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Histopathological Alterations in Ovarian and Uterine Treated with Extract and Fraction of *Biophytum petersianum*

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ABSTRACT

B. petersianum is being used traditionally by the people of West Papua to increase fertility. The effects of extracts and fractions of *B. petersianum* on the estrus cycle duration and estrus phase in rats were evaluated. The present work was designed to investigate the effects of administration of *B. petersianum* extract and its fraction on the histopathological changes in the ovaries and uterine of rats. The female rats were treated with carboxy methyl cellulose sodium (CMC sodium) 1 % (group I), ethynyl estradiol (group II), extract (group III), n-hexane fraction (group IV), ethyl acetate fraction (group V), and water fraction (group VI) of *B. petersianum* via gavage daily for 7 days. At days 7th and 14th, 3 rats in each group were euthanized. Uterine and ovarian collected and weighed. Hematoxylin-Eosin staining and the paraffin technique were used for histological preparation. Data were analyzed by One Way ANOVA followed by the Duncan test. *B. petersianum* has an effect to increase ovarian and uterine weight, increasing most of the number of follicles especially primary follicle and uterine thickness. The treatment with ethyl acetate fraction of *B. petersianum* (group V) constantly showed more effective results than the other treatment.
Keywords: Biophytum petersianum; Phytoestrogens; Ovaries; Uterine; Histopathology

INTRODUCTION

Menopause, a typical occurrence in women, is characterized by the cessation of menstruation due to depleted estrogen and progesterone levels caused by the cessation of ovarian follicular activity (Pourjafari et al., 2019). The cessation of menstruation is frequently accompanied by several unpleasant symptoms, such as fatigue, anxiety, depression, urinary problems, vaginal dryness, headaches, vasomotor symptoms (hot flashes and night sweats), difficulties concentrating, disturbed sleep, and changes in bone metabolism (D'alonzo et al., 2019). After the menopause transition, these symptoms may persist for several years. This stage is difficult for most women, and hormone replacement therapy (HRT) is commonly used to treat menopausal symptoms. However, there are several risks related to HRT, including thromboembolic occurrences, heart disease, and some types of cancer (such as breast, ovarian, and womb cancer) (Marjoibanks et al., 2017; Huber et al., 2021).

Considering the risks associated with pharmaceutical medications, medicinal herbs are becoming more and more popular today. One of the beneficial and healthy ways to reduce the symptoms of menopause in women who have low estrogen is by using phytoestrogens (Ascenzi et al., 2006). *Biophytum petersianum*, known as kebar grass, is commonly used by the people of West Papua to improve reproductive health,

increase fertility, and reduce painful and irregular menstrual cycles (Unity and Inara, 2011). Several studies have shown that *B. petersianum* extract can be a source of multiple antioxidants with an LC50 of 27.7 and reduce serum MDA levels in a rat model of endometriosis (Aminudin et al., 2020; Trisetiyono et al., 2020). Such antioxidant activity is assumed to contribute to pharmacological effects, such as improving fertility (Alok et al., 2014).

The previous study reported that *B. petersianum* had an estrogenic effect, could lengthen the estrous phase, and shorten the estrous cycle, as well as to thicken the uterine wall and promote vascularization in the ovary and uterine, increased the level of 17β-estradiol in mice blood (Sukarsono et al., 2012; Claudia, 2018; Aprilia et al., 2020; Mulyati et al., 2022).

Some effects have been pharmacologically demonstrated but are still necessary for determining their effect on histology ovaries and uterine. The amount of active chemicals each plant produces and accumulates in its tissues differs depending on the polarity of the solvents it attracts (Masaya et al.,2017). The present study was to determine the effect of the extract and fraction of *B. petersianum* on the histology of the ovaries and uterine. *B. petersianum* was fractionated with different solvents.

