Anti-obese effects of mulberry (Morus alba L.) root bark through the inhibition of digestive enzymes and 3T3-L1 adipocyte differentiation

Yong-Xiang Wu¹, You-Jeong Kim, Sha Li¹, Myung-Chul Yun¹, Jin-Mi Yoon¹, Jin-Young Kim¹, Sung-II Cho¹, Kun-Ho Son², Taewan Kim¹*

¹Department of Food Science and Biotechnology, Andong National University, Andong 760-749, Korea
²Department of Food and Nutrition, Andong National University, Andong 760-749, Korea

Abstract

Anti-obese effects of mulberry (Morus alba L.) root bark was investigated in vitro by measuring its inhibitory effect against 3T3-L1 preadipocyte differentiation and digestive enzymes such as α-amylase, α-glucosidase and pancreatic lipase. Ethanol extract of mulberry root bark (MRE) showed the potent inhibitory activities on α-amylase, α-glucosidase and pancreatic lipase with IC₅₀ values of 7.86±0.36, 0.12±0.03 and 7.93±0.11 mg/mL, respectively. Furthermore, MRE significantly suppressed cellular lipid accumulation in 3T3-L1 cells in a dose-dependent manner. To elucidate the mechanism of MRE, we performed qRT-PCR and Western blotting for the expression of genes related with adipogenesis and lipogenesis. Treatment of MRE markedly suppressed the protein expression of PPARγ, C/EBPα and SREBP-1c, as well as FAS and ACC, which are the key transcription factors and metabolic enzymes in adipogenesis and lipogenesis. In addition, qRT-PCR analysis indicated that the anti-adipogenesis effect of MRE might be due to its inhibition at transcription levels. These results demonstrate that MRE can effectively suppress adipocyte differentiation and inhibit key enzymes related to obesity. Our findings suggest that mulberry root bark may have a potential benefit in preventing obesity.

Key words: mulberry root bark, adipogenesis, 3T3-L1 preadipocyte, obesity

Introduction

Obesity has become a serious global health problem in recent decades. Obesity can lead to many chronic diseases such as dyslipidemia, type 2 diabetes mellitus (T2DM), cardiovascular diseases, cancer and hypertension (1-5). Among them, T2DM is the most closely associated with obesity and they seem to share common causative factors, chemical abnormalities and clinical complications (6). Obesity is mainly caused by excessive food calorie intake and an abnormal accumulation of body fat (7). Consumption of a high-fat diet is a major risk factor that increases the chances of developing obesity and T2DM. Inhibition of digestive enzymes such as α-amylase, α-glucosidase and pancreatic lipase can decrease carbohydrate digestion and retard fat absorption, and therefore may be one of the most effective approaches to controlling obesity and related metabolic disorders.

Adipocyte, also known as fat cell, is associated with lipid homeostasis and energy balance (8). Therefore it has been widely used as an effective in vitro model for the evaluation of adipocyte differentiation. Adipocyte differentiation is the
process by which undifferentiated preadipocytes are converted to differentiated adipocyte. Adipocyte differentiation is regulated by two key transcription factors, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) (9). C/EBPα and PPARγ are activated during the differentiation process and promote together the expression of downstream adipose specific genes involved in adipose phenotype as well as in glucose and lipid metabolism (10, 11). Sterol regulatory element binding protein-1c (SREBP-1c) is a well-known upstream regulator of PPARγ in the adipogenesis pathway (12). Moreover, SREBP-1c could regulate the genes involved in lipogenesis such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) (13). Thus, controlling adipocyte differentiation has become a major target to prevent and treat obesity and related metabolic disorders.

The medicinal use of the root bark of mulberry has been documented in the Pharmacopoeia of People’s Republic of China and British Herbal Pharmacopoeia for a long history. Recently, it has been primarily used as antioxidant (14–15), antiasthmatic (16), antidepressant (17) and skin whitening ingredients (14). Besides, it was reported to have anti-inflammatory and antiviral effects in vitro (18, 19) and a hypolipidemic activity in vivo (15, 20, 21). Moreover, plants of this genus were known to be rich in flavonoids and prenylated flavonoids. The prenylated flavonoids such as morusin, kuwanon C, sanggenon D and kuwanon G isolated from mulberry root bark were reported to possess antioxidant, antimicrobial and anti-inflammatory activities (14, 22, 23). Recently, the phenolic compounds including maclurin, rutin, isoquercitrin, resveratrol and morin isolated from mulberry root bark showed potential antioxidant and whitening activities (24). It was reported that moracin M has a hypoglycemic effect on alloxan-diabetic mice (21). Additionally, morusabalanol A, a Diels-Alder type adduct with a new carbon skeleton, significantly attenuated the H2O2-induced cell damage and could be used as an euro-protective agent (25).

