The Role of Fennel Infusion on Estrous Cycle 
and Follicles Development of White Rats

(Peran Infusa Buah Adas terhadap Siklus Estrus dan Perkembangan Folikel pada Tikus Putih)

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Abstract: This study aims to describe the estrous cycle and follicles development in female rats given 
infusion of fennel fruit that known to have phytoestrogen compound. Twenty five of female rats were 
used in this research and were divided into 5 groups. Group I was negative control group (NC), given 
1 mL/100g bw of distilled water, group II was positive control group (PC), given 0.0045 mg/100g bw 
of ethinyl estradiol, groups III, IV and V were treatment groups (T1, T2 and T3), given fennel infusion 
at 36.5; 73 and 146 mg/100g bw respectively. All treatments were conducted every morning for 20 
days by oral route. Changes of vaginal epithelium were observed through vaginal swabs previously 
stained with Giemsa stain. Histopathological examination of ovarian were examined to reveal follicles 
development. Results showed that fennel fruit infusion extended the duration of estrous and metestrous 
phases, while shortened the proestrous and diestrous phases. Even though the longest estrous phase 
was found in T3 group, there was no significant effect on the lengthening of estrous cycle. Moreover, 
infusion of fennel fruit had no effect on the development of ovarian follicles, except tended to increase 
the number of corpus luteum.

Keywords: phytoestrogen, fennel fruit, estrous cycle, follicles development, rat.

Abstrak: Penelitian ini bertujuan untuk mengkaji siklus estrus dan perkembangan folikel tikus betina 
yang diberi infusa buah adas yang diketahui memiliki kandungan fitoesrogen. Sebanyak 25 ekor tikus 
betina dipergunakan di dalam penelitian ini, dan dibagi menjadi 5 kelompok. Kelompok I yaitu kontrol 
negatif (KN) yang diberi air suling 1 mL/100g bb, kelompok II yaitu kontrol positif (KP) yang diberi 
etinil estradiol 0,0045 mg/100g bb, kelompok II, IV dan V adalah kelompok perlakuan (D1, D2 dan D3) 
yang diberi infusa adas dosis 36,5; 73 dan 146 mg/100 g bb. Pemberian infusa adas dilakukan setiap 
pagi hari dengan cara pencokokan selama 20 hari. Perubahan gambaran epitel vagina diamati secara 
mikroskop pada preparat ulas vagina yang diwarnai dengan pewarna Giemsa. Pengamatan histopatologi 
ovarium dilakukan untuk mengobservasi perkembangan folikel. Hasil penelitian menunjukkan bahwa 
permeninfusa mempengaruhi periode fase estrus dan metestrus serta memperpendek durasi fase 
proestrus dan diestrus. Meskipun durasi fase estrus tampak paling panjang pada kelompok D3, tetapi 
merupakan pengaruh yang signifikan terhadap perpanjangan durasi siklus estrus. Lebih lanjut, 
infusi adas tidak berpengaruh terhadap tingkat perkembangan folikel ovarium tikus putih, kecuali 
kekacauan meningkatkan jumlah luteum.

Kata kunci: fitoestrogen, adas, siklus estrus, perkembangan folikel, tikus.
INTRODUCTION

MENOPAUSE occurs when the ovaries are ageing and no longer give respond to the signals of gonadotropin to synthesize and secrete estrogen so that estrogen levels become decreasing. This phenomenon is due to the loss of follicle that in line with age by atresia and monthly ovulation. Loss of follicles resulting in reduction secretion of estrogen and progesterone. Decreased levels of estrogen and progesterone disrupt the axis of the hypothalamic-pituitary-ovarian and negative feedback mechanisms. Cycles halt although a small number of ovarian hormone is still secreted by the adrenal glands\(^{(1,2)}\).

Low level of estrogen has serious impact to women because it can cause a decrease in bone density, increase the risk of cardiovascular disease, dryness of skin and vaginal mucos as well as hot flashes or hot sensation throughout the body, sweating, tachycardia followed by redness of the skin. To overcome the effects caused by menopause stage, lots of women use hormone replacement therapy. However, given various negative impacts as a result of hormone replacement therapy, researchers begin to find substances that are safe for the health as an estrogen replacement\(^{(3,4)}\).

Phytoestrogens are chemical substances found in plants and have similar effect as endogenous estrogen. It has structure that similar to 17\(\beta\) estradiol, so that it can bind both estrogen receptor alpha (ER\(\alpha\)) and estrogen receptor beta (ER\(\beta\)). Although it can bind estrogen receptor, effects of phytoestrogens are less active compared to natural estrogen\(^{(5)}\).

