The Optimal Dose of the Green Tea and Coffee Extracts to Suppress the Expression of PPAR-γ and C/EBP-α on Differentiated 3T3-L1 Adipocytes

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Abstract

Obesity is the excess fat content in the body caused by the expansion of white adipose tissue. This condition begins with the differentiation of adipose tissue that is known to be controlled by 2 main transcription factors, such as Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) and CCAAT Enhancer-Binding Protein-α (C/EBP-α). Their activation and collaboration are critical for developing functioning adipocytes and preserving metabolic balance in adipose tissue. Research on the benefits of natural bioactive components that regulate adipogenesis has recently become an exciting focus. Foodstuffs reported to affect this condition positively include green coffee and green tea. The primary substance in green coffee is chlorogenic acid (CGA), meanwhile in green tea is epigallocatechin gallate (EGCG). Based on medical benefit potential, the present study evaluated the dose that produced the best effect on PPAR-γ and C/EBP-α expression between single and combined doses compared to undifferentiated adipocytes (3T3-L1). 3T3-L1 were cultured and divided into negative (NEG) and positive (DIF) groups. The DIF group is obtained by induction of differentiation, then divided into seventeen therapeutic doses. At the end of therapy, cells were fixed to measure the expression of PPAR-γ and C/EBP-α using the immunocytochemistry method. The DIF group produced the highest expression of PPAR-γ and C/EBP-α. Among the single-dose group, the lowest PPAR-γ was found in C320 and C/EBP-α in the T320 group. Meanwhile, the dose with the lowest PPAR-γ and C/EBP-α expression was found in the combination of green tea and coffee (TC) 160/80 (*p*-value = 0.00). The findings of this study showed that a combination of green tea and coffee extracts at doses of 160/80 has the potential for anti-obesity by suppressing the differentiation of 3T3-L1 into adipocytes by reducing the expression of PPAR-γ and C/EBP-α.

Keywords: C/EBP-α, Green tea, Green coffee, Obesity, PPAR-γ, 3T3-L1

Introduction

Obesity is a disease with a rapid increase, with the most significant prevalence worldwide. The majority of obesity is estimated to continue to increase, as seen from the increasing trend of overweight in adults and children. In 2016, the WHO reported that more than 1.9 billion individuals over 18 were overweight. Obesity was reported in 39 % of adults over 18, 39 % of men and 40 % of women [1]. In the same year, more than 340 million kids and teenagers aged 5 to 19 were overweight. This ever-increasing number causes obesity to become a double burden of malnutrition in both developing and developed countries. Exposure to foods high in fat-sugar-salt, energy-dense, and micronutrient-poor nutrition, together with a lack of physical activity, is a burden for the state because it increases the occurrence of related diseases, will be accompanied by the issue of undernutrition, which remains unsolved [2,3].

An energy imbalance between calories ingested and expended is the primary cause of obesity [4,5]. Increased consumption of foods high in fat and sugar, which are not matched by physical activity, is one of the leading causes of an increase in body mass index (BMI). Initially, obesity was defined as a BMI increase of ≥ 30 kg/m^2 [6]. However, BMI is no longer a measure of obesity due to the limitation between muscle composition and fat percentage in each individual's body [7]. The clinical understanding of the term obesity

then sharp on the fat content in the body, which is caused by adipose tissue [8,9]. This condition was later described as adipogenesis, with the knowledge that adipocyte tissue has a range of essential roles in metabolism and the immune system. Thus, obesity can link several non-communicable diseases and become a risk factor for several diseases, such as metabolic syndrome, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and cancer [10-12].

Obesity is a condition that occurs due to the accumulation of excess white adipose tissue (WAT) [8,13]. Adipocyte tissue is a collection or mix of several cells: Adipocytes, stromal preadipocytes, immune cells, and endothelium [14]. Adipocyte tissue secretome consists of several specific proteins such as hormones, chemokines, to vasoactive and angiogenic molecules. WAT expansion is also related to the abundance of adipocyte tissue that begins with adipocyte differentiation. Adipocyte differentiation is defined as increasing adipocyte cell size and developing mature adipocyte cells from adipocyte precursors [14,15]. This differentiation process can cause changes in the shape of preadipocyte cells to mature adipocyte cells that are round and more voluminous. Adipocyte differentiation that occurs intensively will lead to the accumulation of mature adipose cells, which can be spread in several parts of the body, such as the stomach (visceral fat) and the tissues under the skin (subcutaneous fat) [15-17]. WAT expansion and differentiation in the visceral or abdominal setting are most strongly correlated to insulin resistance and CVD [11,12].

