Analysis of Functional Components of Burdock (Arctium lappa root) Standard Water Extract and its Aphrodisiac Effect in Experimental Rats

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Summary: High performance liquid chromatography (HPLC) analysis showed that the content of chlorogenic acid in burdock standard water extract (BSWE) was about 1.22±0.07 mg/g and no araciin and arctigenin was detected. The inulin was determined to be 174.33±3.68 mg/g in BSWE by colorimetry method. The amino acids were analyzed by the amino acid analyzer and showed that asparagine and arginine are higher and determined to be 3021.00±13.53mg/100g, 2042.33±8.62mg/100g in BSWE, respectively. The results of aphrodisiac pharmacological experiments showed that the number of riding, insertions and the latency of rats in BSWE groups showed significant differences as compared with control, at the 7th day and 15th day after drug administration. Further study indicated that the aphrodisiac effect of BSWE is mostly associated with its regulation on NO, sialic acid and antioxidative ways and arginine should be one of the active components in BSWE.

Keywords: Burdock standard water extract; Aphrodisiac effect; Inulin; Chlorogenic acid.

Introduction

The fruit of *Arctium lappa* L. is a traditional Chinese medicine with antipyretic effect commonly used in China and arctiin is regarded as one of its active constituents. Research indicated that arctigenin, the metabolite of arctiin, is the real compound taking effect for the pharmacological actions of arctiin [1-2].

Burdock is the root of *Arctium lappa* L. regarded as an excellent supplementary foods in Dietetic Materia Medica [3]. More than a hundred years ago, it was introduced to Japan from China and was bred as a kind of vegetable. In the latter half of 20th century, burdock gradually obtains international recognition for its nutritional value due to the increasing popularity of the macrobiotic diet. In the 1990s, cultivated burdock was introduced into Lanling, Shandong Province and Xuzhou, Jiangsu province of China. Presently its anti-diabetes, anti-hyperlipidemia, and anti-aging actions were examined [4]. Burdock has tonic action like ginseng and "Oriental Ginseng" was called in Japan [5]. In Asian folks, burdock was regarded as aphrodisiac agent [6]. In Hawaii, it was used as an aphrodisiac gift for a newly-married couple [7]. Now the burdock has been developed into standard water extract and used as a solid drink in China. This paper deals with the determination of functional components of burdock standard water extracts (BSWE) and its aphrodisiac action so as to elucidate its active constituents and possible aphrodisiac mechanism.

Experimental

Materials and reagents

BSWE (Yuan Sheng He Jin Biotechnology Co., Ltd. Shandong, China); NanBao capsule (Tianjin Li Sheng Pharmaceutical Co., Ltd. China); Arctiin and arctigenin (China Food and Drug Administration Institute. Lot number: 110819-201611); Chlorogenic acid (China Food and Drug Administration Institute. Lot number: 110753-201415); Progesterone injection, estradiol benzoate (Ningbo Second Hormone Plant, China), Chromatographic Methanol (Tianjin Kemiou Chemical Reagent Co., Ltd. lot number: 20161002); 95% ethanol (Tianjin Fuyu Fine Chemical Co., Ltd. Lot number: 20160411); Glucose (Xiwang Pharmaceutical Co., Ltd. lot number: 20160822); Concentrated sulfuric acid (Shenyang Xinxing Factory, lot number: 20161023); Phenol (Shanghai Huyu Biotechnology Co., Ltd. lot number: 20160213); other reagents were purchased from various chemical reagents company. Rat testosterone Elisa Kit (Shanghai Qiao Du Biological Technology Co., Ltd. Lot number: 201706), other kits were purchased in Nanjing Jiancheng Bioengineering Institute.

