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by Epy M. Luqman

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RESEARCH ARTICLE

The Effect of Kebar Grass Extract (*Biophytum petersianum* Klotzch) on Total Growth of Ovarian Follicles on Mice (*Mus Musculus*) during Lactation period were Exposed by Carbofuran

Athaya K. Mulyadi[1], Tri W. Suprayogi[1], Maslichah Mafruchati[1], Imam Mustofa[1], Eka P. Hestianah[1], Juliano M. Ntoruru[2], Epy M. Luqman[1]*

[1]Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga Muyorejo Surabaya, 60115 Indonesia.

[2]Research Assistant, Meru University of Science and Technology, Kenya.

*Corresponding Author E-mail: **epy-m-l@fkh.unair.ac.id**

ABSTRACT:

The study aimed to know the effect of Kebar grass extraction amounts of mice (Mus musculus) follicles ovary exposed by carbofuran. This study was an experimental study with a completely randomized design. Forty two lactation mice used in this study were divided into seven groups, that were: C was control (Aquadest), P1 was injected with carbofuran 1/4 LD_{50} (0.0125mg/day), P2 was injected with carbofuran 1/8 LD_{50} (0.00625mg/day), P3 was injected with carbofuran 1/4 LD_{50} (0.0125mg/day) and Kebar grass extract (0.135mg/day), P4 was injected with carbofuran 1/8 LD_{50} (0.00625mg/day) and Kebar grass extract (0.135mg/day), P5 was injected with carbofuran 1/4 LD_{50} (0.0125mg/day) and vitamin C (0.2ml/day) and P6 was injected with carbofuran 1/8 LD_{50} (0.00625mg/day) and vitamin C (0.2ml/day) with a treatment period of 14 days. Then on the 15day of treatment, mice were sacrificed, and the ovary was taken. Then, histology preparation with HE (Hematoxylin-eosin) staining was made, and follicles ovary were counted. Data were analyzed by One Way ANOVA followed by the Duncan test. This study showed that exposed carbofuran was a significant decrease in the growth of ovarian follicles ($p<0.05$). The administration of the Kebar grass extraction exposure to carbofuran is proven to increase primary and secondary follicles. The Kebar grass extraction administration gave a better effect than vitamin C ($p<0.05$).

KEYWORDS: Kebar grass extract, Follicles, Ovary, Carbofuran, Lactation, Pesticide stress.

INTRODUCTION:

The insecticide has an essential role in agriculture activities to protect plants from pests. By the rules, the application should not cause environmental pollution, health problem, poisoned, and residues in food products[1]. According to World Health Organization (WHO), every year occur cases of insecticide poisoned by farmers cause dead. 80% of poisoned reported happening in a developing country. Insecticide groups of carbamates have many uses in Indonesian to eradicate the pest. Group of carbamates, which is often used carbofuran, carbaryl, and aldicarb.

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The risk of carbofuran can causes residues on rice, rice field water, and soil field in Java islands in 1997. The resulting residues on rice are 0.5-1.3 ppb with maximum residue limit according to Codex Alimentarius commission is 0.1 ppm[2]. Carbofuran residue risk can cause teratogenic, adrenal damage, and reproductive system disorders on Passer domesticus[3,4].

Damage due to exposure carbofuran affects Reactive Oxygen Species (ROS) activities that cause dead brain cells during the mother lactation period[5]. ROS or free radicals is a molecule that is not pairs and stable that molecule will take an electron from another molecule and very reactive[6,7]. Free radicals induce reproduction toxicity and infertility because insecticide exposure causes oxidative stress who induce lipid peroxidation and DNA damage on fish, rats, and pregnant women[8]. Exposure carbofuran shows an increase in the total

atretic follicle and decreases in a total of healthy follicles[9]. A decrease in healthy follicles' total resulted from granulosa cells in the ovary have apoptosis and necrosis[10] causes atretic follicles. Insecticide also affects vacuolar degeneration on cells follicle, so that looks increase empty follicles in the ovary[11].

The lactation period is when mother secretion milk from the mammary gland after giving birth to their child[12]. In this period, mothers need lots of nutrition compared when pregnant[13,14]. Environmental pollutions like insecticide and heavy metal cause a change in the immune system. Toxic substances cause the suppression of immunomodulatory components, which indirectly affect organs as nerve, reproduction, respiratory, and endocrine[15]. Insecticide causes damage to the mother and their child before, during, and after birth[16]. Measurement of follicles during the lactation period is thought to have the function of identifying mothers that are potentially relatively infertile after weaning[17].

