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In vitro and *in vivo* investigation of natural compounds from seed extract of *Mucuna pruriens* lacking L-DOPA for the treatment of erectile dysfunction

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ABSTRACT

Objective: To investigate the biological effects of the *Mucuna pruriens* (*M. pruriens*) seed extracts that lacked L-DOPA, which was formerly reported as the active ingredient, on erectile dysfunction (ED) both *in vitro* and *in vivo*.

Methods: Seed of *M. pruriens* plant that cultivated in Mae Taeng District, Chiang Mai Province, Thailand, was collected. Component of its seeds were extracted and isolated into 2 fractions using methanol, polar and nonpolar. Each fraction was investigated for phytochemicals using gas chromatography and mass spectroscopy and was screened for biological activity *in vitro* using three different cell lines. The most biological active fraction was used to treat both streptozotocin (STZ)-induced diabetes mellitus-erectile dysfunction (DM-ED) male Wistar rats and normal rats (n = 6 per groups) to compare the effect on sexual behavior parameters, including number of intromission, mounting and ejaculation, with that of rats given Sildenafil by individually pairing with their female counterparts. Penile tissues and serums were collected to determine histological structure, related gene expression and biomolecules.

Results: The phytochemicals of the polar fraction were possibly catechol and its derivatives plus polyphenols, whereas the nonpolar fraction consisted of lipid derivatives. L-DOPA was not detected in either of the extracts. The polar fraction was able to up-regulate the expression of ED-related genes including eNOS and nNOS *in vitro* which subsequently promotes nitric oxide production and maintains intracellular cyclic guanosine monophosphate levels. When administrated to DM-ED rats, the polar extract significantly improved all sexual behavior parameters in DM-ED rats compared to untreated group (18.3 ± 1.8 to 10.8 ± 2.9 for intromission, 9.8 ± 2.2 to 5.7 ± 1.3 for mounting, and 1.8 ± 0.6 to 0.2 ± 0.4 for ejaculation). That effect might due to the ability of the extract to stimulate the expression of eNOS and nNOS which results in nitric oxide production and subsequently maintains cyclic guanosine monophosphate levels in penile tissue. Moreover, this extract may also prevent penile tissue deterioration due to diabetes.

Conclusions: The polar extract of *M. pruriens* seed can be used for ED therapy, especially in patients with metabolic diseases including diabetes. The action of the extract might be due to catechol and its derivatives and polyphenols.

1. Introduction

Erectile dysfunction (ED) is one of the most common disabilities found in elderly men [1]. It has been defined as the incapability to achieve or maintain a penile erection adequate for satisfactory sexual intercourse [2]. The prevalence of ED may involve a complex interplay among vascular, neurogenic, hormonal and psychological factors which are important for erection [3]. In pathophysiology terms, the condition of impotence is caused by impaired function of arteries and corpora cavernosa within penis [4]. Impairment of cavernosal arteries, lack of smooth muscle tone and defects in neuronal stimuli can lead to unsuccessful penile erection [5]. The occurrence of ED is also related to other cardiovascular and neuronal diseases including atherosclerosis and diabetes [6,7]. The major biomolecule which plays an important role in penile erection is nitric oxide (NO) [8]. NO is synthesized through the activity of nitric oxide synthase (NOS) enzymes which are

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expressed in the endothelium of arteries and by neuron cells. NO stimulates production of cyclic guanosine monophosphate (cGMP) and results in smooth muscle relaxation and vasodilation through the NO/cGMP pathway [9,10]. Shortage of intracellular NO and/or cGMP can lead to ED. Retaining intracellular cGMP levels by inhibiting its regulating enzyme cGMP-specific phosphodiesterase (PDE5) is considered to be the most therapeutically important target [11]. Sildenafil (Viagra®) is the most well-known PDE5 inhibitor and is the most common oral medication for ED therapy [12]. However, sildenafil has some potentially life-threatening side effects, such as severe hypotension and cardiac arrhythmia [13], and has limitations in usage benefits, *e.g.*, it becomes less effective when used by patients with a vascular or neuronal deficiency such as diabetes [14]. Alternative therapeutic agents which can be used as sildenafil substitutions to avoid the disadvantages are needed.

Natural products have been considered as an alternative medicine for ED [15,16]. One of the most promising plants which have been studied for ED treatment properties is *Mucuna pruriens* (*M. pruriens*). *M. pruriens*, also known as velvet bean, is a tropical legume which is mentioned in ancient Ayurvedic literature as a versatile medicinal plant [17]. Its seed is known to have benefits for Parkinsonism as it contains high amount of levodopa (L-DOPA), a precursor for numerous neurotransmitters including dopamine, noradrenaline and adrenaline [18–20]. A number of scientific papers report the benefit of *M. pruriens* seed for diabetes, as its extract can significantly reduce blood glucose levels [21,22]. In research studies of ED, *M. pruriens* seed extract has been reported to be effective. Ethanolic extract of *M. pruriens* seed has been found to pharmacologically improve sexual performance in both normal rats [23] and diabetes-induced ED rats [24]. The extract is also reported to help penile tissue recover from and protect against oxidative stress in animal models [25]. The effects of *M. pruriens* have been described as relying on L-DOPA which is reported to stimulate aphrodisiac activity and to recover spermatogenic loss in infertile subjects [26]. The potency of L-DOPA in *M. pruriens* has been confirmed in an animal model using lipid-based extraction to enhance the amount of L-DOPA obtained [27]. However, L-DOPA has been shown to be involved only in energetic aspects of appetitive sexual behavior in men, but not genital or subjective sexual arousal [28]. Moreover, there are phytochemicals other than L-DOPA which have biological activities, including alkaloids, phenolic compounds, saponins and oligosaccharides which might be useful on ED [29,30]. In this study, we investigated active ingredients of *M. pruriens* seed other than L-DOPA for ED therapy using a solvent extraction process and confirming their activities using *in vitro* models. Phytochemicals in each fraction were identified using gas chromatography and mass spectroscopy (GC/MS). The proper fraction was applied to streptozotocin (STZ)-induced diabetes mellitus-induced ED (DM-ED) Wistar rats, which is a general model for ED research *in vivo* [31], to compare the biological effects to those of sildenafil.

2. Materials and methods

2.1. Chemicals and reagents

Sildenafil citrate (Viagra®) 100 mg tablets were purchased from Pfizer (Thailand) Ltd. 2,2-Diphenyl-1-picrylhydrazyl, Griess's reagent and STZ were purchased from Sigma–Aldrich® Inc., Singapore. cGMP Parameter Assay Kit was purchased from R&D Systems, Inc., USA. Illustra RNAspin Mini RNA Isolation Kit was purchased from GE Medical Systems (Thailand) Ltd. Tetro cDNA Synthesis Kit and SensiFAST™ SYBR® Lo-ROX Kit were purchased from Bioline Singapore. Reagents used in cell culture were acquired from Gibco®, Thermo Fisher Scientific Co., Ltd., Thailand. Other chemicals used in this study were analytical grade.

2.2. M. pruriens seed extract preparation

M. pruriens seeds were harvested from Mae Taeng District, Chiang Mai Province, Thailand, and were blended into a fine powder using an industrial blending machine. The crude powder, which weighed about 1 kg, was macerated with 4 L of methanol at ambient temperature for 7 days with occasional stirring. The methanolic solution was filtered with a Whatman No.1 filter membrane and evaporated using a vacuum evaporator. The extraction was repeated four times and all the extracts were pooled. An appropriate amount of warm distilled water was used to reconstitute the extract. Diethylether in a volume equal to the distilled water was used for solvent partitioning. Solvent extraction using diethylether was repeated four times and all the diethylether layers were pooled to be evaporated. The water fraction was evaporated to eliminate any remaining organic solvent and the residue was dried using the lyophilization technique. The extract from the water layer was designated the 'polar fraction' while the extract from the diethylether layer was designated the 'nonpolar fraction'. Antioxidant activity of each fraction was determined using 2,2-diphenyl-1-picrylhydrazyl assay as described above [32,33].

2.3. Phytochemical identification using the GC/MS technique

Both the polar and nonpolar fractions of the *M. pruriens* seed extracts were weighed and dissolved in methanol to make a 1 mg/mL solution. One microliter of each sample was injected in split less mode into a GC-FID apparatus (GC 7890A Agilent Technology) attached to an MS instrument (MSD 5975C [EI] Agilent Technology) located at the Science and Technology Service Center (STSC), Faculty of Science, Chiang Mai University. Chromatographic analysis was conducted using the method described above [34]. All peaks were identified using the MS spectra library provided with Agilent GC/MS software.