Animals

Six weeks SD rats (180-220g) were purchased from the laboratory animal center of Changsheng Bio-

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Technique Co. Ltd. (Benxi, Liaoning, China), qualified No. SCXK 2010-0001. Animals were well cared in clean metabolic cages, the room temperature and relative humidity was set as 22±11~23℃ and 55%; photoperiod was controlled as 12 h natural light and 12 h dark. The animals were fed ad libitum with standard feed and water during the experimental period. The experiment strictly adhered to the Guide for the Care and Use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The research protocol was approved by the ethics committee of Liaoning University of Traditional Chinese Medicine, China (131/2010).

Instruments

UV-2100 UV-Vis spectrophotometer (Unocal Shanghai Instrument Co., Ltd，USA); Agilent 1260 High Performance Liquid Chromatography (Agilent Technologies, Inc.， USA), equipped with a quaternary gradient pump, DAD detector; FA-1004 electronic balance (Shanghai Jingke balance plant); Hitachi high-speed automatic amino acid analyzer L-8900 (Guangzhou City of Science and Technology Instrument Co., Ltd.，Japan); Enzyme analyzer KC-100 (Shenzhen Kate Biological Medical Technology Co., Ltd.).

Preparation of BSWE

The BSWE were produced in Yuan-Sheng-He -Jin Biotechnology Co. Ltd.，China and its preparation procedure was briefly described as follows. After rinsing with tap water, 1 kg of burdock was cut into thin slices and then boiled with 10 L of distilled water for 4 hours, then, after filtration, the filtrates were concentrated and lyophilized to give the dried powder (3.2% for the yield). 3 batches of BSWE (No. 20161012, 20161115, 20161215) were analyzed.

Component analyses of BSWE

Arctiin and arctigenin detection

The arctiin and arctigenin were detected with HPLC as described previously [8].The chromatographic conditions are: Agilent TC-C18 column (4.6mm × 250mm, 5μm) were used with mixture of methanol-water (Water: 0 min, 55%; 10 min, 46%; 20 min, 30%; linear gradient, 1.0 ml min^{-1} of flow rate) as mobile phase, the detection wavelength was set at 280 nm and the column temperature was with 25 °C.

Chlorogenic acid determination

An HPLC method was established to determine the chlorogenic acid by the phenomenex C18 column (4.6mm × 250mm, 5μm) eluting with mixture of acetonitrile-0.4% phosphoric acid solution (8:92, 1.0 ml min-1 of the flow rate), the detection wavelength was set at 327 nm and the column temperature was monitored at 35 °C.

Sample preparation

Weigh the BSWE 401.8mg in a 10ml volumetric flask and fill with 60% methanol to obtain 40.18mg / ml of the sample solution.

Methodological validation

The quantitative analysis was performed using an external calibration method. The linearity calibration were constructed by six different concentrations of chlorogenic acid to ben y=3002.1x+32.31 (r2=0.9998, 0.04-1.2 μg). The variation coefficients of chlorogenic acid were 0.81% for the intra-day assays. The repeatability test was also carried out with five independent sample solutions prepared and the RSD was determined to be 0.91%. The accuracy of the method was performed according to the recovery experiments. Chlorogenic acid stock solutions were added before the extraction with the half amount of chlorogenic acid in the BSWE, mean recoveries were 98.8%, which presented good accuracy for the analysis.

Inulin determination

The inulin was determined by the difference of total saccharides and reducing saccharides as for the contents of inulin [9]. The determination of total saccharides was performed by using the phenol-sulfuric acid method and the reducing saccharides was determined by the DNS colorimetry. The methodological validation showed good results with 1.30% of RSD for precision, 1.35% of RSD for repeatability in determination of total saccharides and 1.30% of RSD for precision, 1.35% of RSD for repeatability and 1.71% of RSD for stability in the determination of reducing saccharides, indicating the feasibility of the method.

Amino acid determination

Amino acid was determined as a method in the national standard with the amino acid analyzer [10]. The chromatographic conditions are: Ion exchange column (4.6 mm × 60 mm, 3 μm) was used with the separation column temperature at 57 °C and the reaction column temperature was 135 °C, the buffer flow rate was 0.40

mL min-1 (1.0-22.0 MPa) and the ninhydrin flow rate was 0.35mL min-1 (0.2-2.5 MPa), channel 1: the detection wavelength was 570 nm, channel 2: the detection wavelength was 440 nm.

