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ADVISIOR: PROF. GUN-HEE KIM

Anti-obesity Effect of *Selaginella tamariscina* on Inhibition of Pancreatic Lipase and Adipocyte Differentiation

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ABSTRACT

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Obesity is a disorder of lipid metabolism and continues to be a global health problem because it is known to be implicated in various diseases, such as hypertension, coronary heart diseases, and type II diabetes. Digestion and absorption of dietary lipids by pancreatic lipase, which mainly controls the energy intake of fat into monoglycerides and fatty acids, can be targeted for development of anti-obesity agents. *Selaginella tamariscina* have been used in traditional oriental herbal medicine on hemorrhage and recent studies show that it also has anti-inflammatory and anti-cancer effects. The purpose of this study was designed to evaluate new usability as natural anti-obesity agent of *Selaginella tamariscina*. The effect of *Selaginella tamariscina* extracts on pancreatic lipase inhibition activity as well as its lipid accumulation effect of in 3T3-L1 cells was assessed by examining Oil Red O staining and lipolysis assay. The pancreatic lipase inhibition activity of 30 different natural plants extract showed a wide range between 17 ~ 91% and among the

samples, *Selaginella tamariscina* was chosen for further research due to shortage of previous studies on its anti-obesity efficacy. Lipid accumulation in cells were determined by Oil red O staining and measurement of free glycerol release. While the incubation of 3T3-L1 adipocytes with *Selaginella tamariscina* extract inhibited differentiation of preadipocytes, the level of free glycerol released into cultured medium was increased in several concentrations.

All these results indicate that *Selaginella tamariscina* extract inhibit adipogenesis through controlling mechanism that via inhibition of pancreatic lipase and involves the direct downregulation of adipogenic differentiation. Thus, *Selaginella tamariscina* is a possible candidate for regulating lipid accumulation in obesity.

Keywords: *Selaginella tamariscina*, pancreatic lipase, adipocyte differentiation, lipolysis, anti-obesity

1 INTRODUCTION

Selaginella, also known as spikemoss and Keoun Back in Korean, has been used as traditional medicine in Korea and China for various diseases, including bloody feces, prolapse of the anus, hematuria, stanching, amenorrhea, dysmenorrhea, metrorrhagia and chronic hepatitis (Lee et al., 2008; Zheng et al., 2013). Some pharmacological studies have demonstrated that *Selaginella tamariscina* lowers blood glucose levels, antioxidant, anti-inflammatory and antitumor (Miao et al., 1996; Lee et al., 2008; Li et al., 2014). Secondary aromatic plant metabolites that has one or more hydroxyl substituents are called phenolic compounds and polyphenols from natural resources such as flavonols and tannins from various plants including fruits and vegetables, also reduced obesity in vivo together with lipase inhibition (Kurihara et al., 2006; McDougall et al., 2009; Sosnowska et al., 2015).

Obesity is a major chronic metabolic disorder caused by an imbalance between the intake of food and energy use. Energy intake starts from fat absorption through digestion of fat into monoglycerides and fatty acids mainly by pancreatic lipase (Lee et al., 2013), which can be targeted for development of anti-obesity agents. Absorbed fat is further accumulated into adipose tissue via abnormal growth of adipocytes characterized by increased

numbers of fat cells storing their lipids through excessive adipocyte differentiation (Lee et al., 2013). An increase in adipose tissue mass occurs through the triggering of adipocyte differentiation by two-step processes: the proliferation of preadipocytes and the differentiation of these cells into mature adipocyte cells (Hutley, 2004). The adipocyte is known to play an active role in many physiological and pathological processes regarding lipid metabolism which is formed by adipogenesis from precursor cell. The transition from preadipocyte to adipocyte involves four stages directed by PPARγ and C/EBPα. PPARγ maintains adipocytes in the terminal differentiation stage. The major cellular compartment for fat accumulation is called "lipid droplet" that plays crucial roles in lipid metabolism. The balance of lipids stored within lipid droplets is controlled by the net balance of triglyceride (TG) synthesis (lipogenesis) and degradation (lipolysis) (Lee, 2008).

In this study, we examined 30 different natural plant materials for their pancreatic lipase inhibition activity, and *Selaginella tamariscina* was screened as a highly inhibitory plant. We also report the effects of *Selaginella tamariscina* extracts on lipid accumulation during adipogenesis in 3T3-L1 cells and releasing free glycerol in to the serum.

2 LITERATURE REVIEW

2.1. *Selaginella tamariscina*

Selaginella, also known as spikemoss and Keoun Back in Korean, is the only surviving genus within the plant family Selaginellaceae. Selaginella includes more than 700 species widely distributed around the globe (Nguyen et al., 2015a). It has been used as traditional medicine in Korea and China for various diseases, including bloody feces, prolapse of the anus, hematuria, stanching, amenorrhea, dysmenorrhea, metrorrhagia and chronic hepatitis (Lee et al., 2008; Zheng et al., 2013).

Some pharmacological studies have demonstrated that *Selaginella tamariscina* lowers blood glucose levels and to facilitate the repair of pancreatic islet β-cells injured by alloxan (Miao et al., 1996). Also, crude extracts of *Selaginella tamariscina* reduced the production of proinflammatory cytokines, interleukin-1β and tumor necrosis factor-α in human mesangial cells (Kuo et al., 1998). Furthermore, the water extract of *Selaginella tamariscina* significantly reduced receptor activator for the nuclear factor-κB ligand-induced osteoclastogenesis by inhibiting the activation of signaling molecules and the expression of relevant transcription factors which may have positive effects on bone-destructive disease (Shim et

al., 2012). Studies of the phytochemical constituents of *Selaginella tamariscina* showed that it contains a number of flavonoids, lignans, selaginellins, alkaloids, sterols, aliphatic acid and phenols (Li et al., 2014; Nguyen et al., 2015b). Amentoflavone, sumaflavone, robustaflavone, ginkgetin, hinokiflavone and isocryptomerin are some examples of bioflavonoids, which is flavonoid dimers connected with a C-C or a C-O-C bond, that is rich in *Selaginella tamariscina* and known to have various pharmacological activities including antioxidant, anti-inflammatory and antitumor (Lee et al., 2008; Li et al., 2014).

