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FERTILITY ENHANCING EFFECT OF ETHANOL EXTRACT OF *Carica papaya* L. SEED IN MICE

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Abstract

This paper reports the fertility effects of the ethanol extract of *C. papaya* L. seeds for the evaluation of determining the estrous cycle which plays an important role in increasing fertility success. This study aims to assess the length of the estrous cycle time through vaginal cytology images. The papaya seed extract dosages used consisted of: the control group (C) was given papaya seed extract 0 mg/40 g BW in 0.4 ml aquabidest; treatment 1 (T1) was given papaya seed extract 2 mg/40 g BW in 0.4 ml aquabidest; treatment 2 (T2) was given papaya seed extract 4 mg/40 g BW in 0.4 ml aquabidest; Treatment 3 (T3) was given papaya seed extract 8 mg/40 g BW 0.4 ml aquabidest. Samples from vaginal cytology were collected every 4 hours for three months, and stained with Giemsa and the data obtained were tabulated and analyzed descriptively. The results showed that giving papaya seed extract to female mice could prolong the estrous time for the pro-estrous phase and the estrous phase at a dose of 2 mg/40 g BW, 4 mg/40 g BW, and 8 mg/40 g BW. In the proestrous phase, the longest mean duration of the proestrous phase occurred in the 8 mg/40 g BW treatment (25 hours) and in the estrous phase the longest average duration of the estrous phase occurred in the 8 mg/40 g BW treatment. (80.4 hours), compared to controls. Meanwhile, papaya seed extract did not show any changes in the composition and structure of the epithelial cells of the vaginal swab. The conclusion of this study is that the estrous cycle time of mice can be determined by the method of vaginal cytology analysis.

Keywords: Papaya seed extract, estrous cycle, vaginal cytology, female mice.

Introduction:

The determination of the estrous cycle, the length of the estrous period and the time of insemination can be determined based on changes in behavior (Mauget et al., 2007), invasive (blood plasma) and non-invasive (urine and fecal) hormone profile analysis (Möhle et al., 2002); Pereira et al., 2006; Hesterman et al., 2008) as well as observations of vaginal cytology (Zen 1983; Durrant et al., 2002; Durrant et al., 2003; Tsiligianni et al., 2004; Hesterman et al., 2008).

Physiological changes can affect follicular growth, increased growth of the endometrium, uterin and cervix as well as increased vascularity and keratinization of the vaginal epithelium in some species. The vaginal smear preparation in the proestrous phase is characterized by a decrease in the number of nucleated epithelial cells and white blood cells, replaced by cornified epithelial cells. In the luteal phase, the epithelial cells from the vagina will be dominated by parabasal cells, while entering the estrous phase the epithelial cells turn into superficial cells and cornified cells which indicate the animal is in an estrous peak state (Seier et al., 1991).

Papaya contains alkaloid compounds that are anti-fertility and can be used as an ingredient for male and female contraceptives (Naggayi et al., 2015) tannins, and terpenes and enzymes such as papain and chymopapain (Adeneye et al., 2009). The active chemical compounds of papaya, namely triterpenoids, are a derivative of steroids, active chemical compounds of steroids and triterpenoids are thought to be compounds that work as antifertility factors. The two active ingredients are thought to be capable of causing disruption in the the hypothalamic pituitary pathway which subsequently results in disruption of GnRH secretion which then affects the formation, development and maturation of follicles (Borrow et al., 2001; Garor et al., 2009). Based on this, a study to determine the estrous cycle in the proestrous and estrous phases was conducted on female mice as experimental animals to determine the length of the estrous cycle through vaginal cytology observations.

Materials and Methods

Plant materials

Papaya seeds were collected from Bandar Lampung city area, Lampung Province, Indonesia. The collected papaya seeds were labeled and transferred to the laboratory, washed with tap water, air dried, and stored until further investigations.

Preparation of the plant extract

Papaya seeds have been obtained are then cleaned and then processed to dry using a 70ºC oven for 15 minutes, after drying the plants are milled using a blender to produce a powder obtained 283.9 grams. Papaya seeds powder which was crushed was put into a 2000 ml glass beaker then macerated using 96% ethanol solvent for 3 x 24 hours to obtain macerate. Filtrate that has been obtained will be concentrated or thickened using a rotary evaporator at a temperature of 50 ^{0}C for 1 hour.

