



Moringa oleifera extract enhances sexual performance in stressed rats^{*}

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Abstract: Aphrodisiacs are required to improve male sexual function under stressful conditions. Due to the effects of oxidative stress and dopamine on male sexual function, we hypothesized that *Moringa oleifera* leaves might improve male sexual dysfunction induced by stress. Therefore, the effects on various factors playing important roles in male sexual behavior, such as antioxidant effects, the suppression of monoamine and phosphodiesterase type 5 (PDE-5) activities, serum testosterone and corticosterone levels, and histomorphological changes in the testes, of a hydroethanolic extract of *M. oleifera* leaves were investigated. Various doses of extract including 10, 50, and 250 mg/kg body weight (BW) were given orally to male Wistar rats before exposure to 12 h-immobilization stress for 7 d. The results demonstrated that the extract showed both antioxidant and monoamine oxidase type B (MAO-B) suppression activities. At 7 d of treatment, the low dose of extract improved sexual performance in stress-exposed rats by decreasing intromission latency and increasing intromission frequency. It also suppressed PDE-5 activity, decreased serum corticosterone level, but increased serum testosterone, numbers of interstitial cells of Leydig and spermatozoa. The increased numbers of interstitial cells of Leydig and spermatozoa might have been due to the antioxidant effect of the extract. The increased sexual performance during the intromission phase might have been due to the suppression of MAO-B and PDE-5 activities and increased testosterone. Therefore, *M. oleifera* is a potential aphrodisiac, but further research concerning the precise underlying mechanisms is still needed.

Key words: *Moringa oleifera*, Sexual behaviors, Stress
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1 Introduction

Sexual feeling and sexual activity are inevitable parts of life, which play an important role in the sur-

vival of the human race (Kothari, 2001). Since sex is a most intimate, indispensable, and integral part of every individual's life and can be a source of pleasure and fulfillment, sexual dysfunction can induce depression, anxiety, and debilitating feelings of inadequacy (Kennedy *et al.*, 1999; Baldwin, 2001). Although male sexual dysfunction is not a life threatening disorder, it can greatly affect the quality of life. It has been reported that about 20%–30% of men suffer from sexual dysfunction (Lewis *et al.*, 2004). Because of its high impact and high prevalence, a great effort has been made to search for effective interventions to protect against sexual dysfunction.

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Sexual function is a complex process involving the brain, hormones, emotions, nerves, muscles, and blood vessels. It is very sensitive to stress. Therefore, stressful lifestyles or events usually induce sexual dysfunction. It has been reported that chronic physical and psychological stresses modulate neurotransmission in the median preoptic area and decrease penile blood flow, resulting in erectile dysfunction (Santosh *et al.*, 2011). Experimental stress induced by restraint can suppress testicular steroidogenesis, giving rise to a reduction in plasma testosterone (Orr and Mann, 1990). Long term exposure to stress destroys interstitial cells of Leydig and decreases serum testosterone and spermatogenesis (Rai *et al.*, 2004). Stress also increases the activity of the hypothalamo-pituitary-adrenal axis (HPA-axis), leading to the enhanced plasma cortisol and increased sympathetic system function (Carrasco and van de Kar, 2003). This leads to excessive oxidative stress and stress-related disorders (Ahmad *et al.*, 2012) including sexual dysfunction (Orr and Mann, 1990).

To date, most available drugs in the market target at the intromission phase, the most common phase of sexual dysfunction. The most popular drugs are sildenafil citrate, vardenafil and tadalafil citrate, which target the suppression of phosphodiesterase type 5 (PDE-5), which in turn increases penile blood flow and penile tumescence. Unfortunately, these drugs have serious side effects such as sudden hypotension, hypersensitivity reaction, myalgia, abnormal vision, and infertility (Fauci *et al.*, 2005). Since the current therapeutic drugs do not target all phases of the sexual responsive cycle and produce serious side effects, a novel strategy is required, which is cheap, easy to apply, and less toxic.

Various herbs known to be aphrodisiacs have long been used for enhancing sexual desire and sexual performance in traditional folklore. They can exert their actions at various targets such as the neuro-endocrine system, which plays a crucial role in sexual motivation and function (Hull *et al.*, 1997; Meston and Frohlich, 2000), and PDE-5, an important enzyme in the signal pathway which regulates penile erection via the regulation of cavernous smooth muscle tone, which in turn controls penile blood flow (Andersson, 2001). Several lines of evidence show that testosterone and dopamine can control male sexual function both at the nervous system and at the

penis (Hull *et al.*, 1997). In addition, a recent study has shown that oxidative stress also plays a role in the impairment of cavernosal function and the pathophysiology of erectile dysfunction (Minhas *et al.*, 2002; Hamed *et al.*, 2003; de Young *et al.*, 2004), and that antioxidants can improve erectile function (Zhang *et al.*, 2011).

Moringa oleifera Lam. syn. *M. pterygosperma*, or the drumstick tree, a widely consumed vegetable in Thailand, belongs to the Moringaceae family. It has long been used in nutritional, industrial, and medical fields. *M. oleifera* leaves are used for treating various ailments including constipation, headache, fever, and diabetes (Makonnen *et al.*, 1997). In addition, it has been demonstrated that *M. oleifera* leaf extracts inhibit 6- β -hydroxylation of testosterone (Monera *et al.*, 2008). Therefore, the sexual enhancing potential of this plant has gained attention. On the basis of the effects of dopamine and oxidative stress on male sexual function mentioned earlier, we hypothesized that *M. oleifera* leaves, which have antioxidant (Sreelatha and Padma, 2009) and monoamine modulation effects (Ganguly and Guha, 2008), might have beneficial effects on male sexual dysfunction induced by stress. Due to the limited supporting scientific data, we aimed to evaluate the sexual enhancing potential of *M. oleifera* by determining the effect of a hydroethanolic extract from *M. oleifera* leaves on various aspects of male sexual behavior in stressed rats, and to determine the effect of the extract on various factors involved in male sexual function, including the antioxidant effect, monoamine and PDE-5 suppression activities, serum testosterone and corticosterone levels, and histomorphological changes in the testes.

