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Inhibitory effects of *Porphyra dentata* extract on 3T3-L1 adipocyte differentiation

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Abstract

This study was aimed to investigate the inhibitory effects of *Porphyra dentata* (*P. dentata*) extract on the adipogenesis of 3T3-L1 cells and evaluate its anti-obesity effect. The proliferation of 3T3-L1 cells and differentiation of adipocytes under treatment of *P. dentata* extract was examined by measuring the cell viability using alamarBlue assay and lipid droplets by Oil Red O staining. Results showed that *P. dentata* extract has no cytotoxicity effect and lipid droplets formation decreased in a concentration-dependent manner in 3T3-L1 cells. It has been confirmed that transcription factors affecting lipid accumulation and anti-adipogenic effects during cell differentiation are linked to *P. dentata* extract. We observed that *P. dentata* shows lowering the mRNA expression of peroxisome proliferator-activated receptor γ2 (PPARγ2), CCAAT/enhancer binding protein α (C/EBPα) that adipogenesis-associated key transcription factors and inhibiting adipogenesis in the early stages of differentiation. Treating the cells with *P. dentata* did not only suppressed PPARγ2 and C/EBPα but also significantly decreased the mRNA expression of adiponectin, Leptin, fatty acid synthase, adipocyte protein 2, and Acetyl-coA carboxylase 1. Overall, the *P. dentata* extract demonstrated inhibitory property in adipogenesis, which has a potential effect in anti-obesity in 3T3-L1 cells.

Keywords: 3T3-L1, Adipocyte differentiation, Adipogenesis, *Porphyra dentata*, Anti-obesity

INTRODUCTION

Obesity is one of the biggest health problems in the world today and the number of obese people are increasing in all over the world [1,2]. Obesity is defined as an increase in body weight [3] caused excessive accumulation of fat cells due to adipocyte differentiation [4]. Hence, it is also closely linked to metabolic diseases such as type 2 diabetes (T2D), liver disease, cardiovascular disease (CVD), cancers, hypertension and other disorders which increased these disease occurrence [5,6]. Accumulation of fat cells that cause obesity is by the differentiation of adipocytes called adipogenesis. Peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein (C/EBP) transcription factor family are key players to regulate the differentiation of preadipocytes by inducing adipogenic related

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Competing interests
No potential conflict of interest relevant to this article was reported.

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Cho JY, Kim SH.
Data curation: Cho JY, Kim SH.
Formal analysis: Choi SY, Lee SY, Cho JY, Kim SH.
Methodology: Choi SY, Lee SY, Jang Dh, Lee SJ.
Writing - original draft: Choi SY.
Writing - review & editing: Cho JY, Kim SH.

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This article does not require IRB/IACUC approval because there are no human and animal participants.

genes including adiponectin (ADIPOQ), leptin, fatty acid synthase (FAS), adpocyte protein 2 (aP2), and acetyl-coA carboxylase 1 (ACC) [7–10].

Laver, an edible seaweed species belonging to the genus Porphya, is commonly grown and consumed in Korea, China, and Japan. Laver is a rich source of vitamins, minerals, polysaccharides, phenolic compounds and mycosporine-like amino acids (MAAs) [11]. The polysaccharides components include laminarin and fucoidan while phenolic compounds present includes epigallocatechin gallate (EGCG) and catechin. Moreover, MAAs present in laver are mycosporine, shinorine, and porphyra-334. Several studies have shown that laver has antioxidant [12], anti-ultraviolet [13], anti-inflammatory [14], and antitumor [15] effects because of its bioactive compounds present. Marine algae, especially seaweeds are a promising source of anti-obesity agent [16] and anti-obesity effects are reported in various kinds of seaweed (brown, red and green) [17]. Also polysaccharides and phenol compounds are also reported to have anti-obesity effects [18,19].

Porphyra dentata (*P. dentata*) used in this study, is a kind of red algae and belongs to the Bangiaceae and Pyropia genus and an edible red seaweed in eastern Asian countries [20]. *P. dentata* contains polysaccharides and phenolic compound such as fucoidan, EGCG, and catechin [21]. It was reported that it has anti-inflammatory activity by suppressing nitric oxide production in LPS-stimulated macrophage [21]. As reported, although the components of *P. dentata* have a potential to regulate adipogenesis, anti-obesity effect on this extract have not been addressed.

