A Thesis

For the Degree of Master of Science

Anti-adipogenic and Anti-diabetic effects of Flavonoids

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List of Abbreviations

ACC	Acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
FA	Fatty acid
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
GSK3β	Glycogen synthase kinase 3 beta
HMGCR	Hydroxy-3-methylglutaryl coenzyme A reductase
IR	Insulin resistance
ITT	Insulin tolerance test
LDL	Low-density lipoprotein
MET	Metformin
PDK1	Phosphoinositide-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PPI	Protein-protein interaction
TC	Total cholesterol
TG	Triglyceride

General Introduction

Adipogenesis and obesity

Obesity arises from an imbalance in energy intake and energy expenditure that eventually leads to the pathological growth of adipocytes. Obesity contributes to several health issues including hyperglycemia, hyperlipidemia, insulin resistance and chronic inflammation [1]. The research of obesity have mainly focused on the adipogenesis which is the process of the development of pre-adipocytes into mature adipocytes [2]. Obesity is known to be depend on both hypertrophy (size of the adipocytes) of pre-existing adipocytes and hyperplasia (number of adipocytes) due to formation of new adipocytes from precursor cells [3].

Diabetes

Diabetes mellitus (DM) characterized by elevated blood sugar levels over a prolonged period resulting from either destruction or impairment of insulin-secreting pancreatic β cells and insulin action in target tissues [4]. Diabetes increased risk of cardiovascular and cerebrovascular disease, limb amputations, blindness, and even death [5]. T1DM is caused by β-cell destruction, and leads to absolute insulin deficiency. This type of diabetes only accounts for 5–10% of diabetes [6]. In T2DM, even though the normal production of insulin takes place in the β cells of the pancreas, insulin receptors or insulin-signaling molecules in the cell membrane do not respond normally to insulin, known as “insulin resistant” [7].

AMPK pathway in adipogenesis

AMP-activated protein kinase (AMPK) is an energy sensor that modulates cellular metabolism. It regulates glucose and lipid metabolism to modulate energy levels [8]. AMPK leads to the phosphorylation and the inactivation of its substrate acetyl-CoA carboxylase (ACC), which catalyzes the conversion of acetyl-CoA to malonyl-CoA in the de novo lipid synthesis pathway [9]. Phosphorylation of AMPK inhibits cholesterol synthesis via inactivation of key metabolic enzyme involved in cholesterol synthesis including hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) [10]. Activation of AMPK inhibits lipolysis, and down-regulate key adipogenic genes including peroxisome proliferator-activated receptor (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα) [11].

AMPK pathway in glucose uptake

AMPK activity supports whole body glucose homeostasis and by enhancing the insulin independent glucose uptake in cells [12]. In addition, AMPK activation enhances the phosphorylation of GSK3β that leads to subsequent dephosphorylation and activation of glycogen synthase (GS), and ultimately increases the glycogen synthesis [13]. In addition, AMPK activation can suppress G6Pase and phosphoenolpyruvate carboxykinase (PEPCK), and then decreases hepatic glucose production [14].

Naringenin

Naringenin is known to have several therapeutic benefits including anti-hyperlipidemic and anti-hyperglycemic effects [15, 16]. Previous study reported that naringenin lowered levels of plasma cholesterol and hepatic triacylglycerol in rats fed with a high-cholesterol diet [17]. In addition, naringenin inhibits adipogenesis and exerts anti-inflammatory effects in adipocytes, and decreased blood glucose levels in diet induced insulin resistance mice [18, 19, 20] .

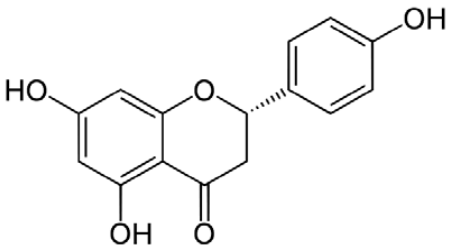


Figure 1. Chemical structure of Naringenin

Naringin

Naringin is flavanone glycoside abundantly found in grapefruits, and possesses several pharmacological properties including anti-oxidant, anti-inflammatory, anti-apoptotic, anti-osteoporotic, and anti-carcinogenic properties [21]. In addition, naringin is known to prevent adipogenesis via inhibiting the adipose tissue growth and adipocyte differentiation, and reduces plasma lipid in a mice model by changing the activities of hepatic-lipid metabolizing enzymes [22, 23].

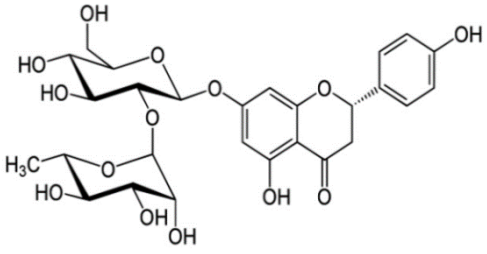


Figure 2. Chemical structure of Naringin

Hesperetin

Hesperetin is the aglycone of hesperidin which exhibits beneficial properties including anti-oxidant, anti-inflammation, and anti-lipidemic effect [24, 25]. Hesperetin has shown protective effects against fatty liver disease and diabetes attributed by its lipid-lowering efficacy, insulin-sensitizing, and stimulating glucose utilization by the cells [26, 27]. In addition, hesperetin exhibits a protective effect on the cardiovascular system as well as metabolic homeostasis of lipid and glucose [28].

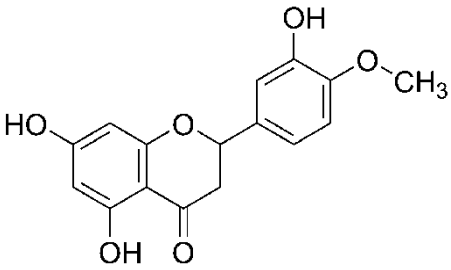


Figure 3. Chemical structure of Hesperetin

Differential gene expression

Differential gene expression profiling is used in molecular biology research to observe the transcription of genes to elucidate the mechanisms underlying a biological pathway [29]. RNA-sequencing can be used for genome-wide gene expression analysis, and the technique is rapidly becoming important research tools for identifying potential biomarkers [30, 31]. Investigation of the relation among the gene expression profiles enable an assessment of drug specificity, safety, and efficacy, thereby improving the efficiency and the cost effectiveness of the drug development process [32]. Therefore, the present study observed the expression of genes related to lipid metabolism to investigate the anti-adipogenic effect of flavonoids in 3T3-L1 adipocytes.

Protein-protein interaction

Protein-protein interactions (PPIs) illustrated the biochemical interactions among the protein molecules [33]. The data of PPIs provide the useful information for the biological mechanisms and the development of therapeutic drugs [34]. In particular, its applications contributed largely to recent advances in biomedical research [34]. PPIs network formed by the gene products illustrate the interaction with each other to accomplish particular cellular functions including metabolism and signal transduction [35]. Previous study reported that the highly connected network genes so-called “hub genes” are more likely to be involved in disease processes [36]. Therefore, the present study investigated the effects of flavonoids on the expression of hub genes related to lipid metabolism which are not fully discovered yet.

Molecular docking

Molecular docking is a computational binding analysis that estimates its binding affinity of ligand in the binding site to its macromolecular target (receptor-protein) [37]. This technique has become useful tool for the drug discovery as it is efficient for the finding of more potent, selective, and efficient drug candidates [38]. The goal of molecular docking is to predict molecular recognition by estimating binding affinity [39]. The present study investigated the binding affinities of naringenin, naringin, and hesperetin to γ-subunit of AMPK in order to confirm whether the flavonoids are potent AMPK activators.

Hypothesis

The present study hypothesized that flavonoids (Naringenin, Naringin, and Hesperetin) exert anti-adipogenic by normalizing the gene expression related to lipid metabolism. In addition, the study hypothesized that these flavonoids exert ant-diabetic and anti- adipogenic effects via activation of AMPK which is important for both lipid metabolism and glucose uptake.

Objectives

The major objectives of the present study are:

- To evaluate the anti-adipogenic effect of naringenin, naringin, and hesperetin by restoring the expression of genes related to lipid metabolism to normal level in 3T3-L1 adipocytes.
- To investigate the anti-diabetic effect of naringenin, naringin, and hesperetin by enhancing the glucose uptake via phosphorylation of AMPK in insulin resistant HepG2 cells.

PART-I

Anti-adipogenic effect of Flavonoids

1.1 Abstract

The present study examined the anti-adipogenic effect of flavonoids-Naringenin, naringin, and hesperetin on 3T3-L1 adipocytes. Naringenin, naringin, and hesperetin inhibited both lipid accumulation and TG content with increased phosphorylation levels of both AMP-activated protein kinase (AMPK) and acetyl Co-A carboxylase (ACC), and decreased expression level of HMG-CoA reductase (HMGCR) in 3T3-L1 adipocytes. The treatment of naringenin, naringin, and hesperetin increased the 2-NBDG uptake by differentiated 3T3-L1 adipocytes. RNA sequencing analysis revealed that up-regulated (>2-fold) 32 genes and down-regulated (< 0.6-fold) 17 genes related to lipid metabolism including *Acaca, Fasn, Scd1, Mogat1, Dgat, Lipin1, Cpt1a,* and *Lepr* genes were normalized to the control level in naringenin treated adipocytes. In addition, up-regulated (>2-fold) 25 genes and down-regulated (< 0.6-fold) 25 genes related to lipid metabolism including *Acaca, Fasn, Fabp5, Scd1, Srebf1, Hmgcs1, Cpt1c, Lepr,* and *Lrp1* genes were normalized to the control level by naringin. Hesperetin normalized up-regulated (>2-fold) 26 genes and down-regulated (< 0.6-fold) 24 genes related to lipid metabolism including *Acaca, Elovl3, Mogat2, Fabp5, Lipin1,* and *Pnpla3* in differentiated 3T3-L1 adipocytes. Results suggest that flavonoids naringenin, naringin, and hesperetin have anti-adipogenic potentials by normalizing the expression levels of genes related to lipid metabolism that were perturbed in differentiated 3T3-L1 cells.

Keywords: **Flavonoids, Anti-adipogenic, Gene expression, RNA sequencing**

1.2 Introduction

Obesity, a serious consequence of a prolonged disruption in energy homeostasis caused by an imbalance of energy intake and expenditure [40]. The surplus energy is stored as lipid, specifically triglyceride (TG), in white adipose tissues (WATs) [41]. Obesity is known to be a major contributor to the global burden of chronic diseases and complications, including cardiovascular diseases, diabetes, and cancers [42].

AMPK is an energy sensor that regulates glucose and lipid metabolism to modulate energy homeostasis in the body. The activation of AMPK inhibits pre-adipocyte differentiation, and suppresses transcription factors including PPARγ, C/EBPα, and SREBP-1c which are necessary for adipogenesis to occur [8]. In addition, the activation of AMPK inactivates key metabolic enzymes involved in fatty acid and cholesterol synthesis, including ACC and HMGCR [43]. Therefore, AMPK is considered to be a target for the treatment of obesity and associated metabolic diseases.

Previous studies investigated anti-adipogenic agents as potential therapeutics for preventing obesity and associated disorders [44]. Recently, the use of natural bioactive compounds is trending as alternative methods for the treatment of obesity and related diseases because of low risks of side effects than synthetic drugs [8].

Flavonoids are plant-derived compounds with multiple therapeutic effects including anti-hyperglycemic and anti-hyperlipidemic activities [45]. Flavonoids mediate these biological effects via cell signaling pathways based on molecular interactions with numerous enzymes [46]. For naringin or its aglycon naringenin, which are citrus-derived flavonoids, diverse therapeutic interests have been described including anti-diabetic and anti-lipidemic effects [47]. Hesperetin is the aglycone form of hesperidin commonly present in citrus fruits with trihydroxyflavone and an additional methoxyl substituent at the C-4`-position [48]. Hesperetin has various beneficial properties including anti-oxidant, anti-inflammation, and

anti-adipogenic activities [49]. In addition, hesperetin known to be a promising bioactive compound against lipid biosynthesis, hyperlipidemia, and hyperglycemia [50]. However, the detailed mechanism for the action of these flavonoids on anti-adipogenesis is poorly understood so far.

In this context, the present study hypothesized that naringenin, naringin, and hesperetin exerts anti-adipogenic potential by normalizing the activities of key enzymes related to lipid metabolism, and by restoring the expression of genes related to lipid metabolism to normal level. For this, the effects of these flavonoids on the phosphorylation levels of AMPK, ACC, and the expression levels of HMGCR were observed. In particular, RNA sequencing analysis was performed to observe the anti-adipogenic effect of naringenin, naringin, and hesperetin by normalizing the expression of genes related to lipid metabolism which was perturbed in differentiated 3T3-L1 adipocytes.

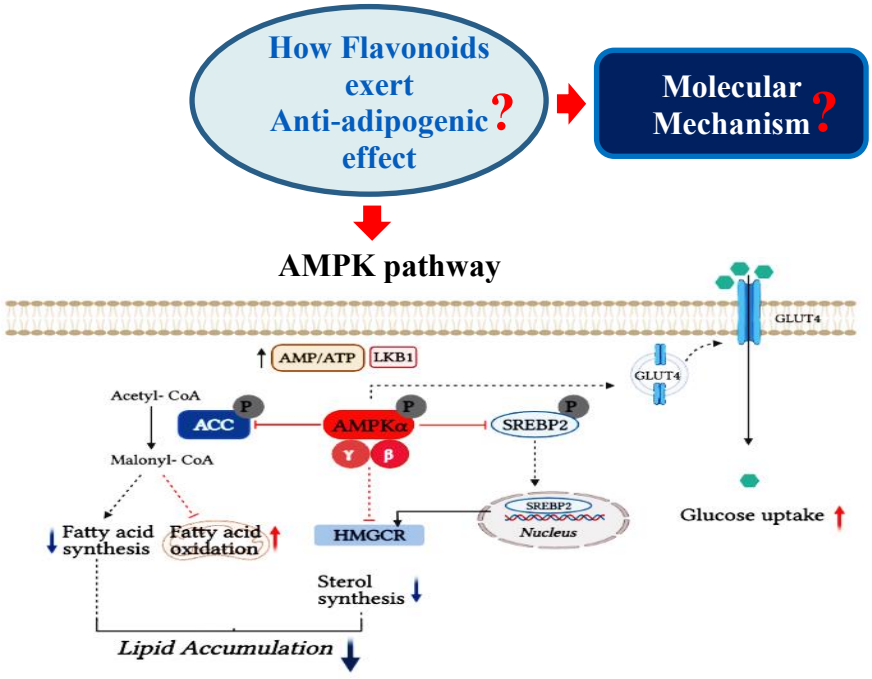


Figure 4. Hypothetical model for the Anti-adipogenic effect of flavonoids

1.3 Materials and Methods

1.3.1 Culture and differentiation of adipocytes

3T3-L1 fibroblasts (KCLB 42835) from the Korean Cell Line Bank (KCLB, Korea) were cultured Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% BS (Bovine Serum), 100 U/mL penicillin, and 100 μg/mL streptomycin (1% PS) (Gibco, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C.

3T3-L1 cells were cultured in 96 well plates (1×10^5 cells/well) after reaching the post confluence, cells were induced for differentiation by adding DIM- Differentiation Initiation Media containing DMEM, 1% PS, 10% FBS (Gibco, USA), 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma, USA), 0.5 μM Dexmethasone (Sigma, USA), 2 μM Rosiglitazone (Sigma, USA) and 10 μg/mL insulin (Sigma, USA) for 48 hrs, and then the cell medium was changed to a normal medium (DMEM containing 1% PS and 10% FBS) containing 10 μg/mL insulin. The media were changed to normal growth media after 48 hrs and every 2 days thereafter until differentiation to mature adipocytes. To examine effects of test samples on the lipid accumulation, cells were cultured with DIM with or without test samples 5 μM of simvastatin (Sigma, USA), different concentrations of flavonoids (Sigma, USA) diluted in DMSO until differentiation to mature adipocytes. On day 8, completely differentiated adipocytes were used to observe the lipid accumulation through Oil Red O staining and Western blot analysis.

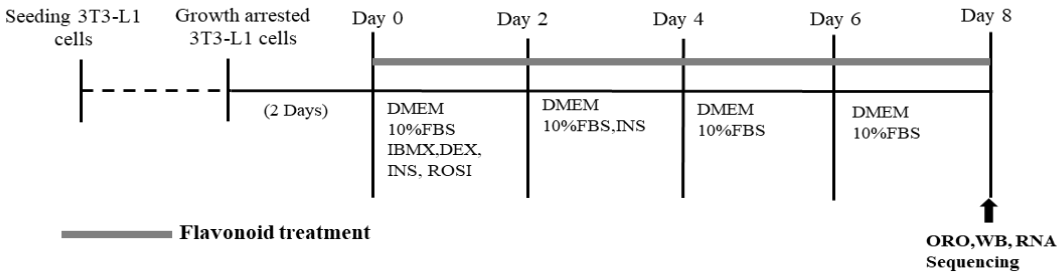


Figure 5. Differentiation procedure and assay schedule

1.3.2 Monitoring cellular viability –MTT assay

3T3-L1 cells were seeded in 96-well plates at the cell density of 1×10^5 cells/well and cultured overnight using routine culture media. After reaching the confluence, the cells were treated with different concentrations (0-100 µM) of flavonoids for 48 hrs. Cell culture media was removed and added fresh media containing 10% (Ez-cytox DogenBio, Korea) into each well, according to the manufacturer's instructions. Plates were incubated for 3 hrs at 37°C and 5% CO_2. Cell viability indicated by the formazan production was measured with an ELISA microplate reader (TECAN, Austria) at 450 nm wavelength.

