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Inhibition of Adenovirus 36 Replication and Lipid Accumulation by *Distylium racemosum*

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Abstract : Obesity is a worldwide disease and one of the major risk factors. Virus among many factors can lead to obesity. Adenovirus 36 (Ad-36) is the adipogenic virus linked with human obesity. Nevertheless, there is no drug to treat both Ad-36 infection and obesity associated with virus. For the precedent study on anti-cholesterol test, *Distylium racemosum* (*D. racemosum*), *Quercus salicina* (*Q. salicina*) and *Raphiolepis indica* (*R. indica*) were selected. This study was carried out to evaluate the anti-cholesterol effects, anti-lipid effects and inhibition of Ad-36 replication from three extracts. *D. racemosum* (50 μg/mL) inhibited lipid accumulation on 3T3-L1 adipocyte. *D. racemosum* inhibited adipocyte differentiation through suppression of regulator peroxisome proliferator-activated receptor-γ (PPARγ) genes and adipocyte-specific genes such as adipocyte protein 2 (aP2). *D. racemosum* inhibited replication of Ad-36 at 50 μg/mL of concentration. Therefore, the extract of *D. racemosum* could be a candidate for development of anti-Ad-36 and anti-obesity drugs.

Keywords : Distylium racemosum, Adenovirus 36, Anti-lipid, Anti-cholesterol, Anti-obesity

1. Introduction

Obesity is defined as having an abnormal or excessive amount of body fat accumulation and is one of the major risk factors for chronic diseases including diabetes, cardiovascular disease and cancer [1]. At least 2.8 million of the world popularity are dying due to obesity and it has reached epidemic proportions worldwide [2]. Therefore, obesity is the most serious public health problem declared from the World Health Organization (WHO) [3].

Obesity is occurred by many causes. Recent studies have been made actively about correlation between virus infection and obesity. Viruses such as canine distemper virus, Rous-associated virus 7, Borna virus and SMAM-1 cause obesity in animals, and nearly every known Adenovirus 36 (Ad-36) is associated with obesity in humans [4]. Adenoviruses are contained of 50 different types and causes acute upper respiratory infections. Ad-36 was first isolated from the

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slurry of a six year old girl affected by diabetes and enteritis in 1978 [5]. Eleven percent of non-obese people of American adults, 30% of obese people of American adults, and 30% of obese children of South Korea were reported that they were infected with Ad-36 [6]. Fat cells infected with Ad-36 have been known for the differentiation to more fat cells and more lipid accumulation in cells. When E4orf1 gene of Ad-36 was inserted into preadipocyte, adipogenic transcription factors were stimulated [7]. Group infected with Ad-36 has shown significantly low serum cholesterol and triglyceride levels compared with the control group [8].

Obesity is generated by adipocyte differentiation and fat accumulation. Adipocyte differentiation is controlled by specific gene expression [9]. Major specific genes associated with fat accumulation are peroxisome proliferator-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein-α (C/EBPα). Especially, adipogenesis are mainly controlled by the PPARγ, belonged to nuclear receptor superfamily [10, 11]. PPARγ induces the expression fatty acid binding protein such as adipocyte protein 2 (aP2) as well as many other genes [12].

Jeju Island of the Korea has a valuable ecosystem, and is known for the variety and richness of plants and over 7800 species of them have been classified to date [13]. Therefore, natural extracts from Jeju Island were tested by anti-cholesterol screening test. Three natural extracts among them were selected as cholesterol inhibitors. The extracts are extracted from the leaves of *Distylium racemosum* (*D. racemosum*), *Quercus salicina* (*Q. salicina*) and *Raphiolepis indica* (*R. indica*) which are ones of oriental medical plants grown wildly in Jeju. *D. racemosum* is known for anti-oxidant effects [14]. *Q. salicina* is known for anti-inflammatory, anti-edemic, diuretic and litholytic activities [15]. *R. indica* has been a decorative form of art. However, scientific data on biological and physiological effects of those are insufficient.

