RESEARCH ARTICLE

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The Ethanol Extract of Tangse Liberica Coffee (*Coffea liberica*) Inhibited the Reduction of Leydig Cell Number and Testosterone Levels in Male Wistar Rats (*Rattus norvegicus*) Exposed to Ultraviolet B Light

Dhea Frisilla, Wimpie Pangkahila, and Agus Eka Darwinata

ABSTRACT

***Background*: Aging is a complex mechanism related to various factors including free radicals. Free radicals that occur due to exposure to ultraviolet light can cause oxidative stress and damage the spermatogenesis process, including Leydig cells and testosterone. Therefore, antioxidants play a very important role in inhibiting these reactions, which are abundant in coffee. This study aimed to analyze the effect of the ethanol extract of Tangse liberica coffee (Coffea liberica) in inhibiting the decrease in Leydig cell number and testosterone levels in male Wistar (Rattus norvegicus) rats exposed to Ultraviolet B light.**

***Methods*: This study includes a post-test-only control group type of laboratory experimental study. The research group was divided into three groups, namely positive control (without giving coffee), treatment 1 (giving coffee extract 216 mg/kgBW), and treatment 2 (giving coffee extract 432 mg/kg BW). A total of 30 rats were used in this study and exposed to UV-B 0.225 MW/cm² 8 hours per day for 28 days. Testosterone assessment was carried out by examining blood specimens and Leydig cell counts were carried out histologically with Hematoxylin-Eosin staining. One-way ANOVA analysis and Kruskal Wallis test followed by post hoc test were performed in this study with a significance level of 95%.**

***Results*: The result showed a significant difference in the number of Leydig cells between the study groups ($p = 0.016$) with the highest mean in group 1, 48.27 9.12 cells/LFoV. The same thing also happened to the difference in testosterone levels between groups ($p < 0.001$) with the most dominant mean in treatment group 2 being 76.42 nmol/L.**

***Conclusion*: The conclusion of this research is the administration of the ethanol extract of Tangse liberica coffee (Coffea liberica) can inhibit the decrease in Leydig cells number and testosterone levels in male Wistar (Rattus norvegicus) rats exposed to Ultraviolet B light.**

Keywords: Ethanol extract, leydig cells, tangse liberica coffee, testosterone, ultraviolet B.

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D. Frisilla *
Udayana University, Indonesia.
(e-mail: dheafrisilla90@gmail.com)
W. Pangkahila
Udayana University, Indonesia.
A. E. Darwinata
Udayana University, Indonesia.

Corresponding Author

I. Introduction

Aging is a biological process characterized by the occurrence of structural damage, functional decline, depression, and typical phenotypic changes and increases the likelihood of death [1]. Several theories explain the process of aging occurs such as wear and tear theory, neuroendocrine, genetic control, and free radicals. These theories are believed to be factors that cause humans to grow old through an aging process that then becomes ill and eventually leads to death [2].

The free radical theory is a theory that is believed to be the main cause of aging. The theory explains that high free radicals can trigger the formation of Reactive Oxygen Species (ROS). One of the causes of high free radicals is exposure to ultraviolet (UV) rays. UV radiation especially UVB (280-320 nm) results in DNA damage both directly and indirectly [3], [4]. Directly, the double helix on DNA can absorb energy from UV photon shortwave and undergo covalent modification, indirectly the damage occurs through the production of oxidative free radicals [5].

If not strictly controlled, oxidative stress is responsible for the induction of several diseases ranging from sunburn, skin cancer, cataracts, and immune suppression can even involve various other organs such as cardiovascular disease, neurology, and reproductive or sexual maturity. In reproduction, oxidative stress can cause damage to spermatogenesis including in Leydig and testosterone cells [6].

