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Aphrodisiac Property of the Aqueous Extract of *Cynanchum wilfordii*

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Abstract The purpose of the current study was to investigate the total phenolic content and the antioxidant activity of the aqueous extract of *Cynanchum wilfordii* Radix (CWW). In addition, we conducted *in vitro* and *in vivo* tests to examine whether the aqueous extract of CWW has an aphrodisiac property. The results indicated significant increases in testosterone synthesis in Leydig TM3 cells when the cells were treated with CWW at concentrations of 50, 100, and 200 μg/mL. In the *in vivo* study, CWW (50, 100, and 200 mg/kg body weight/day) and tadalafil (2 mg/kg body weight/day) were administered by oral gavage to male Sprague-Dawley (SD) rats for 15 days. On day 15, the rats were evaluated for sexual behavior parameters (mount latency, ML; mounting frequency, MF; intromission latency, IL; intromission frequency, IF; ejaculation latency, EL; post-ejaculatory interval, PEI) by pairing them with estrus females. Following the sexual interactions, blood samples were collected from the rats to evaluate their serum hormone levels. In the rats administered 200 mg/kg body weight/day of CWW, MF ($p < 0.05$) and IF ($p < 0.01$) significantly increased, while ML, IL, EL, and PEI significantly ($p < 0.05$) decreased. In addition, the serum levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone increased. CWW improved sexual motivation, libido, and potency in the male SD rats by stimulating LH, FSH, and testosterone secretion. The results indicated that *Cynanchum wilfordii* has an aphrodisiac effect.

Keywords: *Male sexual behavior, Aphrodisiac, Testosterone, Cynanchum wilfordii*

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1. Introduction

Reactive oxygen species (ROS) are highly reactive oxidizing agents that include peroxides, superoxide, hydroxyl radical, and singlet oxygen. During mitochondrial electron transport reactions, ROS, which are the natural byproducts, can be generated and accumulated continuously [1,2,3]. Prolonged exposure to ROS can cause accumulative oxidative damage, which is thought to be one of the major contributors to cellular aging and functional decline during the production of testosterone in Leydig cells [2,3]. The latter is associated with loss of sperm motility and defects in membrane integrity [4,5]. In general, antioxidants are substances that protect against the damages caused by ROS and lipid peroxidation. Hence, ROS scavengers can be administered to improve sperm function [6]. Vegetables and fruits are rich in polyphenols, which are known to reduce oxidative stress induced by ROS. Phenolic antioxidants are important components of pharmaceutical and food products. Substantial evidence has shown that the use of herbal medicine supplements in humans improves male infertility and erectile dysfunction (ED) [7,8,9,10,11]. The Leydig cells in the interstitium of the testis are the main source of testosterone, which stimulates the differentiation of male sexual characteristics and steroidogenesis in the testes [12,13]. Functional deficiency of Leydig cells is highly associated with age-related decline in serum testosterone levels [14,15]. A substantial decrease in serum testosterone level may affect spermatogenesis, result in loss of bone strength, or cause a decline in muscle performance or physical function, which can reduce the quality of life of an aging human [16,17]. A dominant proportion of aging males have reduced serum testosterone levels, which is the leading cause of andropausal symptoms [15]. The definition of andropause is controversially debated; however, according to the most recent literature, andropause consists of at least three sexual symptoms: loss of morning erection, low libido, and ED [18]. Male sexual dysfunction (MSD) affects not only sexual interactions, but also, the overall satisfaction of life. MSD is characterized by ED, ejaculation dysfunction, and hypogonadism. It also induces serious public health concerns [19]. Sexual behavior in male rats is partially controlled by the hypothalamic-pituitary-gonadal (HPG) axis. Gonadotropin releasing hormone released from the hypothalamus activates the release of luteinizing hormone (LH), which in turn stimulates the release of testosterone from the Leydig cells in the testes [20]. Male rats reflexively secrete testosterone when they smell (anticipatory release) or mate (ejaculatory release) with an estrus female rat. The secreted testosterone is in

