RESEARCH ARTICLE | JULY 10 2017

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AIP Conf. Proc. 1862, 030112 (2017)
https://doi.org/10.1063/1.4991216

Eurycoma Longifolia Extract Increases Intracellular Production Activity of Luteinizing Hormone (LH) in Pituitary

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Abstract. Administration of the boiled water (extract) of *Eurycoma longifolia* (*E. Longifolia*) 18 mg/200 g body weight (bw) actually increases basophil cells in the anterior pituitary. Meanwhile, it is observed that basophil cells in anterior pituitary are producer cells of LH and FSH. Cell activity rate producing intracellular FSH does not increase in the amount significantly after administration of the *E. longifolia* onto the third day. The research attempts to prove the performance of *E. longifolia* to producer cells of luteinizing hormone (LH) in the anterior pituitary. Applied approach by a technical method of immunohistochemistry staining uses an antibody anti-LH. Observation is established to the treatment group of the *E longifolia* in a dose of 18 mg/200 g a bw on the 1st day and 3rd day, compared to control group of 1 ml distilled water on the 1st day and 3rd day. Research results that administration of the extract of *E longifolia* onto the third day has increased the activity of producer cells of LH in the pituitary, the synthesizing intracellular LH obviously. It can be concluded that *E longifolia* constitutes strong trigger in producer cells of LH to synthesize LH hormone.

INTRODUCTION

Wijayakusuma [1] and Kardono *et al.* [2] suggest that powdered *E. longifolia* which is brewed with water served as beverages is believed to increase libido and man's erection endurance. Chemical substances in *E. Longifolia* are used overall to recover the ability of man's erection and body fitness [3, 4]. *E. Longifolia* powder weighs in 1 g combined with 100 ml brewing water which is consumed by adult man for three-day treatment can enhance libido and appetite. Dosage conversion amounts 1 g/man's body weight to white rats weight as the subject of experiment rates in 18 mg/200 g bw [2, 5].

The role of *E. longifolia* in the increase of the subject 24-month male white rats libido with low sexuality is examined by Ang *et al.* [6]. In the experiment, it is employed such administration of drinking with a dose of 100 mg/200 g of body weight given twice a day for 10 days followed by an observation of the yawning and stretching behaviors. Both behaviors are considered as motion reflecting the rise of libido to the male white rats. It results that *E. longifolia* play a role as an aphrodisiac among an old male white rat [6].

Pratomo *et al.* [7] explain that the administration of *E. longifolia* with brewed dose of 18 mg/200 g bw in 1 ml distilled water heighten libido into highest rate compared other brewed doses respectively 100 mg/200 g bw, 200 mg/200 g bw, and control (1 ml distilled water). Libido behavior of male, white rats shows prominently due to the presence of estrus female that is separated/insulated by wire net partition in the cage leading to the observation: 1) approaching the partition/female, 2) positioning face to face, 3) pawing/biting the partition. Brewed dose of 18 mg/200 g bw is secure dose because methanol-water fraction of *E. longifolia* equals to 34.65 g/kg bw of the rats [8]. Any supply having higher LD50 than 15 g/kg is determined practically nontoxic [9]. In other words, *E. longifolia* constitutes nontoxic substance due to its LD50 higher than 15 g/kg bw [8].

In accordance Pratomo *et al.* [10] ascertains that the spread of basophil cells in anterior pituitary with *E. longifolia* treatment in brewed dose of 18 mg/200 g bw in 1 ml distilled water on the 1st day until 3rd day causes

