RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2022.102

ScienceAsia 48 (2022): **718–725**

Anabolic effect of *Butea superba* Roxb. on improving skeletal muscle atrophy and strength via androgen receptors and increasing parvalbumin levels in orchidectomized rats

Fadeela Binalee[a], **Wandee Udomuksorn**[b], **Ekkasit Kumarnsit**[b], **Uraporn Vongvatcharanon**[b], **Surapong Vongvatcharanon**[c,*]

[a] Department of Anatomy, Faculty of Medicine, Princess of Naradhiwas University, Narathiwat 96000 Thailand
[b] Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Songkhla 90112 Thailand
[c] Division of Oral and Maxillofacial Surgery (Anesthesiology Section), Faculty of Dentistry, Prince of Songkla University, Songkhla 90112 Thailand

*Corresponding author, e-mail: surapong.v@psu.ac.th

Received 20 Nov 2021, Accepted 15 May 2022
Available online 15 Jul 2022

ABSTRACT: *Butea superba* Roxb. (BS) has been used in Thai traditional medicine for the promotion of male sexual vigor. BS may have androgenic or anabolic activity in male animals. This study investigated the effect of BS on toxicity and androgenic and anabolic activities including alteration of androgen receptor immunoreactivity (AR-ir) and parvalbumin (PV) levels in an extensor digitorum longus (EDL) and gastrocnemius in orchidectomized rats. Adult male Wistar rats were divided into 6 groups: (1) sham-operated (SHAM), (2) orchidectomized and double distilled water treated (BS-0), (3) orchidectomized and 6 mg/kg of testosterone propionate treated (TP), orchidectomized and BS treated for 12 weeks at various doses: (4) 5 mg/kg (BS-5), (5) 50 mg/kg (BS-50), and (6) 500 mg/kg (BS-500). All the BS groups led to a significant decrease in testosterone level and the reproductive organ weight compared to those of the SHAM ($p < 0.05$). In addition, the treatment in all doses of BS may have no toxicity. Interestingly, the treatment with any dose of BS significantly increased strength, endurance, muscle mass, PV levels ($p < 0.05$), and AR-ir of both EDL and gastrocnemius. Our data also demonstrated that the BS had no androgenic effects but showed anabolic effects by increasing mass, strength, and endurance of skeletal muscles. An increase of PV levels induced by BS may be an important mechanism for improving muscle strength and endurance of skeletal muscles in orchidectomized rats. Therefore, the anabolic effects of BS may be a piece of useful information for an alternative treatment of sarcopenia in andropause.

KEYWORDS: *Butea superba* Roxb., muscle strength, muscle atrophy, androgen receptors, parvalbumin

INTRODUCTION

Sarcopenia is referred to the age-related loss of skeletal muscle mass and strength. People with sarcopenia face an increased risk of falls, which affects the quality of life [1]. Previous studies reported factors associated with sarcopenia including motor neuron loss [2], malnutrition [3], and hormonal change [4]. The andropause exhibits a decline in serum testosterone associated with loss of muscle mass and strength. Testosterone and its metabolites operate their effects through binding to the androgen receptors [5]. Long-term testosterone administration reduces age-associated sarcopenia in men [6]. However, the adverse effects of testosterone administration to older men such as prostate cancer, edema, and high prostate-specific antigen remain a concern [7]. As reported by Nilwik et al [8], only a decline of fiber size, but not fiber number, was found in age-related loss of muscle mass. Interestingly, sarcopenia was characterized by a significant low fiber type-2 diameter in men. The fast switching type-2 muscle fibers are essential to maintain posture for preventing falls and fractures. Parvalbumin (PV) is a main soluble Ca^{2+}-binding protein and found only in fast-twitch fibers. PV knowingly acts as a relaxation factor in mammal skeletal muscles [9].

Butea superba Roxb. (BS), known as "Kwao Khruea Daeng" in Thai, is a big climber plant from the family *Papillionaccae*. As traditional medicine, the plant tubers are applied for rejuvenation and treatment of impotence in men [10]. According to the work of Cherdshewasart et al [11], the treatment with BS at a dose of 150 and 200 mg/kg/BW for 90 days to intact male rats significantly decreased serum testosterone levels and slightly reduced serum luteinizing hormone (LH) levels with a normal appearance of the testis by histological examination. These suggested that BS functioned as an androgen disruptor through modifying the testosterone biosynthesis or metabolism. Therefore, BS may have androgenic activity in male animals, but there is no strong evidence to support the conclusion [12]. Therefore, this study aims to investigate the effect of subchronic treatment of BS on toxicity, androgenic activity, mass, strength, and endurance including alteration of AR-ir and PV levels in EDL and gastrocnemius muscles. Both EDL and gastrocnemius contain more than 90% of fast switching type-2 muscle fibers and a high level of PV [13]. Therefore, obtained data may provide a basic mechanism of testosterone and BS on improving skeletal muscle strength which

will be a useful piece of information for an alternative treatment of sarcopenia in andropause.

