ผลของน้ำมันไพลต่อแรงตึงตัวของหลอดเลือดแดงเอออร์ตาที่แยกจากกายของหนูขาว

นาง รุ้งนภา มีศรีผ่อง

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EFFECTS OF OIL FROM ZINGIBER CASSUMUNAR ROXB. ON VASCULAR TONE OF ISOLATED RAT AORTA

Mrs. Rungnapa Mesripong

สถาบันวิทยบริการ
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รุ้งนภา มีศรีผ่อง: ผลของน้ำมันไพลต่อแรงตึงตัวของหลอดเลือดเอออร์ตาที่แยกจากกายของหนูขาว (EFFECTS OF OIL FROM ZINGIBER CASSUMUNAR ROXB.ON VASCULAR TONE OF ISOLATED RAT AORTA) อ.ที่ปรึกษา: ผศ. ดร. สุรีย์ เจียรณ์มงคล. 90 หน้า.

ไพล (*Zingiber cassumunar* Roxb.) เป็นสมุนไพรไทยชนิดหนึ่งซึ่งนำเอาน้ำมันไพลมาใช้บรรเทาอาการปวด ดังนั้นการศึกษานี้จึงศึกษาผลของน้ำมันไพลต่อการหดและคลายตัวของกล้ามเนื้อเรียบหลอดเลือดเอออร์ตาทั้งที่มีและไม่มีเยื่อบุผนังหลอดเลือด จากหนูขาวสายพันธุ์ Wistar เพศผู้ น้ำหนัก 250-300 กรัม โดยวัดแรงตึงตัวของกล้ามเนื้อแบบ Isometric ผลการศึกษาพบว่าน้ำมันไพลที่ความเข้มข้น 50 - 200 µg/ml สามารถยับยั้งการหดตัวของหลอดเลือดที่ไม่มีเยื่อบุผนังที่ถูกกระตุ้นด้วย phenylephrine (1 µM) และ KCl (40 mM) ได้ อย่างมีนัยสำคัญทางสถิติ โดยความแรงในการยับยั้งขึ้นกับความเข้มข้นของน้ำมันไพล ทั้งนี้น้ำมันไพลในช่วงความเข้มข้นดังกล่าวไม่มีผลยับยั้งการหดตัวของหลอดเลือดที่มีเยื่อบุผนังที่ถูกกระตุ้นด้วย phenylephrine นอกจากนี้ น้ำมันไพลที่ความเข้มข้น 40 และ 100 µg/ml ยับยั้งการหดตัวของกล้ามเนื้อเรียบหลอดเลือดที่ถูกกระตุ้นด้วย phenylephrine ในสภาวะที่ปราศจาก Ca^{2+} แต่ไม่มีผลต่อการหดตัวของกล้ามเนื้อเรียบหลอดเลือดที่ถูกกระตุ้นด้วย caffeine (10 mM) และผลของน้ำมันไพลที่ความเข้มข้น 50 และ 100 µg/ml สามารถลดการตอบสนองของกล้ามเนื้อเรียบหลอดเลือดต่อ $CaCl_2$ เมื่อกล้ามเนื้ออยู่ในสภาวะ depolarization โดยมีค่า pD2 เท่ากับ 3.76 ± 0.09 และ 3.57 ± 1.35 ตามลำดับ นอกจากนี้จากการศึกษาพบว่าน้ำมันไพลมีผลทำให้กล้ามเนื้อหลอดเลือดคลายตัวผ่านทางกลไกที่เกี่ยวข้องกับเยื่อบุผนังหลอดเลือด โดยมีค่า EC 50 เท่ากับ 32.80 ± 4.43 µg/ml ผลของน้ำมันไพลที่ความเข้มข้น 40 µg/ml ที่ทำให้เกิดการคลายตัวของหลอดเลือดที่มีเยื่อบุผนังนั้น ถูกยับยั้งได้ด้วย methylene blue (10 µM), L-NAME (10 µM) , glibenclamide (10 µM), indomethacin (10 µM), atropine (1 µM), propranolol (10 µM), และ tetraethylammonium chloride (10 µM) จึงอาจสรุปได้ว่า น้ำมันไพลมีผลต่อแรงตึงตัวกล้ามเนื้อเรียบหลอดเลือดโดยขึ้นกับทั้งที่มีเยื่อบุและไม่มีเยื่อบุผนังหลอดเลือด โดยส่วนหนึ่งอาจเกี่ยวข้องกับการรบกวน การเคลื่อนที่ของ Ca^{2+} จากภายนอกเข้าสู่ภายในเซลล์ หรือมีผลต่อการเคลื่อนที่ Ca^{2+} ภายในเซลล์ นอกจากนั้นน้ำมันไพลอาจไปรบกวนการทำงานของหลอดเลือดผ่านทาง endothelium factors เช่น NO/cGMP pathway, hyperpolarizing, cyclooxygenase, muscarinic receptors และ β-adrenoceptor.

ภาควิชา.....เภสัชวิทยา .....ลายมือชื่อนิสิต.....

สาขาวิชา.....เภสัชวิทยา.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

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RUNGNAPA MESRIPONG: EFFECTS OF OIL FROM ZINGIBER CASSUMUNAR ROXB.ON VASCULAR TONE OF ISOLATED RAT AORTA: ASST. PROF. SUREE JIANMONGKOL, Ph.D., 90 pp.

Plai (*Zingiber cassumunar* Roxb.) is one of Thai herbal plants, which is well recognized for relieving muscle pain. In this study, the effects of plai oil on the contraction and relaxation of vascular smooth muscle in the endothelium-intact and endothelium-denuded rat aorta were investigated. The thoracic aorta was isolated from male Wistar rats (250-300 g), and the vascular tensions were measured isometrically. The results showed that plai oil (50 - 200 μg/ml) significantly inhibited the PE- and KCl-induced contraction of endothelium-denuded aorta in concentration-dependent manner, but had no effects in endothelium-intact aorta. In addition, plai oil (40 and 100 μg/ml) significantly inhibited the PE-induced contraction in Ca^{2+}- free condition, but not the caffeine-induced contraction. Furthermore, plai oil (50 and 100 μg/ml) suppressed $CaCl_2$ – induced contraction in high K^+- depolarizing solution with the apparent pD2 values of 3.76 ± 0.09 and 3.57 ± 1.35, respectively. The results also demonstrated that plai oil caused vasodilatation in endothelium-intact aorta with the apparent EC 50 values of 32.80 ± 4.43 μg/m. Various compounds including methylene blue (10 μM), L-NAME (10 μM), glibenclamide (10 μM), indomethacin (10 μM), atropine (1 μM), propranolol (10 μM), and tetraethylammonium chloride (10 μM) significantly inhibited the relaxant effect of plai oil. In conclusion, plai oil modulated the vascular tone via endothelium-dependent and endothelium-independent pathways. The direct actions on smooth muscle were possibly linked to non-specific inhibition of Ca^{2+} influx as well as inhibition of PE-mediated Ca^{2+} release from sarcoplamic reticulum. Moreover, plai oil may influences the vascular contractility through endothelium factors including NO-cGMP pathway, hyperpolarizing, cyclooxygenase, muscarinic receptors and β–adrenoceptor.

Department.....Pharmacology.....Student's signature.....Rungnapa Mesripong.....

Field of study.....Pharmacology .....Advisor's signature.....Suree Jianmongkol.....

Academic year..... 2006.....Co-advisor's signature.....Basan Dhuemyakon.....

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LIST OF ABBREVIATIONS

$[Ca^{2+}]_i$	Intracellular calcium ion concentration
Ca^{2+}	calcium ion
AC	adenylate cyclase
IP_3	inositol 1, 4, 5- trisphosphate
ATP	adenosine 5'- triphosphate
cAMP	cyclic adenosine 3',5'- monophosphate
cGMP	cyclic guanosine 3',5'- monophosphate
NO	nitric oxide
SR	sarcoplasmic reticulum
PE	phenylephrine
K^+	potassium ion
KCl	potassium chloride
ACh	acetylcholine
M	molar
ml	millilitre
μM	micromolar
μg	microgram
ROC	Receptor-operated calcium channels
VOC	Voltage-operated calcium channels
TEA	tetraethylammonium chloride
L-NAME	N^G-nitro -L-arginine methyl ester
KHS	Krebs-Henseleit solution
PO	Plai oil
COX	cyclooxygenase

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EDRF	endothelium-derived relaxing factors
VSM	vascular smooth muscle
DMPBD	(E)-1-(3, 4-dimethoxyphenyl) butadiene
S.E.M	standard error of mean
ANOVA	one- way analysis of variance
Z. cassumunar Roxb.	*Zingiber cassumunar* Roxb.

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CHAPTER I

INTRODUCTION

Herbal medicine has long been proven useful for human health. Plai (*Zingiber cassumunar* Roxb.) is one of herbal plants, which is well recognized for relieving muscle pain. Plai extract and plai oil have been used as an ingredient in topical creams, ointment and massage oils for muscle analgesic action and improved topical blood circulation.

In Thailand and many Asian countries, plai is widely used in folklore remedies as a single plant or as a component of herbal medicine. For example, Prasaplai, which is Thai traditional medicine, has been used for alleviation of colicky pain and abnormal menstrual period (วิชัย ริ้วตระกูล, 2546). Various compounds in plai oil have been identified including sabinene, γ-terpinene, α-terpinene, terpinen-4-ol, and (E)-1-(3, 4-dimethoxyphenyl) butadiene (DMPBD) (Cosey, 1971).

The pharmacological studies have been demonstrated that the rhizomes of *Z. cassumunar* have antioxidant (Lertsatitthanakorn, *et al*., 2006) and antimicrobial activities (นันทวัน บุยยะประภัศร, 2523). The hexane extract elicit anti-inflammatory activity (Jeenapongsa, *et al*., 2003). (*E*)-1-(3, 4-dimethoxyphenyl) butadiene (DMPBD), which was isolated from the hexane extract, exhibited a strong inhibitory action on the edema formation in carrageenan-induced rat paw edema (Panthong, *et al*., 1990; Jeenapongsa, *et al*., 2003). In addition, phenylbutenoids, which are typical non-polar substances in the rhizomes of plai, have insecticidal activity (Nugroho, *et al*., 1996).

Plai oil is one of essential ingredients in herbal compress (มาโนช วามานนท์ และคณะ, 2537), massage oil and skin care products for anti-inflammatory effect and muscle relaxation (Wanauppathamkul, 2003). It has been demonstrated that plai extracts and its constituents inhibited agonist-induced contraction of smooth muscle in several *in vitro* models of isolated organs including guinea pig and rat trachea, guinea -pig ileum and rat uterus (เรณู โกยสุโข และคณะ, 2533). In addition, plai oil caused relaxation of smooth muscles (วัลภา อนันตศานต์, 2525; สุวรรณา เวชอภิกุล, 2547). It has been reported that

plai-induced relaxation in isolated rat uterus and intestine was antagonized by acetylcholine, calcium chloride, but not by either alpha-blocker, beta- blocker or histamine (วัลภา อนันตศานต์, 2525).

As known, intracellular Ca^{2+} ($[Ca^{2+}]_i$) is a key element in controlling vascular smooth muscle contraction (Aaronson *et al*., 2004; Horowitz, 1996). An increase of sufficient magnitude of $[Ca^{2+}]_i$ fully induces contraction of vascular smooth muscle (VSM). Under physiological conditions, an increasing in $[Ca^{2+}]_i$ is attributed to a change in membrane potential (electromechanical coupling) or to an activation of specific receptor (pharmaco-mechanical coupling), resulting in an increase in Ca^{2+} influx (Orallo, 1996; Katz, 1997). On the other hand, muscle relaxation results from a decrease in $[Ca^{2+}]_i$ which may be due to a blockade of voltage-operated calcium ion channels (VOC) or activation of Ca^{2+}efflux system such as Ca^{2+}- ATPase and Na^{+}/ Ca^{2+} exchanger (Felder, *et al*., 1994; Martin, *et al*.,1999). In addition, endothelium plays a crucial role on vasorelaxation by releasing several factors such as nitric oxide (NO) and other endothelium-derived relaxing factors (EDRF) (Klabuade, 2005; Busses, *et al*., 2002). Although there is evidence of plai-induced relaxation on several models of smooth muscle, the mechanisms of plai actions have not been extensively investigated.

This study aimed to investigate the action of oil from *Zingiber cassumunar* Roxb. on modulation of vascular tone. It is possible that oil from *Z. cassumunar* Roxb. may exert its vasorelaxant action via endothelium-dependent and –independent pathways. Hence, this study is designed to examine the direct effect of oil from *Z. cassumunar* Roxb. on smooth muscle contractility including the interference of Ca^{2+}influx as well as Ca^{2+}release from sarcoplasmic reticulum (SR). In addition, the influence of oil from *Z. cassumunar* Roxb. on endothelium function is also investigated, including the NO-cGMP pathway, the hyperpolarization factors and activation of β_2-adrenergic system.

Hypothesis

Oil from *Zingiber cassumunar* Roxb. modulates vascular tone via endothelium-dependent and –independent pathways. It is possible that oil from *Z. cassumunar* Roxb. directly affects smooth muscle by disrupting calcium influx through voltage-operated calcium ion channels, and Ca^{2+} mobilization within smooth muscle cells. Moreover, oil from *Z. cassumunar* Roxb. may activate endothelium to release certain relaxing factors such as NO.

Objectives

1. To investigate the effects of oil from *Z. cassumunar* Roxb. on the contraction and relaxation of vascular smooth muscle in the endothelium intact and endothelium-denuded rat aorta.
2. To examine the mechanisms of action of oil from *Z. cassumunar* Roxb. in modulating vascular tone.

Expected Benefit and Application

This study will provide new pharmacological knowledge on the effects of oil from *Z. cassumunar* Roxb. on the contraction of vascular smooth muscle as well as its mechanisms of action. The information from this study will be useful for the further application in herbal and traditional medicine development as well as for prediction the potential adverse effects on the vascular system.

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CHAPTER II

LITERATURE REVIEWS

Plai (*Zingiber cassumumar* Roxb.) is a Thai herbal plant which has been used for medicinal purposes in Thailand and Southeast Asia for centuries (สมสุข มัจฉาชีพ, 2534). The rhizome has a yellow to green color with fleshy thick texture containing multiple sessile tubers. The characteristic of plai include as follow: distichous leaves, oblong-lanceolate, very short ligule, bilobed, pubescent. Spike ovoidellipsoid are bracts greenish red, narrowly obovate or rhomboid and calyx truncate, glabrous. Corolla tube has pale yellow, dorsal lobe cymbiform, lateral lobe linearlanceolate. Labellum is pale yellow, suborbicular, apex emarginate, lateral lobe ovate-oblong, appendage slightly longer than anther (มาโนช วามานนท์ และคณะ, 2537) (Figure. 1). The odor is reported as strong and reminiscent of a mixture of ginger, camphor and turmeric. The taste is hot and camphoraceous, pleasant aromatic and taste are pungent. Essential oil of plai can be steam distilled from the rhizome and has a pale amber color. The scent is a cool, green peppery one with a touch of a bite.

Figure 1 *Zingiber cassumumar* Roxb. aerial and underground parts (From; http://www.tistr.or.th/pharma/Zingiber%20cassumunar.htm and http://www.rspg.thaigov.net/plants_data/use/herbs14.htm)

Rhizome of plai has essential oil of 8 % (มาโนช วามานนท์ และคณะ, 2537). A major part of the oil consists of monoterpenes with sabinene and terpinen-4-ol as main constituents. The main active constituents of the oil are sabinene, γ- terpinene, α-terpinene, and the other active chemicals are curcuminoids derivatives (curcumin, cassumunarin A, B and C), β-sitosterol and cyclohexane derivatives, naphthoquinone derivatives, butanoid derivatives such as (E)-1-(3',4'-dimethoxyphenyl) butadiene; DMPBD (สุวรรณา เวชอภิกุล, 2547 ; Masuda and Jitoe,1995). The major constituents in essential oil of rhizome of *Z. cassumunar* are shown in table 1.

Table1. Essential oil composition (%) of the different source rhizomes of *Zingiber cassumunar* Roxb.

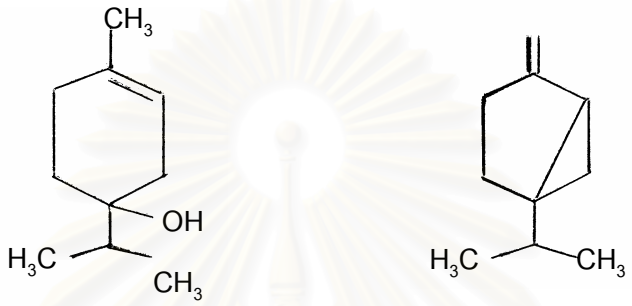
(A): Indonesia, (B): Thailand-Prachinburi, (C): Thailand – TISTR

Compound	A	B	C
Sabinene	10.1	44	34.7
Terpinen-4-ol	10.2	24	32.3
γ-Terpinene	3.6	9	6.7
α-Terpinene	2.0	6	3.7
DMPBD	9.8	6	7.2

(From; Wanauppathamkul , 2003.)

Major compositions of plai oil are terpinen-4-ol, sabinene, terpinene and DMPBD. Sabinene is a natural bicyclic monoterpene with the molecular formula $C_{10}H_{16}$. It is isolated from the cyclopentane ring fused to a cyclopropane ring. Sabinene is one of the chemical compounds that contributes to the spiciness of black pepper and is a major constituent of carrot seed oil (Wikipedia, 2006). It also occurs in tea tree oil at a low concentration. Terpinen-4-ol is terpene. It is considered the primary active ingredient of tea tree oil (Nascimento, 2005). The terpinenes are three isomeric hydrocarbons that are classified as terpenes. They each have the same molecular formula and carbon framework, but they differ in the position of carbon-carbon double bonds. α-terpinene

has been isolated from cardamom and marjoram oils, and from other natural sources. β-terpinene has no known natural source, but can be synthesized from sabinene. γ-terpinene is natural compound which can be isolated from a variety of plant sources (Wikipedia, 2006).



Terpinen-4-ol Sabinene

Figure 2 Chemical structures of terpinen-4-ol and sabinene

Plai has long been regarded by Thai massage therapists as one of those oils necessary to have in their kit to combat joint and muscle problems (Wanauppathamkul, 2003). On joints inflamation from injury, plai is best combined with other oils such as black pepper (*Piper nigrum* L.) and lemon (*Citrus limo*n Burm.) or neroli (*Citrus aurantium* L.), Himalayan cedarwood (*Cedrus deodora* G.) and orange (*Citrus aurantifolia* Swingle) (Chamratpan and Homchuen, 2005). These combinations decreased the swollen, eased the pain and considerably speeded up the healing intima for digestive upset had been used to counter irritable bowel syndrome (พฤฒมา-จารย์ วิพุธโยคะ รัตนรังษี, 2534). The essential oils from plai have also been shown to cure acne, bruises, burnt skin, inflammation, muscle pain, insect bite and asthmatic symptoms. Plai oil has been even proven to cope with cough and respiratory symptoms as well. A number of pure compounds isolated from the plants have been shown to possess anti-microbial, topical and oral anti-inflammatory, analgesic and anti-oxidative activities

(สุวรรณา เวชอภิกุล, 2547 ; Osaki, 1991). The fresh rhizome of plai is used in traditional Thai massage for muscle relaxant and joint pains (Wanauppathamkul, 2003).

(E)-1-(3, 4-dimethoxyphenyl) butadiene (DMPBD) which was extracted and isolated from plai exhibited antiinflammatory activity both *in vivo* and *in vitro* models. DMPBD inhibited the rat ear edema induced by ethyl phenylpropiate, arachidonic acid and 12-o-tetradecanoylphorbol 13-acetate. DMPBD possesses a potent anti-inflammatory activity through the inhibition of both cyclooxygenase (COX) and lipoxygenase (LO) pathways (Jeenapongsa, *et al*., 2003).

Pharmacological studies of plai on smooth muscle relaxation have been reported. For example, Prasaplai has been used for colicky pain, abnormal menstrual period (วิชัย ริ้วตระกูล, 2456). The rhizome of plai has been used as antiasthmatic drug in Thai traditional medicine for a long time (สุวรรณา เวชอภิกุล, 2547). Moreover, plai was shown to reduce the size of wheal developing from intracutaneous injection of histamine in healthy volunteer. However, this antihistamic activity of plai was less potent than that of chlorpheniramine ($P< 0.05$) (Piromrat, and Tuchinda., 1986).

Pure compounds from plai such as (E)-4-(3', 4'-dimethoxyphenyl) but-3-en-1-ol and (E)-4-(3', 4'-dimethoxyphenyl) but-3-en-1-ol acetate exhibited antagonistic effects on contraction of isolated guinea-pig and rat trachea in the presence of histamine and methacholine. The bronchodilator effect of plai was not attributed to stimulation of beta-adrenergic receptor (เรณู โกยสุโข และคณะ, 2533). Furthermore, (E)-4-(3', 4'-dimethoxyphenyl) but-3-en-1-ol acetate and (E)-4-(3', 4'-dimethoxyphenyl)but-3-en-1-ol acetate exhibited antagonistic effects on acetylcholine (0.06 µg/ml), histamine (0.3 µg/ml), serotonin (5 µg/ml) and barium chloride (0.2 µg/ml)-induced contraction of isolated guinea-pig ileum. In addition, (E)-4-(3', 4'-dimethoxyphenyl) but-3-en-1-ol exhibited relaxant effect in uterine muscle (เรณู โกยสุโข และคณะ, 2533). In other studies, plai water extract caused smooth muscle relaxation in several isolated organ preparations including rat uterus, rat intestine and human umbilical artery (วัลภา อนันต-ศานต์, 2525). In addition, the plai induced-muscle relaxation was antagonized by acetylcholine, calcium chloride, but not by alpha-blocker, beta- blocker or histamine.

Although there are some evidences of plai-induced relaxation in several models of smooth muscle, the mechanisms of plai actions have not been extensively investigated. This study aimed to investigate the modulations action of plai oil on rat vascular tone. It is possible that plai oil may exert its vasorelaxant action via endothelium-dependent and –independent pathways. Hence, the study is designed to examine the direct effect of plai oil on rat smooth muscle including the interference of Ca^{2+}influx as well as Ca^{2+}release from sarcoplasmic reticulum (SR). In addition, the influence of plai oil on endothelium function is also investigated, including the NO-cGMP pathway, the hyperpolarization factors and activation of β_2-adrenergic system.

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CHAPTER III

MATERIALS AND METHODS

Chemicals

Major chemicals used in this study include phenylephrine (PE), potassium chloride (KCl), acetylcholine (ACh), caffeine, tetraethlammonium (TEA), indomethacin, glibenclamide, N^G-nitro -L-arginine methyl ester (L-NAME), methylene blue, atropine, propranolol, dimethyl sulfoxide (DMSO) 99.5%, terpinen – 4 - ol 98%. All chemicals were purchased from Sigma-Aldrich (St.Louis, MO) and standard sabinene from Thailand Institute of Scientific and Technological Research (TISTR).

Test compounds

Plai oil was obtained from Thailand Institute of Scientific and Technological Research (TISTR). The major constituents included α–pinen 1.68%, sabinene 39.13%, α -terpinene 2.44%, γ-terpinen 5.67%, terpinen-4-ol 34.12% and (E)-1-(3,4-dimethoxyphenyl)butadiene (DMPBD) 2.40%. Plai oil was dissolved in 99.5% DMSO and the final concentration of DMSO in is less than 0.07% (v/v). This concentration of DMSO had no effect on rat vascular smooth muscle contractility.

Preparation of aortic rings

Sixty adult male Wistar rats of body weight between 250-300 g were used in this study. They were obtained from National Laboratory Animal Center, Salaya, Nakornpathon. The animals were housed in the animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University for 1-2 weeks before experimentation. Animals were housed under condition of controlled temperature of 25 ± 2 ºC and exposed to a daily 12 hours light-dark cycle. Animals were supplied with pellets diet from National Laboratory Animal Center and water ad libitum. This study was approved by Animal Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University on the use of laboratory animals in teaching and research.

Rats were anaesthetized by ether and killed by cervical dislocation. The thoracic aorta was removed and placed in Petri-dish containing Krebs-Henseleit solution (KHS) of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.64, KH_2PO_4 1.18, $NaHCO_3$ 7 and glucose 5.5. Then, the thoracic aorta was cleaned and cut in to 4 segments of approximately 0.3- 0.5 cm long. Each of ring segments was suspended in double walled organ baths (Harvard type Organ bath) and attached to an isometric force transducer (Harvard Apparatus Ltd.) under a resting tension of 1.0 g. The bath was contained 15 ml of KHS at 37°C and bubbled with a mixture of 95% 0_2 and 5% CO_2. The tension was recorded on model Gilson N_2 coupled to an amplifier (Harvard Apparatus Ltd, England). During an initial stabilization period of approximately 60 min, the solution was replaced every 15 min. The aortic rings were tested for functional endothelium by addition of acetylcholine (ACh 10 µM) after precontracted with phenylephrine (PE 10 µM). The relaxation of at least 60-80 % was considered endothelium intact for further experiment. In some preparations, the endothelium was removed by rubbing the lumen with a cotton swab. The absence of the functional endothelium was confirmed by a relaxant response of less than 10% after challenge with ACh (10 µM).

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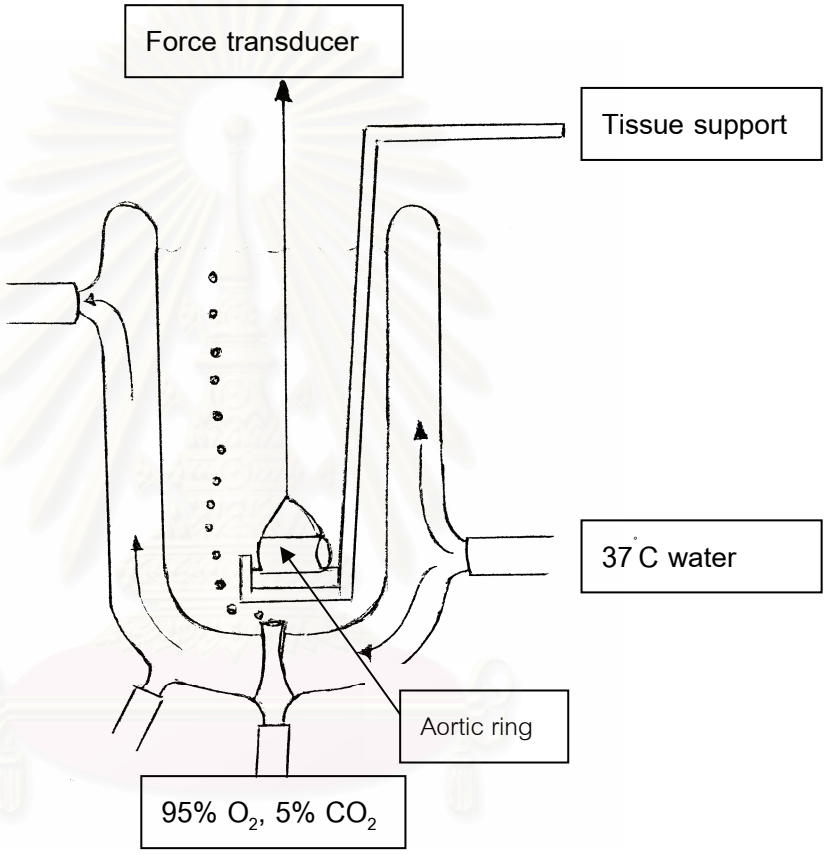

Figure 3 Illustration of instrument and organ bath for isolated rat aorta.

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Experiments

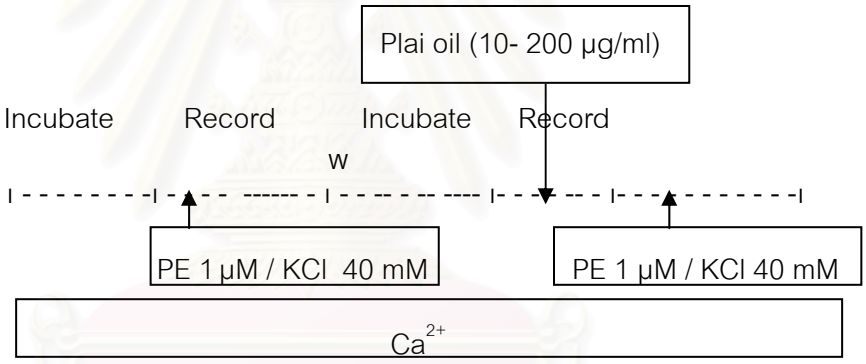
1. Effect of plai oil on aortic contraction

1.1 Effect of plai oil on aortic contraction.

The aortic ring was placed in normal KHS until the tension was stable. Then, PE (1 μM) or KCl (40 mM) was added to induce contraction. The tension was recorded for 20 minutes. The effects of Plai oil on PE- or KCl- induced contraction were determined by incubating plai oil at concentration of 10- 200 μg/ml for 20 minutes prior to addition of either PE or KCl. Responses to each concentration of the plai oil were expressed as a percentage of the maximal contraction induced by either PE (1 μM) or KCl (40 mM).

In separated experiments, the effect of terpinen - 4 - ol (34 μg/ml) or sabinene (39 μg/ml) on aortic contraction were also tested.

