IN VITRO ANTIOXIDANT AND ANTI-ADIPOGENIC EFFECTS OF SLENDESTA, STANDARD
POTATO EXTRACTS CONTAINING 5% PROTEASE INHIBITOR II

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Abstract

Background: The objective of the present study is to observe the anti-adipogenic effects of Slendesta (SLD), a standard potato protein extracts containing 5% potato protease inhibitor II (PI2) on the 3T3-L1 preadipocytes which are able to differentiate into mature adipocytes and accumulate lipids, as an obesity model with cytotoxicity and antioxidant effects.

Materials and Methods: The cytotoxicity of SLD was observed against 3T3-L1 preadipocyte cell line by MTT assay, and also anti-adipogenic effects were observed through lipid accumulation assay during 3T3-L1 differentiation as comparing with *N*-Acetyl-L-cysteine (NAC). In addition, antioxidant effects of SLD were detected by free radical scavenging capacity and superoxide dismutase (SOD)-like activity as comparing with ascorbic acid.

Results: The SLD showed obvious cytotoxicity against 3T3-L1 pre-adipocyte cell line at higher concentrations, from 1.5 mg/ml for 72 h treatment, and the cytotoxic IC_{50} of SLD after 24, 48 and 72 h treatment times were detected as 10.11 ± 0.67, 5.71 ± 0.37 and 5.34 ± 0.21 mg/ml, respectively. The SLD also concentration-dependently inhibited the lipid accumulations formatted during 3T3-L1 cell differentiations. The adipogenic specific genes including PPARγ, C/EBPα, C/EBPβ and leptin were found to be reduced in SLD and NAC-treated cells compared to control cells. Furthermore, the SLD effectively showed DPPH radical scavenging activity ($IC_{50} = 161.98 \pm 64.65$ μg/ml) and SOD-like effects ($IC_{50} = 284.54 \pm 54.47$ μg/ml), and the cellular ROS was significantly inhibited in the SLD-treated cells compared to control cells.

Conclusion: The results suggest that SLD effectively inhibit the differentiations of 3T3-L1 preadipose cell probably through antioxidant activities and direct cytotoxicity in case of higher concentration, along with satiety effects mediated by increases of circulating cholecystokinin. These findings are considered as direct evidences that SLD may serve as a predictable functional ingredient for obesity as an alternative therapy.

Key words: Slendesta, potato protease inhibitor II, 3T3-L1 cell, cytotoxicity, anti-adipogenic effects, antioxidant effects.

Abbreviations: BCS = Bovine calf serum; CCK = Cholecystokinin; MEM = Dulbecco's modified Eagle's medium; DPPH = 1,2-diphenyl-2-picrylhydrazyl; FBS = Fetal bovine serum; FDA = Food and drug administration; HPLC = High-performance liquid

chromatography**;** IBMX = 3-isobutyl-1-methylxanthine**;** IC_{50} = 50% inhibitory concentrations**;** MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide**;** NAC = N-acetyl-L-cysteine**;** NADPH = Nicotinamide adenine dinucleotide phosphate**;** OD = Optical density**;** P/S = Penicillin-streptomycin**;** PBS = Phosphate-buffered saline**;** PI2 = Potato protease inhibitor II**;** ROS = Reactive oxygen species**;** SLD = Slendesta, Standard potato protein extracts contains 5% PI2, test substance**;** SOD = Superoxide dismutase

