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MECHANISM OF ANTIPLATELET AGGREGATION OF *ANGELICA DAHURICA* EXTRACT IN H UMAN PLATELETS

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วิวรรณ พรหมประดิษฐ์ : กลไกการออกฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดมนุษย์ของสารสกัดโกฐสอ (MECHANISM OF ANTIPLATELET AGGREGATION OF *ANGELICA DAHURICA* EXTRACT IN HUMAN PLATELETS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภญ. ดร. รัตยา ลือชาพุฒิพร, 58 หน้า.

สารสกัดโกฐสอมีฤทธิ์ในการต้านการเกาะกลุ่มของเกล็ดเลือด ซึ่งนำมาใช้ในการรักษาโรคระบบไหลเวียนเลือดผิดปกติ วัตถุประสงค์ของการศึกษานี้เพื่อศึกษากลไกการยับยั้งการเกาะกลุ่มกันของเกล็ดเลือดของสารสกัดโกฐสอในเกล็ดเลือดของมนุษย์เมื่อกระตุ้นด้วยอะดีโนซีนไดฟอสเฟต (ADP) (40 ไมโครโมลลาร์) และคอลลาเจน (20 ไมโครกรัมต่อมิลลิลิตร) และวิเคราะห์การแสดงออกของโปรตีนที่เกิดขึ้นในเกล็ดเลือด จากผลการทดลองแสดงให้เห็นว่าสารสกัดโกฐสอที่ความเข้มข้น 0.1, 0.25, 0.5 และ 1 มิลลิกรัมต่อมิลลิลิตรยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่กระตุ้นด้วย ADP อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับตัวทำละลายควบคุมมีค่า 38.2±13.7% (p<0.05), 50.2±11.6% (p=0.01), 73.4±8.5% (p<0.001) และ 79.8±7.4% (p<0.001) ตามลำดับ โดยสารสกัดโกฐสอที่ความเข้มข้น 0.1, 0.25, 0.5 และ 1 มิลลิกรัมต่อมิลลิลิตรมีการเพิ่มการเติมหมู่ฟอสเฟตบน VASP ที่ตำแหน่ง Ser^{157} (6.7±2.3 และ 8.3±2.2; p<0.05) มากกว่าที่ตำแหน่ง Ser^{239} (3.0±1.4; p=0.054 และ 5.1±1.5; p=0.054) ตามลำดับอย่างมีนัยสำคัญทางสถิติ แสดงให้เห็นว่าการเติมหมู่ฟอสเฟตบน VASP น่าจะผ่านทางวิถีไซคลิกอะดีโนซีนโมโนฟอสเฟต/โปรตีนไคเนสเอ (cAMP/PKA) โกฐสอที่ความเข้มข้น 0.1, 0.25, 0.5 และ 1 มิลลิกรัมต่อมิลลิลิตรยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่กระตุ้นด้วยคอลลาเจนอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับตัวทำละลายควบคุม มีค่า 52.8±14%, 60.6±8.9%, 84.3±2% และ 85.8± 2.3% (p<0.001) ตามลำดับ แต่ไม่ลด PI3K p 85 และการเติมหมู่ฟอสเฟตของ Akt ที่ตำแหน่ง Thr^{308} โดยสรุปแล้วสารสกัดโกฐสอมีกลไกการออกฤทธิ์ยับยั้งการเกาะกลุ่มของเกล็ดเลือดโดยเพิ่มการเติมหมู่ฟอสเฟตบน VASP ผ่านวิถี cAMP/PKA

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต .....

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VIVAN PROMPRADITH: MECHANISM OF ANTIPLATELET AGGREGATION OF *ANGELICA DAHURICA* EXTRACT IN HUMAN PLATELETS. ADVISOR: ASST. PROF. RATAYA LUECHAPUDIPORN, Ph.D., 58 pp.

