journal homepage: https://ojs3.unpatti.ac.id/index.php/biopendix

B I O P E N D I X P-ISSN: 2407-4969
Volume 12, Number 2, October 2025 E-ISSN:2684-8341
Pages: 102-109 DOI: https://doi.org/10.30598/biopendixvol12issue2page102-109

Phytoestrogenic Activity of Fresh Bitter Melon (*Momordica charantia* L.) Fruit Extract on Ovarian Folliculogenesis in Female Mice

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Submitted: May 15, 2025; Revised: June 19, 2025; Accepted: June 22, 2025; Published: October 31, 2025

Abstract. Bitter melon (*Momordica charantia* L.**)** is a medicinal plant that contains bioactive compounds such as flavonoids, saponins, tannins, triterpenoids, and alkaloids, which have potential as antifertility agents. This study aimed to evaluate the effect of bitter melon extract on the fertility of female mice (*Mus musculus L.*). A total of 18 female mice were divided into three groups: a control group (K0), treatment group 1 (P1) received 5% bitter melon extract, and treatment group 2 (P2) received 10% extract. The extract was administered orally for 16 days. The ovaries were collected and analyzed using the paraffin method with Hematoxylin-Eosin staining. The observed parameters included the number of primary, secondary, tertiary, and Graafian follicles. The results showed that the 5% extract increased the number of primary and secondary follicles, while the 10% extract reduced all follicle types, including Graafian follicles. These findings suggest that a low dose of bitter melon extract may stimulate follicular development, whereas a high dose has antifertility potential through a phytoestrogenic mechanism.

Keywords: *Momordica charantia* L; Phytoestrogen; Ovarian Follicles; Ovarian Histology

INTRODUCTION

Indonesia is the fourth most populous country in the world. Based on the 2020 Population Census conducted by Statistics Indonesia, the total population reached 270.20 million people, with an average population density of 141 individuals per square kilometer. The annual population growth rate is 1.25%, indicating a rapid increase that presents various challenges in key sectors such as public health, education, and social welfare (Badan Pusat Statistik, 2019).

The Government of Indonesia has implemented various policies to curb the rate of population growth, one of which is through the Family Planning (FP) program, as stipulated in the Law of the Republic of Indonesia Number 52 of 2009 concerning Population Development and Family Development. This policy reflects the government's commitment to improving population quality by controlling birth rates and ensuring equitable access to family planning services (Frans & Djasri, 2023). Furthermore, according to (Ahmed *et al.* 2012), the successful design and implementation of appropriate family planning strategies can contribute to a global reduction in maternal mortality rates by up to 29%.

Contraception refers to efforts or methods aimed at preventing pregnancy. However, long-term use of contraceptives may lead to side effects, such as hormonal imbalances, irregular menstrual cycles, metabolic alterations, and increased risk of certain diseases. These conditions highlight the need for safer and more acceptable natural alternatives to conventional contraceptives. One potential plant with antifertility properties is *Momordica charantia L.*, commonly known as bitter melon, which is traditionally used as herbal medicine and contains various bioactive compounds, including flavonoids, saponins, and alkaloids. Several studies have shown that these compounds may exert antifertility effects by modulating reproductive hormones and inhibiting folliculogenesis. Bitter melon also contains phytoestrogens, which support its potential as a natural antifertility agent. According to (Rezady *et al*., 2019), bitter melon extract has been shown to reduce the number of ovarian

follicles. Additionally, a study by (Cholifah *et al*. 2014), demonstrated that bitter melon extract could decrease sperm production, affecting both sperm quality and quantity.

Momordica charantia L., commonly known as bitter melon, is a tropical plant widely distributed across Asia, India, East Africa, and South America. Bitter melon fruit possesses a wide range of pharmacological properties, including antihyperglycemic, antimicrobial, antioxidant, antiviral, antitumor, anthelmintic, antimutagenic, antifertility, and immunomodulatory activities. Its bioactive constituents include flavonoids, alkaloids, saponins, tannins, triterpenoids, cucurbitacins (bitter compounds), momordicosides, momorcharins, momordicins, momordins, tricosapic acid, resins, charantin, and hydroxytryptamine (Aydin & Kaya, 2020). Among these, cucurbitacins are known to exert biological effects that inhibit ovarian function through two primary pathways: direct cytotoxicity to ovarian tissues and disruption of gonadotropin hormone regulation, which is essential for follicular growth and maturation.