However, to date, there have been no previous studies on the anti-adipogenic activity of mulberry root bark using 3T3-L1 cells and on the investigation of its molecular mechanism.

This study was aimed to investigate the inhibitory effects of mulberry root bark on digestive enzymes and 3T3-L1 preadipocytes differentiation. MRE was further evaluated for the potential mechanism underlying the regulation of cell differentiation, in which the gene expression patterns involved in adipogenesis and lipogenesis of 3T3-L1 adipocyte cells were analyzed.

**Materials and methods**

**Materials**

3T3-L1 cells were purchased from American Type Culture Collection (ATCC). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), calf bovine serum (Cals), phosphate-buffered saline (PBS, pH 7.4) and other tissue culture reagents were purchased from PAA Laboratories GmbH (Pasching, Austria). Type II crude porcine pancreatic lipase, p-nitrophenyl palmitate (PNP), a-amylase from porcine pancreas Type VI-A, a-glucosidase from saccharomyces cerevisiae, p-nitrophenyl-a-D-glucopyranoside (pNPG) and all other chemicals were bought from Sigma-Aldrich Co (St. Louis, MO, USA). Antibodies for target proteins (PPARγ, C/EBPα, SREBP-1c, FAS, ACC and β-actin) were acquired from Cell Signaling Technology (Beverly, MA, USA). For qRT-PCR test, the TRizol Reagent was obtained from Invitrogen (Carlsbad, CA), PrimerScript™ 1st Strand cDNA Synthesis Kit from TaKaRa Bio Inc. (Otsu, Japan) and SYBR Green from PhileKorea Technology (Daejeon, Korea).

**Preparation of mulberry root bark extracts**

Dried root barks of mulberry were purchased from Omni Herb Company and extracted with 70% ethanol (MRE) and hot water (MRW). The extracts were filtered and evaporated under reduced pressure using a vacuum rotary evaporator. The extracts were then freeze-dried and stored at -70°C until used. The extraction yield obtained from MRE and MRW were 16.71% and 17.23% (w/w), respectively.

**Digestive enzymes inhibitory assays**

The a-amylase inhibitory activities of samples (MRE and MRW) were measured according to the method described by Ali et al. (26) and their a-glucosidase inhibitory activities were determined based on the method of Kim et al. (27). Pancreatic lipase inhibitory activities were determined according to the method of Kim et al. (28). In this study, acarbose and orlistat were used as positive controls. The percent inhibition of digestive enzymes was calculated as follows: Inhibition (%)=[(OD\text{control}-OD\text{sample})/OD\text{control}]×100. The IC50 values were calculate by linear regression analysis.

**Cell culture and adipocyte differentiation**

3T3-L1 cells were cultured in T-flask with Dulbecco’s modified Eagle’s medium (DMEM) containing 10% Calf and 10 μg/mL of penicillin/streptomycin at 37°C in 5% CO2 atmosphere. Then, cells were subcultured after cells grown
to more than 80-90% confluence. Two days after confluence (day 0), cells were stimulated to differentiate with differentiation medium containing DMEM with 10% FBS, 0.5 mM 3-isobutyl-1-methyl-xanthine, 5 μg/mL insulin and 1 μM dexamethasone for 2 days (day 2). Cells were then maintained in DMEM supplemented with 10% FBS and 5 μg/mL insulin for another 2 days (day 4), followed by culturing with DMEM with 10% FBS for an additional 4 days (day 8). Test samples were added in medium at various concentrations throughout the whole culture period (day 0-8).

**Cell viability assay**

The cytotoxicity of the sample was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method (29). Briefly, the cells were seeded at 10³ cells/well onto flat bottomed 96-well culture plates and treated with test samples at various concentrations. After incubation for 48 hr, the cells were incubated with MTT solution (5 mg/mL) at 37°C for 3 hr. The MTT-containing media was removed and dimethyl sulfoxide was added to dissolve formazan precipitates, then the optical density was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Oil red O staining**

Cells were washed three times with phosphate buffered saline (PBS) and fixed for 30 min with 4% formaldehyde. The fixed cells were washed three times with distilled water. Oil red O (0.5% in 60% isopropanol) was diluted with water (3:2), filtered through Whatman paper and incubated with the fixed cells for 1 hr at room temperature. Plates were rinsed three times with distilled water and the stained fat droplets in the adipocytes were visualized by inverted microscopy and photographed. Spectrophotometric analysis of the stain was performed by dissolving the stained lipid droplets with isopropanol and measuring at 520 nm.

