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***Kaempferia parviflora*, a plant used in traditional medicine to enhance sexual performance contains large amounts of low affinity PDE5 inhibitors**

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Abstract

Aim of the study—A number of medicinal plants are used in traditional medicine to treat erectile dysfunction. Since cyclic nucleotide PDEs inhibitors underlie several current treatments for this condition, we sought to show whether these plants might contain substantial amounts of PDE5 inhibitors.

Materials and methods—Forty one plant extracts and eight 7-methoxyflavones from *Kaempferia parviflora* Wall. ex Baker were screened for PDE5 and PDE6 inhibitory activities using the two-step radioactive assay. The PDE5 and PDE6 were prepared from mice lung and chicken retinas, respectively. All plant extracts were tested at 50 µg/ml whereas the pure compounds were tested at 10 µM.

Results—From forty one plant extracts tested, four showed the PDE5 inhibitory effect. The chemical constituents isolated from rhizomes of *Kaempferia parviflora* were further investigated on inhibitory activity against PDE5 and PDE6. The results showed that 7-methoxyflavones from this plant showed inhibition toward both enzymes. The most potent PDE5 inhibitor was 5,7-dimethoxyflavone (IC₅₀ = 10.64 ± 2.09 µM, selectivity on PDE5 over PDE6 = 3.71). Structure activity relationship showed that the methoxyl group at C-5 position of 7-methoxyflavones was necessary for PDE5 inhibition.

Conclusions—*Kaempferia parviflora* rhizome extract and its 7-methoxyflavone constituents had moderate inhibitory activity against PDE5. This finding provides an explanation for enhancing sexual performance in the traditional use of *Kaempferia parviflora*. Moreover, 5,7-dimethoxyflavones should make a useful lead compound to further develop clinically efficacious PDE5 inhibitors.

Keywords

Kaempferia parviflora; Phosphodiesterase5; Methoxyflavones; Traditional sexual performance enhancer; Leguminosae

1. Introduction

Phosphodiesterase5 (PDE5) is one of eleven isozymes in PDE family. Its role is to regulate tissue cGMP levels by cGMP hydrolysis (Essayan, 1999). It is abundant in vascular smooth muscle cells and plays a significant role in generating tone in smooth muscle of the penile corpus cavernosum. The PDE5 inhibitor, sildenafil, is currently used for erectile dysfunction as it increases the level of cGMP which induces vascular smooth muscle relaxation, vasodilation and increases blood flow to penile tissue (Corbin et al., 2002). However, sildenafil also inhibits PDE6 which is present in the retina and this inhibition may give rise to visual disturbances as an unavoidable side effect of this treatment (Uckert et al., 2006).

Many medicinal plants including some from Thailand have been used traditionally to enhance sexual performance by treating erectile dysfunction and therefore might contain compounds that act via PDE5 inhibition. In a previous study, we found that some of these plant extracts could indeed inhibit a mixture of PDEs a major component of which was PDE1 (Temkitthawon et al., 2008). Therefore, in this study we aimed to determine the PDE5 inhibitory effect of these medicinal plant extracts and to identify the compounds responsible for this action.

2. Materials and methods

2.1. Plant materials

The plant materials were collected from Northern Thailand. Nineteen voucher specimens of the collected medicinal plants used as sexual performance enhancers (Table 1) are kept at Queen Sirikit Botanic Garden, Chiangmai. The dried stem of *Mucuna colettii* was a gift from Professor Wichai Cherdshewasart, Department of Biology, Faculty of Sciences, Chulalongkorn University, Bangkok, Thailand. *Butea superba* and 20 voucher specimens of leguminoseous plants (Table 2) are kept at Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok and at the PBM Herbarium, Faculty of Pharmacy, Mahidol University, Bangkok.

2.2. Chemicals

cGMP, crude snake venom (*Crotalus atrox*), histone, bovine serum albumin (BSA), ethylene glycol tetraacetic acid (EGTA), imidazole, tris(hydroxymethyl)aminomethane (Tris), magnesium chloride (MgCl₂), diethylaminoethyl sephadex (DEAE-Sephadex) and

dipyridamole were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). [^3H]cGMP was obtained from Perkin Elmer (Boston, MA, U.S.A.).