This study investigated and compared anti-cholesterol effects of extracts from *D. racemosum, Q. salicina* and *R. indica.* Quercetin was used as single component and positive control. Quercetin is well known as scavenge superoxide radicals and protect from lipid peroxidation [16]. The effect of extracts on the adipocyte differentiation of 3T3-L1 cells were investigated by measuring lipid accumulation and the expression major adipogenic transcriptional factor such as PPAR γ and adipocyte-specific genes such as aP2. Moreover, Ad-36 replication in A549 cell line treated with the extracts was examined by comparing Ad-36 hexon DNA expression. Inhibition of Ad-36 by medicinal herbs having anti-cholesterol and anti-lipid effects, might be used for drug development to prevent obesity and to treat viruses.

2. Materials and Methods

2.1. Extraction of medicinal plants

Ethanol extraction approach is employed to extract medical plant materials which are five hundred forty eight species of medical plants from Jeju, Korea. The fresh leaves of them were collected, washed with tap water, air dried, homogenized to a fine powder and stored in air-tight containers. The air dried powder (100 g) was extracted with ethanol (40-60° C) in a extractor for 18-20 hours and solution was evaporated to dryness under reduced pressure and controlled temperature by using rotary evaporator. The extract was stored in a refrigerator at 4℃ in air-tight bottles until further use. The concentration of extracts was prepared to be 10 mg/mL in ethanol and used. Quercetin was used as positive control. The extracts were tested by anti-cholesterol screening test.

2.2. Cholesterol adsorptivity

Cholesterol adsorptivity was measured by total cholesterol kit (Asan-Pharm, Seoul, Korea). Briefly, 100 μL of different concentration of nature extracts ranging from 0.1 to 10 mg/mL were mixed with 1.8 mg/mL cholesterol (Sigma-Aldrich, St. Louis, MO, USA) in Ethanol, and reacted at 20℃ for 20 minutes. 50 μL of 0.1 M hexadecyl-trimethylammonium bromide (Sigma-Aldrich, St. Louis, MO, USA) was added and centrifuged at 4° C for 10 minutes at 15,000 rpm. After supernatant was collected, it was reacted with enzyme solution at 37° C for 5 minutes. Observance density (OD) was measured at 500 nm using Spectronic Genesys 5 (Milton Roy Company, New York, USA).

2.3. Cell viability assays

3T3-L1 preadipocyte cells (Korean Cell Line Bank, Seoul, Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose containing a 10% bovine calf serum (BCS), 1% penicillin and streptomycin (PS) for proliferation at 37° C and 5% CO_2. Human epithelial (A549) cells (Korean Cell Line Bank, Seoul, Korea) were cultured in DMEM high glucose a 10% fetal bovine serum (FBS), 1% non essential amino acids, 1% PS. After cell was counted, it was diluted to 1×10^5cells/mL. Then, it was pipetted to 96 well plate each 100 μL. It was incubated for one night. The extracts from natural materials were diluted 2, 10, 50, 100 and 200 μg/mL concentrations, it was pipetted each 100 μL. It was shaked at 150 rpm for 5 minutes and incubated at 37° C and 5% CO_2 for 3 days. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra zoliumbromide (MTT) solution (Ambresco, Ohio, USA) concentrated on 5 mg/mL in phosphate-buffered saline (PBS) was pipetted each 20 μL. After shaked at 150 rpm for 5 minutes, it was incubated for 2 hours. Medium were deleted completely. dimethyl sulfoxide (Ambresco, Ohio, USA) were pipetted each 200 μL and it was shaked at 150 rpm for 5 minutes. OD was measured at 560 nm using ELISA reader (Amersham Life Science, Buckinghamshire, UK).

2.4. Differentiation of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were grown in DMEM high glucose with 10% BCS and 1% PS. At 2 day post confluence, cell differentiation was induced with 10% FBS, 10 μg/mL insulin (INS), 1 μM dexamethasone (DEX), 0.5 μM isobutylmethylxanthine (IBMX) and 1% PS. After 3 days, cells were then maintained in DMEM high glucose with 10% FBS, 10 μg/mL INS and 1% PS for 4 additional days. This medium was changed every 2 days. The adipocytes were treated into the culture medium containing the extracts at 2 day. Cells were treated with 0, 2, 10 and 50 μg/mL of extracts. After treatment with extracts for 7 days, the 3T3-L1 adipocytes were staining and RT-PCR.