Leydig cells are cells that play an important role in many vital physiological processes in men such as testosterone

production, controlling sexual development, and maintaining secondary sexual characteristics and behaviors. Leydig cells are rich in lipids and can be found in the interstitial tissue around the seminiferous tubules. The location of Leydig cells in testicular tissue allows Leydig cells to interact with testicular macrophages. Inflammatory mediators such as ROS and cytokines produced by activated macrophages are known to interfere with steroidogenesis through inhibition of StAR protein expression. The aging of Leydig cells is also closely related to ROS in particular the production of ROS associated with the P450 system in steroidogenic cells [7].

In previous studies, oxidative stress resulted in Leydig cell death in experimental animals mediated by activation of intrinsic pathway apoptosis, independent p53 pathway, and endoplasmic reticulum stress [8]. The decline in Leydig and testosterone cells that continue to run is directly proportional to the process of aging so the decline in Leydig and testosterone cells must be prevented to prevent aging, especially in the reproductive system [9].

Antioxidants can now be obtained from natural ingredients, one of which is coffee. Coffee contains chlorogenic, ferulic, caffeic, n-coumaric, melanoidin, caffeine, trigonelline, and phenylalanine acids that show high antioxidant activity [10]. Kopi liberica has higher antioxidant activity, this is following a study conducted by Saw [11] who looked at the antioxidant activity of three different types of coffee beans using the DPPH method showing that liberica coffee beans have the highest antioxidant activity, followed by arabica coffee and robusta.

Aceh Province has been known worldwide for Gayo arabica coffee, but there is another type of coffee, namely liberica which is now starting to be cultivated in the Tangse area, Pidie so that it is known as Tangse liberica coffee. Based on BPS data in 2019, as many as ± 3,831 ha of Tangse land was planted with coffee with a total production of ± 2,234 tons/year [12].

This study aims to analyze the effect of giving Tangse liberica coffee ethanol extract (*Coffea liberica*) in inhibiting the decrease in Leydig cell count and testosterone levels in male Wistar rats (Rattus norvegicus) exposed to ultraviolet B light.

II. METHODS

A randomized post-test-only controlled group design was used in this study. This study is being carried out from February 2022 to May 2022 at Udayana University's Medical Faculty's Integrated Biomedical Laboratory in Bali. The extraction process of Tangse liberica coffee is carried out at the Faculty of Agricultural Technology, Integrated Service Laboratory, Udayana University.

A total of 30 male Wistar rats were used as research subjects, then randomly divided into three groups, each group containing ten Wistar rats. All groups were exposed to UV-B ray intensity of 0.255 MW/cm². Treatment 1 and Treatment 2 group received Tangse liberica coffee extract 216 mg/kgBW and 432 mg/kgBW for 28 days. While the control group did not get Tangse liberica coffee extract.

The basis for determining the dose of intervention in this study is to refer to the average coffee consumption as 1 cup of coffee/day with an average amount of coffee of 12 g dissolved in hot water with a volume of 200 ml. Using the dose conversion formula of experimental animals, the dose of administration to rats was 12×0.018=216 mg/kgBW and based on the calculation of making a test preparation of Tangse liberica coffee ethanol extract was given a dose preparation of 12.96 g dissolved with a solvent of 300 cc aquabidest through gastric sonde for 10 rat experimental animals (Rattus norvegicus) for 28 days.

A. Extract of Tangse Liberica Coffee (Coffea liberica)

Dried Tangse liberica coffee beans are blended into small flakes and ground until smooth. Furthermore, coffee grounds were weighed as much as 300 gr using a scale balance sheet and macerated in a 97% ethanol solution of 1200 ml for 2×24 hours using a shaker bath. After macerating, the coffee is filtered using a vacuum pump. The solvent is evaporated using a rotary evaporator so that a concentrated extract is obtained in the form of a paste.

B. Leydig Cell Examination

Examination of Leydig cell levels in this study was carried out by histopathological method using hematoxylin Eosin (HE) staining. The number of Leydig cells is calculated by looking for areas with a relatively even distribution of Leydig cells and then using an objective lens 40 times, the image of the preparation was photographed with a magnification of 400 times. The calculation data on the numerical scale are the number of Leydig / LPB cells (per large field; magnification 400 times).

