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Methanol Extract of Rue Leaves on Body Fat Composition, Insulin Resistance, and Leptin Gene Expression

Florensia Syntesa Haryta[1]✉, Shanti Listyawati[1,2], Dono Indarto[3,4]

[1]Master Program of Biosciences, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia
[3]Biomedical Laboratory, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia
[4]Department of Physiology, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia
Full Affiliation

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Abstract

Obesity is a multifactorial and complex disorder characterized by excessive accumulation of adipose tissue, with negative health outcomes. Rue leaves (Ruta graveolens) contain active compounds that have activity as anti-obesity, such as rutacridone. Therefore, this study aims to determine the effect of Rue Leaves Methanol Extract (MER) on Obesity Index (OI), Body Fat Content (BFC), Wet Fat Weight (WFW), Insulin level (IL), Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), Homeostatic Model Assessment for β-cell Function (HOMA-β) and Leptin Gene Expression (LGE) in obesity model rats (OR). This study was an experimental animal study with a pretest-posttest control group design using 24 male Sprague-Dawley rats, which were randomly divided into six groups. The normal group (N) was male rats + standard rat feed (BR-2). The negative and positive groups (NC and PC) were OR + BR-2 + 1 mL 0.9% NaCl or 0.6 mg/kgBW pure rutacridone, respectively. Treatment groups (T1-T3) were OR + BR-2 + 80, 100, or 200 mg/kg BW MERL for 30 days. Our results demonstrated that MER administration significantly reduced OI, BFC, IL, and HOMA-IR also significantly increased HOMA-β level with $p < 0.05$, compared to the NC group in a dose-dependent manner. Our data also showed low expression of the leptin gene and WFW in all treatment groups. In conclusion, MER administration reduces OI, BFC, WFW, IL, HOMA-IR, and LGE while increasing HOMA-β in male obese rats.

Introduction

Obesity is among the worldwide health challenges, disrupting the art of living with an impact on short- and long-term bodily functions, including daily energy expenditure. In fact, by 2035, the World Obesity Atlas estimates that almost 4 billion individuals will have entered this weight status-obese (BMI ≥25 kg/m²). Aside from the role in energy storage, adipose tissue plays a significant role in secreting hormones that have been implicated in negative metabolic effects, including insulin resistance and long-term metabolic diseases like hyperinsulinemia, hyperglycaemia, hypertension, and cardiovascular disease. Indonesia is among the countries with a rising trend in obesity cases in adults, and the increase has been projected at 5.8% from 2020 to 2035 (World Obesity Atlas, 2023). Besides, based on data in 2021 and forecasts for 2045, Indonesia holds fifth place out of the top 10 countries with the highest number of diabetes-19.5 and 28.6 million people, respectively (International Diabetes Federation, 2021).

✉ Correspondence Address:
Master Program of Biosciences, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Indonesia
Email: shantilistyawati@staff.uns.ac.id

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The mechanism of obesity pathogenesis has two major theoretical models: the energy balance model (EBM) and the carbohydrate-insulin model (CIM) (Hall *et al*., 2022). According to the CIM theory, increased adiposity stimulates the hunger centre in the hypothalamus. Following high glycaemic load (GL) foods, absorption of these foods in the gastrointestinal tract induces the release of ghrelin (the hunger hormone) from the gastric fundus. Ghrelin activates the arcuate nucleus of the hypothalamus by binding to neurons of neuropeptide Y (NPY) and agouti-related peptide (AgRP) origin, leading to increased hunger and feeding. On the other hand, according to EBM, obesity is brought about when energy intake exceeds energy expenditure, resulting in fat deposition in adipose tissue. Management of obesity according to EBM looks at reducing energy intake while increasing energy expenditure to save excess energy within the body. In contrast, according to CIM, a low-carbohydrate diet would prevent this fat accumulation (Ludwig *et al*., 2022).

Obesity is a multifactorial and intricate disorder that is marked by the excessive accumulation of adipose tissue, with ensuing negative health outcomes (Lin & Li, 2021). The disequilibrium between energy expenditure and caloric intake initiates the secretion of endocrine hormones that play a key role in the preservation of energy homeostasis. The gene known as leptin (LEP) plays a significant role in energy and appetite regulation. The leptin gene encodes for leptin, which is the hormone secreted by adipocytes with the function of controlling hunger. Leptin, as an energy status signalling molecule, decreases its secretion when fasting and rises after taking in nutrients (Friedman, 2019). Hunger will be increased, and the consequence will be the overconsumption of energy, with the result being fat storage in adipose tissue. Fat deposition in different adipose tissues can lead to body weight (BW) gain (Goossens, 2017). The Obesity Index (OI) is a commonly used anthropometric parameter for measuring the level of obesity in rodent populations; however, it is not able to determine the percentage of body fat. Additional anthropometric measurements Obesity Index (OI) and Body Fat Content (BFC), are thus necessary since they are exact calculations of the percentage of body fat. Furthermore, Wet Fat Weight (WFW) measurement can be employed to determine visceral fat mass in rodent models as another anthropometric measure. Body weight gain (BW) also leads to the onset of obesity and insulin resistance, which can result in complications like diabetes mellitus (Masood & Moorthy, 2023). The insulin resistance is calculated using the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR), and pancreatic beta-cell function is estimated using the Homeostatic Model Assessment for β-cell Function (HOMA-β). These techniques measure insulin resistance and β-cell function by calculating blood glucose levels in relation to insulin levels (IL).

