Relaxant mechanism of *Eulophia macrobulbon* ethanolic extract and 1-(4´-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol on human corpus cavernosum

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ABSTRACT
Background: *Eulophia macrobulbon* (E.C.Parish and Rchb.f.) Hook.F. has been shown to be a promising phosphodiesterase-5 (PDE5) inhibitor that relaxes rat isolated pulmonary artery.

Objective: To test this plant's possible application in human erectile dysfunction (ED) using an ethanolic extract of *E. macrobulbon* tubers (EM extract), and an isolated constituent, 1-(4´-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (HDP).

Methods: Dried tubers of *E. macrobulbon* (EM) were extracted with 95% ethanol and the HDP was isolated by open column chromatography and high-performance liquid chromatography (HPLC) methods. The relaxant mechanism of the EM extract and the HDP was studied on isolated human cavernosal (HC) strips.

Results: EM extract relaxed HC strips, and this effect was not modified by N^G-nitro-L-arginine (L-NNA), ODQ, tetraethylammonium, nor glybenclamide. HDP relaxed HC strips to the same extent as that of sildenafil. EM extract and HDP potentiated relaxation of the HC strips to glyceryl trinitrate in a similar manner to that of sildenafil. EM extract and sildenafil, but not HDP, increased cGMP content of the HC strips in a concentration-dependent manner. In the

thapsigargin-pretreated HC strips, nifedipine or EM extract, but not HDP, suppressed the contractile response of the HC strips to phenylephrine. When nifedipine and/or SKF 96365 were added, followed by EM extract or HDP, further suppression was found in the case of HDP but not with EM extract. Ca^{2+} free Krebs solution, suppressed the phenylephrine contraction on HC strips and further suppression was found when adding EM extract or HDP.

Conclusion: These results indicate that EM extract causes a relaxation of HC strips by serving as an inhibitor of PDE5, of voltage- and stored-operated Ca^{2+} channels, and of intracellular Ca^{2+} mobilization. Thus, EM extract might be a good choice for development as a functional food for erectile dysfunction in men. However, further studies are needed to identify other PDE5 and the Ca^{2+} channel inhibiting components of the extract.

Keywords: *Eulophia macrobulbon*; human cavernosum; phosphodiesterase-5 inhibitor; sildenafil; Ca^{2+}channel inhibitor

Human Ethic Number: EC.57/B 06-004

BACKGROUND

Erectile dysfunction (ED) or impotence is the persistent inability to attain and maintain an erection during intercourse [1]. ED is recognized throughout the world as a serious problem for men. Medications such as inhibitors of phosphodiesterase 5 (PDE5), agents that inhibit cGMP hydrolysis, are often sought to treat this condition since they could promote corpus cavernosum relaxation resulting in prolongation of penile erection [2-5].

Penile erection is a hemodynamic process involving relaxation of smooth muscle of the corpus cavernosum, which results to increase flow of blood into the trabecular space of the corpus cavernosa [6-7]. Smooth muscle relaxation is mediated by nitric oxide (NO) released from endothelial cell lining blood vessels, the lacunar space of the corpora cavernosa, and the parasympathetic non-adrenergic, non-cholinergic neuron in the penis [8-11]. NO activates soluble guanylate cyclase that increased conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) [8] provides the signal which leads to relaxation of smooth muscle of the corpus cavernosum and penile arterioles. PDE5 is the predominant cyclic guanosine monophosphate (cGMP) degrading enzyme found in platelets, penile corpus cavernosum, smooth muscle cells, and all vascular smooth muscle cells particularly in the pulmonary vessels [12-13]. Inhibition of PDE5 increasesc GMP, thus enhancing the nitric oxide (NO)/ cGMP signaling pathway leading to vascular smooth muscle relaxation of penile corpus cavernosum [3, 14-15]. Although various PDE5 inhibitors e.g. sildenafil, vardenafil and tadalafil have been clinically used for erectile dysfunction, these standard synthetic inhibitors may cause nausea, headache, and cutaneous flushing [16]. Thus, interest turns to natural substances with perceived lower side effects [17].

Our previous study showed that the tubers of *Eulophia macrobulbon* (E.C. Parish and Rchb.f.) Hook. f., a plant in the Orchidaceae family, inhibited PDE-5. This plant can be found throughout Asia. The major PDE-5 inhibitor in the plant has been isolated and identified as 1-(4'-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (HDP) (Figure 1) [18]. Both an ethanolic extract of *E. Macrobulbon* tuber and HDP relax pulmonary [19] and mesenteric arteries [20] relieve pulmonary hypertension in rats [19]. Since the same signaling mechanisms operate in penile erection, EM extract may find an application in ED. Here, we use standard pharmacological techniques to investigate the potential role of EM extract and HDP in the

cellular pathway using the relevant tissue, human corpus cavernosum, as a prelude to their possible development for clinical application to ED.

