EBP-α expressions are dosage dependent.

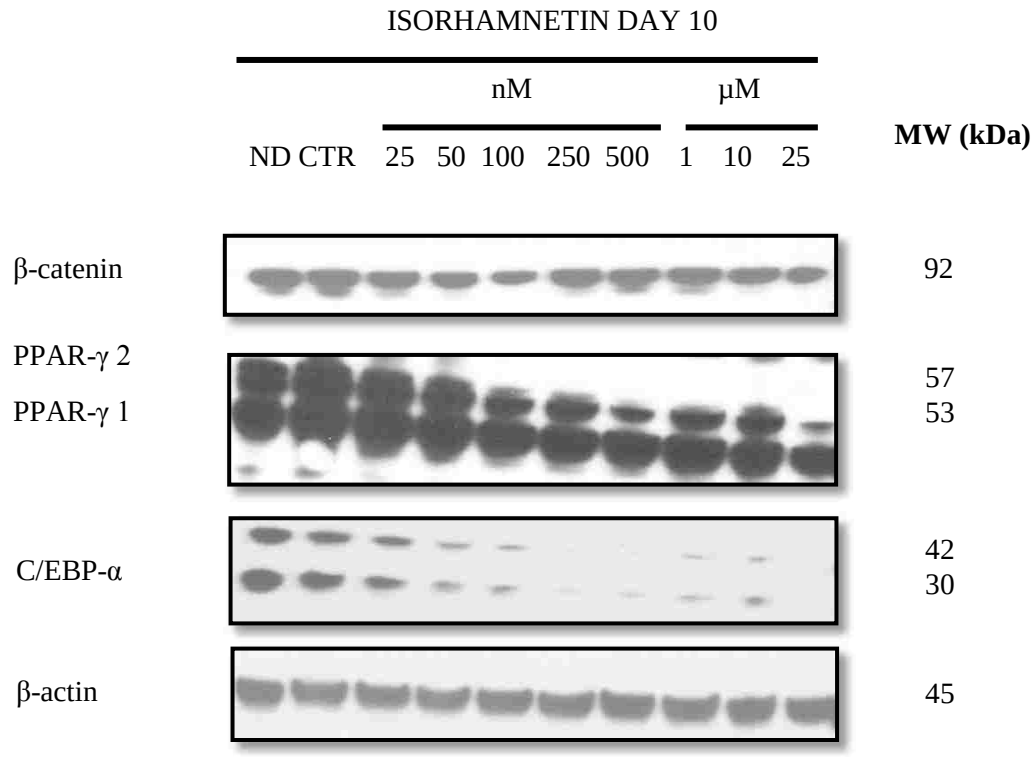


Figure 4.17. Western blot analysis of β-catenin, PPAR-γ, and C/EBP-α on day 10 in response to isorhamnetin concentration from 25 nM to 25 μM.

β-catenin expression in response of isorhamnetin concentration from 25 nM to 25 μM, control and non-differentiated cells is shown in Figure 4.18. Highest expression of β-catenin level was in control cells, and the lowest in 100 nM isorhamnetin treated cells. Level of β-catenin does not follow a specific trend, these suggest it expression is not dosage dependent. There was significant difference for β-catenin expression ($P < 0.0001$; $P < 0.05$) among isorhamnetin treatments. Quercetin maintained levels of β-catenin over 61.80 ± 0.30%, while isorhamnetin levels were above 49.04 ± 0.95% of control. These results suggested that quercetin had better effects to maintain β-catenin level than isorhamnetin.

The results of C/EBP-α level in cells treated with 25 nM to 25 μM are shown in Figure 4.19. There was significant difference ($P < 0.0001$; $P < 0.05$) among at increasing concentrations of isorhamnetin from 25 nM to 25 μM. C/EBP-α p30 and p42 levels decreased with increasing concentration

of isorhamnetin. C/EBP-α p 30 and p4 2levels decreased dramatically by 72.68 ± 1.54% and 69.31 ± 1.22% respectively, until 25 µM isorhamnetin compared to control In contrast with quercetin results we can state that isorhamnetin had a similar response to inhibition of C/EBP-α p30 and p42 expression. Lee et al. reported significant decrease of C/EBP-α p30 and p42 levels in cells that were treated with 25 or 50 µM isorhamnetin during adipocyte differentiation compared to control cells [9].

