CHAPTER I

INTRODUCTION

The rationale for the study

According to the great leap forward of medical sciences, the average human life span of people is increasing. Consequently, health problems from degenerative diseases in elderly people are highly found. The sexual inability such as erectile dysfunction (ED) is one of the most common degenerative symptoms. ED is the condition which one cannot achieve or sustain an erection for satisfactory sexual performance [1-5]. The consequences of ED are losing self-esteem which may cause social problem. At the present, almost 50% of the men over 40s are suffering from ED [2]. In Thailand, the prevalence of ED in men aged between 40 and 70 years increased from 37.50% in 2000 to 42.18% in 2008 [6]. Moreover, it is predicted that over 300 million men around the world will have this condition in 2025 [7]. One of pathologies of ED is related to cyclic guanosine monophosphate (cGMP) level which has several profound effects in human body. In penile tissue, cGMP plays a crucial role for regulation of penile erection. It is produced by converting of guanosine triphosphate (GTP) via stimulating soluble guanylate cyclase (sGC) by nitric oxide (NO). The cGMP affects to calcium (Ca^{2+}) ion channel permeability. Owing to the increasing level of cGMP, the Ca^{2+} influx across the cell membrane is diminished resulting in the reduced smooth muscle tone. Eventually, cavernous smooth muscle relaxes and blood flow to penis increases resulting in penile erection. The penis will be flaccid after cGMP is decreased by phosphodiesterase 5 (PDE5) which abundantly localizes in cavernosal tissue [8]. In the case of presence of PDE5 inhibitors (PDE5-Is) such as sildenafil (Viagra®) (**Figure 1**) thus, the erection is retained. After sildenafil was launched to market in 1998, it rapidly achieved success as drug for ED treatment [9-11]. However, sildenafil has several adverse effects such as headache (16%), flushing (10%), dyspepsia (7%), nasal congestion (4%), urinary tract infection (3%), abnormal vision (3%), diarrhoea (3%), dizziness (2%)[12,13]. The visual side effect of sildenafil may relate to inhibition of PDE6 in retinas because the structures of both PDE5 and PDE6 are similar [12-14]. The sildenafil's successful

story intensely brought the scientists to seek for the new PDE5 inhibitors from both synthetic and natural sources. Their goal is to find the selective PDE5 inhibitors with improved therapeutic benefits/undesirable side effect ratios.

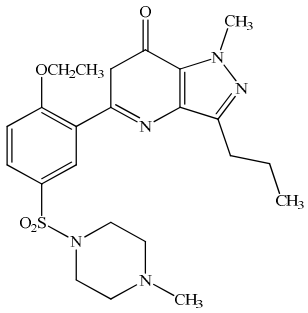


Figure 1 Sildenafil (Viagra®), a PDE5 inhibitor

The main purpose of this study is to investigate for PDE5 inhibitors either from natural products or compounds derived from natural products.

Our first attempt was using ethnopharmacology approach for the investigation. In spite of the increasing availability of effective medical treatments, the alternative medicines mainly herbal remedies claimed for improving sexual performance have been popularly used among Thai men. Many medicinal plants such as *Betula alnoides* Buch.-Ham. ex G.Don, *Butea superba* Roxb., *Elephantopus scaber* L. and *Kaempferia parviflora* Wall. ex. Baker, L. have been traditionally claimed for enhance male sexual performance (aphrodisiac agent). Since cyclic nucleotide PDEs inhibitors underlie several current treatments for erectile dysfunction, we sought to show whether these plants contain PDE5 inhibitory activity. In the previous study, we found that some of the plant extracts could indeed inhibit a mixture of PDEs of which a major component was PDE1 [15]. This discovery had profoundly supported our hypothesis on PDE5 inhibitory activity from these plants. In this study, Thai aphrodisiac plants were investigated on PDE5 inhibitory activity. Besides, some plants from Leguminosae were also explored on this activity because this family contained flavonoids which are known to inhibit PDE5 [16,17]. Moreover, we randomized some plants in Zingerberaceae and Orchidaceae family for on PDE5 inhibitory activity screening.

K. parviflora, is one of those herbs which has been widely used as male sexual enhancer for a long time [18]. There are a few scientific data to support its aphrodisiac activity in males. The ethanolic rhizhome extract of this plant was reported to improve endothelial function by nitric oxide production activation which is related to erection circumstance [19,20]. In recent year, the *in vivo* study showed that alcoholic extract of *K. parviflora* could increase blood flow to the testis [21]. Besides activation of NO production, PDE5 inhibitory activity is the one of the possible mechanisms of penile erection. For this reason, we were interested in methoxyflavone derivatives (**Figure 2**), the major constituents in this plant [18,19,22-26], for PDE5 inhibition. Moreover, their selectivity over PDE6 was evaluated.

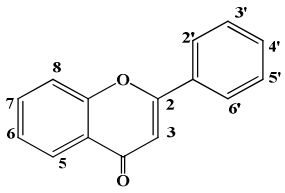


Figure 2 Flavone skeleton

From our random screening, *Eulophia macrobulbon* (Parish & Rchb. f.) Hook. f. belonging to Orchidaceae family, showed PDE5 inhibitory activity. The chemical constituents of this plant have not been report yet. From the study of related species i.e. *Eulophia nuda* Lindl [27-31], *Eulophia petersii* Rchb. f. [32] and *Eulophia ochreata* Lindl [33], phenanthrene compounds (**Figure 3**) were reported as common constituents in this genus. This group of compounds might be responsible for PDE5 inhibitory activity.

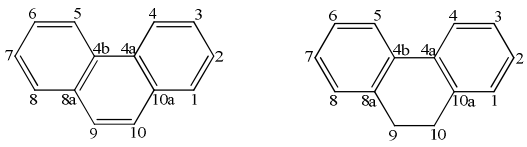


Figure 3 Phenanthrene skeleton

Natural substances can be used as significant templates to synthesis the promising compounds for treatment of various diseases. The isoquinoline alkaloids i.e. papaverine (**Figure 4**) which was isolated from *Papaver somniferum* L., was reported as non selective PDE inhibitors [34]. Papaverine was also used for ED treatment but it showed frequent side effects due to poor selectivity among PDE isozymes. The compound was usually administered by direct penile injection to reduce the side effects which found from oral papaverine such as polymorphic ventricular tachycardia. However, up to 50% of men eventually discontinue the treatment because of needle phobia and tissue damage at the area of injection [35]. The mechanism of penile injected papaverine might associated with PDE5 which is plentiful in this tissue [8]. So that isoquinolines might be the structure which plays the important role as PDE5 inhibitors. In this study, some isoquinoline derivatives, i.e. tetrahydroisquinolines and dihydroisoquinolines, were screened for PDE inhibitory activity to investigate for the new PDE5 inhibitors.

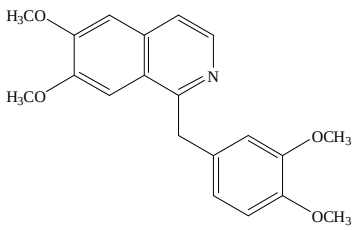


Figure 4 Papaverine

The objectives of this study

1. To screen the PDE5 inhibitory activities from some Thai medicinal plants
2. To explore the structure activity relationship (SAR) of 7-methoxyflavones isolated from *K. parviflora* on PDE5 and PDE6 inhibitory activities
3. To isolate and identify PDE5 inhibitors from the *E. macrobulbon*
4. To determine the selectivity on PDE5 over PDE6 of compounds isolated from *E. macrobulbon*
5. To study the relaxant effect on rat pulmonary artery from *E. macrobulbon* extract
6. To investigate some isoquinoline derivatives on PDE5 inhibitory activity

The expected outputs and outcomes

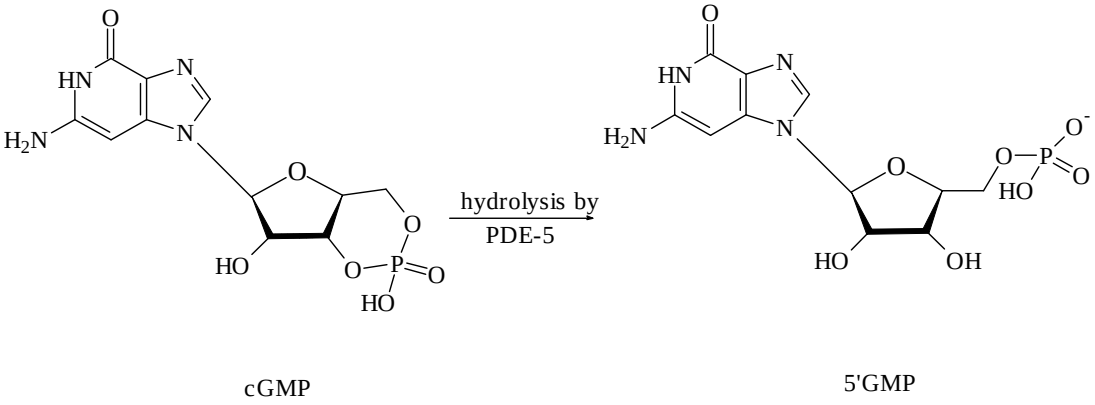
1. The scientific data to support the efficacy of *K. parviflora* for improving the male sexual performance via PDE5 inhibitory effect
2. The PDE5 inhibitors from *E. macrobulbon*
3. The SAR of isoquinoline derivatives on PDE5 inhibitory activity

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Cyclic nucleotide phosphodiesterases (PDEs)

PDEs are a group of enzymes that have powerful effect on cellular signal because they regulate the intracellular second messengers, cAMP and/or cGMP. These enzymes urge the breakdown either of cAMP or cGMP to their biologically inactive 5′ derivatives (**Figure 5**)



cGMP 5'GMP

Figure 5 The hydrolysis reaction of cGMP by PDE5

The present, PDEs are classified to 11 isozymes (PDE1 to PDE11) on the basis of different specificity and sensitivity to endogenous and exogenous substances. By substrate specificity, PDEs can be classified into 3 groups. cAMP specificity group includes PDE4, PDE7 and PDE8 while cGMP specificity group contains PDE5, PDE6 and PDE9. The rest PDE isozymes are specific for both cAMP and cGMP (**Figure 6**). [36-38]. PDEs can regulate various cellular functions since they are specifically distributed in many tissues (**Table 1**). Many physiological functions such as cardiac contractility, smooth muscle relaxation, platelet aggregation, visual response, fluid homeostasis and immune responses are controlled by PDEs. Therefore, inhibition of PDEs is designated as a new therapeutic target for many pathologies such as inflammation, cardiovascular, asthma, chronic obstructive pulmonary disease (COPD), pulmonary hypertension (PH) and erectile dysfunction (ED).

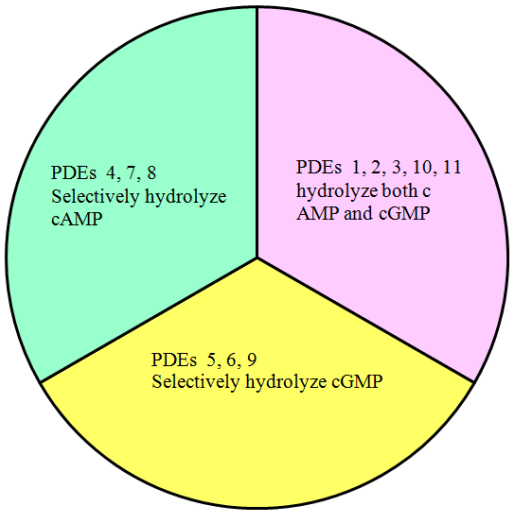


Figure 6 The three major types of mammalian PDEs classified according to their substrate selectivities

Table 1 Human cyclic nucleotide PDE families

Family	Characteristics [37,39]	Substate specificity [8]	Tissue distribution [8,37]	Target Disease [38]	Examples of inhibitors [37]
PDE 1	CaM-dependent	cAMP,cGMP (cGMP>cAMP)	Heart, Brain, Lung, Smooth muscle	Acute ischemic stroke Hypertension	Vinpocetine,
PDE 2	cGMP-stimulated	cAMP,cGMP	Adrenal cortex, Heart, Brain, Lung,Liver, Platelets	ND	EHNA
PDE 3	cGMP-inhibited (cAMP>cCGMP specific)	cAMP>cGMP	Heart, Lung, Liver, Platelets, Adipose tissue, Immunocytes	Intermittent claudication	Milrinone Cilostazol
PDE 4	cAMP-specific/ cGMP-insensitive	cAMP	Sertoli cells, Kidney, Brain, Liver, Lung, Immunocytes	Asthma COPD Allergic rhinitis	Rolipram, Cilomilast Roflumilast

Table 1(cont.)

Family	Characteristics [37,39]	Substate specificity [8]	Tissue distribution [8,37]	Target Disease [38]	Examples of inhibitors [37]
PDE 5	cGMP-specific	cGMP	CC, Lung, Platelets,	Erectile dysfunction PH	Sildenafil, Vardenafil Tadalafil
PDE 6	Photoreceptor	cGMP	Rod and cone photoreceptor	ND	Zaprinast, Dipyridamole
PDE 7	High affinity-cAMP-specific	cAMP	Skeletal muscle, T-cells, Heart, Kidney, Brain	ND	None
PDE 8	cAMP-specific	cAMP	Testis, liver, Thyroid	ND	Dipyridamole
PDE 9	cGMP-specific	cGMP	Kidney	ND	SCH-51866
PDE 10	cAMP,cGMP-specific	cAMP,cGMP	Testis, Brain	ND	Sildenafil, Zaprinast, Dipyridamole
PDE 11	cAMP,cGMP-specific	cAMP,cGMP	Skeletal muscle, Prostate	ND	Zaprinast, Dipyridamole

Note: ND means no data.

Phosphodiesterase 5 (PDE5)

PDE5 is a dimeric molecule and single subunit of which regulatory and catalytic domains are an N-terminal and a C-terminal regions, respectively. Each region shows the different role such as N-terminal region is a site for phosphorylation by cAMP and cGMP dependent protein kinase, GAF A and GAF B are involved in cGMP binding [40]. PDE5 is present in variety of tissues such as platelets, lung, vascular smooth muscle especially in vascular smooth muscle of corpus cavernosum (CC) which represents a target for erectile dysfunction. When PDE5 is inhibited, cGMP level is raised and causes smooth muscle relaxation in corpus cavanosum and then penile erection. Thus, PDE5 inhibitors can use in treatment for ED effectively. Beside predominant amount in CC, PDE5 is also easily found in lung. This evidence led to new indication of PDE5 inhibitors for treatment of pulmonary arterial hypertension [41].

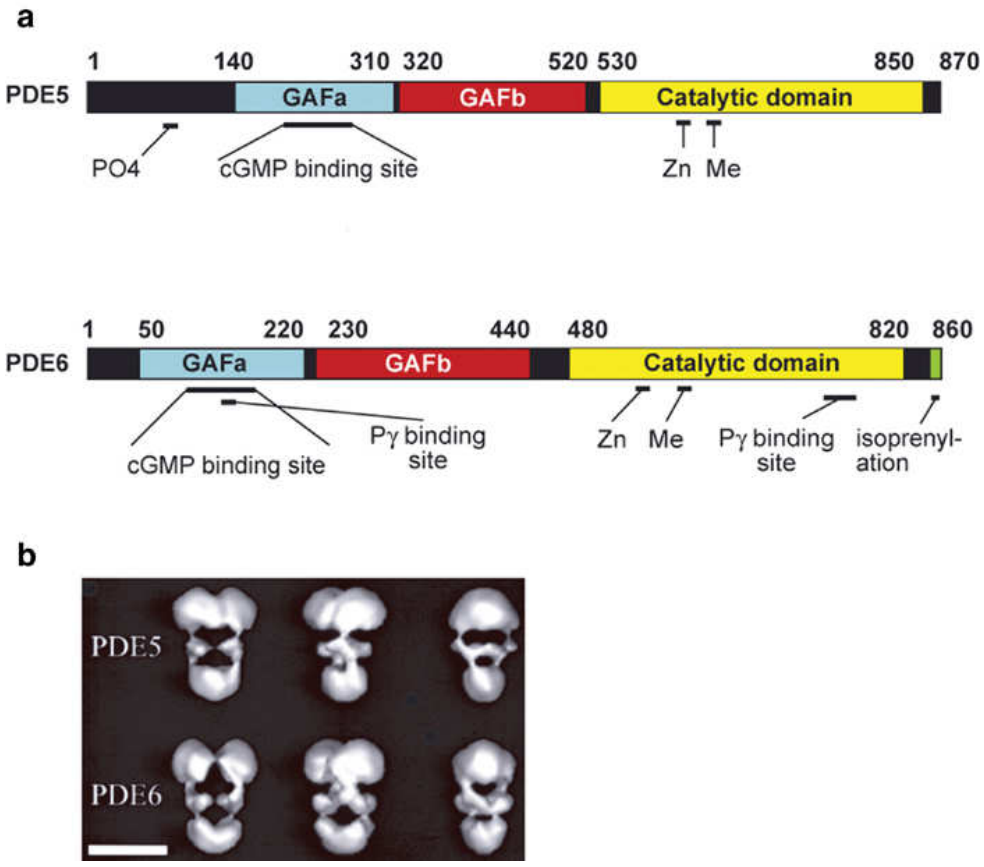
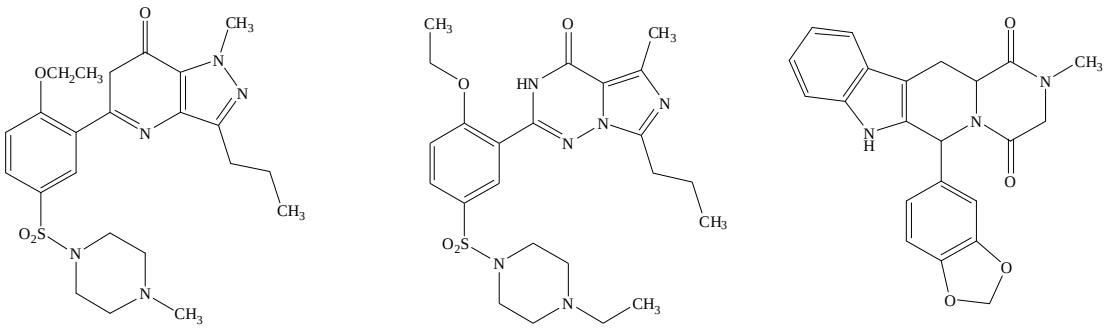


Figure 7 Similarities and differences between PDE5 and PDE6 [14]

PDE5 inhibitors

Sildenafil was firstly developed for treatment of hypertension. Due to erection side effect of sildenafil, the Pfizer's researchers decided to change study of sildenafil effect on trial as a drug for ED. In 1998, sildenafil, marketed as Viagra® was approved as a drug for ED treatment [39]. Soon after that, the second and third PDE-5 inhibitors, vardenafil (Levitra®) and tadalafil (Cialis®) were developed and launched to the market for the same indication [42]. Their structures show in **Figure 8**. However, PDE5-selective inhibitors (sildenafil and vardenafil except tadalafil) are most potent in inhibiting PDE6 according to structural and biochemical similarities of PDE5 and PDE6 (**Figure 7**) [14]. The result from PDE6 inhibition of both sildenafil and vardenafil is cyanopsia, usually described as blue vision. Up to 11% of patients who took sildenafil could difficultly distinguished between blue and green color [42]. For reduction of this undesirable effect, the new generation of PDE5 inhibitors should be more selectivity on PDE5 over PDE6.



