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***In vivo* efficacy of new polyherbal combination in sexual behavior male rat model**

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ABSTRACT

To investigate the aphrodisiac potential of polyherbal formulations prepared from different parts of *Angelica sinensis*, *Astragalus membranaceus*, *Cistanche tubulosa*, *Cynomorium songaricum*, *Epimedium sagittatum*, *Salvia miltiorrhiza*, *Paeoniae alba*, and *Peliosanthes micrantha* in inexperienced male Sprague-Dawley rats. The different concentrations of prepared polyherbal formulations i.e. 430 and 860 mg/kg and testosterone injection positive control were administered orally to rats (n = 11 animals per group) for 7 days. The following sexual performances were evaluated: mount frequency (MF), mount latency (ML), intromission frequency (IF), intromission latency (IL), ejaculation frequency (EF), post-ejaculatory interval (PEI), ejaculation latency (EL); and two indexes such as copulatory efficiency (CE), intromission ratio (IR) for single dose and repeated doses of 7 days. Mating behavior parameters in male rats were monitored on days 1 and 7 of treatment pairing with receptive females. After termination of drug treatment, the body weights, weights of reproductive organs, and levels of testosterone were also evaluated. The present results over 7 days of the repeated dose experiment showed increases in body weights, up to 6.13% at a high dose of 860 mg/kgP, significantly

increased weight gains in reproductive organs, and significantly increased levels of serum testosterone in treated rats. The tested prescription showed different effects of sexual behavior parameters between acute single dose and subacute repeated doses during 7 days of administration. In the acute study, the prescription improved the immediate performance of sexually inexperienced male rats. In the subacute research, the administration at high dose rats improved the mount frequency (MF) and ejaculation frequency (EF). On the contrary, the increase of mount latency (ML) and post-ejaculatory interval (PEI) might indicate long use of the formulation can decrease its ED treatment efficiency. In conclusion, the polyherbal formulation showed a significant increase in serum testosterone levels, weights of reproductive organs as well as mating behaviors as compared to controls, suggesting good aphrodisiac activity on male Sprague-Dawley rats in the above experimental model. However, the repeated use of polyherbal formulation is less effective in ED treatment.

Keywords: Polyherbal combination, *Peliosanthes micrantha*, rhizome, testosterone, sexual behaviors, male Sprague-Dawley rats

1. INTRODUCTION

Sexuality is related to reproduction involving conjugation, conception, and procreation. Sexual dysfunction concerns the inability to have normal sexual activity, including the loss or partial erection, inability to keep an erection, premature ejaculation, reduced libido, orgasm, and arousal disorder. Erectile dysfunction (ED) is a common disorder affecting the life quality of male patients and their partners. ED is currently considered a social-psychological-physiological disease with complex etiology and various treatment methods (Sangiorgi et al. 2021) [1]. A worldwide rising trend of ED-men affected by ED is projected to be a staggering 322 million men globally (Ayta et al. 1999) [2]. The occurrence of ED is associated with many comorbidities and risk factors, such as aging, obesity, decreased androgen levels, and cardiovascular diseases (Tirado et al. 2016; Zeleke et al. 2023; Fujita et al. 2024) [3-5].

Androgens are important hormones for the development and maintenance of the male phenotype. The most abundant circulating androgen is testosterone ($\pm 90\%$), produced in the testis's Leydig cells and converted by the enzyme 5α -reductase to produce a more potent androgen, 5α -dihydrotestosterone. The low testosterone levels generally characterized the male ED. Testosterone disturbances impair sexual performance, male secondary sexual characteristics development, spermatogenesis, and subsequent male infertility. The above symptoms can be cured by restoring serum testosterone to the normal range (Van den Broeck et al. 2020) [6]. Currently, twelve treatments are available and classified as internal or external. The most popular internal treatments relying on oral pharmacotherapy are PDE5 inhibitors, hormonal replacement therapy, testosterone, and soluble guanylate cyclase activators. In comparison, the external treatments commonly used are intracavernosal and intraurethral penile injections, vacuum devices, and endovascular tools (Mobley et al. 2017; Pyrgidis et al. 2021) [7, 8]. Current drugs available including avanafil, sildenafil, tadalafil, and vardenafil, which are used to treat ED, can cause side effects (Aversa et al. 2019; Gul et al. 2019) [9, 10].

It has been shown that traditional medicines have undoubtedly continued to gain attention as an alternative for sexual life's improvement or enhancement. This is due to their affordability, accessibility, and fewer side effects (Wada et al. 2023) [11]. In the world and Vietnam, several medicinal plant species and polyherbal formulations have been reported as

stimulants of sexual activities. *Angelica sinensis* roots are popularly used in Traditional Chinese Medicine (TCM). Its solution was injected to avoid the decrease of NOS activity in penile tissues of rats caused by cavernous nerve injury in rats (Hu et al. 2001) [12]. *A. sinensis* is one component used in two polyherbal formulations, Huoxue Tongluo Decoction (Wang et al. 2021) [13] and Hongjing I granules (Ma et al. 2020) [14], which are effective in ED treatment in the male Wistar rats models. Hydroalcoholic extracts of *Cistanche tubulosa* at a dose of 800 mg/kgP administrated for 20 days in the male rats model revealed there were the increases in serum level of androgenic progesterone and testosterone; sperm count (2.7-folds) and sperm motility (1.4-folds) and the decrease in abnormal sperm (0.6-folds) in rats (Wang et al. 2016) [15]. The radix extract from *Cynomorium songaricum* after administration to 8-week-old male Wistar rats for 56 consecutive days (at a dose of 1000 mg/kgP showed significant increases in epididymal sperm count and absolute testes weights (Yang et al. 2010) [16].

Hydroethanolic extract of *Epimedium grandiflorum* leaves orally administrated at the doses up to 200 mg/kgP for 42 days after CCl₄ intoxication on male albino rats restored liver enzymes, renal profiles, and stress markers and increased androgenic reproductive hormones like testosterone, luteinizing hormone, follicle stimulating hormone and prolactin while decreased progesterone and estradiol toward normal. It also improved the structural architecture of testicular tissue particularly in high dose group of 200 mg/kgP (Munir et al. 2020) [17]. The *E. grandiflorum* herb was also one component of the ED effective Hongjing I granules (Ma et al. 2020) [14]. The root extract of *Astragalus membranaceus*, in a vitro experiment at 10 mg/ml, increased the motility of sperm in semen and washed sperm (Hong et al. 1992) [18] while enhancing sperm values such as sperm count and motility in an in vivo five-week-old male ICR mice model (Kim et al. 2015) [19].

