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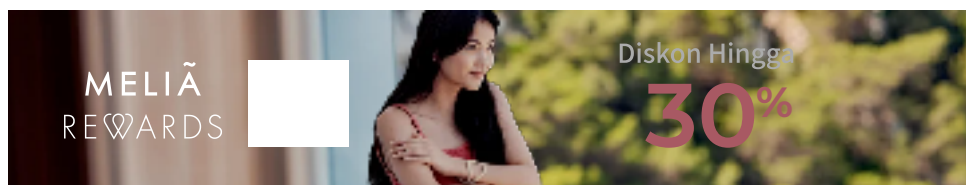
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Efficacy of Ethanol Extract of Water Clover (*Marsilea crenata*) Leaves on Estradiol Levels in Wistar Rats Post Ovariectomy Skin Aging Model

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ABSTRACT

Background: estrogen itself consists of three different types of hormones, namely estrone, estradiol, and estriol. Decreased levels of estrogen can occur in individuals who have disorders of the pituitary gland, Turner syndrome and in women who have gone through menopause. This can cause changes in many body systems and organs including the skin. Water clover plants contain bioactive isoflavones which include flavonoid glycosides including genistein and daidzein, both of which have body activities similar to estrogen. The estrogenic activity is related to the structure of isoflavones which can be transformed into equol which has a structure similar to the hormone estrogen. **Objective:** to analyze the effect of water clover leaf extract (*Marsilea crenata*) on estradiol levels in post ovariectomy wistar rats with skin aging models. **Methods:** experimental research with the type of true experiment posttest only control group. The design of this study used five groups consisting of a control group (K) and a treatment group (P1, P2, P3, and P4). **Results:** giving clover leaves can increase the level of estradiol in the blood. indicated by the results of the Kruskal-Wallis non-parametric test showing the P value = 0.016 (<0.05). That is, there are differences in the influence of the five research groups. **Conclusion:** there is a significant effect of giving water clover leaf ethanol extract on estradiol blood levels in post ovariectomy rats.

Keywords: water clover leaf extract, estradiol, rats, ovariectomy.

INTRODUCTION

Estrogen is a sex hormone produced by the ovaries to develop sexual organs and regulate the female menstrual cycle (Trisunuwati, 2016). Decreased levels of estrogen can occur in individuals who have disorders of the pituitary gland, Turner syndrome and in women who have gone through menopause. This can cause changes in many body systems and organs including the skin.

Estrogen function is mediated by estrogen receptors (REs), which belong to the nuclear receptor superfamily. REs itself has two types, namely estrogen receptors alpha (RE α) and estrogen receptors beta (RE β). RE α and RE β are located in the nucleus where they are bound to binding proteins (companions) when they are not bound to estrogen (Fitriani, 2018). An important releasing factor is Gonadotropin Releasing hormone (GnRH). GnRH secreted by the hypothalamus gland will trigger FSH and LH by the anterior pituitary gland (Guyton and Hall, 2008). Phytoestrogens are thought to work as estrogen agonists by filling the estrogen receptor site when estrogen is not available in the body.

Clover plants are very familiar to the people of Indonesia. Clover plants contain bioactive isoflavones which include flavonoid glycosides including genistein and daidzein, both of which have body activities similar to estrogen (Trisunuwati, 2016). The estrogenic activity is related to the isoflavone structure that can be transformed into a similar structure similar to the hormone estrogen (Trisunuwati, 2016). In previous studies, consuming water clover was able to increase estrogen levels in the blood (Titisari et al., 2019). Research conducted by Trisunuwati (2016) said that water clover leaf juice can play a role in increasing estrogen blood levels in experimental animals, due to the estrogenic effect of water clover leaves.

MATERIAL AND METHODS

Animals

The materials of this research were healthy female Wistar rats (*Rattus norvegicus*), aged 12 months with average body weight between 100-150 grams. Standard animal care and experimental procedure were approved by the Ethics Committee of the Health Polytechnic of the Ministry of Health of Malang, Indonesia. Rats were randomly divided into five groups.

Ovariectomy

The rats were anesthetized with ketamine-HCl at a dose of 100 mg/mL as much as 0.1 mL/head, then surgery was performed on the previously shaved lumbar region (left/right) to remove hair on the dorsal lumbar region. Both left and right ovaries were cut and removed, then sutured and given antibiotics. **Preparation and Intervention**

Those groups were: ovariectomized group with decoction of the ethanol extract of clover leaf water at a dose of 20 mg/KgBW in 3 cc of solvent. (Group P1); ovariectomized group with decoction of the ethanol extract of clover leaf water at a dose of 30 mg/KgBW in 3 cc of solvent (Group P2); ovariectomized group with decoction of the ethanol extract of clover leaf water at a dose of 40 mg/KgBW in 3 cc of solvent (Group P3); ovariectomized group with decoction of the estradiol at a dose 30 µg/100 gBB in 3 cc of solvent (Group P4); and ovariectomized group with given standard food and drink (Group K).

