Combination effect of Centella asiatica (L.) urban and Pluchea indica (L.) urban on uterus weight and uterus and oviduct histological profiles of Rattus norwegicus

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Combination Effect of *Centella asiatica* (L.) Urban and *Pluchea indica* (L.) Urban on Uterus Weight and Uterus and Oviduct Histological Profiles of *Rattus norwegicus*

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Abstract. The use of herbs as a medicinal material is currently increasing because they are considered to have lower negative effects than synthetic drugs. *Centella asiatica* and *Pluchea indica* allegedly contain compounds that can affect the female reproductive organs, such as the uterus and oviduct, and reproductive hormone levels. The main function of the uterus is as a place of implantation and the oviduct as a place of fertilization, so that if there is interference in either of these organs reproductive function will also be disrupted. This study used completely randomized design with six treatments and four replicates. The treatment used was a combination of *Centella asiatica* and *Pluchea* indica at dosages of 0 mg/kg bw (control), 25 mg/kg bw, 50 mg/kg bw, 75 mg/kg bw, 125 mg/kg bw and 200 mg/kg bw for 18 days. Estrus synchronization was performed by prostaglandin (Lutalyse) injection 3 days before treatment. The parameters observed were uterine weight, endometrial thickness, myometrial thickness, number of endometrial glands, oviduct mucosal thickness, oviduct smooth muscle thickness and estrogen and progesterone levels. The uterus and oviduct were prepared with hematoxylin and eosin staining. Data were analyzed by ANOVA and with LSD test. The results showed that the combination of leaf extracts of *Centella asiatica* and *Pluchea indica* had an effect on the histological profile of uterus and oviduct and estrogen and progesteron levels, but did not have an effect on the weight of uterus.

Keywords: Mucosal gland, reproductive, synthetic drug.

INTRODUCTION

The use of natural materials both as medicine and other purposes is tending to increase, especially with the issue of back to nature and the prolonged economic crisis that resulted in the decline in purchasing power. Traditional medicines and medicinal herbs are widely used by communities because they are considered relatively cheaper, affordable by all walks of life, efficient and the side effects are lower than synthetic drugs.[1] The use of natural materials as traditional medicine in Indonesia has been done by the community since many centuries ago. Traditional medicines derived from plants are a major source of new medicines.[2] Many studies show that thousands of chemical components are contained in plants.[1]

Indonesia is the country with the second largest biodiversity in the world after Brazil. Indonesia has 25,000-30,000 plant species that make up 80% of the world's crops and 90% of Asia's crops.[3,4] *Centella asiatica* and *Pluchea indica* are two examples of medicinal plants in Indonesia.[5]

Compounds in *C. asiatica* include triterpenoids, asiatic acid, essential oils, flavonoids and other components such as amino acids, fatty acids, sesquiterpenes, alkaloids, sterols, carotenoids, tannins, inorganic salts and others.[6] One of the active compounds of *C. asiatica* that is known to affect female reproductive organs is the triterpenoid group.[7] High doses of *C. asiatica* cause antifertility effects, suspected to be due to cytotoxic asiatic acid contents. Excessive levels of asiatic acid in the blood cause cell apoptosis in the ovary follicle.[8]

P.indica contains a variety of active compounds, such as alkaloids, flavonoids, tannins and essential oils.[9] The active compounds in *P.indica* that are thought to affect reproductive activity include tannins, alkaloids and flavonoids.[10,11] Fajriaty reported that the decoction of beluntas leaf (945 mg/kg bw) had antifertility effects in female rats in the form of decreasing the number of fetal births.[12]

Some compounds, which are widely used as antifertility agents, usually have estrogen-like structures. They can occupy the position of estrogen receptors in the reproductive organs and, most importantly, interfere with the hypothalamic-pituitary-ovarian or testicular axis.[13] The antifertility agent can inhibit implantation, characterized by unpreparedness of the uterus to receive a zygote as indicated by decreased thickness of the myometrial lining and uterine endometrium. Estrogen receptors are found in brain cells and target cells specific to female reproductive organs, such as the uterus and breast.[14] Hormonal regulation may result in changes in the thickness of layers of the walls of the uterus and of the oviduct, especially the endometrium and myometrium.

