Pharmaceutical Sciences Asia

Pharm Sci Asia 2024; 51(4), 336-348

DOI:10.29090/psa.2024.04.24.AP0965

Research Article

EVALUATE THE ACUTE, SUBCHRONIC TOXICITY, AND PROTECTIVE EFFECT OF *BUTEA SUPERBA* ROXB. EXTRACT IN THE SODIUM VALPROATE-INDUCED HYPOGONADISM IN SWISS ALBINO MALE MICE

Huu Lac Thuy Nguyen[1], Bao Kim Nguyen[2], Minh Hien Khuu[2], Dang Thuy Hien Nguyen[2], Nguyen Hoang Linh Phan[2], Minh Nhut Truong[3], Minh Thai Nguyen[4], Thanh Binh Nguyen[5,6], Huynh Nhu Mai[2,*]

1 Department of Analytical Chemistry and Drug Quality Control, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Vietnam
2 Department of Pharmacology, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Vietnam
3 Unit of Traditional Medicine and Pharmacy, Faculty of Traditional Medicine, University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Vietnam
4 Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City, Ho Chi Minh City, Vietnam
5 Biomedical engineering training program, Faculty of Medicine and Pharmacy, Thu Dau Mot University, Binh Duong Province, Vietnam
6 Tissues and Cells Unit, Biomedical Research Center, Pham Ngoc Thach University of Medicine, Ho Chi Minh City, Vietnam

ABSTRACT

Butea superba Roxb. *(BS)* has been used in traditional medicine to treat liver malfunction, enhance vitality, and have an anti-aging effect. However, limited research shows its hypergonadistic effects. This study aimed to assess the acute, subchronic toxicity, and the protective effects of *BS* stem extract in treating hypogonadism induced by sodium valproate (SV) in male mice. The acute and subchronic toxicity tests were conducted following the Guidelines for Preclinical and Clinical Trials of Traditional Medicine and Herbal Medicine by the Vietnam Ministry of Health and OECD guidelines. To investigate the ameliorative effect of *BS* extract, mice were divided into 5 groups (n = 8): control group (distilled water, p.o.), SV group (500 mg/kg, p.o.), testosterone group (2 mg/kg, p.o.), and treated groups given *BS* doses of 10 mg/kg and *BS* 100 mg/kg, orally two hours after using SV (500 mg/kg, p.o.). Viability and sperm concentration were assessed by flow cytometry. Additionally, testosterone levels and testicular H&E staining were determined. The *BS* extract produced no toxic effects at the maximum dose administered orally (36.3 g/kg). The subchronic toxicity at doses of 10 mg/kg and 100 mg/kg showed no signs of toxicity during 28-day treatment. In the androgenic study, groups treated with *BS* extract at both doses showed a significant increase in serum testosterone, relative weight and diameter of the testis, sperm count, relative weight of levator ani-bulbocavernosus muscles, a notable decrease in the death rate of sperm, and an improvement in the histology of the testis compared to the SV group. These results were similar to those of the testosterone group.

BS extract did not produce acute or subchronic toxicities at the chosen doses. In addition, *BS* exhibited protective effects against SV-induced hypogonadism, with the best results seen in the group treated with the dose of 100 mg/kg.

Keywords:
Butea superba Roxb.; Androgenic effect; Acute; Subchronic; Toxicity; Mice.

1. INTRODUCTION

The rate of hypogonadism in men over 45 years old in the US when examined at a medical facility is estimated to be 38.7%[1]. In Vietnam, the rate of men with hypogonadism increases with age: 9% under 50 years old,

***Corresponding author:**
* Huynh Nhu Mai Email: mhnhu@ump.edu.vn

29% between 50 and 59 years old, 44% between 60 and 69 years old, 70% between 70 and 79 years old, and 80% over 80 years old[2]. The results of a survey study by Van Zyl J.A. showed that ranging from 8.9% to 68.7% of men experience at least one sexual dysfunction during their lifetime[3,4].

The decrease in testosterone hormone causes a series of syndromes in reproductive organs, as testosterone is responsible for sexual differentiation, testicular descent, spermatogenesis, enlargement of testes[5] , and the enhancement of muscle regeneration[6].

Because the use of drugs in modern medicine has adverse effects on liver and kidney, the search for another treatment method that is both effective and less expensive was promoted[7]. *Butea superba* Roxb. belongs to the bean family (Fabaceae), grows and develops in secondary forests with a high humus content and a pH of 5.5-6.5. This species is widely distributed in India, China, and some Southeast Asian countries, including Thailand. Research around the world targeted the roots of the *Butea superba* Roxb. on enhancing male physiology[8], hepatocellular injury[9] and ameliorating depression-like symtoms[10]. However, there has not been any comprehensive research on the toxicity data of BS stem extract as well as its protective effect on male reproductive organs against hypogonadism, although the use of stem would allow for more output as well as high economic efficiency.

Many research showed sodium valproate's ability to induce reproductive disorders through a reduced level of serum FSH and LH, a reduction of testes' weights, sperm count, and sperm motility, an increase of abnormal sperm morphology. Therefore, our study used sodium valproate to induce hypogonadism and assess the ameliorative activity of *Butea superba* on the reproductive function of male mice.

Therefore, to provide scientific evidence about the safety and effectiveness of BS stem, we conducted acute and subchronic tests along with investigating its ability to improve the hypogonadism induced by sodium valproate in male Swiss albino mice.

2. MATERIALS AND METHODS

2.1 Animals

All animals were treated in accordance with the Institute for Laboratory Animal Research (ILAR) Guidelines for the Care and Use of Laboratory Animals. All the experiments were conducted in the Laboratory Animal House, Department of Pharmacology, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh City. The experimental study was approved by the Ethics Committee (decision no. 589/GCN-HĐĐĐNCTĐV) on June 12th, 2023.

Male Swiss albino mice aged 6-7 weeks, each weighing approximately 24.5 ± 2.5 g, were obtained from the Pasteur Institute in Ho Chi Minh City. Animals were randomized and acclimated to housing for 2 weeks prior to the experimental protocols. They were fed a standard diet and water ad libitum.

2.2 Extract preparation

Butea superba Roxb. was identified, propagated and exploited by the Forest Science Institute of Central Highlands and South of Central Vietnam (FSIH) in the National project (NVQG.2013/13) directed by the Ministry of Agriculture and Rural Development[11]. The ecological characteristics and distribution of this species were published in the Agriculture and Rural Development Journal[12]. In December 2022, *Butea superba* Roxb. stem was provided by the Forest Science Institute of Central Highlands and South of Central Vietnam (FSIH) in Da Lat province, Vietnam[13].

The stems were cut into small slices, dried, and ground into coarse powder. 50g of *Butea superba* powder (the ratio of powder to solvent is 1 to 6) was moistened for 30 minutes with 70% ethanol solvent. The powder was cold soaked for 24 hours, then drained at a drip rate of 50-60 drops per minute. Subsequently, the alcohol was evaporated (the evaporation temperature was 45 °C) to obtain the extract. The extract was stored at -20 °C before use.

2.3 Acute toxicity test

The acute toxicity test was conducted according to the Guidelines for Preclinical and Clinical Trials of Traditional Medicine and Herbal Medicine by the Vietnam Ministry of Health[14] and OECD with slight modifications[15]. Prior to the start of the experiment, the body weights of the animals were recorded to calculate the proper treatment dosage. 10 male mice were divided into two groups, receiving treatments as follows:

- Control group (n = 5): distilled water;
- *BS* extract (n = 5): *Butea superba* extract 36.3 g/kg body weight.

The animals were then observed for any abnormalities in the first 24 hours and changes in general behaviors, physical condition, waste, and mortality rate in the 72 hours after the administration.

If any death was recorded, macroscopic analysis would be conducted, and the lethal dose of 50% of the animals tested (LD_{50}) would be determined by testing on new groups of mice with a different dose range. Based on the mortality rate within each dose, the LD_{50} would be calculated using the Karber-Behrens formula. If no death was recorded in 72 hours, observation would continue within 14 days to record the animals' body weights and abnormalities.