C. Testosterone Examination

Examination of testosterone levels of experimental animals in this study was carried out using an ELISA Kit under the brand *Bioassay Technology Laboratory* with Cat No. E0259Ra. The inspection procedure follows the instructions provided on the KIT.

D. Data Analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) software for Windows version 24. All data were tested for normality using Shapiro-Wilk. The significance test used One-Way Annova (parametric test) with post hoc Tukey test.

III. RESULTS

The number of Leydig cells was histologically examined in this study with testicular tissue preparations. The calculation was carried out by observing the number of Leydig cells in the right and left testicles of each of the 3 field views in a zig-zag direction, with a microscope magnification of 400 X then the results obtained were averaged.

It appears that the number of Leydig cells in the interstitial part of the testicle located between the seminiferous tubules, is round in shape and has a nucleus in the middle marked with a yellow arrow. Descriptive analysis showed that Leydig cell mean, and testosterone levels were greater and higher in the treatment group.

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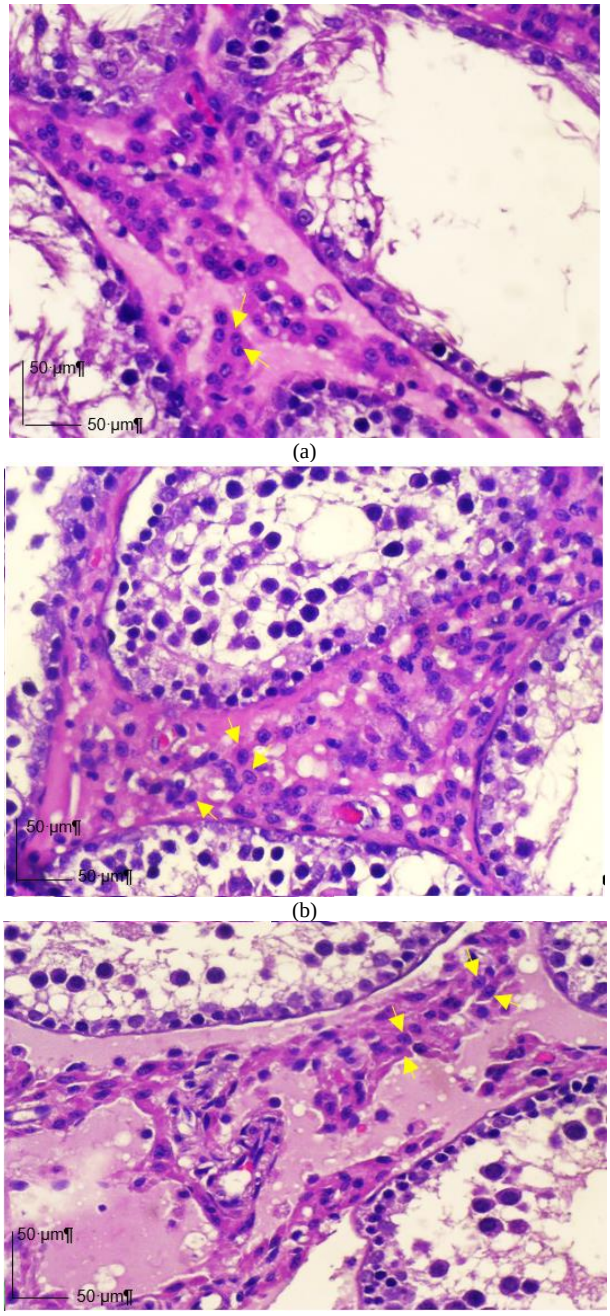

Fig. 1. Leydig cells in hematoxylin Eosin (HE) staining:
(a) Control group; (b) Treatment 1; (c) Treatment 2.

