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The effect of wungu leaf extract (*Graptophyl lumpictum* (L.) Griff) against vaginal wall thickness and collagen density in the ovariectomized mice (*Mus musculus)*

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ABSTRACT

Menopause is part of a woman's reproductive physiological condition which causes a decrease in the estrogen hormone synthesis and caused decrease in cell proliferation, and collagen synthesis in the vagina. The purpose of this study was to determine the effect of wungu leaf extract with its estrogenic substance on the thickness and the collagen density of mice vaginal wall that has been ovariectomized. In this study, 24 female mice were divided into 6 groups: normal control group (without ovariectomy and given CMC solution), positive control ovariectomy (esthero solution 0.0104 mg/kg BW), negative control ovariectomy (CMC solution 1g/L), and wungu leaves {*Graptophyl lumpictum* (L.) Griff} extract treatment groups (10, 20, and 30 mg/kg BW). The mice were given orally once a day for 40 days. All mice were sacrificed, vaginal organs were taken, then histological preparations were made with paraffin method using *van Gieson* and *Weigert's Iron Hematoxylin* staining. Finally, treatment, the parameter analysis was performed statistically at $\alpha = 0.05$. The results showed that the given of wungu leaves extract could increase the wall thickness and the collagen density of vagina and the optimal dose was 20 mg/kg BW. The conclusion of this study is that wungu leaves extract could prevent vaginal wall damage in pasca ovariectomy or menopause condition.

Key words: *Graptophyl lumpictum, Vaginal wall, Collagen density, Mus musculus*

Introduction

Menopause is part of the physiological condition of female reproduction. Menopause in humans generally occurs between the ages of 45-55 years. About 4-5 years before menopause, the levels of follicle stimulating hormone (FSH) begin to increase slightly, while the production of estrogen, inhibin, and ovarian progesterone decreases (Smart, 2010). Estrogen is a steroid hormone produced by ovarian follicles. Lack of estrogen hormone can cause some physical complaints such as vaginal canal becomes thinner, drier and less elastic, painful intercourse, increased body temperature, decreased cell proliferation, and decreased collagen synthesis (Manuaba, 2009). The decrease of estrogen, among others, causes atrophy of epithelial cells and tissue cells in the vagina and causes thinning of the vaginal wall. Pain copulation (dyspareunia) is one of the complaints that are often found in menopausal

women caused by thinning vaginal wall, dry and less elastic (vaginal dryness) (Al-Baghdadi and Ewies, 2009).

The decrease of estrogen in menopausal conditions also affects the synthesis of collagen by fibroblasts. The decrease will cause an increase in the activity of the matrix metalloproteinase-1 (MMP-1) enzyme which is a collagen degrading enzyme. The increase of MMP-1 activity will increase collagen degradation, thus resulting in a decrease in collagen density (Rhein and Santiago, 2010).

One of the way to improve the quality of cells making up the vaginal wall by using HRT (Hormone Replacement Theraphy). HRT is done to overcome the lack of the hormone estrogen. HRT generally uses synthetic estrogens as contained in several drugs including ovolumun and esthero, but the use of synthetic estrogens such as estrogen in contraceptive drugs, has a negative effect that is increasing the risk of breast and uterine cancer in long-term use (Nani, 2009). As a substitute, estrogen is sought from herbal sources, namely phytoestrogens, because phytoestrogens do not affect the increased incidence of breast and uterine cancer (Raden, 2011). Natural phytoestrogens have a lower potential than synthetic estrogens but have fewer and safer side effects (Huang *et al.,* 2013).

One of the plants that can be used as traditional medicine that contains phytoestrogens is wungu leaf (*Graptophyl lumpictum* (L.) Griff). Wungu (*Graptophyl lumpictum* (L.) Griff) leaf plants contain phytostrogens such as flavonoids and phytosterols. Phytoestrogen effects from wungu leaves have been shown to increase the proliferation of cells in the endometrium and myometrium from ovariectomy mice (Suhargo, 2005). The extract of wungu leaf can become phytoestrogens because the content of flavonoids can bind to estrogen receptors in the body and the content of phytosterols can be used as a precursor to almost all steroid hormones including sex hormones namely estrogen. Flavonoids contained in the leaves of wungu are kaempferol. Kaempferol has a role to induce phosphorylation of the estrogen receptor α (ER-α) and activation of the estrogen receptor. Kaempferol will trigger osteoblast differentiation and mineralization through ER signaling induced by ER-α phosphorylation (Guo *et al.,* 2012).