2.4 Subchronic toxicity test

In the subchronic toxicity test, male mice were divided into three groups (n = 7-8) and received oral treatment for 28 consecutive days as follows:
- Control group: distilled water;
- *BS* extract 10 mg/kg: *Butea superba* extract 10 mg/kg body weight;- *BS* extract 100 mg/kg: *Butea superba* extract 100 mg/kg body weight.

The body weights of mice were measured before and during the treatment. Any mortality and toxicity signs of the animals were also recorded daily throughout the experiment. After 28 days, mice were sacrificed to collect blood samples for the assessment of hematological and biochemical parameters. The liver and kidneys were collected for microscopic analysis via Hematoxylin and Eosin staining.

2.5 Experiment design to evaluate the protective effects of *Butea superba* extract

In this study, mice were divided into five groups (n = 8). The experiment design is shown in Table 1.

All of the reagents. testosterone, sodium valproate were made fresh. *BS* extract was dissolved in distilled water prior to use. The doses of *BS* extract (10 mg/kg and 100 mg/kg) were selected based on our pilot study and the previous research[8,16]. Mice were given the treatment at a volume of 0.1 ml/10 g body weight. The body weight was recorded once every 3 days over the course of 35 days. On the 36th day, mice were sacrificed, and blood was collected to assess the serum testosterone level; testis and levator ani plus bulbocavernosus muscles to record the relative weight and testis histological structure; and sperm to assess the sperm count and vitality.

Table 1. Experiment design in 35 days

Group	Treatment
Control	Distilled water (DW), p.o.
Sodium valproate 500 mg/kg	Sodium valproate (500 mg/kg/day, p.o.)
BS 10 mg/kg[8]	*Butea superba* extract (10 mg/kg/day, p.o.) 2 hours prior to sodium valproate (500 mg/kg/day, p.o.)
BS 100 mg/kg[8]	*Butea superba* extract (100 mg/kg/day, p.o.) 2 hours prior to sodium valproate (500 mg/kg/day, p.o.)
Testosterone 2 mg/kg	Testosterone (2 mg/kg/day, p.o.) 2 hours prior to sodium valproate (500 mg/kg/day, p.o.)

2.6 Serum testosterone analysis

The blood sample was taken and centrifuged at 6000 rpm for 15 minutes at room temperature, then 200 µl of serum was collected and measured by the DRG Testosterone ELISA Kit (EIA-1559) based on the manufacturer's instructions

2.7 Weights of the testes and the levator ani plus bulbocavernosus muscles

2.7.1 Dissection of the testes

After the separation of testicles, their weights were determined by using an analytical balance (Kern ASL 220-4N). The testes were then dimensioned horizontally and vertically by using a metric micrometer (Moore and Wright, Sheffield, UK)[17]. Testicular length (L) from pole to pole and maximal width in two perpendicular planes (W1 and W2) were measured in millimeters (mm)[18].

2.7.2 Dissection of the levator ani plus bulbocavernosus muscles (LABC)

The skin and adnexa from the perianal region were removed to reveal the levator ani plus bulbocavernosus (LABC) muscles and penile bulbs. Remove the fat using forceps and micro dissecting 400 µl of NaCl to obtain a 15-fold dilution. Sperm scissors. Dissecting the bulbocavernosus (BC) muscle from the penile bulb, removing the white connective tissue and the reddish corpus spongiosum. Lift the bulbocavernosus (BC) muscles and cut the colon in two. Pull the levator ani plus bulbocavernosus (LABC) upward, remove the fat and adnexa, and trim the fat and adnexa[19].

2.8 Sperm concentration and sperm vitality

2.8.1 Preparation of sperm samples

On the 36th day, mice were sacrificed to collect the vas deferens. According to the research by Gray et al.[19], a small incision was made in the mouse scrotum, and the seminal vesicles were exposed. The bilateral ducts after separation were stored in 250 µl of Ferticult Flushing Medium (FFM).

With sperm concentration, 100 µl of sperm was added to 400 µl NaCl (0,9%), centrifuged for the first time at 250 rpm for 7 minutes. The supernatant was discarded, and 400 µl NaCl (0,9 %) were added, then centrifuged a second time at 250 rpm for 7 minutes.

After discarding the supernatant, 400 µl NaCl (0,9%) was added and vortexed for 1 second. Similarly, to assess the sperm vitality, 1 µl of PI was added and incubated for 5 minutes after adding 400 µl of NaCl (0,9%) at the second centrifugation. 100 µl out of the 250 µl mixture was contained in eppendorf and then concentration and percentage of dead sperm were

determined by flow cytometry. Samples were diluted in NaCl (0,9%) until total events/µl < 2000.

2.8.2 Sperm concentration

Using a C6 Plus Flow Cytometer, all fluorescence signals from tagged spermatozoa were examined

With the fluidic system set to "slow" and the flow rate of about 100 cells per second, a total of 10,000 spermatozoa were detected. Red fluorescence (640 nm) in the PerCP-A channel is excited by a 14.7 mW diode red laser at a single wavelength of 640 nm. The flow cytometer software was used to compute the proportion of positive cells (positive) on a 1023-channel scale. Similar methods were used for the study on the C6 Plus flow cytometer, and the BD Accuri software (BD Biosciences, Ann Arbor, MI, USA) produced dot plots[20].

2.8.3 Sperm vitality

The same set of samples was run simultaneously with C6 Plus flow cytometers. We used the BD Accuri workspace (BD Biosciences, San Jose, CA, USA) and gating strategy, which was standardized using two plots and sequential gating as described in a study by Ribeiro S et al[20]. The following plots were used: PI fluorescence and FSC, or "Plot 2" – The PI-positive event gating (R1) application selected the PI-positive spermatozoa and excluded the debris[21]. PI Fluorescent Positive Gate, or "Plot 3" – PI-positive samples are depicted in the upper right quadrant. Areas with PerCP-A or PI channel values > 10^5 are defined as dead spermatids. Results are expressed as a percentage of dead sperm (PI++) and live sperm (PI+)[22].

2.9 Hematoxylin - Eosin staining (H&E)

The testicles were collected and fixed in formalin 10% for 24 hours at room temperature. According to a standard protocol, paraffin-embedded specimens were prepared, and 5 µm sections were stained with hematoxylin and eosin (Sigma-Aldrich, St. Louis, MO, USA) and assessed by Olympus BX40 microscope[23]. The severity of gem cell damage was assessed by the Johnsen score[24].

2.10 Statistical analysis

Statistical analysis was performed using IBM SPSS 20 software. One-way analysis of variance (ANOVA) followed by Fisher's LSD post-hoc test was used to assess the statistical differences among means. $p < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1 Acute toxicity test

Effects of *Butea superba* (BS) extract on the body weights of male mice in 14 days were shown in Figure 1.

