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สัญญาเลขที่ R2561B005

สำนักหอสมุด

รายงานวิจัยฉบับสมบูรณ์

การศึกษาฤทธิ์ของสารสกัดเถาวัลย์เปรียงต่อพฤติกรรมการสืบพันธุ์ในหนูที่ถูกเหนี่ยวนำให้เกิดภาวะสมรรถภาพทางเพศเสื่อมจากการทำให้เกิดความเครียดโดยการจำกัดการเคลื่อนไหว

Effect of *Derris scandens* (Roxb.) Benth. extract on mating behavior of male rat induced erectile dysfunction by chronic immobilization-induced stress

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กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณนิสิตบัณฑิตศึกษา อาจารย์ เจ้าหน้าที่ คณะเภสัชศาสตร์ และสถานวิจัยเครื่องสำอางและผลิตภัณฑ์ธรรมชาติ มหาวิทยาลัยนเรศวร รวมทั้งสมาชิก Bioscreening units ซึ่งมีส่วนให้งานวิจัยครั้งนี้สำเร็จลงด้วยดี

การศึกษาครั้งนี้ ได้รับการสนับสนุนงบประมาณ จากงบประมาณแผ่นดินมหาวิทยาลัยนเรศวร ปีงบประมาณ 2561 จึงขอขอบคุณมา ณ ที่นี้ด้วย

บทคัดย่อ

ภาวะสมรรถภาพทางเพศเสื่อมในเพศชายเป็นภาวะที่อวัยวะเพศชายไม่สามารถแข็งตัวหรือแข็งตัวได้ไม่นานระหว่างการมีเพศสัมพันธ์ ซึ่งมักพบความเกี่ยวข้องกับภาวะทางจิตใจและโรคต่างๆ โดยเป็นภาวะที่พบได้เกือบ 50% ในผู้ชายที่มีอายุตั้งแต่ 40 ปีขึ้นไป และมีแนวโน้มที่จะพบเพิ่มขึ้น ปัจจุบันยาชนิดรับประทานที่ใช้รักษามีเพียงกลุ่มเดียวคือ ยาต้านเอนไซม์ฟอสโฟไดเอสเทอเรส 5 ตัวอย่างเช่น sildenafil, vardenafil, tadalafil และ avanafil ซึ่งนอกจากจะเป็นยาที่มีราคาสูงแล้วยังพบผลข้างเคียงในผู้ใช้ยาอีกด้วย

จากการศึกษาวิจัยในครั้งนี้พบว่า สารสกัด 95% เอทานอลของเถาวัลย์เปรียงและสารบริสุทธิ์ที่แยกได้จากเถาของเถาวัลย์เปรียงมีฤทธิ์ต้านเอนไซม์ฟอสโฟไดเอสเทอเรส 5 ได้ในเกณฑ์ดี คือมีค่าความเข้มข้นที่ยับยั้งการออกฤทธิ์ของเอนไซม์ฟอสโฟไดเอสเทอเรส 5 ร้อยละ 50 ที่ 7.66 ไมโครกรัม/มิลลิลิตร และจากการทดสอบฤทธิ์ต้านเอนไซม์ฟอสโฟไดเอสเทอเรส 5 ของสารไอโซฟลาโวนและสารคูมารินที่แยกได้จากเถาวัลย์เปรียงจำนวน 8 ชนิด พบว่า สาร Osajin (8), 4',5,7 – trihydroxybiprenylisoflavone (4) และ derrisisoflavone A (2) มีฤทธิ์ต้านเอนไซม์เอนไซม์ฟอสโฟไดเอสเทอเรส 5 ได้ในระดับสูง โดยมีค่าความเข้มข้นที่ยับยั้งการออกฤทธิ์ของเอนไซม์ฟอสโฟไดเอสเทอเรส 5 ร้อยละ 50 ที่ 4, 8 และ 9 ไมโครโมลาร์ ตามลำดับ นอกจากนี้ การศึกษาความจำเพาะของการออกฤทธิ์ต้านเอนไซม์ฟอสโฟไดเอสเทอเรส 5 เปรียบเทียบกับเอนไซม์ฟอสโฟไดเอสเทอเรสอื่นได้แก่ เอนไซม์ฟอสโฟไดเอสเทอเรส 1 และเอนไซม์ฟอสโฟไดเอสเทอเรส 6 พบว่า สารทั้ง 3 ชนิดนี้มีฤทธิ์จำเพาะต่อการยับยั้งฟอสโฟไดเอสเทอเรส 5 สูงเมื่อเทียบกับเอนไซม์ฟอสโฟไดเอสเทอเรส 1 แต่มีความจำเพาะอยู่ในระดับต่ำเมื่อเทียบกับเอนไซม์ฟอสโฟไดเอสเทอเรส 6

วิธีลิควิดโครมาโทกราฟี แบบอิเล็กโตรสเปรย์ แมสสเปกโทรเมทรี ชนิดควอดดรูโพล (LC-QTOF-MS) ได้ถูกพัฒนาขึ้น สำหรับการควบคุมคุณภาพและการวิเคราะห์เชิงปริมาณของสารสำคัญในเถาวัลย์เปรียง โดยระดับต่ำสุดที่สามารถวิเคราะห์สารทดสอบทั้ง 8 ชนิดได้ (Limit of quantitation) อยู่ในช่วง 0.1 – 5 ไมโครกรัม/มิลลิลิตร ค่าพารามิเตอร์ของการทดสอบ ได้แก่ ค่าความเที่ยงตรง (Accuracy) และค่าความแม่นยำ (Precision) อยู่ในเกณฑ์การยอมรับได้ แสดงว่าวิธีทดสอบดังกล่าวมีความเหมาะสมสำหรับการควบคุมคุณภาพและวิเคราะห์หาปริมาณสารสำคัญในเถาวัลย์เปรียง ซึ่งจากการวิเคราะห์พบว่าในสารสกัดเถาวัลย์เปรียงพบสารทดสอบทั้ง 8 ชนิด โดยมีสาร derrisisoflavone A (2) และสาร lupalbigenin (3) เป็นองค์ประกอบหลัก

จากการทดสอบฤทธิ์ของสารสกัดเถาวัลย์เปรียงต่อการฟื้นฟูสมรรถภาพทางเพศในหนูแรทเพศผู้สายพันธุ์ Wistar ที่ได้รับความเครียดจากการจำกัดการเคลื่อนไหว พบว่าการได้รับสารสกัดเถาวัลย์เปรียงขนาด 200, 400 และ 800 มิลลิกรัม/กิโลกรัม/วัน ติดต่อกัน 14 วัน มีผลฟื้นฟูสมรรถภาพทางเพศ โดยลดระยะเวลาการขึ้นคร่อมหนูเพศเมีย (Mount latency) และระยะเวลาการสอดใส่อวัยวะเพศของหนูเพศผู้ (Intromission latency) นอกจากนี้สารสกัดเถาวัลย์เปรียงยังมีฤทธิ์ในการเพิ่มประสิทธิภาพของการ

แข็งตัวของอวัยวะเพศของหนูแรทโดยสามารถเพิ่มจำนวนของการสอดใส่ (Intromission number) อีกด้วย อย่างไรก็ตามฤทธิ์ของสารสกัดเถาวัลย์เปรียงต่อสารสื่อประสาท ได้แก่ Norepinephrine, Dopamine และ Serotonin รวมถึงฮอร์โมนเพศ คือ Testosterone นั้นยังจำเป็นที่จะต้องศึกษาเพิ่มเติม

การศึกษาวิจัยนี้รายงานฤทธิ์ต้านเอนไซม์ฟอสโฟไดเอสเทอเรสและฤทธิ์ฟื้นฟูสมรรถภาพทางเพศในหนูแรทเพศผู้ของสารสกัดเถาวัลย์เปรียงเป็นครั้งแรก วิธีลิควิดโครมาโทกราฟี แบบอิเล็กโตรสเปรย์แมสสเปกโทรเมทรี ชนิดควอดดรูโพล ได้ถูกพัฒนาขึ้นและมีความเหมาะสมสำหรับใช้ในการวิเคราะห์เชิงปริมาณและการควบคุมคุณภาพของสารสกัดเถาวัลย์เปรียง

Abstract

Erectile dysfunction (ED) is defined as an inability to achieve and sustain penile erection sufficient for sexual performance. Organic factors and psychogenic factors are known to be a cause of ED. Recently, phosphodiesterase 5 (PDE5) inhibitors have been used as a first line medicine for the treatment of ED. In the search for new PDE5 inhibitors from natural sources, *Derris scandens* (Roxb.) Benth. stem and its chemical constituents were evaluated for their inhibitory activity against PDE5 as well as other isozymes, PDE1 and PDE6. It was found that the 95% ethanol extract of *D. scandens* stem (DE) showed PDE5 inhibitory activity with the IC_{50} values about 7 μg/mL. Among seven isoflavones and a coumarin isolated from this plant, osajin **(8)**, 4',5,7 – trihydroxybiprenylisoflavone **(4)** and derrisisoflavone A **(2)** had the highest ability to inhibit PDE5 (IC_{50} values of 4, 8 and 9 μM, respectively). These 3 compounds exhibited selectivity on PDE5 over PDE1, however, the selectivity on PDE5 over PDE6 were low. In order to quantitatively determine these bioactive constituents in *D. scandens*, the liquid chromatography-quadrupole-time-of-flight-mass spectrometry (LC-QTOF-MS) method has been developed and validated. The LOQ values in the range of 0.1 – 5 μg/mL were obtained. The assay showed satisfactory precision and accuracy. The results from our method indicated that DE comprised of all eight compounds with derrisisoflavone A **(2)** and lupalbigenin (3) presenting as the major constituents.

The effect of DE on the mating behaviour of stress-induced ED male rats was investigated. Oral administration of the extract at the doses of 200, 400 and 800 mg/kg/day for 14 days significantly motivated the mating behaviour by reducing the mount latency and intromission latency. The increase of efficiency of penile erection and penile orientation by DE were also observed from the increase of intromission number. However, the effects of DE on the levels of testosterone and neurotransmitters are still not clear.

In conclusion, this study reports the PDE5 inhibitory activity of DE and its isoflavonoid constituents for the first time. LC-QTOF-MS was successfully used for the quantitative analysis of these compounds in DE and the plant materials. This study also indicated the potential of DE as an aphrodisiac in stress-induced ED rats. More research is needed to improve the underlying mechanisms of the extract.

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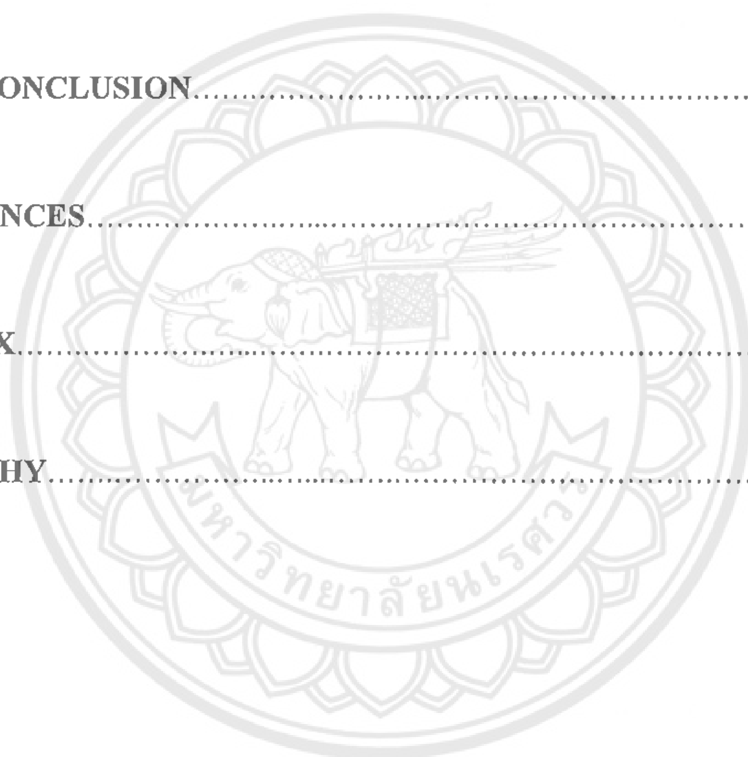

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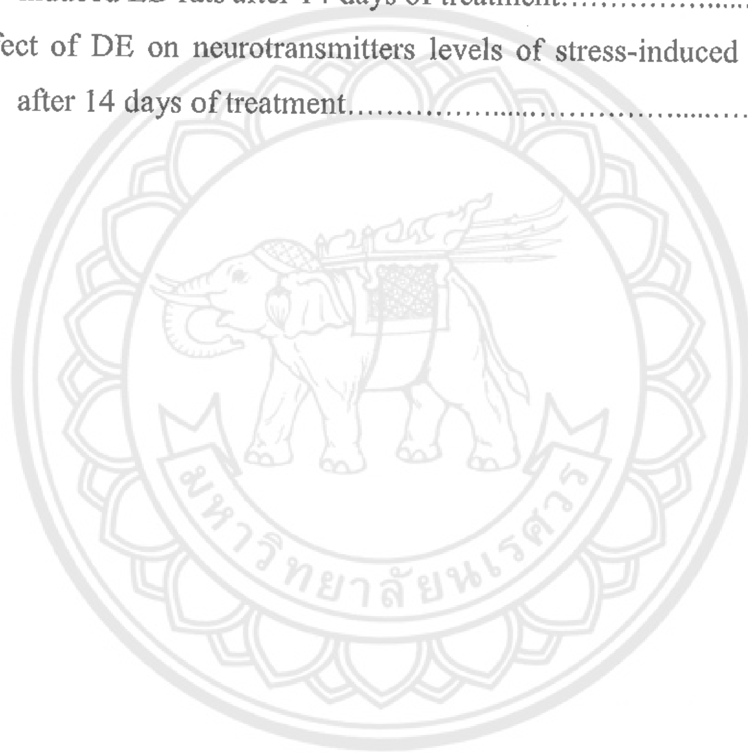

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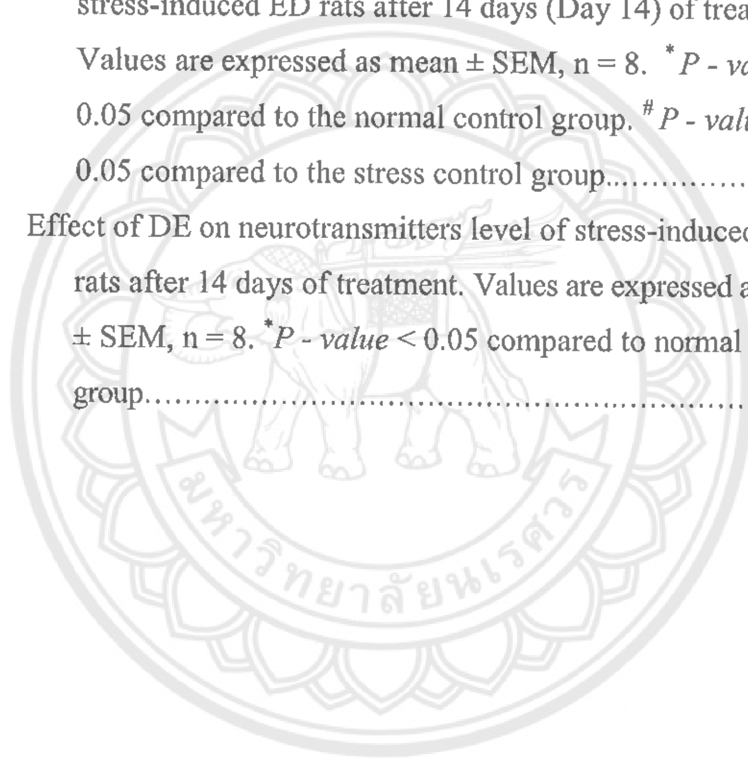


CHAPTER I

INTRODUCTION

Background and rationale

Erectile dysfunction (ED), also known as impotence, is defined as 'an inability to achieve and sustain penile erection sufficient for sexual performance' [1]. Currently, it is estimated that almost half of Thai males between the ages of 40 to 70 experience ED. Statistics have indicated that the prevalence rate of Thai males experiencing ED has increased from 37.50% in 2000 to 42.18% in 2008 [2]. The results from the Massachusetts male aging study (MMAS) between 1987 and 2004 reported an overall prevalence of 52% ED in men aged 40 to 70 years in the United States [3].

Erectile function involves the integration of complex processes between neurologic, vascular, smooth muscle, hormonal, and psychological factors. The achievement and sustaining of erection requires a good arterial inflow of blood into erectile tissue as well as the potential reduction of venous outflow. Disease or risk factors that affect arterial or venous function, would therefore, be expected to influence erectile function [4]. Pharmacological influences on male rat sexual behaviour are reviewed in an attempt to identify neurotransmitters and their respective receptor types that regulate various factors comprising the behavioural pattern. Evidences show that dopamine, oxytocin, adrenocorticotropic hormone (ACTH), and melanocyte-stimulating hormones (MSH) appear to increase sexual arousal. On the other hand, serotonin, norepinephrine, GABA, opiate and prolactin appear to inhibit sexual response [5]. In the past, psychogenic issues were believed to be a cause of ED. Recently organic factors have been emphasized and identified as one of the major etiologies of sexual dysfunction. There are several pathophysiological factors involved in developing ED including vascular disorders such as atherosclerosis, ischemic heart disease and cavernosal disorders; neurological disorders such as cerebral diseases, spinal cord injury, spinal disease and pudendal nerve injury; hormonal disorders such as hypogonadism, hyperprolactinemia, and Addison's disease. In addition, some

medications, such as antihypertensives, antidepressants, diuretics, cardiac medications, and hormones can cause ED. Also, marijuana use, alcohol abuse, narcotics use and cigarette smoking can induce ED [6, 7]. The study of cross-sectional results from MMAS by Araujo et al [8] concluded that ED is related to depressive symptoms, that is psychological disorders.

Currently, the treatment of ED has focused mainly on the mechanisms involved in the relaxation mechanism of the corpus cavernosum smooth muscle. The biochemical and physiological functions related to the mechanism could enhance erectile function sufficiently for normal sexual activity, especially phosphodiesterase 5 (PDE5) enzymes [7]. The PDE5 inhibitor drug is the first-line drug used for treating ED [4, 7].

PDE5 is the predominant cGMP degrading enzyme in platelets, the penile corpus cavernosum, smooth muscle cells, and all vascular smooth muscle cells mainly in the pulmonary vessel [9, 10]. A cGMP, the second messenger, plays a role in the regulation of penile erection through the relaxation of smooth muscle cells in the penile corpus cavernosum [11, 12, 13]. To date, four PDE5 inhibitors were approved for erectile dysfunction (ED) treatment by FDA and available in a market including sildenafil [14], vardenafil [15], tadalafil [16] and avanafil [17]. Sildenafil was granted FDA approval for treating pulmonary artery hypertension [14] and tadalafil was approved for treating benign prostatic hyperplasia [16]. The selectivity of PDE5 inhibitor drug is in concern as some side effects are presented in patients who take non-selective PDE5 inhibitor drugs. Non-selectivity inhibition of PDE5 inhibitor can cause visual abnormalities from the inhibition of PDE6, tachycardia, vasodilation and flushing from the inhibition of PDE1. Moreover, other adverse effects were also reported including headache, dyspepsia, nasal congestion and nasopharyngitis [18, 19, 20]. Therefore, the search for a new selective PDE5 inhibitor drug is still on-going, especially from natural sources.

In our preliminary study, 37 plants used as a tonic or rejuvenating agents in Thai traditional medicine, were screened and extracted with 95% ethanol. The result showed that the extract of leaves and stems of *Derris scandens* (Roxb.) Benth exhibited more than 90% PDE5 inhibition at the final concentration of 50 μg/mL and the IC_{50} values of the leaf and stem extracts were 4.18 ± 0.79 and 5.17 ± 1.45 μg/mL,

respectively. In this study, the potential of *D. scandens* as a PDE5 inhibitor for ED therapy was to be examined.

D. scandens is a woody vine that belongs to the family of Fabaceae. It has been used in Thai traditional medicine for a long time as a muscle pain reliever, diuretic, antidysentery, expectorant, antitussive and rejuvenating agent [21, 22]. The powder and hydroalcoholic extract of *D. scandens* stem were recorded in the National list of essential medicines (NLEM) of Thailand for the treatments of muscle pain, low back pain and knee osteoarthritis [23]. The clinical studies indicated the ability of the hydroalcoholic extract of the *D. scandens* stem on pain reduction for knee osteoarthritis [24] and low back pain patients [25]. So far, no studies of the effect of *D. scandens* on PDE5 or on sexual behaviour have been reported.

In the present work the PDE (PDE1, 5 and 6) inhibitory activities of *D. scandens* extracts and isolated compounds were determined. The method for quantitative determination of PDE5 inhibitors in *D. scandens* using liquid chromatography-quadrupole-time-of-flight-mass spectrometry (LC-QTOF-MS) was developed and validated. The contents of PDE5 inhibitors in *D. scandens* extract and dried plant materials were measured. The extract which showed the highest PDE5 inhibitory activity was tested for the effect on mating behaviour of stress induced ED rats. The results obtained from these studies could indicate the ability of *D. scandens* in treating ED.

Objectives of the study

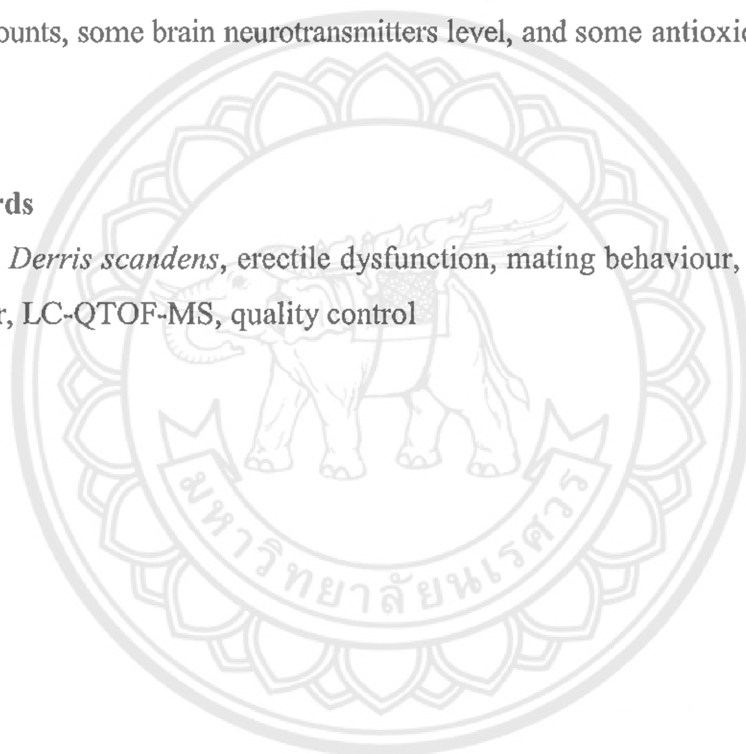
1. To evaluate PDE 1, 5 and 6 inhibitory activities of the extracts and isolated compounds of *D. scandens*.
2. To develop the methods for quantitative determination of PDE5 inhibitors in *D. scandens* by using LC-QTOF-MS analysis.
3. To evaluate the effects of *D. scandens* extract in male rats induced erectile dysfunction by chronic immobilization-induced stress on mating behaviour, hormones, sperm counts, some brain neurotransmitters level, and some antioxidant enzyme level in testes.

Scope of the study

D. scandens were extracted using various solvents including water, 50% ethanol and 95% ethanol. The extracts and isolated compounds of *D. scandens* were evaluated for the PDE (PDE1, 5 and 6) inhibitory activities. The structure-activity relationships (SAR) of the compounds were discussed. The LC-QTOF-MS method for quantitative determination of PDE5 inhibitors in *D. scandens* extracts and dried plant materials was developed and validated. The extract which showed the highest PDE5 inhibitory activity was determined for the effects on mating behaviours of stress induced ED rats, hormones, sperm counts, some brain neurotransmitters level, and some antioxidant enzyme level in testes.

Keywords

Derris scandens, erectile dysfunction, mating behaviour, phosphodiesterase 5 inhibitor, LC-QTOF-MS, quality control



CHAPTER II

LITERATURE REVIEW

Erectile and erectile dysfunction (ED)

Prevalence of ED

The prevalence of ED in Asian populations between 1986 and 2006 was analysed by Cheng et al [26] using computer-based searches through MEDLINE, PubMed, PsycINFO and others. The results showed that the prevalence of ED in Asian populations increased with age. Pooled random effects age-specific prevalence rates were 15.1%, 29.6%, 40.6%, 54.3% and 70.0% for the age groups of 20–29, 30–39, 40–49, 50–59, and 60–69 years, respectively. The prevalence of ED in Thailand was carried out by Kongkanand et al [27] 1,250 males from each area of Thailand, including north, south, eastern, central, and Bangkok were interviewed. The results showed that 37.5% of men aged 40 – 70 years experienced ED. Severe ED occurs in 4.7%, moderate ED in 4.7% and mild ED in 19.1%. Among another, the Massachusetts male aging study between 1987 to 2004 estimated that more than half of men aged 40 – 70 years in the United States experienced erectile dysfunction (ED). The overall prevalence of mild, moderate and complete ED were 16.5%, 17.5% and 4.9%, respectively, at age of 40 years, and 18%, 34% and 15% at age of 70 years [28].

