Estrogen hormone level of prepubertal female rat treated with Calliandra calothyrsus ethanolic leaf extract

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Abstract. This research examined the phytoestrogen potential of Calliandra calothyrsus leaf extract in prepubertal female rat (Rattus norvegicus). Sixty weaned female rats (21 days old) were divided into five groups i.e. control (K), negative control which was given 0.5% Na CMC suspension (KN) and treatment groups which were given with C. calothyrsus ethanolic leaf extract doses 25 mg/kg bw (P1), 50 mg/kg bw (P2) and 75 mg/kg bw (P3). The treatment suspension was administered 0.5 mL/rat/day by gavage for 28 days, started at the age of 21st days old. The rats were sacrificed and the blood samples were collected from 4 rats/group at the age of 28th, 42nd and 56th days old, each. The concentration of estrogen hormone levels were measured from blood serum by ELISA kit and were read at 450 nm wavelength with an ELISA Spectrophotometer. Data was analyzed statistically by General Linear Model with 95% of confidence. The result showed that rat’s body weight decreased significantly with the higher doses and the longer the treatment of C. calothyrsus leaf extract due to the anti-nutritive activity of calliandra tannins. The estrogen hormone level was significantly increased at the highest dose. The highest estrogen levels were found in the group of female rats which were given the extract of 75 mg/kg bw until the age of 42nd days. This results showed that there was a phytoestrogen potential in the C. calothyrsus leaf extract.

1. Introduction

The reproductive hormones estrogen and progesterone are produced by the ovaries, corpus luteum, placenta and adrenal cortex of the females. These hormones play important roles by stimulating the reproductive organs growth and development, the secondary sexual characteristics and mammary gland development, and also prepare estrus cycles for the mating behavior [1]. Estrogen greatly contributes to female puberty, an early function of reproductive organs, which is characterized by the occurrence of estrous and ovulation cycles [2].

In female, estrogen hormone deficiency caused reproductive disorders, menstrual cycle disruption and fertility rate decrease while the low levels of estrogen in prepubertal female caused miometrium atrophy and inactivity [2]. Estrogen stimulates bone epifiseal growth and maturation especially during the female prepubertal period [3].

Nowadays, many experiments have been done to explore exogenous estrogen sources, some compounds derived from plants are known to have estrogenic properties (phytoestrogens).
Phytoestrogens are potentially used as an alternative hormone replacement therapy to reduce symptoms due to drastic hormonal changes [4].

Red calliandra plants (*Calliandra calothyrsus*) is a potential herbal medicine, the availability of this plant is quite abundant in the highlands area of Indonesia. *C. calothyrsus* contains flavonoids, quercetin, saponins, caffeine acids, and alkaloids [5, 6, 7]. However, there has been no research on the phytoestrogens potential of *C. calothyrsus*. In this study, prepubertal female rats were given *C. calothyrsus* leaf extract which contains estrogenic flavonoids. The rat estrogen levels were expected to increase in the body which in turn could increase the reproductive organ growth of the female rats.

2. Methods

2.1. *Calliandra calothyrsus* leaf extraction

*C. calothyrsus* leaves (the dark-green color) were collected in the afternoon along the Mekarsari Highway of Baturiti Bedugul at Tabanan District of Bali Province. The leaves were air-dried, blended, sieved and stored in a dry place. The powdered substance was weighed (500 g) using a digital electronic scale (Scout, Ohaus), then was soaked in 2500 mL of 100% ethanol using an extractor, stirred and allowed to stand for 48 hours. The maserate obtained by filtration was collected and evaporated with a vacuum rotary evaporator at 50°C. The viscous extract obtained, then weighed using an analytical scale. The crude extract was ready for preparing an appropriate concentrations for the treatments.

The 0.5% Na CMC was prepared by weighing 50 mg Na CMC and spread it over warm water in a mortar and crushed homogeneously. The mixture was put into a 100 mL measuring flask and distilled water was added up to 100 mL. The 0.5% Na CMC suspension was used as the carrier and was administered to the negative control group.

2.2. Animals

Sixty female rats (*Rattus norvegicus*), aged 21 days (prepubertal), each group were housed in a 30x20x12 cm metabolic cage under standard laboratory conditions (26-27°C temperature, 50-60% relative humidity, and 12 hours light/dark cycle). Rats were given standard feed and drink water ad libitum. They were weighed at age 21st, 28th, 42nd and 56th days old. The experiment was approved by The Animal Ethical Committee, Faculty of Veterinary Medicine, Udayana University.