The utilization of wungu leaves as a treatment for menopause has not been much studied. Therefore, researchers want to find out how much influence the leaves of wungu on wall thickness and collagen density in the vagina of bilateral ovariectomy mice. The Bilateral ovariectomy purposes to test the presence of estrogenic activity and also substances that are antiestrogenic from a particular substance.

Materials and Methods

Materials

The Materials used for this study include: Wungu leaves (*Graptophyl lumpictum* (L.) Griff) , grown in the Faculty of Science and Technology and have been identified in the Purwodadi Botanical Garden, 70% ethanol, carboxymethylcellulose (CMC), esthero (conjugated estrogen) 0.625 mg/L tablet, distilled water, betadine, entellan, ketamine solution, intestinal paint yarn, silk thread, cotton bud, 0.9% NaCl solution, cotton, tissue paper, van Gieson staining (picric acid & fuchsin acid) and Weigert's Iron Hematoxylin , neutral buffered formalin (NBF) 10%, rat feed, graded alcohol (50% alcohol; 60% alcohol; 70% alcohol; 80% alcohol: 96% alcohol; absolute alcohol), pure xylol, paraffin, Mayer albumin (chicken eggs white: glycerin 1: 1), xylol: paraffin (1: 1). The tools used for this study include: 40 x 30 x 15 cm plastic tubs, tub lids made of wire mesh, rat feed trays, rat drink bottles, chemical cups, measuring cups, blenders, surgical sets, disposable syringes 1 mL, micrometers, scales, rotary evaporators, glass objects, glass lids, sliding boxes, closed containers. Microscope, ImageJ version 1.52a computer program, microtome, paraffin block, cutter, bunsen, oven, brush, water bath, paraffin mold, staining container, network cassette, objective micrometer, Optilab camera microscope.

The Making of Wungu Leaves Extract

The process of making the ethanol extract of the wungu leaves is done by means of fresh wungu leaves prepared and weighed, the wungu leaves are put into an oven at 40 °C, the leaves are blended with a blender and leaf powder is obtained, the leaf powder is soaked with 70% ethanol for one night, separation of ethanol fraction from the powder by filtering, ethanol evaporation was carried out with a rotary evaporator for 2 days, the results obtained were thick leaves extract of gel in the form of gel (Suhargo *et al.,* 2003).

The ethanol extract of Wungu's leaves was made by mixing CMC. CMC is used as a solvent because it can homogenize ethanol extracts from Wungu

leaves. Making CMC solution is done by weighing 1 g of CMC powder and dissolving it with 1 L of distilled water, so that the dose obtained is 1 g/L of CMC solution. Wungu leaf extract solution in this study was made with a concentration of 10 mg/kgBW, 20 mg/kgBW, 30 mg/kgBW dissolved with 1 g/L CMC solution.

The use of Wungu's leaf ethanol extract concentration is based on its use in humans. In humans, 7 wet leaves are usually used or equivalent to 10 g for 1-time use (Wijayakusuma *et al.,* 1998). From the 10 g, 0.04 g of extract was obtained, so the concentration given to mice required 0.0026 x 0.04 = 0.000104 g or 0.1 mg for one use. Mice have an endurance of up to 9x more resistant than humans (Ghosh, 1971), so to find out anti-estrogenic test, the dose used is 10 mg/kgBW, 20 mg/kgBW, 30 mg/kgBW in 1 g/L CMC solution.

The Making of Esthero Solution

Esthero is a tablet drug with synthetic estrogen that is consumed by humans. Each esthero tablet contains 0.625 mg of synthetic estrogen. Use in mice is used at 0.0026 x 0.625 mg = 0.001625 mg = 0.001 mg and dissolved in 0.1 mL of CMC solution at a dose of 0.0104 mg/kgBW.

