

# Effect of *Butea Superba*. Roxb root extract on male hamster fertility

Chainarong Tocharus, Ph.D.,<sup>1</sup> Dawan Shimbhu, Ph.D.,<sup>2</sup> Jiraporn Tocharus, Ph.D.<sup>3</sup> and Arampa Ruchiratanti-angkoor, M.D.<sup>1</sup>

<sup>1</sup>Department of Anatomy, Faculty of Medicine, Chiang Mai University, <sup>2</sup>Department of Biochemistry, Faculty of Medical Science, Naresuan University, <sup>3</sup>Department of Physiology, Faculty of Medicine, Chiang Mai University

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**Objective** To investigate the long term effects of ethanolic extracts of *Butea superba* Roxb. root on sperm number and fertility, sperm quality and histology of testis in male hamsters.

**Methods** Male hamsters (100-120 g) were divided into four equal groups: one control group and three different treatment groups. The control group received distilled water orally each day for 6 months. The treatment groups were fed with alcoholic extract suspensions of *B. superba* root at doses of 0.1, 1 or 10 mg/kg BW/day for 6 months. The fertility of sperm was assessed by determining the number of 2-cell embryos collected from super-ovulated female hamsters after mating. The quality of sperm in male hamsters treated with *B. superba* was evaluated by counting the number of sperm, and the number of intact acrosomes on the sperm head. Histopathologic study of the testes was performed to evaluate the number of spermatogonia.

**Results** After six months of daily oral treatment with root extract, the sperm count was increased in the treated male hamsters in a dose-dependent manner. The number of 2-cell embryos in super-ovulated females, after mating with treated males, was significantly greater in *B. superba*-treated groups than in the control group. There was no change between the treated and control group in the percentage of sperm with acrosome. No pathology of reproductive organs was observed.

**Conclusion** *B. superba* root extract increases spermatogenesis among male hamsters, without affecting fertility and acrosome integrity, after 6 months of oral treatment. **Chiang Mai Med J 2012;51(2):39-44.**

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**Keywords:** acrosome; *Butea superba* Roxb., fertilization, sperm fertility, triple stain

## INTRODUCTION

*Butea superba* Roxb. (known locally as Kwao Khrua Dang) is found in northern Thailand as a climbing tree with long tuberous roots and red sap [1]. Products from this plant have been used

by humans as a physical and mental tonic and for the prevention of age-related health problems [2]. Thai traditional medicine uses *B. superba* as a rejuvenating herb for men. Compounds

extracted from *B. superba* have been effective in vitro in inhibiting cAMP phosphodiesterase; the mechanism that plays an important role in penile erection [3]. Previous studies have shown that ethanolic extract of *B. superba* induced penile erection by increasing intracavernous pressure with the maximum effective dose of 1 mg/kg, and also relaxed the corpus cavernosal smooth muscle in rat models [4]. The clinical trial of *B. superba* powder in Thai males showed effective treatment of erectile dysfunction [5]. The study on chronic toxicity of *B. superba* examined its effects on body weight, food consumption, relative organ, hematology, blood chemistry and histopathology [6]. Although there have been a considerable number of investigations into the effects of reproductive toxicants on the histology of the testis and ovary, there are no reports on damage to the fertility of sperm. Fertilization in vivo requires adequate numbers of spermatozoa with normal morphology and motility to be ejaculated [7, 8]. Since spermatogenesis involves a complex process of cellular development, impairment at any stage may lead to a reduction in fertility. Spermatogenesis has been shown to be susceptible to damage from a variety of chemical agents [9].

The aim of this study was to examine the effect on fertility of long-term treatment of alcoholic extract of *B. superba* root, as shown by sperm concentration and acrosome integrity of sperm, in correlation to the testicular architecture in hamsters.

## Methods

### Animals

Male hamsters (100-120 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. They were housed under a 12:12 h light-dark cycle and maintained at  $25 \pm 3$  °C. The experimental protocol was approved by the Committee on Animal Experimentation, Faculty of Medical Science, Naresuan University.

### Preparation of root extract

Tuberous roots of *B. superba* were collected from Phrae province, Thailand, in November, 2006. This plant was identified by the Botany and Weed Science Division, Depart-

ment of Agriculture, Kasetsart University, Bangkok, with the sample voucher code of MFLU 310. The roots were sliced into small pieces and extracted with ethanol. The ethanolic extract was evaporated under reduced pressure at 45 °C, using a rotary evaporator, and stored at -20 °C until use.

