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"PENGARUH PEMBERIAN EKSTRAK DAUN SEMBUNG *(Blumea balsamifera)* TERHADAP KADAR MALONDIALDEHYDE (MDA) DAN SUPEROKSIDA DISMUTASE (SOD) TIKUS JANTAN HIPERLIPIDEMIA"

Peneliti Utama	: I Gede Widhiantara, S.Si.,M.Biomed
Peneliti Lain	: A.A. Ayu Putri Permatasari, S.Si.,M.Biomed
Unit/Lembaga/Tempat Penelitian	: Lab. Sains Univ. Dhyana Pura, Lab. Animal Unit Bag. Farmakologi FK. Unud. dan Balai Besar Venteriner Denpasar
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Prof. Dr. dr. I Gde Raka Widiana, Sp.PD-KGH
NIP. 195607071982111001

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The Potential of *Blumea balsamifera* L. Extract to Maintain Antioxidant Enzyme and Luteinizing Hormones in Rats Induced by High Cholesterol Diets

Abstract

Background: The discovery of herbal ingredients for antihypercholesterolemic as well as improving male reproductive function is very necessary due to high cholesterol diet factors. This study was to determine the levels of total cholesterol, body weight, plasma malondialdehyde (MDA), Superoxide Dismutase (SOD), Luteinizing Hormone (LH) in high-cholesterol-fed male rats provided with *Blumea balsamifera* leaves extract (BBLE).

Methods: This research utilized a randomized post-test only control group. Ethanol was used to extract the BBLE, which was then evaporated. For 21 days, 16 Wistar rats that met the inclusion criteria were given a high cholesterol diet. To determine the effect of BBLE on the HC-diet, the samples were divided into two groups (control and BBLE group) on day 22. The treatments lasted 30 days. SOD, plasma MDA, LH, and total cholesterol were measured.

Results: The results showed that the SOD and LH parameters were significantly higher in the treatment of BBLE compared to the control group *($p<0.05$)*. The parameters of total cholesterol levels, bodyweight, and MDA of rats given BBLE were significantly lower than those of the control group *($p<0.05$)*.

Conclusion: Our findings highlight the BBLE has antihypercholesterolemic and antioxidant properties. The BBLE also has the potential to be used as a therapy to maintain male reproductive function because it has a positive effect on the hypothalamic-pituitary axis through increasing LH secretion.

Keywords: Malondialdehyde, Superoxide dismutase, Luteinizing hormone, *Blumea balsamifera* extract, High-cholesterol diet

Introduction

Increased levels of cholesterol in the blood, known as Hypercholesterolemic, are linked to a variety of health issues, including diabetes, cancer, and obesity, as well as having an influence on reproductive abnormalities. Hypercholesterolemic is characterized by oxidative stress caused by elevated malondialdehyde (MDA) levels and reduced superoxide dismutase (SOD). MDA, on the other hand, is an oxidative damage marker of physiological cell damage (1). A study showed that the hypercholesterolemic group had higher plasma MDA concentrations than the non-hypercholesterolemic group. This indicates the correlation between oxidative stress and hypercholesterolemic progression (2). As a result, the availability of antioxidant ingredients is critical in maintaining a balance between antioxidant enzymes and oxidative stress levels, (3–6) especially in people with hypercholesterolemic.

At the tissue level, hypercholesterolemic increases Reactive Oxygen Species (ROS) production and the lipid peroxide formation imbalance (7,8). Oxidative stress may also cause disruption of the hypothalamic-pituitary-testis axis in the male reproductive system, resulting in reduced luteinizing hormone release. This disorder causes a reduction in Leydig cells number in the testes as well as an impairment of the spermatogenesis process (9). Our previous study showed a significant reduction in the number of Leydig cells, spermatogonia A cells, primary spermatocytes, spermatids 7, spermatids 16, and Corvus cavernosum muscle thickness significantly in rats induced by a high-cholesterol diet for 50 days (10). The administration of a high-fat diet also significantly decreased the lumen diameter and thickness of the dorsal penile artery endothelium in Wistar rats (11).

