

Antifertility activities of ethanol extract of sugar apple leaves (*Annona squamosa* L.) in the reproductive system: spermatogenesis and sperm quality study

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ABSTRACT

The use of male contraceptives is still limited to condoms and vasectomy. However, their applications have frequently led to complaints, making male participation in family planning programs persistently low. Therefore, developing contraceptive drugs from parts of plants with antifertility activity becomes necessary. This study aimed to identify the antifertility activity of sugar apple leaves ethanol extract in spermatogenesis and sperm quality in test animals. There were four treatment groups: control (suspension PGA 1%), Dose I (24.2 g/Kg BW rat), Dose II (48.4 mg/Kg BW rat), and Dose III (96.8 mg/Kg BW rat). The test preparation was administered orally for 48 days. The effect on spermatogenesis was observed from the testicular index (%), seminiferous tubule diameter (μm), and the number of spermatogenic cells and spermatocytes. The sperm quality was determined from sperm motility, concentration, and morphology. All of these parameters were statistically analyzed using ANOVA. The results showed that the testicular index and the number of spermatogenic cells and spermatocytes in all groups of rats were significantly reduced. The administration of the ethanolic extract of sugar apple leaves also decreased the seminiferous tubule diameter with a significant size shrinkage in Dose III. Sperm quality analysis revealed a reduction in sperm count and motility and a significant increase in sperm abnormalities in all doses. The antifertility activity (i.e., the reduction of sperm count and motility) in rats receiving Dose III was similar to the ones in Dose II. Therefore, the best dose of antifertility activity is Dose III (96.8 mg/Kg BW rat), implying that it has the potential as male contraceptive.

Keywords: Ethanol extracts, sugar apple leaves, spermatogenesis, sperm quality

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INTRODUCTION

The Indonesian government has been promoting a family planning program for married couples of childbearing ages to suppress and control the population. The program provides many services, including the ongoing provision of contraceptives (Aulanni'am *et al.*, 2007). The principle of contraceptive application is preventing pregnancy by inhibiting the union of sperm cells and eggs, as well as the formation and maturation of sperm in the ejaculatory ducts, and by disturbing reproductive hormones (Rusmiati, 2007).

The male contraceptive method remains limited to condoms and vasectomy and male participation in the program is still low (Rusmiati, 2007). The use of condoms causes psychological complaints, while vasectomies have permanent side effects (Bagia *et al.*, 2011). Natural ingredient-based contraceptives through traditional medication can be an alternative to overcome this problem. The community prefers traditional medicine because it is relatively cheap, its ingredients are easily available and safe for human's body, and it has smaller side effects than modern medicines (Rusmiati, 2007).

Many studies have claimed the potential use of natural substances as candidates for male contraceptive materials. Barbados nut (*Jatropha curcas* L.) has been reported to induce an antispermatic mechanism (Larasaty, 2013). Other plant species are scientifically proven antifertility agents as they can decrease the quality of spermatozoa. They are Roscoe zedoary (*Curcuma zedoaria*) (Ashafani *et al.*, 2010), willow-leaved justicia (*Justicia gendarussa*) (Bagia *et al.*, 2011), and red guava leaves (*Psidium guajava* L.) (Hartini, 2011).

Empirical data show that sugar apple leaves can function as antifertility (Dalimartha, 2003). These leaves contain several secondary metabolites, including alkaloids, flavonoids, saponins, quinones, tannins, and steroids/triterpenoids (Mulyani *et al.*, 2013). The steroid groups are precursors of antifertility elements (Simbolon *et al.*, 2013). Also, active compounds such as alkaloids, flavonoids, triterpenoids, saponins, and tannins are thought to have antifertility potentials (Nurliani, 2007). Therefore, testing the antifertility activity of sugar apple leaves extract is essential for finding new natural ingredient-based contraceptives.

RESEARCH METHOD

Tools and Materials

The tools used in this study were macerator, rotary evaporator (Eyela), microtome (Leica), microscope (Olympus BX41), oven incubator (Memmert), analytical scales (Shimadzu), surgical instruments (Gold Cross), hand counters, a hemocytometer—the Neubauer Chamber (Assistant Germany), and cavity slide (Sail Brand).

The research materials included sugar apple leaf, Dragendorff reagent, Lieberman-Burchard reagent, Mayer reagent, NBF (Neutral Buffer Formalin), 70% ethanol, 80% ethanol, 90% ethanol, 96% ethanol, 1% PGA, Hematoxylin-Eosin stain, and eosin 2%.