### Treatment of hamsters with *B. superba* root extract

Male hamsters were divided randomly into four groups of six hamsters per group. The control group received distilled water orally, whereas the treatment groups were fed with 1-mL suspension of *B. superba* root extract at doses of 0.1, 1 and 10 mg/kg BW/day through 17-gauge tubing for 6 months.

### Measurement of body and reproductive organ weight

Body weight was recorded before treatment, at weekly intervals during treatment and at the end of treatment. Testes, epididymis, seminal vesicle and prostate were excised and weighed after removal of surrounding adipose tissue.

### Semen analysis

Epididymal sperm counts were determined, as described previously [10, 11]. Briefly, the cauda epididymis was removed, cut and weighed, then finely minced with scissors to release the epididymal content into a 35-mm Petri dish containing 2 mL of Tyrode's solution (pH 7.4). The sperm was counted using a Neubauer hemocytometer.

The acrosome status was assessed by triple stain procedure [12]. A 200  $\mu$ L sample from each treatment suspension was incubated with an equal volume of TALP-HEPES (HEPES 10 mM, NaCl 127 mM, KCl 3.16 mM,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  2.0 mM,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  0.5 mM, Na lactate 10 mM,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  0.35 mM,  $\text{NaHCO}_3$  2.0 mM, Glucose 5.0 mM) containing 2% Trypan Blue at 37 °C for 10 min, and then centrifuged at 200 g for 10 min. The pellet was resuspended with 0.5 mL of TALP-HEPES. Three smears were made from each sample and fixed with 2.5% glutaraldehyde for 1 h. The fixed sperm was air-dried and stained overnight with 0.8% Bismark Brown for 5 min at 40 °C, and 0.8% Rose Bengal for 30 min at 24 °C. Live and dead sperm were distinguished in this procedure by staining with Trypan Blue, in the presence of acrosome on the sperm head resulting in uptake of Rose Bengal at the sperm tip. Slides were evaluated by counting 400 cells, using bright field microscopy, and results were expressed as percentage of sperm with an intact acrosome.

### Testicular histology study

After the hamsters were sacrificed, their testes were dissected, cut into small slices and fixed in a 10% formaldehyde buffer for 24 h. The tissue was washed free of fixative, dehydrated in alcohol series, and then embedded in paraffin. Tissue sections of 5- $\mu$ m thickness were prepared and placed on glass slides. The sections were stained with hematoxylin and eosin and mounted in a mounting medium. The slides were observed under a light microscope for qualitative changes in the seminiferous tubules.

**Table 1.** Effect of *B. superba* extract on body weight changes and reproductive organs weight

Group (N=6)	Dose of extract (mg/kg BW)	Body weight in g (Mean ± SD)	Organ weights in mg (Mean ± SD)			
			Testis	Epididymis	Seminal vesicle	Prostate
1	None	128.12 ± 0.60	4.15 ± 0.49	0.82 ± 0.16	1.59 ± 0.58	0.22 ± 0.09
2	0.1	130.33 ± 0.41	4.02 ± 0.50	0.78 ± 0.10	1.51 ± 0.60	0.20 ± 0.09
3	1.0	126.81 ± 0.33	4.17 ± 0.27	0.77 ± 0.07	4.15 ± 0.49	0.19 ± 0.09
4	10.0	129.59 ± 0.28	3.39 ± 0.57	0.78 ± 0.09	1.48 ± 0.57	0.22 ± 0.18

### Fertility test

A male was mated with two superovulated females at the end of the six-month treatment period. Superovulation was achieved by intraperitoneal injection of 25 µL of pregnant mare serum gonadotropin, followed 72 h later by administration of 25 µL of human chorionic gonadotropin. In the following morning, presence of vaginal plug and sperm in the vaginal smear was considered positive indices of mating. After 48 h, the mated females were sacrificed and their oviducts removed. The 2-cell stage embryos were recovered by flushing the oviducts with Dulbecco's phosphate buffered saline and counted under a microscope.

### Statistical analysis

Statistical comparison between different treatment results was analyzed by one-way analysis of variance followed by the LSD test. Differences with a *p*-value of 0.05 or less were considered statistically significant.

