ผลของสารสกัดโกฐสอที่มีต่อการเกาะกลุ่มของเกล็ดเลือด

นางสาวปริญญาพร ปราชญ์เมธีกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE EFFECT OF *ANGELICA DAHURICA* EXTRACTS ON PLATELET AGGREGATION

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ปริญญาพร ปราชญ์เมธีกุล: ผลของสารสกัดโกฐสอที่มีต่อการเกาะกลุ่มของเกล็ดเลือด. (THT EFFECT OF *ANGELICA DAHURICA* EXTRACTS ON PLATELET AGGREGATION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. รัตยา ลือชาพุฒิพร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. พลภัทร โรจน์นครินทร์, 100 หน้า.

การเกาะกลุ่มของเกล็ดเลือดเป็นกลไกหนึ่งที่สำคัญในการห้ามเลือด ในสภาวะที่เกล็ดเลือดทำงานผิดปกติอาจนำไปสู่ภาวะเลือดไหลหรือภาวะลิ่มเลือดอุดตันหลอดเลือดแดง โกฐสอถูกนำมาใช้เป็นส่วนประกอบในยาหอมซึ่งเป็นตำรับยาไทยที่ใช้ในการรักษาความผิดปกติของระบบไหลเวียนเลือด อิมเพอราโทรินเป็นองค์ประกอบทางเคมีหลักตัวหนึ่งในโกฐสอ วัตถุประสงค์ของงานวิจัยในครั้งนี้เพื่อศึกษาฤทธิ์ของสารสกัดโกฐสอและอิมเพอราโทรินที่มีต่อการเกาะกลุ่มของเกล็ดเลือดที่ถูกกระตุ้นด้วยอะดีโนซีนไดฟอสเฟต กรดอะราชิโดนิก และคอลลาเจน และกลไกการออกฤทธิ์ที่มีต่อการเกาะกลุ่มของเกล็ดเลือด เมื่อบ่มสารสกัดโกฐสอที่ความเข้มข้น 0.1 - 1 มิลลิกรัมต่อมิลลิลิตรและอิมเพอราโทรินที่ความเข้มข้น 10 - 300 ไมโครโมลาร์ในพลาสมาที่มีเกล็ดเลือดสูงเป็นเวลา 3 นาทีก่อนใส่ตัวกระตุ้น พบว่าโกฐสอที่ความเข้มข้น 1 มิลลิกรัมต่อมิลลิลิตรยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่ถูกกระตุ้นด้วยอะดีโนซีนไดฟอสเฟตและคอลลาเจนอย่างมีนัยสำคัญทางสถิติ มีเปอร์เซ็นต์ยับยั้ง 62.82% (p=0.000) และ 66.28% (p=0.002) ตามลำดับ อิมเพอราโทรินที่ความเข้มข้น 300 ไมโครโมลาร์ยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่ถูกกระตุ้นด้วยอะดีโนซีนไดฟอสเฟต มีเปอร์เซ็นต์ยับยั้ง 33.96% (P=0.012) เปรียบเทียบกับตัวทำละลายควบคุม แต่ไม่ยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่ถูกกระตุ้นด้วยคอลลาเจน ทั้งโกฐสอและอิมเพอราโทรินมีฤทธิ์ยับยั้งในช่วง secondary phase โกฐสอเพิ่ม lag phase เมื่อกระตุ้นเกล็ดเลือดด้วยคอลลาเจนเปรียบเทียบกับตัวทำละลายควบคุม (367.78 *vs.* 116.35 วินาที, p=0.008) ทั้งโกฐสอและอิมเพอราโทรินไม่มีฤทธิ์ยับยั้งเกล็ดเลือดที่ถูกกระตุ้นด้วยกรดอะราชิโดนิกแต่สามารถชะลอการเกาะกลุ่มของเกล็ดเลือดได้ เมื่อกระตุ้นเกล็ดเลือดด้วยอะดีโนซีนไดฟอสเฟต โกฐสอสามารถเพิ่มระดับไซคลิกอะดีโนซีนโมโนฟอสเฟต (cAMP) ได้อย่างมีนัยสำคัญทางสถิติ (p=0.027) แต่ไม่มีผลต่อระดับทรอมโบเซนบีสอง (TxB_2) โดยสรุปฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดของสารสกัดโกฐสอและอิมเพอราโทรินอย่างน้อยที่สุดน่าจะเกี่ยวข้องกับการส่งสัญญาณผ่านวิถีอะดีนิเลต ไซเคลส – ไซคลิกอะดีโนซีนโมโนฟอสเฟต

สาขาวิชาเภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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PRINYAPORN PRADMETHEEKUL: THE EFFECT OF *ANGELICA DAHURICA* EXTRACTS ON PLATELET AGGREGATION. ADVISOR: ASST. PROF. RATAYA LUECHAPUDIPORN Ph.D., CO-ADVISOR: PROF. PONLAPAT ROJNUCKARIN Ph.D., 100 pp.

Platelet aggregation is one of the important mechanisms in hemostasis. Improper platelet function may lead to bleeding or atherothrombosis. *Angelica dahurica* (AD) has been used in the mixture of Ya-Hom, a Thai herbal formulation, which is used for treatment of circulatory disorder. Imperatorin is one of the major chemical compounds in AD. The aims of this study were to investigate the effects of AD extracts (ADE) and imperatorin on arachidonic acid (AA)-, adenosine diphosphate (ADP)-, and collagen-induced platelet aggregation and their mode of action on platelet aggregation. ADE at 0.1 – 1 mg/ml and imperatorin at 10 – 300 μM were preincubated in platelet rich plasma (PRP) for 3 min before adding agonists. The results demonstrated that ADE at 1 mg/ml inhibited ADP- and collagen- induced platelet aggregation significantly by 62.82% (p=0.000) and 66.28% (p=0.002), while 300 μM of imperatorin inhibited ADP-induced platelet aggregation by 33.96% (p=0.012) compared with vehicle control but not inhibited collagen-induced platelet aggregation. Both ADE and imperatorin inhibited secondary phase of ADP-induced aggregation. ADE increased lag phase in collagen-induced platelet aggregation compared with vehicle control (367.78 *vs.* 116.35 sec, p=0.008). Both ADE and imperatorin could not inhibit AA-induced platelet aggregation but they could delay platelet aggregation. When induced platelet aggregation by ADP, ADE significantly increased cAMP levels (p=0.027), but did not affect on thromboxane B_2 level. In conclusion, the antiplatelet property of ADE and imperatorin at least take part in adenylate cyclase – cAMP pathway.

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

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Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

ºC	Degree celcius
5-HT	5-Hydoxytryptamine
AA	Arachidonic acid
ADP	Adenosine 5' -diphosphate
AMP	Adenosine monophosphate
ASA	Acetylsalicylic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3´, 5´-monophosphate
cGMP	Cyclic guanosine 3´, 5´-monophosphate
Cm	Centimeter
COX	Cyclooxygenase
DAG	Diacylglycerol
EDTA	Ethylenediamine tetraacetic acid
eNOS	Endothelial nitric oxide synthase
Etc.	Et cetera
FcRγ	Fc receptor γ-chain
GP	Glycoprotein
GPCRs	G protein-coupled receptors
IC_{50}	The half maximal inhibitory concentrations
IP3	Inositol (1, 4, 5)-triphosphate
LPS	Lipopolysaccharide
MLCK	Myosin light chain kinase
µg	Microgram
µl	Microliter
µM	Micromolar
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter

mM	Millimolar
MLCK	Myosin light-chain kinase
nmol	Nanomole
nM	Nanomolar
NO	Nitric oxide
PARs	Protease-activated receptors
PI3K	Phosphoinositide 3-kinases
PIP2	Phosphotidylinositol (4, 5)-biphosphate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PGI_2	Prostacyclin
PKC	Protein kinase C
PPP	Platelet poor plasma
PRP	Platelet rich plasma
rpm	Revolution per minute
TP	Thromboxane receptor
TxA_2	Thromboxane A_2
VASP	Vasodilator-stimulated phosphoprotein
vWF	von Willebrand factor

CHAPTER I

INTRODUCTION

1. Background and rationale

Platelets are responsible for the first line body defense following injury. Adhesion and activation of platelets at the site of injury leads to platelet aggregation in order to forms platelet plugs that help preventing blood loss. Thus, their role is essential for hemostasis. However, inappropriate platelet function contributes to various health problems including bleeding disorders and atherothrombotic diseases. Nowadays, it is well known that antiplatelet agents are effective in both prevention and treatment of cardiovascular diseases, such as myocardial infarction, ischemic stroke, etc., which are the main causes of death worldwide. The targets of current antiplatelet drugs are mainly the signaling pathways in order to inhibit platelet aggregation.

Platelet possesses various receptors on its membrane. They bind various specific agonists and mediate signaling pathways. Therefore, the mechanism underlying platelet aggregation is complex. Initially, platelets adhere at a site of injury after exposure to collagen and activate phospholipase C (PLC) *via* GPVI receptor. This leads to formation of two secondary messengers inositol triphosphate (IP3), which is required for calcium release from internal store, and diacylglycerol, which activates protein kinase C (PKC) resulting in shape change and secretion of soluble agonists, such as adenosine triphosphate (ADP) and thromboxane A_2 (TXA_2). These soluble agonists act as amplifiers of platelet response by recruit others platelets at injured vessel wall. For ADP, it binds two distinct G-protein coupled receptors, $P2Y_1$ and $P2Y_{12}$, which activates PLC and inhibits adenylate cyclase activity, respectively. TXA_2, a lipid mediator, is synthesized by the conversion of arachidonic acid to prostaglandin and TXA_2 using cyclooxygenase (COX) and thomboxane synthase, respectively. It binds to prostanoid receptor on

platelet membrane. Then, Rho/Rho kinase is activated *via* $G_{12/13}$ coupled receptor resulting in cytoskeletal reorganization leading to platelet shape change. Activation of PLC leads to increase intracellular calcium, which is a key messenger to activate platelet function. All signaling pathways culminate in activated integrin α_{IIa}/β_3 receptor that binds fibrinogen. This final common pathway results in the crosslink between adjacent platelets and then ensues on platelet aggregation for arrest bleeding.

Nowadays, antiplatelet agents are used for treatment and prevention of cardiovascular disease. However, antiplatelet drugs still have limitations. Aspirin, a standard antiplatelet drug, has side effects, such as gastric ulcers, renal failure and bleeding. Thienopyridines including clopidrogrel are effective but occasionally cause bleeding and neutropenia with high cost (Barrett, 2008). A search of antiplatelet agents from medicinal plants is an attractive field. In this study, we are interested in *Angelica dahurica* (AD). The dried root of *Angelica dahurica* is used for treatment of headache, toothache, menstrual disorders, and neuralgia, in Chinese traditional medicine (Lee, 2011). In Thailand, AD is in the mixture of many Thai traditional medicines such as YA-HOM, which are used for treatment of circulatory disorder (บัญชียาจากสมุนไพรแห่งชาติ , 2554). However, previous studies of antiplatelet activity of AD have been limited. Among over 20 furanocoumarins isolated from this plant. Imperatorin exhibits biological activities such as anti-inflammation, anti-tumor, anti-convulsant, vasodilatation, antihypertension and prevention of myocardial hypertrophy, etc. Thus, the aims of this study were to investigate the antiplatelet activity and the mode of actions of the ethanolic extract from the root of *Angelica dahurica* and imperatorin which may act as an active compound of this plant.

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2. Objectives

2.1 To study the effects of the ethanolic extract from the root of *Angelica dahurica* (ADE) and imperatorin on platelet aggregation induced by ADP, arachidonic acid and collagen.

2.2.1 To study the modes of actions of ADE and imperatorin on platelet aggregation.

3. Hypothesis

The ethanolic extract from the root of *Angelica dahurica* and imperatorin have an antiplatelet activity. The mode of actions might be mediated *via* adynylate cyclase pathway.

4. Expected Benefits and Application

This study provides the antiplatelet activity of the ethanolic extract from the root of *Angelica dahurica* and imperatorin and theirs possible mechanisms of action.

CHAPTER II

LITERATURE REVIEW

1. Platelets

Platelets are the smallest type of blood cells in circulating blood. They have discoid shape, averaging 2-4 μm in diameter and 0.5 μm in thickness. Platelets are anucleate cells that derived from cytoplasm of megakaryocytes in bone marrow. Therefore, they lack of genomic DNA but contain megakaryocyte-derived messenger RNA for protein synthesis. The development of megakaryocyte and production of platelets are controlled by thrombopoietin, an important cytokine that is secreted from liver and spleen. After leaving the bone marrow, platelets circulate in blood for the lifespan of 9-10 days. Subsequently, macrophages will eliminate dead platelets by phagocytosis in liver and spleen. Platelets are produced approximately $35x10^9$ daily. Normal platelet counts are 150-450 x 10^9 platelets/liter and one-third of platelets are stored in spleen. Platelets play a pivotal role in hemostasis by surveying blood vessel wall for integrity and preventing blood loss after vascular injury.

2. Platelet structure and function

2.1 Platelet structure (White, 2007)

Platelet structure can be divided into three zones as follows.

2.1.1 Peripheral zone

Peripheral zone consists of surface membrane and the surface-connected open canalicular system. Platelet membrane provides adhesion area for cell-cell

interaction. It can be further divided into three distinct domains: the exterior coat, the unit membrane and the submembrane area.

The exterior coat is a 15-20 nm thick glycocaclyx. This domain is rich in glycoprotein receptors, for instance, glycoprotein (GP) Ia, Ib, Ic, IIb, IIIa, IV, V and XI. These receptors have an anionic charge, which make repulsive force on platelet surface. Therefore, platelets cannot attach with normal vascular surface or themselves in blood circulation. The unit membrane is a lipid bilayer, rich in phospholipids. This domain support strength of platelet shape. It contains anion and cation pumps, which helps to maintain transmembrane ionic gradients. It also provides catalyst for fluid-phase coagulation. The submembrane area are lining beneath the unit membrane. It contains microfilaments network associated with cytoplasmic filament system. This domain plays an important role in signaling events from all transmembrane receptors to cytoplasmic domains.

2.1.2 Sol-gel zone

Sol-gel zone is the matrix of the cytoplasm, which consists of coiled microtubule system, actomyosin microfilament system and dense tubular system. The coiled microtubule system helps platelets to maintain discoid shape. The microfilament system is involved in platelet change shape, internal transformation, contraction of the hemostatic plug and retraction of clots.

2.1.3 Organelle zone

Organelle zone contains mitochondria, peroxisomes and three types of secretory organelles including α granules, dense bodies (δ granules) and lysosomes. Dense granules contain adenosine diphosphate (ADP), 5-hydroxytryptamine (5HT) or serotonin, Ca^{2+}, Mg^{2+}, catecholamines, etc. These substances are secreted for amplifying

platelet activation. α Granules contain adhesion molecules, chemokines, coagulation factors and fibrinolytic proteins. Examples are thrombospondin, ATPase, von Willebrand factor (vWF), fibronectin, platelet factor 4 (PF4), plasminogen, β-thromboglobulin (β-TG), platelet derived growth factor (PDGF), coagulation factor (FV, FVIII). Lysosomes contain acid hydrolases which are secreted only after exposure to strong agonist, like thrombin.

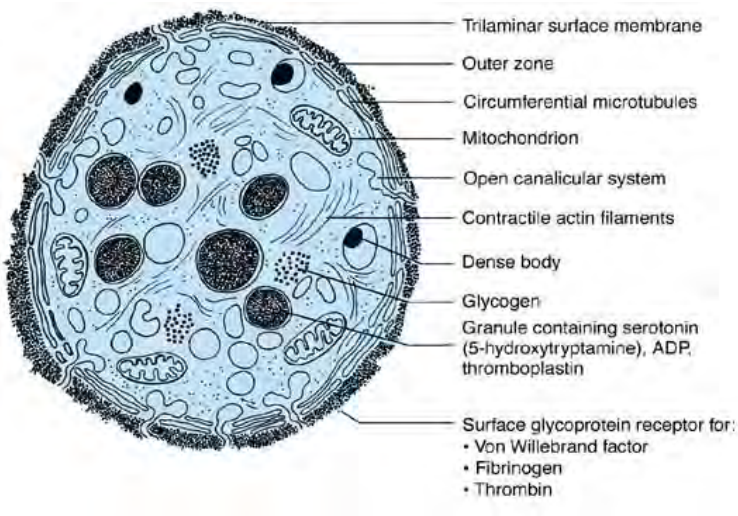


Figure 2.1 Structure of platelet (Chandrasoma, 1998 : online)

2.2 Platelet function

The primary role of platelets is prevention of blood loss after vascular injury. In normal state, platelets circulate in close proximity to vascular wall without interacting with one another or endothelial cells. This is due to endothelial production and secretion of nitric

oxide (NO), prostacyclin (PGI_2) and ecto-nucleosidase to inhibit platelet activation (Davi and Patrono, 2007).

When blood vessel injury occurs, this leads to formation of hemostatic plug. Initially, platelets exposed to subendothelial matrix that contacts vascular lumen, such as fibrillar collagen type I, III and VI, von Willebrand factor (vWF) and fibronectin, at the site of injured vessel. The response of platelets occurs rapidly by tethering along vessel wall and then adhering to collagen or vWF. This event is mediated by adhesive receptors on platelet that bind collagen and vWF firmly. After that, platelet activation leads to shape change from discoid shape to pseudopodia and release of substances from their storage granules *via* open canalicular system. Dense granules release soluble agonists such as ADP, 5-HT and ATP which help amplifying platelet activation. α granules release procoagulant substances to promote thrombin generation in coagulation cascade. After secretion within seconds, the released agonists will bind to its receptors on platelet membrane resulting in platelet activation *via* multiple downstream intracellular signaling. This is followed by an activation of phospholipase A_2 (PLA_2), the formation of thromboxane A_2 (TxB_2) and release. Platelet activation leads to increasing cytoplasmic calcium that promotes platelet shape change, secretion and aggregation. Calcium can triggers a conformation change of integrin $\alpha_{IIb}\beta_3$ or GP IIb/IIIa receptors on platelet membrane. The final event is platelet aggregation requiring an interaction between activated forms of integrin $\alpha_{IIb}\beta_3$ and fibrinogen. This allows multiple crosslink between adjacent platelets in platelet aggregates. The soluble agonists will further activate and recruit other platelets to form hemostatic plugs, so-called primary hemostasis. Thrombin, an end product from coagulation cascade, will cleave fibrinogen to form fibrin which can stabilize platelet plaque, so-called secondary hemostasis (Ruggeri, 2002).

3. Signaling event during platelet aggregation

Platelet possesses a variety of receptors on surface membrane (table 2.1).

Table2.1 Platelet agonists and its surface membrane receptors (Brass et al., 2007; Gibbins, 2004; Woulfe, 2005).

Agonist	**Source**	**Receptor**	**Effectors**
Collagen	Subendothelium component	GPIa/IIa or Integrin $\alpha_2\beta_1$	-*/ Adhesion
		GP VI	FcR_γ / $PLC_\gamma 2$
vWF	Subendothelium component, plasma protein	GPIb/V/IX complex and integrinα_{IIb}/β_3	-* / Adhesion
			- / adhesion
Fibrinogen	α granule and plasma	integrinα_{IIb}/β_3	- / adhesion
ADP	Dense granule	$P2Y_1$	G_q / PLC_β
		$P2Y_{12}$	G_{i2} / $\downarrow$ cAMP,PI3K
ATP	Dense granule	P2X1	
Epinephrine	Dense granule	α_{2A} adrenergic	G_z / $\downarrow$ cAMP
Serotonin	Dense granule	$5-HT_2$	G_q / PLC_β
Thrombin	End product of coagulation cascade	PAR 1	G_q / PLC_β , G_{13} / Rho
		PAR 4	G_q / PLC_β, G_{13} / Rho
TXA_2	Produced by arachidonic acid metabolism pathway	TP_α	G_q / PLC_β
		TP_β	G_{13} / Rho
PGI_2	Lipid mediator Endothelium cell	IP	G_S / $\uparrow$ cAMP

*Mechanism not clear. **Abbreviations:** ADP = adenosine diphosphate; ATP = adenosine triphosphate; cAMP = cyclic AMP; PAR = protease activated receptor; FcR_γ = Fc receptor γ-chain; GP = glycoprotein; 5-HT = 5-hydroxytryptamine; PLC = phospholipase; PGI_2 =

prostacyclin; PI3K = phosphatidylinositol 3-kinases; IP = prostacyclin receptor, TXA_2 = thromboxane A_2; TP = thromboxane receptor

Platelet aggregation is stimulated *via* agonist binding its specific receptor mediating intracellular signaling events. Initially, platelet becomes adhere to exposed subendothelium at the site of injured vessel wall by an interaction with platelet membrane receptors. This event requires adhesive receptors such as integrin $\alpha_2\beta_1$, GP VI and GPIbα-V-IX complex (figure 2.2). Integrin $\alpha_2\beta_1$ and GP VI bind directly to collagen, whereas GPIbα-V-IX complex requires cofactor vWF, which helps supporting platelet adhesion. GPVI receptor can activate platelet by stimulating tyrosine kinase activity, due to GPVI association with the Fc receptor γ-chain (FcRγ). GPVI receptors are clustered after stimulation by collagen. This results in the phosphorylation of FcRγ by tyrosine kinase in the Src family kinase, Lyn and Fyn. Then, the phosphorylation of the transmembrane adaptor protein LAT leads to the assembly of signaling proteins, such as phospholipase Cγ2 (PLCγ2). The activation of PLCγ2 hydrolyzes phosphotidylinositol (4, 5)-biphosphate (PIP2) to form two secondary messenger, inositol (1, 4, 5)-triphosphate (IP3) and diacylglycerol. IP3 is responsible for calcium mobilization from intracellular store, dense tubular system, raising the intracellular Ca^{2+} concentration and also activate Ca^{2+} influx across platelet membrane. DAG is responsible for activation of protein kinase C (PKC) isoforms. PKC can phosphorylate multiple proteins on serine and threonine residues, such as activate phospholipase A_2 (PLA_2). These signaling molecules trigger the cytoskeletal reorganization that allows change in discoid to pseudopodia shape, the secretion of ADP from dense granules and the production and release of TXA_2 (Giroux et al., 2009).

The release of soluble agonists is required for amplifying platelet response by recruiting additional circulating platelets (figure 2.2). Due to an interaction of collagen and platelet to form a monolayer at injured vessel lining is not sufficient to stop bleeding. Most of soluble agonists such as ADP and TXA_2 interact with G protein-coupled receptors (GPCRs) *via* one or more receptors on platelet membrane. G-proteins consist of three

different subunits, α, β and γ, and can be divided into four major families which are G_q, G_{12}, G_i and G_s. ADP, released from dense granule, is a weak agonist but play an important role in amplifying platelet response. ADP possesses two distinct purinergic receptors, denoted $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is coupled to G_q whereas $P2Y_{12}$ is coupled to a G_i family member. The $P2Y_1$ receptor coupled to G_q mediates signaling *via* activation of phospholipase C_β (PLC_β), which hydrolyzes membrane PIP2 to produce IP3 and DAG, as mentioned above. The $P2Y_{12}$ receptor coupled to G_i mediates signaling pathway that inhibits adenylate cyclase activity. Thus, this inhibits cyclic AMP formation resulting in decreased phosphorylation of vasodilator-stimulated phosphoprotein (VASP) which helps decreasing intracellular Ca^{2+} by restore it in dense tubular system. Inhibition of cAMP is also involved in phosphoinositide 3-kinases (PI3K) activation. PI3K mediates signals *via* stimulation of Akt phosphorylation in platelet. These lead to promotion of platelet aggregation. Both $P2Y_1$ and $P2Y_{12}$ receptors are required for performing full platelet aggregation stimulated by ADP. Furthermore, another purinergic receptor on platelet membrane is P2X1, which is an ATP-Ca^{2+}gated channel receptor. ATP releases from dense granule and binds to P2X1 receptor causing of calcium influx into platelet (Garcia et al., 2010).