Figure 1. ***Selaginella tamariscina***

(Flora of China, *Selaginella tamariscina*)

2.2 Phenolic compounds

Secondary aromatic plant metabolites that has one or more hydroxyl substituents are called phenolic compounds and polyphenols from natural resources have beneficial health effect which are based on their strong antioxidant activities which can delay or inhibit oxidative damage related diseases in human body (Gülçin, 2012; Yang et al., 2014; Zhang et al., 2015). Epidemiological and interventional studies showed that consuming phenolic-rich foods is related to the prevalence of several chronic diseases (Kris-Etherton et al., 2002). In addition, phenolic compounds may also play a key role to the management of type 2 diabetes by inhibition of α-glucosidase which reduces intestinal glucose digestion and absorption (Zhang et al., 2010; Balasubramaniam et al., 2013). Furthermore, phenolic compounds such as flavonols and tannins from various plants including fruits and vegetables, also reduced obesity in vivo together with lipase inhibition (Kurihara et al., 2006; McDougall et al., 2009; Sosnowska et al., 2015). These results imply that materials with phenolic compounds may be useful to decrease dietary fat absorption and accumulation.

2.3 Obesity

2.3.1 Pancreatic lipase

Obesity is a major chronic metabolic disorder caused by an imbalance between the intake of food and energy use; it is no longer considered to be cosmetic problem but a risk factor for several clinical disorders such as hypertension, hyperglycemia, insulin resistance, endothelial dysfunction, elevated triglycerides, and high cholesterol levels (Nicolai et al., 2009; Pasini et al., 2010; Shuster et al., 2012; Cordeiro et al., 2013; Lim et al., 2014; Vanella et al., 2016).

Energy intake starts from fat absorption through digestion of fat into monoglycerides and fatty acids mainly by pancreatic lipase (Lee et al., 2013). Absorbed fat is further accumulated into adipose tissue via abnormal growth of adipocytes characterized by increased numbers of fat cells storing their lipids through excessive adipocyte differentiation (Lee et al., 2013). Orlistat, a specific pancreatic lipase inhibitor, has been clinically used for the prevention of obesity by blocking approximately 30% absorption of dietary fat (Borgström 1988; Hill et al., 1999; Ballinger & Peikin, 2002). However, the uses of orlistat may cause some side effects which include oily stools, flatulence, diarrhea, bloating, abdominal pain, dyspepsia, and fecal spotting (Glazer, 2001; Filippatos et al., 2008). Therefore, anti-obesity treatment

derived from natural products using synergic inhibitory action on both lipase and adipocyte differentiation are needed (Yun, 2010; Kim et al., 2012).

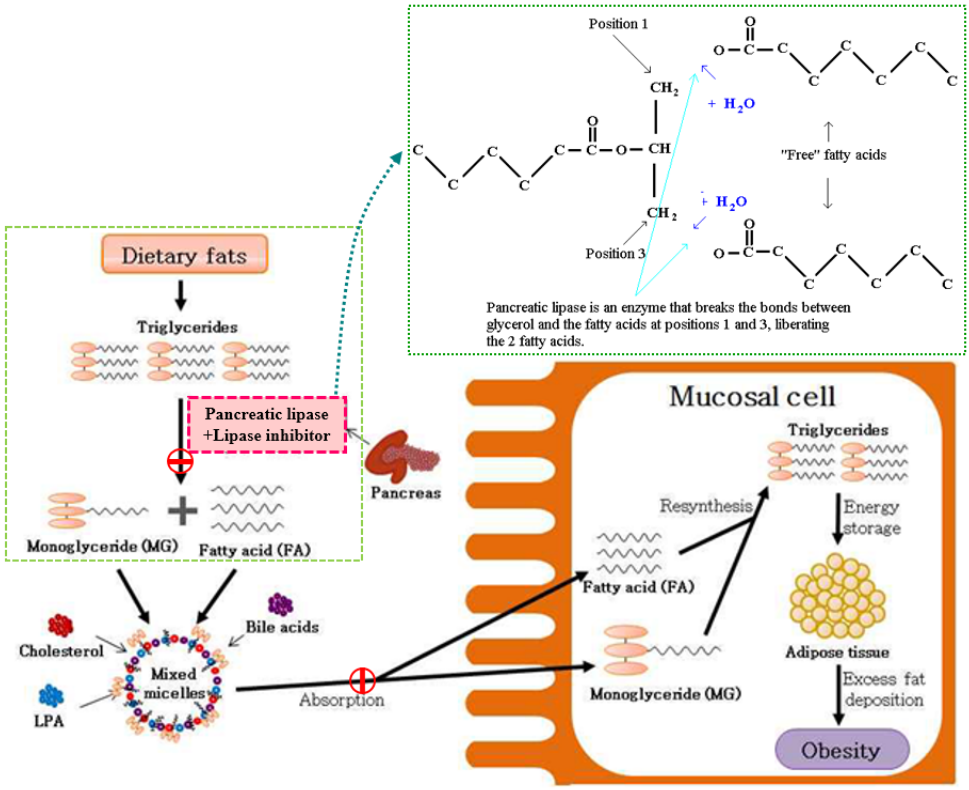

Figure 2. Lipase inhibitor system

2.3.2 Adipose tissue and adipogenesis

Obesity can be described as an abnormal increase in the adipose tissue. In humans, two types of adipose tissue exist, which are known as white adipose tissue (WAT, white fat) and brown adipose tissue (BAT, brown fat) (Choi, 2015). WAT is the predominant type of commonly known adipose tissue. Adipose tissue is formed by adipocytes, which play a critical role in energy balance and lipid homeostasis. The most-known function of adipocyte is to store energy in the form of triglycerides when energy intake exceeds total body energy expenditure (Spiegelman & Flier. 2001). Obesity, the development of WAT, is the result of two processes: the increase in number of adipocytes that develop from precursor cells, and the growth of individual fat cells due to formation of triglycerides (Klyde and Hirsch, 1979; Choi, 2015).