Herbal plants contain various bioactive compounds, such as flavonoids, saponins, tannins, triterpenoids, and alkaloids, which have potential as antifertility agents. These antifertility compounds generally act through two main mechanisms: inducing cytotoxicity in reproductive cells and modulating the hormonal system, thereby disrupting the balance of reproductive hormones. Such disturbances can directly affect the differentiation and maturation processes of germ cells, including ovarian follicle development in females.

Phytoestrogens are naturally occurring bioactive compounds commonly found in a variety of daily dietary sources, including soy products, legumes, fruits, vegetables, and whole grains. These compounds are classified into several major groups, namely isoflavones, coumestans, flavanols, and lignans. Due to their structural similarity to 17β-estradiol, phytoestrogens are capable of binding to estrogen receptors (ERα and ERβ), thereby eliciting estrogenic or anti-estrogenic biological responses in target cells (Suhaimi *et al*., 2023). The phytoestrogen compounds found in bitter melon have potential antifertility effects. Phytoestrogens are naturally occurring plant-derived compounds that possess structural and functional similarities to estrogen, the hormone produced by the ovaries and placenta (Whitten & Patisaul, 2001). These compounds belong to the phenolic group and are known to exert estrogen-like activity; however, the precise dosage that may disrupt the reproductive system remains unclear. Since all reproductive tissues—including the ovaries, uterus, hypothalamus, and pituitary gland express estrogen receptors (ERα and ERβ), phytoestrogens have the potential to interact with these tissues (Cevik *et al*., 2015).

The hypothalamus and pituitary gland play essential roles in responding to estrogenic signals by regulating the secretion of gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which control the ovulation process (McGee & Hsueh, 2000). Increased estrogen stimulation can trigger a negative feedback mechanism, ultimately suppressing ovulation. In the ovaries, estrogenic signaling is required to regulate the expression of genes involved in follicular growth and the formation of FSH and LH receptors, which are necessary for responding to gonadotropin signals. Continuous excessive estrogen stimulation may inhibit implantation. Therefore, disturbances or excessive estrogenic activity—including those induced by phytoestrogens—may potentially lead to fertility disorders, ranging from subfertility to infertility (Jefferson, 2010).

MATERIALS AND METHODS

The equipment used included mouse cages, a digital scale, a gavage needle, a scalpel (split), mortar and pestle, gauze, dissecting set, Petri dish, graph paper, measuring glass, surgical tray, digital camera, balsam bottles, sample cups, beaker glasses, forceps, small brush, Winkler bottles, Canada balsam bottles, microtome knife, microtome, oven, hot plate, holder, microscope slides, cover glasses, staining jars, microscope, basin, cutter, and reagent bottles. The materials used were formalin buffer, Bouin's solution, hematoxylin, eosin, 30% alcohol, 40% alcohol, 50% alcohol, 60% alcohol, 70% alcohol, 80% alcohol, 90% alcohol, 96% alcohol, absolute alcohol (100%), distilled water (aquadest), xylene, paraffin, 0.9% NaCl, planting boxes, hot water, cold water, Canada balsam, *Mus musculus* L., bitter melon (*Momordica charantia* L.), label paper, tissue paper, and food and water for mice.

Research Procedure

Preparation of Experimental Animals

This study used female mice (*Mus musculus* L.) aged approximately 8 to 10 weeks, with an average body weight of 25 grams. All test animals were housed in mouse cages, with each cage containing six mice from the same treatment group. A total of 18 mice were used in this study, randomly divided into three groups: one control group and two treatment groups. Before treatment administration, all mice underwent a seven-day acclimatization period. During this period, the mice were provided with food and drinking water *ad libitum*.

Preparation of Fresh Bitter Melon Extract (*Momordica charantia* L.)

Fresh bitter melon (*Momordica charantia* L.) extract was prepared by weighing 100 grams of the fruit, which was then ground using a mortar and pestle to produce a concentrated extract. From this concentrate, 5 ml and 10 ml aliquots were taken and each diluted in 100 ml of distilled water (aquadest).