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The Preparation of Animals Experimental

The experimental animals used in this study were female mice (*Mus musculus*) from strains of BALB/C which were 2 months old and weighed around 20-30 g, totaling 24 animals. Mice are used as experimental animals, because starting from their genetic, biological and behavioral characteristics, they are very similar to humans. Many symptomatic conditions in humans can be replicated in mice, and the price of mice is relatively cheap, and can be bought in large quantities. Mice were placed in plastic cages (40 x 30 x 18 cm) covered with gauze, the condition of the room/animal enclosure was ventilated with a 12-hour bright and 12-hour dark lighting system then acclimated for two weeks. The ethics test was conducted at the Faculty of Dentistry, Airlangga University, Surabaya, Indonesia.

Experimental Design

Mice (*Mus musculus*) acclimatized for 14 days, after which the weighing process of mice was carried out. Then 24 mice were ovariectomized and weighed their body weight. Mice were rested for 14 days for the healing process of ovariectomy scars. After 2 weeks of rest, mice were randomly divided into 6 groups (K1: normal control without ovariectomy only given 0.1 mL CMC solution at a dose of 1 g/L; K2: positive control of ovariectomy given 0.1 mL esthero solution at dose 0, 0104 mg/kgBW; K3: negative control of ovariectomy given 0.1 mL of CMC solution at a dose of 1 g/L; P1, P2, and P3 were given a larvae of ethanol extract of Wungu leaves with a dose of 10, 20 and 30 mg/respectively kgBB) orally for 40 days. After 40 days, weighing the mice was carried out, then the mice were surgically removed to remove the vaginal organs. The vaginal organs are made into histological preparations by the paraffin methods.

The Measurement of Mice Vaginal Wall Thickness

The measurement of vaginal wall thickness in various groups is done by measuring the height of the vaginal wall starting from the highest epithelium stratificatum squamous over until the layer of tunica adventisia. In measuring the thickness of a mouse's vaginal wall, the ImageJ 1.52a computer program will be used. The histology of vagina was observed with an Olympus CX41 microscope, and be photographed with the Optilab Pro camera at 100x magnification in four different areas. To measure the thickness of the mice's vaginal wall an objective micrometer photo was taken at 100 times magnification for calibration on the ImageJ computer program version 1.52a. Measurement of the thickness of the vaginal wall of mice was repeated 4 times and carried out in different measurement areas by shifting the preparations to the right and left sides. The unit used in measuring the thickness of a mouse's vaginal wall is a micrometer (μm).

The Measurement of Mice Vaginal Collagen Density

The Collagen Density Observation is done by observing the percent of the area that displays collagen in the preparations, then done by digital analysis methods. Histology of mice vaginal was observed by enlarging 400 times using an Olympus CX41

microscope, and photographed with an Optilab Pro camera. After being photographed, then analyzed with ImageJ software. Part of the vaginal wall is observed is the area of lamina propria where at 40x magnification, lamina propria is divided into 4 regions. Then each area was enlarged at 100x magnification and divided into 4 regions by sliding the microscope on the left and right sides, then each area was enlarged at 400x magnification to observe the collagen density. So that there will be 16 regions that will be analyzed the density of collagen in each treatment group. The density of collagen is calculated as the percentage of pixel area of collagen that is red compared to the pixel area of the entire area.

Results

The Thickness of The Vaginal Wall of Mice

The results of measurement of the thickness of the vaginal wall of mice in various treatment groups can be seen in Figure 1.