2.4. Cell culture and treatment

Human endothelial hybrid cell line Ea.hy926, human colorectal carcinoma cell line HCT-15 and rat glioma cell line C6 were acquired from ATCC® number CRL-2922, CCL-225 and CCL-107, respectively. Ea.hy926 was cultured in Dulbecco's Modified Eagle Medium complemented with 10% fetal bovine serum, 2% hypoxanthine-aminopterin-thymidine (HAT) medium, 100 U/mL penicillin G sodium and 100 μg/mL streptomycin. C6 and HCT-15 were cultured in RPMI 1640 medium with the same supplements as Ea.hy926 with the exception of HAT medium. The cells were kept in an incubator with 5% CO_2 at 37 °C. For determination of NO production, remaining intracellular cGMP and gene expression, cells were plated in 6-

well plates at a concentration of 2×10^5 cells per well. The cell densities were required to reach their 80% confluence before being used in the experiments. Phytochemicals at indicated concentrations from MTT assay were used to treat the cells. Sildenafil at a concentration 25 μM was used as the positive control in the *in vitro* study. After the treatment period, cell lysates were collected for the determination of gene expression or intracellular cGMP level, while culture supernatants were collected for the measurement of NO.

2.5. Animal management and substance treatment

Male Wistar rats of 250–300 g, 6–8 weeks old were purchased from the National Laboratory Animal Center, Mahidol University, Thailand, and kept at a temperature of 25 ± 2 °C with a light–dark cycle of 12/12 h at the Laboratory Animal House, Faculty of Medicine, Chiang Mai University. All rats were allowed standard rodent chow and clean tap water *ad libitum*. All procedures involving handling of the animals in this study were in accordance with the Council for International Organizations of Medical Sciences guidelines and were approved for Animal Research Ethics by the Animal Research Committee of the Faculty of Medicine, Chiang Mai University, Thailand (Protocol No. 37/2547).

The rats were divided into 8 groups: 1) control group, 2) DM-ED group, 3) sildenafil group, 4) DM-ED with sildenafil group, 5) low concentration of *M. pruriens* extract (LMP) group, 6) DM-ED with LMP group, 7) high concentration of *M. pruriens* extract (HMP) group and 8) DM-ED with HMP group. Each group consisted of 6 rats which were allowed to acclimate in the laboratory for a week prior to initiation of the experiment. STZ was intraperitoneally injected into the DM-ED group at a dose of 60 mg/kg body weight. Total body weight and blood glucose levels were measured before and after STZ administration. Rats were confirmed as diabetic when their blood glucose levels rose to more than 2.0 g/L and they lost a significant amount of body weight. One week after the rats had been successfully induced to become diabetic, the apomorphine (APO) test was performed to confirm penile erection ability. All rats in each groups were subcutaneously injected at the back of the neck with 80 μg/kg of APO. They were then kept in a dark inspection box for 10 min. Following the injection and the period in the dark box, erection of the glans penis was observed for 20 min for the frequency and duration. This APO test was performed to confirm that the generation of DM-ED rats had been successful [31]. In the sildenafil-treated group, a solution of 5 mg/kg body weight of standard sildenafil was fed orally to the rats daily for 3 weeks after the diabetic condition had been successfully generated. The concentration of *M. pruriens* extracts used was 20 mg/kg and 200 mg/kg body weight in the LMP group and the HMP group, respectively. An oral suspension of *M. pruriens* extract was prepared in distilled water and administrated to specimens daily during the treatment period as well. Female Wistar rats were compatibly arranged related to male rat numbers and fed the same as the male rats but without substance treatments. Prior to the mating experiment, 2 μg/kg estradiol benzoate and 500 μg/kg progesterone were subcutaneous injected into the female rats. After observation of sexual behavior, all male experimental subjects were euthanized using pentobarbital (Nembutal®, Liboume, France) at concentration of 100 mg/kg of body weight. Penile tissue was immediately placed in liquid nitrogen and stored at −80 °C until used. Tissue sample examination included Masson's trichrome staining, gene expression determination with real-time PCR and intracellular cGMP determination. Blood samples were also collected in heparin tubes and centrifuged at 2 000 *g* for 10 min to isolate serum. That serum was collected under standard protocol and then used for NO determination [35].

2.6. Sexual behavior observation

The male rats in each group were mated individually with a single female rat in an isolated inspection box. The observation period for each pair, which was recorded by digital video recorder, was 20 min. The observation was performed by inspectors using double-blinded experiment. The sexual parameters consisted of the number of mountings, intromissions and ejaculations. Mounting number is the number of mounts without intromission from first introduction to the female. Intromission number is the number of attempts at sexual intercourse by the male rat after introduction to the female. Ejaculation number is the number of incidents of ejaculation during intercourse by the male rat [36].

2.7. Masson's trichrome staining

This histological technique was used to evaluate the effect of the different treatments on the cavernosal tissues by determining the number and arrangement of smooth muscle and collagen in the cavernosa. Isolated penises from rats in each group were fixed in 10% formaldehyde and kept at −80 °C until the tissue section process. Masson's trichrome staining was performed [37] by the Department of Pathology, Faculty of Medicine, Chiang Mai University. Tissues were observed under fine microscope where the collagen fiber was presented in blue color, smooth muscle cells were red and nuclei showed as black spots. Determination of the differences between each group was done qualitatively.

2.8. NO determination

Released NO was measured via nitrite, the stable oxidized form of NO which was distributed in the culture supernatant or serum samples [38]. One microliter of culture supernatant or diluted serum samples was then immediately collected and Griess reagent (0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4) was added in an equal volume. The mixture was incubated at room temperature for 10 min and absorbance was measured at 540 nm using a microplate reader. The concentration of nitrite in the samples, which ranged from 1–100 μM, was calculated using the standard curve generated from the reaction of Griess reagent with the sequence of sodium nitrite concentrations. An appropriate dilution was required with some of the serum samples to produce suitable results.

2.9. Intracellular cGMP determination

The level of intracellular cGMP was measured using a Parameter™ cGMP Competitive ELISA Kit. The treated Ea.hy926 or HCT-15 cells were lysed using lysis buffer

provided with the test kit; 100 μL of each sample was transferred onto the analytical plate. For penile tissues, samples were homogenized using homogenizer and lysed by RIPA buffer [39]. The experimental procedure was performed following the manufacturer's guidelines. After that process, absorbance was measured at UV 450 nm. The concentration value of PGE2 in the samples was determined using Genesis software and the value was compared to the data obtained from the standard cGMP at a concentration range of 0–500 pmol/mL.

2.10. Gene expression analysis using real-time reverse transcriptase polymerase chain reaction (real-time RT PCR)

Total RNA was extracted using the Illustra RNAspin Mini RNA Isolation Kit. Reverse transcription reaction was performed using 1.0 μg of total RNA which was then reverse-transcribed into cDNA using a Tetro cDNA Synthesis Kit. Real-time RT-PCR was conducted using a SensiFAST™ SYBR® Lo-ROX Kit according to the manufacturer's protocol. PCR primers were obtained from Invitrogen™. The reactions were conducted to determine the reaction of denaturation, annealing and extension on the Real-Time PCR detection system using a 7500 Fast Real-Time PCR instrument. The data were collected and the fold changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method [40]. The genes of interest in the human cells are iNOS, eNOS and PDE5. The genes used in the rat cell study were eNOS, nNOS and PDE5. β-Actin of both species was used as the reference constitutive gene. The primer sequences used in this experiment are as follows:

Homo sapiens nitric oxide synthase 2 (NOS2) forward CTGCTTGAGGTGGGCGG.
Homo sapiens nitric oxide synthase 2 (NOS2) reverse GTGACTCTGACTCGGGACGCC.
Homo sapiens nitric oxide synthase 3 (NOS3) forward TGGACCTGGATACCCGGAC.
Homo sapiens nitric oxide synthase 3 (NOS3) reverse TGGTGACTTTGGCTAGCTGG.
Homo sapiens phosphodiesterase 5A (PDE5A) forward GAAAAGGACTTTGCTGCTT.
Homo sapiens phosphodiesterase 5A (PDE5A) reverse TGATTTTGTTTGCATCATGT.
Homo sapiens actin beta (ACTB) forward CGTGAAAAGATGACCCAGATCA.
Homo sapiens actin beta (ACTB) reverse CACAGCCTGGATGGCTACGT.
Rattus norvegicus nitric oxide synthase 3 (Nos3) forward CATGGAAAGGAAGTGCAGCA.
Rattus norvegicus nitric oxide synthase 3 (Nos3) reverse AGCTGCTGTGCGTAGCTCT.
Rattus norvegicus nitric oxide synthase 1 (Nos1) forward ACTGACACCCTGCACCTGAAGA.
Rattus norvegicus nitric oxide synthase 1 (Nos1) reverse GTGCGGACATCTTCTGACTTCC.
Rattus norvegicus phosphodiesterase 5A (Pde5a) forward GCTGGATGGCTGCAGGAA.
Rattus norvegicus phosphodiesterase 5A (Pde5a) reverse CCCCATTAAGGAGCGTCTTCT.
Rattus norvegicus actin, beta (Actb) forward TTCAACACCCCAGCCATGT.
Rattus norvegicus actin, beta (Actb) reverse TGGTACGACCAGAGGCATACAG.