Treatment of Experimental Animals

The experimental animals were divided into three groups, each consisting of six female mice. Group I served as the control group and received no treatment. Groups II and III were treatment groups that received bitter melon (*Momordica charantia* L.) extract at different concentrations. All mice were provided with food and water *ad libitum* throughout the study. The extract was administered orally using the gavage method every two days during the treatment period. The dosage was adjusted according to body weight, with 0.3 mL of extract given per 30 grams of body weight. The treatment details for each group were as follows:

- Group I (Control): no treatment
- Group II (Treatment 5%): 0.3 mL bitter melon extract / 30 g BW
- Group III (Treatment 10%): 0.3 mL bitter melon extract / 30 g BW

Administration of Bitter Melon Extract

On day 0 of the estrous phase, all mice were weighed before treatment. Bitter melon extract was administered orally from day 0 to day 14 of the estrous phase. On day 16, the mice were euthanized for further examination.

Dissection Procedure

On day 16 of treatment, the experimental animals were euthanized via cervical dislocation. The euthanized mice were placed on a surgical tray, and laparotomy was performed by making an incision along the abdomen using a dissecting set until the uterus became visible. The ovaries, kidneys, liver, and spleen were then carefully removed and rinsed with 0.9% physiological saline solution. The collected organs were subsequently fixed in Bouin's solution.

Histological Preparation of Ovarian Tissue (Paraffin Method)

Ovarian tissues were obtained through surgical biopsy of the female mice (*Mus musculus* L.). The first step was tissue fixation, which aimed to preserve cellular structures in a state as close as possible to physiological conditions. The excised ovaries were fixed in Bouin's solution. After fixation, the samples were washed with 70% alcohol to remove residual fixatives before undergoing dehydration.

Dehydration was performed by immersing tissue samples sequentially in graded alcohol concentrations: 70%, 80%, 90%, 96%, and 100%, each for 60 minutes. After dehydration, tissues were cleared in pure xylene for approximately 4 hours. The clearing step was followed by infiltration in an oven at 56 °C. The tissue samples were gradually immersed in a mixture of xylene and paraffin with ratios of 3:1, 1:1, and 1:3 for 30 minutes each. This step was continued by immersing the tissues in pure paraffin for 60 minutes to ensure complete infiltration.

Embedding was then performed using rectangular molds prepared from thick calendar paper. The infiltrated tissue was placed into the mold, and molten paraffin was poured in until the tissue was completely submerged. The paraffin blocks were labeled and left overnight in a freezer until completely solidified.

Once solidified, the paraffin blocks were removed from the molds and mounted on wooden holders (2 × 2 × 2 cm) using melted paraffin as adhesive. The mounted blocks were then installed on a rotary microtome equipped with a blade. Tissue sections were cut at a thickness of 6 µm. To prevent paraffin waste, cutting was performed over newspaper or manila paper.The paraffin ribbons were collected using a fine brush and floated on warm water to help them adhere to glass slides. The slides were then placed on a hot plate to enhance adhesion. The slides were left to dry overnight and labeled appropriately.

Deparaffinization was performed by immersing the slides in xylene for 3 to 5 repetitions until paraffin was completely removed. De-alcoholization was then carried out by sequentially immersing the slides in alcohol:xylene mixtures (1:3, 1:1, 3:1), followed by absolute alcohol, and then in 90%, 80%, 70%, and 50% alcohol, each for 3 to 5 repetitions. The slides were then rinsed in distilled water.

The first staining step involved immersion in safranin for 1 minute. The slides were rinsed with distilled water and dried. The tissue sections were then rehydrated by sequential immersion in 30%, 50%, 70%, 80%, 90%, and absolute alcohol (each for 3 to 5 repetitions), followed by immersion in alcohol:xylene mixtures (3:1, 1:1, 1:3), and finally in pure xylene. Excess alcohol was removed using absorbent paper.

The prepared slides were mounted with Canada balsam and covered with a cover glass. Observations were made under a light microscope. Each slide was labeled with the species name, organ/tissue type, section

orientation (transverse or longitudinal), staining method, and date of preparation. All slides were stored in special slide storage boxes. Prepared slides were examined under a microscope to quantify the number of primary, secondary, tertiary, and Graafian follicles in both the right and left ovaries.

Analysis Data

Quantitative data obtained from the observation of follicle counts were analyzed using statistical software version 21. The data were subjected to One-Way Analysis of Variance (ANOVA) to evaluate the differences among treatment groups.

RESULTS AND DISCUSSION

Microscopic Observation of Ovarian Follicles in Female Mice Following Administration of Bitter Melon (*Momordica charantia* L.) Extract

The effect of bitter melon extract on ovarian follicle count was evaluated through histological analysis of ovarian tissue using Hematoxylin-Eosin (H&E) staining. Histological examinations were carried out under a light microscope at 100× magnification to identify and quantify the number of primary, secondary, tertiary, and Graafian follicles in each treatment group.