Erectile tissue

The erectile tissue (Figure 1) span the length of the penis shaft, which consists of three cylindrical bodies including the single corpus spongiosum and the paired corpora cavernosa as well as the fibrous tissue, nerves, lymphatics, and blood vessels. Corpus spongiosum is the single column of sponge-like tissue running along the front of the penis which ends at the glans penis and also surrounds the urethra within the penis. The corpus spongiosum fills with blood to protect the fine tissues of the urethra, and generate an erection. Corpora cavernosa is the paired column of sponge-tissue running side by side of the penis shaft and surrounded by elastic fibres known as tunica albuginea. Corpora cavernosa have the ability to engorge with blood from arteries in the penis. The increasing of blood inside the corpora generates the

pressure against the veins, which surrounds the chambers, and stops the veins from draining the blood out of the penis, which facilitates penile rigidity and erection. The tunica albuginea of corpora cavernosa is a bilayer-fibroelastic structure surrounding each of the corpora cavernosa and the corpus spongiosum. It is composed of the outer longitudinal muscle fibres that insert proximally on the inferior pubic rami, and the inner circular layer, which contains cavernous tissue to support the sinusoids and form the vertical septum to separate each cavernosum. This septum and the tunica albuginea together, support penile rigidity when erection occurs [29, 30, 31]. The pudendal artery is the major carrier of the blood supply to the penis, dividing into three branches including the bulbourethal artery, dorsal artery, and the cavernous artery. The cavernous artery supplies the corpora cavernosa, the dorsal artery supplies the skin, subcutaneous tissue and the glans penis while, the bulbourethal artery supplies the corpus spongiosum [32].

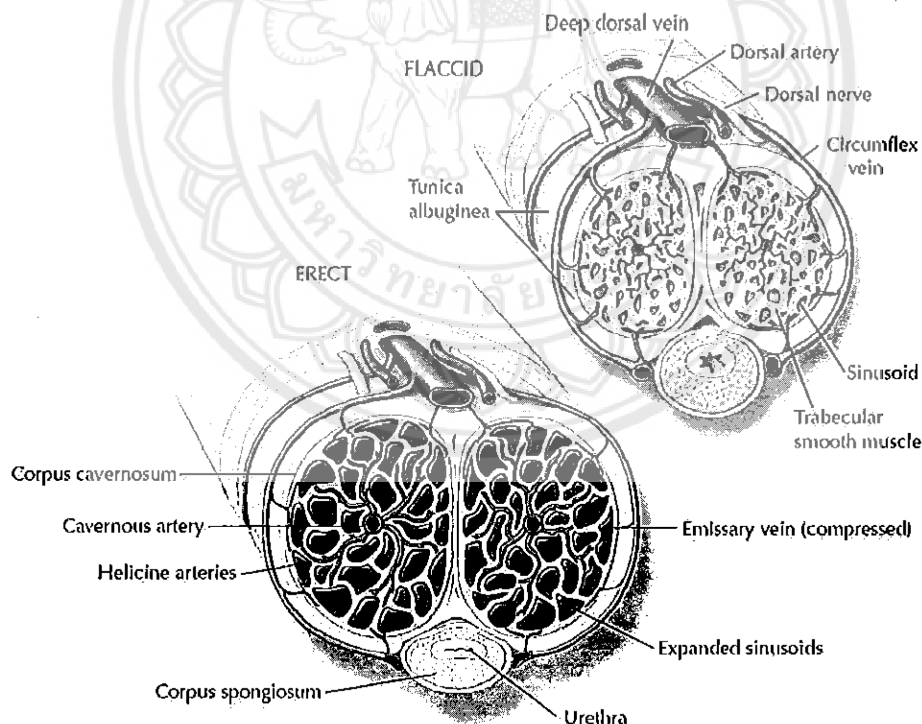


Figure 1 Structure of erectile tissue [7]

Hormones

Androgens are essential for male sexual desire, particularly testosterone (T). The functions of testosterone include the promotion spermatogenesis in testes, muscle development, bone growth, the development of secondary sexual characteristics, and the maintenance of libido in both males and females. The regulation of testosterone occurs when testosterone levels are low. The hypothalamus releases the gonadotropin-releasing hormone (GnRH). GnRH stimulates the anterior pituitary to release two gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates the Leydig cells in the testes to increase the production of testosterone. FSH stimulates the Sertoli cells within the seminiferous tubules to promote spermatogenesis, release androgen-binding protein (ABP), and produce inhibins, a hormone that inhibits FSH release from the anterior pituitary. High levels of testosterone in the bloodstream cause negative feedback to decrease the production and release of GnRH, LH and FSH. To achieve these events, testosterone will bind to androgen receptors in the hypothalamus and the anterior pituitary. The decrease of GnRH causes a reduction in the amounts of LH and FSH released, resulting in a decrease in the levels of testosterone in the bloodstream [29].

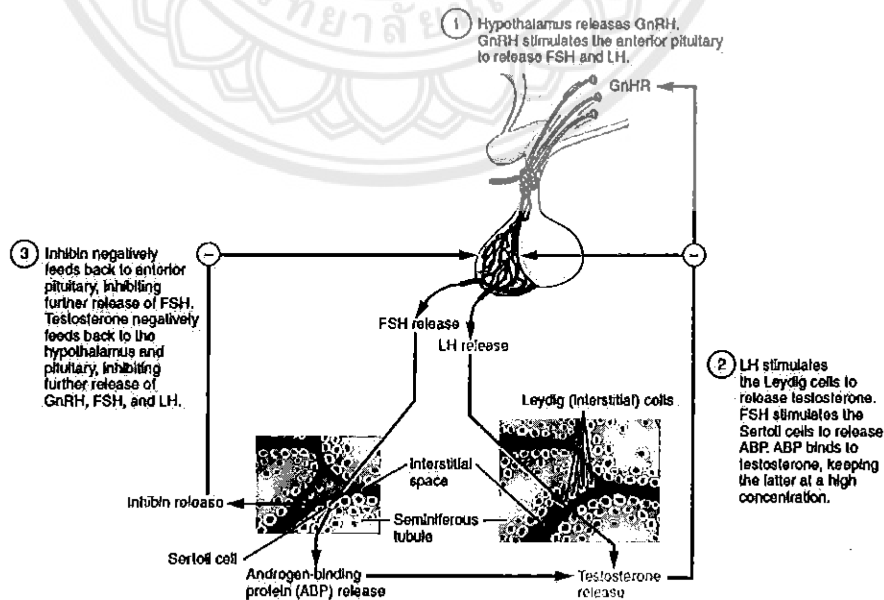


Figure 2 Regulation of Testosterone Production [29]

Neurotransmitters mediate penile erection

The regulation of sexual function is a complex process involving the integration of the peripheral nervous system (PNS), central nervous system (CNS), and the endocrine system [5].

Central neurotransmitters and neuropeptides that control penile erection include: serotonin, dopamine, oxytocin, nitric oxide (NO), noradrenaline, and opioid peptides. They regulate penile erection, both facilitate and inhibit, by acting in several brain areas i.e., the medial preoptic area (MPOA), the paraventricular nucleus (PVN), the hippocampus, the amygdala, the hypothalamus, the medulla oblongata and the spinal cord. Serotonin or 5-Hydroxytryptamine (5-HT) may facilitate or inhibit penile erection depending on the receptor subtype involved. 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2C} receptor subtypes have been identified and found at different levels of the spinal cord. Stimulation of 5-HT_{2C} receptors support erection and inhibit ejaculation. Stimulation of 5-HT_{1A} receptors has the opposite effects: support ejaculation and inhibit erection. Dopamine is the main catecholamine in the CNS. Dopaminergic neurons found in MPOA, PVN and have also been identified traveling from the caudal hypothalamus to innervate the lumbosacral spinal cord. It has facilitative effects on sexual motivation, copulatory proficiency, and genital reflexes. Oxytocin present in the PVN of hypothalamus. It plays a key role in motivation of penile erection and sexual behaviour by the activation of nitric oxide synthase (NOS). NO found in the PVN that is the primary site for interaction of NO with the oxytocinergic neurons to facilitate penile erection. NO releases in MPOA have also been associated with copulatory behaviour. Noradrenaline can control the penile erection both at the brain and spinal cord level. It has been found that α-adrenoceptor subtypes can regulate sexual function, activate α-1 adrenoceptors and inhibit α-2 adrenoceptors facilitates. Opioid peptides have long been assumed to be related to the regulation of male sexual function, because sexual dysfunction has been observed in men with long-term opiate use. It has already been indicated that opioid μ-receptor stimulation centrally inhibits penile erection by inhibiting mechanisms involved with oxytocinergic neurotransmission.

The peripheral neurotransmitters including autonomic (sympathetic and parasympathetic) and somatic nervous system control the vascular and smooth muscle of

corpus cavernosum, respectively. The nerves, that support the penile, have been identified as adrenergic, cholinergic, and non-adrenergic, non-cholinergic nerves (NANC). The tone of penile is regulated by several neurotransmitters and mediators that stimulate the contraction and relaxation of penile smooth muscle. The mediators that involved in regulation the contraction of penile are noradrenaline, endothelin, and angiotensin. The relaxation mediators are acetylcholine, nitric oxide and cyclic nucleotide, vasoactive intestinal polypeptide (VIP) and prostanoid. The contraction and relaxation of penile smooth muscle, result in penile erection and penile flaccid, respectively [5, 33].

Physiology of penile erection

Penile erection involves; an engorgement of sinusoidal inside the corpus cavernosa, vasodilation of penile arterial leading to an increasing of penile blood flow, and compression of the emissary veins resulting in reducing of the venous outflow. The erection occurs when the parasympathetic nervous system stimulates the release of nitric oxide (NO) from nerve ending (non-adrenergic, non-cholinergic nerves: NANC) and endothelium. NO diffused into trabecular smooth muscle and arterial smooth muscle cells to activate guanylyl cyclase (GC). GC is a catalyst for the second messenger, cyclic guanosine monophosphate (cGMP). cGMP stimulates the protein kinase G (PKG), leading to a decrease in intracellular calcium concentrations, and smooth muscle relaxation. This is achieved through the influence of several cellular processes including 1) the opening of the K+ channel causing hyperpolarization; and 2) the sequestration of intracellular calcium by the endoplasmic reticulum (ER) and the inhibition of the voltage-dependent calcium channels to block the influx of calcium, thus reducing intracellular calcium. 3) The activation of myosin light chain phosphatase (MLCP) to detach the myosin head from actin. Another second messenger involved in smooth muscle relaxation is cyclic adenosine monophosphate (cAMP) which is activated by cAMP-signaling molecules such as adenylyl cyclase (AC), vasoactive intestinal peptide (VIP), calcitonin gene-related peptides (CGRP) and prostaglandins E_1 (PGE_1). The smooth muscle relaxation results in an increase of penile blood flow in the corpora cavernosa through the cavernosal artery (pudendal artery), an increase in intracavernosal pressure (ICP) and an expansion of the sinusoids therein. The increase of ICP causes the compression of the subtrunical venular plexuses between the tunica albuginea and the peripheral sinusoids which reduce

venous outflow and keep the blood in the corpora cavernosa resulting in penile erection [6, 7, 32, 34, 35].

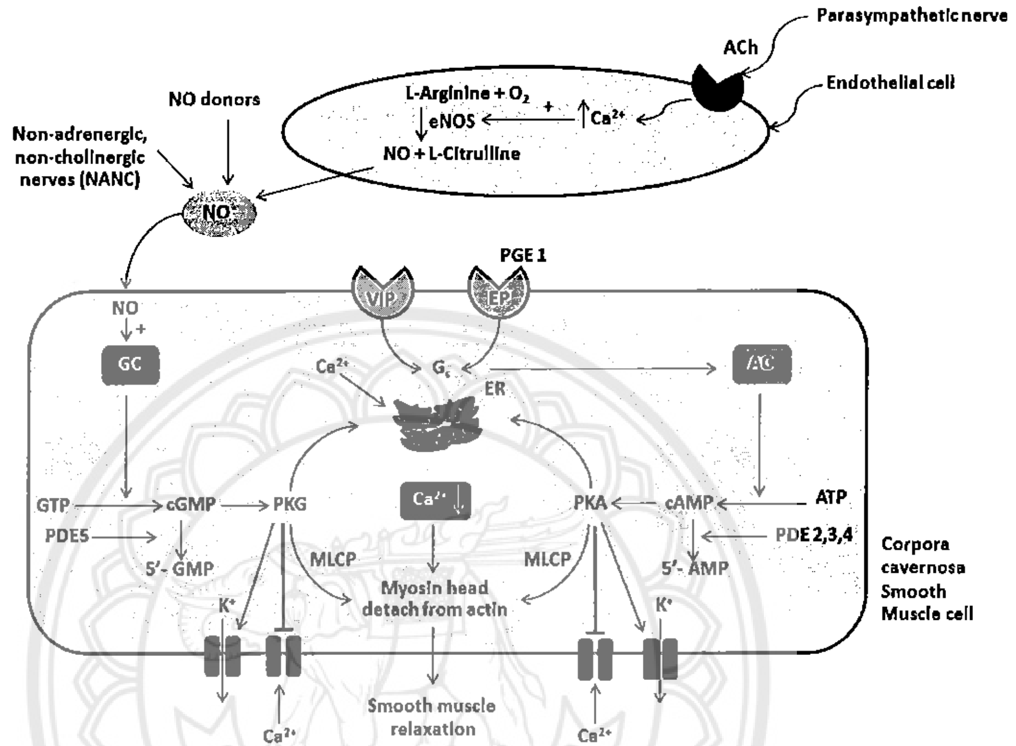


Figure 3 Mechanism of the regulation of corpus cavernosum smooth muscle tone and penile erection [6, 34]

cAMP and cGMP are substrates of phosphodiesterase enzyme (PDE) with the production of inactive compounds. The PDEs family consists of at least eleven isozymes that differ for substrate specificity, sensitivity to inhibitors and organ localization. Several members of PDEs found in the human corpus cavernosum have been identified. They include the cAMP/cGMP non-selective PDE2, the cAMP-selective PDE3 and PDE4, and cGMP selective PDE5 [6, 35, 36]. PDE5 is abundant in human corpus cavernosum which specifically hydrolyzes the phosphodiester bond of cGMP resulting in decreased rigidity. Even though other PDEs appear in the corpus cavernosum, they do not significantly modulate the level of cGMP associated with the ability to achieve penile erection [12].

Pathophysiology and etiology of ED

ED is the inability to achieve and maintain a penile erection sufficient for sexual performance. Causes of ED can be classified into two types; 1) primary, i.e. of developmental origin and 2) secondary, i.e. deriving from vasculogenic (or organic) or psychogenic diseases. For primary ED, the sex hormone disturbances may play an important role, while in secondary ED; failures in the basic activating mechanisms of the erection may be the cause [37]. The basic mechanisms involved in ED may result from 1) initiation failure which may be associated to neurogenic, psychogenic or endocrinologic; 2) failure to fill which may result from arteriogenic; and 3) failure to keep the blood inside the corpora cavernosa which may from cavernosal disorders [32].

The pathophysiological factors involved in developing psychogenic ED including anxiety and depression, which might be the results in loss of libido, over-inhibition and impaired nitric oxide release. The pathology of both the central and the peripheral nervous system from some diseases such as cerebral diseases, spinal cord injury, spinal disease and pudendal nerve injury, can cause the neurogenic disorder. Pathologic processes in the MPOA, the PVN and the hippocampus areas, such as Parkinson's disease, stroke, encephalitis, or temporal lobe epilepsy, are often associated with ED. The pathology on vascular such as atherosclerosis, ischemic heart disease and cavernosal disorders are affected to the blood supply to the penis. These can increase the time to achieve penile erection. Other conditions such as smoking, hypertension, hyperlipidaemia and diabetes, are also associated with the development of vasculogenic ED. In addition, some medications, such as antihypertensive, antidepressants, diuretics, cardiac medications, and hormones can cause ED. ED can also be induced by marijuana, alcohol, narcotics and cigarette [6, 7].

Treatment of ED

In the past, intracavernous and intraurethal pharmacotherapy was the first line treatment for ED [6]. Recently, it has been replaced by oral therapies including oral PDE5 inhibitors, oral α-adrenoceptor antagonist, and oral dopamine receptor agonist. Intracavernous injection and transurethal injection therapies has become the second-line therapies [5].

Phosphodiesterase (PDE)

PDEs family

PDEs are a family of enzymes that selectively catalyse the hydrolysis of adenosine 3',5'-cyclic monophosphate (cAMP) bond and/or guanosine 3',5'-cyclic monophosphate (cGMP) bond. They regulate the intracellular levels, localization and duration of action of these secondary messengers by controlling a balance between their production and degradation [9]. There are eleven families of mammalian PDEs (named PDE1 to PDE11), each family share a conserved catalytic domain but different splice variants. These are characterized by difference in their three-dimensional structure, kinetic properties, substrate specificity, intracellular localization, tissues expression, and inhibitor sensitivities (Table 1). PDEs expressed in specific tissues and play an important role in many signal transduction pathways [9, 11, 13, 18, 19, 38].

Table 1 **Substrate specificity, mainly tissue localization and functions of PDEs family** [9, 12, 18].

PDE family	Substrate specificity	Mainly tissue localization	Functions
PDE1	cGMP > cAMP	Brain, heart, vascular smooth muscle	Involved in dopaminergic signalling, regulates vascular smooth muscle proliferation and contraction
PDE2	cGMP = cAMP	Adrenal cortex, heart, brain, corpus cavernosum	Regulates aldosterone secretion from the adrenal gland, cAMP and PKA phosphorylation of Ca^{2+} channels in the heart, cGMP in neurons, long-term memory
PDE3	cAMP > cGMP	Heart, corpus cavernosum, liver, pancreas, vascular smooth muscle, platelets	Regulates cardiac contraction, vascular smooth muscle contraction, mediates insulin signalling, regulates cell cycle/proliferation, platelet aggregation

Table 1 (cont.)

PDE family	Substrate specificity	Mainly tissue localization	Functions
PDE4	cAMP	Lung, mast cells, vascular smooth muscle	Monocyte and macrophage activation, neutrophil infiltration, vascular smooth muscle proliferation, vasodilation, cardiac contraction
PDE5	cGMP	Corpus cavernosum, lung, vascular smooth muscle, platelets, brain, heart, kidney, skeletal muscle	Regulates vascular smooth muscle contraction, (especially in penis and lung), platelet aggregation
PDE6	cGMP > cAMP	Retina	Involved in signal transduction of the photoresponse in the eye, may also regulate melatonin release from the pineal gland
PDE7	cGMP >> cAMP	Skeletal muscle, T cells	Activates T-cell and other inflammatory cells
PDE8	cAMP	Testis, thyroid	Activates T cell, sperm, or leydig cell function
PDE9	cGMP	Broadly expressed, not well characterized	Unknown
PDE10	cGMP > cAMP	Brain, testes	Learning and memory
PDE11	cGMP = cAMP	Skeletal muscle, prostate, liver, kidney, pituitary, testis	Sperm development and function

PDE5 and its inhibitors

PDE5 previously named cyclic GMP-specific phosphodiesterase as it specifically hydrolyses the phosphodiester bond of cGMP. It has been found in several species and one gene, PDE5A, was identified with three isoforms being expressed: PDE5A1, PDE5A2 and PDE5A3 [9]. PDE5A1 and PDE5A2 are ubiquitous, whereas PDE5A3 is specific to vascular smooth muscle [9, 10, 18]. PDE5 is abundant in penile corpus cavernosum, vascular smooth muscle cells and platelet; and is the predominant cGMP metabolizing PDE in these cells [10, 12]. cGMP is a second messenger that induces vasorelaxation through the relaxation of vascular smooth muscle cells. Nitric oxide (NO) and PDE5 normally regulate the levels of intracellular cGMP. NO binds to soluble guanylyl cyclase (sGC) to activate the synthesis of cGMP from guanosine triphosphate (GTP) resulting in elevation of intracellular cGMP level. An increase in cGMP bind to cGMP-selective sites on several types of proteins, including cGMP-dependent protein kinase (PKG), cGMP-gated cation channels, and allosteric sites on PDE5. cGMP binding to PKG to phosphorylate cellular proteins involved in Ca^{2+} homeostasis [13]. This causes a reducing level in intracellular calcium leading to smooth muscle relaxation [13], platelet disaggregation [39], vasodilation [11] including penile erection [12]. cGMP is inactivated by phosphorylation at a catalytic site of PDE5. The decreased level of cGMP could cause many diseases such as platelet aggregation [39], pulmonary hypertension and erectile dysfunction (ED) [11]. Thus, the intracellular levels of cGMP can be increased by NO or by inhibiting PDE5 [12].

PDE5 inhibitors

In the 1990s, sildenafil, a PDE5 inhibitor was clinically investigated as a treatment for hypertension and angina pectoris. The results indicated that the drug was not successful in these conditions, but many men who took part in the clinical trials, experienced erections and priapism (erection lasting longer than 4 hours). This led to the possibility that sildenafil could be used as a treatment for ED. Sildenafil was approved by the FDA in 1998, marketed as Viagra® [14]. At the present time, the four oral PDE5 inhibitors commercially available are sildenafil (Viagra®) [14], vardenafil (Levirate®) [15], tadalafil (Cialis®) [16] and avanafil (Stendra®) [17]. They have been used for treating ED [19], anti-platelet aggregation [38, 39] and pulmonary hypertension [40]. The structure of PDE5 inhibitors are similar to cGMP and compete with cGMP at the catalytic site of PDE5 (Figure 4).

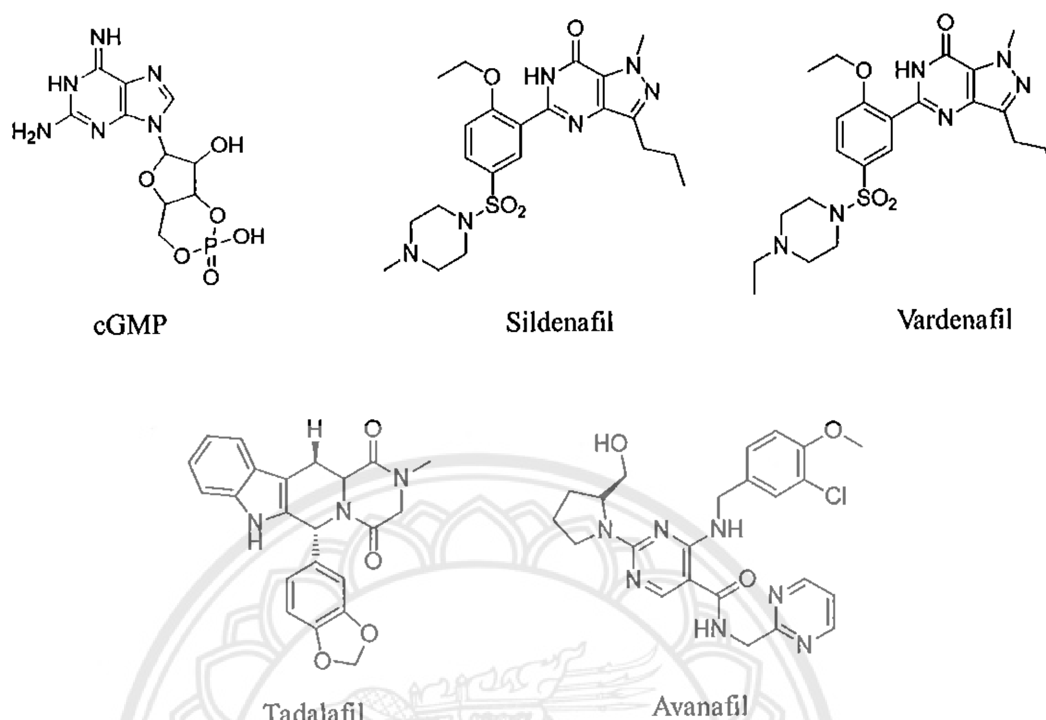


Figure 4 Chemical structures of cGMP, sildenafil, vardenafil tadalafil, and avanafil

The efficacy of each PDE5 inhibitor drug depends on their potency and selectivity to PDE5. Potency is normally expressed as IC_{50} or the drug concentration required inhibiting the activity of the PDE by 50%. PDE5 inhibitor drugs were evaluated according to the inhibitory activity in all PDEs isoforms (PDE1-11). Avanafil shows the highest potency in PDE5 inhibitory activity at the IC_{50} value of 5.2 nM. It also shows the highest selectivity against PDE1 and 11. For PDE6, tadalafil shows the highest selectivity against PDE6. However, for other PDEs, all PDE5 inhibitors show the low value of IC_{50} and the rank orders of selectivity are same [18, 41].

Table 2 Selectivity of PDE5 inhibitor drugs [18, 41]

IC_{50} (nM)	PDE5	PDE1	PDE6	PDE11
Sildenafil	8.5	350	49	1, 725
Ratio: x/PDE5	1	41	7.4	203
Vardenafil	0.89	121	11	308
Ratio: x/PDE5	1	136	15	346
Tadalafil	5	>10, 000	5, 100	67
Ratio: x/PDE5	1	>2, 000	1, 020	13.4
Avanafil	5.2	-	-	-
Ratio: x/PDE5	1	>10, 000	121	>19, 000

PDE1 is mainly expressed in brain, heart, and vascular smooth muscle cell. Three subtypes of PDE1 were identified; include PDE1A, PDE1B, and PDE1C, as Ca^{2+}-calmodulin activated. PDE1A and PDE1B affect to cGMP greater degree than cAMP. PDE1C has the equivalent effect on cGMP and cAMP. Inhibition of PDE1 could induce vasodilatation, flushing, and tachycardia. PDE6 is strictly localized in the retina and plays an important role in the signal transduction of vision. Inhibition of PDE6 can cause visual disturbances such as changes in acuity, colour or brightness. PDE11 has a wide tissue distribution, including skeletal muscle, liver, kidney, testis, prostate, and pituitary. It plays a role in the development and function of sperm. Inhibition of PDE11 could produce alteration in the quality of sperm [18, 19, 20]. As some side effects are presented in patients who take non-selective PDE5 inhibitor drugs, the search for a new selective PDE5 inhibitor drugs is still interesting, especially from natural sources.