The aqueous extract solution of radix *Salvia miltiorrhiza* was intraperitoneally injected at a dose of 1 mL/kgP for 6 weeks to the diabetic rats. The *S. miltiorrhiza* injection improved erectile function via increased erection times and ameliorated the morphological abnormalities of the corpora cavernosum, and suppressed caspase-3 activation in penile tissue (Zhang et al. 2018) [20]. A study showed that the radix extract of *P. alba* may treat diabetes mellitus-induced ED via the HIF-1 α /HMOX1 pathway (Jie et al., 2020) [21]. The *Paeoniae alba* radix is also composed in the ED effective Hongjing I granules (Ma et al. 2020) [14]. The *P. micrantha* was found in Vietnam and traditionally used for male impotence (Averyanov et al. 2013) [22]. A few research of chemistry and biological activities have recently been published for the *P. micrantha* species. In the alcoholic extract of its rhizomes, UHPLC-Q-TOF-MS identified four compounds, namely pumilaside A, pumilaside C, β -sitosterol, and glycoside J-3 (Do et al. 2024) [23]. The extract was standardized and showed mild DPPH antioxidant activity (Le et al. 2024) [24]. In the unpublished data, the extract showed different sexual behavior effects between acute single dose and subacute repeated doses during 7 days in male Sprague-Dawley rat model and indicated good effects in mount latency and intromission frequency.

In the practical traditional medicine, most of the medicinal plants are used in the formulation forms combining of two or more plants. A US herbal formulation, VigRX, combined of *Panax ginseng* root, *Serenoa repens* powder, *Ginkgo biloba* leaf, *Crataegus laevigata*, *Ptychopetalum olacoides* bark extract 4:1, *Erythroxylum catuaba* bark extract 4:1 powder, *Cuscuta chinensis* seed extract 4:1, and *Epimedium sagittatum* extract 20:1 was patented and tested upon oral administration of 30 mg/kgP in 14-day rats model study. The study results demonstrated a marked enhancement in sexual behavior, including decreased intromission and ejaculation latencies, increased intromission, ejaculation, and mounting

frequencies, and increased blood testosterone levels (Smitasirib et al. 2010) [25]. One polyherbal formulation prepared from different parts of *Tribulus terrestris*, *Curculigo orchoides*, *Allium tuberosum*, *Cucurbita pepo*, *Elephant creeper*, *Mucuna pruriens*, and *Terminalia catappa* was made and orally tested at the doses up to 600 mg/kgP and sildenafil citrate as standard in Albino rats model for 3 weeks, and showed a significant increase in mating behavior as well as mating performance, serum hormonal levels, sperm count, and testes-body weight ratio (Sahoo et al. 2014) [26].

Another polyherbal formulation, Poweromin X Ten, is composed of extracts from *Tribulus terrestris* fruit, *Withania somnifera* root, *Asparagus racemosus* root, *Punica granatum* seed, *Myristica fragrans* mace, *Chlorophytum arundinaceum* root, *Curculigo orchoides* rhizome, *Celastrus paniculatus* seed, *Orchis mascula* root, and *Shilajit exudate* and was proved to enhance male sexual function (Bojja et al. 2024) [27]. Huoxue Tongluo polyherbal prescription contains 12 medicinal plants including *Achyranthis bidentatae Radix*, *Angelica sinensis Radix*, *Bupleuri Radix*, *Citri reticulatae*, *Curcuma longa Radix*, *Epimrdii Herba*, *Morindae officinalis Radix*, *Paeoniae Radix Rubra*, *Pericarpium viride*, *Tribuli Fructus*, *Vaccariae Semen*, and *Heamadipsa*, *Scolopendra*. Its decoction improved erectile dysfunction caused by ischemic stroke and the structure of the corpus cavernosa. It also inhibited the expression of TNF and VEGF proteins in penile tissue and enhanced the expression of eNOS protein in penile tissue (Wang et al. 2021) [13].

The Hongjing I granule herbal formulation is composed of *Achyranthis bidentatae Radix* (1,5 g), *Angelica sinensis* (4,8 g), *Radix Astragali* (6 g), *Cistanche deserticola* (1 g), *Codonopsis pilosula* (4,5 g), *Cyathula officinalis* (3,6 g), *Epimedium brevicornu* (0,5 g), *Fructus Lycii* (6 g), *Morindae officinalis Radix* (1,5 g), *Radix Paeoniae alba* (1,5 g), *Rhodiola rosea* (1,5 g), *Salvia miltiorrhiza* (3 g), and *Tribuli fructus* (2 g). Its in vivo administration in the male Wistar rats ameliorated the shape of the penis and induced protein synthesis, showing its effectiveness in ED treatment (Ma et al. 2020) [14]. One polyherbal tea leave blend from *Hibiscus sabdariffa*, *Annona muricata*, and *Moringa oleifera* (ratio of 2:2:6) was shown able to repress the activity of PDE-5, thus, to prevent the conversion of cGMP to 5'GMP, and to adversely affect the penile erection/rigidity process in streptozotocin (STZ)-induced diabetic male rats. Its treatment reduced arginase activity while arginase, on the other hand, competes with NO synthase in endothelial cells, thus, reducing NO availability. The presence of NO is critical to the erectile process, and it is created in the erectile tissue. This formula can be used to ameliorate both diabetes mellitus and also diabetes mellitus erectile dysfunction (Idowu et al. 2023) [28].

In Vietnam, the ED treatments using polyherbal formulations have been published. One decoction, TXCB was for formulated from a Vietnamese traditional medicine containing eight medicinal herbs: *Balanophora laxiflora* (10 g), *Epimedium sagittatum* (10 g), *Curculigo orchoides* (8 g), *Morindae officinalis* (8 g), *Cornus cervipar* (5 g), *Millettiae speciosae* (10 g), *Dendrobium officinale* (10 g), and *Lycium barbarum* (10 g) was investigated in rabbits model and showed the enhancement on the quantity and quality of sperm whose reproductive ability was impaired by fluconazole in vivo (Vu et al. 2020) [29].