Sembung *(Blumea balsamifera)* is a traditional plant with a high antioxidant content and is commonly used as a traditional medicine in Indonesia and many other Asian countries (12). *B. balsamifera* leaf is mostly used to make a traditional drink called "Loloh" by Balinese people in Indonesia. *B. balsamifera* contains bioactive substances such as essential

oils (cineol, borneol, landerol, and camphor), flavonols, tannins, resin, and cantoxillin. The flavonoid compounds can serve as immunomodulators and antioxidants preventing the body from producing so much oxidative stress (13). The results of our preliminary research indicate that *B. balsamifera* leaf extract (BBLE) is able to increase the number and diameter of Leydig cells in rats given high-fat feed (14).

Based on the above, this study aims to study the antihypercholesterolemic and antioxidant potential of BBLE and their relation to the improvement of the work of the hypothalamic-pituitary axis in secreting LH. This findings may be utilized to develop pharmacological formulations, particularly for the treatment of male infertility related with hypercholesterolemic risk factors.

Methods

Research design

This study is included in an experimental study with a randomized posttest only control group design. The sample is taken randomly and divided into 2 groups, namely the control group (high cholesterol feed and sterile distilled water) and the BBLE group (high cholesterol feed and 4 mg/mL/BW rats of BBLE orally) daily for 30 days. The treatment dosage was determined using a previous study (15) which was converted to rats as well as our preliminary study in high-cholesterol feed rats (unpublished data). The number of samples in each group was added by 1 rat as a reserve (16).

Preparation of BBLE

B. balsamifera plants were obtained from plantations in Luwus Village, Tabanan Regency, Bali which had previously been identified/determined at the Indonesian Institute of Science (LIPI), Bali Botanic Garden, Candikuning, Baturiti, Tabanan-Bali. The extraction process follows the extraction method described in previous studies (14). Fresh *B.*

balsamifera leaves, washed, dried, and crushed using a blender. A total of 250 grams of *B. balsamifera* powder was then macerated for 24 hours in ethanol. The ethanol as solvent was macerated for 24 hours. The macerated extract is furthermore filtered, and the filtrate is concentrated using a rotary evaporator to separate the extract material's alcohol content. The extract was evaporated until forming the thick green sticky paste. The production of BBLE was carried out at the Laboratory of Science, Universitas Dhyana Pura.

Phytochemical screening

The BBLE was screened for phytochemicals qualitatively and quantitatively. Phytochemical compounds analyzed qualitatively include alkaloids, flavonoids, triterpenoids, saponins, phenols, tannins, and steroids. Meanwhile, the quantitative test included total phenol (mg / 100 g GAE), flavonoids (mg / 100 g), and tannin content (mg / 100 g TAE) (17). The standard indicator of BBLE is also adjusted to the 2017 Indonesian Herbal Pharmacopoeia Standardization Edition II (18).

Sample and treatment

Test animals were approved by The Research Ethics Committee of the Faculty of Medicine, Udayana University, and the Sanglah Central General Hospital (RSUP) in Denpasar, Bali, with number: 2020.02.2.2.0937. The samples in this study were adult male Wistar rats *(Rattus norvegicus)* aged 3-4 months with a body weight of 150-200 grams. Samples were taken randomly to get the number of samples and divided into two groups. During the acclimatization phase, rats were given a standard diet that included 20-25 % protein, 5% fat, 45-50 % starch, 5% crude fiber, 4% ash, and vitamins and minerals. While the composition of the high-cholesterol diet used in this study contained a mixture of 10% pork oil (100 grams = 108.7 ml; oil density 0.92 mg/ml), 5% duck egg yolk (50 grams), and standard feed up to 1,000 grams.