Similar to ADP, TXA_2, a short half-lived lipid mediator, is produced and secreted from platelets as autocrine or paracrine to amplify platelet response. Arachidonic acid is a precursor for TXA_2 formation that is cleaved from membrane phospholipid by the activation of cytosolic PLA_2. Then, AA is metabolized by the enzyme activity of cyclooxygenase-1 (COX-1) and thromboxane synthase leading to the formation of TXA_2. This agonist binds to its GPCRs on platelet membrane known as thromboxane receptor. There are two splice variants for TP receptor, TPα and TPβ. Both TP receptor subtypes mediate predominantly through G_q and $G_{12/13}$. G_q activates PLC_β to increase intracellular Ca^{2+} and stimulates PKC, while $G_{12/13}$ mediates Rho/Rho-kinase pathway, which activates RhoA, and then phosphorylates myosin light chain participating in cytoskeletal reorganization during platelet shape change.

Among agonists that activate platelet, thrombin is the most potent activator. Thrombin is the end product from coagulation cascade, which is mediated *via* protease-activated receptors (PARs) on platelet membrane. There are two PARs expressed on human platelet membrane, PAR1 and PAR4, that couple to G_q and G_{12}/G_{13}. Both receptor-mediated intracellular signals converge with responses to other agonists, which signal through the same G proteins. Thrombin can increase intracellular Ca^{2+} concentration to a 10-fold over baseline, exceed 1 μM, within seconds. Basal Ca^{2+} concentration level is approximately 100 nM. Furthermore, PAR1 can activate platelet response induced by thrombin at low concentration (approximately 0.1nM), while PAR4 contributes at high concentration (Woulfe, 2005).

Others agonists, such as epinephrine and serotonin, are weak agonists that potentiate other agonists. Epinephrine mediates signal *via* α_{2A} adrenergic receptor, which couple to G_z, a G_i family member. This receptor is similar to $P2Y_{12}$ ADP receptor that inhibits cAMP activity but does not increases intracellular Ca^{2+} and platelet shape change like other agonists. Serotonin, or 5-Hydoxytryptamine (5-HT), is taken up by platelets and stored in dense granule. 5-HT mediates signals *via* $5\text{-}HT_{2A}$ receptor which is G_q-coupled.

All signaling pathways stimulate released intracellular calcium from internal store or calcium influx. Subsequently, calcium, a key messenger, activates other signaling protein including protein kinase A (PKA) in order to produce TXA_2 and secrete soluble agonists to activate additional platelets. The activation of integrin $\alpha_{IIb}\beta_3$ receptor is the final common pathway stimulated by all agonists. Thus, activated integrin $\alpha_{IIb}\beta_3$ receptors can bind fibrinogen and vWF to form platelet plug but it is not stable. The final step requires thrombin, which is not only an activator of platelets but also cleave fibrinogen to form fibrin that can help stabilizing platelet plugs by clot retraction (Krotz et al., 2008).

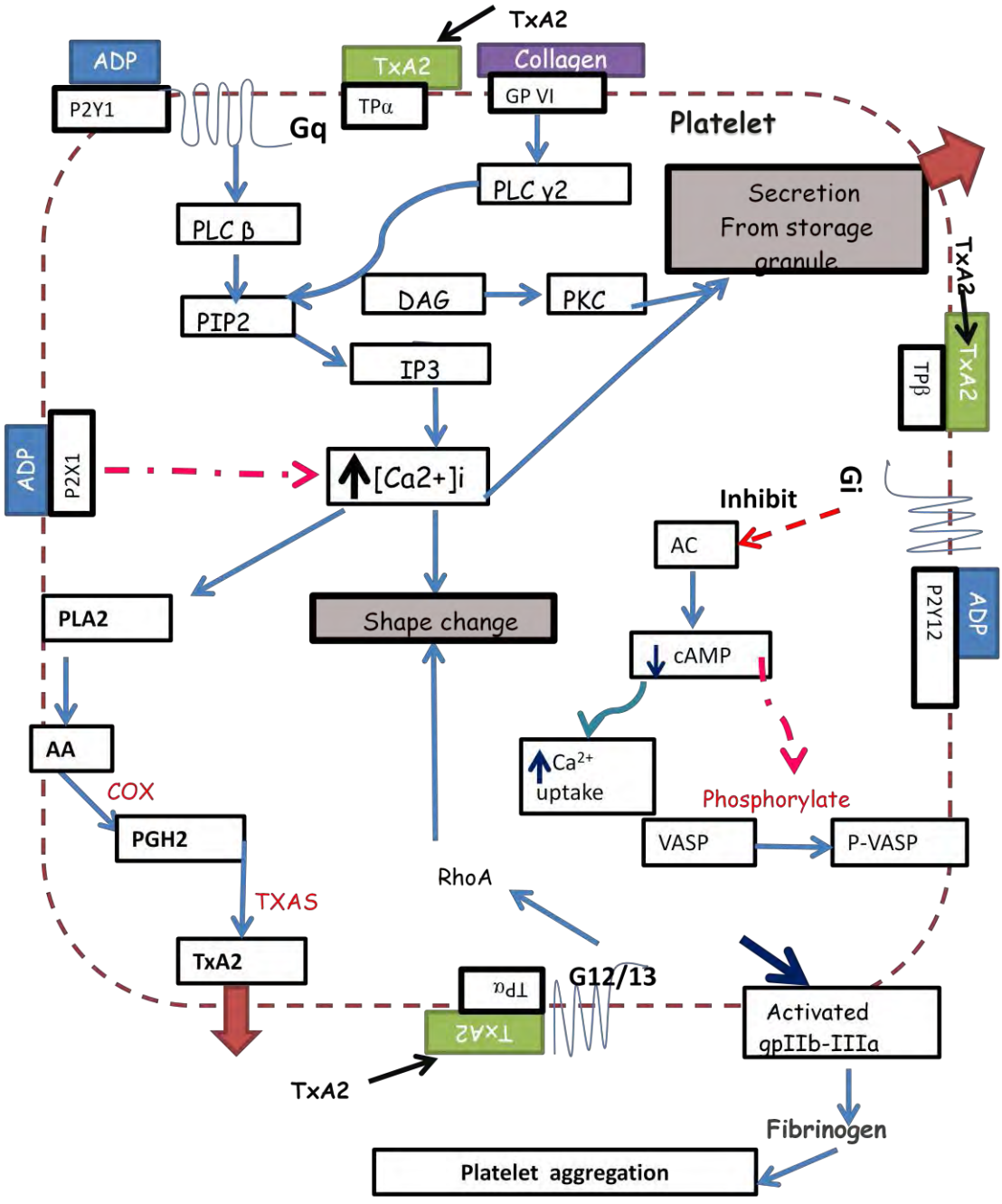


Figure 2.2 Signaling events of platelet activation by ADP, arachidonic acid and collagen in human platelet (Xiang et al., 2008).

4. Inhibition of platelet function by the endothelium

In order to maintain normal hemostasis, the balance between prothrombotic and antithrombotic are required for prevention pathological state. The endothelium plays an important role in this part by limitation of platelet plugs, thrombus sizes or platelet reactivity. The endothelium contains three thromboregulators such as NO, PGI_2 and ecto nucleosidase CD39 pathway. Free radical, NO, can induce vasodilation. NO is synthesized through the conversion of L-arginine to citrulline. Then it is catalyzed by endothelial nitric oxide synthase (eNOS). NO is released into blood circulation, and then, across plasma membrane to binds and activates soluble guanylyl cyclase (sGC) in cytoplasm. This leads to the increasing of cyclic guanosine 3´, 5´-monophosphate (cGMP). Subsequently, protein kinase G (PKG) is activated. The subsequence of this leads to increase in cAMP and thereby inhibits platelet aggregation. Similar to NO, PGI_2, is a potent inhibitor of platelet activity. AA is a precursor for PGI_2 and is converted by COX and prostacyclin synthase to form PGI_2 in vascular endothelium, and then released into bloodstream. After released, PGI_2 binds to the prostacyclin receptor on platelet membrane. PGI_2, coupled to G_s, mediates signaling *via* activation of adenylate cyclase, which increases cyclic adenosine 3´, 5´-monophosphate (cAMP) and leads to activation of PKA. PKA causes the phosphorylation of several proteins, such as myosin light-chain kinase (MLCK), IP3 receptor and VASP Consequently, PGI_2 leads to the inhibition of Rho – kinase mediates MLCK activation, decreasing granule secretion, inhibition of PKC activation, decreasing intracellular calcium and inhibition of integrin $\alpha_{IIb}\beta_3$ activation. Both NO and PGI_2 have short half life. Ecto – nucleosidase or CD 39 found on variety of cells including endothelial cells and platelets. CD 39 is able to inhibit platelet activation by hydrolysis from ADP and ATP, which are released from activated platelets, to adenosine monophosphate (AMP). Consequently, CD 39 prevents further excessive platelet recruitments (Jones et al., 2012).

5. *Angelica dahurica* (Fisch. Ex Hoffm.) Benth. & Hook.f. ex Franch & Sav

Family	: Umbelliferae
Synonym	: *Callisace dahurica* Franch & Sav., *Angelica macrocarpa* H. Wolff, *Angelica porphyrocaulis* Nakai & Kitag., *Angelica tshiliensis* H. Wolff
Chinese name	: Bai Zhi
Common name	: Dahurian angelica

Angelica dahurica (AD) is a perennial herb that grows to 1-2.5 meters in height. The root has a brown cylindrical shape with 2 - 5 cm in diameter and approximately 30 cm in length. The stem is purplish – green in color with 2 – 8 cm in diameter. The leaf is tripinnate with 40 cm in width and 50 cm in length. Its leaf and root have special fragrance. It has flower approximately in July to August and is ripen in August to September. This plant distributes in Northeastern China, Japan, Korea, Taiwan and Far East Russia. This plant thrives in moist and shady environments, such as near riverside, on high mountain or edge of forest (ชยันต์และวิเชียร, 2548). Bai Zhi or Kot – Sor, named in Thailand, is the root of AD which has been widely used in traditional medicine. Chinese traditional medicine uses Bai Zhi as antipyretic, analgesic for cold, toothache and headache (Ketai et al., 2001). In Thailand, YA-HOM, a mixture of many herbs including the root of AD, is used for treatment of circulatory disorder. In addition, it should be aware of bleeding when taking this Thai traditional medicine with anticoagulants or antiplatelet (บัญชียาจากสมุนไพรแห่งชาติ, 2554).

The chemical constituents of AD are a group of volatile oils, glycosides and coumarins, of which furanocoumarins are major active constituents. Furanocoumarin is able to increase skin sensitivity to sunlight (Wei and Ito, 2006). Thus, furanocoumarins are not widely used for skin treatment. There are more than 20 furanocoumarins found in this plant including 5-methoxy-8-hydroxypsoralen, bergapten, byakangelicin, imperatorin, isoimperatorin, isopimpinellin, knidilin, osthenol, oxypeucedanin methanolate, oxypeucedanin hydrate, phellopterin, scopoletin, xanthotoxol, etc. (Baek et al., 2000; Kang et al., 2008; Ketai, Huitao et al., 2001; Zhang et al., 2009; Zheng et al., 2010)

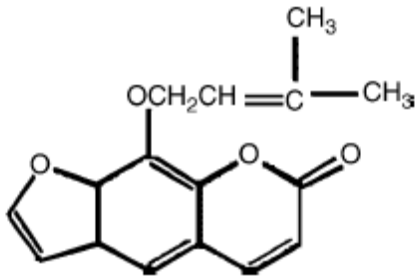


Figure 2.3 Chemical structure of imperatorin (Li, 2006)

5.1 Biological activity

There are several previously reported biological activities of the root of AD as follows.

5.1.1 Antimicrobial activity and anti-staphylococcal activity

Coumarins isolated from the ethyl acetate extracts of the dried root of AD had antimicrobial activity against bacteria and fungi. Isoimperatorin (62.5 μM/ml) inhibited

growth of *Bacillus subtilis* and byakangelicin (62.5 μM/ml) also inhibited growth of *Aspergillus candidus* (Kwon et al., 1997). Hexane extracts of the dried root of AD at the concentrations of 6.57 and 2.05 g were screened for antibacterial activity and was found that 6.57 g of this extract to be active against bacteria. Falcarindiol, which was isolated from 6.57 g of this extract, had the minimum inhibitory concentrations against multidrug resistance (MDR) strains of *Staphylococcus aureus* ranged from 8 to 32 μg/ml (Lechner et al., 2004).

5.1.2 Anti-inflammation, anti-histamine and anti-sepsis

Furanocoumarins isolated from the root of AD, including byakangelicin, phellopterin, imperatorin, isoimperatorin and oxypeucedanin methanolate, could inhibit lipopolysaccharide (LPS) induced prostaglandin E_2 (PGE_2) production in rat peritoneal macrophage. Among these compounds, imperatorin (3-30 μM) showed the most potent inhibition by suppression of cyclooxygenase (COX-2) expression and microsomal prostaglandin (PG) synthase expression (Ban et al., 2003). On the mechanism study, ethyl acetate of ADE (50 and 100 μg/ml) inhibited LPS-induced tumor necrosis factor -α (TNF-α), NO and PGE_2 in murine macrophage, RAW264.7 cells, as well as decreased inducible nitric oxide synthase (iNOS) and COX-2 expression. These inhibitory effects might mediate by the inhibition of translocation of NF- KB, a transcription factor, into nucleus and prevention its inhibitory protein I-KB degradation (Kang et al., 2007).

70% ethanolic ADE at concentrations of 25-200 μg/ml suppressed iNOS and COX-2 expression in LPS-stimulated macrophage leading to the inhibition of COX-2-derived PGE_2 and iNOS-derived NO production. ADE (25-200 μg/ml) also inhibited inflammatory mediators, such as NO, TNF – α, interleukin 6 (IL-6) and PGE_2 production in RAW264.7. ADE (200 μg/ml) showed the inhibition of I-KB/NF- KB activity (Lee et al., 2011). These inhibitory effects might act *via* the suppression of the NF-KB pathway.

In addition, byakangelicol (10-50 μM), isolated from the dried root of AD, inhibited COX-2 expression and PGE_2 released induced by interleukin-1β (IL-1β) in human pulmonary cell lines, A549. However, byakangelicol did not inhibit the activity and expression of COX-1 at concentrations up to 200 μM (Lin et al., 2002). 5-methoxy-8-(2-hydroxy-3-buthoxy-3-methylbutyloxy)-psoralen (MP), isolated of this plant, also mediated anti-inflammatory activity by inhibition of both cyclooxygenase-2 and 5-lipoxygenase, at concentrations ranged from 32 to 72 μM, and leukotriene $C_{4,}$ at concentrations ranged from 2 to 16 μM in a dose dependent manner (Hua et al., 2008).

Histamine is one of the important chemical mediators in allergic reaction. It is released from mast cells or basophils. Bergapten, oxypeucedanin hydrate, and byakangelicin, isolated from AD, at the concentrations of 25 mg/kg could inhibit histamine release in the peritoneal cavity fluid induced by compound 48/80 in male mice from the in vivo study (Kimura and Okuda, 1997).

Song and colleague (2005) demonstrated that ethyl acetate fraction of the root of AD (30 mg/ml) could prevent lethality from sepsis induced by LPS and D-gactosamine (D-GaIN) in mice. Increased survival rate was a result from decreasing TNF – α level in plasma from macrophage. In addition, it also prevented liver damage by reducing alanine aminotransferase in LPS/D-GaIN induced sepsis (Song et al., 2005).

5.1.3 Liver protective activity

In the screening for hepatoprotective agents, it was found that methanolic extract from AD could protect hepatotoxicity induced by tacrine, an acetylcholinesterase inhibitor, in Hep G2 cells. Isolation of compounds from this extract found that imperatorin (36.6 μM) and byakangelicin (47.9 μM) exhibited strong hepatoprotective activity (Oh et al., 2002).

5.1.4 Tyrosinase inhibition and melanogenesis activity

Tyrosinase catalyzes tyrosine to 3, 4-dihydroxyphenylalanine (L-DOPA), which is used for the formation of melanin. Melanin is used for the color of mammalian skin. Consequently, an agent that inhibits tyrosinase activity may be used for skin whitening agent. In search of a new skin whitening agent, it was found that 9-hydroxy-4-methoxypsoralen, isolated from the ethyl acetate extract of AD, showed a potent inhibitory activity against mushroom tyrosinase activity. The half maximal inhibitory concentration (IC_{50}) of 9-hydroxy-4-methoxypsoralen was 2 μg/ml inhibition of tyrosinase activity (Piao et al., 2004a). Similarly, 25 μg/ml of the ethanolic extract of the dried root of AD had no effect on melanogenesis (Matsuda et al., 2005).

5.1.5 Anti-proliferation and anti-tumor activity

On the screening research for anti-tumor activity, it was found that six furanocoumarins, isolated from the hexane soluble part of the methanol extracts of AD, exhibited cytotoxicity against various cultured tumor cell lines, such as non small cell lung, ovary, melanoma, central nervous system and colon tumor cells. All six compounds exhibited cytotoxicity against five tumor cell lines after exposure to each of tumor cell lines for 48 hours, which had the half maximal inhibitory concentrations (IC_{50}) as follows. Isoimperatorin had IC_{50} ranged from 5 to 12 μg/ml. Cnidicin had the IC_{50}, ranged from 6.8 to 7 μg/ml. Imperatorin had the IC_{50}, ranged from 12.3 to 19.4 μg/ml. Oxypeucedanin had the IC_{50}, ranged from 3.4 to 19.8 μg/ml. Byakangelicol had the IC_{50}, ranged from 14.3 to 28 μg/ml. Oxypeucedanin hydrate had the IC_{50}, more than 30 μg/ml (Kim et al., 2007).

Pae et al. (2002) studied effects of imperatorin isolated from dried root of AD on apoptosis in human promyelocytic leukemia (HL-60 cells). The study demonstrated that imperatorin was able to induce apoptosis in HL-60 cells at concentrations of 5 and 10 μM after 48 h incubation. Its mechanism might act *via* cytochrome C dependent pathway by

activating caspase-9 and caspase-3 and also cleaving poly (ADP-ribose) polymerase (Pae et al., 2002). While Luo et al. (2011) demonstrated another underlying mechanism that imperatorin (90 μM) could inhibit caspase–8 and its effect was suppressed by caspase-8 inhibitor in human cancer cell lines, HepG2 cells. Thus, its mode of action acts through both receptor and mitochondria pathway, which are the major apoptotic pathway (Luo et al., 2011).

5.1.6 Central nervous system and Anti-dementia activity

During screening medicinal plants for activity on central nervous system (CNS), it was found that 8 furanocoumarins isolated from the methanol extract of dried root of AD had affinity to brain benzodiazepine receptors in vitro. Phellopterin (IC_{50} = 0.36 μM) inhibited on the binding [^{3}H] diazepam to CNS benzodiazepine receptors in vitro (Bergendorff et al., 1997).

There is one report about effects of AD as an anti-dementia agent. Marumoto and Miyasawa (2010) found that the chloroform extract of dried root of AD could inhibit β-secretase 1 (BACE1). Aspartic – acid protease help forming myelin sheaths and also inhibiting amyloid plaques, which is related to Alzeimer disease. Among coumarins that isolated from this chloroform extract, imperatorin (91.8 μM) and byakangelicin (104.9 μM) showed the inhibitory effects against β-secretase (Marumoto and Miyazawa, 2010).

5.1.7 Antioxidant activity

Piao and his colleagues (2004) studied the methanol following methylene chloride extract of the root of AD. They found that 9-hydroxy-4-methoxypsoralen and alloisoimperatorin at the concentration 200 μg/ml, among 11 furanocoumarins isolated from this plant, showed antioxidant effects against DPPH free radical and protected renal

epithelial cell damage induced by 2,2 -azobis (2-aminodinopropane) dihydrochloride (AAPH) (Piao et al., 2004b).

Xu et al. (2011) studied polysaccharides and its fractions from the root of AD. The study demonstrated that the antioxidant activity of the fractions, isolated from the polysaccharides extracts, at the concentrations approximately 250 μg/ml with inhibitory effects on different parameters, such as malondialdehyde (MDA) production, the ferrous ion ($Fe2^{+}$) chelation, and the $HO^{\cdot}$ radical-scavenging. The study suggested that the chemical composition contributed to their antioxidant activity in polysaccharides extracts (Xu et al., 2011).

5.1.8 Vasodilation and cardioprotective activity

Nie and his colleagues (2009) showed that cyclohexane extract (IC_{50} = 40.5 mg/ml) from dried root of AD had potent vasodilatory activity on mouse thoracic aorta. The mode of action of this plant involves at least NO system without effecting β adrenergic receptor on vascular smooth muscle cells. From HPLC analysis, this extract contains highest imperatorin and isoimperatorin compared with other extract consistent with the finding that imperatorin exhibited 4 time stronger activity than isoimperatorin. Thus, imperatorin might be an active compound with vasodilatory activity (Nie et al., 2009). According to the other study, imperatorin exhibited a potent vasodilatory effect. Imperatorin (1 μM-1 mM) relaxed vasoconstriction at concentrations dependent manner with or without endothelium. Imperatorin, at concentrations of 10 and 30 μM, were shifted parallel rightward of the concentration-response curves of 5-HT. The vasodilatory effects of imperatorin possible involved the inhibition of voltage dependent calcium channels and receptor-mediated calcium influx and inhibition of releases. However, its mechanism might not involve the inhibition of store operated calcium channels (SOCCs) (He et al., 2007; Zhang et al., 2010).

Oxypeucedanin at concentrations 0.1 - 1 μM, isolated from root of AD, prolonged cardiac action potential by blocking human voltage – gated K^+ (hKv) 1.5, one of eight subclasses of Kv channels. The Kv channels play an important role in regulating action potential duration contributed by repolarization. The hKv 1.5 gene is predominantly expressed in atrium. Thus, hKv 1.5 blocking resulted in prolongation of atrial action potential, which had a benefit for treatment of atrial fibrillation (Eun et al., 2005). Besides, Zhang et al. (2011) recently reported that imperatorin (25mg/kg) also acted as a hypotensive agent in spontaneous hypertensive rats (SHRs) model by blocking L-type calcium channels on vascular smooth muscle cells (Zhang et al., 2011).

Aside from these biological activities, there is only one study related to the effect from the root of AD on hemostasis. From the study, it reveals that the methanolic extract of this plant has antithrombotic activity in the mouse model of thrombosis. It was found that the butanol fraction of this extract at the concentration of 500 mg/kg could increase survival rate by 35% (Chen et al., 1996).

