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RESEARCH

Anti-diabetic and Anti-obesity Effects of *Citrus amblycarpa* Hassk. Peels Extract in High Fat Diet Rats Model

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■ Abstract

OBJECTIVES: Obesity is an abnormality of fat accumulation or adipose tissue in the body that affects human health via its association with the risk of development of metabolic syndrome diseases such as diabetes mellitus. This is the first study of *C. amblycarpa* Hassk. peel extract supplementation effect againts obese and obesity markers in Indonesia. **METHODS:** We divided rats into 6 groups, the normal diet group (ND), the high-fat diet (HFD) group with orlistat, and various doses of *Citrus amblycarpa* Hassk. Peels extract (CAPE) 200, 300, and 400 mg/kg BW. The whole blood was collected to measure blood glucose level (BGL). Abdominal adipose tissues were examined for histopathological evaluation using Hematoxylin eosin, and gene expression was analyzed using RT-PCR. **RESULTS:** The data shows a decline of BGL in HFD rats with 200 mg/kg BW CAPE supplementation compared to negative control. The presence of hyperplasia and hypertrophy in visceral fat tissue in HFD rats were indicated by the expansion of adipose cell size but can be suppressed by giving CAPE with various doses which are indicated by the lower adiposity cell area compared to HFD rats without supplementation, but with no statistical significance. The CAPE suppressed *Leptin* and *Ghrelin* in the HFD rats, as well as the HFD rats model with Orlistat, but this difference was not statistically significant. **CONCLUSION:** Six weeks of CAPE supplementation was beneficial in reducing BGL, adipocyte cell size, *Leptin*, and *Ghrelin* expression in HFD rats.

Keywords: Adipocyte, Extract, Gene, Herb Medicine, Diabetes, Obesity.

1. Introduction

Obesity is an abnormality of fat accumulation or adipose tissue in the body that affect human's healthy via its association to the risk of development of metabolic syndrome disease such as diabetes mellitus, cardiovascular disease, hypertension, and hyperlipidemia [1]. It is a significant public health epidemic which has progressively worsened over past a half-decade [1]. In South Kalimantan in 2018 the incidence of obesity was 19.52% in adults aged > 18 years [2]. This is due to the lifestyle of the people of South Kalimantan, especially in big cities like Banjarmasin who like to eat outside the home. This community habit is one of the triggers for high obesity in South Kalimantan.

Increased body mass has a strong association with diabetes and insulin resistance. In obese individuals, the amount of nonesterified fatty acids, glycerol, hormones, cytokines, proinflammatory markers, and other substances involved in the development of insulin resistance will also increase [3]. The proportion of obesity in adults >18 years of age in Indonesia is increasing, namely 10.5% (2007), 14.8% (2013), and 21.8% (2018) [2]. The prevalence of nutritional status based on BMI (Body Mass Index) categories in South Kalimantan Province in 2018 in adults >18 years of age with obesity was 19.52% [2].

The high calorie diet in modern society can lead to fat accumulation in adipose tissue. Adipose tissue dysfunction and increases the risk of cardiovascular disease and diabetes [1]. Obesity is characterized by increased free fatty acids (FFA) in the blood and the accumulation of adipose tissue around the abdomen, *especially* in the visceral part of the body and subcutaneous abdominal adipose tissue [4].

Increased FFA will reduce the expression of insulin receptor substrate-1 (IRS-1), reduce the activation of phosphatidylinositol 3-kinase-serine/threonine kinase (PI3K-AKT) in the liver and skeletal muscle, and increase the expression of c-Jun N-terminal kinase (JNK) in the pancreas. This causes insulin resistance in the liver and skeletal muscle and apoptosis of pancreatic β-cells, leading to hyperglycaemia [5].

