

ANTISPERMATOGENIC ACTIVITY OF ETHANOLIC LEAVES EXTRACT OF CALINA PAPAYA ON SEMINIFEROUS TUBULES WISTAR RATS

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ABSTRACT

Indonesia is a country with a high birth rate which continues to increase each year. Methods to reduce the high birth rate are needed, especially herbal based contraceptives. This research aims to study the possibility of Calina papaya leaf ethanolic extract as an anti-spermatogenic agent which hopefully could be expanded into a natural antifertility drug candidate. Research was done using 24 white male Wistar rats divided into four groups. Dosages were given in 30 day intervals as follows: 0 mg/kg body weight (control group), 100 mg/kg body weight (P1), 200 mg/kg body weight (P2), and 300 mg/kg body weight (P3). Rats were euthanized on day 31 and the testes were processed histologically and stained using hematoxylin-eosin (HE). The diameter and surface area of seminiferous tubules, lumen surface area, and spermatogenesis index were then observed. Data were analysed using one way analysis of variance (ANOVA) and followed by Duncan multiple range test. Results showed that there was a significant difference ($P < 0.05$) in lumen surface area and spermatogenesis index between the control and treatment groups. It was determined that the optimum dosage given to attain the desirable effect was 300 mg/kg body weight. There was no significant difference found on surface area and diameter of tubulus seminiferous between the control and treatment group ($P > 0.05$). The conclusion from this research was that papaya leaf (Calina variety) ethanolic extract has potential as a natural anti-spermatogenic with an optimum dose of 300 mg/kg body weight.

Key words: anti-spermatogenic, Calina papaya leaf, seminiferous tubules, spermatogenesis index, testes

ABSTRAK

Indonesia merupakan negara dengan angka kelahiran yang relatif tinggi dan terus naik setiap tahunnya. Alternatif dalam menurunkan angka kelahiran adalah penggunaan alat kontrasepsi berbahan herbal. Penelitian bertujuan mengetahui potensi tanaman herbal ekstrak etanol daun pepaya varietas Calina sebagai agen antispermatogenik alami. Percobaan dilakukan selama 30 hari menggunakan 24 ekor tikus dibagi menjadi 4 kelompok yang terdiri atas kontrol (dosis 0 mg/kg BB), P1 (dosis 100 mg/kg BB), P2 (dosis 200 mg/kg BB), P3 (dosis 300 mg/kg BB). Pembedahan dilakukan pada hari ke-31 untuk mengambil testis. Testis dibuat sediaan histologis diwarnai dengan pewarnaan haematoxylin-eosin. Parameter yang diamati berupa diameter tubulus seminiferus, luas tubulus seminiferus, luas lumen dan indeks spermatogenesis. Data dianalisis menggunakan One Way Anova dengan uji lanjut Duncan multiple range test. Hasil yang diperoleh menunjukkan bahwa terdapat perbedaan signifikan ($P < 0,05$) pada luas lumen dan indeks spermatogenesis antar perlakuan dengan hasil yang optimum pada dosis 300 mg/kg BB dibandingkan dengan kontrol. Pada luas dan diameter tubulus seminiferus tidak menunjukkan perbedaan secara signifikan ($P > 0,05$). Kesimpulan dari penelitian ini adalah ekstrak etanol daun pepaya varietas Calina berpotensi menjadi agen antispermatogenik alami dengan pemberian dosis optimal pada 300 mg/kg BB.

Kata kunci: antispermatogenik, daun pepaya varietas Calina, testis, tubulus seminiferus, indeks spermatogenesis

INTRODUCTION

The population growth rate in Indonesia continues to increase each year (Akmal *et al.*, 2011). In 2018, the total amount of the population of Indonesian reached 265 million people (BPS, 2019). Uncontrolled growth rates could potentially lead to ill-favored effects such as a decline in overall life expectancy and public health, as well as an increase in poverty. The Indonesian government currently attempts to control the increasing population growth rate with contraception methods and infertility management (Bernadus *et al.*, 2013).

The utilization of contraceptives determines the successfulness of family planning programs. Contraceptives in men function as anti-spermatogenic agents which suppress the spermatogenesis in order to inhibit spermatozoa maturation and hinder spermatozoa transport in the vas deferens preventing fertilization (Setiawan *et al.*, 2015). However, the involvement of men in applying contraceptive methods is still poor, hence family planning programs are still not quite a success. There are some issues that arise for men in applying contraceptive methods, especially when

dealing with vasectomies. This method is reported to have caused disorders in the immune systems of men who have had the procedure. Another method is the utilization of condoms which some men find inconvenient.