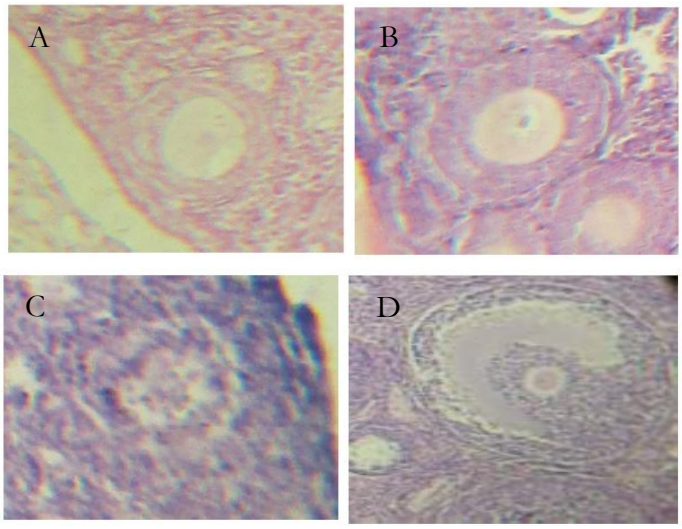

Figure 1. Ovarian follicle development stages**:** A. Primary follicle, B. Secondary follicle, C. Tertiary follicle, D. Graafian follicle.

Figure 1 illustrates follicular development within the ovarian tissue. The observed follicle types include primary, secondary, tertiary, and Graafian follicles, representing sequential stages of folliculogenesis. According to (Erickson, 2009), ovarian follicles are generally classified into four developmental stages: primordial, primary, secondary, and tertiary. Primordial follicles are characterized by a single thin layer of granulosa cells (<10 cells) surrounding an immature oocyte measuring approximately 30 µm in diameter and lacking a zona pellucida. The total follicle diameter is typically less than 40 µm. Primary follicles exhibit one to two layers of cuboidal granulosa cells (10–40 cells) encircling a round oocyte with a diameter of 25–45 µm. At this stage, the zona pellucida begins to form unevenly around the oocyte. A cellular connection in the form of adherens junctions is observed between the oocyte and granulosa cells.

During follicular development, primary follicles migrate toward the ovarian cortex. At the secondary follicle stage, small cavities begin to form among the granulosa cell layers, which subsequently coalesce and fill with follicular fluid (antrum). This fluid contains a variety of essential components, including high-molecular-weight hyaluronic acid, growth factors, and proteins of the fibrinolytic system such as plasminogen and fibrinogen. In addition, anticoagulant substances such as heparan sulfate, proteoglycans, and high concentrations of steroid hormones—such as progesterone, androstenedione, and estrogen—bound to their carrier proteins, are also present (Mescher, 2018).

The secondary follicle represents the subsequent stage of follicular development following the primary follicle. This phase is characterized by active cellular proliferation, indicated by the formation of additional granulosa cell layers surrounding the oocyte. Compared to the primary follicle, the secondary follicle exhibits a larger diameter (Kangawa *et al*., 2017). The key distinction between these two stages lies not only in follicular

size but also in the increased number of granulosa cell layers, with the secondary follicle possessing two or more. Histologically, secondary follicles are surrounded by irregular spaces formed through the differentiation of ovarian stromal cells. The epithelial cells constituting this structure develop into the theca folliculi. As follicular growth progresses, the theca folliculi differentiates into two distinct functional layers: the theca interna and theca externa (Cushman *et al*., 2000).

The tertiary follicle stage is characterized by the displacement of the oocyte into the cumulus oophorus and a marked enlargement of the follicular structure due to extensive granulosa cell proliferation. In its late phase, the tertiary follicle exhibits a substantial accumulation of eosinophilic follicular fluid within the antral cavity (Dekrismar *et al*., 2022). The progression into the preovulatory, or Graafian follicle, is defined by further expansion of the antral space and peripheral positioning of the oocyte, which remains connected to the mural granulosa cells via the corona radiata. This stage also involves increased stratification of granulosa cells, indicative of follicular maturity and impending ovulation.