In addition, there are hormones that also change function when obesity occurs such as Leptin and Ghrelin. Leptin is an important peripheral hormone that plays a role in the satiety response in the brain after eating. In obesity, its expression and secretion also increase. Adult adipocytes will accumulate triglycerides which have a hypertrophic effect, causing leptin overexpression and resulting in leptin resistance [6]. Ghrelin is an orexigenic peripheral hormone produced by the stomach which initiate eating response and digestion of food. Ghrelin is an upregulation in conditions of malnutrition such as anorexia, and a downregulation of positive energy such as obesity [7]. Obesity can be cured by efforts to inhibit fat accumulation. This obstacle can be tackled by herbal plants as an alternative medicine because of wide distribution, low cost and promotion of local wisdom.

In South Kalimantan, there is an endemic citrus plant namely the kuit lime (*Citrus amblycarpa* Hassk). This citrus has similarities with kaffir lime, but the surface of the fruit is wrinkled, not rough like kaffir lime. Citrus plants have active ingredients that are essential for health, including vitamin C, flavonoids, carotenoids, limonoids, and minerals. The main flavonoids in citrus are naringin, narirutin, and hesperidin found in fruit peels, seeds, and pulp [8]. In several studies in both in vivo and in vitro, in the citrus juice has multiple components that can play a role in inhibiting fat accumulation. In the study of Montalbano *et al.* [9], the flavonoid of orange juice can act as an antiobesity and has lipolytic activity [9]. The flavonoids can inhibit lipid peroxidation and cell fragility [10]. There was a decrease in COX-2, ICAM-1 and TNF-a levels in the adipose tissue of obese model mice treated with orange peel extract containing high flavonoids [11].

This study emphasizes the potential of kuit lime peel extract as anti-diabetic and anti-obesity through some molecular and histological evaluation. There have been previous studies stating that the flavonoid and essential oil content is high enough in the *Citrus amblycarpa* Hassk. peel so that it has the potential to control appetite and dissolve fat [12]. However, there is no research proving glicemic control of *C. amblycarpa* peels extract, histology and molecular genetics analysis in high fat diet animal models treated with lime peel extract. The aims of this study is to analyze blood glucose level, expression of *Leptin*, *Ghrelin* Genes and adipocyte cell size in adipocyte tissue by administering *Citrus amblycarpa* Hassk. peel extract with various doses.

2. Methods

2.1. Preparation of C. amblycarpa Hassk. Peel Extracts (CAPE)

Lime peel (*Citrus amblycarpa* Hassk.) which had been dried and cut into pieces as much as 1500 g was extracted by maceration method using 70% ethanol solvent for 5 days and the residue was macerated again for 3 days. After the extract was collected, it was evaporated using a rotary evaporator to obtain a thick extract.

2.2. Distribution Experimental Animal Group

Thirty male white rats (*Rattus norvegicus*) Wistar strain aged 2-3 months were randomly divided into 6 groups, namely the normal diet group (ND), the high-fat diet group + aquadest (HFD), the high-fat diet + orlistat group (HFD + Orlistat), and the high-fat diet group + *C. amblycarpa* Hassk. peel extract (200 mg/kg BW (HFD+ CAPE 200mg/kg BW), 300 mg/kg BW (HFD+ CAPE 300mg/kg BW), 400 mg/Kg BW (HFD+ CAPE 400mg/kg BW)). Male rats were chosen because they are not influenced by hormonal cycles that can confound the analysis of non-reproductive traits [13]. Before being given the induction treatment with a high-fat diet, the rats were given 7 days acclimatization period. Giving a high-fat diet for 6 weeks until the rats' body weight reaches 20% of normal rats' body weight [14]. Then, followed by treatment with *C. amblycarpa* Hassk. peel extract for 6 weeks. Measurements of body weight was measured every week until the end of the treatment period to ensure the addition of rats bodyweight. This study was performed with a protocol approved by the Animal Research Ethics Committee of Faculty of Medicine, University of Lambung Mangkurat (approval No. 18-KE-253).

2.3. Induction of High Fat Diet (HFD) in Research Rats

The negative control group rats were fed a normal (standard) rat diet and tap water ad libitum. Meanwhile, the rats in the other group received a high-fat diet and tap water by ad libitum. A high-fat diet is made with a special composition that contains high fat consisting of a mixture of milled corn, krosvet, Hi Pro-vite feed, duck egg yolk, and wheat flour.