addition to normal surges called 'spontaneous release', which occur throughout the day [21,22]. Several recent studies have demonstrated that restoration of testosterone levels improves andropausal symptoms, including sarcopenia, loss of muscle mass, depression, cognitive disorders, increased body fat mass, sexual dysfunction, and fatigue [23,24,25]. The purpose of testosterone replacement therapy (TRT) is to relieve the depressive symptoms and problems of late-onset hypogonadism by restoring serum testosterone concentrations to within the normal biological range [26]. Unfortunately, TRT can simultaneously induce many side effects including prostate cancer, liver toxicity, benign prostatic hyperplasia, and cardiovascular problems [27]. Therefore, development of natural products that can protect Leydig cells and support the continued production of endogenous testosterone is very crucial for alleviating the hypogonadism. Recent studies are focused on finding new natural substances to replace TRT to avoid the side effects associated with the latter [18].

Cynanchum wilfordii is used in traditional herbal medicines in Korea for the prevention and treatment of various diseases such as rheumatic arthritis, geriatric diseases, atherosclerotic vascular diseases, and ischemia-induced diseases [28]. Research is ongoing on the use of *C. wilfordii* to improve menopausal symptoms. However, the effect of *C. wilfordii* on male sexual behavior has not been reported. To the best of our knowledge, this is the first study to report the effects of the aqueous extract of *C. wilfordii* Radix (CWW) on enhancing the sexual behavior of male SD rats. This research therefore evaluated the effects of CWW on the sexual behavior and serum hormone levels of male SD rats, which were allowed to copulate with estrus female SD rats for 15 days.

2. Materials and Methods

2.1. Preparation of *C. wilfordii* Extract

C. wilfordii Radix was collected from the Jecheon-si area (Chungcheongbuk-do, Republic of Korea). Fresh *C. wilfordii* roots were washed with tap water and air-dried at room temperature (25-30°C). Two kilograms of the roots were sliced and extracted with distilled water (DW) (DW:material, 20:1, v/w) twice using a soxhlet extractor at 100°C for 4 h. The aqueous extract was then filtered through a No. 4 filter paper (Advantec, Dublin, CA, USA). The combined filtrates were concentrated using a rotary evaporator (R-210; Buchi, Flawil, Switzerland) and the resulting filtrate was freeze-dried. The freeze-dried product was stored at -80°C until use. Tadalafil, progesterone, and estradiol were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Phytochemical Analysis

2.2.1. Total Phenolic Content (TPC)

The TPC of CWW was determined using the Folin-Ciocalteu colorimetric method according to the procedure stated by Singleton et al [29] with slight modifications. Gallic acid was used as the standard and TPC was expressed as mg GAE/g CWW. Briefly, appropriate dilutions of the extract were oxidized with Folin-Ciocalteu reagent. The reaction was neutralized with sodium carbonate at room temperature for 90 min. Absorbance was measured at 750 nm using a microplate reader (SpectraMax® 190; Molecular Devices, Sunnyvale, CA, USA).

2.2.2. Determination of the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity of CWW

The free radical scavenging activity of CWW was determined according to the method stated by Brand-Williams et al [30]. Briefly, 40 µL of appropriately diluted concentrations of CWW were added to 200 µL of 0.1 mM DPPH in methanol, after which the mixtures were incubated at room temperature for 90 min. Absorbance was then measured at 517 nm.

2.2.3. Determination of the Ferric Reducing Antioxidant Power (FRAP) of CWW

The FRAP assay was performed according to procedure stated by Benzie and Strain [31]. Briefly, 10 µL of known concentrations of CWW were mixed with 200 µL of FRAP reagent and incubated at 37°C for 10 min. Change in absorbance was measured at 593 nm.

2.3. *In vitro* Study

2.3.1. Cell Line

TM3 cells (ATCC, Manassas, VA, USA), a type of murine Leydig cell line, were used for the study. The cells were maintained in Dulbecco's Modified Eagle Medium/Ham's F-12 supplemented with 2.5% fetal bovine serum and 5% horse serum (GIBCO®; Life Technologies Co., Carlsbad, CA, USA) at 37°C. For binding and competition assays, the cells (5×10^5 cells/mL of medium/well) were seeded in a 48-well plate, unless otherwise specified.