Two key transcription factors, especially Peroxisome Proliferator-Activated Receptor (PPAR) and CCAAT Enhancer-Binding Protein (C/EBP), are known to regulate the adipocyte differentiation process. Genes involved in adipocyte development, lipid metabolism, and insulin sensitivity are expressed more often when PPAR-γ is activated. Adipocyte-specific genes, including adiponectin, leptin, and fatty acid-binding protein 4 (FABP4), are expressed in a way controlled by PPAR-γ's transcriptional activity, which contains the adipogenic programme [13]. PPAR-γ activation is also associated with improved insulin sensitivity in adipocytes. It enhances insulin-mediated glucose uptake by increasing the expression of glucose transporters, such as GLUT4, and improves insulin signalling and adipocyte response to insulin [14]. Moreover, PPAR-γ also controls the expression of various adipokines, which are bioactive molecules secreted by adipocytes. It positively regulates adiponectin expression, an adipokine involved in insulin sensitivity and metabolic homeostasis. PPAR-γ also influences the expression of other adipokines, such as leptin and resistin [15]. Obesity, insulin resistance, and type 2 diabetes are a few metabolic diseases linked to the dysregulation of PPAR-γ activity [13-15].

Another vital transcription factor involved in Adipogenesis is C/EBP-α. It belongs to the family of CCAAT/enhancer-binding proteins, and adipose tissue exhibits high expression levels. During the early phases of adipocyte differentiation, C/EBP-α is activated and works with PPAR-γ to promote adipogenesis [16]. Essential adipogenic genes, such as adipocyte protein 2 (aP2), lipoprotein lipase (LPL), and fatty acid synthase (FAS), are regulated by C/EBP-α [17]. Additionally, C/EBP-α helps to keep mature adipocyte functions like lipid storage and insulin sensitivity functioning correctly [18]. Adipocyte development is hampered, and adipocyte bulk is decreased when C/EBP-α function is lost [16-18].

PPAR-γ and C/EBP-α coordinated activity is essential for the proper growth and operation of adipose tissue. Their activation and interaction control adipocyte differentiation, lipid metabolism, adipokine production, and insulin sensitivity [19-21]. The involvement of C/EBP-β and C/EBPδ in the early phase induces PPAR-γ [22,23]. The expression of PPAR-γ and C/EBP-αwill be positively regulated by one another. In the initial stages of adipocyte cell development, PPAR- is more important. C/EBP-, meanwhile, plays a significant role in differentiation's termination phase [24]. In addition, GLUT 4, a glucose transporter in adipose tissue, skeletal muscle, and cardiac muscle, can have its gene expression regulated by C/EBP-α [25]. So, the transcription factors PPAR-γ and C/EBP-α play crucial roles in the control of adipogenesis. Their activation and collaboration are critical for developing functioning adipocytes and preserving metabolic balance in adipose tissue. Knowledge of regulating adipocyte differentiation by genetic and environmental factors is an insight that obesity can be inhibited by using potential strategies to prevent and treat obesity [26].

Research on the benefits of natural bioactive components that can regulate adipogenesis. Foodstuffs reported to affect this condition positively include green coffee and green tea. Coffee is extensively consumed worldwide, primarily because of its unique organoleptic qualities and bioactive components, which positively impact human health. The amount of coffee consumption worldwide has steadily climbed over the past 40 years, according to data from International Coffee Organization [27]. There are several alleged health advantages of coffee as a source of polyphenols, including chlorogenic acid. Green coffee beans, or unroasted coffee, contain chlorogenic acid. Green, black, and oolong teas are the most famous varieties of tea made from the Camellia sinensis plant, also used to make coffee. Green tea is one of the three tea varieties with the most significant impact on human health [28]. Green tea, popular in Asia, some

of North Africa, America, and Europe, makes up 20 % of the estimated 2.5 million tonnes of tea leaves globally produced yearly [29].

The primary phenolic substance in green coffee is chlorogenic acid (CGA), which is known to prevent the development of adipocyte cells [30]. According to Park *et al.* [31], CGA can decrease the release of adipokines like leptin and adiponectin and restrict the growth of lipid droplets and the accumulation of intracellular triglycerides. Also, giving CGA to 3T3-L1 cells might lessen the expression of the genes for fatty acid synthase (FAS), sterol regulatory element binding protein-1c (SREBP-1c), and peroxisome proliferator-activated receptor gamma (c) at the protein level. Meanwhile, the flavonoid epigallocatechin gallate is found in green tea (EGCG) [32,33]. Several studies have proven the potential of EGCG as an anti-preadipocyte cell anti-differentiator. Kim and Sakamoto reported the results of their research that administering EGCG to 3T3-L1 preadipocytes could reduce intracellular lipid accumulation [34]. Cell cycle analysis showed that EGCG could inhibit cell proliferation by interfering with the cell cycle during the clonal expansion 3T3-L1 [35]. Based on this potential, the present study evaluated the dose that produced the best effect on PPARγ and C/EBP-α protein expression between single and combined doses compared to undifferentiated adipocytes (3T3-L1).