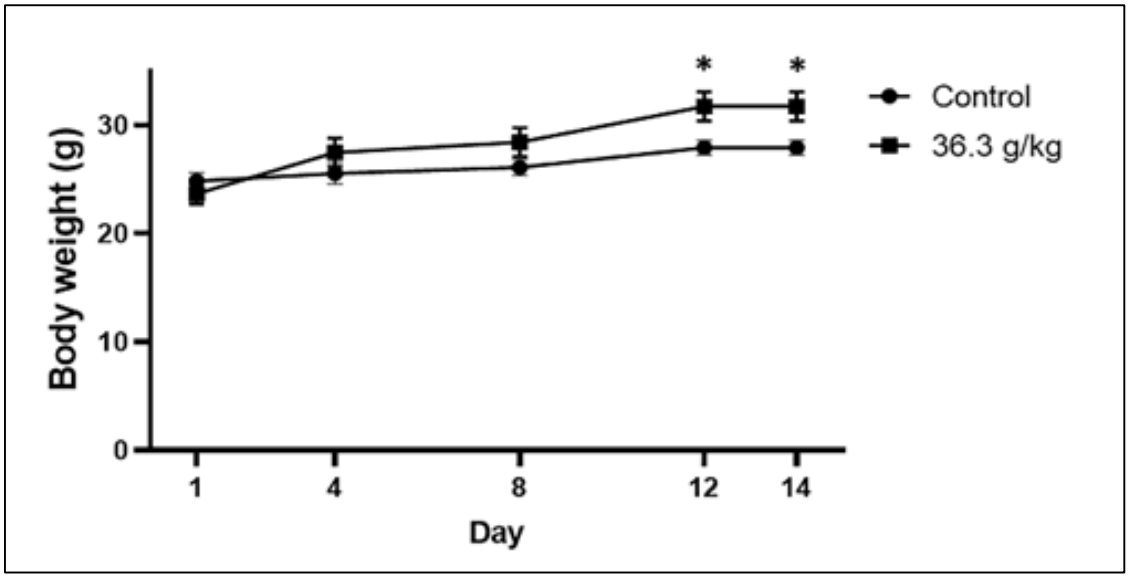

Figure 1. Body weights of male mice in 14 days. Data are expressed as Mean ± S.E.M. and analyzed using Student's t-test (n = 8 per group).

Oral administration of the *BS* extract at the dose of 36.3 g/kg resulted in no mortality or signs of acute toxicity in mice, as observed for a short period of 72 hours and a prolonged period of 14 days. As shown in Figure 1, the body weights of the treated group gradually increased during the 14-day period. On the 12th and 14th day of the experiment, the body weight of the *BS* group were significantly higher compared to the control group ($p<0.05$). However, the organ showed no macroscopic abnormalities. Therefore, the LD50 dose could not be determined, and *BS* ethanolic extract produced no acute toxicity according to the Guidelines for Preclinical and Clinical Trials of Traditional Medicine and Herbal Medicine by the Vietnam Ministry of Health[14]. Regarding the Loomis and Hayes classification (1996)[25], *BS* extract can be classified as "relatively harmless" since the LD50 surpassed 15000 mg/kg and categorized in group 5 according to the Globally Harmonized Classification System[26]. The research of Manosroi et al. in 2000 on the acute toxicity of *BS* in rats reported that the LD50 dose was 20 g/kg[27], making the equivalent dose for mice approximately twice this value, which supported our results.

3.2 Subchronic toxicity test

3.2.1 Effects of *Butea superba* (BS) extract on general behaviors and body weights of male mice in 28 days

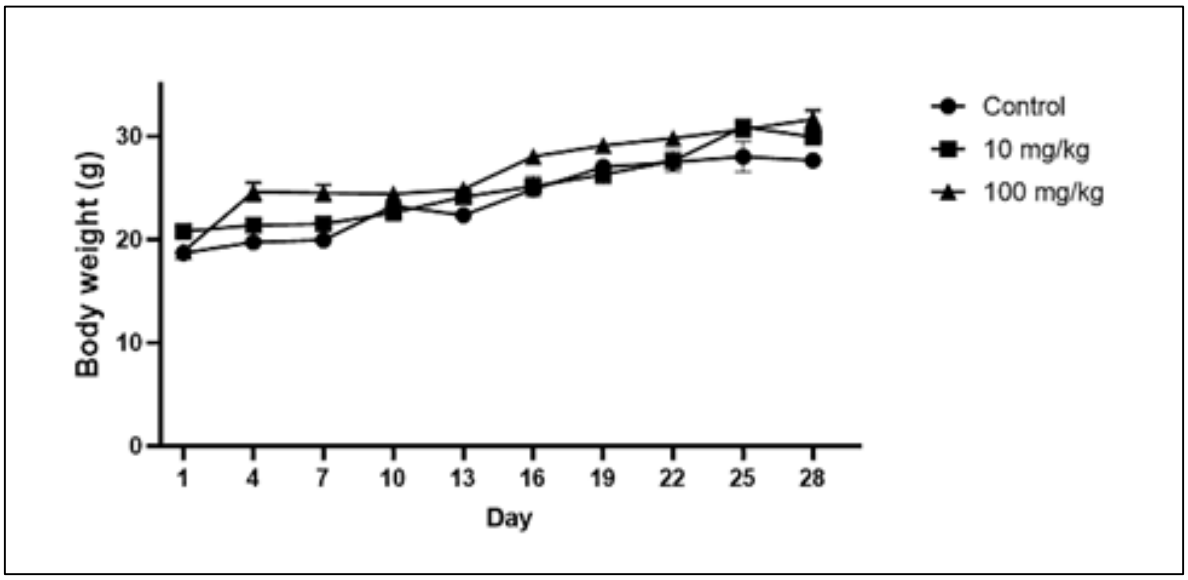

Figure 2. Body weights of male mice in 28 days. Data are expressed as Mean ± S.E.M. and analyzed using ANOVA and Fisher's LSD post-hoc test (n = 8 per group).

Oral administration of the BS extract at doses of 10 mg/kg and 100 mg/kg for 28 days did not result in any significant changes in the general behaviors of treated mice compared to the control group. Overall, both groups appeared healthy, and no mortality was observed throughout the 28-day treatment period. Figure 2 shows that the body weights of both the BS 10 mg/kg group and the BS 100 mg/kg group gradually increased during 28 days with no significant difference in comparison to the control group ($p > 0.05$).

3.2.2 Effects of *Butea superba* (BS) extract on the serum biochemical parameters

As shown in Table 2, no significant difference in the other biochemical parameters was recorded between the control and treated groups ($p > 0.05$), therefore treatment with the dose of alcohol extract at 10 mg/kg and 100 mg/kg continuously for 28 days did not affect the biochemical parameters (Table 2).

Table 2. Effects of *Butea superba* (*BS*) extract on the serum biochemical parameters.

Parameters	Control	*BS* 10 mg/kg	*BS* 100 mg/kg
AST (U/I)	121.57 ± 13.36	113.48 ± 13.32	103.30 ± 19.84
ALT (U/I)	62.18 ± 4.62	56.91 ± 7.69	62.31 ± 4.61
Total bilirubin (mg/dL)	0.29 ± 0.03	0.35 ± 0.28	0.34 ± 0.01
Direct bilirubin (mg/dL)	0.06 ± 0.003	0.07 ± 0.001	0.07 ± 0.001
Indirect bilirubin (mg/dL)	0.22 ± 0.03	0.27 ± 0.02	0.26 ± 0.01
Urea (mg/dL)	27.49 ± 4.63	33.37 ± 2.69	28.63 ± 1.20
Creatinine (mg/dL)	0.49 ± 0.06	0.67 ± 0.04	0.60 ± 0.01

Data were expressed as Mean ± S.E.M, n = 7-8 mice each group. Comparisons between the groups were made by one-way analysis of variance (ANOVA) followed by Fisher's LSD post-hoc test. *$p < 0.05$, **$p < 0.01$ vs. control group

3.2.3 Effects of *Butea superba* (BS) extract on the hematological parameters

The hematological profiles of the control and the BS treated groups are summarized in Table 3. A significant decrease was observed in the white blood cell (WBC) level of treated mice in BS 10 mg/kg group ($p < 0.01$) and BS 100 mg/kg group ($p < 0.05$). The study of Wichai Cherdshewasart in 2008 showed the powder suspension of tuberous BS at the dose of 200 mg/kg decreased the percentage of neutrophils[28], which supported our results. Many medicinal plants were used for anti-inflammatory effects also reduced total WBC and neutrophils, including Zataria multiflora Boiss, Portulaca oleracea L., etc.[29].

In addition, the red blood cell count (RBC) of the treated groups were significantly higher than those of the control group. To the best of our knowledge, no previous study has assessed the effect of BS on RBC. However, more studies are necessary to investigate the mechanism behind the RBC. In terms of other hematological parameters, no significant difference was observed between the control and treated groups ($p > 0.05$).