2.11. Statistical analysis

The statistical significance of differences between the groups was assessed using one-way analysis of variance; all results are expressed as mean ± SEM of triplicate independent experiments. Statistical significance is assumed at $P < 0.05$.

3. Results

3.1. Qualitative analysis of M. pruriens seed extract

After the extraction process, 50.70 g of the polar fraction (5.07% w/w) and 40.17 g of the nonpolar fraction (4.02% w/w) were obtained. Phytochemical components of each fraction were determined using the GC/MS technique. As displayed in Figure 1A, a number of phytochemicals in the polar fraction were observed in the first half of GC chromatogram while most of substances in the nonpolar fraction were found in the second half of the chromatogram. Peaks found in both fractions were identified using the MS spectra library and are displayed in Table 1 as probability percentages. Surprisingly, L-DOPA which had been reported as an active ingredient in *M. pruriens* seed was not found in either fraction in this study. We determined the peak L-DOPA from Sinemet® tablets. The L-DOPA peak could be observed at a retention time of 12.294 min which did not match with any peaks in either the polar or the nonpolar fractions. In the polar fraction, the substances of interest were catechol (1), 2,4-bis(1,1-dimethylethyl)-phenol (5), protocatechuic acid, methylester (7) and 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (9). Major components of the polar fraction were likely to be glycerol, probably glycerol-1-palmitate (14). 7,9-Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione was the substance in the polar fraction with a percentage most nearly identical to the spectra library. For the nonpolar fraction, most of the constituents might have been fatty acids and lipid compounds. Some lipid compounds were found in the polar fraction, but lower amounts than in the nonpolar fraction. This finding correlates with previous studies which reported on the amount of lipid compounds in *M. pruriens* seeds [29,41]. The antioxidant activities of each fraction were related to components found in the GC chromatogram (Figure 1B). The polar fraction showed higher antioxidant activity than the nonpolar fraction, and even higher than the crude extract. This activity was due to components in the polar fractions, most of which might be potent antioxidants.

3.2. Effect of M. pruriens extract on penile erection-related biomolecules in vitro

In this study, we wanted to simulate penile erection *in vitro*. NO produced from neuron cells through the action of nNOS initiates penile erection. Releasing NO triggers endothelial cells to supply more NO through activity of the eNOS enzyme. NO enters the target cells and stimulates the synthesis of cGMP which, in turn, activates numerous cellular responses and eventually causes muscle relaxation. This cell activation can be

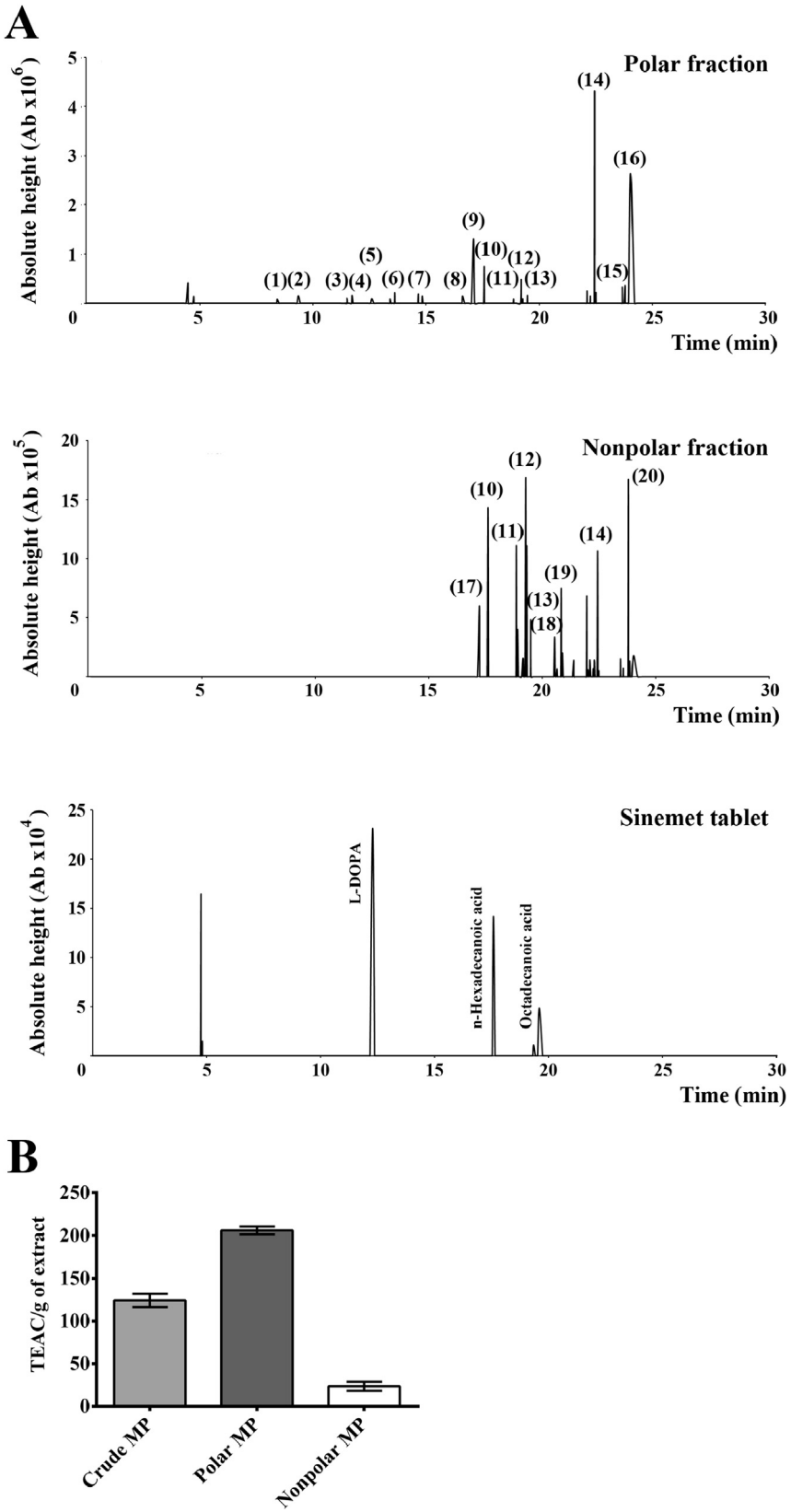

Figure 1. (A) GC chromatogram of *M. pruriens* seed extract with peaks from polar and nonpolar fractions.
Identification of major peaks is shown in Table 1. GC chromatogram of Sinemet®, which has L-DOPA as an active compound, is displayed below the chromatograms of extracts. Retention time of L-DOPA was 12.294 min. (B) Antioxidant properties of *M. pruriens* seed extract were measured by DPPH assay. Antioxidant properties or Trolox equivalent antioxidant capability (TEAC) are in Trolox Equivalents (TE) per gram of dry weight of extract.

Table 1

Identification of major peaks in the chromatogram of each fraction using MS spectra library. Probability was measured by comparing with the MS spectra in NIST library.

