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ANTIOXIDANT EFFECT OF MORINGA (*Moringa oleifera*) LEAF ETHANOL EXTRACT ON SPERMATOGENESIS IN OLD WISTAR RATS (*Rattus norvegicus*)

Efek Antioksidan Ekstrak Etanol Daun Kelor (*Moringa oleifera*) terhadap Spermatogenesis pada Tikus (*Rattus norvegicus*) Galur Wistar Tua

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ABSTRACT
*Infertility is the failure of pregnancy after regular sexual intercourse for 6-12 months without contraception. Infertility in man is primarily caused by damaged sperm production, for example, impairments in the spermatogenesis process, low spermatozoa concentrations, morphological factors, and abnormal sperm motility. The purpose of this study is to ascertain the antioxidant effect of moringa (*Moringa oleifera*) leaves ethanol extract on spermatogenesis (spermatogonia, spermatocytes, and spermatids counts) in old Wistar rats (*Rattus norvegicus*). This study took healthy old Wistar rats aged 18-19 months with a body weight of 200-250 g and with no physical disabilities. A total of 36 was Wistar rats divided into two groups, namely the treatment group (supplied with Moringa leaf ethanol extract of 50 mg/kgBW/ and 0.5% Carboxymethyl cellulose (CMC) of 0.5 mL per day) and the control group (only with 0.5% CMC of 0.5 mL per day) for 30 days. The results showed that there was a significant difference in the spermatogonia, spermatocyte, and spermatid counts between the group treated with Moringa leaf ethanol extract and the control group, resulting in a p-value of 0.000. Therefore, it can be concluded that Moringa leaf ethanol extract had a significant influence on the spermatogonia, spermatocyte, and spermatid counts in old Wistar rats*.

Keywords: Moringa leaf, ethanol extract, old, spermatogenesis, white rats

ABSTRAK
Infertilitas adalah kegagalan kehamilan setelah melakukan hubungan seksual secara teratur selama 6-12 bulan tanpa alat kontrasepsi. Penyebab infertilitas pada pria disebabkan karena produksi sperma yang rusak misalnya, gangguan pada proses spermatogenesis, konsentrasi spermatozoa rendah, morfologi serta motilitas sperma yang abnormal. Penelitian ini bertujuan untuk mengetahui efek antioksidan ekstrak etanol daun kelor (*Moringa oleifera*) terhadap spermatogenesis (Spermatogonia, Spermatosit, dan Spermatid) pada tikus (*Rattus norvegicus*) galur Wistar tua. Tikus yang digunakan adalah tikus tua yang berusia 18-19 bulan dengan berat badan 200-250 g, dengan kondisi sehat dan tidak cacat fisik sejumlah 36 dibagi menjadi 2 kelompok, yaitu kelompok perlakuan (ekstrak etanol daun kelor 50 mg/kgBW/0,5 mL Carboxymethyl cellulose (CMC) 0.5% per hari) dan kelompok kontrol (CMC 0.5% 0.5 mL per hari) selama 30 hari. Hasil penelitian menunjukkan terdapat perbedaan jumlah spermatogonia, spermatosit, dan spermatid yang signifikan antara kelompok yang diberikan ekstrak etanol daun kelor dengan kelompok kontrol yang tidak diberikan dengan nilai p 0,000, sehingga dapat simpulkan ekstrak etanol daun kelor dapat memberikan pengaruh signifikan terhadap jumlah spermatogonia, spermatosit, dan spermatid pada tikus galur Wistar tua.

Kata Kunci: *Daun kelor, ekstrak etanol, spermatogenesis, tikus putih, tua*

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INTRODUCTION

Fertility disorders or infertility are a scourge for married couples who want children since it can cause disharmony in the household. This condition often leads to polygamy, as well as medical, psychological, social, and economic problems, to the point that it can result in divorce. Infertility is the failure of pregnancy after regular sexual intercourse for 6-12 months without contraceptives (El Adlani et al. 2021; Zhu et al. 2022). About half of the causes of infertility in men include damaged sperm production, for example, impairments in the process of spermatogenesis, low spermatozoa concentrations, morphology as well as abnormal sperm motility. According to (Dimitriadis et al. 2017), the causes of male infertility are divided into three categories: Pre-testicular, Testicular, and Post-testicular factors. Pre-testicular factors include conditions outside the Testicle and affect the spermatogenesis process.