Clinically, several formulations have been examined in the world

The formula for Shao-Fu-Zhu-Yu-Tang consisted of the following components and proportions: *Angelica sinensis* (3 g), *Cinnamomum cassia* (1 g), *Commiphora* (1 g), *Corydalis Yanhusuo* (1 g), *Foenicunum Vulgare* (0.1g), *Ligusticum chuanxiong* (2g), *Paeonia veitchii* (2 g), *Trogopteris canthipes* (2 g), *Typha angustata* (3 g), and *Zingiber officinale* (0.2 g).

Its clinical trial of 36 patients with chronic prostatitis before and after treatment for 60 days showed an increase in sperm quantity and quality, and the motility and quality of human semen due to an increase in sperm acrosin activity. This formulation can also work as a sperm antioxidant and antiaging agent (Yang et al. 2003) [30]. A clinical study of one proprietary blend consisting of herbal extracts such as *Crataegus laevigata*, *Cuscuta chinensis*, *Epimedium sagittatum*, *Erythroxylum catuaba*, *Ginkgo biloba*, Korean *Panax ginseng*, *Ptychopetalum olacoides*, *Serenoa repens*, and *Turnera diffusa* showed excellent activity (Shah et al. 2012) [31].

A new polyherbal formulation was prepared by combining the sap of *Acacia senegal*, root of *Astragalus membranaceus*, sap of *Bombax ceiba*, root of *Chlorophytum borivilianum*, root of *Eurycoma longifolia*, seeds of *Hygrophila spinosa*, seeds of *Mimosa pudica*, seeds of *Mucuna pruriens*, seed coat of *Plantago ovate*, and rocky candy in equal ratio. Its clinical trial in thirty-six oligospermia male patients for 90 days showed the increases in sperm concentration, in semen volume, and sperm motility, and the improvement and regulation in serum hormone levels. The new formulation demonstrated the evidence on synergistic spermatogenic effect for the treatment of oligospermia leading to infertility (Hussain et al. 2018) [32]. A Korean herbal medicine, MYOMI-14, is composed of Cinnamomi Cortex, Corni Fructus, Cuscutae Semen, Cynomorii Herba, Dioscoreae Rhizoma, Epimedii Herba, Ginseng Radix, Lycii Fructus, Morindae Radix, Poria Sclerotium, Plantaginis Semen, Rehmanniae Radix Preparata, Rubi Fructus, and Schisandrae Fructus.

The formulation was used to treat 17 infertile patients with low semen quality for 10 weeks. The clinical results showed that MYOMI-14 improved the semen quality of infertile men without adverse effects (Jo et al. 2019) [33]. Another clinical study of one traditional Thai polyherbal formulation, Sao Thong Tai, containing of equal water decoction combination from *Boesenbergia rotunda*, *Sida acuta*, *Dactyloctenium aegyptium*, and *Oryza sativa* showed its effectivity for treating mild to moderate ED in elderly men without causing serious side effects with daily dose of 1,600 mg for 8 weeks (Lin et al. 2022) [34]. The Hongjing I granule herbal formulation was studied in some multicentre, randomized, double-blind, placebo-controlled clinical trial protocols. One study over the 122 men enrolled during eight weeks post-randomization showed significantly improved symptoms in patients with mild to moderate ED with no adverse events (Xu et al, 2023; Xu et al, 2023) [35, 36].

Although there have been many studies on the separate use of *Angelica sinensis*, *Astragalus membranaceus*, *Cistanche tubulosa*, *Cynomorium songaricum*, *Epimedium sagittatum*, *Salvia miltiorrhiza*, *Paeoniae alba*, and *Peliosanthes micrantha* in the field of male infertility and erectile disorders as mentioned above, no studies have been found on the combined use of these herbs. In the previous studies, a polyherbal formulation, AM, was prepared and standardized. Each AM capsule composed of the herbal extract powders (512 mg) from Radix *Angelica sinensis* (ASE: 68 mg), Radix *Astragalus membranaceus* (AME: 68 mg), *Cistanche tubulosa* stem (CTE: 50 mg), Radix *Cynomorium songaricum* (CSE: 50mg), Herba *Epimedium sagittatum* (EBE: 40 mg), Radix *Salvia miltiorrhiza* (SME: 56 mg), Radix *Paeoniae alba* (PAE: 68 mg), and *songaricum* (CSE: 50 mg), Herba *Epimedium sagittatum* (EBE: 40 mg), Radix *Salvia miltiorrhiza* (SME: 56 mg), Radix *Peliosanthes micrantha* (PME: 100 mg), and suitable weights of other pharmaceutical ingredients (Le et al. 2024; Nguyen et al. 2024) [24, 37]. The formulation showed non-acute toxicity at dose of 17.5 g/kgP and non-semi-chronic toxicity was at dose of 400 mg/kgP during consecutive 28 days (Le et al. 2024) [38]. The aim of our study was to determine the sexual behavior effect of immediate and 7 day oral

administration of the polyherbal formulation, AM in experienced male Sprague-Dawley rat model.

2. MATERIALS AND METHODS

Chemicals and reagents

Testosterone, estradiol benzoate, thiopental, diethyl ether, and Natri Carboxymethyl Cellulose were procured from Sigma-Aldrich Chemical (USA). Elikine Testosterone Elisa kit was purchased from Abbkine Co. (USA). Progesterone was from Glentham Life Sciences (Germany). Analytical grade ethanol and other chemicals were procured from Merck (USA).

Plant materials, extract and AM capsules preparation

The radix of *C. songaricum* and *S. miltiorrhiza*, aerial parts of *E. sagittatum*, and stem of *C. tubulosa* were purchased from Lao Cai (Vietnam) in 2021. The radix of *A. sinensis*, *A. membranaceus*, and *P. alba* were purchased in Ninh Hiep Commune Medicinal Plants Market of Hanoi (Vietnam) in 2021. All the samples were authenticated by Dr. Hai T.V. (VAST). *P. micrantha* rhizomes were collected in Dak Nong Province, Vietnam in October 2021 and identified by Dr. Nguyen Sinh Khang, Institute of Ecology and Biological Resources, VAST. Their voucher specimens (LC21CS, LC21SM, LC21ES, LC21CT, NH21AS, NH21AM, NH21PA, and DN21PM) were deposited in the CHTD (VAST). The plant materials were dried and powdered. The samples were stored in a dry environment before use.

