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Original Article

The Effect of *Eucalyptus globulus* Hydroalcoholic Extract on LH, FSH and Testosterone Concentrations and Sperm Morphology

Widjaja, G[1 *], Ibrahim, N. J[2], Hadi, S. J[2], Cababat, F[3], Turki Jalil, A[4], Al-Charak, A. G. H[2], Yasin, G[5], Sultan, M. Q[6], Fakri Mustafa, Y[7]

1. Faculty of Public Health, Indonesia University, Depok, Indonesia
2. Veterinary Medicine Collage, Al-Qasim Green University, Al-Qasim, Iraq
3. College of Arts and Sciences, Cebu Technological University, Moalboal, Cebu, Philippines
4. Faculty of Biology and Ecology, YankaKupala State University of Grodno, 230023 Grodno, Belarus
5. Department of Botany, Bahauddin Zakariya University, Multan, Pakistan
6. College of Dentistry, Al-Ayen University, Thi-Qar, Iraq
7. Department of Pharmaceutical Chemistry, College of Pharmacy, University of Mosul, Mosul-41001, Iraq

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Corresponding Author: nadya1122@gmail.com

Abstract

Scientists have been paying attention to the life-giving properties of medicinal plants for many years. Among these plants is the eucalyptus plant. This plant has various compounds such as cineole and terpenes. It also contains compounds such as flavonoids, aliphatic aldehydes, sesquiterpene, quinotanen, catechins, salts, and vitamins. In the present study, the hydroalcoholic extract of Eucalyptus leaves with concentrations of 175, 350, and 700 mg/kg body weight, and spermatogenesis were studied in 40 adult Wistar rats in five groups of eight. Adult male mice received the extract at the above concentrations by gavage for 28 days. Control mice received only solvent and water, while control mice received no substance other than municipal tap water and normal food. After the last administration of the drug, the animals were weighed and anesthetized, and then blood samples were taken from their hearts. Concentrations of LH, FSH, and testosterone were measured by an ELISA kit. The results showed that body weight and testis, seminiferous tube diameter, Leydig cell diameter, epithelium thickness, number of Leydig cells, spermatogonium, spermatocytes, spermatids, sperm, and testosterone concentration increased significantly with the group. But no significant difference was observed in the concentration of FSH and LH hormones or the number of Sertoli cells. Therefore, it can be concluded that eucalyptus leaf extract may increase the proliferation of sex cells in the seminiferous tubules of rats.

Keywords: Hydroalcoholic extract, Eucalyptus leaves, Reproductive hormone, Sperm morphology

1. Introduction

Eucalyptus is an evergreen tree that is native to Australia (1). This tree grows very fast, and its size varies according to its different species. Among eucalyptus species, globulus is the main source of eucalyptus oil worldwide (2-4). Flavonoids and tannins (5) are plant antioxidants found in eucalyptus leaves, and tannins help reduce inflammation. The properties of eucalyptus are many, such as anti-igflatulence (6), fever (7), laxative (8), and hypoglycemia (9), treatment of colds (10), respiratory infections (11), and antibiotic properties (12).

Eucalyptus leaves are a rich source of antioxidants, especially flavonoids, which protect the body against oxidative stress and free radical damage (13). Diets rich in these flavonoids can fight cancer, heart disease, and

dementia. Research has shown that eucalyptus can reduce mucus and help the lungs. This plant has anti-inflammatory properties, and one of its main components is eucalyptol (cineole), which is found in eucalyptus oil or extract (14).

Eucalyptus can improve dry skin and increase ceramide levels (15). Ceramides are a type of fatty acid in the skin that helps maintain skin moisture (16). People with dry or scaly skin or skin conditions such as dermatitis and psoriasis usually have lower ceramide levels in their skin than people with healthy skin. Topical eucalyptus can increase ceramide production and enhance moisture retention and skin protection capacity. The extract of this leaf contains a compound called macrocarpal-A, which stimulates the production of ceramide (17). Inhalation of eucalyptus oil can reduce symptoms. Eucalyptus contains anti-inflammatory compounds such as cineole and limonene that act as analgesics (18). This plant can reduce the activity of the sympathetic nervous system—the stress response system—and increase the activity of the parasympathetic nervous system, causing relaxation.