Materials

The following drugs were used, acetylcholine chloride, nifedipine, N^G-nitro-L-arginine (L-NNA), phenylephrine hydrochloride, tetraethylammonium (TEA) obtained from Sigma, St. Louis, MO, USA.1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) was obtained from Trocis, UK. Sildenafil citrate (Viagra) was from Pfizer. Glyceryl trinitrate (GTN) was from Mycomed, Denmark. L-NNA, GTN and sildenafil were dissolved in distilled water, nifedipine was dissolved in DMSO (20%) and the other chemicals were dissolved in a solution (1 liter) containing NaCl (9g), NaH_2PO_4 (0.19 g), and ascorbic acid (0.03 g). Methanol and dichloromethane were purchased from Carlo Erba, France. All solvents were analytical grade purity. A standard HDP was isolated and purified from *E. Macrobulbon* [18].

MEDTHODS
Preparation of plant extractand isolation of HDP

Fresh tubers of EM were collected from Prachinburi province, Thailand. The voucher specimen (No.002716) was identified by Associate Professor Dr. Anupan Kongbungkerd, Department of Biology, Naresuan University, Thailand and therein lodged. EM extract was prepared from 2 kg of dried, powdered tubers, macerated with 95% ethanol (14 L, 3 d) twice and filtered and evaporated by vacuum to yield 15.8% (w/w) of extract. The extract contained 0.52% (w/w) of HDP analyzed by HPLC. The HPLC separation was performed on a Gemini-NX C18 column (150 x 4.0 mm i.d., 5 μm particle size) with gradient elution of a mobile phase comprising 0.05 M sodium acetate (pH 3.7) and acetonitrile. The injection volume was 20 μl. The flow rate was adjusted to 1 ml/min and detection was performed at 265 nm. The TLC fingerprint of the extract was also recorded [21].

HDP was isolated from EM extract as described by Temkitthawon et al. [18] and Changwichit et al. [21]. The purity of the compound was more than 95% analyzed by NMR and HPLCas described previously [18] and as shown in Figure 1B. The EM extract and HDP were stored at -20°C until used.

The chemical profile of EM extract and HDP were established using high performance liquid chromatography (HPLC). The extract was analyzed by using HP 1100 system (Agilent) comprising of a photodiode array detector and C18 column (Waters) with gradient flow of methanol:water (5:95→100:0) containing $C_2HF_3O_2$ 0.05% at 1 ml/min flow and detected by UV- DAD (200-600 nm).

Pharmacological studies
Preparation of human cavernosal strips
A) Patients

Cavernosal tissue was obtained from20potent healthy men (age 22–30 years) who had elected for penile amputations for gender changeand whohad been discontinued treatment with antiandrogens and estrogens two months before surgery. All protocols were approved by the Human Ethics Committee Faculty of Traditional Thai Medicine Review Board, Prince of Songkla University (EC. 57/B 06-004). Research carried out on human was incompliance with the Helsinki Declaration. All participants provided informed written consent.

B) Tissue preparation

Cavernosal strips were prepared using a method modified from Mirone et al. [22]. The amputated penis was carefully placed in cold Krebs-Heinseleit solution equilibrated with carbogen (O_2/ CO_2) and washed twice with this solution. The penes were opened along the median section, and longitudinal strips of about 2x7 mm (WxL) were dissected (8 strips per patient). These cavernosal strips were mounted in 20 ml tissue baths maintained at $37^{\circ}C$ containing Krebs-Heinseleit solution (mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 0.45, KH_2PO_4 1.18, glucose 11.7, Na_2EDTA 0.024, $NaHCO_3$ 25.0, ascorbic acid 0.09). The HC strip was connected to a force-transducer (FT03C) and bridge amplifiers (Grass Instruments, Quincy, Mass, USA), basal tension adjusted to 1.0 g, maintained at 37 $^{\circ}C$ and continuously bubbled with O_2/ CO_2 mixture (95%: 5%). The strips were equilibrated for 60 min in Krebs-Heinseleit (K-H) solution refreshed every 15 min. After equilibration, the presence of functional endothelium of the HC strip was assessed through the following methods: 1µM phenylephrine was added to produce a sustained contracture (after 10 min) on which a relaxation was superimposed by application of 10µM Ach. HC strips having a relaxation >80% were deemed usable. Phenylephrine/ Ach were removed by several washouts with K-H solution until contraction returned to baseline and equilibrated for 45 min before the experiment began. Each experiment proceeded by adding phenylephrine. The drugs were added cumulatively, and the effect of drug-induced relaxation was measured as the decline from the maximal steady tension produced by phenylephrine (0.3 µM for the ones with L-NNA and 3 µM for the ones without L-NNA).