2 Materials and methods

2.1 Chemicals and reagents

Folin-Ciocalteu reagent, sodium carbonate, sodium acetate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, aluminum chloride, potassium acetate, bovine serum albumin, tannic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (Fluka), clorgyline (selective MAO-A-I), tyramine, vanillic acid, and 4-aminoantipyrine were obtained from the Sigma-Aldrich Corporation, Ltd., Thailand. Gallic acid,

ascorbic acid, and quercetin used were of analytical grade. A PDE-Glo™ phosphodiesterase Assay Kit was purchased from the Promega Corporation, Thailand. Sildenafil citrate used in this study was from the Pfizer Company. All other substances were of analytical grade.

2.2 Plant collection and extraction

Fresh leaves of *M. oleifera* were harvested during November and December, 2011 from the Khon Kaen Province in Thailand. The plant specimen was authenticated by Assoc. Prof. Dr. Panee SIRISA-ARD, Faculty of Pharmaceutical Sciences, Chiangmai University, Thailand.

The leaves were homogenized as powder after being cleaned and dried at 60 °C. The powder was extracted in 50% hydro-ethanolic solution using a maceration technique for 72 h. The non-soluble part was separated using Whatman No. 1 filter paper. The filtrated extract was evaporated using a rotary evaporator. The yielded extract of 17.49% dry plant tissue was kept at -20 °C until used. A voucher specimen was deposited at the Integrative Complementary Alternative Medicine Research and Development Center, Khon Kaen University, Thailand.

2.3 Evaluation of total phenolic compounds and flavonoid content

The total content of phenolic compounds was determined using the Folin-Ciocalteu method (Basma *et al.*, 2011). In brief, the mixture, which contained 500 µl of Folin-Ciocalteu reagent (10%), 0.8 ml of sodium carbonate (7.5%, 0.075 g/ml) and 0.5 ml distilled water (DW), was mixed with 0.2 ml of 1 mg/ml *M. oleifera* leaf extract and left at room temperature in the dark for 45 min. The optical density was read with an ultraviolet-visible spectrophotometer at 765 nm. DW was used as a blank. The accuracy of data analysis was enhanced by using a triplicate-sample method. The total phenolic content (TPC) is expressed as mg of gallic acid equivalent (GAE)/g of dry weight, using the standard calibration line of gallic acid.

The total flavonoid content (TFC) was measured by aluminum chloride colorimetry (Pourmorad *et al.*, 2006). A mixture containing 50% alcohol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 mol/L potassium acetate, and 2.8 ml of DW was mixed with 0.5 ml of

the plant extract (2 mg/ml) and left at room temperature for 40 min in the dark. The absorbance at 415 nm was read using an ultraviolet-visible spectrophotometer. DW containing all the chemicals mentioned earlier except the plant extract, was used as the blank and each sample was prepared in triplicate. The mean value of the triplicate was used for analysis. The TFC is presented as mg of quercetin equivalent (QE)/g of dry weight, using the standard calibration line of quercetin.

2.4 Antioxidant assays

Both DPPH and ferric reducing antioxidant power (FRAP) assays were carried out to determine the antioxidant activity of the extract. Stable free radical scavenging capacity was measured via the DPPH method (de Ancos *et al.*, 2002) with a slight modification. In brief, 2.96 ml of a 0.1 mmol/L solution of DPPH in methanol was incubated with 40 µl of various concentrations of extract (1.0, 2.0, 5.0, 10.0, 20.0, and 25.0 mg/ml) at room temperature for 30 min. The decrease in DPPH radicals was evaluated by measurement of optical density at 515 nm. The stable free radical scavenging capacity is presented as the percentage of inhibition of DPPH radicals (I_{DPPH}), calculated according to the following equation: $I_{DPPH} = (A_c - A_s) / A_c \times 100\%$, where A_c and A_s are absorbances of control and sample, respectively.

To determine the FRAP, the reaction mixture containing FRAP solution (300 mmol/L sodium acetate buffer (pH 3.6), 10 mmol/L TPTZ solution in 40 mmol/L HCl, and 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution at a ratio of 10:1:1 (v/v)) (Thaipong *et al.*, 2006) and 0.15 ml of the dilution of the sample (0.5 mg/ml), was incubated at 37 °C in the dark for 30 min. The optical density of the colored product (ferrous tripyridyltriazine complex) was measured at 593 nm. Data are presented as µmol ascorbic acid equivalent (AAE)/g extract.

2.5 Amino acid determination

Amino acid evaluation was performed according to the method of Nwidi *et al.* (2012). To hydrolyze peptide bonds, 1 ml of 6 mol/L HCl and 0.08 ml of 5% (v/v) phenol/water solution were mixed with 5 mg of extract and heated in a Pyrex tube with plastic Teflon-coated screw caps (13 cm×1 cm) at 110 °C for 72 h. The solution was reheated in an oven at 70 °C,

diluted with 1.0 ml of sodium citrate buffer (pH 2.2) and filtered through a GV Millex Unity filter (Millipore). The amino acid profile analysis was carried out by cation-exchange chromatography using an automated amino acid analyzer, Shimadzu LC-10A/C47A. The sample was eluted by sodium, and post-column derivatization was performed using *o*-phthaldialdehyde (OPA). The retention time and area obtained by each amino acid were compared with those of standards to identify and quantify the amino acid profile.

