Antifertility Effect of the Ethanol Extract of *Centella asiatica* L. Urban Against the White Rat (*Rattus norvegicus* L.) in the Early Post-Implantation

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Abstract. *Centella asiatica* is commonly known as centella or pegagan is one of the biological resources that have many benefits for mankind. Clinical and pharmacological study of *Centella asiatica* has been widely proven by scientists of the world, but there are a few properties that require further investigation in particular its effect on fertility. This research aims to know the influence of the ethanol extract of pegagan (*Centella asiatica*) towards the birth rates or fertility in the early post-implantation. On the research, white rat (*Rattus norvegicus* L.) is used as animal tested. The method that used in this research was an experiment by using randomly complete design. The tested animals were grouped into 4 treatment i.e. group I (control group), group II (dose 175 mg/kg body weight), group III (a dose of 200 mg/kg body weight) and Group IV (dose 225 mg/kg body weight). The tested animals were given the exraction of *Centella asiatica* leaf orally with different doses for 15 days after post-implantation. The value of post-implantation death percentage is calculated by counting the number of implantation, either containing a live fetus, or dead fetus and embryo resorb. The results showed that *Centella asiatica* gave the influence on white white rat fertilisation. On the grup IV treatment with the dose of 225 mg/kg body weight showed the highest KPI val-ue i.e. 57.23%.

1. Introduction

Biological resources have been widely merits by people for various purposes such as medicines, cosmetics, furniture, and other industries [1], [2]. Until now the treatment of allopathik is still attractive to the majority of people for various reasons [3]–[5]. Most of the medicines in the world are produced from plants [6], [7]. But not a few plants also contain ingredients that negatively effect conferring in humans. One of the plants are quite important and has taken advantage of the general public are pegagan plants (*Centella asiatica*) [8], [9]. Pegagan (Indonesia) also known as pennywort (Asia), guta kola (India) [4] and bua-bok (Thailand). This small creeping plants have been used long time in traditional medical herb [1]. In the tropical area this plant has a long history of being used for therapy in a variety of conditions such as skin disorders, blood vessel disease, inflammatory, and microangiopathy [10]. *Centella asiatica* can grow in a shady and damp environment and also marshy land [9], [11].

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*Centella asiatica* is a medicinal plant that is already widely used and very popular in South Asia [12]. According to the results of some research, *C. asiatica* can be utilized as a remedy of healing wounds, inflammations, rheumatism, asthma, hemorrhoids, tuberculosis, leprosy, fever, appetite enhancer and as well as improve the blood circulation [12], [13]. The efficacy of this herbal plant is to give a good effect in the improving of nerve stimulation to the brain power and capable of waging a blood flow to the brain vessels [7], [14], [15]. However, *C. asiatica* is presumed have a negative effect to the human body either. Unfortunately the information is still uncertain because there have been no clinical trials of these negative effects.

According to the some results of the research, *C. asiatica* contains triterpenoid saponin which mainly composed of some compounds such as asiaticoside and madecassoside, triterpene saponin genins, essential oil, flavonoids, phytosterols, sugars and other active ingredients such as tannins, amino acids, fatty acids, alkaloids and mineral salts [8], [16]–[18]. Saponins or flavonoids are active compounds in *C. asiatica* that have antiestrogen or can be synthesised onto antiestrogen in the body [19]. Antiestrogen is a compound that can inhibit or modify some of the effects of estrogen. The effect of antiestrogen can cause womb lining (endometrial atrophy). Despite the fertilization takes place, the process of implantation will be disturbed [9], [20]. Phytochemical analysis results showed that the content of alkaloids, saponins, tannins, flavonoids and triterpenoid of *C. asiatica* in air more stronger than *C. asiatica* by in vitro. However the results of *C. asiatica* in vitro culture contain steroids that are not produced from *C. asiatica* which grown the ground field [21].