The Shapiro-Wilk was used for the normality test. Leydig cells in the entire study group showed a normal data distribution (p>0.05). Referring to the variable testosterone, testosterone levels in treatment group 2 showed values (p<0.05) indicating that the data were not normally distributed.

Statistically using the One-Way ANOVA test, it can be concluded that there are differences in the number of meaningful Leydig cells between groups (p<0.05). The average number of Leydig cells in the group with Tangse liberica coffee extract administration of 216 mg/kgBW for 28 days showed the highest average compared to doses of 432 mg / kgBW and control of 48.27 cells/LFoV (Table I).

Post hoc analysis showed that the comparison of control and treatment groups (1 and 2) showed a significant difference (p<0.05). However, the comparison of treatment group 1 (dose 216 mg/kgBW) and treatment 2 (dose 432 mg/kgBW) did not show a significant difference with a difference of 2.54 cells/LFoV. Comparison between the control group and treatment group 1 (dose 216 mg/kgBW) showed the largest average difference compared to other groups of 10.57 cells/LFoV (Fig. 2).

TABLE I: ANALYSIS OF THE EFFECT OF GIVING LIBERICA COFFEE ETHANOL EXTRACT (*COFFEA LIBERICA*) TANGSE IN INHIBITING THE DECREASE IN THE NUMBER OF LEYDIG CELLS

Group	n	Mean SD±	P-value
Control	10	37.7 7±	
Treatment 1	9	48.27 9.12±	0.016
Treatment 2	10	45.73 7.12±	

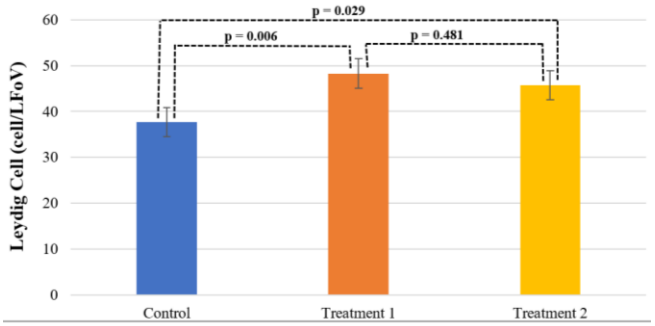

Fig. 2. Leydig cell counts between research groups.

Meanwhile, testosterone levels in the group with the administration of Tangse liberica coffee extract of 432 mg/kgBW for 28 days showed the highest average compared to a dose of 216 mg / kgBW and control of 76.42 nmol/L (Table II). Statistically, it can be concluded that there are significant differences in testosterone levels between groups (p<0.05).

TABLE II: ANALYSIS OF THE EFFECT OF GIVING LIBERICA COFFEE ETHANOL EXTRACT (*COFFEA LIBERICA*) TANGSE IN INHIBITING THE DECLINE OF TESTOSTERONE LEVELS

Group	n	Median	Min-Max	P-value
Control	10	31.7	30.47–32.44	<0.001
Treatment 1	9	51.71	51.31–52.39	
Treatment 2	10	76. 42	70.01–85.44	

Statistically, all group comparisons showed a significant difference (p<0.05). A comparison between the control group and treatment group 2 (dose 432 mg/kgBW) showed the largest average difference compared to other groups, which was 44.72 nmol/L (Fig. 3).

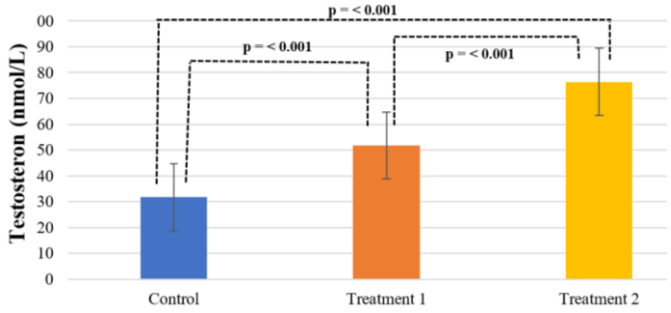

Fig. 3. Testosterone levels between groups.