Materials and methods

Green coffee preparation and extraction

Robusta green coffee (*Coffea robusta*) beans were obtained from the Dampit, Malang, Indonesia. To separate the coffee beans from dirt or low-quality coffee beans, perform a sorting process. This green coffee extraction and decaffeination method are modified from [36]. The coffee bean was roasted in an oven at 180 °C for 3 min or until the first crack appeared. Coffee beans were ground using a grinder to optimize the extraction results. Coffee beans were extracted using the infusion method with demineralized water with a coffee bean ratio: solvent of 1:15, at a temperature of 90 °C, for 10 min. The extracts were filtered using filter paper. Activated carbon for decaffeination of green coffee extract was carried out by adding sugar cane to distilled water to as much as 2.5 %w/v water, formic acid to 0.5 %w/v water, and activated carbon to as much as 25 %w/v water. Then the mixture was mixed in a water bath shaker, at a temperature of 80 °C, for 6 h. After that, activated carbon was separated from the solvent and rinsed with water (200 %v/w carbon). Activated carbon is decaffeinated with a ratio of activated carbon: green coffee extract 1:75 (w/v). The decaffeination process was reacted using a shaker water bath at 80 °C for 8 h. After the process, the decaffeinated coffee extract was filtered using coarse filter paper. The decaffeinated coffee extract was added with 5 % maltodextrin (w/v). The mixtures were dried using a food dehydrator at 60 °C for 5 h. The decaffeinated coffee extract powder was size reduced using a dry blender and sieved through an 80-mesh sieve.

Green tea extraction and decaffeination

The green tea (*Camelia sinensis*) was first-grade quality tea from the top of 3 shoots planted in Ciwidey, Bandung, Indonesia. A sorting process was carried out to separate dry green tea samples from impurities or low-quality green tea. Dried green tea was ground using a grinder. Green tea was extracted using the infusion method with demineralized water with a ratio of green tea and water was = 1:15; at a temperature of 90 °C; for 30 min [37]. After that, it was filtered using coarse filter paper. Green tea decaffeination was done by adapting the blanching method [37]. Green tea decaffeination was carried out at 50 °C and 5 min. The decaffeinated green tea extract was added with 5 % maltodextrin and then dried using a food dehydrator at 60 °C for 5 h. The dried decaffeinated green tea was size reduced using a dry blender and filtered through an 80-mesh sieve.

Maintenance and differentiation of 3T3-L1 culture cells

3T3-L1 mouse preadipocytes from ATCC passage number 10 until 15 were cultured in Dulbecco's Modified Eagle's Medium (DMEM high glucose) containing 10 % Fetal Bovine Serum (FBS, Gibco) and 1 % Penicillin/Streptomycin (P/S, Gibco) in a 5 % CO_2 incubator at 37 °C using a 60 mm dish culture. Before seeding, a 12 mm round cover glass was placed on the bottom of the plate. The medium was changed every 2 days. This method was a modification of [51]. To induce cell differentiation, 3T3-L1 preadipocyte cultures obtained 2 days after confluent were stimulated with 0.5 mM 3-isobutyl-1-methyl-xanthine, 0.25 M Dexamethasone, 2 M Rosiglitazone, and 1 g/mL of insulin (MDI differentiation medium) for one day. Then the cells were kept in a 10 % FBS/DMEM medium with 1 g/mL insulin for 10 days. The medium was replaced every two days. By this time, more than 90 % of the cells will become mature adipocytes with lipid-filled droplets. On the last day, the fixation process was carried out by removing the medium and

replacing it with PBS 2 times, then 10 % formalin was added before proceeding to the immunocytochemistry stage.

Determination of stock and dosage dilution

The determination of the dose used for therapy in this study is based on a preliminary study [38-40]. The stock solution was prepared by dissolving 64 mg of green tea or green coffee in 1 mL of sterile PBS to obtain a stock solution with a 64 mg/mL concentration or 64,000 mg/µL. To get a sub stock solution with a dose of 320 mg/µL, the dilution method was carried out by taking 42 µL of tea or coffee stock solution to be diluted with DMEM until the total volume became 4,200 µL. The second, third, and fourth sub-stock solutions with doses of 160, 80, and 40 mg/µL are the dilutions of the first, second, and third sub-stock solutions with a dilution factor 2×. At a combination dose of tea: coffee 1:1, taken from the sub-stock solution at 320 mg/µL. The dilution method took 42 µL of tea and coffee stock solutions each to be diluted with DMEM to a total volume of 4,200 µL. The second, third, and fourth sub-stock solutions with doses of 160, 80, and 40 mg/µL are the dilutions of the first, second, and third sub-stock solutions with a dilution factor 2×. Meanwhile, for the combined dose of tea: coffee 2:1, the sub-stock solution with an amount of 320 mg/µL, diluted by taking 42 µL of tea stock solution and 21 µL of coffee stock solution to be diluted with DMEM until the total volume becomes 4200 µL. The second and third sub-stock solutions with a dose of 160 and 80 mg/µL are the dilutions of the first and second sub-stock solutions with a dilution factor 2×.

In this study, we used 17 doses of single doses and combinations. A single amount of tea consists of 4 doses; 40 mg/µL (T40), 80 mg/µL (T80), 160 mg/µL (T160), and 320 mg/µL (T320). A single dose of coffee consists of 4 doses; 40 mg/µL (C40), 80 mg/µL (C80), 160 mg/µL (C160), and 320 mg/µL (C320). For the combined dose of tea: coffee with a ratio 1:1 consisting of 4 doses; 40 mg/µL: 40 mg/µL (TC40), 80 mg/µL: 80 mg/µL (TC480), 160 mg/µL: 160 mg/µL (TC160), and 320 mg/µL:320 mg/µL (TC320). While the combined dose of tea: coffee with a ratio 2:1 consisted of 4 doses; 80 mg/µL: 40 mg/µL (TC80/40), 160 mg/µL: 80 mg/µL (TC160/80), and 320 mg/µL: 160 mg/ µL (TC320/160).

