

Efficacy of *Allium sativum*, *Curcuma mangga* and *Acorus calamus* Extract Combination on Rat Fertility

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ABSTRACT

Objective: to evaluate the efficacy of *Allium sativum*, *Curcuma mangga*, and *Acorus calamus* extract combination on female rat fertility. **Methods:** Thirty-six female rats were randomized divided into 9 groups (n=4): C- (no treatment); C+ (clomiphene citrate); composition 1 (*A. sativum* 36%: *C. mangga* 36%: *A. calamus* 28%) with 3 doses i.e T1, T2 & T3 of 50, 75 & 100 mg/kg BW; composition 2 (*A. sativum* 35%: *C. mangga* 40%: *A. calamus* 25%) with 3 doses i.e T4, T5 & T6 of 50, 75 & 100 mg/kg BW and T7 (Subur Kandungan herb™). Treatment was given every day for 15 days. **Results:** The results revealed that all treatments affected reproductive hormone and uterine histology profile. However, T1 produced the highest estrogen and progesterone levels, and T2 produced the highest endometrial and myometrium thickness, as well as the number of endometrial glands. Furthermore, the hormonal profile in T1 and histological profile of uterus in T2 were not significantly different from those of T7 group. **Conclusions:** Combined *A. sativum*, *C. mangga*, and *A. calamus* extracts could be used to increase fertility in female rat. The most effective treatment in this study was dose of 50-75 mg/BW in both compositions.

Key words: *Acorus calamus*; *Allium sativum*; *Curcuma mangga*; Fertility; Rat.

INTRODUCTION

Infertility is a reproductive problem experienced by 1 of 6 couples of male and female in the world.¹ Infertility is defined as the inability of the couples to conceive after one year trying of unprotected sex.² Many factors that can cause infertility and involve male and female. Several factors, including changes in the menstrual cycle and ovulation, hormonal problems,² oxidative stress³ and deformity of reproductive organs⁴ were common factors that affect female fertility. However, 25% of infertility causes are still unknown.² Infertility evaluation sometimes involves many procedures such as physical examination⁵, blood tests (to detect hormonal levels)⁶ and laparoscopy (detection of abnormalities of reproductive organs).²

Estrogen and progesterone are essential hormones related to fertility. The primary function of estrogen is coupling behavioral estrus, regulation of menstrual cycle, development of mammary glands, thickening endometrium and the development of female secondary sexual characteristics.^{7,8} Furthermore, the concentration of serum estrogen and progesterone can be used to determine pregnancy and pathological conditions.⁹

There are several options used primarily to address infertility including adopting a healthy lifestyle, using assisted reproductive technology (ART) involves procedure of in vitro fertilization and cryopreservation, using ovulation induction drugs, hormone therapy, and herbs. However, in the recent year's herbs have been used to treat infertility and it is an inclusive practice based on theory, beliefs and experience.^{10,11}

Complementary and alternative medicine has become increasingly popular in developed countries.¹² Even, around 80% of the total population in developed countries depend on herbal medicines for their primary healthcare because they are cheap, affordable and available in large quantities.¹³ Besides, the use of herbal medicines has been shown to have lower negative effects for the body compared to synthetic drugs.¹⁴ Therapeutic interventions in modern medicine are based on an understanding of the processes and mechanisms of disease while the use of multicomponent herbal formulas is based on theory and practical experience from thousands of years.¹⁵

Indonesia is mega biodiversity country that had diverse plant natural resource for herbal medicine. For the people of some area in Indonesia like Java and Madura, traditional medicine better known as herbal medicine (Jamu). One of the herbal medicine that was believed and primarily used to increase fertility by Madurese people of Indonesia is "Jamu Subur Kandungan." It consists of a combination of three materials, namely garlic bulb (*Allium sativum*), mango ginger rhizome (*Curcuma mangga*), and sweet flag rhizome (*Acorus calamus*). Herbal medicines including "Jamu Subur Kandungan" need to be examined scientifically to find out their efficacy with appropriate dosage. Therefore, this study aimed to evaluate the effect of combined *Allium sativum*, *Curcuma mangga*, and *Acorus calamus* extract on female rat fertility at different composition and doses by investigating serum estrogen and progesterone levels and uterine histology profile.