International Symposium on Current Progress in Mathematics and Sciences 2016 (ISCPMS 2016)
AIP Conf. Proc. 1862, 030112-1–030112-6; doi: 10.1063/1.4991216
Published by AIP Publishing. 978-0-7354-1536-2/$30.00

raising amount significantly from average 27.9 cells into 35.2 cells (Duncan Test, $\alpha = 0.05$). Meanwhile, the spread of basophil cells in pituitary in control group with distilled water treatment, on the 1st day until 3rd day shows decreasing indication, from average 28.8 cells becomes 28.1 cells (not significant statistically, Duncan Test, $\alpha = 0.05$). Basophil cells in anterior pituitary are known to function in synthesizing LH hormone and *Follicle stimulating hormone* (FSH). A pituitary with haematoxylin eosins (HE) staining differs into three types cells, they are 1). Chromophobe cell (neutrophil) uneasy to stain, 2). Chromatin cell taking acid stain (eosinophil) such as cell producing prolactin is acidophil, and 3). Chromatin cell taking alkalic the stain (basophil). The cell producing LH and FSH in anterior pituitary is basophil cell [11-13].

According to the findings stating that basophil cell increasing in real circumstance after administering *E. longifolia*, Pratomo *et al.* [14] continues the research reporting that the rate of cell activity producing intracellular FSH does not significantly rise up after given *E. longifolia* treatment until the 3rd day. The research concludes that *E. longifolia* treatment given once every morning for 3 days does not influence any cell activity producing FSH in the anterior pituitary. Making the continual process physiologically in the related body with stable FSH function does not increase. The FSH in the woman and female animals functions: 1). to smooth follicle growth, 2). to prepare follicle for LH function, and 3). to help estrogen synthesis which is stimulated by LH. [11, 15]. Meanwhile, FSH hormone in man and male animals stimulates seminal and testis, as well plays important role in the beginning of spermatogenesis. Plasma FSH concentration increases during puberty from a lower level in childhood [11, 13, 15].

The cell producing FSH is not enhanced for its production activity by administering the *E. longifolia* [14], whereas the portion of basophil cell increases after adding *E. longifolia* for three days [10]. In another word, there are other basophil cells which are increased. In harmony with the theoretical framework that basophil cell in anterior pituitary is cell producing FSH and LH. Then there should be another basophil cell (except cell producing FSH) having its activity increased by *E. longifolia*; LH producer cell. Based on the notion above, the future research should be established in order to prove the virtue of *E. longifolia* in the enhancement of hormonal cells activity synthesizing intracellular *luteinizing hormone* (LH) in the anterior pituitary.

The objective of this research is to evaluate the *E. longifolia* performance in rising hormonal cell activity synthesizing LH in the anterior pituitary.

Potency of *E. longifolia* to be applied as standardized Herbs; the extract water or brewed *E. longifolia* with dose of 1 g/body weight of adult man (from conversion 18 mg/200 g body weight (bw) of white rats) is to be served as drinking for once in every morning for 3 days to increase LH hormone. The escalation of LH in men will fix the physical condition, libido, and his reproduction, since it is firmly related to the function of the testosterone increase. Besides, the rise of LH in women will repair physically brightness condition and libido (sexual desire) because it is strongly connected with estrogen escalation.

MATERIALS AND METHODS

This research is established in Histology Laboratory of Anatomy, Physiology and Pharmacology Department of Bogor Agricultural Institute (IPB) Veterinary Faculty.

Research Materials. The experiment makes use of white adult male white rats *Rattus norvegicus* strain Sprague-Dawley ages 3.5 months with weighs 121-194 g. The white rats are obtained from Animal Experiment Laboratory of BPOM, 30 males and 6 females with details as following: 15 males with *E. longifolia* treatment in which 10 males will be victimized using anesthesia severally 5 white rats (n = repetition = 5) on the 1st day and 5 white rats on the 3rd day. Other 15 males are used as a control group with distilled water administration. In this control group, there are 10 that will be victimized respectively, 5 white rats on the day-1 and 5 white rats on the 3rd day. Any estrus females will be taken amounting 6 females to be used as the seducer. Before the *E. longifolia*, treatment is given, the males are adapted in the experimental cage in anatomy and histology laboratory, of the veterinary faculty of IPB. The adaptation period takes respectively 2 weeks. During the observation, all the white rats are fed and given drinks ad libitum.