Sildenafil Vardenafil Tadalafil

Figure 8 PDE5 inhibitors

Erectile dysfunction (ED)

Erectile dysfunction (ED) is defined as “the persistent inability to achieve or maintain an erection adequate for sexual intercourse” [2,42-44]. At the present, the incidence of ED is increasing. Nearly 50% of men over the age of 40 had ever experienced to ED. In the US, eighteen million men (18%) are expected to experience ED [3,45]. In Thailand, prevalence of ED in men aged between 40 and 70 years was 42.18% of which 17.50% is mild dysfunction group, 13.13% is moderate dysfunction group and 11.55% is severe dysfunction group [42-44]. In 2025, the occurrence of ED will be as 322 million men around the world [43]. This disorder is correlated with physical and psychological health. Beside aging, several factors (**Figure 9**) have been related to ED.

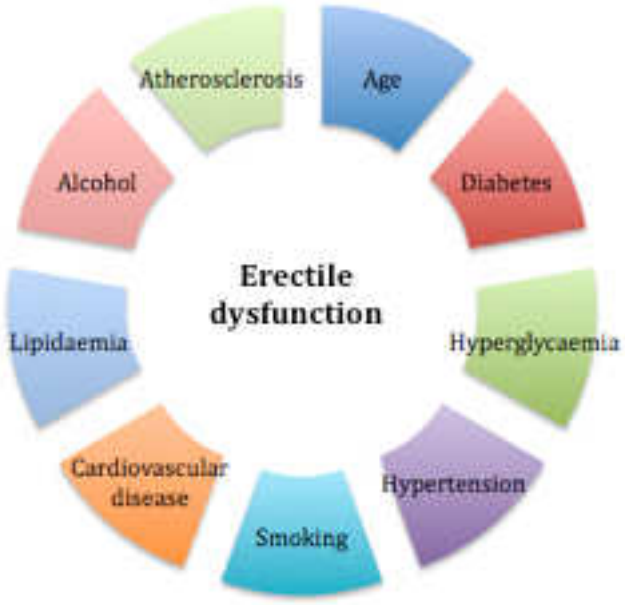


Figure 9 Factors that cause erectile dysfunction

The penile erection is the complex interaction of neuronal, vascular and myogenic events which are related by cholinergic and non-cholinergic factors such as nitric oxide (NO) [18]. During sexual stimulation, NO is released from the non-adrenergic, non-cholinergic cavernous nerve and endothelial cell and diffuses into the

smooth muscle of corpus cavernosum (CC). Then, it binds to soluble guanylate cyclase (sGC) enzyme which has role in changing nucleotide guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). The cGMP affects to calcium ion (Ca^{2+}) channel permeability. Owing to the increasing level of cGMP, the Ca^{2+} influx across the cell membrane is diminished resulting in the reduced smooth muscle tone. Eventually, cavernous smooth muscle relaxes and blood flow to penis increases resulting in penile erection. The penis will be flaccid after cGMP is decreased by phosphodiesterase 5 (PDE5) in cavernosal tissue. In the case of presence of PDE5 inhibitors such as sildenafil, thus the erection is retained. The mechanism of PDE5 inhibitors in penile tissue is shown in **Figure 10**.

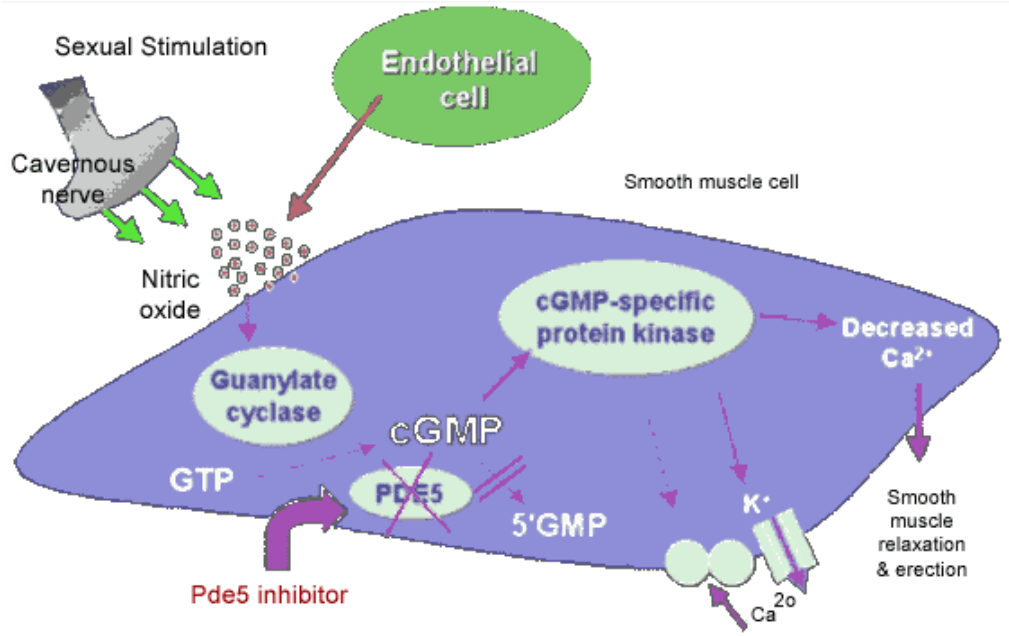


Figure 10 The mechanism of PDE5 inhibitors in corpus cavernosum

Pulmonary hypertension (PH)

Pulmonary hypertension (PH) begins when tiny arteries in your lungs, called pulmonary arteries, and capillaries become narrowed, blocked or destroyed. This makes it harder for blood to flow through your lungs, which raises pressure within the arteries in your lungs. As the pressure builds, your heart's lower right chamber (right ventricle) must work harder to pump blood through your lungs, eventually causing

your heart muscle to weaken and eventually fail completely. PH is a serious illness that becomes progressively worse and is sometimes fatal [46]. As PDE5 abundantly localized in lung, the new indication of PDE5 inhibitors is for PH treatment [41,46-49]. In recent, only sildenafil (Revatio®) and tadalafil (Adcirca®) has been approved by US FDA for treatment of PH.

Natural substances against PDEs activity

There are many phytochemical products which improve sexual dysfunction including ED in folk treatment, for example, icariin (flavonoids) [50], sophoflavescenol (flavonoids) [17], and forskolin (diterpenes) [51]. Additionally, some Leguminosae plants which contain several flavonoid compounds [52] have also been used as aphrodisiac agents. The study of cAMP PDE inhibitory activity from some Thai plants revealed that 3, 7,3′ trihydroxy-4′-methoxyflavone and 3, 3′ dihydroxy-4′-methoxyflavone-7-O-β-D-glucopyranoside isolated from the tuber root of *Butea superba* Roxb. showed *in vitro* cAMP PDE inhibitory activity [53]. Besides *B. superba* , several Thai herbs have been served for improving male sexual performance or aphrodisiac agents in traditional medicine. The one popular example of those plants is *Kaempferia parviflora* Wall. ex Baker therefore, it has been reviewed in pharmacological activity and its chemical constituent in next section. Ko and colleagues reported that many flavonoids had inhibitory effect against various PDE isozymes (**Table 2**). For example, Luteolin and genistein had non-selective PDE inhibitory effect whereas eriodictyol and hesperitin showed selectively inhibitory activity on PDE3 and PDE4, respectivelyIn addition, flavones i.e., diosmetin and luteolin express the inhibition of PDE5 [16].

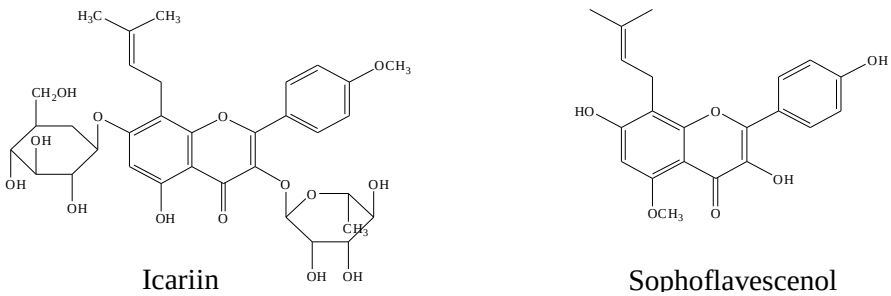


Figure 11 Natural substances against PDE5

Table 2 IC_{50} (μM) values of flavonoids on phosphodiesterase isozymes [16]

Class	Name	PDE isozyme				
		1	**2**	**3**	**4**	**5**
flavones	luteolin	21.5 ± 2.9 (3)	13.3 ± 0.8 (3)	10.1 ± 1.8 (5)	19.1 ± 2.4 (6)	19.3 ± 3.2 (4)
	luteolin-7-glucoside	>100 (3)	35.1 ± 0.2 (3)	>100 (3)	43.0 ± 5.3 (4)	>100 (3)
	diosmetin	14.4 ± 6.2 (3)	4.8 ± 0.8 (4)	>100 (3)	20.2 ± 2.4 (3)	15.3 ± 3.6 (3)
	apigenin	25.4 ± 3.7 (3)	16.7 ± 6.3 (5)	10.5 ± 3.5 (4)	>100 (3)	>100 (3)
	chrysin	>100 (3)	>100 (4)	>100 (3)	>100 (4)	>100 (4)
flavonols	quercetin	27.8 ± 5.7 (3)	17.9 ± 3.4 (4)	5.6 ± 1.0 (4)	9.9 ± 2.5 (3)	>100 (3)
	myricetin	24.9 ±3.6 (3)	12.8 ± 0.6 (4)	12.4 ± 3.3 (4)	39.8 ± 2.1 (6)	>100 (3)
flavanones	eriodictyol	>100 (3)	>100 (3)	52.5 ±17.7 (4)	>100 (3)	>100 (3)
	hesperetin	>100 (3)	>100 (3)	>100 (3)	28.2 ± 1.1 (3)	>100 (3)
isoflavones	genistein	16.8 ± 2.3 (3)	1.7 ± 0.2 (4)	12.9 ±5.2 (3)	9.5 ± 1.9 (4)	73.9 ± 7.1 (3)
	daidzein	>100 (3)	>100 (4)	28.6 ± 8.5 (3)	>100 (4)	>100 (3)

Table 2 (cont.)

Class	Name	PDE isozyme				
		1	**2**	**3**	**4**	**5**
	biochanin A	29.1 ± 0.3 (3)	27.9 ± 4.1 (4)	>100 (3)	8.5 ± 0.1 (4)	>100 (3)
	prunetin	>100 (3)	>100 (4)	>100 (3)	61.9 ± 17.3 (4)	>100 (3)
		122.8 ± 44.9 (3)	4.4 ± 1.0 (5)	2.4 ± 0.5 (3)	11.4 ±1.6 (8)	3.3 ±0.9 (7)

Note: All values are expressed as the mean ± S.E.M. (n), where n is the number of experiments. [a] Reference drugs for PDE isozymes 1, 2, 3, 4, and 5 were vinpocetine, EHNA, milrinone, Ro 20-1724, and zaprinast, respectively.

Isoquinoline alkaloids for ED

The isoquinoline alkaloids are a large class of medicinally active alkaloids whose properties are variable. Papaverine is one of isoquinoline derivatives which isolated from *Papaver somniferum* L. It is a well known smooth muscle relaxant agent with multiple activities as PDEs inhibitor. For treatment of ED , papaverine has a potential to be used as a ED drug for intravenous injection therapy. However, this gave rise to side effects such as priapism, local fibrosis and pain due to their route of administration. [35,51]. The mechanism of penile injected papaverine might relate to PDE5 which abundantly localizes in this tissue. Therefore, if we take isoquinoline as a model, we may find that small structural differences could lead to more useful compounds with a more definite mechanism of action as PDE5-inhibitors.

***Kaempferia parviflora* Wall. ex Baker**

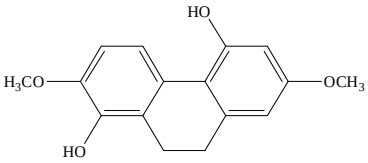
K. parviflora (Krachaidam) belongs to Zingiberaceae family. It is a typically grown in Thailand. In Thai traditional medicine, rhizomes of *K. parviflora* have been used for treatment of wide variety of illnesss including erectile dysfuntion [18]. Several of its biological activities were revealed as anti-oxidant, anti-inflammatory, anti-allergic, antispasmodic and antifungal [54]. Wattanapitayakul and co-worker [20] suggested that *K. parviflora* might improve endothelial function by activation of NO production which correlate with penile erection phenomenon. Additionally, the its ethanolic rhizhome extract could increase the blood flow to the testis in *in vivo* study [21]. In our previous study [15], *K. parviflora* could inhibit mixture PDE which mainly contain PDE1. The major constituents in rhizhome of *K. parviflora* are methoxyflavones. Among them, 5,7-dimethoxyflavone is the main component [25]. There is no report on biological activity related to erection mechanism of these compounds. Thus, it was interesting to explore the PDE5 inhibitory activity of them.

***Eulophia macrobulbon* (Parish & Rchb. f.) Hook. f.**

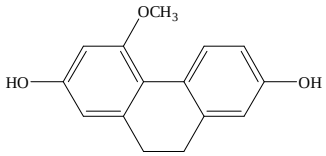
Figure 12 Flowers and pseudo-bulbs of *E. macrobulbon*

E. macrobulbon (**Figure 12**) is typical distributed in south-east asia especially in Thailand, Myanamar, Laos, Cambodia and Vietnam. It is terrestrial orchids which has a large tuber-like pseudobulb carrying oblong to elliptic lanceolate, acuminate leaves that blooms in the spring on an erect, robust, to 18" [to 45 cm] long, many flowered, racemose, inflorescence with very slender bracts [55]. On the chemical constituents, there was no report in this plant. However, one of relative species *E. nuda* Lindl, had been popularly studied on chemical constituents and bioactivities.

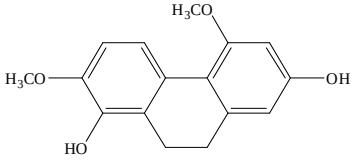
Tuchinda *et. al.* revealed crude extract of *E. nuda* tuber exhibited anti-inflammatory activity in carrageenan- induced paw edema test. In addition, some phenanthrenes were isolated from this plant. The other relative plants are *Eulophia petersii* Rchb. f. [32] and *Eulophia. ochreata* Lindl. [33] were study on chemical content which are summarized in **Figures 13-14**.



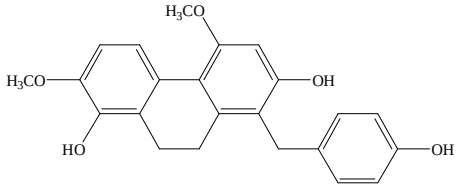
Eulophiol [30]



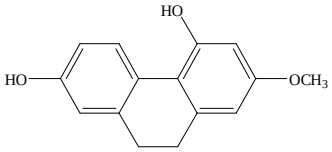
9,10-dihydro-4-methoxyphenanthrene-2,7-diol [30]



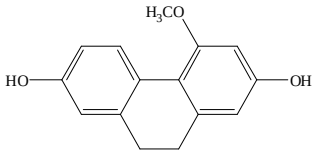
9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol [30, 33]



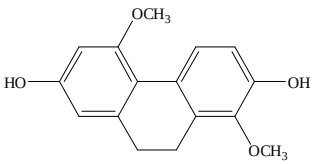
9,10-dihydro-1-(4′-hydroxybenzyl)-4,7-dimethoxyphenanthren-2,8-diol [31]



lusianthridin [32]

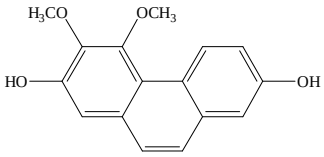


coelonin [32]

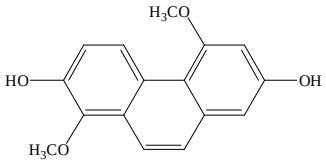


9,10-dihydro-1,5-dimethoxyphenanthrene-2,7-diol [32]

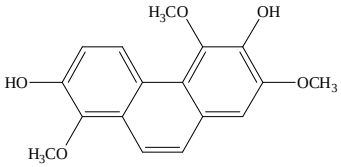
Figure 13 9,10 dihydrophenanthrene compounds in *Eulophia* spp.



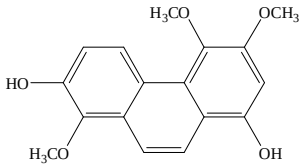
Nudol [30]



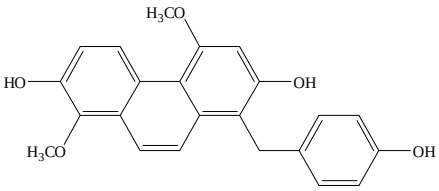
1,5 dimethoxyphenanthrene-2,7-diol [30]



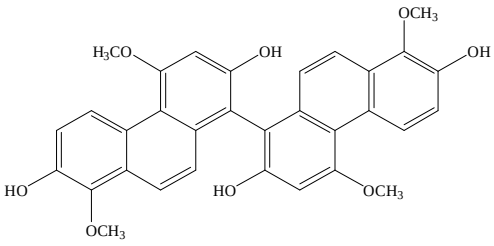
1,5,7-trimethoxyphenanthrene-2,6-diol [30]



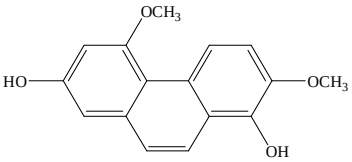
1,5,6-trimethoxyphenanthrene-2,8-diol [30]



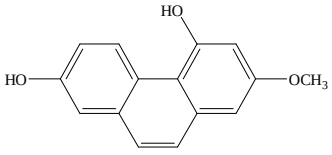
1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthren-2,7-diol [31]



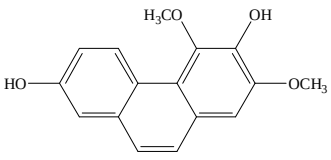
4,4′,8,8′-tetramethoxy-[1,1′]biphenanthrene [30]



2,5 dimethoxyphenanthrene-1,7-diol [32]



Lusianthrin [32]



2,4 dimethoxyphenanthrene-3,7-diol [33]

Figure 14 9,10 dehydrophenanthrene compounds in *Eulophia* spp.

CHAPTER III

RESEARCH METHODOLOGY

Plant materials

In this study, the plants selected for screening were divided into 3 groups. The first group was plants used in traditional aphrodisiac and neurotonic remedies (**Table 3**). The second group was Leguminosae family plants (**Table 4**). The third group was miscellaneous plants (**Table 5**). Some plant extracts in the first and second groups had been screened on PDE1 inhibitory activity in the previous work. These plants were identified by Mr. Wittaya Pongamornkul, Queen Sirikit Botanic Garden, Chiangmai, Thailand and Professor Wongsatit Chuakul, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The herbarium specimens were kept at Queen Sirikit Botanic Garden, Chiangmai and PBM herbarium, Faculty of Pharmacy, Mahidol University, Bangkok. The plants in third group were bought from Chatujak Market, Bangkok, Thailand. These plants have not been identified yet, except for *Asparagus racemosus* Willd., *Curcuma aeruginosa* Roxb, *Eulophia macrobulbon* (Parish & Rchb. f.) Hook. f, and *Stephania suberosa* Forman.

Preparation of the extracts for screening PDE5 inhibitory activity

The materials were cut into small pieces and dried in a hot air oven at 50-60˚C. The dried materials were macerated in 95% EtOH for 3 days and filtered. The filtrate was evaporated under reduced pressure until dryness. The residue from the filtration were macerated in the same solvent again for 3 days and filtered. The filtrate was evaporated with the same procedure and combined with the extract from the first extraction. The extracts were kept in refrigerator at 2-8˚C until the test day.