Experiment 1.1



1.2 Effect of plai oil on aortic contraction in calcium free medium.

1.2.1 Contraction induced by phenylephrine

The aortic ring was placed in normal KHS until the tension was stable. The PE (1μM) was added to induce aortic contraction. The tension was recorded 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 PE (1μM). The effect of plai oil on PE-induced contraction in Ca^{2+}-free KHS was studied by addition of plai oil 5 minutes prior to addition of PE. The response was expressed as a percentage of PE-induced contraction in Ca^{2+}-free KHS in the absence of tested compounds

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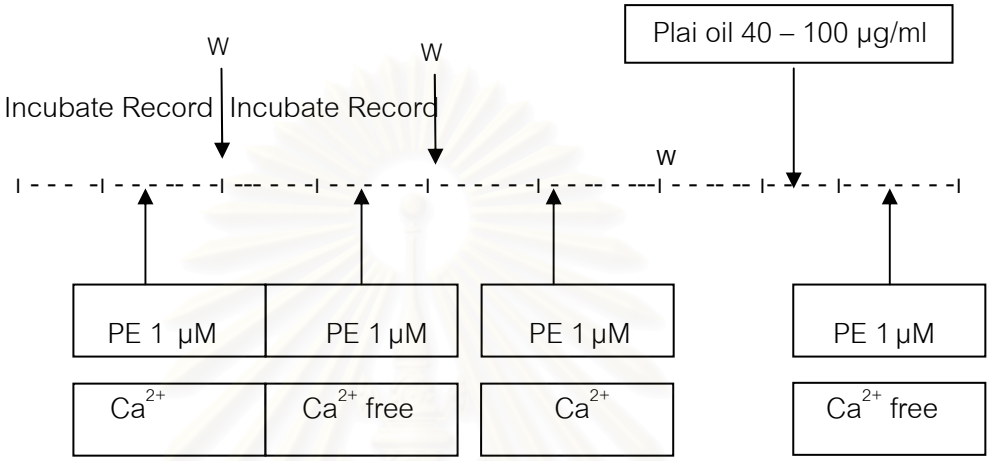
In separated experiments, the effect of terpinen - 4 - ol (34 µg/ml) or sabinene (39 µg/ml) on aortic contraction were also tested.

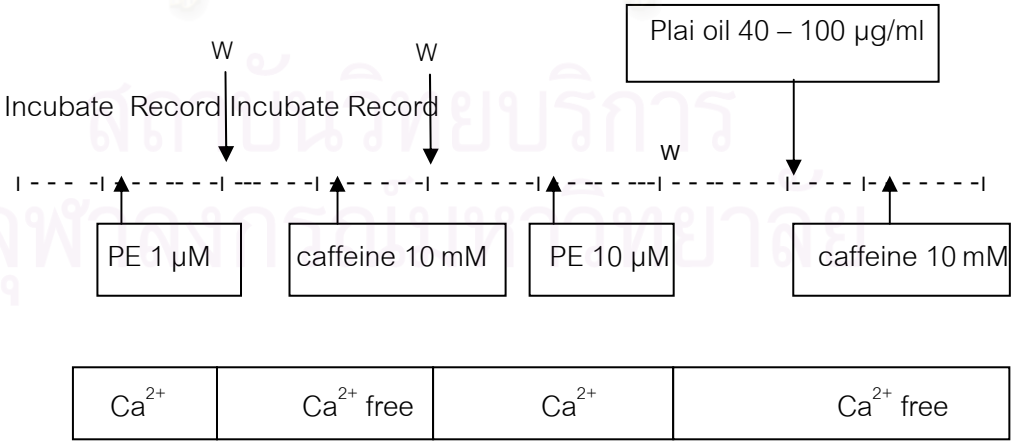
Experiment 1.2.1



1.2.2 Contraction induced by caffeine

The experiment procedures were similar to those in section 1.2.1, except using caffeine (10 mM) instead of PE to induce contraction.

Experiment 1.2.2

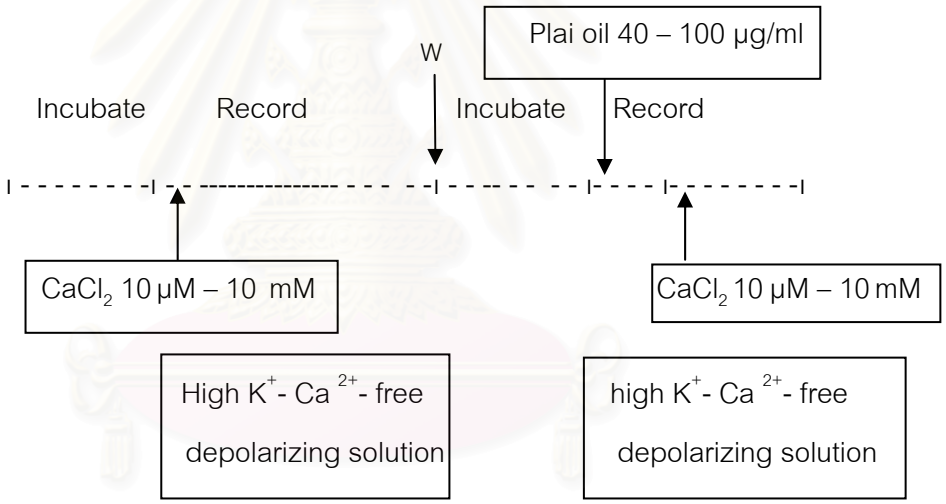


1.3 Effect of plai oil on contraction induced by Ca^{2+} in high K^{+} - Ca^{2+}- free depolarizing solution

The aortic ring was placed in normal KHS until the tension was stable. After the equilibration period, the normal KHS was replaced by high K^{+} - Ca^{2+}- free depolarizing solution and incubated until the tension was stable. Then, $CaCl_2$ (10 µM to 10 mM) were added cumulatively to induce aortic contraction. The effect of plai oil was studied by addition of plai oil 5 minutes prior to $CaCl_2$. The contraction was expressed as a percentage of the maximum contraction induced by $CaCl_2$.

In separated experiments, the effect of terpinen - 4 - ol (34 µg/ml) or sabinene (39 µg/ml) on aortic contraction were also tested.

Experiment 1.3



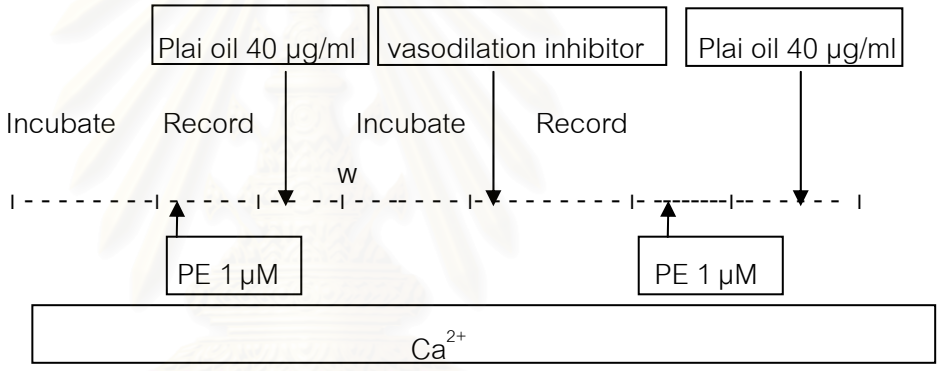
2. Relaxant effects of plai oil

The aortic ring was placed in normal KHS until the tension was stable. The ring was precontracted with either PE (1 µM) or KCl (40 mM). When the contraction reached plateau state, plai oil (1.25 µg/ml to 100 µg/ml) was added cumulatively to produce relaxation. The tension was recorded and expressed as a percentage of the PE - or KCl -induced contraction. In separated experiments, the effect of terpinen - 4 - ol (1.36×10^{-3} µg/ml to 136 µg/ml) or sabinene (1.36×10^{-3} µg/ml to 136 µg/ml) on aortic contraction were also tested.

In order to investigate the mechanism of plai oil on vasorelaxation, several specific inhibitors were added 30 minutes before the precontraction with PE. These inhibitors included atropine (1 μM), indomethacin (10 μM), tetraethylammonium (TEA) (10 μM), and propranolol (10 μM), L-NAME (10 μM), methylene blue (10 μM), or glibenclamide (10 μM). The relaxations were expressed as the percentage of the PE-induced contraction.

In separated experiments, the effect of terpinen - 4 - ol (13 μg/ml) or sabinene (15 μg/ml) on aortic contraction were also tested.

Experiment 2.



Statistical Data Analysis

Results were expressed as the mean ± standard error of the mean (S.E.M) for 4-6 separated experiments. The EC50 was calculated from dose-response curves by linear regression. Statistical significances were tested either by one- way analysis of variance (ANOVA) followed by post-hoc Scheffer test. The *p* values of less than 0.05 were considered statistically significant.

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.

CHAPTER IV

RESULTS

1. Effect of oil from *Zingiber cassumunar* Roxb. (plai oil) on aortic contraction

1.1 Effect of plai oil on aortic contraction in Ca^{2+}-containing solution.

As demonstrated in Figure 4, the contraction profiles of endothelium-intact and endothelium-denuded aortic rings in response to PE were similar, consisting of phasic and tonic phases. The magnitude of tension induced by PE (1 μM) was 0.990 ± 0.024 g (*n* = 37) for endothelium-intact aortic rings, and 0.867 ± 0.021 g (*n* = 40) for endothelium-denuded aortic rings. The responsiveness of aortic rings toward PE in the presence of plai oil as well as its major constituents including terpinen-4-ol and sabinene was also shown in Figure 5 - 16. Pretreatment of the endothelium-denuded aortic rings with plai oil at concentration of 100 and 200 μg/ml resulted in the significant decrease of contractile responses to PE by 26 % and 35 % (*n* = 6), respectively (Figure 17). Plai oil inhibited the contraction effect of PE on aortic muscle in concentration-dependent manner. However, plai oil up to 200 μg/ml had no significant inhibitory effect on PE-induced contraction of endothelium-intact aortic ring, suggesting the protective effect of endothelium. In the intact preparations, terpinen-4-ol (34 μg/ml) was more potent than plai oil (100 μg/ml) and sabinene (39 μg/ml) in suppressing PE-induced contraction. Terpinen-4-ol caused a significant reduction in PE - induced contraction by 21 % (*n* = 4). In contrast, sabinene was the most potent compound in inhibiting PE-induced contraction in endothelium-denuded aortic ring (Figure 18).

The contraction profiles of aortic preparations in response to KCl (40 mM) were similar to those of PE–induced contraction, but with less magnitude (Figure 19). In this study, the aortic tension in response to KCl was 0.867 ± 0.021 g (*n* = 27) in endothelium-intact preparations and 0.710 ± 0.024 g (*n* = 30) in endothelium-denuded preparations. Plai oil was able to suppress KCl-induced contraction of both endothelium-intact and endothelium-denuded aortic rings in concentration-dependent manner (Figure 20 - 27).

In addition, the inhibitory action of plai oil was more potent against KCl-induced contraction than PE-induced contraction. At the concentrations of 50 and 100 µg/ml, plai oil was able to suppress KCl - induced contraction of endothelium-intact aortic rings by 33 % and 58 %, respectively. However, an increase of concentration to 200 µg/ml had no effect on the degree of inhibition, suggesting the maximum inhibitory effect of plai oil was 60% approximately. Removal of endothelium intensified the inhibitory effect of plai oil on KCl-induced contraction (Figure 28). At the concentration of 10 and 50 µg/ml, plai oil significantly decreased the endothelium-denuded aortic contraction in response to KCl by 31 % and 51 %, respectively. The inhibitory effects of plai oil at the concentration of 10 and 50 µg/ml were significantly higher in endothelium-denuded aortic preparation. Nevertheless, the maximum inhibitory actions of plai oil were quite comparable in endothelium-intact and endothelium-denuded preparations.

Furthermore, plai oil (at the concentration of 50 and 100 µg/ml) and sabinene (at the concentration of 39 µg/ml) significantly increased baseline tensions of endothelium-denuded aortic rings by 28.31 ± 6.18 % (n = 8), 10.87 ± 5.44 % (n = 8) and 22.03 ± 4.43 % (n = 5), respectively (Figure 10, 11, 16). These effects were not observed in endothelium-intact aortic rings (Figure 6, 7, 15), suggesting the influence of endothelium on vascular response to plai oil.

1.2 Effect of plai oil on aortic contraction in Ca^{2+}-free medium.

In Ca^{2+}-free solution, PE (1 µM) and caffeine (10 mM) produced a small, transient contraction in endothelium-denuded (Figure 29, 33). The observable tensions were 0.134 ± 0.019 g (n = 6) for PE-induced contraction and 0.051 ± 0.017 g for caffeine-induced contraction. In this study, plai oil (at concentration of 40 µg/ml and 100 µg/ml) and sabinene (at concentration of 39 µg/ml) significantly inhibited PE-induced contraction whereas terpinen-4-ol (at concentration of 34 µg/ml) had no inhibitory effects (Figure 29, 30). The muscle tension decreased by 28 % (n = 6) in the presence of plai oil at concentration of 40 µg/ml. Plai oil at the concentration of 100 µg/ml had not resulted in a significant increase in the inhibitory effect on PE-induced contraction in Ca^{2+}-free solution (Figure 31). In contrast to the effect of plai oil, upon increasing the concentration of sabinene to 39 µg/ml, the inhibitory effect significantly increased by 36

% (Figure 32). Interestingly, the inhibitory actions of plai oil were not observed when caffeine, instead of PE, was a contractant. As shown in figure 33-34, plai oil at concentration of 100 µg/ml had no effect on caffeine-induced aortic contraction.

1.3 Effects of plai oil on contraction induced by addition of Ca^{2+} in high K^{+}-Ca^{2+}-free depolarizing solution

The contraction profile of endothelium-denuded aortic rings upon cumulative addition of $CaCl_2$ (10 µM to 10 mM) in high K^{+}-Ca^{2+}-free depolarizing solution was shown in figure 35. Under this condition, plai oil at concentration of 50 and 100 µg/ml significantly shifted the dose response curve of $CaCl_2$-induced contraction rightward and downward (Figure 36). The maximum contractions of aortic muscles reduced to 72.03 ± 2.31 % (*n* = 6), and 67.38 ± 3.30 % (*n* = 6) in the presence of plai oil at concentration of 50 µg/ml and 100 µg/ml, respectively. In the presence of terpinen-4-ol (34 µg/ml) and sabinene (39 µg/ml), the maximum contractions of aortic muscles were 102.51 ± 3.01 % (*n* = 6), and 38.19 ± 3.77 % (*n* = 6), respectively (Figure 37). The apparent pD2 values were 3.76 ± 0.09 for plai oil at concentration of 50 µg/ml and 3.57 ± 1.35 for plai oil at concentration of 100 µg/ml. The pD2 values of terpinen-4-ol and sabinene were 2.94 ± 0.13 and 3.36 ± 0.13, respectively.

2. Effects of Plai oil on the relaxation of rat aorta

2.1 Effects of plai oil on the relaxation of rat aorta precontracted with PE (10 µM) and KCl (40mM).

The relaxation profiles of endothelium-intact and endothelium-denuded aortic rings precontracted with PE (10 µM) were demonstrated in Figure 38-43. In this study, Plai oil (1.25-60 µg/ml) and terpinen-4-ol (1.36×10^{-3} µg/ml to 136 µg/ml) were able to dose-dependently relax the vascular tensions of both endothelium-intact and endothelium-denuded aortic rings pretreated with PE (Figure 44-45). However, the vasodilation effects of plai oil and terpinen-4-ol were more prominent in endothelium-intact aortic rings than in endothelium-denuded preparations. Sabinene (1.36×10^{-3} µg/ml to 136 µg/ml) elicited different relaxation profiles (Figure 42 - 43). Sabinene at the concentration up to 1.36 µg/ml was able to cause vasorelaxation of endothelium-intact aortic rings precontracted with PE. However, at the high concentration of more than 1.3

µg/ml, sabinene induced contraction (Figure 45). This phenomenon was not observed in the experiment using endothelium-denuded aortic rings. As shown in Figure 42, sabinene at concentration of 1.36×10^{-3} µg/ml to 136 µg/ml caused vasorelaxation of PE-precontracted rings in dose-dependent manner.

The apparent EC50 values for plai oil-induced relaxation were 32.80 ± 4.43 µg/ml (n = 6) in endothelium-intact aortic rings and 47.75 ± 3.46 µg/ml (*n* = 6) in endothelium-denuded aortic rings (Figure 44). Terpinen-4-ol at the highest concentration in this study (136 µg/ml) caused 34.44 ± 3.80 % (*n* = 6) and 73.92 ± 9.72 % (*n* = 7) relaxation for endothelium-intact and endothelium-denuded aortic rings, respectively. Moreover, sabinene at the highest concentration in this study (136 µg/ml) produced 36.00 ± 3.97% vasorelaxation in endothelium-denuded aortic rings (Figure 45).

The relaxation profiles of endothelium-intact and endothelium-denuded aortic rings precontracted with KCl were shown in Figure 46-47. Plai oil (1.25-60 µg/ml) was able to produce vasorelaxation in both endothelium-intact and endothelium-denuded aortic rings precontracted with KCl. The apparent EC50 values of plai oil-induced vasorelaxation were 29.05 ± 6.78 µg/ml (*n* = 6) in endothelium-intact aortic rings and 36.42 ± 5.66 µg/ml (*n* = 7) in endothelium-denuded aortic rings (Figure 48).

2.2 The influence of endothelium on plai oil – induced relaxation.

In this study, plai oil (40 µg/ml), terpinen-4-ol (13 µg/ml), sabinene (15 µg/ml) and mixture of trepinen-4-ol (13 µg/ml) and sabinene (15 µg/ml) were able to relax the vascular tensions of endothelium-intact aortic rings precontracted with PE (1 µM) (Figure 49 – 50). At the concentration of 40 µg/ml, plai oil caused 33.18 ± 2.84 % (*n* = 18) relaxation. Terpinen-4-ol (13 µg/ml), sabinene (15 µg/ml) and mixture of terpinen-4-ol (13 µg/ml) and sabinene (15 µg/ml) significantly different from plai oil induced relaxation at 45 ± 1.22 % (*n* = 5), 5 ± 0.70 % (*n* = 5), and 29 ± 0.33 % (*n* = 5), respectively (Figure 51). In contrast, the vasodilation in endothelium effects of plai oil was more prominent in endothelium-intact aortic rings than in endothelium-denuded preparations and terpinen-

4-ol, sabinene and mixture had not resulted in a significant induced relaxation effects on PE-induced contraction in endothelium-denuded aortic rings.

2.3 The endothelium-dependent relaxant mechanism of the Plai oil

The endothelium-dependent relaxation of plai oil reduced significantly in the presence of certain vasodilators including indomethacin, atropine, propranolol, glibenclamide, tetraethylammonium (TEA), L-NAME and methylene blue. In this study, plai oil at concentration of 40 µg/m caused endothelium-dependent relaxation of 33.18 ± 2.84% (*n* = 18). The presence of indomethacin (10 µM), atropine (1 µM), propranolol (10 µM) and glibenclamide (10 µM) significantly reduced plai oil-induced relaxation (Figure 55-58). Atropine produced the highest inhibition of plai oil-induced relaxation by 97.59% in endothelium-intact aortic rings. In contrast, methylene blue (10 µM), L-NAME (10 µM) and tetraethylammonium (10 µM) exhibited less influence on plai oil-induced relaxation (Figure 52-54). Methylene blue was the less potent inhibitor of plai oil-induced relaxation. The relaxation reduced by 55.13 % in the presence of methylene blue endothelium-intact aortic rings (Figure 59).

The endothelium-independent mechanism of plai oil induced relaxation was also investigated. Plai oil at concentration of 40 µg/m caused relaxation of endothelium-denuded aortic rings pretreated with PE at the magnitude of 21.18 ± 2.43 % (*n* = 17). The results showed that neither of methylene bule, atropine, propranolol or glibenclamide could interfere plai oil-induced vasorelaxation in endothelium-denuded aortic rings (Figure 60-64).

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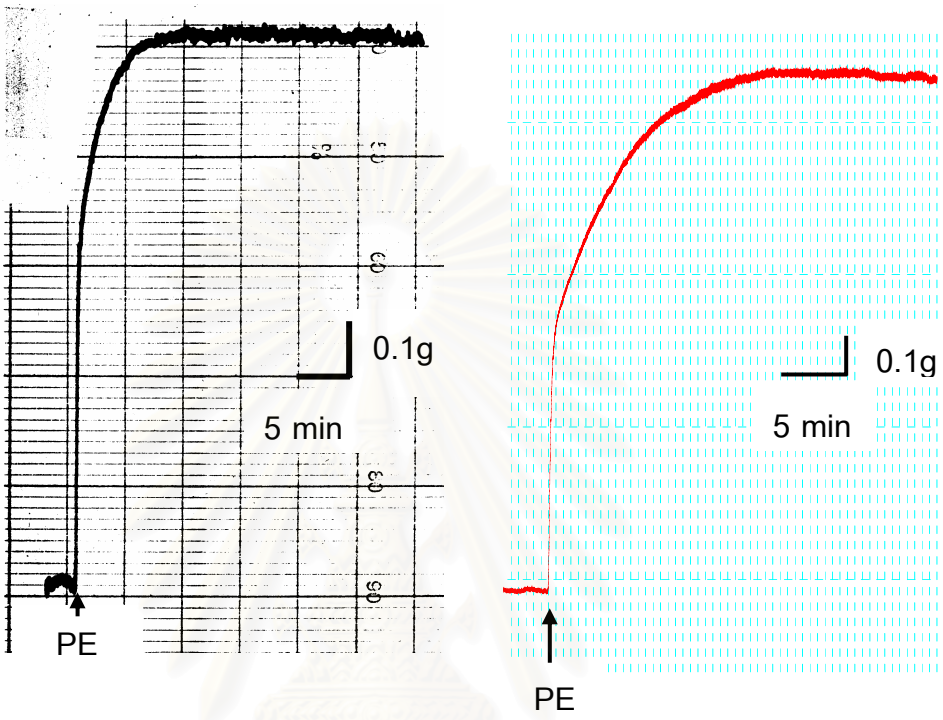

(1a) (1b)

Figure 4 Representative tracing shows the PE-induced contraction of endothelium-intact aortic ring (1a) and endothelium-denuded aortic ring (1b) in Ca^{2+}-containing solution.

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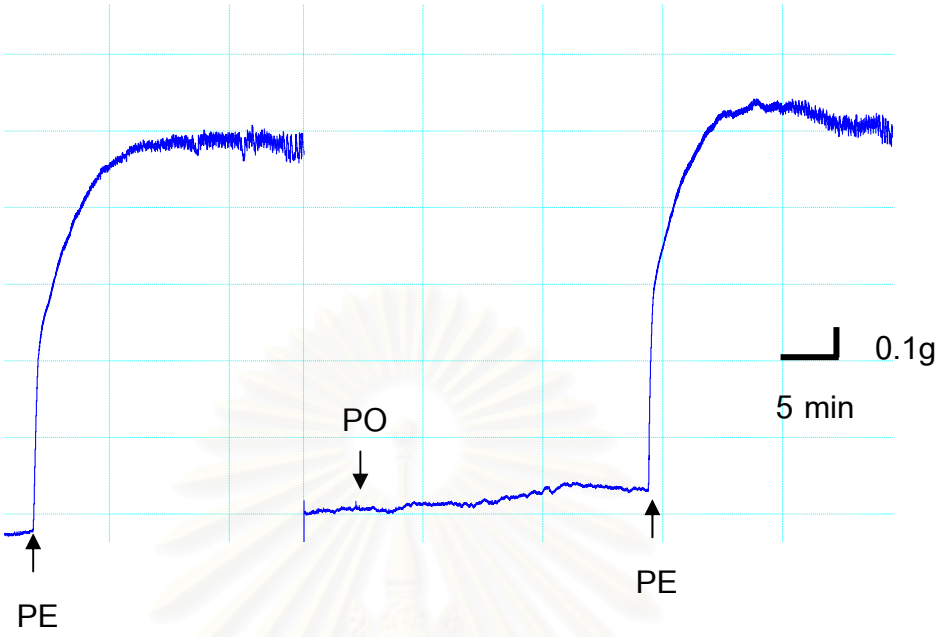

Figure 5 Representative tracing shows the effect of plai oil (10 µg/ml) on the PE-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

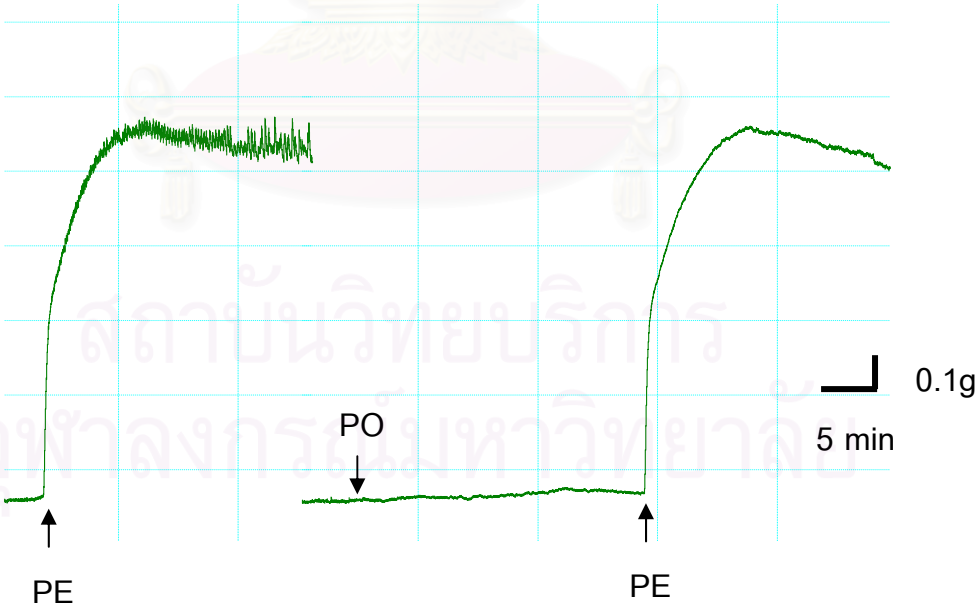

Figure 6 Representative tracing shows the effect of plai oil (50 µg/ml) on the PE-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

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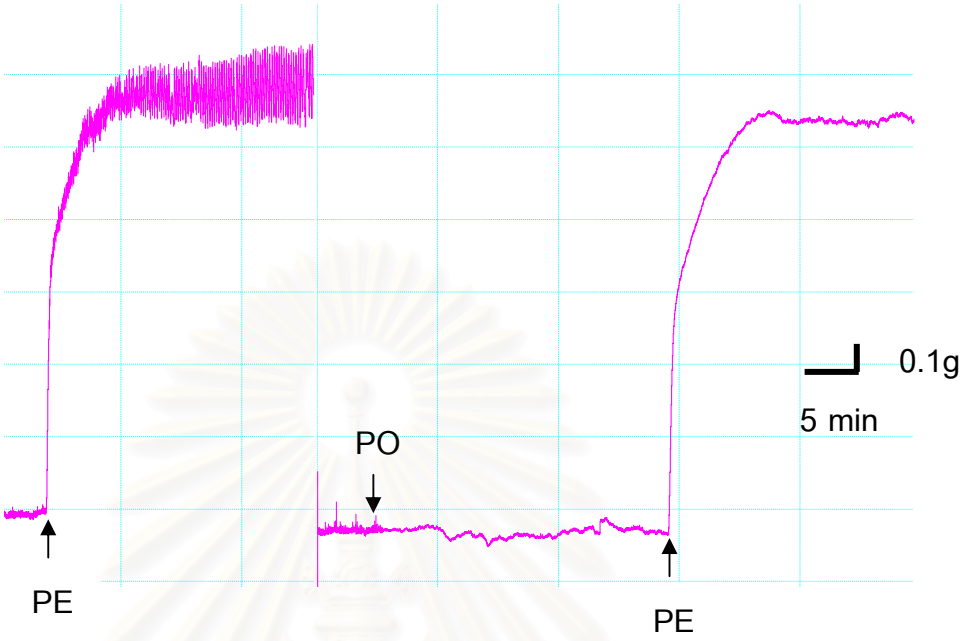

Figure 7 Representative tracing shows the effect of plai oil (100 µg/ml) on the PE-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

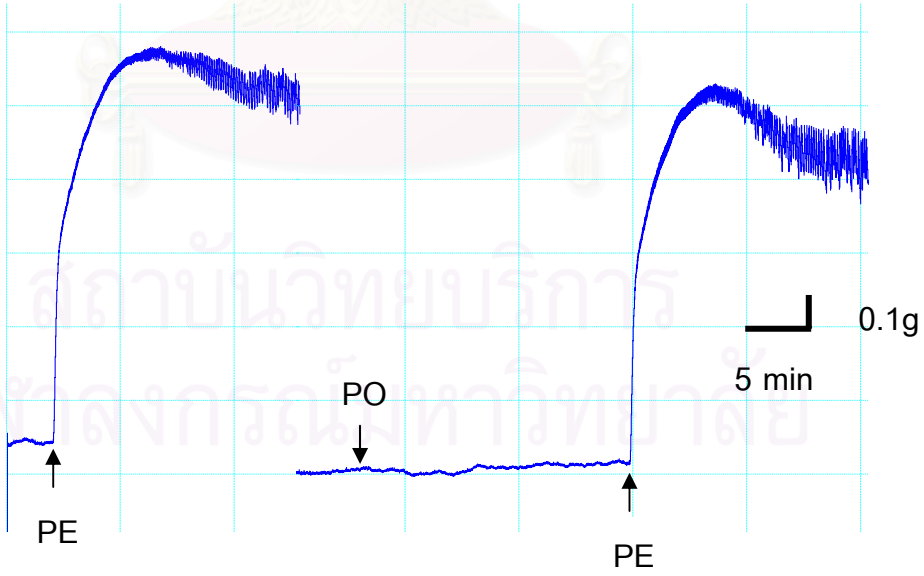

Figure 8 Representative tracing shows the effect of plai oil (200 µg/ml) on the PE-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

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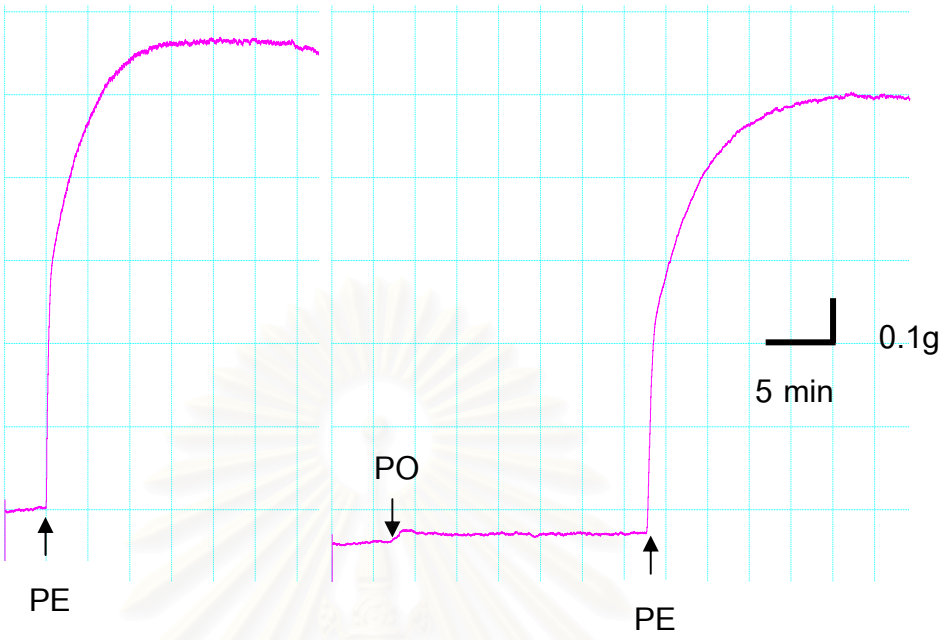

Figure 9 Representative tracing shows the effect of plai oil (10 µg/ml) on the PE-induced contraction of endothelium-denuded aortic ring in Ca^{2+}-containing solution.

