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Folliculogenesis Effect of *Allium sativum*, *Curcuma mangga* and *Acorus calamus* Extracts on Rats (*Rattus norvegicus*)

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ABSTRACT

This study aims at determining the effect of the combination of *Allium sativum, Curcuma mangga* and *Acorus calamus* on ovarian folliculogenesis of rats (*Rattus novergicus*). This study used a completely randomized design (CRD) consisting of nine treatments and four replications. Treatment consisted of K- (negative control), K+ (clomiphene citrate dose of 0.9 mg/kg BW), P_1, P_2, P_3 (composition 1, dose of 50, 75 and 100 mg/kg BW), P_4, P_5, P_6 (composition 2, dose of 50, 75 and 100 mg/kg BW) and P_7 (Subur kandungan herb™, dose of 75 mg/kg BW). The combination of *A. sativum, C. mangga* and *A. calamus* affected the amount of primary, secondary, tertiary follicle, total follicle and the amount of ovulation. The highest number of primary, secondary, tertiary follicle, total follicle and the number of ovulation obtained in P_7 were not significantly different from P_2 and K+, while the lowest was indicated by K-. Giving a combination of the three extracts affected the maturity of ovarian follicles, thereby increasing the fertility of female rats.

Key words : Folliculogenesis, *Allium sativum, Curcuma mangga, Acorus calamus, Rattus norvegicus*

INTRODUCTION

Infertility is a reproductive problem that often occurs in 1 of 6 couples of males and females worldwide. Couples are said to experience infertility problems if they do not deliver a baby for a year (Kashani and Akhondzadeh, 2017). The problem can occur in both men and women. Infertility in women reaches 50% of the total cases found (Hanson *et al.*, 2017). Efforts to overcome infertility problems include living a healthy lifestyle, using reproductive technology such as *in vitro* fertilization, using ovulation induction drugs, hormone therapy and herbs. Herbs have been used to overcome infertility problems for thousands of years ago, and it is an inclusive practice based on theory, beliefs and experiences (Akour *et al.*, 2016).
Complementary and alternative medicine is conventional in both developing and developed countries. Even, up to now around 80% of the total populations in developing countries still use herbal medicines to cure some diseases because they are cheap, affordable and available in large quantities. Besides, the use of herbal medicines has low side effects for the body, compared to synthetic drugs (Ekor, 2013).
One of the herbal medicines that is often used to increase fertility by Madurese people of Indonesia is "Jamu Subur Kandungan". It consists of a combination of three material, namely, *Allium sativu*m bulbs (*Allium sativum*), Curcuma rhizoma (*Curcuma mangga*) and Calamus rhizoma (*Acorus calamus*). Previous study reported that *A. sativum* extract indicated the presence of alkaloid, terpenoids, flavonoids, steroid, phenol, anthraquinones, saponin, tannin and glycoside, carbohydrate, protein, fats, crude fibre, calcium, potassium, magnesium, zinc, phosphorous, iron and copper (Ali and Ibrahim, 2019), while *C. mangg*a contained polyphenols, flavonoids, triterpenes, sterols, curcumin, demethoxycurcumin and bisdemethoxy curcumin (Malek *et al.*, 2011). *A. calamus* contained glycosides, carbohydrate, phenolic compound, alkaloids, flavonoids, tannin, saponin, steroids and triterpenoids (Mamta and Jyoti, 2012). The three plants have the potential as phytoestrogens, which can increase estrogen and progesterone. One of the reproductive

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organs that are influenced by estrogen is the ovary. The ovary produces steroid hormones that allow the development of secondary female sexual characteristics and support pregnancy. This study aimed at determining the effect of ethanol extract of *A. sativum* (L.), *C. mangga* Val. and *A. calamus* (L.) on ovarian folliculogenesis of rats (*Rattus norvegicus* L.).

