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Effect of Ethanol Extract of Cherry (*Muntingia calabura* L.) Leaves on the Estrous Cycle in Female Mice (*Mus musculus*)

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Abstract

Cherry (*Muntingia calabura* L.) leaves contain primary secondary metabolites in the form of flavonoids that have the potential to inhibit fertility by interfering with ovarian, uterus, or vaginal function. One of the parameters that can be used to assess the antifertility potential of a compound or herbal extract is the estrous cycle. This study aimed to determine the effect of cherry leaves ethanol extract intake on the estrous cycle in female mice (*Mus musculus*). This study used a Complete Random Design (CRD) with 3 treatments, namely control of Na CMC 1% (C), cherry leaves extract at a dose of 125 mg/kg of mice body weight (T1), and 250 mg/kg of mice body weight (T2), each with 3 replicates. The results showed that cherry leaves ethanol extract did not have a significant effect on the length of the estrous cycle of mice analyzed by the Kruskal–Wallis test (*Asymp value. Sig* > 0.05), although there is a tendency for the estrous cycle in the treatment group to be longer than the control group. This study provides insights for follow-up research with more diverse doses, treatment duration, and reproductive parameters in determining the antifertility activity of an herbal metabolite compound.

Keywords: antifertility; estrous cycle; *Muntingia calabura*; *Mus musculus*.

INTRODUCTION

Indonesia is a developing country with a fairly high population density. The family planning (KB) program is one of the government's steps to suppress the high rate of population growth. Hormonal contraceptives are a type of contraception that is commonly used in Indonesia. However, hormonal contraceptives often cause uncomfortable side effects, so it is necessary to develop natural contraceptives. Cherry (*Muntingia calabura* L.) is one of the plants with potential as an antifertility agent. In Rezeki et al (2023), The cherry plant contains alkaloid compounds, tannins, saponins, and flavonoids. Flavonoids are the main constituents contained in cherry (*Muntingia calabura*) leaf.

Flavonoids have estrogenic effects and can function similarly to estrogen by binding to estrogen receptors. As phytoestrogens, flavonoids can prevent natural estrogens from binding to their receptors, thereby increasing the amount of free estrogen in the bloodstream (Shanmugaloga & Shilpa, 2024). An increase in the hormone estrogen provides a positive feedback to the anterior pituitary to produce LH thus helping to speed up the release of eggs (ovulation) (Ma et al., 2023).

The menstrual cycle in primates is identical to the non-primate estrous cycle. The estrous cycle is a physiological cycle that occurs in non-primate female animals and can be used as a parameter to assess the antifertility potential of a compound or herbal extract (Kasyoki et al., 2022). The estrous cycle is divided into four phases, namely proestrus, estrus, metestrus, and diestrus (Das et al., 2023). Phase identification is carried out with vaginal smears, which is taking vaginal fluid from mice, which is then microscopic observation of cell conditions to find out the phase that the mice are experiencing. The proestrus phase is characterized by an abundance of nucleated epithelial cells. The estrus phase is characterized by epithelial cells that have undergone cornification (*cornified cells*) so that the nucleus is no longer visible. The metestrus phase is characterized by the discovery of cornified epithelial cells, nucleated epithelial cells, and a small number of leukocytes. The diestrus phase is characterized by the predominance of leukocytes (white blood cells) and a small sized nucleated epithelium cells (Sekulovski et al., 2025).

According to previous research by Kiyama (2023), The content of flavonoids has an estrogenic effect that can accelerate the estrus phase by accelerating the release of eggs (ovulation) and prolonging the diestrus phase in one cycle. This study aimed to determine the effect of ethanol administration of cherry (*Muntingia calabura*) leaves extract on the estrous cycle of female mice (*Mus musculus*).