**Western blotting analysis**

Extracted and quantified proteins (30 μg) from 3T3-L1 cells were separated with 10% SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked in 5% skim milk containing TBST (Tris-buffered saline/Tween) for 3 hr. The membranes were probed overnight with primary antibodies (PPARγ, C/EBPα, SREBP-1c, FAS and ACC). After being washed with TBST buffer, membranes were incubated with secondary antibodies for 1 hr. The expression levels of PPARγ, C/EBPα, SREBP-1c, FAS and ACC were determined using SuperSignal West Pico chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with monoclonal mouse β-actin antibody was performed for comparative control.

**RNA extraction and qRT-PCR**

Confluent cultures of 3T3-L1 cells in 60 mm dish were induced as previously described. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. After cDNA was prepared from isolated RNA by using PrimerScript™ 1st Strand cDNA Synthesis Kit (TaKaRa), the cDNA was used as a template for real-time PCR with the illumine ECO™ qPCR system (PhileKorea Technology, Daejeon, Korea). The specific primers are given in Table 1. The mRNA level was normalized using β-actin as an internal control. Analysis was carried out in triplicates.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward (F); Reverse (R) primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>F: 5'-CTG CTA CGT GAC TCA TCT TG-3' R: 5'-GAG AGG TCC ACA GAG CTG ATT-3'</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>F: 5'-GCC TCA CAC AGT TCC TG-3' R: 5'-TGG CCT TCT CCA GTC-3'</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>F: 5'-GGG GAC ATC GGA AAC AAG CTG A3' R: 5'-CAG ACT GCA GGC CAG ATC CA3'</td>
</tr>
<tr>
<td>FAS</td>
<td>F: 5'-ATG CGA CGG GAA GAC CAC GTG CT-3' R: 5'-AGA GAC GTC CTA CTC GCT CGC CT-3'</td>
</tr>
<tr>
<td>ACC</td>
<td>F: 5'-GAG TGA CTG CGG AAA CAT CTC TG-3' R: 5'-GTC CCT TCC TGA CAA ACC AGT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: 5'-GGG GCA CCT GAC AGA CTA CCT CA-3' R: 5'-GTT GCC ATT AGT GAC GAT C3'</td>
</tr>
</tbody>
</table>

**Statistical analysis**

All values were expressed in terms of mean±standard deviation (SD) of at least three independent experiments, tested by analysis of variance (ANOVA) with Duncan’s multiple range tests. Differences were considered to be statistically significant when the p-value was less than 0.05.

**Results and Discussion**

**Effects of mulberry root bark extracts on α-amylase and α-glucosidase activities**

α-amylase and α-glucosidase, the two important starch-hydrolyzing enzymes, are essential during digestion of carbohydrate. Inhibitors of these enzymes can decrease
carbohydrate digestion and increase overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (30,31). In addition, inhibition of those enzymes may reduce excessive food caloric intake and retard fat accumulation. Therefore, development of α-amylase and α-glucosidase inhibitors may be one of the most effective approaches to controlling obesity and T2DM. As shown in Table 2, MRE showed dose-dependent inhibition on α-amylase activity with IC₅₀ value of 7.86±0.36 mg/mL. MRW also exhibited some α-amylase inhibitory activity with IC₅₀ value of 20.10±0.86 mg/mL. Acarbose was used as a positive control in this study, with IC₅₀ value of 0.09 mg/mL. In addition, both MRE and MRW had appreciable inhibitory activity against α-glucosidase with IC₅₀ values of 0.12±0.03 mg/mL and 1.15±0.02 mg/mL, respectively (Table 3). Especially, MRE inhibited α-glucosidase activity much stronger than positive control acarbose (IC₅₀ value was 0.49±0.05 mg/mL). These results indicate that mulberry root bark extracts could be utilized as natural sources for potential anti-obesity and anti-diabetic application through inhibition of α-amylase and α-glucosidase activities.