Introduction

Obesity contributes to the etiologies of various comorbid conditions, such as cardiovascular disease, hypertension and type 2 diabetes (Wendel et al., 2008; Costanzo et al., 2015). In addition to storing lipid for energy, adipose secretes a variety of adipokines, many of which affect metabolism and inflammation in adipose and non-adipose tissues. Modulation of endocrine functions in the adipose tissue can contribute to a chronic state of inflammation, which leads to pathogenesis of the associated disorders, specifically insulin resistance (Tilg and Moschen, 2006). Recent studies show that obesity may induce systemic oxidative stress; oxidative species increased in fat accumulation could be an early instigator in the obesity-associated metabolic syndrome (Calzadilla et al., 2011; Alcala et al., 2015). A number of medicines are currently used or developed for obesity, including orlistat or sibutramine, the only FDA approved drug therapies for obesity (Komarnytsky et al., 2011). However, currently available pharmacological agents for obesity have strict limitations due to various adverse effects (Viner et al., 2010). Therefore, the novel therapeutics to reduce food intake and body weight with minimal adverse reactions are highly desirable, and natural antioxidants have been especially highlighted because of lower side effects (Calzadilla et al., 2011; Lee et al., 2014). Until now, various antioxidants showing elimination effects of reactive oxygen species (ROS) and free radical scavenger activities like ascorbic acid, α-tocopherol, carotenoid, flavonoid, butylated hydroxy toluene, butylated hydroxy anisole and propylene glycol, have been used as ingredients for various functional foods (Cho et al., 2011; Kang et al., 2014; Kang et al., 2015; Yang et al., 2015). Potato tuber is the source of potato protease inhibitor II (PI2) active in eliciting a satiety response (Rawlings et al., 2008) and delayed gastric emptying in humans (Schwartz et al., 1994). Potato protease inhibitors inhibit food intake and increase circulating cholecystokinin (CCK) levels by a trypsin-dependent mechanism, and may be used as an effective and alternative anti-obese agent, with less toxicity (Komarnytsky et al., 2011). Although several methods to isolate and purify PI2 have been developed over the years on a laboratory scale, all of them are laborious or expensive (Kim et al., 2006b; Rawlings et al., 2008). Potato protein recovery is also often complicated by interactions with non-protein components of potato tubers that lead to poor solubility and reduced biological activity of the protein fraction, thus hampering the potential therapeutic applications (Komarnytsky et al., 2011). Given the low yield and complexity of PI2 isolation, a crude potato protease inhibitor concentrate that contains several thermostable protease inhibitors, including PI2 were developed and it also showed potential satiety-promoting activity *in vivo*, by elevated the CCK circulations similar to PI2 (Komarnytsky et al., 2011). Slendesta (SLD) is a standard potato protein extracts containing 5% PI2. In this study, we intended to observe the anti-adipogenic effects of SLD on the 3T3-L1 preadipocytes as an obesity model which are able to differentiate into mature adipocytes and accumulate lipids, with cytotoxicity and antioxidant effects (Calzadilla et al., 2011; Yang et al., 2014; Nie et al., 2015).

Materials and Methods

Materials

A light beige colored powder of SLD, containing 5% PI2 as an active ingredient, was prepared and supplied by AriBio (Seoul, Korea) as a test material. Briefly, raw potatoes were grinded in bleach based sanitizing solution with citric acid, and

centrifuged for removing fiber and undissolved solid (4000 rpm, 60 L/min). The potato supernatant was heated to 70 °C for 1 h to obtain stable potato protein. The resultant potato juice was filtered through a 10 kDa pore for removing excessive water and undissolved solids, and increasing the purity. Then, it was dried and the acquired powders were finally formulated with microcrystalline cellulose (45% in SLD) to adjust PI2 contents as 5% under high-performance liquid chromatography (HPLC) analysis (Fig. 1). White to light yellow solid of L-Ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and white powder of *N*-Acetyl-L-cysteine (NAC; Sigma-Aldrich, St. Louis, MO, USA) were used as standard references for antioxidant and anti-adipogenic effects.
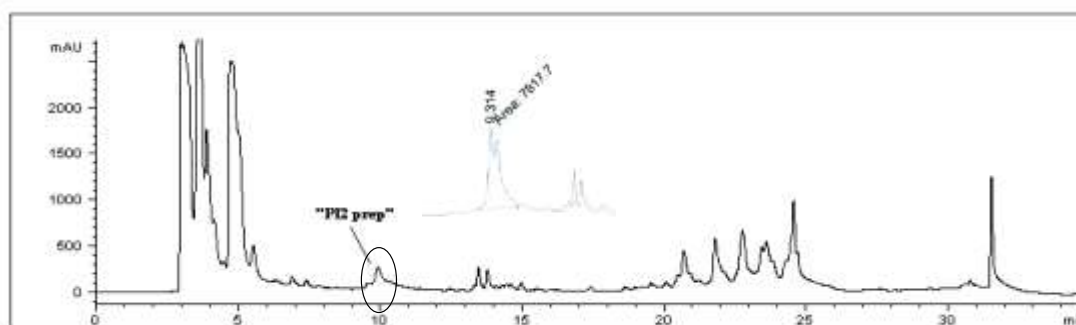
Figure 1: Representative HPLC Chromatogram of PI2 in SLD. Note that SLD used in this study containing 5% PI2 (ranged in 5.02~5.14%) in HPLC system based analysis. PI2 = potato protease inhibitor II, SLD = Slendesta, standard potato protein extracts as a test material, HPLC = High-performance liquid chromatography.