Natural PDE5 inhibitors

Flavonoids

Many studies indicate that flavonoid compounds have the ability to inhibit PDE [42, 43, 44]. Ko and colleagues [42] reported the PDE1-5 inhibitory activity of thirteen flavonoid compounds (Table 3) measured according to a two-step procedure using cAMP with [^{3}H]-cAMP or cGMP with [^{3}H]-cGMP as substrate. All compounds show non-selective PDE inhibitory activity that could cause side effects such as

flushing and tachycardia from the inhibition of PDE1. The inhibition of PDE3 and PDE4 are beneficial to asthma or chronic obstructive pulmonary disease (COPD) patients [45]. Genistein, isoflavonoid compound found in *D. scandens,* was also evaluated the PDE activity in this study. It showed the mild PDE5 inhibitory activity with the IC_{50} value of 73.9 ± 7.1 μM, but potently inhibited PDE1-4 with the IC_{50} value of 16.8 ± 2.3, 1.7 ± 0.2, 12.9 ± 5.2 and 9.5 ± 1.9, respectively [42]. Shin et al [44] reported the PDE5 inhibitory activity of five flavonoids isolated from *Sophora flavescens* including kushenol H, kushenol K, kurarinol, sophoflavescenol and kuraridine. Sophoflavescenol (Figure 5) showed the highest PDE5 inhibitory activity with the IC_{50} value of 0.013 μM. It exhibited the PDE5 selectivity over PDE3 and PDE4 with 31.5- and 196.2-fold, respectively.

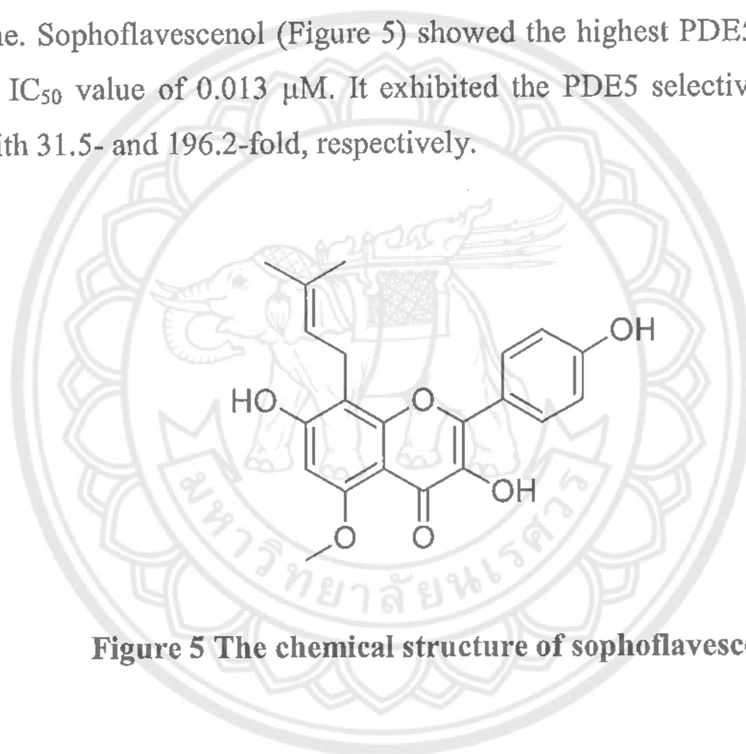
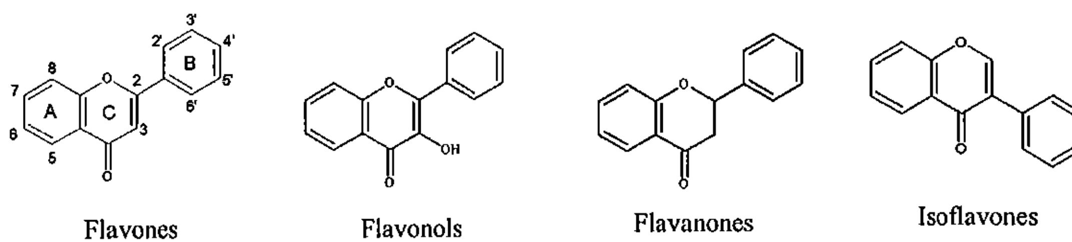


Figure 5 The chemical structure of sophoflavescenol

Table 3 Structures of flavonoids investigated for the inhibition of the activities of PDE1 – 5.



Class	Name	Substitution				
		5	7	3'	4'	5'
Flavones	Luteolin	OH	OH	OH	OH	H
	Luteolin-7-glucoside	OH	O-glu	OH	OH	H
	Diosmetin	OH	OH	OH	OCH_3	H
	Apiginin	OH	OH	H	OH	H
	Chrysin	OH	OH	H	H	H
Flavonols	Quercetin	OH	OH	OH	OH	H
	Myricetin	OH	OH	OH	OH	OH
Flavanones	Eriodictyol	OH	OH	OH	OH	H
	Hesperetin	OH	OH	OH	OCH_3	H
Isoflavones	Genistein	OH	OH	H	OH	H
	Daidzein	OH	OH	H	OH	H
	Biochanin A	OH	OH	H	OCH_3	H
	Prunetin	OH	OCH_3	H	OH	H

Curcuminoids

Kruangtip et al [46] studied the PDE5 and PDE 6 inhibitory activity of three natural curcuminoids including curcumin, demethoxycurcumin and bisdemethoxycurcumin (Figure 6). The results showed that all compounds, showed a moderate inhibitory activity on PDE5 with the IC_{50} values of 18.8 ± 2.1, 50.6 ± 3.3 and 94.4 ± 5.2 μM, respectively

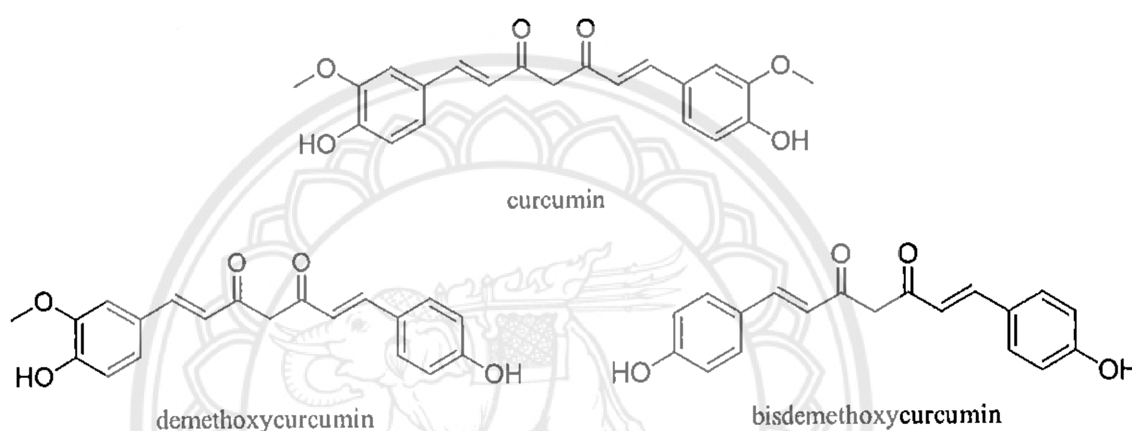


Figure 6 Chemical structures of curcuminoids

Xanthones

Sabphon et al [47] reported 1,3,5-trihydroxy-4-prenylxanthone (Figure 7), xanthone isolated from *Anaxagorea luzonensis*, as a potent PDE5 inhibitor with an IC_{50} value of 3.0 μM

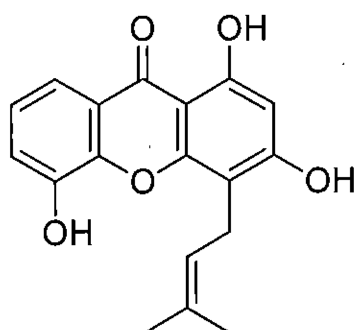


Figure 7 Chemical structure of 1,3,5-trihydroxy-4-prenylxanthone

***Derris scandens* (Roxb.) Benth.**

General information

Derris scandens (Roxb.) Benth., is known in Thailand as thao-wan-priang. Its common names include jewel vine, hog creeper and Malay jewel vine. It is a woody vine of the family of Fabaceae. The leaves are green, and compound with alternate, leaflets ovate or elliptic, odd-pinnate, 1 – 2.5 cm wide and 3 – 5 cm in length. The flowers are inflorescence in axillary pendulous raceme, pea-shaped and light pink or white. The vine grows up to 30 m in length, with a trunk of up to 40 cm in diameter. It has been used in Thailand for a long time as a pain killer, diuretic and in rejuvenating agents [21, 22].

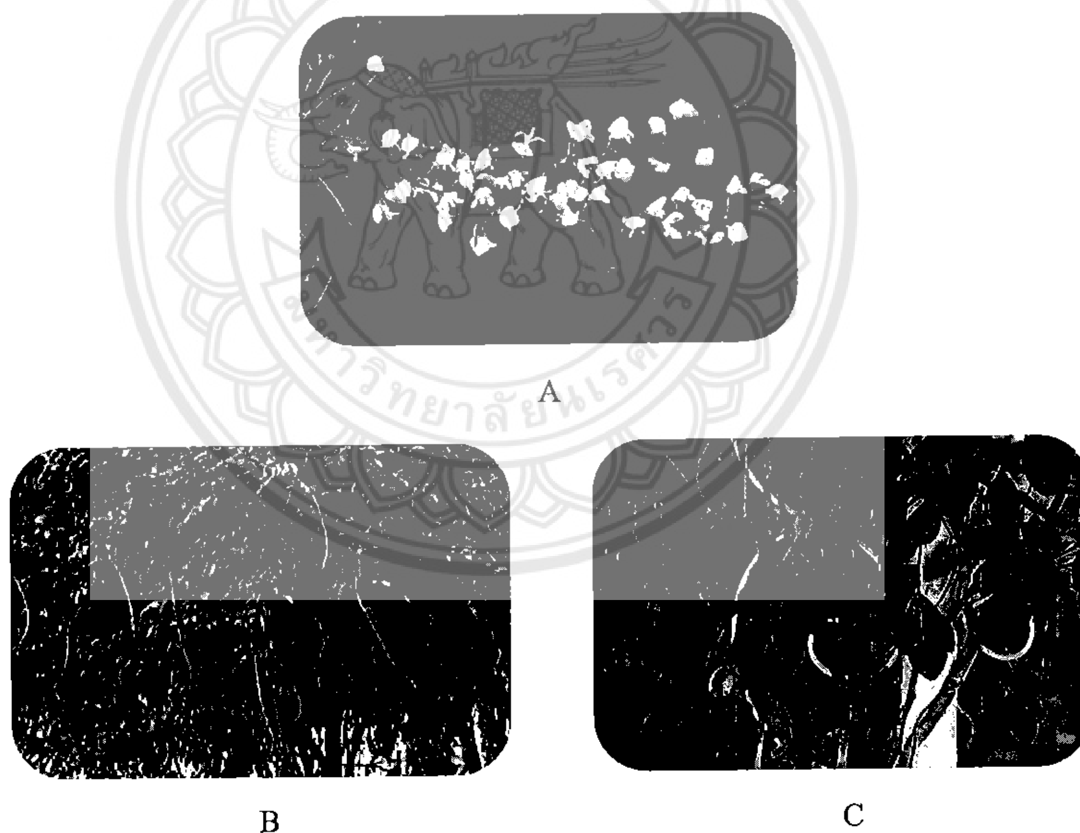


Figure 8 Parts of *D. scandens*; A. Flower B. Whole plant C. Leaf

Chemical constituents

The chemical constituents are benzil derivatives (derrisdione and scandione) [48, 49], pterocarpens (flemichapparin B-C and maackian) [48], coumarins (scandenin, scandenin A-B, and robustic acid) [49, 50, 51], steroids (β-sitosterol and β-sitosterol glucopyranoside) [51], isoflavones (derrisisoflavone A-F, erysenegalensein E, genistein, lupalbigenin, lupinisol A, osajin, scandenone, scanderone, scandinone, 4',5,7-trihydroxybiprenylisoflavone) [48, 49, 50, 52], and isoflavone glycosides (derriscandenosied A-E and genistein 7-O-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside) [53, 54]. Several scientific studies indicated that scandenin, genistein, genistein 7-O-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside and lupalbigenin have an anti-inflammatory effect by inhibition of the eicosanoid synthesis [54]. Derrisisoflavone A-F, erysenegalensein E, lupinisoflavone G, lupinisol A, scandenin and 4',5,7 -trihydroxybiprenylisoflavone have an antidermatophyte effect against Trichophyton mentagrophytes [52]. Scandenin, scandenin A-B, scandinone and lupalbigenin have an anti-oxidant effect [50, 54], while chandalone, sental, scanderone, flemichapparin B and maackian have a vasorelaxant effect by the increasing of mean arterial pressure [48]. The prenylated isoflavone scandenone or warangalone was identified as a selective and potent inhibitor of rat liver cyclic AMP-dependent protein kinase catalytic subunit (cAK) with the median inhibitory concentration or IC_{50} value 3.5 μM [55]. Some of the compounds found in *D. scandens* shown in Figure 9, below.

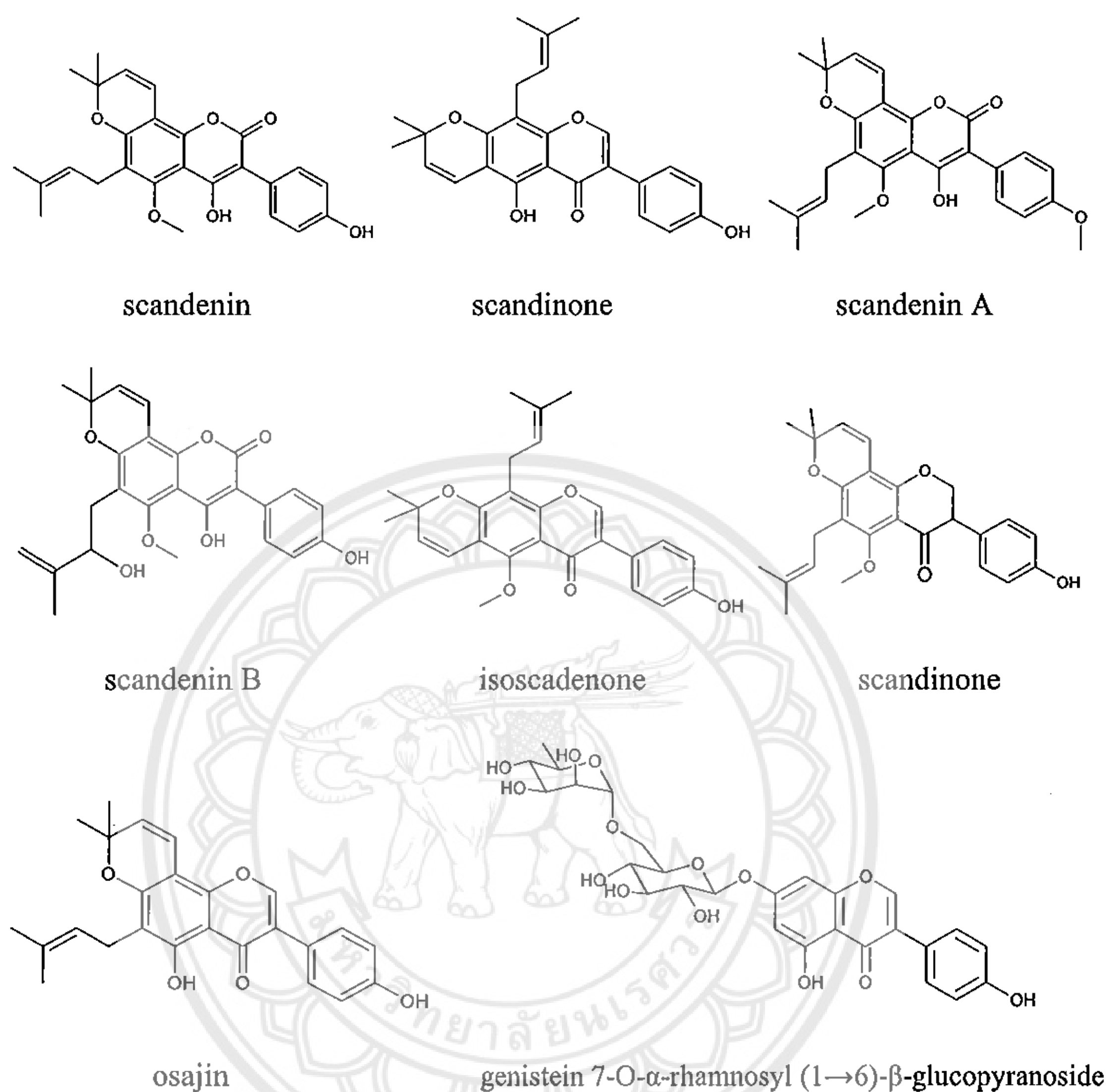


Figure 9 Some of compounds found in *D. scandens*

Chemical constituents of 50% ethanol extract of the stem from *D. scandens* were reported by Wongsinkongman et al [56]. The extract was separated on X-Terra RP18 column using a gradient mixture of acetic acid, water and acetonitrile as the mobile phase and the eluant was detected at 260 nm. The phenolic compound, piscidic acid and a isoflavone glycoside, genistein 7-O-α-rhamnosyl (1→6)-β-glucopyranoside, were found as the major components with the retention times of 1.453 and 8.752 min, respectively.

Moreover, Rao and colleagues found that methanol extract of *D. scandens* contained scandinone, scadenone, scandenin A, scandenin B and 4′, 5′, 7-trihydroxybiprenyl isoflavone, which showed moderate effect on free radical scavenging and α-glucosidase inhibitory activity [50]. The separation was done using HPLC method with a phenomenex column (250×4.6 mm i.d. particle size; 5 μm) as the stationary phase, a gradient mixture of water and acetonitrile as the mobile phase, and a PDA detection. The flow rate was maintained at 1.0 mL/min during the run. Retention times of scandinone, scadenone, scandenin A, scandenin B and 4′, 5′, 7-trihydroxybiprenyl isoflavone are 15.81, 22.01, 17.05, 4.33 and 12.52 min, respectively

Bioactivities of *D. scandens*

Several bioactivities of the stem of *D. scandens* were reported, such as anti-hypertension [57], anti-inflammation [54, 58], anti-oxidation [48, 59], antinociception [60], anti-migration of cancer cell on hepatoma cell line (HepG2), breast cancer cell line (MCF-7) and cholangiocarcinoma cell line (KKU-M139 and KKU-M213) [61]. Radiosensitivity of Hep-2 laryngeal cancer cells [62], stimulate lymphocyte proliferation and inhibit the effect of NK cell on HIV-infected patients [63], immunostimulation , cox-1 enzyme inhibition [56], α-glucosidase enzyme inhibition [50], free radical scavenging activity [50] and anti-microbials [48, 59, 64]. In this review, only the activities related to the project are discussed.

Hypotensive and negative chronotropic activities

The study of Jansakul and colleagues reported that the intravenous injection via jugular vein of hypotensive fraction (0.04 – 1.60 mg/kg) of water extract from the stem of *D. scandens,* caused a decrease in the mean arterial blood pressure and heart rate in anesthetized rats, in dose dependent manner. This result was significant when compared to rats that were given a saline injection. The mechanism of the hypotensive fraction was proved by pre-treating the rat with 1.5 mg/kg atropine; a non-specific muscarinic receptor antagonist of parasympathetic nervous system, or 0.6 mg/kg propranolol; β-adrenergic receptor antagonist of the cardiovascular system both blood vessel and the heart. The result showed that rats pre-treated with atropine and propranolol did not experience an effect of the blood pressure, while in the case of heart rate, blocking β-adrenergic receptor by propranolol, significantly reduced the

decreasing heart rate. This result suggests that the hypotensive activity of hypotensive fraction may act as β-adrenergic receptor antagonist of the cardiovascular system. In the in vitro preparation using isolated thoracic aorta and atria, they also found that the hypotensive fraction caused a dose dependent vasodilation of endothelium – intact thoracic aortic rings, but did not affect the thoracic aortic ring without endothelium. For the effect on the rate of isolated atria contraction, the hypotensive fraction caused a dose dependent decrease the rate of atrial contraction but did not affect to the force of contraction. They also found that pre–incubation the atria with 0.1 mg/mL of the hypotensive fraction caused a negative chronotropic effect with no changes in force of the atrial contraction. These results suggest that the mechanism of hypotensive fraction from *D. scandens* for the hypotensive and negative chronotropic activities may involve a direct effect, as a β-adrenergic receptor antagonist at the atria, and an indirect effect by causing the vasodilation via stimulating the release of nitric oxide from the vascular endothelial cells [57].

Antioxidant activity

Prommee et al [59] studied the antioxidation effect of the 50% ethanol extract of *D. scandens* using DPPH radical scavenging assay. The extract showed less antioxidant activity than butylated hydroxytoluene (BHT), a positive control, with the median effective concentration or EC_{50} values of 61.97 ± 0.81 and 10.66 ± 0.97 μg/mL, respectively. Mahabusarakam et al [48] studied the antioxidant effect of pure compounds isolated from *D. scandens* using DPPH radical scavenging assay. The result showed that three compounds including scandinone, derrisisoflavone A and santal show the high activity with the IC_{50} value of 8.75, 3.63 and 2.75 μM, respectively. Moreover, derrisisoflavone A and santal showed greater antioxidant activity than the positive control (BHT) which showed an IC_{50} value at 6.88 μM.

Anti – inflammatory activity

Laupattarakasem et al [54] reported that an aqueous extract of *D. scandens* showed the ability to inhibit the generation of myeloperoxidase (tissue-damage enzyme), thromboxane B_2 and leukotriene B_4. The researchers also investigated the effect of the extract in rat induced paw edema by carrageenan. The effect was evaluated by measuring the volume of edema by water displacement using plethysmometer. The result showed that an aqueous extract given intraperitoneally,

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caused a significant dose-dependent reduction in edema (82% for 100 mg/kg and 91 for 500 mg/kg). This result did not apply to orally administered dosages [58]. They also reported the anti-inflammatory effect of four isoflavone from *D. scandens* including genistein glycoside (7-O-α-rhamnosyl (1→6)-β-glucosyl glycoside), genistein, 5, 7, 4′-trihydroxy-6, 5′-diprenyliso-flavone and scandenin by using radioimmunoassay. The result indicated that the isolated compounds can inhibit both thromboxane and leukotriene. The IC_{50} value of the compounds on COX inhibition were 1500 μM, 100 μM, 3 μM and 8 μM, while the IC_{50} value on 5-LOX inhibition were 2500 μM, 80 μM, 6 μM and 8 μM for genistein glycoside (7-O-α-rhamnosyl (1→6)-β-glucosyl glycoside), genistein, 5, 7, 4′-trihydroxy-6, 5′-diprenylisoflavone and scandenin respectively.

COX – 1 inhibitory activity

Wongsinkongman et al [56] published the bioactive compounds of D.scandens extract, piscidic acid and genistein 7-O-α-rhamnosyl (1→6)-β glucopyranoside, and evaluated their effect on inflammatory (COX-1 and COX-2) inhibitory activity using radioimmunoassay. The result showed that only genistein 7-O-α-rhamnosyl (1→6)-β glucopyranoside show the effect on COX-1 inhibitory activity with the IC_{50} value 4 μg/mL.

Antinociception

Punjanon and colleague determined the antinociceptive effect of 50% ethanol extract of *D. scandens,* which was performed using a writhing test in mice with acetic acid (0.75% v/v) induced abdominal constriction. In the writhing test model, the extract at the doses of 1, 10, 100 and 1, 000 mg/kg BW were orally administered to the animals, while intraperitoneal injection of aspirin (50 mg/kg BW) was used as a standard drug. The result indicated that the extract at the dose of 10, 100 and 1,000 mg/kg BW and the standard drug significant ($p < 0.05$, n = 6) decreased the number of abdominal constrictions and stretching of the hind limbs. The extract also produced a dose-dependent manner by increasing the percentage inhibition of writhing. The median effective dose (ED_{50}) value of the extract was 35.5 mg/kg BW. The 50% ethanol extract of *D. scandens* indicated a potent antinociceptive effect [60].