1.3.3 Oil red O staining

Postconfluent pre-adipocytes were differentiated into adipocytes as described above. According to the previous method after adipocyte differentiation, on day 8, cells were stained with Oil Red O [51] with slight modifications. The fully differentiated adipocytes were fixed in 10% (v/v) formaldehyde (Biosesang, Korea) for 1 hr at room temperature. Next, the cells were rinsed twice with PBS (Gibco, USA) and stained with 0.5% Oil red O solution (Sigma, USA) (60% of oil red o stock solution and 40% distilled water) for 1 hr in the dark. The cells were washed with distilled water to remove the unbound dye, and the images were captured using the IncuCyte®ZOOM (Essen BioScience, Inc.USA) at 20X magnification. The quantitative analysis was performed by using IncuCyte®ZOOM processing software.

1.3.4 Measurement of Triglyceride content

Cellular TG contents were quantified using a commercially available triglyceride colourimetric assay kit (BioAssay Systems, USA). The differentiated 3T3-L1 cells at day 8 were washed with PBS and harvested to cell lysis buffer containing 5% Triton X100 (Bio-

Rad Laboratories). Cells were homogenized and centrifuged at 3000 × g for 5 min. The TG content of the diluted supernatants was analyzed according to the manufacturer's instructions. The protein concentration of each sample was measured using the Bio-Rad DC protein assay (Bio-Rad Laboratories, USA). TG contents were normalized with the respective protein concentration as detected by bovine serum albumin (Sigma, USA) as the calibration standard.

1.3.5 2-NBDG glucose uptake assay

Glucose uptake assays were performed on fully differentiated 3T3-L1 adipocytes. Briefly, pre-adipocytes were cultured in 96 well plates and induced to differentiate using the DIM protocol, as described above. Adipocytes were serum-starved for 12 hrs and then incubated with samples for 24 hrs. The cells were incubated with 40 μM 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose (2-NBDG) (Carlsbad, USA) for 30 min at 37° C. Cells were then washed three times with ice-cold PBS for 3 times and the fluorescent images were taken from IncuCyte® ZOOM Fluorescent Microscope (Essen BioScience, Inc. USA). The IncuCyte® ZOOM Fluorescent Processing Software was used to analyze the total fluorescent intensities of each well.

1.3.6 Western blotting

Cells were washed with PBS and lysed with ice-cold RIPA buffer containing protease inhibitor mixture. The whole-cell lysates were centrifuged at 12,000 rpm for 10 min. The supernatant was separated, and the amount of protein was assessed by Bradford assay (Bio-Rad Laboratories, USA). Equal amounts of protein was mixed with 20% of loading buffer and separated by SDS PAGE and subject to Western blot with AMPK (Cell signalling technology, USA), ACC (Cell signalling technology, USA), p-AMPK (Cell signalling technology, USA), and p-ACC (Cell signalling technology, USA) and β-actin (Thermofisher,

USA) antibodies. A Chemi-luminescence Bioimaging Instrument (NeoScience Co., Ltd., Korea) was used to detect proteins of interest. Densitometry analysis was performed using the ImageJ analysis software.

1.3.7 RNA sequencing analysis

RNA sequencing analysis was done as performed previously [52]. Briefly, total RNA was isolated from 3T3-L1 adipocytes using an Easy-blue RNA extraction kit (iNtRON Biotechnology, Korea). The RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Netherlands). Based on the manufacturer's instructions, libraries for RNAs were constructed using Ouantseq 3'mRNA-Seq Library Prep Kit (Lexogen, Austria). High-throughput sequencing was performed as single-end 75 sequencings using NextSeq 500 (Illumina, USA). QuantSeq 3' mRNA-Seq reads were aligned using Bowtie2 version 2.1.0 [53]. Differentially expressed genes were determined based on counts from unique and multiple alignments using EdgeR within R version 3.2.2 using Bioconductor version 3.0 [54]. The RT (Read count) data were processed based on Quantile normalization method using the Genowiz^{TM} version 4.0.5.6 (Ocimum Biosolutions, India). Gene classification was performed using the Medline database (National Centre for Biotechnology Information, USA).

1.3.8 Statistical analysis

Values were expressed as means ± S.E. of three independent experiments. Data were statistically analyzed with the aid of IBM SPSS Statistics (Ver.17.0; USA). The statistical differences among groups were analyzed with one-way analysis (ANOVA) followed by Turkey's test. The $P < 0.05$, $P < 0.005$, and $P < 0.0005$ indicate statistically significant differences from the differentiated control group.

1.4. Results

1.4.1 Cell viability of 3T3-L1 cells

MTT assay was performed to assess the effect of naringenin, naringin, and hesperetin on 3T3-L1 cell viability (Fig. 6). Significant cellular toxicity was not observed up to 25 μM of concentration of flavonoids in 3T3-L1 cells. Accordingly, the experiments were conducted with the non-toxic concentrations of flavonoids (10 and 20 μM).

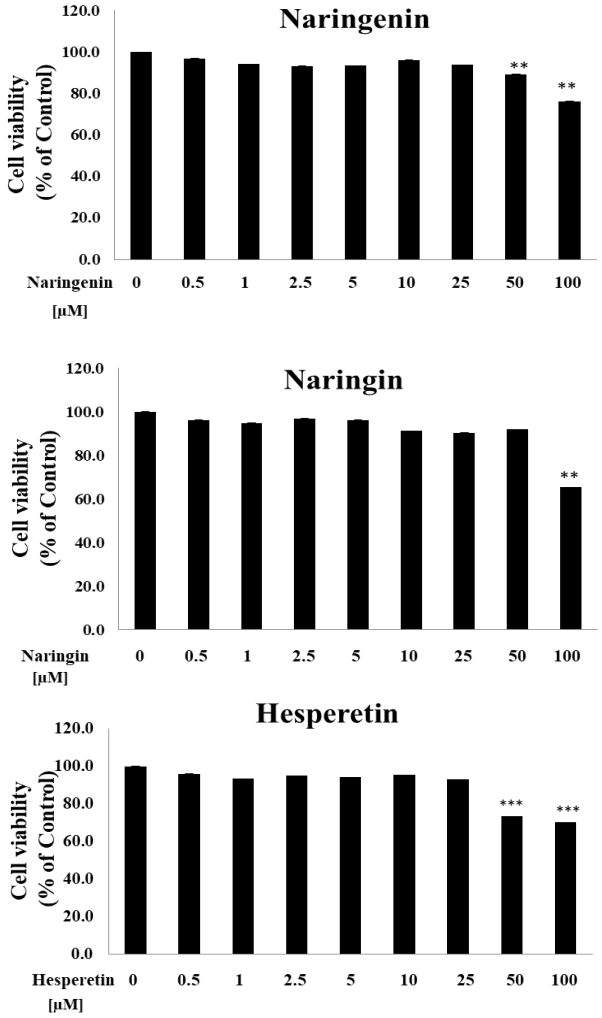


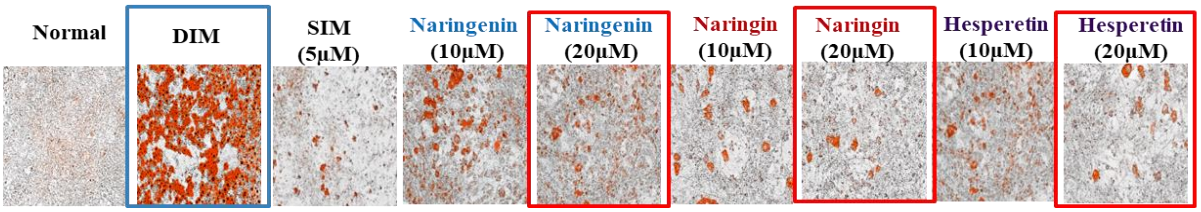
Figure 6. Effect of Naringenin, Naringin, and Hesperetin on 3T3-L1 cells viability.

3T3-L1 cells were cultured at a density of 1×10^{5} in 96 well plates, after reaching the confluence, cells were treated with 0-50 μM of flavonoids for 48 hrs, and cell viability was measured by the MTT assay (C). Values represent mean ± SE. $^{**}P < 0.005$ and $^{**}P < 0.0005$ vs. the control.

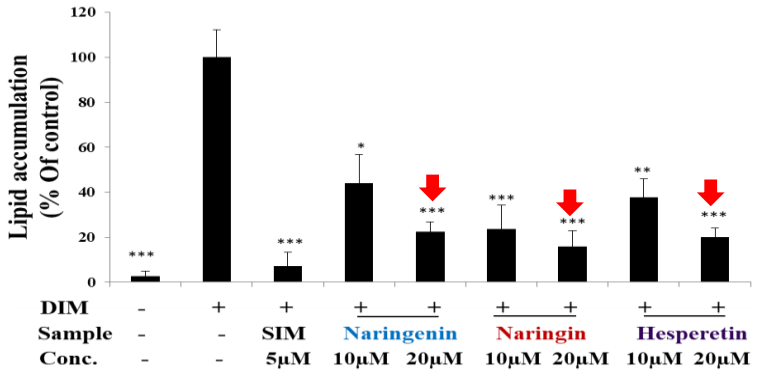
1.4.2 Lipid accumulation and TG content

The effect of naringenin, naringin, and hesperetin on lipid accumulation and TG content in 3T3-L1 adipocytes were observed by Oil Red O staining and TG assay (Fig. 7). Differentiated adipocytes significantly increased both lipid accumulation and TG content compared to undifferentiated adipocytes (Fig. 7B and 7C). In contrast, the treatment of simvastatin (positive control) and higher concentration (20 μM) of naringenin, naringin, and hesperetin significantly inhibited ($P < 0.0005$) both lipid accumulation and TG content (Fig. 7B and 7C). Therefore, the results suggest that naringenin, naringin, and hesperetin suppressed both lipid accumulation and TG content in differentiated 3T3-L1 adipocytes.

(A)



(B)



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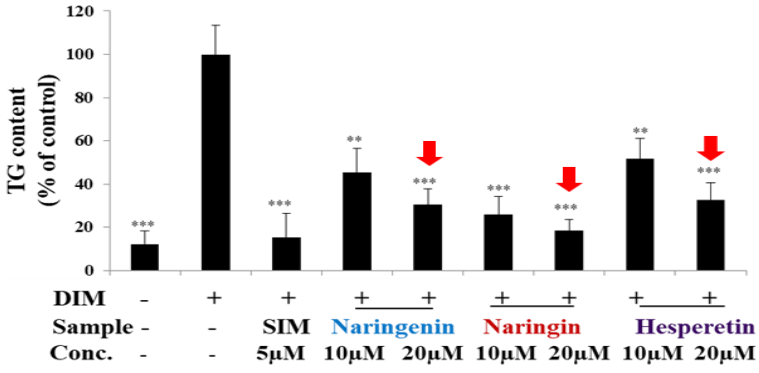


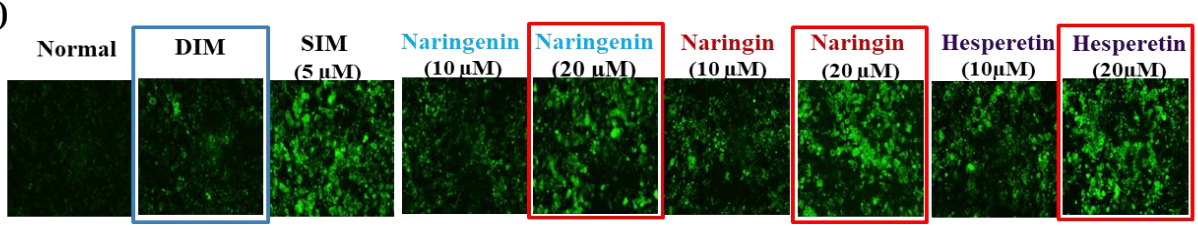
Figure 7. Effect of Naringenin, Naringin, and Hesperetin on intracellular lipid accumulation.

Cells were differentiated and treated with samples as described in Material and Methods. The lipid accumulation was measured by Oil-Red O staining on day 8. Representative images were captured using the IncuCyte Zoom at 20X magnification (A). Quantitative analysis of lipid accumulation in 3T3-L1 adipocytes calculated by IncuCyte ZOOM® fluorescence processing software (B). Effect of naringenin, naringin, and hesperetin on triglyceride accumulation in 3T3-L1 adipocytes. Total intracellular TG concentration was determined using commercial kits (C). Values represent mean ± SE. *P < 0.05, **P < 0.005 and ***P < 0.0005 vs. the differentiated control.

1.4.3 Glucose uptake in 3T3-L1 adipocytes

The glucose uptake effect of naringenin, naringin, and hesperetin in 3T3-L1 adipocytes was assessed via 2-NBDG glucose uptake assay (Fig. 8). Differentiated 3T3-L1 adipocytes showed slightly increased glucose uptake when compared with undifferentiated adipocytes. However, the treatment of simvastatin significantly increased (P < 0.005) the glucose uptake compared to the differentiated control. In particular, naringenin, naringin, and hesperetin significantly increased (P < 0.005) glucose uptake compared to the differentiated control when used at 20 µM of concentration (Fig. 8A and 8B). These results indicate that naringenin, naringin, and hesperetin stimulated the cellular glucose uptake in differentiated 3T3-L1 adipocytes.

(A)



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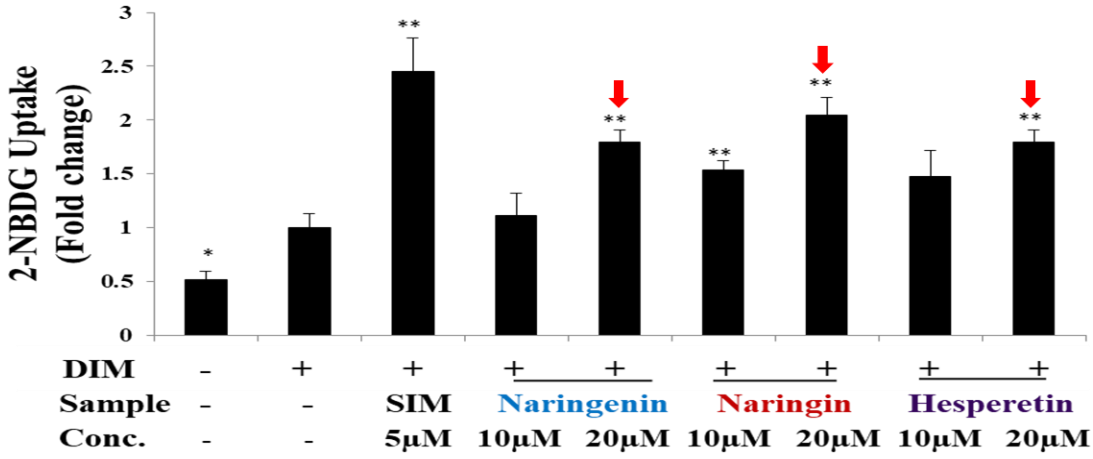


Figure 8. Effect of Naringenin, Naringin, and Hesperetin on glucose uptake.