Cell Cultures

3T3-L1, a preadipocyte cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured according to the manufacturer's protocols. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) with 10% Bovine calf serum (BCS; Gibco, Gaithersburg, MD, USA) and 1% penicillin-streptomycin (P/S; Hyclone, Logan, UT, USA) at 37°C in a fully humidified atmosphere of 5% CO_2. After the 3T3-L1 cells reached to 90% confluency, the adipocyte differentiation was induced in the post-confluent cells via 2 day-treatments with DMEM containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and a hormonal mixture (MDI) (0.5 mM IBMX, 1 μM dexamethasone and 10 μg/ml insulin; Sigma-Aldrich, St. Louis, MO, USA). Then, the culture medium was replaced with DMEM containing 10% FBS and 10μg/ml insulin only as a supplement, and it was changed every other day. For the treatments, 2-day post-confluent cells were differentiated with or without SLD (0.75 and 1 mg/ml) and NAC (10 mM).

Cell Viability

Cell viability was determined using MTT assay. The 3T3-L1 cells were seeded in 24-well plates at a density of 2×10^3 cells per well, and cultured at 37°C in 5% CO_2. After one day, a fresh medium containing 10% FBS was added, and the cells were incubated in a CO_2 incubator at 37°C in the presence of samples (0.01, 0.1, 0.75, 1, 1.5, 2.5, 5 and 10 mg/ml) for 24, 48 and 72 h, before being treated with 100 μl of 2.5 mg/ml of MTT (Sigma-Aldrich, St. Louis, MO, USA). The cells then were incubated at 37°C for an additional 4 h. The medium containing MTT was discarded, and MTT formazan was extracted with 1 ml of dimethyl

sulfoxide. The absorbance of optical density (OD) was read at 570 nm with a reference wavelength of 650 nm with a micro-plate reader (Sunrise; Tecan, Männedorf, Switzerland). The cell viability was calculated as follow Equation; Cell viability (%) = (ODs/ODc)×100, ODs is the absorbance of the treated cells, whereas ODc is the absorbance of the non-treated cells at 570 nm as a negative control. The results were expressed in terms of IC_{50} (the concentration reach the cell viability as 50% of control).

Anti-Adipogenic Effects

Oil Red O Staining

After the induction of adipocyte differentiation, the cells were stained with Oil Red O. Briefly, cells were washed with phosphate-buffered saline (PBS), and fixed with 10% formalin for 30 min. The cells were washed with distilled water, and stained with Oil Red O working solution (0.5% Oil Red O in 70% isopropanol; Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Then the excessive stain was washed with distilled water, and the stained cells were dried. The differentiation was determined via elution with 100% isopropanol and the OD was measured at 490 nm.

Total RAN extraction and RT-PCR analysis: The mRNA expressions for PPARγ, C/EBPα, C/EBPβ and leptin on the harvest cells were detected by real-time RT-PCR according to previously established methods (Jeon et al., 2004; Park et al., 2012). Briefly, RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentrations and quality were determined by CFX96[TM] Real-Time System (Bio-Rad, Hercules, CA, USA). To remove contaminating DNA, samples were treated with recombinant DNase I (DNA-free; Ambion, Austin, TX, USA). RNA was reverse-transcribed using the reagent High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, the cDNA strand first was synthesized from the total RNA and then the mixture of the primers and the cDNA products was amplified by PCR, and the conditions of PCR amplification were 58°C for 30 min, 94°C for 2 min, 35 cycles of 94°C for 15 sec, 60°C for 30 sec, 68°C for 1 min, and then 72°C for 5 min. Primers were synthesized by Bioneer (Daejeon, South Korea) and the sequences are: PPARγ, 5'-GGTGAAACTCTGGGAGATTC-3' and 5'-CAACCA-TTGGGTCAGCTCTT-3'; C/EBPα, 5'-AGGTGCTGGAGTTGACCAGT-3' and 5'-CAGCCTAGAGATCCAGCGAC-3'; C/EBPβ, 5'-TGAAGAGGTCGGCGAAGAGTTCG-3' and 5'-GGCGGTCATTGTCACTGGTCAAC-3'; Leptin, 5'-CCAAAACCCTCATCAAGACC-3' and 5'-CTCAAAGCCACCACCTCTG-T-3'; G3PDH, 5'-CCATCAACGACCCCTTCAT-3' and 5'-GTCCTCAGTGTAGCCCAAGA-3'. Analysis was carried out using ABI Step One Plus Sequence Detection System (Applied Biosystems, Foster City, CA, USA), and their expression levels were calculated as relative to vehicle control. The expression of glyceraldehydes-3- phosphate dehydrogenase (G3PDH) mRNA was used as a control for tissue integrity in all samples.