Fraction	No.	Retention time	Name of phytochemicals	% Probability
Polar	1	8.417	Catechol	62.9
	2	9.363	Caprolactam	94.0
	3	11.268	Vanillin lactoside	57.0
	4	11.723	Sucrose	64.6
	5	12.608	2,4-Bis (1,1-dimethylethyl)-phenol	95.0
	6	13.609	Diethyl phthalate	55.9
	7	14.655	Protocatechuic acid, methyl ester	94.8
	8	16.612	1-Butyl-2-octyl phthalate	90.0
	9	17.099	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	99.4
	10	17.597	n-Hexadecanoic acid	98.5
	11	18.867	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	95.0
	12	19.261	9,12-Octadecadienoic acid (Z,Z)-	99.0
	13	19.468	Octadecadienoic acid	96.5
	14	22.447	Glycerol 1-palmitate	87.8
	15	23.673	γ-Sitosterol	74.9
	16	24.039	2-Hydroxy-1-(hydroxymethyl) ethyl stearate	51.1
Nonpolar	17	17.236	Hexadecanoic acid, methyl ester	78.5
	10	17.602	n-Hexadecanoic acid	99.0
	11	18.867	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	98.0
	12	19.256	9,12-Octadecadienoic acid (Z,Z)-	99.5
	13	19.490	Octadecadienoic acid	91.3
	18	20.633	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	29.3
	19	20.829	Pyrrolidine, 1-(1-oxo-7, 10-hexadecadienyl)-	50.2
	14	22.431	Glycerol 1-palmitate	51.3
	20	23.805	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	96.0

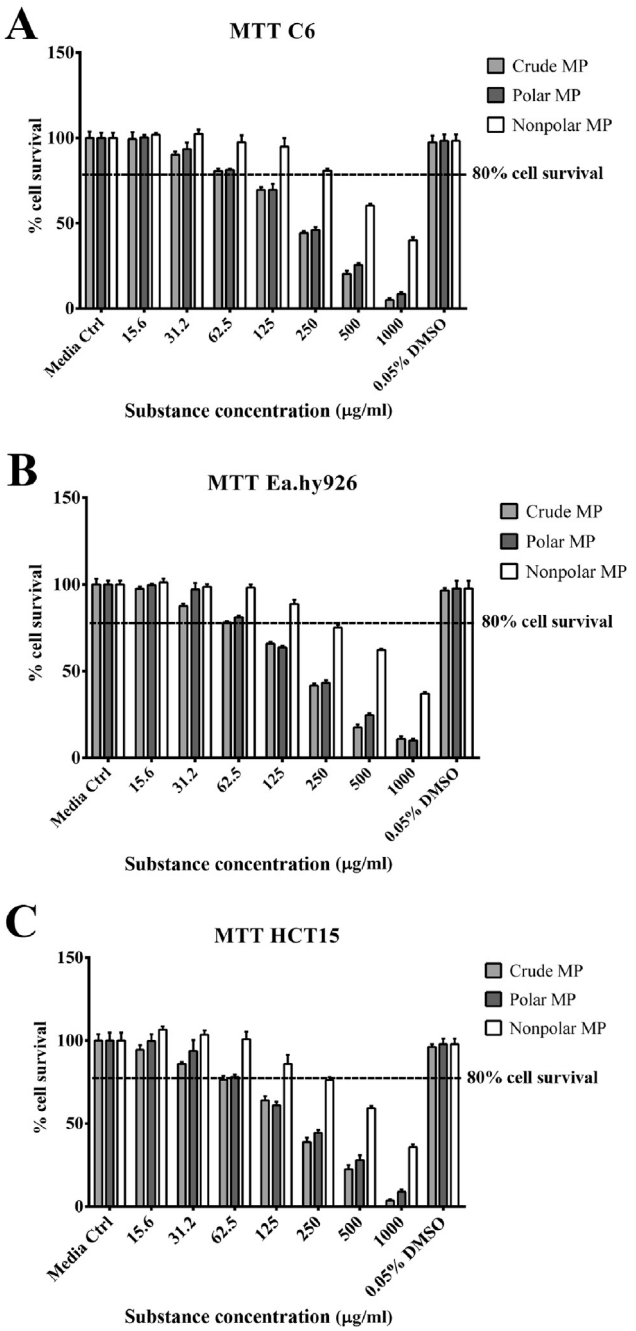

Figure 2. Cytotoxicity of cell lines used in this study.
(A) C6, (B) Ea.hy926 and (C) HCT-15. The concentration of extract which allowed more than 80% of the cells to survive was applied in further studies. At a concentration of 62.5 μg/mL, most of tested substances allowed more than 80% of the cells to survive, so a concentration of 50 μg/mL was chosen.

neutralized by the activity of PDE5 enzyme [9,10,42]. Therefore, cell lines which portray related cells in the process including Ea.hy926 which represents endothelial cells, C6 substitute neuronal cells and HCT-15 colorectal adenocarcinoma cells where PDE5 is highly expressed and has been studied on sildenafil [43,44] were chosen. Prior to the experiments, MTT assay was performed to determine appropriate concentrations of each fraction for each type of indicator cell. All fractions were found to be suitable for further experiments at concentration of less than 50 μg/mL, a level which provides a cell survival rate of more than 80% (Figure 2). Test substances were administrated to all cell lines for 24 h. Sildenafil, which was used as a positive control, was able to significantly elevate the production of NO by endothelial cells; that might be due to upregulation of eNOS and iNOS gene expression (Figure 3B). As expected, sildenafil clearly preserved intracellular cGMP maintenance through its mechanism of action which consequently enhanced PDE5 gene expression in both Ea.hy926 and HCT-15 cells (Figure 3B and C). Although sildenafil could stimulate NO production by C6 cells, its action tended to be slightly involved with nNOS gene upregulation (Figure 3A). For the *M. pruriens* seed extracts, the polar fraction significantly stimulated nNOS gene expression which in turn triggered C6 cells to produce NO at greater levels than sildenafil (Figure 3A). *M. pruriens* seed extract and its polar fraction also elevated the expression of

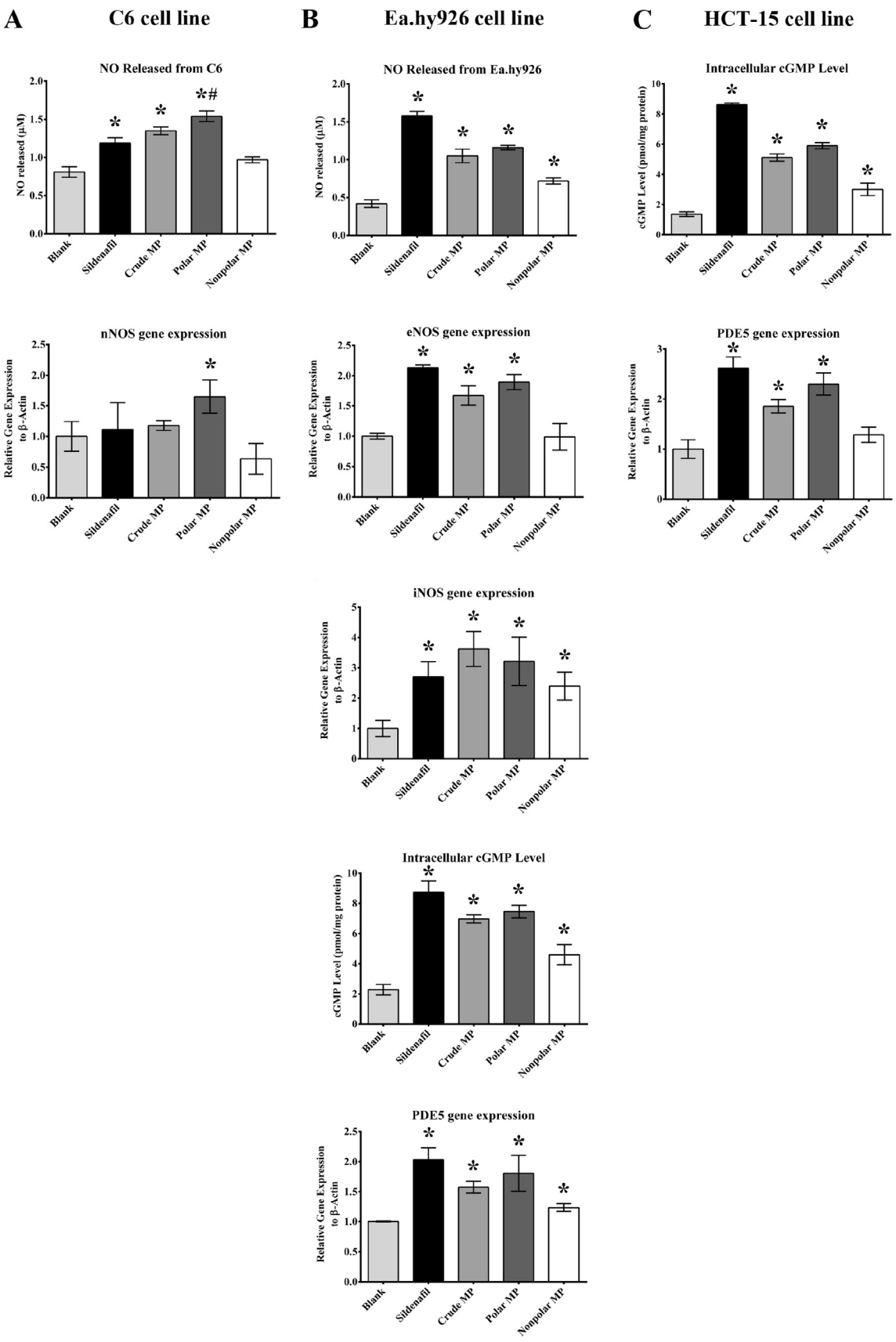

Figure 3. The effect of *M. pruriens* seed extract *in vitro*.
(A) Released NO and nNOS gene expression in C6 cell line. (B) Released NO, gene expression of eNOS, iNOS, intracellular cGMP level and PDE5 gene expression in Ea.hy926 cells. (C) Intracellular cGMP level and PDE5 gene expression in HCT-15 cells. *$P < 0.05$ compared with control group, #$P < 0.05$ compared with sildenafil-treated group.

