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The Quality of Male Mice (*Mus musculus* L.) Spermatozoes Which is given Ethanol Leaves Extract of *Piper caninum*

Dewa Gede Nanda Artha Suyasa[1], Ni Made Rai Suarni[1], Ni Luh Suriani[1*], Ni Gusti Ayu Manik Ermayanti[1], Ni Wayan Sudatri[1], Gusti Ayu Mayani Kristina Dewi[2], Rusdianasari[3]

[1]*Biology Study Program, Faculty of Mathematics and Natural Sciences, Udayana University Bali 80361 – Indonesia*
[2]*Department of Animal Husbandry Undergraduate Study Program, Faculty of Animal Husbandry, Udayana University, Bali 80361 – Indonesia*
[3]*Politeknik Negeri Sriwijaya, 30139, Bukit besar Palembang, Indonesia*

Corresponding Author: Ni Luh Suriani, E-mail: nandasuyasa@gmail.com; *niluhsuriani@unud.ac.id*

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ABSTRACT

Piper caninum) is one of the plant species of Piperaceae family which contain various of secondary metabolism products including alkaloids, flavonoids, steroids and essential oil. The objective of this experiment is to find if mice (*M. Musculus L.*) that were given the *P. caninum* ethanol leaf extract would react with spermatozoa quality. For this experiment, we used a Completely Randomized Design (CRD) which consists of 4 handlings, and each handling cconsists of 6 repeats. In this experiment, there are vary handling such as P0 or Control where the mice just only fed and given water and P1 or Handling 1 where the mice are not only fed they are also given 500 mg/kg body weight dosage of *P. caninum* ethanol leaf extract, P2 or Handling 2 where the mice are given 1000mg/kg body weight dosage of *P. caninum* ethanol leaf extract. And P3 or Handling 3 where the mice are given 1500mg/kg body weight dosage of *P. caninum* ethanol leaf extract. The treatment continues for 9 days and the ethanol extract is given to the male mice by gavage method. The observation parameter consists of the number of spermatozoa, spermatozoa viability, and spermatozoa morphology in male mice's cauda epididymis. ANOVA is used for observation results and if there is any significant difference between handling this experiment and continuing with the LSD test. The result shows that 500, and 1000 mg/kg body weight dosage of *P. caninum* ethanol extract improved the motility of spermatozoa significantly rather than control. The most effective dosage for spermatozoa motility test is 1000 mg/kg body weight. While it's proven effective in spermatozoa motility tests, the ethanol extract doesn't effective for mice's testicle weight, spermatozoa quantity, spermatozoa viability, and spermatozoa morphology.

1. Introduction

Nowadays, infertility is a frightening spectre for most people because it can cause economic and psychological problems. Infertility is the inability of a couple to produce offspring (Hadi, 2011). Infertility in married couples is caused by various factors with proportions: female factors 45%, male factors 40%, and idiopathic (spontaneous) factors 15% (Lestari, 2015).

Factors that cause male infertility include genetics, age, infection, autoantibodies, testosterone deficiency, hypogonadism, cancer, environmental factors, side effects of treatment, retrograde ejaculation, vasectomy, varicocele, and sperm quality. Infertility can be identified by sperm examination or semen analysis (Diarti et al., 2016).

Infertility in men can be treated with various types of treatment such as treating infections using antibiotics, drugs, hormone therapy, and surgery. However, drugs that use hormones and antibiotics have side effects such as seizures, headaches, seizures, nausea, increased blood pressure, blurred vision, narrow spectrum, and the presence of resistance (World Health Association, 2018). So other safer alternative treatments are needed, such as using traditional medicines derived from natural plants.

Indonesia is a country that has abundant natural wealth. Many types of plants can grow in this country, both useful plants and wild plants (weeds), and most of the plants that live in Indonesia can be used by the community as traditional medicine. The use of natural plants as medicinal plants is believed to be safer and the side effects are relatively smaller than using drugs derived from chemicals (BPOM RI, 2010). One of the plants that can be used as medicine is *P. caninum*.

According to Wan et al. (2011) *P. caninum* belongs to the Piperaceae family and is one of the flowering and fruiting plants. *P. caninum* contains alkaloids, flavonoids, polyphenols, and steroids (Salleh et al., 2015). Several studies have stated that flavonoid compounds are antioxidants that improve sperm quality by preventing damage to spermatozoa membranes (Safwan, 2016).

