Inhibitory effects of *Orostachys malacophyllus* var. *iwarenge* extracts on reactive oxygen species production and lipid accumulation during 3T3-L1 adipocyte differentiation

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Abstract *Orostachys malacophyllus* var. *iwarenge* was extracted with water, 30 and 70% ethyl alcohol. The ethyl alcohol extracts showed higher total phenol contents and greater antioxidant effects than the water extract. Treatment with the 70% alcohol extract inhibited reactive oxygen species (ROS) production and lipid accumulation during 3T3-L1 adipogenesis. Furthermore, the 70% extract inhibited the mRNA and protein expressions of the pro-oxidant enzyme NADPH oxidase 4 and of the NADPH-producing enzyme glucose-6-phosphate dehydrogenase. The mRNA and protein expressions of antioxidant enzymes, such as copper-zinc superoxide dismutase and manganese superoxide dismutase increased in cells treated with the 70% alcohol extract. In addition, this extract suppressed the mRNA and protein levels of adipogenic transcription factors and of their marker genes. These results indicate that *O. malacophyllus* extracts inhibit lipid accumulation and ROS production by controlling adipogenic factors and pro-/anti-oxidant enzyme responses.

Keywords *Orostachys malacophyllus* · Antioxidant · 3T3-L1 cells · Reactive oxygen species · Adipocyte differentiation

Introduction

Obesity is defined as excessive body fat accumulation in adipose tissue due to increases in the sizes and numbers of adipocytes. In the contemporary society, obesity is a recognized health problem and known to be associated with chronic diseases including hypertension, coronary heart disease, type 2 diabetes, and cancer (Kopelman, 2000). Adipogenesis is a differentiation process whereby pre-adipocytes become mature adipocytes that accumulate triacylglycerol in the abdomen (Seo et al., 2016). Adipogenesis is accompanied by coordinated changes in cell morphology, hormone sensitivity, and gene expression, and these changes are regulated by several transcription factors, including PPAR (peroxisome proliferator-activated receptor) γ and C/EBP (CCAAT/enhancer-binding proteins) α (Rosen et al., 2000). PPAR γ and C/EBP α are two main transcriptional regulators induced during the early process of adipocyte differentiation, which regulate the adipocyte development and glucose homeostasis (Tontonoz et al., 1994). PPAR γ and C/EBP α work cooperatively to enhance the expressions of several other adipogenic markers (Fève, 2005). aP2 transports fatty acids in cytoplasm and is involved in the intracellular transport and metabolism of fatty acids (Storch and Thumser, 2000). In addition, under PPAR γ and C/EBP α control, aP2 plays an important role as a mediator of fatty acid synthesis during adipogenesis.

Recently, several studies have shown that reactive oxygen species (ROS) status is closely correlated to the pathogenesis and developments of metabolic diseases (Lee et al., 2016; Seo et al., 2016). During adipogenesis, adipocytes generate a variety of adipogenic cytokines, ROS, and fatty acids (Adachi et al., 2009), and recent research suggests that fat cell derived ROS functions are major

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modulators of metabolic syndrome, and in particular of insulin resistance and obesity (Furukawa et al., 2004; Vincent and Taylor, 2006). ROS also plays a critical role in the regulation of adipocyte differentiation due to its involvement in the up-regulations of adipogenic transcription factors (Lee et al., 2012), though it has been shown that anti-oxidative enzymes are down-regulated during adipogenesis (Liu et al., 2012). In adipocytes, cellular ROS levels depend on a balance between pro-oxidant and anti-oxidant enzymes, which are responsible for producing ROS (e.g., nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase 4 and NOX4) or eliminating ROS (e.g., Cu/Zn-SOD (copper-zinc superoxide dismutase), Mn-SOD (manganese superoxide dismutase), catalase, and GPx (glutathione peroxidase) (Evans et al., 2003; McCord and Fridovich, 1988). NOX 4 is a plasma enzyme that utilizes cytosolic NADPH as an electron donor for ROS production. NADPH is generated by malic enzyme and hexose monophosphate (HMP) shunt enzymes, such as, G6PDH (glucose-6-phosphate dehydrogenase) and 6-phosphogluconate dehydrogenase (6PGD) (Park et al., 2005). G6PDH, the rate-limiting HMP shunt enzyme, plays a critical role in redox potential control and cell maintenance by controlling the production of NADPH (Furukawa et al., 2004; Meister, 1988). Previous studies suggest that natural antioxidants usefully control cellular ROS generated by the pro-oxidant system in adipocytes (Furukawa et al., 2004; Lee et al., 2009).