MATERIAL AND METHODS

Materials

B. petersianum herb was obtained from West Papua, Indonesia, formalin 10%, normal saline, alcohol, methanol, paraffin, xylol, Giemsa staining, aqua dest, Ethinyl estradiol, CMC-Na (sodium carboxy methyl cellulose), VCO, ketamine-xylazine, Hematoxylin-Eosin stain, Giemsa stain, n-Hexan, ethyl acetate, animal feed, analytical scale (LabPro DT224), and rat cages.

Instruments

Rotary evaporator, microtome blade, gavage, syringes with a needle, staining jar, hot plate, spirit lamp, dropper, filter paper, cotton swab, vacuum dryer, Separatory funnel (Pyrex®), object glass and cover glass, surgical instruments, oven (Memmert®), furnaces (DAIHAN Scientific Furnace®), microscope (Olympus®).

Preparation of the Extract and Fraction of *B. petersianum*

The *B. petersianum* herb was washed under running water to remove dust and dirt. The herb was oven-dried at 50°C and at 60°C and pulverized using a grinder to obtain coarse powder. For three days, the powder was macerated with 90% (v/v) ethanol. The extract was filtered through the Buchner funnel. The filtrates were collected and concentrated using a rotary evaporator and vacuum dryer to eliminate the water content.

Fractionation of Crude Extract with Different Solvent

Fractionation was performed by suspending each extract in 250 mL of water separately and partitioning with different organic solvents (hexane, and ethyl acetate) in sequence of increasing polarity using a separating funnel. By evaporating the appropriate solvent using a vacuum dryer. All three fractions of each plant extract were dried. All extracts were kept at 4 °C pending additional analysis.

Experimental Animals

The protocol in this study refers to the research of Effendi et al. (2022). The protocol was approved by The Animal Research Ethics Committee, Faculty of Mathematics and Natural Sciences, Pakuan University, with reference number 80/KEPHP-UNPAK/01/2020. The animal use in the study were 36, three months female Sprague Dawley rats with an average body weight of 220 g (Coefisien of Variation 13%).

The rats was divided into groups and acclimated to laboratory conditions for a week. The rats were fed with commercial

pellets and had unlimited access to food and water.

Sprague Dawley rats were randomly selected from the total population and divided into six groups, with six rats in each group. Group I (negative control) received 1% carboxy methyl cellulose sodium (CMC sodium), Group II (positive control) was treated with standard drugs; Ethynil estradiol 0,045 mg/kg BW, Groups III to VI received the extract ethanol (2.16 mg/kg BW), n-hexane fraction (0.094 mg/kg BW), ethyl acetate fraction (0.1035 mg/kg BW), and aqueous fraction (0.199 mg/kg BW of *B. petersianum* respectively (Effendi et al., 2022).

All group of rat were administered orally for seven days, starting during the estrus phase. Synchronization of the estrus phase was done by the Whitten Effect method by placing the male rat cage on top of the female rat cage. Vaginal smears were collected daily and evaluated microscopically every 12 h. On days 7^{th} and 14^{th}, three rats in each group were euthanized with Xylazine 15 mg/kg BW intraperitoneally injection and dissection. The ovaries and uterine were surgically removed for histopathological investigations.

Histopathological Sample Preparation

The ovarian and uterus were freed of fatty tissue and rinsed with saline before being fixed in 10% formaldehyde, dried, and embedded in paraffin. A histologist and pathologist confirmed the preparation and evaluation of ovarian and uterine pathological changes at the Indonesian Institute for Veterinary Sciences (BB Litvet). Histological parameters were observed using an Olympus microscope. The number of follicles includes the corpus luteum, primary follicles, secondary follicles, tertiary and de Graff follicles, the diameter of the ovarian follicles, the thickness of the uterine, and the number of uterine glands between groups.

Statistical Analysis

The data were presented as mean ± standard deviation (mean ± S.D.) and analyzed through a one-way analysis of variance (ANOVA) Duncan to find the differences between groups.

RESULTS AND DISCUSSION

Effect of *B. petersianum* on Ovaries and Uterine Weight

The effect of *B. petersianum* at the stage of cell and tissue development will affect the weight of the ovaries and uterine organs. Table 1 show the effect of extracts and all fractions at day-7^{th} and 14^{th} on ovaries and uterine weight.