Efforts to increase the participation of men in the use of contraceptives requires several alternatives, such as the use of herbal ingredients which have the potential to inhibit spermatogenesis and fertilization. Herbal ingredients which have the potential as anti-spermatogenic agents contain several secondary metabolite compounds such as alkaloids, papain, saponins, flavonoids, and tannins which might affect and disrupt physiological processes during fertilization (Milind and Gurdita, 2011).

Alkaloid compounds in some herbal plants can suppress the secretion of reproductive hormones which are needed for spermatogenesis (Akmal *et al.*, 2008). Tannin has the ability to coagulate sperm, thereby reducing sperm motility and viability. Secondary metabolites such as flavonoids can affect sperm quality and fertilization mechanisms (Prasetyaningrum *et al.*, 2015). Ashfahani *et al.* (2010) states that flavonoids

can reduce the motility and viability of spermatozoa. Papain can also interfere with the mechanisms responsible for Follicle Stimulating Hormone (FSH) and Leutinizing Hormone (LH) in spermatogenesis (Augustine *et al.*, 2019).

Indonesia is home to a wide range of herbal plants that contain natural secondary metabolites which have the potential to be candidates for anti-fertility drugs (Walansendow, 2016). One of the plants that thrives in Indonesia and is rich in secondary metabolite content is the papaya plant. Papaya plants (*Carica papaya* L.) are one of the plants that have been extensively used in traditional medicine (Milind and Gurdita, 2011). The papaya plant originates from the tropical Americas and is a plant that is widely cultivated in Indonesia. One of the superior papaya plant varieties is the Calina papaya. The Calina papaya variety is the result of plant breeding from the tropical fruit study center Institut Pertanian Bogor (PKBT-IPB), with the name IPB-9 or Calina (Ardiansyah, 2020).

Papaya leaves contain a wide range of metabolite compounds including: alkaloids, triterpenoids, steroids, flavonoids, saponins and tannins. Several types of secondary metabolites have similarities with other herbal plants that have the potential to be natural anti-spermatogenic agents (A'yun and Laily, 2015). As of now, there is no information and research development regarding the potential of Calina papaya leaves as a natural anti-permatogenic agent. Therefore, this research was conducted to determine the anti-spermatogenic potential of Calina papaya leaves ethanolic extract in Wistar rats.

MATERIALS AND METHODS

Preparation of Calina Papaya Leaves Ethanolic Extract

Papaya leaves were identified in the Laboratory of Botany, Universitas Ahmad Dahlan with the identification number: 149/Lab.Bio/B/X/2019 and name *Carica papaya* Var. Calina. Leaf simplicia weighing 11 kg was extracted using the maceration method with 96% ethanol as a solvent. The macerated product was evaporated using a rotary evaporator at a temperature of 60° C. The liquid extract obtained was evaporated using a water-bath to obtain a thick extract. The thick extract weighed 83.78 g with a yield of 8.37%.

Maintenance and Treatment of Experimental Animals

The study has received approval from the UAD Ethics Committee (No: 012002007). Experimental animals used were male Wistar rats (*Rattus norvegicus* Berkenhout, 1769). Rats were ± 12 weeks old and weighed 245-247 g. Acclimatization was carried out for 4 weeks. Feeding and drinking were done *ad libitum*. Twenty-four rats were divided into 4 treatment groups consisting of control (0 mg/kg body weight), P1 (100 mg/kg body weight), P2 (200 mg/kg body weight) and P3 (300 mg/kg body weight). Rats were administered

ethanolic extract of papaya leaf by gavage for 30 days. Weighing was done once a week as growth observation.

Preparation of Histological Specimen

On Day 31, rats were euthanized and the testicular organs were removed. The testicular organs were washed using 0.9% NaCl to remove debris and blood, then fixed using 10% Buffer Neutral Formalin (BNF) to make histological preparations. Paraffin blocks and hematoxylin-eosin staining were done to make a histological specimen.