The *A. sinensis* roots were ground into powder, extracted in boiling water with the solid-water ratio of 1 : 19 for 2 h, filtered, re-extracted twice, and then lyophilized with a high yield of 50.78%. The powdered extract was dissolved, filtered through a 0.22 µm syringe filter, and then lyophilized to final ASE powder (Hu et al. 2001) [12]. The hydroalcoholic extracts of *C. tubulosa* were prepared as follows: Dried *C. tubulosa* stems were sliced, and ground into powder. The extraction conditions were as follows: boiling in 50% ethanol with the solid-liquid ratio of 1 : 19 for 2 h, filtered, re-extracted twice, and then lyophilized to final CTE powder (Wang et al. 2016) [15]. The radix extract from *C. songaricum* was prepared by maceration. Their fragmented roots were mixed with 1:20 w/v in purified water for three weeks. The mixture was filtered by woven texture to collect the liquid. The fluid was evaporated by warming up the vessel containing the fluid in hot water. The resulting concentrated liquid was dried in the oven and kept at 70 °C. The solidified product was milled to fine CSE powder yielding a light brownish-colored extract of 26.0% (Yang et al. 2010) [16].

Dry *E. grandiflorum* leaves were cut, and sonicated in an ultrasonic extractor at 20 kHz and 1 kW with the aqueous ethanol (30:70 v/v) thrice with the solid-liquid ratio of 1 : 10 for 4 hours at room temperature. Then the solution was filtered by Whatman filter paper number 1 and evaporated using a rotary evaporator under reduced pressure yielding powdered EGE extract (Munir et al. 2020) [17]. The roots of *A. membranaceus* were boiled in water with the solid-water ratio of 1 : 2 for 2 h at 100 °C, and then the suspension was filtered and concentrated under reduced pressure. The filtrate was lyophilized and yielded 18.92% of AME powder (Hong et al. 1992) [18]. The radix of *S. miltiorrhiza* was added to water with the solid-water ratio of 1 : 10 and decocted twice. The filtrate was combined and concentrated at 70 °C yielding powdered SME extract (Zhang et al. 2018) [20]. The dry *P. alba* radix was ground to powder, immersed in water with a solid-water ratio of 1 : 8, and extracted in a reflux water bath 2 times,

2 h each time. The proper amount of 95% ethanol was added to the extracts to adjust the ethanol concentration to 70%. The solution was kept at 4 °C for 12 h and then filtered. The cake was dried and went through 80 mesh sieve to yield PAE powder (Jie et al., 2020) [21]. The *P. micrantha* rhizomes (8.90 kg) were extracted with 96% ethanol (17.5L × 3 times) at room temperature in an ultrasonic extractor at 20 kHz and 1 kW as described previously (Do et al. 2024) [23]. The combined extract was concentrated under reduced pressure to dryness, yielding a PME residue (123,30g). The *S. miltiorrhizae* Radix was also extracted with 96% ethanol (17.5L × 3 times) at room temperature in an ultrasonic extractor at 20 kHz and 1 kW as for *P. micrantha* rhizomes and yielded the SME fine powder.

Each AM capsule composed of the herbal extract powders (512mg) from Radix *A. sinensis* (ASE: 68 mg), Radix *A. membranaceus* (AME: 68 mg), *C. tubulosa* stem (CTE: 50 mg), Radix *C. songaricum* (CSE: 50 mg), herb *E. sagittatum* (EBE: 40 mg), Radix *S. miltiorrhiza* (SME: 56 mg), Radix *P. alba* (PAE: 68 mg), and Rhizomes *P. micrantha* (PME: 100mg). Five hundred and twelve milligrams of the formulated combination of dry extract powders and suitable weights of pharmaceutical excipients are enough to fill in each hard capsule (Le et al. 2024) [24].

Experimental animals

Sixty 12-13 weeks-old Sprague-Dawley rats (60 males and 50 females; 200-250g) were obtained from the National Institute of Hygiene and Epidemiology, Vietnam. Housing conditions and experimentation were in the standard laboratory conditions at 25 ±2 °C, relative humidity of 44-56%, and 12:12 hours of light and dark cycles. They were maintained 5 days before the experiment in the VIPTAM Institute of Technology Application (VIPTAM). To identify the rats, a designated number was marked on the tail of each rat using a permanent marker. The design and performance of animal experiments were approved by the VIPTAM's Ethical Committee (reference number No. 220815/Vip/Pre).

Acute single dose study

Female rats were anesthetized by thiopental and xylazine hydrochloride, ovariectomized and kept fed during 14 days. Then, they were brought into oestrous by sequential subcutaneous injection with estradiol benzoate (0.25 mg/kg body weight) and followed with progesterone (2.50 mg/kg body weight) at 52 h and 4 h prior to aphrodisiac studies, respectively (Tang et al. 2017) [39]. They were checked for sexual receptivity with sexually active adult males (different from the ones used in the study). The female rat was brought out and replaced by another one if she did not accept the male, and only those showing copulatory behavior were used in the study. A preliminary screening was carried out to identify the sexually sluggish males. Briefly, the male rats were placed singly with sexually receptive females for 4-5 minutes before the exposures, then trained by exposing them to sexually receptive females once daily in the dark cycle for 3 consecutive times with an interval of 4 days. Male animals that failed to achieve ejaculation during any of the last three exposures were considered to be sexually sluggish and used in the present study (Hull et al. 2007) [40]. Tested doses were chosen following our unpublished data to reflect the current recommended doses of a new herbal combination in the form of capsules which were used for adult human males (3 or 6 capsules per day), using an average body weight of 50 kg. The calculated pre-clinical doses were equivalent to 430 and 860 mg/kgP, respectively.

A total of 44 sexually inexperienced and sluggish male rats were selected, housed separately, and randomly divided into four groups.

