Vasorelaxant effects of 3,5,7,3',4'-pentamethoxyflavone isolated from *Kaempferia parviflora*: partly stimulating the release of NO and H_2S by rat thoracic aorta

Somruedee Yorsin, Yauwapa Sukpondma, Chaweewan Jansakul

Abstract

Pentamethoxyflavone (PMF) was isolated from the rhizomes of *Kaempferia parviflora*, a plant that is claimed to have anti-hypertensive effects. We have investigated the activity of the PMF on isolated rat thoracic aortic rings. PMF caused a relaxation of phenylephrine precontracted aortic rings, and this effect was inhibited by N^G-nitro-L-arginine (LNA), ODQ (guanylyl cyclase inhibitor), or by removal of the vascular endothelium. In the presence of LNA or removal of the endothelium, ODQ potentiated the relaxant activity of the PMF, and this effect was inhibited by DL-propargylglycine (PAG, a cystathionine-γ-lyase inhibitor) and SQ22536 (an adenylyl cyclase inhibitor). Glybenclamide, but not tetraethylammonium, potentiated the relaxation of the PMF whether LNA was present or not, and the potentiation was inhibited by PAG and SQ22536. In normal Krebs solution with nifedipine, or in a Ca^{2+}-free Krebs solution, PMF caused a further inhibition of the phenylephrine concentration- response (*C-R*) curve of the aortic ring. In the aortic ring treated with thapsigargin, PMF suppressed the phenylephrine *C-R* curve and a further suppression was found when nifedipine, SKF-96365 (store-operated Ca^{2+} channel inhibitor) and/or Y-27632 (Rho-kinase inhibitor) was also added. These results revealed that PMF caused a relaxation of thoracic aortic rings by stimulating the release of nitric oxide and H_2S, that act as an adenylyl cyclase stimulator, and an inhibitor of intracellular calcium mobilization.

J Physiol Biomed Sci. 2015; 28(1): 5-14

Keywords: *Kaempferia parviflora, pentamethoxyflavone, thoracic aorta, cAMP, nitric oxide, H_2S*

Quercetin has attracted numerous beneficial health claims[1] and is the most abundant flavonoid polyphenolic compound present in human dietary vegetables and fruits.[2] In the cardiovascular system, it has been shown to be antihypertensive in various rat models of hypertension.[1,3] *In vitro*, quercetin is a vasorelaxant independent of the endothelium[4] yet in addition also increases endothelial NO production.[5] On the other hand, quercetin itself is poorly absorbed, has a high susceptibility to metabolic conjugation, and exists mostly in a conjugated form in the systemic circulation, that results in its low bioavailability.[6-7] Thus quercetin is largely ineffective *in vivo*. This low bioavailability is mainly due to an efficient system for conjugation of its polyhydroxylated groups with glucuronic acid and sulfate in normal situations. However, it has been reported that the intestinal transportation of this flavonoid could be much improved through methylation. In an *in vivo* experiment in the rat, oral administration of one methylated flavone resulted in a higher bioavailability and tissue distribution with no detectable levels of its unmethylated analogue. Thus, methoxylation appears to be a simple and effective way to increase both the metabolic resistance and the transport of the flavonoid into the circulatory system.[8-9] Some methoxylated flavonoids occur naturally including methoxylated quercetins, quercetin 3,7,3',4'-tetra-methylether and quercetin 3,5,7,3',4'-pentamethyl-ether (3,5,7,3',4'-pentamethoxyflavone) found in some medicinal plants such as *Achyrocline satureioide*[10] and the rhizomes of *Kaempferia parviflora.*[11-12] A further ten other polymethoxy-flavones are found in the peel of *Citrus* fruits that are used in several traditional medicines from Eastern China.[13-15] At least three of these i.e., tangeretin, nobiletin (5,6,7,8,3,4́–hexamethoxyflavone) and sinensetin, have biological activity on G1 cell cycle arrest in human breast and colon cancer cells, on cardiovascular protection via its antihypertensive actions, and show attenuation of atherosclerosis, are anti-inflammatory, have antiangiogenesis activity, inhibit platelet adhesion, and have anti-adipogenic properties on mature 3T3-L1 adipocytes.[16-21] To date, there seem to have been no reports on the effects of polymethoxyflavone on the endothelial-vascular smooth muscle unit of isolated blood vessels.

Rhizomes of *Kaempferia parviflora* (Zingiberaceae family) have been used in Thai traditional

From the Department of Biomedical Science, Faculty of Medicine (S.Y.), Department of Chemistry, Faculty of Science (Y.S.), and Faculty of Traditional Thai medicine (C.J.), Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.

Corresponding author:
Chaweewan Jansakul, Ph.D.
Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand .
E-mail: chaweewan.j@psu.ac.th

medicine for many purposes, including its use as an aphrodisiac, as an anti-hypertensive compound, to improve blood flow, and to promote longevity with good health and well being.[11-12] There is some limited scientific support for these therapeutic claims. Chaturapanich *et al*.[22] demonstrated that a *Kaempferia parviflora* alcohol extract produced a significant increase in blood flow to the testis in male rats. Later Murata *et al*.[23] showed that a methanolic extract of *Kaempferia parviflora* caused an improvement to the blood clotting mechanism where the active principles were methoxyflavones. One of these compounds, 3,5,7,3',4'-pentamethoxyflavone (PMF), relaxed isolated human cavernosum by inhibiting both voltage-dependent Ca^{2+} channels and intracellular calcium mobilization.[24] Hnatyszyn *et al*.[10] showed that quercetin 3,7,3',4'-tetramethylether, quercetin 3,5,7,3',4'-pentamethylether and quercetin itself were all equally effective relaxants of isolated guinea-pig cavernosum. For isolated blood vessels, quercetin molecules with an increasing number of methoxylations showed different activity profiles for potency and mechanism of action on vasorelaxation.[25] Thus in rat thoracic rings, 5,7-dimethoxyflavone (DMF) produced an endothelium-dependent relaxation via an increased K^+ efflux and by inhibition of extracellular Ca^{2+}entry through the NO-cGMP and cyclooxygenase pathways. Trimethoxyflavone had similar effects except that it failed to activate the cyclooxygenase pathway.[26]

Thus, polymethoxylated flavonols promise to offer a quantal leap forward in the efficacious cardiovascular treatment regimens hitherto unrealised by quercetin and other polyphenols in the clinic. The simplest starting point would be 3,5,7,3',4'-pentamethoxyflavone since it works in other tissues and complete methoxylation improves absorption, allows for greater systemic stability and improves cellular uptake.[8-9,11,27] Although we have already studied corpus cavernosum, the greatest clinical impact is likely to be on vascular function. Therefore, in the present work we aim to analyze the action of PMF on isolated rat aorta with such an application in mind.

Materials and Methods

Plant material

Fresh rhizomes of *Kaempferia parviflora* (black ginger or kra-chai-dum in Thai) were collected in Phurua District, Loei Province, Thailand. Authentication was achieved by comparison with the herbarium specimen in the Department of Biology Herbarium, Faculty of Science, Prince of Songkla University, Thailand, where a voucher specimen (Collecting No. 2548-03) of the plant material has been deposited.

