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Antioxidant and anti-adipogenic activities of acorn shells

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Abstract Antioxidant and anti-adipogenic activities of water extract (WE) and methanol extract (ME) of acorn shells (AS), from *Quercus acutissima* Carruth. grown in Korea, were investigated. At a concentration of 50 μg/mL, the WE had a scavenging activity of 53.84% for the DPPH and 76.09% for the ABTS radical, while the ME had corresponding scavenging activities of 29.09 and 48.43%. Total phenolic contents of WE and ME were 375.96 and 288.01 mg gallic acid equivalents/g of extracts, respectively. Both extracts significantly inhibited 3T3-L1 adipogenesis in a dose-dependent manner, and concomitantly decreased the size and number of intracellular lipid droplets. Furthermore, the anti-adipogenic activities of WE and ME are largely limited in the pre- and early stages of adipogenesis. The results suggest that AS may be a promising source of antioxidants and anti-obesity compounds.

Keywords: acorn, shell, antioxidant, anti-adipiogenic

Introduction

Acorn is the fruit of the *Quercus* tree, belonging to the Fagaceae family, and has been traditionally used as an astringent, antidiarrheal, and antidote in Asian countries (1). It is mainly composed of carbohydrates (2), and contains various phenolic compounds that have shown beneficial properties such as antioxidant activity (3), prevention of degenerative diseases (4), improvement of the lipid profile in obese rats (5), and anti-diabetic activity (6-8). Although there are many studies in which the physiological activities of acorn have been reported, most of these studies were focused on the edible parts of the acorn kernel. In Korea, acorn has been widely consumed in the form of jelly, using its starch, and the acorn shell is generated as byproducts after processing at an industrial scale. These byproducts comprise approximately 20% (w/w) acorn, and are either discarded or underutilized. In this study, we evaluated the potential of AS as a source of high-value antioxidants and anti-obesity materials. We performed this study to explore the total phenolic and tannin contents and ability to scavenge DPPH and ABTS radicals, and evaluated anti-adipogenic activity in 3T3-L1 cells.

Materials and Methods

Reagents Folin-Ciocalteu reagent was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's Modified Eagle's Medium, penicillin-streptomycin, and bovine calf serum were obtained from Welgene Inc. (Daegu, Korea). Fetal bovine serum was purchased from GE Healthcare Bio-Sciences Co. (Piscataway, NJ, USA). Other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Preparation of extracts Acorns of *Quercus acutissima* Carruth. were purchased at local shop (Changwon, Korea) in November 2014, and their shells were removed and collected. The acorn shells (AS) were dessicated in a dry oven (Sangwoo Co., Incheon, Korea) at 50°C for 12 h, and the shells were then ground using a blender (Samyang Co., Gimpo, Korea). The resulting powder, with a particle diameter smaller than 710 μm (25 mesh), was used in the following extraction preparation. Water extract (WE) was prepared with deionized water (1 g/100 mL) at 100°C for 30 min, and methanol extract (ME) was made with methanol (1 g/100 mL) at 25°C for 12 h according to the method described by Ishida *et al*. (9). Three different extraction processes were carried out, and the extracts were combined. Average extraction yields of WE and ME were 11.6 and 6.8% (w/w powder), respectively. The extracts were then dissolved in dimethlsulfoxide (DMSO) and stored in a deep freezer (Operon Co., Seoul, Korea) at −70°C for further experiments.

DPPH and ABTS radical scavenging activity (RSA) and ABTS RSA The DPPH and ABTS RSAs of the extracts were determined by the methods of Lee *et al*. (10) and Re *et al*. (11), respectively. Ascorbic acid was used as a positive control and all tests were carried out in triplicate.

Total phenolic content (TPC) and tannin content (TC) TPC in the extracts were determined according to the method of Gutfinger (12), and TC of dried AS was determined according to the method of Yonemori *et al.* (13). TPC and TC were expressed as μg gallic acid equivalents (GAE).

Cell culture and adipocyte differentiation The second day after confluency was designated as Day 0. 3T3-L1 preadipocytes were treated with 10, 50, 100, and 200 μg/mL of the WE and ME during Day −2 to Day 6. 3T3-L1 cell culture and adipocyte differentiation was conducted according to our previously reported method (14).

