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The Effects of Calina Papaya Leaf Ethanol Extract On Estrus Cycle And Uterus Morphology of Wistar Rats

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Abstract. Calina papaya leaf is an Indonesian papaya plant type that has the potential to be used as a medicinal plant. Phytochemical compounds found in papaya leaves act as antifertility agents. The research aimed to study the antifertility effect of Calina papaya leaf ethanol extract on estrus cycle and uterine morphology of wistar rats. The experiment lasted 30 days and involved 25 female wistar rats. The treatment consisted of 5 groups, namely K (distilled water), K+ (contraceptive pill), P1 (Calina papaya leaf ethanol extract 200mg/KgBW), P2 (Calina papaya leaf ethanol extract 300mg/KgBW), and P3 (Calina papaya leaf ethanol extract 400mg/KgBW). The parameters of estrus cycle consisted of estrus cycle phase, total cycle and estrus cycle ratio. Uterus morphological parameters consist of wall thickness of the endometrium, myometrium, and perimetrium. The estrus cycle examination was carried out every day using the vaginal smear method (Giemsa 5%). On the 31st day, surgery was performed and the uterus was taken for histological observation using the paraffin method (HE staining). Data were analyzed using One Way ANOVA with further Duncan's test. The results showed that a dose of 400 mg/KgBW could affect the estrus cycle with accelerate the estrus phase and increase the estrus cycle ratio compared to the control ($P<0.05$). There was a decrease in endometrium and myometrium wall thickness at a dose of 400 mg/KgBW compared to the control ($P<0.05$). The conclusion of the study is that the ethanol extract of Calina papaya leaves at a dose of 400 mg/KgBW has the potential to be a natural antifertility agent on female wistar rats.

Key words: Estrus Cycle, Uterus, Antifertility, Calina Papaya Leaf Extract, Wistar Rat

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INTRODUCTION

Indonesia is the fourth most populous country in the world after China, India and the United States. In 2020 Indonesia has a projected population growth of 270 million people and an increase of 32.56 million people with an annual population rate of 1.25% (Badan Pusat Statistik, 2020). The increase in population year after year has caused problems with clothing, food, and housing. Jobs that are difficult to obtain or pay a low wage might lead to a lot of unemployment, poverty, and unfulfilled life needs. The government has implemented Family Planning (KB) initiatives in an attempt to control population density. One of these projects is to use contraception to minimize pregnancy rates. There are several methods of contraception, namely by using a device or by taking drugs. Condoms, IUDs or spirals, and implants are all regularly used methods of contraception (Zaman et al., 2019; David et al., 2019). Drug-based contraception is available in the form of injections and contraceptive pills. The usage of numerous contraceptive methods is still being complained by the public because some of them induce side effects such as menstrual disorder obesity, stomach discomfort, and headaches (Adani et al., 2017).

One of the efforts to reduce the side effects of contraceptive drugs is to look for alternatives such as herbal-based contraceptives. The use of herbal medicines has low side effects and toxicity, and can be cultivated continuously (Shaik et al., 2017). *Pandanus odoratissimus* extract contains alkaloids, flavonoids, tannins, terpenoids, and saponins that can be used as natural contraceptives by antiimplantation and antiestrogenic in test animals (Kumar et al., 2017). Triterpenoid compounds and steroids in herbal plants can suppress fertility levels by disrupting the ovaries, uterus, and vagina (Putriani & Sumarmin, 2018). The content of benzyl isothiocynate (BITC) in papaya seed extract has also an effect on female rats' uterine tissue, reducing the thickness of the myometrium wall (Adebiyi et al., 2003). The composition of the endometrial epithelium can change from ciliated stratified cuboidal epithelium to stratified cuboidal epithelium when *Gossypium herbaceum* extract is used (Dolatab et al., 2014).

