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Research Article

The Effect of Ethanol Extract of *Piper nigrum* L. Fruit on Reproductive System in Adult Male Wistar Rats: A Study of FSH, LH, Testosterone Level and Spermatogenic Cells

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ABSTRACT

Exploration to find out new natural contraceptive agent for male is still being developed. Black pepper (*Piper nigrum* L.) and its main alkaloid piperine have potential antifertility because of sitotoxic and hormonal effects. The aim of this study is to find out the effect of ethanolic extract of black pepper (*Piper nigrum* L.) fruit of on reproductive hormone serum level, sperm quality, and spermatogenic cell populations in adult male Wistar rat. Twenty five male rats were divided into five groups consisting of two control group, i.e. $K_{(-)}$ (Na-CMC 0.5%), $K_{(+)}$ (finasteride 0.45mg/kg BW), and three groups received different doses of black pepper fruit ethanolic extract, i.e. $D_{(1)}$ (3.33mg/kg BW), $D_{(2)}$ (6.66mg/kg BW) and $D_{(3)}$ (13.32mg/kg BW) respectively. The treatment were given to each group for 55 days. Reproductive parameters were measured, including serum level of reproductive hormones (FSH, LH, dan testosteron), quality of cauda epididymal sperm (spermatozoa concentration, motility and morphological abnormality), spermatogenic cell populations (primary spermatocyte and spermatid count) and seminiferous tubules diameter. Ethanol extract of black pepper fruit at doses of 3.33mg/kg BW, 6.66mg/kg BW, and 13.32mg/kg BW increased serum FSH level. Extract at dose of 13.32mg/kg BW decreased serum LH level, while extract at doses of 6.66mg/kg BW and 13.32mg/kg BW decreased serum testosterone level. The number of primary spermatocytes, spermatozoa concentration, and spermatozoa motility were decreased by administration of ethanol extract of black pepper fruit with dose of 6.66mg/kg BW and 13.32mg/kg BW. Ethanol extract of black pepper fruit at dose of 6.66mg/kg BW and 13.32mg/kg BW had a negative impact on the male reproductive system and showing potential antifertility in male rat.

Keyword: black pepper (*Piper nigrum* L.), FSH, LH, male rat, testosterone, spermatogenic cells

INTRODUCTION

Based on 2012 Indonesia Demographic and Health Survey (IDHS) data, the prevalence of contraceptive methods use in Indonesia among married women aged 15-49 is 61.9%, while among married men is 4.7% (Anonim, 2013). The low rate of contraceptive use in men due to the lack of contraceptive information, limited contraception, and limited Family Planning services for men (PUBIO, 1999). Application of medicinal plants in traditional treatment is a local wisdom of Indonesia society. Medicinal plants considered to have less toxicity and minimal side effects. It was found that 74 plant species empirically used by Indonesian people as traditional contraceptives (Nurhuda *et al.*, 1995). Through the results of the study, there are 13 of them potentially used as male contraceptive because they have antispermatogenic effect. Some of these plants are cantel (*Androphogon sorghum*), kayu api-api leaf and fruit (*Avicinia officinale*), papaya seed (*Carica papaya*), pacing seed (*Costus speciosus*), mangosteen leaf (*Garcinia mengostana*), hibiscus flower (*Hibiscus rosa-sinensis* L.), and oyong seed (*Luffa acutangula*) (Winarso and Sundari, 1997).

Black pepper (*Piper nigrum* L.) and its main alkaloid piperine have previously been shown in study to have antifertility and antispermatogenic effects in male rats characterized by degenerative changes on seminiferous tubules, epididymis, and sperm parameters (Mishra and Singh, 2009). Black pepper has 5α-reductase inhibitor activity which can affect reproductive hormone imbalance, so black pepper maybe a potential natural contraceptive agent for male (Hirata *et al.*, 2007). The study will examine the effect of black pepper on reproductive parameters by giving ethanol extract of black pepper fruit at various doses to adult male rats and evaluate their impact on reproductive hormone levels and spermatogenic cell populations.

