

The Effect of Bitter Extracts (*Andrographis Paniculata* Nees.) of Ovarium Histologys (*Mus Musculus* L.)

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Abstract - Bitter is widely used in the treatment of various diseases such as fever, gastric infections, respiratory infections, malarial fever, insect repellent, diabetic complications, protects against liver diseases, antiviral, immunostimulators and suppresses retenosis in angiosplastic patients. However, its effect on the reproductive system, especially on the female reproductive system is controversial. The asrographolide isolated from bitter (*A. paniculata*) has anticancer activity through the mechanism of apoptosis against cancer cells HeLa. Ovarium is a very important reproductive organs in the animal reproductive system. as a producer of ova, estrogen and progesterone.

The research was conducted at Zoology Laboratory and Animal Home Laboratory of FMIPA UNP. The design used was Completely Randomized Design with 4 treatments and 6 repetitions. The treatment used was control, given with bitoto extract of 0.2 g / BB / head / day, 0.4 g / BB / head / day, and 0.6 g / BB / head / day. The treatment was carried out for 12 days referring to the cogenesis cycle in the mouse, then made histology of each treatment and observations included measurement of ovarian weight and size, number of primary follicles, secondary follicles, tertiary follicles, De Graaf follicles, corpus luteum and abnormalities damage to the ovarian histology of mice. The data were analyzed using the Analysis of Variable Fingerprint (ANOVA), if significantly different then it will be continued with BNT advanced test with 5% level.

The results of this study showed that, giving oral administration of oral extract for 12 days did not affect the histology of the ovaries of mice statistically, but tended to have an effect on increasing the number of tertiary follicles in the ovaries of mice.

Keywords - *Andrographis paniculata* Ness; *Andrografolide*; *Mus musculus* L.

I. INTRODUCTION

Bitter plants (*A. paniculata*) consist of leaves, stems, flowers and dried fruit. The leaves are green, odorless, taste very bitter. Crossed leaves opposite, generally apart from the stem, the shape of the lanceol to the shape of tongue spear, fragile, thin, hairless, the base of pointed leaves, pointed tip, flat leaf edge. The upper surface is dark green or brownish green, the bottom surface is pale green. Short leaf stalk, jorong-shaped fruit, base and sharp edges. The hairless trunk with 2-6 mm thick, rectangular. The upper trunk is often with a slightly ribbed angle. The outer surface of the skin is dark green to brownish green. The inner surface is white or white in gray. Seed is rather hard, the outer surface is light brown with bulge [23].



Pict 1. Bitter (*Andrographis paniculata* Nees.)
(Private collection)

All parts of plants bitter (*A. paniculata*) consisting of leaves, stems, roots, and flowers can be used as an anticancer drug. However, the leaves and stems of bitter

(*A. paniculata*) are the most widely used parts. This plant tastes bitter and cold. Currently part of the plant is already widely processed and made in the form of tablets and capsules so that its utilization is more practical [16]. Andrografolide is the main ingredient of this herb. Andrographolide has a water-soluble hardness, it is very bitter and rectangular shaped [24]. Andrografolide is the main active component in the herb bitter (*A. paniculata*). This active component has several pharmacological activities such as immunosuppressant, antithrombotic, antiviral, antioxidant and anti inflammatory. Andrographolide as an anti-inflammatory and antioxidant works against various body cells with specific mechanisms [2].

The use of high doses can cause unpleasant stomach, vomiting, nausea and loss of appetite due to the bitter taste of andrographolide, whereas in women it can cause antifertility effects. Antifertility is a term used for compounds or substances that can interfere with the reproductive system. Antifertility compounds are compounds that have the ability to prevent fertility by disrupting some of the normal reproductive mechanisms, both male and female [5]. In study showed that bitter extract from bitter (*A. Paniculata*) was found to decrease spermatozoa in mice during ejaculation and decrease normal sperm count from mice so that it can be used as antifertility (*Mus musculus* L.) [28].