The thickness of the vaginal wall in the K1 group (488.90 ± 30.873 µm) showed no significant difference with K2 and P2, but significantly different from the K3, P1 and P3 groups. In the K2 group (501.60 ± 27,653 µm) showed no significant difference with K1 and P2, but significantly different from the K3, P1, and P3 groups. In the K3 group (455,45 ± 13,193 µm) showed significant differences with groups K1, K2, P1, P2, and P3. The results of the wall thickness in the P1 group (402.23 ± 11.575 µm) showed significantly different from K1, K2, K3, P2, and P3. The results of the wall thickness in the P2 group (506.67 ± 21.487 µm) showed no significant difference from the normal group by administering a non-ovariectomy (K1) CMC solution, an ovariectomy positive control group giving esthero larvae (K2), but significantly different from the negative ovariectomy control group. administration of CMC (K3), treatment group 1 (P1) and treatment group 3 (P3). The results of the wall thickness in the P3 group (566.70 ± 16,922 µm) showed significantly different from K1, K2, K3, P1, and P2 (figure 2).

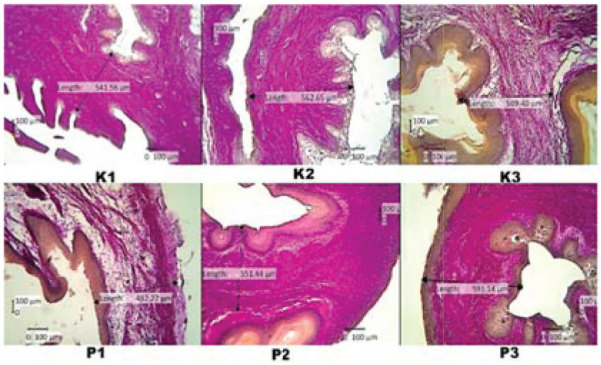

Fig. 1. Histology of the thickness of the vaginal wall of mice in various treatment groups. K1: normal control group by giving CMC solution. (non-ovariectomy). K2: ovariectomy control group by administering esthero solution. K3: ovariectomy control group by giving CMC solution. P1: Ovariectomy treatment group with the administration of a leaf extract with a dose of 10 mg/kg BW. P2: Ovariectomy treatment group by administering Wungu leaf extract with a dose of 20 mg/kg body weight. P3 ovariectomy treatment group with the administration of a leaf extract extract of 30 mg/kg BW. Lu = Lumen, SqEp = Stratified squamous Epthitelium, VM = Vaginal Mucosa, LP = Lamina Propria, M = Muskularis, A = Adventist Staining van Gieson; 100x magnification).

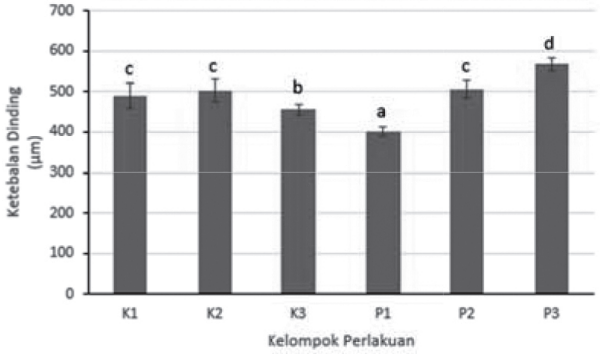

Fig. 3. Mice vaginal wall thickness diagram (µm) in all groups. Different letters indicate there are significant differences based on Duncan's test. K1: normal control group by giving CMC solution. (non-ovariectomy). K2: ovariectomy control group by administering esthero solution. K3: ovariectomy control group by giving CMC solution. P1: Ovariectomy treatment group with the administration of a leaf extract with a dose of 10 mg/kg BW. P2: Ovariectomy treatment group by administering Wungu leaf extract with a dose of 20 mg/kg body weight. P3 ovariectomy treatment group with the administration of a leaf extract of 30 mg/kg BW.

Collagen Density of Mice Vaginal

The results of collagen density of the vaginal wall of mice in various treatment groups can be seen in Figure 3.