Lot 1, normal control (NC) (n = 11): were given Na-CMC 0,5 %, 10 ml/kgP;

Lot 2, positive control (n=11): were subcutaneously injected with testosterone, 0.4 mg/kgP, 2 times per week;

Lot 3 (n = 11): was given with the powder of polyherbal combination at a dose of 430 mg/kgP in Na-CMC 0,5 %;

Lot 4 (n = 11): was given with the powder of polyherbal combination at a dose of 860 mg/kgP in Na-CMC 0,5 %.

The rats were orally gavaged with the powder of polyherbal combination once using a metal oropharyngeal cannula. After an adaptation period of 10 min, a sexually receptive female was presented to the male by dropping into the cage.

Measurement of sexual parameters

Two hours after the dropping of the female, behavioral parameters were recorded for 30 minutes by a trained observer unaware of the treatment given to each group during the reversed dark phase using a dim red light for illumination. An 8MP camera recorded all the activities. The following sexual behavior parameters were analyzed including Mount frequency (MF): Total number of mounts preceding ejaculation at a specified period; Mount latency (ML): Time duration (in seconds) from the introduction of the female into the cage till the first mount by the male with pelvic thrusting; Intromission frequency (IF): Total number of intromission preceding ejaculation; Intromission latency (IL): Time duration (in seconds) taken from the introduction of the female into the cage till the first mount and vaginal penetration or intromission; Ejaculation frequency (EF): Number of ejaculations from the time of introduction of the female rats to the male within a given time frame; Post ejaculatory interval (PEI): Time duration taken from ejaculation until the next intromission; and Ejaculation latency (EL): Time duration (in seconds) from the first intromission till ejaculation. Tests were terminated immediately after the first post-ejaculatory intromission; or if intromission did not occur within 15 minutes of female introduction; or if ejaculation latency exceeded 30 minutes.

Two rating behavior indexes were computed as following:

Copulatory efficiency = (number of intromissions / number of mounts) × 100 (%) or
 $CE = (IF/MF) \times 100 (\%)$;

Intromission ration = number of intromissions / (number of mounts + number of intromissions) × 100 (%) or $IR = IF/(IF+MF) \times 100 (\%)$ (Kenmogne et al. 2016) [41].

Subacute multi-dose study

The same set of rats was used for the chronic study. The drug/vehicle treatment was continued for 7 more days. Ten minutes after the drug/vehicle administration on day 7, sexual behavior of rats was measured as described above under 'Acute single dose study'. At the end of the behavioral study, all rats were weighed, anesthetized with diethyl ether, and sacrificed. Serum samples were separated by heart puncture and stored at -20 °C, which was used for the testosterone level estimation by using an ELISA kit following the manufacturer's guidance. Reproductive organs including testis, seminal vesicles, bulbourethral glands, levator ani,

prostate glands, and glans penis, were collected immediately after dissection by open castration. Then, the samples were completely trimmed off any attached tissues or visible fat at the organs, washed thrice in normal saline and dried with filter paper to blot away any blood and fluid. The weights of dried organs were determined (Yakubu et al. 2011) [42].

Data analysis

Data were expressed as the mean, standard deviation of the mean value ($M \pm SD$), minimum, and maximum. Statistical analysis was performed using SPSS (version 16.0, SPSS Inc., Chicago, IL, USA), two-way analysis of variance (ANOVA), and followed by Dunnett's and Fisher's LSD analysis. Frequency comparison tables of the parameters of sexual behaviors were generated along with the p-value from Fisher's exact test (Chi-square) and assessed for statistical significance by Mann-Whitney U-test. Differences were considered significant at $p > 0.05$.

3. RESULTS

Effects on body weights, reproductive organs' weights and testosterone levels

Attention was given to body weight evaluation before and at the experiment end as presented in Table 1. After 7 days of repeated-dose experiments, the mean weights of the dosed rats were not statistically different in comparison to NC. Results showed that all the increases in body weights were observed for all the groups of tested animals till the end of treatment (day 7). In body weights, the control rats (NC), the positive control rats (P) and two extract-dosed rats (AM-1 and AM-2) showed significant increases (~2.27, 5.50, 5.18, and 6.13%, respectively ($p > 0.05$)).

Table 1. Weighting evaluation of body weights in tested rats.

Lot/dose (mg/kg)	Body weight (g)		Body weight change (%)
	Before administration	7th day	
Lot 1, normal control (NC): Na-CMC 0.5 %	244.44 $\pm$ 13.02	250.00 $\pm$ 11.67	2.27
Lot 2, positive control (P): testosterone, 0.4 mg/kgP	234.28 $\pm$ 14.77	247.14 $\pm$ 20.55	5.49
Lot 3: AM-1, 430 mg/kgP	233.14 $\pm$ 12.61	245.21 $\pm$ 18.62	5.18
Lot 4: AM-2, 860 mg/kgP	229.18 $\pm$ 13.51	243.24 $\pm$ 17.65	6.13

$p > 0.05$ means that the difference is statistically significant.

After 7 days of the repeated-dose experiments, mean weight changes in the reproductive organs of tested rats in different experimental groups were presented in the Table 2. Results

showed that the mean weights of the testicles, seminal vesicle, bulbourethral glands, levator ani, prostate glands, and glans penis from all the rats significantly increased in comparison to NC rats ($p>0.05$).

All rats of the testosterone- and AM-treated animal groups presented higher mean weights of all the reproductive organs than the NC rats, except the mean glans penis weights of the P rats were lower. In comparison to the P rats, all rats of the AM-treated animal groups presented higher mean weights of the testicles, bulbourethral glands, and glans penis while the lower mean weights of seminal vesicle, levator ani, and prostate glands were observed. For AM-treated animals, the mean weights of the testicles, bulbourethral glands, and levator ani were changed similarly, while the values of the seminal vesicle and glans penis were higher, and the values of the prostate glands were lower at high dose.

Table 2. Weighting evaluation of reproductive organs in tested rats.