Histological Observation of Seminiferous Tubule

Specimens were observed using Opti lab with 100x and 400x magnification. Parameters observed were: seminiferous tubule diameter, seminiferous tubule area, lumen area, and spermatogenesis index. Measurements and counting of sperm cells were done using Image raster 3 (Setiawan *et al.*, 2020). This is the formula that was used for calculating the spermatogenesis index (SI) (Kaspul, 2016).

$$SI = \frac{(\sum \text{Spermatocytes} + \sum \text{Spermatids})}{\sum \text{Spermatogonia} + \sum \text{Spermatocytes} + \sum \text{Spermatids}} \times 100\%$$

Data Analysis

Data obtained was processed using statistical analysis with One Way Anova, followed by Duncan multiple range test at the 5% confidence level.

RESULTS AND DISCUSSION

Body Weight

Measurements of body weight were taken to determine whether there were any growth affecting toxicity. It was stated that phytochemical compounds can produce side effects such as loss of weight and appetite in experimental animals. Mardiaty and Sitaswi (2016) stated that an increase in body weight is influenced by feed content. Variations in feed content and active ingredients at certain doses can affect animal growth and behavior. However, in this study, ethanolic extract of papaya leaf did not affect nutrient metabolism, cause any anabolic changes, or have any effect on appetite. This was proven by statistical analysis findings that determined there was no significant difference in body weight among treatments ($P > 0.05$) (Table 1).

During the 30 days of treatment, it was observed that all experimental animals gained weight in weekly intervals. Presumably, metabolic substances in all dose variations do not affect feed nutrient absorption. All experimental animals received identical feed with the same nutritional content. The insignificant weight gain of experimental animals between treatments showed that the secondary metabolite content in the papaya leaf ethanolic extract did not affect the digestive systems of the rats or interfere with the absorption of nutrients during the treatment. This indicates that papaya leaf ethanolic extract is safe for consumption and does not cause symptoms related to toxicity.

Testicular Weight

The testes are the main glands in the male reproductive system which is responsible in the production of spermatozoa and synthesis of androgen hormones (Hughes and Acerini, 2008). Spermatozoa are the result of the spermatogenesis in the seminiferous tubules which is influenced by testosterone (Susetyarini *et al.*, 2015). The seminiferous tubule is the largest constituent component in the testes. If there is a decrease in testicular weight, it can be a result of damage and or atrophy of cells making up the seminiferous tubules (Musfirah *et al.*, 2016).

Results of testicular weight observation showed that there was a significant difference in the mean of testicular weight between the control and treatment groups ($P < 0.05$). The highest mean of testicular weight was in the control group, while the lowest was in the P3 group (dose 300 mg/kg body weight) (Table 2). Testicular weight and body weight ratios showed normal ratios in all treatment groups ($P > 0.05$).

Jahan *et al.* (2009) stated that microscopically, spermatogenesis can be seen from changes in testicular weight. Reduced cell size can be caused by a decrease in the number of organelles in the cytoplasm and a

decrease in metabolism. Proliferation imbalance and cell death can render decreases in cell numbers. According to Cholifah *et al.* (2014), deflation of FSH and LH production might induce a decrease in testicular weight. The two hormones do not work optimally due to inhibition of Gonadotropin Releasing Hormone (GnRH) secretion by the testicular pituitary hypothalamus. Therefore, FSH affects the Sertoli cells to stimulate the formation of germinal cells in spermatogenesis and LH affects the Leydig cells to secrete testosterone. Setiawan and Yunianto (2016) stated that tannins might have cytotoxic effect in cells, therefore affecting the number of cells that constitute the testes. It is suspected that secondary metabolite components such as tannins in the papaya leaf ethanolic extract affect testicular weight by cytotoxic effects in the testicular organs.

Research by Lohiya *et al.* (2005) proved that the active components in the papaya ethanolic can cause vacuolization in the cytoplasm of Sertoli cells and damage some organelles in the cytoplasm, thereby reducing their metabolic activity. The Sertoli cells abnormality will inhibit the differentiation and maturation of spermatogenic cells, especially spermatids and spermatozoa, thereby causing a

Table 1. Experimental animal body weight after treatment with papaya leaf ethanolic extract for 30 days

Week	Body weight (g)			
	K	P1	P2	P3
0	256.16±27.80 ^a	260.66±44.31 ^a	250.16±34.02 ^a	259.00±32.56 ^a
1	258.50±32.04 ^a	283.16±54.31 ^a	256.50±28.34 ^a	268.50±28.20 ^a
2	277.33±29.13 ^a	285.83±35.47 ^a	275.66±31.02 ^a	272.83±32.43 ^a
3	280.66±20.43 ^a	301.50±31.49 ^a	290.33±38.28 ^a	292.50±30.70 ^a
4	300.16±20.51 ^a	309.00±41.11 ^a	295.16±38.14 ^a	293.00±22.33 ^a