Presently, several treatment options for obesity are available, but with minimal efficacy and multiple side effects (González-Muniesa *et al*., 2017). Lifestyle and dietary changes that promote weight loss are considered effective ways of managing obesity. Yet, most obese individuals do not adhere to diet plans, leading to weight regain (Bray *et al*., 2016). Pharmacologic drugs, like orlistat, lorcaserin, and naltrexone, are widely applied in the treatment of obesity. Such drug therapy has side effects that may range from dizziness to nausea, hypertension, insomnia, and hypoglycaemia, particularly for diabetic patients (Kishore *et al*., 2018).

The inclination toward treatments that are derived from natural sources, like plant extracts, is now being researched extensively. The *Ruta graveolens* plant is known for its medicinal value and has more than 120 bioactive compounds that are responsible for its pharmacological activity (Kannan & Babu, 2012). One of the bioactive compounds found in Ruta graveolens is the alkaloid rutacridone. Computational studies have revealed that rutacridone can be a TCPTP inhibitor, which has the potential to decrease leptin resistance and help in obesity control (Fitrianingrum *et al*., 2019). Nevertheless, there has been no report on the impact of *Ruta graveolens* leaves on obesity, either in vivo or in vitro. Thus, the present study aims to investigate the role of the methanol extract of Rue (*Ruta graveolens*)

leaves (MER) in obesity prevention through an animal model of obesity.

Methods

The Rue leaves used were obtained from B2P2TOOT Tawangmangu, young leaves picked in the morning. The leaves were dried in an oven at 60°C for one day. The dried leaves were then ground using a blender to produce a powder (simplicia). The Rue simplicia was soaked in 70% methanol solvent at room temperature for three days. The filtrate obtained was then concentrated using a rotary evaporator at 60°C with a speed of 80 rpm and a pressure of 175 mbar (Ratheesh *et al*., 2011). After cervical dislocation, euthanasia was completed for all rats. Surgical procedures were performed on the animals. Sub-abdominal adipose tissue was excised and weighed using an analytical balance to determine the wet fat weight. The weighed wet fat was then stored at -80°C for subsequent use in leptin gene expression analysis.

This study is quantitative research with an experimental design using a pretest and posttest control design (OI, BFC, IL, HOMA-IR, and HOMA-B) and post-test only (WFW and LGE). The target population of this study is all male white rats (*Rattus norvegicus*) of the Sprague Dawley strain, aged 8-10 weeks, with a body weight of approximately 300 g. The sample size will be estimated according to the Federer formula, $(n-1)(t-1) > 15$, in which n refers to the minimal sample size for each treatment group and t refers to the number of treatments, establishing a minimum of four rats per treatment group. The study thus rounded up the total to 4 rats per treatment group. Ethical clearance was provided by the Commission Ethics of Health Research, General Hospital (RSUD) Dr. Moewardi, Surakarta, number 100/I/HREC/2023, on January 30, 2023.

The normal group (N) consists of rats that were not subjected to obesity modeling and were given drinking water (aquades) for 30 days. The negative control group (NC) consists of rats that were confirmed obese and then given a diet intervention and placebo (0.9% NaCl) at 1 mL using a sonde every morning for 30 days. The positive control group (PC) consists of rats that were confirmed obese and then given a diet intervention and rutacridone solution at 0.6 mg/kgBW using a sonde every morning for 30 days. Treatment group 1 (T1) consists of rats that were confirmed obese and then given a diet intervention and MER at a dose of 80 mg/kgBW using a sonde every morning for 30 days. Treatment group 2 (T2) consists of rats that were confirmed obese and then given a diet intervention and MER at a dose of 100 mg/kgBW using a sonde every morning for 30 days. Treatment group 3 (T3) consists of rats that were confirmed obese and then given a diet intervention and MER at a dose of 200 mg/kgBW using a sonde every morning for 30 days.

The preparation of the obese rat model follows Sundari *et al*. (2022). Before the rats were subjected to obesity intervention, they were acclimatized for 7 days in a cage environment with 12 hours of light and 12 hours of darkness at 25°C, with *Comfeed Br-2 feed and drinking water (aquades). After* adaptation, the rats' body weight, body length, and subcutaneous fat thickness were measured. A total of 40 rats were then subjected to obesity intervention for 30 days by feeding them a High Fat High Fructose (HFHFr) diet, consisting of beef fat, duck egg yolk, chicken liver, and butter, along with 10% fructose in their drinking water. The normal group was only given BR-2 feed and drinking water (aquades). The amount of feed given to the obese and normal groups was 10% of the rats' body weight. After 30 days of obesity intervention, the rats' body weight, body length, and subcutaneous fat thickness were measured again. The rats were considered obese if they had an obesity index ≥ 300 g/$cm^{1/3}$(Lee *et al*., 2011).

The OI was calculated using the Rohrer Index formula = {BW (g)/Naso-anal length $(cm)^3$} x 10^3, where rats were considered obese if they had an index ≥ 30 g/cm^3. BW and PB measurements were conducted weekly in the morning using a body weight scale (Sonic, Jakarta) and a stadiometer (Measure King, Jakarta). Meanwhile, body fat percentage was calculated using a formula = 0.581 x TM Index-22.03, in which the TM Index was = BW (g)/Naso-anal length $(cm)^{2.823}$ x 10^3. At the end of the study, blood was collected from the rats via the orbital vein to measure

insulin levels using a Rat Insulin ELISA kit. The obtained insulin levels were subsequently used for the calculation of HOMA-IR and HOMA-β. Fasting blood glucose levels were obtained from blood glucose measurements taken from the data in the dissertation of Dr. Sulistyani Kusumaningrum, Sp.Rad (K), who is part of this research project. The formulas used for the calculations are as follows:

$$\text{HOMA-IR} = \frac{(insulin\ (\mu\frac{U}{ml})\ X\ fasting\ blood\ glucose\ (\frac{mg}{dl})}{405}$$

$$\text{HOMA-}\beta = \frac{(360\ X\ insulin\ (\mu\frac{U}{ml})}{fasting\ blood\ glucose\ (\frac{mg}{dl})-63}$$