Lot/dose (mg/kg)	Weight of organs (g/100gP rats)					
	Testicles	Seminal vesicle	Cowper's glands	Levator ani	Prostate glands	Glans penis
Lot 1, NC: Na-CMC 0.5 %	0.656 ±0.064	0.298 ±0.016	0.026 ±0.002	0.298 ±0.009	0.080 ±0.009	0.045 ±0.002
Lot 2, P: testosterone, 0.4 mg/kgP	0.671 ±0.066	0.669 ±0.086	0.035 ±0.003	0.406 ±0.015	0.163 ±0.018	0.026 ±0.005
	p=0.13	p=0.52	p=0.66	p=0.15	p=0.87	p=0.01
Lot 3: AM-1, 430mg/kgP	0.78 ±0.07	0.49 ±0.10	0.04 ±0.00	0.39 ±0.04	0.14 ±0.03	0.07 ±0.01
	p=0.2	p=0.4.0	p=0.00	p=0.04	p=0.01	p=0.00
Lot 4: AM-2, 860 mg/kgP	0.78 ±0.08	0.53 ±0.14	0.04 ±0.01	0.39 ±0.06	0.12 ±0.04	0.10 ±0.10
	p=0.18	p=0.18	p=0.01	p=0.09	p=0.47	p=0.17

$p>0.05$ means that the difference is statistically significant.

Table 3. Serum testosterone levels in tested rats.

Lot/dose (mg/kg): 2 h	Testosteron (ng/dl)	
	Before administration	7 days
Lot 1, NC: Na-CMC 0.5 %	15.12 ±3.54	6.92 ±1.78
Lot 2, P: testosterone, 0.4 mg/kgP	16.16 ±3.31	49.23 ±1.87

Lot 3: AM-1, 430 mg/kgP	6.36 ±0.98	11.90 ±3.39
Lot 4: AM-2, 860 mg/kgP	8.01 ±1.08	15.35 ±9.89

p>0.05 means that the difference is statistically significant.

In the present study, the serum testosterone concentrations of the tested rats were detected by ELISA and presented in Table 3. After 7 days of the repeated-dose experiments, the mean testosterone levels increased significantly with the testosterone- and two AM-treated animal groups while NC rats had lower serum testosterone concentrations.

Acute single dose administration effects on sexual behavior parameters

Acute sexual behaviors of the tested rats were observed after 2 hours of the extract administration. The female rats displayed proceptivity as observed by their ear-wiggling, lordosis, and hopping while the treated male rats showed signs of enhanced sexual activity towards the female rats as witnessed by their eager and quick movement towards the female rats as well as some distinctly visible signs of pre-copulatory actions such as body-sniffing, anogenital exploration, circling, which finally culminated in mounting. In Table 4, the results of acute rating behaviors were presented. It was shown that the mount and intromissions were increased in two AM-treated animal groups compared to testosterone-treated and NC rats.

Table 4. Acute rating behaviors in tested rats.

Lot/dose (mg/kg): 2 h	Mount (%)	Intromission (%)
Lot 1, NC: Na-CMC 0,5 %	34.40	25.60
Lot 2, P: testosterone, 0,4 mg/kgP	70.30	64.80
Lot 3: AM-1, 430 mg/kgP	75.00	72.40
Lot 4: AM-2, 860 mg/kgP	76.80	75.10

p>0.05 means that the difference is statistically significant.

In Table 5, the results obtained from the acute sexual behavior analysis (after 2h) relating to male rat sexual characteristics of both AM-treated rats groups compared with the rats in the untreated NC group indicated that AM significantly decreased mean ML, MF, IL, IF, and EF, and AM significantly increased mean PEI (p>0.05). Meanwhile, mean EL was increased in low doses but decreased in high dose.

However, in comparison to the P rats, both AM-treated rats decreased mean ML, IL, IF, and EL while increasing mean MF, EF, and PEI (p>0.05). When compared between the two AM-treated rats groups, the high dose showed higher mean ML, MF, EF, PEI, and lower mean EL with similar mean IL and IF values. Both the CE and IR indexes were higher in the treated rats in comparison to the NC rats.

Table 5. Acute sexual behaviors in tested rats.

Lot/dose (mg/kg)	ML (s)	MF (times)	IL (s)	IF (times/30 min)	EL (s)	PEI (s)	EF (times/30 min)	CE (%)	IR (%)
Lot 1, NC: Na-CMC 0,5 %	132.11 ±361.02	3.56 ±3.54	35.33 ±46.47	45.67 ±16.39	566.11 ±330.2	390.78 ±56.15	2.00 ±0.71	1282.87	92.77
Lot 2, P: testosterone, 0,4 mg/kgP	74.09 ±194.55	1.73 ±1.68	37.82 ±37.84	58.09 ±17.82	1035.82 ±708.81	330.00 ±51.73	1.09 ±0.94	3357.80	97.11
	p=0.65	p=0.15	p=0.90	p=0.13	p=0.08	p=0.05	p=0.03		
Lot 3: AM-1, 430 mg/kgP	0.00 ±0.00	2.60± 2.37	16.00 ±14.94	40.80± 12.43	660.00 ±390.12	421.50 ±69.10	1.70 ± 0.82	1569.23	94.01
	-	p=0.49	p=0.23	p=0.47	p=0.58	p=0.31	p=0.41		
Lot 4: AM-2, 860 mg/kgP	10.25 ±13.32	3.42 ±3.42	15.83 ±7.32	40.75 ±8.38	554.83 ±373.26	428.45 ±94.87	1.83 ±0.58	1191.52	92.26
	p=0.25	p=0.93	p=0.17	p=0.38	p=0.94	p=0.31	p=0.56		

p>0.05 means that the difference is statistically significant.

Subacute multi-dose administration effects on sexual behavior parameters

Subacute multi-dose sexual behaviors of the tested rats were observed after 7 days of the repeated extract administration. Table 6 presented the results of subacute rating behaviors. It showed that the rates of mounts and intromissions were increased in two AM–treated animal groups compared to testosterone–treated and NC rats. Similar rates were observed between two AM–treated animal groups.

Table 6. Subacute rating behaviors in tested rats.

Lot/dose (mg/kg): 7 days	Mount (%)	Intromission (%)
Lot 1, NC: Na-CMC 0.5 %	34.70	24.90
Lot 2, P: testosterone, 0.4 mg/kgP	73.60	72.10
Lot 3: AM-1, 430 mg/kgP	83.10	80.50
Lot 4: AM-2, 860 mg/kgP	85.40	81.60

p>0.05 means that the difference is statistically significant.