More than 20 furanocoumarins isolated from the root of AD. Imperatorin, isoimperatorin, oxypeucedanin, and scopoletin have been widely studied for their biological activities and are considered as reference standards in the quality control (Nie et al., 2009). Among these compounds, imperatorin (figure 2.3) at the concentration of 300 mg/mg, given by single injection into intraperitoneal in mice, had anti-convulsant against electroshock-induced seizures (Luszczki et al., 2010). Imperatorin had been extensively studied and showed various biological activities, such as anti-inflammation (Ban, Lim et al., 2003), anti-hypertension (Zhang, et al., 2011), potent vasodilation (He, Zhang et al., 2007), potent antiproliferative and anti-tumor (Kawaii et al., 2001) as mentioned above. In addition, other biological activities of imperatorin were to inhibition of HIV-1 replication (Sancho et al., 2004). Imperatorin at the concentrations of 50 and 100 μM inhibited of intercellular adhesion-molecule -1 (ICAM-1) in a macrophage-derived foam cells (Yang et al., 2002). Imperatorin inhibited myocardial hypertrophy in mice that treated with imperatorin 15 mg

$kg^{-1}d^{-1}$, by a NO-mediated pathway (Zhang et al., 2012). Although little is known on the effect of imperatorin on platelet, there was a study in search for antiplatelet activity of coumarins. The study demonstrated that imperatorin isolated from *Peucedanum Japonicum*, which was in the same family as AD, had antiplatelet aggregation activity. imperatorin at the concentration of 100 μg/ml inhibited AA- and collagen-induced platelet aggregation in washed rabbit platelets (Chen, et al., 1996). Consequent of these effects, imperatorin might be an active gradient of AD.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Chemicals

All chemicals were obtained commercially and used without further purification. The following chemicals were purchased from Sigma Chemical Co., St Louis, U.S.A.: Adenosine diphosphate (ADP), apyrase grade VII, arachidonic acid, bovine serum albumin (BSA), brilliant cresyl blue, calcium chloride, citric acid, collagen solution type I, dimethyl sulfoxide (DMSO), ethylene diamine tetraacetic acid (EDTA), fibrinogen, d-glucose, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), heparin, imperatorin, magnesium chloride, potassium chloride, potassium hydrogen phosphate, prostacyclin I_2, sodium chloride, sodium hydrogen phosphate, sigma coat, trichloroacetic acid (TCA), trisodium citrate.

Other chemicals were purchased from commercial sources as follows: diethyl ether from Lab Scan Co., Ltd Thailand. Cyclic AMP enzyme immuno assay kit from Cayman chemical company Michica America, TxB_2 biotrak enzyme immuno assay (EIA) kit from GE healthcare, UK.

All other chemicals and reagents were obtained from commercial sources with analytical grade available.

ADE was supported by Assoc. Prof. Uthai Sotanaphan; Pharmacognosy, Faculty of Pharmacy, Silpakorn University. The extracts were dissolved in DMSO for stock solution and diluted in normal saline before uses in the study.

1.2 Instruments

1. Aggregometer Aggram™, Helena laboratory, USA

2. Microplate reader Wallac model 1420, PerkinElmer Ltd.,USA

3. Table Top Refrigerated Centrifuge Rotina 380 model, Andreas Hettich GmbH & Co. KG, Germany

4. pH meter CG842 Schott, Scientific Promotion, co., Ltd., Japan

5. Ultrasonic bath bandelin, Bandelin GmbH & Co. KG, Germany

6. Water bath memmert, Chatcharee Holding co.,Ltd., Thailand

2. Methods

2.1 Blood collection

Blood was drawn from healthy volunteers, aged from 18 to 55 years, who did not take any medicines and vitamin supplements for least 14 days before participation in the study. All volunteers provided informed consent, and the study was approved by the Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Whole blood was collected using a 21G needle and rapidly transferred to a 50 ml polystyrene tube containing 3.2% sodium citrate (1 ml: 9 ml blood) to avoid coagulation and mixed thoroughly.

2.2 Platelet preparation

25-30 ml of blood was collected into a 50 ml polystyrene tube containing 3.2% sodium citrate and was rested for 10 min (figure 3.1). Then, a blood sample was

centrifuged at 200 x *g* for 10 min at 21ºC. The supernatant was collected by a plastic pipette into 15 ml polystyrene tube as platelet rich plasma (PRP). Remaining blood was respun at 1,500 x *g* for 15 min at 21ºC and the supernatant was collected by a plastic pipette as platelet poor plasma (PPP). PRP must be use within 4 hours after blood draws in each experiment. PPP was used as a blank for platelet aggregation study.

2.3 Preparation of washed platelets

To prepare washed platelets (figure 3.2), blood was collected into acid/citrate/glucose (ACD) solution (85 mM sodium citrate, 78 mM citric acid, and 11 mM d-glucose; blood: ACD 6:1 v/v). Then, samples were centrifuged at 1,000 x *g*, 8 min at 21ºC. After centrifugation, the supernatant (PRP) was supplemented with PGI_2 (0.5 μM) and heparin (10 U/ml) and then centrifuged at 1,000 x *g*. Platelet pellets were resuspended in Tyrode-HEPES buffer (136 mm NaCl, 5 mm Hepes, 2.7 mm KCl, 2 mm $MgCl_2$, 0.42 mm Na_2HPO_4, 5 mm glucose, and 3.5 mg/ml BSA). Platelet suspensions were supplemented with PGI_2 (0.5 μM) and apyrase (0.02 U /ml) and centrifuged again at 1,000 x g, 8 min at 21ºC. The washed platelets were finally suspended in Tyrode-HEPES's solution containing 1 mM $CaCl^{2+}$.

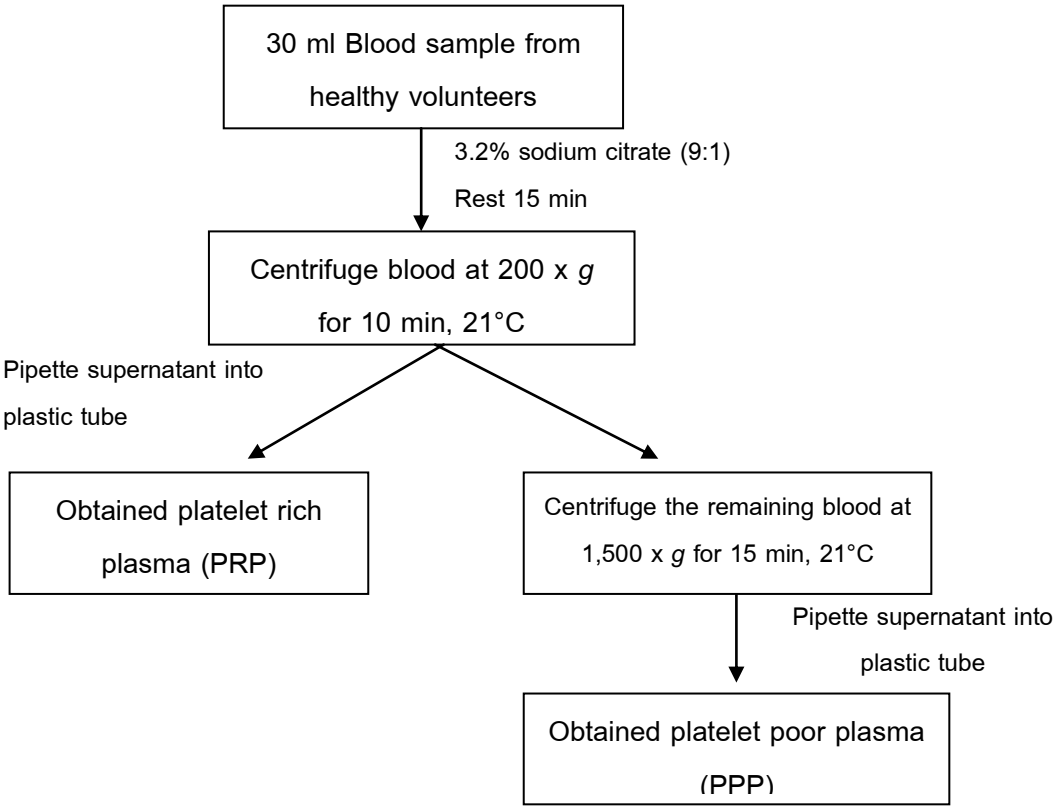


Figure 3.1 Preparation of platelet rich plasma isolated from blood by centrifugation

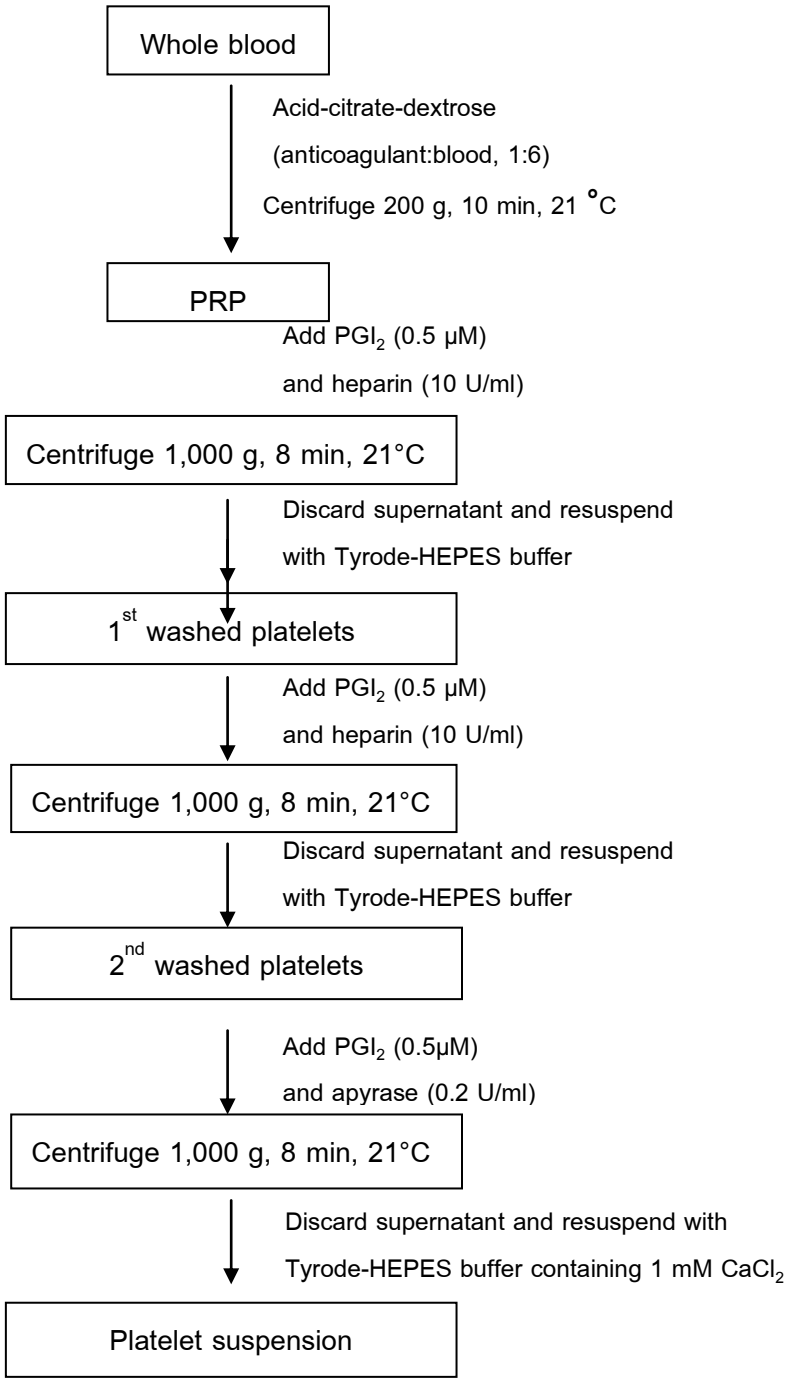


Figure 3.2 Preparation of washed platelets by centrifugation

3. Experimental procedure

3.1 Platelet aggregation study (Dutta-Roy et al., 1999)

Platelet aggregation was studied by the turbidimetric method (Born and Cross, 1963). PRP (200 µl) was incubated at 37 °C in the aggregometer for 1 min under continuous stirring with the speed of 600 rpm and measured its turbidity as baseline (figure 3.3). Then, ADE at concentrations of 0.1, 0.25, 0.5 and 1 mg/ml, imperatorin at concentrations of 10 (2.7 µg/ml), 25 (6.8 µg/ml), 37.5 (10.12 µg/ml), 75 (20.25 µg/ml), 150 (40.5 µg/ml) and 300 µM (81.1 µg/ml) or the vehicle (0.5% DMSO) were added to PRP before the addition of the aggregating agents and were incubated for 3 min. Acetylsalicylic acid (ASA) at the concentration of 500 µM (90 µg/ml) was used as a positive control. DMSO, which was used as a vehicle control, did not have antiplatelet aggregation activity. Platelet aggregation was induced by ADP at the concentration of 5 or 10 µM (2.1 or 4.2 µg/ml, respectively), AA at the concentration of 0.4 or 0.8 mM (120 or 240 µg/ml, respectively) or collagen at the concentration of 30 µg/ml. For ADP-induced platelet aggregation, the concentration of ADP either 5 or 10 µM that induced maximum aggregation was selected for the further experiments. The following parameters of aggregation tracings were analyzed as: the maximum aggregation, the slope of aggregation tracings and the lag-time. The maximum aggregation expressed in percentage of light transmittance, as an index of rate of aggregation which is expressed in millimeters/minute (mm./min). The slope of aggregation tracings determined the speed of platelet aggregation. The lag-time or onset of aggregation is the delaying time for aggregation after addition of collagen agonist, which is expressed in unit of seconds. Measurement changes of light transmittance were recorded for 6 min. Values are presented as a percentage of maximum aggregation compared with the control. The percentage of inhibition of platelet aggregation was calculated using the following equation.

$$\% \text{ Inhibition} = [(A-B) \div A] \times 100$$

A is the maximum aggregation rate of vehicle-treated PRP, whereas B is the maximum aggregation of sample-treated PRP.

3.2 Antagonism on ADP induced platelet aggregation (Jin et al., 2005)

The log concentration – response relationship of ADP was determined in this study. The percentages of platelet aggregation were increased in a dose dependent manner. The action of antagonists is to prevent agonists from activating receptors. So, sufficiently high concentrations of ADP can surmount the effect of a given concentration of the antagonist; that is, the maximum platelet aggregation affects for ADP remains the same for any fixed concentration of antagonist. If the antagonism is competitive, the presence of antagonist increases the agonist concentration required for a given degree of response, and so the agonist concentration-effect curve is shifted to the right. In order to investigate the competitive antagonism of ADE and imperatorin on ADP-induced platelet aggregation, ADE or imperatorin at a fixed concentration was added for 3 min before the initiation of platelet aggregation by increase the concentrations of ADP. Analysis of the relationship between increasing the concentrations of ADP and percentage of platelet aggregation in the presence of antagonist was detemined. Data were represented as graphical presentation of concentration – response curve. Simillar to platelet aggregation study, the process of study was followed.

PRP (200 µl) was incubated at 37 °C in the aggregometer for 1 min under continuous stirring with the speed of 600 rpm and measured as baseline (figure 3.4). Then, ADE was used at concentrations of 0.42 and 0.84 mg/ml, which were IC_{25} and IC_{50}, respectively. Imperatorin at the concentration of 150 µM (40.5 µg/ml), ASA at the concentration of 250 µM (45 µg/ml) or the vehicle (0.5% DMSO) were added to PRP before

the addition of ADP and were incubated for 3 min. Acetylsalicylic acid (ASA) was used as a positive control. Platelet aggregation was induced by ADP at concentrations of 1, 2.5, 5, 10, 20, 40 and 80 μM or 0.525, 1.05, 2.1, 4.2, 8.4, 16.8 and 33.6 μg/ml. The results were measured as the changes in light transmission and were recorded for 6 min. Values are presented as a percentage of maximum aggregation compared with the control. Then, data were plot between concentration of ADP and percent platelet aggregation of pretreatment PRP with 0.5%DMSO, ASA, ADE and imperatorin. Usually, an axis of concentrations of ADP displayed as a log scale.

3.3 Measurement of cAMP level (Lazarus and Garg, 2004)

Adenosine 3', 5' cyclic monophosphase or cAMP is a ubiquitous cellular secondary messenger, which is a component of a signaling pathway linking to the internal cellular enzymatic activity. cAMP is synthesized from ATP by membrane-bound adenylate cyclase and is neutralized by hydrolysis of cAMP to AMP by phosphodiesterases. In this experiment, the cAMP level was determined using competitive enzyme immune assay kit. The assay is based on a competition between free cAMP and cAMP-acetylcholinesterase (AChE) conjugate (cAMP tracer) for a limited number of cAMP-specific rabbit antibody binding sites. The amount of cAMP tracer that was able to bind to the rabbit antibody would be inversely proportional to the concentration of cAMP in the well. After adding substrate to AChE, the product would be developed showing a yellow color. The intensity of this color was inversely proportional to the amount of free cAMP present in the well.

PRP (450μl) was incubated with ADE at concentrations of 0.1, 0.25, 0.5 and 1 mg/ml or imperatorin at concentrations of 10, 75, 150 and 300 μM (2.7, 20.25, 40.5 and 81.1 μg/ml, respectively) for 3 min at 37°C with continuous stirring at speed 1,200 rpm before adding ADP (figure 3.5). A phosphodiesterase inhibitor, 3-Isobutyl-1-methylxanthine

(IBMX), was used as a positive control at the concentration of 200 μM or 44.5 μg/ml. Six minutes after the addition of ADP; the reaction was stopped with 0.5% ice-cold TCA. The samples were vortexed for 10 sec, sonicated for 1 min, and centrifuged at 10,000 x *g* for 10 min. After that, the supernatant were transferred to 1.5 ml eppendorf and 100 μL of 1 M HCl was added. Subsequently, the supernatant were washed 10 times with 750 μL of diethyl ether. The solvent was removed under a stream of nitrogen and the samples were stored at -80°C until analysis.

Prior to analysis, the samples were reconstituted in 100 μL assay buffer (figure 3.6). Blank, total activity (TA), non-specific binding (NSB), maximum binding (B_0) and cAMP standards were measured as controls. The cAMP standards were prepared in 8 concentrations, which are 0.3, 1.0, 3.1, 9.3, 27.8, 83.3, 250 and 750 μL, respectively. For NSB, assay buffer and cAMP tracer were added into the well. For samples and standard, cAMP tracer and antibody were added into the well, respectively. For B_0, assay buffer were added instead of sample into the well. Then, all samples and controls were incubated overnight at 4 ºC. After that, all wells were washed with washing buffer for 5 times. Then Ellman's reagent, which is substrate for AChE, was added to every well. The plate was covered and incubated for 120 min with gentle shaking. The yellow color was developed and the plate was read under the microplate reader at the wavelength of 450 nm.

3.4 Measurement of thromboxane B_2 (TxB_2) level (Liu et al., 2009)

TxA_2 has a short half life of 37 seconds before it is metabolized to the stable TxB_2. Therefore, this study measured TxB_2 levels instead.

Washed platelets (200 μL) were incubated at 37ºC with ADE at concentrations of 0.1, 0.25, 0.5 and 1 mg/ml or with aspirin at 500 μM or (90 μg/ml) for 3 min before adding ADP (figure 3.7). Six minutes after adding ADP, the reactions were stopped by

adding 2 mM EGTA with 50 μM indomethacin. Then, the samples were centrifuged at 12,000x*g* for 3 min and the supernatant was collected for TxB_2 measurement using enzyme immune assay (EIA) kit.

The EIA kit was based on competitive binding method. The samples were diluted with assay buffer (0.1M phosphate buffer pH 7.5 containing 0.9% sodium chloride, 0.1% bovine serum albumin) at 1:10 dilution factor. For control measurements, blank, total activity (TA), non-specific binding (NSB), maximum binding (B_o) and standards were measured. The TxB_2 standards were prepared at 8 concentrations, which are 0, 0.5, 1, 2, 4, 8, 16, 32 and 64 pg per well. For NSB and B_o, assay buffer was added for 100 and 50 μL, respectively. Samples (50 μL) were added into well and, then, antiserum (50 μL) was added to all wells except NSB and blank wells. After that, all wells except the blank were added with 50 μL of peroxidase conjugate. Then, the plate was incubated at 15 – 30 ºC for 1 hour with gentle shaking. The plate was washed with washing buffer (0.01 M phosphate buffer pH 7.5 containing 0.05% Tween 20) 4 times and, then the substrate (150 μL), tetramethylbenzidine, was added to all wells. All wells were incubated for exactly 15 min with gentle shaking before stopping reaction by adding 1 M sulfuric acid (H_2SO_4). After the reaction stopped, yellow color was developed and light absorbance was measured at 450 nm using a microplate reader.

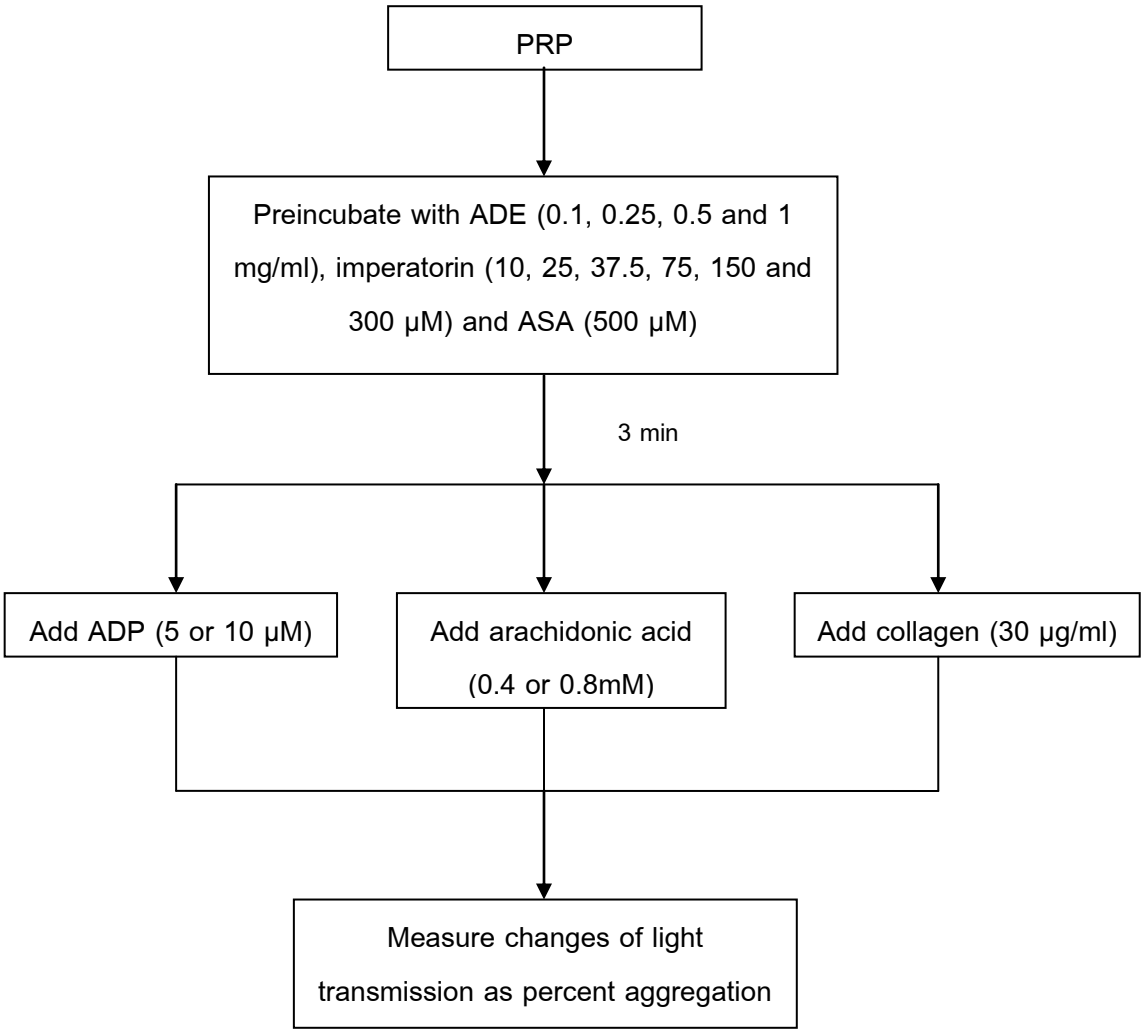


Figure 3.3 Study the effects of ADE and imperatorin on platelet aggregation.