Orostachys malacophyllus belongs to the family Crasulaceae, and is indigenous in Korea, China and Japan. Plants can be collected during the summer or autumn and after removing roots and sun drying are used as a medicinal ingredient in traditional medicines (Park et al., 1991, Yoon et al., 2000). Previous studies on *O. malacophyllus* have reported the presence quercetin, friedelin, kaempferol, epicatechin, epi-friedlanol, grutinone, glutinol, campesterol, β -sitosterol, triterpenoid, fatty acid ester and aromatic acids (Jeong et al., 2011a, b). Furthermore, *O. malacophyllus* has been demonstrated to have anti-inflammatory, anti-febrile, hemostatic, and anti-cancer effects (Ryu et al., 2009). However, more scientific research is required to establish the signaling pathways responsible for its physiological activity.

In this study, we evaluated the total phenolic contents and antioxidant capacities of water and 30 and 70% aqueous ethanolic extracts of *O. malacophyllus*, and investigated their inhibitory effects on ROS production and lipid accumulation during adipocyte differentiation and examined their effects on the expressions of pro- and anti-oxidant enzymes and adipogenic transcription factors.

Materials and methods

Chemicals

Fetal bovine serum (FBS), Dulbecco's modified eagle's medium (DMEM) and antibiotic solution (100X, 100unit) were purchased from Welgene Inc. (Daegu, Korea). Gallic acid, Folin-Ciocalteu phenol reagent, ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, sodium phosphate, sodium carbonate, isobutylmethyl-xanthine (IBMX), insulin, dexamethasone (DEX), dimethylthiazol-2-yl-2,5-diphenyl-tetrazolium-bromide (MTT), dimethyl sulfoxide (DMSO), isopropanol, *N*-acetyl-L-cysteine (NAC), nitroblue tetrazolium (NBT), fluorescein sodium salt (FL) and Oil Red O were purchased from Sigma (St. Louis, MO, USA). Anti-NOX4, G6PDH, Cu/Zn-SOD, Mn-SOD, PPAR γ , C/EBP α , α P2, β -actin and horseradish peroxidase (HRP)-conjugated anti-rabbit were obtained from Cell Signaling Technology (Danvers, MA, USA).

Plant materials and extraction

Orostachys malacophyllus var. *iwarenge* was supplied by Professor Kuen-Woo Park of the Department of Life Sciences and Biotechnology, Korea University (Seoul, Korea). It has been cultivated at Korea University farm (Namyangju, Gyeonggi-do, Korea) *O. malacophyllus* was extracted by boiling with water, 30 and 70% ethanol for 4 h three times. The extracts were concentrated by rotary evaporation under 45 °C. The extract was concentrated by rotary evaporation under 45 °C and stored at – 20 °C until experiment.

Determination of total phenolic contents

Total phenolic contents were measured with the Folin-Ciocalteu assay (Singleton and Rossi, 1965). Briefly, 20 μ L of sample or gallic acid standard at various concentrations were placed in a 96-well plate, and 100 μ L of 2 N Folin-Ciocalteu agent added. After 3 min, 180 μ L of 2% sodium carbonate was added, and the mixture was placed for 1 h at room temperature. The absorbance at 750 nm was measured using a spectrophotometer (SpectraMax M2, Molecular Devices, USA). The results were expressed as mg gallic acid equivalents (GAE)/g and were based on a calibration curve of gallic acid.

DPPH radical scavenging activity

The scavenging activity against DPPH free radical was measured using the method used by Li et al. (2005). The sample at various concentrations was mixed with 100 μ L of 0.2 mM DPPH solution and placed for 30 min at room temperature. The absorbance was measured at 515 nm and DPPH scavenging capacity were expressed as mg ascorbic acid equivalents (AAE)/g.

