

Downregulation of Hepatic De Novo Lipogenesis and Adipogenesis in Adipocytes by *Pinus densiflora* Bark Extract

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Received: July 13, 2017
Revised: August 21, 2017
Accepted: September 4, 2017

First published online
September 5, 2017

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pISSN 1017-7825, eISSN 1738-8872

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Korean red pine (*Pinus densiflora*) bark extract, PineXol (PX), was investigated for its potential antioxidant and anti-inflammation effects in vitro. It was hypothesized that PX treatment (25–150 µg/ml) would reduce the lipid synthesis in HepG2 hepatocytes as well as lipid accumulation in 3T3-L1 adipocytes. Hepatocytes' intracellular triglycerides and cholesterol were decreased in the PX 150 µg/ml treatment group compared with the control ($p < 0.05$). Consequently, de novo lipogenic proteins (acetyl-CoA carboxylase 1, stearoyl-CoA desaturase 1, elongase of very long chain fatty acids 6, glycerol-3-phosphate acyltransferase 1, and sterol regulatory element-binding protein 1) were significantly decreased in hepatocytes by PX 150 µg/ml treatment compared with the control ($p < 0.05$). In differentiated 3T3-L1 adipocytes, the lipid accumulation was significantly attenuated by all PX treatments ($p < 0.01$). Regulators of adipogenesis, including CCAAT-enhancer-binding proteins alpha, peroxisome proliferator-activated receptor gamma, and perilipin, were decreased in PX 100 µg/ml treatment compared with the control ($p < 0.05$). In conclusion, PX might have anti-obesity effects by blocking hepatic lipogenesis and by inhibiting adipogenesis in adipocytes.

Keywords: PineXol (PX), anti-obesity, de novo lipogenesis, adipogenesis, in vitro

Introduction

Obesity has been a serious health issue for decades, not only in Western countries but also in Asian countries [1, 2]. Major causes of obesity are related to the imbalance between energy intake and energy expenditure [2]. The worldwide increase in obesity rates leads to a rise in the incidence of chronic diseases, such as cardiovascular disease, cancer, type 2 diabetes, and dyslipidemia [3–5]. Korea has not been exempted from the ongoing trend as mortalities attributed to the same chronic diseases have also been rising. According to the Korean Society for the Study of Obesity, one-third of Korean adults and one-tenth of Korean children are obese [6]. Therefore, the importance of obesity prevention has been a rapidly emerging topical issue in Korea.

Obesity is tentatively related to dyslipidemia, such as

increased triglyceride, total cholesterol, LDL-cholesterol, and/or decreased HDL-cholesterol. The dyslipidemia prevalence was reported to be around 47.8% in adults over 30 years of age in Korea in 2015 [7]. The continuously reported increase in the prevalence of dyslipidemia is associated with functional abnormalities of the liver and white adipose tissue. The liver is a site where metabolic indications leading to obesity reveal well, apart from being the most important lipid synthetic site. Investigations have been conducted to show that regulating de novo lipogenesis can be a remedial mark for obesity and liver diseases [8–10]. Furthermore, the adipose tissue's main function is lipid accumulation, but lipogenesis and lipolysis also take place [11]. Thus, obesity is basically a consequence of overloading white adipose tissue with lipids [12].

Anti-obesity drugs such as orlistat, phentermine, and sibutramine have flourished with anti-augmentation of the

obesity rate through appetite suppression and caloric intake reduction. However, their applications have remained restricted because of side effects, including headache, abdominal pain, and sweating [13, 14]. Management of obesity through dietary alterations has proved harmless, more cost effective, and with few side effects compared with medication [15]. Henceforth, the prevention of obesity by natural functional substances and not by treatment with drugs has become a subject of interest scientifically.