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MATERIALS AND METHODS

Materials

Female rat was obtained from the Animal Physiology Laboratory, Faculty of Science and Technology, Universitas Islam Negeri Maulana Malik Ibrahim Malang, Indonesia. Symplisia of *A. sativum*, *C. mangga*, and *A. calamus* were purchased from Balai Materia Medika, Batu Malang, Indonesia. Subur kandungan herb™ (PJ. Ribkah Maryam Jokotole, Madura), clomiphene citrate (ClomiPHENE, Teva Pharmaceuticals US), hCG (Chorulon, Intervet), PMSG (Folligon, Intervet), estrogen, progesterone, elisa kit (FineTest).

Animal procedure

Thirty-six fertile female *Rattus norvegicus* (wistar strain) were prepared with the ages around three months old and about 180-230 g body weight. They were placed in the Animal House of Animal Physiology Laboratory Universitas Islam Negeri Maulana Malik Ibrahim Malang maintained temperature (24-26 °C) and humidity (40-50 %) under light/dark cycle (light on 12/12 h). They fed with pellet and given water ad libitum. All treatment has been tested through the Health Research Ethics Committee (KEPK) of the Faculty of Medicine and Health Sciences of the Universitas Islam Negeri Maulana Malik Ibrahim Malang (Approval Reference Number: 016/EC/KEPK-FKIK/2018).

Experimental design and research procedure

The animals were allowed to acclimatize for seven days. Synchronization of the estrus cycle in rats using 10 IU of Pregnant Mare's Serum Gonadotropin (PMSG) by intraperitoneal injection, following by injection of 10 IU Human Chorionic Gonadotropin (hCG) after 48 h. The estrus phase was confirmed by vaginal smears examination and Giemsa staining observed under a microscope with 400x magnification.¹⁶

This research using completely randomized design with 9 treatments and 4 replications. The groups of treatments were categorized according to the following composition and dosage: Composition 1 consisted of *A. sativum* 36 %: *C. mangga* 36%: *A. calamus* 28% and composition 2 consisted of *A. sativum* 35 %: *C. mangga* 40 %: *A. calamus* 25%. The treatment consisting of negative control (C-): no treatment, positive control (C+): clomiphene citrate dose of 0.9 mg/kg BW; T1: composition 1 with dose of 50 mg/kg BW, T2: composition 1 with dose of 75 mg/kg BW, T3: composition 1 with dose of 100 mg/kg BW, T4: composition 2 with dose of 50 mg/kg BW, T5: composition 2 with dose of 75 mg/kg BW, T6: composition 2 with dose of 100 mg/kg BW, T7: Subur kandungan herb™ with dose of 75 mg/kg BW. All treatments were mixed with 0.5 ml Na CMC 0.5% as a solvent.

Extract was prepared following the method of Muchtarmah et al.¹⁷ and Muchtarmah et al.¹⁸ The extract was administered according to oral gavage technique. Each rats on treatment T1-T6 received 2 ml extract continuously for 15 days (3 times of estrus cycle) after estrus phase while 2 group rats in positive control and T7 were administered according to the prescribed dose which has been explained above. After 15 days, the rats were sacrificed by cervical dislocation, and blood was taken from the aorta for the hormonal assay. The uterus was taken from the abdominal cavity for histology preparations using Hematoxylin-eosin staining.

Estrogen and progesterone assay

Measurement of estrogen and progesterone levels was carried out using elisa kit. Blood samples were incubated for 2 h at room temperature and then centrifuged at 1000 rpm for 15 min. The supernatant obtained was separated from the pellet and put in 2 ml of Eppendorf, then stored in a freezer -70 °C.

