Effects of Root Extracts of *Eurycoma longifolia* Jack on Corpus Cavernosum of Rat

Bae Huey Tee See Ziau Hoe Swee Hung Cheah Sau Kuen Lam

Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Significance of the Study

- In this study, dichloromethane subfraction-I, a more purified extract of *Eurycoma longifolia* was able to antagonize angiotensin-converting enzyme and angiotensin II that induced contraction which caused corpus cavernosum relaxation via inhibition of angiotensin II type I receptor. This phenomenon could be developed into an alternative form of therapy for erectile dysfunction without the attendant reported side effects of phosphodiesterase type 5 inhibition.

Keywords

Eurycoma longifolia Jack · Erectile dysfunction · Corpus cavernosum · Angiotensin II · Angiotensin-converting enzyme · Bradykinin

Abstract

Objective: This study was conducted to investigate the mechanisms of action of *Eurycoma longifolia* in rat corpus cavernosum. **Materials and Methods:** Tincture of the roots was concentrated to dryness by evaporating the ethanol in vacuo. This ethanolic extract was partitioned into 5 fractions sequentially with hexane, dichloromethane (DCM), ethyl acetate, butanol, and water. The corpus cavernosum relaxant activity of each fraction was investigated. The DCM fraction which showed the highest potency in relaxing phenylephrine-precontracted corpora cavernosa was purified by column chromatography. The effects of the most potent DCM subfraction in relaxing phenylephrine-precontracted corpora cavernosa, DCM-I, on angiotensin I- or angiotensin II-in-

duced contractions in corpora cavernosa were investigated. The effects of DCM-I pretreatment on the responses of phenylephrine-precontracted corpora cavernosa to angiotensin II or bradykinin were also studied. An in vitro assay was conducted to evaluate the effect of DCM-I on angiotensin-converting enzyme activity. **Results:** Fraction DCM-I decreased the maximal contractions (100%) evoked by angiotensin I and angiotensin II to $30 \pm 14\%$ and $26 \pm 16\%$ ($p < 0.001$), respectively. In phenylephrine-precontracted corpora cavernosa, DCM-I pretreatment caused angiotensin II to induce $82 \pm 27\%$ relaxation of maximal contraction ($p < 0.01$) and enhanced ($p < 0.001$) bradykinin-induced relaxations from $47 \pm 8\%$ to $100 \pm 5\%$. In vitro, DCM-I was able to reduce ($p < 0.001$) the maximal angiotensin-converting enzyme activity to $78 \pm 0.24\%$. **Conclusion:** Fraction DCM-I was able to antagonize angiotensin II-induced contraction to cause corpus cavernosum relaxation via inhibition of angiotensin II type 1 receptor and enhance bradykinin-induced relaxation through inhibition of angiotensin-converting enzyme.

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Introduction

Eurycoma longifolia Jack (*E. longifolia*) is a Southeast Asian shrub tree. The decoction of the roots has been used frequently by native populations as a tonic to enhance virility [1]. Scientific studies on the erectogenic properties of this plant revealed that its root extracts appear to be able to increase penile erection in rats [2, 3] and humans [4], in which markedly higher scores in the International Index of Erectile Function (IIEF) have been obtained compared to placebo [4]. However, the mechanisms of action underlying the erectogenic properties of this plant still remain unclear.

Penile erection requires the coordination of vasodilatation and corpus cavernosal smooth muscle relaxation [5]. Vascular smooth muscle relaxation (vasodilation) reduces the resistance of inflowing blood to the penis whilst corpus cavernosal relaxation causes the expansion of sinusoids, thus increasing the capacity to store blood [6]. Furthermore, sinusoidal expansion also leads to the compression of emissary veins by the tunica albuginea that would subsequently decrease the venous outflow to a minimum so as to achieve penile rigidity [6]. In addition, the local renin-angiotensin system that exists in the corpus cavernosum plays an important role in maintaining the corpus cavernosal tone by balancing the bradykinin (BK)-induced relaxation with the angiotensin II (Ang II)-induced contraction [7].