Angelica dahurica extract (ADE) has been shown to exhibit antiplatelet aggregation properties, which promise to be used as a therapeutic agent for circulatory disorder. The aim of the present study was to investigate the mechanism of ADE in antiplatelet aggregation in human platelets. The effect of ADE on human platelet aggregation induced by adenosine diphosphate (ADP, 40 μM) and collagen (20 μg/ml) were explored in vitro, and the potential mechanism such activity were investigated using western blot analysis. The result showed that ADE at the concentration of 0.1, 0.25, 0.5 and 1 mg/ml significantly inhibited ADP-induced platelet aggregation by 38.2±13.7% (p<0.05), 50.2±11.6% (p=0.01), 73.4±8.5% (p<0.001) and 79.8±7.4% (p<0.001), respectively when compare to vehicle control. ADE at the concentration of 0.5 and 1 mg/ml significantly activated the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser^{157} (6.7±2.3 and 8.3±2.2; p<0.05), more than those at Ser^{239} (3.0±1.4; p=0.054 and 5.1±1.5; p=0.054), respectively, suggesting that VASP phosphorylation could mediated mainly through cAMP/PKA pathway. ADE at concentration of 0.1, 0.25, 0.5 and 1 mg/ml significantly inhibited collagen-induced platelet aggregation by 52.8±1%, 60.6±8.9%, 84.3±2% and 85.8± 2.3% (p<0.001), respectively when compare to vehicle control. ADE did not decrease phosphorylation of Akt at Thr^{308}. In conclusion, the mechanism of antiplatelet aggregation of ADE may include the increase of VASP phosphorylation and mediated through cAMP/PKA pathway.

Department:	Pharmacology and Physiology	Student's Signature .....
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LIST OF ABBREVAITION

AC	Adenylate cyclase
ACD	Acid citrate dextrose
AD	*Angelica dahurica*
AD	*Angelica dahurica* extract
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
°C	Degree celcius
cm	Centimeter
CVD	Cardiovascular disease
COX	Cyclooxygenase
DAG	Diacylglycerol
eNOS	Endothelial nitric oxide synthase
FcRγ	Fc receptor γ –chain
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GP	Glycoprotein
GPCRs	G protein-coupled receptors

5-HT	5-Hydoxytryptamine
h	hour
IP3	Inostol (1, 4, 5)-triphosphate
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
mg	Milligram
min	minute
ml	milliliter
mm	millimeter
mM	Millimolar
mU	milliunit
kDa	kilo dalton
kU	kilounit
Nmol	Nanomol
NO	Nitric oxide
PARs	Protease-activated receptors
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PI3Ks	Phosphoinositide 3-kinases
PIP_2	phosphatidylinositol-4,5-bisphosphate

PLC	Phospholipase C
PGI_2	Prostacyclin I_2
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PRP	Platelet rich plasma
PVDF	Polyvinyllidene fluoride
β-TG	β-thromboglobulin
Ser	Serine
Thr	Threonine
TxA_2	Thromboxane A_2
VASP	Vasodilator-stimulated phosphoprotein
V	Volt
vWF	von Willebrand factor
WHO	World Health Organization

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Platelets have an essential role in hemostasis by preventing blood loss and maintain vascular integrity. When vascular injury has occurred, platelets become exposed to subendothelial matrix, in particular collagen; induce platelet adhesion, activation and aggregation into a stable hemostatic plug to heal the vessel wall (1). On the other hand, the formation of platelet aggregation may lead to pathophysiological of cardiovascular diseases, such as thrombosis, atherosclerosis and myocardial infarction, and then increase the mortality (2). According to the World Health Organization (WHO), cardiovascular diseases (CVD) are the biggest cause of deaths globally. More than 17 million people died from CVD in 2012, approximated 31% of all global death due to coronary heart disease and stroke. Ministry of Public Health of Thailand also reported that death rate from CVD was increasing in 2011-2013 from approximately 62 to 84 deaths per 100,000 population (3).

In normal circulation, most platelets never interact with the endothelial surface during their entire lifetime. However, if vascular is injured, the subendothelial extracellular matrix such as collagen and von Willebrand factor (VWF) are exposed, and then platelets promptly adhere in order to stop hemorrhage and promote tissue healing. Collagens are the most potent mediators of platelet adhesion. After platelets exposure to collagen *via* glycoprotein-VI (GP-VI) receptor lead to activation of phospholipase Cγ (PLCγ), which by hydrolyzing membrane phosphatidylinositol-4,5-bisphosphate (PIP_2) generate two secondary messengers: inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Then release calcium and activate

protein kinase C (PKC), resulting in shape change and secretion of soluble platelet agonists from dense granules such as adenosine diphosphate (ADP), and thromboxane A_2 (TxA_2). ADP is the prominent amplifier of platelet activation. ADP induces platelet aggregation *via* two G-protein coupled receptors (GPCRs): $P2Y_1$ and $P2Y_{12}$ which activate PLCβ and inhibit adenylate cyclase (AC), respectively. The $P2Y_1$ receptor mediates calcium mobilization, shape change and transient aggregation. The $P2Y_{12}$ receptor potentiates platelet secretion and involve in irreversible aggregation by decrease cyclic adenosine monophosphate (cAMP) level and vasodilator-stimulated phosphoprotein (VASP) phosphorylation.