Relaxant activity of EM extract, sildenafil and HDP

Phenylephrine (3 µM) was added to HC strips until the contraction reached a plateau (10 min), and then either EM extract (0.1-3mg/ml), HDP (0.1-3 mM), or sildenafil (0.1-3 mM) were added in accumulating concentrations. The EM extract, sildenafil and HDP induced relaxation (%) was calculated as the percentage of the maximal contraction at the plateau state established byphenylephrine.

Role of nitric oxide, guanylate cyclase and K^+ channels

To test the role of these pathways, HC strips were precontracted with phenylephrine (3 µM or 0.3µM when L-NNA used) and EM extract (0.1-3 mg/ml) added accumulatively. After several washings followed by re-equilibration of the HC strips for 1 h, the HC strips were incubated with 1H-[1,2,4] oxadiazolo [4,3-a] quinoxaline-1-one (ODQ, 10 µM, a guanlylate cyclase inhibitor [23]), glybenclamide (10µM, a K_{ATP}-blocker [24]) or tetraethylammonium (TEA,1 mM, a K_{Ca}-channel blocker [25]) and/or N^G-nitro-L-arginine (L-NNA, 3 mM, a nitric oxide synthase inhibitor [26]) for 20min. Then, the impact of EM extract on *C-R* relationship was determined in the presence of the respective drug beginning with the appropriate concentration of phenylephrine.Percentage relaxation of each drug was determined as described above.

Phosphodiesterase inhibition

Nitric oxide is a direct activator of guanylate cyclase which converts GTP to cGMP which is constitutively hydrolyzed by PDE5.

    To assess the role of EM extract or HDP as a PDE5 inhibitor in comparison to the sildenafil (a PDE5 inhibitor [27]), a cumulative relaxation *C-R* curve to glyceryl trinitrate, a nitric oxide donor, on the HC strip precontracted with phenylephrine (3 µM) was performed. Following several washings, the HC strip was incubated with sildenafil (3mM), EM extract (3 mg/ml) or HDP (3mM) for 20 min and the relaxation *C-R* relationship to glyceryl trinitrate on the phenylephrine-precontracted HC strip was obtained in the presence of the incubating drugs. Percentage relaxation of each drug was determined as described above.

cGMP measurement by ELISA

HC strips were incubated in tissue baths containing L-NNA (3 mM) alone (control) or with (1)1 mM sildenafil, (2) 3 mM sildenafil, (3) 1 mg/ml EM, (4) 3 mg/ml EM, (5) 1 mM HDP or (6) 3 mM HDP, for 30 min. The HC strip of each incubating medium was collected and kept in -70°C until used. Each frozen HC strip was chopped on ice and homogenized in 0.1 M HCl (0.4 g of tissue in 1 ml of 0.1 M HCl). The lysates were centrifuged (14,000 rpm for 10 min) and supernatants were collected for cyclic nucleotide competitive ELISA (Enzo, USA), and was performed according to the manufacture's guidelines. Cyclic nucleotide content was normalized to sample total protein content of each sample (by Bradford assay).

Role of voltage-or stored-operated Ca^{2+}channels

HC strips were treated with L-NNA for 60 min then incubated with thapsigargin (1µM), a SERCA pump inhibitor, for 40min to deplete the sarcoplasmic Ca^{2+}intracellular store which, in turn, opens of the plasma membrane store-operated Ca^{2+} channels. Then, the contractile responses to phenylephrine (3µM) were determined before and after adding of the EM (3mg/ml), HDP (3 mM) or nifedipine (1 mM, a voltage-Ca^{2+} channel inhibitor) alone or in combination of EM or HDP with nifedipine and/or SKF 96365 (3mM, a store-operated Ca^{2+} channel inhibitor). Percent contraction to phenylephrine in the presence of each drug or in combinationwas calculated against the maximal plateau contraction established by the phenylephrine alone.

Inhibition of intracellular Ca^{2+}mobilization

To determine whether EM or HDP plays a role as an inhibitor of an intracellular Ca^{2+} mobilization, contractile responses to phenylephrine (3 µM) on the HC strips were performed in the Ca^{2+} free Krebs solution before and after incubating with EM (3 mg/ml) or HDP (3mM) in the presence of L-NNA.Percent contraction to phenylephrine in the Ca^{2+} free Krebs solution, EM extract or HDP was calculated against the maximal plateau contraction established by phenylephrine alone in normal Ca^{2+} Krebs solution.

Statistical analysis

Results are expressed as a mean ± standard error of the mean (SEM) where n indicates number of HC strips obtained from different patients. Statistical comparison was performed using the statistical differences between 2 measurements as determined by the two-tailed unpaired or paired Student's t-testand among the group was determined by one-way ANOVA. Post-hoc

analysis was performed with Tukey's range testusing GraphPad Prism 5. A *p* value ≤ 0.05 was considered to indicate a significant difference between values.