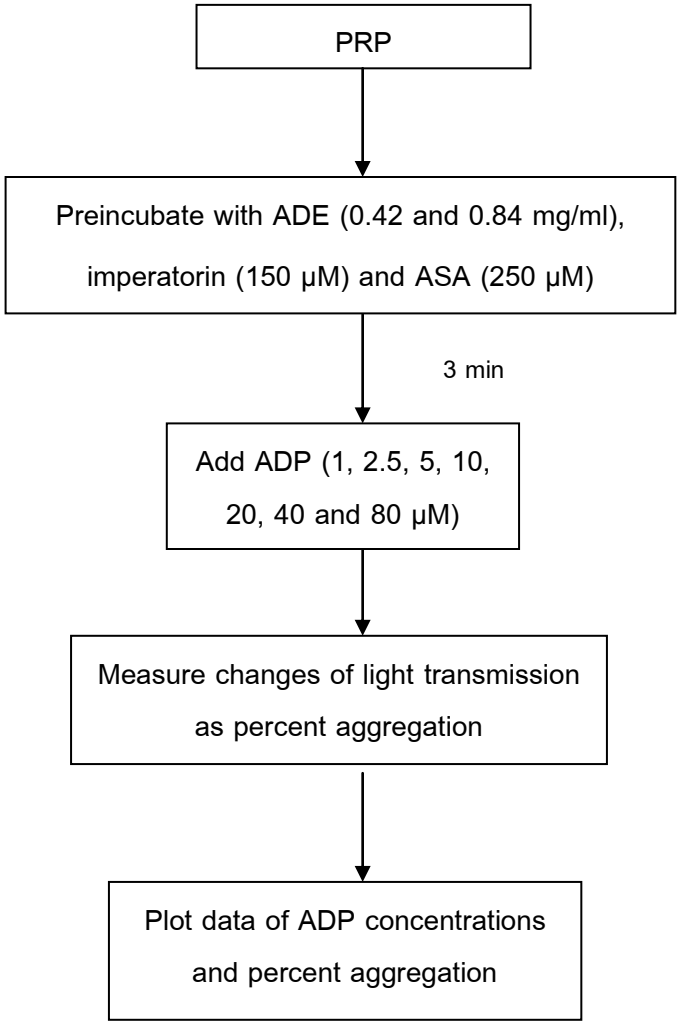


Figure 3.4 Study the antagonism effect of ADE and imperatorin on ADP-induced platelet aggregation.

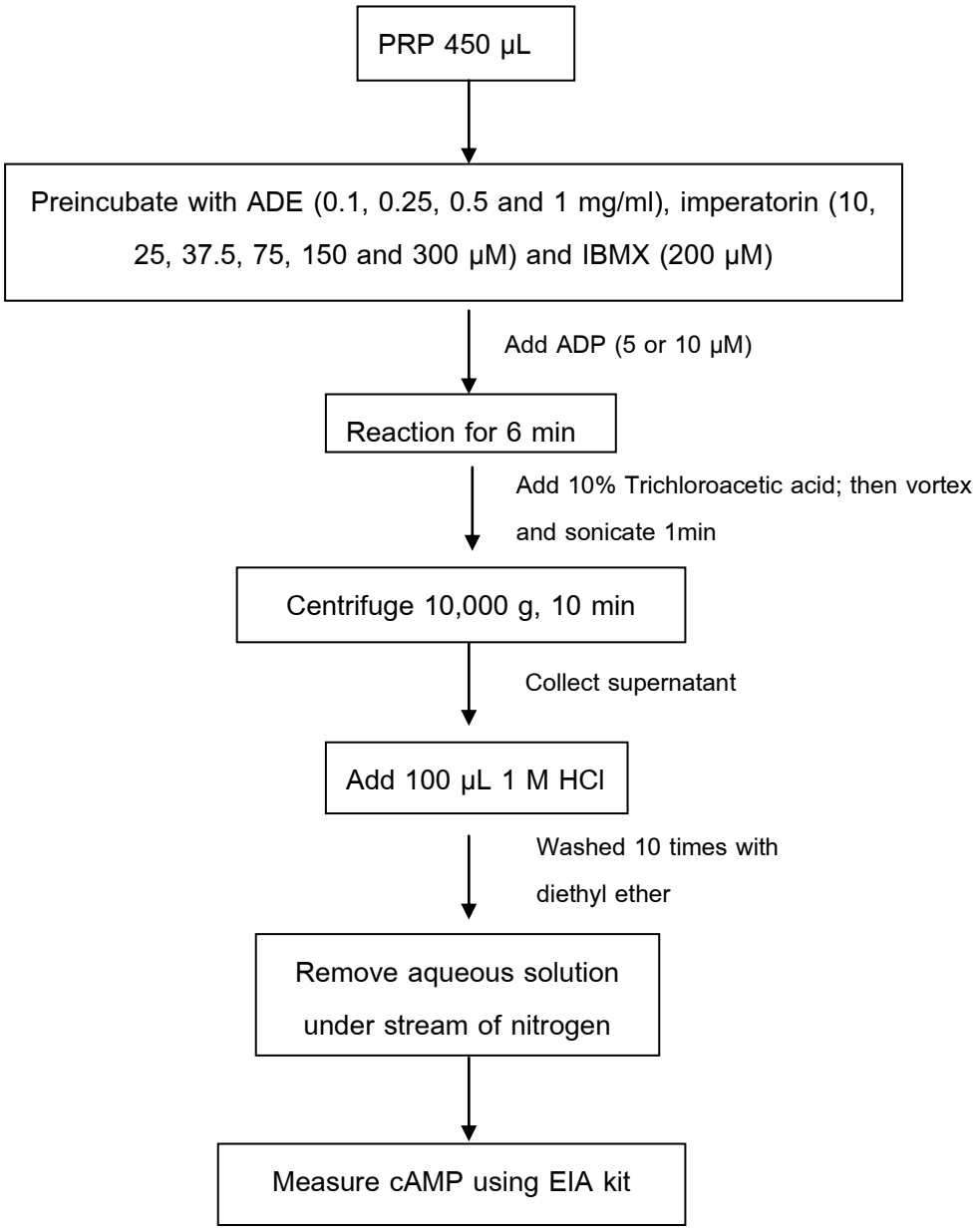


Figure 3.5 Study the effects of ADE and imperatorin on cAMP levels with the extraction of cAMP process from pretreated PRP.

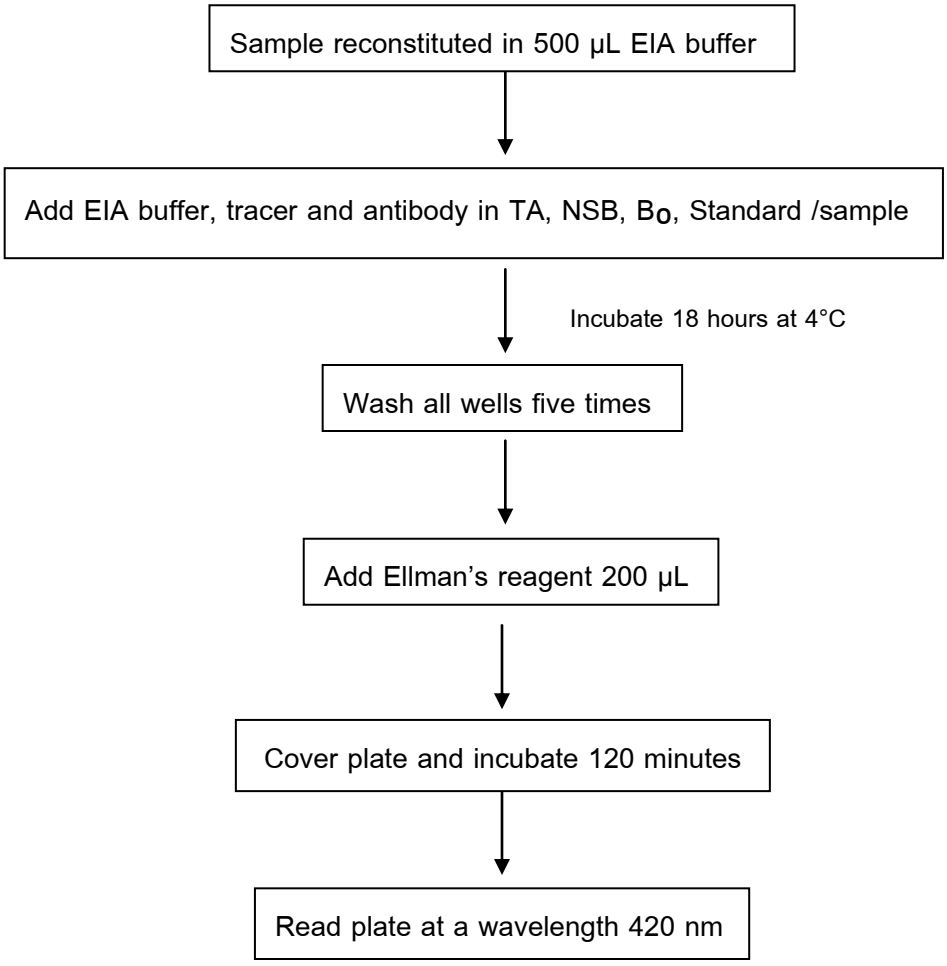


Figure 3.6 Measurement of cAMP level from pretreated PRP with 0.5%DMSO, ASA IBMX (200 µM), ADE (0.1-1 mg/ml) and imperatorin (10-300 µM).

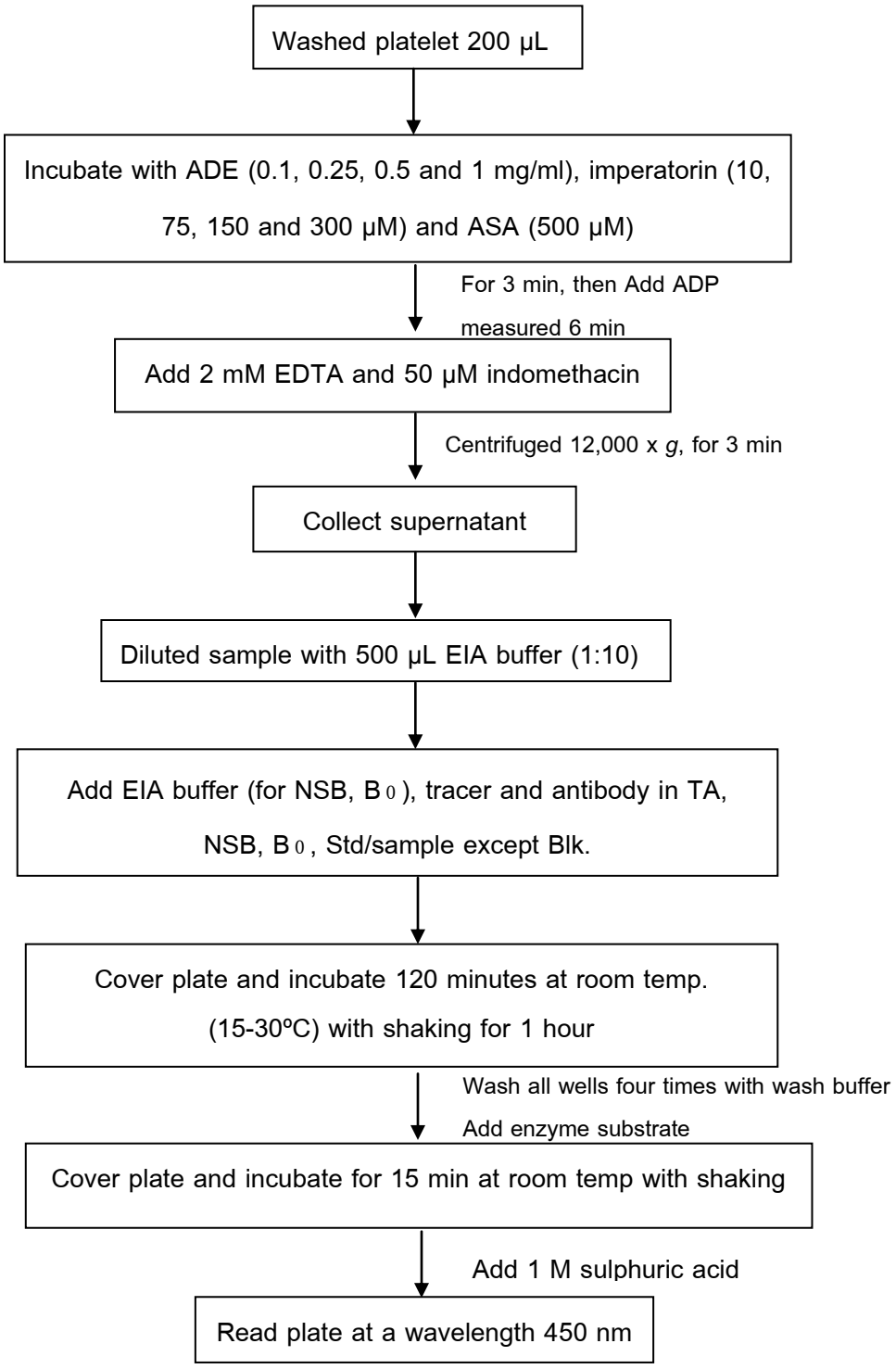


Figure 3.7 Study the effect of ADE and imperatorin on thromboxane B_2 level from pretreated PRP.

4. Statistical Analysis

All continuous data are expressed as the means ± standard errors of mean (S.E.M.). All experiments were done in triplicate determinations from three or four separate experiments. Differences between groups were assessed by one-way analysis of variance. If there are significant differences among group means, then each group was compared using Tukey method using the PASW statistics 18 for window software. P-values less than 0.05 were accepted as statistical significance.

CHAPTER IV

RESULTS

1. The effect of ADE and imperatorin on platelet aggregation.

1.1. Platelet aggregation induced by ADP

To investigate the antiplatelet activity of ADE and imperatorin on platelet aggregation, ADP, a weak agonist, was used in this study. PRP was incubated at 37°C under stirring condition. It was pretreated with 0.5% DMSO (vehicle), 500 μM of ASA, ADE at concentrations of 0.1, 0.25, 0.5 and 1 mg/ml or imperatorin at concentrations of 10, 25, 37.5, 75, 150 and 300 μM for 3 min. Then, ADP (5 or 10 μM) were added and were measured changes in light transmission, which represents as percent platelet aggregation, for 6 min. ADP-induced platelet aggregation exhibit two phase of aggregation, which are primary and secondary phase as shown in figure 4.1.

Results were shown in figure 4.1, 4.2, and 4.3. From figure 4.2, ADE at 1 mg/ml significantly decreased the percentage of ADP-induced platelet aggregation by 28.17% ($p=0.000$) compared with vehicle control (77.36%). The percentage of inhibition of ADE did not significantly differences from 500 μM ASA (90 μg/ml). Similar to ADE, imperatorin at 300 μM (81.1 μg/ml) significantly decreased ADP-induced platelet aggregation by 48.23% ($p=0.044$) and did not significantly difference in the percentage of inhibition compared with 500 μM ASA as shown in figure 4.3. Conforming to percentage of aggregation, slopes were also decreased. ADE at 1 mg/ml significantly decreased slopes by 65.04 mm./min ($p=0.001$) compared with 0.5%DMSO (131.63 mm./min), whereas imperatorin at 300 μM (81.1 μg/ml) did not significantly decrease slope. In accordance with ASA, ADE and imperatorin could inhibit secondary phase of ADP-induced platelet aggregation but could not inhibit first phase and platelet shape change still occurred.

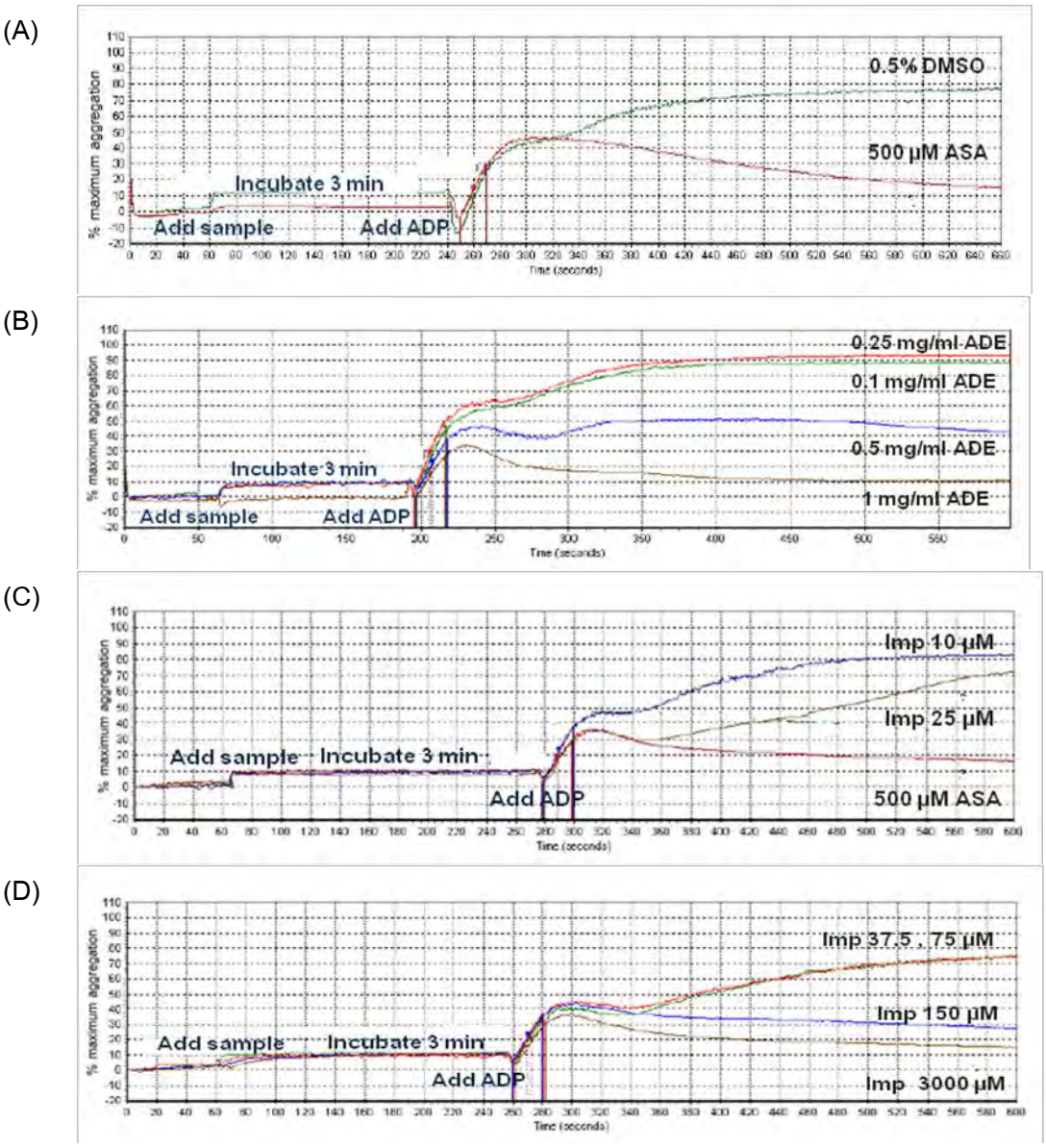
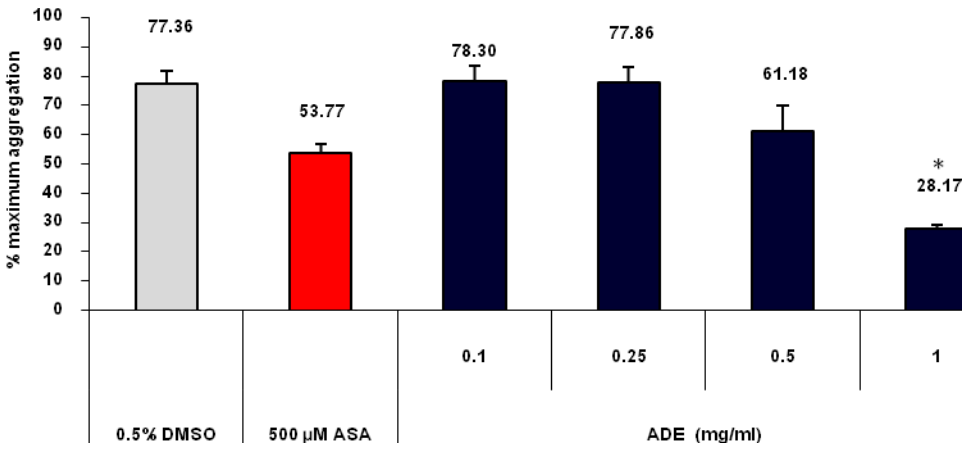


Figure 4.1 Representative aggregogram showed the effects of ADE and imperatorin on ADP-induced platelet aggregation. After adding ADP, 500 µM ASA (A) inhibited secondary phase of ADP-induced aggregation compared with 0.5%DMSO. ADE (B) at concentrations of 0.5 and 1 mg/ml also inhibited platelet aggregation. Imperatorin (C and D) at concentrations of 150 and 300 µM inhibited secondary phase of ADP-induced platelet aggregation as well as 500 µM ASA (C).

