ฤทธิ์ในการคลายกล้ามเนื้อของสารสกัดด้วยเอทานอลจากผลิตภัณฑ์ตำรับยาประสะไพลต่อกล้ามเนื้อมดลูกที่แยกจากกายหนูแรท

นางสาว สุรีรัตน์ รังสิมันตุชาติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTISPASMODIC EFFECTS OF ALCOHOLIC EXTRACTS FROM ORAL FORMULATION “PRASAPLAI” ON ISOLATED RAT UTERINE MUSCLE

Miss Sureerat Rangsimantuchat

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สุรีรัตน์ รังสิมันตุชาติ : ฤทธิ์ในการคลายกล้ามเนื้อของสารสกัดด้วยเอทานอลจากผลิตภัณฑ์ตำรับยาประสะไพลต่อกล้ามเนื้อมดลูกที่แยกจากกายหนูแรท. (ANTISPASMODIC EFFECTS OF ALCOHOLIC EXTRACTS FROM ORAL FORMULATION “PRASAPLAI” ON ISOLATED RAT UTERINE MUSCLE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. สุรีย์ เจียรณ์มงคล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร. ประสาน ธรรมอุปกรณ์, 107 หน้า.

การศึกษาครั้งนี้ได้ศึกษาฤทธิ์ในการคลายกล้ามเนื้อของสารสกัดด้วยเอทานอลจากผลิตภัณฑ์ตำรับยาประสะไพล (PSP01 และ PSP02) โดยเปรียบเทียบกับน้ำมันไพลมาตรฐาน (PSPoil) ต่อการหดตัวของกล้ามเนื้อมดลูกที่แยกจากกายหนูแรท ผลการศึกษา พบว่าสารทดสอบทุกชนิดสามารถทำให้มดลูกเกิดการคลายตัวทั้งใน spontaneous และจากการกระตุ้นให้หดตัวด้วย ACh (1μM), oxytocin (2 nM), $PGF_{2\alpha}$ (1μM) และ KCl (50 mM) ได้อย่างมีนัยสำคัญทางสถิติ และสารทดสอบทุกชนิด ที่ความเข้มข้น 100 μg/ml สามารถยับยั้งการหดตัวจากการกระตุ้นด้วยแคลเซียมคลอไรด์แบบสะสมขนาด ในสภาวะ high K^+-Ca^{2+}-free และ vanadate ในสภาวะที่ปราศจาก Ca^{2+} ได้ จากผลการศึกษาแสดงให้เห็นว่าตำรับยาประสะไพลทั้ง 2 ตำรับมีฤทธิ์ในการยับยั้งการหดตัวของมดลูก และมีความแรงในการยับยั้งมากกว่าน้ำมันไพลและสารมาตรฐาน อาจเกิดจากฤทธิ์ของสมุนไพรหลายชนิดที่เป็นองค์ประกอบในตำรับยาร่วมกัน โดยฤทธิ์ในการคลายตัวของมดลูกนั้นเกี่ยวข้องกับกลไกของแคลเซียม (Ca^{2+}-dependent pathway) และไม่เกิดจากการกระตุ้นที่ β_2-adrenoceptors อย่างไรก็ตามควรมีการศึกษาถึงผลต่อปริมาณแคลเซียมภายในเซลล์เพื่อให้ทราบถึงการออกฤทธิ์ที่แน่ชัดต่อไป

สาขาวิชา.....เภสัชวิทยา..... ลายมือชื่อนิสิต.....

ปีการศึกษา.. 2552..... ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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SUREERAT RANGSIMANTUCHAT : ANTISPASMODIC EFFECTS OF ALCOHOLIC EXTRACTS FROM ORAL FORMULATION “PRASAPLAI” ON ISOLATED RAT UTERINE MUSCLE. ADVISOR : ASST. PROF. SUREE JIANMONGKOL, Ph.D., CO- ADVISOR : ASSOC. PROF. PRASAN DHUMMA-UPAKORN, Ph.D., 107 pp.

This study investigated the spasmolytic effect of the alcoholic extracts from polyherbal formulation “Prasaplai” (PSP01 and PSP02), in comparison with the alcoholic extract from *Zingiber cassumunar* Roxb. (PSPoil), on the *in vitro* model of isolated rat uterine horn. The result indicated that all of the test materials significantly relaxed the muscle tension either developed spontaneously or developed by pretreatment with ACh (1μM), oxytocin (2 nM), $PGF_{2\alpha}$ (1μM) and KCl (50 mM). The inhibitory effects were concentration-dependent. In addition, all of the test extracts (100 μg/ml) significantly inhibited the contraction induced by $CaCl_2$ in high K^+-Ca^{2+}-free condition and vanadate-induced contraction in Ca^{2+}-free condition. Both PSP01 and PSP02 were equivalent in inhibiting uterine contraction with the higher potency than plai oil (PSPoil) or its major herbal ingredient (sabinene and terpinen-4-ol). Thus, it was likely that there were other ingredients in the whole preparation involving in its relaxation effects. The plausible mechanisms of actions of the alcoholic extracts involved either inhibiting a rising of free cytosolic Ca^{2+} or Ca^{2+}-dependent signaling pathway. Furthermore, the actions of these extracts were not mediated through β_2-adrenoceptors. Further studies could be in need to clarify its influence on intracellular Ca^{2+}level.

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

Academic Year : 2009 Advisor’s Signature .....

Co-Advisor’s Signature .....

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LIST OF ABBREVIATIONS

AC	Adenylate cyclase
ACh	Acetylcholine
ANOVA	One-way analysis of variance
AT	Atropine
ATP	Adenosine 5'- triphosphate
ATPase	Adenosine triphosphatase
Ca^{2+}	Calcium ion
$[Ca^{2+}]i$	Intracellular calcium ion concentration
Ca-CAM	Calcium-calmodulin complex
CaM	Calmodulin
CaMK	Calcium calmodulin-dependent protein kinase
cAMP	Cyclic adenosine 3',5'- monophosphate
CICR	Calcium induced calcium release
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CRAC	Calcium release-activated calcium channel
DAG	Diacylglycerol
DMPBD	(E)-1-(3,4-dimethoxyphenyl) butadiene

DMSO	Dimethylsulfoxide
G	Heterotrimeric G proteins
H	Hormone
IC_{50}	50% Inhibitory concentration
InsP	Inositol phosphate
IP_3	Inositol 1,4,5-trisphosphate
K^+	Potassium ion
KCl	Potassium chloride
LC20	Myosin light chain
LO	Lipoxygenase
M	Muscarinic
M	Molar
ml	Mililitre
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
NSAIDs	Non-steroidal anti-inflammatory drugs
OXY	Oxytocin
PGE_2	Prostaglandin E_2
$PGF_2\alpha$	Prostaglandin $F_2\alpha$
PIP_2	phosphatidylinositol bis-phosphate

PKA	Protein kinase A
PLC	Phospholipase C
Ptase	Protein phosphatase
ROC	Receptor - operated calcium channels
RyRs	Ryanodine receptors
S.E.M	Standard error of mean
SMOC	Second messenger-operated cation channels
SR	Sarcoplasmic reticulum
VOC	Voltage-operated calcium channels
µg	Microgram
µM	Micromolar

CHAPTER I

INTRODUCTION

Background and rationale

Prasaplai preparation has been used in Thailand for a long time. Currently, it is on the list of the Thai traditional common household drug announced by Ministry of Public Health for relieving muscle pain, postpartum uterine involution and abnormal menstrual cycle (National drugs committee, 2006). This preparation composes of ten medicinal plants and two minerals. The major medicinal plant in this preparation is *Zingiber cassumunar* Roxb. or "plai" (approximately 80%). Other medicinal plants include *Acorus calamus* (Linn.), *Allium sativum* (Linn.), *Citrus hystrix* DC., *Curcuma zedoaria* Roscoe, *Eleutherine palmifolia* (Linn.) Merr, *Nigella sativa* (Linn.), *Piper chaba* Hunt, *Piper nigrum* (Linn.) and *Zingiber officinale* Roscoe. The two minerals are sodium chloride and camphor.

There were a few pharmacological studies of "Prasaplai" preparation. It has been demonstrated that aqueous and alcoholic extracts of "Prasaplai" contained anti-inflammatory and spasmolytic activities. These extracts could inhibit uterine contraction provoked by ACh, oxytocin and PGE_2 (Nualkaew *et al*., 2003; 2004). In addition, there were certain investigations on the actions of "Plai", a major component of the preparation. It was reported that aqueous extract of plai *(Zingiber cassumunar* Roxb.) 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 (Koysooko *et al*., 1988). The relaxation caused by aqueous extract of plai in isolated rat uterus and intestine could be antagonized by acetylcholine, calcium chloride but not by alpha-blocker, beta-blocker or histamine (Arj-am, 2008).

As known, intracellular Ca^{2+} ($[Ca^{2+}]_i$) is a major element regulating the contraction of smooth muscle (Karaki *et al*., 1997). The $[Ca^{2+}]_i$ increases via a release of Ca^{2+} from internal store as well as via an influx of extracellular Ca^{2+} through voltage-operated Ca^{2+} channel (VOC) and receptor operated Ca^{2+}channel (ROC) (Vander *et al*., 2001; Wray *et al*., 2003; Guyton and Hall, 2006; Sanborn, 2007). A decrease in $[Ca^{2+}]_i$ could result in muscle relaxation. This process could be initiated via several pathways such as blockade of voltage-operated calcium ion channels (Luckas *et al*., 1999) or activation of Ca^{2+}efflux system such as Ca^{2+} ATPase and Na^{+} / Ca^{2+} exchanger (Felder *et al*., 1994). Although there were certain reports about the spasmolytic effects of alcoholic extract of Prasaplai preparation in the *in vitro* model of isolated rat uterus, the underlying mechanisms of action especially the handling of intracellular calcium have not been investigated. In addition, it was interesting to know whether the pharmacological actions of plai (a major ingredient in formulation) could equally represent those of the whole preparation. Thus, this study aimed to investigate the mechanisms of spasmolytic actions of the alcoholic extract of Prasaplai preparation in the model of the isolated rat uterus. The designs of this study were to examine the direct effect of Prasaplai preparations on smooth muscle contractility and its interferences on Ca^{2+} handling. In

addition, the effects of alcoholic extract of Prasaplai were to compare with those of alcoholic extract of *Zingiber cassumunar* Roxb. (plai).

Hypothesis:

The alcoholic extracts of Prasaplai preparations and *Zingiber cassumunar* Roxb. (Plai oil) were comparable in their inhibitory effects on the contraction of uterus muscle in estrus cycle. It was possible that its mechanisms of action involved with an interference on calcium movement into the cells.

Objective:

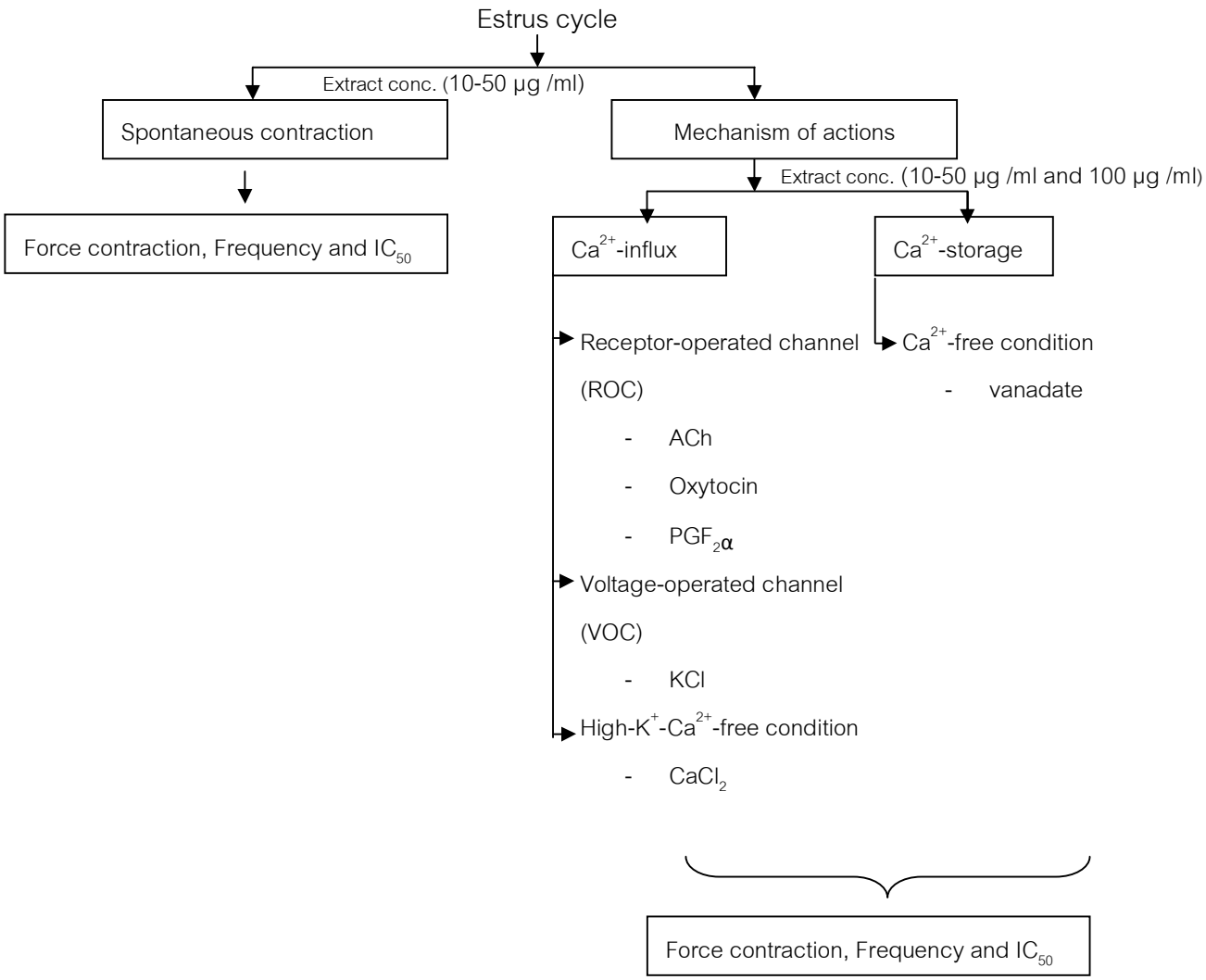
1. To examine the spasmolytic effects of alcoholic extract from prasaplai preparation in comparison with plai oil, using the model of the isolated rat uterus in estrus cycle.

2. To define the plausible mechanisms of the alcoholic extract from Prasaplai preparation and plai oil, in particular an involvement of calcium handling in the isolated rat uterus.

Expected Benefit and Application:

This study clarified the effects of alcoholic extract from Prasaplai preparation on the contraction of isolated rat uterus as well as its mechanisms of action. The result was useful for further application in traditional medicine development as well as for prediction the potential adverse effects on the reproductive system.

Research Plan



CHAPTER II

LITERATURE REVIEW

Anatomy and Physiology of the Uterus

The human uterus is a hollow, thick-walled, pear-shaped muscular organ in the female reproductive system. It is located between the urinary bladder in front and the rectum behind and sits above the vagina. The human uterus can be divided into two parts. One is cervix which is the lower part of the uterus that opens into the vagina. The other is corpus which is the main body of the uterus. Uterus muscle is composed of three layers as follows.

1. **Endometrium:** This is the innermost layer of the uterus. The thickness of the endometrium is regulated by hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH), and gonadotropin releasing hormone (GnRH) (Parker and Schimmer, 2006). Hence, the function and thickness of this layer depend on the phase of the menstrual cycle.

2. **Myometrium**: This middle layer is a thick wall made of smooth muscle cells. During pregnancy, this layer expands to accommodate a developing embryo (Thibodeau and Patton, 2007).

3. **Serosa**: This layer is the outermost layer which merges with connective tissue (ligaments) to suspend the uterus in the pelvis (Martin *et al*., 2004; Thiodeau and Patton, 2007).

In the uterus of rat or other rodents have different shapes from human uterus. Rat uterus can be divided into 3 parts, including left and right uterine horns and the body of uterus (Figure 1). The uterine horns are composed of vascular, glandular mucosa and smooth muscle. The functions of muscle in uterine horns are comparable to those of human uterus (Kittel *et al.,* 2004).

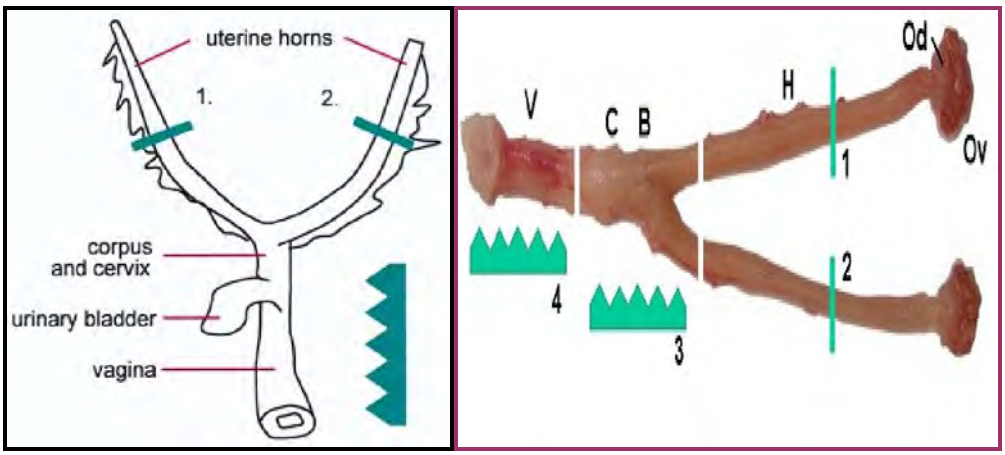


Figure 1: Rat uterine and vagina (V: vagina, C: cervix, H: uterine horn, Od: oviduct, Ov: ovary). Localization at 1 and 2 are the middle regions of rat uterine horn, 3 indicate uterine bodies and cervix and 4 is anterior portion of vagina (Kittel *et al.,* 2004).

The Oestrous Cycle in Rats

This section was to describe the oestrous cycle in rats and its effects on myometrial contractility and excitability. Similar to human' s cycle, the menstrual cycle in rats is regulated by the complex interaction of hormones including LH, FSH and the female sex hormones estrogen and progesterone (Parker and Schimmer, 2006). The

duration of rat reproductive cycle (or oestrous cycle) lasts 4-5 days. The cycle can be characterized by four distinct periods, including prooestrous, oestrous, metoestrous (or dioestrous I) and dioestrous (or dioestrous II) (Freeman, 1994). In pro-oestrous period which lasts only 12-14 h, the rats have high levels of FSH to stimulate the follicle and endometrium proliferation. Then, the cycle moves into oestrous period which lasts 25-27 h. In the early stage of oestrous period, estrogen level remains high until the ovulation is over. Then, the cycle undergoes into the next metoestrous period which lasts only 6-8 h. In the metoestrous phase, the corpus luteum proliferates and produces progesterone. Consequently, the thickness of endometrium progressively increases. The last phase is dioestrous period of 55-57 h. Without the pregnancy term, this phase will be terminated with the regression of the corpus luteum. The muscle undergoes reorganization for the next cycle (Houdeau *et al*., 2003).

Control of Uterine Contraction

The contractility of myometrium is regulated by an interaction of myogenic, neurogenic and hormonal control systems (Sanborn, 2007). According to myogenic control, it is generally accepted that Ca^{2+}- dependent regulation of interaction between myosin and actin filaments is fundamental to smooth muscle contraction. Myometrial contraction is initiated by an increase in the concentration of intracellular free calcium. Consequently, a Ca^{2+}-CAM complex which is an activator of myosin light chain kinase (MLCK) increases. Then, the active MLCK catalyzes the phosphorylation of serine at position 19 (Ser 19) of the regulatory light chain (myosin light chain, MLC_{20}) to allow the

activation of myosin ATPase. Finally, the contraction of muscle occurs (Somlyo and Somlyo, 1994; Karaki *et al*., 1997; Wray *et al*., 2003).

$Ca^{2+}$and Smooth Muscle Contraction

Similar to other smooth muscle cells, the level of intracellular Ca^{2+}in uterus muscle cell depends on the entry of Ca^{2+}across the plasma membrane and the release of $Ca^{2+}$from sarcoplasmic reticulum (Figure 2) (Webb, 2003; Wray *et al*., 2003). The mechanism pathways of Ca^{2+} release and Ca^{2+} influx were described below.

1. Ca^{2+} Influx through Voltage-Operated Calcium Channel

In smooth muscle, L-type Ca^{2+} channels are considered to be a major Ca^{2+} influx pathway in myometrium (Riemer *et al*., 2000; Sanborn, 2000; Miyoshi, 2004). They are opened by depolarization or changes in action potential or slow wave discharge (Bolton *et al*., 1999). Under resting condition (-40 to -50 mV), these channels are close and inactivated (Parkington and Colemman, 1990). Upon excitation, membrane rapid depolarization occurs and voltage-dependent Ca^{2+}channels open. The channel opening leads to Ca^{2+} flux into the cell (Wray *et al*., 2003). In addition, it has long been known that high potassium solution can depolarize plasma membrane, leading to Ca^{2+}channel opening (Bolton, 1979). Treatment muscle with potassium-rich solution induces contraction that is dependent on extracellular Ca^{2+} (Godfrained *et al*., 1986). Moreover, chemicals such as Bay K8644 that directly activate L-type Ca^{2+} channels are able to provoke contraction of smooth muscle (Vaghy, 1998; Wray *et al*., 2003).

2. Ca^{2+} Influx through Receptor-Operated Ca^{2+}Channel

The receptor-operated Ca^{2+}channel (ROC) allows Ca^{2+}entry into smooth muscle cells through mechanisms that are independence of membrane depolarization (Somlyo and Somlyo, 1994). ROC is usually coupled to either excitatory receptors or G proteins linked receptors (Sanborn *et al*., 1998). Activation of G protein (G_q) increases phospholipase C activity, leading to the breakdown of phosphatidylinositol bisphosphate (PIP_2) into inositol triphosphates (IP_3) and diacylglycerol (DAG). The binding of IP_3 to its receptors on the sarcoplasmic reticulum (SR) results in the release of Ca^{2+} into the cytosol (Webb, 2003). On the other hand, DAG activates protein kinase C (PKC), which subsequently phosphorylates L-type Ca^{2+}channels. Finally, L-type Ca^{2+} channels open, leading to Ca^{2+} influx (Webb, 2003).

3. Ca^{2+}Release from Sarcoplasmic reticulum (SR)

In myometrial cells, intracellular $Ca^{2+}$is contained mainly in the sarcoplasmic reticulum (SR) (Wray *et al*., 2005). There are two types of intracellular Ca^{2+} channels in the uterine muscles. One is the ryanodine receptors (RyR) which can be activated by Ca^{2+}. This is a process referred as Ca^{2+}- induced Ca^{2+}- release (CICR) (Lynn *et al*., 1995; Martin *et al*., 1999). There are numerous concerns about the functional expression of RyR receptors in the myometrium. It has been reported that the RyR may express, but not function, except at the end of pregnancy (Wray *et al*., 2003; 2005; Wray and Shmygol, 2007). Thus caffeine which is an activator of RyR has no effect in freshly isolated cells (Arnaudeau *et al*., 1994). The other type of channel is IP_3-coupled

receptors which compose of three isoforms including type1, type2 and type3. All of three isoforms share about 60-80% similarity in their amino acid sequences and express on luminal side of the SR membrane (Wray and Shmygol, 2007). The IP_3 receptor mediated-Ca^{2+} release are a common pathway in oxytocin, prostaglandin, α-adrenergic and M-cholinergic-induced calcium signaling. Several types of spasmogens, which are classified by their receptor binding on the surface membrane, share the same mechanisms of contraction through activation of PLC, leading to the release of calcium from the SR (Shmigol *et al*., 2001; Wray and Shmygol, 2007).

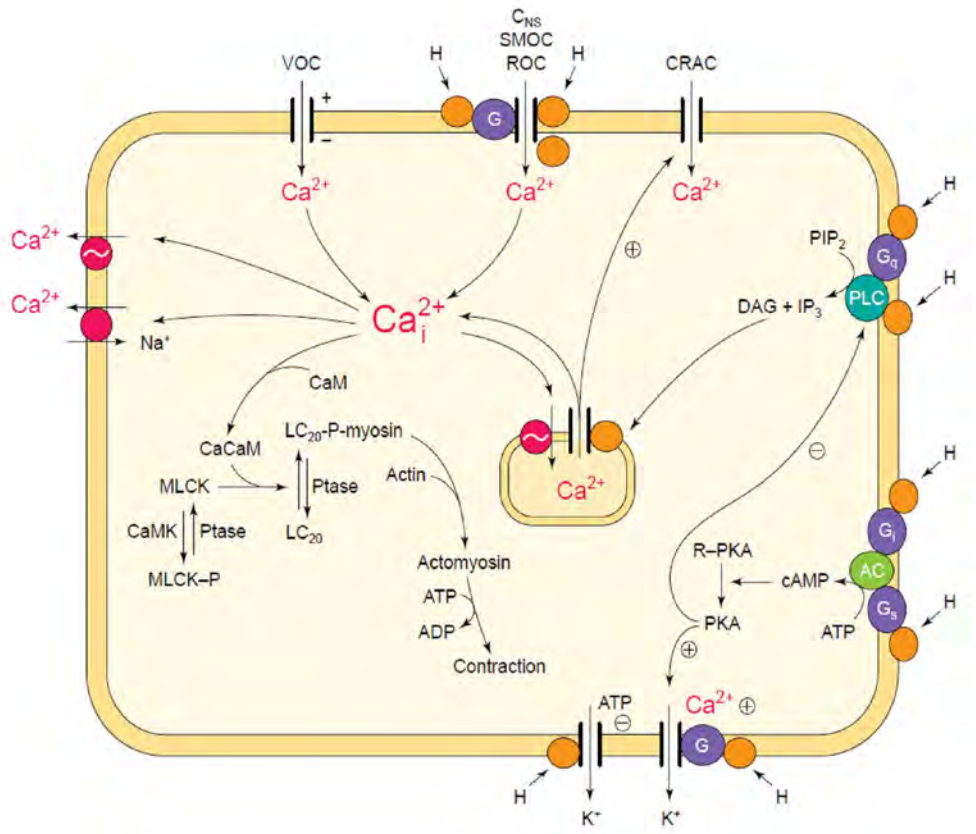


Figure 2: Pathways leading to control of [Ca^{2+}] in smooth muscle. Components include hormones or other ligands (H), calmodulin (CaM), myosin light chain kinase (MLCK), myosin light chain (LC20), protein phosphatase (Ptase), a voltage-operated Ca^{2+} channel (VOC), receptor-operated channel (ROC), nonselective (CNS) and second messenger-operated (SMOC) cation channels, a calcium release-activated calcium channel (CRAC), heterotrimeric G proteins (G), phospholipase C (PLC), inositol 1,4,5-trisphosphate (IP_3), diacylglycerol (DAG), adenylyl cylase (AC), protein kinase A (PKA), calcium calmodulin-dependent protein kinase (CaMK), phosphatidylinositol bis-phosphate (PIP_2), Ca^{2+} transport ATPases, Na^+-Ca^{2+} exchanger, a calcium-activated potassium channel and an ATP-sensitive potassium channel (Modified from Sanborn *et al.*, 1995.)

$Ca^{2+}$and Smooth Muscle Relaxation

The relaxation of smooth muscle is initiated by termination of Ca^{2+}entry and dephosphorylation of myosin light chain (Wray *et al*., 2003). There are mainly two mechanisms for removal of cytosolic Ca^{2+}, including plasmalemma and sarcoplasmic reticulum Ca^{2+}-Mg^{+}-ATPase and Na^{+}/Ca^{2+} exchanger (Matthew *et al*., 2004). The sarcoplasmic reticulum and the plasma membrane contain Ca^{2+}, Mg^{+}-ATPases that remove cytosolic Ca^{2+}. This enzyme has an autoinhibitory domain that can be bound by calmodulin (CAM). The binding of CAM activates the plasma membrane Ca^{2+} pump. The activity of Ca^{2+}, Mg^{+}-ATPases can be inhibited by several different pharmacological agents such as vanadate, thapsigargin, and cyclopiazonic acid (Webb, 2003). The other channel for cytosolic Ca^{2+}removal is the Na^{+}/ Ca^{2+} exchangers. The exchanger can be found on the plasma membrane, closely coupled to intracellular Ca^{2+} level.

As previously mentioned, receptor-operated and voltage-operated Ca^{2+} channels located in the plasma membrane are important in regulation of Ca^{2+} influx and muscle contractility. During relaxation, both types of these Ca^{2+}channels are close, resulting in a decrease of Ca^{2+}entry into the cell. The decrease of intracellular Ca^{2+} leads to the dissociation of Ca^{2+}-CAM complex and the inactivation of MLCK. Meanwhile, the phosphorylated myosin light chains (MLC) are dephosphorylated by myosin light chain phosphatase (MLCP), leading to smooth muscle relaxation (Wray *et al*., 2003). This event was simplified by the diagram in Figure 3.

