ผลของฟิลแลนทิน และไฮโปฟิลแลนทินต่อแรงตึงตัวของหลอดเลือดแดงใหญ่ที่แยกจากกายหนูแรท

นางสาว มาริสา อินทร์ชู

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญา วิทยาศาสตรมหาบัณฑิต
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EFFECTS OF PHYLLANTHIN AND HYPOPHYLLANTHIN ON VASCULAR TENSION OF ISOLATED RAT AORTA

Miss Marisa Inchoo

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Pharmacology
(Interdisciplinary Program)
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By	Miss Marisa Inchoo
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Suree Jianmongkol, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…….. Dean of the Graduate School

(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

…….. Chairman

(Assistant Professor Wacharee Limpanasithikul, Ph.D.)

……. Thesis Advisor

(Assistant Professor Suree Jianmongkol, Ph.D.)

…….. Examiner

(Assistant Professor Naowarat Suthamnatpong, Ph.D.)

…….. External Examiner

(Associate Professor Prasan Dhumma-upakorn, Ph.D.)

มาริสา อินทร์ชู : ผลของฟิลแลนทิน และไฮโปฟิลแลนทิน ต่อแรงตึงตัวของหลอดเลือดแดงใหญ่ที่แยกจากกายหนูแรท . (EFFECTS OF PHYLLANTHIN AND HYPOPHYLLANTHIN ON VASCULAR TENSION OF ISOLTED RAT AORTA) อ. ที่ปรึกษา วิทยานิพนธ์หลัก : ผศ. ดร. สุรีย์ เจียรณ์มงคล , 98 หน้า.

การศึกษาครั้งนี้ได้ศึกษาผลของฟิลแลนทิน และไฮโปฟิลแลนทินต่อแรงตึงของหลอดเลือดแดงใหญ่ที่แยกจากกายหนูแรท ผลการศึกษาพบว่าฟิลแลนทิน และไฮโปฟิลแลนทิน (1-100 μM) ทำให้หลอดเลือดที่ถูกกระตุ้นด้วย phenylephrine (PE) คลายตัวได้แบบ concentration-dependent manner นอกจากนี้การขูดเซลล์เยื่อบุผิวออกไม่มีผลต่อการคลายตัวของหลอดเลือดที่เป็นผลจากสารทดสอบทั้ง 2 ชนิด ซึ่งผลของฟิลแลทิน และไฮโปฟิลแลนทิน (100 μM) ที่ทำให้หลอดเลือดที่ไม่มีเซลล์เยื่อบุผิวคลายตัวนั้นไม่ถูกยับยั้งโดย atropine (1 μM), $BaCl_2$ (10 μM), glybenclamide (10 μM), indomethacin (10 μM), L-NAME (100 μM), methylene blue (10 μM), propranolol (10 μM), 4-aminopyridine (100 μM) และ TEA (10 μM) ผลการทดลองแสดงให้เห็นว่า ลิกแนนทั้ง 2 ชนิดมีผลเหนี่ยวนำให้หลอดเลือดหดตัว และถูกยับยั้งได้โดย tyramine หรือ prazosin นอกจากนี้สารทั้ง 2 ชนิดยับยั้งการหดตัวของกล้ามเนื้อเรียบหลอดเลือดที่ถูกกระตุ้นด้วย PE และ KCl ได้แบบ concentration-dependent manner และสารทั้ง 2 ชนิด (100 μM) ยังสามารถยับยั้ง การหดตัวของกล้ามเนื้อเรียบที่เหนี่ยวนำด้วยแคลเซียม ที่เกิดจากแคลเซียมภายในเซลล์ถูกไล่ออกหมด อย่างไรก็ตามฟิลแลนทินเท่านั้นมีผลยับยั้งการหดตัวของกล้ามเนื้อเรียบหลอดเลือดในสภาวะที่มี K^+ สูงและปราศจาก Ca^{2+} แต่ไฮโปฟิลแลนทินไม่มีผลยับยั้งการตอบสนองนี้ นอกจากนี้ สารทั้ง 2 ชนิด (100 μM) ยับยั้งการหดตัวของกล้ามเนื้อเรียบหลอดเลือดที่ถูกกระตุ้นด้วย PE ในสภาวะที่ปราศจาก Ca^{2+} แต่ไม่มีต่อการหดตัวของกล้ามเนื้อเรียบหลอดเลือดที่ถูกกระตุ้นด้วย caffeine ซึ่งผลการทดลองทั้งหมดแสดงให้เห็นว่าฟิลแลนทิน และไฮโปฟิลแลนทินมีผลต่อแรงตึงตัวของหลอดเลือดผ่านกลไกที่ไม่เกี่ยวข้องกับเซลล์เยื่อบุผิว โดยอาจเกี่ยวข้องกับการยับยั้งการเคลื่อนที่ของ Ca^{2+} จากภายนอกเข้าสู้ภายในเซลล์ และการยับยั้งหลั่ง Ca^{2+} จาก sarcoplasmic reticulum ผ่านทางการกระตุ้นด้วย PE

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษา วิทยานิพนธ์หลัก.....

5187238020 : MAJOR PHARMACOLOGY

KEYWORDS : VASORELAXATION / PHYLLANTHIN / HYPOPHYLLANTHIN

MARISI INCHOO : EFFECTS OF PHYLLANTHIN AND HYPOPHYLLANTHIN ON VASCULAR TENSION OF ISOLATED RAT AORTA. THESIS ADVISOR : ASST. PROF. SUREE JIANMONGKOL, Ph.D., 98 pp.

The purpose of this study was to investigate the modulating effects of phyllanthin and hypophyllanthin on vascular tension, using in the *in vitro* model of isolated rat aorta. The results indicated that both phyllanthin and hypophyllanthin (1-100 μM) significantly relaxed the sustained contraction induced by phenylephrine (PE) in a concentration-dependent manner. In addition, endothelial removal had no significant influence on the vasorelaxation responses of the aortic rings toward these two compounds. In endothelium-denuded rings, vasorelaxant inhibitors [atropine (1 μM), $BaCl_2$ (10 μM), glybenclamide (10 μM), indomethacin (10 μM), L-NAME (100 μM), methylene blue (10 μM), propranolol (10 μM), 4-aminopyridine (100 μM) and TEA (10 μM)] did not inhibit the vasorelaxation responses induced by either phyllanthin or hypophyllanthin (100 μM). The data also demonstrated that the two lignans were able to induce aortic contraction which could be suppressed by either tyramine or prazosin. In addition, both compounds inhibited the PE- and KCl-mediated contraction of aortic muscle in concentration-dependent manner. Furthermore, these two compounds (100 μM) also significantly inhibited the Ca^{2+}- induced contraction of aortic muscles that were depleted of Ca^{2+}. However, only phyllanthin (100 μM), but not hypophyllanthin was able to inhibit the Ca^{2+}- mediated contraction in high K^+ - Ca^{2+}- free condition. Both compounds (100 μM) significantly inhibited PE-induced contraction in Ca^{2+}- free condition, but could not affect caffeine-induced contraction. Taken together, phyllanthin and hypophyllanthin could modulate the vascular tension via the endothelium-independent mechanisms. The modulating effect of the test compounds were likely involved with the inhibition of Ca^{2+} influx as well as with the inhibition of PE-mediated Ca^{2+} release from sarcoplasmic reticulum.

Field of Study : Pharmacology Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

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Finally, I would like to thanks and indescribable gratitude goes to my family and my friends for their love and care, this inspired me to succeed my academic achievement and I am very appreciated to all experiment animals for their valuable life

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LIST OF ABBREVIATIONS

4-AP	4-aminipyridine
AC	adenylate cyclase
ACh	acetylcholine
ANOVA	one-way analysis of mean
ATP	adenosine 5'-triphosphate
$BaCl_2$	barium chloride
Ca^{2+}	calcium ion
cAMP	cyclic adenosine 3', 5' monophosphate
cGMP	cyclic guanosine 3', 5' monophosphate
cm	centimeters
COX	cycloxygenase
DG	diacylglycerol
DMSO	dimethyl sulfoxide
EDHF	endothelium-derived hyperpolarizing factor
GTP	guanosine 5'-triphosphate
IC_{50}	50% Inhibitory concentration
IP_3	inositol 1, 4, 5-trisphosphate
IP_3R	inositol 1, 4, 5-trisphosphate receptor
IRT	increase in the resting tone
KHS	Krebs-Henseleit solution
K^+	potassium ion
KCl	potassium chloride
L-NAME	N^G-nitro-L-arginine methyl ester
M	molar
MLC	myosin light chain
MLCK	myosin light chain kinase
ml	milliltre
mm	millimeters

mM	millimolar
μM	micromolar
NO	nitric oxide
NOS	nitric oxide synthase
PE	phenylephrine
PGI_2	prostacyclin
PIP_2	phosphatidylinositol bis-phosphate
PKA	protein kinase A
PKG	protein kinase G
PLC	phospholipase C
RhoGEF	guanine nucleotide exchange factor
ROC	receptor-operated Ca^{2+} channel
RyR	ryanodine receptor
S.E.M.	standard error of the mean
sGC	soluble guanylyl cyclase
SR	sarcoplasmic recticulum
TEA	tetraethylammonium
VOC	voltage-operated Ca^{2+} channel

CHAPTER I

BACKGROUND AND INTRODUCTION

Lignans are a group of dimeric phenylpropanoids (Figure 1) found in various plants such as flaxseed, soybeans, cereals, gains, oats, garlic, carrot, tomato, vegetables and fruits. Examples of lignans are secoisolariciresinol, matairesinol, eudesmin and sesamin. These compounds were reported to induce vasorelaxation in various isolated organ systems and hypotension in rat and rabbit models (Nakano *et al*. 2006; Prasad, 2004; Raimundo *et al*., 2009). The modes of actions of these compounds were linked to activation NO/cGMP pathway (Nakano *et al*. 2006; Prasad, 2004) as well as activation of histamine H_1 receptors in endothelial cells (Raimundo *et al*., 2009).

Phyllanthin (diarylbutane) and hypophyllanthin (aryltetrahydronapthalene) (Figure 1) are major components found in *Phyllantus amarus* Schum. and Thonn. (Euphorbiaceae) [also known as "Luk Tai Bai" in Thai] (Bagalkotkar *et al*., 2006). In Thailand, this plant has been used in traditional medicine for a number of diseases in cardiovascular and gastrointestinal tract systems (Abdulla *et. al.,* 2010; Shokunbi and Odetola, 2008; Thippeswamy *et al*., 2011). It has been reported that the alcoholic extract of *P. amarus* induced smooth muscle relaxation in various tissues including vascular system, uterine, intestine, stomach and trachea (Kitisin, 1952). In addition, the aqueous extract from its leaves exerted its myocardial suppression and hypotensive effects in rabbits, which was possibly linked to muscarinic receptor-mediated mechanism and calcium channel ion blockade (Amaechina and Omogbai, 2007). It was likely that either phyllanthin or hypophyllanthin, which were the two major lignans isolated from this plant, possessed the intrinsic pharmacological actions similar to those of the *P. amarus* extracts. However, a few researches have been conducted to investigate the modulating effects of these two lignans on vascular tension and the involved mechanism.

The purpose of this study was to investigate the modulating effects of phyllanthin and hypophyllanthin on vascular tension. Furthermore, the involvement of endothelium in its vascular actions was also examined.

Hypothesis

Phyllanthin and hypophyllanthin modulated the vascular tension via both endothelium-dependent and endothelium-independent pathways. In endothelium-dependent pathway, these two compounds exerted its effects via activation of the NO/cGMP pathway. In addition, these two compounds could affect the alteration of cytosolic Ca^{2+} in smooth muscle, independently from the endothelium.

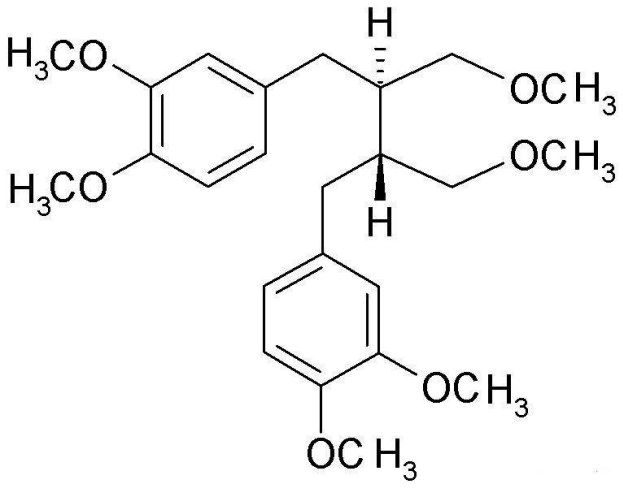
Objectives

1. To investigate the modulating effects of phyllanthin and hypophyllanthin on vascular tension.

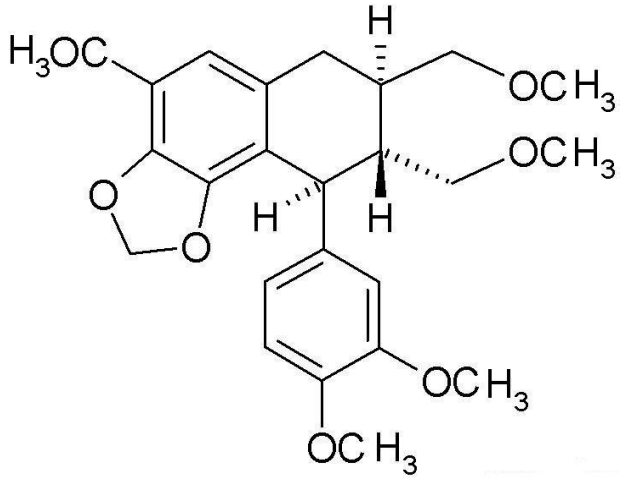
2. To investigate the mechanisms and influence of endothelium on either phyllanthin or hypophyllanthin mediated-contractility of vascular smooth muscle. In addition, it was possible that the endothelium-independent effects of these two compounds involved with alteration in extracellular Ca^{2+} entry and intracellular Ca^{2+} release in the smooth muscle.

Expected Benefits and Application

This study provided the new pharmacological effects on vascular tension of phyllanthin and hypophyllanthin and the involved mechanisms of action. The information would be useful for further application in herbal and traditional medicine development.



Phyllanthin



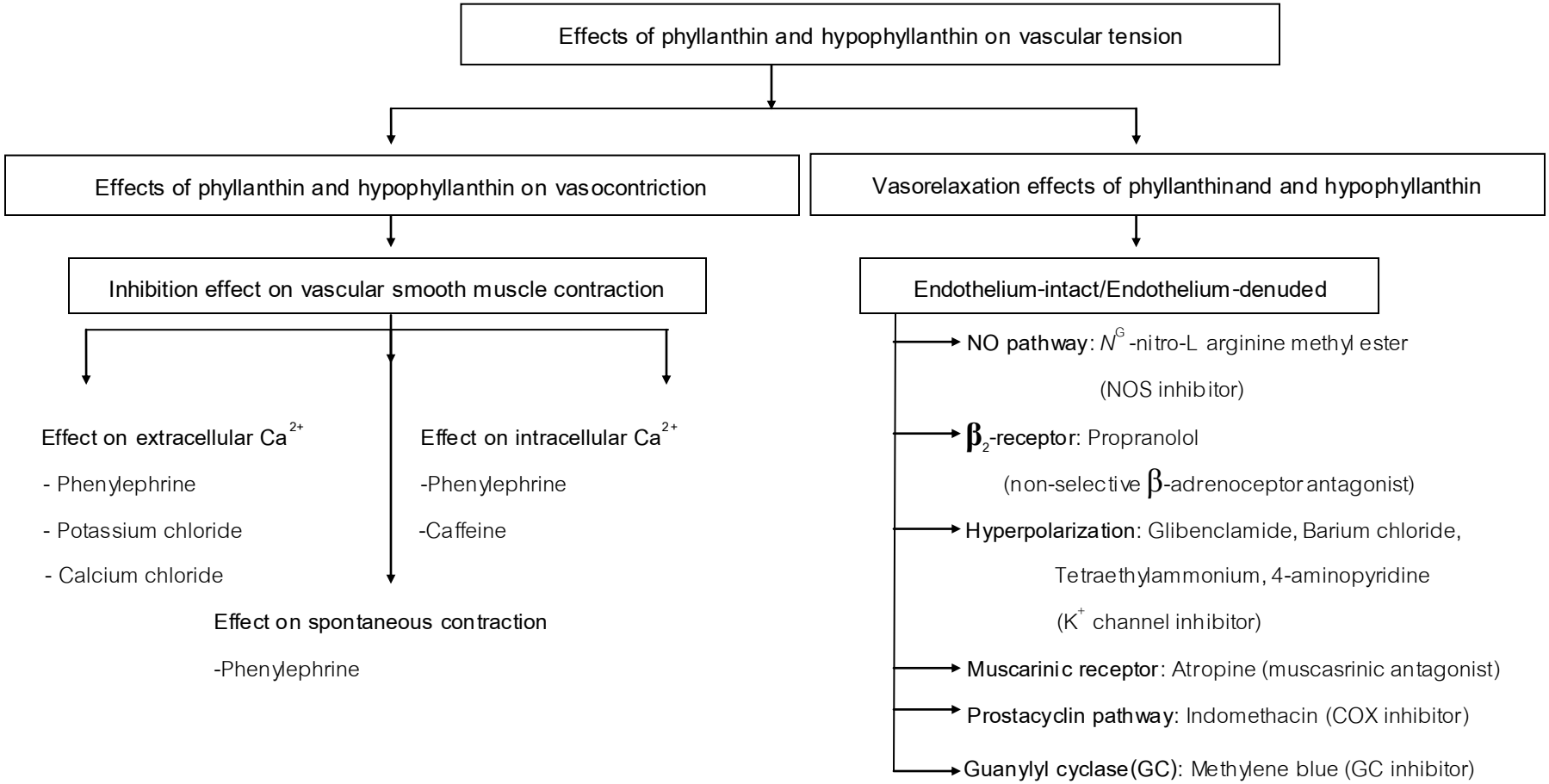
Hypophyllanthin

Figure 1 Structure of phyllanthin and hypophyllanthin

(http://www.alibaba.com/product-tp/11106_hin.html)

Study design

Test compounds: Phyllanthin and Hypophyllanthin

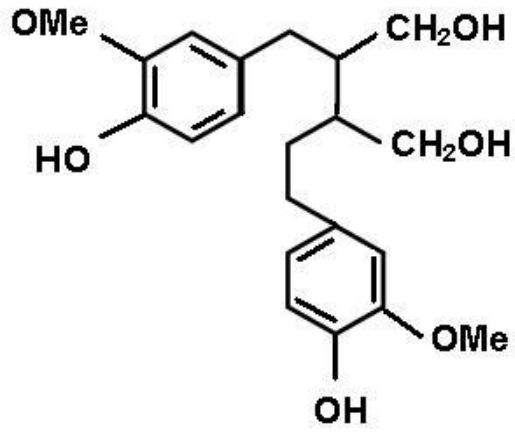


CHAPTER II

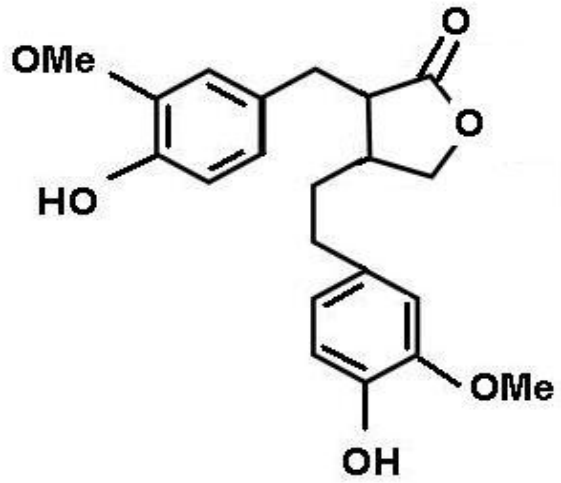
LITERATURE REVIEWS

Lignans are a group of phytochemicals found in various herbal plants such as *Phyllanthus* species, *Forsythia intermedia*, *Valeriana* species, *Arctium lappa, Schisandra chinensis* (Bagalkotkar *et al*., 2006; Park *et. al*., 2009; Rahman *et al*., 1990). In addition, lignans can be counted as a class of secondary plant metabolites, which are produced by oxidative dimerization of two phenylpropanoid units (Sok *et al*., 2009). The aromatic rings of lignans can be oxygenated and bear hydroxyl, methoxyl, or methylenedioxy groups (Hearon and MacGregor, 1955). The basic molecular structure of lignans was shown in Figure 2. Lignans can be further modified by cyclization to tetrahydrofuran or tetrahydronaphthalene derivatives (Hearon and MacGregor, 1955). Examples of lignans include secoisolariciresinol diglucoside (SDG), pinoresinol-4, 40-di-O-b-D-glucoside (PDG), pinoresinol, pinoresinol glycoside, podophyllotoxin, picropodophyllin, eudesmin and sesamin (Figure 3). Several pharmacological studies have demonstrated the beneficial effects of lignans such as antioxidant, antitumour, antimitotic, antiviral, antimicrobial and hepatoprotective effects (Macrae and Towers, 1984; Sung and Kim, 2000). In addition, pinoresinol-4, 40-di-O-b-D-glucoside (PDG) was reported to stimulate calcium mobilization and chemotactic migration of mouse embryo fibroblasts (Do *et* al., 2009). Moreover, lignans also have protective effects on the cardiovascular system (Miyawaki *et al*., 2009; Oh *et al*., 2008) and antihypertensive effect (Oh *et al*., 2008; Prasad, 2004; Raimundo *et al*., 2009). For example, secoisolariciresinol diglucoside (SDG) exerted its antihypertensive effect through activation of guanylate cyclase enzyme (GC). This effect was not due to nitric oxide synthase (NOS) activity (Prasad, 2004). Saucerneol and saucerneol D, which were lignans from the ethanol extract of the roots of *S. chinensis*, caused vasorelaxation via NO/cGMP pathway (Oh *et al*., 2008). Furthermore, sesamin, another lignan from sesame seeds and oil, could also induce vasorelaxation through activation of NO/cGMP pathway (Nakano *et al*, 2006). The vasorelaxant activity of certain lignans such as eudesmin may involve activation of

histamine H_1 receptors in endothelial cells, leading to synthesis and release nitric oxide (NO) and prostracyclin (PGI_2) (Raimundo *et al*., 2009).

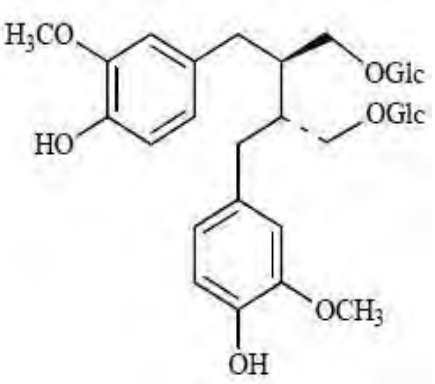


Secoisolariciresinol

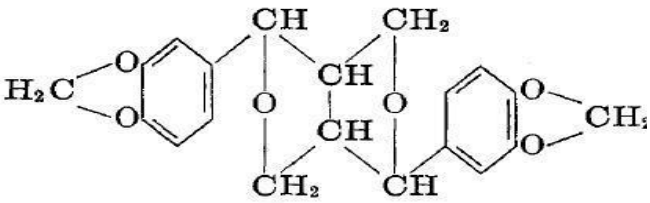


Matairesinol

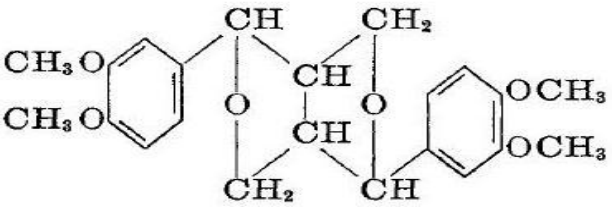
Figure 2 Basic structures of lignans



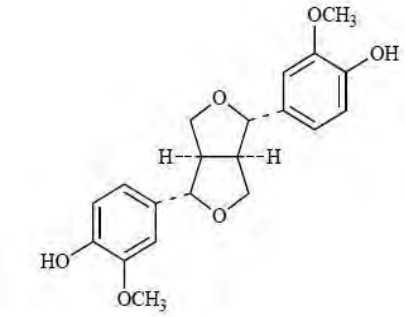
Secoisolariciresinol diglyside



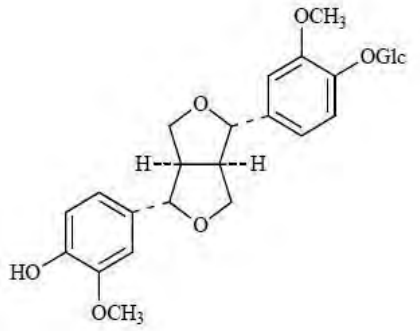
Sesamin



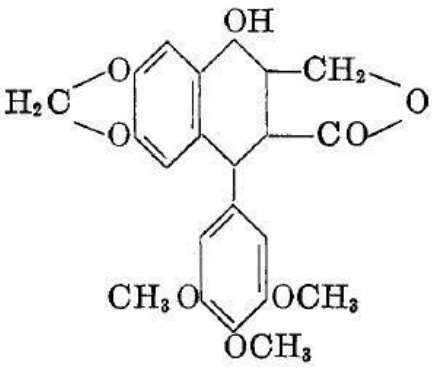
Eudesmin



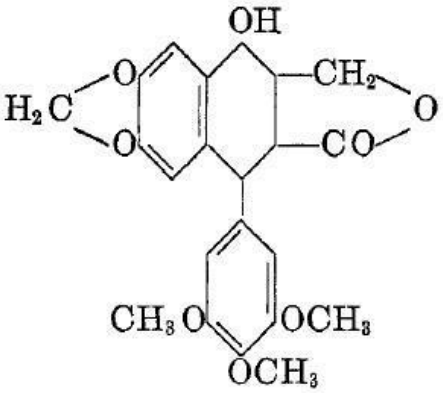
Pinoresinol



Pinoresinol gycoside



Picropodophyllin



Podophyllotoxin

Figure 3 Chemical structures of lignans (Do *et* al., 2009; Macrae and Towers, 1984).

Phyllanthus amarus Schum. and Thonn. (Euphorbiaceae; synonym: *Phyllanthus nanus* Hook. f. and *Phyllanthus niruri* Thw.) [also known as "Luk Tai Bai" in Thai], is a small herb, found in most tropical and subtropical regions, including Amazon rainforest, South East Asia, Southern India and China. *P. amarus* has been used in herbal medicine such as Ayurveda, Traditional Chinese Medicine and Indonesian Jamu (Bagalkotkar *et al*., 2006). The whole plant is also useful for tradition medicine to treat several diseases. In Thailand, this plant has been used in traditional medicine for a number of diseases in cardiovascular and gastrointestinal tract systems (Abdulla *et. al.,* 2010; Shokunbi and Odetola, 2008; Thippeswamy *et al*., 2011).

The plants grow up to 10-60 cm tall. Its branchlet is 2-6 cm long with 10-20 leaves arranging in alternate fashion. The leaves can be elliptic, oblong to ovate and obtuse or apiculate at apex. This plant has small off-white-greenish flowers. The fruits are tiny, smooth capsules containing seeds. (Figure 4) (Khatoon *et al*., 2006; Bagalkotkar *et al*., 2006).

P. amarus has a number of constituents such as lignans, terpenoids, alkaloids, flavonoids, phenols and tannins (Bagalkotkar *et al*., 2006). Phyllanthin and hypophyllanthin are the two major lignans found in this plant.

Figure 4 *Phyllanthus amarus* Schum. and Thonn. (http://www.learners.in.th/moo_lita/list; http://www.stuartxchange.org/SampaSampa_kan.html).

The yield of phyllanthin (a diarylbutane type lignan) and hypophyllanthin (a aryltetrahydronaphthalene type lignans) were approximately 0.5% and 0.3% of dried whole plant, respectively (Tripathi *et al*., 2006). Other lignans included niranthin, nirtetralin, phyltetralin, lintetralin, isolintetralin, 2, 3-desmethoxy seco-isolintetralin, 2, 3-des-methoxy seco-isolintetralin diacetate, linnanthin, demethylenedi-oxyniranthin, nirphyllin and phyllnirurin. Other identified compounds in *P. amarus* are flavonoids [rutin, quercetin, quercitrin, astragalin, gallocatechin, nirurin, quercetol, niruriflavone], terpenes [limonene, p-cymene, lupeol], coumarins [ellagic acid, methyl brevifolincarboxylate], tannins [repandusinic acid, geraniin, corilagin], saponins, alkaloids [norsecurinine], 1-O-galloyl-6-O-luteoyl-a-D-glucose and b-glucogallin (Bagalkotkar *et al*., 2006)

Major lignans compounds from *P. amarus* such as phyllanthin and hypophyllanthin have been shown to reverse the plasma uric acid level of hyperuricemic animals to its normal level (Murugaiyah and Chan, 2006). In addition, it was reported that the antioxidative and hepatoprotective activities of phyllanthin and hypophyllanthin were able to protect CCl_4- and galactosamine-induced cytotoxicity in primary cultured rat hepatocytes and HepG2 cell line. The data showed that these compounds could prevent leakage of cytosolic enzyme [alanine aminotransferase (ALT); lactate dehydrogenase (LDH)], could reduce lipid peroxidation and improve glutathione status of the cell treated with either CCl_4 or galactosamine (Krithika *et al*., 2009; Syamasundar *et al*., 1985). Moreover, phyllanthin could protect against ethanol-induced oxidative stress in rat liver cells. Consequently, cellular damages could be prevented (Chirdchupunseree, 2009). In other studies, phyllanthin and hypophyllanthin were effective multidrug resistance reversing agents, as evidenced by its inhibition p-glycoprotein function *in vitro* cancer cell lines (Leite *et al*., 2006; Somanabandhu, 1993).

It has been reported that the alcoholic extract of *P. amarus* could induce relaxation in smooth muscles of various organs including vascular smooth muscle, uterus, intestine, stomach and trachea (Kitisin, 1952). In addition, the aqueous extract from its leaves reduced mean arterial pressure significantly (Etuk, 2006). The aqueous extract of leaves of *P. amarus* suppressed the myocardial function and caused

hypotensive effects in rabbits and rat. These effects were possibly linked to muscarinic receptor-mediated mechanisms and calcium channel ion blockade (Amaechina and Omogbai, 2007). Methyl brevifolincarboxylate from the leaves of *Phyllanthus niruri* inhibited NE-induced vasocontraction which, in part, was due to inhibition of intracellular Ca^{2+} flux through ROC (Iizuka, Moriyama and Nagai, 2006). Furthermore, n-butanol extract from leaves of *Phyllanthus acidus* induced vasorelaxation via NO/cGMP pathway and opening of K_{ATP} and BK_{Ca} channels in the vascular smooth muscle of isolated rat thoracic aorta (Leeya *et al*., 2010).

Control of vascular tone: contraction and relaxation

Vascular contraction

Vascular tone is regulated by a number of factors such as endothelium-derived factors, autonomic nervous system, neurohormone and local mechanisms (Taohjian *et al*., 2008). As known, contraction of vascular smooth muscle largely depends on the levels of intracellular Ca^{2+}. The rising of free cytosolic Ca^{2+} depends on the influx of Ca^{2+} from the extracellular environment and the release of Ca^{2+} from sarcoplasmic reticulum (SR) stores (van Breemen and Saida, 1989; Carl, Lee and sanders, 1996). The pathways of Ca^{2+} entry and the release from SR stores were described below (Figure 6).

The Ca^{2+} influx from an extracellular source

The Ca^{2+} influx into smooth muscle cell occurs mainly through two types of transmembrane Ca^{2+} channels: voltage-operated calcium channels (VOC) and receptor-operated calcium channels (ROC) (van Breemen and Saida, 1989). In smooth muscle cells, the L-type Ca^{2+} channels, which can be either VOC or ROC, are considered to be a major Ca^{2+} influx. As the VOC, the channels open by changes in action potential of plasma membrane, leading to depolarization and open of the channels (Karaki *et al*., 1997; McFadzean and Gibson, 2002). Generally, the *in vitro* models have provided evidence of a principal role of membrane depolarization by using, high potassium

solution to depolarize plasma membrane. Consequently, contraction of smooth muscle can be observed due to Ca^{2+} influx through the open VOC (McFadzean and Gibson, 2002). For the ROC, the channels open through activation of receptor coupled to signaling cascade that eventually activate the channels. In vascular smooth muscle, an activation of α_1- adrenoceptor stimulates the formation of inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DG). Subsequently, IP_3 binds to its receptors on SR stores, leading to the release Ca^{2+} from SR whereas DG activates protein kinase C (PKC) and its signaling cascade. Consequently, the phosphorylation of these L-type Ca^{2+}channels occurs and the channels open (van Breemen and Saida, 1989; McFadzean and Gibson, 2002).

In general, either a number of receptor agonists or depolarizing solution are used to open of ROC or VOC in order to provoke vascular contraction in the vitro model of isolated organ preparation (van Breemen and Saida, 1989). There is another mechanism to induce the Ca^{2+} movement across plasma membrane in the absence of any contractant, as described by other investigators (Noguera *et al.*, 1992, 1993, 1997). In this model of spontaneous contraction, α-1 agonist was used repetitively to induce contraction in Ca^{2+}- free condition until the contractile response was not observed. As known, the α-1 agonist-mediated contraction contains two phases which include an initial phasic response and a tonic contraction. The initial phasic response is triggered by a rising of Ca^{2+} released from SR via inositol 1, 4, 5-trisphosphate (IP_3) pathway. The tonic contraction subsequently maintained by an influx of extracellular Ca^{2+} (van Breemen and Saida, 1989; McFadzean and Gibson, 2002). Thus, the depletion of intracellular Ca^{2+} pools can be obtained in the muscle repetitively treated with α-1 agonists in Ca^{2+}- free solution. Moreover, it was hypothesized that in this specific condition, Ca^{2+}- depleted capacitative Ca^{2+} stores triggered the signaling cascade to activate membrane Ca^{2+} channels. The presence of extracellular Ca^{2+} then could rapidly flux into the cells in order to replenish the capacitative Ca^{2+} stores. Hence, spontaneous

contraction or increase in the resting tone could be observed in this pnenomenon (Figure 5) (Noguera *et al.*, 1997).