Table 3 Thai medicinal plants used as aphrodisiac and neurotonic remedies used in this study

No	Thai name	Scientific name	Family	Part used	Collection number
1	ว่านน้ำ	*Acorus calamus* L.	Araceae	Root	WP.1907
2	สังกรณี	*Barleria strigosa* Willd.	Acanthaceae	Whole plant	WP.1900
3	ฮ่อสะพายควาย	*Berchemia floribunda* Wall.	Betulaceae	Stem bark	WP.1899
4	กำลังเสือโคร่ง	*Betula alnoides* Buch.-Ham. ex G.Don	Rhamnaceae	Stem	WP.1893
5	กระชาย	*Boesenbergia rotunda* (L.) Mansf.	Zingiberaceae	Rhizome	QSBG. 4152
6	กวาวเครือแดง	*Butea superba* Roxb.	Leguminosae	Root bark	Fansai0021
7	ฝาง	*Caesalpinia sappan* L.	Caesalpiniaceae	Stem	WP.1903
8	จอกบ่หวาย	*Drosera burmannii* Vahl	Droseraceae	Arial part	QSBG. 9202
9	โด่ไม่รู้ล้ม	*Elephantopus scaber* L.	Asteraceae	Whole plant	WP.1895
10	กำลังช้างเผือก	*Hiptage benghalensis* (L.) Kurz	Malpighiaceae	Stem	WP.1896
11	กระชายดำ	*Kaempferia parviflora* Wall. ex Baker	Zingiberaceae	Rhizome	QSBG. 25275

Table 3 (cont.)

No	Thai name	Scientific name	Family	Part used	Collection number
12	กะตังใบ	*Leea indica (Burm.f.)* Merr.	Leeaceae	Root	QSBG. 15194
13	กวาวเครือดำ	*Mucuna collettii* Lace	Leguminosae	Stem	BKF 115495
14	ฝนแสนห่า	*Myxopyrum smilacifolium* Blume subsp.	Oleaceae	Root	WP.1904
15	ม้าแม่กำ่า	*Polygala chinensis* L.	Polygalaceae	Whole plant	QSBG. 10073
16	ช้าพลู	*Piper sarmentosum* Roxb.	Piperaceae	Root	WP.1902
17	มะเขือแจ้เครือ	*Securidaca inappendiculata* Hassk	Polygalaceae	Stem	WP.1897
18	เนระพูสี	*Tacca chantrieri* André	Taccaceae	Root	WP.1901
19	บอระเพ็ด	*Tinospora crispa* (L.) Miers ex Hook.f.& Thomson	Menispermaceae	Stem	QSBG. 11325
20	โสมไทย	*Talinum paniculatum* (Jacq.) Gaertn.	Portulacaceae	Rhizome	QSBG. 9524
21	รางแดง	*Ventilago denticulata* Willd.	Rhamnaceae	Stem	WP.1894

Table 4 Plants from Leguminosae family used in this study. The leaves were used for the test

No.	Thai Name	Scientific name	Collection number
1	กระถินณรงค์	*Acacia auriculaeformis* A. Cunn.	Sirikul 013
2	ส้มป่อย	*Acacia concinna* (Willd.) DC.	Sirikul 014
3	ชะอม	*Acacia pennata* (L.) Willd. Subsp. Insuavis (Lace) I.C. Nielsen	Sirikul 012
4	กาหลง	*Bauhinia acuminate* L.	Sirikul 011
5	ชงโค	*Bauhinia glauca* (Wall. ex Benth.) Benth.	Sirikul 009
6	อรพิม	*Bauhinia winitii* Craib	Sirikul 010
7	ทองกวาว	*Butea monosperma* (Lam.) Taub.	Sirikul 019
8	ตันหยง	*Caesalpinia coriaria* (Jacq.) Willd.	Sirikul 020
9	ฝาง	*Caesalpinia sappan* L.	Sirikul 015
10	คูณ	*Cassia fistula* L.	Sirikul 004
11	หางนกยูงฝรั่ง	*Delonix regia* (Bojer ex Hook.) Raf.	Sirikul 016
12	กระถิน	*Leucaena leucocephala* (Lam.) de Wit	Sirikul 006
13	มะขามเทศ	*Pithecellobium dulce* (Roxb.) Benth.	Sirikul 008
14	ก้ามปู	*Samanea saman* (Jacq.) Merr.	Sirikul 005
15	โสกเหลือง	*Saraca thaipingensis* Cantley ex Prain	Sirikul 018
16	ชุมเห็ดเทศ	*Senna alata* (L.) Roxb.	Sirikul 003
17	ขี้เหล็ก	*Senna siamea* (Lam.) Irwin & Barneby	Sirikul 001

Table 4 (cont.)

No.	Thai Name	Scientific name	Collection number
18	ทรงบาดาล	*Senna surattensis* (Burm.f.) Irwin & Barneby	Sirikul 017
19	แค	*Sesbania grandiflora* (L.) Desv.	Sirikul 002
20	มะขาม	*Tamarindus indica* L.	Sirikul 007

Table 5 Miscellaneous plants used in this study

No.	Thai Name	Scientific Name	Family	Part used	Collection number
1	รากสามสิบ	*Asparagus racemosus* Willd.	Asperagaceae	Root	RKT0005
2	ว่านมหาเมฆ	*Curcuma aeruginosa* Roxb.	Zingiberaceae	Rhizhome	ganniga001
3	ขมิ้น	*Curcuma longa* L.	Zingiberaceae	Rhizhome	NI
4	ขมิ้นอ้อย*	*Curcuma zedoaria* (Berg) Roscoe	Zingiberaceae	Rhizhome	NI
5	ห้าร้อยนาง*	*Curcuma petiolata* Roxb.	Zingiberaceae	Rhizhome	NI
6	ชักมดลูก*	*Curcuma xanthorrhiza* Roxb.	Zingiberaceae	Rhizhome	NI
7	นางคำ*	*Curcuma* sp.	Zingiberaceae	Rhizhome	NI
8	หัวใหญ่*	*Curcuma* sp.	Zingiberaceae	Rhizhome	NI
9	เพชรม้า*	*Curcuma* sp.	Zingiberaceae	Rhizhome	NI
10	ม้าเหลือง*	*Curcuma* sp.	Zingiberaceae	Rhizhome	NI
11	ม้าขาว*	*Curcuma* sp.	Zingiberaceae	Rhizhome	NI

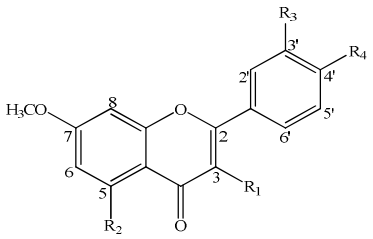
Table 5 (cont.)

No.	Thai Name	Scientific Name	Family	Part used	Collection number
12	ม้าห้อ*	*Curcuma* sp.	Zingiberaceae	Rhizhome	NI
13	มหากำลัง*	*Curcuma* sp.	Zingiberaceae	Rhizhome	NI
14	หนุมานยกทัพ*	*Curcuma* sp.	Zingiberaceae	Rhizhome	NI
15	ว่านอึ่ง	*Eulophia macrobulbon* (Parish & Rchb. f.) Hook. f	Orchidaceae	Pseudobulb	002716
16	ทิพยเนตร*	*Kaempferia rotunda* Linn.	Zingiberaceae	Rhizhome	NI
17	บัวบกนา*	*Stephania pierrei* Diels	Menispermaceae	Bulb	NI
18	บอระเพ็ดพุงช้าง	*Stephania suberosa* Forman	Menispermaceae	Root	Fansai0026
19	ไพลดำ*	*Zingiber ottensii* Valeton	Zingiberaceae	Rhizhome	NI

Note: NI means no identification. All of these plants are to be identified later.

7-methoxyflavone from *K. parviflora*

7-methoxy flavones from *K. parviflora* were obtained from Assistant Professor Dr. Pattara Sawasdee, Faculty of Chemistry, Chulalongkorn University. They were isolated as reported previously. [20] The structures are shown in **Figure 15**.



Compound	Substitution			
	R_1	R_2	R_3	R_4
Tectochrysin **(1)**	H	OH	H	H
5,7-dimethoxyflavone **(2)**	H	OCH_3	H	H
5-hydroxy-7,4′-dimethoxyflavone **(3)**	H	OH	H	OCH_3
5,7,4′-trimethoxyflavone **(4)**	H	OCH_3	H	OCH_3
5-hydroxy-3,7-dimethoxyflavone **(5)**	OCH_3	OH	H	H
3,5,7-trimethoxyflavone **(6)**	OCH_3	OCH_3	H	H
5-hydroxy-3,7,4′-trimethoxyflavone **(7)**	OCH_3	OH	H	OCH_3
3,5,7,3′,4′-pentamethoxyflavone **(8)**	OCH_3	OCH_3	OCH_3	OCH_3

Figure 15 The 7-methoxyflavone constituents from rhizomes of *K. parviflora*

Extraction and isolation of PDE5 inhibitors from *E. macrobulbon*

1. General experiment procedures for isolation and structure elucidation

For column chromatography, three techniques including vacuum column chromatography, open column chromatography and gel filtration chromatography were used in this study. A silica gel 60 (0.040-0.063 mm granule size; Merck, Germany) was used for the first and second techniques while sephadex LH-20 (GE Healthcare Life Sciences, USA) was used for gel filtration chromatography. Preparative thin layer chromatography (prep-TLC) was performed on commercial Silica gel 60 F_{254} precoated glass plate (1 mm thick, Merck, Germany). The analytical

thin layer chromatography (TLC) was carried out on TLC silica gel 60 F_{254} aluminium sheet (5 x 10 cm, Merck, Germany) using the ultraviolet light at wavelength of 254 and 366 nm detection and sprayed with vanillin reagent and heating at 100ºC for a few minutes.

Nuclear Magnetic Resonance (NMR) spectra were recorded in deuterated acetone (CD_3COCD_3), deuterated methanol (CD_3OD) and deuterated DMSO (CD_3SOCD_3) on a Bruker AV400 (USA) spectrometer at 400 MHz (^1H) and 100 MHz (^{13}C) with tetramethylsilane (TMS) as the internal standard. Two-dimensional experiments (COSY, HSQC, HMBC, NOESY) were performed at Science Lab Center, Faculty of Science, Nareusuan University, Thailand. Mass spectra were obtained using and electron ionization mass spectrometric (EI-MS) (MAT 95 XL, Thermo Finnigan, Germanyes) from Scientific Equipment Center, Prince of Songkla University, Songkla, Thailand. The Infrared (IR) absorption spectra were recorded using KBr technique on Fourier Transform Infrared Spectrometer (FT-IR) (Spectrum GX Series, PerkinElmer) at Science Lab Center, Faculty of Science, Naresuan University, Thailand.

2. Extraction

The fresh pseudo-bulbs of *E. macrobulbon* (40 kg) were finely chopped and dried in a hot air oven at 55°C. The dried material was ground until it had fine powder. Consequently, the powder pseudo-bulbs of *M. macrobulbon* (2 kg) were successively extracted in the same protocol in the section of preparation of the extracts for screening PDE5 inhibitory activity. The EtOH extract (200 g) was dissolved with methanol (MeOH) (200 ml) then solvent-solvent partition was carried out by starting with hexane (6 x 200 ml). After that water (800 ml) was added to MeOH layer. The mixture was separated into 2 groups (400 ml) and each was poured into 1000 ml separating funnel. Each separating funnel was partitioned in the same way. Each mixture was partitioned with dichloromethane (CH_2Cl_2) (8 x 400 ml), ethylacetate (EtOAc) (8 x 400 ml) and n-buthanol (BuOH) (5 x 400 ml). The procedure for solvent-solvent partition showed in **Figure 16**. All extracts had been concentrated in vacuum until dryness. After removal of solvent, the final concentration of 50 µg/ml of each extract was determined for PDE5 inhibitory activity using the Method II of PDE5 inhibitory activity assay.

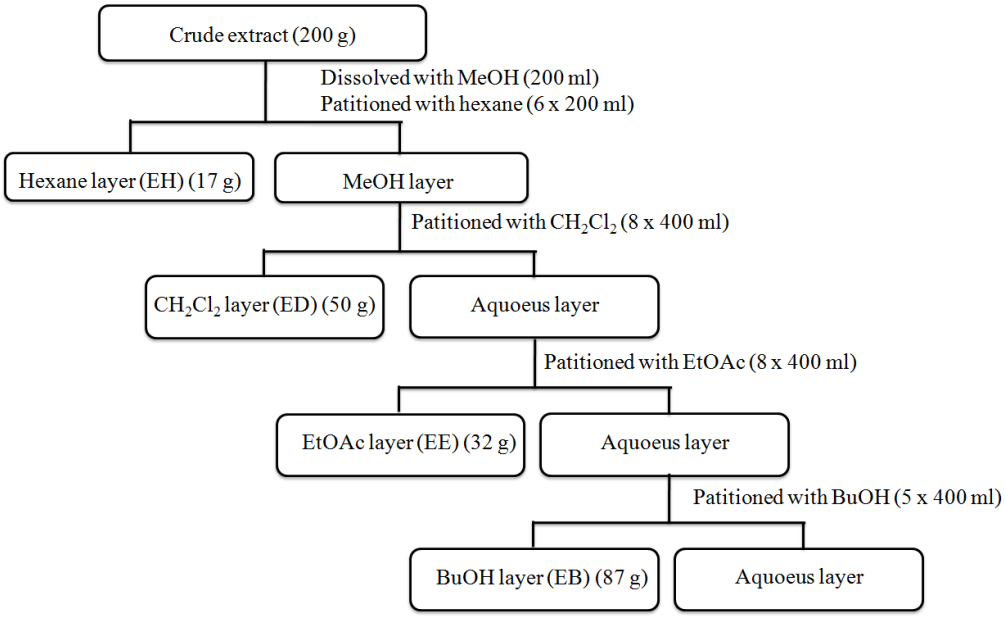


Figure 16 Solvent-solvent partition procedure of *E. macrobulbon* extract.

3. Isolation and purification of the PDE5 inhibitors

The PDE5 inhibitors isolation procedure, involving a combination of various chromatographic techniques, was illustrated in **Figure 17**. The dichloromethane extract (50 g) was chromatographed by vacuum column chromatography (VCC) (13 x 7 cm, i.d. x h) on silica gel and eluted with a gradient mobile phase of CH_2Cl_2 and MeOH and was final eluted with 1% of 1 N HCl in MeOH. The fractions were monitored by TLC (CH_2Cl_2-MeOH (98:2) and petroleum ether:ethylacetate (EtOAc) (1:1). Thirteen pooled fractions (ED-Q1 - ED-Q13) were obtained. All fractions were submitted to PDE5 inhibitory activity screening test. The active fractions were further fractioned.

3.1 Isolation of **9** (9,10-dihydro-2,5-imethoxyphenanthrene-1,7-diol)

The first separation step of fraction ED-Q4 (1.2 g) was done by VCC (8 x 6 cm, i.d. x h) on silica gel. Six pooled fractions were obtained after monitoring on TLC. ED-Q4-2, 37 mg was subjected to silica gel preparative TLC (prep-TLC) with petroleum ether (pet.Ether)-EtOAc (27:13) as a solvent system to afford **9** (24 mg).

3.2 Isolation of **10** (9,10-dihydro-4-(4′-hydroxybenzyl)-2,7-dimethoxyphenanthrene-1,7-diol) and **11** (1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol)

Fraction ED-Q6 (1 g) was separated by gel chromatography on Sephadex LH-20 (2.2 x 105 cm) with MeOH to yield fraction ED-Q6-3 (100 mg) and ED-Q6-5 (200 mg). Fraction ED-Q6-3 (100 mg) was further fractionated on a silica gel column (3.5 x 14 cm) using gradient form of pet.Ether and EtOAc as a mobile phase to give 10 fractions. ED-Q6-3-4 (40 mg) was isolated by gel chromatography on Sephadex LH-20 (1.5 x 90 cm) with MeOH to give fraction ED-Q6-3-4-3 which was finally purified by prep-TLC on siliga gel with the use of hexane-EtOAc (3:7) as mobile phase. These separations yielded **10** (8 mg). Fraction ED-Q6-5 (200 mg) was further separated on a silica gel column (3.5 x 12 cm) using gradient form of pet.Ether and EtOAc as a mobile phase to give **11** (110 mg).

3.3 Isolation of **12** (1,5,7-trimethoxyphenanthrene-2,6-diol)

Fraction ED-Q2 (50 mg) was chromatographed on a silica gel column (3.5 x 14 cm) using hexane and EtOAc as a mobile phase (7:3, isocratic system) to give fractions ED-Q2-1 to 8. Fraction ED-Q2-8 were further purified by prep-TLC on silica gel using hexane-EtOAc (6:4) as a solvent system to afford **12** (2 mg).

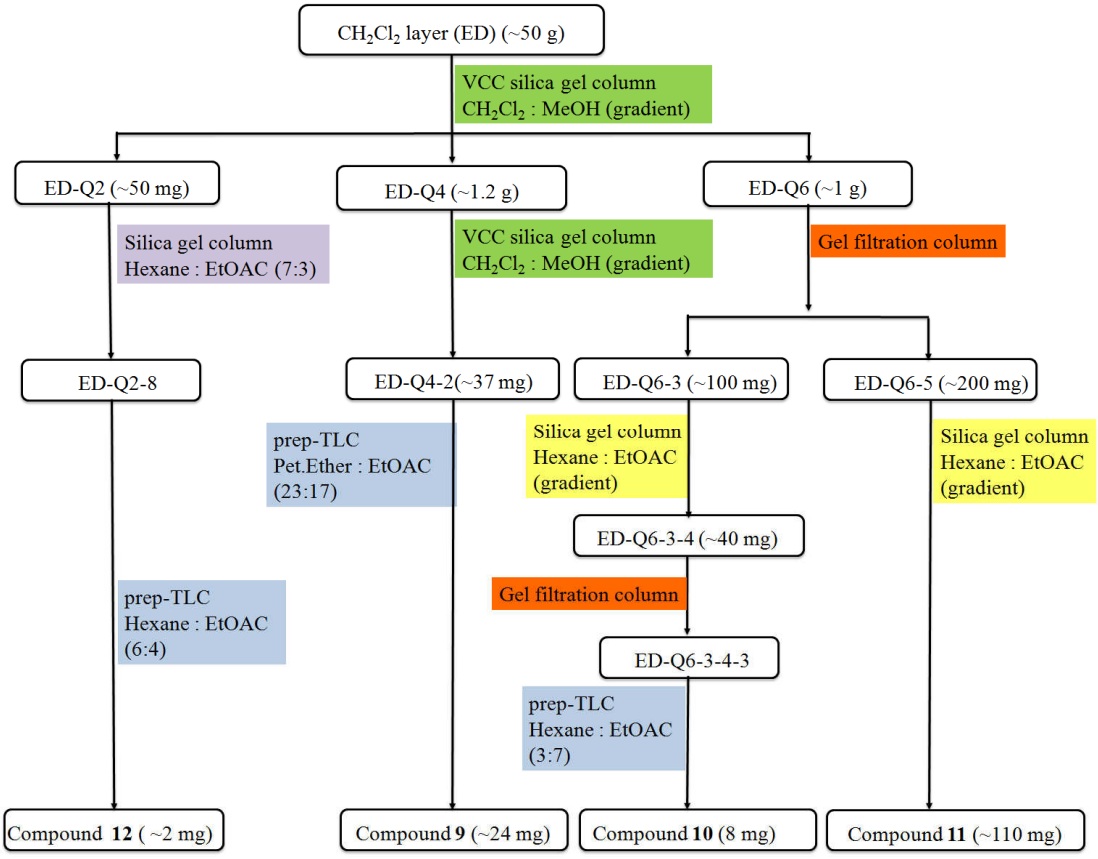


Figure 17 Isolation procedure of PDE5 inhibitors from *E. macrobulbon*

4. Spectral data of isolated compounds

The isolated compounds were characterized by their spectroscopic data including NMR, MS and IR spectra.