Antioxidant Effects

The antioxidant effects were measured based on the free radical scavenging capacity (Kedare and Singh, 2011) and SOD-like activity (Marklund and Marklund, 1974) in this experiment.

DPPH radical scavenger activity test: The assay for free radical scavenging capacity was carried out according to the method that has been reported previously (Kedare and Singh, 2011). The DPPH radical shows a deep violet color due to its unpaired electron, and the radical scavenging capacity can be followed spectrophotometrically by the loss of absorbance at 525 nm. Briefly, 95% ethanolic solution with 0.2 mM DPPH (Sigma-Aldrich, St. Louis, MO, USA) was added to a sample of the stock. Each sample solution was diluted with distilled water for final concentrations at 6.25, 12.5, 25, 50, 100 and 200 μg/ml in L-Ascorbic acid, or 12.5,

167

25, 50, 100, 200 and 500 μg/ml in SLD, and they were then agitated. After 10 min, their ODs were measured at 525 nm with a UV/Vis spectrophotometer (OPTIZEN POP, Mecasys, Daejeon, Korea). The free radical scavenging activity was calculated according to the following Equation; DPPH radical scavenging activity (%) = 100-[(ODs/ODc) ×100], ODs is the absorbance of the experimental sample, whereas ODc is the absorbance of the vehicle treated control at 525 nm. The results were expressed in terms of IC_{50} (the concentration needed to reduce 50% of DPPH).

SOD-like activity test: The assay for free radical scavenging capacity was carried out according to the method that has been reported previously (Marklund and Marklund, 1974). Each sample solution was diluted with distilled water for final concentrations at 6.25, 12.5, 25, 50, 100 and 200μg/ml in L-Ascorbic acid, or 12.5, 25, 50, 100, 200 and 500 μg/ml in SLD, and they were then agitated with Tris-HCl buffer [50 mM Tris(hydroxymethyl) aminomethane and 10 mM EDTA , pH 8.5] and 7.2 mM pyrogallol (Merck, Whitehouse Station, NJ, USA) for 10 min at 25°C. After agitation, the reactions were stopped by adding 1N HCl (Daejung, Siheung, Korea). Among the solutions, the oxidative pyrogallol detected at 420 nm was measured after 10 min with a UV/Vis spectrophotometer. The SOD-like activity was calculated according to the following Equation; SOD-like activity (%) = 100-[(ODs/ODc)×100], ODs is the absorbance of the experimental sample, whereas ODc is the absorbance of the vehicle treated control at 420nm. The results were expressed in terms of IC_{50} (the concentration needed to reduce 50% of pyrogallol oxidations).

Measurement of cellular ROS levels: To assess the effect of SLD treatment on ROS generation, 3T3-L1 cells were treated with SLD (0.75 and 1 mg/ml) and NAC (10 mM). After seeding cells in 6-well plates, the production of ROS was measured by membrane permeable 2'-7'-dichlorofluorescin diacetate (H_2DCFDA) (Sigma-Aldrich, St. Louis, MO, USA) which is oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) by ROS following cleavage of its acetate groups by intracellular esterases. The cells were treated with 10 μM H_2DCFDA for 30 min at 37°C and then analyzed with image-based cytometry with a Tali[®] Image-Based Cytometer (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analyses

All *in vitro* data were expressed as means ± SD in three or six independent experiments, respectively. Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If the Levene test indicated no significant deviations, the obtained data were analyzed by one way ANOVA test followed by Tukey's multi-comparison (Tukey) test to determine which pairs of group were significantly different. In case of significant deviations at Levene test, a non-parametric comparison test, Kruskal-Wallis H test was conducted. When a significant difference is observed in the Kruskal-Wallis H test, the Mann-Whitney U (MW) test was conducted to determine the specific pairs of group. IC_{50} values in each in vitro assay were calculated by Probit methods, and statistical analyses were conducted using SPSS for Windows (Release 14.0K, SPSS Inc., Chicago, IL, USA) (Kim et al., 2014).

