



Original Research

## Effect of Isoflavone Genistein Daidzein from *Pueraria lobata* Extract on Vascular Endothelial Growth Factor Expression in The Aorta of Hypoestrogenic Rat

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### KEYWORD

Bithok (*Pueraria lobata*); Hypoestrogenic rat; Isoflavone Genistein Daidzein; VEGF



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### ABSTRACT

**Introduction:** Currently, there is no research about the effect of isoflavone-based phytoestrogens on the expression of VEGF in arteries which play a role in preventing vascular diseases in menopause women (hypoestrogenic state). We want to know the effect of *Isoflavone Genistein Daidzein* in *Pueraria lobata* extract on VEGF expression in the arterial endothelium of hypoestrogenic rats.

**Methods:** This is an analytical, experimental study with a post-test-only control group design. Subjects were Wistar strain rats (*Rattus norvegicus*) randomized into five groups of different treatments by simple random sampling. The aorta was then examined for VEGF expression. Statistical analysis used were ANOVA, followed by multiple comparison tests by the Tukey Honestly Significant Difference and regression test with SPSS series 11.0. The analysis is significant if  $p < 0.05$ .

**Results:** *Isoflavone Genistein Daidzein* (IGD) in *Pueraria lobata* extract will increase the expression of hypoestrogenic rat endothelial VEGF. IGD dose of 15 mg/kg BW/day resulted in VEGF expression with an average of  $6.67 \pm 2.94$ , 30 mg/kg BW/day with an average of  $9.83 \pm 2.71$ , and 60 mg/kg BW/day with an average of  $9.83 \pm 2.71$  ( $p < 0.05$ ).

**Conclusion:** VEGF in arteries of hypoestrogenic rats without IGD supplementation was not significantly different from VEGF in the average rats. There was a significant positive effect on VEGF expression in hypoestrogenic rat arteries due to the administration of IGD in *Pueraria lobata* extract.

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## INTRODUCTION

The impaired quality of life that occurs in postmenopausal women is thought to be due to a decrease in ovarian function, which causes a reduction in estrogen. The decline in estrogen levels in the blood affects physiological conditions and increases the risk of several diseases, especially vascular disease. In several studies, reproductive women are less susceptible to vascular disease caused by atherosclerosis, such as coronary heart disease, myocardial ischemia, and stroke. This is thought to be inseparable from the role of estrogen as a vasculoprotective agent [1–3].

Several lines of evidence imply that estrogen influences angiogenesis directly through its effects on endothelial cells. Estradiol increases functional endothelium repair following arterial injury, according to Beach et al.'s research [4]. Endothelial proliferation and migration are induced by estradiol [5], which is mediated by the traditional estrogen receptor expressed by endothelial cells [6,7]. Several studies have suggested that vascular endothelial growth factor (VEGF) may be responsible for some of the angiogenic effects of estradiol. Estradiol has been demonstrated to boost VEGF expression in uterine and vascular tissue [8–11].

Like other steroid hormones, estrogen enters the cell through diffusion and binds to the estrogen receptor found in the cytoplasm and nucleus. The complex will bind to coactivator proteins that facilitate gene expression and have a high affinity for DNA binding sites to activate gene transcription. Transcription produces mRNA, which will later be translated into the cytoplasmic ribosomes to make proteins. Estradiol will bind to the estrogen response element, which is located at 1.5 kb from the transcription start that estradiol can directly increase the transcription process of the VEGF gene.

Vascular endothelial growth factor (VEGF) is a protein-ligand that stimulates the formation of new blood vessels, promotes vascular endothelium growth, and can prevent endothelial apoptosis. VEGF is also vasculoprotective by preventing endothelial damage due to oxidized LDL preventing atherosclerosis. Estrogen levels influence VEGF production. Estrogen will increase the amount of VEGF mRNA, which is then translated by the nucleus, and then VEGF is produced [5,6].

Experts have agreed on the use and benefits of estrogen replacement therapy (ERT) to reduce complications in postmenopausal women. However, the use of ERT has resulted in several side effects that are considered detrimental to the patients. Using estrogen as long-term ERT will cause various complications, including an increased risk of breast cancer.

Considering the significant side effects, efforts have been made to look for alternative compounds such as estrogen with mild side effects. One of the compounds' resembling estrogen is phytoestrogens which contain the active ingredient isoflavones. Isoflavone phytoestrogens have long been used as functional foods, but not much research has been done, especially on local foods.