MATERIAL AND METHODS

Plant extract

Dried fruit of black pepper obtained from farmer in Lampung province is determined by Department of Pharmaceutical Biology, Faculty of Pharmacy UGM. Determination is done to confirm the desired species of pepper, *Piper nigrum* L. as evidenced by the certificate No: UGM/FA/0080/M/03/02. The extraction of black pepper is conducted at Research Laboratory, Department of Pharmaceutical Biology, Faculty of Pharmacy UGM. The extraction was performed by maceration technique (Sutyarso *et al.*, 2016). Each 50g of pepper powder were macerated with 500mL of 96% ethanol solvent for 3×24h at room temperature. The macerate thus obtained was filtered with Whatman No.1 filter paper and the filtrate was evaporated using water bath until yellowish brown paste was obtained. Then the extract was weighed for oral administration of individual rat according to the doses used, ie 3.33, 6.66, and 13.32mg/kg BW of rat.

Experimental design and animals

An experimental post-test only randomized controlled study was design among twenty five adult male Wistar rats divided into five groups consisting of negative control group or $K_{(-)}$ (Na-CMC 0.5%), positive control group or $K_{(+)}$ (finasteride 0,45mg/kg BW) and three groups received different doses of black pepper fruit ethanolic extract, i.e. $D_{(1)}$ (3.33mg/kg BW), $D_{(2)}$ (6.66mg/kg BW) and $D_{(3)}$ (13.32mg/kg BW). The treatments were administered once daily for 55d. All of the research procedure was done with the approval and supervision of Animal Ethics Committee LPPT UGM No: 00022/04/LPPT/IV/2017.

Hormone assay

Orbital sinus blood sample was collected about 3mL in microtube for each rat. Blood is centrifuged at 3000rpm (rounds per minute) for 10m then stored in refrigerator at -20°C. Serum hormone levels of Testostron, LH and FSH was measured at the Parasitology Laboratory, Faculty of Medicine UGM using ELISA (Enzym-Linked Immunosorbent Assay) method according to the procedures listed in the ELISA Elabscience manual book.

Sperm analysis and testis histological study

The animals were sacrificed by intramuscular ketamine injection dose 100 mg/kg BW following cervical dislocation. Right testis and cauda epididymis were taken from the animals using disecting kit. Spermatozoa concentration analysis was done according to Ilyas method (Ilyas, 2007). Motility and morphological abnormality examination of spermatozoa were performed according to WHO (WHO, 2010). For histological examination (seminiferous tubular diameter and number of primary spermatocyte as well as spermatid), testicular tissue were stained with haematoxylin-eosin (HE) conducted at Anatomical Pathology Laboratory, Faculty of Medicine UGM.

Statistical analysis

Statistics that applied are programmed in SPSS version 17. The data were analysed with one way-ANOVA and continued with post hoc LSD (Least Significant Different) test. Non-normal distributed or non-homogeneous data were tested with alternative nonparametric statistics, Kruskal-Wallis test and continued with Mann-Whitney test.

RESULTS AND DISCUSSION

Body weight gain and testis weight

Table I show that percentage of body weight gain at the end of treatment (medial-final) of D(2) and D(3) are significantly less than K(-), K(+) and D(1), respectively. Significant decrease was observed in absolute testis weight of D(2)

Table I. Effect of etanol extract black pepper of on body weight and body weight gain

Group	Values are mean±SD of 5 rats				
	Initial BW Week-0 (g)	**Medial BW Week-4 (g)**	**Final BW Week-8 (g)**	**BW gain initial-medial (%)**	**BW gain medial-final (%)**
K(-)	200.2±21.9	257.4±33.8	283.8±19.4	28.8±12.5	11.4±13.3
K(+)	207.0±7.8	261.4±18.5	282.4±16.5	26.3±7.1	8.2±6.3
D(1)	225.4±12.5	272.0±24.6	298.4±20.6	20.7±8.6	10.8±16.1
D(2)	241.2±21.6	290.2±28.7	238.2±22.3	20.3±6.4	-16.8±14.7 [a,b,c]
D(3)	209.0±28.2	264.4±22.8	233.4±29.4	27.4±12.8	-11.7±8.5 [a,b,c]

a: significantly different than K(-); b: significantly different than K(+); c; significantly different than D(1); d: significantly different than D(2); e: significantly different than D(3); statistically significant as $p < 0.05$ analyzed by one way Anova followed by LSD test.