PPAR-γ 1 and 2 levels in isorhamnetin treated cells are shown in Figure 4.20.; there were significant different ($P < 0.0001$; $P < 0.05$). PPAR-γ 1 and 2 level decreased with increasing concentration of isorhamnetin. These results suggest a dependent dosage relation between PPAR-γ and isorhamnetin. PPAR-γ 1 and 2 lowest levels were observed at 25 µM isorhamnetin, on overall the PPAR-γ 1 and 2 level decreased by 41.48 ± 9.51% and 2.01 ± 32.46 respectively compared to control. Results suggest that quercetin have a highest effect on PPAR-γ 1 and 2 than isorhamnetin. Quercetin decreased PPAR-γ 1 and 2 expressions by 40.55 ± 6.34% and 25.57 ± 20.07% more than isorhamnetin. Lee et al. reported a decreased expression of PPAR-γ 1 and 2 by 25 or 50 µM isorhamnetin [9].

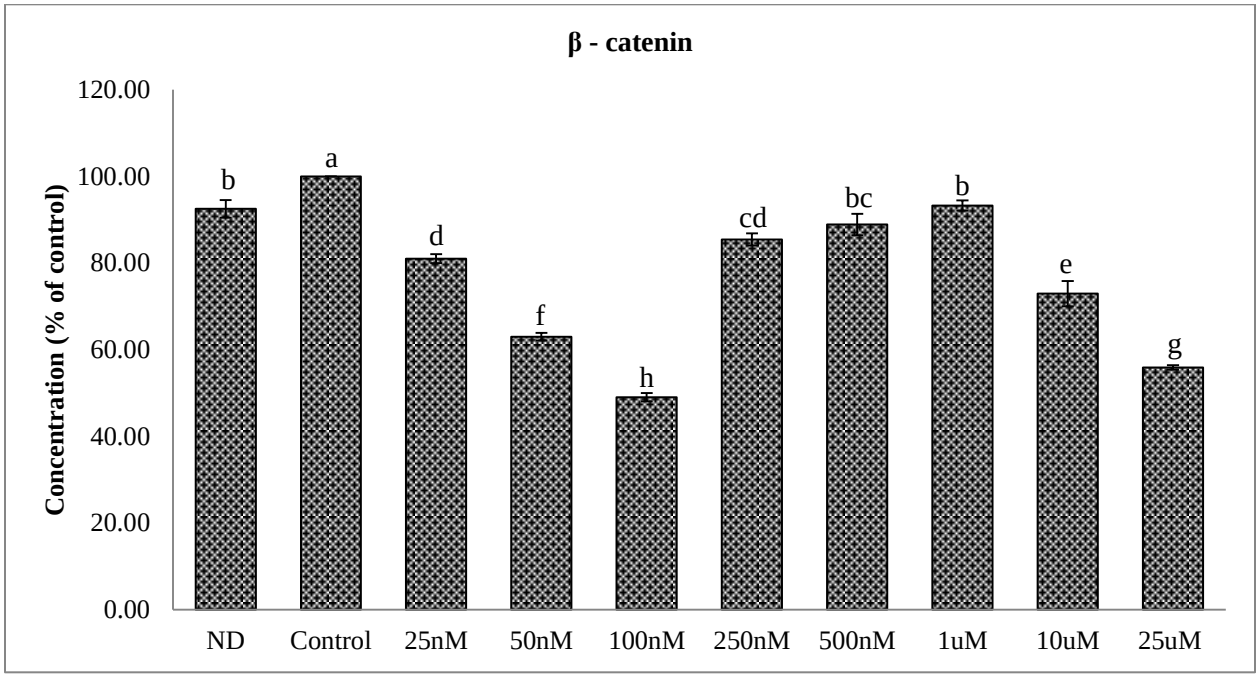


Figure 4.18. β-catenin expression in isorhamnetin (25 nM to 25 µM) treated cells. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different ($P < 0.05$).