2.3.1. Data Collection Rat Body Weight

Weighing rats using Krischef brand scales in grams. Weighing was carried out after the acclimatization process was completed and before starting the treatment, namely day 8 to week 6 in the obesity induction period, then continued until the next 6 weeks of treatment.

2.4. Blood Sampling and Blood Sugar Measurement

Rats were previously fed for 12 hours. Rats were injected with ketamine 50 mg/kg as much as 0.2 cc using a 1 cc syringe in the quadriceps musculus intramuscularly. Rats were waited until they were limp with the characteristics of inactive rat movements when moved. Blood was taken from the heart (intracardium) of the rat by aspiration with a 3 cc syringe. A whole blood sample of 1-2 drops is touched to the right side

of the blood glucose strip until the device beeps. The EasyTouch® GCU Meter will count down in 10 seconds. The blood glucose result will appear on the screen of the EasyTouch® GCU Meter.

2.5. Quantitative real-time polymerase chain reaction (PCR)

Total RNA from brain and intestine tissue were isolated with NucleoZOL (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocols. cDNA was synthesized using 0.5 µg total RNA with a ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan). Synthesized cDNA was amplified with the target gene primers and THUNDERBIRD SYBR qPCR Mix (Toyobo) using the Quant-Studio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression data were normalized to β- actin gene expression. Primer sequences are as listed below:[15]

Leptin

Forward: 50- GGA AGC CTC GCT CTA CTC CA-30
Reverse: 50- GAA TGT CCT GCA GAG AGC CC-30

Ghrelin

Forward: 50- CAG AGC ACC AGA AAG CCC AGC AG -30
Reverse: 50- CCA ACA TCG AAG GGA GCA TTG AA -30

β- Actin

Forward: 5'-ATCCGTAAAGACCTCTATGCCAACA-3'
Reverse: 5'-GCTAGGAGCCAGGGCAGTAATCT-3'

2.6. Histological Analysis

Abdominal adipose tissues were examined for histopathological evaluation. The fixed tissues were subjected to general histological processes, such as dehydration, paraffin embedding, and cutting. Hematoxylin and eosin staining was performed, and histopathological changes were evaluated by observation using an optical microscope (Olympus BX53, Tokyo, Japan).

2.7. Statistical Analysis

All experimental results are expressed as the mean ± standard deviation, and One-way analysis of variance (ANOVA) and Dunnett post-test were performed to identify significant differences between animal groups to analyze adipocyte cell size and gene expression. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of CAPE on Blood Glucose Level in HFD Rats Model