Histological preparation of uterine

Uterus were fixed in bouin fixative for 24 h. Specimens were dehydrated, embedded in paraffin wax and serially sectioned at 1-6 µm thickness on rotary microtome, followed clearing, hydration, hematoxylin and eosin staining. Slides were observed under light microscope (Olympus CX21, Japan).¹⁹

Statistical analysis

The data from this study were analyzed with normality and homogeneity test followed by analysis of variance (ANOVA) with Duncan's Multiple Distance Test. All test used SPSS 15 (SPSS Inc., USA). The differences were considered significant when $p < 0.05$.

RESULTS

Level of estrogen

Combination of *A. sativum*, *C. mangga*, and *A. calamus* extract and Subur Kandungan herb increased estrogen levels significantly ($p < 0.05$) compared to negative control/C- (525.40 ± 4.37 ng/mL) (Figure 1). The highest estrogen level was found in T7 group (971.40 ± 9.84 ng/mL) but not significantly different ($p > 0.05$) with T1 (967.73 ± 3.51 ng/mL), T3 (943.07 ± 4.80 ng/mL), T4 (892.40 ± 3.22 ng/mL), T5 (884.07 ± 5.38 ng/mL) and T6 (878.07 ± 9.80 ng/mL). However, T7 group significantly different from T2 (811.73 ± 8.30 ng/mL) and C+ (794.40 ± 1.51 ng/mL) and C- (Figure 1).

Levels of progesterone

The results revealed clomiphene citrate group (C+) increased significantly ($p < 0.05$) the levels of progesterone compared to control group/C- (11.45 ± 1.76 ng/mL vs 5.17 ± 1.74 ng/mL) (Figure 2). Interestingly, all treatment with combined *A. sativum*, *C. mangga*, and *A. calamus* extract at all dose treatment increased the progesterone levels T2 (8.58 ± 2.64 ng/mL), T3 (10.49 ± 2.95 ng/mL), T4 (13.46 ± 1.72 ng/mL), T5 (12.59 ± 2.42 ng/mL), T6 (11.75 ± 3.28 ng/mL). The highest progesterone levels were found in T1 (13.78 ± 2.39 ng/mL) and in Subur Kandungan herb™ (8.91 ± 3.16 ng/mL). In this study, the use of low doses of 50 mg/kg BW (T1 and T4) were able to increase progesterone levels significantly, while the use of higher doses of 75-100 mg/kg BW (T2, T3, T5, T6, T7) tended to reduce progesterone levels (Figure 2). This finding proved that the use of low doses of combined *A. sativum*, *C. mangga*, and *A. calamus* extract could induce the optimal production of progesterone levels.

Uterine histological profile

The endometrium and myometrium thickness

Figure 3 showed the histological structure of the uterus, which consisted of three layers. Perimetrium or serosa tunica was the outermost layer composed of loose connective tissue. The middle layer was the myometrium, which included thick circular muscles. The inner layer was endometrium which consisted of columnar epithelial layer. Endometrium was in direct contact with uterine lumen where there were glands containing nutrient fluids for the embryo. Thickening of the endometrial and myometrial layers increased the diameter of uterus. Endometrium is an uterine layer that is most responsive to hormonal changes.

The study found that the extract combination at dose of 75 mg/kg BW on T2 (74.62 ± 2.41), T5 (74.03 ± 13.24), and T7 (84.53 ± 5.74) optimally increased endometrial thickness ($p < 0.05$) followed by dose of 100 mg/kg BW on T6 (68.40 ± 2.42) and T3 (60.36 ± 3.04), dose of 50 mg/kg BW on T1 (56.91 ± 6.71) and T4 (53.79 ± 1.94). Extract combination also increased significantly myometrial thickness compared to C- ($p < 0.05$). Treatment 7 (31.05 ± 2.91) exhibited the highest myometrial thickness

followed by T2 (30.57 ± 1.92), T1(27.87 ± 8.65), T5 (23.34 ± 6.01), T3 (20.60 ± 4.15), C+ (20.18 ± 2.01), T4(19.44 ± 1.99), T6 (18.40 ± 1.20) and C- (15.61 ± 2.39) (Figure 4).