Spermatogenesis is the formation process of Spermatozoa (male gamete cells) in seminiferous tubules (Baptissart et al. 2013). Spermatogonium in mice requires four cycles to form Spermatozoa. The required time is relatively consistent, ranging from 48-52 days. The duration of the spermatogenic cycle (Spermatogonia, Spermatocytes, and Spermatids) in rats is 12 days. Spermatogonium in mice requires four cycles to produce Spermatozoa in the lumen of the tubules. Spermatozoa take about eight days to cross the cauda epididymis from the lumen of the seminiferous tubules to the caput epididymis. Temperature can affect the duration of *spermatogenesis* (Lara et al. 2016). Spermatogenesis in humans begins at the age of 14, then decreases along with the aging process.

The results of a study conducted by Luceri et al. (2018) indicated that an increase in systemic ROS during aging occurred in 15-month-old mice, resulting in systemic oxidative stress as indicated by an increase in the amount of protein carbonyl in the plasma of 15-month-old animals.

According to Henkel et al. (2019), oxidative stress is closely related to various pathologies, such as aging and male infertility. Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and antioxidant production (Luceri et al. 2018). Physiological levels of ROS are necessary to regulate sperm capacity processes, acrosome reactions, hyperactivation, and sperm-oocyte fusion (Lee et al. 2017, Fatima 2018). Supraphysiological levels of ROS can affect spermatogenesis, decrease the motility of spermatozoa, and damage mitochondria and DNA integrity (Lucio et al. 2013, Morielli and O'Flaherty 2015, Fatima 2018).

Antioxidants or reducing agents prevent oxidation or neutralize compounds that have been oxidized by donating hydrogen and or electrons (Henkel et al. 2019, Martin-Hidalgo et al. 2019). The body has several antioxidant mechanisms that protect itself from cell damage caused by free radicals. Glutathione peroxidase, catalase, and superoxide dismutase (SOD) are antioxidant enzymes that can counteract free radicals. To work perfectly, they require cofactor micronutrients such as selenium, iron, copper, zinc, and manganese. All these micronutrients are contained in Moringa leaves (Krisnadi 2015, Ighodaro and Akinloye 2018)

Moringa contains 46 powerful antioxidants, including Vitamins A, C, E, K, B (choline), B1 (thiamin), B2 (riboflavin), B3 (niacin), B6, alanine, beta-carotene, alpha-carotene, arginine, beta-cystoterol, caffeoylquinic acid, campesterol, carotenoids, chlorophyll, chromium, delta-5-avenasterol, Delta (Krisnadi 2015). These compounds can protect the body from the adverse effects of free radicals.

The results of the analysis of *Moringa* leaves in the South Denpasar area of Bali indicated that it has the antioxidant capacity, including phenolics, flavonoids, tannins, ascorbic acid, alkaloids, and saponins (Widiastini et al. 2021). Based on this background, this study aims to determine the effect of the ethanol extract of *M. oleifera* leaves against the spermatogenic cells (spermatogonia, spermatocytes, and spermatid cells) of white Wistar rats (*Rattus norvegicus*).

MATERIALS AND METHODS

Location and time

This study was conducted from January to March, 2021, at the Integrated

Biomedical Laboratory of the Faculty of Medicine, Universitas Udayana.

Materials

Male Wistar rats *(R. norvegicus*) with a body weight of 200-250 g aged 18-19 months, given with ethanol extracts of Moringa leaves of 50 mg/kgBW per day for 30 days, 0.5% CMC, ketamine: xylazine*,* 1 set of surgical tools, surgical board, 0.9% NaCl solution, 1% Eosin and 10% Nigrosin, drop pipette, petri dish, glass object, and (Olympus) light microscope.

Method

This study applied an experimental design, namely the randomized post-test only control group design. The samples in this study include old male Wistar rats (*R. norvegicus*). The inclusion criteria included a body weight of 200-250 g an age range of 18-19 months. Meanwhile, the exclusion criteria include sick appearance and inactive movement. The samples belonged to the drop-out criteria when they died during the study and did not get a weight loss of more than 10% after the acclimatization period in the laboratory. The sample size in this study was 36 rats, which were divided into two groups, namely 18 for the treatment group and 18 for the control group. To determine the samples, the researchers utilized the random sampling technique.

Production ethanol extract

The Moringa leaf extract was made by macerating 50 g of dried Moringa leaves, crushed using a blender, added with 96% ethanol solvent, put in a container, tightly closed, and left for two days away from sunlight. This mixture was filtered to generate macerates. The pulp was macerated with 96% ethanol using the same procedure. The maceration was conducted until clear macerates were obtained. The macerates were evaporated using a rotary vacuum evaporator at a temperature of 40ºC (Putra et al. 2016, Cahyani and Sukadana 2017, Wasonowati et al. 2019, Widiastini et al. 2021).