2.3. Plant extraction and isolation

Plant materials were cut into small pieces and dried in a hot air oven at 55 °C. The dried materials were macerated in ethanol twice, for 3 days each, and filtered. Both filtrates were combined and evaporated under reduced pressure until dried.

Flavonoids from *Kaempferia parviflora* were isolated as reported previously (Sawasdee et al., 2009).

2.4. Enzyme preparation

PDE5 was obtained from mouse lung tissue. Briefly, fresh tissue was minced and homogenized in 1 ml of Tris buffer (50 mM Tris, pH 7.5, 2 mM EDTA, 1 mM DTT and 1:100 of 100 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 14,000 rpm for 20 min at 4 °C and the supernatant was used as a source of PDE5. The PDE5 inhibitor, sildenafil, was used to confirm that the supernatant contained abundant PDE5. PDE6 was prepared from chicken retinas as previously described (Huang et al., 2004). All of the tissues were obtained from animals for which we have approval by Institutional Animal Care and Use Committee (IACUC) Committee of University of Washington.

2.5. PDE assay

PDE activity was measured according to the method 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 PDE 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 1 μM [^3H]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 20 mM 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 [^3H]guanosine. The eluant was mixed with a scintillant cocktail and the radioactivity was measured using a β -counter. The PDE in the study was standardized to have a hydrolysis activity of 15–20% of the total substrate counts. The calculation of hydrolysis is shown in Eq. (1). The PDE inhibitory activity is calculated from Eq. (2).

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

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

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

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 IC₅₀s were determined. For each enzyme preparation, the assay was performed in triplicate.

3. Results and discussion

In this study, forty one plant extracts were screened for PDE5 inhibitory activity. These plant extracts were selected by either 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 (Shin et al., 2002). In the screening, two traditional sexual performance enhancer plants, i.e. *Caesalpinia sappan* and *Kaempferia parviflora*, and two leguminoseous plants, i.e. *Acacia auriculaeformis* and *Senna surattensis* showed moderate effects on PDE5 (60–70% inhibitions) (Tables 1 and 2). These extracts were further tested to determine their IC₅₀s (Table 3). The IC₅₀s were in the range of 10–50 µg/ml which was significantly higher than sildenafil. The selectivity on PDE5 of the sample was expressed as the high IC₅₀ ratio of PDE6/PDE5 (Table 3). Three of these extracts showed some PDE5 selectivity similar to sildenafil (4.85) but the *Caesalpinia sappan* extract showed clear PDE6 discrimination.

Kaempferia parviflora, a member of Zingiberaceae, was selected for further study because of its popularity as a sexual performance enhancer (Yenjai et al., 2004). There are some scientific evidences for this activity. Thus an ethanolic extract of the rhizomes improves endothelial cell function via nitric oxide production on which penile erection depends (Wattanapitayakul et al., 2007). *In vivo*, the extract was able to increase blood flow to the testis (Chaturapanich et al., 2008). 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 (Fig. 1) which are the major constituents in this plant (Sutthanut et al., 2007). Eight 7-methoxyflavones (Fig. 1) were isolated from *Kaempferia parviflora* as reported previously (Sawasdee et al., 2009). These compounds were tested on PDE5 and PDE6 and the results are shown in Tables 4 and 5. Compounds **2**, **4**, **6**, and **8** showed inhibitory activities against both PDE5 and PDE6 higher than 35% at 10 µM. These compounds were further examined their IC₅₀s for PDE5 and PDE6 inhibitions (Table 5). Among the tested flavones, **2** is the most potent PDE5 inhibitor and **8** is the most potent PDE6 inhibitor. Most noteworthy is that **2** (IC₅₀ 10.64 µM) was 3-fold more potent than **8**

(IC₅₀ 30.41 μ M) for PDE5 inhibitory activity while **8** (IC₅₀ 7.83 μ M) was 5-fold more potent than **2** (IC₅₀ 39.45 μ M) for PDE6 inhibitory activity. These IC₅₀s are in the same range as that reported from other flavonoids (Ko et al., 2004) which was much lower than sildenafil. The inhibitory selectivity of **2** and **8** is, respectively, expressed as the PDE6/PDE5 IC₅₀ ratios of 3.71 and 0.16 while that of sildenafil is 4.85 (Table 5). Therefore, PDE5 and PDE6 inhibitory results of *Kaempferia 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 toward 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 (Ko et al., 2004). However, this is the first report focusing on SAR of 7-methoxyflavones on PDE5.