Table 3. Effects of *Butea superba* (*BS*) extract on the hematological parameters.

Parameters	Control	*BS* 10 mg/kg	*BS* 100 mg/kg
RBC (10^{12}/L)	8.54 ± 0.19	9.40 ± 0.13**	9.56 ± 0.15**
Hemoglobin (g/dL)	13.68 ± 0.33	14.84 ± 0.55	15.16 ± 0.38
Hematocrit (%)	44.55 ± 0.97	48.09 ± 1.95	48.44 ± 1.32
MCV (μm^3)	52.23 ± 0.89	51.16 ± 1.47	50.84 ± 0.95
MCH (pg)	15.88 ± 0.32	15.87 ± 0.42	15.69 ± 0.39
MCHC (g/dL)	30.64 ± 0.28	31.11 ± 0.33	30.51 ± 0.29
RDW (%)	17.76 ± 0.63	17.64 ± 0.45	17.56 ± 0.51
WBC (10^{12}/L)	10.26 ± 0.97	6.47 ± 0.86**	7.15 ± 0.92*
Platelets (10^9/L)	629 ± 22	813 ± 133	768 ± 113

Data were expressed as Mean ± S.E.M, n = 7 - 8 mice each group. Comparisons between the groups were made by one-way analysis of variance (ANOVA) followed by Fisher's LSD post-hoc test. *$p < 0.05$, **$p < 0.01$ vs. control group

The hematological profiles of the control and the *BS* treated groups are summarized in Table 3. A significant decrease was observed in the white blood cell (WBC) level of treated mice in *BS* 10 mg/kg group ($p < 0.01$) and *BS* 100 mg/kg group ($p < 0.05$). The study of Wichai Cherdshewasart in 2008 showed the powder suspension of tuberous *BS* at the dose of 200 mg/kg decreased the percentage of neutrophils[28], which supported our results. Many medicinal plants were used for anti-inflammatory effects also reduced total WBC and neutrophils, including *Zataria multiflora* Boiss, *Portulaca oleracea* L., etc.[29].

In addition, the red blood cell count (RBC) of the treated groups were significantly higher than those of the control group. To the best of our knowledge, no previous study has assessed the effect of *BS* on RBC. However, more studies are necessary to investigate the mechanism behind the RBC. In terms of other hematological parameters, no significant difference was observed between the control and treated groups ($p > 0.05$).

3.2.4 Effects of *Butea superba* (BS) extract on the histology of liver and kidney

The photomicrographs of the liver and kidney of the control and *BS* treated groups showed normal morphological architecture. Similar to the control group, no signs of hepatocellular injury, necrosis, congestion, fat accumulation, or hemorrhagic regions were observed around the central vein (Fig. 3A). The result is consistent with the study by Wichai Cherdshewasart in 2008. which also recorded no changes in the histology of the liver[28]. Renal tissue samples from the control and treated groups: glomeruli, tubules, and interstitial tissue had normal structure (Fig. 3B).

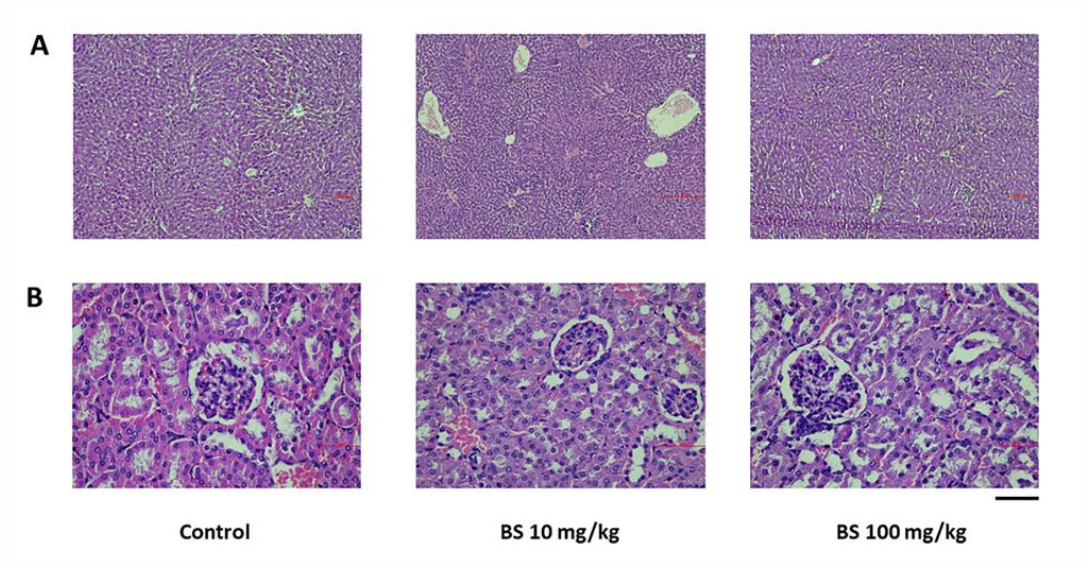

Figure 3. Photomicrographs of (A) liver and (B) kidney of mice. Hematoxylin and eosin (H&E); Scale bar - 50 µm. *BS* = *Butea superba* Roxb.

3.3 Effects of *Butea superba* ethanolic extract on serum testosterone level

Figure 4 shows that the concentration of serum testosterone in the sodium valproate group decreased significantly ($p < 0.01$) compared to that of the control group. After being treated with the extracts in 35 days, the *BS* extract 10 mg/kg group and *BS* extract 100 mg/kg group demonstrated a significant increase in the serum testosterone level compared to the untreated mice of the sodium valproate group ($p < 0.01$), which is similar to the Testosterone 2 mg/kg group.

In terms of serum testosterone level, the treated group with BS extract showed a significant increase when compared to the sodium valproate group.

This result corresponds with earlier findings by Eumkeb et al., which suggested that genistein, biochain A, and Un1 in the tuberous root of *Butea superba* extract helped raise the testosterone level[30,31]. Moreover, the later results of testicular micromorphology suggest that the Leydig cells and Sertoli cells, which are solely responsible for the synthesis of testosterone, increased in the treated group.

However, a recent report showed that the powder suspension of *BS* at doses of 150 and 200 mg/kg body weight significantly reduced serum testosterone levels and slightly decreased serum luteinizing hormone (LH) with a normal testicular micromorphology when being treated in 90 consecutive days[28]

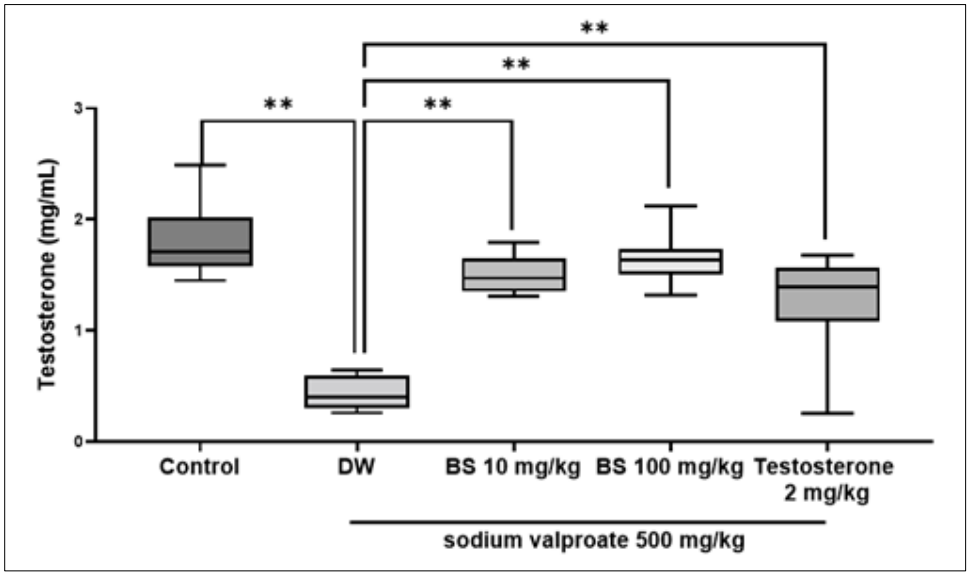

Figure 4. Level of serum testosterone in mice. Data are expressed as box plots with medians, and whiskers indicate minimal and maximal values and analyzed using ANOVA and Fisher's LSD post-hoc test (n = 8 per group), ** $P < 0.01$. DW = distilled water, BS = *Butea superba* Roxb.