Eukaryote protein kinase inhibitory activity

The numerous species of *Derris* have been used as insecticides. They have been investigated for insecticidal compounds, especially rotenone. For *D. scandens,* various compounds have been isolated and structurally characterized. Wang and colleagues investigated the possible sites of action for compounds isolated from the stems of *D. scandens.* These sites of protein kinases are involved in signal transduction in eukaryotes, which consist of rat liver cyclic AMP-dependent protein kinase (cAK), rat brain Ca $^{2+}$-phospholipid-dependent protein kinase C (PKC), wheat embryo Ca^{2+}-dependent protein kinase (CDPK) and avian gizzard calmodulin-dependent myosin light chain kinase (MLCK). They found that the prenylated isoflavone, warangalone, had a selective and potent inhibitory activity of cAK with the IC_{50} value 3.5 μM whilst, the high concentration of warangalone did not inhibit PKC, CDPK and MLCK. The mechanism of inhibition of rat liver cAK by warangalone was studied using Lineweaver-Burk reciprocal plots of v_0^{-1} ((initial velocity) $^{-1}$) vs (substrate) $^{-1}$ from enzyme kinetic data obtained in the presence or absence of warangalone, which indicated that it is a non-competitive inhibitor of cAK with respect to the ATP and synthetic peptide substrate kemptide of cAK. Other isoflavones including 8-γ-γ-dimethyl allylwighteone, 3'-γ-γ-dimethylallylwighteone, nallanin, biochanin A and eturunagarone, inhibit cAK with the IC_{50} values of 20, 24, 33 100 and 248 μM, respectively but are not inhibitors of the other protein kinases tested. Genistein is a relatively poor cAK inhibitor with the IC_{50} value of 126 μM but is a much more effective MLCK inhibition with the IC_{50} value of 14 μM. Other coumarins including robustic acid and 4, 4'-di-*O*-methylscandenin were also investigated. Robustic acid is a potent and specific cAK inhibitor with the IC_{50} value 10 μM while, 4, 4'-di-*O*-methylscandenin is a poor cAK inhibitor with the IC_{50} value 150 μM [55]. All of these compounds' structures are shown in Figure 10.

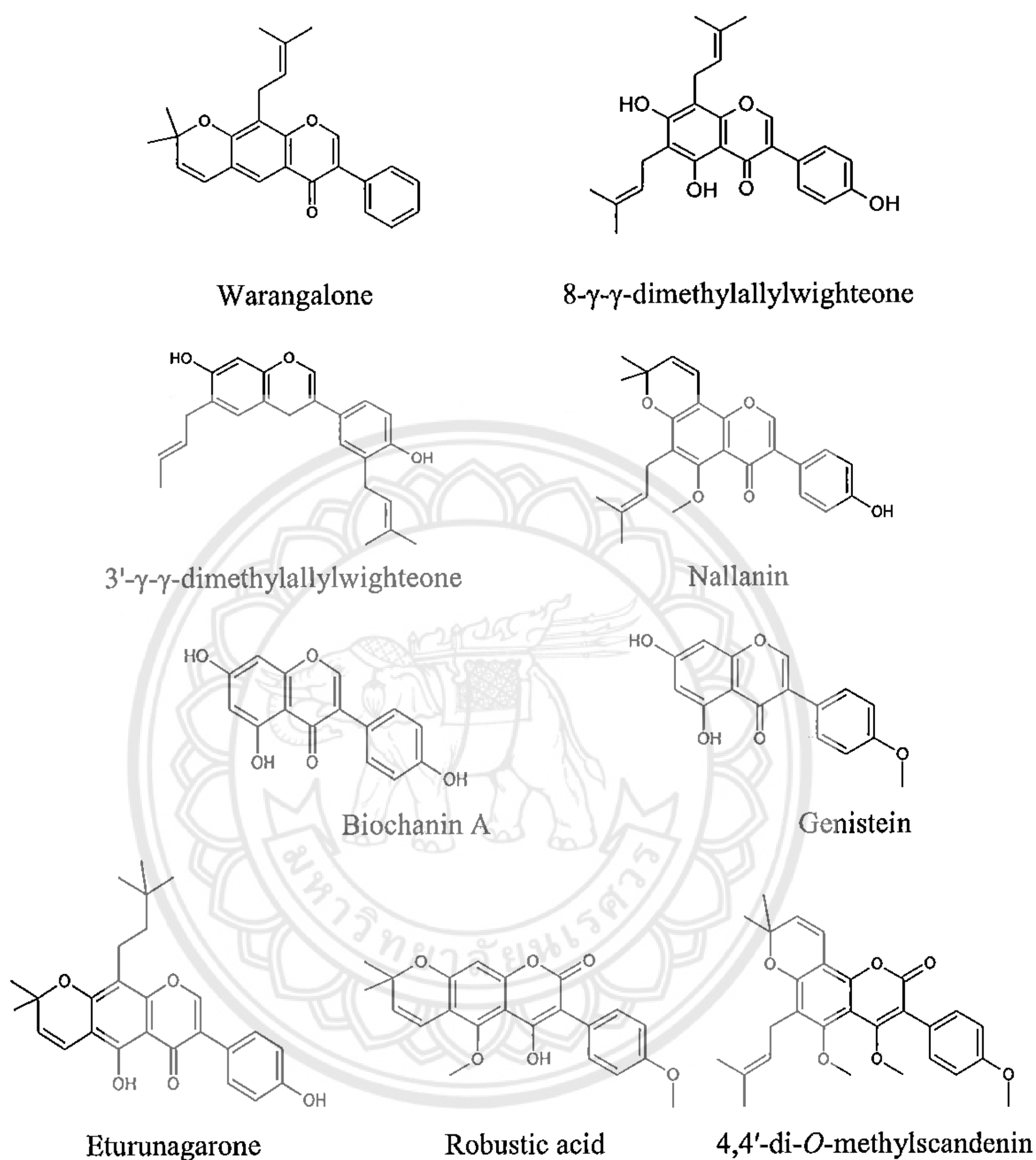


Figure 10 The structures of prenylated isoflavones and coumarins isolated from stems of *D. scandens*.

Toxicity and safety studies

The study of Chavalittumrong et al [65] reported that the chronic toxicity of 50% ethanol extracts of *D. scandens* in both male and female Wistar rats. The study included 80 male and 80 female rats. The animals were randomly divided into 4 groups of 20 animals. The control group was received 10 mL/kg BW of water. The treated groups were given extracts at doses of 6, 60 or 600 mg kg^{-1} BW for a period of 6 months. The results showed no significant difference of initial or final body weight and hematological parameters or serum chemistry between control and treated groups. Histopathological studies of internal organs revealed no remarkable lesions related to extract toxicity. These results suggest that there is no toxicity effect from 50% ethanol extract of *D. scandens* in rats.

The safety study of 50% ethanol extract of *D. scandens* in twelve healthy volunteers (phase I trial) was reported by Chavalittumrong et al [66]. The oral administration of the extract at the dose of 400 mg/day was given to the volunteers for 2 months. No major side effects were reported from the volunteers. Although, some of hematological and biochemical parameters such as hemoglobin, hematocrit and basophil were significantly changed but within normal limits. It was additionally found that the amounts of interleukin (IL) 2, 4 and 6 were significantly increased. These results suggested that the 50% ethanol extract of *D. scandens* at the dose of 400 mg/day given to healthy volunteers for 2 months was safe and could modulate the immune system by increasing the level of cytokines.

Clinical studies

Lower back pain

Srimongkol and colleges studied the efficacy of 50% ethanol extract of *D. scandens* compared with diclofenac in patients with lower back pain. Thirty-seven patients in each group received 600 mg/day of *D. scandens* extract or 75 mg/day of diclofenac for 7 days. The result showed that the level of pain by the Visual Analog Scale significantly decreased from the base line in both groups. They also found that in *D. scandens* treated group, the white blood cell count significantly decreased, but remained within the normal accepted range. No side effects from both treated groups were found and these results concluded that 50% ethanol extract of *D. scandens* showed an analgesic effect in patients suffering from low back pain and was safe as diclofenac [25].

Knee osteoarthritis

A single-blinded randomized control trial study was conducted on one hundred twenty-five human subjects with knee osteoarthritis (OA) to determine the analgesic effect of *D. scandens* extract compared to naproxen by Kuptniratsaikul et al [24]. The study found that after twice-daily oral administration of 50% ethanol extract of *D. scandens* at the dose of 400 mg for 4 weeks, the reduction of pain and the improvement of knee function in OA patients were not significantly different to when compared to naproxen patients. Adverse events occurred in both groups including dizziness, nausea, vomiting, headache, constipation, GI irritation, dyspepsia or rash. GI irritation and dyspepsia were observed in naproxen patients more often than in the *D. scandens* group. These results concluded that orally administered 50% ethanol extract of *D. scandens* at the dose of 400 mg (800 mg/day) for 4 weeks was demonstrated to have as efficacious analgesic effect on knee OA and to be as safe as naproxen (500 mg/day).

Another double-blinded, randomized study compared the efficacy and side effects of *D. scandens* extract, and ibuprofen in patients suffering knee OA by Benchakanta et al [67]. The study conducted on one hundred seventy-eight human subjects with knee OA. After oral administration of the extract at the dose of 1, 000 mg three times per day (3, 000 mg/day) for seven days, the pain reduction in OA patients was not significantly different when compared to ibuprofen (1, 200 mg/day) patients. GI irritation was observed in *D. scandens* extract group more often than ibuprofen group. The result suggested that *D. scandens* extract had the ability to exert analgesic effect in knee OA and was as safe as ibuprofen.

Determination of PDE inhibitory activity

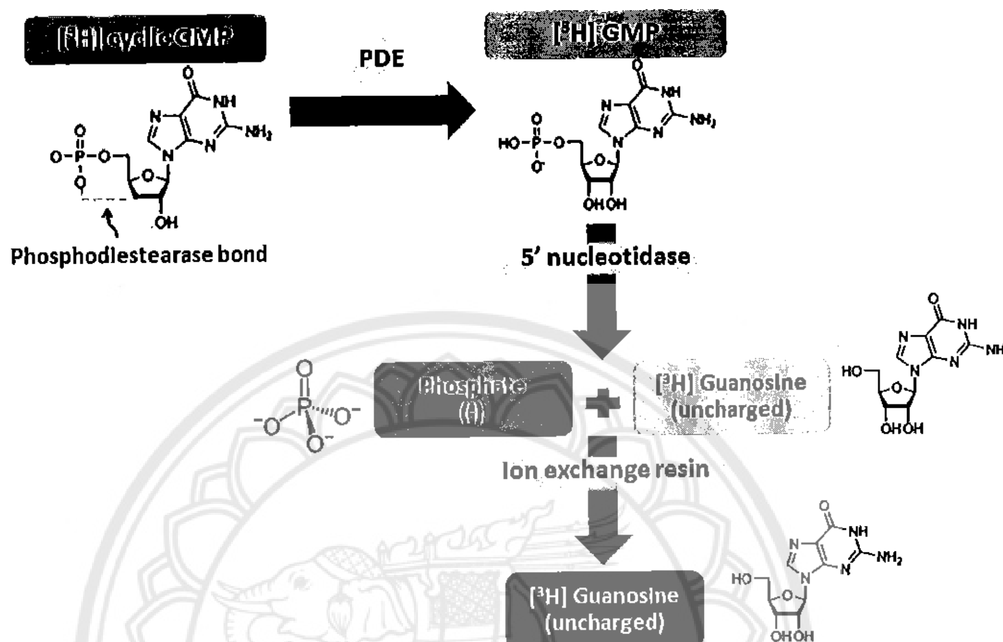


Figure 11 The principle of PDE assay

PDE assay was developed in the first time by Sutherland and Butcher in 1962. The principle of PDE assay is based on two steps of enzymatic – radioactive reaction (Figure 11). In 1970, Beavo and colleagues modified this method by label the substrate (cGMP) with radioactive agent (tritium). The first reaction occurs when phosphodiesterase hydrolyses the [^{3}H] cGMP and change to [^{3}H]-5'GMP. Second reaction needs snake venom containing 5'nucleotidase, to hydrolyze [^{3}H]-5'GMP into phosphate and [^{3}H]- guanosine. The activity of enzyme will be determined from [^{3}H]-guanosine which is uncharged and will be separated from other radioactive compounds in the assay by anion exchange resin [68].

Determination of mating behaviour in rats

Male rat mating behaviour consists of three behaviours including mounting, intromission and ejaculation. A male rat normally mounts a female from the back. His forelegs will pose on the top of the female's back. Male makes rapid anteroposterior shallow pelvic thrusts. The female will have a lordosis posture. During mounting, the penis does not enter into the female's vagina. For intromission; male makes a deep pelvic thrust to insert his penis into female's vagina, and dismounts rapidly from the female. After intromission, male will always licks his genital and never immediately mount or intromission again. Ejaculation can occur with intromission. It can be observed when a male rat remains on the female for 1 – 3 second. He then slowly lifts his forelegs. After ejaculation, the female then moves from the male, while the male licks his genital and becomes inactive for 4 – 7 minutes. Mating behaviour are assessed using parameters as follows [69]:

1. Mount latency: The time from the introductions of the female to the first mount by the male.

2. Mounting number: The number of mounts without the intromission from the time of the introduction of the female until ejaculation.

3. Intromission latency: The time from the introduction of the female to the first intromission by the male.

4. Intromission number: The number of the intromissions from the time of female introduction to ejaculation.

5. Ejaculation latency: The time from the first intromission to ejaculation.

6. Ejaculation number: The number of ejaculations characterized by longer, deeper pelvic thrusting and slow dismount followed by a period of inactivity.

7. Postejaculatory interval: The time from ejaculation to the next intromission.

Mount latency and mount number represent the libido and sexual motivation. Intromission needs penile erection so intromission latency represents a libido and sexual motivation. Intromission number represents the potency of erection and penile orientation. Ejaculation latency and ejaculation number indicate the copulatory performance. Post-ejaculation interval represents sexual motivation [69].

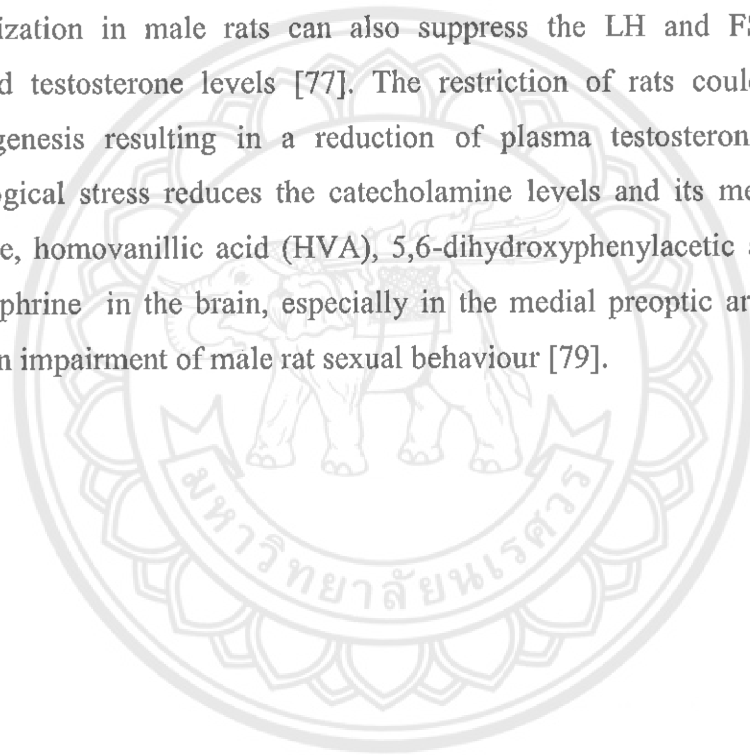
Animal mating behaviour models used for ED screening

The understanding of sexual medicine has derived from the studies in animals. Many current medical and surgical therapies in sexual medicine have been tried based on these animal models. Some of animal models used for screening ED such as cavernous nerve injury, aged-induced ED, diabetic models (type 1 and type 2), hypercholesterolemia or hyperlipidaemia model, castration model, smoking model, organic model, priapism model, psychological model (non-organic model) i.e. stress by immobilization (IMB), stress by electric foot shocks (EFS) and stress by immersion in cold water (ICW) [70].

Stress-induced ED rats

Almeida and colleagues studied the chronic intermittent immobilization-induced stress in male rats. The prepubertal (40-days-olds) male Wistar rats (n=10 each group) were immobilized inside plastic tube for 6 hours daily, for 15 days (killed at early puberty), and for 60 days (killed at full maturity). The results indicated that plasma LH in both groups was lower than control. The testosterone level was higher than control (2-fold) in pubertal rats, but was lower than control in adult rats [71]. Another report of them showed that androgen testicular function was observed in adult rats submitted to immobilization-induced stress from puberty. Male Wistar rats were immobilized inside plastic tube, 6 hours a day for 60 days. The results indicated that plasma prolactin in immobilized group was higher than control. Plasma LH and testosterone in immobilized group were lower than control. No significant difference of plasma FSH between immobilized and control groups was observed [72]. They also investigated the plasma hormone levels in chronic and acute immobilization-induced stress in male pubertal rats. Male Wistar rats were immobilized inside plastic tube, 6 hours a day for 15 days for chronic immobilization (n=10), and 5 min of immobilization for acute immobilization before sacrifice. The results indicated that plasma prolactin in both groups was higher than in the control group. No significant changes were observed in plasma FSH according chronic or acute immobilization. Plasma LH in the chronic group was lower than in the control group while was not significantly different in the control and acute groups. Plasma testosterone was significantly higher than the control in both groups [73]. They also reported sexual behaviours of male rats submitted to chronic immobilization-induced stress. Stress

was attained by immobilizing rats inside a plastic tube for 6 hours a day, over 15 days. The results indicated that prolonged immobilization increased the mount latency and the number of thrusting. No changes in the testicular weight or histological pathology were observed. Immobilization causes a significant reduction of mature spermatid in testes and the spermatozoa concentration in cauda epididymidis [74]. It has been also reported that chronic physical and emotional stress suppresses the reproductive function [75]. Among other studies, stress-induced in male rats can reduce the testosterone secretion, spermatogenesis, and libido [72, 75, 76]. Stress induced by immobilization in male rats can also suppress the LH and FSH responses, and decreased testosterone levels [77]. The restriction of rats could reduce testicular steroidogenesis resulting in a reduction of plasma testosterone [78]. Long-term psychological stress reduces the catecholamine levels and its metabolites including dopamine, homovanillic acid (HVA), 5,6-dihydroxyphenylacetic acid (DOPAC) and norepinephrine in the brain, especially in the medial preoptic area (MPOA). These lead to an impairment of male rat sexual behaviour [79].



CHAPTER III

RESEARCH METHODOLOGY

Evaluation of PDE inhibitory activities of *D. scandens* extracts and isolated compounds

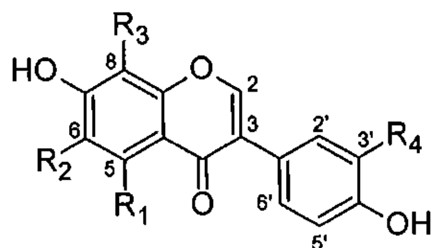
Chemicals and standards

Acetonitrile, water and methanol were of LC-MS grade and purchased from RCI labscan. Formic acid was of analytical grade and purchased from Merck. cGMP, crude snake venom (Crotalus atrox), calmodulin from bovine heart, histone, bovine serum albumin (BSA), diethylaminoethyl sephadex (DEAE-Sephadex), dipyridamole (purity > 98%), IBMX (purity > 99%), ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), imidazole, magnesium chloride ($MgCl_2$), crude phosphodiesterase 3',5'-cyclic nucleotide (PDE1) from bovine heart, tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich. Sildenafil citrate (purity > 98%) was purchased from European Directorate for the Quality of Medicines & HealthCare (EDQM) – Council of Europe. [^{3}H]cGMP was purchased from Perkin Elmer.

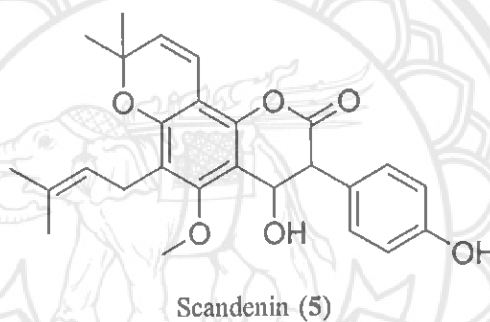
Compound **1** (Genistein; purity > 98%) was purchased from Apex biotechnology. Other isoflavones (**2** – **4**, **6** – **8**) and a coumarin (**5**) isolated from *D. scandens* were provided as gifts from Prof. Dr. Apichart Suksamrarn, Faculty of Sciences, Ramkhamhaeng University, Thailand. The purities of these compounds were more than 95% determined by NMR and LC-QTOF-MS.

Plant materials

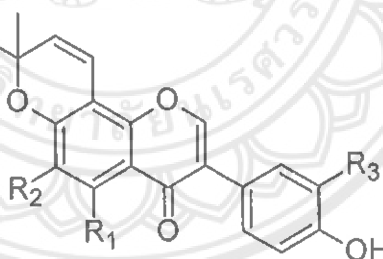
Leaves and stems of *D. scandens* were collected from locations in Phitsanulok Province, Thailand, in April 2016, and identified by Dr. Pranee Nangngam from the Department of Biology in the Faculty of Science, Naresuan University, Thailand. A voucher specimen with the catalogue No. 004331 is deposited at the PNU Herbarium in the Faculty of Science, Naresuan University.



Compound	Substitution			
	R_1	R_2	R_3	R_4
Genistein (1)	OH	H	H	H
Derrisisoflavone A (2)	OMe	Prenyl	Prenyl	H
Lupalbigenin (3)	OH	Prenyl	H	Prenyl
4',5,7-Trihydroxybiprenylisoflavone (4)	OH	Prenyl	Prenyl	H



Scandenin (5)



Compound	Substitution		
	R_1	R_2	R_3
Scandinone (6)	OMe	Prenyl	H
Scanderone (7)	OH	H	Prenyl
Osajin (8)	OH	Prenyl	H

Figure 12 Chemical structures of seven isoflavones and a coumarin in *D. scandens*

Plant extraction

The collected leaves and stems were dried in a hot air oven at 55°C for three days and were then pulverized into powder with a grinder prior to extraction. The aqueous extracts were prepared by infusing the powdered materials (10 g) in hot water at 80°C (100 mL) for 15 min. After filtration, the water was then sublimated in a freeze dryer to obtain 0.91 g and 0.89 g for aqueous extracts of leaves and stems, respectively. The 50% ethanol extracts were obtained by maceration the powdered materials (10 g) in 50% ethanol (100 mL) for 24 hours at room temperature under shaking at 300 – 350 rpm. After filtration, the filtrate was then evaporated under reduced pressure at 30 – 35°C to remove the ethanol. The solution without ethanol was then sublimated in a freeze dryer to obtain 1.37 g for 50% ethanol leaf extract and 1.08 g for 50% ethanol stem extract. The 95% ethanol extracts were prepared by maceration of the powdered materials (10 g) in 95% ethanol (100 mL) for 24 hours at room temperature under shaking at 300 – 350 rpm. After filtration, the filtrate was then concentrated under vacuum at 30 – 35°C until dryness and the dried extracts was obtained (1.01 g and 0.28 g for 95% ethanol extract of leave and stems, respectively). The extracts were stored at -20°C awaiting further study.

Measurement of PDE inhibitory activities

Enzymes preparation

PDE1, which is the crude PDE isolated from bovine heart, was purchased from Sigma-Aldrich. PDE5 was extracted from fresh rat lung tissue. Briefly, rat lung tissue was minced and homogenized in 2 mL of buffer PP (150 mM Tris, pH 7.5, 6 mM EDTA, 3 mM DTT and 1:100 of 100 mM phenylmethylsulfonyl fluoride). The homogenate were centrifuged at 14,000 rpm for 15 min at 4°C and the supernatant were used as a source of PDE5. PDE6 was extracted from chicken retina. The preparation method of PDE6 was described in the report of Huang and colleagues [80]. All experiments using the animal tissues were approved and performed in accordance with the guidelines provided by the Naresuan University Animal Care and Use Committee (NUACUC) (No. 6002003, approval date: January 25, 2017).

PDE assays

The PDE1 and 5 inhibitory activity measurements were modified from the report of Sonnenburg and colleagues [81] whereas the PDE6 assay was modified from the method of Huang and colleagues [80]. The assay consisted of 25 μL of 10 mM EGTA, 25 μL of buffer C (100 mM Tris–HCl (pH 7.5), 100 mM imidazole, 15 mM $MgCl_2$ and 1.0 mg/mL BSA), 25 μL of PDE enzyme, 25 μL of test sample or only solvent as a blank. Then a substrate, 25 μL of 1 μM [^{3}H] cGMP, was added and the solution incubated at 30°C for 10 min. The reaction was stopped by placing the tube in boiling water for 1 min and then cooled. In the next step, 25 μL of 2.5 mg/mL of snake venom was added and the solution incubated at 30°C for 5 min. The assay was diluted with 250 μL of low salt buffer (20 mM Tris–HCl, pH 6.8) and transferred to a DEAE ion exchange resin column. The [^{3}H] guanosine was eluted from the resin with 500 μL of low salt buffer, four times, and the eluates collected in a scintillation vial. Four mL of the scintillant cocktail was added to the vial and mixed completely. The radioactivity of the cocktail was measured by using a liquid scintillation counter (Tri-Carb 2910 TR, Perkin Elmer). In case of the PDE1 inhibitory activity assay, EDTA was used instead of EGTA, and 4 mM $CaCl_2$ and 16 μg/mL calmodulin were added into buffer C, while 2.5 mg/mL of histone was added for PDE6 assay. The PDE enzymes in the study were standardized to have a hydrolysis activity of 20–30% of the total substrate counts. The calculation of hydrolysis is shown in Eq.1-2 and the PDE inhibitory activity is calculated from Eq.3.

$$\text{\% hydrolysis of sample} = \left[\frac{(\text{CPMsample} - \text{CPMblank})}{(\text{CPMtotal count} - \text{CPMblank})}\right] \times 100 \qquad \text{Eq.1}$$

$$\text{\% hydrolysis of control} = \left[\frac{(\text{CPMcontrol} - \text{CPMblank})}{(\text{CPMtotal count} - \text{CPMblank})}\right] \times 100 \qquad \text{Eq.2}$$

$$\text{\% PDE inhibition} = \left[1 - \left(\frac{\text{\% hydrolysis of sample}}{\text{\% hydrolysis of control}}\right)\right] \times 100 \qquad \text{Eq.3}$$

Where CPM_{sample} is the radioactive count rate of the assay with enzyme; $CPM_{control}$ is the radioactive count rate of the assay with enzyme but without sample: and CPM_{blank} is the same but without enzyme. $CPM_{total\ count}$ is the count rate of 25 μL of substrate

plus 2 mL of low salt buffer. IBMX, sildenafil and dipyridamole were used as positive controls for PDE1, PDE5 and PDE6 inhibitory activity, respectively.