The body has natural defenses to neutralize free radicals by increase the production of antioxidants endogenous. However, if there are more free radical concentrations in the body, it is necessary to balance with antioxidants exogenous[18,19]. Flavonoids are antioxidants derived from phenolic compounds derived from plants[20,21]. These compounds are useful in counteracting free radicals and are also proven to fight diseases caused by free radicals[22]. Kebar grass (*Biophytum petersianum Klotzch*) is a plant that contains flavonoids. Other antioxidants found in Kebar grass are vitamin E and A. Vitamin E is the primary defense against free radicals and can also attack lipid peroxide, which is thought to be a degenerative cause of cells[23]. In contrast, vitamin A plays an essential role in stabilizing free radicals[22].

Research on the Kebar grass extraction on the reproduction of mother during lactation has not been widely studied, especially the effect on the number of follicles in the ovaries exposed to carbofuran. This study was conducted to show that giving Kebar grass extraction can increase the number of healthy ovarian follicles and decrease the number of atretic follicles exposed by carbofuran.

MATERIAL AND METHODS:

The research tools that are used is experimental animal cages, feed and drinking containers, sonde needles, syringe 3ml, surgical equipment (surgical scissors, scalpel, tweezers), object glass, cover glass, ointment pots, tissue processor automatic, water bath, hot plate, microtome, blade and Olympus® CX-23 microscopes. The research materials that are used is mice, carbofuran (2, 3-Dihydro-2,2-dimethyl-7-benzofuranol N-methyl-carbamate 98%) from Aldrich Chemistry dengan Bellstain Registry number 1428746, Product of USA, dry Kebar grass in packaging from Papua, vitamin C used as a reference substance, pellets, drinking water, aquadest, CMC Na, 70% etanol, husks for cage tools, ether solution, 10% buffer formalin and alcohol.

Preparation of Experimental Animals:

Pregnant mice strain Balb/C were divided into seven groups is K, P1, P2, P3, P4, P5, and P6. There are six repetitions for each group.

Method:

The research procedure was conducted by testing the code of ethics of experimental animals with the number 1.KE.107.06.2019 at the Faculty of Veterinary Medicine, Airlangga University. Mice were divided into seven groups, that were: K was control (aquadest), P1 was injected with carbofuran 1/4 LD_{50} (0.0125mg/day), P2 was injected with carbofuran 1/8 LD_{50} (0.00625 mg/day), P3 was injected with carbofuran 1/4 LD_{50} (0.0125mg/day) and Kebar grass extract (0.135mg/day), P4 was injected with carbofuran 1/8 LD_{50} (0.00625 mg/day) and Kebar grass extract (0,135mg/day)[24, 25], P5 was injected with carbofuran 1/4 LD_{50} (0.0125mg/day) and vitamin C (0.2ml/day) and P6 was injected with carbofuran 1/8 LD_{50} (0.00625mg/day) and vitamin C (0.2ml/day) with a treatment period of 14 days. Then on the 15day of treatment, mice were sacrificed and the ovary was taken. Then, histology preparation with HE (Hematoxylin-eosin).

Histopathological preparations:

Counting the number of ovarian follicles was each treatment group in five different fields of view on each histopathological preparation. Microscopic observation of the ovary in histopathology preparations using an Olympus® CX-23 microscope with a magnification of 100X. Observations were made by counting the number of the primary, secondary, tertiary, Graafian, and atretic follicles. The calculation of each field of view in one preparation is added up, then the average is calculated, then statistical analysis.

Data Analysis:

The data were processed and analyzed statistically using the ANOVA (Analysis of Variance) test if normally distributed data were obtained, followed by the Duncan test. If it is found that the data is not normally distributed, the data is analyzed using the Kruskal Wallis test.

RESULTS:

Primary Follicle:

The analysis results with the ANOVA test showed that the primary follicle was significantly different ($p<0.05$), so it was continued using the Duncan test. Table 1 shows

that group C has a significant difference in the number of primary follicles in groups P1, P2, and P4. Group P1 did not significantly differ with P2, but there was a significant difference with P5, P6, K, P3, and P4. Group P4 did not have a significant difference with P3, but there was a significant difference with P1, P2, P5, P6, and C. Group P6 did not have a significant difference with P5, C, and P3. However, there was a significant difference between P1, P2, and P4.