Eucalyptus leaf extract contains eucalyptol (19), which can improve dental health (20). Eucalyptus leaves also contain large amounts of macrocarpal-C, a type of polyphenol (21). This compound reduces the bacteria that cause caries or the bacteria that cause gum disease. Eucalyptol in eucalyptus extract is used as a natural insecticide that can repel insects.

Eucalyptus (Eucalyptus globules Labill) has relatively strong alien effects due to its plant secondary compounds. Ebrahimi Kia (22) reported that eucalyptus leaf essential oil had inhibitory effects on germination and seed growth of leeks, sorghum, barley, tomatoes, and corn; increased oxygen uptake by carrot parenchyma and reduced reduction of indole 2- and 6-dichlorophenol. (DCPIP) in spinach chloroplasts (22).

Consumption of eucalyptus extract significantly increased the activity of ACP and ALP SOD enzymes in the liver and increased MDA activity in both the liver and serum (23). The hydroalcoholic extract of Eucalyptus leaf has inhibitory activity against tyrosinase and hyaluronidase enzymes and therefore can be useful in the treatment of allergies and inflammation (24). This extract has anti-hyperglycemic effects that do not directly affect blood glucose concentration in normal mice. Therefore, it modulates insulin secretion or insulin function (25, 26). This study aimed to investigate the effect of eucalyptus extract on the physiology of the male reproductive system in rats.

2. Materials and Methods

2.1. Experimental Animals and Treatments

In this experiment, 40 adult male Wistar rats with an average body weight of 180.40±10.26 g were used. The rats were kept in polycarbonate cages measuring 42×15×42 cm^3 with a steel mesh roof. The floors of the cages were covered with sawdust and wood chips. Eight cages were selected, and five rats were placed in each cage. The cages were washed with water and disinfectant every two days, and the floor was cleaned at the same time. The ambient temperature during the experimental period was approximately 22±2°C. Irradiation of light from the windows of the laboratory was done indirectly and uniformly by the ventilation device in it. The light frequency for the animals was adjusted to 12 hours of light and 12 hours of darkness. Animal drinking water (urban tap water) and feeding pellets were provided to rats throughout the study period without restriction.

The animals were weighed with a digital scale with an accuracy of 0.001 g and were randomly divided into 5 separate groups based on the weight range as follows.

- The control group consisted of eight adult male rats who consumed only intensive food and water daily for 28 days, and the animals did not receive any medication.
- The control group consisted of eight adult male rats that were gavaged with solvent (distilled water and alcohol) daily for 28 days.
- Experimental group 1 consisted of eight adult male rats who received 175 mg/kg body weight of hydroalcoholic extract of Eucalyptus leaf by gavage daily.

● Experimental group 2 consisted of eight adult male rats who received 350 mg/kg body weight of hydroalcoholic extract of Eucalyptus leaf by gavage daily.

● Experimental group 3 consisted of eight adult male rats who received 700 mg/kg body weight of hydroalcoholic extract of Eucalyptus leaf by gavage daily.

2.2. Preparation of Hydroalcoholic Extract of Eucalyptus Leaves

The leaves of the eucalyptus plant were purchased from the local market, and after cleaning and washing, they were powdered with an electric mill. Eucalyptus leaf powder was mixed with 500 mL of distilled water and 96 ° C ethyl alcohol (50:50) and kept in the dark for 48 hours. During this time, Erlenmeyer mixes the powder, water, and alcohol for 20 minutes each day until the extract is completely dissolved in the alcohol. The contents of the Erlenmeyer flask were then smoothed with filter paper. The filtered liquid was concentrated using an evaporator at 50 °C under a vacuum. The concentrated extract from the extractor was placed in sterile Petri dishes. Petri dishes were placed at 40 ° C to evaporate as much water and alcohol as possible. In the last step, the obtained dry matter was used to prepare different concentrations of eucalyptus leaf extract (175, 350, and 700 mg/kg) in sterile distilled water.

The method of administration of the extract was gavage. All ethical principles with laboratory animals were observed in this study. In this method, the mouse is first restrained, and then slowly, with one hand, the skin of the mouse's neck is held in place so that the mouse's head and neck are completely restrained, leaving the rat's mouth relatively open, while at the same time injecting the mouth without injury to the lower limbs (lungs, *etc*.) was done slowly by inserting a gavage into the beginning of the oesophagus and finally the stomach of the rat.

The weight of each mouse was recorded twice a week before the experiment and during the experiment. The weighing method was to set the digital scale to zero (digital scale accuracy: 0.001 g) then the mice were weighed one by one.