IC_{50} values were calculated from the nonlinear regression of the plot of values of percent inhibition versus log concentration of the sample using GraphPad Prism 5 software. IC_{50} values are shown as the mean and standard deviation (SD). All experiments were performed in triplicate.

LC-QTOF-MS analysis for quantitative determination of PDE5 inhibitors in *D. scandens*

Preparation of samples and standards solutions

The powder of dried plant materials and extracts were weighed and dissolved with methanol at the concentration of 10 – 20 mg/mL and 2 – 20 mg/mL, respectively. The solutions were then sonicated for 15 min and filtered through nylon syringe filters with a 0.45 µm pore size. The analysis was performed in triplicate.

The stock solution of each standard compound was freshly prepared by dissolving in methanol to obtain the concentration of 1 mg/mL. These solutions (**1** – **8**) were further mixed and diluted with methanol to make standard calibration curves, LOD and LOQ for the eight compounds.

Instruments and chromatographic conditions

LC-QTOF-MS analysis used an Agilent 1260 infinity Series HPLC system coupled to an Agilent-6540 QTOF mass spectrometer equipped with an electrospray interface (ESI). The LC-QTOF-MS systems consist of a binary pump, an online degasser, an auto sampler and a column thermostat. Chromatographic separations were performed on a Luna C18(2) LC column (4.6 mm x 150 mm) with a particle size of 100Å 5 µm (Phenomenex) maintained the temperature at 35°C. Chromatographic conditions are including: The mobile phase was the mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with a flow rate of 0.5 mL/min. The gradient elution was established as follows: 0 – 3 min, 25:75, (A: B v/v) and 3 – 13 min, 5:95. Post-time was 5 min for re-equilibration of the system prior to the next injection. The injection volume was 5 µl. The MS system was operated in positive electrospray ionization mode with the conditions as follows: gas temperature of 350°C, drying gas 10 L/min, nebulizer 30 psig, capillary voltage 3500 V, fragmentor

100 V, skimmer 65 V, OCT 1RF Vpp 750 V. All acquisition and analysis of data were analysed using MassHunter software (Agilent Technologies).

Method validation

The method was validated according to the ICH guidelines by determining linearity, selectivity, LOD, LOQ, precision and accuracy. The linearity range of the standards was determined on five concentration levels (5 – 25 μg/mL). Calibration curves were measured in every analysis day and each sample was determined in triplicate. The linearity of calibration curve was assessed by calculating the coefficient of determination (r^2). LOD and LOQ under the present chromatographic conditions were determined by injecting the standard solutions until a signal-to-noise ratio of each compound was 3 for LOD and 10 for LOQ. The selectivity was confirmed from the typical *m/z*, the mass spectra and retention times of each reference standard. The intra-day precision of the method was evaluated by repeating the measurements of three concentration levels (7.5, 12.5 and 22 μg/mL) of the eight analytes for 5-times within one day. This procedure was repeated on two more days for determining the inter-day precision (n = 3, each level). Precision is expressed as %RSD calculated from standard deviation/mean × 100. The accuracy and recovery were determined by spiking the known concentration of the mixed standard solution to 95% ethanol stem extract to obtain three different concentrations (7.5, 12.5 and 22 μg/mL) of each analyte. These experiments were done in triplicate. The accuracy is expressed as % recovery which was calculated from 100 × (measured analyte concentration – analyte concentration in the non-spiked extract / analyte concentration spiked.

Evaluation of *D. scandens* extract (DE) on mating behaviours of stress-induced ED rats

Chemicals and reagents

Estrogen (β-estradiol 3-benzoate), progesterone (4-pregnene-3, 20-dione) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, USA). Phosphate buffered saline (PBS) was purchased from Gibco (Maryland, USA). Pentobarbital sodium (Nembutal) was purchased from Ceva Sante Animate (Libourne, France). Acetonitrile, water and methanol were of LC-MS grade for LC-MS analysis or HPLC grade for HPLC analysis and purchased from RCI Labscan (Bangkok, Thailand). Formic acid was of analytical grade and purchased from Merck.

Plant material

Stems of *D.scandens* were collected from Phitsanulok Province, Thailand, in April, 2016. The plant was identified by Dr. Pranee Nangngam from the Department of Biology in the Faculty of Science, Naresuan University, Thailand. The voucher specimen (No. 004331) has been deposited at the PNU Herbarium in the Faculty of Science, Naresuan University, Thailand.

Preparation of the extract

The dried and powdered *D. scandens* stems were macerated with 95% ethanol in the ratio of 1:10 (w/v) for 24 h at the room temperature, under shaking at 300 – 350 rpm and filtered through filter paper. After filtration, the filtrate was concentrated under reduced pressure at controlled temperature of 30 – 35°C until dry. The extracts were weighed and kept at -20°C until used.

Determination of active components in the extract using LC-QTOF-MS

Preparation of sample solution

The extract was weighed and dissolved with methanol at the concentration of 2 – 20 mg/mL. The solution was then sonicated for a period of 15 min and filtered through nylon syringe filters with a 0.45 µm pore size. The analysis was measured in triplicate.

LC-QTOF-MS condition

The analysis was performed on LC-QTOF-MS which consisted of an Agilent 1260 infinity series HPLC system equipped with an Agilent-6540 QTOF mass spectrometer coupled to an electrospray interface (ESI). The LC-QTOF-MS systems consisted of a binary pump, an online degasser, an auto sampler and a column thermostat. The active components of *D. scandens* extract were separated on Luna C18(2) LC column (4.6 mm x 150 mm) with a particle size of 100Å 5 µm (Phenomenex) maintained at a temperature of 35°C. Chromatographic conditions include: The gradient mixture of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile was used as a mobile phase. The flow rate during the run was maintained at 0.5 mL/min. The gradient profile of solvent A and solvent B was established as follows: 0 – 3 min, 25:75, (A: B v/v) and 3 – 13 min, 5:95. Post-time was 5 min for re-equilibration of the system prior to the next injection. The injection volume was 5 µl. The MS system was operated in positive electrospray ionization mode with the conditions as follows: gas temperature of 350°C, drying gas 10 L/min, nebulizer 30 psig, capillary voltage 3500 V, fragmentor 100 V, skimmer 65 V, OCT 1RF Vpp 750 V. MassHunter software (Agilent Technologies) was used for data acquisition and data analysis. The percent content of the active components in DE is presented as mg of the compound per gram of dry plant, using the standard calibration curve of the standards.

Animals

Male and female Wistar rats (5 – 7 months old) were obtained from M-CLEA Bioresource Co., Ltd (MCBC), Bangplee, Samutprakan. They were housed, three to four rats per cage, at the Naresuan University Center for Animal Research (NUCAR). The animal room was maintained under a constant temperature at 22 ± 1°C with relative humidity of 55 + 10% and 12-hour reverse dark - light cycle (light on at 09:00 PM - 09:00 AM and light off at 09:00 AM - 09:00 PM). They were fed with pellet food and water *ad libitum*.

All experiments were conducted according to the ethical guidelines stated in The Care and Use of Animals for Scientific Purposes Act 2015 & Standard of Institutional Animal Care and Use Committee.The protocols were approved and performed in accordance with the guidelines provided by the Naresuan University Animal Care and Use

Committee (NUACUC) (No. 590712, approval date: October 31, 2016).

Preparation of female rats for studying their mating behaviour

To prevent pregnancy, fourteen female rats were induced by a bilateral ovariectomy before studying their mating behaviour for at least 2 weeks. For ovariectomy, the animals were anaesthetised with 80 mg/kg ketamine and 10 mg/kg xylazine, administered by intraperitoneal injection. The skin surface on the middle part of abdomen were sterilised with 70% ethanol. A small incision, approximately 1 cm in length was made. The ovaries were pulled out through the muscle incision, and removed. The uterine tubes were tied, and the horns were put back into the peritoneal cavity. The wound was closed by suturing and applied with povidone iodine (Betadine®) for disinfection. The wound was covered by Fixomull stretch®. After the surgery, the rats were placed on a warm patch to allow recovery from the anaesthetics and then returned to their cages. They were closely observed until their wound healed.

After full recovery and before mating behaviour for 48 hours, these female rats were induced into the oestrus cycle by an injection of 0.1 mL of 25 μg estradiol benzoate, subcutaneously. They were given 0.1 mL of 500 μg progesterone, subcutaneously, before studying their mating behaviour for 4 hours. The experiment was done according to NUCAR SOP no. 05 – 10 (Subcutaneous Injection to Mouse and Rat).

Stress introduction procedure

The method was modified from Almeida et al [71]. Briefly, stress was attended by immobilizing the animal inside a plastic tube, 15.5 cm in length and 6.0 cm in diameter, for 6 hours a day during light-off period starting from 8:00 AM each day for 14 consecutive days.

Male rat treatment

The male rats were randomly divided into 6 groups, 8 rats in each group. Group I animals served as a normal control and received only vehicle (propylene glycol) by oral gavage. Group II animals served as stress control and received vehicle plus stress. Group III to V animals received stress and administered with DE (*D. scandens* extract) suspended in propylene glycol by oral gavage at the dose of 200, 400 and 800 mg/kg/day, respectively. Group VI animals served as positive control and received stress plus sildenafil citrate at the dose of 5 mg/kg.

The treatments and the stress-exposure were performed once a day. After being exposed to stress, the animals were removed from their restraint-plastic tubes and given a 30 minute-refreshment period. They were then given the test substances by oral gavage administration and returned to their cages. This experiment was done for 14 days.

Mating behaviour evaluation

The mating behaviour was evaluated on day 1 and 14 after the rats received the substance administration for 30 minutes. The male rat was individually placed in the observation cage (clear glass box, 70 x 35 x 35 cm) and allowed to get acclimated to the new environment for about 5 minutes. An oestrous female rat was then introduced into each observation cage and the mating behaviour was continuously recorded by digital video camera for 30 minutes. The mating behaviours were evaluated by two experts. The assessed mating parameters including the following parameters:

1. Mount latency: The time from the introductions of the female to the first mount by the male.
2. Mounting number: The number of mounts without the intromission from the time of the introduction of the female until ejaculation.
3. Intromission latency: The time from the introduction of the female to the first intromission by the male.
4. Intromission number: The number of the intromissions from the time of female introduction to ejaculation.
5. Ejaculation latency: The time from the first intromission to ejaculation.
6. Ejaculation number: The number of ejaculations characterized by longer, deeper pelvic thrusting and slow dismount followed by a period of inactivity.
7. Postejaculatory interval: The time from ejaculation to the next intromission.

Effect on body weight loss

Body weight (BW) of each rat was measured before starting the experiments (day 0), day 7 and day 14 after finishing the experiments. The percentage of body weight loss was calculated from 100 × ((average of BW in day 0 - average of BW in day 14)/ Average of BW in day 0).

Effect on hormones

On day 15 (9:00 AM – 11:00 AM),the male rats were euthanized by an i.p. of Nembutal (100 mg/kg BW) and blood was collected from the heart, kept into heparinized tubes and stored at 4°C for hormonal assay. The experiments were conducted according to NUCAR SOP no. 05 – 12 (blood collection in rodent). Testosterone and cortisol were assayed by Biolab medical technic clinic, Phitsanulok, Thailand.

Effect on sexual organ weight

The sexual organs; penis, testis, epididymis, and vas deferens were removed and weighed. The organ weights were normalised with 100 g BW and expressed as percentage of organ weight per BW.

Effect on sperm

The spermatozoa samples were collected from the both sides of the caudal epididymis and vas deferens. The collected samples were suspended in 1 mL of 1×PBS which was incubated at 37°C for 30 minutes. Ten μL of the samples solution were then transferred to the haemocytometer (Counting Chamber, Makler, USA) which were observed under a light microscope. The samples were analysed immediately after collection and evaluated by two experts. The sperm analyses were done for evaluating sperms' number and sperm motility. For sperm number, sperm was randomly counted from three rows and the calculation was performed according to the number of counted cells and haemocytometer dimensions. The results are expressed as mean count in millions of sperm per millilitre. For motility evaluations, the motile and immotile sperm were classified. The results were then calculated and expressed as the percentage of motile sperm.

Effect on neurotransmitters level in rat brain

Preparation of sample solution

After euthanizing the rat, the brain tissue was removed and washed in cold (4°C) normal saline. It was weighed and stored at -80°C until used. The whole brain tissue of each rat was separately homogenized (Ultra-turex T8, Ika-werke GMBH & CO., Germany) in 0.1 M of perchloric acid (0.1 g/mL) and centrifuged (Labofuge 400R, Heraeus instruments, Germany) at 14,000 rpm for 20 min at 4°C. The supernatant was then used as a source for measuring neurotransmitter levels in the rats' brain i.e.

norepinephrine, dopamine and serotonin (5-hydroxytryptamine, 5-HT).

HPLC condition

Chromatographic analysis was performed using a Shimadzu CBM-20A HPLC system equipped with Shimadzu LC-20AD pumps, DGU-20A degassing unit, SIL-20AC prominence HPLC auto-samplers, CTO-10ASVP column oven and coupled to an ESA Coulochem III electrochemical detector. The temperature of the auto-sampler was maintained at 4°C. The optimal electrical potential settings were: E1 -175 mV and E2 +220 mV. Chromatographic separations were achieved on ACE C18-AR HPLC column (4.6 mm x 250 mm).The mobile phase consisted of 3% methanol in water, 3% acetonitrile in water, 0.15 mM ammonium acetate and 0.1 mM EDTA, buffered to a pH of 6 with 1 M acetic acid. The flow rate was set at 1 mL/min. Post-time was 3 min for re-equilibration of the system prior to the next injection. The injection volume was 20 µL. The stock solution of each standard compound was freshly prepared by dissolving in 0.1 M perchloric acid to obtain the concentration of 10 mg/mL. These standard solutions were further mixed and diluted with water to make standard calibration curves which were ranged from 1024 – 16 ng/mL. Peaks in the sample solution were identified by comparing the retention time of each peak in the standard solution of norepinephrine, dopamine and 5-HT.

Statistical analysis

All values were expressed as the mean ± standard error of measurement (SEM). Statistical analysis were conducted using Student's unpaired *t*-test and one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test in order to detect inter-group differences. *P*-values of <0.05 were considered statistically significant.

CHAPTER IV

RESULTS AND DISCUSSION

Plant extracts

The leaves and stems of *D. scandens* were extracted using 95% ethanol, 50% ethanol and water. The percent yields of each part are shown in Table 4.

Table 4 Percent yields of leaves and stems of *D. scandens* extracted with various solvents.

No.	Solvent	Weight of powdered plant (g)	Weight of crude extract (g)	% yield
Leaves				
1	95% Ethanol	10	1.01	10.10
2	50% Ethanol	10	1.37	13.70
3	Water	10	0.91	9.10
Stems				
1	95% Ethanol	10	0.28	2.80
2	50% Ethanol	8	0.86	10.75
3	Water	10	0.89	8.90

PDE inhibitory activities of *D. scandens* extracts and isolated compounds

The extracts of *D. scandens* were determined for their PDE 1, 5 and 6 inhibitory activities using 3-Isobutyl-1-methylxanthine (IBMX), sildenafil and dipyridamole as positive controls. The results are shown in Table 5 (for dose-response curves, see Figure 13 – 15). The extract with the highest potency on PDE5 inhibition was 95% ethanol stem extract with the IC_{50} value about 7 µg/mL. It also showed the high PDE6 inhibition with the IC_{50} value about 15 µg/mL. However, the extract showed low PDE1 inhibition with a percent inhibition value about 59% at the final concentration of 50 µg/mL.

Table 5 Percentage of PDE5 inhibition at the final concentration of 50 μg/mL and the IC_{50} values against PDE5 and 6 of leaf and stem extracts of *D. scandens*.

Extract	% PDE5 inhibition (at the concentration of 50 μg/mL)	IC_{50} (μg/mL)		Selectivity ratio PDE6/PDE5
		PDE5	PDE6	
Leaf				
95% Ethanol extract	74.29 ± 3.80	11.43 (9.06-14.41)	ND	ND
50% Ethanol extract	53.51 ± 3.80	ND	ND	ND
Water extract	0.38 ± 7.10	ND	ND	ND
Stem				
95% Ethanol extract	82.98 ± 0.85	7.33 (5.95 -9.02)	15.01 (12.11-18.61)	2.05
50% Ethanol extract	71.12 ± 4.37	22.61 (18.06-28.32)	ND	ND
Water extract	0.67 ± 5.73	ND	ND	ND
Sildenafil	ND	0.001 (0.0009-0.0018)	0.0062 (0.0056-0.0068)	6.2
Dipyridamole	ND	0.44 (0.43-0.45)	0.53 (0.48-0.58)	1.20

%PDE5 inhibition is expressed as mean ± SD and IC_{50} values against PDE5 and 6 are presented as mean with the 95% confidence interval inside the parentheses (n=3). Sildenafil and dipyridamole were used as positive controls. ND: Not determined.

The PDE inhibitory activities of isoflavonoids and a coumarin constituents of *D. scandens* (**1** – **8**) and the standard PDE inhibitors are shown in Table 6. Amongst the tested compounds, derrisisoflavone A (**2**), 4',5,7-trihydroxybiprenylisoflavone (**4**) and osajin (**8**) exhibited high PDE5 inhibitory effect with the IC_{50} values about 9, 8 and 4 μM, respectively. Lupalbigenin (**3**) displayed weaker inhibition with an IC_{50} value about 16 μM whereas genistein (**1**), scandenin (**5**), scandinone (**6**), and scanderone (**7**) did not show obvious inhibition of PDE5. Compounds **2** and **8** also inhibited PDE6 with the IC_{50} values in the micromolar range while compounds **4** inhibited PDE6 with the IC_{50} values about 14 μM. As for PDE1, all tested compounds except compounds **2** and **4** show IC_{50} more than 100 μM. Most of the isoflavones in

this study were reported for the inhibitory activity on PDE1, 5 and 6 for the first time. Only **1**, has been reported to show a moderate inhibition effect on PDE1 – 5 [42] and **8** has been tested the PDE-5A inhibition using microplate non-radioactive assay based on cleavage of Fluor-labelled cGMP [82].

Table 6 Effect of compounds isolated from *D. scandens* on PDE 1, 5 and 6 inhibitory activities.

Compound	IC_{50} (μM)			Selectivity ratio	
	PDE5	PDE1	PDE6	PDE1/PDE5	PDE6/PDE5
(1) Genistein	> 100	> 100	16.70 (13.61-20.69)	ND	ND
(2) Derrisisoflavone A	8.55 (7.25-10.08)	95.07 (90.92-99.41)	8.07 (6.84-9.52)	11.12	0.94
(3) Lupalbigenin	16.25 (14.28-18.42)	> 100	85.49 (80.83-90.43)	> 6.15	5.26
(4) 4',5,7-Trihydroxybiprenylisoflavone	7.68 (6.55-9.01)	42.06 (41.26-42.88)	13.90 (13.48-14.35)	5.48	1.81
(5) Scandenin	108.13 (104.76-111.61)	> 100	> 100	> 0.93	> 0.93
(6) Scandinone	> 200	> 100	> 200	ND	ND
(7) Scanderone	> 100	> 100	> 100	ND	ND
(8) Osajin	4.10 (3.06-5.49)	> 100	1.30 (0.84-2.02)	> 24.39	0.32
IBMX	12.64 (10.78-14.82)	7.15 (6.32-8.08)	10.15 (9.19-11.21)	0.57	0.80
Sildenafil	0.002 (0.001-0.004)	1.18 (1.02-1.35)	0.013 (0.012-0.014)	590	6.50
Dipyridamole	0.87 (0.85-089)	> 100	1.05 (0.97-1.14)	> 114.94	1.21

IC50 values are presented as mean with the 95% confidence interval inside the parentheses (n=3). IBMX (3-Isobutyl-1-methylxanthine), sildenafil and dipyridamole are positive controls. ND: Not determined.

The structures of tested compounds in this study can be divided into three groups; isoflavone analogues (**1-4**), cyclized isoflavone analogues (isoflavones with the extra ring at C-7 and C-8, **6-8**) and cyclized coumarin structure (coumarins with the extra ring at C-7 and C-8, **5**). Among the isoflavones (**1-4**), the importance of prenyl-substitution on PDE5 inhibitory activity was observed (Table 6). The unprenyl-substituted molecule (**1**) completely loses its PDE5 activity. This result was in agreement with the study of Dell'Agli et al [43] and Shin et al [83] which reported that

prenyl groups of phenolic compounds were important for PDE5 inhibition. The position of prenyl-substitution might also affect PDE5 inhibitory activity. Compounds **2** and **4** with the prenyl groups at C-6 and C-8 showed higher inhibitory activity against PDE5 about 2-folds more than compound **3** which has a prenyl at C-6 and C-3'. The similar results on PDE1 and PDE6 inhibitory activities were found. The demethylation of **2** to provide **4** showed no effect on PDE5 and PDE6 inhibitions but slightly increased PDE1 inhibition. For the cyclization of isoflavones in **6** – **8**, the extra ring might result from the intramolecular interaction between hydroxyl group at position-7 and double bond of prenyl at position-8. The cyclization analogues were likely to add rigidity to the molecules. The substitution of the prenyl group at C-6 in **8** showed higher inhibition on PDE5 over C-3' in compound **7**. This confirmed the preference of more lipophilic or steric groups on the benzene site. Interestingly, the different effect of methoxy substitution at C-5 was found in **6** which might due to the restrict conformation of cyclized isoflavones. The addition of larger groups at C-5 in **6** was not preferred as it might increase the steric property. Changing the isoflavone structure of **6** to the bioisostere coumarin ring (**5**) might cause the difference in conformation of the molecule and the activity of **5** on PDE5 inhibition was slightly improved.

None of the isolated compounds from *D. scandens* gave the higher PDE5 inhibitory activity and selectivity against PDE1 or PDE6 over the positive controls (sildenafil and dipyridamole). However, compounds **2**, **4** and **8** showed higher activity on PDE5 and 6 inhibitions more than IBMX. Compound **8** showed the highest activity on PDE5 among the tested compounds and the selectivity ratio on PDE1 was more than 24 although it gave the lowest selectivity ratio for PDE6 (0.32). The results obtained from this study could be useful for the future modification of the active compounds to improve the selectivity and potency of PDE5 inhibitors.

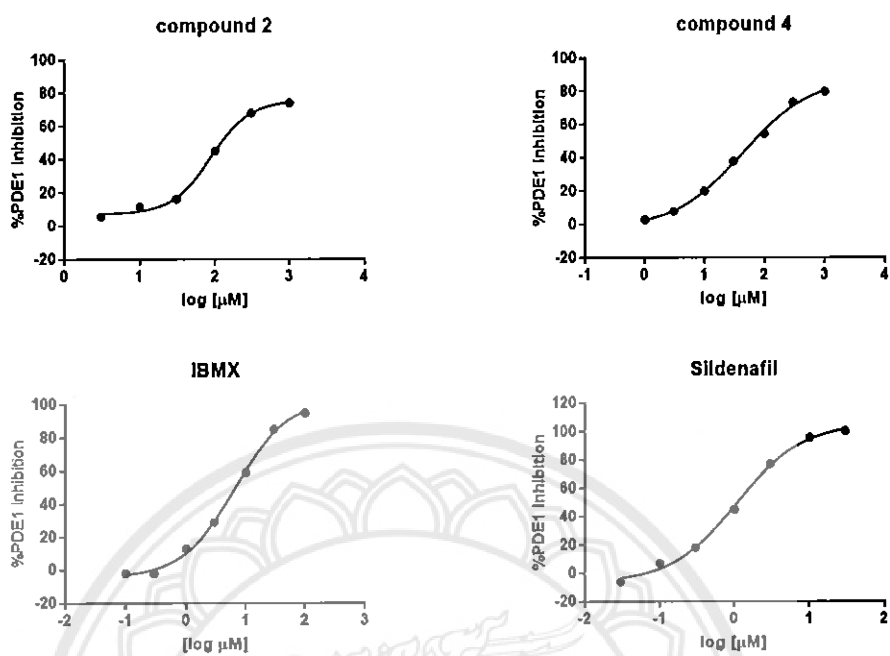


Figure 13 Representative dose – response curves of the active compounds on PDE1 inhibitory activity.

