ORIGINAL RESEARCH

Moringa oleifera extract affects the diameter of the Graafian follicles in female *Mus musculus*

Amiruddin[1], Sriyana Herman[2]*, Musthamin Balumbi[1], Marwia Rahawarat[1], Lili Darlian[1], Julia Fitrianingsih[2], Rika Handayani[2], Rusli[3]
[1]Department of Biology Education, Faculty of Teacher Training and Education, Universitas Halu Oleo, Kendari, Indonesia
[2]Department of Reproductive Health, Faculty of Health Technology, Universitas Megarezky Makassar, Indonesia
[3]Department of Nutrition, Faculty of Sport Science and Health, Universitas Negeri Makassar, Indonesia

Article Info
Received Aug 29, 2023
Revised Oct 3, 2023
Accepted Oct 13, 2023
Published Apr 1, 2024

***Corresponding author:**
Sriyana Herman
SriyanaH@unimerz.ac.id

Keywords:
Fertility
Ovarian follicle
Granulosa cells
Graafian follicles
Moringa extract
Maternal health

ABSTRACT

Objective: This study aimed to determine the effect of Moringa leaf extract (*Moringa oleifera* Lam.) on the diameter of Graafian follicles in female mice (*Mus musculus*).

Materials and Methods: This study used experimental design, employing a cohort of 24 female mice of 20-25 grams in weight, aged between 2-3 months, and in good health. These subjects were divided into three treatment groups and subjected to oral doses of Moringa leaf extract at 300 mg/kg BW, 400 mg/kg BW, and 500 mg/kg BW over a 14-day period. The study procedures involved the preparation of the experimental animals, preparation of Moringa leaf extract, treatments administration, and the preparation of histological specimens. Subsequently, the diameters of Graafian follicles within each treatment group were measured. Data analysis were performed using the ANOVA test ($p < 0.05$) followed with the Least Significance Difference (LSD) test utilizing the IBM SPSS software.

Results: There were variations in Graafian follicle diameters across the experimental groups. The average diameters were 180.944 µm in the control group, 239.942 µm in treatment group 1, 315.006 µm in treatment group 2, and 396.650 µm in treatment group 3. This indicated that dose administration starting from 300 mg/kg, 400 mg/kg, and 500 mg/kg had an effect on the size of the follicle and antrum diameter. The number of granulosa cells was found to increase, as well as the size of the ovum.

Conclusion: Moringa leaf extract in different doses has a significant positive effect on increasing the diameter of the Graafian follicles in female mice.

How to cite: Amiruddin, Herman S, Rahawarat M, et al. *Moringa oleifera* extract affects the diameter of the Graafian follicles in female *Mus musculus*. Majalah Obstetri & Ginekologi (Journal of Obstetrics & Gynecology Science). 2024;32(1):14-21 doi: 10.20473/mog.V32I12024.14-21.

Highlights:

1. This study unveils a dose-dependent relationship between Moringa leaf extract and increased Graafian follicle size in female mice.
2. The significant positive effect of Moringa leaf extract on Graafian follicles suggests promising implications for fertility interventions, offering hope for individuals seeking natural treatment to address infertility challenges.

INTRODUCTION

Fertility refers to the ideal functioning of the reproductive organs in facilitating the development of egg follicles, including primary, secondary, tertiary, and Graafian follicles, leading to the formation of ova, ovulation, and the synthesis and release of steroid hormones.[1] Graafian follicles consist of a central nucleus cell surrounded by a cell membrane and many follicular cells. The cell's nucleus possesses the capability to undergo development and maturation, ultimately resulting in the formation of an egg, also known as an ovum.[2] Insufficient nutrition disrupts follicle growth, which in turn affects the fertilization process.