Erectile dysfunction (ED), one of the more common forms of malfunction of the male reproductive system, markedly affects the quality of life in men and their partners [8]. It is estimated that ED will affect approximately 322 million men worldwide by 2025, an increase of 170 million from 1995 [8]. Although ED can be ameliorated by phosphodiesterase type 5 (PDE 5) inhibitors like sildenafil [9], reported side effects of the oral therapies due to the unselective inhibition of PDE isoforms are still of major concern [10]. One of the major concerns appears to be that these PDE 5 inhibitors could also inhibit PDE 6 that are expressed in retina and might cause visual disturbances [10] ranging from abnormal color perception to photophobia [11]. Moreover, PDE 5 inhibitors may also cause blindness [12, 13] and hearing loss [14, 15]. Due to these potential side effects or nonresponders to these therapies, alternative treatments that can act on different mechanisms are necessary.

Many studies have been conducted in humans and animals to investigate the effects of Ang II on erectile function such as the finding that levels of Ang II in cavernous blood of healthy men are higher in the detumescence phase compared to the other penile phases of flaccidity,

tumescence, and rigidity [7, 16]. Higher levels of Ang II in the cavernous blood during penile flaccidity have been reported in patients with organic ED compared to healthy men [16]. Kifor et al. [17] showed that intracavernous injections of Ang II were able to terminate the spontaneous erections in anesthetized dogs. Hence, it is expected that inhibitors that suppress the formation or function of Ang II such as angiotensin-converting enzyme (ACE) inhibitors (ACEi) or Ang II type 1 receptor (AT₁R) blockers (ARBs) could vastly improve erectile function [6]. Currently, ACEi and ARBs that target local renin-angiotensin system in the corpus cavernosum are still not available on the market. One of the plausible ways to seek for the suitable candidates is by exploring the diverse range of natural products found in the environment. Therefore, this present study was conducted to investigate the probability of *E. longifolia* to inhibit the formation or function of Ang II at the tissue level to bring about relaxation in the rat corpus cavernosum.

Materials and Methods

Materials

Plant Materials

Roots of *E. longifolia* were collected from Peninsular Malaysia. A specimen (No. KLU 47214) was authenticated and deposited at the Rimba Ilmu Herbarium, University of Malaya.

Animals

Sixty-three adult male Sprague-Dawley rats aged 2–3 months and weighing 200–210 g were obtained from the Animal Experimental Unit, University of Malaya. All the rats were cared for and used in accordance with the Guide for the Care and Use of Laboratory Animals. They were kept under standard temperature and relative humidity laboratory conditions with 12-h light and dark cycles. Clean water and standard rat chow (Altromin, Germany) were made available ad libitum. All experimental procedures were approved by the University of Malaya Animal Care and Ethics Committee.

Chemicals and Drugs

All chemicals used in this study were of analytical grade and purchased from Merck KGaA (Darmstadt, Germany), except the 95% aqueous ethanol and sulfuric acid which were purchased from R&M Marketing (UK) and RCI Labscan Ltd. (Bangkok, Thailand), respectively. Phenylephrine (PE) hydrochloride, acetylcholine (ACh) chloride, angiotensin I (Ang I), Ang II, BK, ACE, hippuryl-L-histidyl-L-leucine (HHL), *p*-anisaldehyde and Tween[®] 80 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Methods

Extraction and Purification of Plant Material

Fresh roots were cleaned, dried, and then pulverized to powder. The powder was soaked in 95% aqueous ethanol that produced a tincture that was separated by filtration. The crude ethanolic ex-

tract was obtained after the ethanol was evaporated to dryness in vacuo.