Extraction and isolation of the 3,5,7,3',4'-pentamethoxyflavone (PMF) from rhizomes of *Kaempferia parviflora*

The method used to isolate the pure PMF and the HPLC method used for its assay has been previously fully described by Jansakul *et al*.[24]

Preparation of thoracic aortic rings

Adult female Wistar rats were supplied by the Southern Laboratory Animal Facility, Faculty of Science, Prince of Songkla University. The animals were housed in controlled environmental conditions at 24-26 $^{\circ}$C on a 12 h dark and 12 h light cycle with access to standard rat food and tap water *ad libitum*. The methods employed and the experimental protocols were approved by the Prince of Songkla University Ethical Committee (Ethic No. 166/1724) and the investigation conformed to the Guide for the Care and Use of Laboratory Animals.

Adult female Wistar rat in estrous weighing 220-250 g were killed by cervical dislocation. The thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue. Two adjacent rings of 4-5 mm in length were cut. In one ring the endothelium layer was removed mechanically by gently rubbing the intimal surface with a stainless steel rod, using the method of Jansakul *et al*.[28] The aortic rings with or without a functional endothelium were mounted horizontally between two parallel stainless steel hooks, with extreme care not to damage the endothelium of the endothelium-intact aortic rings, and resuspended in a 20-ml organ bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4 \cdot 7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at 37 $^{\circ}$C and continuously bubbled with a 95% O_2 and 5% CO_2 gas mixture. One hook was fixed at the bottom and the other was connected to a force displacement transducer that was connected to a Grass polygraph for the recording of changes in the isometric tension. Prior to testing, tissues were equilibrated for 60 min under a resting tension of 1 g and the bath solution was replaced with pre-warmed oxygenated Krebs-Henseleit solution every 15 min.

As a prelude to testing, a functional endothelium of the intact thoracic aortic rings was verified in every preparation as follows: The thoracic aortic ring was precontracted with 3 μM phenylephrine until the response reached a plateau (5-8 min), and then acetylcholine was added (30 μM). Ring viability was judged by a > 80% vasorelaxation back to the tension generated by the ring before adding phenylephrine. Denudation was confirmed by the complete absence of vasorelaxation following the response to the addition of acetylcholine. The preparations were then washed several times with Krebs-Henseleit solution, and allowed to fully relax for 45 min before the experimental protocol began.

Experimental protocol

Effects on nitric oxide, guanylyl cyclase, adenylyl cyclase, H_2S stimulation, and K^+ channels

After equilibration, the thoracic aortic rings were

precontracted with 3 μM phenylephrine for 10 min (plateau reached), and the cumulative concentration-response (*C-R*) relationships of the thoracic aortic ring to PMF (0.0003-0.1 mM) was determined. Following several washings and re-equilibration of the thoracic aortic ring for another 60 min with changes to the Krebs-Henseleit solution every 15 min, the thoracic aortic rings were incubated with N^G-nitro-L-arginine (LNA, 0.3 mM, inhibitor of nitric oxide synthase); 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 0.01 mM, a soluble guanylyl cyclase inhibitor); glybenclamide (0.01 mM, an ATP sensitive K^+ channel inhibitor); tetraethylammonium (TEA, 1 mM, a voltage activated K^+ channel blocker) for 40 min; and/or by an addition of DL-propargylglycine (PAG, 10 mM, a cystathionine-γ-lyase inhibitor or an H_2S inhibitor); or 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, 0.1mM, an adenylyl cyclase inhibitor) as required. Then the PMF (0.003-0.1 mM) cumulative *C-R* relationships were determined on the phenylephrine-induced contraction in the continuous presence of each drug.

Inhibition of voltage-dependent calcium channels, intracellular Ca^{2+} mobilization, store-operated calcium channels, and Rho-kinase

In all the experiments described in this section, the thoracic aortic rings were first equilibrated for 60 min in Krebs solution, followed by a functional assessment of the endothelium as above, after which they were incubated with Krebs solution in the presence of LNA (0.3 mM) for 60 min with a replacement of the incubation medium every 20 min. Thereafter LNA was present throughout.

To determine whether PMF played a role as a voltage-dependent Ca^{2+} channel blocker, the *C-R* curve to phenylephrine was studied in the normal Krebs medium. This was followed by several washings and re-equilibration for 40 min, then the thoracic aortic rings were incubated with nifedipine (3 μM) for 20 min and again the *C-R* curve to phenylephrine was determined in the presence of nifedipine. The same procedure was repeated in the presence of nifedipine with PMF (0.03 mM).

To determine whether PMF inhibited intracellular Ca^{2+} mobilization, a similar protocol was carried out with another set of thoracic aortic rings in Ca^{2+}-free Krebs solution before and after incubating the thoracic aortic ring with PMF.

To determine the role of PMF on the store-operated Ca^{2+} channel and/or the Rho-kinase inhibitor, eight sets of thoracic aortic rings were used. Each set of the thoracic aortic rings was first constructed with the *C-R* curve to phenylephrine in the presence of LNA, followed by several washings and re-equilibration for another 40 min. The thoracic aortic rings were then incubated with thapsigargin (3 μM), a sarcoplasmic reticulum ATPase inhibitor, for 40 min by which time the small contraction (~ 0.1-0.2 g) of the thoracic aortic rings had reached a plateau, in one set of these the *C-R* relationship to phenylephrine was determined in the presence of thapsigargin. For the others, PMF (0.03 mM), nifedipine (3 μM), SKF-96365 (100 μM, a store-operated Ca^{2+} channel blocker) and/or Y-27632 (30 μM, a Rho-kinase inhibitor) were added sequentially as required and incubated for 20 min, at which time the thoracic aortic ring had relaxed to its original basal level, then the cumulative *C-R* relationship to phenylephrine was obtained in the presence of thapsigargin together with their corresponding cocktails.

Drugs

The following drugs were used: acetylcholine chloride, nifedipine, N^G-nitro-L-arginine (LNA), phenylephrine hydrochloride, tetraethylammonium (TEA), DL-propargylglycine (PAG, cystathionine-γ-

Table 1 E_{Max} and EC_{50} values of the relaxation of the endothelium-intact (Endo) or denuded- (No endo) thoracic aortic ring precontracted with phenylephrine to 3,5,7,3´,4´-pentamethoxyflavone (PMF) either in the presence of N^G-nitro-L-arginine (LNA), ODQ, Glybenclamide (Glyben), TEA, DL-propargylglycine (PAG) and/or SQ22536 (SQ).