Simplicia of the root of *E. longifolia* plant (*Eurycomae longifolia Jack*) is peeled in the way of making powdered traditional health drinks by modification [16, 17]. Xylem section of the root which is relatively hard should be washed and drained before being sliced. The pieces of the root are dried up in the oven at 50 °C for 5 days. Next, they are cut into smaller pieces by a stainless sharp knife. All of the material next in line is ground into smooth grains using a special grinder to make flour and then the material is filtered using a Mesh 50 sieve (a pore size = 300 μm). The following Simplicia is saved in jars in a dry cabinet. The fraction of liquid extract or brewing of the *E. longifolia* is made in several steps such as a distilled water after heated in 80 °C is rested for 1 minute then poured

into powdered *E. longifolia* with dosing 18 mg/200 g body weight per 1 ml distilled water. The solution is stirred well and rested until it is cool. The next step is separate supernatant to be used in the observation.

Before taking pituitary tissue in the male white rat's brain to analyze: how high activity of cell producing LH hormone, the next step is to give brewed *E. longifolia* and control per oral at 09.00 western Indonesian times (WIB). And then, at 14.00 WIB both groups (the experimental group and control group) are observed related to the libido behavior using the estrus females. After that, the sample of pituitary tissue should be taken using experimental design as describe below.

Control group accepts 1 ml distilled water treatment per oral for each white rat by repetition 5 white rats every day on the 1st day until the 3rd day. A sampling of tissue for control the white rats is performed in the same way as to the *E. longifolia* group, it means on the 1st and 3rd day. All of the tissue samples obtained is fixated in formaldehyde solution 4 % in the NaCl physiologic 0.9 and saved for 1 week before further step in analyzing the micromorphology of basophil cell producing LH.

In another word, an *E. longifolia* group is prepared for sampling the tissue on the 1st day and the 3rd day as described at Table 1.

Before sampling the pituitary tissue, the white rats is anesthetized intraperitoneally using Ketamil which contains Ketamine 100 mg/ml with 0.2 cc dose for 100-200 g body weight. Using victimized white rats for sampling the tissue is described in the following section:

- **Day 1st**. 15 white rats are given *E. longifolia* and then 5 white rats are victimized to take the tissue sampling.
- **Day 2nd**. 10 white rats are given *E. longifolia* with no white rats is victimized.
- **Day 3rd**. 5 white rats are given *E. longifolia* and then 5 white rats are victimized for tissue sampling.

After the sample of pituitary tissue being fixated for 1 week, dehydration with degrees alcohol is employed for 4 days, and then the samples continue to clearing with xylol and blocking with paraffin histo. Next, the samples are saved in the fridge for a couple of days. After finishing with the storage, the sample is sliced using a microtome carefully into 4-5 μm size. Micromorphology analyze is performed toward activity of anterior pituitary cells playing role in production of LH hormone using immunohistochemistry staining.

Steps to immunohistochemistry follow Kiernan method [18] which is modified below:

1. Pituitary preparation slide result from cutting with microtome (has been through the dehydration stage, clearing, paraffining, embedding and blocking) which has been stored in the incubator 40 °C is taken out and then placed in distance of metal rack soaking boxes in the fall down position in the range of metal box rack of submerge in supine position in the incubator 65 °C for 3 minutes for paraffin melting.
2. Rehydration/deparaffination, with such steps: immersion each slide for 3 minutes is moved to one bottle to following bottle respectively xylol 3, xylol 2, xylol 1 and then alcohol absolute 3, alcohol absolute 2, alcohol absolute 1 and continue to alcohol 95 %, 90 %, 80 %, 70 % and distilled water (immersion in distilled water approximately ±20 minutes).
3. The bottom part of the slide is drained using filter paper and then marked in a circle on the lower slide of a pituitary using a marker, (the marker used is immEdge vector laboratories Inc., Bulingame CA 94010), immersed once again in distilled water for 30 minutes.
4. After finishing the immersion, slide is parallelized in line with marked sign face down and dripped with phosphate buffered saline (PBS) solution 0.1 N, rested for 5 minutes, this is performed three times (by dripping PBS 0.1 N and resting it for 5 minutes, removing PBS then dripping PBS for the twice and resting it for 5 minutes, removing PBS and then dripping it for the third time and giving it 5 minutes to rest), and further step.
5. Adding / dripping normal goat serum (NGS) 5-10 % in PBS and giving interval time for 30 minutes in the immersion of NGS. It has to be made sure that storage box of the slide is closed to prevent external contamination.
6. The washing with PBS is done for three times like in the number 4,
7. Adding/dripping antibody primary hormone (LH) which is diluted 1000 times, and let resting it in interval time in the closed box without light for one night/overnight with room temperature 27 °C.
8. After passing through overnight incubation, the material enters to next step which is washing with PBS again for two times.
9. The material will be washed again with PBS three times, on the third washing; PBS immersion takes 20-30 minutes.
10. Next, PBS is removed and then the material is added by a secondary antibody of LH and immersed, allowed to rest for 45 minutes in the closed box.

11. Washing with PBS again for 5 minutes three times.
12. PBS is removed and the material is added with chromogenic staining deamine benzedin (DAB), it should be rested for 1 second and then DAB staining immediately is removed.
13. Slide with its rack is immersed in distilled water for 15-20 minute.
14. Dehydration process is performed in one bottle to next bottle, bottle alcohol 70 %, 80 %, 90 %, 95 % respectively, and the bottles are shaken gently three times and then allowed to rest for 1 minute.
15. Next step, bottles alcohol absolute are shaken three times and allowed to rest for 2 minutes respectively alcohol absolute 1, 2, 3.
16. Then xylol bottles are shaken three times and allowed to rest for 2 minutes respectively xylol 1, 2, and 3.
17. The final step is mounting by dripping or adding entelan and closing with cover glass carefully.

Performance analysis of *E. longifolia* on the rate of cell activity producing LH is established in every treatment, analyzed from ten points of view are on several repetition slides (three repetitions) chosen. Cell calculation is begun from the most top point over a circle, continued to the right clock-wisely circulates to the inner part. The data is measured the averaging for each treatment. Observation and micromorphology analysis of cell producing LH hormone in pituitary use a binocular light microscope with magnification 400 times. Data of the cell amount with certain rate activity (positive 3, 2 and 1) between control and treatment of *E. longifolia* is analyzed using a Duncan test with a reliance 95 % ($\alpha = 0.05$).

RESULTS AND DISCUSSION

The degree of cell activity producing LH hormone is expressed in the gradation of chromogenic colors bound by complex antigen-antibody LH on pituitary cells producing LH hormone [18]. Cell activity producing LH shows as the strongest / most active is signed by visualization of dark brown color (its score as positive 3 or 3+++) which means in such cells contained synthesized LH hormone in maximal concentration qualitatively. Next order is 2++ score: the degree shows a little bit lower, the substance of intracellular LH hormone is showed by visualization of brown color indicating medium activity, while 1+ score show in light brown color for the group with weak LH synthesize.

Figure 1 describes the increase of cell activity producing LH to synthesize intracellular LH. *E. longifolia* treatment until 3rd day obviously increases the amount of dark brown cells, such cells are the producers of LH positive 3 (3+++).

The average amount of cells producing LH according to the degree of the activity group on the control group (distilled water) and treatment group (E. longifolia) is described in Table 2.