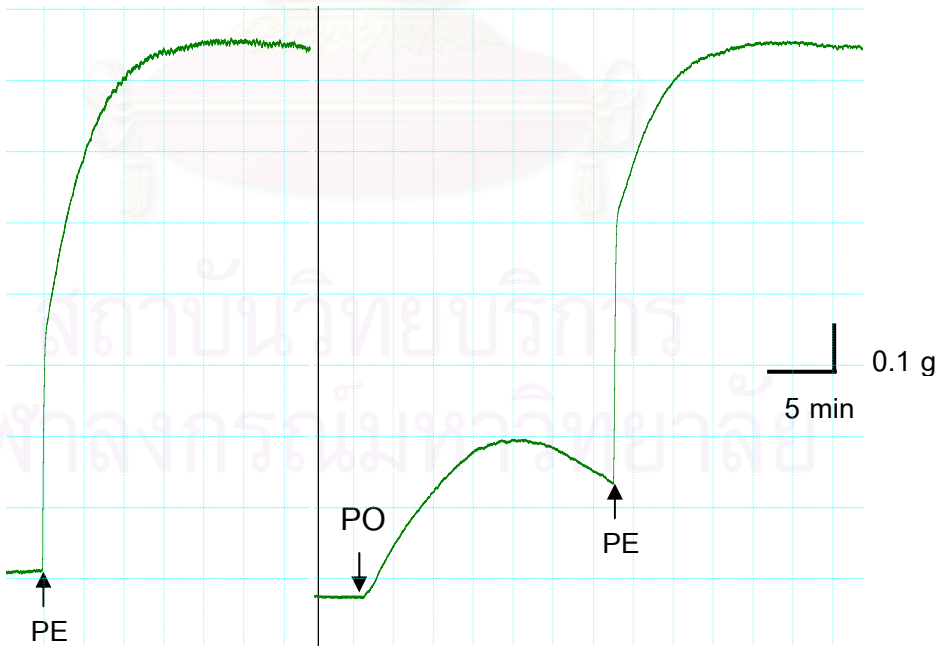

Figure 10 Representative tracing shows the effect of plai oil (50 µg/ml) on the PE-induced contraction of endothelium-denuded aortic ring in Ca^{2+}-containing solution.

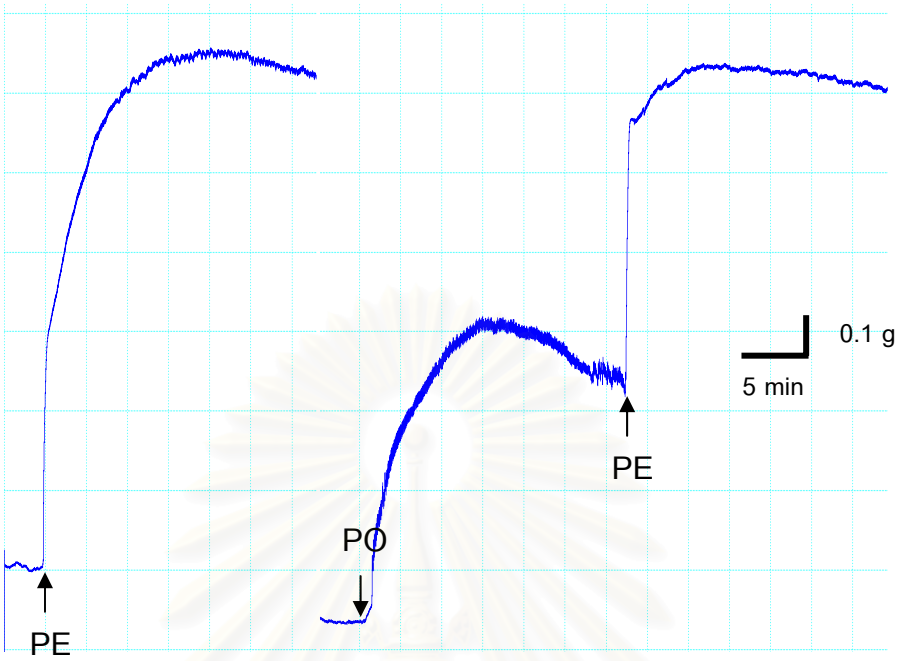

Figure 11 Representative tracing shows the effect of plai oil (100 µg/ml) on the PE-induced contraction of endothelium-denuded aortic ring in Ca^{2+}-containing solution.

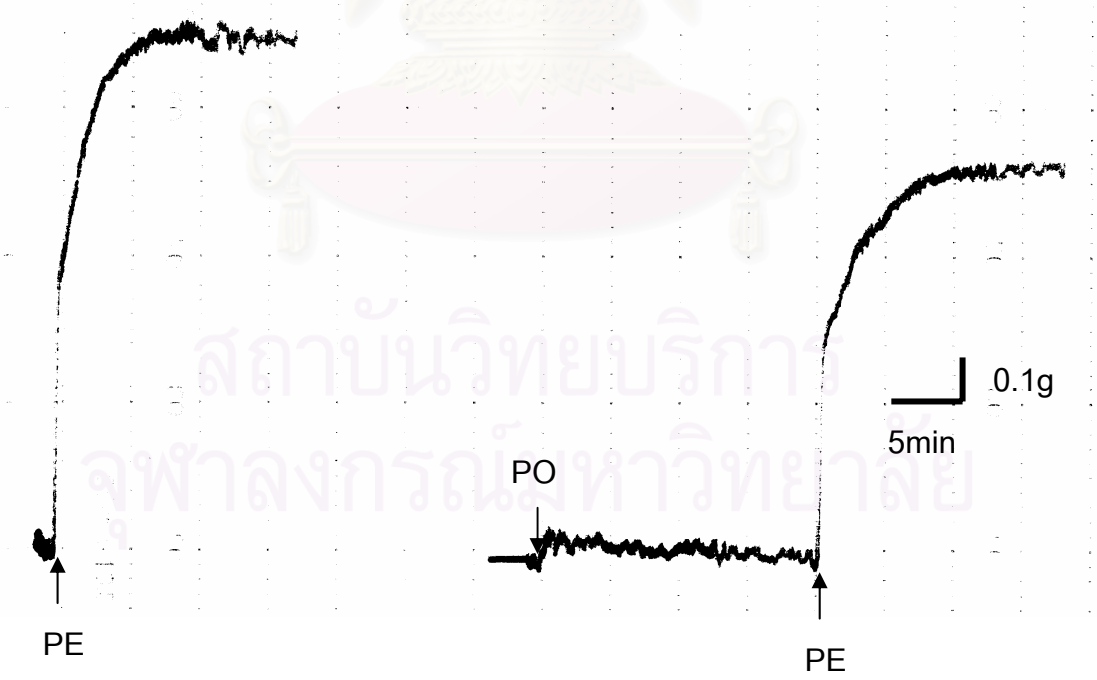

Figure 12 Representative tracing shows the effect of plai oil (200 µg/ml) on the PE-induced contraction of endothelium-denuded aortic ring in Ca^{2+}-containing solution.

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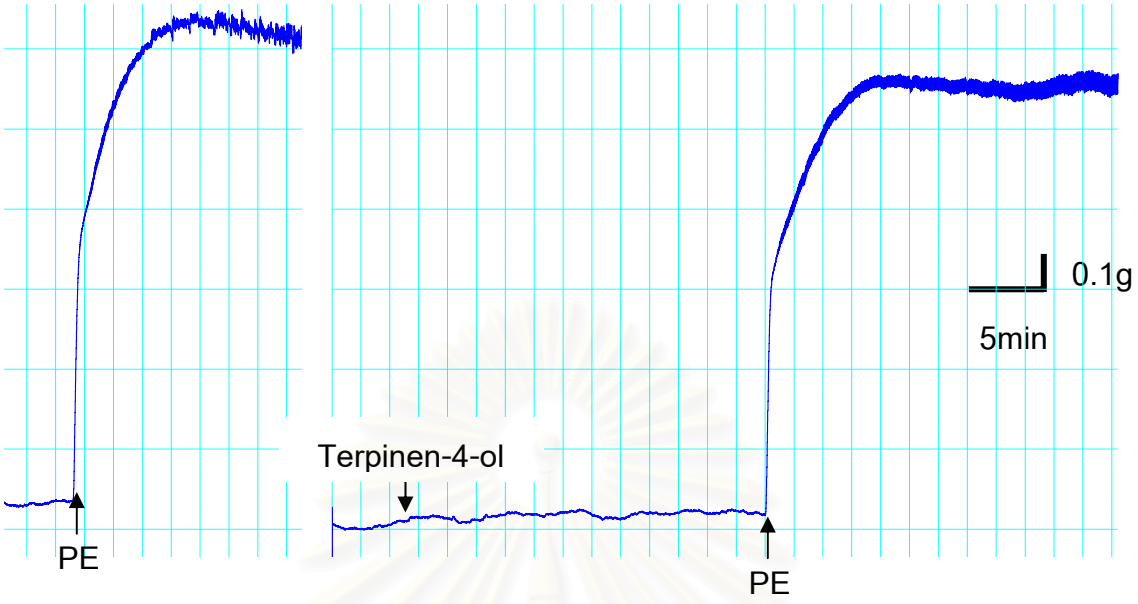


Figure 13 Representative tracing shows the effect of terpinen-4-ol (34 µg/ml) on the contraction on the PE-induced contraction of endothelium-intact aortic ring in Ca^{2+}- containing solution.

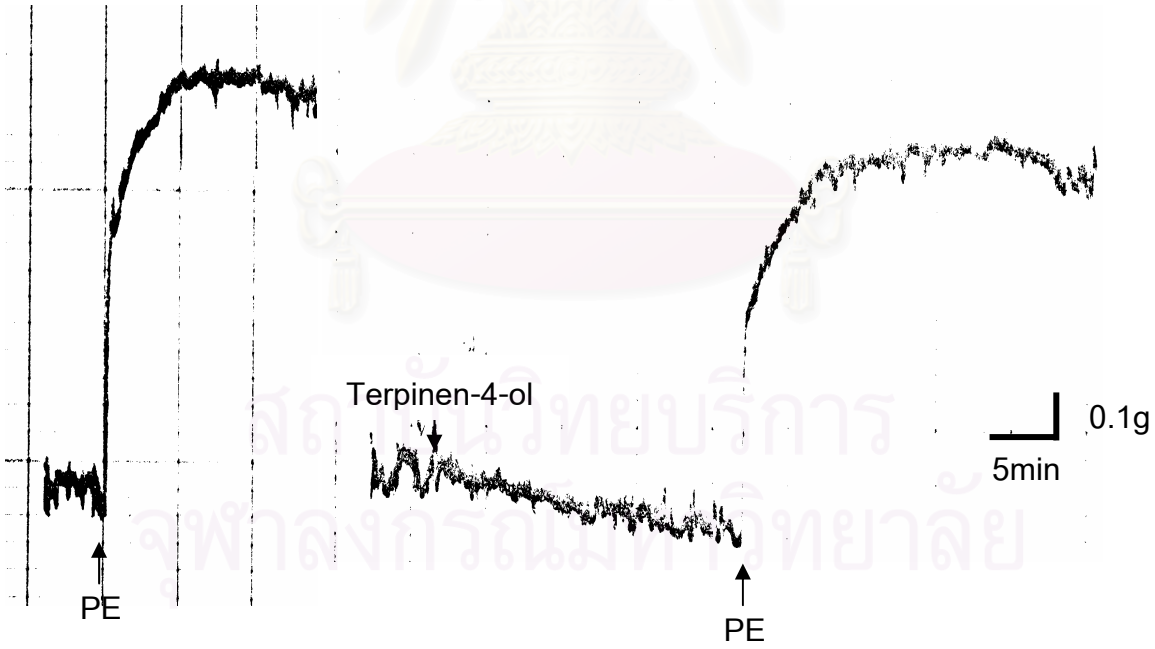


Figure 14 Representative tracing shows the effect of terpinen-4-ol (34 µg/ml) on the contraction on the PE-induced contraction of endothelium-denuded aortic ring in Ca^{2+}- containing solution.

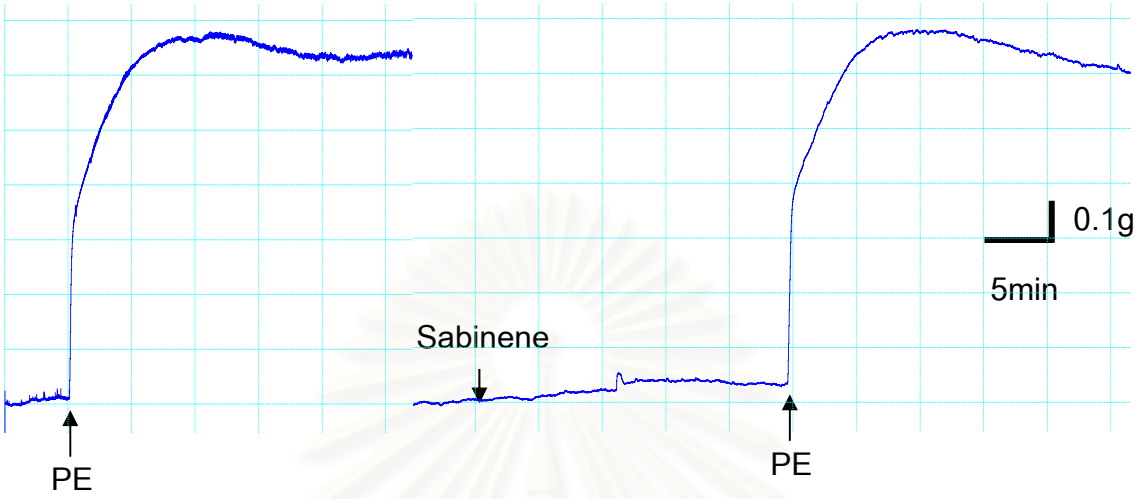


Figure 15 Representative tracing shows the effect of sabinene(39µg/ml) on the contraction on the PE-induced contraction of endothelium-intact aortic ring in Ca^{2+}- containing solution.

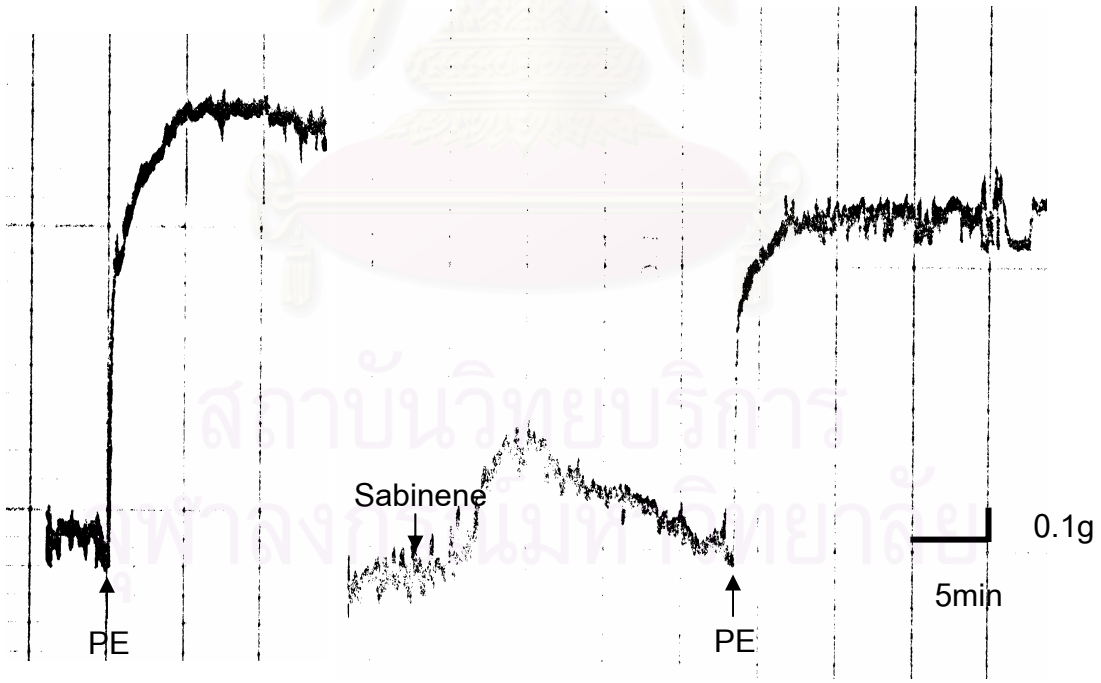


Figure 16 Representative tracing shows the effect of sabinene (39 µg/ml) on the contraction on the PE-induced contraction of endothelium-denuded aortic ring in Ca^{2+}- containing solution.

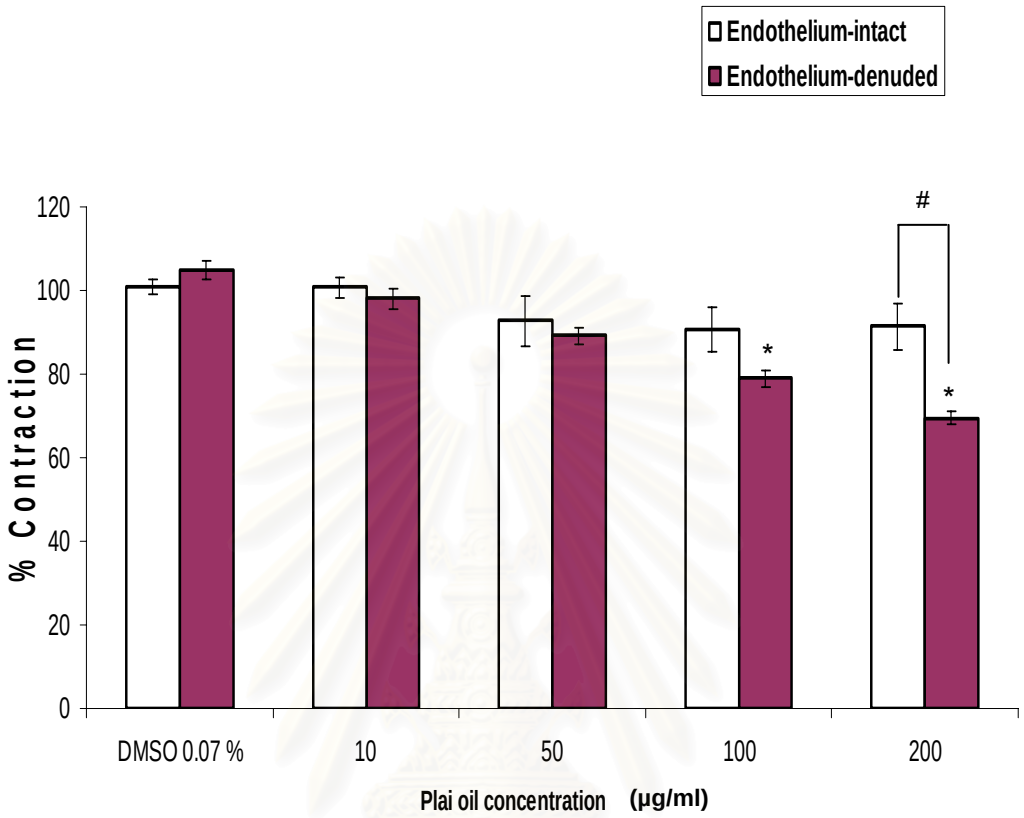


Figure17 Effects of plai oil on contraction of endothelium-intact and endothelium-denuded aortic rings induced by PE (1 µM) in Ca^{2+}-containing solution.

Data were presented mean ± S.E.M, n = 5 – 6.

* p<0.05 showed significant difference from DMSO 0.07 % (v/v).

# p<0.05 showed significant difference from endothelium-intact group.

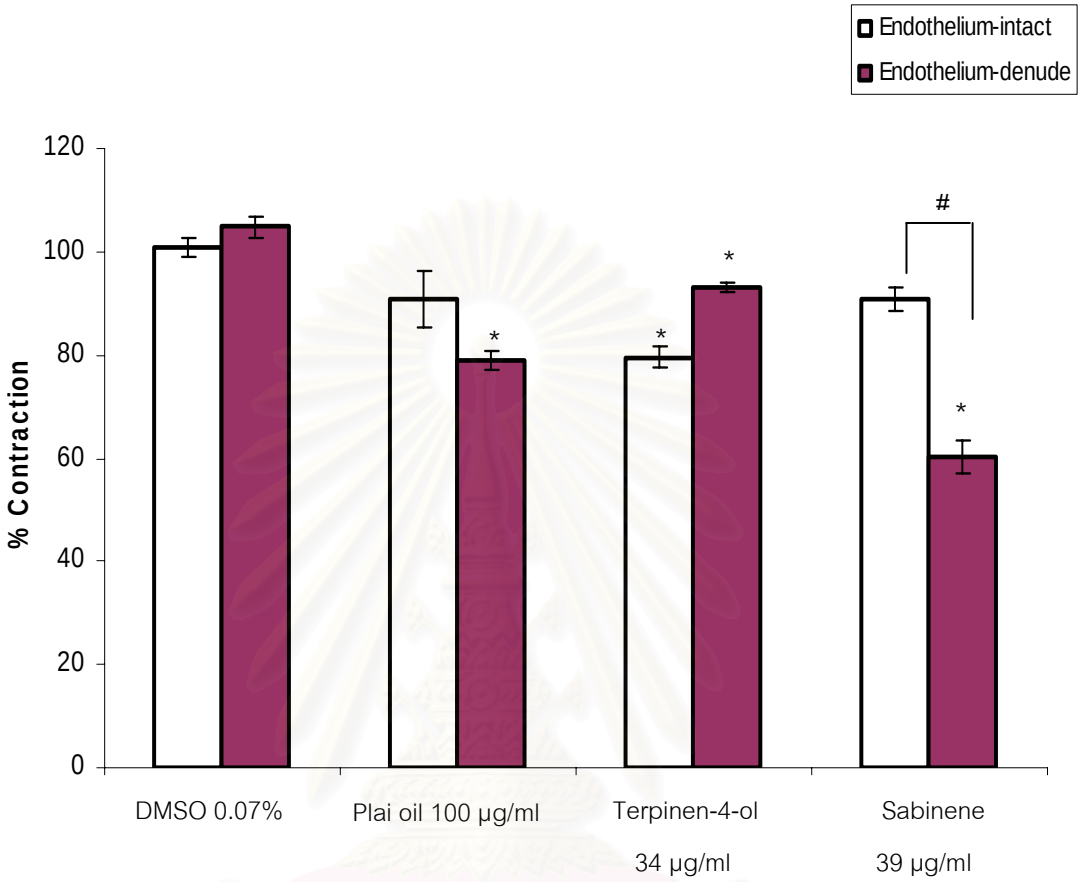
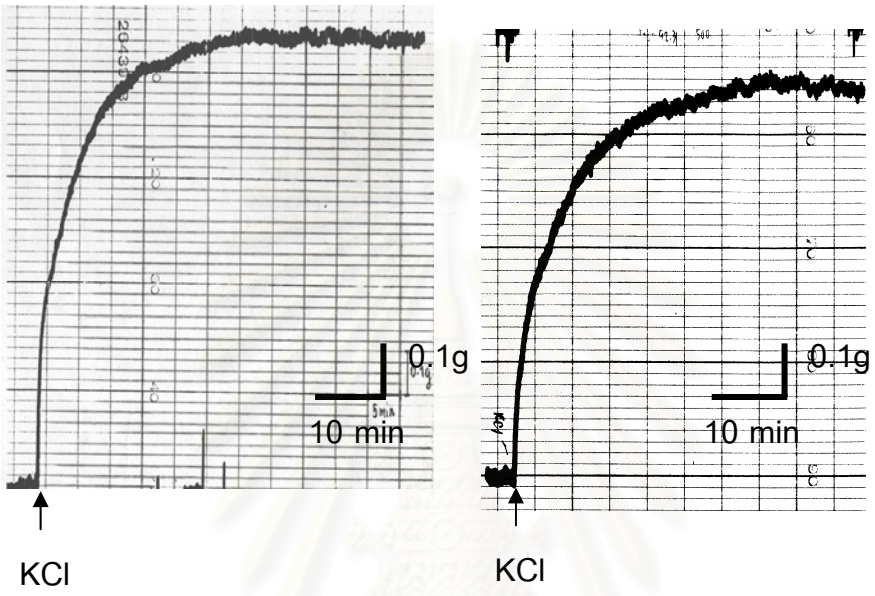


Figure18 Effects of plai oil, terpinen-4-ol and sabinene on contraction of endothelium-intact and endothelium-denuded aortic rings induced by PE (1 µM) in Ca^{2+}- containing solution.

Data were presented mean ± S.E.M, *n* = 5 - 6.

* p<0.05 showed significant difference from DMSO 0.07 % (v/v).

# *p*<0.05 showed significant difference from endothelium-intact group.



(1a) (1b)

Figure 19 Representative tracing shows the KCl -induced contraction of endothelium-intact aortic ring (1a) and endothelium-denuded aortic ring (1b) in Ca^{2+}- containing solution.