Antiplatelet agents are used for prevention and treatment of CVD to reduce mortality. The well-known meta-analysis, the Antiplatelet Trialists' Collaboration (ATC) found a 25% relative risk reduction of vascular death, myocardial infarction or stroke for aspirin, versus placebo (4). In the Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) trial, an overall relative risk reduction of 8.7% in vascular death, ischemic stroke or myocardial infarction was found in clopidogrel versus aspirin. Clopidogrel showed clinically benefit over aspirin in patients at high risk of atherothrombotic with a relative risk reduction of 14.9% (5). However, limitations of current antiplatelet therapy are drug resistance, less efficacy in some patients and adverse effects, such as gastric ulcer, renal failure and bleeding (6). Therefore, the development of new antiplatelet agents still needed and traditional medicine may be an alternative source for therapeutic drug in CVD.

Angelica dahurica (Fisch. Ex Hoffm.) Benth. & Hook.f.ex Franch & Sav is in Umbelliferae Family. *Angelica dahurica* (AD) is called Bai Zhi, named in China or Kot-Sor, named in Thailand. Chinese traditional medicine uses Bai Zhi as antipyretic, analgesic or cold, toothache and headache (7). The root of AD has been widely used and composed in Thai traditional medicine including YA-HOM which is used for

treatment of circulatory disorder (8). AD extract (ADE) is contained of more than 20 furanocoumarins such as 5-methoxypsoralen, bergapten, byakangelicin, imperatorin, isoimperatorin, isopimpineline, ostenol, oxypeucedanin hydrate (9, 10). The previous study showed that ADE at 1 mg/ml inhibited platelet aggregation induced by ADP and collagen. ADE increased lag phase in collagen induced platelet aggregation. In addition, ADE significantly increased cAMP level indicating that the antiplatelet activity of ADE may take part in adenylate cyclase – cAMP pathway (11). Therefore, this study was focus on investigating the potential mechanism of *Angelica dahurica* extract in antiplatelet aggregation via cAMP pathway in human platelets.

CHAPTER II
LITERATURE REVIEWS

2.1 Platelet morphology

Platelets are small, anucleate cells in the circulating blood. They are produced from cytoplasm of megakaryocytes in a process called thrombopoiesis in bone marrow, the same as the red cells and the white blood cells. Platelets have discoid shape like a plate and range in size between 2.0 and 5.0 μm in diameter. They are only about 20% of the diameter of red blood cells. Platelets typically circulate in the blood for 7-10 days. Subsequently, dead platelets were eliminated by macrophages, called phagocytosis in liver and spleen. Platelets are produced approximately 35×10^{9} platelets daily. In blood circulation, normal platelet counts are about 150,000 to 350,000 platelets per microliter and one-third of platelets are stored in spleen (12).

2.2 Platelet structure

Platelet structural elements can be divided into four zones: peripheral zone, sol-gel zone, organelle zone and membrane system as shown in figure 1 (13).

(1) Peripheral zone

This zone can be divided into 3 domains: exterior coat, unit membrane and submembrane region.

- Exterior surface of the platelet or glycocalyx, this area is the first site of contact and is covered with receptors such as glycoprotein (GP) Ia, Ib, Ic, IIb, IIIa, IV, V

and XI. The GPs are the receptor, that sense changes in the platelet environment and facilitate adhesion adhesion and aggregation.

- Unit membrane is a lipid bilayer, similar to that of other cell types. The platelet cytoskeleton is important for regulating the platelet shape. It contains an anion and cation pumps, which maintain transmembrane ionic gradients.

- Submembrane region is the area lying under the unit membrane. It contained of numerous protein which regulated the signaling process of platelet activation.

(2) Sol-gel zone or structural zone

Sol-gel zone is the matrix of the cytoplasm under the submembrane region, which consists of the platelet microtubule coil. It is formed from a single microtubule that is wound 8 to 12 times into a coil, which supports the discoid shape of the resting platelet, internal transformation of activated platelet, contraction of the platelet plug and hemostatic.

(3) Organelle zone

The cellular organelles contain mitochondria and golgi apparatus that are common to other cell types. The organelle zone of platelets contains three major types of granules in the cytoplasm including α-granules, dense granules, and lysosomes.

- α-Granules contain chemokines, adhesion molecules, coagulation factors and fibrinolytic protein such as ATPase, vWF, plasminogen, coagulation factor, β-thromboglobulin (β-TG), platelet derived growth factor (PDGF).