eNOS and iNOS genes which led to an increment in NO production by Ea.hy926 but below the level reached with sildenafil (Figure 3B). The nonpolar fraction of *M. pruriens* extract was unable to stimulate eNOS gene expression. *M. pruriens* seed extract and its polar fraction could significantly maintain intracellular cGMP and upregulate PDE5 gene expression in both Ea.hy926 and HCT-15 cells (Figure 3B and C). The polar fraction of *M. pruriens* seed extract could perform biological functions quite similar to sildenafil but in addition was able to stimulate nNOS gene expression in neuron cells. Based on these results, it appears that the effect of *M. pruriens* seed extract might involve compounds found in the polar fraction which are not found in the nonpolar fraction. For those reasons, only the polar fraction of *M. pruriens* seed extract was selected for the *in vivo* experiments.

3.3. Effect of the polar fraction of M. pruriens extract on sexual behavior in animals

ED has been shown to be related to diabetes and other cardiovascular diseases [45]. In this study, we generated DM-ED rats as a model for ED. STZ was used to induce DM in the rats. Basic parameters including bodyweight, amount of food and water intake per day and serum glucose were measured for a week following STZ injection (Table 2). Serum glucose levels of the DM-ED group were significantly increased up to almost 3.0 g/L, while bodyweight of the DM-ED rats was reduced indicating that DM had been successfully induced in the rats. One week after the DM status of the rats was confirmed, APO test, used to confirm penile erection efficacy in animal models using APO to stimulate sexual response through the dopaminergic pathway, was performed [46]. The effectiveness of penile erection in the DM-ED rats was significantly reduced compared to the normal rats which confirmed the ED condition in the DM rats. During the 3-week treatment period, the rats were administered one of three substances: sildenafil at 5 mg/kg bodyweight, polar fraction of *M. pruriens* seed extract at a low concentration (20 mg/kg bodyweight) (LMP), or *M. pruriens* seed extract at a high concentration (200 mg/kg bodyweight) (HMP). Three parameters of sexual behavior were observed following the treating period: number of mountings, number of intromissions and number of ejaculations [31,47] (Figure 4A–C). All three types of sexual behavior were significantly reduced in the DM-ED group compared to normal group (10.8 ± 2.9 to 18.3 ± 3.2 for intromission, 5.7 ± 1.3 to 10.3 ± 1.7 for mounting and 0.2 ± 0.4 to 2.2 ± 0.5 for ejaculation). Sildenafil did not show a significant effect on any of the indicator parameters in either the normal or the DM-ED rats (parameter of intromission, mounting and ejaculation from normal group were 19.0 ± 2.8, 9.3 ± 1.7 and 2.3 ± 0.4, respectively, while the parameters from DM-ED group were 11.8 ± 2.5, 3.2 ± 2.1 and 0.0 ± 0.0, respectively). *M. pruriens* seed extract at the low concentration did not express efficacy in either the normal or the DM-ED rats (parameter of intromission, mounting and ejaculation from normal group were 17.0 ± 2.0, 8.8 ± 1.9 and 1.7 ± 0.4, respectively, while the parameters from

Table 2
Basic profile of rats after STZ-induction and APO test results.

Parameter	Normal group (n = 24)	DM-ED group (n = 22)
Body weight (g)	367.78 ± 6.41	320.00 ± 10.93*
Food intake (g/d)	20.89 ± 2.13	25.78 ± 2.26*
Water intake (mL/d)	38.89 ± 2.55	135.56 ± 7.87*
Serum glucose (mg/dL)	122.73 ± 13.83	293.55 ± 29.89*
No. of hyperaemia	21.94 ± 6.35	7.56 ± 4.28*
Average time of hyperaemia (s)	18.06 ± 2.77	7.07 ± 1.55*

All parameters were determined a week after STZ injection. APO test was performed a week after DM condition was confirmed in the DM group. *$P < 0.05$ compared with normal control group.

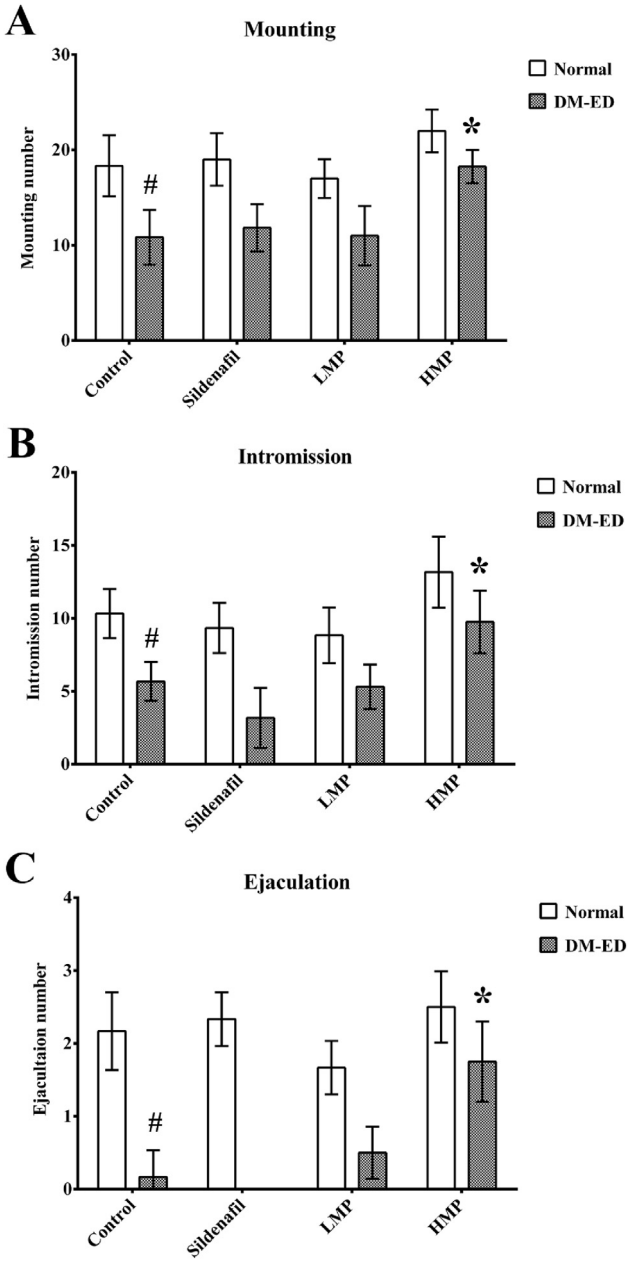

Figure 4. Average number of occurrences of sexual behavior observed in 20 min.
(A) Intromissions, (B) mountings and (C) ejaculations. Sexual behavior test was performed following a 3-week treatment period. *$P < 0.05$ compared with DM-ED control group, #$P < 0.05$ compared with normal control group. The sildenafil group was treated with sildenafil 5 mg/kg BW once daily, the LMP group was treated with *M. pruriens* seed extract 20 mg/kg BW once daily and the HMP group was treated with *M. pruriens* seed extract 200 mg/kg BW once daily.

DM-ED group were 11.0 ± 3.1, 5.3 ± 1.5 and 0.5 ± 0.4, respectively). At the high concentration, *M. pruriens* seed extract showed a slight elevation of efficacy, but not significantly different from the control or the sildenafil treated groups (parameter of intromission, mounting and ejaculation from normal group were 22.0 ± 2.3, 13.2 ± 2.4 and 2.5 ± 0.5, respectively). The polar fraction of *M. pruriens* seed extract did significantly increase the number of mountings, intromissions and ejaculations in the DM-ED rats compared to the untreated and the sildenafil-treated group (parameter of intromission, mounting and ejaculation from normal group were 18.3 ± 1.8, 9.8 ± 2.2 and 1.8 ± 0.6, respectively). This result suggests that *M. pruriens* seed extract polar fraction at a high concentration might be effective in improving ED in diabetic rats.