2.3. Anaesthetize Rats

It anesthetized the animals 24 hours after the last administration of the drug using ether and inhalation. For this purpose, a cotton ball is soaked in a suitable amount of ether and placed in an animal anesthesia container. Animals were placed in it one by one for about 1-2 minutes to observe mild anesthesia symptoms in the animals. The animal was then taken out of the enclosure and placed on the dissection table. At this time, the heart rate and respiration of rats per minute decreased significantly. Ether was used for mild anesthesia and had less effect on blood flow velocity, which is important for proper blood collection.

2.4. Blood Sampling

After placing the animal on the dissection table, first cut the sternum and ribs with pliers and a scalpel, then cut the sternum and ribs, and cut the diaphragm with pliers by pulling aside the sternum and ribs to expose the heart. Then a 5 mL syringe was inserted into the ventricle, and blood sampling was performed carefully.

2.5. Preparation of Serum

The collected blood was poured into the test tube without anticoagulant, and after coagulation, the tubes were centrifuged for 15 minutes at 3000 rpm. After removing the tube from the centrifuge, the blood serum was carefully separated by a pipette and transferred to other test tubes. Then their lids were sealed with parafilm, and after labeling, they were kept in the freezer at -20 °C until the measurement of FSH, LH, and testosterone.

2.6. Measure the Concentrations of LH, FSH, and Testosterone

Concentrations of FSH, LH, and testosterone in the blood serum of animals were measured using standard kits by the ELISA method in the university laboratory.

2.7. Preparation of Testicular Tissue Samples

After collecting blood from the animal's heart, the left and right testicles were removed from the scrotum, as

well as the epididymis in the lower part of the scrotum. Other connective tissues were isolated with a surgical razor, and then the testes were placed in physiological serum. All additional tissues were isolated. They were then dried with sterile gauze, and the left and right testes were weighed on a digital scale with an accuracy of 0.0001 g. After weighing, the left and right testicles were placed separately in containers containing a 10% formalin fixator to prepare for tissue preparation and cross-section.

2.8. Counting Testicular Tissue Cells

After preparing tissue samples and slides, spermatogonia, spermatocytes, spermatids, spermatozoa, Sertoli, and Leydig cells within 6 tubules in three primary, middle, and terminal regions were randomly prepared from three sections. They were counted from each testicular tissue with ×40 magnification, and after calculating the means, they were statistically analyzed by the software.

2.9. Statistical Analysis

The results were compared based on SPSS (Ver. 19) statistical software using the T-test, ANOVA, and Duncan tests at a 5% level. Then, based on the obtained analysis, the relevant diagrams were drawn in Excel.

3. Results

In this study, the effects of a hydroalcoholic extract of Eucalyptus leaves were compared after 28 days in experimental group 1 with a minimum dose of 175 mg/kg, experimental group 2 with an average dose of 350 mg/kg, and experimental group 3 with a maximum dose of 700 mg/kg. The control and control groups examined 40 adult male rats, and factors such as changes in body weight during the experiment, changes in the weight of the male reproductive organs (testes), the concentrations of FSH, LH, and testosterone, and changes in sperm count, spermatocytes, spermatids, spermatozoid, Sertoli, and Leydig were examined.

Body weight changes in all groups are shown in figure 1. The mean body weight was not affected by different treatments (P>0.05). Changes in testicular weight in different groups are shown in figure 2, respectively. In this experiment, the mean testicular weight did not show a statistically significant difference (P>0.05). Different levels of eucalyptus extract had no effect on the mice's testicular weight to body weight ratio, which was in the range of 0.58 to 0.61.

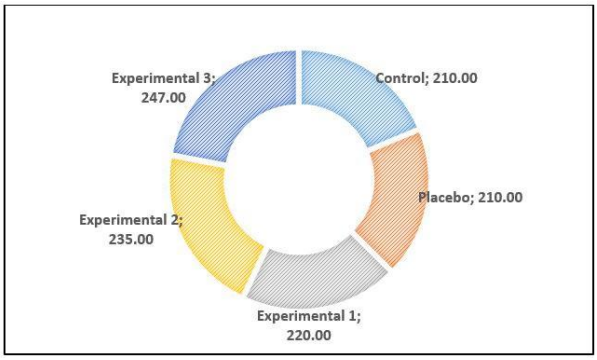

Figure 1. The effect of different experimental treatments on body weight (g)