Phytoestrogens may reduce the activity of estrogen by inhibiting the activity of the receptors when estrogen levels are very high. On the contrary, when estrogen levels are low, phytoestrogens which is bound to the receptor can provide effects as natural estrogen\(^{(6)}\). Several studies have shown the effects of phytoestrogens which slows menopause in women and reduce the symptoms. The presence of estrogenic agent in the early stages of development can spur a variety of reactions in the body of young rats. One is by stimulating the growth spurt of the reproductive organs, besides the possibility of the onset of puberty\(^{(7)}\).

Phytoestrogens have three main groups namely isoflavones, lignans and coumestane. These three groups of phytoestrogens can be found in at about 300 species of plants, especially legumes family. Soybeans, seeds, grains, legumes and vegetables are a source of phytoestrogens. Fennel fruit (\textit{Foeniculum vulgare} Mill.) is known to contain trans-anetol, fenchone and estragol that assumed to be potential as phytoestrogens\(^{(8)}\). Anethol is an active estrogen agent contained in fennel essential oils. Several other studies found that the actual pharmacologically active agent is a polymer of anethol, such dianethol and photoanethol. Fennel fruit used in this research was sweet fennel (\textit{Foeniculum vulgare}, subspesies vulgare varietas dulce Mill.). Sweet fennel contains anethol (50-80\%), limonen (5\%), phenicon (5\%), alpha-pinen (0.5\%), estragol (methyl-chavicol), safrol, camphene, beta-pinen, beta-myrcenedan \(p\)-cymen\(^{(9)}\).

Fennel have pivotal role in increasing immunity, healing flu, overcoming early ejaculation, and stimulate erection. Fennel is also to have anesthetic effect, diaphoretic, stimulate central nervous system, and stimulate androgen secretion. Fennel also have role in inhibiting secretion of certain enzyme such as aldose-reductase, phosphodiesterase and lipoksiginase. Another effects of fennel are delaying of ageing, stimulating ovulation and protecting the liver from any toxins (antihepatotoksic)\(^{(10-13)}\).

Based on the previous research about the content of phytoestrogens in fennel, this research aims to study the effect of fennel infusion at various doses on the estrous cycle and ovarian follicles development of white rats (\textit{Rattus novergicus}) at productive age to explore its potential for overcoming condition caused by low level of endogenous estrogen or for anti ageing agent.

MATERIALS AND METHOD

METHOD. Preparation of Fennel Fruit Infusion. Plant determination was conducted at Herbarium Bogoriense, Bogor. Dried simplisia of fennel fruit was grinded and filtered using mesh 8 and 24 filters. Infusion given as treatment was made everyday (fresh) by boiling 10 mg of fennel fruit in 100 mL of water at 60\(^\circ\)C for 15 minutes. The solution was then filtered using mesh 30 filter, and kept in a clean bottle.

Animals Housing. This research used 25 female rats strain Rattus novergicus, 200-250 g of body weight (bw), aged 5 months, and have delivered offspring twice. All rats were maintained in cages containing 2-3 individuals. Prior to treatment, all animals were acclimatized for 2 weeks. Feedstuff and water were given \textit{ad libitum}. After acclimatization, the animals were divided into 5 groups. Group I was negative control group (NC) given 1 mL/100 g bw of distilled water, group II was positive control group (PC) given 0.0045 mg/100 g bw of ethinyl estradiol, groups III, IV and V were treatment groups (T1, T2 and T3) given fennel infusion at three different doses of 36.5; 73 and 146 mg/100 g bw respectively\(^{(14)}\).

Experimental Design. Administration of distilled water, ethinyl estradiol and fennel infusion were completed by oral route for 20 days. Vaginal swabs
were taken twice a day, in the morning and in the afternoon with a period of 12 hours and stained with Giemsa. The changes of vaginal epithelium were examined to determine estrous cycle phases using a light microscope. At the end of the treatment, the animals were ether-anaesthezied, and subjected to cervicalis dislocation before necropsy. Ovarians were then collected, kept in Buffer Neutral Formalin (BNF) 10% and prepared for histopathological examination after Hematoxylin and Eosin (HE) staining. Follicles development was evaluated by calculating the number of follicles of both right and left ovaries in various phases of its development.

