

Research Article

Evaluation of the Antifertility Effect and Toxicity of Areca nut as Oral Contraceptive: Study on Male Rats

Evaluasi Efek Antifertilitas dan Toksisitas Areca Catechu sebagai Oral Kontraseptif: Studi pada Tikus Jantan

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ABSTRACT

Currently, very few male contraceptive methods are available. Areca nuts are known to have an antifertility effect, but the effective, safe dose is not certainly known. Data on its effectiveness and safety are needed to develop the potential of the areca nuts as an herbal contraceptive in men. This study determines the antifertility effect of areca nut administration at doses of 40, 30, and 20 mg/kgBW and their safety on the kidneys and liver. The green-colored raw areca nuts were used. The treatment groups were male rats whose fertility status was known before treatment. The control group were given distilled water. The treatment were given one dose a daily dissolved for 28 days of treatment, then male rats were bred again. Rats were terminated for histopathological examination of liver, kidneys, testes, and for analysis of spermatozoa. The administration of areca nuts caused a decreasing number of litters, but only the group given 40 mg/kgBW dose had a significant decrease ($p < 0.05$). There were a damaged testes, a decreasing number and motility of spermatozoa as well as increasing spermatozoa abnormalities. There were no changes in liver tissue, but there was a mild grade cast in kidney tissue. Areca nut have antifertility effect in male rats with dose dependent manner, histopathological examination revealed cast nephrotoxic but not hepatotoxic.

Keywords: Antifertility, areca, fertility, hepatotoxic, nephrotoxic

ABSTRAK

Saat ini, metode kontrasepsi pria masih sangat sedikit. Biji pinang diketahui mempunyai efek antifertilitas pria, akan tetapi dosis efektif dan aman belum diketahui secara pasti. Data efektifitas dan keamanan diperlukan untuk pengembangan potensi biji pinang sebagai kontrasepsi herbal pada pria. Penelitian ini bertujuan mengetahui efek antifertilitas pemberian biji pinang dosis 40, 30 dan 20 mg/kgBB dan keamanannya pada organ ginjal dan hepar. Biji pinang yang digunakan adalah dari buah yang berwarna hijau. Perlakuan menggunakan tikus jantan yang telah diketahui status fertilitasnya sebelum perlakuan. Kelompok kontrol diberikan aquadest. Perlakuan diberikan satu kali sehari selama 28 hari, kemudian tikus dikawinkan kembali dengan betina pasangannya. Tikus diterminasi untuk pemeriksaan histopatologi testis, ginjal dan hepar serta analisis spermatozoa. Pemberian biji pinang menyebabkan penurunan jumlah anakan postest, akan tetapi hanya kelompok dosis 40 mg/kgBB yang mengalami penurunan secara bermakna ($p < 0,05$). Pemeriksaan histopatologi testis didapatkan penurunan diameter testis dan peningkatan degeneratif tubulus, sedangkan analisis sperma didapatkan penurunan jumlah spermatozoa dan motilitas spermatozoa serta peningkatan abnormalitas spermatozoa. Tidak ditemukan perubahan jaringan hepar pada kelompok perlakuan dibandingkan kelompok kontrol, sedangkan pada ginjal didapatkan gambaran cast dengan derajat ringan. Biji pinang mempunyai efek antifertilas pada tikus jantan yang bersifat dose-dependent dan pemeriksaan histopatologi didapatkan gambaran cast nefrotoksik akan tetapi tidak ditemukan tanda hepatotoksik.

Kata Kunci: Antifertility, areca, fertility, hepatotoksik, nefrotoksik

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INTRODUCTION

Male contraceptive availability is limited to only condoms and vasectomy, and the development of new reversible male contraception has been slow. The availability of more options for safe, reversible, and non-hormone-influenced contraception can equalize the contraceptive burden. Currently, if a couple wants to delay pregnancy, most of the contraception is done by the women because of the options available for the contraceptive methods. However, several hormonal and non-hormonal male contraceptive drugs are currently being developed. Some have been marketed but are withdrawn from the circulation considering the side effects, while others are in the clinical trial stage (1). A study shows that more men are willing to use condoms than hormonal contraceptives (2). The future of the development of male non-hormonal contraceptives is more promising, so that efforts to find and develop oral contraceptive drugs should be directed to non-hormonal contraceptives (3).