The purpose of this study is to evaluate whether the *P. dentata* extract has inhibitory effects on adipogenesis from preadipocyte to mature adipocyte in 3T3-L1 cells.

MATERIALS AND METHODS

Chemicals and cell

Dulbecco's Modified Eagle's Medium (DMEM; high glucose) was purchased from Hyclone™ (Logan, UT, USA). Fetal bovine serum (FBS) were purchased from Gibco-BRL (Gaithersburg, MD, USA). 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), pioglitazone, insulin, Oil Red O powder, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formaldehyde Solution for 4% formalin was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). 2-propanol-GR and ethanol were purchased Merck (Kenilworth, NJ, USA). The 3T3-L1 cells were purchased from American Type Culture Collection (Rockville, CT, USA).

Preparation of the *Porphyra dentata* extract

Dried *P. dentata* was obtained from Mokpo Marine Food-industry Research Center (Mokpo, Korea). The dried *P. dentata* (50 g) was extracted with 1.5 L of 50% aqueous methanol (MeOH) at room temperature for one day and then filtered. The residues were re-extracted with 0.75 L 50% methanol and then filtered. The combined filtrates were evaporated at 38℃ under a vacuum. The 50% MeOH extracts of *P. dentata* were deposited at −20℃ until use in experiment.

Cell culture and differentiation

3T3-L1 preadipocytes (ATCC®, CL-173™) were cultured in DMEM (high glucose) supplemented with 10% FBS, 1% penicillin and streptomycin (Welgene, Gyeongsan, Korea) at 37℃ in 5% CO_2. For experiment, cells were seeded in 6-well plates at a density of 1.0×10^5 cells/well and grown to confluence. Forty-eight hours after confluence (day 0), adipogenesis was induced by adding differentiation medium (DMEM; high glucose containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine; IBMX, 1 μM Dexamethasone; DEX, 1 μM Pioglitazone, 10 μg/mL insulin,

1 μL/mL dimethyl sulfoxide; DMSO) for 48 h. Every two days, the medium was changed with DMEM; high glucose supplemented 10% FBS, 10 μg/mL insulin, 1 μL/mL DMSO until 8 days. The pre-adipocytes were maintained and changed medium for every 48 h with DMEM; high glucose, 10% FBS, and 1 μL/mL DMSO medium. To investigate the effects of *P. dentata* on adipocyte differentiation, cell culture was treated *P. dentata* 50% MeOH extract in different concentrations (6.25, 12.5, and 25 μg/mL) in the differentiation medium for every two days, from the beginning to the end of the experiment. After 5 days of treatment with *P. dentata* 50% MeOH extract. 3T3-L1 adipocyte cells were harvested for Real-time quantitative polymerase chain reaction (RT-qPCR) and after 8 days the 3T3-L1 adipocyte cells were fixed in 4% formalin for Oil Red O staining.

Cell viability assay

3T3-L1 cells were seeded in 96-well plates at a density of 7.5×10^2 cells/well containing 200 μL of 10% FBS-DMEM; high glucose. After cell seeding, *P. dentata* 50% MeOH extract was added by concentration dependent (6.25, 12.5, and 25 μg/mL). After 24 and 48hr after addition of the extract, alamarBlue™ Cell Viability Reagent (ThermoFisher Scientific, Waltham, MA, USA) was added and then fluorescence value was measured by SYNERGY multi-mode reader (BioTek, Seoul, Korea). Viability of cells was measured using alamarBlue assay according manufacturer instructions.

Oil Red O staining of lipid droplets

To measure the cell lipid droplets, the 3T3-L1 cells were stained with Oil Red O solution. 3T3-L1 cells were washed twice with PBS and adherent cells were fixed in 4% formalin for 10 min at room temperature. The 4% formalin was discarded and fresh 4% formalin was added and incubated for 1h at room temperature. After 1hr, is was washed with tertiary distilled water. The cells were added with 60% isopropanol and let it stand for 5 min at room temperature. After 5 min, 60% isopropanol was discarded and the cells were allowed dry completely at room temperature. After drying, 1 ml Oil Red O solution was added to each well and incubated at room temperature for 20 minutes. The cells were washed three times with tertiary distilled water and photographed using a Leica Microscopy, DE/Polyvar SC (Leica, Wetzlar, Germany).

