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Antifertility Effect of Sirih Leaf (*Piper betle* L.) Ethanol Extract on Male Wistar Rats Spermatogenesis

(*Efek Antifertilitas Ekstrak Etanol Daun Sirih (Piper betle L.) pada Spermatogenesis Tikus Wistar Jantan*)

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ABSTRACT

Background: Currently, there are still very limited male contraceptive options. The ideal male contraceptive is still needed and ideally has the characteristics of having sufficient effectiveness, fully reversible and safe for long-term use. Several studies have been conducted to explore *Piper betle* as a contraceptive. **Objectives:** This study aims to examine the effect of 96% ethanol extract of sirih leaf (*Piper betle* L.) in reducing rat spermatogenesis quality, which includes number, concentration, motility and morphology of male rat sperm. **Material and Methods:** This study used a posttest design with 28 white male rats. The rats were divided into four groups, each consisting of 7 rats. Group I was a control group. The test groups were group II, III, and IV, and each received an ethanolic extract of *Piper betle* leaves with various dosages of 200, 400, and 800 mg/kg body weight (BW), respectively, for 30 days. On day 31, all mice were sacrificed and analyzed for sperm count and concentration, sperm motility and sperm morphology. **Results:** The administration of 96% *Piper betle* leaves ethanol extract (PBEE) found to decrease the number and concentration of rat's sperm, decrease the progressive sperm motility and reduce the proportion of normal morphological rat sperm. PBEE at dose of 800 mg/kg BW showed the greatest decreasing effect with the significantly different with the other treatments ($p<0.05$). **Conclusions:** PBEE has contraceptive ability with a mechanism to reduce sperm count and concentration, sperm motility and sperm morphology.

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ABSTRAK

Latar Belakang: Kontrasepsi pria masih terbatas dan memiliki sedikit pilihan. Kontrasepsi pria yang ideal tetap dibutuhkan dan idealnya harus memiliki efektivitas yang cukup, reversibel dan aman untuk penggunaan jangka panjang. Beberapa penelitian telah dilakukan untuk mengeksplorasi daun sirih (*Piper betle* L.*)* sebagai alat kontrasepsi. **Tujuan:** Penelitian ini bertujuan untuk melihat pengaruh ekstrak etanol 96% daun sirih terhadap penurunan kualitas spermatogenesis tikus yang meliputi jumlah, konsentrasi, motilitas dan morfologi spermatozoa tikus jantan. **Bahan dan Metode:** Penelitian ini menggunakan desain *post-test only group design*. Sebanyak 28 ekor tikus putih jantan galur Wistar (*Rattus norvegicus*) dibagi dalam 4 kelompok dan setiap kelompok terdiri dari 7 ekor tikus. Kelompok I merupakan kelompok kontrol, kelompok II, III dan IV masing-masing diberi perlakuan PBEE 96% 200 mg/kg BB, 400 mg/kg BB dan 800 mg/kgBB selama 30 hari. Pada hari ke 31, semua tikus dikorbankan dan dilakukan analisa terhadap jumlah dan konsentrasi sperma, motilitas sperma dan morfologi sperma. **Hasil:** Pemberian ekstrak etanol daun *Piper betle* 96% menurunkan jumlah dan konsentrasi sperma tikus, menurunkan motilitas sperma progresif dan menurunkan proporsi sperma tikus dengan morfologi normal. Ekstrak etanol daun sirih dosis 800 mg/ kg BB menunjukkan efek penurunan paling besar dengan nilai yang berbeda signifikan diantara semua dosis ($p<0.05$). **Kesimpulan:** Ekstrak etanol daun sirih berpotensi sebagai kontrasepsi dengan mekanisme menurukan jumlah dan konsentrasi sperma, motilitas sperma dan morfologi sperma.

Kata kunci: Daun sirih (*Piper betle* L.), kontrasepsi, antifertilitas, konsentrasi sperma, motilitas sperma, morfologi sperma.

INTRODUCTION

Contraception is one of the strategic tools used to improve the quality of the family (Badan Kependudukan dan Keluarga Berencana Nasional, 2020). Use of contraception significantly reduced unintended pregnancies and birth, and set birth spacing. Ideal birth interval length, improves the physical and mental health of mothers and children is more guaranteed (Barclay & Kolk, 2018). Ideally, contraception can be used by both men and women. Currently, there are still very limited male contraceptive options. The development of male hormonal contraception continues to progress slowly. This is partly due to the side effects of impaired libido, or enlargement of the prostate gland. The ideal male contraceptive is still needed and ideally has the characteristics of having sufficient effectiveness, fully reversible and safe for long-term use. (Chao, Page, & Anderson, 2014)

Piper betle is a traditional medicinal plant that has various properties (Biswas et al., 2022). Several studies have been conducted to explore its efficacy as a contraceptive, using various methods and there were some differences in results (Sarkar et al., 2001; Wuwungan, Queljoe, & Wewengkang, 2017). This study aims to examine the effect of 96% ethanol extract of *Piper betle* leaves in reducing rat spermatogenesis quality, which includes the number, concentration, motility and morphology of male rat sperm.