Enzyme-linked immunosorbent assay

Standard kits are set at graded concentrations, namely: 25, 50, 100, 250, 500, 1000 and 2000 pg mL⁻¹. A total of 100 L standard kit, control and sample were pipetted into each ELISA microplate well in duplicate, covered and labeled and then incubated for two hours at 37 C. Then 200 L of horse radish peroxidase conjugate enzyme was added into each well of the microplate and incubated for 60 minutes at a temperature of 20–25 C. After incubation, washing was carried out with 400 L of wash solution for each well. A total of 100 L of substrate solution was added to each well and incubated for 15–30 minutes at 37 C. At this point the microplate must be protected from direct light. A stop solution containing 0.5 mol H₂SO₄ was added to each well as much as 50 L to stop the enzymatic reaction. The optical density of each well was determined and the plate reading was adjusted at a wavelength of 450 nm for 10 minutes using an ELISA reader.

Statistical analysis

Data were collected from the research status, then performed cleaning, editing and coding. The data that was collected were entered in the *Statistical Package for the Social Sciences* (SPSS) data format version 20.0 (SPSS, Inc., Chicago, Illinois). The statistical test used was the normality test with the *Shapiro-Wilk* test because the sample number was smaller than 30 per group and the data were normally distributed ($p > 0.05$). For comparison analysis, data that were normally distributed and homogeneous were analysed with parametric statistical tests with *One Way Anova* test.

RESULTS

In this study, female Wistar rats aged 12 months were ovariectomized treated with topical treatment for three weeks. Subjects were divided into five groups, ovariectomized + ethanol extract of clover leaf water at a dose of 20 mg/KgBW. (Group P1); ovariectomized + ethanol extract of clover leaf water at a dose of 30 mg/KgBW (Group P2); ovariectomized + ethanol extract of clover leaf water at a dose of 40 mg/KgBW (Group P3); ovariectomized + estradiol at a dose 30 µg/100 gBB (Group P4); and

ovariectomized + standard food and drink (Group K). Water clover extract and estradiol treatments, were given once a day for three weeks.

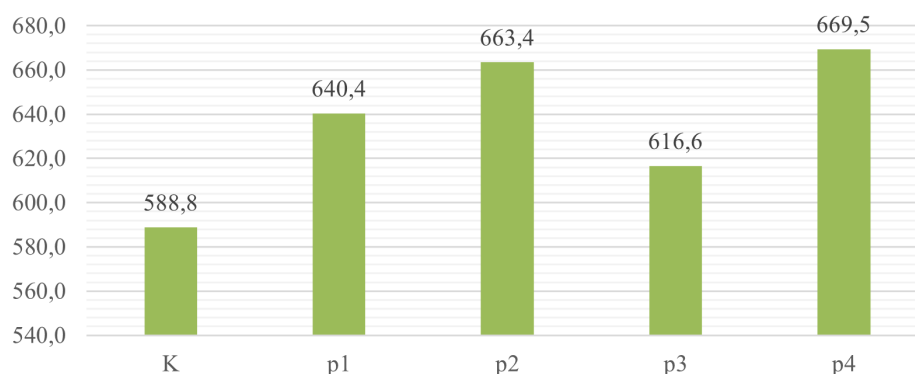
Estradiol Blood Levels

In this study, measurements of estradiol levels were carried out in the control and treatment groups. The results of the measurement of estradiol levels in the control and treatment groups are described in Figure 1.

The results of the study of estradiol levels in the control group above showed that the control group had the lowest mean value compared to the other groups. The results of estradiol levels in the treatment group showed that the group with the highest mean estradiol level was found in the P4 group given estradiol tab. 30 g/100 gBW, while the treatment group with the lowest mean estradiol was found in the P3 group which was given ethanol extract of clover leaf 40 mg/kgBW. The most optimal dose of ethanol extract was found in group P2 which was given 30 mg/kgBW ethanol extract of clover leaf with an increase in the mean estradiol which almost resembled oral exogenous estradiol therapy.

