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## Estrogenicity of the isoflavone genistein pigeon pie seeds (*Cajanus cajan* L. Mill sp.) on reproductive organs in rat

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**Abstract.** Genistein is a compound with structures and properties similar to 17 $\beta$  estradiol which can be extracted from pigeon pie. The aim of the study was to identify the genistein profiles of pigeon pie in blood, urine, and feces and their effects on uterine and vaginal tissue structures of rats. Nine female white rats of Sprague Dawley, 6-7 months, weighing 150-180 g, were used and given pigeon pie seed solution for 36 days, genistein profiles were analyzed using HPLC, uterine and vaginal tissue structures were analyzed with HE staining. The results showed that the genistein contained in pigeon pie was lower than that in the synthetic genistein; vaginal epithelials and uterine proliferations were indicated. To conclude, pigeon pie was found to contain genistein which is a potential alternative for a natural estrogen source.

**Keywords:** genistein, estrogenic, uterine, vaginal

### 1. Introduction

The use of estrogen hormone can naturally be developed from various plants or processed food products which contain estrogenic compounds or similar potential estrogen-like compounds. Many research results have promoted Leguminosae family plants such as soybeans and soy products as natural sources of estrogen hormone. It is also known that soybean seeds and soybean processed products contain various phytoestrogen compounds [1]. Phytoestrogen compounds have a chemical structure similar to 17 $\beta$ -estradiol, and often referred to as "estrogen-like molecules". Some other estrogen-like compounds are: 1) isoflavonoid (genistein, daidzein, biochanine A, formononetin), 2) flavonoids (chrysin, apigenin, naringenin, kaempferol, quercetin), 3) coumestans (coumestrol, 4-methoxycoumestrol), and 4) lignans (enterolactone, enterodiol, matairesinol, secoisolariciresinol-diglucoside) [2].

Isoflavone is a group of non-steroid estrogen compounds with the chemical structures and properties similar to 17 $\beta$  estradiol and has estrogenic activity [3,4]. Isoflavone compounds are commonly found in Leguminosae family and one of its members is pigeon pie. Isoflavones in plants fall into the group of phytoestrogens because they have chemical structures and properties similar to estrogen hormones. Further, Phytoestrogen group compounds in plants can bind to estrogen receptor [5], making their activity resembles estrogen hormone. Isoflavones in plants, processed foods and fermented foods are usually in the form of glycoside derivatives chemical structures [6]. There are 12 isoflavone isomers comprising three aglycone compounds (daidzein, genistein and glycitein) and glucoside (daidzine, genistin, glycitin, acetyl-daidzin, acetylgenistin, and acetylglycitin) [7].



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Genistein is one isoflavone compound with the chemical structure similar to  $17\beta$  estradiol [8,9,10]. In regard to the fact that pigeon pie is categorized in the Leguminosae species, [11] found that pigeon pie contains flavonoids (quercetin, luteolin, apigenin), isoflavonoids (cajanin, cajanol) and stilbene (longistylin A and C). Further research conducted by [12] also found that pigeon pie (*Cajanus cajan* L. Mill sp.) contained genistein and daidzein compounds of 247.89828  $\mu\text{g/g}$  and 188.61309  $\mu\text{g/g}$ , respectively. Later, it is possible that estrogenic activity to be increased after the genistein undergoes metabolism completed by intestinal microorganisms [13].

Phytoestrogen compounds also have similar chemical structures with  $17\beta$  estradiol. Thus, although unclassified as hormones, they are capable of functioning as estrogen receptors and producing effects similar to endogenous estrogen [14]. It is possible for genistein to promote ovarian development because it can increase the hypophysis and hypothalamic hormone actions [15]. The study results of [16] mentioned that the administration of isoflavone compounds caused epithelialization of vaginal tissue structure and proliferation of uterine endometrium tissue structure.