MATERIALS AND METHODS

Animals and experiment design

Adult male Wistar rats (12 weeks old, 250–300 g weight) were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Thailand. The rats were given standard rat chow and tap water ad libitum and were maintained at 22 °C with a 12/12-dark/light cycle (lighting on at 06:00 am). The experimental protocols described in this study were approved by the Animal Ethical Committee of the Prince of Songkla University for the care and use of experimental animals (MOE 0521.11/76). The rats were randomly divided into 6 groups ($n = 10$ per group): (1) sham-operated (SHAM), (2) orchidectomized and distilled water treated (BS-0), (3) orchidectomized and 6 mg/kg of testosterone propionate treated (TP), orchidectomized and ethanolic extract of BS treated for 12 weeks at various doses: (4) 5 mg/kg (BS-5), (5) 50 mg/kg (BS-50), and (6) 500 mg/kg (BS-500) as a subchronic treatment. Doses of testosterone and BS treatments were modified from the study by Malaivijitnond et al [12].

Preparation of BS suspensions

The BS jet spray-dried extract was obtained from St. Herb Cosmetics International Co., Ltd., Thailand (Lot No. A55050501). The extract contained 7.32 mg puerarin, 3.75 mg daidzein, and 13.33 mg/100 g genistein as determined by HPLC analysis. (Certificate of Analysis and Test Report Lot No. A55050501 of St. Herb Cosmetics International Co., Ltd., Thailand).

Measurement of muscle strength

At the end of the treatment, the contractile performances of EDL and gastrocnemius were evaluated. The procedure for measurement of contractile performance of the muscles was modified from the work of Inthanuchit et al [14]. Briefly, rats were anesthetized by an intraperitoneal injection of 70 mg/kg of thiopental (Jagsonpal Pharmaceuticals, New Delhi, India). The EDL and gastrocnemius were dissected and directly stimulated using a bipolar electrode. The contractile force was converted to analogue signal through a force transducer (Model 1030; AD Instruments, NSW, Australia). The signal was amplified using Bridge Amplifier (Model 110, AD Instruments) and transferred to the chart program of the PowerLab system (Model 4/20; AD Instruments) for converting into digital signals. Data were stored in a personal computer for offline analysis.

Sample preparation

Blood samples were obtained from the retro-orbital sinus to determine serum testosterone and sex hormone-binding globulin (SHBG) levels using an Elecsys Testosterone II reagent kit and Elecsys SHBG reagent kit (Cobas®, Roche Diagnostics GmbH, Basel, Switzerland). The serum glutamic oxaloacetic transaminase (SGOT) and serum glutamate-pyruvate transaminase (SGPT) levels were evaluated for a toxicity test. The EDL, gastrocnemius muscle, epididymis, prostate gland, seminal vesicle, vas deferens, liver, kidney, and spleen were removed and weighed. The EDL and gastrocnemius then were divided into 2 parts at their mid-belly, rapidly frozen, and stored at −80 °C for cryosectioning, immunofluorescence and Western blotting.

Measurement of muscle fiber size

The procedure for measurement of muscle fiber size was modified from the work of Inthanuchit et al [14]. Briefly, the EDL and gastrocnemius were cut into 20 μm cross-sections using a cryostat microtome. Every fifth section was used for systematic random sampling and coded such that all subsequent analyses were carried out in a blind manner. The frozen sections were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin. The representative sections were captured by a digital camera (DP50, Olympus, Tokyo, Japan) at ×40 magnification. The cross-sectional area of each fiber from all groups was measured using Image-pro Plus 6.0 analysis software (Media Cybernetics, Bethesda, MD, USA).

Immunofluorescence for androgen receptors (AR)

Adjacent series of EDL and gastrocnemius sections from each group were placed in a microwave oven at 700 W for antigen retrieval using 0.01 M citric buffer pH 6 for 10 min. These steps were followed by incubating the sections with 0.3% Triton X-100 in 0.1 M phosphate buffer (PB) for 60 min and in 2% normal horse serum (Vector Laboratories, Burlingham, CA, USA) in 0.1 M PB for 2 h at 4 °C. After washing in 0.1 M PB, the sections were incubated 48 h at 4 °C with AR antibody (N-20): sc-816 (Santa Cruz Biotechnology, Inc., USA) at a dilution 1:50 in 0.1 M PB followed by incubating with fluorescence-labeled anti-rabbit IgG secondary antibodies (1:200 dilution, Vector Laboratories) for 1 h. The sections were finally mounted with Vectashield (Vector Laboratories). The coded sections were examined by fluorescence microscopy (BX 50, Olympus). Images were captured with an Olympus DP-73 digital camera (Olympus) at ×40 magnification by an observer unaware of the experimental protocols. Controls were performed by either omitting the first or secondary antibody. None of these controls showed any labeling.