One of the most important reproductive organs is the ovary. The ovaries are a pair of glands consisting of the right ovary located behind the right kidney and left ovary located behind the left kidney. The distance between the ovaries and the kidneys varies by species [9].

Ovaries have 3 functions, namely: ovum production, estrogen production and progesterone production [21]. The development of ovarian follicles is influenced by the hormone estrogen. Estrogen is primarily produced by granulosa cells that convert androgens produced by the cells into the estrogen interna estrogen. Growth and ripening of ovarian follicles and estrogen secretion are controlled by the pituitary gonadotropin hormones FSH and LH. The secretion of estrogen by the ovaries triggers the release of LH for ovulation in the estrous time. LH stimulates the formation of the corpus luteum. While estrogen affects the secretion of pituitary gonadotropin hormone through the feedback effect on the hypothalamus [17].

II. REVIEW OF LITERATURE

Some tests of the efficacy of bitter (*A. paniculata*) against diseases such as fever, gastric infections, respiratory infections, malaria fever, insect repellent, diabetic complications, protects against liver, antiviral, immunostimulatory and suppresses retenosis in angiosplastic patients [23]. This plant contains many active chemical constituents that exhibit definite pharmacological effects. It can be easily predicted from the article's findings of *Andrographis paniculata*, has anti-inflammatory, antidiabetic, anticancer, antimalarial, antiangiogenic, anti venom and antimicrobial activity. So this plant has important medicinal value in traditional therapy [6]. This plant is also very useful in curing various human health diseases especially in diseases by viruses such as respiratory problems, HIV, liver damage etc., phytochemical tests reveal the presence of glycosides, saponins, tannins and alkaloids, but not from anthraquinone. Overdose of this plant leads to some side effects such as nausea, vomiting and loss of appetite. Therefore, researchers must perform various formulations for bitter (*A. paniculata*) and develop new drug molecules. This plant is considered a very important, safe and medicinal plant for mankind [7].

The main compounds contained in the plant bitter (*A. paniculata*) are andrografolide, neoandrografolide, didehydroandrografolide and 14-deo-xyandrographolide [3]. Andrographolide can be easily dissolved in methanol, ethanol, pyridine, acetic acid and acetone, and slightly soluble in ether and water. The extraction of andrografolide which has been widely applied is extraction using alcohol. The process leads to degradation of andrografolide [22].

Andrografolide isolated from bitter (*A. paniculata*) has anticancer activity through the mechanism of apoptosis against HeLa cancer cells. It can also induce cell apoptosis of TD-47 cell strains from human breast cancer, which is affected by concentration and exposure time with indicators of increased expression of p53, bax, caspase-3 and decreased bcl-2 expression. The aquades extract from bitter (*A. paniculata*) administered on cell cultures of mammary adenocarcinoma from C3H mice showed an increase in apoptosis at a concentration of 1 mg / L. An in vitro study of PC-3 prostate cancer cell line cells administered with bitter extract (*A. paniculata*) showed a tendency to decrease VEGF levels according to dose increase. Bitter extract (*A. paniculata*) and andrografolide may also inhibit the specific angiogenesis

of tumors in B16F-10 melanoma either in vitro or in vivo [19].

Based on research proved bitter (*A. paniculata*) can increase the number of white blood leukocytes of male rats (*Rattus norvegicus*) exposed to benzene with the dose of the best bitter (*A. paniculata*) filtrate is 0.45 ml dose [12]. The results of the study showed that the disruption of the spermatogenesis stage in the seminiferous tubules, namely degradation and the spread of spermatogenic cells (spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, spermatozoa), sertoli cell destruction, lysis of basal lamina, interstitial tissue, and Leydig cells still seem intact. The mechanism of action of ethanol extract of the herb bitter (*A. paniculata*) is probably cytotoxic and antimitotic. Thin Layer Chromatography (ECT) of ethanolic extract of the herb bitter (*A. paniculata*) showed the presence of at least four patches of terpenoid compounds suspected to be related to andrographolide [11].