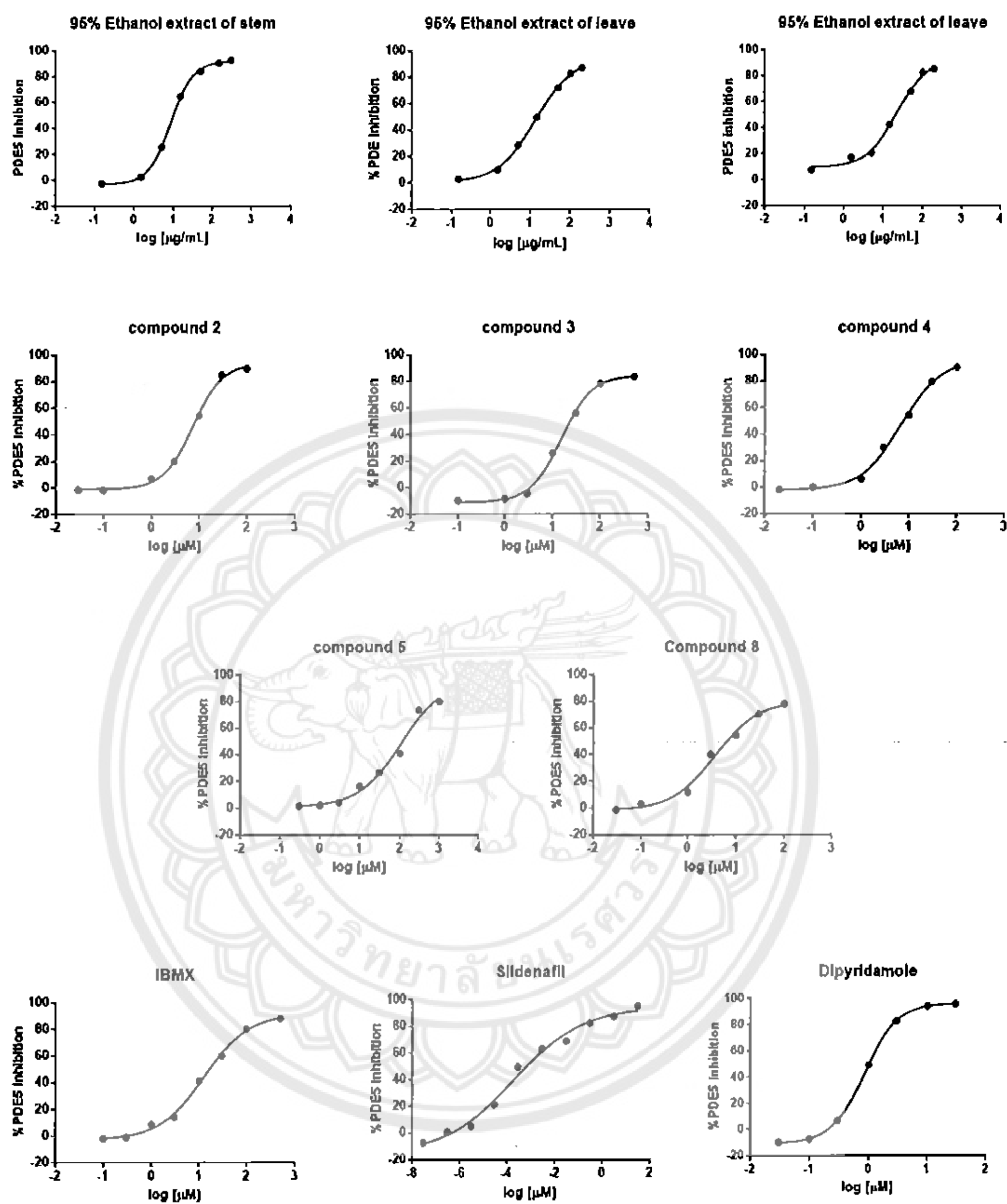


Figure 14 Representative dose – response curves of the active compounds on PDE5 inhibitory activity.

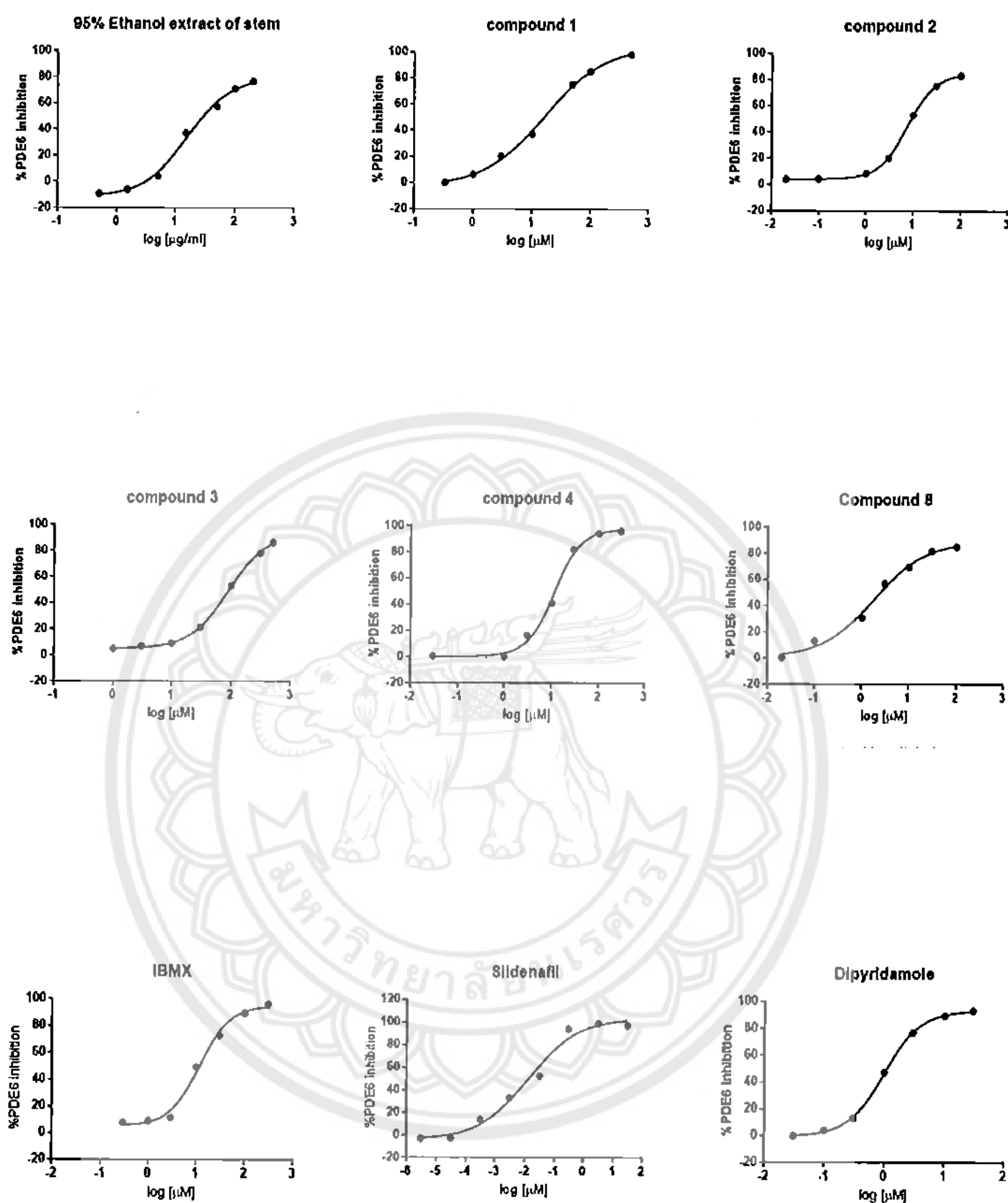


Figure 15 Representative dose – response curves of the active compounds on PDE6 inhibitory activity.

LC-QTOF-MS method for quantitatively determination of PDE5 inhibitors in *D. scandens*

LC-QTOF-MS method validation

The LC-QTOF-MS method for quantitative determination of the seven isoflavones and a coumarin in *D. scandens* were developed and validated according to the ICH guidelines. The separation of the eight compounds was done on C18(2) column with gradient elution of water and acetonitrile, both acidified with 0.1%(v/v) formic acid. The tailing factor and resolution of each compound met the ICH guidelines. The identification of each of the reference standards were confirmed by the typical *m/z*, the mass spectra and retention times using the extracted ion chromatograms (EIC) mode that specified only the selected *m/z*. The calibration equations, linearity, limit of detection (LOD) and limit of quantitation (LOQ) values are presented in Table 7.

Table 7 Measured mass, calibration equations, retention time (t_R), LODs and LOQs, of eight reference standards (1 – 8) analysed by LC-QTOF-MS.

Compound	Measured mass *m/z*$[M+H]^+$	t_R (min)	Calibration equation	Curve Weighting	Linearity	LOD (μg/mL)	LOQ (μg/mL)
1	271.0551	3.604	Y = 31600.66X + 42726.76	1/X	0.9992	1	5
2	421.1949	6.442	Y = 133355.51X + 945251.12	$1/X^2$	0.9993	0.05	0.1
3	407.1861	6.838	Y = 23329.46X + 238863.02	$1/X^2$	0.9998	0.25	1
4	407.1797	7.173	Y = 15704.32X + 169422.79	$1/X^2$	0.9996	0.25	1
5	435.1746	7.476	Y = 33815.54X + 371665.84	1/X	0.9991	0.1	0.5
6	419.1791	8.454	Y =66136.42X + 723944.85	$1/X^2$	0.9994	0.25	1
7	405.1638	9.152	Y = 28213.46X + 178947.29	1/X	0.9994	1	5
8	405.1638	9.717	Y = 14362.82X + 106219.86	1/X	0.9998	1	5

For determination of the 8 analytes, LOQ values in the range of 0.1 – 5 mg/mL were obtained. The coefficients determination (r^2) for the calibration curves of all compounds were more than 0.9990. The intra-day and inter-day precision of all eight compounds were not more than 6. The analytical method developed for the quantitation of all eight compounds had good accuracy with overall recovery in the range from 96 – 104 (%RSD ≤ 6.08). All data are shown in Table 8. The representative of LC chromatogram of the standard compounds and 95% ethanol extract of *D. scandens* stem are shown in Figure 16.

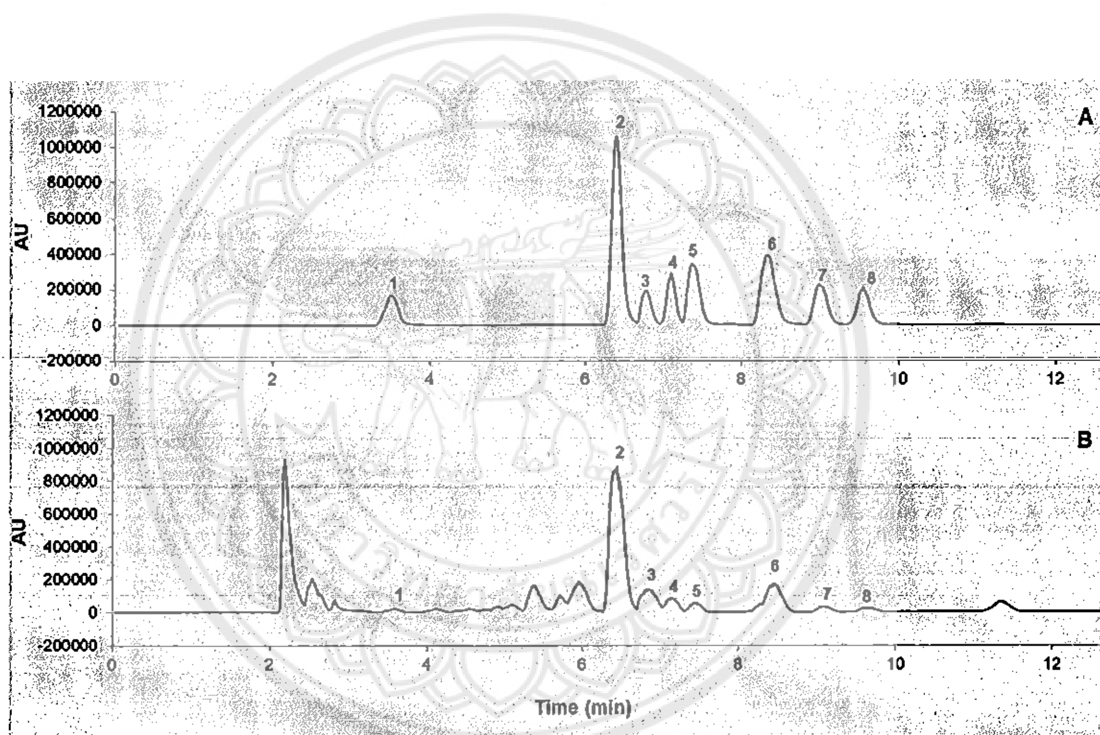


Figure 16 **Representative LC chromatograms of (A) eight standard compounds (1 – 8; 25 µg/mL) isolated from *Derris scandens* and (B) the 95% ethanol extract of *D. scandens* stem (20 mg/mL). The following constituents were assigned: 1 = genistein, 2 = derrisisoflavone A, 3 = lupalbigenin, 4 = 4',5,7-trihydroxybiprenylisoflavone, 5 = scandenin, 6 = scandinone, 7 = scanderone, and 8 = osajin**

Table 8 Intra-day and inter-day precision, and accuracy of LC-QTOF-MS method for determination of compound 1 – 8 assessed at three different concentration levels (n = 3) in three consecutive days. Accuracy was expressed as the % recovery of each compound in three different concentrations in *D. scandens* stem extract.

Compound	Spiked level (µg/mL)	Accuracy (n=9)			Intra-day precision (n=5)		Inter-day precision (n=9)	
		Measured concentration (µg/mL)	%Recovery	%RSD	Measured concentration (µg/mL)	%RSD	Measured concentration (µg/mL)	%RSD
1	7.5	7.72	101.41	2.39	7.46	5.17	7.58	4.03
	12.5	12.80	103.85	3.67	12.28	2.27	12.42	3.82
	22	21.63	99.89	4.55	21.86	2.36	21.88	3.42
2	7.5	7.32	96.63	3.58	7.39	3.68	7.51	3.26
	12.5	12.59	100.41	3.74	12.43	0.84	12.55	1.41
	22	21.29	96.28	3.28	21.58	1.28	21.80	1.69
3	7.5	7.72	103.50	2.08	7.64	2.67	7.51	2.48
	12.5	12.24	98.72	3.54	12.61	1.90	12.55	1.76
	22	21.62	99.17	2.10	21.94	1.32	21.80	1.20
4	7.5	7.34	98.02	1.46	7.56	3.26	7.72	2.51
	12.5	12.30	98.41	3.21	12.46	2.71	12.51	0.78
	22	21.78	99.03	2.15	22.10	2.34	22.45	0.79
5	7.5	7.55	100.70	6.29	7.62	3.11	7.63	3.12
	12.5	12.37	99.01	3.09	12.59	1.32	12.55	1.33
	22	22.18	100.88	2.21	22.38	0.41	22.33	0.70
6	7.5	7.38	98.27	2.98	7.55	2.69	7.49	2.46
	12.5	12.72	101.43	1.50	12.55	1.65	12.61	1.61
	22	22.00	99.81	3.80	22.15	1.14	22.04	1.92
7	7.5	7.49	99.39	5.76	7.72	2.32	7.64	3.59
	12.5	12.40	99.90	1.98	12.73	1.57	12.55	2.16
	22	21.98	99.35	1.12	22.26	0.97	22.15	1.45

Table 8 (cont.)

Compound	Spiked level (μg/mL)	Accuracy (n=9)			Intra-day precision (n=5)		Inter-day precision (n=9)	
		Measured concentration (μg/mL)	%Recovery	%RSD	Measured concentration (μg/mL)	%RSD	Measured concentration (μg/mL)	%RSD
8	7.5	7.37	98.18	6.08	7.74	1.98	7.66	1.92
	12.5	12.74	100.93	2.26	12.61	1.95	12.68	2.17
	22	21.94	98.93	2.42	22.48	2.19	22.28	2.13

Quantitatively determination of PDE5 inhibitors content in *D. scandens* extracts and dried plant materials

The contents of seven isoflavones and a coumarin in *D. scandens* extracts and dried plant materials were investigated using our validated LC-QTOF-MS method. The results in Table 9 showed that these compounds are found mainly in the stems and almost absence in the leaves. The 95% ethanol extract of stems contained the highest amount of the compounds tested compared to the extracts from other solvent (Table 3). It was correlated to the fact that the highest PDE5 inhibitory activity was found in 95% ethanol extract (Table 5). The activity might mainly be contributed from **2, 3** and **4** which were found as major components in the 95% ethanol extract of stems. On the other hand, none of the compounds tested were found in the aqueous extracts corresponding to no PDE5 inhibitory activity belonging to these extracts. The results suggest 95% ethanol should be used to prepare *D. scandens* stem extract to achieve high PDE5 inhibitory activity.

Table 9 Contents of seven isoflavones and a coumarin in *D. scandens* extracts and dried plant materials expressed in milligrams per gram.

Compound	Leaves extract			Stems extract			Dried plant material	
	95% Ethanol extract (n=9)	50% Ethanol extract (n=9)	Water extract (n=9)	95% Ethanol extract (n=9)	50% Ethanol extract (n=9)	Water extract (n=9)	Leaves (n=3)	Stems (n=3)
1	ND	ND	ND	0.5 ± 0.1	0.19 ± 0.2	ND	ND	0.20 ± 0.00
2	< LOD	ND	< LOD	30.3 ± 10.1	2.88 ± 3.1	< LOQ	< LOD	1.53 ± 0.07
3	5.3 ± 2.59	< LOD	ND	34.9 ± 11.2	2.71 ± 0.6	ND	0.06 ± 0.01	1.54 ± 0.03
4	ND	< LOD	ND	13.4 ± 4.5	4.49 ± 0.4	ND	ND	0.63 ± 0.03
5	ND	ND	ND	3.9 ± 0.6	< LOD	ND	ND	< LOQ
6	ND	ND	ND	1.0 ± 2.1	< LOQ	< LOD	ND	< LOQ
7	ND	ND	ND	4.1 ± 0.4	< LOD	ND	ND	0.32 ± 0.02
8	ND	ND	ND	6.6 ± 1.7	< LOD	ND	ND	0.25 ± 0.00

The results were expressed as mean ± S.D.
ND: No detection.

Evaluation of the 95% ethanol extract of *D. scandens* stem (DE) on mating behaviours of stress-induced ED rats

Determination of active components in DE

Based on our finding, the major active components on PDE5 inhibitory activity of DE are derrisisoflavone A (2) and lupalbigenin (3). Using our validated LC-QTOF-MS method, the contents of these two active components in DE are 3.03 ± 1.01 and 3.49 ± 1.12% w/w, respectively.

Effect on mating behaviours of stress-induced ED Rats

The mating behaviours were studied on day 1 and day 14 after immobilization. The parameters include mount latency (ML), mount number (MN), intromission latency (IL), intromission number (IN), ejaculation latency (EL), ejaculation number (EN) and post-ejaculation interval (PEI) were evaluated. As shown in Table 10 and Figures 18 – 21, ML and IL in day 1 and day 14 of stress control group were significantly longer than that of normal control group (*P-value* < 0.05).

The IN of stress control group was significantly lower than that of the normal control group. These indicated the ED suffering of rats in stress control group [74]. The reduction in ML and IL of stress rats treated with DE 200, 400 and 800 mg/kg, compared to stress rats treated with vehicle, suggested an increase in sexual motivation [69]. This might be due to the PDE5 inhibitory effect of the extract leading to the increase of blood flow to male reproductive organs [12]. On day 1, IN of stress rats treated with DE 200 and 800 mg/kg were significantly higher than stress control group while the dose of 400 mg/kg tended to increase IN even not significantly different from the stress control group. This indicated that DE affected the efficiency of penile erection and penile orientation [69] of stress rat. The EL, EN and PEI were not significantly different in each group. The similar results have shown from previous studies of some plant extracts on stress-induced ED by IMB [84, 85]. Treating stress rats with 200 mg/kg of *Cynodon dactylon* methanol extract for 30 days could improve sexual function by increase MN and decrease IL without the effect on ML, IN, EL and EN [84]. Seven-day oral administration of 10 mg/kg *Moringa oleifera* leaf extract could enhance sexual activities by decreasing IL and increasing IN without affecting ML, MN, EL and EN [85]. These studies indicated that plant extracts could improve sexual functions of stress rats in different ways. Some plant extracts may increase arousal by increasing MN, reducing ML or reducing IL while other plants may affect to the efficiency of penile erection by increasing IN. Our present study indicated the sexual motivation effects of DE in stress rats by reducing ML and IL. In addition, DE improved to the efficiency of penile erection by increase IN.

Table 10 Effect of an oral administration of DE on mating behaviours in stress-induced ED rats after a single dose (Day 1) and after 14 day (Day 14) of treatment.

Group	ML (sec)	MN	IL (sec)	IN	EL (sec)	EN	PEI (sec)
Day 1							
Normal control	37.3 ± 10.9	8.9 ± 3.1	61.9 ± 21.4	46.8 ± 8.2	985.0 ± 160.6	0.8 ± 0.3	284.7 ± 34.8
Stress + Vehicle	134.0 ± 19.3*	4.1 ± 1.4	142.8 ± 19.1*	18.8 ± 6.6*	798.0 ± 250.7	0.5 ± 0.3	294.3 ± 27.2
Stress + DE 200	43.9 ± 10.7$^{\#}$	6.1 ± 1.3	60.7 ± 9.8$^{\#}$	40.3 ± 6.1$^{\#}$	856.0 ± 207.4	1.1 ± 0.4	338.8 ± 52.3
Stress + DE 400	38.6 ± 7.6$^{\#}$	4.8 ± 1.2	59.5 ± 10.2$^{\#}$	36.9 ± 6.6	1,056.3 ± 199.3	1.4 ± 0.4	313.6 ± 23.7
Stress + DE 800	36.1 ± 5.2$^{\#}$	3.4 ± 1.2*	50.8 ± 11.3$^{\#}$	45.6 ± 6.9$^{\#}$	804.0 ± 254.3	1.0 ± 0.4	344.0 ± 58.5
Stress + sildenafil	19.1 ± 5.1$^{\#}$	3.8 ± 0.8	28.3 ± 5.2$^{\#}$	32.8 ± 4.4	883.2 ± 190.1	1.3 ± 0.3	346.5 ± 39.6
Day 14							
Normal control	5.9 ± 0.9	8.1 ± 4.1	7.5 ± 0.7	52.5 ± 10.7	923.7 ± 228.8	1.3 ± 0.4	293.8 ± 32.5
Stress + vehicle	21.4 ± 6.9**	4.1 ± 0.7	27.5 ± 7.1**	48.1 ± 9.1	793.6 ± 148.8	1.8 ± 0.4	309.1 ± 34.7
Stress + DE 200	6.1 ± 1.5$^{\#\#}$	6.1 ± 1.3	7.9 ± 1.4$^{\#\#}$	61.1 ± 8.8	1,010.0 ± 270.2	1.1 ± 0.4	268.3 ± 18.1
Stress + DE 400	4.9 ± 0.6$^{\#\#}$	5.9 ± 1.1	8.0 ± 1.4$^{\#\#}$	45.6 ± 7.3	756.0 ± 138.8	0.9 ± 0.4	288.8 ± 28.1
Stress + DE 800	4.8 ± 0.7$^{\#\#}$	8.3 ± 2.1	5.6 ± 1.0$^{\#\#}$	54.5 ± 7.2	890.3 ± 156.5	1.4 ± 0.3	261.8 ± 21.2
Stress + sildenafil	6.0 ± 0.7$^{\#\#}$	4.4 ± 1.3	7.4 ± 0.9$^{\#\#}$	38.1 ± 5.9	1,026.5 ± 278.1	1.0 ± 0.5	314.5 ± 25.1

Values are expressed as mean ± SEM, n = 8.

*,** *P - value* < 0.05 compared to the normal control group in day 1 and day 14, respectively.

$^{\#,\#\#}$ *P - value* < 0.05 compared to the stress -vehicle group in day 1 and day 14, respectively.

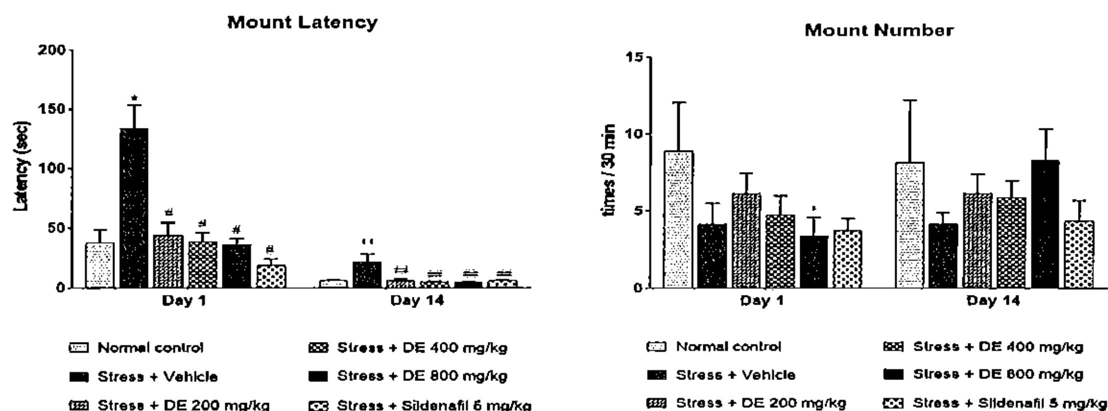


Figure 17 Effect of an oral administration of DE on mount latency and mount number of stress-induced ED rats after a single dose (Day 1) and after 14 days (Day 14) of treatment. Values are expressed as mean ± SEM, n = 8. *, ** *P - value* < 0.05 compared to the normal control group in day 1 and day 14, respectively. #, ## *P - value* < 0.05 compared to the stress control group in day 1 and day 14, respectively.