## Results

### Effect of *B. superba* root extract on body and reproductive organ weight

After 6 months of daily oral treatment with *B. superba* root extract, the mean body weight in all of the treatment groups did not differ significantly from that in the control group. Similarly, the mean weight of reproductive organs (testes, epididymis, seminal vesicle and prostate) was comparable between the control and treated groups (Table 1).

### Changes in sperm concentration and acrosome integrity

In evaluating the effect of long term *B. superba* root extract treatment on hamster sperm quality, the concentration and acrosome status of epididymal sperm were determined. As shown in Fig. 1, the sperm concentration increased significantly in hamsters treated for 6 months with *B.*

*superba* root extract in a dose-dependent manner (*p*<0.05). When the integrity of acrosome on the sperm head was assessed by triple staining (Fig. 2), the percentages of viable sperm with intact acrosome were similar in the control and *B. superba* - treated groups. The percentages of dead sperm with intact acrosome were lower than those of the viable sperm. However, there was no difference between the groups in the percentages of sperm with acrosome, indicating that the treatment with *B. superba* root extract does not affect acrosome integrity of the spermatozoa.

### Testicular histology

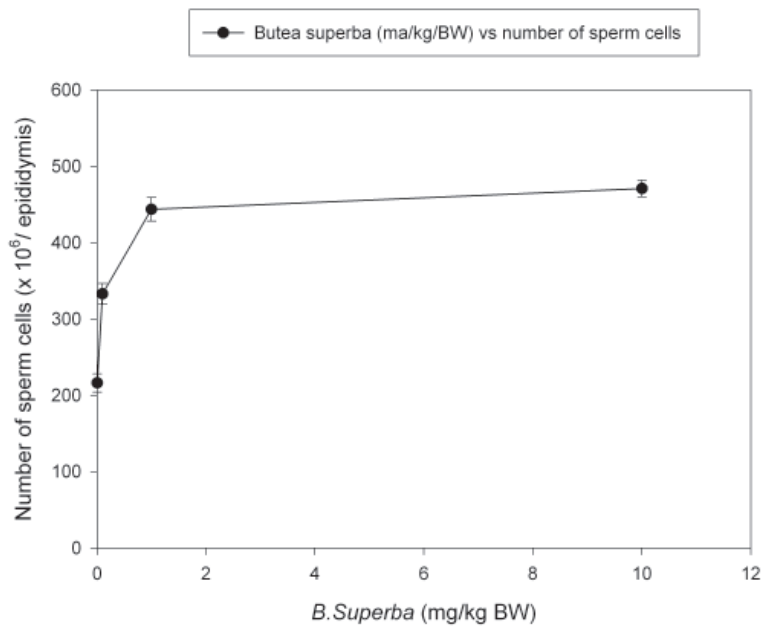
Histological analysis of the testes revealed that a greater abundance of primary spermatocytes and spermatids was observed in the *B. superba* - treated groups than in the control group (Fig. 3a and 3b). No abnormal pathological change was detected in either the control or treated groups.

### Change in fertility

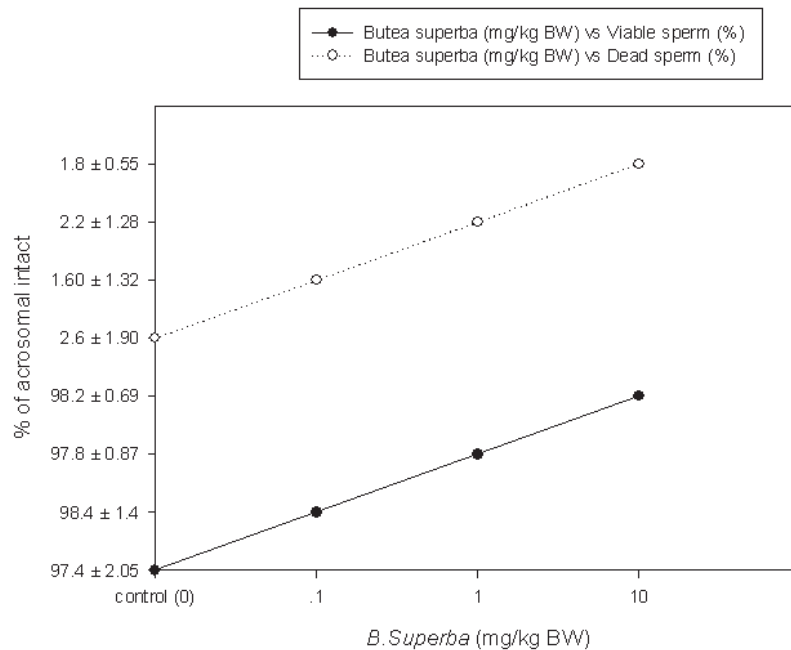
In evaluating the fertility of male hamsters after 6 months treatment with *B. superba* root extract, the percentages of 2-cell embryos collected from the super-ovulated females, after mating with treated males, were compared between the control and treated groups. The percentage of 2-cell embryos was higher at the dose of 1 mg/kg BW as compared with doses of 0.1 and 10 mg/kg BW and the control group (*p*<0.05) (Table 2).

