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Effect of protein fractions from *Pithecellobium jiringa* on secretions of interleukin-6 and leptin in 3T3-L1 preadipocytes

*Yanti, Woenardhy, K., Widjaja, A.Y. and Agustinah, W.

Food Technology Program, Faculty of Biotechnology, Atma Jaya Catholic University, Jalan Jenderal Sudirman 51, Jakarta 12930, Indonesia

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Abstract

Obesity is a prevalent metabolic disorder that is induced by inflammatory responses that lead to the increased levels of pro-inflammatory mediators, including interleukin-6 (IL-6) and leptin. *Pithecellobium jiringa* or jengkol, is a functional tropical plant in Southeast Asia regions including Indonesia that has been for culinary and traditional medicines. This research was aimed to identify chemical constituents of protein fractions from *P. jiringa* peel and seed by using pyrolysis gas chromatography mass spectrometry (py-GC/MS), and their inhibitory effects at various concentrations (5-100 µg/ml) on IL-6 and leptin expression in mouse 3T3-L1 preadipocytes by conducting the enzyme-linked immunosorbent assay (ELISA). Both protein fractions from *P. jiringa* peel and seed consisted of amino acid derivatives, including alanine ethyl ester (peel part), dimehylamine, piperidine, aziridine, and phenyl acetyl glutamine (seed part). Protein fractions from *P. jiringa* peel at 25 µg/ml decreased >50% leptin expression in 3T3-L1 preadipocytes compared to that of seed protein fractions. Meanwhile, seed protein fraction dose-dependently inhibited IL-6 expression. At 10 µg/ml, seed protein fraction reduced up to 50% IL-6 production, respectively. Our results suggest that protein fractions from both *P. jiringa* peel and seed may have potential anti-obesity effects on 3T3-L1 preadipocytes via attenuating IL-6 and leptin expression. *P. jiringa* protein fractions can be used as alternative food supplements for reducing the risk of obesity.

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Introduction

Obesity is a condition when body fat has been accumulated excessively that may occur due to genetically or by other factors, such as unhealthy diets and physical habits. When obesity occured, adipose tissues increased (hyperplasia) and swollen. The excess storage of triacylglycerols from the dietary intake resulted in an excessive influx of free fatty acid into blood vessels and caused several problems in blood circulation (Teng *et al.*, 2014). Cytokines and pro-inflammatory proteins, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), adiponectin, resistin, retinol-binding protein 4 (RBP4), plasminogen activator inhibitor-1 (PAI-1), and leptin were regulated as inflamatory response and their concentration increased when obesity occured. These factors determined chronic low grade-inflammation that occured in the body and the overexpression of these factors could lead to insulin resistance (Friedman and Halaas, 1998; Qatanani and Lazar, 2007).

An alternative way to cure obesity is the use of anti-obesity herbal supplements. The basic principle of anti-obesity herbal supplement is to regulate the energy intake and expenditure. In several studies, herbal treatments have been proven to be safe and effective to reduce body weight. *Coleus forskohlii* (Indian Coleus) and *Garcinia camborgia* (Kudam Puli) have showed their effectiveness to reduce body weight significantly (Chandrasekaran *et al.*, 2012). Some plants, such as *Zingiber officinale* and *Alpina galanga* had been reported to have strong anti-inflammatory properties by inhibiting the induction of several genes that encoded the inflammatory responses, including chemokines, cytokines and inducible cyclooxygenase-2 enzyme (Grzanna *et al.*, 2005).

Pithecellobium jiringa, known as jengkol in Indonesia, is a tropical plant that has been used not only for foods, but is also believed to be able to cure various diseases, such as skin ailment, chest pain, gum pains, toothache, hypertension, and diabetes (Muslim *et al.*, 2012). *P. jiringa* seed extract has been reported to possess antibacterial and antifungal effects (Bakar *et al.*, 2012). Meanwhile, *P. jiringa* peel is rich in polyphenols with high antioxidant activity (Ibrahim *et al.*, 2012). However, there is no information about

*Corresponding author.
Email: *yanti@atmajaya.ac.id*

the efficacy of biomolecule fractions derived from *P. jiringa* for management of metabolic disorders, including obesity. This study was focused on identification of chemical constituents in protein fractions from peel and seed parts of *P. jiringa* protein fraction and determination of anti-obesity activity of protein fractions on modulation of IL-6 and leptin expression in 3T3-L1 preadipocytes *in vitro*.