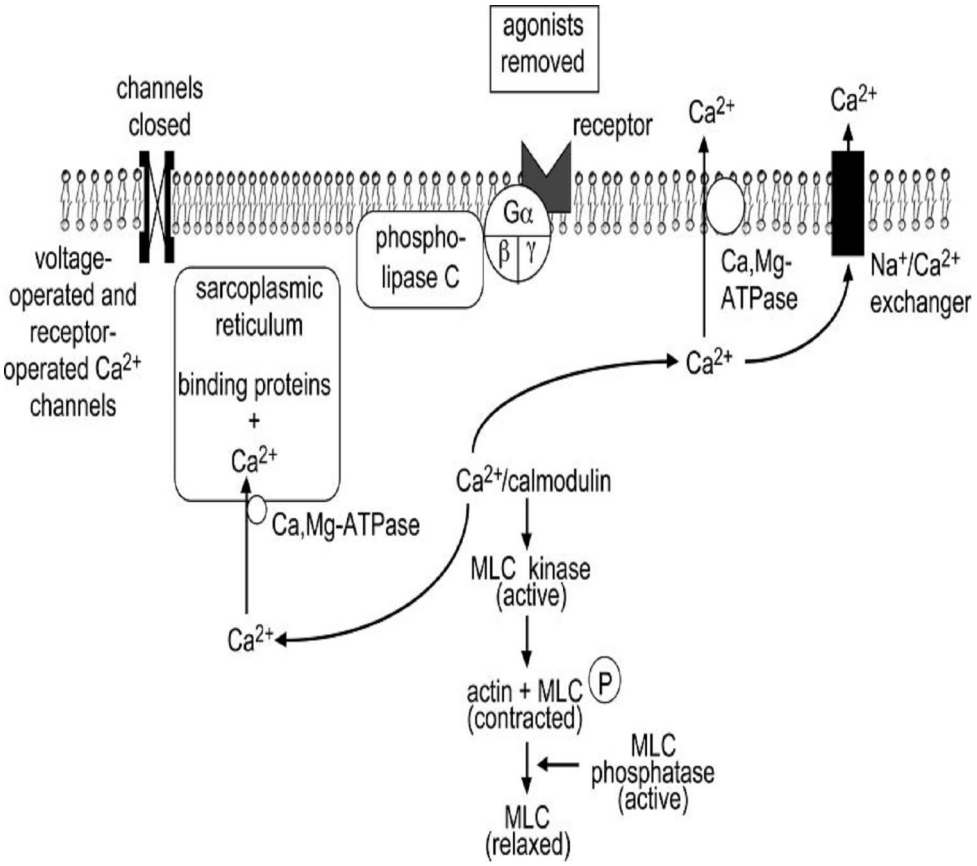


Figure 3: Mechanism of smooth muscle relaxation (Modified from Webb, 2003)

Modulators of Uterine Contraction

Uterine contraction can be modulated by several hormones, neurotransmitter, electrolyte and chemicals. These modulators can influence the frequency, duration, and amplitude of the contractions. This section provided a brief overview of certain modulators influencing uterine contraction.

1. Hormones

1.1 Estrogen and Progesterone

Estrogen plays a significant role in increasing the sensitivity of the contractile response. In late pregnancy, estrogen promotes uterine contractility via its effects on contractile proteins, gap junction formation and increased responsiveness of

uterus to contractant such as oxytocin and prostaglandin $F_{2\alpha}$ (Eagland and Cooper, 2001). The effects of estrogen include the increases in receptor numbers, density and sensitivity, as well as an increase in gap junction expression in myometrium (Sato *et al*., 1996; Eagland and Cooper, 2001). An increase in gap junction formation provides partial cytoplasmic continuity between adjacent cells and thus serves as an ideal anatomical substrate for coordinating tissue responses (Christ and Brink, 2000). Progesterone plays a role in maintaining the uterus in a quiescent stage. It inhibits gap junction formation and down regulates oxytocin receptors (Cole and Garfield, 1986; Sato *et al*., 1996; Eagland and Cooper, 2001).

1.2 Oxytocin

Oxytocin is synthesized in hypothalamic neurons and transported down axons of the posterior pituitary for secretion into blood (Sultatos, 1997; Eagland and Cooper, 2001). Oxytocin receptors are G-protein-coupled receptors that bind with oxytocin on the myometrial membrane. The binding of oxytocin receptor stimulates phospholipase C signaling pathway. In term, estrogen increases uterine sensitivity to oxytocin through induction of receptors densities (Zingg and Laporte, 2003). In post partum, oxytocin receptor densities rapidly decrease

2. Autacoids

2.1 Prostaglandin (PGs)

Prostaglandins, in particular $PGF_{2\alpha}$ and PGE_2, have roles in muscle contractility and modeling. The actions of the PGs are mediated by specific membrane

receptors. $PGF_{2\alpha}$ involves with an induction of parturition during late pregnancy whereas PGE_2 plays an important role in ovulation and fertilization (Tsuboi *et al*., 2002). $PGF_{2\alpha}$ induces uterine contraction through its involvement in phosphatidylinositol signaling pathway. Consequently, intracellular Ca^{2+} increases via IP_3-mediated Ca^{2+} release from its internal store. Furthermore, $PGF_{2\alpha}$ may also open nonselective cation channel, leading to sodium influx to the cell. In turn, plasma membrane depolarizes and membrane Ca^{2+} channels become activated (Bolton, 1979).

3. Neurotransmitters and their receptors

3.1 Adrenergic receptor

Adrenergic signaling pathways are under influence of progesterone and estrogen (Dupuis *et al*., 2004). These two hormones regulate the expression of β-adrenergic receptor, heterotrimeric G proteins ($G_{s\alpha}$, $G_{i\alpha}$ and $G_{q\alpha}$) and PLC_β enzymes (Dupuis *et al*., 2004). The β-adrenoceptors in myometrium is β_2-adrenoceptor which is present mainly in longitudinal muscle of rostral uterine horn (Houdeau *et al*., 1998). The agonist binding to β-adrenoceptors leads to activation of G_s proteins which is coupled to adenyl cyclase (AC) system. The activated adenylyl cyclase catalyzes the conversion of cytosolic ATP to cyclic AMP (cAMP), which then activates protein kinase A (PKA). Consequently, PKA phosphorylates a member of proteins and causes muscle relaxation by decrease Ca^{2+} sensitivity of contractile element (Wray *et al*., 2003; Faber, 2009). Generally, high progesterone levels increase both receptor number and sensitivity of β_2-

receptor. However, at term, progesterone is rapid desensitization, which causes a loss of myometrium response to β_2- agonist (Sanborn, 2007).

3.2 Cholinergic receptor

ACh is the principal excitatory neurotransmitter of the parasympathetic nervous system that produces smooth muscle contraction via activation of muscarinic receptor. Muscarinic receptor has many subtypes including M_1-M_5. In uterine smooth muscle, M_3 subtype is predominantly expressed (Bolton, 1979). These G-protein coupled receptors, when stimulate, result in activation of membrane bound PLC and increase IP_3 production. Eventually, the intracellular Ca^{2+}rise and muscle contract (Sanborn, 2007).

Prasaplai Preparation

Prasaplai preparation is a Thai traditional medicine. It is widely used for postpartum uterine involution, relieving dysmenorrhea and adjusting the cycle of menstruation (Nualkaew *et al*., 2003; 2004). This preparation composes of ten medicinal plants and two minerals. The major medicinal plant in this preparation was *Zingiber cassumunar* Roxb. or “plai” (approximately 80%). Other medicinal plants included *Acorus calamus* (Linn.), *Allium sativum* (Linn.), *Citrus hystrix* DC., *Curcuma zedoaria* Roscoe, *Eleutherine palmifolia* (Linn.) Merr, *Nigella sativa* (Linn.), *Piper chaba* Hunt, *Piper nigrum* (Linn.) and *Zingiber officinale* Roscoe. The two minerals were sodium chloride and camphor.

Pharmacological and Toxicological Evaluation of Prasaplai Preparation

It has been reported that aqueous extract of Prasaplai preparation contained antispasmodic activity against rat uterine contractions induced by acetylcholine, oxytocin and PGE_2. Furthermore, this extract had anti-inflammatory action with ability to inhibit COX-1 and COX-2 activities (Nualkaew *et al*., 2003; 2004). It was reported that water extract and ethanolic extracts of Prasaplai (up to 20 g/kg, oral) had no observed toxic effects in experimental rats. In addition, both extracts had no estrogenic activity (Nualkaew *et al*., 2003; 2004). The pharmacological studies of a whole "Prasaplai" preparation were quite limited. Most studies investigated the effects of plai (*Zingiber cassumunar* Roxb.), of which the fresh rhizome was used in traditional Thai massage for muscle relaxant, antiasthmatic drug and joint pains (Wanauppathamkul, 2009). In addition, a number of studies on the aqueous extract of plai revealed its antispasmodic effects in several isolated organ preparations including rat uterus, rat intestine and human umbilical artery (Anuntasarn, 1982). The action of plai in isolated organ of rat uterus, rat intestine and human umbilical artery were antagonized by acetylcholine, calcium chloride, but not by alpha-blocker, beta-blocker or histamine (Anuntasarn, 1982). In addition, it has been reported that ethanolic extract of plai oil showed relaxant effect on isolated vascular smooth muscle model. The action of plai oil may influences on endothelium factors including NO-cGMP pathway, hyperpolarizing, cyclooxygenase, muscarinic receptors and β-adrenoceptor (Mesripong, 2006; Arj-Am, 2008).

Several chemical components which were isolated from plai oil include α-pinen , sabinene, α-terpinene, γ-terpinen, terpien-4-ol, (E)-1-(3, 4-dimethoxyphenyl) butadiene (DMPBD) and (E)-4-(3',4'-dimethoxyphenyl) but-3-en-1-ol (or compound D) . It has been a few studies of these compounds. Recently, there were reported that sabinene and terpinen-4-ol showed relaxant effect on the isolated of vascular smooth muscle model. The action of sabinene and terpinen-4-ol may involve with the endothelium-dependent and endothelium-independent pathways (Mesripong, 2006). DMPBD showed its antiinflammatory activities in both *in vitro* and *in vivo* models (Jeenapongsa *et al*., 2003). When locally injected, DMPBD was able to inhibit the edema induced by ethylphenylpropiate, arachidonic acid and 12-o-tetradecanoylphorbol 13-acetate (Panthong *et al*., 1990; Panthong *et al*., 1997; Jeenapongsa *et al*., 2003). Compound D which was isolated from hexane extract of plai exhibited a strong inhibitory activity on the edema formation in carrageenan-induced rat paw edema and also exhibited its relaxant effect in isolated uterine muscle (Koysooko *et al*., 1988; Panthong *et al*., 1990). Furthermore, compound D antagonized the effects of various spasmogens including acetylcholine (0.06 µg/ml), histamine (0.3 µg/ml), serotonin (5 µg/ml) and barium chloride (0.2 µg/ml) in isolated guinea-pig ileum (Koysooko *et al*., 1988).

CHAPTER III

MATERIALS AND METHODS

1. Animals

Virgin female Wistar rats (200-300 g) were purchased from The National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand. The animals were housed in animal care facility at the Faculty of Pharmaceutical Sciences, Chulalongkorn University. They were kept in a room under controlled environment (12 hours light/dark cycle, lights on at 7:00 am; temperature at 25±1^{o} C) at least 1 week prior to each experiment. Animals were provided with standard diet and water ad libitum.

The following study protocol was approved by the Ethics Committee on Animal Experiment, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. Chemicals and test materials

The major chemicals include acetylcholine chloride, atropine sulfate, isoproterenol hydrochloride, diethylstilbestrol, Dimethyl sulfoxide (DMSO, 99.5 %), propranolol hydrochloride, Ethylene glycol bis (β-aminoethylether)-N, N', N'-tetra acetic acid (EGTA), oxytocin, prostaglandin $F_{2\alpha}$ and verapamil hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All chemicals were dissolved in distilled water. The Locke-Ringer solution contains (mM): NaCl, 154; KCl, 5.63; $CaCl_2$ 2.16; $NaHCO_3$, 5.95; $MgCl_2$, 2.10 and D-glucose, 5.55. The high-K^+-calcium-free

depolarizing solution contains (mM): NaCl, 154; KCl, 5.63; $NaHCO_3$, 5.95; D-glucose, 25.55 and EDTA 0.01. All chemicals are analytical grades.

Test materials

The alcoholic extracts of Prasaplai formulation were commercially available from two different sources with the same processes of alcoholic extraction (PSP01 and PSP02) and the alcoholic extract of *Zingiber cassumunar* Roxb. (PSP oil) were obtained from Thailand Institute of Scientific and Technological Research. The major constituents of plai oil included α-pinen 1.68%, sabinene 39.13%, α-terpinene 2.44%, γ-terpinen 5.67%, terpien-4-ol 34.12% and (E)-1-(3, 4-dimethoxyphenyl) butadiene (DMPBD) 2.40%.

All test materials were dissolved in DMSO at a concentration of 500 mg/ml and kept as a stock solution at 2-8 ^{o}C until use (not more than 1 month). The final concentration of DMSO in each experiment was less than 0.5% (v/v). At this concentration, DMSO had no effect on contractility of rat uterus smooth muscle.

3. Experimental procedures

3.1 Tissue preparations

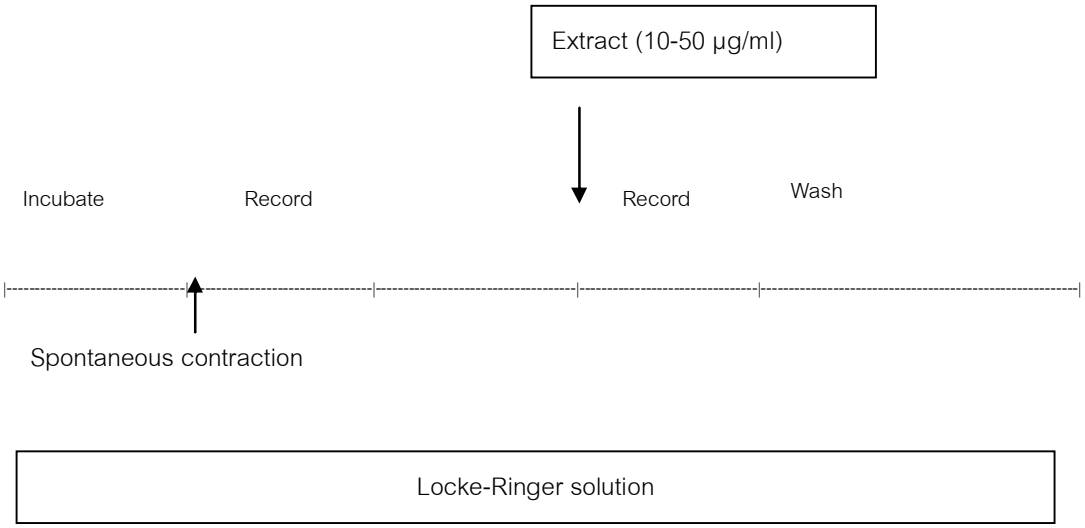
The animals were given estradiol-17-benzoate (1mg/kg), intraperitoneally at 24-48 h before the experiments in order to induce the estrus stage. The estrus stage was confirmed by microscopic examination of vaginal smear. Cornified cells were dominant in the estrus cycle (Marcondes *et al*., 2002).

The animals were anesthetized by CO_2 and killed by cervical dislocation. The uterine horns were isolated and placed in petri-dish containing Locke-Ringer solution. The uterine horn was cleaned and cut to 4 segments of approximately 10-15 mm long. Each of segments was suspended in double walled organ baths (Harvard type Organ bath) and attached to an isometric force transducer UFI 1030 (ADInstruments, Australia) under a resting tension of 1.0 g. The organ bath contained 20 ml of Locke-Ringer solution at 37 ^{o} C and bubbled with a mixture of 95% O_2 and 5% CO_2. The other end of force transducer was connected to PowerLab /4SP equipped connected to a computer with program SCOPE CHART 5 V. 2.0 (ADInstruments, Australia). Each uterine horn was allowed to equilibrate for 1 h before experimental protocol. During this equilibration period, the tissues were washed with 20 ml of Locke-Ringer solution every 15 min.

3.2 Effects of the test materials on spontaneous contraction

The uterine segment was placed in normal Locke-Ringer solution until the tension was stable. Then, each alcoholic extract (concentration 10-50 µg/ml) was added cumulatively to the bathing solution. The response was expressed as percentage of the maximal response of the spontaneous contraction (100%). In parallel experiments, the effects of DMSO were also determined.

Experiment 3.2

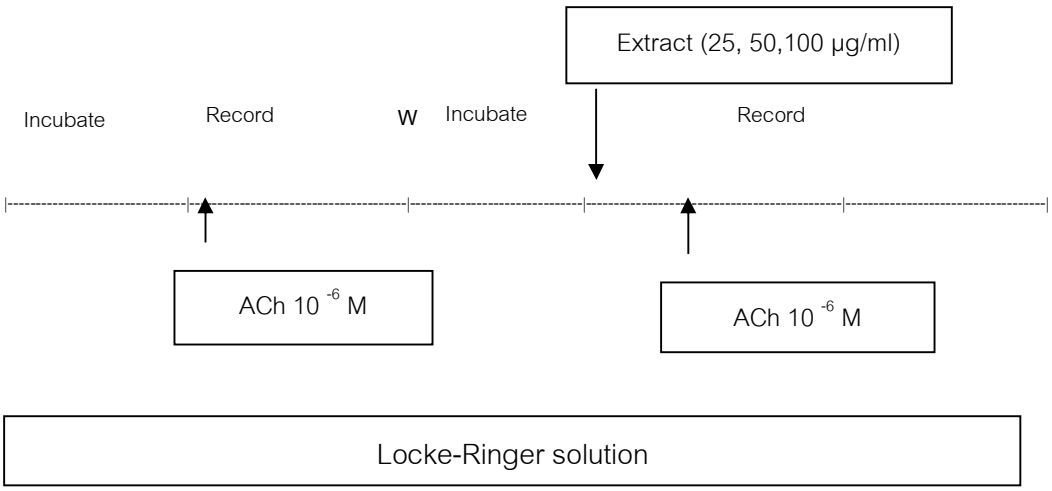


3.3 Effects of the test materials on agonist-induced contraction

3.3.1 Contraction-induced by acetylcholine

Acetylcholine (ACh) induced transient contraction of smooth muscle. In this experiment, the uterine segment was placed in normal Locke-Ringer solution. When the tension was stable, ACh at the maximal concentration (10^{-6}M) was used to provoke contraction, which was referred as 100% tension. The effects of the test extract were determined by incubating the test extract at various concentrations (25, 50 and 100 µg/ml) for 10 minutes prior to addition of ACh. The response was expressed as percentage of the maximum contraction induced by ACh (10^{-6}M). In parallel experiments, the effects of DMSO were also determined.

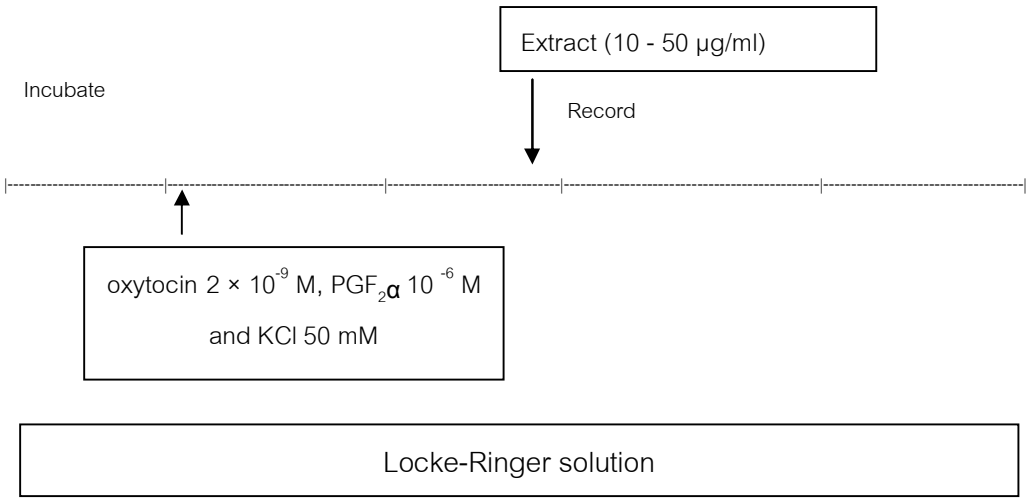
Experiment 3.3.1



3.3.2 Contraction-induced by oxytocin, $PGF_{2\alpha}$ and KCl

The test material was added cumulatively in order to determine IC_{50}. The agonists in this experiment included oxytocin (2 x 10^{-9} M), $PGF_{2\alpha}$ (10^{-6}M) and KCl (50 x 10^{-3} M). When the contraction reached plateau state, the test materials (10-50 µg/ml) were added cumulatively to produce relaxation. The tension was recorded and expressed as percentage of the agonist-induced contraction. In separated experiments, the effects of DMSO were also determined.

Experiment 3.3.2

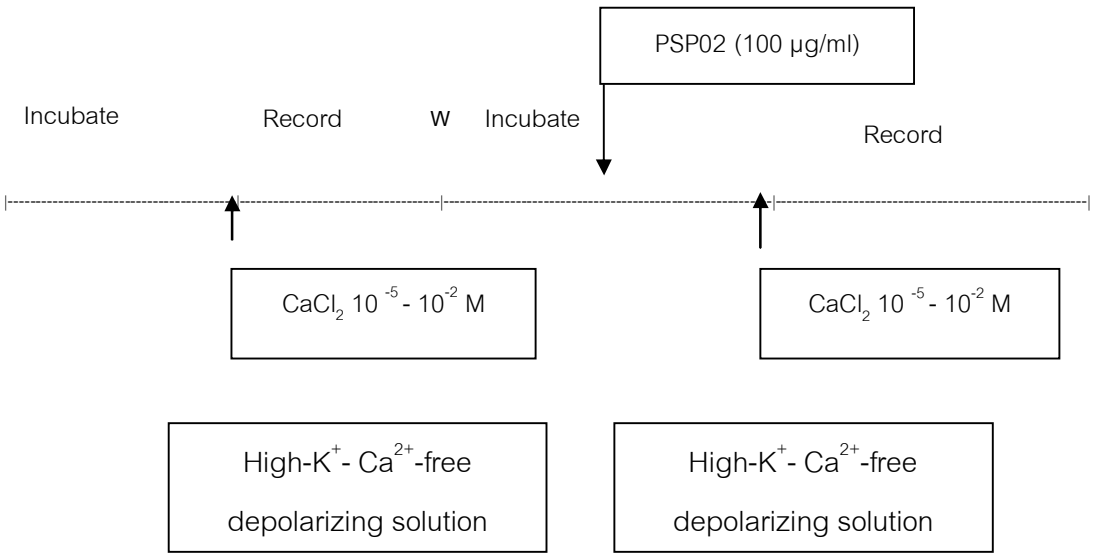


Furthermore, single treatment of the test materials was also applied to determine the sensitivity of uterine response toward specific agonist. In addition, the major components in plai (sabinene and terpinen-4-ol) were also used to compare the potencies with the test materials. Briefly, the uterine horns were stimulated with specific agonist (ACh, $PGF_{2\alpha}$, oxytocin and KCl) at the maximal concentration. After washing and reequilibrating the tissue, the test materials (PSP01, PSP02 and PSPoil) 100 µg/ml, sabinene 39.13 µg/ml and terpinene-4-ol 34.12 µg/ml were incubated with the tissue for 10 minutes prior to addition of agonist. The response was expressed as percentage of the maximum contraction induced by each stimulus.

3.4 Effects of the test materials on Ca^{2+}-influx

In order to investigate the effect of test materials on Ca^{2+} influx, the experiments were performed in high-K^{+}- Ca^{2+}-free depolarizing solution. After 30 min equilibration period in normal Locke-Ringer solution, the uterine strip was exposed to a high-K^{+}- Ca^{2+}-free depolarizing solution for 60 min. Then, the solution was changed every 10 min interval with the fresh depolarizing solution. The experiment started with adding $CaCl_2$ (10^{-5}-10^{-2}M) cumulatively to the bathing solution at 5 min interval to establish the reference baseline values. After washing and reequilibrating the tissue, the test materials were incubated with the tissue for 10 min prior to addition of $CaCl_2$. The contraction response was expressed as percentage of the maximum contraction induced by $CaCl_2$. In parallel experiments, the effects of DMSO, PSPoil (100 µg/ml), sabinene (39 µg/ml) or terpinen-4-ol (34 µg/ml) were also determined.

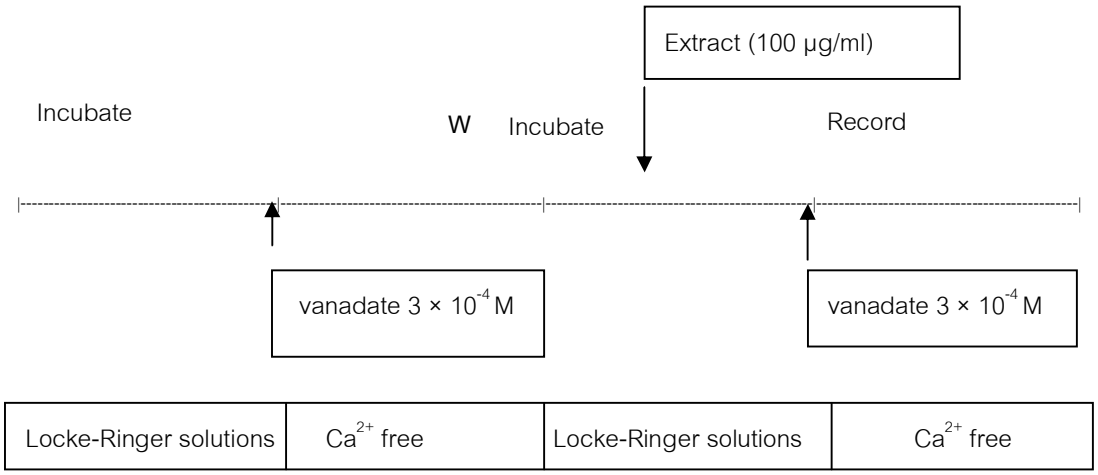
Experiment 3.4



3.5 Effect of the test materials on Ca^{2+}-storage

In order to investigate the effect of test materials on Ca^{2+}-storage in sarcoplasmic reticulum (SR), the experiments were conducted in Ca^{2+}-free solution with the use of vanadate to provoke the contraction (Perez-Guerrero *et al.,* 1996). In brief, uterine segment was equilibrated in normal Locke- Ringer solution for 60 minutes, followed by changing the medium to Ca^{2+}-free solution containing 0.01 mM EDTA. After the resting tension was stable, the uterine segment was incubated with the test materials (100 µg/ml) for 10 min prior to addition of vanadate ($3x10^{-4}$ M). The contraction response was expressed as percentage of the maximum contraction induced by vanadate. In separated experiments, the effects of DMSO, PSPoil (100 µg/ml), sabinene (39 µg/ml) and terpinen-4-ol (34 µg/ml) were also determined.

Experiment 3.5



4. Data Analysis

The values of force contractions were represented as the percentage (changes in contraction recorded in comparison with 100% contractions induced by the spasmogens in the absence of the extract). The frequency of contraction was also calculated by the number of stroke within 10 min (Sawasdipanich, 1994). The pD2' value was calculated according to Van Rossum (1963).

$pD2' = -\log [B] + \log ([E_{AM}] / [E_{AMB}]-1.$

[B] was concentration of non competitive antagonist.

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

The results were expressed as mean ± standard error of mean (S.E.M) for 4-5 separated experiments. The individual IC_{50} values were obtained by linear regression from the concentration response curve. Statistical significance were tested by one-way

analysis of variance (ANOVA) followed by post-hoc Dunnett's. The *p* values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

1. Effects of the test materials on spontaneous contraction

The spontaneous contraction profiles of uterine horn were demonstrated in Figure 4A, with the basal force at 3.64±0.27 g (n=4) and basal frequency at 6.37±0.94 stroke/10min (n=4). In this experiment, DMSO had no significant effect on spontaneous contraction. Cumulative addition of the test materials (PSP01, PSP02 and PSPoil) 10-50 µg/ml significantly reduced the basal tone and frequency of spontaneous contraction, as demonstrated in Figure 4B-4D. The concentration response curves were plotted in Figure 5A for force of contraction and Figure 5B for frequency, respectively. The results demonstrated that all test extracts reduced force and frequency of spontaneous contraction in concentration-dependent manner. In addition, the inhibitory potency of PSP01 and PSP02 were equivalent and higher than PSPoil. The linear regression concentration response curve allowed the calculation of IC_{50}, as shown in Table 1.

