Anti-oxidant and Anti-adipogenic Effects of Acorn (*Quercus acutissima* CARR.) Shell Extracts via Regulation of Wnt Signaling in 3T3-L1 Cells

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Abstract Acorn (*Quercus acutissima* CARR.) is a nut from the Fagaceae family that has been used in traditional medicine for many years. However, shells from acorns are regarded as a by-product and are mostly discarded. Anti-adipogenic activities of acorn shells were investigated using 3T3-L1 cells and methanol shell extracts (AE-M). AE-M demonstrated Cu²⁺-chelation activities and anti-oxidant activities via reduction of oxidative stress levels induced using AAPH. Six days after adipocyte differentiation, 50 and 100 µg/mL AE-M completely suppressed 3T3-L1 adipogenesis and the anti-adipogenic effect was stronger than for the positive control 50 µM quercetin. Treatment with AE-M in 3T3-L1 cells reduced mRNA expression levels of adipogenic genes. AE-M-inhibition was found in pre-adipogenic, early, and intermediate stages of adipogenesis in 3T3-L1 cells. The Wnt/β-catenin signaling pathway is required for AE-M-inhibition of 3T3-L1 adipogenesis.

Keywords: acorn shell, adipogenesis, anti-oxidant, obesity, wnt signaling

Introduction

Obesity, characterized as an accumulation of fat due to an increase in the size and number of adipocytes, has become a serious public health problem and an important worldwide issue (1). Obesity, which results from an imbalance between energy intake and expenditure, is a metabolic disorder commonly associated with hypertension, type 2 diabetes, and coronary heart disease (2). Thus, prevention of obesity can contribute to enhancement of the quality of human life.

Adipogenesis is defined as the differentiation process of preadipocytes into adipocytes. The process of adipogenesis is characteristically described as a cascade of gene expressions regulated by adipogenic transcriptional factors. As cells undergo adipogenesis in response to adipogenic signals, the initial event is rapid induction of C/EBPβ expression. Induction of C/EBPβ is immediately followed by increases in PPARγ and C/EBPα expressions (3,4). Adipogenesis is reportedly regulated by cell signaling pathways and endocrines that either stimulate or inhibit cell differentiation (5).

Well known factors that stimulate adipogenesis in pre-adipocytes include insulin signaling, PPARγ agonist, glucocorticoids, and cyclic adenosine monophosphate (cAMP) (5). In contrast, Wnt signaling,

epidermal growth factor, prostaglandin F2α, tumor necrosis factor-α (TNF-α), and transforming growth factor-beta (TGF-β) signaling are known to play negative roles in adipogenesis. Recent studies have indicated that activation of Wnt signaling can suppress adipogenesis in pre-adipocytes via inhibition of the adipogenic transcriptional factor families C/EBP and PPAR. Thus, elevation or sustainment of these negative factors can be an important strategy for obesity prevention.

Excessive production of intracellular lipid accumulation in adipocytes is closely associated with generation of ROS. Increased ROS production during lipid accumulation in fat tissue reportedly accelerated oxidative stress in biological systems. The pentose phosphate pathway (PPP) is a biochemical pathway that generates nicotinamide adenine dinucleotide phosphate (NADPH), an essential cofactor used for lipid synthesis. Produced NADPH is converted to NADP⁺ via activation of NADPH oxidase and unstable NADP⁺ causes excessive ROS production via over-production of intracellular superoxide radicals (6). Generated ROS subsequently accelerate adipogenic differentiation and relatively high ROS levels are caused by oxidative stress in adipose tissues. Furthermore, an imbalance in cellular redox homeostasis can lead to the physiological diseases of obesity, inflammation, and cancer (7).