Furthermore, a research study found that the phytoestrogens genistein and equol from soy can increase the ovarian development of *Huso huso* species [17]. Also, giving genistein compounds 26.6 mg/hr within 6 months to apes (equivalent 0.625 mg/hr to humans) was found to cause vaginal maturation [18].

Isoflavone compounds in the circulatory system are in the form of aglycone molecule, most isoflavones undergo excretion through urinary process, while some of them get to the circulation through enterohepatic process [19]. Genistein compounds experience a process of absorption inside the intestine and undergo the process of excretion through the bile. Similarly, daidzein compounds undergo metabolism in the gastrointestinal tract by the microflora in the intestine and become equol [20]. Similar to genistein, daidzein gets through the enterohepatic circulation process and undergoes the process of excretion in bile and continues to the excretion process through urine and feces slowly [21].

There are still many species of Leguminosae which have not been widely researched and used as natural estrogen sources and one of them is pigeon pie (*Cajanus cajan* L. Mill sp.). In this respect, an estrogenic study of phytoestrogen compounds in pigeon pie has not been conducted so that the promising benefits in the health sector have not been optimally explored and used. The objective of the study is then to analyze the potential of isoflavone genistein compounds contained in pigeon pie through a preclinical research study on the reproductive system of rat.

## 2. Materials and methods

### 2.1 Materials

The pigeon pie seeds were obtained from a plantation, bicolor variety, and the selected ripe pod seeds were those in black color.

### 2.2 Sample preparation of Pigeon pie and synthetic genistein solution

The dried pigeon pie seeds were washed and dried. It was later refined with a blender, and weighed 240 g, dissolved with 240 ml aquades, and squeezed to get 1.5 ml of solution. The HPLC analysis on the 1.5 ml of pigeon pie seed solution was 40.45506  $\mu\text{g/ml}$ . Meanwhile, the synthetic genistein was purchased from Sigma-Nachalai Tesque Inc. Kyoto Japan.

### 2.3 Laboratory animal treatment

Nine Sprague Dawley female rats aged 6-7 months, 150-180 g weight, were obtained from an animal husbandry laboratory in Blitar, Indonesia. The animals were kept in the animal laboratory of Bioscience Institute in Brawijaya University, Indonesia. The room temperature was maintained at  $\pm 27^\circ\text{C}$  with the relative humidity of 50-60% (ethical clearance) no. 168-KEP-UB. The rats were later grouped into 3 treatment groups. Group I which was the control group ( $P_0$ ), group II which contained rats given pigeon pie seed solution with the comparison of 24g: 24ml ( $P_1$ ) and group III which contained rats given synthetic genistein ( $P_2$ ). The giving of pigeon pie seed solution and genistein employed the



*gavage* method using a sonde into the animals' stomach in the morning for 36 days. The animals were placed in a metabolite cage for 24 hours, and the sampling processes for the blood, urine and feces were carried out every 8 hours. Fraction I was at the 8<sup>th</sup> hour, fraction II was at the 16<sup>th</sup>, and fraction III was at the 24<sup>th</sup> hour.

#### 2.4 Organ collection and organ staining

The surgeries and removals of the animals' vaginal and kidney organs were performed on the 37<sup>th</sup> day. The organs were soaked for fixation in 4% PFA solution. The making of the histology slides were then conducted in compliance with the procedure described in [22] and the staining was completed using Hematoxylin-Eosin.

#### 2.5 Measurement of genistein in blood, urine, and feces

The measurements of genistein in the blood, urine, and feces using HPLC method were performed in Fraction I at the 8<sup>th</sup> hour, Fraction II at the 16<sup>th</sup> hour, and Fraction III at the 24<sup>th</sup> hour. The Genistein Analysis of the blood, urine, and feces used HPLC method, which was developed by [23,24].