Materials and Methods

Plants materials

P. jiringa samples (code: DJ01 for peel and DJ03 for seed) were collected from traditional market at Balige, North Sumatera (Indonesia). Sample vouchers were stored at Faculty of Biotechnology, Atma Jaya Catholic University, Jakarta (Indonesia). It was dried under the sun and separated into peel and seed. Each dried part of *P. jiringa* peel and seed was crushed separately and freeze-dried until it became powder.

Extraction of protein fractions

Protein fractions from *P. jiringa* peel and seed were extracted using isoelectric precipitation (Bollag *et al*., 1996). Each part was weighed 100 g, crushed, and mixed with 25% (w/v) distilled water. A 1 M sodium hydroxide (NaOH) was added until pH of solution reached 8.6, and stirred for 30 minutes at temperature of 50°C. The solution was centrifuged at 3000 *x* g for 30 minutes and the supernatant was taken. The supernatant was added with 2 M hydrogen chloride (HCl) until pH of 4.5 for protein precipitation, followed by centrifugation at 1500 *x* g for 20 minutes. The pellet was then dried in incubator at 37°C overnight. Protein fractions from *P. jiringa* peel and seed were further identified using pyrolysis-gas chromatography mass spectrometry (py-GC/MS).

Identification of chemical constituents in P. jiringa *protein fractions through pyrolysis gas chromatography mass spectrometry (py-GC/MS)*

Py-GC/MS was carried out to analyse the chemical constituents found in each fraction. Py-GC/MS analysis was done using QP2010 (Shimadzu, Duisburg) and conducted at the Center for Research for Development on Forest Engineering and Forest Product, Bogor (Indonesia). Sample was injected to the capillary column (Phase Rtx-5MS) with a film thickness of 60 m x 0.25 mmID. Pyrolysis temperature was set to 280°C. Helium was used as the carrier gas.

Cell culture and viability

The mouse 3T3-L1 preadipocytes (American Type Culture Collection (ATCC) CL-173) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, and 100 µg/ml of streptomycin. Cells were incubated at 37°C in 5% CO_2 to confluence. Cells were treated with protein fractions from *P. jiringa* peel and seed at various concentrations (1-100 µg/ml) in 96-well plate. Cell viability was evaluated with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay, then absorbance was measured at 550 nm.

Sample treatment

Cells were seeded at a concentration of 2 x 105 cells/ml in 12-well plates and cultured for 24 hours in DMEM-FBS. After washing with Dulbecco's Phosphate-Buffered Saline (DPBS), the cells were incubated in serum-free DMEM (positive control group), DPBS (negative control group), or serum free DMEM plus treatment for 48 hours. The treatment groups were protein fractions from *P. jiringa* peel and seed at various safe concentrations based on viability results ≥ 80%. Cell media were collected for further assay.

Determination of IL-6 and leptin expression by enzyme-linked immunosorbent assay (ELISA)

IL-6 and leptin expression were determined by Mouse IL-6 and Leptin Quantikine ELISA Kits (RD Systems, Minneapolis, MN) with sandwich principle. The specific antibody for each target protein (IL-6 and leptin) had been pre-coated into microplate. Cell media from each protein fraction from *P. jiringa* peel and seed in various concentrations (5-100 µg/ml) and control were added onto wells to bound target protein that contained in the media by the immobilized antibody. All the wells of the microplate were washed to remove any unbound substances and added with conjugate from the kit. The wells were washed again to remove any unbound substances and added with a color substrate reagent. The color was changed in the presence of protein-bound enzyme and the color intensity was showed the amount of bounded protein. The reaction was stopped after the addition of stop solution and optical density of each well was measured at 450 nm using ELISA microplate reader.

Statistical analysis

Triplicate experiments were performed for MTT assay, and duplicate experiments were performed for ELISA. All data were presented as the mean ±

standard deviation (SD). The significant difference between control and treated groups were analyzed by the paired Student's t-test ($p<0.05$).