Quantification of adipogenic gene expression using Real-time quantitative polymerase chain reaction

Total RNA was isolated from cells using Hybrid R™ (GeneAll Biothechnology, Seoul, Korea) including RiboEX™ treatment of samples to eliminate genomic DNA, protein, and lipid. Quality of RNA was determined by using Nanodrop 2000 spectrophotometer (ThermoFisher Scientific) and RNA gel electrophoresis. cDNA was synthesized from the total RNA using a RevertAid First Strand cDNA Synthesis kit (ThermoFisher Scientific). The real-time PCR was conducted using a CFX96™ Real-Time PCR Detection System (Bio rad, Hercules, CA, USA). The level of target gene cDNA was measured by TB Green® Premix EX Taq™ (Tli Rnase H plus) (Takara Bio, Kosatsu, Japan). All samples were analyzed in triplicate and quantified by the relative standard curve method using the gene expressions of L32 as a housekeeping gene. The sequences of the primer pairs used in this study are listed in Table 1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 0.8 (GraphPad Software, San Diego, CA, USA). All the data were analyzed using one-way analysis of variance (ANOVA) with multiple comparisons. Differences between groups were analyzed using *t*-test and values of $p < 0.05$ were

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Table 1. Primer sequence used in the RT-qPCR experiment

Gene	Forward (5'-3')	Reverse (3'-5')
L32	TCTGGTGAAGCCCAAGATCG	CTCTGGGTTTCCGCCAGT
PPARy2	GTGCTCCAGAAGATGACAGAC	GGTGGGACTTTCCTGCTAA
C/EBPa	TGGACAAGAACAGCAACGAG	TCACTGGTCAACTCCAGCAC
ADIPOQ	CCGTTCTCTTCACCTACGAC	TCCCCATCCCCATACAC
Leptin	TCAACTCCCTGTTTCCAAAT	TCTTCACGAATGTCCCACGA
FAS	CCCAGCCCATAAGAGTTACA	ATCGGGAAGTCAGCACAA
aP2	TGGAAGCTTGTCTCCAGTGA	AATCCCCATTTACGCTGATG
ACC1	GACGTTCGCCATAACCAAGT	CTGTTTAGCGTGGGGATGTT

considered statistically significant. All experiments were performed triplicate and data were expressed as mean ± SEM.

RESULTS

Porphyra dentata 50% MeOH extract shows no cytotoxicity in 3T3-L1 pre-adipocytes

We first performed the alamarBlue assay to test the effect of *P. dentata* 50% MeOH extract on cell viability. As shown in Fig. 1, *P. dentata* 50% MeOH extract at 6.25, 12.5, and 25 μg/mL showed no significant effect on cell viability in 3T3L1 mouse preadipocytes after 24 h and 48 h treatment. These results indicate that the *P. dentata* 50% MeOH extract have no cytotoxicity on cells.

Porphyra dentata 50% MeOH extract inhibits differentiation and lipid accumulation of 3T3-L1 cells

To investigate the effect of *P. dentata* extract on 3T3-L1 preadipocytes adipogenesis, we treated *P. dentata* extract with various concentrations for 8 days and stained the lipid droplets with Oil Red O during adipocyte differentiation (Fig. 2). Oil Red O staining assay revealed that *P. dentata* dramatically reduced lipid accumulation in a concentration dependent manner. These results indicate that *P. dentata* 50% MeOH extract suppressed adipocyte differentiation and lipid droplets formation in 3T3-L1 preadipocytes.