III. METHODOLOGY

The study was conducted from October to December 2017 at the Zoology Laboratory and animal Home of Biology Department of FMIPA UNP. The type of this research is experiment with Randomized Complete Random Design (RAL) design with 4 treatments and 6 replications.

A. Tools and Materials

The tools used in this study include animal cages, digital analytical scales, blenders, porcelain spoons, a set of surgical instruments, cuvac (syringe), watch glass, vial bottles, glass objects, cover glass, erlenmeyer, dropper drops, measuring flask, stirrer, light microscope, incubator, refrigerator, stopwatch, paraffin block paper, micro pipette, micro tube, petri dish, measuring cup, waterbath, desiccator, hotplate, microtom, gavage needle, cotton bud, ruler and kater.

The materials used in this study were intermittent (*Mus musculus* L.), dry bitter (*A. paniculata*), serial alcohol (95%, 90%, 80%, 70%), 0.9% NaCl, absolute alcohol, liquid paraffin, aquabides, PBS (*Phosphate Buffered Saline*), CMC1% (tap water, mineral water, methanol, hematoxylin solution, eosin solution, polish and gloves.

B. Making Bitter extract

Bitter (*A. paniculata*) which has been dried in the branched branches and leaves, then mashed with a blender so that the form of dry powder. For preparation of

the extract of bitter (*A. paniculata*) is made by taking 100 grams of dried dried powder (*A. paniculata*) then immersed in 200 ml of methanol and let stand for 48 hours. Further filtered by using filter paper so that filtrate (sari) obtained. The filtrate is evaporated in the waterbath at 50 ° C until the solvent has not dripped to produce a concentrated extract. The extract of bitter (*A. paniculata*) which can be a solid plate is stored in the desiccator.

To dissolve the extract 1% CMC solution was used. At the time of first use weighed with a digital analytical scale in accordance with the treatment dose then dissolved with aquabides that have been prepared and stirred until homogeneous.

C. Test Animal Preparation

Animals used were mice (*Mus musculus* L.) female with adult age 11-12 weeks obtained from breeder of mice (*Mus musculus* L.) in Andaleh area of Padang City. Mice (*Mus musculus* L.) were placed in a rectangular plastic enclosure of 30 cm (p) x 20 cm (l) x 10 cm (t) covered with a wire and given a wooden residue base replaced twice a week. Feed and drinking water are given on ad libitum.

D. Making Vaginal Animal Smears

The method used in the manufacture of vaginal smears is the topical method. Cotton bud was immersed in 0.9% NaCl, then the tip was inserted into the vaginal opening of the mouse and rotated slowly. The tip of the cotton bud is applied to the glass object that has been dropped 0.9% NaCl solution then made a thin smear evenly. The preparations were fixed with 70% alcohol for 5 minutes, stained with Giemsa and left for two minutes. The preparation is then washed with aquades and allowed to dry. Preparations are observed under a microscope with magnification of 10 × 10.

E. Giving of bitter extract (*A. paniculata*)

The administration of the extract of bitter (*A. paniculata*) was done orally using the tuberculin syringe and the gavage needle of the test animal according to the prescribed treatment that is 0.2 gr, 0.4 gr, and 0.6 gr, performed daily for 12 days referring to the old cycle of oogenesis in mice (*Mus musculus* L.).

F. Mice Surgery (*Mus musculus* L.)

Mice (*Mus musculus* L.) first dislocated part of the neck, then dissected and taken the organ ovarium for histology then made. Ovarian weight measurements were performed by weighing fresh ovarian organs using

analytic scales, while the size of the ovaries was measured using a measuring instrument (millimeter paper). The calculation of ovarium mice follicles and the corpus luteum was performed by using shooting of the BX 51 olympus light microscope at 40x (objective 40x) and 400x (objective 40x) magnification. While the count is done manually.

