

ESTRUS CYCLE OF *Rattus norvegicus* EXPOSED TO CIGARETTE SMOKE AFTER TREATMENT ETHANOL EXTRACT OF GRASS (*Biophytum petersianum* Klotzsch)

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ABSTRACT

Kebar grass is a plant that contains phytoestrogens which have the same effect as endogenous estrogens which are thought to be able to increase endogenous estrogen levels in the blood due to the adverse effects of free radicals in the form of cigarette smoke. The purpose of this study was to determine the effect of the ethanol extract of kebar grass (*Biophytum petersianum* Klotzsch) on the length of the estrous cycle of *Rattus norvegicus* rats exposed to cigarette smoke. The research used an experimental method, namely a completely randomized design with four treatment groups and three replications. Rats the negative group was fed normally, the positive control group was exposed to cigarette smoke for 28 days, while the rats in the Kebar grass ethanol extract dosed group were given cigarette smoke exposure for 28 days, and were given Kebar grass ethanol extract for 28 days. Vaginal smears were taken in the morning with a span of 24 hours for 28 days. Changes in the vaginal epithelium are examined to determine the phase of the estrous cycle using a microscope. The data obtained were analyzed by Analysis of Variance (ANOVA)-SPSS version 16.0 followed by Duncan test with a 95% confidence interval ($\alpha=0.05$). The results showed that administration of Kebar grass ethanol extract was able to extend the estrous cycle time at a dose of 0.135 mg/head/day due to prolongation of the proestrus and estrus phases.

Keywords: *smoke, phytoestrogens, kebar grass, estrus.*

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INTRODUCTION

Reproduction is a physiological process that occurs in all living things in order to maintain offspring and survival. Things that support the success of the reproductive process are the reproductive organs and normal reproductive hormone levels. The reproductive organs will function when they reach puberty which is marked by the occurrence of the estrous cycle (Ganong, 1995).

The estrous cycle is a phase from estrus to the next estrus (Mulyono, 2005). Throughout the estrus cycle, there are reproductive hormones that have an important role in regulating them which are interconnected with one another. One of the hormones that plays an important role in regulating the estrous cycle is the hormone estrogen. If there is a decrease in the hormone estrogen, it will result in disruption of the estrous cycle (Ganong, 2003).

The decrease in the hormone estrogen can be caused by the presence of free radicals. Free radicals are reactive oxygen compounds which are compounds with unpaired electrons. One of the free radicals is cigarette smoke which contains a complex mixture of toxins including tar, nicotine, and CO which affect nerve cells in the brain. These nerve cells can influence the secretion of the hormone estradiol. Nicotine in cigarettes causes ovum maturation disorders, ovulation disorders which are characterized by a decrease in

the LH hormone which can affect estrogen metabolism. Disturbances in their metabolism can cause the estrus cycle to become irregular (Wicaksono, 2013). Clove cigarettes are more dangerous than white cigarettes, because the higher levels of tar and nicotine in clove cigarettes cause various diseases for both active smokers and passive smokers, namely healthy people who are not smokers, most of whom are women (Widodo, 2006).

One of the plants that has estrogenic properties include kebar grass (*Biophytum petersianum* Klotzsch), which is a medicinal plant found in Indonesia, especially in West Papua which has been used for generations by the local population as a traditional medicine to improve reproductive performance (Unitly, 2011). Active compounds that act as drugs and fertilizers include steroids, saponins and flavonoids. Kebar grass contains chemical compounds belonging to the alkaloid group, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids and glycosides (Sembiring, 2014), it also contains vitamin E, vitamin A, nutrients and 17 amino acids needed so as to shorten the estrous cycle and prolong the estrous period (Ganong, 199). The content of active compounds contained in Kebar grass can be used as a phytoestrogen that can replace the role of endogenous estrogen (Sadsoeitoeboen, 2005). Phytoestrogens have the effect of normalizing hormones, not only inhibiting excessive absorption of estrogen, but also being able to increase low levels of estrogen. Kebar grass contains substances from the flavonoid group, especially isoflavones and contains antioxidants which function to protect cells from damage caused by free radicals. Isoflavones can bind to estrogen receptors so that they have the potential to replace the function of estrogen (Safrida et al, 2008).