Results

Identification of chemical constituents from P. jiringa *peel and seed*

Chemical constituents of protein fractions from *P. jiringa* peel and seed were identified using py-GC/MS. The chromatograms showed that both protein fractions had various protein derivatives. Peel protein fraction (Figure 1a; Table 1) had major content of alanine ethyl ester (15.4%). Other nonprotein constituent such ammonium carbamate were also found in peel fraction. Meanwhile, seed protein fraction (Figure 1b; Table 1) had four protein derivatives, including dimethylamine (12.6%), piperidine (1.19%), azeridine (2.19%) and phenylacetylglutamine (1.04%). Chromatogram profile also showed that seed fraction consisted of nonprotein constituents, including phenethyl alcohol, mome inositol and levoglucosan.

Effect of protein fractions on cell viability of 3T3-L1 preadipocytes

MTT colorimetric assay was used to determine the safe concentration of sample treatment on 3T3-L1 preadipocytes. Our results showed that *P. jiringa* protein fractions from both peel and seed parts up to 100 µg/ml were safe for 3T3-L1 preadipocytes (Figure 2).

Effect of protein fractions on interleukin-6 and leptin expression in 3T3-L1 preadipocytes

The expression of IL-6 and leptin secreted in cell media of 3T3-L1 preadipocytes after treated with protein fractions from *P. jiringa* peel and seed was quantified by ELISA (Figure 3). For IL-6 profiles, at 10 µg/ml, seed protein fraction significantly inhibited IL-6 expression >50% or ~650 pg/ml compared to that of untreated cells, and it dose-dependently affected the IL-6 secretion in cell media of 3T3-L1 preadipocytes (Figure 3a). Meanwhile, protein fraction from *P. jiringa* peel at 25 µg/ml decreased IL-6 expression up to 50% (~600 pg/ml) in the dose-dependent manner. Leptin profiles showed that at concentration of 25 µg/ml, protein fraction from peel significantly reduced leptin expression in cell media up to 60% compared to that of untreated cells (Figure 3b). However, seed protein fraction at various concentrations only demonstrated slight inhibitory effect (~20%) on leptin secretion in the dose-independent manner.

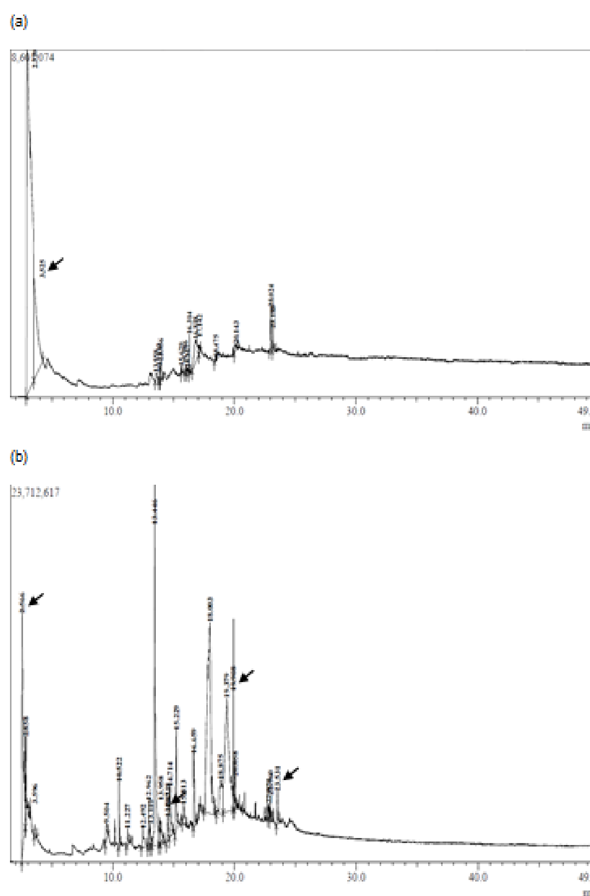

Figure 1. Chromatograms of *P. jiringa* protein fraction extracted from peel (a) and seed (b) by py-GC/MS.

