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Original Research Article

Evaluation of antioxidant capacity and reproductive toxicity of aqueous extract of Thai *Mucuna pruriens* seeds

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ABSTRACT

Objective: In traditional medicine, the seeds of Thai *Mucuna pruriens* (T-MP) are used to treat male dysuria and are believed to enhance fertility. However, information pertaining to the toxicity of T-MP and its interaction with other properties is limited. This study was thus conducted to evaluate the antioxidant capacity and subacute toxicity of T-MP in the reproductive system.
Methods: Total phenolic content and antioxidant capacity of T-MP seed extract were determined using total phenolic content, 2,2-diphenyl-1-picrylhydrazyl and ferric reducing antioxidant power assays. Male and female adult rats were treated orally with T-MP at a dosage of 150 or 300 mg/kg body weight for 14 consecutive days. Sex hormones and functional parameters in the liver and kidney were evaluated. Histopathology of all tissue was conducted using Masson's trichrome staining. Sperm parameters, including concentration, morphology, acrosome reaction status and DNA damage, were also examined. Expression of tyrosine phosphorylated protein (TyrPho), androgen receptor and A-kinase-anchoring protein 4 (AKAP4) were investigated using the Western blot technique.
Results: T-MP seed extract contained phenolic compounds and exhibited high antioxidant capacity with no toxicity at the tested doses. It did not affect liver or kidney function parameters in the male rats, but increased estradiol, aspartate aminotransferase and alanine aminotransferase levels in the females. Additionally, it decreased serum progesterone and alkaline phosphatase levels in female rats. Serum and intratesticular testosterone levels were significantly lower in male rats that received a high dosage of T-MP. Histopathological changes were not observed in any tissue treated with T-MP. T-MP also significantly increased sperm concentration (but did not affect sperm parameters), and enhanced testicular TyrPho protein and androgen receptor and expression of AKAP4 in sperms.
Conclusion: T-MP seed extract exhibited antioxidant capacity and was not harmful to reproductive tissues. It also had a phytoestrogenic effect on females and increased the expression of testicular and sperm markers of male fertility.

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1. Introduction

Mucuna pruriens, also known as the velvet bean, is climbing woody legume that is common in tropical and subtropical regions around the world including Thailand. Various reports have docu-

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mented a range of pharmacological effects of *M. pruriens*, leading it to be called the magic velvet bean [1]. In traditional Ayurvedic medicine, *M. pruriens* is wildly used to treat neurological disorders (especially parkinsonism) and male infertility; it is also used as a natural aphrodisiac because its seeds contain levodopa [2–7]. Additionally, *M. pruriens* has been shown to have antioxidant capacity and to contain various bioactive compounds [8–11]. Its seed extract has also been documented to exhibit neuroprotective [12–16], stroke-preventive [17], cardiovascular-protective [18], hepatoprotective [19], antidiabetic [20,21] and anti-human breast cancer and antineuroblastoma [16,22] effects. Moreover, *M. pruriens* also improves male fertility by facilitating sexual activity and improving sperm parameters [23–26].

Plants of the genus of *Macuna* have been found to vary by region, both botanically and in terms of the pharmacological substances they contain. Patil et al. [27], for example, have described the genetic diversity of *M. pruriens* in various regions of India, including *M. pruriens* var. *pruriens* (L.) DC, *M. pruriens* var. *utilis* (Wall.ex Wight) L. H. Bailey and *M. pruriens* var. *hirsuta* (Wight and Arn.) Wilmot-Dear. A varietal kind of *M. pruriens*, called Thai *M. pruriens* (T-MP; locally known as *mamui*) or *M. pruriens* (L.) DC var. *pruriens*, is commonly found in northeast Thailand. The pods that hold the beans are covered in hairs that are strongly irritating to human skin. According to a previous study [28], its seeds are rich in certain essential amino acids, fatty acids and various minerals. In Thai folk medicine, the roasted seeds of T-MP are commonly used for treatment of male dysuria. In addition, T-MP is believed to improve fertility, mainly by increasing germ cell production and acting as an aphrodisiac. The expression of testicular tyrosine phosphorylated protein (TyrPho) and androgen receptor is involved in spermatogenesis and testosterone production [29–31]. High expression of A-kinase-anchoring protein 4 (AKAP4) in sperm has been shown to improve sperm motility and forward progression [32–34]. However, there have been no studies published that examine the antioxidant activity, safety or reproductive effects of T-MP. This study, therefore, used a rat model to evaluate the antioxidant capacity and subacute toxicity of T-MP seed extract, particularly in the reproductive system.

2. Materials and methods

2.1. Plants used

To minimize natural variations in levels of compounds present in the study plants, they were systemically cultured in one area (Fig. 1). Briefly, T-MP plant samples and seeds were collected from Thailand's Surin Province. Dr. Pranom Chantaranothai, a plant taxonomy expert in the Department of Biology of Khon Kaen University, verified that the specimens were of the *M. pruriens* (L.) DC var. *pruriens* and a T-MP voucher sample was stored (No. S. Iamsaard 01). The seeds were kept in the herbarium database at the Department of Biology, Khon Kaen University. Mature seeds were selected to plant at Ban Arwoot Village (Tael subdistrict, Srikhoraphum District, Surin Province). At about five months after planting, the plants flowered, and subsequently pods formed. The ripe pods of the first crop were harvested at 6 months after planting and were dried in the sun for one week (Fig. 1).

2.2. Plant extraction

Briefly, the sun-dried T-MP seeds were powdered using a stainless-steel, electric grinding machine. Then, T-MP powder (1 kg) was heated in distilled water (3000 mL) at 80–95 °C for 30 min, before being filtered through filter cloth. The aqueous seed filtrate was then frozen and lyophilized (Fig. 2). The percent yield of T-MP seed extract was calculated to be 10.68% ± 0.04%. Preliminary research showed that a 300 mg/kg body weight (bw) dose of T-MP crude powder could be well solubilized in distilled water and serves as a maximal dose. Thus, doses of 300 and 150 mg/kg bw were used in this study for the high- and low-dose treatments, respectively.