(Hirata *et al.*, 2009)

Approximately 100 mg of sub-abdominal adipose tissue was finely minced and homogenized using a homogenizer. Total RNA isolation from the tissue was performed using an RNA Purification Kit. RNA quantification was carried out spectrophotometrically using an ND-1000 Spectrophotometer. cDNA was synthesized from 1 mg of RNA sample using the RevertAid First Strand cDNA Synthesis Kit. qRT-PCR was conducted using a Real-Time PCR System. The target for amplification was the LEP gene, spanning 514 bp. qRT-PCR was performed in duplicate for each sample using Maxima SYBR Green qPCR Master Mix. The total reaction volume of 20 µL contained 4 µL of cDNA (100 ng/µL), 300 nmol/L of each primer set for the target gene, 10 µL of SYBR Green Master Mix, and was filled up to 20 µL with nuclease-free water. Gene expression levels were normalized to the housekeeping gene β-actin. The primer sequences for the leptin gene were based on those used in the study by Metwally *et al.* (2019). The forward primer sequence was CTCAGCATTCAGGCTAAGG, and the reverse primer sequence was AAGCCTCGCTCTACTCCACA. The thermal cycler program was set as follows: 95 °C for 5 minutes, followed by 40 cycles of 94 °C for 15 seconds, annealing for 60 seconds at the primer-specific temperature, and extension at 72 °C for 10 seconds. The 2−ΔΔCT method, a relative quantification approach for mRNA, was used to determine fold changes in gene expression.

The data obtained were then analyzed using IBM SPSS (Statistical Package for the Social Sciences) version 25, and the data were presented as mean ± standard deviation. Univariate analysis used several tests, parametric tests using the Shapiro-Wilk test, One-Way ANOVA followed by the LSD post hoc test, while non-parametric tests used the Shapiro-Wilk test, Kruskal-Wallis's test followed by the Mann-Whitney post hoc test. Multivariate analysis used the dependent T-test to compare means within the same group on different days, and the data were considered significantly different if the p-value was <0.05.

Result and Discussion

The analysis provides grounds for this argument: that administration of MER at 80, 100, and 200 mg/kgBW decreased the indices of obesity, body fat content, wet fat mass, insulin levels, Homeostasis Model Assessment for Insulin Resistance (HOMA-IR), and leptin gene expression ratio in rat models of obesity. All of this shows that MER could raise the Homeostasis Model Assessment for β-Cell Function (HOMA-β) in rat models of obesity. Moving on, the dose levels have been shown to influence the obesity index (OI), body fat content (BFC), wet fat weight (WFW), insulin level (IL), HOMA-IR, HOMA-β, and leptin gene expression ratio (LGE) with dose-response relationships for each of these variables; the higher the MER dose, the stronger is its effect on these variables. The conclusion is thus that MER would seem to offer some effect towards controlling obesity conditions on these variables. After a 30-day intervention period, a significant decrease in the mean Obesity Index (OI) was observed in the NC, PC, T1, T2, and T3 groups, while the N group showed a non-significant increase in mean OI (p=0.583). The smallest reduction in mean OI among the MER groups occurred in T1, with a decrease of -31.91 ± 5.77 g/cm$^{1/3}$, while the highest reduction was observed in T3, with a decrease of -47.69 ± 1.93 g/cm$^{1/3}$. The mean OI of rats across the groups after 30 days of intervention differed significantly (p<0.001). The highest mean OI was found in the NC group (294.66 ± 3.64 g/cm$^{1/3}$), and the lowest mean OI was observed in the T3 group (265.84 ± 2.80 g/cm$^{1/3}$). The mean OI of rats after the 30-day

intervention showed significant differences between groups ($p<0.001$). Based on post-hoc tests, T2 and PC showed no significant difference ($p=0.208$), indicating that MER at a dose of 100 mg/kg BW has comparable efficacy to Rutacridone 0.6 mg/kg BW in reducing OI. Additionally, T2 and PC showed no significant difference compared to the N group ($p=0.274$ and $p=0.861$, respectively), suggesting that MER at 100 mg/kg BW and Rutacridone 0.6 mg/kg BW were able to restore the OI of obese rats to normal levels. The T3 group showed significant differences compared to all other groups, indicating that MER at a dose of 200 mg/kg BW is the optimal dose for reducing the OI of obese rats. This set of methods would show a rise in the obesity index (OI). The higher the value of OI, the more serious the condition of an individual.