G. Data Analysis

Quantitative data were analyzed by using variance analysis (ANOVA) one lane with 5% significant level to know the real difference between treatments. If from the analysis of the variance obtained $F_{count} > F_{table}$, to know the location of the difference followed by DNMRT (*Duncan New Multiple Range Test*). To observe the damage and abnormalities in the histology of the mice ovaries (*Mus musculus* L.) is done descriptively qualitatively.

IV. RESULTS AND DISCUSSION

A. Research Results

After doing research with giving of bitter extract (*A. paniculata*) to female mice (*Mus musculus* L.) for 12 days with dose 0,2 gr / Kg BB, 0,4 gr / Kg BB and 0,6 gr / Kg BB not found a significant difference to ovarian weight and ovary length of mouse (*Mus musculus* L.). For comparison of weight and length of ovary each treatment can be seen in following table.

Tabel 1. The length and weight of the ovary of mice (*Mus musculus* L.) after being given the extract of bitter (*A. paniculata*) for 12 days

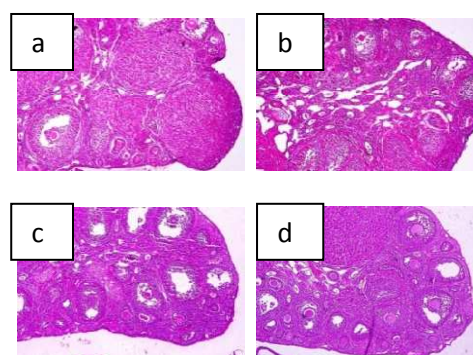
Treatment	Ovary Length (mm)	Ovary Weight (gr)
Control	3,416±0,49	0,015±0,005
0,2 gr/BB	3,683±0,22	0,012±0,002
0,4 gr/BB	3,500±0,67	0,016±0,003
0,6 gr/BB	3,516±0,43	0,017±0,003

Based on the data above can be seen that the average weight of the largest ovarium is the treatment of 0.6 gr and average length of the highest ovarium is the treatment of 0.2 gr is equal to 3.683 mm.

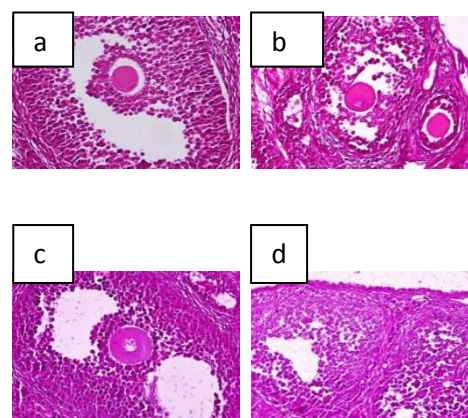
This bitoto extract (*A. paniculata*) also has no significant effect on the number of primary follicles, secondary follicles, tertiary follicles and the corpus luteum of the mice ovary (*Mus musculus* L.). For comparison of number of dam and corpus luteum follicles on each treatment can be seen in the following table.

Tabel 2. Number of ovarian follicles and corpus luteum of mice (*Mus musculus* L.) after being administered extract of bitter (*A. paniculata*) for 12 days

Treatm ent	Primary Follicles	Seconda ry Folikel	Tertiary follicles	The Luteum Corps
Contro l	11,167±3,92	6,334±2,33	11,834±2,71	4±3,03
0,2 gr/BB	9,5±4,84	8,167±1,60	13,667±5,20	3,5±1,51
0,4 gr/BB	13±5,36	7,167±3,76	16,5±4,80	1,667±0,81
0,6 gr/BB	12,667±3,50	9±6	18,334±5,08	2,5±1,37



Pict 2. Mice ovarian histology (*Mus musculus* L.). Control (a), treatment with *Andrographis Paniculata* extract 0.2 g / KgBB / day (b), 0.4 g / KgBB / day (c), 0.6 g / KgBB / day (d).