2.3.2. Cell Viability Assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were treated with different concentrations of CWW (100, 200, 400, and 600 µg/mL) for 24 h in 96-well culture plates. Briefly, the treatment media were replaced with 100 µL of MTT solution (1 mg/mL) and incubated at 37°C for 4 h. The MTT was carefully removed, to which 150 µL of dimethyl sulfoxide was added to dissolve the purple formazan crystals. The absorbance (optical density) of each well was measured at 492 nm using the microplate reader. Cell viability was calculated according to equation (1).

$$\text{Cell viability}\ (\%) = \left[\begin{array}{l}(\text{test sample count} - \text{blank count}) \\ \div(\text{untreated control count} - \text{blank count})\end{array}\right] \times 100. \quad (1)$$

2.3.3. Testosterone Production in the TM3 Cells

After 24-h incubation periods, the culture media were collected and centrifuged at 1000 g for 5 min. Testosterone levels were analyzed using enzyme-linked immunosorbent assay (ELISA) kits (Enzo, Farmingdale, NY, USA) according to the manufacturer's instructions.

2.4. *In vivo* Study

2.4.1. Animals

Three-month-old male (220-250 g) and female (200-220 g) Sprague-Dawley (SD) rats were obtained from Samtako (Osan-si, Gyeonggi-do, Korea). The animals were housed under standard environmental conditions (temperature, 24 ± 2°C; humidity, 50-55%) in a 12 h/12 h dark/light cycle and with free access to standard rat pellets and water.

2.4.2. Animal Groups and Administration of CWW

The study was approved by the Animal Ethics Committee of Jeollanamdo Institute of Natural Resources Research (JINR1505; Jeonnam, Republic of Korea) and was conducted in compliance with international standards on the care and use of experimental animals. The female rats used in the study were prepared using the method reported by Agmo [32].

Forty male SD rats were randomly divided into 5 groups (n = 8 in each group). Group 1 (Normal) was administered 10 mL/kg body weight of DW (the vehicle) while group 2 received 2 mg/kg body weight of tadalafil. Groups 3 (CWW50), 4 (CWW100), and 5 (CWW200) were administered 1 mL of extract containing 50, 100, and 200 mg/kg body weight of CWW, respectively. Each treatment was administered once daily by oral gavage for 15 days.

2.4.3. Study on the Mating Behavior of the Male Rats

The male rats were tested for their sexual behaviors according to a standard procedure. The effects of the 15-day treatment with the various doses of CWW on the sexual behaviors of the male rats were evaluated. Hormone replacement was used to induce estrus behavior in the female rats. The treatment consisted of subcutaneous injections of estradiol benzoate (10 μg/rat) in 0.1 mL arachis oil (administered 48 h before sex testing) and progesterone (500 μg/rat) in 0.1 mL arachis oil (administered 4 h before sex testing). Each male rat was acclimated in a metabolic cage (48.5 × 33.5 × 22.5 cm) for about 5 min, after which a sexually receptive female rat was dropped silently from one side of the cage as a stimulus. On day 15 of the study, the male rats were videotaped or evaluated for their sexual behaviors during a 30-min period. This evaluation was done at 3 h after drug administration. The following male sexual behavior parameters were then recorded: mount frequency (MF) and intromission frequency (IF) (the number of mounts and intromissions, respectively, from the time of introduction of the female rat into the cage), mount latency (ML) and intromission latency (IL) (the time interval between the introduction of the female rat and the first mount or the intromission by the male, respectively), ejaculation latency (EL) (the time from the first intromission to ejaculation, as observed during the 30-min period), and post-ejaculatory interval (PEI) (the time interval between ejaculation and the first intromission of a new series).