The crude ethanolic extract was reconstituted in distilled water and then partitioned with hexane (HX) in a separating funnel. The upper HX layer was collected and evaporated to dryness in vacuo to obtain the HX fraction. The bottom aqueous layer was further partitioned with dichloromethane (DCM) to obtain the DCM fraction. Similarly, the remaining aqueous layer was partitioned with ethyl acetate (EA) and then followed by water-saturated butanol to obtain the EA and water-saturated butanolic (BU) fractions, respectively. Finally, the aqueous layer was freeze-dried to obtain the final aqueous (FA) fraction. Guided by *ex vivo* studies on isolated corpus cavernosum, the muscle relaxant activity of each fraction was investigated on the rat corpus cavernosum to obtain the most potent fraction, which was DCM that was then subjected to further purification.

The DCM fraction was loaded onto a column packed with Merck silica gel 60 (15–40 μ m), then eluted with 6 solvent mixtures of increasing ratios (by 10%) of polar solvents, starting with a mixture of EA and HX (75:25) to obtain DCM-I, the first DCM subfraction. This was then followed by 2 more cycles of elution using EA and HX mixtures of ratios 85:15 and 95:5 to obtain DCM-II and DCM-III, respectively. Subsequently, the column was eluted with EA and methanol mixtures in the ratios of 95:5, 85:15, and 75:25 to obtain DCM-IV, DCM-V, and DCM-VI, respectively. Similar to the above procedure, each DCM subfraction was tested for rat corpus cavernosum relaxant activity to obtain the most potent subfraction which was then used for studies on the probable mechanisms of action.

Ex vivo Studies on Isolated Corpus Cavernosum

Preparation, Validity, and Viability Testing of Corpus Cavernosum. Sixty-three rats were sacrificed by cervical dislocation. From each rat, the penis was dissected out and immediately placed in chilled Krebs-bicarbonate solution containing 118.1 mM NaCl, 4.7 mM KCl, 1.0 mM KH_2PO_4 , 1.0 mM MgSO_4 , 25.0 mM NaHCO_3 , 2.5 mM CaCl_2 , and 11.1 mM glucose, and aerated with a gas mixture of 95% O_2 and 5% CO_2 . The glans penis and urethra were excised and the septum between the corpora cavernosa was then cut to yield 2 strips. The tunica albuginea surrounding each strip was carefully removed to expose the smooth muscle underneath, which was subsequently mounted longitudinally in a 20-mL tissue chamber with one end tied to a tissue holder and the other to a force transducer connected to a physiological data acquisition system (PowerLab[®]) running the Chart[™] version 5.5.5 software program (ADInstruments Pty Ltd., Bella Vista, NSW, Australia). The Krebs-bicarbonate solution in the tissue chamber was maintained at 37°C and aerated with 95% O_2 and 5% CO_2 . Each strip was allowed to equilibrate for 60 min at an optimum resting tension (1 g) with changing of the bath fluid every 15 min before the commencement of all experiments.

In addition, concentration-dependent contraction and relaxation studies were also carried out to establish concentration-dependent contractile and relaxant curves in order to ensure the validity of subsequent experiments. This was done by adding cumulatively increasing amounts of PE (1×10^{-6} to 2.5×10^{-4} M) to the organ bath to cause concentration-dependent contractions of the corpus cavernosum. At contraction induced by maximum concentration of PE (2.5×10^{-4} M), increasing amounts of ACh (5×10^{-2} – 2×10^{-1} M) were then added cumulatively to obtain the concentration-relax-

ation curve. The viability and integrity of endothelia were established by adding ACh (1.1×10^{-1} M) to the PE-precontracted (2.5×10^{-4} M) corpus cavernosum. Strips that were able to relax $\geq 70\%$ of the maximal contraction indicated that the endothelia were intact and were then used for subsequent experiments.

Effects of Partitioned (HX, DCM, EA, BU, and FA) Fractions and DCM (DCM-I to DCM-VI) Subfractions on PE-Precontracted Corpus Cavernosum. Corpora cavernosa were precontracted with PE (2.5×10^{-4} M). After the contractile response stabilized, increasing concentrations of each of the partitioned (HX, DCM, EA, BU, and FA) fractions (0.1–3 mg/mL) or DCM (DCM-I to DCM-VI) subfractions (0.3–2.5 mg/mL) were added cumulatively to the organ bath.