Animal groups

Animal tests conducted acclimatization for 1 week under laboratory conditions in cages that had been prepared. Appropriate 20 female mice age around 6-8 weeks old and weighted around 40 g that splits into 4 groups and can be maintained 100 cm x 50 cm which is placed in a research room or mice cage. The mice cage inside contained a bowl filled with food, rice husks and a place to drink. Mice are fed with small chicken pellets and drink water daily. Husks replaced every 2 days due to moisture due to spillage of food or drink mice in order to prevent the growth of bacteria or fungi.

Treatments

Papaya seed extract was given to each treatment group by force-feeding (orally) using a syringe. Forcing treatment on experimental animals was carried out every day at 10.00 AM for 14 days, then after 14 days vaginal swabs were examined and observed for the histologycal structure of the experimental animals' vagina (to determine the ongoing phase in experimental animals). The papaya seed extract dosages used consisted of: the control group (C) was given papaya seed extract 0 mg/40 g BW in 0.4 ml aquabidest; treatment 1 (T1) was given papaya seed extract 2 mg/40 g BW in 0.4 ml aquabidest; treatment 2 (T2) was given papaya seed extract 4 mg/40 g BW in 0.4 ml aquabidest; Treatment 3 (T3) was given papaya seed extract 8 mg/40 g BW 0.4 ml aquabidest.

Sampling of vaginal cells

Sampling of vaginal cells using a cotton swab method. A cotton swab is moistened with aquabidest and then rubbed on the vaginal wall so that the epithelium cells of the vaginal wall stick to the cotton bud. The cotton bud which already contains epithelium cells is then smeared on a glass object and dripped with one drop of Giemsa dye, then closed with a cover glass. These stained preparations can be directly observed under a microscope at a magnification of 100x. Vaginal epithelial sampling was carried out every 4 hours.

Observed Parameters.

The parameters observed in this study were the length of the estrous cycle time of the fertile phase of the proestrous and estrous of mice (*Mus musculus* L.) and the shape of the vaginal epithelial cells after giving papaya seed extract. The proestrous phase is characterized by the presence of ordinary epithelial cells and leukocytes on histologycal preparations, while the estrous phase is characterized by the presence of cornified epithelial cells. Calculation of the length of the estrous cycle time by observing how long the estrous cycle of mice after treatment was compared to the length of the estrous cycle time before treatment.

Statistical analysis.

Analysis of the data used is Shapiro Wilk for normality test and Levene's Test for variant homogeneity test. Then the One way Anova test was carried out and continued by Least Significant Difference (LSD) test to find out the differences between groups significantly. Then the data obtained is averaged as a result of the study, both on the length of the estrous cycle on proestrous dan estrous phase, and vaginal cytology.

Results

Proestrous and Estrous Phase.

Proestrous and Estrous Cycle's Duration.

The results of this observations, show data on the average length of time of proestrous and estrous in the proestrous phase of mice between control and treatment (Table 1.)

Table 1: The average duration of the estrous cycle in the proestrous and estrous phase of mice after treatment with papaya seed extract ± standard deviation (hours).

Treatment Estrous	The average duration of the estrous cycle in the proestrous phase of mice ± standart deviation (hours)	The average duration of the estrous cycle in the estrous phase of mice ± standart deviation (hours)
Control	17.40 ± 0.962^{c}	32.30 ± 10.65^{b}
2 mg/40 g BB	21.40 ± 2.584^{b}	72.10 ± 24.16^{a}
4 mg/40 g BB	23.30 ± 4.056^{ab}	74.90 ± 18.27^{a}
8 mg/40 g BB	25.00 ± 2.151^{a}	80.40 ± 21.80^{a}

Note: The numbers followed by the same letter show no significant difference at the 5% level of the LSD test.

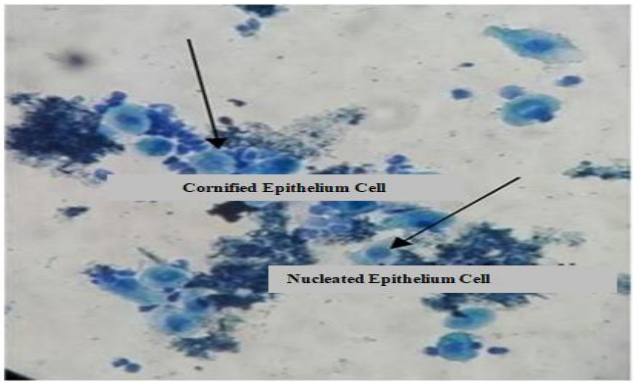
The average yield of the long estrous time period in the proestrous phase after treatment with papaya seed extract had an effect on the length of estrous time in the proestrous phase. The length of time of estrous in the proestrous phase is longer than the control. The longest mean estrous time in the proestrous phase occurred in treatment 8 mg/40 mg BW (25 hours), and the shortest estrous time was found in the control treatment (17.4 hours). The results of ANOVA statistical analysis with a significance level of 5%, showed that there was a significant difference between the control group and the treatment group ($P < 0.05$). Further tests with LSD with a level of 5% between the control and all treatment groups showed a significant difference, while between the treatment groups 2 mg/40 g BW and 4 mg/40 mg BW were not significantly different.

