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## RESEARCH ARTICLE

# The Effect of Gandarusa Leaf Extract (Justicia gendarussa Burm F.) Administration on Estradiol Hormone Level and the Amount of Antral Ovarium Follicle on Female Mice

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***ABSTRACT***

***Background:*** *Physiologically, woman experiencing aging characterized by menopause. Gandarusa leaf contains isoflavon, a phytoesterogen expected to be used as esterogen replacement therapy.* ***Objective:*** *the aim of this research is to investigate the administrative effect of gandarusa leaf extract on the increase of estradiol hormone level and the number of antral ovarium follicle of female mice.*

***Methods:*** *This is experimental study with posttest only control group research design. Samples are 24 balb-c female mice, aged 16-17 months, weighed 19-35 grams, divided into 4 groups. Control group (Ctrl-G) were given 0.48 ml aquadest; group JB10-G, JB20-G, and JB30-G, each were given gandarusa leaf extract with concentration 10%, 20%, dan 30% in volume 0.48 ml orally twice a day. After treatment, dissection were conducted to make ovarium histology preparation with Hematoxylin Ehrlich-Eosin staining, observation were conducted using microscope.*

***Results:*** *Post hoc analysis indicated that the estradiol level and the number of antral ovarium follicle on administration of gandarusa leaf extract with concentration 10%, 20%, dan 30% are significantly higher, $p < 0.05$. The increase of estradiol level with extract concentration of 10% are significantly negatively correlated with the number of antral ovarium follicles $p<0.05$.*

***Conclusion:*** *The administration of gandarusa leaf extract increase the estradiol hormone level and the number of antral ovarium follicle in female mice.*

***Keywords :*** *gandarusa leaves extract, estradiol endogenous, ovary histology*

**ABSTRAK**

**Latar Belakang:** Secara fisiologis wanita yang memasuki proses penuaan (aging) ditandai oleh menopause. Daun gandarusa mengandung isoflavon, suatu fitoestrogen diharapkan dapat dipakai untuk estrogen replacement therapy. **Tujuan:** Tujuan penelitian ini adalah untuk mengetahui efek pemberian ekstrak daun gandarusa terhadap peningkatan kadar hormon estradiol dan jumlah folikel antral ovarium pada mencit betina.

**Metode:** Penelitian ini adalah penelitian experimental dengan rancangan riset posttest only control group design. Sampel adalah 24 mencit betina balb c, umur 16-17 bulan, berat badan 18-35 gram, dibagi menjadi 4 kelompok. Kelompok kontrol (Ctrl-G) diberikan 0,48 ml aquadest; kelompok JB10-G, JB20-G, dan JB30-G, masing–masing diberikan ekstrak daun gandarusa dengan konsentrasi 10%, 20%, dan 30% dalam volume 0.48 ml peroral 2 kali sehari. Sesudah perlakuan dilakukan pembedahan, pembuatan preparat histologi ovarium dengan pengecatan Hematoxylin Ehrlich-Eosin, dilakukan pengamatan menggunakan mikroskop.

**Hasil:** Hasil analisis Pos Hoc menunjukan bahwa kadar estradiol dan jumlah folikel antral ovarium pada pemberian ekstrak daun gandarusa dengan konsentrasi 10%, 20%, dan 30% lebih tinggi bermakna, $p < 0.05$. Peningkatan kadar estradiol dengan konsentrasi ekstrak 10% berkorelasi negatif secara bermakna dengan jumlah folikel antral ovarium, $p<0.05$.

**Simpulan:** pemberian ekstrak daun gandarusa meningkatkan kadar hormon estradiol dan jumlah folikel antral ovarium pada mencit betina.

**Kata kunci:** ekstrak daun gandarusa, estradiol endogen, histologi ovarium.

## INTRODUCTION

Aging is a natural process experienced by every human being with long life as well as the most feared process by most of people. Physiological condition of women who are entering aging process is characterized by menopause due the decrease in esterogen hormone. The symptoms and signs of menopause include hot flushes, night sweats, vaginal dryness, memory loss, insomnia, and depression (Levina, 2002; Adnyana, 2005). Such complains can be reduced by administering synthetic esterogen hormone. Unfortunately, the administration of synthetic estradiol hormone can cause

malignancies such as ovarian cancer, and breast cancer (Safrina, 2009). Gandarusa leaf (Justicia gendarussa Burm. f.) traditionally used for male contraception, contains isoflavonoid expected to increase estradiol level and change the amount of antral ovarium follicle.