4.1 Compound **9** (9,10-dihydro-2,5-imethoxyphenanthrene-1,7-diol)

EIMS *m/z* (relative intensity) : 272 $[M^+]$ (100), 257 $[M-15]^+$ (69), 229 $[M-43]^+$ (15), 197 $[M-75]^+$ (12), 136 $[M-136]^+$ (8). (**Figure 28**, Appendix A)

HREIMS *m/z* : 272.1043 ($C_{16}H_{16}O_4$)

IR (KBr) : ν cm^{-1} 3443, 1601, 1481, 1463, 1441, 1431, 1321, 1276, 1077, 1059, 811 (**Figure 29**, Appendix A)

^{1}H-NMR (400 MHz, in acetone-d_6) : δ ; (**Figure 30-32**, Appendix A)

2.61 (1H, dd, *J* = 4.4, 8.1 Hz, H-9′′), 2.62 (1H, br.d, *J* = 8.1 Hz, H-9′), 2.74 (1H, br.d, *J* = 8.1 Hz, H-10′), 2.75 (1H, dd, *J* = 4.4, 8.11, H-10′′), 3.82 (3H, s, C-

5-OCH_3), 3.85 (3H, s, C-2-OCH_3), 6.38 (1H, d, $J_{8,6}$ = 2.4 Hz, H-8), 6.46 (1H, d, $J_{6,8}$ = 2.4 Hz, H-6), 6.78 (1H, d, $J_{3,4}$ = 8.4 Hz, H-3), 7.73 (1H, d, $J_{4,3}$ = 8.4 Hz, H-4).

^{13}C-NMR (100 MHz, in acetone-d_6) : δ ; (**Figure 33**, Appendix A)

22.18 (CH_2, C-10), 30.99 (CH_2, C-9), 56.24 (C, C-2-OCH_3), 55.71 (C, C-5-OCH_3), 99.15 (CH, C-6), 108.20 (CH, C-8), 108.98 (CH, C-3), 116.33 (C, C-4b), 120.16 (CH, C-4), 124.76 (C, C-10a), 127.61 (C, C-4a), 141.59 (C, C-8a), 142.85 (C, C-1), 146.02 (C, C-2), 157.57 (C, C-7), 159.10 (C, C-5).

4.2 Compound **10** (9,10-dihydro-4-(4′-hydroxybenzyl)-2,7-dimethoxyphenanthrene-1,7-diol)

EIMS *m/z* (relative intensity) : 378 [M^+] (100), 363 [M-15$^+$] (23), 347[M-31$^+$] (22), 335 [M-43$^+$] (11), 272 [M-106$^+$] (11), 107 [M-271$^+$] (19) (**Figure 38**, Appendix B).

HREIMS *m/z* : 378.1478 ($C_{23}H_{22}O_5$)

IR (KBr) : ν cm^{-1} 3412, 1612, 1512, 1493, 1463, 1279, 1252, 1158, 1064, 832) (**Figure 39**, Appendix B)

^{1}H-NMR (400 MHz, in methanol-d_4) : δ ;(**Figure 40-43**, Appendix B)

2.04 (H, ddd, *J* = 14.5, 14.7, 4.0 Hz, H-10′), 2.38 (H, ddd, *J* = 14.7, 14.0, 4.0 Hz, H-9′), 2.61 (H, ddd, *J* = 14.0, 4.0, 2.4 Hz, H-9′′), 3.20 (H, ddd, *J* = 14.5, 4.0, 2.4 Hz, H-10′′), 3.62 (3H, s, C-5-OCH_3), 3.69 (3H, s, C-2-OCH_3), 3.73 (1H, d, *J* = 15.0 Hz, benzylic-CH_2), 3.81 (1H, d, *J* = 15.0 Hz, benzylic-CH_2), 6.36 (H, d, $J_{6,8}$ = 2.0 Hz, H-6), 6.38 (H, d, $J_{8,6}$ = 2.0 Hz, H-8), 6.47 (H, s, H-3), 6.61 (2H, d, $J_{3',2'}$ or $J_{5',6'}$ = 8.4 Hz, H-3′ or H-5′), 6.81 (2H, d, $J_{2',3'}$ or $J_{6',5'}$ = 8.4 Hz, H-2′ or H-6′).

^{13}C-NMR (100 MHz, in methanol-d_4) : δ ; (**Figure 44**, Appendix B)

24.39 (CH_2, C-10), 32.15 (CH_2, C-9), 40.40 (benzylic-CH_2,), 55.29 (C, C-5-OCH_3), 56.21 (C, C-2-OCH_3), 98.55 (CH, C-6), 107.73 (CH, C8), 112.07 (CH, C-3), 115.83 (CH, C-3′), 117.34 (C, C-4b), 127.03 (C, C-10a), 127.36 (C, C-8a), 131.01 (CH, C-2′), 132.69 (C, C-4a), 135.48 (C, C-1′), 140.98 (C, C-4), 144.41 (C, C-1), 147.10 (C, C-2), 156.03 (C, C-4′), 158.09 (C, C-7), 158.30 (C, C-5).

4.3 Compound **11** (1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol)

EIMS *m/z* : 376 (M+1) 376 [M^+] (61), 320 (50), 316 (28), 272 (100), 267 (36), 257 (26), 169 (51), 92 (65) (**Figure 53**, Appendix C).

HREIMS *m/z* : 376.1307 ($C_{23}H_{22}O_5$)

IR (KBr) : ν cm^{-1} 3338, 1614, 1516, 1465, 1378, 1350, 1303, 1223, 1175, 1036, 818 (**Figure 54**, Appendix C)

^{1}H-NMR (400 MHz, in dimethylsulfoxide-d_6) : δ ; (**Figure 55-56**, Appendix C)

3.84 (3H, s, C-8-OCH_3), 4.01 (3H, s, C-4-OCH_3), 4.22 (2H, s, benzylic-CH_2), 6.61 (2H, d, $J_{3',2'}$ = 8.5 Hz, H-3′), 6.93 (1H, s, H-3), 6.97 (2H, d, $J_{2',3'}$ = 8.5 Hz, H-2′), 7.16 (1H, d, $J_{6,5}$ = 9.4 Hz, H-6), 7.79 (1H, d, $J_{10,9}$ = 9.5 Hz, H-10), 7.86 (1H, d, $J_{9,10}$ = 9.5 Hz, H-9), 7.79 (1H, d, $J_{5,}$6 = 9.4 Hz, H-5).

^{13}C-NMR (100 MHz, in dimethylsulfoxide-d_6) : δ ; (**Figure 57**, Appendix C)

29.01 (benzylic-CH_2,), 55.36 (C, C-4-OCH_3), 60.42 (C, C-8-OCH_3), 99.67 (CH, C-3), 113.43 (C, C1), 114.41 (C, C-8a), 114.82 (CH, C-3′), 117.56 (CH, C-6), 120.40 (CH, C-9), 123.11 (CH, C-10), 123.76 (CH, C-5), 124.31 (C, C-4b), 125.93 (C, C-4a), 128.84 (CH, C-2′), 131.8 (C, C-1′), 132.03 (C, C-10a), 140.86 (C, C-8), 145.44 (C, C-7), 152.84 (C, C-2), 155.07 (C, C-4′), 157.08 (C, C-4).

4.4 Compound **12** (1,5,7-trimethoxyphenanthrene-2,6-diol) ($C_{17}H_{16}O_5$)

EIMS *m/z* 300 (M+1) 300 [M^+] (100), 285 (53), 253 (21) (**Figure 64**, Appendix D).

^{1}H-NMR (400 MHz, in methanol-d_4) : δ ;(**Figure 65-6**7, Appendix D)

3.87 (3H, s, C-5-OCH_3), 3.93 (3H, s, C-1-OCH_3), 4.01 (3H, s, C-7-OCH_3), 7.17 (1H, d, $J_{3,4}$ = 8.8Hz, H-3), 7.18 (1H, s, H-8), 7.62 (1H, d, $J_{9,10}$ = 9.0 Hz, H-9), 7.83 (1H, d, $J_{10,9}$ = 9.0 Hz, H-10), 9.09 (1H, d, $J_{4,3}$ = 8.8Hz, H-4).

^{13}C-NMR (100 MHz, in methanol-d_4) : δ ; (**Figure 6**8, Appendix D)

56.34 (C, C-7-OCH_3), 59.77 (C, C-5-OCH_3), 61.36 (C, C-1-OCH_3), 106.09 (CH, C-8), 117.98 (CH, C-3), 118.80 (CH, C-10), 120.39 (C, C-4b), 124.45 (CH, C-4), 125.00 (C, C-4a), 126.96 (C, C-8a), 128.25 (CH, C-9), 128.89 (C, C-10a), 141.23 (C, C-6), 142.50 (C, C-1), 145.80 (C, C-5), 147.72 (C, C-2), 149.00 (C, C-7).

Isoquinoline and piperine derivatives

All of tested compounds in this study were obtained from Assoc Professor Nantaka Khorana, Department of Pharmaceutical chemistry and Pharmacognosy, faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok. Piperine **(13)** and it derivatives i.e., piperic acid (**14**) and piperonylic acid (**15**), were tested on the mixture PDEs activity. Tetrahydroisoquinoline (THIQ) derivatives and their intermediate (amide form and dihydroisoquinoline (DHIQ)) which were synthesized as shown in **Figure 18** for the acetylcholinesterase inhibitory activity study [56,57], were investigated on PDEs (the mixture of crude PDE enzyme) and PDE5 inhibitory activity in this study.

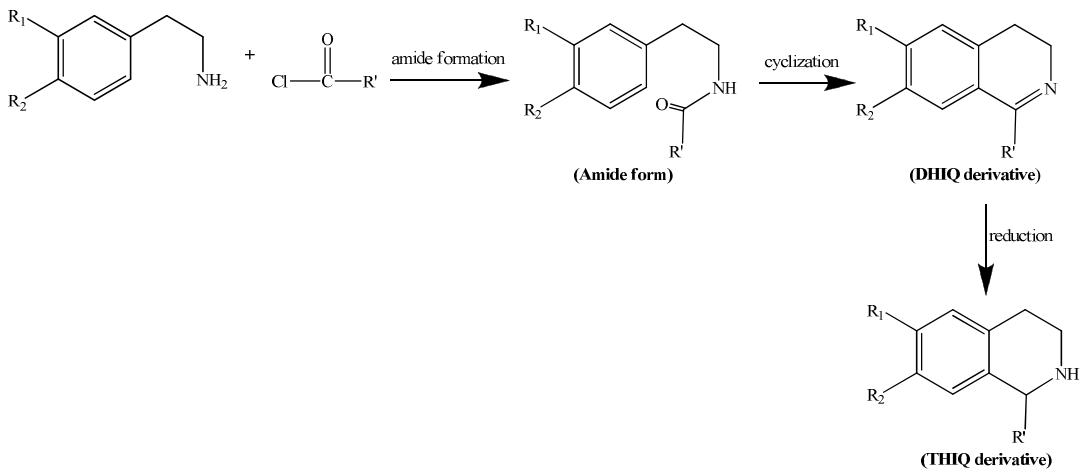


Figure 18 Synthetic pathway for amide, DHIQ and THIQ derivatives [56,57]

Bioassays

1. PDE assays

1.1 Chemicals

Table 6 Chemical list in this study

Chemical name	Company
[8-^{3}H]cGMP	Perkin-Elmer, USA
Bovine serum albumin (BSA)	Sigma, USA
Calcium chloride	Ajax Finechem, USA
Calmodulin from bovine heart	Sigma, USA
cGMP	Sigma, USA
Diethylaminoethyl sephadex (DEAE-Sephadex)	Sigma, USA
Diethyl-(2-hydroxypropyl) aminoethyl sephadex (QAE-Sephadex)	Sigma, USA
Dimethyl sulfoxide (DMSO)	Sigma, USA
Dithiothreitol (DTT) solution	Sigma, USA
EDTA	Bio-rad, USA
Ethylene glycol tetraacetic acid (EGTA)	Sigma, USA
Glycerol	Sigma, USA
Histone from calf thymus, Type VIII-S	Sigma, USA
Imidazole	Sigma, USA
Magnesium acetate	Qrec, Newzealand
Magnesium chloride	Sigma, USA
3-(N-Morpholino) propanesulfonic acid, 4-Morpholinepropanesulfonic acid (MOPS)	Sigma, USA
Phosphodiesterase 3′,5′-Cyclic Nucleotide Activator-deficient from bovine heart	Sigma, USA
Phenylmethylsulfonyl fluoride (PMSF)	Sigma, USA
Snake venom, *Crotalus atrox* (Western Diamondback Rattlesnake)	Sigma, USA

Table 6 (cont.)

Chemical name	Company
Sodium chloride	Ajax Finechem, USA
Tris (hydroxymethyl) aminomethane	Merck, USA
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen, USA
Fetal Bovine Serum (FBS)	Invitrogen, New Zealand
Trypsin	Invitrogen, USA
Pen-step	Invitrogen, USA
Lipofectamine 2000 reagent	Invitrogen, USA
Opti-MEM I (Reduced Serum Medium 1X)	Invitrogen, New Zealand
Ampicillin	Sigma, USA
Kanamycin	Sigma,USA
Bacteriological Agar	Amresco, USA
Luria Broth base (Miller's LB Broth base)	Invitrogen, USA
Qiagen Plasmid Maxi Kit	Qiagen, USA

1.2 Enzyme preparation

PDE1

In this study, the crude PDE isolated from bovine heart (Sigma, USA) was used as a source of PDE1.

PDE5

1. PDE5 from mice lung [41]

Mice lung was used as the source of PDE5 . Briefly, fresh tissue was minced and homogenized using homogenizer in 1 ml of Tris buffer (50 mM Tris pH7.5, 2mM EDTA, 1mM DTT and 1:100 of 100 mM PMSF). The homogenate was centrifuged at 4°C for 20 min and the supernatant was used as a source of PDE5. A PDE5 inhibitor, sildenafil, was used to confirm the presence of PDE5.

2. PDE5 from human recombinant PDE5 [40]

2.1 Cell culture

Human embryonic kidney (HEK) 293 cells were obtained from Dr. Phanchana Sanguansermsri, Faculty of Medical Sciences, Naresuan University. They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin on 100 mm plates at 37°C in a humidified 5% CO_2 atmosphere.

2.2 Plasmid DNA transfection

Human PDE5A, a gift from Professor Joseph A Beavo, University of Washington, USA, was subcloned into pcDNA3 vector which contained ampicillin resistant gene. The plasmids for transfection were purified using a Qiagen plasmid maxi kit (Qiagen®). Cells were transfected based on the Lipofectamine 2000™ instruction. After 2 days of transfection, cells were harvested using scraper and lysed in the same condition of PDE5 preparation from lung tissue. The supernatant from the centrifugation was used in PDE5 assay.

PDE6

The PDE6 was prepared from chicken retinas as previously describe [58]. In brief, the retinas were removed from chicken eyes and homogenized in hypotonic buffer (10 mM Tris, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.2 mM PMSF) using a Potter- Elvehjem tissue grinder. The homogenate was centrifuged at 100,000 g for 1 h and then the supernatant was loaded on a DE52 anion-exchange column and eluted with a gradient NaCl in Tris-HCl buffer, pH 7.5 (20-300 mM NaCl) at 4°C. Each fraction (5 ml) was determined for PDE6 activity. The fractions with high PDE6 activity were pooled and concentrated using centrifugal concentrator. The concentrate PDE6 was dissolved in 50% glycerol and kept at -20°C.

1.3 PDE inhibitory activity assay

The general principles of PDE assay (**Figure 19**) is the coupled enzymatic reaction. Phosphodiesterase cleaves cyclic nucleotide compound to leave 5′-monophosphate product (intermediate product). Then the second enzyme, nucleotidase in snake venom, will react with 5′-monophosphate and produce phosphate and nucleoside (guanosine or adenosine). Since both cyclic nucleotide and

5′-derivative contain a negative charge while the nucleoside does not, the radiolabled nucleosides can be separated from other compounds using ionic exchange resin.

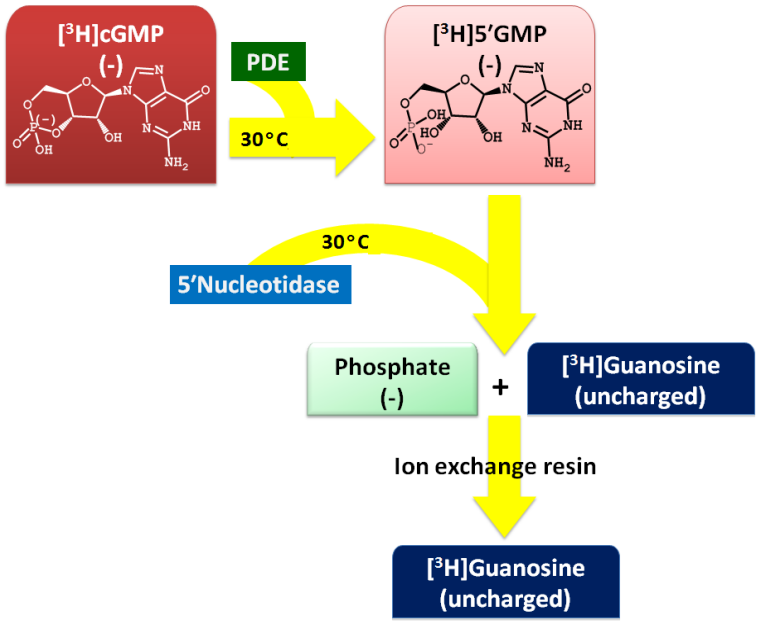


Figure 19 Principle of radioactive PDE assay

In this study, the radioactive assays for evaluation of PDE1, PDE5 and PDE6 inhibitory activity were modified from the methods reported by Sonnenburg *et al*. [59] and Huang *et al*. [58], respectively.

PDE5 inhibitory activity assay

1. Method I

This method was used for determination of PDE5 inhibitory activity from the Thai plants used as aphrodisiac and neurotonic agents, Leguminosae plants, 7-methoxyflavones isolated from *K. parviflora*, and isoquinoline derivatives. PDE5 from mice lung was used in the study.

PDE5 activity was measured based on two-step radioactive procedure as described by Sonnenburg et al. (1998). The reaction mixture was composed of 25 µl of Buffer A (100 mM Tris-HCl (pH 7.5), 100 mM imidazole, 15 mM $MgCl_2$ and 1.0 mg/ml BSA), 25 µl of 10 mM EGTA, 25 µl of PDE5 solution and 25 µl of test sample or only solvent (5%DMSO) as a control. The reaction mixture

was mixed with a substrate, 25 µl of 5 µM [^{3}H]cGMP and incubated at 30°C for 10 min. After that, the reaction was stopped by placing the tube in boiling water for 1 min and cooled for 5 min. For the second enzymatic reaction, 25 µl of 2.5 mg/ml snake venom containing 5'-nucleotidase enzyme was added to the reaction mixture**,** incubated at 30°C for 5 min. Then, 250 µl of 20mM Tris-HCl, pH 6.8 (buffer 1) was added. The reaction mixture was transferred to a DEAE ion exchange resin column and eluted 4 times with 500 µl of buffer 1 to obtain the hydrolysis product, uncharged [^{3}H]guanosine. The eluant was mixed with a scintillant cocktail and the radioactivity was measured using a β-counter. The PDE5 activity in the study was standardized to have a hydrolysis activity of 20-25% of the total substrate counts. The calculation of hydrolysis is shown in equation (1). The PDE5 inhibitory activity was calculated from equation (2).

$$\%\,\text{hydrolysis}_{\text{sample}} = \left[\frac{\left(\text{CPM}_{\text{sample}} - \text{CPM}_{\text{background}}\right)}{\left(\text{CPM}_{\text{total count1}} - \text{CPM}_{\text{background}}\right)} \right] \text{x}\,100 \quad \text{--------(1)}$$

where CPM_{sample} is the radioactive count rate of the assay with enzyme and $CPM_{background}$ is the same but without enzyme. $CPM_{total\ count}$ is the count rate of 25 µl of substrate plus 2 ml of buffer 1.

$$\%\,\text{PDE inhibition} = \left[1 - \left(\frac{\%\text{hydrolysis}_{\text{sample}}}{\%\text{hydrolysis}_{\text{control}}} \right) \right] \text{x}\,100 \quad \text{----------(2)}$$

where $\%hydrolysis_{sample}$ and $\%hydrolysis_{control}$ were the enzyme activities of the sample and solvent (1% DMSO) used in the assay, respectively.