	EC_{50} in μM (95% confidence limit)			E_{Max} (%)		
	Endo	Endo+LNA	No endo	Endo	Endo+LNA	No endo
PMF	3.6 (2.1-6.2)	28.7 (21.4-38.4)	31.5 (26.9-35.9)	95.6±9.9	94.6±6.6	95.3±4.4
PMF+ODQ	11.7 (9.5-14.5)[b]	11.8 (9.0-15.5)[a]	13.5 (10.5-17.3)[a]	107.8±4.5	99.8±4.2	99.5±3.8
PMF		29.4 (20.2-43.1)			116.1±9.7	
PMF+PAG		32.2 (25.4-40.1)			111.3±5.6	
PMF+PAG+SQ		28.6 (19.6-39.2)			109.5±7.0	
PMF		33.4 (28.3-39.4)			95.3±3.4	98.5±3.8
PMF+ODQ		10.2 (8.9-11.6)[a]			100.2±2.6	101.4±2.5
PMF+ODQ+PAG		25.1 (20.2-28.8)			112.4±1.8[b]	100.8±1.4
PMF+ODQ+PAG+SQ		36.5 (31.5-42.6)			103.4±1.9	103.5±3.1
PMF	8.1 (5.9-11.0)	31.3 (27.2-36.1)		102.5±11.6	96.3±5.2	
PMF+Glyben	3.3 (2.2-5.1)[a]	13.9 (9.9-16.5)[a]		92.1±4.6	101.2±2.9	
PMF+Glyben+PAG		23.6 (18.8-27.4)			105.8±3.5	
PMF+Glyben+PAG+SQ		29.7 (26.5-35.9)			99.6±3.4	
PMF+TEA	7.8(5.1-10.4)			106.6±3.1		

Values were obtained from 6 aortic rings each from a different rat (n = 6). [a] Significantly lower than control and [b] significantly higher than control.

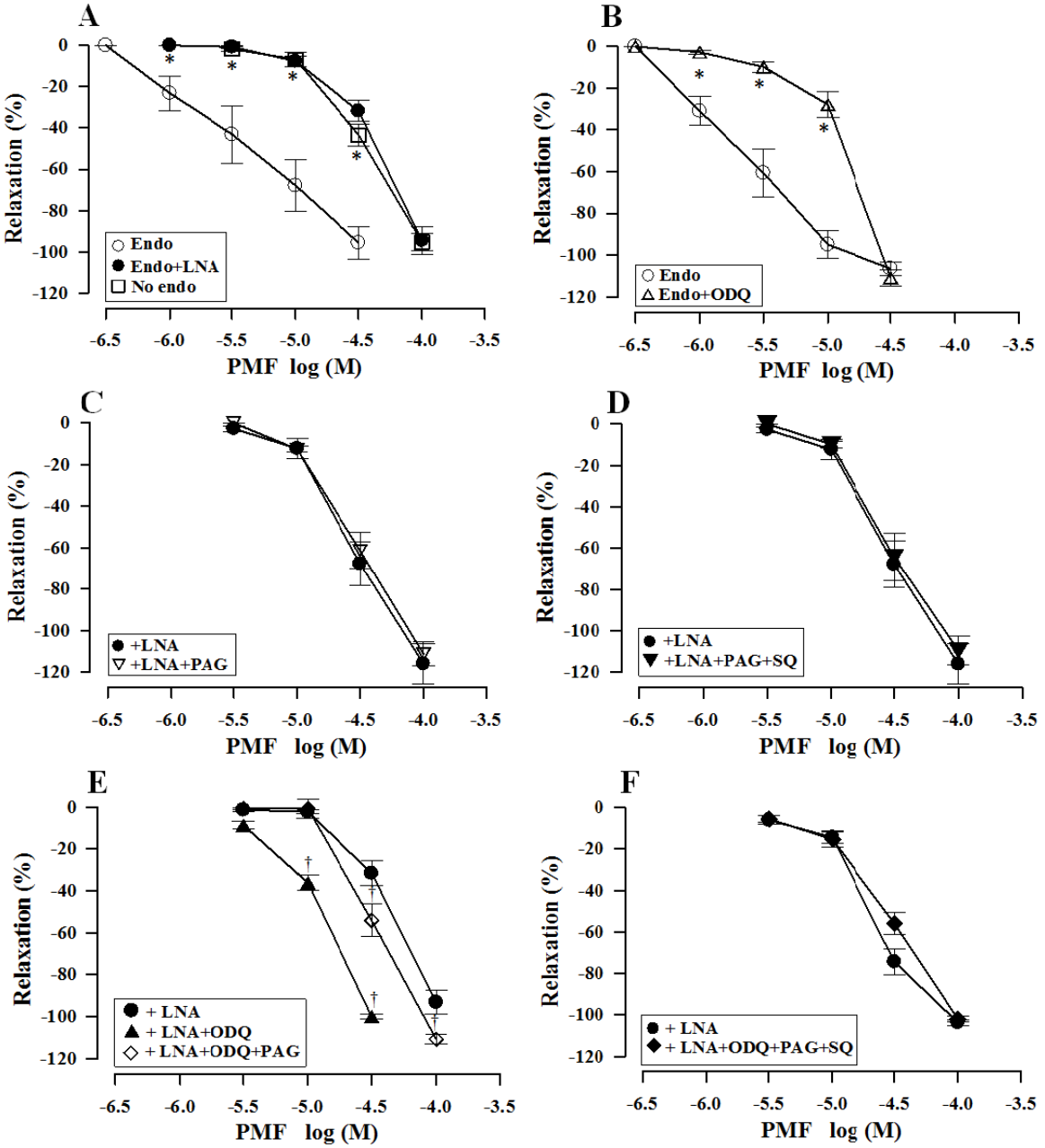

Figure 1 Effects of the removal of endothelium (no endo), N^G-nitro-L-arginine (LNA, 0.3 mM), ODQ (0.01 mM), DL-propargylglycine (PAG,10 mM) and/or 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536: SQ, 0.1 mM) on the relaxation of the thoracic aortic rings that had been precontracted with phenylephrine (3 μM for endothelium-intact, 0.3 μM for that with LNA, ODQ and/or -denuded vessel) to 3,5,7,3',4'-pentamethoxyflavone (PMF). Drug-relaxation is expressed as the percentage inhibition of the maximum phenylephrine tension. Each point represents a mean value ± SEM of 6 aortic rings each from a different animal (n = 6). *Significantly higher than the control (○) group, $P < 0.05$ and †significantly lower than the one with LNA (●), $P < 0.05$.

lyase inhibitor) and 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, adenylyl cyclase inhibitor) were all from Sigma, U.S.A. 1*H*-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), thapsigargin, SKF-96365 and trans-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride (Y-27632) were from Trocis, UK. LNA, thapsigargin, SKF-96365, Y-27632 and PAG were dissolved in distilled water, nifedipine and SQ22536 were dissolved in 20% DMSO, and the remainder were dissolved in a solution (1 liter) containing NaCl 9 g, NaH_2PO_4 0.19 g and ascorbic acid 0.03 g.