(A)



(B)

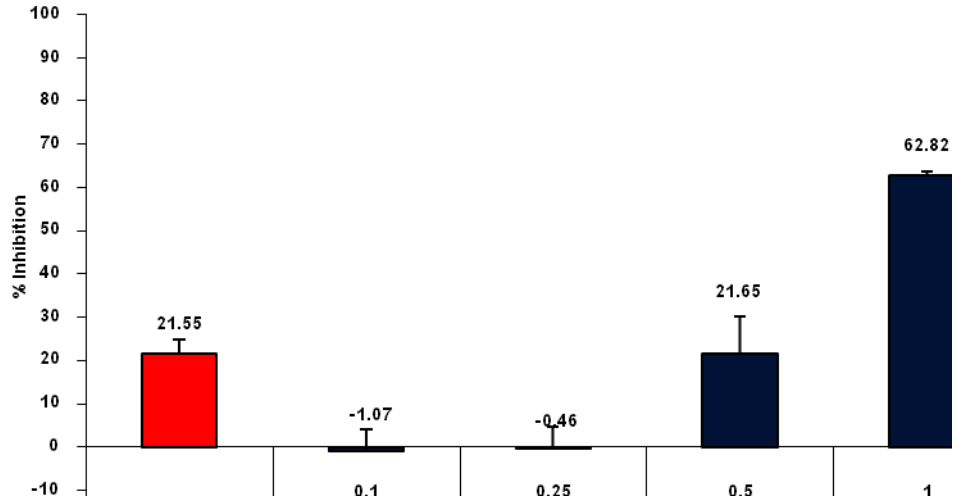
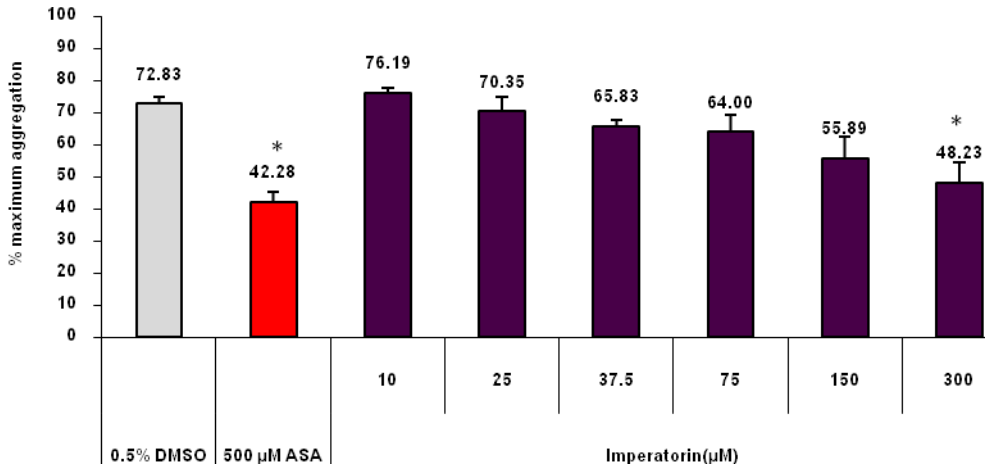


Figure 4.2 Effects of ADE pretreatment on ADP-induced platelet aggregation. PRP were preincubated for 3 min with ADE at concentrations of 0.1-1 mg/ml, 500 µM ASA or 0.5% DMSO, followed by the addition of ADP. Data are represented as percent maximum aggregation (A) and percent inhibition (B). Valves expressed as means. * Significant difference compared with vehicle control at p = 0.000 (n=4).

(A)



(B)

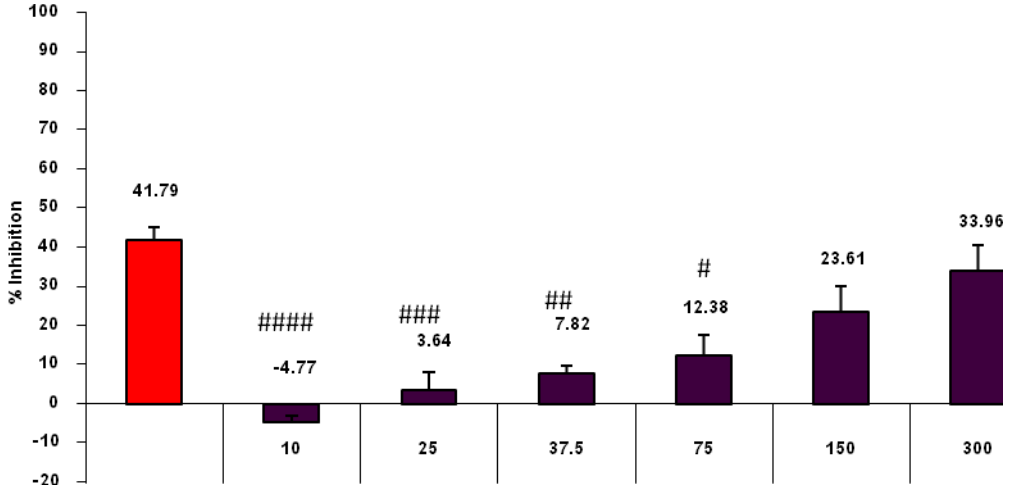


Figure 4.3 Effects of imperatorin pretreatment on ADP-induced platelet aggregation. PRP were preincubated for 3 min with imperatorin at concentrations of 10-300 μM, ASA 500 μM or vehicle control (0.5% DMSO), followed by the addition of ADP. Data are represented as percent maximum aggregation (A) and percent inhibition (B). Valves expressed as means. * p=0.044 Significant difference compared with vehicle control at (n=4), #, ##, ### and #### significant difference compared with 500 μM ASA at p = 0.040 0.024, 0.004, and 0.000, respectively.

Table 4.1 The effects of ADE on rate of aggregation represented as slopes and the percentage reduction of slope of ADP-induced platelet aggregation.

Sample + ADP	Slope (mm./min)	% reduction of slopes
0.5% DMSO	131.6 ± 11.06	100 ± 0.00
ASA 500 μM	118.6 ± 5.06	5.4 ± 3.13
ADE 0.1 mg/ml	115.6 ± 10.47	7.4 ± 2.31
ADE 0.25 mg/ml	112.3 ± 6.97	9.3 ± 1.88
ADE 0.5 mg/ml	99.2 ± 8.87	19.6 ± 7.03
ADE 1 mg/ml	65.0 ± 3.67 *	45.6 ± 2.22

PRP was pretreated with ADE (0.1-1 mg/ml), 500 μM ASA or 0.5%DMSO. Values are means ± SEM of slope, * P = 0.001 *vs* control (n = 4).

Table 4.2 The effects of imperatorin on rate of aggregation represented as slopes and the percentage reduction of slope of ADP-induced platelet aggregation.

Sample + ADP	Slope (mm./min)	% reduction of slope
0.5% DMSO	103.3 ± 5.16	100 ± 0.00
ASA 500 μM	99.5 ± 7.21	3.7 ± 4.18
Imperatorin 10 μM	104.6 ± 3.94	6.7 ± 8.19
Imperatorin 25 μM	104.7 ± 5.68	- 1.3 ± 0.95
Imperatorin 37.5 μM	95.9 ± 4.21	1.5 ± 4.91
Imperatorin 75 μM	104.4 ± 4.41	-1.2 ± 1.37
Imperatorin 150 μM	103.5 ± 3.84	-0.4 ± 1.32
Imperatorin 300 μM	104.9 ± 6.48	-1.3 ± 2.06

PRP was pretreated with imperatorin (10-300 μM), 500 μM ASA or 0.5%DMSO. Values are means ± SEM of slope (n = 4).

1.2. Platelet aggregation induced by arachidonic acid

Arachidonic acid is the precursor of TxA_2 which is one of the strong agonists used as an aggregating agent. PRP was preincubated at 37°C with 0.5% DMSO (vehicle), 500 µM ASA, ADE at concentrations of 0.1, 0.25, 0.5 and 1 mg/ml or imperatorin at concentrations of 10, 25, 37.5, 75, 150 and 300 µM for 3 min.

Figure 4.6, 4.7 and 4.8 showed that both ADE and imperatorin could not decrease the maximum aggregation of AA-induced platelet aggregation. While 500 µM ASA significantly decreased AA-induced platelet aggregation compared with 0.5%DMSO (24.56% *vs.* 81%; p=0.002). The percentage inhibition of ADE and imperatorin were significantly lower than 500 µM ASA (p=0.000) (figure 4.5B and 4.6B). In accordance with the maximum of aggregation, 1 mg/ml ADE and 300 µM imperatorin (81.1 µg/ml) could not decrease the rate of aggregation or slope when compared with control as shown in table 4.3 and 4.4., whereas, 500 µM ASA (90 µg/ml) significantly decreased slope when compared with 0.5%DMSO (1.2 mm./min *vs.*141.4 mm./min; p=0.000).

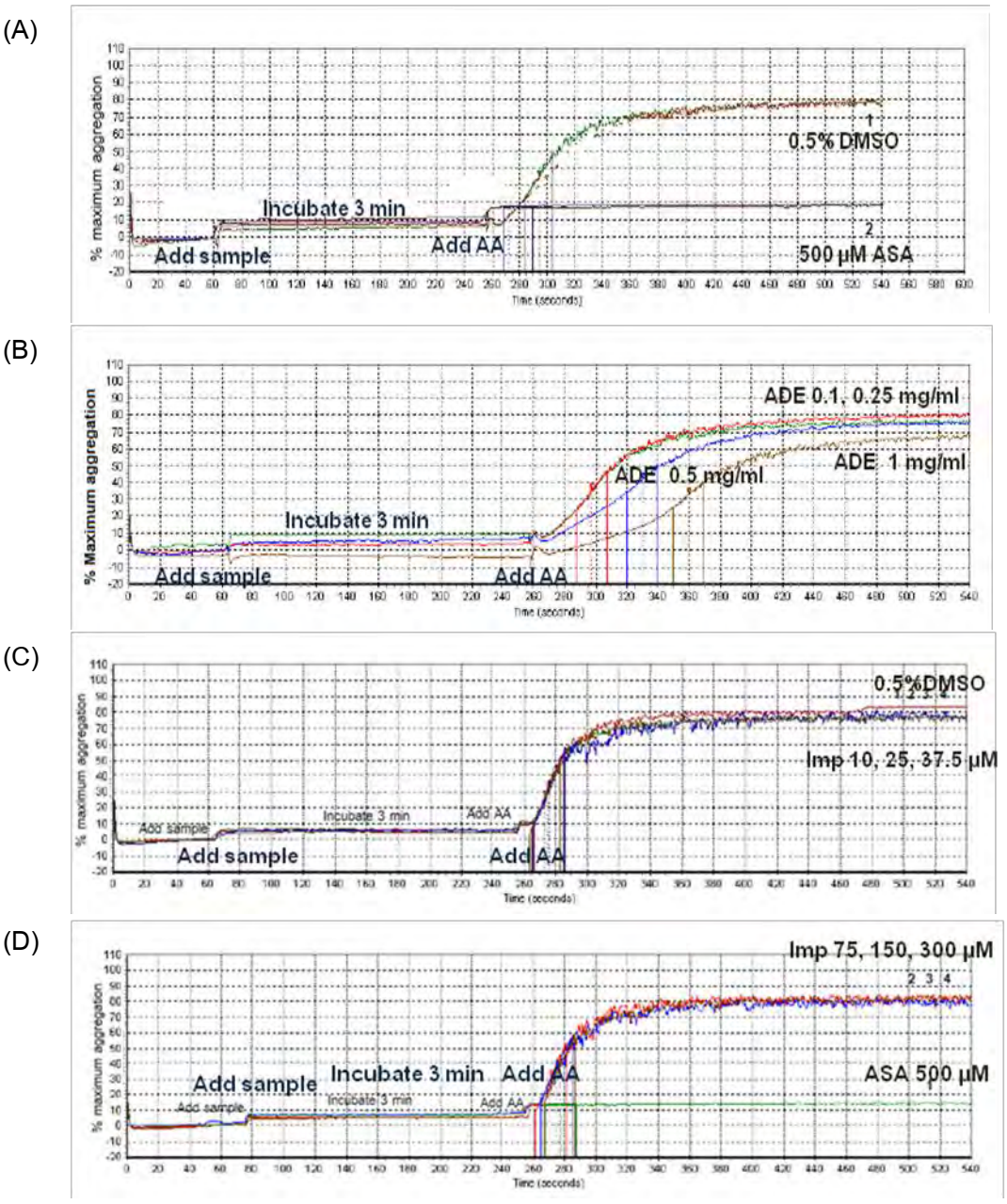


Figure 4.4 Representative aggregogram tracing of the effects ADE and imperatorin on AA-induced platelet aggregation. After adding AA, 500 µM ASA (A) inhibited platelet aggregation compared with 0.5%DMSO (A). ADE (B) could not inhibit aggregation but decreased the slope. Imperatorin (C and D) could not inhibit AA-induced platelet aggregation compare with 0.5%DMSO (C), whereas 500 µM ASA (A and D) inhibited AA-induced platelet aggregation.

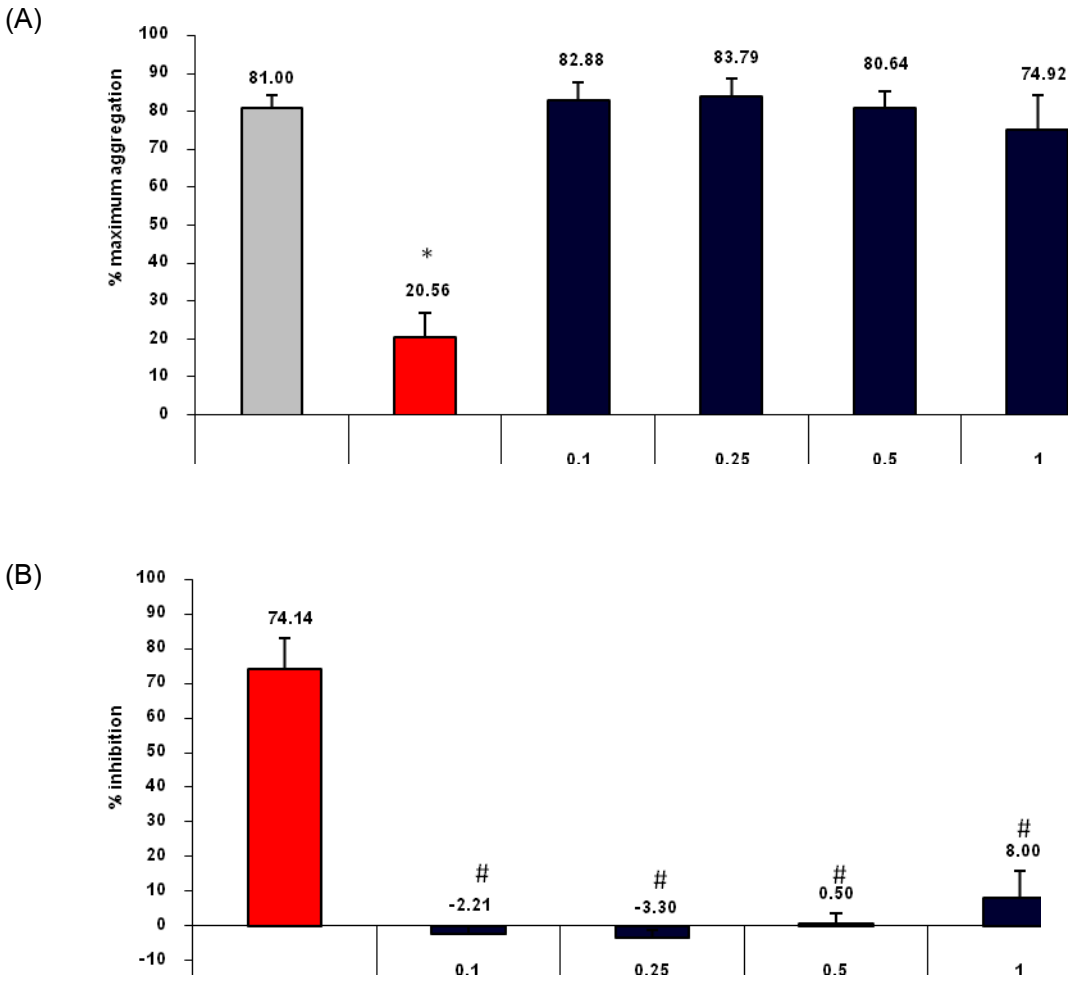


Figure 4.5 Effects of ADE pretreatment on AA-induced platelet aggregation. PRP were preincubated for 3 min with ADE at concentration 0.1-1 mg/ml, 500 μM ASA or vehicle control (0.5% DMSO), followed by the addition of AA. Data represents as percent maximum aggregation (A) and percent inhibition (B). * Significant difference compared with vehicle control at p=0.012, # Significant difference compared with 500 μM ASA at p=0.000 (n=3).

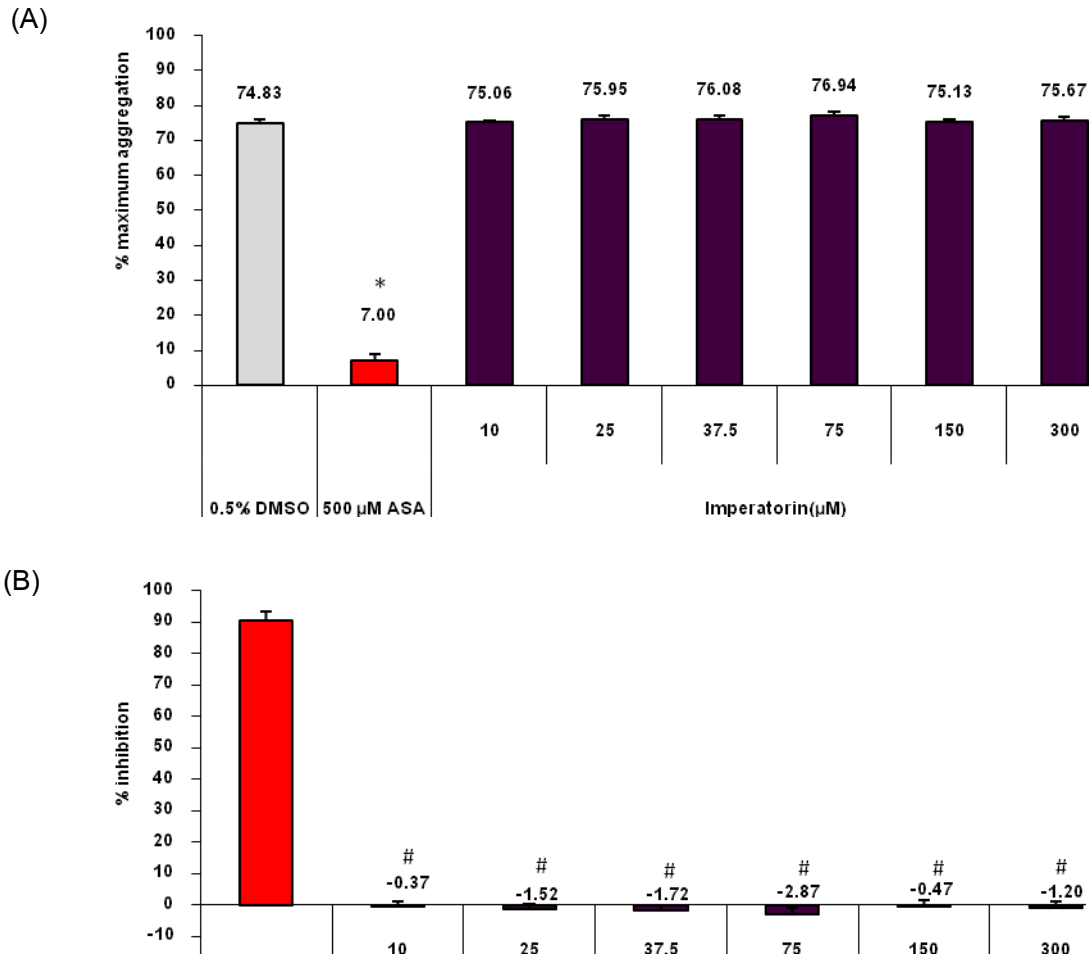


Figure 4.6 Effects of imperatorin pretreatment on arachidonic acid induced platelet aggregation. PRP were preincubated for 3 min with imperatorin at concentrations of 10-300 µM, 500 µM ASA or 0.5% DMSO, followed by the addition of AA. Data are represented as percent maximum aggregation (A) and percent inhibition (B). * Significant difference compared with vehicle control at p=0.000 (n=4). # Significant difference compared with 500 µM ASA at p=0.000

Table 4.3 The effects of ADE on rate of aggregation represented as slopes and the percentage reduction of slope of AA-induced platelet aggregation.

Sample + AA	Slope(mm./min)	% reduction of slopes
0.5% DMSO	107.9 ± 27.39	100 ± 0.00
500 μM ASA	0.8 ± 0.64	36.5 ± 26.50
ADE 0.1 mg/ml	113.7 ± 22.30	1.1 ± 4.27
ADE 0.25 mg/ml	99.8 ± 23.39	16.1 ± 2.70
ADE 0.5 mg/ml	84.8 ± 23.50	33.8 ± 0.94
ADE 1 mg/ml	57.1 ± 11.88	54.1± 3.18

PRP was pretreated with ADE (0.1-1 mg/ml), 500 μM ASA or 0.5%DMSO. Values are means ± S.E.M. of slopes (mm./min). (n = 3).

Table 4.4 The effects of imperatorin on rate of aggregation represented and the percentage reduction of slope as slopes of AA-induced platelet aggregation.

Sample + AA	Slope(mm./min)	% reduction of slopes
0.5% DMSO	141.4 ± 3.99	100 ± 0.00
500 μM ASA	1.2 ± 0.66 *	99.2 ± 0.40
Imperatorin 10 μM	129.6 ± 4.02	9.4 ± 1.49
Imperatorin 25 μM	123.6 ± 4.39	13.4 ± 2.52
Imperatorin 37.5 μM	120.6 ± 5.15	22.5 ± 6.98
Imperatorin 75 μM	116.2 ± 7.42	18.3 ± 6.21
Imperatorin 150 μM	104.9 ± 10.69	26.4 ± 7.99
Imperatorin 300 μM	121.6 ± 12.18	17.6 ± 13.28

PRP was pretreated with imperatorin (10-300 μM), 500 μM ASA or 0.5%DMSO. Values are means ± S.E.M. of slopes (mm./min) p=0.000 compared with 0.5%DMSO (n=4).

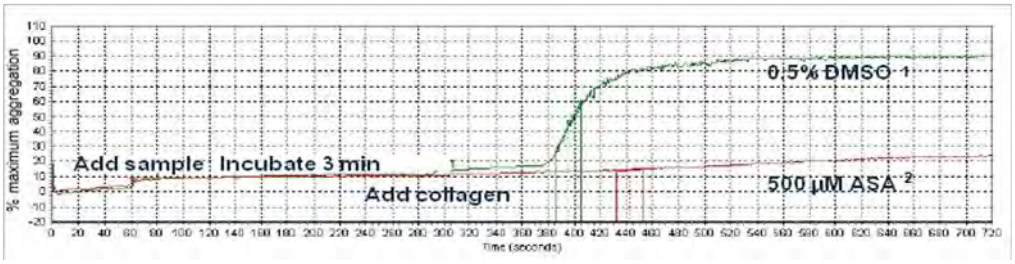
1.3. Platelet aggregation induced by collagen

To further investigate antiplatelet activity of ADE and imperatorin, collagen was used as the agonist in this study. Platelet aggregation induced by collagen usually has a lag time longer than other agonists. The lag time is the delaying time from adding agonist until platelet response in shape change,

After adding collagen, pretreatment of PRP with 1 mg/ml of ADE and 500 μM ASA significantly decreased maximum collagen-induced platelet aggregation by 34.89% ($p=0.011$) and 25.35% ($p=0.002$) compared with vehicle control (81.75%) as shown in figure 4.8. The percent inhibition of collagen-induced platelet aggregation of 1 mg/ml ADE was comparable to 500 μM ASA (90 μg/ml). On the other hand, imperatorin did not decreased maximum collagen-induced platelet aggregation at all concentrations, indicating that imperatorin cannot inhibit platelet aggregation induced by collagen when compare with 500 μM ASA ($p \leq 0.001$) (Figure 4.9).

From table 4.5, ADE at the concentration of 1 mg/ml significantly decreased the slope of collagen-induced platelet aggregation compared with 0.5%DMSO (11.3 mm./min *vs.* 59.3 mm./min; $p=0.008$). In addition, a lag time was increased significantly compared with 0.5%DMSO (367.8 *vs.* 116.4 seconds; $p=0.008$). While 500 μM ASA (90 μg/ml) significantly decreased the slope of collagen-induced platelet aggregation (3 mm./min, $p=0.002$) but did not increased a lag time. Imperatorin did not affected on the slope and lag time of collagen-induced platelet aggregation (table 4.6).