Pict 3. Mice ovarian histology (*Mus musculus* L.) shows the morphology of tertiary follicles. Control (a), treatment with *Andrographis paniculata* extract 0.2 g / KgBB / day (b), 0.4 g / KgBB / day (c), 0.6 g / KgBB / day (d).

B. Discussion

The weight and length of the ovary are not significantly affected after the treatment of the extract of bitter (*A. paniculata*), illustrates that the extract of bitter (*A. paniculata*) does not significantly affect the size and weight of the ovaries. Kolibianakis et al. (2005) in his study said changes in ovarian development can be influenced by the amount of circulating hormones such as luteinizing hormone (LH), follicle stimulating hormone (FSH), and growth hormone (GH). In addition, the development of this ovary is also influenced intake of nutrients and individual physical conditions. The size and weight of the ovaries that are not significantly affected are likely to occur because the amount of these hormones that are affected by the extract of bitter (*A. paniculata*) is not enough to affect the development of the ovaries.

Although the statistical test showed no significant difference ($p > 0,05$), but from the calculation result, there was a tendency that the length of the ovary with the treatment dose of 0.2 g / Kg BW was greater than the control treatment, the treatment dose was 0.4 gr / Kg BB and treatment dose 0,6 gr / Kg BB. However, in the calculation of ovarian weight from the beginning of the control treatment decreased the weight at the treatment dose of 0.2 gr / kg body weight and again experienced an average weight increase at the dose of treatment of 0.4 gr / kg and dose of 0.6 gr / kg body weight.

The absence of significant differences from ANOVA results after the experiments was most likely due to changes in estrogen levels in the blood causing the secretion of FSH by the hypothalamus to continue so that no effect on the development of follicles in the ovaries. The development of ovarian follicles is influenced by the hormone estrogen. Estrogen is mainly produced by granulosa cells that convert androgens produced by the cells of the internal tract to estrogen. The presence of correlation between the number of follicles with estrogen concentrations was found to be a positive correlation that the higher the number of follicles the higher the estrogen concentration [17].

Growth and ripening of ovarian follicles and estrogen secretion are controlled by the pituitary gonadotropin hormones FSH and LH. The secretion of estrogen by the ovaries triggers the release of LH for ovulation in the estrous time. LH stimulates the formation of the corpus luteum. While estrogen affects the secretion of pituitary gonadotropin hormone through feedback effects on the hypothalamus [17]. Cooperation of these hormones can

sustain egg life. Ovulation is affected by the concentration between FSH and LH. Hormones can affect the egg because of the receptor protein in the target cell. The receptor protein reacts with the hormone molecule so that it stimulates adenylcyclase to become active in the cytoplasm. Adenylcyclase reacts with ATP molecules to form cyclic AMP. This molecule activates kinases, which can ultimately affect the cell nucleus for transcription [27]. The difference in the average number of follicles produced from each dose of giving bitter extract proves the amount of estrogen hormone that is activated by andrographolide which amounts differ also between treatment groups.

Females are born with primordial follicles, after puberty throughout the ovaries and their follicles will begin to grow [10]. The first stage of follicle growth is the development of the ovum followed by the growth of layers of granulosa cells in some follicles, these follicles are known as primary follicles. Since these follicles are present before the experimental animals are given treatment, the number of primary follicles is not significantly affected. In this research, it was found that the highest number of primary follicles was in ovarium of mice with a dose of 0.4 g / kg and the lowest treatment was at a treatment dose of 0.2 g / Kg BW.

In secondary and tertiary follicles there is also no significant effect of these bitter extracts. The contents of the control treatment (not given the bitter extract) had the lowest average number of secondary follicles compared to the other treatment groups. Although not significantly influenced by statistical test, based on the results of the research table, it is known that the bitter extract tends to increase the number of tertiary tertiary follicles of mice. The tertiary follicle in animals with the treatment shows the increase in size, the increase of granulosa cell thickness, but observed multiple and small-sized antrum follicles. Microscopic images give the impression of disturbance unification of antrum follicles and maturation to de-graaf follicles. In the treatment group we can observe the dominance of follicles in the tertiary follicle phase, but there is a decrease in the number of follicles in the more mature phases (de-graaf follicles and corpus luteum). Reduced proportions of these components give the impression of the rise of follicular maturation or the occurrence of maturation arrest at the tertiary follicle stage.