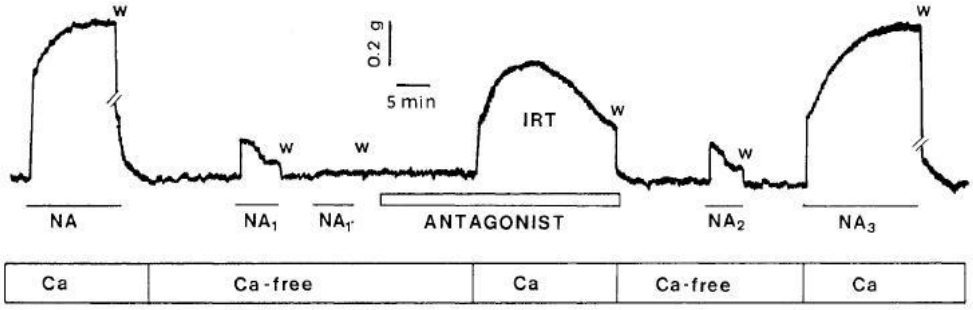


Figure 5 Spontaneous contraction or increase in the resting tone (Noguera *et al.*, 1997).

The release of intracellular Ca^{2+} from the sarcoplasmic reticulum (SR)

SR is a major storage of Ca^{2+} within the smooth muscle cells. The Ca^{2+} release from SR is generally signaled by activation of either inositol 1, 4, 5-trisphosphate receptor (IP_3R) or ryanodine receptor (RyR). Caffeine is a known RyR agonist, which can mediate the release of Ca^{2+} from SR through RyR activation (van Breemen and Saida, 1989; Hill *et al.*, 2001). The IP_3-induced Ca^{2+} release occurs from the production of IP_3, a second messenger in the activation of α-adrenergic receptor. The binding of IP_3 to IP_3R on SR stores leads to the release Ca^{2+} from SR (van Breemen and Saida, 1989; Hill *et al.*, 2001; Harnett and Biancani, 2003). Furthermore, the Ca^{2+} release from SR can be resulted from extracellular through voltage-operated Ca^{2+} channel (VOC), receptor-operated calcium channels (ROC) and Na^{+}/Ca^{2+} exchanger, leading to the increase of intracellular Ca^{2+} and release Ca^{2+} from SR (Karaki *et al.*, 1997).

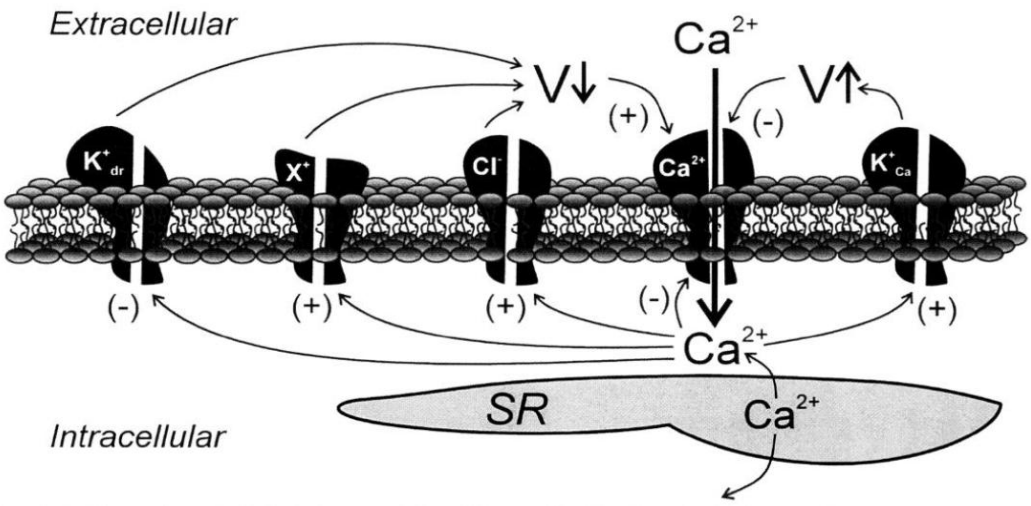


Figure 6 Pathways involving the control of Ca^{2+} entry through Ca^{2+} channels and release of Ca^{2+} from sarcoplasmic reticulum (SR) (Carl, Lee and sanders, 1996).

As increasing of intracellular Ca^{2+} and then, Ca^{2+} binds to calmodulin to form a Ca^{2+}- calmodulin complex, an activator of myosin light chain kinase (MLCK). Subsequently, MLCK phosphorylates myosin light chain (MLC) to enable myosin-actin interaction, which leads to cross-bridge cycling and contraction (Webb, 2003). At the same time, RhoA is activated by guanine nucleotide exchange factor (RhoGEF) via small GTP-binding protein RhoA, leading to migration of RhoA to the plasma membrane. Then, RhoA activates Rho kinase and lead to inhibit of myosin phosphatase activity and this promotes the contraction (Webb. 2003). This event was simply illustrated by the diagram in Figure 7.

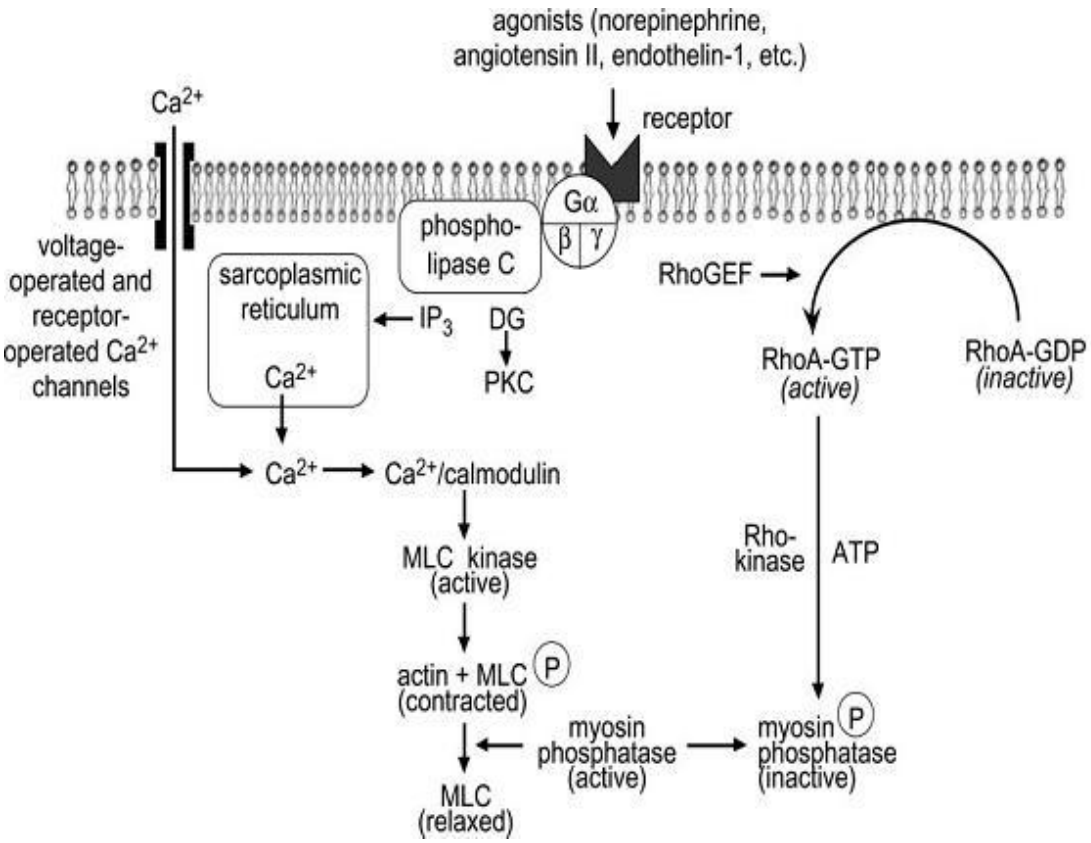


Figure 7 Pathways to illustrate signaling in smooth muscle contraction (Webb, 2003)

Smooth muscle relaxation

Generally, the vasorelaxation can be governed by either endothelium-dependent or endothelium-independent mechanisms. The mechanisms that involve with smooth muscle relaxation were shown in Figure 8.

The endothelium-dependent relaxation has been linked to the production of endothelium-derived relaxing factors (Suzuki, Chen and Yamamoto, 1992). The activation of the specific receptor such as muscarinic receptor on endothelial cell, can eventually lead to an increase of intracellular Ca^{2+} and an activation of endothelial cells synthesis and secretion of various vasodilators such as nitric oxide (NO), prostacyclin

(PGI_2), epoxyeicosatrienoic acids and endothelium-derived hyperpolarizing factor (EDHF) (Ignarro, 2002; Stankevicius *et al.*, 2003; Vallance, 1992).

Endogenous NO is generated in endothelium cells via the enzyme nitric oxide synthase (NOS). After production, NO diffuses to vascular smooth muscle and activates soluble guanylyl cyclase (sGC) to generate cyclic quanosine monophosphate (cGMP) (Denninger and Marletta, 1999). As known, cGMP activates cGMP-dependent protein kinase/protein kinase G (PKG), which subsequently phosphorylates Ca^{2+}- activated K^+channels (K_{Ca} channels) and ATP-sensitive K^+channels (K_{ATP} channels) (Ko *et al.*, 2008). The phosphorylation causes an open of these K^+ channels and K^+ efflux, followed by membrane hyperpolarization, closure of voltage-operated Ca^{2+}channel, decrease in intracellular Ca^{2+} and vasorelaxation (Lee and Severson, 1994; Hecker, 2000; Ignarro, 2002; Stankevicius *et al.*, 2003).

Other vasorelaxing substances released from endothelium cells are prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF). Prostracyclin (PGI_2) is product of arachidonic acid from cyclooxygenase (COX) catalysis. In smooth muscle cell, PGI_2 activates adenylate cyclase (AC) and increases cyclic adenosine monophosphate (cAMP). Subsequently, cAMP activates cGMP-dependent protein kinase/protein kinase A (PKA), resulting in phosphorylation of Ca^{2+}- dependent K^+ (K_{Ca}) channels, ATP-sensitive K^+ (K_{ATP}) channels, voltage-dependent K^+ (K_v) channels, inward rectifier K^+ (K_{IR}) channels and vasorelaxation eventually (Ko *et al.*, 2008). Substrate of Endothelium-derived hyperpolarizing factor (EDHF) has yet known, but its production involve with Ca^{2+}- dependent process in the endothelial cell. Although the mechanism of action is unclear, it is likely that EDHF mediates vasorelaxation via suppression of intracellular Ca^{2+} in smooth muscle (Suzuki, Chen and Yamamoto 1992; Stankevicius *et al.*, 2003).

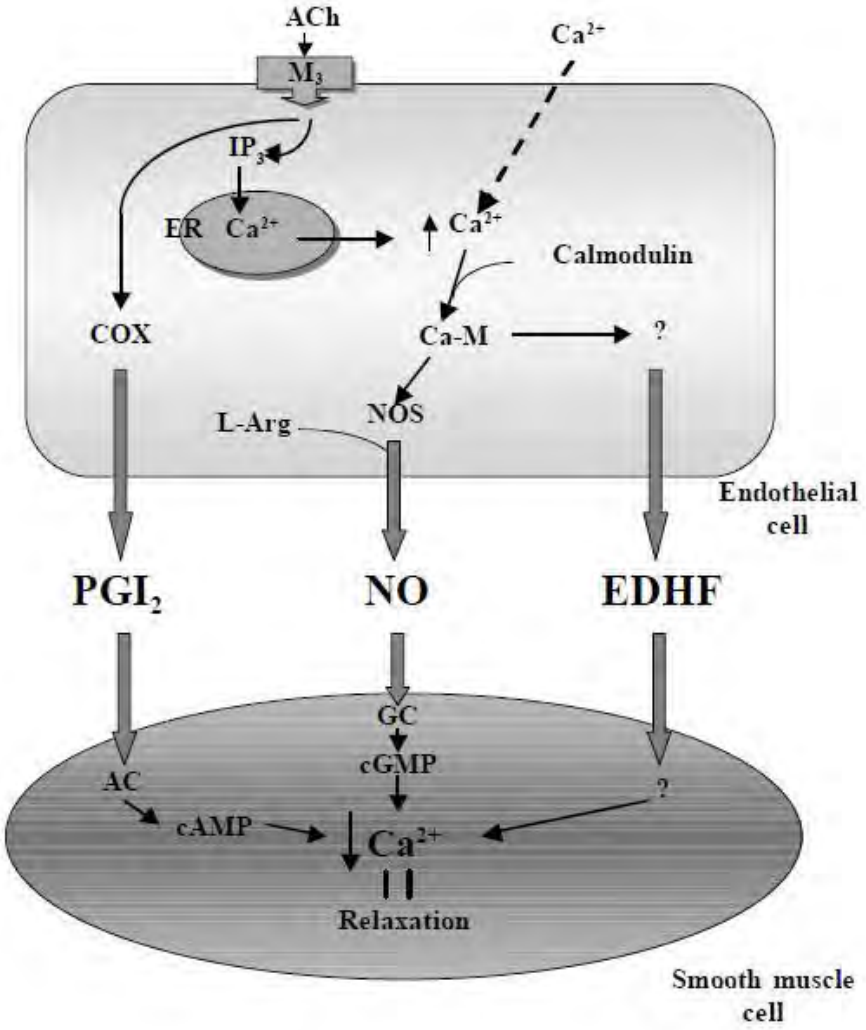


Figure 8 Mechanism of smooth muscle relaxation (Stankevicius *et al.*, 2003)

The endothelium-independent pathways involve with β-adrenoceptor and K^+ channels in smooth muscle. Stimulation of β-adrenoceptor in vascular smooth muscle leads to the activation of adenylate cyclase (AC) and production of cyclic adenosine monophosphate (cAMP). As known, an increasing of cAMP further activates protein kinase A (PKA), which subsequently phosphorylates K^+ channels and vasorelaxation (Baloglu *et. al.*, 2007; Ericsson and Lundholm, 1975).

K^+ channels play important roles in the regulation of the membrane potential in vascular smooth muscle cells. To date, four distinct types of K^+ channel have been identified in vascular smooth muscle: ATP-sensitive potassium (K_{ATP}) channels, voltage-dependent K^+ (K_v) channels, Ca^{2+}- activated K^+ (BK_{Ca}) channels and inward rectifier K^+ (K_{IR}) channels. Each of K^+ channels is responsive to protein kinase G (PKG) and protein kinase A (PKA). The activation of these K^+ channel leads to the membrane hyperpolarization, closure of voltage-operated Ca^{2+} channels, decrease in intracellular Ca^{2+} and vasodilation. Whereas K^+ channels are closed, decreased efflux of K^+ leads to membrane depolarization and vasoconstriction (Figure 9) (Jackson, 2000; Ko *et al.*, 2008).

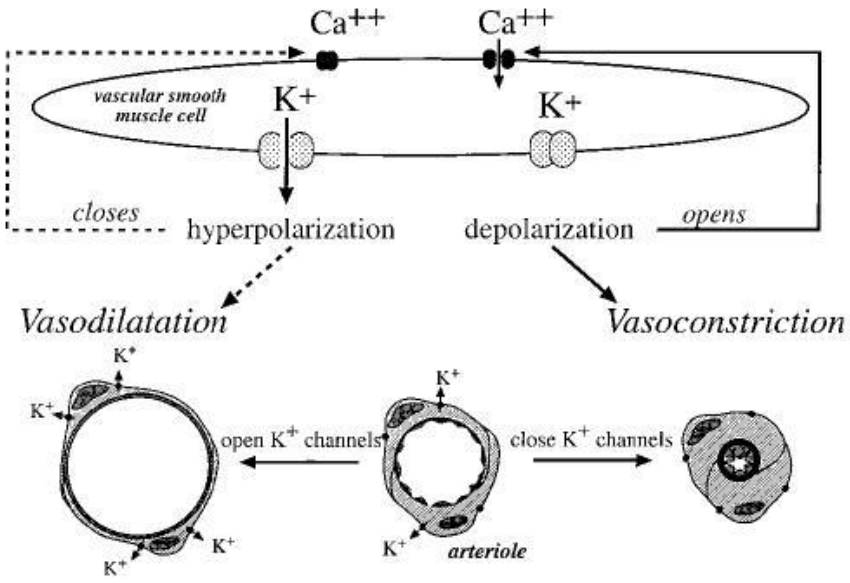


Figure 9 K^+ channels and vascular tone (Jackson, 2000)

CHAPTER III

MATERIALS AND METHODS

Animals

Adult male Wistar rats of body weight between 250-300 g were obtained from National Laboratory Animal center, Mahidol University, Salaya, Nakornpathom. The animals were acclimatized for 1-2 week before the experimentation in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University. Animals were housed under environment-controlled conditions: at room temperature 25 ± 2 ˚C with 12-hours light/dark cycle, and 60% humidity. The animals received standard rat pellets and tap water *ad libitum*. This study was approved by the Ethics Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. (Approval number 10-33-004)

Chemicals

Major chemicals in this study included phenylephrine (PE), potassium chloride (KCl), acetylcholine (ACh), caffeine, calcium chloride ($CaCl_2$), atropine, nifedipine, tyramine, prozosin, barium chloride ($BaCl_2$), glibenclamide, indomethacin, methylene blue, N^G-nitro-L-arginine methyl ester (L-NAME), propranolol, tetraethylammonium (TEA), 4-aminopyridine (4-AP), dimethyl sulfoxide (DMSO) 99.5%. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The high K^+- Ca^{2+}- free depolarizing solution contains (mM): NaCl 27; KCl 100; $NaHCO_3$ 14; KH_2PO_4 14; D-glucose 10 and $MgCl_2$ 0.54. The Ca^{2+}- free solution contains (mM): NaCl 119; KCl 4.7; $NaHCO_3$ 25; D-glucose 11.1; $MgSO_4$ 1.0; $KH_2PO_4$1.2 and EDTA 0.2. All chemicals were analytical grades.

Test compounds

Phyllanthin and hypophyllanthin were kindly provided by Assoc. Prof. Pornpen Pramyothin, Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The isolation and purification were described in Chirdchupunseree (2009). In brief, the two compounds were isolated from the fresh aerial part of *Phyllanthus amarus* which was collected in and around Faculty of Pharmaceutical Sciences, Chulalongkorn University. After pulverization and extraction with hexane, the compounds were isolated and further purified by column chromatography, using n-hexane and ethyl acetate as mobile phase. Phyllanthin and hypophyllanthin were identified by IR spectrum and HPLC, comparing with standard references (Chromadex, Inc., U.S.A.). The purities of these two compounds were at least 98%.

Phyllanthin and hypophyllanthin were dissolved in 99.5% DMSO throughout the studies. The final concentration of DMSO in the system was less than 0.2% (v/v). At this concentration, DMSO had no vasoactive effects on rat vascular smooth muscle.

Preparation of aortic rings

Rats were anaesthetized by carbon dioxide (CO_2) and sacrificed by cervical dislocation. The thoracic aorta was carefully removed and placed in petri-dish containing Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 119; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.2; $NaHCO_3$ 25; D-Glucose 11.1 and $MgSO_4$ 1.0. Then, the thoracic aorta was cleaned and cut into 4 segments of approximately 2-3 mm long. Each segment was suspended in double walled organ baths (Harvard type Organ bath) which contained 10 ml of KHS bubbled with carbogen gas, pH 7.4 ± 0.5 at 37°C. The vascular tension was recorded with an isometric force transducer model MLT 050/A (ADInstruments, Australia) that was connected to computer equipped with Software Chart 5.0 of PowerLab 4/SP data acquisition system (ADInstruments, Australia). During an initial stabilization period of approximately 60 min, KHS was replaced every 15 min.

In some preparations, the endothelium was removed by gently rubbing the lumen with cotton swab. Generally, the aortic rings were tested for the presence of functional endothelium by addition of acetylcholine (ACh 10 μM). The preparations were considered as endothelium-intact when the relaxation responses to ACh were greater than 60%. The endothelium-denuded preparations were applied when the relaxation responses were less 10%.

Experimental procedures

1. Vasorelaxation effects of the test compounds

The aortic rings were pre-contracted with PE (1 μM). At plateau state, both phyllanthin and hypophyllanthin were added cumulatively in the concentration ranges of 1-100 μM (Figure 10). In another experiments, a number of known vasodilation inhibitors [atropine (1 μM), $BaCl_2$ (10 μM), glibenclamide (10 μM), indomethacin (10 μM), L-NAME (100 μM), methylene blue (10 μM), propranolol (10 μM), 4-aminopyridine (100 μM) and TEA (10 μM)] were added 10 minutes prior to the test compounds. In separated experiments, the effect of DMSO was also determined, and used as control group. The relaxation responses were calculated in relative to the tensions developed from addition of PE (1 μM) in the absence of the test compounds. The data were expressed as the percentages of the PE-induced contraction.

Experiment 1

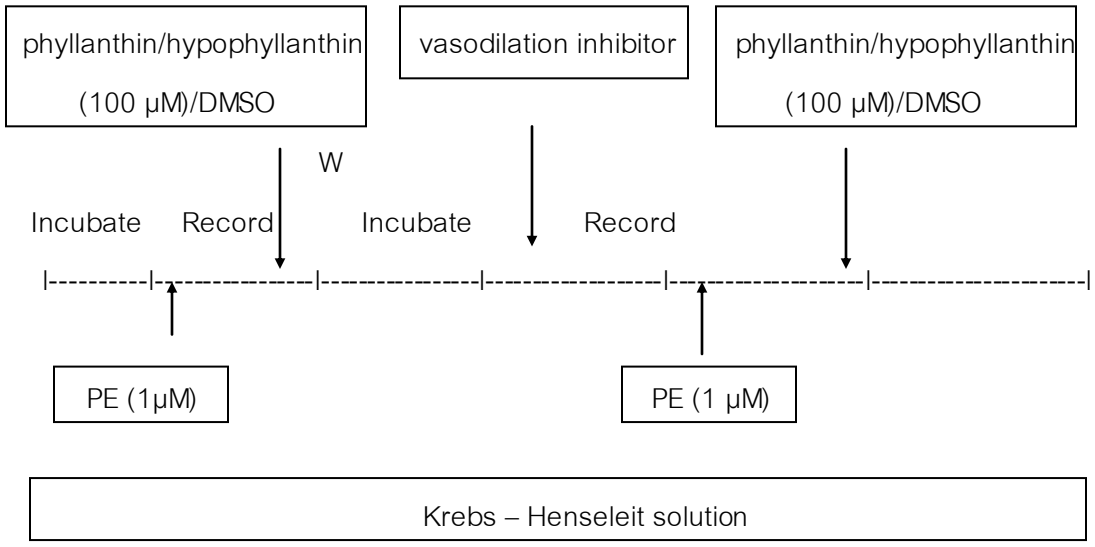


Figure 10 Experimental diagram for testing the vasorelaxation responses of the test compounds.

2. Effects of the test compounds on vascular contractions

2.1 Vasopressive actions of the test compounds

The aortic rings were suspended in normal KHS until the baseline tension was stable. Then, the test compounds (in the concentration range of 1 μM -100 μM) were added cumulatively to induce contraction. After washing, either tyramine (1 μM), its can release NE from presynaptic storage vesicles, leading to muscle contraction (Krishnamurty and Grollmax, 1972) or prazosin (1 μM), a known α_1-adrenoceptor antagonist was added to the test system for 10 minutes prior to addition of either phyllanthin or hypophyllanthin (Figure 11)

Experiment 2.1

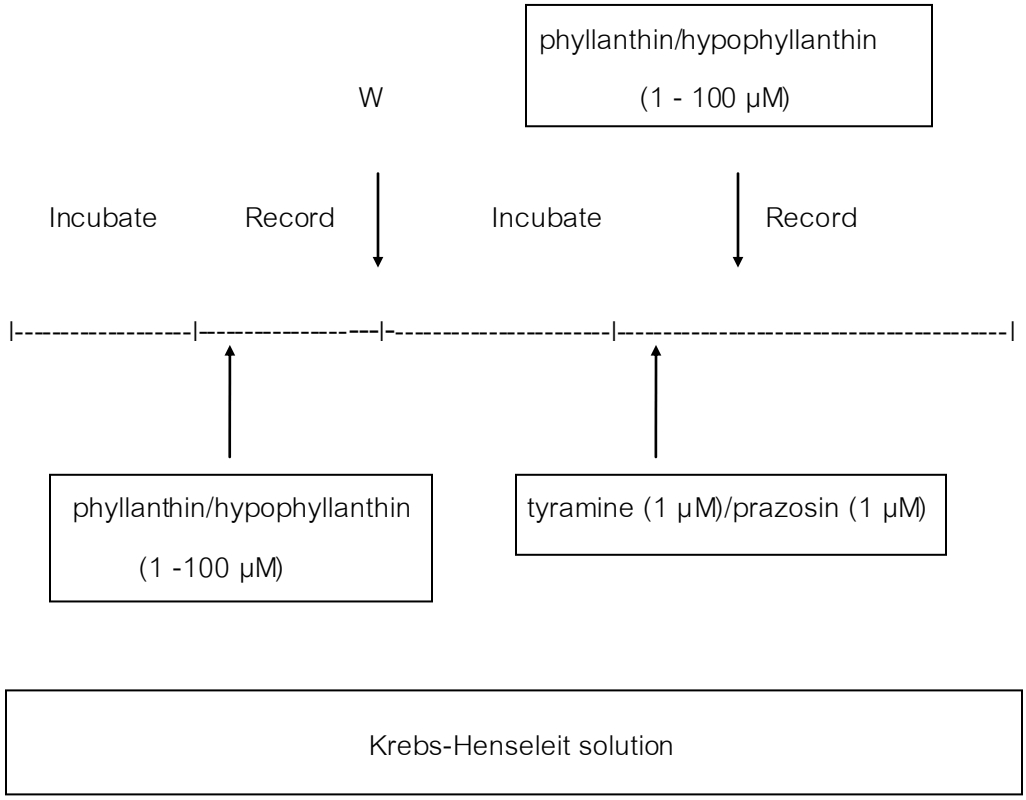


Figure 11 Experimental diagram for testing the vasopressive effects of the test compounds.

2.2 Agonist-induced aortic contraction

The aortic rings were suspended in normal KHS containing tyramine (1 μM) until the baseline tension was stable. Then, vasoactive agonist [either PE (1 μM) or KCl (40 mM)] was added to provoke contraction. The tension was recorded for 15 minutes. The effect of the test compounds (phyllanthin and hypophyllanthin) on agonist-induced contraction were determined by incubating the compound at a selected concentration (ranging 1-200 μM) for 20 minutes prior to addition of either agonist (Figure 12). The residual tension in the presence of the test compound was expressed as a percentage of the maximal contraction induced by each specific agonist. In separated experiments, the effect of DMSO was also determined, and used as control group.

Experiment 2.2

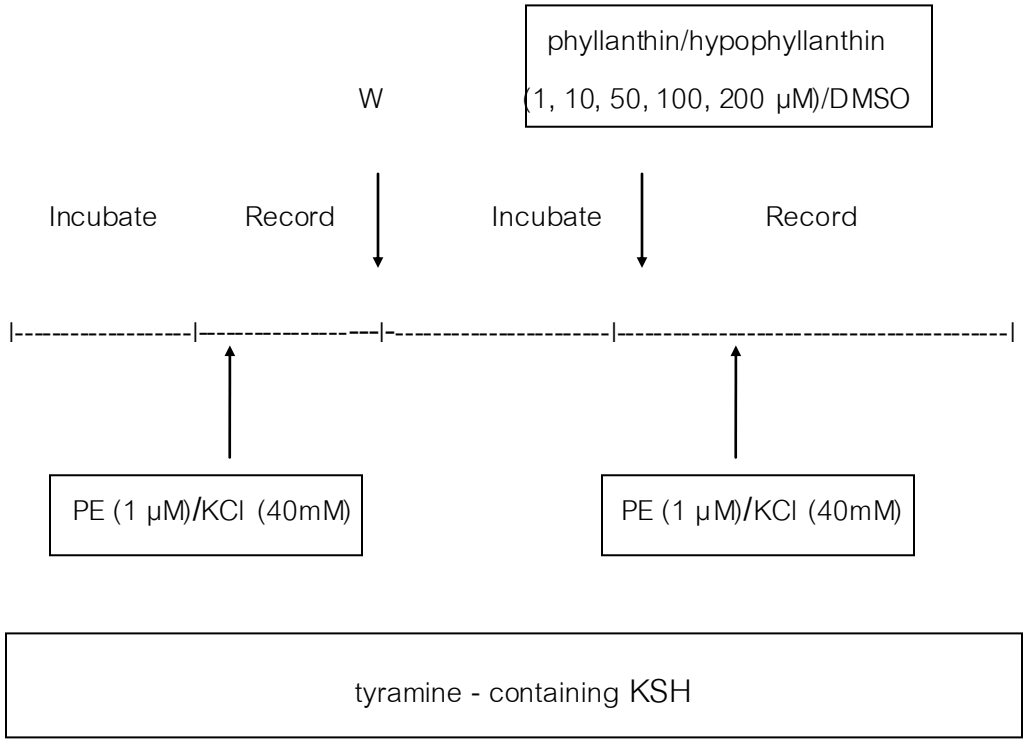


Figure 12 Experimental diagram for testing the effects of the test compounds on agonist-induced aortic contraction.

2.3 Spontaneous contraction

The spontaneous contraction could be induced under certain specific condition (Noguera and D'Ocon, 1992). A spontaneous rising of the resting tension could be obtained in the rings that were depleted of intracellular Ca^{2+} by PE (10 μM), as previously described by Noguera *et al.*, 1997. In brief, PE (10 μM) was added into Ca^{2+}-containing solution to provoke aortic contraction for 15 minutes. Then, the rings were washed with Ca^{2+}- free KHS, and incubating in this Ca^{2+}-free buffer for another 15 minutes. Next, either PE (10 μM) or KCl (40 mM) was repetitively applied until no contraction was observed. Then, upon changing the bathing solution from Ca^{2+}- free to Ca^{2+}- containing KHS, a spontaneous contraction of the aortic rings were observed (Figure 13). The effect of the test compounds (phyllanthin and hypophyllanthin) on spontaneous contraction was examined by addition the test compounds at concentration of 100 μM for 5 minutes in Ca^{2+}- free KHS prior to changing it to normal KHS. The spontaneous contraction was expressed as percentage residual contraction which was calculated as the percentage of PE-induced contraction in normal KHS. In separated experiments, the effect of DMSO was also determined, and used as control group.

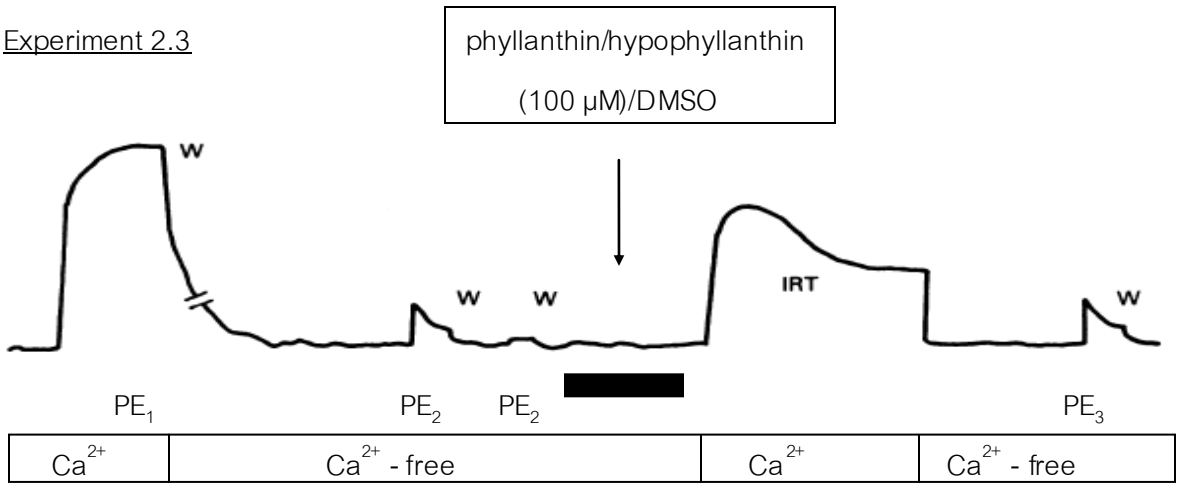


Figure 13 Experimental diagram for testing the effects of the test compounds on spontaneous contraction.

2.4 Aortic contraction in high K^+- Ca^{2+}- free depolarizing solution

The aortic rings were suspended in normal KHS until the baseline tension was stable. Next, the normal KHS was replaced by high K^+- Ca^{2+}- free depolarizing solution and further incubated until the baseline tension was stable. Then, $CaCl_2$ (in the concentration range of 10 µM-10 mM) were added cumulatively to induce contraction (Figure 14). The effect of the test compounds (phyllanthin and hypophyllanthin) was examined by addition the test compound at concentration of 100 µM for 10 minutes prior to addition of $CaCl_2$. The contraction was expressed as a percentage of the maximum contraction induced by $CaCl_2$. In separated experiments, the effect of DMSO (solvent control) and nifedipine (positive control) were also determined.

The pD_2 value was calculated according to van Rossum (1963).

$pD_2 = -\log [B] + \log ([E_{AM}] / [E_{AMB}]-1)$

[B] was concentration of non competitive antagonist

$[E_{AM}]$ and $[E_{AMB}]$ were maximum contraction in the presence of antagonist and absence of antagonist.