RESULTS

Biochemical analysis of the EM extract

The HPLC chromatogram of EM extract was shown in Figure 1. There are 4 HPLC major peaks detected at the wavelength of 254 nm at the retention time (min) of 14.58, 18.50, 20.35, and 20.91 for the HPLC peak 1 to 4, respectively. On UV spectral analysis, HPLC-peak 4 is equivalent to the HDP which has retention time at 20.97 with a similar of peaks UV spectrums.

A   EM extract, 10 mg/ml
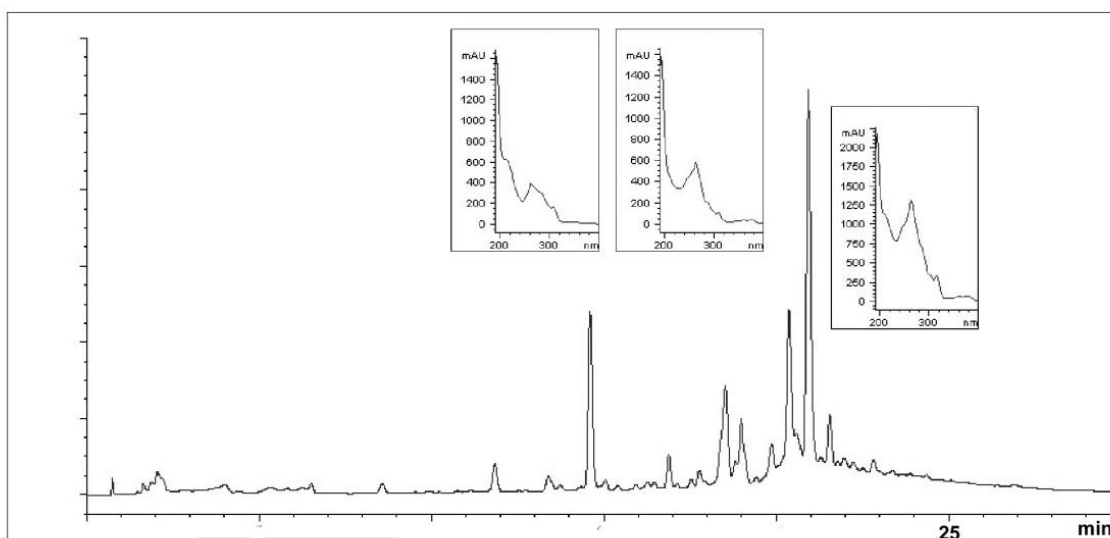
B   HDP, 0.1 mg/ml
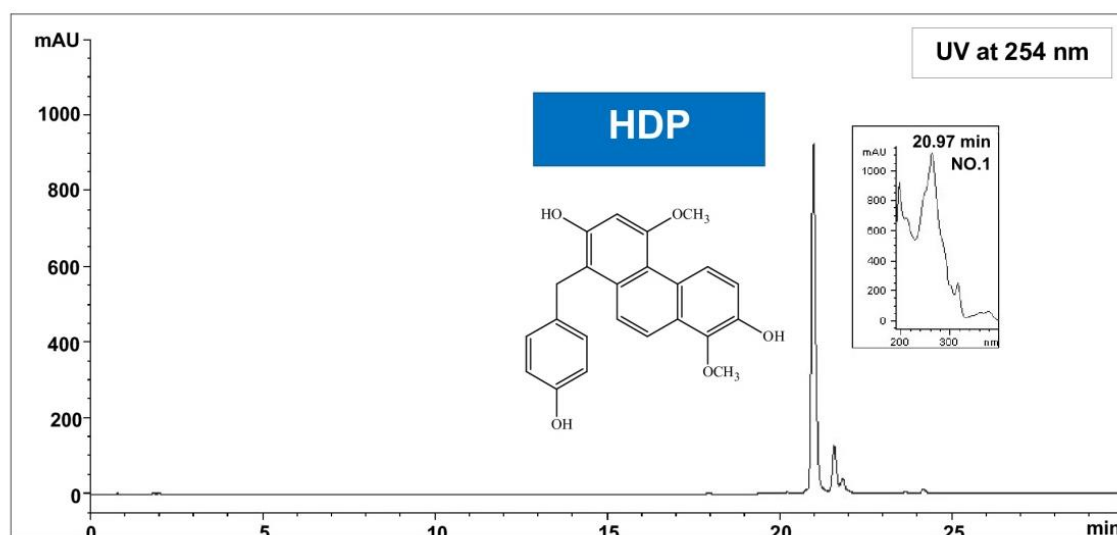
Figure 1. HPLC chromatogram of EM extract (A) and 1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol) HDP (B). The column eluent for each of the 4 EM extract peaks was scanned at the wavelength of 254 nm. Retention time in minute of each peak is shown as a miniature of each peak of its UV spectra.