2.5. Oil Red O staining of 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated in culture media containing with various concentration of the extracts, and then fixed in 10% formaldehyde prepared for 1 hour. The cells were washed with distilled water (DW) 3 times, and were stained with the Oil red O (Sigma-Aldrich, St. Louis, MO, USA) solution. The cells were then washed in DW three times for 1 hour each time. 3T3-L1 adipocytes were eluted into isopropanol.

2.6. Reverse transcription-PCR (RT-PCR)

RNA was isolated by accuzol RNA extraction solution (Bioneer, Daejeon, Korea). Isolated RNA was reverse transcribed to cDNA for 60 minutes at 42° C and then 10 minutes at 72° C by RT-PCR. The primers were designed following as: GAPDH forward - GCT AGG ACT GGA TAA GCA GGG, GAPDH reverse - GAT GGG CTT CCC GTT

GAT GA, PPARγ forward-GTG AGA CCA ACA GCC TGA CG, PPARγ reverse - ACA GAC TCG GCA CTC AAT GG, aP2 forward - GAA ATC ACC GCA GAC GAC AG, aP2 reverse - AAC TCT TGT GGA AGT CAC GCC. The amplification cycles were 94° C for 1 minute, 56° C for 1 minute and 72° C for 1 minute. After 35 cycles, PCR products were separated by electrophoresis on 1.5% agarose gel for 25 minutes at 100 V. Gels were stained with ethidium bromide visualized by Alpha Innotech imaging system (San Leandro, CA, USA).

2.7. Inhibition of Adenovirus 36 replication

A549 cells were infected of adenovirus 36 for 2 hours. After infection, the cells were cultured in complete DMEM containing 10% FBS and 1% PS in an incubator with the condition of 37° C and 5% CO_2. After 48 hours, the infected cells were collected for further analyses. Genomic DNA was extracted using an *AccuPrep* Genomic DNA extraction Kit (Bioneer, Daejeon, Korea). The primers were designed following as: GAPDH forward primer- CCC ACC ACA CTG AAT CTC CC, GAPDH reverse primer - CTC ACC TTG ACA CAA GCC CA , Ad-36 hexon forward primer - ATG GCC AGC TAC TTT GA, Ad-36 hexon reverse primer - TGA GGT TCT GGC TGG AAA GT. The amplification cycles were 94° C for 1 minute, 56° C for 1 minute and 72° C for 1 minute. After 35 cycles, PCR products were separated by electrophoresis on 1.5% agarose gel for 25 minutes at 100 V. Gels were stained with ethidium bromide visualized by Alpha Innotech imaging system (San Leandro, CA, USA).

2.8. Statistical analysis

Statistical analysis of data was performed with ANOVA followed by *P*-values less than 0.05 were considered significant.

3. Results and Discussion

3.1. Cholesterol reduction effects of the natural extracts

The medical plants are *D. racemosum, Q. salicina* and *R. indica* which are ones of oriental medical plants grown wildly in Jeju. Cholesterol adsoptivity of the extracts (10 mg/mL) from *D. racemosum*, *Q. salicina, R. indica* and quercetin were 69%, 39%, 32% and 84%, respectively. Cholesterol adsorptivity of the extracts from *D. racemosum*, *Q. salicina* and *R. indica* were not very high compared to that of quercetin, the single component. Cholesterol adsoptivities by the all extracts were gradually increased depending on concentration. Cholesterol adsorptivity of the extracts from *D. racemosum* and quercetin was increased compared to those of *Q. salicina* and *R. indica* through dose-dependent increase. Cholesterol adsoptivity of the extract from *D. racemosum* was highest among the natural extracts (Fig. 1).

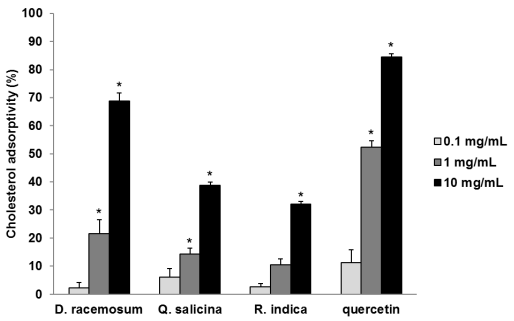

Fig. 1. Cholesterol adsorptivity of extracts from *D. racemosum, Q. salicina, R. indica* and quercetin. Cholesterol adsoptivity of *D. racemosum*, *Q. salicina, R. indica* and quercetin were 69, 39, 32 and 84%, respectively, at 10 mg/mL of concentration. quercetin as material and positive control material was physiologically active. *D. racemosum* among the natural extracts showed the highest cholesterol reduction effects ($p<0.05$).