Analysis of protein PPAR-γ and C/EBP-α expression using immunocytochemistry

Cells were prepared on a slide glass. The method of measuring PPAR-γ and C/EBP-α expression using green coffee immunocytochemistry was modified from Horvai *et al.* [41]. The formalin on the well plate containing the 3T3-L1 culture was discarded and rinsed with PBS 3 times. Then added with 3 % H_2O_2 and reacted for 20 min, then rinsed with PBS 3 times. Nonspecific protein blocking was carried out by dripping approximately 1 drop of blocking buffer (0.5 mL), and then incubation was carried out for 60 min at room temperature. The slides were then rinsed with PBS 3 times for 2 min of each rinsing. Primary antibody staining was done by dripping the primary antibody dissolved in a blocking buffer on the specimen. Incubation was carried out overnight at 4 ºC. After overnight incubation, the specimens were left at room temperature and then rinsed with PBS 3 times, each waiting for 2 min. Secondary antibody staining was carried out by dripping Biotin-Conjugated-Anti-globulin approximately 0.5 mL on each slide, then incubating for 60 min at room temperature. The slides were then rinsed with PBS three times for 2 min of each rinsing. The specimen slides were continued with Strepavidin-Horse Radish Peroxidase (SA-HRP) substrate and incubated for 40 min at room temperature. The slides were then rinsed with PBS three times each, waiting for 2 min, and then rinsed with sufficient distilled water. Chromogen administration was done by giving DAB chromogen dissolved in DAB buffer (1:40) approximately 1 drop (0.5 mL) on each slide and incubated for 20 min at room temperature. The slides were then rinsed with distilled water thrice for 2 min each. Specimens that have been set from the previous process, followed by background staining (other than target cells) by dripping Mayer's Hematoxillen, which has been diluted with distilled water (1:3). Incubation was carried out for 10 min at room temperature. The slides were then rinsed with distilled water. The specimens were dried until they were completely free of water, then specimen dripped with Entellan™ mounting medium and covered with a cover glass. The slide preparations were then observed under a microscope and scanned with a Trinocular Microscope Nikon Eclipse LV100D-U, with the aid of NIS-Element D 4.40.00 64-bit.

Statistical analysis

Protein expression data were analyzed using ImageJ software and expressed as the mean fold change in relative expression. Average fold change and standard deviation (SD) were obtained from 3 biological replicate samples per condition. Statistical analysis was performed using SPSS 20.0 and GraphPad Prism 9.0 software. The mean ± standard deviation indicated the evaluation of statistical data. All data were analyzed by one-way ANOVA, followed by the Duncan test to determine the significance of the differences

between the treatment groups to test the significance value of $p < 0.05$. The Spearman test was used to analyze the correlation between PPAR-γ and C/EBP-α.

Ethical clearance

This experimental design has been fulfilled and approved by the Health Research Ethics Committee of Saiful Anwar General Hospital, Malang, Indonesia, by registered number: 400/211/K.3/302/2021.

Results and discussion

Decreased PPAR-γ and C/EBP-α protein expressions in the group given a combination of green coffee and green tea extracts

This research analyzed the caffeine content in green coffee and green tea raw materials used for therapy on 3T3-L1 cells. Analysis of caffeine content in green coffee and green tea raw materials was 2.155 and 3.03 %, respectively. In comparison, the caffeine content after the extraction and decaffeination processes in green coffee and green tea were 1.42 and 1.53 %, respectively. This result means the reduction rate is 34.11 % for the green coffee sample and 49.5 % for the green coffee sample.

This study found that the highest average PPARɣ protein expression in the positive control group (DIF) was 0.861 ± 0.0746. Meanwhile, the lowest average PPAR-γ protein expression was in the negative control group (NEG), 0.043 ± 0.023. In the single-dose T group, the lowest average value was found at the T40 dose, with an average value of 0.064 ± 0.015. In the single dose C group, the lowest average value was found at the C320 dose with an average value of 0.055 ± 0.018. In the TC 1:1 combination group, the lowest average value was found at the TC320 dose with an average value of 0.157 ± 0.031. In the combination group with a 2:1 TC ratio, the lowest average value was found at the TC160/80 dose with an average value of 0.141 ± 0.024. The single-dose group showed the lowest average value of PPARɣ protein expression in the C320 treatment group. In the combination dose group, the lowest mean PPARɣ protein expression was found in the TC160/80 group. Meanwhile, the lowest average value of PPARɣ protein expression from all groups was in the C320 treatment. Based on the results of the one-way ANOVA test, it can be seen that the F significance value is 0.00 (p-value < 0.05), so it can be stated that there is a significant difference in PPAR-γ protein expression between groups (**Table 1**, **Figure 1A**, and **Figure 2**). This study also proves that the average expression of protein C/EBP-α in the positive control group (DIF) is 1.649 ± 0.163.