Indonesia has a very high and diverse biodiversity and potential of herbal plants. Papaya plant is an example of a plant that grows in Indonesia and has potential as a natural herbal ingredient. One of the papaya varieties that are widely developed in Indonesia is the California or Calina papaya. The Calina papaya was developed by the Bogor Agricultural University's Center for Tropical Fruit Studies (PKHT-IPB) (Ismaya et al., 2019). Papaya leaves contain alkaloid compounds, flavonoids, tannins, and glycosides (Mahatriny et al., 2014; Ashok et al., 2017). The phytochemicals such as flavonoids and alkaloids has phytoestrogen effect by causing negative feedback on reproductive hormones. The phytoestrogens effect can inhibit the follicle stimulating hormone (FSH) and luteinizing hormone (LH) on the reproductive cycle (Setiawan et al., 2021). Inhibited hormone production can affect the reproductive cycle and inhibit fertilization, such as accelerating the estrus phase and decreasing the thickness of the uterine endometrium. A long estrus cycle with a fast estrus phase (ovulation) causes a shorter maturity time. The inhibition of fertilization is also caused by the thin morphology of the uterine endometrium, which results in failure of implantation of the embryo. There is no preclinical evidence that Calina papaya leaf has the ability to influence female rat reproduction. The aim of this study was to determine Calina papaya leaf extract effect on the estrus cycle and uterine morphology in wistar rats as a candidate for natural antifertility medication molecules. The advantage of the research was expected to be used as a source of information that Calina papaya extract can be used as a contraceptive agent for women.

METHODS

The research was conducted from January 2021 to June 2021 at the Laboratory of Animal Structure and Physiology, Universitas Ahmad Dahlan, Yogyakarta. This research has been approved by the Ethics Committee of Universitas Ahmad Dahlan with letter number 012102008. Calina papaya leaves were taken at Sewon papaya Farm, Bantul, DIY. The Calina papaya plant was identified at the Plant Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada (No: 014900/S.Tb/x/2020) with the scientific name *Carica papaya* L.

Calina papaya leaves ethanol extract

Papaya leaves with dark green characteristics were sorted, then washed under running water to remove the dirt and dust and drained. The leaves were cut into small pieces, then dried and blended, so that 770 gr of simplicia powder was obtained. The simplicia powder was weighed and extracted with 96 % ethanol using the maceration process. The simplicia was sosaked for 3 x 24 hours before being filtered to obtain the macerate. Macerate had already been evaporated with a rotary evaporator at 50-70°C, yielding a semi-solid viscous extract weighing 193 grams and having a blackish green color. The extract was then analyzed for phytochemical compounds in the Pharmacy Biology laboratory, Faculty of Pharmacy, Universitas Gadjah Mada using Thin Layer Chromatography (TLC). TLC was used to look for phytochemicals in papaya leaf extract, which included alkaloids, flavonoids, triterpenoids, tannins, and saponins.

Preparation of test animals and provision of treatment

The test animals used were 25 female wistar rats aged 15 weeks weighing ± 200 grams. The rats were acclimatized for 30 days in a 50x40cm rearing room, temperature 19-25℃, humidity 40-70%, and husk changes were carried out every 3 days. The feeding was in the form of BR2 pellets and drinking water *ad libitum*. The treatment consisted of 5 groups, i.e. K (distilled water), K+ (The "Andalan" brand contraceptive pill with an ethinylestradiol 0.03 mg and levonorgestrel 0.15 mg) by following the daily dose in humans and converted to a rat dose, P1 (Calina papaya leaf ethanol extract 200mg/KgBW), P2 (Calina papaya leaf ethanol extract 300mg/KgBW), and P3 (Calina papaya leaf ethanol extract 400mg/KgBW). All treatment doses were dissolved using 1 ml of distilled water oral gavage for 30 days. Wistar rats were weighed once a week using a digital scale to determine body weight.

Observation of the estrus cycle

The estrus cycle was observed using a vaginal smear by applying a cotton bud that had been moistened with 0.9% NaCl (Ngadjui et al., 2013). Vaginal smear treatment was carried out at every 10.00 WIB. The vaginal walls of the rats were gently rubbed and smeared using cotton bud. For 30 days, the findings of vaginal reviews were smeared in one direction on the object glass every 24 hours. After that, the item glass was dripped with 5% Giemsa and left for 5-10 minutes (Sitasiwi & Mardiati, 2016). The vaginal smears were then cleaned and dried under running water. The vaginal smear were then examined with an Olympus Cx21 microscope (100x magnification) and photographs were made with Optilab and Images raster (Mohammed & Sundaram, 2018). Observations consisted of the phase of the estrus cycle, the total length of the estrus cycle, and the ratio of the estrus cycle (Adeniyi & Agoreyo, 2019). The estrus cycle ratio was calculated by dividing the number of proestrus and estrus phases by the number of metestrus and diestrus phases with the following formula:

Estrous Cycle Ratio (ECR) = $\frac{(proestrus+estrus)}{(metestrus+diestrus)}$ (Adeniyi & Agoreyo, 2019).