Until now, there has been no research on the effect of coriander grass on the estrus cycle in female rats exposed to smoke. Therefore, researchers are interested in conducting research on the description of the estrus cycle of *Rattus norvegicus* rats exposed to cigarette smoke after being treated with ethanol extract of coriander grass (*Biophytum petersianum* Klotzsch).

METHOD

This research is an experimental laboratory at the Zoology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Pattimura University, Ambon. This study used an experimental method using a completely randomized design consisting of four treatments and each was repeated three times. The treatment studied was the administration of graded doses of coriander grass ethanol extract given to rats, namely 3 rats that were not given the extract solution and were not exposed to cigarette smoke (control -) (P0), 3 rats that were not given the extract solution, but were exposed to cigarette smoke 10 sticks/head/day (+ control) (P1), 3 rats that were given an extract solution of 0.0675 mg/head/day and were exposed to cigarette smoke 10 sticks/head/day (P2), and 3 rats that were given a solution of 0.135 extract mg/head/day; exposed to cigarette smoke 10 cigarettes/head/day (P3). The research variable is the length of each phase of the estrous cycle (estrous cycle length).

Material

The model animals used in this study were 12 *Rattus norvegicus*, clove cigarettes, distilled water, 70% ethanol, physiological fluids (0.9% NaCl), 10% giemsa, 70% methanol, kebar grass extract and rat feed. The equipment used in this study was a microscope, 4 unit rat cages with a cover of wire mesh covered with husks on the bottom, sonde, object glass, smoking chamber, smoking pump, vacuum, pipette, label paper, cotton buds, blender, Erlenmeyer, filter paper, tissue and rotary evaporator.

Procedures

Preparation of Animal Models:

Twelve rats divided into four treatment groups were placed in plastic cages covered with ram wire and covered with husks. Feed in the form of pellets and drinking water. The cage environment is made so that it is not damp, adequate ventilation and irradiation. Before treatment the animals are adapted to the atmosphere of the cage for 1 week.

Cigarette Smoke Exposure:

Cigarette smoke exposure to rats in the P1, P2 and P3 treatment groups was carried out every day for 28 days. Smoking was given twice a day for 28 days, 5 cigarettes in the morning and 5 cigarettes in the afternoon. Rat were put into the smoked chamber individually through the top of the smoking chamber, then closed again. One cigarette is attached to a pipe connected to a pump, the cigarette is burned using a match and the pump is turned on, so that the cigarette smoke enters the vacuum and then flows into the smoking chamber.

Kebar Grass Extraction:

Kebar grass is taken as much as 1 kg and air-dried then the Kebar grass is ground using a blender. After obtaining Kebar grass powder then proceed with the extraction process using the maceration method. The manufacturing procedure is as follows: 250g of Kebar grass powder is weighed and put into an Erlenmeyer, after that, 1 liter of 70% ethanol is added and allowed to stand for 24 hours. After 24 hours, it was filtered using filter paper to obtain a liquid extract of Kebar grass. The extraction residue was repeated 3 times. The

obtained liquid extract of Kebar grass was then concentrated using a rotary evaporator. From the concentration results, a concentrated ethanol extract of Kebar grass was obtained.

Determination of Dosage of Kebar Grass Ethanol Extract:

Based on empirical use in society, especially in the Kebar district, women with an average body weight of 50 kg use Kebar grass ± 5.25 mg. To get the dose of Kebar grass to be used, the body weight of the converted rats is multiplied by dose of kebar grass in humans. If using the animal model of female rats, the female body weight is 50 kg, divided by the male body weight according to the conversion table, which is 70 kg [8], consumes 5.25 mg of coriander grass, where the conversion factor is for humans with a body weight of 70 kg to mice with a body weight of ± 200 gr is 0.018 so that: $= 50/70 \text{ kg} \times 5.25 \times 0.018 = 0.0675$ mg.

Based on the results above, the first dose was used as much as 0.0675 mg/head/day, then the dose was made in stages so that the second dose was 0.135 mg/head/day.