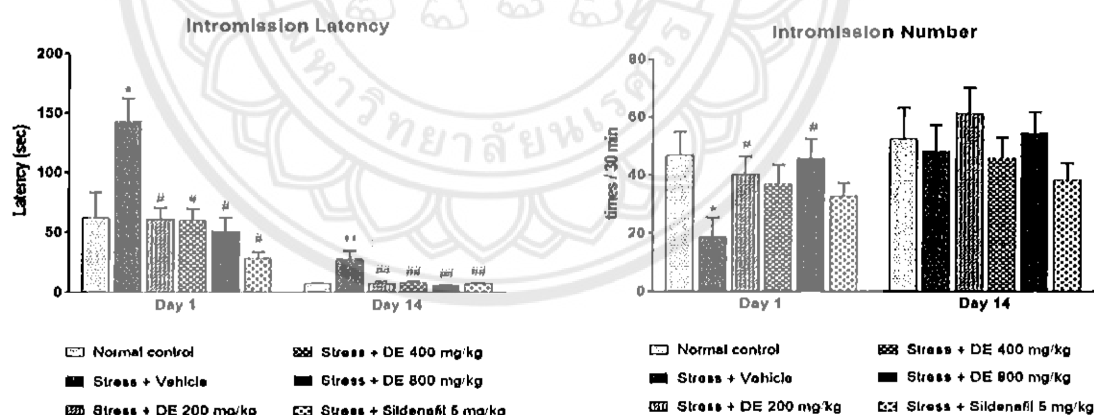


Figure 18 Effect of an oral administration of DE on intromission latency and intromission number of stress-induced ED rats after a single dose (Day 1) and after 14 days (Day 14) of treatment. Values are expressed as mean ± SEM, n = 8. *, ** *P - value* < 0.05 compared to the normal control group in day 1 and day 14, respectively. #, ## *P - value* < 0.05 compared to the stress control group in day 1 and day 14, respectively.

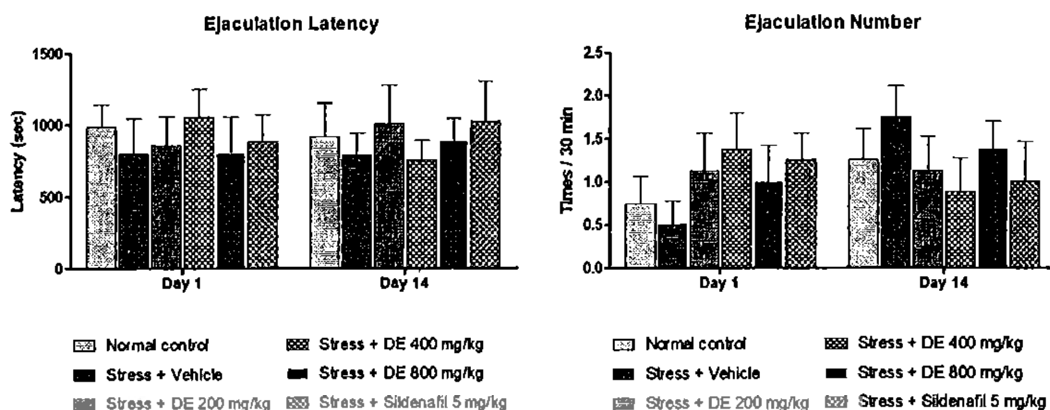


Figure 19 Effect of an oral administration of DE on ejaculation latency and ejaculation number of stress-induced ED rats after a single dose (Day 1) and after 14 days (Day 14) of treatment. Values are expressed as mean ± SEM, n = 8.

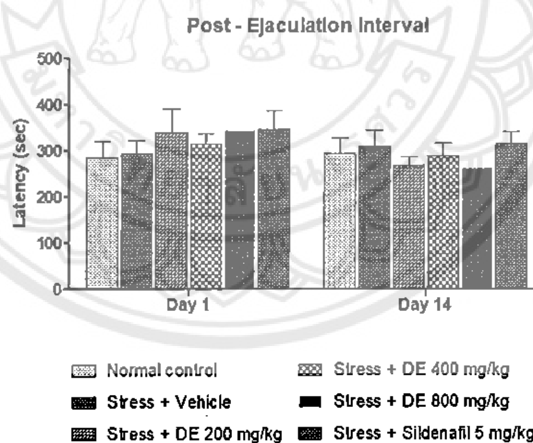


Figure 20 Effect of an oral administration of DE on post-ejaculation latency of stress-induced ED rats after a single dose (Day 1) and after 14 days (Day 14) of treatment. Values are expressed as mean ± SEM, n = 8.

Effect of stress on body weight loss and sexual organ weight

To investigate the effect of stress on body weight and sexual organs, rats were treated with IMB for 6 hours a day for 14 days. We found that a significant reduction in body weight was observed in all treated-rats with stress, except rats-treated with sildenafil compared with the normal control group. The relative weights of penis, testis, epididymis, and vas deferens of all groups were not significantly different from that of the normal control group. This suggested that stress had no effect on sexual organs of rats. All results are shown in Table 11 and Table 12.

Table 11 **Effect of stress on BW (g) before starting the experiments (day 0), after 7 days (Day 7) and after 14 days (Day 14) of treatment and percentage of weight loss of stress-induced ED rats after 14 days treatment.**

Group	Day 0	Day 7	Day 14	% Weight loss
Normal control	451.4 ± 24.51	455.6 ± 27.27	441.9 ±22.74	2.10 ± 1.44
Stress + vehicle	498.9 ± 39.52	471.8 ± 42.79	457.9 ± 46.10	8.22 ± 1.71*
Stress + DE 200 mg/kg	510.3 ± 7.63	468.3 ± 22.07	464.2 ± 22.83	9.03 ± 1.26*
Stress + DE 400 mg/kg	508.7 ± 25.47	485.2 ± 24.58	475.5 ± 25.28	6.52 ± 1.07*
Stress + DE 800 mg/kg	503.6 ± 16.69	473.8 ± 12.66	460.3 ± 20.29	8.61 ± 1.42*
Stress + sildenafil 5 mg/kg	482.1 ± 41.85	459.9 ± 40.27	459.2 ± 48.23	4.95 ± 1.00

Values are expressed as mean ± SEM, n = 8.

* *P - value* < 0.05 compared to the normal control group.

Table 12 Effect of DE on percentage of percentage of organ weight per BW of stress-induced ED rats after 14 days of treatment.

Group	Penis	Testis	Epididymis	Vas deferens
Normal control	0.10 ± 0.00	0.81 ± 0.02	0.13 ± 0.00	0.07 ± 0.01
Stress + vehicle	0.09 ± 0.00	0.81 ± 0.03	0.12 ± 0.01	0.07 ± 0.01
Stress + DE 200 mg/kg	0.09 ± 0.01	0.77 ± 0.01	0.12 ± 0.01	0.08 ± 0.01
Stress + DE 400 mg/kg	0.08 ± 0.00	0.76 ± 0.01	0.11 ± 0.01	0.08 ± 0.00
Stress + DE 800 mg/kg	0.10 ± 0.00	0.80 ± 0.01	0.12 ± 0.00	0.08 ± 0.01
Stress + sildenafil 5 mg/kg	0.08 ± 0.00	0.78 ± 0.02	0.12 ± 0.00	0.07 ± 0.00

Values are expressed as mean ± SEM, n = 8.

Effect on sperm

Sperm were collected from the both sides of the caudal epididymis and vas deferens. The spermatozoa concentration in cauda epididymidis generally declines following immobilization [74]. This finding showed that the mean value of sperm counts in the stress control group was significantly lower than that in the normal control group (Table 13).

Interestingly, 400 mg/kg DE could attenuate the reduction of the value of sperm counts of rats induced by stress, but it did not affect sperm motility. Stress did not affect rat sperm motility in this experiment. It is noted that the rats treated with a higher dose of DE (800 mg/kg) showed a significantly lower percentage of sperm motility compared to that of other groups. Previous studies have investigated whether plant extracts could increase the sperm count of stressed rats. *C. dactylon* methanol extract at a dose of 200 mg/kg could increase the sperm count of stressed rats, while rats treated with another dose (100 mg/kg) and the other solvent extracts i.e. aqueous and benzene extracts, did not [84]. The increase of spermatozoa was also observed from stressed rats treated with *M. oleifera* leaf extract at a high dose (250 mg/kg), but not with the lower doses (10 mg/kg and 50 mg/kg) [85].

Table 13 Effect of DE on the mean count of sperm and percentage of sperm motility of stress-induced ED rats after 14 days of treatment.

Group	Mean count ($\times 10^6$ sperms/mL)	% Sperm motility
Normal control	125.00 ± 15.84	40.42 ± 1.18
Stress + vehicle	56.50 ± 8.28*	36.17 ± 2.12
Stress + DE 200 mg/kg	92.33 ± 7.69	29.59 ± 3.76*
Stress + DE 400 mg/kg	109.04 ± 20.89$^{\#}$	38.28 ± 2.17
Stress + DE 800 mg/kg	88.58 ± 12.11	27.57 ± 2.16$^{*,\#}$
Stress + sildenafil 5 mg/kg	88.67 ± 11.91	35.72 ± 2.39

Values are expressed as mean ± SEM, n = 8.
**P - value* < 0.05 compared to the normal control group.
$^{\#}$*P - value* < 0.05 compared to the stress control group.

Effect of DE on hormones in blood of stress-induced ED rat

After 14 days of treatment, all blood samples were collected from the rats' hearts and analysed. As shown in Table 14 and Figure 21, the cortisol hormone level of the stress control rats was significantly higher than that of the normal control group. The effect of DE at the doses of 200, 400, 800 mg/kg and sildenafil 5 mg/kg to attenuate the increase of cortisol induced by stress were clearly observed. There have been reports that the increase of cortisol levels induced by stress can directly inhibit testosterone production [86, 87]. On the contrary, testosterone levels of rats treated with stress was decreased but not significantly different to that of the control group. All doses of DE did not affect to the level of testosterone in stress induced ED rats.

Table 14 Effect of DE on cortisol and testosterone levels in blood of stress-induced ED rats after 14 days of treatment.

Group	Cortisol (ng/mL)	Testosterone (ng/mL)
Normal control	9.1 ± 0.8	3.5 ± 0.8
Stress + Vehicle	11.8 ± 1.0*	2.9 ± 0.7
Stress + DE 200 mg/kg	8.4 ± 0.5#	4.0 ± 0.6
Stress + DE 400 mg/kg	7.8 ± 0.6#	3.1 ± 0.5
Stress + DE 800 mg/kg	8.8 ± 1.0#	2.7 ± 0.4
Stress + sildenafil 5 mg/kg	8.0 ± 1.0#	2.2 ± 0.2

Values are expressed as mean ± SEM, n = 8.
* *P - value* < 0.05 compared to the normal control group.
\# *P - value* < 0.05 compared to the stress control group.

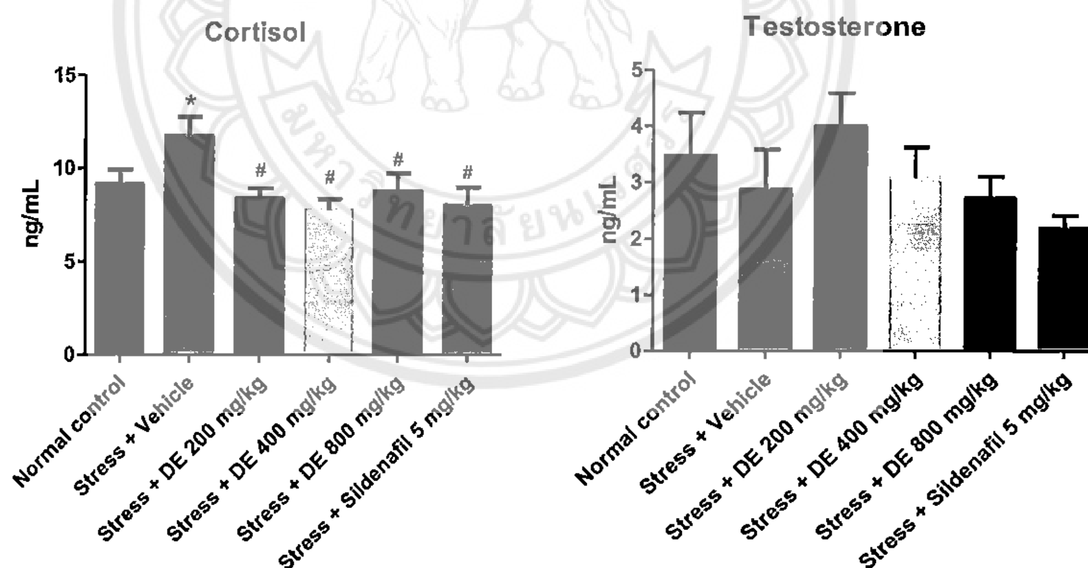


Figure 21 Effect of DE on cortisol and testosterone level in blood of stress-induced ED rats after 14 days (Day 14) of treatment. Values are expressed as mean ± SEM, n = 8. * *P - value* < 0.05 compared to the normal control group. # *P - value* < 0.05 compared to the stress control group.

Effect of DE on neurotransmitters level in brain of stress-induced ED rat

The level of neurotransmitters, norepinephrine, dopamine and serotonin in the whole brains of all groups were studied. There has been reports that stress can increase the release of norepinephrine [87, 88] while decreasing dopamine and serotonin levels in animals. The results showed that the stress induced by immobilization in our experiment did not significantly change the level of these neurotransmitters (Table 15 and Figure 22). It is noted that the decrease of norepinephrine level and the increase of dopamine levels in stress rats treated with DE (800 mg/kg BW) was observed. Further study needs to be done on this in order to clarify these observations.

Table 15 Effect of DE on neurotransmitters levels of stress-induced ED rats after 14 days of treatment.

Group	Norepinephrine (ng/gram tissue)	Dopamine (ng/gram tissue)	Serotonin (ng/gram tissue)
Normal control	687.4 ± 18.8	1151.1 ± 78.0	790.7 ± 68.7
Stress + vehicle	753.8 ± 62.4	1333.1 ± 115.8	692.4 ± 28.4
Stress + DE 200 mg/kg	765.5 ± 29.1	1273.2 ± 62.2	783.4 ± 35.2
Stress + DE 400 mg/kg	720.6 ± 26.3	1326.4 ± 69.4	800.8 ± 33.2
Stress + DE 800 mg/kg	541.9 ± 46.9*	1405.0 ± 31.8*	775.9 ± 37.9
Stress + sildenafil 5 mg/kg	656.8 ± 44.8	1299.8 ± 68.3	764.9 ± 49.2

Values are expressed as mean ± SEM, n = 8.
* *P - value* < 0.05 compared to the normal control group.

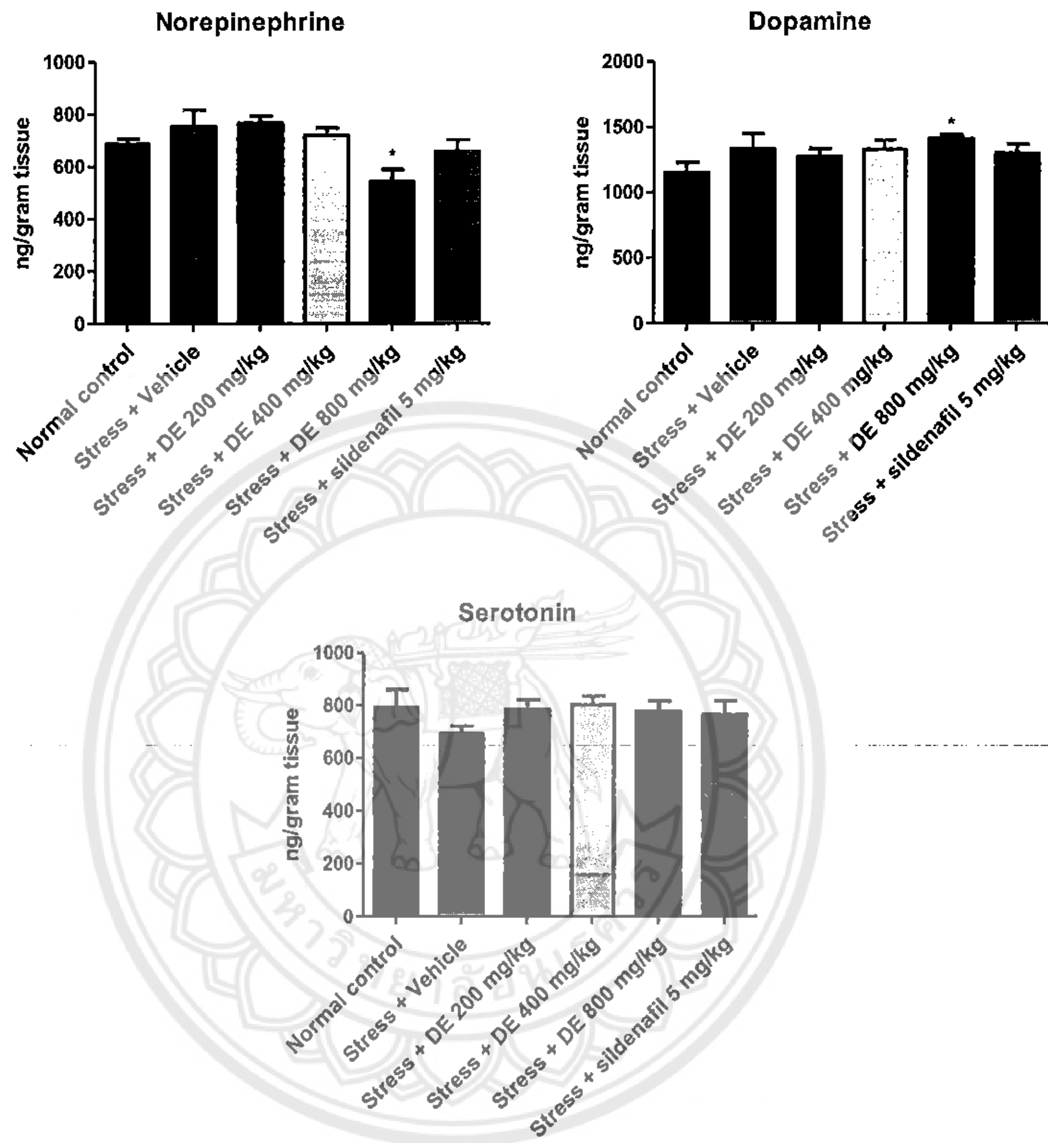


Figure 22 Effect of DE on neurotransmitters level of stress-induced ED rats after 14 days of treatment. Values are expressed as mean ± SEM, n = 8. **P* - *value* < 0.05 compared to normal control group.

CHAPTER V

CONCLUSION

These research studies involved the PDE (PDE1, 5 and 6) inhibitory activity of *D. scandens* extracts and isolated compounds, SAR discussion of the compounds, quantitative determination of PDE5 inhibitors in *D. scandens*, and *in vivo* study of the 95% ethanol extract of *D. scandens* stem on mating behaviour of stress-induced ED male rats.

The PDE5 inhibitors found in *D. scandens* were reported here for the first time. The stem of *D. scandens* were extracted with various solvents, water, 50% ethanol and 95% ethanol. The 95% ethanol extract of *D. scandens* stem (DE) showed the highest PDE5 inhibitory activity. Among seven isoflavones and a coumarin isolated from this plant, the most potent compounds on PDE5 inhibition are **2**, **4** and **8**. Another major compound, **3**, showed moderate activity on PDE5 with the highest selectivity on PDE5 over PDE6 compared with other isoflavones studied. Some SAR have been observed such as the importance of prenyl-substitution on PDE5 inhibitory activity. LC-QTOF-MS analysis for the quantitative determination of PDE5 inhibitors in *D. scandens* have been developed and validated for the first time. The method was successfully used for the quantitative analysis of these compounds in the plant materials and the extracts of *D. scandens*. Using our validated LC-QTOF-MS method, we found that compound **2** and **3** were present as the major compounds in DE.

There has been reported that stress can induced ED by increasing the corticosteroid level leading to an increase of norepinephrine level, a decrease of luteinizing hormone and a decrease of spermatogenesis [86, 87, 88]. We were then interested to study the effect of our PDE5 inhibitor extract, DE, on sexual behaviour of stress induced ED male rats. The study indicated that oral administration of the extract significantly motivated mating behaviour by reducing the mount latency and intromission latency. The increase of efficiency of penile erection and penile orientation by DE were also observed from the increase of intromission number. DE had no effect on sexual organ weight. An increase of number of sperm and number of live sperm were observed in stress-rats subjected to 400 mg/kg of DE whereas treating

stress-rats with 800 mg/kg can increase dopamine levels. However, the effect of DE on testosterone and neurotransmitters are still not clear. Even the extract showed PDE5 inhibitory effect in *in vitro* study, but the underlying mechanism of the extract on PDE5 level in penis tissue was not evaluated. This study indicated the potential of DE as an aphrodisiac. However, more studies of the effect of DE on the level of testosterone and neurotransmitters especially dopamine and norepinephrine during mating performance, the level of cGMP or PDE5 in the penis tissue, penile blood flow, and an intracavernous pressure are needed.

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Appendix

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Thieme

Phosphodiesterase 5 Inhibitors from *Derris scandens*

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ABSTRACT

Phosphodiesterase 5 inhibitors have been used as a first-line medicine for the treatment of erectile dysfunction. In the search for new phosphodiesterase 5 inhibitors from natural sources, we found that the 95% ethanol extract of *Derris scandens* stem showed phosphodiesterase 5 inhibitory activity with an IC_{50} value of about 7 µg/mL. Seven isoflavones and a coumarin constituent isolated from this plant were investigated for phosphodiesterase 5 inhibitory activity. The results showed that osajin (8), 4′,5,7-trihydroxybiprenylisoflavone (4), and derrisisoflavone A (2) had the ability to inhibit phosphodiesterase 5 with IC_{50} values of 4, 8, and 9 µM, respectively. These compounds exhibited selectivity on phosphodiesterase 5 over phosphodiesterase 1, however, the selectivity on phosphodiesterase 5 over phosphodiesterase 6 was low. In order to quantitatively determine these bioactive constituents in *D. scandens* extract, LC-QTOF-MS method has been developed and validated. The limit of quantitation values in the range of 0.1–5 µg/mL were obtained. The assay showed satisfactory precision and accuracy. The results from our method showed that the 95% ethanol extract of *D. scandens* stem was comprised of all eight compounds, with derrisisoflavone A (2) and lupalbigenin (3) presenting as the major constituents.

Introduction

PDE5 is the predominant cGMP degrading enzyme in platelets, the penile corpus cavernosum, smooth muscle cells, and all vascular smooth muscle cells, mainly in the pulmonary vessel [1,2]. cGMP, the second messenger, plays a role in the regulation of penile erection through the relaxation of smooth muscle cells in the penile corpus cavernosum [3–5]. To date, four PDE5 inhibitors were approved for ED treatment by the FDA and are available on the market, including sildenafil, vardenafil, tadalafil, and avanafil. Sildenafil was granted FDA approval for treating pulmonary artery hypertension [6] and tadalafil was approved for treating benign prostatic hyperplasia [7]. The selectivity of the PDE5 inhibitor drug is of concern as some side effects have presented in patients who take nonselective PDE5 inhibitor drugs. Nonselectivity inhibition of the PDE5 inhibitor can cause visual abnormalities from the inhibition of PDE6, tachycardia, vasodilation, and flushing from the inhibition of PDE1. Moreover, other adverse effects were also reported including headache, dyspepsia, nasal congestion, and nasopharyngitis [8–10]. Therefore, the search for a new selective PDE5 inhibitor drug is still ongoing, especially from natural sources.