Isoflavones have weak estrogenic properties in molar terms, with in vivo activity between  $1 \times 10^4$  and  $1 \times 10^{-2}$  of estradiol. However, in people who consume soy foods, serum isoflavone levels are up to 10,000 times the levels of endogenous estrogens. High concentrations in serum and tissue will compensate for the weakness. The active components of isoflavones are genistein (4'-methoxy-7 dihydroisoflavone), daidzein (4'-7-dihydroxyisoflavone), biochanin (4'-methoxy-5-7-dihydroisoflavone), and formononetin (4-methoxy-7-dihydroisoflavone). Because the structure and function of phytoestrogens resemble that of estrogen, its use is expected to increase VEGF [7,8].

This study determined the effect of *Isoflavone Genistein Daidzein* in *Pueraria lobata* extract on VEGF expression in the arterial endothelium of hypoestrogenic rats.

## MATERIAL AND METHODS

This is an analytical, experimental study to know the effect of IGD phytoestrogens on aortic endothelial VEGF expression in hypoestrogenic rats. The research design was a post-test-only control group. All methods were approved by the Ethical Committee of Medicine Faculty, Universitas Brawijaya.

Hypoestrogenic rat samples were made at the Pharmacology Laboratory of the Medical Faculty Universitas Brawijaya. *Bithok* extract was made in the chemical laboratory of Universitas Brawijaya. The slides were made in the Anatomical Pathology Laboratory of Saiful Anwar Hospital. The staining process and analysis of beta-receptor expression were carried out at the Biomedical Laboratory of Medical Faculty Universitas Brawijaya. This study used 30 rats (*Rattus norvegicus*) Wistar strain with predetermined inclusion and exclusion criteria; then, simple random sampling was used to determine the treatment obtained. There were five groups of treatment; normal control group, control group with oophorectomy only, and oophorectomy group with various IGD doses (15mg/kg BW/day; 30mg/kg BW/day; 60mg/kg BW/day).

Oophorectomy is performed to create a hypoestrogenic condition. Oophorectomy was performed according to the modified Ingle DJ and Grith JQ method. *Bithok* extraction was carried out according to the Figallo method. The isoflavones genistein and daidzein levels in *bithok* extract were analyzed using high-performance liquid chromatography (HPLC) techniques. Wistar rat aortic tissue after treatment was stained using Hematoxylin-Eosin and VEGF immunocytochemistry (Santa Cruz). VEGF expression examinations were performed by two examiners separately.

The data were analyzed statistically using the independent sample T-test to compare mean VEGF expression in each treatment group with control group, ANOVA test for multiple comparisons, followed by the Tukey Honestly Significant Difference (BNJ) multiple match and regression tests. The statistical test is said to be significant if  $p < 0.05$ . The calculation process is carried out with the help of SPSS series 11.0 computer software.

### Ethics

All techniques in this study were carried out in compliance with the appropriate manuals and regulations and were approved by Health Research Ethics Committee, Faculty of Medicine, Brawijaya University, Malang, Indonesia.

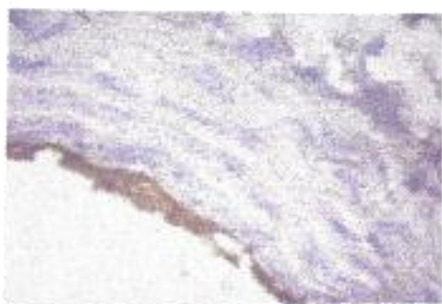


Fig. 1A.



Fig. 1B.

**Fig. 1.** VEGF expression in the aortic endothelium of oophorectomized rats. Purple in color is aortic smooth muscle cells with darker nuclei. Endothelial cell VEGF expression is shown in brown. Fig. 1A is the examination result with 400x magnification, while Fig. 1B with 1000x magnification.

**Table 1.** Baseline Descriptive Statistics of VEGF Expression in Hypoestrogenic Rat Arteries in 5 Treatments

Treatment	Min (%)	Max (%)	Average (%)	SW.Deviation (%)
Control normal (N)	2	6	3.67	1.51
Control oophorectomy (OVX)	0	4	1.8	1.64
OVX + IGD 15 mg/kgBW/day	3	11	6.67	2.94
OVX + IGD 30 mg/kgBW/day	7	14	9.83	2.71
OVX + IGD 60 mg/kgBW/day	8	19	12	4.29

OVX : oophorectomy. IGD : Isoflavones, Genistein, Daidzein

## RESULTS

In this research, VEGF expression in arterial blood vessels of hypoestrogenic rats varied at various doses of the *Isoflavone Genistein Daidzein* in *Bithok* (*Pueraria lobata*). The expression of VEGF in the arterial blood vessels of hypoestrogenic rats is described in the following figure.