Determination of the Estrus Cycle:

Collecting data to determine the length of the estrous cycle by carrying out vaginal examination. Vaginal swabs were carried out every 24 hours during the 28 days of observation during the force-feeding period. The following are the steps for making a vaginal smear: Vaginal smears were taken using a cotton bud dipped in 0.9% physiological NaCl, then applied to the vaginal wall of the rat and rotated 360°. After that, the results of the vaginal smear were smeared on the object glass. The glass object was immersed in 70% methanol for 10 minutes, removed, then allowed to dry. Giemsa staining was then performed for 30 minutes, washed in running water, then dried. The vaginal swab sample was observed under a microscope.

Data analysis

The data obtained was analyzed using ANOVA, and if there is a significant difference it is continued with a further test.

DISCUSSION RESULT

The results of this study were observations of the length of the proestrus, estrus, metestrus, and diestrus phases in female rats with several treatments showing varying times (hours) (Table 1).

Table 1. The average length of each estrous cycle phase and the total estrus cycle in each treatment group.

| Ethanol extract (mg/days/Rat) | Cycle Length (Hours) | | | | |
|----------------------------------|--------------------------------|-------------------------------|-------------------------------|--------------------------------|---------------------------------|
| | Proestrus | Estrus | Metestrus | Diestrus | Total (hours) |
| Control- | 17,20 \pm 4,84 ^{ab} | 19,86 \pm 1,80 ^a | 23,20 \pm 2,43 ^a | 58,53 \pm 9,63 ^a | 118,79 \pm 13,54 ^a |
| Control + | 12,00 \pm 00,00 ^a | 8,00 \pm 6,92 ^b | 35,20 \pm 1,38 ^b | 85,60 \pm 1,38 ^c | 140,80 \pm 5,543 ^a |
| Dose 0.0675 | 20,00 \pm 3,66 ^b | 37,86 \pm 5,61 ^c | 22,00 \pm 3,46 ^a | 49,60 \pm 5,54 ^{ab} | 129,46 \pm 16,20 ^a |
| Dose 0.135 | 21,66 \pm 2,08 ^b | 49,60 \pm 2,77 ^d | 21,60 \pm 2,40 ^a | 39,33 \pm 5,85 ^b | 132,19 \pm 7,738 ^a |

Note: Different superscript letters in one line show significantly different results ($P < 0.05$) between treatment groups.

Based on Table 1, it can be seen that the proestrus phase in the negative control group did not show a significant difference with the ethanol extract group of twin grass at doses of 0.0675 mg/head/day and 0.135 mg/head/day. The negative control group was not significantly different from the positive control group, but the positive control group showed a significant difference from the ethanol extract group of coriander grass at doses of 0.0675 mg/head/day and 0.135 mg/head/day. The coriander grass ethanol extract group at a dose of 0.135 mg/head/day had the highest proestrus phase compared to the other treatments, while the positive control group had the lowest proestrus phase, 12.00 hours. The length of the estrous phase of each treatment group showed significant differences from each other. The coriander grass ethanol extract group at a dose of 0.135 mg/head/day had the highest estrus phase of 49.60 hours compared to the other treatments, while the positive control group had the lowest estrus phase of 8.00 hours.

In the metestrous phase, administration of ethanol extract of kebar grass at doses of 0.0675 mg/head/day and 0.135 mg/head/day did not show a significant difference with the negative control group, however in the positive control group it showed a significant difference and had the highest metestrous phase duration. when compared to other treatment groups. In the diestrus phase, the negative control group was not significantly different from the ethanol extract group of coriander grass at a dose of 0.0675 mg/head/day. The ethanol extract group of coriander grass at a dose of 0.135 mg/head/day did not show a significant difference from the ethanol extract group for coriander grass at a dose of 0.0675 mg/head/day, but the positive control group

showed a significant difference from the negative control group and the ethanol grass extract group. twins dose of 0.135 mg/head/day. The positive control group had the highest diestrus phase of 85.60 hours compared to other treatments, while the ethanol extract group of 0.135 mg/head/day had the lowest diestrus phase. The estrous cycle phases of rats in this study were presented in the form of microscopic images of confirmed cells, nucleated epithelial cells, and leukocyte cells (Figure 1). Based on the results of observations on the four phases of the rat estrus cycle (Figure 1), it can be seen that during the proestrus phase there is a dominance of nucleated epithelial cells, these cells are oval in shape and have a clear nucleus in the middle. In the estrous phase, there are many cornified cells (horn cells) that appear singly or in layers (layers). In the metestrus phase, there are nucleated epithelial cells, conification cells, and leukocytes, which qualitatively predominate in almost equal numbers. During the diestrus phase, there are a large number of leukocytes and a small number of nucleated epithelial cells.