Table 1: Duncan test results for the average number of the primary, secondary, tertiary, and atretic follicles.

Group	Primary Follicle	Secondary Follicle	Tertiary Follicle	Atretic Follicle
C	$5.13^{c}\pm0.70$	$4.86^{c}\pm0.64$	$1.13^{a,b}\pm0.41$	$1.93^{d}\pm0.41$
P1	$2.93^{a}\pm0.61$	$2.26^{a}\pm0.41$	$0.40^{a}\pm0.34$	$3.93^{f}\pm0.30$
P2	$3.67^{a,b}\pm0.90$	$2.73^{a}\pm0.30$	$0.46^{a}\pm0.41$	$3.46^{f}\pm0.50$
P3	$5.67^{c,d}\pm0.23$	$5.20^{c}\pm0.40$	$1.20^{a,b}\pm0.20$	$2.26^{d,e}\pm0.30$
P4	$6.40^{d}\pm0.34$	$5.93^{d}\pm0.30$	$1.60^{b}\pm0.52$	$2.00^{d,e}\pm0.20$
P5	$4.60^{b,c}\pm0.20$	$4.06^{b}\pm0.50$	$0.67^{a}\pm0.57$	$2.60^{e}\pm0.20$
P6	$4.86^{c}\pm0.64$	$4.46^{b,c}\pm0.11$	$0.80^{a,b}\pm0.40$	$2.46^{d,e}\pm0.30$

Note: Different superscripts show significant differences ($p<0.05$).
C (Control), P1 (Carbofuran 1/4 LD_{50}), P2 (Carbofuran 1/8 LD_{50}), P3 (Kebar grass extraction + Carbofuran 1/4 LD_{50}), P4 (Kebar grass extraction + Carbofuran 1/8 LD_{50}), P5 (Vitamin C + Carbofuran 1/4 LD_{50}), P6 (Vitamin C + Carbofuran 1/8 LD_{50}).

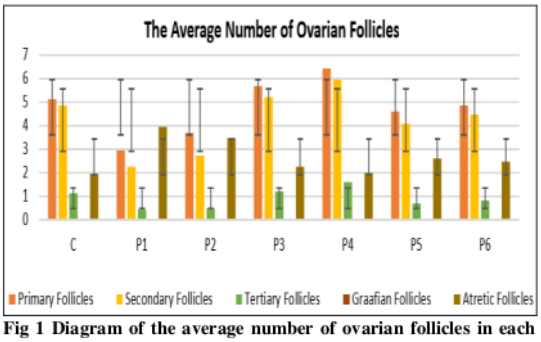

Fig 1 Diagram of the average number of ovarian follicles in each treatment.

Secondary Follicle:

The analysis results with the ANOVA test showed significantly different ($p<0.05$) and then continued using Duncan test. Based on Table 1 shows that in group C there was a significant difference in the number of secondary follicles in groups P1, P2, P5, and P4. Group P1 did not have a significant difference with P2, but there was a significant difference with groups P5, P6, C, P3, and P4. Group P6 did not significantly differ with C, P3, and P5, but there was a significant difference between P1, P2, and P4.

Tertiary Follicle:

The ANOVA test, it was found that were significantly different ($p<0.05$) then continued by using the Duncan test. Table 1 shows that there was no significant difference in the number of tertiary follicles in the P1, P2, P3, P4, P5, and P6 groups in the C group. The P4 group had a significant difference with P1, P2, and P5.

Atretic Follicle:

The analysis results with the ANOVA test showed that they were significantly different ($p<0.05$), so it was continued using the Duncan test. Based on Table 1 show that in group C there were differences in the number of atretic follicles that were significant with P5, P2, and P1. Group P5 did not significantly differ with P4, P3, and P6, but there was a significant difference between C, P2, and P1. Groups P1 and P2 had significant differences with C, P4, P3, P6, and P5.