2.4.4. Determination of Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH), and Testosterone Levels in Serum

On day 15 of the study, blood samples were collected to measure serum FSH, LH, and testosterone levels using the following procedure. The animals were placed in a plastic restrainer and Cetacaine®, a topical anesthetic, was applied to their tails. Approximately 1 mm of the tip of each rat's tail was then cut using a sterile blade and 0.3 mL of blood was collected into a microcentrifuge tube within 6 min. Following blood collection, the tips of the rats' tails were pinched to induce blood clotting. For male rats in the experimental group, blood was collected within 15 min at the end of the sex testing. The blood samples were centrifuged at 12,000 rpm for 10 min using a Sartorius centrifuge (Model 1-15PK; Göttingen, Germany). The serum concentrations of testosterone, LH, and FSH were then measured by following an immunoenzymatic method. The assays were conducted using a microplate reader according to the kit manufacturer's instructions (Enzo).

2.5. Statistical Analysis

The results have been expressed as mean ± standard error of the mean (S.E.M). Data from the groups were compared by analysis of variance (ANOVA), followed by Dunnet's *post hoc* test. All statistical analyses were performed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, California, USA). *P* value < 0.05 was considered as statistically significant.

3. Results

3.1. Phytochemical Analysis

The TPC (mg GAE/100 g CWW), %DPPH inhibition (IC_{50}, mg/mL), and FRAP value($EC_{0.5}$, mg/mL) were obtained as 514 ± 15, 0.16 ± 0.04, and 0.6 ± 0.2, respectively. The extraction yield of CWW was found to be 18.5 ± 3.1% (w/w) (Table 1).

Table 1. Total phenolic content and antioxidant activity.

Sample	Extraction yield (%)	Total phenolic content (mg GAE/100g)	DPPH (IC_{50}mg/mL)	Reducing power ($EC_{0.5}$mg/mL)
CWW	18.5±3.1	514±15	0.16±0.04	0.6±0.2

Each value represents mean ± S.D., n=3.

3.2. Effect of CWW on Testosterone Synthesis in Leydig TM3 Cells

It was observed that the CWW treatment did not decrease cell viability (Figure 1). As shown in Figure 2, after the TM3 cells were treated with CWW for 24 h, testosterone synthesis increased significantly.

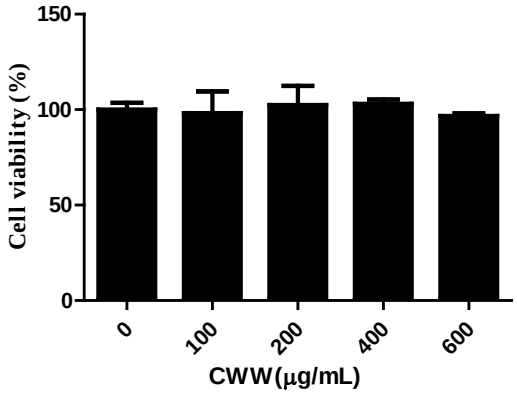

Figure 1. Cell viability on TM3 Leydig cells treated with varying concentrations of CWW for 24h. Values represent mean ± S.D. CWW ; aqueous extract of *C. wilfordii*

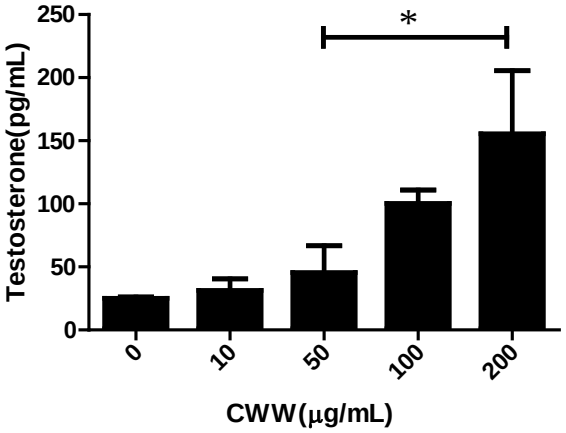

Figure 2. Testrosterone production in TM3 Leydig cells treated with varying concentrations of CWW for 24h. Values represent mean ± S.D. * p<0.05. Considered significant as compared to normal. CWW ; aqueous extract of *C. wilfordii*