Groups of follicles in the ovary that can develop are divided into preantral or early antral with size of 0.2-2 mm which is not dependent to gonadotropin, small follicle antral size 2-6 mm some are sensitive to gonadotropin. All healthy follicles showed granulose cells activities and with diameter of less than 6 mm (Deb, Campbell and Clewes, 2010).

Phytochemistry analysis indicated that gandarusa leaves contain 12 flavonoid components with major component of 6,8-di-□-L-arabinopiranosil-4,5,7-trihidroksiflavon or 6,8-diarabinosilapigenin or Gendarusin A (Prajogo, 2002). During physiological condition, isoflavonoid in the gandarusa acts as active ingredient which can influence the estrogen level directly or by negative feedbacks (Susetyarini, 2009; Satyaningtyas and Estiasih, 2014). Isoflavonoid in the body can be agonistic if the environmental estrogen is low. On the contrary, it will be antagonistic if environmental estrogen is high (Ludwig et al., 2004; Stubert and Gerber, 2009). Isoflavonoid physiologically can be interconnected with estrogen □ receptor in the hypothalamus creating negative feedback, causing Gonadotropin Releasing Hormone (GnRH) secretion, Folicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) lowered, which followed by ovarium follicle changes and the decrease of endogenic estrogen level (Markaverich et al., 1995). Menopause is marked by the increase of FSH and the decreased estrogen level due to the decrease of ovarium follicle (Santoro N, 2005). The decrease of estrogen level is excpected to be replaced by isoflavonois so all symptoms due to the menopause can be reduced.

The purpose of this research is to prove that the administration of gandarusa leave extract can have impact on ovarium diameter changes and the increase of estradiol hormone level on female mice.

## METHODS

This is experimental research using posttest only control group design (Pocock, 1983). Samples used for this research are female mice balb-c strain aged 16–17 months weighed 18-35 gram obtained from preclinical laboratory service and test animal development UGM Yogyakarta. The age of the mice is determined by the date of birth obtained during the sampling. One month old of mice is equivalent to 3 years old of a human, so 16 to 17 months old mice are equivalent to 58-51 years old or during menopause (Safrida S et al., 2013). The number of sample for this research is 24 mice randomly divided into 4 groups, each consist of 6 mice. Control group (Ctrl-G), were given 0.48 ml aquadest. Gandarusa 10% group (JB10-G), received gandarusa leaves extract with 10% concentration of 0.48 ml. Gandarussa 20% group (JB20-G), administed 0.48 ml gandarusa leaves extract with 20% concentration. Group gandarussa 30% (JB30-G), administered 0.48 ml gandarusa leaves extract with 30% concentration. All treatments were administered orally twice a day on 8 am and 5 pm for 28 days. During the research, the mice were fed and given water ad libitum with food using pellets with same volume and composition. In the final stage of research, blood were drawn to check the estrogen level and ovarium to examine the diameter of antral follicle. This research was conducted after ethical approval from ethical committee faculty of medicine Unissula Semarang.

### Gandarusa Leaves Extract Dosage

This reseach using gandarusa leave extract which extracted using ethanol solvent 70% conducted in PAU laboratory UGM Yogyakarta. The administration dosage were obtained from the dosage conversion result from rabbits to mice dosage with mice conversiton factor (0.08) according to Lawrence's conversion table formula. Then, the 0.08 mice dosage is multiplied with the previous research dosage (0.08 x 6) = 0.48 ml (Darmayasa, 2008).