Experiment 2.4

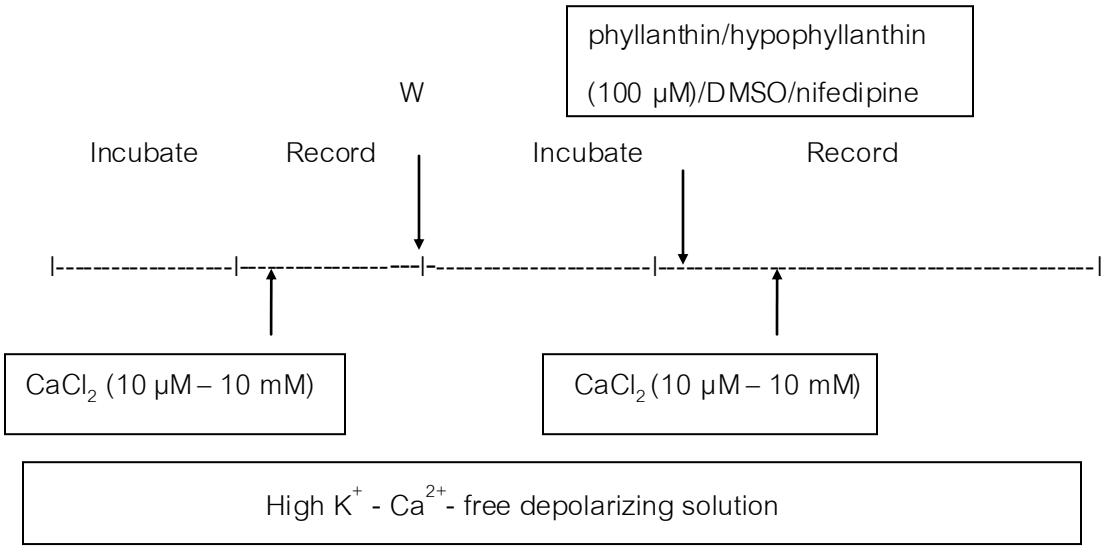


Figure 14 Experimental diagram for testing the effects of the test compounds on aortic contraction in high K^+- Ca^{2+}- free depolarizing solution.

2.5 Aortic contraction in Ca^{2+}- free solution

After the baseline tension was stable in normal KHS, PE (1 μM) was added to induce aortic contraction for 15 minutes. Then, the aortic ring was washed with Ca^{2+}- free KHS 3 times, followed by incubating the ring in Ca^{2+}- free KHS for 15 minutes prior to addition of either PE (1 μM) or caffeine (10 mM). The effect of the test compounds (phyllanthin and hypophyllanthin) on contraction in Ca^{2+}- free KHS were studied by addition of the compound at concentration of 100 μM for 10 minutes prior to addition of either PE (1 μM) or caffeine (10 mM) (Figure 15). The response was expressed as a percentage of either PE- or caffeine-induced contraction in Ca^{2+}- free KHS in the absence of the test compounds. In separated experiments, the effect of DMSO was also determined, and used as control group.

Experiment 2.5

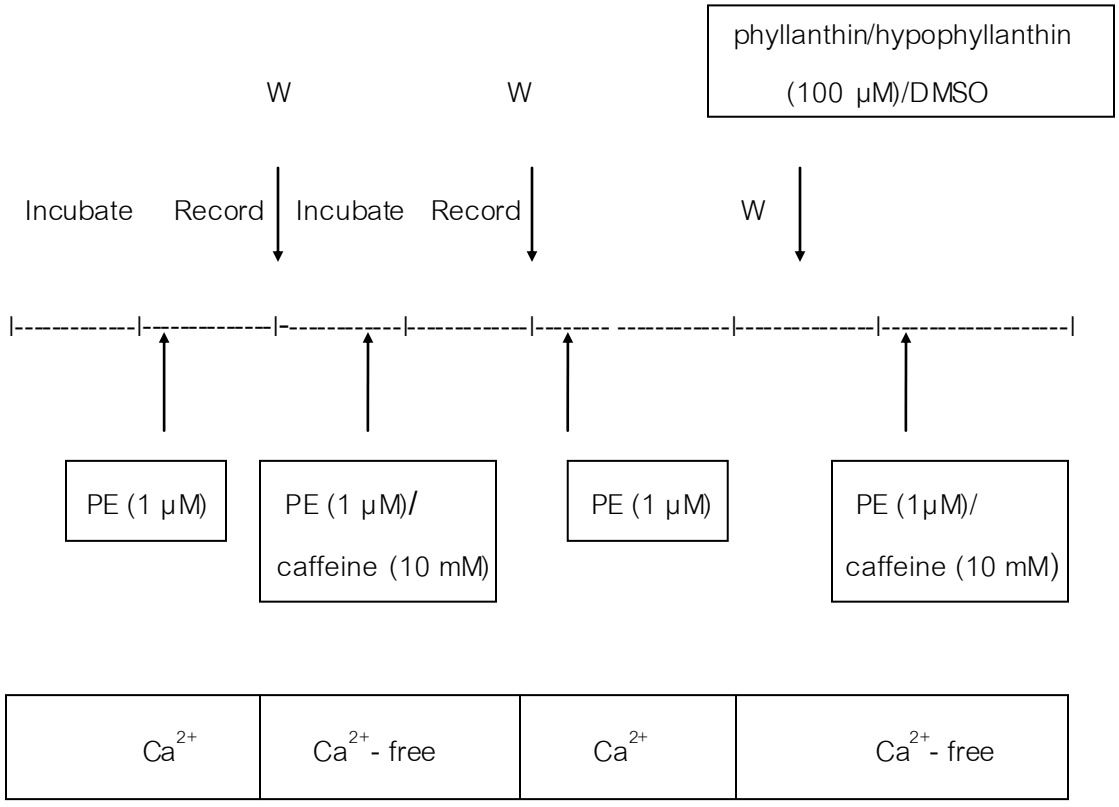


Figure 15 Experimental diagram for testing the effects of the test compounds on aortic contraction in Ca^{2+}- free solution.

Statistical Data Analysis

The results were expressed as the mean ± standard error of the mean (S.E.M.) for 4-6 separated experiments. The individual IC_{50} values were calculated from dose-response curves by linear regression. Statistical significances were tested by one-way analysis of variance (ANOVA) followed by post-hoc Dunnett *t*-test, where appropriate. The *p* values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

1. Vasorelaxation effects of the test compounds

The relaxation profiles of endothelium-intact and endothelium-denuded aortic rings were demonstrated in Figure 16-18. In this study, both phyllanthin and hypophyllanthin (1-100 μM) significantly induced vasorelaxation in the aortic rings that were pretreated with PE (1 μM). The vasorelaxation effects of these two compounds were concentration-dependent. In addition, the relaxation responses to each compound in either endothelium-intact or endothelium-denuded aortic rings were comparable (Figure 19), suggesting that removal of the functional endothelium had no significant influence on the intrinsic vasorelaxing activity of these compounds. The results also revealed that phyllanthin was more potent than hypophyllanthin in modulating the vascular tension, as evidenced by the apparent EC_{50} values of phyllanthin and hypophyllanthin induced relaxation were 56.55 ± 2.91 μM and 80.08 ± 5.05 μM in endothelium-intact aortic rings, respectively and 55.40 ± 5.50 μM and 84.02 ± 5.93 μM in endothelium-duded aortic rings, respectively.

In parallel studies, a number of known vasorelaxant inhibitors [atropine (1 μM), $BaCl_2$ (10 μM), glybenclamide (10 μM), indomethacin (10 μM), L-NAME (100 μM), methylene blue (10 μM), propranolol (10 μM), 4-aminopyridine (100 μM) and TEA (10 μM)] were preincubated with the endothelium-denuded rings prior to the relaxation experiment as abovementioned. The results showed that none of these inhibitors could influence the vasorelaxation responses induced by both phyllanthin and hypophyllanthin (Figure 35-45 in appendix).

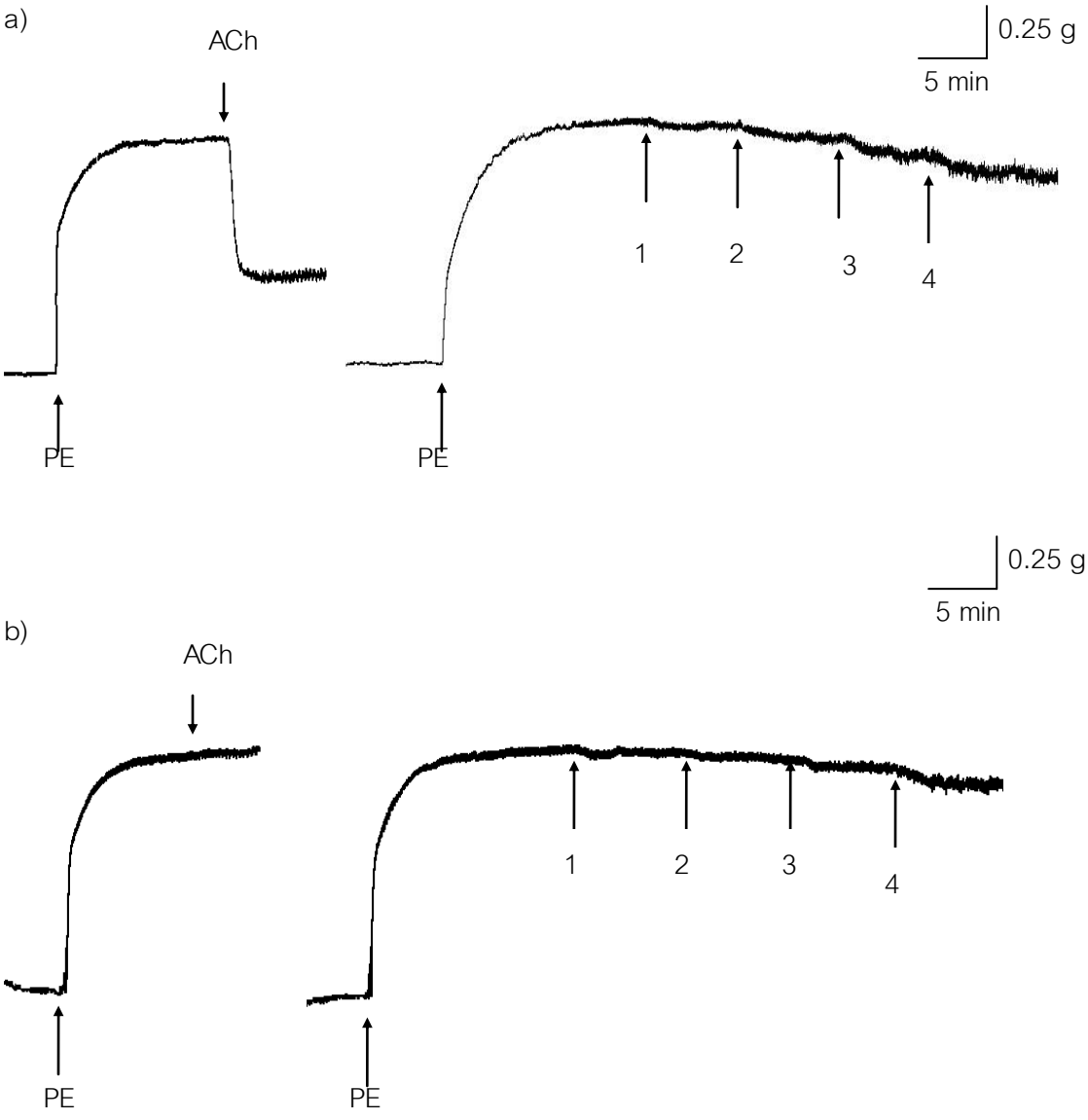


Figure 16 Representative tracing showed the relaxation induced by cumulative addition of DMSO 0.2% v/v on PE-induced contraction of endothelium-intact (a) and endothelium-denuded aortic rings (b).

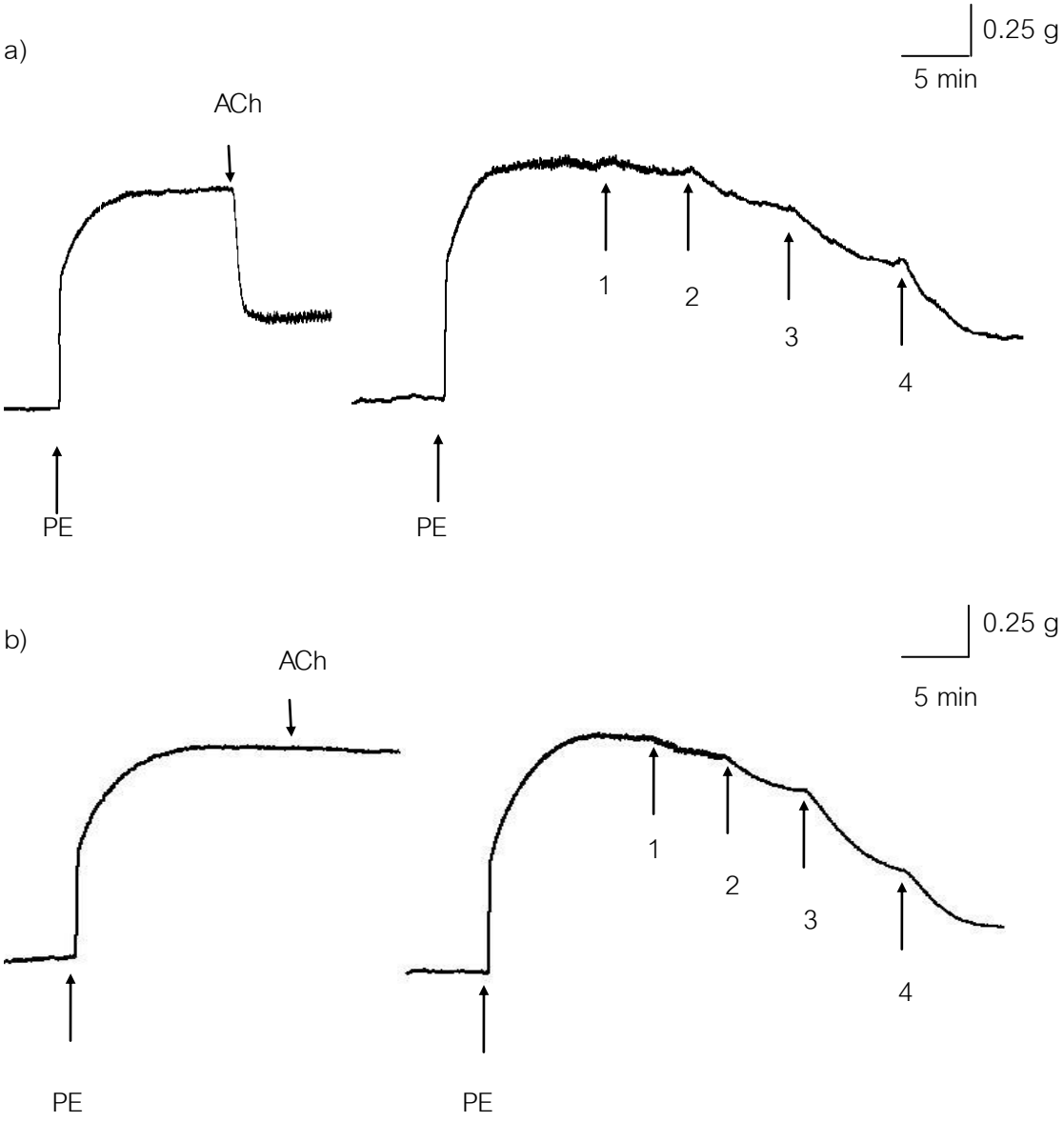


Figure17 Representative tracing showed the relaxation induced by cumulative addition of phyllanthin on PE-induced contraction of endothelium-intact (a) and endothelium-denuded aortic rings (b). Phyllanthin concentrations were 1=1, 2=10, 3=50, 4=100 μM.

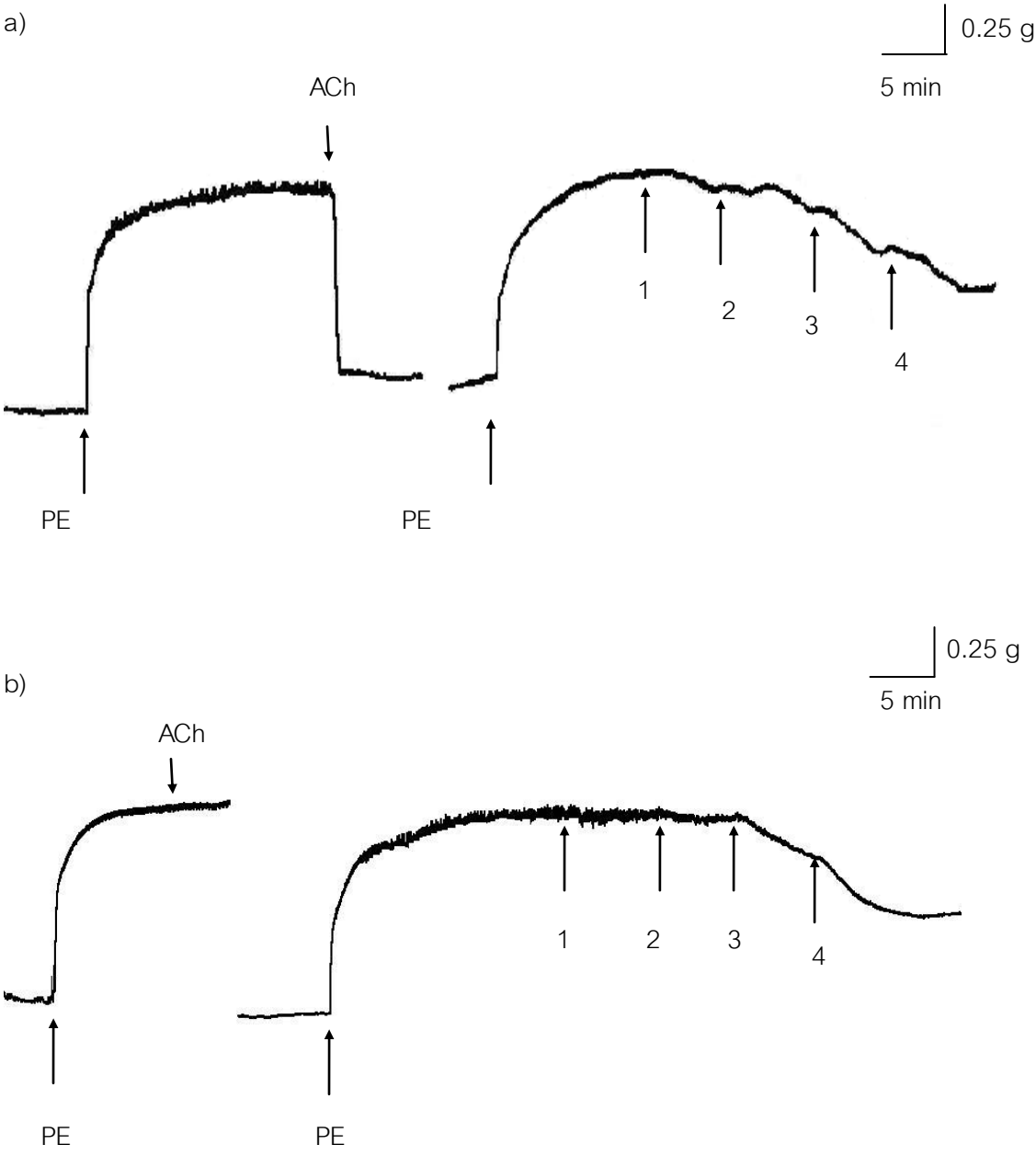


Figure 18 Representative tracing showed the relaxation induced by cumulative addition of hypophyllanthin on PE-induced contraction of endothelium-intact (a) and endothelium-denuded aortic rings (b). Hypophyllanthin concentrations were 1=1, 2=10, 3=50, 4=100 μM.

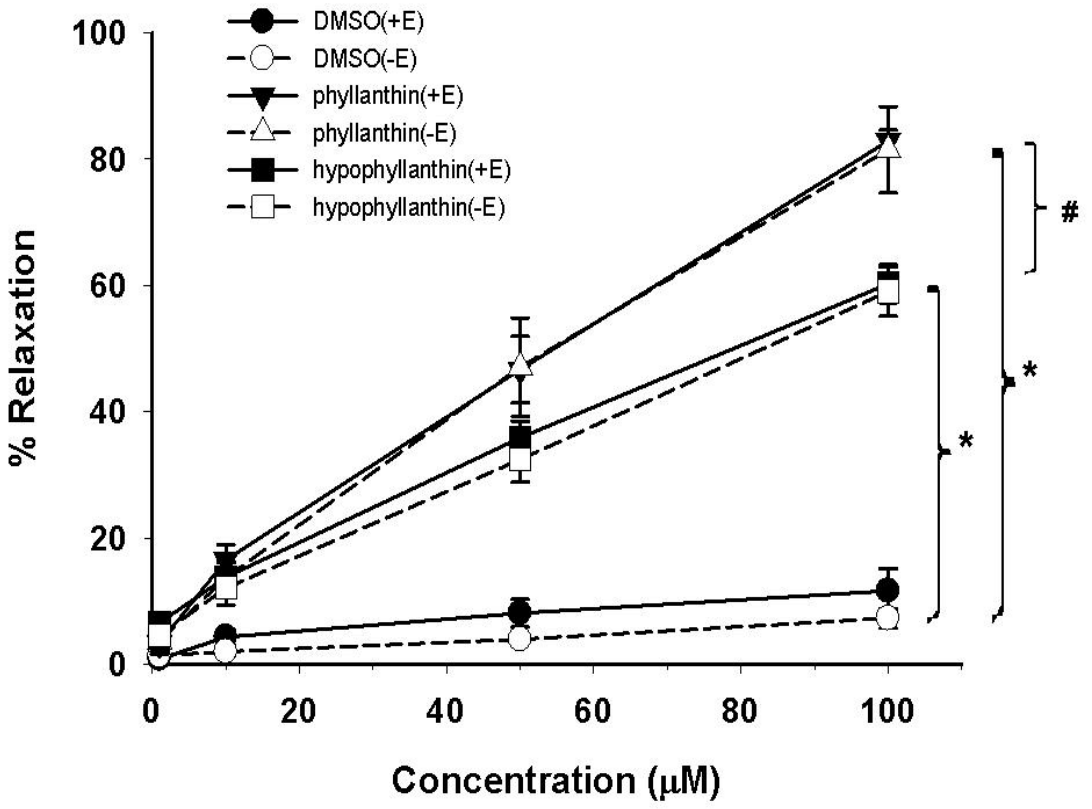


Figure 19 Vasorelaxation effects of phyllanthin and hypophyllanthin (1-100 μM) in endothelium-intact and -denuded aortic rings that were precontracted with PE (1 μM).

Data were presented as mean ± S.E.M., n = 6

*p<0.05 showed significant difference from DMSO control group.

#p<0.05 showed significant difference between phyllanthin and hypophyllanthin.

2. Effects of the test compounds on vascular contractions

2.1 Vasopressive actions

The contraction profiles of endothelium-denuded aortic rings upon cumulative addition of phyllanthin or hypophyllanthin (1-100 μM) in normal KHS were shown in Figure 20. The results demonstrated that phyllanthin and hypophyllanthin at concentration 10 and 50 μM were able to induce aortic contraction. Further addition of these two compounds at the concentration of 100 μM did not increase or sustain the contractility, instead it relaxed the vascular tension. In another experiment, preincubation of the aortic muscle with tyramine caused transient, but significant contraction with the tension of 0.31 ± 0.04 g (n=20). The results were in agreement with other reports (Krishnamurty and Grollmax, 1972) on tyramine-mediated release of NE and muscle contraction, suggesting that there were presynaptic storage vesicles of NE in the aortic preparations. It was interesting that the vasopressive effects of the test compounds observed in this study could be abolished in the presence of tyramine (1 uM) (Figure 21). Furthermore, preincubation with prazosin (1 uM), a known α_1-adrenoceptor antagonist, could also prevent the vasopressive actions of both phyllanthin and hypophyllanthin (Figure 22). Taken together, the vasopressive action of both phyllanthin and hypophyllanthin were not directly from its intrinsic property. It was likely that these two compounds were able to release NE from endogenous presynaptic storages which consequently induced muscle contraction.

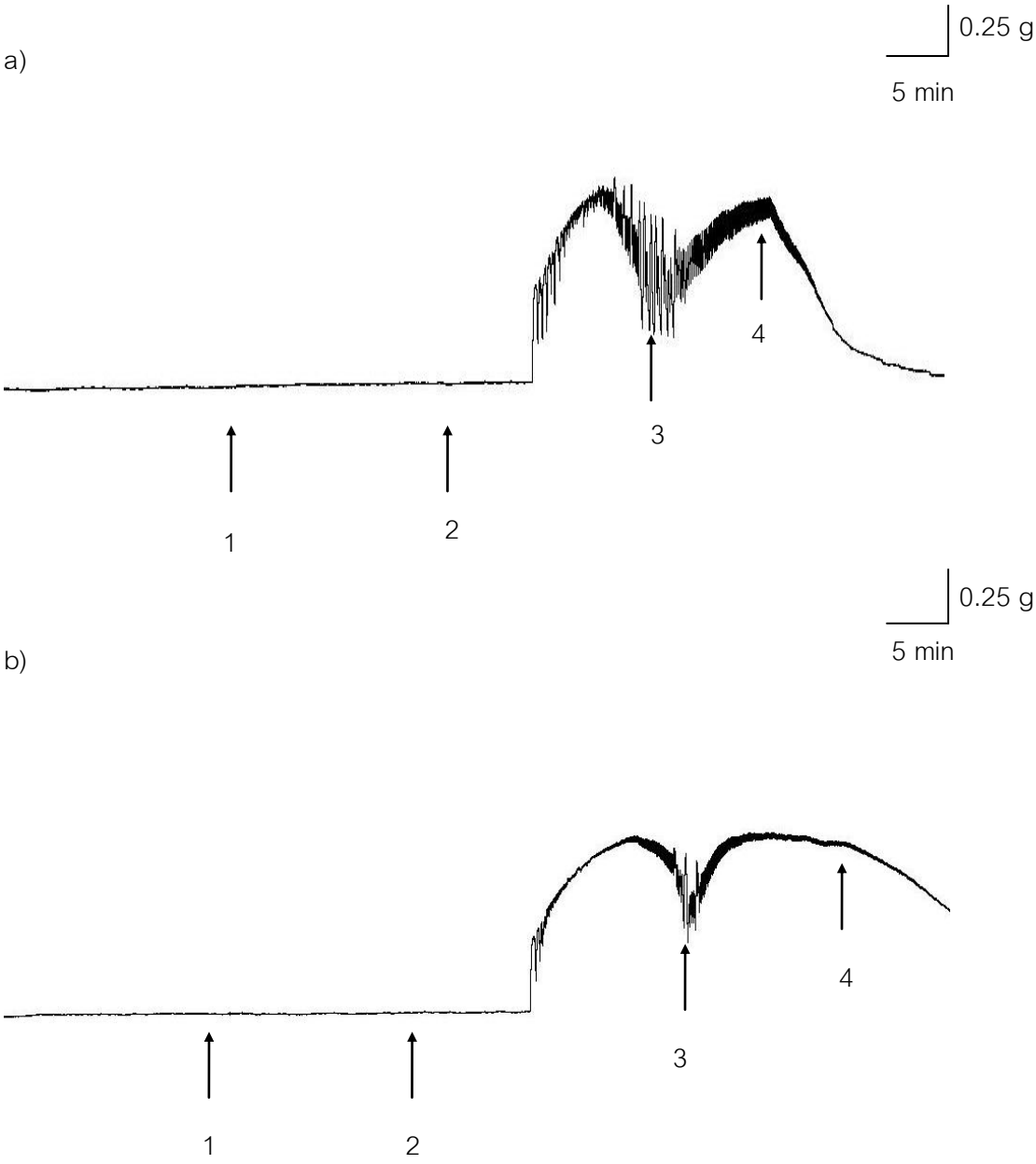


Figure 20 Representative tracing showed the contraction induced by cumulative addition of phyllanthin (a) and hypophyllanthin (b) on resting tension in KHS. Phyllanthin and hypophyllanthin concentrations were 1=1, 2=10, 3=50, 4=100 μM.

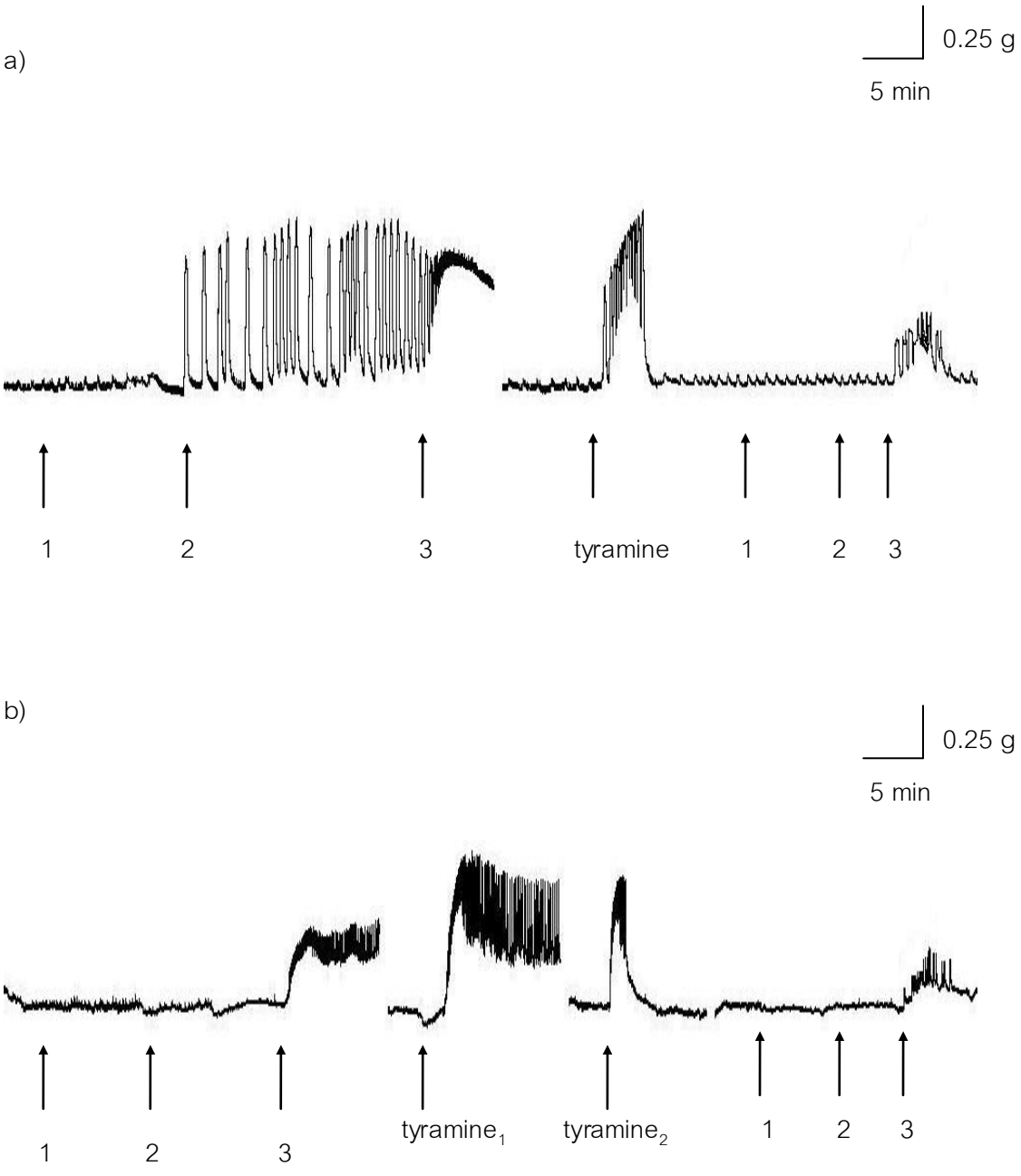


Figure 21 Representative tracing showed the effect of tyramine on phyllanthin (a) and hypophyllanthin (b) induced contraction in KHS. Phyllanthin and hypophyllanthin concentrations were 1=1, 2=10, 3=50 μM.

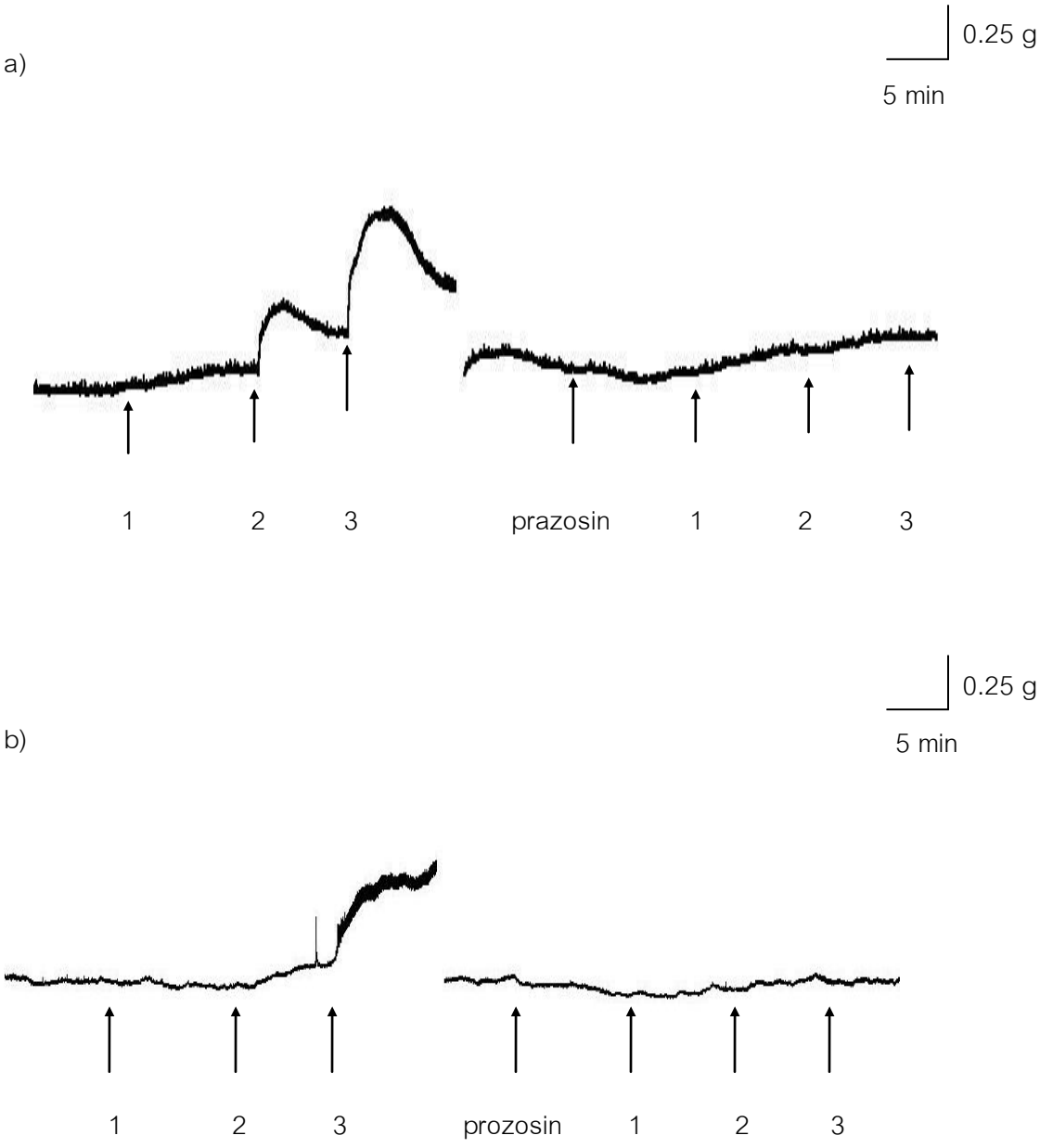


Figure 22 Representative tracing showed the effect of prazosin on phyllanthin (a) and hypophyllanthin (b) induced contraction in KHS. Phyllanthin and hypophyllanthin concentrations were 1=1, 2=10, 3=50 μM.