The adipocyte plays active role in many kinds of physiological and pathological processes regarding lipid metabolism. Adipocyte differentiation, known as adipogenesis, is a process of differentiation into adipocytes from preadipocytes. Gain of function associated with adipocyte differentiation includes (1) an increase in lipogenic capacity and the appearance of cytoplasmic lipid droplets, (2) acquisition of insulin sensitivity with regard to glucose uptake, and (3) the expression and secretion of numerous bioactive molecules (Morrison R.F. et al., 1999; Choi, 2015).

The majority of energy stored in body originated from ingested triglycerides appearing in the circulation incorporated in chylomicrons. Some fatty acid are synthesized in the liver and adipocytes through lipogenesis. Triglycerides synthesis requires glycerol and fatty acids which are transformed into glycerol-3P and fatty acyl-CoA to be used as substrates (Park, 2011). The glycerol-3P is therefore produced from glucose through the first step of glycolysis and from glycerolgenesis (Reshef, 2003). During intracellular lipolysis, triglyceride are broken down via diacylglycerol and monoacylglycerol to form three moles of free fatty acid and one mole of glycerol per mole of completely hydrolyzed triglyceride (Hla, 2001). Regulation of lipolysis is essential to ensure an adequate supply of lipid to the tissues that utilized free fatty acid. Dietary fat is absorbed by the intestine and secreted into the circulation in the form of chylomicrons (Park, 2011).

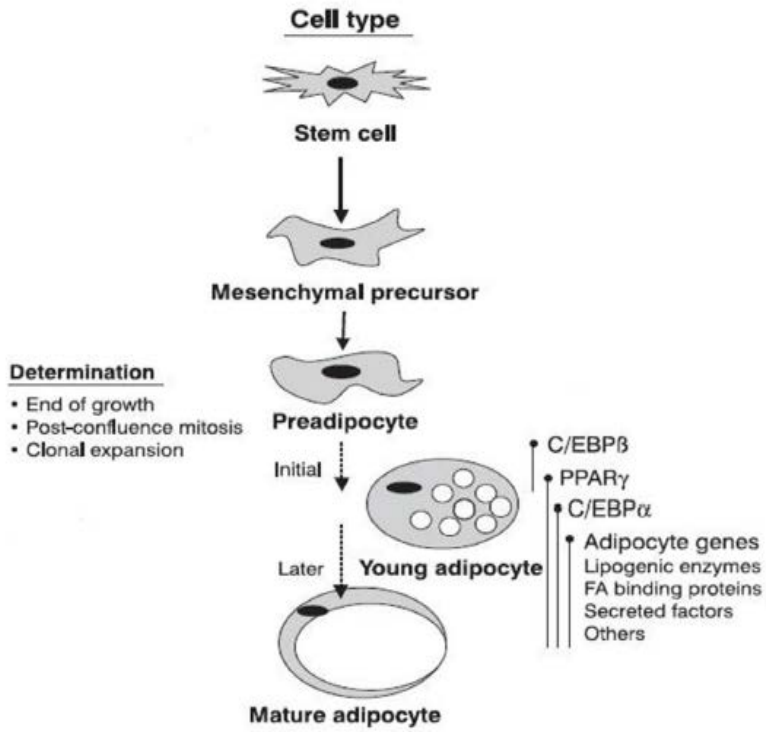

Figure 3. Schematic diagram of the adipocyte differentiation process.

(Fonseca-Alaniz et al., 2007)

3 MATERIALS AND METHODS

3.1 Experimental materials

3T3-L1 fibroblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) and antibiotic/antimycotic solution (100X, 100unit) were purchased from Gibco (Waltham, MA, USA). Isobutylmethyl-xanthine (IBMX), insulin, dexamethasone (DEX), Dimethylthiazol-2-yl-2,5-diphenyl tetrazoliumbromide (MTT), Dimethyl sulfoxide (DMSO), isopropanol, N-acetyl-L-cysteine (NAC), Oil Red O solution, free glycerol reagent and glycerol standard solution were purchased from Sigma (St. Louis, MO, USA).

3.2 Methods

3.2.1 Extraction of 30 natural plants

Selaginella tamariscina were purchased from Seoul Oriental Medicine Industry District in Korea. 40 g of dried *Selaginella tamariscina* were added to 80 ml of 70% ethanol. Extraction were performed for 3 hours at 70℃ and filtered. The ethanolic extract was evaporated under vacuum (ELISA EVAPORATOR NVC-2000, SB-1000, DPE-1210, CA-

1112, ELISA, Japan) for making the powders which were used as sample.