2.6 Determination of monoamine oxidase type B (MAO-B) inhibition

The MAO-B suppression effect was determined by spectrophotometry (Schurr and Livne, 1976). In brief, the rats were sacrificed by cervical dislocation and the cerebral cortices of healthy rats were removed and prepared as an homogenate with RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 2 mmol/L ethylene diamine tetraacetic acid (EDTA), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% Triton X-100) and centrifuged at 14000g at 4 °C for 20 min. After the centrifugation, the supernatant was harvested and served as the source of MAO-B. To initiate the enzyme reaction, 50 µl of brain homogenate, preincubated with 50 µmol/L clorgyline (a selective inhibitor of MAO-A) at a ratio of 100:1 (v/v), was added to the solution containing 2.75 ml Tris buffer (0.1 mol/L, pH 7.4) and 100 µl of 0.1 mol/L benzylamine (Holt *et al.*, 1997; Dhingra and Goyal, 2008). The absorbance changes in the tested samples in the double beam spectrophotometer at a wavelength of 249.5 nm within 5 min were recorded against blanks containing Tris buffer and 5-hydroxytryptamine (Xu *et al.*, 2005). The MAO-B suppression effect of Selegiline, a standard MAO-B inhibitor, was also studied using the same procedure, except that Selegiline was assayed instead of the plant extract.

2.7 Stress exposure

Since immobilization or restraint stress has been recognized as a valid test for studying stress-induced physical and psychological alterations and stress-related disorders (Al-Mohaisen *et al.*, 2000; Xu *et al.*, 2006; Zaidi *et al.*, 2014), it was used as the experimental model in this study. Immobilization stress was induced using a modified method of Retana-Márquez

et al. (2003) and Ahmad *et al.* (2012). In brief, immobilization stress was applied between 6:00 a.m. and 6:00 p.m. The rats were put into a transparent perforated plastic tube (20 cm long and 7 cm in diameter). Since the animal fitted tightly in the tube, it could not move or turn around.

2.8 Male sexual behavior evaluation

Fertile male Wistar rats were used as experimental animals. They were randomly divided into seven groups each with six animals. (1) Group I: naive control (non-stress group); rats received no treatment. (2) Group II: vehicle plus stress; rats were given the vehicle (DW) orally, 45 min before being subjected to 12 h-restraint stress exposure for 7 d. (3) Group III: sildenafil; this group served as a positive control based on the sexual enhancing effect of sildenafil citrate. All rats in this group were administered sildenafil citrate at a dose of 5 mg/kg 45 min before being subjected to 12 h-restraint stress exposure for 7 d. Since this drug has a maximum mode of action of 4 h after administration, an oral suspension of this drug at a dose of 5 mg/kg was given to all rats 1 h before the mating behavior assessment. (4) Group IV: tianeptine; this group also served as a positive control based on the dopamine enhancing effect of this drug (Invernizzi *et al.*, 1992) and the positive modulation effect of dopamine on sexual dysfunction (El-Shafey *et al.*, 2006). All rats in this group were given tianeptine orally at a dose of 15 mg/kg 45 min before being subjected to 12 h-restraint stress exposure for 7 d. (5) Groups V–VII: *M. oleifera* leaf extract treated groups; rats in these groups were given orally various doses of *M. oleifera* leaf extract, including 10, 50, and 250 mg/kg 45 min before being subjected to 12 h-restraint stress exposure for 7 d.

The treatments and the stress-exposure were carried out once daily. After being exposed to stress, the animals were given a 3 h-refreshment period before their sexual behavior evaluation (Prabsattroo *et al.*, 2012). The sexual behavior assessments were performed by an experienced observer blind to the treatments, at room temperature between 9:00 p.m. and 12:00 midnight after a single intervention and after one week of interventions.

Male rats subjected to single or repeated doses of the plant extract were paired with estrous female rats induced by estradiol benzoate (Sigma, St. Louis, MO,

USA) at a dose of 2 µg/kg body weight (BW) and progesterone (Sigma, St. Louis, MO) at a dose of 500 µg/kg BW subcutaneously at 48 and 6 h, respectively, before the determination of copulatory behavior. The sexual behavior of the rats in a clear plastic box was monitored for 3 h using video recording (Gauthaman *et al.*, 2002). The assessed sexual parameters were latencies and frequencies of mounting, intromission, and ejaculation.

2.9 Determination of PDE-5

The level of PDE-5 was determined using a PDE-Glo™ Phosphodiesterase Assay Kit (Promega Corp., Thailand). The penis was washed with phosphate buffer solution (PBS), cut into small pieces, and prepared as homogenate using lysate RIPA buffer. The sample was centrifuged at 14000g at 4 °C for 15 min. The supernatant was separated and served for the determination of PDE-5 activity. The PDE-Glo™ phosphodiesterase assay was carried out in a 96-well plate. The assay was performed according to the guidelines of the kit. In brief, the penis was incubated with cyclic guanosine monophosphate (cGMP) substrate in reaction buffer until the phosphodiesterase reaction was complete. PDE-Glo™ termination buffer was incubated with PDE detection solution containing adenosine triphosphate (ATP) and protein kinase A (PKA). The amount of ATP consumed by this reaction, which is directly correlated with the cGMP level, was evaluated using the luciferase-based Kinase-Glo reagent. After a 10-min incubation period at room temperature, the optical density of the sample was determined using a SpectraMax® L microplate luminometer (MDS AT (US) Inc.) and expressed as relative light units (RLUs) and as a percentage of the control.