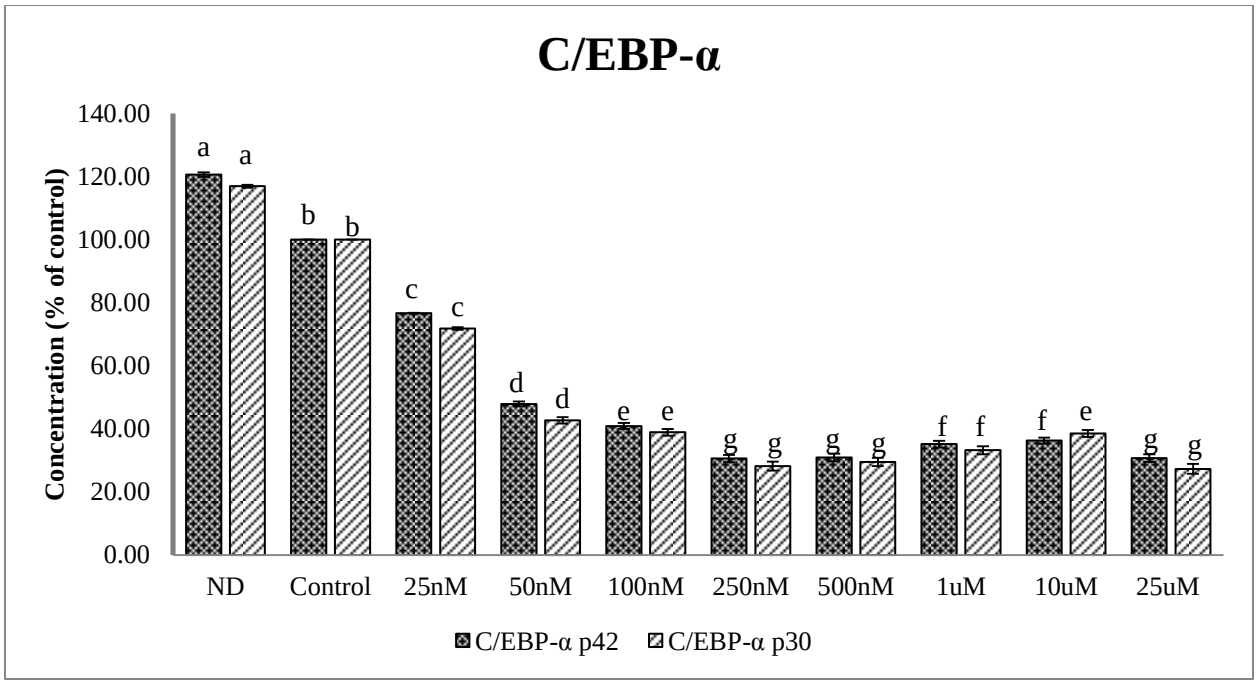


Figure 4.19. C/EBP-α expression in isorhamnetin treatments from 25 nM to 25 µM. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different (P < 0.05).