Many herbs studied have antifertility effects in animal tests, but some are less effective in humans (4). Some advantages of herbs are that they are cheaper, easy to obtain, and easy to buy. The development of herbal contraception should be carried out on plants that are socially and culturally accepted by the community (5).

Areca nuts (*Areca catechu*) are made into traditional beverages in several cities in Sumatra, where the trees are widely cultivated. Local people believe that areca nuts increase male libido. The efficacy of areca nuts on the male reproductive organs has been investigated, including their effect on fertility. Several studies have shown that areca nuts can cause infertility in animal trials. Administration of high doses of areca nuts can cause motility and morphology disorders of rat spermatozoa or testicular tissue abnormalities (6-11).

Although herbal ingredients are often considered safe, some herbal ingredients are known to damage the liver and kidneys, especially at high doses or given for long term (12-15). Kidneys and the liver are organs often used as parameters for assessing toxicity. Several studies show that extract of areca nuts or arecoline also cause damages to other organs, such as the liver and kidneys, especially in long-term administration and high doses (9,16-20). Determination of an effective and safe dose range is needed to develop areca nuts as a candidate for an oral herbal male contraceptive. Few studies were done regarding a specific dose of areca nuts on infertility and its safety. A study showed that administration of areca nuts at a dose of 50 mg/kgBW causes infertility, and there is no effect on testosterone levels in rats (10), but it caused hepatotoxic and nephrotoxic. However, this study used only one dose, so the dose dependent pattern was unknown. This study aimed to examine the effect of areca nut administration on the fertility status of male rats, testicular organs, kidneys, and liver at lower doses, that is dose of 40, 30, and 20 mg/kgBW in 28 days.

METHOD

Preparation of Areca

Simplicia was made by drying chopped green betel nuts in an oven at 50°C for 24 hours. The nuts were obtained from

one of the areca nut plantations in Jambi City. The dried areca nuts were then powdered, stored in a dark bottle, and dissolved with distilled water before being administered. The test results of the three main phytochemical compounds that were determined by spectrophotometer in the areca nuts used in this study were alkaloids 1.524 ± 0.049 %w/w, phenol 33.82 ± 0.245 %w/w, and tannins 22.606 ± 0.873 %w/w.

Experimental Animal

This research has received ethical approval from the Ethics Commission of the Faculty of Medicine and Health Sciences, Jambi University, through the ethical clearance number 1545/UN21.8/PT.01.04/2021.

This research was conducted at the Biomedical Laboratory, Faculty of Medicine and Health Sciences, Jambi University. As many as 20 male rats, *Rattus Norvegicus* Sprague Dawley, aged three months, fertile, 200-250 grams of weight, were randomly divided into four groups. Rats were caged in groups. Dark and light cycles, temperature, and humidity were maintained, and the rats received standard food and drink ad libitum.

Group 1, 2, and 3 were given betel nut simplicia doses of 40, 30, and 20 mg/kgBW, respectively. In contrast, the control group, group 4, was given the solvent used to dissolve the simplicia, which was distilled water. The treatment was given once a day using a nasogastric tube.

Male rats used were fertile male rats proven by their ability to impregnate female rats that were mated before the treatment with a ratio of 1 male: 3 females. Females with the highest number of litters were used in the fertility test after treatment. On day 29, male rats were mated for 7 days with their female partners while still being given betel nuts until day 35. The numbers of litters born before and after treatment were used to assess the changes in the fertility status of male rats. On day 36, male rats were sacrificed, and the testes, liver, and kidneys were taken.