In preliminary screening, the plant extracts were tested at the final concentration of 50 µg/ml whereas pure compounds were tested at the final concentration of 10 µM. All samples were dissolved in DMSO and diluted with water. The final concentration of DMSO was 1% in the assay medium. For extracts that gave >60% PDE5 inhibition, the IC_{50}s were determined. Each experiment was carried out in duplicate in scintillant vial.

2. Method II

This method was used for determination of PDE5 inhibitory activity of the extract, fractions and chemical constituents from *E. macrobulbon* and some plant extracts (**Table 5**). The human recombinant PDE5 was used in this method.

The PDE5 inhibitory activity was determined according to the method of Sonnenburg *et al*. with some modification. In brief, the reaction mixture was composed of 20 µl of Buffer AV (100 mM Tris-HCl (pH 7.5), 100 mM imidazole, 15 mM $MgCl_2$, 1.0 mg/ml BSA and 2.5 mg/ml snake venom), 20 µl of 10 mM EGTA, 20 µl of PDE5 solution and 20 µl of test sample or only solvent (5%DMSO) as a control. The reaction was started by adding of 20 µl of 5 µM [^{3}H]cGMP (~50,000 cpm) and performed at 30°C for 40 min. Then, reaction was stopped by 100 µl of 50% QAE resin. After shaking for 10 min, the resin was left to be settled for 20 min. The supernatant was transferred to a new 100 µl of 50% QAE resin, shaken for 10 min and the resin was left to be settled for 20 min again. The radioactive value was counted on TopCount NXT (PerkinElmer, USA) after shaking of 100 µl of supernatant with 200 µl of Microscint® 20 for 2 hr. The PDE5 activity in the study was standardized in the same range in method I. The calculation of hydrolysis was slightly different from Method I as can be seen in equation (3). Each experiment was carried out in duplicate in 96-well plate. The IC_{50}s were determined when extracts gave >80% PDE5 inhibition.

$$\%\,\text{hydrolysis}_{\text{sample}} = \left[\frac{\left(\text{CPM}_{\text{sample}} - \text{CPM}_{\text{background}}\right)}{\left(\text{CPM}_{\text{total count1}} - \text{CPM}_{\text{background}}\right)} \right] \text{x}\,100 \quad \text{--------(3)}$$

where CPM_{sample} is the radioactive count rate of the assay with enzyme and $CPM_{background}$ is the same but without enzyme. $CPM_{total\ count}$ is the count rate of 20 µl of substrate plus 100 ml of buffer 1.

PDE1 and PDE6 inhibitory activity assays

The PDE1 inhibitory activity assay was gradually modified from Sonnenburg *et al*.[59] whereas the PDE6 assay was adapted from the method of

Huang and colleagues [58]. Both assays were similar to PDE5 assay except the additional step of calcium-calmodulin and histone activations because PDE1 and PDE6 are needed to be activated with calcium-calmodulin and histone, respectively. For PDE1 assay, the final concentration of $CaCl_2$ and calmodulin for PDE1 activation was 0.8 mM and 4 µg/ml, respectively while 0.5 mg/ml of histone was used in PDE6 assay.

The plant extracts and compounds were tested at the same final concentration to that in PDE5 inhibitory activity assay (50 µg/ml and 10 µM, respectively). In case of > 80% PDE1 or PDE6 inhibition, the IC_{50}s were measured. In this work, the %hydrolysis of PDE1 and PDE6 enzyme was in the range of 20-25% for a control group.

PDEs mixture inhibitory activity assay

This assay was used for investigation of PDEs inhibitory activity from isoquinoline and piperine derivatives.

The PDEs inhibitory activity assay was modified from PDE [^{3}H]cGMP SPA enzyme assay instruction, code TRKQ 7100 (GE Healthcare, USA). The ethanolic plant extracts were screened at 0.1 mg/ml. The extracts giving more than 95% PDE inhibitory activity were further investigated for their concentration that could inhibit 50% of enzyme activity (IC_{50}). In that case, the extracts with various concentrations ranging from 1 mg/ml to 1 ng/ml were tested. Each experiment was carried out in triplicate in 96-well plates. Briefly, 5 µl of tested samples dissolved in 10% DMSO, 5 µl of assay buffer (50 mM Tris-HCl pH 7.5, 8.3 mM $MgCl_2$, and 1.7 mM EGTA), 5 µl of 7.5 mU/ml PDEs, 5 µl of 244 nM [^{3}H]cGMP (around 100,000 cpm) and 30 µl of deionized water mixed in a well. The reaction was carried out at 30°C for 40 min and stopped by the addition of 25 µl PDE SPA beads (0.5 mg) suspended in 18 mM zinc sulfate. The reaction mixture was left to be settled at a room temperature for 20 min before counted in TopCount NXT (Perkin-Elmer, USA).

2. Vasodilator effects [60]

Rats were sacrificed after being completely anesthetized with 65 mg/kg sodium pentobarbital by intraperitoneal injection. The pulmonary artery was excised, removed of loose connective tissue and cut into rings of 3 mm width. The vascular rings are mounted on a pair of intraluminal wire in organ bath chambers containing

Krebs'solution. After equilibration of vessel rings were allowed for 1 hr at 1 g of resting tone. Krebs'solution was replaced every 15 min period. After an equilibration period, the arterial ring is determined for viability by adding 10 µM of phenylephrine (PE). When the contraction is stable, 10 µM of acetylcholine (ACh) was used to evaluate endothelium-dependent relaxation. When ACh promotes more than 80% relaxation, the arterial ring could be used in this experiment. Consequently, the effect of ACh was washed out by replacing of Krebs'solution every 15 min period for 1 hr. At the beginning of evaluation of vasodilator effect from *E. macrobulbon*, 10 µM of PE was added into the bath for making the contraction of vessel. After steady state of PE contraction, *E. macrobulbon* extract (1-200 µg/ml) is cumulatively added into the bath. High concentration of potassium (80 mM KCl) was added in the last step of experiment for proving of vessel alive. The protocol showed in **figure 20**.

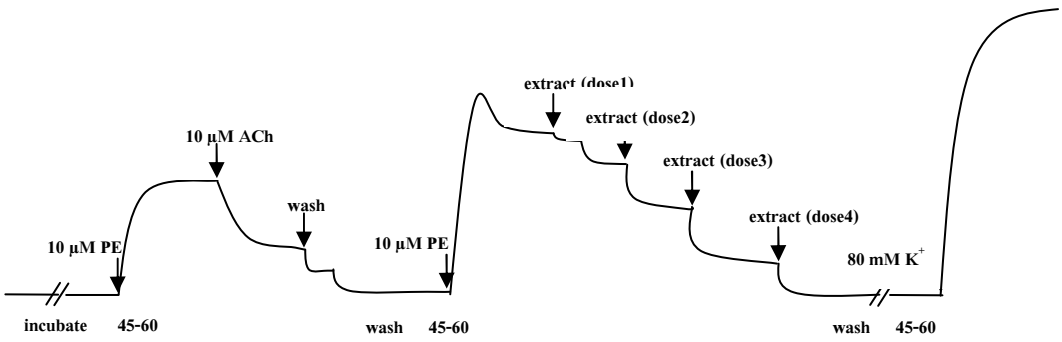


Figure 20 Vasodilator effect protocol

CHAPTER IV

RESULTS AND DISCUSSION

Screening of PDE5 inhibitory activity from some Thai medicinal plants

In this study, sixty plant extracts were screened for PDE5 inhibitory activity. In the beginning, plant extracts were selected by their ethnopharmacology or chemotaxonomy. Twenty one plants are used as sexual performance enhancers in the traditional medicines used in Northern Thailand. The other extracts came from plants of the Leguminosae family which are known to be a rich source of flavonoids and some of these have been reported to exhibit PDE5 inhibitory activity [17]. The results from these strategies of plant selection were revealed that two traditional sexual performance enhancer plants, i.e. *Caesalpinia sappan* and *Kaempferia parviflora*, two leguminoseous plants, i.e. *Acacia auriculaeformis* and *Senna surattensis* and showed moderate effects on PDE5 (60-70% inhibitions) (**Tables 7 and 8**). These extracts were further tested to determine their IC_{50}s. The selectivity on PDE5 of the sample was expressed as the high IC_{50} ratio of PDE6/PDE5 (**Table 9**). Three of these extracts showed some PDE5 selectivity similar to sildenafil (4.85) but *C. sappan* extract showed clear PDE6 discrimination. However, the potencies of these extracts were significantly lower than sildenafil. Unfortunately, from our preliminary experiment, the PDE5 inhibitors from these active plants were present in highly polar fractions which were not easy to be fractionated. Therefore, some plants were randomly selected for screening of PDE5 inhibitory activity (**Table 10**). From 19 randomized plants, 5 ethanolic plant extracts i.e. *Curcuma longa* Linn., *Curcuma zedoaria* (Berg) Roscoe, *Curcuma petiolata* Roxb., *Curcuma* sp. (นางคำ) and *Eulophia macrobulbon* (Parish & Rchb. f.) Hook. f could inhibit PDE5 more than 80% inhibition at 50 µg/ml of extracts. Curcuminoids were major components of these *Curcuma* spp. In recent year, curcumin, one of curcuminoids, was able to inhibit PDE activity as non selective PDE inhibit. It showed IC_{50} values in range of micro molar on PDE1-PDE5 isozymes. [61] Therefore, the PDE5 inhibitory activity from *Curcuma* species might be corresponding with curcumin. The one of promising plant for PDE5 inhibitors was *E.*

macrobulbon was further studied on chemical constituents using bioassay guided fractionation.

Table 7 Percentage PDE5 inhibition by ethanolic extracts from some plants used as traditional sexual performance enhancers (*n*=3) (the final concentration of the extracts was 50 μg/ml)

No.	Scientific name	Part used	%PDE5 inhibitory activity
1	*Acorus calamus* L.	Root	0.87 ± 2.49
2	*Barleria strigosa* Willd.	Whole plant	2.13 ± 5.75
3	*Berchemia floribunda* Wall.	Stem bark	7.33 ± 0.59
4	*Betula alnoides* Buch.-Ham. ex G.Don	Stem	36.40 ± 8.07
5	*Boesenbergia rotunda* (L.) Mansf.	Rhizome	40.86 ± 3.94
6	*Butea superba* Roxb.	Root bark	7.88 ± 5.59
7	*Caesalpinia sappan* L.	Stem	60.23 ± 1.81
8	*Drosera burmannii* Vahl	Arial part	4.52 ± 0.40
9	*Elephantopus scaber* L.	Whole plant	4.12 ± 0.75
10	*Hiptage benghalensis* (L.) Kurz	Stem	32.31 ± 3.77
11	*Kaempferia parviflora* Wall. ex Baker	Rhizome	62.63 ± 7.17
12	*Leea indica (Burm.f.)* Merr.	Root	31.36 ± 7.47
13	*Mucuna collettii* Lace	Stem	1.13 ± 5.06
14	*Myxopyrum smilacifolium* Blume subsp.	Root	0.46 ± 0.69
15	*Polygala chinensis* L.	Whole plant	3.85 ± 9.35
16	*Piper sarmentosum* Roxb.	Root	4.19 ± 6.70
17	*Securidaca inappendiculata* Hassk	Stem	9.50 ± 7.27
18	*Tacca chantrieri* André	Root	0.49 ± 7.75

Table 7 (cont.)

No.	Scientific name	Part used	%PDE5 inhibitory activity
19	*Tinospora crispa* (L.) Miers ex Hook.f.& Thomson	Stem	2.54 ± 7.88
20	*Talinum paniculatum* (Jacq.) Gaertn.	Rhizome	1.16 ± 4.19
21	*Ventilago denticulata* Willd.	Stem	36.43 ± 7.25

Table 8 Percentage PDE5 inhibition by ethanolic leaf extracts from some leguminoseous plants (*n=3*) (the final concentration of the extracts was 50 μg/ml)

No.	Scientific name	%PDE5 inhibitory activity
1	*Acacia auriculaeformis* A. Cunn.	73.66 ± 4.87
2	*Acacia concinna* (Willd.) DC.	28.70 ± 3.19
3	*Acacia pennata* (L.) Willd. subsp. *insuavis* (Lace) I.C. Nielsen	13.97 ± 4.62
4	*Bauhinia acuminata* L.	36.94 ± 4.23
5	*Bauhinia glauca* (Wall. ex Benth.) Benth.	23.89 ± 3.06
6	*Bauhinia winitii* Craib	45.92 ± 4.36
7	*Butea monosperma* (Lam.) Taub.	41.48 ± 0.64
8	*Caesalpinia coriaria* (Jacq.) Willd.	47.50 ± 4.67
9	*Caesalpinia sappan* L.	25.87 ± 6.49
10	*Cassia fistula* L.	10.60 ± 0.25
11	*Delonix regia* (Bojer ex Hook.) Raf.	31.03 ± 10.19

Table 8 (cont.)

No.	Scientific name	%PDE5 inhibitory activity
12	*Leucaena leucocephala* (Lam.) de Wit	29.82 ± 8.64
13	*Pithecellobium dulce* (Roxb.) Benth.	30.25 ± 0.84
14	*Samanea saman* (Jacq.) Merr.	47.59 ± 6.59
15	*Saraca thaipingensis* Cantley ex Prain	21.53 ± 2.63
16	*Senna alata* (L.) Roxb.	19.78 ± 5.29
17	*Senna siamea* (Lam.) Irwin & Barneby	19.94 ± 5.18
18	*Senna surattensis* (Burm.f.) Irwin & Barneby	65.08 ± 0.78
19	*Sesbania grandiflora* (L.) Desv.	32.97 ± 5.39
20	*Tamarindus indica* L.	55.79 ± 2.68

Table 9 IC_{50} values of some ethanolic plant extracts against PDE5 and PDE6 (*n=3*)

Plant extract	IC_{50} (µg/ml)		IC_{50} Ratio
	PDE5	PDE6	PDE6/PDE5
C. sappan	45.95 ± 3.62	4.96 ± 2.16	0.11
K. parviflora	12.24 ± 0.99	13.74 ± 1.83	1.12
A. auriculaeformis	12.72 ± 2.27	41.28 ± 3.47	3.25
S. surattensis	12.00 ± 3.68	30.45 ± 1.04	2.54

Table 10 Percentage PDE5 inhibition of some Thai medicinal plants (*n=3*) (the final concentration of the extracts was 50 μg/ml.

No.	Scientific Name	%PDE5 inhbition
1	*Asparagus racemosus* Willd.	18.94 ± 2.35
2	*Curcuma aeruginosa* Roxb.	20.74 ± 1.18
3	*Curcuma longa* Linn.	84.01 ± 1.29
4	*Curcuma zedoaria* (Berg) Roscoe	88.27 ± 4.12
5	*Curcuma petiolata* Roxb.	81.92 ± 3.53
6	*Curcuma xanthorrhiza* Roxb.	49.57 ± 4.12
7	*Curcuma* sp. (นางคำ)	81.73 ± 4.71
8	*Curcuma* sp. (หัวใหญ่)	41.78 ± 2.35
9	*Curcuma* sp. (เพชรม้า)	19.56 ± 1.76
10	*Curcuma* sp. (ม้าเหลือง)	52.10 ± 3.53
11	*Curcuma* sp. (ม้าขาว)	14.82 ± 2.24
12	*Curcuma* sp. (ม้าห้อ)	27.08 ± 3.29
13	*Curcuma* sp. (มหากำลัง)	18.69 ± 2.00
14	*Curcuma* sp. (หนุมานยกทัพ)	21.88 ± 1.76
15	*Eulophia macrobulbon* (Parish & Rchb. f. Hook. f)	86.33 ± 2.35
16	*Kaempferia rotunda*	48.79 ± 4.12
17	*Stephania pierrei* Diels	14.77 ± 1.18
18	*Stephania suberosa* Forman	68.43 ± 2.71
19	*Zingiber ottensii Valeton*	38.51 ± 2.12

SAR of 7-methoxyflavones isolated from *K. parviflora* on PDE5 inhibitory activity

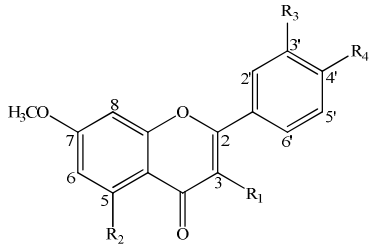
K. parviflora, a member of Zingiberaceae, was selected for further study because of its popularity as a sexual performance enhancer [18]. There are some scientific evidences for this activity. An ethanolic extract of the rhizomes improves endothelial cell function via nitric oxide production on which penile erection depends [20]. *In vivo*, the extract was able to increase blood flow to the testis [21]. However, the obvious test on the important target molecules in penile erection has not previously been undertaken. Thus we sought to show whether PDE5 inhibitory activity could be detected using both the extract and some methoxyflavone derivatives which are the major constituents in this plant [25]. Eight 7-methoxyflavones were isolated from *K. parviflora* as reported previously [62]. These compounds were tested on PDE5 and PDE6 and the results are shown in **Tables 11-12**. Compounds **2**, **4**, **6**, and **8** showed inhibitory activities against both PDE5 and PDE6 higher than 35% at 10 µM. The IC_{50}s for PDE5 and PDE6 of these compounds were further examined (**Table 12**). Among the tested flavones, **2** is the most potent PDE5 inhibitor and **8** is the most potent PDE6 inhibitor. Most noteworthy is that **2** (IC_{50} 10.64 µM) was 3-fold more potent than **8** (IC_{50} 30.41 µM) for PDE5 inhibitory activity while **8** (IC_{50} 7.83 µM) was 5-fold more potent than **2** (IC_{50} 39.45 µM) for PDE6 inhibitory activity. These IC_{50} values are in the same range as that reported from other flavonoids [16] which was much lower than sildenafil. The inhibitory selectivity of **2** and **8** is respectively expressed as the PDE6/PDE5 IC_{50} ratios of 3.71 and 0.16 while that of sildenafil is 4.85 (**Table 12**). Therefore, PDE5 and PDE6 inhibitory results of *K. parviflora* crude extract might mainly come from **2** and **8**, respectively.

The structure activity relationship (SAR) on PDE5 and PDE6 inhibitory activity of eight 7-methoxyflavones can be tentatively illustrated from the % inhibitory activity in Table 4 as follows. The fact that PDE5 and PDE6 inhibitions of **6** was much higher than **5** suggested that the methoxyl group at C-5 of 7-methoxyflavone was essential for such the activities and the substitution of the hydroxyl group at C-5 diminished the inhibition activities. The same results were also observed between **2** and **1** as well as between **4** and **3**. On the other hand, the addition of the methoxyl group at C-4' was not beneficial for PDEs inhibition as can

be seen from the similar potency between **1** and **3, 2** and **4,** as well as **5** and **7**. In addition, the methoxyl substitution at C-3 slightly decreased the PDE5 inhibitory activity while maintained the PDE6 inhibition activity as seen in **2** and **6**. Finally, it is noteworthy to mention that the methoxyl substitution at C-3' increases the inhibitory activity towards PDE6 as in **8**. The SAR of some flavonoids on PDE isozymes was studied by Ko and colleagues. Flavones could inhibit PDE5 while the other group of flavonoids such as flavonols, flavones and isoflavones had no effect on PDE5 [16]. However, this is the first report focusing on SAR of 7-methoxyflavones on PDE5.

Table 11 % Inhibitory activity of 7-methoxyflavone derivatives from *K. parviflora* on PDE5 and PDE6 (*n=3*). The final concentration of the compounds was 10 μM.