Statistical analysis

Results are expressed as mean ± SEM where n indicates the number of thoracic aortic rings. Each thoracic aortic ring was obtained from a different rat. Drug-induced relaxation was measured as the decline from the maximal steady tension produced by phenylephrine. The steady decline achieved at each drug concentration was expressed as a percentage of the initial maximum produced by phenylephrine. The

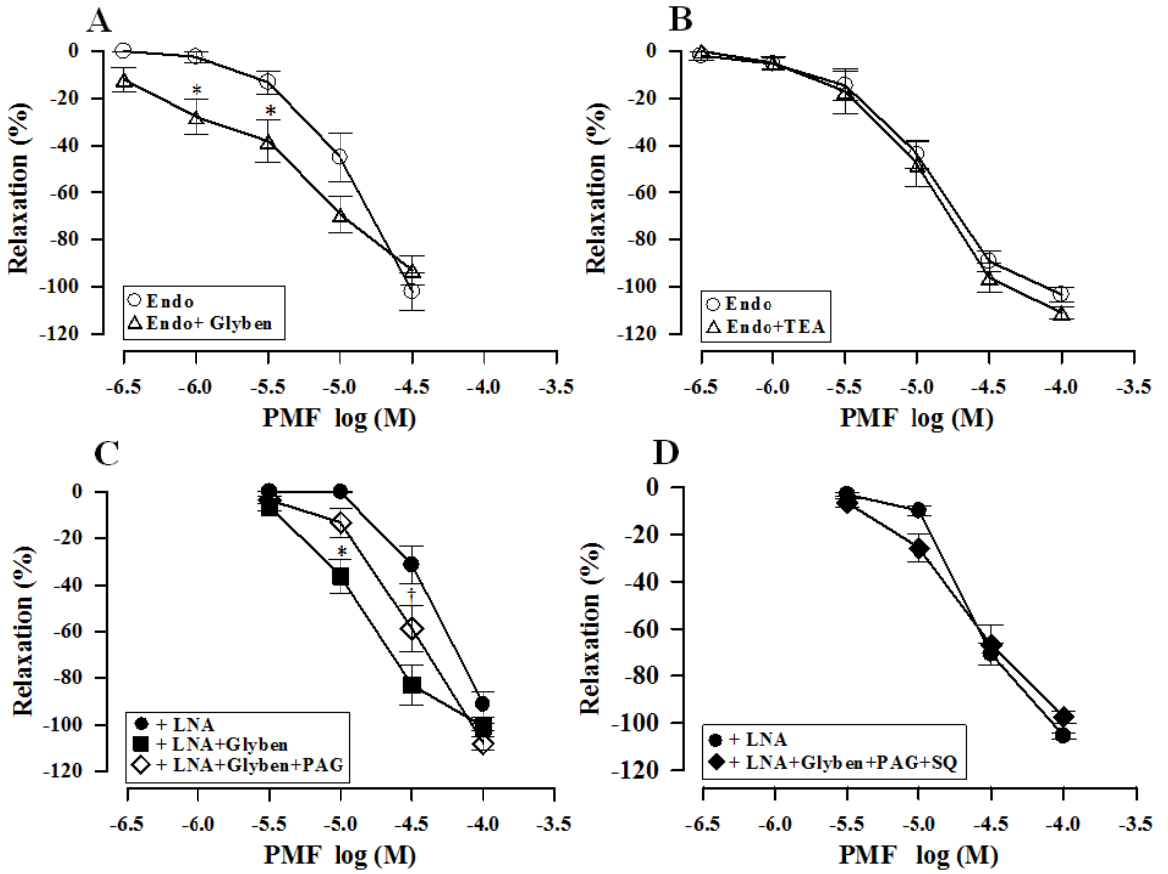

Figure 2 Effects of N^G-nitro-L-arginine (LNA, 0.3 mM), glybenclamide (Glyben, 0.01 mM), TEA (1 mM), DL-propargylglycine (PAG, 10 mM) and/or 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536:SQ, 0.1 mM) on the relaxation of thoracic aortic ring precontracted with phenylephrine (3 μM for the control, 0.3 μM for that with LNA) to 3,5,7,3',4'-pentamethoxyflavone (PMF). Drug-relaxation is expressed as the percentage inhibition of the maximum phenylephrine tension. Each point represents a mean ± SEM of 6 aortic rings each from a different animal (n = 6). *Significantly lower than their control (○) groups and †significantly lower than the one with LNA (●), $P < 0.05$.

contractile cumulative *C-R* curve to phenylephrine, and the steady increase achieved at each phenylephrine concentration was expressed as a percentage of the E_{max} obtained from their control group. The drug concentration that produced 50% of the maximal response for the drug (EC_{50}) was derived from regression analysis over the linear portion of the concentration-response curve. Statistical differences between two measurements was determined by two-tailed unpaired Student's *t*-test; differences among groups was determined by one way ANOVA and post hoc analysis was performed with a Duncan test. A P value ≤ 0.05 was considered to be a significant difference in all experiments.

Results

Effects on nitric oxide, guanylyl cyclase, adenylyl cyclase, H_2S stimulation, and K^+ channels

PMF caused a relaxation of the thoracic aortic ring precontracted with phenylephrine in a concentration-dependent manner. LNA, ODQ or removal of the vascular endothelium significantly shifted the *C-R* curve of the PMF to the right (Figure 1A and B) and increased the EC_{50} values 3-8 fold (Table 1). In the endothelium-intact aortic ring in the presence of LNA, PAG alone or together with SQ22536 there was no change in the relaxant activity of the PMF (Figure 1C and D), whereas ODQ caused a potentiation of the relaxant activity of the PMF with a decrease in its EC_{50} values (Table 1). The potentiating effect of ODQ on the PMF of these blood vessels was significantly inhibited by PAG and when SQ22536 was also added the potentiating effect of the ODQ was restored to the same level as their control groups (Figure 1E-H).

TEA did not modulate the PMF *C-R* curve (Figure 2B) whereas glybenclamide potentiated the vasorelaxant *C-R* curve of the PMF with a decrease in the EC_{50} values of the endothelium-intact thoracic aortic rings no matter whether LNA was present or not (Figure 2A and C and Table 1). The potentiating effect of glybenclamide on the PMF *C-R* curve of the endothelium-intact thoracic aortic ring with LNA (Figure 2C) was significantly inhibited by PAG and when SQ22536 was also added the *C-R* curve of PMF was restored to the same level as that of the control groups (Figure 2D).

Block of voltage-dependent calcium channels, and/or intracellular calcium mobilization