K= 0 mg/kg body weight, P1= 100 mg/kg body weight, P2= 200 mg/kg body weight, P3= 300 mg/kg body weight

Table 2. Testicular weight of experimental animals after treatment with papaya leaf ethanolic extract for 30 days

Treatment	Testicular weight (g)	Testicular weight ratio
K	1.38±0.11 ^b	0.45±0.03 ^a
P1	1.37±0.13 ^b	0.44±0.09 ^a
P2	1.24±0.06 ^{ab}	0.42±0.05 ^a
P3	1.21±0.13 ^a	0.41±0.05 ^a

^{a, b, ab} Different superscripts within the same column indicate significant differences ($P < 0.05$). K= 0 mg/kg body weight, P1= 100 mg/kg body weight, P2= 200 mg/kg body weight, P3= 300 mg/kg body weight

Table 3. Diameter of seminiferous tubule, surface area of seminiferous tubule (μm^2) and lumen surface area of seminiferous tubule after papaya leaf ethanolic extract treatment for 30 days

Treatment	Seminiferous tubule diameter (μm)	Surface area of seminiferous tubule (μm^2)	Lumen surface area of seminiferous tubule (μm^2)
K	279.21±26.15 ^a	60,860.60±12,033.62 ^a	7,143.47±655.67 ^a
P1	279.29±23.49 ^a	62,626.07±12,956.11 ^a	7,409.04±2,232.98 ^a
P2	255.26±18.10 ^a	52,431.90±9,293.33 ^a	8,847.34±1,284.88 ^a
P3	261.46±14.82 ^a	55,373.44±6,578.65 ^a	14,013.98±3,023.19 ^b

^{a, b} Different superscripts within the same column indicate significant differences ($P < 0.05$). K= 0 mg/kg body weight, P1= 100 mg/kg body weight, P2= 200 mg/kg body weight, P3= 300 mg/kg body weight

Table 4. Number of spermatogenic cells after papaya leaf ethanolic extract treatment for 30 days

Treatment	Number of spermatogenic cell			Spermatogenesis index (%)
	Spermatogonia	Spermatocyt	Spermatid	
K	45.83±7.65 ^c	66.83±7.60 ^b	62.00±7.97 ^d	75.42±2.00 ^a
P1	33.83±3.48 ^b	59.33±11.18 ^b	45.66±5.31 ^c	74.38±3.57 ^{ab}
P2	31.00±2.28 ^{ab}	47.83±8.79 ^a	33.00±5.05 ^b	71.97±2.82 ^{ab}
P3	27.50±4.03 ^a	41.00±7.12 ^a	26.16±2.92 ^a	70.89±4.49 ^c

^{a, b, c} Different superscripts within the same column indicate significant differences ($P < 0.05$). K= 0 mg/kg body weight, P1= 100 mg/kg body weight, P2= 200 mg/kg body weight, P3= 300 mg/kg body weight

decrease in testicular weight. According to Setiawan and Yunianto (2015) flavonoid content in medicinal plants can affect the maturation of spermatogenic cells and has an impact on reducing testicular weight. It is suspected that flavonoids contained in papaya leaves of Calina variety have the same effect in reducing testicular weight.

Histology of Seminiferous Tubules

Observation of seminiferous tubule histology aims to determine microscopic changes seen from seminiferous tubule diameter, seminiferous tubule surface area, surface area of the lumen and number of spermatogenic cells due to the administration of papaya leaf ethanolic extract. Based on the observation of seminiferous tubule tissue (Figure 1), it can be seen that

there are differences in surface area of the lumen and spermatogenic cell arrangement. The control group showed that the spermatogenic cells in the seminiferous tubules were arranged normally, which is neat and tight. The lumen was completely filled with spermatozoa; however, the P1 group showed that the arrangement of spermatogenic cells was less neat and tight compared to the control group. P2 and P3 groups showed increasing damage to the seminiferous tubules. The spermatogenic cells in the seminiferous tubules had begun to spread irregularly, the lumen became wider, and the spermatozoa in the lumen were almost completely absent.

