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The Effect of Methanol Extract of Soybean *Glycine max L. Merr.* on Serum Steroid Hormone in Rat (*Rattus norvegicus L.*)

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Abstract : This research was to explore the genistein compound in soybean methanol extract and to study the effect of soybean methanol extract on the level of testosterone and estradiol of serum. Twenty male rats (*Rattus norvegicus L.*) were divided into 4 groups, the first group as a control, the second to fourth groups were given soybean extract as much as 250, 500, and genistein 0.3 mg/kg of body weight respectively. Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) were used to measure genistein compound in the extract. Measurement of testosterone and estradiol serum level were conducted using ELISA. Genistein identification result using TLC showed R_f spot value 0.43. Chromatogram peak of HPLC showed time retention 2.050 minutes and genistein level in 1 gram sample of soybean extract 0.6356 mg of genistein. Results *invivo* showed that 250 and 500 mg/kg of body weight of methanolic extract of soybean seed decreased the level of testosterone and estradiol serum. It could be concluded that soybean methanol extract significantly decreased the level of testosterone and estradiol serum for 52 days.

Key Words : *Soybean, genistein, testosterone, estradiol, male rat.*

Introduction

Soybean (*Glycine max L. Merr.*) is one of *Leguminoceae* and already consumed by human in daily life usually in the form of processed soybean as a flour or soybean liquid. In the past years, many researchs had been conducted regarding the effect of *Leguminoceae*, Research had been conducted because the plant has a number of compounds that have been isolated and has many important roles in human life. In pharmacology and medicine, it widely used as prevention and treatment of disease.

Based on the benefits of soybean, in order to meet a demand of the food industry of soybean, some local varieties of soybean had been released lately, such as Argomulyo, Bromo, Burangrang, Wilis, Anjasmoro and Grobogan¹. Local soybean varieties of Grobogan is soybean varieties which had reached 2.2 tons per

hectare productivity and is also above the national level, that only 1.49 tons per ha. This variety has the advantages of short life (76 days), the size of large peas, weight seed (18 g/100 seeds), high production, protein content 43.9 percent higher than the imported soy and Wilis varieties which had been cultivated by farmers².

One compound that has important aspect of soybeans as a source of functional food is isoflavone. It is a secondary metabolites produced by plants that synthesis by 2- hydroxyisoflavone synthase (IFS). The compounds are not synthesized by microorganisms. Therefore, the plant is a major source producing isoflavone compounds in nature. Isoflavones contain in soybeans present in high amount, and the highest is in the seeds, especially in the hypocotyl (germ)³. The amount and composition of isoflavones in the seed varies, depending on the morphology of the seeds (cotyledons, hypocotyl, and integument), genotype and cultivation environment⁴.

Most isoflavones in soybean are found in the form of genistein and daidzein. Therefore isoflavones as bioactive compounds in food supplement works to prevent some chronic diseases, such as cardiovascular, prevent osteoporosis, and antioxidants and the importance is to prevent cancer⁵⁻⁸. Genistein and daidzein include in phenol heterocyclic group that has structure similar to estrogen, so isoflavones often referred to phytoestrogen⁹. Therefore isoflavones may not only have a variety of physiological effects beneficial but also act as an endocrine disruptor. This is because they can act like estrogen and have activity antiestrogen which affect the metabolism of sex hormones and related biological activity¹⁰.

Therefore, the objective of this study was to determine the content of genistein in soybean extracts Grobogan varieties by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) method and to determine the effect of soybean methanol extract on the levels of testosterone and estradiol serum of rat.

Experimental

Materials

Soybean were acquired from Balitkabi (Balai Penelitian Aneka Tanaman Kacang dan Umbi) Malang Indonesia, isoflavon-genistein standard solution (Sigma Chemical Co.), methanol p.a (E. Merck), hexana p.a (E. Merck), ethyl acetate p.a (E. Merck), toluen p.a (E. Merck), aseton p.a (E. Merck), formiat acid p.a (E. Merck), silica plate gel 20x10, kits enzyme-linked immunosorbent assay (ELISA) testosterone and estradiol.

Soybean extract preparation

Soybeans were dried and then crushed made into powder form. Soy powder as much as 100 grams were defatted by soaking in 200 ml of n-hexane. Nonfat soy powder was dried overnight subsequently extracted by maceration method using methanol to 80% with a ratio (1:5) and were closed for 2 x 24 hours. The solution was filtered and then the pellet were dried using waterbath until concentrated extract was obtained. Finally the extracts stored in the refrigerator for later analyzed by TLC and HPLC and used for in vivo tests.