Estrogen causes proliferation of the endometrial stroma and promotes development of the endometrial glands that will later nourish the implanted zygote.[15] The hormone estrogen can also stimulate oviduct motility that will produce peristaltic movement that serves to help move the embryo to the uterus.[16] Estrogen also causes marked proliferation in the mucosa of the oviduct, increasing the number of secretory epithelial cells.[15]

This study used a combination of leaf extracts of *C. asiatica* and *P. indica* because they have similar main compounds that were expected to work synergistically. Thus, it was necessary to do research to determine the effects of leaf extracts of *C. asiatica* and *P. indica* on the reproduction status of female rats through the histology of the uterus and oviduct and reproductive hormone levels.

EXPERIMENTAL DETAILS

This study used completely randomized design with six treatment groups, in which each group consisted of four female Wistar rats (*Rattus norvegicus*) with body weight of 100-150 g and 2-3 months of age. Group 1 (C-): rats were given 2.5 mL 0.5% NaCMC, group 2 (T1): rats were treated with *C. asiatica* extract at a dose of 25 mg/kg bw + *P. indica* extract at a dose of 25 mg/kg bw + 2.5 mL 0.5% NaCMC, group 3 (T2): *C. asiatica* extract at dose of 50 mg/kg bw + *P. indica* extract at a dose of 50 mg/kg bw + 2.5 mL 0.5% NaCMC, group 4 (T3): *C. asiatica* extract at dose of 75 mg/kg bw + *P. indica* extract at a dose of 75 mg/kg bw + 2.5 mL 0.5% NaCMC, group 5 (T4): *C. asiatica* extract at a dose of 125 mg/kg bw + *P. indica* extract at a dose of 125 mg/kg bw + 2.5 mL 0.5% NaCMC and group 6 (T5): *C. asiatica* extract at a dose of 200 mg/kg bw + *P. indica* extract at a dose of 200 mg/kg bw + 2.5 mL 0.5% NaCMC.

The rats were acclimatized for 1 week, fed standard pellet BR1 and provided water *ad libitum*. The powder of *C. asiatica* and *P. indica* was finely macerated with 70% ethanol solvent then filtered with a Buchner funnel. The extraction procedure referred to was that of Muchtaromah et al.[18] for *C. asiatica* leaves. The synchronization of estrus cycle in rats was performed prior to treatment with an intramuscular injection of 0.1 mL of prostaglandin (Lutalyse). The estrus cycle was evaluated twice a day at 6:00 and 18:00 by vaginal smears and Giemsa staining, then observed under a microscope at 400x magnification.

The combination of *C. asiatica* and *P. indica* extract was given orally for 18 days, 3 days after prostaglandin injection according to the dose and time specified. After 18 days treatment, rats were sacrificed with cervical dislocation. The uterus and oviduct were removed at the time of surgery, washed with PBS and sterile water and cleaned with sterile paper. Furthermore, the uterus was weighed to obtain the data of wet weight of uterus. The uterus and oviduct were then fixed in a 10% formalin solution. The preparation of histologic preparations used the paraffin method with the hematoxylin and eosin staining.

Histological profiles of uterus (endometrial thickness, myometrial thickness, number of endometrial glands) and oviduct (thickness of the oviduct mucosa and the thickness of oviduct smooth muscle) were measured using OptiLab Software and observed by light microscope with 400x and 100x magnifications. Blood samples of 2-3 mL were taken from aorta for testing estrogen and progesterone levels. The blood was incubated for 2 h at room temperature and centrifuged at 1000 rpm for 15 min. The supernatant obtained was separated from the pellet and stored in a freezer at a temperature of -70 °C. Estrogen and progesterone levels were tested using ELISA Kits (Elabscience).

Data of parameters were analyzed using one-way ANOVA (analysis of variance) with significance at 5%. If $F_{count} > F_{table}$ then analysis continued with LSD (least significant difference) again with significance at 5%. The histological profile was described in the form of photographs.