Results

Cytotoxicity of SLD on 3T3-L1 Preadiopocytes

Significant (p<0.01) decreases of viabilities were detected from 5 mg/ml concentration of the SLD treated 3T3-L1 cells at 24 and 48 h treatment times, and accordingly, the cytotoxic IC_{50} of SLD for 24 and 48 h treatment on the 3T3-L1 preadipocytes were calculated as 10.11 ± 0.67 and 5.71 ± 0.37 mg/ml, respectively, In addition, the 3T3-L1 cell viabilities of SLD for 72 h

treatment were significantly ($p<0.01$) decreased from 1.5 mg/ml concentration, and they showed the IC_{50} value of 5.34 ± 0.21 mg/ml, in this experiment. However, no significant changes on the 3T3-L1 cell viabilities were demonstrated at 0.01, 0.1, 0.75 and 1 mg/ml concentrations of SLD treated cells as compared with non-treated vehicle control, regardless of treatment times; 24, 48 and 72 h (Fig. 2).
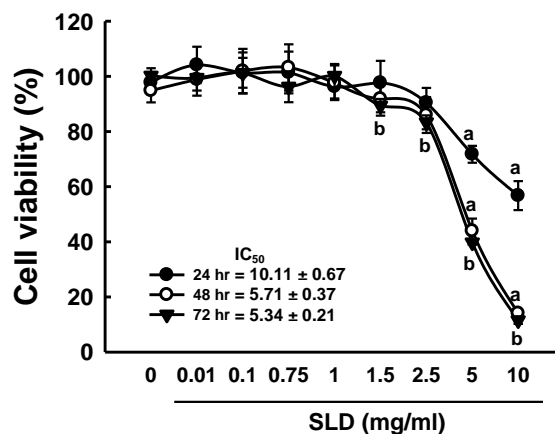
Figure 2: Effects of SLD on 3T3-L1 Preadipocyte Viabilities. Values are expressed as means $\pm$ SD in six independent experiments. SLD = Slendesta, standard potato protein extracts, a test substance, MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, IC_{50} = concentration reach to cell viability as 50% of control. [a] $p<0.01$ as compared with control by Tukey test, [b] $p<0.01$ as compared with control by MW test.

Effect of SLD on Lipid Accumulation in 3T3-L1 Cells

The anti-adipogenic effects of SLD were measured by lipid accumulation during the differentiation of 3T3-L1 cells using Oil Red O staining. The SLD concentrations at 0.75 and 1 mg/ml, that did not affect cell viability, were selected in this anti-adipogenic assay. As shown in Fig 3, in the differentiated 3T3-L1 cells, the control cells showed lipid accumulation strongly, whereas the SLD-treated cells showed significant ($p<0.01$) decreases of the intracellular lipid accumulation, concentration-dependently. In addition, 10 mM of NAC-treated cells also resulted in significant decreases of lipid accumulation compared with control cells (Fig. 3).
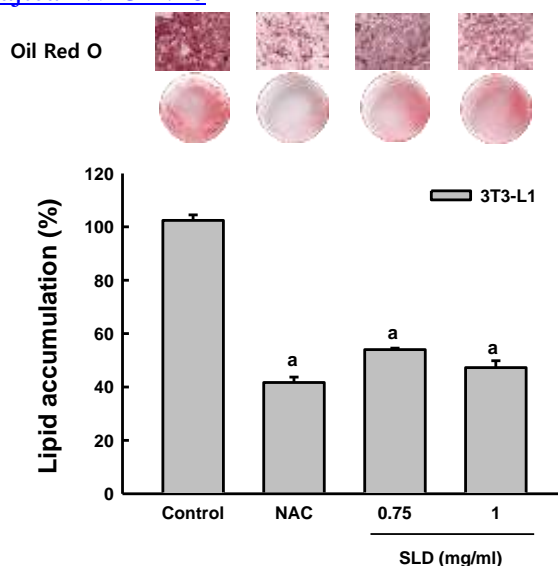
Figure 3: Effects of SLD and NAC on Lipid Accumulations during 3T3-L1 Cell Differentiations. Values are expressed as means ± SD in three independent experiments. SLD = Slendesta, standard potato protein extracts, as a test material, NAC = *N*-acetyl-L-cysteine. [a] p<0.01 as compared with control.