2. Effects of the test materials on agonist-induced contraction

2.1 Contraction-induced by acetylcholine (ACh)

ACh produced transient contractions of isolated rat uterus as shown in Figure 6. Hence, the cumulative effects of the test materials could not be achieved. In this study, single treatment of the test materials was applied to determine its effect on ACh-induced contraction. The average amplitude of force induced by ACh was 6.35±0.30 g at the rate

of 6.93±0.42 stroke/10 min (n=4). Pretreatment of the uterine horn with all the test materials (PSP01, PSP02, and PSPoil) significantly inhibited both force and frequency in concentration-dependent manner (Figure 6-8). The effects of the test materials on uterine tension were summarized in Figure 9. The result showed that both PSP01 and PSP02 at concentration of 25 µg/ml significantly suppressed ACh-induced contraction by 20%. At the higher concentration (50 and 100 µg/ml), all the test extracts exerted its inhibition in concentration-dependent manner. However, the inhibitory effect of PSP01 appeared to reach maximal level of approximately 40% at the concentration of 50 µg/ml. PSP02 elicited its maximal force reduction of approximately 70% at the concentration of 100 µg/ml. The result also revealed that PSPoil was the least potent extract in this study with the maximal inhibition of approximately 20%. Hence, PSP02 was the highest potent force inhibitor against ACh-induced contraction. The apparent descending order of potency on force reduction was PSP02>PSP01>PSPoil. The effects of the tests materials on frequency of ACh-induced contractions were also demonstrated in Figure 10. All test extracts significantly reduced the frequency of contraction in concentration-dependent manner. It was noteworthy to mention that all of the test materials (PSP01, PSP02 and PSPoil) had high potencies in reducing frequency than force of ACh-induced contraction. The maximum effects on frequency reduction were approximately 60%, 80% and 70% for PSP01, PSP02 and PSPoil at the concentration of 100 µg/ml, respectively. The descending order of rate inhibition was PSP02>PSPoil>PSP01.

2.2 Contraction-induced by oxytocin, $PGF_{2\alpha}$ and KCl

The characteristic of contraction induced by oxytocin differed from those induced by ACh. Oxytocin (2 nM) caused rhythmic contractions of isolated rat uterus with the basal force at 7.12± 0.53 g and frequency at 7.85 ± 0.48 stroke/10 min (n=4). As shown in Figure 11, oxytocin provoked a strong sustainable contraction for both force and frequency. Hence, the effects of the test materials in cumulative concentration (10-50 µg/ml) were carried out to determine their inhibitory potencies. The responsiveness of uterine horn toward oxytocin in the presence of test materials was shown in Figure 11A-11D. DMSO had no effect on uterine contraction in this experiment (Figure 11A). The cumulative addition of the test materials (10-50 µg/ml) significantly reduced both force and frequency of oxytocin-induced contraction in the concentration-dependent manner (Figure 11B-11D). The concentration response curves of force and frequency of contraction were plotted as shown in Figure 12A and B, respectively. The descending order of potency on force reduction was PSP01>PSP02>PSPoil. The results demonstrated that the inhibitory actions of all the test materials were quite comparable in force reduction. In addition, PSP01 and PSP02 were apparently more potent in frequency reduction than PSPoil. The linear regression concentration response curve allowed the calculation of IC_{50}, as shown in Table 1.

The contractions profile of isolated uterine preparations in response to $PGF_{2\alpha}$ (10^{-6} M) were similar to those of oxytocin-induced contraction (Figure 13A-13D). The basal force at 7.36± 0.43 g, and basal frequency at 5.56 ± 0.54 stroke/ 10 min (n=4).

Cumulative addition of the test extracts inhibited force and frequency of $PGF_{2\alpha}$-induced contraction in concentration-dependent manner, as shown in Figure14A and B respectively. The descending order of potency on force reduction was PSP02>PSPoil>PSP01. The result demonstrated that the force reduction of all the test materials were quite comparable. In addition, PSP01 and PSP02 were apparently more potent in frequency reduction than PSPoil. The linear regression concentration response curves allowed the calculation of IC_{50}, as shown in Table 1.

The contractile response of uterine horn toward KCl stimulation contained two phases including initial rapid, phasic contraction followed by sustained tonic contraction (Figure 15A-15D). In this study, the baseline value of the contractile amplitude were 4.63±0.40 g (n=5). Cumulative addition of each alcoholic extracts (PSP01, PSP02 and PSPoil) at the concentration ranging from 10-50 µg/ml during the sustained tonic contraction inhibited the contraction in concentration-dependent manner (Figure 16). The concentration response curves, as shown in Figure 16, revealed that the inhibitory potencies of PSP01 and PSP02 were equivalent against KCl-induced contraction. In addition, both extracts (PSP01 and PSP02) were more potent than PSPoil. The linear regression concentration response curves allowed the calculation of IC_{50}, as shown in Table 1.

The effects of the single treatment of the test materials (PSP01, PSP02 and PSPoil) at the concentration of 100 µg/ml were also determined to compare with the major ingredient of plai oil (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml). The

results indicated that all test materials inhibited both force and frequency of contractions provoked by ACh, oxytocin, $PGF_{2\alpha}$ and KCl, as shown in Figure 17-24. In ACh-induced contraction, PSP02 was the most potent in force reduction, as shown in Figure 18A. The apparent descending order of potency in force reduction was PSP02>PSP01>PSPoil>terpinen-4-ol>sabinene. The effects of the tests materials on frequency of ACh-induced contractions were demonstrated in Figure 18B. All the test materials were equivalent in reducing the frequency of contraction. Pretreatment of the test materials (100 µg/ml), sabinene 39.13 µg/ml or terpinen-4-ol 34.12 µg/ml inhibited force and frequency of contractions provoked by oxytocin as shown in Figure 19 and 20, respectively. The descending order of potency on force reduction was PSP02>PSPoil>PSP01>terpinen-4-ol>sabinene. In addition, PSP01, PSP02 and PSPoil were apparently more potent in frequency reduction than terpinene-4-ol and sabinene. Single addition of the test extracts (100 µg/ml), sabinene 39.13 µg/ml or terpinen-4-ol 34.12 µg/ml inhibited force and frequency of $PGF_{2\alpha}$-induced contraction as shown in Figure 21 and 22, respectively. The descending order of potency on force reduction was PSPoil>PSP01>PSP02>terpinen-4-ol>sabinene. The result demonstrated that the inhibitory actions of all the test materials were quite comparable in force reduction. In addition, PSP01, PSP02 and PSPoil were apparently more potent in frequency reduction than terpinen-4-ol and sabinene. Pretreatment of the test materials (100 µg/ml), sabinene 39.13 µg/ml or terpinen-4-ol 34.12 µg/ml reduced force of KCl-induced

contraction as shown in Figure 23-24. The descending order of potency on force reduction was PSPoil>PSP02>PSP01>terpinene-4-ol>sabinene.

3. Effects of the test materials on Ca^{2+}-influx

The contraction profiles of uterine horn upon cumulative addition of $CaCl_2$ (10^{-5}-10^{-3}M) in high K^+-Ca^{2+}-free depolarizing solution were shown in Figure 25A-25F. Under this condition, PSP02 at concentration of 100 µg/ml significantly suppressed the dose response curves of $CaCl_2$-induced contraction (Figure 26). The maximum contraction of uterine horn muscle reduced to 43.51±8.10% (n=5) and 57.31±11.70% (n=5) in the presence of PSP02 and PSPoil 100 µg/ml, respectively. The effects of sabinene (39.13 µg/ml) and terpinen-4-ol (34.12 µg/ml) were not statistically significant from those of the DMSO control group. The maximum contraction of uterine horn muscle reduced to 89.62±6.76% and 84.36±2.00% in the presence of sabinene and terpinen-4-ol, respectively. As seen in Figure 26, the concentration response curves revealed that PSP02 was more potent than PSPoil, sabinene and terpinen-4-ol to suppress $CaCl_2$-induced contraction. The apparent pD2 values of PSP02 and PSPoil 100µg/ml were 3.08±0.10 and 3.28±0.10, respectively. In addition, the pD2 values of sabinene (39.13 µg/ml) and terpinen-4-ol (34.12 µg/ml) were 3.49±0.43 and 3.39±0.09, respectively.

4. Effects of the test materials on Ca^{2+}-storage in sarcoplasmic reticulum (SR)

In Ca^{2+}- free solution, vanadate (0.3 mM) induced sustained uterine contraction with the tension of 4.25±0.46 g (n=4) (Figure 27). In this study, the test materials (100 µg/ml), sabinene 39.13 µg/ml or terpinen-4-ol 34.12 µg/ml significantly inhibited

vanadate-induced contraction (Figure 28). The descending order of potency on force reduction was PSP02>terpinen-4-ol>PSPoil>sabinene. PSP02 and terpinen-4-ol completely inhibited the contraction provoked by vanadate, suggesting their high effects on Ca^{2+}storage in SR.

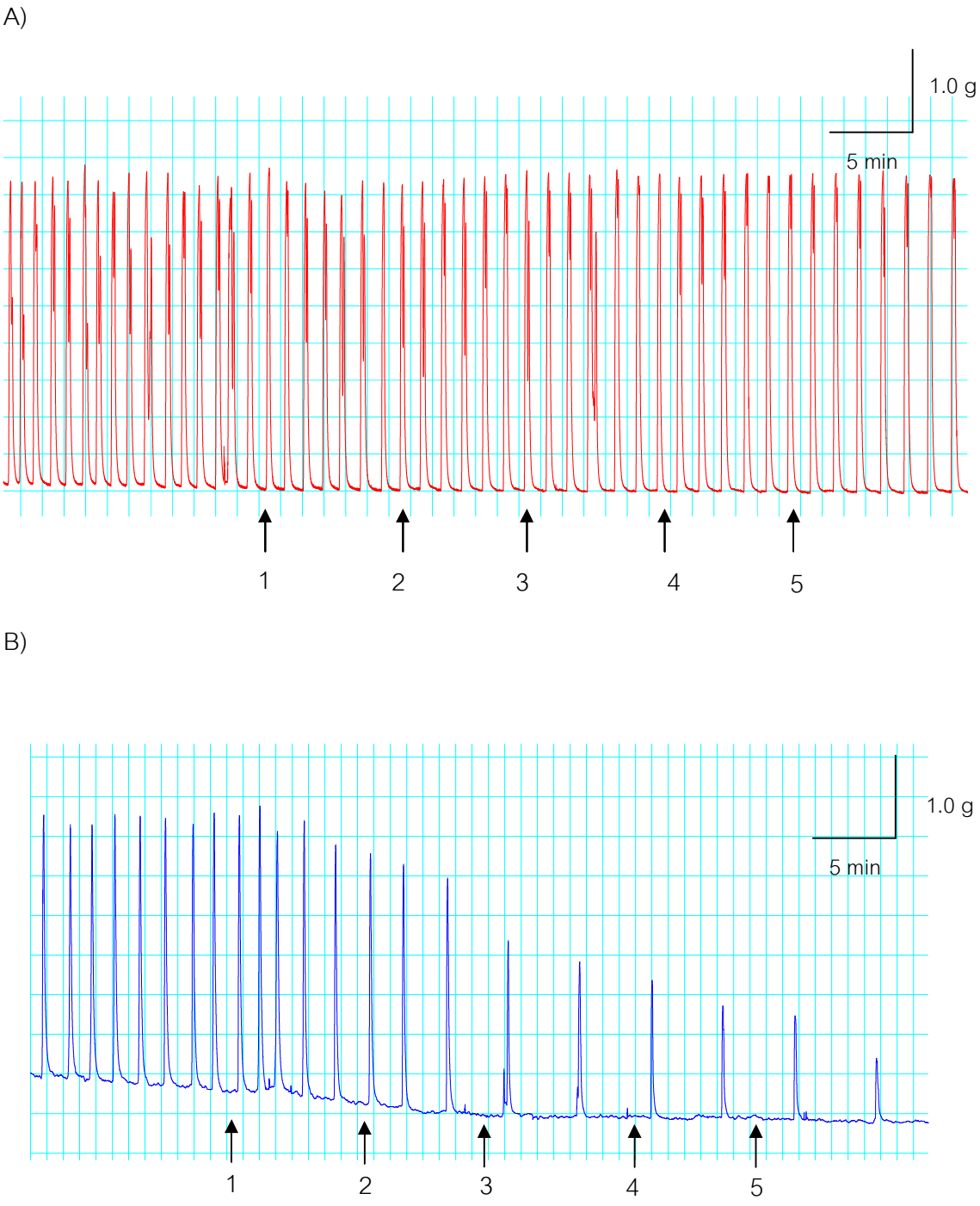
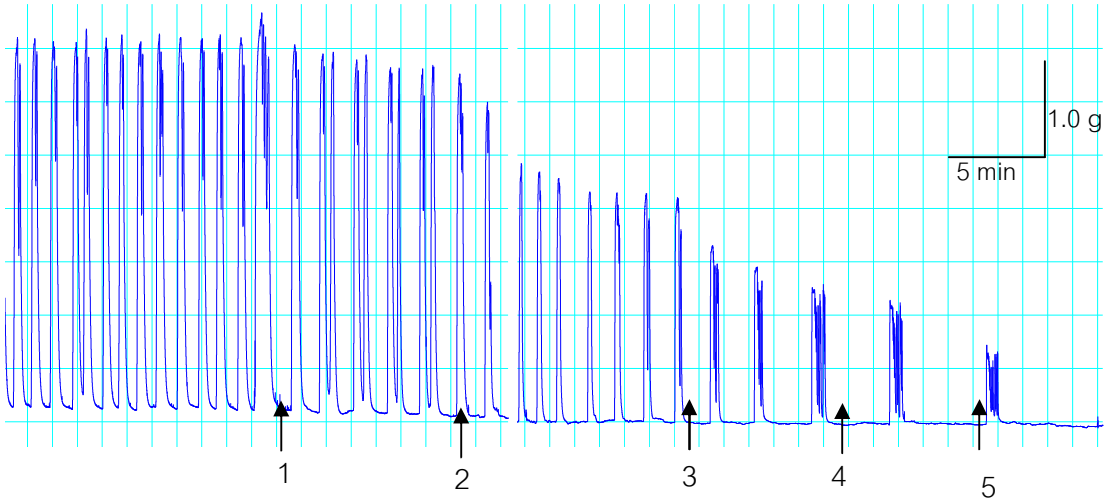
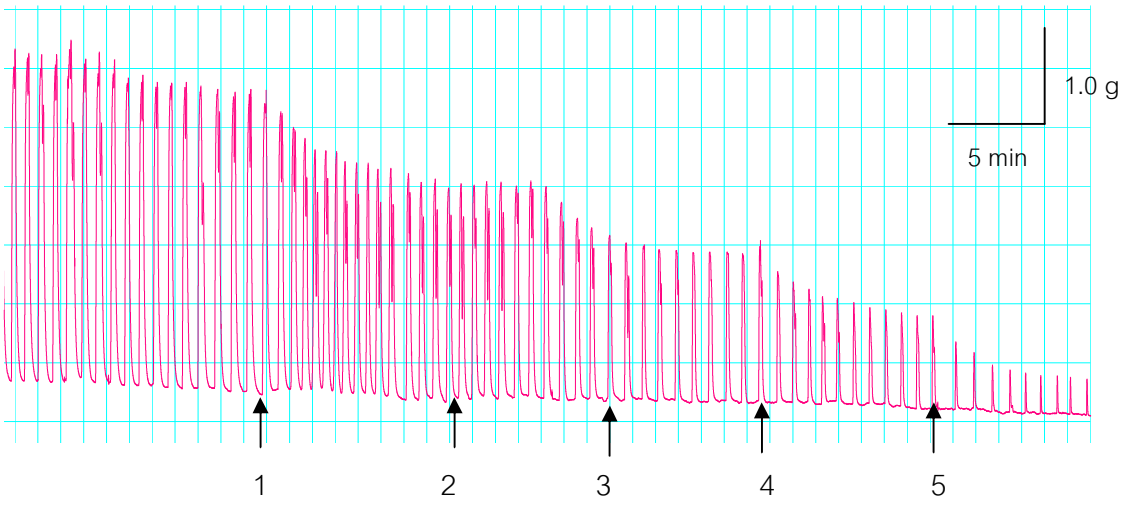


Figure 4: Representative tracing showed the relaxation induced by cumulative addition of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil on spontaneous contraction of isolated rat uterine horn. The test materials concentrations were 10 µg/ml (1), 20 µg/ml (2), 30 µg/ml (3), 40 µg/ml (4) and 50 µg/ml (5).

C)



D)



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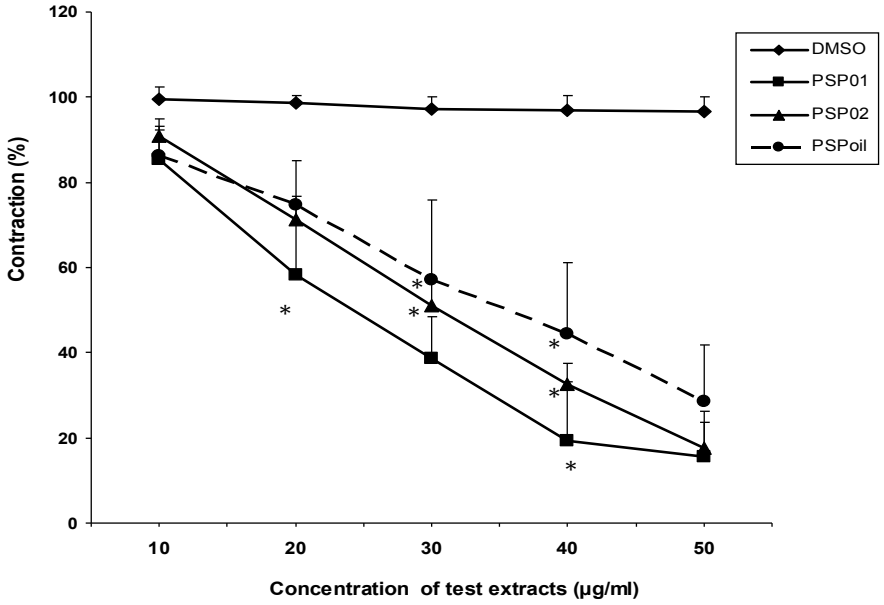
A)



B)

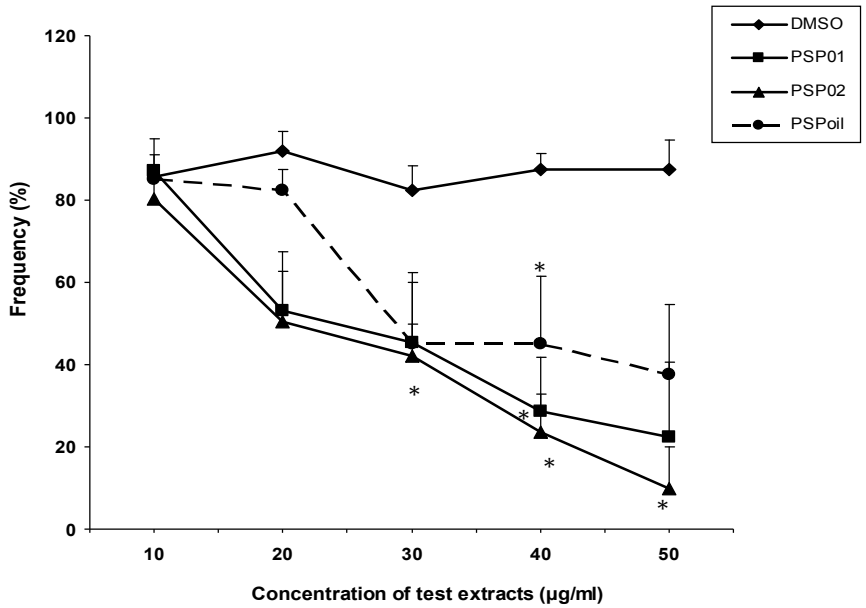


Figure 5: Concentration response curves of the test materials (10-50 µg/ml) on the contractile of amplitudes (A) and frequency (B) of spontaneously-contracting rat isolated uterine horns. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test, $*p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

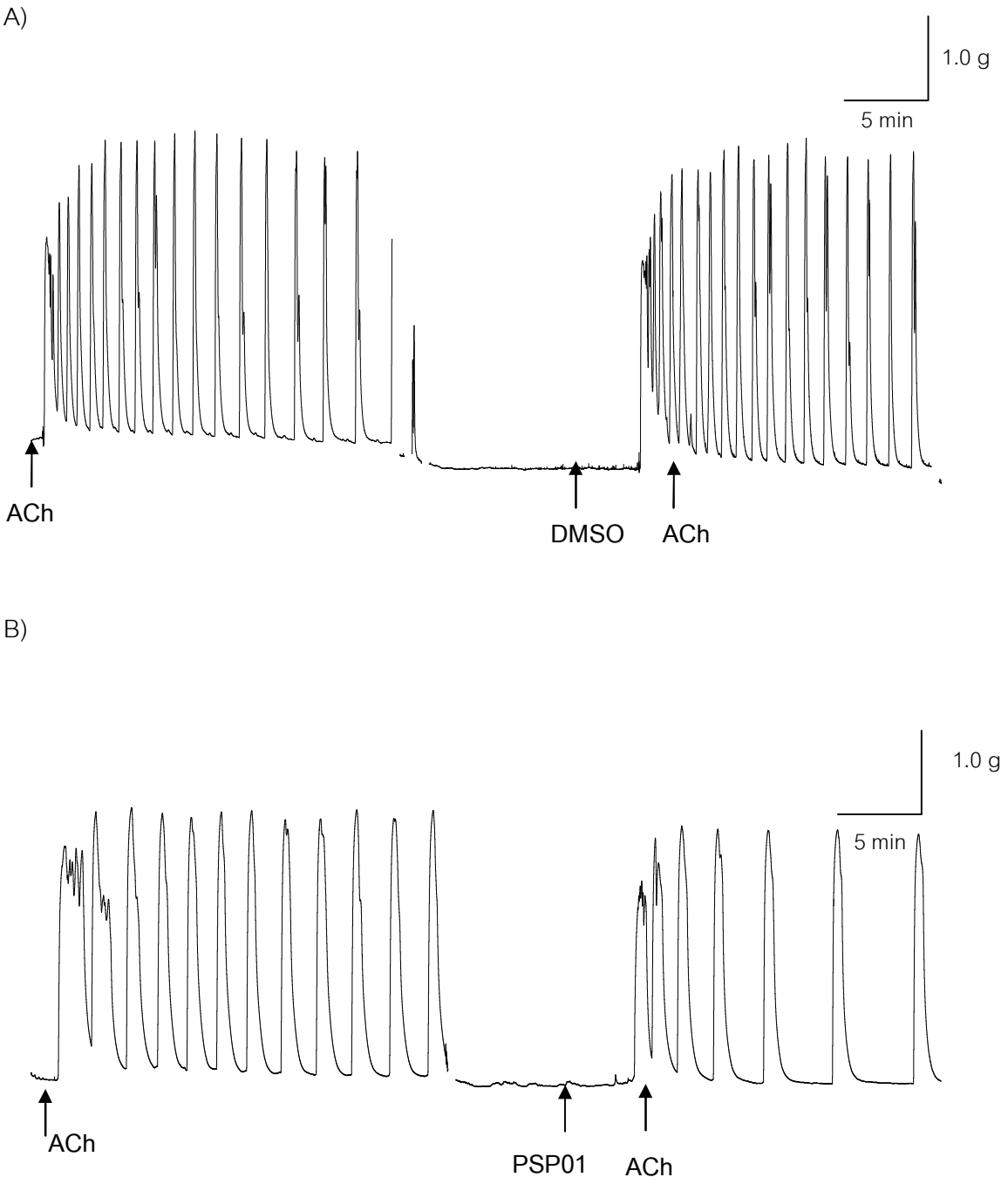
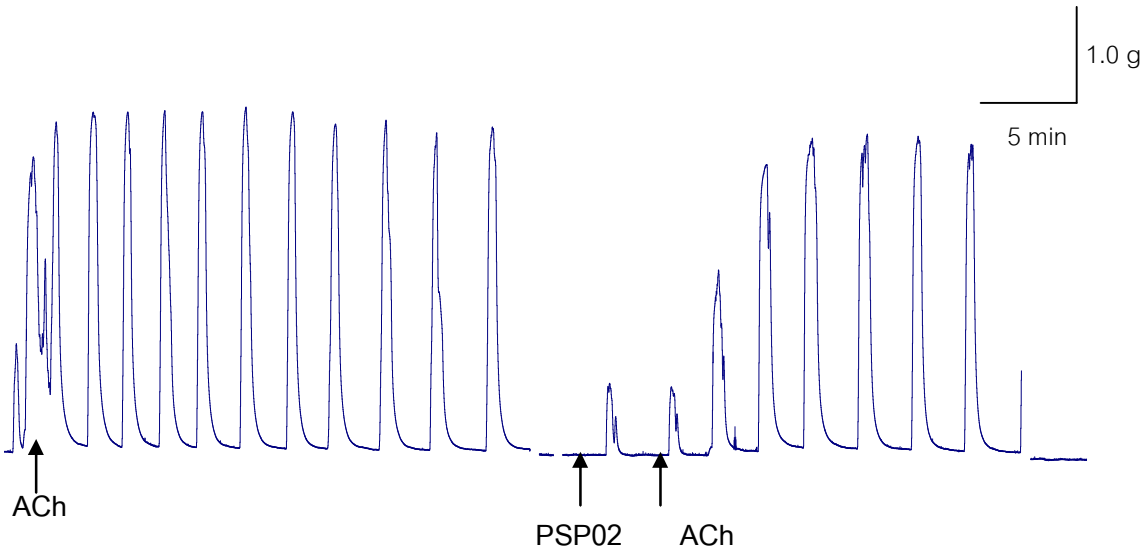


Figure 6: Representative tracing showed the effect of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil 25 µg/ml on ACh-induced contraction in isolated rat uterus.

C)



D)

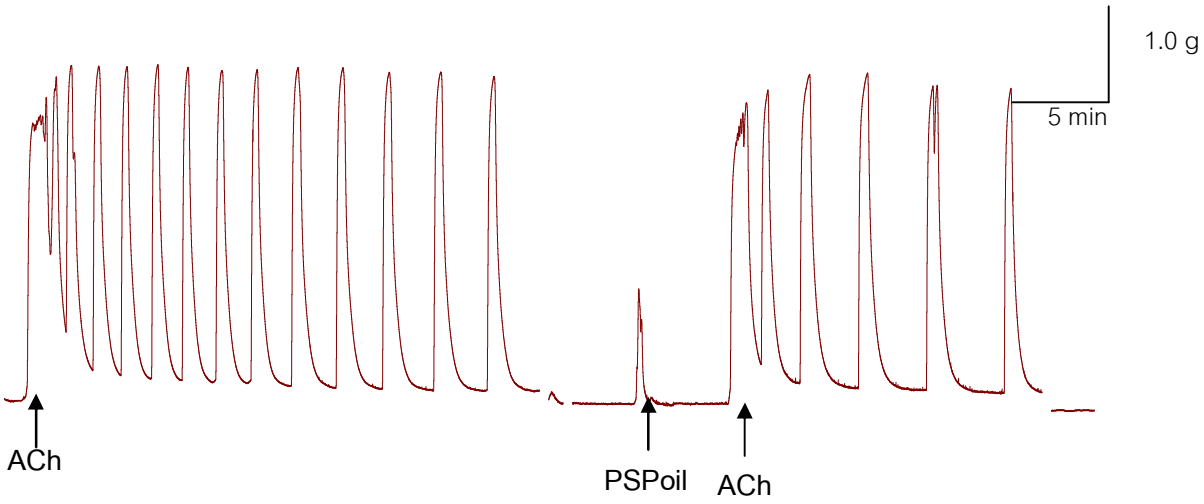


Figure 6: Representative tracing showed the effect of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil 25 µg/ml on ACh-induced contraction in isolated rat uterus.