Follicles that are easily observed and easily differentiated from other follicles are de Graaf follicles.

This is because the De Graaf follicle has a large size compared to other follicles and there is a large follicular (antrum) cavity [15]. De Graaf's mature follicles release the hormone estrogen. The increase of estrogen concentration during follicle growth also affects the cervix. Thus the important role of FSH and LH is shown to the release of estrogen hormone by the De Graaf follicle. It will retain the acidic properties of the cervix causing sperm to not survive due to an unsuitable environment so that sperm will die and no fertilization occurs [29].

In this study there were no De Graaf follicles in ovarian histology. In this bitter plant (*A. paniculata*) contain secondary metabolite compounds such as glycosides, saponins, tannins, flavonoids and alkaloids. These substances have cytotoxic, anti-androgen or estrogenic effects. Saponins have anti-estrogen effects that interfere with natural estrogen bonds to their receptors [8]. States that flavonoids are compounds that have similar structures with estrogen hormones but do not stimulate these receptors. This causes the amount of free estrogen to increase in the blood so that will cause negative feedback on GnRH secretion. GnRH secretion disorder will suppress the FSH hormone that stimulates the growth of granulosa cells in the ovary follicle so that follicle development is impaired [13]. States that alkaloids can decrease the concentration of FSH and estradiol in the body. Alkaloids cause FSH can not bind to receptors present in granulosa cells so that FSH action is disrupted. This causes the process of folliculogenesis disrupted. This is also due to the mice used in the diestrus phase where no ovulation occurs so that the De Graaf follicle will not be encountered [18].

Mice used in this study were mice (*Mus musculus* L.) in the diestrus phase. Diestrus phase is a slow growth period, characterized by the presence of mucosa in the vaginal smear, microscopically visible leucocytes and epithelium. This phase lasts 2 to 4 days [25]. Diestrus is the longest phase among the phases in the lethal cycle [20]. Diestrus is the last and longest phase in the estrus cycle of mammalian cattle. The corpus luteum becomes mature and the influence of progesterone becomes dominant. The endometrium thickens, the uterine gland enlarges, and the uterine muscle shows an increase in development. This change is intended to supply food substances to the embryo in the event of pregnancy. This condition will continue during pregnancy and the corpus luteum will be maintained until the end of pregnancy [27]. This results in the discovery of the corpus luteum in the histologic ovaries of mice.

In the ANOVA test on the number of corpus luteum from histology ovarium of mice that have been given the extract of bitter (*A. paniculata*) is also not found any significant difference. This means that the extract of bitter (*A. paniculata*) has no effect on the number of corpus luteum from the ovaries of mice. In the control treatment, the treatment dose of 0.2 gr / KgBB and the treatment dose of 0.4 g / KgBB decreased the number of corpus luteum. However, the number of corpus luteum again increased at a treatment dose of 0.6 gr / KgBB. This occurs because of less optimal FSH and LH secretion by the pituitary causes an increase in the amount of estrogen occurs slowly so that estrogen levels are maintained in the blood because there is no increase in estrogen. Estrogen levels maintained in the blood result in inhibition of LH surges leading to inhibition of ovulation [4]. Inhibition of the ovulation process leads to inhibition of corpus luteum formation resulting in decreased progesterone levels. Progesterone and LH hormones affect the luteinization phase [1].

V. CONCLUSION

Bitter extract (*A. paniculata*) had no significant effect on the weight, length, number of primary follicles, the number of secondary follicles, the number of tertiary follicles and the number of corpus luteum ovaries of mice (*Mus musculus* L.), but the bitoto extract (*A. paniculata*) tended to increase the number tertiary ovarian follicles of mice (*Mus musculus* L.).

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