Next, we investigated the effect of SLD on adipogenic genes expression. Transcript levels for adipogenic specific genes including PPARγ, C/EBPα, C/EBPβ and leptin were assessed in the SLD-treated cells by RT-PCR analysis. The results indicated that the SLD reduced the expression of PPARγ, C/EBPα, C/EBPβ and leptin compared to differentiated 3T3-L1 cells (Figs. 4A-D).
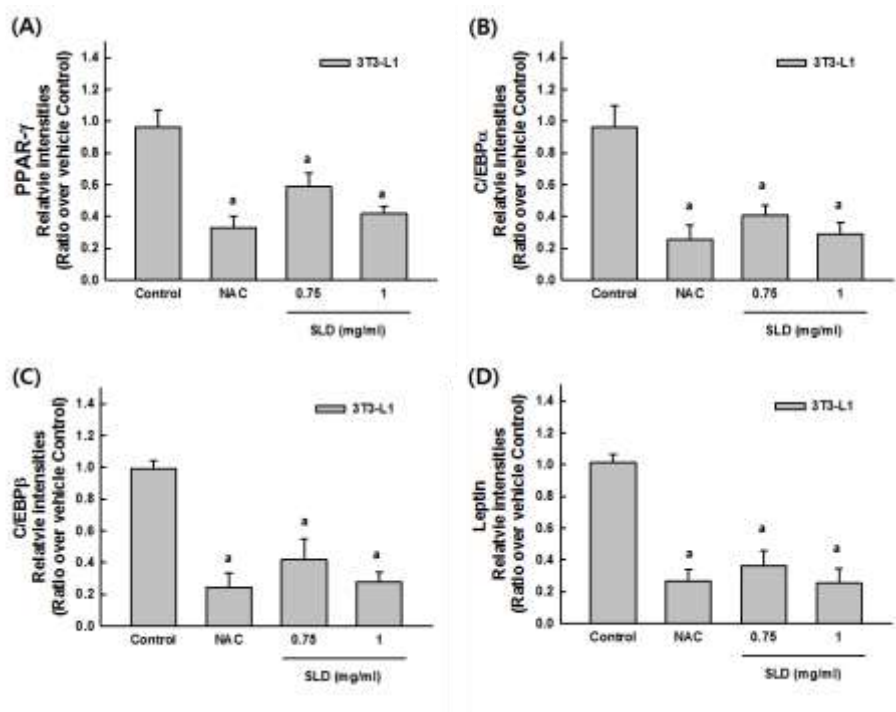

Figure 4: Effects of SLD and NAC on Adiogenesis-related Gene Expression during 3T3-L1 Cell Differentiations. Transcript levels for (A) PPARγ, (B) C/EBPα, (C) C/EBPβ, (D) leptin, and G3PDH were measured using RT-PCR analysis following the treatment of 3T3-L1 cells with NAC or SLD (0.75 and 1 mg/ml). Values are expressed as means ± SD in three independent experiments. SLD = Slendesta, standard potato protein extracts, as a test material, NAC = *N*-acetyl-L-cysteine. [a] p<0.01 as compared with control.

Antioxidant Effects

DPPH radical scavenger activity: Significant ($p < 0.01$ or $p < 0.05$) increases of the DPPH radical scavenger activities were detected from 6.25 and 25 μg/ml concentration of L-Ascorbic acid and SLD treated samples, respectively, and accordingly, the IC_{50} of L-Ascorbic acid and SLD on DPPH radical scavenger activity were detected as 18.98 ± 7.08 and 161.98 ± 64.65 μg/ml, respectively (Fig. 5).