### Relaxations induced by EM extract, sildenafil and HDP

EM extract (0.1-3 mg/ml), sildenafil (0.1-3 mM), and HDP (0.1-3 mM), each reproducibly and reversibly relaxed HC strips precontracted by phenylephrine in a concentration-dependent manner (Figure2).
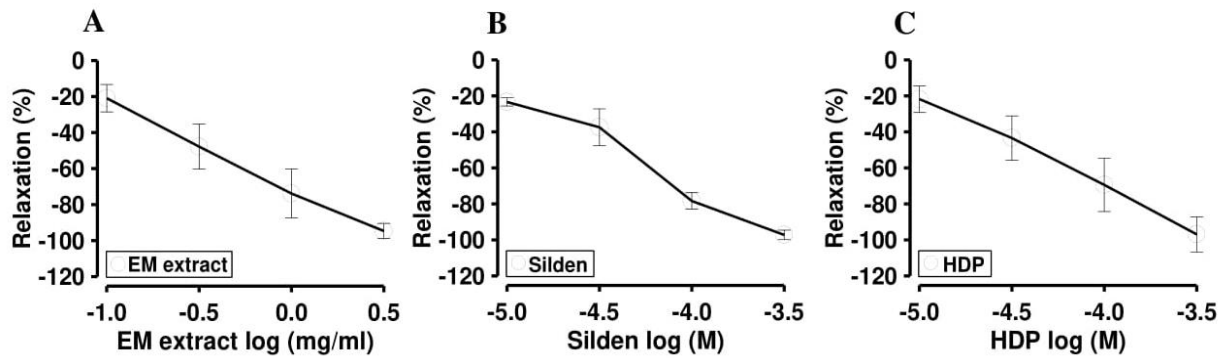
Figure 2. Relaxation of HC strips precontracted with phenylephrine to EM extract (A), sildenafil (B) or HDP(C). Each point represented mean ± SEM, n=6.

### Role of nitric oxide, guanylate cyclase and K$^+$ channels

The blockers of NOS (L-NNA), guanylate cyclase (ODQ), and the BK- (TEA) and K$_{ATP}$ channels (glybenclamide) that predominantly mediate relaxation of the HC muscle [28], did not modulate the EM extract- induced relaxation (Figure3).
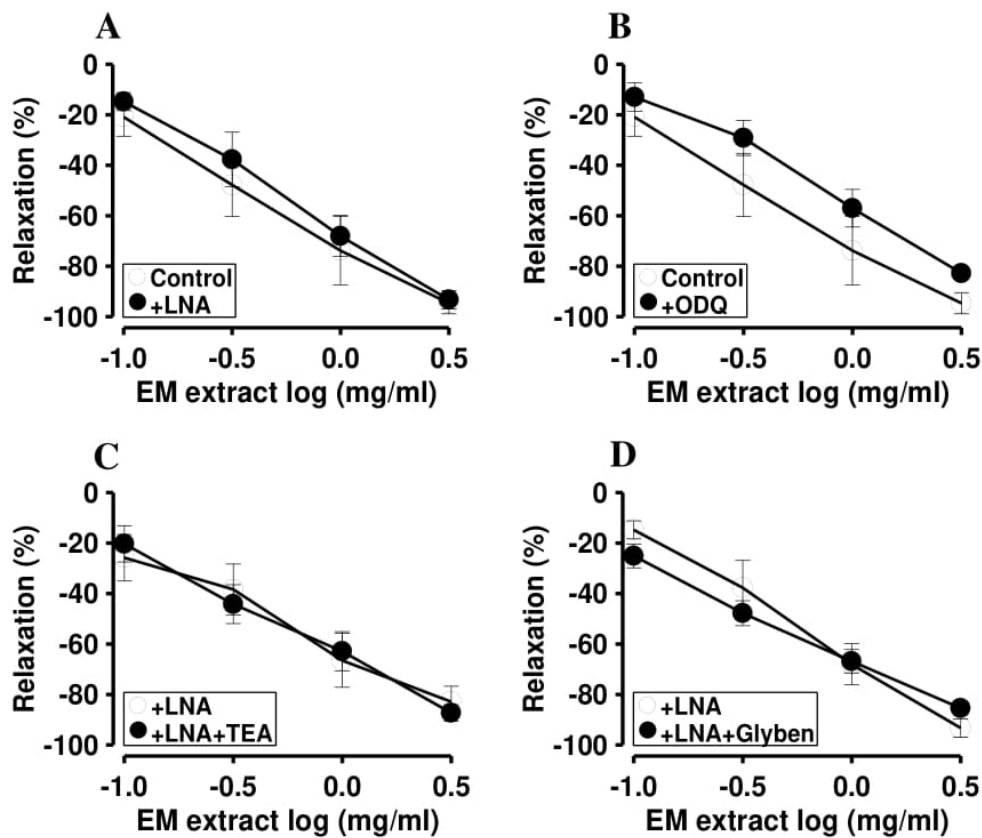
Figure3. Relaxations of HC strip precontracted with phenylephrine. The roles of nitric oxide synthase, guanylate cyclase, and K$^+$ channels were tested using N-nitro-L-arginine (L-NNA), ODQ, and TEA or glybenclamide. Each point represented mean ± SEM, n=6.