2.2 Agonist-induced aortic contraction

The contraction profiles of endothelium-denuded aortic rings in responses to PE (1 μM) were similar to those of KCl (40 mM) induced contraction (Figure 46, 53 in appendix). The magnitude of the developed tension provoked by PE (1 μM) was 1.41 ± 0.06 g (n = 45) and that provoked by KCl (40 mM) was 1.21 ± 0.05 g (n=58), respectively. In addition, tyramine did not interfere with the aortic contraction provoked by either PE or KCl. The developed tensions in the presence of tyramine (1 μ) were 1.27 ± 0.08 g (n=15) for PE-mediated contraction and 1.22 ± 0.08 g (n=23) for KCl-mediated contraction, respectively. Pretreatment of aortic rings with phyllanthin and hypophyllanthin were able to inhibit the aortic contraction provoked by either PE or KCl in concentration-dependent manner (Figure 47-52, 54-59 in appendix). It was interesting that hypophyllanthin at concentration 200 μM did not significantly inhibit the KCl-mediated contraction instead it significantly increased the aortic tension.

The inhibitory effects of the test compounds on vascular tension were summarized in Figure 23, 24. The inhibition profiles suggested that phyllanthin was more potent than hypophyllanthin in suppressing the contractile responses of vascular smooth muscle upon challenges with either PE or KCl. The apparent IC_{50} values of phyllanthin were 57.67 ± 8.85 μM for PE-mediated contraction, and 63.30 ± 2.69 μM for KCl-mediated contraction (Table 7 in appendix).

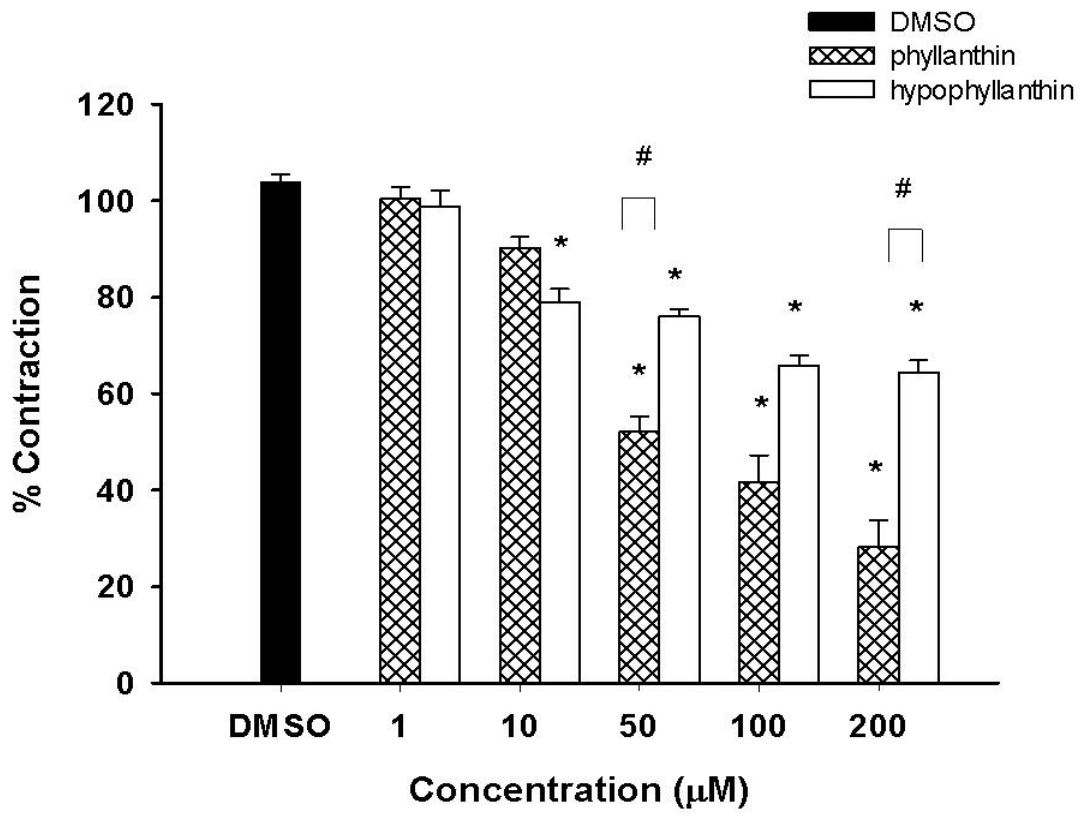


Figure 23 Effects of phyllanthin and hypophyllanthin (1-200 μM) on vascular contraction induced by PE (1 μM) in endothelium-denuded aortic rings.

Data were present as mean ± S.E.M., n = 4-6

*p<0.05 showed significant difference from DMSO control group.

#p<0.05 showed significant difference between phyllanthin and hypophyllanthin.

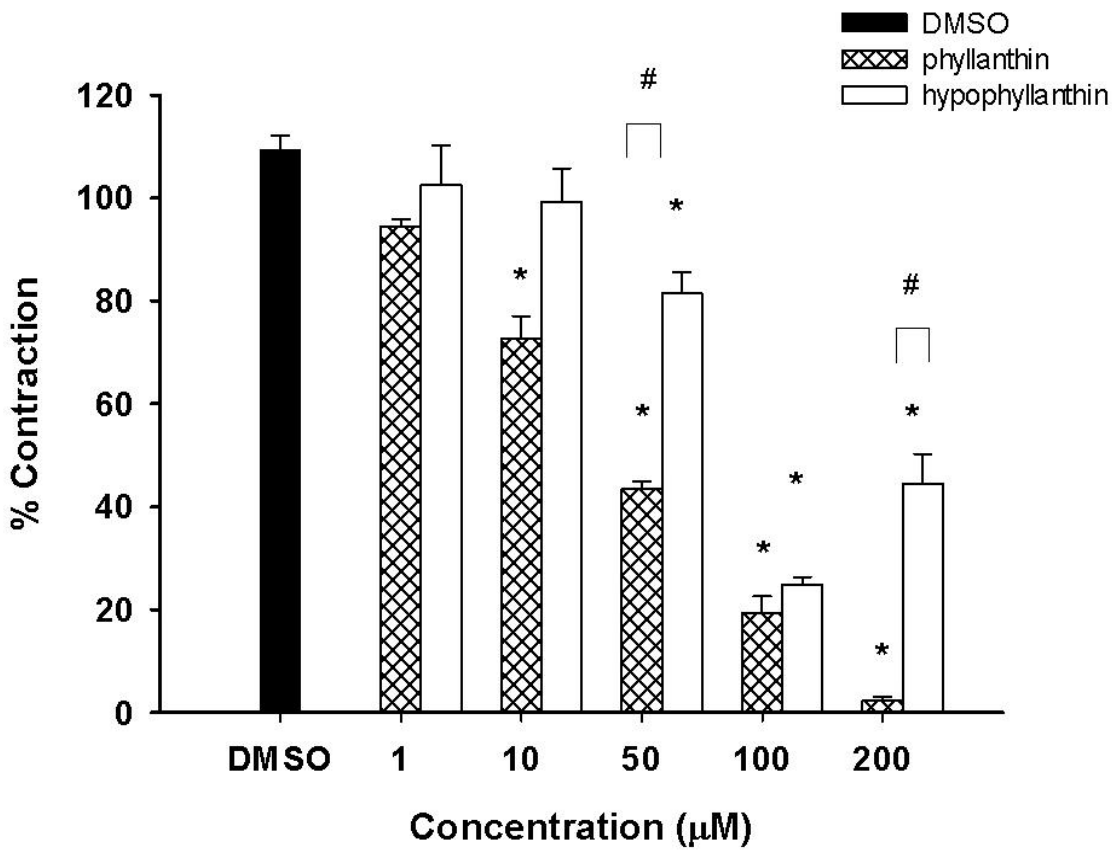


Figure 24 Effects of phyllanthin and hypophyllanthin (1-200 μM) on vascular contraction induced by KCl (40 mM) in endothelium-denuded aortic rings.

Data were present as mean ± S.E.M., n = 4-6

*p<0.05 showed significant difference from DMSO control group.

#p<0.05 showed significant difference between phyllanthin and hypophyllanthin.

2.3 Spontaneous contraction

Upon changing of the physiological solution from Ca^{2+}- free solution to Ca^{2+}-containing solution, a spontaneous contraction was observed, suggesting the Ca^{2+} flux into the smooth muscle through the membrane Ca^{2+} channel as previously reported by other investigators (Noguera *et al.*, 1997). In this study, the maximum of spontaneous contraction was 52.82 ± 2.30 % (n=16) in relative to the contractile responses to PE (1 μM) in Ca^{2+}- containing normal KHS. On the contrary, a spontaneous contraction was not observed under similar condition, except that KCl (40 mM) was used to provoke the contraction (Figure 60 in appendix). Pretreatment of aortic rings with DMSO (0.2% v/v) did not affect on spontaneous contraction in this experimental condition (Figure 25).

The results showed that both phyllanthin and hypophyllanthin (100 μM) significantly inhibited a spontaneous contraction of the Ca^{2+}- depleted rings upon changing Ca^{2+}- free KHS to Ca^{2+}- containing buffer (Figure 26, 27). The inhibition profiles suggested that these two compounds might affect capacitative Ca^{2+} entry and refilling of Ca^{2+}- internal stores.

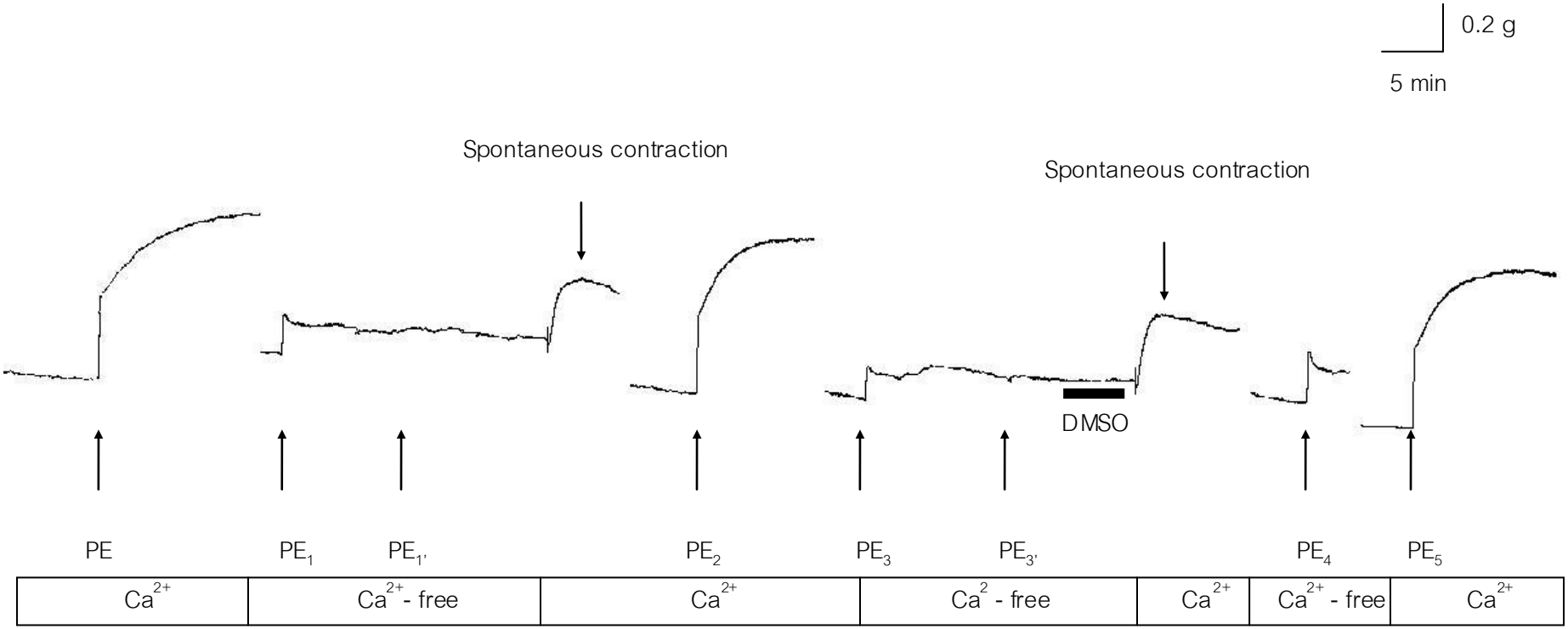


Figure 25 Representative tracing showed the effect of DMSO 0.2% (v/v) on spontaneous contraction in Ca^{2+}- containing KHS.

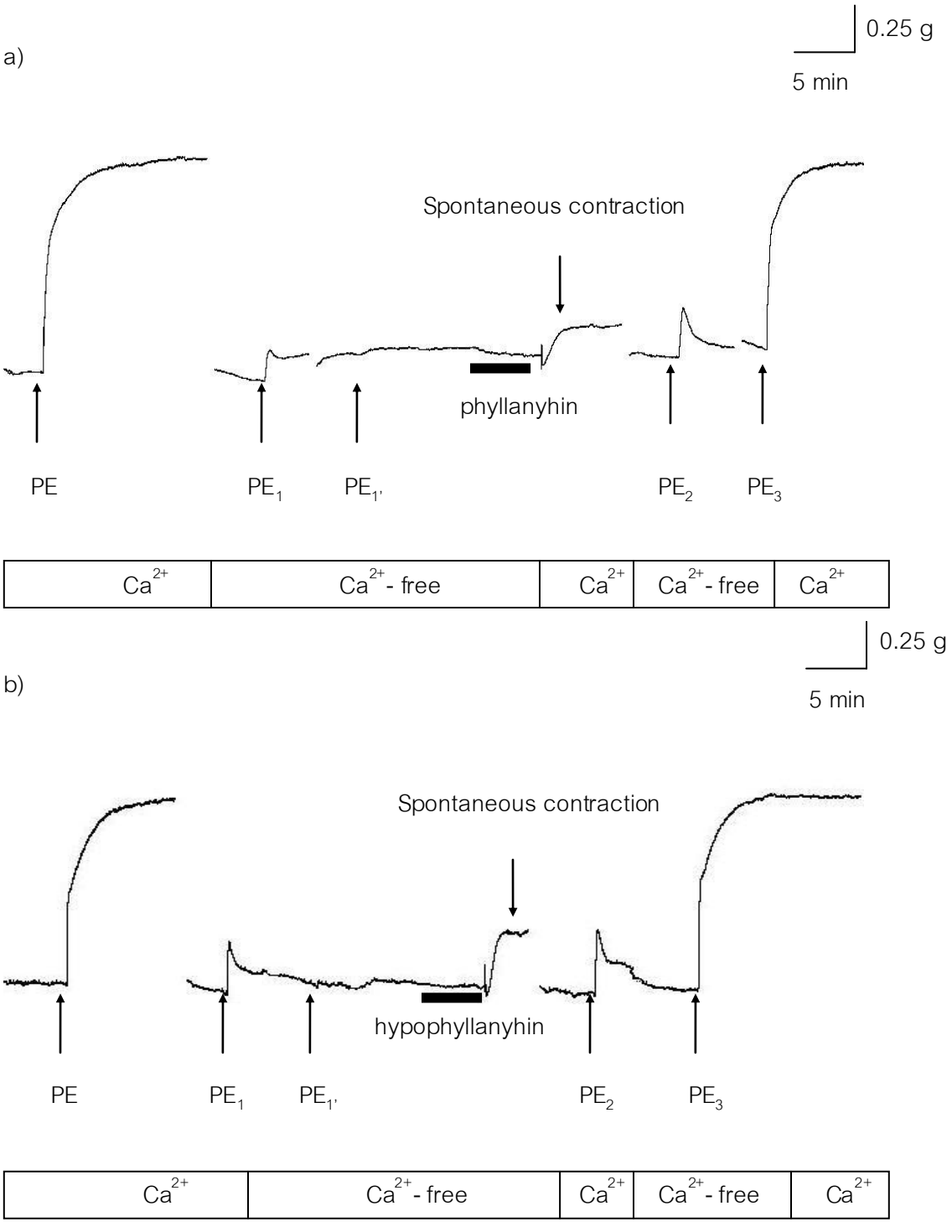


Figure 26 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (100 μM) on spontaneous contraction in Ca^{2+}-containing KHS.

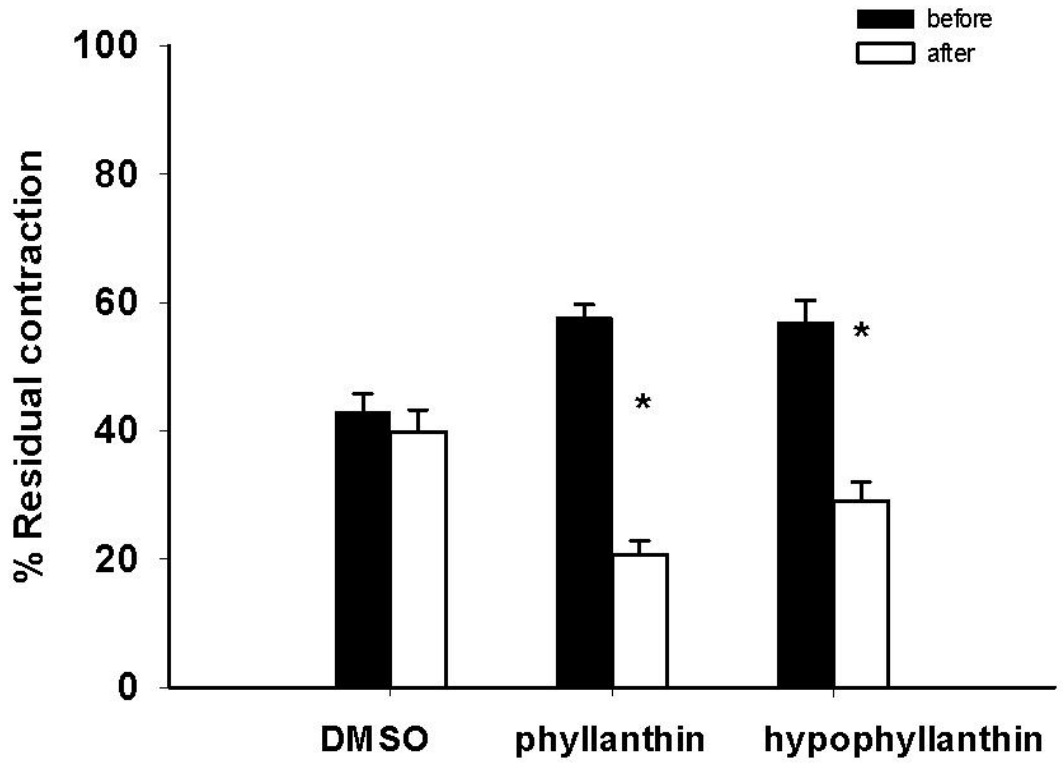


Figure 27 Effects of phyllanthin and hypophyllanthin (100 μM) on spontaneous contraction in Ca^{2+}- containing KHS.

Data were present as mean ± S.E.M., n = 5-6

*p<0.05 showed significant difference from DMSO control group.

2.4 Aortic contraction in high K^+ - Ca^{2+}- free depolarizing solution

The contraction profiles of endothelium-denuded aortic rings upon cumulative addition of $CaCl_2$ (10 μM – 10mM) in high K^+ - Ca^{2+}- free depolarizing solution were shown in Figure 28-30. In this study, nifedipine, a known Ca^{2+} blocker, was used as a positive control in inhibiting the $CaCl_2$-mediated contraction of depolarized aortic muscle. The results demonstrated that phyllanthin (100 μM) and nifedipine (10 μM) significantly inhibited the $CaCl_2$-induced contraction (Figure 31), with the apparent pD_2 values of 2.53 ± 0.13 and 2.78 ± 0.09 for phyllanthin and nifedipine, respectively. In addition, the inhibitory effect of phyllanthin was surmountable with the cumulative addition of $CaCl_2$, suggesting its reversibility. By contrast, the contractile responses in the presence of hypophyllanthin at the equimolar concentration were not significantly different from those of DMSO control group. These findings suggested that phyllanthin, but not hypophyllanthin, was able to directly inhibit Ca^{2+} influx to the smooth muscle through voltage-operated Ca^{2+} channels (VOC).

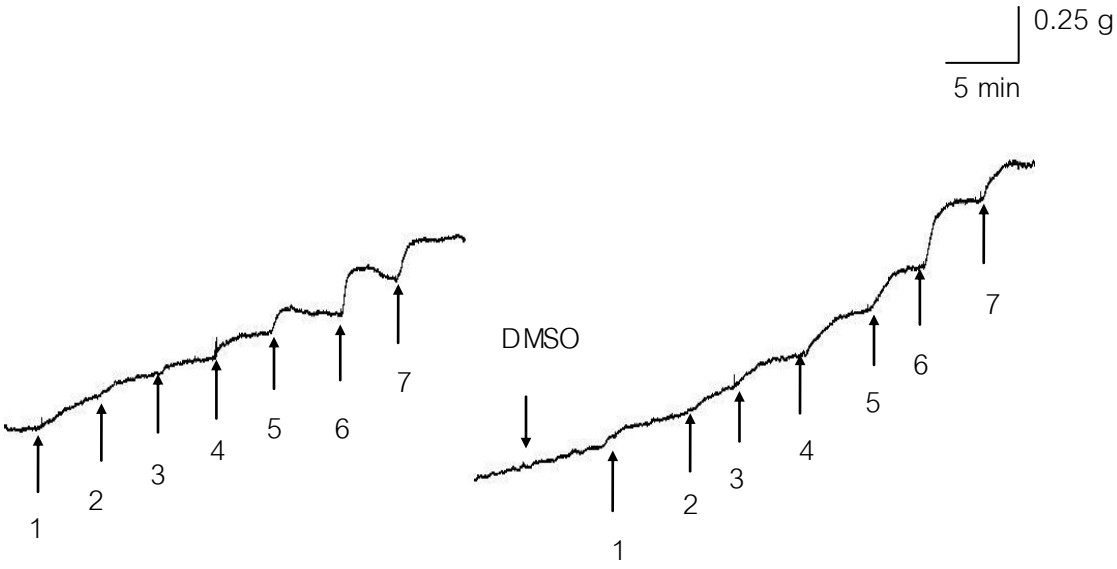


Figure 28 Representative tracing showed the effect of DMSO 0.2% (v/v) on $CaCl_2$ -induced contraction of endothelium-denuded aortic rings in high K^+- Ca^{2+}- free depolarizing solution. $CaCl_2$ concentrations were 1=10^{-5}, 2=5x10^{-5}, 3=10^{-4}, 4=5x10^{-4}, 5=10^{-3}, 6=5x10^{-3}, 7=10^{-2} µM.

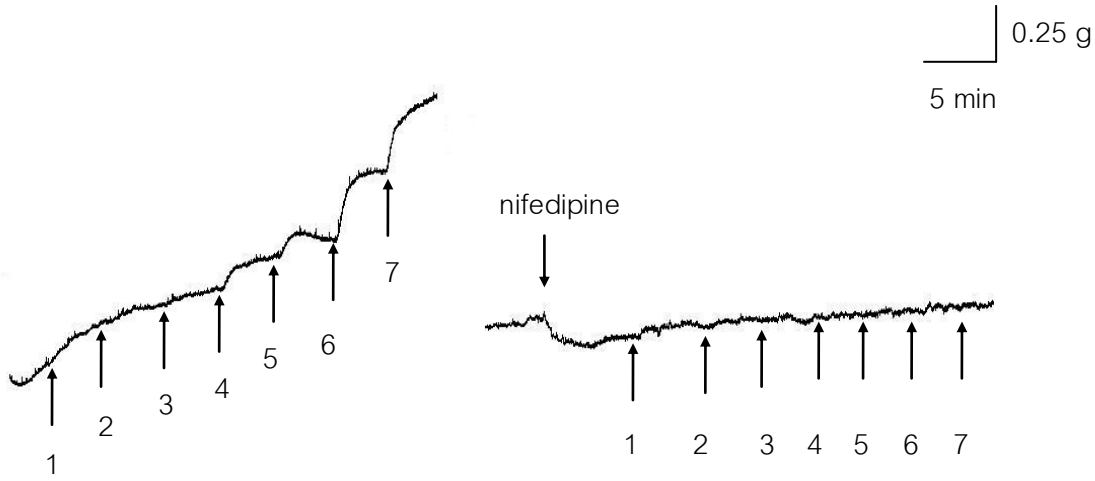


Figure 29 Representative tracing showed the effect of nifedipine (positive control) on $CaCl_2$ -induced contraction of endothelium-denuded aortic rings in high K^+- Ca^{2+}- free depolarizing solution. $CaCl_2$ concentrations were 1=10^{-5}, 2=5x10^{-5}, 3=10^{-4}, 4=5x10^{-4}, 5=10^{-3}, 6=5x10^{-3}, 7=10^{-2} µM

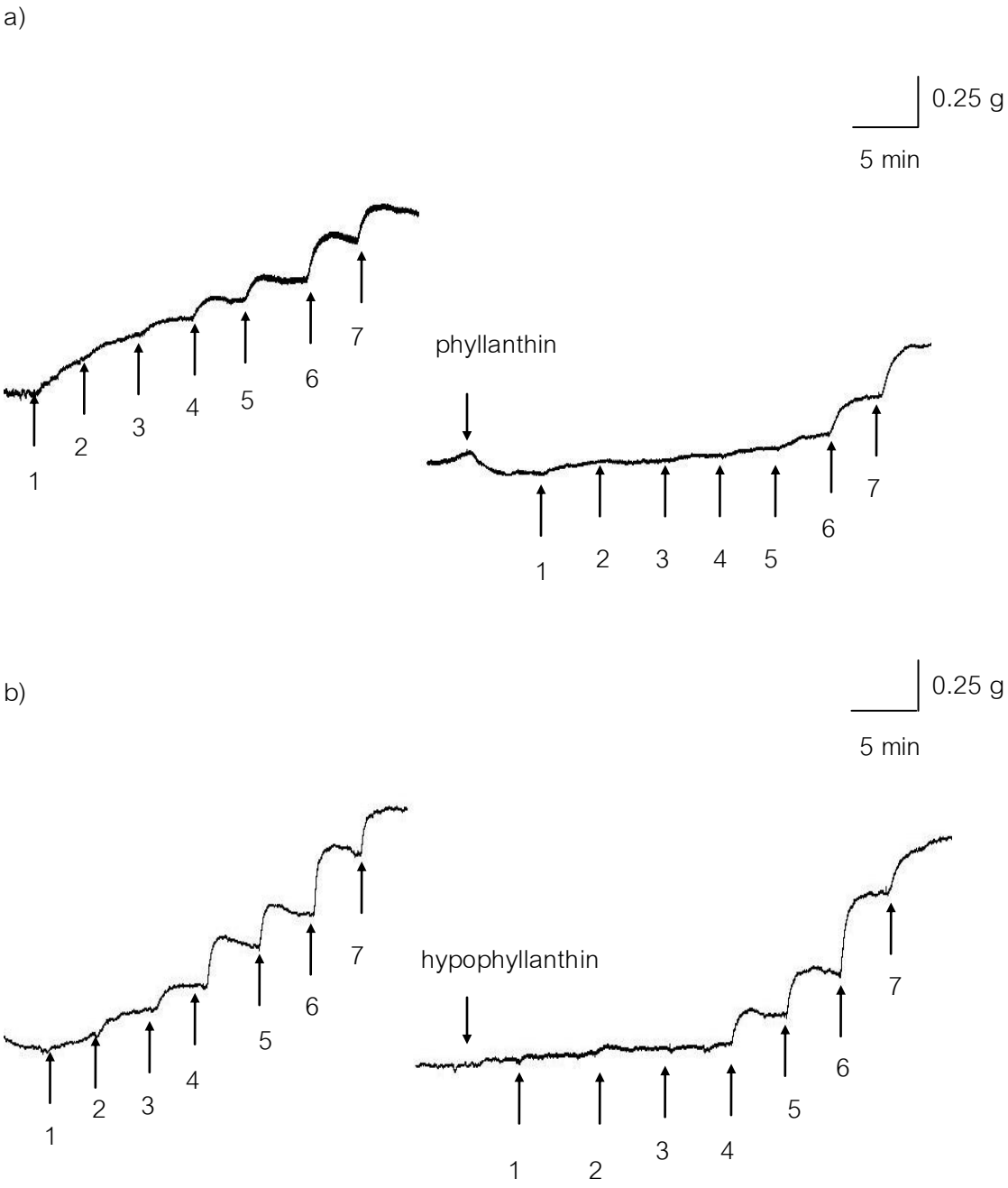


Figure 30 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (100 μM) on $CaCl_2$ -induced contraction of endothelium-denuded aortic rings in high K^+- Ca^{2+}- free depolarizing solution. $CaCl_2$ concentrations were 1=10^{-5}, 2=5x10^{-5}, 3=10^{-4}, 4=5x10^{-4}, 5=10^{-3}, 6=5x10^{-3}, 7=10^{-2} μM.

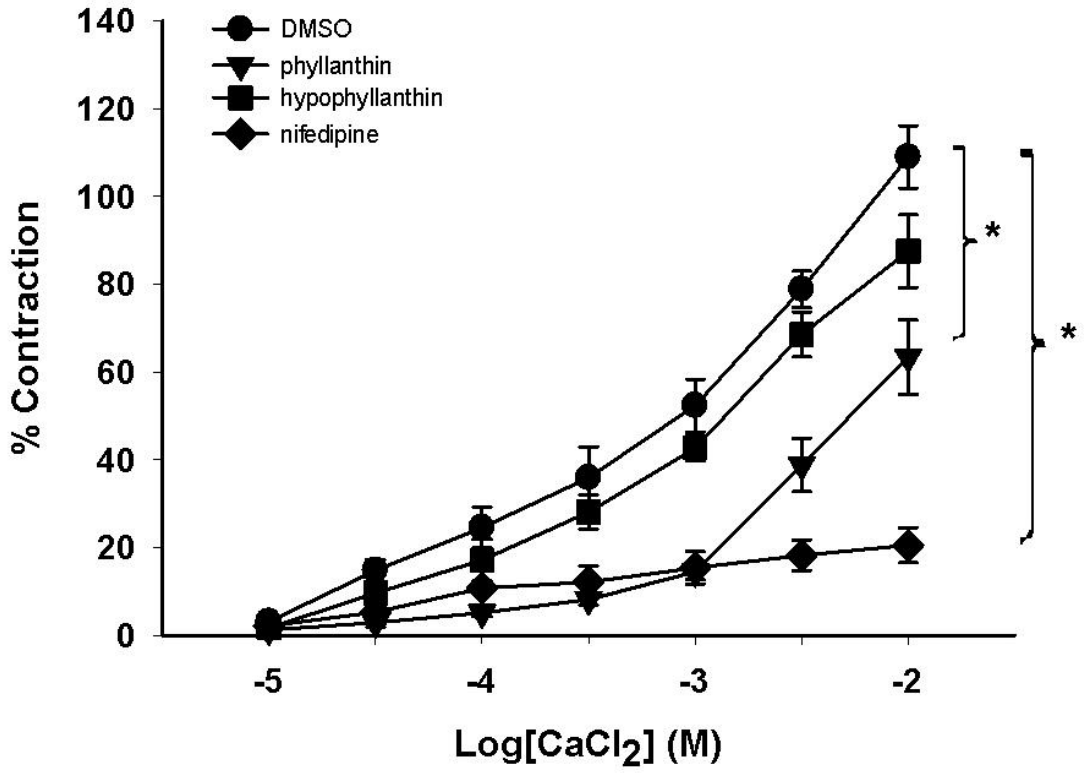


Figure 31 Effects of phyllanthin and hypophyllanthin (100 μM) on vascular contraction induced by cumulative addition of $CaCl_2$ in high K^+- Ca^{2+}-free depolarizing solution.

Data were present as mean ± S.E.M., n = 5-6

*p<0.05 showed significant difference from DMSO control group.