Nifedipine significantly inhibited the PMF *C-R* curve

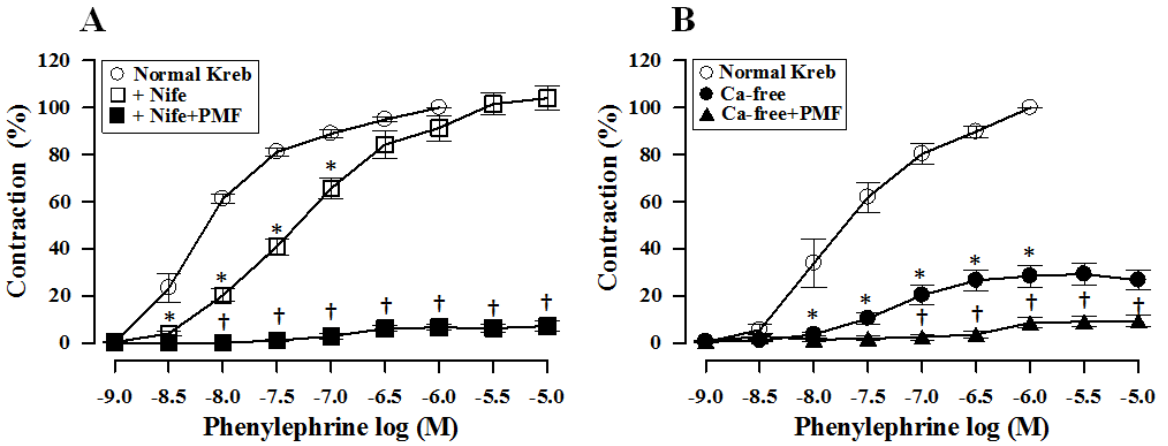

Figure 3 Contractile responses of the endothelium-intact thoracic aortic ring in the presence of LNA to phenylephrine in normal Krebs solution before and after blocking the L-type Ca^{2+} channel with nifedipine (3 μM, left) for 20 min or in a Ca^{2+}-free medium (right) and with 3,5,7,3',4'-pentamethoxyflavone (PMF, 0.03 mM). Each point represents a mean value ± SEM of 6 aortic rings each from a different rat (n = 6). * Significantly lower than the control (○) group, $P < 0.05$. †Significantly lower than that with nifedipine (□) or in Ca^{2+}-free medium (●) and the control groups, $P < 0.05$.

and a further inhibition was found when PMF was also added (Figure 3A). A similar result was found in the Ca^{2+} free Krebs medium (Figure 3B).

Store-operated calcium channels, Rho-kinase

In the normal Krebs solution in the presence of LNA and thapsigargin, nifedipine (3 μM), SKF-96365 (100 μM), Y-27632 (30 μM) or PMF (0.03 mM) caused a significant inhibition of the phenylephrine *C-R* curves on the thoracic aortic ring. In this situation, the depression of the phenylephrine *C-R* curve by PMF was bigger than the one produced by nifedipine and SKF-96365 at a low concentration of the phenylephrine but not at its high concentrations where the maximal responses were the same (Figure 4A and B). When compared to the effect of Y-27632, the *C-R* curve of the phenylephrine in the presence of PMF was significantly lower than the one with Y-27632 throughout (Figure 4C). When nifedipine and/or SKF-96365 were added together with PMF, a further depression of the phenylephrine *C-R* curve was obtained (Figure 4D). A further significant inhibition of the phenylephrine *C-R* curve was found when Y-27632 was also added together with nifedipine, SKF-96365 and PMF (Figure 4E).

Discussion

The present study has clearly demonstrated that PMF has a relaxant activity on the isolated thoracic aortic rings in an endothelium-dependent and -independent manner. Possible mechanisms responsible for the relaxation were explored to determine if PMF acted 1) as a nitric oxide stimulator, a guanylyl cyclase-, an adenylyl cyclase- or H_2S stimulator and/or by opening of K^+ channels, 2) by blocking voltage-dependent calcium channels, 3) by inhibition of intracellular calcium mobilization, 4) as a store-operated calcium channel inhibitor, or 5) as a Rho-kinase inhibitor.

Possible actions as a nitric oxide stimulator, a guanylyl cyclase-, an adenylyl cyclase- or H_2S stimulator and/or by the opening of K^+ channels

Our finding that LNA inhibited the relaxant activity of the PMF indicated that PMF stimulated nitric oxide release. ODQ alone was also found to inhibit the relaxant activity of the PMF on the endothelium-intact thoracic aortic rings, however no further additional inhibition was obtained when LNA was also present. These results indicated that PMF would not act directly via a stimulation of the guanylyl cyclase in the vascular smooth muscle. The inhibitory effect of the ODQ could be indirect via the soluble guanylyl cyclase that was activated by the endothelial NO that resulted from the PMF stimulation. These results are different from our previous reports on the human cavernosum, in which it was found that PMF showed very little effect on the nitric oxide stimulation with no effect on the soluble guanylyl cyclase stimulation.[24] The reason for this is most likely due to the different types of blood vessels, and human cavernosum might have a different underlying mechanism for its relaxant activity to the PMF: the relaxation of the cavernosum is predominantly a response as the result of neuronal NO transmission.[29-30] However, a further study would be needed to clarify this possibility. In addition, it was a surprise to see that in the presence of LNA with the endothelium-intact thoracic aortic ring, ODQ potentiated the relaxant activity of the PMF, in a situation when the nitric oxide and soluble guanylyl cyclase was being inhibited. Thus, it is possible that PMF might also activate other signalling pathways: such as the H_2S and/or adenylyl cyclase pathways.

It has now been accepted that H_2S is the third endogenous gasotransmitter with pivotal roles in regulating vascular homeostasis.[31-34] H_2S is released from the vascular endothelium as well as from the vascular smooth muscles by cystathionine-γ-lyase (CSE), the key enzyme that utilizes L-cysteine as a