#### 2.6 Preparation of blood samples

The serum samples were taken as much as 50 µl and placed in a closed Erlenmeyer flask, being added 10 ml of acetonitrile, 2 ml of HCl 0.1 M and 5 ml of aquades and stirred using a stirrer for 2 hours at room temperature. The solution was filtered with a Whatmann filter paper No.42 to extract the filtrate. The filtrate was evaporated using a rotary evaporator with the temperature of less than 30° C. The residue of the evaporation was dissolved with 10 ml of 85% HPLC grade methanol in the water and filtered with 0.45 µm polytetrafluoroethylene filter to be later analyzed by HPLC.

#### 2.7 Determination of genistein serum levels

The determination of blood genistein serum levels employed HPLC Shimadzu method with C18 specification. The mobile phase solution used 0.1% glacial acetic acid in water and 0.1% glacial acetic acid in acetonitrile. Later, 20 µl samples were injected. The flow rate of the solution was 1 ml/min. The detector used photodiode at  $\lambda$  255-300 nm, column temperature 25° C, flow rate 0.8 ml/ min., wavelength 255 nm, running time 40 minutes, with post running time 15 minutes.

#### 2.8 Determination of urinary and feces genistein level

Each urine sample taken was as much as 0.5 ml (urin) or 0.5 g (feces) placed in a closed Erlenmeyer flask being added 10 ml of acetonitrile, 2 ml of HCl 0.1 M and 5 ml of aquades and stirred using a stirrer for 2 hours at room temperature. The solution was then filtered using a Whatmann filter paper No.42 and the filtrate was taken. The filtrate was evaporated using a rotary evaporator with a temperature of < 30° C. The residue of the evaporation was dissolved with 10 ml of 80% HPLC grade in water, then filtered using a filter device with 0.45 µm polytetrafluoroethylene filter (Alltech associates, Deerfield, IL) to be later analyzed using HPLC Shimadzu.

#### 2.9 Data analysis

Analyses of genistein data in the blood, urine, and feses samples were based on chromatographic results. Changes in the structure of vaginal and renal tissue were observed using an optical microscope.

### 3. Results

#### 3.1 Analysis of isoflavone genistein in the blood samples (Fraction I, II, and III)

The measurement result of genistein compound in the blood samples using HPLC method. Fraction I, II, and III showed that the genistein level in pigeon pie observed from the blood samples was lower than that in the synthetic genistein level (Fig. 1). The genistein pigeon pie at fraction I was 119831.334

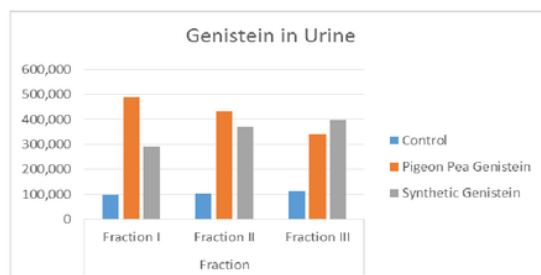
pg/l, at fraction II was 125036.489 pg/l, and at fraction III was 110627.338 pg/l. Meanwhile, the genistein synthetic at fraction I was 121327.404 pg/l, at fraction II was 173059.532 pg/l, and at fraction III was 215355.581 pg/l (Fig. 1).



**Figure 1.** Genistein in the blood samples of control-treated mice, pigeon pie and synthetic genistein at Fraction I, II, and III.

### 3.2 Analysis of isoflavone genistein in the urine samples (Fraction I, II, and III)

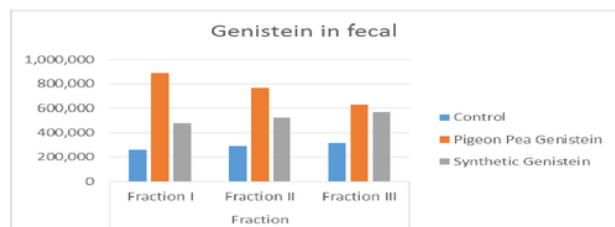
The measurement result of genistein compound in the urine samples using HPLC method at Fraction I, II, and III showed that the genistein level of pigeon pie at Fraction I was 490.465 pg/l, at Fraction II was 432.044 pg/l, and at fraction III was 338.527 pg/l. Meanwhile, the genistein synthetic at Fraction I was 292.487 pg/l, at Fraction II was 369.471 pg/l, and at Fraction III was 397.623 pg/l (Fig. 2).