The collagen density of the vaginal wall in the K1 group (80.35 ± 3.684 present pixel area) showed no significant difference with K2, K3, P1 and P2, but

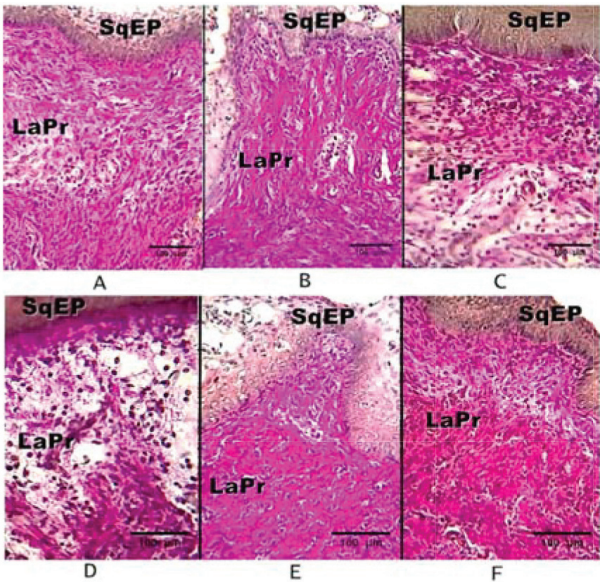

Fig. 2. Histology of lamina propria on the vaginal wall of mice in various groups. A = normal group with non-ovariectomy CMC solution (K1), B = ovariectomy control group giving esthero larvae (K2), C = ovariectomy control group giving CMC solution (K3), D = treatment group 1 (P1), E = group treatment 2 (P2), and F = treatment group 3 (P3). SqEp = Stratified squamous epthitelium, LaPr = Lamina propria (400x magnification)

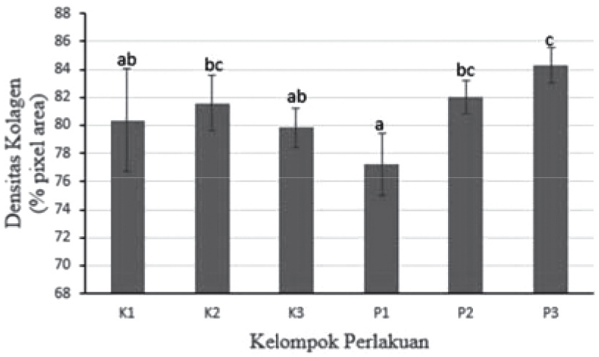

Fig. 4. Collagen density diagram on the vaginal wall of mice (% pixel area) in all groups. Different letters indicate there are significant differences based on Duncan's test. K1: normal control group by giving CMC solution. (non- ovariectomy). K2: ovariectomy control group by administering esthero solution. K3: ovariectomy control group by giving CMC solution. P1: Ovariectomy treatment group with the administration of a leaf extract with a dose of 10 mg/kg BW. P2: Ovariectomy treatment group by administering Wungu leaf extract with a dose of 20 mg/kg body weight. P3 ovariectomy treatment group with the administration of a leaf extract extract of 30 mg/kg BW

significantly different from the P3 groups. In the K2 group (81.57 ± 2.002 present pixel area) showed no significant difference with K1, K3 and P2, P3 but significantly different from the P1. In the K3 group (79.80 ± 1.452 present pixel area) showed significant differences with groups K1, K2, P1, P2, but significantly different from the P3 group. The results of collagen density in the P1 group (77.21 ± 2.203 percent pixel area) showed no significant difference with K1 and K3, but showed significant differences with groups K2, P2 and P3. The results of collagen density in the P2 group (82 ± 1.166 percent pixel area) showed no significant difference with K1, K2, K3, and P3 but significantly different from the P1 group. The results of collagen density in the P3 group (84.25 ± 1.265 percent pixel area) showed significantly different from K1, K3, and P1 (Fig. 4).

Discussion

Menopause is a progressive decline in ovarian function associated with specific changes in organs and tissues including atrophy of the labia, vagina and uterus. In addition, due to reduced estrogen causes complaints about disruption of the vaginal epithelium, supporting tissue, and elasticity of the vaginal wall. Vaginal epithelium contains many estrogen receptors which are very helpful in reducing the pain caused especially during menopause.

The hormone estrogen has a big influence in optimizing the function of the reproductive organs. Estrogen receptors in the body consist of two receptors namely RE α which are spread in female reproductive tissue and RE β found in bone, kidney, endothelial cells and blood vessels (Bustamam, 2008).