### Gandarusa Leaves Extract

The making of gandarusa leave extract is done in the PAU laboratory UGM Yogyakarta. Gandarusa leave extract were dried in the oven 40 $^0$C temperature for 7 hours of 1500 gram powder (strained) gandarusa leave were extracted with ethanol solvent 70% as much as 7,5 liter (1:5) using maserasi method by stirring using stirrer for approximately 1 hour and then left for 24 hours. Maserat then separated and strain using flannel cloth. Maserat were evaporated using vacuum ratory evaporator temperature 60$^0$C with rpm speed of 100 rpm. The thick extract were weighed and the immersion were counted. Then solution with concentration of 10%, 20%, and 30% were prepared.

### Ovarium Histology Preparation

In the final stage of the research, mice were terminated by inserting mice into the tube which contains 100% chloroform until the mice is dead. The mice then dissected to obtain left and right ovarium and make histology preparation. The making of ovarium

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**Table 1. Mean of Estradiol concentration and Antral Follikel of Left and Right Ovary**

| Variables | Groups | | | | p Value (Anova) |
|---|---|---|---|---|---|
| | Ctrl-G N=6 χ (±SD) | (JB10-G) N=6 χ (±SD) | (JB20-G) N=6 χ (±SD) | (JB30-G) N=6 χ (±SD) | |
| Estradiol Concentration (pg/mL) | 192.83 (±59.21) | 589.50 (±42.87) | 813.0 (±11.86) | 1288.0 (±10.73) | 0.000 |
| Diameter of Antral Follicle of Ovary (cm) | 5.50 (±0.517) | 9.76 (±0.612) | 13.06 (±0.30) | 14.13 (±0.206) | 0.000 |

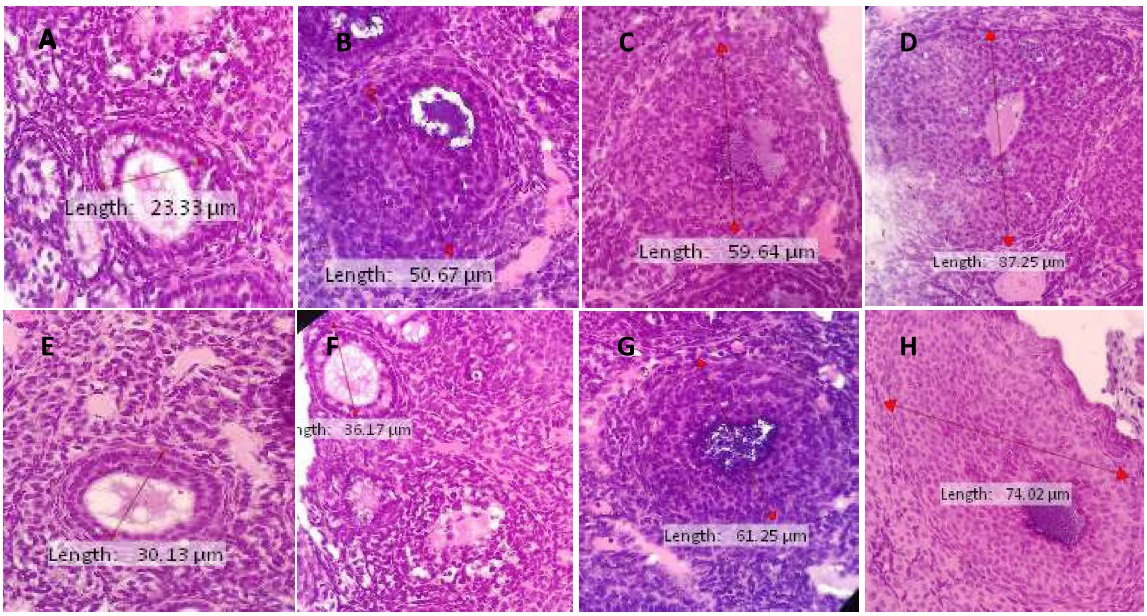


**Figure 1. Diameter of Antral Follicle Left and Right Ovary were statained hy HE.**
**Note: Left Ovary A: Ctrl-G; B: JB10-G; C: JB20-G; D: JB30-G. Right Ovary:**
**E: Ctrl-G; F: JB10-G; G: JB20-G; H: JB30-G**