Average Number of Ovarian Follicles in Female Mice

In this study, bitter melon (*Momordica charantia*) extract was administered orally to the experimental animals for 16 consecutive days. Follicular development was assessed by quantifying the number of ovarian follicles, including primary, secondary, tertiary, and Graafian follicles. Based on statistical analysis using one-way analysis of variance (ANOVA), administration of the extract at a 5% concentration resulted in a significant increase ($p < 0.05$) in the number of primary, secondary, and Graafian follicles compared to the control group. This suggests that the 5% concentration may promote folliculogenesis in female mice. Conversely, administration of the extract at a 10% concentration led to a decrease in the number of primary and secondary follicles relative to the control group, indicating a potential inhibitory or cytotoxic effect at higher concentrations. Meanwhile, no statistically significant differences were observed in the number of tertiary follicles across all experimental groups. The complete observational and statistical data are presented in Table 1.

Table 1. Mean Number of Ovarian Follicles in Mice Observed During Folliculogenesis in Each Treatment Group

Treatment	Mean number of ovarian follicles in Mice			
	Primary Follicle	Secondary Follicles	Tertiary Follicles	Graafian Follicles
K0	7,5 ± 1,97	6,3 ±2,07	2,7 ± 0,82	2,7 ± 0,52
P1	10,7 ± 4,41	8,8 ± 3,92	4,0 ± 3,22	9,0 ± 2,45
P2	4,5 ± 2,26	4,7, ± 2,66	2,8 ± 1,83	4,3 ± 1,97

Note: K0 = Control group (no treatment); P1 = Treatment with 5% *Momordica charantia* extract; P2 = Treatment with 10% *Momordica charantia* extract.

Based on Table 1, the administration of *Momordica charantia* fruit extract was found to influence the increase in the average number of ovarian follicles in female mice. Treatment with a 5% extract concentration (P1) showed a higher number of primary, secondary, and Graafian follicles compared to the control group. However, the number of tertiary follicles did not differ significantly among all treatment groups. These findings indicate that low-dose *Momordica charantia* extract is capable of stimulating follicular development, particularly at the primary, secondary, and Graafian stages. *Momordica charantia* fruit contains various secondary metabolites with potential antioxidant properties, such as flavonoids, alkaloids, saponins, and tannins. These compounds are known to scavenge free radicals and prevent oxidative stress, which could otherwise lead to cellular damage, including damage to ovarian cell

Phenolic compounds are known to possess antioxidant activity that plays a crucial role in neutralizing free radicals in the body (Husna *et al*., 2022) Flavonoids, one of the major phenolic constituents found in *Momordica charantia* extract, act as free radical scavengers by donating electrons to reactive molecules, thereby inhibiting oxidative activity and preventing cellular damage. In addition to their antioxidant properties, *M. charantia* also exhibits immunomodulatory effects by reducing cortisol (a stress hormone) levels and protecting immune cell function from oxidative stress-induced damage. A study conducted by (Rezady *et al*.,2019) demonstrated that the administration of *M. charantia* extract significantly increased the number of ovarian follicles in rats induced with Streptozotocin, indicating its potential role in enhancing folliculogenesis.

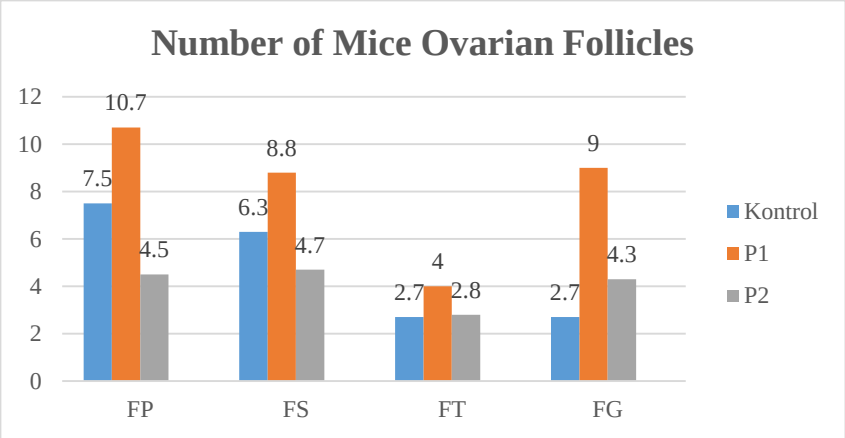

Figure 2. Diagram illustrating the average number of ovarian follicles in mice at each stage of folliculogenesis across treatment groups. Description**:** K0: Untreated (Control), P1: Treatment with 5% *Momordica charantia* extract, P2: Treatment with 10% *M. charantia* extract, FP: Primary follicle, FS: Secondary follicle, FT: Tertiary follicle, FG: Graafian follicle.