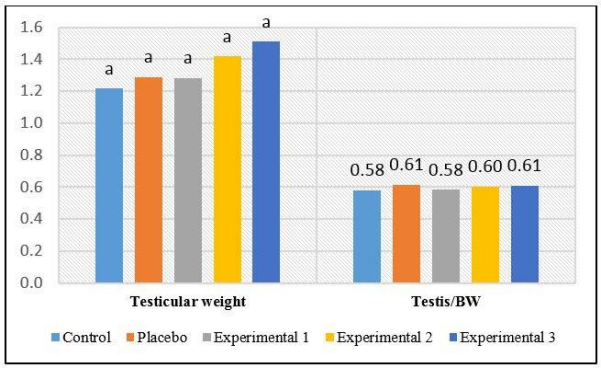

Figure 2. The effect of different experimental treatments on testicular weight (g) and testicular to body weight ratio
BW: Body weight

Changes in the number of spermatogonial cells in all groups after the experiment are shown in figure 3. The mean number of spermatogonia in different groups receiving the extract (experimental with the minimum, medium, and maximum doses) increased compared to the control and control groups. The experimental group 3 had the most Spermatogonia cells (P>0.05), while the control and placebo groups had the fewest.The ratio of the number of spermatogonia cells produced to testicular weight was 47.78 to 59.22, and the lowest ratio belonged to the control groups (50.21) and placebo (47.87). Changes in spermatocyte count and spermatocyte ratio to testicular weight in all groups

after the test are shown in figure 3. The mean number of spermatocytes and their ratio to testicular weight in different groups receiving the extract (experimental with the minimum, moderate, and maximum doses) increased compared to the control and control groups (87.15 to 98.44) (P<0.05). The results of the spermatid cell count in all groups after the experiment are presented in figure 3. The mean number of spermatid cells in the experimental groups was affected by the experiments, and the treatments with moderate and maximum doses increased compared to the control and control groups (P<0.05). The results showed that the experimental groups had a spermatid count to testicular weight ratio of 2, 90.02, while the placebo group had a ratio of 83.71.The consumption of eucalyptus extract exhibited a significant effect on the number of sperm cells and their ratio to testicular weight in different groups compared to the control group. This ratio was estimated to be more than 75% in all three experimental groups (Figure 3).

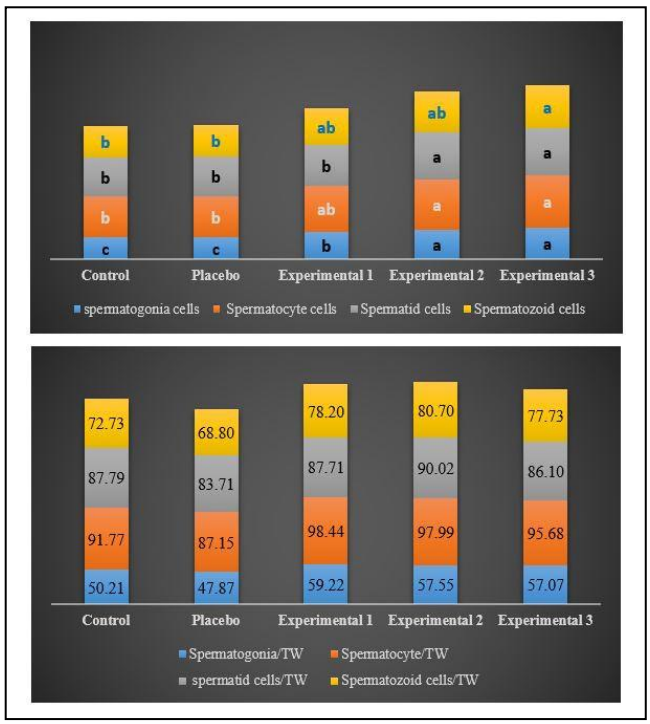

Figure 3. The effect of different experimental treatments on reproductive cells and reproductive cells to testis weight ratio
Columns of the same color and with different letters showed a significant difference at the level of 5%

Figure 4 indicated that the number of Sertoli and Leydig cells and their ratio to testicular weight were not affected by the experimental treatments (P>0.05). Figures 5 and 6 showed that the mean diameter of Leydig cells, seminiferous tubules, and epithelial thickness of seminiferous tubules increased significantly (P<0.05) but lumen diameter was not affected by different levels of experimental treatments (P>0.05).