Table II. Effect of etanol extract black pepper of on testis weight

Group n=25	Values are mean±SD of 5 rats	
	Absolute (g)	**Relative (%)**
K(-)	1.26±0.05	0.47±0.04
K(+)	1.28±0.06	0.45±0.04
D(1)	1.28±0.07	0.43±0.02
D(2)	0.97±0.14 [a,b,c]	0.41±0.04
D(3)	1.04±0.09 [a,b,c]	0.45±0.10

a: significantly different than K(-); b: significantly different than K(+); c; significantly different than D(1); d: significantly different than D(2); e: significantly different than D(3); statistically significant as $p<0.05$ analyzed by one way Anova followed by LSD test.

and D(3) compared to K(-), K(+) and D(1), respectively (Table II). Reduction in relative testis weight was detected at K(+), D(1), D(2), and D(3) compared to K(-), but not statistically significant. The administration of black pepper extract affects body weight. Towards the end of treatment at week 8, the percentage of body weight gain of D(1), D(2), and D(3) was reduced even body weight loss occured in D(2) and D(3) (Table I). Black pepper can lead to body weight lose by decreasing fat mass. Study reveals decrease in total cholesterol, free fatty acids, phospholipids and triglycerides in mice given black pepper powder or piperine, but the final body weight did not change significantly because mice always administered high-fat diet (Vijayakumar *et al*., 2002).

Overview of testis histological

Cross-sectional view of testis histology indicates testicular degeneration in K(+) and D(3), while K(-) and D(1) is normal. Histologycal changes of D(3) show the most damage, including disorganized spermatogenic cells arrangement, mononuclear cells accumulation in tubular lumen, lysis of basal lamina tissue, and damaged interstitial tissue of testis (Figure 1).

Chemical compounds threaten male fertility such as defect in spermatogenesis are related to their effect on sexual hormonal imbalance and/or ROS production in the testicular and epididymal environment (Choobineh *et al.,* 2016). Infertility caused by certain chemical compounds can be reversed or may be permanent, so further investigations are necessary before certain compounds used for contraceptives (Alonso-Alvarez *et al*., 2007). Black pepper extract affects absolute testis weight but not relative testis weight because decrease in testis weight is associated with body weight loss (Table II). Testis weight is influenced by condition of testicular tissue. Histologic changes were shown in seminiferous tubules testis which treated with black pepper extract compared with negative control (Figure 1).

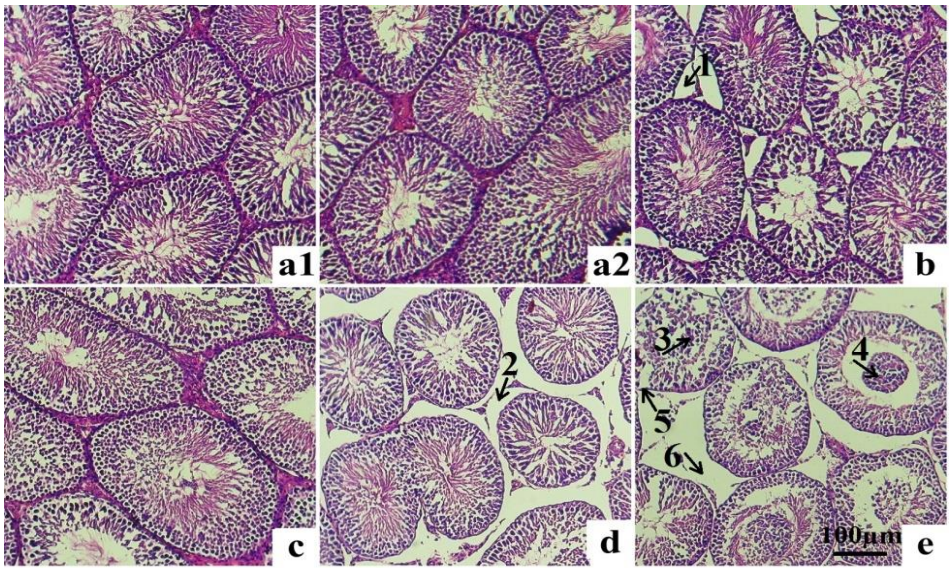

Figure 1. Effect of etanol extract black pepper of on testis histological a1 and a2: K(-) treated with 0.5% Na-CMC; b: K(+) treated with finasteride 0.45mg/kg BW showed interstitial tissue damage [1]; c: D(1) treated with extract dose 3.33mg/kg BW; d: D(2) treated with extract dose 6.66mg/kg BW showed interstitial tissue damage [2]; e: D(3) treated with extract dose 13.32mg/kg BW showed disorganized spermatogenic cells arrangement [3], mononuclear cells accumulation in tubular lumen [4], lysis of basal lamina tissue [5], interstitial tissue damage [6]; 100x magnification, hematoxylin-eosin (HE) staining