Western blot analysis

Western blot method was modified from Bunratsami et al [15]. Briefly, the samples of EDL and the

gastrocnemius muscles were homogenized using a homogenizer (Polytron Aggregate, Kinematica, Lucerne, Switzerland) in lysis buffer (Tissue extraction reagent I, Invitrogen™, CA, USA, including protease inhibitor cocktail, Amresco®, OH, USA) and then centrifuged at 14 000×g for 30 min at 4 °C to remove cellular debris. The protein concentration of the supernatant was determined using BCA™ Protein Assay Kit (Thermo Scientific, Rockford, USA) and performed according to the manufacturer's protocol. Five micrograms of protein from each of the EDL and gastrocnemius muscles in all treatment groups were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% (w/v) polyacrylamide gel by electrophoresis. For Western blot, the parvalbumin protein was transferred from the SDS-PAGE gel to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). After a non-specific binding blocking with 2.5% blotting-grade blocker, Nonfat dry milk (Bio-Rad Laboratories) in TBS (150 mM NaCl, 100 mM Tris-HCl, pH 8), the membranes were incubated with 1:1000 diluted anti-PV antibody (Chemicon Temecula, California, USA) as primary antibody in 2.5% blotting-grade blocker, non-fat milk in TBS for 2 h, followed by 1:10 000 diluted horseradish peroxidase-conjugated anti-mouse IgG (Cell Signaling, Danvers, MA, USA) as secondary antibody in 2.5% blotting-grade blocker, non-fat milk in TBS for 2 h. Immunoblotting products were visualized with an enhanced chemiluminescence detection kit according to the manufacturer's instructions (Pierce® ECL Plus Western Blotting Substrate, Thermo Scientific, Rockford, USA). The PV protein bands that corresponded to a molecular weight of 12 kDa were visualized by BioSpectrum® Imaging System Chemi 410 and the intensity of each band was measured using VisionWorks®LS analysis software (Ultra-Violet Products Ltd., Cambridge, UK).

Statistical analysis

Data are expressed as mean values ± standard error of the mean. The statistical evaluation of the data was analyzed by one-way ANOVA and a least significant difference test (LSD) to determine a significant difference between the means at $p < 0.05$.

RESULTS

Effects of subchronic treatment with BS on testosterone levels and weights of body, reproductive organ, EDL, gastrocnemius, liver, kidney, and spleen

Our data demonstrated that in the BS-0 group, orchidectomy resulted in a significant decrease of testosterone, free testosterone, and bioavailable levels compared to the SHAM group ($p < 0.05$). In contrast to testosterone treatment group (TP), a significant increase of testosterone, free testosterone, and bioavailable levels was observed compared to the BS-0 group ($p < 0.05$). Interestingly, all doses of the BS treatment (5 mg, 50 mg, and 500 mg/kg) in the BS-5, BS-50 and BS-500 groups did not increase the testosterone, free testosterone, and bioavailable levels. Furthermore, there were no significant changes in SHBG and albumin levels (Table 1). A significant reduction of weight gain and male reproductive organs was observed in the BS-0 groups compared to that of the SHAM ($p < 0.05$). Although the testosterone treatment did not induce the increase of weight gain in the TP group, all male reproductive organ weights were significantly increased ($p < 0.05$). In contrast to the BS treatment group, only the 50 mg/kg of BS treatment group (BS-50) demonstrated significant increase in weight gain compared to the orchidectomized group (BS-0) ($p < 0.05$). In all with BS treated groups (BS-5, BS-50, and BS-500), the testosterone level and the weight of male reproductive organs were significantly decreased compared to those of the SHAM. However, the EDL and gastrocnemius weight was not significantly different between each group. Furthermore, the liver, kidney, and spleen weight were not significantly different between SHAM and all the BS treated groups (Table 1).

Toxicity assessment of BS subchronic treatment

The TP group demonstrated a significant increase in SGOT (184.80 ± 5.46 U/l) and SGPT (110.70 ± 7.56 U/l) compared to that of the SHAM (179.80 ± 5.33 and 91.30 ± 5.47 U/l, respectively) ($p < 0.05$). In contrast, all BS treatment groups (BS-5, BS-50, and BS-500) showed no significant difference in SGOT (172.70 ± 5.86, 177.50 ± 5.86, and 182.10 ± 5.59 U/l, respectively) and SGPT levels (95.00 ± 5.18, 92.60 ± 5.72, and 83.90 ± 2.48 U/l, respectively) compared to that of the SHAM and BS-0 (184.6 ± 3.98 and 96.30 ± 6.12 U/l, respectively).

Effects of subchronic treatment of BS on strength and endurance of the EDL and gastrocnemius

The strength of the EDL and gastrocnemius was demonstrated by measuring peak twitch tension, peak tetanic tension, and contraction time whereas endurance of EDL and gastrocnemius was shown by time to fatigue. In the BS-0 group, the peak twitch tension, peak tetanic tension, and time to fatigue of both the EDL and gastrocnemius were significantly decreased, but the contraction time and one-half relaxation time were significantly increased compared to those of the SHAM ($p < 0.05$) (Tables 2 and 3). In comparison, the TP, BS-5, BS-50, and BS-500 groups demonstrated a significant increase in the peak twitch tension and peak tetanic tension time but a significant reduction in the contraction time and one-half relaxation time compared to those of the BS-0 group ($p < 0.05$) of both the EDL and gastrocnemius (Tables 2 and 3). For the endurance, the TP group showed a significant increase in the time to fatigue of both the EDL and gastrocnemius compared to those of the BS0 group

Table 1 Testosterone, free testosterone, bioavailable testosterone, SHBG, and albumin levels in blood plasma, body weight, reproductive organ weight, EDL weight, gastrocnemius weight, liver weight, kidney weight, and spleen weight of all treatment groups.