The Table 7 presented the subacute sexual behaviors in tested rats after repeated doses during 7 days. In comparison to NC rats, the testosterone– and two AM–treated rats increased MF and EF, and decreased IL and EL significantly while only two AM–treated rats significantly

decreased IF ($p>0.05$). Between the two AM-treated rats groups, the ML, IF and PEI were changed adversely compared to NC rats. In comparison to P rats, two AM-treated rats increased IL and EF, and decreased IF and EL significantly ($p>0.05$). When compared between the two AM-treated rats groups, the high dose showed higher mean ML, IF, EL, and PEI, and lower mean MF, IL and EF values. Both the CE and IR indexes were lower in the treated rats in comparison to the NC rats.

Table 7. Subacute sexual behaviors in tested rats.

Lot/dose (mg/kg)	ML (s)	MF (times)	IL (s)	IF (times/30 min.)	EL (s)	PEI (s)	EF (times/30 min.)	CE (%)	IR (%)
Lot 1, NC: Na-CMC 0.5 %	75.00 ± 120.33	4.82 ± 3.03	94.82 ± 255.54	42.00 ± 20.21	872.91 ± 600.55	390.13 ± 84.12	1.45 ± 0.93	871.37	89.71
Lot 2, P: testosterone, 0.4 mg/kgP	151.82 ± 222.47	6.45 ± 4.41	19.27 ± 14.38	49.09 ± 20.79	624.09 ± 590.91	354.89 ± 70.71	1.55 ± 0.93	761.09	88.39
	p=0.33	p=0.32	p=0.34	p=0.43	p=0.34	p=0.36	p=0.82		
Lot 3: AM-1, 430 mg/kgP	14.73 ± 17.61	7.27 ± 5.37	36.09 ± 32.61	32.4 ± 9.17	434.27 ± 381.89	337.91 ± 171.51	2.36 ± 0.67	446.35	81.70
	p=0.12	p=0.20	p=0.46	p=0.17	p=0.05	p=0.44	p=0.02		
Lot 4: AM-2, 860 mg/kgP	117.70 ± 303.48	5.60 ± 9.16	31.20 ± 37.06	38.40 ± 17.95	527.70 ± 483.1	491.44 ± 157.12	1.80 ± 0.79	685.71	87.27
	p=0.67	p=0.79	p=0.45	p=0.67	p=0.17	p=0.13	p=0.37		

$p>0.05$ means that the difference is statistically significant.

4. DISCUSSION

Effects on body weights, reproductive organs' weights and testosterone levels

Male sexual dysfunction including erectile dysfunction and premature ejaculation, is the most common problem that contributes to infertility, distress, relationship problems and quality of life. More people believe that the herbal medicines, which can increase libido, potency, and sexual pleasure, are safe to consume and harmless. Numerous plants/natural products/combinations are used as sex enhancers without any scientific evidence. For ED treatment purposes, we have combined eight medicinal plants including one new *P. micrantha* species traditionally employed in the Dak Nong province of Vietnam for its supposed male sexuality-improving activities in one polyherbal formulation, AM. To understand the scientific basis, we investigated the effects of the prescription on the serum testosterone and sexual behavior parameters using the male Sprague-Dawley rat model in this study.

Our results showed that there were increases in body weights, up to 6.13% over 7 days of the experiment in all the tested animal groups. Body weight changes indicate occurring physiological and metabolic reactions within an organism. Weight gain is often associated with increased anabolic and metabolic processes while weight loss is associated with disease

conditions. In our results, the body weight gains in all the tested rats can be influenced by but not limited to feed consumption rate, proximate composition of the polyherbal combination, and disease conditions (metabolic disorders). Gain in body weight may lead to obesity, which in turn leads to poor male reproductive health (He et al, 2021) [43].

The male reproductive system consists of the internal structures: the testicles, levator ani, epididymis, vas deferens, seminal vesicle, prostate glands, Cowper's glands, and the external structures: glans penis promoting the ejaculation of sperm for fertilization, and producing important androgens for male development (Mark, 2014) [44]. The weights of reproductive organs are an important index to indicate the weight changes for physiological and pathological status as the weight changes in reproductive organs could influence the function of those organs. The reproductive organs of males are crucially essential for producing male gametes and their transportation to the female reproductive tract for successful fertilization (Woldemeskel 2017) [45]. In our study, the reproductive organ weights and the major male androgen, testosterone, were assessed.

The weight changes of the reproductive organs indicate the changes in physiological and pathological status as they could influence the function of these organs. In the present study, the higher weights of all the reproductive organs were observed in all rats of the testosterone- and AM-treated rats, except the lower glans penis weights of the P rats in comparison to the NC rats. The testicles act as both endocrine and exocrine organs, responsible for androgen production and sperm production and transport. In our study, the weight gain in the testicles can reflect changes in seminiferous tubules where its addition indicates a significant increment in sperm production (Zaidi et al. 2023) [46]. During ejaculation, levator ani muscle contraction facilitates semen ejection (Ada et al. 2019) [47].

Thus, the weight gain in the levator ani muscles can stimulate the semen ejection. The seminal vesicles in males were associated with infertility as the seminal plasma is mainly produced in the seminal vesicles (approximately 65-75%), with approximately 20-30% made by the prostate, and small amounts secreted by the Cowper's glands (Takahiro et al. 2024) [48]. The weight gain in the seminal vesicles, prostate glands, and Cowper's glands can stimulate more seminal plasma production supporting male fertility. Penis's main function is to get and keep an erection, ejaculate and reproduce. The angle of the erect penis is determined by its size. The glans penis essentially functions as a large arteriovenous shunt (Dean & Lue, 2005) [49]. Our results showed the weight gain in the glans penis of the AM-treated rats that can lead to good erection. In conclusion, the weight gains of the reproductive organs in the tested rats after the AM oral administration for 7 days indicate a better influence on the functions of these organs towards ED treatment and infertility. The male reproductive system has the function to produce androgens such as testosterone. Weight changes of reproductive organs can influence the androgenic activity. Androgen levels contribute to improved copulatory and sexual performance (Besong et al. 2023) [50]. In our study, serum testosterone levels in AM-treated rats were found to significantly increased, as compared with the NC rats. Our results suggested that the AM may have testosterone mimetic properties and may increase testosterone secretion in Leydig cells by affecting cAMP in vitro (Zhang et al, 2006) [51].