RESULTS AND DISCUSSION

Uterus Weight, Uterus and Oviduct Histology of the Rat

The results revealed that combination of *C. asiatica* and *P. indica* affected the thickness of endometrium, myometrium, oviduct mucosa layer and oviduct smooth muscle layer as well as the number of glands, but not uterine weight (Table 1). Data of uterus weight were C (154.9 ± 3.4 mg), T1 (199.1 ± 1.2 mg), T2 (157.7 ± 5.4 mg), T3 (208.6 ± 9.5 mg), T4 (133.0 ± 4.7 mg) and T5 (143.9 ± 4.9 mg). Although they have different average weights, statistically there was no difference among the treatments. The uterine weight is strongly affected by the thickness of endometrium and the mucus produced by the uterine gland. The thickness of endometrium is influenced by the reproductive hormones, especially estrogen, whereas it is one of the factors that affects the weight of the uterus. Although the thickness of endometrium layer and amount of endometrial glands were elevated, it did not affect the weight of the uterus, since the uterus is an organ composed of many constituent tissues.

The highest endometrial thickness was obtained in T3 (371.6 ± 3.5 µm) followed by T2 (318.9 ± 6.8 µm) and T1 (307.7 ± 3.0 µm), while T4 (290.5 ± 5.3 µm), C (264.6 ± 3.8 µm) and T5 (262.1 ± 34.4 µm) decreased (Table 1 and Fig. 1). This is influenced by the triterpenoid content in the extract. Triterpenoids can work through two ways, namely through the hormonal system or directly on the reproductive organs.[6] The target reproductive organs consist of the ovaries, oviduct, uterus, cervix, vaginal tract and the outside of the genitals. The triterpenoid saponin, although not a hormone, has a structure similar to estrogen and can also occupy estrogen receptors is and capable of causing effects like endogenous estrogen itself. It affects ovaries, uterus, oviduct, vagina and several other organs. Affinity to estrogen receptors is not as high as estradiol but because its structure is similar to estrogen it has an estrogenic effect.[18] Farooq argued that the activity and clinical implications of this active compounds is highly dependent on the number of estrogen receptors, the location of estrogen receptors and the concentrations of endogenous estrogens.[19]

TABLE 1. The effect of *C. asiatica* and *P. indica* combination on uterine weight, uterus and oviduct histology

| Treatment	Uterus				Oviduct	
	Uterine weight (mg)	Endometrial thickness (µm)	Miometrial thickness (µm)	Endometrial glands number	Mucosa layer (µm)	Smooth muscle layer (µm)
Control	154.9± 3.4	264.6±3.8[a]	60.5± 5.8[ab]	11.7± 1.7[a]	57.5± 1.6[a]	93.2±1.4[b]
T1 (25 mg/kgbw)	199.1 ±1.2	307.7±3.8[ab]	69.5± 1.4[bc]	12.6± 6.0[a]	112.8± 3.0[b]	96.0±1.5[b]
T2 (50 mg/kgbw)	157.7±5.4	318.9±6.8[ab]	74.8± 5.5[c]	12.2± 3.8[a]	72.7± 1.5[a]	105.7±1.2[b]
T3 (75 mg/kgbw)	208.6±9.5	371.6±3.5	74.0± 8.5[c]	19.5± 4.6[b]	71.6± 1.5[a]	99.1±1.3[b]
T4 (125 mg/kgbw)	133.0±4.7	290.5± 5.3[a]	52.2± 6.9[a]	10.2± 0.9[a]	71.8± 1.4[a]	87.5±6.7[ab]
T5 (200 mg/kgbw)	143.9±4.9	262.1± 34.4[a]	60.4± 4.5[ab]	12.5± 2.8[a]	65.7 ± 1.2[a]	74.2 ± 6.0[a]

The largest number of endometrial glands was found in T3 (19.5 ± 4.6), different from other treatments T1 (12.6 ± 6.0), T5 (12.5 ± 2.8), T2 (12.2 ± 3.8), C (11.7 ± 1.7) and T4 (10.2 ± 0.9). The greatest thickness of the myometrial layer was in T2 (74.8 ± 5.5µm) followed by T3 (74.0 ± 8.5µm), T1 (69.5 ± 1.4µm), which were different from C (60.5 ± 5.8µm), T5 (60.4 ± 4.5µm) and T4 (52.2 ± 6.9µm).