Acorn (*Quercus acutissima* CARR.) is mainly distributed in subtropical regions of Asia and has demonstrated physiological effects, including anti-oxidant, anti-dementia, anti-inflammatory, and anti-asthma activities (8). Acorns contain gallic acid, digallic acid, gallotannin, and tanninpolyphenols. Gallic acid in particular is abundant in acorns (9). Extracts from acorns reportedly exhibited anti-oxidant activities against DPPH and diets containing acorns reportedly have a preventative effect against oxidative damage in the liver via inhibition of lipid peroxide formation and an increase in anti-oxidant enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (Gpx), and catalase in Sprague Dawley (SD) rats (10).

Administration of acorn extracts concurrent with a high fat diet in SD rats significantly reduced levels of total lipids and cholesterol, compared with rats on a normal diet and showed both an increased speed for movement of food residues through the small intestine and a decreased absorption rate of triglycerols and total cholesterol (11). However, the anti-adipogenic effect of acorn shells and the molecular mechanisms associated with adipogenesis remain unclear. The purpose of this study was investigation of anti-oxidant and anti-adipogenic activities of methanol extracts from acorn shells (AE-M) using 3T3-L1 cells, and evaluation of the molecular mechanism underlying the anti-adipogenic effect of AE-M.

Materials and Methods

Materials Acorns were collected from the ground in the experimental forest of Chubu University (Kasugai, Japan) in October of 2013. Dulbecco's Modified Eagle's Medium (DMEM), phosphate-buffered saline (PBS), and penicillin-streptomycin were obtained from Welgene Inc. (Daegu, Korea). Fetal bovine serum (FBS) and fetal calf serum (FCS) were purchased from GE Healthcare Bio-Sciences Co. (Piscataway, NJ, USA). TRIzol was purchased from Life Technologies (Carlsbad, CA, USA). All other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Preparation of AE-M Acorns were separated into flesh and shells, which were cryo-milled in liquid nitrogen using a freezer mill (Spex 6750; Spex, Metuchen, NJ, USA) into fine powders with particle sizes smaller than 60 μm . Five g of the resulting shell powder was mixed with 50 mL of methanol at 25°C for 24 h, then filtered (Whatman No. 2) and evaporated using a rotary evaporator. The relative amount of extract to powdered acorn shell used for the extraction procedure

was estimated to be 9% (w/w) for methanol extraction.

Peroxy radical scavenging activity The scavenging activity of AE-M against peroxy radicals was measured using oxygen radical absorbance capacity (ORAC) assay. One hundred μL of an 80 nM fluorescein solution was mixed with 1, 5, and 10 $\mu\text{g/mL}$ AE-M. Fifty μL of 80 mM 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was then added to fluorescein and the AE-M mixture. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm at 37°C using a fluorescence reader (GENios; Tecan, Salzburg, Austria). One ORAC value was equivalent to the net protection area provided by 1 μmol of Trolox.

Reduction potential A concentration of 1 mM CuCl_2 was mixed with 80 μL of 625 μM neocuproin in a pH 7.4 potassium phosphate buffer. The CuCl_2 -neocuproin mixture was then mixed with 1, 5, and 10 $\mu\text{g/mL}$ AE-M, incubated for 1 h at room temperature, then the absorbance was measured at 454 nm using a microplate reader (Sunrise; Tecan). The number of copper (I) ions (μmol) was calculated using an extinction coefficient of $7.95 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for Cu^+ /neocuproin.

Cu^{2+} -chelation activity The Cu^{2+} -chelation activity of AE-M was evaluated using calcein as a specific probe for reaction with metal ions. One hundred μL of 1, 5, and 10 $\mu\text{g/mL}$ AE-M was mixed with 200 μL of a 0.4 μM CuSO_4 solution and added to a 50 nM calcein solution. The fluorescence intensity was determined at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a fluorescence reader. The Cu^{2+} -chelation activity was calculated as a percentage vs. a control that contained only calcein.