**Effect of mulberry root bark extracts on pancreatic lipase activity**

Pancreatic lipase, also known as pancreatic triacylglycerol lipase, is very important in hydrolysis of triacylglycerols (32). Pancreatic lipase inhibition is a valuable pathway to retard fat absorption and therefore attenuates obesity (33). The pancreatic lipase inhibitory activity of ethanol and water extracts from mulberry root bark is shown in Table 4. MRE exhibited pancreatic lipase inhibitory activity, with IC₅₀ value of 7.93±0.11 mg/mL. And the IC₅₀ value of MRW was 75.13±0.81 mg/mL. The reference compound of atorvastatin was used in this study, with IC₅₀ value of 0.01 mg/mL. Several studies also suggest that the extract of plants such as tea, soybean and ginseng inhibit pancreatic lipase activity (31,33). These results demonstrate that mulberry root bark has potential as a pancreatic lipase inhibitor.

**Table 3. Effect of mulberry root bark extracts on α-glucosidase activity**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Concentration (mg/mL)</th>
<th>α-glucosidase inhibitory activity (%)</th>
<th>IC₅₀ (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRE</td>
<td>0.1</td>
<td>40.2±3.46</td>
<td>0.12±0.03</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>81.55±3.54</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>98.03±0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>99.39±0.07</td>
<td></td>
</tr>
<tr>
<td>MRW</td>
<td>0.1</td>
<td>11.99±4.50</td>
<td>1.15±0.02</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>22.15±2.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>33.05±0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>43.09±4.33</td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>-</td>
<td>-</td>
<td>0.49±0.05</td>
</tr>
</tbody>
</table>

IC₅₀: Concentration required for 50% inhibition of α-glucosidase activity.

**Table 4. Effect of mulberry root bark extracts on pancreatic lipase activity**

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Concentration (mg/mL)</th>
<th>Lipase inhibitory activity (%)</th>
<th>IC₅₀ (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRE</td>
<td>1</td>
<td>22.95±0.99^d</td>
<td>7.93±0.11</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>38.6±3.43^d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>57.72±1.30^d</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>68.16±2.01^d</td>
<td></td>
</tr>
<tr>
<td>MRW</td>
<td>1</td>
<td>ND^b</td>
<td>75.13±0.81</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>ND^b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5.54±3.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10.76±3.39</td>
<td></td>
</tr>
<tr>
<td>Acarbose</td>
<td>-</td>
<td>-</td>
<td>0.01</td>
</tr>
</tbody>
</table>

IC₅₀: Concentration required for 50% inhibition of pancreatic lipase activity.

ND: Not detected.

**Effect of mulberry root bark extracts on adipogenic differentiation in 3T3-L1 cells**

Many current understandings of adipogenesis are based on 3T3-L1 cells. 3T3-L1 preadipocytes cells have been served as a well-established in vitro model to search for new health benefit food or agents for the obesity and related metabolic...
Anti-obese effects of mulberry (*Morus alba* L.) root bark

(A) Cell viability (% of control)

![Graph showing cell viability](image)

(B) Relative fat accumulation (% of MDI)

![Graph showing relative fat accumulation](image)

(C) Lipid accumulation was quantified by measuring the absorbance at 520 nm. Data are expressed in terms of mean ± standard deviation (SD) of at least three independent experiments, tested by analysis of variance (ANOVA) with Duncan’s multiple range tests. Differences were considered statistically different at p<0.05.

**Fig. 1.** Effect of mulberry root bark extracts on adipogenic differentiation in 3T3-L1 cells.

(A) Cell viability of 3T3-L1 cells exposed to increasing concentration of MRW and MRE. Cells were treated with different concentration of MRW and MRE for 48 h and cell viability was analyzed using an MTT assay. (B) 3T3-L1 preadipocytes were induced to differentiate into adipocytes by the MDI [0.5 mM 1-isobutyl-3-methylxanthine, 1 μM dexamethasone, and 5 μg/mL insulin]. Following 8-d differentiation, differentiated adipocytes were fixed and stained with Oil Red O in order to visualize lipid droplets.

(C) Lipid accumulation was quantified by measuring the absorbance at 520 nm. Data are expressed in terms of mean ± standard deviation (SD) of at least three independent experiments, tested by analysis of variance (ANOVA) with Duncan’s multiple range tests. Differences were considered statistically different at p<0.05.
disorders in numerous studies (34). In this study, first of all, the effects of mulberry root bark extracts (MRE and MRW) on cell viability and the differentiation of 3T3-L1 cells were evaluated by MTT assay and Oil Red O staining assay. As evidenced by the MTT assay, no significant cytotoxicity was observed at concentrations up to 400 μg/mL, as compared with the non-treated control (Fig. 1A). Treatment of 3T3-L1 cells with MRE decreased adipocyte differentiation, in a dose-dependent manner, as indicated by a decrease in Oil Red O incorporation (Fig. 1C). The lipid accumulations were significantly decreased by 12.52% and 22.41% (p<0.05) at 100 μg/mL and 200 μg/mL of MRE, respectively (Fig. 1B). However, the inhibition of relative fat accumulation was not detectable in MRW. Many plant extracts and their derivatives showed the potent inhibitory activities on adipocyte differentiation and have been used as anti-obesity dietary supplements (35). These results indicate that mulberry root bark extract can efficiently block adipocyte differentiation and may have a potential benefit in preventing obesity.