In general, ovarian and uterine weights were higher than the negative control (group I). Ovarian weight was a significant difference ($P<0.05$) between the seventh day compared to group 1 (control negative). However, it was a decrease in ovarian weight on 14 days, and no significant ($P>0,05$) compared to group I (negative control). This result showed that *B. petersianum* could increase ovarian weight, especially in group V. The effect of this treatments on the ovaries was short-term and reversible after treatment was discontinued, except in group V (ethyl acetate fraction), where the effects were generally stable up to 7 days after administration was discontinued.

B. petersianum also functions as phytoestrogen, which is reported to replace the role of endogenous estrogen (Simatauw and Unity. 2019). Phytoestrogen compound was predicted to stimulate the gonadotropin hormone (GnRH). Stimulation of gonadotropin will increase the size of the ovaries, uterine, vagina, and fallopian tubes (Hall. 2015). Thus, it stimulates the ovarian tissue to release estrogen and progesterone hormones. The integrity of the follicle was closely correlated with the density of healthy ovarian follicles. According to a previous study (Murasawa et al., 2005), ovarian follicular density may affect ovarian weight and reflect an ovary's capacity to produce mature follicles.

Table 1. Weight of the Ovaries and Uterine

Treatment	Ovarian Weight (g±SD)		Uterine Weight (g±)	
	7th Days	14th Days	7th Days	14th Days
Group I	0.1472±0.03^{ab}	0.1114±0.01^{a}	0.8832±0.19^{xyz}	0.6584±0.00^{x}
Group II	0.2220±0.02^{d}	0.1381±0.04^{a}	0.9410±0.12^{yz}	0.9170±0.09^{yz}
Group III	0.2224±0.02^{cd}	0.1578±0.16^{a}	0.9710±0.16^{yz}	0.9370±0.06^{xy}
Group IV	0.2274±0.03^{d}	0.1871±0.01^{abc}	0.9803±0.15^{yz}	1.0425±0.14^{yz}
Group V	0.2710±0.01^{d}	0.1955±0.04^{bcd}	0.9997±0.01^{yz}	1.0760 ±0.10^{z}
Group VI	0.2010^{b}±0.00^{e}	0.1216±0.03^{cd}	0.9370±0.25^{yz}	0.7520±0.03^{z}

Values are Mean ± S.D. P *< 0.05 Different superscript letters following the value in the same column indicate significant difference (P<0.05). Group I (negative control), Group II (positive control;Ethynil estradiol 0,045 mg/kg BW), Groups III (extract 2,16 mg/kg BW), Group IV (n-hexane fraction 0,094 mg/kg BW), Group V (ethyl acetate fraction 0,1035 mg/kg BW), and water fraction (0,199 mg/kg BW) of Biophytum petersianum*

Tabel 1 showed that the uterine weight was no significant difference (p>0.05) on the seventh day and decreased on the 14th day except for groups IV and V. However, it was a significant difference from group I (p<0.05). The result showed that *B. petersianum* could increase uterine weight, although it needs longer to affect the uterine than the ovarian.

Uterine is an organ that is very responsive to estrogen changes. Estrogen plays a role in cell proliferation and tissue growth related to reproductive function. If estrogen concentration increases in the body, it will change the uterine weight in response to the luteinizing hormone (LH) released by the pituitary. Previous research data showed that providing *B. petersianum* extract can also increase the level of 17β-estradiol in mice blood (Pasaribu and Indyastuti. 2004).

The Number of Follicles in The Ovaries of Rats After Treatment With Biophytum petersianum

The number of follicles in an ovary can be used to examine ovarian function, particularly the link between follicle development and the factors that control it. Table 2 shows the number of follicles in the ovaries of rats after the administration of Biophytum petersianum.