Analysis and identification of genistein

Analysis of genistein by Thin Layer Chromatography (TLC) was performed using stationary phase silica gel GF 254, the mobile phase toluene - ethyl acetate - acetone - formic acid (20: 4: 2: 1)¹¹ and stain spotter was checked by ultra violet rays. Plague formed observed in visible light, the UV lamp 254 nm and 366 nm then calculated its R_f. R_f extract samples was measured, then compared with the value of a genistein standard R_f.

Analysis of High Performance Liquid Chromatography (HPLC)

Standard genistein were dissolved in methanol (1 mg/ml) and then made a series of several concentrations with a concentration of 10 ppm, 8 ppm, 6 ppm, 4 ppm and 2 ppm. Analysis by HPLC instruments column LiChrospher (R) 100 RP-18 (non-polar) HP 1050 Liquid Chromatograph System equipped with a Hypersil 120-S ODS column (250 mm × 4.6 mm, 10 μm) and a UV detector; column length 10 cm; mobile phase methanol:water (70:30, v / v); a flow rate of 1 ml/min; injection volume 20 μL; UV detector at wavelength of 261 nm; oven temperature to room temperature. To detect the genistein present in the mixture,

the retention time of the sample chromatograms were compared with standard chromatogram retention time. Data obtained in the form of an area, then the values of a, b and r were determined by comparing the sample concentration (ppm) to the area.

Ethical clearance

Methods used in this study was approved by the Ethical Commission clearance Integrated Research and Testing Laboratory of the University of Gadjah Mada No. 279/KEC-LPPT/VI/2015.

Animal Experiments

Twenty males Wistar rat (*Rattus norvegicus* L.) weight 100-150 g were obtained. Animals were acclimatized under laboratory conditions (12:12h day/night cycle at a temperature of $25^{\circ}\text{C}\pm 2$) for 1 week. Standard pellet and water were given ad libitum.

Rats were weighed and divided into 4 groups of 5. The first groups as a control, the second to fourth groups were given soybean extract as much as 250, 500, and genistein 0.3 mg/kg of body weight respectively. Extract were given orally for 52 days. At the end of treatment, the animals were euthanized and surgery was conducted. Blood samples were collected from the intra-orbital plexus. Collected blood was centrifuged at 3000 rpm for 10 min at 4°C for serum. Serum was taken, then stored in a freezer at -20°C until measurement of hormone levels were performed. Supernatant was taken and stored in a refrigerator at temperature of -20°C to measured levels of testosterone and estradiol serum. Spectra kits testosterone and estradiol were used according to the instructions in the ELISA kits.

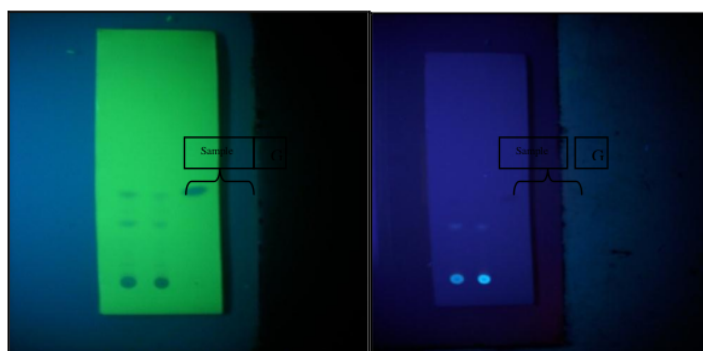
Statistic analysis

Data were analyzed using SPSS statistical software and average data was compared using one-way ANOVA and Duncan test with a confidence level of 0.05.

Results and Discussion

Analysis and identification of genistein in soybean extract

Result of TLC on soybean extract and standard genistein detected with UV 254 nm and 366 nm were showed in Figure 1 and Table 1.



Detection with UV 254 nm

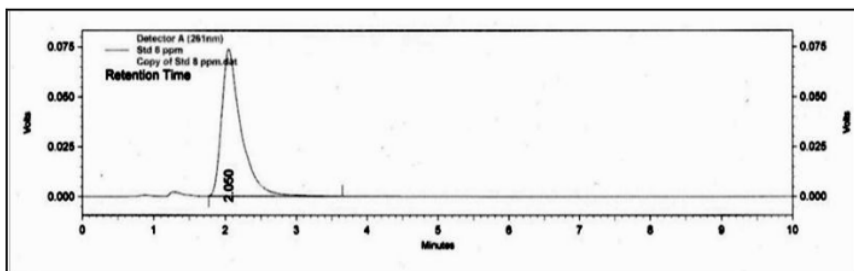
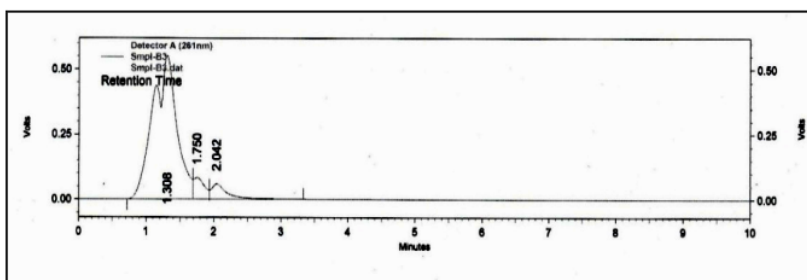
Detection with UV light 366 nm