This suggested that administration of *C. asiatica* and *P. indica* at doses of 25 to 75 mg/kg bw (T1, T2 and T3) led to increased uterine receptivity. The presence of active compounds from the combination of extracts such as the triterpenoid saponin, asiatic acid, and madecassoside at low doses can elevate cell proliferation in uterine tissue resulting in an increase especially of the lining of the endometrium and myometrium. The triterpenoid compound saponin in glycans is known to contain the steroid diosgenin, which can be synthesized into estrogen through a series of chemical reactions.[20,21]. Triterpenoid saponins in *C. asiatica* and *P. indica* work in the same way as estrogen, which is to attach to the receptor of estrogen so the ligand-receptor complex will induce the expression of genes that are responsive to estrogen, resulting in increased uterine mass. While active compounds such as

flavonoids in *P. indica* could increase the thickness of the endometrium and myometrium through their antioxidant activities.

Zhao and Mu showed that estrogenic activity will affect proliferation of stromal and epithelial cells. Estrogen affects the activity of cell proliferation by binding to receptors on the target cell, which can alter hormone receptor conformation.[22] Pan reports that conformational changes cause the estrogen-receptor complex to become active so as to bind to the binding site on the DNA chain, especially on the acceptor side. The interaction between the estrogen-receptor complex and the side of the DNA acceptor causes gene expression to increase. The expression of these genes is catalyzed by the enzyme RNA polymerase that causes an increase in mRNA. On the other hand, the synthesis of tRNA will also increase so that in the end the synthesis of cell material becomes increased, which supports cell proliferation.[23]

C. asiatica and *P. indica* at doses of 125-200 mg/kg bw (T4 & T5) decreased layer thickness and number of uterine glands (Table 1 and Fig. 1). Muchtaromah in a similar study reported that administration of a high dose *C. asiatica* extract (125 mg/kg bw, 200 mg/kg bw or 275 mg/kg bw) in mice had antifertility effects. The result was not finding any follicles that reached the stage of a graafian follicle. As a result, theca cells at ovary follicles cannot produce estrogen optimaly.[20] Furthermore, low estrogen levels inhibit the proliferation of endometrial cells, glands and myometrial cells. Whirledge and Cidlowski reported that an adult animal exposed to an estrogen precursor from the outside will have high levels of estrogen in the blood. Moreover, endogenous estrogen production still continues. It causes negative feedback on the hypothalamus to decrease FSH secretion. As a result, estrogen secretion also decreases and affects several reproduction organs.[24]

(a)   (b)
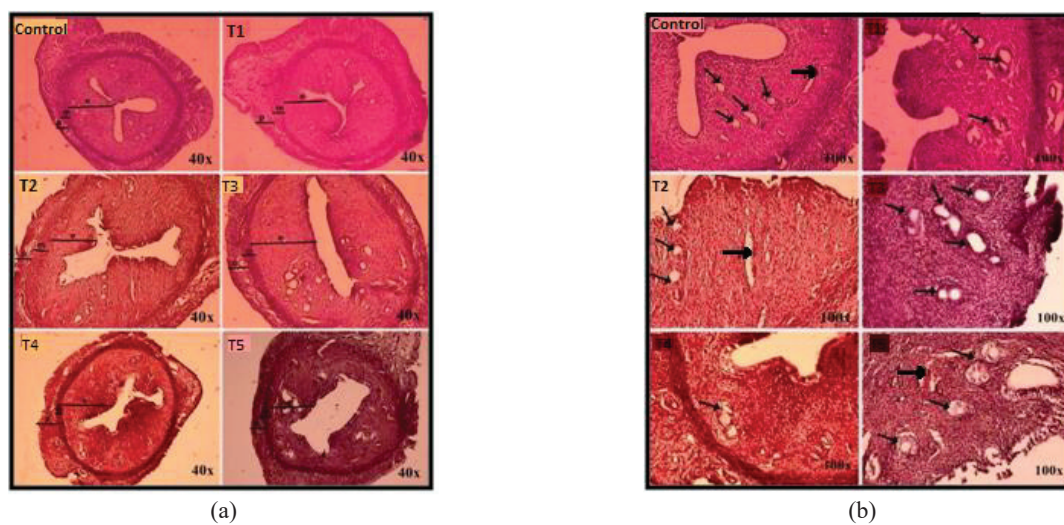
FIGURE 1. Histological profile of uterus (a) e: endometrium; m: myometrium; p: perimetrium
(b) →: endometrial gland.