- Dense granules contain ADP, 5-hydroxytryptamine (5-HT) or serotonin, Ca^{2+}, Mg^{2+} and catecholamines which may be released during platelet activation.

- Lysosomes contain acid hydrolases which are released after exposure to strong agonist (14).

(4) Membrane system

Two distinct membrane systems in platelets are open canaliculi system (OCS) and dense tubular system (DTS). They play a dynamic role in platelet physiology, activation, shape change, internal transformation, and uptake of foreign particles (15).

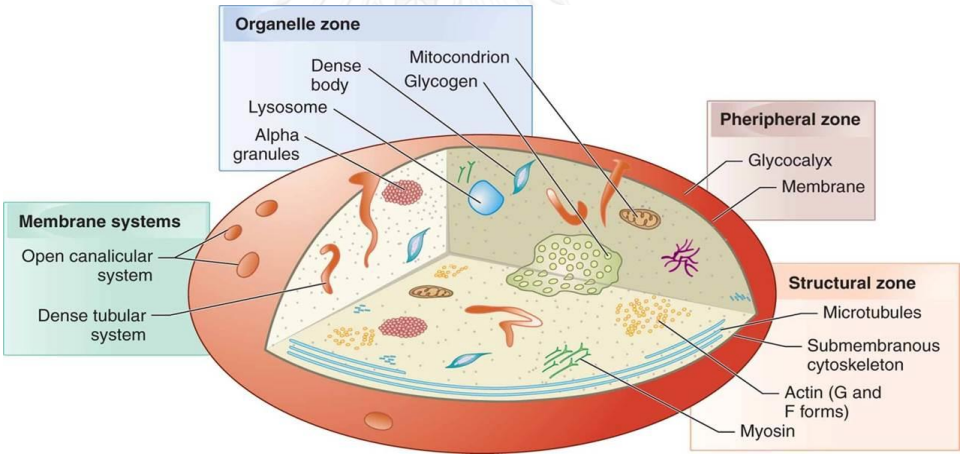


Figure 1. Platelet structure (16)

2.3 Platelet function

Platelet activation is contained of three major processes (17) .

1. Platelet adhesion

In normal conditions of flow, platelets have to slow down to stop at sites of vascular injury. Under physiological conditions it is exposed that binding of vWF to collagen enables binding to GPIb/V/IX. The static or low shear conditions, platelets adhere predominantly to collagen of the subendothelium. Collagen binds initially to GPIa/IIa, cross-links many of these integrin molecules, and in this way activates platelets. Other collagen receptors, such as CD36 and GPVI, play crucial roles in collagen-induced signaling. GPVI, the major signaling receptor, is a member of the immunoglobulin superfamily and is linked to the Fc receptor γ chain. Stimulation of platelets as a result of adhesion leads to spreading, activation of GPIIb/IIa, enabling binding of soluble fibrinogen, and granule secretion (14).

2. Platelet secretion

Activated platelets release several granule components which modulate functions of interacting platelets and blood and vascular cells. Several secretion products of immobilized platelets stimulate additional circulating platelets which are recruited to form aggregates. The dense granules of platelets contain important secondary agonists such as ADP or serotonin. ADP is predicted to be the prominent amplifier of initial platelet activation. There are two important ADP receptors on the platelet surface. The $P2Y_1$ receptor mediates mobilization of Ca^{2+} and shape change and transient aggregation. The $P2Y_{12}$ receptor potentiate platelet secretion and to be involved in sustained irreversible aggregation. Serotonin (5-hydroxytryptamine, 5-HT), a strong vasoconstrictor, binds to the Gq-coupled $5HT_{2A}$-receptor and amplifies with ADP the platelet response. The dense tubular system contains a Ca^{2+} pool which is mobilized during platelet activation. Ca^{2+} fluxes are central triggers in platelet activation, platelet attraction, and platelet aggregation (17).