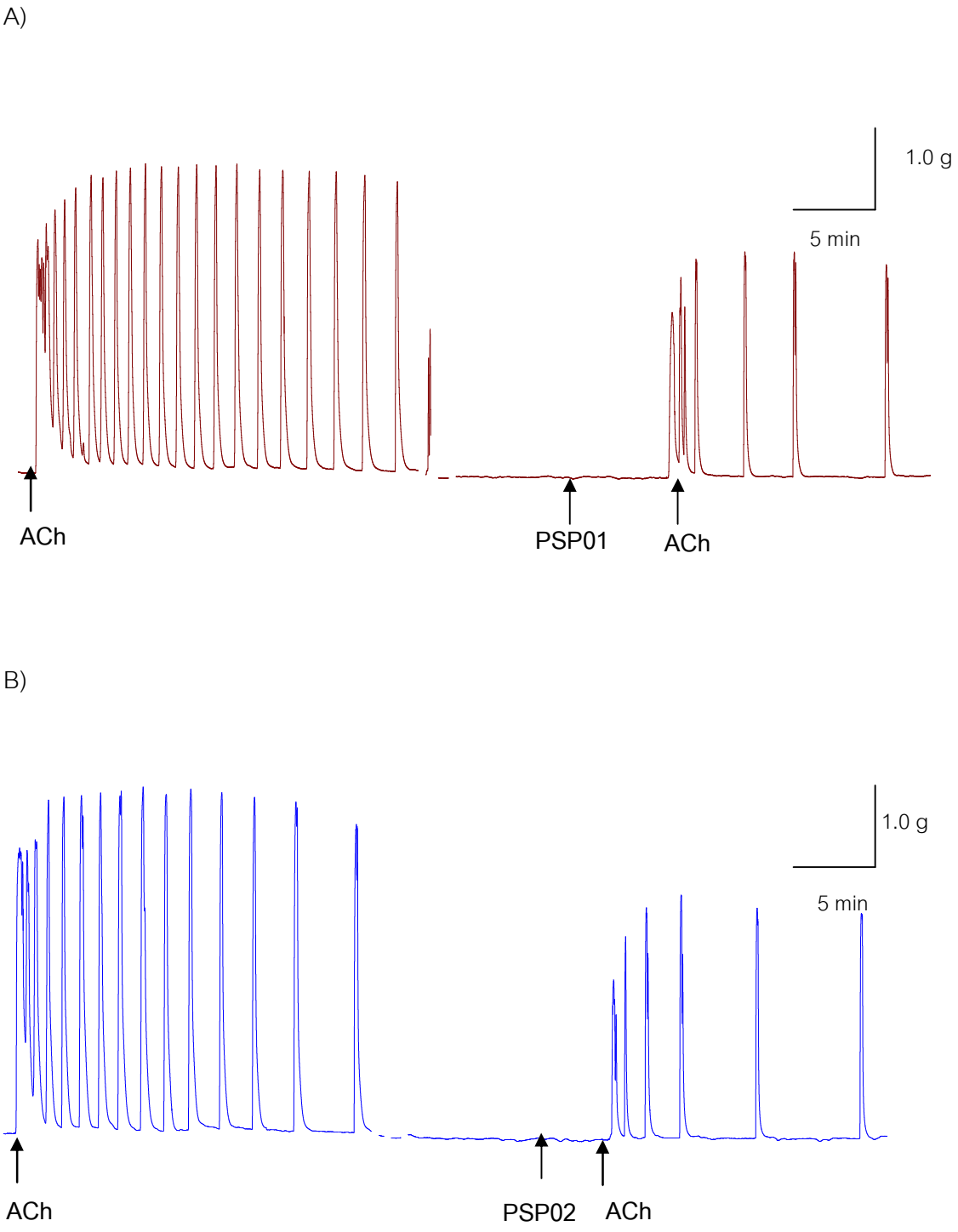


Figure 7: Representative tracing showed the effect of the test materials (A) PSP01, (B) PSP02 and C PSPoil 50 µg/ml on ACh-induced contraction in isolated rat uterus.

C)

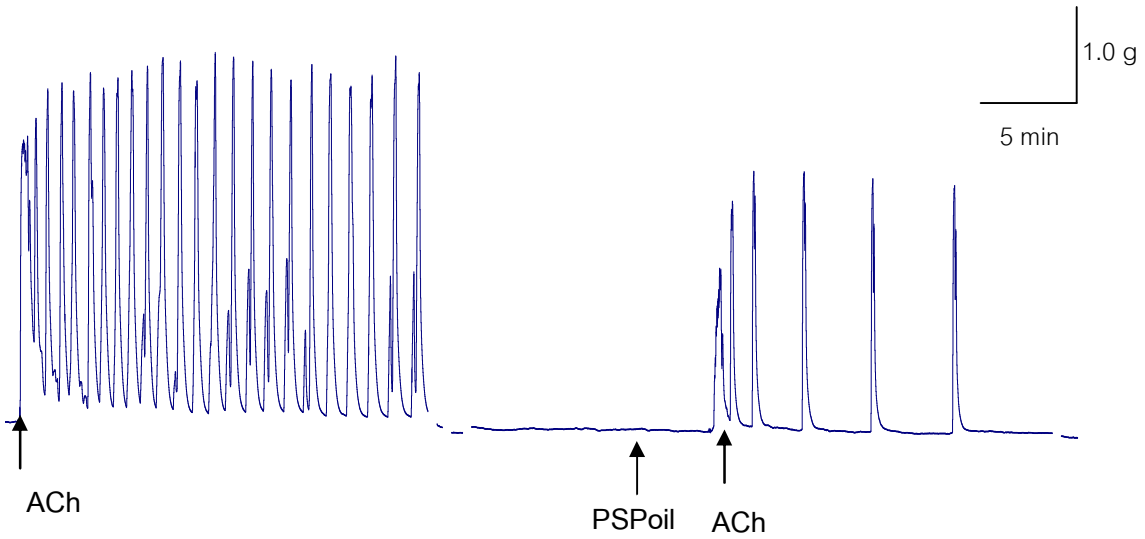


Figure 7: Representative tracing showed the effect of the test materials (A) PSP01, (B) PSP02 and C PSPoil 50 µg/ml on ACh-induced contraction in isolated rat uterus.

A)

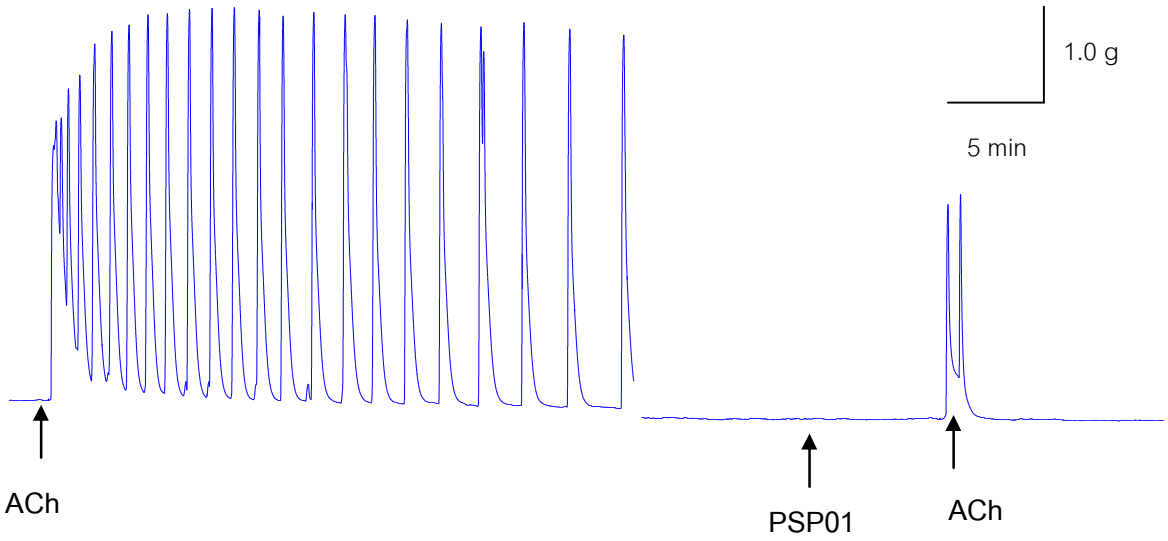
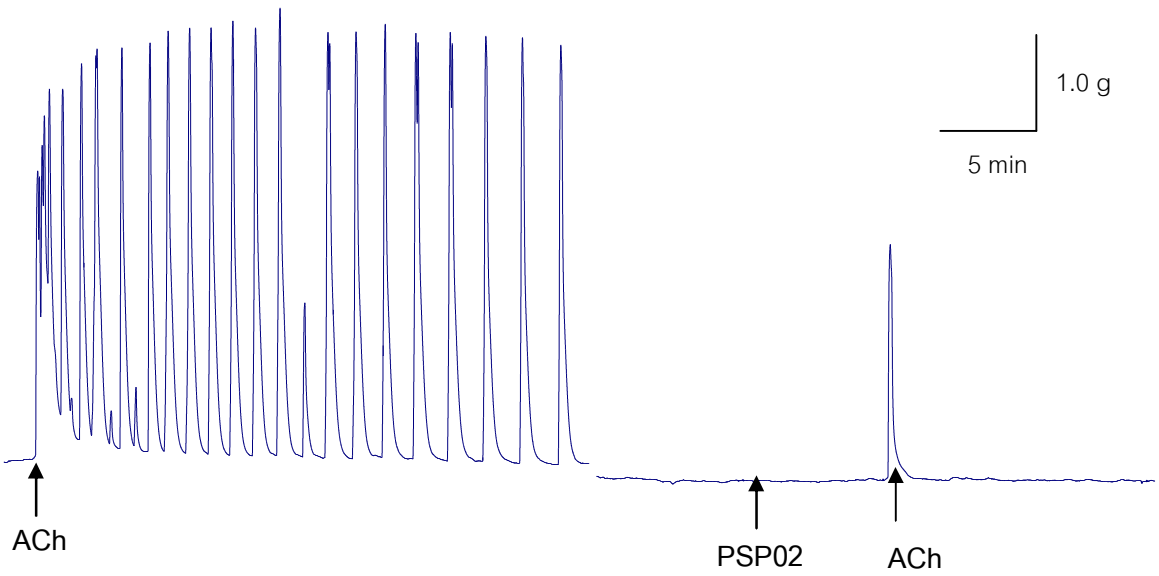


Figure 8: Representative tracing showed the effect of the test materials (A) PSP01, (B) PSP02 and C PSPoil 100 µg/ml on ACh-induced contraction in isolated rat uterus.

B)



C)

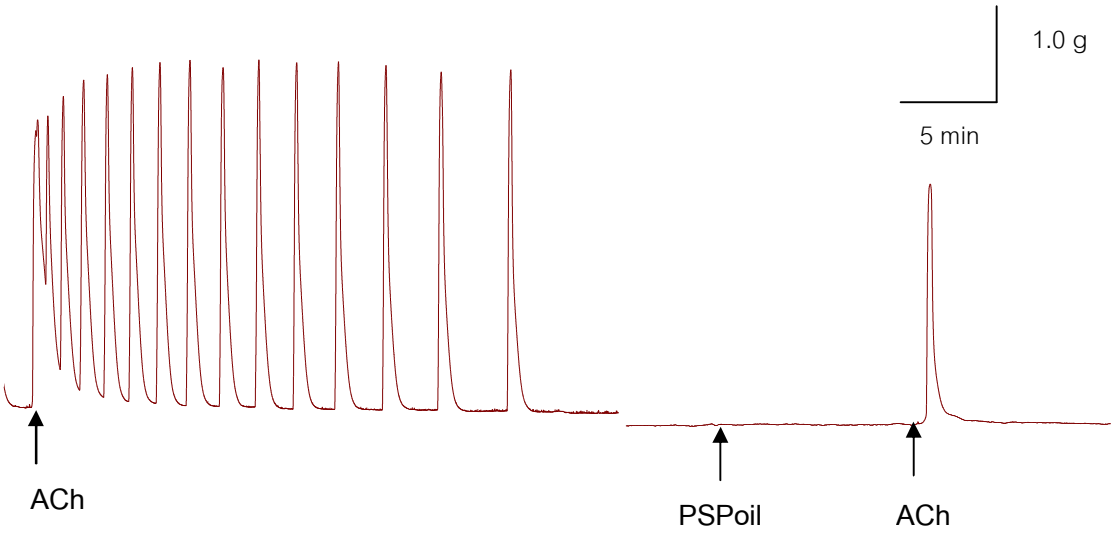


Figure 8: Representative tracing showed the effect of the test materials (A) PSP01, (B) PSP02 and C PSPoil 100 µg/ml on ACh-induced contraction in isolated rat uterus.

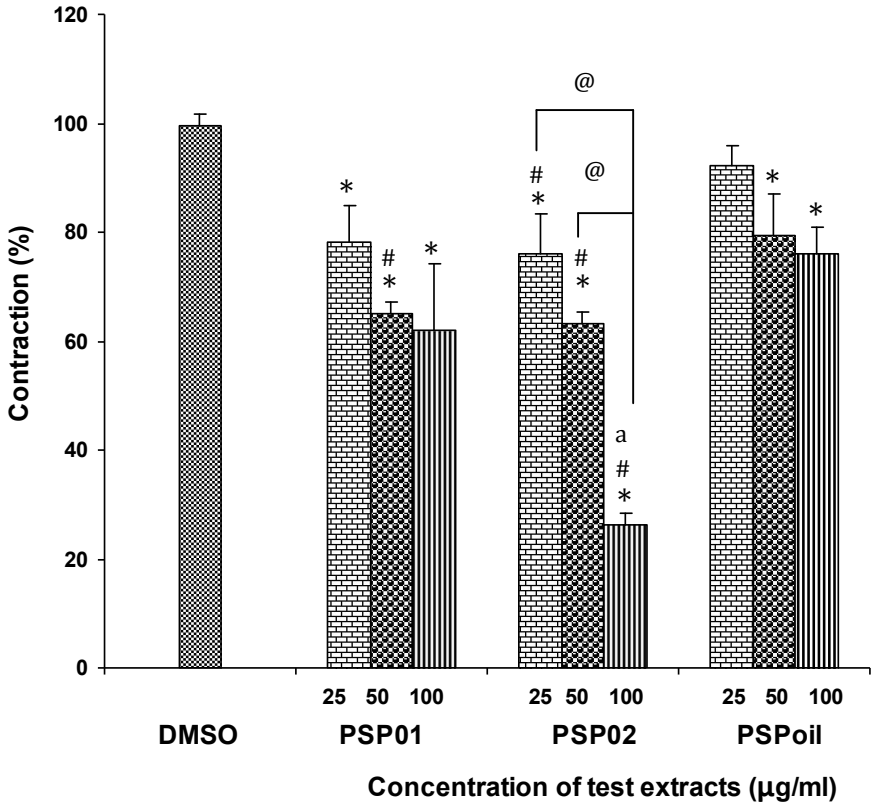


Figure 9: Effects of the test materials (25, 50 and 100 µg/ml) on force of ACh-induced contractions. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

#$p < 0.05$ denoted statistically significant difference from PSPoil.

@$p < 0.05$ denoted statistically significant difference between 25 and 50 µg/ml compared with 100 µg/ ml of the test materials.

a$p < 0.05$ denoted statistically significant difference between PSP01 and PSP02.

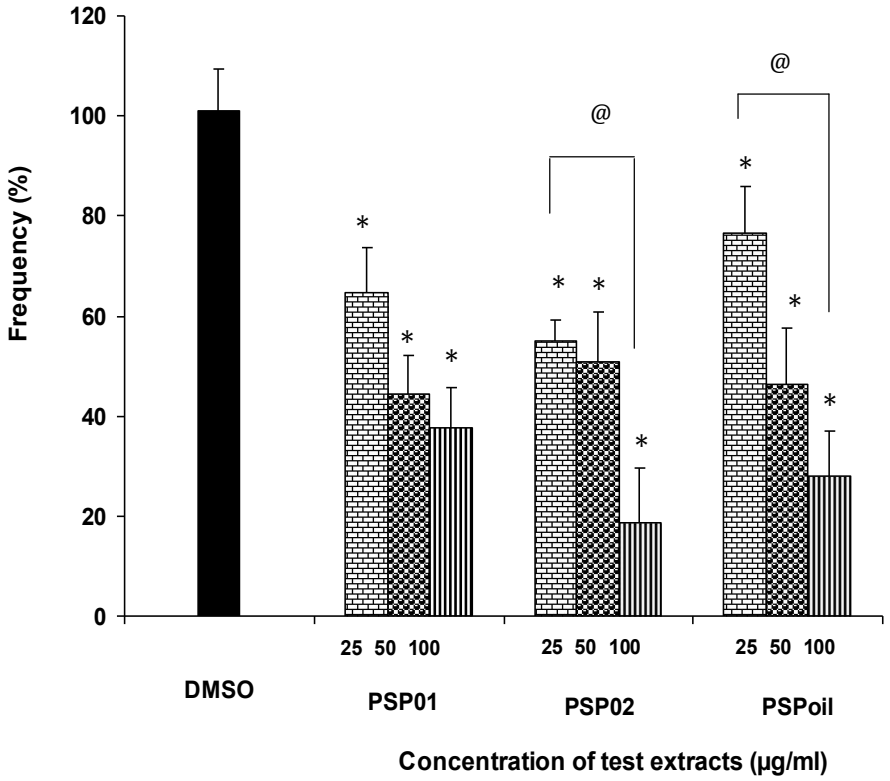
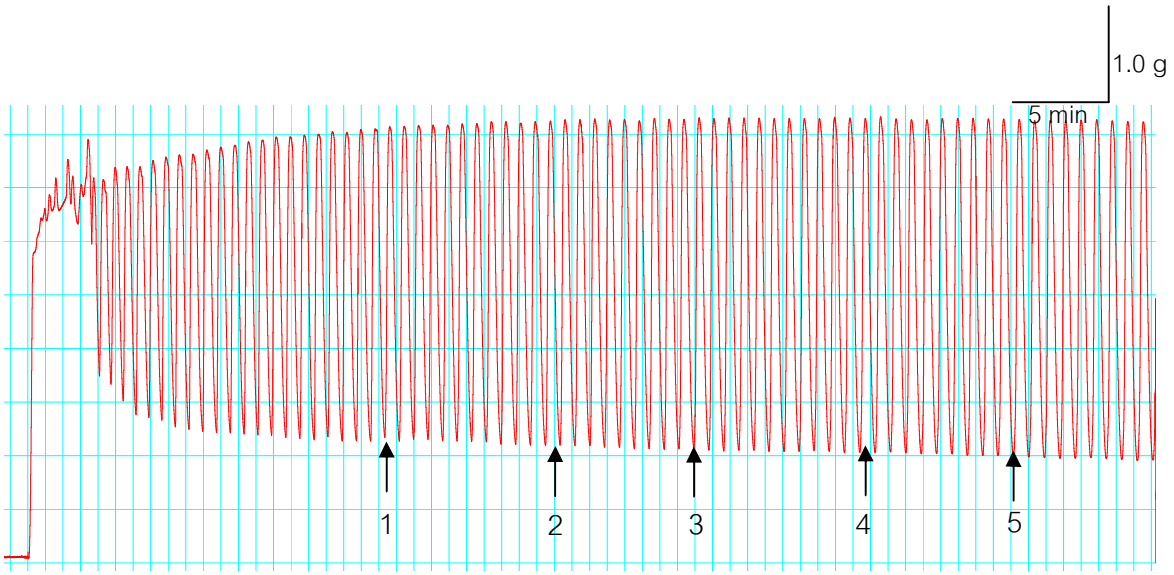


Figure 10: Effects of the test materials (25, 50 and 100 µg/ml) on frequency of ACh-induced contractions. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.

$^{*}p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

$^{@}p < 0.05$ denoted statistically significant difference between 25 and 50 µg/ml compared with 100 µg/ml of the test materials.

A)



B)

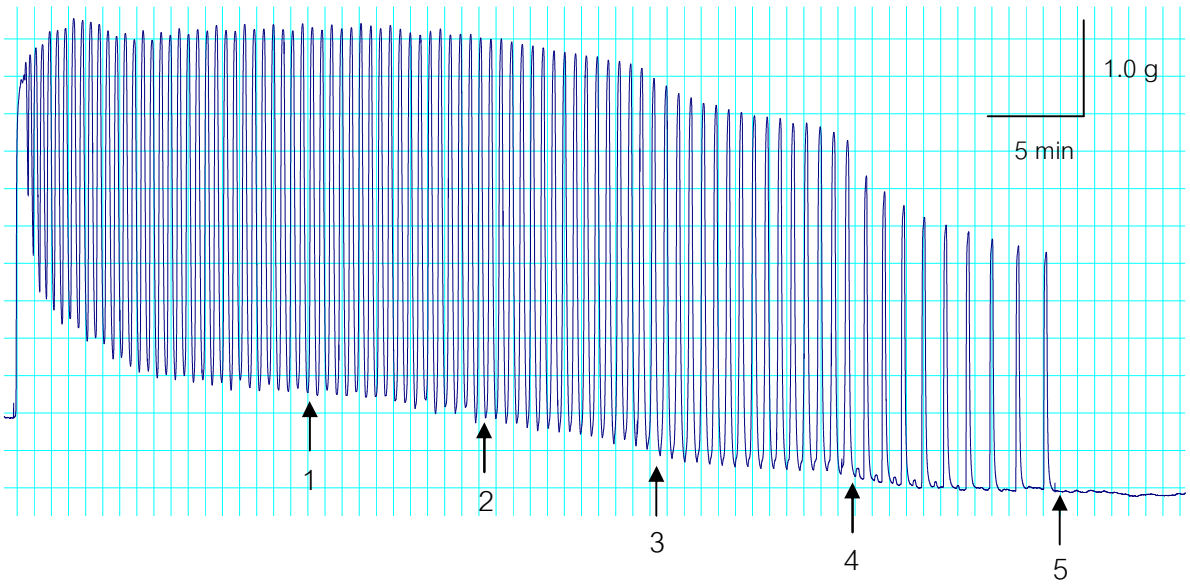
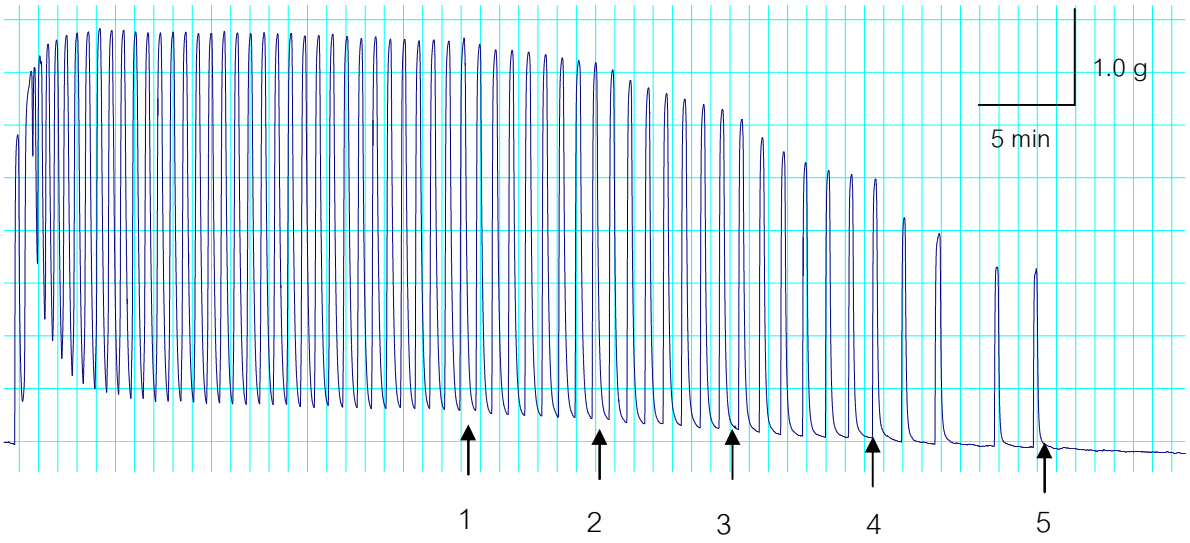


Figure 11: Representative tracing showed the relaxation induced by cumulative addition of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil on oxytocin-induced contraction of isolated rat uterine horn. The test materials concentrations were 10 µg/ml (1), 20 µg/ml (2), 30 µg/ml (3), 40 µg/ml (4) and 50 µg/ml (5).

C)



D)

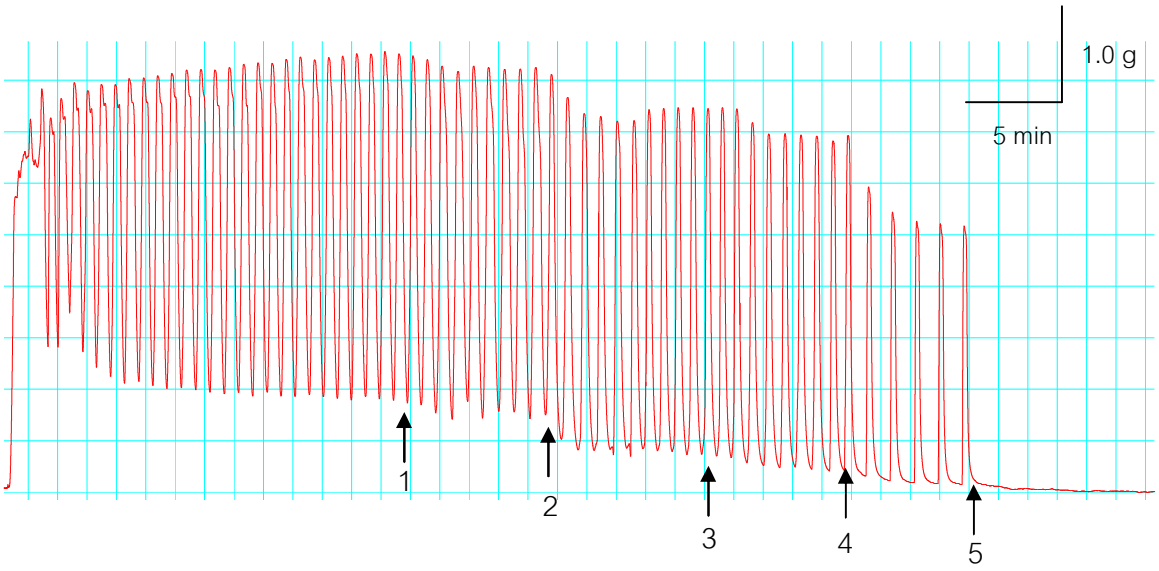
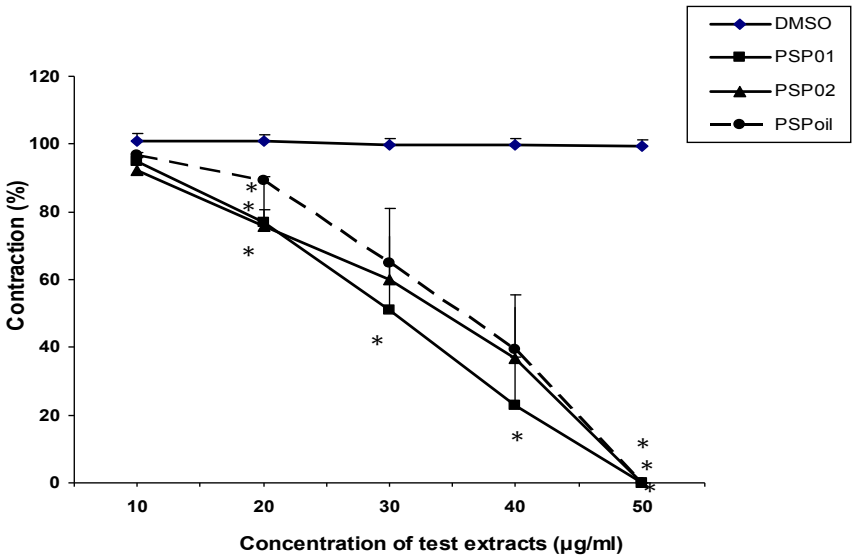


Figure 11: Representative tracing showed the relaxation induced by cumulative addition of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil on oxytocin-induced contraction of isolated rat uterine horn. The test materials concentrations were 10 µg/ml (1), 20 µg/ml (2), 30 µg/ml (3), 40 µg/ml (4) and 50 µg/ml (5).