## Discussion

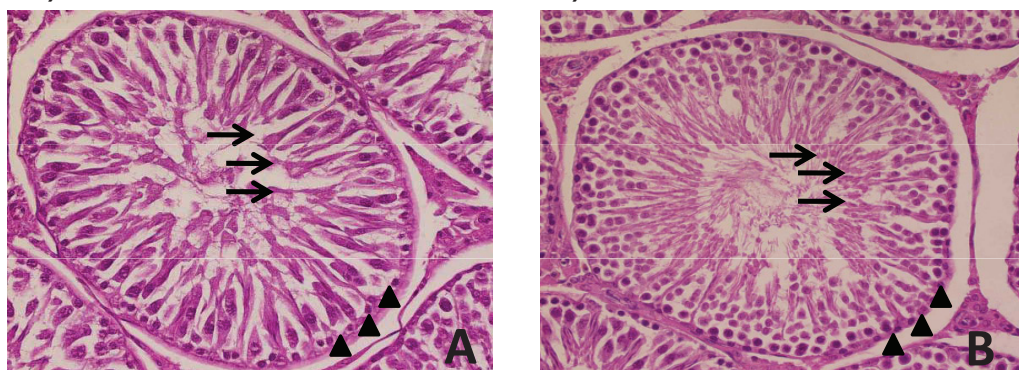
This study investigated the effects of long term oral treatment of *B. superba* root extract on sperm quality in the epididymis, and fertility of



**Figure 1.** Concentration of sperm in the epididymis of *B. superba*-treated male hamsters. Data represent mean and SD of sperm concentration per epididymis derived from 6 hamsters per group \*,  $p < 0.05$ .



**Figure 2.** Percentage of sperm with intact acrosome. Sperm was separated into viable and dead cells by Trypan Blue exclusion. The presence of acrosome in both groups of sperm was determined by Rose Bengal uptake. Data represent the mean percentages and SD of sperm with intact acrosome in each sperm category in different treatment groups.



**Figure 3.** Hematoxylin and eosin-stained testicular sections from a control hamster (A) and hamster receiving *B. superba* root extract at a dose of 10 mg/kg BW/day (B) (40x). Arrows indicate spermatozoa. Arrowheads indicate spermatogonia.

**Table 2.** The number of 2-cell embryo collected from male hamsters treated with various concentration of *B. superba* extract for 6 months

Group (N=6)	Treatment with <i>B. superba</i> extract (mg/kg BW)	Number of superovulated female hamsters	Number of two-cell embryos <sup>a</sup> (%)
1	None	12	26.00 ± 2.10
2	0.1	12	27.00 ± 3.52
3	1.0	12	28.33 ± 2.73 <sup>b</sup>
4	10.0	12	26.50 ± 2.17 <sup>b</sup>

<sup>a</sup> Number of 2-cell embryos is presented as percentage of untreated controls; <sup>b</sup> Significant difference ( $p < 0.05$ ) as compared with normal control.

male hamsters. The results revealed that sperm concentration in the epididymis increased significantly in *B. superba* treated-hamsters in a dose-dependent manner. These results are consistent with previous reports of rats treated with crude *B. superba* powder [13]. Furthermore, histological analysis of testes indicated an increase in number of spermatogonia and spermatozoa in seminiferous tubules in the *B. superba* - treated groups. These results indicate that *B. superba* has a positive effect on the process of spermatogenesis.