Meanwhile, the lowest mean protein expression in the negative control group (NEG) was 0.303 ± 0.038. In the single-dose T group, the lowest average value was found at the T320 dose, with an average value of 0.434 ± 0.0411. In the single dose C group, the lowest average value was found at the C80 dose with an average value of 0.564 ± 0.011. In the combination group with a 1:1 TC ratio, the lowest average value was found at the TC320 dose with an average value of 0.472 ± 0.0127. Among the single-dose group, the lowest average value of C/EBP-α protein expression was the T320 group. In the combined dose group, the lowest average C/EBP-α protein expression was found in the TC160/80 treatment. While of all groups, the lowest average value of C/EBP-α protein expression was in the T320 treatment. In the combination group with a 2:1 TC ratio, the lowest average value was found at the TC160/80 dose with an average value of 0.438 ± 0.036. Based on the test results, it can be seen that the significance value of F is 0.000 (p-value < 0.05), so it was proved that there were significant differences in C/EBP-α protein expression between groups (**Table 1**, **Figure 1B**, and **Figure 3**).

Adipocytes undergo a number of maturation stages, cell remodelling, and vascularization with obesity. Several transcription factors that progressively work together to control the expression of hundreds of genes necessary for forming mature adipose cells are involved in preadipocyte differentiation [42,43]. Peroxisome Proliferator-Activated Receptor (PPAR) and CCAAT/enhancer-binding protein (C/EBP) are 2 of these transcription factors that are important for preadipocyte development [44]. The C/EBP group, which consists of the transcription factors C/EBP-α, C/EBP-β, C/EBP-ɣ, C/EBP-δ, and C/EBP-ε, has a significant amount of basic-leucine zipper (bZIP) near the end of C [45]. By starting the mitotic clonal proliferation of preadipocytes and activating PPAR and C/EBP, C/EBP-α and C/EBP- δ, immediately (within 2 - 4 h) active and operate in the early stages of differentiation [42,43]. Early in the process of adipocyte maturation, C/EBP homologous protein (CHOP-10) is produced. Creating a heterodimer with other C/EBP groups is a negative regulator of the C/EBP transcription factor [46]. The expression of C/EBP during adipogenesis is regulated by several transcription factors, including cyclic adenylyl monophosphate (cAMP) response element-binding protein (CREB), early growth response 2 (EGR2, also known as KROX20), and Kruppel-like factor 4 (KLF4) [42,47,48].

C/EBP-α is needed both for the process of adipogenesis and in maintaining adipocyte cells to function normally. Previous research conducted by Linhart *et al.* [49] showed that rats deficient in C/EBP-α had

abnormal WAT formation. On the other hand, a study conducted by Freytag *et al.* showed an expression of C/EBP-α in some fibroblast cells known to trigger adipogenesis [50]. It is further understood that C/EBP-α cannot induce adipogenesis in the absence of PPAR-γ [43,51]. It is understood that PPAR-γ can promote adipocyte development in cells lacking C/EBP-α [43]. This circumstance suggests that PPAR-γ can trigger adipogenesis even without C/EBP-α. Positive feedback between C/EBP-α and PPAR-γ, which control each other's expression and create various adipocyte-specific genes such as aP2 (fatty acid binding protein), CD36 (receptor lipoprotein), and FATP-1 (protein one transport of fatty acids), is crucial in late-stage adipocyte development. It is well known that the transcription factors C/EBP-α and PPAR-γ will promote the production of adipocyte-specific proteins like glucose transporter throughout this adipocyte development phase (GLUT 4). Adipocytes will also combine glucose and free fatty acids to produce and store fat as energy and expand their cell size [42,43,52].

Table 1 Results of ANOVA Analysis on PPAR-γ and C/EBP-α expression.