3.4 Effects of *Butea superba* ethanolic extract on the diameter and the relative weight of the testes

Figure 5 shows that the relative weights of the testes of mice in the sodium valproate group were significantly lower compared to those in the control group ($p < 0.01$), those in the treated group with testosterone 2 mg/kg ($p < 0.01$), and *Butea superba* extract at the dose of 10 mg/kg ($p < 0.01$)and 100 mg/kg ($p < 0.01$).

Similarly, the diameters of testes of mice in both groups treated with *Butea superba* extract increased in comparison to those of the sodium valproate group However, there are no signs of dose-dependent efficacy on this indicator since the mice treated with a high dose of 100 mg/kg did not show a significant difference from those treated with a lower dose of 10 mg/kg.

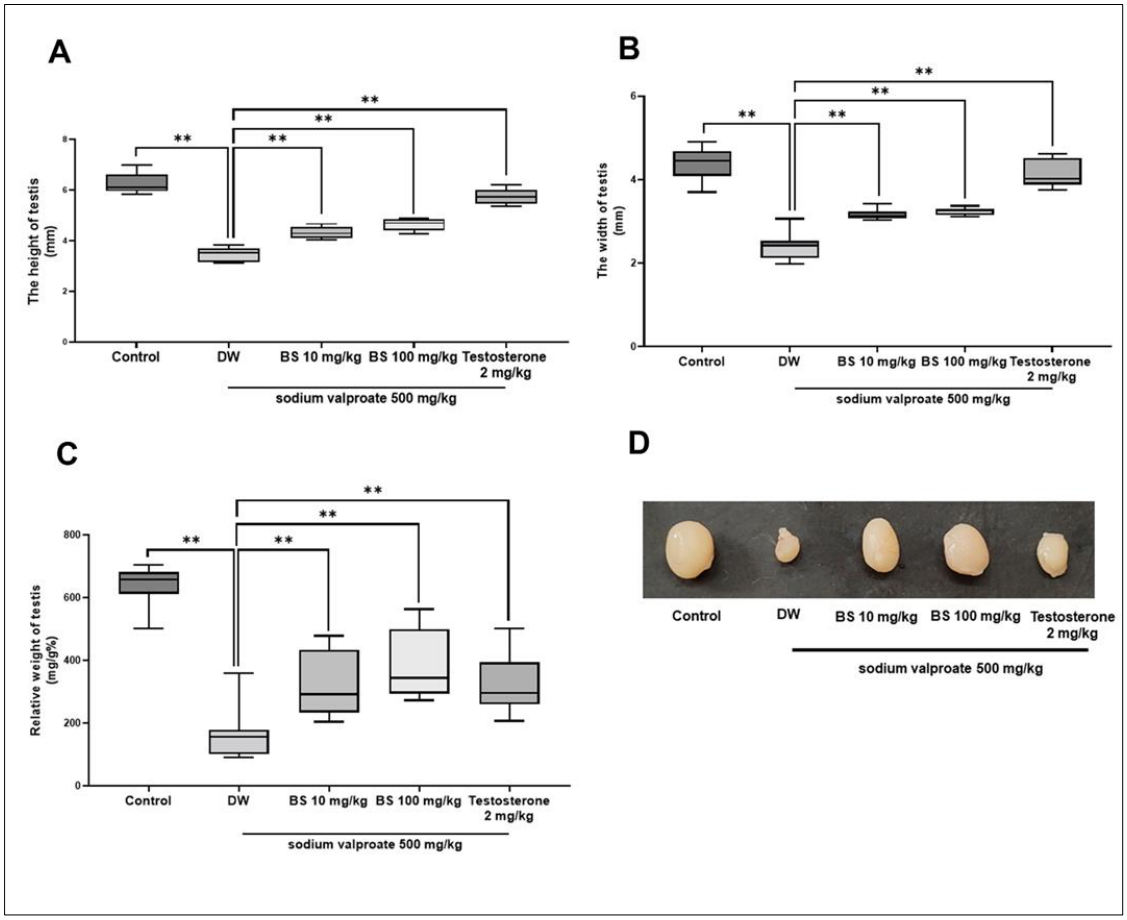

Figure 5. After 35 days, testicles were obtained from adult male mice and the height (A) and width (B) of testis were collected, as were the relative weight of testis (C), and image of testis (D). Data are expressed as box plots with medians, and whiskers indicate minimal and maximal values, and analyzed using ANOVA and Fisher's LSD post-hoc test (n = 8 per group), ** $P < 0.01$. DW = distilled water, *BS* = *Butea superba* Roxb.

3.5 Effects of *Butea superba* (*BS*) ethanolic extract on the relative weight of levator ani - bulbocavernosus muscle

As shown in Figure 6, after 35 days, a significant reduction was recorded in the relative weights of levator ani - bulbocavernosus muscles of the sodium valproate group in comparison to those of the control group ($p < 0.01$). Similar to the Testosterone 2 mg/kg group, the group treated with *Butea superba* extract of 10 mg/kg and 100 mg/kg, respectively, had a significantly higher index compared to those of the untreated mice in the sodium valproate group ($p < 0.01$ and $p < 0.01$).

The levator ani-bulbocavernosus (LABC) muscle is responsible for elevating intracavernous pressure (ICP), producing and maintaining erection[31]. This muscle is sensitive to the alteration of testosterone levels. To the best of our knowledge, this is the first time the protective effect of *Butea superba* extract on LABC muscle against atrophy due to hypogonadism[32]. The weight of this body section increased almost twofold after treatment with both doses, yet these increases were not significant compared to the untreated group. The result indicated that BS extract may improve the weight of the levator ani - bulbocavernosus muscle, but further studies would be necessary.