3.2.2 Pancreatic lipase inhibition activity

The procedure for lipase activity determination was determined using the method of Eom et al. (2013) with some modifications. Briefly, an enzyme buffer (10 mM MOPS, 1 mM EDTA, pH 6.8) was prepared by adding 30 µL porcine pancreatic lipase (Sigma, St. Louis, MO, USA) in 850 µL Tris buffer (100 mM Tris-HC1 and 5 mM CaCl2, pH 7.0). Then, 100 µL of the compounds at the test concentration or orlistat (Sigma, USA) was mixed with 880 µL of the enzyme buffer and incubated for 15 min at 37℃. After 15 mins, 20 µL of the substrate solution (10mM of *p*-nitrophenylbutyrate in dimethyl formamide) was added and the enzymatic reactions were allowed to proceed for 15 min at 37℃. Pancreatic lipase activity was determined by measuring the hydrolysis of *p*-nitrophenylbutyrate to *p*-nitrophenol at 405 nm with the use of an ELISA reader (SpectraMax M2, Molecular Devices, USA). The inhibition of lipase activity was expressed as the percentage decrease in the optical density (OD) when porcine pancreatic lipase was incubated with the test compounds.

3.2.3 Evaluation of phenolic compounds

3.2.3.1 Measurement of total phenolic contents

Total phenolic contents were measured by the method of Folin-Ciocalteu assay (Florence et al., 1992). 70 µL of the sample at different concentration was placed in 96-well plate and 70 µL of 2 N Folin-Ciocalteu agent added. Three minutes later 70 µL of 2% sodium carbonate was added and the mixture was incubated for 1 h at room temperature. The absorbance was measured by spectrophotometer (SpectraMax M2, Molecular Devices, USA) at 760 nm. The results were expressed as gallic acid equivalent (GAE) per mg of extract, based on the calibration curve of gallic acid.

3.2.3.2 Measurement of total flavonoid contents

The total flavonoid contents were determined according to the method of Quettier assay. 50 µL of the sample at different concentration was placed in 96-well plate and 150 µL of 2% aluminum chloride was added into each of the wells. After 15 minute at room temperature, the absorbance was measured by spectrophotometer (SpectraMax M2, Molecular Devices, USA) at 430 nm. The results were expressed as quercetin equivalent (QE) per mg of extract, based on the calibration

curve of quercetin.

3.2.4 Evaluation of adipocyte differentiation

3.2.4.1 Measurement of cell viability

To assess the cell viability two days after confluence, 3T3-L1 preadipocytes were seeded in DMEM supplemented with 10% FBS into 96-well plates and incubated at 37 °C in a 5% CO_2 incubator. After 24 hours later, 3T3-L1 preadipocytes were treated with media containing 6 concentrations of sample (31.25, 62.5, 125, 250, 500 and 1000 μg/mL). After 72 hours, 5 mg/mL of MTT solution was added to the each well. The treatment medium was removed and the resulting formazans were dissolved with DMSO. The absorbance was measured at 570 nm in a microplate reader.

3.2.4.2 Cell culture and differentiation

3T3-L1 cells were maintained in DMEM supplemented with 10% Bovine Serum and 100 unit antibiotics at 37°C in a 5% CO_2 incubator. For the experiment of adipogenesis, 3T3-L1 cells were grown until 2 day post-confluence. To induce differentiation, 2 days post-confluent 3T3-L1 cells were maintained with DMEM containing 10% FBS, 10 μg/ml insulin, 0.5

μM DEX and 0.5 mM IBMX (MDI) for 36 hours. After that culture, medium was changed with DMEM supplemented with 10% FBS and 10 μg/ml of insulin every 3 days. During differentiation, the cells were treated with samples every 3 days, for 8 days.

3.2.4.3 Oil Red O staining

Adipocyte differentiation was typically monitored by staining the cultured cells with Oil Red O solution. Briefly, cells were washed gently with PBS and fixed in 10% paraformaldehyde for one hour. Oil red O staining solution (3:2 mixture of 0.5% Oil Red O-Isopropanol solution and water) was add to the cells and kept at room temperature for 15 min and then cells were washed with PBS. For the quantification of Oil red O uptake, cells were incubated with isopropanol. After 10 min incubation at room temperature, solution was transferred to a 96-well plate and absorbance was determined at 500 nm.

3.2.4.4 Lipolysis assay

The differentiated 3T3-L1 adipocytes were incubated with various concentration of extracts for 72 hours. The incubation medium was transferred to another set of tubes and heated at 70℃ for 10 min to

inactivate any enzymes released by the cells. Then, incubated medium was assayed for free glycerol using glycerol reagent and the absorption was measured at 540 nm.

3.2.5 Statistical analysis

All data are presented as mean ± standard deviation (S.D) from at least three independent experiments performed in triplicate. Statistical significance was determined by analysis of variance (ANOVA) and Duncan's multiple range tests were used to determine the significance of differences between groups using SPSS (Statistical Package for Social Science, ver. 19, Chicago, IL, USA). The level of $p<0.05$ was considered to indicate.

4 RESULTS AND DISCUSSION

4.1 Pancreatic lipase inhibition activity of 30 different natural plant extracts

Obesity is caused by the imbalance between energy intake and expenditure. Newer approaches for the treatment of obesity have involved suppression of dietary triglyceride absorption by inhibiting pancreatic lipase of excess calories (Birari and Bhutani, 2007). Phytochemicals identified from traditional medicinal plants present an exciting opportunity for the development of newer therapeutics (Kim et al., 2010).

The pancreatic lipase inhibition activity of 30 different natural plants extract showed a wide range between 17 ~ 91%. *Coix lacryma-jobi* L. var. *mayuen* Stapf and *Selaginella tamariscina* each showed 91.2% and 90.4% which were the most effective of all, and there were 10 samples that their extracts inhibition rate was over 50%. Among the plants that showed a high inhibition activity, *Selaginella tamariscina* was chosen for further research due to shortage of previous studies on its anti-obesity efficacy.