Groups	PPAR-γ		C/EBP-α	
	Min	Max	Min	Max
NEG	0.03	0.07	0.27	0.34
DIF	0.79	0.94	1.47	1.80
T40	0.05	0.08	0.92	0.96
T80	0.37	0.43	0.61	0.74
T160	0.14	0.23	0.37	0.78
T320	0.10	0.19	0.39	0.46
C40	0.41	0.55	0.67	0.87
C80	0.13	0.16	0.55	0.57
C160	0.42	0.87	0.87	1.06
C320	0.04	0.07	0.55	0.62
TC40	0.21	0.47	0.92	1.03
TC80	0.20	0.21	0.46	0.56
TC160	0.24	0.44	0.54	0.58
TC320	0.13	0.19	0.46	0.49
TC80/40	0.17	0.30	0.65	0.87
TC160/80	0.11	0.16	0.40	0.47
TC320/160	0.24	0.44	0.58	0.64
Mean Square	0.139		0.298	
F	20.229		40.906	
p-value	0.000		0.000	
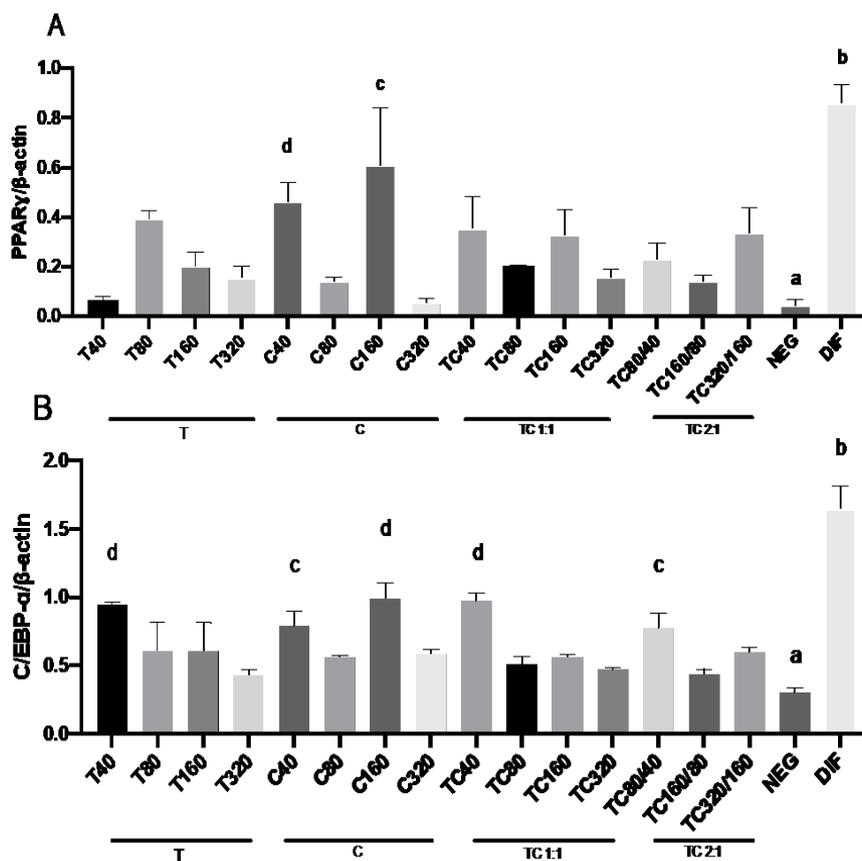
Figure 1 The result findings on PPAR-γ and C/EBP- α expression. The expression level was normalized based on β-actin level-one-way ANOVA and Tukey posthoc test was used. $p < 0.05$ was considered to indicate a statistically significant difference. Groups that appear in the same homogeneous subset are not significantly different.
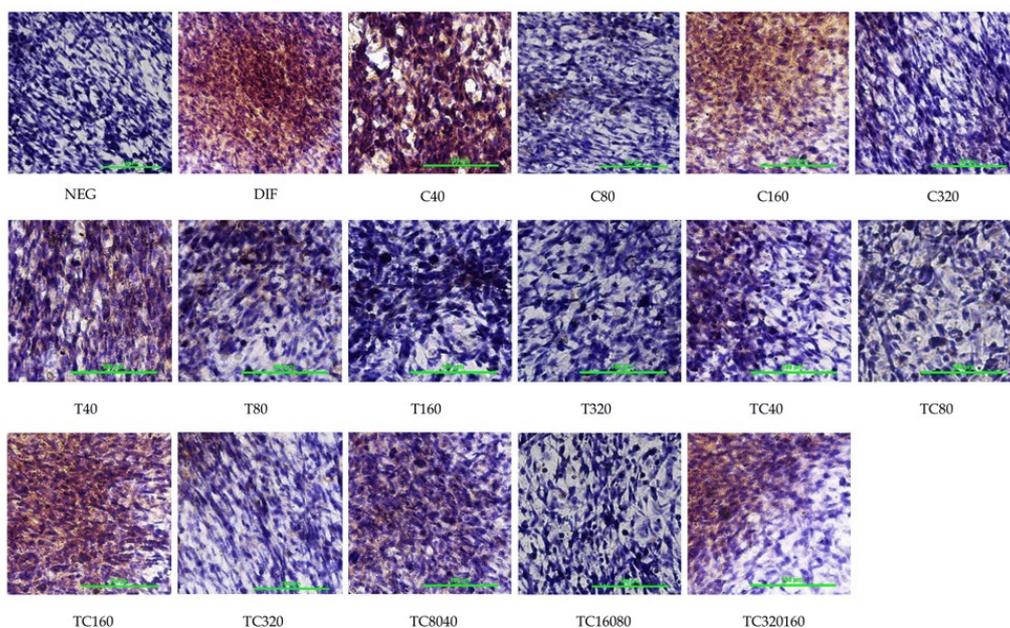