The sample preparation briefly introduced as follows. Weighed 0.2000 g BSWE into the hydrolysis tube and 6 mol/L hydrochloric acid for 10 ml was added, then 3 drops of phenol were also added. The hydrolysis tube was frozen for 5min and then filled with the test tube with nitrogen, sealed and put it into an oven at 110°C to hydrolyze for 22 h. After the hydrolysis, the hydrolyzates were filtered and transferred to a 50 ml volumetric flask and filled with distilled water. 1ml of hydrolysate solution was pipetted into a 5 ml volumetric flask and dried in a vacuum drier at 40-50 °C to dryness and dissolved in pH 2.2 buffer. The amino acids in BSWE were determined with an amino acid automatic analyzer by the external standard methods with authentic amino acids as a reference control.

Pharmacology experiments

Animal grouping

Based on the literature [4, 11], 48 male rats were randomly divided into four groups and twelve rats in each group. There were control, NanBao (350mg/kg), BSWE (500mg/kg) and BSWE (1000mg/kg), respectively. They were administrated with distilled water (10 ml/kg body weight), NanBao capsule (350mg/kg body weight), BSWE (500mg/kg body weight) and BSWE (500mg/kg body weight) by gastric perfusion respectively.

NanBao capsule is a traditional Chinese medicine formula, and the main ingredients of NanBao capsules are *Epimedium brevicornu* Maxim, *Morinda officinalis* How, *Curculigo orchioides* Gaertn., *Panax ginseng* C. A. Mey, *Angelica sinensis* (Oliv.) Diels and so on. It is a vital funaction promoter, possess the similar function to Viagra. It can strengthen the Yang and invigorates the kidney. It is used for treatment of insufficiency of kidney-Yang, impotence, premature ejaculation, lumbago, soreness of extremities, wetness and coldness of scrotum, lassitude and anorexia.

Experimental observation

Male rats received training three times with female rats for the aphrodisiac sexual experience. Each male rat was allowed 30 minutes activity with a female rat with behavioral estrus for copulatory behavior, as described previously [12-13]. In aphrodisiac experiment of BSWE, the mating behavior test was observed at 3rd, 7th and 15th day of drug administration. The test was conducted between 19 o'clock and 23 o'clock under dim light. Four male rat randomly selected from each group for mating behavior test. Firstly, one qualified male rat was placed in a metabolic cage (48.5 cm × 33.5 cm × 22.5 cm). After 10 minutes of adaptation, one female rat was placed into the cage, and then, their mating behavior of male rats was observed and recorded for 20 min. The experimental procedure was repeated four times. The female rat estrus was induced by subcutaneous injection of estradiol benzoate (10 μg/100 g body weight) and progesterone (0.5 mg/100 g body weight) 48 h and 4 h before the mating experiment. The following male sexual behavior parameters were recorded or calculated after monitoring for the 20 min observation period: mount frequency (MF, the number of mounts from the time of introduction of the female until ejaculation) and intromission frequency (IF,the number of intromissions from the time of introduction of the female until ejaculation), mount latency (ML, the time interval between the introduction of the female and the first mount by the male) and intromission latency (IL,the time interval between the introduction of the female and the first intromission by the male), ejaculatory latency (EL, the time interval between the first intromission and ejaculation).

Rats were sacrificed 28 days after intragastric administration of the drug and the testis, epididymis, prostate and seminal vesicles were harvested and weighed. The blood of abdominal aorta was collected into serum preparation tube. The separated serum was collected and divided into aliquots, stored at -20°C for further assay.

Organ biochemical assay

Sialic acid and MDA activity were measured in testis tissues samples using biochemical kits according to the manufacture's instruction (Nanjing Jiancheng Bioengineering Institute, China); Fructose was measured in semen samples using biochemical kits according to the manufacture's instruction (Nanjing Jiancheng Bioengineering Institute, China); NOS activity was measured in penile tissue samples using biochemical kits according to the manufacture's instruction (Nanjing Jiancheng Bioengineering Institute, China).