(A)



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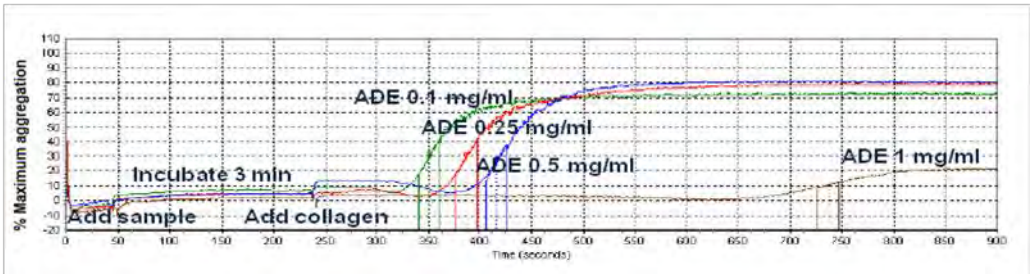

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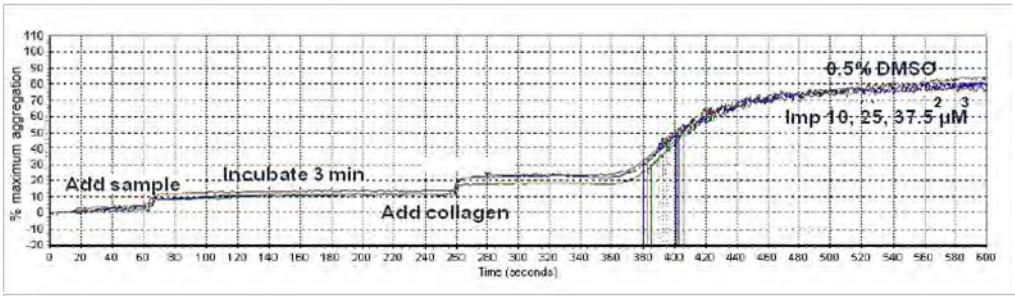

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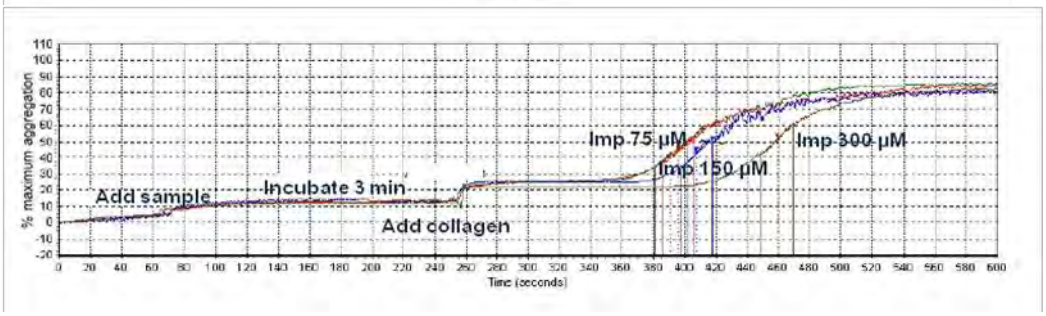


Figure 4.7 Representative aggregogram tracing of the effects ADE and imperatorin on collagen-induced platelet aggregation. After adding collagen, 500 µM ASA (A) inhibited platelet aggregation compared with 0.5%DMSO (A). ADE (B) at concentrations of 1 mg/ml inhibited collagen-induced platelet aggregation with decreasing slopes and lags time. Imperatorin (C and D) could not inhibit collagen-induced platelet aggregation compared with 0.5%DMSO (C).

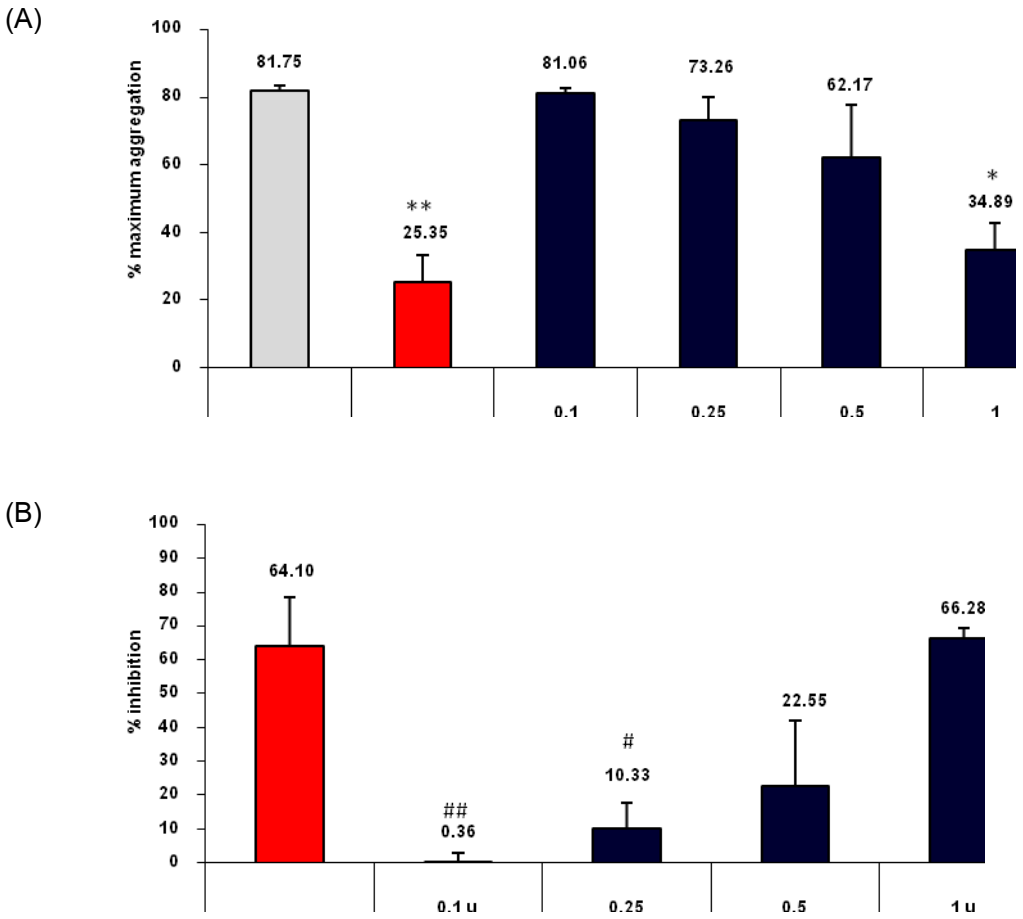


Figure 4.8 Effects of ADE pretreatment on collagen-induced platelet aggregation. PRP were preincubated for 3 min with ADE at concentrations of 0.1-1 mg/ml, 500 μM ASA or 0.5% DMSO, followed by the addition of collagen. Data are represented as percent maximum aggregation (A) and percent inhibition (B). *,** significant difference compared with 0.5%DMSO at p=0.011 and 0.002, respectively. #,## significant difference compared with 500 μM ASA at p=0.021 and 0.005, respectively.

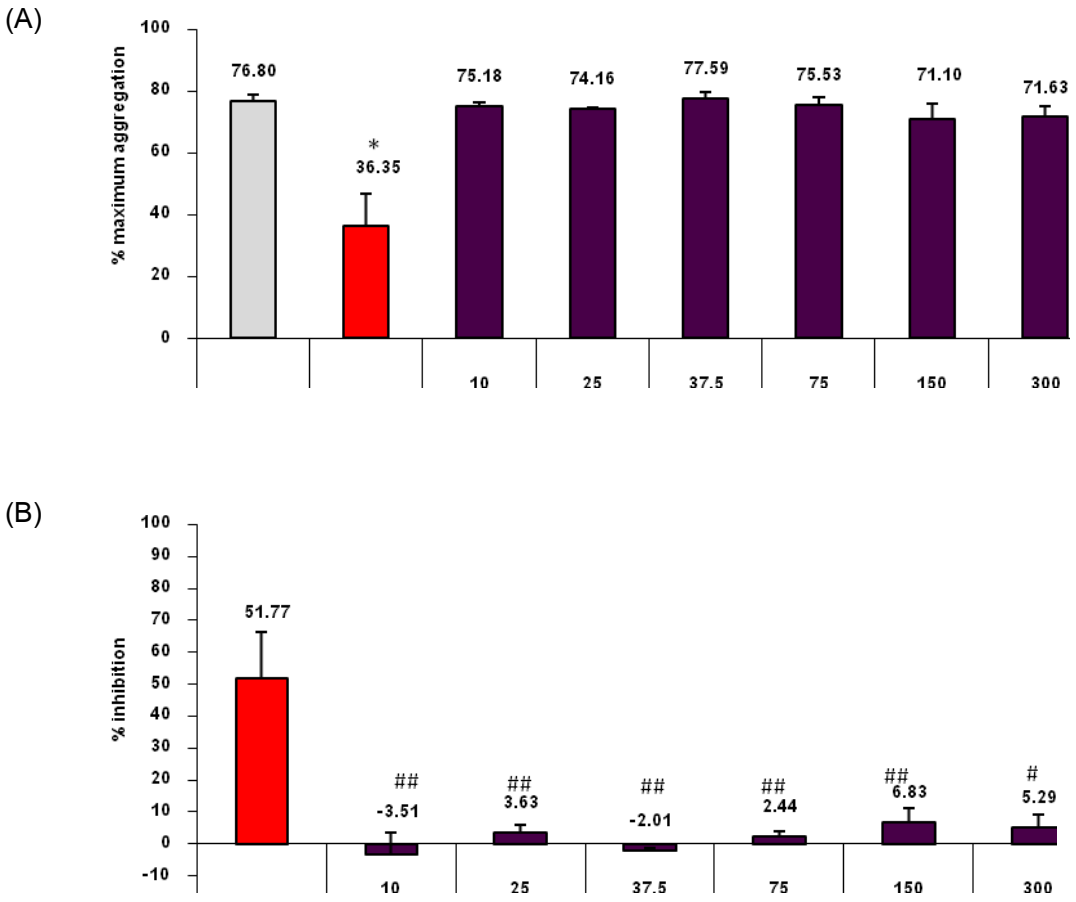


Figure 4.9 Effects of imperatorin pretreatment on collagen-induced platelet aggregation. PRP were preincubated for 3 min with imperatorin at concentrations of 10-300 μM, 500 μM ASA or 0.5% DMSO, followed by the addition of collagen. Data are represented as percent maximum aggregation (A) and percent inhibition (B). * Significant difference compared with vehicle control at p=0.000. #, ## significant difference compared with 500 μM ASA at p=0.001 and 0.000, respectively (n=4).

Table 4.5 The effects of ADE on rate of aggregation represented as slopes, the percentage reduction of slope and lag time of collagen-induced platelet aggregation.

Sample + collagen	Slope (mm./min)	% reduction of slope	Lag time (second)
0.5% DMSO	59.3 ± 1.56	100 ± 0.00	116.4 ± 17.25
ASA 500 μM	3 ± 1.65 **	94.8 ± 3.40	300.8 ± 36.13
AD 0.1 mg/ml	70.8 ± 1.82	-19.6 ± 1.13	106.2 ± 30.75
AD 0.25 mg/ml	57.8 ± 8.67	1.0 ± 16.90	140.3 ± 48.11
AD 0.5 mg/ml	45.3 ± 13.33	21.8 ± 23.42	198.4 ± 60.10
AD 1 mg/ml	11.3 ± 7.34 *	80 ± 15.35	367.8 ± 8.28 *

PRP was pretreated with ADE (0.1-1 mg/ml), 500 μM ASA or 0.5%DMSO. Values are means ± S.E.M. * p=0.008, ** p=0.002 *vs* control (n = 4).

Table 4.6 the effects of imperatorin on rate of aggregation represented as slopes, the percentage reduction of slope and lag times of collagen-induced platelet aggregation.

Sample + collagen	Slope (mm./min)	% reduction of slope	Lag time (second)
0.5% DMSO	70.8 ± 10.43	100 ± 0.00	106.6 ± 35.67
ASA 500 μM	22.6 ± 15.57	71.2 ± 14.04	143.3 ± 57.03
Imperatorin 10 μM	76.6 ± 14.26	-1.31 ± 14.46	58.8 ± 9.92
Imperatorin 25 μM	79.1 ± 12.33	-12.4 ± 6.18	62.1 ± 11.81
Imperatorin 37.5 μM	74.2 ± 17.44	-11.89 ± 6.95	55.0 ± 11.73
Imperatorin 75 μM	74.5 ± 14.53	-4.49 ± 9.71	61.7 ± 11.29
Imperatorin 150 μM	73.3 ± 14.87	-1.35 ± 9.73	69.2 ± 13.13
Imperatorin 300 μM	75 ± 12.38	-6.82 ± 7.73	76.3 ± 19.24

PRP was pretreated with imperatorin (10-300 μM), 500 μM ASA or 0.5%DMSO. Values are means ± S.E.M. (n = 4).

2. **The antagonism effects of** ADE **and imperatorin on ADP-induced platelet aggregation.**

To evaluate the competitive antagonism of ADE and imperatorin on ADP-induced platelet aggregation, concentration-response relationships were observed.

ASA is a COX inhibitor and its modes of action do not directly act *via* ADP receptor. When 250 μM ASA (45 μg/ml) was preincubated with PRP for 3 min, it reduced maximum ADP-induced platelet aggregation. The antiplatelet activity of 250 μM ASA was reduced after increase concentrations of ADP at 1-20 μM or 0.525-8.4 μg/ml. 250 μM ASA (45 μg/ml) did not produced a parallel shift to the right of the concentration-response curve of ADP, confirming ASA did not an antagonism on the ADP receptor as shown in figure 4.10.

When ADE at concentrations of 0.42 and 0.84 mg/ml (figure 4.11 and 4.12) were preincubated with PRP for 3 min, they reduced maximum ADP-induced platelet aggregation at low concentrations (below 10 μM). Their reductions of maximum ADP-induced aggregation were reduced after increasing concentrations of ADP at 1-80 μM or 0.525-33.6 μg/ml. The percentage of maximum aggregation was plotted against the concentrations of ADP. The results demonstrated that the fixed concentration of ADE at 0.42 and 0.84 mg/ml produced a parallel shift to the right of the concentration-response curve of ADP. High ADP concentrations can overcome inhibition by ADE. However, pretreatment the PRP with imperatorin at the concentration of 150 μM could not inhibit ADP-induced platelet aggregation (figure 4.13).

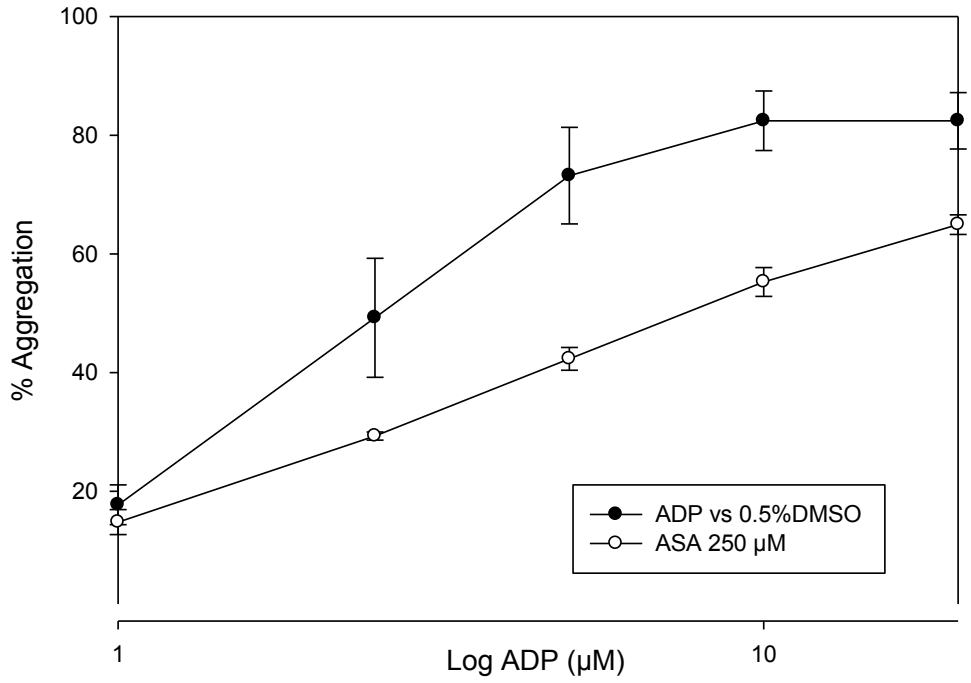
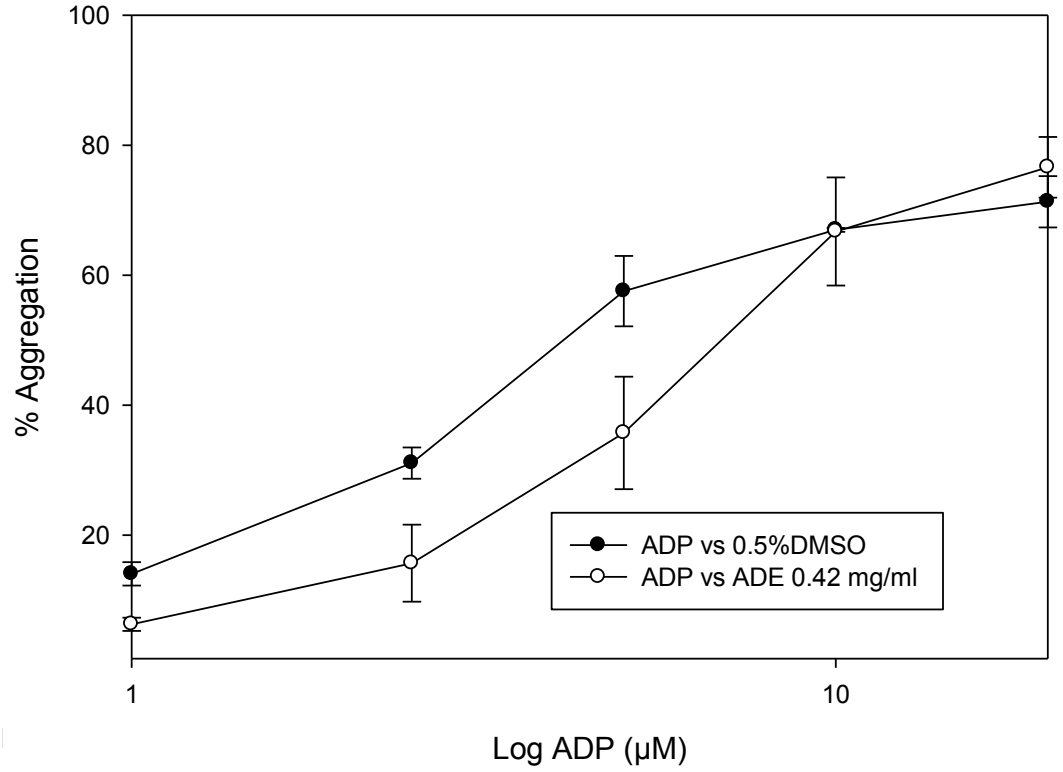


Figure 4.10 Change in log concentration effects curve of ADP (1-20 μM) produced by the fixed concentration of tested compounds, ASA (250 μM). Each point represents as mean ± S.E.M. (n = 4).



e

fixed concentration of ADE at 0.42 mg/ml. Each point represents as mean ± S.E.M. (n = 4).

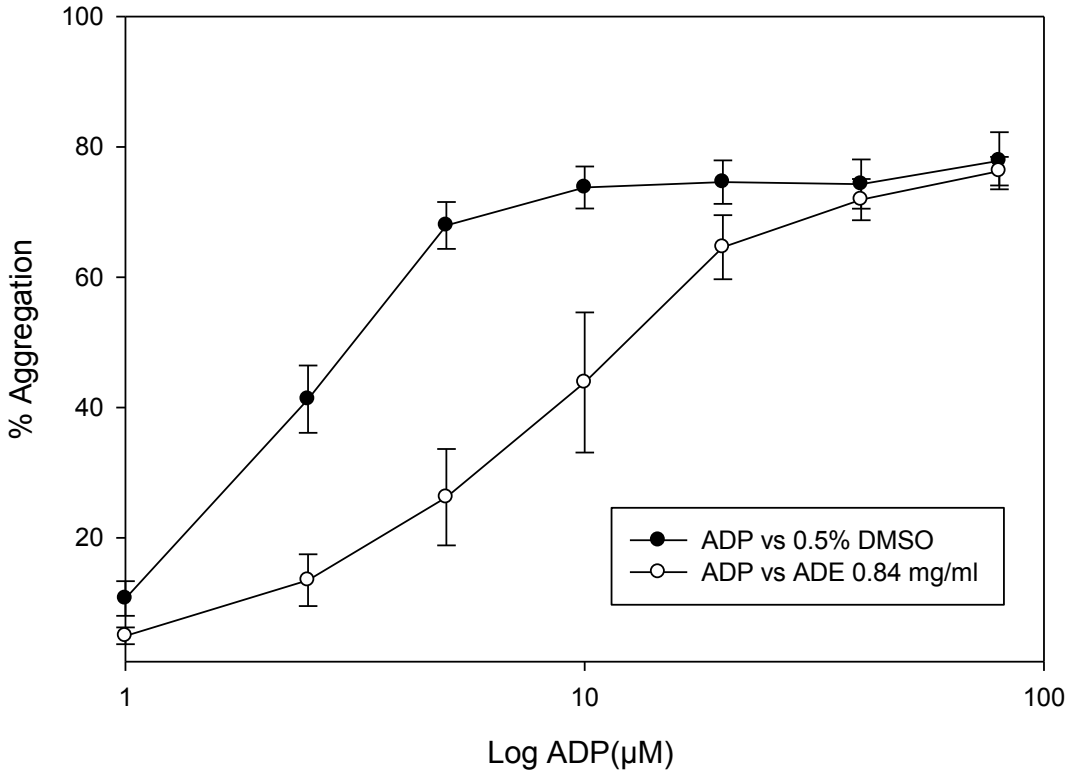


Figure 4.12 Change in log concentration effects curve of ADP (1-80 μM) produced by the fixed concentration of ADE at 0.84 mg/ml. Each point represents as mean ± S.E.M. (n = 4).