The treatment results of the long estrous cycle time in the estrous phase after treatment with papaya seed extract had an effect on the length of time of the estrous of estrous phase. The length of time for the estrous phase was longer compared to the control. The longest average estrous time in the estrous phase occurred in the treatment of 8 mg/40 g BW (80.4 hours), while the shortest was in the control treatment (32.3 hours). However, in this study, the estrous phase in the female mice used had a longer estrous cycle phase time.

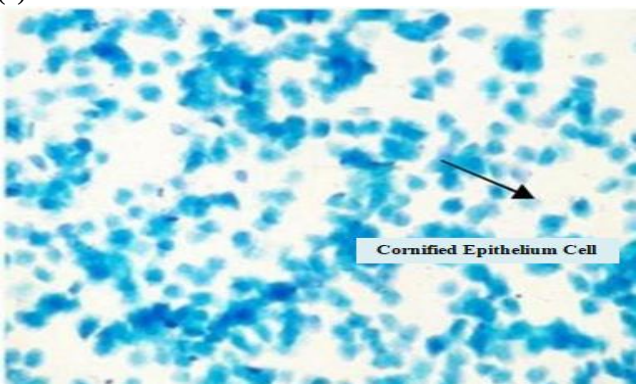
This can be seen in the control, the control has an average estrous time in the estrous phase of 32.3 hours and will increase when the papaya seed extract is added with increasing doses as well. The results of the ANOVA statistical analysis with a significant level of 5%, showed that there was a real or significant difference between the control and treatment groups ($P<0.05$). Further tests with LSD with a level of 5% between control and all treatment groups showed a significant difference. Based on available data, the effect of papaya seed extract can continue to increase according to the increasing dose used. There was no significant difference between the treatment doses of 2 mg/40 g BW, 4 mg/40 g BW and 8 mg/40 g BW. The higher the dose used, the longer the estrous phase will take longer.

The structure of vaginal epithelial cells.

It was found that there is no difference showed on the epithelial cell structure of the proestrous phase mice vaginal swab between control and treatment and known nucleated epithelial cells and cornified epithelial cells (Figure 1 a) and the presence of cornified epithelial cells (Figure 1 b)



(a)



(b)

Figure 1: The epithelial cell structure of female mice (*Mus musculus* L.) in the proestrous phase after treatment with papaya seed extract. Magnification of 100x and staining with methylene blue 1% (a). The epithelial cell structure of female mice (*Mus musculus* L.) during estrous phase after treatment with papaya seed extract. Magnification of 100x and staining with methylene blue 1% (b).

From the observation of the epithelial cell structure in the vaginal swab of mice (*Mus musculus* L.) in the proestrous phase, the results obtained were analyzed descriptively by looking at changes in the structure and composition of epithelial cells between treatments compared to controls. Based on the results presented in Figure 1 (a), it can be seen that the histologycal structure of the vaginal swab of the proestrous phase between control and treatment did not show a significant difference, it appears that there are nucleated epithelial cells and cornified epithelial cells.

From the observation of the epithelial cell structure in the estrous phase of the vaginal swab of mice the results obtained were analyzed descriptively by looking at changes in the structure and composition of epithelial cells between treatments compared to controls. Based on the results presented in Figure 1 (b), it can be seen that the histologycal structure of estrous phase mice vaginal swabs between control and treatment did not show a significant difference, seen the presence of cornified epithelial cells.

Discussion

In this study, it was found that giving papaya seed extract to female mice could prolong the oestrous time of the proestrous phase and the oestrous phase at a dose of 2 mg / 40 g BW, 4 mg / 40 g BW, and 8 mg / 40 g BW. In the prooestrous phase, the longest mean duration of the proestrous phase occurred in treatment 8 mg / 40 mg BW (25 hours) and in the oestrous phase the longest mean duration of the oestrous phase occurred in treatment 8 mg / 40 mg BW. (80.4 hours), compared to controls.