Among the sample plants, some showed a different results in comparison with other studies. In other studies, *Astragalus membranaceus* Bunge showed -11.0±2.2% (Zheng et al., 2010) and 7.4±1.4% (Roh & Jung,

2012) of pancreatic lipase inhibition activity meanwhile our study result was 24.6±5.3%. This indicates that even with same plant material, different experimental design such as sample extraction methodology and procedure can affect the result. Also, some studies used plant samples that people consume globally such as legumes, fruits and vegetables (Sreerama et al., 2012; Sosnowska et al, 2015).

Table 1. Pancreatic lipase inhibition activity of 30 different natural plant extracts

Scientific name	Lipase inhibition (%)
***Coix lacryma-jobi* L. var. *mayuen* Stapf**	**91.2**
***Selaginella tamariscina* (Beauv.) Spring**	**90.4**
Cinnamomum aromaticum	**81.1**
***Morus alba* L.**	**69.3**
***Foeniculum vulgare* Mill.**	**63.7**
***Elshotzia ciliata* Hylander.**	**59.6**
***Atractylodes macrocephala* Koidzumi**	**58.9**
***Rubus coreanus* Miquel**	**57.1**
***Curcuma aromatica* Salisb.**	**56.2**
***Poria cocos* Wolf**	**50.2**
***Agrimonia pilosa* Ledebour**	**47.7**
***Lindera obtusiloba* Blume**	**47.7**
***Polyporus umbellatus* Fries**	**47.0**
***Zizyphus jujuba* var. inermis**	**46.3**
***Gardenia jasminoides* Ellis**	**45.8**
***Zingiber officinale* Roscoe**	**45.6**
***Conyza canadensis* Erigeron canadensis L.**	**44.7**
Nelumbo nucifera	**43.3**
***Syzygium aromaticum* (L.)**	**42.4**
***Platycodon grandiflorum* (Jacq) Nakai**	**39.8**
***Eisenia bicyclis* (Kjellman) Setchell**	**39.1**
***Schizandra chinensis* Baillon**	**38.7**
***Glycyrrhiza uralensis* Fisch**	**35.3**
***Citrus unshiu* S.Marcov.**	**32.2**
***Rehmannia glutinosa* (Gaertn.) Libosch. ex Steud.**	**31.2**
***Mentha arvensis* Linne var. *piperascens* Malinvaud**	**28.0**
***Scutellaria baicalensis* Georgi**	**25.7**
***Astragalus membranaceus* Bunge**	**24.6**
Allium hookeri	**19.6**
***Arctium lappa* L.**	**17.0**

Each value represented mean of triplicate experiments.

4.2 Pancreatic lipase inhibition activity of *Selaginella tamariscina*

Pancreatic lipase inhibition of *Selaginella tamariscina* extracts is expressed as percentage (%) in Fig. 4. *Selaginella tamariscina* extract showed concentration dependent increase inhibitory effect and high concentration of Selaginella tamariscina extract showed a similar or highr inhibition activity than the Orlistat control (50 ng/mL). Orlistat has serious side effects, such as abdominal gas with oily spotting, stomach pain, irregular menstrual periods, and headaches (Bray, 2009). *Selaginella tamariscina*, a Korean medicinal herb, its total flavonoids significantly decreased the concentration of fasting blood glucose, total cholesterol, triglycerides and low-density-lipoprotein cholesterol, while it increased the levels of insulin and high-density-lipoprotein cholesterol in diabetic mice (Zheng et al., 2013). Also, chemical compounds isolated from *Selaginella tamariscina* not only showed stimulatory potency on 2-NBDG uptake but also showed potent inhibitory effects on PTP1B enzyme activity, which suggested the potential of the isolates as insulin mimetics for developing antidiabetic agents (Nguyen et al., 2015b).

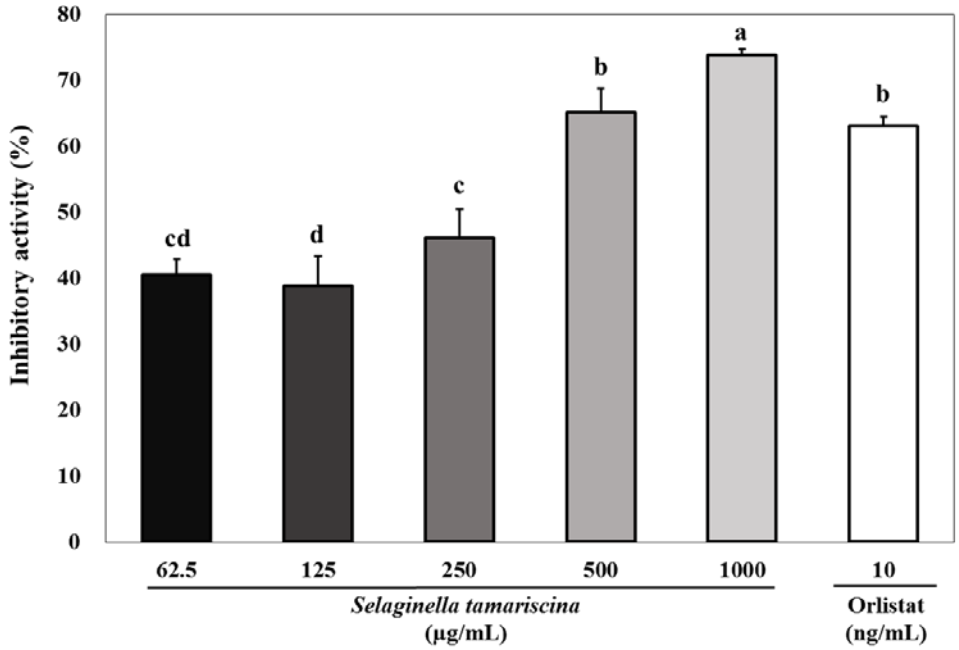

Figure 4. Pancreatic lipase inhibition activity of *Selaginella tamariscina*.