Sperm Analysis

Sperm analysis was conducted by obtaining the Cauda epididymis; one of the cauda epididymis used for calculating the number of spermatozoa and their motility was placed in 10 mL 0.1M PBS, while the other cauda for morphological examination of spermatozoa was placed in 1 mL 0.1M PBS. Each cauda was carefully cut in each solution to allow the spermatozoa to come out into the solution. The number of spermatozoa was calculated using a hemacytometer under a light microscope by calculating the total number of spermatozoa with heads in 5 squares (4x4 or 0.04 square mm) using a formula. Spermatozoa motility was calculated when the spermatozoa entered the 4x4 squares but categorized as immotile if the spermatozoa were not moving. The percentage of immotile spermatozoa was calculated by comparing the total spermatozoa. Spermatozoa morphology was classified as normal if it had a head, neck, and tail that were straight and not folded. The percentage of spermatozoa with abnormal morphology was calculated by comparing it with the total spermatozoa.

Histopathology Analysis

The weight of the organs was measured using a digital scale. The liver weight was the entire lobe, while the weight

of the kidney and testis was the average weight of the two kidneys or testes. Histopathological examinations for testes, liver, and kidneys were using Hematoxylin-Eosin staining. Histopathological assessment was performed by an anatomical pathologist using the blinded method. Testicular assessment parameters included the diameter of the seminiferous tubules and the percentage of degenerative tubules. The number of tubules read was 100 tubules in the entire field of view. The degree of tubular seminiferous degeneration was assessed as minimal if <5%, mild if 5-25%, moderate if 25-50%, severe if 50-75%, and very severe if >75%. The diameter of the seminiferous tubule was measured using the shortest diameter if the tubule was not spheric. Renal assessment parameters included interstitial congestion, interstitial inflammation, tubular cast, tubular vacuolization, and tubular degeneration. Liver assessment parameters included bleeding, sinusoidal dilatation, lobular disarray, lobular inflammation, portal inflammation, necrosis, interface hepatitis, microsteatosis, cholestasis, accumulation of lipofuscin, and fibrosis. Semi-qualitative assessment was using the following scoring: (0) no change, (1) mild, <30%, (2) moderate, 30-50%, and (3) severe, >50%.

Statistical Analysis

Data were presented in mean \pm standard deviation for normal data distribution. Different tests were done using the Anova test and LSD test. Meanwhile, the non-normal distribution data were presented in median, minimum, and maximum. The different test used was the Kruskal Wallis test, followed by the Mann Whitney test. The difference in the mean number of litters before and after treatment was assessed using paired T-test. The level of confidence used was 95%, with a p-value of <0.05.

RESULTS

Fertility Status

The numbers of litters before and after areca nut administration in each group are presented in Table 1. The difference test showed no significant difference between the number of litters among the groups before treatment, so the baseline fertility status of male and female rats did not differ among groups. The numbers of litters after treatment in groups receiving areca nut dose 40 mg/kgBW were significantly lower than before the treatment, although there were no females that were not pregnant (zero litters) in these groups. The decrease in the number of litters before and after treatment in this group was also significantly greater than the control group ($p=0,15$). It showed that areca nut dose of 40 mg/kgBW can significantly decrease the fertility status.

Effect on Testis and Spermatozoa

Assessment results of testicular weight, seminiferous tubule, number and morphology, and spermatozoa motility after treatment are presented in Table 2. The effects of areca nuts on testes and spermatozoa are shown in Table 2. Areca nuts have an impact on spermatogenesis and seminiferous tubule.

The group that received areca nut dose of 40 mg/kg BW showed significantly smaller seminiferous tubule diameter with an increase in degenerative tubule (Figure 2) and an increase in the percentage of abnormal spermatozoa morphology compared to the control group, similar to the group receiving a dose of 30 mg/kgBW.

This study found several abnormal features of spermatozoa morphology (Figure 1).

Table 1. Number of litters before and after treatment in each group

Group	Number of litters						Mean \pm SD Differences Before and After	p-value*
	Before treatment			After treatment				
	Minimum	Maximum	Mean \pm SD	Minimum	Maximum	Mean \pm SD		
I	7	10	8,50 \pm 1,29	5	8	6,25 \pm 1,25	2,25 \pm 0,50**	0,003
II	5	10	8,25 \pm 2,21	6	8	6,75 \pm 0,95	1,50 \pm 1,91	0,215
III	7	12	9,00 \pm 2,16	7	9	7,75 \pm 0,95	1,50 \pm 1,29	0,141
IV	6	9	7,5 \pm 1,29	5	8	6,75 \pm 1,25	0,75 \pm 0,5 0	0,580