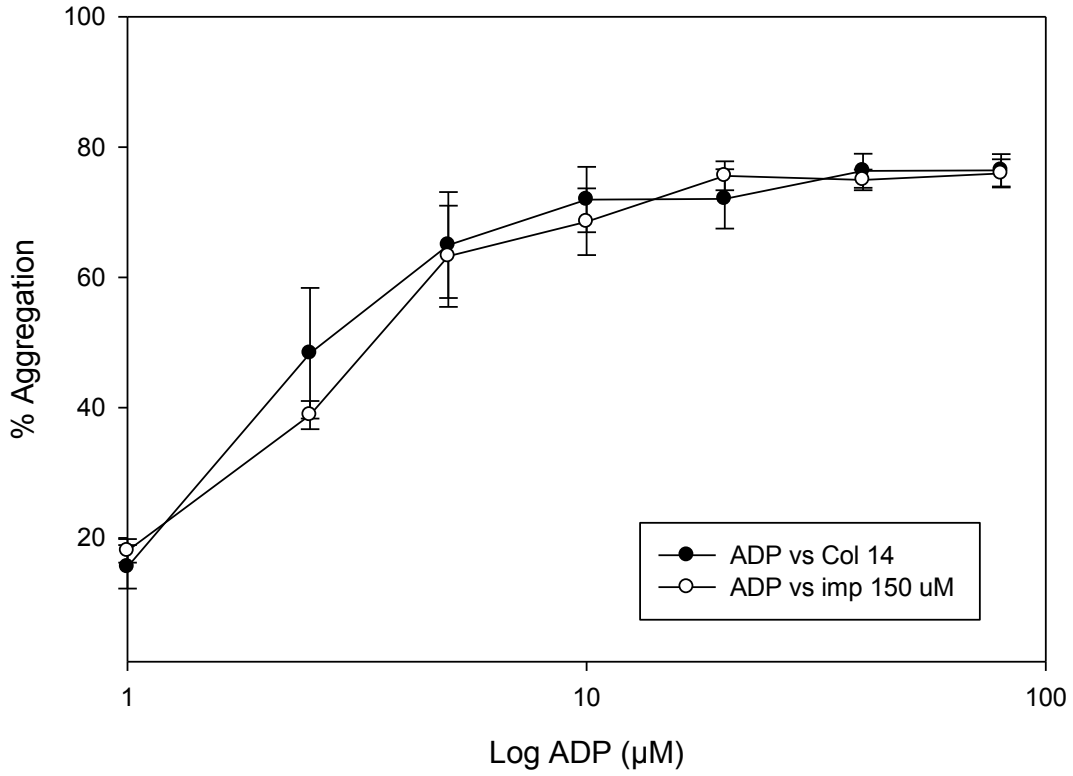


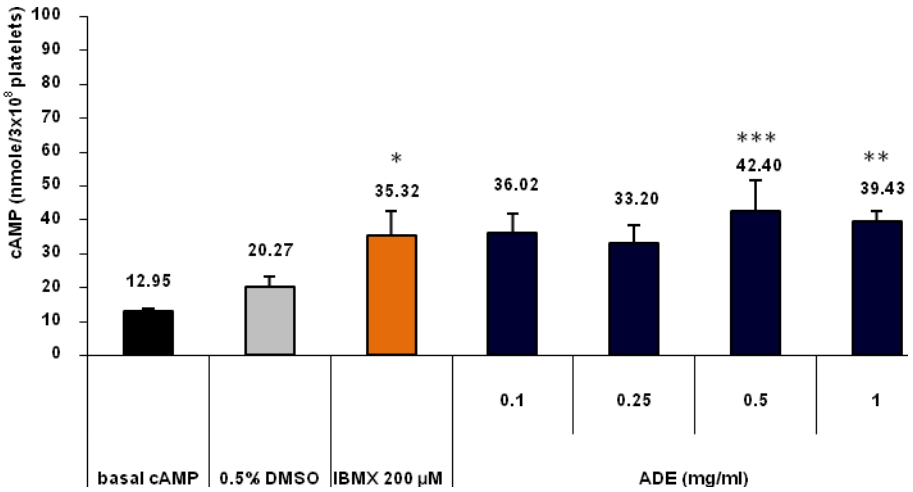
Figure 4.13 Change in log concentration effects curve of ADP (1-80 µM) produced by the fixed concentration of imperatorin (150 µM). Each point represents as mean ± S.E.M. (n = 4).

3. The effect of ADE and imperatorin on cAMP level.

To explore underlying antiplatelet mechanisms of ADE and imperatorin in ADP-induced platelet aggregation, the platelet cAMP levels were measured. 3-isobutyl-1-methylxanthine (IBMX) is a phosphodiesterase inhibitor, which prevents metabolism of cAMP and used as positive control.

From figure 4.11, preincubation of IBMX at the concentration 200 μM (44.46 μg/ml) significantly increase cAMP level by 35.32 pmole/ $3x10^8$ platelets ($p=0.029$) compared with 0.5%DMSO 20.27 pmole/ $3x10^8$ platelets. Similarly, ADE at the concentrations of 0.5 and 1 mg/ml significantly increased cAMP levels ($p=0.007$ and $p=0.027$). Imperatorin did not increase cAMP levels in the range of concentrations from 10 to 300 μM.

(A)



(B)

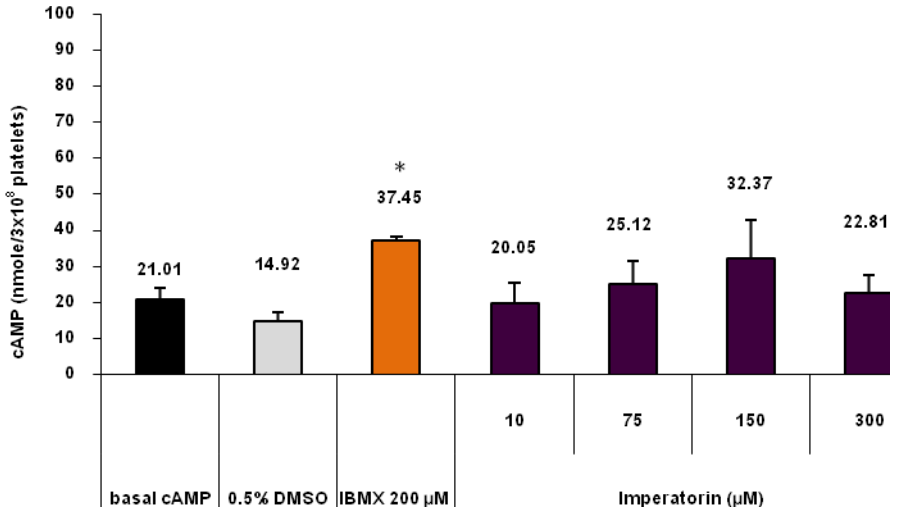


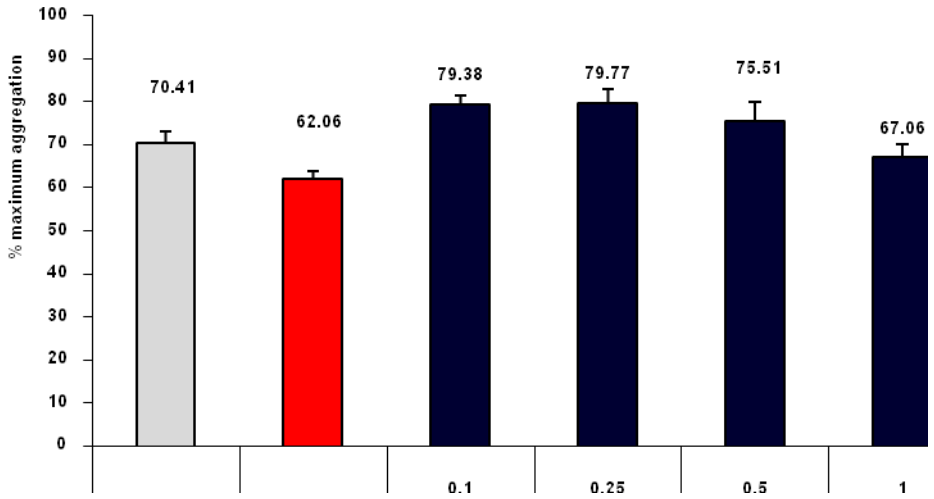
Figure 4.14 Effects of ADE (0.1-1 mg/ml) and imperatorin (10-300 µM) on platelet cAMP levels when induced platelet aggregation by ADP (10 µM). PRP was incubated in the presence or absence of ADE or imperatorin for 3 min at 37ºC with stirring. IBMX served as a positive control. Results are expressed as mean±SEM. * p=0.029, **p=0.027 and ***p=0.007 compared with vehicle control. Experiments were done in triplicate from three separated experiments.

4. **The effects of** ADE **and imperatorin on platelet TxB_2 levels.**

To explore underlying whether there is another antiplatelet mechanisms of ADE and imperatorin in ADP- induced platelet aggregation or not. ADP signaling pathways are one of the upstream involving the activation of PLA_2 leading to the production and secretion TxA_2.

In this study, we used washed platelets instead of PRP in order to prevent color disturbance. Pretreatment PRP with ADE at 0.1 to 1 mg/ml for 3 min, and then ADP was added. The reaction was stopped and measured the platelet TxB_2 levels. From figure 4.12A, 500 μM ASA (90 μg/ml) significantly decreased TxB_2 levels by 2.42 pg/3x10^8 platelets ($p=0.004$) compared with control 12.67 pg/3x10^8 platelets. However, ADE at 0.1 to 1 mg/ml did not decreased TxB_2 levels on ADP-induced platelet aggregation.

(A)



(B)

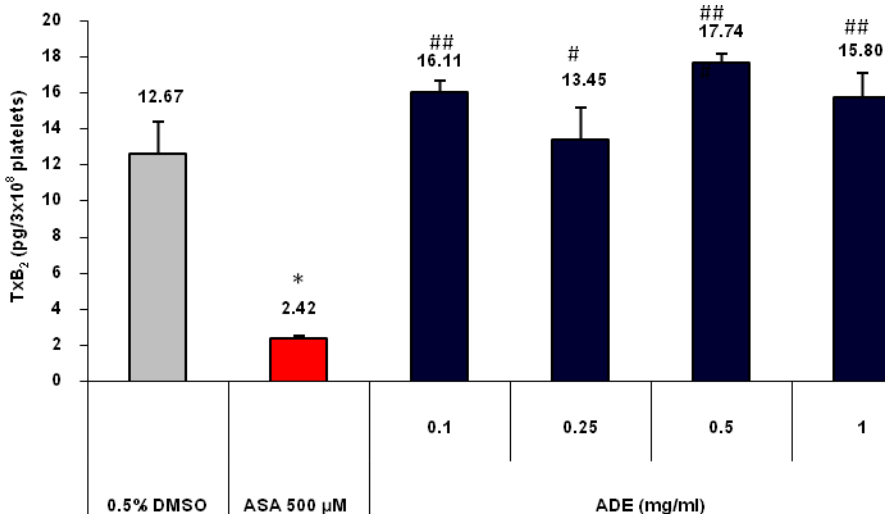


Figure 4.15 The effects of ADE (0.1-1 mg/ml) on TxB_2 levels after induction of platelet aggregation by ADP (10 µM). PRP was incubated in the presence or absence of ADE for 3 min at 37ºC with stirring. ASA 500 µM served as positive control. Data are represented as percent maximum aggregation of washed platelet (A) and platelet TxB_2 levels (B). Results are expressed as mean ± SEM. * Significant difference compared with vehicle control at p=0.004. #, ## significant difference compared with 500 µM ASA at p=0.002 and 0.000, respectively (n=3).

CHAPTER V

DISCUSSION AND CONCLUSION

Platelet aggregation can be induced by several agonists, such as ADP, collagen, epinephrine, platelet activating factor, serotonin, thrombin and thromboxane A_2 (TxA_2). Among these agonists, collagen mediates the initial response of platelet activation to injured vessel wall leading to generate and secrete auto-activating agonists, such as ADP and TxA_2. Both ADP and TxA_2 are an amplifier of platelet activation by sustain the cascade of aggregation and recruit other platelets to the site of injury (Krotz et al., 2008). All three agonists are essential for platelet aggregation, each of which activates specific receptor signaling pathways on platelet membrane. These agonists also share a common pathway leading in platelet secretion substances from their granule, the increase of intracellular calcium, the formation of TxA_2 and the activation of final pathway, activated fibrinogen receptors. In this study, ADP, arachidonic acid which is metabolized to TxA_2 and collagen were used as aggregating agents to explore antiplatelet activity of ADE and imperatorin.

The activation of ADP-induced platelet aggregation involves two purinergic receptors on platelet membrane, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$, coupled to $G_{\alpha q}$, activation of phospholipase C (PLC) and $P2Y_{12}$, coupled to G_i, inhibition of adenylate cyclase. ADP-induced platelet aggregation normally produces two phase of aggregation which are reversible and irreversible phase of aggregation as detected by turbidimetry (Zhou and Schmaier, 2005). The co-activation of both $P2Y_1$ and $P2Y_{12}$ is required for produce full response of platelet aggregation (Storey et al., 2000). In the study of $G_{\alpha q}$ deficient mouse platelets, ADP could not produce platelet aggregates compare with wild-type mouse platelets in which $G_{\alpha q}$ is the stimulatory regulator for PLC_β (Offermanns et al., 1997). In accordance with Jin and Kanapuri (1998), they reported that both G_q, coupled to PLC, and G_i, coupled to inhibition of adenylate cyclase activity, are required for full

response of ADP-induced platelet aggregation. ARL 66096, a $P2Y_{12}$ antagonist, inhibits secondary wave of ADP-induced platelet aggregation with remaining shape change, while A3P5PS, a $P2Y_1$ antagonist, abrogates both primary and secondary wave. $P2Y_1$ receptor coupled to $G_{\alpha q}$ is responsible for mediating platelet shape change, intracellular calcium mobilization, and initiates aggregation, while $P2Y_{12}$ receptor coupled to G_i, adenylate cyclase inhibition, is responsible for complete and amplification, which involve secretion and production of TxA_2 of ADP-induce platelet aggregation (Jin and Kunapuli, 1998). The results of the present study demonstrated that ADE at 1 mg/ml and imperatorin at 300 μM (81.1 μg/ml) inhibited ADP-induced platelet aggregation. Both ADE and imperatorin inhibited irreversible phase of ADP induced platelet aggregation (figure 4.1). Thus, their effects did not directly inhibit PLC *via* G_q couple to $P2Y_1$ but might be involved the inhibiton of Gi couple to adenylate cyclase activity *via* $P2Y_{12}$ or arachidonic acid-thromboxane pathway.

In contrast to ADP-induced platelet aggregation, the results demonstrated that ADE and imperatorin did not inhibit AA-induced platelet aggregation. On the other hand, ASA at the concentration of 500 μM (90 μg/ml) showed the most potent inhibitory effects on AA-induced platelet aggregation. The mode of action of aspirin is to inhibit cyclooxygenase activity resulting in blocking the formation of TxA_2 (Armstrong et al., 2008). Ban et al. (2003) demonstrated that imperatorin at concentrations of 3 to 30 μM (0.81 to 8.12 μg/ml) decreased prostaglandin E_2 (PGE_2) levels on lipoploysaccharide (LPS)-induced PGE_2 production, which was a product from arachidonic acid, in rat peritoneal macrophage. Its inhibitory possible might be involved AA-PGE pathway. However, they also found that imperatorin at 3 to 30 μM did not inhibit the LPS-induced released of radioactivity of [$_3$H]-arachidonic acid-labeled macrophage. In accordance with Lee *et al.,* (2011), the study demonstrated that the effects of 70% ethanolic of ADE at the concentration of 200 μg/ml inhibited COX-2 expression resulting in decreasing of PGE_2 levels on LPS-induced

inflammation in murine macrophage. Therefore, The results of present study showed that both ADE and imperatorin did not directly affect arachidonic acid - TxA_2 pathway, indicating that the inhibitory effect possible involve upstream of platelet aggregation mechanism. The modes of action of ADE and imperatorin were differenced from ASA.

Collagen mediated signaling through activation of $PLC_{\gamma 2}$ and subsequent produce inositol 1, 4, 5 triphosphate (IP3) and 1, 2-diacylglycerol (Giroux et al.). IP_3 promotes calcium release from dense tubular system and calcium, in turn, stimulates phospholipase A_2 (PLA_2) leading to increased intracellular calcium and TxA_2 production, while DAG stimulates PKC. Both IP3 and DAG are mediated the response of platelets such as shape change, granule secretion and aggregation. The effects of collagen-induced platelet aggregation can be enhanced by the production of TxA_2. The pretreatment of platelets with COX inhibitors such as aspirin could decrease the response of collagen-induced platelet aggregation by inhibiting the production of TxA_2 (Roberts et al., 2004). Platelet aggregations in response to collagen typically display longer lag time than other agonist in order to increase intracellular calcium, which stimulate granule secretion and the production of TxA_2. The results demonstrated that 500 μM ASA inhibited collagen-induced platelet aggregation with decreasing slope. ASA inhibited collagen-induced platelet aggregation with prolonged lag time (150% of control) *via* inhibition of TxA_2 production (Englyst et al., 2003). ADE, at the concentration of 1 mg/ml, inhibited collagen-induced platelet aggregation. The results in this study demonstrated that ADE inhibited collagen induced platelet aggregation by prolonged lag time and decreased slope (table 4.5). Therefore, the antiplatelet activity of ADE might affect on calcium mobilization with affecting granule secretion such as ADP, 5-HT, epinephrine, etc. The modes of action of ADE whether it could inhibit calcium still unreveal. In the further study, it is interesting to investigate on calcium mobilization.

However, imperatorin at the concentration of 300 μM (81.1 μg/ml) did not show the inhibitory effect on collagen-induced platelet aggregation. In contrast to the present study,

Chen et al. (1996) studied the coumarins isolated from *Peufanum Japonicum* on washed rabbit platelets and reported that imperatorin at the concentration of 100 μg/ml inhibited AA- and collagen-induced platelet aggregation. This contradictory effect of imperatorin may be due to the lower concentration used in the present study or the differences between the platelet membrane protein and GP composition of rabbit, and human platelets, which may explain some of the differences observed with aggregation responses in these species (Toor et al., 1982).

From the results of this study, there were differences between ADE and imperatorin effects. Imperatorin could not inhibit collagen-induced platelet aggregation, whereas ADE inhibited collagen-induced platelet aggregation. Zheng et al. (2010) purified and quantified 11 coumarins from 12 samples of *Angelica dahurica* in different parts of China using HPLC-ESI-MS/MS method. Among these coumarins, ADE had imperatorin by 20%. The study of Chen et al. (1996) demonstrated that bergapten and cnidilin could inhibit AA- and collagen-induced platelet aggregation. ADE had cnidilin and bergapten approximately 14% and 4%, respectively. Therefore, there might have other chemical compounds in ADE, which are resposible for antiplatelet activity.

Both ADP and collagen have a common pathway *via* difference subtypes of phospholipases C (PLC) causing the formation of IP_3 and DAG as mention above. In addition, ADP can inhibit adenylate cyclase activity inducing platelet aggregation. Dutta-Roy demonstrated that Ginkgo biloba extract (GBE) at 4 mg/ml inhibited ADP- and collagen-induced platelet aggregation in vitro using platelet rich plasma (PRP) but not inhibited AA-induced platelet aggregation. GBE did not act as specific antagonist to platelet receptors. GBE at 4 mg/ml was able to increase cAMP levels in platelets and inhibited TxB_2 production (Dutta-Roy et al., 1999). Lazarus and Garg (2004) demonstrated that tomato extracts inhibited ADP- and collagen-induced platelet aggregation but did not inhibited AA-induced platelet aggregation using PRP. The studied found that tomato extracts (40 μL) did not increase cAMP. The study suggested that its inhibitory effects might act through the

PLC pathway upstream of COX (Lazarus and Garg, 2004). There was a crosslink between ADP and collagen signaling pathway. Both ADP and collagen was able to promoted $\alpha_{IIA}\beta_3$ activation. GPVI, a main mediated signaling platelet collagen receptor, dependent on secreted ADP signaling especially through $P2Y_{12}$ ADP coupled to G_i dependent pathway (Larson et al., 2003; Lova et al., 2002). In this study, the results demonstrated that ADE could inhibit ADP- and collagen-induced platelet aggregation but ADE did not inhibited AA-induced platelet aggregation. These results suggested that the inhibitory effects of ADE might act *via* common pathway. The modes of action of ADE and imperatorin might mediate from inhibition of ADP signaling pathway. The results in this demonstrated the antagonism of ADE (0.42 and 0.84 mg/ml) showed the rightward shift of ADP concentrations - response curve, indicating that ADE might be an antagonist of ADP. The antiplatelet activity of ADE might mediate *via* adenylate cyclase – cAMP pathway.

Prostaglandin (PGE_1) is one of the most potent inhibitors of platelet aggregation. It directly stimulates adenylate cyclase causing increased intracellular cAMP. On the other hand, the inhibitors of phosphodiesterase inhibit the conversion of cAMP to AMP and also increase cAMP level. Increased cAMP leads to inhibition of calcium influx and secretion by suppression of IP_3, DAG and TxA_2 activated calcium influx, and thus, inhibits platelet aggregation. IBMX, a nonselective phosphodiesterase inhibitor, at the concentration of 0.5 mM could raise intracellular cAMP (15 pmole/ $5x10^5$ platelets) in resting washed human platelets with in absence of PGE_1. In addition, IBMX (6 μM) could inhibit platelet aggregation induced by thrombin (0.01U) in washed human platelets (Liu et al., 2009). Our results showed that IBMX (200μM or 44.5 μg/ml) could increase cAMP levels in ADP induced platelet aggregations, similar to ADE at 1 mg/ml. Thus, the antiplatelet activity of ADE may be mediated by increased cAMP leading to a decrease in secretion. This effect might act *via* stimulation of adenylate cyclase or *via* inhibition of phosphodiesterase. On the other hand, our results showed that ADE could not inhibit thromboxane formation (figure

4.15), suggesting that antiplatelet activity of ADE did not mediated *via* COX pathway. However, ADE decreased the slope of AA-induced platelet aggregation, indicating that ADE slowed the initiation of platelet ggregation but its action was not strong enough to reduce the AA-induced platelet aggregation since ADE did not inhibit COX activity.

The overall results, confirmed that the inhibitory effects of ADE might act *via* common pathway at least involved the cAMP pathway either by inhibiton of Gi couple to adenylate cyclase activity *via* $P2Y_{12}$ or by inhibition of intracellular signaling leading to increase cAMP level but not arachidonic acid-thromboxane pathway. However, the antiplatelet activity of imperatorin was lower than ADE and imperatorin can inhibit platelet aggregation induced by ADP but not by collagen or AA. So the antiplatelet activity of ADE might come from more than one compound other than imperatorin.

ADE at the concentration of 1 mg/ml was a high concentration to produce its inhibitory effects in ADP- and collagen-induced platelet aggregation. While most of the previously biological activities of the root of AD reported in a much lower concentration such as; 50 and 100 µg/ml of ethyl acetate of ADE for the inhibition of LPS induced tumor necrosis factor -α (TNF-α), nitric oxide (NO) and PGE_2 expression in murine macrophage (Kang et al., 2007); 25-200 µg/ml of 70% ethanolic ADE for the suppression of the NF-KB pathway and inhibition of COX-2 expression and decrease inflammatory mediators (Kang, Lee et al., 2007; Lee et al., 2011); approximately 200 µg/ml of the 50% inhibition concentration of furanocoumadine compounds from ethanolic extract of dried root of AD for antioxidant activity (Piao et al., 2004) So, the concentration of ADE used for antiplatelet activity has approximately 5-10 times more than those of the previous reported for other biological activities, indicating a wide margin of safety because it needed high amount of ADE to produce its inhibitory effects on platelets. Therefore, consuming Thai traditional medicine that contained ADE is safe enough for therapeutic use. However, it should be used with precaution when administration with antiplatelet agents or anticoagulants.

