

The extract of "shoe flower" (*Hibiscus rosea sinensis* L) leaves inhibit the spermatogenesis of ddy strain mice

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Abstrak

Penelitian ini dilakukan dalam rangka mencari bahan kontrasepsi pria yang bersumber dari tanaman, khususnya daun kembang sepatu (*Hibiscus rosea sinensis* L). Penelitian ini bertujuan untuk melihat apakah ekstrak daun kembang sepatu (*Hibiscus rosea sinensis* L) dapat menghambat proses spermatogenesis mencit strain ddy. Penelitian ini dilakukan pada tiga kelompok yang masing-masing terdiri dari delapan ekor mencit. Kelompok kontrol diberikan karboksimetil selulose (CMC) 1% dalam 0,5 ml aquabides, kelompok perlakuan I diberikan ekstrak daun kembang sepatu (*Hibiscus rosea sinensis* L), dosis 700 mg/kg BB ditambahkan CMC 1% dalam 0,5 ml aquabides dan kelompok perlakuan kedua diberikan ekstrak daun kembang sepatu (*Hibiscus rosea sinensis* L), dosis 800 mg/kg BB ditambahkan CMC 1% dalam 0,5 ml aquabides. Perlakuan ini diberikan selama 40 hari sesuai dengan siklus spermatogenesis. Setelah itu dilakukan pembuatan preparat histologis testis mencit, diikuti pengamatan preparat testis dengan mikroskop cahaya pembesaran 100x dan 400x untuk menghitung sel-sel spermatogenik. Terakhir, dilakukan pemotretan tubulus seminiferus ketiga kelompok yang terdiri dari sel-sel spermatogenik melalui mikroskop cahaya dengan pembesaran 100x dan 400x dengan memakai kamera Fuji dan film Fuji, ASA 200. Hasil menunjukkan perbedaan yang sangat bermakna antara kelompok kontrol dan kelompok perlakuan I dan II, yaitu terjadi penurunan jumlah sel-sel spermatogonia, spermatosit primer pakhten dan spermatid pada kelompok perlakuan ($P < 0,01$). Dari hasil penelitian ini, dapat disimpulkan bahwa ekstrak daun kembang sepatu (*Hibiscus rosea sinensis* L) menghambat proses spermatogenesis. Hasil penelitian ini diharapkan dapat dikembangkan sebagai bahan kontrasepsi pria. (*Med J Indones* 2008; 17: 157-62)

Abstract

This study was conducted in order to develop male contraception from plants, namely the "shoe flower" (*Hibiscus rosea sinensis* L) leaves. The objective of this study was to find out whether the extract of "shoe flower" leaves could inhibit the process of spermatogenesis on ddy strain mice. This research was performed in 3 groups and each group consisted of 8 mice. The control group was given 1% carboxy methyl cellulose (CMC) in 0.5 ml aquabides. The treatment group I was given the extract of "shoe flower" leaves 700 mg/kg BW and 1% CMC in 0.5 ml aquabides, and the second treatment group was given the extract of "shoe flower" leaves, 800 mg/kg BW and 1% CMC in 0.5 ml aquabides. The treatment were given for 40 days in accordance with the spermatogenesis cycle. Then, the production of histological slides of the mice testis and the observation of the slides using light microscope with magnification of 100x and 400x were done. Further, counting of the spermatogenic cells was done. At last the pictures of seminiferous tubulus cross-section of the three groups which consisted of spermatogenic cells were taken through light microscope with magnification of 100x and 400x using Fuji camera and Fuji film, 200 ASA. The results showed significant differences between the control, treatment I, and treatment II group. There were decreased numbers of spermatogonia, pachyten primary spermatocytes and spermatids in treatment groups ($P < 0.01$). The result of this study showed that the extract of "shoe flower" (*Hibiscus rosea sinensis* L) leaves, inhibited the process of spermatogenesis of ddy strain mice. It is hoped that the result of this study can be developed into a male contraception. (*Med J Indones* 2008; 17: 157-62)

Keywords: spermatogonia, Pachyten primary spermatocytes, spermatids

Indonesia is the fourth largest populated country in the world after China, India, and USA, with 215 millions inhabitants, and the growth rate in 1990-2000 was 1.48%.¹ The national birth control program has

decelerated the growth rate. However, population control still became national concern due to the reduced death rate as the consequence of economic development. To suppress the population explosion it is necessary to re-decelerate the population growth.² Furthermore, men's participation is not merely just as motivator, but it is important for them to be involved in birth control. Men are only 5.6% of total acceptors. Low men's

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participation rate is due to social, cultural, and religious factors. Among the reasons is the limited selection of male contraception available.² With wide selections of contraception methods there would be more alternative for men.