Effects of MRE on adipogenic and lipogenic gene expressions in 3T3-L1 cells

To gain a better understanding of the molecular mechanisms underlying the anti-adipogenic effect, we conducted qRT-PCR and Western blotting analysis to examine the effect of MRE on the expression of key transcriptional factors. PPARγ and C/EBPα are necessary and should be sufficient for adipogenesis in vivo and in vitro (10,11). We observed that PPARγ and C/EBPα were strongly inhibited by MRE at the transcriptional levels (Fig. 2). The mRNA level of PPARγ was reduced up to 70.97% and that of C/EBPα mRNA was decreased by up to 55.09%, respectively. Consistent with the mRNA expression, the protein expression levels of transcription factors (PPARγ and C/EBPα) showed a similar alteration pattern (p<0.05, Fig. 3). We also determined the gene expression of SREBP-1c, a key transcription regulator which is well-known upstream regulators of PPARγ in the adipogenesis pathway (12). It showed that the SREBP-1c mRNA was suppressed significantly by MRE (Fig. 2). Furthermore, treatment of MRE induced down-regulation of SREBP-1c protein expression (p<0.05, Fig. 3). These results indicate that MRE suppresses SREBP-1c as the upstream regulator of PPARγ at the transcriptional level. However, the deep investigation of molecular mechanism of MRE on PPARγ upstream signaling pathway need to be studied further. Consequently, these observations suggest that MRE inhibits adipogenesis during adipocyte differentiation.

FAS and ACC are the two key enzymes in lipogenesis, a fact that renders they are important targets of anti-obesity research (36). Therefore, we further studied whether MRE regulated the genes expression of FAS and ACC. Treatment with MRE inhibited the transcription of FAS in a dose-dependent manner (p<0.05, Fig. 2). It also induced down-regulation of ACC transcription compared with fully differentiated adipocytes. Moreover, the protein level of FAS was fallen dramatically up to 97.14% (p<0.05, Fig. 3). ACC protein expression was decreased by up to 57.08% upon MRE addition (p<0.05, Fig. 3). These results indicate that MRE inhibits lipogenesis during adipocyte differentiation.

Conclusion

Our investigations indicate that mulberry root bark is an effective inhibitor of α-amylase, α-glucosidase and pancreatic lipase. In addition, MRE has potential of inhibiting adipogenic differentiation in 3T3-L1 cells. These effects of MRE may work on multiple molecular targets and complex mechanisms modulating the expression of transcription factors such as PPARγ, C/EBPα and SREBP-1c, and suppressing the expression of lipogenesis related proteins such as FAS and ACC. Consequently, based on these findings, we suggest that mulberry root bark can be utilized as a functional resource having pharmacological and health-promoting effects for anti-obesity. Additional studies of active compounds responsible for the anti-obese effect of MRE are currently underway.
Fig. 3.Suppressive effects of MRE on adipogenesis and lipogenesis protein expression in 3T3-L1 cells.

Western blotting was performed using 30 μg of each sample. The loading control was assessed using β-actin antibody. The relative intensities of PPARγ, C/EBPα, SREBP-1c, FAS, ACC expression compared with the β-actin expression were determined using Quantity One software. Data are expressed in terms of mean±standard deviation (SD) of at least three independent experiments, tested by analysis of variance (ANOVA) with Duncan's multiple range tests. Differences were considered statistically different at p<0.05.

요 약
본 연구에서는 상백피의 소화효소 저해활성과 3T3-L1 전지방세포의 분화 억제능을 기반으로 항비만 효능소재로 (MRE)는 α-amylase와 α-glucosidase, pancreatic lipase를 활용한 소화효소 저해활성 평가 실험에서 각각 7.86±0.36, 0.12±0.03, 7.93±0.11 mg/mL의 IC50 값을 보이며 우수한 악제 활성을 나타냈다. 또한 3T3-L1 전지방세포를 활용한
Acknowledgement

This research was partially supported by the ministry of trade, industry and energy through the R&D supporting program for reginal industry (R0003475).

References


20. El-Beshbishy HA, Singab ANB, Sinkkonen J, Pihlaja