SOD-like activity

Significant ($p < 0.01$) increases of the SOD-like activities were detected from 25 and 50 μg/ml concentration of L-Ascorbic acid and SLD treated samples, respectively, and accordingly, the IC_{50} of L-Ascorbic acid and SLD on the SOD-like activity were detected as 111.99 ± 22.23 and 284.54 ± 54.47 μg/ml, respectively (Fig. 6).
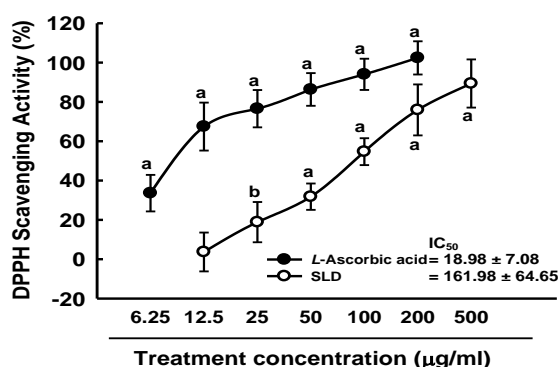
Figure 5: Effects of SLD and L-Ascorbic Acid on DPPH Radical Scavenger Activity. Values are expressed as means ± SD in six independent experiments. SLD = Slendesta, standard potato protein extracts, a test material, DPPH = 1,1-diphenyl-2-picrylhydrazyl, IC_{50} = concentration needed to reduce 50% of DPPH. Vehicle control set as base 0%. [a] $p < 0.01$ and [b] $p < 0.05$ as compared with control.
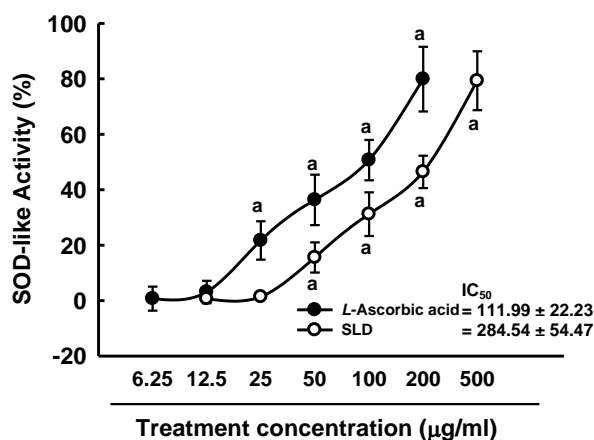
Figure 6: Effects of SLD and L-Ascorbic Acid on SOD-like Activity. Values are expressed as means ± SD in six independent experiments. SLD = Slendesta, standard potato protein extracts, a test material, SOD = superoxide dismutase, IC_{50} = concentration needed to reduce 50% of pyrogallol oxidations. Vehicle control set as base 0%. [a] $p < 0.01$ as compared with control.

171

Measurement of cellular ROS

To investigate whether SLD could inhibit 3T3-L1 cells against ROS generation, 3T3-L1 cells were treated with SLD at 0.75 and 1mg/ml. The results are shown as the proportions of ROS positive cells against negative ones. The significant decreases of ROS generation were observed in the SLD and NAC treated cells compared to control cells (Fig. 7).

Discussion

In this experiment, cytotoxicity of SLD containing 5% PI2 which effective in reducing food intake and body weight gain, when administered orally, by increasing circulating CCK levels through a trypsin-dependent mechanism (Komarnytsky et al., 2011), was observed in 3T3-L1 preadipocyte cell line by MTT assay, and also anti-adipogenic effects were observed through lipid accumulation assay during 3T3-L1 differentiation (Calzadilla et al., 2011; Yang et al., 2014; Nie et al., 2015) as comparing with NAC, a well-known antioxidant, reduces body weight and visceral fats by down regulation of metalothionein expression and inhibit differentiation in 3T3-L1 adipocytes (Kim et al., 2006a; Calzadilla et al., 2011). In addition, the antioxidant effects of SLD were detected by free radical scavenging capacity and SOD-like activity as comparing with ascorbic acid, a representative antioxidant vitamin C (Cho et al., 2011; Kim et al., 2014), as a series of investigations to develop a potential functional ingredient for obesity.
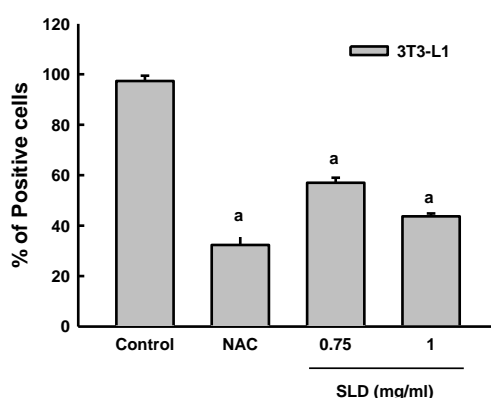
Figure 7: Effects of SLD against ROS Generation. 3T3-L1 cells were treated with NAC (10mM) or SLD (0.75 and 1 mg/ml). The ROS generation was measured by H_2DCFDA using a Tali[®] system. Values are expressed as means ± SD in three independent experiments. SLD = Slendesta, standard potato protein extracts, as a test material, NAC = *N*-acetyl-L-cysteine. [a] $p<0.01$ as compared with control.