3T3-L1 adipocytes after treated with various *Selaginella tamariscina* extracts in different concentrations (31.25, 62.5, 125, 250, 1000 μg/mL) and Orlistat (10 ng/mL). Each value represented mean ± SD of triplicate experiments. Values with different letters indicate statistically significant differences among groups at $p<0.05$.

4.3 Total phenolic compounds of *Selaginella tamariscina*

Plants are rich in variety of phytochemicals such as phenolics and flavonoids that provides health benefits (Hanasaki et al., 1994). They have many health related properties such as anticancer, antiviral, and anti-inflammatory activities, as well as effects on capillary fragility and inhibition of platelet aggregation in humans (Benavente et al., 1993; Choi, 2015). Thus, we determined the total phenolic and flavonoid contents of *Selaginella tamariscina* extracts. As shown in Table 2, total phenolic contents (60.29 ± 3.11 mg GAE/g) and total flavonoid contents (14.90 ± 0.34 mg QE/g). Other study results show that oriental medically processed *Selaginella tamariscina* extract contained 113.4 mg GAE/g of total phenolic contents and unprocessed extract had 99.3 mg GAE/g. However, total flavonoid contents barely had any difference between the two samples (processed extract 38.6 mg QE/g; unprocessed extract 37.2 mg QE/g). This indicated that depending on the extraction methods or solvent fractions, total phenolic contents result may have different results. Furthermore, distinctions between total phenolic contents can affect a natural derived sample's anti-obesity effect. Further research on the correlation between the total phenolic contents and anti-obesity is needed.

Table 2. Total phenolic compounds of *Selaginella tamariscina* extract

Sample name	TPC (GAE mg/mL)	TFC (QE mg/mL)
Selaginella tamariscina	**60.29 ± 3.11**	**14.90 ± 0.34**

Each value represented mean ± SD of triplicate experiments.
GAE: Gallic Acid Equivalent.

4.4 Effect of *Selaginella tamariscina* on cell viability

MTT assay was done to assess the cell viability of 3T3-L1 preadipocytes treated with various concentrations of *Selaginella tamariscina* extracts. The cell viabilities were ranged from 82.5% to 102.7% after 48, 72 hours incubation with the *Selaginella tamariscina* extracts. The cell viabilities of extracts at the highest concentration (1000 μg/ml) were significantly lower ($p<0.05$) than the control (without treatment). In addition, non-cytotoxicity is defined as viability ≥70% compared to untreated cells (ISO 10993-10995). All the extracts in different concentrations included the highest concentration (1000 μg/ml) were higher than 70%. Thus, all the extracts at concentration of 0-1000 μg/ml were considered as noncytotoxic to 3T3-L1 preadipocytes.

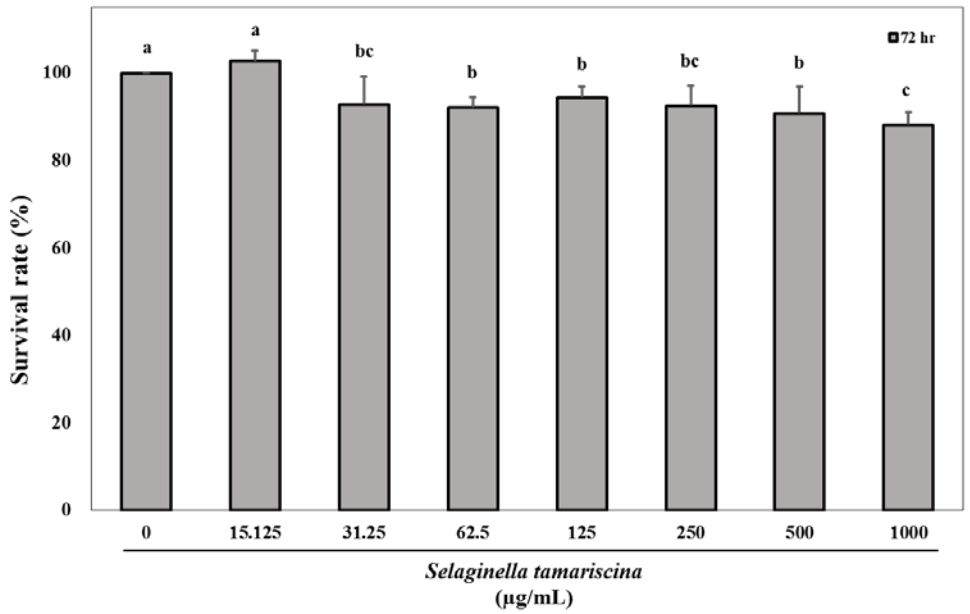

Figure 5. Effect of *Selaginella tamariscina* on cell viability (72 hours).

3T3-L1 cells were treated with different concentration of *Selaginella tamariscina* extracts for 72 hr. Cell viability was determined using the MTT assay. Data were presented as percent growth rate of preadipocytes in present of extracts, compared with control group. Each value represented mean ± SD of triplicate experiments. Values with different letters indicate statistically significant differences among groups at $p<0.05$

4.5 Effect of *Selaginella tamariscina* on adipocyte differentiation

4.5.1 Effect of *Selaginella tamariscina* on the differentiation of preadipocyte 3T3-L1 cell

Adipose tissue plays a significant role in preserving lipid homeostasis and energy balance by storing triglycerides or liberating free fatty acid in response to changes in energy demands (Ahn et al., 2012; Lim et al., 2014). Adipocytes store excess energy in the form of triglycerides which are contained inside lipid droplets (Kim et al., 2010). 3T3-L1 cells are known to differentiate into adipocytes under the appropriate conditions and have been frequently used as a model for adipose cells, which are one of the major sites of lipid and glucose metabolism mechanism (Green & Kehinde, 1975; Choi, 2015).