3T3-L1 cell culture and adipocyte differentiation 3T3-L1 cells, purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea), were cultured in DMEM supplemented with 10% FCS and 100 unit/mL penicillin-streptomycin at 37°C in a humidified incubator with a 5% CO_2 atmosphere. The medium was changed every 2 days. Two days post-confluency was designated as Day 0. 3T3-L1 pre-adipocytes were treated with 10, 50 and 100 $\mu\text{g/mL}$ of extract during Day -2 to Day 2. Two days after confluency (Day 0) cells were cultured using a differentiation medium (DM) supplemented with a hormonal cocktail of 500 μM 3-isobutyl-1-methylxanthine (IBMX), 5.2 μM dexamethasone (DEX), and 167 nM insulin. After differentiation, the medium was replaced with a post-differentiation medium (Post-DM) containing 167 nM insulin for another 2 days. Thereafter, cells were cultured in

Table 1. Primer sequences for PCR analysis

Primer	Forward (5'→3')	Reverse (5'→3')
C/EBP β	GCAAGAGCCGCGACAAG	GGCTCGGGCAGCTGCTT
PPAR γ	CCATTCTGGCCCACTT	CCTTCTGGCCTGTGATCC
C/EBP α	CCAGAGGATGGTTTCGGGTC	TCCCCAACACCTAAGTCCCT
FAS	GTGCACACAGTGCTCAAAGG	GGTATAGACGACGGGCACAG
GAPDH	TGATGGGTGTGAACACGAG	AAGTCGACGAGACAACCTG

Fig. 1. Anti-adipogenic activity of AE-M. 3T3-L1 cells were treated with 10, 50, and 100 μ g/mL AE-M from Day -2 to Day 2. Schemes of 3T3-L1 differentiation and treatment with AE-M (A). ORO-stained lipid droplets in 3T3-L1 adipocytes were quantified at a wavelength of 510 nm (B). Lipid accumulation in 3T3-L1 adipocytes was visualized using ORO staining (C). The size and number of lipid droplets in 3T3-L1 adipocytes were visualized using ORO staining on Day 6 (D). Corresponding letters indicate significant differences based on Student's *t*-test (** $p < 0.01$, and *** $p < 0.001$). Pre, 3T3-L1 pre-adipocytes; Control, 3T3-L1 adipocytes

normal DMEM and the medium was changed every 2 days (Fig. 1A).

Oil Red O (ORO) staining ORO staining was performed on Day 6. Differentiated 3T3-L1 adipocytes were washed using PBS and fixed using 3.7% (v/v) formaldehyde at room temperature for 30 min.

Fixed 3T3-L1 cells were washed three times with tap water and mature adipocytes were stained with a 3 mg/mL ORO solution dissolved in isopropanol at room temperature for 15 min. After ORO staining, cells were washed three times with tap water and completely dried for 10 min. Stained lipid droplets were dissolved in

300 μ L of DMSO and 100 μ L was transferred to a 96 well clear plate. The absorbance of the dissolved ORO was measured at 510 nm using a microplate reader.

Isolation of total RNA and RT-PCR analysis mRNA expression levels of the transcription factors C/EBP α , C/EBP β , PPAR γ , and FAS were estimated using RT-PCR analysis. Total RNA was extracted from AE-M treated 3T3-L1 adipocytes on Day 6 using TRIzol reagent. cDNA synthesis was performed using 2 μ L of total RNA with RT PreMix. Synthesized cDNA was analyzed using RT-PCR with GAPDH as a control. Primer sequences were shown in Table 1.

Inhibitor assay The specific inhibitors IWR-1 (Wnt/ β -catenin inhibitor), SP600125 (JNK inhibitor), LY294002 (PI3K inhibitor), and SB431542 (TGF- β inhibitor) were used for examination of the effects of signalling inhibitors on AE-M inhibited 3T3-L1 adipogenesis. 3T3-L1 cells were exposed to 50 and 100 μ g/mL AE-M in the presence and absence of inhibitors from Day -2 to Day 2. Intracellular lipid accumulation was quantified at 510 nm using ORO staining.