Figure 1. Chromatogram of TLC results from soybean extract samples and genistein standard, seen by UV 254 nm and 366nm

Table 1. Chromatogram of TLC results from soybean extract samples and genistein standard, seen under UV 254 nm and 366 nm

| No | Material | Rf | Color |
|----|--------------------|------|--------------|
| 1 | Standard genistein | 0.43 | Light purple |
| 2 | Soybean extract | 0.42 | Light purple |

HPLC chromatogram results on genistein standard could be seen in Figure 2. It showed genistein retention time 2.050 minutes. While chromatogram in Figure 3. was used as comparison with vivo test. It could be seen there were 4 peaks which 1 of it was the genistein retention time which was 2.042.

**Figure 2. Chromatogram of HPLC genistein standard 8 ppm.****Figure 3. Chromatogram pattern of soybean extract with 1 peak of retention time 2.042 minutes for genistein**

Based on the detection results of the analysis and identification of genistein in soy extracts which conducted by UV 254 nm and 366 nm showed that the sample had a spot parallel to the standard genistein and had same purple color. Based on Table 1, showed that the standard of genistein had value of Rf 0.43 and light purple. In the soybean extract samples had the same color and Rf value was 0.42 approaching standard. This indicates that the sample of the extract contained one type of isoflavones which was genistein.

The results of the HPLC chromatograms of standard genistein showed that genistein had retention time of 2.050 minutes. On the results of chromatogram, there was one peak with retention time corresponding to the standard used which was 2.042 minutes for genistein. This showed that the extract of soybean contained genistein. On the results of genistein standard curve showed a linear regression equation was $y = 178785x - 51817$ with a correlation coefficient $R^2 = 0.9993$. The correlation coefficient was close to 1, means that there is a positive correlation between the levels of genistein and an area of the chromatogram. Determination of soybean extract made by interpolation on a standard curve subsequently acquired levels of genistein in soy extract samples. HPLC analysis results showed that 1 gram samples contained 0.6356 mg of genistein. According to Wang and Murphy¹², the high and low range of isoflavones results due to various factors such as: varieties of soybean, soybean maturity stage, the climate and temperature of a place to grow soybeans, soil conditions, methods of farming, processing and inspection procedures of soy isoflavones.

Effect of soybean extract to testosterone and estradiol serum

The results of testosterone and estradiol serum level with genistein and methanolic extract of soybean seed treatments on rat share well listed in Table 2. Statistically according to Table 2, level of testosterone in control group showed significant difference compared with other groups. Level of testosterone in control group was the highest among the other treatments. Whereas methanolic extract of soybean seed treatments showed the lowest level of estradiol and significant difference compared with control and genistein treatment. These results showed that treatment with methanolic extract of soybean seeds had the biggest impact in reducing testosterone and estradiol level. This showed that even though in the extract contained genistein, where genistein was known as estrogenic an antiestrogenic, there were other compounds affected the testosterone and estradiol level. Methanolic extract of soybean seed contain genistein and the other isoflavon. It can be concluded that pure genistein can decrease testosterone level but it will have a better effect if genistein is used with other nature compound in soybean seeds.

Table 2. The result of hormone testosterone level (ng/mL) and hormone estradiol level (pg/mL)

| Group | Testosterone (ng/mL) Mean \pm SD | Estradiol (pg/mL) Mean \pm SD |
|--------------------------|---------------------------------------|------------------------------------|
| Control | 3.23 \pm 0.84 ^a | 47.42 \pm 0.98 ^a |
| Soy Extract 250 mg/kg bw | 1.17 \pm 0.39 ^b | 44.98 \pm 1.49 ^b |
| Soy Extract 500 mg/kg bw | 1.40 \pm 0.54 ^b | 44.97 \pm 0.65 ^b |
| Genistein 0.3 mg/kg bw | 1.29 \pm 0.26 ^b | 47.16 \pm 2.02 ^a |

The numbers followed by the same letter on each column are not significantly different (n=5, p> 0.05).