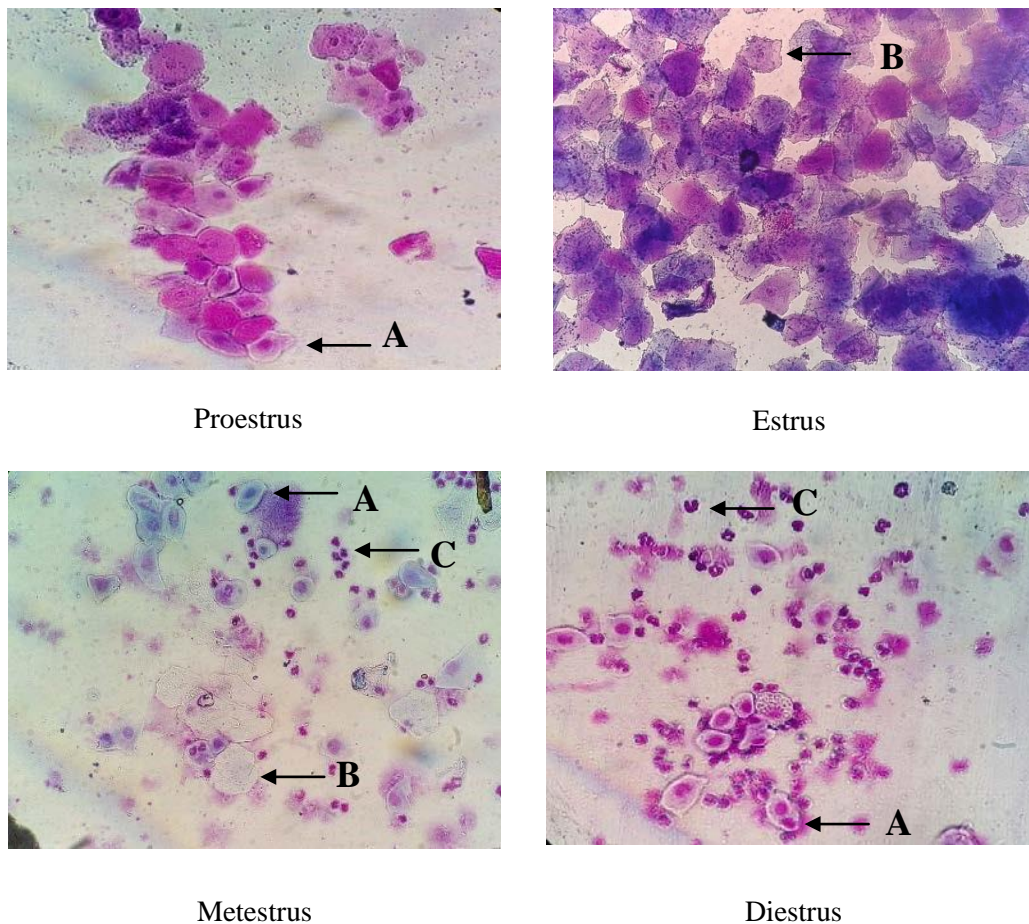


Figure 1. The phases of the estrus cycle of *Rattus novergicus* after being treated with ethanol extract of kebar grass (*Biophytum petersianum* Klotzsch) for 28 days. (A) Nucleated epithelium; (B) Confirmed cells; and (C) Leukocytes. 400x magnification.