2.3. Experimental design

This experiment used a completely randomized design of 5x3 factorial design. The first factor was the dosage of treatment and the second factor was the length of treatment. Weaned female rats were divided into 5 groups i.e. control (K), negative control that was given 0.5% Na CMC suspension (KN) and treatment groups which treated with *C. calothyrsus* ethanolic leaf extract doses 25 mg/ kg bw (P1), 50 mg/ kg bw (P2) and 75 mg/kg bw (P3), each. The treatment suspension was administered 0.5 mL/rat/day by gavage for 28 days, started at the age of 21st days old. The rats were sacrificed and the blood samples were collected from 4 rats/ groups at the age of 28th, 42nd and 56th days old, each.

2.4. Estrogen hormone level analysis

The blood was taken from the retro-orbital plexus by a microcapillair, fed into an appendorf tube and centrifuged (3000 rpm) for 10 minutes. Serum was taken 50 mL followed by deproteinase process with addition of 500 μL uranil acetate, centrifuged (3000 rpm) for 5 minutes and 500 μL supernatant was taken. Concentrations of estrogen hormone levels were analysed with the procedure of Mouse/Rat Estradiol ELISA kit (Sigma-Aldrich, USA). The results were read at 450 nm wavelength with an ELISA (Enzyme Linked Immunosorbert Assay) Spectrophotometer and expressed in units of pg/ mL.

2.5. Data analysis

Data analysis was done statistically i.e. descriptive statistic test for characteristics of treatment group and variable frequency distribution, Kolmogorov-Smirnov normality test for the normality of generated
3. Results and discussion

3.1. Body weight
The results of statistical analysis showed no interaction between treatment of dosage with length of treatment on female rats body weight which were treated with *C. calothyrsus* leaf extract. The rats body weight decreased significantly with the higher doses and the longer the treatment (Table 1).

### Table 1. Final weight of female rats treated with *C. calothyrsus* leaf extract

| Treatment            | Dosage               | Final Weight (Mean ± SD), g |
|----------------------|----------------------|-----------------------------|
| Control              | 75.25 ± 5.802        |                             |
| Negative Control     | 72.83 ± 4.435        |                             |
| Dose 25 mg/kg bw     | 63.17 ± 2.516        |                             |
| Dose 50 mg/kg bw     | 60.17 ± 3.202        |                             |
| Dose 75 mg/kg bw     | 54.08 ± 0.500        |                             |

| Age                  | Final Weight (Mean ± SD), g |
|----------------------|-----------------------------|
| 28th days            | 44.05 ± 0.500 a             |
| 42nd days            | 68.75 ± 2.517 b             |
| 56th days            | 82.50 ± 4.435 c             |

Different letters follow (Mean ± SD) values in the same column show significant differences (P<0.05).

*Calliandra calothyrsus* Meissn leaf extract contains tannins and non-tannin phenol compounds. The phenol compounds of *C. calothyrsus* act entirely as tannins. The condensed tannins content of *C. calothyrsus*, has an anti-nutritive activity which decrease animal body weight. The interaction of tannins to bind various nutrients, thereby, reduced the digestibility and absorption of nutrients by the body. The main ability of tannins to form complexes with proteins, decreased protein digestibility and inhibited digestive enzymes [8]. These mechanism explained why the body weight of rats treated with *C. calothyrsus* leaf extract decreased significantly in line with the higher doses and longer treatment.

3.2. Estrogen Hormone Levels
The results showed that there was interaction between the treatment dosage and the age to female hormone estrogen level treated with *C. calothyrsus* leaf extract. The higher the dose of the extract, the estrogen hormone level increased, but the significant increase was at the highest dose (75 mg/kg bw). However, the estrogen levels did not differ significantly based on the age of rats when the blood sample were taken (as well as the length of extract treatment). The highest estrogen levels were found in the group of female rats which were given a dose of 75 mg/kg bw of *C. calothyrsus* leaf extract for 21 days or when the blood sample was taken at the age of 42nd days (Table 2).