2.5 Aortic contraction in Ca^{2+}- free solution

The contraction profiles of endothelium-denuded aortic rings in Ca^{2+}- free solution upon addition of specific contraction were shown in Figure 32. PE (1 μM) produced a biphasic contractile response whereas caffeine (10 mM) produced a small transient contraction. In Ca^{2+}- free buffer, PE could increase intracellular Ca^{2+} by activating IP_3 receptor at sarcoplasmic reticulum. The tension induced by PE in this Ca^{2+} -free condition was 0.24 ± 0.03 g (n = 15). The results showed that both phyllanthin and hypophyllanthin at concentration of 100 μM significantly inhibited PE-induced contraction to 24.08 ± 1.29% and 55.72 ± 2.89% (n=4-5), respectively (Figure 34). The findings suggested that both compounds could interfere the smooth muscle contraction involving with the IP_3-mediated release of Ca^{2+} from its internal stores.

The effect of the test compounds on the caffeine-mediated contraction was also investigated (Figure 33). As known, caffeine could increase intracellular Ca^{2+} by activating ryanodine receptor at sarcoplasmic reticulum (SR). In this study, the tension induced by caffeine was 0.12 ± 0.02 g (n = 12). As shown in Figure 34, both phyllanthin and hypophyllanthin at concentration of 100 μM had any effect on caffeine-induced contraction. The results suggested that both test compounds had any influcence on the release of Ca^{2+} from SR via activation of ryanodine receptor.

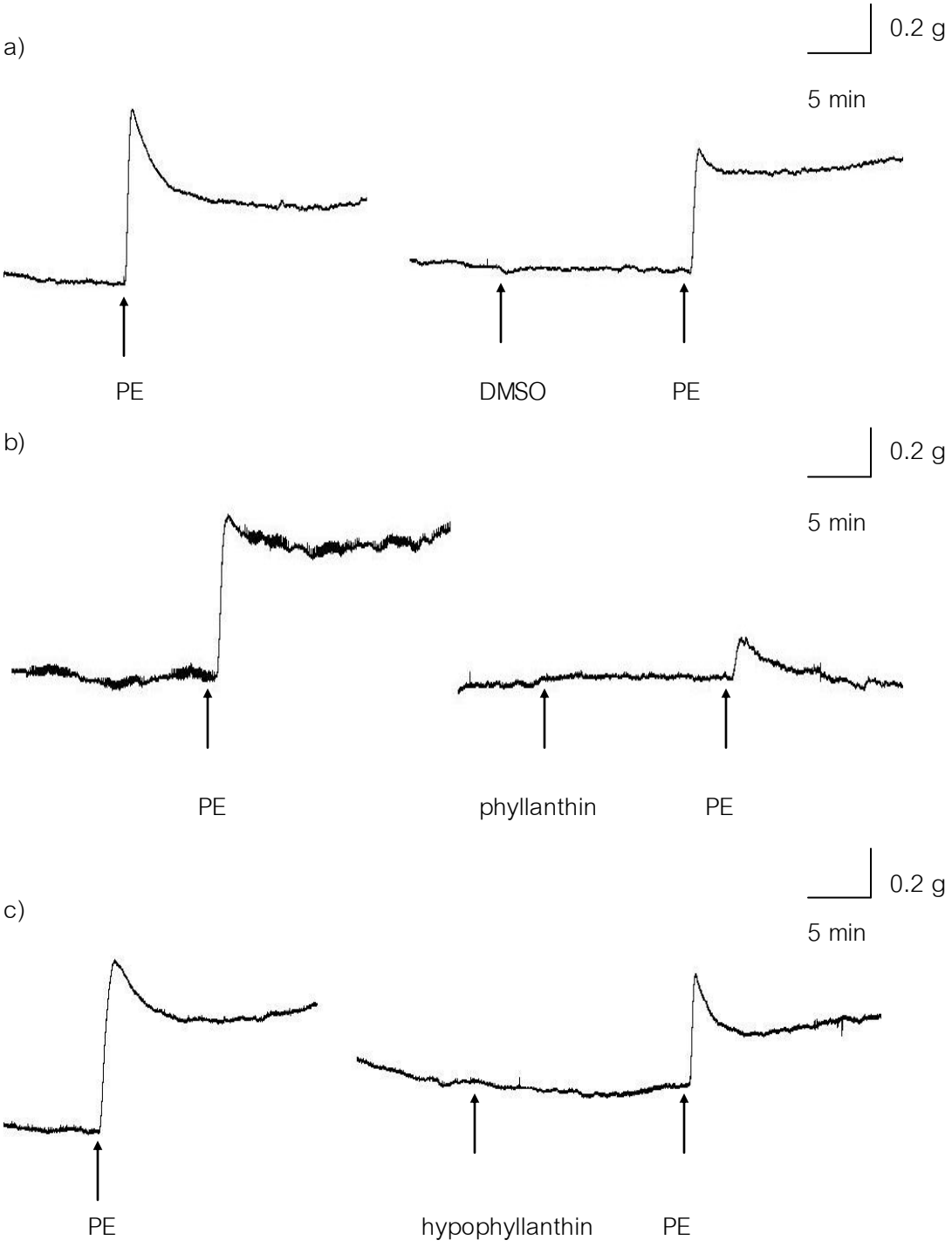


Figure 32 Representative tracing showed the effects of DMSO 0.2% (v/v) (a), phyllanthin (b) and hypophyllanthin (c) (100 µM) on PE-induced contraction of endothelium-denuded aortic rings in Ca^{2+} - free solution.

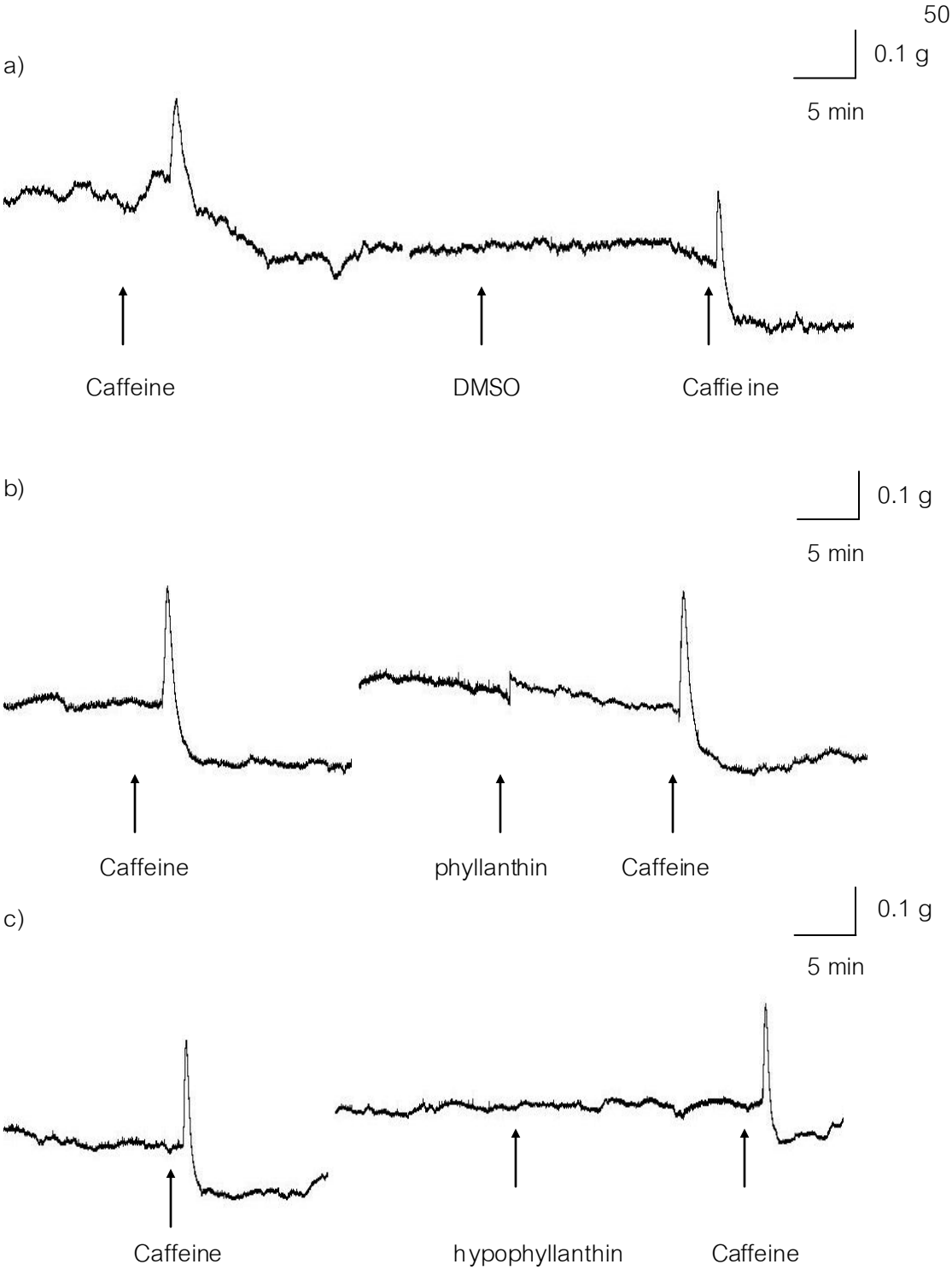


Figure 33 Representative tracing showed the effects of DMSO 0.2% (v/v) (a), phyllanthin (b) and hypophyllanthin (c) (100 µM) on caffeine-induced contraction of endothelium-denuded aortic rings in Ca^{2+} - free solution.

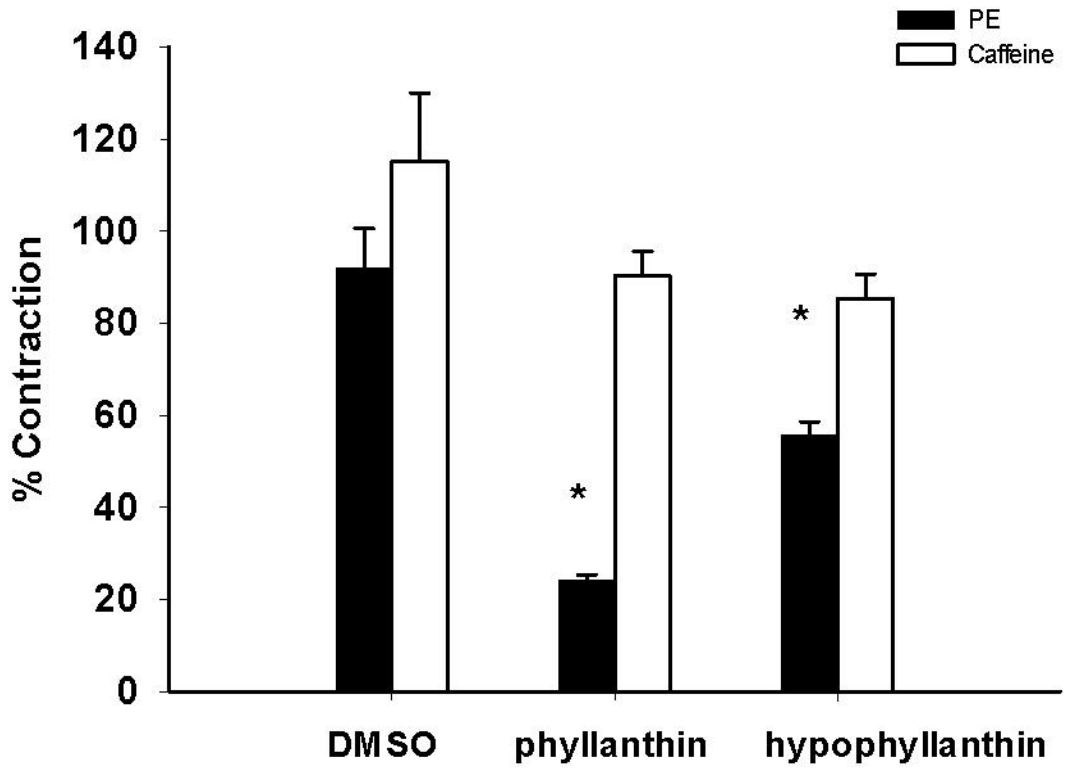


Figure 34 Effects of phyllanthin and hypophyllanthin (100 μM) on vascular contraction induced by PE and caffeine in Ca^{2+} - free solution.

Data were present as mean ± S.E.M., n = 4-5

*p<0.05 showed significant difference from DMSO control group

CHAPTER V

DISCUSSION AND CONCLUSION

The purpose of this study was to investigate the effects of phyllanthin and hypophyllanthin on vascular tension. The study was also examined the influence of endothelium on the vascular actions of these two compounds.

The effects of phyllanthin and hypophyllanthin induced vasorelaxation were determined in endothelium-intact and endothelium-denuded aortic rings. In this study, both phyllanthin and hypophyllanthin significantly induced vasorelaxation in the aortic rings that were pretreated with PE. The relaxation responses of aortic muscle to each compound in the presence and absence of endothelium appeared to be comparable. These findings suggested that the intrinsic vasorelaxing activity of these two compounds was independent of functional endothelium. As known, endothelial cells released a number of vasodilators such as nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) (Ignarro, 2002; Stankevicius *et al.*, 2003; Suzuki, Chen and Yamamoto, 1992). It was unlikely that both phyllanthin and hypophyllanthin could enhance the release of these endothelium derived relaxing factors (EDRFs). Moreover, treatment the aortic muscles with methylene blue and specific K^+ channel inhibitors such as glibenclamide, tetraethylammonium, 4-aminopyridine and barium chloride had no influence on the relaxation mediated by these two lignans.

As known, the key action of endothelial derived NO is to activate soluble quanylyl cyclase (sGC) in smooth muscle cells, leading to relaxation. In this study, methylene blue, a known sGC inhibitor had no significant effects on the relaxation induced by both phyllanthin and hypophyllanthin. These results suggested that the actions of the two lignans were not related to activation of the NO/cGMP pathway. These findings distinguished the modes of action of phyllanthin and hypophyllanyhin from those of other lignans such as secoisolariciresinol diglucoside (SDG) is a lignan isolated from flaxseed which has the molecular structure similar to phyllanthin and hypophyllanthin (Figure 1). It was reported that the hypotensive effect of SDG was

mediated through NO/cGMP pathway (Prasad, 2004). Furthermore, n-butanol extract from leaves of *Phyllanthus acidus* induced vasorelaxation via NO/cGMP pathway (Leeya *et al*., 2010). In addition, the vasorelaxation effects of these two compounds could not be inhibited by atropine, a muscarinic receptor antagonist. The findings supported that the two compounds had no influence on the NO/cGMP pathways.

The effects of both phyllanthin and hypophyllanthin on K^+ channel were also determined in this study. It is well established that K^+ channels play important roles in the regulation of membrane potential in vascular smooth muscle cells. The open of K^+ channels causes the membrane hyperpolarization, leading to the closure of voltage-operated Ca^{2+} channels and decrease of intracellular Ca^{2+} and vasodilation (Jackson, 2000; Ko *et al*., 2008). To date, four distinct types of K^+ channel have been identified in vascular smooth muscle: including ATP-sensitive potassium (K_{ATP}) channels, voltage-dependent K^+ (K_v) channels, Ca^{2+}- activated K^+ (BK_{Ca}) channels and inward rectifier K^+ (K_{IR}) channels. (Jackson, 2000; Ko *et al*., 2008). It was shown that n-butanol extract from leaves of *Phyllanthus acidus* induced vasorelaxation via opening of K_{ATP} and BK_{Ca} channels in the vascular smooth muscle of isolated rat thoracic aorta (Leeya *et al*., 2010). In this study, treatment with various K channels blockers including gli benclamide (an ATP-sensitive K^+blocker), tetraethylammonium (a Ca^{2+}- activated K^+ blocker), 4-aminopyridine (a voltage-dependent K^+ blocker) and barium chloride (an inward rectifier K^+ blocker) had no significant inhibitory effect against vasorelaxation induced by both phyllanthin and hypophyllanthin. Hence it might be concluded that the mechanisms of action of these two lignans were not related to K^+ channels. Furthermore, the vasorelaxant effects of these two compounds could not be blocked by propranolol, a non-selective β-adrenoceptor antagonist, suggesting that these lignans had no interaction with β_2-adrenoceptors.

It was interesting that phyllanthin and hypophyllanthin were able to induce aortic contraction in the absence of endothelium. This vasoconstriction effect was transient and reached maximum values at the cumulative concentration of the lignans up to 50 μM. Further addition of these two compounds at the concentration of 100 μM did not

increase or sustain the contraction. In order to determine whether the two lignans contain the intrinsic vasoconstrictive activity, pretreatment the aortic muscle with tyramine prior to the test compounds were performed. Tyramine can release NE from presynaptic storage vesicles, leading to muscle contraction (Krishnamurty and Grollmax, 1972). In this study, tyramine treatment caused transient, but significant contraction of aortic muscle, suggesting the presence of presynaptic storage vesicles of NE in the aortic preparations. It was interesting that the vasopressive effects of both phyllanthin and hypophyllanthin could be abolished in the presence of tyramine. Furthermore, prazosin, a known α_1-adrenoceptor antagonist, could also prevent the vasopressive actions of these two lignans. Taken together, the vasopressive action of both phyllanthin and hypophyllanthin were not directly mediated from its intrinsic property. It was likely that these two compounds were able to release NE from endogenous presynaptic storages, which consequently resulted in muscle contraction.

It is widely known that the influx of extracellular Ca^{2+} occur through transmembrane Ca^{2+} channels, which can be either receptor-operated Ca^{2+} channel (ROC) or voltage-operated Ca^{2+} channel (VOC), leading to vasoconstriction (van Breemen and Saida, 1989; Karaki *et al.*, 1997). Activation of α_1- receptors eventually results in activation of receptor-operated Ca^{2+} channel (ROC) whereas KCl-induced depolarization of plasma membrane causes Ca^{2+} influx through voltage-gated Ca^{2+} channel (VOC) (Ko *et al*, 2008; Nelson and Quayle, 1995). In this study, both phyllanthin and hypophyllanthin were able to inhibit the aortic contraction provoked by either PE or KCl in concentration-dependent manner. These findings suggested that these two lignans might be able to interfere a rising of cytosolic Ca^{2+} which is a key element of muscle contraction. One possibility was that the two compounds might affect extracellular Ca^{2+} influx through plasma membrane Ca^{2+} channels including both ROC and VOC.

This hypothesis was tested in mechanistic entry of Ca^{2+} across plasma membrane in the absence of agonist in the spontaneous contraction model (or increase in the resting tone; IRT) as previously described by other investigators (Noguera *et al.*,

1992, 1993, 1997). In this IRT model, the Ca^{2+} flux through plasma membrane Ca^{2+} channels can occur in order to replenish capacitative Ca^{2+} stores in the Ca^{2+}- depleted smooth muscle. The depletion of intracellular Ca^{2+} pools caused by repetitive stimulation the muscle with α_1-adrenoceptor agonists in Ca^{2+} free condition. Upon addition of Ca^{2+} back to the test system, Ca^{2+} influx from the extracellular environment occur rapidly, resulting in the spontaneous contraction or IRT. In this study, both phyllanthin and hypophyllanthin were able to inhibit spontaneous contraction under this specific condition. Hence, the mechanical refilling process of Ca^{2+} in this condition was disrupted by these two lignans. It was quite certain that membrane Ca^{2+} channels coupled to α_1- adrenoceptor could be affected by these two compounds. Furthermore, the effects of the two compounds on VOC were demonstrated in the $CaCl_2$-mediated contraction in high K^+ depolarizing solution. The principle of method involved the influx of Ca^{2+} from extracellular environment into intracellular pool through the fully open VOC induced by membrane depolarization. In this condition, phyllanthin, but not hypophyllanthin, significantly inhibited the $CaCl_2$-induced contraction. The results demonstrated that the contractile response in the presence of hypophyllanthin at the equimolar concentration to that of phyllanthin were not significantly different from DMSO control group. Hence, it might be concluded that phyllanthin was more effective than hypophyllanthin in inhibiting Ca^{2+} movement through the VOC. As described above, phyllanthin and hypophyllanthin had the inhibitory effects on both PE- and KCl-induced contraction. Therefore it could be suggested that two compounds may directly disrupt the increase of intracellular Ca^{2+} in vascular smooth muscle. Phyllanthin was able to block both VOC and ROC effectively whereas hypophyllanthin preferred ROC to VOC.

The potential effects of these two compounds on intracellular release of Ca^{2+} were also investigated in the experimental contraction model in Ca^{2+}- free solution. It is well established that PE and caffeine produced a small transient contraction of smooth muscle in Ca^{2+}- free solution through Ca^{2+}- release from SR. The mechanism of PE - induced Ca^{2+}- release from SR is mediated through the binding of IP_3 to and the IP_3 receptor (IP_3R) on the SR, resulting in a release internal Ca^{2+} from SR (Noguera and

D'Ocon, 1992). Caffeine-induced a transient contraction through the binding to the ryanodine receptor (RyR), leading to stimulation of Ca^{2+}- release from SR (Herrmann-Frank *et al*., 1991; Meissner, 1994). Both phyllanthin and hypophyllanthin were able to inhibit the PE-induced transient contraction, but it had no effect on caffeine-induced contraction in Ca^{2+}- free solution. These findings suggested that these two lignans could interfere the smooth muscle contraction involving with the IP_3-mediated release of Ca^{2+} from its internal stores.

In conclusion, phyllanthin and hypophyllanthin induced aortic relaxation via endothelium-independent mechanism. Its direct actions on vascular smooth muscle may be linked to non-specific inhibition of Ca^{2+} influx and inhibition of PE-mediated Ca^{2+} release from SR.

REFFERENCES

Abdulla, M. A., Ahmed, K. A - A., AL-Bayaty, F. H., and Masood, Y. Gastroprotective effect of Phyllanthus niruri leaf extract against ethanol-induced gastric mucosal injury in rats. African Journal of Pharmacy and Pharmacology 4(5) (2010): 226-230.

Amaechina, F. C., and Omogbai, E. K. Hypotensive effect of aqueous extract of the leaves of *Phyllanthus amarus* Schum. and Thonn. (Euphorbiaceae). Acta Poloniae Pharmaceutica - Drug Research 64(6) (2007): 547-552.

Baloglu, E., Kiziltepe, O., and Gurdal, H. The role of Gi proteins in reduced vasorelaxation response to β-adrenoceptor agonists in rat aorta during maturation. European Journal of Pharmacology 564 (2007): 167–173.

Bagalkotkar, G., Sagineedu, S. R., Saad, M. S., and Stanslas, J. Phytochemicals from *Phyllanthus niruri* Linn. and their pharmacological properties : a review. Journal of Pharmacy and Pharmacology 58 (2006): 1559-1570.

Carl, A., Lee, H. K., and Sanders, K. M. Regulation of ion channels in smooth muscles by calcium. American Journal of Physiology 271 (1996): C9-C34.

Cassidy, A., Hanley, B., and Lamuela-Raventos, R. M. Isoflavones, lignans and stilbenes - origins, metabolism and potential importance to human health. Journal of the Science of Food and Agriculture 80 (2000): 1044-1062.

Chirdchupunseree, H. Protective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes. Doctor's Thesis, Faculty of Pharmaceutical Sciences. Chulalongkorn University, 2009.

Cos, P., *et al*. Phytoestrogen: Recent Develoments. Planta Medica 69 (2003): 589-599.

Dai-Eun Sok, D.-E., Hui S. Cui, H. S., and Kim, M. R. Isolation and Boactivities of Furfuran Type Lignan Compounds from Edible Plants. Recent Patents on Food, Nutrition & Agriculture 1 (2009): 87-95.

Denninger, J. W., and Maretta, M. A. Guanylate cyclase and the NO/cGMP signaling pathway. Biochimica et Biophysica Acta 1411 (1999): 334-350.

Do, K. H., *et al*. Pinoresinol-4,40-di-O-b-D-glucoside from Valeriana officinalis root stimulates calcium mobilization and chemotactic migration of mouse embryo fibroblasts. Phytomedicine 16 (2009): 530–537.

Ericsson, E., and Lundholm, L. Adrenergic β-receptor activity and cyclic APM metabolism in vascular smooth muscle; Variations with age. Mechanisms of Ageing and Development 4 (1975): 1-6.

Etuk, E.U. A Review of Medicinal Plants with Hypotensive or Antihypotensive effects. Journal Medical Science 6(6) (2006): 894-900.

Harnett, K. M., and Biancani, P. Calcium-Dependent and Calcium-Independent Contractions in Smooth Muscles. The American Journal of Medicine 115(3A) (2003): 24S-30S.

Hearon, W. M., and MacGregor, W.S. The Naturally Occurring Lignans. Chemical reviews. 55(5) (1955): 957-1068.

Hecker, M. Endothelium-derived hyperpolarizing factor-fact or fiction?. News In Physiological Sciences 15 (2000): 1-5.

Herrmann-Fran, Darling, E., and Meissner, G. Functional characterization of the Ca^{2+}-gated Ca^{2+} release channel of vascular smooth muscle sarcoplasmic reticulum. European Joumal of Physiology 418 (1991): 353-359.

Hill, M. A., Zou, H., Potocnik, S. J. Meininger, G. A., and Davis, M. J. Signal Transduction in Smooth Muscle. Invited Review: Arteriolar smooth muscle mechanotransduction: Ca^{2+} signaling pathways underlying myogenic reactivity. Journal of Applied Physiology 93 (2001): 973-983.

Hypohyllanthin [Online] Available from : http://www.alibaba.com/product-tp/11106_hin.html [2011, March 29]

Ignarro, L. J. Nitric Oxide as a unique signaling molecular in the vascular system: A Historical Overview. Journal of Physiology and Pharmacology 53(4) (2002): 503-514.

Iizuka, T., Moriyama, H., and Nagai, M. Vasorelaxant Effects of Methyl Brevifolincarboxylate from the Leaves of *Phyllanthus niruri*. Biological & Pharmaceutical Bulletin 29(1) (2006): 177-179.

Jackson, W. F. Ion channels and vascular tone. Hypertension 35 (2000): 173-178.

Karaki, H., *et al*. Calcium Movements, Distribution, and Functions in Smooth Muscle. The American Society for Pharmacology and Experimental Therapeutics 49 (1997): 157-230.

Khatoon, S., Rai, V., Rawat, A. K. S., and Mehrotra, S. Comparative pharmacognostic studies of three Phyllanthus species. Journal of Ethnopharmacology 104 (2006): 79–86.

Kitisin, T. Pharmacological studies 3. *Phyllanthihus niruri*. Siriraj Hospital Gazette 4 (1952): 641-649.

Ko, E. A., Han, J., Jung, I. D., and Park, W. S. Physiological roles of K^+channels in vascular smooth muscle cells. Journal Smooth Muscle Research 44(2) (2008): 65-81.

Krishnamurty, V. S. R., and Grollmax, A. Contractile response of rat aorta to norepinephrine and tyramine. The Journal of pharmacology and Experimental Therapeutics 182(2) (1972): 264-272.

Krithika, R., *et al*. Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line. Chemico-Biological Interactions 181 (2009): 351-358.

Lee, M. W., and severson, D. L. Signal transduction in vascular smooth muscle: diacylglycerol second messengers and PKC action. American journal of Physiology 267 (1994): C659-C678.

Leeya, Y., *et al*. Hypotensive activity of an n-butanol extract and their purified compounds from leaves of Phyllanthus acidus (L.) Skeels in rats. European Journal of Pharmacology 649 (2010): 301–313.

Leite, D. F. P., *et al*. The cytotoxic effect and the multidrug resistance reversing action of lignans from *Phyllanthus amarus*. Journal of Planta Medica 72 (2006): 1353-1385.

Lucas, K. A., *et al*. Guanylyl Cyclases and Signaling by Cyclic GMP. Pharmacological Reviews 52(3) (2000): 375-413.

Macrae, W. D., and T.owers G. H. N. Biological avtivities of lignans. Phytochemistry 23(6) (1984): 1207-1220.

Mann, G. E., Rowlands, D. J., Li, F. Y. L., Winter, P. d., and Siow, R. C. M. Activation of endothelial nitric oxide synthase by dietary isoflavones:Role of NO in Nrf2-mediated antioxidant gene expression. Cardiovascular Research 75 (2007): 261-274.

McFadzean, I., and Gibson, A. The developing relationship between receptor-operated andstore-operated calcium channels in smooth muscle. British Journal of Pharmacology 135 (2002): 1-13.

Meissner, G. Rynodine receptor/Ca^{2+} release Channels and their regulation by endogeneous effectors. Annual Reviews Physiology 56 (1994): 485-508.

Murugaiyah, V., and Chan, K.-L. Antihyperuricemic lignans from leaves of *Phyllanthus amarus*. Journal of Planta Medica 72 (2006): 1262-1267.

Nakano, D., *et al*. Sesamin Metabolites Induce an Endothelial Nitric Oxide-Dependent Vasorelaxation through Their Antioxidative Property-Independent Mechanisms: Possible Involvement of the Metabolites in the Antihypertensive Effect of Sesamin. The Journal of Pharmacology and Experimental Therapeutics 318 (2006): 328-335.

Nelson, M. T., and Quqyle, J. M. Physiological roles and properties of potassium channels in arterial smooth muscle. American Journal of Physiology 37 (1995): C799-C822.

Noguera, M. A., and D'Ocon, M. P. Different and common intracellular calcium-stores mobilized by noradrenaline and caffeine in vascular smooth muscle. Naunyn-Schmiedeberg's Archives of Pharmacology 345 (1992): 333-341.

Noguera, M. A., and D'Ocon, M. P. Evidence that depletion of internal calcium stores sensitive to noradrenaline elicits a contractile response dependent on extracellular calcium in rat aorta. British Journal Pharmacology 110 (1993): 861-867.

Noguera, M. A., D'Ocon, P., Ivorra, M. D., and Chulia, S. Capacitative Ca^{2+} entry associated with a1-adrenoceptors in rat aorta. Naunyn-Schmiedeberg's Archives of Pharmacology 356 (1997): 83-89.

Oh, K.-S., *et al*. Cardiovascular Effects of Lignans Isolated from *Saururus chinensis*. Planta Medica 74 (2008): 233-238.

Park, J. Y., *et al*. The mechanism of vasorelaxation induced by Schisandra chinensis extract in rat thoracic aorta. Journal of Ethnopharmacology 121 (2009): 69–73.

Phyllanthin [Online] Available from : http://www.alibaba.com/product-tp/11106_hin.html [2011, March 29]

Phyllanthus amarus [Online] Available from : http://www.learners.in.th/moo_lita/list [2010, December 25]

Phyllanthus amarus [Online] Available from : http://www.stuartxchange.org/SampaSampa_kan.html [2010, December 25]

Prasad, K. Antihypertensive Activity of Secoisolariciresinol Diglucoside (SDG) isolated from Flaxseed: Role of Guanylate Cyclase. International Journal of Angiology 13 (2004): 7-14.

Rahman, M. M. A., Dewick, P. M., Jackson, D. E., and Lucas, J. A. Lignans of Forsythia intermedial. Phytochemistry 29(6) (1990): 1971-198.

Raimundo, J. M., *et al*. The lignan eudesmin extracted from Piper truncatum induced vascular relaxation via activation of endothelial histamine H1 receptors. European Journal of Pharmacology 606 (2009): 150-154.

Rasmussen, H., Takuwa, Y., and Park, S. Protein kinase C in the regulation of smooth muscle contraction. FASEB Journal 1 (1987): 177-185.

Shokunbi, O. S., and Odetola, A. A. Gastroprotective and antioxidant activities of *Phyllanthus amarus* extracts on absolute ethanolinduced ulcer in albino rats. Journal of Medicinal Plants Research 2(10) (2008): 261-267.

Somanabandhu, A. 1H- and 13C-NMR assignments of phyllanthin and hypophyllanthin: lignans that enhance cytotoxic response with cultured multidrug-resistant cells. Journal of Natural Product 56(2) (1993): 233-239.

Stankevicius, E., Kevelaitis, E., Vainorius, E., and Simonsen, U. Role of nitric oxide and other endothelium-derived factors. Medicina 39(4) (2003): 333-341.

Sung, S. H., and Kim, Y. C. Hepatoprotective Diastereomeric Lignans from Saururus chinensis Herbs. Journal of Natural Products 63(7) (2000): 1019-1021.

Suzuki, H., Chen, G., and Yamamoto, Y. Endothelium-Derived Hyperpolarizing Factor (EDHF). Japanese Circulation Journal 56 (1992): 170-174.

Syamasundar, K. V., *et al*. Antihepatotoxic principles of *Phyllanthus niruri* herbs. Journal of Ethnopharmacology 14 (1985): 41-44.

Taohjian, G., and Armstrong, J. Principles of Pharmacology. The Pathophysiology Basic of Drug Therapy. In Chong, D. Y. and Michel T. 2nd ed. Pharmacology of Vascular Tone. pp. 367-383. United states of America, 2008.

Thippeswamy, A. H. M., *et al*. Protective role of *Phyllanthus niruri* extract in doxorubicin-induced myocardial toxicity in rats. Research Article 43 (1) (2011): 31-35.

Tripathi, A. K., Verma, R. K., Gupta, A. K., Gupta, M. M., and Khanuja, S. P. S. Quantitative determination of phyllanthin and hypophyllanthin in phyllanthus species by high-performance thin layer chromatography. Phytochemical Analysis 17 (2006): 394-397.

Vallance, P. Endothelial regulation of vascular tone. Postgraduate Medicine Journal 68 (1992): 697-701.

van Breemen, C., Saida, K. Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. Annual Reviews Physiology 52 (1989): 315-329.

van Rossum, J. M., Hurkmans, J. A., and Wolters, C. J. J. Cumulative dose-response curves. Archives Internationales de Pharmacodynamie et de Therapie 143 (1963): 299–330.

Webb, R. C. Smooth muscle contraction and relaxation. Advances In Physiology Education 27 (2003): 201-206.