Parameter	SHAM	BS-0	TP	BS-5	BS-50	BS-500
Testosterone (ng/dl)	1.236 ± 0.32	0.025 ± 0.00*	15.00 ± 0.00†	0.025 ± 0.00*	0.025 ± 0.00*	0.025 ± 0.00*
Free testosterone (ng/dl)	0.046 ± 0.01	0.001 ± 0.03*	0.587 ± 0.00†	0.001 ± 0.00*	0.001 ± 0.00*	0.001 ± 0.00*
Bioavailable testosterone (ng/dl)	1.220 ± 0.32	0.025 ± 0.00*	14.80 ± 0.00†	0.025 ± 0.00*	0.025 ± 0.00*	0.025 ± 0.00*
SHBG (nmol/l)	0.35 ± 0.00	0.35 ± 0.00	0.35 ± 0.00	0.35 ± 0.00	0.35 ± 0.00	0.35 ± 0.00
Albumin (g/dl)	5.057 ± 0.19	4.829 ± 0.16	4.70 ± 0.22	4.929 ± 0.21	4.614 ± 0.14	4.529 ± 0.23
Weight gain (g/month)	42.69 ± 2.19	26.17 ± 2.05*	20.24 ± 2.15†	29.82 ± 0.61	31.84 ± 1.69†	25.4 ± 1.49
Epididymis weight (g)	1.17 ± 0.10	0.384 ± 0.03*	1.148 ± 0.18†	0.365 ± 0.03	0.494 ± 0.05	0.438 ± 0.03
Epididymis weight/body weight (×100)	0.255 ± 0.02	0.096 ± 0.01*	0.301 ± 0.05†	0.088 ± 0.01	0.116 ± 0.01	0.109 ± 0.01
Prostate gland weight (g)	1.559 ± 0.07	0.529 ± 0.05*	2.43 ± 0.10†	0.422 ± 0.03	0.475 ± 0.04	0.446 ± 0.03
Prostate gland weight/body weight (×100)	0.340 ± 0.01	0.130 ± 0.01*	0.635 ± 0.03†	0.102 ± 0.01	0.112 ± 0.01	0.111 ± 0.01
Seminal vesicle weight (g)	1.814 ± 0.04	0.190 ± 0.03*	4.286 ± 0.20†	0.216 ± 0.02	0.226 ± 0.02	0.181 ± 0.01
Seminal vesicle weight/body weight (×100)	0.396 ± 0.01	0.047 ± 0.01*	1.119 ± 0.05†	0.052 ± 0.00	0.053 ± 0.00	0.045 ± 0.00
vas deferens weight (g)	0.386 ± 0.03	0.168 ± 0.01*	0.446 ± 0.03†	0.204 ± 0.01	0.193 ± 0.01	0.181 ± 0.03
vas deferens weight/body weight (×100)	0.084 ± 0.00	0.042 ± 0.00*	0.117 ± 0.01†	0.049 ± 0.00	0.046 ± 0.00	0.044 ± 0.02
EDL weight (g)	0.159 ± 0.01	0.144 ± 0.01	0.155 ± 0.01	0.159 ± 0.00	0.153 ± 0.01	0.162 ± 0.01
EDL weight/body weight (×100)	0.035 ± 0.00	0.036 ± 0.00	0.041 ± 0.00†	0.038 ± 0.00	0.036 ± 0.00	0.040 ± 0.00
Gastrocnemius weight (g)	4.455 ± 0.13	4.186 ± 0.09	3.973 ± 0.09	4.462 ± 0.10	4.336 ± 0.09	4.341 ± 0.09
Gastrocnemius weight/body weight (×100)	0.972 ± 0.02	1.041 ± 0.03	1.042 ± 0.04	1.079 ± 0.03	1.023 ± 0.02	1.081 ± 0.02
Liver weight (g)	11.557 ± 0.74	9.143 ± 0.49*	11.375 ± 0.72	9.955 ± 0.38	10.044 ± 0.53	10.305 ± 0.48
Liver weight/body weight (×100)	2.518 ± 0.14	2.272 ± 0.13	2.965 ± 0.16†	2.404 ± 0.08	2.364 ± 0.11	2.566 ± 0.12
Kidney weight (g)	3.220 ± 0.21	2.52 ± 0.12*	3.613 ± 0.18†	2.585 ± 0.13	2.601 ± 0.17	2.573 ± 0.08
Kidney weight/body weight (×100)	0.704 ± 0.05	0.623 ± 0.03	0.943 ± 0.05*†	0.624 ± 0.03	0.615 ± 0.04	0.641 ± 0.02
Spleen weight (g)	0.865 ± 0.11	0.724 ± 0.06	0.773 ± 0.07	0.747 ± 0.03	0.645 ± 0.03	0.644 ± 0.03
Spleen weight/body weight (×100)	0.186 ± 0.02	0.179 ± 0.01	0.202 ± 0.02	0.181 ± 0.01	0.152 ± 0.01	0.161 ± 0.01

* Significant difference from SHAM, $p < 0.05$, $n = 10$. † Significant difference from BS-0, $p < 0.05$, $n = 10$.