The beneficial effects of pine bark extract have been known to be resultant from the complex impact of phenolic and polyphenolic flavonoids [16]. Pycnogenol, an extract of Maritime Pine (*Pinus pinaster*) was registered to trade name by Horphag Research (Switzerland) [17]. Pycnogenol has beneficial characteristics, such as anti-inflammatory, anti-oxidant, and anti-obesity effects [18–21]. Pycnogenol has been used as a nutritional supplement with proven efficiency to prevent a variety of chronic diseases [22–24]. Pycnogenol intake inhibits differentiation of pre-adipocytes and stimulates lipolysis in differentiated 3T3-L1 cells [25, 26]. *ob/ob* mice have also been shown to have an anti-obesity effect due to an increase in lipase levels and a decrease in enzymes that regulate de novo lipogenesis in the liver [27].

It is in accordance with the above given scope that PineXol (PX) (Nutrapharm Co., Korea), extracted from Korean Red Pine (*Pinus densiflora*) bark, has been reported to have similar beneficial functions to pycnogenol, including anti-oxidant, anti-inflammation, and anti-lipogenic effects in vitro [28, 29]. According to HPLC analysis, because the phenolic compounds (catechin and taxifolin) and flavonoid (procyanidin B1 and B2) contents are comparable, the similarity of PX and Pycnogenol composition is high [30, 31]. However, there is insufficient information on PX's metabolic mechanisms that lead to its anti-obesity effect. Therefore, this study focused on observing PX anti-obesity effects through performance of further experiments to elucidate its mechanism in vitro using HepG2 and 3T3-L1 cells.

Materials and Methods

Cell Culture and Treatment

The hepatocellular carcinoma cell line HepG2 was obtained from American Type Culture Collection (ATCC, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin (WELLGENE, Inc., Korea). The cells were seeded at 5.0×10^5 cell/well cell density in 6-well plates when they developed 70–80% confluence. The water extract of Korean red pine (*Pinus densiflora*)

bark (commercially known as PineXol) was purchased from Nutrapharm Co. (Korea). The HepG2 cells were treated with 50, 100, or 150 $\mu\text{g/ml}$ PX dissolved in distilled water and were exposed to it for 48 h. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 .

3T3-L1 pre-adipocytes were obtained from American Type Culture Collection. The cells were cultured in DMEM containing 10% bovine calf serum and 1% penicillin (WELLGENE, Inc.) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. The 3T3-L1 cells were seeded at 5.0×10^4 cell/well cell density in 6-well plates until they reached 100% confluence. After 2 days, the medium was changed to DMEM comprising 10% FBS, insulin (10 $\mu\text{g/ml}$), 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine for differentiation (day 0). On day 2, the medium was changed to DMEM containing insulin (10 $\mu\text{g/ml}$) and replaced every 2 days until day 8. PX treatments had concentrations of 25, 50, 75, and 100 $\mu\text{g/ml}$ and were administered to the cells during differentiation.

MTT Assay

Cell viability was evaluated by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, USA). HepG2 cells were seeded at 5.0×10^3 cell/well cell density in 96-well plates. 3T3-L1 pre-adipocytes were seeded at 5.0×10^4 cell/well cell density in 6-well plates. MTT assay was carried out after the above-mentioned PX treatment. First, 5 mg/ml MTT solution was diluted 20 times with medium and then added to the cells at the end of the PX treatment mentioned above. The cells were incubated for 2 h at 37°C to allow formazan-MTT crystal production. After 2 h, the medium was converted to 100% dimethyl sulfoxide to dissolve the formazan with shaking. The solution's absorbance was recorded at 540 nm and cell viability was expressed as a ratio (CTL = 1).

LDH Cytotoxicity Assay

Cytotoxicity induced by PX was measured by lactate dehydrogenase (LDH) leakage into the culture medium. The leakage of LDH in the medium was measured by using a commercial LDH cytotoxicity kit (DoGenbio, Korea). The assay was based on the change of lactate to pyruvate in the presence of LDH with reduction of NAD. The development of NADH from reduction of NAD converted WST (water-soluble tetrazolium salt) formazan, which was evaluated by measuring the absorbance at 450 nm. HepG2 cells were seeded at 2.5×10^4 cell/well in 96-well plates. 3T3-L1 pre-adipocytes were seeded at 1.25×10^4 cell/well in 96-well plates. After a day, a proper concentration of PX was treated into the cells. Following PX treatment, the culture medium was aspirated and centrifuged at 600 $\times g$ for 5 min to obtain cell-free supernatant. Free supernatant was mixed with the LDH reaction mixture in new 96-well plates and left at room temperature for 30 min. Absorbance of the solution was measured at 450 nm. Cell cytotoxicity is presented as percentage values.