Research Procedure

Preparation of test animals

The test animals were white male *Sprague Dawley* rats aged 3-4 months whose identity and health had been confirmed by i-Ratco. They were allowed to first adapt to a new surrounding and given ad libitum access to food and drink.

Preparation of plant samples

The fresh sugar apple leaves were obtained from the Manoko Plantation, Lembang, Bandung. The identity of the leaves was confirmed using the Jatinangor Herbarium in the Laboratory of Plant Taxonomy, Department of Biology, Faculty of Mathematics and Natural Sciences, Padjajaran University, Bandung.

Preparation of simplisia powder

The process of making the simplisia powder began with wet sorting and continued to dry sorting. The clean and dry leaves were cut into small pieces and then crushed to produce sugar apple leaf powder. This powder was later stored in a dry place in a tightly closed container (Kemenkes RI, 1993).

Ethanol extraction from the samples

The extraction employed maceration method with 70% ethanol solvent (Hutasuhut, 2014). The produced extract was weighed (g). Then, it was compared with the weight of the simplisia to obtain the extraction yield (%).

Phytochemical screening

The simplisia and the ethanolic extract of sugar apple leaves were screened phytochemically for secondary metabolites, namely alkaloids, flavonoids, steroids/triterpenoids, saponins, tannins, monoterpenoids, and sesquiterpenoids (Farnsworth, 1966).

Administration of test preparation

The test preparation, which was the ethanolic extract of sugar apple leaves, was administered with the doses presented in Table I. The dose was based on empirical data, i.e., the common usage in the community, which was 15 sheets of leaves or equivalent to 3 grams (Alex, 2011). It was then converted to dosage for a rat. The treatment involved oral administration for 48 days (Larasaty, 2013).

Table I. The treatment and preparation administered to the test animals

Treatments	Administered Preparations
Control	PGA 1%
Dose I	Sugar apple leaf extract in PGA 1% (24.2 mg/Kg BW rat)
Dose II	Sugar apple leaf extract in PGA 1% (48.4 mg/Kg BW rat)
Dose III	Sugar apple leaf extract in PGA 1% (96.8 mg/Kg BW rat)

Research data collection

On Day 49, each test animal was anesthetized with chloroform and then dissected to remove the testicular organ. The antispermatogenic activity was observed in this organ. The epididymis was also removed and observed to determine the sperm quality.

Observation of antispermatogenic activity

Calculation of testicular index (%)

The wet weight of the testicular organ was compared with body weight to obtain the testicular index. The formula is as follows:

$$\text{Organ Index} = \frac{\text{Testicular Organ Weight}}{\text{Body Weight}} \times 100\% \text{ (Larasaty, 2013)}$$

Preparation of testicular histologic specimen

The testes of the white male rats were prepared into histological specimens with Hematoxylin-Eosin (HE) staining (Larasaty, 2013). The preparation consisted of the following steps: fixation, washing, dehydration, clearing, paraffin infiltration, embedding, sectioning, affixing, staining, mounting, and labeling (Suntoro, 1998; Wahyuni, 2012).

Measurement of seminiferous tubule diameter (μm)

The diameter was the average of the three diameter points of three seminiferous tubules. The diameter points were measured with a calibrated ocular micrometer (Wahyuni, 2012)

Enumeration of spermatogenic cells and spermatocytes

The seminiferous tubular cells (i.e., spermatogenic cells and spermatids) were counted, and the average number was determined. The testicular organs were prepared into histological specimens. Then, the seminiferous tubular cells were observed under a microscope and compared with the negative control and the other treatments (Wahyuni, 2012).

Observation of sperm cell quality

Preparation of spermatozoa suspension

The epididymis of the test rats was cut out and dissected to remove the spermatozoa. It was placed in a Petri dish containing 1 mL of physiological saline (0.9% NaCl) and left for 1-2 minutes to allow the spermatozoa to spread out of the epididymis. The parameters observed in this step were motility, concentration, and morphology (Bagia *et al.*, 2011).

Observation of spermatozoa motility

The sperm suspension was dropped to the cavity slide and then added with water to activate the spermatozoa. The motility should be observed immediately after the spermatozoa were removed from the epididymis. It was observed in a stock solution in one field of view (FOV) at 100x magnification (Yulianto, 2013). The motility was scored with 0 to 5, as shown in Table II. It was categorized as normal when 50% of the spermatozoa moved forward, and at least 25% of them moved forward rapidly.