IV. DISCUSSION

This study tried to prove whether the administration of Tangse liberica coffee ethanol extract could inhibit the decrease in the number of Leydig cells in male Wistar rats

(Rattus norvegicus) given exposure to UV-B rays. Based on the results of the study, it was found that the average number of Leydig cells of rats (Rattus norvegicus) Wistar males in the control group were 37.7 cells/large field of view (cells /LFoV) while the average number of Leydig cells in the treatment group with the administration of Tangse liberica coffee ethanol extract was 216 mg/kgBW for 28 days, which was 48.27 cells/LFoV and a dose of 432 mg/kgBW was 45.73 cells / LFoV. Statistically using the One-Way ANOVA test, it can be concluded that there are differences in the number of meaningful Leydig cells between groups ($p<0.05$). From these data, the administration of Tangse liberica coffee ethanol extract (*Coffea liberica*) can inhibit the decrease in the number of Leydig cells in male Wistar rats (Rattus norvegicus) exposed to UV-B rays. This is in line with research conducted by Ismalia [8] which shows that the administration of Balinese robusta coffee extract can prevent a decrease in Leydig cell count and testosterone levels in rats (Rattus norvegicus) due to oxidative stress induced by excessive physical training ($p=0.005$).

The administration of liberica coffee ethanol extract (*Coffea liberica*) Tangse can inhibit the decrease in the number of Leydig cells in male Wistar rats (Rattus norvegicus) exposed to UV-B rays allegedly due to the high content of antioxidants contained in Tangse liberica coffee (*Coffea liberica*). The administration of UV-B rays can trigger the formation of ROS, causing oxidative stress that can cause cell damage (lipid peroxidation and DNA fragmentation), apoptosis, and cell death.[4] If not strictly controlled, oxidative stress is responsible for the induction of several diseases ranging from sunburn, skin cancer, cataracts, and immune suppression can even involve various other organs such as cardiovascular disease, neurology, and reproductive or sexual maturity. In reproduction, oxidative stress can cause damage to spermatogenesis including in Leydig and testosterone cells [6].

Research conducted by Saw [11] showed that liberica coffee beans have the highest antioxidant activity compared to arabica and robusta coffee. Antioxidants found in liberica coffee (*Coffea liberica*) are bioactive compounds of polyphenols, flavonoids, and tannins. The main antioxidant content in coffee is dominated by polyphenols, while flavonoids and tannins are less in percentage. The main compound of polyphenols in coffee, which is as much as 90% of the total polyphenols, is chlorogenic acid followed by caffeic acid and ferulic acid. Chlorogenic acid (chlorogenic acid) is a powerful antioxidant. These compounds work by inhibiting lipid peroxidation, fighting hydroxyl radicals, and fighting superoxide anions. Ferulic acid in coffee has anti-inflammatory, antiallergic, antibacterial, antiplatelet, and antiviral effects. Pharmacologically as an antioxidant. This compound can inhibit lipid peroxidation of biological membranes and inhibit superoxide anions. Caffeic acid works in inhibiting superoxide anions. Flavonoids and tannins act as free radical catchers because they contain a hydroxyl group. Chlorogenic acid (chlorogenic acid) is the main contributor to antioxidants in coffee, in acidic states that have higher antioxidant activity compared to caffeine and other compounds because chlorogenic acid has many hydroxyl groups that affect antioxidant activity. The effective soaking activity for caffeine was obtained by 21.41 ppm and for chlorogenic acid a value of 5.86 ppm was obtained, thus chlorogenic acid has a higher antioxidant activity compared to caffeine. Chlorogenic acid is an ester of caffeine acid and quinic acid. The compound is stable, especially in an acidic state [11].