Intracellular Triglyceride and Cholesterol

HepG2 cell pellets were converged in DPBS using a cell scraper after the PX treatment mentioned above. The DPBS was decanted after centrifugation at 12,000 ×g for 1 min at 4°C. Then, 5% NP-40 (Sigma Aldrich) was added for extraction of proteins and total lipids. The pellet solution was rotated and centrifuged at 12,000 ×g for 10 min at 4°C. First, the supernatant was collected for protein extraction. Subsequently, the remaining solution was heated at 95°C twice. Second, the supernatant was collected after centrifugation at 12,000 ×g for 5 min at room temperature for total lipid extraction. The extracted triglyceride and cholesterol were measured using a commercial kit (Wako Ltd., Japan). The protein quantity was evaluated by Bradford assay (Bio-Rad Co., USA).

Oil Red O Staining Assay

Oil Red O staining was conducted to quantify the intracellular triglyceride content. The 3T3-L1 adipocytes in 6-well-plates were fixed with 10% formalin and rinsed with distilled water. After adding 60% isopropanol for 5 min, a 60% aspirate of the resultant solution was withdrawn and stained with Oil Red O working solution (Oil Red O stock: distilled water = 6:4) for 10 min. Cell images were captured after staining each well using Leica Application Suite EZ (Leica, Germany). For quantification, the Oil Red O reagent was decolorized with 100% isopropanol. The solution was centrifuged at 12,000 ×g for 1 min at 4°C and the absorbance was measured at 500 nm.

Immunoblotting

Cytoplasmic protein was arranged using 4× Laemmli sample buffer (Bio-Rad Co.), and 1× RIPA Buffer (CellNest, Japan) containing a phosphatase inhibitor (Cell Signaling, USA) and a protease inhibitor (Cell Signaling). Lysate was applied to 20% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked in 5% bovine serum albumin (GenDEPOT, Inc., USA) and immunoblotted with target primary antibodies. Secondary antibodies were applied and the blots were quantified with Image Lab (Bio-Rad Co.). Antibodies to phospho-acetyl-CoA carboxylase 1 (ACC1-pS79), acetyl-CoA carboxylase 1 (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), CCAAT/enhancer-

binding protein alpha (C/EBPα), peroxisome proliferator-activated receptor gamma (PPARγ), perilipin, and β-actin were purchased from Cell Signaling. Antibodies to elongase of very long chain fatty acids 6 (ELOVL6), glycerol-3-phosphate acyltransferase 1 (GPAM), and sterol regulatory element-binding protein 1 (SREBP1) were purchased from Santa Cruz Biotechnology (USA).

Statistical Analysis

All data represent the results from independent quadruples. Data are presented as the mean ± standard error. All experiments were evaluated with one-way ANOVA. Dunnett's multiple comparisons test was used to compare means between the PX-treated groups and CTL group. The statistical analysis was carried out using Prism 6 (GraphPad Software, USA). Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Effects of PX on Cell Cytotoxicity in HepG2 Cell and 3T3-L1 Adipocytes

According to the MTT assay in HepG2 cells, cytotoxicity was exhibited by cells that were treated with PX 200 µg/ml only, as shown in Fig. 1A ($p < 0.05$). 3T3-L1 adipocytes did not show any cytotoxicity at all for the PX experimental treatments (Fig. 1B). In the LDH assay test, HepG2 cells represented about 20% cytotoxicity at a concentration of 200 µg/ml PX ($p < 0.05$) (Fig. 1A). 3T3-L1 adipocytes did not exhibit any cytotoxicity from all PX treatments (Fig. 1B). The cytotoxicity observed in HepG2 cells after treatment with PX 200 µg/ml led to the use of three experimental treatments, which were PX 50, 100, and 150 µg/ml. 3T3-L1 adipocytes were treated with PX concentrations of 25, 50, 75, and 100 µg/ml.