ABTS radical scavenging activity

The scavenging activity against ABTS free radical was measured using the by improved ABTS radical scavenging method (Re et al., 1999), with minor modifications. 7.0 mM ABTS (in 20 mM sodium acetate buffer, pH 4.5) and 2.45 mM potassium persulphate mixed for the production of ABTS cation and kept in dark overnight at room temperature. ABTS solution was diluted with phosphate buffer saline (PBS) to an absorbance of 0.7 ± 0.01 at 734 nm. For sample analysis 950 μ L of the diluted ABTS solution was added to 50 μ L of extracts and was mixed thoroughly. Reaction mixture was allowed to stand at room temperature, after reaction, the mixture was then transferred to a 96-well plate and then absorbance was measured at 734 nm. ABTS values were expressed as mg AAE/g.

Cell culture

3T3-L1 cell line were purchased from the ATCC (American Type Culture Collection, Manassas, VA, USA) and were maintained in DMEM supplemented with 10% FBS and 100 unit antibiotics at 37 °C in a 5% CO₂ atmosphere. For adipogenesis experiments, 3T3-L1 cells were grown until 2 days post-confluence. To induce differentiation, 3T3-L1 cells were maintained in DMEM containing 10% FBS, 10 μ g/mL insulin, 0.5 μ M DEX, and 0.5 mM IBMX (MDI) for 36 h. The medium was then changed to DMEM supplemented with 10% FBS and 10 μ g/mL of insulin every 2 days. During differentiation, the cells were treated with or without *O. malacophyllus* extract and NAC (10 mM).

Determination of lipid accumulation using oil red O staining

Adipocyte differentiation was monitored by staining cultured cells with oil red O (Lee et al., 2012). Briefly, cells were washed with PBS and fixed in 3.7% paraformaldehyde. 0.2% oil red O-isopropanol staining solution (3:2 mixture) was added to the cells at room temperature for 1 h. The cells were then washed with deionized water. The stained cells were observed under a microscope (Olympus

SZ61, Olympus Canada Inc., Markham, ON, Canada). To quantify oil red O, cells were incubated with isopropanol. After a 10-min incubation at room temperature, the solution was transferred to a 96-well plate, and absorbance was recorded at 510 nm.

Production of ROS in 3T3-L1 preadipocytes

3T3-L1 preadipocytes were grown to confluence and induced to differentiate into adipocytes. ROS production was detected using the NBT assay. ROS reduce NBT to formazan, which is dark blue and insoluble in water (Furukawa et al., 2004). Eight days after induction, the cells were incubated for 90 min in 0.2% NBT with PBS. The stained cells were observed under a microscope. The formazan was then dissolved in 50% acetic acid, and the absorbance was determined at 570 nm using a spectrophotometer.

RNA extraction and real-time PCR analysis

Total RNA was extracted from cell cultures using the RNeasy kit (Qiagen, Germany). One microgram of total RNA from 3T3-L1 adipocytes was reverse-transcribed with a high-capacity cDNA reverse transcription kits (Applied Biosystems, Warrington, UK) to produce cDNA. Real-time PCR was performed with selective primer sets and Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The reaction was performed for 40 cycles using the following conditions: denaturation at 95 °C for 15 s; annealing at 61 °C for 1 min; and extension at 72 °C for 30 s. β -Actin was used as an internal control. The sample mRNA abundance was calculated according to the $2^{-\Delta\Delta C_t}$ method for β -actin as an internal control. The primer sequences of all tested genes were listed in Table 1.

Protein extraction and western blot analysis

Protein extracts (30 μ g) were separated using SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked in TBS with 0.02% Tween 20 and 5% non-fat dried milk then incubated with primary antibodies specific for NOX4, G6PDH, Cu/Zn-SOD, Mn-SOD, PPAR γ , C/EBP α , aP2, and β -actin. Secondary antibodies conjugated to horseradish peroxidase (1:5000) were applied for 2 h. Bands were visualized using enhanced chemiluminescence (ECL) and Chemidoc image software (Microchemi 4.2, Bio-imaging Systems, USA).