Statistical analysis All data are presented as a mean $\pm$ standard deviation (SD). Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). Significant differences between groups was assessed using a one-way analysis of variance (ANOVA) followed by either Duncan's or Student's *t*-test at $p < 0.05$ for significance.

Results and Discussion

Anti-oxidant activities of AE-M AAPH was used to generate peroxy radicals for determination of the anti-oxidant activities of AE-M against peroxy radicals using ORAC assays. Scavenging activities of AE-M were dose-dependently increased against peroxy radicals (Table 2). Compared with Trolox, a well-known anti-oxidant version of vitamin E, AE-M peroxy radical scavenging activities of 1, 5, and 10 μ g/mL were 2.27 TE (Trolox equivalents), 5.87 TE, and 9.30 TE, respectively. Cu¹⁺ and Cu²⁺ reduction was assessed for confirmation that the anti-oxidant activity of AE-M resulted from a capacity to donate electrons. The reduction potential of AE-M increased dose-

dependently between 1 and 10 μ g/mL.

Inhibition of calcein-Cu²⁺ complex formation by AE-M confirmed a metal chelation activity. Calcein is known as a metal chelator that produces fluorescence. The metal chelation activity of AE-M was investigated for determination of the percentage of the calcein-Cu²⁺ complex inhibited in the presence of AE-M. The Cu²⁺-chelation activity of AE-M was dose-dependently increased from 1, 5, and 10 μ g/mL. The Cu²⁺-chelation activity of AE-M increased by 48.42, 54.75, and 50.95% at 1, 5, and 10 μ g/mL. Thus, anti-oxidant activities of AE-M probably correlated with hydroxyl radical scavenging activities via metal chelation.

A linear relationship between phenolic contents of some plant extracts and anti-oxidant activities is known (12). Methanol extracts from *Quercus robur* and *Quercus cerris* acorn kernels reportedly contained abundant amounts of phenol, gallic acid, and flavonoids. *Quercus robur* and *Quercus cerris* methanol extracts effectively inhibited the Fe²⁺/ascorbate induced lipid peroxidation (13). Karimi et al. (14) reported that the presence of flavonoids in acorn extracts was associated with scavenging activities against DPPH radicals. Furthermore, gallic acid in acorns has a role primarily as an anti-oxidant component against peroxide (15). Thus, gallic acid hydrolysed from tannin in acorn shells probably contributed to the anti-oxidant activity of AE-M.

Inhibitory effect of AE-M against 3T3-L1 adipogenesis 3T3-L1 pre-adipocytes were cultured with 10, 50, and 100 μ g/mL AE-M in DM from Day -2 to Day 2 for examination of AE-M inhibition of adipogenesis of 3T3-L1 cells. AE-M had no effect on the cell viability of confluent 3T3-L1 cells up to 100 μ g/mL (data not shown). Therefore, 10, 50, and 100 μ g/mL AE-M were used in subsequent studies. Inhibitory effects against adipogenesis in AE-M-treated 3T3-L1 cells was visualized using ORO staining on Day 6. The level of lipid accumulation in 3T3-L1 adipocytes was dose-dependently and significantly ($p < 0.05$) inhibited by AE-M treatment from Day -2 to Day 2 (Fig. 1B), compared with controls. The level of lipid accumulation in AE-M-treated 3T3-L1 cells was significantly ($p < 0.05$) inhibited by 44.6, 96.7, and 99.2% at 10, 50, and 100 μ g/mL, respectively, compared with controls. Moreover, intracellular lipid accumulation was strongly inhibited by a 10 μ g/mL AE-M treatment in 3T3-L1