Oil Red O (ORO) staining ORO staining was performed according to our previously reported method (14). The ORO-stained cells were photomicrographed (KI2000; Korea Lab Tech, Seongnam, Korea) at magnifications of 250x for image analysis.

Statistical analysis All data were expressed as mean±SD. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA) and the significance of differences between the groups was verified using a one-way analysis of variance (ANOVA) followed by either a Duncan's or Student's *t*-test (*p*-value <0.05).

Results and Discussions

Antioxidant activity Antioxidant activity of AS was evaluated by determining its scavenging activity against DPPH and ABTS radicals. Figure 1A shows DPPH RSA of the extracts prepared from AS at 50, 100, 150, 200, and 250 μg/mL concentrations. The WE showed stronger DPPH scavenging activity (53.84%) than that shown by ME (29.09%) at 50 μg/mL. The scavenging activity of ascorbic acid, the positive control, was 56.71% (40 μg/mL). DPPH RSA of the ME of Serbian acorn kernels has been reported as 94.29-94.97% at a concentration of 50 μg/mL (15), although the *Quercus* species and extraction method used were different from those in our study. DPPH RSA of WE and ME (40 μg/mL) from shells of Japanese *Quercus acta* was reported as approximately 50 and 60%, respectively (9).

As shown in Fig. 1B, WE showed consistently higher ABTS RSA than ME. At 50 μg/mL, WE and ME scavenged 76.09 and 48.43% of ABTS radicals, respectively. The ABTS RSA of 20 μg/mL ascorbic acid was 70.94%. RSA for ABTS was stronger in both extracts than for DPPH. This resulted from the capacity of ABTS to determine both hydrophilic and hydrophobic antioxidant substances, while DPPH can only be used to measure hydrophobic antioxidant substances (16).

The TPC of WE and ME was determined to be 375.96 and 288.01 mg GAE/g, respectively. The higher phenolic contents of WE may have contributed to its comparatively higher antioxidant activity. The difference in the TPC of WE and ME may be explained in two ways. Firstly, WE was prepared at a higher temperature (100°C) than ME (25°C). Bio-materials are easily extractable at high temperatures.

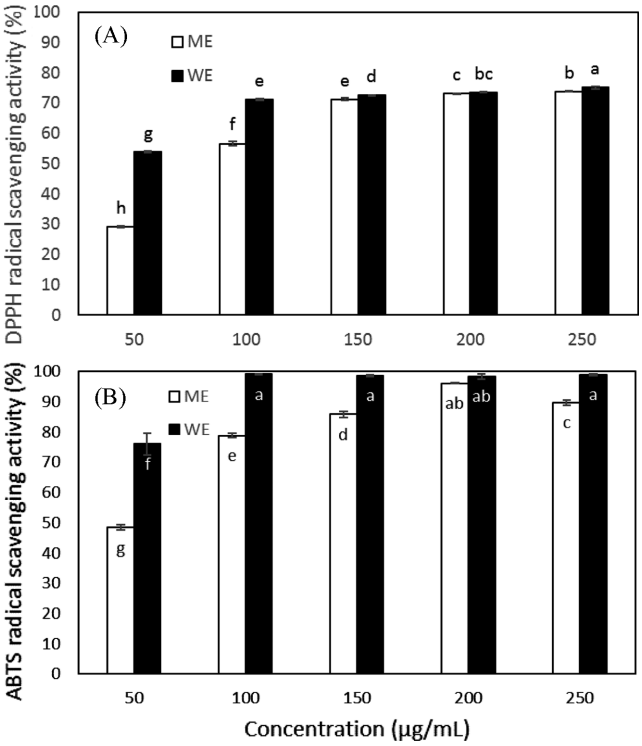

Fig. 1. (A) DPPH and (B) ABTS radical scavenging activities (RSAs) of water extract (WE) and methanol extract (ME). Values with different letters on the bars in each figure are significantly different by Duncan's test at $p<0.05$. WE, water extract of acorn shells; ME, methanol extract of acorn shells

Secondly, water-soluble phenolics are more prevalent than methanol-soluble ones in the AS. On the other hand, WE and ME prepared by the same method with this study using AS of Japanese *Quercus acta* showed a different antioxidant trend, where ME showed higher activity (9). Thus, different oak species show different antioxidant activity and TPC. Gallic acid, in its free or esterified form, has been reported as the main phenolic compound in acorn and its shells (4,9,17). Meanwhile, ME of acorn kernels of *Quercus suber* showed higher antioxidant activity than that shown by WE (4).