In Treatment Group 2 (P2), which received 10% *Momordica charantia* extract, a decrease in the number of primary and secondary follicles was observed compared to the control group. This reduction indicates that higher concentrations of the extract may inhibit follicular development. This inhibitory effect is likely attributable to the presence of bioactive compounds in *M. charantia*, including phytoestrogen such as flavonoids, saponins, and triterpenoids. When consumed in high doses, these compounds may suppress folliculogenesis by reducing Follicle-Stimulating Hormone (FSH) levels. The study by (Zin *et al*., 2013), showed that the administration of plant extracts containing genistein (a type of phytoestrogen) at low doses was able to increase estrogen levels in the blood. In contrast, administration at higher doses led to a decrease in estrogen levels. High-dose genistein intake may enhance its affinity for estrogen receptors in the hypothalamus, which subsequently affects the regulation of growth hormone and growth factor production. The concentration of circulating gonadal hormones in the body is regulated by gonadotropin-releasing hormone (GnRH), which is produced by the hypothalamus. GnRH stimulates the pituitary gland to release gonadotropins, which in turn stimulate the gonads to produce reproductive hormones such as testosterone, estradiol, and progesterone.

Phytoestrogens are secondary metabolites derived from plants that possess a chemical structure similar to endogenous estrogen, particularly 17β-estradiol. Due to this structural similarity, phytoestrogens are capable of interacting with estrogen receptors (ERα and ERβ), which are distributed across various tissues, including ovarian tissue. Endogenous estrogen plays a crucial role in regulating ovarian follicle growth and development by stimulating granulosa cell proliferation, enhancing follicular responsiveness to luteinizing hormone (LH), and supporting the progression of folliculogenesis from the primary follicle stage to the fully mature Graafian follicle (Nuradiah *et al*., 2023).

Flavonoids are a major class of phytoestrogens that can act as estrogenic compounds by directly binding to estrogen receptors. These compounds possess a non-steroidal polyphenolic structure that enables them to recognize and interact with estrogen receptors in the body, thereby mimicking or modulating the action of endogenous estrogen hormones (Kiyama, 2023). Phytoestrogens may competitively inhibit the binding of endogenous estrogen to its receptors, leading to an increased concentration of circulating free estrogen (Patil, 2020). Elevated levels of free estrogen can suppress the secretion of follicle-stimulating hormone (FSH), thereby inhibiting the development of ovarian follicles (Narulita *et al*., 2017). Additionally, triterpenoids and saponins present in plant extracts have been shown to exert cytotoxic effects. Triterpenoids can interfere with the metabolic functions of germ cells due to their cytotoxic activity, while saponins are particularly toxic to cells undergoing active development, such as those in the oogenesis phase.

Phytoestrogens are naturally occurring plant-derived compounds known to influence the regulation of the ovarian cycle, particularly in the processes of follicular development and maturation (Whitten & Patisaul, 2001). One such plant that contains phytoestrogens is bitter melon (*Momordica charantia*). Phytochemical studies have confirmed that *M. charantia* contains a wide array of secondary metabolites, including alkaloids, flavonoids, saponins, tannins, and steroids (Ifeanyi *et al*., 2011). Flavonoids exhibit both structural and functional similarities to endogenous estrogens, enabling them to bind to estrogen receptors within various target tissues. In the ovary, estrogen typically interacts with estrogen receptor subtypes α and β to stimulate granulosa cell proliferation, while in the uterus, the interaction primarily occurs through estrogen receptor α (Satyaningtijas *et al*., 2014). The presence of flavonoids and saponins in *M. charantia* is hypothesized to enhance estrogenic activity and potentially exert antifertility effects by modulating the reproductive endocrine axis. Given their

structural similarity to estrogen, high concentrations of flavonoids may suppress the release of follicle-stimulating hormone (FSH) at the pituitary level, potentially mediated by negative feedback mechanisms involving the hypothalamus

CONCLUSION

The results of this study indicate that administration of *Momordica charantia L.* fruit extract at a 5% concentration effectively enhances the number of primary, secondary, and tertiary ovarian follicles. In contrast, treatment with a 10% concentration tends to reduce the overall follicle count, including Graafian follicles. These findings suggest that low-dose *M. charantia* extract may support ovarian folliculogenesis, whereas higher doses exhibit potential antifertility effects, likely mediated through phytoestrogenic mechanisms that interfere with follicular maturation.

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