Referring to Table 2, all groups had a significantly higher number of follicles than group I (P<0.05), except group III. Generally, the number of follicles decreased on the 14th day in all groups. The corpus luteum increased by treatment of groups II and V, and primary follicles increased by treatment groups IV and VI. Secondary and tertiary follicles increased by treatment group V. The treatment with ethyl acetate fraction (group V) constantly showed the highest average on the number of primaries, tertiary, and de Graaf follicles, and was significantly different from the positive control (ethynyl estradiol). In general, the extract and fraction of *B. petersianum* could increase the number of ovarian follicles in white female rats.

The number of ovarian follicles on day 14 decreased, but the number was still higher than in group I (control negative). The decrease in ovarian follicles is probably due to the reversible effect of *B. petersianum* after discontinuation administration on the seventh day. On the other hand, the decreased number of follicles indicates the number of follicles becoming atresia, leave a few to develop and do not reach ovulation (Camara et al., 2015). The Histomorphology of ovarian tissue in rats can be seen in Figure 1.

Table 2. The Number of Follicles In The Ovaries of Rat After *B. petersianum* Treatment

Treatment	Time (day)	Follicle Number (means±SD)				
		Corpus Luteum	Primary	Secondary	Tertier	De graff
Group I	7th	1.67 ±0.57^{ab}	5.33 ± 4.93^{d}	1.33 ± 0.57^{h}	1.00 ± 1.00^{l}	1.00 ± 1.00^{no}
	14th	1.00 ± 1.00^{a}	6.00 ± 5.56^{d}	3.00 ± 4.35^{hij}	0.67 ± 0.57^{l}	0.67 ± 0.57^{n}
Group II	7th	10.33±7.09^{c}	17.00 ± 3.60^{ef}	7.33 ± 3.05^{ijk}	5.00 ± 3.00^{lm}	7.00 ± 6.08^{no}
	14th	8.67 ± 5.68^{abc}	15.00 ± 5.00^{ef}	6.33 ± 1.52^{ijk}	4.00 ± 3.6^{lm}	5 .00± 4.35^{no}
Group III	7th	5.33 ± 3.21^{abc}	15.33 ± 3.51^{de}	2.67 ± 3.05^{ij}	1.33 ± 1.15^{l}	3.00 ± 3.00^{no}
	14th	3.00 ± 1.00^{abc}	10.33 ± 5.50^{de}	2.00 ± 2.64^{hi}	1.00 ± 1.00^{l}	2.33 ± 0.57^{no}
Group IV	7th	9.00 ± 4.58^{abc}	11.00 ± 3.00^{ef}	3.00 ± 2.64^{hij}	3.67 ± 1.52^{lm}	4.67 ± 3.51^{no}
	14th	7.00 ± 2.00^{abc}	6.67 ± 1.52^{de}	2.33 ± 2.08^{ij}	2.67 ± 2.51^{lm}	2.67 ± 2.51^{no}
Group V	7th	11.00 ± 7.54^{c}	23.33 ± 5.03^{de}	9.00 ± 2.64^{k}	6.33 ± 5.68^{m}	8.67 ± 7.57^{o}
	14th	8.67 ± 4.72^{abc}	20.33 ± 5.5^{d}	6.33 ± 2.08^{ijk}	4.00 ± 1.00^{lm}	6.67 ± 6.11^{no}
Group VI	7th	9.67 ± 2.08^{abc}	12.33 ± 3.05^{g}	1.67 ± 1.52^{hi}	2.33 ± 2.08^{lm}	2.00 ± 2.00^{no}
	14th	8.00 ± 4.58^{abc}	10.33 ± 2.51^{fg}	1.33 ± 0.57^{h}	1.33 ± 1.15^{l}	1.33 ± 1.52^{no}

Values are Mean ± S.D. P < 0.05 Different superscript letters following the value in the same column indicate significant difference (P<0.05. Group I (negative control), Group II (positive control;Ethynil estradiol 0,045 mg/kg BW), Groups III (extract 2,16 mg/kg BW), Group IV (n-hexane fraction 0,094 mg/kg BW), Group V (ethyl acetate fraction 0,1035 mg/kg BW), and water fraction (0,199 mg/kg BW) of Biophytum petersianum