3.4. Polar fraction of M. pruriens seed extract can recover the structure of penile tissue in DM-ED rats

In the DM condition, high serum glucose triggers the formulation of advanced glycation end product and reactive oxygen species which affect the structural and physiological condition of nerves, smooth muscle cells and endothelial cells [48]. This phenomenon also occurs in penile tissue where it alters the collagen composition in corpus cavernosa and endothelial cells [49]. In this study, Masson's trichrome staining was used as a histological technique to clarify the difference between smooth muscle cells, nuclei and collagen fibers which were stained in red, black and blue, respectively (Figure 5). The structural condition of the corpus cavernosum in the diabetic condition (Figure 5E) was clearly changed compared to the physiological condition (Figure 5A). The amount of collagen fiber (white arrows) and size of the sinusoidal spaces (black arrows) were clearly altered. Treatment with sildenafil did not affect the structure of corpus cavernosa in either normal or diabetic conditions (Figure 5B and F). However, significant differences were observed following treatment with a high concentration of the polar fraction of *M. pruriens* extract. In the normal condition rats (Figure 5D), the amount of collagen fiber was observed to increase compared to control group. Moreover, both sinusoidal spaces and collagen fibers recovered in the diabetic rat treated with a high concentration of the polar fraction of *M. pruriens* extract (Figure 5H), indicating that treatment with high concentrations can improve the pathological condition of penile tissue caused by diabetes. The effect of this extract could not be seen in either normal or diabetic rats treated with a low concentration (Figure 5C and G).

3.5. Polar fraction of M. pruriens seed extract can restore penile-related biomolecules and related gene expression in the diabetic condition

The *in vitro* results indicate that the polar fraction of *M. pruriens* seed extract can modulate the production of NO, the expression of NOS isoforms genes, and the intracellular cGMP level. In this experiment, serum and penile tissue samples were collected to determine whether the *in vivo* results were comparable to the *in vitro* results. In the DM-ED rats, serum NO levels were slightly decreased compared to normal rats. The expression of the eNOS and nNOS genes along with the PDE5 gene in penile tissue were also reduced in the DM-ED group (Figure 6B and C) as well as the intracellular cGMP level (Figure 7A). Sildenafil managed to elevate the expression of the eNOS gene in penile tissues in both the normal and the DM-ED group (Figure 6B), but it did not affect nNOS gene expression (Figure 6C). However, sildenafil did not alter the serum NO level in either group (Figure 6A). The PDE5 inhibitor action of sildenafil was able to maintain intracellular cGMP levels (Figure 7A) which consequently increased PDE5 gene expression in both normal and DM-ED rats (Figure 7B). With *M. pruriens* extract, eNOS gene expression in penile tissue was

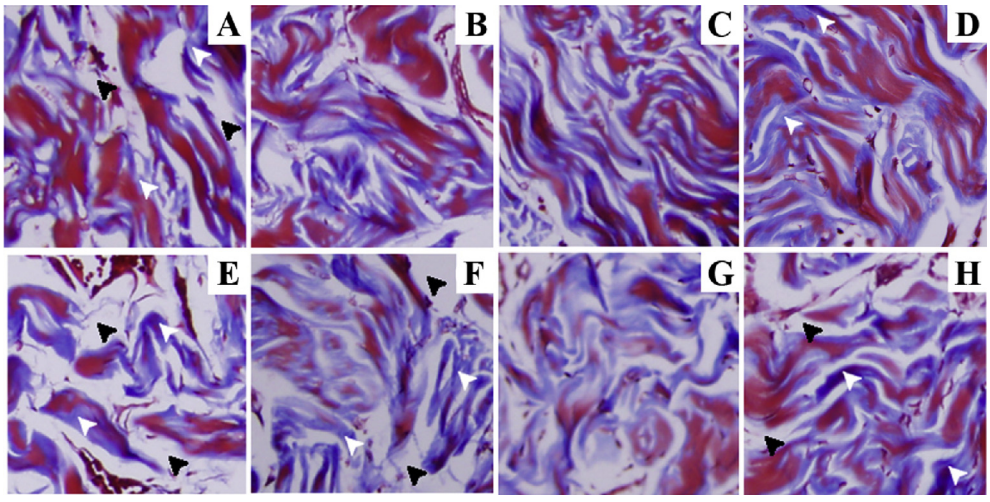

Figure 5. Masson's trichrome staining of penile tissue.
(A) Normal control group, (B) Sildenafil-treated normal group, (C) LMP-treated normal group, (D) HMP-treated normal group (E) DM-ED control group, (F) Sildenafil-treated DM-ED group, (G) LMP-treated DM-ED group and (H) HMP-treated DM-ED group. White arrows indicate blue staining which represents collagen fibers; black arrows indicate unstained areas-sinusoid spaces within the corpus cavernosa. The sildenafil group was treated with sildenafil 5 mg/kg BW once daily, the LMP group was treated with *M. pruriens* seed extract 20 mg/kg BW once daily and the HMP group was treated with *M. pruriens* seed extract 200 mg/kg BW once daily.

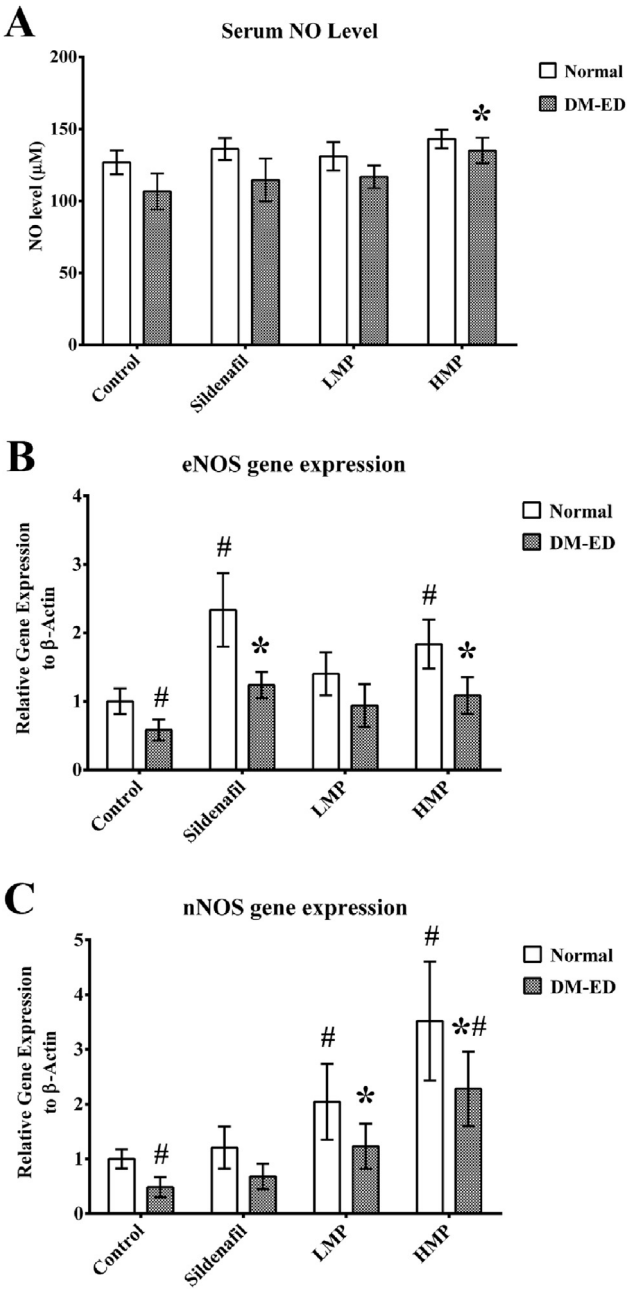

Figure 6. *In vivo* effect of the polar fraction of *M. pruriens* seed extracts on NO related metabolism.
(A) Serum NO level, (B) eNOS gene expression in penile tissues and (C) nNOS gene expression in penile tissues. *$P < 0.05$ compared with the DM-ED control group, #$P < 0.05$ compared with the normal control group. The sildenafil group was treated with sildenafil 5 mg/kg BW once daily; the LMP group was treated with *M. pruriens* seed extract 20 mg/kg BW once daily and the HMP group was treated with *M. pruriens* seed extract 200 mg/kg BW once daily.