A)



B)

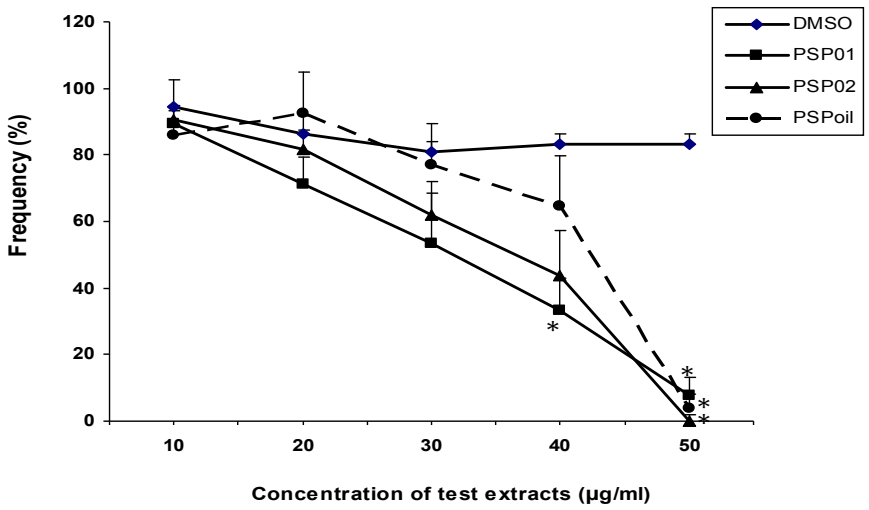


Figure 12: Concentration response curves of the test materials (10-50 µg/ml) on the contractile of amplitudes (A) and frequency (B) of oxytocin-induced contraction on rat isolated uterine horns. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test, *$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

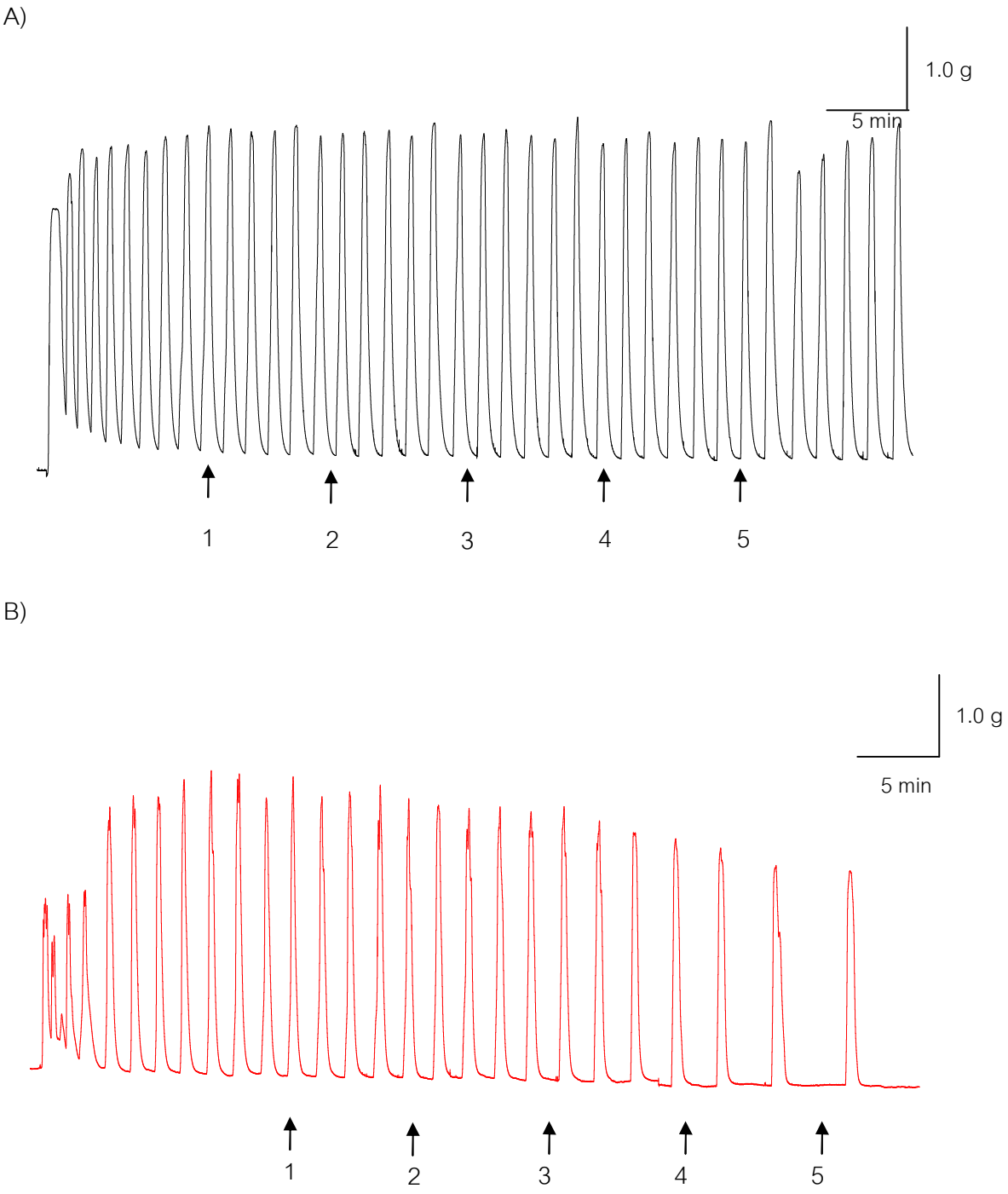


Figure 13: Representative tracing showed the relaxation induced by cumulative addition of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil on $PGF_{2\alpha}$-induced contraction in isolated rat uterus. The test materials concentrations were 10 µg/ml (1), 20 µg/ml (2), 30 µg/ml (3), 40 µg/ml (4) and 50 µg/ml (5).

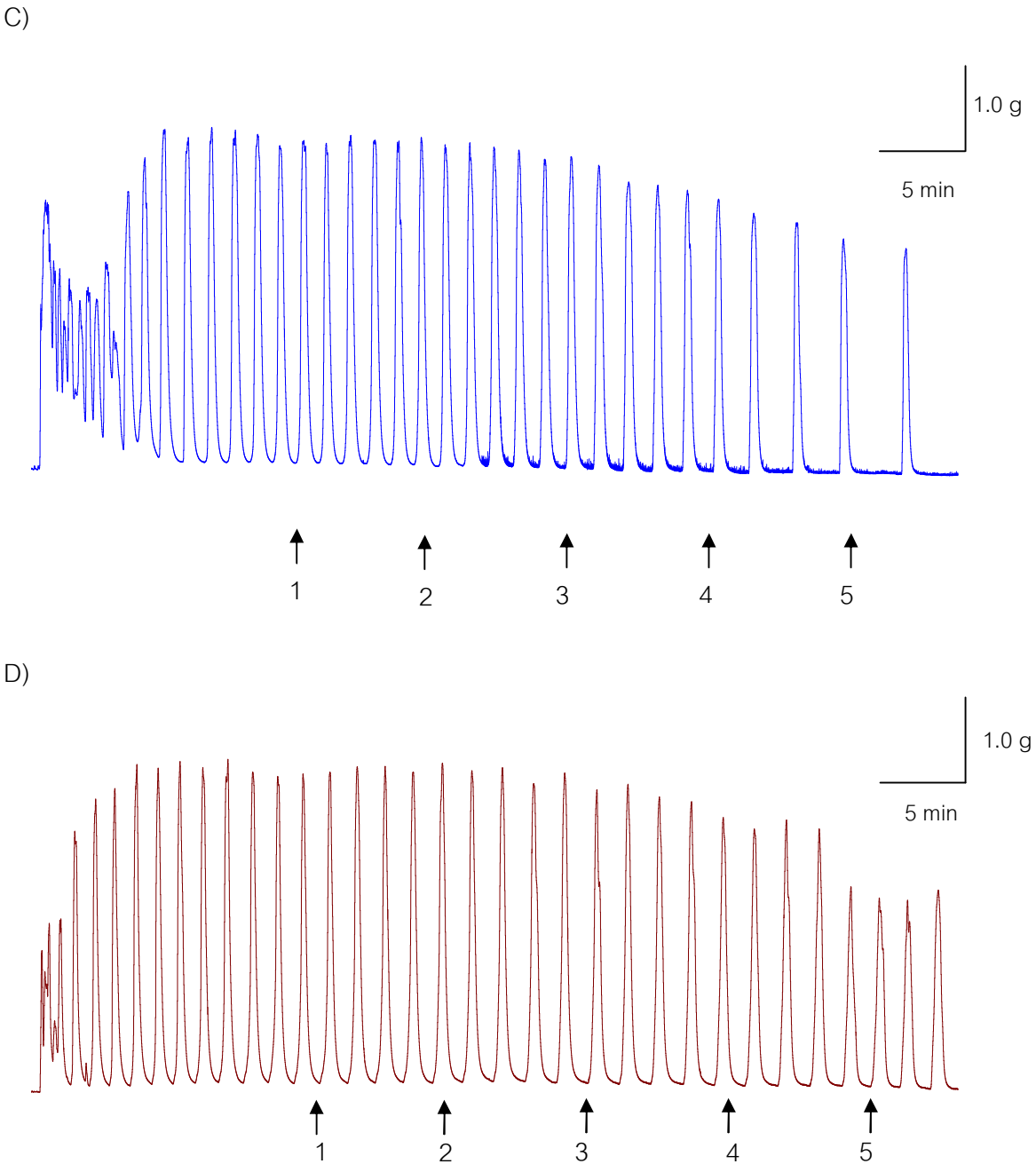
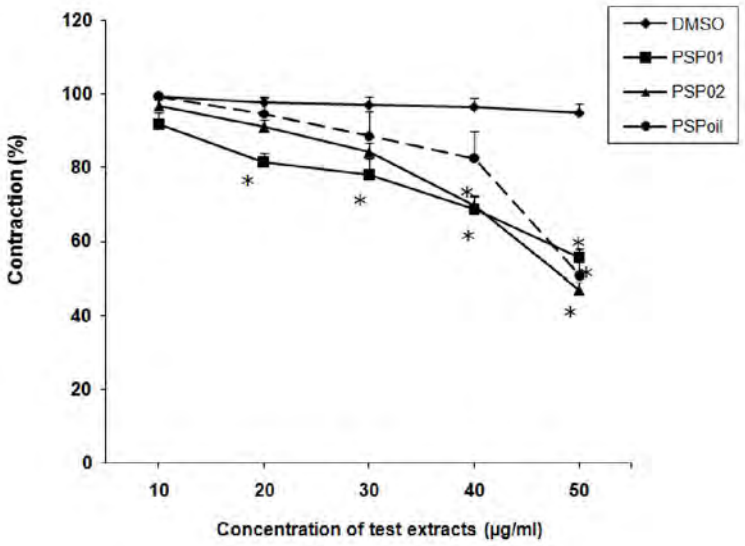


Figure 13: Representative tracing showed the relaxation induced by cumulative addition of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil on $PGF_{2}\alpha$-induced contraction in isolated rat uterus. The test materials concentrations were 10 µg/ml (1), 20 µg/ml (2), 30 µg/ml (3), 40 µg/ml (4) and 50 µg/ml (5).

A)



B)

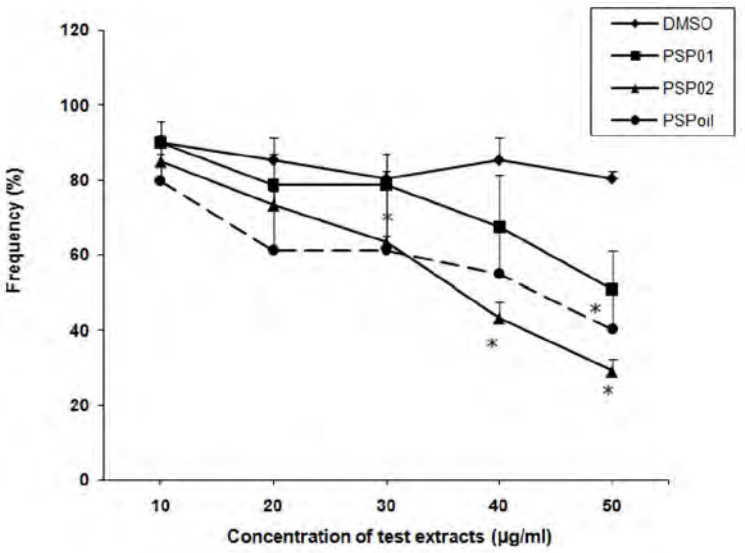


Figure 14: Concentration response curves of the test materials (10-50 µg/ml) on the contractile of amplitudes (A) and frequency (B) of $PGF_{2\alpha}$-induced contraction on rat isolated uterine horns. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test, $*p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

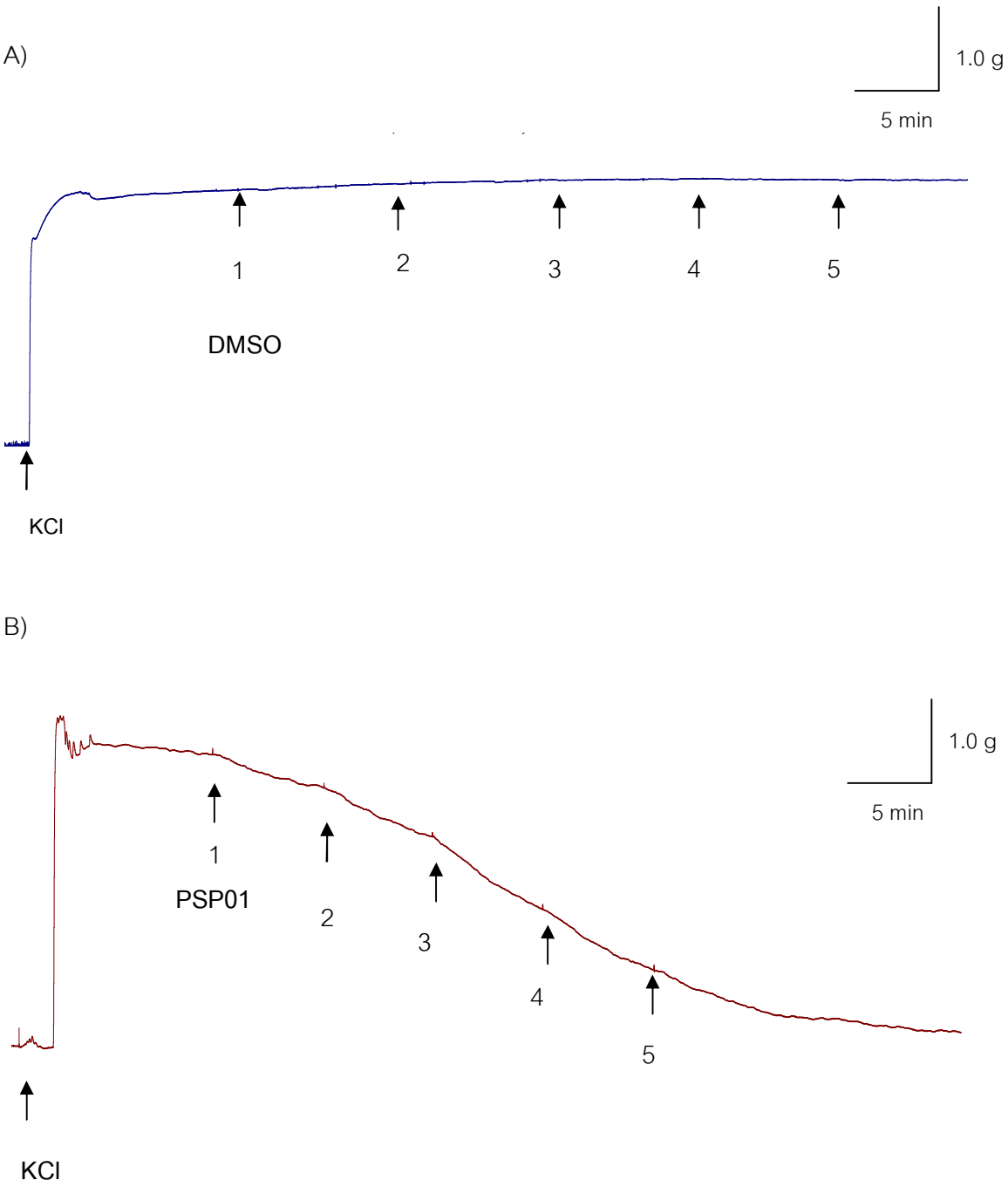
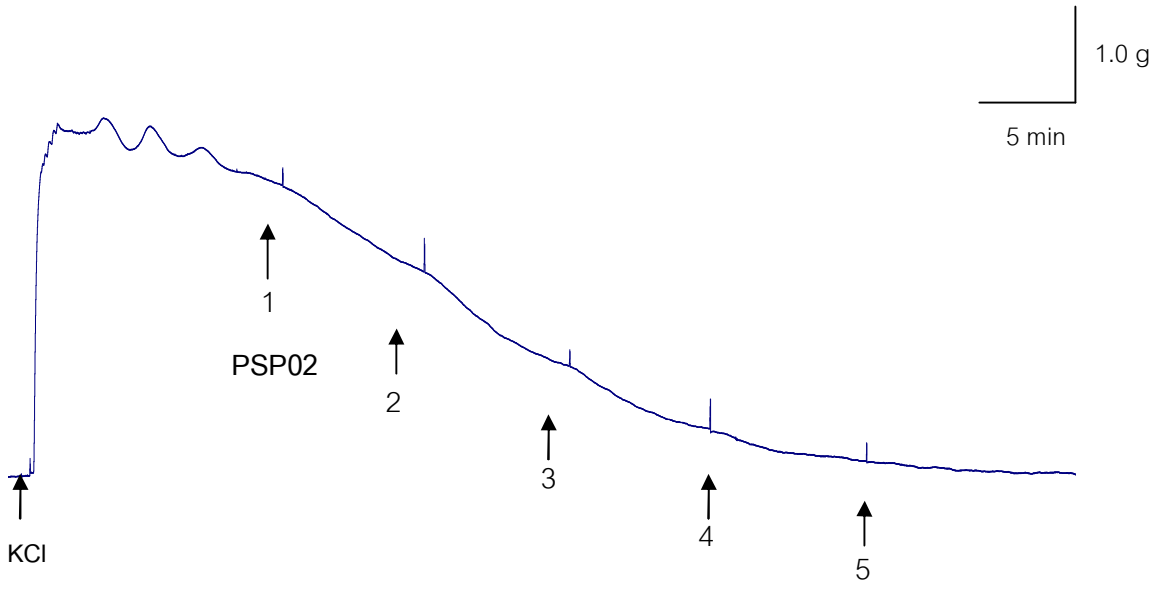


Figure 15: Representative tracing showed the relaxation induced by cumulative addition of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil on KCl-induced contraction in isolated rat uterus. The test materials concentrations were 10 µg/ml (1), 20 µg/ml (2), 30 µg/ml (3), 40 µg/ml (4) and 50 µg/ml (5).

C)



D)

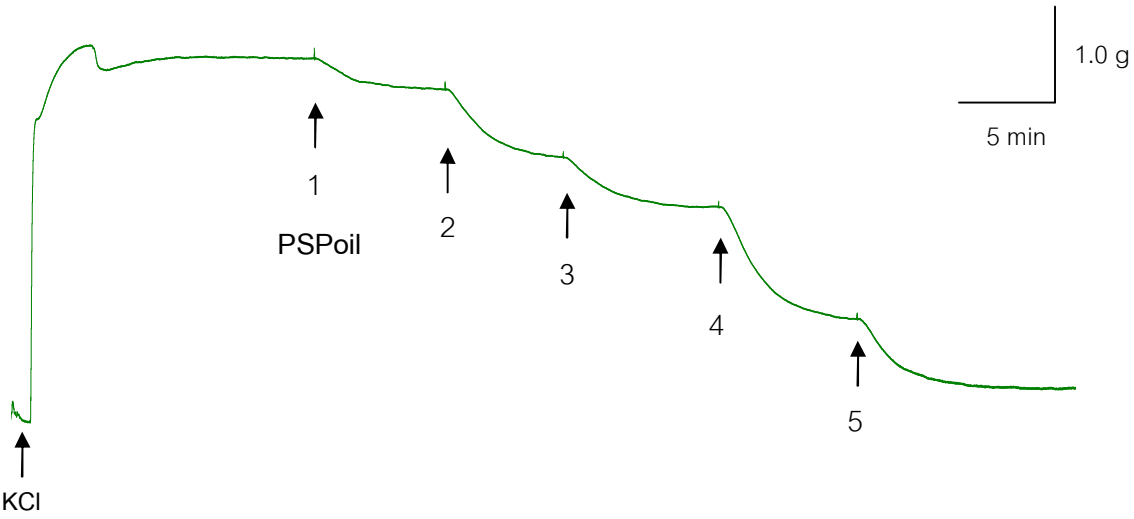


Figure 15: Representative tracing showed the relaxation induced by cumulative addition of the test materials (A) DMSO 0.05%, (B) PSP01, (C) PSP02 and (D) PSPoil on KCl-induced contraction in isolated rat uterus. The test materials concentrations were 10 µg/ml (1), 20 µg/ml (2), 30 µg/ml (3), 40 µg/ml (4) and 50 µg/ml (5).

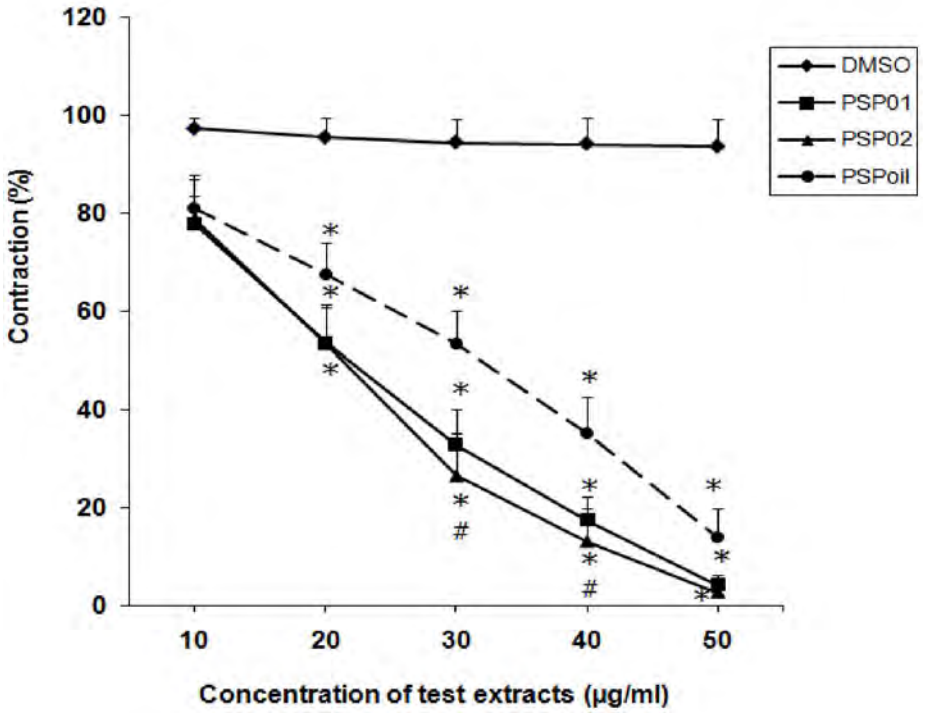


Figure 16: Concentration response curves of the test materials (10-50 µg/ml) on force of KCl-induced isolated rat uterine horns. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

#$p < 0.05$ denoted statistically significant difference from PSPoil.

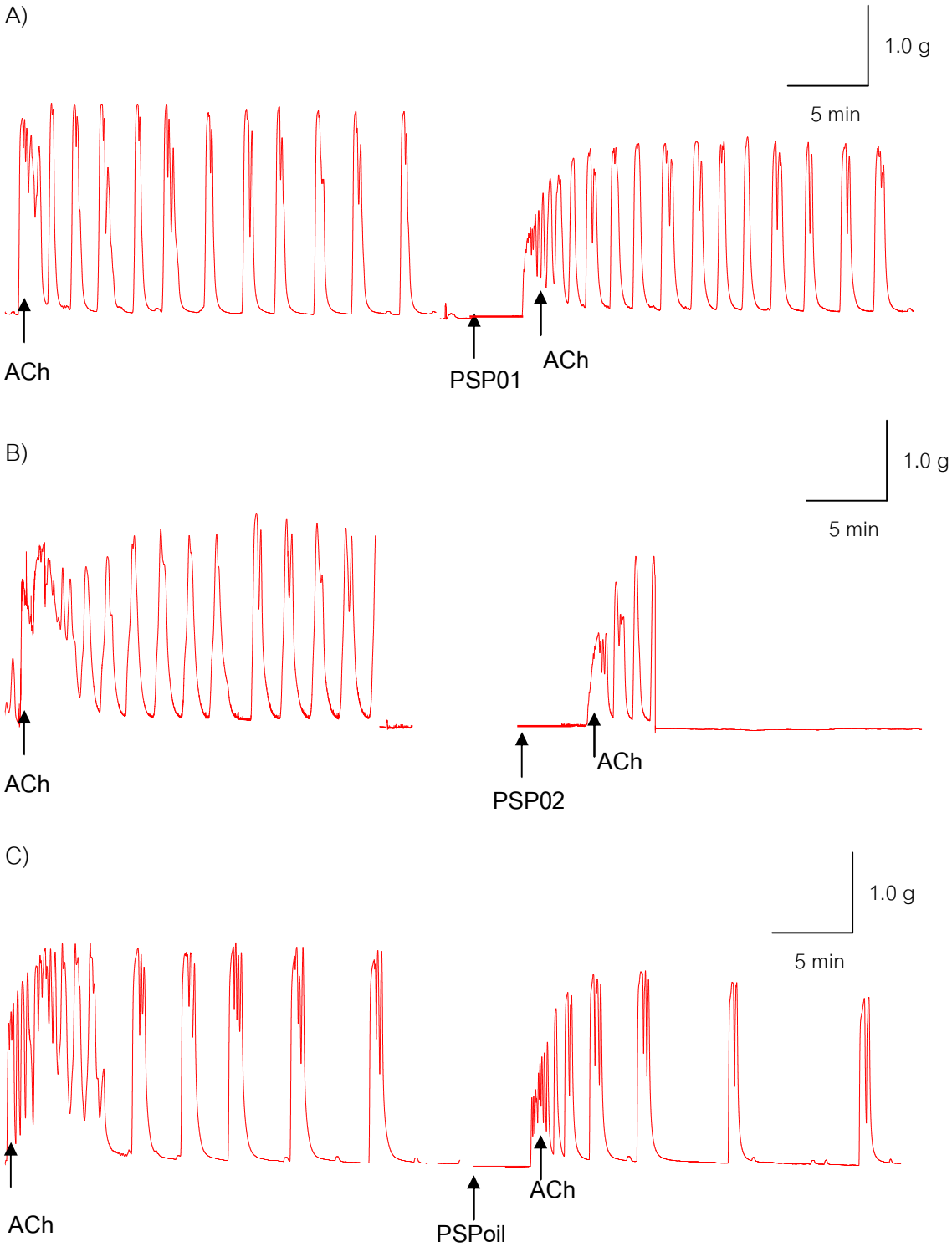


Figure 17: Representative tracing showed the effects of the test materials (A) PSP01, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on ACh-induced contraction.

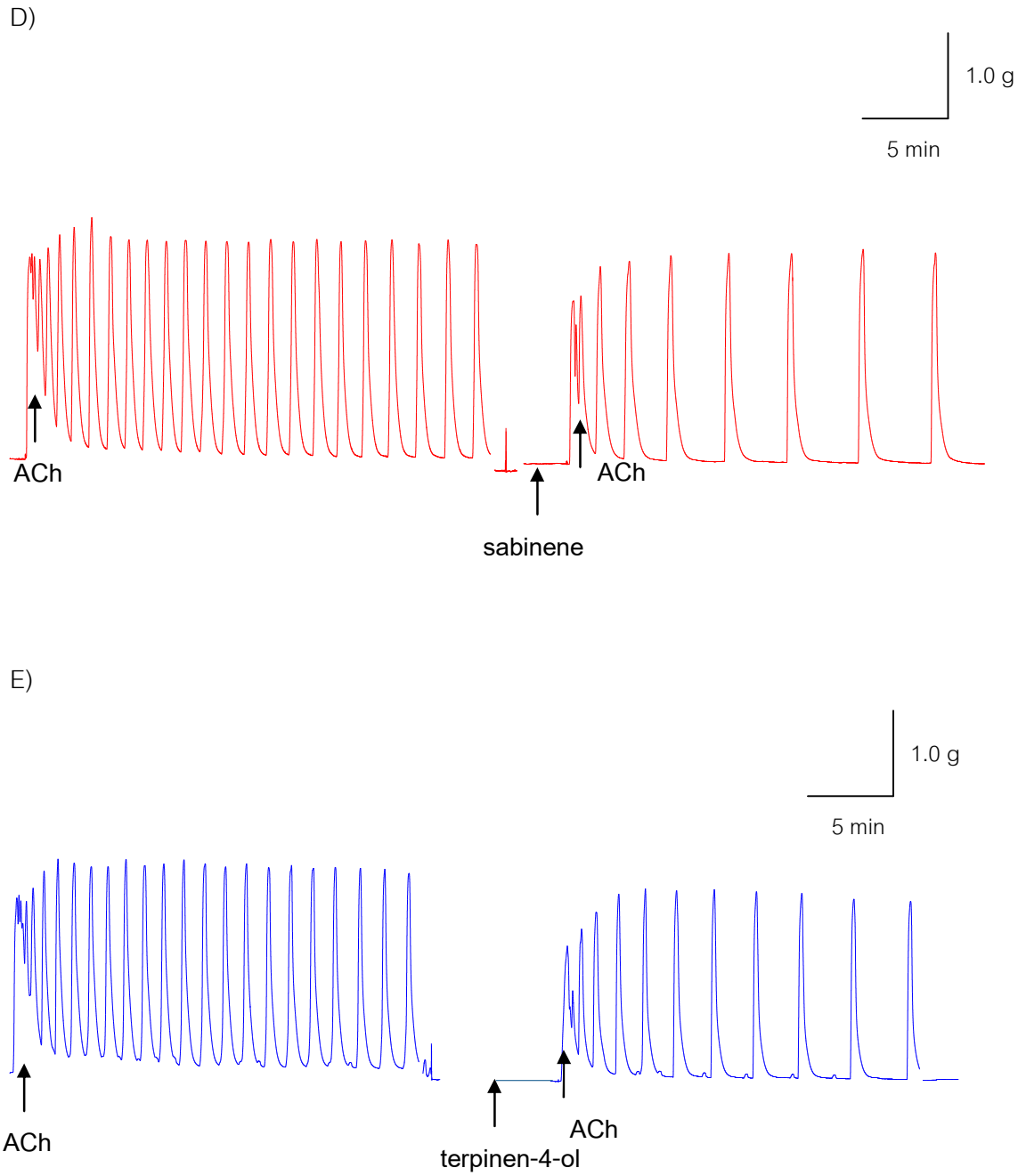
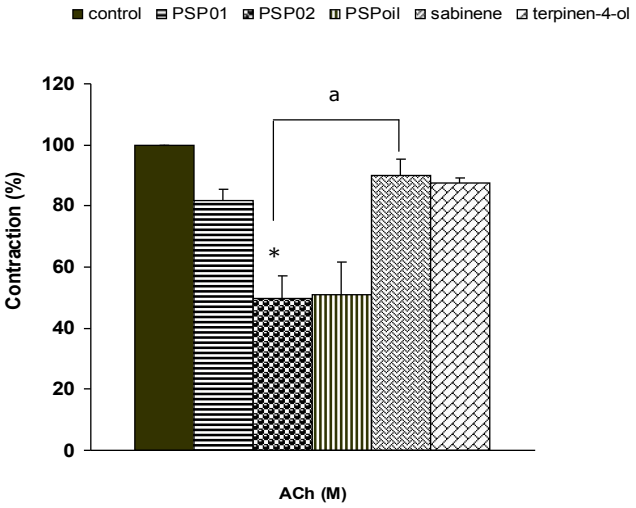


Figure 17: Representative tracing showed the effects of the test materials (A) PSP01, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on ACh-induced contraction.