Flavonoids are also an antioxidant and are proclaimed to have stronger antioxidant properties than vitamim C and E. Flavonid can prevent oxidative stress through the mechanism of direct breaking of ROS, activation of antioxidant enzymes, reduction of α-tocopheryl radicals, inhibition of oxidase, mitigation of oxidative stress caused by nitric oxide, increase in uric acid levels, and increase in low molecular antioxidant properties [13].

In addition to acting as antioxidants, flavonoids generally have a structure consisting of two aromatic rings bound to three carbons and usually in an oxygenated heterocyclic form. Flavonoids will inhibit the aromatase enzyme, which is an enzyme that catalyzes the conversion of androgens into estrogen which will increase the hormone testosterone which also increases the number of Leydig cells [8].

Tangse liberica coffee is one of the high sources of flavonoids. Based on phytochemical tests conducted before this study in the laboratory of the Faculty of Agricultural Technology, Udayana University, Denpasar, Bali, it showed that Tangse liberica coffee extract obtained IC50 results of 48.0914 ppm, antioxidant caps 27215.48 mg/L GAEAC, flavonoid levels 22699.89 mg/100 g. Tannin levels 2912.20 mg/100 g. Total Phenols (polyphenols) as much as 2710.12 mg/100 g. It is also following the literature that, the flavonoid content of Aceh liberica coffee is higher compared to other regions. This shows that various factors cause antioxidant levels of the same type of coffee to be different. The antioxidant content can be affected by temperature, rainfall, and soil type. The average temperature of 18-35 °C is considered very good for plant growth and can increase antioxidant content while temperatures below 18 °C and above 35 °C produce poor growth in terms of leaves, fruits, and antioxidant content. Insanu in his research compared the antioxidant activity of liberica coffee originating from Riau, Jambi, and Aceh and mentioned that liberica coffee originating from Aceh has a higher antioxidant activity than coffee from two other regions with a total phenolic compound (22,585±1,610 g GAE/100 g) and total flavonoid content (4,927±0.355 g QE/100 g) [14].

The results showed that the administration of Tangse liberica coffee ethanol extract (*Coffea liberica*) could inhibit the decrease in testosterone levels in male Wistar rats (Rattus norvegicus) exposed to UV-B rays. The average testosterone content of male Wistar rats (Rattus norvegicus) in the control group was 31.7 nmol/L while the average testosterone content in the treatment group with the administration of Tangse liberica coffee ethanol extract was 216 mg/kgBW for 28 days, which was 51.7 nmol/L and a dose of 432 mg/kgBW was 76.42 nmol/L. Testosterone levels in the treatment group were quite high, for comparison of normal testosterone levels in normal adult mice based on literature ranging from 2-48 nmol/L [15]. Statistically using the Kruskal-Wallis test, it can be concluded that there are differences in the number of testosterone levels between groups ($p<0.05$). This is in line with research conducted by Ismalia [8] which shows that the administration of Balinese robusta coffee extract can prevent

a decrease in Leydig cell count and testosterone levels in rats (Rattus norvegicus) due to oxidative stress induced by excessive physical training p value = 0.005 (< 0.05).

Like the number of Leydig cells, the increase in testosterone levels occurs due to the antioxidant content in Tangse liberica coffee which can inhibit oxidative stress that occurs due to exposure to UVB rays. Because Leydig cells produce testosterone through the process of steroidogenesis, an increase in the number of Leydig cells also increases testosterone levels. The mechanism of action of flavonoids can increase testosterone levels through two mechanisms, namely the inhibition of the enzyme 5-alpha reductase which can convert testosterone into dihydrotestosterone, both flavonoids can also inhibit the work of the aromatase enzyme which catalyzes the conversion of androgens into estrogen, increasing the hormone testosterone.