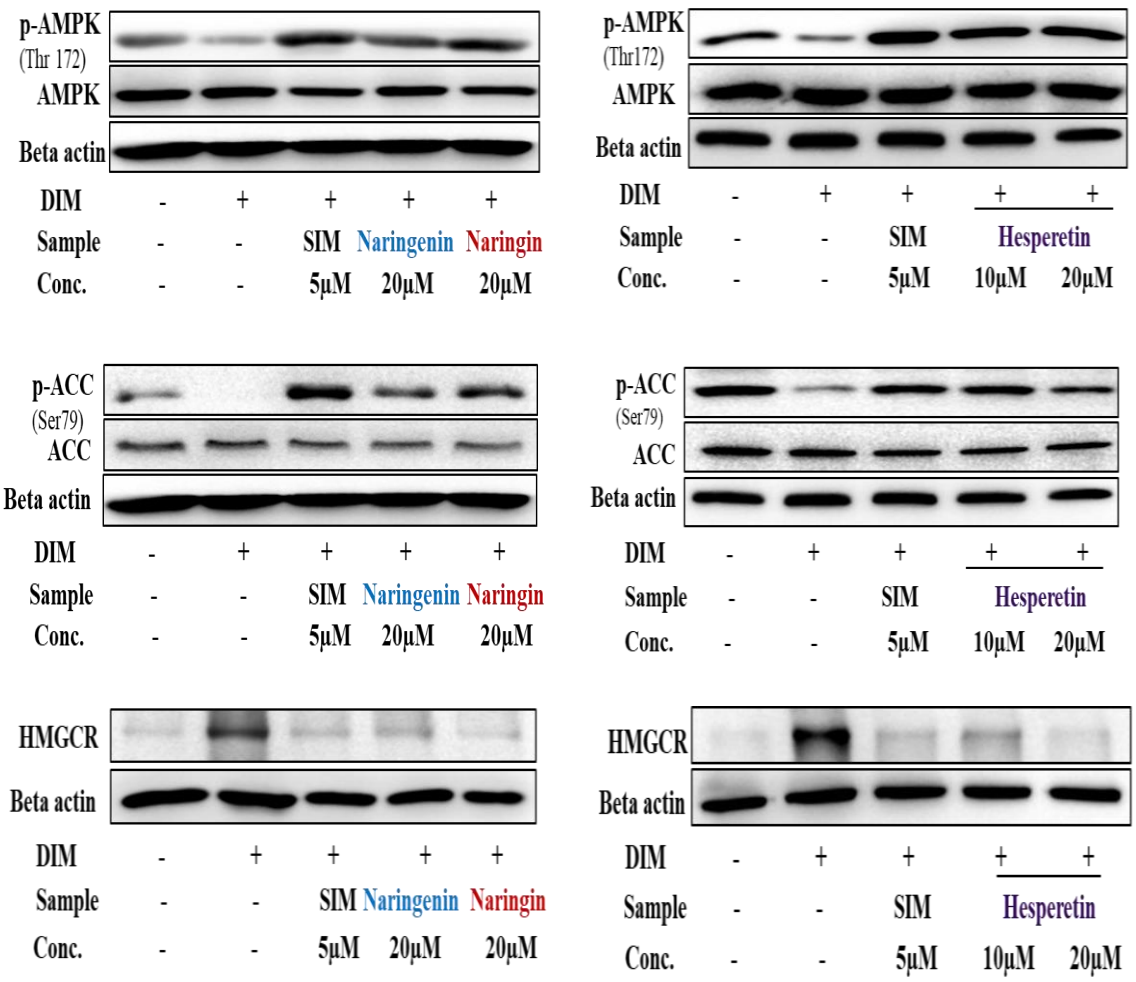
Glucose uptake was assessed by a fluorescent probe, 2-NBDG. Differentiated 3T3-L1 cells were incubated with flavonoids for 24 hrs and incubated with 2-NBDG (40 uM) for 30 min. Cells were washed with PBS for 3 times, and images were taken from IncuCyte ZOOM® fluorescence microscope at 20X magnification (A). Total fluorescent intensity was calculated using IncuCyte ZOOM® fluorescent processing software (B). Values represent mean ± SE. *$P < 0.05$ and **$P < 0.005$ vs. the differentiated control.

1.4.4 Phosphorylation levels of AMPK pathway molecules

Western blot analysis was conducted to observe the effect of naringenin, naringin, and hesperetin on the regulation of key enzymes in the AMPK signaling pathway (Fig. 9). The phosphorylation levels of both AMPK (Thr172) and ACC (Ser79) in 3T3-L1 adipocytes were significantly decreased ($P < 0.05$) in differentiated adipocytes compared to undifferentiated adipocytes. In contrast, the phosphorylation levels of both AMPK (Thr172) and ACC (Ser79) were significantly increased by the treatment of simvastatin, naringenin, naringin, and hesperetin in differentiated 3T3-L1 adipocytes (Fig. 9A and 9B). The effect of naringin on the phosphorylation levels of AMPK (Thr172) and ACC (Ser79) was comparatively higher

than other two flavonoids and was similar to that of simvastatin. In addition, naringenin, naringin, and hesperetin treatment significantly reduced ($P < 0.0005$) the protein expression of HMGCR compared to differentiated control (Fig. 9A and 9B). The results suggest that naringenin, naringin, and hesperetin activated AMPK signaling pathway in 3T3-L1 adipocytes.

(A)



(B)

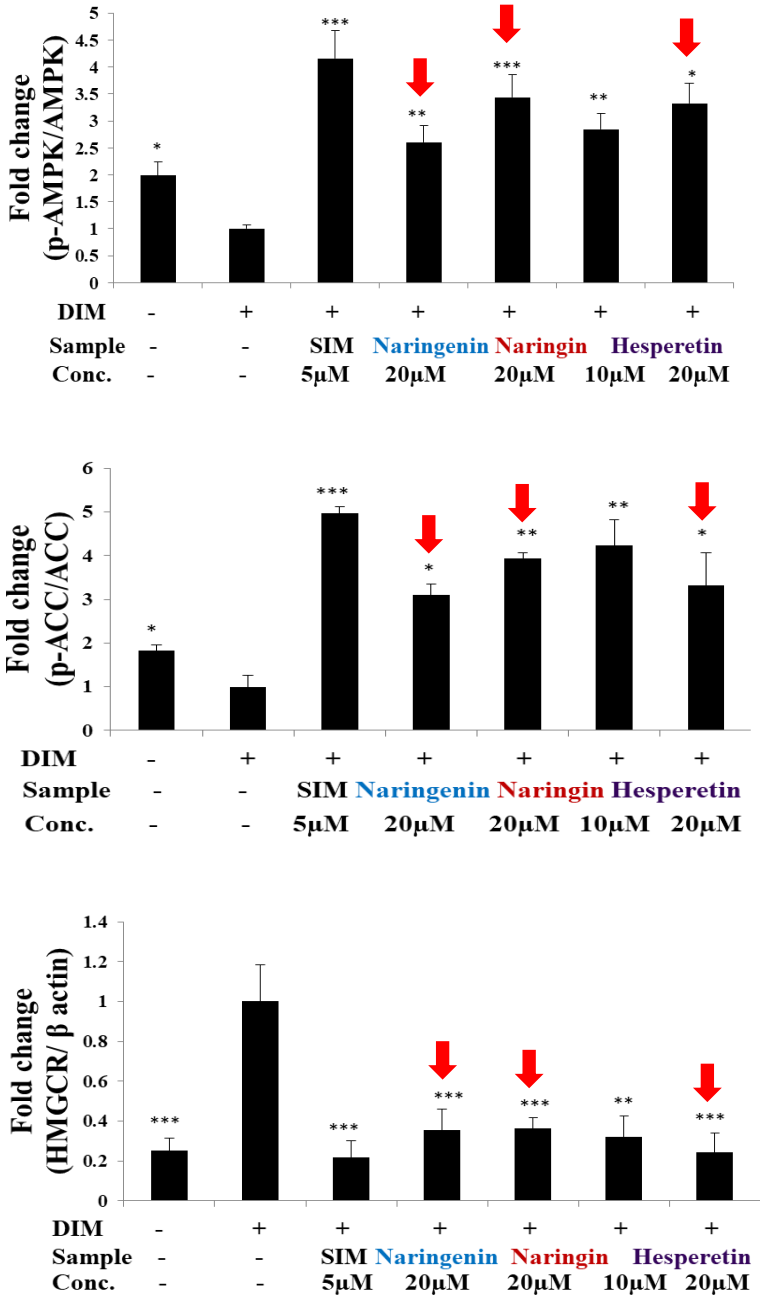


Figure 9. Effect of Naringenin, Naringin, and Hesperetin on expressions of AMPK pathway molecules.

3T3-L1 pre-adipocytes were differentiated in the absence or presence of flavonoids for 8 days. The phosphorylation of AMPK, ACC and HMGCR expression were then determined by Western blot analysis. Immunoblot of p-AMPK, p-ACC, and HMGCR (A). Relative protein levels were quantified using densitometry analysis (B). Values represent mean ± SE. **P* < 0.05, ***P* < 0.005, and ****P* < 0.005 vs. the differentiated control.

1.4.5 Differential gene expression

RNA sequencing analysis was performed to observe the effects of naringenin, naringin, and hesperetin on the expression levels of genes related to lipid metabolism in 3T3-L1 adipocytes. Based on Gene Ontology (GO) analysis, a large proportion of the genes related to lipid metabolism were up- or down-regulated in naringenin, naringin, and hesperetin-treated 3T3-L1 adipocytes (Fig. 10). Up-regulated (> 2-fold) 32 genes including *Scd1*, *Scd3*, *Elovl3*, *Ppara*, *Lipin 1*, *Cebpa* and down-regulated (< 0.6-fold) 17 genes including *Cpt1a*, *Lrp1*, *Lrp5*, *Adh7*, *Lepr* were normalized to the control level in naringenin treated adipocytes (Tables 1 and 2). In addition, up-regulated (> 2-fold) 25 genes including *Scd1*, *Apoa4*, *Ppara*, *Cebpa*, *Fabp5*, *G6pc* and down-regulated (< 0.6-fold) 25 genes including *Pitpnc1*, *Spns2*, *Cpt1c*, *Lepr*, *Pigv* were normalized to the control level in naringin treated adipocytes (Tables 3 and 4). The up-regulated (> 2-fold) 26 genes including *Acaca*, *Elovl3*, *Acadvl*, *Mogat2*, *Fabp5*, *Lipin1*, *Pnpla3*, *Cebpa*, *Cebpb*, *Ppargc1a*, *Pparg*, and *Hmgcs1* and down-regulated (< 0.6-fold) 24 genes including *Lrp1*, *Lrp5*, *Lepr*, and *Cpt1* were normalized to control level by hesperetin treatment in 3T3-L1 adipocytes (Table 5 and 6).

STRING analysis was performed to identify protein-protein interactions (PPI) among the normalized genes induced by naringenin, naringin, and hesperetin and was visualized as a set of nodes and edges (Fig. 10 E-G). The proteins related to lipid metabolism including *Acaca*, *Fasn*, *Scd1*, *Mogat1*, *Dgat*, and *Lipin1* were closely located and interacted directly with each other within the PPI network of naringenin. In addition, normalized down-regulated genes including *Cpt1a*, *Lepr*, and *Lrp1*, were closely located in the functional hub in the PPI network of naringenin. In the PPI network of naringin, the normalized up-regulated genes including *Acaca*, *Fasn*, *Fabp5*, *Scd1*, *Srebf1*, and *Hmgcs1* were closely located and formed a functional cluster. In addition, normalized down-regulated genes including *Cpt1c*,

Lrp1, and *Lepr* were also located in the functional hub of PPI network of narngin. Further, the proteins related to lipid metabolism including *Acaca*, *Elovl3*, *Acadvl*, *Mogat2*, *Fabp5*, *Lipin1*, *Pnpla3*, *Apoa4*, and *Apoc3* were closely located and formed a functional cluster within the PPI network of hesperetin. Overall, RNA sequencing analysis reveals that naringenin, naringin, and hesperetin normalized the expression levels of genes related to lipid metabolism which were perturbed in differentiated 3T3-L1 adipocytes.

Table 1. Up-regulated genes which were normalized by Naringenin

Gene symbol	Differentiated/ Normal	Simvastatin/ Normal	Naringenin /Normal	Gene name
Scd3	188.817	87.202	1.031	Stearoyl-coenzyme A desaturase 3
Elovl3	110.594	74.751	1.002	Elongation of very long chain fatty acids 3
Scd1	95.233	86.417	1.160	Stearoyl-coenzyme A desaturase 1
Ppara	77.170	39.919	1.578	Peroxisome proliferator activated receptor alpha
Lpin1	25.118	21.260	1.283	Lipin 1
Apoa4	21.349	7.713	1.001	Apolipoprotein A-IV
Mogat1	19.342	16.389	1.001	Monoacylglycerol O-acyltransferase 1
Cebpa	18.250	12.830	1.182	CCAAT/enhancer binding protein ,alpha
Cyp2f2	15.210	14.921	1.081	Cytochrome P450, family 2, subfamily f, polypeptide 2
Dgat1	10.944	9.938	1.444	Diacylglycerol O-acyltransferase 1
Pla2g5	7.756	5.796	1.001	Phospholipase A2, group V
Acsf2	6.340	4.254	1.071	Acyl-coa synthetase family member 2
Cyp27a1	5.879	1.000	1.000	Cytochrome P450, family 27, subfamily a, polypeptide 1
G6pc	5.879	1.000	1.000	Glucose-6-phosphatase, catalytic
Pparg	5.716	5.128	1.383	Peroxisome proliferator activated receptor gamma
Dbi	5.654	7.595	1.082	Diazepam binding inhibitor
Adipor2	5.577	4.734	1.244	Adiponectin receptor 2
Fasn	5.001	3.137	1.267	Fatty acid synthase
Awat1	4.885	3.898	1.000	Acyl-coa wax alcohol acyltransferase 1
Hacd2	4.571	4.082	0.945	3-hydroxyacyl-coa dehydratase 2
Acox1	4.282	4.007	1.042	Acyl-Coenzyme A oxidase 1, palmitoyl
Hmgcs1	3.370	3.956	1.214	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
Pik3r6	2.950	1.000	1.000	Phosphoinositide-3-kinase, regulatory subunit
Cyp3a57	2.950	1.000	1.000	Cytochrome P450, family 3, subfamily a, polypeptide 57
Mogat2	2.950	1.000	1.000	Monoacylglycerol O-acyltransferase 2
Apoa2	2.944	1.000	1.000	Apolipoprotein A-II
Scp2	2.776	2.641	0.818	Sterol carrier protein 2, liver
Oxsm	2.656	2.235	0.936	3-oxoacyl-ACP synthase, mitochondrial
Fads2	2.242	1.908	1.197	Fatty acid desaturase 2
Cebpb	2.161	1.279	0.964	CCAAT/enhancer binding protein , beta
Ppargc1a	2.101	2.743	1.129	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
Galc	2.037	1.562	1.056	Galactosylceramidase

Table 2. Down-regulated genes which were normalized by Naringenin

Gene symbol	Differentiated/ Normal	Simvastatin /Normal	Naringenin/ Normal	Gene name
Akr1c14	0.151	0.296	0.792	Aldo-keto reductase family 1, member C14
Gata2	0.163	0.199	1.007	GATA binding protein 2
Npy1r	0.290	0.489	1.072	Neuropeptide Y receptor Y1
Mtmr11	0.291	0.381	0.765	Myotubularin related protein 11
Cpt1a	0.302	0.257	0.789	Carnitine palmitoyltransferase 1a
Adh7	0.316	0.635	1.050	Alcohol dehydrogenase 7 (class IV)
Pld1	0.333	0.462	0.949	Phospholipase D1
Hacd4	0.355	0.211	1.150	3-hydroxyacyl-coa dehydratase 4
Lrp1	0.379	0.387	0.797	Low density lipoprotein receptor-related protein 1
Asah1	0.383	0.467	0.850	N-acylsphingosine amidohydrolase 1
Lepr	0.394	0.984	1.224	Leptin receptor
Lrp5	0.407	0.348	0.918	Low density lipoprotein receptor-related protein 5
Lmna	0.412	0.347	0.726	Lamin A
E2f1	0.440	0.517	1.045	E2F transcription factor 1
Ccnd1	0.454	0.267	0.718	Cyclin D1
Gla	0.474	0.714	1.083	Galactosidase, alpha
Cyb5r3	0.498	0.726	1.009	Cytochrome b5 reductase 3

Table 3. Up-regulated genes which were normalized by Naringin

Gene symbol	Differentiated/ Normal	Simvastatin/ Normal	Naringin/ Normal	Gene name
Scd1	95.233	86.417	1.327	Stearoyl-Coenzyme A desaturase 1
Ppara	77.170	39.919	1.670	Peroxisome proliferator activated receptor alpha
Apoa4	21.349	7.713	1.043	Apolipoprotein A-IV
Cebpa	18.250	12.830	1.161	CCAAT/enhancer binding protein alpha
Fabp5	15.580	12.573	1.001	Fatty acid binding protein 5, epidermal
Lpl	7.613	7.816	1.152	Lipoprotein lipase
G6pc	5.879	1.000	1.012	Glucose-6-phosphatase, catalytic
Pparg	5.716	5.128	1.579	Peroxisome proliferator activated receptor gamma
Adipor2	5.577	4.734	1.174	Adiponectin receptor 2
Fasn	5.001	3.137	1.289	Fatty acid synthase
Bmp4	3.927	1.000	1.008	Bone morphogenetic protein 4
Ghrl	3.901	3.893	1.018	Ghrelin
Hmgcs1	3.370	3.956	1.326	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
Cyp3a57	2.950	1.000	1.005	Cytochrome P450, family 3, subfamily a, polypeptide 57
Mogat2	2.950	1.000	1.005	Monoacylglycerol O-acyltransferase 2
Srebf1	2.907	2.027	1.271	Sterol regulatory element binding transcription factor 1
Lias	2.898	2.812	1.050	Lipoic acid synthetase
Eci3	2.828	1.684	1.234	Enoyl-Coenzyme A delta isomerase 3
Socs2	2.756	2.257	1.058	Suppressor of cytokine signaling 2
G6pdx	2.737	3.247	1.264	Glucose-6-phosphate dehydrogenase X-linked
Oxsm	2.656	2.235	1.020	3-oxoacyl-ACP synthase, mitochondrial
Fads2	2.242	1.908	0.974	Fatty acid desaturase 3
Cebpb	2.161	1.279	1.055	CCAAT/enhancer binding protein (C/EBP), beta
Ppargc1a	2.101	2.743	0.903	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
Galc	2.037	1.562	1.256	Galactosylceramidase