In conclusion, the results of this study demonstrated that the extracts from the root of *Angelica Dahurica* has antiplatelet activity on ADP and collagen induced platelet

aggregation, whereas imperatorin has antiplatelet only on ADP induced platelet aggregation Both ADE and imperatorin could not inhibit arachidonic acid induced platelet aggregation, thus its antiplatelet does not involve AA-TxA_2 pathway. The antiplatelet property of ADE and imperatorin at least take part in adenylate cyclase pathway which mediated in increase cAMP level and might lead to delay or decrease dense granule secretion. ADE has a mild antiplatelet activity at high concentration, so it is safe for use in Thai traditional medicine, however; the beneficial for prevent thrombosis should be further investigated.

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APPENDIX

Identification of *Angelica dahurica* by Thin-layer chromatography (TLC)

Dried-root powder of *Angelica dahurica* 200 mg was extracted with 80% ethanol by ultra-sonication for 30 min. Apply 15 ml of the supernatant to the thin layer chromatographic plate, using silica gel $60F_{254}$ as the coating substance (Merck 5554). Solvent system is Toluene-EtOAc-Formic acid (8.5:1.5:0.5) and run for 15 cm. The plate was sprayed with anisaldehyde spray reagent and visible after heating at 110°C for 10 min.

TLC chromatogram of the 80% ethanolic extract of *Angelica dahurica*

Identification of *Angelica dahurica* by High-performance liquid chromatography (HPLC)

1. Sample preparation: same as TLC

Chromatographic system: Agilent 1100 series pump, on-line solvent degasser, autosampler, photodiode-array detector (DAD) and analysis by Chemstation software Version A.08.01 (Agilent Technologies, USA)

Column: Zorbax Eclipse XDB - C18 (4.6 x 250 mm, 5 micron) (Agilent Part No. 990967-90, S/N USNH007443 Lot No. B05009, USA)

Mobile phase: Gradient system

Solvent A = Methanol

Solvent B = 1% v/v acetic acid in water; pH 2.7

Time (min)	% Solvent A	% Solvent B
0	0	100
5	0	100
45	40	60
55	80	20
60	80	20
65	0	100
70	0	100

Volumn of injection: 20 µl

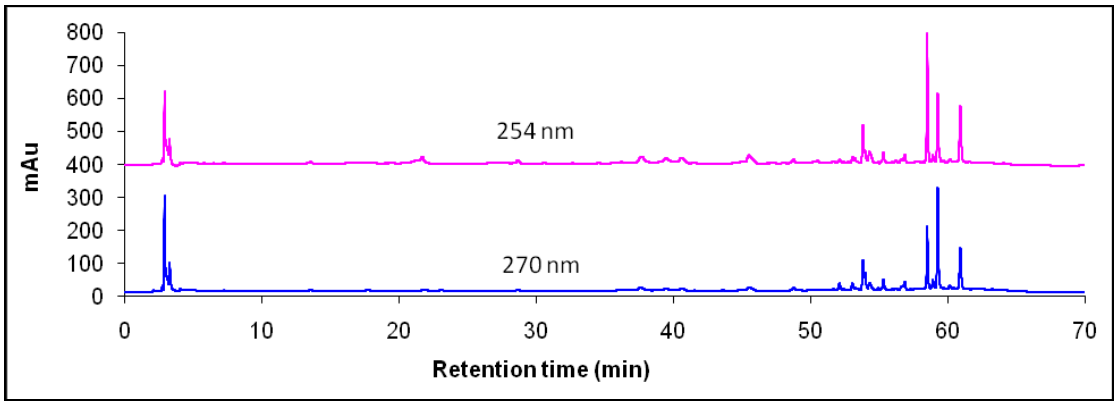
Flow rate: 1.0 ml/min

Detector: Diode-array detector

Wavelenght : 254 and 270 nm

Temperture: 27 ^{o}C

Run time: 70 min



HPLC Chromatogram of the 80% ethanolic extract of *Angelica dahurica*

Study the effect of ADE and imperatorin on platelet aggregation induced by ADP, arachidonic acid and collagen.

1. The effect of ADE on ADP induced platelet aggregation

1.1. The effect of ADE at concentrations 0.1-1mg/ml on ADP induced platelet aggregation after pretreated PRP for 3 min before adding ADP (5 or 10 μM).

A) Percent maximum aggregation of ADE

Sample	mean	SEM
0.5% DMSO	77.36	4.38
500 μM ASA	53.77	2.78
ADE 0.1 mg/ml	78.30	5.03
ADE 0.25 mg/ml	77.86	5.13
ADE0.5 mg/ml	61.18	8.71
ADE 1 mg/ml	28.17	0.95

B) Percent inhibition of ADE compared with vehicle control (0.5% DMSO)

Sample	mean	SEM
500 μM ASA	29.46	5.32
ADE 0.1 mg/ml	-1.07	1.72
ADE 0.25 mg/ml	-0.46	2.07
ADE0.5 mg/ml	21.65	9.28
ADE 1 mg/ml	62.82	3.48

1.2. The effect of imperatorin at concentrations 10-300 μM on ADP induced platelet aggregation after pretreated PRP for 3 min before adding ADP (5 or 10 μM).

A) Percent maximum aggregation of imperatorin

Sample	mean	SEM
0.5% DMSO	72.83	2.11
500 μM ASA	42.28	3.24
Imperatorin 10 μM	76.19	1.73
Imperatorin 25 μM	70.35	4.47
Imperatorin 37.5 μM	65.83	2.00
Imperatorin 75 μM	64.00	5.17
Imperatorin 150 μM	55.89	6.61
Imperatorin 300 μM	48.23	6.45

B) Percent inhibition of imperatorin compared with vehicle control (0.5% DMSO)

Sample	mean	SEM
500 μM ASA	41.79	1.769
Imperatorin 10 μM	-4.77	4.756
Imperatorin 25 μM	3.64	2.117
Imperatorin 37.5 μM	7.82	4.117
Imperatorin 75 μM	12.38	1.207
Imperatorin 150 μM	23.61	5.521
Imperatorin 300 μM	33.96	8.133

1.3. Aggregation rate (slope) of ADE and imperatorin after adding ADP

A) ADE

Sample	mean	SEM
0.5% DMSO	131.63	11.06
500 μM ASA	118.63	5.06
ADE 0.1 mg/ml	115.61	10.47
ADE 0.25 mg/ml	112.30	6.97
ADE0.5 mg/ml	99.15	8.87
ADE 1 mg/ml	65.04	3.67

B) Imperatorin

Sample	mean	SEM
0.5% DMSO	103.33	5.16
500 μM ASA	99.49	7.21
Imperatorin 10 μM	104.57	3.94
Imperatorin 25 μM	104.73	5.68
Imperatorin 37.5 μM	95.92	4.21
Imperatorin 75 μM	104.39	4.41
Imperatorin 150 μM	103.47	3.84
Imperatorin 300 μM	104.86	6.48

2. The effect of ADE and imperatorin on arachidonic acid induced platelet aggregation

2.1 The effect of ADE at concentrations 0.1-1mg/ml on arachidonic acid induced platelet aggregation after pretreated PRP for 3 min before adding arachidonic acid (0.4 or 0.8 mM).

A) Percent maximum aggregation of ADE

Sample	mean	SEM
0.5% DMSO	82.10	2.65
500 μM ASA	26.12	3.95
ADE 0.1 mg/ml	83.47	3.58
ADE 0.25 mg/ml	82.69	4.37
ADE 0.5 mg/ml	81.83	3.20
ADE 1 mg/ml1	60.68	17.13

B) Percent inhibition of ADE compared with vehicle control

Sample	mean	SEM
500 μM ASA	74.14	7.34
ADE 0.1 mg/ml	-2.21	1.59
ADE 0.25 mg/ml	-3.30	1.61
ADE 0.5 mg/ml	0.50	2.51
ADE 1 mg/ml1	8.00	6.41

2.2. The effect of and imperatorin at concentrations 10-300 μM on arachidonic acid induced platelet aggregation after pretreated PRP for 3 min before adding arachidonic acid (0.4 or 0.8 mM).

A) Percent maximum aggregation of imperatorin

Sample	mean	SEM
0.5% DMSO	74.83	1.01
500 μM ASA	7.00	1.69
Imperatorin 10 μM	75.06	0.71
Imperatorin 25 μM	75.95	1.29
Imperatorin 37.5 μM	76.08	0.86
Imperatorin 75 μM	76.94	1.33
Imperatorin 150 μM	75.13	0.87
Imperatorin 300 μM	75.67	0.96

B) Percent inhibition of imperatorin compared with vehicle control

Sample	mean	SEM
500 μM ASA	90.77	2.18
Imperatorin 10 μM	-0.37	1.43
Imperatorin 25 μM	-1.52	1.40
Imperatorin 37.5 μM	-1.72	1.31
Imperatorin 75 μM	-2.87	1.83
Imperatorin 150 μM	-0.47	1.54
Imperatorin 300 μM	-1.20	1.85

2.2 Aggregation rate (slope) of ADE and imperatorin after adding arachidonic acid.

A) ADE

Sample	mean	SEM
0.5% DMSO	107.85	27.39
500 μM ASA	0.84	0.64
ADE 0.1 mg/ml	115.61	22.30
ADE 0.25 mg/ml	112.30	23.39
ADE0.5 mg/ml	99.15	23.50
ADE 1 mg/ml	65.04	11.88

B) Imperatorin

Sample	mean	SEM
0.5% DMSO	141.39	3.99
500 μM ASA	1.18	0.66
Imperatorin 10 μM	104.57	4.02
Imperatorin 25 μM	104.73	4.39
Imperatorin 37.5 μM	95.92	5.15
Imperatorin 75 μM	104.39	7.42
Imperatorin 150 μM	103.47	10.69
Imperatorin 300 μM	104.86	12.18

3. The effect of ADE and imperatorin on collagen induced platelet aggregation

3.1 The effect of ADE at concentrations 0.1-1mg/ml on collagen induced platelet aggregation after pretreated PRP for 3 min before adding collagen (30 µg/ml).

A) Percent maximum aggregation of ADE

Sample	mean	SEM
0.5% DMSO	81.75	6.99
500 µM ASA	25.35	1.25
ADE 0.1 mg/ml	81.06	5.87
ADE 0.25 mg/ml	73.26	13.58
ADE 0.5 mg/ml	62.17	6.99
ADE 1 mg/ml1	34.89	6.99

B) Percent inhibition of ADE compared with vehicle control (0.5% DMSO)

Sample	mean	SEM
500 µM ASA	64.10	12.31
ADE 0.1 mg/ml	0.36	2.41
ADE 0.25 mg/ml	10.33	6.37
ADE0.5 mg/ml	22.55	16.77
ADE 1 mg/ml	66.28	2.78

3.2 The inhibition of imperatorin 10-300 μM on collagen induced platelet aggregation after pretreated PRP for 3 min before adding collagen (30 μg/ml)..

A) Percent maximum aggregation of imperatorin

Sample	mean	SEM
0.5% DMSO	76.80	1.93
500 μM ASA	36.35	9.09
Imperatorin 10 μM	75.18	0.95
Imperatorin 25 μM	74.16	0.48
Imperatorin 37.5 μM	77.59	1.84
Imperatorin 75 μM	75.53	2.18
Imperatorin 150 μM	71.10	4.32
Imperatorin 300 μM	71.63	2.91

B) Percent inhibition of imperatorin compared with vehicle control

Sample	mean	SEM
500 μM ASA	41.58	13.12
Imperatorin 10 μM	-5.60	2.96
Imperatorin 25 μM	-2.80	1.30
Imperatorin 37.5 μM	-4.00	0.30
Imperatorin 75 μM	-3.51	2.08
Imperatorin 150 μM	-4.21	1.57
Imperatorin 300 μM	0.64	1.46

3.3 Aggregation rate (slope) of ADE and imperatorin after adding collagen.

A) ADE

Sample	mean	SEM
0.5% DMSO	59.25	1.56
500 μM ASA	2.96	1.65
ADE 0.1 mg/ml	70.76	1.82
ADE 0.25 mg/ml	57.83	8.67
ADE0.5 mg/ml	45.28	13.33
ADE 1 mg/ml	11.34	7.34

B) Imperatorin

Sample	mean	SEM
0.5% DMSO	70.81	10.43
500 μM ASA	22.63	15.57
Imperatorin 10 μM	76.55	14.26
Imperatorin 25 μM	79.07	12.33
Imperatorin 37.5 μM	74.17	17.44
Imperatorin 75 μM	74.48	14.53
Imperatorin 150 μM	73.28	14.87
Imperatorin 300 μM	74.97	12.38

3.4 Lag time of ADE and imperatorin after adding collagen.

A) ADE

Sample	mean	SEM
0.5% DMSO	116.35	17.25
500 μM ASA	300.75	36.13
ADE 0.1 mg/ml	106.16	30.75
ADE 0.25 mg/ml	140.25	48.11
ADE0.5 mg/ml	198.39	60.10
ADE 1 mg/ml	367.78	8.28

B) Imperatorin

Sample	mean	SEM
0.5% DMSO	106.67	35.67
500 μM ASA	143.33	57.03
Imperatorin 10 μM	58.75	9.92
Imperatorin 25 μM	62.08	11.81
Imperatorin 37.5 μM	55.00	11.73
Imperatorin 75 μM	61.67	11.29
Imperatorin 150 μM	69.17	13.13
Imperatorin 300 μM	76.25	19.24

Study of the antagonism of ADE and imperatorin on ADP-induced platelet aggregation

The effect of 250 μM ASA, ADE at concentration 0.42, 0.84 mg/ml and 150 μM imperatorin on ADP induced platelet aggregation with increasing concentration of ADP (1-80 μM)

A) The antagonism of 250 μM ASA on ADP-induced platelet aggregation

Sample	ADP (μM)	Mean	SEM
0.5% DMSO + ADP	1	17.72	3.37
	2.5	49.23	13.19
	5	73.18	8.50
	10	82.43	1.71
	20	82.42	2.19
250 μM ASA + ADP	1	17.25	1.03
	2.5	29.85	0.74
	5	42.58	2.86
	10	56.15	3.50
	20	66.95	0.20

B) The antagonism of ADE at 0.42 mg/ml on ADP-induced platelet aggregation

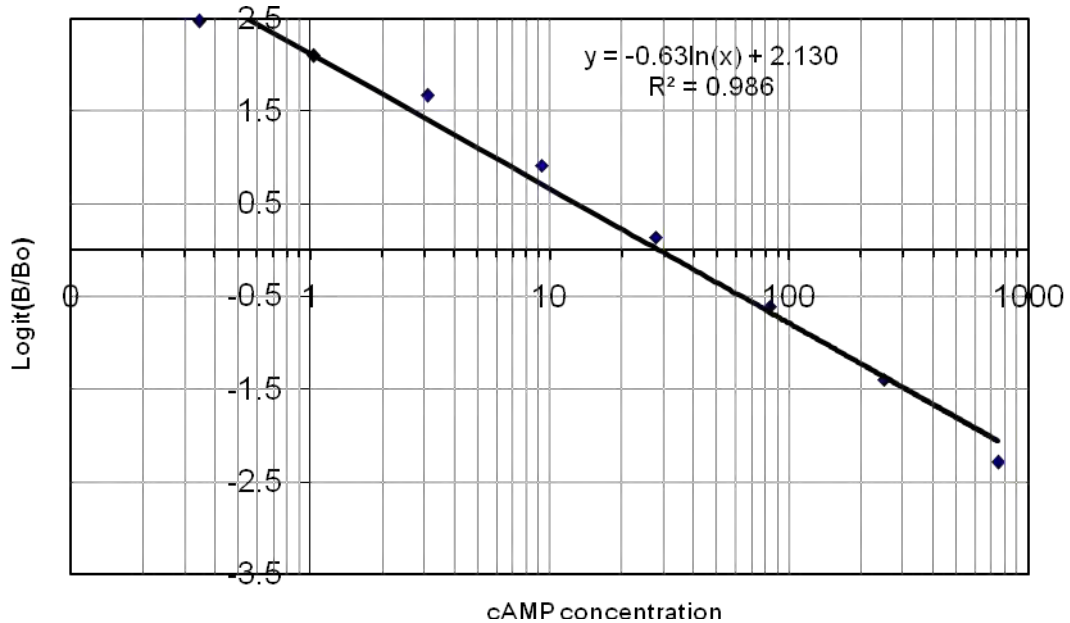
Sample	ADP (μM)	Mean	SEM
0.5% DMSO + ADP	1	14.03	2.75
	2.5	31.08	3.69
	5	57.53	8.27
	10	66.97	0.46
	20	70.18	6.44
ADE 0.42 mg/ml + ADP	1	6.28	1.02
	2.5	15.68	5.93
	5	35.73	8.66
	10	66.72	8.31
	20	76.59	4.66

C) The antagonism of ADE at 0.84 mg/ml on ADP-induced platelet aggregation

Sample	ADP (μM)	Mean	SEM
0.5% DMSO + ADP	1	10.69	2.67
	2.5	41.28	5.16
	5	67.94	3.60
	10	73.76	3.23
	20	74.59	3.34
	40	74.28	3.78
	80	77.87	3.79
ADE 0.84 mg/ml + ADP	1	7.00	2.67
	2.5	22.70	5.16
	5	45.80	3.60
	10	76.07	3.23
	20	80.77	3.34
	40	80.05	3.78
	80	76.40	3.79

D) The antagonism of 150 μM imperatorin on ADP-induced platelet aggregation

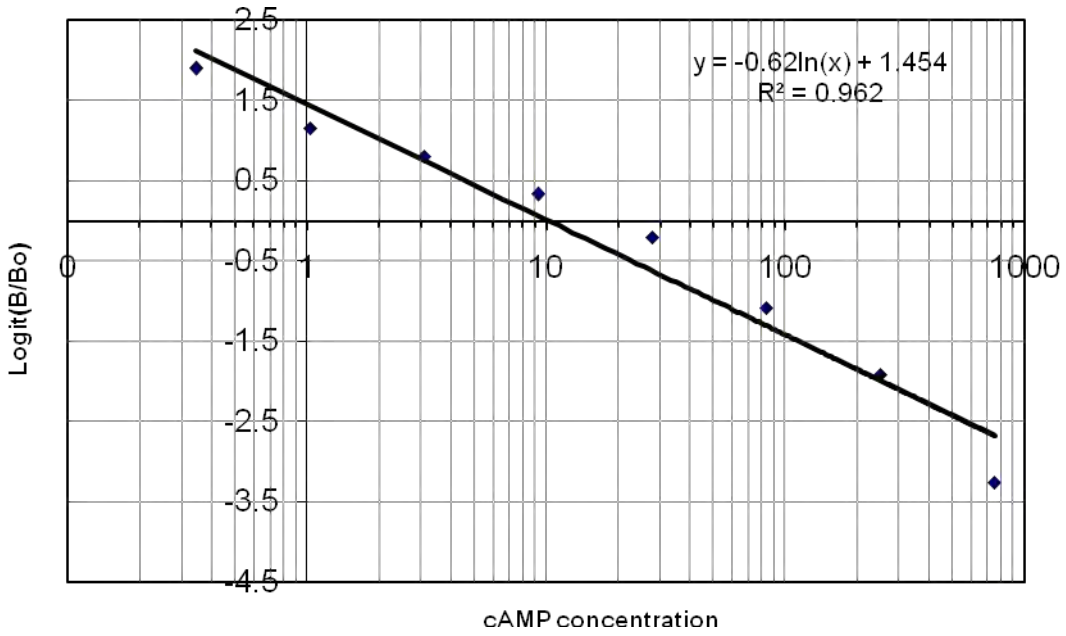
Sample	ADP (μM)	Mean	SEM
0.5% DMSO + ADP	1	15.55	2.54
	2.5	48.35	11.40
	5	64.95	8.90
	10	71.93	6.78
	20	72.05	1.73
	40	76.35	2.76
	80	76.43	2.30
150 μM imperatorin + ADP	1	18.02	1.81
	2.5	38.85	2.16
	5	63.23	7.76
	10	68.53	5.13
	20	75.58	2.22
	40	74.95	1.58
	80	75.95	2.17



Standard curve of cAMP level measurement for ADE

Effect of ADE on cAMP level

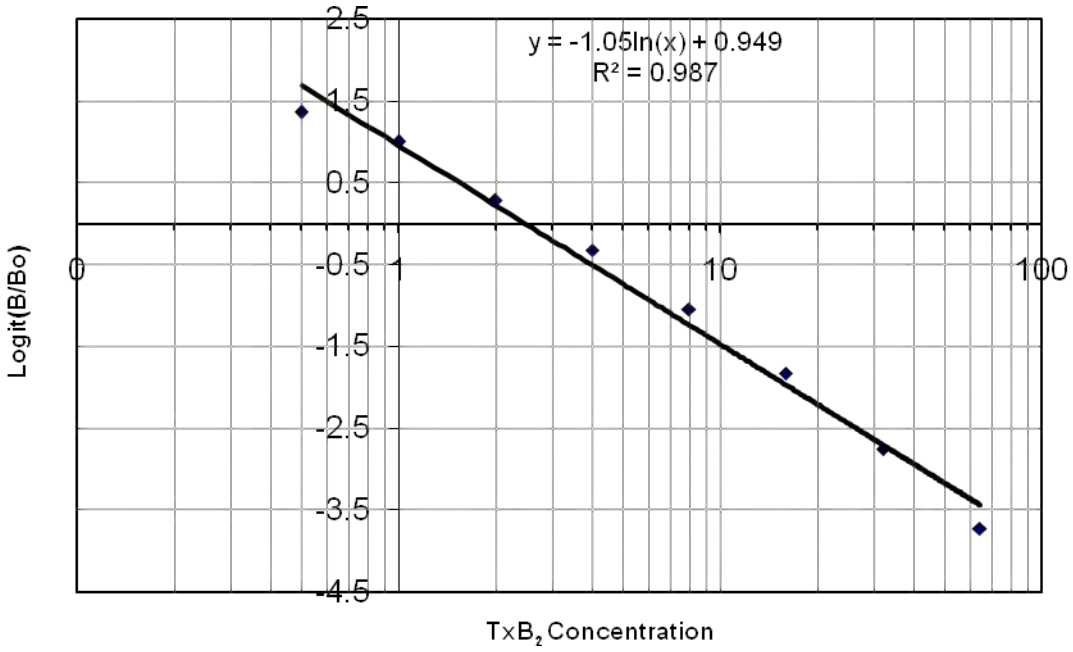
Sample	mean	SEM
basal cAMP	14.73	2.069
0.5% DMSO	26.85	7.913
IBMX 200 μM	47.40	9.979
ADE 0.1 mg/ml	30.37	7.360
ADE 0.25 mg/ml	42.49	10.431
ADE 0.5 mg/ml	38.43	8.340
ADE 1 mg/ml	50.40	14.716



Standard curve of cAMP level measurement for imperatorin

Effect of imperatorin on cAMP level

Sample	mean	SEM
basal cAMP	21.01	3.055
0.5% DMSO	14.92	2.638
IBMX 200 μM	37.45	0.765
Imperatorin 10 μM	20.05	5.493
Imperatorin 75 μM	25.12	6.394
Imperatorin 150 μM	32.37	10.541
Imperatorin 300 μM	22.81	4.966



Standard curve of TxB_2 level measurement for ADE

Effect of ADE on TxA_2 level

Sample	mean	SEM
0.5% DMSO	12.67	1.784
500 μM ASA	2.42	0.165
ADE 0.1 mg/ml	16.11	0.587
ADE 0.25 mg/ml	13.45	1.766
ADE 0.5 mg/ml	17.74	0.443
ADE 1 mg/ml	15.80	1.355

BIOGRAPHY

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