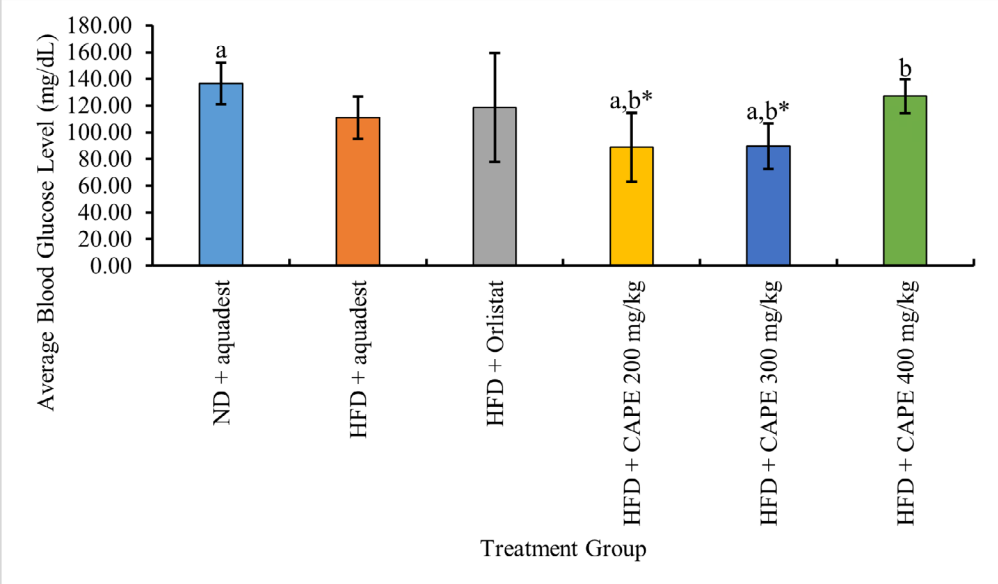

Figure 1: Average of Blood Glucose Level among Groups. CAPE 200 and 300 Treatment Shows Lower BGL Compare to Control Groups All Groups Showed there are no Significant Difference with One-way of Variance Statistically Analysis P > 0.05.

The highest mean blood sugar level was shown in ND which was 136.5±15.631 mg/dL, and the lowest mean blood sugar level was shown in HFD + CAPE 200 mg/kg BW which was 88.75±25.863 mg/dL as shown in Figure 1. There was a decrease in mean blood sugar levels in the HFD + CAPE 200 mg/kg BW and HFD + CAPE 300 mg/kg BW groups, against ND, HFD and HFD + orlistat groups.

One-Way ANOVA test showed that there were significant differences ($p<0.05$) between treatment groups. This indicates that CAPE supplementation can reduce blood sugar levels in rats fed a high-fat diet. The results of statistical tests showed that the CAPE group was not significantly different from the positive control ($p>0.05$). This indicates that CAPE has the same effect on blood sugar levels as the standard drug orlistat.

3.2. Effect of CAPE on Visceral Adipocyte Size in HFD-induced Obese Rats

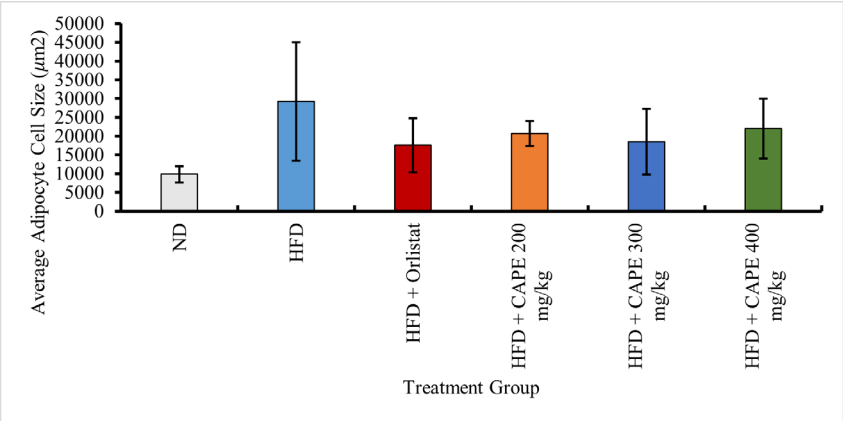

Figure 2: Effect of CAPE on Visceral Adipocyte Size. CAPE Treatment Significantly Reduced Visceral Average Adipocyte Size in HFD Rats Groups (K4, K5, and K6). Bar Graphs Show the Results of the Morphometric Analysis of fat Performed in Visceral (Adipocyte Average Size in the Six Experimental Groups (ND, HFD, HFD + Orlistat, HFD+ CAPE 200, 300 and 400 mg/kg BW). Scale Bars Correspond to 20 µm. Results from the Morphometric Analysis in Graph is Expressed in µm² of Adipocyte Size and Expressed as Mean ± SD of 5 Rats Per each Group. All Groups Showed there are no Significant Difference with One-way of Variance Statistically Analysis P > 0.05 (P= 0.11).