Figure 2 Imaging results using the immunocytochemistry method on 3T3-L1 cells using PPAR-γ antibodies, counterstaining using Mayer-Hematoxylin. Cells were observed at 400$\times$ magnification, scale 1:100 μm
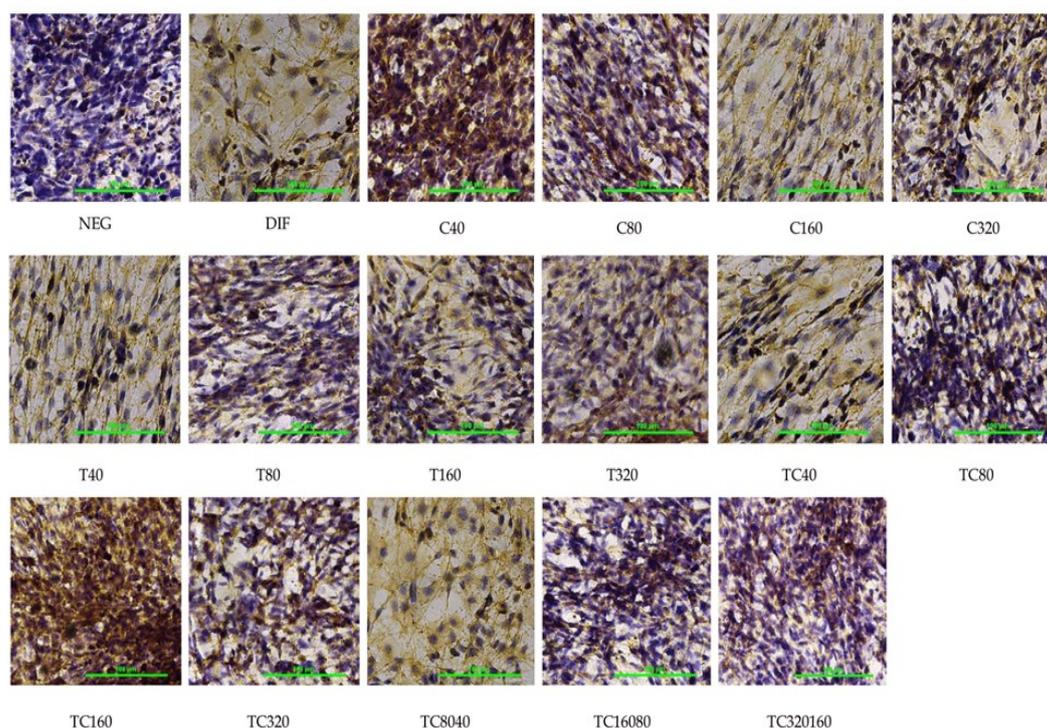
Figure 3 Imaging results using the immunocytochemistry method on 3T3-L1 cells using C/EBP-α antibodies, counterstaining using Mayer-Hematoxylin. Cells were observed at 400× magnification, scale 1:100 μm.

Correlation analysis of PPAR-γ with C/EBP-α protein expression due to the therapy given to the 3T3-L1 cells

Based on **Table 2**, it was found that PPAR-γ and C/EBP-α correlation analysis yielded a *p*-value test of 0.000 which is less than α (0.05). The relationship is positive and linear. That is, it can be concluded that there is a significant relationship between PPAR-γ and C/EBP-α. The correlation coefficient shows a positive relationship, which means that when the PPAR-γ variable is high, the C/EBP-α expression will also be increased, and vice versa. This research discovered that the combination of green tea and green coffee extract treatment effectively prevented the differentiation of 3T3-L1 cells, better than given in single administration. The therapeutic effectiveness of the combination of green coffee and green tea in this study can be seen from the synergistic effect in reducing the expression of PPAR-γ and C/EBP-α. The best impact given by combination therapy requires a lower dose than a single treatment. The lowest PPAR-γ expression was known at the highest coffee dose, namely C320. The same thing was found in the lowest C/EBP-α expression known at the highest dose, T320. When combined, both parameters decreased only with a low dose of green tea and green coffee extract 160/80 (*p*-value < 0.05, **Table 1**).

PPAR-γ and C/EBPα are essential in the early stages of adipocyte differentiation because both are transcription factors of several other genes [53]. Both are known to have the ability to mutually induce expression of each other and work together to activate several other adipocyte genes [54]. In this research, administering single and combination dosages inhibited the expression of PPAR-γ and C/EBP-α related proteins (*p* < 0.05). Several research employing the same cell type has also shown the same situation. In 3T3-L1 preadipocytes, tea extract can lower lipid accumulation during adipogenesis [55-57]. By reducing Akt phosphorylation, green tea extract, they have also decreased the mRNA and protein expression of the transcription factors C/EBP-α and PPAR-γ in 3T3-L1 cells. Another investigation by Kao *et al.* [58] revealed that the catechins in green tea might reduce the activity of glycerol-3-phosphatase, a differentiation marker. These catechins block the production of the primary transcription factors for early cell differentiation, peroxisome proliferator-activated receptor (PPAR) and CCAAT/enhancer-binding protein (C/EBP), which are both followed by the expression of the glucose transporter (GLUT) 4. Subsequent studies by Maki *et al.* [59] revealed that individuals with metabolic syndrome might benefit from green tea extract, which can raise blood adiponectin concentrations and lower visfatin.

Table 2 Pearson correlation analysis results.