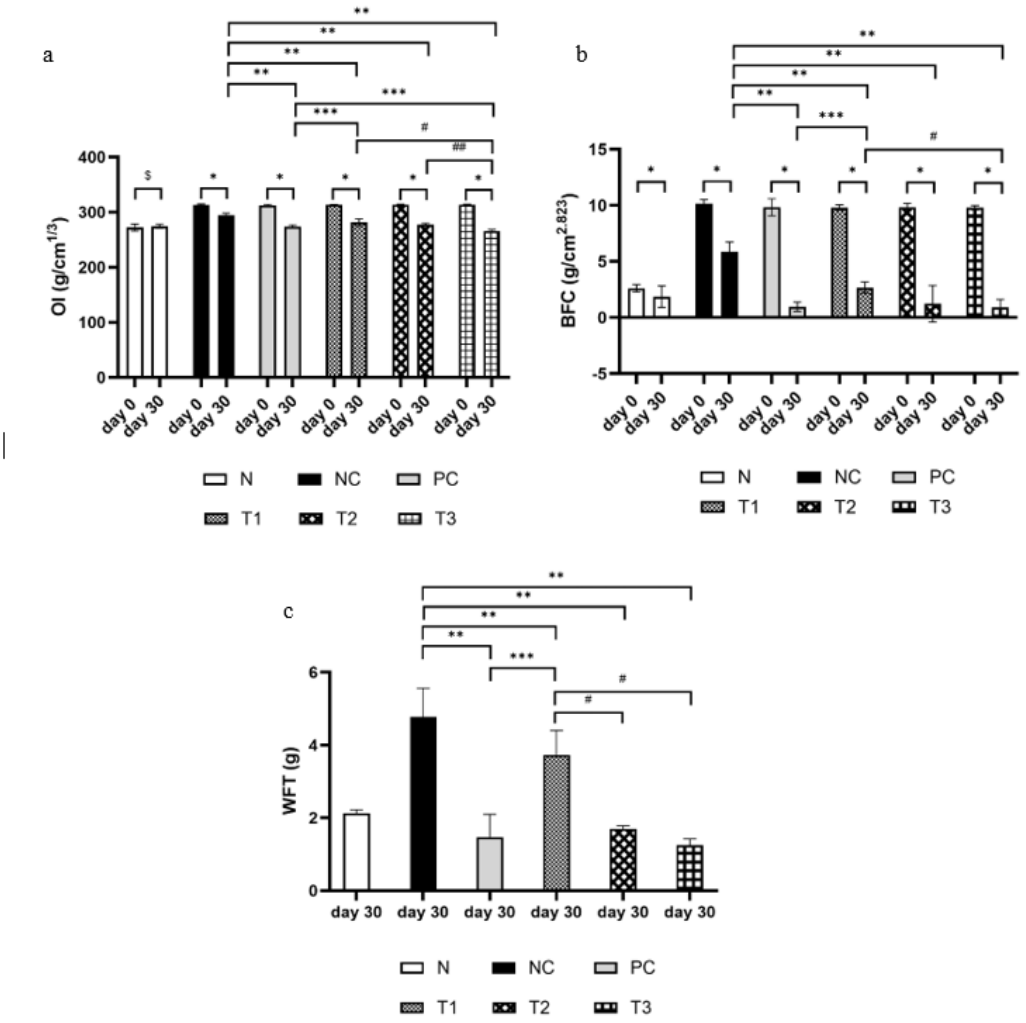

Figure 1. Mean of (a) OI (b) BFC in male obese model rats on day 0 and day 30, also mean of (c) WFW in male obese model rats on day 30, presented as mean ± SD. $) Independent t-test not significant ($p > 0.05$) *) Independent t-test significant ($p < 0.05$) **) Post hoc test significant compared to the negative control (NC). ***) Post hoc test is significant compared to the positive control (PC). #) Post hoc test is significant compared to Treatment 1 (T1). ##) Post hoc test is significant compared to Treatment 2 (T2). Statistical analysis was performed using Repeated-Measures ANOVA (for normally distributed data) and the Friedman test (for non-normally distributed data). N = Normal group (non-obese rats), NC = Negative control group (OR + standard diet + 0.9% NaCl), PC = Positive control group (OR + standard diet + pure rutacridone 0.6 mg/kg BW), T1 = Intervention 1 (OR + standard diet + MER 80 mg/kg BW), T2 = Intervention 2 (OR + standard diet + MER 100 mg/kg BW), T3 = Intervention 3 (OR + standard diet + MER 200 mg/kg BW).

Research conducted has shown a significant drop in the obesity index in NC, PC, T1, T2, and T3 groups following intervention, in keeping with the intervention for each group. This shows that the methanol extract of Rue (*Ruta graveolens*) leaves can significantly reduce the obesity index. However, it is seen that 80 mg/kgBW and 100 mg/kgBW showed a lower drop compared to pure Rutacridone at 0.6 mg/kgBW. Therefore, the average reduction in the OI for the NC group can be attributed to the influence of a standard diet with a lower fat content when compared to the high-fat, high-fructose (HFHFr) diet. Research conducted by Gupta *et al*. in 2012 reported a significant reduction of obesity index in obese rats receiving *Safoof Mohazzil,* a blend of *Ptychotis ajowan, Origanum majorana, Foeniculum vulgare, Carum carvi, Coccus lacca*, and *Ruta graveolens*-for 2 weeks compared to the obese control group. The experimental dose of 200 mg/kgBW was more effective in lowering OI than the positive control group. This indicates that although pure rutacridone could lower OI, the combined effects of osthol, psoralen, quercetin, and rutin that are present in Rue leaves play a far greater role than single ones in lowering OI. Studies report quercetin to reduce obesity by increasing ADP and decreasing leptin and appetite (Zhao *et al*., 2017).