Discussion

This study used adipocytes as one of the experimental cell culture models to investigate the effect of the protein fractions derived from *P. jiringa* peel and seed in regulation of inflammation-related obesity. The 3T3-L1 cell line is a favored model for obesity research because it can be adequately studied in vitro and has similar main characteristics with adipocytes (White and Stephens, 2010). The cell is derived from disaggregated Swiss 3T3 mouse embryos that are differentiating into adipocyte-like cells. During the stage of differentiation, the cells become responsive to insulin. The 3T3-L1 preadipocytes possess mostly mature adipocyte biochemical characteristics and they are easier to be preserved than mature adipocyte. Mature adipocyte is unstable, because the structural integrity of cell organelles are difficult to maintain during the cell fixation (Ntambi and Cheul, 2000).

Protein fractions from *P. jiringa* peel and seed were investigated for their chemical constituents by py-GC/MS (Figure 1; Table 1). Our results demonstrated that both protein fractions contained several amines and amino acids as protein derivatives, including

Table 1. Chemical constituents in protein fractions extracted from peel and seed parts of *Pithecellobium jiringa*

Peak	Constituent	Molecular Formula	Molecular Weight	Concentration (%)
Peel part				
1	Ammonium carbamate	CH_3NO_2	61	73.97
2	Alanine ethyl ester	$C_5H_{11}NO_2$	117	15.40
3	Not identified	$C_7H_{10}O_2$	126	0.70
4	p-Vinylanisole	$C_9H_{10}O$	134	0.46
5	2-Butenethioic acid, 3-(ethylthio)-, S-(1-methylethyl) ester	$C_9H_{16}OS^2$	204	1.21
6	Carvacrol	$C_{10}H_{14}O$	150	0.16
7	2,6-Dimethoxyphenol	$C_8H_{10}O_3$	154	0.29
8	Neric Acid	$C_{10}H_{16}O_2$	168	0.33
9	Monomethylphosphate 2 TMS	$C_7H_{21}O_4PSI_2$	256	0.57
10	2,6-Dihydroxy-4-methoxyacetophenone	$C_9H_{10}O_4$	182	3.96
11	α-Cedrol	$C_{15}H_{26}O$	222	0.32
12	β-Tumerone	$C_{15}H_{22}O$	218	0.15
13	Purin-8-ol, 6-amino-8,9-dihydro-	$C_5H_7N_5O$	153	0.6
14	Cis-octadec-9-enal	$C_{18}H_{34}O$	266	0.96
15	1-Cyclohexyleicosane	$C_{26}H_{52}$	364	0.92
Seed part				
1	Dimethylamine	C_2H_7N	45	12.60
2	Nitrous oxide	N_2O	44	1.90
3	Etyhlene sulfide	C_2H_4S	60	0.45
4	Furfuryl alcohol	$C_5H_6O_2$	98	0.95
5	2-Methtylthiazolidine	C_4H_9NS	103	1.38
6	1-(Azolidin-1-YL)-1-butene	$C_7H_{11}NO$	125	0.51
7	Tert-butyl pentadeuterobenzene	$C_{10}H_9D_5$	134	1.13
8	2,5-Dimethyl-4-hydroxy-3(2H)-furanone	$C_6H_8O_3$	128	1.38
9	Trans-2-methyl-3	$C_7H_{15}N$	113	0.83
10	Phenethyl alcohol	$C_8H_{10}O$	122	13.67
11	N-formylpiperidine	$C_6H_{11}NO$	113	0.75
12	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	$C_6H_8O_4$	144	1.25
13	N-Acetylpiperidine	$C_7H_{13}NO$	127	1.19
14	5-Hydroxy-2,7-dimethyl-4-octanone	$C_{10}H_{20}O_2$	172	2.17
15	Phenylacetic acid	$C_8H_8O_2$	136	3.46
16	N,N-Diethanol-dodecylsulfo	$C_{16}H_{35}NO_4S$	337	1.00
17	2-Phenylacetamide	C_8H_9NO	135	2.56
18	Levoglucosan	$C_6H_{10}O_5$	162	25.92
19	Thymidine	$C_{10}H_{14}N_2O_5$	242	4.02
20	Mome inositol	$C_7H_{14}O_6$	194	17.55
21	1-Benzyl-2-methyl-3-T-butylaziric	$C_{14}H_{21}N$	203	2.19
22	(2R,5R)-5-Acetyl-2-tert-butyl-1-aza-3-oxabicyclo[3.3.0]octan-4-one	$C_{12}H_{19}NO_3$	225	1.06
23	Hyacinthin	C_8H_8O	120	0.47
24	2,6-Diphenylpyridine	$C_{17}H_{13}N$	231	0.58
25	Phenylacetylglutamine	$C_{13}H_{16}N_2O_4$	264	1.04