3.2. Cell viability of the natural extracts on 3T3-L1 and A549 cells

Cell viability of the extracts was determined by MTT assay on 3T3-L1 cells and A549 cells. The cells were treated with various concentrations of *D. racemosum*, *Q. salicina*, *R. indica* and quercetin. The extracts of *D. racemosum* and *Q. salicina* showed cell toxicity over 100 μg/mL concentration on 3T3-L1 cells. The extracts of *R. indica* showed cell toxicity over 200 μg/mL concentration on 3T3-L1 cells. The concentration of quercetin to not affect cell viability is under 10 μg/mL concentration on 3T3-L1 cells (Fig. 2). Extracts of *D. racemosum* and quercetin showed cell toxicity over 100 μg/mL concentration on A549 cells. Extracts of *Q. salicina* and *R. indica* showed cell toxicity over 200 μg/mL concentration on A549 cells (Fig. 3).

3.3. Inhibitory effect on adipogenesis and lipid accumulation by the natural extracts

To examine the anti-lipid effects of *D. racemosum*, *Q salicina*, *R. indica* and quercetin, 3T3-L1 preadipocytes were treated with the extracts for 7 days, and stained with Oil red O. Adipogenesis and lipid accumulation in the cells was inhibited over 50% at 10 μg/mL quercetin and 50 μg/mL *D. racemosum*. Adipogenesis in the cells were