Table 2. Anti-oxidant activity of AE-M

Method	Cu ²⁺	AE-M (μ g/mL)		
		1	5	10
ORAC _{ROO·} (TE [†] , μ M)	-	2.27 $\pm$ 0.20 ^a	5.87 $\pm$ 0.29 ^b	9.30 $\pm$ 0.29 ^c
Reducing potential (Copper(I) ions, μ M)	-	5.70 $\pm$ 0.06 ^a	30.57 $\pm$ 0.28 ^b	42.73 $\pm$ 0.34 ^c
Cu ²⁺ -chelating activity (% of Control)	46.10 $\pm$ 1.26 ^a	48.49 $\pm$ 2.29 ^b	54.75 $\pm$ 3.47 ^c	50.96 $\pm$ 1.05 ^c

[†]TE: Trolox equivalents

Scavenging activity of AE-M against peroxy radicals based on ORAC_{ROO·}. Peroxy radical scavenging activity for AAPH of AE-M was evaluated using ORAC assay. The ORAC value was calculated by dividing the area under an AE-M curve by the area under the Trolox curve, with both areas corrected based on subtraction of the area under a blank curve. One ORAC unit was indicated as the net area of protection provided by 1 μ mol of Trolox (TE). Different corresponding letters indicate significant differences between different concentrations of AE-M (a-c) based on ANOVA with Duncan's multiple range test ($p < 0.05$).

adipocytes (Day 6), compared with 50 μ M quercetin treatment. The AE-M inhibitory effect against lipid accumulation for adipogenesis in 3T3-L1 cells was quantitatively evaluated using ORO staining on Day 6 (Fig. 1C and 1D). In addition, the size and number of intracellular lipid droplets in 3T3-L1 cells decreased in a dose-dependent manner with treatment using 10, 50, and 100 μ g/mL AE-M.

Anti-obesity effects and an increased lipid metabolism have been reported with acorn extracts. Yook *et al.* (10) reported that feeding of acorn powder composed of the flesh or inner skin to high fat diet induced SD rats for 4 weeks reduced levels of plasma and liver lipids, compared with controls, and food intake, body weight gain, the food efficiency ratio, and kidney, epididymis, and spleen weights were no different among experiment groups. Kang *et al.* (16) reported anti-obesity effects of acorn powder in obese rats fed a high fat diet after acorn powder supplementation for 4 weeks with reduced body weights and total cholesterol and triglyceride levels in serum and hepatic tissues. Trimethoxybenzoic acid methyl ester can be formed from free gallic acid via methylation by tetramethylammonium hydroxide (TMAH), and/or esterified gallic acid components can be formed via hydrolysis of tannins, and subsequent methylation in the presence of TMAH (17,18). However, anti-adipogenic activities of acorn shells in 3T3-L1 cells and the underlying molecular mechanism remain unclear. AE-M inhibited levels of intracellular lipid accumulation and adipogenesis in 3T3-L1 cells in this study.

Effects of AE-M treatment on mRNA expression levels of transcriptional factors in 3T3-L1 cells Adipogenesis in 3T3-L1 cells is accompanied by increased expression of transcriptional factors and adipocyte-specific genes (19). Expressions of adipogenic transcriptional factors following AE-M treatment in 3T3-L1 cells were examined for evaluation of the effect of AE-M treatment on mRNA expression levels of transcriptional factors in 3T3-L1 cells. mRNA expression levels of C/EBP α , C/EBP β , PPAR γ , and FAS were analyzed by using RT-PCR with GAPDH as a control. 3T3-L1 pre-adipocytes were treated with a differentiation medium in the presence and absence of AE-M at 50 and 100 μ g/mL from Days -2 to day 2. Quercetin at 50 and 100 μ M was used as a positive control. mRNA expression levels of C/EBP β , C/EBP α , and FAS were completely suppressed by AE-M at 100 μ g/mL (Fig. 2). Furthermore, the mRNA expression level of PPAR γ was dose-dependently inhibited by both 50 and 100 μ g/mL AE-M. Suppression with 50 μ g/mL AE-M was stronger than for 50 and 100 μ M quercetin.