Therefore it is necessary to promote research on male contraception.³ Further development of male contraception has large expectation in the future.⁴

The WHO has form a task force to develop a save, effective, reversible, and acceptable method of male contraception. One of the strategies is to develop herbal male contraception from plants containing active infertility substrates.^{3,4} Indonesia is rich in tropical botanic resources. Botanic contraception has advantages including low toxicity, accessibility, cost efficient and less side effects.^{4,5}

Hibiscus (*Hibiscus rosea sinensis L*) has promising features. The flower, leaves, bark, and roots contain *hibiscetin glycoside*, an anti-spermatogenesis agent, favourable for male contraception. In addition, the bloom contains *hibiscetin glycoside* and calcium oxalate, and the leaves contain some kind of alkaloid. However, the information of its application is still scarce.^{6,7}

Previous reports described that *Hibiscus rosea sinensis L* had anti-spermatogenesis features on male mice.^{5,6}

Purwaningsih (1998)⁷ performed administration of 300 mg/kg *Hibiscus rosea sinensis bloom* extract on AJ strain mice, and the decrease in spermatogenia was significant compared to the control group. However, the treatment group receiving group receiving 200 and 250 mg/kg did not show significant results.

Although the mechanism of the active substrate in *Hibiscus rosea sinensis L* in reducing spermatogenesis is still unknown, it is generally presumed that the bloom and leaf of *Hibiscus rosea sinensis L* inhibit germinal cells through cytotoxic or anti-androgen effect.^{5,6}

The aim of this study was to find out the effect of Hibiscus (*Hibiscus rosea sinensis L*) leaves extract in inhibiting spermatogenesis in ddy strain mice.

METHODS

This study had a randomized post test control group design, and conducted in Sam Ratulangi University, Faculty of Medicine, Joint Research Laboratory. The research period was July to November 2004.

Healthy male mice (mus musculus) ddy strain, 10-16 weeks of age,⁸ 30-45 gr was used in this setting.

The sample number was based on Kirk's equation, 1982,⁹ that concluded that each treatment required minimal 8 mice.

Extraction of hibiscus leaves

The hibiscus leaves were obtained from home yards of Manado and surroundings. The leaves selected were mature and dark green ones.

Extraction: the leaves were cleaned and dried not under direct sunlight or oven dried at 37-40°C for 5 days until the leaves disintegrated. The dry leaves were blended, shaken, and then weighted. The powders were extracted with 90% ethanol in Soxhlet and Weaton instrument for 4-5 hours at 50-60°C. The liquid extract was distilled until free from alcohol.^{10,11}

The extract weighted and then kept in bottles in a refrigerator. The extract was weighted according to the necessity based on the body weight and then carboxy methyl cellulose (CMC) 1% was added and liquefied with aquabides 0.5 ml.

ddy strain mice selection and treatment

Selection

Male mice, 10-16 weeks, 30-45 grams (weighted with special scale, Tanita, Japan). The selected mice are caged in a plastic basin, one for each. The basins were labeled according to the treatment start, weight, and age.

Feeding

The mice were fed with pellets containing corn starch, soy starch, grain powder, fish powder, rice beads, vitamin C and B, non fat milk, coconut oil, and water. Sometimes the menu included carrots and long bean. Tap water was used for hydration.

Treatment

Hibiscus leaves (*Hibiscus rosea sinensis L*) extract was administered orally to male ddy strain mice in treatment groups, i.e.:

First treatment group: 700 mg/kgBW with CMC 1% in 0.5 ml aquabides.

Second treatment group: 800 mg/kgBW with CMC 1% in 0.5 ml aquabides.

Control group: received CMC 1% in 0.5 ml aquabides, orally.

The treatment of treatment and control groups was continued for 40 days.

Spermatogenic cell-counting and sizing of the tubulus seminiferus diameter

Mice testicle slides, was made and the preparation was observed under light microscope with 100 and 400 times magnification.

Every slices containing cross sectioned seminiferus epithelium stage VII were examined. This selection is due to the long duration of the stage VII and the large number of the spermatogenic cells. The spermatogonia, pakhiten primary spermatocytes, and spermatids were counted, and their diameters were measured.