3. Platelet aggregation

The aggregation of platelets is characterized by the accumulation of platelets into a hemostatic plug. The central platelet receptor in this process is the GPIIb/IIIa ($\alpha_{IIb}\beta_3$-integrin) linking activated platelets through fibrinogen bridges. A resting platelet presents about 40,000 to 50,000 GPIIb/IIIa complexes on its surface. In its nonactive state this integrin cannot bind soluble ligands like plasma fibrinogen, vWF, fibronectin, or vitronectin. Only stimulation of a platelet leads to an increase in GPIIb/IIIa molecules, via α-granule exocytosis, and to activation of surface-exposed GPIIb/IIIa, enabling binding of soluble ligands. On the other hand, immobilized fibrinogen on stimulated platelets serves as an adhesive substrate for resting platelets through GPIIb/IIIa that leads to amplification of primary aggregation. Interaction between GPIIb/IIIa and its ligand is associated with molecular conformational changes, resulting in a firm connection. In high shear environments, as found in arterioles and stenos arteries, platelet activation/aggregation can be induced by shear itself. In this case, platelets are first linked by vWF bridges via the GPIb/V/IX complex. This interaction leads to activation of GPIIb/IIIa and in turn to stable vWF-mediated platelet aggregates (18).

2.4 Receptors and signaling in platelets

Activated platelets cause the release of soluble platelet agonists such as ADP thrombin, 5-HT and TxA_2. Platelet agonists induced signal transduction via their receptors and their effects on platelets (Table 1) (19).

Table 1. Agonists involved in platelet activation and their effects on platelets

Platelet activator	Receptor(s)	Effect on platelets
ADP	$P2Y_1$ $P2Y_{12}$	Platelet shape change ($P2Y_1$) Transient aggregation ($P2Y_1$) Sustained irreversible aggregation ($P2Y_{12}$) Expression of P-selectin ($P2Y_{12}$) Release of thromboxane A_2 ($P2Y_1$,$P2Y_{12}$) Platelet recruitment to sites of injury ($P2Y_{12}$) Induction of procoagulation activity and aggregation ($P2Y_{12}$)
Thromboxane A_2	$TP\alpha$ $TP\beta$	Platelet recruitment and aggregation to a primary platelet plug ($TP\alpha$)
Serotonin	5-HT-2A	Platelet recruitment to sites of injury Induction of procoagulant activity via retention of fibrinogen and thrombospondin on platelet surface
Epinephrine	α_{2A}	Supplementary role overlapping $P2Y_{12}$ receptor signaling
Collagen	GPIb (high shear via vWF) GPIIb/IIIa (high shear via vWF) GPIa/IIa (low shear) GPVI (low shear)	Activation of GPIIb/IIIa Release of ADP and thromboxane A_2 Platelet spreading Platelet aggregation Induction of procoagulant activity via release of Ca^{2+}
Thrombin	PAR-I PAR-4	Platelet aggregation (PAR-I) Release of ADP, Thromboxane A_2 (PAR-4), serotonin (PAR-I) and epinephrine (PAR-I) Activation/mobilization of P-selectin and CD-40 ligand (PAR-I) Induction of platelet procoagulant activity (PAR-I)

In platelet structures are contained of a matrix protein. Collagen is synthesized by macrophage, smooth muscle cell, endothelial cell, keratinocytes, epithelial cells and fibroblasts. Collagen in blood vessels contains about 40% of total protein which help to maintain blood vessel integrity and elasticity. When blood vessel injury, resulting in exposure of platelets to subendothelial protein collagen, collagen is a strong agonist which induces of platelet adhesion, secretion and aggregation by binding to platelet receptors such as GPIa/IIa (integrin $\alpha_2\beta_1$), GP-VI and GPIbα-V-IX complex (14, 20). Integrin $\alpha_2\beta_1$ and GP-VI bind directly to collagen whereas GPIbα-V-IX complex requires cofactor vWF. GP-VI is the important platelet-collagen receptor which induced platelet activation and platelets adhesion by $\alpha_2\beta_1$ intregrin to vascular wall (20). GP-VI is coupled to a Fc receptor (FcR) γ chain that is essential for its expression and function in platelets (14). This protein complex is responsible for the activation of Phospholipase Cγ2 (PLCγ2). The activation of PLCγ2 hydrolyzes phosphatidylinositol (4,5)-biphosphate (PIP_2) to two secondary messenger, inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is responsible for increasing of intracellular Ca^{2+} concentration and also activate Ca^{2+} influx across platelet membrane. DAG is responsible for activation of PKC isoforms lead to phosphorylate multiple proteins on serine (Ser) and threonine (Thr) residues. These signaling molecules trigger the cytoskeleton reorganization that allows change in discoid to pseudopodia shape, the secretion of ADP and TXA_2 (14) (Figure 2). The previous study, downstream from PI3 Kinases (PI3Ks), Akt1 is also required to promote platelet secretion when induced by collagen (21). PI3Ks have been shown a common and key signaling mediator in platelet activation (22).