Table 4. Up-regulated genes which were normalized by Naringin

Gene symbol	Differentiated/ Normal	Simvastatin/ Normal	Naringin/ Normal	Gene name
Akr1c14	0.151	0.296	0.993	Aldo-keto reductase family 1, member C14
Pitpnc1	0.164	0.204	0.899	Phosphatidylinositol transfer protein, cytoplasmic 1
Spns2	0.280	0.232	0.775	Spinster homolog 2
Gba	0.288	0.379	0.707	Glucosidase, beta, acid
Npy1r	0.290	0.489	1.069	Neuropeptide Y receptor Y1
Mtmr11	0.291	0.381	0.956	Myotubularin related protein 11
Cpt1c	0.302	0.257	1.012	Carnitine palmitoyltransferase 1c
Adh7	0.316	0.635	1.001	Alcohol dehydrogenase 7 (class IV)
Pld1	0.333	0.462	0.972	Phospholipase D1
Hacd4	0.355	0.211	1.025	3-hydroxyacyl-coa dehydratase 4
Pigv	0.360	0.735	0.802	Phosphatidylinositol glycan anchor biosynthesis, class V
Hexa	0.375	0.491	0.870	Hexosaminidase A
Lrp1	0.379	0.387	0.861	Low density lipoprotein receptor-related protein 1
Lepr	0.394	0.984	1.056	Leptin receptor
Fgf10	0.403	0.615	0.998	Fibroblast growth factor 10
Lrp5	0.407	0.348	1.096	Low density lipoprotein receptor-related protein 5
Lmna	0.412	0.347	0.784	Lamin A
Scpep1	0.438	0.533	0.772	Serine carboxypeptidase 1
E2f1	0.440	0.517	1.112	E2F transcription factor 1
Lima1	0.457	0.334	1.008	LIM domain and actin binding 1
Fgf7	0.463	0.480	1.167	Fibroblast growth factor 7
Gla	0.474	0.714	1.101	Galactosidase, alpha
Pigt	0.491	0.587	1.124	Phosphatidylinositol glycan anchor biosynthesis, class T
Socs5	0.494	0.584	0.921	Suppressor of cytokine signaling 5
Tmem150a	0.497	0.640	0.782	Transmembrane protein 150A

Table 5. Up-regulated genes which were normalized by Hesperetin

Gene symbol	Differentiated/ Normal	Simvastatin /Normal	Hesperetin/ Normal	Gene name
Pnpla3	201.769	86.212	1.075	Patatin-like phospholipase domain containing 3
Elovl3	110.594	74.751	1.050	Elongation of very long chain fatty acids like 3
Adora1	66.632	4.773	1.040	Adenosine A1 receptor
Lipin1	25.118	21.260	1.274	Lipin1
Apoa4	21.349	7.713	1.026	Apolipoprotein A-IV
Cebpa	18.250	12.830	1.008	CCAAT/enhancer binding protein (C/EBP), alpha
Agpat2	15.837	19.861	0.739	1-acylglycerol-3-phosphate O-acyltransferase 2
Fabp5	15.580	12.573	0.931	Fatty acid binding protein 5, epidermal
Cyp2f2	15.210	14.921	1.031	Cytochrome P450, family 2, subfamily f, polypeptide 2
Apoc3	14.610	2.898	1.018	Apolipoprotein C-III
Fa2h	9.710	7.743	1.021	Fatty acid 2-hydroxylase
G6pc	5.879	1.000	1.007	Glucose-6-phosphatase, catalytic
Pparg	5.716	1.000	1.007	Peroxisome proliferator activated receptor gamma
Abca1	5.150	4.390	1.119	ATP-binding cassette, sub-family A, member 1
Akr1d1	4.904	1.000	1.006	Aldo-keto reductase family 1, member D1
Awat1	4.885	3.898	1.010	Acyl-coa wax alcohol acyltransferase 1
Ugt1a7c	3.927	1.000	1.005	UDP glucuronosyltransferase 1 family,polypeptide A7C
Ces1e	3.915	2.930	1.007	Carboxylesterase 1E
Ces4a	3.915	2.930	1.007	Carboxylesterase 4A
Hmgcs1	3.370	3.956	1.163	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
Acadvl	3.280	2.574	1.193	Acyl-coenzyme Adehydrogenase, very long chain
Mogat2	2.950	1.000	1.003	Monoacylglycerol O-acyltransferase 2
Pik3r6	2.950	1.000	1.003	Phosphoinositide-3-kinase, regulatory subunit 6
Acaca	2.884	2.600	1.125	Acetyl-coenzyme Acarboxylase alfa
Acsf3	2.810	2.605	1.128	Acyl-coa synthetase family member 3
Cebpb	2.161	1.279	1.018	CCAAT/enhancer binding protein (C/EBP), beta

Table 6. Up-regulated genes which were normalized by Hesperetin

Gene symbol	Differentiated / Normal	Simvastatin/ Normal	Hesperetin/ Normal	Gene name
Lmna	0.163	0.347	0.774	Lamin A
E2f1	0.164	0.517	1.120	E2F transcription factor 1
Ccnd1	0.203	0.267	0.814	Cyclin D1
Gata2	0.291	0.199	0.927	GATA binding protein 2
Cpt1	0.302	0.615	0.830	Carnitine palmitoyltransferase 1
Plscr1	0.346	0.593	0.829	Phospholipid scramblase family,
Pld1	0.375	0.462	1.006	Phospholipase D1
Gla	0.379	0.714	0.948	Galactosidase, alpha
Asah1	0.383	0.467	0.874	N-acylsphingosine amidohydrolase 1
Hexa	0.383	0.491	0.796	Hexosaminidase A
Htra2	0.394	0.451	0.738	Htra serine peptidase 2
Plbd2	0.403	0.646	1.207	Phospholipase B domain containing 2
Lepr	0.407	0.984	0.842	Leptin receptor
Adh7	0.421	0.635	1.067	Alcohol dehydrogenase 7
Pigt	0.440	0.587	0.822	Phosphatidylinositol glycan anchor biosynthesis, class T
Aldh1a1	0.454	0.573	0.924	Aldehyde dehydrogenase family 1, subfamily A1
Lrp5	0.457	0.348	0.886	Low density lipoprotein receptor-related protein 5
Socs5	0.463	0.584	0.786	Suppressor of cytokine signaling 5
Cyb5r3	0.474	0.726	1.014	Cytochrome b5 reductase 3
Akr1c14	0.490	0.296	0.866	Aldo-keto reductase family 1, member C14
Egfr	0.494	0.275	0.710	Epidermal growth factor receptor
Pitpnc1	0.494	0.204	0.797	Phosphatidylinositol transfer protein, cytoplasmic 1
Lrp1	0.498	0.387	0.861	Low density lipoprotein receptor-related protein

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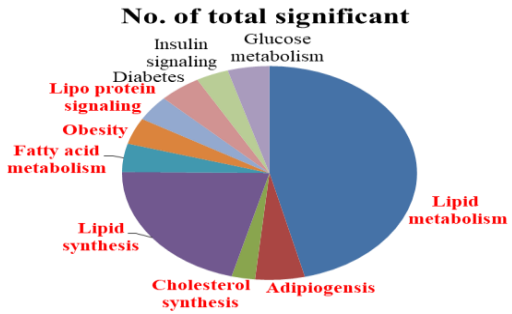

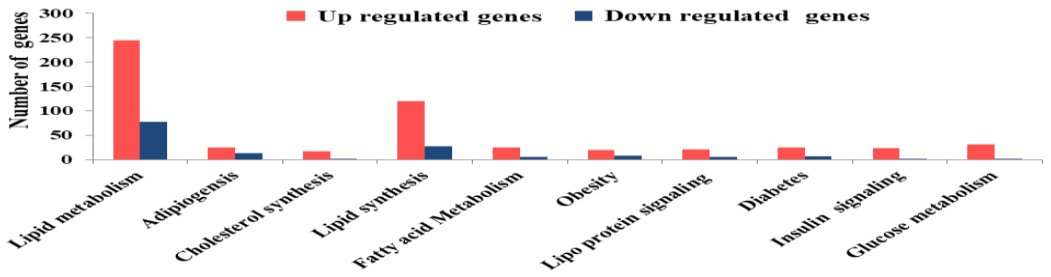

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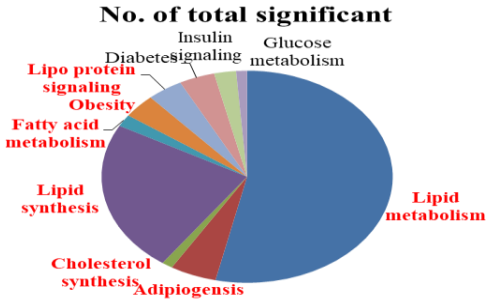

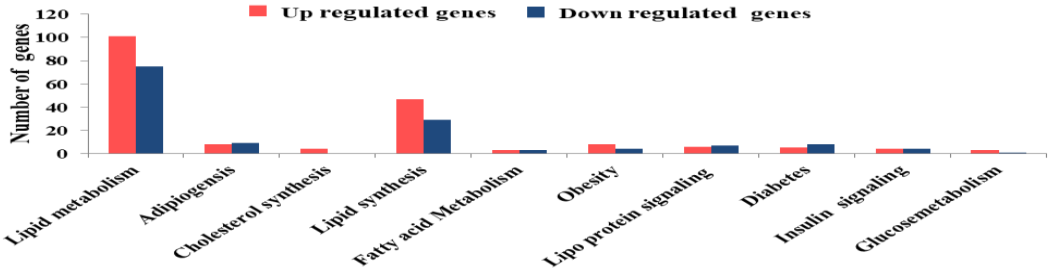

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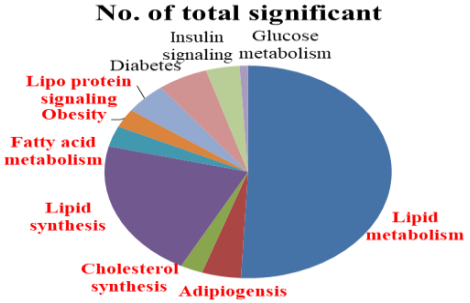

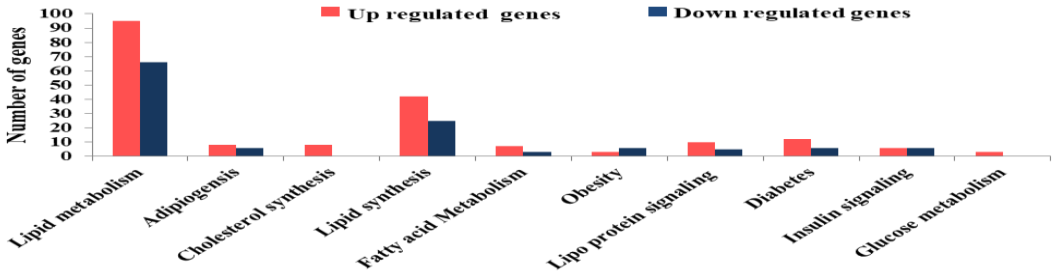

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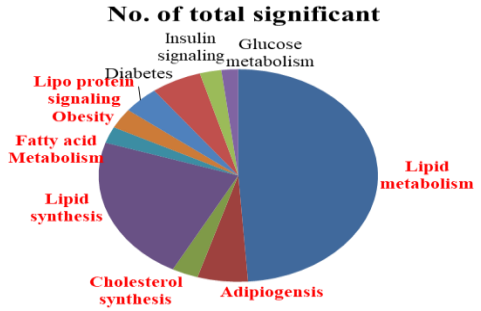
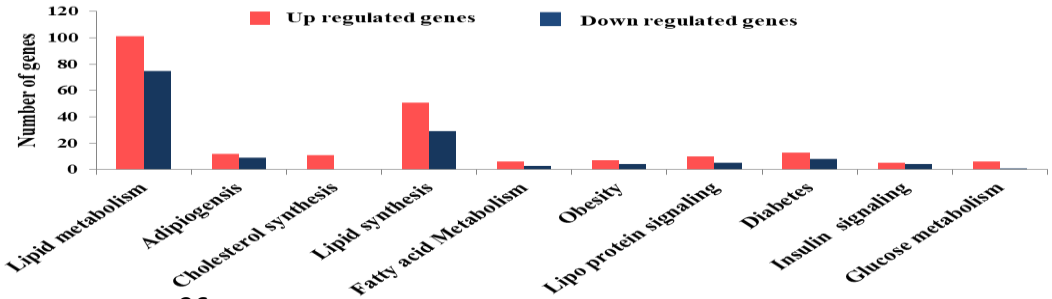




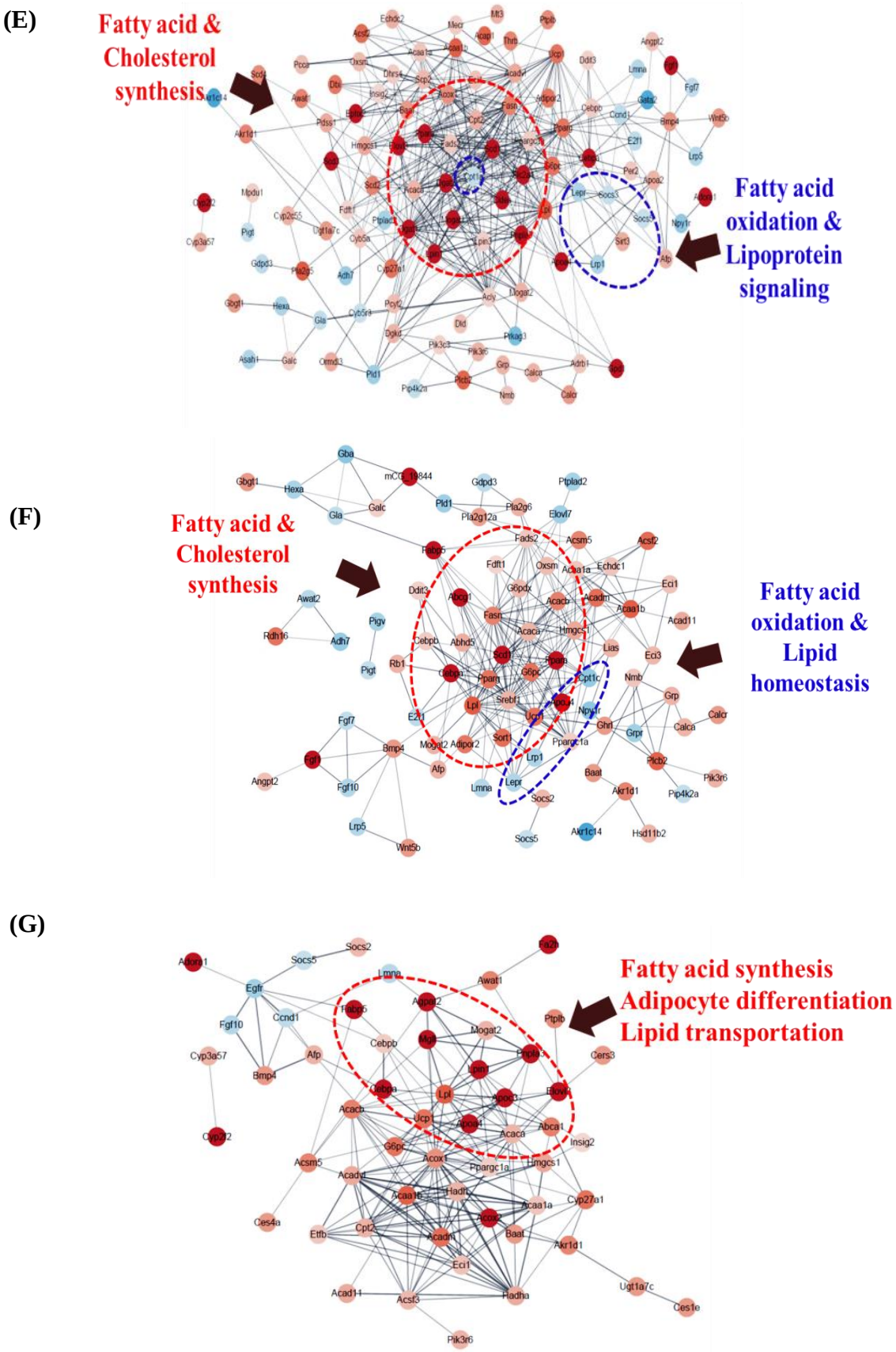


Figure 10. Effect of Naringenin, Naringin, and Hesperetin on differential gene expression.