Histological preparation of uterus

On day 31, rats were sacrificed under anesthesia using 10% ether and neck dislocation was performed. Rats were dissected using a dissection set and uterine organs were taken. The uterus was then weighed using a digital scale and cut crosswise for ± 1 cm. The uterus was inserted into Buffer Neutral Formalin 10% (BNF) solution for 12 hours. Furthermore, it was then prepared using the paraffin method which consisted of stages of dehydration, infiltration, sectioning, and mounting. A rotary microtome with a thickness of 5-6 m was used to cut the uterus. Hematoxylin and Eosin stain were used to stain the slides. The uterus were then examined under a 100x magnification using an Olympus Cx21 microscope. Optilab and Image Raster were used to measure the endometrium, myometrium, and perimetrium thickness.

Data Analysis

All data parameters were analyzed using one way ANOVA (P < 0.05) and followed by a post hoc test using Duncan's test (P < 0.05) to determine significant differences between treatments.

RESULTS AND DISCUSSION

Phytochemical compounds of Calina papaya leaf ethanol extract

The results of the phytochemical compound content test showed that Calina papaya leaf ethanol extract was known to contain several compounds such as alkaloids, flavonoids, triterpenoids, tannins, and saponins (Table 1). Ethanol extract of papaya leaves also contains groups of alkaloids, flavonoids, glycosides, tannins, triterpenoids, steroids, and saponins (Mahatriny et al., 2014; A'yun et al., 2015). Compounds such as flavonoids, tannins, and saponins have the potential and ability as natural antifertility agents. These compounds can accelerate the estrus phase, inhibit ovulation, and affect the endometrial wall of female rats (Singh et al., 2018).

Table 1. Phytochemical compounds of Calina papaya leaf ethanol extract

Compound	Detection	Results	Description
Alkaloid	Dragendorff	+	Orange color
Flavonoid	Citroborate	+	Yellow color
Triterpenoid	Anisaldehyde	+	Brick red color
Tanin	FeCl3	+	Green color
Saponin	Liebermann-Burchard (LB)	+	Black color

Note: (+) positive: contains a class of phytochemical compound

Estrus cycle

Research showed that the ethanol extract of Calina papaya leaves affects the length of each phase of the estrus cycle of adult female wistar rats. The treatment of papaya leaf ethanol extract affected each of the cycle phases consisting of proestrus, estrus, diestrus, and metestrus (Figure 1).

Table 2. Estrus cycle of female wistar rats after receiving Calina papaya leaf ethanol extract

Variable	Treatment				
	K	K+	P1	P2	P3
Proestrus (%)	20.67 ± 2.78[a]	46.00 ± 2.25[c]	32.67 ± 5.48[b]	39.34 ± 7.60[bc]	46.00 ± 10.65[c]
Estrus (%)	24.00 ± 2.78[c]	14.67 ± 6.50[ab]	19.33 ± 5.48[bc]	16.00 ± 2.78[ab]	10.00 ± 5.27[a]
Metestrus (%)	18.00 ± 8.70[a]	33.33 ± 6.67[b]	16.67± 5.27[a]	24.67 ± 5.01[ab]	32.67 ± 7.02[b]
Diestrus (%)	34.67 ± 10.16[c]	8.00 ± 5.57[a]	32.00 ± 11.20[c]	19.33 ± 5.47[ab]	25.33 ± 9.30[bc]
Estrus Cycle (times/month)	6.00 ± 0.70[c]	4.20 ± 0.83[ab]	5.00 ± 1.22[bc]	3.80 ± 0.83[ab]	3.20 ± 0.83[a]
Estrus Cycle Ratio	0.83 ± 0.12[a]	1.63 ± 0.42[b]	1.23 ± 0.52[ab]	1.34 ± 0.48[ab]	1.63 ± 0.39[b]