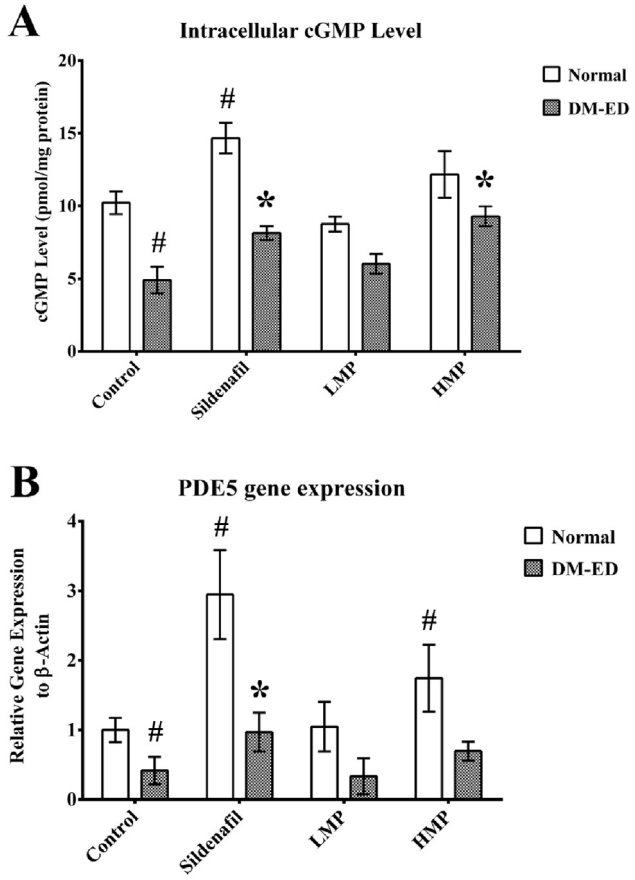

Figure 7. *In vivo* effect of the polar fraction from *M. pruriens* seed extracts.
(A) Intracellular cGMP level and (B) PDE5 gene expression in penile tissue collected from each group. *$P < 0.05$ compared with DM-ED control group, #$P < 0.05$ compared with normal control group. The sildenafil group was treated with sildenafil 5 mg/kg BW once daily; the LMP group was treated with *M. pruriens* seed extract 20 mg/kg BW once daily and the HMP group was treated with *M. pruriens* seed extract 200 mg/kg BW once daily.

not affected when administrated at the low concentration. However, eNOS gene expression was elevated in both groups when *Mucuna pruriens* extract was treated at the high concentration (Figure 6B). Moreover, *M. pruriens* seed extract was able to increase the expression of nNOS in penile tissues even at the low concentration in both the normal and the DM-ED groups (Figure 6C). The effect of this extract could be only observed at high concentration in DM-ED rats (Figure 6A). This result might be related to intracellular cGMP maintenance in penile tissue which was observed in HMP-treated DM-ED group but not the normal group (Figure 7A). PDE5 gene expression was not affected in DM-ED penile tissue, but in the normal group this gene expression was significantly increased (Figure 7B). There was no significant difference between the normal and the DM-ED condition rats when *M. pruriens* seed extract was administrated at the low concentration. These effects of *M. pruriens* seed extract were not involved with its antidiabetic properties as the baseline serum glucose was not decreased at the time of euthanization (Figure 8A). Moreover, serum insulin levels were not altered following treatment with *M. pruriens* seed extract in either the normal or the DM-ED group (Figure 8B). The effect of this extract was not involved with serum testosterone modification as the level of that hormone was not changed in either of the groups with any of the treatments (Figure 8C). These results suggest that treatment with the polar fraction of *M. pruriens* seed extract could be beneficial for a diabetic-induced ED condition.

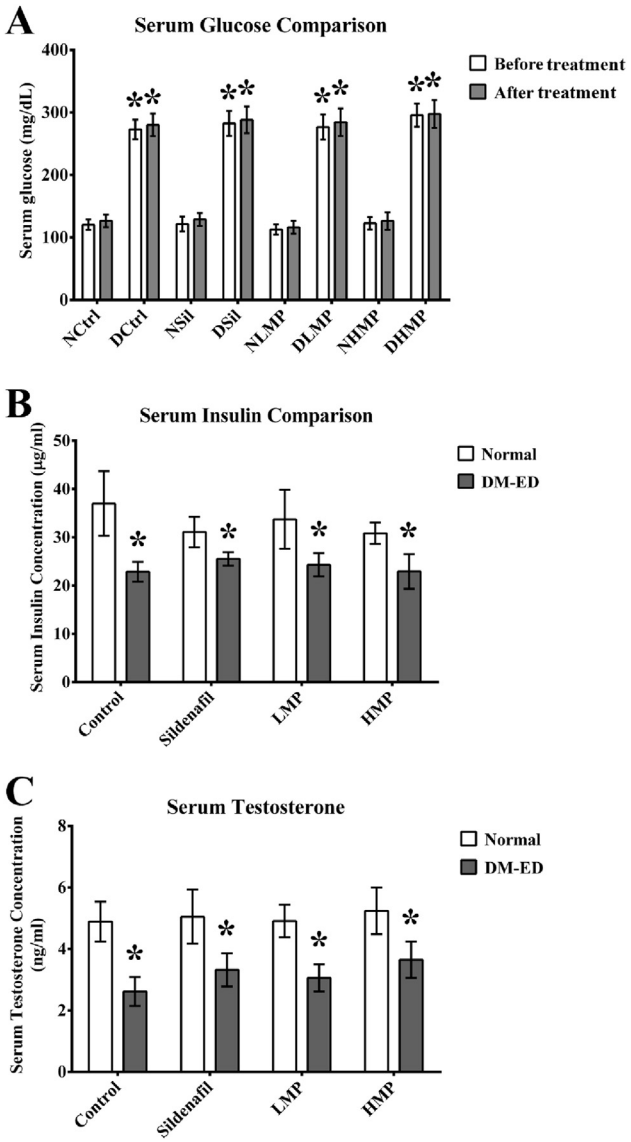

Figure 8. Comparative profile of serum biomolecules.
(A) Glucose, (B) insulin and (C) testosterone levels. Serum glucose was determined on the first day just before initial substance treatment and at euthanization after final treatment. *$P < 0.05$ compared with normal control group. For serum glucose comparison, the white bar shows serum glucose in samples collected on the first day of treatment while the grey bar shows serum glucose at euthanization. *N* = normal, D = DM-ED, Ctrl = untreated group, Sil = sildenafil-treated group, LMP = low concentration *M. pruriens* seed extract treated group and HMP = high concentration *M. pruriens* seed extract treated group. For serum insulin and testosterone determination, the sildenafil group was treated with sildenafil 5 mg/kg BW once daily, the LMP group was treated with *M. pruriens* seed extract 20 mg/kg BW once daily and the HMP group was treated with *M. pruriens* seed extract 200 mg/kg BW once daily.

4. Discussion

NO, small molecule which is distributed throughout human body, is considered to be a crucial component in a number of physiological and pathological conditions including the penile erection mechanism [50]. Physiologically, NO is produced by two cell types: neuron cells in which nNOS triggers penile erection, and vascular endothelial cells in which eNOS plays a role in penile erection maintenance [8,51]. NO activates soluble guanylyl cyclase within target cells which catalyze the production of intracellular cGMP from GTP, leading to smooth muscle relaxation. However, this activation is regulated through the function of the enzyme PDE5 which is specific to cGMP. Impairment of any related enzymes or biomolecules will trigger the ED condition. Based on this information, NO level at target organs, activity of nNOS, eNOS and/or guanylyl cyclase in target cells as well as levels of intracellular cGMP and PDE5 enzyme inhibition are considered to be therapeutic targets for ED treatment [52]. A prime therapeutic target is PDE5 inhibition as it prevents cGMP degradation. However, this medication becomes less effective when used in cases of neuronal or vascular deterioration such as diabetes [53]. Augmentation of NO levels and related NOS enzyme activity is regarded as an alternative therapeutic target for ED as it compensates for the limitations of PDE5 inhibition. There have been a number of scientific research efforts investigating the effect of natural products on ED [15,54,55]. Quercetin, resveratrol and other phenolic compounds have been reported to provide an advantage with ED and cardiovascular diseases due to their effect on eNOS upregulation and activation [56–63]. Sildenafil has also been reported to elevate the expression of the eNOS gene in *in vitro* study [64] which emphasize the use of eNOS gene activation or upregulation for ED therapy.