The thickness of the mucosa layer of oviduct was highest in T1 (112.8 ± 3.0 µm) followed by T2 (72.7 ± 1.5 µm), T3 (71.6 ± 1.5 µm), T4 (71.8 ± 1.4 µm), T5 (65.7 ± 1.2 µm) and control (57.5 ± 1.6 µm), while the most smooth muscle thickness was obtained in T2 (105.7 ± 1.2 µm) followed by T3 (99.1 ± 1.3 µm), T1 (96.0 ± 1.5 µm), C (93.2 ± 1.4 µm), T4 (87.5 ± 6.7 µm) and T5 (74.2 ± 6.0 µm). The thickness of mucosa and muscular layers are influenced by estrogen. A combination of low doses (25-75 mg/kg bw) worked optimally to produce sufficient estrogen levels. The combination of high doses (125-200 mg/kg bw) actually led to negative feedback, which subsequently decreased estrogen levels in the blood.
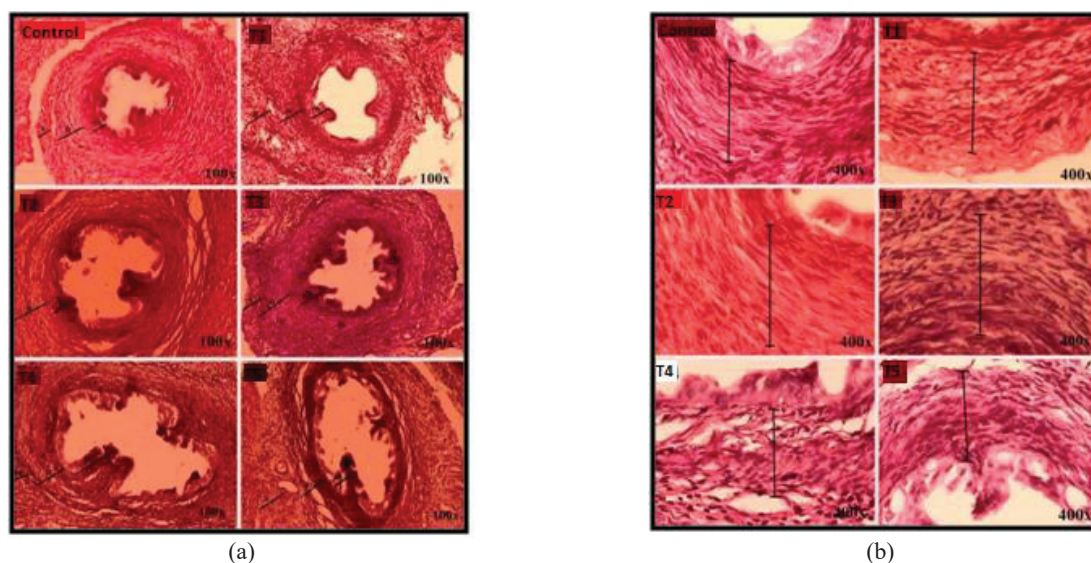
FIGURE 2. Histological profile of oviduct. (a) m: tunica mucosa; o: tunica muscularis; s: tunica serosa (b) ─: tunica muscularis.

Kress & Monson reported that estrogen caused proliferation of glandular tissue in the mucosa layer so the number of ciliary epithelial cells increased. Estrogen also affected the multiplication of smooth muscle tissue so that the smooth muscle mass of the oviduct increased.[24] The oviduct mucosa is composed of ciliary and secretory epithelial cells. An increase in the number of cells, both secretory and ciliary epithelial cells, could cause a thicker mucosal layer of the oviduct.[24]

The thickness of the mucosa and smooth muscle layers of the oviduct will affect the individual's fertility as it helps transport ovum and embryo through smooth muscle contraction. The oviduct muscular layer consists of two layers of smooth muscle, the inner circular layer and the outer longitudinal layer. Smooth muscle contractions will produce a peristaltic movement that helps to transport the ovum to the ampulla (fertilization) and transport the embryo to the uterus (implantation).[15]

In addition, the antioxidant activity contains in the combination plays a role to protect damaged cells. Giving extracts in combination is expected to provide a synergistic effect so expected to obtain the beneficial effects. *P. indica* contains some polyphenols and flavonoids that have the ability as an antioxidant, which protect cells from oxidative damage by neutralizing reactive oxidants and help the proliferation process.[25]