2.10 Determination of testosterone and corticosterone levels

At the end of study, the venous blood of each animal was prepared as serum by centrifugation at 2000g at 4 °C for 15 min. The serum was stored at -80 °C until used. Testosterone levels were measured using a radioimmunoassay (RIA) Kit (TESTO-CT2, Cisbio International, France) and corticosterone levels were measured using a Corticosterone Double Antibody Radioimmunoassay Kit (MP Biomedicals) for the quantitative determination of corticosterone in

rat and mice serum. The results are expressed as ng/ml. Both assays were performed at the Radiology Department, Srinakarindhra Hospital, Faculty of Medicine, Khon Kaen University, Thailand.

2.11 Histological study

The testes were dissected out, freed from surrounding tissues and weighed quickly on a sensitive balance. They were fixed, embedded in paraffin, cut into 10 µm thick sections, and stained using hematoxylin and eosin (H&E). Histomorphological analysis was performed using a light microscope.

2.12 Statistical analysis

The experimental data are presented as mean± standard error of the mean (SEM). The statistical significance was evaluated using analysis of variance (ANOVA) followed by Duncan's test. A *P*-value of <0.05 was considered to be statistically significant.

3 Results

3.1 Phenolic compound and flavonoid contents

The total phenolic compounds in a 50% (v/v) hydro-ethanolic extract of *M. oleifera* leaves were determined by the Folin-Ciocalteu method and expressed as GAE. The concentration of phenolics found in the *M. oleifera* leaf extract used in this study was (62.333±0.008) mg GAE/g extract. The flavonoid concentration of the extract was also determined using the aluminum chloride colorimetric method and was found to be (29.900±0.001) mg QE/g extract.

3.2 DPPH radical scavenging activity and FRAP

In this study, the 50% hydro-ethanolic extract of *M. oleifera* leaves was tested for its antioxidant scavenging effects on DPPH radicals. The results obtained at different concentrations of extract are given in Fig. 1. The results show that the extract showed dose-dependent activity. The concentration required for a 50% (v/v) DPPH reduction (inhibitory concentration 50%; IC₅₀) by the extract was determined using ascorbic acid as reference. The IC₅₀ of ascorbic acid was (0.124±0.009) mg/ml, whereas that of the *M. oleifera* leaf extract was (8.270±0.023) mg/ml.

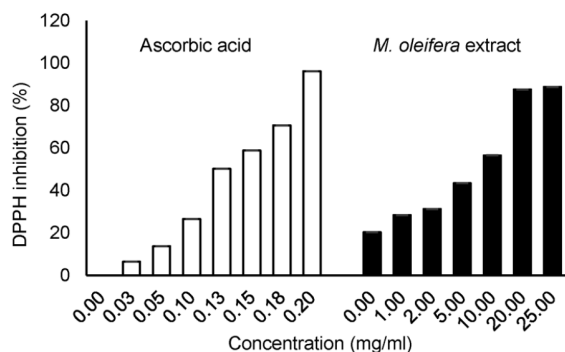


Fig. 1 DPPH radical scavenging activities of *M. oleifera* leaf extract and ascorbic acid (standard control)

Reducing power has been reported to be a validated indicator reflecting antioxidant activity (Oktay *et al.*, 2003). The reducing power of *M. oleifera* leaf extract was assessed via an FRAP assay. The reducing power of the extract at various concentrations is shown in Fig. 2. The IC_{50} of the extract evaluated using the FRAP assay was $(399.00 \pm 0.010) \mu\text{mol/L}$ AAE/mg extract.

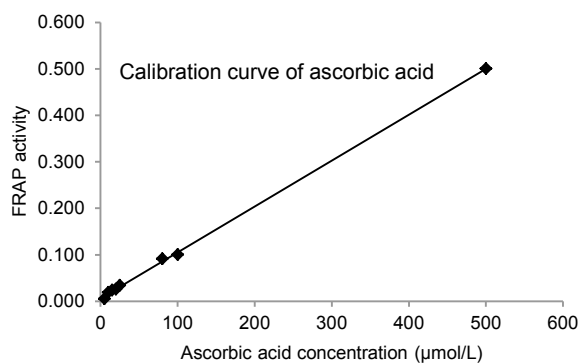


Fig. 2 Ferric reducing antioxidant power (FRAP) of *M. oleifera* leaf extract using ascorbic acid data to provide a calibration curve

FRAP activity of *M. oleifera* is $399 \mu\text{mol/L}$ AAE/mg extract

3.3 Amino acid composition of *M. oleifera* leaf extract

The amino acid composition of the leaf extract of *M. oleifera* is shown in Table 1. Phenylalanine showed the highest concentration (2981.00 mg/100 g). Serine, glutamic acid, leucine, lysine, isoleucine, tyrosine, histidine, valine, alanine, aspartic acid, proline, tryptophan, glycine, cysteine, methionine, threonine, arginine, hydroxylysine, and hydroxyproline were also observed.