Table 1 Primer sequences for real time-PCR

Primers	Sequences	
	Forward	Reverse
PPAR γ	5'-CTCCTGTTGACCCAGAGCAT-3'	5'-AGCCTTGCATCCTTCACAAG-3'
C/EBP α	5'-GCACAAGAACAGCAACGAGTA-3'	5'-GGCGGTCAATTGTCACCTGGTC-3'
aP2	5'-GACCTGGAAACTCGTCTCCA-3'	5'-CACATTGGCCACACCGTCCT-3'
NOX4	5'-GAAGCCCATTGAGGAGTCA-3'	5'-GGGTCCACAGCAGAAAATC-3'
G6PDH	5'-CGATGGCAGAGCAGGT-3'	5'-GATCTGGTCCTCACG-3'
Mn-SOD	5'-CAGCATGGGTTCACGTCCA-3'	5'-CACATTGGCCACACCGTCCT-3'
Cu/Zn-SOD	5'-GGGTTGGCTTGGTTTCAATA-3'	5'-AGGTAGTAAGCGTGCTCCCA-3'
β -actin	5'-CCGTCTCCCTCCATCGT-3'	5'-ATCGTCCCAAGTTGGTTACAATGC-3'

Statistical analysis

All data are presented as mean $\pm$ standard deviation of three independent experiments. Statistical significance was determined using ANOVA, and Duncan's multiple range test was used to determine significant differences between groups. All calculations were performed in Statistical Package for Social Science (SPSS, ver. 19, Chicago, IL, USA). A p value of < 0.05 was considered to indicate statistical significance.

Results and discussion

Total phenolic contents and antioxidant properties in *O. malacophyllus* extracts

The total phenolic contents and antioxidant capacities of the water, 30 and 70% ethanol extracts of *O. malacophyllus* (hereafter OMW, OME 30 and OME 70, respectively) are shown Table 2. These results revealed the OME 70 most effectively extracted phenolics from *O. malacophyllus*. Total phenolic contents of the OMW, OME 30 and OME 70 were 68.44, 72.56 and 112.31 mg GAE/g extract, respectively.

The lower yields of phenolic compounds obtained by water extraction were possibly due to ineffective tissue wall disruption (Hong and Kim, 2010). Ethanol was selected as the extraction solvent due to its wide use for the

extraction of phenolics from various food materials, and because the uses of ethanol and water are preferred on health and safety grounds (Westcott and Muir, 1998). In a previous study, various ethanol/water ratios were investigated, and 70% ethanol provided extracts with the highest total phenolic contents (Hong and Kim, 2010).

In DPPH assay, the OME 70 had the highest antioxidant capacity at 26.11 mg AAE/g extract (Table 2). Antioxidant capacities as determined by DPPH assay in descending order were as follows: OME 70 > OME 30 > OMW. ABTS assay also showed the OME 70 had the highest antioxidant capacity at 36.19 mg AA/g extract. Antioxidant capacities as determined by ABTS assays in descending order were: OME 70 > OME 30 > OMW. The antioxidant capacity of the OME 70 was attributed to its higher total phenolic content, as it has been reported that antioxidant activity is directly proportional to total phenolic content (Son et al., 2014). Therefore, the OME 70 was deemed optimal for the extraction of phenolic compounds and was used to evaluate the inhibitory effect of *O. malacophyllus* on ROS production and adipocyte differentiation in 3T3-L1 cells.