Note: Numbers followed by different letters in the same line shows that there is a significant difference (P>0.05); K (distilled water), K+ (Contraceptive pills 14.184mg/KgBW), P1 (Papaya Leaf Ethanol Extract 200 mg/KgBW), P2 (Papaya Leaf Ethanol Extract 300 mg/KgBW), P3 (Papaya Leaf Ethanol Extract 400 mg/KgBW). Mean ± SD

The results (Table 2) shows that the dose of 400 mg/Kg BW is optimum in prolonging the proestrus phase of wistar rats (P<0.05) by 46.00 ± 10.65%. The metestrus phase also showed that the dose of 400 mg/KgBW showed the highest result (32.67 ± 7.02%) compared to the other treatments. The shortest percentage of the diestrus phase was found in the doses of 300 mg/BW and K+ compared to the other treatments (P <0.05). It is suspected that the administration of papaya leaf ethanol extract prolongs the proestrus, metestrus, and speed up diestrus phases. The duration of the three phases in one cycle shows that the folliculogenesis stage is relatively long, so that the ovulation mechanism is inhibited and affects the fertility of rats (Sharma et al., 2013).

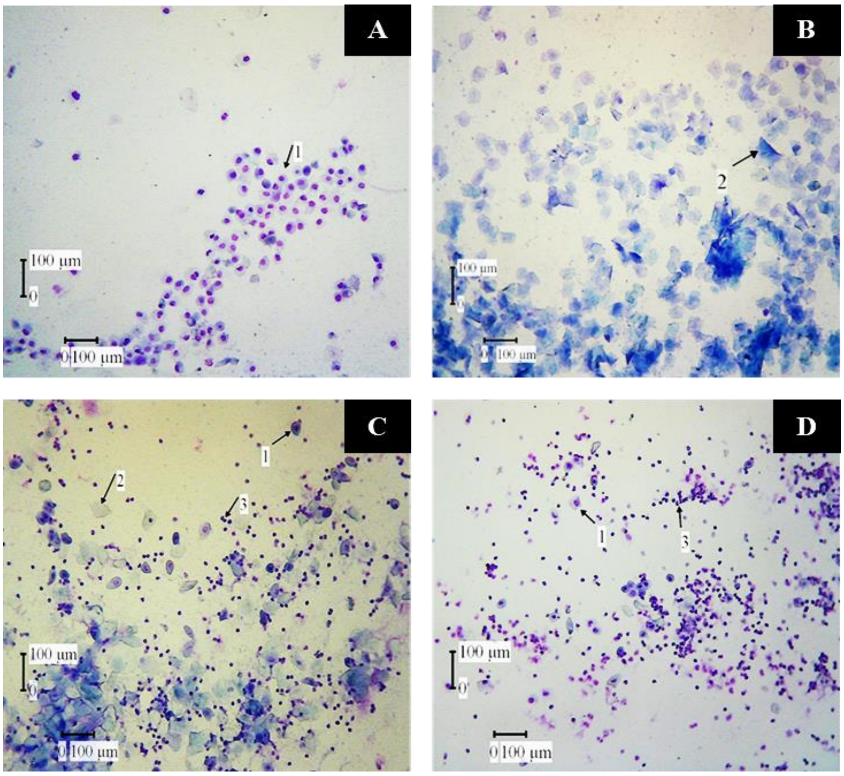

Figure 1. Vaginal smears of adult female rats treated with papaya leaf ethanol extract with Giemsa staining at 10x10 magnification (A. Proestrus; B. Estrus C. Metestrus; D. Diestrus; 1. Nuclear epithelium; 2. Cornified epithelium; 3. Leukocytes)

The data results in the estrus phase showed that the dose 400mg/KgBW (10.00 ± 5.27 %) had the shortest estrus phase compared to the control (P<0.05). These data show that papaya leaves accelerate the estrus phase which plays a role in rat fertility (ovulation period). The relatively fast estrus phase makes ovulation time and fertility period relatively shorter. The results showed that the ethanol extract of Calina papaya leaves shortened the estrus phase and extended the proestrus, metestrus, and diestrus phases for 30 days of treatment. The results of the data also showed that the ethanol extract of papaya leaves had the same effectiveness as contraceptive pills of ethinylestradiol 0.03 mg and containing levonorgestrel 0.15 mg to shortening the estrus phase (Table 2).