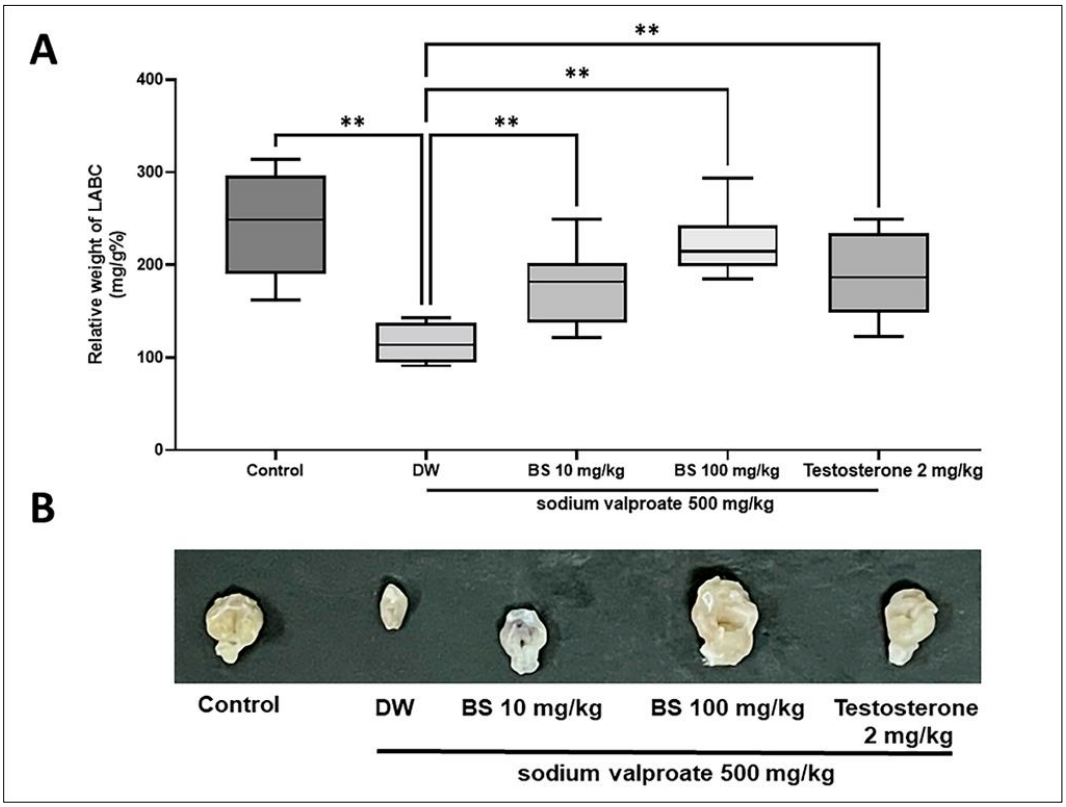

Figure 6. After 35 days, levator ani plus bulbocavernosus muscles (LABC) were obtained from adult male mice, and the relative weight of levator ani plus bulbocavernosus muscles (LABC) (A) was determined; image of testis (B). Data are expressed as box plots with medians, and whiskers indicate minimal and maximal values, and analyzed using ANOVA and Fisher's LSD post-hoc test (n = 8 per group), ** P < 0.01. DW = distilled water, *BS* = *Butea superba* Roxb.

3.6 Effects of *Butea superba* (*BS)* ethanolic extract on sperm count

The sperm count in the sodium valproate group decreased sharply by 85%, which was statistically significant ($p < 0.01$) compared to the control group. The sperm density of the Testosterone group significantly increased in comparison to that of the sodium valproate group ($p<0.01$). Moreover, the figures increased after the oral administration of BS extract at both doses, and the increase was considered significant compared to the sodium valproate group ($p<0.01$).

However, to the best of our knowledge, no research on androgenic activity has yet used BS stems to treat mice with sodium valproate-induced hypogonadism. Moreover, we also applied the flow cytometry method in our study of androgenic activity to assess the sperm count and sperm vitality, which helped raise the accuracy and credibility of this research[33]

3.7 Effects of *Butea superba* (BS) ethanolic extract on sperm vitality

Figure 8 showed a significant increase in the percentage of dead sperm of the control group compared to that of the sodium valproate group ($p<0.01$), which dropped significantly after a 35-day treatment of *Butea superba (BS)* extract at doses of 10 mg/kg ($p<0.01$) and 100 mg/kg ($p<0.01$). The percentage of dead sperm in the Testosterone group decreased significantly compared to the sodium valproate group ($p<0.05$).

The *BS* extract at both doses of 10 mg/kg and 100 mg/kg increased sperm count and sperm vitality compared to the untreated group. The result is consistent with some previous studies that *BS* powder suspension helps increase the sperm concentration of mice[16,34]. In addition, the percentage of dead sperm significantly decreased after being treated with *BS* extract. This result suggested that *BS* extract could protect the sperm, thereby preventing the permeability of the PI stain. Sabbir Khan et al. [16] suggested that sodium valproate decreased GSH levels, leading to an imbalance of the antioxidant enzymes: glutathione reductase and glutathione peroxidase. Therefore, the ROS level increased, causing the peroxidation of lipids and oxidative DNA damage responsible for germ cell toxicity[35]

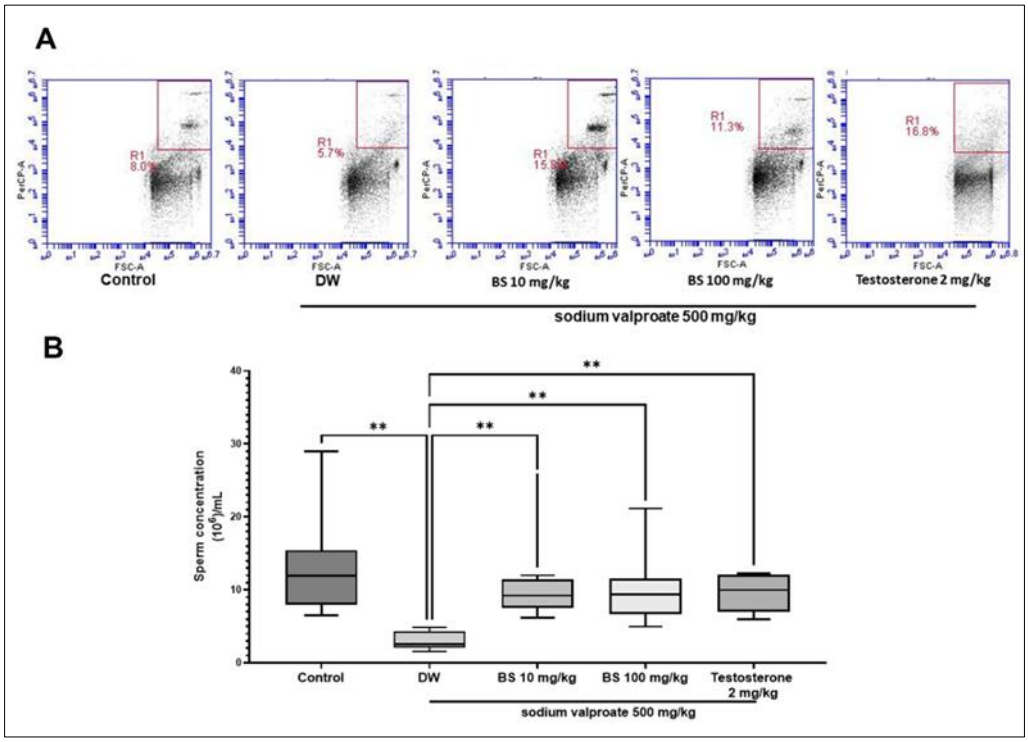

Figure 7. Flow cytometry analysis of sperm concentration. (A) Sperm were isolated from the epididymis, stained with propidium iodide, and analyzed using flow cytometry. (B) Sperm concentration. Data are expressed as box plots with medians, and whiskers indicate minimal and maximal values and analyzed using ANOVA and Fisher's LSD post-hoc test (n = 8 per group), ** P < 0.01. DW = distilled water, *BS* = *Butea superba* Roxb

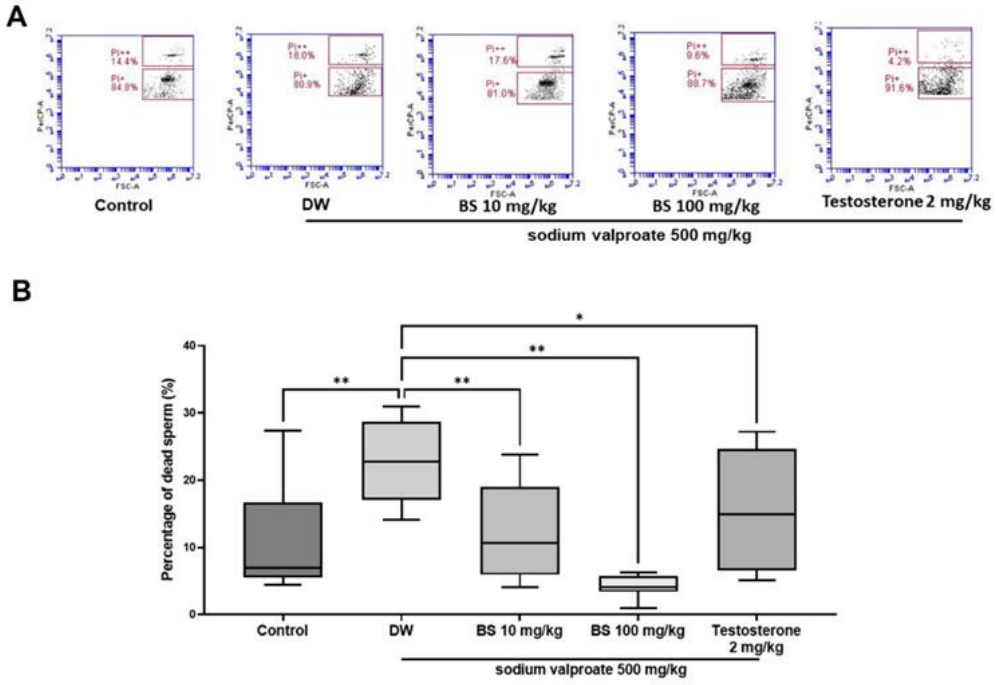

Figure 8. Flow cytometry analysis of dead sperm. (A) Sperm were isolated from the epididymis, stained with propidium iodide, and analyzed using flow cytometry. The percentages displayed in the lower right represent the dead cells. (B) Percentages of dead sperm. Data are expressed as box plots with medians, and whiskers indicate minimal and maximal values and analyzed using ANOVA and Fisher's LSD post-hoc test (n = 8 per group), *P < 0.05, ** P < 0.01. DW = distilled water, *BS* = *Butea superba* Roxb.