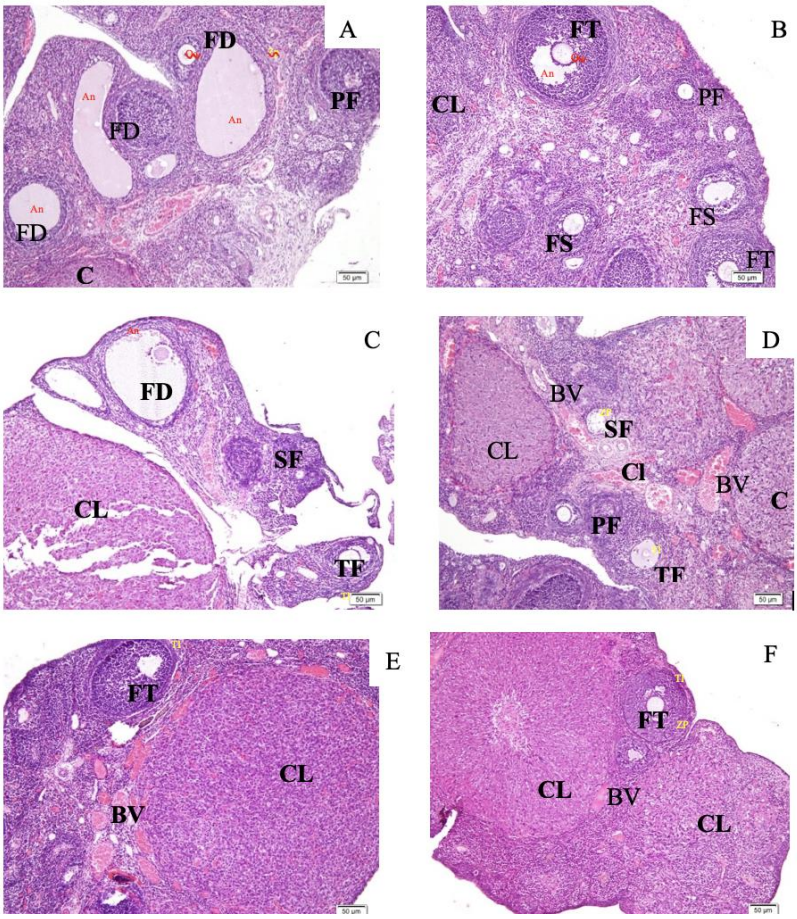

Figure 1. Classification of follicular development ;

A) Group I ovarian on 7thday, B) Group I ovarian on 14thday, C) Group II ovarian on 7thday, D) Group II ovarian on 14thday, E) Group V ovarian on 7thday, F) Group I ovarian on 14thday, HE Staining, 100x Magnification.CL: Corpus luteum (the oocyte had been ovulated), FP: Primary follicle, oocytes surrounded with a single layer of qubodial granulosa cells, FS: secondary follicle, has two cuboidal granulosa cell layers with antrum, FT: Follicle tertier, has more cuboidal granulosa cell layers with antrum FD: Follicle de graff, has large antrum and, C: congestion, V: blood vessel.

Numerous studies reported that the accumulation of *B. petersianum* affects the number of follicles actively developing (Gadelha et al., 2014; Talakua et al., 2020). Folliculogenesis, the process of follicle growth and development in the ovaries, is related to the influence of estrogen on the ovaries. The increase in follicle number may be due to the presence of specific substances in *B. petersianum* that affect the secretion of gonadotrophin hormones, such as Follicular-stimulating hormone (FSH) and Luteinizing hormone (LH), and phytoestrogenic effect followed by antioxidant properties from *B. petersianum* substances (Simatauw & Unity, 2019; Talakua et al., 2020; Effendi., 2022).