**Data analysis.** Data obtained were analyzed using Analysis of Variant (ANOVA) and continued by Duncan’s test with two variables to evaluate the effect of fennel infusion and the effective dose.

### RESULTS AND DISCUSSION

**Role of Fennel Infusion to The Length of Estrous Cycle.** Phases in estrous cycle in rat showed in Figure 1. In general, the length of estrous cycle in female white rats is 4-5 days. Normal duration for proestrus, estrous, metestrous and diestrous phases are 12, 12, 21 and 57 hours respectively. Determination of the stage of the phases referred to as Figure 1. Data obtained from this research showed that the total length of the estrous cycles in the positive control group (PC) and in all treating groups (T1, T2 and T3) were extended compared to the negative control group (NC) as shown in Table 1. This was assumed to be due to the activity of ethinyl estradiol in rats of positive control group, while in the treatment groups, lengthened of the cycle was possibly due to the activity of the substance similar to estrogen in the fennel. This consistent with the previous reseach, stated that the occurrence of the changes in vaginal cytology after treatment was dependent on dosages and the assumed active compound in the herb.

Rats in NC, PC and all treatment groups had vary in its estrous cycle and this is related to the length of each phases within the cycle. In PC and treatment groups, administration of fennel infusion gave rise an extended time in the estrous phase. This condition has positive impact to the fertilization process because during this phase the female tend to receive the male thus give advantage in success breeding program. Different doses of phytoestrogen given would influence the effects, stated that the administration of phytoestrogen with high dose, could block estrogen effect. Biological response of phytoestrogens depends on factors such as species, age, gender, dose, mode of administration, and metabolism.

The length of proestrus phase in PC, T1, T2 and T3 groups were significantly shorter than the period of proestrus phase in NC group. Proestrus phase in T1 group was the shortest compared to T2, T3 and PC groups. Estrous phase in T1, T2, T3 and PC groups showed an extended period than the period of estrus in NC group. Moreover, estrous phases in T2, T3 and PC were significantly longer (p<0.05) compared to NC and PC groups.

![Figure 1. Phases in estrous cycle in rat: A. nucleated epithelium cells; B. cornified and pavement cells; C. pavement cells and leucocyte; D. leucocyte and nucleated epithelium cells.](image)

### Table 1. Length of estrous cycle and its phases of female white rats (hours) after fennel infusion dmisntration (hours).

<table>
<thead>
<tr>
<th>Phase</th>
<th>NC</th>
<th>PC</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proestrus</td>
<td>7.68 ± 3.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4 ± 3.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.96 ± 1.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.88 ± 3.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.88 ± 1.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Estrus</td>
<td>17.76 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.76 ± 18.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.6 ± 14.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.28 ± 18.39&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>41.76 ± 9.08&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Metestrus</td>
<td>15.36 ± 4.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.08 ± 6.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.24 ± 11.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.04 ± 13.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.56 ± 8.07&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diestrus</td>
<td>27.36 ± 6.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.76 ± 9.075&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2 ± 3.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.32 ± 4.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8 ± 4.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total length</td>
<td>68.16 ± 8.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72 ± 24.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72 ± 16.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.52 ± 18.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.00 ± 18.15&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: NC= negative control group given 1 ml/100g bw of distilled water, PC= positive control group given 0.0045 mg/100g bw of ethinyl estradiol, T1= treatment group given 36.5 mg/100g bw of fennel infusion, T2= treatment group given 73 mg/100g bw of fennel infusion, and T3= treatment group given 146 mg/100g bw of fennel infusion.
with NC group, however, estrous phase in T1 group tended to be longer than NC (p > 0.05). Even though estrous phases in T1, T2 and T3 were extended, but the longest phase was found in PC that given ethinyl estradiol orally. This phenomenon may be due to the effect caused by fenell infusion was less strong compared to the synthetic estrogen. In humans and animals, the use of synthetic estrogen in birth control pills and other drugs are very potent, although the potential of phytoestrogens are known to be small(23,24).

At the time before estrus, follicular de Graaf reaches its maximum size that is able to synthesize and secrete estrogen in large quantities. In the vaginal swab examination was seen high number leukocytes and the formation of nucleated cells was also found(25). Administration of ethinyl estradiol and fenell infusion caused shortened of diestrous phase compared with the negative control group. In the contrary, phases that seemed to be extended were estrous and metestrous phases. Extension of estrous period is beneficial as the extension of the estrous cycle gave important effects on reproduction because it could reduce the cumulative number of cycles and was very potential in terms of fertility(26).