In a study by Khalaji *et al*. [16], male albino mice exposed to compact fluorescent lamps with a power of 40 W, 8 hours per day for 45 days with intensities of UVA (1.06 W/m^2) and UVB (0.02 W/m^2) showed a significant decrease in FSH, prolactin, testicular weight, sperm motility, tubular differentiation index, and spermiation index [16]. In addition to affecting Leydig and testosterone cells, coffee administration can increase the motility of Wistar rat spermatozoa exposed to UV rays, so coffee is very beneficial for the reproductive system (p = 0.02) [17].

UV radiation especially UVB (280-320 nm) results in DNA damage both directly and indirectly. Directly, the double helix on DNA can absorb energy from UV photon shortwave and undergo covalent modification, indirectly the damage occurs through the production of oxidative free radicals [5]. If not strictly controlled, oxidative stress is responsible for the induction of several diseases ranging from sunburn, skin cancer, cataracts, immune suppression can even involve various other organs such as cardiovascular disease, neurology, and reproductive or sexual maturity. In reproduction, oxidative stress can cause damage to spermatogenesis including in Leydig and testosterone cells [6].

Due to its important role in vital physiological processes in men such as testosterone production, a decrease in the number of Leydig cells due to apoptosis and degenerative can also have an impact on the reduction of testosterone levels directly so that it can induce aging and infertility [7]. Based on previous studies, exposure to UV-B rays in experimental mice may also decrease the motility of spermatozoa [17]. The decline in Leydig cells and testosterone that continues to run is directly proportional to the process of aging so the decline in Leydig cells and testosterone must be inhibited to prevent aging, especially in the reproductive system. This prevention is considered very beneficial, especially for community groups who are vulnerable to exposure to UV-B rays and outdoor activities [9].

Pathogenesis of decreasing Leydig cell count and testosterone levels are mediated by free radicals, so the administration of antioxidants is one of the right steps to inhibit oxidative stress due to the presence of these free radicals. At the time of oxidative stress due to ROS, antioxidants can stop this chain reaction and avoid or repair biochemical injuries caused by oxidized compounds through 5 mechanisms, namely direct cooling of ROS, recovery of stable molecules by donating H^+ atoms to the formed free radical compounds, resulting in more stable radical compounds, inhibition of oxidative enzymes such as xanthine oxidase, protein kinase C, inhibits Fe^{+2} and Cu^+ involved in the conversion of O_2 and H_2O_2 into HO^- radicals as well as repairs damage that has occurred to important cell components, such as membrane lipids, proteins, and deoxyribonucleic acid (DNA) [18].

Liberica coffee contains chlorogenic acid, caffeine, flavonoids, and tannins which show high antioxidant activity. Previous studies have also shown a fairly good antioxidant activity of iberica coffee, where the administration of Lliberica coffee histologically can reduce the degenerative liver cells and reduce the glucose level of mice (Mus musculus webster). The administration of Tangse liberica coffee ethanol extract in this study showed inhibition from the decrease in Leydig cell count and Testosterone levels, thus supporting the concept of anti-aging medicine [10].

V. Conclusion

The administration of liberica coffee ethanol extract (*Coffea liberica*) Tangse can inhibit the decrease in the number of Leydig cells in male Wistar rats (Rattus norvegicus) exposed to Ultraviolet B light with the most effective dose of 216 mg/kgBW. The administration of liberica coffee ethanol extract (*Coffea liberica*) Tangse can inhibit the decrease in testosterone levels in male Wistar rats (Rattus norvegicus) exposed to Ultraviolet B light with the most effective dose of 432 mg/kgBW. It is necessary to conduct further studies that assess the effect of Tangse liberica extract coffee (*Coffea liberica*) administration on other biological markers of the reproductive system such as FSH levels, prolactin, and testicular weight, and sperm motility. It is also necessary to conduct toxicity tests to see any biochemical, physiological, and pathological reactions useful for obtaining hazard information after exposure. And it is necessary to test antioxidant indicators to assess the effect of Tangse liberica extract coffee (*Coffea liberica*) on the research sample.

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