A significant reduction in mean Body Fat Content (BFC) was observed in the NC, PC, T1, T2, and T3 groups, while the N group showed a non-significant reduction in mean BFC ($p=0.297$). The smallest reduction in mean BFC among the MER groups occurred in T1, with a decrease of -7.13 ± 0.25 g/cm$^{2 \cdot 823}$, while the highest reduction was observed in T3, with a decrease of -8.92 ± 0.55 g/cm$^{2 \cdot 823}$. The mean BFC of rats across the groups after 30 days of intervention differed significantly ($p<0.001$). The highest mean BFC was found in the NC group (5.85 ± 0.89 g/cm$^{2 \cdot 823}$), and the lowest mean BFC was observed in the T3 group (0.88 ± 0.74 g/cm$^{2 \cdot 823}$). Body Fat Content (BFC) is one of the primary variables for assessing obesity in rats. It refers to the total amount of fat in the body compared to total body weight. The increasing BFC will increase the obesity index, argued Crescenzo *et al*. (2018). The increase in the Lee Index represents an increase in body weight gain in conjunction with the redistribution of body fat, especially visceral fat, which is considered more harmful than subcutaneous fat. This finding establishes that obese rats had a markedly increased BFC compared to normal rats. After 30 days of intervention, a significant decrease in the average body fat was observed for the five groups: NC, PC, T1, T2, and T3. The extent of BFC reduction in the groups administered methanol extract of Ruta graveolens leaves at doses of 80, 100, and 200 mg/kgBW corresponded to the dose levels (-7.13 ± 0.25 g/cm², -8.74 ± 1.38 g/cm², and -8.92 ± 0.55 g/cm², respectively). The methanol extract of *Ruta graveolens* leaves at a dose of 200 mg/kgBW was the most effective in reducing BFC in obese rats, even outperforming pure Rutacridone at 0.6 mg/kgBW. A study conducted by Adel Mehraban *et al*. (2024) reported that the administration of Persian Medicine, a combination of *Cuminum cyminum*, *Apium graveolens*, *Ruta graveolens*, and *Trachyspermum ammi*, significantly reduced body fat content in obese rats over a 4-week intervention period compared to rats given a placebo capsule. The reduction in the Persian Medicine group was comparable to that observed in rats receiving acupuncture treatment. Another study by Valizadeh *et al*. (2019) reported that the administration of capsules containing *Origanum vulgare, Carum carvi, Trachyspermum copticum*, and *Ruta graveolens* over 8 weeks significantly reduced body weight, BMI, and body fat mass in adults. Several compounds, such as polyphenols, flavonoids, saponins, and terpenoids, have demonstrated anti-obesity effects. This reduction is hypothesized to result from the synergistic interaction between polyphenols and other compounds that can enhance thermogenesis and reduce appetite.

The average Wet Fat Weight (WFW) among the groups of rats on day 30 was statistically different ($p<0.001$). The T3 group exhibited the lowest average WFW (1.25 ± 0.17 g) when compared to the NC group, whose highest average VFM was recorded at (4.77 ± 0.79 g). In addition, it was observed that PC, T2, and T3 groups presented lower VFM when compared to the N group, showing that

these groups were more effective in managing VFM in obese rats compared to the N group. According to the post-hoc test, T2 presented no significant difference when compared to the N, PC, and T3 groups (p=0.250, p=0.537, and p=0.224, respectively). Similarly, the T3 group showed no significant difference compared to the PC group (p=0.537) but was significantly different from the N group (p=0.025). These findings suggest that MER at 100 and 200 mg/kg BW is effective in controlling VFM in obese rats, as effective as pure Rutacridone at 0.6 mg/kg BW, while MER at 200 mg/kg BW demonstrated better control of VFM compared to the other groups. Therefore, MER at 200 mg/kg BW is considered the best dose to treat VFM in obese rats. Wet fat refers to the adipose tissue removed from the bodies of rats, and it denotes adipose collected from the vicinity of internal organs, also known as visceral fat. From the evaluation, it was noted that the wet fat weight of the normal rats was less than that of the PC, T1, and T2 groups. The T3 group (1.25 ± 0.7 g) had a lower wet fat weight compared to the other two groups. This implies that the methanol extract from *Ruta graveolens* leaves at the dose of 200 mg/kgBW is the most efficient in giving rise to lower wet fat weight than the pure Rutacridone at 0.6 mg/kgBW. *Ruta graveolens* leaves contain some compounds like quercetin, rutin, osthol, and psoralen, which act at the anti-obesity level. Fat accumulation and lipid metabolism are tightly governed by adipose tissue and the liver. AMPK (AMP-activated protein kinase) is a key molecule for maintaining the energy balance. Once activated, AMPK inhibits lipogenesis and adipocyte differentiation while stimulating fatty acid oxidation and lipolysis via regulation of downstream targets. Studies by Jiang *et al*. (2020) have revealed that quercetin and its glycosides can reduce lipogenesis and enhance fatty acid oxidation and lipolysis via AMPK regulation in white adipose tissue (WAT) and the liver. In addition, ACC (acetyl-CoA carboxylase), which is a target of AMPK, can lead to the inhibition of its activity and a reduction in malonyl-CoA levels, thereby increasing the activity of CPT-1 (carnitine palmitoyltransferase-1) by counteracting the inhibitory effect of malonyl-CoA on CPT-1. Quercetin also suppresses the increased expression of SREBP1 (sterol regulatory element-binding protein 1) and FAS (fatty acid synthase) induced by a high-fat diet. SREBP1 is a key transcriptional regulator of lipogenesis, playing a central role in lipid synthesis (Zhang *et al*., 2024). ACC, CPT1, and PPARα (peroxisome proliferator-activated receptor alpha) are also involved in fatty acid oxidation (Cho *et al*., 2018).