The observations of seminiferous tubule diameter, seminiferous tubule surface area, surface area of the lumen and number of spermatogenic cells showed that

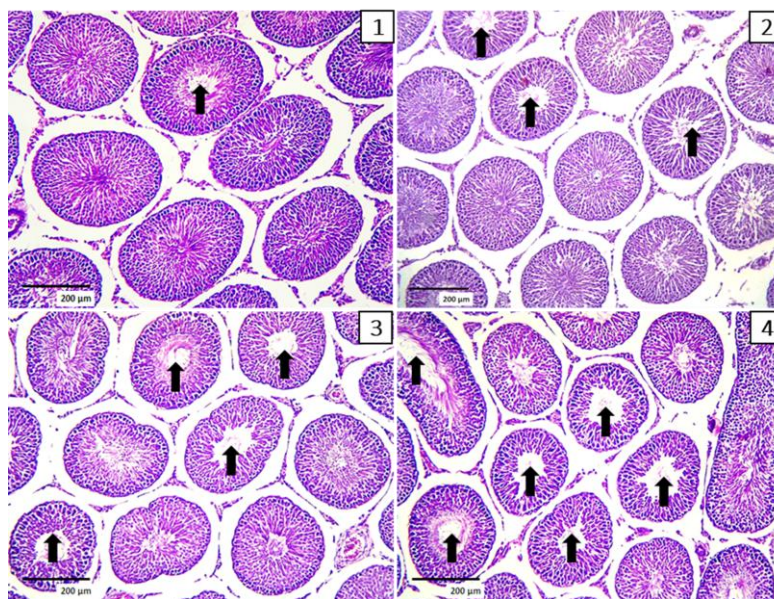


Figure 1. Histology of rat testes after papaya leaf ethanolic extracts treatment. 1= K (0 mg/kg body weight), 2= P1 (100 mg/kg body weight), 3= P2 (200 mg/kg body weight), 4= P3 (300 mg/kg body weight). The arrows show the expansion of the lumen in the seminiferous tubule tissue. Scale bare represent 200 µm. HE. 100x

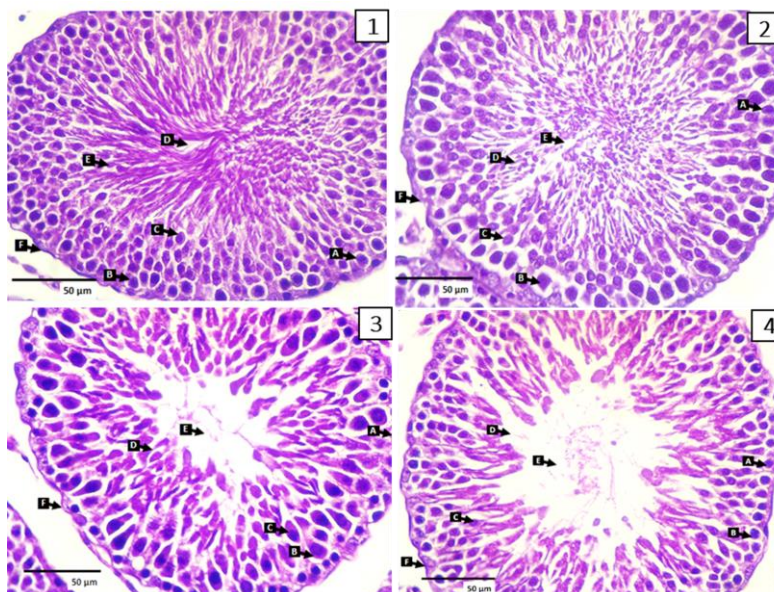


Figure 2. Histology of rat testes after papaya leaf ethanolic extract treatment. 1= K (0 mg/kg body weight), 2= P1 (100 mg/kg body weight), 3= P2 (200 mg/kg body weight), 4= P3 (300 mg/kg body weight). A= Spermatogonia, B= Spermatocytes, C= Spermatid, D= Spermatozoa, E= Lumen, F= Basal lamina. Scale bare represent 50 µm. HE. 400x

the administration of papaya leaf ethanolic extract did not significantly affect the diameter and area of seminiferous tubules ($P>0.05$) (Table 3). This proves that papaya leaf ethanolic extract does not have a significant effect on the diameter and area of seminiferous tubules. The diameter and area of the seminiferous tubules in all treatments were relatively the same and there was no visible inflammation or hemorrhage upon microscopic observation (Table 3). This shows that the papaya leaf ethanolic extract did not affect the size of seminiferous tubule in rats.