GO analysis of 3T3-L1 adipocytes compared to the normal cells (A). Naringenin treated 3T3-L1 adipocytes compared to the normal cells (B). Naringin treated 3T3-L1 adipocytes compared to the normal cells (C). Hesperetin treated 3T3-L1 adipocytes compared to the normal cells (D).The pie chart indicates functional categorization of the differentially expressed genes in 3T3-L1 adipocytes, and the bar graph represents the number of genes up and down-regulated. PPI networks of naringenin (E), naringin (F), and hesperetin (G). Red circles represent up-regulated genes, and blue circles represent down-regulated genes in 3T3-L1 adipocytes.

1.5 Discussion

The present study demonstrated the anti-adipogenic effects of naringenin, naringin, and hesperetin on differentiated 3T3-L1 adipocytes. The treatments of naringenin, naringin, and hesperetin suppress both lipid accumulation and TG content with increased glucose uptake in 3T3-L1 adipocytes. Naringenin, naringin, and hesperetin increase the phosphorylation levels of AMPK and ACC, and inhibit the expression level of HMGCR which are the key enzymes in lipogenesis and cholesterol synthesis. RNA sequencing results reveal that naringenin treatment normalize the expression of genes involved in lipid metabolism including *Acaca*, *Fasn, Scd1, Mogat1, Dgat, Lipin1, Cpt1a,* and *Lepr*. In addition, the treatment of naringin normalize the expression of genes related to lipid metabolism including *Acaca, Fasn, Fabp5, Scd1, Srebf1, Hmgcs1, Cpt1c, Lepr,* and *Lrp1*. Hesperetin treatment also normalized the expression of genes including *Acaca, Elovl3, Mogat2, Fabp5, Lipin1, Pnpla3, Lrp1, Lrp5, Lepr,* and *Cpt1* which are involved in lipid metabolism in differentiated 3T3-L1 adipocytes.

The present study shows that the treatments of naringenin, naringin, and hesperetin have reduced the intracellular lipid accumulation and TG content in 3T3-L1 adipocytes as observed previously [19, 55, 56]. Naringenin reduces diet-induced weight gain, and improves glucose and lipid metabolism in animal models [57]. In addition, naringin rich pomelo (*Citrus grandis* (L.) Osbeck) peel extract is shown to reduce body weight, TG, and total cholesterol level in obese rats [58]. A recent study reported that hesperetin supplementation inhibits the cholesterol synthesis and enhances the clearance of circulating Low Density Lipoprotein (LDL) particles by triggering the expression of LDL receptors [59]. Hesperetin reduces the secretion of apolipoprotein (apo) B-containing lipoproteins in HepG2 cells [60] [61]. The reduced hepatic secretion of apo B-containing lipoproteins, such as Very Low Density Lipoprotein (VLDL), showed lower plasma TG and cholesterol [26]. Therefore, the

present study confirmed that naringenin, naringin, and hesperetin play a significant role in the inhibition of lipid accumulation and TG synthesis in differentiated 3T3-L1 adipocytes.

The present study confirmed that naringenin, naringin, and hesperetin significantly increases glucose uptake in 3T3-L1 adipocytes. Many flavonoids are known to increase the glucose uptake by the activation of AMPK which enhances GLUT4 translocation in 3T3-L1 adipocytes [62, 63]. Naringenin, naringin, and hesperetin are known to enhance glucose utilization by increased glycolysis which ultimately reduce the utilization of lipids [64, 65]. Therefore, the previous and present findings suggest that naringenin, naringin, and hesperetin have anti-adipogenic effect by increasing glucose uptake that might enhance glucose metabolism in differentiated 3T3-L1 adipocytes.

In the present study, the treatment of naringenin, naringin, and hesperetin increase the phosphorylation levels of both AMPK and ACC, and decreased the expression level of HMGCR. Activation of AMPK suppresses lipid synthesis [66] through down-regulation of key adipogenic factors including PPARγ, C/EBPα, and SREBP-1c [8]. Phosphorylation of ACC inhibits fatty acid synthesis by blocking the conversion of acetyl CoA to malonyl CoA [9]. Naringenin is known to reduce the levels of plasma cholesterol and hepatic TG accompanying by the decrease expression of HMGCR in animal models [67]. In addition, naringin is known to activate AMPK and suppress HMGCR expression in type 2 diabetic mice [66, 68]. Previous studies reported study reported that hesperetin treatment attenuated adipose tissue fat by up-regulation of AMPK [69] and hesperetin lowered the plasma cholesterol and TG levels by inhibition of hepatic HMGCR activity [70]. The previous and the present findings suggest that treatments of naringenin, naringin, and hesperetin regulate lipid metabolism by activating AMPK pathway in 3T3-L1 adipocytes, which potentially contributes to reduce adipogenesis.

RNA sequencing results reveal that naringein, naringin, and hesperetin treatment normalized the expression of genes which are involved in lipid metabolism in 3T3-L1 adipocytes. The treatment of naringenin normalized the expressions of up-regulated genes including *Acaca*, *Fasn*, *Scd1*, *Mogat1*, *Dgat*, and *Lipin1*. *Acaca* encoding enzyme catalyses the carboxylation of acetyl-CoA to malonyl-CoA [71]. *Fasn* catalyses the conversion of malonyl CoA into palmitate [72]. *Scd1* is the rate-limiting enzyme in synthesis of mono-unsaturated fatty acids [73]. *Mogat* catalyzes the synthesis of diacylglycerol, the precursor of triacylglycerol [74]. *Dgat* catalyzes the covalent addition of a fatty acyl chain to diacylglycerol [75] and reduces TG levels in *Dgat* knockout mice [76]. *Lipin1* is a key regulator of TG metabolism and lipoprotein synthesis [77]. In this regard, normalization of genes related to lipid metabolism including *Acaca, Fasn, Scd1, Mogat1, Lipin1,* and *Dgat* by naringenin treatment contributes to the reduction of adipogenesis in 3T3-L1 adipocytes.

Naringenin treatment normalized the expressions of down-regulated genes including *Cpt1a*, and *Lepr*. *Cpt1* catalyze the fatty acid oxidation by converting acyl-CoAs into acylcarnitines [78]. *Cpt1* knockout mice are reported to be susceptible to high-fat diet induced obesity [79]. *Lepr* regulates energy expenditure and reduces lipid accumulation in the body [80]. Previous study showed the attenuation of obesity via *Lepr* gene therapy [81]. Naringenin stimulated mRNA expression of *Cpt1* [82] which supports the present findings. Therefore, the previous and present findings suggest that naringenin reduce the adipogenesis by normalizing genes related to lipid metabolism in 3T3-L1 adipocytes.

The treatment of naringin normalized the expressions of up-regulated genes including *Acaca, Fasn, Scd1, Fabp5, Srebf1,* and *Hmgcs1*. As described previously, the expression of *Acaca* catalyses the first committed step of fatty acid synthesis [71]. *Fasn* is the central enzyme involve in de novo lipogenesis [72], and *Scd1* the rate-limiting enzyme in synthesis of mono-unsaturated fatty acids [83]. In addition, *Fabp5* involves in lipid trafficking and

intracellular fatty acid storage [84]. *Srebf1* regulates SREEBP1a and SREBP1c involves in fatty acid and cholesterol synthesis [85]. *Hmgcs1* involve in cholesterol synthesis by converting acetyl CoA and acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) [86]. In this regard, normalization of genes related to lipid metabolism including *Acaca*, *Fasn*, *Fabp5*, *Scd1*, *Srebf1*, and *Hmgcs1* by naringin treatment contributes to the reduction of adipogenesis in 3T3-L1 adipocytes.

The treatment of naringin normalized the expressions of down-regulated genes including *Cpt1c*, *Lepr*, and *Lrp1*. *Cpt1* involves in fatty acid oxidation [78] and *Lepr* regulates energy expenditure which reduces lipid accumulation [80]. *Lrp1* is key factor in maintaining lipid homeostasis, insulin sensitivity, and glucose homeostasis [87]. Overall, results suggest naringin restores the adipogenesis to the normal level by normalizing the expression of *Cpt1c*, *Lepr*, and *Lrp1* genes in 3T3-L1 adipocytes.

Hesperetin treatment in 3T3-L1 adipocytes has normalized up-regulated genes related to lipid metabolism including, *Acaca*, *Elovl3*, *Mogat2*, *Fabp5*, *Lipin1*, and *Pnpla3*. *Acaca* encoding enzyme catalyses the first committed step of fatty acid synthesis [71]. *Elovl3* is known to catlyse the elongation of saturated and monounsaturated fatty acids up to 24 carbons [88]. *Mogat* is involved in the synthesis of diacylglycerol, the precursor of triacylglycerol [74]. *Fabp5* act as intracellular fatty acid transporters which regulates lipid trafficking and fatty acids storage in cells [89]. *Lipin1* is a key regulator of TG and phospholipid metabolism, and lipoprotein synthesis [77]. *Pnpla3* is involved in the hydrolysis of triglycerides and enhances lipogenesis in adipose tissues [90]. In this regard, normalization of genes related to lipid metabolism including *Acaca*, *Elovl3*, *Mogat2*, *Fabp5*, *Lipin1*, and *Pnpla3* by hesperetin treatment contributes to the reduction of lipid accumulation in 3T3-L1 adipocytes.

In addition down-regulated genes including *Lrp1*, *Lrp5*, *Lepr*, and *Cpt1* were normalized to control level by hesperetin. Based on previous study, both *Lrp1* and *Lrp5* play important roles in lipoprotein metabolism and cholesterol homeostasis [91]. *Lepr* regulates energy expenditure and reduces lipid accumulation in the body [92]. Previous studies observed that morbid obesity in *Lepr* knockout mice [80] and showed the attenuation of obesity via *Lepr* gene therapy [93]. Actually, hesperetin is known to increase the mRNA level of *Lrp* in HepG2 cells [94]. In addition, the present study observed that hesperetin normalized down-regulated *Cpt1*, which is fatty acid oxidation related gene [95]. Therefore, hesperetin restores the lipid metabolism to the normal level by normalizing the expression of *Lrp1*, *Lrp5*, *Lepr*, and *Cpt1* genes in 3T3-L1 adipocytes.

In the present study, naringenin, naringin, and hesperetin showed significant anti-adipogenic effects, still naringin has higher potent than naringenin and hesperetin in mitigating obesity. Glycosides are known to be more biologically active than the respective aglycone, since the bound sugar moiety of O-glycosides is known to influence their bioavailability [96]. Attaching the glycosidic moiety increases its hydrophilicity which influences the pharmacokinetic properties of the respective compounds [97, 98]. O-glycosylation can enhance some biological benefits including anti-adipogenic activity [44]. Therefore, naringin being a 7 O-glycoside shows higher anti-adipogenic potential than the aglycones naringenin and hesperetin in 3T3-L1 adipocytes.

The result of the present study results indicate that naringenin, naringin, and hesperetin treatment reduces the lipid accumulation and TG content in 3T3-L1 adipocytes by increasing the phosphorylation of both AMPK and ACC while reducing the expression of HMGCR. In particular, naringenin, naringin, and hesperetin normalize the expression of genes involved in lipid metabolism in 3T3-L1 adipocytes (Fig. 11). Further studies are needed to delineate the role of these genes as therapeutic targets in obesity. The overall

results suggest that treatment of naringenin, naringin, and hesperetin have potent anti-adipogenic effect by normalizing the expression of genes related to lipid metabolism which are perturbed in differentiated 3T3-L1 adipocytes.

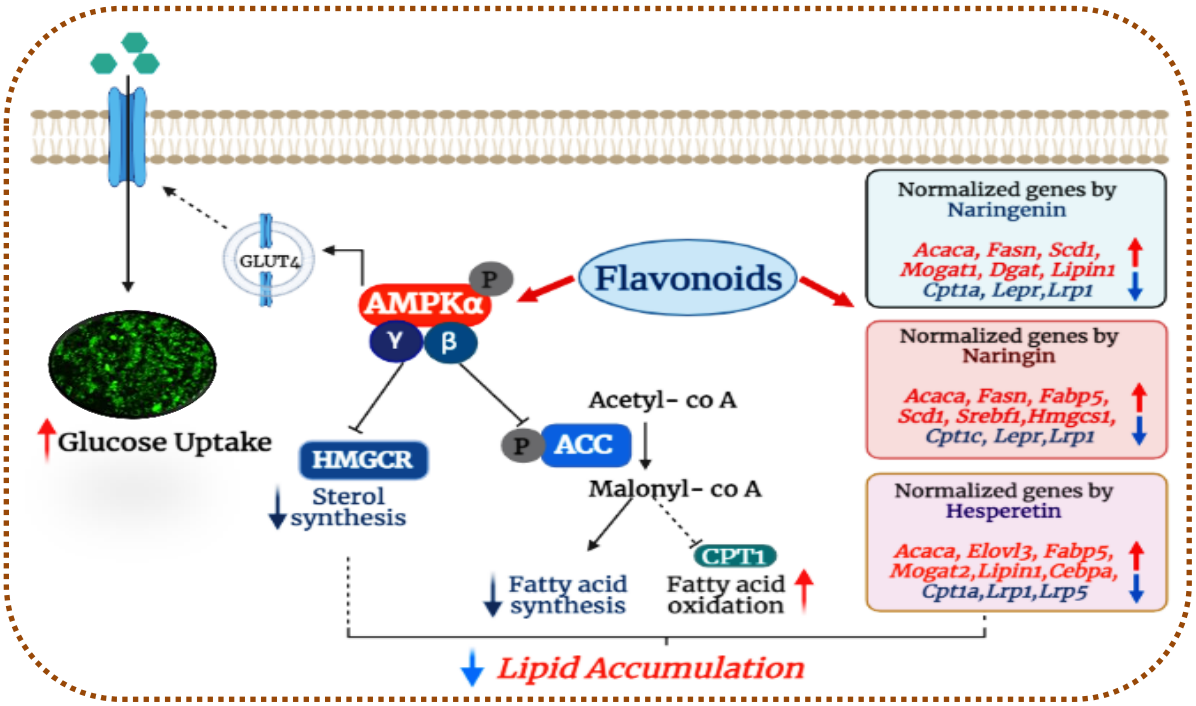


Figure 11. Proposed model for Anti-adipogenic effect of flavonoids

PART– II

Anti-diabetic effect of Flavonoids

2.1 Abstract

The present study examined the anti-diabetic effect of naringenin, naringin, and hesperetin on HepG2 cells. The treatment of naringenin, naringin, and hesperetin stimulated the glucose uptake regardless of insulin stimulation in high glucose treated HepG2 cells. Western blot analysis revealed that naringenin, naringin, and hesperetin increased the glucose uptake by promoting the phosphorylation of AMP-activated protein kinase (AMPK) at Thr172 and with increased phosphorylation of Glycogen synthase kinase 3 beta (GSK3β) in HepG2 cells. In addition, the molecular docking studies reveals that naringein, naringin, and hesperetin bind to site 4 of γ-subunit of AMPK with high binding affinities; naringenin -7.6 kcal/mol, naringin -9.1 kcal/mol, and hesperetin -8.0 kcal/mol closer to that of modulator (e.g. AMP -8.1 kcal/mol) of AMPK. Therefore, naringenin, naringin, and hesperetin could be positive modulators for AMPK activation which enhance glucose uptake regardless of insulin stimulation in high glucose treated HepG2 cells. Overall results suggest that the phosphorylation of AMPK at Thr172 by naringenin, naringin, and hesperetin might enhance glucose uptake regardless of insulin stimulation in high glucose treated HepG2 cells.

***Keywords*: Flavonoids, Glucose uptake, AMPK phosphorylation, Molecular docking analysis**

2.2 Introduction

Diabetes is characterized by elevation in blood sugar level for prolonged period of time [99]. Among the two available forms of diabetes, Type 2 diabetes (T2DM) is considered to be the most severe chronic diseases caused by increased insulin resistance [100].

Perturbation of an insulin-independent signaling cascade is one potential strategy to enhance glucose uptake in an insulin resistant state [12]. Activation of AMPK regulates the insulin-independent glucose uptake via stimulating the GLUT2 translocation and regulates the systemic glucose homeostasis [101]. In addition, AMPK activation is responsible for maintain glucose homeostasis via phosphorylation of downstream targets including GSK3β [13]. Phosphorylation and inhibition of GSK3, activates glycogen synthase (GS), promotes the conversion of glucose-6 phosphate (G6P) to G1P then uridine diphosphoglucose (UDP-G), which is targeted towards glycogen synthesis [102]. Therefore, AMPK is known to be an attractive pharmaceutical approach for the treatment of diabetes [12]. AMPK is inactive unless phosphorylated on the α-subunit activation loop at Thr172 [103]. However, AMP binding to the γ-subunit enhances Thr172 phosphorylation and suppresses dephosphorylation of Thr172. In addition, upstream kinases, liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) phosphorylate AMPK Thr172 through AMP binding [103].

As pharmacological management of diabetes is associated with undesirable side effects dietary intervention through intake of polyphenol-rich plant compounds that can modulate glucose metabolism is targeting modality of diabetes management [104, 105]. Further, with the progress of modern technology, the binding phenomena of protein with small compounds can be analyzed via computational simulation. These methods make it

possible to participate in computer-aided drug design (CADD) which is an effective strategy in drug discovery [106].