Table 1 Amino acid profile of *M. oleifera* leaf extract detected by gas chromatography (ion exchange chromatographic method)

Essential	Concentration (mg/100 g)	Non-essential	Concentration (mg/100 g)
Alanine	416.67	Arginine	<5.00
Histidine	475.27	Aspartic acid	368.14
Hydroxylysine	<5.00	Cysteine	133.39
Isoleucine	631.02	Glutamic acid	1422.00
Leucine	910.44	Glycine	131.33
Lysine	652.61	Hydroxyproline	<5.00
Methionine	91.32	Proline	283.88
Phenylalanine	2981.00	Serine	1554.00
Threonine	79.72	Tyrosine	546.81
Tryptophan	263.80	Valine	422.97

3.4 MAO-B suppression effect of *M. oleifera*

The MAO-B suppression effect of the extract was determined and used as an indicator to reflect the available dopamine, an important neurotransmitter which plays a pivotal role in male sexual function. The IC_{50} of MAO-B was $(0.255 \pm 0.008) \text{ mg/ml}$ while that of selegiline, a standard MAO-B inhibitor, used for enhancing the dopamine level, was $(0.005 \pm 0.001) \text{ mg/ml}$.

3.5 Male sexual enhancing effect of *M. oleifera* leaf extract

The effects of *M. oleifera* leaf extract on sexual behavior are presented in Table 2. The current data confirm the reputation of *M. oleifera* as a sexual stimulating agent. Rats that received either tianeptine (Group IV) or sildenafil citrate (Group III) and were subjected to the 12-h immobilization stress showed significantly decreased mounting latency ($P < 0.05$, compared to vehicle control plus stress treated group (Group II) after a single intervention). However, only those rats in Group III showed a decreased intromission latency after the 7-d intervention period ($P < 0.05$, compared to Group II). Unfortunately, no changes in latencies or the numbers of mounting, intromission, or ejaculation events were observed in rats of Group IV. After a single administration, rats treated with the extract at a dose of 50 mg/kg and exposed to immobilization stress (Group VI) showed a significantly decreased mounting latency ($P < 0.05$, compared to Group II). Only rats subjected to the extract at a dose of 10 mg/kg and exposed to immobilization stress (Group V) significantly increased mounting numbers

($P<0.05$, compared to Group II) after a single administration. Interestingly, rats subjected to the extract at a dose of 10 mg/kg (Group V) and exposed to immobilization stress showed a significant decrease in intromission latency, but an increase in the number of intromissions ($P<0.05$, compared to Group II) after the 7-d intervention period. In addition, rats subjected to a high dose of extract plus stress (Group VII) also showed increased mounting numbers after the 7-d intervention period ($P<0.05$, compared to Group II). No significant changes in latency or the number of ejaculations were observed throughout the 7-d study period.

3.6 PDE-5 activity of *M. oleifera* leaf extract

PDE-5 activity in the penis of rats given *M. oleifera* leaf extract was determined (Fig. 3). The extract at a dose of 10 mg/kg significantly attenuated the enhanced PDE-5 activity in the penis of stress-exposed rats ($P<0.05$; compared to the control (Group I)). Both

sildenafil citrate and tianeptine produced significant reductions in PDE-5 activity in the penis ($P<0.01$ and $P<0.05$, respectively; compared to Group II). Rats in Group V showed a significant decrease in PDE-5 activity in the penis ($P<0.05$; compared to Group II).

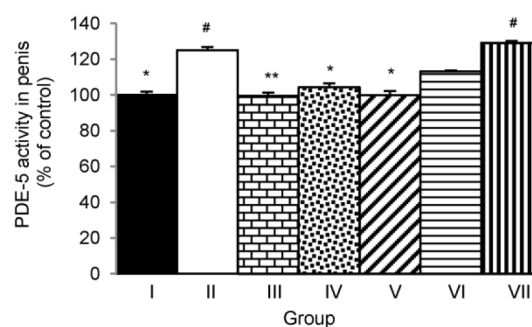


Fig. 3 Effect of hydro-ethanolic extracts of *M. oleifera* leaves on phosphodiesterase type 5 (PDE-5) activity in the penis of stress-exposed rats after 7 d of treatment. Data are expressed as mean \pm SEM ($n=6$). * $P<0.05$, ** $P<0.01$, compared with Group II. # $P<0.05$, compared with Group I

Table 2 Effect of hydro-alcoholic extracts of *M. oleifera* leaves on male sexual behavior of stress-exposed rats at baseline, after a single dose, and after 7 d of treatment