A high cholesterol diet in this study consisted and distilled water were given to the Control group (C). Whereas the BBLE group (P) was supplemented a high cholesterol diet and BBLE of 4 mg/mL/body weight rats per day (each rat was given 1 mL containing 4 mg of extract) for 30 days orally (15). After 30 days, blood was taken through the canthus medial orbitalis sinus for examination of plasma MDA levels, SOD, Luteinizing hormone, and total cholesterol.

Total cholesterol and Body weight

Total cholesterol of rats was analyzed using the Cholesterol FS with Catalogue No: 1 1300 99 10 021 (Diagnostic Systems, Germany) based on the procedure. In summary, 1 ml of the sample was centrifuged at 3,000 rpm for 15 minutes. Then, the plasma was taken using a micropipette and put in an Effendorf tube, and stored at -20°C. The centrifuged plasma was mixed with the reagent. The entire sample mixture was incubated for 5 min at 37°C. Cholesterol levels were read on a spectrophotometer with a wavelength of 500 nm. Calculation of the results according to the following equation (19) :

$$Total\ cholesterol\ level = \frac{Sample\ Absorbance}{Standard\ Absorbance} \times Standard\ Concentration$$

Rats in both treatments were weighed after 30 days of intervention using 1 kg analytical balance (20) (Mettler Toledo AL-204 Analytical Balance). This was performed to determine body weight mass due to exposure to high-cholesterol feed and BBLE.

Plasma MDA

MDA levels in plasma were determined using ab238537 (MDA Assay Kit) (Bioassay Kit System, USA). The first two columns currently offer standard solutions. Each concentration was replicated up to 50 µL/well. The samples were mixed at the same concentration. Each well-received 50 µL of biotinylated detection is a working solution. For 45 minutes at 45°C, the plates were closed. Then each well-received 350 µL wash buffer was

soaked for 1-2 minutes and dried sterile. The plates were then cleaned three times and re-incubated at 37°C for 30 minutes with a 100 µL HRP conjugate working solution. Incubation of each well for 25 minutes at 37°C with 90 µL of substrate reagent. Then 50 µL of stop solution was added to each well. Each well's optical density was measured using an ELISA Reader at 450 nm.

SOD levels

SOD levels were checked using the SOD Assay Kit with Catalog No: ESOD-100 (Bioassay Kit Systems, USA). In short, 20 µL of plasma sample solution was added to each sample and 20 µL of H_2O_2 was added to blank 1 and blank 2.200 µL of WST working solution and 20 µL of Dillution buffer were added to each blank. 20 µL of Enzyme solution was added to each sample. The well-plates were incubated at 37°C for 20 minutes. The absorbance was obtained using a microplate reader at a wavelength of 450 nm. The results obtained were analyzed using the SOD standard in the form of U/mL.

Luteinizing Hormone (LH) levels

The LH levels were measured using the MBS764675 Rat LH ELISA Kit (MyBioSource, US). In brief, all reagents operating standards. It was incubated at 4°C with 20 µL of standard, control, and sample solutions. Each well got 100 µL HRP and 100 µL biotin conjugate. The wells were drained and tin-papered. This was followed by an hour of cooling. Then the tin paper was removed and the well was aspirated. It was cleaned 3 times with 300 µL 1X washing solution, soaking for 5 seconds between each wash. After the final wash, the 1X Washing Solution was aspirated or decanted. With a dry sterile cloth, the surplus liquid was wiped off the plate-wells. Then, all wells were incubated for 15 minutes at room temperature in the dark with 100 µL TMB substrate solution. All wells received 100 µL stop solution in the same sequence and pace as the TMB Substrate Solution. The plates were

thoroughly shaken. During incubation, the hue changes from blue to yellow. After 30 minutes, the sample's absorbance was measured at 450 nm.