The ethanolic extract of papaya leaves contains compounds from the group of alkaloids, flavonoids, glycosides, tannins, triterpenoids, steroids, and saponins (Mahatriny et al., 2014; A'yun et al., 2015). The content of flavonoids has an estrogenic effect by increasing estrogen levels in the blood, thereby causing negative feedback to the hypothalamus to inhibit the formation of Gonadotropin Releasing Hormone (GnRH), suppress pituitary stimulation and inhibit the FSH secretion (Jain et al. 2015, et al. 2017; Adani et al. 2017). Low FSH levels result in delayed development and reduce the number of ovarian follicles so that LH levels are low (Mustapha et al., 2011). The short estrus phase can be influenced by triterpenoid and saponin compounds contained in the ethanol extract of papaya leaves by hormonal disturbances (Hakameri et al., 2020). Triterpenoids and saponins can bind to estrogen receptors and trigger interactions with Estrogen Response Element (ERE) in triggering the expression of estrogen responsive genes in estrogen synthesis (Men & Zong., 2019). Increased levels of estrogen in the blood that are too high can cause negative feedback, thereby suppressing the ovulation mechanism (Jain et al., 2015; Zhang et al., 2020). The ethanol extract of papaya leaves contains flavonoids, triterpenoids, and saponins, which are thought to be able to lengthen the proestrus, metestrus, and diestrus phases, as well as shorten the period between ovulation and the estrus phase.

The estrus cycle with a dose of 400 mg/Kg BW had fewer cycles than the other treatments (P<0.05) with 3.20 ± 0.83 times/month (Table 2). These results indicate that the estrus cycle at dose 400 mg/kg BW was relatively long, ie for 30 days experienced 3.20 ± 0.83 cycles compared to the control group which experienced 6.00 ± 0.70 cycles. The relatively long total estrus cycle is related to hormonal disruptions that cause the duration of each phase of the estrus cycle to be irregular, thereby limiting ovulation and decreasing the chances of conception (Mustapha et al., 2011). During the 30 day treatment, it is suspected that the estrus cycle, which is disrupted by reproductive hormones, causes an extension of the overall estrus cycle. The estrus cycle ratio was used to predict the intervals of folliculogenesis, luteogenesis, and luteolysis. An increase in the ratio of the estrus cycle can indicate the follicular phase and delayed ovulation (Adeniyi & Agoreyo, 2019). The results of the data showed that the dose of 400 mg/Kg BW had a higher ratio than the control ($P<0.05$). It is suspected that the ethanol extract increases the luteal phase compared to the follicular phase, so that the development of new folliculogenesis can be inhibited. The results of the data (Table 2) that the Calina papaya leaf ethanol extract has the same ability to affect the estrus cycle with a short estrus phase (follicular phase) and increase the ratio of estrus cycles compared to contraceptive pill.

Uterus morphology

The thickness of the endometrium and myometrium of uterus female wistar rats was influenced by an ethanolic extract of Calina papaya leaves, according to the study. The results revealed that the optimum dose of 400 mg/KgBW had an effect on endometrium and myometrium thickness (Table 3) (Figure 2). Thick changes in the endometrium are thought to be due to the content of antifertility compounds in the ethanol extract of papaya leaves. Several phytochemical compounds in plants are thought to have the potential to suppress fertility levels by interfering the uterus development (Malpani et al., 2020). The alkaloid compound in the ethanolic extract of Calina papaya leaves are estrogenic and antiproliferative. Antiproliferative properties are thought to inhibit the proliferative process in the endometrium, thereby causing a decrease in endometrium thickness (Kumar & Devi, 2017). The estrogenic properties of the alkaloids are thought to be similar to the synthetic base materials used for contraceptive pill contraception. The estrogenic effect occurs because of the binding between phytoestrogens and the estrogen receptor and triggers the synthesis of estrogen. Increased levels of estrogen will reduce the secretion of FSH and LH. FSH and LH are involved in the production of estrogen and progesterone in the ovaries (Yang et al., 2019). Inhibition of the proliferation of cells that make up the uterus wall, as well as interference with the function of the hormone progesterone in the thickness of the endometrium and the development of blood vessels in the endometrium, would arise from disrupted estrogen synthesis (Hakameri et al., 2020).