Blood biochemical assay

Serum testosterone was determined using ELISA kit, according to the manufacture's instruction

(Shanghai Qiao Du Biological Technology Co., Ltd. China), ELISA kits were rat specified. Serum NOS was determined using biochemical kit, according to the manufacture's instruction (Nanjing Jiancheng Bioengineering Institute, China).

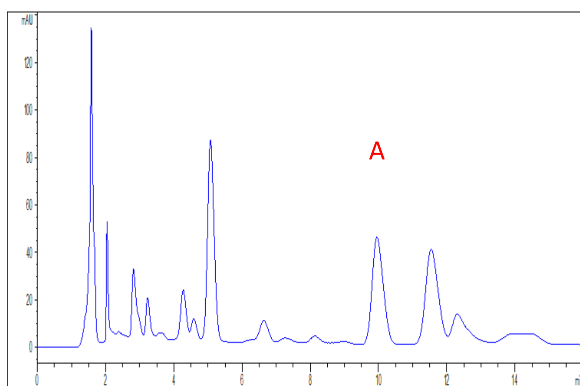
Statistical analysis

Results were as mean ± S.D.M. The data were statistically treated with SPSS 17.0 for windows software, and the significance of difference between the means was determined by one way analysis of variance (ANOVA) with post hoc tests, and values were considered significant when $p < 0.05$.

Result and Discussion

Chlorogenic acid and arctiin

Arctiin and arctigenin in different parts of burdock were investigated previously [14]. The wild burdock contains a small amount of arctiin - 0.39mg / g, but it does not contain arctigenin. The cultivated burdock does not contain arctiin and arctigenin. In this time, arctiin and arctigenin could also not be detected in our HPLC analysis. Further, HPLC analysis indicated that chlorogenic acid in BSWE was 1.22±0.07 mg / g and its chromatogram is shown in Fig 1.



A: chlorogenic acid

Fig. 1: HPLC Chromatogram of chlorogenic acid of BSWE.

Inulin

Inulin in BSWE was determined by the difference of total saccharides and reducing saccharides, indicating that the inulin was about 174.33 ± 3.68 mg /g in BSWE.

Amino acid

The amino acids in BSWE were determined accordance to a national standard and the results are shown in Table-1. Among them, asparagine and arginine are higher and accounts for over 40% of total amino acids.

Table-1: Determination of amino acid content ($\bar{x} \pm s$).

Amino acids (mg/100g)	
Aspartic acid	3021.00±13.53
Threonine	228.00±4.00
Serine	229.00±3.61
Glutamic acid	577.67±13.01
Glycine	144.33±3.51
Alanine	415.33±4.04
Cystine	44.67±2.08
Valine	256.33±8.50
Methionine	86.00±2.65
Isoleucine	127.33±6.11
Leucine	130.33±3.21
Tyrosine	236.00±3.61
Phenylalanine	308.00±13.11
Lysine	305.67±13.58
Histidine	372.00±11.00
Arginine	2042.33±8.62
Proline	800.00±8.89

Mating behavior test

The observations of sexual behavior are presented in Table-2. In the aphrodisiac experiment, BSWE with two doses showed in a dose-dependent manner on the effect of the animal behavior, revealing that 1000 mg/kg of BSWE administration significantly increased sexual behavior as compared with the control.

The low dose group of BSWE had no significant effect on MF or IF on the 3th day of administration ($p > 0.05$), but the MF was significantly increased in both high and low doses groups on the 7th day and 15th day of drug administration as compared with the control group ($p < 0.05$). Administration of either 500 or 1000 mg/kg BSWE for 7 and 15 days both significantly decreased ML as compared with the control group ($p < 0.05$). In addition, both doses of BSWE showed a tendency to delay EL too.