The mechanism of estrogen is directly bound to the estrogen receptor (RE α) contained in the target cell (cells in the vagina) to influence cell proliferation activity, thereby changing the estrogen receptor conformation. Changes in conformation cause the bonds between estrogen and estrogen receptors to become active. So that it binds to the site binding on the side of the DNA chain acceptor the interaction between the DNA acceptor causes gene expression to increase. Gene expression is catalyzed by the RNA polymerase enzyme which causes an increase in mRNA (Cunningham *et al.*, 2010). Equations should be centered and should be numbered with

the number on the right-hand side.

In the results of research on the thickness of the vaginal wall of mice it is known that the normal non-ovariectomy (K1) group is significantly different from K3, P1, and P3, but not significantly different from K2 and P2. In normal conditions, the ovary will produce the hormone estrogen normally, where the estrogen hormone functions for the proliferation of cells in the vagina. Estrogen binds to alpha receptors that are in the vaginal epithelium. Then the receptor activates genes in the ribosome for activation of protein synthesis and then from protein synthesis the collagenase enzyme will be produced for cell proliferation.

The measurement results of the wall thickness of the ovariectomy group giving synthetic estrogen (esthero solution) K2 showed almost the same results as the K1 group. This is because the administration of esthero causes an increase in the hormone estrogen, so that the hormone estrogen that enters the target cell will bind to the estrogen receptor (RE) in the nucleus and cause the estrogen receptor to become active.

In the ovariectomy (K3) control group, Carboxymethylcelluloce (CMC) was administered which served as a stabilizer, thickener, and emulsifier (Lestari *et al.*, 2013). The K3 group tends to gain weight due to increased fat deposits in fat tissue (Nurdin, 2002), where the administration of CMC in the ovariectomy (K3) control group causes the fat in the body to be emulsified so that the fat is easily degraded by lipases to fatty acids and facilitates fat absorption by the blood to be circulated to and fill the fat tissue. so the results of measurement of wall thickness are still lower than group K1. In this fat tissue estrogen synthesis also occurs, so the estrogen can bind to the alpha estrogen receptor (RE α), but the type of estrogen produced is a type of weak estrogen (estron). When compared with the normal non-ovariectomy (K1) group, the administration of CMC in the K3 group still could not provide a significant improvement in the vaginal wall thickness of the ovariectomy mice.

In the measurement results of the thickness of the treatment group 1 (P1) who were given a leaf extract with a dose of 10 mg/kg BW showed a decrease in wall thickness, this can be caused because the leaves have a weak estrogenic effect, where by giving too low a dose will cause adverse conditions.

The results of the measurement of the wall thickness of the treatment group 2 (P2) who were given a leaf extract with a dose of 20 mg/kg BW gave an improvement effect on the vaginal wall thickness of the ovariectomy mice. The content of flavonoids in the leaves extract at the right dose can interact with receptors to produce weak estrogenic or antiestrogenic effects because flavonoids have phenolic rings as a prerequisite for binding estrogen receptors, so that by attaching flavonoids to alpha estrogen receptors (RE α) will be able to help repair and repair increased cell proliferation in the vaginal wall.

In the measurement results of the wall thickness of the treatment group 3 (P3) who were given a leaf extract with a dose of 30 mg/kg BW showed an increase in wall thickness exceeding the normal group K1, this meant that giving a leaf extract with a dose that was too high could result in an adverse effect disadvantage like tumorigenesis. When compared with the K2 and K3 groups, the results of the measurement of the wall thickness of the P3 group are still higher than the K2 and K3 groups, but the dose given in the P3 group must also be considered because there are results that exceed the normal level which can trigger tumorigenesis.

Based on research data on collagen density, the normal non-ovariectomy (K1) group was significantly different from the treatment group 3 (P3). This is because the ovariectomy condition occurs a decrease in the hormone estrogen in the body which will trigger a decrease in the activity of fibroblast cell proliferation which will cause an increase in collagen degradation. Estrogen can affect collagen metabolism; estrogen deficiency can stimulate collagen degradation by increasing MMP-2 activity and causing a decrease in TIMP.