Reduction of Intracellular Lipids in HepG2 by PX

Intracellular levels of triglycerides and cholesterol in HepG2 are reported in Fig. 2. The triglyceride content in

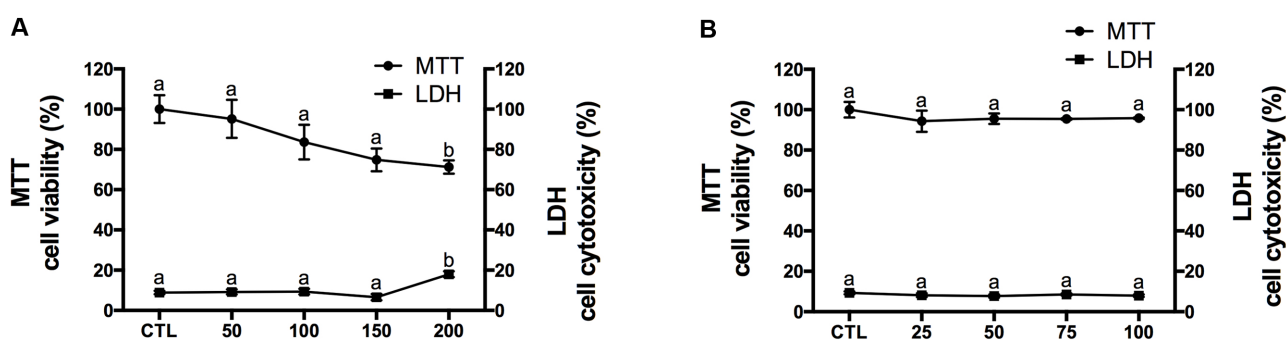


Fig. 1. Cell viability and cytotoxicity of (A) HepG2 and (B) 3T3-L1 adipocytes by MTT and LDH assay.

Data represent the mean ± standard error. $p < 0.05$.