Several plant compounds offer advantages in addressing reproductive issues. *Moringa oleifera* Lam., also known as Moringa, is a very nutritious plant that plays a key role in enhancing fertility. It is has been recognized that a quantity of 25 grams of Moringa leaf powder is sufficient to fulfill the daily nutritional requirements of children as follows: 42% protein, 125% calcium, 60% magnesium, 41% potassium, 71% iron, 272% vitamin A, and 22% vitamin C.[3] Furthermore, Moringa is rich in zinc, vitamin C, and antioxidants, including flavonoids. It also includes β-carotene, which is a precursor to vitamin A, as well as vitamins B complex, C, D, and K.[4]

Several studies have investigated the effect of Moringa leaf extract on the reproductive abilities of male and female mice (*Mus musculus*). A study conducted by Gunawati et al.[4] examined the impact of Moringa leaf on male mice (*Mus musculus*). This study observed a rise in the generation of spermatogenic cells.

Balumbi et al.[5] did a study utilizing Moringa leaf extract to determine the duration of the estrus cycle in mice. The study demonstrated that the administration of Moringa leaf extract resulted in a normal length of the estrus cycle in the treatment group, as opposed to the control group which had a cycle length of 4-5 days. A study conducted by Narulita et al.[1] demonstrated that the leaves of the Moringa plant had the highest nutritional value. Moringa leaves provide a superior nutritional composition compared to other plant components, hence it is reasonable to anticipate its usefulness in increasing the size of the ovaries and enhancement of their functionality.

In a study conducted by Alfian,[6] the diameter of the Graafian follicles was evaluated using papaya seed extract. The findings indicated that there was no statistically significant difference between the treatment and control groups. This study also measured the diameter of the Graafian follicles but, different from the previous studies, this study used Moringa leaf extract. This study aimed to assess the effect of Moringa leaves on the diameter of Graafian follicles in female mice.

MATERIALS AND METHODS

This study was a true experimental study using a completely randomized design (CRD).[7] The period of the study was four months, from July to October 2022 at the Laboratory of the Department of Biology, Faculty of Teacher Training and Education, Halu Oleo University, Kendari, Southeast Sulawesi, Indonesia. The protocol of this study was approved by the Research Ethics Committee of the Institute for Research and Community Services, Halu Oleo University with number: 185d/ UN29.20.1.2/PG/2022.

The population in this study was 45 female mice acclimatized for 2 weeks at the Laboratory of the Department of Biology Education of Halu Oleo University. The mice were subsequently randomized to 24 female mice using simple random sampling with the sample criteria of 20-25 grams of weight, age of 12-13 weeks, in good health and not physically disabled. The rats were allocated into four groups of control group (K), treatment group 1 (P1), treatment group 2 (P2), and treatment group 3 (P3), each consisting of 6 mice. The mice were acclimatized for 1 week, kept in rectangular cages of 38 cm x 27 cm x 13 cm covered with wire. The pedestal in the cage was wood shavings that were changed twice a week. During maintenance, the mice were given an intake of 6 grams of pellets/day and water ad libitum through a drinking bottle.

Preparation of extract from *Moringa oleifera* leaves

The Moringa leaves were dried in an oven for 12 hours at a temperature of 50ºC. The dried Moringa leaves were blended until becoming powder. The dry powder was measured at 1000 grams, added with 96% ethanol as much as 3 liters, then the extraction process was carried out by maceration method for 24 hours. The extract was filtered, then the Moringa leaf filtrate was concentrated using a rotary evaporator.

Animal treatment

Moringa leaf extract was administered in the morning (07.00 am) with different doses. Healthy adult female mice (2-3 months) were randomized into four groups and treated as follows: Control group (K), comprising 6 samples, receiving distilled water only; treatment group 1 (P1), consisting of 6 samples receiving a dose of 300 mg/kg BW; treatment group 2 (P2), 6 samples receiving

a dose of 400 mg/kg BW; and treatment group 3 (P3), consisting of 6 samples receiving a dose of 500 mg/kg BW. The extract was administered for 14 days which was carried out directly by the researchers and on day 15, the female mice were dissected to remove their ovaries. The treatment was given for 14 days considering three estrus cycles. This was in accordance with research conducted by Nugroho[8] where the normal average estrous cycle of female mice was 4-5 days. Dosage refers to previous studies conducted by Balumbi, et al.[5] in female mice test animals to see ovarian morphometry and estrus cycle. Moringa leaf extract (*Moringa oleifera* Lam.) was mixed with 0.2% Na-CMC. This was because Moringa leaf extract can be dispersed well in Na-CMC.