Data analysis

The collected data were analyzed using the Statistical Package for the Social Sciences (SPSS) 23 software program (IBM, USA). The results were evaluated using the Independent-Samples T-Test with a confidence interval of $P<0.05$ to determine the significant differences between each treatments. Observation results were displayed in the form of Figures using GraphPad Prism 8.0.1 for Windows (GraphPad Software, Inc, USA) and Tables.

Results

Phytochemical compounds

Overall, the results of the phytochemical screening of the BBLE used as a treatment in our study were positive for flavonoids, saponins, phenols, tannins, and steroids (Table 1).

Likewise, the quantitative results showed that the highest levels of flavonoid compounds were obtained in the extracted material, namely 22083.88 mg / 100 g (22.083%). The percentage of total flavonoids and yield reported in this study are accordance with the Indonesian Herbal Pharmacopoeia (18). The results of the quantitative test for phytochemical compounds are shown in Table 2 below.

The effect of BBLE on total cholesterol levels and body weight

There were significant differences ($p<.05$) between the both groups based on total cholesterol levels. The control group's average cholesterol level was 72.92±17.13 mg/dL higher than the BBLE group, which was 35.86±1.15 mg/dL lower (Figure 1). Weight parameters also differed substantially between the two groups ($p<0.05$). The average

bodyweight of the control rats after 30 days of high-cholesterol feeding was up to 272±9.85 grams, which was higher than the rats supplemented with BBLE, which was 165.38±5.73 grams (Figure 2).

The effect of BBLE on plasma MDA and SOD levels

The mean plasma MDA and SOD levels of Wistar rats following high-cholesterol diet and BBLE intervention for 30 days revealed significant differences between the both groups *($p < .05$)*. When compared to the BBLE group, the average MDA plasma levels in the control group were higher, namely 29.40±8.60 mol/mL compared to 12.41±5.17 mol/L. (Figure 3). In contrast to plasma MDA, SOD levels in the control group were lower at 19.2± 5.98 U/mL compared to 37.69 ±4.82 U/mL in the BBLE group (Figure 4). This implies that BBLE has a positive effect on lowering plasma MDA levels in the body as a result of a high-cholesterol diet and functions as an essential anti-oxidative agent in rats.

The effect of BBLE on LH levels

The levels of luteinizing hormone (LH) varied substantially across treatments ($p<.05$). The control group's average of 25.34±2.18 mIU/mL was lower than the BBLE group average of 47.84±3.62 mIU/mL (Figure 5), indicating that BBLE plays an important role in the mechanism of maintaining the hypothalamic-pituitary axis by stimulating LH.

Overall, all test variables in the BBLE group were close to the baseline levels measured in pre-intervention (Figure 1-4). Table 3 summarizes the findings from our observations.

Discussion

The BBLE has the highest flavonoid concentration of the three major kinds of phytochemical compounds (21). Flavonoids are a class of natural phenols found in many fruits and vegetables as well as traditional medicine (22) that offer health advantages via cell-mediated signaling, antioxidants, anti-inflammatory, neurological, and cardiovascular effects (23). Besides from that, that tannin is the second most abundant in our extract components.

The BBLE used in this study was effective at lowering total cholesterol levels in Wistar rats fed a high-cholesterol diet. High cholesterol levels may increase the amount of fat in adipose tissue, leading to a increase in body weight. Consequently, the flavonoids in BBLE act as PPAR (peroxisome proliferator-activated receptor) ligands, decreasing the expression of the sterol regulatory element-binding protein 1c (SREBP-1c) in the liver and, as a result, decreasing the formation of triglycerides in tissues (24).

Plasma MDA and SOD levels are essential indicators for monitoring oxidative stress in the body induced by free radical toxicity, such as high-cholesterol diets (3,25,26). In our findings, we hypothesized that flavonoid levels had a substantial beneficial impact on high SOD levels, resulting in lower plasma MDA levels in the BBLE group. The flavonoid concentration of the BBLE is high, which may serve as antioxidants and promote cell regeneration caused to free radical damage. Flavonoids have a metabolic mechanism in cell membranes that includes trapping free radicals of unsaturated fatty acids and converting them into non-free radicals. As a consequence of these processes, lipid oxidation is decreased. In the chemical structure of flavonoids, ring B has a hydroxy group capable of donating hydrogen, which influences free radical stability (27).