The biochemical activities of the *C. asiatica* such as triterpenoid saponins, flavonoids, essential oils, and phytosteroler allegedly might be affecting on the metabolism of females reproductive organs such as ovary, uterus and ovarian follicles [22], [23]. Triterpenoid saponins on the *C. asiatica* in low doses could increase the number of primary, secondary and tertier follicles on the ovary of the female white rat [21]. In terms of biochemical activities antifertility, triterpenoid saponins namely asiaticoside and madecassoside were able to repair of the damaged cells and collagen fibers formed quickly. The active ingredients are also able to improve the granulosa of cells which inhibit the gonadotropin hormones secretion that cause the follicles cannot develop [24]. Granulosa cells on the ovarian follicle produces a compound which acts as an inhibitor of gonadotropin synthesis and secretion in particular follicle-stimulating hormone (FSH) [25], [26]. At high doses, *C. asiatica* extract tend to lower the amount of the primary, secondary, tertier follicle and deGraff on the ovaries of the female white rat [21], [26], [27]. A similar statement was also mentioned that high doses of extract *C. asiatica* ovarian follicle development may decrease in neonatal white rat. The aim of this research is to know the influence of the granting *C. asiatica* leaves extract in the early post-implantation against fertility of rats (*Rattus norvegicus*) white strain Sprague Dawley.

2. Materials and Methods

This research was conducted in the laboratory of Biology UHAMKA Jakarta. The research design used was Complete Randomized Design with 4 treatments and 6 replications respectively. A total of 24 adult female rats were distributed into 12 trays/cages grouped into 4 groups. Mice in Group I were given a normal diet (placebo) that was used as a control. In Group II, III and IV were extracted *C. asiatica* at a dose of 175 mg/kg of body weight, 200 mg/kg of body weight, 225 mg/kg of body weight respectively.

2.1 The extract preparation

Leaf extraction of *C. asiatica* was done by maceration method. This method uses solubility principle in which the solvent used was 70% polar ethanol. The *C. asiatica* leaf that has been dried then grinded into powder by grinding it into a grinding machine. The next step was the powder will be macerated for 24 hours and repeatedly 3 times to obtain soluble and soft. The chopped leaf extract of *C. asiatica* form was used for the treatment.
2.2 Preparation of the animals test
The tested animal was white mouse (*Rattus norvegicus*) adult (± 250 gram) bought from Animal laboratory, Faculty of Animal Husbandry, Bogor Agricultural University (IPB). The tested animals used in this study were female white rats Sprague Dawley that have been approved by the UHAMKA ethics committee (protocol number 027/KEPU/XI/2016). The white mice were acclimatized for 1 week to be adapted to their environment. During the acclimation process, rats were fed pellets and given drinking water by ad libitum.

2.3 Reagents
The materials used in this study were pellet, sterile aquades, chloroform, NaCl 0.9%, alcohol 70%, alcohol 90%, ethanol 70%, ethanol 90%, amoniac 1%, amoniac 25%, HCL, Dragendrof, Mayer reagent, Mg powder, amyl alcohol, NaOH, FeCl3, ether, petroleum ether, Hrmatoxilin, Bouin solution, Xylol, Eosin solution, George solution and paraffin.

2.4 Research procedure
The first procedure to do is to mate the tested animals until pregnancy occurs. Female rats were inserted into a cage with male rats in the ratio of three female mice and one male rat. The mated female white rats are those in the proestrus phase or the final proestrus. The pregnancy is check by taking a vaginal fluid with a blunt pipette which is given a 0.9% NaCl liquid [24]. Rats are considered to have been mated if there is sperm in vaginal smears [23]. Once the Rats were identified have been pregnant, the administration of *C. asiatica* leaf extract in the early post-implantation phase was administered on the 6th day until the 9th day of pregnancy (3 days in a row) [14]. *C. asiatica* leaf extract was given to fertil females orally by volume 1 ml/100mg of the female white rats weight [23], [24], [28].

