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Coffee pulp ethanolic extract ameliorates palmitic acid-induced inflammation in RAW 264.7 macrophage and insulin resistance in 3T3-L1 adipocyte

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Abstract

Coffee pulp is a waste material from the coffee industry. Major compounds of coffee pulp are chlorogenic acids (CGAs) which possess anti-oxidant and anti-inflammatory properties. Obesity-induced macrophage recruitment in adipocyte results in chronic low grade inflammation followed by insulin resistance in adipocyte and palmitic acid (PA) release which further increases inflammation. Limiting the effects of PA may slow this cycle which creates diabetes. This study aims to determine the effect of coffee pulp ethanolic extract (CPEE) both on PA-stimulated inflammation in RAW 264.7 macrophage and insulin resistance in 3T3-L1 adipocyte cell lines. The CPEE was prepared and determined CGAs profile using HPLC analysis. The major CGAs of CPEE was 3-caffeoylquinic acid (12.93 mg/g extract). Cytotoxicity study revealed that 5-200 µg/mL of CPEE was not toxic to both cell lines. CPEE (50-200 µg/mL) significantly decreased pro-inflammatory cytokines, IL-6 and TNF-α protein expression in a dose dependent manner in PA-treated RAW 264.7 cells. Moreover, CPEE significantly ameliorates insulin resistance in PA-treated 3T3-L1 cells by increasing glucose uptake and decreasing lipolysis. Coffee pulp could be a promising source for agents of anti-inflammatory and anti-insulin resistant effects. Therefore, scientific evidence suggest the use of coffee pulp as an agent for obesity-induced diabetes prevention.

Introduction

Obesity is a serious health problem worldwide. It is a risk factor for non-communicable diseases (NCD) including insulin resistance which cause a type 2 diabetes mellitus (T2DM). The pathogenesis of insulin resistance involves obesity-induced inflammation in adipose tissue. The over expansion of adipocyte in obesity that can recruit macrophages and other immune cells in to adipose tissue.[1,2] The activated macrophage produces various pro-inflammatory cytokine such as tumor necrosis factor-alpha (TNF-α) and interluekin-6 (IL-6), which in turn recruit augmentative macrophages resulting in chronic low-grade inflammation in adipose tissue.[3] Moreover, free fatty acid are elevated in circulating blood which also promotes macrophage inflammation and increases pro-inflammatory cytokines both from macrophage and adipose tissue.[4] Pro-inflammatory cytokine, TNF-α and IL-6 and free fatty acid can impede insulin signaling in adipocyte which interfere tyrosine phosphorylation on insulin receptor substrate (IRS) lead to cellular insulin resistance.[3,5] With insulin resistance of adipocyte, glucose and free fatty acid increase in blood circulation. Furthermore, elevated TNF-α, IL-6 and free fatty acid exert paracrine effects to activate inflammatory pathways within insulin target cells including muscle and liver resulting in systemic insulin resistance and

progress to T2DM .[6] Therefore, repression of obesity-induced inflammation and insulin resistance maybe a strategy to protect development of T2DM.

Coffee pulp is a main byproduct after removal of the bean from coffee cherry *(Coffeaarabica* L.). It is an abundant waste material in the coffee industry. One ton of coffee pulp (dry weight) are produced from two tons of coffee cherry.[7] Coffee pulp is highly acidic and toxic in soil and water. Research has found a way to utilize this waste. Coffee pulp contains phenolic acid and flavonoids; 42.2% chlorogenic acids (CGAs) and 21.6 % epicatechinrespectivly.[8] CGAs have various isomers and potential bioactivity such as antioxidant and anti-inflammatory activities in LPS -induced RAW 264.7 cells.[9] In addition, there are anti-obesity properties and improved lipid metabolism in high fat diet-induced-obese mice.[10]

However, these isomer types of CGAs and bioactivities in coffee pulp have not been reported. Thus, this study aims to investigate CGAs profile in coffee pulp ethanolic extract (CPEE) and also examines anti-inflammation in RAW 264.7 macrophage and anti-insulin resistance in 3T3-L1 adipocyte.