Increased levels of antioxidant enzymes induced by BBLE are capable of maintaining the hypothalamus-performance pituitary's in generating LH. SOD generation, by lowering negative feedback, was able to promote an increase in HPA axis activity (28). The pituitary

gland to release LH, which in turn stimulates Leydig cells to produce testosterone through Steroidogenesis (29).

Based on the parameters tested, there was a correlation between a near-baseline decrease in total cholesterol and an increase in antioxidant enzymes and LH secretion. Clinical investigations/research regarding the synergistic mechanism of the herbal ingredients that we use are still needed to be able to verify the combination of phytochemical compounds reported against side effects that can be caused and increase their pharmacological potential as new phytomedicine substances (30).

Conclusion

The BBLE phytochemical compounds act as antihypercholesterolemic and antioxidant. In this study, the increase in antioxidant enzymes enhanced the pituitary's function in secreting LH. Further research is needed, particularly on the role of BBLE on several molecular markers in testicular tissue, to provide a comprehensive knowledge about the impact of BBLE on the pathophysiology of male infertility caused by hypercholesterolemic.

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Author Contribution

IGW were research concept, supervisor, and proofread manuscript, AAAPP were extraction, research guideline, and conducted the study, IWR processed the experimental design and collecting the data, PAW and LPW conducted the study, collected samples, data analysis, writing manuscript

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382

Figures/Tables

Table 1. Phytochemical compounds, their reactions and result

No.	Phytochemical compounds	Material	Reactions	Result	References
1	Alkaloid	Mayer	Green	-	(31)
2	Flavonoid	NaOH 10%	Brown	+	(32)
3	Triterpenoid	Liebermen-Burchard H_2SO_4 10%	Green	-	(31)
4	Saponin	Hot water + HCl	Foam	+	(33)
5	Phenol	Hot water + $FeCl_3$	Dark green	+	(32)
6	Tannin	FeCl 1%	Dark green	+	(34)
7	Steroid	Lieberman-Burchard H_2SO_4 10%	Dark green blackish	+	(31)

Note : + : positive and - : negative result

Table 2. Levels of phytochemical compounds of *B. balsamifera* leaf extract

Total phenol (mg/100 g GAE)(	Flavonoid (mg/100 g)(%)	Tannin (mg/100 g TAE)
6820.04	22083.88 (22.083%)	696.99
-	Not less than 1.31%*	-

Note: *percentage based on the Indonesian Herbal Pharmacopoeia Standardization II edition (18)

Table 3. Test parameters in Wistar rats observed in this study

Parameter	Control	BBLE	*p-value*
Malondialdehyde (MDA) (µmol/mL)	29.40±8.60	12.41±5.17	$p < 0.05$
Superoxide dismustase (SOD) (U/mL)	19.20±5.98	37.69±4.82	$p < 0.05$
Luteinizing hormones (LH) (mIU/mL)	25.34±2.18	47.84±3.62	$p < 0.05$
Total cholesterol (mg/dL)	72.92±17.13	35.86±1.15	$p < 0.05$
Body weight (gram)	272±9.85	165.38±5.73	$p < 0.05$

Result are presented as mean±SD.