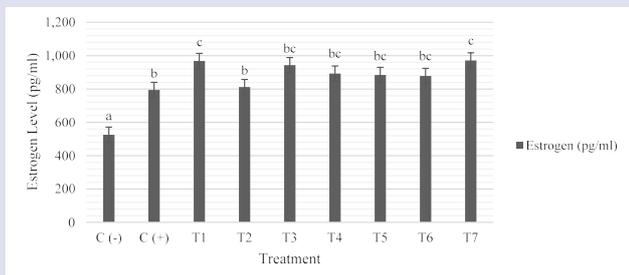


Figure 1: Profile of estrogen levels after treatment.

Note: C- (no treatment), C + (clomiphene citrate 0.9 mg/kg BW), composition 1: T1 (50 mg/kg BW), T2 (75 mg/kg BW), T3 (100 mg/kg BW), composition 2: T4 (50 mg/kg BW), T5 (75 mg/kg BW), T6 (100 mg/kg BW), T7 (Subur kandungan herb™ of 75 mg/kg BW). The bars represent the mean ± SD of estrogen level.

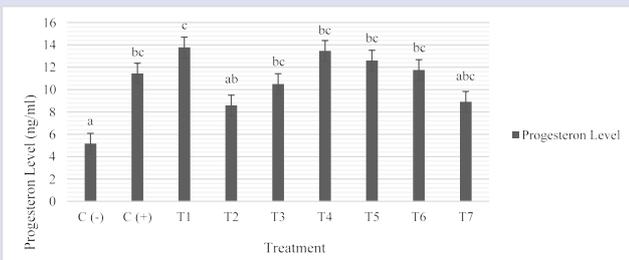


Figure 2: Profile of progesterone level after treatment.

Note: C- (no treatment), C + (clomiphene citrate 0.9 mg/kg BW), composition 1: T1 (50 mg/kg BW), T2 (75 mg/kg BW), T3 (100 mg/kg BW), composition 2: T4 (50 mg/kg BW), T5 (75 mg/kg BW), T6 (100 mg/kg BW), T7 (Subur kandungan herb™ of 75 mg/kg BW). The bars represent the mean ± SD of progesterone level.

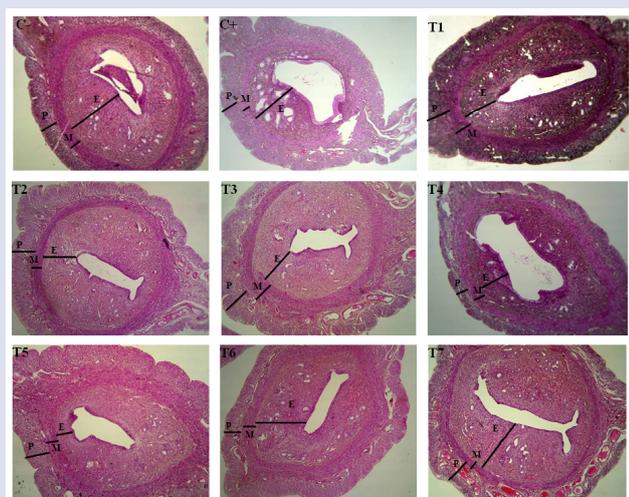


Figure 3: Uterine histological structure after treatment (M: 100x).

E: endometrium, M: myometrium, P: perimetrium C- (no treatment), C + (clomiphene citrate 0.9 mg/kg BW), composition 1: T1 (50 mg/kg BW), T2 (75 mg/kg BW), T3 (100 mg/kg BW), composition 2: T4 (50 mg/kg BW), T5 (75 mg/kg BW), T6 (100 mg/kg BW), T7 (Subur kandungan herb™ of 75 mg/kg BW).