**Figure 2.** Genistein in the urine samples of control-treated mice, pigeon pie and synthetic genistein at Fraction I, II, and III.

### 3.3 Analysis of isoflavone genistein in the fecal samples (Fraction I, II, and III)

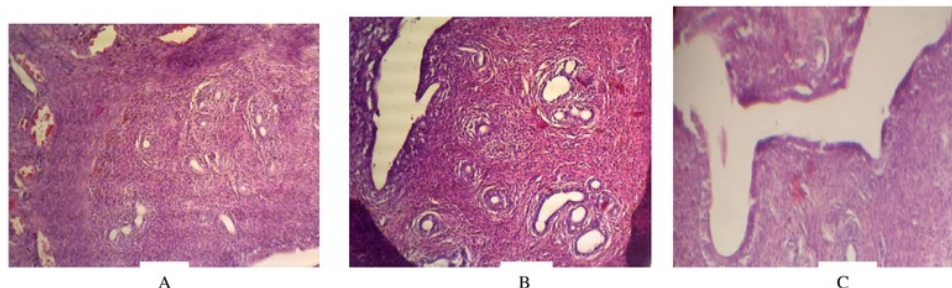
The measurement result of genistein compound in the fecal samples using HPLC method for each fraction showed that the genistein level of pigeon pie at Fraction I was 888.769 pg/l, at Fraction II was 768.301 pg/l, and at Fraction III was 627.177 pg/l. Meanwhile the genistein level in the synthetic genistein at Fraction I was 477.845 pg/l, at Fraction II was 526.925 pg/l, and at Fraction III was 573.515 pg/l (Fig. 3).



**Figure 3.** Genistein in the fecal samples of control-treated mice, pigeon pie and synthetic genistein at Fraction I, II, and III.

### 3.4 Observation of uterine, vaginal and renal histopathology

The structure of the uterine tissue consists of a layer of perimetrium, myometrium, and endometrium. The uterine tissue structures of the mice appeared normal in the control treatment ( $P_0$ ) (Fig. 4A). Proliferation of myometrial muscle tissue, proliferation of endometrial tissue and uterine gland, and narrowed uterine lumen were indicated ( $P_1$ ) (Fig. 4B). Meanwhile, proliferation of endometrial and myometrial lining was less extensive, and wider lumen of the uterus were also indicated ( $P_2$ ) (Fig. 4C).



**Figure 4.** Uterine tissue structure of the white rats, HE staining, 100x

(A) Control; (B) Treatment of pigeon pie seeds (C) Treatment of synthetic genistein

(A) The layer of endometrium with the uterine gland, myometrium; (B) The uterine lumen got narrower due to the proliferation of epithelial cells, the uterine gland develops, while the endometrial and myometrial layer experienced proliferation and the epithelial cells experienced cornification resulting in solidity; (C) wide uterine lumen due to absence of epithelial cell proliferation, the uterine glands did not experience much proliferation, the endometrial layer and myometrium were less extensive

Structure of the vaginal tissue consists of three layers, namely tunica mucosa, muscularis, and adventitia. In the tunica mucosa there is a complex tissue of squamous epithel and lamina propria. In the tunica mucosa, there are also layers of circular and longitudinal muscles, while in the Tunica adventitia there are fibroelastic tissues. With regards to this, the structure of the mice vaginal tissue appeared normal in the control treatment ( $P_0$ ) (Fig. 5A). Meanwhile, there was an increased proliferation of the vaginal epithelium, proliferation of the circular and longitudinal muscle tissue, multiple leucocyte cells in the lamina propria, and proliferation of the adventitia layer were all indicated, with the muscular layer border section was quite dense and contained many elastic fibers in pigeon pie ( $P_1$ ) treatment (Fig. 5B). Proliferation of the vaginal epithelium, and quite dense muscular layer occurred in the treatment of synthetic genistein ( $P_2$ ) although it was lower than that in the pigeon pie group (Fig. 5C).