dimethylamine, piperidine, aziridine, phenyl acetyl glutamine, and alanine ethyl ester. Piperidine from *Piper retrofractum* Vahl. has anti-obesity activities through activating peroxisome proliferator-activated receptor γ (PPARγ) and regulating lipid metabolism (Kim *et al*., 2011). In addition, other piperidine alkaloids had also been found to possess anti-obesity and AMPK activating effects investigated in 3T3-L1 adipocytes. Aziridine can be used as active substances in pharmaceutical for treatment of obesity and diabetes mellitus (Alig and Muller, 1983).

MTT profile showed that up to 100 µg/ml, both protein fractions from *P. jiringa* seed and peel had no toxic effect to 3T3-L1 preadipocyte viability, indicating their safe dosages for further assay (Figure 2). According to Hostanka *et al.* (2007), samples were considered to have cytotoxic effects if they caused the decreased cell viability more than 80%, respectively. Moreover, cell morphology also demonstrated the disruption of cell membrane if samples were toxic against cells (Li *et al*., 2011).

Obesity-associated proinflammatory cytokine such as IL-6 can be used as a biomarker because obese people exhibit a higher magnitude of this cytokine expression when compared with non obese people (Rieusset *et al*., 2004; Goyal *et al*., 2012; Ruiz *et al*., 2014). IL-6 is a major proinflammatory cytokine that played a great role in obesity and insulin resistance. An increased level of IL-6 showed the occurance of inflammation and adipocyte hyperplasia (Hotamisligil *et al*., 1995; Yoshida and Tanaka ,2014). In this study, IL-6 was used to examine the anti-obesity effect of *P. jiringa* protein fractions targeting obesity prevention in preadipocyte model.

Our results showed that protein fractions from *P. jiringa* seed and peel reduced the secretion of IL-6 in mouse 3T3-L1 preadipocytes in the dose-independent manner (Figure 3a). It is implied that seed protein fraction is more effective as IL-6 inhibitor compared to that of peel protein fraction in preadipocyte model *in vitro*. At lowest concentration (10 µg/ml), seed protein fraction significantly blocked >50% of IL-6 secreted from cell media of preadipocytes. However, the IL-6 inhibition by seed protein fraction was less effective than that of pentamethylquercetin (PMQ), a synthetic polymethoxilated flavonoid (Chen *et al*., 2011). The PMQ at concentration of 0.01 µg/ml strongly downgraded IL-6 secretion up to 80% in 3T3-L1 preadipocyte cells. Considering that protein fraction of *P. jiringa* seed is more natural than the synthetic PMQ, it is assumed that *P. jiringa* seed fraction offer potential anti-obesity effect. Recent

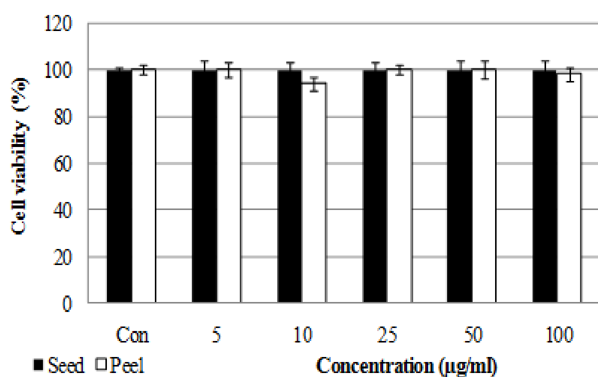

Figure 2. Cell viability of 3T3-L1 preadipocytes after treated with protein fractions derived fom *P. jiringa* peel and seed at concentrations of 5-100 µg/ml by MTT assay ($p<0.05$).