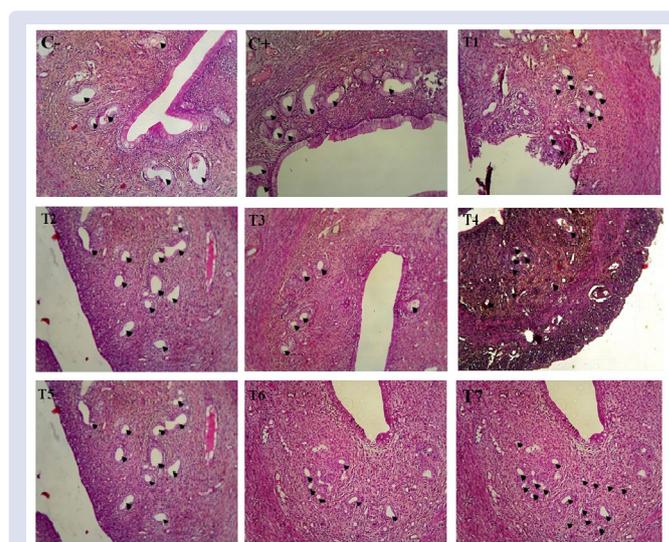


Figure 4: Number of endometrial glands in the cross-section of the uterus. E: Endometrial gland, M: 100 times.

Endometrial glands

In this study, T7 (20.66 ± 2.08) showed the highest number of endometrial glands had no significant different with T2 (20.00 ± 2.64) and T5 (19.66 ± 6.11) (p>0.05), but different from other groups (p<0.05). The lowest number of endometrial glands was found at C- (9.66 ± 2.51) had no different with T4 (10.33 ± 3.51) and T1 (12.33 ± 3.05).

DISCUSSION

Level of estrogen

Clomiphene citrate was used as a positive control in this study. Clomiphene citrate is a standard drug used to improve female fertility through ovulation induction. However, prolonged use of Clomiphene citrate more than 12 menstrual cycles may increase the risk of ovarian cancer.²⁰ Our finding indicated that the combination of *A. sativum*, *C. mangga*, and *A. calamus* extract with different ratios and doses and Subur kandungan herb™ could increase estrogen levels better than clomiphene citrate. Moreover, the combination of *A. sativum*, *C. mangga*, and *A. calamus* extract at the lowest dose of 50 mg/kg BW (T1 and T4) tended to increase the estrogen levels, while the highest dose of 100 mg/kg BW (T3 and T6) tended to reduce the estrogen levels. This finding was in line with the previous study that combination of *C. asiatica* extract and *P. indica* with doses above 75 mg/kg BW decreased the estrogen levels.²¹ It is revealed that both combination extracts contain phytoestrogens compound resulted in the similar hormonal response.

We have previously reported that *A. sativum*, *C. mangga*, and *A. calamus* extract contain phytochemical including flavonoid, alkaloid, and triterpenoid compounds.¹⁸ Similarly, Krizova *et al.*²² reported that some phytoestrogen compounds contained in plants included isoflavones, lignans, coumestans, triterpenic glycosides, and other compounds such as alkaloids, triterpenoids, and chalcone.

Isoflavones belong to the flavonoid group and the largest group in flavonoids. Genistein is one of the derivatives of isoflavone compounds. Lacy²³ reported that the structure of genistein and estrogen is almost similar and has an estrogenic effect that can bind to estrogen receptors (ER). According to Primiani *et al.*²⁴ genistein is an isoflavone derivative and is the most estrogenic phytoestrogen while the isoflavone derivative content of combined *A. sativum*, *C. mangga* and *A. calamus* is not elucidated yet and still needs further research.