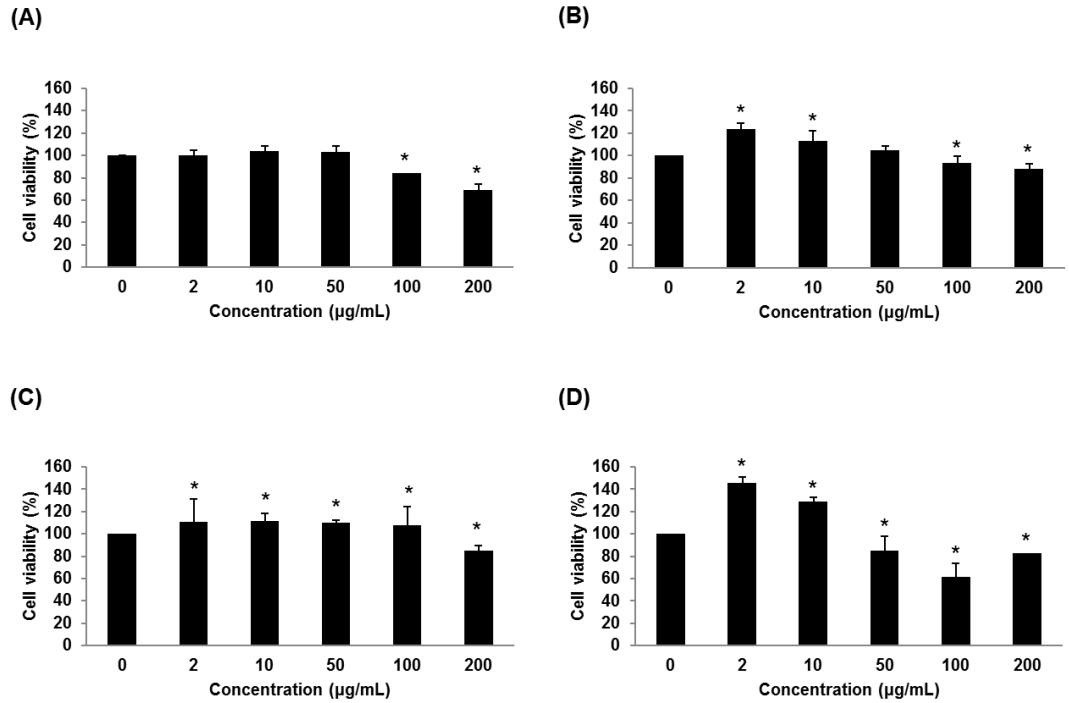

Fig. 2. Cell viability of natural extracts from *D. racemosum*, *Q. salicina*, *R. indica* and quercetin in 3T3-L1 cells. (A) *D. racemosum*, (B) *Q. salicina*, (C) *R. indica*, (D) quercetin. 3T3-L1 cells were treated with various concentrations of quercetin, *D. racemosum*, *Q. salicina* and *R. indica*. Cell viability was measured by the MTT assay after 72 hours. The concentration of the three natural extracts and quercetin to not affect cell viability is 50 μg/mL and 10 μg/mL, respectively. Therefore, the concentration is determined as maximum concentration in every experiment ($p<0.05$).

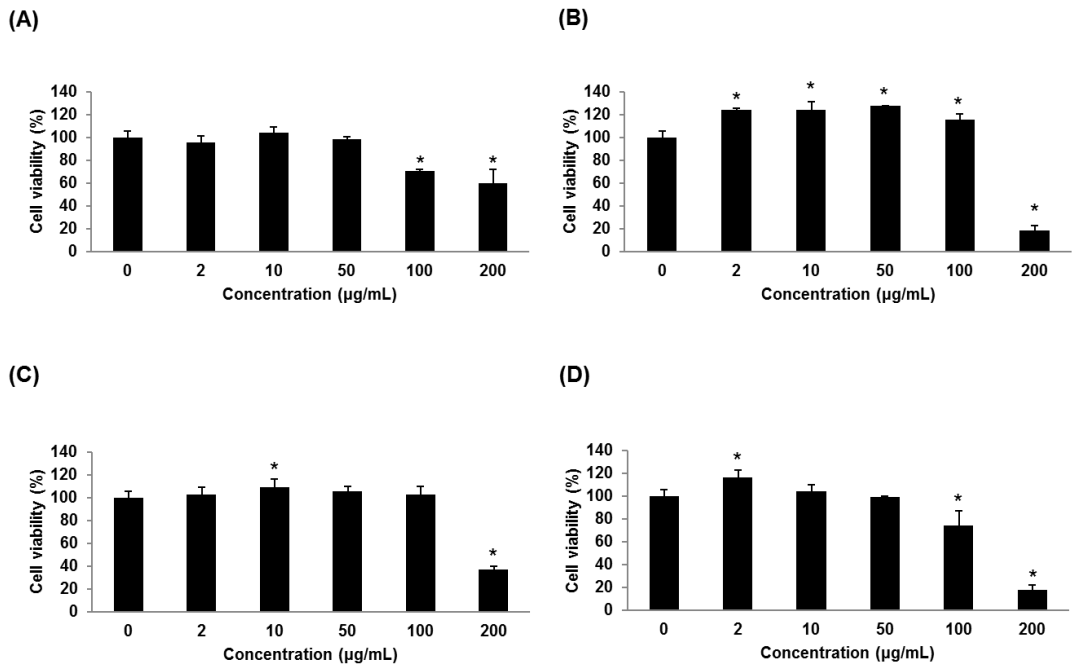

Fig. 3. Cell viability of natural extracts from *D. racemosum, Q. salicina, R. indica* and quercetin in A549 cells. (A) *D. racemosum*, (B) *Q. salicina*, (C) *R. indica,* (D) quercetin. A549 cells were treated with various concentrations of *D. racemosum, Q. salicina, R. indica* and quercetin. Cell viability was measured by MTT assay after 72 hours. The concentration of the three natural extracts and quercetin to not affect cell viability is 50 μg/mL. Therefore, the concentration is determined as maximum concentration in every experiment ($p<0.05$).

not inhibited by the extracts of both *Q. salicina* and *R. indica.* These results indicate that the extract from *D. racemosum* might be used effectively for development of anti-lipid drugs. The extracts from *Q. salicina* and *R. indica* showed no anti-lipid effects (Fig. 4).