Methodology

Preparation and HPLC analysis of coffee pulp ethanolic extract (CPEE)

Dried coffee pulp was provided by HILLKOFF CO.Ltd.. It was extracted twice with 80% ethanol (1:10 w/v). After filtration, solvent was removed using an evaporator. The extract was freeze dried and kept at -20 °C until used. The CGAs profile of CPEE was examined by HPLC analysis according to A.P. Craig, 2016.[11]

Cell culture and differentiation

RAW 264.7 macrophage cell lines (ATCC, USA) were maintained as a suspension culture in the ultra low attachment culture dish in Gibco™ DMEM, High Glucose (Life technology, USA) with L-glutamine supplemented with 10% HyClone™ Fetal Bovine Serum (FBS) (GE Healthcare Life Sciences, USA) and 1% antibiotic under 5% CO_2 at 37 °C.

3T3-L1 pre-adipocytes cell lines (ATCC, USA) was cultured in DMEM containing 10% calf serum and 1% antibiotic, and incubated at 37 °C in a humidified 5% CO_2. After full confluence, 3T3-L1 pre-adipocyte was differentiated by cultured in DMEM containing 0.5 mM 3-isobutyl-1-metylxanthine (IBMX), 0.5 µg/ml dexamethasone (Dex), 5 µg/ml insulin and 10% FBS for 3 days, followed by being cultured 3 days in the same medium without IBMX and Dex. Differentiation of mature 3T3-L1 was completed by incubating the cell in DMEM containing 10% FBS for 7-14 days

Palmitic acid-BSA complex

Inflammation in RAW 264.7 macrophages and insulin resistance in mature 3T3-L1 adipocytes was induced with palmitic acid (PA) (Sigma Aldrich, USA). PA needs to be complexed with BSA to solubilize and passed to the cell. Briefly, PA was dissolved in 100% ethanol at 70 °C and combined with BSA solution with a molar ratio of 1 mM PA to 0.18 mM BSA.

Non-toxic concentration determination

Cytotoxicity test was performed in the 96-well plate. RAW 264.7 cells were seeded at 1.5×10^4 cells/well. 3T3-L1 adipocytes were seeded at 2×10^3 cells/well and grown to maturation as mentioned above. Then cells were cultured in various concentrations (5-400 µg/mL) of CPEE. This assay is usually performed for 24 hours. Macrophage cytotoxicity was determined using Sulforhodamine B (SRB) assay (Sigma Aldrich, USA). The adipocyte cytotoxicity was determined using WST-1 assay (Santa Cruz Biotechnology, USA). Non-toxic concentrations of the extract were used in the further experiment.

Effect of coffee pulp ethanolic extract (CPEE) on palmitic acid (PA)-induced RAW 264.7 macrophages inflammation

RAW 264.7 macrophages (1.5×10^5 cells/well) were plated in 24-well plate. The CPEE (50-200 µg/mL) were pre-treated for 4 hours followed by 200 µM PA-BSA complex or BSA vehicle for control and incubated for 20 hours. Then, conditioned media were collected to determine TNF-α and IL-6 protein level using ELISA kits (BioLegend ELISA MAX™ Deluxe Set, USA).

Effect of coffee pulp ethanolic extract (CPEE) on palmitic acid (PA)-induced 3T3-L1 adipocytes insulin resistance

3T3-L1 adipocytes (2×10^4 cells/well) were plated in 24 well-plate and grown to maturation. The CPEE (50-200 µg/mL) were pre-treated for 4 hours followed by 200 µM PA-BSA complex or BSA vehicle for control and incubated for 20 hours. Then, cells and conditioned media were collected to determine glucose uptake and lipolysis activities.

Glucose uptake assay

Cells were washed with phosphate-buffered saline (PBS) and then incubated with Gibco™ DMEM, Low Glucose (Life technology, USA) for 3 hours. The DMEM media was replaced by 80 mM 2-NBD-glucose (Invitrogen, USA), 100 mM insulin, and the cells were incubated for 1 hour at 37 ºC. Fluorescence was measured at λex =466 nm and λem = 540 nm using a microplate reader.

Lipolysis assay

Glycerol content was determined in the conditioned medium of matured 3T3-L1 adipocyte in the presence of insulin using a free glycerol determination kit (Sigma Aldrich, USA) following the instruction of the manufacture.