MATERIALS AND METHODS

Materials collected were : *A. calamus*, *C. mangga*, *A. sativum* simplisia (Balai Materia Medika Batu), Subur kandungan herb™ (Ribkah Jokotole Home Industry, Madura), 70% ethanol (Bratachem), Na CMC (Himedia), PMSG (Intervet), hCG (Intervet), clomiphene citrate (Sande Farma), 10% formalin, paraffin, xylol, Hematoxylin, Eosin (Roche Diagnostics).

This research used completely randomized design with nine treatments and four replications. Rats with three months old and 180-230 g body weight were acclimatized for seven days. Synchronization of estrus cycle was done by use of 10 IU of PMSG for 48 h following injection of 10 IU HCG. The estrus cycle was confirmed by vaginal smears test and Giemsa staining (Cora *et al*., 2015).

The treatment consisted of negative control (K-) : no treatment, positive control (K+) : clomiphene citrate dose of 0.9 mg/kg BW; P_1, P_2, P_3 : composition 1 with dose of 50, 75 and 100 mg/kg BW, P_4 : composition 2 with dose of 50, 75 and 100 mg/kg BW and P_7 : 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. Composition 1 consisted of *A. sativum* 36%: *C. mangga* 36%: *A. calamus* 28% and composition 2 consisted of *A. sativum* 35% : *C. mangga* 40% and *A. calamus* 25%. Each rat received 2 ml extract continuously for 15 days. After 15 days, the rats were sacrificed by cervical dislocation, and the ovarium was taken from the abdominal cavity for histology preparations. All treatments were tested through the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the Universitas Islam Negeri Maulana Malik Ibrahim Malang (016/EC/KEPK-FKIK/2018).

Ovarium 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; El-Zahraa and Abd-Elhafez, 2018). 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 AND DISCUSSION

The combination of *A. sativum*, *C. mangga* and *A. calamus* extracts increased the number of primary follicles, secondary follicles, tertiary follicles, total follicles and ovulation in the experimental group compared to the control group (K-) significantly ($P<0.05$) as presented in Fig. 1. The highest numbers of primary follicles, secondary follicles, tertiery follicles and total follicles were found in Subur Kandungan herb, followed by Clomiphen citrate (standard fertile medicine), and extract dose of 75 mg/kg, while corpus luteum number was obtained in K+, which was not significantly different from extract dose of 75 mg/kg and P_7 but significantly different from other treatments. The more corpus luteum in the ovary, the higher was the ovulation rate. Subur Kandungan (Trade Mark) herb as a traditional fertility drug standard as in its packaging had the efficacy of increasing the number of eggs, increasing fertility, strengthening the muscles of the uterus, healthy reproductive organs, balancing reproductive hormones and healthy bodies (Adji, 2012).

Giving a combination of *A. sativum, C. mangga* and *A. calamus* at low dose 50-75 mg/kg (P_1, P_2, P_4 and P_5) noted a trend of increasing the number of follicles of all stages and ovulation, whereas a higher dose of 100 mg/kg (P_3 and P_6) actually showed the opposite trend. The decrease of follicle number, presumably due to the role of active substances on hormonal metabolism, was mainly related to the metabolism and synthesis of reproductive hormones. The presence of triterpenoid saponin active substances at high doses was suspected of causing negative feedback in the hypothalamus which further resulted in impaired GnRH secretion, thus affecting follicle formation, development and maturation and ovulation process (Wigglesworth *et al*., 2014).