The inhibition effects of *Selaginella tamariscina* extracts on fat droplet formation in 3T3-L1 cells, through the quantification method of Oil red O staining, are presented. Oil red O staining material was used as a marker of adipogenesis where the higher the Oil red O staining material value, the higher the lipid accumulation inside the cells. Higher concentration of *Selaginella tamariscina* extracts (125, 250 μg/ml) significantly reduced ($p<0.05$) lipid accumulation in 3T3-L1 adipocytes compared to control (without treatment).

In this study, *Selaginella tamariscina* extracts at concentration of 250 μg/ml showed the highest inhibition in lipid accumulation while extracts at concentration of 31.25 μg/ml and 62.5 μg/ml were not significant compared to untreated control in reducing lipid accumulation of adipocytes. This implied that *Selaginella tamariscina* extracts at 125, 250 μg/ml were able to reduce the lipid accumulation in the adipocytes and 250 μg/ml concentration was comparable to 10 mM N-acetyl-L-cysteine (NAC) which was used as positive control in the study. The active compounds that is contained in the extract might suppress one or more of the pathways in adipogenesis that lead to the decrease lipid accumulation inside the adipocyte differentiation through inhibition of growth arrest in cell cycle.

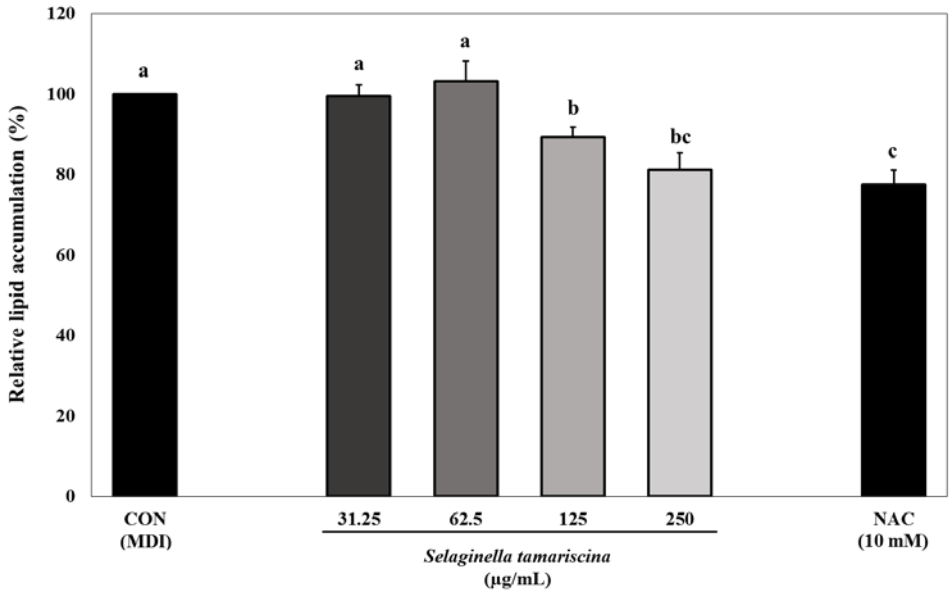

Figure 6. Effect of *Selaginella tamariscina* on the differentiation of preadipocyte 3T3-L1 cell.

Preadipocyte cells were differentiated with medium containing MDI presence or absence *Selaginella tamariscina* extracts for 8 days. Cells were stained with Oil-red O to determine the lipid accumulation of the cells. All values were calculated as a percentage of intracellular lipid accumulation compared with MDI group. Data are mean of three independent experiment ± SD values. Values with different letters indicate statistically significant differences among groups at $p<0.05$

4.5.2 Effect of *Selaginella tamariscina* on the Lipolysis of adipocyte cell

To determine the lipolytic effect of the *Selaginella tamariscina* extract in adipose tissues, glycerol release was examined as indicator of lipolysis. Lipid accumulation is controlled by a balance between lipogenesis and lipolysis (Rayalam, 2008). Lipolysis is a catabolic process that hydrolyzes stored triglycerides in fat tissue to release free fatty acid and glycerol (Lim et al., 2014). Therefore, lipolysis of fat cells can regulate the homeostasis of energy by controlling the release of fatty acids and glycerol into plasma (Kim et al., 2009). Lipolysis in adipocytes is known to be signaled by the activation of intracellular cAMP level, which in turn activates protein kinase A and substrates such as hormone-sensitive lipase and perilipin (Holm, 2003). Hormone-sensitive lipase is a key enzyme in the mobilization of free fatty acids from adipocytes (Holm, 2003).

To determine whether *Selaginella tamariscina* exerts lipolytic effects in 3T3-L1 adipocytes, differentiated adipocytes were treated with various concentrations of extract for 72 hours then, cell supernatant was collected for free glycerol release assay. The level of glycerol secretion into the medium increased by treatment of *Selaginella tamariscina* extract (31.25,

125, 250 µg/ml).