The diagram above shows a comparison of the average size of adipocytes in the treatment group of rats with a normal diet and rats with HFD supplemented with CAPE or not. The average adipocyte cell size in HFD + CAPE groups showed decline value almost similar to positive control. However, the result is insignificantly different compared to among all groups (P= 0.11) (Figure 2). The HFD rats group which are including HFD without CAPE, HFD+Orlistat, HFD+CAPE 200 mg/kg BW, HFD+CAPE 300 mg/kg BW and HFD+CAPE 400 mg/kg BW showed a wider increase in adipocyte cell area compared to the normal feed group, with an area reaching above 10,000 µm². Giving CAPE to HFD rats group for 6 weeks showed a reduction in the size of adipocyte cell size compared to HFD group alone without supplementation. HFD + orlistat showed there was not significantly change from HFD + CAPE 300 mg/kg BW group roughly 1750 µm². Administration of the CAPE 200 and 400 mg/kg showed a similar decrease in adipocyte cell size about 22500 µm². The data shows the presence of hyperplasia and hypertrophy in visceral fat tissue in HFD groups indicated by the expansion of adipose cell size, but can be suppressed by giving CAPE with various doses. This result is indicated by the lower adiposity cell area compared to HFD rats without supplementation. Statistical analysis revealed insignificant differences in adipocytes cell size between group of either HFD or non-HFD rats (Figure 2).

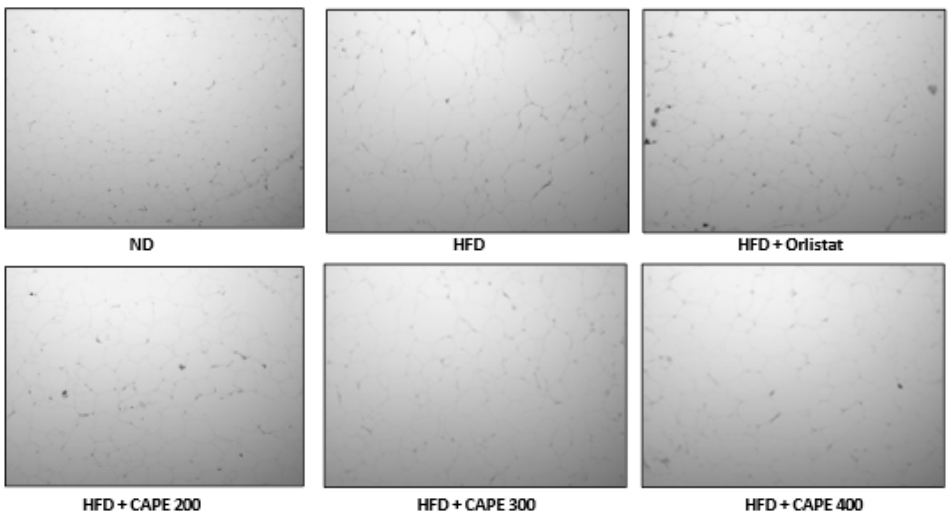

Figure 3: Effect of CAPE on Visceral Adipocyte Size (HE). Histological Features of Abdominal Adipose Tissue in Sagittal Sections (Hematoxylin and Eosin Staining) Showing Visceral Adipocyte Size in the Six Experimental Groups. Representative Light Microscopy Images from each Group Were Used to Measure Adipocyte Area. The Average Adipocyte Cell Size in HFD + CAPE Groups Showed Decline Value Almost Similar to Positive Control. However, The Result is Insignificantly Different Compared to among All Groups (P= 0.11).