Effects of DCM Subfraction-I on Ang I- or Ang II-Induced Contractions. Corpora cavernosa were incubated with DCM-I (2.5 mg/mL) or vehicle (0.15% Tween[®] 80 in distilled water) for 15 min, after which increasing concentrations of Ang I or Ang II (3.16×10^{-7} – 3.16×10^{-4} M) were added cumulatively.

Effects of DCM Subfraction-I on Ang II-Induced Contractions and BK-Induced Relaxations in PE-Precontracted Corpus Cavernosum. This study was conducted according to the method of Arun et al. [18]. Corpora cavernosa were incubated with DCM-I (1.25 mg/mL) or vehicle for 15 min, after which PE (2.5×10^{-4} M) was added to precontract the corpora cavernosa. Once the contractile response had stabilized, increasing concentrations of Ang II (3.16×10^{-7} – 3.16×10^{-4} M) were added cumulatively.

In another experiment, BK (3.16×10^{-5} – 1×10^{-3} M) was added cumulatively instead of Ang II once the PE-induced contraction had stabilized.

In vitro Studies

Effects of DCM Subfraction-I on ACE Activity. The ACE inhibitory assay was conducted according to the method of Hoe et al. [19]. In brief, DCM-I (0.3–2.5 mg/mL) or vehicle was added to the incubation buffer containing ACE and the substrate, HHL. In the reaction, HHL was cleaved by ACE, and the resultant hippurate produced was mixed with cyanuric chloride to form chromogens that were quantified by measuring the absorbance at 382 nm. Blanks were treated in the same manner except that the terminating solution (hydrochloric acid) was added before the addition of substrate.

Phytochemical Analyses of DCM Subfraction-I. Thin-layer chromatography plates (Merck KGaA; silica gel 60) were spotted with DCM-I and then developed with a solvent mixture of methanol and EA (75:25). Once developed, the plates were allowed to dry completely before being sprayed with either commercially available Dragendorff reagent (Merck KGaA) or anisaldehyde-sulfuric acid reagent and heated at 105°C for 10 min to visualize the compounds present under daylight [20]. Alkaloids will appear as orange spots, while terpenoids will appear as purple, blue, or red spots in the plate [20].

Statistical Analyses

Values are expressed as means $\pm$ SEM. Data were analyzed by SPSS[®] Statistics version 17.0 (SPSS Inc., Chicago, IL, USA) and graphed using GraphPad Prism[®] version 5 for Windows (GraphPad Software Inc., San Diego, CA, USA). The Student *t* test was used to compare the differences between 2 groups while a 1-way ANOVA Tukey test was used to perform multiple group comparisons. A probability value of less than 0.05 ($p < 0.05$) was considered to be statistically significant.

Fig. 1. **a** Cumulative concentration-response curve of PE-induced contraction in corpora cavernosa of Sprague-Dawley rats. **b** Cumulative concentration-relaxation curve of ACh in PE-precontracted corpora cavernosa of Sprague-Dawley rats. Values are means $\pm$ SEM ($n = 6$).

Results

Ex vivo Studies on Isolated Corpus Cavernosum

PE and ACh were able to contract and relax corpora cavernosa, respectively, in a dose-dependent manner as shown in Figure 1a and b. The maximum concentration of PE was 2.5×10^{-4} M (Fig. 1a). The concentration of ACh that induced 70% of relaxation in PE-precontracted corpora cavernosa was 1.1×10^{-1} M (Fig. 1b). The results of these preparatory procedures indicated that the bioassay setup was valid and viable.