From the basic descriptive statistics above, VEGF expression in the arterial blood vessels of hypoestrogenic rats in normal control group ranged between 2 - 6% with an average of  $3.67 \pm 1.51\%$ , while in the oophorectomy-only group, ranged between 0 - 4% with an average of  $1.80 \pm 1.64\%$ . The difference between mean expression of VEGF in normal control treatment and oophorectomy-only treatment was not statistically significant ( $p=0.081$ ). Treatment of IGD with a dose of 15 mg/kg BW/day resulted in VEGF expression in the arterial blood vessels of hypoestrogenic rats in the range of 3 - 11% with an average of  $6.67 \pm 2.94\%$ . Compared to the control group, this mean expression of VEGF was also not statistically different ( $p=0.050$ ).

Increasing the IGD dose at 30 mg/kg BW/day resulted in VEGF expression in the arteries of hypoestrogenic rats in the range of 7-14%, with an average of  $9.83 \pm 2.71\%$ . Compared to the control

group, this mean expression of VEGF was statistically different ( $p=0.001$ ). While a dose of 60 mg/kg BW/day resulted in VEGF expression in the arterial blood vessels of hypoestrogenic rats in the range of 8-19% with an average of  $12, 00 \pm 4.29\%$  and statistically different from the control group ( $p=0.001$ ).

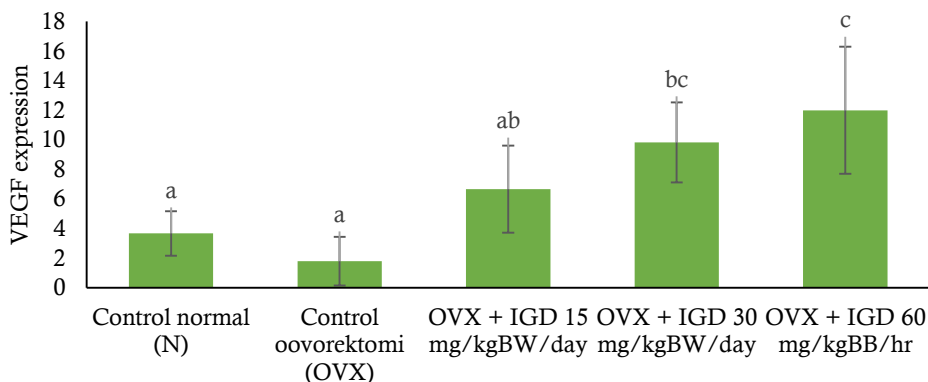
Differences in the dose of the *Isoflavone Genistein Daidzein* in *Pueraria lobata* (*Bithok*), especially at a dose of 60 mg/kg BW/day, have shown a very sharp difference in VEGF expression in the arterial blood vessels of hypoestrogenic rats against the control (-). The relationship between the dose of the *Isoflavone Genistein Daidzein* in *Pueraria lobata* (*Bithok*) and VEGF expression in the arterial blood vessels of hypoestrogenic rats is described in Fig. 2.

The dose-effect of the isoflavone extract of genistein daidzein in *Pueraria lobata* (*Bithok*) on VEGF expression in the arterial blood vessels of hypoestrogenic rats carried out by analysis of variance in one-way ANOVA, which is described in Table 2.

The one-way ANOVA results explained a significant effect (P-value <0.05) of the IGD dose treatment on VEGF expression in the arterial blood vessels of hypoestrogenic rats. The interpretation of these results is that there are at least two treatment groups with different mean VEGF expressions in the

arterial blood vessels of hypoestrogenic rats. A follow-up analysis to examine the difference in the mean of VEGF expression in the arterial blood vessels of hypoestrogenic rats in the five treatments was carried out using the Tukey Honestly Significant Difference test.

carried out at a 30 mg/kg BW/day. Based on the one-way ANOVA results, the mean value of VEGF expression in the arterial blood vessels of hypoestrogenic rats at a 95% confidence interval for each group can be described in Table 2. Values in this interval can be used