Table 2 Muscle performance of EDL.

Parameter	SHAM	BS-0	TP	BS-5	BS-50	BS-500
Peak twitch tension (N)	0.079 ± 0.006	0.057 ± 0.004*	0.073 ± 0.004†	0.072 ± 0.003†	0.070 ± 0.002†	0.062 ± 0.006†
Peak tetanic tension (N)	0.386 ± 0.044	0.159 ± 0.038*	0.430 ± 0.019†	0.319 ± 0.032†	0.344 ± 0.020†	0.279 ± 0.029†
Contraction time (s)	0.043 ± 0.0009	0.047 ± 0.0011*	0.043 ± 0.0005†	0.043 ± 0.0014†	0.043 ± 0.0005†	0.044 ± 0.0017†
One-half relaxation time (s)	0.023 ± 0.0008	0.028 ± 0.0016*	0.023 ± 0.0007†	0.023 ± 0.0006†	0.023 ± 0.0009†	0.022 ± 0.0005†
Time to peak tension (s)	0.442 ± 0.065	0.575 ± 0.056	0.535 ± 0.054	0.548 ± 0.051	0.515 ± 0.082	0.628 ± 0.065
Time to fatigue (s)	20.269 ± 1.469	14.530 ± 2.182*	20.583 ± 1.727†	13.447 ± 1.348	16.558 ± 0.590	16.734 ± 1.204

* Significant difference from SHAM, $p < 0.05$, $n = 10$. † Significant difference from BS-0, $p < 0.05$, $n = 10$.

($p < 0.05$). In addition, the BS-50 and BS-500 groups tend to show an increase in the time to fatigue of the EDL. The TP and all the BS groups demonstrated a significant increase in the time to fatigue of the gastrocnemius compared to that of the BS-0 group ($p < 0.05$) (Tables 2 and 3).

Effects of subchronic treatment of BS on the EDL and gastrocnemius fiber size

Morphology of cross-sectional EDL and gastrocnemius fibers was demonstrated in Figs. S1 and S2. The nuclei were at the periphery of the fiber and purplish blue-stained whereas the cytoplasm was pink-stained. Measurement of the cross-sectional area of EDL and

Table 3 Muscle performance of gastrocnemius.

Parameter	SHAM	BS-0	TP	BS-5	BS-50	BS-500
Peak twitch tension (N)	0.296 ± 0.021	0.233 ± 0.011*	0.251 ± 0.019†	0.276 ± 0.017†	0.280 ± 0.014†	0.256 ± 0.016
Peak tetanic tension (N)	0.717 ± 0.050	0.494 ± 0.050*	0.710 ± 0.043†	0.685 ± 0.049†	0.708 ± 0.047†	0.635 ± 0.051
Contraction time (s)	0.046 ± 0.0009	0.050 ± 0.0011*	0.046 ± 0.0005†	0.047 ± 0.0014†	0.046 ± 0.0005†	0.046 ± 0.0017
One-half relaxation time (s)	0.026 ± 0.0006	0.032 ± 0.0030*	0.025 ± 0.0007†	0.025 ± 0.0007†	0.027 ± 0.0008†	0.026 ± 0.0007
Time to peak tension (s)	0.237 ± 0.018	0.283 ± 0.031	0.248 ± 0.017	0.267 ± 0.016	0.231 ± 0.014	0.271 ± 0.009
Time to fatigue (s)	48.623 ± 7.373	26.007 ± 2.893*	46.869 ± 7.552†	58.116 ± 4.140†	63.116 ± 8.352†	56.908 ± 8.253

* Significant difference from SHAM, $p < 0.05$, $n = 10$. † Significant difference from BS-0, $p < 0.05$, $n = 10$.

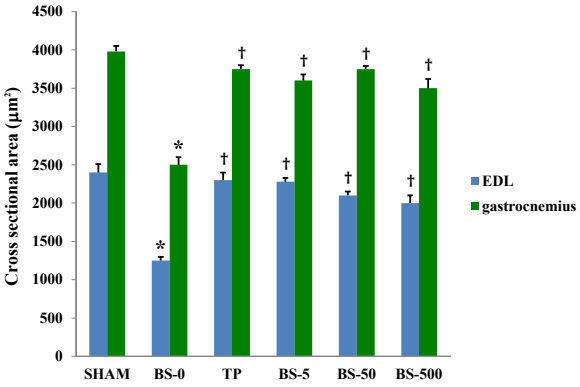

Fig. 1 Histogram of the cross-sectional area of the EDL and gastrocnemius of the SHAM, BS-0, TP, BS-5, BS-50, and BS-500, $n = 10$. * Significantly different from the SHAM, $p < 0.05$. † Significantly different from the BS-0, $p < 0.05$.