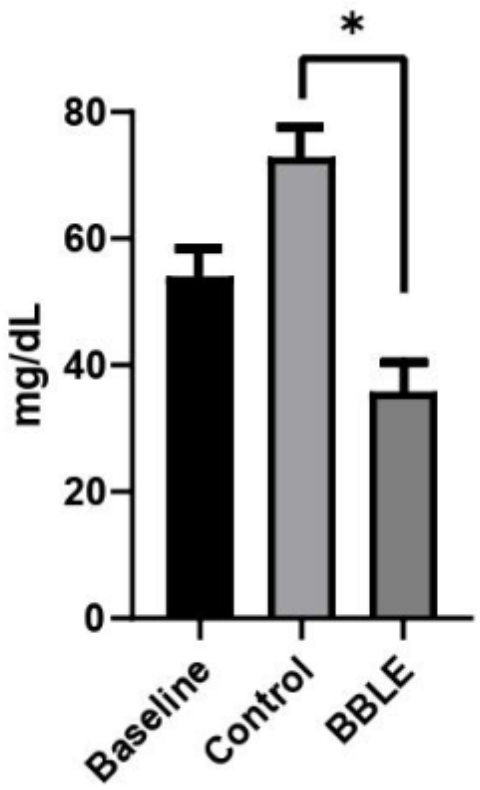


Figure 1. Total cholesterol levels of hypercholesterolemic Wistar rats (n = 8) (Mean ± SD). Sign * indicates that there is a significant difference ($p < 0.05$) between the two groups. Baseline is the measurement result in the both groups before intervention.

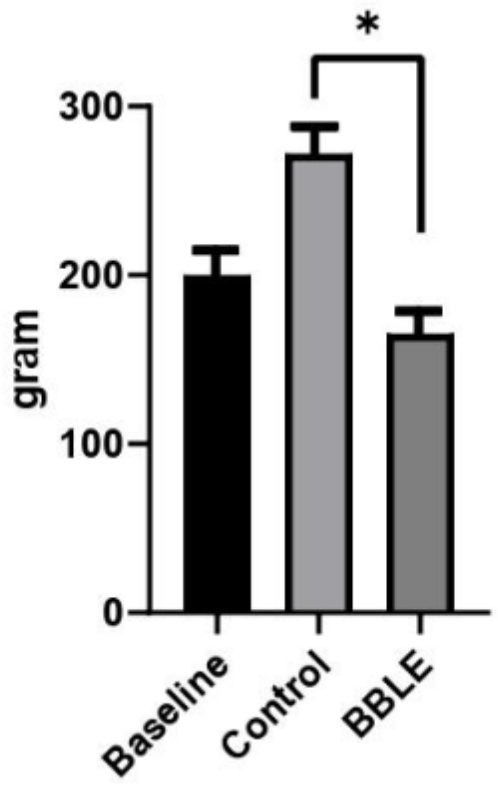
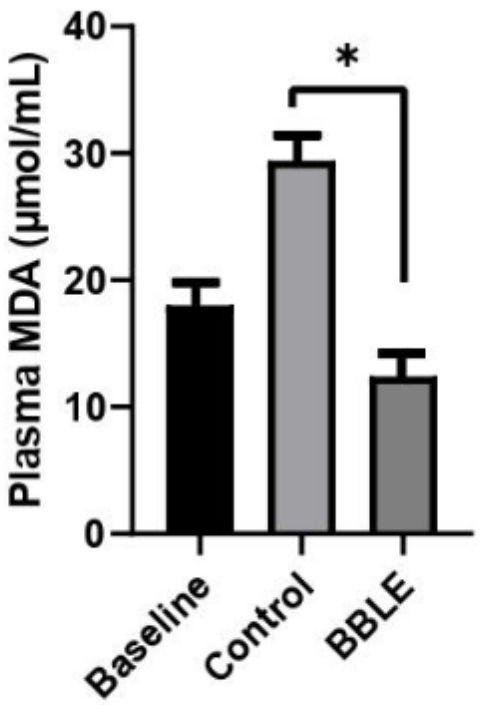
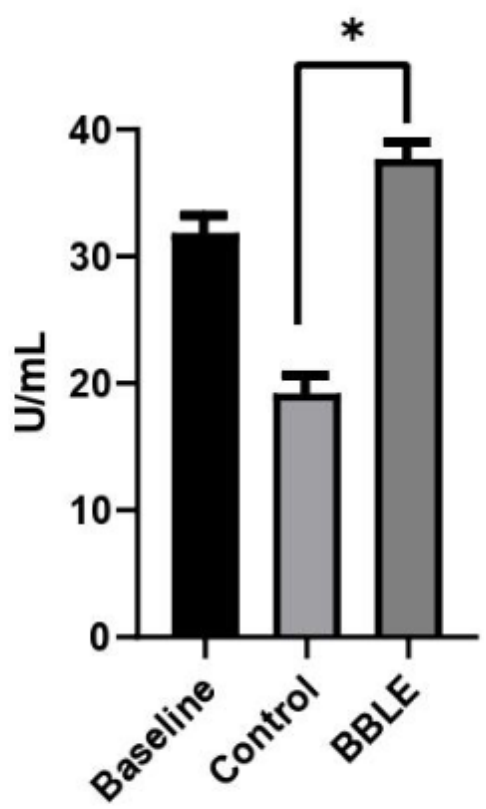