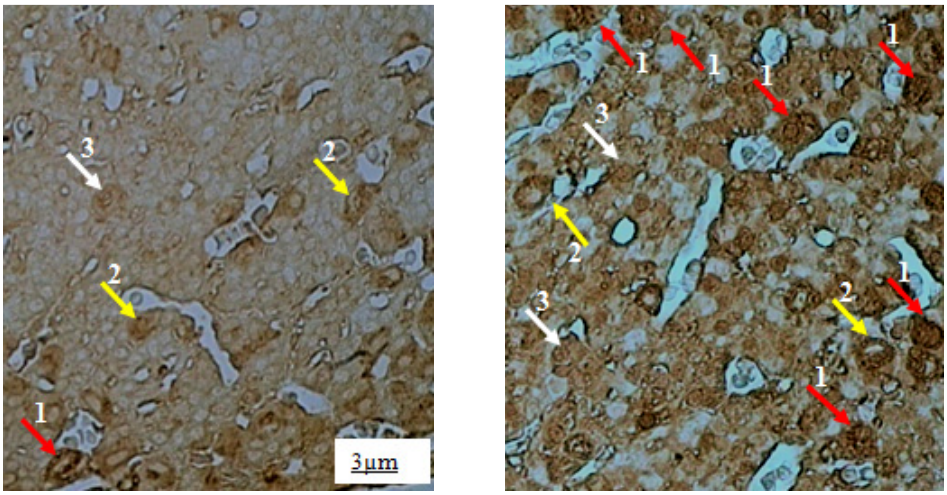
Data in Table 2 shows that administration of brewed dose of *E, longifolia* 18 mg/200 g bw increases the activity of cells producing LH to synthesize LH in actual on the 3rd day (Duncan test $\alpha = 0.05$). Cells producing LH is activated strongly shown by the increasing amount of LH cells positive 3 on the 1st to the 3rd day (8.0 becomes 32.4 cells), which becomes double compared to the control (distilled water administration) from 7.2 cells becomes 14.4 cells. The same notion also happens that the increase of cells amounts producing LH positive 2 and positive 1 on the 1st day until the 3rd day compared to the control from the day 1st until the 3rd day. Overall, it shows an increase of intracellular LH hormone production on the pituitary after the administration of an *E. longifolia* until the 3rd day. Total cells which are positive LH from the 3rd day are 82.8 cells compared to the control on the 3rd day, which 56.7 cells (the Duncan test, $\alpha = 0.05$).

Synthesis of LH production on pituitary on the day1st after administration of *E. longfolia* shows development compared to the control. The increase of LH synthesis on the 1st day is shown on all stages response group / positive 3, 2 and 1. The amount of LH cells on all treatment of activity stages on the 1st day is 8.0; 21.2; 17.1; and 46.3 cells, higher than the control (distilled water treatment) on the 1st day, they are 7.2; 19.9; 15.6; and 41.8 cells.

According to the finding (Table 2), it can be concluded that, *E. longifolia* constitutes strong trigger to synthesize/produce LH hormone. Mechanism of activating performance response pituitary cells producing LH

TABLE 1. Design when sampling the tissue

Time	9.00 WIB	5 hours	14.00 WIB	14.12 WIB
Day 1st, 2nd, 3rd	Administration of *E. longifolia*	♂White rats take a rest	Observation on libido behavior for 10 minutes	Anesthesia, sampling the tissue



(a) (b)

FIGURE 1. Spread of cell producing LH hormone in pituitary on: (a) control 3rd day and (b) *E. longifolia* treatment until the 3rd day. Red arrow, 1. Dark brown cells = positive 3 = strong activity of intracellular LH production; Yellow arrow, 2. Brown cells = positive 2 = intermediate intracellular LH production; White arrow, 3. Light brown / obscure color cells = positive 1 = weak intracellular LH production (using immunohistochemistry staining with antibody anti LH).