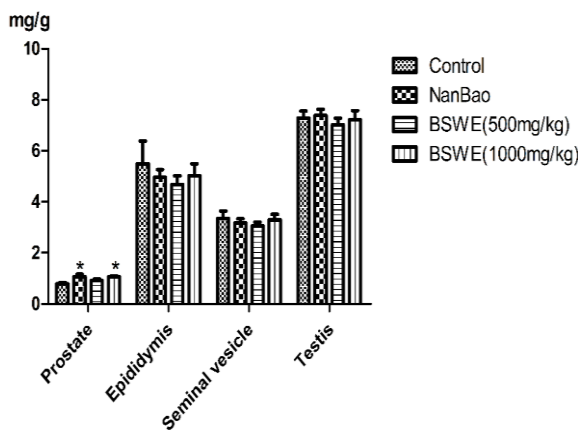
Weight of sexual organs

There was a significant effect on the weight of the prostate in the BSWE high-dose group ($P < 0.05$), but almost no effect on the weight of other sexual organs in both positive and BSWE groups. The results are shown in Fig 2.

Table-2: Effects of BSWE on sexual behavior in rats (x ± s).

Sexual behavior Parameters	Days of treatment	Control	NanBao (350 mg/kg)	BSWE(500 mg/kg)	BSWE (1000 mg/kg)
	3 day	131.25±32.50	137.25±33.49	129.25±17.46	117.75±12.37
Mount Latency	7 day	154.00±17.21	108.00±11.52**	130.00±25.92*	121.25±16.50**
	15 day	140.75±40.43	60.25±30.38**	101.25±6.60*	93.75±29.95*
	3 day	171.75±30.57	165.75±25.20	152.25±20.42	137.00±17.46*
Intromission Latency	7 day	183.75±11.15	125.00±12.19**	155.75±13.91	156.00±28.60
	15 day	167.25±30.30	88.00±35.95**	146.25±20.01	142.00±29.43
	3 day	275.75±33.73	250.25±11.03	251.25±16.74	240.25±14.01*
Ejaculatory Latency	7 day	287.00±9.80	275.75±10.50*	281.25±16.74	282.00±13.37
	15 day	278.75±28.22	278.25±64.12	297.75±3.40	315.25±5.44
	3 day	14.00±0.82	20.00±1.63**	15.25±0.96	16.75±0.96**
Mount Frequency	7 day	13.25±1.26	20.00±1.63**	19.00±0.82**	20.00±0.82**
	15 day	13.00±0.82	23.00±6.53**	23.00±0.82**	26.75±1.26**
	3 day	11.00±1.63	15.00±3.74*	12.00±2.83	13.00±1.41
Intromission Frequency	7 day	11.25±1.26	15.75±1.26**	15.00±1.41**	16.00±1.63**
	15 day	11.00±0.82	18.25±3.86**	14.25±2.50	19.25±4.92**

For the comparison between the experimental and control groups, * indicated a significant difference at $p < 0.05$. All values were presented as mean ± SD of four independent experiments (n =4).



For the comparison between the experimental and control groups, * indicated a significant difference at $p < 0.05$. All values were presented as mean ± SD of twelve independent experiments (n = 12).

Fig. 2: Effects of BSWE on weight of sexual organs in rat.