2.3. Total phenolic content and antioxidant capacity assays

2.3.1. Determination of total phenolic content

The total phenolic content (TPC) in T-MP extract was measured using the Folin-Ciocalteu reagent method, as described previously

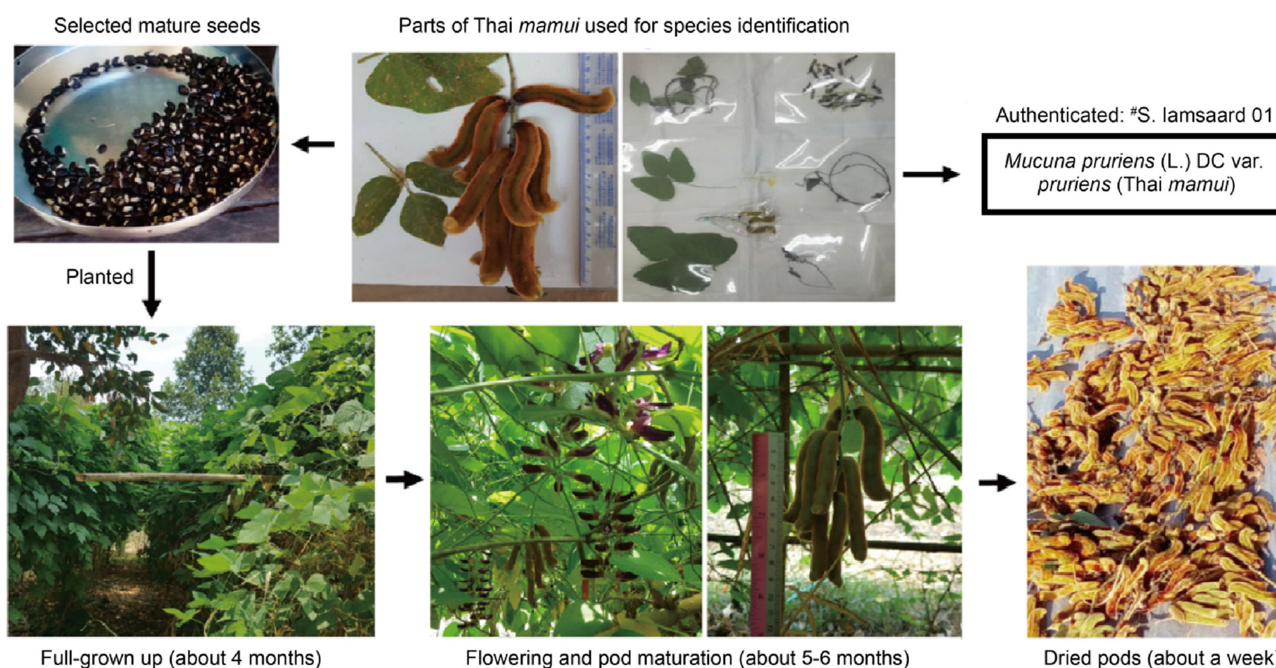

Fig. 1. Plant collection for authentication, seed selection from mature pods, and planting and pod harvesting of *Mucuna pruriens* (L.) DC var. *pruriens* (Thai *mamui*).

[35,36]. Gallic acid was used as a positive control, and five concentrations of gallic acid were used to construct a standard curve. Then, 0.2 mL of T-MP aqueous solution (2 mg/mL) was incubated with the Folin-Ciocalteu reagent (1 mL, 10%, v/v) for 5 min. A 3 mL aliquot of sodium carbonate (0.2 g/mL) was added to the mixture and incubated for 2 h, at 25 °C, in a dark room. The absorbance was read at 765 nm using an ultraviolet spectrophotometer (Jasco V530, Japan) in triplicate; and the absorbance of the test solution was expressed as gallic acid equivalents using the regression equation defined by the calibration curve.

2.3.2. 2,2-Diphenyl-1-picrylhydrazyl radical-scavenging assay

The evaluation of T-MP radical-scavenging activity was performed following published protocols [36,37]. The radical-scavenging ability of T-MP extract (5 concentrations) was compared to the standard compound, ascorbic acid ($y = 15.8420x + 3.9382$, $R^2 = 0.9929$). In brief, each T-MP concentration was mixed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol and incubated for 30 min at 25 °C, in a dark room. Then, the absorbance was measured at 517 nm in triplicate. The percentage of radical-scavenging capacity was calculated as described by Brand-Williams et al. [38]: DPPH radical scavenging (%) = ([DPPH absorbance − sample absorbance] × 100)/DPPH absorbance.

Additionally, ascorbic acid equivalent antioxidant capacity (AEAC) of T-MP (100 g) was calculated by a half maximal inhibitory concentration (IC_{50}) of standard ascorbic acid as described by Chew et al. [39].

2.3.3. Reducing power capacity assay

Reducing power capacity was measured using the ferric reducing antioxidant power (FRAP) assay as described previously [35,36]. Briefly, 0.1 mL of T-MP solution (1 mg/mL) was incubated with FRAP reagent (3 mL, 300 mmol/L acetate buffer; 20 mmol/L $FeCl_3$ [10:1:1, v/v/v]; and 10 mmol/L of 2,4,6-tripyridyl-*S*-triazine) in a dark room for 20 min. Subsequently, the absorbance was measured at 593 nm using an ultraviolet–visible spectrophotometer. The reducing power capacity of T-MP extract was compared to a standard curve plotted from 5 concentrations of ascorbic acid and reported as μg/mg ascorbic acid equivalent.