microanatomy were done by paraffing method with the following stages: (1) Fixation, ovarium is fixated insite buffer formalin solution, continued by Bouin solution for 3 hours (2) Washing, dehidration and clearing, the ovarium organ were washed with alcohol 70% several times. Dehidration was conducted using increased concentration absolute alcohol from 70%, 80%, 90%, 95%. To clarify the ovarium organ, it is soaked in toluol overnight. (3) Infiltration and embedding, paraffin infiltration into the tissues by soaking organovarium into the mixture of toluol and paraffin for 30 minutes, and then followed by pure paraffin I, II, III each for 50 minutes followed by embedding which is organ planting into solid paraffin. (4) Incission and attachment, paraffin block contains ovarium organ were slice 6 µm in the miccle using Microm microtome type HM 351. And then it is attached on the object glass which was smeared with Mayers albumin. It is left for 24 hours so the attachment of the ovarium organ slices can strongly. (5) Staining and mounting, preparations were stained with Hematoxylin Ehrlich-Eosin with the following sequence: xylol I selama 5 menit; xylol II for 5 minutes; xylol III for 5 minutes; alcohol 100% I for 5 minutes; alcohol 100% II for 5 minutes; aquadest (few dips), Harris-Hematoxylin for 1 5 minutes;aquadest for 1 minute (dipped up and down); acid alcohol 1 % as much as 5-7 dips (not to pale); aquadest I for 1 minute; aquadest II for 15 minutes; eosin for 2 minutes; alcohol 96% I for 3 minutes; alcohol 96% II for 3 minutes; alcohol 100% I for 3 minutes; alcohol 100% II for 3 minutes; xylol IV for 5 minutes; xylol V for 5 minutes. The stained preparations were sealed, attached using permount. (6) Observation, observation conducted to see the diameter of antral ovarium follicle on each left and right ovariums which made to blocks by paraffin method by HE staining and then observed into five viewing fields using Olympus microscope type BX51 with magnification 40x10 (400x), the observation begin from the left and then shifted to the right then downwards clockwise. The observation result on the number of left and right antral ovarium follicles then added all the follicles.

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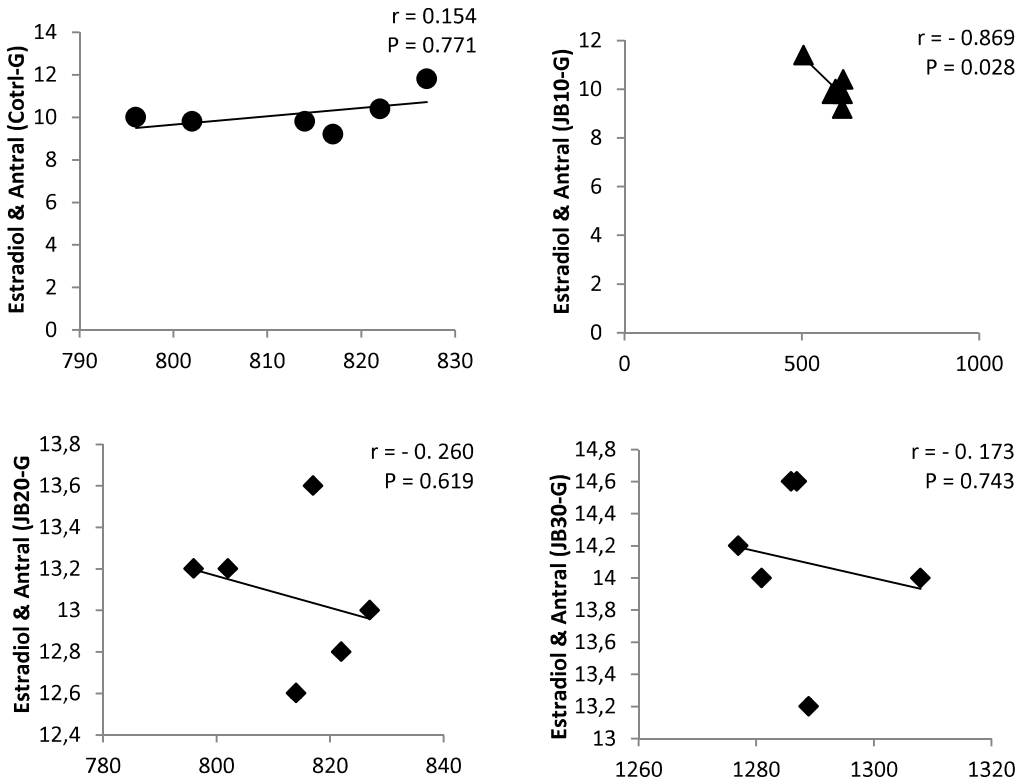


**Estrogen Hormone Examination**

Blood collection were conducted during the end of the research in the morning through medial cantus snus orbitalis of the right eye as much as 0.5 ml to examine the endogenic estradiol level using ELISA method.