A)



B)

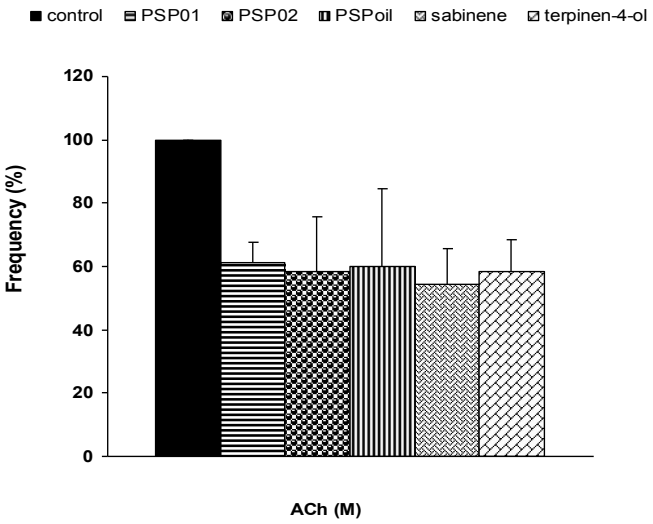


Figure 18: Comparative effects of the test extracts 100 µg/ml with the major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on ACh-induced contractions (18A) force, (18B) frequency. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test. *p<0.05 denoted statistically significant difference from control. [a]p<0.05 denoted statistically significant difference from sabinene.

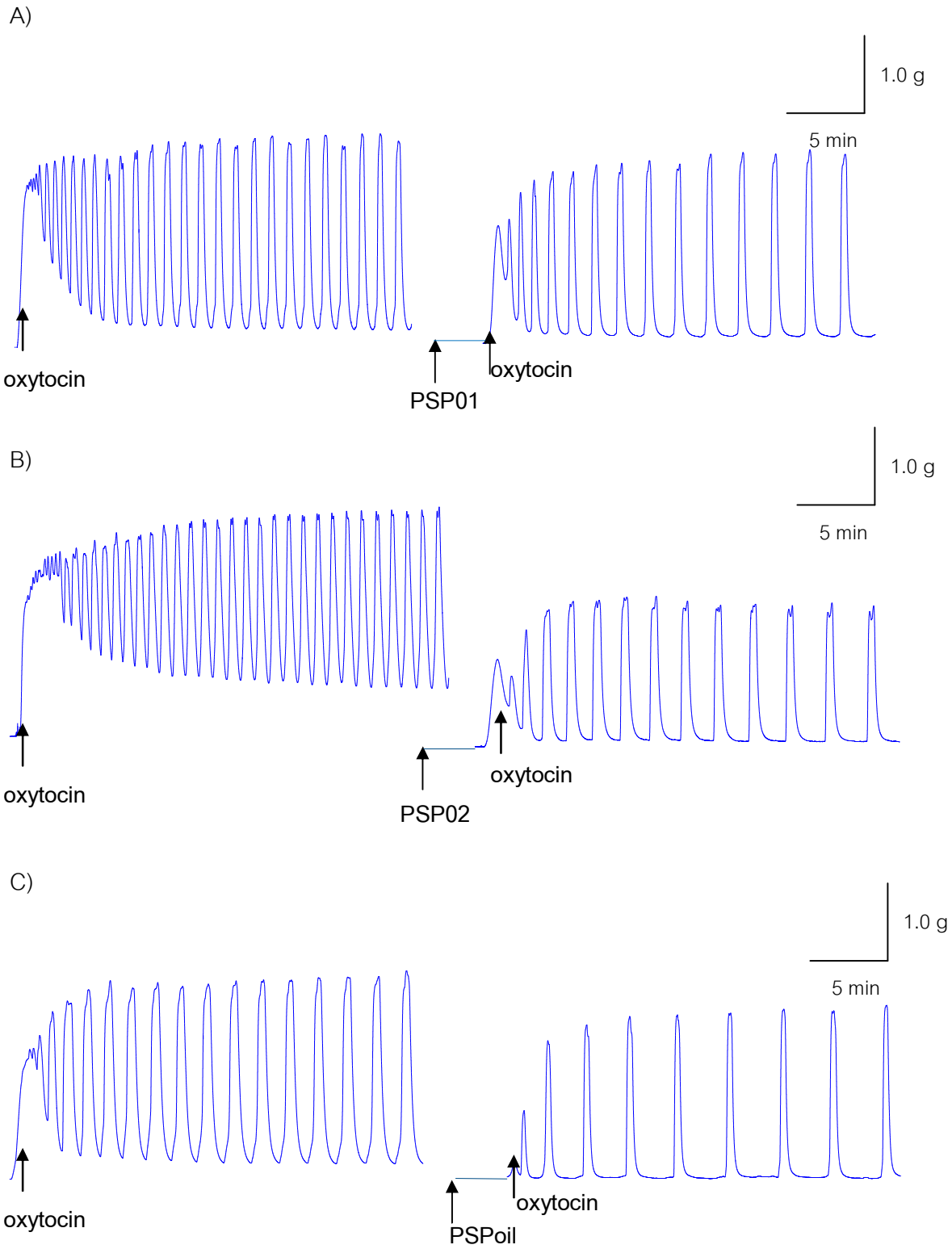


Figure 19: Representative tracing showed the effects of the test materials (A) PSP01, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on oxytocin-induced contraction.

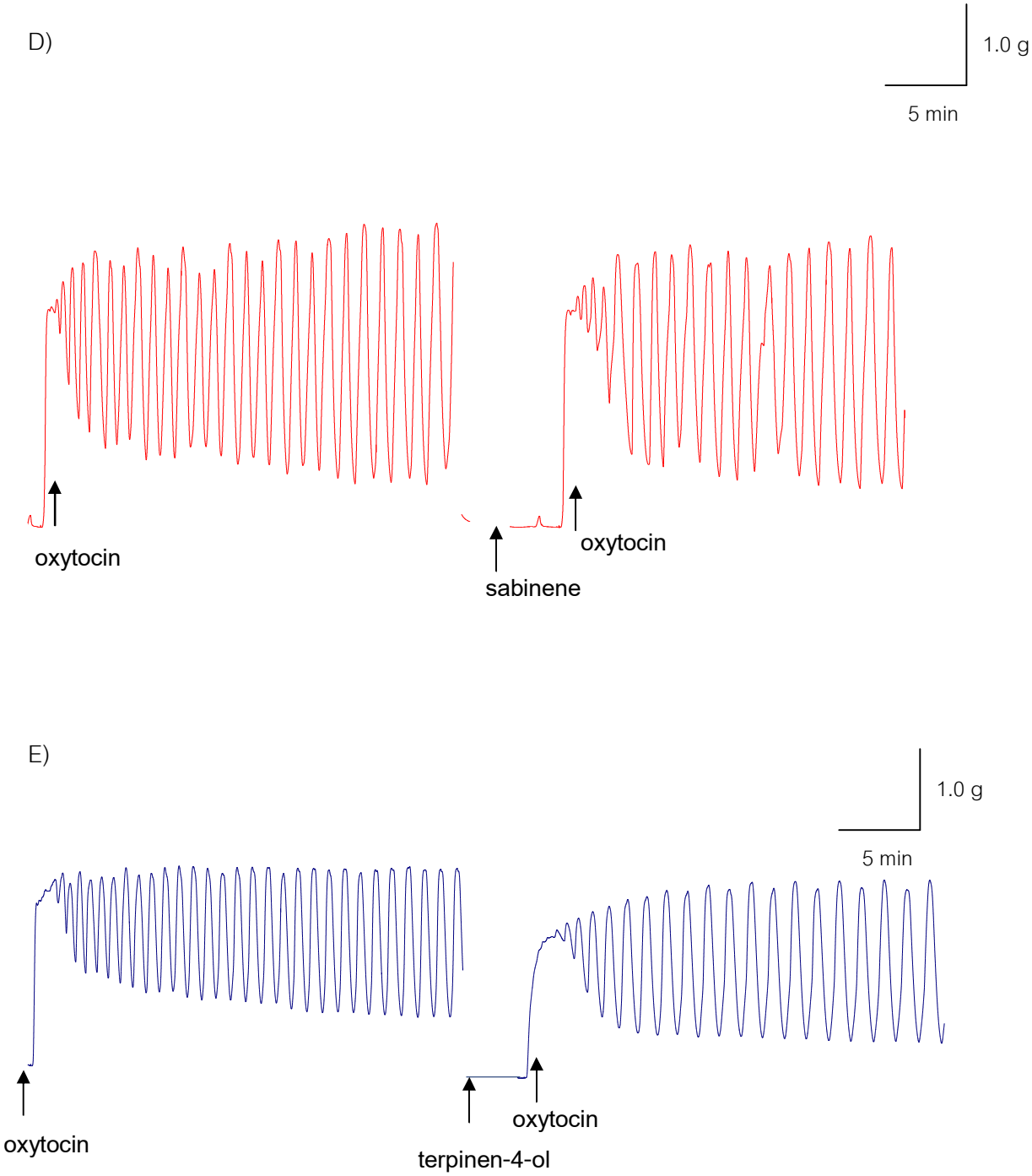
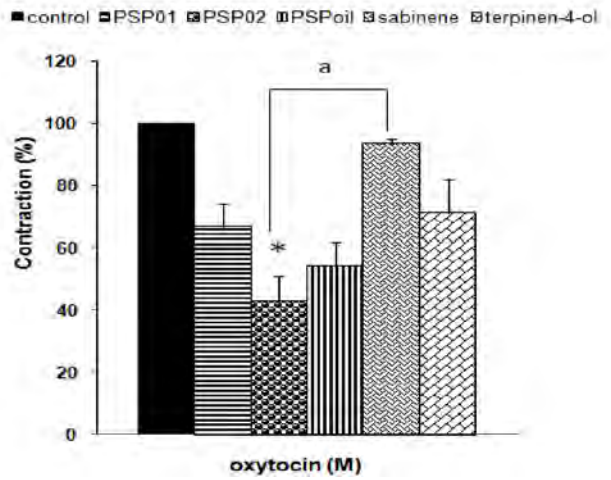


Figure 19: Representative tracing showed the effects of the test materials (A) PSP01, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on oxytocin-induced contraction.

A)



B)

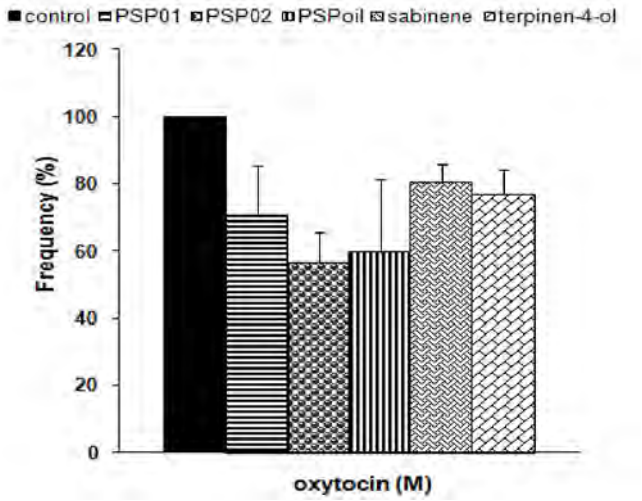


Figure 20: Comparative effects of the test extracts 100 µg/ml with major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on oxytocin-induced contractions (20A) force, (20B) frequency. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test. $^{*}p<0.05$ denoted statistically significant difference from control. $^{a}p<0.05$ denoted statistically significant difference from sabinene.

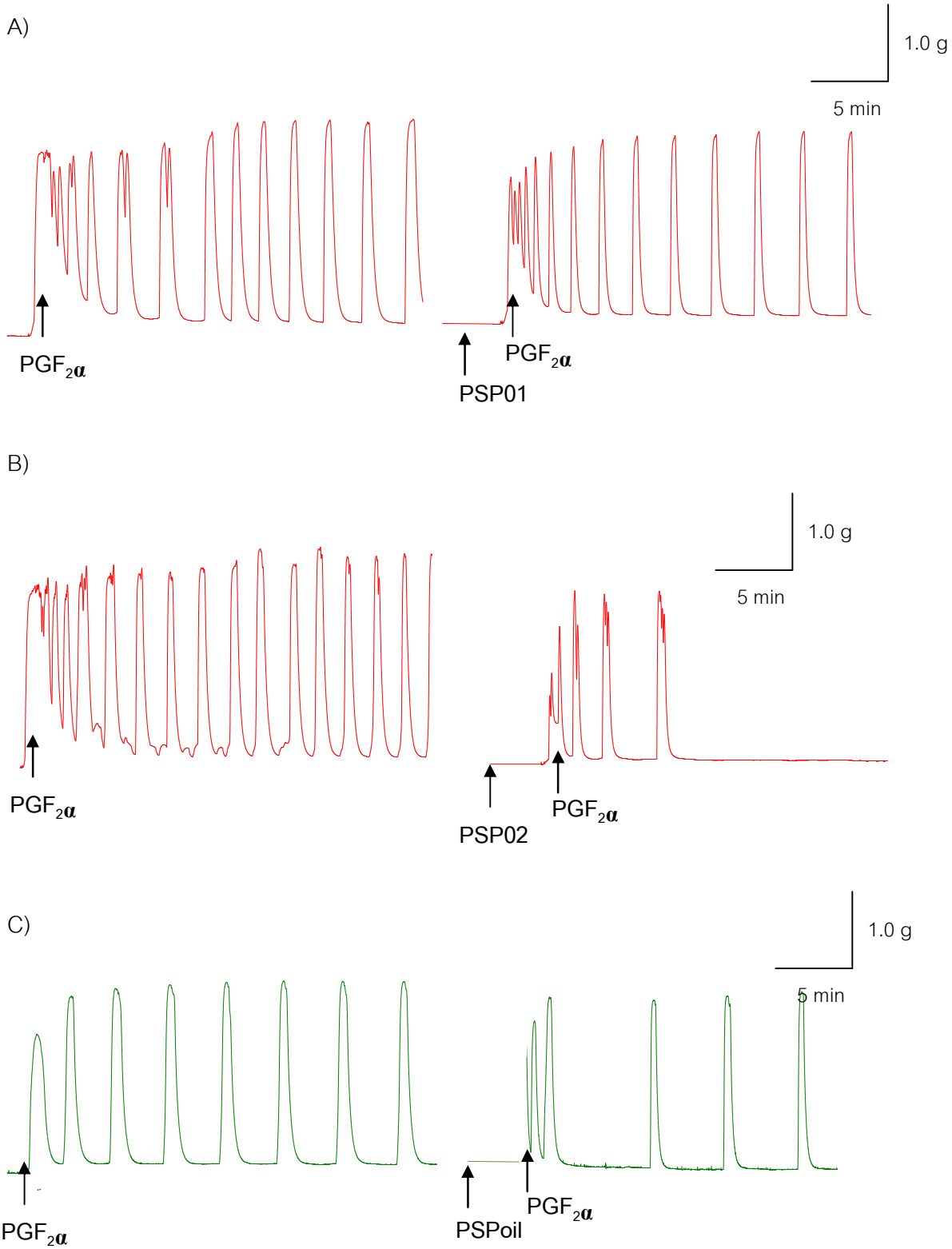


Figure 21: Representative tracing showed the effects of the test materials (A) PSP01, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on $PGF_{2\alpha}$-induced contraction.

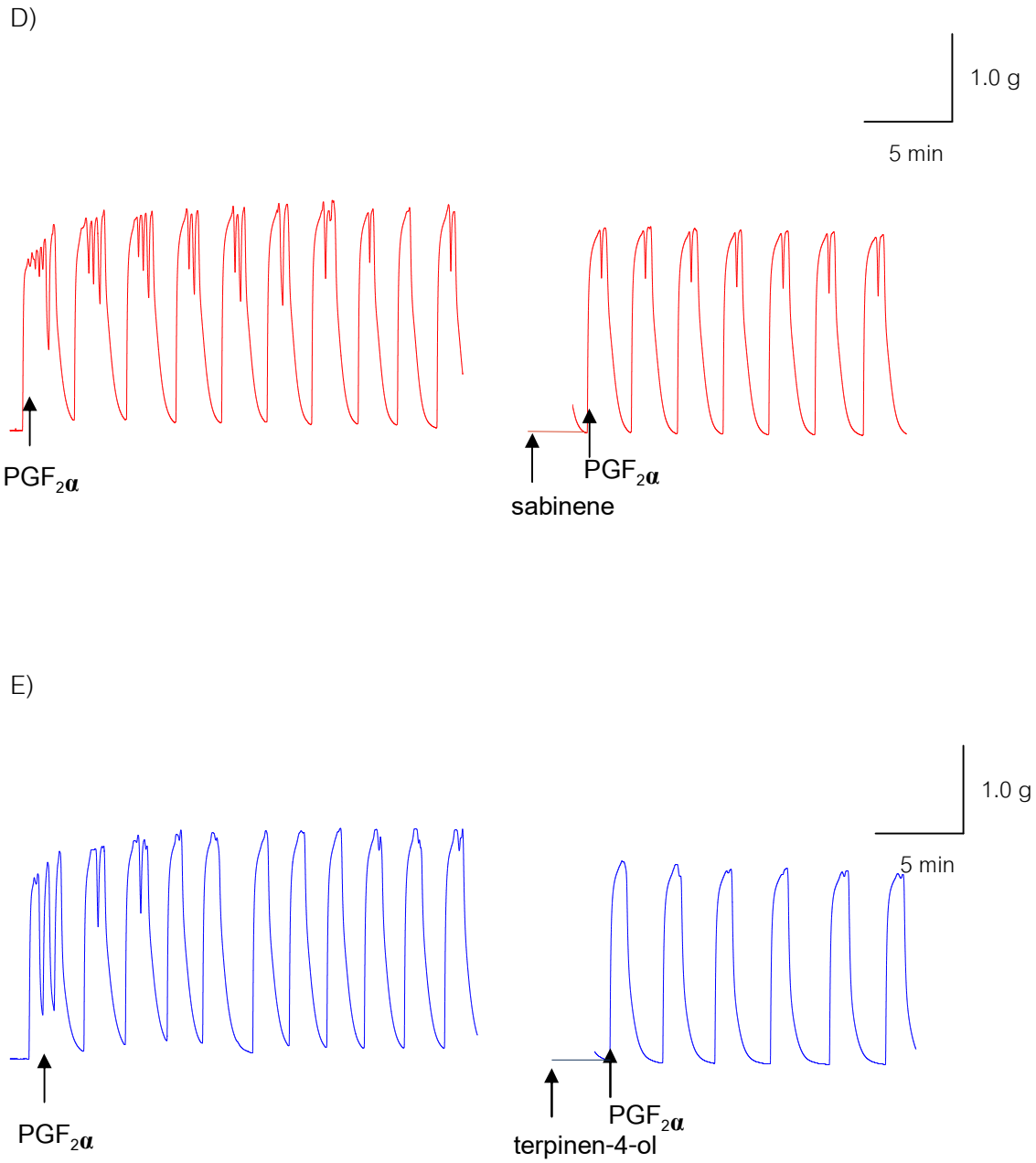
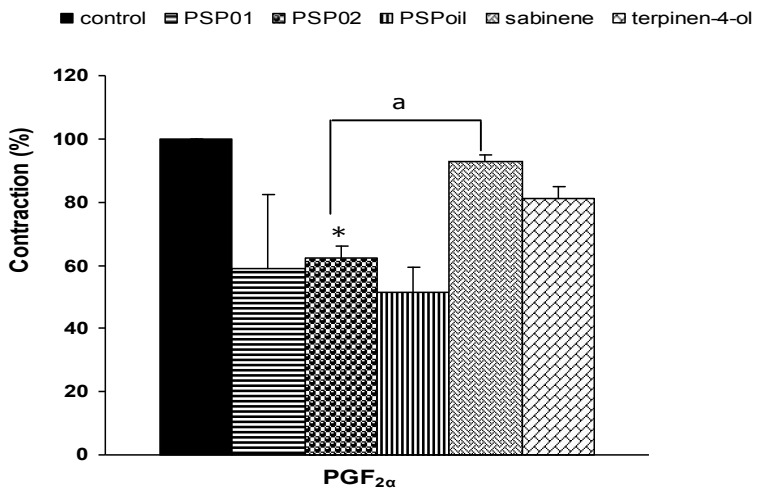


Figure 21: Representative tracing showed the effects of the test materials (A) PSP01, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on $PGF_{2\alpha}$-induced contraction.

A)



B)

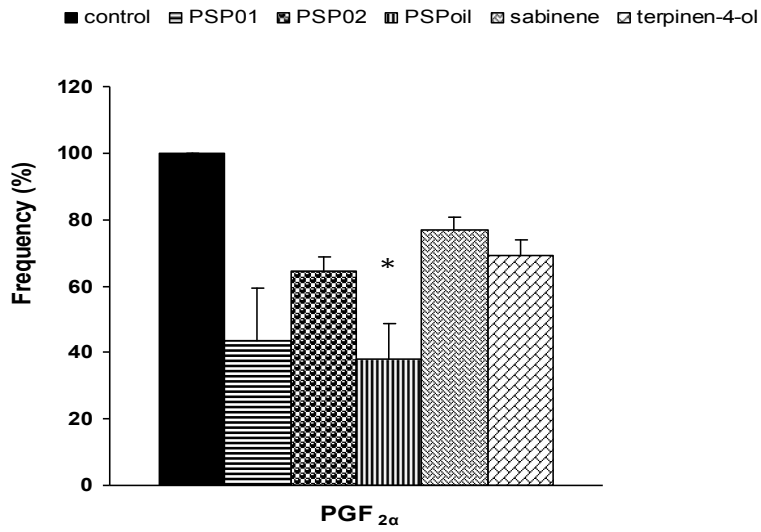


Figure 22: Comparative effects of the test extracts 100 µg/ml with the major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on $PGF_{2\alpha}$-induced contractions (22A) force, (22B) frequency. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test. $^{*}p<0.05$ denoted statistically significant difference from control. $^{a}p<0.05$ denoted statistically significant difference from sabinene.

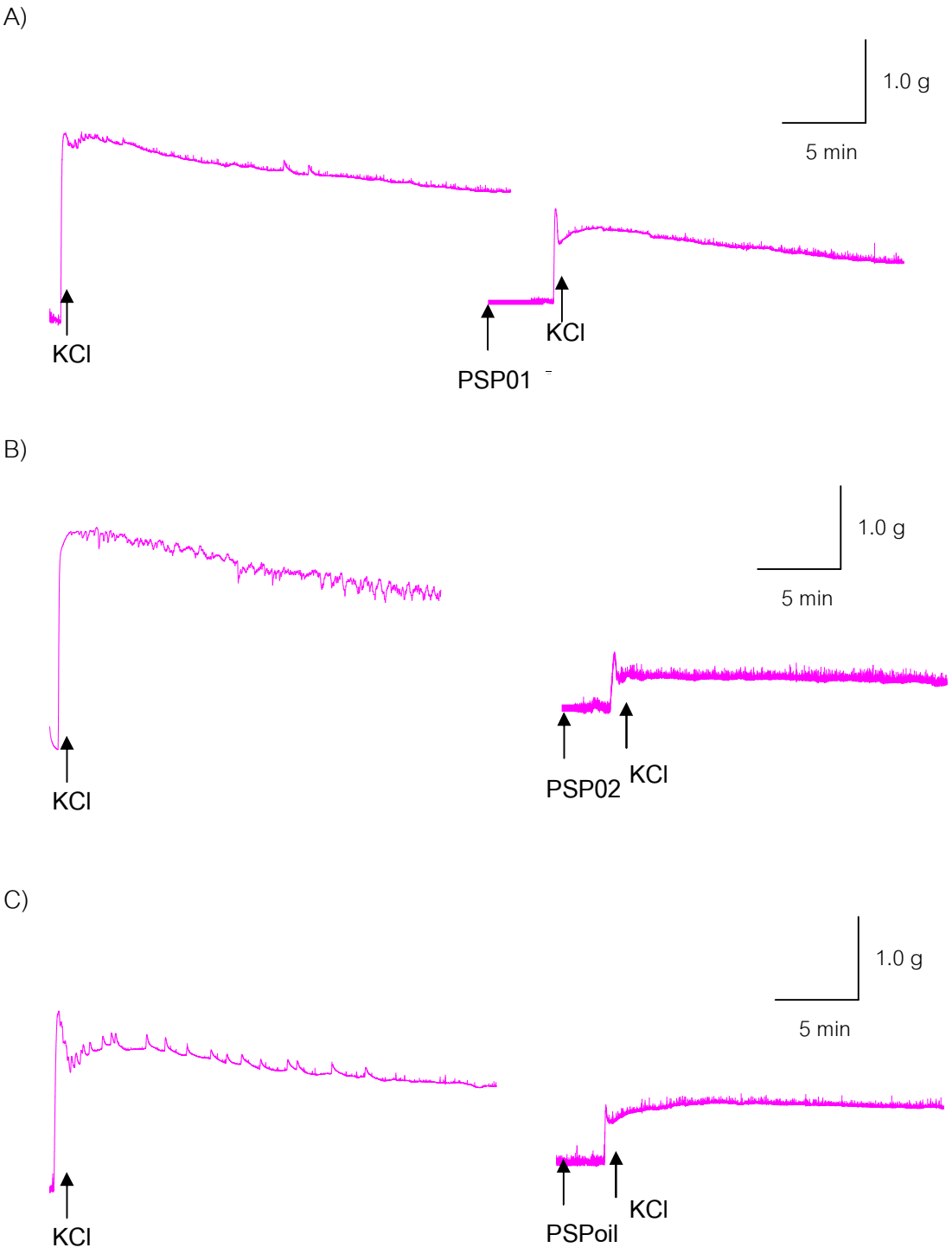


Figure 23: Representative tracing showed the effects of the test materials (A) PSP01, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on KCl-induced contraction.

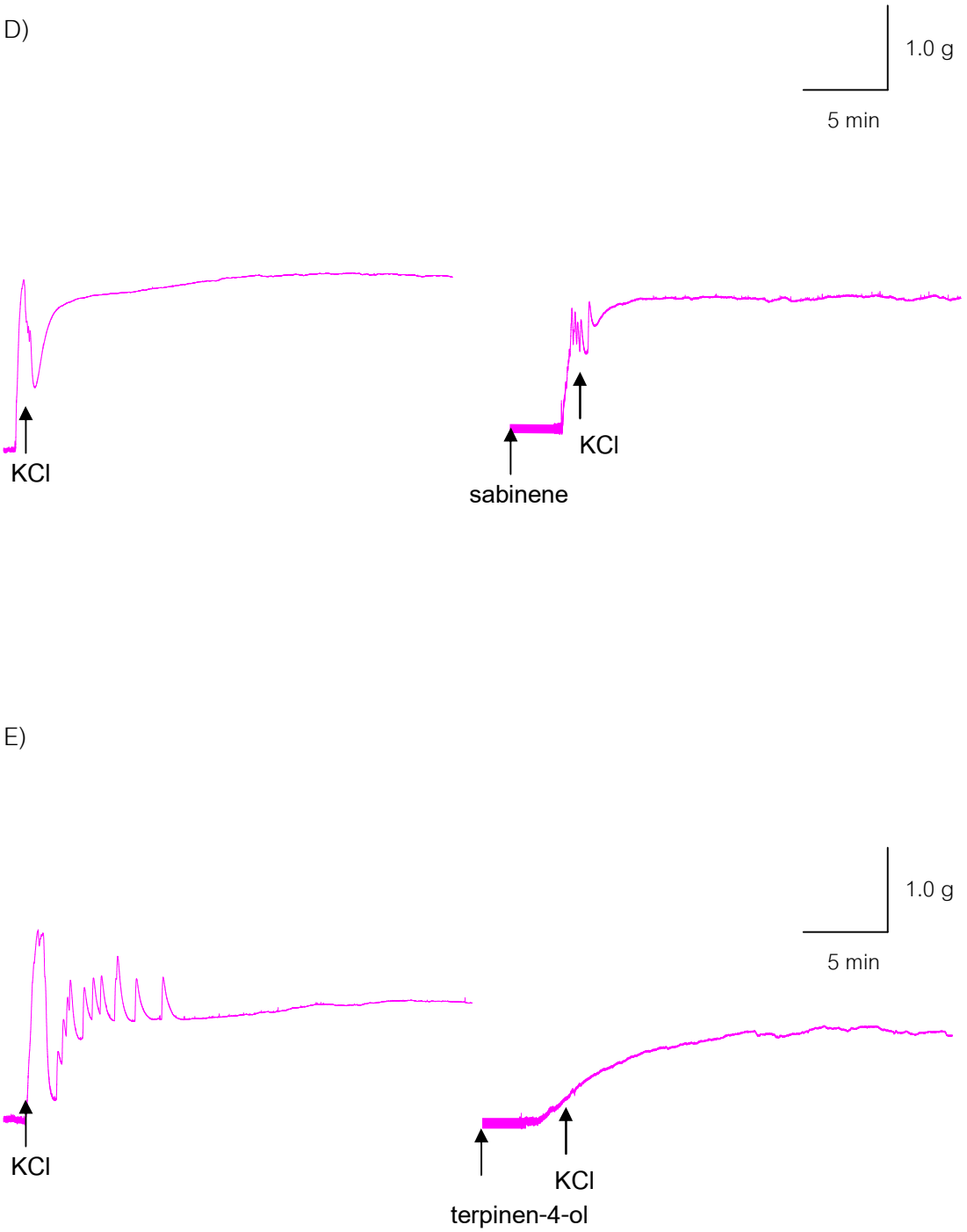


Figure 23: Representative tracing showed the effects of the test materials (A) PSP01, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on KCl-induced contraction.