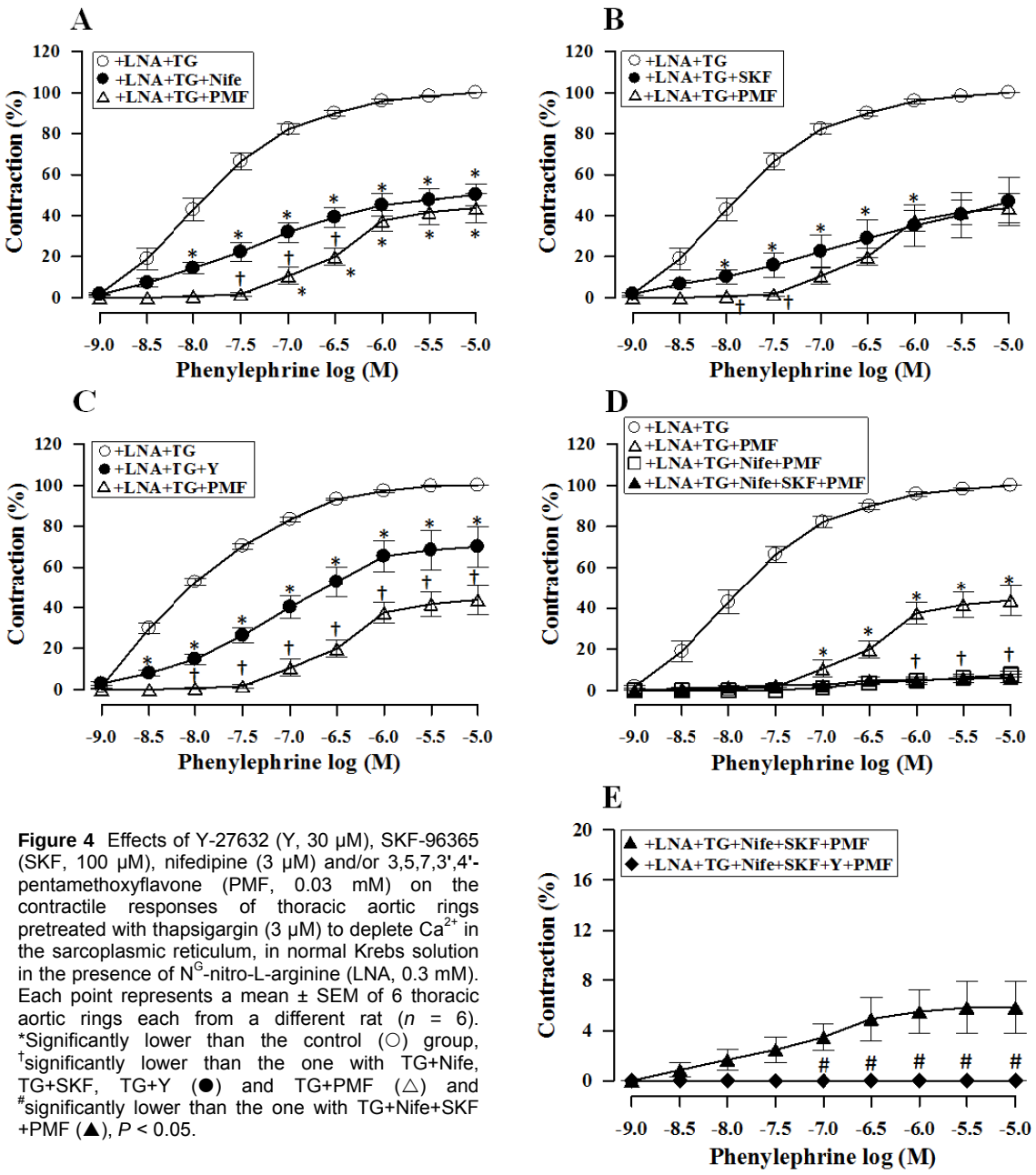

Figure 4 Effects of Y-27632 (Y, 30 μM), SKF-96365 (SKF, 100 μM), nifedipine (3 μM) and/or 3,5,7,3',4'-pentamethoxyflavone (PMF, 0.03 mM) on the contractile responses of thoracic aortic rings pretreated with thapsigargin (3 μM) to deplete Ca^{2+} in the sarcoplasmic reticulum, in normal Krebs solution in the presence of N^G-nitro-L-arginine (LNA, 0.3 mM). Each point represents a mean ± SEM of 6 thoracic aortic rings each from a different rat (n = 6). *Significantly lower than the control (○) group, †significantly lower than the one with TG+Nife, TG+SKF, TG+Y (●) and TG+PMF (△) and #significantly lower than the one with TG+Nife+SKF +PMF (▲), $P < 0.05$.

substrate to form H_2S.[35-37] Within the vascular wall, the NO and H_2S pathways coexist and serve similar functions.[38] In the case of the nitric oxide pathway, Li *et al*.[39] reported that quercetin induced a rapid eNOS phosphorylation that enhanced the production of NO and promoted vasodilatation of the endothelium-intact thoracic aortic ring via the cAMP/PKA-mediated pathway. They found that the production of the intracellular cAMP was quickly increased by stimulation with quercetin concomitantly with the induction of eNOS phosphorylation at Ser 1179. In addition, the effect of cAMP was also mediated through the allosteric activation of the protein kinase A (PKA), which has been shown to decrease the intracellular Ca^{2+} concentration and lead to a relaxation of the vascular smooth muscle.[40-42] In a separate finding, Chiwororo and Ojewole[43] found that quercetin-induced relaxation of the rat isolated portal vein, partly via the cAMP-dependent protein kinase pathway. Thus, it is possible that PMF might stimulate the release of H_2S when nitric oxide had been removed and when the soluble guanylyl cyclase had been inhibited by ODQ, so the augmentation of the H_2S and/or cAMP by the PMF could also induce vasodilatation. In order to prove these possibilities, the endothelium-intact thoracic aortic rings in the presence of LNA and/or ODQ were preincubated with PAG, a cystathionine- γ-lyase inhibitor (inhibits H_2S generation), alone or together with SQ22536 (an adenylyl cyclase inhibitor) before performing the *C-R* curve to the PMF. As shown in Figure 1C and D, in the presence of LNA, PAG alone or together with SQ22536 did not modify the PMF *C-R* curve when compared to that of the one with LNA. However,

when ODQ was added together with LNA, the PAG significantly inhibited the PMF *C-R* curve, and when the SQ22536 was also added with the PAG, the PMF *C-R* curve was restored to the same level as that of the control groups (Figure 1E and F). These results indicated that the PMF might stimulate the release of H_2S as well as affecting an increase in the generation of cAMP by the adenylyl cyclase from the vascular smooth muscle and this resulted in vasodilatation.

TEA did not modify the PMF *C-R* curve, whereas glybenclamide potentiated the PMF *C-R* curve of the endothelium-intact thoracic aortic rings whether LNA was present or not. This indicated that PMF did not open the Ca^{2+} sensitive K^+ channel (K_{Ca}), nor did it open the ATP sensitive K^+ channel (K_{ATP}).[44-46] These results are similar to those found in the human cavernosum.[24] The potentiation of the PMF *C-R* curve by glybenclamide would result from a stimulated release of H_2S and/or cAMP by the PMF from the thoracic aorta (Figure 2C and D). This confirmed the above finding that the PMF stimulated H_2S release and cAMP formation. The finding that the stimulated release of H_2S by the PMF on the thoracic aortic ring was not inhibited by glybenclamide, a K_{ATP} channel blocker, was analogous to what occurred in the middle cerebral artery where it was found that the exogenous H_2S mediated relaxation, yet received no contribution from the K_{ATP} channel.[36] Although a few investigators have reported that the mechanism responsible for the vasodilatory effect of the H_2S, involved K_{ATP} channel, because its effect was inhibited by glybenclamide,[32,47-48] this was different from our finding. However, in a recent report, an H_2S-mediated relaxation of the endothelium-denuded blood vessels did occur by inhibition of the L-type calcium channels with an additional contribution by the K^+ channels, probably K_v7 but not K_{ATP}, K_{Ca}, or K_{ir} subtypes.[31, 34, 36] Therefore, a further study would be needed to confirm the present finding.

Possible reactions via voltage- or store-operative calcium channels, Rho-kinase and intracellular Ca^{2+} mobilization

Our finding that PMF antagonized the phenylephrine-induced contraction of the thoracic aortic ring in the normal Krebs solution with Ca^{2+}channel blocker, nifedipine, as well as in the Ca^{2+} free Krebs medium indicated that PMF might inhibit Ca^{2+} mobilization from the intracellular stores.[49-52] These results were also similar to those found in the isolated human cavernosum.[24]

PMF might act as a store-operated Ca^{2+} channel inhibitor, and further experiments were carried out in the presence of thapsigargin, a specific sarcoplasmic-endoplasmic reticulum Ca-ATPase (SERCA) pump inhibitor,[53-54] to deplete the intracellular Ca^{2+} store, which then would stimulate the opening of the plasma membrane store-operated Ca^{2+} channels to add to the refilling of the intracellular stores.[55-56] Thus it would be expected that if PMF played a role as a voltage-, store-operated Ca^{2+}-channel blocker, or a Rho-kinase inhibitor, the phenylephrine *C-R* curve of the thapsigargin that induced the intracellular Ca^{2+} depleted-thoracic aorta could not be further inhibited by addition of the PMF after having been pre-incubated with nifedipine, SKF-96365, or Y-27632 respectively. As shown in the result section, when PMF was added together with nifedipine, SKF-96365 and/or Y-27632, a complete inhibition of the phenylephrine *C-R* curve was obtained. These results indicated that PMF did not act as an L-type Ca^{2+} channel inhibitor, a store-operated Ca^{2+} channel inhibitor or a Rho-kinase inhibitor. These results are consistent with those for human cavernosum except for the blockade of the voltage operated Ca^{2+} channel that was found in the isolated human cavernosum.[24] This also confirmed that different types of smooth muscle from different animals can have different mechanism of reaction to PMF.