Phosphodiesterase inhibition

Glyceryltrinitrate, a nitric oxide donor, caused a relaxation of precontracted HC strips in a concentration dependent manner.The relaxation by the glyceryl trinitrate was potentiated by incubating the HC strip with sildenafil, the EM extract or the HDP (Figure 4A-C).
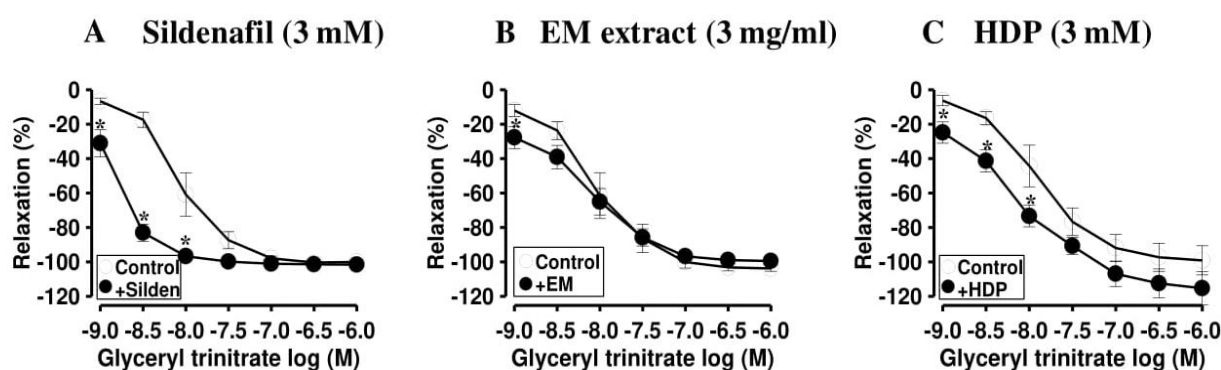
Figure 4. Effects of sildenafil, EM extract or HDP on relaxation of HC strip precontracted with phenylephrine to glyceryltrinitrate. Each point represented mean ± SEM, n=6. *significantly lower than the control group, $p < 0.05$.

cGMP levels

Sildenafil and EM extract increased cGMP contents of HC strips in a concentration-dependent manner, whereas HDP only had a modest effect (Figure 5).
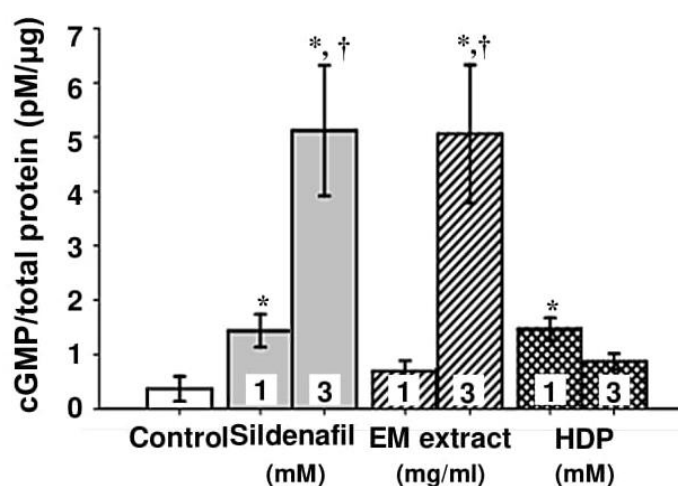

Figure 5. cGMP contents of human cavernosal muscle strips after 30 min of preincubation with L-NNA (control) alone, or L-NNA with sildenafil (1 or 3 mM), EM extract (1 or 3 mg/ml) or HDP (1 or 3 mM). Each bar graph represented mean ± SEM, n=6.*significantly higher than the control group, †significantly higher than their own group with lower concentration, $p < 0.05$.

Inhibition of voltage-or stored-operated calcium channels

If EM extract or HDP plays a role as a voltage-gated Ca^{2+} channel inhibitor and/or a store-operated Ca^{2+} channel inhibitor, the contractile response of the HC strip to phenylephrine in the presence of EM extract or HDP would not be modified by the addition of nifedipine and/or SKF-96365 under conditions where store-operated Ca^{2+} channels are also activated. In order to stimulate opening of the store-operated Ca^{2+} channels, the HC strips were incubated with thapsigargin (1 µM), a SERCApump inhibitor, to deplete the intracellular Ca^{2+}store in the sarcoplasmic reticulum and this inturn stimulated the opening of the plasma membrane store-operated Ca^{2+} channel [29-32].