3.3. Effect of CWW on Sexual Behavior

The results of the sexual behavior study have been presented in Figure 3. The results confirmed the potential of CWW as a sexual stimulant. A decrease in ML (p < 0.05) was observed in the rats in the CWW200 group. In the CWW200 group, MF significantly (p < 0.05) increased, whereas IL, EL, and PEI significantly (p < 0.05) decreased. Furthermore, IF significantly increased in the CWW100 (p < 0.05) and CWW200 (p < 0.01) groups. Throughout the experiment, precoital sexual behavior was notable among the rats that received the highest dose of the extract (CWW200); however, tadalafil was more effective than the extract was. The results of the study showed that, treatment with CWW at a dose of 200 mg/kg body weight/day decreases ML, IL, EL, and PEI but significantly increases MF and IF.

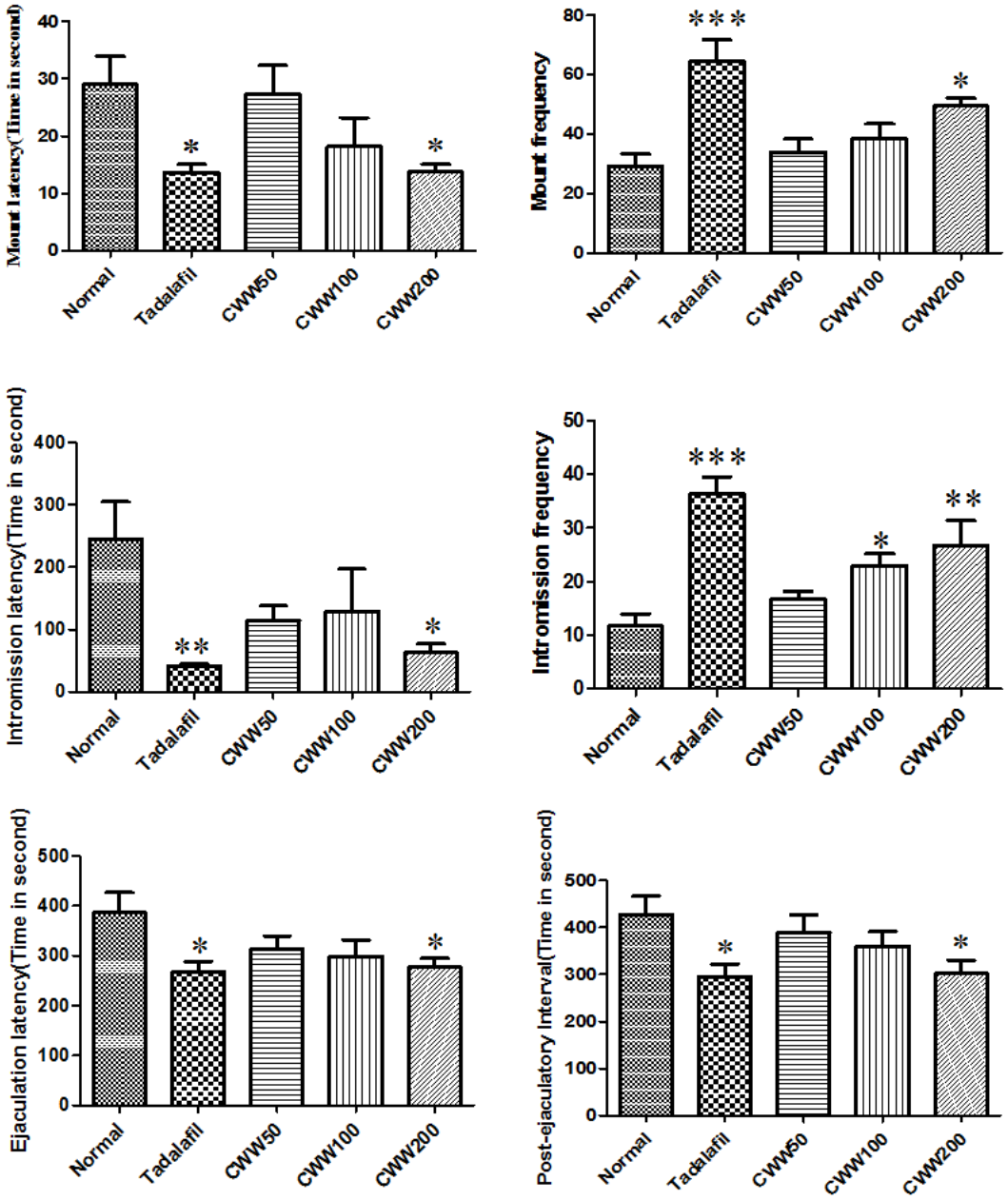

Figure 3. Effects of CWW on sexual behavior in male rats during treatment. All values are expressed as mean ± S.E.M. Data are analysed by one-way ANOVA followed by Dunnett's test. Normal (vehicle); CWW50 (50mg/kg b.w.) p.o.; CWW100 (100mg/kg b.w.) p.o.; CWW200 (200mg/kg b.w.) p.o., Tadalafil (2mg/kg b.w.) p.o. * p<0.05., ** p<0.01., *** p<0.001. Considered significant as compared to normal. CWW ; aqueous extract of *C. wilfordii*