Effects of *O. malacophyllus* extract on ROS production and lipid accumulation during adipogenesis

To evaluate the cytotoxic effect of the OME 70, 3T3-L1 adipocytes were treated with 10, 25, 50, 100, 150, and

Table 2 Total phenolic contents and antioxidant capacities of *O. malacophyllus* extract

Extract solvent ($\mu\text{g/mL}$)	TPC (mg GAE/g extract)	DPPH (mg AAE/g extract)	ABTS (mg AAE/g extract)
Water	68.44 $\pm$ 1.52 ^c	19.46 $\pm$ 0.04 ^c	23.07 $\pm$ 0.04 ^c
30% Ethanol	72.56 $\pm$ 1.20 ^b	21.07 $\pm$ 0.28 ^b	28.82 $\pm$ 0.04 ^b
70% Ethanol	112.31 $\pm$ 1.20 ^a	26.11 $\pm$ 0.41 ^a	36.19 $\pm$ 0.92 ^a

Each value represents the mean $\pm$ SD of triplicate experiments

Values with different letters indicate statistically significant differences in same column at $p < 0.05$

200 µg/mL for 72 h. OME 70 at 150 and 200 µg/mL significantly and dose-dependently suppressed cell viability ($p < 0.05$) (data not shown). Thus, we used the OME 70 at concentrations of 10, 50, and 100 µg/mL in subsequent experiments.

The effect of OME 70 on ROS production in 3T3-L1 cells was determined using the NBT assay (Fig. 1). The production of dark blue formazan crystals was significantly

reduced by treatment with OME 70 as compared with MDI treated controls. OME 70 extract at 100 µg/mL decreased formazan formation by 72.39% (Fig. 1A), which was similar to that achieved by NAC (10 mM) the positive control (71.94%) ($p < 0.05$). These results illustrate OME 70 inhibited ROS production during 3T3-L1 preadipocyte differentiation.

We also examined the effect of OME 70 on lipid accumulation during adipocyte differentiation. OME 70 at 100 µg/mL inhibited lipid accumulation by 40.02% as compared with MDI (Fig. 1B). These results show OME 70 inhibited lipid accumulation by decreasing adipogenesis in differentiated 3T3-L1 adipocytes. In addition, OME 70 reduced ROS production during adipocyte differentiation. As shown by Lee et al. (2009), ROS are generated in fat cells in response to adipocyte differentiation and arrest growth by increasing mitotic clonal expansion. *Orostachys malacophyllus* extracts scavenge or inactivate ROS in the adipocytes and suppress adipocyte differentiation by inhibiting growth arrest.

Effects of OME 70 on the mRNA and protein expressions of ROS-generating genes

Recent reports suggest ROS production in adipose tissues of obese mice is related to the activation NADPH oxidase (NOX) and inhibition of antioxidant enzymes (Furukawa et al., 2004; Park et al., 2006). NADPH is regulated by G6PDH and affects lipid synthesis and ROS generation. G6PDH is a main intracellular source of NADPH, which is required as a cofactor for NOX enzyme and for lipid syntheses (Adachi et al., 2009; Vincent et al., 2006). Thus, we examined the inhibitory effect of OME 70 on ROS production by determining the mRNA levels of the ROS-generating genes NOX4 and G6PDH (Fig. 2). NOX4 mRNA levels were more reduced by OME 70 than MDI group (Fig. 2A). Furthermore, the effect of OME 70 at 10 µg/mL was greater than that of NAC at 10 mM. The expression of G6PDH mRNA was also greatly suppressed by OME 70 at all concentrations tested (Fig. 2B). Furthermore, the inhibitory effect of OME 70 on the mRNA levels of NOX4 and G6PDH paralleled its effect on ROS production. Consistent with decreases in these gene expressions, OME 70 suppressed the NOX4 (Fig. 2C) and G6PDH (Fig. 2D) protein levels during adipocyte differentiation. More specifically, these proteins levels were markedly more reduced by OME 70 at 100 µg/mL than by the MDI group. These findings show that OME 70 significantly reduced ROS production suppressing NOX4 and G6PDH protein levels during adipogenesis.