Organ biochemical assay

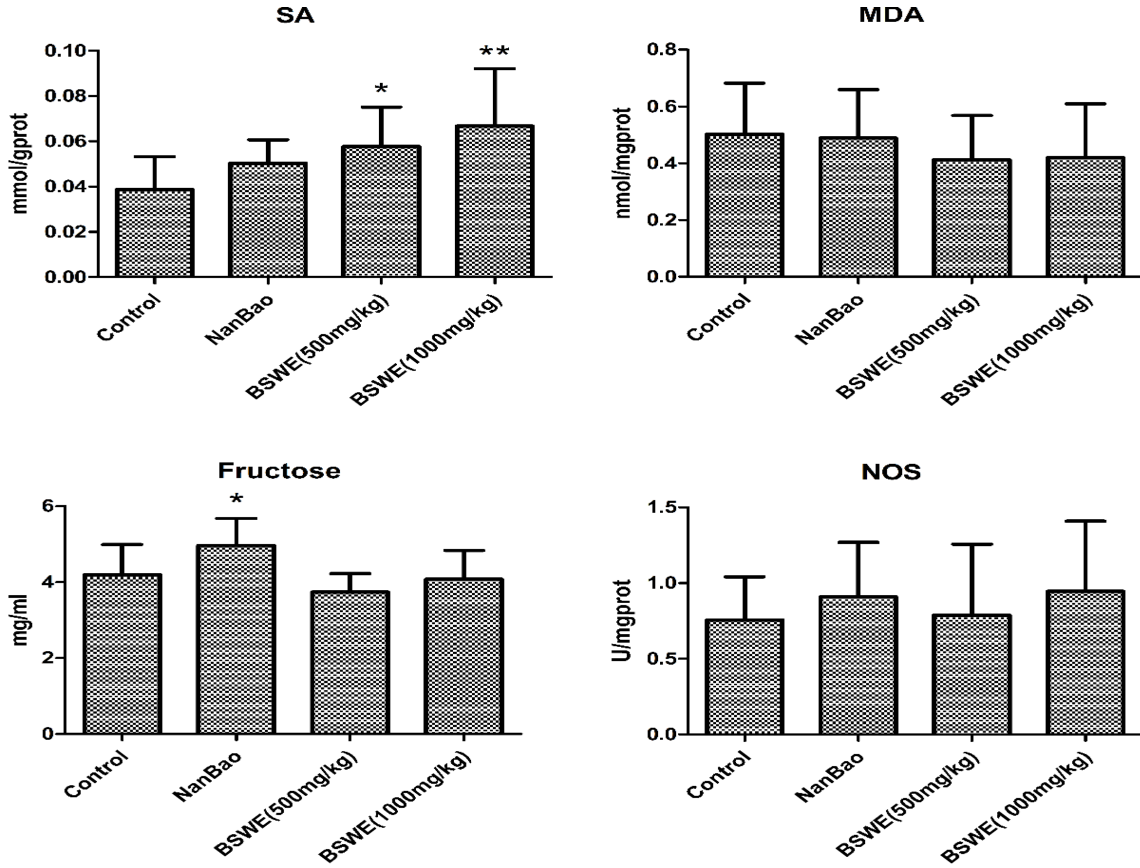
Compared with the control group, the sialic acid in the testes of the BSWE group was significantly increased (P <0.05 or P <0.01), MDA in the testes showed a decreased tendency, but NOS activity in the penile tissue exhibited an increasing trend. The results are shown in Fig. 3.

Blood biochemical assay

Compared with the control group, the testosterone and the NOS in the BSWE groups were significantly increased (P <0.05 or P <0.01). The results are shown in Fig. 4.