Table III. Effect of etanol extract black pepper of on seminiferous tubules diameter, primary spermatocyte, and spermatid number

Group	Values are mean±SD of 5 rats		
	Seminiferous Tubules Diameter (µm)	Primary Spermatocyte Number	Spermatid Number
K(-)	231.3±15.0	57.4±7.9	165.0±30.4
K(+)	232.3±19.1	58.6±9.9	180.9±30.1
D(1)	248.5±18.0	52.5±3.6	183.6±18.0
D(2)	212.7±22.1 [c]	39.6±9.8 [a,b,c]	147.5±41.2
D(3)	210.3±20.4 [c]	39.8±6.5 [a,b,c]	138.3±29.8

a: significantly different than K(-); b: significantly different than K(+); c; significantly different than D(1); d: significantly different than D(2); e: significantly different than D(3); statistically significant as $p<0.05$ analyzed by one way Anova followed by LSD test.

Administration of finasteride and black pepper extract in D(2) and D(3) has negative effect on germinal epithelium, such as necrotic cells in the lumen of the seminiferous tubules in D(3). However, degenerative changes in testicular tissue did not occur in the group given extract of low-dose black pepper D(1). Thus, black pepper has dose-dependent effect on testicular tissue damage.

Seminiferous tubules, spermatogenic cells and sperm analysis

Table III shows significantly decreased seminiferous tubules diameter of D(2) and D(3) compared to D(1). The number of primary spermatocytes of D(2) and D (3) are significantly lower than K(-), K(+) and D(1), respectively. Reduction in number of spermatid is detected at D(2) and D(3) compared to K(-), K(+), and D(1),

Table IV. Effect of black pepper etanol extract on concentration, motility, morphological abnormality of cauda epididymal spermatozoa

Group	Values are mean±SD of 5 rats		
	Concentration (cells/mL suspension)	Motility (%)	Morphological Abnormality (%)
K(-)	182.50±20.15	47.9±2.5	31.6±5.8 [b]
K(+)	200.00±28.77	34.1±4.2 [a,c]	46.3±3.0
D(1)	192.00±19.63	53.3±5.9	27.8±6.0 [b]
D(2)	128.50±46.05 [a,b,c]	36.9±6.0 [a,c,e]	30.3±2.2 [b]
D(3)	121.00±38.95 [a,b,c]	27.3±6.9 [a,c,d]	29.8±2.8 [b]

a: significantly different than K(-); b: significantly different than K(+); c; significantly different than D(1); d: significantly different than D(2); e: significantly different than D(3); statistically significant as $p<0.05$ analyzed by one way Anova followed by LSD test.

respectively but not statistically significant. Significant decrease was observed in spermatozoa concentration of D(2) and D(3) compared to K(-), K(+) and D(1), respectively. Spermatozoa motility of K (+), D(2) and D(3) are significantly lower than K(-) and D(1), respectively. Spermatozoa morphologycal abnormality of K(+) is significantly higher than all the rest of groups (Table IV).