Cell growth inhibition assay using MTT is generally used *in vitro* assay to detect possible cytotoxic activities of test materials, and has been widely used for screening the basic safety of candidates (Sumantran, 2011). In MTT assay, favorable obesity control agent show lower cytotoxic effects on normal cell lines, but show appropriated cytotoxicity to preadipocytes or adipocytes (Cao et al., 2013; Wang et al., 2014). In the present study, the SLD showed obvious concentration and treatment time-dependent cytotoxicity against the 3T3-L1 cell line.

The murine 3T3-L1 fibroblastic cell line can be induced to differentiate into mature adipocytes in cell culture using

172

dexamethasone and insulin, and it has been used as a model system to study the mechanisms involved in the adipogenic process through observation of lipid accumulation during differentiation into matured adipocytes (Calzadilla et al., 2011; Yang et al., 2014; Nie et al., 2015), and also in this study; the control cells had lipid accumulation and strongly induced differentiated 3T3-L1 cells. Whereas, the SLD-treated cells showed significant decreases of intracellular lipid accumulation, concentration dependently, suggesting SLD's potent inhibitory effects on adipocyte differentiation. NAC-treated cells also cause a decrease of lipid accumulation compared with the control cells, similar to those of previous studies by other investigators (Kim et al., 2006a; Calzadilla et al., 2011). Furthermore, the SLD treatment prevented the adiopocyte differentiation by attenuating the expression of PPARγ, C/EBPα, C/EBPβ, and leptin.

It has been believed that oxidative stresses are involved in adipocyte differentiation and lipid accumulations (Atashi et al., 2015). During differentiation into matured adipocyte, 3T3-L1 cell produce nicotinamide adenine dinucleotide phosphate (NADPH) to storage of absorbed glucose, mediated by pentose phosphate pathway. These NADPH were changes as $NADP^+$ by NADPH oxidae-4, and produced large amounts of superoxide, which facilitate the differentiation of preadipocytes into matured adipocytes, simultaneously irritate macrophages to release of another oxidative stresses. These cascade responses initiate by superoxide released from preadipocytes, finally involved in the pathogenesis of obesity and related various complications (Lee et al., 2013). So far as investigated, antioxidant effects can be easily detectable by free radical scavenger effects (Kim et al., 2008; Cho et al., 2011). In the normal healthy conditions, enough endogenous antioxidants are exist and, adequately eliminated the ROS but cellular deconstructions are accelerated in aging conditions as decreases of endogenous antioxidants. SOD is one of the endogenous antioxidant enzymes that contribute to enzymatic defense mechanisms (Cheeseman and Slater, 1993). The increase of SOD or SOD-like activities means antioxidant potentials (An et al., 2004). In the present study, *L*-Ascorbic acid used as a standard antioxidant reference drug, showed potent DPPH radical scavenger effects and SOD-like activities, ranged in reference values of previous studies (Cho et al., 2011; Kim et al., 2014), and the SLD also showed favorable DPPH radical scavenger and SOD-like effects. In addition, the cellular ROS levels were reduced in SLD-treated cells, therefore, these results suggest that SLD effectively prevented the ROS generation during differentiation into matured adipocyte.

Taking together the results of this experiment, it suggests that SLD effectively inhibited 3T3-L1 adipose cell differentiations may be through antioxidant activities and direct cytotoxicity in case of higher concentration, at least partially in a condition of this experiment, along with satiety effects already known to be mediated by increases of circulating cholecystokinin. These findings are considered as direct evidences that SLD may serve as a predictable functional ingredient for obesity as an alternative therapy. In addition, standard references used in this experiment – NAC and Ascorbic acid also showed favorable anti-adipogenic and antioxidant effects, ranged in reference values, suggesting the experiment protocol and results of the present study are acceptable.

Acknowledgments

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174

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