MATERIALS AND METHODS

Study area

This study used the complete random design method (CRD) with female mice (*Mus musculus*) as model animals. The female mice (*Mus musculus*) used amounted to 9 animals with criteria such as healthy, not physically disabled, 3-4 months old, weighing 27-33 grams, and not pregnant. The study lasted for 30 days, consisting of 15 days of acclimatization and 15 days of treatment. The research was conducted in the sub-biology and sub-chemistry laboratory of the UNS Integrated Laboratory. This research used tools and materials to support its implementation. The tools used were knives, digital scales, basins, ovens, blenders, glass jars, aluminum foil, glass stirrer, strainers, glass funnels, rotary evaporators, bottles, microscopes, sondes, watch glass, glass objects, glass covers, microscopes, cotton buds, mice cage. The materials used in this study are cherry leaves, 96% ethanol, 1% Na CMC, 70% alcohol, methylene blue, aquades, husks (wood powder/rice), mice feed.

Procedures

Cherry leaves extraction

Cherry leaves extract was obtained using the maceration method. Maceration is a method of obtaining phytochemical active ingredients in which plants are extracted using solvents (Gori et al., 2020). Extraction began with washing the leaves with running water to remove dirt and dust. The leaves were cut into smaller pieces, and dried in an oven at 40-50°C, then mashed with a blender and sifted with a 40-mesh sieve. After that, it was macerated using 96% ethanol liquid, that was, the leaves are weighed 300 grams then put into a maceration container then 96% ethanol is added until it was submerged and protected from sunlight, then closed and left for 5 days with occasional stirring in a closed glass jar to ensure that the solvent permeates evenly throughout the leaves powder. After that it was filtered to separate the filtrate and the pulp. The filtrate obtained was then collected and evaporated with a rotary evaporator at a speed of 60 rpm at a temperature of no more than 50°C so that a thick extract was obtained and stored.

Preparation of animals

The animals used were female mice aged 3-4 months with a body weight of 27-33 grams, in a healthy physical condition, not deformed, and in a condition that was not pregnant. Animals were adapted by being fed in the form of pellets and drinking water adlibitum (without limits) for 15 days in the biology sub-lab cage of the UNS integrated laboratory before being given treatment. All research procedures were analyzed and have been approved by the Health Research Ethics Commission of Dr. Moewardi Hospital, Surakarta, Indonesia No. 1.334/ VI/ HREC/ 2025.

Treatments of animals

Treatment was carried out for 15 days after the acclimatization period. Treatment was given using an oral probe and was divided into 3 groups, namely

- Control (C): Na CMC 1 %
- Dose 1 (T1): 125 mg/kg body weight of mice in solvents Na CMC 1%
- Dose 2 (T2): 250 mg/kg body weight of mice in solvents Na CMC 1%

To determine the dosage according to body weight, test animals were weighed daily.

Making vaginal smear preparations

The preparation was carried out every day for 15 days after the acclimatization period and the administration of the extract, the preparation of vaginal smear was carried out by applying a cotton swab (cutton bud) that has been moistened with 0.9% physiological NaCl into the vagina of mice. Then the results obtained were applied lightly to the one-way object glass. The spread was made 3 spreads. Then the preparation was dripped with *methylene blue*, left for 5-10 minutes. Then washed with running aquades and dried on bunsen. After that, the preparation was observed under an Optilab microscope with a magnification of 4-10x to determine the estrus phases that occur, namely estrus, metestrus, diestrus and proestrus.

Data analysis

After the preparation of the vaginal smear preparation, the identification of phases in the estrous cycle was carried out through the presence and qualitative number of vaginal epithelial cells (Ekambaram et al., 2017). The proestrus phase is a phase characterized by a large number of small to medium-sized nucleated epithelial cells and a few leukocytes. This phase signifies the coming of lust. The estrus phase is a phase characterized by the formation of cornified cells (horned cells) as an illustration of the many mitoses that occur in the vaginal mucosa. When the estrus phase is about to end, the vaginal lumen forms horned cells with degenerate nuclei. The metestrus phase is a phase characterized by the discovery of cornified epithelial cells and a few leukocytes. In this phase, the ovary contains the corpus luteum which contains lutein cells and small follicles that do not have a nucleus. The diestrus phase is a phase characterized by the dominance of leukocytes (white blood cells) and the appearance of nucleated epithelial cells (Ajayi & Akhigbe, 2020).