**STATISTICAL ANALYSIS**

Data were collected and presented in descriptive form and then data normality were tested using Shapiro Wilk test and homogeneity test using Levene Test. The result of normality and homogeneity indicated that the data of estradiol level and diameter of antral ovarium follicle showing normal distribution and homogen, the One Way Anova were run, followed by LSD Post hoc test. The complete result of analysis statistic is significant if $p < 0.05$.

**RESULTS**

After the administration of gandarusa leaves extract for 28 days, twice a day, the histology of antral ovarium follicle images were obtained (figure 1), the average diameter of antral ovarium follicle and estradiol level were illustrated on table 1.

The result of the study indicated that the highest mean value of estradiol level and diameter of antral ovarium follicle is in group JB30-G, followed by group JB20-G, then group JB10-G, the lowest value of estradiol hormone value and the diameter of ovarium follicle is in group Ctrl-G. To investigate whether there are significant difference between groups, the Anova test were run considering the data were normally distributed and homogen. The result of Anova test indicated the significant difference between estradiol level and antral ovarium follicle between groups, $p < 0.001$.

**Estradiol Level**

The post hoc analysis on estradiol level show that the estradiol level on group JB10-G, JB20-G, and JB30-G were significantly higher compate to control, $p < 0.001$. Estradiol level on group JB30-G were significantly higher compared to JB10-G and JB20-G, $p< 0.001$. And so does the estradiol level on JB20-G compared to group JB10-G, $p < 0.001$ (figure 2).

**Diamater of Antral Ovarium Follicle**

The post hoc analysis on diameter of antral ovarium follicle indicated that diameter of antral ovarium on group JB10-G, JB20-G, andJB30-G were significantly higher compared to control $p < 0.001$. Diameter of antral ovarium follicle on group JB30-G were significantly higher compared to JB10-G and JB20-G, $p< 0.001$. And so does the diameter of ovarium follicle on group JB20-G if compared to group JB10-G, $p < 0.001$ (figure 3).

## DISCUSSION

The result of this research indicated that the estradiol level on the administration of gandarusa leaves extract with 10%, 20%, and 30% concentration are proven to increase the estradiol levels compared to the control. The result of this research illustrated that isoflavonoid in gandarusa leaves extract play important role in the increase of estradiol level. This makes sense, considering isoflavonoid is a natural estrogen which comes from plants (fitoestrogen), just like estradiol hormone in the human body which can bind with α and  estrogen receptors which distributed inside the body (Susetyarini, 2009) so it can produce various effects caused by estradiol eventhough with lower potentials.

The administration of gandarusa leaves extracts with 30% concentrations was proven as the most effective to increase the estradiol level compared to 20% and 10% concentrations. The administration of 20% concentration also can increase estradiol level higher than 10% concentration (figure 2). This result illustrated that the effect of gandarusa leave extract administration according to the amount of the dosage administered. The higher the dosage the higher the estradiol within the blood. Therefore, the increase of high estradion level due to the administration of gandarusa leaves extract can effect the decline of productive female mice fertility level which mediated by negative feedback effect on hypothalamus, hypofisis, and ovarium axis so the folliculogenesis did not take place. However, in this research, the mice used were mice on menopause age so the folliculogenesis were not expected.