In the presence of L-NNA and thapsigargin, either nifedipine or EM extract, but not HDP, each suppressed the contractile responses of the HC strips to phenylephrine. When EM extract was added together with nifedipine, or nifedipine with SKF96365, no further suppression of the phenylephrine contraction obtained (Figure 6A). On the other hand, when HDP was added together with nifedipine or nifedipine with SKF96365, a further suppression was found (Figure 6B).
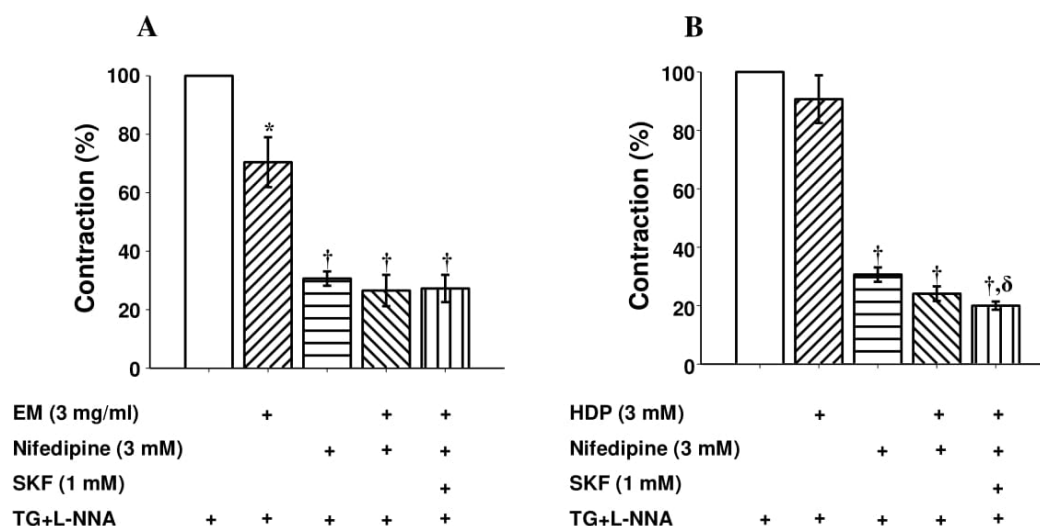
Figure 6. Effects of EM extract, HDP, nifedipine and/or SKF 96365 on contractile responses of the phenylephrine on the HC strips. Each point represented mean ± SEM, n=6. *significantly lower than the one with L-NNA+TG) control group, †significantly lower than the one with L-NNA + TG and the one with L-NNA + TG and with EM extract or with HDP and δsignificantly lower than the one with L-NNA + TG + nifedipine, $p < 0.05$.

Inhibition of intracellular Ca^{2+} mobilization

In the Ca^{2+} free Krebs solution, the contractile responses to phenylephrine on the HC strip in the presence of L-NNA were depressed. Further suppression was found when EM extract or HDP was also added into the incubation medium (Figure 7).
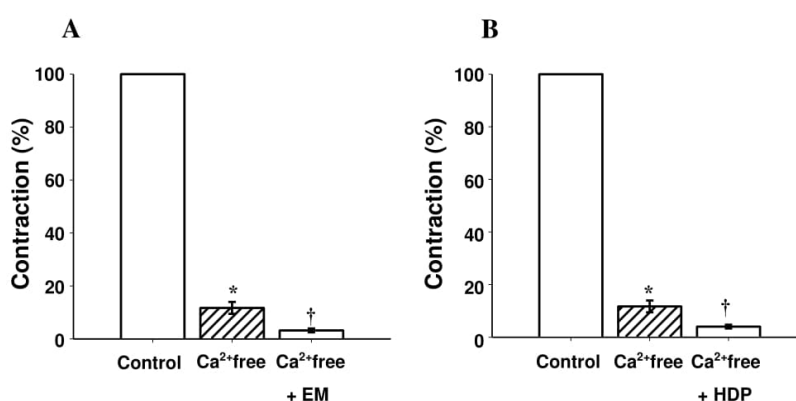
Figure 7. Contractile responsiveness of the HC strips to 3μMphenylephrinein normal Krebs solution with Ca^{2+} (control) or in Ca^{2+} free Krebs solution alone, in the presence of EM extract or HDP. L-NNA (30 mM) was present throughout. Each point represented mean ± SEM, n=6. *significantly lower than control and †significantly lower than the other groups, $p < 0.05$.

DISCUSSION

EM extract or HDP clearly relaxed isolated HC strips. Further experiments explored the underlying mechanisms for the relaxation activity on nitric oxide synthase stimulator, a guanylate cyclase stimulator, opening of K^+ channels, a PDE-5 inhibitor, inhibiting the voltage- or stored-operated calcium channels, or inhibiting the intracellular Ca^{2+} mobilization.