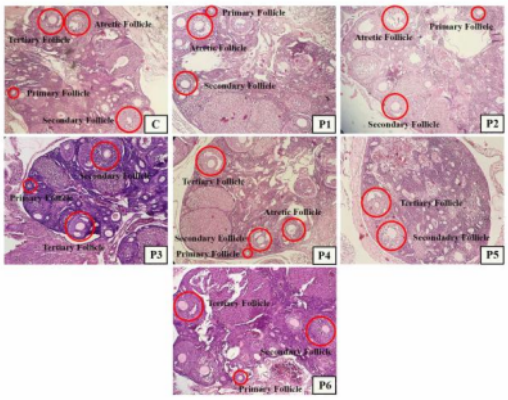

Fig 2 The histopathology of ovaries with Hematoxylin Eosin staining using an Olympus CX-23 microscope with 100x magnification in groups K, P1, P2, P3, P4, P5, and P6.

DISCUSSION:

Primary Follicle:

Primary follicles are the beginning of folliculogenesis. They are characterized by granulosa cells that are cuboidal and oocyte in the middle. The process of development is influenced by extra ovarian and intrafollicular signals. The intrafollicular signals from growth factors, while the extra ovarian signals from gonadotropin hormones[26]. The receptors receive these signals on the granulosa cells of the ovarian follicles. Primary follicles express FSH receptors are on granulosa cells[27,28].

The results showed that giving carbofuran caused a decrease in the number of primary follicles in the P1 and P2 groups allegedly because the toxic effects caused an increase in ROS that formed free radicals. P1 experienced a lower decrease than P2; this could be due to the higher carbofuran dose at P1 (0.0125mg) compared to P2 (0.00625mg). Carbofuran entered the body and metabolized cytochrome P_{450} to produce 3-hydroxycarbofuran, causing increased toxic activity because it is systemic[29]. Insecticides can indirectly affect gonadal function by influencing the action of the hypothalamus-pituitary-gonads or directly by oxidative stress mechanisms[30]. Oxidative stress results from an increase in free radicals that can damage cell membranes

through lipid peroxidation[20]. This cell damage causes the primary follicle to decline to not develop into the next stage, namely the secondary follicle.

The increase in the number of primary follicles in the P4 group was due to the antioxidants contained in Kebar grass, namely flavonoids, vitamins E and A. Flavonoids have a role in reducing the effects of ROS by trapping free radicals such as superoxide and hydroxyl anions[31]. Vitamins E and A are non-enzymatic antioxidants that protect membranes cell and improve reproductive function. Vitamin E changes free radicals into hydroperoxides and neutralizes ROS, likes superoxide radicals, hydrogen peroxide, and hydroxyl radicals. Vitamin A is a fat-soluble antioxidant that scavenges free radicals from plasma lipoproteins and cell membranes[7] and can protect cells from superoxide radicals[32]. The P3 group showed inhibition in decreasing the number of primary follicles. The difference in giving the Kebar grass extract with the same dose (3.375mg) could be due to differences in the dose of exposure to carbofuran in each group. This study showed that the Kebar grass extract could ward off free radicals caused by carbofuran, but the P3 group could not increase the number of primary follicles.

The administration of vitamin C showed inhibition in reducing the number of primary follicles, both in the P5 and P6 groups. Vitamin C is a hydrophilic antioxidant that can trap free radicals to protect cell membranes from oxidative stress[33]. Administration of vitamin C in aging mice showed an increase in the number of the primary, secondary, and antral follicles[34]. This study indicates that the antioxidants from Kebar grass extract provide a more significant effect than giving vitamin C because the compounds contained in Kebar grass are flavonoids. Flavonoids are phenolic compounds with more than one hydroxyl group to reduce free radicals such as superoxide anion radicals, hydroxyl radicals, peroxyl radicals, and alkoxyl radicals[31]. The antioxidant ability of flavonoids is more effective than vitamin C and vitamin E[22,35].

Secondary Follicle:

Secondary follicles are the next developmental stage of primary follicles, consisting of granulosa cells, internal theca cells, zona pellucid, and oocytes. Like primary follicles, secondary follicles in their development are influenced by intrafollicular signals and extra ovarian signals. Secondary follicles express FSH receptors on granulosa cells and express LH receptors on theca cells[27]. Granulosa cells and theca cells in ovarian follicles have an essential role in synthesizing the hormone estrogen. Besides, granulosa cells play a role in the growth and maturation process of follicles[36]. If there is damage to these cells, it can cause impaired growth and maturation of ovarian follicles.