Structure



Compound	Substitution				%Inhibitory activity at 10 μM	
	R_1	R_2	R_3	R_4	PDE5	PDE6
1	H	OH	H	H	18.23 ± 3.26	-1.87 ± 1.19
2	H	OCH_3	H	H	53.65 ± 1.15	38.50 ± 1.06
3	H	OH	H	OCH_3	17.64 ± 3.19	2.45 ± 1.08
4	H	OCH_3	H	OCH_3	37.82 ± 4.08	16.63 ± 5.23
5	OCH_3	OH	H	H	0.76 ± 1.26	0.73 ± 2.34
6	OCH_3	OCH_3	H	H	44.96 ± 2.43	36.83 ± 3.45
7	OCH_3	OH	H	OCH_3	6.02 ± 5.94	0.95 ± 2.85
8	OCH_3	OCH_3	OCH_3	OCH_3	37.55 ± 2.07	58.06 ± 3.52

Table 12 IC_{50} values of 7-methoxyflavone derivatives from *K. parviflora* on PDE5 and PDE6 (*n=3*)

Compound	IC_{50} (μM)		IC_{50} Ratio
	PDE5	PDE6	PDE6/PDE5
2	10.64 ± 2.09	39.45 ± 1.00	3.71
4	37.38 ± 1.15	27.33 ± 2.46	0.73
6	16.32 ± 1.93	42.58 ± 2.11	2.61
8	30.41 ± 2.34	7.83 ± 1.12	0.26
Dipyridamole	1.46 ± 0.81	0.231 ± 0.03	0.16
Sildenafil	0.0068 ± 0.00	0.033 ± 0.01	4.85

Isolation of PDE5 inhibitors from *E.macrobulbon*

The fresh pseudo-bulbs of *E.macrobulbon* were extracted with 95% EtOH at room temperature and evaporated under reducing pressure. After evaporation, the extracts were subjected to solvent-solvent partitioning yielding n-hexane, CH_2Cl_2, EtOAc, BuOH and H_2O fractions (**Figure 16**). These fractions, together with the original EtOH extract, were tested for their PDE5 inhibitory activity using method 2 (**Figure 21**). Both CH_2Cl_2 and EtOAc fractions exhibited a high PDE5 inhibitory activity at 50 μg/ml final concentration. Moreover, it was observed that these fractions were more active than original crude EtOH extract. Hence, solvent-solvent partitioning technique was capable of concentrating PDE5 inhibitory activity into some fractions. Then, CH_2Cl_2 extracts was submitted to phytochemical isolation using bioassay guided fractionation.

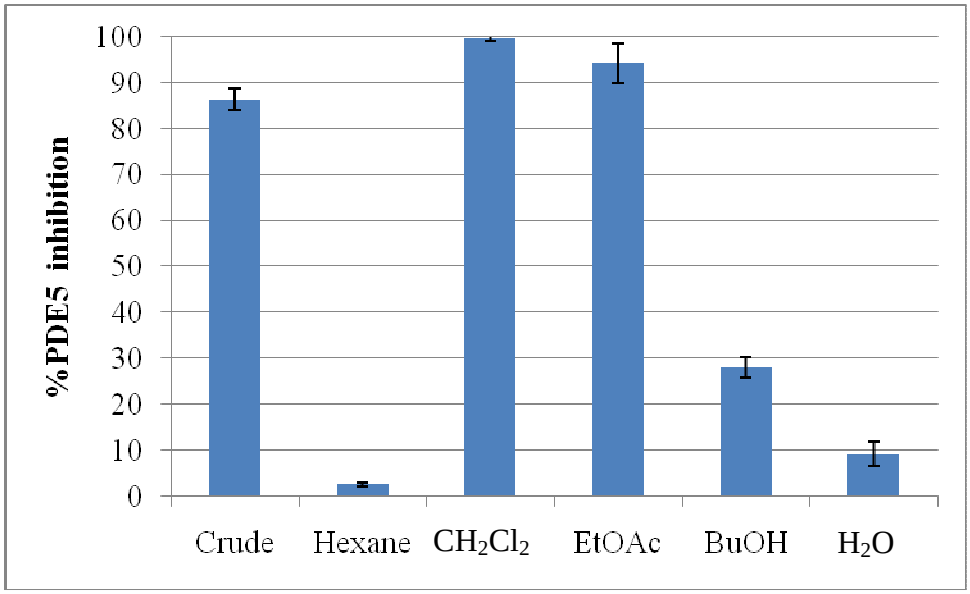
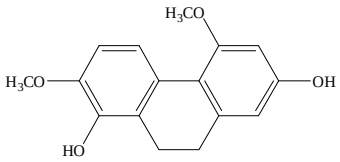
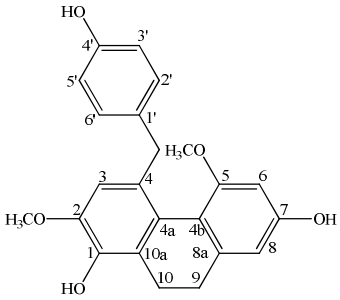


Figure 21 PDE5 inhibitory activity of crude EtOH extract of *E. macrobulbon* and its partitioned extracts at a final concentration of 50 μg/ml (*n*=3)

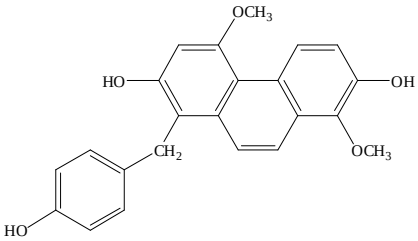
The CH_2Cl_2 extract of *E. macrobulbon* was fractionated using the combination of various chromatographic methods (**Figure 17**) to give compounds **9 - 12** which were identified as phenanthrenes (**Figure 22**). The structure elucidations of these four compounds are described as follows.



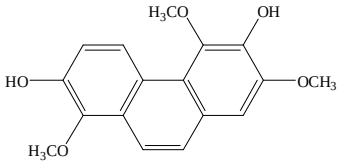
9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol **(9)**



9,10-dihydro-4-(4′-hydroxybenzyl)-2,5-dimethoxyphenanthrene-1,7-diol **(10)**



1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol **(11)**



1,5,7-trimethoxyphenanthrene-2,6-diol **(12)**

Figure 22 Structures of phenanthrenes isolated from *E. macrobulbon* extract

Compound **9** was obtained as colorless needle-shaped crystals, mp 202-203ºC [30]. The HREIMS of compound **9** exhibited a molecular ion peak at *m/z* 272.1043 suggesting the molecular formula $C_{16}H_{16}O_4$ (D.B.E. = 9). In IR spectrum, the characteristic band of hydroxyl group was shown at 3443 cm^{-1} (O-H stretching). The band at 3003 - 2948 cm^{-1} and the band at 1601 - 1431 cm^{-1} represented C-H stretching and C=C stretching of aromatic rings, respectively. ^{1}H and ^{13}C NMR spectral data of **9** are shown in **Table 13**. 2D NMR including COSY, HMQC, HMBC and NOESY experiments were performed. The degree of protonation of each carbon was measured by DEPT experiment. In ^{1}H NMR spectrum, four protons at δ 2.61 (dd, *J* = 4.4, 8.1 Hz), 2.62 (br.d, *J* = 8.1 Hz), 2.74 (dd, *J* = 4.4, 8.1 Hz) and 2.74 (br.d, *J* = 8.1 Hz) which are typical H-9 and H-10 signals of 9, 10 dihydrophenanthrenes were observed. The presence of two 3H-singlet at 3.82 and 3.85 was referred to two methoxy groups. A pair of doublets at δ 6.39 and 6.45 with a coupling constant of 1.98 indicated the *meta* coupling between H-8 and H-6, while a pair of doublets at δ 6.78 and 7.73

showed *ortho* coupling of the protons of H-3 and H-4. In addition, HMBC was used for creation of the ring substitution patterns and linkage site (**Figure 23 (a)**). The confirmation of assignment of the methoxy groups was based on the observation in NOESY experiment (**Figure 23(b)**). These spectra were compared with reported data, and the structure was thus identified as 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol (**9**) which was previously found in pseudo-bulb of *E. nuda* [30]. However, this is the first report on the isolation of **9** from *E. macrobulbon*.

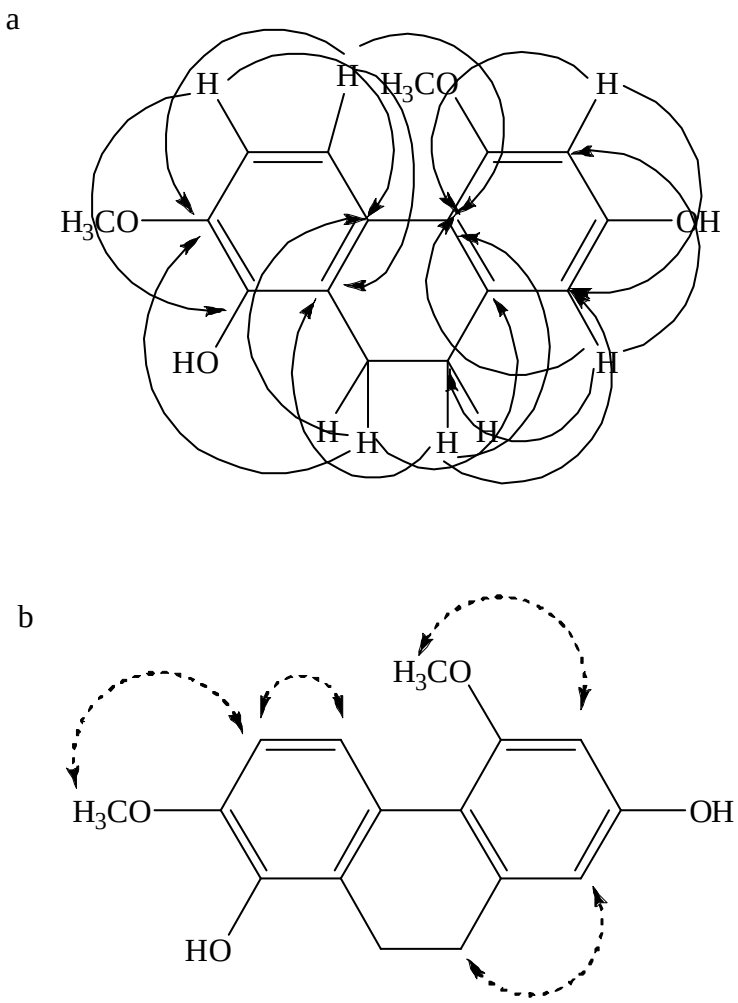


Figure 23 Diagnostic HMBC (a) and NOESY (b) correlations of 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol (9)

Table 13 ^{1}H -NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data (δ in ppm) of 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol (9) (in acetone-d_6)

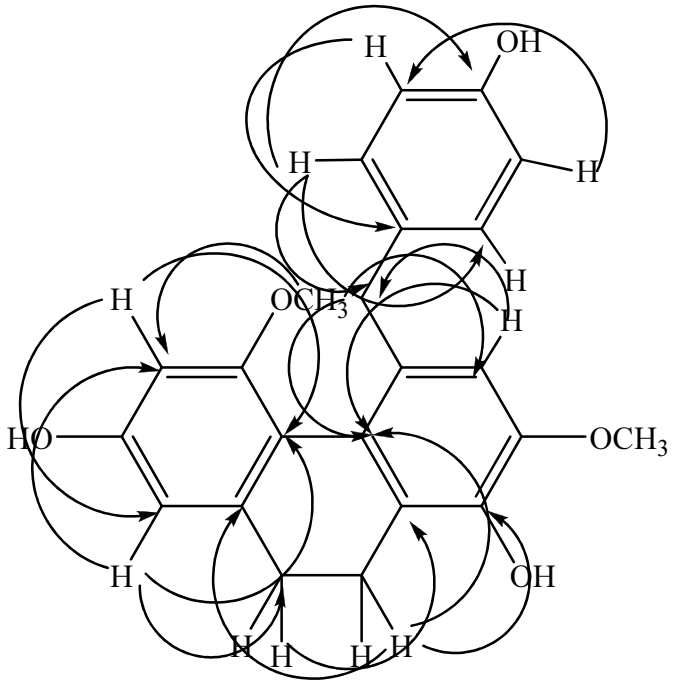
Position	Sub-position	δ_C	C-species	δ_H, (multiplicity, J in Hz)	HMBC	NOESY
1	-	142.85	C	-	-	-
2	-	146.02	C	-	-	-
3	-	108.98	CH	6.78 (1H, d, $J_{3,4}$ = 8.4)	C-1, C-2, C-4a,C-4b, C-10a	C-2-OC<u>H</u>$_3$, H-4
4	-	120.16	CH	7.73 (1H, d, $J_{4,3}$ = 8.4)	C-2, C-3, C-4b, C-9a,C-10, C-10a	H-3
4a	-	127.61	C	-	-	-
4b	-	116.33	C	-	-	-
5	-	159.10	C	-	-	-
6	-	99.15	C	6.46 (1H, d, $J_{6,8}$ = 2.4)	C-4a, C-5, C-4b, C-7,C-8	C-5-OC<u>H</u>$_3$
7	-	157.57	C	-	-	-
8	-	108.20	CH	6.38 (1H, d, $J_{8,6}$ = 2.4)	C-4a, C-4b, C-6, C-7,C-9	H-9
8a	-	141.59	C	-	-	-
9	9′	30.99	CH_2	2.62 (1H, br.d, J = 8.1)	C-4b, C-7,C-8, C-9a,C-10 C10a	H_{-8}
	9′′	30.99		2.61 (1H, dd, J = 4.4, 8.1)	C4b, C7,C8, C9a,C10 C10a	H_8
	10′′	22.18		2.75 (1H, dd, J = 4.4, 8.1)	C2, C4a, C8, C9, C9a, C10a	-

Table 13 ^{1}H (cont.)

Position	Sub-position	δ_C	C-species	δ_H, (multiplicity, *J* in Hz)	HMBC	NOESY
10	10′	22.18	CH_2	2.74 (1H, br.d, *J* = 8.1)	C-2, C-4a, C-8, C-9, C-8a, C-10a	-
10a	-	124.76	C	-	-	-
C-2-O<u>C</u>H$_3$	-	56.24	C	3.85 (3H, s)	C-2	H-3
C-5-O<u>C</u>H$_3$	-	55.71	C	3.82 (3H, s)	C-5	H-6

The HREIMS data of compound **10** exhibited a molecular ion peak at *m/z* 378.1478 which represented MH^+ for $C_{23}H_{22}O_5$ (D.B.E. = 13). IR spectrum showed a typical characteristic band of hydroxyl group at 3338 (O-H stretching) and aromatic at 1614, 1516 (C=C stretching). The whole NMR spectrum of **10** was summarized in **Table 14**. The ^{13}C NMR spectrum of **10** in methanol-d_4 showed 21 peaks which represented 23 carbon atoms. The carbons were determined as two methyl, three methylene, seven methine and eleven quaternary carbons by DEPT and HSQC experiments. The ^1H NMR indicated four proton at δ 2.04, 3.20, 2.38, 2.61 (ddd) which are typical of the protons of the two methylene groups of 9, 10 dihydrophenanthrene derivatives. Two methoxyl groups were observed at δ 3.62 and 3.69. The presence of two doublets for benzylic-CH_2 at δ 3.72 and 3.81 (J = 15.0 Hz) indicated a benzyl substitution. A pair of doublets (2H) at δ 6.81 and 6.61 with *ortho* coupling constant of 8.4 Hz were assigned to H-2′, H-6′ and H-3′, H-5′, respectively. These data suggested a *para*-substituted aromatic. One singlet aromatic proton signal at δ 6.47 was found as well as a pair of doublets at δ 6.36 and 6.38 with a coupling constant of 2.0 Hz (*meta* coupling of H-3 and H-1, respectively). The ^1H and ^{13}C assignments were based on HMBC and NOESY experiments (**Figure 24**). The presence of a cross peak between H-3 and benzylic-CH_2 in HBMC experiment could support the connectivity between 1-4 hydroxybenzyl group and phenanthrene structure at C-4. The methoxy positions in the structure were assigned using NOESY (**Figure 24 b**). Methoxy at δ 3.62 was located at C-5 since it displayed cross peaks with H-6 while the other methoxy (δ 3.69) showed a cross peak with H-3 in NOESY experiment suggesting that it located at C-2. Finally, this compound (**10**), was unambiguously identified as 9,10-dihydro-4-(4′-hydroxybenzyl)-2,5-dimethoxyphenanthrene-1,7-diol, a new 9,10-dihydrophenanthrene derivatives.

a



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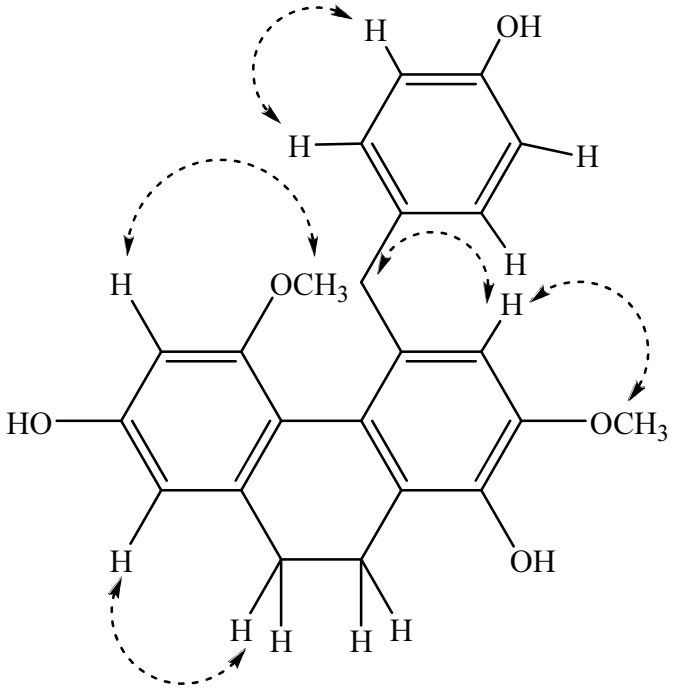


Figure 24 Diagnostic HMBC (a) and NOESY (b) correlations of 9,10-dihydro-4-(4′-hydroxybenzyl)-2,5-dimethoxyphenanthrene-1,7-diol (10)

Table 14 ^{1}H -NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data (δ in ppm) of 9,10-dihydro-4-(4′-hydroxybenzyl)-2,5-dimethoxyphenanthrene-1,7-diol (10) (in methanol-d_4)

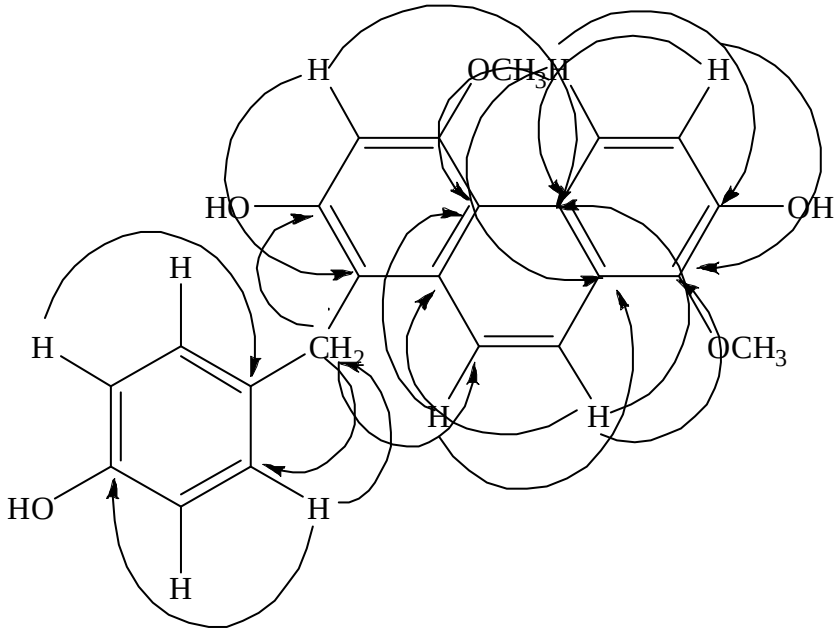
Position	Sub-position	δ_C	C-species	δ_H, (multiplicity, J in Hz)	HMBC	NOESY
1	-	144.41	C	-	-	-
2	-	147.10	C	-	-	-
3	-	112.07	CH	6.47 (H, s, H-3)	C-4-$\underline{C}H_2$, C-2, C-4, C-4a, C-4b, C-10a	C-4-C$\underline{H}_2$, C-2-OC$\underline{H}_3$
4	-	140.98	C	-	-	-
4a	-	132.69	C	-	-	-
4b	-	117.34	C	-	-	-
5	-	158.30	C	-	-	-
6	-	98.55	CH	6.36 (H, d, $J_{6,8}$ = 2.0)	C-4b, C-5, C-8	C-7-OC$\underline{H}_3$
7	-	158.09	C	-	-	-
8	-	107.73	CH	6.38 (H, d, $J_{8,6}$ = 2.0)	C-4b, C-6, C-7, C-9	H-9
8a	-	127.36	C	-	-	-
9	9′	32.15	CH_2	2.38 (H, ddd, J = 14.7, 14.0, 4.0)	C-1, C-4b, C-7, C-8, C-10, C-10a	-
	9′′			2.61 (H, ddd, J = 14.0, 4.0, 2.4)	C-1, C-4b, C-8, C-10, C-10a	-

Table 14 (cont.)