Estrogen and Progesterone Levels

The levels of rat estrogen and progesterone after administration of *C. asiatica* and *P. indica* are presented in Figure 3. Data for estrogen levels showed highest were, respectively, T3 (241.00 ± 17.88 ng/mL), T2 (238.17 ± 10.93 ng/mL), T1 (236.32 ± 11.04 ng/mL) and C (234.12 ± 17.88 ng/mL), which were different from T4 (233.52 ± 17.88 ng/mL) and T5 (212.9 ± 17.88 ng/mL). The combination of extracts at a dose of 75 mg/kg bw was optimal for increasing estrogen levels, while at high doses of 200 mg/kg bw it actually lowered estrogen levels in the blood.

Andria reported that *C. asiatica* leaf at high doses of 560, 630 and 700 mg/kg bw reduced estrogen levels in rats with successive results, i.e. (41.83 ± 0.70 ng/mL), (34.07 ± 0.73 ng/mL) and (30.90 ± 9.51 ng/mL). *C. asiatica* leaf contains the triterpenoid saponin. It contains genes that control conversion into progesterone through a chemical process that produces testosterone and estrogen. Progesterone is formed from pregnenolone by removal of hydrogen atoms from C3 and a double bond shift from ring B at position 5-6 to ring A at position 4-5. This change occurs because of the enzymes 3β-hydroxy dehydrogenase and $\Delta^{4\text{-}5}$ isomerase. Additionally, with the help of 17α-hydroxylase enzyme, progesterone is converted to 17-hydroxy progesterone, which then interacts with desmolase to form testosterone. Furthermore, testosterone leads to aromatization, i.e. the formation of phenolic hydroxy groups in atoms (C3) into estrogens.[20]
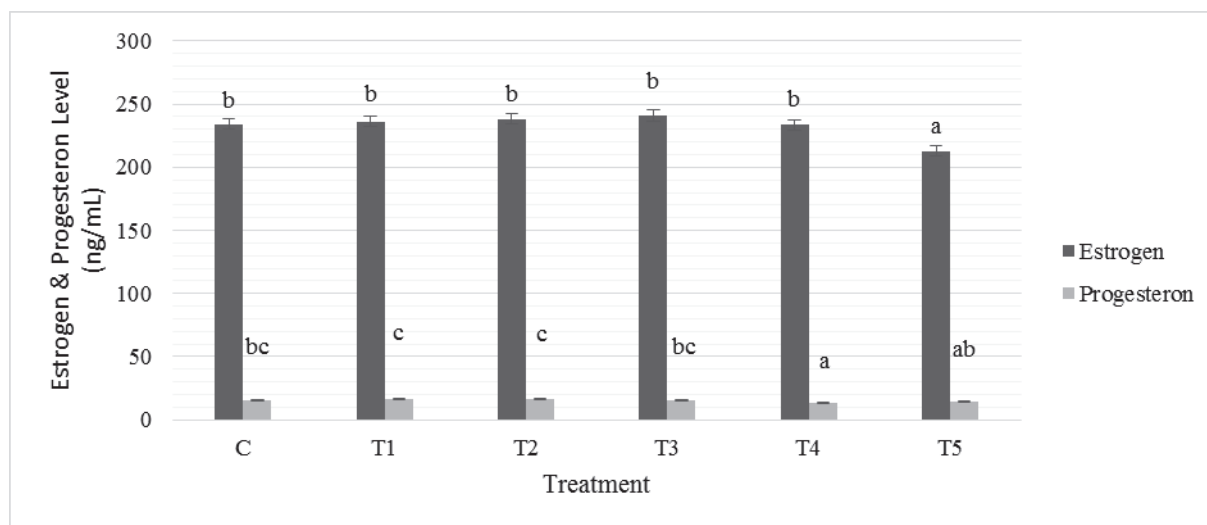
FIGURE 3. Level of Estrogen and Progesterone.