Estrogen works in the anterior pituitary and hypothalamus to regulate feedback mechanism. The high concentration of estrogen in a long time can affect a positive feedback mechanism to secrete LH. With high phytoestrogen levels and recurrent circulation can lead to effects such as its potential to extend the estrus phase. This phenomenon is also caused by blocking of estrogen receptor by phytoestrogens so that the receptor can not be occupied by estrogen. In other words, phytoestrogens can compete and replace the function of estrogen(27).

Application of phytoestrogens at appropriate dose is hoped to give advantageous effect on the hormonal balance in the body, especially in menopause patients. Phytoestrogens may play a role in stabilizing the hormonal function, by inhibiting excessive estrogen activity that can induce cancer and also can substitute when estrogen levels in the body are low(28).

Role of Fennel Infusion on The Development of Ovarian Follicle in Female Rats. From the histopatological evaluation of the ovary, it was found different stages of follicle development in all group of the animals, starting from the very beginning follicle or primordial follicle, primary, secondary, tertiary

| Tabel 2. Mean of percentage of primordial follicle, primary, secondary, tertiary, de Graaf follicle, corpus luteum, and atresia follicle in female rats. |
|-------------------------------------------------|-----------------|---------------|-------------|-------------|-------------|-------------|
| Follicles                                      | NC              | PC            | T1          | T2          | T3          |
| Primordial                                    | 43.40±24.33a    | 39.90±8.31a   | 51.66±6.50a | 37.71±6.73a | 30.39±3.81a |
| Primary                                       | 15.19±4.80a     | 28.58±8.56a   | 9.01±3.90a  | 10.84±2.82a | 20.41±10.86a|
| Secondary                                     | 7.80±5.41a      | 5.99±2.64a    | 6.07±0.26a  | 11.26±3.00a | 3.98±2.93a  |
| Tertiary                                      | 1.85±3.71a      | 1.42±1.23a    | 4.51±1.94a  | 2.53±2.14a  | 3.46±2.51a  |
| De Graaf                                      | 1.47±2.94a      | 1.39±2.41a    | 0.00±0.00a  | 1.62±1.95a  | 1.76±2.48a  |
| C. Luteum                                     | 9.03±8.31a      | 11.87±7.22a   | 16.64±1.43a | 19.71±7.36a | 20.20±9.41a |
| Atresia                                       | 21.26±16.79a    | 10.86±5.23a   | 12.13±0.52a | 16.33±2.42a | 19.80±6.47a |

Note: Different superscript(a,b) in the same row refer to as significantly different.
and de Graaf follicles. Different stages of the follicle development could be seen in Figure 2, 3 and 4 which was taken from different groups of treatment (T2 and T3 groups). Percentage of primordial follicle, primary, secondary, tertiary, de Graaf follicles, corpus luteum and atresia follicle found from histopathological observation presented in Table 2.

In the prenatal period, oogonia proliferates during embryonic period and become the origin of primary oocytes. Each primary oocyte enveloped by a single layer of follicular cells and at this stage is referred to as primordial follicles. Primary oocytes will remain at the stage of prophase I of meiosis during embryonic life, after birth until puberty. In rat, soon after birth, the number of oocytes approximately 8,000 pieces, in a state of rest and covered by a layer of follicle cells. The number of primordial follicles may decrease with age due to atresia (follicle regression and degeneration(29).

Based on the data in Table 2, the percentage of primordial follicles in all groups of rats was not significantly different. This is because the age of the rats used was the same and so that the stage of reproductive status. In addition to that, the administration of ethinyl estradiol and fennel infusion at the given dose did not affect the growth of primordial follicles, furthermore did not stimulate the occurrence of atresia. Towards the puberty, primary oocytes begin to grow, while the surrounding epithelial cells changed from flat into cuboidal form, and so called called primary follicles. The transition from primordial follicles into primary follicles involves changes in the oocyte, follicular cells and stromal cells adjacent(30).

The results revealed that there was no significant difference between treatment groups in terms of the percentage of primary follicles. It assumed that the dose of ethinyl estradiol and fennel infusion did not affect the development of primary follicles. GnRH from the hypothalamus induces the anterior pituitary to produce and secrete FSH that stimulate follicular development. In other words, the primary follicles develop into secondary follicles due to the stimulation of FSH(31). Secondary follicles are growing follicles, has its antrum, cumulus oophorus and corona radiata. Cumulus oophorus is a pile of granulosa cells surrounding the oocyte and support it in secondary follicles, whereas the corona radiata is formed by the granulosa cells surrounding the oocyte. This follicle consists of a fully grown oocytes and surrounded by the zona pellucida, 5-8 layers of granulosa cells, the basal lamina, theca interna and theca externa which contains a number of small blood vessels. Under the influence of FSH, the cells of secondary follicles begin to secrete estrogen(32).