Determination of spermatogonia, primary spermatocytes and spermatids

Spermatogonia are attached on the basement membrane. In spermatogonia A, the nucleus exhibits fine smooth chromatin. Spermatogonia B exhibits condensed irregular chromatin.¹²

Primary spermatocytes perform meiosis to become secondary spermatocytes, and the processes include: a long profase leptoten, zigoten, pakhiten, diploten, and diakinesis. In pakhiten phase, the chromosomes wave shortened presenting rough and solid appearance. The pakhiten stadium is the longest (175.3 hours).¹³ The primary spermatocytes demonstrate a large and obvious nucleus. Generally the nucleus is located in the center. The oldest generation of the spermatocyte is the pakhiten.¹³

The spermatid determination was based on the groups and cap that is generally eccentric. The head cap gains the maximum size on stage VII.¹³

Taking picture of the tubulus seminiferus

The pictures were taken from 100 and 400 magnification of the microscope using Fuji camera and Fuji ASA 200 films, printed on 3R paper.

Data Analysis

Normality assessment was done using Kolmogrov-Smirnov test and variance homogeneity using F test.

If the data is normal and the variance homogenous, analysis of variance (ANOVA) is used to test the research hypothesis followed by Dunnett test when the result is significant.

If the result is the contrary from above, Kruskal-Wallis test are used followed by multiple comparison test when the result is significant.

RESULTS

Spermatogonia number in the three groups

The data of spermatogonia did not meet the criteria for ANOVA.

The median and the Kruskal-Wallis test result are presented in Table 1.

Spermatogonia median of the control group was higher than the 700 mg/kgBW and the 800 mg/kgBW group ($P \leq 0.000$).

Pakhiten primary spermatocyte number in the three groups

The mean and standard deviation (SD) of the pakhiten primary spermatocyte and the result of analysis of variance are presented in Table 2.

Table 1. Kruskal-Wallis test: spermatogonia number difference in the 3 groups

Treatment	Median	Mean Rank	X ²	P
Control group	292.5	20.50	17.899	0.000
700 mg/kgBW group	196.5	11.31		
800 mg/kgBW group	144.0	5.69		

Table 2. ANOVA: pakhiten primary spermatocyte number difference in the 3 groups

Treatment	Mean	DS	F
Control group	423.63	33.814	114.810
700 mg/kgBW group	204.88	42.927	
800 mg/kgBW group	187.25	25.269	

Table 3. Dunnett test : treatment to control group difference

Treatment group	Control group	Mean discrepancy	P
700 mg/kgBW group	Control	218.750	0.000
800 mg/kgBW group	Control	236.375	0.000

The F in table 2 indicated a statically significant difference, therefore Dunnet test was performed.

The test results in Table 3 show significant difference of the control group compared to the 700 mg/kgBW group and the 800 mg/kgBW group ($P < 0.01$).

Spermatid number of the three groups

The median and the Kruskal-Wallis test result are presented in Table 4.

Spermatid median of the control group was higher than the 700 mg/kgBW and the 800 mg/kgBW group ($P \leq 0.000$).

Table 4. Kruskal-Wallis test : spermatid number difference in the 3 groups

Treatment	Median	Mean Rank	X ²	P
Control group	546.0	20.50	15.411	0.000
700 mg/kgBW group	187.0	8.19		
800 mg/kgBW group	206.5	8.81		

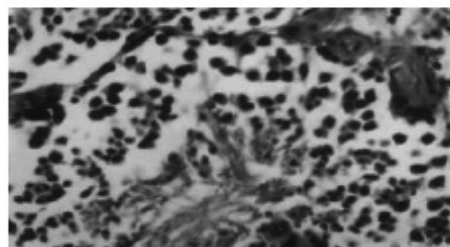
Microphotographs of seminiferous tubules in the 3 groups are presented in Figure 1.

DISCUSSION

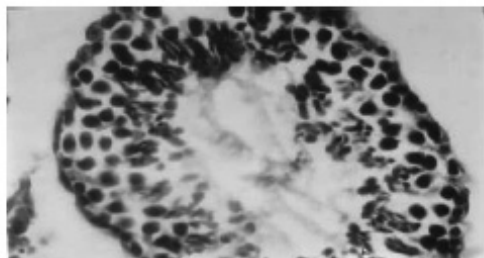
From this study we observed that administration of the *Hibiscus rosea sinensis L leaves* extract reduced the number of the spermatogenic cells (spermatogonia, pakiten primary spermatocyte, and spermatid) very significantly.