Position	Sub-position	δ_C	C-species	δ_H, (multiplicity, *J* in Hz)	HMBC	NOESY
10	10′	24.39	CH_2	2.04 (H, ddd, *J* = 14.5, 14.7, 4.0)	C-1, C-2, C-4, C-8a, C-9	-
	10′′			3.20 (H, ddd, *J* = 14.5, 4.0, 2.4)	C-1, C-4, C-8a, C-9	-
10a	-	127.03	C	-	-	-
1′	-	135.48	C	-	-	-
2′	-	131.01	CH	6.81 (H, d, $J_{2',3'}$ = 8.4)	C-4-$\underline{C}H_2$, C-3′, C-4′,C-6′	H-3′
3′	-	115.83	CH	6.61 (H, d, $J_{3',2'}$ = 8.4)	C-1′, C-2′, C-4′ , C-5′	H2′
4′	-	156.03	C	-	-	-
5′	-	115.83	CH	6.61 (H, d, $J_{5',6'}$ = 8.4)	C-1′, C-3′, C-4′ ,C-6′	-
6′	-	131.01	CH	6.81 (H, d, $J_{6',5'}$ = 8.4)	C-4-$\underline{C}H_2$, C-2′, C-4′, C-5′	-
C-4-$\underline{C}H_2$	-	40.40	benzylic-CH_2	3.73 (H, d, *J*= 15.0)	C-3, C-4a, C-10a, C-1′,C-2′	H-3
	-			3.81 (H, d, *J*= 15.0)	C-3, C-4a, C-10a, C-1′,C-2′	H-3
C-2-O$\underline{C}H_3$	-	56.21	C	3.69 (3H, s,)	C-2	H-3
C-5-O$\underline{C}H_3$	-	55.29	C	3.62 (3H, s)	C-5	H-6

The HREIMS data of compound **11** disclosed a molecular ion peak at *m/z* 376.1307 which represented MH^+ for $C_{23}H_{20}O_5$ (D.B.E. = 14). The IR spectrum showed O-H stretching band at 3338cm^{-1} and C=C of aromatic streching band at 1614and 1516 cm^{-1}. The summarized data from NMR was illustrated in **Table 15**. From ^{13}C NMR spectrum, 21 carbon peaks processed 23 carbon atoms. In DEPT and HSQC experiments, the carbons were determined as two methyl, one methylene, nine methine and eleven quaternary carbons. Unlike compounds 1 and 2, the signals of two methylene protons which are the characteric of 9,10 dihydrophenanthrene were missing. This suggested that **11** was 9,10 dehydrophenanthrene derivative. As for substitution groups, benzylic-CH_2 (δ 4.22) was observed in **11**. The presence of two aromatic methoxyl groups in **11** was evident from three-proton singlet at δ 3.84 and 4.01. Furthermore, the ^1H NMR displayed two pairs of doublet protons at δ 7.79 and 7.86 and δ 9.12 and 7.16 with *ortho* coupling constant of 9.4 and 9.5 Hz, respectively. They were assigned to H-10, H-9 and H-5, H-6, respectively. The singlet proton at δ 6.93 which belong to phenanthrene skeleton was observed. In the benzyl unit, doublet signals of H-3′ and H-5′ as well as that of H-2′ and H-6′ were magnetically equivalent as they resonated at the same chemical shifts (δ 6.61; H-3′, H-5′ and δ 6.97; H-2′, H-6′). Both signals showed the *ortho*-coupling constant of 8.5. It suggested that the benzyl moiety should be *para*-substituted. The assignment of the position of the methoxy and other structural connectivities were finally accomplished by using HMBC and (**Figure 25a**) and NOESY (**Figure 25b**) experiments. The connectivity between of benzyl and phenanthrene structure was deduced from the cross peak between proton of benzylic-CH_2 and C-2 and C-10. In addition, the arrangement of benzyl substitution had also been confirmed by the evidence of the cross peak between H-6′ and H-10 in NOESY spectra. Therefore, **11** was identified as 1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol. This compound had been reported in *E. nuda* [30] but this is the first finding in *E. macrobulbon*.

a



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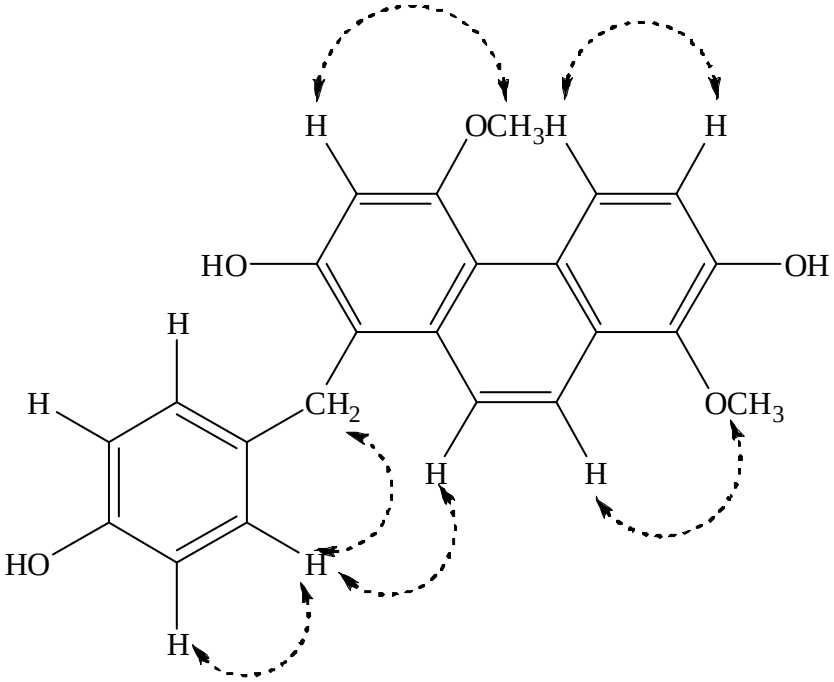


Figure 25 Diagnostic HMBC (a) and NOESY (b) correlations of 1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol (11)

Table 15 ^{1}H -NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data (δ in ppm) of (1-(4′-hydroxybenzyl)-4,8-dimethoxyphenanthrene-2,7-diol) (in dimethylsulfoxide-d_6) (11)

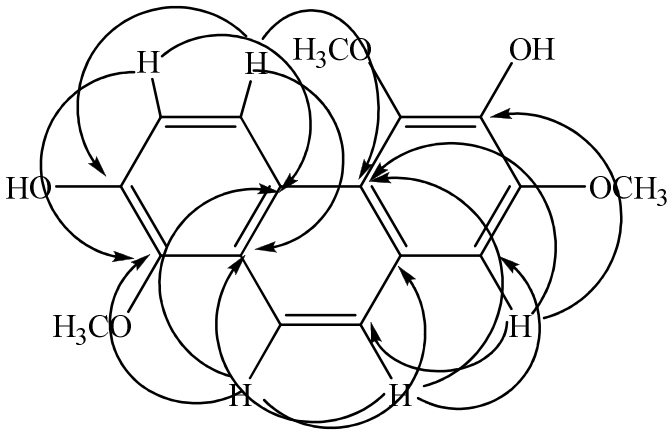
Position	δ_C	C-species	δ_H, (multiplicity, J in Hz)	HMBC	NOESY
1	113.43	C	-	-	-
2	152.84	C	-	-	-
3	99.67	CH	6.93 (1H, s, H-3)	C-1, C-2, C-4, C-4b	C-4-OC$\underline{H}_3$
4	157.08	C	-	-	-
4a	125.93	C	-	-	-
4b	124.31	C	-	-	-
5	123.76	CH	9.12 (1H, d, $J_{5,6}$ = 9.4)	C-4a, C-7, C-8, C-8a	H-6
6	117.56	CH	7.16 (1H, d, $J_{6,5}$ = 9.4)	C-4b, C-7, C-8, C-8a	H-5
7	145.44	C	-	-	-
8	140.86	C	-	-	-
8a	114.41	C	-	-	-
9	120.40	CH	7.86 (1H, d, $J_{9,10}$ = 9.5)	C-4a, C-4b, C-8, C-8a, C-10a	C-8-OC$\underline{H}_3$
10	123.11	CH	7.79 (1H, d, $J_{10,9}$ = 9.5)	C-4a, C-4b, C-8a, C-10a	H-6′
10a	132.03	C	-	-	-
1′	131.80	C	-	-	-

Table 15 (cont.)

Position	δ_C	C-species	δ_H, (multiplicity, *J* in Hz)	HMBC	NOESY
2′	128.84	CH	6.97 (1H, d, $J_{2',3'}$ = 8.5)	C-1-CH_2, C-4′, C-6′	C-1-CH_2, H-3′
3′	114.82	CH	6.61 (1H , d, $J_{3',2'}$ = 8.5)	C-1′, C-2′, C-4′, C-5′	H-2′
4′	155.07	C	-	-	-
5′	114.82	CH	6.61 (1H , d, $J_{5',6'}$ = 8.5)	C-1′, C-3′, C-4′, C-6′	H-6′
6′	128.84	CH	6.97 (1H , d, $J_{6',5'}$ = 8.5)	C-1-CH_2, C-4′, C-2′	H-10, C-1-CH_2, H-5′
C-1-$\underline{C}H_2$	29.01	benzylic-CH_2	4.22 (2H, s)	C-1, C-2, C-10a, C-1′, C-2′, C-6′	H-2′ or H-6′
C-4-O$\underline{C}H_3$	55.36	C	4.01 (3H, s)	C-4	H-3
C-8-O$\underline{C}H_3$	60.42	C	3.84 (3H, s)	C-8	H-9

The EIMS of **12** exhibited a molecular ion at *m/z* 300 corresponding to the molecular formula of $C_{17}H_{16}O_5$ (D.B.E=10). The NMR spectral data of **12** are summarized in **Table 16**. The ^{1}H NMR spectrum indicated resonances for three methoxy groups at δ 3.86 (s, C5-OCH_3), 3.93 (s, C1-OCH_3) and 4.01 (s, C7-OCH_3) and five aromatic protons. The signals of a pair of *ortho* coupling protons at δ 7.17 and 9.09 were assigned to H-3 and H-4, respectively while another pair of *ortho* coupling proton were assigned to H-9 and H-10 (δ 7.62 and 7.83). In addition, the presence of downfield aromatic proton (9.09 ppm) of H-4 of 9,10-dehydro-phenanthrene derivatives implied that C-5 might be substituted with hydroxyl or methoxyl group [63]. The remaining one aromatic proton appeared as singlet at δ 7.18. The ^{13}C NMR spectrum of **12** showed only 9 carbon peaks due to small amount of the sample. However, complete carbon peaks could be obtained from HMBC experiment. The carbons were determined as three methyl and five methine carbons by DEPT and HSQC experiments whereas nine quaternary carbons were found from HMBC experiment. Among the nine quaternary carbons, five downfield signals at δ 149.00, 147.72, 145.80, 142.50 and 141.23 were contributed to the three methoxyl and two hydroxyl-substituted aromatic carbons. The connectivity was based on HMBC and NOESY experiments. The assignments of quaternary carbons in ring B of phenanthrene were deduced from the cross peaks found between H-3 (δ 7.17) and C-4a (δ 125.00), H-4 (δ 9.09) and C-5a (δ 120.39) and C-10a (δ 128.89), H-9 (δ 7.62) and C-10a (δ 128.89), H-10 (δ 7.83) and C-9a (δ 126.96) (**Figure 26a**). The NOESY spectra showed the correlation between the three methoxy protons with aromatic protons (**Figure 26b**). This evidence was useful for the assignments of methoxy groups in the structure. On the basic of the above data, **12** was finally identified as 1,5,7-trimethoxyphenanthrene-2,6-diol. This compound was reported for the first time from *E. macrobulbon*. Previously, it was described as the component in the other orchids i.e., *E. nuda* [30], *Dendrobium densiflorum* [64] and *D. thyrsiflorum* [65].

a



b

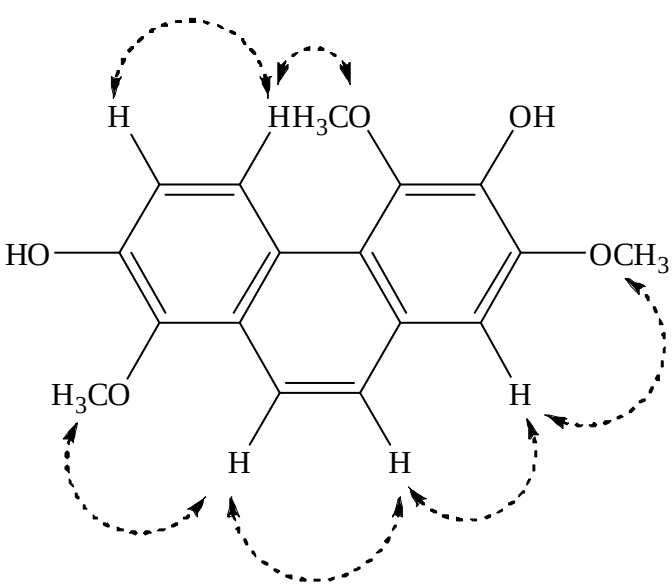


Figure 26 Diagnostic HMBC (a) and NOESY (b) correlations of (1,5,7-trimethoxyphenanthrene-2,6-diol) (12)

Table 16 ^{1}H -NMR (400 MHz) and ^{13}C NMR (100 MHz) spectral data (δ in ppm) of (1,5,7-trimethoxyphenanthrene-2,6-diol) (in methanol-d_4) (12)

Position	δ_C	C-species	δ_H, (multiplicity, J in Hz)	HMBC	NOESY
1	142.50	C	-	-	-
2	147.72	C	-	-	-
3	117.98	CH	7.17 (1H, d, $J_{3,4}$ = 8.8)	C-1, C-2, C-4a	H-4
4	124.45	CH	9.09 (1H, d, $J_{4,3}$ = 8.8)	C-1, C-2, C-4b, C-10a	C-5-OC$\underline{H}_3$, H-3
4a	125.00	C	-	-	-
5a	120.39	C	-	-	-
5	145.80	C	-	-	-
6	141.23	C	-	-	-
7	149.00	C	-	-	-
8	106.09	CH	7.18 (1H, s)	C-5, C-4b, C-6, C-7, C-9	C-7-OC$\underline{H}_3$, H-9
9a	126.96	C	-	-	-
9	128.25	CH	7.62 (1H, d, $J_{9,10}$ = 9.0)	C-4a, C-4b, C-8, C-8a, C-10, C-10a	H-8, H-10
10	118.80	CH	7.83 (1H, d, $J_{10,9}$ = 9.0)	C-1, C-4a, C-9, C-9a, C-10a	H-9, C-1-OC$\underline{H}_3$,
10a	128.89	C	-	-	-

Table 16 (cont.)

Position	δ_C	C-species	δ_H, (multiplicity, *J* in Hz)	HMBC	NOESY
C-1-O<u>C</u>H$_3$	61.36	C	3.93 (3H, s)	C-1	H-10
C-5-O<u>C</u>H$_3$	59.77	C	3.87 (3H, s)	C-5	H-4
C-7-O<u>C</u>H$_3$	56.34	C	4.01 (3H, s)	C-7	H-8

PDE5 inhibitory activity of isolated phenanthrenes from *E. macrobulbon*

From preliminary screening, *E. macrobulbon* expressed a promising activity on PDE5 enzyme. This plant had not never been reported for bioactivities and chemical constituents yet. Therefore, *E. macrobulbon* was selected for the further study. Most of chemical constituents of relative species i.e., *E. nuda* [27-31], *E. petersii* [32] and *E. ochreata* [33,66] were phenanthrene compounds. Some phenanthrenes were reported on pharmacological properties, for example, cytotoxicity, antioxidant, antiplatelet aggregation and smooth muscle relaxant [67]. The elevation of cGMP in cell causes smooth muscle relaxation thus phenanthrenes might relate to the increasing of cGMP level in the cell which is regulated by PDE5. The isolated phenanthrenes from *E. macrobulbon*, therefore, were tested for PDE5 inhibitory activity and its selectivity over PDE6 and PDE1(**Table 17**). Compounds **10**, **11** and **12** showed inhibitory activities against both PDE5 and PDE6 higher than 70% at 50 μg/ml. The IC_{50}s for PDE5 and PDE6 of these compounds were further examined (**Table 18**). Among the tested compounds, **11** was the most potent PDE5 and PDE6 inhibitor. All compounds could not inhibit PDE1 which is abundant in heart [68]. From this data, it could be implied that these compounds have no effect on heart. Sildenafil (IC_{50} 0.03 μM) was only 55-fold more potent than **11** (IC_{50} 1.67 μM) for PDE5 inhibitory activity while the IC_{50} values of other compounds were much lower than sildenafil (> 2000 fold). The inhibitory selectivity of **11** was slightly less than sildenafil (**Table 18**) while compound (**10**) showed more selectivity on PDE5 over PDE6 (PDE6/PDE5 IC_{50} ratio of 1.51).

According to the limited numbers of the compounds, the structure activity relationship (SAR) could not be concluded from these experiments. We noticed that the aromaticity properties of the compound might be possible for PDE5 and PDE6 inhibitory activity. However, it requires the further study for the complete SAR.