Note: *Paired T-test, ** Mann whitney Test for group 1 and group IV (control) had significant difference ($p=0,15$)

Table 2. Assessment of testicular weight, seminiferous tubule, number of spermatozoa , and its morphology and motility after treatment

Parameter	Group				p-value
	1	2	3	4	
Weight of testis (gram) ^a	1.43(1.35-1.58)	1.50 (1.02-1.71)	1.50(1.49-1.52)	1.53(1.42-1.65)	0.339 ^a
Diameter of seminiferous tubule (μ m) ^a	35.80 (34.07-37.67)	36.88 (34.46-37.77)	39.52(38.54-42.08)	40.30(39.2-41.88)	0.007 ^a
Percentage of degenerative seminiferous tubule ^b	26.5 \pm 5.91	16 \pm 2.16	8.5 \pm 1.29	4.75 \pm 0.95	0.000 ^b
Number of spermatozoa ^b (10^6 cell/mL)	320.75 \pm 124.29	278.75 \pm 119.39	316.25 \pm 234.39	333.75 \pm 36.37	0.975 ^b
Percentage of abnormal morphology of spermatozoa ^b (%)	42.00 \pm 7.78	34.25 \pm 4.27	28.50 \pm 8.8	27.50 \pm 20.33	0.035 ^b
Percentage of immotile spermatozoa ^b (%)	70.83 \pm 20.36	54.55 \pm 15.30	46.44 \pm 21.96	54.09 \pm 22.10	0.410 ^b

Note: a Kruskal-Wallis test; b ANOVA test

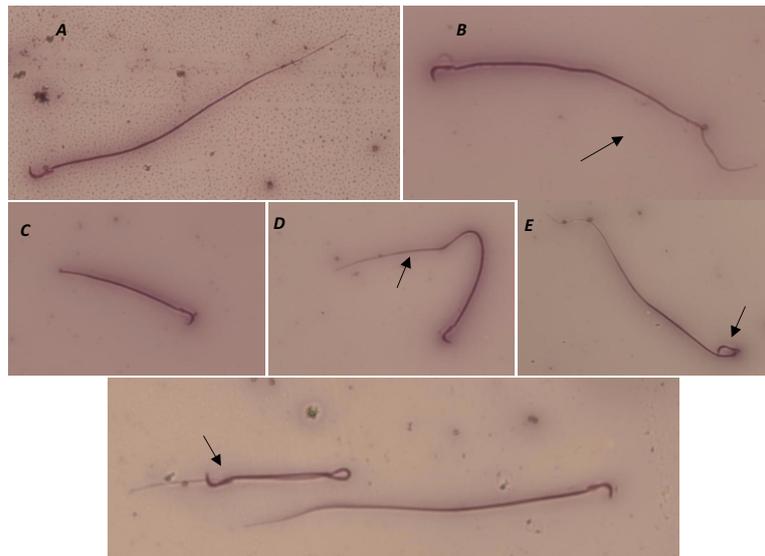


Figure 1. Morphology of spermatozoa

Note: (A) normal morphology of spermatozoa, it has head, neck, and long straight tail, (B) abnormal morphology of spermatozoa, curled tail, (C) abnormal morphology of spermatozoa, short tail, (D) abnormal morphology of spermatozoa, bent tail, (E) abnormal head or neck morphology of spermatozoa, (F) flipped spermatozoa (arrow), normal spermatozoa (under).