Research on the effect of the ethanolic extract of *P. caninum* leaves on the quality of spermatozoa in mice has never been done, so research needs to be done.

2. Literature Review

3. Methodology
3.1 Time and place of study

The research was conducted from July 2021 to September 2021. Treatment of the experimental animals is carried out at the Experimental animal maintenance room of the Faculty of Mathematics and Natural Sciences, Udayana University. Extracts of *P. caninum* were made at the Biochemistry Laboratory and the Laboratory of Genetic Resources and Molecular Biology, Udayana University. The surgery was carried out at the Laboratory of Animal Structure and Development in the Biology Study Program, Udayana University.

3.2. Tools and materials

The materials used in the study were mice (*M. Musculus*), *P. caninum*, 70% ethanol, aquadest, 0.9% NaCl, 1% eosin, 2% formalin, filter paper, cotton, tissue, and husks. The tools used were microscopes, glass bottles, glass slides, surgical instruments (scissors, knives, tweezers, needles), glass objects, trays, sonde, a rectangular cage (plastic tub) with a size of 33x22x15 cm), the place for Mice to eat and drink.

3.3. Methods

Determining LD_{50}
Determination of LD50 was carried out before the study to determine the toxicity of the ethanol extract of *P.caninum* leaves in order to determine the dose used in this study. The LD50 value was determined by counting the number of sample deaths for 24 hours caused by a single administration of the test preparation to 10 mice at a dose of 2000 mg/kg bw. Each was given orally with a suspension of 2000 mg/kg bw of *P.caninum* leave ethanol extract with a volume of 0.5 mL by gavage. Clinical symptom observation was carried out in the first 24 hours after treatment (Fadli, 2015).

P. caninum leaf extract-making process
leaves are sorted wet to separate from the dirt that is attached and then washed thoroughly. After that, it is dried by aerating and not exposed to direct sunlight until it dries, after that it is made into powder by means of a blender. Then 150 grams of extract powder was macerated using 1.5 L 70% ethanol (1:10) for 3 days while stirring. The results are filtered with filter paper to obtain the filtrate and concentrated using a rotary evaporator until the solvent evaporates so that in the end a thick extract is obtained then the results are weighed and several concentrations are made which can improve sperm quality in mice (Rikkit, 2017).

P caninum leave extract dosage measurement
In this study, the dosage of the extract was made with the calculation used according to Olsen *et al.* (2008) as:

$$\text{Dosage} = \frac{\text{Dosage x } Mice's\ weight}{\text{Concentration}}$$

P caninum extract application on mice
Piper caninum extract that has been dissolved with distilled water, then given by gavage with a volume not exceeding the intragastric volume (0.5 ml) of mice. The treatment was carried out on mice once every day, namely in the morning at 08.00-10.30 WITA for 9 days with a dose of 500 mg/kg BW, 1000 mg/kg BW, and 1500 mg/kg BW mice. On the 10th day, observations were made with the same parameters. Observations included the number, motility, viability, and abnormalities of the spermatozoa of the cauda epididymis of male mice.

Right testicular weight measurement on mice
After 9 days of treatment, on the 10th day the male mice were sacrificed and then the right testicle was taken. The organ was taken and washed using 0.9% NaCl, the next step the testes were placed on filter paper to remove the remaining NaCl, then weighed with digital scales and expressed in grams (g).

The number of motile cauda epididymal spermatozoa
The left cauda epididymis was placed in a petri dish that already contained 1 ml of physiological saline solution (NaCl 0.9%) at 37^{o}C, then cut into pieces with scissors so that the liquid came out, then stirred until a homogeneous suspension was formed. The spermatozoa suspension was placed on a hemocytometer and observed under a microscope (Wibisono, 2010). According to Abu *et al,* (2013) calculations were carried out on motile and non-motile spermatozoa from 100 spermatozoa and the results were in percent form.

Observation of the cauda epididymal spermatozoa viability
Observation and calculation of spermatozoa viability were carried out using sperm smear preparations stained with 1% eosin and observed using a light microscope with 400 x magnification. Viability was determined by not absorbing the eosin dye in spermatozoa cells (Wibisono, 2010).