2.4. Animal grouping and T-MP treatment

Adult male and female Wistar rats ($n = 48$; males = 24 and females = 24; 180–200 g) were purchased from the Nomura Siam International Co. Ltd., Pathumwan, Bangkok, Thailand. All animals were transferred to the Animal Care Unit at the Faculty of Medicine, Khon Kaen University, Thailand, and acclimatized for a week, before grouping into six groups of 8 individuals: control (male), control (female), 150 T-MP (male), 150 T-MP (female), 300 T-MP (male) and 300 T-MP (female). For the 150 and 300 T-MP groups, rats were treated with T-MP extract at a dose of 150 or 300 mg/kg bw via oral gavage, while control animals were given distilled water for 14 consecutive days, to study the subacute toxicity. All rats were housed in stainless steel cages, maintained in the air-conditioned room at (25 ± 2) °C, with 12/12 h light/dark cycle and fed with standard chow diet (Chareon Pokapan Co. Ltd., Thailand). This study was approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (Ethic record No. AEMDKKU 005/2019).

2.5. Sex hormone and biochemical serum analysis

At the end of the experiment, all animals were euthanized and 3 mL of blood was collected from the left ventricle by cardiac puncture with a hypodermic needle. Blood samples from each group were immediately centrifuged at 13,000 r/min for 15 min at 4 °C, to separate the supernatant plasma from the blood cells. The levels

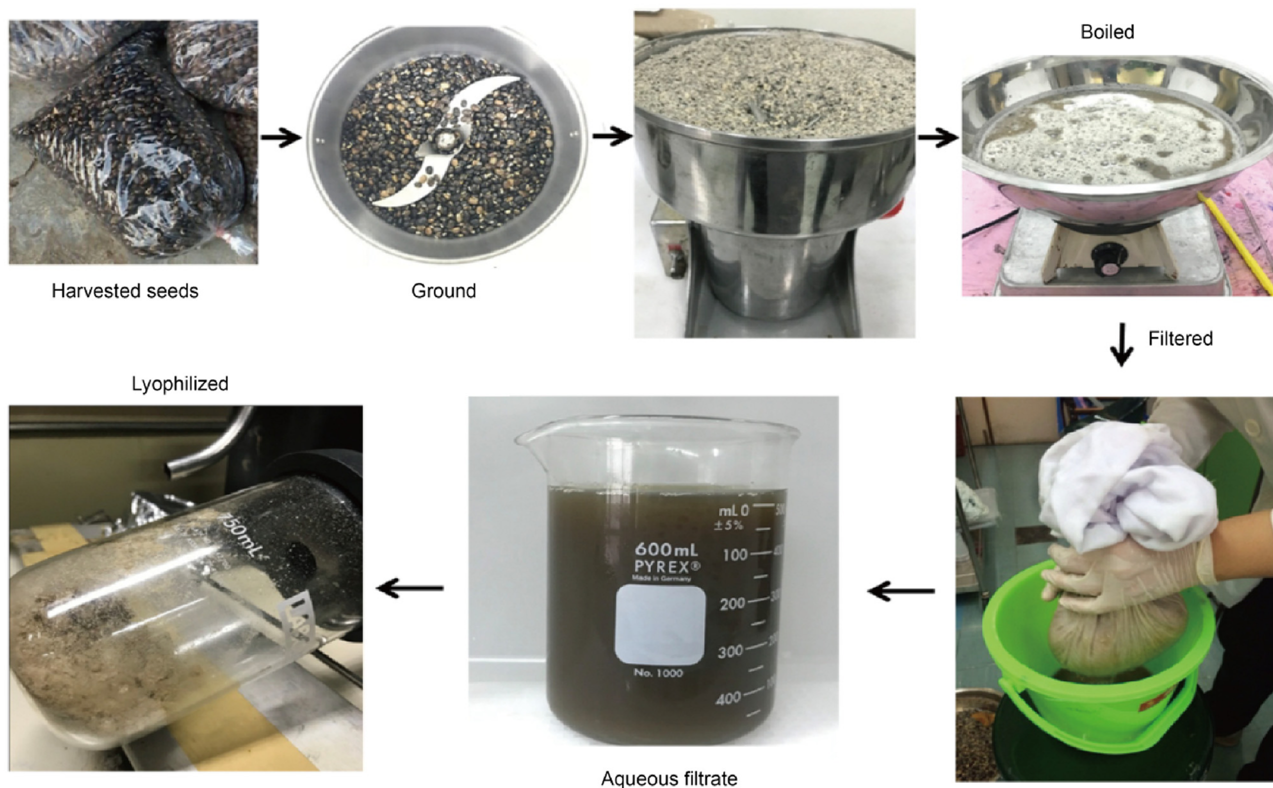

Fig. 2. The process of aqueous extraction from Thai *Mucuna pruriens* seeds.

of estradiol, progesterone and testosterone from blood serum or intratesticular lysate were measured using radioimmunoassay at the Radiology Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. Subsequently, the levels of liver and kidney function parameters, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, creatinine and blood urea nitrogen, were determined using the Roche/Hitachi Cobas c 501 system (Cobas® 6000 modular analyzer series, Roche Diagnostics Corporation) at the Diagnosis Clinical Chemistry Laboratory, Khon Kaen University.

2.6. Weights, morphological and histopathological examinations

After blood collection, the liver, kidney and reproductive organs, including ovary, oviduct, uterus, testis and epididymis plus vas deferens, were removed and weighted (to be calculated as absolute [g] and relative weights [g/kg bw] as previously described [40]). To investigate histopathological conditions such as cirrhosis or fibrosis, the organs were immediately fixed in 10% formalin, in phosphate buffered saline (PBS; pH = 7.4), for 48 h and processed for light microscopy, following standard protocols. All paraffinized sections (5–7 μm) were stained using Masson's trichrome kits (Sigma-Aldrich Inc., USA). The histopathological micrographs were taken using a Nikon ECLIPSE E200 light microscope (Nikon Inc., Japan) with a digital camera (Nikon Inc., Japan). The ImageJ program (version 1.49p, W. Rasband, the National Institutes of Health) was used to measure the seminiferous tubule diameter and germinal epithelial height in photomicrographs.