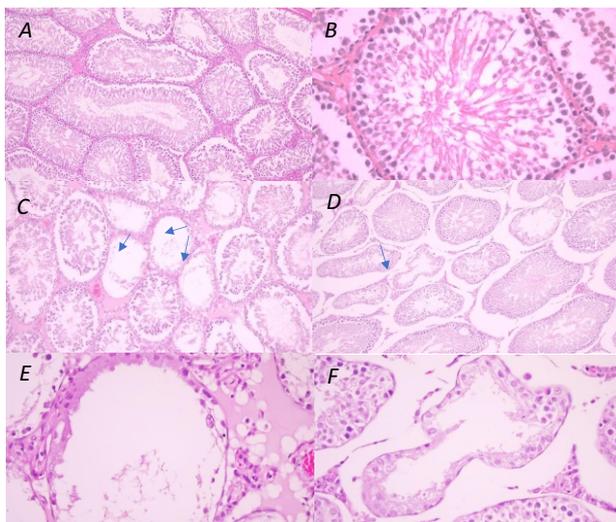


Figure 2. Histopathology of the testis; the seminiferous tubules

Note: A, B are images of the seminiferous tubules that appear the most in the treatment group, especially in groups 2 and 3. (100x and 400x magnification). There were uniformity of histological features and average of tube diameter size. Seminiferous tubules are lined by Sertoli and germ cells arranged in layers and surrounded by a basement membrane consisting of an outer thin parietal epithelium overlying peritubular myoid cells, collagen and an inner layer of the extracellular matrix; C-F are images of the seminiferous tubules that appear the most in the treatment group, especially in groups 1, 40mg/kgBW group (100x and 400 x magnification). There were abnormal of seminiferous tubules showed loss of cell and shrunken (arrow). There was a loss of typical germ cell arrangement—healthy Leydig cells and irregular tubular shape.

Safety Evaluation

Based on the daily monitoring, there were not a sign of sick or unhealthy rats. But, on day 20th, there was one rat that died in the group 2. We thought that the death of the rat had nothing to do with areca. We excluded rats with the same code as the dead mouse code in the other groups from the all analysis.

Histopathological assessment of liver tissue after treatment in each group did not reveal any signs of sinus dilatation, necrosis, steatosis, cholestasis, lipofuscin accumulation, lobular disarray, or fibrosis in all groups (Figure 3). Based on morphology, hemorrhage and inflammation was found with the mean score in each group, as shown in Table 3. The different tests showed no significant differences in liver weight, hemorrhage, and inflammation in the liver tissue between the treatment group and the control group. It indicates that the administration of areca nut dose of 40-20 mg/kgBW does not cause significant damage to the liver.

Table 3. The mean of liver weight and histopathological scores

Parameter	Mean score				Nilai p
	1	2	3	4	
Liver weight (gram)	9.15±1.21	9.81±0.97	9.18±1.05	9.02±1.06	0.740*
Hemorrhage	0.75±0.50	0.50±0.57	0.25±0.50	0.25±0.50	0.454
Portal inflammation	1.00	0.75±0.50	0.75±0.50	1.00	0.543
Lobular inflammation	1.00	0.50±0.57	0.75±0.50	1.00	0.238
Interface hepatitis	1.00	0.50±0.57	0.50±0.57	1.00	0.172

Note: Kruskal Wallis test, * Anova test

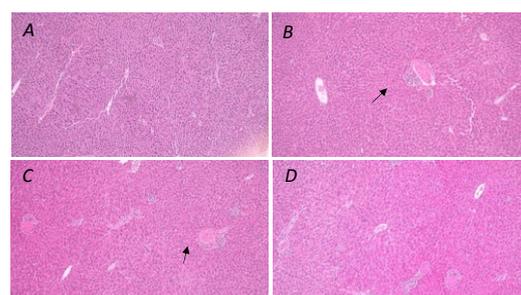


Figure 3. Histopathology of the liver

Note: A the control group; C-D the treatment group; 40 mg/kgBW, 30 mg/kgBW and 20 mg/kgBW (100 x magnification). There was inflammatory cell infiltration (arrow) in all treatment groups, both in the lobular parenchyma, around the central vein and around the portal vein.

Assessment of organ weight and kidney tissue histopathology after treatment in each group can be seen in Table 4. There were no significant differences in congestion, inflammation, vacuolization, and degeneration among the groups, except for tubular cast (Figure 4).