MATERIAL AND METHODS

Materials

Piper betle leaves was obtained from pajak sukaramai, a local market in Medan, 96% ethanol , phosphate buffer solution (PBS). All chemicals are procured from local companies.

Methods

Animals

Experimental animals used in this study were male Wistar rats with the age of 3.5 - 4 months obtained from the Unit of Laboratory Animals Research, Faculty of Medicine, University of Muhammadiyah, Sumatera Utara. The animals undergo 7 days of acclimatization before the research was conducted. During the study, mice in the control and treatment groups received regular food and drink ad libitum. The research protocol was approved by Ethical Commission of Health Research, Faculty of Medicine, University of Muhammadiyah, Sumatera Utara (No 791/ KEPK/FKUMSU/2022).

Extraction

Piper betle leaves ethanolic extract (PBEE) was made by maseration technique. One kilogram of fresh *Piper betle* leaf, were cleaned with running water, then the leaves were dried. Leaves that have been dried, crushed into powder. The process produces 100 grams of dry weight of *Piper betle* leaves. *Piper betle* leaf powder is put into a closed container and then soaked using 96% ethanol solvent until it crosses the powder surface of about three fingers. Immersion was carried out for 3 days and occasionally stirred at room temperature. Then the maserate was evaporated with a Vacuum Rotary Evaporator to get a thick extract of *Piper betle* leaf.

Treatment of PBEE on animals

As much as, 28 male rats of the Wistar strain (*Rattus norvegicus*) were used which were divided into 4 groups, namely the negative control group and 3 treatment group which were given PBEE at a dose of 200mg/grBW/day, 400mg/grBW/day. ,800 mg/grBW/day for 30 days. Rats were given PBBE using a probe with disposable syringe once a day. After 30 days, then on the 31st day surgery was performed. The rats were anesthetized with ether, then terminated and dissected. The testes and cauda epididymis were taken. The cauda epididymis is separated from the testis by cutting the proximal part of the corpus epididymis and the distal part of the vas deferens. Next, the cauda epididymis was put into a plastic pot containing 1.5 ml of PBS then the pot with the cauda epididymis was vibrated by hand so that the sperm came out into the solution. to form a spermatozoa suspension.

Sperm Concentration Test

Sperm concentration test was carried out by diluting 20 µL of liquified semen into 380 µL of PBS. Then we put a small amount of the mixture (1-2 drops of pipette) into the improved Neubauer's chamber and wait for 5 minutes to calculate the concentration. Calculations were carried out on 4 large squares. Sperm concentration was calculated by multiplying the number of sperm in the four squares times 50,000. Calculation results were expressed in $x10^6$ cells/ml.

Sperm Motility Test

Sperm motility test was carried out by dripping the spermatozoa suspension on the object glass using a dropper and closed using a cover glass. The sperm movements were observed under a microscope with a magnification of 60x. Sperm motility was classified into 3 categories as follows:

A: movement of spermatozoa forward straight and fast (progressive)
B: twisting spermatozoa movement, difficulty advancing straight/slow (non-progressive)
C: sperm were stationary or did not appear to move (immotile).

Motile sperm (non stationary) were the percentage from categories A and B.

Sperm Morfology Test

Safranin and crystal violet dyes were used to see the morphology of normal and abnormal sperm cells (spermatogenic cells). After epididymal sperm fluid smeared on an object glass, the crystal violet dye was dropped on the smear for 30 seconds. Then, the iodine solution was dropped without rinsing the previous dye, and were waited for 30 seconds. After that the smear was rinsed with distilled water. Finally, the smear was dripped by safranin dye and were waited for 30 seconds. After 30 second, the smear was read under a microscope with an objective magnification of 60x.

Data Analysis

Analysis was done using Oneway Anova with SPSS since all the data were normally distributed. The analysis was continued with Duncan test, to determine the difference between groups with a 95% confidence level, since the data were homogen.

RESULTS AND DISCUSSION

The sperm concentration of male rats in this study can be seen in Table 1. After 30 days of treatment, there was a decrease in the number and concentration of rat sperm. The larger the dose of PBEE, the lower the sperm count and concentration of the rats. Oneway Anova statistical test showed significant results ($p < 0.01$). The Duncan post hoc test also showed differences between all control and treatment groups. The best dose that lowers sperm concentration is PBEE 800 mg/g BW group.