Group	ML (s)	MN	IL (s)	IN	EL (s)	EF
Baseline						
I	15.6 \pm 10.7	29.2 \pm 5.2	102.5 \pm 64.4	23.2 \pm 2.9	582.3 \pm 114.8	2.3 \pm 0.5
II	13.0 \pm 3.3	31.0 \pm 2.0	292.6 \pm 257.0	17.2 \pm 4.2	1026.6 \pm 318.7	1.6 \pm 0.7
III	33.0 \pm 12.2	44.3 \pm 6.2	155.8 \pm 86.2	18.3 \pm 3.1	946.5 \pm 198.7	1.5 \pm 0.3
IV	11.5 \pm 4.8	38.2 \pm 6.7	45.0 \pm 8.3	23.2 \pm 1.5	750.8 \pm 218.1	1.8 \pm 0.4
V	20.5 \pm 10.9	34.0 \pm 5.1	149.6 \pm 65.6	14.5 \pm 2.3	707.0 \pm 78.3	1.4 \pm 0.4
VI	19.7 \pm 7.5	32.2 \pm 5.2	173.3 \pm 86.1	22.4 \pm 3.2	1015.1 \pm 268.3	2.0 \pm 0.7
VII	29.8 \pm 13.8	34.7 \pm 3.3	68.7 \pm 26.5	21.7 \pm 3.7	1143.8 \pm 163.4	1.3 \pm 0.4
Single dose						
I	15.7 \pm 8.0	39.0 \pm 6.3	38.7 \pm 8.3	27.6 \pm 5.0	613.0 \pm 129.9	2.0 \pm 0.4
II	29.0 \pm 11.2	32.2 \pm 3.7	111.4 \pm 70.2	26.2 \pm 5.2	326.6 \pm 196.5	2.2 \pm 0.2
III	3.2 \pm 0.9*	37.5 \pm 5.1	75.3 \pm 24.2	20.7 \pm 2.9	568.3 \pm 139.8	2.7 \pm 0.5
IV	6.0 \pm 2.3*	28.8 \pm 6.4	38.3 \pm 8.7	23.7 \pm 3.1	502.3 \pm 87.1	2.0 \pm 0.3
V	28.6 \pm 7.3	49.8 \pm 6.4*	78.0 \pm 21.8	21.2 \pm 2.8	447.4 \pm 62.9	2.5 \pm 0.3
VI	6.4 \pm 2.0*	44.8 \pm 6.2	58.6 \pm 20.1	22.0 \pm 4.5	883.2 \pm 137.3	1.3 \pm 0.3
VII	34.6 \pm 11.3	41.0 \pm 7.1	46.3 \pm 13.8	17.6 \pm 4.3	895.2 \pm 254.4	1.4 \pm 0.4
7-d						
I	2.0 \pm 0.8	31.0 \pm 1.5	15.0 \pm 3.8*	29.8 \pm 1.9*	421.5 \pm 112.5	2.5 \pm 0.2
II	15.8 \pm 7.7	28.2 \pm 4.0	81.8 \pm 49.7#	20.3 \pm 3.9#	482.5 \pm 225.1	2.4 \pm 0.4
III	3.3 \pm 1.3	33.8 \pm 4.9	13.8 \pm 3.2*	29.2 \pm 1.4*	319.7 \pm 137.6	3.0 \pm 0.4
IV	18.2 \pm 7.3	33.7 \pm 7.9	47.3 \pm 9.5	26.2 \pm 1.2	639.0 \pm 55.4	2.3 \pm 0.2
V	14.2 \pm 7.4	29.7 \pm 2.4	12.6 \pm 4.4*	32.7 \pm 3.0**	397.8 \pm 44.5	2.2 \pm 0.2
VI	15.2 \pm 6.9	44.4 \pm 6.6	27.8 \pm 10.8	23.6 \pm 3.0	672.3 \pm 243.9	2.0 \pm 0.6
VII	18.2 \pm 7.2	46.2 \pm 9.2*	39.8 \pm 14.2	24.6 \pm 4.3	711.8 \pm 111.8	1.8 \pm 0.2

ML: mount latency; MN: mount number; IL: intromission latency; IN: intromission number; EL: ejaculation latency; EF: ejaculation frequency. Data are expressed as mean \pm SEM ($n=6$). * $P<0.05$, ** $P<0.01$, compared with Group II. # $P<0.05$, compared with Group I

3.7 Effect of *M. oleifera* leaf extract on testosterone and corticosterone levels

Fig. 4 shows that the male rats receiving sildenafil citrate and *M. oleifera* leaf extract at doses of 10 and 250 mg/kg and exposed to immobilization stress for 7 d showed elevated levels of serum testosterone ($P < 0.001$, $P < 0.05$, and $P < 0.01$, respectively; compared to Group II). We also evaluated the effect of the extract on the serum corticosterone level (Fig. 5). Rats subjected to the extract treatment at doses of 10, 50, and 250 mg/kg and exposed to immobilization stress for 7 d showed elevated serum corticosterone levels ($P < 0.01$, $P < 0.001$, and $P < 0.05$, respectively; compared to Group II).

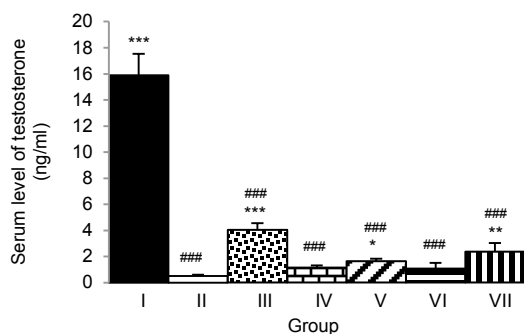


Fig. 4 Effect of hydro-ethanolic extracts of *M. oleifera* leaves on serum testosterone levels of stress-exposed rats after 7 d of treatment

Data are expressed as mean \pm SEM ($n=6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with Group II. ### $P < 0.001$, compared with Group I

3.8 Effect of *M. oleifera* leaf extract on testis histology

The histological morphology of the testis was evaluated and the results are shown in Fig. 6. In the vehicle plus stress treated group (Group II), the seminiferous epithelium was disorganized and fewer interstitial cells of Leydig were observed. Rats subjected to stress and sildenafil, or stress plus tianeptine, or stress plus *M. oleifera* extract at doses used in this study showed a more organized seminiferous epithelium than those in Group II. In addition, rats in all these groups appeared to have more interstitial cells of Leydig and more spermatozoa in the lumen of the seminiferous tubules than those in Group II.