After 15th days, the rats were anesthetized by inhalation using chloroform and then surgically followed the direction of a duplex-shaped uterine horn and then continued to the left and right of the uterine horn line. So the contents of the abdominal cavity will be visible, including ovaries and uterus [24], [29]. Then observe the amount of implantation containing live fetus, dead fetus or embryo resection. Inside the amniotic sac, the live fetus is pink and the dead fetus appears pale white, a black blood clot with the rest of the aspirated embryonic tissue (in the absence of an embryo) and expressed as an embryo is absorbed [24], [28].

2.5 Data analysis
Data analysis was done by collecting data to calculate the percentage of Death Post-implantation (KPI) by the following formula:

\[
KPI = \frac{\sum \text{Implantation} - \sum \text{Living fetus}}{\sum \text{Corpus luteum}} \times 100\%
\]

Furthermore, a homogeneity test and a t statistic test were performed to determine the effect of each treatment. The data obtained were then analyzed using SPSS version 21 [14].

3. Results and Discussion

3.1 Result
In this research, we have done a test on giving of *C. asatica*'s leaf extraction at early stage of post-immunization to the fertility of white rat. The test was conducted to determine the effect of leaf extract of *C. asiatica* on the death of white mouse fetus. Examination is performed on day 15 or 6 days after treatment (*Post-implantation of Early*). Observations were made by dissecting the test animals and observing the uterus. The observations of uterine morphology of the four treatments are as follows.
Figure 1 above represents the result of the effect of *C. asiatica* extraction in one replication of the rat uterus in all four treatment groups. Morphologically can be seen the comparison of living fetus in Group I, Group II with Group III and Group IV. In Group III and Group IV treatments, it was clear that there were no living fetuses and there was an acceptably higher embryo rate than Group I and Group II treatments. The small amount of implantation of the fetus in a uterine sac will have a larger and heavier volume (Fig. 1b) than the large number of implants (Figure 1a).

The next observation is to calculate the value of KPI. The value of KPI is the value of the average percentage of resorb embryos that occur in the uterus. Results of research on mortality in early white mouse post-implantation (KPI) were displaced in Table 1.

| Treatment | sd | Post-implantation death (KPI) (%) |
|-----------|----|----------------------------------|
| Grup I    | 0  | 0\(^a\)                          |
| Grup II   | 12.67 | 9.72\(^a\)                    |
| Grup III  | 35.21 | 56.23\(^b\)                    |
| Grup IV   | 46.87 | 57.23\(^b\)                    |

Description: Figures with different letter superscript show significant differences (\(\alpha = 0.05\)).

Based on the data in Table 1 it can be seen that the administration of ethanol extraction of *C. asiatica* leaf at treatment of Group II, Group III and Group IV showed difference of result with Group I (control). From the result of statistical test using pair t test at 5% significance level, it can be seen that in Group I treatment with Group II showed no significant effect difference. While in the treatment of Group III and Group IV with the dose of 200 mg/kg of body weight and 225 mg/kg of body weight, showed a significant difference to the increase in the average percentage of KPI. This shows that administration of ethanol extract of *C. asiatica* leaf with the dose of 200 mg/kg of body weight and 225 mg/kg of body weight gave a significant effect on post-implantation mortality of white rat.
3.2 Discussion
The granting of ethanol extraction of *C. asiatica* has done toward the white rat (*Rattus norvegicus* L.). Based on the four groups the treatment, it can be noted that the granting of extractions of *C. asiatica* can affect to the fertility of white rat which shown the embryos resorbs at an early stage of post-implantation (Group III and Group IV). Based on the analysis of variant with 95% confidence levels, there is no noticeable difference to the percentage of life fetus, dead fetus and embryo resorption (disappear) among the control group with the treatment. It was proved by the value (P>0.05). Dead fetus can be founded in group III and IV at a dose of 200 mg/kg body weight. and 225 mg/kg body weight. The granting of *C. asiatica* extraction in group III and IV (Figure 1c, 1d) in this research lead to the freezing of the embryo implantation and higher level resorption than the treatment in group I and group II. In the treatment of group II (dose 175 mg/kg body weight.), although there are changes but those changes have yet to give a significant influence of the death of post-implantation white rats. It is allegedly due to compounds in the extract of the leaves of *C. asiatica* can still be tolerated by the white rat fetus cells. While at a dose of 200 mg/kg body weight. and above, the cells of the fetus have been unable to tolerate compounds in the extract of the leaves of *C. asiatica* causing embryonic resorbs.