At the testicular level, it has been observed that black pepper extract dose 6.66 and 13.32mg/kg WB acted as antispermatogenic caused significantly decrease in seminiferous tubules diameter and number of primary spermatocytes (Table III). Similar results have been previously reported by Mishra and Singh using black pepper powder (Mishra and Singh, 2009). The study by Malini *et al.,* revealed that administration of piperin dose 5mg/kg BW caused partial damage of spermatogenic cells, whereas piperine dose 10mg/kg BW caused seminiferous tubular damage and decrease in diameter of seminiferous tubules, number of spermatocyte as well as number of spermatid (Malini *et al*., 1999). Black pepper extract not only affected spermatogenic cells at the testicular level, but also in the epididymal environment and then caused decrease in concentation of cauda epididymal spermatozoa in D(2) and D(3). Administration of finasteride 0.45mg/kg WB caused decrease in motility and increase in morphological abnormality of spermatozoa (Table IV). Results of this study is consequent with the results obtained by Serga who gave the same dose of finasteride in mice for two months. Finasteride administration significantly reduced plasma glutathione levels associated with an increase in ROS level that triggered oxidative stress. Finasteride is known to inhibit 5α-reductase that converts testosterone to DHT, so circulating testosterone levels may increase (Serga, 2009). Clinical studies of the finasteride effects on fertility problems has been conducted and revealed that the use of low-dose Finasteride in humans (1mg) not associated with spermatogenesis. However, the use of finasteride at higher dose (5mg) has a negative effect on spermatogenesis (Amory *et al.,* 2007; Laborde and Brannigan, 2010). The finasteride dose we used was 0.45mg/kg BW in mice equivalent to 5mg dose for humans, but our experiment did not thoroughly affect spermato-genesis and hormonal abnormalities compared to negative control. Causes of spermatogenesis include oxidative stress in the testicular and epididymal environments and decrease in sialic acid levels secreted epididymic epithelium are under androgen control (Mishra and Singh, 2009; D'cruz *et al.,* 2008; Janarthanan *et al.,* 2014). Administration of high-dose piperine 100mg/kg BW for 30d can decrease antioxidant enzymes in the testis and epididymis such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase and increase free radical levels such as hydrogen peroxide which is a reactive oxygen species (ROS) in mice. Hydrogen peroxide is converted by the reaction of fenton to hydroxyl radical which causes lipid peroxidation as the main manifestation of oxidative stress (D'cruz *et al*., 2008).

Table V. Effect of etanol extract black pepper of on serum FSH, LH, and testosterone hormone levels

Group n=25	Values are mean ± SD of 5 rats		
	FSH[1] (ng/mL)	LH[2] (ng/mL)	Testosterone[2] (ng/mL)
K(-)	33.7±9.1	38.1±23.3	6.8±2.2
K(+)	51.4±7.9 [a]	22.1±13.1	3.7±1.7
D(1)	50.8±9.7 [a]	45.4±23.3	4.8±2.1
D(2)	48.9±12.7 [a]	24.7±4.3	3.8±0.8 [a]
D(3)	51.5±6.9 [a]	17.0±7.4 [c]	4.4±1.9 [a]

a: significantly different than K(-); b: significantly different than K(+); c; significantly different than D(1); d: significantly different than D(2); e: significantly different than D(3); statistically significant as $p<0.05$ analyzed by [1] one way Anova followed by LSD test and [2] *Kruskal-Wallis test* followed by *Mann-Whitney test*.

Serum FSH, LH, testosterone hormone levels

Table V shows significantly decreased serum FSH level of K(-) compared to K(+), D(1), D(2) and D(3), respectively. Serum LH level of D(3) is significantly lower than D(1). Serum testosterone level is significantly higher compared to D(2) and D(3).

Black pepper has an impact on hormone imbalance (Table V). The decrease of LH and testosterone may be related to the effect of piperine on the hypothalamus-testis axis. D(2) and D(3) received medium-dose and high-dose black pepper extract contained more piperine. Piperine is able to inhibit the activity of 5α-reductase in vitro, so there is the possibility of excessive testosterone levels in the testicular area (Hirata *et al.*, 2007). The amount of testosterone are released from the testis to the bloodstream and stimulus of testosterone levels above normal is accepted by receptors in the central nervous system. These signals play a role in decreasing LH secretion directly from the anterior pituitary and decreasing GnRH secretion from the hypothalamus resulting in reduced LH level indirectly. In response to the lack of LH levels, the production of testosterone by Leydig cells is inhibited (Cheng, 2008). Thus in this study, their level may decrease.Our data show that not all gonadotropin hormone levels was reduced. An elevated FSH occurs in K(+), D(1), D(2), and D(3) compared to K(-). FSH is a hormone that works on Sertoli cells to maintain the development of spermatogenesis and produce ABP (androgen binding protein). Administration black pepper extract and finasteride with high-doses and in the long term can stimulate increasing serum FSH levels. It was probably caused by necrosis or apoptosis occured in Sertoli cells and FSH failed to bind to its receptors. Sertoli cells damage may lead to limited inhibin production which affect imbalance of circulating FSH levels. However, D(1) with elevated FSH did not show signs of histological testis damage due to the compensation of the decreased testosterone levels but not as low as K(+), D(2) and D(3). In this study, finasteride effect on testosterone levels was similar to K (-) in accordance with the study of (Soni *et al.,* 2017). Finasteride administration in rat for 90d increased dihydrotestosterone significantly. However, testosterone levels in normal rats and finasteride group was similar.