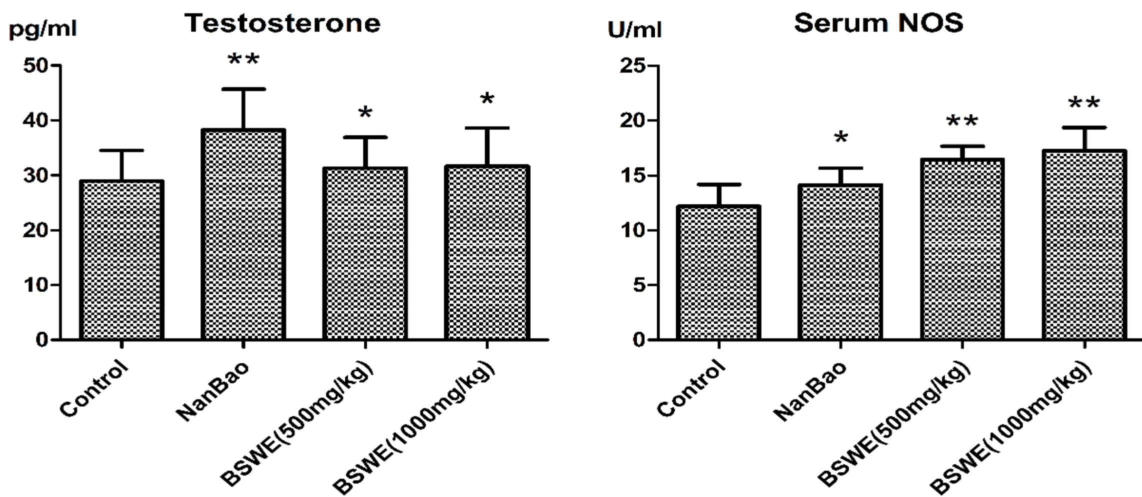
Our results indicated that the cultivated burdock does not contain arctiin and arctigenin. However, in recent years, some references mistakenly believed that burdock contains arctiin and arctigenin [15], and we again confirmed that the burdock contained almost no arctigenin and arctiin, so the other constituents of burdock are responsible for its various pharmacological actions. Chlorogenic acid has a wide range of pharmacological effects, such as anti-diabetes, protection of blood vessels and liver, inhibition of platelet aggregation, anti-inflammatory, immune suppression [16]. Asparagine and arginine account for over 40% of total amino acid in BSWE.

This study analyzed the effect of BSWE on aphrodisiac competence in male rats with NanBao capsule as a positive reference drug. Our results indicated that BSWE could enhance the sexual competence of male rats compared with controls group with distilled water. These results in a scientific way to prove the evidence of burdocks for enhancing male sexual competence. The mating behavior test revealed that the BSWE significantly increased MF and IF as compared with the control group, though the intensities were less than that of the positive. BSWE also caused significant decreasing in ML and IL compared with control group, this is the same as the effect of the NanBao capsule in ML and IL. MF and IF are considered to be indexes for libido (sexual desire), while ML and IL are regarded as indicators of sexual arousal [17-20]. The significant increases in MF and IF and the decreases in ML and IL indicate that male rats libido was enhanced by BSWE.



For the comparison between the experimental and control groups, * indicated a significant difference at $p < 0.05$, ** indicated a significant difference at $p < 0.01$. All values were presented as mean ± SD of twelve independent experiments (n = 12).

Fig. 3: Effects of BSWE on biochemical assay in organ of rats.



For the comparison between the experimental and control groups, * indicated a significant difference at $p < 0.05$, ** indicated a significant difference at $p < 0.01$. All values were presented as mean ± SD of twelve independent experiments (n = 12).

Fig. 4: Effects of BSWE on blood assay in rats.

Sialic acid is a neuraminic acid, which is widely present in animal tissues and microbes，it is an important component for cell membrane glycoprotein and a glycolipid. The sialic acid in the testis has the role for sperm lubricant; it can reduce the friction between sperms and promote sperm acrosome membrane integrity [21]. The increased of sialic acid indicated that BSWE could stimulate male reproductive function, especially the gonadal secretion function. On the other hand, the prostate is a hormone-dependent gland [22, 23], and the weight gain of the prostate is due to the effects of androgens because androgens have the effect of promoting anabolic activity [24]. These results indicate that BSWE has an effect of promoting androgen secretion. Sperm motility is hormone-dependent [25, 26], so BSWE can increase sperm volume and sperm motility.