M. pruriens seed extract has been reported to be effective in improving sexual behavior both in animal models and in human patients due to its major component L-DOPA [23–26]. However, other substances found in *M. pruriens* seed were not considered. We first explored *M. pruriens* seed extract to determine whether active compounds other than L-DOPA were present. We collected *M. pruriens* seeds from plants cultivated in Mae Taeng District, Chiang Mai Province, and used the extraction method used in previous studies which claim to have identified most of the constituents of *M. pruriens*, including L-DOPA [27]. We then separated the phytochemicals found in *M. pruriens* seed extract into 2 fractions, polar and nonpolar, using a solvent extraction technique. The phytochemicals were identified using a GC/MS technique. Based on the MS spectra library, the polar fraction was found to contain catechol and protocatechuic acid derivatives which are metabolites of phenolic compounds such as anthocyanin and procyanidin [65]. 2,4-Bis(1,1-dimethylethyl)-phenol, or 2,6-di-tert-butylphenol, was also found in the polar fraction. This phenolic compound, which is also found in the seeds of other plant, exhibits potent antioxidant properties [66]. Another substance found in the polar fraction was 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione which is also found in other plants with antioxidant properties [67]. These four phytochemicals clearly appear to indicate strong antioxidant properties in the polar fraction of *M. pruriens* extract, unlike the nonpolar fraction which consisted primarily of lipid derivatives. Very few scientific research publications on ED have mentioned these four compounds, especially 2,4-bis (1,1-dimethylethyl)-phenol and 7,9-di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione. However, the exact chemical structure of these compounds needs to be clarified in order to verify the identification of the substances and to measure their specific biological activities. Surprisingly, L-DOPA was not detected in the extract from the strain of *M. pruriens* used in the present experiment, indicating that the strain used in this study could have been different from the strain used in previous studies.

The biological activities of both the polar and the nonpolar fractions, along with their crude extracts, were measured using

specific cell lines for each of the types of cells of interest, *e.g.*, C6 for neuron cells, Ea.hy926 for endothelial cells and HCT-15 for smooth muscle cells. Sildenafil enhanced NO production through activation of the eNOS gene in Ea.hy926 cells which subsequently increased synthesis of cGMP and upregulation of the PDE5 gene. In HCT-15 cells, sildenafil, a PDE5 inhibitor, resulted in effective cGMP restoration and PDE5 gene upregulation. However, the effect of sildenafil on NO production from C6 was not likely to have involved nNOS gene expression. Remarkably, gene expression of NOS isoforms were significantly increased after treatment with the polar fraction of *M. pruriens* extract in both neuron cells and endothelial cells. This fraction was also found to be related to the maintenance of intracellular cGMP and PDE5 gene expression in Ea.hy926 cells, but this effect was not comparable to that of sildenafil in HCT-15. This finding indicates that the effect of *M. pruriens* extract might rely on NO production, while the major function of sildenafil is to inhibit PDE5 activity. It further suggests that phytochemicals within the polar fraction might have the same effect as polyphenol which has been previously reported to stimulate the expression of the eNOS gene [57,58]. *M. pruriens* seed extract did, however, show ability to upregulate nNOS gene expression in C6 cells to a significantly greater extent than sildenafil. This finding indicates that some substances in the polar fraction which could stimulate the expression of nNOS, which is found to diminish in ED condition [68]. We suggest that such an effect might involve the presence of catechol and protocatechuic acid [69]. L-DOPA, which was previously reported to be an active compound in *M. pruriens* seed extract, can improve NO production by C6 cells through the upregulation of the nNOS gene. There is no scientific evidence regarding the effect of L-DOPA on the eNOS gene and on endothelial cells or smooth muscle cells [70]. Taken together, these results indicate that the active ingredients in *M. pruriens* seed extract which are beneficial for ED treatment might be not only L-DOPA but also other polar components as well.

The effect of the polar fraction of *M. pruriens* seed extract can be clarified using an animal model. Based on sexual behavior observations, treatment with high doses of *M. pruriens* seed extract polar fraction can apparently restore formerly impaired sexual behaviors in diabetic rats as demonstrated by the significant increase in the number of mountings, intromissions and ejaculations compared to untreated group. We suggest that this might relate to the effect of phytochemicals on nNOS gene expression which can provoke sexual behavior by initiating penile erection. L-DOPA has been demonstrated to be beneficial for ED by increasing the dopamine level in the body, levels which have been found to decline in aging men and ED patients [71]. However, the effect of L-DOPA was reported to be involved only with sexual drive but not to sexual organs or other related biomolecules [28]. The extract, however, was found to enhance endothelial function by stimulating the expression of eNOS which subsequently produces NO in penile tissue and improves the proficiency of penile erection. These results appear to indicate that the active ingredient in *M. pruriens* seed extract is not exclusively L-DOPA, but also includes other polar compounds. Sildenafil surprisingly failed to improve sexual behavior in our study, although the *in vitro* results were promising. We hypothesize that lack of nNOS gene activity stimulation by sildenafil might be the cause of this finding. Furthermore, diabetic or stressed animals have been reported to loss their sexual desire due to a depressive mood which could reduce the efficacy of sildenafil [72]. The recovery outcome in diabetic rats treated with the high dose polar fraction could be clearly observed in penile tissues using histological technique. The amount of collagen fiber and the size of sinusoidal spaces were restored compared to untreated diabetic penile tissue. Losing collagen fiber within the corpus cavernosa has been reported to increase muscle tone for initiation and sustenance of penile erection while increasing sinusoid space related to the increased blood volume required for penile erection initiation [5,73]. The effect on sexual behavior and structural improvement of the corpus cavernosa might be involved with polyphenols or phytochemicals with antioxidant activity which could be due to eNOS activation as has been previously reported [74]. In the diabetic condition, serum NO levels and the expression of NOS genes, PDE5 and intracellular cGMP levels will deteriorate [75,76]. The polar fraction of *M. pruriens* seed extract may have been increased eNOS gene expression in both normal and diabetic rats compared to the untreated group. However, the difference in NO production in animal serum among the groups in our study was difficult to quantify. That difficulty might be due to the short NO half-life and the systemic distribution of NO throughout the body [50]. This limitation could be circumvented by determining serum NO levels at the penile organ or by using the immunohistochemistry techniques. However, in this study greater elevation of serum NO could be observed in HMP-treated DM-ED rats compared to the DM-ED control group. Interestingly, treatment of both normal and diabetic rats with the polar fraction, even at the low concentration, upregulated the expression of the nNOS gene, indicates that the polar fraction of *M. pruriens* seed extract might have an advantage in the initiation stage of penile erection which exceeds the capabilities of sildenafil. PDE5 gene expression and intracellular cGMP levels were also affected by the polar fraction of *M. pruriens* extract. In the sildenafil-treated group, sildenafil inhibited the activity of the PDE5 enzyme to maintain the level of intracellular cGMP. This active mediator was a potent promoter of the PDE5 gene. As a result, sildenafil was able to elevate intracellular cGMP levels, and PDE5 gene expression was found to increase in both normal and DM-ED rats. However, the level of intracellular cGMP and PDE5 gene expression in the high concentration polar fraction group could not be compared to the sildenafil-treated group even with our sexual behavior results. We suggest that the action of *M. pruriens* seed extract might be involved with production of NO, especially by neurons, which is important for erectile initiation. The increase of NO production triggered intracellular cGMP synthesis, and PDE5 gene expression was then upregulated as a cellular compensation mechanism. The polar fraction was not able to elevate serum testosterone levels, an effect which is useful in modulating the ED condition [39], did not alter serum glucose levels or serum insulin levels in either normal or diabetic rats following treatment. These results indicate that the effect of this extract was not due to hypoglycaemic or antidiabetic effects. The different effects of *M. pruriens* on glucose levels might due to differences in the DM induction method and duration of treatment in the rats [22]. Further investigation is required to identify other cellular signaling pathways which might be involved in the activation by *M. pruriens* seed extract.

Active ingredients of *M. pruriens* seed extract are likely to be hydrophilic substances. Preliminary identification using a GC/MS spectra library indicates that these phytochemicals are

possibly catechols, including their derivatives and polyphenol compounds. These phytochemicals can stimulate the expression of eNOS, nNOS and the PDE5 gene both *in vitro* and *in vivo* which can subsequently restore intracellular cGMP levels which is helpful for sustaining penile erection. L-DOPA was not found in *M. pruriens* seed extract. A possible mode of action of *M. pruriens* seed extract is increasing the level of NO through upregulation of the NOS gene, especially nNOS. The substances in the extract were also useful in improving sexual behavior in cases of ED when administered at a high concentration. Moreover, these phytochemicals can prevent the alteration of corpus cavernosa structure which decomposes in case of metabolic disorders including diabetes. The polar extract of *M. pruriens* seed, which lacks L-DOPA, is a potential alternative choice for ED therapy, especially in patients with complications such as diabetes. However, further investigation of the chemical structure of the phytochemicals and the biomolecular responses they induce will be required.

Conflict of interest statement

We declare that we have no conflict of interest.

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