All the fractions of HX, DCM, EA, and BU, except FA, attenuated the PE-induced contractions in a dose-dependent manner; however, marked differences in the potency of each fraction were observed (Fig. 2a). Thus, at the highest dose of fractions used (3 mg/mL), DCM ($94 \pm 7\%$ relaxation) was significantly more potent than HX ($59 \pm 5\%$ relaxation; $p < 0.05$), BU ($29 \pm 7\%$ relaxation; $p < 0.01$), and FA ($4 \pm 9\%$ relaxation; $p < 0.001$), while EA ($93 \pm 15\%$ relaxation) was significantly more effective in attenuating the maximal PE-induced contraction compared to BU ($29 \pm 7\%$ relaxation; $p < 0.05$) and FA ($4 \pm 9\%$ relaxation; $p < 0.01$). Vehicle alone did not elicit any response. There was no significant difference between the maximal relaxant responses of DCM and EA. Overall, DCM and EA showed the highest potency in relaxing corpora cavernosa.

All DCM subfractions except DCM-V and DCM-VI were able to attenuate maximal contractions induced by PE with DCM-I being the most potent subfraction recorded (Fig. 2b). At a dose of 2.5 mg/mL, the relaxation

caused by DCM-I was statistically significant from DCM-IV ($p < 0.01$), DCM-V ($p < 0.001$), and DCM-VI ($p < 0.001$), while those of DCM-II and DCM-III were significantly different from DCM-V and DCM-VI ($p < 0.01$).

Effects of DCM Subfraction-I on Ang I- or Ang II-Induced Contractions

Pretreatment of corpora cavernosa with DCM-I significantly ($p < 0.05$) reduced the contractions evoked by Ang I in a dose-dependent manner (Fig. 3a). Similarly, contraction of corpora cavernosa induced by Ang II was also significantly ($p < 0.05$) reduced in the presence of DCM-I (Fig. 3b).

Effects of DCM Subfraction-I on Ang II-Induced Contractions and BK-Induced Relaxations in PE-Precontracted Corpus Cavernosum

In the presence of DCM-I, not only were the contractions due to Ang II abolished, significant ($p < 0.05$) relaxation of the muscles were observed instead (Fig. 4a), and for BK, the relaxations were further enhanced ($p < 0.001$; Fig. 4b). In the absence of DCM-I, Ang II caused further contractions in PE-precontracted corpora cavernosa, whilst BK produced relaxations.

In vitro Studies

DCM-I inhibited the activity of ACE in a concentration-dependent manner with inhibition being significant at concentrations of 1.25 mg/mL ($p < 0.05$) and 2.5 mg/mL ($p < 0.001$) (Fig. 5).

Fig. 2. Effects of partitioned fractions (a) and DCM subfractions (b) on PE-induced contractions. Values are means $\pm$ SEM ($n = 6$). ^a $p < 0.05$ compared with HX; ^b $p < 0.05$; ^{bb} $p < 0.01$ compared with BU; ^{cc} $p < 0.01$; ^{ccc} $p < 0.001$ compared with FA; ⁺⁺ $p < 0.01$ compared with DCM-IV; ^{**} $p < 0.01$ and ^{***} $p < 0.001$ compared with DCM-V; ^{##} $p < 0.01$ and ^{###} $p < 0.001$ compared with DCM-VI.

Fig. 3. Effects of DCM-I pretreatment on Ang I-induced (a) or Ang II-induced (b) contractions. Values are means $\pm$ SEM ($n = 6$). ^{*} $p < 0.05$, ^{**} $p < 0.01$, and ^{***} $p < 0.001$ compared with control (without DCM-I).

Fig. 4. Effects of DCM-I pretreatment on Ang II-induced contractions (**a**) and BK-induced relaxations (**b**) in PE-precontracted corpora cavernosa. Values are means $\pm$ SEM ($n = 6$). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with control (without DCM-I).

Fig. 5. Effects of DCM-I on activity of ACE. Values are means $\pm$ SEM ($n = 3$). * $p < 0.05$ and *** $p < 0.001$ compared with control (vehicle only).

The thin-layer chromatography plate which was sprayed with anisaldehyde-sulfuric acid reagent revealed a spot that indicated the presence of terpenoids in DCM-I (Fig. 6). For the thin-layer chromatography plate which was sprayed with Dragendorff reagent, no spots were observed.