2.7. Sperm parameter analyses

2.7.1. Sperm count and morphology

Sperm fluid from the left caudal epididymis plus ductus deferens was gently squeezed out and dropped into 1 mL of PBS (pH = 7.4). The sperms were resuspended and centrifuged at 8000 r/min and 25 °C for 5 min, to wash and separate the mature sperm pellet from epididymal fluid. Subsequently, sperm pellets were resuspended with 1 mL of fresh PBS and 100 μL from each sample was fixed with 20 μL of 4% paraformaldehyde in PBS at 4 °C. The remaining sperm suspension (900 μL) was centrifuged again to collect the sperm pellet for protein extraction. The fixed sperm suspension was diluted with PBS (1:5 dilution) before using a Neubauer counting chamber under the light microscope to determine sperm density in triplicate [40]. The number of sperms was calculated as concentration ($\times 10^6$ cells/mL). For the sperm morphological abnormality assay, the fixed sperm suspension was smeared on a gelatin-coated glass slide and air-dried overnight. Then, the dried sperms were further fixed with methyl alcohol for 20 min before staining with hematoxylin for 20 min and eosin for 5 min. Abnormal sperm types (heads and tails) were classified as described by Sakr and co-workers [41], and the percentage of abnormal sperms was calculated from a total of 200 sperms/animal.

2.7.2. Sperm acrosome reaction assay

Briefly, dried sperms smeared on a gelatin-coated slide were stained with 22% Coomassie blue for 5 min [40]. After washing, the slides were mounted using mounting media. For each male rat, 200 sperms were counted and the acrosome was evaluated as being intact (acrosome cap stained with Coomassie blue) or reacted (AcR; acrosome cap unstained). Then, the percentage of AcR sperms was calculated.

2.7.3. Evaluations of incomplete sperm DNA structure and packaging

In brief, the sperm-smeared slides were fixed with 96% ethanol-acetone solution (1:1) at 4 °C for 30 min and then hydrolyzed in 0.1 mol/L HCl at 4 °C for 5 min. Then, the slides were washed in distilled water for 2 min and stained with 0.05% toluidine blue (TB, binding to fragmented DNA via a phosphate group) in 50% McIlvaine's citrate phosphate buffer (pH = 3.5) for 10 min at room temperature [42]. Under light microscope, the sperm not stained by TB was classified as sperm with normal DNA, while sperm stained by TB was deemed to have incomplete DNA structure and packaging [43].

2.7.4. Assessment and visualization of sperm chromatin condensation

Aniline blue (AB), a dye that selectively binds to lysine-rich histones, is commonly used for evaluation of abnormal sperm chromatin condensation. Briefly, smeared sperms were fixed in 2.5% glutaraldehyde buffer for 30 min at room temperature. Each smear was stained with 5% aqueous AB solution in 4% acetic acid (pH = 3.5) for 5 min. Two-hundred sperms in total were counted under a light microscope. The sperms not stained by AB were considered to have normal chromatin condensation, while sperms that were stained were classified as having abnormal chromatin, as described previously [44,45].

2.8. Western blotting analysis

2.8.1. Preparation of total testicular and sperm protein lysates

Total proteins from testicular tissues or sperm cell pellets were extracted using radioimmuno-precipitation assay buffer (Cell Signaling Technology, Inc., USA) containing protease inhibitor cocktails (Sigma, Inc., USA). The sample lysates were sonicated on ice (20 Hz, 60 pulses) using an ultrasonic processor (Cole, Parmer, Vernon Hills, Illinois, USA). Next, the homogenates were centrifuged at 14,000 r/min at 4 °C for 10 min to separate testicular or sperm lysate from the pellet. The total protein concentrations of each lysate were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA) at an absorbance of 280 nm. The total proteins (100 μg) were mixed with loading buffer dye and incubated at 95 °C for 5 min (Digital Dry Bath Incubators Model 113002, Boekel Scientific, USA) before conducting sodium dodecyl sulphate-electrophoresis on a 10% polyacrylamide gel.

2.8.2. Western blotting

After electrophoresis, the separated proteins from testis samples were transferred onto nitrocellulose membranes (Bio-Rad). Membranes were incubated with 5% skim milk in Tris-buffered saline Tween-20 buffer (TBST; 15 mmol/L Tris-HCl [pH = 8.0], 125 mmol/L NaCl and 0.1% [w/v] Tween-20). After washing with 0.05% PBS-Tween (PBS with 0.05% [v/v] Tween-20), the blotted membranes were further incubated with monoclonal antiphosphotyrosine (clone 4G10, 1:2000; Millipore Co., USA) or anti-androgen receptor (N-20) antibody (1: 200 [v/v]; Santa Cruz Biotechnology, Inc., USA) at 4 °C overnight. To detect sperm AKAP4, the sperm protein lysate blot membranes were probed with rabbit monoclonal anti-AKAP4 antibody (diluted 1:10,000 in 5% skim milk/TBST; Ab 230954; Abcam, Cambridge, UK). All membranes were incubated with specific horseradish peroxidase-conjugated secondary antibody diluted 1:5000 in TBST for 1 h at room temperature. Then, membranes were washed for 5 min (3 times) with TBST before detection of TyrPho, androgen receptor and AKAP4 using enhanced chemiluminescence substrate under gel doct 4 (ImageQuant 400, GH Healthcare, USA).

2.9. Statistical analysis

All statistical analyses were carried out using the SPSS statistical software (SPSS for Windows, SPSS Inc., Chicago, IL, USA, version 17.0). The Shapiro-Wilk test was used to confirm normal distribu-

tion of the data. Differences among groups were tested using one-way analysis of variance followed by a posthoc Turkey test. All data were expressed as mean ± standard deviation. A P value ≤ 0.05 was deemed statistically significant.

3. Results

3.1. TPC and antioxidant activity of T-MP

Based on a calibration curve calculated for the gallic acid standard ($y = 0.0044x + 0.1069$, $R^2 = 0.9986$), TPC of T-MP was determined to be (230.00 ± 9.31) µg/mg gallic acid equivalents. The FRAP capacity of T-MP was (561.04 ± 3.30) µg/mg ascorbic acid equivalents. For the DPPH assays, the IC_{50} of this seed extract was determined to be (10.75 ± 0.04) µg/mL. Moreover, the AEAC of T-MP was (27,029.26 ± 936.89) mg ascorbic acid equivalents in each 100 g of powdered extract.