Plasma concentrations of testosterone in different groups are shown in Figure 7. The results obtained in the experimental groups with moderate and maximum doses compared to the control groups show a significant increase. The results of the statistical analysis revealed that the concentrations of LH and FSH hormones in different groups (experimental with the minimum, moderate, and maximum doses) did not demonstrate a significant difference compared to the control and control groups. However, the experimental group's concentration of all three hormones was 3.86 compared to 2.58 in the placebo group.

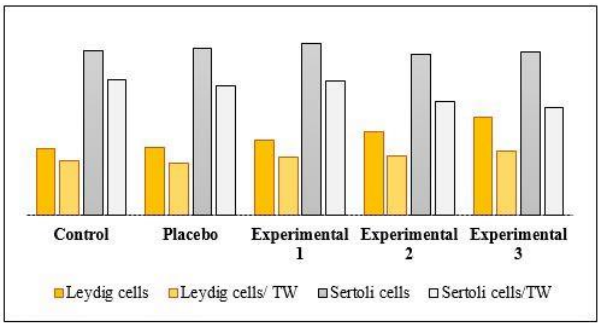

Figure 4. The effect of different experimental treatments on the ratio of Leydig and Sertoli cells and their ratio to testicular weight

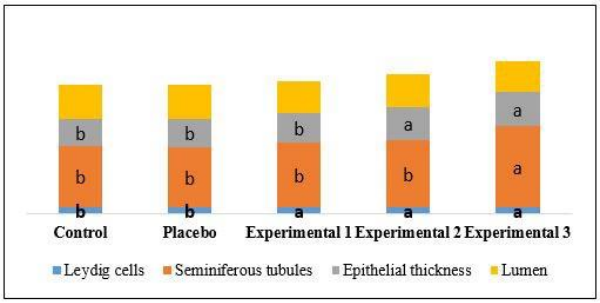

Figure 5. The effect of different experimental treatments on the diameter and thickness of Leydig, epithelial, lumen and seminiferous tubes
Columns of the same color and with different letters showed a significant difference at the level of 5%

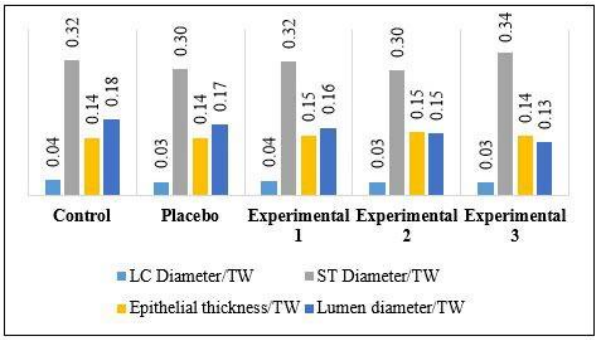

Figure 6. The effect of different experimental treatments on the ratio of diameter and thickness of Leydig, epithelial, lumen and spermatozoa tubes to testicular weight

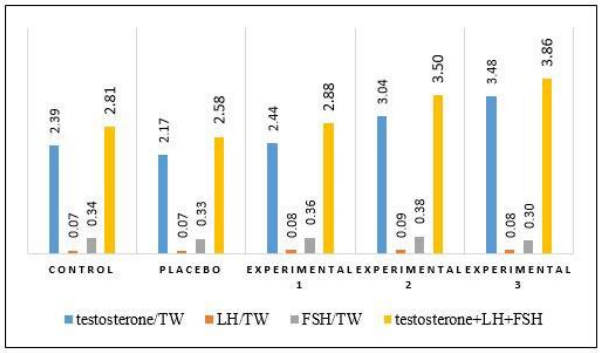

Figure 7. The effect of different experimental treatments on the ratio of reproductive hormones to testicular weight

4. Discussion

It has long been known that men's beliefs are proportional to their sperm concentration. The critical number of spermatozoids necessary for pregnancy is estimated at 20 million spermatozoids per cubic millimeter of semen, and men whose sperm count is less than 20 million sperm per milliliter are less likely to believe it (27).