The results showed that the administration of kebar grass ethanol extract led to an extension of the length of the estrous cycle due to the proestrus and estrus phases which tended to be longer, but the metestrus and diestrus phases tended to be shorter. The proestrus and estrus phases in the negative control, ethanol extract of coriander grass at doses of 0.0675 mg/head/day and 0.135 mg/head/day tended to be longer than the positive control. The proestrus and estrus phases of the ethanol extract of coriander grass at a dose of 0.135 mg/head/day tended to be longer when compared to the ethanol extract of coriander grass at a dose of 0.0675 mg/head/day. Kebar grass which can act as a phytoestrogen is thought to be able to bind to receptors ER- β estrogen resulting in estrogenic effects on the vaginal epithelium, namely the proliferation and cornification of vaginal epithelial cells. Reference (Safrida, 2008), states that phytoestrogens have a chemical structure similar to estrogen and work by imitating estrogen, the results that will be obtained are very dependent on the dose given, so it can be assumed that administration of kebar grass ethanol extract can increase levels of 17 β -estradiol in the blood mouse. This is in line with reference (Wajo, 2005) which states that the administration of kebar grass extract causes the development of free-range chicken follicles, because it is

suspected to contain saponins which are the basic material for the synthesis of steroid hormones. Kebar grass belongs to the steroid group which can turn into estrogen through the aromatization process so that it can increase and extend the estrus time (Wicaksono, 2013). During the proestrus phase, the influence of the gonadotropin hormone in the form of Follicle Stimulating Hormone (FSH) predominates in stimulating follicular development. These growing follicles will secrete more and more estrogen as seen in the ethanol extract of the kebar grass at doses of 0.0675 mg/head/day and 0.135 mg/head/day having a longer proestrus phase than the negative control. This shows that the activity of phytoestrogens from the ethanol extract of Kebar grass causes an extension of time due to an increase in estrogen hormone levels for a long time so that more cell proliferation occurs. This is also in line with the reference statement (Toelihere, 1985) that during the proestrus phase there is an increase in vaginal epithelial vascularization and cornification that occurs in some species, this increased vascularity is caused by higher estrogen.

The estrus phase is characterized by increased secretion of the hormone estrogen, therefore the vaginal epithelial cells turn into cornified cells. This increase in estrogen will cause an increase in Luteinizing Hormone (LH) which can cause ovulation, just before ovulation the follicle enlarges and the ovum in it undergoes maturation. Exposure to high concentrations of estrogen for a long time can cause a positive feedback mechanism to LH (Sherwood, 2001). There was an extension of time in the estrus phase in the ethanol extract of coriander grass at a dose of 0.135 mg/head/day when compared to 0.0675 mg/head/day, the negative control and positive control were influenced by the high concentration of estrogen derived from the ethanol extract of kebar grass at a dose of 0.135 mg/head/day. In the estrous phase, the concentration of estrogen increases according to the development of the Graaf follicles, especially with the addition of ethanol extract of kebar grass which is thought to be able to bind to estrogen receptors, causing the concentration of estrogen to increase and causing signs of estrus to be maintained. The lengthening of the estrus phase provides an opportunity for more follicles to mature and secrete estrogen so that females can receive more frequent mating from males. This is in line with reference (Salisbury, 1985), that the lengthening of the estrus phase indicates an increase in the growth and maturation of ovarian follicles because normally estrous activity will not occur before growing and mature follicles are seen in the ovaries. The results showed an increase in estrogen due to the administration of ethanol extract of kebar grass which is thought to be able to increase the development of follicles so that there are more mature follicles in the ovaries and the hormone estrogen secreted will cause more and more prolongation of the estrus phase. The long estrous phase will affect reproduction and fertility by prolonging the heat of the female so that the mating time will be long. Estrus time extension on the ethanol extract of coriander grass at doses of 0.135 mg/head/day and 0.0675 mg/head/day compared to the negative control was beneficial and potential in terms of fertilization. However, this results in a long total estrous cycle, so one cycle has to wait a long time for estrus to return.