The finding that L-NNA, a nitric oxide synthase inhibitor; or ODQ, a guanylate cyclase inhibitor, did not inhibit the relaxant activity of the EM extract, suggests that the EM extract did not stimulate the nitric oxide synthase nor the soluble guanylate cyclase pathway. However, the main nitric oxide source in cavernosum is not only at the sinus endothelium, but also at the nitrinergic nerve terminals which requires an electrical stimulation at the intact nerve supply to release nitric oxide. Thus, further experiments using electrical stimulation to evoke NO may reveal a more conclusive role for nitric oxide.

It is unlikely that the EM extract activated the K^+ channel either the Ca^{2+} sensitive K^+ channel (K_{Ca}) [25], or the ATP-sensitive K^+ channel (K_{ATP}) [24], as it was found that neither TEA nor glybenclamide inhibited the relaxation C-R curve of the EM extract.

The finding that EM extract and HDP potentiated the relaxant activity of the glyceryl trinitrate on the HC strip similar to what would be produced by sildenafil, a known PDE5 inhibitor [33] suggests that EM extract and HDP might act as a PDE5 inhibitor. To confirm this, we conducted another experiment by measuring cGMP concentration in HC strips by ELISA method, where the HC strips had been incubated with EM extract, HDP or sildenafil in the presence of L-NNA in order to prevent cGMP disturbance produced by the nitric oxide. The results showed that EM extract increased cGMP content in the HC strip in a concentration dependent manner, which was similar to that of the sildenafil. However, HDP caused only a slight increase in HC strip cGMP concentration independent of the HDP concentrations. These results indicated that HDP might have only mild PDE5 inhibitor activity. Thus, EM extract might contain other substance(s) that possess a PDE5 activity besides HDP further study is needed to investigate this.

EM extract and HDP might cause relaxation of the HC strip via a Ca^{2+} channel pathway. In order to identify such activity, the L-NNA treated-HC strips were pre-incubated with thapsigargin, a SERCA pump inhibitor to deplete the stored Ca^{2+}, in the sarcoplasmic reticulum, which would allow opening of the membrane store-operated Ca^{2+} channels [29-32]. In the present study, we expected that if EM extract or HDP acted as a voltage-gated and/or stored-operated Ca^{2+}-channel inhibitor. Thus, the phenylephrine-induced contraction would not be further suppressed in the presence of EM extract or HDP together with nifedipine (a voltage-gated Ca^{2+}-channel inhibitor) and/or SKF 96365 (a store-operated Ca^{2+}channel inhibitor). It was found that nifedipine, a voltagegated Ca^{2+} channel blocker, suppressed the HC strip contraction to phenylephrine. When EM extract was added together with nifedipine or together with nifedipine plus SKF 96365, no further suppression was found, indicating that the EM extract might act in part as inhibitors of the voltage gated and stored-operated Ca^{2+}channels. In case of the HDP, further suppression of the phenylephrine-induced HC strip contraction was observed in the presence of nifedipine, as well as in the presence of nifedipine plus SKF 96365, indicating that HDP might not play a role as a voltage-gated or store-operated Ca^{2+}-channels inhibitors.

In vascular smooth muscle, the α-adrenoceptor-induced contraction in Ca^{2+}-free medium is initiated by Ca^{2+} release from the intracellular (IP_3) store in the sarcoplasmicreticulum[34-35]. In the present study, we investigated the effect of EM extract and HDP onthe HC strip in Ca^{2+}-free medium and found that both substances caused further suppression of the

phenylephrine contractile response on the HC strips. This suggests that EM extract and HDP might inhibit intracellular Ca^{2+} mobility.

CONCLUSIONS

This study used human cavernosal muscle strips, thus providing a reliable basis for preclinical data translation into clinical applications. The EM extract induces relaxant activity on HC strips by acting as a PDE5 inhibitor, which promotes NO/cGMP vascular smooth muscle relaxation signaling pathway and partly inhibiting voltage-gated and a store-operated Ca^{2+} channels. HDP is also a PDE5 inhibitor, and both EM extract and HDP inhibit intracellular mobilization. The study supports the possibility that EM extract would be a good choice as a functional food to limit the development of erectile dysfunction in men. However, further study is still required to identify other PDE5 and the Ca^{2+} channel inhibiting components of the extract.

List of Abbreviations: erectile dysfunction (ED), phosphodiesterase-5 (PDE-5),1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (HDP), *Eulophia macrobulbon* ethanolic extract (EM extract), acetylcholine (Ach), N-nitro-L-arginine (L-NNA), sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), High performance liquid chromatography (HPLC), Human cavernosal (HC)

Authors' contributions: CJ designed and conducted the research. SY, JN, KC and KI conducted the research and performed statistical analysis. KT provided human corpus cavernosum obtained from patients who had undergone sex change. CJ, KI and KC wrote the manuscript. All authors read and approved the final version of the manuscript.

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