Table 17 % Inhibitory activity of isolated phenanthrenes from *E. macrobulbon* on PDE5, PDE6 and PDE1 (*n=3*) (the final concentration of the tested compounds was 50 μg/ml)

Compound	%Inhibitory activity at 50 μg/ml		
	PDE5	PDE6	PDE1
9	44.58 ± 4.13	66.18 ± 8.68	6.25 ± 3.84
10	78.47 ± 3.59	70.13 ± 4.26	25.53 ± 1.27
11	99.23 ± 0.48	104.21 ± 0.24	0.59 ± 2.80
12	72.30 ± 6.80	87.21 ± 4.83	5.80 ± 3.25

Table 18 IC_{50} values of isolated phenanthrenes from *E. macrobulbon* on PDE5 and PDE6 (*n=3*)

Compound	IC_{50}s (μM)		IC_{50} Ratio
	PDE5	PDE6	PDE6/PDE5
10	62.26 ± 3.32	94.11 ± 3.94	1.51
11	1.67 ± 0.54	1.82 ± 0.07	1.09
12	98.07 ± 13.29	50.98 ± 3.84	0.52
Sildenafil	0.03 ± 0.01	0.05 ± 0.02	1.83

Relaxant effect on rat pulmonary artery from *E. macrobulbon*

Recently, PDE5 has been approved for a new indication for treatment of pulmonary artery hypertension (PAH). This suggests us that *E. macrobulbon* might affect to the relaxation of pulmonary artery. The crude EtOH extract of *E. macrobulbon* was investigated for relaxant effect on rat pulmonary artery using organ bath technique. The intact pulmonary artery was used in this study. *E. macrobulbon* extract were cumulatively added into the bath to make the final concentrations of 1-200 µg/ml. The result showed that the intact vessels showed complete relaxation at all the concentration of *E. macrobulbon* extract (**Figure 27a**) while the solvent (DMSO) had no effect on the vessel (**Figure 27b**). The EC_{50} of the extract was calculated as 41.83±8.40 µg/ml (**Figure 27c**). From the literature, some phenanthrenes i.e., 3,4'-dihydroxy-5,5'-dimethoxybibenzyl phenanthrene and 4'-dihydroxy-5,5'-dimethoxybibenzyl phenanthrene had vasorelaxant effect on rat ilium ring [69]. Therefore, this relaxant effect on rat pulmonary artery might be from phenanthrenes which were the major constituents of *E. macrobulbon*. The further study on the mechanism of *E. macrobulbon* and its constituents on pulmonary artery can be useful.

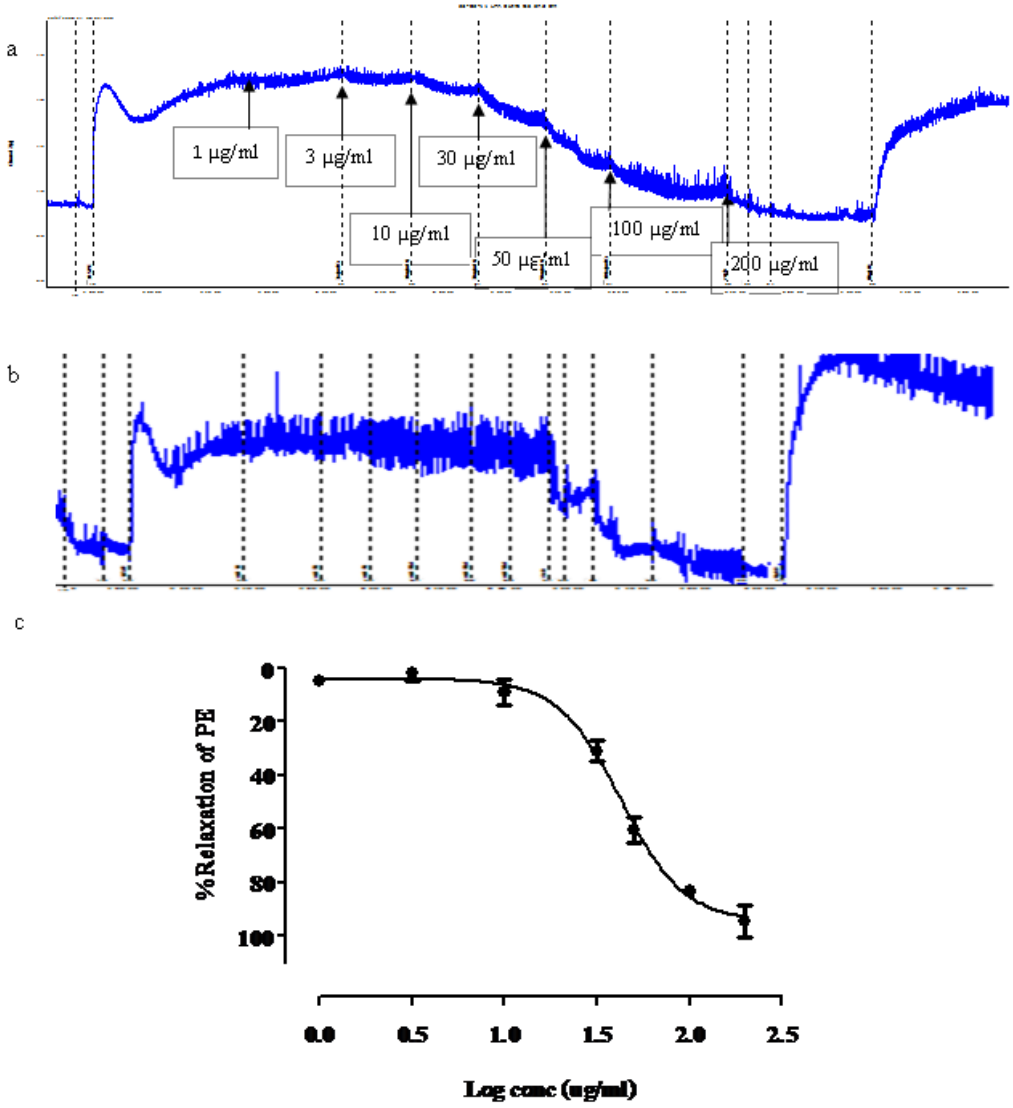
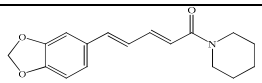
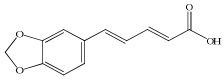
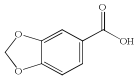


Figure 27 A typical trace of vasorelaxant effect of *E. macrobulbon* extract (1-200 ug/ml) (a), a typical trace of vasorelaxant effect of DMSO (0.2% v/v in bath) (b), Log concentration – relaxation curve of *E. macrobulbon* extract (c)

The investigation of piperine alkaloid on PDEs inhibitory activity

The medicinal plants used as tonic and/or aphrodisiac agents might be a promising source for PDE inhibitors especially PDE5 inhibitors. Therefore, piperine (**13**) which is a major constituent in *Piper nigrum* L [70]. was explored for PDE inhibitory activity. In addition, its derivatives including piperic acid (**14**), the carboxylic acid derivative of piperine and piperonylic acid (**15**), the non-unsaturated double bond derivative of **14** were tested for the observation of the influence of amide functional group and unsaturated double bond on the PDE activity, respectively. The mixture of crude PDEs (which mainly contained of PDE1 isozyme) were used as the preliminary screening. From the results (**Table 19**), **13** could not inhibit this enzyme. When piperidine ring of **13** was hydrolyzed, the %PDEs inhibition was a slightly increased while the reducing of double bond on carbon chain decreased the % inhibition. As **13** and its derivatives gave poor % inhibitory activity on crude PDE enzyme, it is likely that compounds **13-15** might not contain PDE5 inhibitory activity. As for the traditional claim of *P. nigrum* as tonic and aphrodisiac agent, some other mechanisms might be involved; for example, NO production which also stimulates the erection as there were some other components which were responsible for the acticvity [71]. The further study is needed to prove the efficacy of *P. nigrum* and it chemical constituent. .

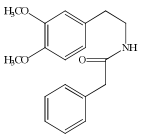
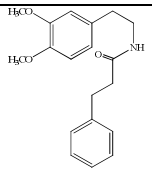
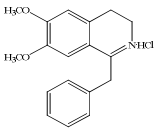
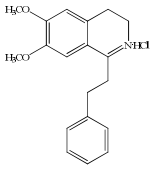
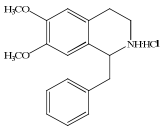
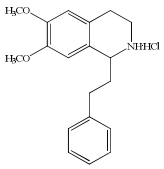
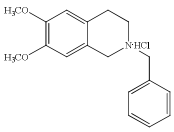
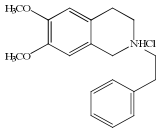
Table 19 The PDEs inhibitory activity of piperine derivatives (*n=3*). The samples were tested at a final concentration of 100 μM

Compounds	%PDEs inhibition
13 	-0.80 ± 3.03
14 	16.85 ± 1.40
15 	1.84 ± 0.18

The investigation of isoquinoline derivatives on PDEs inhibitory activity

From literature reviews, papaverine, isoquinoline derivatives, was a non selective PDE inhibitor [34]. Although, papaverine has some undesirable effects such as priapism (prolong erections) and cavernous fibrosis, intracavernous injection of papaverine has been shown to be an effective means of treatment of ED [35,72] . Isoquinoline structure, therefore, might be a good lead for development of PDE5 inhibitors. In the previous study, isoquinoline derivative have been synthesized (Figure 18 in Chapter 3). Amide and DHIQ analogs were the intermediate substrates in process of THIQ synthesis. The two series of the amide, DHIQ and THIQ with different substitution at 1 position (**16-23**) were investigated on PDEs inhibitory activity to prove the suitable structure (**Table 20**). In comparison to the flexible molecule (**16**, amide) with the similar substitution, the conformational restriction molecule of isoquinoline derivative showed higher PDEs inhibitory activity. The partially reduced isoquinoline (**17**) showed significant improvement in activity over the THIQ analogs (**18**). The same result was also observed in other series of isoquinoline derivatives (**20-22**). These data confirmed that isoquinoline ring might play a crucial role on PDEs inhibitory activity. All DHIQ derivatives showed the higher PDEs activity their corresponding THIQ. In addition, moving of C-1 substituted in THIQ derivatives (**18**, **22**) to N-2 substituted in THIQ (**19**, **23**) improved PDEs inhibitory activity approximately 3-4 folds. These results might be explained by (1) the conformational flexibility of the nitrogen containing ring could render the phenyl ring into the different position and/or (2) the basicity property of nitrogen atom might have effect on binding site of enzyme. Moreover, the addition of alkyl space length between C-1 position and aromatic moiety with two methylene unit seemed to diminish the activity by 2 folds as can be seen in **17** and **21**. The same results were notable in comparison of **18** and **22** and of **19** and **23**, respectively. From this observation, the variation of C-1 position might affect to PDEs inhibition. In order to confirm these results and to understand the relationship between C-1 position of isoquinoline ring and PDEs inhibitory activity, more DHIQ structures with different substitution at position-1 were investigated on this enzyme (**Table 21)**.

Table 20 The PDEs inhibitory activity of amide, DHIQ and THIQ derivatives (*n=3*) (the samples were tested at a final concentration of 100 μM)

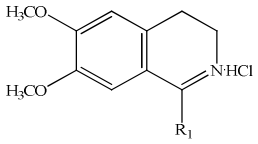
Group	Compounds		%PDEs inhibition	Compounds		%PDEs inhibition
Amide	**16**		2.36 ± 0.34	**20**		4.03 ± 0.10
DHIQ	**17**		68.32 ± 4.22	**21**		34.65 ± 2.38
THIQ	**18**		17.13 ± 2.23	**22**		7.50 ± 1.17
	19		44.39 ± 2.51	**23**		28.38 ± 2.10

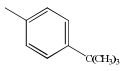
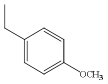
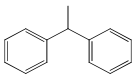
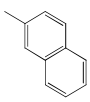
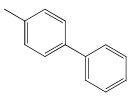
As DHIQ showed the highest PDEs inhibitory activity among the tested compounds, we put emphasized on DHIQ structure. The various substituents on position 1 of DHIQ were explored on PDEs inhibitory activity as can be seen in **Table 21**. When 1-benzyl (**17**) at C-1 position of DHIQ was replaced with small and less lipophilic group, 1-methyl (**24**) and 1-cyclohexyl (**25**), the PDEs inhibitory activities were decreased. This result suggested that the PDEs inhibition might prefer the aromatic moiety at position-1 in the optimal distance. The steric effect of substitutes at position-1 of DHIQ was also observed. The PDEs inhibition of 1-(4′methoxyl)-benzyl DHIQ (**28**) slightly decreased compare to the unsubstituted compound (**17**) whereas the replacement of 1-(3chlorophenyl) DHIQ (**26**) increased the PDEs inhibition. Similar result was also found in the **30** which was 1-(napthalen-1-yl) substitute on C-1 position of DHIQ. However, the replacement of 1-(napthalen-2-yl)

(**31**) expressed lower PDEs inhibitory activity. As the result, it was likely that the substitution at position-1 needed the bulky group but it should be in proper orientation.

Table 21 The PDEs inhibitory activity of DHIQ derivatives (*n=3*)

Structure



Compound	R_1	%PDEs Inhibition
17		68.32±4.22
21		34.65 ± 2.38
24	-CH_3	43.71±4.85
25		33.95±5.58
26		81.96±2.08
27		1.12±1.36
28		43.27±4.60
29		2.04±10.55
30		70.58±5.08
31		44.59±2.26
32		10.76±5.08

The investigation of isoquinoline derivatives on PDE5 inhibitory activity

Due to the different binding site between PDEs crude enzyme (which mainly composed of PDE1) and PDE5, all data above might not be fit with PDE5 enzyme. All DHIQ and THIQ compounds were explored on the PDE5 inhibitory activity, after the PDE5 assay had been successfully established in our laboratory. The results showed in **Table 22**. DHIQ still processed the higher PDE5 inhibition than THIQ. The reason of these observations might depend on (1) weaker base of DHIQ which had more electron density around nitrogen atom and (2) more rigid conformation of DHIQ. The variation of PDE5 inhibition when C-1 position of DHIQ was various suggesting that the appropriate proportion of binding of each compound with active pocket of PDE5 enzyme was a crucial point. This hypothesis could be further confirmed using modeling. The compounds that gave %inhibition >70% were further investigated on IC_{50}s and selectivity over PDE6 (**Table 23**). The result showed that DHIQ showed less potent than sildenafil, a standard PDE5 inhibitor. In addition, there were no compound processed the PDE5 selectivity over PDE6.

Table 22 The PDE5 inhibitory activity of DHIQ and THIQ derivatives (*n=3*)

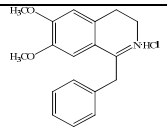
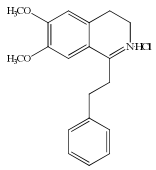
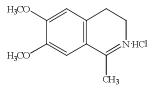
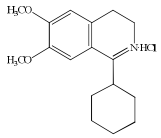
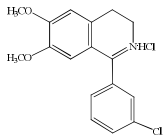
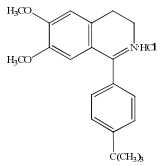
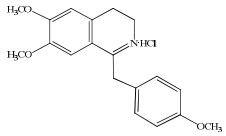
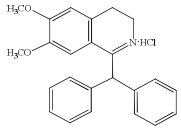
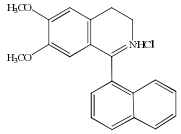
Group	Compounds		%PDE5 inhibiton at 100 μM
DHIQ	**17**		18.12±2.10
	21		15.48±0.38
	24		4.52±1.14
	25		44.07±6.81
	26		80.44±6.12
	27		46.83±1.45
	28		65.86±3.04
	29		41.38±2.26
	30		66.34±2.45

Table 22 (cont.)

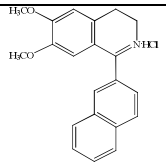
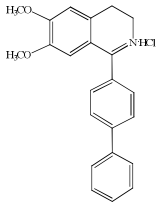
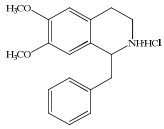
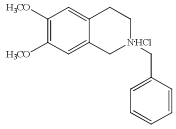
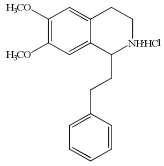
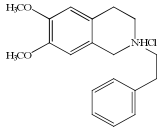
Group	Compounds		%PDE5 inhibiton at 100 µM
THIQ	**31**		81.17±3.45
	32		72.10±1.00
	18		6.52±7.87
	19		8.22±0.56
	22		13.84±9.64
	23		17.29±7.89

Table 23 IC_{50} values of DHIQ derivatives on PDE5 and PDE6 (*n=3*)

Compound	IC_{50} (μM)		IC_{50} Ratio
	PDE5	PDE6	PDE6/PDE5
26	12.57 ± 4.13	23.18 ± 3.96	1.85
31	16.44 ± 3.69	9.34 ± 1.23	0.57
32	19.32 ± 2.42	6.23 ± 2.57	0.32
Dipyridamole	1.46 ± 0.81	0.231 ± 0.03	0.16
Sildenafil	0.0068 ± 0.00	0.033 ± 0.01	4.85

CHAPTER V

CONCLUSION

The main interest of this study was to search for PDE5 inhibitors which might be useful for the treatment of erectile dysfunction and pulmonary hypotension. Natural products were considered to be good sources of drugs. Three strategies were used for plant selection in preliminary screening. The first strategy was based on the ethnopharmacology which was to select the plants used in tradition aphrodisiac recipes. The second strategy was the selection base on plant family that might contain the chemicals of interest which can be called as chemotaxonomic approach. In our case, plants from Leguminosae which were expected to contain flavonoids were collected for the screening. The third strategy was randomly selected plant. In the preliminary screening, two plant extracts from the first strategy i.e., *C. sappan* and *K. parviflora* and two plant extracts from the second strategy i.e., *A. auriculaeformis* and *S. surattensis* showed moderate PDE5 inhibitoryactivity. By randomized selection strategy, four plant extracts belonging to *Curcuma* genus i.e. *C. longa, C. zedoaria, C. petiolata* and *Curcuma* sp. (นางคำ) and one plant extract from Orchidaceae, *E .macrobulbon* showed high PDE5 activity.

K. parviflora, was further investigated for the active components. A series of 7-methoxyflavone derivatives isolated from this plant showed PDE5 inhibitory effect. Even though these flavones and extracts were substantially less potent than sildenafil, this is the first time that the chemical components related to the treatment of erectile dysfunction of *K. parviflora* are reported. The SAR suggests that the methoxy group at C-5 is important for the PDE5 inhibitory activity of 7-methoxyflavone derivatives. Therefore, 5,7-dimethoxyflavone (**2**) might be serving as a potential lead for the development of selectively potent PDE5 inhibitors in clinically efficacious treatments for erectile dysfunction.

By means of bioassay guided fractionation, PDE5 inhibitors from pseudo-bulbs of *E. macrobulbon* were isolated. All of these compounds are belonging to phenanthrene skeleton. One of the four isolated phenanthrenes, 9,10-dihydro-4-(4′-

hydroxybenzyl)-2,5-dimethoxyphenanthrene-1,7-diol (**10**), was a new 9,10-dihydrophenanthrene derivatives. The other isolated compounds had been reported in *E. macrobulbon* for the first time. All compounds showed poor activity on PDE1 so that these compounds might not affect heart function. For PDE5 inhibitory activity, compound **11** was the most potent. The potency of **11** was 55-fold lower than sildenafil and the other compounds isolated were even less active. However, **11** inhibited PDE6 in the same range of potency. This indicated that **11** expressed no selectivity on PDE5 over PDE6. Even though the limited range of tested compounds did not allow a complete SAR on PDE5 inhibitory activity, some remarks could be made. The aromaticity might play a crucial effect on PDE5 inhibitory activity. For the complete SAR study of phenanthrene on PDE5 inhibition, more variety of phenanthrenes should be investigated.

E. macrobulbon crude extract had also exhibited vasorelaxant effect on rat pulmonary artery. This observation might support its PDE5 inhibitory activity since this enzyme plays a crucial role on relaxation of smooth muscle in lung [8,41].

An isoquinoline alkaloid from natural source, papaverine, has been reported as a non selective PDE inhibitor [34,35]. Although it showed efficacy for ED treatment, this drug has not been widely used due to the difficulty of drug administration and some undesirable effects. In this study, we screened series of isoquinoline alkaloid analogs for PDE inhibition effect. From our result, DHIQ derivatives could inhibit PDE5 whereas THIQ showed poor activity on this enzyme. The position of C-1 of DHIQ structure might be beneficial for PDE5 inhibitory activity. However, it requires the further study for the complete SAR.

In conclusion, this study demonstrates the inhibition effects of three groups of natural products i.e. flavones, phenanthrenes and isoquinoline alkaloids on PDE5. Some SAR information was obtained which are useful for further development of drugs for ED and pulmonary hypotension.

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APPENDIX