Observation of the cauda epididymal spermatozoa count.
The suspension from observation 3.6.6 was placed into a counting chamber (hemocytometer), then the hemocytometer which already contained a suspension of spermatozoa was then observed under a light microscope with a magnification of 40 X 10 mediated by optical lab software and then photographed. . Spermatozoa counted were spermatozoa in 25 large hemocytometer boxes. The spermatozoa head is used as a guide for calculation, the number of spermatozoa is expressed in million/ml (modification from Trianty, 2001).

Spermatozoa morphology observation
Observation of the morphology of the spermatozoa of mice was carried out by means of the remaining epididymal suspension from the observation of the motility of the spermatozoa and the observation of the number of spermatozoa by adding 0.9% NaCl so that the volume was 2 ml. Morphological observations were carried out by staining eosin 1% in 70% alcohol. 0.5 ml of spermatozoa suspension was fixed with 2 ml of 2% formalin in distilled water for 10 minutes, then a smear was made on an object glass and air-dried, after drying, 1 drop of 1% eosin was given for 15 minutes, then rinsed with distilled water. Observation of the morphology of the spermatozoa of mice was carried out using a microscope with 400x magnification on 100 spermatozoa, which was carried out 5 times for each mouse. Spermatozoa morphology observations were distinguished into normal and abnormal spermatozoa expressed in percent (Modification from Suarni, 2001 and Trianty, 2001).

Research design
The research design used was Completely Randomized Design (CRD) which consisted of 4 treatments with 6 replications

3.4. Data analysis

The data obtained were analyzed using one-way ANOVA. If there is an effect of forest betel extract on sperm quality, then it is continued with the LSD test (Leas Significant Different) to determine the difference in each treatment. This analysis uses the SPSS version 22 program.

4. Results and Discussion

4.1. LD_{50}

The results of the LD_{50} experiment showed that 10 mice given ethanol extract of P. caninum leaves at a dose of 2000 mg/kg for 24 hours did not die. The ethanol extract of P.caninum leaves in this study can be declared safe so that it can be used as research material.

4.2. Mice's testicle weight

The results of statistical tests on the testicular weight of mice given ethanol extract of P caninum leaves showed that there was no significant difference between treatments (p=0.05) (P=0.000) (Table 1). The average research results can be seen in table 1.

Table 1**.** The results of mice's reproductive organ weight that given *P. caninum* leave extract.

Treatment	N	Testicle weight (gr)
Control	6	0,76 ± 0,013[a]
P1	6	0,76 ± 0,007[a]
P2	6	0,77 ± 0,013[a]
P3	6	0,77 ± 0,009[a]

Note: The average value followed by different letter notations in the same column shows results that aren't significantly different at test level 5% (p>0,05).

4.3. Spermatozoa motility on mice

The results of the statistical test using ANOVA on the motility of spermatozoa in mice showed a significant difference between the treatment and the control (p<0.05) (P=0.000). This indicates that the leaf extract of P. caninum has an effect on the motility of spermatozoa in mice. To find out the real difference in each treatment group, it was continued with the LSD test.

LSD test on sperm motility of mice showed that spermatozoa motility on K was significantly different from that of P1 and P2. Significant differences were also found in P1 and P2, and P2 with P3 while K was not significantly different from P3 (Table 2).

Table 2. The ANOVA analysis result of the mice's spermatozoa motility is given P. caninum ethanol leave extract.

Treatment	Mice's spermatozoa motility
Control	69,16 ± 3,54[a]
P1	72,16 ± 3,71[b]
P2	74,83 ± 4,26[c]
P3	71,16 ± 3,65[ab]

Note: The average value followed by different letter notations in the same column shows results that are significantly different at test level 5% (p<0,05).

4.4. Spermatozoa viability in mice

The results of statistical tests using ANOVA on the viability of spermatozoa in mice showed that the treatment and control were not significantly different (p>0.05) (P=0.000) (Table 3). However, there was an increase in the values of P1, P2, and P3 to the control.

Table 3. The result of spermatozoa viability in mice which is given *P. caninum* ethanol leave extract.

Treatment	Viable Spermatozoa
Control	76,00 ± 6,03[a]
P1	79,16 ± 6,24[a]
P2	79,50 ± 4,03[a]
P3	79,50 ± 7,68[a]

Note: The average value followed by different letter notations in the same column shows results that aren't significantly different at test level 5% (p>0,05).