In the positive control there was a shortening of time in the proestrus and estrus phases. This is thought to be due to the presence of free radicals originating from exposure to cigarette smoke which contain carcinogenic compounds, causing a decrease in estrogen levels so that the proliferation and cornification of vaginal epithelial cells is disrupted and the process of cell swelling does not occur (Toelihere, 1985). Allegedly, nicotine in cigarettes causes impaired ovum maturation. In addition, ovulation disorders are characterized by a decrease in the LH hormone which is known to affect estrogen metabolism. Low estrogen concentrations inhibit the cornification of the vaginal epithelium so that signs of estrus are not found. In the metestrus phase, the hormone estrogen decreases and the progesterone produced by the ovaries increases. There was a lengthening of the metestrus phase in the positive control, presumably due to disturbances in the estrous phase. According to reference (Hidayati, 2015) that the many phases of metestrus are possible due to the inhibition of the estrus phase so that the follicles experience degeneration. This is in line with the results of the study that in positive controls there was a disturbance in the estrus phase due to exposure to cigarette smoke which caused a decrease in estrogen levels in the blood. In the ethanol extract of coriander grass at doses of 0.135 mg/head/day and 0.0675 mg/head/day, the metestrus phase tends to be shorter, but still within the normal range of the metestrus phase. In the metestrous phase, the concentration of estrogen begins to decrease and cornification decreases. The ethanol extract of coriander grass at doses of 0.135 mg/head/day and 0.0675 mg/head/day has an estrogenic effect, seen in the presence of a slight cornification in the vaginal smear preparation. The diestrus phase is the last phase of the estrus cycle which is characterized by no pregnancy, no sexual activity and the animal becomes calm. The positive control had a longer diestrus phase compared to the negative control, the ethanol extract of the coriander grass at a dose of 0.0675 mg/head/day and 0.135 mg/head/day. The prolongation of time during the diestrus phase is thought to be due to low levels

of estrogen so that it cannot trigger the change from the diestrus phase to the proestrus phase. The ethanol extract of coriander grass at a dose of 0.135 mg/head/day had a shorter diestrus phase compared to the negative control, a dose of 0.0675 mg/head/day. This is because during the diestrus phase, the lowest concentration of estrogen lasts only briefly due to exposure to the phytoestrogens of the kebar grass, the high estrogen levels cause the cells to quickly proliferate again. Giving kebar grass ethanol extract causes the body to give the same response as stimulation by estrogen, namely the occurrence of positive feedback which results in a faster return at the beginning of the estrus cycle.

Statistically, the length of the estrus cycle in all treatments was not significantly different from one another, and was still within the normal range for the length of the estrous cycle. In the negative control, the ethanol extract of coriander grass at doses of 0.0675 mg/head/day and 0.135 mg/head/day tended to have shorter estrous cycle lengths, namely 118.79 hours, 129.46 hours and 132.19 hours when compared to the positive control, which was 140.80 hours. The ethanol extract of coriander grass at a dose of 0.135 mg/head/day had a longer estrus cycle compared to the treatment group at a dose of 0.0675 mg/head/day. In the treatment group given the ethanol extract of kebar grass, the estrous cycle was longer, due to the prolongation of the proestrus and estrus phases which are the fertile phases in rats. According to (Gindeland, 2003) references that prolonging the estrous cycle period has an important effect on reproduction and has the potential to increase fertility potential by extending the time of estrus in females so that the mating time will be long. The ethanol extract of coriander grass at a dose of 0.135 mg/head/day has a greater estrogenic effect than the ethanol extract of coriander grass at a dose of 0.0675 mg/head/day because it can prolong the estrous phase. The administration of kebar grass ethanol extract causes high levels of estrogen which is thought to be able to prolong the proestrus and estrus phases. In the group of rats exposed to cigarette smoke (positive control) there was a prolongation of the length of the estrus cycle due to the lengthening of the time in the metestrus and diestrus phases which are non-potential phases in fertility, this is thought to be due to differences in levels of the hormone estrogen in rats exposed to cigarette smoke and mice who were exposed to cigarette smoke were then given Kebar grass ethanol extract. Differences in levels of the hormone estrogen occur because exposure to cigarette smoke is a free radical that causes cell damage, disruption of cell function and even cell death (Fitria, 2013). Decreased estrogen levels result in a negative feedback loop, because cigarette smoke contains a complex mixture of poisons, which affect the nerve cells of the brain which can affect the secretion of the hormone estradiol. Cigarette smoke can also trigger changes in the production of reproductive hormones, namely the hormone estrogen. If there is a deficiency of the hormone estrogen, it will have an impact on the estrus cycle of the rat, low estrogen levels will make the estrus cycle longer or in other words can reduce fertility.

CONCLUSION

The ethanol extract of coriander grass (*Biophytum petersianum* Klotzsch) dose of 0.135 mg/head/day was able to extend the length of the estrus cycle due to the prolongation of the proestrus and estrus phases.

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