3.2. Effects of T-MP on body and organ weight

T-MP did not have an effect on change in body weight over the course of the experiment for male or female rats. Similarly, it had no effect on the absolute and relative weights of the liver and kidney on subacute treatment for either group (Table 1). In addition, the weights of the testis, epididymis plus vas deferens and seminal vesicle plus prostate gland did not differ significantly between control and T-MP-treated rats ($P > 0.05$; Table 2). In the females, T-MP did not affect the relative weights of the ovaries, oviduct or uterus ($P > 0.05$). By contrast, the absolute weights of the ovaries in the 150 and 300 T-MP rats were significantly lower than in the controls ($P < 0.05$; Table 3).

3.3. Histopathology

Masson's trichrome staining revealed no obvious histopathological differences between control and T-MP-treated rats (particularly in terms of increased collagen fibers of the kidney and liver tissue). The microstructures of the renal corpuscles, tubular epithelium, portal triad and hepatic cords had not been altered in any of the groups (Fig. 3).

In addition, T-MP did not cause histological changes to ovary, oviduct or uterine tissue. Ovaries in both groups exhibited normal ovarian cortex events, including growing follicles and some developing corpus lutea (Fig. 4).

In males, atrophic seminiferous tubules and wide interstitial spaces were not observed in any groups (Fig. 5). Moreover, T-MP did not affect the epididymal epithelium, but the density of sperm masses stored in the lumen of T-MP-treated rats was higher than in controls (Fig. 5).

3.4. Effects of T-MP on serum sex hormone levels and biochemical parameters

There were no significant differences in the levels of any biochemical parameters that determine liver and kidney function in the T-MP male rats compared with controls (Table 4). By contrast, both low and high doses of T-MP significantly decreased serum and intratesticular testosterone levels (Table 4). In the females, T-MP significantly increased estradiol but decreased progesterone levels in a dose-dependent fashion (Table 5). In addition, serum AST and ALT levels were significantly higher, while alkaline phosphatase levels were lower, in the T-MP-treated female animals compared with controls. However, creatinine and urea nitrogen levels did not differ significantly (Table 5).

3.5. Effects of T-MP on seminiferous tubule morphometries and sperm parameters

Table 6 shows that neither low nor high doses of T-MP seed extract affected seminiferous tubule diameter or epithelial height. In addition, there were no significant differences in sperm parameters, including percent of sperm morphology, acrosome-reacted sperms, incomplete sperm DNA packaging and chromosome decondensation, between T-MP-treated rats and controls. The higher dosage (300 mg/kg bw) of T-MP increased the concentration of caudal sperms significantly ($P < 0.05$; Table 6).

3.6. Effects of T-MP on expressions of tyrosine phosphorylation, androgen receptor and AKAP4

We found that T-MP increased the expression of testicular Tyr-Pho proteins (50, 43, 34, 29, 26 and 20 kD; Fig. 6A) and androgen receptor (Fig. 6B) in testicular lysate, in a dose-dependent manner. Moreover, the expression of AKAP4 in sperm lysate was also higher in rats treated with T-MP extracts at doses of 150 and 300 mg/kg bw (Fig. 6C).

4. Discussion

The antioxidant capacity of ethanol-based seed extract from *M. pruriens* varieties possessing pods without irritating bristles has been reported [1,45]. This is the first study that has examined the TPC and antioxidant activity in the aqueous seed extract of T-MP. The black seeds of *M. pruriens* demonstrated high antioxidant capacity, as has been shown in previous studies [46]. The bioactive compounds in both water- and methanol-based *M. pruriens* seed extract are proteins, amino acids, tannins, alkaloids, isoquinolines, cyclitols, oligosaccharides, phenols and levodopa [1,8,45–47]. Additionally, *M. pruriens* has been shown to exhibit various pharmacological properties, including antivenom, antimicrobial, neuroprotective and antidiabetic activities [1]. Because *M. pruriens*

Table 1
Effects of T-MP seed extract on body weight change (%), and liver and kidney weight.

Group		Body weight change (%)[a]	Liver weight		Kidney weight	
			Absolute (g)	Relative (g/kg bw)	Absolute (g)	Relative (g/kg bw)
Male	Control	42.02 ± 1.63	13.91 ± 1.56	44.52 ± 4.22	12.38 ± 0.68	3.89 ± 0.23
	150 T-MP	39.12 ± 1.92	11.91 ± 0.57	39.21 ± 1.03	12.12 ± 1.95	3.82 ± 0.31
	300 T-MP	37.50 ± 4.29	12.95 ± 0.96	41.44 ± 2.22	12.32 ± 4.42	4.02 ± 0.37
Female	Control	16.49 ± 3.55	7.77 ± 0.78	34.85 ± 2.59	7.58 ± 0.19	3.41 ± 0.14
	150 T-MP	16.49 ± 0.34	8.54 ± 0.20	39.03 ± 2.69	7.70 ± 0.40	3.10 ± 1.30
	300 T-MP	13.42 ± 1.46	7.73 ± 0.44	37.77 ± 1.86	7.40 ± 0.47	3.67 ± 0.20

Results are expressed as mean ± standard deviations ($n = 8$). Data were analyzed using one-way analysis of variance followed by Tukey's posthoc test. T-MP: Thai *Mucuna pruriens*; bw: body weight.
[a] Compare to initial.

Table 2
Effects of T-MP seed extract on reproductive organ weights among male rats.

Group	Testis weight		Epididymis & vas deferens weight		Seminal vesicle & prostate weight	
	Absolute (g)	Relative (g/kg bw)	Absolute (g)	Relative (g/kg bw)	Absolute (g)	Relative (g/kg bw)
Control	1.68 ± 0.11	5.27 ± 0.36	0.51 ± 0.07	1.66 ± 0.18	1.43 ± 0.25	4.51 ± 0.68
150 T-MP	1.61 ± 0.07	5.18 ± 0.62	0.47 ± 0.02	1.47 ± 0.16	1.30 ± 0.07	4.16 ± 0.29
300 T-MP	1.66 ± 0.10	5.39 ± 0.47	0.51 ± 0.01	1.64 ± 0.09	1.35 ± 0.10	4.49 ± 0.22

Results are expressed as mean ± standard deviations (n = 8). Data were analyzed using one-way analysis of variance followed by Tukey's posthoc test. T-MP: Thai *Mucuna pruriens*; bw: body weight.