Fig. 1 Effects of *O. malacophyllus* extracts on ROS production (A) and lipid accumulation (B) during adipogenesis of 3T3-L1 preadipocytes. 3T3-L1 cells were treated with *O. malacophyllus* extract (10, 50 and 100 µg/mL) during adipogenesis. The control was adipocytes differentiated for 8 days with MDI hormone mixture. NAC was the positive control. Each value represents the mean $\pm$ standard deviation of triplicate experiments. Values with different letters indicate statistically significant differences among groups at $p < 0.05$

Fig. 2 Effects of *O. malacophyllus* extracts on ROS production and lipid accumulation during adipogenesis. 3T3-L1 cells were treated with *O. malacophyllus* extract (10, 50, and 100 µg/mL) during adipogenesis. The control was adipocytes differentiated for 8 days with MDI, a hormone mixture. NAC was the positive control. Total RNA was extracted on day 8, and (A) NOX4 and (B) G6PDH mRNA was measured using quantitative real-time PCR. Total protein

levels were measured using Western blotting with (C) NOX4 and (D) G6PDH antibodies. mRNA and protein levels were quantified and normalized to that of β -actin. The band intensities were measured with software Image J. Each value represents the mean $\pm$ standard deviation of triplicate experiments. Values with different letters indicate statistically significant differences among groups at $p < 0.05$

Effects of OME 70 on the mRNA and protein levels of antioxidant enzymes

The ROS scavengers Cu/Zn-SOD and Mn-SOD are located in cytoplasm and mitochondria, respectively. Generally, the superoxide anion is converted into oxygen and hydrogen peroxide by superoxide dismutase (SOD), or reacts with nitric oxide (NO) to form peroxynitrite. Accordingly, the superoxide anion is believed to be responsible for the formation of other types of ROS (Kruger and von Schaeven, 2003), and Cu/Zn-SOD and Mn-SOD reduce ROS levels by converting superoxide to hydrogen peroxide (McCord and Fridovich, 1988; Seo et al., 2016). Thus, we investigated the effect of OME 70 on the mRNA

expressions of Cu/Zn-SOD and Mn-SOD, which clear ROS from cytoplasm and mitochondria, respectively (Fig. 3). Adipocyte cells treated with OME 70 showed significant, dose-dependent increases in the mRNA levels of Cu/Zn-SOD (Fig. 3A) and Mn-SOD (Fig. 3B). Cu/Zn-SOD and Mn-SOD protein levels were examined by western blot (Fig. 3C, D). OME 70 at 100 µg/mL at increased the levels of Cu/Zn-SOD and Mn-SOD protein compared with MDI (Fig. 3C, D). The antioxidant, NAC, which was used as the positive control, adipocyte differentiation by generating ROS scavenging related factors (Calzadilla et al., 2011). The mRNA levels of Cu/Zn-SOD and Mn-SOD were more increased by NAC than MDI group. A similar result to this study was reported for increasing of SOD mRNA

Fig. 3 Effects of *O. malacophyllus* extracts on mRNA and protein expression of ROS scavenging enzymes. 3T3-L1 cells were treated with *O. malacophyllus* extract (10, 50, and 100 µg/mL) during adipogenesis. The control was adipocytes differentiated for 8 days with MDI, a hormone mixture. NAC was the positive control. Total RNA was extracted on day 8, and (A) Cu/Zn-SOD and (B) Mn-SOD mRNA were measured using quantitative real-time PCR. Total

protein levels were measured using Western blotting with (C) Cu/Zn-SOD and (D) Mn-SOD antibodies. mRNA and protein levels were quantified and normalized to that of β-actin. The band intensities were measured with software Image J. Each value represents the mean ± standard deviation of triplicate experiments. Values with different letters indicate statistically significant differences among groups at $p < 0.05$

expression by NAC. However, NAC greatly decreased protein expressions of Cu/Zn-SOD and Mn-SOD. This result means NAC potentially affect after transcription. RNA from every living organisms can be post-transcriptionally modified by a collection of many distinct biological and chemical modification (Zhao et al., 2017). Moreover, NAC has different mechanism from phenolic extract on antioxidant enzyme levels (Seo et al., 2013; 2016). Wang et al. (2015) reported that NAC did not affect directly antioxidant enzyme activity.