3.4. Inhibition of adipogenic specific genes expression by the natural extracts

To examine whether the expression of adipogenic transcription factors is inhibited by the extracts from *D. racemosum*, *Q salicina, R. indica* and quercetin, 3T3-L1 preadipocytes were treated with various concentration of the extracts and incubated for 7 days. The expression of PPARγ gene was inhibited from 10 μg/mL quercetin and 50 μg/mL *D. racemosum.* The expression of aP2 gene was inhibited from 2 μg/mL quercetin and 50 μg/mL *D. racemosum.* PPARγ and aP2 genes were inhibited by the extract of *D. racemosum* and quercetin in dose-dependent manner. However, PPARγ and aP2 gene were not inhibited by the extracts of both *Q. salicina* and *R. indica. D. racemosum* inhibited adipocyte differentiation through suppression of regulator PPARγ genes and adipocyte-specific genes such as aP2 (Fig. 5). PPARγ was constitutes a expression of adipocyte-specific genes through a master regulator of adipocyte differentiation such as aP2 [17]. Kim *et al.* [18] showed that *Citrus aurantium* flavonoid suppressed adipogenesis by down regulation of

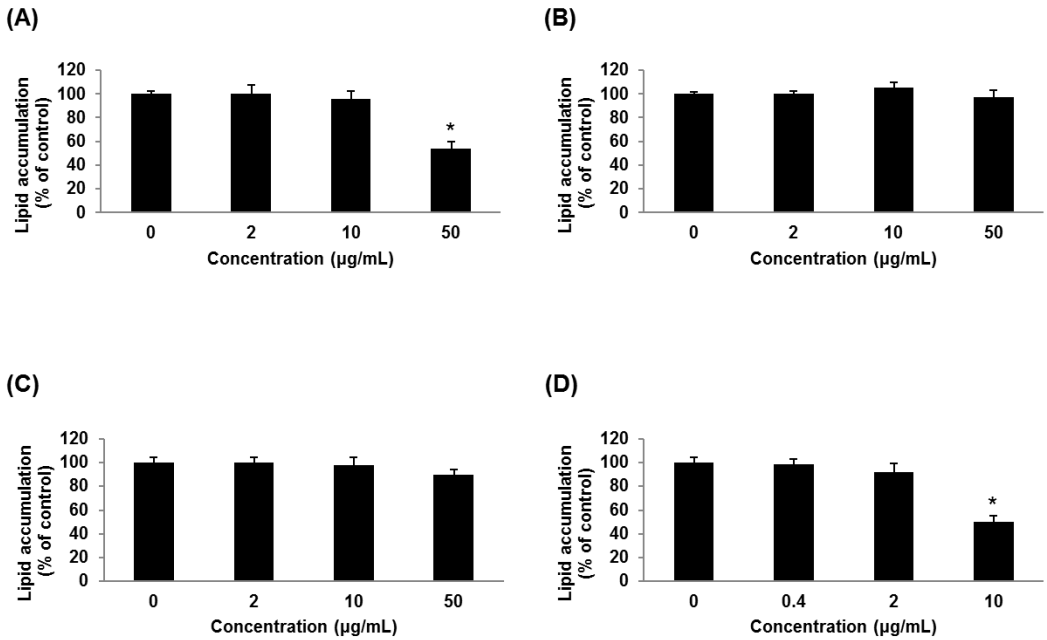

Fig. 4. Inhibitory effects of lipid accumulation by the natural extracts in 3T3-L1 cells. (A) *D. racemosum*, (B) *Q. salicina*, (C) *R. indica,* (D) quercetin. Isopropyl alcohol was used to elute the Oil-red O. Lipid accumulation was decreased in the cell treated with *D. racemosum* and quercetin, significantly ($p<0.05$).

PPARγ and C/EBPα that are relevant to lipid accumulation and lipid metabolism. Song *et al.* [9] showed that the anti-obesity effects of the extracts of blueberry peel on 3T3-L1.