The mechanism of action of phytoestrogens is through the direct genomic mechanism, i.e., phytoestrogens directly bind to estrogen receptors (ER) and affect gene transcription, so that it causes estrogen-like effects (estrogenic effect). Phytoestrogens circulate in the bloodstream in a free form and bind to carrier proteins. Phytoestrogens circulate through the membrane by passive diffusion or active transport.²⁵

Lecomte *et al.*²⁶ stated that estrogen receptors bind to Estrogen Responsive Element (ERE), which activate some proteins for cell division. When transcribing protein synthesis, estrogen/phytoestrogen-receptor complexes not only bind to ERE but also bind to co-regulator. Furthermore, Fuentes & Silveyra²⁵ revealed that the complexes binding of estrogen affect to transcription and translation as well as the maturation process of folliculogenesis, which triggers ovulation and form a corpus luteum to produce estrogen and progesterone.

Levels of progesterone

The flavonoids, alkaloids, and triterpenoids content of the extract may act as a phytoestrogen compound and were assumed to cause steroidogenesis (formation of the steroid hormone) in the ovary. As it has been reported phytoestrogens have steroidogenic effects like increasing progesterone level.² Similarly, Shibeshi *et al.*²⁷ showed that genistein in *Achyranthes aspera* leaf extracts at a dose of 300 and 550 mg/kg BW reduced rat progesterone levels. In other hands, Olayaki *et al.*²⁸ reported that genistein in *Cajanus cajan* extract at dose of 100 and 200 mg/kg BW increased progesterone receptor expression in the rat's uterus. It showed that the phytoestrogens compound could affect the levels of the progesterone through the negative feedback mechanism of Gonadotropin-Releasing Hormones in the hypothalamus. In this study, combination of *A. sativum*, *C. mangga*, and *A. calamus* extract with different ratios and doses and Subur Kandungan herb™ could significantly increase both estrogen and progesterone levels.

The endometrium and myometrium thickness

The appropriate dosage and content of active compounds in combination of *A. sativum*, *C. mangga*, and *A. calamus* extract such as isoflavones, flavonoids, saponins, and alkaloids could affect the endometrium and myometrium thickness (Table 1.). Isoflavones, flavonoids, saponins, and alkaloids stimulate estrogen formation in mammals because their structure is similar to estrogen and bind to estrogen receptors in the uterus, especially the endometrial. The estrogenic effect arises with the bond between phytoestrogens and estrogen receptors, resulting in the activation of estrogen receptors layer.²⁶

Table 1: The effect of *A. sativum*, *C. mangga*, and *A. calamus* extract combination on uterus histology.

Treatment	Uterus		
	Endometrial thickness (µm)	Myometrial thickness(µm)	Endometrial glands number
C- (Normal)	49.29 ± 4.23 ^a	15.61 ± 2.39 ^a	9.66 ± 2.51 ^a
C+ (Clomiphene sitrat)	60.89 ± 2.35 ^{bc}	20.18 ± 2.01 ^a	18.66 ± 3.05 ^c
T1 (50 mg/kg BW)	56.91 ± 6.71 ^{ab}	27.87 ± 8.65 ^{bcd}	12.33 ± 3.05 ^{ab}
T2 (75 mg/kg BW)	74.62 ± 2.41 ^d	30.57 ± 1.92 ^{cd}	20.00 ± 2.64 ^c
T3 (100 mg/kg BW)	60.36 ± 3.04 ^{bc}	20.60 ± 4.15 ^{ab}	15.00 ± 2.64 ^{abc}
T4 (50 mg/kg BW)	53.79 ± 1.94 ^{ab}	19.44 ± 1.99 ^a	10.33 ± 3.51 ^a
T5 (75 mg/kg BW)	74.03 ± 13.24 ^d	23.34 ± 6.01 ^{abc}	19.66 ± 6.11 ^c
T6 (100 mg/kg BW)	68.40 ± 2.42 ^{cd}	18.40 ± 1.20 ^a	17.33 ± 6.11 ^{bc}
T7 (Subur kandungan)	84.53 ± 5.74 ^d	31.05 ± 2.91 ^d	20.66 ± 2.08 ^c