In accordance with the Puspitasari study, triterpenoid compounds should be made at least in small quantities by all creatures that synthesize steroids. In large amounts, the triterpenoids that have a lipid derivative are thought to be capable of causing inhibition of luteinizing hormone (LH) and follicle stimulating hormone (FSH) release.[26] Decreased secretion of FSH and LH would cause estrogen levels also to fall. Estrogen has two types of receptors: alpha (ERα) and beta (ERβ) receptors. The α receptors are present in the ovary, breast, uterus, testicles, pituitary glands, kidney, epididymis and adrenal glands, while β receptors are found in ovarian organs.[13]

The flavonoid in *P. indica* is an estrogenic compound, capable of functioning like estrogen in the body to increase the effects of estrogen.[28] High estrogen levels inhibit the hypothalamus through a negative feedback mechanism, so that FSH and LH are not excreted by the anterior pituitary. This would interfere with the development of ovarian follicle cells, with the result that estrogen levels would decrease and ovulation would not occur.[20]

In addition, the asiatic acid content of *C. asiatica* is thought to be cytotoxic when the levels are excessive in blood, thus causing cell apoptosis in the ovarian follicle. Cell apoptosis in the follicles caused by asiatic acid begins with mitochondrial damage.[20] Singh reported that partial oral administration of the purified fraction of *C. asiatica* extract could induce strong apoptosis.[29] The results of 1,1-diphenyl-2-picrylhydrazyl (DPPH) testing of *P. indica* extract and fractions compared to green tea and rosemary extract, butylated hydroxytoluene (BHT) and alpha-tocopherol succinate showed that the ethyl acetate fraction of *P. indica* was had the most potent natural antioxidants. The use of a combination of *C. asiatica* and *P. indica* extracts is better than the single materials as it is proved to be complementary. Research using a single ingredient (*C. asiatica*) proved to require higher doses to produce antifertility effects than in this study.[8] The content of asiatic acid in *C. asiatica* is suspected to be cytotoxic; however, its effects causing cell apoptosis could be neutralized by flavonoids and phenol of *P. indica* through antioxidant activity.

Progesterone levels in this research were the highest successively in T1 (16.17 ± 1.89 ng/mL), T2 (16.10 ± 0.99 ng/mL), T3 (15.79 ± 0.86 ng/mL) and C (15.52 ± 0.92 ng/mL), which were significantly different from T4 (13.41 ± 1.55 ng/mL) and T5 (13.99 ± 1.34 ng/mL). In the study by Andria, administration of *C. asiatica* at high doses of 560, 630 and 700 mg/kg bw decreased progesterone levels, i.e. 18.13 ± 1.11 ng/mL, 16.49 ± 0.68 ng/mL and 15.33 ± 0.58 ng/mL, compared with control (22.51 ± 0.75 ng/mL).[8]

According to Zhao and Mu, phytoestrogens have to penetrate into the cytoplasm to bind their receptors and then form the special proteins required in cell division. For the transcription process of protein synthesis, the phytoestrogen-receptor complex binds to not only to the estrogen response element (ERE) but also the co-regulator. This will affect the transcription and translation of target proteins as well as the process of follicle maturation to trigger ovulation of graafian follicles. Subsequently, the formation of the corpus luteum will produce estrogen and progesterone.[22]

Progesterone works through the ligand-receptor complex in the target organ. Many progesterone receptors are present in the cytoplasm. Progesterone enters the cell through the diffusion process. There are two types of

progesterone receptors: progesterone receptor A (PRA) and progesterone receptor B (PRB). These two receptors lead to transcription of certain genes that have a specific expression for estrogen. It is presumed that progesterone receptors inhibit the effect of DNA damage response, and this inhibition turns out to affect estrogen. Increased concentrations of progesterone may also occur due to the effect of genistein on rat granulosa cells. Genistein is the most potent phytoestrogen and is known to stimulate progesterone production.[22]

SUMMARY

The combination of *C. asiatica* and *P. indica* in low doses (25-75 mg/kg bw) affected the histology of the uterus and oviduct by increasing the thickness of the endometrium and myometrium as well as the number of glands and estrogen and progesterone levels, but had no effect on uterine weight. Oppositely, at high doses (125-200 mg/kg bw) the combination decreased these parameters. This phenomenon revealed that the combinations are better than the single materials as they proved to be complementary. In low doses it can be used as a fertility drug, while at high doses as an antifertility drug.

ACKNOWLEDGMENTS

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