Effect of *B. petersianum* on The Uterine Thickness

In the overall development of the uterine, estrogen plays an essential role in uterine proliferation (Chung et al., 2015). To find out more about the effect of *B. petersianum* as phytoestrogens on the uterine organs of rats, an analysis of the uterine thickness and histomorphology of the uterine were performed, as shown in Table 4 and Figure 2.

Table 4 showed that the uterine wall thickness of group II and the fraction were higher than group I (negative group). However, only rats from groups II and IV showed significantly different results (P<0,05) compared to group I on the seventh day. Different things were seen on day 14th. Almost all groups were significantly different (P<0.05) compared to group I except group VI. In our study, control positive and a fraction of n-hexane can affect the uterine thickness earlier than the other treatment. The effect is stable until day 14th. However, the highest uterine wall thickness was exhibited by group V, and this result was not significantly different (P<0.05) compared to group II (positive control).

Table 4. The mean values of uterine thickness

Treatment	Uterine thickness (**µm**±sd)	
	H+7	H+14
Group I	441,79±30^{bc}	554,12±72^{bc}
Group II	782,52±79^{f}	717,98±61^{ef}
Group III	417,06±17^{bcd}	688,72±205^{def}
Group IV	462,66±64^{a}	645,29±140^{cde}
Group V	567,26±29^{bcd}	707,56±32^{ef}
Group VI	478,33±52^{ab}	553,40±34^{bc}

Group I (negative control), Group II (positive control;Ethynil estradiol 0,045 mg/kg BW), Groups III (extract grass 2,16 mg/kg BW), Group IV (n-hexane fraction 0,094 mg/kg BW), Group V (ethyl acetate fraction 0,1035 mg/kg BW), and Group VI (water fraction 0,199 mg/kg BW) of Biophytum petersianum. different superscript letters folowing the value (mean ± SD) in the same column indicate significant difference (P<0.05).

The increase of uterine thickness could be to specific substances in *B. petersianum* that increase estradiol to stimulate proliferation in the uterine. Previous research data showed that providing *B. petersianum* extract can also increase the level of 17β-estradiol in mice blood (Pasaribu and Indyastuti. 2004). Estradiol increases by thicker endometrium (Glamour et al., 2018).

Various studies of the effects of plant extracts on fertility have shown that the plant extract may affect one or more sites of the reproductive tract or the reproductive endocrine axis of female animals. This study showed that *B. petersianum* administration affected the ovaries and uterine of rats. The phytoestrogen content is predicted to improve reproductive function (Effendi. 2022). Flavonoids and saponin are reported as phytoestrogen compounds in Biophytum petersianum; according to Mari et al. (2015), flavonoids of this plant have a high affinity for estrogen receptors (ERs) and are beneficial to improve women's sexual health.