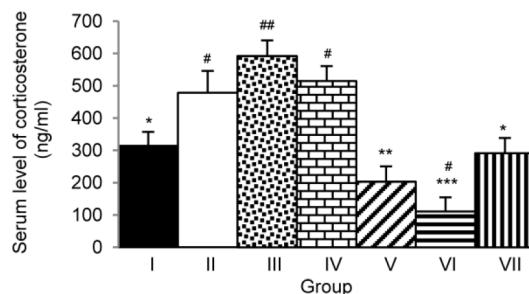


Fig. 5 Effect of hydro-ethanolic extracts of *M. oleifera* leaves on serum corticosterone levels of stress-exposed rats after 7 d of treatment

Data are expressed as mean \pm SEM ($n=6$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with Group II. # $P < 0.05$, ## $P < 0.01$, compared with Group I

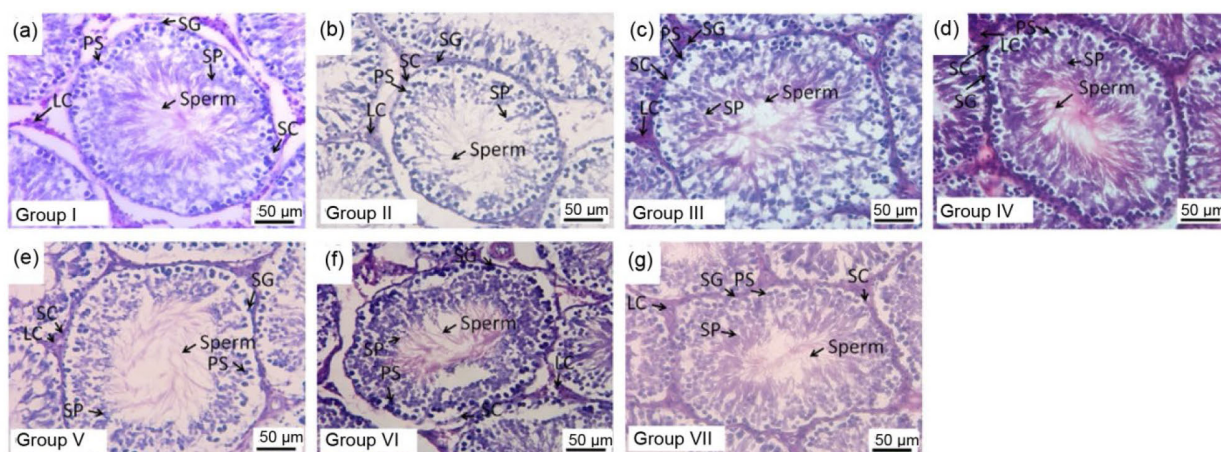


Fig. 6 Haematoxylin and eosin (H&E) stained frozen sections of rat testis showing the histomorphology of seminiferous tubules, Sertoli cells (SC), spermatogonia (SG), primary spermatocytes (PS), spermatids (SP), sperm, and Leydig cells (LC) in the following treatment groups: (a) naive control; (b) vehicle plus stress; (c) sildenafil citrate plus stress; (d) Tianeptine plus stress; (e)–(g) *M. oleifera* at doses of 10, 50, and 250 mg/kg plus stress, respectively

4 Discussion

The current study has revealed that *M. oleifera* leaf extract has antioxidant and MAO-B suppression effects. Phenylalanine was the main amino acid found in the extract. *In vivo* data showed that a single consumption of medium or low doses of extract increased libido. When the treatment was prolonged to 7 d, the low dose of extract enhanced penile erection capacity by decreasing intromission latency and increasing intromission number, decreased both PDE-5 activity and corticosterone levels, increased the numbers of interstitial cells of Leydig and spermatozoa, and increased testosterone levels. The high dose extract also increased the numbers of mountings, interstitial cells of Leydig and spermatozoa, increased testosterone levels, and reduced corticosterone levels.

Male sexual behavior is governed by a complex interaction between different systems in the brain that process sensory inputs, regulate reward and motivation, and integrate hormonal signals (Hull *et al.*, 2004). The regulation of sexual behavior can occur both at the brain and at peripheral sites. It has been reported that testosterone plays an important role in priming brain neural circuits for sexual behavior. The priming effect is achieved partly by the modification of regulating enzymes, receptors, or other proteins which affect neurotransmitter function. Testosterone can increase local dopamine synthesis and metabolism of dopamine (Purves-Tyson *et al.*, 2012), which in turn exerts an influence on various aspects of male sexual function including sexual motivation, copulatory proficiency, and genital reflexes (Hull *et al.*, 2004).

Accumulated evidence has shown that dopamine plays a crucial role in male sexual behavior via the enhanced release of oxytocin from the paraventricular nucleus (PVN) of the hypothalamus, which in turn increases the release of nitric oxide from the cavernosal nerve. Nitric oxide penetrates the cytoplasm of smooth muscle cells and interacts with guanylyl cyclase, catalyzing conformational changes which in turn induce the conversion of guanosine 5'-triphosphate (GTP) to 3'-5'-cyclic guanosine monophosphate. cGMP in turn phosphorylates several proteins, resulting in decreased intracellular calcium levels causing the relaxation of arterial and trabecular smooth muscle, resulting in arterial dilata-

tion, venous constriction, and the rigidity of penile erection. The inactivation of cGMP via PDE-5 decreases the dilation of arterial vessels and the constriction of venous blood vessels and increases penile tumescence (Andersson, 2001).

In this study, stress exposure induced a reduction in the number of interstitial cells of Leydig and in serum testosterone levels. These changes may possibly have been due to enhanced oxidative stress (Rai *et al.*, 2004; Bitgul *et al.*, 2013). *M. oleifera* extract attenuated the reduction in the interstitial cells of Leydig and serum testosterone levels induced by stress exposure. Based on the results of this study, we suggest that the antioxidant effect of the extract alleviates oxidative stress-related cell toxicity, resulting in increased numbers of interstitial cells of Leydig and serum testosterone levels. It has been reported that the elevation of glucocorticoid levels induced by stress can also inhibit the synthesis of testosterone in interstitial cells of Leydig (Gao *et al.*, 1996). Therefore, the mitigating effect of *M. oleifera* extract on the reduction in testosterone induced by stress may occur partly via decreased corticosterone levels.