Figure 2. Body weight of hypercholesterolemic Wistar rats (n = 8) (Mean ± SD). Sign * indicates that there is a significant difference ($p < 0.05$) between the two treatments. Baseline is the measurement result in the two both groups before intervention.



406
407 **Figure 3.** Plasma MDA levels of hypercholesterolemic Wistar rats (n = 8) (Mean ± SD). Sign * indicates that
408 there is a significant difference ($p < 0.05$) between the two treatments. Baseline is the measurement result in the
409 both groups before intervention.
410



411
412 **Figure 4.** SOD levels of hypercholesterolemic Wistar rats (n = 8) (Mean ± SD). Sign * indicates that there is a
413 significant difference ($p < 0.05$) between the two treatments. Baseline is the measurement result in the both
414 groups before intervention.
415 .

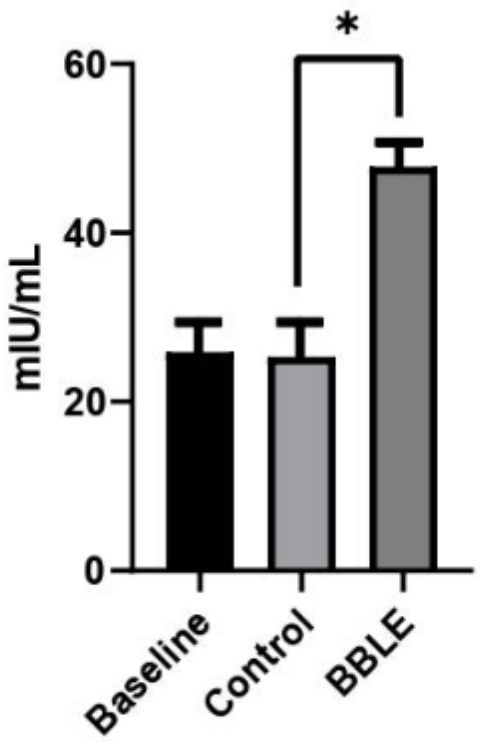


Figure 5. Luteinizing hormones (LH) levels of hypercholesterolemic Wistar rats (n = 8) (Mean ± SD). Sign * indicates that there is a significant difference ($p < 0.05$) between the two treatments. Baseline is the measurement result in the both goups before intervention.

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Enzyme and Luteinizing Hormones in Hypercholesterolemic Rats".

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to reviewers' suggestions, and provide us a revised version of your manuscript and a response letter to t

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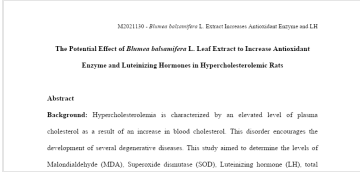

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However, this manuscript needs some minor revisions as mentioned in the manuscript before it can final
1. The consistencies between conclusion and title of the manuscript.
2. Detailed information in the presented tables and figures.
3. Some revisions in the Methods section.

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