3.5. Inhibitory effects of Ad-36 replication by the natural extracts

To examine whether Ad-36 replication is inhibited by the natural extracts, A549 cells were infected with Adenovirus 36 for 2 hours and were cultured for 48 hours in the cell culture media containing the natural extracts. After 48 hours, hexon gene of Adenovirus 36 and GAPDH as cell control gene was analyzed by RT-PCR. Hexon gene of Adenovirus 36 was inhibited in the cell treated with 10 μg/mL quercetin and 50 μg/mL *D. racemosum,* significantly. Hexon gene of Adenovirus 36 was inhibited by the extract of *D. racemosum* and quercetin in dose-dependent manner. However, Hexon gene of Adenovirus 36 was not inhibited by the extracts of both *Q. salicina* and *R. indica* (Fig. 6). Many factors and contributions lead to obesity, but researchers have been pondering the infection obesity for years. Human Ad-36 belonging to Adenovirus subgroup D was first isolated by fecal sample of a girl suffering from enteritis [19]. Dhurandhar *et al.* [20] reported that Ad-36 increased visceral fat, total fat and body weight. Ad-36 enhanced lipid accumulation in 3T3-L1 cells in the presence of adipogenic-inducer such as dexamethasone and insulin [21]. Na *et al.* [22] reported that the anti-obesity and anti-inflammation effects using mulberry extract on Ad-36 were evaluated in vivo.

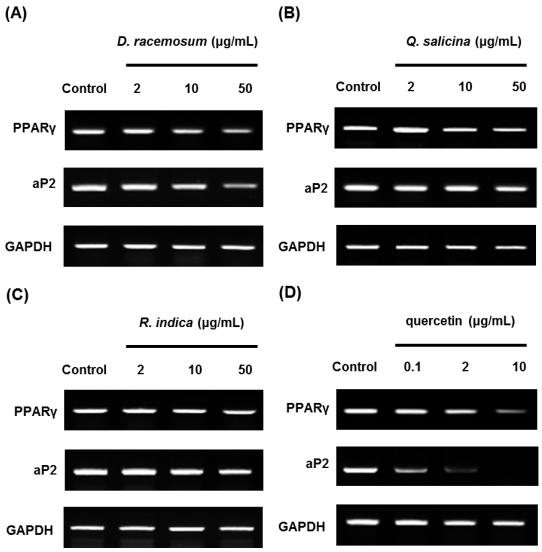

Fig. 5. Inhibitory effects of transcriptional factors and adipocyte-specific genes by the natural extracts. (A) *D. racemosum*, (B) *Q. salicina*, (C) *R. indica,* (D) quercetin. Confluent 3T3-L1 preadipocytes were differentiated into adipocytes in medium containing various concentrations of *D. racemosum*, *Q. salicina* and R. indica and quercetin for 7 days. On day 7, PPARγ and aP2 genes which are transcriptional factors and adipocyte- specific genes, and GAPDH as cell control gene was analyzed by RT-PCR. PPARγ and aP2 gene transcriptions were inhibited in the cell treated with *D. racemosum* and quercetin, significantly.

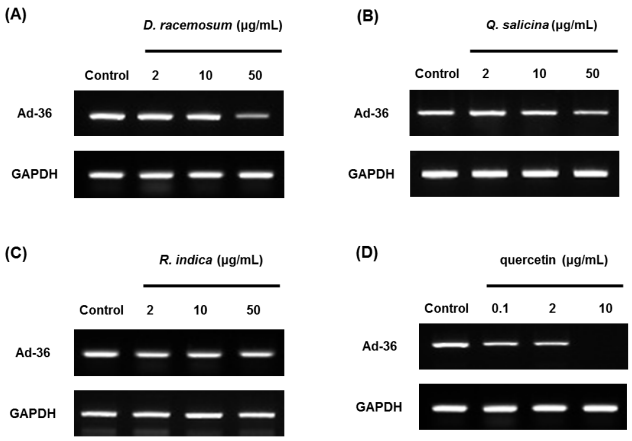

Fig. 6. Inhibitory effects of Adenovirus 36 replication by the natural extracts. (A) *D. racemosum*, (B) *Q. salicina*, (C) *R. indica,* (D) quercetin. A549 cells were infected with adenovirus 36 for 2 hours. After infection, the cells were cultured for 48 hours in the cell culture media containing the natural extracts. After 48 hours, hexon gene of Adenovirus 36 and GAPDH as cell control gene was analyzed by PCR. Hexon gene of Adenovirus 36 was inhibited in the cell treated with *D. racemosum* and quercetin, significantly.

4. Conclusion

This results revealed that the suppression of adipogenesis by *D. racemosum* was caused by expression inhibition of PPARγ and aP2 gene and replication inhibition of Ad-36. Therefore, the extract of *D. racemosum* might be one candidate for development of anti-Ad-36 and anti-obesity drugs.

Acknowledgements

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