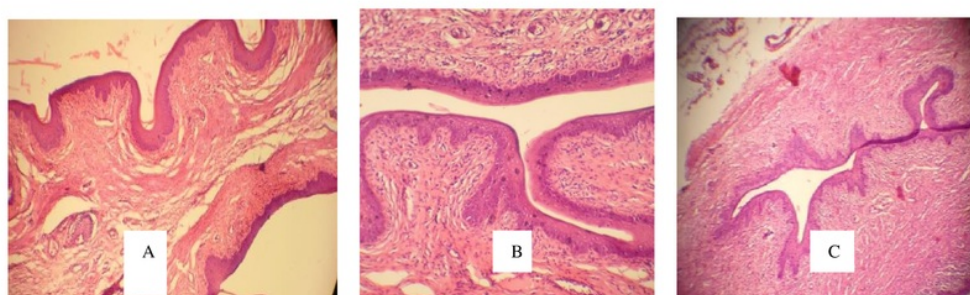




Figure 5. Vaginal tissue structure of the white rats, staining HE, 100x

(A) Control; (B) Treatment of pigeon pie seeds (C) Treatment of pure genistein

(A) The vaginal epithelium remained unchanged, visible mucosal, muscular, and adventitious layers; (B) Proliferation of the epithelial and proliferation of the muscular layer, and proliferation of the adventitia layer; (C) Proliferation of the vaginal epithelium and the muscular layer was quite dense. The observed structures of renal glomerular tissue appeared normal in control treatment ( $P_0$ ) and pigeon pie treatment ( $P_1$ ) (Fig. 6A and 6B), while giving synthetic genistein caused degeneration of the renal fat ( $P_2$ ) (Fig. 6C).

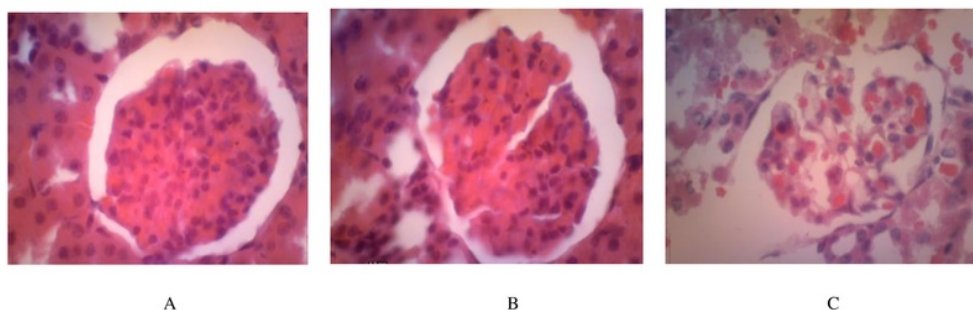


Figure 6. The renal glomerular tissue structure of rats, HE staining, 100x

(A) Control; (B) Treatment of pigeon pie seeds (C) Treatment of pure genistein

(A) The cortex and medulla appeared normal; (B) The cortex and medulla appeared normal; (C) glomerulus underwent degeneration of fat

#### 4. Discussions

Figures 1, 2, and 3 showed the presence of genistein metabolic processes in the body, which were indicated by the levels of genistein in blood, urine, and feces. The contained genistein underwent absorption, distribution, and excretion, every 8 hours. The synthetic genistein levels in the blood, urine and fecal samples were higher than them in the pigeon pie treatment samples. Isoflavone genistein is one component of the pigeon pie chemical compound experiencing absorption and distribution processes in the body system (Fig. 1), followed by excretion process (Fig. 2 and Fig. 3). Meanwhile the distribution of genistein in the tissues showed its potential in the vaginal and renal tissue structures (Fig. 4 and Fig. 5).