Adipogenesis is regulated by expression of adipogenic-specific genes, which are critical during adipogenesis. Down-regulation of the adipogenic transcription factors C/EBP β , C/EBP α , PPAR γ , and aP2 can reportedly prevent adipogenesis (20). Several recent studies have attempted to locate natural functional materials with anti-adipogenic activities (21). Adipogenesis in 3T3-L1 cells is inhibited by regulation of the adipogenic transcriptional factors C/EBP β , C/EBP α , PPAR γ , and FAS (22). Kim and Kim (23) reported that extracts prepared from perilla leaves appeared to mediate 3T3-L1 adipogenesis via

Fig. 2. Effects of AE-M on mRNA expression levels of transcriptional factors in 3T3-L1 cells. RT-PCR was performed for determination of mRNA expression levels of the adipogenic genes C/EBP β , PPAR γ , C/EBP α , and FAS. GAPDH was used as a control. Fifty and 100 μ M quercetin levels were used as positive controls. Pre, 3T3-L1 pre-adipocytes; Control, 3T3-L1 adipocytes

down-regulation of PPAR γ , aP2, and GPDH expressions. Park *et al.* (24) indicated that extracts from centipede grass demonstrated anti-adipogenic activities via inhibition of C/EBP β and C/EBP α , and the PPAR γ and Akt signaling pathways in 3T3-L1 adipocytes. Jeong *et al.* (25) reported that grape skin extracts attenuated expression of the key adipogenic transcription factors C/EBP α , PPAR γ , FAS, and aP2. Thus, AE-M can contribute to inhibition of 3T3-L1 adipogenesis via regulation of adipogenic transcriptional factor expression.

Effects of AE-M treatment at different times during 3T3-L1 adipogenesis Intracellular lipid accumulation at different stages of adipogenesis following AE-M treatment in 3T3-L1 cells was examined. The inhibitory effect against adipogenesis in AE-M-treated 3T3-L1 cells was visualized using ORO dye staining on Day 6. One hundred μ g/mL AE-M was applied at the pre-adipogenic stage (Day -2-0), the early stage (Day 0-2), the intermediate stage (Day 2-4), and the terminal stage (Day 4-6) during adipogenesis in 3T3-L1 cells (Fig. 3A). Lipid accumulation in AE-M-treated 3T3-L1 cells during the pre-adipogenic, early, intermediate, and terminal stages was inhibited by 35.61, 76.20, 91.24, and 95.63% at 10, 25, 50, and 100 μ g/mL, respectively (Fig. 3B and 3C). Lipid accumulation in 3T3-L1 adipocytes on Day 6 was significantly ($p < 0.05$) inhibited by AE-M treatment in the pre-adipogenic, early, and intermediate stages of adipogenesis, but not ($p > 0.05$) in the terminal stage, compared with controls. Thus, acorn extracts are stronger regulators in the pre-adipogenic, early, and intermediate stages of 3T3-L1 adipogenesis.

Expression and activation of transcription factors increased by hormonal and nutritional stimuli can reportedly induce expression of adipocyte-specific genes (26). Pre-adipocyte factor 1 (Pref1) is abundant in undifferentiated pre-adipocytes, whereas decreased Pref1 expression not only accompanies adipocyte differentiation but is also absent from mature adipocytes (27). High expression levels of Wnt-10b are also observed in 3T3-L1 pre-adipocytes (20). Kim *et al.*