Table 3
Effects of T-MP seed extract on reproductive organ weights among female rats.

Group	Ovary weight		Oviduct weight		Uterus weight	
	Absolute (g)	Relative (g/kg bw)	Absolute (g)	Relative (g/kg bw)	Absolute (g)	Relative (g/kg bw)
Control	0.073 ± 0.007	0.270 ± 0.140	0.018 ± 0.004	0.070 ± 0.040	0.224 ± 0.043	1.010 ± 0.210
150 T-MP	0.061 ± 0.006*	0.280 ± 0.030	0.015 ± 0.001	0.060 ± 0.030	0.207 ± 0.065	1.020 ± 0.320
300 T-MP	0.060 ± 0.004*	0.290 ± 0.030	0.016 ± 0.001	0.080 ± 0.010	0.178 ± 0.031	0.860 ± 0.150

Results are expressed as mean ± standard deviations (n = 8). Data were analyzed using one-way analysis of variance followed by Tukey's post hoc test. *$P < 0.05$, *vs* control group. bw: body weight. T-MP: Thai *Mucuna pruriens*.

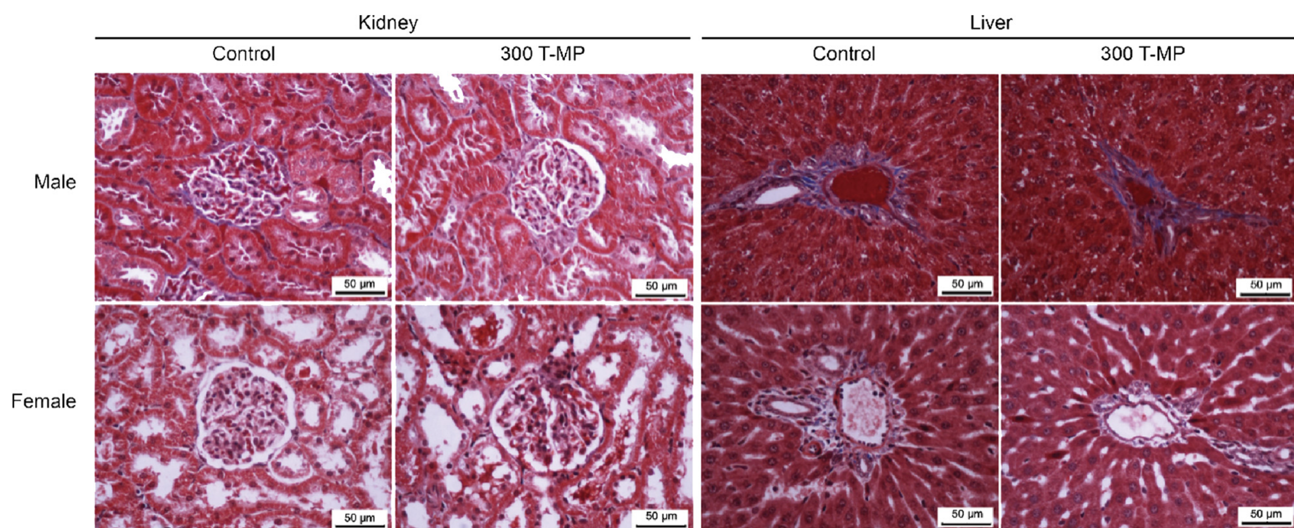

Fig. 3. Representative histological micrographs of kidney and liver tissue from control and high-dose T-MP (300 mg/kg body weight) males and females, stained with Masson's trichrome. T-MP: Thai *Mucuna pruriens*.

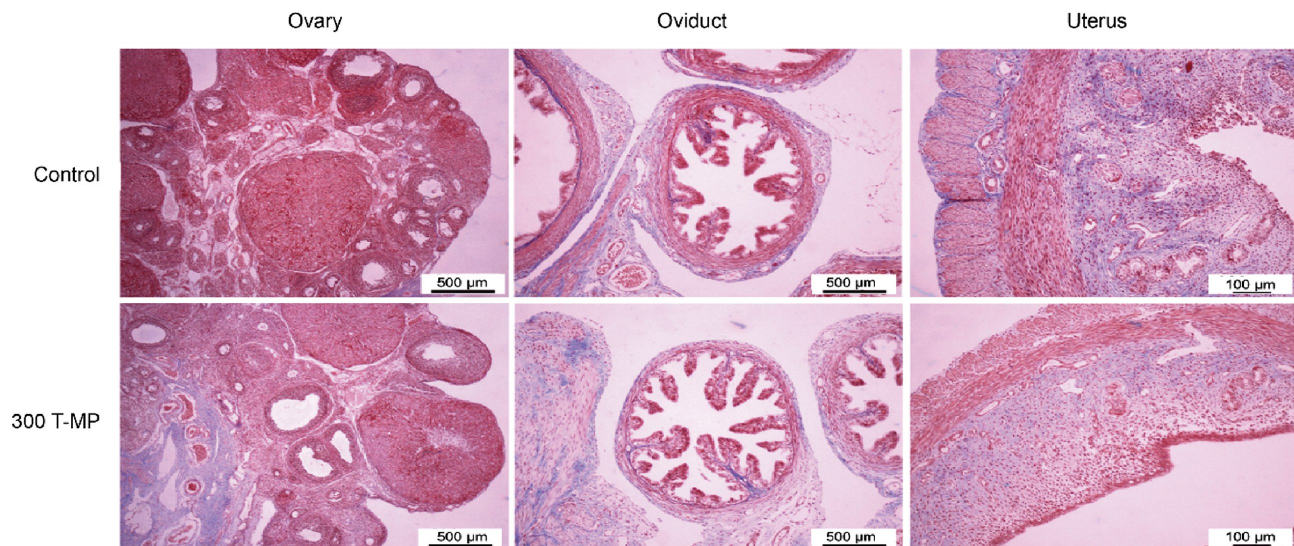

Fig. 4. Representative histological micrographs of ovary, oviduct and uterine tissues from the control and high-dose T-MP (300 mg/kg body weight), stained with Masson's trichrome. T-MP: Thai *Mucuna pruriens*.