The pattern of hormonal levels in this study was similar to previous study with administration of caricapryl-99 alkaloids obtained from papaya seed extract (*Carica papaya*) in male rats caused decreased in levels of LH and testosterone and increased in level of FSH and estrogen (Udoh *et al.,* 2009). There were results of a study reported that serum testosterone levels of male rats treated with aquous extract of *Piper guineense* dose 122 and 245mg/kg BW were elevated, but decreased testosterone level occured in group dose of 245mg/kg BB compared to dose of 122.5mg/kg BW (Mbongue *et al.,* 2005). Ethanol extract of *Piper nigrum* dose 3mg/kg BB can increased serum testosterone in male mice (Sutyarso *et al.,* 2016). Differences in the results of the study are due to different extraction methods and doses.

Black pepper does not only contain piperine but also flavonoids, phenolic acids, and phenolic amides that act as exogenous antioxidants. Flavonoids and phenolic compounds are polyphenols, a group of chemicals that have many phenolic groups. Phenol is a hydroxyl group (-OH) attached to a phenyl ring. Flavonoids and phenolic acids are powerful antioxidant compounds and positively affect fertility parameters. The administration of ethanol extract of *Urtica dioica* (nettle) 100mg/kg BW containing flavonoids and phenolic compounds for 5 days may increase in diameter of seminiferous tubules and seminiferous epithelial thickness in male rats (Golalipour *et al.,* 2011). However, flavonoids and phenolic acids may also negatively affect fertility parameters if these components are given at high-doses (Shehab and Abu-Gharbieh, 2014).Piperine contained in black pepper extract causes spermatogenesis abnormalities because it has properties as an anti-androgen (Hirata *et al.,* 2007; Chinta *et al.,* 2015). Not only piperine, but also fatty acids such as oleic acid and palmitic contained in black pepper fruit have anti-androgen properties (Matsuda *et al.,* 2002). Ethanol and methanol extract of black pepper leaf contained piperine have activity as inhibitor of 5α-reductase tested in vitro and in vivo. 5α-reductase is an enzyme that converts testosterone to DHT (Hirata *et al.,* 2007).There was in vitro and in vitro study investigated docking and molecular dynamics simulation of piperine showed that it was able to interact stable with androgen receptors in the same way as CPA (cyproterone acetate) antagonists. Thus, piperine is able to block the interaction of androgen receptors with their natural ligands (testosterone, DHT). Piperine can interact steadily with ABP (androgen binding protein) and acts like a competitive antagonist for androgen binding (Chinta *et al.,* 2015).

Research on black pepper extract and piperine as an anti-androgenic potential cause fertility problems is limited. This study also has not proven that black pepper extract causes fertility problems. Another study using papaya leaf extract dose 300 and 500mg/kg BW given to male rats showed a decrease in serum testosterone level by 50% compared to control and decreased motility of epididymic spermatozoa to below 30% and caused decrease in the fertility index to 40-100% (Ansah *et al.,* 2016). The result of this research was almost the same as what we observed in D(3) that treated with ethanol extract of black pepper dose 13.32mg/kg BW affected decrease in serum testosterone level and spermatozoa motility. Thus, there is possibility of black pepper extract inducing infertility in male rats. Black pepper ethanol extract has potential antifertility because it negatively affects the reproductive system in adult male rats although previous research results provide different conclusions about the effects of black pepper and piperine on fertility parameters due to differences in extraction methods, dosage, and duration of treatment. Furthermore, an exploration to find out the right dosage of black pepper extract as well as piperine need to be done so the results can provide valid information related to the potential of black pepper extract as a contraceptive for men.

CONCLUSSION

The number of primary spermatocytes, spermatozoa concentration, and spermatozoa motility were decreased by administration of ethanol extract of black pepper fruit with dose of 6.66mg/kg BW and 13.32mg/kg BW, indicating potential antifertility in male rat.

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