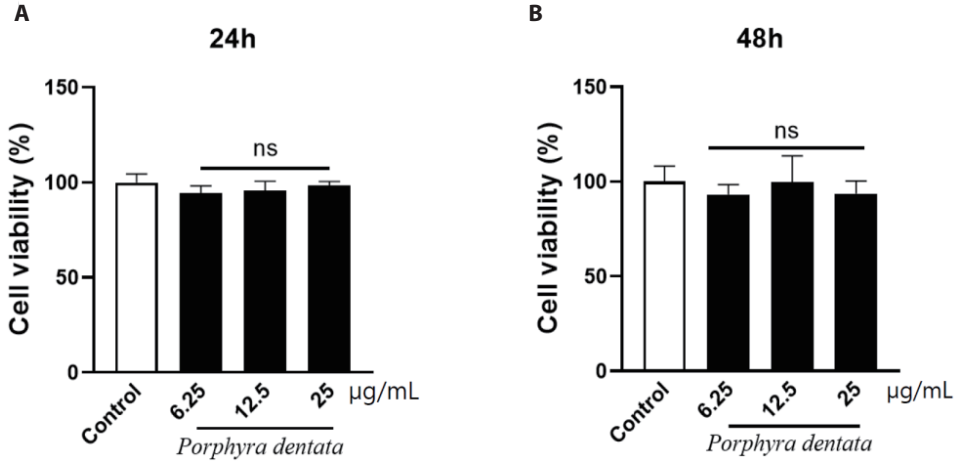

Fig. 1. Cell cytotoxicity of *Porphyra dentata* 50% MeOH extract on 3T3-L1 cells. 3T3-L1 cells were treated with different concentrations (6.25, 12.5, and 25 μg/mL) of *P. dentata* 50% MeOH extract for detection with alamarBlue assay.