Table 3. Uterus ratio and morphology after receiving Calina papaya leaf ethanol extract

Variable	Treatment				
	K	K+	P1	P2	P3
Uterus weight (g)	0.35±0.14^{a}	0.22±0.08^{a}	0.36±0.28^{a}	0.17±0.12^{a}	0.34±0.20^{a}
Final Body Weight (g)	179.00 ± 42,71^{a}	163.80 ± 22.92^{a}	174.00 ± 29.84^{a}	168.00 ± 39.07^{a}	164.40 ± 21.41^{a}
Uterus/Final Body Weight Ratio	0.0020±0.0006^{a}	0.0013±0.0005^{a}	0.0020±0.0017^{a}	0.0009±0.0005^{a}	0.0021±0.0012^{a}
Endometrium thickness (µm)	229.73±4.18^{bc}	261.50±18.70^{c}	210.02±11.73^{ab}	189.93±19.61^{ab}	179.43±15.52^{a}
Miometrium thickness (µm)	87.21±22.97^{b}	82.96±8.71^{b}	68.86±23.74^{b}	63.51±8.81^{b}	54.28±12.66^{a}
Perimetrium thickness (µm)	109.26±24.31^{a}	106.04±13.55^{a}	95.76±29.68^{a}	91.59±12.05^{a}	96.66±27.38^{a}

Note: Numbers followed by different letters in the same line shows that there is a significant difference ($P>0.05$); K (distilled water), K+ (Contraceptive pills 14.184mg/KgBW), P1 (Papaya Leaf Ethanol Extract 200 mg/KgBW), P2 (Papaya Leaf Ethanol Extract 300 mg/KgBW), P3 (Papaya Leaf Ethanol Extract 400 mg/KgBW). Mean ± SD