Effects of OME 70 on the mRNA and protein levels of adipogenic transcription factors

ROS production is closely linked with lipid accumulation in fat cells (Liu et al., 2012), and several researches have reported that fat accumulation and ROS production are regulated by adipogenic transcription factors (Lee et al., 2012; 2016). During adipogenesis, ROS levels are increased by increases in the expressions of PPARγ and C/EBPα (Lee et al., 2009; 2012). Adipocyte differentiation requires the synergistic actions of many transcription factors and adipogenic markers, including PPARγ, C/EBPα, and aP2 (Tanaka et al., 1997). PPARγ and C/EBPα are

induced before most adipocyte-specific genes are transcribed. These two regulators work cooperatively to enhance the expressions of another adipogenic marker, aP2 (Csaki and Reue, 2010). The overexpressions of PPAR γ , C/EBP α , and aP2 can accelerate adipocyte differentiation (Fève, 2005). To determine whether OME 70 inhibits adipocyte differentiation by increasing the expressions of adipogenic transcriptional regulators, the mRNA levels of PPAR γ , C/EBP α , and aP2 were measured in 3T3-L1 cells treated with OME 70 at 10, 50, and 100 $\mu\text{g/mL}$. As shown in Fig. 4, consistent with decreases in lipid accumulation, the expressions of PPAR γ , C/EBP α and aP2 were markedly reduced by OME 70 as compared with the MDI control. Furthermore, at 100 $\mu\text{g/mL}$ OME 70 reduced aP2 mRNA expression by up to 91.53% (Fig. 4C). This finding indicates that decreases in the mRNA expressions of PPAR γ and C/EBP α are followed, in part, by reductions in the mRNA expressions of their target genes. We believe aP2 might have been down-regulated by reductions in the mRNA expressions of these two adipogenic transcription factors, because it is a major target of PPAR γ and C/EBP α .

These results show that the inhibition of adipocyte differentiation by OME 70 might be mediated, in part, by the down-regulations of PPAR γ and C/EBP α and of their target gene, aP2. Consistent with these decreases in gene expression, OME 70 dose-dependently reduced PPAR γ , C/EBP α , and aP2 protein levels during differentiation (Fig. 4). More specifically, at 100 $\mu\text{g/mL}$ OME 70 markedly reduced aP2 protein levels during preadipocyte differentiation as compared with the MDI control (Fig. 4F). This finding suggests OME 70 decreases the differentiation of 3T3-L1 preadipocytes by suppressing expressions of adipogenic transcription factors. These results show that OME 70 suppresses ROS production in cells by regulating ROS-related gene expressions. It was shown recently that ROS promotes mitotic clonal expansion (a crucial stage during adipogenesis) by increasing C/EBP α binding to PPAR γ promoter and up-regulating PPAR γ . Furthermore, it has been shown antioxidants arrest mitotic clonal expansion and inhibit PPAR γ expression (Lee et al., 2009), and thus, it appears the inhibitory effect of OME 70 on lipid accumulation is probably due to ROS suppression.

Fig. 4 Effects of *O. malacophyllus* extracts on mRNA and protein expression of adipogenic transcription factors. 3T3-L1 cells were treated with *O. malacophyllus* extract (10, 50, and 100 $\mu\text{g/mL}$) during adipogenesis. The control was adipocytes differentiated for 8 days with MDI, a hormone mixture. NAC was the positive control. Total RNA was extracted on day 8, and (A) PPAR γ , (B) C/EBP α and (C) aP2 mRNA were measured using quantitative real-time PCR.

Total protein levels were measured using Western blotting with (D) PPAR γ , (E) C/EBP α and (F) aP2 antibodies. mRNA and protein levels were quantified and normalized to that of β -actin. The band intensities were measured with software ImageJ. Each value represents the mean $\pm$ standard deviation of triplicate experiments. Values with different letters indicate statistically significant differences among groups at $p < 0.05$.

In conclusion, the OME 70 contained a higher level of phenolics and had a greater antioxidant capacity than the water or 30% ethanoic *O. malacophyllus* extracts, and inhibited adipogenic transcription factors and reduced lipid accumulation. Moreover, OME 70 reduced ROS levels in adipocytes by decreasing the expressions of ROS generating genes, and it inhibited lipid accumulation by suppressing ROS production. These results indicate OME 70 has antiadipogenic and antioxidative effects during adipocyte differentiation. Further research is required to identify the phenolic components of *O. malacophyllus* responsible for its antiadipogenic and ROS reducing effects.

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