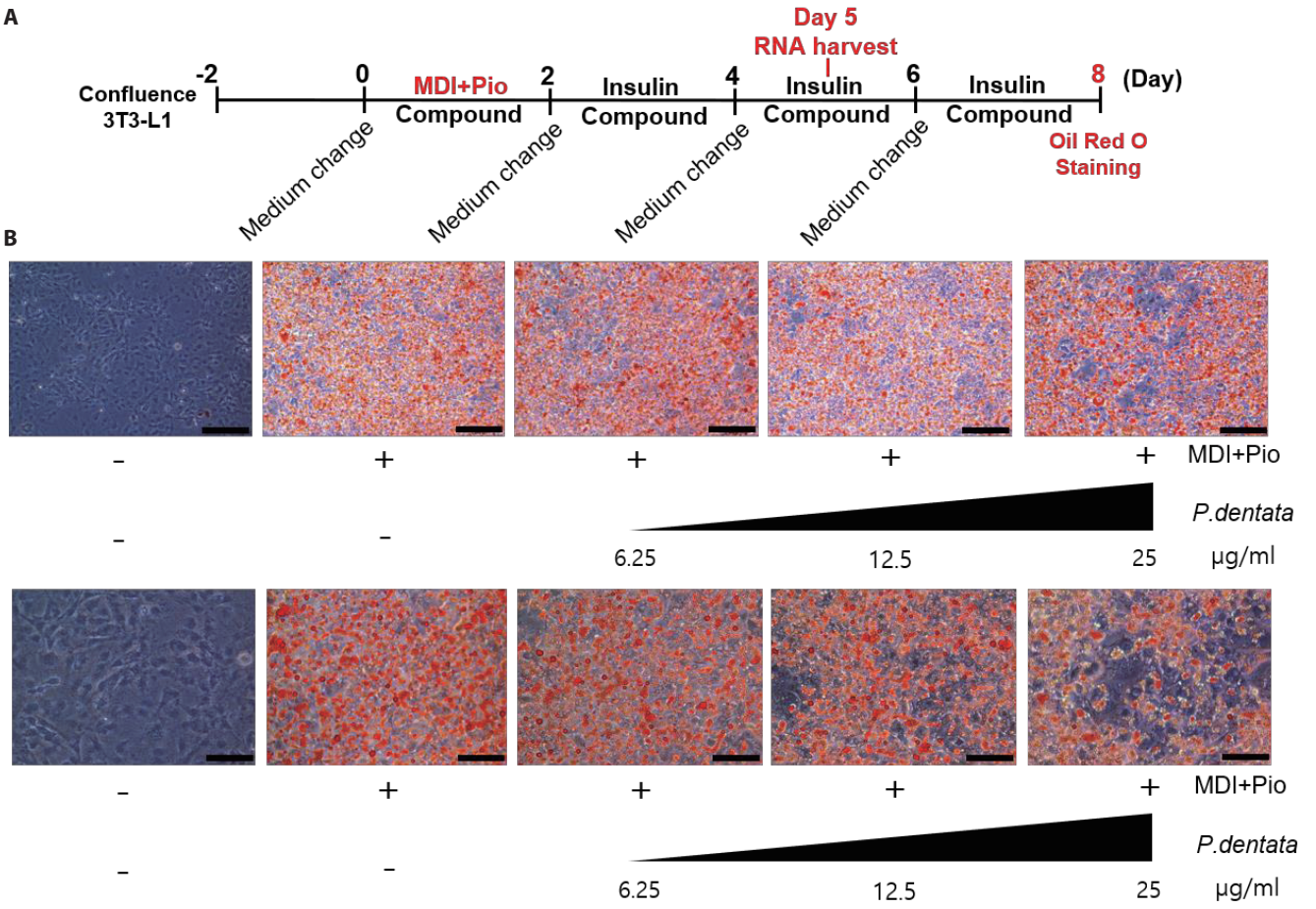

Fig. 2. Decreased accumulation of lipid droplets in differentiated 3T3-L1 cells treatment with *Porphyra dentata* 50% MeOH extract. (A) Time schedule of the culture with *P. dentata* 50% MeOH extract during the differentiation of 3T3-L1 cells. 3T3-L1 cells reach confluence after 2 days then, added MDI+pio medium with *P. dentata* 50% MeOH extract each of different concentration. The medium with *P. dentata* 50% MeOH extract was changed every 48 h containing 10 μg/mL insulin and 1μg/mL DMSO until day 8, followed by staining with Oil Red O. The control 3T3-L1 cells changed every 48 h to fresh medium with 10% FBS, 1 μg/mL DMSO. (B) Effect of *P. dentata* 50% MeOH extract on lipid droplets formation using Oil Red O staining. After 8 days of differentiation, lipid droplet accumulation was stained by Oil Red O staining. Upper panels, scale bar: 100 μm. Lower panels, scale bar: 50 μm. MDI, methylisobutylxantine, dexamethasone, insulin; pio, pioglitazone; FBS, Fetal bovine serum; DMSO, dimethyl sulfoxide.

Porphyra dentata 50% MeOH extract suppressed the expression of adipocyte differentiation marker

Next, we performed RT-qPCR analysis to examine the mRNA expression of adipogenic specific transcription factors such as PPAR-γ2, C/EBPα, and their target genes such as ADIPOQ, Leptin, FAS, aP2, and ACC1 after the treatment of *P. dentata* 50% MeOH extract. The extract decreased the PPAR-γ2, C/EBPα, as well as ADIPOQ, Leptin, FAS, aP2, and ACC1 mRNA expression. Gene expression of the PPAR-γ2, C/EBPα, and their adipogenic related genes following *P. dentata* treatment was significantly lower compared with that of differentiated control adipocytes treated MDI (methylisobutylxanthine, dexamethasone, insulin) plus pioglitazone. *P. dentata* 50% MeOH extract significantly downregulated the expression of adipogenesis associated genes in a dose-dependent manner (Fig. 3).

DISCUSSION

The 3T3-L1 mouse preadipocytes have been widely used for screening the effective agents to regu-