Table 1. Number and Concentration of Rat Sperm

Group	Sperm amount				Sperm Concentration (10^6) cell/ml Mean ± SD	P
	Room 1 Mean ± SD	Room 2 Mean ± SD	Room 3 Mean ± SD	Room 4 Mean ± SD		
Control	311,67 ± 10,41	338,33 ± 7,37	312,33 ± 15,04	322,67 ± 11,015	64.25 ± 0,52[a]	0.01
PBEE 200 mg/g BW	214 ± 4	166 ± 15,1	204 ± 16,37	222 ± 13,11	40.30 ± 0,7[b]	

PBEE 400 mg/g BW	166 ± 4,58	164 ± 5,29	164 ± 7,81	165 ± 10,44	32.95 ± 0,7[c]
PBEE 800 mg/g BW	64 ± 5,29	59 ± 5,29	58,33 ± 6,50	67 ± 3,60	12.42 ± 0,3[d]

Data are shown in mean ± SD with n= 3, Different superscript letters indicate significant difference

In the motility parameters, we assessed whether there was a difference in the percentage of sperm with progressive motility (only counting category A) and assessed the percentage of sperm with motility considered motile (progressive/ category A + non-progressive/ category B). In Table 2, Oneway Anova test shows that there is a significant difference in the percentage of sperm motility in category A and significant difference in the percentage of motile sperm (categories A and B), between the groups (p = 0.01). Duncan's test showed that there was a different effect in all groups. The greatest effect in decreasing progressive or motile sperm motility was in the 800 mg/g BW PEE group.

Table 2. Motility of Rat Sperm

Group	**Sperm Motility (%)**			
	Category A Mean ± SD	**Category B Mean ± SD**	**Category C Mean ± SD**	**Motile (A+B) Mean ± SD**
Control	34,67 ± 0,57[a]	40 ± 0,0	25,33 ± 0,57	74,67 ± 0,577[a]
PBEE 200 mg/g BW	14,33 ± 0,57[b]	54,33 ± 0,57	31,3 ± 0,57	68,67 ± 0,577[b]
PBEE 400 mg/g BW	11 ± 1[c]	59,33 ± 1,15	29,67 ± 0,57	70,33 ± 0,577[c]
PBEE 800 mg/g BW	5,33 ± 0,57[d]	71,33 ± 0,57	23,33 ± 1,15	76,67 ± 1,15[d]
p value	0.01	--	--	0.01

Data are shown in mean ± SD with n= 3, Different superscript letters indicate significant difference

In morphology of the rat sperm parameter, it was found that there was a difference effect in percentage of normal rat sperm morphology. And the greatest decreasing effect on the percentage of normal morphology was obtained in the PBEE 800 mg/g BW group (Table 3).

Table 3. Morphology of Rat Sperm

Group	**Sperm Morphology (%)**	
	Normal Mean ± SD	**Abnormal Mean ± SD**
Control	87,67 ± 0,577[a]	12,33 ± 0,577
PBEE 200 mg/g BW	54,33 ± 0,577[b]	45,67 ± 0,577
PBEE 400 mg/g BW	30,33 ± 0,577[c]	69,67 ± 0,577
PBEE 800 mg/g BW	16,67 ± 0,577[d]	83,33 ± 0,577
P	0.01	--

Data are shown in mean ± SD with n= 3, Different superscript letters indicate significant difference

This study showed that the ethanol extract of betel leaf reduces sperm quality, by reducing the number and concentration of sperm, disrupting sperm motility and increasing abnormal sperm morphology. These results are in line with similar studies regarding the antifertility effect of *Piper betle* leaf extract (Sarkar et al., 2001).

The decrease of sperm count and concentration in PBEE treatment group suggests that PBEE interfering with the sperm production process in the testis. There are many components that play a role in the production process in the testes, including: Sertoli cells, Leydig, and the hormones FSH, LH, testosterone and estrogen. However, it seems that the mechanism of action of PBEE does not interfere with the hormonal profile of the rats, since it has reversible effect and does not change the testosterone level (Shah & Jhade, 2016; Sarkar et al., 2001). Maybe this, due to the antiproliferative effect of hydroxychavicol (HCH) in PBEE.

Piper betle has been attributed for many pharmacological activities. It has anti-proliferation activity, therefore it is a potential agent to be developed as an anti-cancer (Gundala & Aneja, 2014). The phenol content of *Piper betle* leaf, hydroxychavicol (HCH) has been shown to have an inhibitory effect on growth and cell cycles in prostate cancer and oral carcinoma of KB cells (Chang et al., 2007, 2002; Paranjpe et al., 2013). Meanwhile, we also know that there are many mitotic and meosis division activities that occur in the process of spermatogenesis. This may explain the inhibition of the process of spermatogenesis, due to the antiproliferative effect of PBEE (see figure 1).