Giving synthetic estrogen (esthero) in the ovariectomy (K2) control group with the treatment group (P2) with a dose of 20 mg/kg BW did not show a significant difference, but significantly different from the treatment group 1 (P1) and treatment group 3 (P3) given a dose of 10 mg/kg body weight and 30 mg/kg body weight. This condition shows that the administration of synthetic estrogen (K2) and phytoestrogens from wungu leaf extract (P2) can reduce the expression of metalloproteinase matrix on extracellular matrix that is outside the fibroblast cells, so estrogen from esthero causes an increase in tissue inhibitor of metalloproteinase (TIMP) which can inhibit matrix metalloproteinase (MMP) activity in triggering collagen degradation. When compared with the normal K1 group, the

yield of collagen density in the K2 group was slightly higher, but still higher than the K3 group.

In the ovariectomy (K3) control group, Carboxymethylcelluloce (CMC) was administered. Giving CMC causes an increase in fat deposits in fat tissue, fat in the body is emulsified so that the fat is easily degraded by lipases to fatty acids and facilitates the absorption of fat by the blood that will be circulated to and fill the fat tissue. In this fat tissue estrogen synthesis also occurs, so the estrogen can bind to the alpha estrogen receptor (RE α), but the type of estrogen produced is a type of weak estrogen (estron). When compared to the normal non-ovariectomy (K1) group, administration of CMC in the K3 group can inhibit MMP activity and can trigger collagen synthesis and can improve collagen density.

In treatment group 1 (P1) there was an excess of estrogen deficiency resulting in a decrease in TGF-â secretion resulting in a decrease in TIMP and an increase in MMP-2 activity in the wall beyond normal conditions and would stimulate collagen degradation beyond normal conditions. The application of wungu leaf extract with a dose that is too low will result in a decrease in collagen density, whereas when compared with the K2 and K3 groups, the results of the P1 group wall thickness measurements are still below K2 and K3 which can further improve the collagen density of lamina propria in the vaginal walls of mice that are ovariectomy.

The results of the measurement of collagen density in treatment group 2 (P2) which were given wungu leaf extract at a dose of 20 mg/kg BW gave an improved effect on the density of collagen. The results of collagen density measurements in the P2 treatment group were not significantly different from the normal K1 group, K2 group and K3 group. The content of flavonoids in the leaves extract can increase TIMP activity and inhibit MMP activity, so that collagen degradation can be inhibited and collagen synthesis can be increased.

In the study results of the treatment group (P3) an increase in collagen density in the vaginal wall that exceeds the normal group. This causes a dose of 30 mg/kg body weight in treatment group 3 (P3) to be able to work to reduce the decrease in collagen density higher than synthetic estrogen (K2), because at this dose the leaves of the wungu have the maximum ability to increase TIMP activity thereby inhibiting MMP activity -2 and inhibits decreased collagen synthesis by fibroblasts, however collagen densities that are more than this normal group can cause adverse effects such as tumorigenesis

Based on research that has been carried out the risk of decreased wall thickness and collagen density due to artificial menopause (ovariectomy) can be minimized with hormone replacement therapy (HRT), among others, with synthetic estrogens (esthero) in the control group K2 ovariectomy, and also with flavonoids from the extracts of the wungu leaf on treatment group 2 (P2). Drug response in each individual is different, because the response is strongly influenced by the dose given and the metabolic rate and excretion of an individual. Wungu leaves extract must be given appropriately, because if given a dose that is too low will result in increased degradation of collagen that is more than the normal group, whereas if the dose used is too high, can trigger tumorigenesis.

Conclusion

Based on the results of the above study, it can be concluded that the administration of the extract of the leaf wungu (*Graptophyl lumpictum* (L.) Griff) has the effect of improving the thickness of the vaginal wall and the density of vaginal collagen lamina propria in the ovariectomized mice. The optimum dose of leaf extract (*Graptophyl lumpictum* (L.) Griff) is 20 mg/kg BW.

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