		PPAR-γ	**C/EBP-α**
PPARγ	Pearson Correlation	1	0.980**
	Sig. (2-tailed)	-	0.000
	N	18	18
C/EBP-α	Pearson Correlation	0.980**	1
	Sig. (2-tailed)	0.000	-
	N	18	18

Several epidemiological studies have demonstrated a link between coffee drinking and a lower incidence of type 2 diabetes [60,61]. This discovery may result from the inhibition of fat formation in adipocytes. Aoyagi *et al.* [62] showed that coffee impacted 3T3-L1 preadipocyte cells' differentiation ability. Lipid accumulation dropped to half of the control with adding 5 % coffee. Coffee can also suppress PPAR-γ, a transcription factor that controls adipocyte development. Adipocyte development genes such as aP2, adiponectin, CCAAT-enhancer-binding protein (C/EBP-α), glucose transporter 4 (GLUT4), and lipoprotein lipase (LPL) can all be inhibited by coffee [62,63]. Related research by Maki *et al.* [59] revealed that drinking coffee might considerably lower weight growth and adipose tissue accumulation and increase blood levels of glucose, free fatty acids, total cholesterol, and insulin. Moreover, 3T3-L1 preadipocytes' adipogenesis was markedly reduced by the coffee extract. When 3T3-L1 cells were exposed to coffee extract during the early stages of adipogenesis, the cells' ability to reach the G2/M phase or undergo mitotic clonal growth was inhibited (MCE). Moreover, coffee extract stops ERK from phosphorylating CCAAT/enhancer-binding protein (C/EBP-α), preventing C/EBP-α from activating. Furthermore, coffee extract can reduce insulin receptor substrate 1 (IRS1) expression [59,63]. Through proteasomal degradation, the addition of coffee reduced the stability of the IRS1 protein [59]. Our findings suggest that coffee intake has an anti-adipogenic effect, and IRS1 is the major adipogenesis target of coffee extract.

This study also revealed that a combination of green tea and coffee had a better effect than single doses. The combination of green tea-coffee extract (TC 160/80) used in this study enhanced the differentiation delaying development on 3T3-L1 cells by simultaneously decreasing the expression of PPAR-γ and C/EBP-α (*p*-value = 0.00). This improvement is possibly caused by the content of EGCG and CGA, which are known to be the main phenolic components in green tea and green coffee. The results of this study are in line with the research conducted by Kim and Sakamoto, which showed that EGCG inhibited adipocyte differentiation by reducing FoxO1, a class O1 forkhead box transcription factor, via the PI3K/Akt and MEK (MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase) kinases [34]. This result indicates that EGCG can suppress adipocyte clonal expansion by inactivating FoxO1 through insulin signalling and stress-dependent MAPK pathways. Peng *et al* [64] tested the role of chlorogenic acid, a secondary metabolite compound found in many fruits and vegetables, on the differentiation and lipolysis of rat preadipocyte cells [64]. The results showed that regarding the molecular mechanism, CGA could reduce the expression level of genes related to the lipogenic pathway, Srebp1, during the stages of cell differentiation [64]. In addition, CGA is also known to increase the expression of lipolysis, HsI, and does not increase the expression of genes related to lipid synthesis, Dgat1 [65]. Paeng *et al.* [66] stated that CGA could suppress the formation of lipid droplets and the accumulation of intracellular triglycerides and reduce the secretion of adipokines such as leptin and adiponectin [67]. This finding indicates that green tea and coffee at a dose of 160/80 have the potential for anti-obesity by suppressing the differentiation of 3T3-L1 into adipocytes by reducing the expression of PPAR-γ and C/EBP-α. Therefore, the findings of this study can both shed light on drinks linked to anti-obesity and offer an understanding of the molecular processes behind the onset of obesity. Another finding in this study is related to the dose, which is the dose of the combination of green coffee and green tea reduces the expression of PPAR-γ and C/EBP-α in 3T3-L1 cells is the optimal dose of 160/80. So this finding proves that therapy using green tea and coffee has substantial implications for obesity research and anti-obesity interventions. Understanding the molecular mechanisms and signalling pathways regulated by PPAR-γ and C/EBP-α as transcription factors can also guide the development of novel drugs or interventions to modulate adipogenesis and promote healthier adipose tissue function.

This research has several limitations. The number of samples and the time of this investigation are both constrained. Before people can consume it, the combination of green tea and green coffee must first be evaluated on a more significant number of samples and over a more extended period. Additionally,

PPAR and C/EBP-the only two adipogenic markers employed in this study's *in vitro* experiments, could not explain the impact or the mechanistic link. Therefore, in the future, additional investigation will need to measure the protein linked to the downstream end of the pathway.

Conclusions

Green tea and green coffee extract significantly affected the expression of PPAR-γ and C/EBP-α. Green tea and coffee extracts at doses of TC 160/80 have the potential for anti-obesity by suppressing the differentiation of 3T3-L1 into adipocytes by reducing the expression of PPAR-γ and C/EBP-α. The limitation of this study is that it only measured the expression of PPAR-γ and C/EBP-α. There was no measurement of the activation of these transcription factors. Thus, there is a necessity for further research to examine the activation of PPAR-γ and C/EBP-α and their downstream effects on obesity development.

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