Data analysis was performed after tabulation of the average data of estrous cycle length per test animal. The length of estrous cycle was calculated from the time the animal experiences the estrus phase to before the next estrus phase. The data obtained from the results of the study were statistically processed using one way *Anova* to test whether there was a significant difference between

the average scores between groups at a significance level of 0.05. However, the data obtained was tested for normality and homogeneity first as a prerequisite test for the one way *Anova* test.

RESULTS AND DISCUSSION

Length of estrous cycle

The length of the estrous cycle in mice was analyzed by calculating the average length of time one estrous cycle lasted in each mouse in 3 treatment groups. The calculation starts from the estrus phase to the proestrus phase or before the next estrus phase. The average length of the estrous cycle in each group tended to be directly proportional to the dose given. The higher the dose given to the treatment group, the longer the estrous cycle in that group. The average data on the length of the estrous cycle is shown in Table 1.

Table 1. Average estrous cycle length in mice during treatment (15 days).

Number of Replications	Length of Estrous Cycle (X±SD)		
	C (Control)	T1 (125 mg/kg BW of mice)	T2 (250 mg/kg BW of mice)
1	5	5	5
2	4.6	5	6.5
3	4.5	5.5	5
Total	14.1	15.5	16.5
Average	4.7± 0.26	5.17 ± 0.29	5.5 ± 0.87

Note: Data presented as mean (X) ± Standard Deviation (SD)
BW = Body Weight

The data showed that dose group 2 (250 mg/kg of mice body weight) had the highest Mean Rank value, followed by dose group 1 (125 mg/kg of mice body weight) and the control group (lowest Mean Rank). The data was further analyzed using SPSS 16.0 software to test the presence of statistical influence. The first test carried out was the normality test. The results of the normality test showed that the data was not distributed normally, so the prerequisites for the one-way *Anova* test were not met. The next test is the Kruskal-Wallis test. The results of the Kruskal-Wallis test are shown in Table 2.

Table 2. Kruskal Wallis test.

	Length of cycle
Kruskal-Wallis	3.947
df	2
Asymp. Sig.	0.139

The results of the Kruskal-Wallis test show the value of Asymp. Sig (0.139) exceeds the significance level (α = 0.05). Therefore, there was no significant difference in the average length of the estrous cycle between the treatment groups. Although there was a difference in Mean Rank between treatment groups, the results of the Kruskal-Wallis test showed that the difference was not statistically significant.

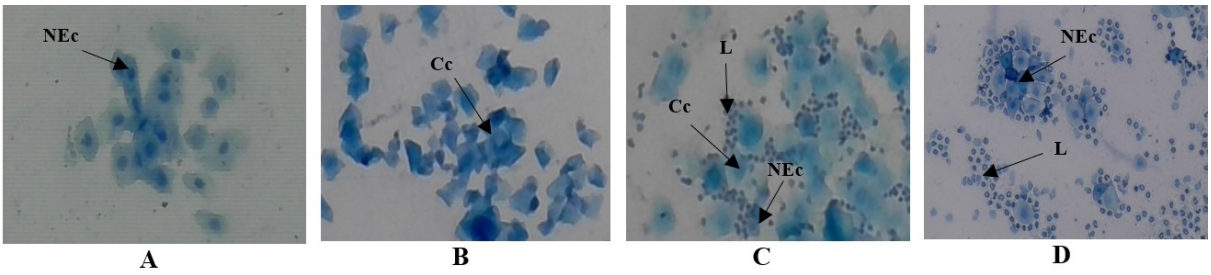

Figure 1. Observation of the phase of the estrous cycle using 10x magnification of Optilab.
Note: (A) Proestrus phase; (B) Estrus phase; (C) Metestrus phase; (D) Diestrus phase; (Nec) Nucleated Epithelial cells; (Cc) Cornified cell; (L) Leukosit.

Body weight of mice

This study used 3 different treatment groups in mice. Another focus of this study was to find out the extent to which treatment variations affected the difference in average weight changes observed in each group. Weight data was taken at the beginning and end of each group's treatment. The data obtained was then analyzed using one way *Anova* test. The weight data of mice at the beginning and end of treatment as well as weight changes are shown in Table 3.

Table 3. Body weight of mice during treatment (15 days).