Table II. The assessment criteria for spermatozoa motility (Ashafani *et al.*, 2010)

Criteria	Scores
Immotile spermatozoa	0
Spermatozoa rotate in one place	1
Spermatozoa move zig-zag or in a circle; < 50% progressive; no wave motion	2
Spermatozoa move progressively and create mass movement (50-80%)	3
Spermatozoa move progressively, travel fast, and immediately form a wave motion with 90% motile sperms	4
Spermatozoa move very progressively and form a wave motion rapidly with 100% motile sperms	5

Spermatozoa morphology

A drop of sperm suspension was placed on the object glass and made into test preparation using the smear method with eosin 2% staining. Through observation, sperms with abnormal morphology (%) were calculated with a hand counter. The spermatozoa were normal if the number of morphologically abnormal sperms was less than 20% (Bagia *et al.*, 2011; Larasaty, 2013).

Spermatozoa concentration

The sperm concentration was identified by counting the number of spermatozoa that entered each of the five Neubauer chambers using a hemocytometer (Sutrisno, 2010). The average number of sperm cells was calculated using the following formula:

$$\text{Sperm Concentration (spz/mL)} = \bar{x} \times 2.5 \times 10^5 \times \text{dilution factor}$$

Data Analysis

The testicular index (%) and spermatozoa abnormality (%) were first subjected to arcsine transformation. Motility (ordinal), number of spermatogenic cells and spermatocytes, and sperm count, as well as the transformation results, were analyzed with ANOVA at a confidence level of 95% (Trihendradi, 2009).

RESULTS AND DISCUSSION

Ethanol extraction

The extraction employed maceration method with 70% ethanol. Ethanol was used as the solvent because of its semi-polar nature, which expectedly attracts polar and non-polar compounds (Hutasuhut, 2014). The yield of the ethanol extraction of sugar apple leaves was 18%.

Phytochemical screening results of simplisia and extract

The phytochemical screening showed that the simplisia and the ethanol extract were positive for alkaloids, flavonoids, steroids, and quinones. Sugar apple leaves contain secondary metabolites, including alkaloids, flavonoids, saponins, quinones, tannins, steroids, and triterpenoids (Mulyani *et al.*, 2013). Alkaloids, flavonoids, and steroids are the metabolite compounds that have antifertility activities (Nurliani, 2007).

Antifertility activities

Testicular Index (%)

The administration of sugar apple leaf extract significantly reduced the average testicular index, indicating antifertility activity (Figure 1). Similar decreased index and antifertility activity were identified in all doses.

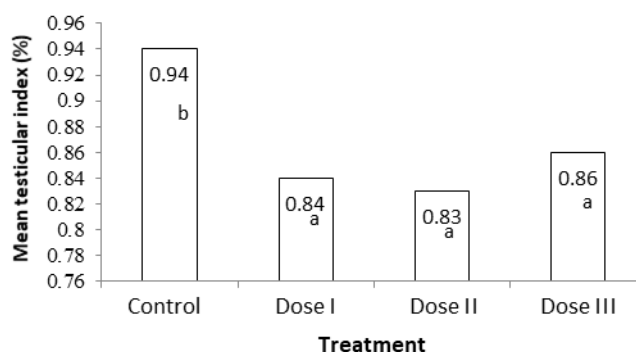


Figure 1. The mean testicular index (%) in every treatment group

Notes: Numbers followed by different letters indicate a significant difference with a confidence level of 95%

The decrease in the average testicular index in test animals is possible because of testicular weight shrinkage. This shrinkage is attributable to the reduction of the size of seminiferous tubules, wherein spermatogenesis takes place (Wahyuni, 2012). Decreased organ weight is assumed to be caused by flavonoids, alkaloids, and steroids found in sugar apple leaf extract that inhibit the secretion of LH (Luteinizing Hormone) and FSH (Follicle-Stimulating Hormone) and, as a result, disrupt spermatogenesis (Rukmana, 2010).