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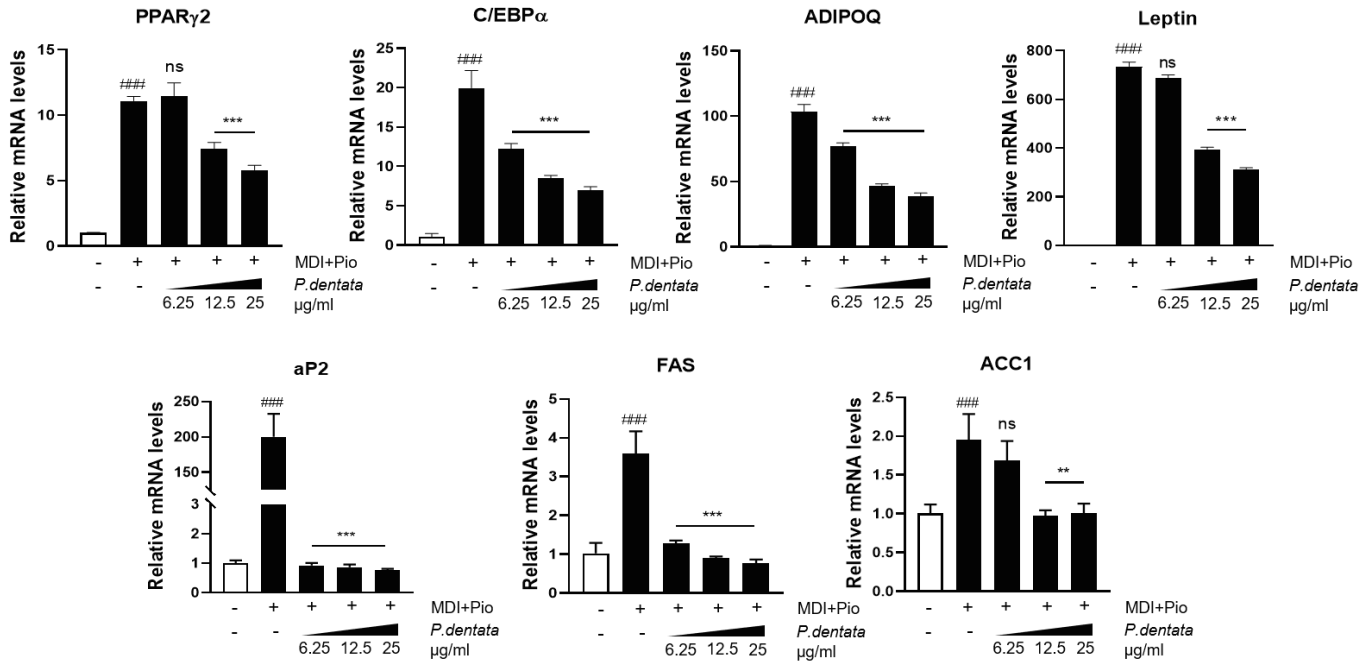

Fig. 3. Expression of adipogenic related genes in 3T3-L1 cells with *Porphyra dentata* 50% MeOH extract. The expression of adipogenic related genes which PPAR-γ2, C/EBPα (adipogenic transcription factors), ADIPOQ, leptin, aP2 (adipokine), FAS and ACC1 (lipogenic enzyme). 3T3-L1 cells cultured with various concentrations of *P. dentata* 50% MeOH extract (6.25, 12.5, and 25 μg/mL) with differentiation media were analyzed on day 5 by RT-qPCR. *P. dentata* 50% MeOH extract treatment decreased adipogenic related genes mRNA expression in a concentration-dependent manner on 5 days. Data were presented as mean and standard errors from three experiments. $^{###}p < 0.001$ vs. preadipocyte, $^{***}p < 0.001$, $^{**}p < 0.01$ vs. MDI+pio. All data are presented as mean ± SD, and experiments were performed three times. PPARγ2, peroxisome proliferator-activated receptor γ2; MDI, methylxanthine, dexamethasone, insulin; C/EBPα, CCAAT/enhancer binding protein α; ADIPOQ, adiponectin; aP2, adipocyte protein 2; FAS, fatty acid synthase; ACC1, acetyl-coA carboxylase-1.

late the adipogenesis. The adipogenesis was determined by Oil Red O staining to show the amount of lipid droplets by specifically staining neutral triglycerides with high levels of adiopocyte-related genes expression in 3T3-L1 cells [22].