The hibiscetin glycoside in the leaves of *Hibiscus rosea sinensis L* might cause these anti-fertility/anti-spermatogenesis effect as was supposed by Purwaningsih (1998, 2003) and Kholkute (1977).⁵⁻⁷

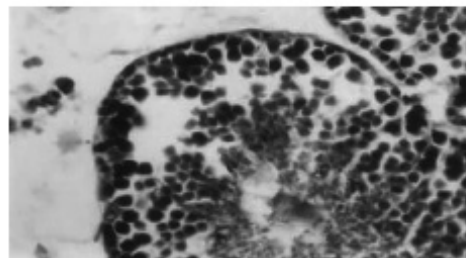
The spermatogenic cell reduction due to hibiscetin glycoside in the *Hibiscus rosea sinensis L* extract might be effected by hormonal mechanism through hypothalamus-pituitary-testis axis. Hormones that directly have effects on the spermatogenesis are gonadotropins, i.e. follicle stimulating hormone (FSH) and lutening hormone (LH) secreted by pituitary gland that is controlled by gonadotropin releasing hormone (GnRH) from hypothalamus.^{12,14-17}



A. Control



B. Dosage 700 mg/kgBW



C. Dosage 800 mg/kgBW

Figure 1. Spermatogenic cells in seminiferous tubulus transverse section of mice testis with H. E. stain, 400x

Hibiscetin was supposed to inhibit FSH and LH, and to cause disruption in testosterone/androgen production in the testis. Lutenizing hormone stimulates Leydig cells to synthesize testosterone and FSH stimulates Sertoli cells to secrete androgen binding protein (ABP).¹⁸ The ABP binds testosterone/androgen to form ABP-androgen complex. Bound testosterone/androgen was carried into the cytoplasm of the target cells. Dissociation process caused the testosterone/ androgen to be released from the complex. Free testosterone/androgen binds cytoplasm receptor forming a new complex, the receptor testosterone/androgen-complex. Dissociation process in the cell releases testosterone/androgen that enters the cell nucleus.^{15,19}

One of the functions of Sertoli cells is to support mitosis and meiosis.^{17,20}

Reduced Sertoli cells in the tubulus seminiferus due to environmental pressure inhibits spermatogonia development. This is because the Sertoli cells can not support the nutrition for all spermatogonia.

During spermatogenesis process, spermatogenic cells are very active, and changes include morphology, biochemistry, and genetic transformation of the cells. The spermatogenic cells depend on glucose for energy. However, pakhiten primary spermatocytes and spermatids depend on lactic and piruvic acid as energy provided by Sertoli cells. Lactic and piruvic acid production is influenced by FSH.^{15,18,21}

Hibiscetin glycoside contained in *Hibiscus rosea sinensis* L extract might influence FSH production and the Sertoli cells causing spermatogenic cell development inhibition.

The mechanism of hibiscetin glycoside in *Hibiscus rosea sinensis* L extract in inhibiting spermatogenesis is still unknown. However previous studies presumed that *Hibiscus rosea sinensis* L extract inhibited germinal cell metabolism and proliferation through cytotoxic or anti-androgen effects.^{6,7}

Hibiscetin glycoside in *Hibiscus rosea sinensis* L extract also has steroid-like features due to *cyclopentanoperhydrophenanthrene* steroid ring. With the feature, androgen receptor of the spermatogenic cell will identify and match with the hibiscetin in the lock and key manner. Androgen and hibiscetin competes to bind with the receptor. If hibiscetin binds the receptor, meiosis, spermiogenesis, and mitosis will be inhibited.^{15,20}

Therefore, hibiscetin glycoside content in *Hibiscus rosea sinensis* L extract might influence spermatogenesis by: cytotoxic effect or hypothalamus-pituitary-testis axis.⁷

However, it is necessary to study whether purified hibiscetin glycoside will cause spermatogenic cell reduction or not.

In conclusion, *Hibiscus rosea sinensis* L leaves extract 700 and 800 mg/kg BW decreased spermatogenic cell number, i.e. spermatogonia, pakhiten primary spermatocytes, and spermatids compared to control ($P = 0.000$).

Suggestion

It is necessary to evaluate the side effects on other organs such as kidney and liver.

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