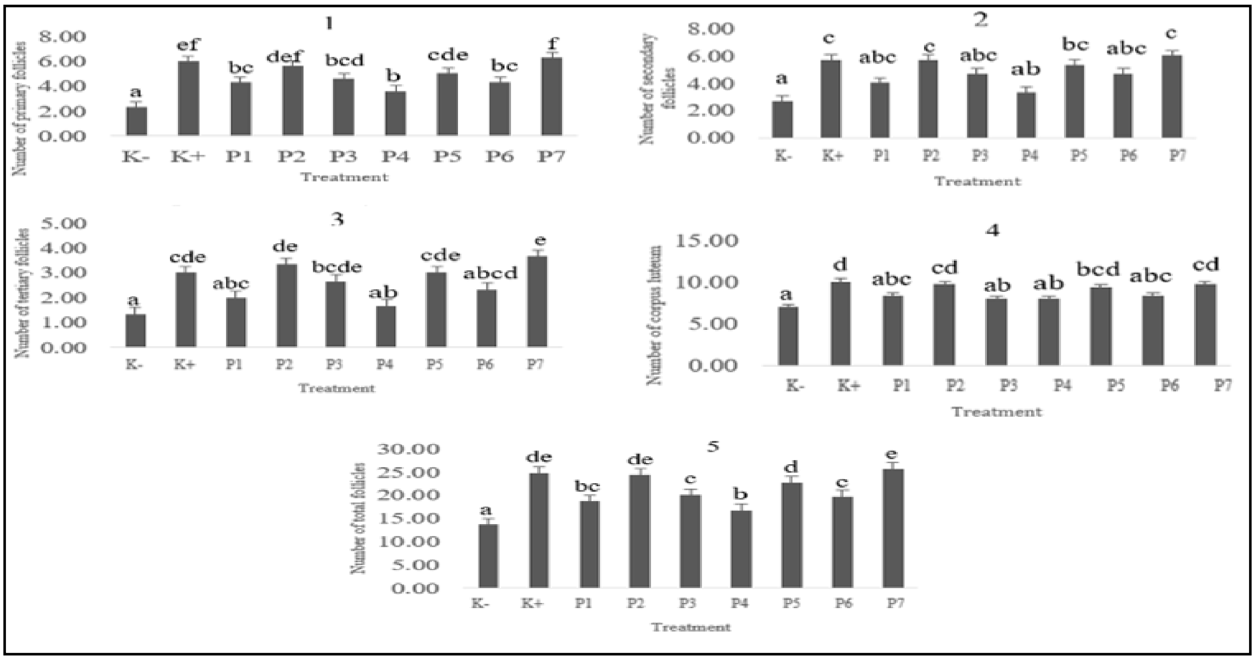

Fig. 1 (1) Effect of treatment on primary follicles number, (2) secondary follicles, (3) tertiary follicles, (4) corpus luteum (ovulation), and (5) total follicles.

The ovary is a female reproductive organ that functions as a place to produce eggs and reproductive hormones. The development of ovarian follicles through several stages, namely, the development of primary follicles, secondary follicles, tertiary follicles, de graff follicles and ovulation, while the former ovulation was the corpus luteum (Shea *et al.*, 2014). Some plants that were often used as medicine and were ingredients of traditional medicine from Madura to increase fertility ("Subur kandungan") are *A. sativum, C. mangga* and *A. calamus*. The results revealed that the combination of *A. sativum, C. mangga* and *A. calamus* increased the number of primary, secondary, tertiary follicles and ovulation.

Increasing the number of ovarian follicles also increased the fertility of women, because one of the parameters of fertility was the amount of ovulation. The development of ovarian follicles was influenced by the production of FSH (follicle stimulating hormone). FSH production in the pituitary caused the follicles to form cavities (antrum) and produce estrogen (Stamatiades and Kaiser, 2018). Estrogen was mostly produced by granulosa cells, which converted androgens produced by internal theca cells into estrogen (Barros *et al.*, 2013).

The function of granulosa cells was feeding eggs and removing inhibiting factors for egg maturation. The follicular development stage was the enlargement of the follicle followed by the formation of a layer of granulosa cells then forming primary follicle. This process occurred because of FSH (follicle stimulating hormone) and LH (Luteinizing hormone). In addition, the intersitium of the ovary produced cells that formed several layers of coils outside the granulosa cells, called theca cells. After the initial stage of growth, the mass of granulosa cells developed and secreted follicular fluid containing estrogen so that the collection of this fluid caused the appearance of antrums during the granulosa cell period (Juengel *et al.*, 2017). If the antrum had formed, granulosa cells and theca cells multiplied rapidly and the rate of secretion increased so that each follicle grew into antral follicle (Guyton and Hall, 2015). The results of observations of ovarian tissue showed follicles in the ovary after being treated with ethanol extract of *A. sativum*, *A. calamus* and *C. mangga* (Fig. 2).