Statistical Analysis

All values were given as mean ± standard derivation (X±SD) from triplicate samples of three independent experiments. Overall, the differences among the treatment groups will be determined by using one-way analysis of variance (ANOVA), followed by Tukey's. *P*-values < 0.01 are regarded as significance.

Results and discussion

Chlorogenic acids profile of coffee pulp ethanolic extract (CPEE)

The chlorogenic acids (CGAs) are phenolic compounds characterized by quinic acid conjugated with caffeic acid.[12] There are various isomers of caffeic acid at other hydroxyl sites on the quinic acid ring including 3-*O*-caffeoylquinic acid (3-CQA), 4-*O*-caffeoylquinic acid (4-CQA) and 5-*O*-caffeoylquinic acid (5-CQA).[13] Moreover, a number of caffeic acid conjugates have more than one group also call dicaffeoylquinic acids (di-CQA) such as 3,4-dicaffeoylquinic acid (3,4-diCQA), 3,5-dicaffeoylquinic acid (3,5-diCQA), and 4,5-dicaffeoylquinic acid (4,5-diCQA).[14] Previous studies have reported that the major CGAs in coffee bean extract are 5-CQA, 4-CQA and 3-CQA respectively.[13,15,16] However, the HPLC analysis of this study found that in CPEE the major compound was 3-CQA and the minor compounds were 4-CQA and 5-CQA (Figure.1). The amount of 3-CQA, 4CQA and 5-CQA was 12.93, 0.93 and 0.74 mg/g extract respectively.

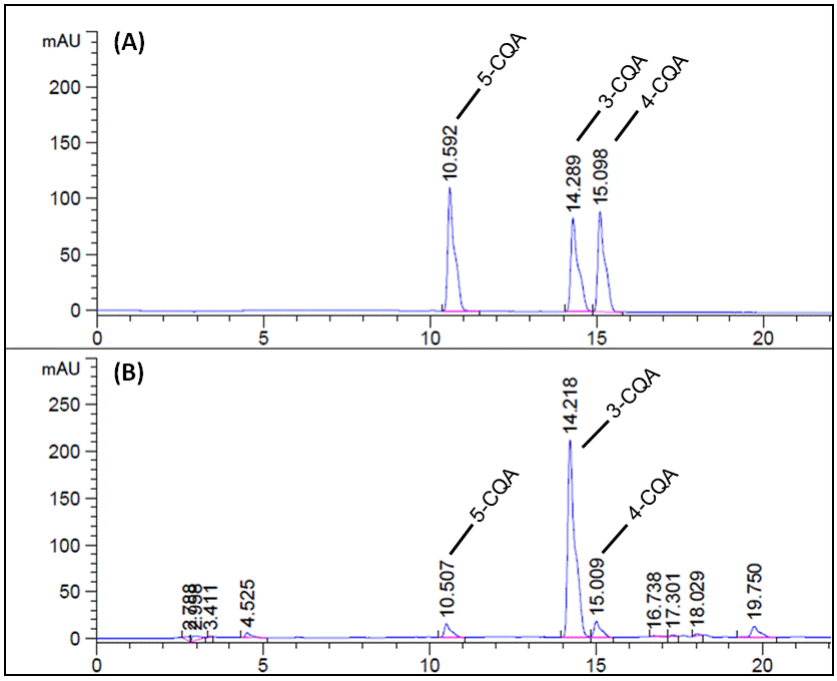

Figure.1 HPLC chromatogram of (A) Standard Chlorogenic acids (3-CQA, 4-CQA, 5-CQA) (B) Coffee pulp ethanolic extract

Effect of CPEE on cell viability

Cell viability of both RAW 264.7 macrophages and 3T3-L1 adipocytes cell lines were examined with various concentrations of CPEE (5-400 µg/mL) for 24 hours. The viability of RAW 264.7 macrophages was slightly decreased by CPEE but 3T3-L1 adipocytes were not affected. However, the cell viability of both cell lines were not significantly affected by CPEE up to 400 µg/mL, relative to untreated control cell (Figure.2). Non-toxic concentrations were used in further experiments.