Flavonoids are plant derived compounds with multiple positive health effects on metabolic disorders including diabetes [107]. Naringin is a flavanone glycoside found in grapes and citrus fruits. Two rhamnose units are attached to its aglycon portion, naringenin, at the 7-carbon position [108]. For naringenin, naringin, and hesperetin diverse biological activities of therapeutic interests have been described including anti-diabetic and anti-dyslipidemic effects [109]. While all these studies exist still the mechanisms by which these flavonoids inhibits diabetic complications and promotes glucose uptake is remained to be clarified further.

The present study examined the glucose uptake effects of naringenin, naringin, and hesperetin on insulin resistant HepG2 cells. For this, we observed the effect of flavonoids on glucose uptake and on the phosphorylation of AMPK and GSK3β in insulin-resistant HepG2 cells. In addition, molecular docking analysis was conducted to observe the binding affinity of naringenin, naringin, and hesperetin to γ-subunit of AMPK.

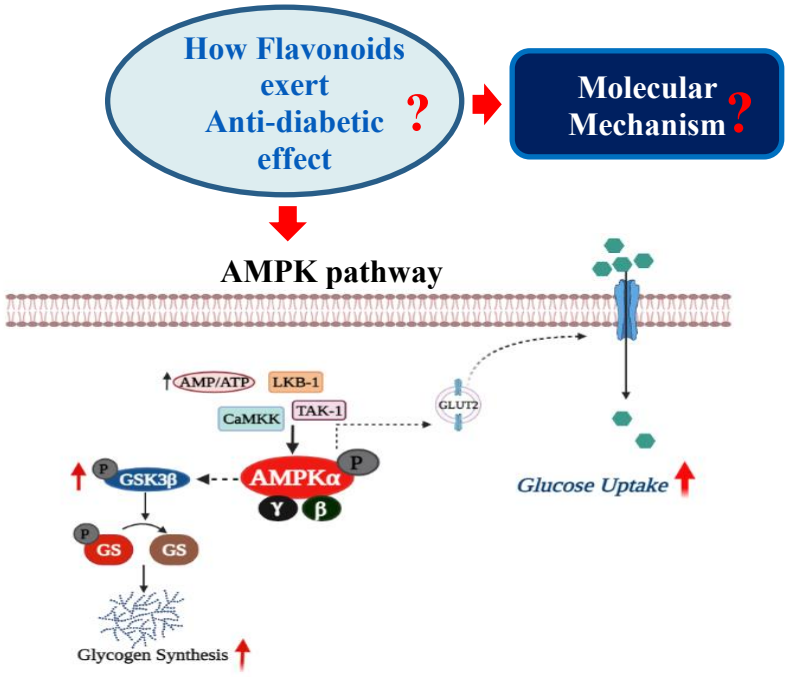


Figure 12. Hypothetical model for the Anti-diabetic effect of flavonoids

2.3 Materials and Methods

2.3.1 Cell culture and treatment

HepG2 cells (KCLB 42707) from the Korean Cell Line Bank (KCLB) (Seoul, Korea) and routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA) in a humidified atmosphere containing 5% CO_2 at 37 °C. After reaching confluence, cells were then seeded in culture plates for further experiments.

The insulin resistant HepG2 cell model was established according to the reported method with slight modifications [110]. In brief, after seeding in 96 well plates, cells were serum-starved for 12 hrs and incubated in serum-free DMEM (Gibco, USA) containing either normal concentrations of glucose (5.5 mM D-glucose) or high concentrations of glucose (30 mM D-glucose) with or without flavonoids (Sigma-Aldrich, USA) (10 and 50μM) and metformin (Sigma-Aldrich, USA) (2 mM) for additional 24 hrs. The high glucose treated cells were used as insulin resistant model. The cells were stimulated with or without 100 nM insulin (Sigma-Aldrich, USA) for 30 min before harvesting.

2.3.2 Cell viability assay

HepG2 cells were seeded in 96-well plates at the cell density of 1×10^5 cells/well and cultured overnight using routine culture media. After reaching the confluence, the cells were treated with different concentrations (0-100 μM) of flavonoids for 24 hrs. Cell culture media was removed and added fresh media containing 10% Ez-cytox (DogenBio, Korea) into each well, according to the manufacturer's instructions. Plates were incubated for 3 hrs at 37°C and 5% CO_2. Cell viability indicated by the production of formazan was measured with an ELISA microplate reader (TECAN, Austria) at 450 nm wavelength.

2.3.3 2- NBDG glucose uptake

HepG2 cells were cultured in 96-well plates. After reaching confluence, the cells were serum-starved for 12 hrs and incubated in a serum-free medium containing either normal or high concentrations of glucose with or without samples (flavonoids 10 µM and 50 µM) for an additional 24 hrs. The cells were incubated with 40 µM 2-NBDG 2-[N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose (Invitrogen, Carlsbad, USA) with or without 100 nM insulin (Sigma-Aldrich, USA) for 30 min at 37°C. Cells were washed with PBS for three times, and the fluorescent images were taken from IncuCyte® ZOOM Fluorescent Microscope (Essen BioScience, Inc. USA). The IncuCyte® ZOOM Fluorescent Processing Software was used to analyze the total fluorescent intensities of each well.

2.3.4 Western blotting

Cells were washed with PBS (Gibco, USA) and lysed with ice-cold RIPA buffer (Tech and Innovation, Korea) containing protease inhibitor mixture. The whole-cell lysates were centrifuged at 12,000 rpm for 10 min. The supernatant was separated, and the amount of protein was assessed by Bradford assay (Bio-Rad Laboratories, USA). Equal amounts of protein from cell homogenates were subjected to SDS PAGE and transferred to PVDF membranes. Membranes were probed with primary antibodies GSK3β, p-GSK3β, AMPK, and p-AMPK (Cell signaling technology, USA) and then detected with peroxidase-conjugated secondary antibodies for 1hr at room temperature. β-actin (Thermofisher, USA) was used as a loading control. Chemi-luminescence Bioimaging Instrument (NeoScience Co., Ltd., Korea) was used to detect proteins of interest Densitometry analysis was performed using the ImageJ analysis software.

2.3.5 Molecular docking

The crystal structure of AMPK in complexes with AMP (PDB ID; 2V8Q) was obtained from Protein Data Bank (PDB). The sitemap tool (Schrodinger Software, Germany) was used to identify the four CBS domains (CBS1, CBS2, CBS3, and CBS4) in the γ-subunit of AMPK. All the AMP molecules in the γ-subunit of AMPK were removed for docking flavonoids. 3D structure of AMP and flavonoids (naringenin, naringin, and hesperetin) were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and energetically minimized using PyRx software (Python Prescription 0.8, The Scripps Research Institute). The grid box used for focused docking was set to 26×44×46 Å to ensure the structure of the γ-subunit of AMPK. The docking experiments were carried out by using the AutoDock Vina module (Molecular Graphics Lab, The Scripps Research Institute, USA). On the basis of binding energy, the best docked pose was selected and the 3D images were generated using the PyMOL (The PyMOL Molecular Graphics System, Ver.2.5.0, Schrodinger, LLC, USA). Docked complex of AMPK were further optimized, validated, and explored using Discovery Studio visualizer (Ver.21.1.0.20298). The hydrogen bond and hydrophobic interactions between receptor and ligand was analysed using the Ligplot program [111].

2.3.6 Statistical analysis

Values were expressed as means ± S.D. of three independent experiments. Data were statistically analyzed with the aid of IBM SPSS Statistics (Ver.17.0; USA). The statistical differences among groups were analyzed with one-way analysis (ANOVA) followed by Turkey's test. $P < 0.05$, $P < 0.005$ and $P < 0.0005$ indicate statistically significant differences from the control group.

2.4 Results

2.4.1 Cell viability

MTT assay was performed to assess the effect of naringenin, naringin, and hesperetin on HepG2 cells viability. Significant cellular toxicity was not observed up to 50 μM of flavonoids concentration in HepG2 cells (Fig. 13). Accordingly, the experiments were conducted with the non-toxic concentrations of flavonoids (10 and 50 μM).

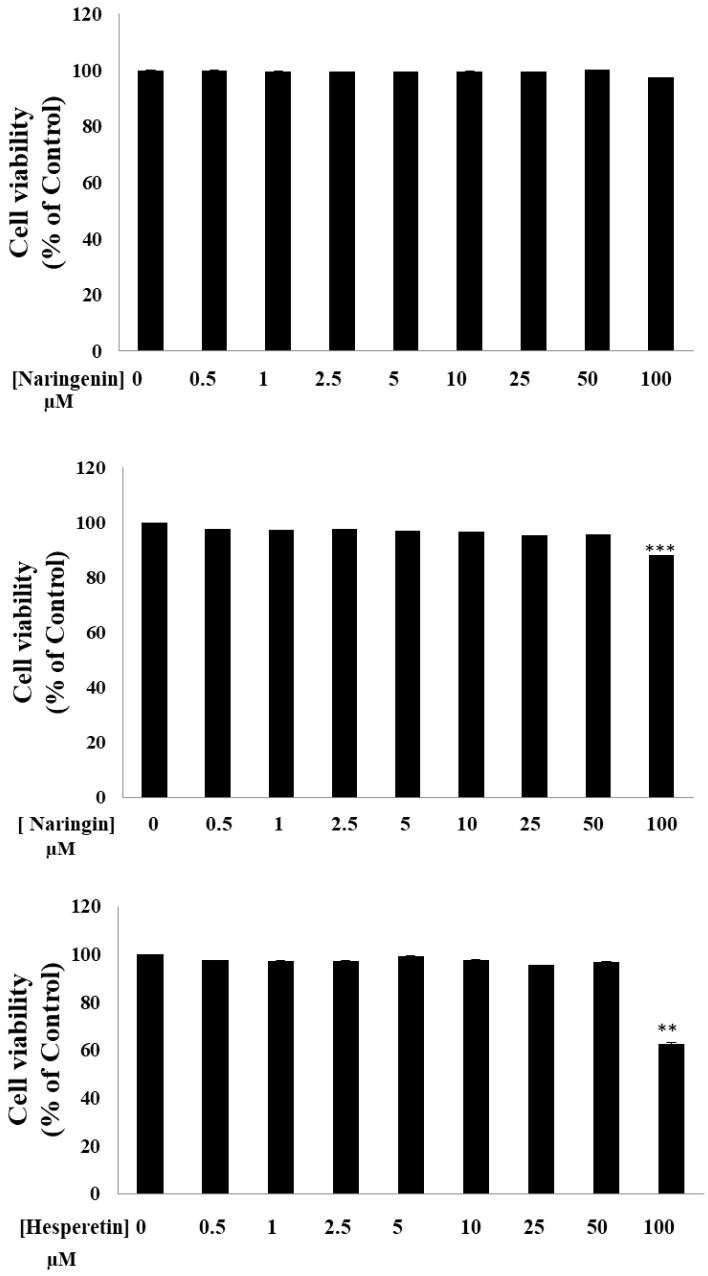


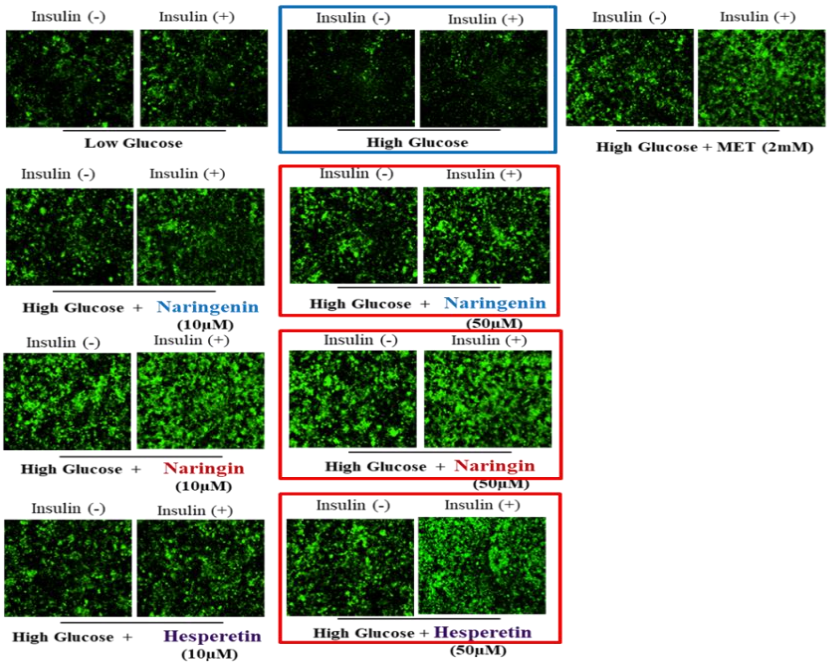
Figure 13. Effect of Naringenin, Naringin, and Hesperetin on HepG2 cells viability.

HepG2 cells were cultured at a density of 1×10^{5} in 96 well plate, after reaching the confluence cells were treated with 0-100 μM of flavonoids for 24 hrs, and cell viability was measured by the MTT assay (B). Values represent mean ± SE. $^{**}P < 0.005$ and $^{**}P < 0.0005$ vs. the control.

2.4.2 Glucose uptake

The effect naringenin, naringin, and hesperetin on glucose uptake in high glucose treated HepG2 cells were determined by 2-NBDG uptake assay (Fig. 14). The results indicated that the glucose uptake was reduced in high glucose treated HepG2 cells compared to low glucose treated cells. In contrast, the treatment of metformin (positive control) showed significantly increased ($P < 0.005$) 2-NBDG uptake compared to high glucose treated HepG2 cells (Fig. 14A and 14B). The treatment of naringenin, naringin, and hesperetin increased the 2-NBDG glucose uptake regardless of insulin stimulation compared to high glucose treated cells (Fig. 14A and 14B). In particular, naringin treatment showed higher glucose uptake than other naringenin and hesperetin treatments in high glucose treated cells. The results suggest that naringenin, naringin, and hesperetin enhanced the glucose uptake in high glucose treated HepG2 cells regardless of insulin stimulation.

(A)



(B)

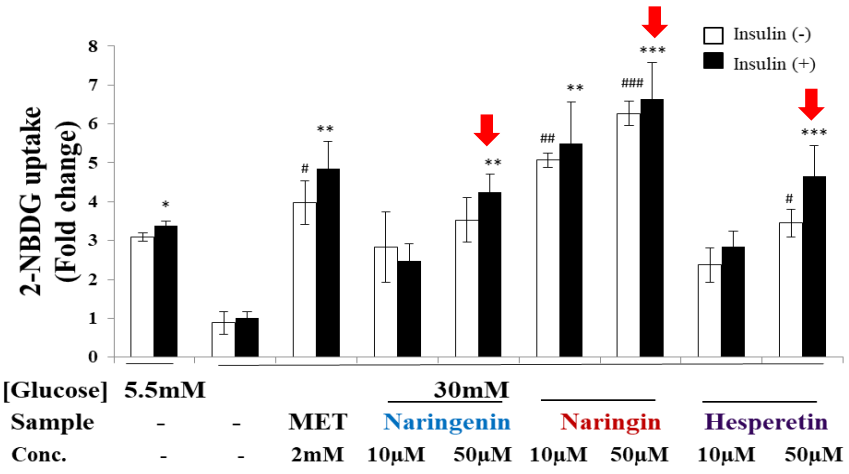


Figure 14. Effect of Naringenin, Naringin, and Hesperetin on glucose uptake.

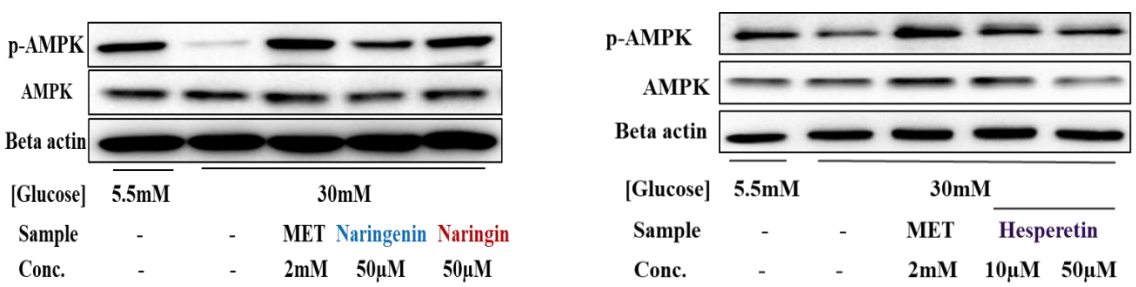
The glucose uptake assay was done using the fluorescent D-glucose analogue 2-NBDG. HepG2 cells were serum-starved for 12 hrs and incubated in serum-free medium containing either normal (5.5 mM) or high (30 mM) concentrations of glucose for an additional 24 hrs in the presence and absence of samples. Then stimulated with or without insulin (100 nM) and 2-NBDG (50 uM) for 30 min. 2-NBDG uptake by cells were detected by IncuZyte Zoom at 20X magnification. (A) Representative images for each sample, (B) Quantitative 2NBDG uptake from each sample. Values are mean ± SE, $*P < 0.05$, $**P < 0.005$, and $*P < 0.0005$,

vs insulin-stimulated high glucose control and $^{\#}P < 0.05$, $^{\#\#}P < 0.005$, and $^{\#\#\#}P < 0.0005$vs without insulin-stimulated high glucose control.