Figure 1. Microscopic photo of spermatozoa viability in mice which is given *P. caninum* ethanol leave extract with 40 x 10 magnification.

Note: Viable spermatozoa (A), not viable spermatozoa (B)

4.5. Amount of spermatozoa

The results of statistical tests using ANOVA on the number of spermatozoa in the cauda epididymis of mice showed that the treatment and control were not significantly different ($p>0.05$) ($P=0.000$) (Table 4). However, judging from the average value, there was an increase in P1, P2, and P3 to the control.

Table 4. Average result of sperm count which is given *P. caninum* ethanol leave extract.

Treatment	N	Amount of spermatozoa (million/ml)
Control	6	368,33 ± 56,08[a]
P1	6	382,16 ± 59,02[a]
P2	6	375,33 ± 57,96[a]
P3	6	351,33 ± 64,75[a]

Note: The average value followed by different letter notations in the same column shows result that aren't significantly different at test level 5% ($p>0,05$).

Figure 2. Photo of Hemocytometer being used to count spermatozoa which is given *P. caninum* ethanol leave extract with 40 x 10 magnification.

4.6. Abnormal spermatozoa morphology

The average results of the observation of abnormal morphology of mice spermatozoa in this study were: K of 4.83%, P1 of 4.50%, P2 of 3.67%, and P3 of 3.67%.

The results of statistical tests using ANOVA on the morphology of spermatozoa in mice showed that the treatment and control were not significantly different ($p>0.05$) ($P=0.000$) (Table 5).

Table 5. Average result of spermatozoa abnormal morphology which is given *P. caninum* ethanol leave extract.

Treatment	Abnormal sperm morphology on mice (%)
Control	4,83 ± 0,98[a]
P1	4,50 ± 1,51[a]
P2	3,67 ± 1,86[a]
P3	3,67 ± 1,50[a]

Note: The average value followed by different letter notations in the same column shows result that aren't significantly different at test level 5% (p>0,05).

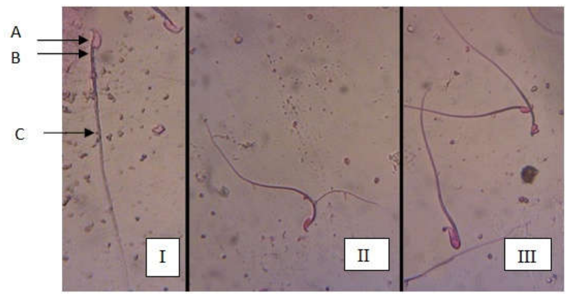

Figure 3. Microscopic photo of spermatozoa morphology in mice treated with *P. caninum* ethanol leaf extract with 40 x 10 magnification.

Note: Image I is normal spermatozoa consisting of head (A), midpiece (B), and tail (C). Image II is an abnormal spermatozoa with 2 pieces of tail. Image III is an abnormal spermatozoa with a broken headpiece.

4.7. Discussion

LD_{50}

According to Meyer et al.(1982) if some extract had a toxic substance then it would kill 50% of test animals. From 10 male mice which given 2000mg/kg BW of *P. caninum* ethanol leaf extract there is no death issue, therefore *P. caninum* ethanol leaf extract is not toxic and proven to be safe to use for this study.

Mice's testicle weight

Study show that *P. caninum* ethanol leave extract don't affect testicle weight in mice. But when looking at the average value there is insignificantly increased mice's testicle weight which is given *P. caninum* ethanol leaf extract, 1000 mg/kg BW and 1500 mg/kg BW dosage capable of increasing testicle weight compare to control

According to Kathrun and Varma (2017) testosterone enhancement is caused by secondary metabolism products such as flavonoids. Flavonoids could also increase sperm cell count by preventing epithelial seminiferous tubules from damage that could lead to spermatogenesis disturbance. The increase in sperm count may increase testicle weight (Anindita and Sutyarso, 2012)

Essential oil can be obtained in *Piper* genus plant that would give an androgenic effect as a well aphrodisiac effect. The aphrodisiac effect would arouse sexual desire and the androgenic effect could increase sperm production by working as a paracrine hormone which is needed by the sertoli cell (Najak and Windhu, 2016). Less significantly sperm weight increased in P2 and P3 presumably because the research conducted was too short so *P. caninum* ethanol leave extract effect isn't optimal.