3.3. Effect of CAPE on Leptin and Ghrelin Gene Expression in HFD-induced Obese Rats

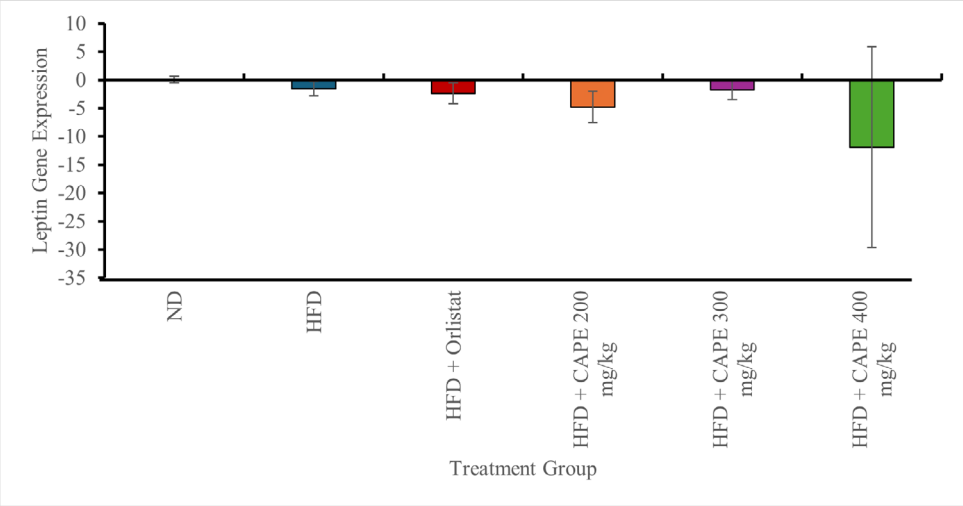

Figure 4: The mRNA Expression of *Leptin* Gene and Reference Genes (ß-actin gene) were Examined by RT-qPCR. The Expression of *Leptin* mRNA in the Brain Tissue Decreased Significantly when Comparing the HFD Group with CAPE to the Control Rats, But Statistical Data by ANOVA Analysis Showed there is no Differences among Groups with P>0.05 (P= 0.08).

Gene expression in the Livak method is described by the mean value of 2-ΔΔCt. *Leptin* gene expression in the normal group had a mean value of 2-ΔΔCt = 1. The normal group was used as a calibrator sample, where the value of -ΔΔCt was considered 0, resulting in a value of 1 (Figure 4). While the HFD group had a lower value, with a negative mean value of 2-ΔΔCt below 1, compared to the normal group. The CAPE 200, 300 and 400 mg/kg rats groups had *Leptin* gene expression with mean 2-ΔΔCt values of -4.76, -1.76, and -11.86 respectively. The HFD + CAPE 400 mg/kg group showed the lowest decrease in *Leptin* gene expression compared to all groups. Orlistat as a positive control showed lower *Leptin* levels than HFD without treatment group and CAPE 300 mg/kg BW group but still higher than CAPE 200 and 400 mg/kg BW. That result revealed no significant different among all groups (P=0.08). However, it is still can be concluded that the expression of the *Leptin* gene in the CAPE treatment group has the lowest value compared to the normal group and HFD group without supplements.

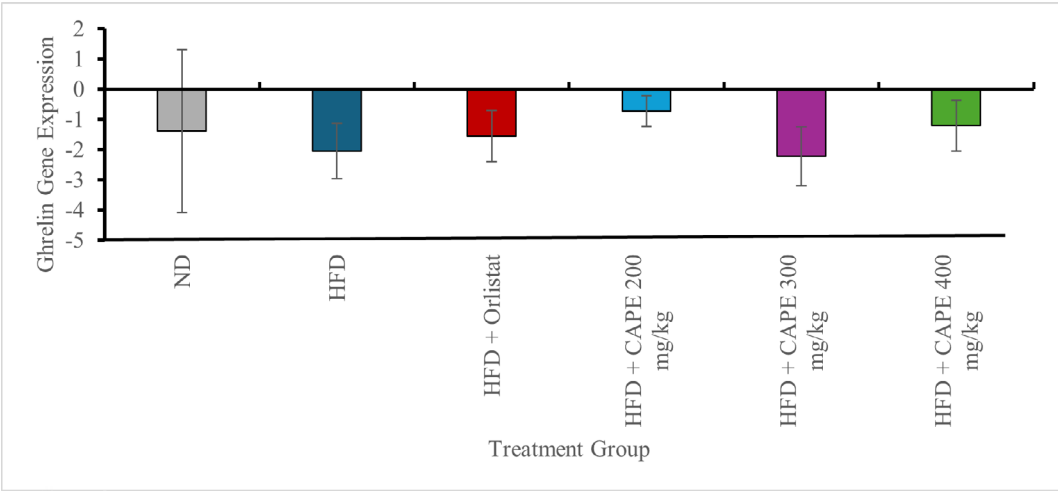

Figure 5: The mRNA Expression of *Ghrelin* Gene and Reference Genes (ß-actin gene) were Examined by RT-qPCR. The Expression of *Ghrelin* mRNA in the Intestine Tissue Decreased Significantly when Comparing the HFD + CAPE 300mg/kg BW to the Control ND and HFD Rats, But Statistical Data Showed there is no Different among Groups with P>0.05 (P= 0.56).