The lowest blood estradiol level in the study group was the control group with an average blood estradiol level of 588.8 pg/ml. The mean levels of estradiol in the control and treatment groups showed significant differences. The highest mean estradiol level was found in the P4 group, which was 669.5 pg/ml given 30 g/100 gBW estradiol tablets and the lowest was in the control group with a value of 588.8 pg/ml which only underwent ovariectomy. There were differences in the mean levels in the control group and the treatment group. The results of the highest mean levels of the treatment group were in group P4 who was given estradiol tablets and the lowest was in group P3 which was given ethanol extract of clover water leaves with the highest dose of 40 mg/KgBW.

Figure 1. Mean Estradiol Levels in Control and Treatment Groups



The Effect of Estradiol Levels by Giving Water Clover Leaf Ethanol Extract

The results showed a significant increase in estradiol levels in post-ovariectomy rats given ethanol extract of clover leaf water significantly. Statistical tests to determine differences between groups showed

a significant difference between the mean estradiol levels of the control group, namely group K vs P1, group K vs P2, and group K vs P4 (Figure 2). So, there was a significant effect on the estradiol levels of post ovariectomy rats given ethanol extract of clover leaf water at doses of 20 and 30 mg/KgBW.

Among the treatment groups given the ethanolic extract of clover leaf, the highest mean level was found in the P2 group with a dose of 30 mg/KgBW and the lowest in the P3 group with a dose of 40 mg/KgBW. So, it can be said that the most effective dose among the three doses of water clover leaf extract in this study was a dose of 30 mg/KgBW. This could be due to several factors that caused the dose and levels of estradiol not to show an interval so that it did not show successive levels of results.

DISCUSSION

Research conducted by Yuliawati et al., (2019) also showed that the ovariectomy and untreated rat group experienced a significant decrease in blood estradiol levels compared to other groups. The results of this study are similar to the previous study by Fernandez et al., (2020) regarding estrogen hormone levels in ovariectomized female rats after administration of Lamtoro Leaf extract said that the group that was ovariectomized and given corn oil (as a control group) had the lowest estrogen levels compared to the treatment group and had significant differences.

Figure 2. The Mann-Whitney non-parametric test data on the mean levels of Estrogen in the five study groups (n=20)

Groups	P4	P3	P2	P1	Mean of Estradiol Levels
P4	-	-	-	-	669,5
P3	0,245	-	-	-	616,6
P2	1,000	0,248	-	-	663,4
P1	0,020 *	0,248	0,021*	-	640,4
K	0,020 *	0,248	0,021*	0,021*	588,8

The theory that supports the results of this study is as research conducted by Hartiningsih (2016) in his research which reported that ovariectomized rats had lower estradiol concentrations than non-ovariectomized rats. This is because the loss of ovaries due to ovariectomy causes estrogen hormone levels to decrease, so that reproductive tissues that have estrogen receptors become atrophic (Zhou, S. *et al.*, 2016).

previous research conducted by Trisunuwati (2017) showed that the presence of water clover leaf squeeze treatment had a significant effect ($P < 0.05$) on the total blood estrogen levels of animal models. The increase in estrogen levels is thought to be due to the effect of phytoestrogens from water clover leaf treatment which is given regularly and for a longer period of time but is still within safe limits. Increased

estrogen levels occur because of the bond between phytoestrogens and receptors, resulting in positive feedback that stimulates the hypothalamus to produce GnRH (Trisunuwati, 2017).

Another similar study was conducted by Trisunuwati (2017) about the efficacy of giving water clover leaf extract to stimulate levels of the progesterone and estrogen hormone in the blood. When estrogen levels in the body decrease, there are many estrogen receptors that are not bound, even though they have low affinity, isoflavones can bind to estrogen receptors. If the body gets a supply of isoflavones or phytoestrogens, there will be an effect of increasing isoflavones with estrogen receptors which can have a beneficial effect on the body (Koswara, 2006).

This research is supported by the statement mentioned by Biben (2012) in the research of Trisunuwati et al. (2017), that the use of phytoestrogen preparations is generally good and useful, but inappropriate or excessive use can interfere with or cause reproductive system dysfunction, especially when used for a long time.

CONCLUSION

Several conclusions can be drawn regarding the effect of giving Ethanol Extract of Water Clover Leaves (*Marsilea crenata*) to Estradiol Levels in Post Ovariectomy Wistar Rats, including:

- a. The estradiol level in the control group was the lowest mean level in the post ovariectomy study group.
- b. The mean estradiol level of the treatment group given the ethanolic extract of clover leaf was higher than the control group.
- c. There is a significant effect of giving water clover leaf ethanol extract on blood estradiol levels in post ovariectomy rats

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