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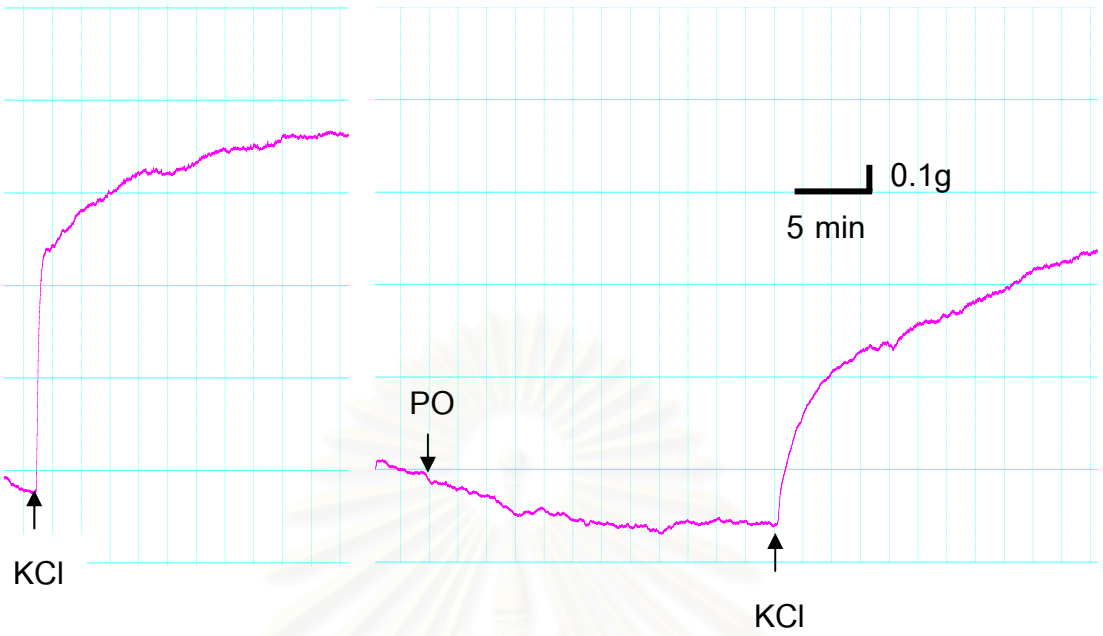


Figure 20 Representative tracing shows the effect of plai oil (10 µg/ml) on the KCl-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

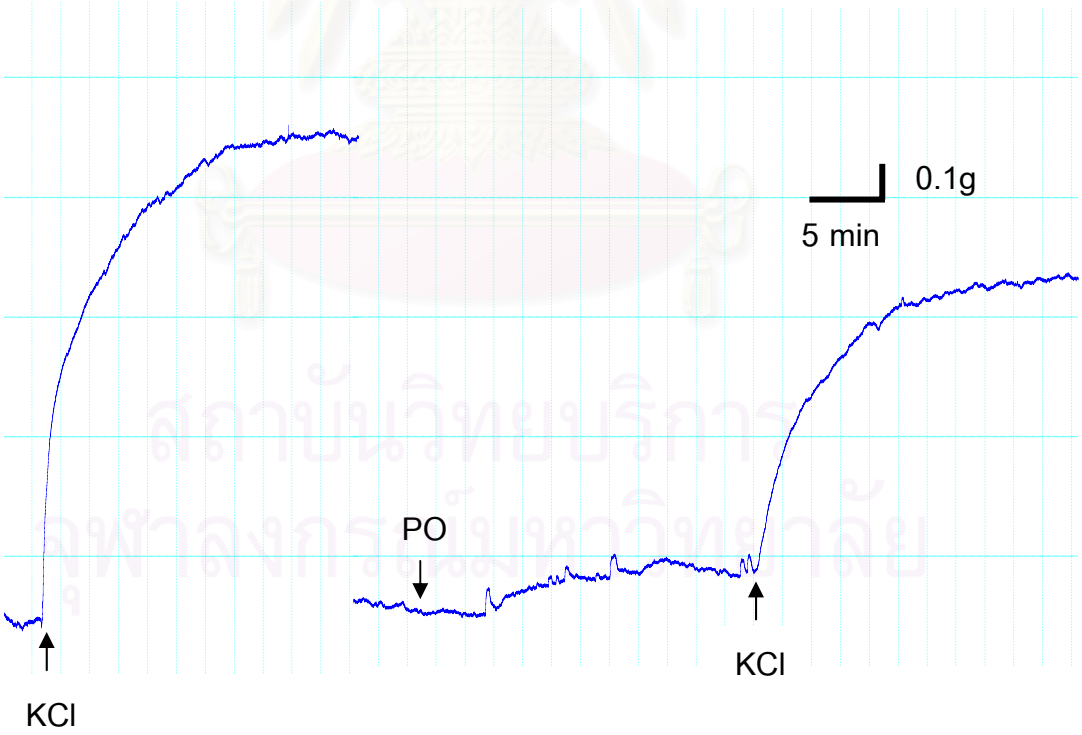


Figure 21 Representative tracing shows the effect of plai oil (50 µg/ml) on the KCl-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

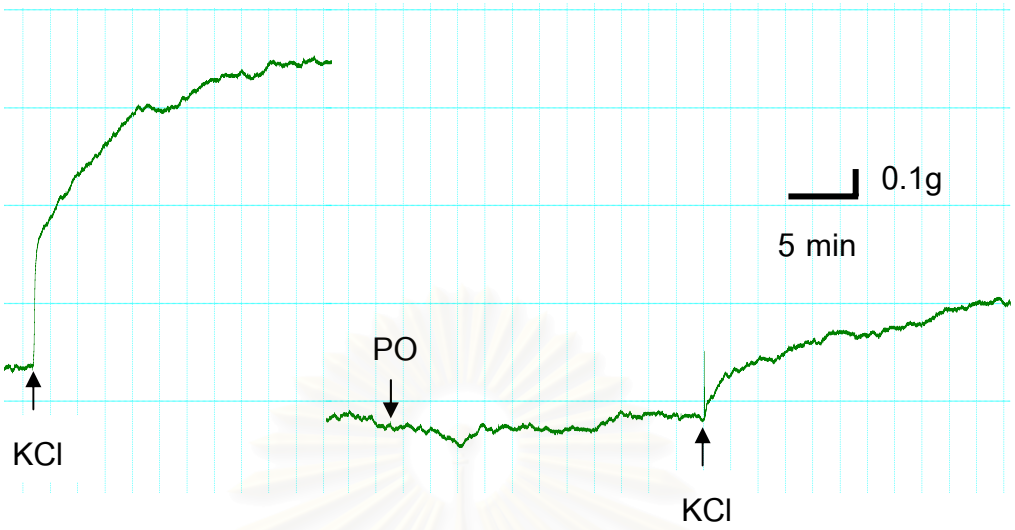


Figure 22 Representative tracing shows the effect of plai oil (100 µg/ml) on the KCl-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

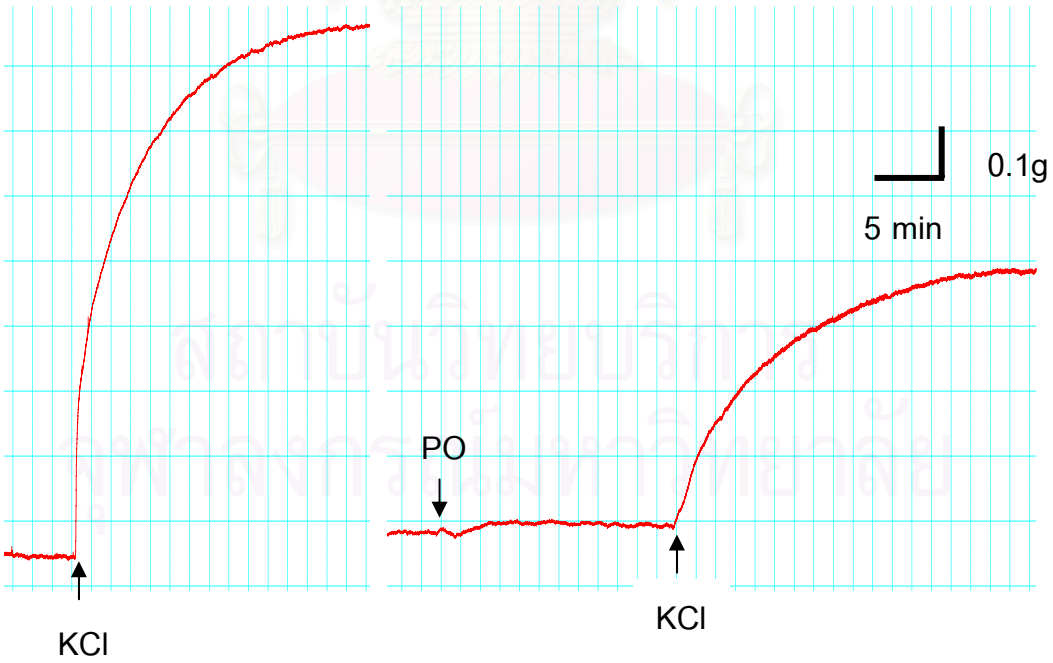


Figure23 Representative tracing shows the effect of plai oil (200 µg/ml) on the KCl-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

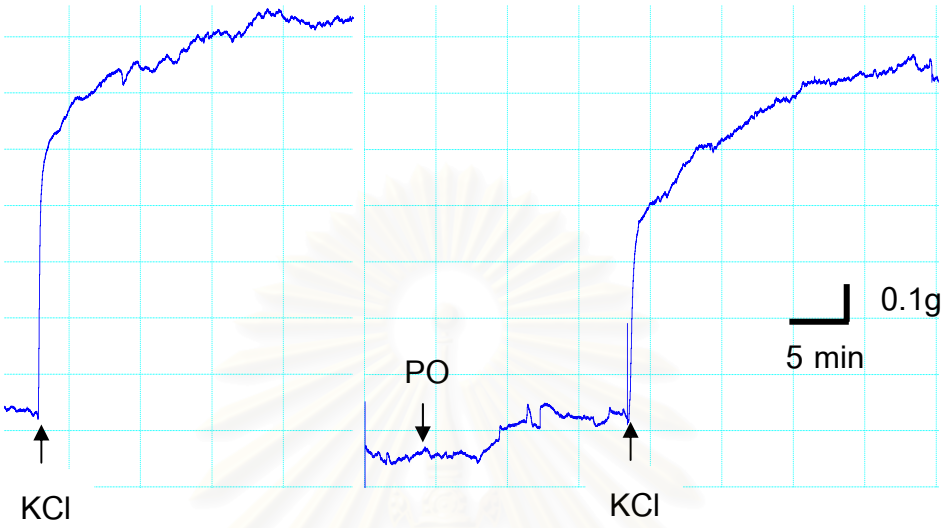


Figure 24 Representative tracing shows the effect of plai oil (10 µg/ml) on the KCl-induced contraction of endothelium-denuded aortic ring in Ca^{2+}-containing solution.

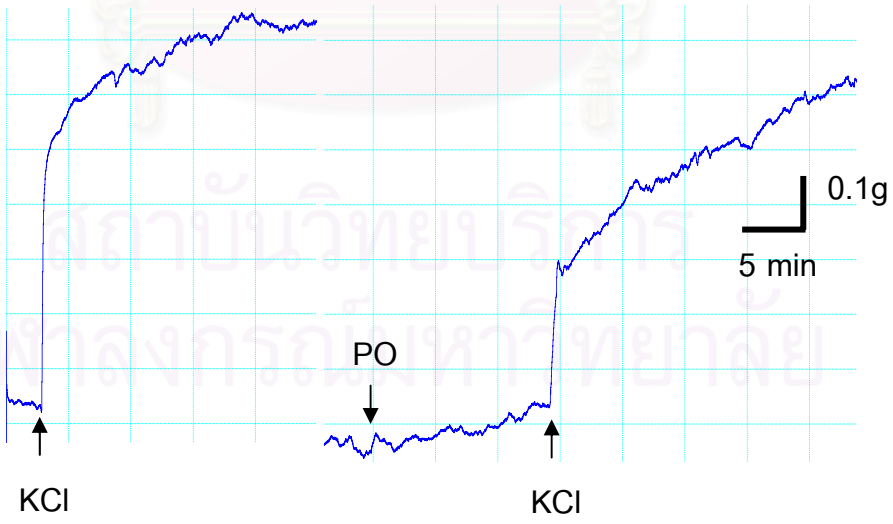


Figure 25 Representative tracing shows the effect of plai oil (50 µg/ml) on the KCl-induced contraction of endothelium-denuded aortic ring in Ca^{2+}- containing solution.

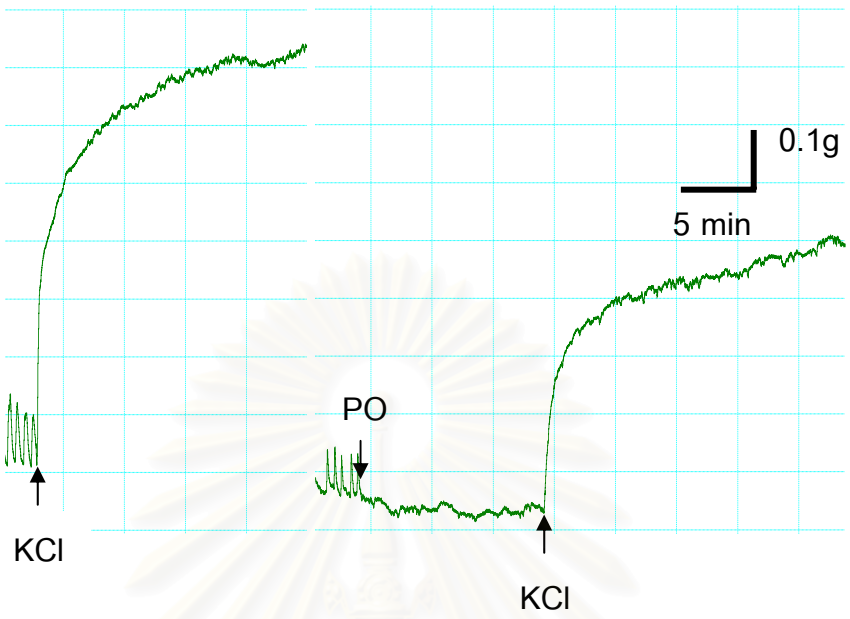


Figure 26 Representative tracing shows the effect of plai oil (100 µg/ml) on the KCl-induced contraction of endothelium-denuded aortic ring in Ca^{2+}- containing solution.

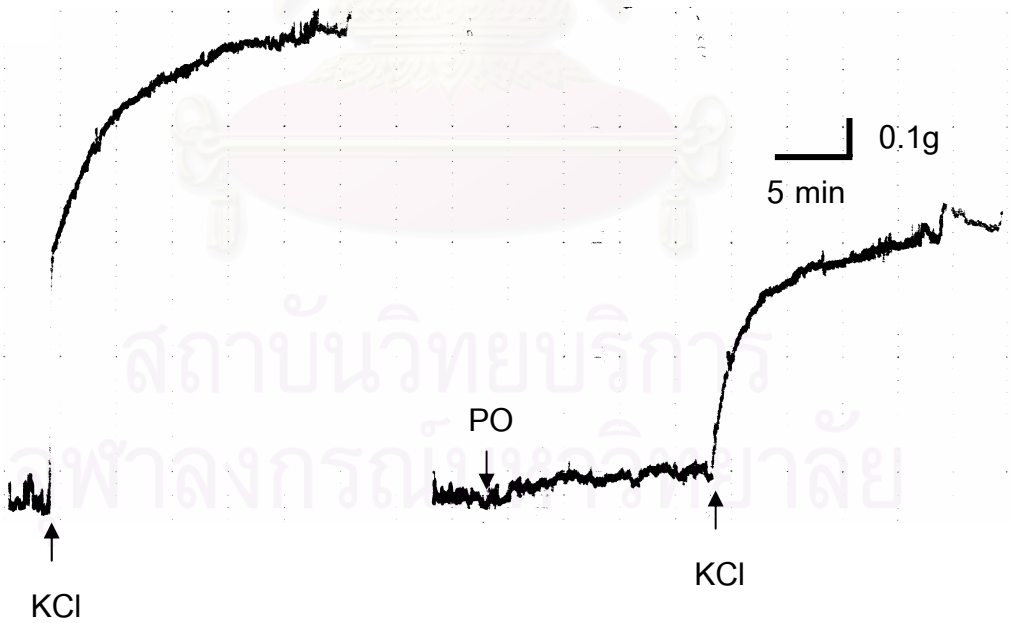


Figure 27 Representative tracing shows the effect of plai oil (200 µg/ml) on the KCl-induced contraction of endothelium-denuded aortic ring in Ca^{2+}- containing solution.

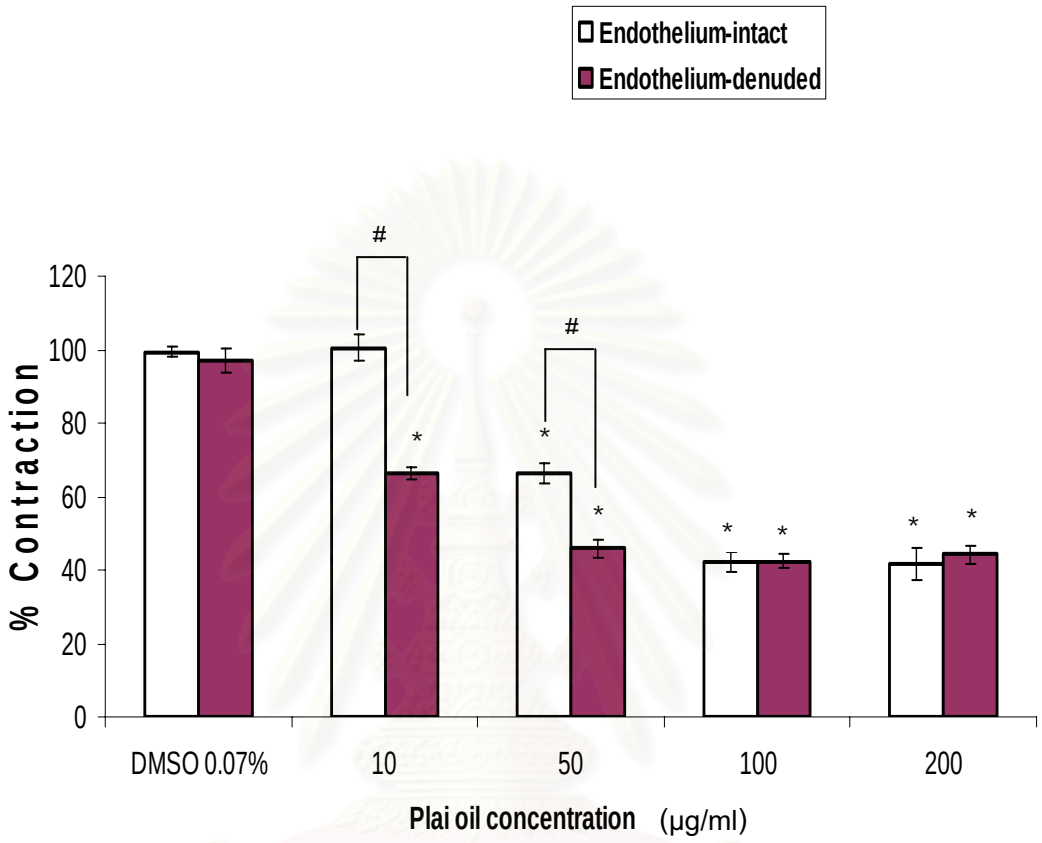


Figure28 Effects of plai oil on contraction of endothelium-intact and endothelium-denuded aortic rings induced by KCl (40 mM) in Ca^{2+}-containing solution.

Data were presented mean ± S.E.M, n = 5 – 6.

* p<0.05 showed significant difference from DMSO 0.07% (v/v), the comparisons were perferned with in the same aortic preparations.

# p<0.05 showed significant difference from endothelium-intact group.

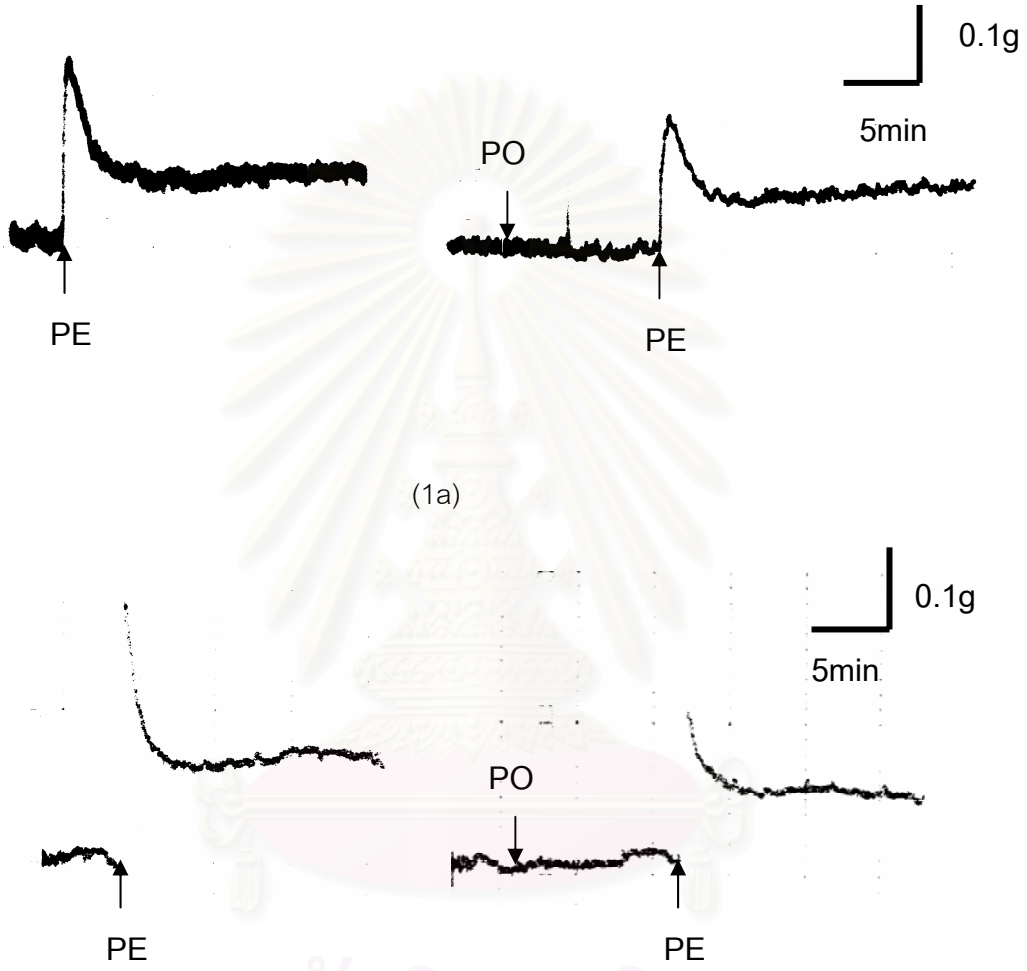
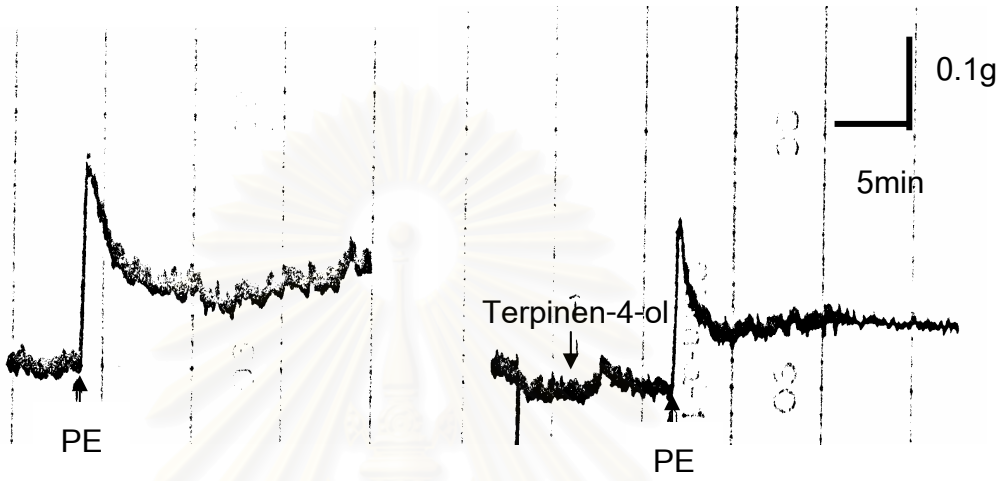
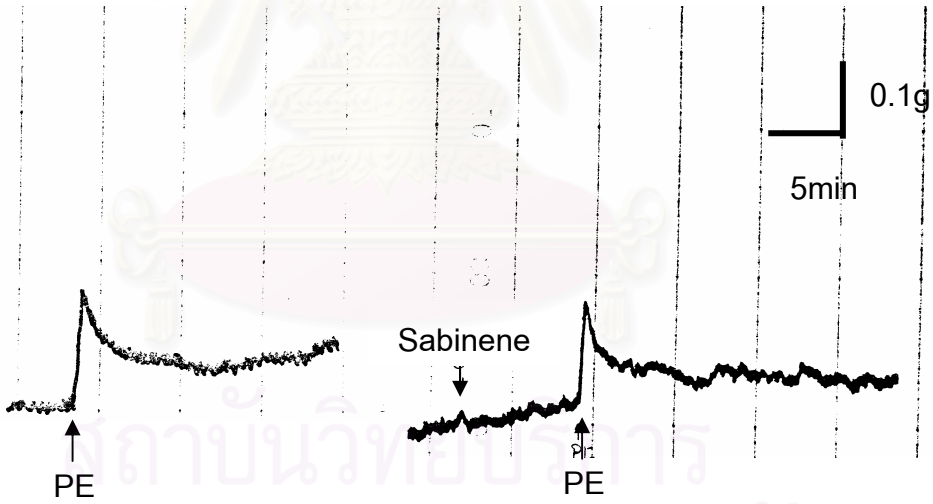


Figure 29 Representative tracing shows the effect of plai oil 40 µg/ml(1a) and 100 µg/ml (1b) on PE-induced contraction of endothelium-denuded aortic ring in Ca^{2+}- free medium.



(1a)



(1b)

Figure 30 Representative tracing shows the effect of terpinen-4-ol (34 µg/ml) (1a), and sabinene (39 µg/ml) (1b) on PE-induced contraction of endothelium-denuded aortic ring in Ca^{2+}- free medium.

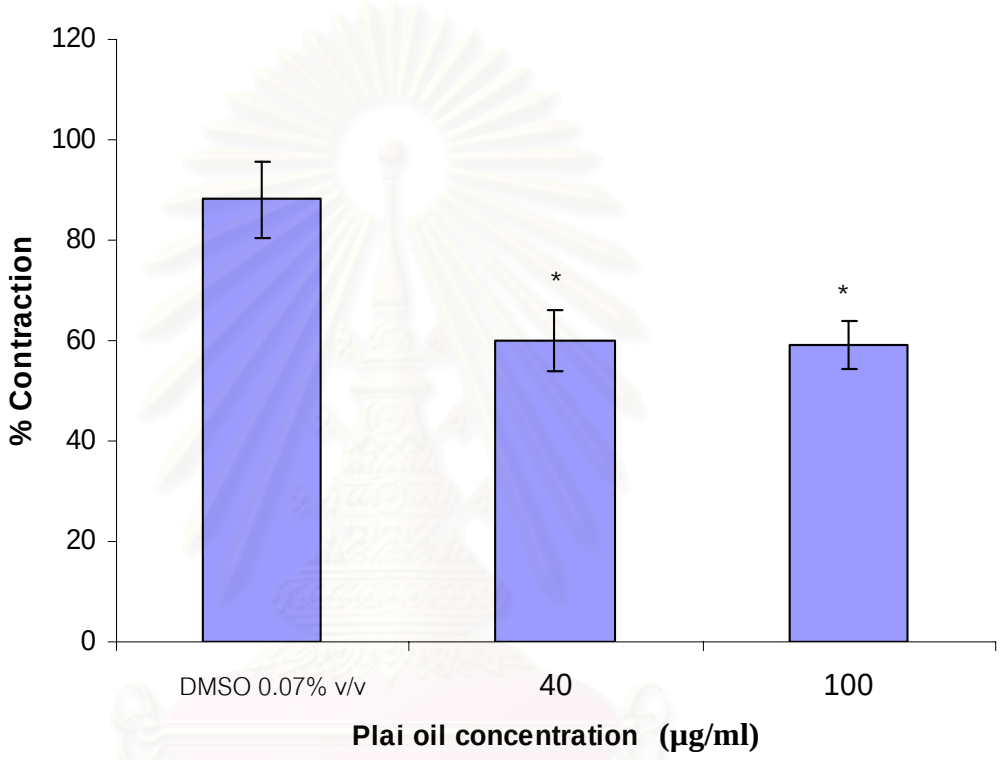


Figure 31 Effect of plai oil on endothelium-denuded aortic contraction induced by PE (1 µM) in Ca^{2+}-free medium.

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

* p<0.05 showed significant difference from DMSO 0.07% (v/v).

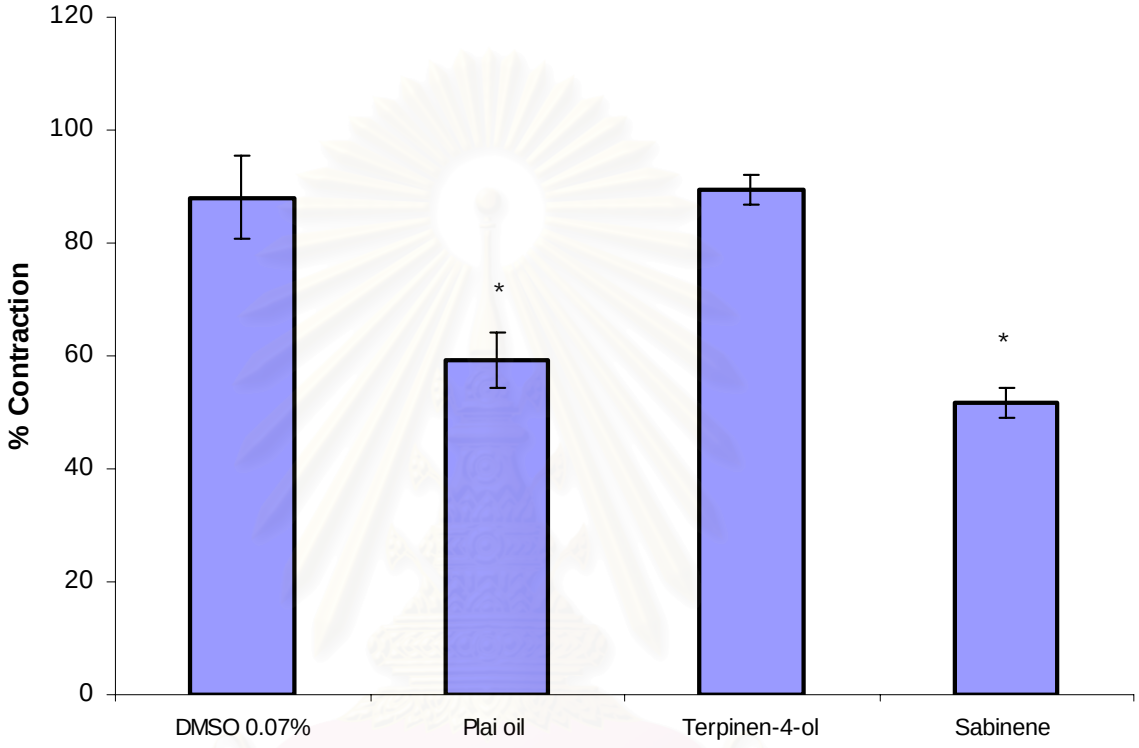
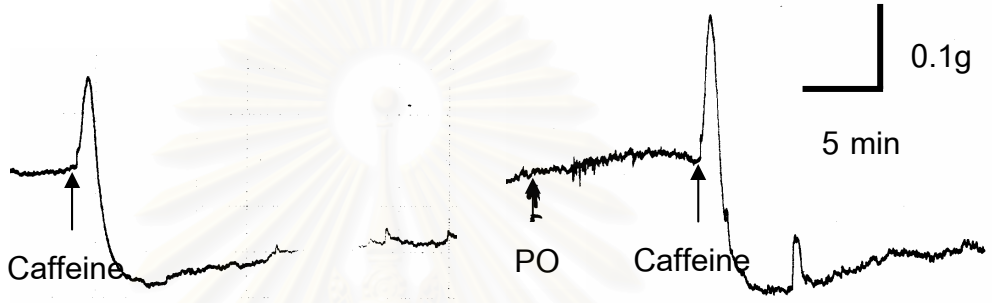


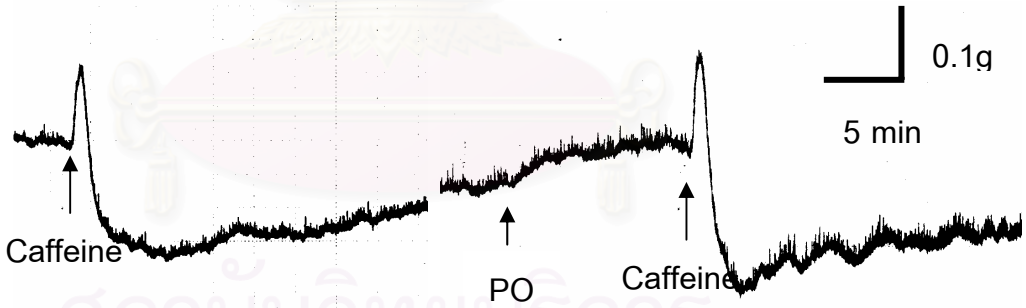
Figure 32 Effect of Plai oil (100 µg/ml), terpinen-4-ol (34 µg/ml), sabinene (39 µg/ml) on endothelium-denuded aortic contraction induced by PE (1µM) in Ca^{2+}-free medium.