**Fig 2.** Relationship of The Isoflavone Extract of Genistein Daidzein in *Pueraria Lobata* (Bithok) with Hypoestrogenic Rat Arterial VEGF Expression

**Table 2.** Average Expression of VEGF in Arterial Vessels of Hypoestrogenic Rats at 95% Confidence Interval

Treatment	Confidence Interval 95%	
	Lower limit (%)	Upper limit (%)
Normal control (N)	1.27	6.06
Oophorectomy control (OVX)	0	4.43
OVX + IGD 15 mg/kgBB/hr	4.27	9.06
OVX + IGD 30 mg/kgBB/hr	7.44	12.23
OVX + IGD 60 mg/kgBB/hr	9.6	14.4

OVX : oophorectomy. IGD : Isoflavones, Genistein, Daidzein

Based on the results of the Tukey test, statistically, there were no significant difference in the mean VEGF expression in the arterial blood vessels of hypoestrogenic rats in the control (+) and control (-) groups. The higher the dose of the *Isoflavone Genistein Daidzein* in *Pueraria lobata* (Bithok) given, the higher the expression of VEGF in the arterial blood vessels of hypoestrogenic rats. There was no significant difference in VEGF expression in the arterial blood vessels of hypoestrogenic rats given the IGD at a dose of 15 mg/kg BW/day with control (-). Comparison with control (-) resulted in a significant difference at a 30 or 60 mg/kg BW/day dose. Furthermore, compared to the average VEGF expression in the arterial arteries of hypoestrogenic rats in the control group (+), there was no significant difference between the IGD group at a dose of 15 mg/kg BW/day.

These results indicate that efforts to restore to normal conditions with a dose of *Isoflavone Genistein Daidzein* in *Pueraria lobata* (Bithok) were successfully

to estimate the average expression of VEGF in arterial blood vessels in the hypoestrogenic Wistar strain (*Rattus Novergicus*) rat population.

The results of this study obtained a cut-off value of 4.43% for the oophorectomy group. This explains that VEGF expression in the arteries of oophorectomized rats has exceeded 4.43%, meaning that the rats are no longer hypoestrogenic. A therapeutic dose of *Isoflavone Genistein Daidzein* in *Pueraria lobata* (Bithok) dose of 15 mg/kg BW/day was proven to normalize the condition because statistically, the amount of VEGF expression in arterial blood vessels in hypoestrogenic rats was the lowest at 4.27%. Furthermore, the relationship between the amount of increased VEGF expression in the arterial blood vessels of hypoestrogenic rats and the dose of the *Isoflavone Genistein Daidzein* in *Pueraria lobata* (Bithok) in specific journals can be estimated using regression analysis.

The regression equation obtained is  $Y = -0.0033 X^2 + 0.3678 X + 1.8255$ . This equation is non-linear with a

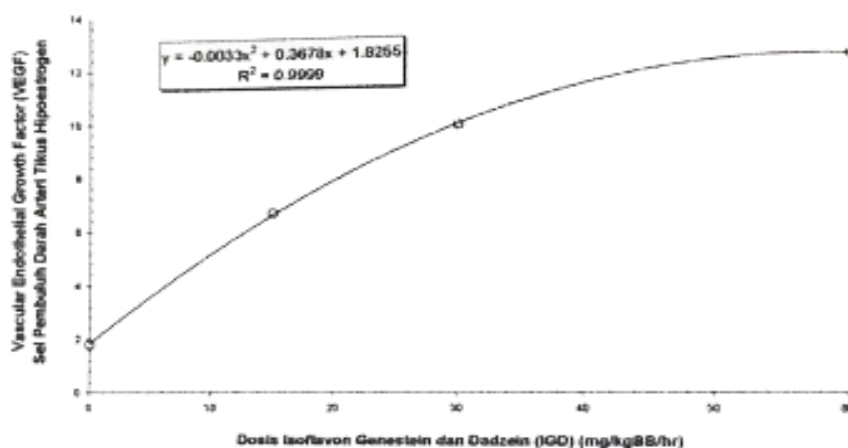


Fig. 3. Effect of IGD Dose on Arterial VEGF Expression in Hypoestrogenic Rats

quadratic pattern, meaning that each dose of *Isoflavone Genistein Daidzein* extract in *Pueraria lobata* (*Bithok*) of 1 mg/kg BW/day will result in an increase in VEGF expression in the arterial blood vessels of different hypoestrogenic rats. Based on Fig. 3, the highest increase occurred at a dose of 60 mg/kg BW/day.