Fig. 3. AE-M inhibition of lipid accumulation was limited to the pre-adipogenic, early, and intermediate stages of 3T3-L1 adipogenesis. 3T3-L1 pre-adipocytes were treated with 100 µg/mL AE-M in the presence and absence of a differentiation medium (A). One hundred µg/mL AE-M applied at different times during the pre-adipogenic stage (Day -2~0, A), early stage (Day 0~2, B), intermediate stage (Day 2~4, C), and terminal stage (Day 4~6, D) in 3T3-L1 cells. Lipid droplets in 3T3-L1 adipocytes were visualized using ORO staining. (B). Inhibitory effects of AE-M treatment based on the size and number of lipid droplets in ORO-stained 3T3-L1 cells were observed on Day 6 (C). Control group (no treatment) vs. treatment group differences were determined based on a one-way ANOVA ($p < 0.05$) followed by Student's *t*-test (***) $p < 0.001$. Pre, 3T3-L1 pre-adipocytes; Control, 3T3-L1 adipocytes

(28) reported that the forkhead box protein (FOXO1) is induced in early stages of adipocyte differentiation and plays an important role for inhibition of adipogenesis via regulation of the insulin signaling pathway. Thus, AE-M probably contributes to FOXO1 anti-adipogenic activities against adipogenic transcriptional factors, particularly in the pre-adipogenic, early, and intermediate stages.

Effect of inhibitors on AE-M-inhibited 3T3-L1 adipogenesis

Selective inhibitors were administered together with 50 and 100 µg/

mL AE-M from Day -2 to Day 2 for examination of AE-M suppression of 3T3-L1 adipogenesis via a specific signaling pathway. AE-M treatment inhibited intracellular lipid accumulation in 3T3-L1 adipocytes by 71.60 and 94.91%, respectively, at 50 and 100 µg/mL (Fig. 4). AE-M-inhibited lipid accumulation was reversed in the presence of IWR-1, whereas 3T3-L1 cells co-treated with AE-M and the inhibitors SP600125, LY294002, and SB431542 showed reduced levels of lipid accumulation, compared with AE-M treatment alone.

Wnt proteins are paracrine and autocrine factors that modulate

Fig. 4. Effects of different signalling inhibitors in AE-M treated 3T3-L1 adipocytes. 3T3-L1 cells were exposed to 50 and 100 µg/mL AE-M in the absence and presence of the inhibitors IWR-1 (Wnt/β-catenin inhibitor, 10 µM), SP600125 (ERK inhibitor, 10 µM), LY294002 (PI3K inhibitor, 5 µM), and SB431542 (TGF-β inhibitor, 5 µM). Levels of lipid accumulation in 3T3-L1 adipocytes were quantified using Oil Red O staining at 510 nm. Different corresponding letters indicate significant differences between groups treated with 50 µg/mL AE-M (a-c) and groups treated of 100 µg/mL AE-M (x-z) at $p < 0.05$.

cell growth and proliferation (29). β-Catenin reportedly acts as an intracellular signal transducer and is also known to play a major regulatory role in the Wnt signaling pathway (30). Expression of dominant β-catenin inhibits adipogenesis via suppression of C/EBPα and PPARγ expressions. Wnt signaling reportedly plays a critical role in regulation of adipogenesis in 3T3-L1 cells and in glucose-induced insulin secretion in pancreatic islets (31). Wnt signaling is also believed to contribute to induction of type 2 diabetes (32). Kim *et al.* (33) reported that kireanol is capable of inhibiting differentiation and lipogenesis in 3T3-L1 adipocytes via increased activation of the Wnt/β-catenin signaling pathway. The increased Wnt signaling in 3T3-L1 maintained pre-adipocytes in an undifferentiated state and inhibited expression of the transcription factors C/EBPα and PPARγ (33). IWR-1 treatment partially, but not definitively, reversed the anti-adipogenic activity of AE-M. Accordingly, factors upstream of Wnt signaling as unreported regulators of Wnt signaling and/or activators of β-catenin proteins may exist. The Wnt signaling pathway is probably required for AE-M inhibition of 3T3-L1 adipogenesis. Methanol extracts from acorn shells have potential applicability as an ingredient for development of nutraceuticals and functional foods. Further study regarding elucidation of a precise mechanism for the anti-adipogenic activity of AE-M is required.

Disclosure The authors declare no conflict of interest.

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