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

* p<0.05 showed significant difference from DMSO 0.07% (v/v).



(1a)



(1b)

Figure 33 Representative tracing shows the effect of plai oil 40 µg/ml (1a), 100 µg/ml (1b) on caffeine–induced contraction of endothelium-denuded aortic ring in Ca^{2+}-free medium.

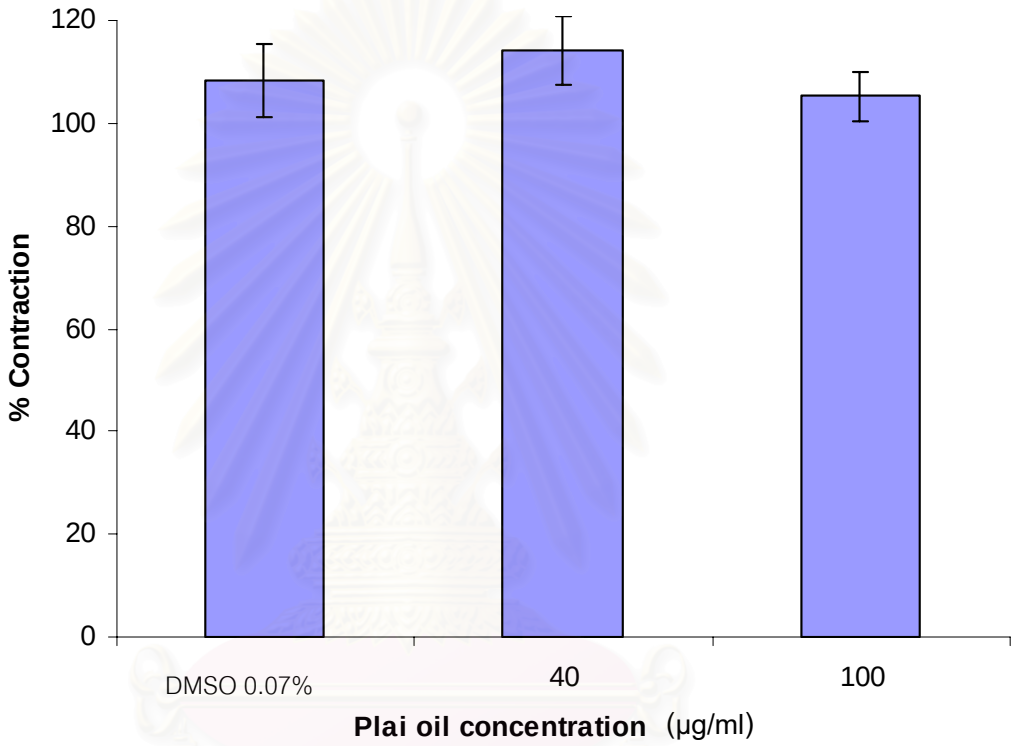
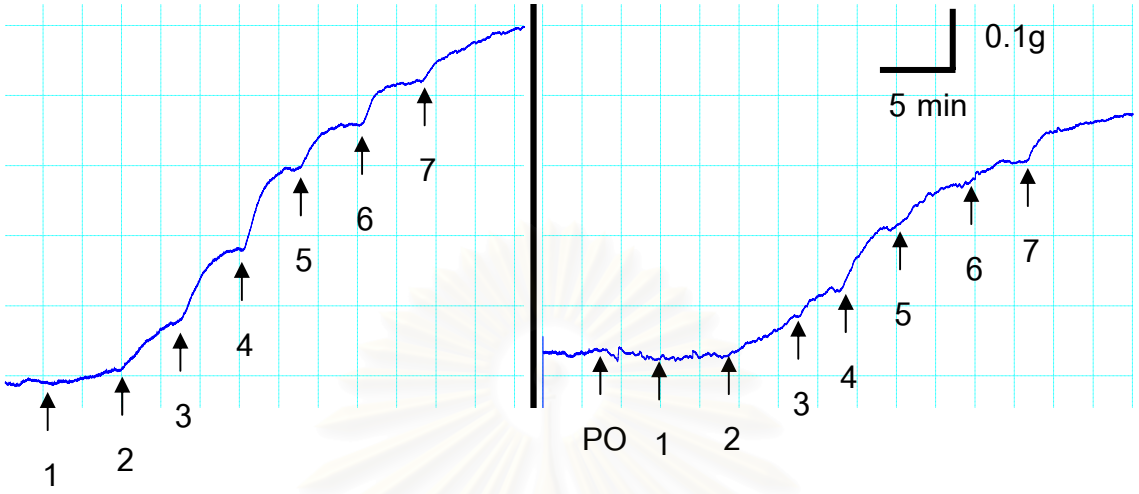
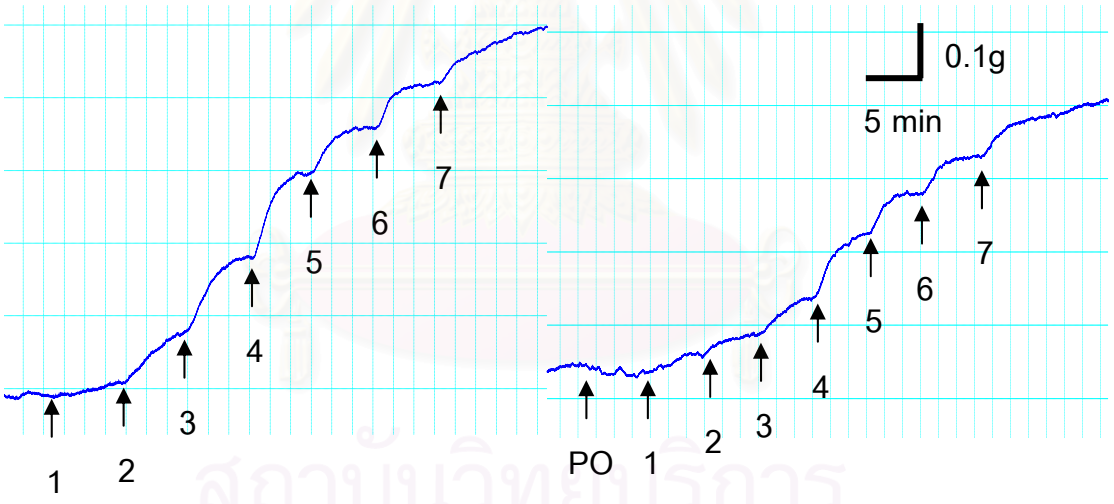


Figure 34 Effect of plai oil on endothelium-denuded aortic contraction induced by caffeine (10 mM) in Ca^{2+}- free medium.

Data were presented as mean ± S.E.M, *n* = 5.



(1a)



(1b)

Figure 35 Representative tracing shows the effect of plai oil 50 µg/ml (1a), and 100µg/ ml (1b) on $CaCl_2$- induced aortic contraction in high K^+-Ca^{2+}-free depolarizing solution.

(1-7; $CaCl_2$ concentration 10^{-5}, 5×10^{-5}, 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2} M)

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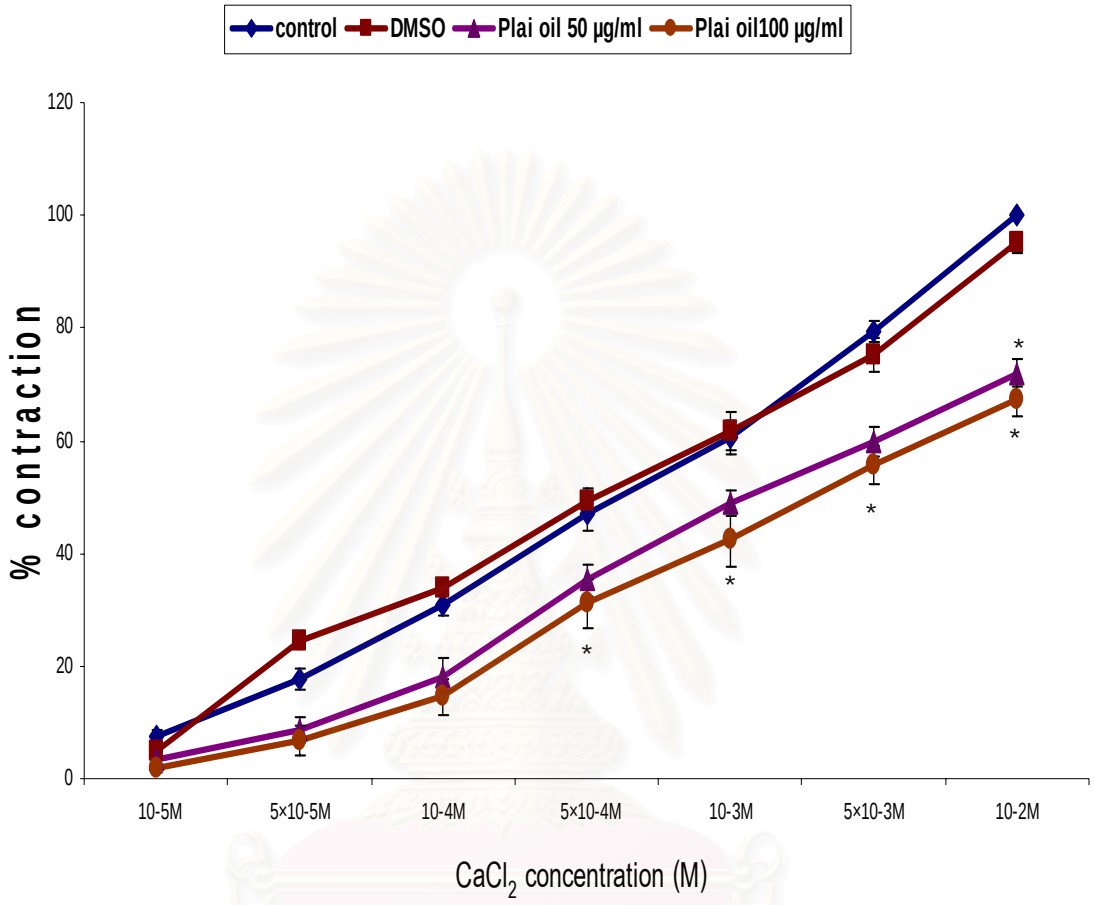

Figure 36 Effect of plai oil on endothelium-denuded aortic contraction induced by cumulative addition of $CaCl_2$ in Ca^{2+}–free depolarizing solution.

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

*p<0.05 showed significant difference from DMSO 0.07% (v/v).

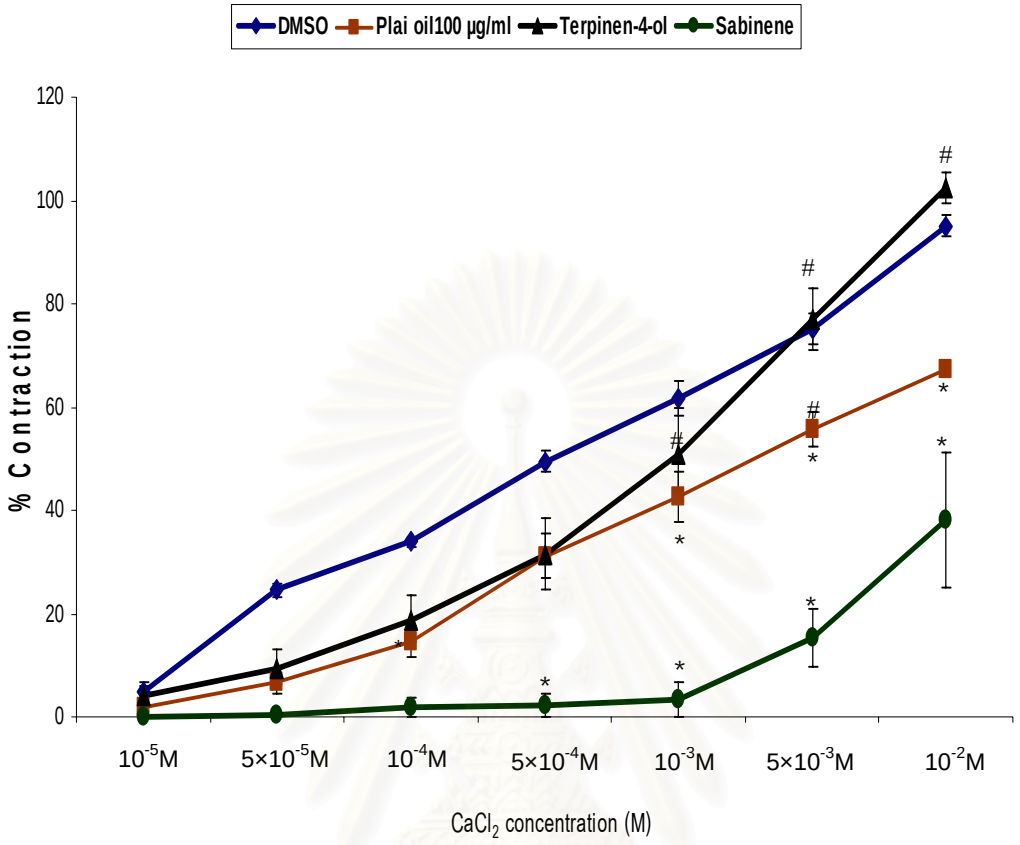

Figure 37 Effect of plai oil (100 µg/ml), terpinen-4-ol (34 µg/ml) and sabinene (39 µg/ml) on endothelium-denuded aortic contraction induced by cumulative addition of $CaCl_2$ in Ca^{2+}–free depolarizing solution.

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

*p<0.05 showed significant difference from DMSO 0.07 % (v/v).

# p<0.05 showed significant difference from sabinene (39 µg/ml).

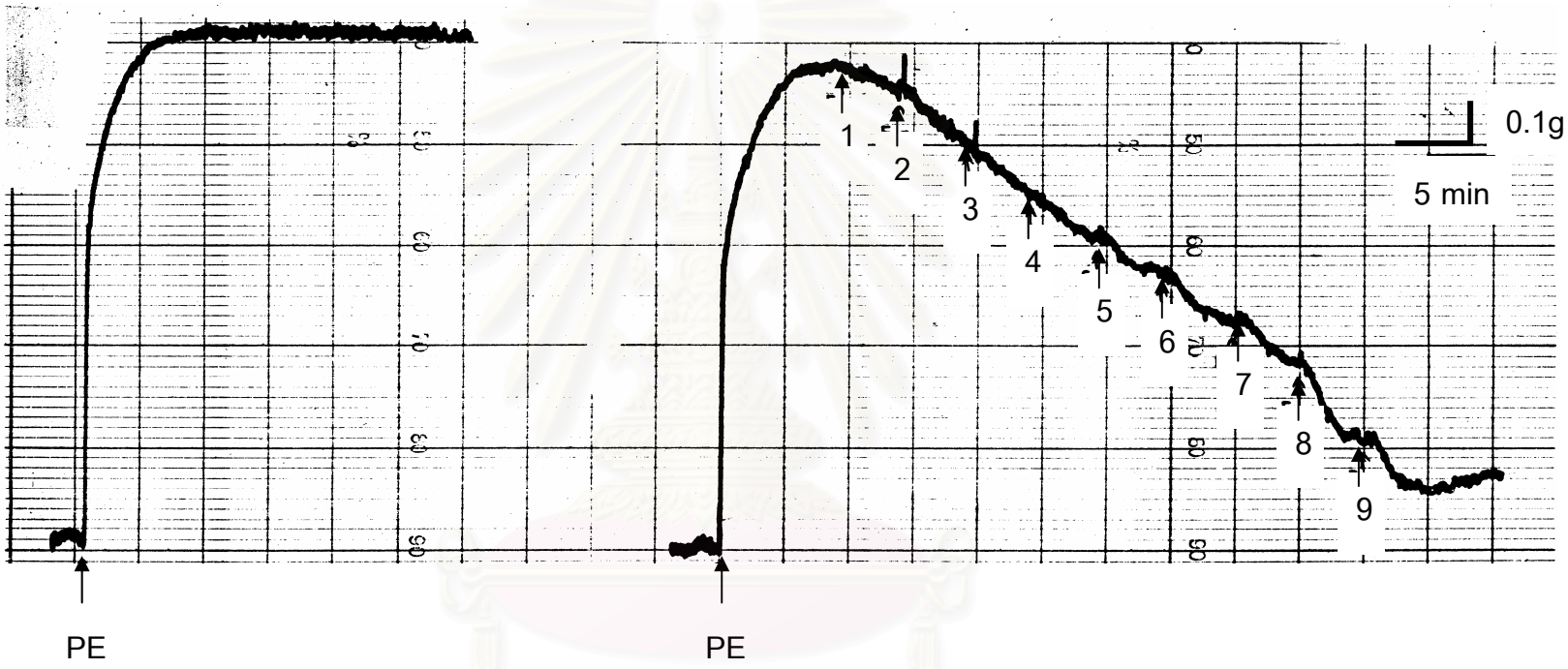

Figure 38 Representative tracing shows the relaxation induced by cumulative addition of plai oil on PE-induced contraction of endothelium-intact aortic rings. Plai oil (PO) concentrations were 1=1.25, 2=2.5, 3=5, 4=10, 5=20, 6=30, 7=40, 8=50, 9=60 µg/ml.

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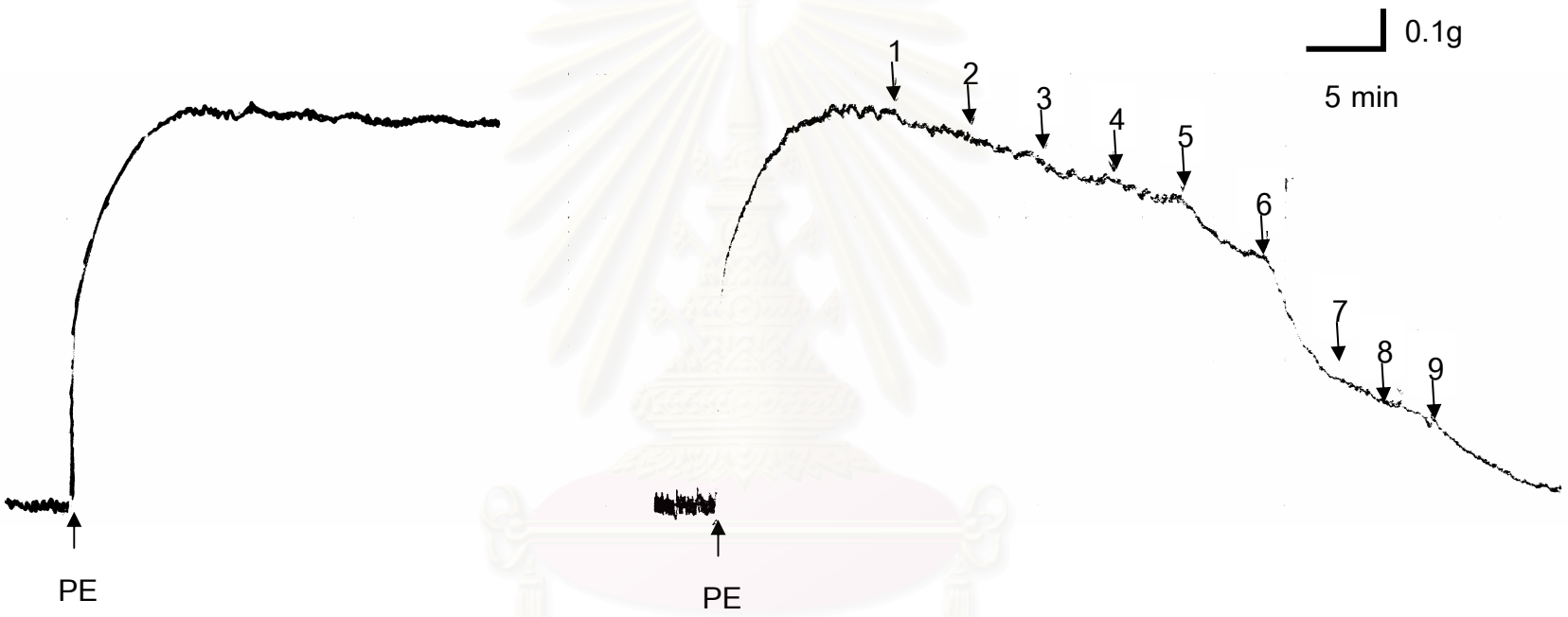

Figure 39 Representative tracing shows the relaxation induced by cumulative addition of plai oil on PE-induced contraction of endothelium-denuded aortic rings. Plai oil (PO) concentrations were 1=1.25, 2=2.5, 3=5, 4=10, 5=20, 6=30, 7=40, 8=50, 9=60 µg/ml.

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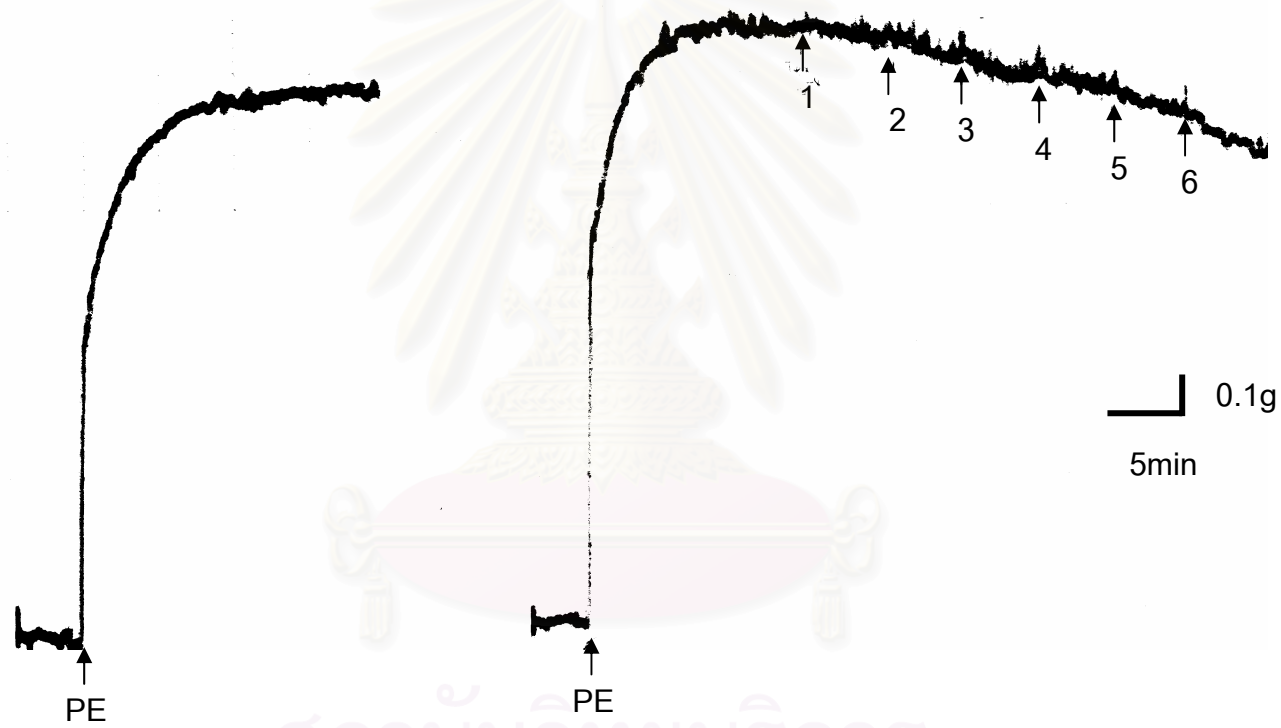

Figure 40 Representative tracing shows the relaxation induced by cumulative addition of terpinen-4-ol on PE-induced contraction of endothelium-intact aortic rings. Terpinen-4-ol concentrations were 1=10^{-8}, 2=10^{-7}, 3=10^{-6}, 4=10^{-5}, 5=10^{-4}, 6=10^{-3}M.

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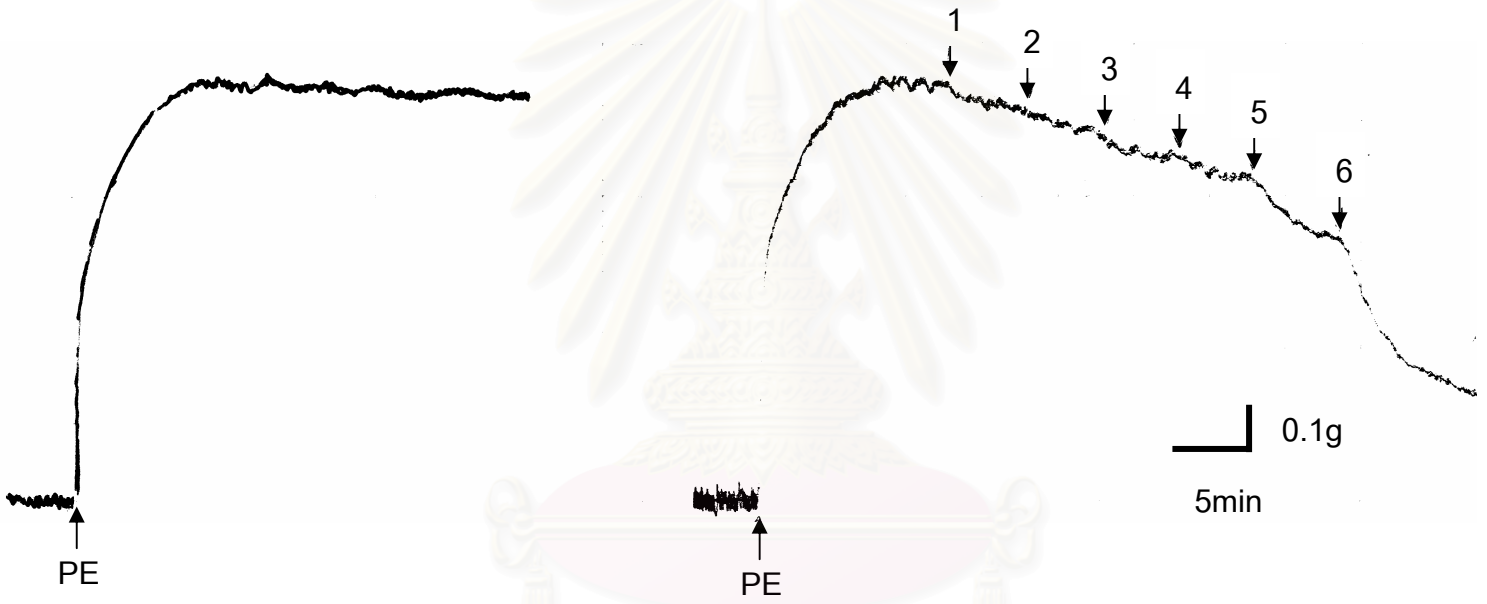

Figure 41 Representative tracing shows the relaxation induced by cumulative addition of terpinen-4-ol on PE-induced contraction of endothelium-denuded aortic rings. Terpinen-4-ol concentrations were 1=10^{-8}, 2=10^{-7}, 3=10^{-6}, 4=10^{-5}, 5=10^{-4}, 6=10^{-3}M.