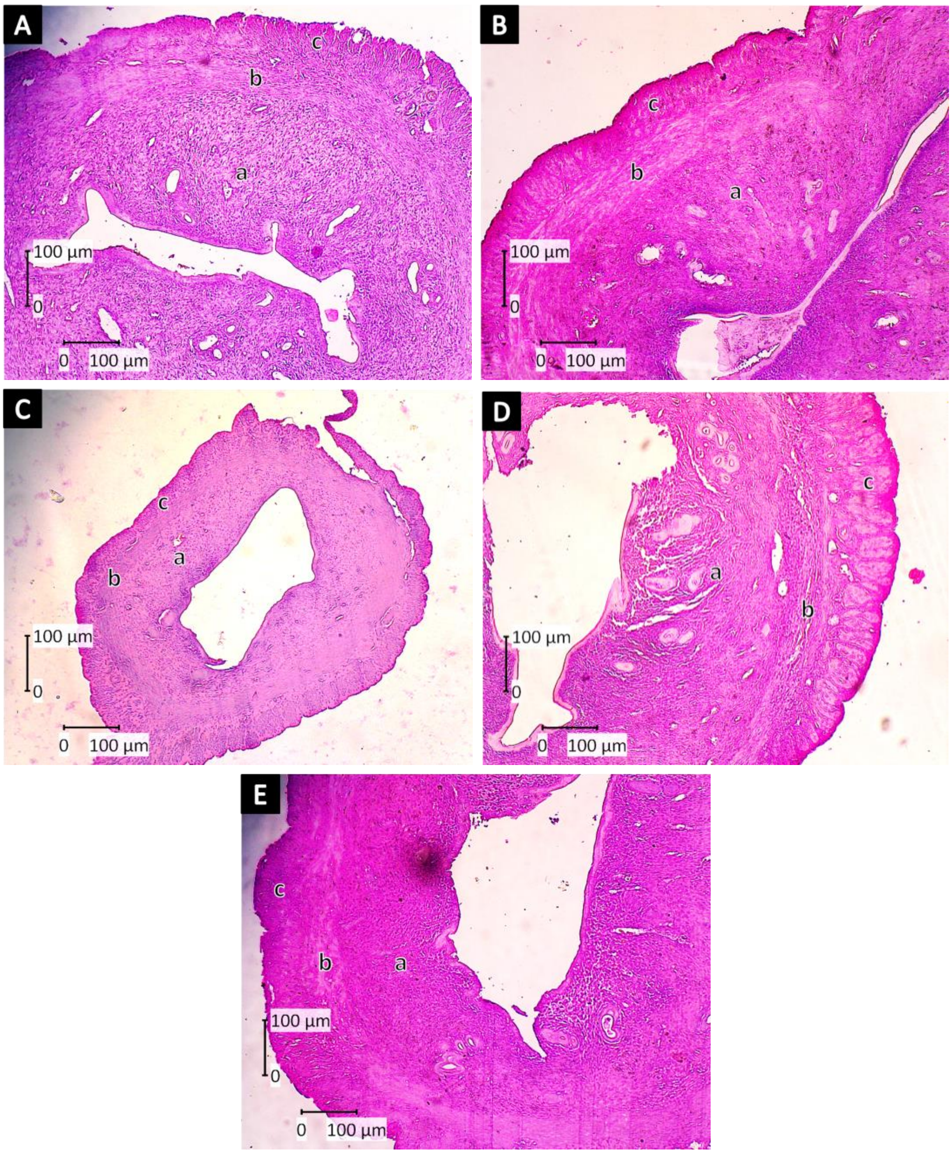

Figure 2. Histological structure of the uterus of wistar rats. HE staining. (A) K (Papaya Leaf Ethanol Extract 0 mg/KgBW); (B) K+ (Contraceptive pill); (C) P1 (Papaya Leaf Ethanol Extract 200 mg/KgBW); (D) P2 (Ethanol Extract of Papaya Leaves 300 mg/KgBW); (E) P3 (Papaya Leaf Ethanol Extract 400 mg/KgBW). (a) Endometrium; (b) Myometrium; (c) Perimetrium.

Triterpenoid compounds can suppress fertility levels by interfering with uterine function. Triterpenoids act on the axis of the hypothalamus, pituitary, and gonads so that they affect the secretion of gonadotropin hormones. Impaired gonadotropin secretion can lead to decreased GnRH production (Akbar et al., 2018). This decrease in GnRH production will affect the secretion of FSH and LH, where FSH and LH secretion can decrease and affect the synthesis of the hormones estrogen and progesterone in the ovaries, so that cell proliferation in the uterine wall is also disrupted and there is a decrease in endometrium thickness (Satria et al., 2018). Antiestrogens are considered to be synthesized in the body from saponins and flavonoids. They work as estrogen antagonists, preventing the hormone's synthesis or effect. Antiestrogens make the ovaries inactive, preventing follicle formation and ovulation due to decreased estrogen release (Kaur et al. 2020) The antiestrogen action also induces endometrial atrophy (Satria et al., 2018).

Myometrium thickness at dose of 400 mg/kg BW was significantly different from the control (P<0.05) (Table 3). The impact of saponin and flavonoid chemicals on papaya leaves is suggested to be the reason for this condition. Saponins are hypothesized to inhibit cell proliferation and cause the myometrium to lose smooth muscle cells. Flavonoid chemicals are considered to cause cell membrane disruptions by altering the components that make up cell membranes and disturbing membrane physiological functions, resulting in membrane damage. Flavonoids, which impede cell proliferation, cause the myometrial smooth muscle membrane to shrink, resulting in a decrease in myometrial thickness (Handayani & Gofur, 2017).

Table 3 shows that there is no significant difference in the final body weight of wistar rats (P>0.05). The administration of papaya leaf ethanol extract did not affect the physiological changes and growth of wistar rats. It is suspected that the content of phytochemical compounds in the extract is still within safe limits and does not affect the appetite of the rats. Table 3 also shows that there is no significant difference in uterine weight and the ratio of uterus to final body weight in all treatments (P>0.05). Many factors affect uterus weight and ratio such as uterus wet weight, endometrial thickness, fat content, age of experimental animals, body weight of experimental animals, and levels of hormones produced by the uterus glands (Memudu & Oluwole, 2021). It is suspected that the ethanol extract of Calina papaya leaves at all doses has not been able to affect the weight and uterine ratio of wistar rats. Calina papaya leaf ethanol extract has potential as a natural antifertility agent in inhibiting the reproductive cycle of female rats. This preclinical research can be a reference for creating herbal contraceptive drugs that are safe to be applied to women in suppressing the population rate in Indonesia.

CONCLUSION

The study concluded that an ethanolic extract of Calina papaya leaves at a dose of 400 mg/kgBW has the potential to be a natural antifertility agent with a short estrus phase (follicular phase), increasing the ratio of estrus cycles, and reducing the thickness of the endometrium and myometrium on female wistar rats.

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