2.4.3 Phosphorylation of AMPK

Western blot analysis was conducted to observe the effect of naringenin, naringin, and hesperetin on the phosphorylation of AMPK (Fig. 15). The phosphorylation levels of AMPK (Thr172) in high glucose treated HepG2 cells were significantly decreased ($P < 0.05$) compared to low glucose treated cells. In contrast, the phosphorylation of AMPK (Thr172) was significantly increased by the treatment of metformin, naringenin, naringin, and hesperetin in high glucose treated HepG2 cells (Fig. 15A and 15B). In particular, the effect of naringin on the phosphorylation levels of AMPK (Thr172) was comparatively higher than other two flavonoids and was similar to that of metformin. The results suggest that naringenin, naringin, and hesperetin increased the phosphorylation of AMPK in high glucose treated HepG2 cells.

(A)



(B)

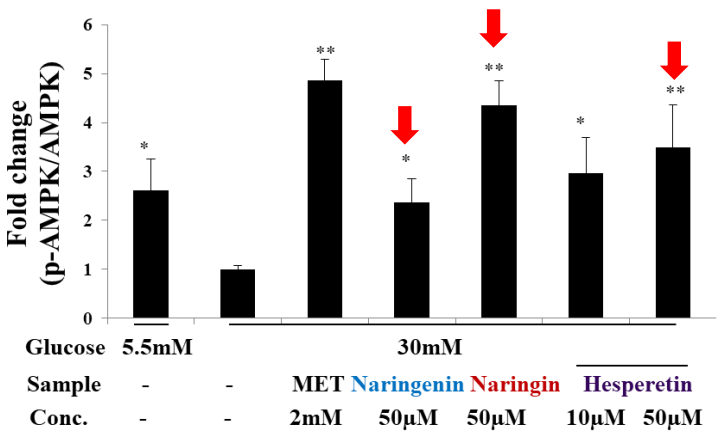
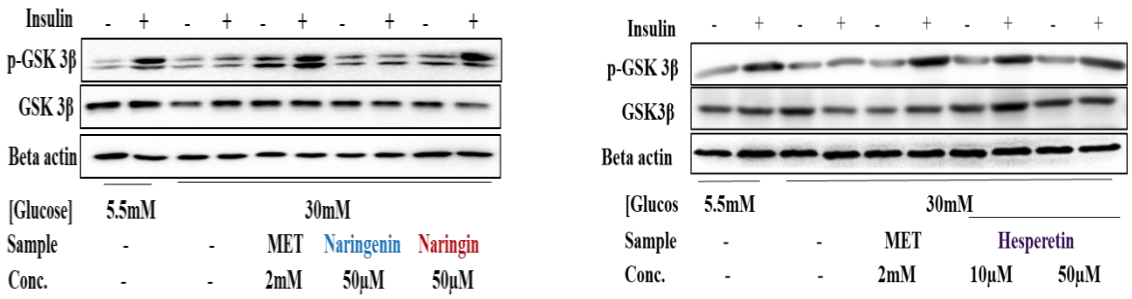


Figure 15. Effect of Naringenin, Naringin, and Hesperetin on AMPK phosphorylation. HepG2 cells were starved in serum-free medium for 12 hrs and incubated in serum-free medium containing either normal (5.5 mM) or high (30 mM) concentrations of glucose with or without different samples for an additional 24 hrs. Total cell extract was subjected to Western blot, and band intensities were quantified using densitometry. Immunoblot band pattern of AMPK (A), and the results of quantitative analysis of the ratio of p-AMPK to AMPK (B). Values are the mean ± SE. **P* < 0.05, ***P* < 0.005 compared with high glucose control.

2.4.4 Phosphorylation of GSK3β

Western blotting was performed to determine the effect of naringenin, naringin, and hesperetin on the phosphorylation level of GSK3β (Ser9) (Fig. 16). The phosphorylation levels of GSK3β (Ser9) in high glucose treated HepG2 cells were significantly decreased (*P* < 0.0005) compared to low glucose treated cells. In contrast, the phosphorylation of GSK3β was increased by the treatment of metformin, naringenin, naringin, and, hesperetin compared to high glucose treated cells in the presence and absence of insulin. However, the treatment of naringin and hesperetin showed significantly higher (*P* <0.0005) phosphorylation of GSK3β compared to naringenin (Fig. 16A and 16B). The results suggest that naringenin, naringin, and hesperetin increased the phosphorylation of GSK3β (Ser9) in high glucose treated HepG2 cells.

(A)



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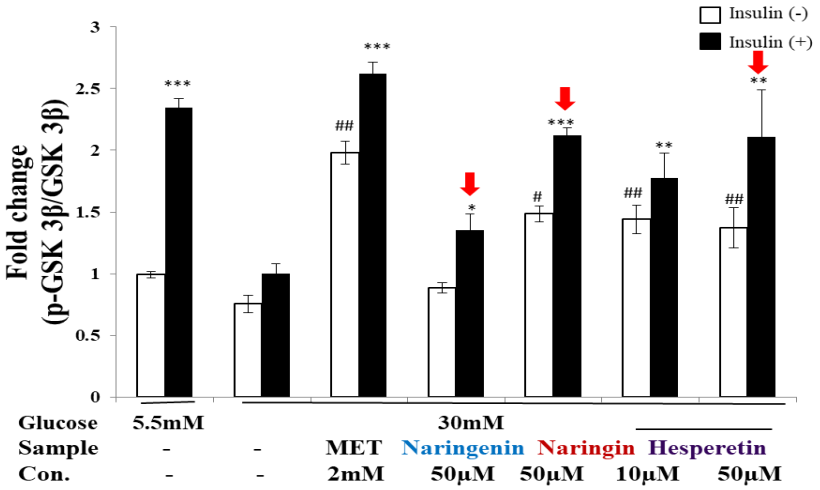


Figure 16. Effect of Naringenin, Naringin, and Hesperetin on phosphorylation of GSK3β.

HepG2 cells were starved in serum-free medium for 12 hrs and incubated in serum-free medium containing either normal (5.5 mM) or high (30 mM) concentrations of glucose with or without different samples for an additional 24 hrs. Before harvesting, the cells were stimulated with or without 100 nM insulin for 30 min. Total cell extract was subjected to Western blot, and band intensities were quantified using densitometry. Immunoblot band pattern of AMPK (A), and the results of quantitative analysis of the ratio of p-GSK3β to GSK3β(B). Values are the mean ± SE. $*P < 0.05$, $**P < 0.005$, and $***P < 0.0005$ compared with high glucose control and and $^{\#}P < 0.05$, and $^{\#\#}P < 0.005$ vs without insulin-stimulated high glucose control.

2.4.5 Molecular docking analysis

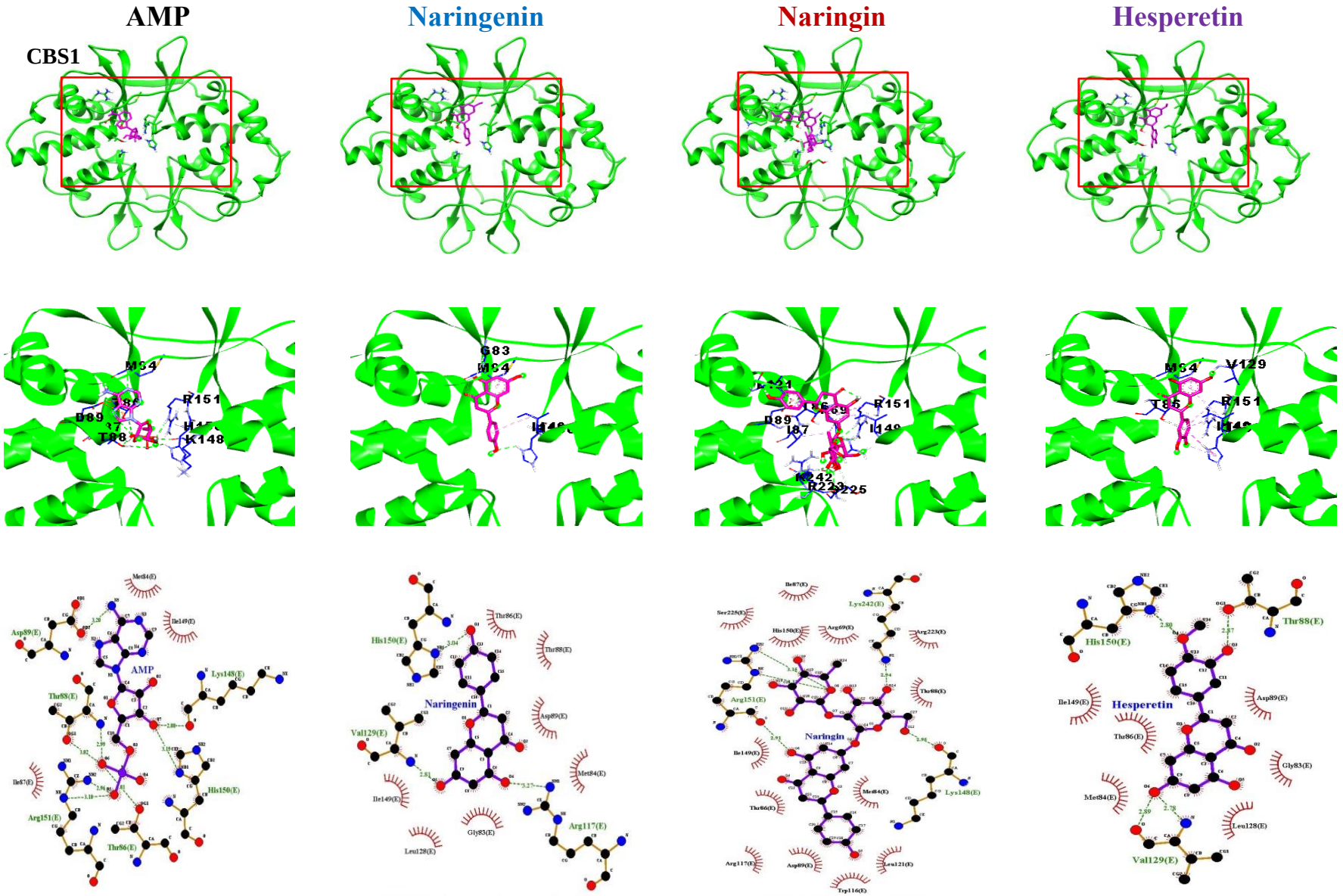
Molecular docking analysis was performed to simulate the binding affinity between flavonoids and γ-subunit of AMPK. As shown in Table 7, flavonoids were bound into each of binding sites (site 1, 3, and 4) in γ-subunit of AMPK with high binding affinities which were close to the affinity of AMP. Overall, AMP and two flavonoids (naringin and hesperetin) showed highest binding score with site 4 than site 1 and 3 in γ-subunit of AMPK. At site 4, naringin has a binding affinity of -9.1 kcal/mol, hesperetin show the binding affinity of -8.0

kcal/mol, and AMP showed the affinity of -8.1 kcal/mol (Table 7). In contrast, naringenin showed the highest binding affinity at site 3 with -8.0 kcal/mol to γ-subunit of AMPK (Table 7). As depicted in Fig.17, naringenin, naringin, and hesperetin interacts with R-groups in γ-subunit of AMPK through hydrogen bonds and hydrophobic interactions. At site 3, naringenin formed hydrophobic interactions with Ile239, Val224, Val296, Leu276, Phe272, Phe243, His270, and Arg268 and formed hydrogen bonds with Arg298 residue. In addition, naringin formed hydrophobic interactions with residues, Ile311, Ala226, Val224, Ser315, His150, Arg223, Lys148, Asp316, and Ile203 and formed hydrogen bonds with Arg298, Asn202, Ala201, Ser313, His297, Ser225, and Thr199 at site 4. Hesperetin formed hydrophobic interaction at site 4 with amino acid residues-Ser315, Ser313, Val224, and Ile203 and formed hydrogen bonds with Arg298, Ala226, Thr199, Ser225, and Asp316. In particular, naringin binds to the all sites in the cavity of γ-subunit of AMPK with high binding affinity than naringenin and hesperetin. The results suggest that naringenin, naringin, and hesperetin might be potential activators of AMPK.

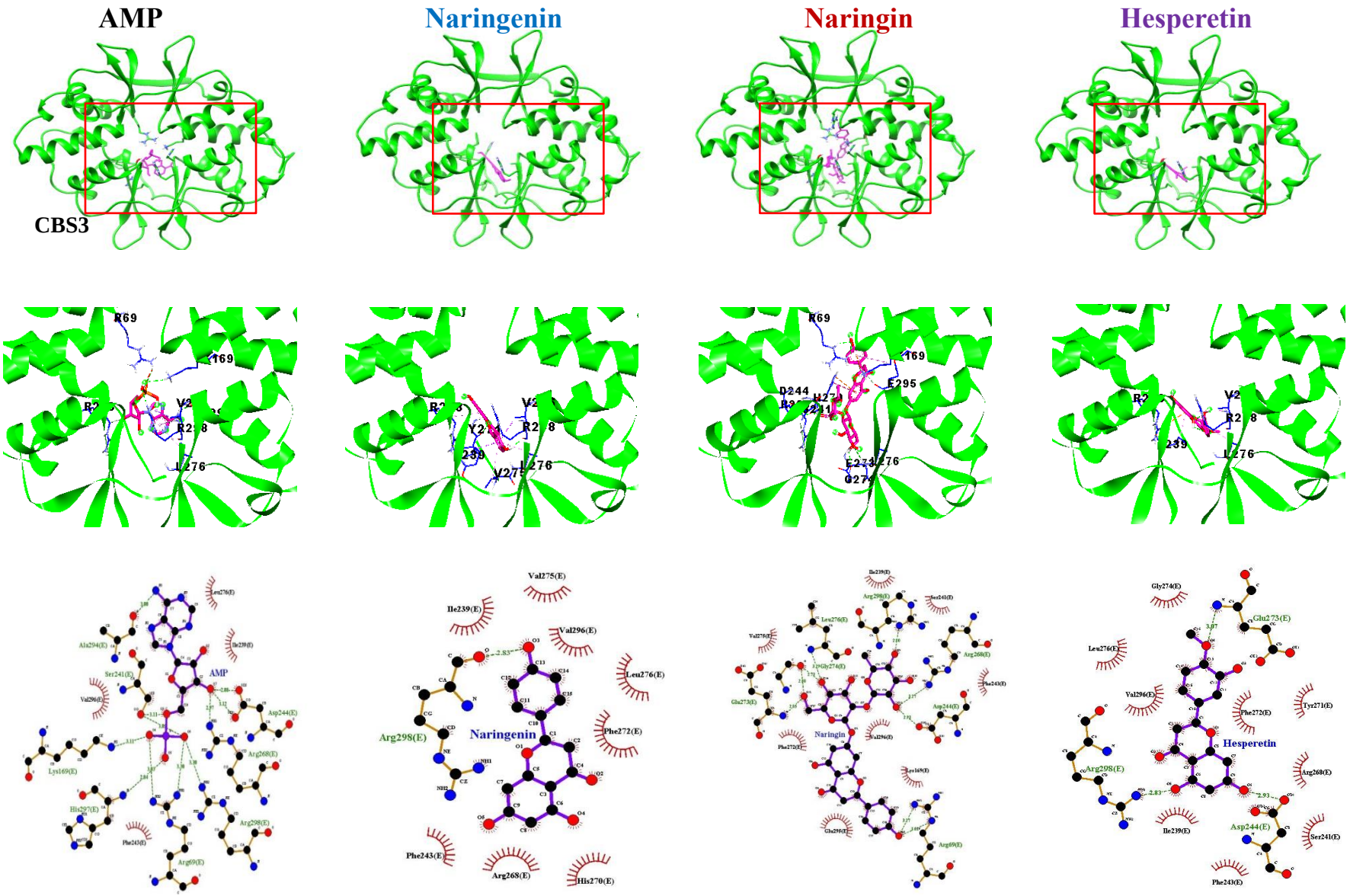
Table 7. Binding energy of AMP and flavonoids at three sites that were identified in the γ-subunit after removing all the co-crystallized ligands