There is a significant difference between groups in the seminiferous tubule lumen ($P<0.05$). Data showed that there was a decrease in lumen surface area the coincided with the increasing dose given. The highest value of lumen surface area was found in P3 group (dose 300 mg/kg body weight) compared to the control. The lumen in the control group was full of spermatogenic cells, whereas in the treatment groups the lumen was more vacuous the more the dose was given (Figure 2). Anti-fertility compounds might work as cytotoxic agents by triggering apoptosis of spermatogenic cells (Husni and Sukesu, 2016). Flavonoids and terpenoids can function as anti-fertility agents, which is indicated by a decrease in the number of spermatogenic cells and an increase in the lumen surface area of the seminiferous tubules (Ain *et al.*, 2018). Spermatocytes are very sensitive to external influences and are easily damaged. If there is damage to spermatocytes, degeneration will occur by means of phagocytosis by Sertoli cells thereby reducing spermatocyte numbers (Johnsons and Everitt, 2018).

De Souza *et al.* (2017) proved that steroids can cause a reduction in the surface area and diameter of seminiferous tubules. Yama *et al.* (2011) also stated that if the levels of testosterone and FSH are reduced, it might cause atrophy of the seminiferous tubules, resulting in a smaller tubular diameter and surface area. The disruption of spermatogenesis will affect the number of germ cells in the testes. The diminishing number of spermatogenic cells leads to morphological changes and an increase in lumen surface area in the seminiferous tubules. The papaya leaf ethanolic extract is thought to have a cytotoxic effect which reduces the number of spermatogenic cells and increases lumen surface area in seminiferous tubules.

Based on the result (Table 4), it is shown that papaya leaf ethanolic extract has an effect on the number spermatogenic cells ($P<0.05$). The active ingredients contained in the ethanolic extract are thought to be able to cause spermatogenesis disorders in rats. Milind and Gurdita (2011) stated that alkaloid compounds, papain, saponins, flavonoids, and tannins can be used as anti-spermatogenic and anti-fertility agents.

LH and FSH are regulated by GnRH. LH functions to stimulate Leydig cells to produce testosterone. FSH functions to stimulate the Sertoli cells in (Cholifah *et al.*, 2014). It is suspected that papaya leaf ethanolic extract decreases FSH and LH levels, thus inhibiting cell proliferation activity and causes a decrease in the number of spermatogenic cells (Sihombing *et al.*, 2015).

Saponins are active secondary metabolites derived from steroids found in plants and might play a role in spermatogenesis (Rajan *et al.*, 2013). Saponins can interfere with hormone performance because they affect the biosynthetic pathway, thereby disrupting testosterone activity (antitestosterone). Saponins bind to testosterone receptors so that testosterone becomes unable to bind to cell receptors in the seminiferous tubules to induce spermatogenesis (Fajria, 2011). Alkaloid compounds can suppress testosterone secretion causing spermatogenesis disruption (Susetyarini *et al.*, 2015). It is suspected that saponins and alkaloids contained in the papaya leaf ethanolic extract have the same effect in disrupting the biosynthetic pathway of testosterone.

Flavonoid compounds can also play a role in stimulating estrogen synthesis. Estrogen and estrogenic substances will suppress FSH and LH (Susetyarini *et al.*, 2015). These estrogenic compounds can stimulate a negative feedback response to the hypothalamus and pituitary gland, causing a decrease in both FSH and LH production. The decrease in LH synthesis will trigger a decrease in the synthesis of testosterone by Leydig cells. The decrease in FSH synthesis will affect the performance of Sertoli cells as nutrient providers for spermatogenic cells. It is suspected that the flavonoids contained in the extract can increase negative feedback responses because they are estrogenic (Alfian *et al.*, 2018).

Reduction of the number of spermatocytes might have a role in the declining number of spermatids. Phagocytosis of damaged spermatogenic cells causes irregular arrangement of spermatogenic cells in the seminiferous tubules. The wide lumen of seminiferous tubule is due to a spermiogenesis disorder which causes a decrease in the number of spermatids and automatically affects the production of spermatozoa (Sukmaningsih, 2009). The papaya leaf ethanolic extract is a potential candidate for anti-spermatogenic agents because it has the ability to reduce the spermatogenesis index and increase the lumen surface area in the seminiferous tubules.

CONCLUSION

Based on the results showed above, papaya leaf ethanolic extract (*Carica papaya* Var. Calina) has the potential to be a natural anti-spermatogenic agent with the optimum dose of 300 mg/kg body weight.

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