Moringa leaf extract administration to the mice was done using syringe equipped with a blunt tip needle, which had been filled with 0.5 ml of Moringa leaf extract liquid. The skin on the nape of the mouse was pulled with the middle finger and thumb of the left hand, and the little finger hold the tail of the mouse. The handheld tool was hold in the right hand and then the extract was injected into the mouse mouth. The administration of Moringa leaf extract to the mice was done by the assigned members of the research team.

Histology preparations

The removed ovaries were put in histological preparations. The preparations were fixed with Bouin's solution for approximately two days, and then washed with 70% alcohol (1 x 60 minutes). The next process was dehydration using 90% alcohol solution for one night, then transferred to 96% alcohol and absolute alcohol for 1 x 60 minutes, respectively. After the dehydration process was complete, it was followed by the purification process by soaking the ovarian organs using a toluol solution for one night, then paraffin infiltration was carried out by immersing the ovarian organs into a mixture of toluol and paraffin in a ratio of 1:1 for 30 minutes followed by pure paraffin I, II, and III for 45 minutes, respectively. The subsequent process was embedding and implanting the ovarian organs into the paraffin, then the position was set in the direction of transverse cutting and they were allowed to freeze, forming a block to be cut with a microtome. Then, the paraffin block was mounted on a holder, placed on a microtome, cut in 6 µm thickness, forming a ribbon. From the slices, the best one was selected, then it was placed on a slide with Mayer's albumin smear and put in a slide warmer for 24 hours to make the attachment stronger.

The procedure of staining and mounting were as follows: First was the deparaffinization. The slide containing the slices of the ovary was dipped in xylol solution until the paraffin was dissolved for one minute, then dried on filter paper. Then the hydration, during which the dried object glass was put into alcohol with decreasing concentrations starting from absolute alcohol, 96%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, and distilled water for one minute each. Subsequently, the objects were placed in a staining jar containing hematoxylin Ehrlich for seven minutes and washed with running water for 10 minutes. They were subsequently being put into 30%, 50%, 60%, and 70% alcohol each for one minute, then into Eosin-Y for ten minutes, rinsed with 70%, 80%, 90%, 96% alcohol, and absolute alcohol for one minute each. Finally, the preparation was put in a xylol solution for 15 minutes, then dried on filter paper, mounted with Canada balsam, and covered with a cover slip.

Graafian follicles diameter calculation

Subsequently, observations were made under a microscope to see and calculate the diameter of de Graff follicles in each treatment group. The data on the number of the Graafian follicles' diameters were then analyzed using SPSS statistics version 25 software. The method of calculation was the same as by Alfian,[6] that was by measuring the outer layer of theca cells (if any) and/or the granulosa cell layer. The formula is:

$$x = \frac{X1 + X2}{2}$$

Notes:
x=Average diameter length
X1=Longest diameter of the follicle
X2=Shortest diameter of the follicle

Statistical analysis

The data on mean and standard deviation were obtained when carrying out the data normality test with the Kolmogorov-Smirnov test. The obtained data were normally distributed and the subsequent data homogeneity test using Levene statistic test showed that the obtained data were homogeneous. This was done as a prerequisite test for using the parametric statistical test (ANOVA test). After testing the hypothesis with the Analysis of Variance, it was found that the result was significant ($p < 0.05$) so that it was continued with the Least Significance Difference (LSD) test which obtained significant differences between each treatment by means of the SPSS application program.