The effect of *B. superba* root extract on spermatogonia may be due to  $\beta$ -sitosterol. This phytochemical component found in *B. superba* may be converted to pregnenolone, an important substrate of testosterone synthesis in the testes [14]. Testosterone may activate the release of GnRH from the hypothalamus in the treated hamsters. Subsequently, FSH and LH, which are released

by the GnRH, may induce spermatogenesis and growth of the spermatozoa during treatment.

Interestingly, the acrosome status of spermatozoa in the *B. superba* – treated groups remained similar to that in the control group. This finding agrees with the effect of *B. superba* root extract on fertility, when tested by mating with superovulated females. The results of this study clearly suggest that 6 months of oral treatment with *B. superba* extract increases spermatogenesis in hamsters without causing adverse effect on the fertility and structural integrity of the reproductive organs.

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### ผลของสารสกัดหัวกวาวเครือแดงในระยะยาวต่อการปฏิสนธิของอสุจิในหนูแฮมเตอร์

ชัยณรงค์ โดจรัส, Ph.D.,<sup>1</sup> ดาวัลย์ นิมกุ, Ph.D.,<sup>2</sup> จิราภรณ์ โดจรัส, Ph.D.<sup>3</sup> และ อารัมภ์ รุจิรัตน์อังกูร, พ.บ.  
<sup>1</sup>ภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่, <sup>2</sup>ภาควิชาชีวเคมี คณะวิทยาศาสตร์-  
 การแพทย์ มหาวิทยาลัยนเรศวร, <sup>3</sup>ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่

**วัตถุประสงค์** เพื่อศึกษาผลของการได้รับสารสกัดหัวกวาวเครือแดงด้วยเอทานอลในระยะยาวต่อการปฏิสนธิ คุณภาพของอสุจิ และพยาธิสภาพของอวัยวะในหนูแฮมเตอร์เพศผู้

**วิธีการศึกษา** แบ่งหนูแฮมเตอร์เพศผู้ น้ำหนัก 100-120 กรัม ออกเป็น 4 กลุ่ม โดยกลุ่มที่ 1 เป็นกลุ่มควบคุม ได้รับการป้อนน้ำทางปากติดต่อกันเป็นระยะเวลา 6 เดือน กลุ่มที่ 2, 3 และ 4 ได้รับการป้อนสารสกัดหัวกวาวเครือแดงที่ความเข้มข้น 0.1, 1 และ 10 มิลลิกรัมต่อกิโลกรัม ของน้ำหนักตัวติดต่อกันเป็นระยะเวลา 6 เดือน แล้วศึกษาความสามารถในการปฏิสนธิของอสุจิด้วยการนับจำนวนของ ตัวอ่อนที่ได้จากหนูตัวเมียในระยะ 2 เซลล์ ศึกษาคุณภาพของอสุจิจากการนับจำนวนอสุจิ และจำนวนของอะโครโซมที่ส่วนหัวของอสุจิ และศึกษาพยาธิสภาพของอวัยวะในหนูแต่ละกลุ่มโดยเทคนิค triple stain

**ผลการศึกษา** สารสกัดหัวกวาวเครือแดงทำให้หนูแฮมเตอร์มีจำนวนของตัวอ่อน และจำนวนเซลล์อสุจิเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ไม่พบความแตกต่างของอะโครโซมที่ส่วนหัวของอสุจิในกลุ่มที่ได้รับสารสกัดหัวกวาวเครือแดงและกลุ่มควบคุม นอกจากนี้ไม่พบความผิดปกติทางพยาธิสภาพของอวัยวะในหนูที่ได้รับสารสกัดหัวกวาวเครือแดง

**สรุปผลการศึกษา** สารสกัดหัวกวาวเครือแดงในปริมาณที่ใช้ศึกษามีฤทธิ์ในการเพิ่มประสิทธิภาพในการปฏิสนธิ เมื่อให้ทางปากแก่หนูแฮมเตอร์เพศผู้ เป็นเวลานาน 6 เดือน โดยไม่ทำให้เกิดพยาธิสภาพในอวัยวะ เชียงใหม่เวชสาร 2555;51(2):39-44.

**คำสำคัญ:** อะโครโซม กวาวเครือแดง การปฏิสนธิ ความสามารถในการปฏิสนธิของอสุจิ ทริปเปิ้ลสแตน