There was a notable decrease in mean Insulin Levels (IL) in groups T2 and T3 with p-values 0.016 and 0.010, respectively. Within the MER groups, T1 showed the slightest decrease in mean IL with a decrease of -3.04 ± 0.08 mIU/L, whereas T3 showed the highest decrease of -3.51 ± 2.90 mIU/L. The 30-day intervention meant interleukin (IL) levels of rats in various groups had a statistically significant difference (p=0.002). The NC group presented the highest mean IL level (23.54 ± 2.04 mIU/L), and the PC group with obese rats presented the lowest mean IL level, but this decrease in IL in the PC group was not statistically significant (19.66 ± 0.90 mIU/L, p=0.141). The IL mean of rats after the 30-day intervention showed between-group differences (p=0.002). Post-hoc tests revealed no significant differences in T1, T2, and T3 compared with the PC group (p=0.432, p=0.80, and p=0.938, respectively). However, only the T3 group was not significantly different from the N group (p=0.069), indicating that MER at 200 mg/kg BW is the most appropriate dose for the recovery of IL to the average values of normal rats. Insulin levels (IL) and obesity are closely linked, particularly through the mechanism of insulin resistance. The accumulation of visceral fat can increase the release of free fatty acids into the bloodstream. Elevated free fatty acids can disrupt insulin signaling, reducing insulin's ability to inhibit glucose production in the liver and decreasing glucose uptake by muscle and adipose tissue. As a result, the pancreas increases insulin secretion to compensate for this resistance, leading to hyperinsulinemia. Based on the research findings, it was observed that obese rats had significantly higher insulin levels compared to normal rats, indicating the presence of hyperinsulinemia due to the accumulation of free fatty acids in obese rats.

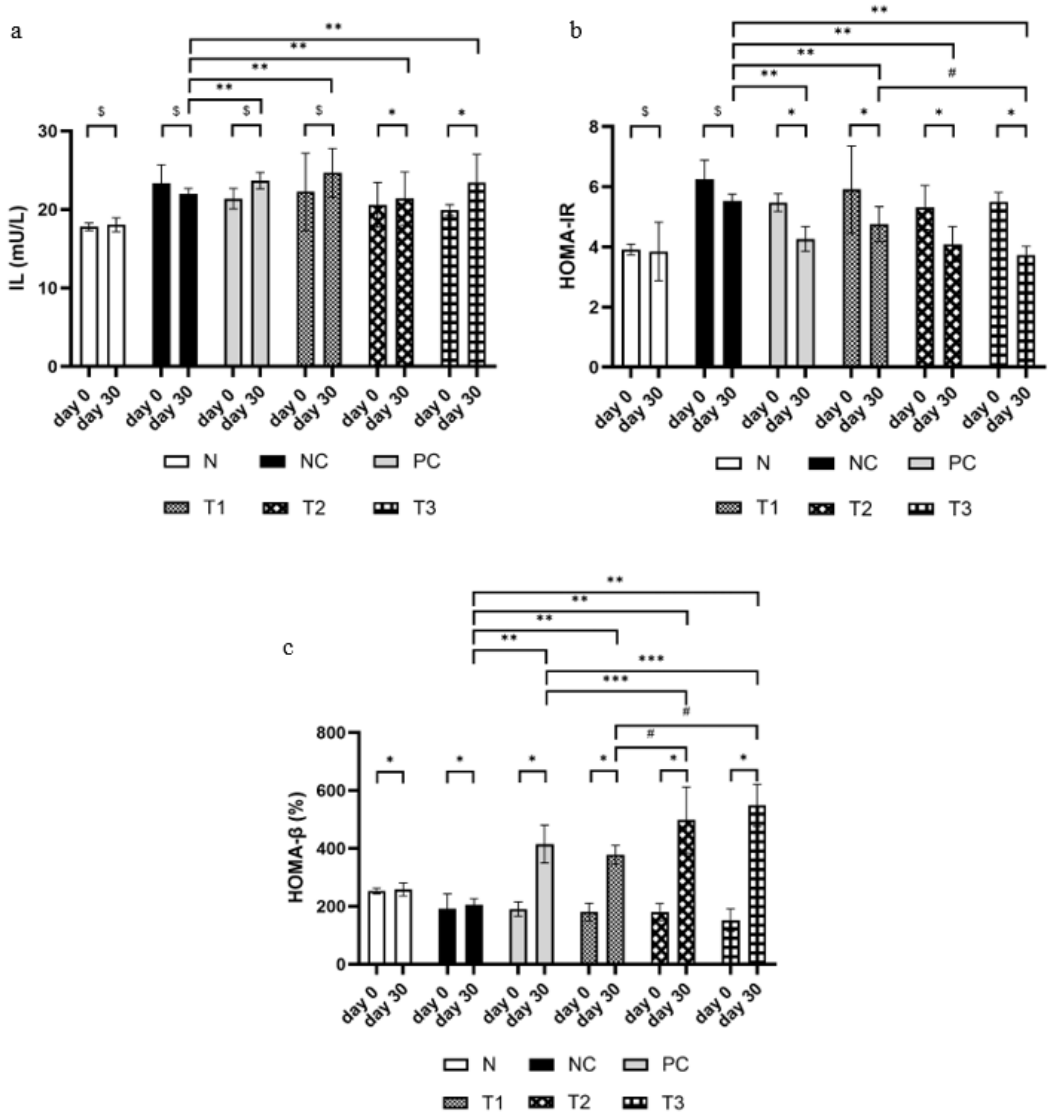

Figure 2. Mean of (a) IL, (b) HOMA-IR, and (c) HOMA-β in male obese model rats on day 0 and day 30, presented as mean ± SD. $) Independent t-test not significant ($p > 0.05$) *) Independent t-test significant ($p < 0.05$) **) Post hoc test significant compared to the negative control (NC). ***) Post hoc test is significant compared to the positive control (PC). #) Post hoc test is significant compared to Treatment 1 (T1). ##) Post hoc test is significant compared to Treatment 2 (T2). Statistical analysis was performed using Repeated-Measures ANOVA (for normally distributed data) and the Friedman test (for non-normally distributed data). N = Normal group (non-obese rats), NC = Negative control group (OR + standard diet + 0.9% NaCl), PC = Positive control group (OR + standard diet + pure rutacridone 0.6 mg/kg BW), T1 = Intervention 1 (OR + standard diet + MER 80 mg/kg BW), T2 = Intervention 2 (OR + standard diet + MER 100 mg/kg BW), T3 = Intervention 3 (OR + standard diet + MER 200 mg/kg BW).