Causes of male infertility include androgen abnormalities associated with normal sperm cell production due to hypothalamic-pituitary defects, interstitial cell defects, or androgen resistance; abnormal sperm cell production with normal levels of androgens due to infection, trauma, congenital duct deformity, immobile sperm production, or abnormal sperm cell production; and combined defects in the production of androgens and sperm cells due to developmental defects such as abnormal testicular descent with acquired testicular defects such as infections or general diseases such as chronic liver and kidney disease (28-30).

Although there was no significant difference in the testicular weights of the rats in this experiment, testicular weight can be increased by increasing testosterone secretion, germ cell growth and division, and the susceptibility of terpenes and flavonoids in the extract (31, 32). The hydroalcoholic extract of eucalyptus leaves has antioxidant properties and eliminates free radicals and active metabolites in the body (33, 34). Vitamins A, B, C, and E prevent DNA breakage and damage. These compounds cause mitotic and meiotic divisions within the testes to continue, leading to increased testicular weight (17, 35-37).

Administration of the extract in different experimental groups compared to control and placebo groups, the mean concentration of LH, but the increase in testosterone, which affects the LH-producing cells in the anterior pituitary gland by creating a negative self-regulatory mechanism, caused a decrease. Serum levels of LH decreased, which in turn reduced GnRH levels. A decrease in this hormone also ultimately affected LH levels (38).

Sesquiterpenes and other flavonoids inhibit the production of arachidonic acid by inhibiting the lipoxygenase and cyclooxygenase pathways, which in turn inhibit the production of prostaglandins. Negative self-regulation of gonadotropins inhibits the secretion of testosterone. Therefore, by increasing the dose of the extract in the experimental groups, an increase in testosterone is observed (39, 40). In addition, testosterone is secreted by interstitial cells, so an increase in the number of interstitial cells can be a factor in increasing the secretion of this hormone. In the experimental groups, increasing the dose of the extract, the secretion of testosterone. In this study, the number and diameter of Leydig cells also increased (41).

Research has shown that in seminiferous tubules, the secretory function is controlled by the number, size, and differentiation of Leydig cells. It has been

suggested that gametes determine the sensitivity of peritoneal cells to androgenic hormones in stages of the spermatogenesis process. According to research, injections of androgenic hormones alter testicular blood circulation, including changes in blood flow, lymphatic flow, and blood vessel permeability. The response to androgens depends on the presence of Leydig cells, which tightly control testicular blood flow (42, 43).

Research has shown that there is a very close relationship between the function of Leydig cells and sex cells in the fallopian tubes, and the differentiation and secretion of Leydig cells are controlled by the fallopian tubes (44, 45). On the other hand, the proliferation of germ cells in spermatozoa is controlled by Leydig cells. Therefore, it must be acknowledged that a significant increase in the number of germ cells in the seminiferous tubules increases the number and secretory function of Leydig cells and increases the progression of spermatogenesis (46).

There is a direct relationship between the secretion of LH and FSH hormones and the proliferation of spermatogonia. Thus, an increase in LH and FSH causes an increase in spermatogonia. In this study, LH and FSH hormones did not show significant changes. Therefore, it can be stated that the increase in cells is due to the increased secretion of testosterone, which is the most important sex hormone (47).

Increasing the number and diameter of interstitial cells can be a factor in increasing the secretion of testosterone. In the experimental groups, with increasing doses, the concentration of testosterone increased. Increased testosterone stimulated spermatogenesis and spermatocyte proliferation (48).

Studies have shown that the enzyme glutathione peroxidase, as an antioxidant, plays a special role in protecting sperm in testicular and epididymal tissues. Deficiency of this enzyme in the body causes infertility, which is located in the plasma membrane of sperm, sperm nucleus, and epididymal fluid protects sperm from free radical bites and causes the final maturation and development of sperm (49, 50). Studies show that consumption of hydroalcoholic extract of Eucalyptus significantly increased the amount of glutathione peroxidase (51).

Authors' Contribution

Study concept and design: G. W. and N. J. I.
Acquisition of data: M. Q. S. and G. Y.
Analysis and interpretation of data: S. J. H.
Drafting of the manuscript: Y. F. M.
Critical revision of the manuscript for important intellectual content: F. C. and A. T. J.
Statistical analysis: A. G. H. A.
Administrative, technical, and material support: G. W., N. J. I. and S. J. H.

Ethics

The study was approved by the Research Ethics Committee of the Indonesia University, Depok, Indonesia.

Conflict of Interest

The authors declare that they have no conflict of interest.

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