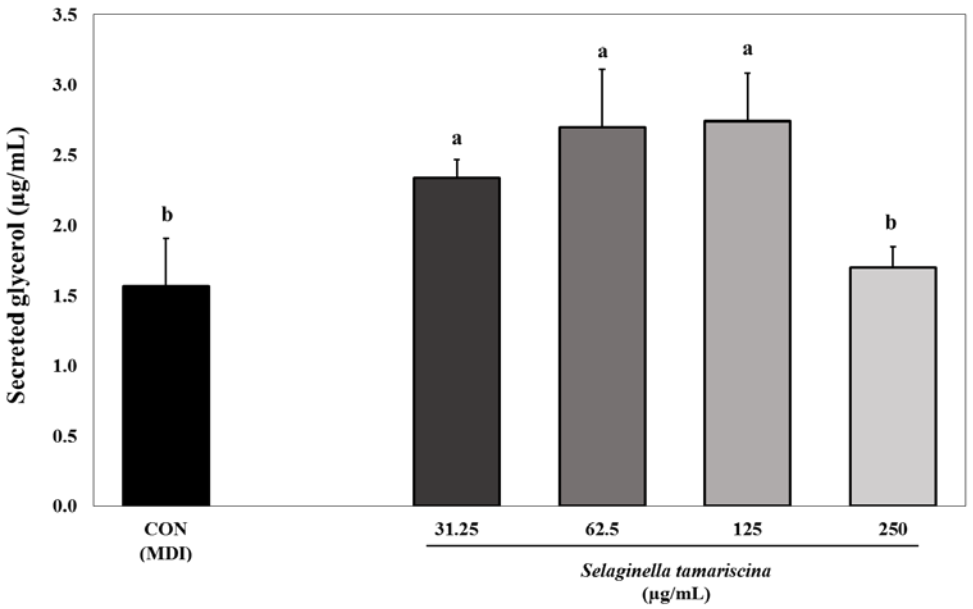

Figure 8. Effect of *Selaginella tamariscina* on glycerol release in 3T3-L1 cell.

Differentiated 3T3-L1 adipocytes were treatment with *Selaginella tamariscina* extract (31.25, 62.5, 125, 250 μg/ml) for 72 hr. Lipolytic effect was evaluated by the release of glycerol to the culture medium. The values were free glycerol contents of the differentiated cells. Data are mean ± SD values, each performed in triplicate. Values with different letters indicate statistically significant differences among groups at $p<0.05$

CONCLUSIONS

Obesity is a disorder of lipid metabolism and continues to be a global health problem because it is known to be implicated in various diseases, such as hypertension, coronary heart diseases, and type II diabetes. Digestion and absorption of dietary lipids by pancreatic lipase, which mainly controls the energy intake of fat into monoglycerides and fatty acids, can be targeted for development of anti-obesity agents. Although few medical supplies with antiobesity effect are available in the market, most of them have adverse side effects (Lim et al., 2014). Orlistat (Xenical™), a specific pancreatic lipase inhibitor clinically approved by Food and Drug Administration could block approximately 30% absorption of dietary fat (Borgstrom, 1988). However, orlistat have been reported with side effects therefore, many recent studies discovered potential anti-obesity effect from natural plant sources with the inhibition of fat absorption by interrupting the lipase and adipocyte activity (Nakai et al., 2005; Conforti et al., 2012).

Selaginella tamariscina have been used in traditional oriental herbal medicine on hemorrhage and recent studies show that it also has anti-inflammatory and anti-cancer effects. The purpose of this study was designed to evaluate new usability as natural anti-obesity agent of *Selaginella*

tamariscina. In this study, the pancreatic lipase inhibition activity of 30 different natural plant extracts were investigated, and among the plants that showed a high inhibition activity, *Selaginella tamariscina* was chosen for further research due to shortage of previous studies on its anti-obesity efficacy by examining lipid accumulation during adipogenesis of 3T3-L1 cells. Our results demonstrate that, *Selaginella tamariscina* extract decreased the cell viability in 3T3-L1 preadipocytes as well as their differentiation to adipocyte cells. In addition, it increased the lipolytic effect that hydrolyzes stored triglycerides in fat tissue to release free fatty acid and glycerol (Lim et al., 2014). Therefore, this study suggest that *Selaginella tamariscina* may be used as potential therapeutic materials or natural resources as a regulator of lipid metabolism in adipocytes.

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ABSTRACT (in Korean)

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건강기능신소재학과

비만은 지질 대사 장애이며 고혈압, 관상 동맥 심장 질환 및 제2형 당뇨병과 같은 다양한 질병의 원인으로 알려져 있다. 섭취한 지방을 monoglycerides와 fatty acid으로 분해하는 췌장 리파아제를 타겟으로 한 항비만제 개발 연구가 활발히 진행되고 있으며, 시중에 유통되고 있는 췌장 리파아제 저해제의 부작용이 보고되면서 천연 유래 기능성물질의 지질대사조절 역할이 주목 받고 있다. 권백은 한방 전통 의학에서 다양한 출혈 등에 사용되어 왔으며 최근의 연구에 따르면 항 염증 및 항암 효과가 있음이 입증되었다. 이 연구에서는 권백 추출물의 췌장 리파아제 억제 및 지방세포 분화에 미치는 영향을 연구함으로써 천연 항 비만 치료제로서의 새로운 유용성을 평가하고자 하였다. 국내 천연 식물 30종을 대상으로 췌장 리파아제 저해능을 측정한 결과, 우수한 활성을 보였으나 아직 항비

만 효과에 대한 보고가 적은 권백을 선택하여 3T3-L1 세포에서의 지질 축적 효과에 미치는 영향을 Oil Red O 염색 및 지방 분해 분석을 통해 평가 하였다. 권백 추출물은 3T3-L1 지방전구세포의 분화 억제 효과를 보였고, 유리 글리세롤의 양을 증가시킴으로써 세포 내 지질을 분해하는 lipolysis에서도 그 효과가 확인되었다.

이상의 결과를 종합해 볼 때, 권백은 비만 등과 같은 지질대사 이상을 개선시키는데 긍정적인 영향을 보임으로써 향후 천연 유래 항비만 기능성소재로써 개발 가능성이 있다고 사료된다.