RESULTS AND DISCUSSION

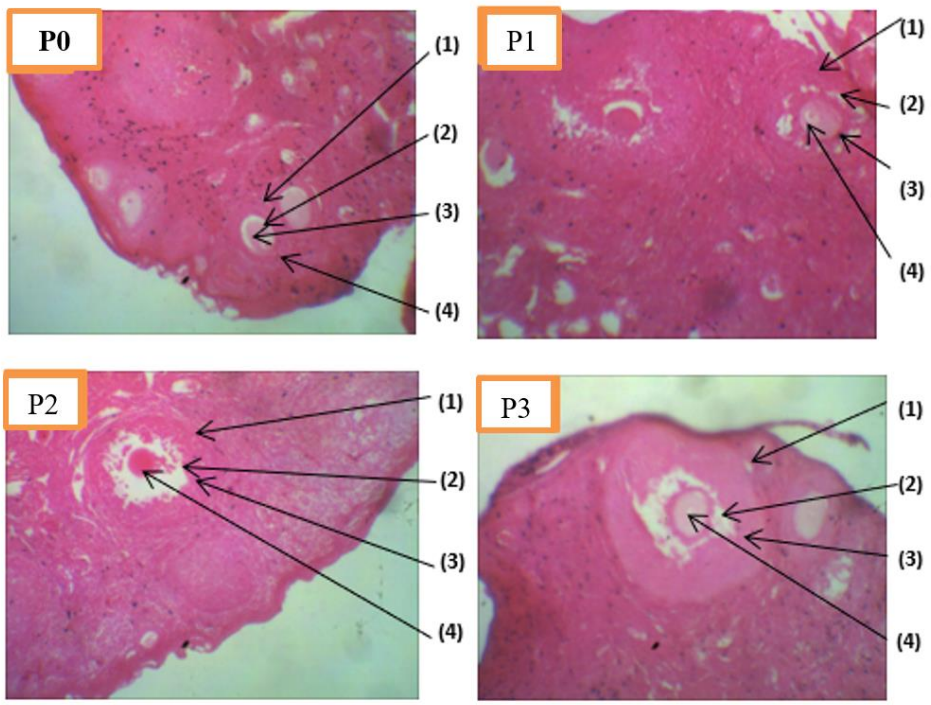
Diameter of the Graafian follicles

Each treatment was assessed by measuring the average diameter of the Graafian follicles in the mice (*Mus musculus*). The administration of Moringa leaf extract has a significant effect on the enlargement of the Graafian follicles' diameter in mice. The control group exhibited the smallest growth, with an average increase of 180.944 µm. In comparison, the groups that received Moringa leaf extract showed larger increases in the average diameter of their follicles. Specifically, the increase was 239.942 µm in the group that received a treatment of 300 mg/kg BW, 315.006 µm in the group receiving a treatment of 400 mg/kg BW, and the highest increase of 396.650 µm was observed in the group receiving 500 mg/kg BW.

However, in the image of the Graafian follicles in group P1 that received an extract dosage of 300 mg/kg BW, the follicle diameter increased due to a modest enlargement of the antrum and an increase in the ovum fiber. In addition, the image of Graafian follicles in group P2, which was administered an extract of 400 mg/kg BW, showed an increase in follicle diameter due to an enlargement of the antrum and an increase in granulosa cells. The ovum also enlarged. In the treatment group P3, the image of Graafian follicles shows a significant rise in the diameter of the follicle and its antrum. Additionally, there was an increase in the number of granulosa cells and the size of the ovum. Subsequently, the testing in this study employed the Analysis of Variance (ANOVA) parametric test, and the outcomes are displayed in Table 2.

Table 1. Effect of *Moringa olifeira* extract on the diameter of the Graafian follicle in mice after 14 days post-treatment

	Treatment Groups				p value
	P0 (n=6)	P1 (n=6)	P2 (n=6)	P3 (n=6)	(0.05)
Average diameter (µm) of the Graafian follicle of mice	180.944	239.942	315.006	396.650	0.000



Notes: (1). Theca externa, (2). Antrum, (3). Granulosa cell, (4). Ovum.

Figure 2. Histology of the Graafian follicles (Zoom 400x). Control (K), 300 mg/kg BW (P1), 400 mg/kg BW (P2), dan 500 mg/kg BW (P3).