De graff follicles were not found in all treatments except in the K-group. This was expected at the time of surgery that ovulation had occurred. The presence of phytoestrogens in a combination of extracts increased LH levels and accelerated ovulation so that ovulation in mice treated groups faster than control.

It has been previously reported that *A. sativum, C. mangga* and *A. calamus* extract contained phytochemical compounds

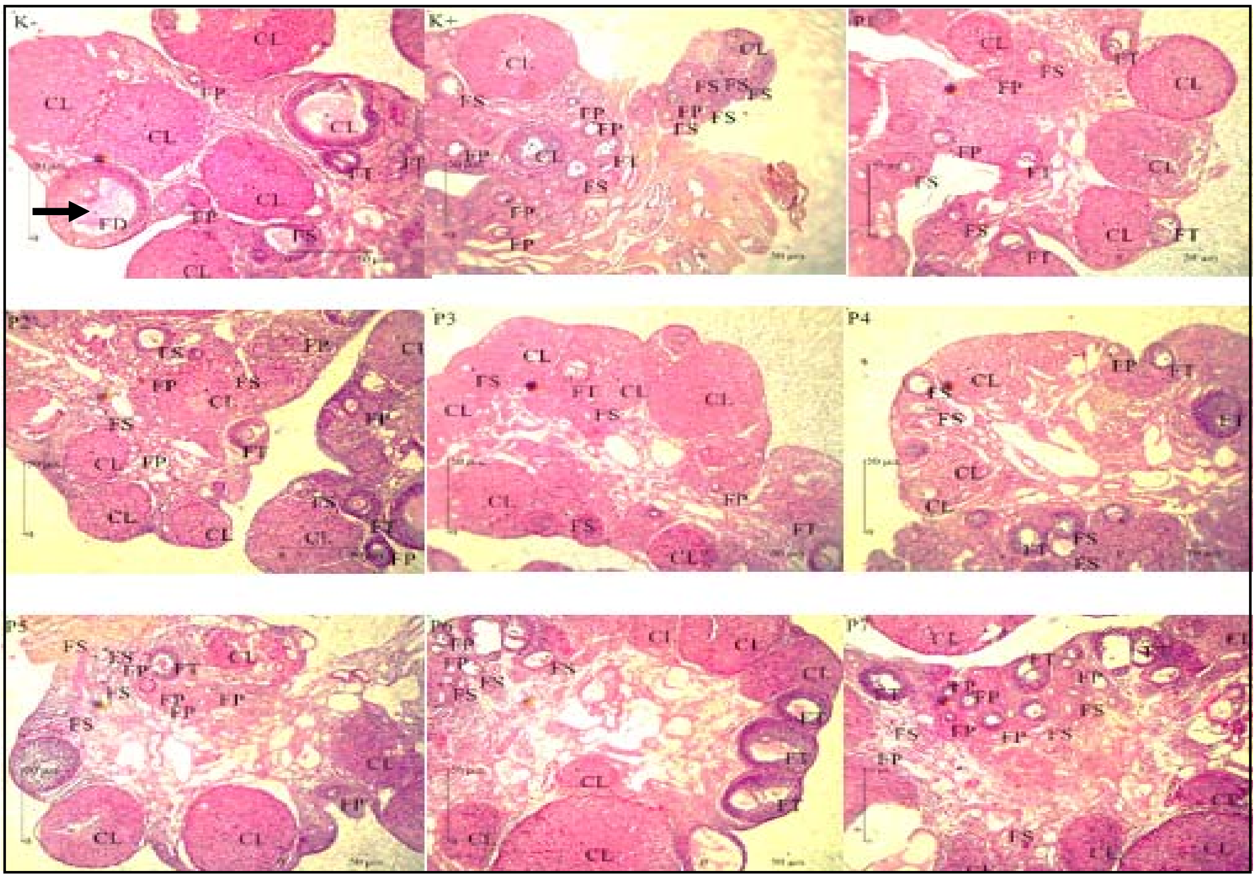

Fig. 2. Cross section of the ovary showing the profile of follicular histology (Magnification 100X). FP (Primary follicle), FS (Secondary follicle), FT (Tertiery follicle) and FD (de Graaf follicle).