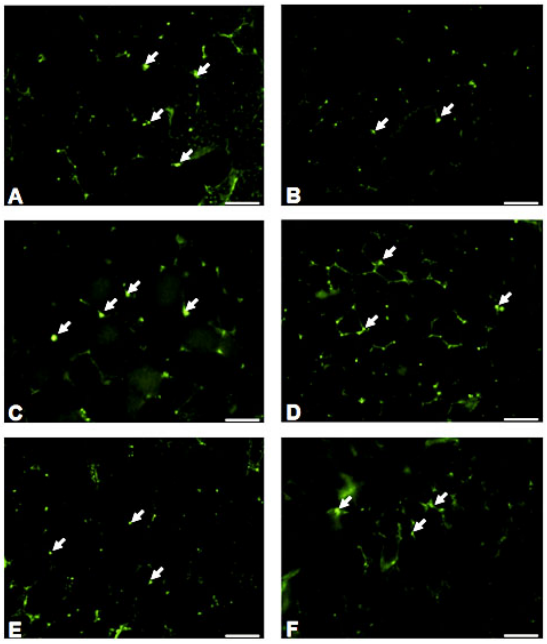

Fig. 2 Cross-sectional micrograph of androgen receptor immunoreactivity (arrows) in the nucleus of the EDL of the SHAM (A), BS-0 (B), TP (C), BS-5 (D), BS-50 (E), and BS-500 (F); scale bar = 50 μm.

gastrocnemius demonstrated a significant decline of the cross-sectional area in the BS-0 compared to that of the SHAM group ($p < 0.05$) whereas the TP group and all the BS groups (BS-5, BS-50, and BS-500), showed a significant increase in the cross-sectional area of both EDL and gastrocnemius ($p < 0.05$) (Fig. 1).

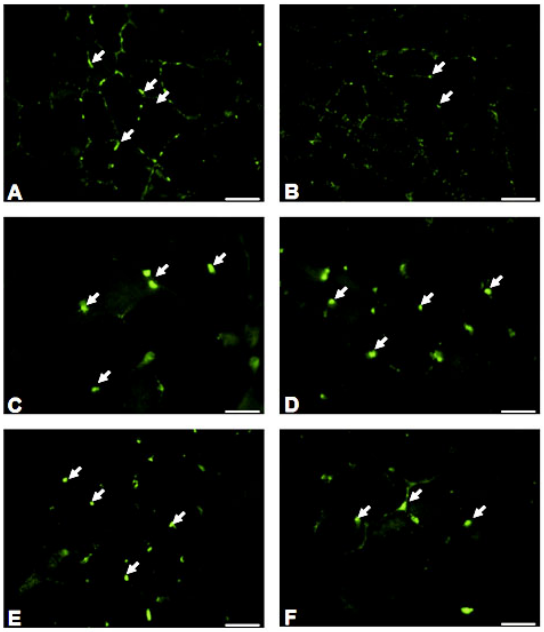

Fig. 3 Cross-sectional micrograph of androgen receptor immunoreactivity (arrows) in the nucleus of the gastrocnemius of the SHAM (A), BS-0 (B), TP (C), BS-5 (D), BS-50 (E), and BS-500 (F); scale bar = 50 μm.

Androgen receptor immunoreactivity (AR-ir) in the EDL and gastrocnemius

The cross sections of EDL and gastrocnemius were stained with H&E to identify the nucleus and cytoplasm as shown in Fig. S3(A) which was identical to the nucleus and cytoplasm of Fig. S3(B) depicting the AR-ir in the nucleus. The AR-ir was detected in the nuclei of the EDL and gastrocnemius. The AR-ir was reduced in the BS-0 group and increased in the TP, BS-5, BS-50, and BS-500 groups of EDL (Fig. 2) and gastrocnemius (Fig. 3).

The parvalbumin (PV) levels of the EDL and gastrocnemius

The size of the PV bands was decreased in the BS-0 groups of both the EDL and gastrocnemius but increased in the TP, BS-5, BS-50, and BS-500 groups compared to those of the BS-0 (Fig. 4A,B). The intensity of PV bands was significantly reduced in the BS-0 group of the EDL and gastrocnemius compared to those of the SHAM ($p < 0.05$) but significantly increased in the TP, BS-5, BS-50, and BS-500 groups ($p < 0.05$) (Fig. 5A,B).

DISCUSSION

Our data demonstrated that orchidectomy caused a decrease of testosterone, free testosterone, and bioavailable levels, resulting in a significant reduction of

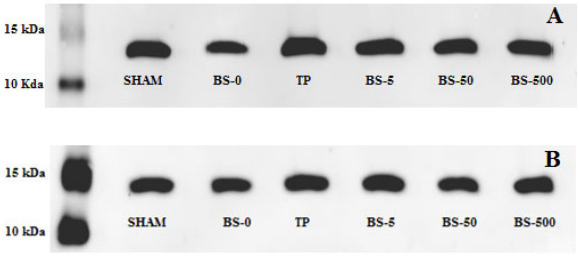

Fig. 4 Western blot showing the bands of parvalbumin (PV) from the EDL (A) and gastrocnemius (B) in the different treatment groups: the SHAM, BS-0, TP, BS-5, BS-50, and BS-500.