The diameter of seminiferous tubules

The average diameter of seminiferous tubules (Figure 2) decreased significantly due to the administration of the ethanolic extract (Figure 3) at the dose of 96.8 mg/Kg BW rat (i.e., treatment in Dose III). The diameter shrinkage activity was equal among the treatment doses. This activity is possible due to the reduction of the population inside the seminiferous tubules. Seminiferous tubules, especially in epithelial tissue, are composed of two cell populations, namely spermatogenic cells and Sertoli cells. The tissue greatly determines the diameter of seminiferous tubules. Decreased diameter is possible due to a reduction in the cell population (Apriliani *et al.*, 2013). It is assumed to be the results of flavonoids, alkaloids, and steroids contained in sugar apple leaf extract that can inhibit the secretion of LH and FSH, disrupting the process of spermatogenesis (Rukmana, 2010).

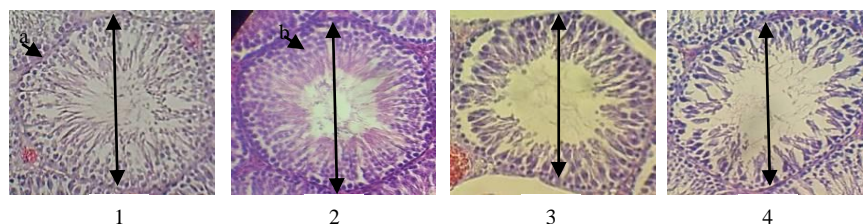


Figure 2. The diameter of the seminiferous tubules in each treatment group (μm)

(HE staining, 160x magnification)

Notes: The seminiferous tubules of the control group (1), Dose I (2), Dose II (3), and Dose III (4); a= spermatogenic cells; b= spermatocytes

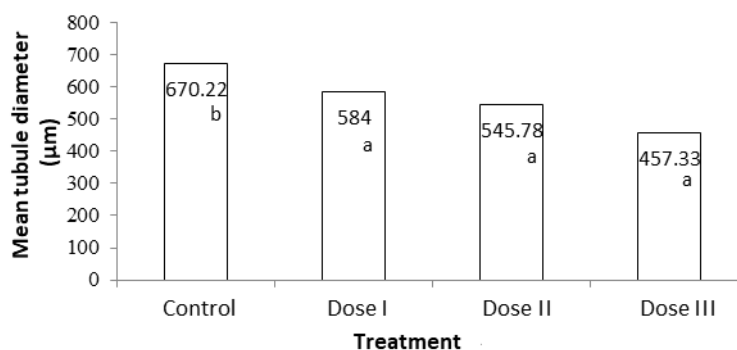


Figure 3. The diameter of seminiferous tubules (μm)

Notes: Numbers followed by different letters indicate a significant difference with a confidence level of 95%

The number of spermatogenic cells

The average number of spermatogenic cells decreased significantly with the administration of the extracts. The reduction became increasingly significant when the dose of the extract was added (Figure 4). Dose III (96.8 mg/Kg BW rat) resulted in the greatest antispermatogenic activity.

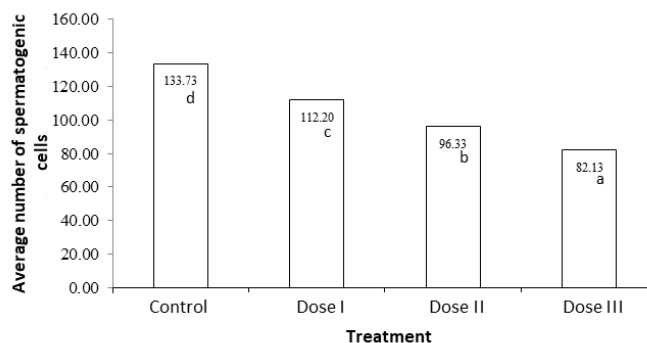


Figure 4. The average number of spermatogenic cells by treatment

Notes: Numbers followed by different letters indicate a significant difference with a confidence level of 95%

The steroids in sugar apple leaves cause the reduction in the number of spermatogenic cells. Steroids are the basic ingredients of testosterone biosynthesis, which stimulates an increase in testosterone. The increase in testosterone in the body stimulates a negative feedback loop in the hypothalamus and the anterior pituitary, which inhibits FSH and LH secretion and, consequently, impede spermatogenesis (Wahyuni, 2012). The function of LH (Luteinizing Hormone) is to stimulate

the Leydig cells to produce testosterone, while FSH (Follicle Stimulating Hormone) acts as a stimulant in spermatogenic cell growth (spermatogenesis) and promotes the Sertoli cells to form androgen-binding proteins (ABP). ABP binds to the testosterone secreted by the Leydig cells that normally help the proliferation of spermatogenic cells to progress to a further stage. Therefore, if FSH production is stopped or reduced due to the negative feedback, then spermatogenesis will also stop and, as a result, reduce the number of spermatogenic cells (Widiyani, 2006; Setyaningsih, 2011). Spermatogenesis begins with the division of spermatogenic cells. Therefore, if the spermatogenic cells are reduced in the first place, then the development of the subsequent cells will be disrupted (Apriliani *et al.*, 2013).