Note: C- (no treatment), C+ (clomiphene citrate 0.9 mg/kg BW), composition 1: T1 (50 mg/kg BW), T2 (75 mg/kg BW), T3 (100 mg/kg BW), composition 2: T4 (50 mg/kg BW), T5 (75 mg/kg BW), T6 (100 mg/kg BW), T7 (Subur kandungan herb™ of 75 mg/kg BW). The bars represent the mean ± SD of uterus histology parameter.

Breinholt *et al.*²⁸ reported that isoflavones, genistein, daidzein, equol, and glycitin were estrogenic in mice uterine. 100 mg/kg body weight of genistein significantly increased uterine weights and the expression of ERα in the uterus. Jefferson *et al.*²¹ compared the estrogenic potential of several phytoestrogens, including genistein, daidzein, and coumestrol in immature mice using different morphological and biochemical tests on the uterus. Genistein and coumestrol showed estrogenic activity in all experiments, others showed estrogenic activity only a single test.

The mechanism of action of estrogen on endometrial thickness can be explained by estrogen activity in the endometrium. Estrogen activity in cells begins following its binding to receptor in the cytosol. Estrogen and receptor complex diffuse into the cell nucleus and attach to DNA. The binding of the estrogen-receptor complex with DNA induces RNA and protein synthesis and cell division occurs approximately 24-48 hours after the initial stimulus.²⁹ The increase of the endometrium and myometrium thickness as well as endometrial glands number was not only caused by hormonal factors but also by cellular mechanisms of the antioxidant activity of extracts. Herbs containing flavonoids have antioxidant potential to eliminate free radicals and reactive oxygen species.³⁰

The phytoestrogens containing in the combination of *A. sativum*, *C. mangga* and *A. calamus* in this study might increase the number of cells and stroma (lamina propria) of the endometrium. Endometrial thickness affects a woman's fertility. Thick of endometrium indicates that female is ready to maintaining the growth of fetus. The increase of endometrium width affects the increasing number of glands because under the epithelial layer there are lamina propria which contains many glands that secrete a lot of mucus. Endometrial mucus is known to have a function as nutrition for the embryo.²¹ Increasing of endometrial thickness usually coincided with an increase in the number of glands.³¹

The administration of extracts at dose 50-75 mg/kg BW (T1, T2, T4, and T5) exhibited an increase in endometrial thickness, myometrium and the number of endometrial glands. The administration of the highest dose of 100 mg/kg BW (T3 and T6) tended to decrease endometrial thickness, myometrium and the number of endometrial glands. Estrogenic activity of phytoestrogens is highly dependent on the dose, number, location of estrogen receptors (Reα and Reβ), and the concentration of estrogen.²⁶ The presence of alkaloid compounds in this study also caused anti-gonadotropic effects in certain doses. Study of Yakubu & Musa reported that alkaloids in aqueous extract of *S. alata* leaves in dose 250, 500, 1000 mg/kgBW exhibit anti-gonadotropic, anti-progesteronic, and feto-maternal toxic activities. Alkaloids show more anti-estrogenic activity (71.43%) compare to estrogenic activity (28.57%). It depends on the dose.³²

Endometrial glands

The combination extracts increased the number of endometrial glands. Phytoestrogen in combination extracts have high antioxidant property, which have the role of protecting and regenerating cells.³⁴ Phytoestrogens also bind to estrogen receptors in the uterus and trigger proliferation and development of the endometrial glands,³⁵ which provide nutrients to the embryo. The number of endometrial glands increases during the secretory stage, while the endometrium thickens due to glandular secretion and stromal fluid activity.³⁶