Spermatozoa quality

The result of statistical analysis showed that *P. caninum* ethanol leaf extract doesn't significantly affect mice's spermatozoa count, spermatozoa morphology, and spermatozoa viability. But seen from the average value in the handling, turn out there is an increase, this indicate that *P. caninum* ethanol leaf extract can improve spermatozoa quality. Less significant influence statistically presumably because the research conducted was too short. The average spermatogenesis duration in mice is 35,5 days with 4 seminiferous epithelial cycles where a single seminiferous epithelial cycle required 207±6 hours or 8,65 days. This research takes 9 days that's mean *P. caninum* ethanol leaf extract only affect 1 of 4 seminiferous epithelial cycles. A notable influence on this research from 4 spermatogenesis cycles is the last cycle specifically spermiation. To be able to observe the quality of spermatozoa as a whole accurately the research must be carried out for at least 36 days.

The significantly different result can be found in the spermatozoa motility test where P1, P2 and P3 significantly increased compared to control. This show that *P. caninum* ethanol leaf extract can improve the quality of mice's spermatozoa especially spermatozoa motility.

The calculation of spermatozoa motility is carried out to determine the quality of spermatozoa because motility greatly affects fertilization. Spermatozoa motility had various grading such as grades A, B, and C. Grade A motility is the best spermatozoa motility out there, a good spermatozoa motility had an agile and straightforward movement. Unlike grade A, grades B and C had poor motility such as twisting, slow-moving, winding, or even immotile (Susilowati *et al.,* 2010).

According to Bintara (2011) spermatozoa, motility is the quality of spermatozoa movement which is consist of the speed and grade of spermatozoa. Progressive propulsion is needed by spermatozoa when in the female reproductive tract to reach the site of fertilization (Sarasstina *et al.*, 2012). According to Nuraini *et al.,* (2012) the greater the immotile sperm percentage the greater possibility of infertility occurrence. , therefore motility is required by sperm to reach the ovum for inducing fertilization.

Rahardianto *et al,*(2012) also said that the motility of spermatozoa is strongly influenced by the normal sperm morphological structure and environmental conditions. Normal spermatozoa morphological structure is related to spermatogenesis which produces normal spermatozoa cells to support their propulsion for reaching the female reproductive organ.

Study showed that the treatment with 500 mg/kg BW, and 1000 mg/kg BW of *P. caninum* ethanol leaf extract significantly increased the number of motile spermatozoa. The most effective dosage is 1000 mg/kg BW with 74,83% motile sperm.

According to Saefudin *et al.* (2013) natural antioxidants in the Piper genus can protect molecules from cell damage caused by oxidation and can improve sperm quality and increase mice's reproduction efficiency so it would increase mice's sperm motility. Tanjung (2013) stated that alkaloid that are obtainable in *P.caninum* leaves act as antioxidants.

Secondary metabolism products such as flavonoid, alkaloid, and essential oil have strong antioxidants which come in handy for increased sperm quality by binding free radicals. The essential oil also have function as a food reserve for spermatozoa. In the process of metabolism and respiration, spermatozoa can also use oxygen to oxidize prime substrates and rebuild ATP then use the ATP to stay viable and motil (Khaki, 2011).

According to Nukman *et al.* (2019) secondary metabolism substrates such as saponin, alkaloid, and tannin can improve blood circulation in the central nervous system (cerebral) and peripheral blood circulation. The effect of increased blood circulation also occurs in male genitalia, this increase in blood circulation will improve the activity of blood tissues so which will indirectly improve organ function. Increased organ function in the male genital causes an increase in testosterone which stimulates a better spermatogenesis process that would increase sperm production. However, in this study, a Higher dosage of *P. caninum* ethanol leaf extract would result in a decrease in sperm count and spermatozoa motility. This happens because diosgenin in the saponin has a steroid core and cholesterol-like molecular structure which is testosterone precursor. This precursor later will occupy the testosterone precursor and will cause negative feedback, so that the secretion of testosterone produced by Leydig cells is also inhibited (Rekha, 2014)

5. Conclusion

The *P. caninum* ethanol leaf extract with 500mg/kg bb and 1000 mg/kg bb dose can improve spermatozoa, most notably its real-time mobility. The optimal dose is 1000 mg/kg bb. The extract doesn't show significant improvement to the mass of the testicle and the quality of spermatozoa, specifically its amount, viability, and morphology

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