Discussion

In this study the root extracts of *E. longifolia* had the ability to cause direct relaxation of rat corpus cavernosum. Furthermore, the more purified form of the root extracts/DCM-I, was able to cause/induce relaxation in

Fig. 6. Thin-layer chromatography plate of DCM-I sprayed with anisaldehyde-sulphuric acid reagent and heated at 105°C for 10 min. Spot observed (arrow) indicates the presence of terpenoids.

corpora cavernosa by counteracting Ang II-induced contractions and enhancing BK-mediated relaxations. This mechanism could probably explain the physiological event necessary for penile erection to occur as previously reported [21].

Ang I-induced contractions observed in the study indicated that Ang I had to be converted by the local corpus cavernosal tissue ACE to Ang II [22] which would then be able to contract the rat corpus cavernosum in a dose-dependent manner as the peptide, Ang I, is known not to have any physiological function [23]. In addition, pretreatment of rat corpus cavernosum with DCM-I was able to significantly inhibit the contractions induced by Ang I, indicating that DCM-I might either inhibit the activity of ACE and thus the conversion of Ang I to Ang II, or directly antagonize the contractile effects of Ang II. It is also plausible that DCM-I could act through both the suggested mechanisms simultaneously to bring about the relaxant effect.

The suggestion that DCM-I might directly counteract the contractile effect of Ang II was shown when pretreatment of corpora cavernosa with DCM-I significantly attenuated the Ang II-induced contractions. The DCM-I pretreatment not only prevented the occurrence of Ang II-induced contractions, but also caused significant Ang II-induced relaxation in the muscle. It is well-established that Ang II elicits diverse physiological effects through 2 subtypes of Ang receptors, namely AT₁R and Ang II type 2 receptor (AT₂R) [24]. The contractile effects of Ang II are mediated via AT₁R, whereas the relaxant effects are brought about by AT₂R [24]. However, Ang II binds to AT₂R when AT₁R is blocked as the expression of AT₂R is relatively low [24]. When Ang II was added to PE-precontracted corpora cavernosa, a contractile response developed, as it was likely that Ang II would have stimulated AT₁R that was abundantly found in cavernous tissue. The finding that Ang II was able to induce relaxation in PE-precontracted corpora cavernosa when the tissue was pretreated with DCM-I could suggest that Ang II would either bind directly to AT₂R [24] and/or be converted to angiotensin-(1-7) by angiotensin-converting enzyme 2 (ACE2) [25] to bring about relaxation through binding with the Mas receptor [26]. However, binding to AT₂R is more likely to occur as the affinity of Ang II for AT₂R is 1,000 times higher than that for ACE2 [27]. For this to occur, AT₁R should be blocked and the blocker appears to be DCM-I.

The presence of DCM-I was found to be able to enhance further the relaxant effect of BK, which has been known to confer beneficial effects on penile erection [7, 28, 29]. BK is able to cause relaxation in human corpus cavernosum [7, 28], and just recently, BK has also been shown to be able to increase intracavernous pressure [29]. The BK potentiation effect of DCM-I could be due to inhibition of ACE alone or simultaneous activation of BK receptors [30].

From the results of the DCM-I, Ang II, and BK studies on rat corpus cavernosum, it appeared that DCM-I was able to antagonize the contractile effects of Ang II while enhancing the relaxant effects of BK. These dual effects of DCM-I could be an outcome of ACE inhibition as ACEi is able to reduce the production of Ang II and at the same time increase the half-life of BK [24], as the activity of ACE was found to be significantly reduced in the presence of DCM-I.

Thus, DCM-I, by acting through entirely different mechanisms, could be developed into an alternative form of therapy without the attendant reported side effects of PDE 5 inhibition. This form of therapy could be an alternative for ED patients, especially those who experience side effects or those who are not responsive to the conventional treatments.

Conclusion

In this study, DCM-I, the semipurified fraction of the roots of *E. longifolia*, was found to be able to cause relaxation in rat corpora cavernosa by counteracting the contractile effects of Ang II via inhibition of ACE and AT₁R as well as enhancing the relaxant effects of BK.

Disclosure Statement

The authors report no conflicts of interest.

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