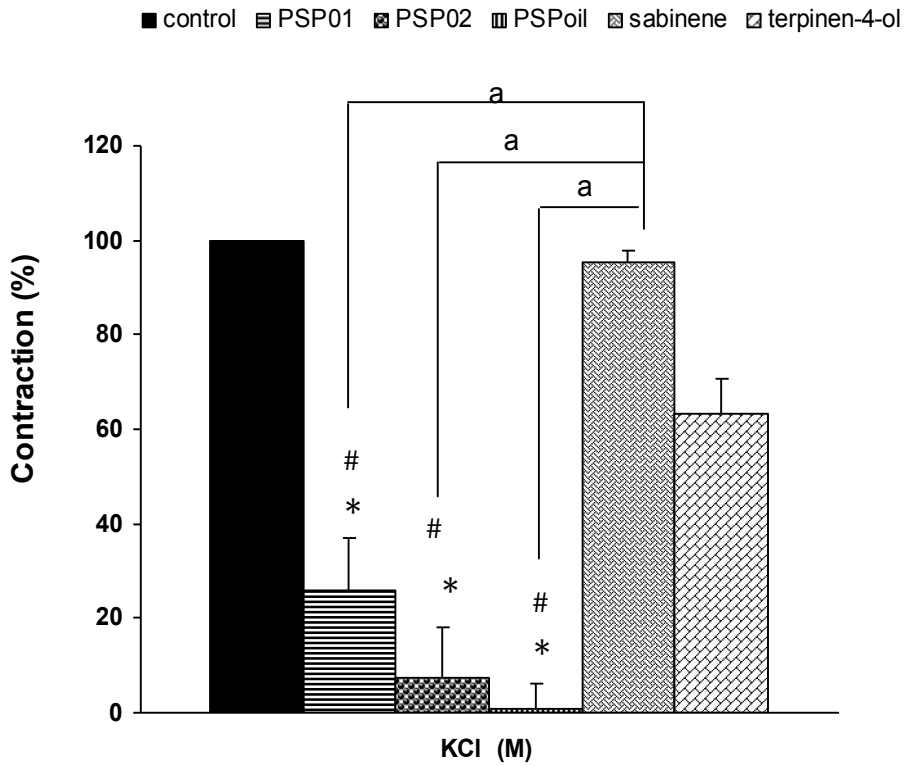


Figure 24: Comparative effects of the test extracts 100 µg/ml with the major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on KCl-induced contractions. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.

$^{*}p < 0.05$ denoted statistically significant difference from control.

$^{a}p < 0.05$ denoted statistically significant difference from sabinene.

$^{\#}p < 0.05$ denoted statistically significant difference from terpinen-4-ol.

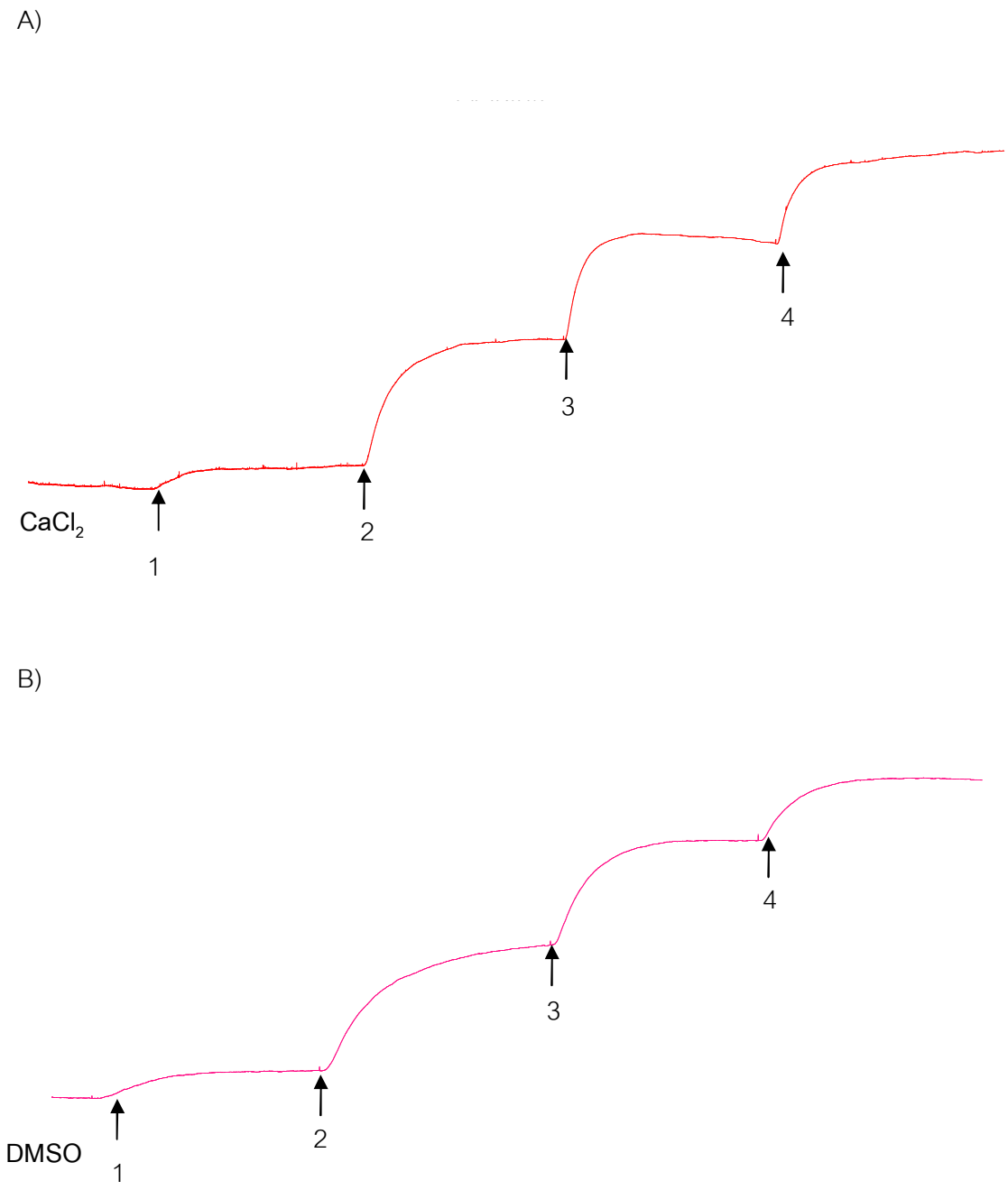
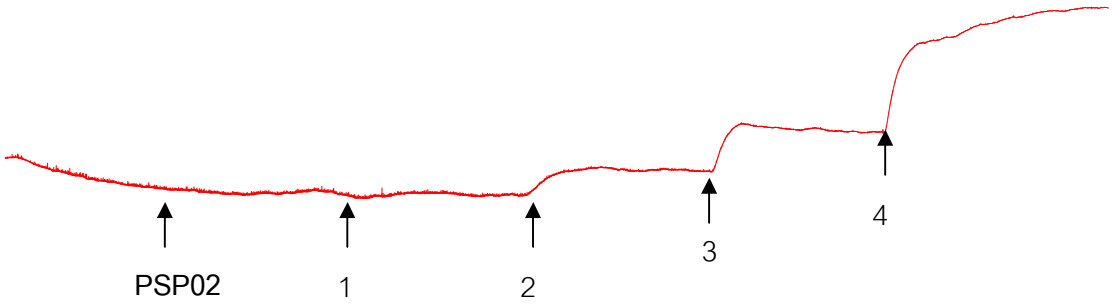


Figure 25: Representative tracing showed the effects of the test materials (A) $CaCl_2$, (B) DMSO 0.05%, (C) PSP02 and (D) PSPoil 100 µg/ml comparative with the major ingredients (E) sabinene 39.13 µg/ml and (F) terpinen-4-ol 34.12 µg/ml on $CaCl_2$-induced contraction in high K^+-Ca^{2+}-free depolarizing solution (1-5; $CaCl_2$ concentration 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M).

C)



D)

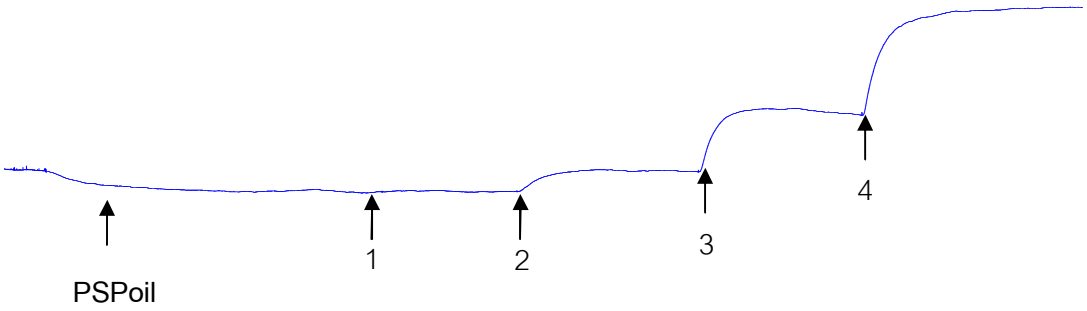
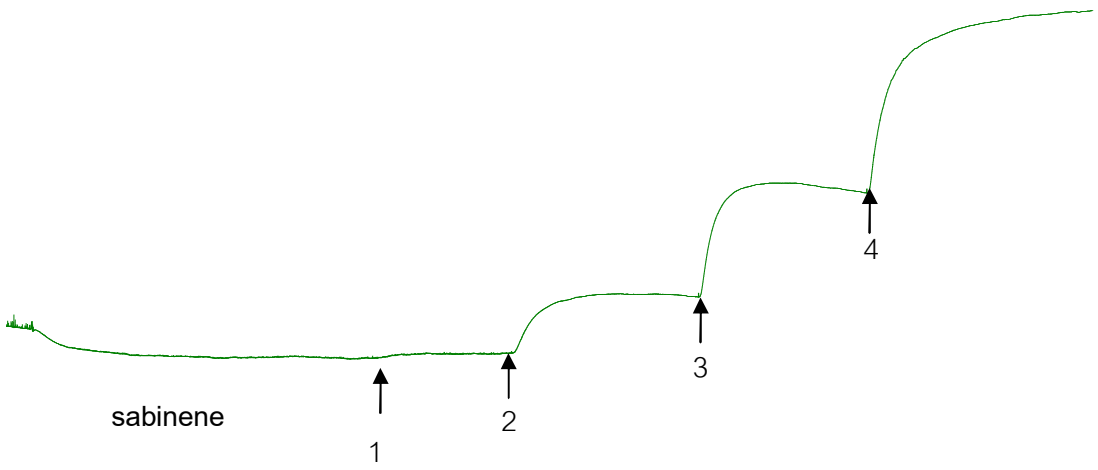


Figure 25: Representative tracing showed the effects of the test materials (A) $CaCl_2$, (B) DMSO 0.05%, (C) PSP02 and (D) PSPoil 100 µg/ml comparative with the major ingredients (E) sabinene 39.13 µg/ml and (F) terpinen-4-ol 34.12 µg/ml on $CaCl_2$-induced contraction in high K^+-Ca^{2+}-free depolarizing solution (1-5; $CaCl_2$ concentration 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M).

E)



F)

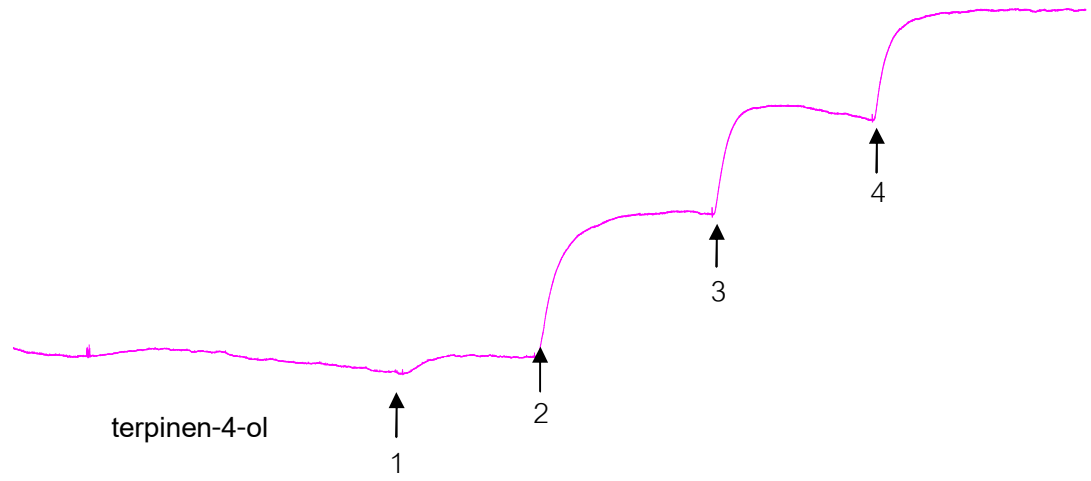


Figure 25: Representative tracing showed the effects of the test materials (A) $CaCl_2$, (B) DMSO 0.05%, (C) PSP02 and (D) PSPoil 100 µg/ml comparative with the major ingredients (E) sabinene 39.13 µg/ml and (F) terpinen-4-ol 34.12 µg/ml on $CaCl_2$-induced contraction in high K^+-Ca^{2+}-free depolarizing solution (1-5; $CaCl_2$ concentration 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M).

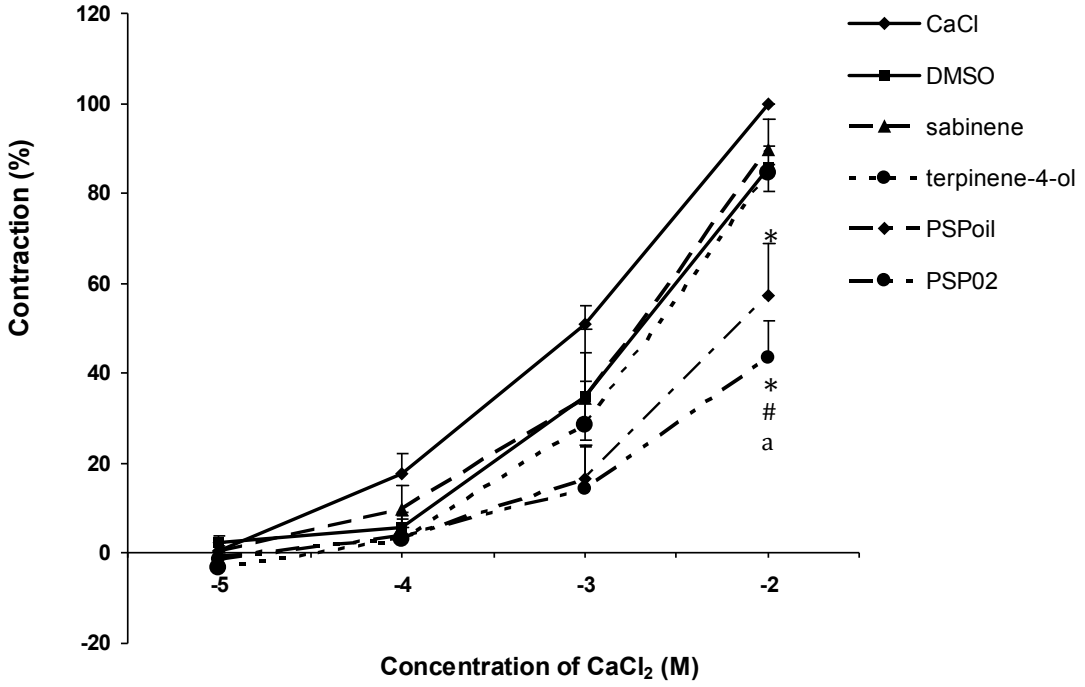


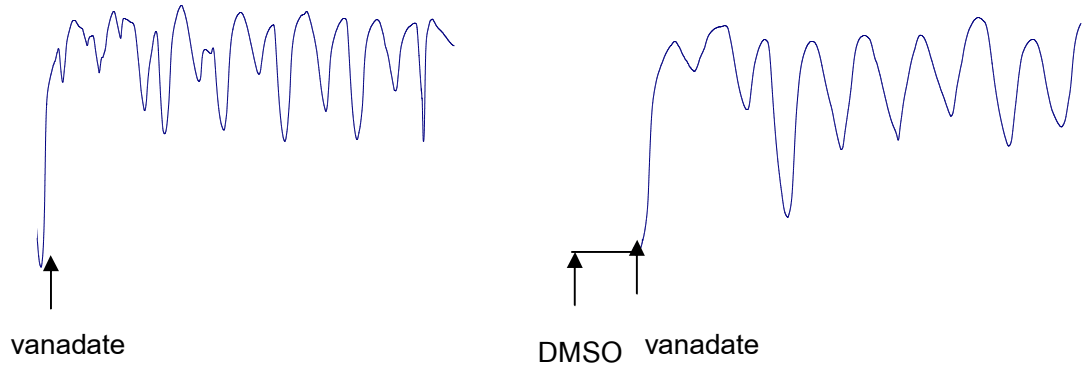
Figure 26: Comparative effects of the test extracts 100 µg/ml with major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on $CaCl_2$-induced contraction in high K^+-Ca^{2+}-free depolarizing solution. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

#$p < 0.05$ denoted statistically significant difference from PSPoil.

a$p < 0.05$ denoted statistically significant difference from sabinene.

A)



B)

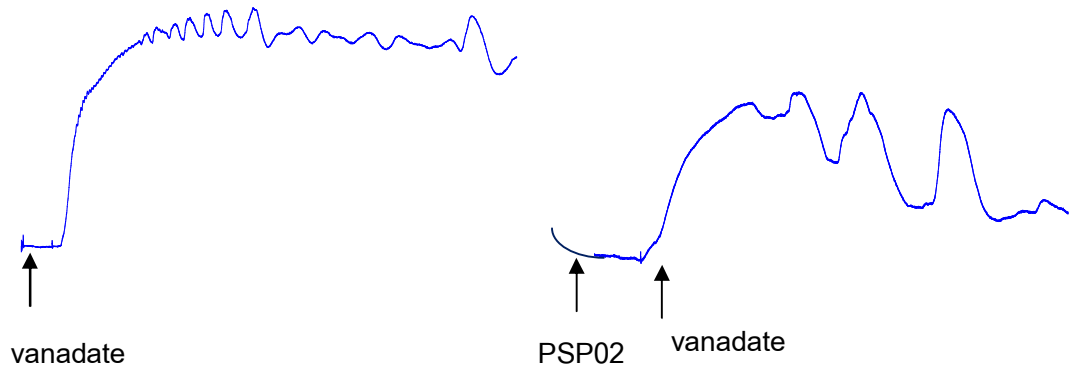
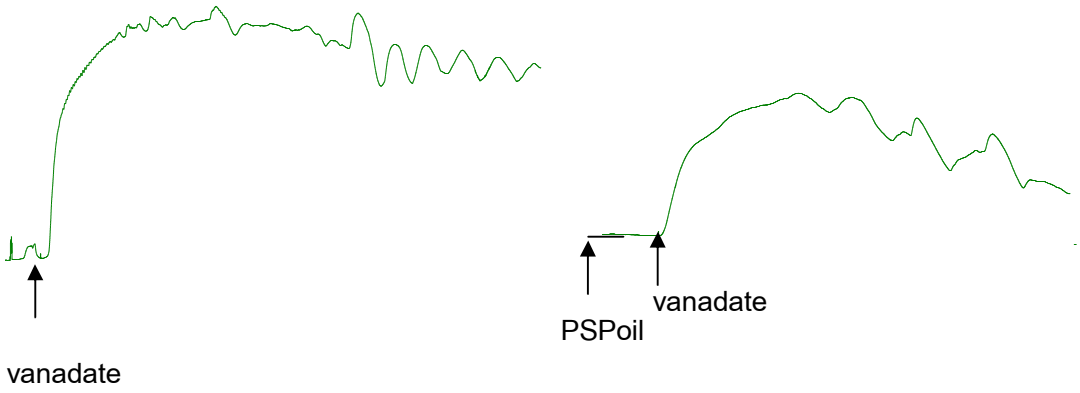


Figure 27: Representative tracing showed the effects of the test materials (A) DMSO 0.05%, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on vanadate-induced contraction in Ca^{2+}-free solution.

C)



D)

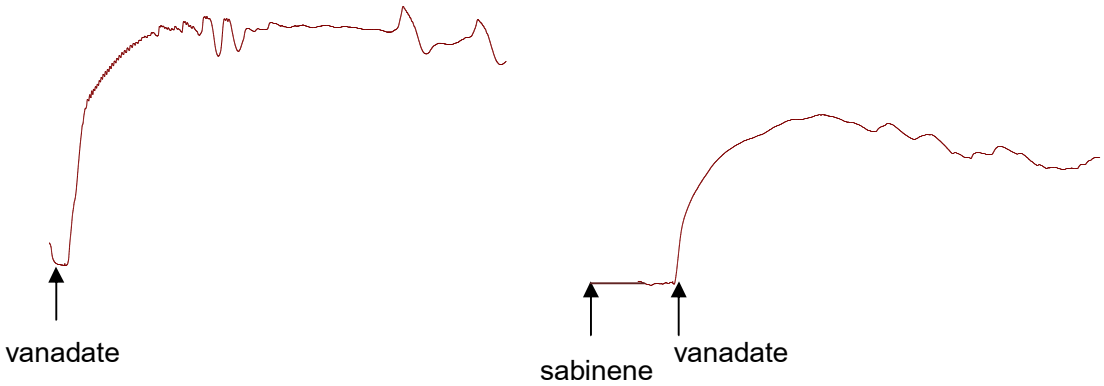


Figure 27: Representative tracing showed the effects of the test materials (A) DMSO 0.05%, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on vanadate-induced contraction in Ca^{2+}-free solution.

E)

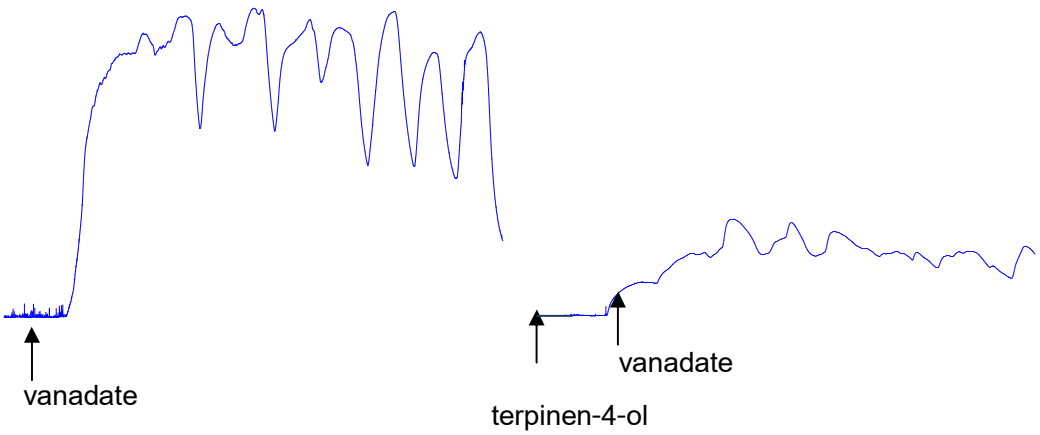


Figure 27: Representative tracing showed the effects of the test materials (A) DMSO 0.05%, (B) PSP02 and (C) PSPoil 100 µg/ml comparative with the major ingredients (D) sabinene 39.13 µg/ml and (E) terpinen-4-ol 34.12 µg/ml on vanadate-induced contraction in Ca^{2+}-free solution.

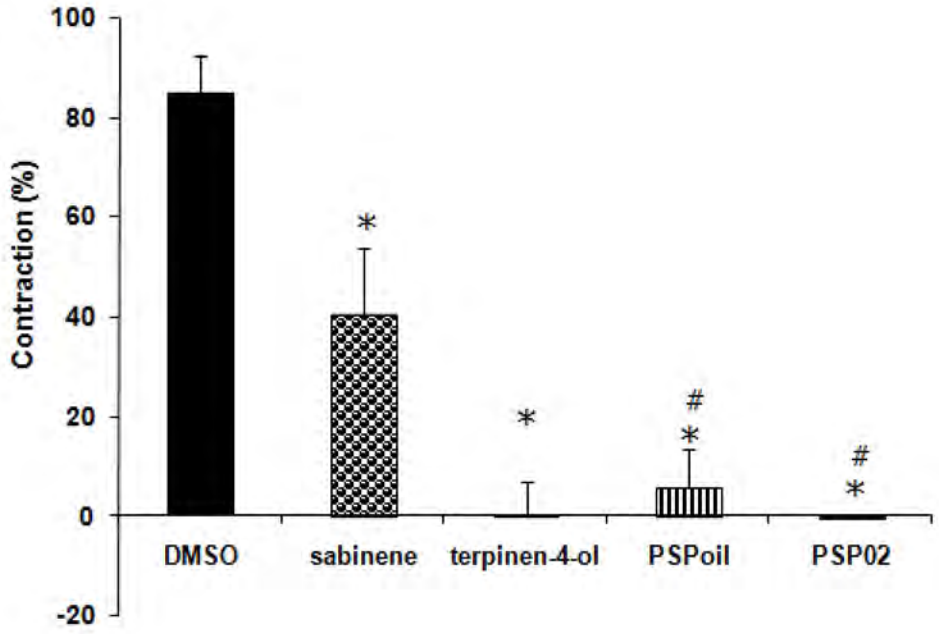


Figure 28: Comparative effects of the test extracts 100 µg/ml with the major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on vanadate-induced contraction in Ca^{2+}-free solution. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Dunnett's test.

$^{*}p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

$^{\#}p < 0.05$ denoted statistically significant difference from sabinene.

Comparison of IC_{50} values (concentration producing 50 % of maximum inhibition of contraction induces by various spasmogens on isolated rat uterus, n= 4-5) of PSP01, PSP02 and PSPoil

Stimulant	IC_{50} (µg/ml)		
	Force of contraction		
	PSPoil	PSP01	PSP02
Spontaneous	41.44±13.50	26.15±6.70	28.88±2.33
Oxytocin	32.55±3.04	27.48±2.89	29.75±4.57
$PGF_{2\alpha}$	56.13±5.01	69.92±4.51	54.42±1.70
KCl	27.80±4.62	20.11±2.72	19.67±2.99
	Frequency of contraction		
	PSPoil	PSP01	PSP02
Spontaneous	52.98±16.44	32.58±10.88	24.42±4.98
Oxytocin	28.53±4.15	35.29±3.91	30.53±3.15
$PGF_{2\alpha}$	48.83±13.39	42.73±4.94	34.45±1.59

Table 1: Comparison of IC_{50} values (concentration producing 50 % of maximum inhibition of contraction induces by various spasmogens on isolated rat uterus, n= 4-5) of PSP01, PSP02 and PSPoil.

CHAPTER V

DISCUSSION AND CONCLUSION

The objective of this study aimed to demonstrate the antispasmodic effect of the alcoholic extract from oral formulation “Prasaplai” (PSP01 and PSP02), comparing with *Zingiber cassumunar* Roxb. (plai oil) in the model of isolated rat uterine horn smooth muscle. In addition, sabinene and terpinen-4-ol which were the major constituents in plai oil were also studied. Although the amount of sabinene and terpinen-4-ol in the extracts were not determined in the “Prasaplai” extracts, it could be assumed that these two compounds could be the major component in Prasaplai extracts because plai was the major component in this formulation. Based on these assumptions, the concentration of sabinene and terpinen-4-ol used in this study were estimated as 39.13 µg/ml and 34.12 µg in the concentration of 100 µg/ml of Prasaplai and plai oil extract.