ABBREVIATIONS

cGMP	guanosine 3',5'-cyclic monophosphate
DEAE	diethylaminoethyl
ED	erectile dysfunction
EGTA	ethylene glycol tetraacetic acid
EIC	extracted ion chromatograms
FDA	U.S. Federal Drug Administration
IBMX	3-isobutyl-1-methylxanthine
ICH	International Conference of Harmonisation
LOD	limit of detection
LOQ	limit of quantitation
PDE 1/5/6	phosphodiesterase 1/5/6
SAR	structure-activity relationship

Derris scandens (Roxb.) Benth. is a woody vine that belongs to the Fabaceae family. It has been used in Thai traditional medicine for a long time as a muscle pain reliever, diuretic, antidysentery, expectorant, antitussive, and rejuvenating agent [11, 12]. The powder and hydroalcoholic extract of *D. scandens* stem were recorded in the National List of Essential Medicines of Thailand for the treatment of muscle pain, low back pain, and knee osteoarthritis [13]. The chemical constituents are benzil derivatives (derrisdione and scandione) [14, 15], pterocarpens (flemichapparin B–C and maackian) [14], coumarins (scandenin, scandenin A–B, and robustic acid) [15–17], steroids (β-sitosterol and β-sitosterol glucopyranoside) [17], isoflavones (derrisisoflavones A–F, erysenegalensein E, genistein, lupalbigenin, lupinisol A, osajin, scandenone, scanderone, scandinone, 4',5,7-trihydroxybiprenylisoflavone) [14–16, 18], and isoflavone glycosides (derriscandenosied A–E and genistein 7-O-[α-rhamnopyranosyl-(1 → 6)]-β-glucopyranoside) [19, 20]. Several scientific studies indicated that scandenin, genistein, genistein 7-O-[α-rhamnopyranosyl-(1 → 6)]-β-glucopyranoside, and lupalbigenin have an anti-inflammatory effect by inhibition of eicosanoid synthesis [20]. Derrisisoflavones A–F, erysenegalensein E, lupinisoflavone G, lupinisol A, scandenin, and 4',5,7-trihydroxybiprenylisoflavone have an antidermatophyte effect against *Trichophyton mentagrophytes* [18]. Scandenin, scandenin A–B, scandinone, and lupalbigenin have an antioxidant effect [16, 20], while chandalone, sehtal, scanderone, flemichapparin B, and maackian have a vasorelaxant effect from the increasing of mean arterial pressure [20]. Clinical studies have indicated the ability of the hydroalcoholic extract of the *D. scandens* stem to reduce pain in knee osteoarthritis [21] and lower back pain patients [22]. However, no scientific studies about the potential of *D. scandens* for treating ED or the inhibition of PDE have been reported.

Isoflavones, a subgroup of flavonoids that are predominantly found in soybeans and other legumes [23], have been reported for PDE5 activities [24, 25]. In the present work, we determined the PDE1, 5, and 6 inhibitory activities of *D. scandens* extracts as well as isoflavones and coumarin isolated from this plant. The selectivity and SARs of the compounds are discussed. To study the content of these compounds in *D. scandens*, an LC-QTOF-MS method was developed and validated.

Results and Discussion

The leaves and stems of *D. scandens* were extracted using 95% ethanol, 50% ethanol, and water. The extracts of *D. scandens* were determined for their PDE1, 5, and 6 inhibitory activities using IBMX, sildenafil, and dipyridamole as positive controls. The results are shown in ▸ **Table 1** (for dose-response curves, see Figs. 1S–3S, Supporting Information). The extract with the highest potency on PDE5 inhibition was the 95% ethanol stem extract, with an IC_{50} value of about 7 µg/mL. It also showed high PDE6 inhibition, with an IC_{50} value of about 15 µg/mL. However, the extract showed low PDE1 inhibition, with a percent inhibition value of about 59% at the final concentration of 50 µg/mL.

The PDE inhibitory activities of the coumarin and isoflavone constituents of *D. scandens* (1–8) (▸ **Fig. 1**) and the standard PDE inhibitors are shown in ▸ **Table 2** (for dose-response curves, see Figs. 1S–3S, Supporting Information). Amongst the tested compounds, derrisisoflavone A (2), 4',5,7-trihydroxybiprenylisoflavone (4), and osajin (8) exhibited a high PDE5 inhibitory effect, with IC_{50} values of about 9, 8, and 4 µM, respectively. Lupalbigenin (3) displayed weaker inhibition with an IC_{50} value of about 16 µM, whereas genistein (1), scandenin (5), scandinone (6), and scanderone (7) did not show obvious inhibition of PDE5. Compounds 2 and 8 also inhibited PDE6, with IC_{50} values in the micromolar range, while compound 4 inhibited PDE6, with an IC_{50} value of about 14 µM. As for PDE1, all tested compounds, except compounds 2 and 4, showed an IC_{50} of more than 100 µM. Most of the isoflavones in this study were reported for inhibitory activity on PDE1, 5, and 6 for the first time. Only 1 has been reported to show a moderate inhibition effect on PDE1 and 5 [26], and 8 has been reported to show PDE-5A inhibition using a microplate non-radioactive assay based on the cleavage of Fluor-labelled cGMP [25].

The structures of the tested compounds in this study can be divided into three groups: isoflavone analogues (1–4), cyclized isoflavone analogues (isoflavones with the extra ring at C-7 and C-8, 6–8), and a cyclized coumarin structure (coumarins with the extra ring at C-7 and C-8, 5). Among the isoflavones (1–4), the importance of prenyl substitution on PDE5 inhibitory activity was observed (▸ **Table 2**). The unprenyl-substituted molecule (1) completely loses its PDE5 inhibitory activity. This result was in agreement with the study of Dell'Agli et al. and Shin et al., who reported that prenyl groups of phenolic compounds were important for PDE5 inhibition [27, 28]. The position of prenyl substitution might also affect PDE5 inhibitory activity. Compounds 2 and 4, with prenyl groups at C-6 and C-8, showed higher inhibitory activity against PDE5 about 2-folds more than compound 3, which has a prenyl at C-6 and C-3'. Similar results for PDE1 and PDE6 inhibitory activities were found. The demethylation of 2 to provide 4 showed no effect on PDE5 and PDE6 inhibitions, but slightly increased PDE1 inhibition. For the cyclization of isoflavones in 6–8, the extra ring might result from the intramolecular interaction between the hydroxyl group at position-7 and the double bond of prenyl at position-8. The cyclization analogues were likely to add rigidity to the molecules. The substitution of the prenyl group at C-6 in 8 showed higher inhibition on PDE5 over C-3' in compound 7. This confirmed the preference of more lipophilic or steric

▶ **Table 1** Percentage of PDE5 inhibition at the final concentration of 50 µg/mL and the IC_{50} values against PDE5 and 6 of leaf and stem extracts of *D. scandens*.

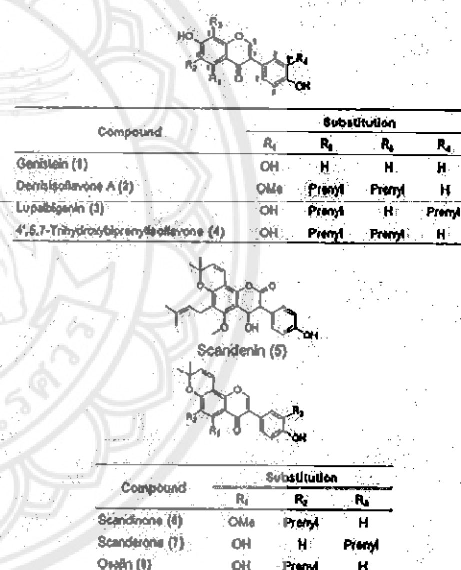
Extract	% PDE5 inhibition (at the concentration of 50 µg/mL)	IC_{50} (µg/mL) PDE5	PDE6	Selectivity ratio PDE6/PDE5
Leaf				
95% Ethanol extract	74.29 ± 3.80	11.43 (9.06–14.41)	ND	ND
50% Ethanol extract	53.51 ± 3.80	ND	ND	ND
Water extract	0.38 ± 7.10	ND	ND	ND
Stem				
95% Ethanol extract	82.98 ± 0.85	7.33 (5.95–9.02)	15.01 (12.11–18.61)	2.05
50% Ethanol extract	71.12 ± 4.37	22.61 (18.06–28.32)	ND	ND
Water extract	0.67 ± 5.73	ND	ND	ND
Sildenafil	ND	0.001 (0.0009–0.0018)	0.0062 (0.0056–0.0068)	6.2
Dipyridamole	ND	0.44 (0.43–0.45)	0.53 (0.48–0.58)	1.20

% PDE5 inhibition is expressed as the mean ± SD, and IC_{50} values against PDE5 and 6 are presented as the mean with the 95% confidence interval inside parentheses (n = 3). Sildenafil and dipyridamole were used as positive controls. ND: Not determined

groups on the benzene site. Interestingly, the different effect of the methoxy substitution at C-5 was found in 6, which might be due to the restrict conformation of cyclized isoflavones. The addition of larger groups at C-5 in 6 was not preferred as it might increase the steric property. Changing the isoflavone structure of 6 to the bioisostere coumarin ring (5) might cause the difference in conformation of the molecule, and the activity of 5 on PDE5 inhibition was slightly improved.

None of the isolated compounds from *D. scandens* gave a higher PDE5 inhibitory activity and selectivity against PDE1 or PDE6 over the positive controls (sildenafil and dipyridamole). However, compounds 2, 4, and 8 showed higher activity on PDE5 and 6 inhibition more than IBMX. Compound 8 showed the highest activity on PDE5 among the tested compounds, and the selectivity ratio on PDE1 was more than 24, although it gave the lowest selectivity ratio for PDE6 (0.32). The results obtained from this study could be useful for the future modification of the active compounds to improve the selectivity and potency of PDE5 inhibitors.

The LC-QTOF-MS method for quantitative determination of the seven isoflavones and a coumarin in *D. scandens* was developed and validated according to ICH guidelines. The separation of the eight compounds was done on a C18(2) column with a gradient elution of water and acetonitrile, both acidified with 0.1% (v/v) formic acid. The tailing factor and resolution of each compound met the ICH guidelines. The identification of each of the reference standards was confirmed by the typical *m/z*, the mass spectra, and retention times using the EIC mode that specified only the selected *m/z*. The calibration equations, linearity, LOD, and LOQ values are presented in Table 1S, Supporting Information. For determination of the eight analytes, LOQ values in the range of 0.1–5 mg/mL were obtained. The coefficient determination (r^2) for the calibration curves of all of the compounds was more than 0.9990. The intraday and inter-day precision of all eight compounds were not more than 6. The analytical method developed for the quantitation of all eight compounds had good accuracy, with overall recovery in the range of 96–104 (%RSD ≤ 6.08). All data are shown in Table 2S, Supporting Information. A representative LC chromatogram of the standard compounds and 95% ethanol extract of *D. scandens* stem are shown in ▶ **Fig. 2**.



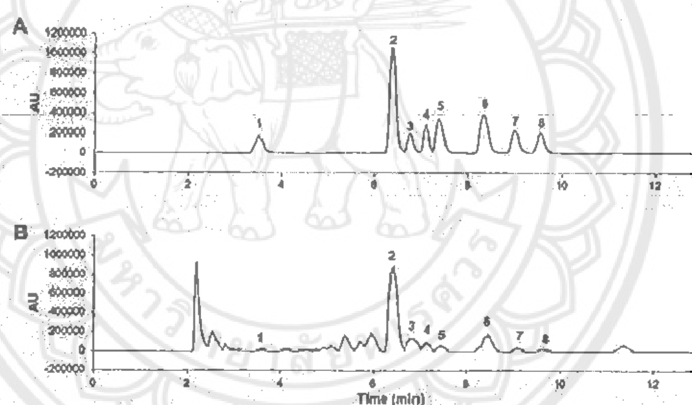
▶ **Fig. 1** Chemical structures of seven isoflavones and a coumarin in *D. scandens*.

The contents of seven isoflavones and a coumarin in *D. scandens* extracts and dried plant materials were investigated using our validated LC-QTOF-MS method. The results in ▶ **Table 3** show that these compounds are found mainly in the stems and are al-

► **Table 2** *Effect of compounds isolated from D. scandens on PDE 1, 5, and 6 inhibitory activities.*

Compound	IC_{50} (µM)			Selectivity ratio	
	PDE5	PDE1	PDE6	PDE1/PDE5	PDE6/PDE5
1	> 100	> 100	16.70 (13.61–20.69)	ND	ND
2	8.55 (7.25–10.08)	95.07 (90.92–99.41)	8.07 (6.84–9.52)	11.12	0.94
3	16.25 (14.28–18.42)	> 100	85.49 (80.83–90.43)	> 6.15	5.26
4	7.68 (6.55–9.01)	42.06 (41.26–42.88)	13.90 (13.48–14.35)	5.48	1.81
5	108.13 (104.76–111.61)	> 100	> 100	> 0.93	> 0.93
6	> 200	> 100	> 200	ND	ND
7	> 100	> 100	> 100	ND	ND
8	4.10 (3.06–5.49)	> 100	1.30 (0.84–2.02)	> 24.39	0.32
IBMX	12.64 (10.78–14.82)	7.15 (6.32–8.08)	10.15 (9.19–11.21)	0.57	0.80
Sildenafil	0.002 (0.001–0.004)	1.18 (1.02–1.35)	0.013 (0.012–0.014)	590	6.50
Dipyridamole	0.87 (0.85–089)	> 100	1.05 (0.97–1.14)	> 114.94	1.21

IC_{50} values are presented as the mean with the 95% confidence interval inside parentheses (n = 3). IBMX (3-isobutyl-1-methylxanthine), sildenafil, and dipyridamole were used as positive controls. ND: Not determined



► **Fig. 2** Representative LC chromatograms of (A) eight standard compounds (1–8; 25 µg/mL) isolated from *D. scandens* and (B) the 95% ethanol extract of *D. scandens* stem (20 mg/mL). The peaks were from the following compounds: 1 = genistein, 2 = derrisisoflavone A, 3 = lupalbigenin, 4 = 4',5,7-trihydroxybiprenylisoflavone, 5 = scandenin, 6 = scandinone, 7 = scanderone, and 8 = osajin.

most absent in the leaves. The 95% ethanol extract of stems contained the highest amount of the compounds tested compared to the extracts from other solvents (► **Table 1**). It was correlated to the fact that the highest PDE5 inhibitory activity was found in the 95% ethanol extract (► **Table 3**). The activity might mainly be contributed from 2, 3, and 4, which were found to be the major components in the 95% ethanol extract of stems. On the other hand, none of the compounds tested were found in the aqueous extracts, corresponding to no PDE5 inhibitory activity belonging to these extracts. The results suggest that 95% ethanol should be used to prepare *D. scandens* stem extract to achieve high PDE5 inhibitory activity.

In conclusion, these studies report on the PDE5 inhibitory activity of *D. scandens* extracts and isoflavones PDE5 inhibitors found in *D. scandens* for the first time. The potent compounds on PDE5 are 2, 4, and 8 of which 2 and 4 were present as the major compounds in the extract. Another major compound, 3, showed moderate activity on PDE5 with the highest selectivity on PDE5 over PDE6 compared with other isoflavones studied. Some SARs have been observed, such as the importance of prenyl substitution on PDE5 inhibitory activity. LC-QTOF-MS was successfully used for the quantitative analysis of these compounds in the plant materials and the extracts of *D. scandens*.

► **Table 3** Contents of seven isoflavones and a coumarin in *D. scandens* extracts and dried plant materials expressed in milligrams per gram.

Compound	Leaf extracts			Stem extracts			Dried plant materials	
	95% Ethanol extract (n = 9)	50% Ethanol extract (n = 9)	Water extract (n = 9)	95% Ethanol extract (n = 9)	50% Ethanol extract (n = 9)	Water extract (n = 9)	Leaves (n = 3)	Stems (n = 3)
1	ND	ND	ND	0.5 ± 0.01	0.19 ± 0.02	ND	ND	0.2 ± 0.00
2	<LOD	ND	<LOD	30.3 ± 1.01	2.88 ± 0.31	<LOQ	<LOD	1.5 ± 0.01
3	5.3 ± 2.59	<LOD	ND	34.9 ± 1.12	2.71 ± 0.06	ND	0.1 ± 0.00	1.5 ± 0.03
4	ND	<LOD	ND	13.4 ± 0.45	4.49 ± 0.04	ND	ND	0.6 ± 0.03
5	ND	ND	ND	3.9 ± 0.06	<LOD	ND	ND	<LOQ
6	ND	ND	ND	1.0 ± 0.21	<LOQ	<LOD	ND	<LOQ
7	ND	ND	ND	4.1 ± 0.04	<LOD	ND	ND	0.3 ± 0.02
8	ND	ND	ND	6.6 ± 0.17	<LOD	ND	ND	0.3 ± 0.00

The results are expressed as the mean ± SD. ND: No detection

Materials and Methods

Chemicals and standards

Acetonitrile, water, and methanol were of LC-MS grade and purchased from RCI Labscan. Formic acid was of analytical grade and purchased from Merck. cGMP, crude snake venom (*Crotalus atrox*), calmodulin from bovine heart, histone, BSA, DEAE sephadex, dipyridamole (purity > 98%), IBMX (purity > 99%), EDTA, EGTA, imidazole, magnesium chloride ($MgCl_2$), crude phosphodiesterase 3′,5′-cyclic nucleotide (PDE1) from bovine heart, and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich. Sildenafil citrate (purity > 98%) was purchased from European Directorate for the Quality of Medicines & HealthCare (EDQM) – Council of Europe. [^{3}H]cGMP was purchased from Perkin Elmer.

Compound **1** (genistein; purity > 98%) was purchased from Apex biotechnology. Other isoflavones (**2**–**4**, **6**–**8**) and a coumarin (**5**) isolated from *D. scandens* were provided as gifts from Prof. Dr. Apichart Suksamrarn, Faculty of Sciences, Ramkhamhaeng University, Thailand. The purities of these compounds were more than 95% as determined by NMR and LC-QTOF-MS.

Plant material

Leaves and stems of *D. scandens* were collected from locations in Phitsanulok Province, Thailand, in April 2016, and identified by Dr. Pranee Nangngam from the Department of Biology in the Faculty of Science, Naresuan University, Thailand. A voucher specimen with the catalogue No. 004331 is deposited at the PNU Herbarium in the Faculty of Science, Naresuan University.

Plant extraction

The collected leaves and stems were dried in a hot air oven at 55 °C for 3 days, and were then pulverized into a powder with a grinder prior to extraction.

The aqueous extracts were prepared by infusing the powdered materials (10 g) in hot water at 80 °C (100 mL) for 15 min. After filtration, the water was then sublimated in a freeze dryer to obtain 0.91 and 0.89 g aqueous extract of leaves and stems, respectively.

The 50% ethanol extracts were obtained by maceration of the powdered materials (10 g) in 50% ethanol (100 mL) for 24 h at room temperature under shaking at 300–350 rpm. After filtration, the filtrate was then evaporated under reduced pressure at 30–35 °C to remove the ethanol. The solution without ethanol was then sublimated in a freeze dryer to obtain 1.37 g of the 50% ethanol leaf extract and 1.08 g of the 50% ethanol stem extract.

The 95% ethanol extracts were prepared by maceration of the powdered materials (10 g) in 95% ethanol (100 mL) for 24 h at room temperature under shaking at 300–350 rpm. After filtration, the filtrate was then concentrated under vacuum at 30–35 °C until dryness and the dried extracts were obtained (1.01 and 0.28 g for 95% ethanol extract of leave and stems, respectively). The extracts were stored at − 20 °C awaiting further study.

LC-QTOF-MS analysis

Preparation of sample and standards solution

The powder of dried plant materials and extracts were weighed and dissolved with methanol at concentrations of 10–20 and 2–20 mg/mL, respectively. The solutions were then sonicated for 15 min and filtered through nylon syringe filters with a 0.45-µm pore size. The analysis was performed in triplicate.

The stock solution of each standard compound was freshly prepared by dissolving it in methanol to obtain the concentration of 1 mg/mL. These solutions (**1**–**8**) were further mixed and diluted with methanol to make standard calibration curves, LOD, and LOQ for the eight compounds.

Instruments and chromatographic conditions

LC-QTOF-MS analysis used an Agilent 1260 Infinity Series HPLC System coupled to an Agilent-6540 QTOF mass spectrometer equipped with an electrospray interface. The LC-QTOF-MS systems consisted of a binary pump, an online degasser, an autosampler, and a column thermostat. Chromatographic separations were performed on a Luna C18(2) LC column (4.6 mm × 150 mm)

with a particle size of 100 Å 5 µm (Phenomenex) and the temperature was maintained at 35 °C. Chromatographic conditions included the mobile phase, which was a mixture of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B), with a flow rate of 0.5 mL/min. The gradient elution was established as follows: 0–3 min, 25:75, (A:B v/v) and 3–13 min, 5:95. Post-time was 5 min for re-equilibration of the system prior to the next injection. The injection volume was 5 µL. The MS system was operated in the positive electrospray ionization mode with the following conditions: gas temperature 350 °C, drying gas 10 L/min, nebulizer 30 psig, capillary voltage 3500 V, fragmentor 100 V, skimmer 65 V, OCT 1RF Vpp 750 V. All acquisitions and analysis of the data were analyzed using MassHunter software (Agilent Technologies).

Method validation

The method was validated according to ICH guidelines by determining linearity, selectivity, LOD, LOQ, precision, and accuracy. The linearity range of the standards was determined on five concentration levels (5–25 µg/mL). Calibration curves were measured on every analysis day and each sample was determined in triplicate. The linearity of the calibration curve was assessed by calculating the coefficient of determination (r^2). LOD and LOQ under the present chromatographic conditions were determined by injecting the standard solutions until a signal-to-noise ratio of each compound was 3 for LOD and 10 for LOQ. The selectivity was confirmed from the typical *m/z*, mass spectra, and retention times of each reference standard. The intraday precision of the method was evaluated by repeating the measurements of three concentration levels (7.5, 12.5, and 22 µg/mL) of the eight analytes 5 times within 1 day. This procedure was repeated on 2 more days for determining the inter-day precision (n = 3, each level). Precision is expressed as %RSD calculated from the standard deviation/mean × 100. The accuracy and recovery were determined by spiking the known concentration of the mixed standard solution to 95% ethanol stem extract to obtain three different concentrations (7.5, 12.5, and 22 µg/mL) of each analyte. These experiments were done in triplicate. The accuracy is expressed as % recovery, which was calculated from 100× (measured analyte concentration – analyte concentration in the non-spiked extract/analyte concentration spiked.

Measurement of phosphodiesterase inhibitory activity

Enzyme preparation

PDE1, which is the crude PDE isolated from bovine heart, was purchased from Sigma-Aldrich. PDE5 was extracted from fresh rat lung tissue. Briefly, the rat lung tissue was minced and homogenized in 2 mL of buffer PP (150 mM Tris, pH 7.5, 6 mM EDTA, 3 mM DTT, and 1:100 of 100 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 14000 rpm for 15 min at 4 °C, and the supernatant was used as a source of PDE5. PDE6 was extracted from chicken retinas. The preparation method of PDE6 was described in the report of Huang et al. [24]. All experiments using the animal tissues were approved and performed in accordance with the guidelines provided by the Naresuan University Animal Care and Use Committee (NUACUC) (No. 6002003, approval date: January 25, 2017).

Phosphodiesterase assay

The PDE1 and 5 inhibitory activity measurements were modified from the report of Sonnenburg et al. [29], whereas the PDE6 assay was modified from the method of Huang and colleagues [24]. The assay consisted of 25 µL of 10 mM EGTA, 25 µL of buffer C [100 mM Tris-HCl (pH 7.5), 100 mM imidazole, 15 mM $MgCl_2$, and 1.0 mg/mL BSA], 25 µL of the PDE enzyme, 25 µL of the test sample or only solvent as a blank. Then a substrate, 25 µL of 1 µM [^{3}H] cGMP, was added and the solution was incubated at 30 °C for 10 min. The reaction was stopped by placing the tube in boiling water for 1 min and then cooled. In the next step, 25 µL of 2.5 mg/mL of snake venom were added and the solution was incubated at 30 °C for 5 min. The assay was diluted with 250 µL of low salt buffer (20 mM Tris-HCl, pH 6.8) and transferred to a DEAE ion exchange resin column. The [^{3}H] guanosine was eluted from the resin with 500 µL of low salt buffer, four times, and the eluates were collected in a scintillation vial. Four mL of the scintillant cocktail were added to the vial and mixed completely. The radioactivity of the cocktail was measured by using a liquid scintillation counter (Tri-Carb 2910 TR; Perkin Elmer). In the case of the PDE1 inhibitory activity assay, EDTA was used instead of EGTA, and 4 mM $CaCl_2$ and 16 µg/mL calmodulin were added to buffer C, while 2.5 mg/mL of histone were added for PDE6 assay. The PDE enzymes in the study were standardized to have a hydrolysis activity of 20–30% of the total substrate counts. The calculation of hydrolysis is shown in Eqs. 1 and 2 and the PDE inhibitory activity is calculated from Eq. 3.

$$\%\ \text{hydrolysis of sample} = \left[\frac{(CPM_{sample} - CPM_{blank})}{(CPM_{total\ count} - CPM_{blank})}\right] \times 100 \quad (1)$$

$$\%\ \text{hydrolysis of control} = \left[\frac{(CPM_{control} - CPM_{blank})}{(CPM_{total\ count} - CPM_{blank})}\right] \times 100 \quad (2)$$

$$\%\ \text{PDE inhibition} = \left[1 - \left(\frac{\%\ \text{hydrolysis of sample}}{\%\ \text{hydrolysis of control}}\right)\right] \times 100 \quad (3)$$

Where CPM_{sample} is the radioactive count rate of the assay with enzyme, $CPM_{control}$ is the radioactive count rate of the assay with enzyme but without sample, and CPM_{blank} is the same but without enzyme. $CPM_{total\ count}$ is the count rate of 25 µL of substrate plus 2 mL of low-salt buffer. IBMX, sildenafil, and dipyridamole were used as positive controls for PDE1, PDE5, and PDE6 inhibitory activity, respectively.

IC_{50} values were calculated from the nonlinear regression of the plot of values of percent inhibition versus log concentration of the sample using GraphPad Prism 5 software. IC_{50} values are shown as the mean with 95% confidence interval. All experiments were performed in triplicate.

Supporting information

The dose-response curves of the active compounds and information from the LC-QTOF-MS method validation are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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