APPENDIX

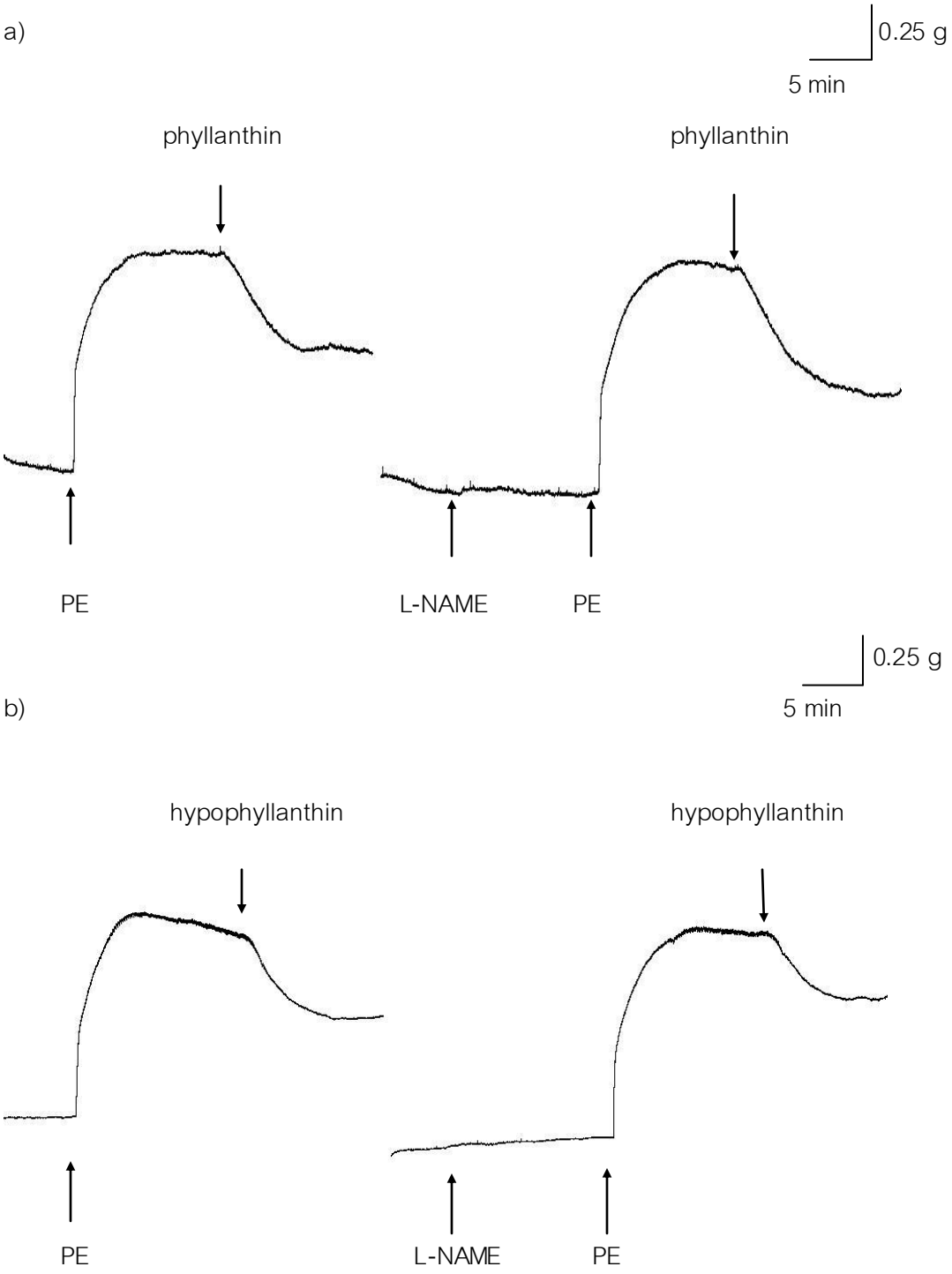


Figure 35 Representative tracing showed the relaxation induced by addition of phyllanthin (a) and hypophyllanthin (b) (100 μM) on PE- induced contraction of endothelium-denuded aortic ring in the presence of L-NAME (100 μM)

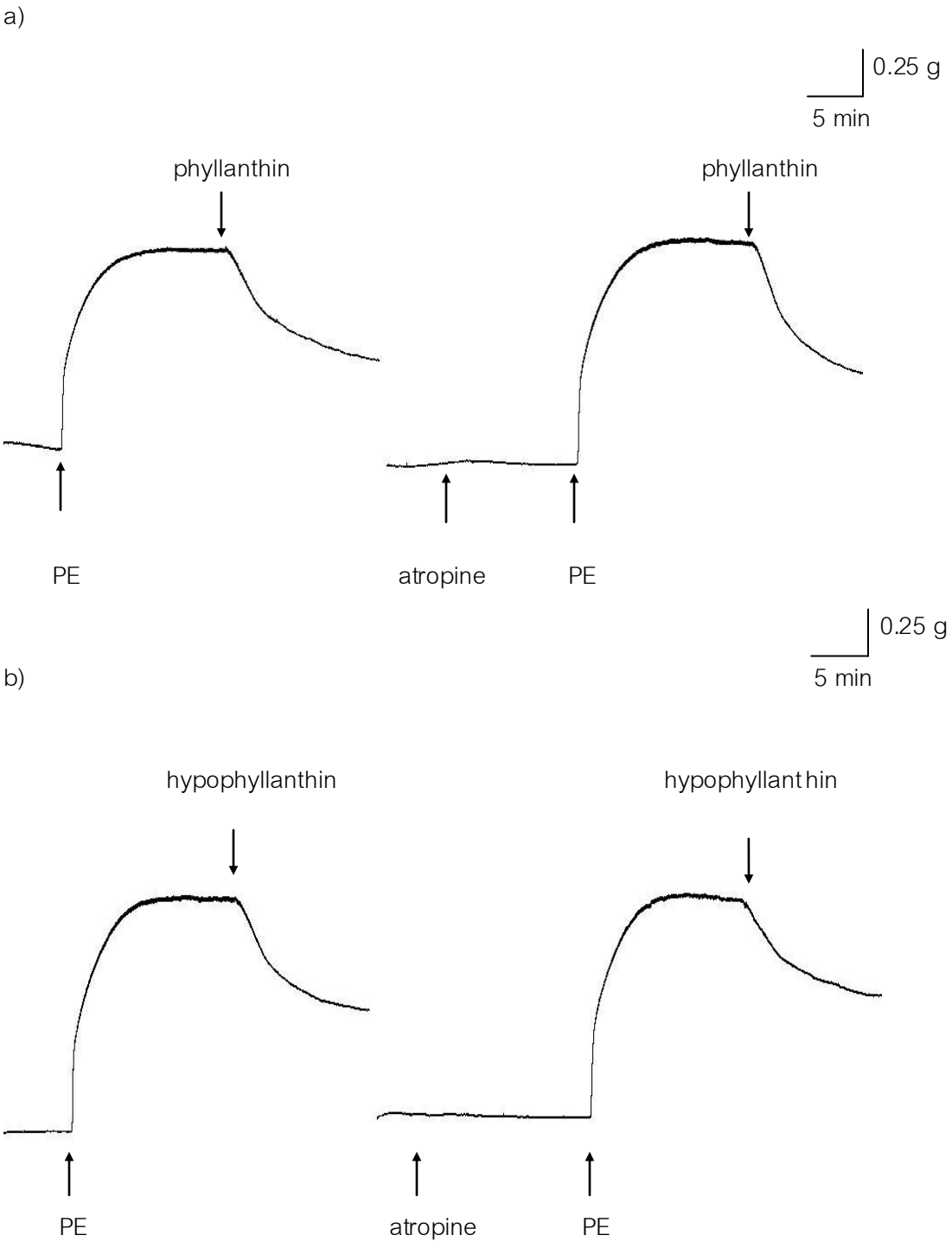


Figure36 Representative tracing showed the relaxation induced by addition of phyllanthin (a) and hypophyllanthin (b) (100 μM) on PE-induced contraction of endothelium- denuded aortic ring in the presence of atropine (1 μM).

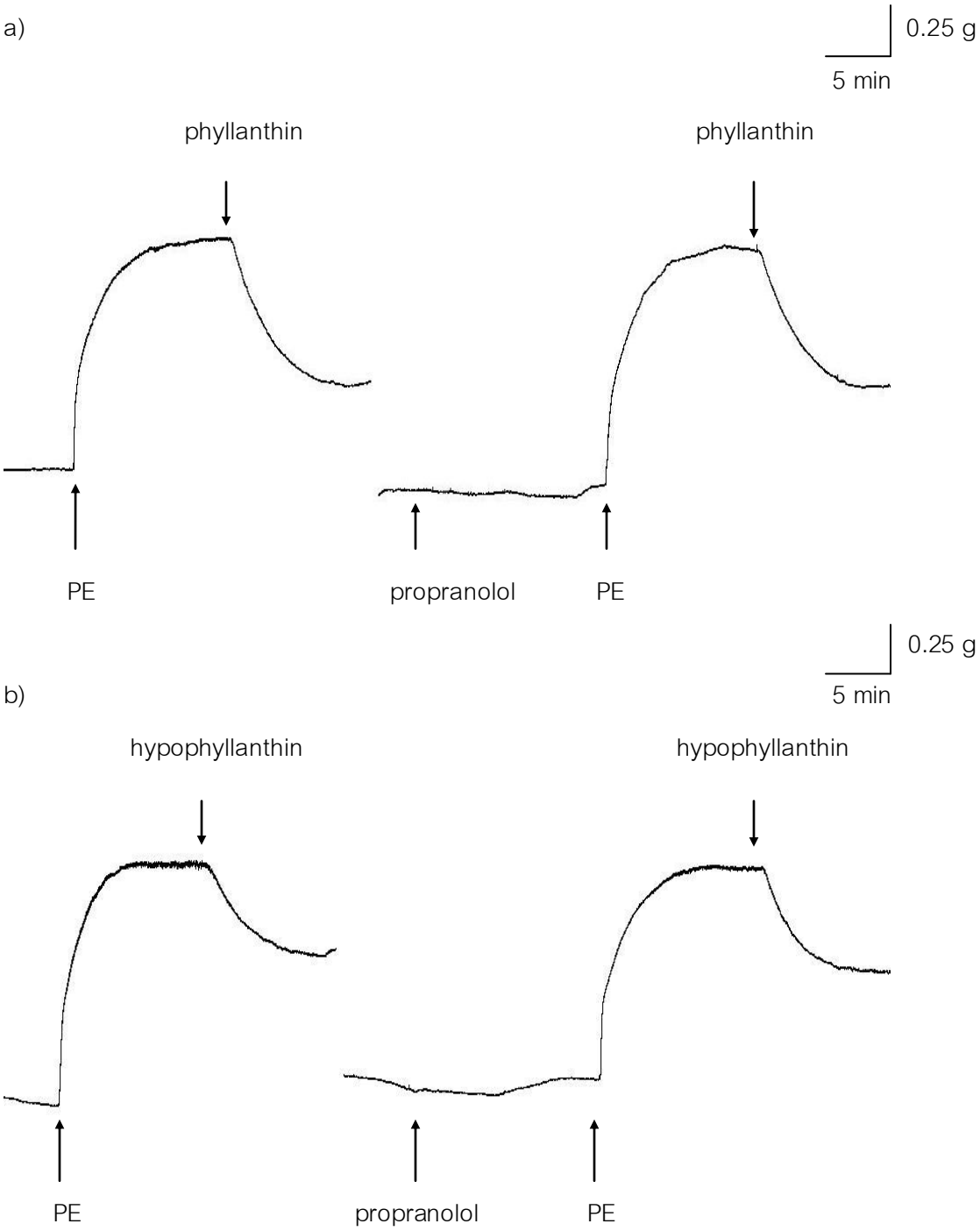


Figure 37 Representative tracing showed the relaxation induced by addition of phyllanthin (a) and hypophyllanthin (b) (100 μM) on PE-induced contraction of endothelium-denuded aortic ring in the presence of propranolol (10 μM).

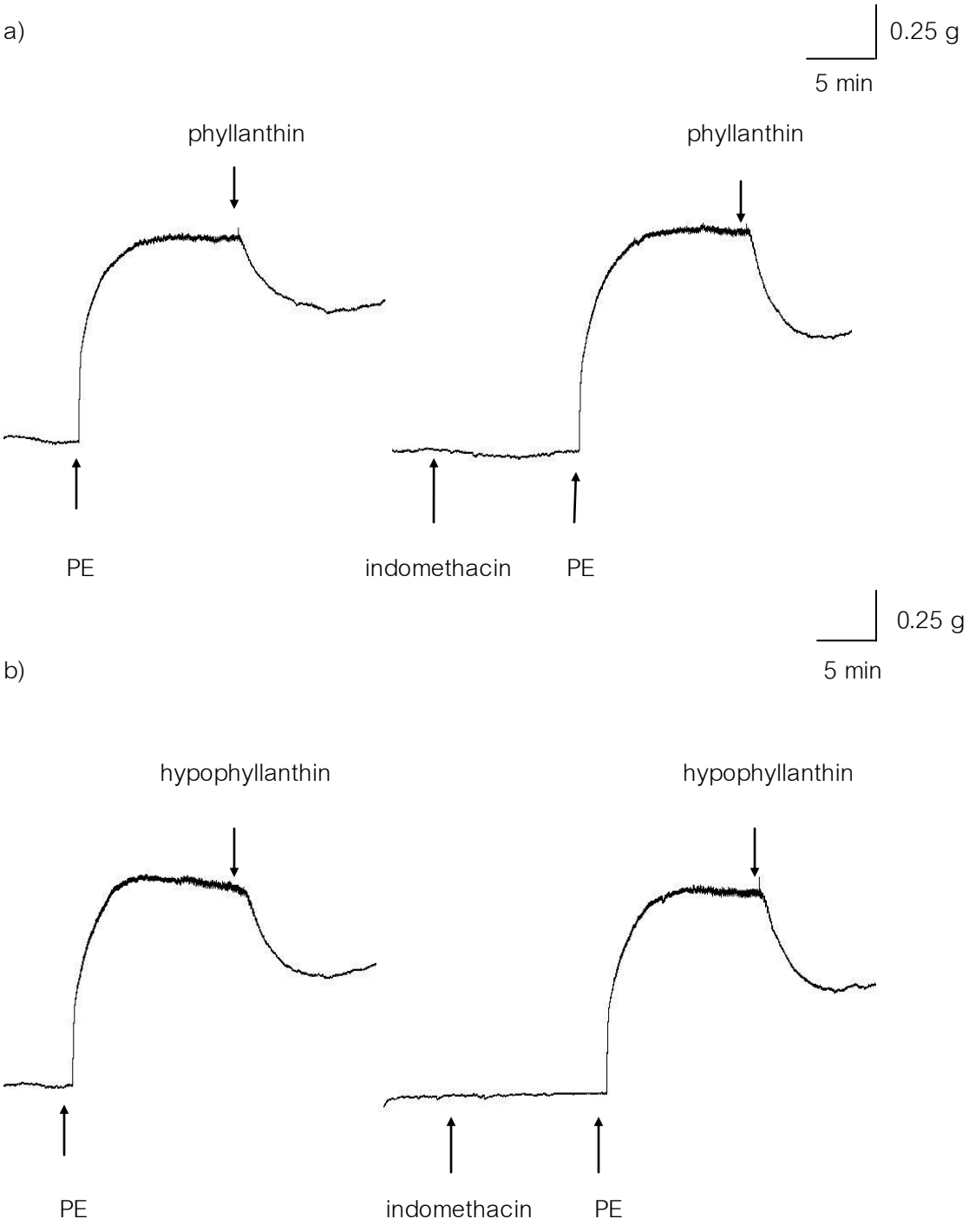


Figure 38 Representative tracing showed the relaxation induced by addition of phyllanthin (a) and hypophyllanthin (b) (100 μM) on PE-induced contraction of endothelium-denuded aortic ring in the presence of indomethacin (10 μM).

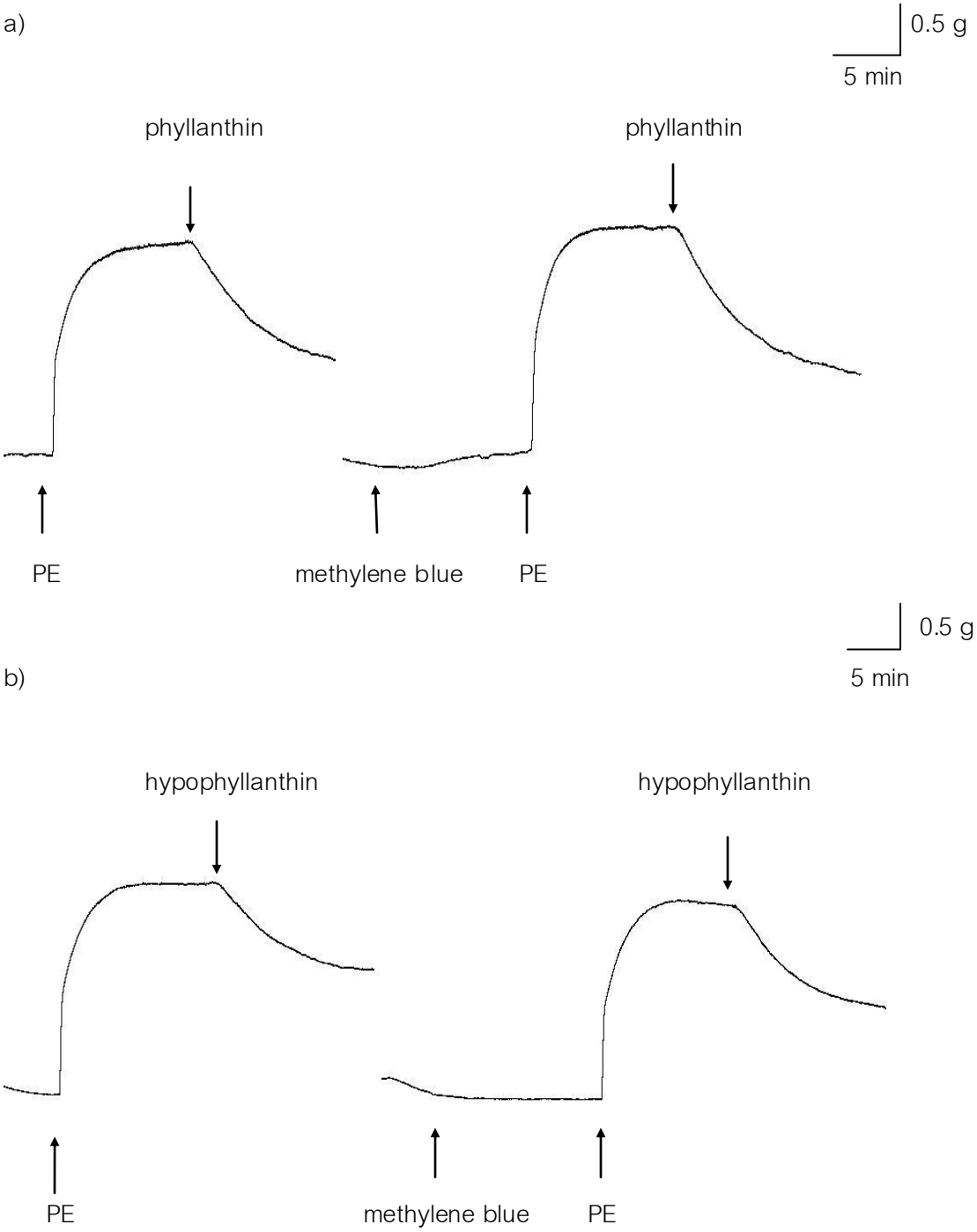


Figure 39 Representative tracing showed the relaxation induced by addition of phyllanthin (a) and hypophyllanthin (b) (100 μM) on PE-induced contraction of endothelium-denuded aortic ring in the presence of methylene blue (10 μM).

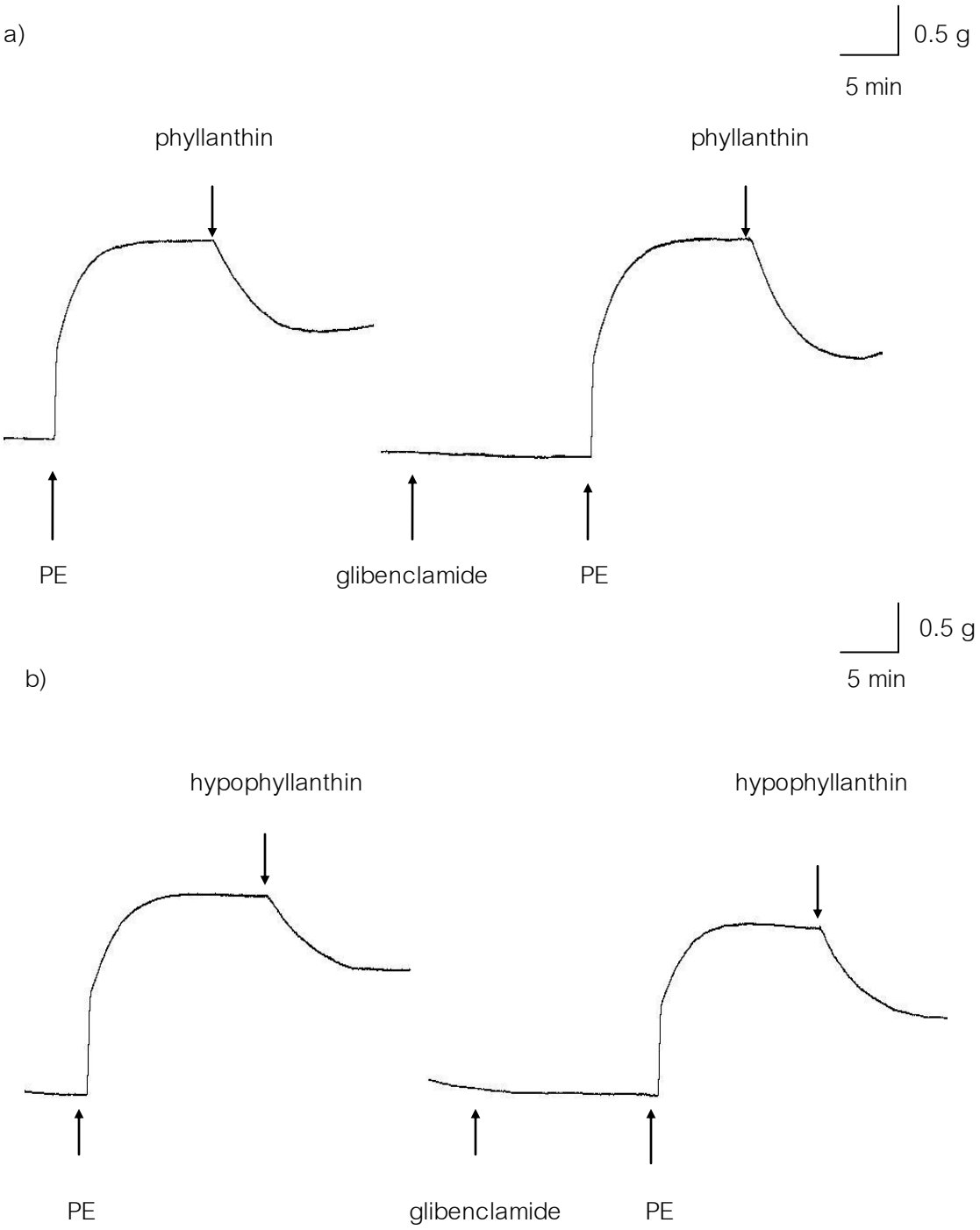


Figure 40 Representative tracing showed the relaxation induced by addition of phyllanthin (100 μM, a) and hypophyllanthin (100 μM, b) on PE-induced contraction of endothelium-denuded aortic ring in the presence of glibenclamide (10 μM).

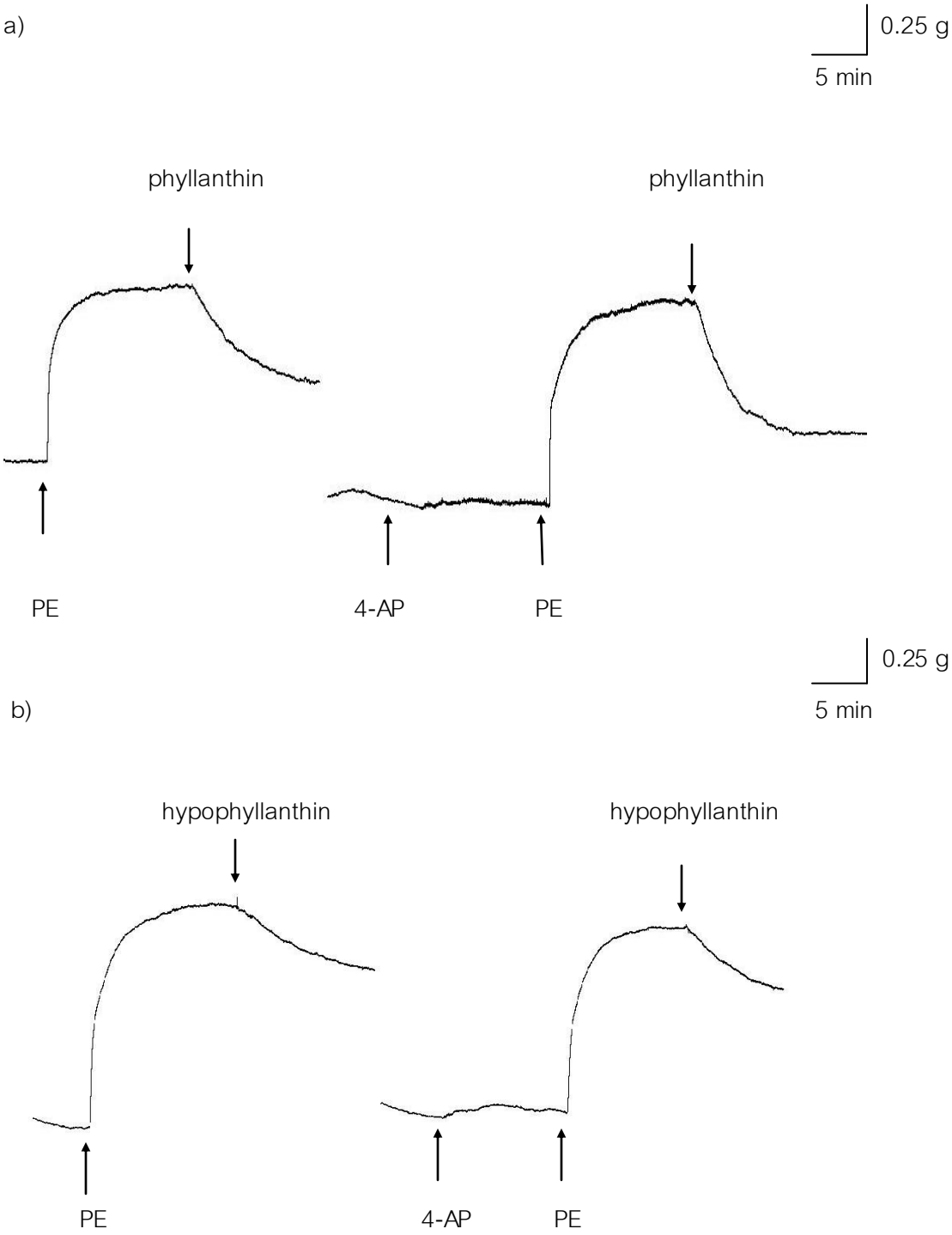


Figure 41 Representative tracing showed the relaxation induced by addition of phyllanthin (100 μM. a) and hypophyllanthin (100 μM, b) on PE-induced contraction of endothelium-denuded aortic ring in the presence of 4-AP (100 μM).

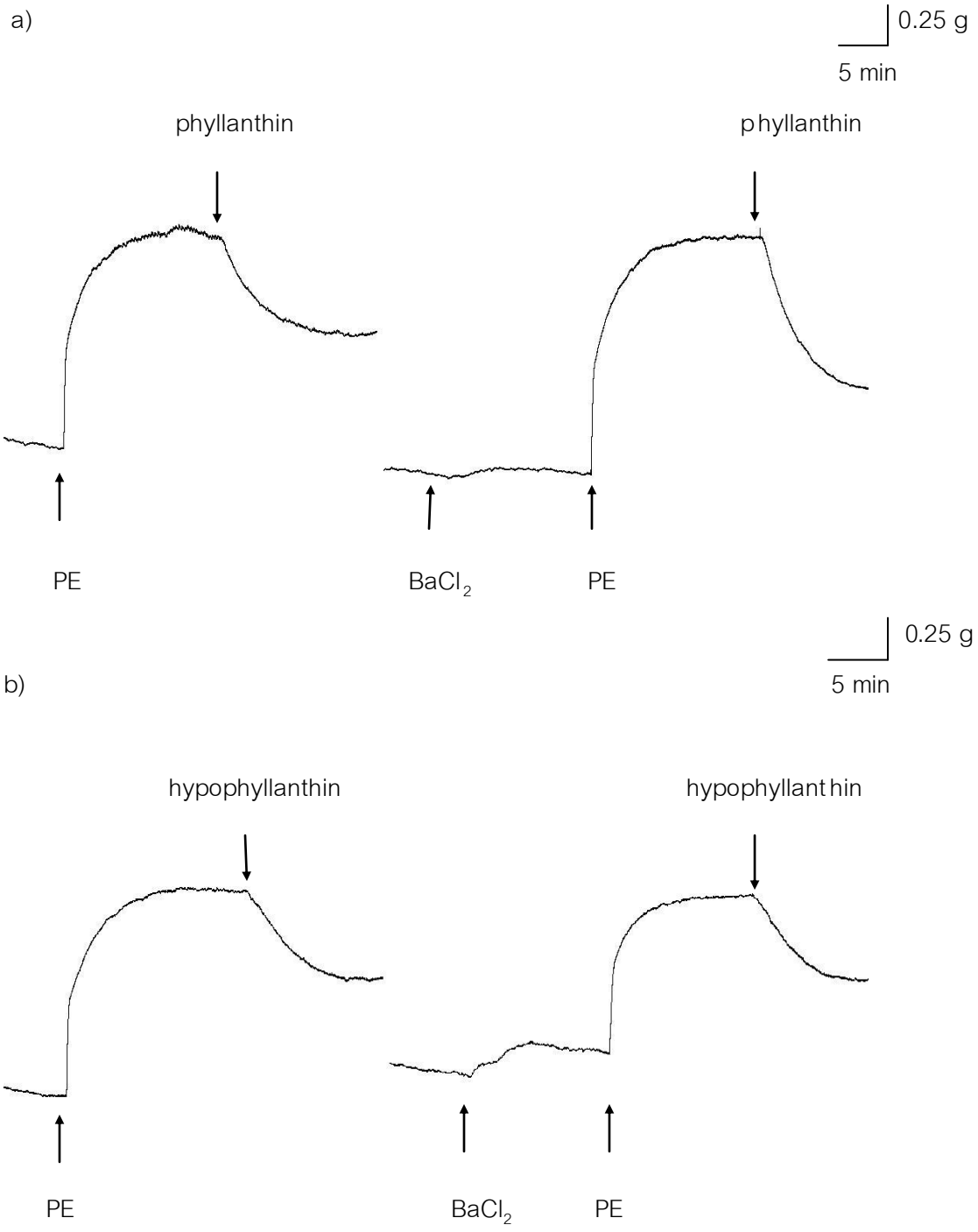


Figure 42 Representative tracing showed the relaxation induced by addition of phyllanthin (100 μM, a) and hypophyllanthin (100 μM, b) on PE-induced contraction of endothelium-denuded aortic ring in the presence of $BaCl_2$ (10 μM).

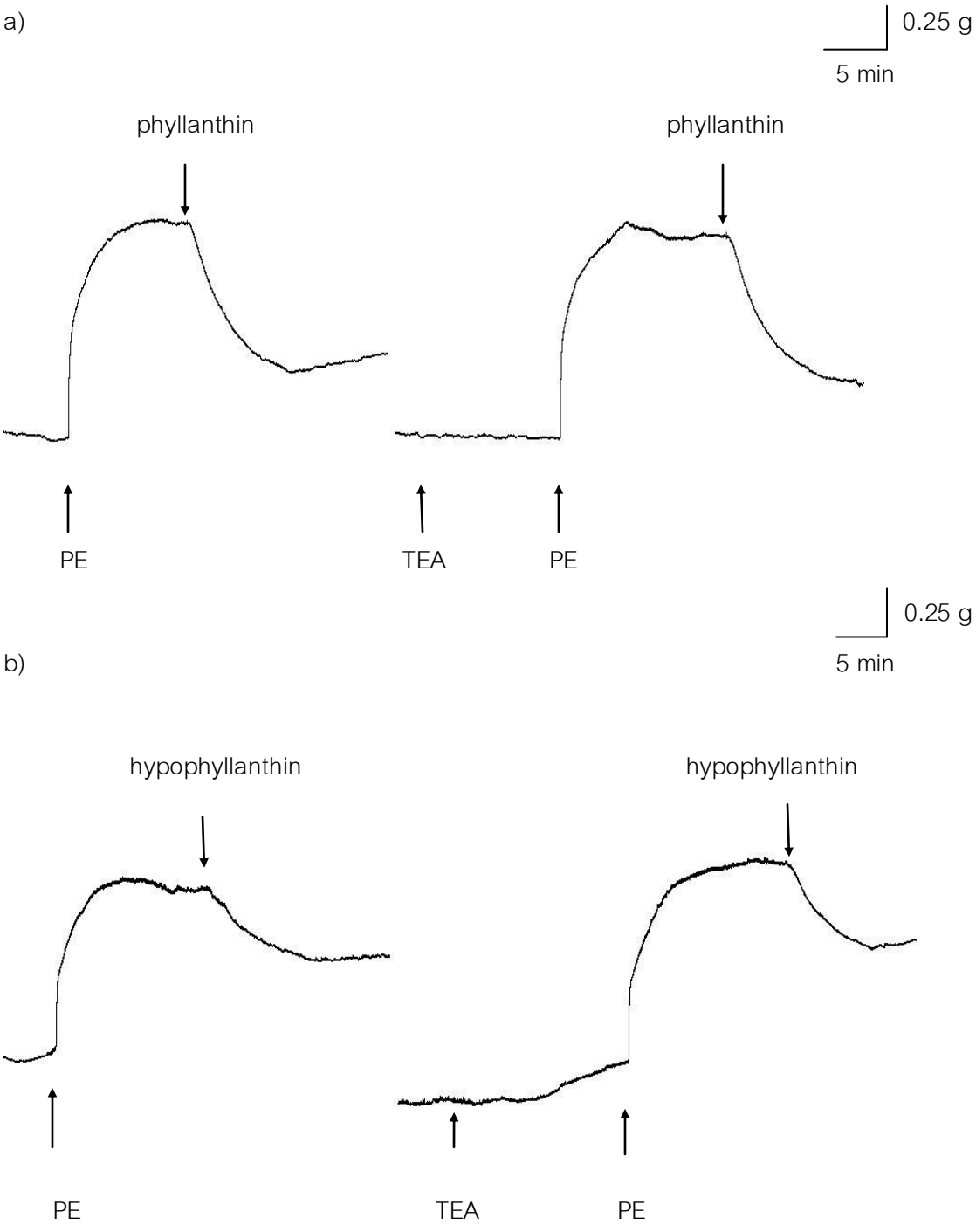


Figure 43 Representative tracing showed the relaxation induced by addition of phyllanthin (100 μM, a) and hypophyllanthin (100 μM, b) on PE-induced contraction of endothelium-denuded aortic ring in the presence of TEA (10 μM).