Acute single dose effects

The sexual behaviors were observed on the days 1st, and 7th after the herbal drug administration. In our experiments, we have examined a complete pattern of male rat sexual behavior, including MF, ML, IF, IL, EF, EL, and PEI. The acute effects were recorded after 2

h of the AM administration at the 1st day. Latency parameters such as ML, IL, EL, and PEI are significant indicators to assess sexual motivation in male rats. When ML and IL decrease, it indicates a shorter time interval between the introduction of a receptive female and the initiation of mounting or intromission behavior. This reduction in latency suggests an increased sexual drive and the potential aphrodisiac activity of the extract (Rahman et al. 2023) [52]. In the present study, AM formulation at both tested doses (430 and 860 mg/kg) significantly decreased ML and IL as compared with the NC rats. Therefore, the decrease in ML and IL indicate the efficacy of the AM in enhancing sexual motivation and performance in male rats. This agreed with the findings on *Massularia acuminata* root in male Wistar rats at the concentrations up to 100 mg/kgP (Yakubu et al. 2010) [53]. On contrary, an increase in another latency parameter, ejaculation latency (EL), indicates higher sexual motivation. The EL demonstrates a significant improvement in the copulatory performance of male rats (Cizmeci et al. 2023; Sotelo-Tapia et al. 2023) [54, 55].

Results of the present study showed the increase in EL values of P and AM-1 treated rats and the decrease in AM-2 treated rats in comparison to the NC rats. The prolongation of the EL by AM-1 treated rats suggests an aphrodisiac action. At higher dose of 860 mg/kgP, the shortening of the EL may indicate an aphrodisiac toxicity. A decrease in post-ejaculatory interval (PEI) in male rats means an increase in potency and libido (Olvera-Roldán et al. 2023) [56]. Our study showed the increase in the PEI of both AM treated groups in comparison to the NC rats, suggesting a decrease of sexual vigor and engagement in subsequent sexual activity. Sexual frequency parameters such as MF, IF, and EF are useful indices of potency, vigor, and libido. Literature shows that MF increase reflects sexual motivation, IF increase indicates the efficiency of erection, penile orientation and easy ejaculatory reflexes. As intromission is possible with adequate erection and coordinated activity of penile muscles, the IF increase also suggests that the mechanism of penile erection was activated. Since an EF increase is an indication of enhanced aphrodisiac effect, the presence of plug in the vagina of the female rats indicates that ejaculation occurs (Agmo 1997) [57].

In our study, AM significantly decreased MF, IF, and EF when compared with the rats in the untreated NC group. When compared between the two AM-treated rats groups, the high dose showed higher mean MF and EF with similar IF values. The EF decrease following the AM administration suggests improved sexual vigor. Similar results were found while working on ethanolic extracts of *Syzygium aromaticum* in male rats (Tajuddin et al. 2003) [58]. All the rats mounted and intromitted without any inhibition of mount-and-intromission frequencies (MF and IF) or copulatory efficiency (CE) or intromission ratio (IE). This suggests that libido, sexual vigor and sexual performance were unimpaired during the aphrodisiac action. Our results showed that AM-treated animal groups increased the rates of mount and intromissions in comparison to testosterone-treated and NC rats. Both the CE and IR indexes were higher in the AM-1 treated rats in comparison to the NC rats. The prolonged EL of the AM-1 dose is an indication that CE performance in the rats was enhanced. Similar performances have been observed on the extracts of *Moringa oleifera* (Zade et al. 2013) [59]. All the present data showed that acute aphrodisiac administration of AM formulation improved the immediate performance of sexually inexperienced male rats.

Subacute multi-dose effects

The sexual behaviors were studied using one repeated dose per day till the day 7th. Our experiments showed that the IL and EL decrease in AM-treated rats using the repeated doses

would indicate the efficacy of the formulation in enhancing sexual motivation and performance in male rats. This agreed with the findings on the aqueous extract of the aerial parts of *Monsonia angustifolia* in male Wistar rats at the concentrations of 300 mg/kgP during 7 days (Fouche et al. 2015) [60]. The EF decrease following the AM-2 administration suggests improved sexual vigor at high dose of 860 mg/kgP. Same results were shown on ethanolic extracts of *S. aromaticum* in male rats (Tajuddin et al. 2003) [58]. Our study showed the increase in the PEI at low dose in comparison to the NC rats, suggesting a decrease of sexual vigor and engagement in subsequent sexual activity (Olvera-Roldán et al. 2023) [56].

The observation of the rats administered with 7 days repeated doses of AM powder showed the MF increase reflecting sexual motivation, and the EF increase enhancing aphrodisiac effect (Agmo 1997) [57]. Similar EF performances were observed on the ethanol extracts of *S. haematodes* in male rats (Bansode et al. 2015) [61]. However, our results indicated the IF decrease reflecting the ED inefficiency (Agmo 1997) [57]. All the present data showed that subacute aphrodisiac administration of AM formulation improved the chronic performance of sexually inexperienced male rats at the 7th day.

Our behavioral study indicated that the effects between acute single dose and subacute repeated doses during 7 days of AM formulation administration were different. For comparisons between the AM-treated rats groups and the normal control group, the study showed the same decreasing trends in intromission latency (IL) and intromission frequency (IF). For comparisons between the AM-treated rats groups in high dose rats, the increases of mount frequency (MF) and ejaculation frequency (EF) were higher in acute study but lower in subacute experiment. Between the acute and subacute rats, the increases of mount latency (ML) and post ejaculatory interval (PEI) were higher in high dose in comparison to low dose. It might indicate the long use of the AM formulation can decrease its ED treatment efficiency.

5. CONCLUSION

In conclusion, the present study demonstrated that the polyherbal formulation, AM, significantly enhances serum testosterone and sexual behavior parameters, thus indicating that the prescription possesses potent aphrodisiac activity. Research exploring other physiological effects such as sperm quality, are needed to better understand the role that the polyherbal formulation, AM, may play in enhancing male sexual function.

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