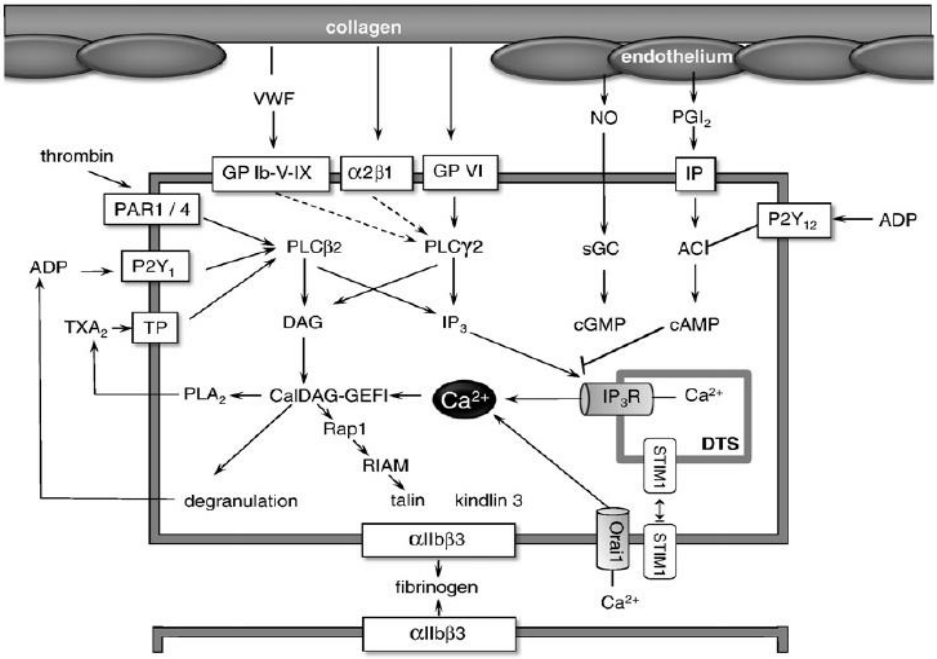


Figure 2. The main receptors and signaling in platelets (14)

ADP has a role of platelet activator. It is secreted from platelet dense granules and also be released from damaged red blood cells at sites of vascular injury. There are three receptors for ADP that members of the purinergic class of GPCRs, including $P2Y_1$, $P2Y_{12}$ and $P2X_1$ (23). $P2X_1$ receptor is ligand-gated ion channels that induces calcium influx (24). $P2Y_1$ receptor is coupled to $G_{\alpha q}$, while $P2Y_{12}$ receptor is coupled to $G_{\alpha i}$. The $P2Y_1$ receptor, by coupling to $G_{\alpha q}$, leads to activation of PLC, generation of IP3, and subsequently mobilization of calcium and also leads to activation of the small G-protein Rho A that mediates platelet shape change. The $P2Y_{12}$ receptor, by coupling to $G_{\alpha i}$, inhibits AC and cAMP synthesis. Cyclic nucleotide synthesis is controlled by AC. The inhibitory effect of $G_{\alpha i}$ on cAMP synthesis leads to the inhibitory effect of cAMP-dependent protein kinase on platelet activation which the cAMP level are regulated by phosphodiesterase (PDE) enzyme (25). Moreover,

ADP receptor signaling is the secondary amplification of platelet activation. Therefore, specifically target of ADP receptors has been developed as anti-thrombotic agents. Clopidogrel and ticlopidine are two commonly prescribed anti-platelet agents that are irreversible antagonists of the $P2Y_{12}$ receptor (Figure 3) (23).