The number of spermatocytes

The administration of the ethanolic extract significantly reduced the average number of spermatocytes (Figure 5). The decrease became increasingly significant when the dose was added. Dose III (96.8 g/200 g BW rat) resulted in the largest spermatocyte decrease. This effect is attributable to the negative feedback that reduces the number of spermatogenic cells, which are mitotically divided into spermatocytes (Hutasuhut, 2014).

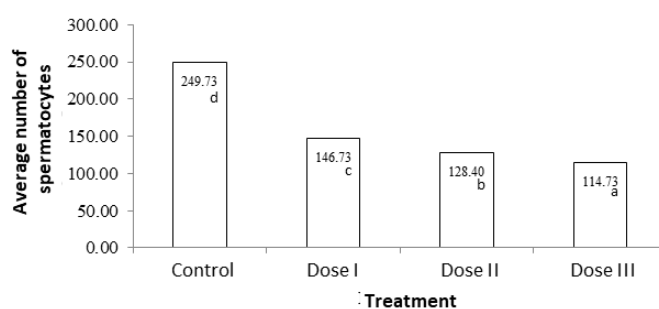


Figure 5. The number of spermatocytes by treatment

Notes: Numbers followed by different letters indicate a significant difference with a confidence level of 95%

Spermatozoa quality

The spermatozoa observed in this study were the ones originating from the epididymis. The epididymis is the site where the maturation of spermatozoa occurs before ejaculation and, therefore, mature spermatozoa are assumed to concentrate the most in it (Hutasuhut, 2014). The parameters of spermatozoa quality in this study included motility, concentration, and morphology.

Spermatozoa motility

Spermatozoa motility is the quality of sperm motion, which includes the type and speed of movement. A good type of movement is straightforward. Sperms that move zig-zag or in a circle represent a structural abnormality (Setyaningsih, 2011). The administration of the ethanolic extract significantly reduced sperm motility (Figure 6), but an increase in dose did not generate different reduction activities. The decreased motility is possible because of the toxic effects of alkaloids. Alkaloids can affect ATPase enzyme activity in spermatozoa cell membranes and further disrupt the homeostasis of sodium and potassium ions (Ashafani *et al.*, 2010).

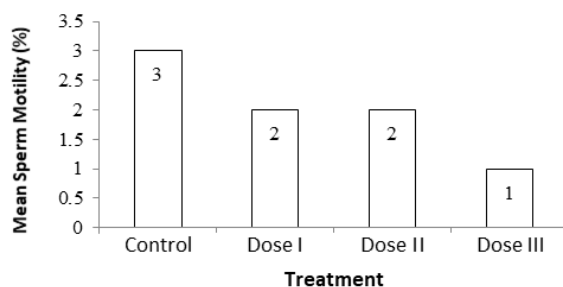


Figure 6. The mean spermatozoa motility (%)

Notes: Numbers followed by different letters indicate a significant difference with a confidence level of 95%

Spermatozoa concentration

The results showed that the administration of the ethanolic extract at different doses significantly reduced the concentration of spermatozoa in all treatment groups (Figure 7). The reduction is caused by the active compounds in the ethanolic extract, namely steroids. Steroids induce a negative feedback mechanism in the hypothalamus-pituitary and testis by inhibiting the secretion of LH and FSH, which subsequently disrupt the spermatogenesis process and decrease the sperm count (Hartini, 2011).