Molecule	Description	Binding site of γ-subunit of AMPK		
		CBS1	CBS3	CBS4
AMP	**Binding energy** (kcal/mol)	- 7.8	-7.4	-8.1
	Hydrogen bonds	Asp89, Arg151, Thr88, Thr86, Lys148, His150	Ala294, Arg69, Arg298, Arg268, Asp244, His297, Lys169, Ser241	Asn202, Ser225, Ser313, Ser315, His150, His297
	Hydrophobic interactions	Met84, Ile149, Ile87	Leu276, Ile239, Phe243, Val296	Ile203, Val224, Ile311, Thr199, Lys148
Naringenin	**Binding energy** (kcal/mol)	-7.7	-8.0	-7.6
	Hydrogen bonds	Arg117, His150, Val129	Arg298	Ala226, Arg298, Thr199, Ser225
	Hydrophobic interactions	Asp89, Ile149, Leu128, Gly83, Thr86, Thr88, Met84	Arg268, Ile239, His270, Phe243, Phe272, Leu276, Val275, Val296	Ser315, Val224, Ile311
Naringin	**Binding energy** (kcal/mol)	-8.5	-8.3	-9.1
	Hydrogen bonds	Arg151, Lys242, Lys148	Arg298, Arg268, Arg69, Asp244, Gly273, Gly247, Leu276	Arg298, Asn202, Ala201, His297, Ser313, Ser225, Thr199
	Hydrophobic interactions	Arg223, Arg117, Asp89, Arg69, Ser225, Thr88, Thr86, Trp116, Met84, His150, Ile87, Ile149, Leu121	Ile239, Ser241, Phe243, Val296, Lys169, Gly295, Phe272, Val275	Ala226, Arg223, Asp316, Ile311, Ile203, His150, Lys148, Ser315, Val224
Hesperetin	**Binding energy** (kcal/mol)	-8.0	-7.7	-8.0
	Hydrogen bonds	His150, Thr88, Val129	Arg298, Asp244, Glu273	Asp316, Arg298, Ala226, Thr199,Ser225
	Hydrophobic interactions	Asp89, Ile149, Thr86, Gly83, Met84, Leu128	Gly274, Leu276, Val296, Ile239, Phe243, Phe272, Tyr271, Arg268, Ser241	Ser315, Ser313, Val224, Ile311

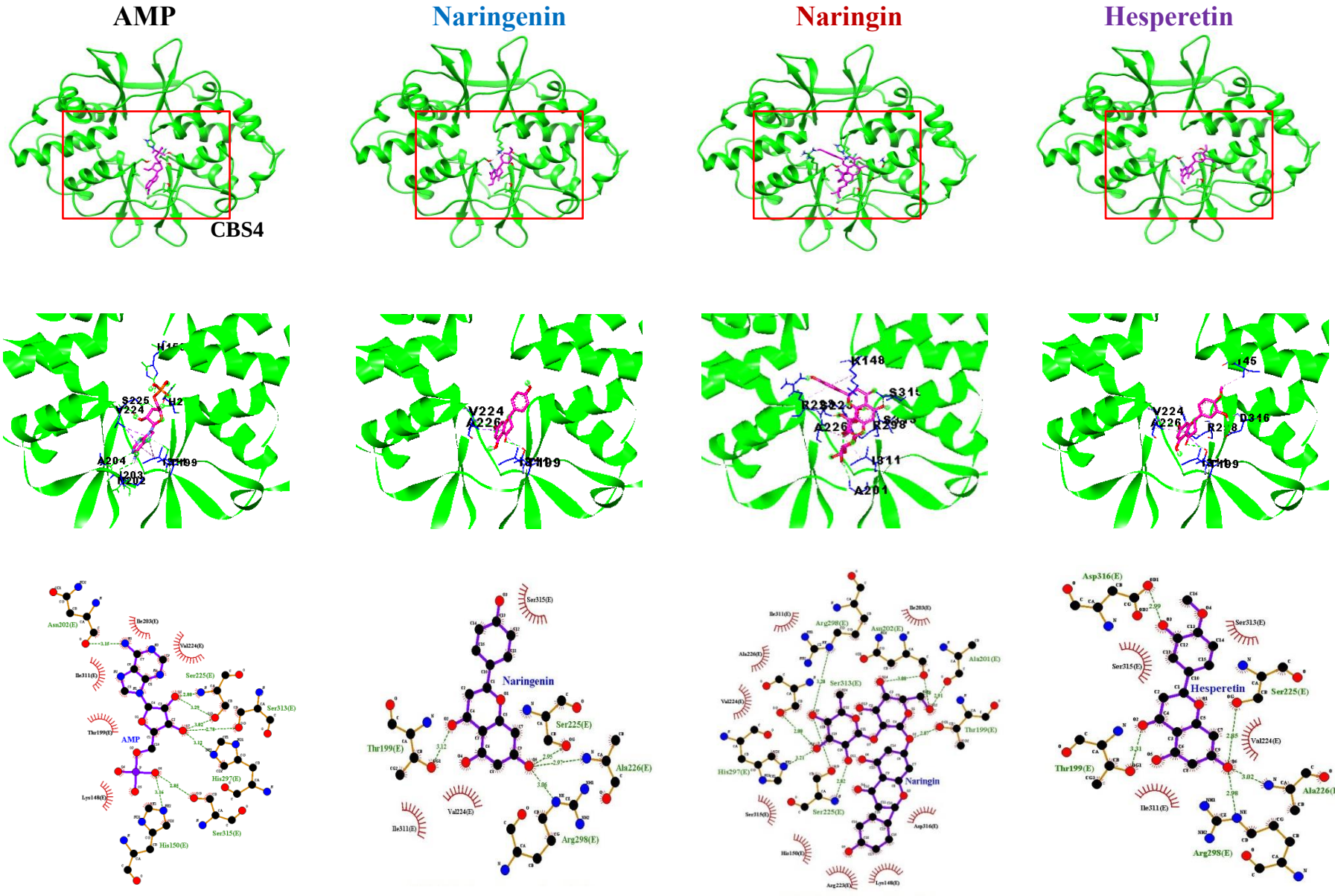
(A)



(B)



(C)



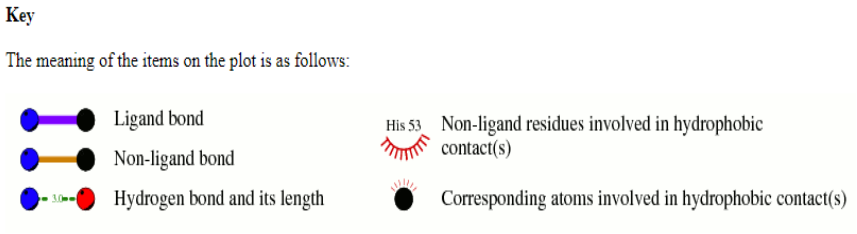


Figure 17. Molecular docking analysis for Naringenin, Naringin, Hesperetin, and AMP binding to AMPK.

Simulated image of AMP, naringenin, naringin, and hesperetin with AMPK complex and hydrogen bonds and hydrophobic interactions between AMPK at site 1 (A), site 3 (B), and site 4 (C) of γ-subunit of AMPK. Half red circles indicate the residues participating in hydrophobic interactions and the dotted lines indicate hydrogen bonds. Full red circles denote the residues present in the binding sites of naringenin, naringin, hesperetin, and AMP.

2.5 Discussion

The present study investigated the anti-diabetic effect of naringenin, naringin, and hesperetin on HepG2 cells. The treatment of naringenin, naringin, and hesperetin showed increased glucose uptake with increase phosphorylation of AMPK (Thr172) and GSK3β. Molecular docking simulation showed that naringenin, naringin, and hesperetin bind to the cavity of γ-subunit of AMPK with high binding affinities which suggest that the flavonoids could be positive modulators of AMPK activation.

The present study demonstrated that naringenin, naringin, and hesperetin significantly enhanced glucose uptake regardless of insulin stimulation in high glucose treated HepG2 cells. Hepatic glucose uptake is largely an insulin-independent process, which contributes to whole-body glucose homeostasis [112]. For example, hepatic glucose uptake can be enhanced by the entry of glucose into the portal vein which stimulates a portal glucose signal [113]. When the hyperglycemia broke out, plasma glucose entered the cytoplasm of hepatocytes through GLUT2 which is an insulin independent system [114]. AMPK has a critical function in this process, because it can suppress gluconeogenesis in the liver and promote glucose uptake peripheral tissues [115].

In liver, AMPK enhances the glucose uptake by up-regulating the expression of GLUT2 [115]. Activation of AMPK suppresses hepatic gluconeogenesis and promotes glycogen synthesis by direct phosphorylation of its substrates, including GSK3β which will ultimately increases the glucose uptake by liver [116, 117]. In addition, the AMPK induced phosphorylation of GSK3β inhibits the transcriptional activity of cAMP response element binding protein (CREB), a key transcription factor which regulates the phosphorylation of gluoneogenic enzymes [116].

AMPK is a heterotrimer consist of catalytic α subunits and regulatory β and γ subunits [117]. Phosphorylation of Thr172 within the activation loop of α subunit can cause the AMPK activation [118]. AMP is a natural activator of AMPK and binds to the allosteric site of cysteine-β synthase (CBS) domains of γ-subunit and thus indirectly promotes the activity at the catalytic domain of α-subunit [119]. AMP binding to AMPK stimulates the phosphorylation of Thr172 in α-subunit by upstream kinases LKB1 and CaMKKβ, and prevents the dephosphorylation of Thr172 [120]. In addition, a previous study showed that transforming growth factor β-activated kinase (TAK1) is a new possible kinase which phosphorylates AMPK on Thr172 through AMP binding [121]. Therefore, binding of adenine nucleotides to the γ-subunit causes conformational changes that regulate the phosphorylation and dephosphorylation of Thr172 which mediates AMPK functioning [120].

In the present study, the molecular docking results indicate that naringenin, naringin, and hesperetin bind to the cavity in γ-subunit of AMPK. AMP and flavonoids bind to and interact with several R-groups of amino acids located in the CBS domains 1, 3, and 4 of γ-subunit. In particular, flavonoids the highest binding scores with CBS 4 domain, where AMP docked with highest binding affinity [122]. The results suggest that naringenin, naringin, and hesperetin enhance the phosphorylation of AMPK at Thr172 which might enhances the glucose uptake via stimulating GLUT2 translocation in HepG2 cells. In addition, naringenin, naringin, and hesperetin increased the phosphorylation of GSK3β which regulates the glycogen synthesis. Overall results suggest that the flavonoids-naringenin, naringin, and hesperetin enhance the glucose uptake by activation of AMPK.

As reported previously, flavonoid glucuronic acids have exhibit beneficial effect in glucose homeostasis with high binding affinities to AMPK [123]. A recent study on roles of plant derived polypheonls against metabolic disorders, reveals that polyphenols in *Lippia citriodora* might act as direct agonists of AMPK, binding to the AMP binding sites of the γ-

subunit and/or the different sites of the interaction zones between the γ and β-subunits [124]. In addition, *in silico* study on Chinese medicinal compounds eugenyl beta-D-glucopyranoside and 6-O-cinnamoyl-D-glucopyranose activates AMPK similar to AMP which is the true modulator of AMPK [125]. Overall, the molecular docking results confirmed that naringenin, naringin, and hesperetin could be positive modulators for AMPK activation.

The present findings suggest that naringin has better anti-diabetic potential than naringenin and hesperetin. Glycosides are known to be more biologically active than the respective aglycone, as the bound sugar moiety influence their bioavailability [96]. Attaching the glycosidic moiety increases its hydrophilicity which influences the pharmacokinetic properties of the compounds [126]. However, the glycoside linkages are less stable than most glucuronide linkages and might not withstand the acidic environment in the stomach, and glycosidase from intestinal bacteria may cleave sugar residues which will generate the agylycone naringenin [97]. Therefore, the absorption of naringin inside the body is remained to be clarified further [127]. However, naringin being a glycoside shows higher anti-diabetic potential than its aglycone naringenin in HepG2 cells.

In conclusion, our findings demonstrated that the glucose uptake by the flavonoids was depend on phosphorylation of AMPK at Thr172 which will enhances the glucose uptake via increased phosphorylation of GSK3β. In addition, molecular docking results depicted that both naringenin and naringin was potential AMPK activators with high binding affinities to γ-subunit of AMPK. Overall results suggest that naringenin, naringin, and hesperetin could be positive modulators for AMPK activation which enhance the glucose uptake regardless of insulin stimulation in high glucose treated HepG2 cells (Fig. 18). However, further efforts are needed to define the precise anti-diabetic role of naringenin, naringin, and hesperetin underlying the AMPK pathway in HepG2 cells.

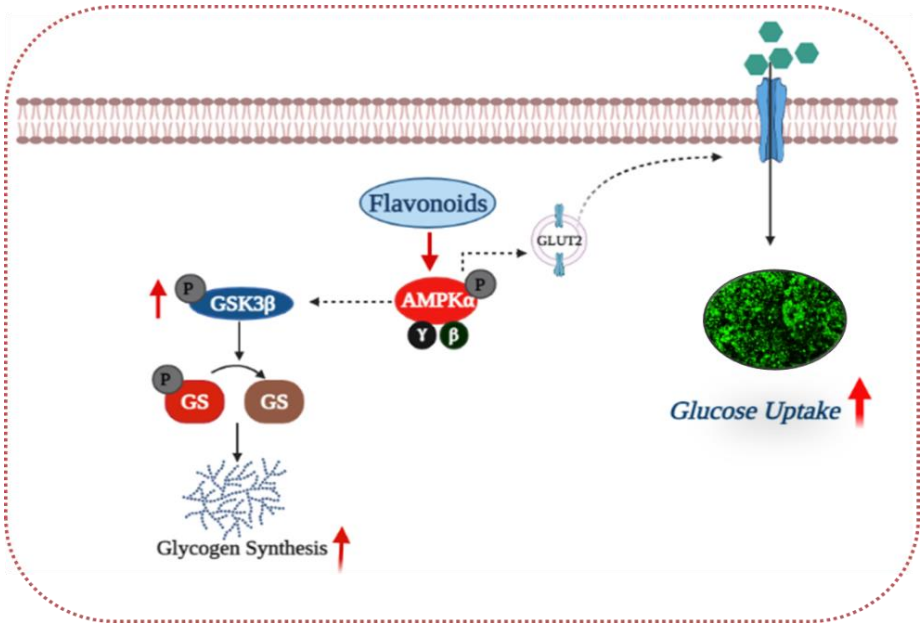


Figure 18. Proposed model for Anti-diabetic effect of flavonoids

General Conclusion

The research evidences suggest that three flavonoids naringenin, naringin, and hesperetin exhibit anti-adipogenic effect via activating the AMPK pathway while normalizing the genes related to lipid metabolism which was up- or down- regulated in 3T3-L1 adipocytes compared to normal cells.

Further, the anti-diabetic study in HepG2 cells exhibited that naringenin, naringin, and hesperetin could be positive modulators for AMPK activation which enhance glucose uptake regardless of insulin stimulation in high glucose treated HepG2 cells.

- **Flavonoids exert Anti-adipogenic effect via activation of AMPK pathway and via normalizing the genes related to lipid metabolism.**

Anti-adipogenic study revealed that naringenin, naringin, and hesperetin reduced lipid accumulation and TG content, and increased glucose uptake by activating AMPK pathway in differentiated 3T3-L1 adipocytes. In particular, differential gene expression analysis revealed that naringenin normalized the genes related to lipid metabolism including *Acaca, Fasn, Scd1, Mogat1, Dgat, Lipin1, Cpt1a,* and *Lepr* to the control level. In addition, naringin normalized the genes related to lipid metabolism including *Acaca, Fasn, Fabp5, Scd1, Srebf1, Hmgcs1, Cpt1c, Lepr,* and *Lrp1* genes. Hesperetin normalized the genes related to lipid metabolism including *Acaca, Elovl3, Mogat2, Fabp5, Lipin1,* and *Pnpla3* in differentiated 3T3-L1 adipocytes. However, further efforts are needed to define the precise anti-adipogenic effect of these flavonoids focusing on these genes related lipid metabolism to underlie the mechanisms of these flavonoids on anti-adipogenesis. The results suggest that these flavonoids might be potent therapeutic targets for obesity.

- **Flavonoids showed potent anti-diabetic effect via being positive modulators for AMPK activation which enhance glucose uptake regardless of insulin stimulation.**

Anti-diabetic study revealed that naringenin, naringin, and hesperetin increase the glucose uptake by promoting the phosphorylation of AMPK at Thr172 and with increased phosphorylation of GSK3β regardless of insulin stimulation in high glucose treated HepG2 cells. Further, the molecular docking studies reveals that naringenin, naringin, and hesperetin were able to activate AMPK at molecular level with binding affinities; naringenin -8.0 kcal/mol, naringin -9.1 kcal/mol, and hesperetin -8.0 kcal/mol which were closer to that of true modulator (e.g. AMP -8.1 kcal/mol) of AMPK. Overall results suggest that the phosphorylation of AMPK at Thr172 by naringenin, naringin, and hesperetin might enhance glucose uptake regardless of insulin stimulation in high glucose treated HepG2 cells. However, further efforts are needed to define the precise anti-diabetic role of these flavonoids underlying the AMPK pathway in HepG2 cells. Overall results suggest that these flavonoids are promising therapeutic targets for diabetes.

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