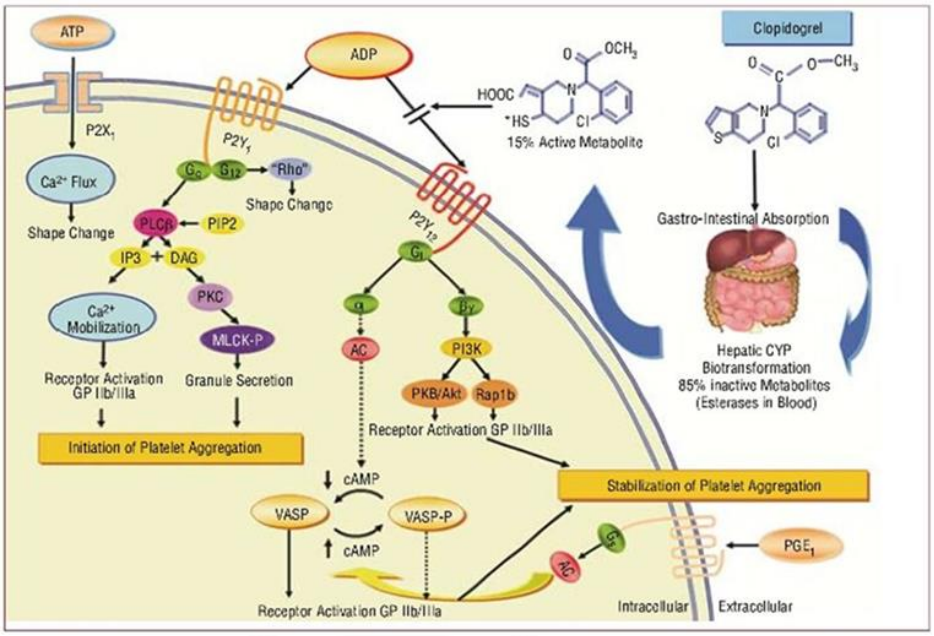


Figure 3. Purinergic receptors and mechanism of action (23)

Consequently, the activation of ADP receptor $P2Y_{12}$ has been shown to be a major mechanism for the stimulation of VASP phosphorylation and the stimulation of the PI3K pathway, as **Gβγ** has been shown to directly bind PI3Ks and induce its activation (26).

Vasodilator-stimulated phosphoprotein (VASP) is an intracellular platelet protein which is non-phosphorylated at basal state, approximate 50 kDa, an actin- and profilin-binding protein and response to vasodilation agents such as prostaglandin I_2 (PGI_2,), and NO which elevated cyclic adenosine monophospate

(cAMP or cGMP) . VASP phosphorylation relates with $P2Y_{12}$ receptor inhibition. It is regulated by the cAMP cascade. cAMP- and cGMP-regulated protein kinases, protein kinase A (PKA) and protein kinase G (PKG). VASP is phosphorylated at three sites including Serine 157 (Ser^{157}), Serine 239 (Ser^{239}) and Threonine 278 (Thr^{278}). VASP phosphorylation by PKA is more prefer at Ser^{157} site, while PKG is at the Ser^{239} site. Phosphorylation at Thr^{278} can be phosphorylated by both PKA and PKG (27). Phosphorylation at Ser^{157} is related with an shift molecular mass from 46 kDa to 50 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (28). Phosphorylation of VASP correlates with reduced activation of integrin αIIbβ3 and inhibition of platelet aggregation (29).

Akt, also known as Protein Kinase B (PKB), is a serine/threonine kinase that contributes to signaling and activation response of human platelet (22). Akt is the downstream signaling which regulated by PI3K. PI3K is a family of lipid kinases that are activated downstream from GPCRs, receptor tyrosine kinases and adhesion receptors (30). PI3K have three families: classes I, II, and III. PI3K p 55-p 85 subunits are in the class IA (α, β and δ) isoforms, and are regulated by tyrosine kinase, while PI3K p101 subunit is in the class IB PI3K (γ) isoform and is activated by GPCR (31). PI3K is responsible for induced PIP_2 and PIP_3, activation of integrin and platelet aggregation (31). There are three isoforms of Akt in mammalian cells: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) (32). Akt1 and Akt2 were found in human platelets and have essential roles in stimulating platelet activation, while Akt3 was sometimes mentioned in platelets. Activated PI3K generates lipid second messengers by phosphorylating the 3' position of phosphoinositides, resulting in PtdIns (3,4) P2 and PtdIns (3,4,5) P3-PtdIns (3,4) P2 and PtdIns (3,4,5) P3 contact with Akt and phosphatidylinositol (32). Akt has been shown to induce thrombus formation in mouse models. Akt plays an essential role in cell survival, growth, migration,

proliferation, and metabolism (lipid and glucose), cell cycle progression, muscle and cardiomyocyte contractility, angiogenesis, and self-renewal of stem cells (33). Lacking of Akt1 or Akt2 in platelets have defect in secretion of dense and α-granule content (22). Akt can be phosphorelated at Ser^{473} and Thr^{308}. Phosphorylation at Ser^{473} is required for full activation of Akt and phosphorylation at Thr^{308} is to partially activate Akt. However, the positions of Ser^{473} and Thr^{308} slightly vary among the Akt isoforms, the sequences surrounding these sited are conserved (32).