HFD group without extract has a lower *Ghrelin* gene expression value than the positive control (HFD + orlistat) and the control group (Normal diet) with the value of gene expression is the average 2-ΔΔCt = -2.04 (Figure 5). While the value of *Ghrelin* gene expression in the positive group, is 2-ΔΔCt = -1.55. The negative control group had a mean

value of 2-ΔΔCt = 1 because the negative group was used as a calibrator sample. The calculation of the 2-ΔΔCt value is the same as that of the *Leptin* gene. The HFD + CAPE 300 mg/kg group had the lowest *Ghrelin* gene expression compared to all treatment groups with a mean of 2-ΔΔCt = -2.2. Then followed by HFD + CAPE 400 mg/kg with a mean value of -1.2 and HFD + CAPE 200 mg/kg with a mean value of -0.71. This shows that CAPE 300 mg/kg has a better effect than orlistat in suppressing *Ghrelin* expression event according to statistics evidence there is no significant difference (P= 0.56).

4. Discussion

In this study, the anti-diabetic effect was detected by measuring blood glucose level among rat models. This shows that the decrease in mean blood sugar levels in the group with CAPE 200 and 300 mg/kgBB is significantly different from the ND group, positive control and negative control. This indicates that CAPE doses of 200 and 300 mg/kgBB have an equivalent effect on reducing the average blood sugar levels of rats fed a high-fat diet.

CAPE is high in flavonoids such as naringin. These compounds have been reported to improve glycaemic status and gene expression of enzymes involved in glucose homeostasis as well as reduce oxidative stress, proinflammation and cytokine production. Naringin and hesperidin can increase glucose storage and insulin sensitivity and reduce lipid accumulation in the liver. In addition, naringin and hesperidin can increase the activity of GLUT4 [16]. Flavonoids act as lipase inhibitors, inhibiting the hydrolysis of triglycerides in the small intestine. This may prevent obesity and hyperglycaemia [17].

The anti-obesity activity of CAPE was determined by measuring body adipose cell size and expression of genes related to adipogenesis, namely Leptin and *Ghrelin* in obese rats treated with CAPE supplement. High-fat diets are used in nutritional research as a strategy to induce weight gain and fat deposition in experimental animals [18, 19]. These results indicate that the supplementation of CAPE was able to modulate visceral through reducing hypertrophy. Progression to obesity is characterized by increased adiposity and increased inflammation of visceral adipose tissue. Weight gain that does not show much inflammation is a bad influence of HFD consumption [20]. HFD feed given during the study for 6 weeks containing high cholesterol could significantly increase the rats' body weight (p=0.035) [21]. Increasing dietary cholesterol increases the size of visceral fat adiposity, free cholesterol and macrophages, as well as the expression of pro-inflammatory genes in primates [22].

On the other hand, in the group of normal rats with regular food, there was no increase in adipose cell size. The expansion of White Adipocyte Tissue (WAT), which includes healthy and normal categories, is often associated with smaller and more numerous adipocytes, accompanied by lower levels of inflammation and fibrosis [23]. Moreover, giving CAPE can reduce the size of adiposity cells in the obese group due to content within the extract. Previous study results revealed within each type of citrus peels contained a higher amount of phenolic compound, flavonoids, vitamin C, and antioxidant activity than those of their inner wasted parts (pulp and seeds) [22]. Vitamin C suppresses visceral adipocyte hypertrophy and glucose intolerance as part of decreased expression of genes involved in lipogenesis [24, 25].