Fetus undergoing resorption characterized by the presence of clots either red or yellow brown. Embryo resorbs is the manifestation of death which results of the conception that can occur due to morphological errors i.e., disability and ends with the death [24]. A dead fetus or embryo resobs is a form of death of intraterus. The deaths of Intrauterus occurred due to not being able to do the repairs or recovery damaged of cells. Defects which occur caused by the large number of damaged cells, so there is no balance between the damaged cells with normal cells. Fetus cells which able to do recovery have the possibility chance to survive bigger than the fetus cells which incapable recovery the damaged cells [30].

The occurrence of embryonic resorbs caused by active compounds contained in *C. asiatica* such as triterpenoid saponins [21], [31]. Active ingredient content of triterpenoid part in fixing the granulosa cells of the ovaries and it will generate a inhibidin [32], [33]. Inhibin functions as an inhibitor of synthesis and secretion of gonadotropins, especially FSH. *C. asiatica* leaf extract contain triterpenoid saponin and flavonoid compounds that can also inhibit the secretion of GnRH [34], [35].

Obstructed of the GnRH secretion causes the anterior pituitary, suppress the Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH) secretion which would cause not generated of estrogen and progesterone hormones, resulting that the flow of the endometrial to blood vessels be reduced [10], [24], [36], [37]. Gonadotropin-Releasing Hormone (GnRH) and Gonadotropins is a hormone which needed to maintain the reproductive function of female animals. In the same way with other endocrine hormones, GnRH and Gonadotropins are controlled by their regulatory principles. Although the synthesis and secretion of GnRH set the LH and FSH, but the GnRH hypothalamus under control of kisspeptin hypothalamus [30], [38]–[40]. GnRH secretion which is inhibited causing anterior pituitary presses FSH and LH secretion where it is possible to cause the production of the hormones estrogen and progesterone are reduced or low, so that the flow of the endometrial blood vessels to be reduced [36], [39]. [41]. The reduced flow of blood vessels on the endometrium impact on decreasing supply of nutrients to the fetus, so that the fetus will be able to see death. Saponins have a negative influence against the reproduction of cattle such as abortion or death, sterile and termination of fertilization process [38].

Ethanol extracts compound of *C. asiatica* leaves containing saponins and flavonoids are antiestrogen because the compounds can lead to decreased estrogen levels [20], [42]. In addition, antiesterogen also can cause ovarian inactive, follicle growth and pepsinogen secretion of endogenous estrogen disturbed. It will cause a decline in the ability of the corpus luteum produces progesterone [23]. If progesterone is not produced, survival fetus will be disturbed [32]. In addition, the endometrial wall originally thickened will be depleted, because the blood supply that transfers oxygen and nutrients to the fetus does not occur resulting in the death of the embryo in the womb.

Progesterone and estrogen balance is important. Progesterone may induce an endometrial shift from the proliferative and apoptotic (influence of estrogen) phase to the nutrients secretion phase, so
that the egg fertilized can develop and become the fetus [24], [27], [43]. The compounds contained in the *C. asiatica* extract can induce apoptosis through a process of activation in mitochondria [43]. The results of this study indicate that the extract of *C. asiatica* triggers apoptosis in the early stages of the embryo which causes a decrease in the number of embryonic cells and disrupts the development of early post-implantation embryo cells.

4. **Conclusions**

Content ethanol extract *C. asiatica* leaf at dose 200 mg/kg body weight and 225 mg/kg body weight give effect on the fertility of rats. The failure of fertilization presented by embryo resorbs. The treatment with the highest dose 225 mg/kg (Group IV) indicates the higher number of embryo resorbs i.e. 57.23%.

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