Tannins, which are found in various plant-based foods, are potentially important biological antioxidants (18). We found that dried AS contained 128.99 and 73.97 mg/g (w/w) soluble and insoluble tannins, respectively, and TC of AS was calculated as 205.96 mg/g. It is difficult to determine individual TC of WE and ME because of methodological problems.

Anti-adipogenic activity To test whether WE and ME treatments can suppress 3T3-L1 adipogenesis, 3T3-L1 cells were exposed to 10, 50, 100, and 200 μg/mL WE and ME from Day −2 to Day 6 (Fig. 2A). The lipid accumulation of 3T3-L1 adipocytes was dose-dependently and significantly ($p<0.05$) inhibited by 70.16, 96.03, 97.79, and 100% in WE and 28.72, 55.34, 84.51, and 100% in ME treatments at 10, 50, 100, and 200 μg/mL from Day −2 to Day 6, respectively (Fig. 2B). This result correlated with decreased intracellular lipid droplets in 3T3-L1

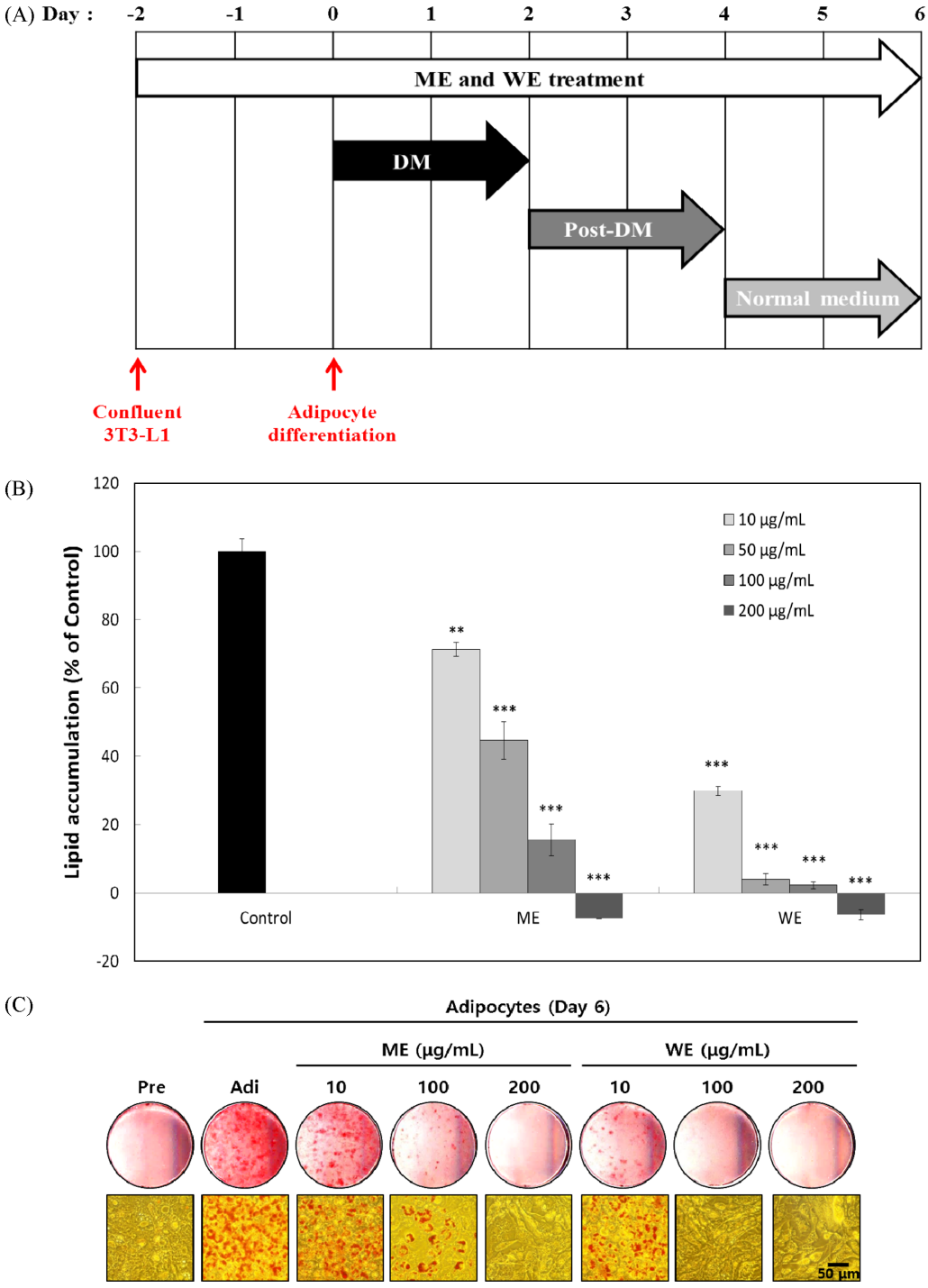

Fig. 2. Anti-adipogenic activities of WE and ME (A) Scheme of 3T3-L1 differentiation. (B) Anti-adipogenic activities of WE and ME. (C) Photomicrographs of decreased lipid droplets. Corresponding letters indicate significant differences based on Student's *t*-test (**p<0.01, and ***p<0.001). WE, water extract of acorn shells; ME, methanol extract of acorn shells; Pre, preadipocytes; Adi, adipocytes

cells as shown in Fig. 2C. Adipogenesis occurs progressively throughout life in humans (19). Thus, to inhibit adipogenesis is crucial for the prevention of obesity (20). In line with the results regarding antioxidant activity, anti-adipogenic activity of WE was stronger than that of ME, suggesting that phenolic compounds, mainly gallic acid, were a pivotal factor in antioxidant and anti-adipogenic activities of AS.

To investigate the adipogenesis stage responsible for mediating the anti-adipogenic activities of WE and ME, 3T3-L1 cells were treated with 200 μg/mL WE and ME at various stages of adipogensis including pre- (A), early (B), intermediate (C), and terminal (D) stages as shown in Fig. 3A. Lipid accumulation in WE- and ME-treated 3T3-L1 cells during the A, B, C, and D stages was inhibited by 68.77, 74.73,