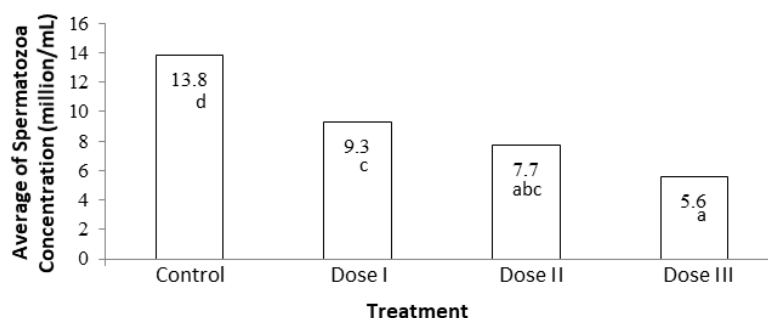


Figure 7. The average of spermatozoa concentration (%)

Notes: Numbers followed by different letters indicate a significant difference with a confidence level of 95%

Spermatozoa morphology

Morphology is one of the parameters determining the quality of spermatozoa. It determines the number of abnormal sperm cells. The results showed that the abnormalities were as follows: spermatozoa with bent tails (Figure 8B), spermatozoa without tails (Figure 8C), headless spermatozoa (Figure 8D), and spermatozoa with double heads (Figure 8E).

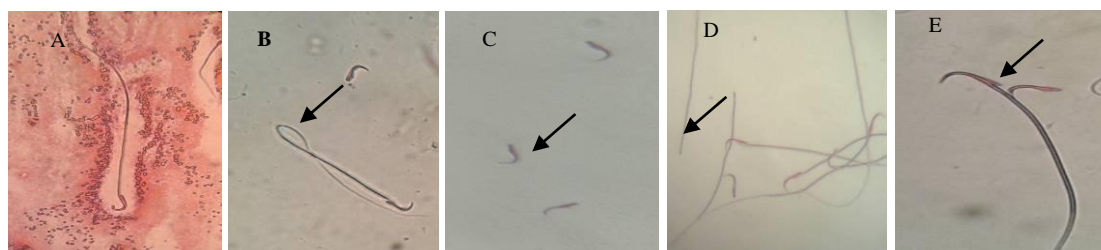


Figure 8. The morphology of the spermatozoa produced in the study

Notes: Staining: HE, microscopic, at 100x magnification

Based on the results of the study, each treatment produced different mean percentages of abnormal sperm cells (Figure 9). The administration of the ethanolic extract significantly increased the percentage of abnormal sperm cells.

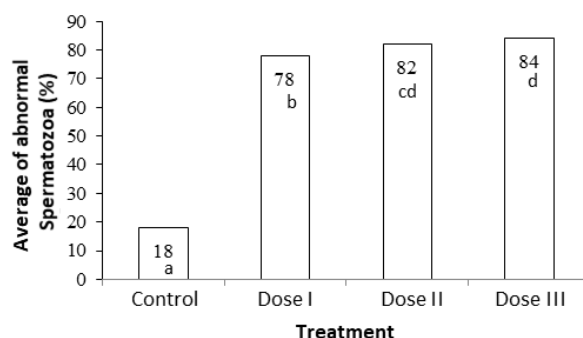


Figure 9. The average percentage of abnormal spermatozoa (%)

Notes: Numbers followed by different letters indicate a significant difference with a confidence level of 95%

An increase in the percentage of abnormal spermatozoa can occur due to various disorders in spermatogenesis, especially in the stage of spermiogenesis. One of the active substances contained in the ethanolic extract of sugar apple leaves (*Annona squamosa* L.) is flavonoid. Flavonoid can inhibit aromatase, i.e., an enzyme that catalyzes the aromatization of androgens into estrogens, which in turn increases androgens and subsequently testosterone. The high concentration of testosterone causes negative feedback to the pituitary, namely the inhibition of FSH and LH secretion and spermatogenesis. Another substance contained in the ethanolic extract of sugar apple leaves (*Annona squamosa* L.) is alkaloid. Alkaloids can affect spermatogenesis by suppressing the secretion of reproductive hormones (FSH and LH) needed in spermatogenesis (Hartini, 2011). Steroids can interfere with spermatogenesis because they are precursors of testosterone (Wahyuni, 2012).

CONCLUSIONS

The administration of the ethanolic extract of sugar apple leaves (*Annona squamosa* L.) can provide antifertility activities by reducing the testicular index, the diameter of seminiferous tubules, and the number of spermatogenic cells and spermatocytes. Besides, it can deteriorate the sperm quality by reducing motility and concentration and increasing morphological abnormalities. At a dose of 96.8 mg/Kg BW rat, the ethanolic extract produced the greatest antifertility activity.

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