3.4. Effect of CWW on Serum LH, FSH, and Testosterone Levels

All the animals treated with CWW showed elevated serum levels of LH, FSH, and testosterone. Compared to the Normal group, the CWW200 group showed significantly higher LH, FSH, and testosterone concentrations in the serum after the 15-day treatment (Table 2). The effects of the extract on LH, FSH, and testosterone levels in the serum were not dose-dependent. Rats that received the tadalafil treatment did not show increases in the serum levels of the hormones.

Table 2. Effect of CWW on FSH, LH and testosterone in male rats.

Group	LH(mlU/mL)	FSH(mlU/mL)	Testosterone(ng/mL)
Normal	4.5±0.2	20.9±0.5	0.5±0.2
Tadalafil	5.1±0.3	22.0±0.5	0.8±0.2
CWW50	4.8±0.4	22.1±0.2	0.6±0.1
CWW100	4.4±0.3	22.5±1.0	0.7±0.1
CWW200	5.7±0.4*	23.0±0.3*	1.0±0.1*

All values are expressed as mean±S.E.M. Data are analysed by one-way ANOVA followed by Dunnett's test. Normal (vehicle); CWW50 (50mg/kg b.w.) p.o.; CWW100 (100mg/kg b.w.) p.o.; CWW200 (200mg/kg b.w.) p.o., Tadalafil (2mg/kg b.w.) p.o. * $p<0.05$. Considered significant as compared to normal. CWW ; aqueous extract of *C. wilfordii*.