4. Conclusion

In preliminary screening of forty one plant extracts, four plant extracts had moderate PDE5 inhibitory activity. One of these extracts, from *Kaempferia 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 *Kaempferia parviflora* are reported. The SAR suggests that the methoxyl 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.

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Compound	Substitution				
	3	5	7	3'	4'
Tectochrysin (1)		OH	OMe		
5,7-dimethoxyflavone (2)		OMe	OMe		
5-hydroxy-7,4'-dimethoxyflavone (3)		OH	OMe		OMe
5,7,4'-trimethoxyflavone (4)		OMe	OMe		OMe
5-hydroxy-3,7-dimethoxyflavone (5)	OMe	OH	OMe		
3,5,7-trimethoxyflavone (6)	OMe	OMe	OMe		
5-hydroxy-3,7,4'-trimethoxyflavone (7)	OMe	OH	OMe		OMe
3,5,7,3',4'-pentamethoxyflavone (8)	OMe	OMe	OMe	OMe	OMe

Fig. 1.

The 7-methoxyflavone constituents from rhizomes of *Kaempferia parviflora*.

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

Table 1

No.	Scientific name	Family	Part used	Collection number	% PDE5 inhibitory activity
1	<i>Acorus calamus</i> L.	Araceae	Root	WP. 1907	0.87 ± 2.49
2	<i>Barleria strigosa</i> Willd.	Acanthaceae	Whole plant	WP. 1900	2.13 ± 5.75
3	<i>Berchemia floribunda</i> Wall.	Betulaceae	Stem bark	WP. 1899	7.33 ± 0.59
4	<i>Betula alnoides</i> Buch.-Ham. ex G. Don	Rhamnaceae	Stem	WP. 1893	36.40 ± 8.07
5	<i>Boesenbergia rotunda</i> (L.) Mansf.	Zingiberaceae	Rhizome	QSBG. 4152	40.86 ± 3.94
6	<i>Butea superba</i> Roxb.	Leguminosae	Root bark	Fansai 0021	7.88 ± 5.59
7	<i>Caesalpinia sappan</i> L.	Caesalpinaceae	Stem	WP. 1903	60.23 ± 1.81
8	<i>Drosera burmannii</i> Vahl	Droseraceae	Aerial part	QSBG. 9202	4.52 ± 0.40
9	<i>Elephantopus scaber</i> L.	Asteraceae	Whole plant	WP. 1895	4.12 ± 0.75
10	<i>Hiptage benghalensis</i> (L.) Kurz	Malpighiaceae	Stem	WP. 1896	32.31 ± 3.77
11	<i>Kaempferia parviflora</i> Wall. ex Baker	Zingiberaceae	Rhizome	QSBG. 25275	62.63 ± 7.17
12	<i>Leea indica</i> (Burm.f.) Merr.	Leeaceae	Root	QSBG. 15194	31.36 ± 7.47
13	<i>Mucuna colletii</i> Lace	Leguminosae	Stem	BKF 115495	1.13 ± 5.06
14	<i>Myxopyrum smilacifolium</i> Blume subsp.	Oleaceae	Root	WP. 1904	0.46 ± 0.69
15	<i>Polygala chinensis</i> L.	Polygalaceae	Whole plant	QSBG. 10073	3.85 ± 9.35
16	<i>Piper samentosum</i> Roxb.	Piperaceae	Root	WP. 1902	4.19 ± 6.70
17	<i>Securidaca inappendiculata</i> Hassk	Polygalaceae	Stem	WP. 1897	9.50 ± 7.27
18	<i>Tacca chantrieri</i> André	Taccaceae	Root	WP. 1901	0.49 ± 7.75
19	<i>Tinospora crispa</i> (L.) Miers ex Hook.f. & Thomson	Menispermaceae	Stem	QSBG. 11325	2.54 ± 7.88
20	<i>Talinum paniculatum</i> (Jacq.) Gaertn.	Portulacaceae	Rhizome	QSBG. 9524	1.16 ± 4.19
21	<i>Ventilago denticulata</i> Willd.	Rhamnaceae	Stem	WP. 1894	36.43 ± 7.25