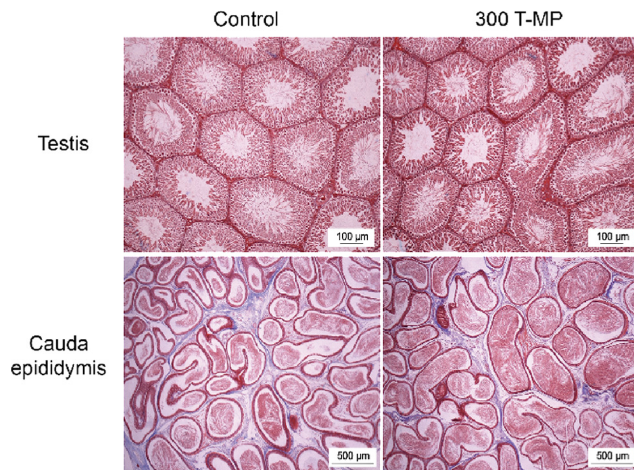

Fig. 5. Representative histological micrographs of seminiferous tubules in rat testis and cauda epididymal tissues from control and high-dose T-MP (300 mg/kg body weight) rats, stained using Masson's trichrome. The density of sperm masses within the epididymis lumens of the T-MP group was greater in control animals (n = 8 for each group). T-MP: Thai *Mucuna pruriens*.

contains levodopa and serotonin, its seed extract has traditionally been used as an aphrodisiac and for treating various nervous disorders, particularly Parkinson's disease. Moreover, it has been shown to enhance male sexual function and sperm parameters [23,24]. The reason for T-MP's use in Thai alternative medicine for treatment of male dysuria may be based on its actions as an aphrodisiac and facilitation of penile erection.

The T-MP crude powder used in this study was easily diluted and well solubilized in distilled water at a dose of 300 mg/kg bw, which is not the case for the ethanol extracts that have been previously described [48,49]. *M. pruriens* seed extract at doses of 2000 and 5000 mg/kg bw has been shown to have no toxicological effects in rats. In our study, although T-MP treatment did not affect liver and kidney histology in either sex, it did alter function parameters in the females, indicating an association with sex hormone control. In addition, T-MP had no effect on the female reproductive organs, but it did significantly increased serum estradiol and decreased progesterone levels. This result was confirmed by the observation of well-developed ovarian follicles, as shown in Fig. 5. This suggests that T-MP exhibits phytoestrogenic activity, which is a novel finding and is supported by the fact that testosterone levels in both serum and intratesticular tissue in the male rats were significantly lower than in the controls (Table 4). However, we found that cauda sperm concentration in the high-dose T-MP (300 mg/kg bw) group was significantly higher. This suggests that the reduced testosterone levels (approximately 4.55 ng/mL) were high enough to maintain spermatogenesis. In addition, other bioactive substances in T-MP extract may be involved in stimulating sperm production, as shown in Table 6. The seed extract did not have any effects on the diameter or epithelial height of seminiferous tubules or on sperm parameters, indicating that T-MP is a safe

Table 4
Effects of T-MP seed extract on sex hormones, and liver and kidney function parameters in male rats.

Group	Serum T (ng/mL)	Testicular T (10^{-2} ng/μg total protein)	AST (U/L)	ALT (U/L)	Alkaline phosphatase (U/L)	Creatinine (mg/dL)	Urea nitrogen (mg/dL)
Control	9.54 ± 0.15	2.30 ± 0.31	21.84 ± 1.11	0.22 ± 0.02	52.00 ± 2.24	0.11 ± 0.07	15.80 ± 1.53
150 T-MP	7.88 ± 0.24*	1.25 ± 0.04*	21.40 ± 1.37	0.23 ± 0.04	53.80 ± 1.79	0.11 ± 0.08	16.06 ± 1.43
300 T-MP	4.55 ± 0.49*	1.00 ± 0.04*	21.68 ± 0.07	0.21 ± 0.02	52.60 ± 2.61	0.12 ± 0.02	16.20 ± 1.19

Results are expressed as mean ± standard deviations (n = 8). Data were analyzed using one-way analysis of variance followed by Tukey's post hoc test. *P < 0.05, *vs* control group. ALT: alanine aminotransferase; AST: aspartate aminotransferase; T: testosterone; T-MP: Thai *Mucuna pruriens*.

Table 5
Effects of T-MP seed extract on sex hormones, and liver and kidney function parameters in female rats.

Group	AST (U/L)	ALT (U/L)	Alkaline phosphatase (U/L)	Creatinine (mg/dL)	Urea nitrogen (mg/dL)	Serum estradiol (ng/mL)	Serum progesterone (ng/mL)
Control	20.00 ± 2.37	25.50 ± 1.19	59.50 ± 1.19	0.29 ± 0.02	21.70 ± 1.35	55.30 ± 0.29	33.10 ± 1.19
150 T-MP	25.00 ± 1.58	28.50 ± 0.40*	53.00 ± 0.79*	0.30 ± 0.03	21.44 ± 1.28	80.38 ± 0.87*	20.48 ± 1.14*
300 T-MP	83.50 ± 0.40*	30.50 ± 0.40*	52.90 ± 0.74*	0.29 ± 0.03	21.60 ± 2.26	84.23 ± 4.17*	15.24 ± 0.08*

Results are expressed as mean ± standard deviations (n = 8). Data were analyzed using one-way analysis of variance followed by Tukey's post hoc test. *P < 0.05, *vs* control group. ALT: alanine aminotransferase; AST: aspartate aminotransferase; T-MP: Thai *Mucuna pruriens*.