including flavonoid, alkaloid and triterpenoid (Muchtaromah *et al.*, 2017a). Similarly, Krizova *et al.* (2019) reported that some phytoestrogen compounds contained in plants included isoflavones, lignans, coumestans, triterpenic glycosides and other compounds such as alkaloids, triterpenoids and chalcone. Isoflavones belonged to the flavonoid group and the largest group in flavonoids. Genistein was one of the derivatives of isoflavone compounds. Lacy (2015) reported that the structure of genistein and estrogen was almost similar and had an estrogenic effect that binded to estrogen receptors (ER). Genistein was an isoflavone derivative and was the most estrogenic phytoestrogen, while the isoflavone derivative content of combined *A. sativum, C. mangga* and *A. calamus* was not elucidated yet and still needs further research (Primiani and Pujiati, 2018).

The flavonoids, alkaloids and triterpenoids content of the extract acted as a phytoestrogen compound and were assumed to cause steroidogenesis (formation of the steroid hormone) in the ovary. The mechanism of action of phytoestrogens was through the direct genomic mechanism i. e. phytoestrogens directly binded to estrogen receptors (ER) and affected gene transcription, so that it caused estrogen-like effects (estrogenic effect). Phytoestrogens circulated in the blood stream in a free form and was binded to carrier proteins. Phytoestrogens circulated through the membrane by passive diffusion or active transport (Fuentes and Silveyra, 2019). Lecomte *et al.* (2017) stated that estrogen receptors binded to Estrogen Responsive Element (ERE), which activated some proteins for cell division. When transcribing protein synthesis, estrogen/phytoestrogen-receptor complexes not only binded to ERE but also binded to co-regulator. Furthermore, Fuentes and Silveyra (2019) revealed that the complexes binding of estrogen affected transcription and translation as well as the maturation process of folliculogenesis, which triggered ovulation and formed a corpus luteum.

This result was supported by the research of Muchtaromah *et al.* (2017b), the combination of *Centella asiatica* and *Plucea indica* influenced the number of follicles, antioxidant activity and hormonal profile of rat ovaries. Control, P_1 (dose of 25+25 mg/kg) and P_2 (dose of 50+50 mg/kg) increased the number of follicles, whereas P_3 (dose of 75+75 mg/kg), P_4 (dose of 125+125 mg/kg) and P_5 (dose of 200+200 mg/kg) decreased the number of follicles. The total follicle was affected by the dose. This was because of the contribution of phytoestrogen in *C. asiatica* and *P. indica.* In high concentrations, phytoestrogen gave negative feedback to the hypothalamus-pituitary-ovary, thus inhibiting the release of LH and FSH. Decreasing LH and FSH reduced the number of follicles and ovulation.

In addition to FSH as the primary regulator of the development of dominant follicles, growth factors produced by follicles worked through autocrine and paracrine mechanisms, modulated the work of FSH, and became an essential influential factor. The presence of FSH and LH was significant for the development of primary, secondary, tertiary and de graff follicles. Follicular growth was influenced by FSH levels in the ovaries so that primary and secondary follicles were developed properly. This was understood because at the beginning of follicular development required FSH in sufficient quantities to encourage follicular development into the next phase. This caused an increase in the number of follicles in the P_2, P_5 and P_7 groups. Ovulatory follicular tissue developed into the corpus luteum, the endocrine tissue that secreted the hormone progesterone during the luteal phase of the ovarian cycle (Barbieri, 2014).

CONCLUSION

The combination of *A. sativum, C. mangga* and *A. calamus* affected the amount of primary, secondary, tertiary follicle, total follicle and the amount of ovulation. The highest number of primary, secondary, tertiary follicle, total follicle and the number of ovulation obtained in P_7 were not significantly different from P_2 and K+, while the lowest was indicated by K-. Giving a combination of the three extracts affected the maturity of ovarian follicles, thereby increasing the fertility of female rats.

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