3.8 Effects of *Butea superba (BS)* ethanolic extract on the histological structure of the testis

As shown in Figure 9, the control group has a normal structure of seminiferous tubules with 6-8 layers of spermatogonial cells, including: spermatocytes, spermatids, spermatogonia, and Sertoli cells connecting the cells. The spermatogonia appears inside the lumen of seminiferous tubules. The interstitial tissue contains many Leydig cells.

In comparison to the control group, the seminiferous tubules of the mice in the sodium valproate group have a significant reduction in size, in the number of Leydig cells and Sertoli cells, and a degradation in the number of spermatogonia, spermatocytes, and spermatids, along with the absence of sperm in all tubules. The interstitial tissue lessens in some sections, making the seminiferous tubules seem discrete

In general, the treated groups of mice showed an increase in the size of the tubules, in the number of tubular layers, Sertoli cells, Leydig cells, and spermatozoa; moreover, less seminiferous epithelial vacuolation was observed compared to the sodium valproate group. In the *BS* extract 10 mg/kg group, some seminiferous cells have an enlarged lumen with fewer spermatogonial cells and Leydig cells than the *BS* extract 100 mg/kg group and the testosterone group. In the group treated with Testosterone 2 mg/kg, most of the seminiferous tubules have the same shape as the control group, some tubules have an enlarged lumen, and some sections have sparse interstitial tissue. The *BS* extract 100 mg/kg group has tubular structures similar to the control group; however, some tubules still have seminiferous epithelial vacuolation.

The Johnsen's score reflects the histological injury of the testis, which is significantly higher in the treated group with *Butea superba* extract at both doses of 10 and 100 mg/kg in comparison to the sodium valproate group (Fig. 9).

We furthermore investigated the efficacy of both doses of *BS* on the histology of the testicles via Johnsen's score, which was a quantitative histological grading system used in several studies to prognosis for male reproductive capacity[34]. The results showed that the *BS* ethanolic extract made a significant improvement on the testicular tissues of the treated group, which suggests that the extract may contain compounds with androgenic activity. These compounds can increase the release of GnRH (gonadotropic hormone) from the hypothalamus, thereby improving the release of male sex hormones, which are responsible for the growth of sertoli cells and leydig cells that can augment the size of the testis[21,22] and produce testosterone.

Many studies proved that the *Butea superba* ethanolic extract from tuberous roots could increase serum testosterone level and sperm concentration of healthy mice[21,22].

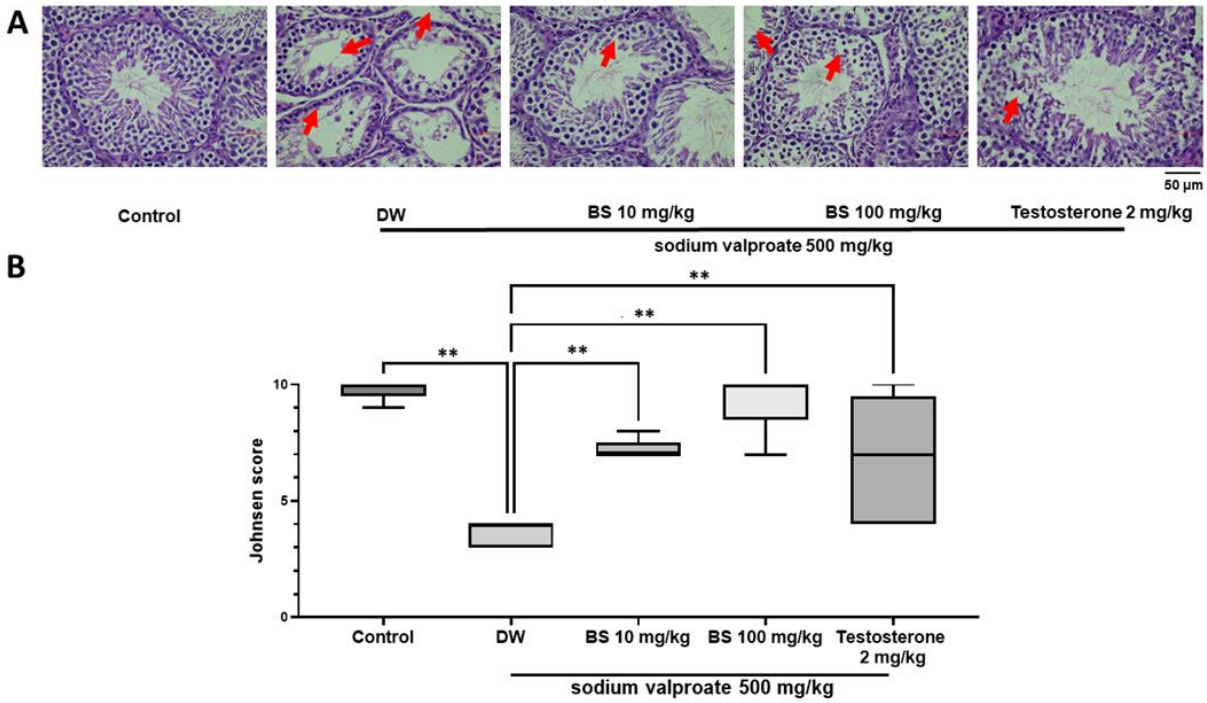

Figure 9. (A) Photomicrographs of testicular (seminiferous tubule) sections of mice. Hematoxylin and eosin (H&E); Scale bar - 50 µm. (B) Johnsen score of seminiferous tubulars. Arrowhead pointed to the abnormal structure of seminiferous tubules: decreased layers of spermatogonial cells, Leydig cells, and enlarged lumen. Data are expressed as box plots with medians, and whiskers indicate minimal and maximal values and analyzed using ANOVA and Fisher's LSD post-hoc test (n = 8 per group), ** $P < 0.01$. DW = distilled water, BS = *Butea superba* Roxb

4. CONCLUSION

Butea superba (BS) extract did not produce any acute or subchronic toxicity at the chosen doses. Treatment of BS ethanolic extract in 35 consecutive days at both doses of 10 mg/kg and 100 mg/kg body weight exhibited protective activity on male mice's reproductive system through the increase in serum testosterone level, relative weights of testes, and the levator ani plus bulbocavernosus muscles.

In addition, the *BS* extract at both doses also helped protect testis, and spermatozoon against the degenerative effect of sodium valproate by increasing sperm count and decreasing the percentage of dead

sperm in male Swiss albino mice. Our findings could provide a useful foundation for studying the efficacy of products made from the stem of *Butea superba* Roxb.