In the present study, we demonstrated that *P. dentata* extract dramatically inhibited lipid accumulation during adipocyte differentiation with decreasing PPARγ2, C/EBPα, ADIPOQ, leptin, FAS, aP2, and ACC1 expression. One of the alternative splicing forms of PPAR, PPARγ2, is a lipid-activated transcription factor which specifically expressed in adipose tissue [23]. In response to fatty acids, PPARγ2 leads to fat accumulation in adipocytes by modulating target genes involved in lipid metabolism [24]. However, PPARγ2 does not function alone but cooperatively with transcription factors in the C/EBP family to induce adipocyte differentiation [9,24]. The C/EBPs belong to the basic-leucine zipper class of transcription factors and has several forms including C/EBPα, C/EBPβ, C/EBPγ, and C/EBPδ [25]. The temporal expression of these factors during adipocyte differentiation indicates a cascade whereby early induction of C/EBPβ and C/EBPδ leads to induction of C/EBPα, which C/EBPα induces expression of many adipogenic related genes directly [26]. In this study, the mRNA expression of PPARγ2 and C/EBPα decreased significantly after treatment of *P. dentata* extract compared with that in differentiated control cells. It has been reported that PPARγ2 and C/EBPα cooperates to increase adipogenic genes using a positive feedback loop between them leading to adipogenesis [27]. ADIPOQ, leptin, and aP2 investigated in this study, are adipokine which cytokine secreted by adipose tissue and in obesity [28,29]. ADIPOQ is an adipocyte-specific factor, which adipocyte-derived hormone, it is abundantly produced and secreted by adipose tissues and widely recognized for its anti-inflammatory effects [30]. Leptin is also

adipocyte-derived hormone that circulates in proportion to fat mass and acts as a negative regulator of energy homeostasis [31]. aP2 called fatty acid binding protein 4 (FABP4) is one of the only genes characterized by sufficient regulatory sequences to direct adipose-specific expression *in vivo* [32,33]. Having played an important role as adipocytes differentiation marker, as it can lead to the development of increasingly large adipocytes by leptin resistance and contribute to the accumulation of excessive fat masses found in obese states [34]. These adipokine levels were increased during differentiation from preadipocytes to maturation adipocytes [34,35]. The other adipogenic related genes, FAS and ACC1 are lipogenic enzymes. FAS is the key enzyme in lipogenesis, catalyzing the reactions for the synthesis of long-chain fatty acids [36]. ACC1 is a multi-subunit lipogenic enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA for the biosynthesis of fatty acids [37]. Plants have been used as traditional natural medicines for healing many diseases [38] and many studies have shown that plant-derived foods have the potential to reduce obesity [39]. A plant belonging to the genus *Porphyra*, called laver, also consumed mainly as processed food or used a source of health-enhancing substances and this group have a unique active substances which provide health benefits [39,40]. *Porphyra* species contain biological active compounds, including polysaccharides, carotenoids, phenolic compounds, and MAAs. These compounds have been reported to have antioxidant [41], anti-inflammatory [42], anti-cancer [43,44], prevention of nervous system [45], and bone disease [46]. In particular, fucoidan, carotenoids, and phenolic compounds (EGCG) inhibited lipid accumulation in 3T3-L1 cells.

Previously, Kim et al. [22] indicated that *Pyropia yezoensi* (*P. yezoensis*) methanol extract, one types of laver contain high MAAs content (120 mg/g dried extract) reduces the contents of accumulation lipid determined by Oil Red O staining in a dose-dependent manner [22]. However, they demonstrated that treatment with high concentration (5 mg/mL) of the *P. yezoensis* methanol extract inhibited adipogenesis with decrease of preadipocytes proliferation via oxidative stress and proapoptotic effects. Our result indicates that *P. dentata* 50% MeOH extract at low concentration of ~25 μg/mL significantly suggest anti-adipogenesis in a dose-dependent manner with no cytotoxicity in 3T3-L1 cells. Hence, further study is needed to identify whether bioactive compounds (polysaccharides, carotenoids, phenolics, etc.) contained in laver contribute suppression of lipid accumulation in adipocyte.

In conclusion, *P. dentata* extract inhibited the accumulation of lipid droplets in concentration-dependent manner. Moreover, *P. dentata* extract inhibits the expression of adipogenic related genes involved in the adipogenesis from preadipocytes to mature adipocytes in 3T3-L1 cells. Especially, in all experiments, lipid droplets formation and gene expression are inhibited in a concentration-dependent manner (6.25, 12.5, and 25 μg/mL) of *P. dentata* extract. Thus, the result revealed that *P. dentata* has an effect of anti-obesity that inhibits adipogenesis. Since pharmacological effects of the *Porphyra* species are proven [47], and *P. dentata* belonging to that species is also consumed as food, it has the potential to be used as a dietary supplement and medicinal food item to suppress obesity.

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