Based on the results obtained, percentage of secondary follicles was not significantly different between treatment groups. This might be because phytoestrogens in fennel infusion and ethinyl estradiol given were not sufficient to supply as estrogen so that gave positive feedback on FSH secretion from the pituitary(33). Thus, administration of ethinyl estradiol and infusion fennel could not affect the development of secondary follicles at 20 days of exposure.

Tertiary follicles surrounded by two layers of tissue, the theca interna and theca externa. Theca interna is the inner layer that produces estrogen and rich in blood vessels, while the theca externa is the outer layer that will gradually merge with the ovarian stroma. Antral follicular will continue to grow in line with the development of tertiary follicles. Follicular antrum filled by a clear fluid (liquor folliculi) that is rich in protein and estrogen. The development of this follicle is under the regulation of FSH from the anterior pituitary gland(34).

De Graaf follicle is follicle that will ovulate with antrum that become bigger and the size of the follicle is also greater than the other follicles. Before ovulation, the oocyte inside the follicle completes the first meiotic division, which will form a secondary oocyte and the first polar body. After the first meiosis cleavage, the oocyte undergoes the second meiotic division and stops at metaphase II stage. These follicles are formed due to an increase in FSH in the ovary. The mature de Graaf follicles has folliculi liquor containing estrogen and ready to ovulate. Estrogen suppresses FSH and stimulate secretion of LH from the anterior pituitary. LH is the hormone responsible for the maturation of follicles and the occurrence of ovulation(35).

The percentage of de Graaf follicles was not significantly different between treatment groups. This is presumably because the dose of phytoestrogens given for 20 days of exposure had not been able to influence the pituitary gland to inhibit the release of FSH that stimulate follicular development(36). Phytoestrogens work by binding to estrogen receptors and will cause estrogenic activity. Based on statistical test, there was no significant difference of the average percentage de Graaf follicles between treatments.

Immediately after ovulation, the follicle cavity filled with blood and lymph, forming the corpus hemorrhagicum. Furthermore corpus hemorrhagicum in the form of a blood clot will develop into the corpus luteum that contain lots of lutein cells that produce progesterone. The number of corpus luteum represents the number of oocyte successfully ovulated. The increase of the estradiol level towards its peak, will further stimulate LH thus increase the intrafollicular pressure for ovulation(37).
Based on the data, it is shown that the percentage of corpus luteum between treatment groups was not significantly different. Even though there was no significant difference between treatment group, however, in groups given ethinyl estradiol (PC) and fennel infusion (T1, T2 and T3) seemed to be higher than that of negative control group (NC). It is presumably that fennel infusion would give effect in the process of ovulation, or in other words it could trigger ovulation. Phytoestrogens could increase the number of corpus luteum. High estrogen levels trigger the surge of LH so mature follicles ovulate. Previous finding also revealed that ethanolic-extracted fennel could increase blood estrogen in mouse [38].

Atresia is a degenerative process that causes the ovum is not ovulate. It is estimated that approximately 99.9% of oocytes in the ovary at birth was destined to disappear by atresia at a certain stage of its development. Atresia can be influenced by several factors, such as age, season, nutrition, stage of reproductive cycle, pregnancy and lactation, hypophysectomy, unilateral ovariectomy, exogenous hormones and disruption of the blood supply in the ovaries [39].

The results of this study showed that there was no significant difference in the number of follicular atresia among treatment groups. This suggests that the effects of phytoestrogens given for 20 days did not affect the exposure of the follicle to undergo atresia. Phytoestrogens could affect the development of follicles, the decrease of serum estradiol and the increase of the number of follicular atresia and corpus luteum [38].

**CONCLUSION**

In general, it can be concluded, that the administration of fennel infusion at a dose of 36.5, 73 and 146 mg/100 bw could prolong estrous and metestrous phases, on the contrary shortened the diestrous and proestrous phases of the estrous cycle. The longest estrous phase was found at the administration of fennel infusion at a dose of 146 mg/100 bw, while the length of the cycle did not show any significant difference between treatments. In terms of follicle development, fennel infusion had no significant effect. This might be due to the low dose and the period of the time.

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