The oxygen free radicals can attack the polyunsaturated fatty acids in the biofilm to triggers lipid peroxidation and thereby forms lipid peroxides to damage the tissue cells. The antioxidant defense system of testis is important to ensure the normal structure and function of testes. Under normal conditions, the antioxidant defense mechanism of testicular tissues and secretions can quench reactive oxygen and protect testes and mature sperm cells from injury [27]. Peroxidation damage is considered to be an important factor contributing to testicular dysfunction under pathological conditions (such as testicular pathological damage caused by diabetes and injury) [28]. Oxidative damage in testis often leads to decrease the number of spermatozoa, inadequate sperm motility or sperm motility disorders or infertility. BSWE has an amount of chlorogenic acid. Chlorogenic acid is formed by esterification of caffeic and quinic acids. It is one of the most abundant polyphenols found in various agricultural products such as coffee, beans, potatoes and apples [29]. Research demonstrated that chlorogenic acid exhibits various biological properties, including anti-bacterial, anti-oxidative, and anti-carcinogenic activities [30-33]. The decrease of MDA caused by BSWE indicated that it has an effect of modulating the reproductive function and anti-oxidation on rats, which is consistent with the anti-oxidative action of chlorogenic acid. It is speculated that chlorogenic acid may be one of the anti-oxidative ingredients in BSWE.

Arginine plays an important role in all amino acids due to its extensive biological functions. It is involved in the organization of cellular protein, urea, creatine, creatinine, nitric oxide (NO), glutamine, pyrimidine and another synthesis in the body [34]. Arginine has two direct metabolic pathways in animals: one is the decomposition of ornithine and urea under the action of arginase; another, under the action of nitric oxide syntheses (NOS), arginine is decomposed into molecules such as citrulline and NO [35]. The delayed EL and increased penile erection in treated male rats indicated the involvement of NO in the intervention [36]. Penile erection is a hemodynamic change caused by the neuromuscular relaxation of the corpus cavernosum and related vascular smooth muscle [37]. The NO-cGMP pathway plays an important role in the regulation of the relaxation of the smooth muscle of the corpus cavernosum. During sexual stimulation, parasympathetic, non-cholinergic non-adrenergic (NANC) nerve endings and vascular endothelial cells in the penile cavernous synthesize and release NO under the action of NOS. NO activates guanylate cyclase in smooth muscle cells, increases cGMP synthesis, or activates protein kinase G (PKG), PKG enables calcium channel closure and potassium channel opening, both of these effects can cause spontaneous smooth muscle relaxation induced penile erection. NOS is a synthetase that catalyzes the production of NO from L-arginine and O_2, which are widely found in the nerve tissue, vascular endothelium, respiratory tract, intestinal epithelium, myocardium and kidney. In the penile tissue, NO could relax the cavernosal artery and cavernous sinus smooth muscle and increase blood perfusion to induce penile erection. In the blood, NO could remove the thrombosis and prevent clotting and cardiovascular diseases. NOS of BSWE in blood showed significantly difference from control ($P <0.01$), indicating that the BSWE could promote the blood circulation through generation of NO and vasodilation, which could improve sexual competence. It is speculated that arginine may be one of the aphrodisiac ingredients in BSWE.

BSWE could increase the level of rat testosterone, indicating BSWE could enhance the GnRH-LH signalling and the involvement of the stimulation of hypothalamic-pituitary-gonadal axis [38]. Testosterone is the main male gonadal hormone produced by the interstitial leydig cells of the testis. The action of testosterone is to increase the rate of target cell protein formation in testies. Testosterone could convert to dihydrotestosterone which further bound to the cytoplasmic protein receptor of the nucleus under the action of 5a-reductase. It then stimulates DNA and RNA transcription, activates

RNA polymerase, and promotes protein production. Finally, resulting in increasing the weight of the second gonadal organ [39]. Sperm production also involves complex interactions between testicular structure and secretory function. Testosterone increase blood flow to stimulate testicular tissue growth and sperm production [40]. In addition, testosterone is responsible for the penile tumescence and rigidity [41]. Our results indicated that the increase of testosterone in rats maybe one of mechanism for enhancing male sexual behavior and increasing weight of prostate of BSWE.

Conclusion

This study demonstrated that BSWE could enhance sexual competence and behavior in male rats. It is speculated that arginine and chlorogenic acid should be the chief aphrodisiac ingredients of BSWE. These results further supported the acclaimed use of this plant as an aphrodisiac in Chinese folk medicine and provided the scientific basis for the health benefit of burdock.

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