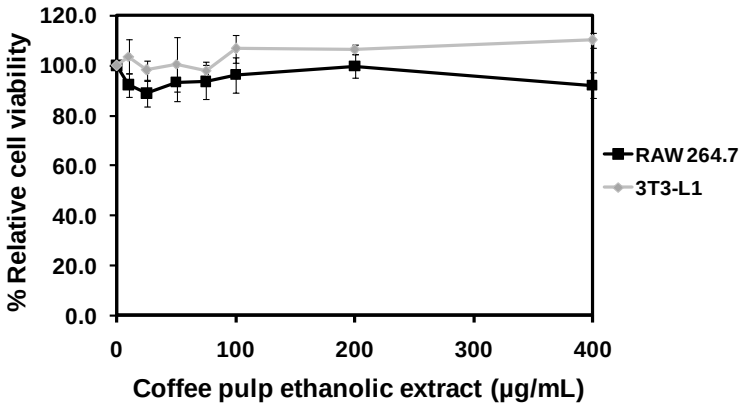

Figure.2 Effects of CPEE on cell viability of RAW 264.7 macrophages and 3T3-L1 adipocytes. The cell lines were incubated with various concentration of CPEE (5, 10, 25, 50, 75, 100, 200, 400 µg/mL) for 24 hours. The RAW 264.7 macrophages cell viability was determined using SRB assay and the 3T3-L1 adipocytes cell viability was determined using WST-1.

Anti-inflammatory activity of CPEE on PA-induced RAW 264.7 macrophages inflammation

In obesity, saturated free fatty acids (SFA) are increased in blood level. The majority of circulating SFA is palmitic acid.[17] It is one inflammatory molecule which can bind Toll-like receptors 4 (TLR-4) and activate NF-kB and JNK pathway, resulting in increased pro-inflammatory cytokine such as TNF-α as well as IL-6.[6,18] In this study , we examined the effect of CPEE on PA-treated RAW 264.7 cell macrophages. Pro-inflammatory cytokine, TNF-α and IL-6 protein levels were analyzed using ELISA kits. The PA-treated RAW 264.7 cell macrophages significantly increased TNF-α and IL-6 protein level compared with untreated control. However, CPEE reduced the inflammation caused by PA-treated RAW 264.7 cell macrophages by significantly reducing TNF-α and IL-6 protein level in dose dependent manner (Figure.3A, 3B).

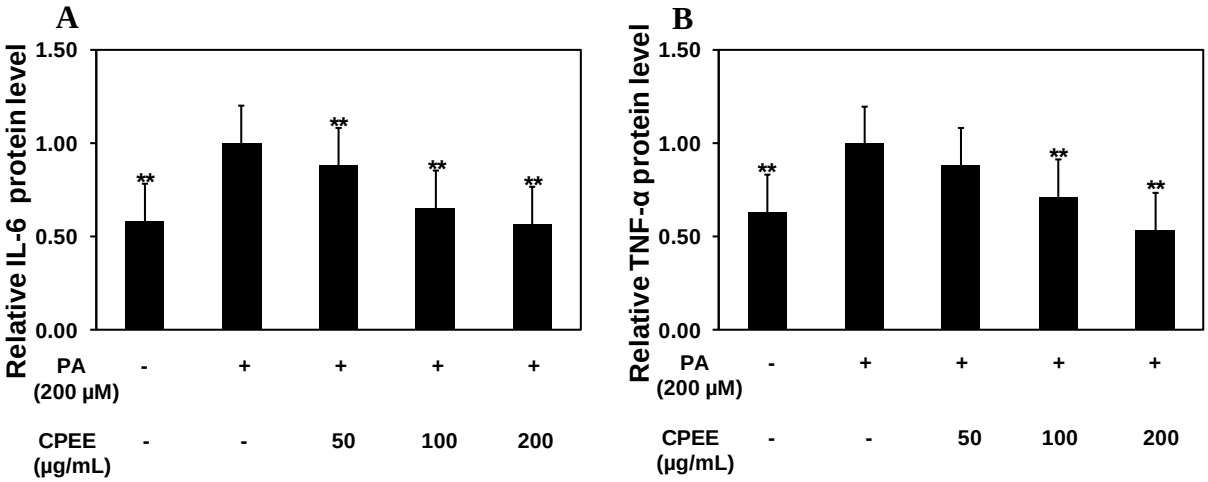

Figure.3 The anti-inflammatory activity of CPEE on PA-induced RAW 264.7 macrophages inflammation. The cells were treated with presence or absence CPEE (50, 100, 200 μg/mL) for 4 hours followed by 200 μM PA or BSA-complex vehicle for 20 hours and then conditioned medium were collected to investigate pro-inflammatory cytokine protein level, IL-6 protein (A) TNF-α (B) (**p < 0.01 relative to the control treated with PA alone)