Table 4. Mean of kidney weight and renal histopathological score

Parameter	Mean of score				Nilai p
	1	2	3	4	
Kidney weight (gram)	1.04±0.13	1.36±0.31	1.06±0.12	1.10±0.11	0.216
Interstitial congestion	0.5±0.57	0.12±0.25	0.25±0.5	0.625±0.25	0.278
Interstitial inflammation	1.00	0.87±0.25	0.87±0.25	0.87±0.25	0.764
Tubular cast	1.12±0.25	0.75±0.5	0.62±0.47	0	0.027
Tubular vacuolization	1.00	1.00	1.00	0.87±0.25	0.392
Tubular degeneration	1.00	1.00	1.00	0.87±0.25	0.392

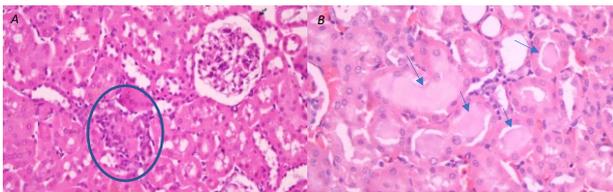


Figure 4. Histopathology of the kidney of all treatment group
Note: A and B Morphology of kidney around cortex at 400x magnification; There was inflammation (circle) and tubular casts (arrow)

DISCUSSION

This study was conducted to assess the potential effects of infertility and the toxicity effect on the liver and kidneys. Kidneys and the liver are organs that are often studied regarding the toxicity of a compound in long-term administration. Herbs are considered safe for consumption, but there are many reports of hepatotoxic and nephrotoxic events due to the use of herbs, especially for usage in long-term or high doses. This study shows that the areca nut dose of 40 mg/kgBW could cause a decrease in fertility status, where the number of litters born was lower than before treatment. However, the dose of 40 mg/kgBW did not cause the female rats not to get pregnant at all as stated in the previous study, the administration of areca nut dose of 50 mg/kgBW caused the female partner not to become pregnant (10).

The infertility effect of areca nuts is caused by the damage to the seminiferous tubule, in which this study found an

increase in degenerative seminiferous tubules. This tubular degeneration will cause spermatogenesis disorders and a decrease in the diameter of the seminiferous tubule. The blood testicular barrier formed by Sertoli cells and epithelioid cells located in the seminiferous tubules creates a microenvironment that supports the process of spermatogenesis. In vitro studies have shown that arecoline can cause an impaired blood-testicular barrier by disrupting protein that involved in tight junction formation (11). The histopathological picture of the testes in the treatment group given areca nuts showed empty tubules where there was no spermatogenesis process.

The results of the histopathological examination showed no difference in the histopathological features of the liver, but there was a mild degree of a renal tubular cast in the treatment group compared to the control group. These results indicate that the areca nut dose of 40 mg/kgBW does not cause hepatotoxicity. Meanwhile, in the kidney, areca nuts at a dose of 40 mg/kgBW caused a mild increase in tubular cast. The presence of casts in this study needs to be investigated further, because the presence of casts can be pathological or non-pathological. Determination of the type of cast can be done by special staining or immunohistochemistry (21). Casts can cause tubular obstruction in large quantities. Decreased kidney function is one of the nephrotoxic parameters. The limitation of this study is that renal function tests were not performed.

This study uses all parts of the areca nuts since processing herbal ingredients is not complicated and the public can reach it easily. On the other hand, in modern medicine, active compound is needed for standardization because of the significant variations in the content of active compounds in herbal plants that are influenced by environment such as soil conditions, weather, or harvest time (22,23). Arecoline is active compound that believed have cytotoxicity role. The limitation of this study is that the arecoline compound quantification test was not carried out. Phytochemical tests were only carried out for the quantification of alkaloids, phenols, and tannins. The requirement for contraception is to know the nature of its reversibility (1). Further research to assess the reversibility of the effects of areca nut infertility needs to be done.

In conclusion, although several studies have shown that arecoline or areca nuts have an infertility effect, this study shows that areca nut at lower doses still have an infertility effect. Lower doses have a lower risk of side effects. The use of whole components of herbal plant rather than the extract or single active component allows synergistic interactions between the phytochemical components that contained in it (24). This study showed that all parts of areca nuts at a dose of 40 mg/kgBW could cause a decrease in male rat fertility, and its safety profile did not reveal hepatotoxic and nephrotoxic histopathological parameters, except for mild tubular cast.

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