In this study, the uterus muscles were isolated from estrogen-primed animals in order to acquire a good standardized contractility with a small animal-to-animal variation. As known, estrogen influences uterine contractility by reducing the membrane potential of myometrial cells and by increasing gap junction between adjacent cells (Eagland and Cooper, 2001). An increase in gap junction formation provides partial cytoplasmic continuity between adjacent cells. Consequently, these muscles serve as an ideal anatomical substrate for coordinating tissue responses (Christ and Brink, 2000). Moreover, estrogen downregulates cytosolic NOS activity of uterine muscle while

upregulates the expression of the oxytocin receptor (Batra *et al*., 2003; Parker and Schimmer, 2006).

Spontaneous rhythmic contraction is under influences of pacemaker activity in myometrial cells as well as hormonal and sympathetic/parasympathetic activators (Wray *et al*., 2003; Sanborn, 2007). The results of this study demonstrated that the alcoholic extracts from oral formulation Prasaplai (PSP01, PSP02) and plai oil (PSPoil) significantly inhibited both amplitude and frequency of spontaneous contraction of uterus muscle.

Next, the effects and the possible mechanisms of actions of the extracts were determined with the use of various spasmogens including ACh, oxytocin, $PGF_{2\alpha}$ and KCl. The spasmogens were chosen based on its different receptor binding on the cell surface to provoke the contraction. Oxytocin and $PGF_{2\alpha}$ provoke myometrium contractions by binding to oxytocin and prostaglandin receptors, respectively (Phillippe *et al*., 2002; Shmygol *et al*., 2006). Activation of these receptors stimulates the G-protein signaling pathway, resulting in stimulation of phospholipase C (PLC) activity and release of Ca^{2+} from internal store (Wray *et al*., 2003; Shmygol *et al*., 2006). Besides the intracellular source, the influx of extracellular Ca^{2+} is the major source of an increase in intracellular Ca^{2+}. It was demonstrated that calcium channel blockers completely abolished the contractions induced by either oxytocin or $PGF_{2\alpha}$ (Perusquia and Kubli-Garfias, 1992; Kawarabayashi *et al*., 1997; Phillippe *et al*., 1997; Sultatos, 1997; Ruttner *et al*., 2002). In the whole cell patch clamp experiment, oxytocin was found to induce an inward current through activation of nonselective cation channels (Shimamura *et al*.,

1994). These currents could depolarize the plasma membrane and enhanced excitability (Carl *et al*., 1996). The depolarization enhanced the probability of opening of the voltage-dependent Ca^{2+} channels and increased the influx of Ca^{2+}. In addition, ACh provokes contraction via activation of muscarinic (M3)-G protein-coupled receptor on the myometrium, causing the release of Ca^{2+} through the G_q-PLC-IP_3 system (Papano, 2007). Furthermore, KCl-induced muscle contractility results from membrane depolarization which enhances excitability and opening of the voltage operated Ca^{2+}channels. Consequently, the influx of Ca^{2+} from extracellular space increases (Wray *et al*., 2005).

As known, a rising of intracellular $Ca^{2+}$is the key component in activating the contractile elements. All of agonists in this study (ACh, oxytocin, $PGF_{2\alpha}$ and KCl) lead to increase of intracellular Ca^{2+}either through Ca^{2+} influx from extracellular space or Ca^{2+} release from internal store. It was possible that the actions of the extracts were mediated through either inhibiting intracellular Ca^{2+}rising, or disturbing Ca^{2+}-mediated downstream signaling or disrupting the contractile elements.

In this study, the effects of the extracts on β_2-adrenoceptors could not be blocked by propranolol, a known β-blocker. This result suggested that the effects of the extracts on uterine relaxation were unlikely mediated through β_2-adrenoceptors stimulation and its Ca^{2+}-independent signaling pathway. The result also implicated that the contractile elements were not cellular targets of these extracts. Furthermore, the underlying mechanisms largely depended on Ca^{2+}-mediated pathways.

The physiological approaches to determine the effects on muscle tensions in isolated organ system could not provide the direct information about the intracellular Ca^{2+}levels. However, the contractile effects of spasmogens in this study were mainly due to a rising of intracellular Ca^{2+}. Hence, the actions of the extracts were likely involved with inhibition of Ca^{2+}-dependent muscle contraction, possibly by either hindering an increase of intracellular Ca^{2+}or inactivating Ca^{2+}-dependent signaling cascade. As known, free cytosolic Ca^{2+}increases through either an influx from extracellular space or a release from sarcoplasmic reticulum. The results showed that the alcoholic extracts were able to inhibit $CaCl_2$-induced contraction in a high K^+-Ca^{2+}-free depolarizing solution in a concentration-dependent manner. In high K^+-Ca^{2+}-free depolarizing solution, voltage-operated calcium channel (VOC) fully open through membrane depolarization, allowing the influx of $Ca^{2+}$from extracellular source. Consequently, the muscle contracts increasingly upon cumulative addition of $CaCl_2$. The findings in this study suggested that the extracts might directly affect Ca^{2+}-influx across the plasma membrane through voltage-operated calcium channels.

In Ca^{2+} free condition, the alcoholic extracts were able to inhibit vanadate-induced contraction. It has been well established that vanadate, an inhibitor of protein tyrosine phosphatase, increases myosin light chain phosphorylation in rat uterus (Karaki *et al.*, 1997). In addition, vanadate exerts it spasmogenic effects on smooth muscle through inhibition of Ca^{2+}-ATPase, which can be located on the plasma membrane and sarcoplasmic reticulum (Sunano *et al.*, 1987; 1988). Consequently, Ca^{2+}accumulate

within the cells. It has been reported that vanadate produced sustained contraction for more than 1 hour in Ca^{2+}-free condition (Mironneau *et al*., 1984). In the present experiment, vanadate was demonstrated to produce sustained contraction in Ca^{2+}-free solution. All of the test extracts were able to suppress the vanadate-induced contraction, suggesting that these extract could affect Ca^{2+}-handling within the cells through stimulate Ca^{2+}-ATPase activity and neutralize the vanadate effects. In addition, these findings also suggested that all of the extracts might affect Ca^{2+}-signaling pathway in muscle contraction.

The effects of both PSP01 and PSP2 were equivalent in inhibiting uterine contraction. The result showed that the extracts of whole preparation (PSP01 and PSP02) were more potent than plai oil (PSPoil). The relaxation effects were also observed from incubating the muscle with sabinene and terpinene-4-ol. These two compounds slightly inhibited the uterus contraction provoked by various spasmogens (ACh, oxytocin, $PGF_{2\alpha}$, KCl, $CaCl_2$ and vanadate), with the inhibitory effects less than 30%. The results revealed that these two compounds were less potent than the alcoholic extracts of Prasaplai preparations. In this study, the concentrations of these two compounds were the highest concentrations that were able to dissolve in DMSO. Thus, it was likely that there were other ingredients in the whole preparation involving in its relaxation effects. Furthermore, the effects of these two compounds were more selective to the vanadate-induced contraction in Ca^{2+}-free condition (appendix D). Hence, it was possible that Ca^{2+}-ATPase might be their cellular target.

In summary, the alcoholic extracts of Prasaplai preparation directly modulated the uterus contraction via Ca^{2+}-dependent pathway. The alcoholic extract of Prasaplai apparently have a potential value in the treatment of certain diseases, including gynecological disturbances and dysfunctions associated with an increase in uterine muscular activity such as dysmenorrhea and premature deliveries. However, further studies are in need to clarify it influence on intracellular Ca^{2+}level.

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APPENDICES

APPENDIX A

Table 2: Effects of the test materials (10-50 µg/ml) on the contractile of amplitudes (A) and frequency (B) of spontaneously-contracting rat isolated uterine horns.

.A.

Extracts	Force of contractions (%)				
	concentration (µg/ml)				
	10	20	30	40	50
DMSO	99.61±2.93	98.52±1.90	97.20±2.96	96.85±3.49	96.55±3.58
PSP01	85.46±6.78	58.16±16.90*	38.72±9.87	19.44±13.77*	15.49±10.85
PSP02	90.86±2.19	71.30±5.30	50.98±5.60*	32.47±5.17*	17.62±6.07
PSPoil	86.39±8.57	74.79±10.41	57.08±18.85*	44.41±16.72*	28.44±13.27

B.

Extracts	Frequency (%)				
	concentration (µg/ml)				
	10	20	30	40	50
DMSO	85.62±5.44	91.87±4.93	82.50±5.95	87.50±3.75	87.50±7.22
PSP01	87.10±7.81	53.04±14.50	45.41±16.88	28.75±13.15*	22.50±18.07
PSP02	80.41±7.08	50.41±12.37	42.08±7.92*	23.54±9.43*	10.00±10.00*
PSPoil	85.00±2.50	82.50±5.00	45.00±15.00	45.00±16.52*	37.50±17.08

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

Table 3: Effects of the test materials (25, 50 and 100 µg/ml) on the contractile of amplitudes (A) and frequency (B) of ACh-induced contractions.

A.

Extracts	Force of contractions (%)		
	concentration (µg/ml)		
	25	50	100
DMSO	100.65±1.52	99.44±2.09	99.69±2.91
PSP01	78.31±6.44*	64.99±2.28*#	61.95±12.32*
PSP02	75.92±7.30*#@	63.22±2.12*#@	26.12±2.28*#a
PSPoil	92.27±3.70	79.45±7.44*	75.91±5.11*

B.

Extracts	Frequency (%)		
	concentration (µg/ml)		
	25	50	100
DMSO	100.89±10.95	108.33±8.33	111.90±7.90
PSP01	64.65±8.90*	44.25±7.92*	37.50±8.29*
PSP02	55.12±3.95*@	50.83±10.13*	18.75±10.96*@
PSPoil	76.49±9.45*@	46.25±11.43*	28.10±8.82*@

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

#$p < 0.05$ denoted statistically significant difference from PSPoil.

@$p < 0.05$ denoted statistically significant difference between 25 and 50 µg/ml compared with 100 µg/ ml of the test materials.

a$p < 0.05$ denoted statistically significant difference between PSP01 and PSP02.

Table 4: Effects of the test materials (10-50 µg/ml) on the contractile of amplitudes (A) and frequency (B) of oxytocin-induced contraction on rat isolated uterine horns.

A.

Extracts	Force of contractions (%)				
	concentration (µg/ml)				
	10	20	30	40	50
DMSO	101.05±1.49	100.87±0.90	99.65±1.73	99.66±1.66	99.43±1.61
PSP01	94.81±1.34	77.03±3.47*	51.18±13.06*	22.88±14.33*	0.00±0.00*
PSP02	92.40±4.55	75.88±12.99*	60.16±12.54	36.87±15.01	0.00±0.00*
PSPoil	96.56±0.81	89.42±1.02*	64.85±16.21	39.31±16.04	0.04±0.03*

B.

Extracts	Frequency (%)				
	concentration (µg/ml)				
	10	20	30	40	50
DMSO	94.64±7.83	86.43±5.49	80.71±3.27	83.21±2.97	83.21±2.97
PSP01	89.43±5.30	71.24±8.02	53.52±14.93	33.14±9.97*	7.71±5.56*
PSP02	90.64±2.48	81.79±5.54	61.93±10.24	43.59±13.63	0.00±2.00*
PSPoil	85.85±4.38	92.38±12.40	76.92±12.34	64.50±15.24	4.00±4.19*

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

Table 5: Effects of the test materials (10-50 µg/ml) on the contractile of amplitudes (A) and frequency (B) of $PGF_{2\alpha}$-induced contraction on rat isolated uterine horns

A.

Extracts	Force of contractions (%)				
	concentration (µg/ml)				
	10	20	30	40	50
DMSO	99.39±0.89	97.80±1.43	97.02±2.27	96.51±2.53	94.89±2.59
PSP01	91.79±3.02	81.50±2.45*	77.99±4.46*	68.77±3.50*	55.78±2.22*
PSP02	96.77±1.45	91.06±2.12	84.18±2.68	69.77±2.77*	46.95±2.13*
PSPoil	99.34±0.12	94.57±4.37	88.53±6.53	82.49±7.36	50.92±6.95*

B.

Extracts	Frequency (%)				
	concentrations (µg/ml)				
	10	20	30	40	50
DMSO	90.00±5.77	85.36±6.06	80.36±2.14	85.36±6.06	80.36±2.14
PSP01	90.00±5.77	78.75±8.26	78.75±8.26	67.50±13.77	50.83±10.31
PSP02	85.00±7.01	73.33±3.85	63.33±1.92*	43.33±4.08*	29.17±3.15*
PSPoil	79.85±7.25	61.21±16.80	61.21±16.80	54.96±12.64	40.38±9.22*

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

Table 6: Effects of the test materials (10-50 µg/ml) on the contractile of amplitudes of KCl-induced isolated rat uterine horns.

Extracts	Force of contractions (%)				
	concentrations (µg/ml)				
	10	20	30	40	50
DMSO	97.32±2.28	95.49±3.92	94.46±4.92	94.16±5.25	93.61±5.53
PSP01	78.00±5.59	53.68±7.72*	32.98±7.14*	17.53±4.94*	4.26±2.06*
PSP02	79.09±7.72	53.48±7.45*	26.66±8.70*#	13.24±6.63*#	2.78±2.07*
PSPoil	81.12±6.80	67.48±6.49*	53.39±6.91*	35.26±7.33*	14.10±5.93*

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

#$p < 0.05$ denoted statistically significant difference from PSPoil.

Table 7: Comparative effects of the test extracts 100 µg/ml with major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on force (A) and frequency (B) of various agonist-induced contractions.

A.

Extracts	Force of contractions (%)			
	Agonists			
	ACh	oxytocin	$PGF_{2\alpha}$	KCl
Control	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
PSP01	81.94±3.68	66.53±7.70	59.05±23.25	26.00±12.64*#a
PSP02	49.69±7.29*a	42.78±8.27*a	62.18±3.95*a	7.35±10.81*#a
PSPoil	51.01±10.69	54.12±8.01	51.35±8.19	-11.20±9.09*#a
sabinene	90.08±5.18	93.31±1.55	92.84±2.13	95.40±2.48
terpinen-4-ol	87.48±1.79	70.98±11.22	81.15±3.64	63.30±7.32

*$p < 0.05$ denoted statistically significant difference from control.

#$p < 0.05$ denoted statistically significant difference from terpinen-4-ol.

a$p < 0.05$ denoted statistically significant difference from sabinene.

Table 7: Comparative effects of the test extracts 100 µg/ml with major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on force (A) and frequency (B) of various agonist-induced contractions.

B.

Extracts	Frequency (%)		
	Agonists		
	ACh	oxytocin	$PGF_{2\alpha}$
Control	100.00±0.00	100.00±0.00	100.00±0.00
PSP01	61.14±6.61	70.56±15.00	43.75±15.73
PSP02	58.33±17.35	56.52±9.24	64.36±4.61
PSPoil	60.00±24.49	59.58±21.61	38.21±10.32#
sabinene	54.42±11.39	80.31±5.41	76.98±3.91
terpinen-4-ol	58.53±10.07	76.67±7.71	69.15±4.65

*$p < 0.05$ denoted statistically significant difference from control.

#$p < 0.05$ denoted statistically significant difference from terpinen-4-ol.

a$p < 0.05$ denoted statistically significant difference from sabinene.

Table 8: Comparative effects of the test extracts 100 µg/ml with major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on $CaCl_2$-induced contraction in high K^+-Ca^{2+}-free depolarizing solution.

$CaCl_2$ concentration (M)	Force of contractions (%)					
	Control	DMSO (0.05%)	PSPoil (100µg/ml)	PSP02 (100µg/ml)	sabinene (100µg/ml)	terpinene-4-ol (100µg/ml)
10^{-5}	0.39±1.23	2.28±1.62	-0.44±0.30	-1.27±1.11	0.61±1.68	-3.36±2.13
10^{-4}	17.88±4.21	5.70±1.96	3.47±2.21	4.07±4.19	9.72±5.33	3.02±6.04
10^{-3}	50.76±4.28	34.99±9.76	16.74±7.36	14.26±9.51	34.59±15.31	28.41±9.72
10^{-2}	100.00±0.00	85.53±5.05	57.31±11.70*	43.51±8.10*#a	89.62±6.76	84.37±2.00

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

#$p < 0.05$ denoted statistically significant difference from PSPoil.

a$p < 0.05$ denoted statistically significant difference from sabinene.

Table 9: Comparative effects of the test extracts 100 µg/ml with major ingredients (sabinene 39.13 µg/ml and terpinen-4-ol 34.12 µg/ml) on vanadate-induced contraction in Ca^{2+}-free solution.

Extracts	Contraction (%)
DMSO (0.05%)	84.82±7.48
Sabinene (39.14 µg/ml)	40.61±13.15*
Terpinen-4-ol (34.12 µg/ml)	0.25±6.69*
PSPoil (100 µg/ml)	5.76±7.56*#
PSP02 (100 µg/ml)	-0.04±0.57*#

*$p < 0.05$ denoted statistically significant difference from DMSO 0.05% (v/v).

#$p < 0.05$ denoted statistically significant difference from sabinene.

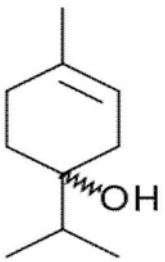
APPENDIX B

Table 10: Chemical compositions of physiological salt solution

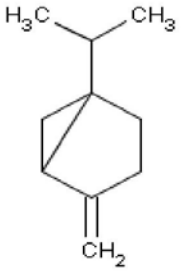
Chemical composition	MW	Physiological salt solution					
		Locke-Ringer		High K^+ (56.3mM) Ca^{2+} free (HKFCa)		Ca^{2+}-Mg^{2+} free EDTA (FCa)	
		mM	1 x (g)	mM	1 x (g)	mM	1 x (g)
NaCl	58.44	154.00	9.00	99.60	5.82	154.00	9.00
KCl	74.55	5.63	0.42	56.30	4.47	5.63	0.42
$CaCl_2$	110.99	2.16	0.24	-	-	-	-
$NaHCO_3$	84.01	5.95	0.50	5.95	0.50	5.90	0.50
D(+) Glucose	198.17	5.55	1.10	2.77	0.55	5.50	1.10
$MgCl_2$	95.30	2.10	0.20	-	-	-	-
EDTA	292.24	-	-	-	-	0.01	0.0029

(Source: Perez-Guerrers *et al*., 1996)

APPENDIX C



A.



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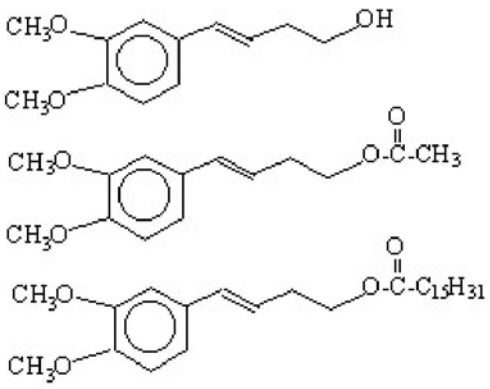
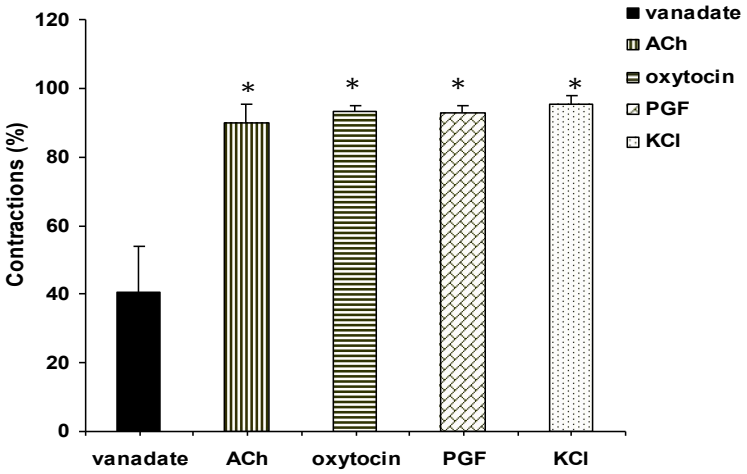


C.

Figure 29: Chemical structures of sabinene (A), terpinen-4-ol (B) and compounds D [(E)-4-(3', 4'-dimethoxyphenyl) but-3-en-1-ol] (C) (Mesripong, 2006)

APPENDIX D

A.



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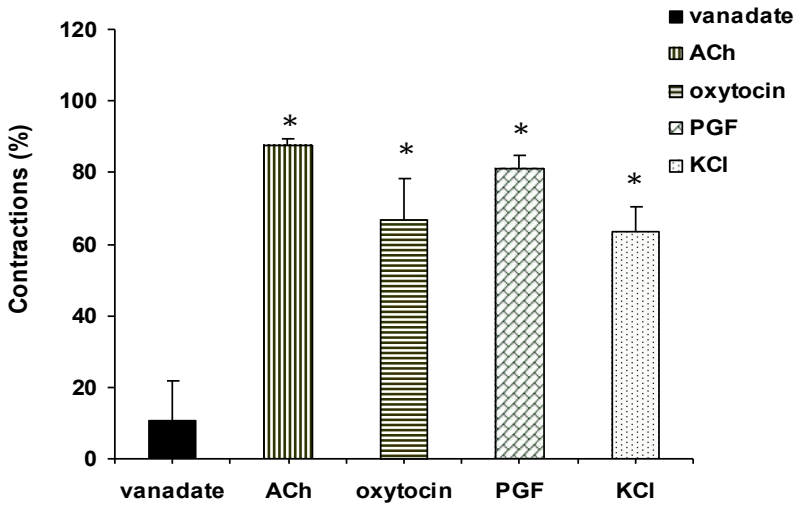
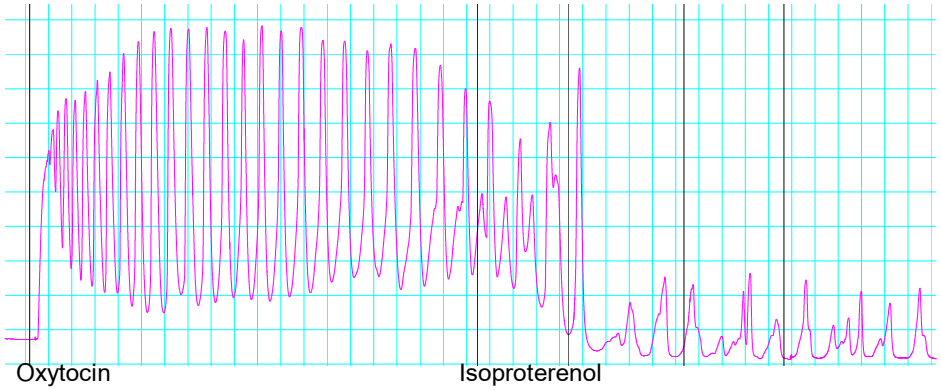


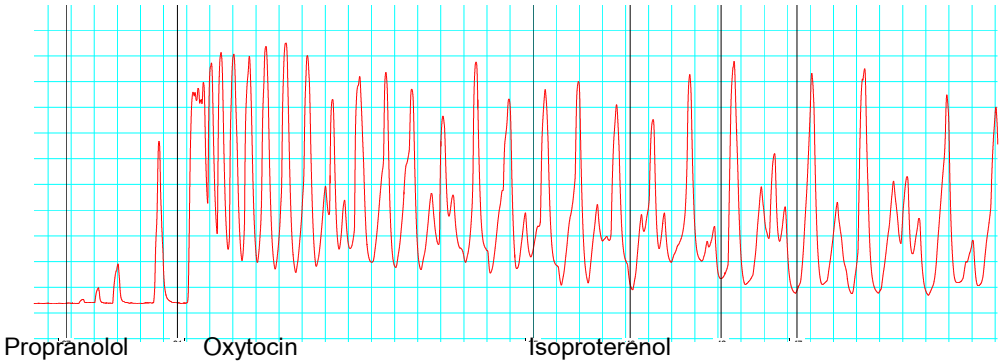
Figure 30: Comparative effects of the major ingredients sabinene 39.13 µg/ml (A) and terpinen-4-ol 34.12 µg/ml (B) on various spasmogens-induced contractions. Vanadate is an inducer of Ca^{2+} free solution. ACh, oxytocin, $PGF_{2\alpha}$ and KCl are inducer of Ca^{2+} solution. Each point represented mean±S.E.M. of 4-5 experiments. Statistical comparison was performed by ANOVA analysis followed by Bonferroni test. $^{*}p < 0.05$ denoted statistically significant difference from vanadate.

APPENDIX E

A.



B.



C.

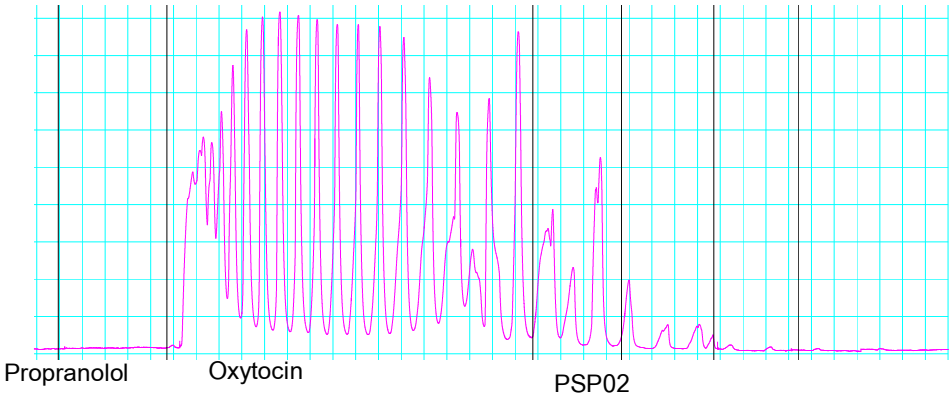


Figure 31: Representative tracing showed the effects of the test materials (A) isoproterenol, (B) propranolol and (C) PSP02 100 µg/ml on isoproterenol induced relaxation.

APPENDIX F

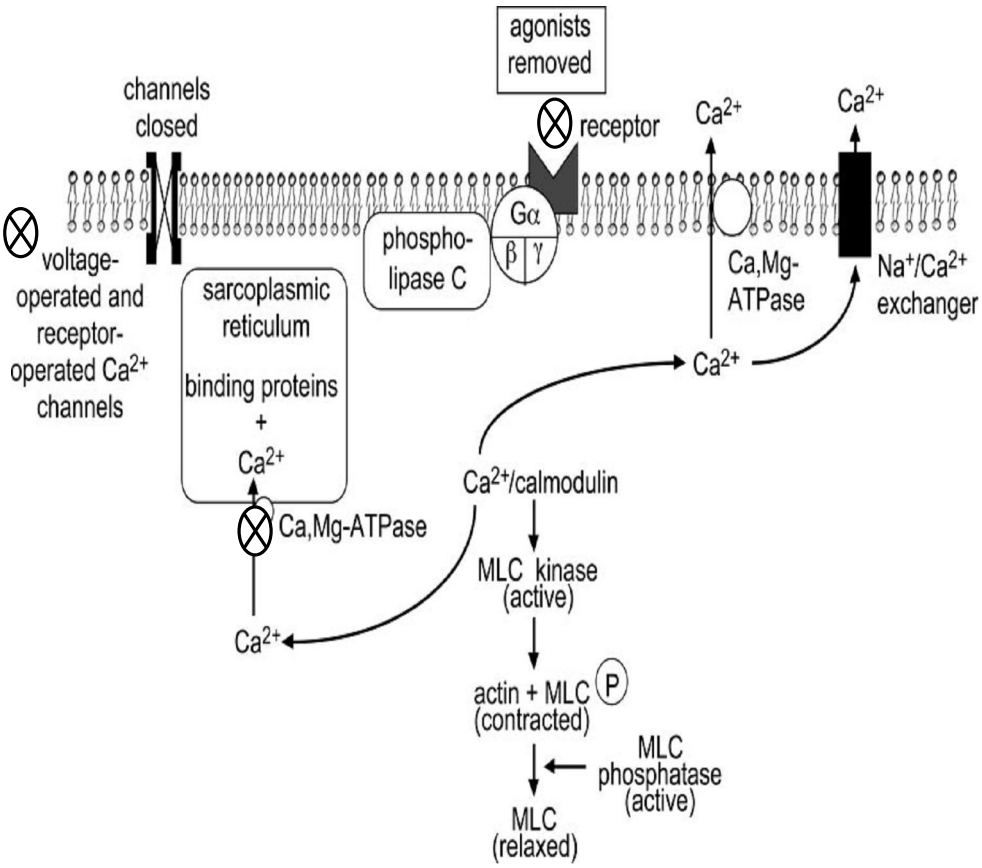


Figure 32: Plausible mechanism of actions of Prasaplai formulation.

BIOGRAPHY

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