4. Discussion

The results we obtained from the phytochemical analysis showed that the aqueous extract of CWW possesses an antioxidant capacity. Several studies have demonstrated that bioactive agents such as flavonoids, alkaloids, saponins, and phenolic acids are implicated in improving sexual behavior in male rats [33,34,35,36]. Flavonoids alter androgen levels, which play an important role in sexual stimulation. They also enhance the activities of antioxidants, thereby imparting an indirect potentiating effect on sexual behavior parameters [37]. Similarly, alkaloids enhance vascular relaxation by promoting nitric oxide production and by protecting the synthesized nitric oxide against ROS [36]. Saponins exhibit a sex-stimulating activity by enhancing androgen levels via binding to hormone receptors to provoke conformational changes or via binding to enzymes that are affected by the synthesis of such hormones [35]. In addition, phenols stimulate the secretion of FSH and testosterone [38]. *C. wilfordii* contains steroidal alkaloids (such as gagaminine) [39], pregnane glycosides, cynanchone, acetophenones (such as cynandione A) [40], and anthraquinones (such as emodin, chrysophanol, rhein, and physcion) [41]. Gagaminine has been confirmed to have inhibitory effects on aldehyde oxidase activity and lipid peroxidation [42]. Furthermore, pregnane glycosides, such as wilfoside K1N, have been found to inhibit angiogenesis and tumor cell invasion [43,44]. Cynandione A has also been reported to have a neuroprotective effect against oxidative stress-induced toxicity [45]. The TM3 Leydig cell line used in this study was derived from mouse testes. The Leydig cells are located in the interstitial space and account for about 95% of androgen synthesis and secretion [46]. The present study examined the efficacy and cytotoxicity of CWW. TM3 cells were continuously treated with various concentrations of CWW aqueous extract. An increase in testosterone synthesis was observed in the TM3 cell line after the CWW treatment. Testosterone synthesis in the TM3 cells is similar to that in the Leydig cells in the testes; therefore, treatment with CWW may increase testosterone synthesis *in vivo*. In addition, we researched the aphrodisiac effect of CWW on male rats, using tadalafil as the positive control. Results from the sexual behavior test showed that CWW significantly increased MF and IF; however, its effect was less than that of tadalafil. CWW also caused significant reductions in ML and IL; however, tadalafil caused higher reductions in the two parameters. In *in vivo* studies, ML and IL are used as indicators of copulatory motivation since ML and IL are inversely proportional to copulatory motivation. On the other hand, MF and IF are indices of libido and potency, while IF and EF are considered as behavioral indications of copulatory performance and facilitation [47]. Therefore, the increases in MF and IF and the decreases in ML and IL that were observed after administering CWW to the rats demonstrate that CWW enhances copulatory motivation, libido, and potency. On the other hand, the increase in IF and the reductions in IL and EL indicate that CWW improves and sustains penile erection, which is necessary for intromission and results in an enhancement in mating performance. PEI is a parameter that indicates the rate of recovery from exhaustion after the first series of mating. It is therefore a sign of potency and libido [48]. Thus, the significant decrease in PEI that was observed in the animals treated with CWW shows that CWW causes a faster recovery from and a lesser exhaustion after ejaculation, which are signs of an increased sex drive (libido and potency). Previous reports have shown that sexual behavior and penile erection are androgen-dependent (acting both centrally and peripherally) and that treating orchiectomized rats with testosterone restores both sexual behavior and penile erectile capacity [49,50]. In addition, androgens control corporal nitric oxide synthase activity [51,52], which induces penile erection. Androgens have stimulatory influences on several aspects of the male sexual behavior, including penile erection. Copulatory behavior is reliant on the normal functioning of the HPG axis. In most studies in mammalian species, castration has been discovered to decrease considerably the erectile responses to a variety of stimuli, whereas androgen replacement therapy reduces these effects [53]. Testosterone also enhances male sexual behavior by improving dopamine release in the medial preoptic area and by potentiating nitrergic neurotransmission [54,55,56,57]. Therefore, an increase in testosterone concentration should improve androgen-dependent indices such as sexual behavior and maintenance of spermatogenesis. FSH stimulates spermatogenesis in the Sertoli cells, whereas LH stimulates the synthesis and secretion of testosterone by the Leydig cells [58]. According to our results, elevated levels of testosterone were observed in the sera of the CWW-treated animals. The serum levels of FSH and LH in the rats were also increased after treatment with CWW. Testosterone is produced by the Leydig cells of the testes in response to LH, under the control of the hypothalamic–pituitary–testicular axis. It therefore seems that the increases in the serum levels LH and testosterone after the CWW

treatment may be responsible for the observed enhancement in the sexual behavior of the male rats. In this study, we observed significant increases in the serum levels of testosterone, LH, and FSH in the CWW-treated animals.

In conclusion, this study has demonstrated that CWW enhances sexual motivation, libido, and potency in male SD rats by stimulating the secretion of testosterone, LH, and FSH. The mechanism underlying the secretion of the hormones appears to be via the stimulation of gonadotropin release, probably as a result of activation of the hypothalamus.

Disclosure

The authors declare no conflict of interest.

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