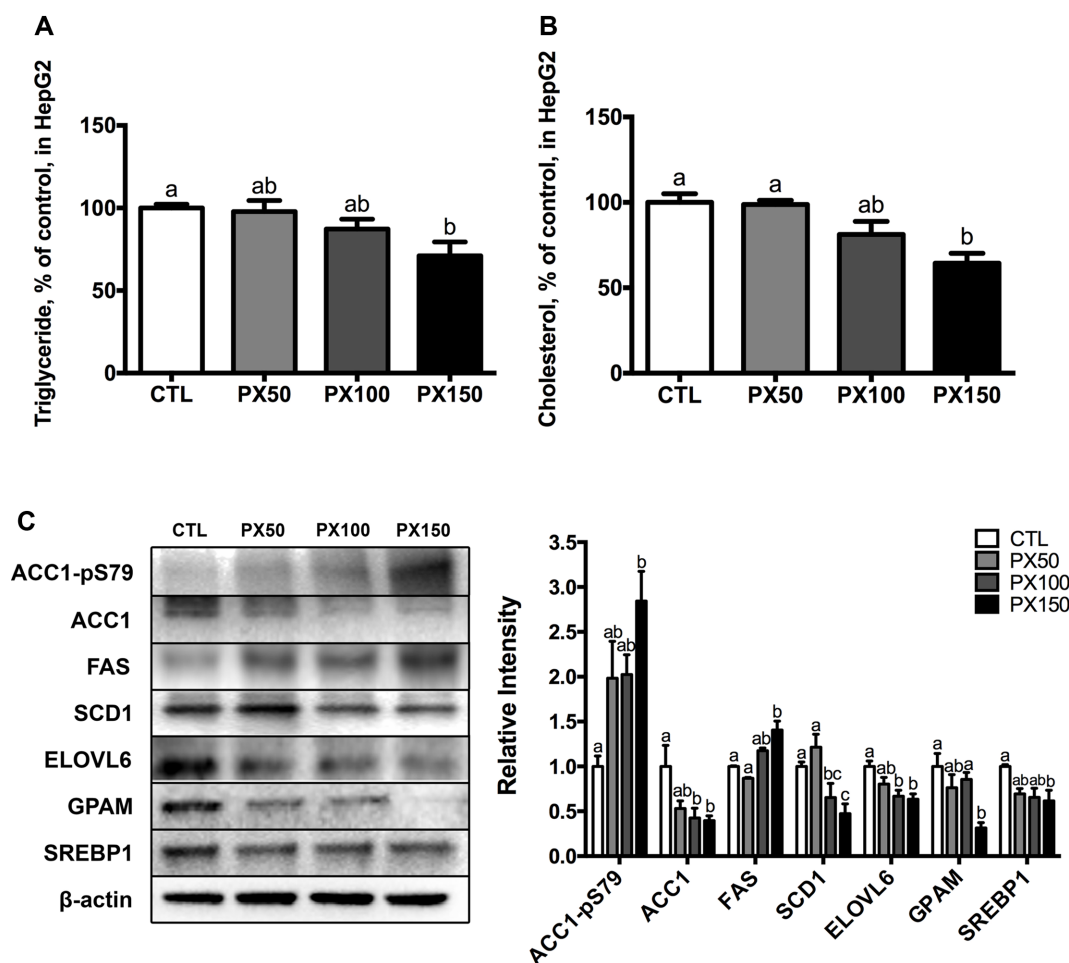


Fig. 2. (A) Triglyceride and (B) cholesterol levels of HepG2 cells and (C) western blot analysis of the activities of hepatic de novo lipogenesis pathways in HepG2 cell compared with the CTL group.

Data represent the mean \pm standard error. $p < 0.05$.

the PX 150 group was reduced by approximately 28% compared with the CTL group ($p < 0.05$) (Fig. 2A). The cholesterol content of the PX 150 group was restrained at approximately 36% compared with the CTL group ($p < 0.05$) (Fig. 2B). No significant difference in triglyceride and cholesterol levels among the CTL, PX 50 $\mu\text{g}/\text{ml}$, and PX 100 $\mu\text{g}/\text{ml}$ groups was noticed. Guerrero *et al.* [32] stated that cholesterol ester synthesis of HepG2 was significantly decreased by Pycnogenol at a concentration of 25 mg/ml. Free cholesterol and triglyceride were also reduced by half for the same treatment [32]. NMuLi liver cells treated with 50 $\mu\text{g}/\text{ml}$ Pycnogenol for 4 days had significantly suppressed intracellular lipid droplet formation [33]. In this research, PX inhibited triglyceride and cholesterol increases in hepatocytes, thus indicating its potential use as an anti-obesity nutraceutical.

Inhibition of De Novo Lipogenesis in HepG2 by PX

Fig. 2C shows the protein levels of enzymes and transcriptional regulatory factors related to de novo lipogenesis that were analyzed using western blot assay. β -Actin was used for normalization of the expression levels. ACC1 facilitates the pivotal step of the fatty acid synthesis pathway. ACC1-pS79, which is an inactivation form of ACC1, was markedly increased in the PX 150 $\mu\text{g}/\text{ml}$ group compared with the CTL group ($p < 0.05$). FAS, which catalyzes the synthesis of palmitate from acetyl-CoA, was increased by approximately 40% in the PX 150 $\mu\text{g}/\text{ml}$ group compared with the CTL group ($p < 0.05$). The reasons for how these results occurred are unknown and further studies are needed. Levels of ACC1 were decreased by approximately 57% and 61% in the PX 100 $\mu\text{g}/\text{ml}$ and PX 150 $\mu\text{g}/\text{ml}$ groups, respectively, compared with the CTL