Table 2

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	Collection number	% PDE5 inhibitory activity
1	<i>Acacia auriculaeformis</i> A. Cunn.	Sirikul 013	73.66 ± 4.87
2	<i>Acacia concinna</i> (Willd.) DC.	Sirikul 014	28.70 ± 3.19
3	<i>Acacia pennata</i> (L.) Willd. subsp. <i>insuavis</i> (Lace) I.C. Nielsen	Sirikul 012	13.97 ± 4.62
4	<i>Bauhinia acuminata</i> L.	Sirikul 011	36.94 ± 4.23
5	<i>Bauhinia glauca</i> (Wall. ex Benth.) Benth.	Sirikul 009	23.89 ± 3.06
6	<i>Bauhinia winitii</i> Craib	Sirikul 010	45.92 ± 4.36
7	<i>Butea monosperma</i> (Lam.) Taub.	Sirikul 019	41.48 ± 0.64
8	<i>Caesalpinia coriaria</i> (Jacq.) Willd.	Sirikul 020	47.50 ± 4.67
9	<i>Caesalpinia sappan</i> L.	Sirikul 015	25.87 ± 6.49
10	<i>Cassia fistula</i> L.	Sirikul 004	10.60 ± 0.25
11	<i>Delonix regia</i> (Bojer ex Hook.) Raf.	Sirikul 016	31.03 ± 10.19
12	<i>Leucaena leucocephala</i> (Lam.) de Wit	Sirikul 006	29.82 ± 8.64
13	<i>Pithecellobium dulce</i> (Roxb.) Benth.	Sirikul 008	30.25 ± 0.84
14	<i>Samanea saman</i> (Jacq.) Merr.	Sirikul 005	47.59 ± 6.59
15	<i>Saraca thaipingensis</i> Cantley ex Prain	Sirikul 018	21.53 ± 2.63
16	<i>Senna alata</i> (L.) Roxb.	Sirikul 003	19.78 ± 5.29
17	<i>Senna siamea</i> (Lam.) Irwin & Barneby	Sirikul 001	19.94 ± 5.18
18	<i>Senna surattensis</i> (Burm.f.) Irwin & Barneby	Sirikul 017	65.08 ± 0.78
19	<i>Sesbania grandiflora</i> (L.) Desv.	Sirikul 002	32.97 ± 5.39
20	<i>Tamarindus indica</i> L.	Sirikul 007	55.79 ± 2.68

Table 3

IC₅₀ values of some ethanolic plant extracts against PDE5 and PDE6 ($n = 3$).

Plant extract	Part used	IC ₅₀ (μg/ml)		IC ₅₀ ratio PDE6/PDE5
		PDE5	PDE6	
<i>Caesalpinia sappan</i>	Stem	45.95 ± 3.62	4.96 ± 2.16	0.11
<i>Kaempferia parviflora</i>	Rhizome	12.24 ± 0.99	13.74 ± 1.83	1.12
<i>Acacia auriculaeformis</i>	Leaf	12.72 ± 2.27	41.28 ± 3.47	3.25
<i>Senna surattensis</i>	Leaf	12.00 ± 3.68	30.45 ± 1.04	2.54

Table 4

% Inhibitory activity of 7-methoxyflavone derivatives from *Kaempferia parviflora* on PDE5 and PDE6 ($n = 3$). The final concentration of the compounds was 10 μ M.

Compound	% Inhibitory activity at 10 μ M	
	PDE5	PDE6
1	18.23 $\pm$ 3.26	-1.87 $\pm$ 1.19
2	53.65 $\pm$ 1.15	38.50 $\pm$ 1.06
3	17.64 $\pm$ 3.19	2.45 $\pm$ 1.08
4	37.82 $\pm$ 4.08	16.63 $\pm$ 5.23
5	0.76 $\pm$ 1.26	0.73 $\pm$ 2.34
6	44.96 $\pm$ 2.43	36.83 $\pm$ 3.45
7	6.02 $\pm$ 5.94	0.95 $\pm$ 2.85
8	37.55 $\pm$ 2.07	58.06 $\pm$ 3.52

Table 5

IC₅₀ values of 7-methoxyflavone derivatives from *Kaempferia parviflora* on PDE5 and PDE6 (*n* = 3).

Compound	IC ₅₀ (μM)		IC ₅₀ ratio PDE6/PDE5
	PDE5	PDE6	
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