The male reproductive system is a complex that consists of the hypothalamus, the anterior pituitary gland, testes and related organs such as the prostate gland, seminal vesicles and Cowper glands¹³. These organs work together to maintain strength, fertility, and secondary sexual characteristics of male. Testes consist of two compartments: the tubules seminiferous and intertubular tissue, which forms the interstitium. Tubulus seminiferous is coated by a layer of germ cells in various stages of development (spermatogonia, spermatocytes, spermatids, and spermatozoa) and supporting Sertoli cells. Interstitium consists of loose connective tissue, blood and lymphatic vessels, and a variety of cell types, including Leydig cells, fibroblasts, macrophages and leukocytes.

In Leydig cells, testosterone is partly metabolized to estradiol by aromatase. This enzyme sustains the balance between testosterone and estradiol levels in testicles. Estradiol is also important in the development and maintenance of reproductive function of male with regard to animal deficiency in receptor estrogen gene or aromatase gene¹⁴⁻¹⁶, and male with deficiency congenital aromatase¹⁷. Testosterone is essential for several physiological processes in humans. Some of the biological effects of testosterone are mediated through aromatization to estradiol^{14, 18-19}. Aromatase seems to be served in a variety of tissues, and its activity is essential for male reproductive function^{17, 20}.

Although there have been evidence that the development of the reproductive tract of male were influenced by the action of estrogen^{15, 21-22}, scientific attention to the role of estrogen in reproductive activity. Recently the most public concern that exposure to environment chemicals can affect the endocrine and reproductive systems. Exposure of high levels of estrogenic chemicals cause in a number of disorders, including reducing the size of the gonads, feminization of genetic males, and the number and quality of sperm are low. In this case, the estrogenic activity has been associated with a variety of steroidal and non-steroidal compounds, including one of them is phytoestrogen (such as genistein, daidzen)²³.

The study by Tabrizi *et al.* on the effects of phytoestrogens on testosterone support these results²⁴. McGarvey *et al.* found that LH levels in rats decreased as a result of exposure to genistein²⁵. This study revealed a decrease in serum testosterone levels. Decreasing levels of testosterone serum may be due to the decreasing levels of LH/ICSH serum. Leydig cells secretes testosterone by stimulating effects of LH²⁶⁻²⁷. Decrease in testosterone occur due to estrogen receptors α and β which are found in the brain²⁸. Activation of the estrogen receptor by soybean phytoestrogens may cause negative feedback responses in those organs. Next will lead to decreased levels of gonadotropin releasing hormone (GnRH), causing decrease in the biosynthesis of pituitary gonadotropins²⁹.

The decreasing in testosterone serum concentrations can also occur due to inhibition of androgenic testicular enzyme activity, because this enzyme is responsible for the regulation of testosterone biosynthesis³⁰⁻³¹. In addition, exposure to estrogenic chemicals have proven to decrease the volume of Leydig cells that responsible for the secretion of testosterone and damage the mRNA and also protein levels of important enzymes steroidogenesis³¹⁻³². While the soybean extract is also known as the lowering factor in blood cholesterol levels (both human and animal) and inhibiting the biosynthesis of cholesterol³³. The production of testosterone is not associated with cholesterol metabolism³⁴, it depends on modulation of steroidogenic enzymes. For example, the conversion of cholesterol into biologically active testosterone is enzyme process (including an enzyme that controls the transfer of cholesterol from the outer to the inner mitochondrial membrane)³⁵⁻³⁶.

In this study, serum estradiol levels showed significant reduction. The study by Weber³⁷ and Glazier and Boman³⁸ showed that phytoestrogens did not produce a significant reduction in estradiol levels. With regard to these results, there is a possibility that the different effects of phytoestrogens on male reproductive system is due to the effects of estrogenic and anti-estrogenic, as a function of phytoestrogens with estrogen receptors, which both have the ability of agonistic and antagonistic. Depending on the type of phytoestrogens and locations, the effects can be different. For example, a very weak agonist isoflavones bind to estrogen receptors less than the estradiol³⁹.

When estradiol levels are low in the body and bind less competitive, isoflavones showed strong agonistic effect. On the other hand, the anti-estrogenic effect of isoflavones depends on the relative concentrations of phytoestrogens endogenous and estrogen, and it is possible that when estrogen is high, phytoestrogens make estradiol receptors are not available for estradiol. Because they are consumed in large quantities in the diet, metabolic effects usually from antiestrogenic, because they compete with the much more powerful endogenous estradiol receptors, thus blocking their estrogenic activity⁴⁰⁻⁴¹. Phytoestrogens produce a variety of physiological effects in both humans and animal models. Their effects on the male reproductive system depend on the type of phytoestrogen, concentration and models studied⁴².

Conclusion

In 1 gram soybean (variety Grobogan) contains genistein 0.6356 mg. Exposure of methanol extract of soybean on male rats with dose 250 and 500 mg/kg bw for 52 days could decrease serum testosterone and estradiol significantly.

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