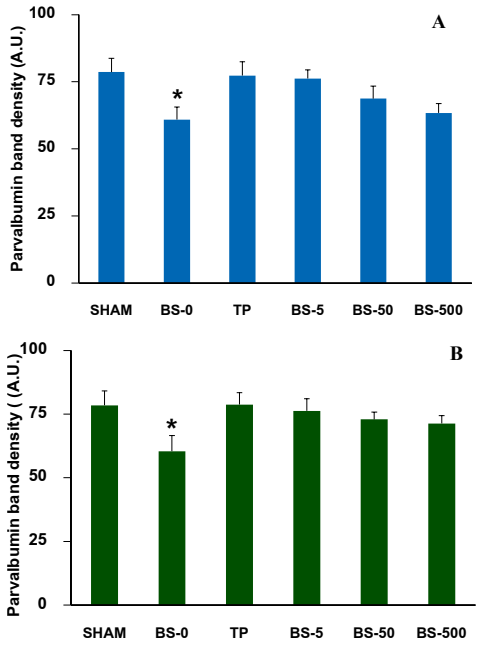

Fig. 5 (A) Histogram showing the densitometric quantification of blots of EDL PV. (B) gastrocnemius PV of the SHAM, BS-0, TP, BS-5, BS-50, and BS-500 groups. A.U., arbitrary units, $n = 10$. * Significantly different from the SHAM, $p < 0.05$. † Significantly different from the BS-0, $p < 0.05$.

weight gain and male reproductive organs. In contrast, in all BS treated groups (BS-5, BS-50, and BS-500), the testosterone level and the weight of male reproductive organs were low, suggesting that BS has no androgenic biological effects. This correlates to the work of Malaivijitnond et al [12] which demonstrated the decrease of testosterone level in the orchidectomized rats and BS treatment at the dose of 10 mg, 50 mg, and 250 mg/kg body weight. For toxicity test, the testosterone treatment showed an increase in SGOT and SGPT. When compared all doses of BS treatments, none induced an increase in SGOT and SGPT levels, and no alteration of liver, kidney, and spleen weight was observed. These findings suggest that the treatment with BS at the dose of 5 mg, 50 mg, and 500 mg/kg for 12 weeks may have no toxicity. Our data also demonstrated that orchidectomy resulted in a decline of the strength, endurance, and cross-sectional area of EDL and gastrocnemius, suggesting that the reduction of testosterone leads to an atrophy of EDL and gastrocnemius, whereas the testosterone treatment induced an increase in the strength, endurance, and the cross-sectional area of both the EDL and gastrocnemius. According to the work of Ting and Chang [16], testosterone replacement in hypogonadal men improved muscle mass and strength. The supplementation of androgen increases protein synthesis, RNA polymerase activity, and the uptake of 2-deoxyglucose and enhances glycogen synthesis in skeletal muscle. Interestingly, all doses of the BS treatments increased the strength, endurance, and the cross-sectional areas of both EDL and gastrocnemius similar to those found in the testosterone treatment. In a previous work by Roengsumran et al [17], flavonoid (3,7,3′-Trihydroxy-4′-methoxyflavone) and flavonoid glycoside (3,3′-dihydroxy-4′-methoxyflavone-7-O-β-D-glucopyranoside) were isolated from the tuber root of *Butea superba* Roxb., and the function of the compounds was investigated. They found that both flavonoid and flavonoid glycoside were influential in inhibiting cyclic adenosine monophosphate (cAMP) phosphodiesterase. It was demonstrated that the activation of cAMP signaling resulted in distinct hypertrophic responses in skeletal myofibers through complicated molecular mechanisms. These pathways are involved in stimulating hypertrophy and resisting atrophy in animal models of disorder including muscular hypertrophy, age-related atrophy, and disuse atrophy [18].

The data of AR-ir in the EDL and gastrocnemius demonstrated a reduction of AR-ir in the BS-0 group but an increase of AR-ir in the TP and all BS groups, suggesting that testosterone and *Butea superba* Roxb. (BS) may activate AR-ir in both the EDL and gastrocnemius. In addition, the PV levels of both the EDL and gastrocnemius were declined in the BS-0 group but increased in the TP and all the BS groups (BS-5, BS-50, and BS-500). From our data, we proposed the mechanism of testosterone via AR-ir on improving skeletal muscle strength by increasing PV levels. The enhancement of PV resulted in a fast contraction relaxation cycle which affected muscle strength and endurance. Interestingly, all doses of the BS treatment induced an increase of mass, strength, endurance, AR-ir, and PV levels of the EDL and gastrocnemius similar to those of testosterone treatment. The main components of BS are puerarin (7.32 mg/100 g), daidzein (3.75 mg/100 g), and genistein (13.33 mg/100 g). Therefore, the amount of 3 active compounds in the BS-5, BS-50