Table 6
Effects of T-MP seed extract on diameter and epithelial height of the seminiferous tubule and on sperm parameters.

Group	Seminiferous tubule diameter (μm)	Seminiferous epithelial height (μm)	Cauda sperm concentration (10^6/mL)	Abnormality of sperm morphology (%)	Acrosome-reacted sperms (%)	Incomplete sperm DNA structure & packaging (%)	Decondensed sperm chromatin (%)
Control	187.45 ± 3.15	41.75 ± 5.59	6.60 ± 1.15	17.13 ± 1.80	5.50 ± 0.05	2.33 ± 0.29	1.00 ± 0.50
150 T-MP	188.52 ± 3.45	40.85 ± 5.84	7.23 ± 0.85	18.83 ± 0.76	6.75 ± 1.71	2.60 ± 0.47	1.60 ± 1.14
300 T-MP	187.30 ± 3.49	39.81 ± 5.32	13.99 ± 0.75*	15.90 ± 2.88	6.80 ± 2.49	2.50 ± 0.10	1.50 ± 1.27

Results are expressed as mean ± standard deviation, with n = 8. Data were analyzed using one-way analysis of variance followed by Tukey's post hoc test. *P < 0.05, *vs* control group. T-MP: Thai *Mucuna pruriens*.

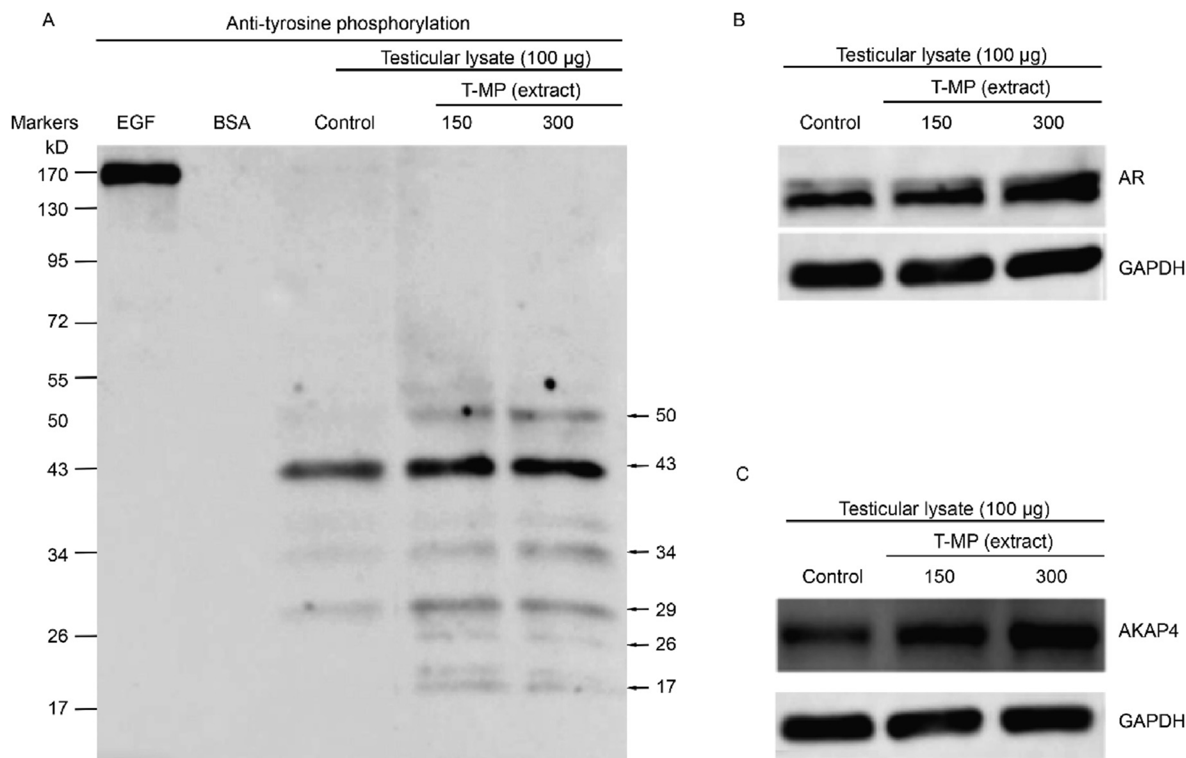

Fig. 6. Expression of tyrosine phosphorylated proteins (A), AR (B) and sperm AKAP4 (C). EGF: epidermal growth factor; BSA: bovine serum albumin; AR: androgen receptor; AKAP4: A-kinase-anchoring protein 4; GAPDH: reduced glyceraldehyde-phosphate dehydrogenase.

and effective treatment for enhancing sperm production. Testicular TyrPho proteins and androgen receptor are localized in the testes and involved in spermatogenesis and androgen synthesis [29–31,50]. Furthermore, the presence of AKAP4 on the principal piece of the sperm tail is essential for forward motility in the female reproductive tract [32–34,51]. T-MP extract exhibited fertility-enhancing effects on sperm concentration, morphology and progressive motility, due to its ability to augment testicular TyrPho protein, androgen receptor and sperm AKAP4 expression (Fig. 5).

5. Conclusion

We found that T-MP seed extract has high antioxidant capacity and is nontoxic to the reproductive system. Moreover, it has phytoestrogenic effects on female rats and fertility-enhancing effects on males. It is thus possible to develop T-MP seed extract as a natural fertility supplement to promote reproductive health.

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Authors' contribution

SI contributed to experimental design, developing manuscript and critical discussion; SA contributed to data collection and statistical analysis; JB contributed to antioxidant and phenolic component analyses; SY contributed to sperm morphology analyses and figure arts; ST contributed to data collection and histological analyses; SB contributed to blood collection and sex hormone determination; NL contributed to data collection, plant extraction and animal handling; PC contributed to Western blotting; NT contributed to semen analysis and acrosome reaction assays; CC contributed to data collection and animal handling; WS contributed to blood collection and serum biochemical determinations.

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Conflicts of interest

The authors declare that that they have no conflicts of interest.

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