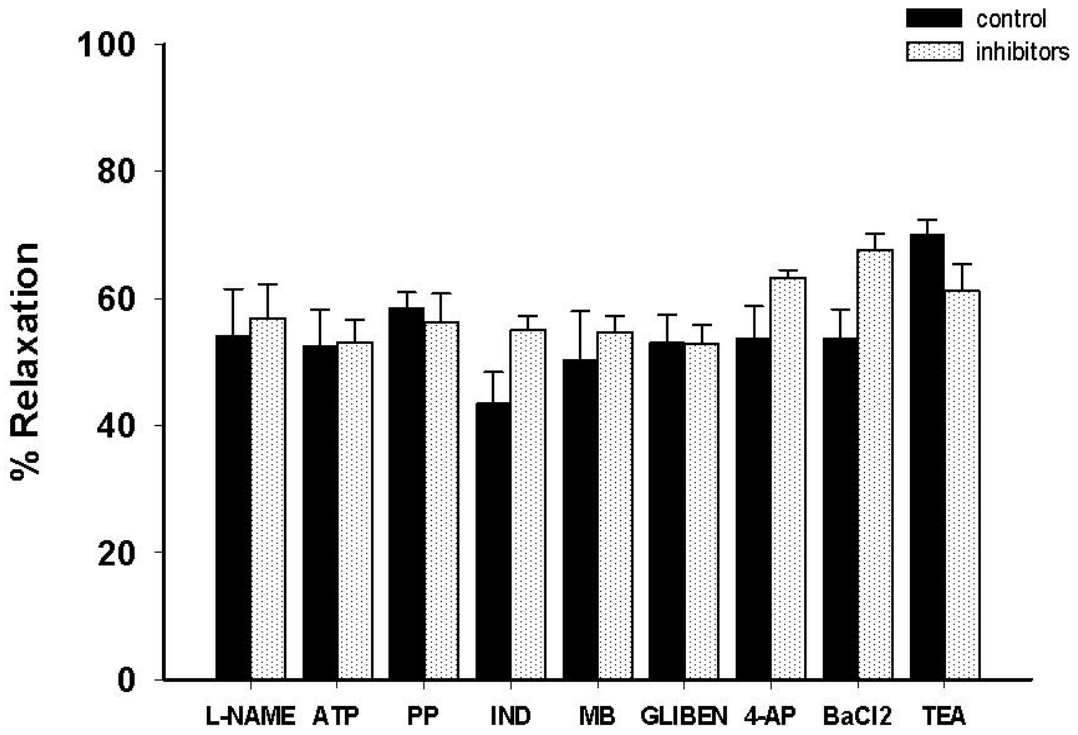


Figure 44 The effects of vasodilator inhibitors: ATP (atropine), PP (propranolol), IND (indomethacin), MB (methylene blue), GLIBEN (glibenclamide), 4-AP (4-aminipyridine), $BaCl_2$ (barium chloride), TEA (tetraethylammonium) on phyllanthin (100 μM) induced relaxation of endothelium-denuded aortic rings precontracted with PE (1 μM).

Data were presented as mean ± S.E.M., n = 4-6

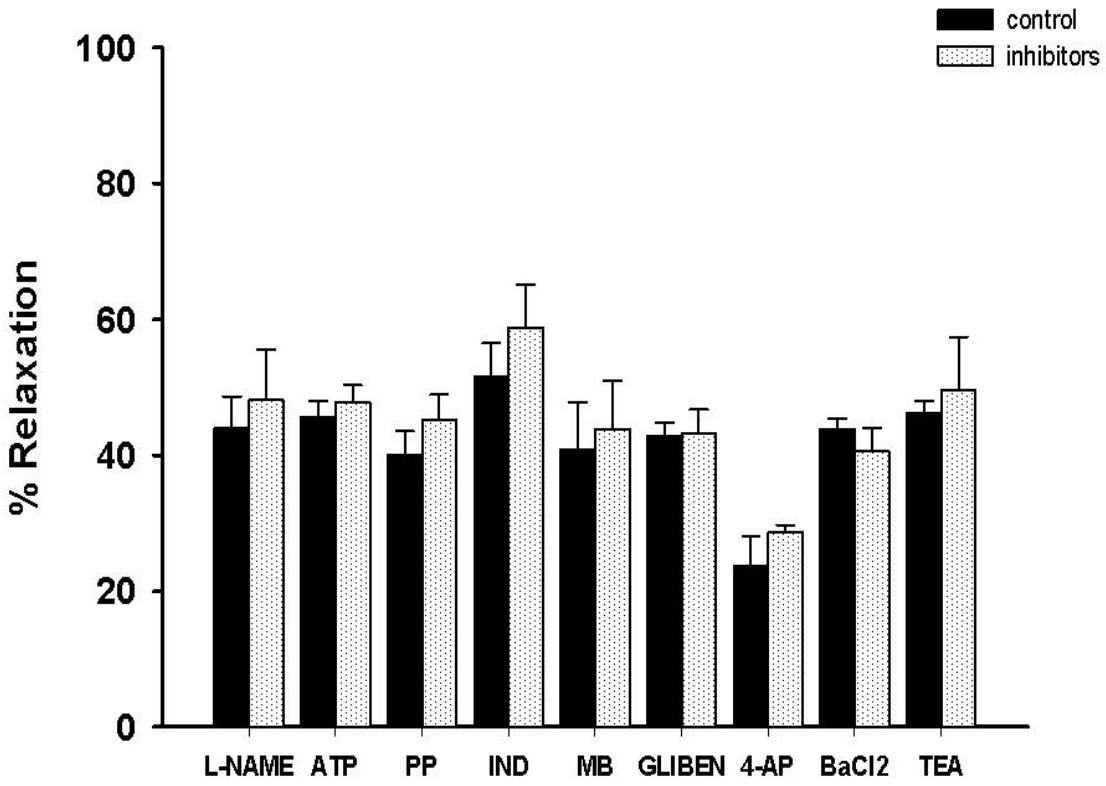


Figure 45 The effects of vasodilator inhibitors: ATP (atropine), PP (propranolol), IND (indomethacin), MB (methylene blue), GLIBEN (glibenclamide), 4-AP (4-aminipyridine), $BaCl_2$ (barium chloride), TEA (tetraethylammonium) on hypophyllanthin (100 μM) induced relaxation of endothelium-denuded aortic rings precontracted with PE (1 μM).

Data were presented as mean ± S.E.M., n = 4-6

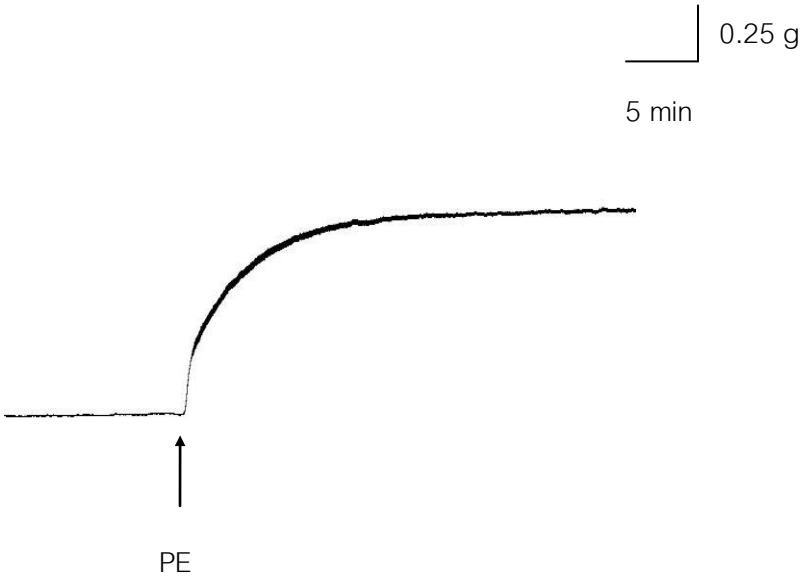


Figure 46 Representative tracing showed the PE-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

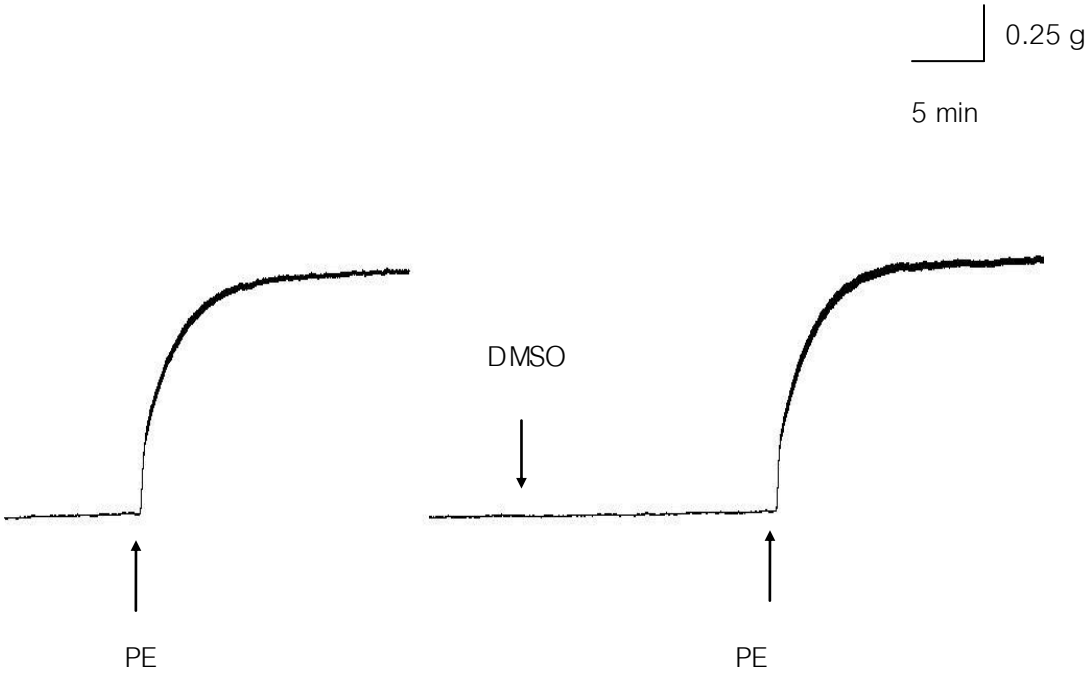


Figure 47 Representative tracing showed the effect of DMSO 0.2% (v/v) on PE-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

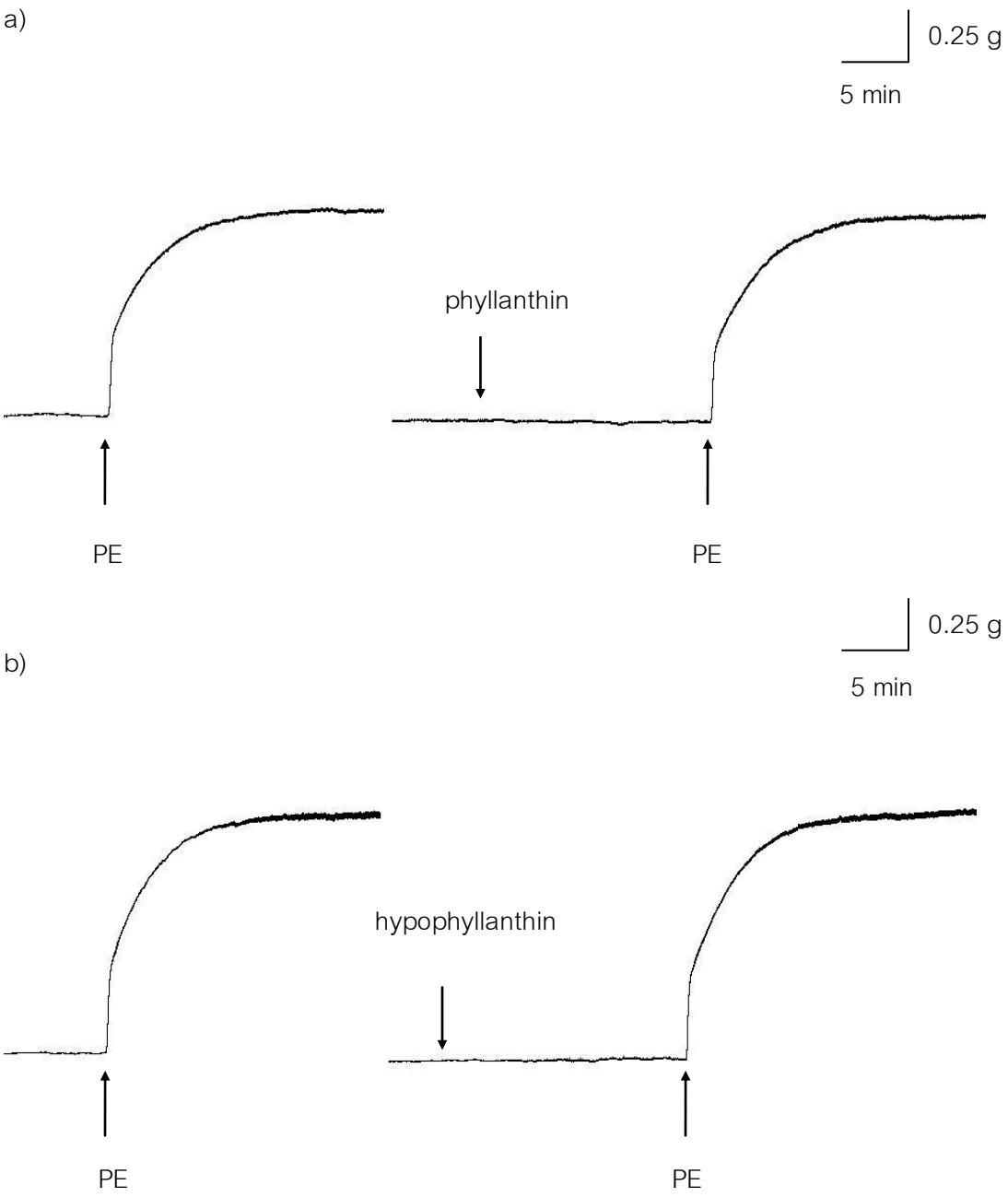


Figure 48 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (1 μM) on PE-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

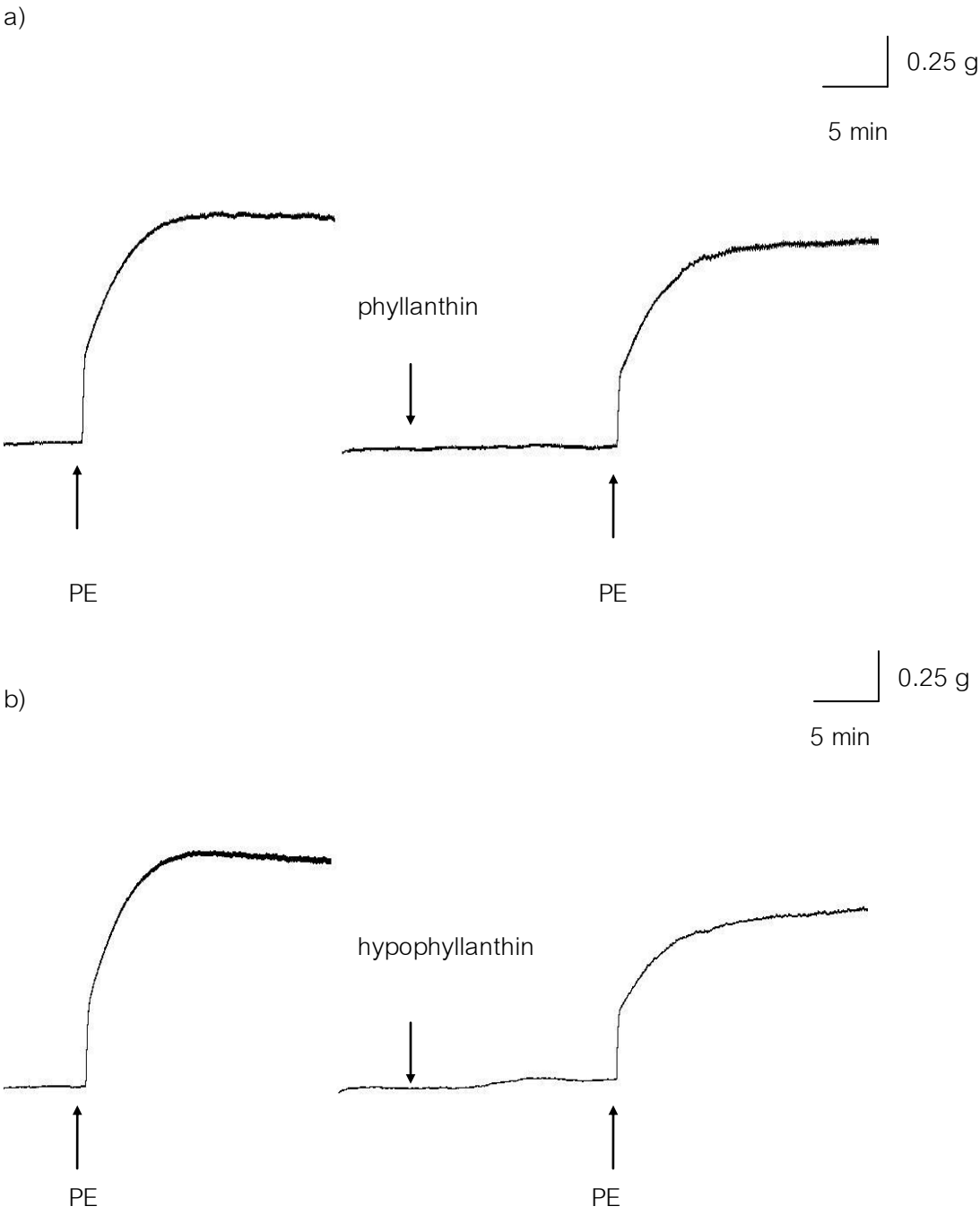


Figure 49 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (10 μM) on PE-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

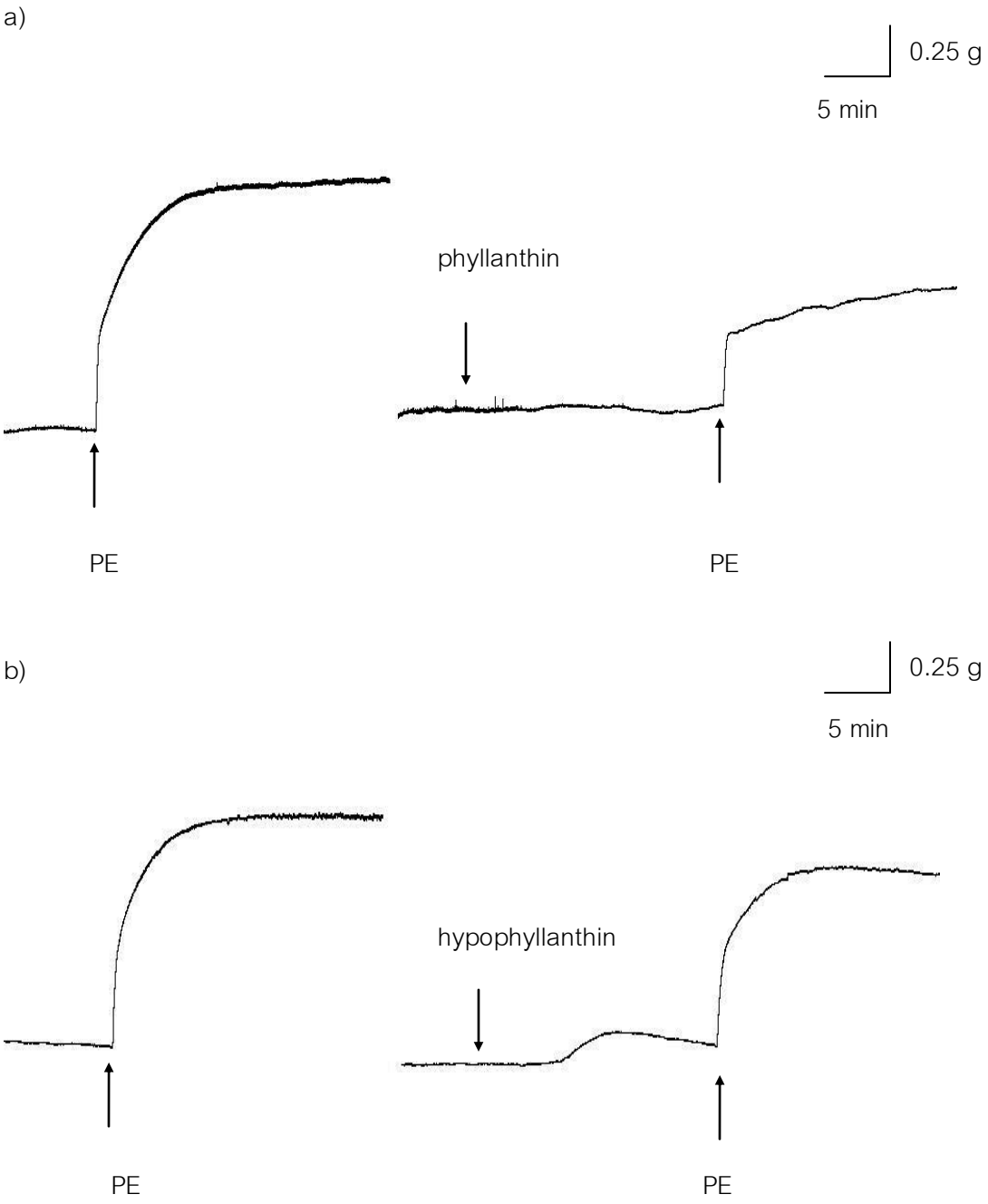


Figure 50 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (50 μM) on PE-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

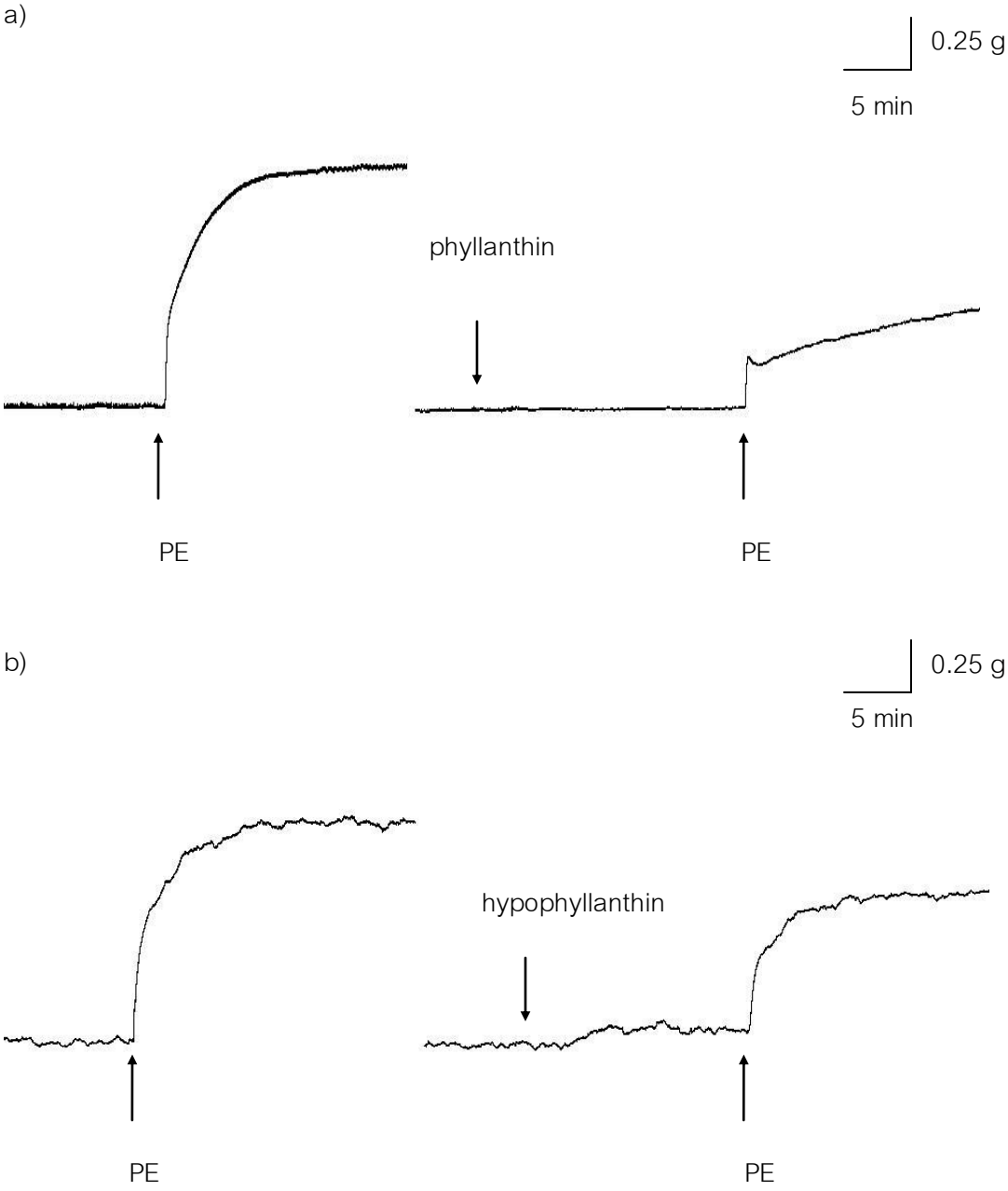


Figure 51 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (100 μM) on PE-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine .

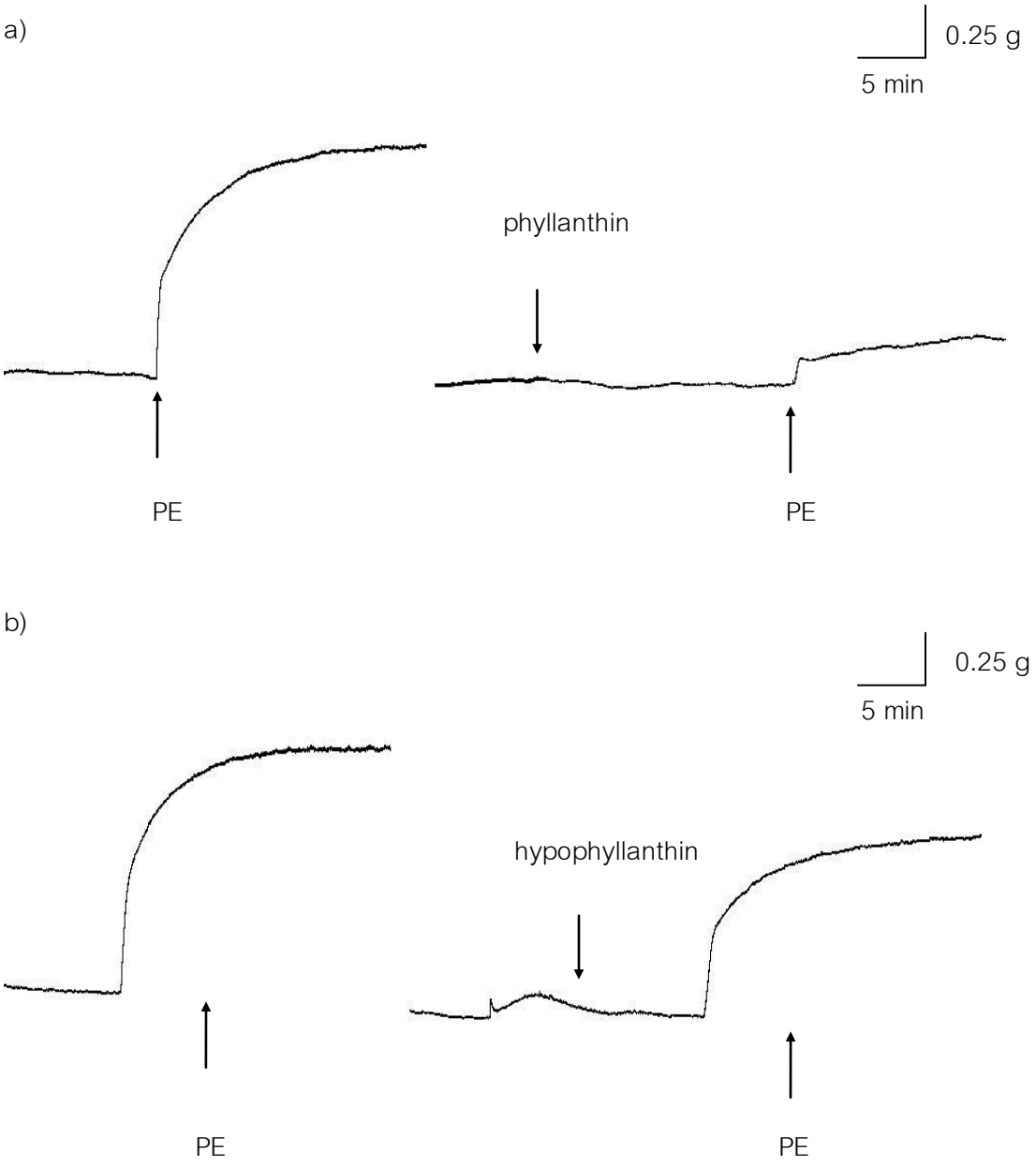


Figure 52 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (200 μM) on PE-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

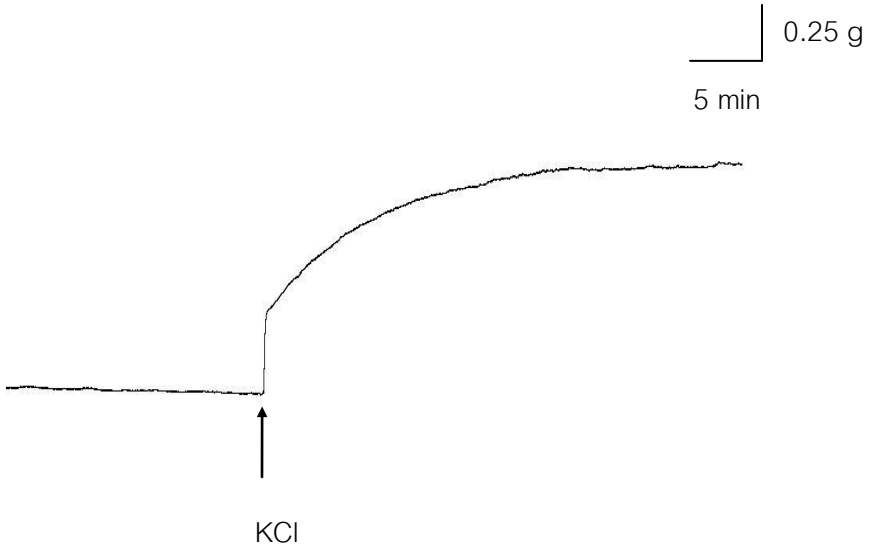


Figure 53 Representative tracing showed the KCl-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

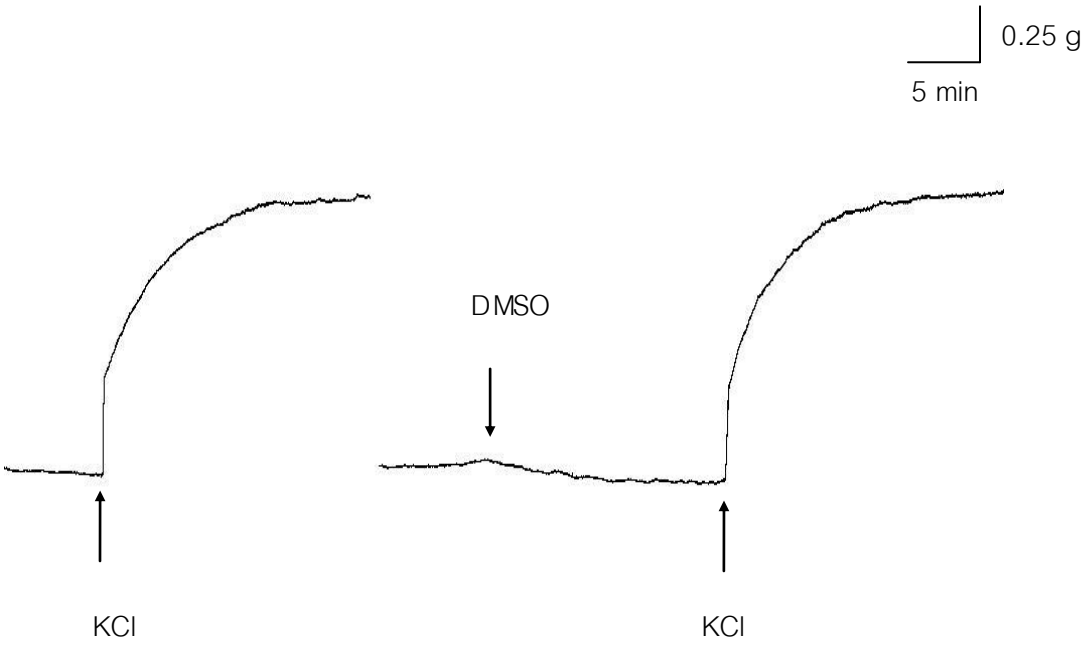


Figure 54 Representative tracing showed the effect of DMSO 0.2% (v/v) on KCl-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