Research procedure

This study began with weighing the experimental animals. The treatment group was administered with 50 mg/kgBW *Moringa* leaf ethanol extract dissolved with 0.5% CMC of 0.5 mL per day. The control group was administered with a CMC of 0.5% of 0.5 mL per day. The administration was performed through sonde at 08.00-09.00 a.m. Central Indonesian Time (WITA) and given for 30 days.

After passing the treatment period, namely on the 30th day, the old *(R. norvegicus*) Wistar rats were terminated by anesthesia using *ketamine*: *xylazine* of 100 mg/kg: 10 mg/kg (10 : 1 ratio) intra-muscularly (IM), and then euthanized with the cervical dislocation method. The testicles were separated from the cauda epididymis and then put into a petri dish containing 0.9% NaCl of 5 mL, the cauda epididymis was cut as smoothly as possible and then put into a petri dish and stirred until homogeneous. The bodies of the rats were buried.

***Spermatogenic* cell examination**

Histological preparations were made by fixating the testicular organs in a 10% formalin buffer solution for 24 hours, followed by the same treatment in Bouin solution for 3 hours. Then, the testicles were washed several times with a 70% alcohol solution. The dehydration process was performed with an alcohol solution of stratified concentration, and to purify the preparation, it was introduced into toluene solution for 24 hours. The infiltration of paraffin into the tissues was carried out by soaking the testicles using a mixture of toluene and paraffin solutions for 30 minutes, as well as the embedding stage for planting the testicles into solid paraffins. Paraffin blocks containing the testicles were slashed using microtomes with a thickness of 3-5 µm. The resulting slices were attached on the object glass that had been smeared with Mayer's albumin and left for 24 hours to be strong enough. The last step was staining the histological preparations using hematoxylin-eosin reagents, closed, and glued together with permount. The quantitative data include the total spermatogonium A cell, pakhiten primary spermatocyte cell, and spermatid cell from both sample groups. The observation was conducted using an Olympus® light microscope and an OptiLab® camera with a magnification of 40 x 10. The observation was performed by

sweeping the histological preparation starting from the upper left corner of the preparation, then moving spirally towards the bootom-right to get the five best viewing fields on the right and left testicles.

The data were analyzed using descriptively by displaying the frequency and average distribution of Spermatogenic Cells: Spermatogonia, Spermatocyte, and Spermatid cells. The data normality analysis was carried out using the Shapiro Wilk test. The data were distributed normally if the p-value > 0.05 after obtaining data before and after treatment in both the treatment and control groups. After obtaining the results of the data normality test, a comparative analysis was performed. If the data were distributed normally, the analysis test used was an independent sample t-test at a meaningfulness level of $\alpha = 0.05$ to determine the difference between the treatment and control groups. The data were analyzed using a 95% confidence level (95% CI/Confidence Interval is one of the other parameters to measure how accurate a sample's Mean represents (includes) the actual Population Mean value) or was expressed when $p < 0.05$. Meanwhile, if the data were not distributed normally, the analysis test used was the Mann Whitney test. To determine the differences between the treatment and control groups, the data were analyzed using a 95% confidence level or declared different when $p < 0.05$.

RESULTS AND DISCUSSION

This study used 36 white rats (R. norvegicus) which met the eligibility criteria, were male, aged 18-19 months. The following are the results of the analysis carried out: The average body weight of the rats used was 229.35 g ±11.4%, with the minimum weight of 202 g and the maximum weight of 250 g (Table 1). Randomization was conducted on all rats using the simple random allocation method.

The table 2 illustrates that the treatment group had the largest mean of 39.83, 95% CI 37.76 – 41.91, the largest minimum number was 31, and the largest maximum number was 48. Data on spermatogonia count in both the treatment and control groups were normally distributed with a p-value of 0.669 for the treatment group and 0.096 for the control group. Since the data were distributed normally, an independent t-test analysis was performed and a p-value of 0.000 was obtained (p-value < 0.05), meaning that there was a significant difference in spermatogonia count between the group administered with Moringa leaf ethanol extract and the control group, which did not undergo the same treatment. Therefore, it can be concluded that Moringa leaf ethanol extract has a significant influence on the number of spermatogonia.