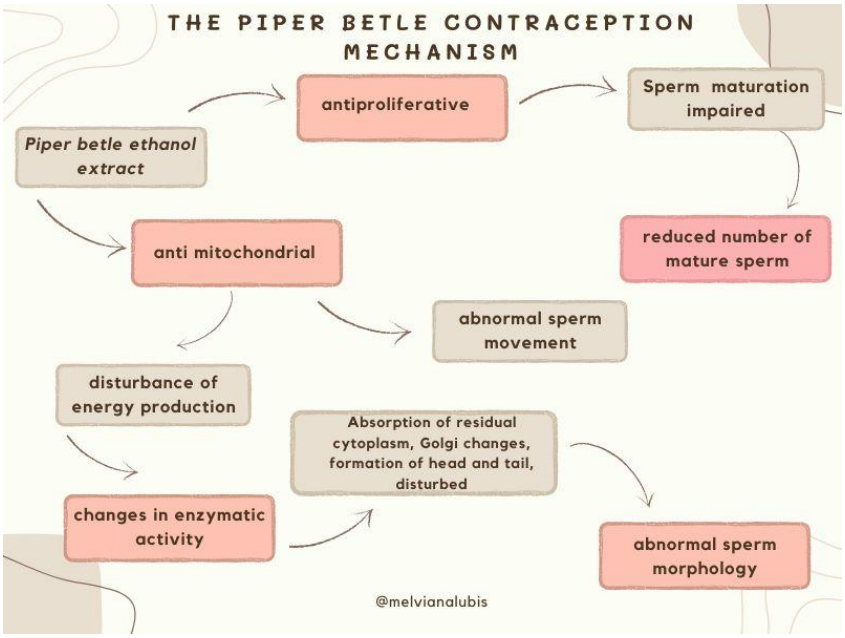

Figure 1. *Piper betle* ethanol extract contraception mechanism hypothesis

There are many factors that affect sperm movement. Increasing in non-progressive sperm could be caused by disruption of the sperm mitochondria (Neisi, Noorbakhsh, Hashemitabar, Afroogh, & Cheraghian, 2022). A study revealed that 50% ethanol extract of *Piper betle* leaves suppresses the mitochondrial activity of sperm in humans (Singh et al., 2011). Concentration and exposure time of PBEE are inversely related to mitochondrial activity. This anti-mitochondrial activity is also mediated by HCH. HCH causes early but transient increase of mitochondria-derived reactive oxygen species

which then triggers a cascade of reactions that cause loss of mitochondrial membrane potential and leading to apoptosis (Chakraborty et al., 2012). In addition, there is also evidence showing enzymatic changes associated with energy utilization and mitochondrial oxidation-reduction events in rat reproductive organs. Hence, sperm metabolic processes are disrupted. (Naik & Changamma, 2015).

Spermiogenesis is a process of maturation of spermatids into mature spermatozoa. Here changes in Golgi organelles, formation of caps and tails, occur . In addition, there is absorption of excess cytoplasm which is phagocytized by Sertoli cells. Changes in enzyme activity and disturbances in mitochondria as described above, indicate a change in the ongoing process of spermatogenesis. Hence, disturbances in the processes of spermatogenesis and spermiogenesis may cause abnormal sperm morphology.

Another plant that has potential as a male contraceptive is papaya (*Carica papaya* L.) seed extract. Alkaloids in the n-hexane fraction of papaya seeds are cytotoxic so they inhibit the development of leydig cells in producing testosterone. In addition, triterpenoids and steroids inhibit the feedback mechanism in the hypothalamus pituitary to decrease the production of LH (Luteinizing Hormone) and FSH (Follicle Stimulating Hormone). As a result of the feedback mechanism being disrupted, the levels of FSH and LH entering the blood circulation decrease, so that the process of spermatogenesis stops and the number of spermatozoa produced decreases in the seminiferous tubules. (Nita et al., 2019). Unlike the *Piper betle* leaf extract, the papaya seed extract causes a decrease in testosterone and a decrease in organ size. Decreased testosterone tends to cause decreased libido and other undesirable effects. Therefore, *Piper betle* leaf extract is superior to user convenience than papaya seed extract.

CONCLUSION

Ethanolic extract of *Piper betle* leaves with dosages of 200 mg/kg BW, 400 mg/kg BW, and 800 mg/kg BW can effectively decrease concentration, motility and morphology of rat sperm. The most optimal dose is 800 mg/kg BW. This study still requires histopathological, enzymatic and hormonal evaluation to explain exactly how the contraceptive mechanism is achieved.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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