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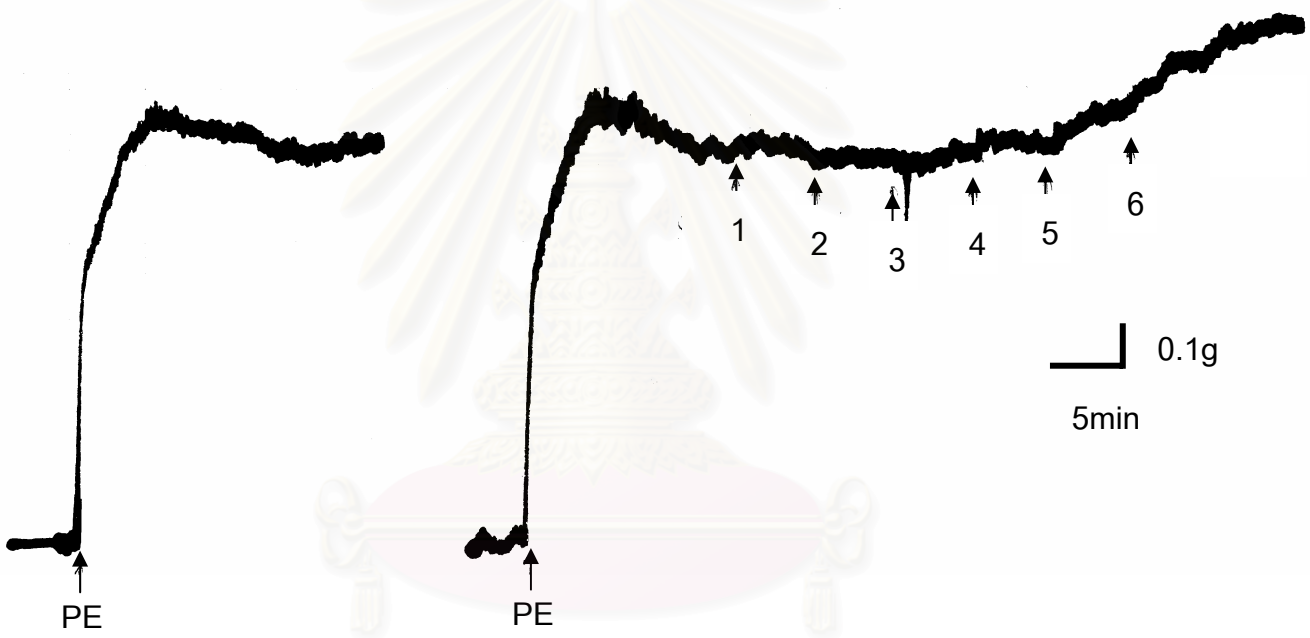

Figure 42 Representative tracing shows the relaxation induced by cumulative addtion of sabinene on PE-induced contraction of endothelium-intact aortic rings. Sabinene concentrations were 1=10^{-8}, 2=10^{-7}, 3=10^{-6}, 4=10^{-5}, 5=10^{-4}, 6=10^{-3}M.

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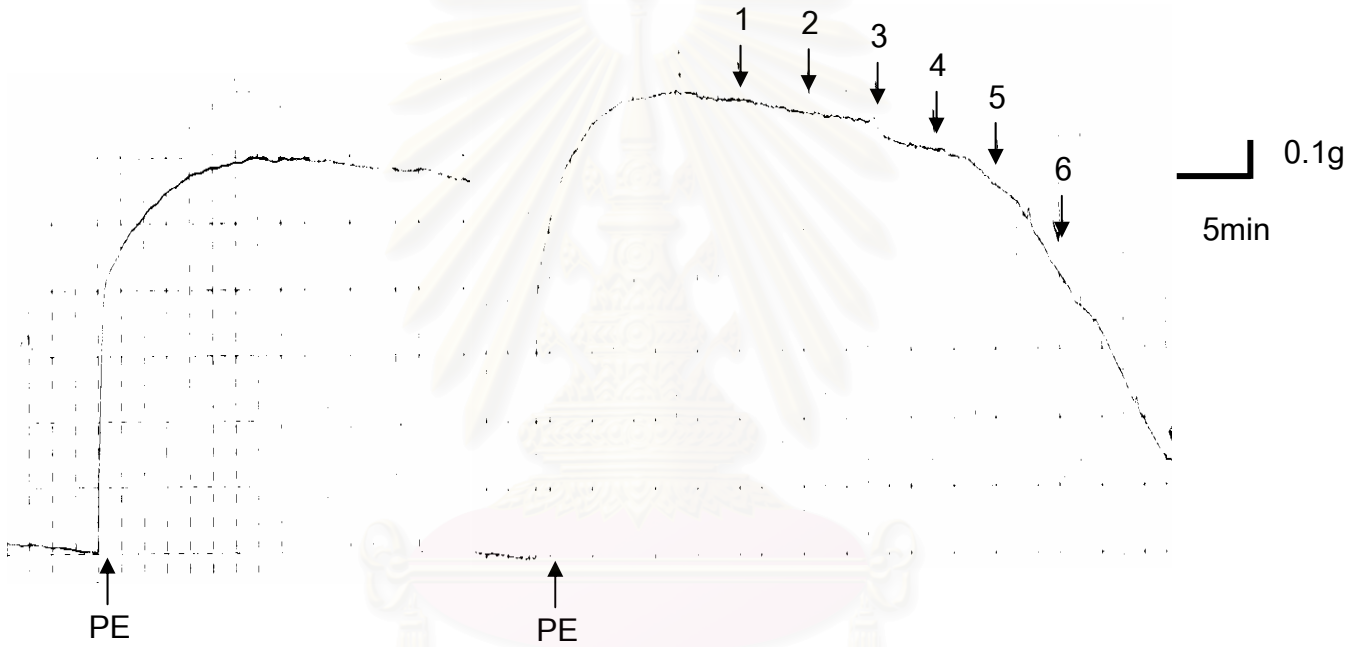

Figure 43 Representative tracing shows the relaxation induced by cumulative addition of sabinene on PE-induced contraction of endothelium-denuded aortic rings. Sabinene concentration were 1=10^{-8}, 2=10^{-7}, 3=10^{-6}, 4=10^{-5}, 5=10^{-4}, 6=10^{-3}M.

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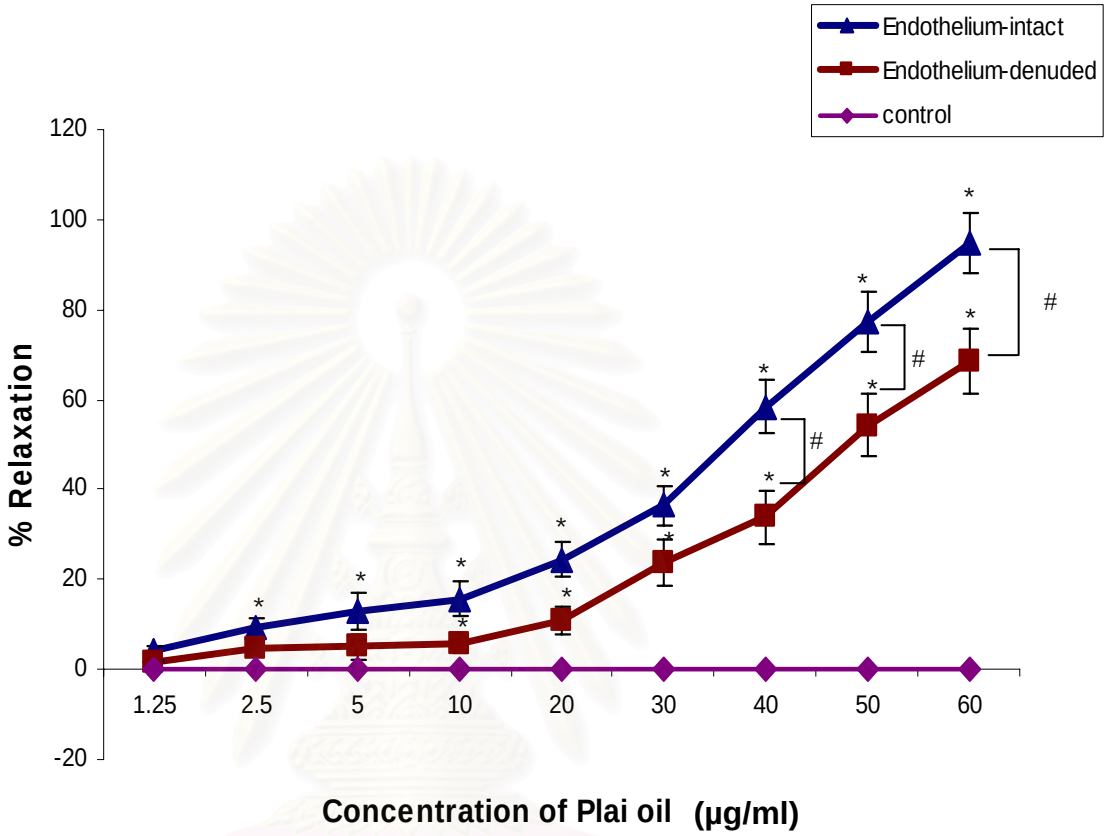

Figure 44 Concentration response curves of endothelium-intact and endothelium-denuded aortic rings precontracted with PE (1 µM).

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

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

\# $p < 0.05$ showed significant difference from endothelium-intact group.

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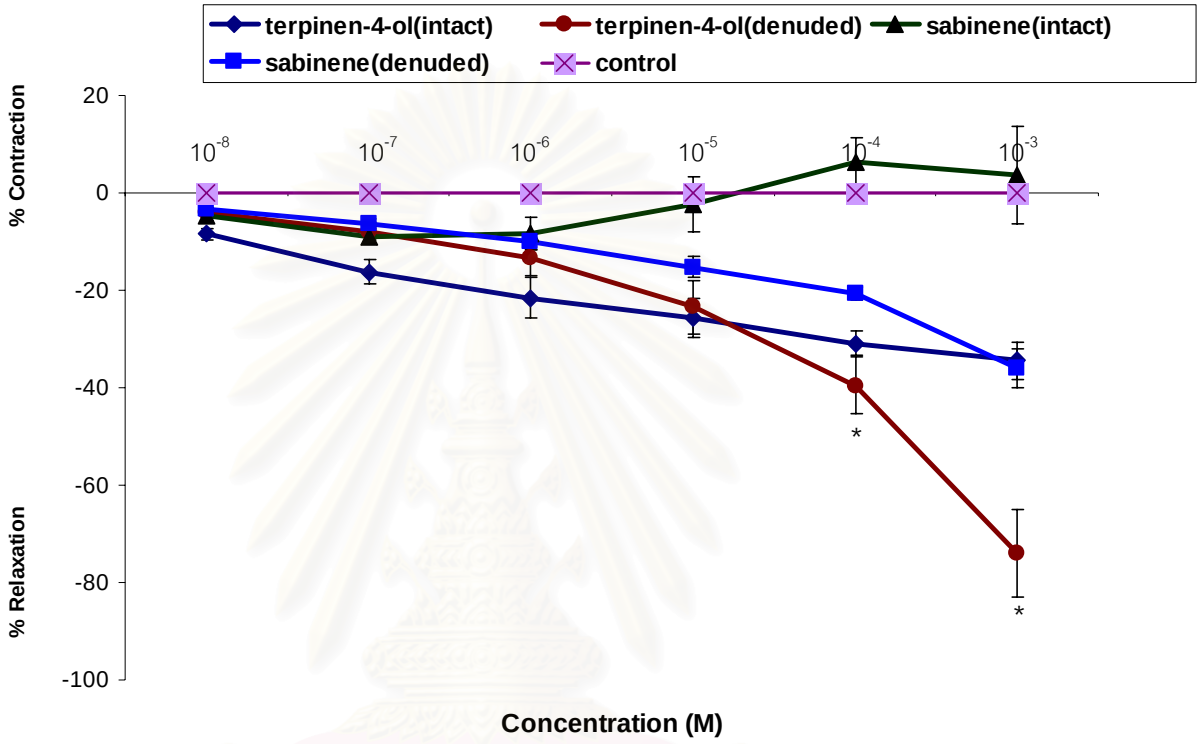


Figure 45 Concentration response curves of terpinen-4-ol (1.36×10^{-3} µg/ml to 136 µg/ml) and sabinene (1.36×10^{-3} µg/ml to 136 µg/ml) of endothelium-intact and endothelium-denuded aortic rings precontracted with PE (1 µM).

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

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

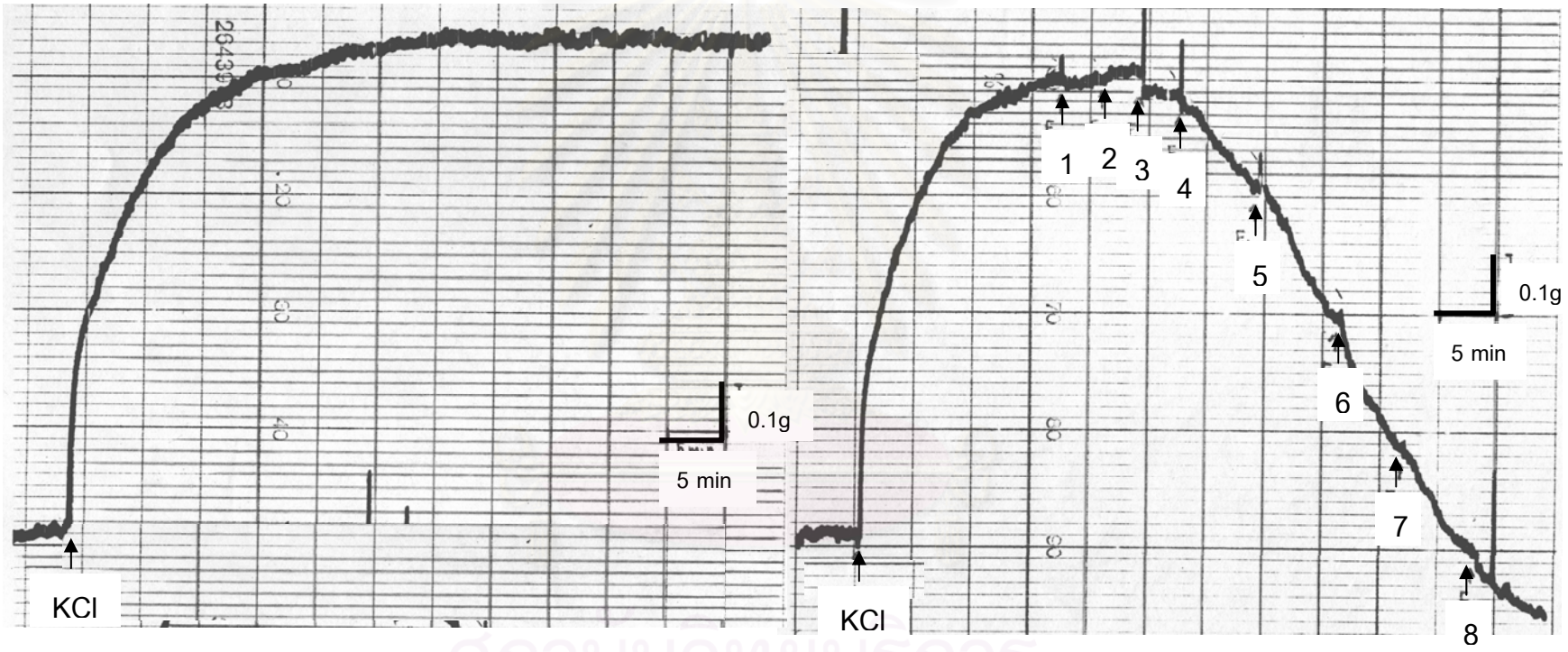


Figure 46 Representative tracing shows the relaxation induced by cumulative addition of plai oil on KCl-induced contraction of endothelium-intact aortic rings. Plai oil (PO) concentrations were 1=1.25, 2=2.5, 3=5, 4=10, 5=20, 6=30, 7=40, 8=50 μg/ml.

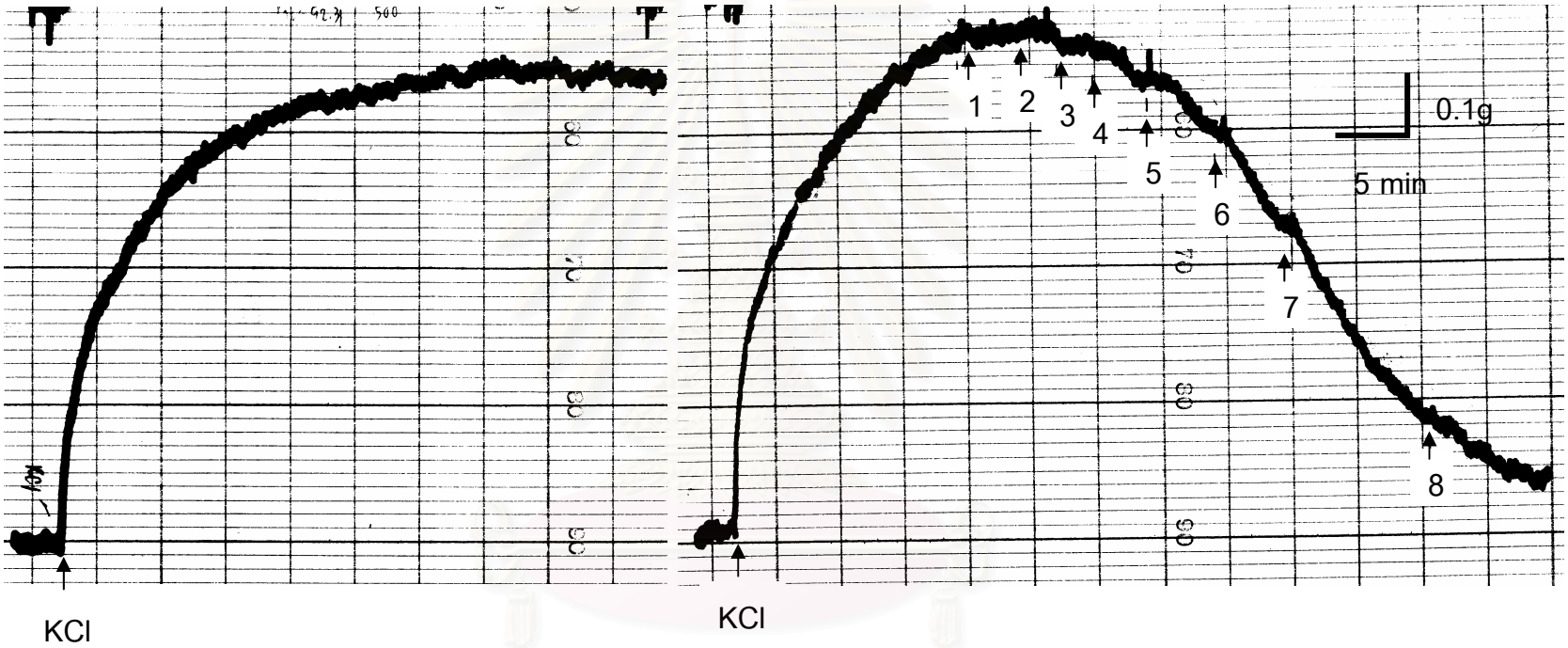


Figure 47 Representative tracing shows the relaxation induced by cumulative addition of plai oil on KCl-induced contraction of endothelium-denuded aortic rings. Plai oil (PO) concentrations were 1=1.25, 2=2.5, 3=5, 4=10, 5=20, 6=30, 7=40, 8=50 µg/ml.

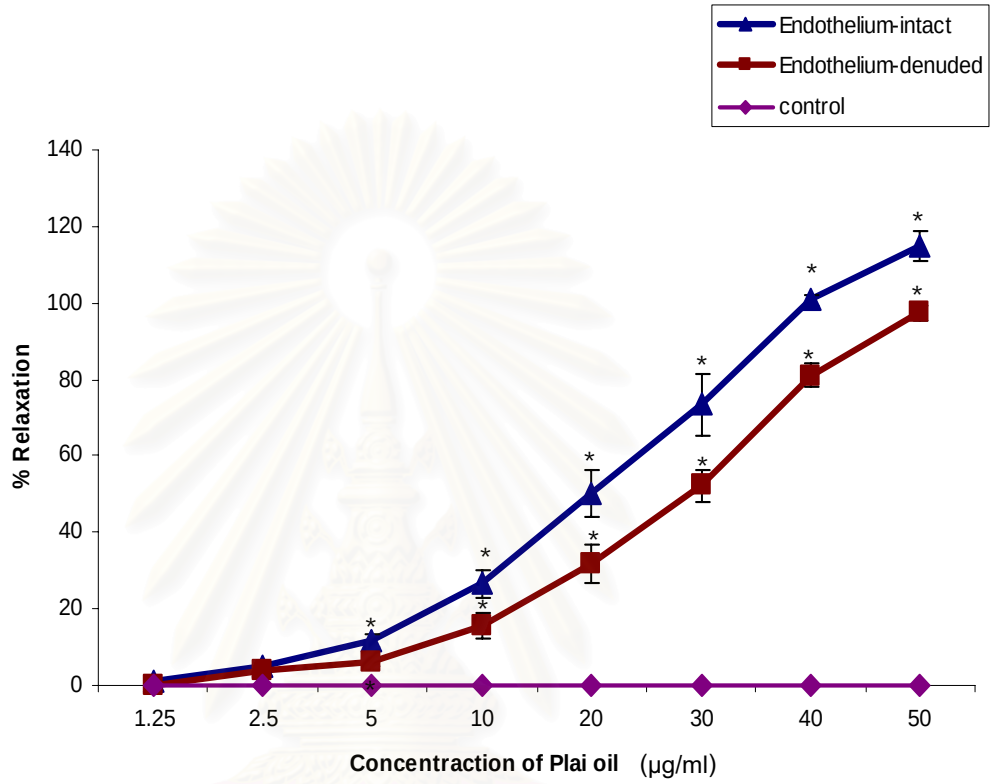
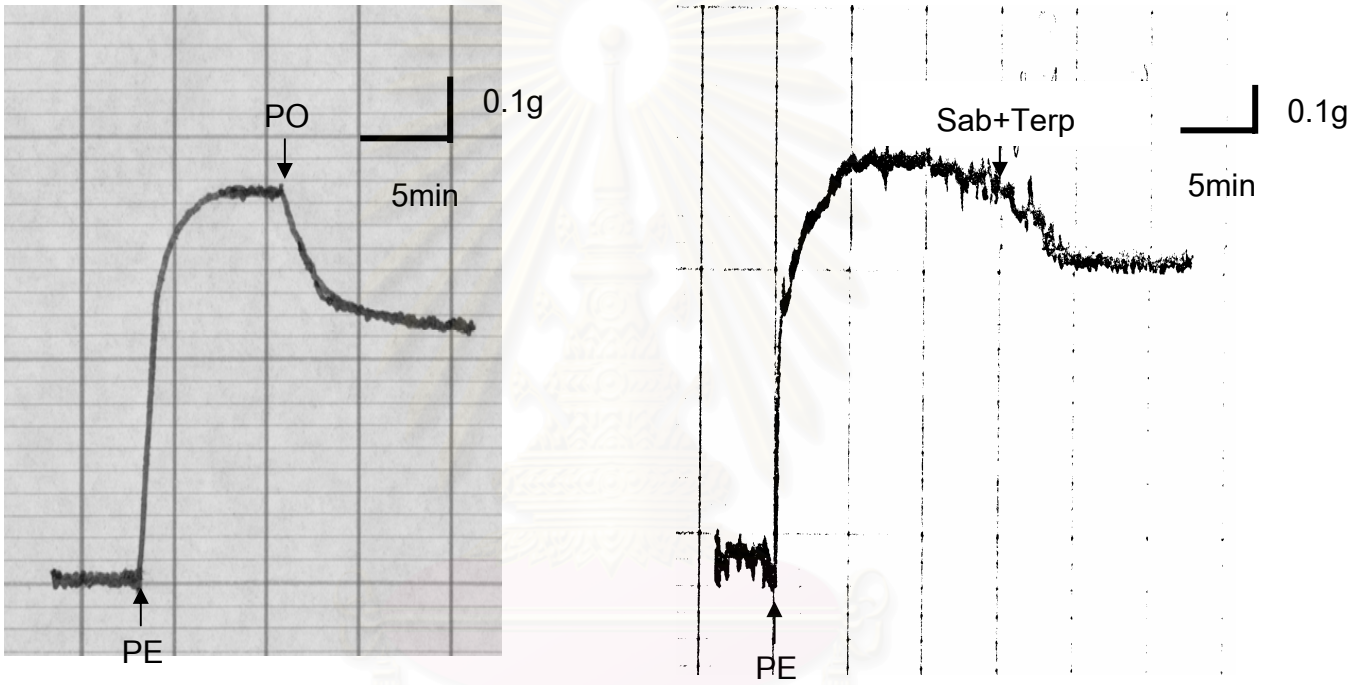


Figure 48 Concentration response curves of endothelium-intact and endothelium-denuded aortic rings precontracted with KCl (40 mM).

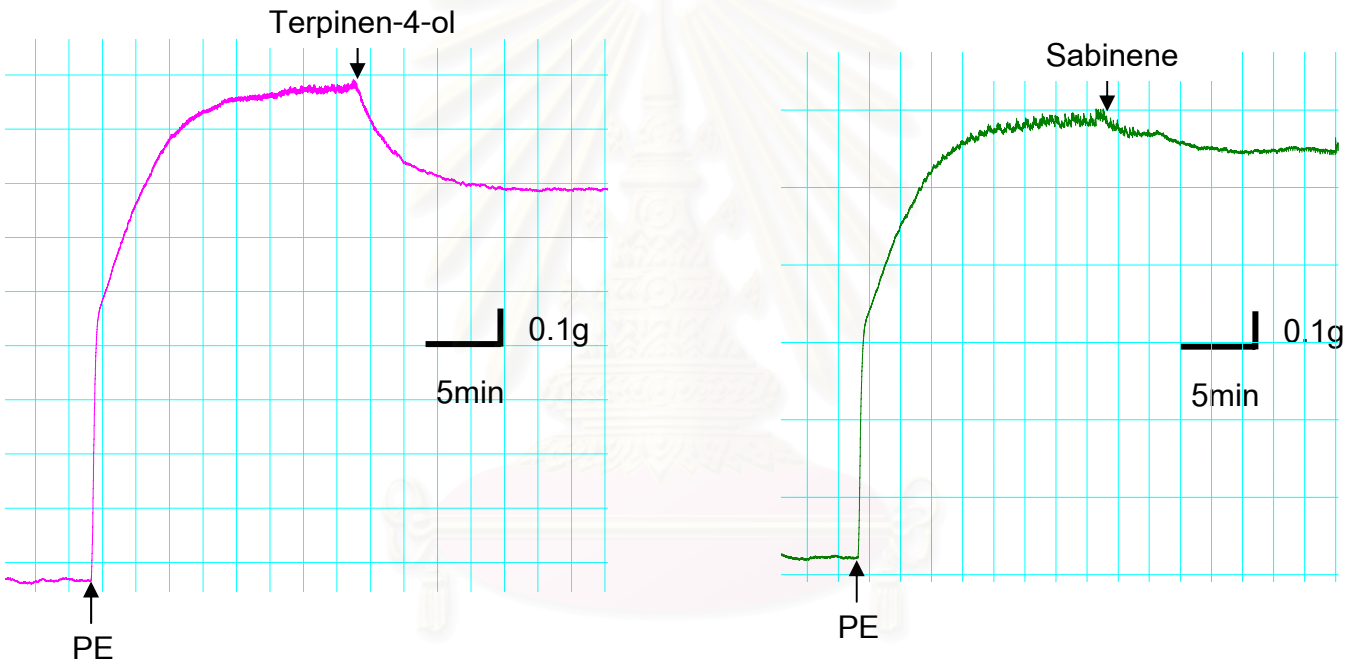
Data were presented as mean ± S.E.M, $n = 6$.

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



(1a) (1b)

Figure 49 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) (1a) and mixture of terpinen-4-ol (13 µg/ml) and sabinene (15 µg/ml) (1b) on the PE-induced contraction of endothelium-intact aortic ring in Ca^{2+}- containing solution.



(1a) (1b)

Figure 50 Representative tracing shows the relaxation induced by addition of terpinen-4-ol (13 µg/ml) (1a) and sabinene (15 µg/ml) on the PE-induced contraction of endothelium-intact aortic ring in Ca^{2+}-containing solution.

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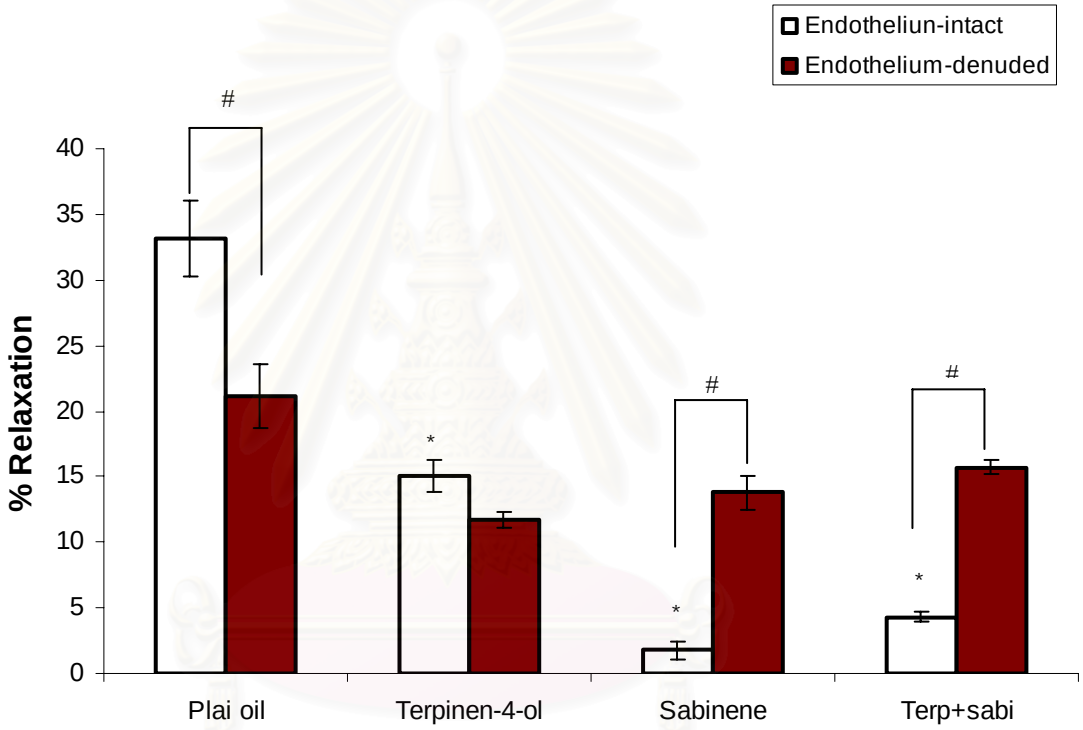

Figure 51 The effect of plai oil (40 µg/ml), terpinen-4-ol (13 µg/ml) and sabinene (15 µg/ml) -induced relaxation of endothelium intact on PE-induced contraction.