Treatment	Initial treatment (X±SD)	Final treatment (X±SD)	Body weight changes
C (Control)	29.7±0.58	32±1.0	+2.3
T1 (Cerry leaves extract 125 mg/kg BW of mice)	29±1.0	31.3±2.08	+2.3
T2 (Cerry leaves extract 250 mg/kg BW of mice)	29.3±3.21	29±2.0	-0.3

Note: Data presented as mean (X) ± standard deviation (SD)
BW = Body Weight

The mice weight data showed a different response in each treatment group. The control and treatment group of dose 1 showed the same weight gain seen from the beginning and end of the treatment, which was 2.3 grams. Meanwhile, dose 2 (T2) group showed a reduction in the weight of mice by 0.3 grams.

The results of the one way *Anova* test showed that the average weight change between groups did not different significantly with *p-value* of 0.487 (*p-value* > 0.05). In statistic, the results of the analysis showed that the treatment given did not result in significant changes in body weight, and that the variation in the weight of the mice was more likely to be caused by natural variation between individuals than by the effects of the treatment.

Discussion

The results of the study showed that the treatment given had the potential to modulate reproductive activity in mice. However, the mechanism depends on the dose given. Although statistically there is no significant difference between treatment groups. The average cycle length in dose 2 group tends to be higher than that of dose 1 and control groups. This gives an indication that the bioactive components in cherry leaves extract may affect the hormonal dynamics that regulate the etruss cycle. Cherry leaves contain the main secondary metabolites, namely flavonoids (Sadino et al., 2022). Flavonoids have phytoestrogenic activity with the ability to bind to the ERα/ERβ estrogen receptor and have the potential to disrupt endogenous estradiol balance (Bolt et al., 2024). At high concentrations, such interactions can trigger different cellular responses than endogenous estradiol, be it proliferation stimulation (agonist effect) or inhibition of natural estrogen signals (antagonistic effect) depending on the cell type and dose (Patisaul & Jefferson, 2010).

Other secondary metabolites in cherry leaves are alkaloids, tannins, saponins, and terpenoids (Sadino et al., 2022). Alkaloids work as neuroactivators that are not directly related to the reproductive system and tend to play a role in cell protective and analgesic mechanisms (Zakaria et al., 2007). Meanwhile, tannins can interfere with the ovulation and implantation process in experimental animals through a decrease in the absorption of minerals such as Fe and Zn. Saponin compounds have detergent activity that affects ovarian cells and can modulate reproductive hormones. Terpenoids have a structure that can resemble steroids so they can act as phytoestrogens that affect the secretion of experimental animal reproductive hormones (Kuchekar et al., 2021)

Based on research (Mittelman-Smith et al., 2017), Modulation of estrogen receptors can alter gonadotropin secretion and affect the timing of ovulation including the duration of the estrus and diestrus phases in the test animals. This pattern is in line with the tendency to increase cycle length at dose 2 (250 mg/kg body weight of mice) which can physiologically be caused by disruption of LH peaks and delayed ovulation. Although the pattern of increasing estrous cycle length appears to follow the given dose level, statistically significant significance has not been achieved.

From the results of the analysis body weight of mice, it is known that the treatment given does not have a significant effect on the change in the weight of the mice. Although there was weight loss in dose 2 (250 mg/kg body weight of mice), the data on differences between treatment groups could not be said to be statistically significant. This insignificant difference can be caused because the dose given is still below the toxicity threshold and the dose given does not sufficiently affect the *appetite center* in the hypothalamus (Nasir et al., 2020). Body weight stability between treatment groups showed that cherry leaves extract at the tested doses did not cause acute systemic toxicity or massive disruption of nutrient metabolism in mice.

CONCLUSIONS

The use of cherry leaves ethanol extract in this study did not have a significant effect on the difference in the length of the estrous cycle of mice between groups. Although there was a tendency for the estrous cycle in the treatment group to be longer than the control group. Meanwhile, in the weight parameters, the treatment with the highest dose caused a weight loss of 0.3 grams. However, the weight loss cannot be called statistically significant difference.

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Authors' Contributions: Maulidatuz Zahro as a writer who designs research, conducts research, analyzes research data and writes research manuscripts. Harlita as a supervisor in every stage of research and manuscript writing. All authors read and approved the final version of the manuscript.

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