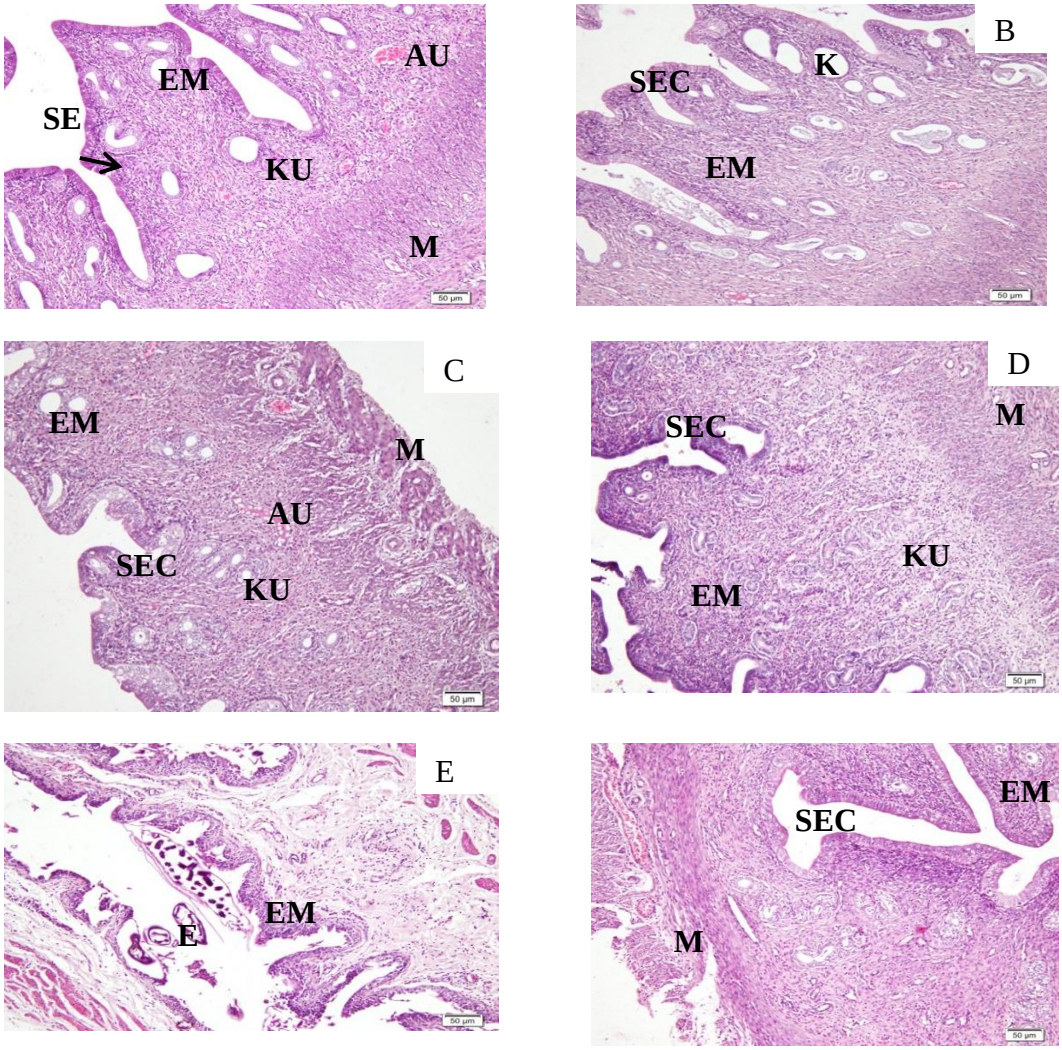

Figure 2. Histology of the Rat Uterine

HE Staining, 100x Magnification, A) Group I ovarian on 7th day, B) Group I ovarian on 14th day, C) Group II ovarian on 7th day, D) Group II ovarian on 14th day, E) Group V ovarian on 7th day, F) Group I ovarian on 14th day, EM: endometrium, M: Myometrium, SEC: mucosal cuboid epithelial cells, AU: uterine arteries, KU: uterine glands, E: embryo

B. petersianum has various antioxidant properties, namely flavonoids, saponins, tannins, vitamin E, and vitamin A (Unity & Inara, 2011; Lefaan. 2014). These bioactive compounds have antioxidant characteristics, can regulate several enzymes in steroidogenesis, act as reactive oxygen species (ROS) scavenging agents in ovary cells, or can regulate the production of hormones in the ovary (Mbemya et al.,2017). In addition, nutrients and amino acids contained play a role estrogenic effect.

The active components contained in *B. petersianum* have different levels of polarity. The amount of active chemicals each plant produces and accumulates in its tissues differs depending on the polarity of the solvents it attracts (Masaya et al.,2017). In our study, Group V (fraction of ethyl acetate) generally showed the highest effect on the ovaries and uterine parameters. It indicates that semipolar compounds play an essential role in the effectiveness of *B. petersianum* grass as a phytoestrogen. Unfortunately, any information related to types of secondary metabolites partaking in such a mechanism still needs to be reviewed further.

CONCLUSION

In this study, the administration of *B. petersianum* extract and fraction affected ovarian and uterine weights and ovarian and uterine histology compared to negative controls. The ethyl acetate fraction of the (group V) treatment showed more effectiveness than the other treatment.

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