The table 3 represents that the treatment group had a good spermatocyte with the largest mean of 61.39, 95% CI 59.16-63.61, the largest minimum number

Table 1. Descriptive analysis of body weight of male Wistar rats (*R. norvegicus*) in the treatment and control groups

Groups	Minimum	Maximum	Mean
Treatment and control	202	250	229.35 g ±11.4%

Table 2. Spermatogonia count in male Wistar rats (*R. norvegicus*) after the application of *Moringa* leaf ethanol extract

Groups	Mean	95% CI	Minimum	Maximum	Normality Test	Independent Sample T Test
Treatment	39.83 ± 4,17	37.76 – 41.91	31	48	0,669	0,000
Control	25.72 ± 5.33	23.07 – 28.37	19	35	0,096	

Description: P < 0.05, there was a significant effect using the independent sample t test

Table 3. Spermatocyte count in male Wistar rats (*R. norvegicus*) after the application of *Moringa* leaf ethanol extract

Group	Mean ± SD	95% CI	Minimum	Maximum	Normality Test	Mann Whitney test
Treatment	61.39 ± 4.47	59.16-63.61	54	69	0,612	0,000
Control	49.33 ± 8.26	45.22-53.44	38	62	0,032	

Description: P <0.05, there was a significant effect using the Mann Whitney test

was 54, and the highest maximum number was 69. Spermatocyte count data in both the treatment and control groups were not normally distributed with a p-value of 0.612 for the treatment group and 0.032 for the control group. For this reason, the Mann Whitney test analysis was carried out, resulting in a p-value of 0.000 (p value < 0.05), meaning that there was a significant difference in sperm morphology between the group administered with Moringa leaf ethanol extract and the control group, which did not undergo the same treatment. Therefore, it can be concluded that Moringa leaf extract has a significant influence on spermatocyte count.

Table table 4 shows that the treatment group had a good Spermatid Count with the largest mean of 89.94, 95% CI 87.04-92.85, the largest minimum number was 79, and the highest maximum number was 99. Spermatid count data in both the treatment and control groups were normally distributed with a p-value of 0.789 for the treatment group and 0.640 for the control group. Since the data were distributed normally, an independent sample t-test analysis was carried out and resulted in a p-value of 0.000 (p value < 0.05), meaning that there was a significant difference in spermatid count between the group administered with Moringa leaf ethanol extract and the control group, which did not undergo the same treatment. Therefore, it can be concluded that Moringa leaf extract has a significant influence on the spermatid count.

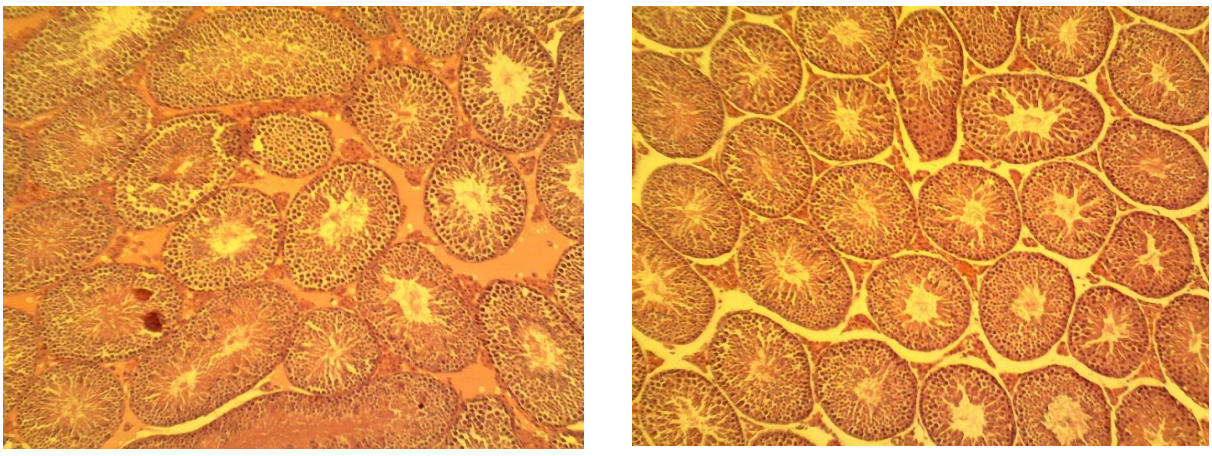
Figure 1 illustrates that the seminiferous tubules in the treatment group had more normal shape than that in the control group, which were mostly oval.