5. ACKNOWLEDGMENT

Author contribution

H.L.T.N: methodology, conceptualization, writing-original draft, validation; B.K.N: methodology, writing-original draft, data curation; M.H.K, D.T.H.N, N.H.L.P: methodology, writing-original draft, visualization; M.N.T: methodology, data curation, validation; M.T.N: flow cytometry method, validation, data curation; T.B.N: visualization, validation, H&E staining; H.N.M: writing-review & editing, supervision, formal analysis, validation, conceptualization"

Conflict of interest

The authors declare that they have no conflict of interest

Funding

This research received no external funding.

Ethics approval

This research was reviewed and approved by the Ethical Committee, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam under the approval number no.589/GCN-HĐĐĐNCTĐV on June 12th, 2023.

Article info:

Received February 5, 2024
Received in revised form July 17, 2024
Accepted July 29, 2024

REFERENCES

1. Mulligan T, Frick MF, Zuraw QC ,Stemhagen A, McWhirter C. Prevalence of hypogonadism in males aged at least 45 years: the HIM study. Int J Clin Pract. 2006;60(7):762-69.
2. Anh TQ. Male reproductive pathology. Medical Publishing House. 2009; 24-30:232-46.
3. Lotti F, Maggi M, Sexual dysfunction and male infertility. Nat Rev Urol. 2008;15(5):287-307.
4. Van Zyl JA, Sex and infertility. Part I. Prevalence of psychosexual problems and subjacent factors. S Afr Med J. 1987;72(7):482-84.
5. Nassar GN, Stephen WL. Physiology. Testosterone. StatPearls Publishing. 2024.
6. Ye F, McCoy SC, Ross HH, Bernardo JA, Beharry AW, Senf SM, et al. Transcriptional regulation of myotrophic actions by testosterone and trenbolone on androgen-responsive muscle. Steroids. 2014;87:59-66.
7. Carpentier M, Sahpaz S, Bailleul F. Plantes et dysfonction érectile. Phytotherapie. 2004;66-71.
8. Tocharus C, Sooksaen P, Shimbhu D, Tocharus J. *Butea superba* (Roxb.) improves penile erection in diabetic rats. Andrologia. 2012;44(1):728-33.
9. Kaur V, Kumar M, Kaur P, Kaur S, Singh AP, Kaur S. Hepatoprotective activity of Butea monosperma bark against thioacetamide-induced liver injury in rats. Biomed Pharmacother. 2017;89:332-41.
10. Mizuki D, Matsumoto K, Tanaka K, Thi Le X, Fujiwara H, Ishikawa T, et al. Antidepressant-like effect of *butea superba* in mice exposed to chronic mild stress and its possible mechanism of action. J Ethnopharmacol. 2014;156:16-25.
11. Exploitation and development of genetic resources of silkworm (Urceola minutiflora Mid.) and bloodworm (*Butea superba* Roxb.) in the central highlands as raw material for medicine production. State level project. 2013-2017.
12. Giang TT, Nguyen TN, Hoang TNH. Distribution, ecological and natural regeneration characteristics of *butea superba* roxb, in Lam Dong Province. Agriculture and Rural Development Journal. 2020;9:116-23.
13. Thanh GT, Nguyen NT, Huong HTN. Distribution, ecological and natural regeneration characteristics of *Butea superba* Roxb. in Lam Dong province. 2020;9:116-23
14. Vietnam Ministry of Health. Guidelines for Preclinical and Clinical Trials of Traditional Medicine and Herbal Medicines, in: V.M.o. Health (Ed.), 2015.
15. OECD guideline for the testing of chemicals. The Hershberger.2001;601:858.
16. Khan S, Ahmad T, Parekh CV, Trivedi PP, Kushwaha S, Jena G. Investigation on sodium valproate induced germ cell damage, oxidative stress and genotoxicity in male swiss mice. Reprod Toxicol. 2011;32(4):385-94.
17. Yang ZW, Wreford NG, de Kretser DM. A quantitative study of spermatogenesis in the developing rat testis. 1990;43(4):629-35.
18. Spears J, Matthews, Hartwig S. Evaluation of noninvasive methods of predicting testicular weight and development in an inbred and outbred strain of laboratory mouse. Theriogenology. 2013;79(3):566-73.
19. Gray LE, Jr, Furr J, Ostby JS. Hershberger assay to investigate the effects of endocrine-disrupting compounds with androgenic or antiandrogenic activity in castrate-immature male rats. Curr Protoc Toxicol. 2005;16:16.9.
20. Ribeiro S, Sharma R, Gupta S, Cakar Z, De Geyter C, Agarwal A. Inter- and intra-laboratory standardization of TUNEL assay for assessment of sperm DNA fragmentation. Andrology. 2017;5(3):477-85.
21. Muratori M, Tamburrino L, Marchiani S, Cambi M, Olivito B, Azzari C, et al. Investigation on the origin of sperm DNA fragmentation: Role of apoptosis, immaturity and oxidative stress. Mol Med. 2015;21(1):109-22.
22. Agarwal A, Gupta S, Sharma R. Measurement of DNA fragmentation in spermatozoa by TUNEL assay using bench top flow cytometer. Andrological Evaluation of Male Infertility.2016:181-203.
23. Huynh DTM, Huynh T, Le MNT, Mai HN, Investigation of acute and sub-chronic oral toxicity and effects of Allium ascalonicum L. extract on Triton WR1339-induced hyperlipidemia on Swiss albino mice, Pharmacological Research - Modern Chinese Medicine, 10, 2024; 100407.
24. Nguyen Thanh T, Phuoc D, Thuan D, Le MT, Nguyen Vu Quoc H. Assessment of testis histopathological changes and spermatogenesis in male mice exposed to chronic scrotal heat stress. Journal of Animal Behaviour and Biometeorology.2020;8:174-180.
25. Hayes, Wallace A, and Loomis TA. Loomis's essentials of toxicology (4th ed.). Academic Press. CA. 1996:208-45.
26. United Nations. Globally Harmonised System of Classification and Labelling of Chemicals (GHS, Rev. 7); United Nations: New York and, NY, USA; Geneva, Switzerland, 2017;7.
27. Manosroi J, Manosroi A, Aritajat S. The research and development of natural products for Thai traditional medicine. 1999.
28. Cherdshewasart W, Bhuntaku P, Panriansaen R, Dahlan W, Malaivijitnond S. Androgen disruption and toxicity tests of

Butea superba Roxb., a traditional herb used for treatment of erectile dysfunction, in male rats. Maturitas. 2008;60:131-37
29. Beigoli S, Behrouz S, Memarzia A, Ghasemi SZ, Boskabady M, Marefati N, et al. Effects of allium cepa and its constituents on respiratory and allergic disorders: A comprehensive review of experimental and clinical evidence. Evidence-Based Complementary and Alternative Medicine. 2021; 2021: 5554259.
30. Eumkeb G, Tanphonkrang S, Sirichaiwetchakoon K, Hengpratom T, Naknarong W, The synergy effect of daidzein and genistein isolated from *butea superba* Roxb. on the reproductive system of male mice. Natural product research. 2017;31(6):672-75.
31. Eumkeb G, Naknarong W, Sirichaiwetchakoon K. The effects of red kwao krue (*butea superba* roxb.) extract on sperm quality and testosterone level in mice. Thai Journal of Pharmaceutical Sciences. 2013;38:120-23.
32. Wespes E, Nogueira, Herbaut AG, Caufriez M, Schulman CC. Role of the bulbocavernosus muscles on the mechanism of human erection. Eur Urol. 1990;18(1):45-8
33. de Lima Rosa J, de Paula Freitas Dell'Aqua C, de Souza FF, Missassi G, Kempinas WG. Multiple flow cytometry analysis for assessing human sperm functional characteristics. Reprod Toxicol. 2023;117:108353.
34. Glander HJ, Horn Lc, Dorschner W, Paasch U, Kratzsch J. Probability to retrieve testicular spermatozoa in azoospermic patients. Asian journal of andrology. 2000;2(3):199-205.
35. Sikka SC. Relative impact of oxidative stress on male reproductive function. Current medicinal chemistry. 2001; 8 (7): 851-62.