Within the body, the high level of exogenic isoflavonoid can bind with estrogen receptor in the hypothalamus and skin. Towards hipothalamus, isoflavonoid can give negative feedback so the Gonadotropin Releasing Hormone (GnRH) and gonadotrophin (Gn) secretions are decreased. The decrease of GnRH and Gn cause the decline in Folicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) secretions, which followed by decline of Gn (FSH and LH), so the primary ovarium follicle unable to grow to become follicle degraf which produces estradiol, as the consequence the endogenic estradiol decrease (Deb, Campbell and Clewes, 2010). Referring to the work mechanism of the isoflavonoid, then to prove whether the high level of estradiol in this reseach is endogenic estradiol or exogenic estradiol due to the isoflavonoid enhancement from gandarusa it is necessary to examine the number of follicles and the diameter of antral ovarium follicles.

This research indicated that the administration of gandarusa leaves extract with concentrations of 10%, 20%, and 30% are significantly proven to increase the ovarium follicle compared to the control. The antral ovarium follicle are also significantly higher with 20% dosage if compared to the 10% concentration (figure 3). This result illustrated that the dosage of gandarusa leaves extract administration have positive correlations with the effect caused. The higher the dosage administered the higher the effect caused. However, the Pearson's correlation analysis of this research result describing that the increase of the hight estradiol level due to the gandarusa leaves extract administration are not linearly followed by the increase amount of antral ovarium follicle, on the contrary inverted correlation tend to occur (negative correlation; figure 4).

Referring to the Pearson analysis, it is clear that the estradiol level and the number of antral ovarium follicle on the control group eventhough not significant tent to indicate to positive correlation. On the contrary, the gandarusa leaves administration with concentrations 10%, 20%, and 30% tend to indicate the negative correlations. Even from those various dosage, the administration of gandarusa leaves extract with 10% concentration indicated the most significant negative correlations between estradiol level and the antral ovarium follicle (figure 4). This research result illustrated that the administration of gandarusa leaves extract concentration 10% are proven to increase estradiol level but not followed by the same increase from the number of antral ovarium follicle. This is stronglu assumed due to the negative feedback mediated by the increased of estradiol level. The increase of high estradiol level cause estrogen  (ER) receptor in hypothalamus (COUSE et al., 1997) binds estrogen and cause the decline of GnRH secretion, followed by the decline of FSH, and LH from hypophisis. However, the limitation of the study did not examine the GnRH, FSH, and LH. Furthermore, to ensure the role of the negative feedback it is necessary to conduct further research.

The administration of gandarusa leaves extract with 20% and 30% concentrations, also have negative correlations between estradiol levels and the number of antral ovarium follicle, eventhough not significant. But there is interesting thing, the correlation of estradiol level with the number of antral ovarium follicle on the gandarusa extract administration with 30% concentration were lower compared to 20% concentration, and 20% concentration were lower compared to 10% concentration. This research result gives the image that the gandarusa leaves extract administration with high concentration cand also produce the higher estradiol level but not followed by the increase number of antral ovarium follicle liniearly so the corretation is lower. Referring to the negative

correlation on gandarusa leaves extract administration with 10% correlation, then the increase of estradiol level happen on the high dosage (20% and 30%) gandarusa extract cause the areas around hypothalamus become very estrogenic so the isoflavonoid experience changes from agonistic to antagonistic (Stubert and Gerber, 2009) as the result, the increase level of GnRH, FSH, and LH also few increase of antral follicle ovarium. Thus, the agonistic and antagonistic isoflavonoid depends on the are's estradiol level so it is frequently considered as selective estrogen receptor modulators (SERMs) family (Chang et al., 2008).

The age of the mice used for this research were 16-17 months old equivalents to the human's 48-51 years (menopause period). Therefore, the relative increase of estradiol not followed by the number of antral ovarium follicles due to the administration of gandarusa leaves extract then it is considered as hormone replacement therapy on menopause women after the clinical trials. Furthermore, as the result of the study it also can indocated that administration of isoflvonoid as estrogen hormone replacement therapy is safely proven and even acts as cancer protector on various organs with estrogen receptors (Stubert and Gerber, 2009).

## CONCLUSION

This research indicated that the administration of gandarusa leaves extract with 10%, 20% 30% concentrations for 28 days are evidently proven on the effect of the increase of estradiol level and the increase of antral ovarium follicle on female mice.

## CONFLICT OF INTEREST

There is no conflict of interest.

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