The one-way ANOVA statistical method has several assumptions that must be met, including homogeneity of variance and normal distribution of residual data. The homogeneity of variance was tested using the Lavene test, while the residual data distribution was tested using the Kolmogorov Smirnov test. The results of Lavene's test resulted in an F value of 2.079 (p-value = 0.115), which concluded that the value of the variance of VEGF expression in the arterial blood vessels of hypoestrogenic rats from the five treatments in this experiment was statistically homogeneous. While the results of the Kolmogorov-Smirnov test for the normal distribution of residual data, the Z value of 0.816 (p-value = 0.518) has concluded that the residual value is normally distributed.

## DISCUSSION

In Table 1, it can be explained that the administration of the IGD at a dose of 15 mg/kg BW/day can increase the expression of VEGF in the arterial blood vessels of hypoestrogenic rats by 3 - 11%, while a higher increase was obtained after the IGD dose was increased to 30 mg/kg BW/day with a range of 7-14% and 60 mg/kg BW/day with a range of 8-19%. VEGF expression in arteries without IGD supplementation at a 95% confidence interval will range from 0.00% - 4.43%.

One-way ANOVA was used to prove the effect of IGD on VEGF expression in arteries. The analysis showed a significant effect of the IGD on VEGF expression in the arterial blood vessels of hypoestrogenic rats (p-value <0.05). This conclusion explains that the different doses of IGD in *Pueraria lobata* (*Bithok*) extract will affect the expression of VEGF in the arteries.

The difference in VEGF expression in the arterial blood vessels of the normal group and the oophorectomy group was not significant (notation letter a). This can be explained because estrogen is not the only trigger for VEGF expression. There is still a state of hypoxia and several growth factors, such as epidermal growth factor, TGF-a, TGF-b, IGF-1, and FGF, which also play a role in VEGF expression. The results showed that at a dose of 15 mg/kg BW/day, the average expression of VEGF in arteries was 6.67%, and if it were increased at a dose of 30 or 60 mg/kg BW/day, it would increase the average up to 9.83 % and 12%. The results of the Tukey follow-up test concluded that there was no significant difference in the expression of VEGF in arteries at a dose of 15 to 30 mg/kg BW/day (notation letters ab and b). The results of the comparison of VEGF expression in the arterial blood vessels of rats in the normal group against the 30 mg/kg BW/day group showed a significant difference (notation of letters a and b). So it can be concluded that the treatment that gave significant VEGF expression started at a dose of 30 mg/kg BW/day.

The relationship between IGD administration and VEGF expression in hypoestrogenic rat arteries was quadratic. The results of the regression analysis shown in Fig. 3 produce the regression equation  $Y = -0.0033 X^2 + 0.2678 X + 1.8255$ . (Y = VEGF expression in arteries and X = IGD dose).

Despite the fact that this study has a different target, it is consistent with a study by Huh J-E et al., who found that formononetin, a phytoestrogen, promotes early fracture healing and increases the number of vessels as well as the expression of VEGF and VEGF receptor 2 in the early stages of chondrogenesis in rats [9]. However, Sasamura's other research suggests that phytoestrogens may inhibit angiogenesis. In human renal cancer cells injected into rats, genistein decreases cell growth, causes apoptosis, and suppresses *in vivo* angiogenesis [10]. In HUVECs, genistein also suppresses oxyLDL-induced angiogenesis [11]. Genistein's anti-angiogenic action may be attributed to the downregulation of cell adhesion-related genes and cell adhesion impairment. Anti-angiogenic substances such as tissue factor, endostatin, and angiostatin may be activated by genistein.

## CONCLUSION

VEGF expression in arterial hypoestrogenic rats without IGD supplementation in *Pueraria lobata* (Bithok) extract was not significantly different from VEGF expression in normal rats. The treatment that gave a significant expression of VEGF was starting at a dose of 30 mg/kg BW/day. There was a significant positive effect on VEGF expression in hypoestrogenic rat arteries due to the administration of IGD in *Pueraria lobata* extract. Looking at the research process and the current results, we suggest that further research is needed to prolong the menopause/hypoestrogenic period in rats for more than three weeks and increase the dose of the IGD to more variable doses.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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