and BS-500 treatments was puerarin (0.366 μg/kg, 3.66 μg/kg, and 36.6 μg/kg, respectively), daidzein (0.1875 μg/kg, 1.875 μg/kg, and 18.75 μg/kg, respectively), and genistein (0.6667 μg/kg, 6.667 μg/kg, and 66.67 μg/kg, respectively). The effects on skeletal muscles were reported in many previous studies. The eight-week oral administration of 100 mg/kg puerarin extracted from *Pueraria lobate* was demonstrated to ameliorate muscle wasting, improve skeletal muscle weight and strength in type-1 diabetic rats by upregulation of protein synthesis via the Akt/mTOR signaling pathway and autophagy inhibition in skeletal muscle [19]. The soy isoflavone daidzein was shown to increase in the soleus muscle mass to body weight ratio in female mice by promoting protein synthesis via activating mTOR signaling in C2C12 myotubules [20]. Furthermore, the study by Chen and Chang [21] reported that daidzein may function as a phytoandrogen by modulating androgen receptor coactivators. According to the work of Aoyama et al [22], they demonstrated that 24-day treatment with 0.5 g genistein/kg diet prevents denervation-induced muscle atrophy in male rodents via the effect on estrogen receptor-alpha-mediated modulation of *Atrogin 1* and *Murf1*. Recent molecular docking study demonstrated that flavonoid conjugates such as glucuronide or sulfate of genistein present in plasma after ingestion of dietary flavonoids showed a high binding affinity with estrogen receptor and epidermal growth factor receptor [23]. Therefore, these 3 main components of BS may induce skeletal protein synthesis including parvalbumin protein which contributed to increased skeletal muscle mass, strength, and endurance. Although the amount of the 3 active compounds in all doses of the BS treatment was low, the treatment with all BS doses induced an improvement of the EDL and gastrocnemius mass, strength, and endurance. This may be due to a synergistic effect of the 3 active compounds. A low dose of the 3 active compounds may result in a low toxicity effect.

CONCLUSION

Subchronic treatment with 5, 50, and 500 mg/kg of BS improved muscle strength and endurance by increasing parvalbumin levels in both EDL and gastrocnemius muscles. In addition, the treatment with BS prevented skeletal muscle atrophy. An anabolic biological effect of BS may act through androgen receptors without inducing an increase of testosterone level. Furthermore, treatment with 5, 50, and 500 mg/kg BS for 12 weeks may have no toxicity.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2022.102.

Acknowledgements: This study was financially supported by a grant of Prince of Songkla University under DEN570366S and Faculty of Science Research Fund, Prince of Songkla University, contract no. 1-2554-02-004. We thank Mrs. Anna Chatthong for the assistance with the English editing.

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Appendix A. Supplementary data

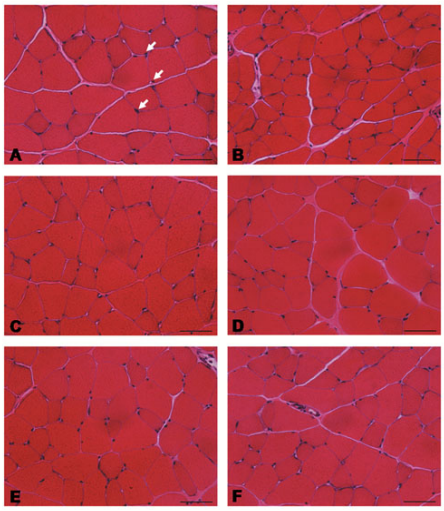

Fig. S1 H&E-stained cross-sectional micrographs of EDL in the SHAM (A), BS-0 (B), TP (C), BS-5 (D), BS-50 (E), and BS-500 (F) groups. The nucleus of each fiber was purplish blue (arrows); scale bar = 50 μm.

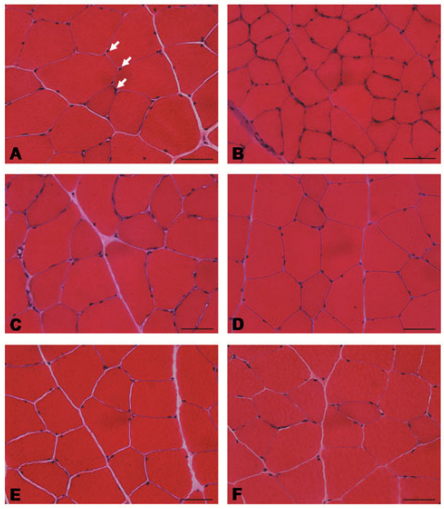

Fig. S2 H&E-stained cross-sectional micrographs of gastrocnemius in the SHAM (A), BS-0 (B), TP (C), BS-5 (D), BS-50 (E), and BS-500 (F) groups. The nucleus of each fiber was purplish blue (arrows); scale bar = 50 μm.

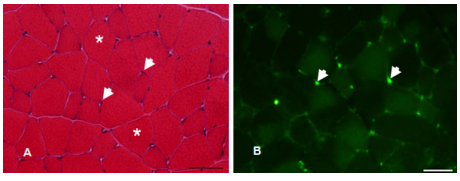

Fig. S3 (A) H&E-stained cross-sectional micrographs of EDL showing nucleus (arrows) and cytoplasm (*). (B) Cross-sectional micrograph of androgen receptor immunoreactivity in the nucleus of the EDL (arrows); scale bar = 50 μm.