In conclusion, the present study has demonstrated that PMF exerted a relaxant activity on the isolated thoracic aorta by stimulating the release of nitric oxide and H_2S from the blood vessel. In addition, it also acted as an adenylyl cyclase stimulator, and might act as an inhibitor of the intracellular Ca^{2+} mobilization from the sarcoplasmic reticulum. It does not appear to act as an opener of K_{ATP}- or K_{Ca} channel, an inhibitor of Rho-kinase or an L-type- or a store-operated Ca^{2+}channel. At the present time, this is the first report to demonstrate that PMF also stimulates the release of H_2S, the third endogenous vasodilatory gasotransmitter, in addition to the nitric oxide of the rat thoracic aorta. Taken together PMF caused a vasorelaxation of the thoracic aortic ring via concerted reactions with several different pathways that provided significant advantages in that one could compensate if another signalling pathway had undergone defection. Therefore, PMF is a novel vasodilatory compound to be considered for the development of an alternative treatment for hypertension.

Acknowledgements

This work was supported by the National Research Council of Thailand under the Prince of Songkla University (Research grant No. Sci520037s). The authors thank Prof. Poungpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University, Thailand for her kind help in identifying the plant, Miss Srisurat Duangsai for technical assistance and Dr. Brian Hodgson, Dr. Norman Scholfield and Dr. Alan Gueter for English assistance.

Conflict of interest

None to declare.

References

1. Perez-Vizcaino F, Duarte J, Jimenez R, Santos-Buelga C, Osuna A. Antihypertensive effects of the flavonoid quercetin. Pharmacol Rep. 2009; 61:67-75.
2. Boots AW, Haenen GR, Bast A. Health effects of quercetin: From antioxidant to nutraceutical. Eur J Pharmacol. 2008; 585: 325-37.
3. Duarte J, Perez-Palencia R, Vargas F, Ocete MA, Perez-Vizcaino F, Zarzuelo A, *et al.* Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. Br J Pharmacol. 2001; 133: 117-24.
4. Duarte J, Perez-Vizcaino F, Zarzuelo A, Jimenez J, Tamargo J. Vasodilator effects of quercetin in isolated rat vascular smooth muscle. Eur J Pharmacol. 1993; 239: 1-7.
5. Monori-Kiss A, Monos E, Nadasy GL. Quantitative analysis of vasodilatory action of quercetin on intramural coronary resistance arteries of the rat in vitro. Plos One. 2014; 9: e105587.
6. Ader P, Wessmann A, Wolffram S. Bioavailability and metabolism of the flavonol quercetin in the pig. Free Radic Biol Med. 2000; 28: 1056-67.
7. Manach C, Texier O, Morand C, Crespy V, Regerat F, Demigne C, Remesy C. Comparison of the bioavailability of quercetin and catechin in rats. Free Radic Biol Med. 1999; 27: 1259-66.
8. Walle T. Methylation of dietary flavones greatly improves their hepatic metabolic stability and intestinal absorption. Mol Pharm. 2007; 4: 826-32.
9. Wen X, Walle T. Methylated flavonoids have greatly improved intestinal absorption and metabolic stability. Drug Metab Dispos. 2006; 34: 1786-92.
10. Hnatyszyn O, Moscatelli V, Rondina R, Costa M, Arranz C, Balaszczuk A, *et al.* Flavonoids from *Achyrocline satureioides* with relaxant effects on the smooth muscle of guinea pig corpus cavernosum. Phytomedicine. 2004; 11: 366-9.
11. Mekjaruskul C, Jay M, Sripanidkulchai B. Pharmacokinetics, bioavailability, tissue distribution, excretion, and metabolite identification of methoxyflavones in *Kaempferia parviflora* extract in rats. Drug Metab Dispos. 2012; 40: 2342-53.
12. Yenjai C, Sutthanut K, Sripanidkulchai B, Mungkhun N, Wongma C, Mukdasai S, *et al.* Further studies of bioactive flavonoids from *Kaempferia parviflora*. KKU Sci J. 2007; 35: 37-41.
13. Li S, Lo CY, Ho CT. Hydroxylated polymethoxyflavones and methylated flavonoids in sweet orange (*Citrus sinensis*) peel. J Agric Food Chem. 2006; 54: 4176-85.
14. Lu Y, Zhang C, Bucheli P, Wei D. Citrus flavonoids in fruit and traditional Chinese medicinal food ingredients in China. Plant Foods Hum Nutr. 2006; 61: 57-65.
15. Mizuno M, IInuma M, Ohara M, Tanaka T, Iwamasa M. Chemotaxonomy of the genus citrus based on polymethoxyflavones. Chem Pharm Bull (Tokyo). 1991; 39: 945-9.
16. Ikemura M, Sasaki Y, Giddings JC, Yamamoto J. Protective effects of nobiletin on hypertension and cerebral thrombosis in stroke-prone spontaneously hypertensive rats (SHRSP). Food Nutr Sci. 2012; 3: 1539-46.
17. Kang SI, Shin HS, Ko HC, Kim SJ. Effects of sinensetin on lipid metabolism in mature 3T3-L1 adipocytes. Phytother Res. 2013; 27: 131-4.
18. Lam IK, Alex D, Wang YH, Liu P, Liu AL, Du GH, *et al.* In vitro and in vivo structure and activity relationship analysis of polymethoxylated flavonoids: identifying sinensetin as a novel antiangiogenesis agent. MolNutr Food Res. 2012; 56: 945-56.
19. Lin N, Sato T, Takayama Y, Mimaki Y, Sashida Y, Yano M, *et al.* Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. Biochem Pharmacol. 2003; 65: 2065-71.
20. Mulvihill EE, Assini JM, Lee JK, Allister EM, Sutherland BG, Koppes JB, *et al.* Nobiletin attenuates VLDL overproduction, dyslipidemia, and atherosclerosis in mice with diet-induced insulin resistance. Diabetes. 2011; 60: 1446-57.
21. Robbins RC. Flavones in citrus exhibit antiadhesive action on platelets. Int J Vitam Nutr Res. 1988; 58: 418-21.
22. Chaturapanich G, Chaiyakul S, Verawatnapakul V, Pholpramool C. Effects of *Kaempferia parviflora* extracts on reproductive parameters and spermatic blood flow in male rats. Reproduction. 2008; 136: 515-22.
23. Murata K, Deguchi T, Fujita T, Matsuda H. Improvement in blood fluidity by *Kaempferia parviflora* rhizome. J Nat Med. 2012; 67: 719-24.
24. Jansakul C, Tachanaparuksa K, Mulvany MJ, Sukpondma Y. Relaxant mechanisms of 3,5,7,3',4'-pentamethoxyflavone on isolated human cavernosum. Eur J Pharmacol. 2012; 691: 235-44.
25. Tep-Areenan P, Sawasdee P, Randall M. Possible mechanisms of vasorelaxation for 5,7-dimethoxyflavone from *Kaempferia parviflora* in the rat aorta. Phytother Res. 2010; 24: 1520-5.
26. Tep-areenan P, SawasdeeP. Vasorelaxant Effects of 5,7,4́-trimethoxyflavone from *Kaepmferia parviflora* in the rat aorta. Int J Pharm. 2010; 6: 419-24.
27. Yorsin S, Kanokwiroon K, Radenahmad N, Jansakul C. Effects of *Kaempferia parviflora* rhizomes dichloromethane extract on vascular functions in middle-aged male rat. J Ethnopharmacol. 2014; 156: 162-74.
28. Jansakul C, Boura AL, King RG. Effects of endothelial cell removal on constrictor and dilator responses of aortae of pregnant rats. J Auton Pharmacol. 1989; 9: 93-101.
29. Ghalayini IF. Nitric oxide-cyclic GMP pathway with some emphasis on cavernosal contractility. Int J Impot Res. 2004; 16: 459-69.
30. Hurt KJ, Sezen SF, Lagoda GF, Musicki B, Rameau GA, Snyder SH, *et al.* Cyclic AMP-dependent phosphorylation of neuronal nitric oxide synthase mediates penile erection. Proc Natl Acad Sci USA 2012; 109: 16624-9.
31. Al-Magableh MR, Hart JL. Mechanism of vasorelaxation and role of endogenous hydrogen sulfide production in mouse aorta. Naunyn Schmiedebergs Arch Pharmacol. 2011; 383: 403-13.
32. Cheng Y, Ndisang JF, Tang G, Cao K, Wang R.

Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. Am J Physiol Heart Circ Physiol. 2004; 287: H2316-23.
33. Hart JL. Role of sulfur-containing gaseous substances in the cardiovascular system. Front Bio Sci. 2011; (Elite Ed) 3: 736-49.
34. Martelli A, Testai L, Breschi MC, Lawson K, McKay NG, MiceliF, *et al.* Vasorelaxation by hydrogen sulfide involves activation of K_v7 potassium channels. Pharmacol Res. 2013; 70:27-34.
35. Bhatia M. Hydrogen sulfide as a vasodilator. IUBMB Life. 2005; 57: 603-6.
36. Streeter E, Hart J, Badoer E. An investigation of the mechanisms of hydrogen sulfide-induced vasorelaxation in rat middle cerebral arteries. Naunyn Schmiedebergs Arch Pharmacol. 2012; 385: 991-1002.
37. Sun Y, Tan CS, Jin HF, Du JB. The vasorelaxing effect of hydrogen sulfide on isolated rat aortic rings versus pulmonary artery rings. Acta Pharmacol Sin. 2011; 32: 456-64.
38. Bucci M, Papapetropoulos A, Vellecco V, Zhou Z, Pyriochou A, Roussos C, *et al.* Hydrogen sulfide is an endogenous inhibitor of phosphodiesterase activity. Arterioscler Thromb Vasc Biol. 2010; 30: 1998-2004.
39. Li PG, Sun L, Han X, Ling S, Gan WT, Xu JW. Quercetin induces rapid eNOS phosphorylation and vasodilation by an Akt-independent and PKA-dependent mechanism. Pharmacol. 2012; 89: 220-8.
40. Jiang H, Colbran JL, Francis SH, Corbin JD. Direct evidence for cross-activation of cGMP-dependent protein kinase by cAMP in pig coronary arteries. J Biol Chem. 1992a; 267: 1015-9.
41. Jiang H, Shabb JB, Corbin JD. Cross-activation: overriding cAMP/cGMP selectivities of protein kinases in tissues. Biochem Cell Biol. 1992b; 70: 1283-9.
42. Murthy KS, Makhlouf GM. Differential regulation of phospholipase A2 (PLA2)-dependent Ca^{2+} signaling in smooth muscle by cAMP- and cGMP-dependent protein kinases. Inhibitory phosphorylation of PLA2 by cyclic nucleotide-dependent protein kinases. J Biol Chem. 1998; 273: 34519-26.
43. Chiwororo WD, Ojewole JA. Dual effect of quercetin on rat isolated portal vein smooth muscle contractility. Cardiovasc J Afr. 2010; 21: 132-6.
44. Brayden JE. Functional roles of K_{ATP} channels in vascular smooth muscle. Clin. Exp. Pharmacol Physiol. 2002; 29: 312-6.
45. Ko EA, Han J, Jung ID, Park WS. Physiological roles of K^+ channels in vascular smooth muscle cells. J Smooth Muscle Res. 2008; 44: 65-81.
46. Stojnic N, Gojkovic-Bukarica L, Peric M, Grbovic L, Lesic A, Bumbasirevic M, *et al.* Potassium channel opener pinacidil induces relaxation of the isolated human radial artery. J Pharmacol Sci. 2007; 104: 122-9.
47. Moccia F, Bertoni G, Pla AF, Dragoni S, Pupo E, Merlino A, *et al.* Hydrogen sulfide regulates intracellular Ca^{2+} concentration in endothelial cells from excised rat aorta. Curr Pharm Biotechnol. 2011; 12: 1416-26.
48. Tang G, Wu L, Liang W, Wang R. Direct stimulation of K(ATP) channels by exogenous and endogenous hydrogen sulfide in vascular smooth muscle cells. Mol Pharmacol. 2005; 68: 1757-64.
49. Abebe W, Harris KH, MacLeod KM. Enhanced contractile responses of arteries from diabetic rats to alpha 1-adrenoceptor stimulation in the absence and presence of extracellular calcium. J Cardiovasc Pharmacol. 1990; 16: 239-48.
50. Akata T. Cellular and molecular mechanisms regulating vascular tone. Part 1: Basic mechanisms controlling cytosolic Ca^{2+} concentration and the Ca^{2+}-dependent regulation of vascular tone. J Anesth. 2007; 21: 220-31.
51. Nelson MT, Standen NB, Brayden JE, Worley JF. Noradrenaline contracts arteries by activating voltage-dependent calcium channels. Nature. 1988; 336: 382-5.
52. Noguera MA, Madrero Y, Ivorra MD, D'Ocon P. Characterization of two different Ca^{2+} entry pathways dependent on depletion of internal Ca^{2+} pools in rat aorta. Naunyn Schmiedebergs Arch Pharmacol. 1998; 357: 92-9.
53. Quinn T, Feighery R, Baird AW. Role of Rho-kinase in guinea-pig gallbladder smooth muscle contraction. Eur J Pharmacol. 2006; 534: 210-7.
54. Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc Natl Acad Sci USA. 1990; 87: 2466-70.
55. Parekh AB, Putney JW. Store-operated calcium channels. Physiol Rev. 2005; 85: 757-810.
56. Putney JW. The physiological function of store-operated calcium entry. Neurochem Res. 2011; 36: 1157-65.