* $p<0.05$, significantly different from plai oil group.

# $p<0.05$, significantly different from endothelium-intact group.

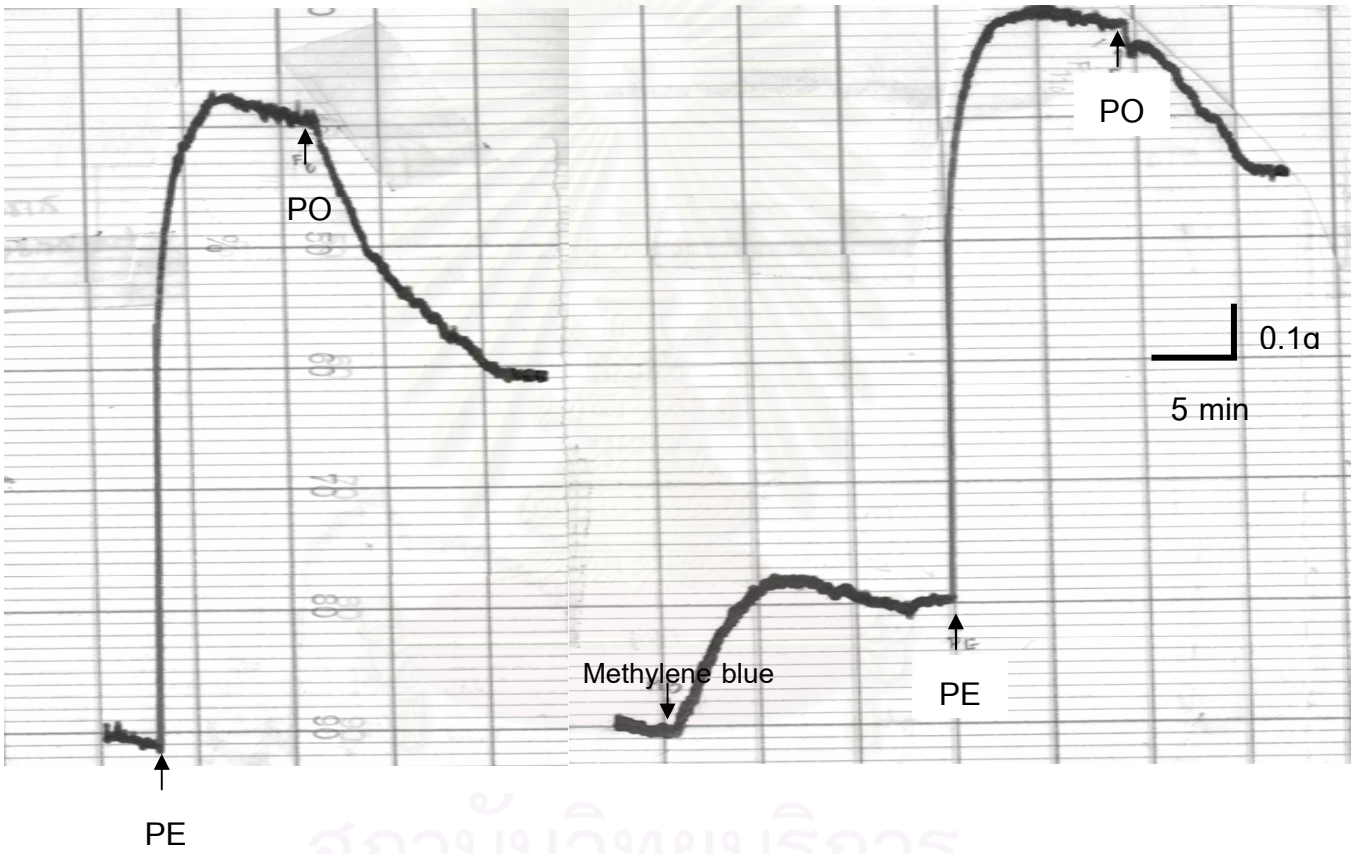

Figure 52 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-intact aortic rings in the presence of methylene blue (10 µM).

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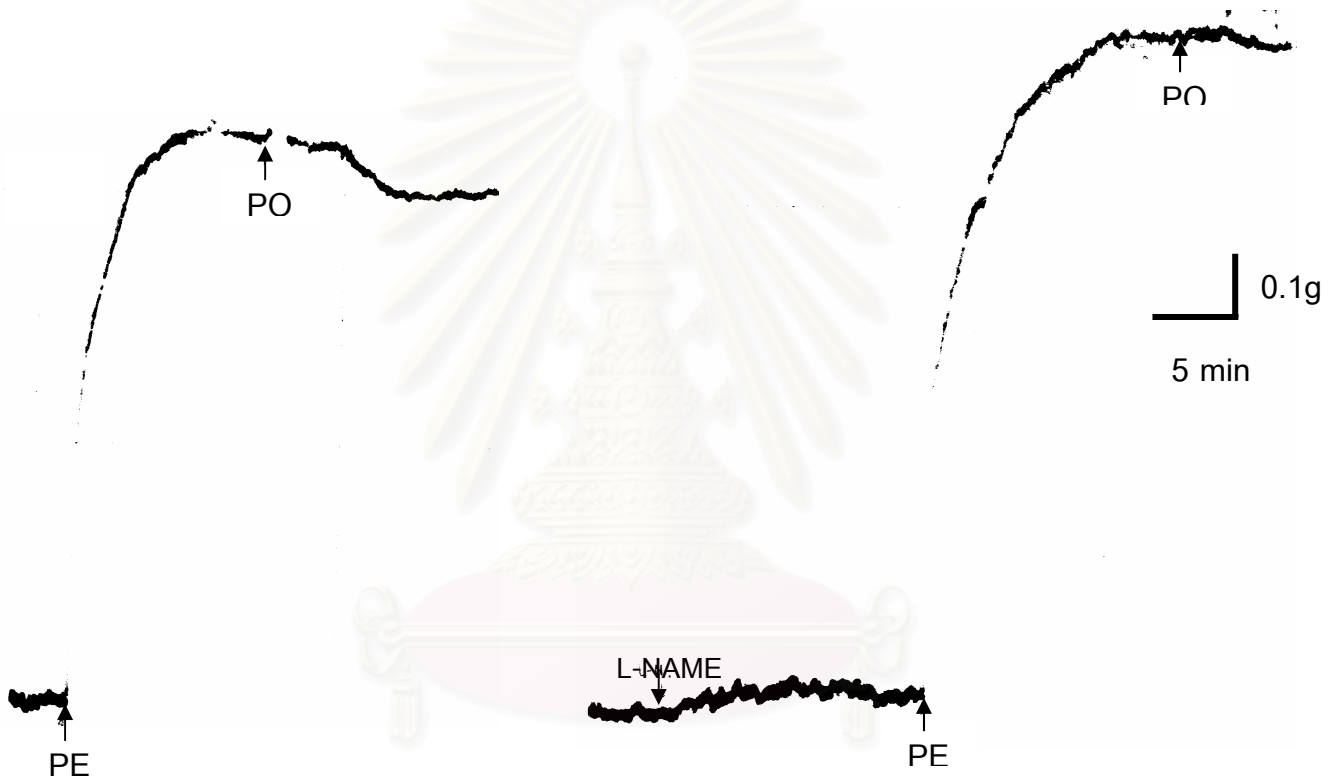

Figure 53 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-intact aortic rings in the presence of L-NAME (10 µM).

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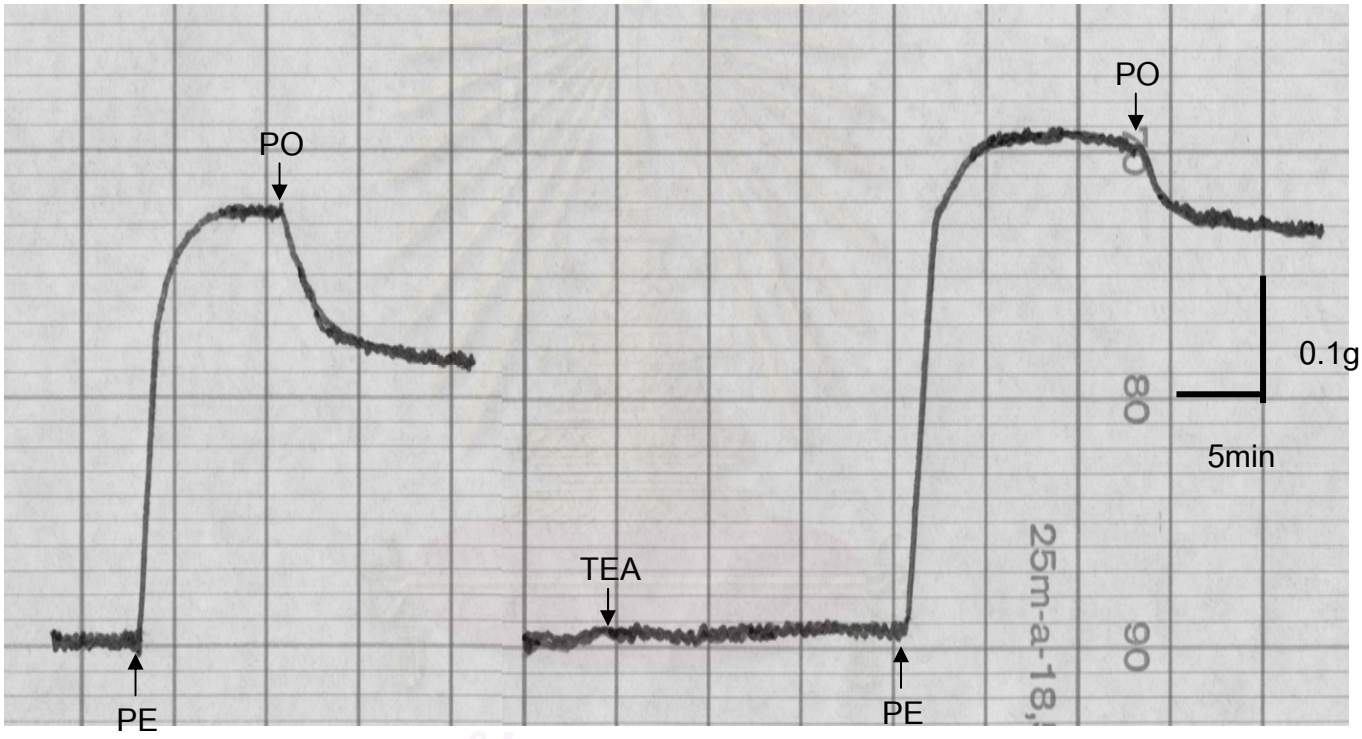

Figure 54 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-intact aortic rings in the presence of TEA (10 µM).

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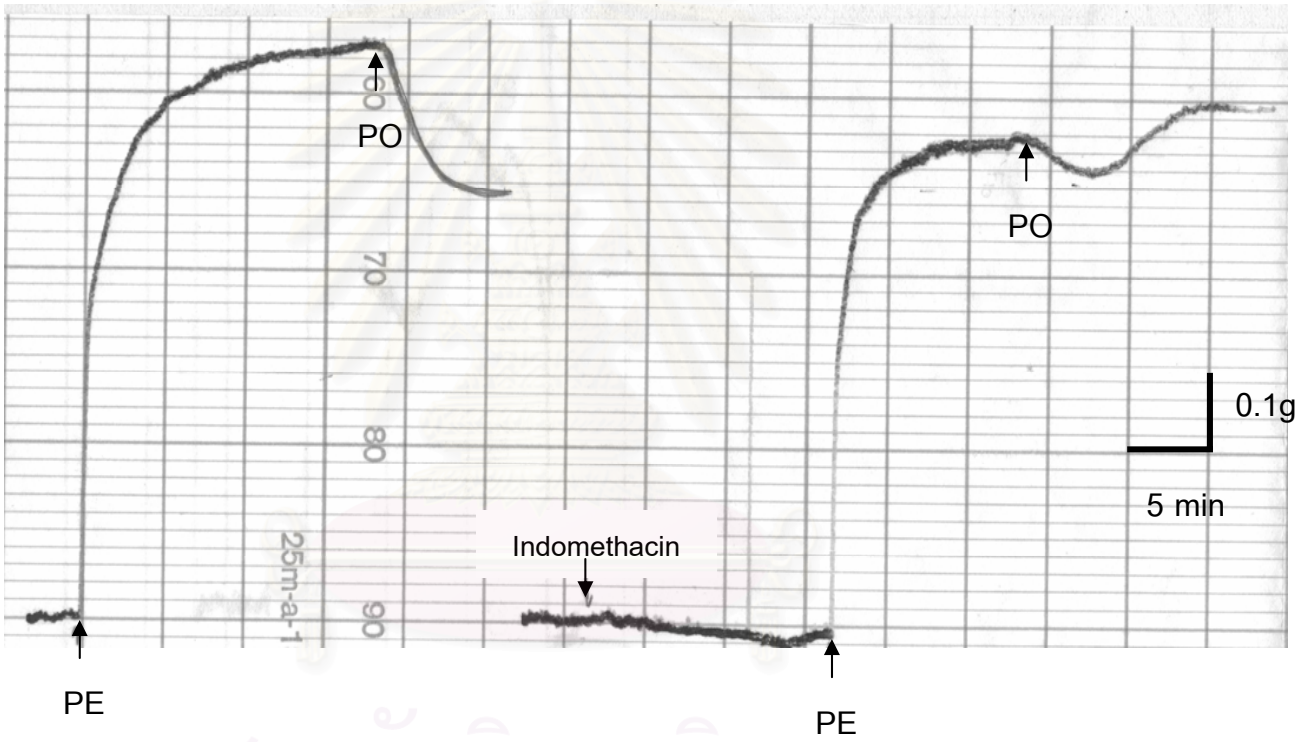

Figure 55 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-intact aortic rings in the presence of indomethacin (10 µM).

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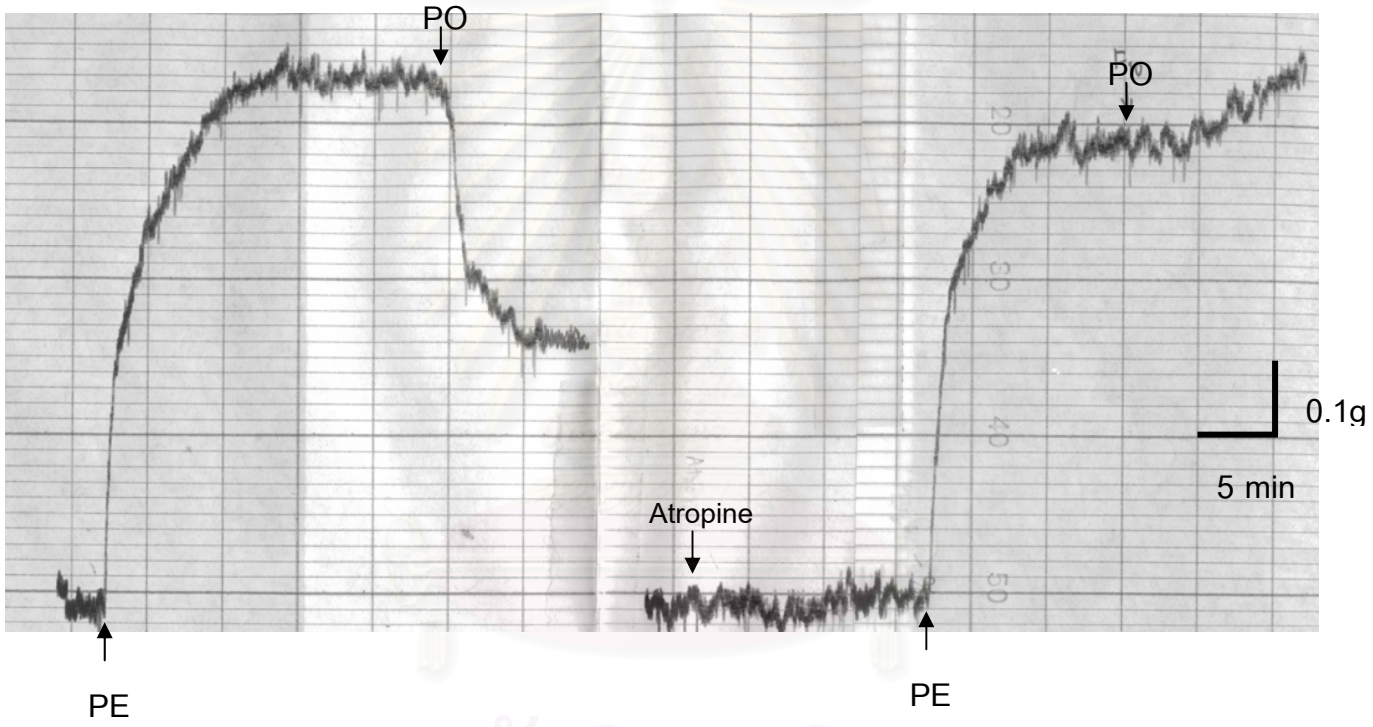

Figure 56 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-intact aortic rings in the presence of atropine (1 µM).

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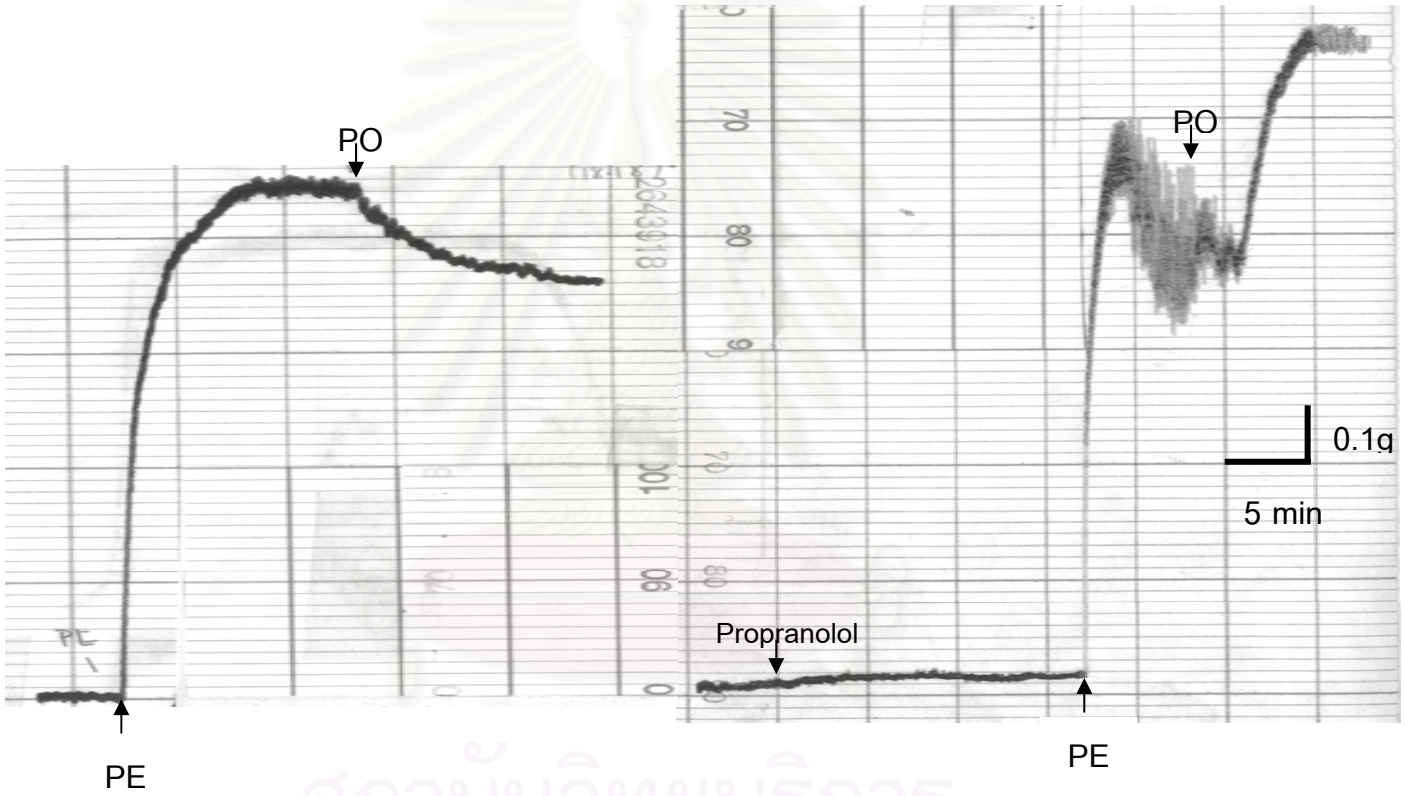

Figure 57 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-intact aortic rings in the presence of propranolol (10 µM).

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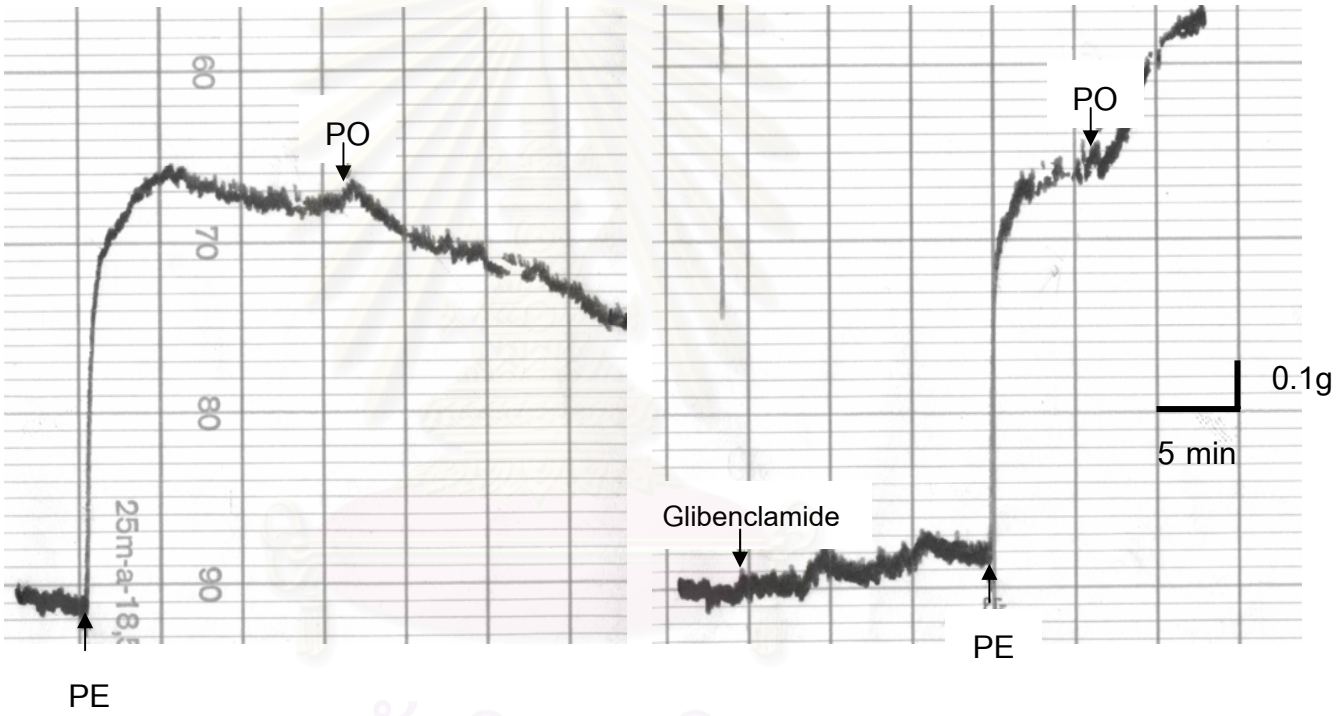

Figure 58 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-intact aortic rings in the presence of glibenclamide (10 µM).

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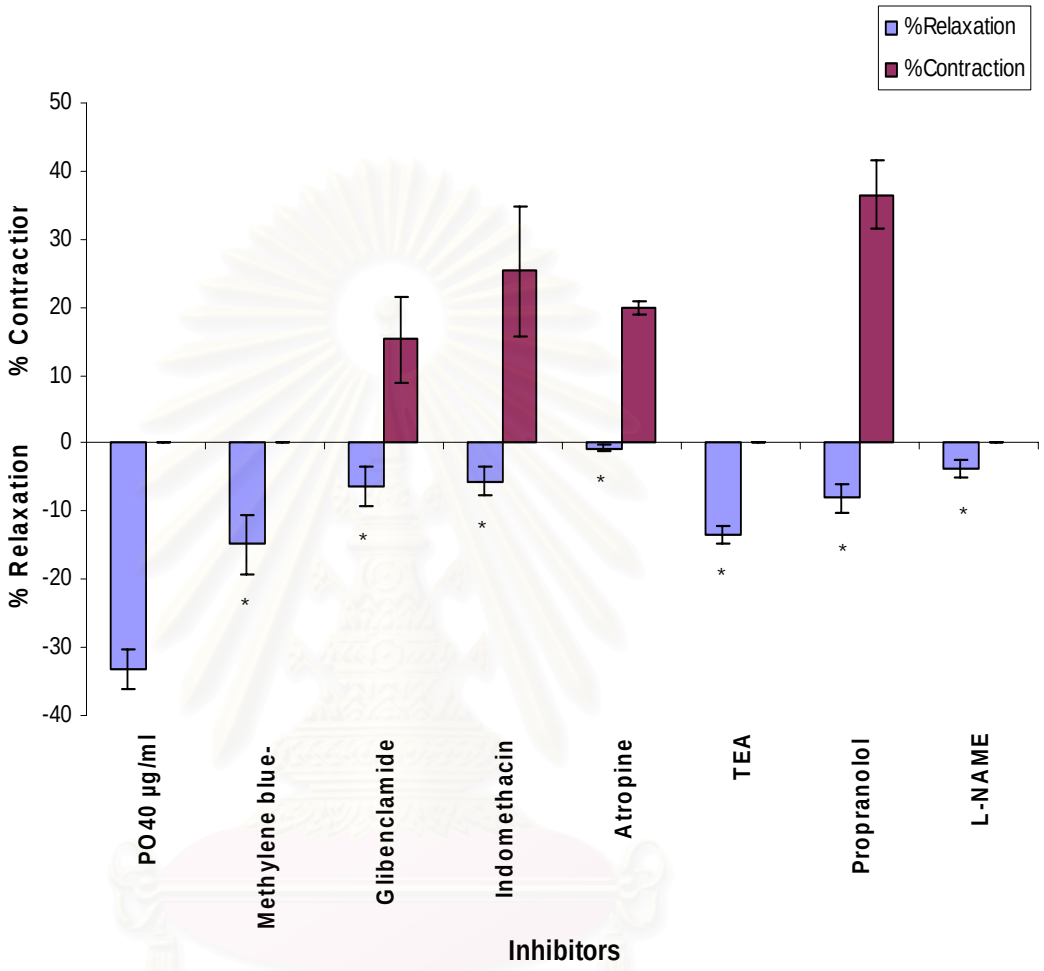

Figure 59 The effect of various vasodilator inhibitors on plai oil induced relaxation of endothelium-intact aortic rings pre contracted with PE.

The responses are expressed as the percentage of PE-induced contraction.

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

*p<0.05, significantly different from control group (plai oil 40 µg/ml).