Leptin acts as an inhibiting factor for obesity by regulating energy use, food intake and adiposity. The main effect of leptin on adipose tissue is probably mediated through the peripheral nervous system. *Leptin* increases sympathetic efferent signals to adipose tissue to increase lipolysis [26]. Overconsuming a high-fat diet increases *Leptin* gene expression, even though it has no correlation with a change in body weight. However, activation of the signaling pathway for body weight regulation has synergy mechanism with amount of leptin reaching the brain [27].

In an acute fasting reduces circulating leptin, whereas in a fed state increases leptin synthesis which is mediated due to an increase in insulin during feeding. Plasma leptin depends on gender, fat distribution and adiposity size [28]. In obesity, there is a leptin resistance causing disrupted leptin signaling and increasing food intake [26]. In this study implies a decline in *Leptin* gene expression which could be a first reflection of the initial lower hyperphagia in HFD rats with CAPE supplementation. Previous in vivo study revealed lipolytic effect of leptin are facilitated by the neuro adipose junctions. This effect has correlation to adipocyte innervation in white adipose tissue [29]. The fluctuate of *Leptin* levels have to be clarified by leptin serum level and *Leptin* receptor gene expression.

Ghrelin increases blood glucose and appetite leading imbalance condition related to obesity. Ghrelin's food intake-stimulatory effects, peripherally injected ghrelin increases abdominal white adipose tissue in rodents [30]. If there is inhibition of ghrelin or its receptors, it will reduce blood glucose levels and trigger fat metabolism in the body [31]. The evidence showed in the group with CAPE treatment in HFD rats having lower *Ghrelin* expression compared to obese rats without extract supplements. mRNA expression of *Leptin* was risen through *Ghrelin* expression as an effect of adiposity and bad progression of glycemic control under a high fat diet [32]. In other words, the higher *Ghrelin* expression hence higher *Leptin* expression in order to control food intake and energy expenditure.

In this study, we demonstrated that CAPE suppressed *Leptin* and *Ghrelin* in the HFD rats, as well as the HFD-induced obesity rat model with Orlistat. Previous study revealed the pool of flavonoids in orange juice extract exerts an anti-obesity effect on fat accumulation in diet-induced obese zebrafish which are marked by reduction of leptin and *Ghrelin* gene expressions [7]. Orange juice extract and CAPE are similar come from Citrus family which have flavonoids as one of major

compounds. Flavonoids such as naringin, hesperidin, and nobiletin have been experimentally proven can reduce lipid levels through blockade of hepatic fatty acid synthesis and induction of increased fatty acid oxidation [33]. The previous data from clinical trials showed the beneficial of citrus flavonoids in the decline of proinflammatory cytokines in humans, being useful to inhibit the complications present in obesity [34].

A limitation of our study is that, although CAPE can reduce BGL, it needs to be confirmed by GLUT-4 activity marker. In addition, the expression of *Leptin* and *Ghrelin* have to be clarified by measure leptin and ghrelin serum level in some organs such as liver, kidney, skeletal, pancreas, and brain and also adipokine such as adiponectin [34]. Adiponectin is one of adipocyte cytokine (adipokine) which act contradictory as anti-inflammatory cytokine.

5. Conclusion

In conclusion, we have shown that 6 weeks of CAPE supplementation was beneficial in reducing blood glucose level, adipocyte cell size, *Leptin* and *Ghrelin* expression in HFD rats. The CAPE 200 mg/kg BW has best effect to decline BGL. Besides, CAPE 400 mg/kg BW can reduce *Leptin* mRNA level into the lowest level, and CAPE 300 mg/kg BW can reduce *Ghrelin* mRNA level better than positive control and also reduce adipocyte cell size better than HFD rats group without supplementation.

6. Declaration

6.1 Acknowledgements

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6.2 Conflict of Interest Statement

The authors have no conflict of interest to report.

6.3 Ethical Approval

Approval for this study was provided by the Ethical Clearance Committee, Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia (approval No. 18-KE-253).

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