Glandular hypertrophy caused by excessive secretion which the gland becomes dilated, the lumen widens and fills with secretions. If the presence of estrogen in the female body is lacking, then phytoestrogens can be a support for endogenous estrogen. The presence of phytoestrogens in small amounts helps to bind to estrogen receptors that are still empty so that there is good support between endogenous estrogens and phytoestrogens in increasing cellular response.³⁵

CONCLUSION

The combination of *A. sativum*, *C. mangga*, and *A. calamus* extracts could be used to increase fertility. The combination extract increased estrogen and progesterone levels with an optimal dose of 50 mg/kgBW (T1 & T4) and also increased the thickness of the endometrium, myometrium, and the number of endometrial glands with a dose of 75 mg/kgBW (T2 & T5), but decreased thickness of endometrium, myometrium and the number of glands at a dose of 100 mg/kg BW (T3 & T6).

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CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

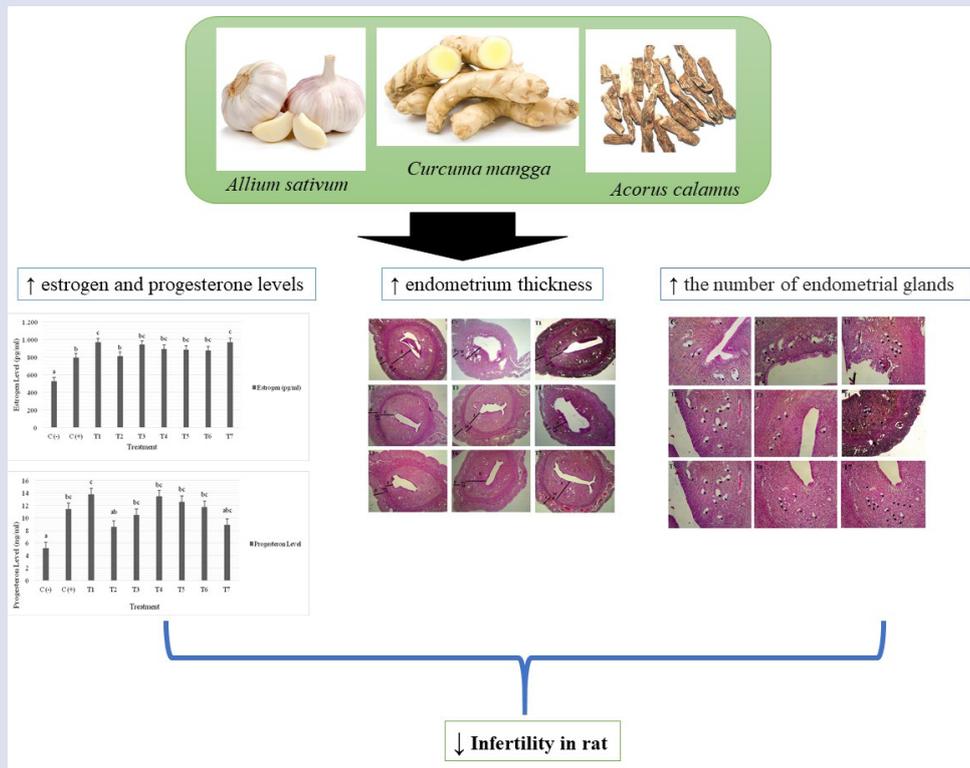
CONTRIBUTION OF AUTHORS

We declare that this work was done by Bayyinatul Muchtarmah, Roihatul Muti'ah, Desy Rahma Yusmalasari, Putri Mardiyana, Tanjina Sharmin, Amaq Fadholly and all liabilities pertaining to claims relating to the contents of this article will be borne by the authors. Bayyinatul Muchtarmah performed the study design and drafting the manuscript, Roihatul Muti'ah performed the data analysis and Desy Rahma Yusmalasari, Putri Mardiyana, Tanjina Sharmin, Amaq Fadholly performed the final approval of the manuscript.

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GRAPHICAL ABSTRACT



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