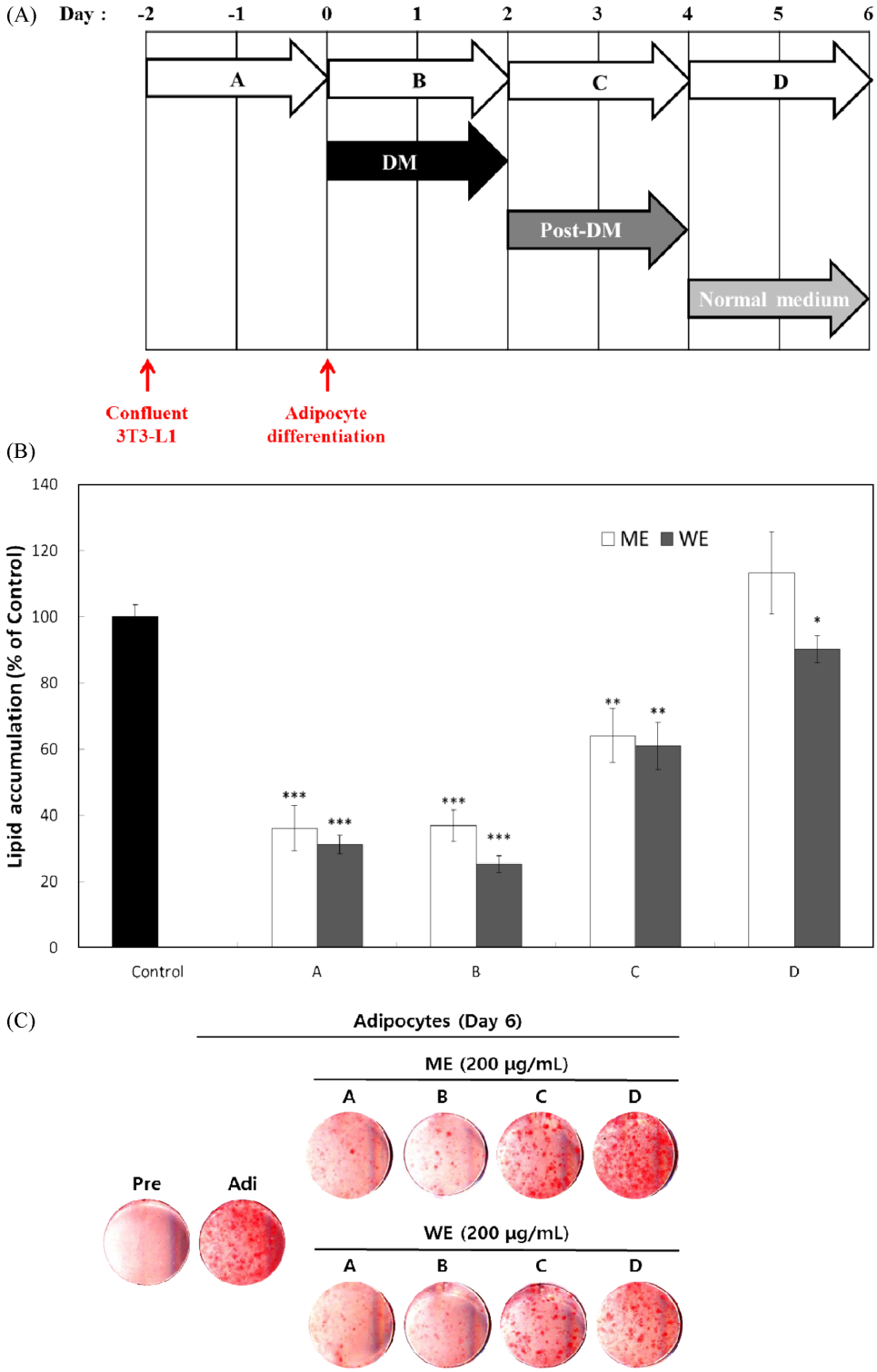

Fig. 3. Inhibitory effects of WE and ME in the various stages of 3T3-L1 adipogenesis. (A) Scheme of 3T3-L1 differentiation. The letter of A, B, C, D in arrow indicate the ME- and WE-treated stages (pre-, early, intermediate, and terminal stages) in 3T3-L1 adipogenesis (B) Lipid droplets were quantified at a wavelength of 510 nm. (C) The decreased lipid droplets were visualized using ORO staining. Corresponding letters indicate significant differences based on Student's *t*-test (**p*<0.05, ***p*<0.01, and ****p*<0.001). WE, water extract of acorn shells; ME, methanol extract of acorn shells; Pre, preadipocytes; Adi, adipocytes

39.07, and 9.85% in WE and 63.84, 63.07, 35.92, and 0% in ME treatment, respectively (Fig. 3B). In WE and ME treatment (200 μg/mL) during A and B stages, stronger inhibition of lipid accumulation was observed than during treatments in C and D stages. Similarly, a dramatic decrease in lipid droplets was observed, particularly in A and B stages (Fig. 3C). Together, these results suggest that inhibitory

action of WE and ME was largely limited to the pre and early stages of adipogenesis. Adipogenesis is dependent on coordinated regulation of hormones, adipokines, nutrients and transcriptional factors. Here, we observed that WE and ME are strong regulators of adipogenesis although direct molecular targets are still unclear. However, a partial explanation is that WE and ME could be acting as regulators in pre and early stages of adipogenesis, since it was recently reported that the resveratrol inhibited adipogenesis through modulation of mitotic clonal expansion by suppression of the cell cycle entry in the early stage of adipogenesis (21). In addition, inhibition of preadipocytes into G2/M phase in the early stage requires phosphorylation of the insulin receptor, insulin receptor substrate-1, and the Akt pathway (22). Therefore, we speculated that inhibitory activities of WE and ME, particularly the gallic acid abundant in AS, is mediated by regulation of the cell cycle and insulin signaling in the early stage of adipogenesis. In conclusion, our evidence supports the potential of AS as natural antioxidant and anti-adipogenic materials for the development of functional foods.

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Disclosure The authors declare no conflict of interest.

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