Spermatogenesis in the seminiferous tubules in the testes of rats consists of three main phases, including spermatogonia mitosis, spermatocyte meiosis, and spermiogenesis, in which spherical spermatids turn into elongated spermatids. The peritubular myoid cells, Sertoli cells, and male germ cells are the three main cell types that make up the seminiferous tubules. The tissue that connects the seminiferous tubules, which contains Leydig cells, is known as the testicular stroma (Zhou et al. 2019). Aging is characterized by a progressive decrease in physiological integrity that provokes impaired functioning caused by free radicals as a result of oxidative stress plus genetic and environmental modifications. Interventions that limit or inhibit free radical reactions will reduce the rate of change due to aging. so it is expected to reduce the rate of aging and pathogenesis of the disease (Balin and Allen 2018). Spermatogenesis is a lifelong process that also occurs in elderly men; However, the productive capacity of spermatogenic tissue seems to decrease with age. The reproductive capacity of men decreases with

Table 4. Spermatid count in male Wistar rats (*R. norvegicus*) after the application of *Moringa* leaf ethanol extract

Groups	Mean ± SD	95% CI	Minimum	Maximum	Normality Test	Independent sample T-test
Treatment	89.94 ± 5.84	87.04-92.85	79	99	0,789	0,000
Control	68.89 ± 7.20	65.31-72.47	56	83	0,640	

Description: P <0.05, there was a significant effect using the independent sample t-test



A **B**

Figure 1. Picture of the testes under light microscope at 400× magnification in (A) the control group, (B) the treatment group

age. Testicular morphology is one of the effects of aging on male reproductive system. Age changes affect testicular changes (Gunes et al. 2016).

This study indicated that the spermatogonia, spermatocyte, and spermatid counts in the group administered with Moringa leaf ethanol extract of 50 mg/kgBW were higher than those in the control group, with a p-value of 0.000 (p-value < 0.05), meaning that Moringa leaf ethanol extract can increase spermatogonia, spermatocyte, spermatid counts.

More than 90 different types of nutrients, including important vitamins, minerals, amino acids, anti-aging compounds, and anti-inflammatory compounds, are known to be present in moringa. More than 300 ailments have been prevented by the traditional medicinal plant moringa, which includes 539 components used in traditional African and Indian medicine (Toripah et al. 2014).

The results of the analysis of Moringa leaves in the South Denpasar area of Bali indicated that it has antioxidant capacity, including Phenolics, Flavonoids, Tannins, Ascorbic acid, Alkaloids, and Saponins (Widiastini et al. 2021). Most biomolecules are protected greatly against oxidative damage thanks to antioxidants included in moringa leaves, which help to neutralize free radicals (Ceci et al. 2022, Ezz El-Din Ibrahim et al. 2022).

Flavonoid content in Moringa leaves can affect the spermatogenesis process, which is indicated by changes in the diameter of the seminiferous tubules followed by the thickness of the tubule epithelium. Flavonoids can serve as antioxidants that can inhibit OS (Oxidative Stress), fight the dangers of free radicals, and improve the spermatogenesis process. Flavonoids can also improve cell regeneration process by destructing free radicals, providing a competitive substrate for unsaturated lipids in membranes, and/or accelerating the repair mechanism of damaged cell (Dasgupta and Klein 2014).

Ascorbic acid or Vitamin C contained in Moringa leaves is one of the water-soluble vitamins that acts as a key cofactor in various hydroxylation and amidase processes (Wibawa et al. 2020). Vitamin C plays a role in chemical reactions in the body as an electron carrier. The action mechanism of vitamin C in counteracting free radicals is by donating one electron to make them semidehydro ascorbic acids or ascorbic radicals. These radicals are more stable and can interact with other free radicals to make them non-reactive free radicals called scavenging or quenching free radicals (Padayatty and Levine 2016). The results of a study by (Zhang et al. 2022), indicated that vitamin C provided a protective effect on male reproduction such as reducing testicular damage, sperm abnormalities, and malondialdehyde. Based on the results of a literature study conducted by Widiastini et al. 2022, Increased libido is one aspect of sexual behavior that is positively impacted by moringa. Additionally, it has a favorable impact on spermatogenesis and spermatozoa quality. Additionally, it restores the activity of endogenous antioxidant enzymes, lowers ROS levels, boosts sperm motility, sperm count/volume, and germ cell count, and protects against testicular damage and Leydig cells.

CONCLUSION

The results of this study showed that the old Wistar rats (*R. norvegicus*) in the treatment group administered with Moringa (*Moringa oleifera*) leaf ethanol extract of 50 mg/kgBW for 30 days produced higher spermatogonia, spermatocyte, and spermatid counts compared to those in the control group administered with only 0.5% CMC of 0.5 mL, resulting in a p-value of 0.000 (p-value < 0.05). It indicated that there was a significant difference in the spermatogonia, spermatocyte, and spermatid counts between the group administered with Moringa leaf ethanol extract and the control group, which did not undergo the same treatment. Therefore, it can be concluded that Moringa leaf ethanol extract has a significant influence on the spermatogonia, spermatocyte, and spermatid counts.

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