### Table 2. Estrogen hormone levels of female rats after leaf extract treatment *Calliandra calothyrsus*

| Treatment         | Dosage               | Age | Average   |
|-------------------|----------------------|-----|-----------|
|                   |                      | 28th days | 42nd days | 56th days |           |
| Control           | 36.28 ± 3.285        | 35.79 ± 1.057 | 38.41 ± 1.527 | 36.83 ± 2.240 A |
| Negative Control  | 41.31 ± 3.981        | 41.98 ± 3.915 | 40.71 ± 2.455 | 41.33 ± 3.098 B |
| Dose 25 mg/kg bw  | 49.17 ± 1.280        | 39.33 ± 1.127 | 41.76 ± 4.913 | 43.42 ± 5.143 B |
| Dose 50 mg/kg bw  | 42.04 ± 5.439        | 44.25 ± 0.134 | 47.18 ± 8.796 | 44.49 ± 5.634 B |
| Dose 75 mg/kg bw  | 46.98 ± 1.974        | 53.20 ± 3.682 | 46.13 ± 2.291 | 48.77 ± 4.104 C |

Different letters follow (Mean ± SD) values in the same column show significant differences (P<0.05).
The increase of estrogen levels in line with the dosage and the length of *C.calothyrsus* leaf extract treatments in this study showed the potential of *C.calothyrsus* as phytoestrogens. Phytoestrogen’s effects might be pro-estrogenic or anti-estrogenic depend on the concentration. Phytoestrogens derived from plant have similar structures and functions to endogenous estrogens produced by mammalian body [9].

The receptor affinity of phytoestrogen is much lower than estrogen. However, in estrogen deficiency, many receptors are not bound by estrogen hormone. Therefore, phytoestrogens can bind the empty estrogen receptors [10]. Estrogen plays a vital role in a normal postnatal female physiology or pathology. Estrogen level changes may cause direct or indirect dysfunction in the female sexual and reproduction [11].

In this study, weaned female rats at the age of 21st days old (prepubertal period) normally have lower estrogen hormone levels than the adults. Some compounds of *C.calothyrsus* leaf extract increased estrogen hormone levels in the prepubertal blood serum. This phenomena lead to various estrogenic effects. The estrogenic effects range from ovarian and uterine histological changes, teratogenicity, alteration in the estrous/menstrual cycle and implantation, or some other effects. Therefore, it is necessary to do more research about the estrogenic effects of *C.calothyrsus* leaf extract on reproductive organ of prepubertal female rats.

The genus calliandra contains flavonoids and saponins, glycosides, steroids, fatty acids, alkaloids, polyphenols, anthraquinon, 2-hydroxy-4-methoxy benzoic acid, gallic acid, methyl gallate, myricitrin, quercitrin, myricetin 3-O-B-D-4Cl-lucopyranoside, afzelin, isoquercitrin, myricitrin 2’-O-gallate, myricetin 3-O-(6’-O-galloyl)-a-D-glucopyranoside, quercitrin 2’-O-gallate, quercetin 3-O-methyl ether, myrictrin2’-3’“-di-O-gallate, afzelin 2’-O-gallate, 1,2,3,4,6-penta-O-galloyl-â-D-4Cl-glucopyranose, caffeine and betulinic acid [5,6,7]. It is important to further find out which bioactive compounds or substances of calliandra leaf extract that act or have the phytoestrogen ability lead to pro-hormonal or antihormonal effects.

Phytoestrogens show their biological effects by their interaction with some enzymes in the biosynthesis and metabolism of estradiol/estrogen hormone [12] i.e. aromatase [13], steroid sulfatase [14], and 17h-hydroxysteroid oxidoreductase [15]. Phytoestrogens could have a significant impact on development in ways that affect later reproductive health [16].

4. Conclusion
Prepubertal female rat body weight decreased significantly with the higher doses and the longer the treatment of *C.calothyrsus* leaf extract due to the anti-nutritive activity of calliandra tannins. The significant increase of estrogen hormone level was at the highest dose of *C.calothyrsus* leaf extract (75 mg/kg bw). The highest estrogen levels were found in the group of female rats which were given a dose of 75 mg/kg bw of *C.calothyrsus* leaf extract for 21 days (when the rat blood samples were taken at the age of 42nd days). The results of this study showed the phytoestrogen potential of *C. calothyrsus* leaf extract.

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