Table 2. The results of Analysis of Variance (ANOVA) test on the Graafian follicles diameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	157260.559	3	52420.186	97.504	.000
Within Groups	10752.447	20	537.622		
Total	168013.006	23			

Table 3. The results of Tukey's honestly significant difference (HSD) test

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	P1	-85933.33333*	23459.24872	.008	-151594.2743	-20272.3924
	P2	-144330.66667*	23459.24872	.000	-209991.6076	-78669.7257
	P3	-242641.66667*	23459.24872	.000	-308302.6076	-176980.7257
P1	Control	85933.33333*	23459.24872	.008	20272.3924	151594.2743
	P2	-58397.33333	23459.24872	.092	-124058.2743	7263.6076
	P3	-156708.33333*	23459.24872	.000	-222369.2743	-91047.3924
P2	Control	144330.66667*	23459.24872	.000	78669.7257	209991.6076
	P1	58397.33333	23459.24872	.092	-7263.6076	124058.2743
	P3	-98311.00000*	23459.24872	.002	-163971.9409	-32650.0591
P3	Control	242641.66667*	23459.24872	.000	176980.7257	308302.6076
	P1	156708.33333*	23459.24872	.000	91047.3924	222369.2743
	P2	98311.00000*	23459.24872	.002	32650.0591	163971.9409

*. The mean difference is significant at the 0.05 level.

The statistical analysis presented in Table 2 demonstrates that the administration of Moringa leaf extract, at varying doses, had a considerably favorable impact on the diameter of the Graafian follicles in mice (*Mus musculus*). There were significant differences seen in each treatment group.

Table 3 demonstrates that the control group does not show a statistically significant effect on the diameter of the Graafian follicles. The treatments administered at doses of 300 mg/kg BW and 400 mg/kg BW exhibited had resulted a slightly different effect. However, the administration of a dose of 500 mg/kg BW in treatment group 3 had a significant effect on the size of the Graafian follicles.

The treatment was administered for a duration of 14 days, by considering the three estrus cycles. According to Nugroho et al.[8], their research revealed that the typical estrous cycle of female mice lasts for 4-5 days. The doses administered in this study were based on a previous research conducted by Balumbi, et al.[5], which examined the ovarian morphometry and estrus cycle in female mice experimental animals. The Moringa (*Moringa oleifera* Lam.) leaf extract was combined with a 0.2% solution of sodium carboxymethyl cellulose (Na-CMC) because of the favorable dispersibility of the Moringa leaf extract in Na-CMC.

The findings indicated that there were different in the Graafian follicles among the different treatments. The treatment group P3 exhibited the greatest disparity when administered a dosage of 500 mg/kg BW of Moringa leaf extract. In addition, the diameter of the Graafian follicles demonstrated a progressive increase in correlation with the increasing dosage of Moringa leaf extract, as compared to the control group. The results of our study indicated that the administration of Moringa leaf extract at a dosage of 500 mg/kg BW led to an enlargement of the longest Graafian follicle in the left ovary of the mice. The average diameter of the Graafian follicles was measured to be 396.65 micrometers.

The alterations in the ovaries are influenced by the hormonal response that occurs as a result of the changes in the estrous cycle. Balumbi, et al.[5] reported that the inclusion of Moringa leaf extract in the diet enhances the estrous cycle due to the presence of essential elements in Moringa leaves that support development and growth. Rani et al.,[3] discovered that the inclusion of vitamin E in Moringa leaves can enhance reproductive success. The growth of the follicle is determined by its diameter and the oocyte. Vitamin E acts as an antioxidant and enhances the production of steroid hormones during the oocyte maturation.[9,10] The increase of the follicle's antrum is a result of the proliferation of granulosa cells, leading to an increase in diameter of the follicle's basement membrane. The antrum is filled with fluid that contains the estrogen hormone secreted by the granulosa cells. The estrogen hormone is produced by the theca interna cells of the ovarian follicles. The estrogen influences the period of puberty in female animals. Female animals undergoing puberty are

showing signs of sexual desire or estrus, and ovulate their egg cells.[11]