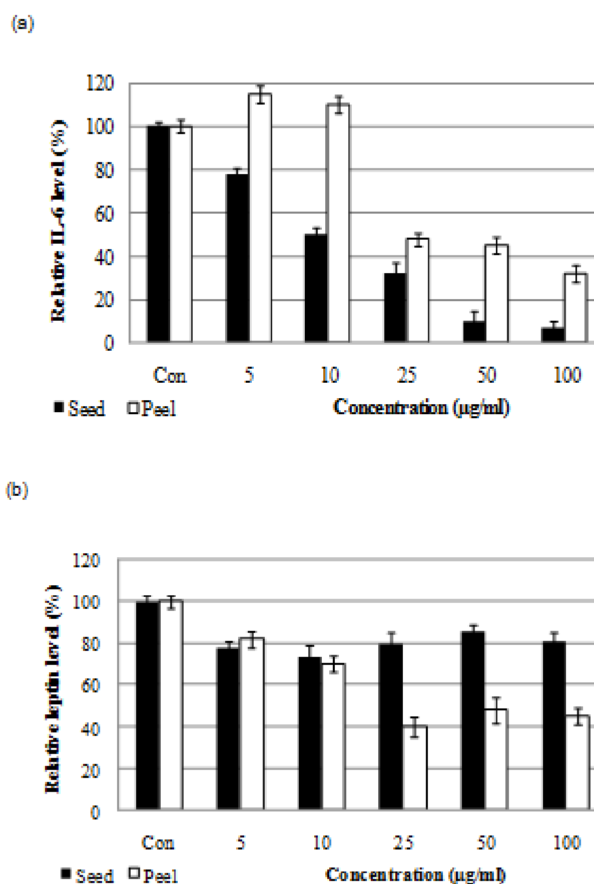

Figure 3. Effect of protein fractions derived from *P. jiringa* peel and seed (5-100 µg/ml) on IL-6 (a) and leptin (b) expression in mouse 3T3-L1 preadipocytes by ELISA ($p<0.05$).

study demonstrated that ethanolic extract of *P. jiringa* also exerted antiangiogenesis and antioxidant activities (Muslim *et al*., 2012). *P. jiringa* seed extract was reported to effectively reduce blood sugar level in balb/c mice in vivo model. Some plant extracts, including sea-buckthorn (*Hippophae rhamnoides* L.), bamboo, and paprika also showed their efficacies to reduce IL-6 concentration significantly in preadipocytes in vitro (Chen *et al*., 2011; Higa and Panee, 2011; Maeda *et al*., 2013).

Obesity is related with the development of inflammation. Expansion of the adipose tissue depot in obesity may increase the production of proinflammatory cytokines and influence the gene expression of adipose tissue as an endocrine organ (Godwill and Isaac, 2012). In obese person, the body produces leptin, which is an adipose tissue hormone that functions as an signal in a negative feedback that maintains adipose tissue mass. Leptin stimulates lipolysis, inhibits the expression of genes of lipid synthesis such as fatty acid synthase, and upragulates adipocytes genes involved in lipid oxidation (Vangipuram *et al*., 2007). Leptin plays an important role in regulation of body weight. According to our data, the decrease of leptin level indicated anti-obesity mechanism in jengkol protein fractions in particular peel part (Figure 3b). Recent report demonstrated that *Lysimachiafoenum* extract exerted antiobesity effect by decreasing leptin and restored it to normal levels (Seo *et al*., 2011). *Sphenocentrum jollyanum* extract has an anti-obesity potential by significantly decrease leptin levels up to 26% (Anyanwu, 2014). Coffee bean extract significantly decreased expression of leptin, indicating its potential used as a therapeutic agent to cure obesity (Song *et al*., 2014).

Leptin is a peptide that is produced mainly by fat tissue because of that in obese subjects induced high level of leptin protein. Normally leptin reduces food intake and body weight, but elevation in leptin level with obese subjects may cause leptin resistance which leads to increased food intake and becoming more overweight and obese (Azizian *et al*., 2012). Obesity is positively correlated with leptin resistance (Bahathiq, 2010). Our study indicated that protein fraction from jengkol peel significantly reduced expression of leptin probably through the increase of leptin sensitivity.

Conclusion

Our results suggest that protein fractions derived from *P. jiringa* peel and seed have potential anti-obesity effects through reducing proinflammatory mediators of IL-6 and leptin expression in mouse 3T3-L1 preadipocytes in vitro. Interestingly, *P. jiringa* seed can be suggested to be consumed as a healthy diet for reducing obesity. Further study is needed to do purification and characterization the purified proteins from the fractions that are responsible for anti-obesity target.

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