After a 30-day intervention period, a significant reduction in average insulin levels was observed in the T2 and T3 groups. The extent of insulin reduction in the groups administered methanol extract of *Ruta graveolens* leaves at doses of 80, 100, and 200 mg/kgBW corresponded to the dose levels (-3.04 ± 0.08 mU/L, -3.25 ± 0.10 mU/L, and -3.51 ± 2.90 mU/L, respectively). The methanol extract of *Ruta graveolens* leaves at a dose of 200 mg/kgBW was the most effective in reducing insulin levels in obese rats, even outperforming pure Rutacridone at 0.6 mg/kgBW. This suggests that the combination of various compounds in the methanol extract of *Ruta graveolens* leaves works more effectively than a single pure compound. *Ruta graveolens* has been reported to contain rutin. Rutin has numerous benefits, including strong antioxidant properties, radical-scavenging effects, antidiabetic effects, and anti-inflammatory effects (Fitzpatrick and Woldemariam, 2017). Several studies have demonstrated that rutin inhibits two enzymes that catalyze carbohydrate digestion, namely α-glucosidase and α-amylase. The inhibition of these enzymes prevents the small intestine from absorbing glucose molecules, thereby preventing a sharp rise in blood glucose levels (Ghorbani, 2017).

A significant reduction in mean HOMA-IR was observed in the PC, T1, T2, and T3 groups, with p=0.012, p=0.011, p=0.001, and p<0.001, respectively. The smallest reduction in mean HOMA-IR among the MER groups occurred in T1, with a decrease of -1.87 ± 0.24, while the highest reduction was observed in T3, with a decrease of -2.56 ± 0.41. The mean HOMA-IR of rats across the groups after 30 days of intervention differed significantly (p<0.001). The highest mean HOMA-IR was found in the NC group (5.92 ± 0.64), while the lowest mean HOMA-IR in obese rats was observed in the T3 group (3.86 ± 0.18). Insulin resistance refers to producing cells in any part of the body that tend not to respond to insulin effectively. Such a situation leads to elevated blood glucose levels and is responsible for giving rise to T2DM (Smith *et al*., 2021). Findings of research indicate that obese rats exhibit significantly higher HOMA-IR values as compared to the normal ones. Hence, a lowering of receptor sensitivity to insulin is associated with continuous increases in insulin production. After a 30-day intervention, significant reductions in mean HOMA-IR were observed in the PC, T1, T2, and T3 groups. The magnitude of HOMA-IR reduction in the groups treated with methanol extract of Rue leaves at doses of 80, 100, and 200 mg/kg BW corresponded to the dosage levels (-1.87 ± 0.24, -2.10 ± 0.04, and -2.56 ± 0.41, respectively). The 200 mg/kg BW dose of methanol extract of Rue leaves was the most effective in reducing HOMA-IR in obese rats, outperforming even pure Rutacridone at 0.6 mg/kg BW. One active compound in *Ruta graveolens* is quercetin. Studies report that quercetin (75 mg/kg/day) reduces serum resistin in a non-alcoholic fatty liver disease rat model induced by an 8-week high-fat (HF) diet. Administration of quercetin and Ruta graveolens-a traditional European medicinal plant-inhibits the expression of resistin in fat by downregulating resistin-encoding genes. When quercetin (QC) and metformin (MF) are administered together, they reduce levels of hyperglycemia and insulin resistance in patients with type 2 diabetes mellitus by increasing GLUT-4 expression in skeletal muscles and adipose tissues while reversing oxidative stress and inflammatory conditions. Thus, type 2 diabetes patients are advised to use quercetin as a complementary therapy with metformin and consume quercetin-rich foods (Eldamarawi & Abdelazeem, 2020). The observed HOMA-IR reduction in this study is hypothesized to result from improved insulin sensitivity in diabetic rats treated with methanol extract of Rue leaves, attributed to quercetin's ability to lower plasma free fatty acids and lipids, increase intracellular cAMP, and activate protein kinase A (PKA) (Shi *et al*., 2021).

A significant increase in mean HOMA-β was observed in the PC, T1, T2, and T3 groups, while the N and NC groups showed an increase in mean HOMA-β that was not significant (p=0.844 and p=0.526, respectively). The smallest increase in mean HOMA-β among the MER groups occurred in T1, with an increase of -125.47 ± 27.75%, while the highest increase was observed in T3, with an increase of 285.32 ± 49.48%. The mean HOMA-β of rats across

the groups after 30 days of intervention differed significantly ($p<0.001$). The highest mean HOMA-β was found in the T2 group (470.52 ± 39.12%), while the lowest mean HOMA-β was observed in the NC group (219.07 ± 18.49%). The Homeostasis Model Assessment for Beta Cell Function (HOMA-β) is a variable used to assess pancreatic β-cell function. The HOMA-β index measures the capacity of pancreatic β-cells to secrete insulin based on fasting glucose and insulin levels. Previous studies indicate that obesity affects pancreatic β-cell function, reflected in altered HOMA-β values (Chen *et al*., 2021). Results show that obese rats have significantly lower HOMA-β values than normal rats, indicating that metabolic and inflammatory stress can cause pancreatic β-cell dysfunction, marked by reduced HOMA-β. After 30 days of intervention, significant increases in mean HOMA-β were observed in the PC, T1, T2, and T3 groups. The magnitude of HOMA-β improvement in groups treated with methanol extract of Rue leaves at 80, 100, and 200 mg/kg BW doses corresponded to dosage levels (125.47 ± 27.75%, 265.72 ± 11.37%, and 285.32 ± 49.48%, respectively). The 200 mg/kg BW dose of methanol extract of Rue leaves was most effective in enhancing HOMA-β in obese rats, surpassing pure Rutacridone at 0.6 mg/kg BW. A study by Khamchan *et al*. (2018) reported that quercetin in *Syzygium samarangense* improved HOMA-β and insulin sensitivity in rats with damaged pancreatic β-cells by boosting antioxidant defenses. In pancreatic β-cells, hyperglycemia activates reactive oxygen species (ROS) and reactive nitrogen species (RNS) production via pathways such as mitochondrial superoxide overproduction, glycation, hexosamine pathway activation, PKC activation, sorbitol metabolism, and alpha-ketoaldehyde generation. Consequently, an imbalance between excessive free radical production and impaired cellular antioxidant defenses leads to oxidative stress accumulation in pancreatic β-cells. Previous studies report that 300 mg/kg BW of methanol extract of Rue leaves reduced MDA levels compared to controls in male rats Asgharian *et al*. (2020).