Yuliani et al.[12] conducted phytochemical analysis and found that Moringa leaves contain antioxidants such as flavonoids, saponins, tannins, and terpenoids. Flavonoids belong to the isoflavone group. Isoflavones are compounds that promote the synthesis of estrogen and possess a structural similarity to the hormone estrogen. Flavonoids are a group of isoflavone compounds that directly bind to the estrogen receptor due to their chemical structure, which closely resembles that of 17-ß estradiol in the body. The most prevalent compound is 17-ß estradiol. In a separate study, both female and male mice demonstrated an increase in the width of their seminiferous tubules when administered a dose of 0.55 mg/kg of Morinaga leaf extract. This effect was attributed to the presence of flavonoids.[13] Additionally, it can impede NFkβ and apoptosis via the TNF-α pathway, hence facilitating the development of Graafian follicles.[14] Furthermore, it is worth noting that not only are the leaves of the Moringa plant highly beneficial, but the extract derived from the bark of Moringa can also serve as an active element with antifertility properties.[15]

The hormone is produced by the theca interna cells, granulosa cells of the ovarian follicles, and the corpus luteum.[16] Dafaalla et al.[17] reported that Moringa leaf extracts had a substantial impact on the synthesis of testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). The Moringa leaf is rich in ascorbic acid and possesses potent antioxidant properties. Ascorbic acid has a crucial function in safeguarding the brain and cerebrospinal fluid against the harmful effects of free radicals, thereby ensuring their protection from damage. Additionally, it supports the regular production of LH and FSH hormones by the pituitary gland in the brain. The *Moringa oleifera* is thought to possess therapeutic properties, functioning as an antioxidant, and exhibiting anti-inflammatory, antibacterial, antifungal, and various other advantages.[18]

A study conducted by Balumbi et al.[5] revealed that Moringa leaf extract can act as estrogen receptors in the body, leading to an increase in estrogen levels in the blood. This increase in estrogen stimulates the hypothalamus to secrete GnRF (Gonadotropin Releasing Factor), which in turn increases the production of FSH hormone. The FSH hormone then stimulates the development of primary and secondary follicles, transforming them into Graafian follicles. Furthermore, increased blood estrogen levels result in a sudden increase of LH (luteinizing hormone) in the bloodstream. This disorder causes an increase in the number of cells in the ovaries, leading to a change in the size of the follicle. According to a study conducted by Amelia et al.[19], the administration of Moringa leaf ethanol extract to mice was found to have a positive effect on enhancing the development of the folliculogenesis process. However, in mice with an endometriosis model, there was also an increase in folliculogenesis when Moringa leaf ethanol was administered at a dosage of 0.35 mg/kg BW.[10] A separate investigation carried out by Fathil demonstrated that Moringa enhances the concentrations of reproductive hormones (testosterone, luteinizing hormone, follicle stimulating hormone) in mice.[20]

The main limitation of this study was in the production of the histological preparations. Prior to conducting the main investigation, it would have been advisable to conduct a preliminary study in order to improve the process of sample preparation and enhance the accuracy of microscopy observations. The findings of this study suggest that incorporating *Moringa oleifera* Lam. leaves into the diet or using them as herbal supplements (traditional herbal medicine) can have positive effects on reproductive performance, particularly in humans. This implies that there is a practical application for the consumption of *Moringa oleifera* Lam. leaves in improving reproductive health.

CONCLUSION

The extract of Moringa leaves (*Moringa oleifera* Lam.) has a significant effect on increasing the diameter of the Graafian follicles in mice (*Mus musculus*). This is due to the presence of vitamin E in Moringa leaves, which stimulates granulosa cells to produce estrogen hormone, thereby facilitating the process of folliculogenesis.

DISCLOSURES

Acknowledgments

The authors wish to thank to, first; the Head of the Laboratory of Universitas Halu Oleo for generously supporting this project; second, to all laboratory staffs of Universitas Halu Oleo for their support as volunteers and members of data collection team.

Conflict of interests

The authors had no conflict of interests regarding with respect to the authorship and/or publication of this paper.

Funding

No funding or sponsorship was received for this study or publication of this article.

Author contributions

AD and SH contributed to preparing and checking the completeness of tools and materials for laboratory sample examination; MB and MR contributed to controlling the condition of the samples and treating the samples according to the research design; LD and JF, contributed to observing, measuring, and administering respective doses of Moringa leaf extract up to the completion of the study; RH and R contributed to assisting data collection, data measurement, tabulation of research results, editing and administering research approval, and the documentation of research results. All authors have read and approved the contents of the final manuscript.

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