The results of this study showed a decrease in the number of secondary follicles in the P1 and P2 groups. This decrease is thought to occur in the same way as primary follicles due to exposure to carbofuran, causing free radicals that increase ROS production in the body, resulting in oxidative stress. Reactive oxygen species compounds such as O_2-, H_2O_2, and OH- have an essential role in oxidative damage to cells[7]. Carbofuran is lipid-soluble that will form lipid peroxidation after binding to lipids from the cell membrane[37]. The final result of lipid peroxidation is malondialdehyde (MDA). Increased MDA level is one factor that indicates a decrease in the number of ovarian follicles leading to infertility[38]. It is known that giving carbofuran to mother mice during lactation resulted in an increase of MDA levels, necrosis of Purkinje cells, and a decrease in ChE[5].

The increase in the number of secondary follicles in the P4 group is thought to contain in content Kebar grass is flavonoids, vitamins E and A; this is following a study that states that the flavonoids found in plants can increase the number of antral follicles exposed to the insecticide cypermethrin[39]. Administration of insecticides with vitamin E antioxidant therapy can reduce MDA levels and increase rat ovaries' GSH levels. Vitamin E can defend cells from oxidative stress[40] by acting as a protector against lipid peroxidation through its chain-breaking action[8]. Vitamin A in Kebar grass has been shown to protect cells from free radical attack and maintain the epithelial layer of the reproductive tissue[41]. As with primary follicles, the P3 group study results showed that it could maintain the number of secondary follicles; this could be because the dose of exposure to carbofuran in the P3 group was lower than the P4 group.

Administration of vitamin C showed a decrease in the number of secondary follicles in the P5 group, while the group maintained the secondary follicle count compared to the control group. The P5 group had the highest exposure dose in this study. Based on antioxidants' actions, it is better to give antioxidants in combination compared with a single form[7]. In the P5 group, there was a decrease in the number of secondary follicles. Antioxidants in vitamin C have not been able to provide a good effect in the P5 group. However, the P6 group showed that vitamin C could maintain the number of secondary follicles; this is the same as the research results from the number of primary follicles.

Tertiary Follicle:

Tertiary follicles are the next development stage of secondary follicles, which consist of granulosa cells, internal and external theca cells, zona pellucid, oocytes, and small spaces between granulosa cells called call Exner bodies. Its development of these follicles is affected by intrafollicular signals and extraovarian signals[27].

The results of this study showed a decrease in the number of tertiary follicles in the P1 and P2 groups, but there was no significant difference with the control group. The decrease in the number of tertiary follicles is the same as the decrease in the number experienced by primary and secondary follicles, which is thought to occur because carbofuran's toxic effect causes an increase in ROS in oxidative stress. ROS binds to plasma lipoproteins and cell membrane unsaturated fats, resulting in lipid peroxidation formation[7]. There was no significant difference with the control group, presumably because the mice used in this study were in the lactation period. During lactation, the follicles in the ovaries experience a decrease in the release of gonadotropin hormones from breastfeeding children[42].

The administration of Kebar grass extract showed that it maintained the number of tertiary follicles in the P3 and P4 groups compared to the control group because the antioxidants present in Kebar grass are flavonoids that can inhibit lipid peroxidation by reducing peroxyl radicals and overcoming superoxide anions[31]. Vitamin E, a lipid antioxidant, can overcome free radicals and functions to stabilize cell membranes[43] and vitamin A which can stabilize free radicals[7]. The provision of vitamin C showed the same results as the P1 and P2 groups. There was no significant difference with the control group; this can be affected because vitamin C's antioxidant action has not worked maximum. After all, it is given in a single form[7].

Graafian follicle:

Graafian follicles are mature follicles and ready to be ovulated. The extra ovarian signal (gonadotropin hormone) plays an essential role in reaching the mature follicle. Granulosa cells have FSH receptors and theca cells have LH receptors[27]. A large space is formed at this stage called follicular antrum, which contains follicular fluid rich in protein and estrogen. Ovulation occurs when the LH level increases, causing the oocyte to leave the follicle ready for fertilization[42]. Based on Table 1, the results showed no Graafian follicle development in each treatment group; this is thought to be due to cell damage in primary, secondary, and tertiary follicles so that the follicles do not reach the maturation stage. A study showed that giving carbofuran decreased growing follicles, Graafian follicles, and increased atretic follicles[9]. Another study conducted on mice exposed to carbofuran showed the results of fewer developing follicles, more atretic follicles, and hemorrhage[44].