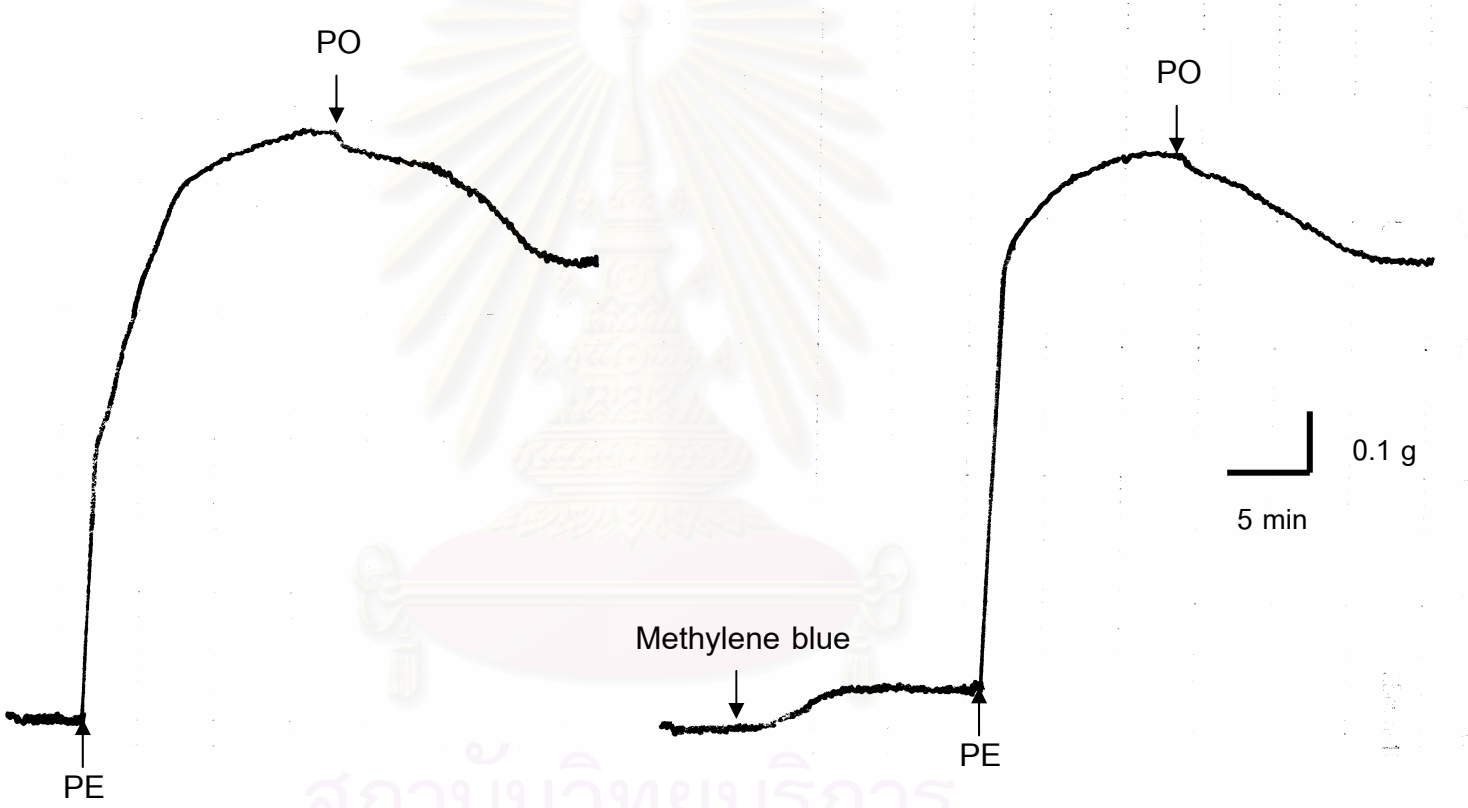

Figure 60 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-denuded aortic rings in the presence of methylene blue (10 µM).

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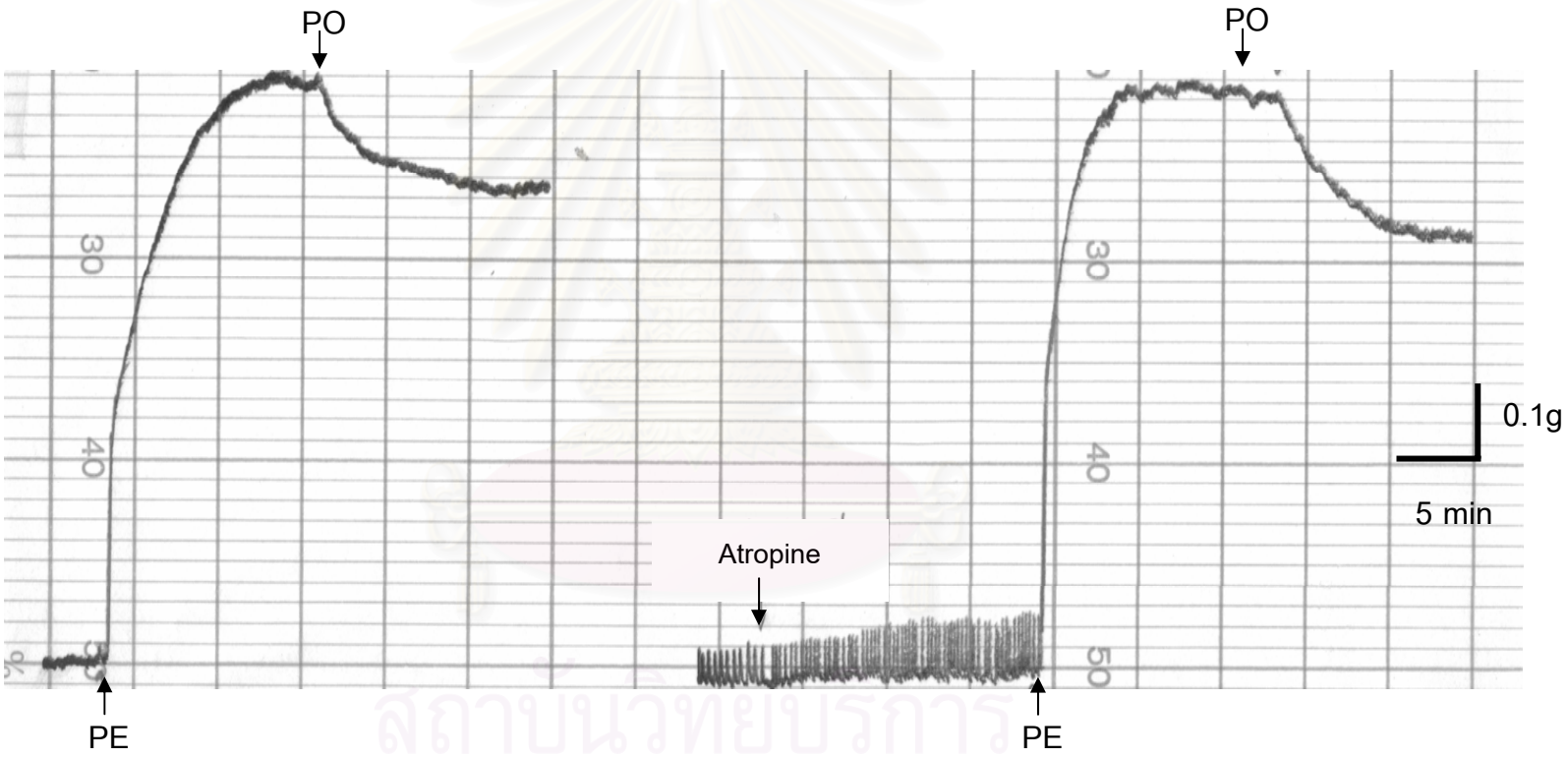

Figure 61 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-denuded aortic rings in the presence of atropine (1 µM).

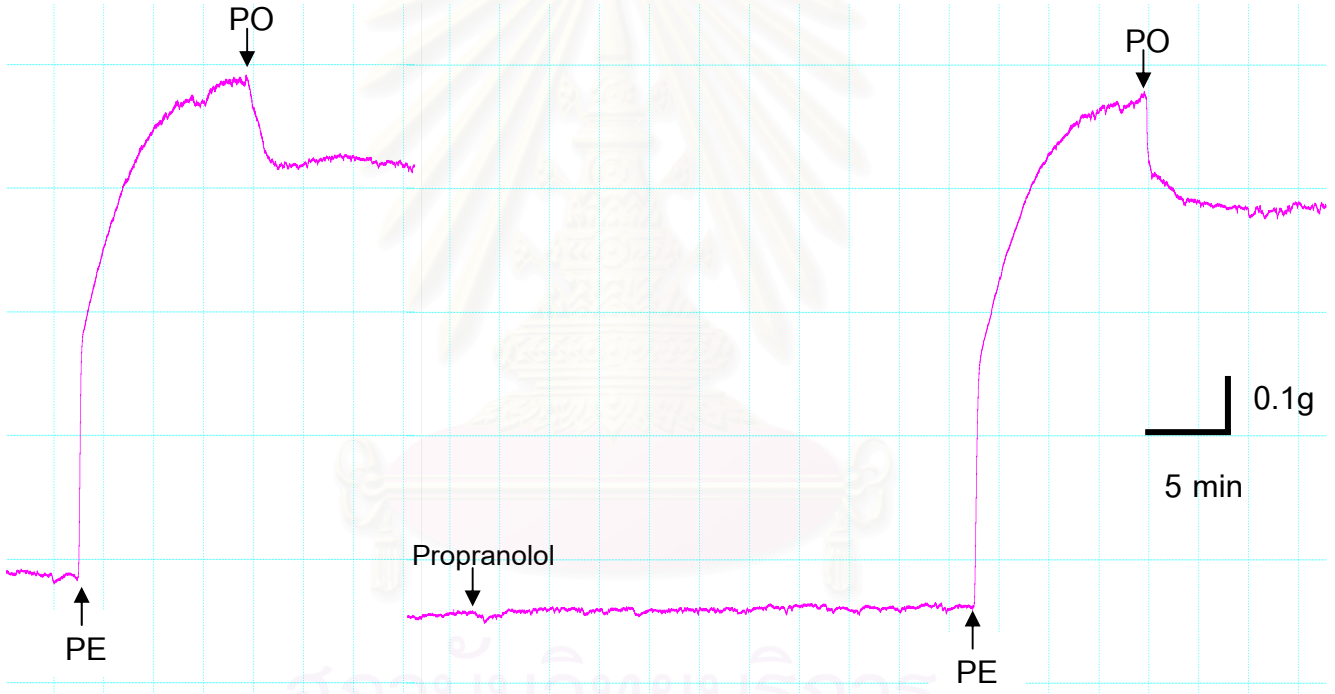

Figure 62 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-denuded aortic rings in the presence of propranolol (10 µM).

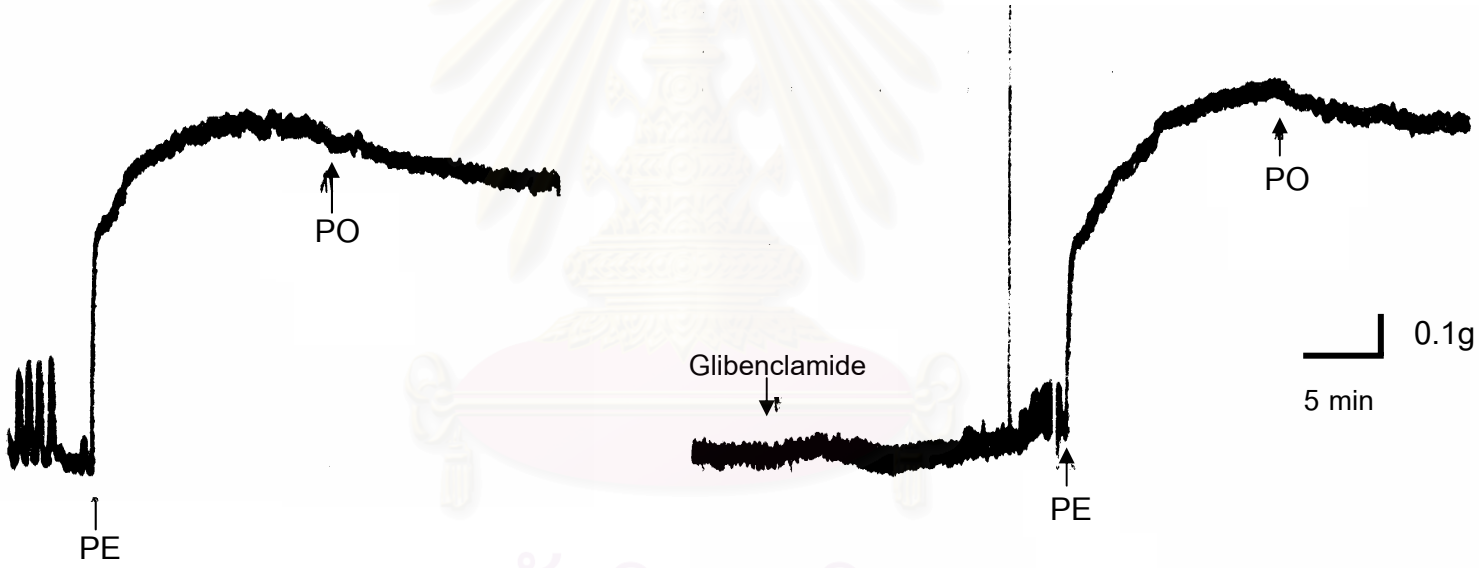

Figure 63 Representative tracing shows the relaxation induced by addition of plai oil (40 µg/ml) on PE-induced contraction of endothelium-denuded aortic rings in the presence of glibenclamide (10 µM).

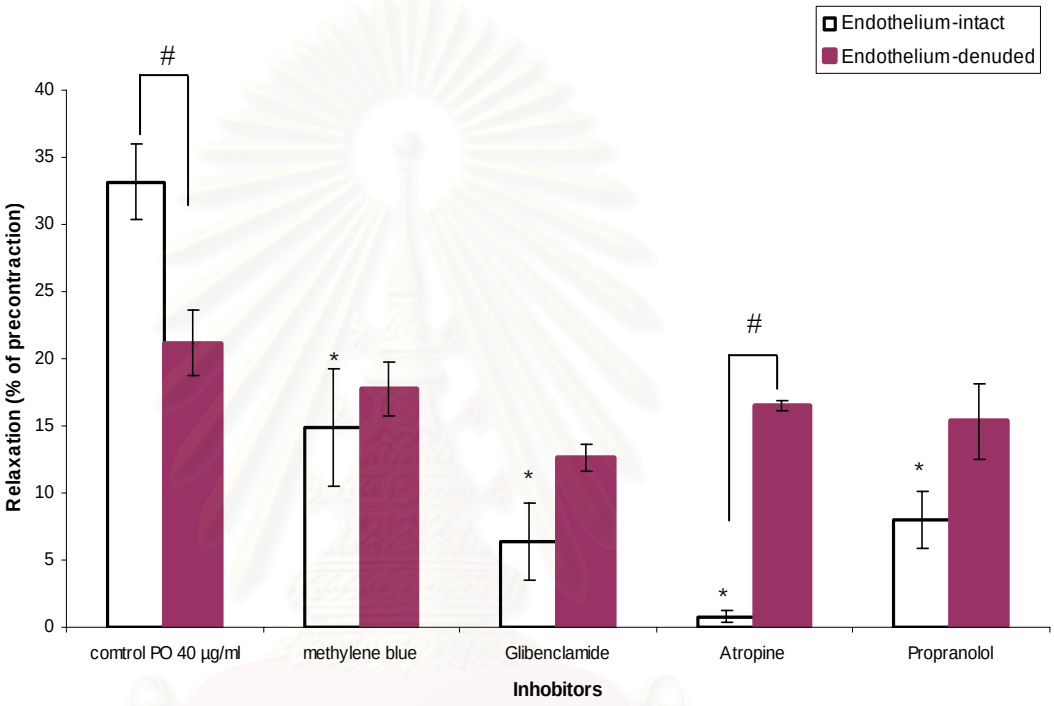

Figure 64 The effects of various vasodilator inhibitors on plai oil (40 µg/ml) induced relaxation of endothelium- intact and endothelium-denuded aortic rings precontracted with PE.

The responses are expressed as the percentage of PE-induced contraction. Data were presented mean± S.E.M, *n* = 4.

*p<0.05, significantly different from plai oil group.

# p<0.05, significantly different from endothelium- intact group.

CHAPTER V

DISCUSSION AND CONCLUSIONS

This study aimed to investigate the action of oil from *Zingiber cassumunar* Roxb. (plai oil) on the modulating of vascular tone. The study was also designed to determine the influence of endothelium on the responsiveness of vascular smooth muscle to plai oil. As known, intracellular Ca^{2+} ($[Ca^{2+}]_i$) is a key element in contraction of smooth muscle (Aaronson, *et al*., 2004; Karaki, *et al*., 1997). The $[Ca^{2+}]_i$ increases via a release of Ca^{2+} from internal store (Orallo, 1996; Komaru, 2000; Richard, 2002) as well as an influx of external Ca^{2+} through voltage-operated Ca^{2+} channel (VOC) and receptor-operated Ca^{2+} channel (ROC) (Kanmura, 1998); Marin, *et al*., 1999; Sobey, 2001). Relaxation of smooth muscle is usually initiated by a decrease in $[Ca^{2+}]_i$ via several mechanisms such as blockade of Ca^{2+} influx (Klabuade, 2005). In addition, endothelium plays a crucial role on vasorelaxation by releasing endothelium-derived relaxing factors (EDRF) such as nitric oxide (NO) (Luscher, 1995; Vanhoutte, 2004).

The result of this study demonstrated that plai oil had an intrinsic activity to influence vascular contractility. It was able to inhibit PE- and KCl-induced contraction of rat aortic rings in Ca^{2+}- containing solution. In the absence of endothelium, the inhibitory action of plai oil on PE-induced contraction was concentration-dependent. Endothelium appears to abolish the inhibitory effect of plai oil on PE-induced contraction. However, this observation was not evidenced in KCl-induced contraction. Plai oil elicited its concentration-dependent inhibition of both endothelium-intact and endothelium-denuded aortic rings in response to KCl although the effect of plai oil was more potent in the denuded preparation. Taken together, the inhibitory effects of plai oil were attenuated by the presence of endothelium. The results suggested the critical role of endothelium in protecting smooth muscle from plai oil effects. Unexpectedly, plai oil at concentration of 50 and 100 µg/ml caused endothelium-denuded aortic rings to contract. This plai oil-induced contraction was unnoticeable in endothelium-intact rings. This phenomenon suggested the multiaction of plai oil on modulating the vascular tone.

It was possible that plai oil may directly affect the contraction of smooth muscle independent of endothelium.

Plai oil had the inhibitory effects on both PE- and KCl-induced contraction. Therefore it could be suggested that plai oil may directly disrupt the increase of $[Ca^{2+}]_i$ in smooth muscle. It was possible that the actions of plai oil were mediated through nonspecific inhibition of Ca^{2+} influx from extracellular or to an inhibitory effect on intracellular Ca^{2+} release. To confirm these suggestions, the aortic contraction in the experimental model of Ca^{2+}- free medium and high K^+-Ca^{2+}- free depolarizing solution were performed. In this study, plai oil was able to inhibit the PE-induced transient contraction in Ca^{2+}- free medium, but not the caffeine – induced contraction. In Ca^{2+}-free medium, the transient contraction of smooth muscle is largely attributed to Ca^{2+} release from sarcoplasmic reticulum (SR) (Gonzales, *et al*., 2000). It has been well established that PE and caffeine, via their different mechanism, cause Ca^{2+} release from SR. The mechanism of PE–induced Ca^{2+}- release from SR is mediated through inositol triphosphate (IP_3), which binds to its receptors on the SR and caused release Ca^{2+} (Gonzales, *et al*., 2000; Abdel-Latif, 1986). The caffeine is ability to induce contractile response. Caffeine-induced a transient contraction is attributable to release of Ca^{2+} from SR (Gonazales, *et al*., 2000; Karaki and Weiss, 1988; Watanabe, *et al*., 1992). The mechanism of caffeine is considered to be due to its binding to the ryanodine receptor Ca^{2+}- release channel. This phenomenon occurs depleting intracellular Ca^{2+}store, that increases muscle tone and depolarizes the membrane (Marin, *et al*., 1999). Hence, plai oil selectively inhibits Ca^{2+} release from SR via PE-mediated mechanism.

In high K^+- Ca^{2+}- free depolarizing solution, this method was able to determine an inhibition of Ca^{2+} entry from extracellular pool when $CaCl_2$ was added cumulatively. In this study, plai oil inhibited $CaCl_2$-induced contraction in a concentration-dependent manner. This finding suggests that plai oil directly affected Ca^{2+} influx across the membrane through VOC.

Furthermore, the present study was performed to investigate the mechanisms involved in plai oil-induced vasorelaxation. The results show that plai oil cause endothelium - dependent relaxation of aortic rings precontracted with PE and KCl. In addition, endothelium was significantly influenced on the actions of plai oil on vasorelaxation. It is possible that plai oil may cause endothelium to release its vasodilation factors such as NO. Consequently, these factors attenuate the contraction of vascular smooth muscle.

The potential mechanisms of plai oil-induced vasorelaxation were evaluated in endothelium-intact and endothelium-denuded aortic rings. In this study, the results demonstrated that endothelium significantly influenced on the actions of plai oil on vasorelaxation. Several mechanisms were probed for the relaxant effect of plai oil on endothelium cells, including NO-cGMP pathway, hyperpolarizing, cyclooxygenase, muscarinic receptors and β–adrenoceptor. NO is produced from L- arginine by NO syntase enzyme (Luscher, 1995). The presence of L-NAME, an inhibitor of NOS, abolished the vasorelaxant effect of plai oil, suggesting that plai oil exerted its vasorelaxation via an increase in NO production. Moreover, the presence of methylene blue, an inhibitor soluble guanylyl cyclase (sGC), (Wanstall, *et al*., 2005), significantly inhibited the relaxation induced by plai oil. Taken together, the result confirmed that the relaxant effect of plai oil on rat aortic rings was mediated through NO-cGMP pathway.

The vasorelaxant activity of plai oil was mediated through multi-mechanisms. Hyperpolarizing factor was another potential mechanism determined in this study. The results demonstrated that glibenclamide, an ATP-sensitive potassium channel blocker, inhibited plai oil-induced relaxation (Parkington, *et al*., 2004). In addition, the effect of plai oil was attenuated by tetraethylammonium chloride (TEA), a non selective-specific potassium channel inhibitor. Hence, plai oil may also cause vasorelaxation via hyperpolarizing mechanism. Furthermore, plai oil-induced relaxation was also attenuated by certain compounds including atropine, indomethacin and propranolol, suggesting the involvement of muscarinic receptor, cyclooxygenase and β–adrenoceptor.

Taken altogether, plai oil influenced vascular tone via several mechanisms. The actions of plai oil were integrative effect of each constituent in this mixture. Two major constituent of plai oil are terpinen-4-ol and sabinene. Other constituents include α –terpinen,γ -terpinen, α-pinen, and (E)-1-(3, 4-dimethoxyphenyl) butadiene (DMPBD) (Baker, and Nabney, 1975). This study demonstrated that terpinen-4-ol and sabinene had different actions on the vascular tone. In endothelium-denuded rings, sabinene was more potent than terpinen-4-ol against PE-induced contraction. In contrast, the action of sabinene in endothelium-intact rings was opposite to those of terpinen-4-ol. Moreover, both sabinene and terpinen-4-ol were able to cause endothelium independent relaxation of aortic smooth muscle, with less potency than plai oil. The reconstitution of sabinene and terpinen-4-ol did not increase its effects to the comparable degree of plai oil relaxant action. This result revealed the disparity between plai oil and its major constituents.

In conclusion, plai oil can modulate the vascular tone via endothelium dependent and endothelium independent pathways. Its direct actions on smooth muscle may be linked to non-specific inhibition of Ca^{2+} influx as well as inhibition of PE-mediated Ca^{2+} release from SR. Moreover, plai oil influences the vascular contractility through endothelium factors including NO-cGMP pathway, hyperpolarizing, cyclooxygenase, muscarinic receptors and β–adrenoceptor.

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APPENDICES

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Table 2 Compound of Physiological solution (mM).

Chemical	Physiological solution		
	Kreb Henseleit	Ca2+-Free Kreb Henseleit	Potassium Depolarizing
NaCl	119	119	27
KCl	4.7	4.7	100
$CaCl_2$	2.5	-	-
$MgSO_4$	1.0	1.0	-
KH_2PO_4	1.2	1.2	14.0
D-glucose	11.1	11.1	10
EDTA	-	0.1	-
$MgCl_2$	-	-	0.54
$NaHCO_3$	25	25	14

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Table 3 The effect of plai oil on the percentage of contraction induced by PE (1μM) in endothelium-intact and endothelium-denuded.

	Endothelium-intact	Endothelium-denude
DMSO (0.07%)	100.89±1.73	104.72±2.24
Plai oil(10μg/ml)	100.71±2.35	98.01±2.25
Plai oil (50μg/ml)	92.85±5.97	89.17±1.88
Plai oil (100μg/ml)	90.79±5.32	78.91±1.89*
Plai oil (200μg/ml)	91.41±3.14	69.46±1.51*

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

* p<0.05 showed significant difference of plai oil-treated group from DMSO 0.07% (v/v) group.

Table 4 The effect of plai oil (100 μg/ml), terpinan-4-ol (34 μg/ml) and sabinene (39 μg/ml) on the percentage of contraction induced by PE (1 μM) in endothelium-intact and endothelium-denuded aortic rings.

	Endothelium-intact	Endothelium-denude
DMSO 0.07% (v/v)	100.89±1.73	104.72±2.24
Plai oil (100 μg/ml)	90.79±5.32	78.91±1.88*
Terpinan-4-ol (34 μg/ml)	79.58±2.15*	93.10±0.96
Sabinene (39 μg/ml)	90.60±2.28	60.29±3.14*

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

* p<0.05 showed significant difference from DMSO 0.07% (v/v) group.

Table 5 The effect of plai oil on the percentage of contraction induced by KCl (40 mM) in endothelium-intact and endothelium-denuded.

	Endothelium-intact	Endothelium-denude
DMSO 0.07% (v/v)	99.37±1.51	96.96±3.16
Plai oil (10μg/ml)	100.49±3.42	66.32±1.83*
Plai oil (50μg/ml)	66.31±2.52*	45.86±2.32*
Plai oil (100μg/ml)	42.26±2.54*	42.42±1.83*
Plai oil (200μg/ml)	41.60±4.39*	44.23±2.36*

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

* $p<0.05$ showed significant difference of plai oil-treated group from DMSO 0.07% (v/v) group.

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Table 6 The effect of plai oil (40µg/ml) and 0.07% (v/v) DMSO on the percentage of contraction induced by PE (1µM) and caffeine (10 mM) in endothelium-denuded aortic rings in Ca^{2+}- free KHS.

Contractants	n	Plai oil (40 µg/ml)	Plai oil (100 µg/ml)	DMSO 0.07 % (v/v)
PE (1µM)	6	60.14±6.05 *	59.10±4.97 *	88.11.±7.5
Caffeine (10 mM)	6	114.12±6.78	105.18±4.94	108.20±7.25

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

* p<0.05 showed significant difference of contraction from DMSO 0.07% (v/v) group.

Table 7 The effect of plai oil (100 µg/ml), terpinan-4-ol (34 µg/ml) and sabinene (39 µg/ml) on the percentage of contraction induced by PE (1 µM) in endothelium-denuded aortic rings in Ca^{2+}-free KHS.

	% Contraction
DMSO 0.07 % (v/v)	88.11±7.55
Plai oil (100 µg/ml)	59.10±4.97*
Terpinan-4-ol (34 µg/ml)	89.32±2.73
Sabinene (39 µg/ml)	51.70±2.70*

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

* p<0.05 showed significant difference of contraction from DMSO 0.07% (v/v) group.

Table 8 The percentage of contraction induced by adding cumulatively $CaCl_2$ in endothelium-denuded aortic rings.

Concentration of $CaCl_2$(M)	Control (n=10)	Plai oil 50µg/ml (n=6)	Plai oil 100µg/ml (n=6)	DMSO 0.07% (n=6)
10^{-5}	3.63±0.87	3.38±1.48	1.86±0.77	4.82±1.06
5×10^{-5}	16.36±2.40	8.67±2.36	6.80±2.51	24.56±1.03
10^{-4}	33.12±2.39	18.24±3.15	14.50±3.05	33.84±1.03
5×10^{-4}	54.04±2.66	35.21±2.75	31.15±4.28*	52.78±1.60
10^{-3}	70.03±1.33	48.73±2.36	42.56±4.89*	65.11±2.65
5×10^{-3}	83.14±1.08	59.86±2.53	55.79±3.36*	78.50±2.86
10^{-2}	100±0.00	72.03±2.31*	67.38±3.30*	96.76±1.53

Data were present as mean ±S.E.M, *n* = 6 - 10.

* $p<0.05$ showed significant difference of contraction from DMSO 0.07% (v/v) group.

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Table 9 The effects of plai oil (40 µg/ml), terpinan-4-ol (13 µg/ml) and sabinene (15 µg/ml) on percentage of induced relaxation in endothelium-intact and endothelium-denuded aortic rings.

	Endothelium-intact	Endothelium-denuded
Plai oil (40 µg/ml)	33.18±2.84	21.18±2.43#
Terpinan-4-ol (13 µg/ml)	15.11±1.22*	11.77±0.62
Sabinene (15 µg/ml)	1.79±0.70*	13.81±1.32#
Terpinan-4-ol + sabinene	4.33±0.33*	15.70±0.53#

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

* $p<0.05$ showed significant difference of contraction from plai oil group.

# $p<0.05$ showed significant difference of contraction from endothelium-intact.

Table 10 The effects of inhibitors on percentage of relaxation induced by plai oil (40 µg/ml) in endothelium-intact aortic rings.

	Mean of %relaxation	N
Plai oil (40 µg/ml)	33.18±2.84	18
Methylene blue (10µM)	14.89±4.35*	5
Glibemclamide (10µM)	6.35±2.86*	6
Indomethacin (10µM)	5.58±2.02*	6
Atropine (1µM)	0.80±0.46*	4
TEA (10µM)	13.46±1.44*	5
Propranolol (10µM)	8.00±2.08*	6
L-NAME (10µM)	3.79±1.26*	5

Data were present as mean ±S.E.M, *n* = 4- 18.

* p<0.05 showed significant difference of contraction from plai oil group.

Table 11 The effects of inhibitors on percentage of relaxation induced by plai oil (40 µg/ml) in endothelium-denuded aortic rings.

	Mean	N
Control plai oil (40 µg/ml)	21.19±2.43	17
Methylene bule (10 µM)	17.80±2.00	4
Glibenclamide (10 µM)	12.60±0.99	4
Atropine (1 µM)	16.50±0.42	4
Propranolol (10 µM)	15.32±2.76	4

Data were present as mean ±S.E.M, n = 4 - 17.

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