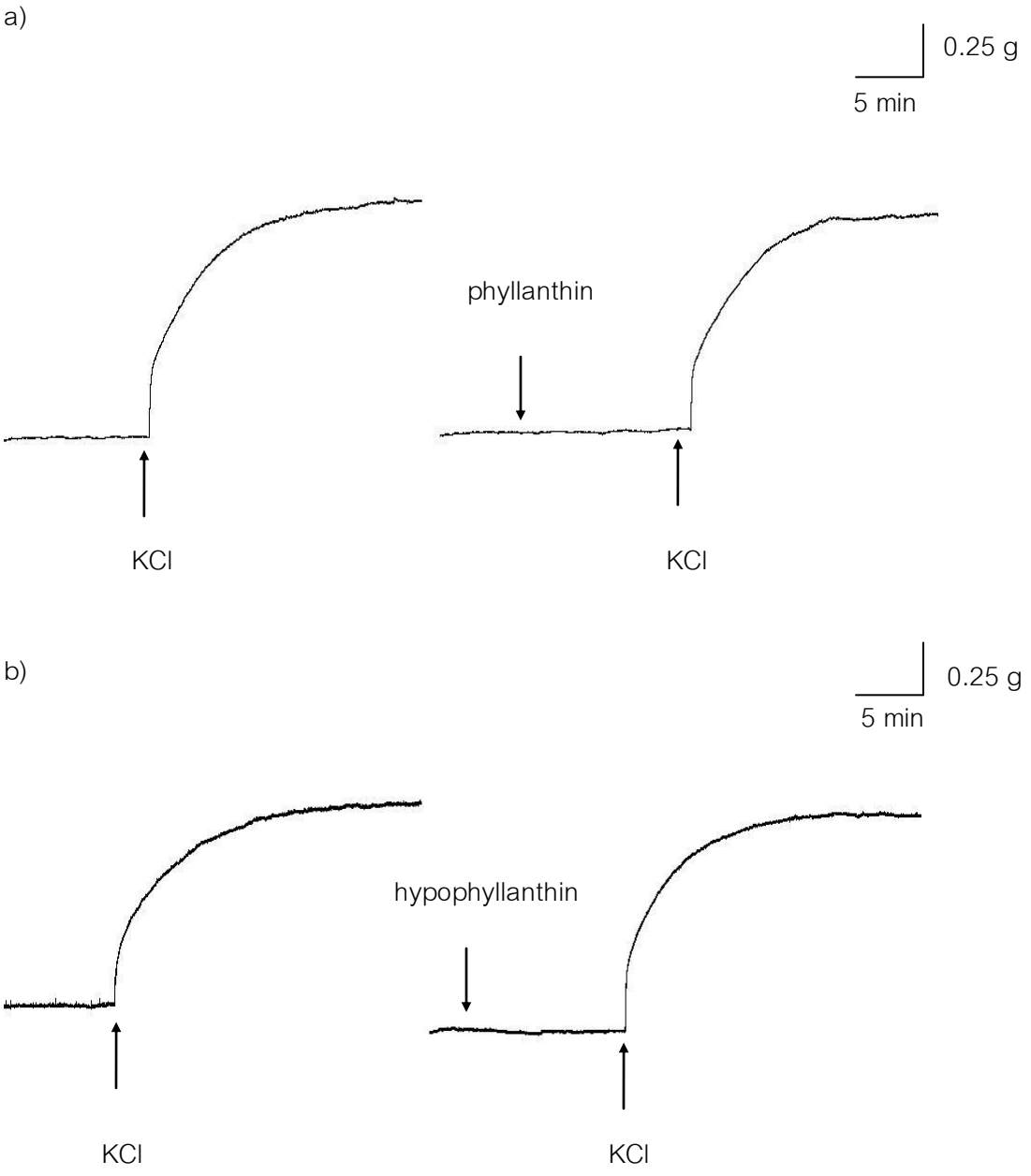


Figure 55 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (1 μM) on KCl-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

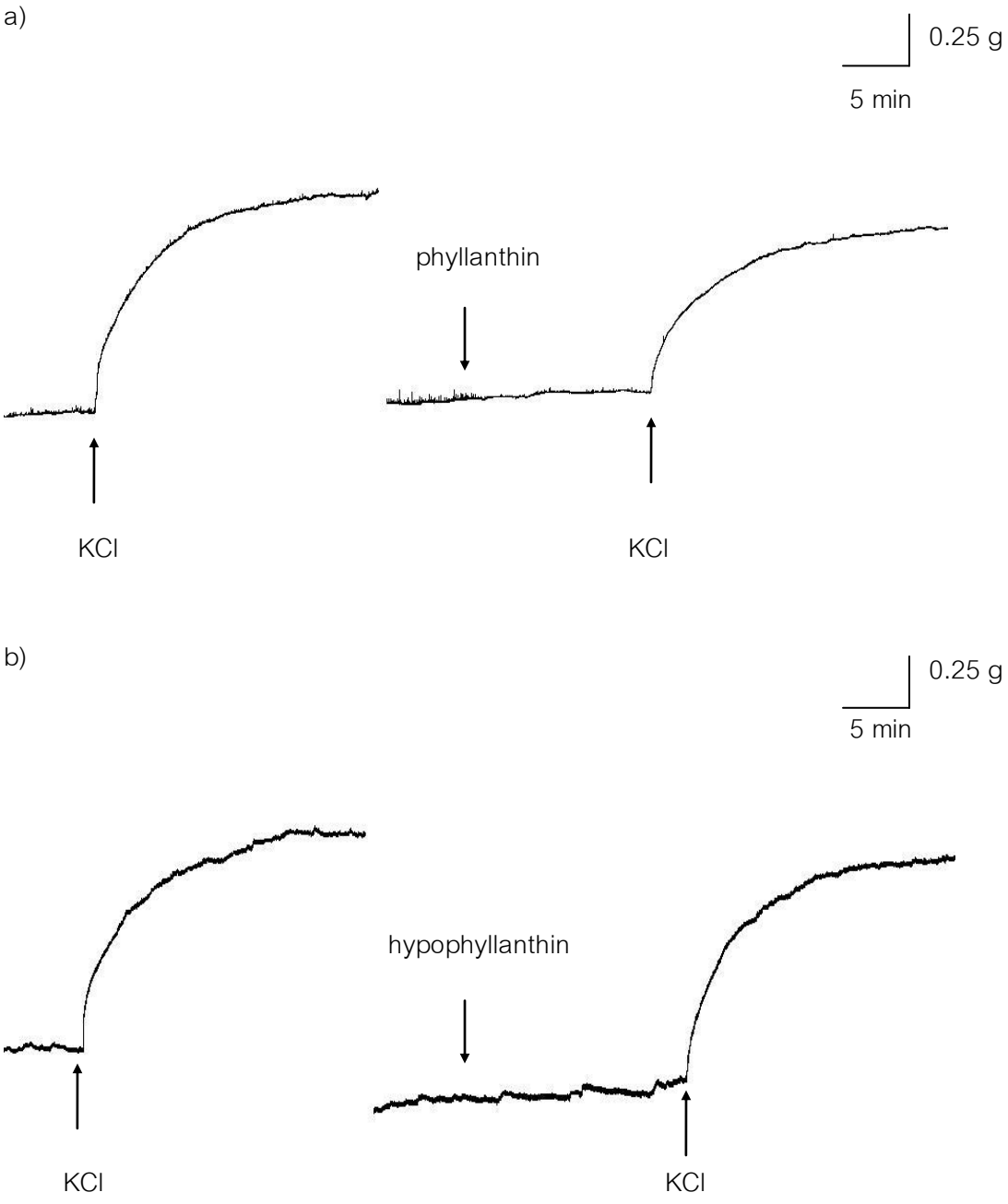


Figure 56 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (10 μM) on KCl-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

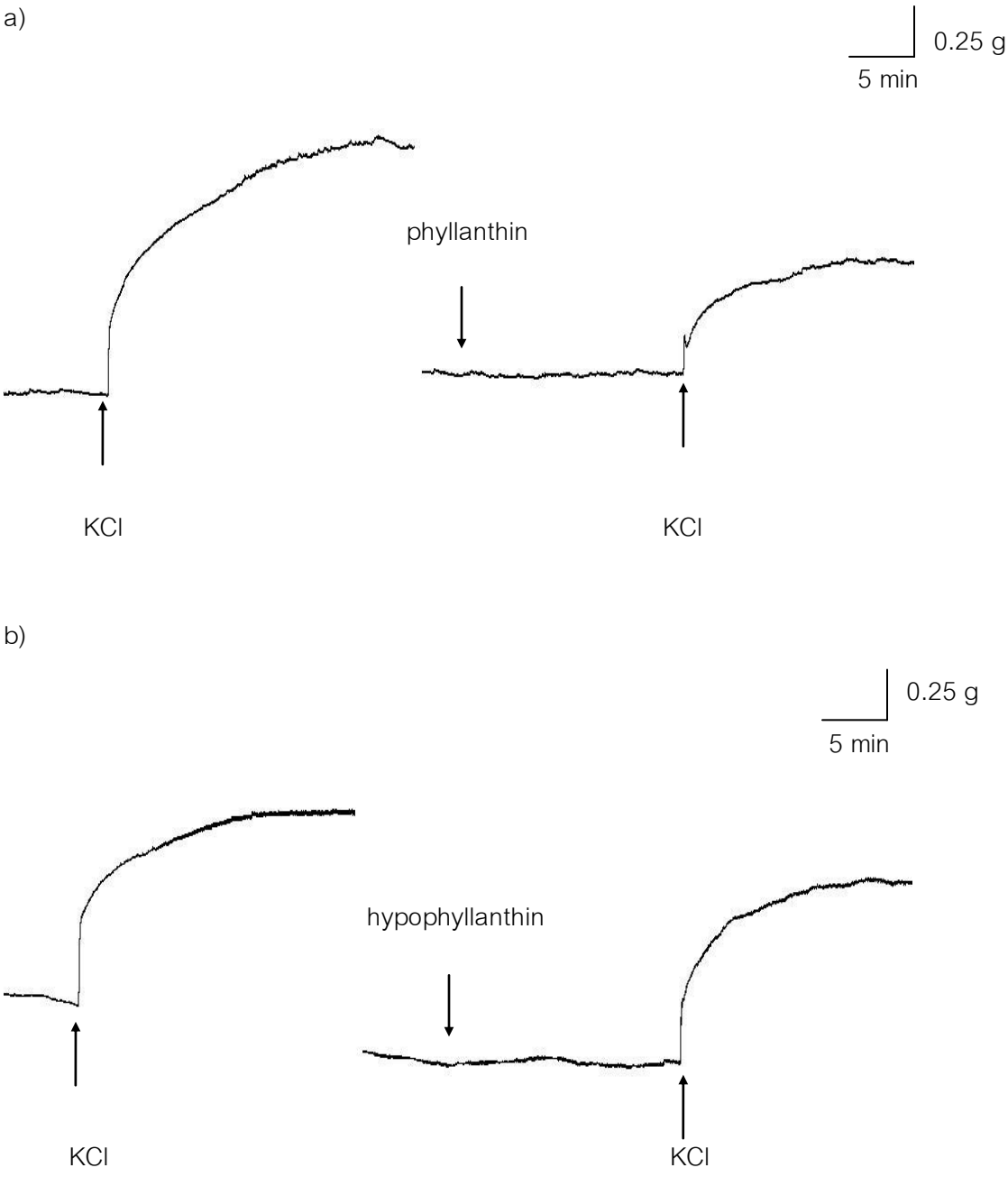


Figure 57 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (50 μM) on KCl-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

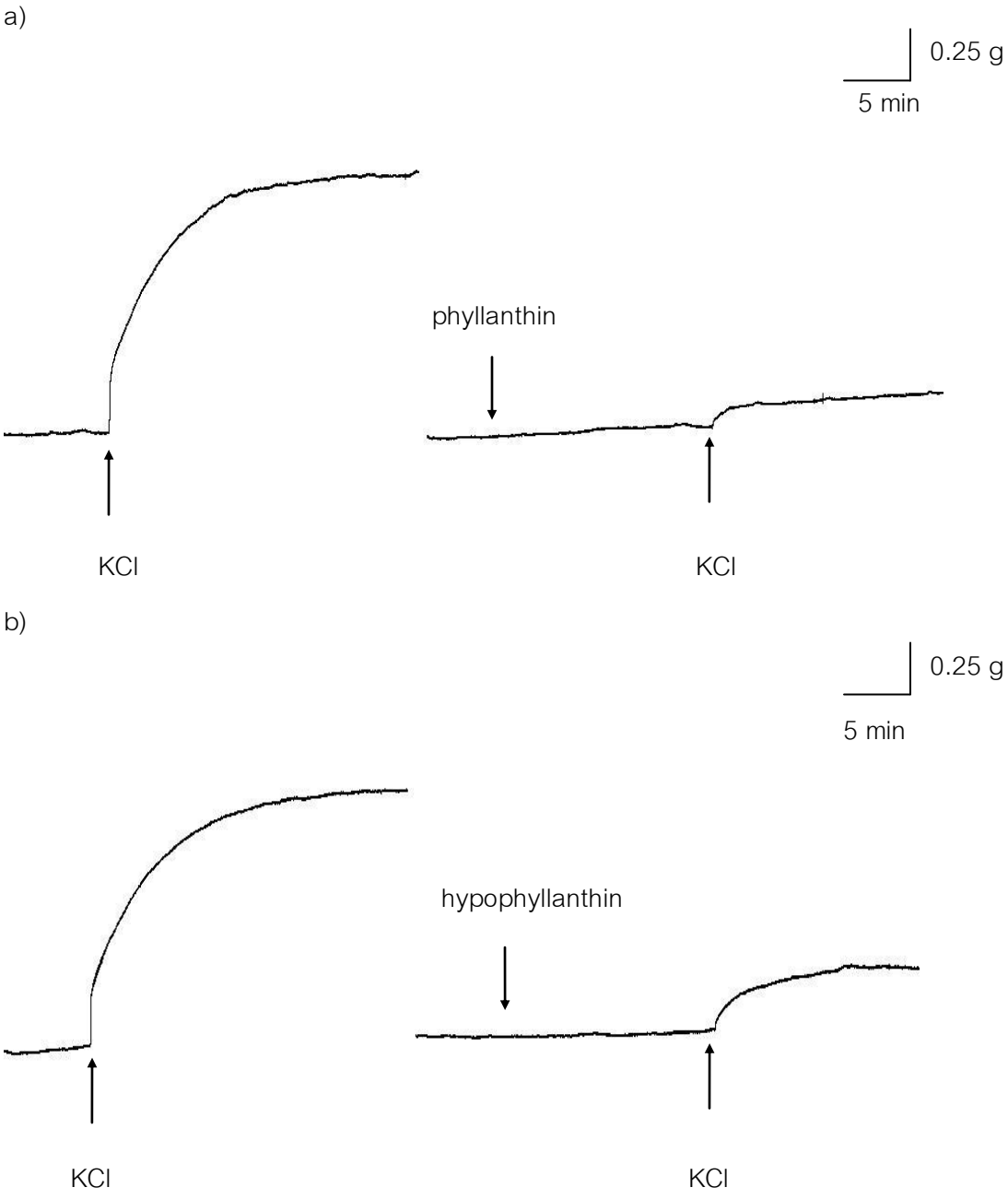


Figure 58 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (100 μM) on KCl-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

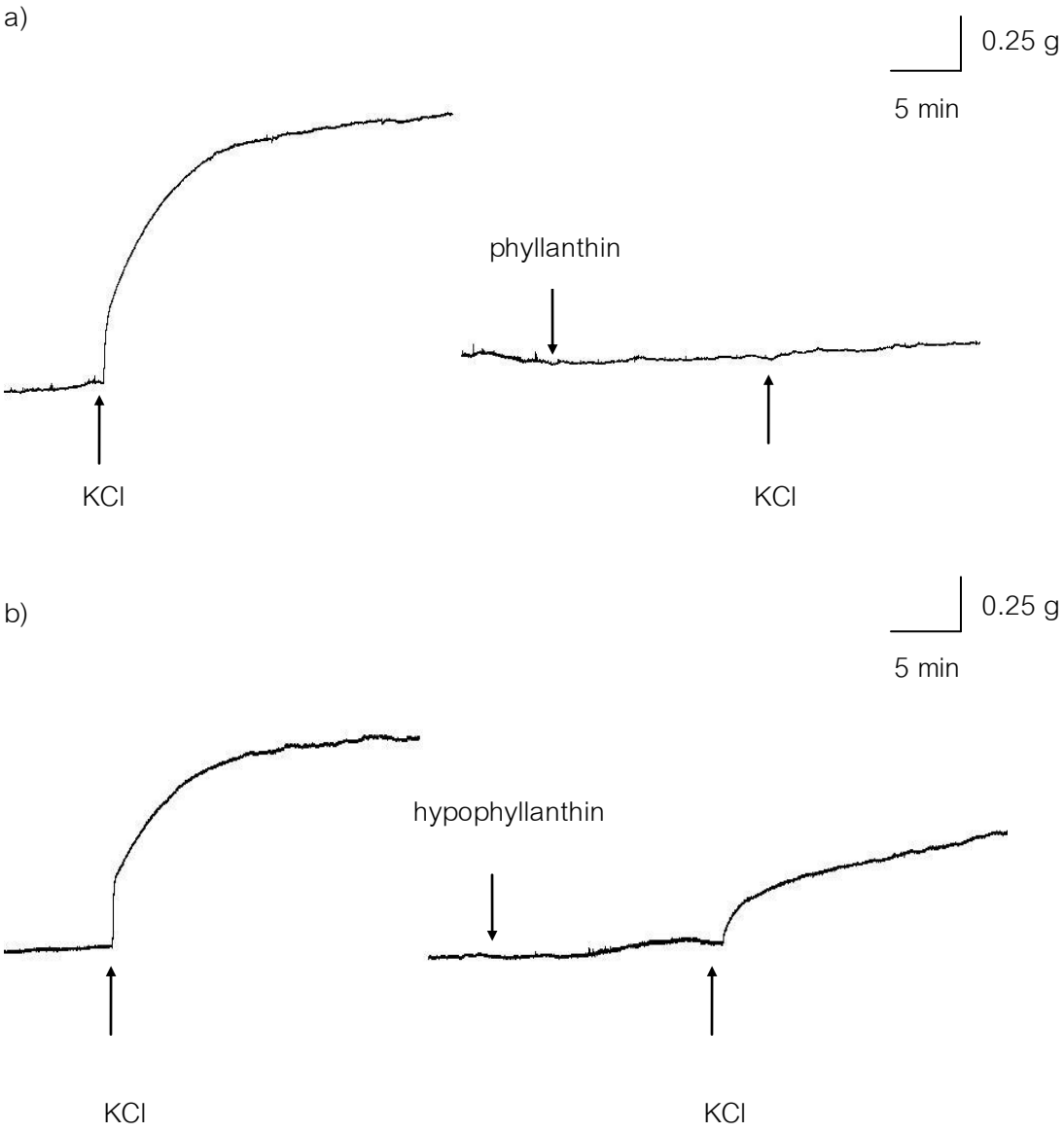


Figure 59 Representative tracing showed the effects of phyllanthin (a) and hypophyllanthin (b) (200 μM) on KCl-induced contraction of endothelium-denuded aortic rings in KHS containing tyramine.

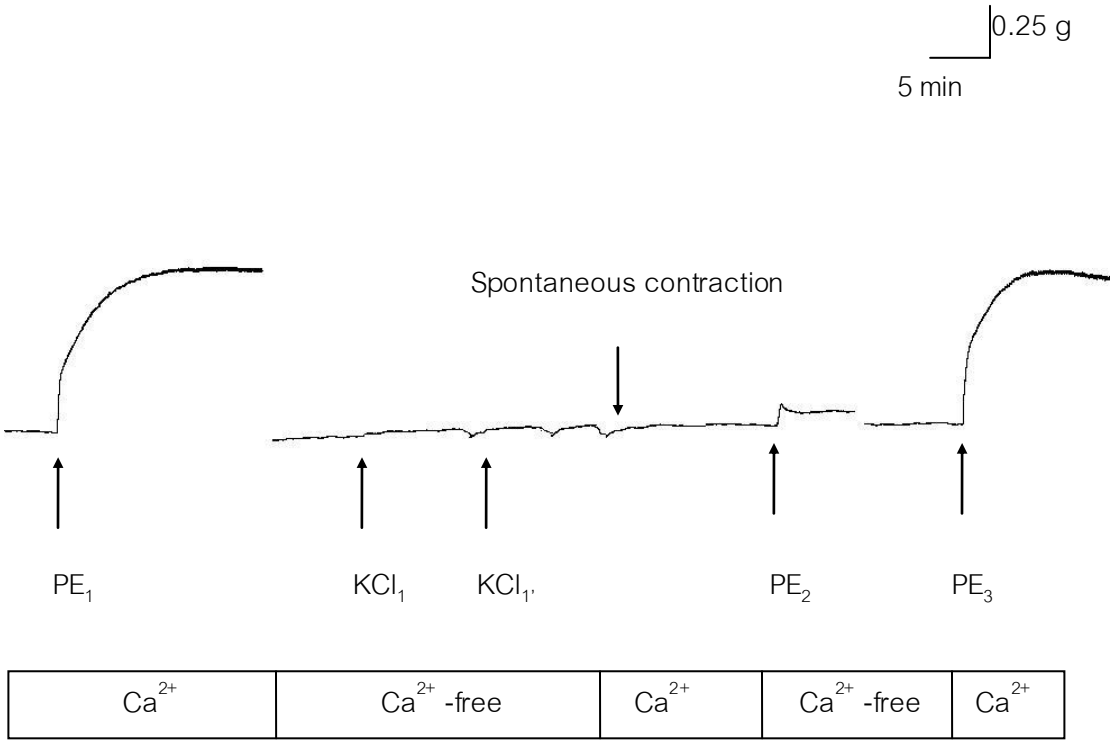


Figure 60 Representative tracing showed the effects of KCl (40 mM) on spontaneous contraction in Ca^{2+}- containing KHS.

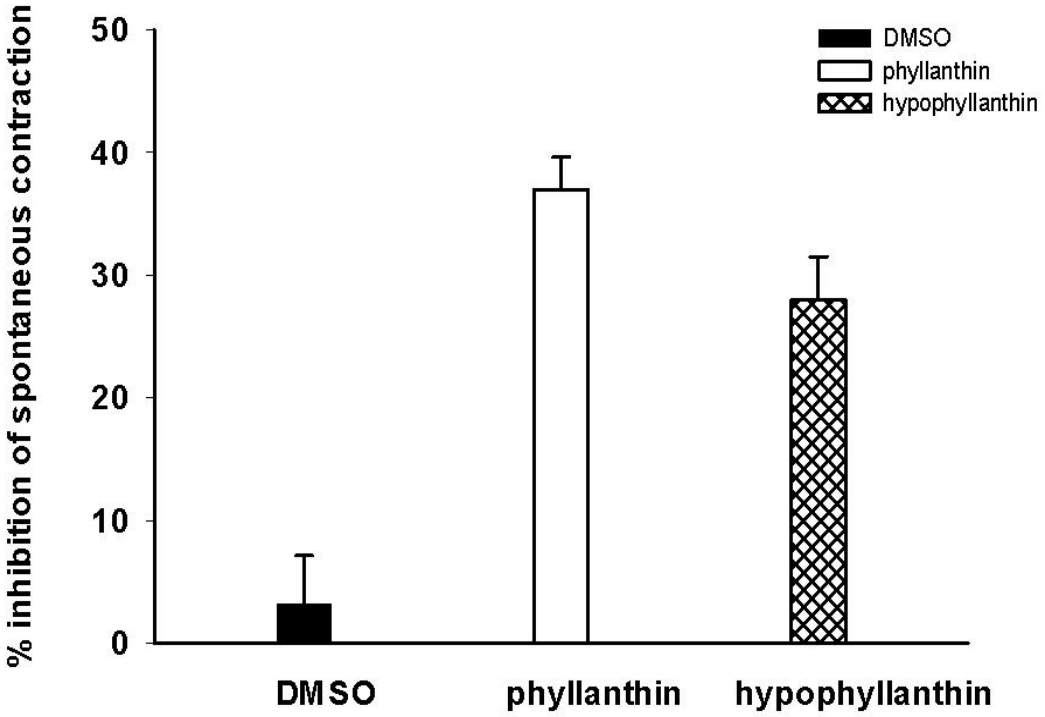


Figure 61 The effects of phyllanthin and hypophyllanthin on spontaneous contraction in Ca^{2+}- containing KHS.

Data were present as mean ± S.E.M., n = 5-6

*p<0.05 showed significant difference from DMSO control group.

Teble1 Compound of Physiological solution (mM)

Chemical	Physiological solution		
	Kreb Henseleit	Ca^{2+}-free Solution	High K^{+}- Ca^{2+}- free Depolarizing Solution
NaCl	119	119	27
KCl	4.7	4.7	100
$CaCl_2$	2.5	-	-
$MgSO_4$	1.0	1.0	-
$NaHCO_3$	25	25	14
KH_2PO_4	1.2	1.2	14
Glucose	11.1	11.1	10
EDTA	-	0.2	-
$MgCl_2$	-	-	0.54

Table 2 Vasorelaxation effects of the test compounds in endothelium-intact and endothelium-denuded aortic rings.

Test compounds	Concentration (μM)	% Relaxation	
		Endothelium-intact	Endothelium-denuded
Phyllanthin	1	3.28 ± 1.63	3.57 ± 1.53
	10	16.53 ± 2.42	13.73 ± 2.54
	50	46.67 ± 5.24*	47.08 ± 7.82*
	100	82.88 ± 1.73*,#	81.51 ± 6.83*,#
Hypophyllanthin	1	6.55 ± 1.47	4.58 ± 2.04
	10	13.78 ± 2.32	12.06 ± 2.70
	50	35.87 ± 2.61*	32.43 ± 3.57*
	100	60.25 ± 3.01*,#	59.03 ± 3.90*,#
DMSO	0.2% (v/v)	6.20 ± 1.32	3.64 ± 0.64
Positive control (Ach)	10	61.85 ± 3.64	3.00 ± 0.73

Data were present as mean ± S.E.M., n = 6

*p<0.05 showed significant difference from DMSO control group.

#p<0.05 showed significant difference between phyllanthin and hypophyllanthin.

Table 3 The apparent EC_{50} of the test compounds on vasorelaxation

Test compounds	EC_{50} values (μM)	
	Endothelium-intact	Endothelium-denuded
Phyllanthin	56.55 ± 2.91	55.40 ± 5.5
Hypophyllanthin	80.08 ± 5.05	84.02 ± 5.93

Data were presented as mean ± S.E.M., n=6

Table 4 Effects of inhibitors on vasorelaxation induced by the test compounds in endothelium-denuded aortic rings.

Vasodilation inhibitors	% Relaxation			
	Phyllanthin		Hypophyllanthin	
	control	inhibitor	control	inhibitor
L-NAME (100 μM)	54.19 ± 7.29	56.87 ± 5.39	43.98 ± 4.69	48.14 ± 7.48
Atropine (10 μM)	52.54 ± 5.67	53.09 ± 3.58	45.60 ± 2.47	47.83 ± 2.46
Propranolol (10 μM)	58.51 ± 2.40	56.33 ± 4.39	39.92 ± 3.62	45.21 ± 3.72
Indomethacin (10 μM)	43.48 ± 4.89	54.99 ± 2.25	51.51 ± 5.01	58.78 ± 6.37
Methylene blue (10 μM)	50.27 ± 7.71	54.70 ± 2.47	40.80 ± 7.05	43.86 ± 7.17
Glibenclamide (10 μM)	53.06 ± 4.33	52.77 ± 3.04	42.79 ± 2.10	43.28 ± 3.42
4-AP (10 μM)	53.75 ± 4.99	63.29 ± 1.17	23.66 ± 4.43	28.56 ± 1.20
$BaCl_2$ (100 μM)	53.81 ± 4.49	67.62 ± 2.53	43.87 ±1.59	40.60 ± 3.44
TEA (100 μM)	70.05 ± 2.39	61.22 ± 4.16	46.17 ± 1.80	49.66 ± 7.74

Data were present as mean ± S.E.M., n = 4-6

Table 5 Effect of the test compounds on vascular contraction induced by PE (1 μM) in endothelium-denuded aortic rings.

Test compounds	Concentration (μM)	% Contraction
		PE 10 μM
Phyllanthin	1	100.38 ± 2.51
	10	90.22 ± 2.27
	50	52.09 ± 3.19*,#
	100	41.70 ± 5.54*
	200	28.22 ± 5.63 *,#
Hypophyllanthin	1	98.81 ± 3.37
	10	78.92 ± 2.80*
	50	75.98 ± 1.45*,#
	100	65.81 ± 2.13*
	200	64.48 ± 2.46 *,#
DMSO	0.2% (v/v)	104.04 ± 1.53

Data were present as mean ± S.E.M., n = 4-6

*p<0.05 showed significant difference from DMSO control group.

#p<0.05 showed significant difference between phyllanthin and hypophyllanthin.

Table 6 Effect of the test compounds on vascular contraction induced by KCl (40 mM) in endothelium-denuded aortic rings.

Test compounds	Concentration (μM)	% Contraction
		KCl 40 mM
Phyllanthin	1	94.43 ± 1.45
	10	72.75 ± 4.32*
	50	43.45 ± 1.45*,#
	100	19.44 ± 3.14*
	200	2.31 ± 0.66 *
Hypophyllanthin	1	102.53 ± 7.71
	10	99.20 ± 6.56
	50	81.42 ± 4.14 *,#
	100	24.93 ± 1.45*
	200	44.44 ± 5.84 *
DMSO	0.2% (v/v)	109.40 ± 2.70

Data were present as mean ± S.E. M., n = 4-6

*p<0.05 showed significant difference from DMSO control group.

#p<0.05 showed significant difference between phyllanthin and hypophyllanthin.

Table 7 The apparent IC_{50} of the test compounds on vasoconstriction

Test compounds	IC_{50} values (μM)	
	PE-mediated contraction	KCl-mediated contraction
Phyllanthin	57.67 ± 8.85	63.30 ± 2.69
Hypophyllanthin	N/D	N/D

Data were presented as mean ± S.E.M., n= 4-6

Table 8 The apparent percentage inhibition of the spontaneous contraction

Test compounds	% Inhibition of spontaneous contraction
DMSO 0.2 % (v/v)	3.18 ± 3.94
Phyllanthin	36.98 ± 2.65*
Hypophyllanthin	28.01 ± 3.50*

Data were present as mean ± S.E.M., n = 5-6

*$p<0.05$ showed significant difference from DMSO control group.

Table 9 Effect of the test compounds on vascular contraction induced by $CaCl_2$ (10 µM - 10 mM) in high K^+- Ca^{2+}- free depolarizing solution.

$CaCl_2$ concentration (M)	% Contraction				
	Control	Phyllanthin (100 µM)	Hypophyllanthin (100 µM)	DMSO **0.2%** **(v/v)**	Nifedipine (10 µM)
10^{-5}	6.21 ± 1.33	1.26 ± 0.32	1.68 ± 0.58	3.14 ± 1.01	2.18 ± 0.94
$5x10^{-5}$	19.54±2.43	2.91 ± 0.49*	9.60 ± 4.76	14.77 ± 2.53	5.28 ± 3.36
10^{-4}	29.29±2.23	5.17 ± 0.92*	17.12 ± 4.73	24.56 ± 4.64	10.76 ± 3.82
$5x10^{-4}$	45.51±3.02	8.17 ± 1.31*	28.09 ± 3.88	35.88 ± 7.07	12.15 ± 3.67*
10^{-3}	54.98±1.70	14.47 ± 1.75*	42.67 ± 2.95	52.33 ± 6.00	15.37 ± 3.71*
$5x10^{-3}$	83.52±2.01	38.82 ± 6.06*	68.54 ± 5.13	78.82 ± 4.14	18.23 ± 3.48*
10^{-2}	100 ± 0	63.36 ± 8.49	87.49 ± 8.28	108.94 ± 7.08	20.51 ± 3.99*

Data were present as mean ± S.E.M., n = 5-6

*p<0.05 showed significant difference from DMSO control group.

Table 10 The apparent pD_2 values of the test compounds

Test compounds	The pD_2 values
Phyllanthin	2.53 ± 0.13
Hypophyllanthin	N/D
Nifedipine	2.78 ± 0.09

Data were present as mean ± S.E.M., n = 5-6

Table 11 Effect of the test compounds on vascular contraction induced by either PE (1 μM) or caffeine (10 mM) in Ca^{2+}- free solution.

Agonist	% Contraction		
	DMSO 0.2%(v/v)	Phyllanthin	hypophyllanthin
PE 1 μM	92.11 ± 8.42	24.08 ± 1.29*	55.72 ± 2.89*
Caffeine 10 mM	114.99 ± 14.96	90.22 ± 5.42	85.23 ± 4.89

Data were present as mean ± S.E.M., n = 4-5

*p<0.05 showed significant difference from DMSO control group.

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 10-33-004	**Approval No. 10-33-004**
Protocol Title Effects of phyllanthin and hypophyllanthin on vascular tension of isolated rat aorta	
Principal Investigator Suree Jianmongkol, Ph.D.	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval March 31, 2010	**Date of Expiration** March 31, 2011
Applicant Faculty/Institution Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Rd., Pathumwan BKK-THAILAND. 10330	
Signature of Chairperson	**Signature of Authorized Official**
Name and Title THONGCHAI SOOKSAWATE, Ph.D. Chairman	**Name and Title** PARKPOOM TENGAMNUAY, Ph.D. Associate Dean (Research and Academic Service)
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

BIOGRAPHY

NAME	Miss Marisa Inchoo
DATE OF BIRTH	April 4, 1985
PLACE OF BIRTH	Lumpang
HOME ADDRESS	543/14, Moo 2, Kolak District, Aumphoe Mueang, Prachuapkhirikhan Province, 77000
EDUCATION	Bachelor of Science (Veterinary Technology) with Second Class Honours Kasetsart University, 2008