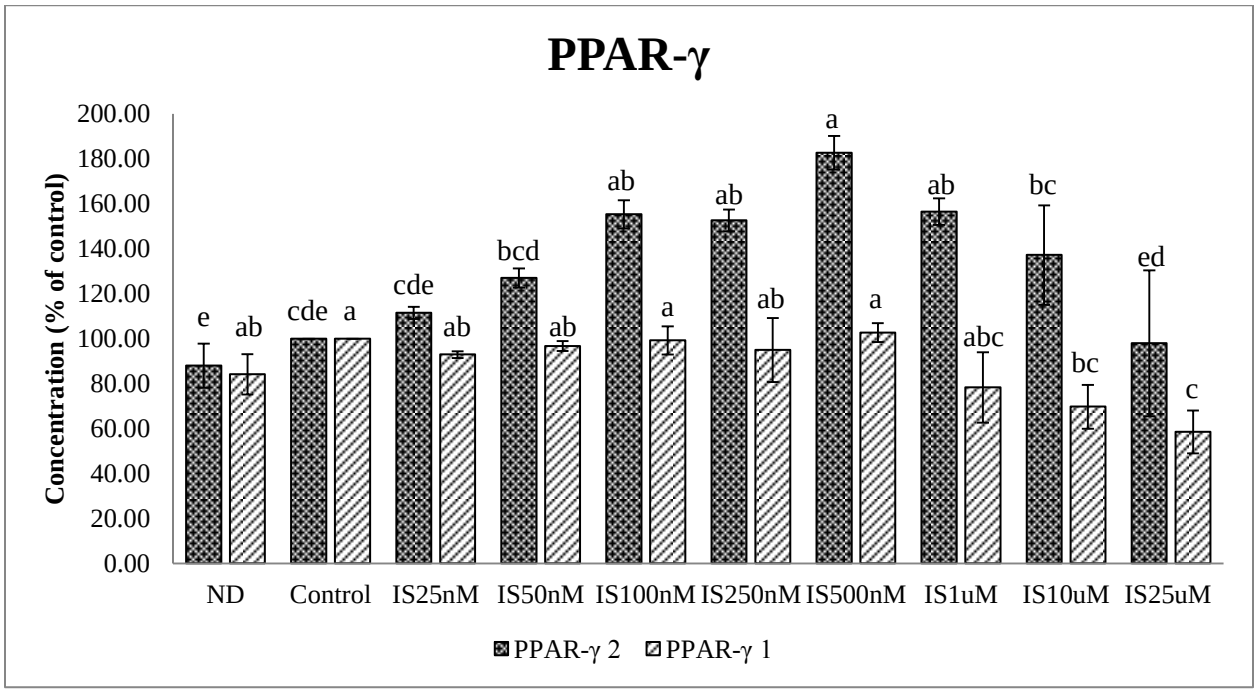


Figure 4.20. PPAR-γ expression in isorhamnetin treatments from 25 nM to 25 µM. Results are presented as mean ±S.D. (n = 3). Letters with different superscripts are significantly different (P < 0.05).

CHAPTER 5

CONCLUSIONS

This study demonstrated quercetin or isorhamnetin are not cytotoxic to 3T3-L1 cells at physiological or supraphysiological concentrations. During 3T3-L1 adipocyte differentiation in the presence of quercetin or isorhamnetin, average cell viability was 94.84% and 97.63%, respectively. Compared to control 3T3-L1 cells, the following results were obtained:

1. Isorhamnetin suppressed lipid accumulation more than quercetin during 3T3-L1 cell differentiation. Isorhamnetin down regulated lipid accumulation by 41.75% and 43.29% at 250 nM and 25 μM, respectively.
2. Quercetin at 25 μM down regulated lipid droplet accumulation only by 8.64 ± 9.54%.
3. Quercetin or isorhamnetin did not have any effect on MCP-1 levels during adipocyte differentiation.
4. Quercetin reduced β-catenin to 61.80 ± 0.30% of control untreated 3T3-L1 cells, while isorhamnetin reduced it to 49.04 ± 0.95% of control.
5. C/EBP-α p30 and p42 levels decreased dramatically by 72.68 ± 1.54% and 69.31 ± 1.22% using 250 nM and 25 μM isorhamnetin, respectively. C/EBP-α p30 and p42 levels decreased by 68.73 ± 2.66% and 54.48 ± 2.60% respectively using 25 μM quercetin compared to control.
6. Quercetin decreased PPAR-γ 1 and 2 expressions by 45.03 ± 3.17% and 27.58 ± 12.39%, while isorhamnetin decreased PPAR-γ 1 and 2 expressions by 41.48 ± 9.51% and 2.01 ± 32.46%, respectively. These results suggest that isorhamnetin at low concentrations can inhibit lipid accumulation in 3T3-L1 cells.

These findings show how quercetin or isorhamnetin at physiological and supraphysiological concentrations affect the regulation of fat cell volume and number, and further suggest that isorhamnetin at physiological concentrations might inhibit lipid droplet accumulation during adipocyte differentiation by down-regulating the expression of C/EBP-α and PPAR-γ.

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