group ($p < 0.05$). In the PX 150 $\mu\text{g}/\text{ml}$ group, levels of SCD1 and ELOVL6, which are important lipogenic enzymes, were reduced by approximately 53% and 37%, respectively, compared with the CTL group ($p < 0.05$). The level of GPAM, which catalyzes the first step in triglyceride biosynthesis, was diminished by approximately 69% in the PX 150 $\mu\text{g}/\text{ml}$ group in comparison with the CTL group ($p < 0.05$). The levels of ELOVL6 and GPAM in the PX 100 $\mu\text{g}/\text{ml}$ group were also significantly decreased ($p < 0.05$). SREBP1 is a transcriptional factor that plays a crucial role in the regulation of de novo lipogenesis. Levels of SREBP1 in the PX 150 $\mu\text{g}/\text{ml}$ group were decreased by approximately 38% compared with the CTL group ($p < 0.05$).

Hwang *et al.* [34] evaluated the effects of red pine tree (*Pinus densiflora*) extract at a concentration of 100 $\mu\text{g}/\text{ml}$ on lipogenesis. A significant decrease of target gene expression factors of de novo lipogenesis such as ACC, SCD1, and SREBP1 were noted compared with oleic acid-treated groups in HepG2 cells. It was also discovered that the protein level of SREBP1 was also suppressed. In this study, PX restrained de novo lipogenesis in vitro through reduction of target gene expression, hence subsequently diminishing lipogenic enzymes.

Lipid Accumulation Reduction in 3T3-L1 Adipocytes by PX

The amount of lipids in 3T3-L1 adipocytes was determined by Oil Red O reagent, which stained triglycerides to red in color (Fig. 3A). The degree of absorbance was therefore an indicator of lipid accumulation, which was indicated as a ratio (CTL = 1). Lipids were significantly suppressed in the PX 50 $\mu\text{g}/\text{ml}$ treatment in comparison with the CTL group ($p < 0.05$). The PX 75 $\mu\text{g}/\text{ml}$ and PX 100 $\mu\text{g}/\text{ml}$ treatment groups showed a decline in lipid accumulation by approximately 36% and 40%, respectively, in comparison with the CTL group ($p < 0.05$). Lipid accumulation in the PX-treated groups (50, 100, and 200 $\mu\text{g}/\text{ml}$) was considerably more inhibited than in the control group for 3T3-L1 [29]. Western blot analysis for proteins related to adipogenesis was conducted to examine the given mechanism.

PX Retardation of Adipogenesis in 3T3-L1 Adipocytes

Western blotting was carried out on 3T3-L1 adipocytes to identify PX effects on adipogenesis. Protein levels of CAAT (controlled amino acid therapy)/C/EBP α , PPAR γ , and perilipin, related to differentiation of adipocyte cells, are reported in Fig. 3B. β -Actin was used for normalization of