TABLE 2. The Amount of cells producing LH in pituitary after distilled water and *E. longifolia* treatment on the 1st until the 3rd day

No	Treatment	Cells Amount (average ± SD) LH producer			
		Positive 3	Positive 2	Positive 1	Total
1	Distilled water 1st day	7.2±0.3 ^{a}	19.9±0.5 ^{a}	15.6±2.2 ^{a}	41.8±2.1 ^{a}
2	Distilled water 3rd day	14.4±0.7 ^{b}	24.5±1.6 ^{a}	17.8±1.0 $^{a.b}$	56.7± 1.6 ^{b}
3	*E. longifolia* 1st day	8.0± 0.3 ^{a}	21.2±1.8 ^{a}	17.1±1.6 $^{a.b}$	46.3±1.2 ^{a}
4	*E. longifolia* 3rd day	32.4±4.5 ^{c}	28.0± 2.7 ^{b}	23.4±3.4 ^{b}	82.8±4.9 ^{c}

Information: superscript lowercase differs from the same column indicating the obvious difference on the rate of 5 %, the Duncan test α = 0.05.

usually is preceded by an increase of a GnRH for LH (GnRH-LH) which is secretion of the hypothalamus. Relating to the *E. longifolia* treatment until day-3 that increases LH degree, it can be predicted it is caused by such substance in Eurycoma longifolia (separately or synergized) playing an important role of GnRH-LH function from hypothalamus so that stimulating cells producing LH in pituitary to heighten LH synthesize. Finally, such *E. longifolia* substances are eurycomanone with its derivate [2] and/or longilactone playing as GnRH-LH. The other mechanism of *E. longifolia* probably goes to other ways: After it is detected that there is pheromone from female estrus white rats boosted up with the presence of such female, the pheromone receptor on the olfactory mucosa of the male white rats is strengthened in its performance. The strengthening of response/performance is predicted as the responsibility of one of or several substances in *E. longifolia*, example eurycomanone with its derivate and or longilactone. Component of longilactone substance and eurycomanone with its derivate constitute a polar substance which will be diluted in a polar solvent such as water [4]. Signal reaction from pheromone receptor which has been strengthened by substances contained in *E. longifolia* to medial preoptic area (MPO) areal, medial amygdala (MEA) and part of bed nucleus from stria terminalis (BST) functioning as libido controller, can stimulate GnRH-LH release from hypothalamus and furthermore stimulate or trigger gonadotropin LH hormone from anterior pituitary glands [19, 20].

*Luteinizing hormon*e (LH) is a reproduction hormone with molecule weight 28.500-30.000 Dalton (28.5-30.0 kDa). LH in women and female animal stimulate final-stage maturation from follicle graaf and ovulation process, the corpus luteum development as well. Estrogen and progesterone secretions are stimulated by LH. In other words, LH in men and male animals stimulate testosterone formation by testicle. LH stimulates nongerminal elements

consisting of interstitial cells (Leydig cells) to produce androgen, dehydroepiandrosterone and testosterone. Another function of LH is to endure the further process of final spermatogenesis, meanwhile, on the embryonic stages, it functions in the preparation of additional genitals such as ductus deferen, prostate and vesica seminals [11, 15].

The reaction is begun with LH bonded with membrane receptors which are specific to interstitial cells or Leydig cells in men and male animals and luteal cells in women and female animals (those cells are not influenced by FSH). LH activation triggers steroid genesis on Leydig cells which furthermore leads to changing process of acetate becoming squalene, a substance which will be cholesterol in the synthesize. It also applies in the acceleration of cholesterol change into 20 alphahidroxy cholesterol, an inter substance needed in testosterone synthesize or progesterone in women and female animals [11, 15].

Herb therapy to increase LH may not be allowed to be applied in long term usage because the increase of LH in long period will cause severe effect, such as phenomena of azoospermia in men and infertility in women [21]. Corresponding to the finding of this research indicating that in the 3rd-day of the administration of *E. longifolia* dose 18 mg/200 g body weight of male white rat which is conversed to adult men weight 1 g in 100 ml water is a safe method to increase libido.

CONCLUSIONS

Administration of *E. longifolia* until three days obviously increases the production of intracellular LH in the pituitary of the male white rats. Related to that notion it can be concluded that *E. longifolia* constitute powerful trigger to synthesize LH hormone.

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