Anti-insulin resistance activity of CPEE on PA-induced 3T3-L1 adipocytes insulin resistance

Previous study have been reported that PA could be one contributing mechanism for adipose tissue inflammation and dysfunction in obesity.[19,20] PA can be also activated NF-*k*B and JNK pathway via TLR-4 and leaded to cytokine production which impairs insulin signaling in adipocyte. Insulin resistance in adipocyte reduced glucose uptake resulting in increased blood glucose levels. Moreover, free fatty acids also increased by stimulated lipolysis. In this study, we aim to investigate anti-insulin resistance activity of CPEE on PA-induced 3T3-L1 adipocytes. Glucose uptake was decreased in PA-treated 3T3-L1 adipocytes, 40% relative with untreated control (Figure.4A), while glycerol level was increased (Figure.4B). The CPEE could improve insulin sensitivity in PA-treated cells. Glucose uptake was increased nearly as much as in untreated control group at 200 μg/mL of CPEE (Figure.4A). Moreover, lipolytic activity was suppressed by significant decreasing of glycerol level at 100 and 200 μg/mL of CPEE (Figure.4B).

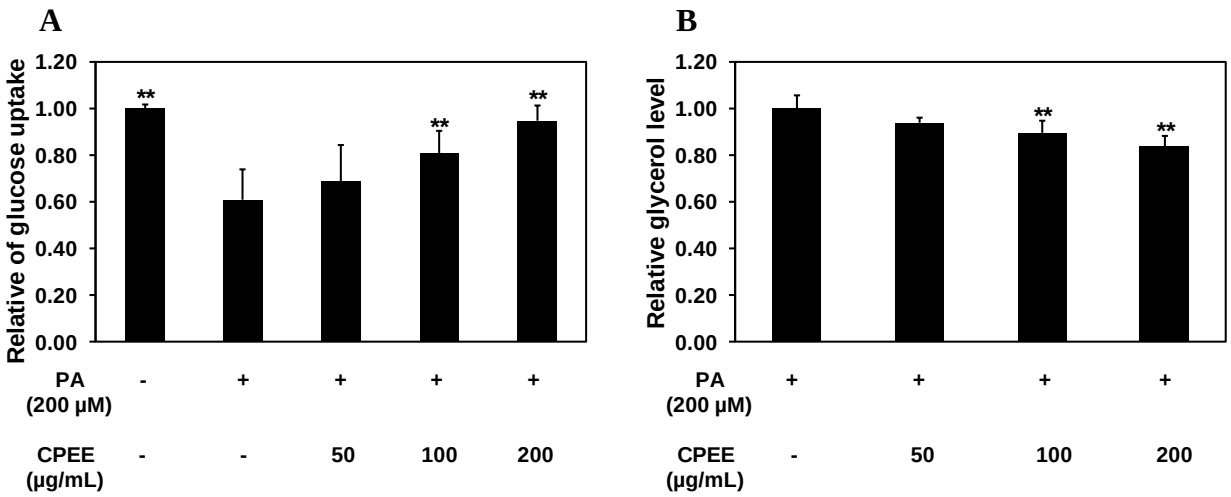

Figure.4 The anti-insulin resistance activity of CPEE on PA-induced RAW 264.7 macrophages inflammation. The cells were treated with presence or absence of CPEE (50, 100, 200 µg/mL) for 4 hours followed by 200 µM PA or BSA-complex vehicle for 20 hours. The cells were collected to determine glucose uptake (A) and conditioned medium were collected to measure glycerol level (B) (**$p < 0.01$ relative to the control treated with PA alone)

Conclusion

3-caffeoylquinic acid is the major CGA in coffee pulp. The ethanol extract of coffee pulp showed anti-inflammatory and anti insulin-resistant activities in macrophages and adipocytes respectively. Thus, scientific evidence suggest the potential of coffee pulp as an agent for obesity-induced diabetes prevention.

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