The results showed that the prolongation of the oestrous cycle in the proestrous and oestrous phases was due to the effect of the ethanol extract of papaya seeds. It is suspected that this disturbance occurs at the hormonal level and results in disruption of the ovarian cycle which in turn disrupts the oestrous cycle because these two cycles occur in parallel.

The compounds contained in papaya seed extract are saponins, tannins, flavonols, glycosides, terpeoids, alkaloids, reducing sugars, amino acids, fats, proteins, phenols, vitamins, sterols and triterpene. Saponins, tannins, flavonoids, terpenoids, alkaloids, sterols and triterpene compounds can suppress fertility levels by disrupting the function of the ovaries, uterus or vagina. Naggayi et al., (2015). Raji et al., (2005) stated that the water extract of papaya seeds has anti-fertility ability by influencing the secretion of the hormone estrogen, causing disruption of the estrous cycle in Wistar rats.

In addition, Nalbandov (1990) added that the length of time for the proestrous phase and the oestrous phase in the oestrous cycle is controlled by the reproductive hormone system through the hypothalamus-pituitary-ovarian axis. If there is a change in the length of the oestrous cycle, it means that there has been a disturbance in the control mechanism of the hypothalamus axis.

The mechanism of estrus cycle disruption that occurs due to exposure to water extract of papaya seeds can occur in several ways, including disturbances in reproductive hormone secretion. Udoh (2005) states that triterpenoid and saponin anti-fertility agents act on the hypothalamus-pituitary-gonad axis, thereby affecting the secretion of gonadotropin hormones.

The oestrous cycle begins with preparation for follicular development (proestrus), then continues with oestrous, metoestrous and dioestrous phases (Hafez and Hafez, 2000). The ovarian cycle is influenced by the gonadotropin hormone secreted by the anterior pituitary. The destruction of neuron cells results in a disruption of the hypothalamus to stimulate the secretion of GnRH which stimulates the pituitary to secrete FSH and LH (Uke, 2008).

The progesterone hormone is a steroid hormone secreted by the corpus luteum, placenta and drenal glands (McDonald, 2000; Hafez 2000). The secretion of the hormone progesterone depends on the status of the oestrous cycle, the highest level of progesterone is in the luteal phase because the corpus luteum is the main source of progesterone and the lowest level is in the follicular phase (McDonald, 2000). The hormone progesterone is a hormone that is very important in regulating the function of the normal female reproductive cycle (Hafez, 2000). To determine the changes that occur in one estrous cycle, it can be seen by describing changes in the epithelial cells in the vagina, including a picture of cell changes during the prooestrus and oestrus phases.

The results showed that the ethanol extract of papaya seeds (*Carica papaya* L.) did not show changes in the composition and structure of the epithelial cells of vaginal swabs, both in the prooestrous and oestrous phases. Determination of the estrous cycle, the length of the estrous period and the time of insemination can be determined based on changes in vaginal cytology (Zen 1983; Durrant et al., 2002; Durrant *et al*., 2003; Tsiligianni *et al*., 2004; Hesterman et al., 2008). In the proestrus phase, large ornified epithelial cells are found. During the estrous phase, almost the entire surface of the epithelial cells is cornified, some are nucleated and some are without nuclei. Towards the end of estrus, some leukocyte cells appear to multiply on the second or third day after the end of estrus (McDonald, 1980; Johnston et al., 2001; Davidson, 2004). Furthermore, Rugh (1968) added that in mice that were in the phase of proestrus, metestrus and diestrus, nucleated epithelial cells and leukocytes were found. In the oestrus phase only cornified epithelial cells were found, while in mice that were in the proestrous, metestrous and diestrous phases, nucleated epithelial cells and leukocytes were found (Rugh, 1968).

In the prooestrous phase there is also an LH surge which is needed to induce ovulation. In the vaginal smear preparations, intermediate cells were found, namely the transition from parabasal cells and intermediate cells to superficial cells. This phase is characterized by the discovery of many superficial cells. Superficial cells are the largest cells that can be seen on a vaginal smear, they are polygonal and appear very flattened. The nucleus is sometimes missing or found but very small and dark (picnotic). The superficial cells without the nucleus often undergo cornification. In this phase, sometimes very small numbers of leukocytes are also found (Cox et al., 1994). The hormone estrogen causes increased mitosis and proliferation of epithelial cells and signaling processes in surface epithelial cells. The high concentration of estrogen at the time of estrus results in thickening of the vaginal wall and causes the epithelial cells to bulge and release from the vaginal epithelial wall. Mark cells were seen to be dominant in vaginal swabs (Kumar et al., 2005).

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