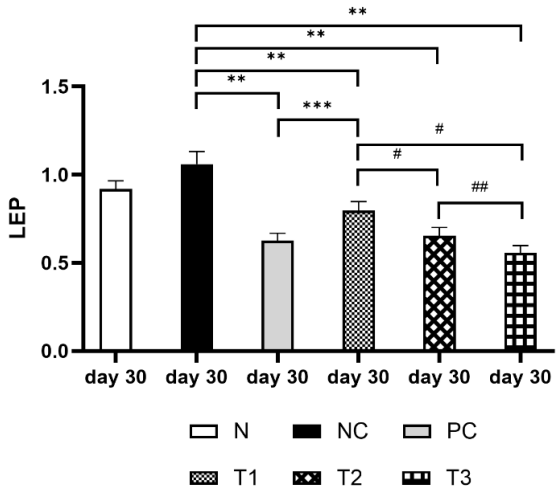

Figure 3. Mean Leptin Gene Ratio Measurement (LGE) in male obese model rats on day 0 and day 30, presented as mean ± SD. [$]) Independent t-test not significant ($p > 0.05$) *) Independent t-test significant ($p < 0.05$) **) Post hoc test significant compared to the negative control (NC). ***) Post hoc test is significant compared to the positive control (PC). [#]) Post hoc test is significant compared to Treatment 1 (T1). [##]) Post hoc test is significant compared to Treatment 2 (T2). Statistical analysis was performed using Repeated-Measures ANOVA (for normally distributed data) and the Friedman test (for non-normally distributed data). N = Normal group (non-obese rats), NC = Negative control group (OR + standard diet + 0.9% NaCl), PC = Positive control group (OR + standard diet + pure rutacridone 0.6 mg/kg BW), T1 = Intervention 1 (OR + standard diet + MER 80 mg/kg BW), T2 = Intervention 2 (OR + standard diet + MER 100 mg/kg BW), T3 = Intervention 3 (OR + standard diet + MER 200 mg/kg BW).

The mean Leptin Gene Expression Ratio (LGE) among the rat groups on day 30 showed significant differences ($p<0.001$). The lowest mean LGE was observed in the T3 group (0.56 ± 0.04), while the highest mean LGE was found in the NC group (1.06 ± 0.07). It was also noted that the PC, T1, T2, and T3 groups had lower mean LGE compared to the N group, indicating that the Rutacridone and MER groups were effective in controlling LGE in obese rats, resulting in a gene expression ratio like that of normal rats. Based on post-hoc analysis, the PC, NC, T1, T2, and T3 groups showed significant differences compared to the N group. Meanwhile, the PC group showed no significant difference compared to T2 and T3 ($p=0.534$ and $p=0.119$, respectively), but T2 was significantly different from T3 ($p=0.039$). This suggests that T3 (MER at a dose of 200 mg/kg BW) is the most effective dose of MER in achieving a low LGE. The leptin gene (LEP) is one of the genes involved in how someone utilizes energy and appetite regulation. The gene for leptin encodes the leptin hormone, which is secreted by adipose cells to regulate energy balance through positive feedback in the brain. Besides its role in energy metabolism, leptin also plays a role in insulin homeostasis and glucose metabolism through interactions with pancreatic beta cells. Based on the findings of the study mentioned, T3 exhibited LGE less than the obese and normal rat groups. The LGE in the group administered with methanol extract of Rue (*Ruta graveolens*) leaves (MER) at doses of 80, 100, and 200 mg/kgBW decreased in a dose-dependent manner (0.80 ± 0.05, 0.65 ± 0.04, and 0.56 ± 0.04, respectively). The methanol extract at doses of 200 mg/kgBW was found to be most effective in reducing LGE in the case of obese rats-even more effective than pure rutacridone at 0.6 mg/kgBW. This indicates that the activities of the various compounds in MER override the inhibiting activity of pure rutacridone, which works through several mechanisms that include inhibiting the increase of TCPTP under undrained over-nourishment conditions, which enhances the leptin receptor function, and finally aids leptin receptor conditions. High leptin levels are proportional to increased leptin gene expression and excess body weight and fat levels.

Conclusion

The methanol extract of Rue leaves (common rue / *Ruta graveolens*) demonstrates significant anti-obesity effects. Administration of the extract effectively reduces OI, BFC, IL, and HOMA-IR while increasing HOMA-β. Furthermore, the extract downregulates the relative expression of the leptin gene (LGE) and WFW. Analysis of variance showed that the intakes produced a dose-dependent response in certain parameters like OI, BFC, WFW, IL, HOMA-IR, HOMA-β, and LGE. This study suggested that the methanol extract of Rue leaves could serve as a potential natural management for obesity. Further studies would elucidate the mechanisms through which they act and the potential clinical applications.

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