The control group did not show Graafian follicles' development; this can be caused because the mice used in this study were mother mice during the lactation period. During lactation, the release of gonadotropin hormones decreases and inhibits estrus during labor because the anterior pituitary secretes the hormone prolactin for breastfeeding[42]. After post-partum estrus, the rats do not re-estrus until the weaning period is complete, called postweaning estrus[45]. The weaning period in mice ranged from 18-21 days[46]. The lactation period will suppress fertility for some time after birth; this causes limited follicular growth because, in this period, there is recovery from the pituitary gonadotropins due to the suppressive effect of increasing steroid hormones during pregnancy. During breastfeeding activity, LH hormone levels' secretion is erratic and lower than that of the standard follicular phase[47,48]. This statement is presumably the reason why this study does not show any development of the Graafian follicle.

Atretic Follicle:

The analysis results with the ANOVA test showed that they were significantly different ($p < 0.05$), so it was continued using the Duncan test. Based on Table 1 show that in group C there were differences in the number of atretic follicles that were significant with P5, P2, and P1. Group P5 did not significantly differ with P4, P3, and P6, but there was a significant difference between C, P2, and P1. Groups P1 and P2 had significant differences with C, P4, P3, P6, and P5.

An atretic follicle is a degenerative process that causes oocyte, not ovulation. It is suspected that the ovarian oocyte will disappear at a particular stage of development. Atretic follicles occur affected by nutrition, impaired ovarian blood supply, pregnancy, and breastfeeding[49]. Atretic follicles occur accompanied by damage to cell membranes, reduced granulosa cells, and pycnosis[50].

The results of this study indicated an increase in the number of atretic follicles in the P1 and P2 groups. This increase occurs because exposure to carbofuran results in apoptosis of granulosa cells caused by oxidative stress, thereby increasing free radicals' formation[8]. Oxidative stress in the ovaries causes folliculogenesis disorders, follicles do not develop and atretic[51]. The decreasing number of ovarian follicles is thought to be due to an increase in atretic follicles so that only a few follicles develop[52]. In addition to an increase in atretic follicles caused by oxidative stress, this increase can result from disorders of the hypothalamus-pituitary-ovarian system with a hormonal imbalance so that the developing follicles were decreased and atretic follicles increased in mice exposed by carbofuran[9]. This is consistent with the statement that ovarian toxicity caused by insecticides can be affected by endocrine disruption and oxidative stress[30]. Other insecticides such as malathion show decreased antioxidant enzyme activity in healthy antral follicles. They exerted an inhibitory effect on SOD and

CAT activity with increased lipid peroxidation leading to atretic follicles[53].

The inhibition of increasing the number of atretic follicles in the P3 and P4 groups was thought to be due to the antioxidant effect of the Kebar grass, which can prevent cell damage in ovarian follicles due to oxidative stress. The combination of the antioxidants present in Kebar grass has a good effect on the number of ovarian follicles. Antioxidant administration is preferably given in combination based on its antioxidant action[7]. The combination of primary antioxidants that act in scavenging/reducing free radicals with phenolic antioxidants that reduce free radicals and as a chelating agent gives maximum results in oxidative stress[22,54]. In the Kebar grass, the primary antioxidants are vitamin A and vitamin E, while those that act as phenolic antioxidants are flavonoids. A study conducted using local rabbits showed that the extract of Kebar grass could stimulate follicles' formation in the ovaries[55,56].

Administration of vitamin C showed inhibition of increasing the number of atretic follicles in the P6 group. In contrast, the P5 group showed an increase in atretic follicles, presumably due to the higher carbofuran exposure dose in this group. Oxidative stress causes follicles that do not develop into atretic follicles[51]. In its single form, the administration of vitamin C causes this antioxidant not to work optimally[22]. Vitamin C in the P6 group showed inhibition in increasing the number of atretic follicles. This dose showed that the antioxidant vitamin C gave a better effect than the P5 group.

This study indicated that giving carbofuran was shown to reduce follicular development significantly ($p<0.05$). The administration of Kebar grass extraction to mice exposed to carbofuran increased the number of primary and secondary follicles at a carbofuran dose of 0.00625 mg. The application of Kebar grass extract gave a better effect than vitamin C. The extract of Kebar grass with a dose of 3.375mg had a more significant effect on exposure to carbofuran with a dose of 0.00625mg compared to a dose of 0.0125mg.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7