It has been shown that *M. oleifera* extract also suppresses MAO-B. Due to the crucial role of dopamine in male sexual behavior mentioned earlier, it is possible that suppression of MAO-B activity may enhance the available dopamine, which in turn increases oxytocin release from the PVN and induces the release of nitric oxide from the cavernosal nerve, triggering the elevation of cGMP in the penis. The elevation of cGMP induced by *M. oleifera* extract and the suppression effect of the extract on PDE-5 may enhance the cGMP-dependent vasodilation effect of nitric oxide, leading to enhanced penile blood flow and erection. Since the high dose of *M. oleifera* extract failed to show a PDE-5 suppression effect, no significant change in the intromission phase was observed in stress-exposed rats that received a high dose of extract. In addition, it has been reported that the elevation of testosterone can regulate PDE-5 function (Aversa *et al.*, 2009) and modify dopamine inactivation via MAO-B, leading to increased penile erection (Sanna *et al.*, 2011). Therefore, the enhanced intromission phase observed in this study may have been related to MAO-B suppression activity, the ability to increase the testosterone level or the PDE-5 suppression effect.

In this study, an increased number of spermatozoa was also observed in stress-exposed rats subjected to *M. oleifera* leaf extract treatment. It has been reported that spermatozoa are vulnerable to oxidative stress because of the high concentration of polyunsaturated fatty acids involved in the regulation of sperm maturation, spermatogenesis, capacitation, acrosome reaction, and eventually membrane fusion, and their low antioxidant capacity. An attack of oxidative stress may lead to structural damage and decreased viability. Therefore, the increased spermatozoa density induced by the *M. oleifera* leaf extract may possibly be due to the antioxidant effect of the extract.

Our data also suggest that the enhanced dopaminergic function induced by the *M. oleifera* leaf extract may occur not only via the MAO-B suppression effect but also via the high concentration of phenylalanine in the plant extract. Since phenylalanine serves as a precursor for tyrosine and dopamine synthesis, the enhanced dopamine function observed in this study may also occur partly via the increased dopamine precursor supplement. Moreover, the *M. oleifera* leaf extract used in this study also contained abundant phenolics and flavonoids. Therefore, the antioxidant effect of the extract may have been due to these ingredients.

This study is the first to demonstrate the possible benefit of *M. oleifera* active amino acids on dopaminergic and sexual function besides the possible role of phenolics and flavonoids in the extract, and the biochemical and histological changes related to stress and sexual function. However, the determination of the effect of the plant extract on serum testosterone and PDE-5 activity in the penis at only a single time point and the lack of nitric oxide and penile vascular blood flow measurement, were limitations of this study. The effect of the plant extract on serum testosterone before and during sexual performance should be determined to confirm the modulating role of the extract on the sexual enhancement effect of testosterone. The effect of the extract on PDE-5 activity in testis both before and during the sexual intromission phase should also be determined together with the levels of nitric oxide, cGMP, and penile blood flow, to confirm that the suppression of PDE-5 induced by the plant extract can enhance the cGMP-dependent vasodilation effects of nitric oxide, penile blood flow, and penile erection.

Therefore, further investigations of the effect of *M. oleifera* leaf extract on the alterations of testosterone and dopamine levels before and during sexual performance, together with evaluation of the cGMP-dependent vasodilation effects of nitric oxide, penile blood flow and penile erection, are required to improve understanding of the precise underlying mechanisms of the sexual enhancing effect of *M. oleifera* leaf extracts.

5 Conclusions

The use of *Moringa oleifera* leaf extract may provide a cheap and simple approach for improving sexual function under stressful conditions. Therefore, the extract appears to be a potential aphrodisiac, particularly for people with stressful daily lifestyles. However, more research is needed concerning the possible underlying mechanisms of action of *M. oleifera* leaf extracts, such as the determination of testosterone levels before and during sexual performance, and the evaluation of the cGMP-dependent vasodilation effects of nitric oxide, penile blood flow, and penile erection.

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Compliance with ethics guidelines

Thawatchai PRABSATTROO, Jintanaporn WATTAN-ATHORN, Sitthichai IAMSAARD, Pichet SOMSAPT, Opass SRITRAGOOL, Wipawee THUKHUMMEE, and Supaporn MUCHIMAPURA declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题目: 辣木提取物对应激大鼠性能力具有增强作用

目的: 探讨辣木叶提取物对改善应激条件下雄性性功能障碍的作用。

创新点: 根据氧化应激和多巴胺对雄性性功能的影响, 推测具有抗氧化和单胺调节功能的辣木叶能改善应激条件下的雄性性功能障碍。

方法: 本文以雄性 Wistar 大鼠作为实验对象, 通过对影响雄性性功能的各种因素进行测量及统计学分析, 包括抗氧化性、B 型单胺氧化酶 (MAO-B) 和 5 型磷酸二酯酶 (PDE-5) 活性、血清睾酮和皮质甾酮水平、睾丸内组织形态学变化。

结论: 研究结果表明, 辣木叶提取物作为一种抗氧化剂具有抑制 MAO-B 和 PDE-5 活性的作用, 同时能降低血清皮质酮水平, 提高血清睾酮水平, 增加睾丸间质细胞和精子的数量。睾丸间质细胞和精子的数量的增加可能是由于辣木叶提取物的抗氧化作用, 插入阶段性功能增强可能是由于 MAO-B 和 PDE-5 活性的抑制和睾酮水平的增加。因此, 辣木可以作为一种潜在的壮阳药。

关键词: 辣木; 性功能; 应激