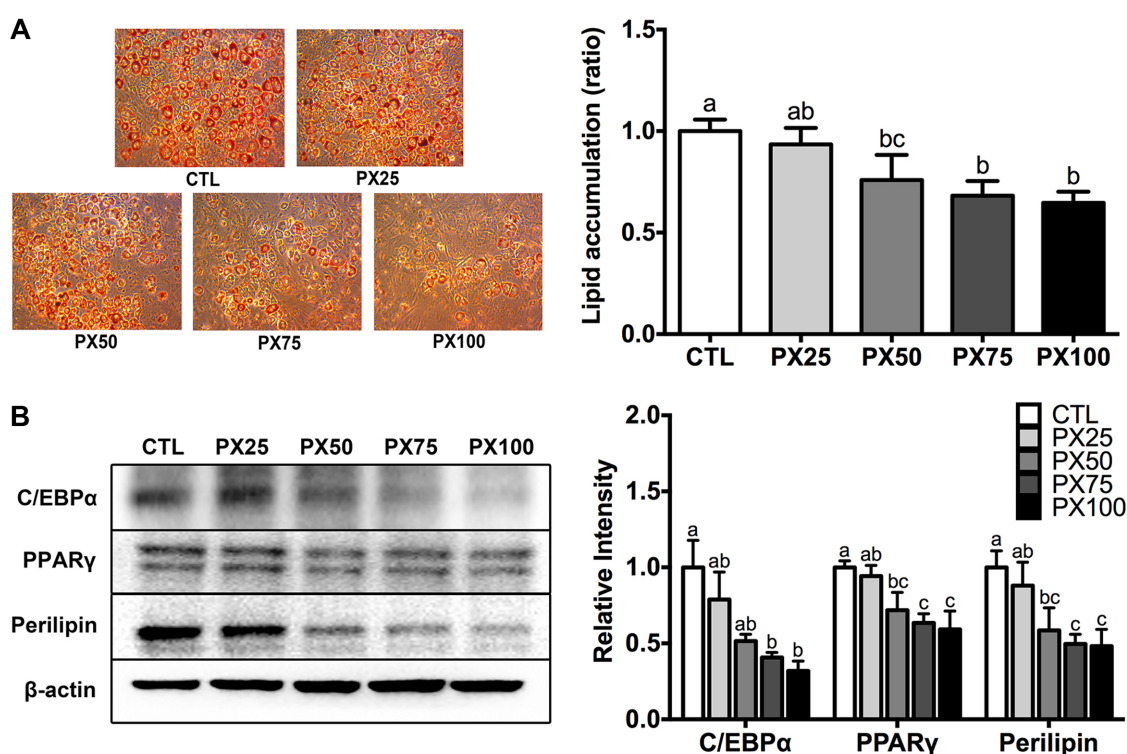


Fig. 3. (A) Cell images after Oil Red O staining and the relative quantification data and (B) western blot analysis representing the activities of proteins related to adipogenesis in 3T3-L1 adipocytes compared with the CTL group.

Data represent the mean \pm standard error. $p < 0.05$.

the expression levels. The level of C/EBP α was dramatically reduced by more than 50% in the PX (75 and 100 μ g/ml) groups compared with the CTL group ($p < 0.05$). The level of PPAR γ in the PX 100 μ g/ml group was significantly decreased by approximately 38% against that in the CTL group ($p < 0.05$). The perilipin level in the PX 50 μ g/ml, PX 75 μ g/ml, and PX 100 μ g/ml groups was clearly reduced in comparison with that in the CTL group ($p < 0.05$). The effect of PX on adipogenesis proved to be stronger at higher concentrations.

Comparably, 10–200 μ g/ml Pycnogenol markedly diminished adipogenesis by virtue of restraining PPAR γ and adiponectin expression in 3T3-L1 adipocytes [35]. Lee *et al.* [18] reported that mRNA expression of C/EBP α and PPAR γ was attenuated in Pycnogenol-treated groups at concentration levels of 50, 100, and 200 μ g/ml for 3T3-L1 adipocytes. The decline of perilipin A in *ob/ob* mice and primary mouse adipocytes was observed at 50 and 100 μ g/ml Pycnogenol treatment [27].

In conclusion, despite displaying cell cytotoxicity in HepG2 at a concentration of 200 μ g/ml, PX conclusively proved to be very capable of reducing lipid levels in HepG2 through a cocktail of mechanisms. PX reduced HepG2 intracellular lipid levels by inactivating ACC1. It also reduced SCD1 and ELOVL6, which are lipogenic enzymes, as well as SREBP1, which is a transcriptional factor associated with de novo lipogenesis. Further to that, PX decreased lipid accumulation in 3T3-L1 cells by downregulating the differentiation of pre-adipocytes, hence blocking adipogenesis. This research thus shows that PX can be a novel nutraceutical with excellent natural anti-lipogenic and anti-adipogenic properties. The study has also generated convincingly useful data for the future use of PX as an obesity antagonistic agent, bearing in mind the paradigm global shift towards natural medication.

Acknowledgments

This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through “High Value-added Food Technology Development Program,” funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (#115015-03-1-SB010).

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