Since the synthetic genistein levels in blood were higher than the genistein levels in pigeon pie seeds, it suggested that, within the body system, genistein was suspected to interact or experience chemical structure changes. The components of the isoflavones pigeon pie such as daidzein, genistein, glicitein, daidzin, genistin, glycitin, malonyl daidzin, malonyl genistin and malonyl genistin had the isomer and enantiomer chemical structures so that their position in the body system was more stable than single/synthetic compounds. Daidzein was entirely converted to the structure of dihydrodaidzein both cis- and trans-, tetrahydrodaidzein, O-desmethyldaidzein (ODMA), dehydroequol, and equol by intestinal microflora after consumption [21,25]. Isolation of enzymes from *Slakia* sp. NATTS strains had the potential to convert daidzein to equol [26]. Additionally, the structure of cis-tetrahydrodaidzein and trans-tetrahydrodaidzein can be identified from the human urine [27,28].

The estrogenic compounds of isoflavone in pigeon pie (daidzein, genistein, glicitein, daidzin, genistin, glycitin, malonyl daidzin, malonyl genistin, and malonyl genistin) experienced absorption and distribution processes, thus increasing the number of genistein-like compounds entering actively into the blood and bound to estrogen receptors in the tissues. In the presence of a competitor, the level of genistein in the blood of pigeon pie treatment was lower than the genistein blood level of synthetic



genistein treatment. However, further research is needed to confirm the truth of a competitor's guess and the genistein structure changes in the body.

The chemical structure of genistein is similar to estrogen, as is endogenous estrogen, genistein undergoing enterohepatic circulation secreted in bile and excreted slowly through urine and feces [21]. Genistein in the body's biological system will experience direct absorption or further metabolism by intestinal microflora. Genistein in urinary excretion is found in about 3-10% [29]. Daidzein undergoes metabolic processes into equol in the digestive tract by intestinal microbes and the ability of daidzein to produce equol depending on the presence of intestinal microbes [20]. The bacterial species namely *Lactobacillus* and *Bifidobacteria* are thought to play an important role in daidzein metabolism to equol [30,31].

The estrogenic potential of pigeon pie compounds in the body is stronger than the synthetic compound. The structure of the vaginal tissue undergoes proliferation and cornification of its epithelial layer, the proliferation of the muscular layer, and the adventitia layer (Fig. 5B). Genistein and daidzein are suspected of having estrogen-like hormones because they have estrogen-like chemical structures, binding to estrogen receptors in ER  $\alpha$  and ER  $\beta$  [32].

Natural ingredients for medicine usually consist of one or more mixtures of ingredients that are processed together Skalli et al. [33] resulting in increasingly complex compounds, as well as the increasingly complex pharmacokinetic and pharmacodynamic [34], this is due to the interactive patterns between multi component compounds in the body's biological system. Metabolism of complex compounds of natural materials in the body system makes the characteristics of natural materials to be more stable than synthetic materials.

Giving synthetic genistein results in necrosis and degeneration of the renal fat (Fig. 6C). Ren's are organs susceptible to toxic effects, drugs, and chemicals. Toxicity tests of a drug or food substance can be identified through the changes in the structure of the kidney tissue. Examination of glomerular necrosis and tubules is an evidence of kidney cell damage. Prolonged administration of pigeon pie did not cause damage to renal tissue structure (Fig. 6B). Also, the complex compounds in the natural materials interact synergistically [34,35,36], thus providing optimum and safer effects than synthetic compounds.

## 5. Conclusion

The level of genistein in pigeon pie seeds is lower than that of synthetic genistein in the blood. Genistein extracted from pigeon seeds pie provides estrogenic potential in the uterus and vagina better than synthetic genistein. Also, the provision of pigeon pie in a long time does not damage the renal glomerulus.

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