Phosphodiesterase (PDEs) are enzymes containing of isozyme families that hydrolyze the 3'-phosphoester bond on cAMP and/or cGMP, converting into biologically inactive 5'-nucleotide metabolites. Cyclic nucleotide levels are degraded by PDEs enzyme. PDEs have three different types in platelets such as PDE2, PDE3 and PDE5. PDE2 and PDE3 are able to degrade cAMP and cGMP. PDE2 hydrolyzes both cAMP and cGMP, PDE3 preferentially hydrolyzes cAMP. PDE5 is highly specific for cGMP hydrolysis. PDE3A is also activated by cAMP-dependent protein kinase (protein kinase A [PKA])-mediated phosphorylation, suggesting a negative feedback loop for cAMP signaling (25). The elevation of cAMP is regulated by inhibition of adenylyl cyclase and phosphodiesterase (34).

2.5 *Angelica dahurica*

Angelica dahurica (Fisch. Ex Hoffm.) Benth. & Hook.f.ex Franch & Sav is in Umbelliferae family, and has synonym such as *Callisace dahurica* Franch & Sav., *Angelica macrocarpa* H. Wolff, *Angelica porphyrocaulis*, *Angelica tshiliensis* H. Wolff. *Angelica dahurica* (AD) is a biennial herb that grows to 1-2 meters and 6 foots 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 and roof have special fragrance. It has the seeds ripen from

August to October and flower approximately in July to September and. This plant distributes in Northeastern China, Japan, Korea, Taiwan. This plant prefers moist soil and shady environment, such as near riverside, on high mountain or forest edge of China (35).

AD is called Bai Zhi, named in China or Kot-Sor, named in Thailand, is the root of AD which has been widely used in traditional medicine. Dried-root powder of AD was extracted with 50% ethanol. AD extract (ADE) is contained of an essential oil and more than 20 furanocoumarins such as 5-methoxypsoralen, bergapten, byakangelicin, imperatorin, isoimperatorin, ostenol. Chinese traditional medicine uses Bai Zhi as antipyretic, analgesic or cold, toothache and headache (7). 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 (8).

2.6 Pharmacological activity of *Angelica dahurica*

The previous study showed that ADE at 1 mg/ml inhibited platelet aggregation induced by ADP and collagen by 62.82% (p=0.000) and 66.28% (p=0.002) respectively, while imperatorin is one of the chemical compound in AD at concentration 300 μM can inhibited ADP induced platelet aggregation by 33.96% (p=0.012) compared with vehicle control. Imperatorin cannot inhibit collagen induced platelet aggregation. ADE increased lag phase in collagen induced platelet aggregation. In addition, ADE significantly increased cAMP level. Both of ADE and imperatorin inhibited secondary phase of ADP- induced aggregation. ADE increase lag phase in collagen induce platelet aggregation compare with vehicle control (367.78 *vs.* 116.35 sec, p=0.008). ADE can significantly increase cAMP level (p=0.027) (11). In

addition, Butanol extract of dried root of AD has been reported enhanced increase survival rate in the mouse model of thrombosis (36). AD has the liver protective activity, antimicrobial activity, anti-inflammatory activity and anti-tumor activity.

Liver protective activity

The fractionation of the MeOH extract of *Angelica dahurica* Benth et Hook resulted in the isolation of imperatorin and byakangelicin exhibited strong hepatoprotective activities, showed EC_{50} values of 36.6±0.98 and 47.9±4.6 μM, respectively (37).

Antimicrobial activity

Coumarins isolated from the ethyl acetate extract of the dried root of AD, inhibited *Bacillus subtilis* growth and byakangelicin also inhibited *Asperagillus candidus* growth (38).

Anti-inflammatory activity

The isolation of the root of AD is furanocoumarins, including byakangelicin, imperatorin, isoimperatorin, oxypeucedanin methanolate, and phellopterin could inhibited lipopolysaccharide (LPS) induced prostaglandin E_2 (PGE_2) in rat peritoneal macrophage. Among these compounds, imperatorin showed the most potent inhibition by suppression of cyclooxygenase (COX-2) expression and microsomal prostaglandin (PG) synthase (10).

Anti-tumor activity

The research for anti-tumor activity was found that furanocoumarins, isolated from the methanol extract of AD, exhibited cytotoxicity against various cultured tumor cell lines, such as non-small cell lung, colon tumor and melanoma (39) .

Imperatorin has been extensively studied and shown potent pharmacological activities such as induces vasodilatation by mechanisms of the vasodilatation are mainly involved inhibiting voltage dependent calcium channel and receptor-mediated Ca^{2+} influx and release, and might be partly due to opening calcium-activated potassium channel and competitive antagonism of 5-HT receptors (40).

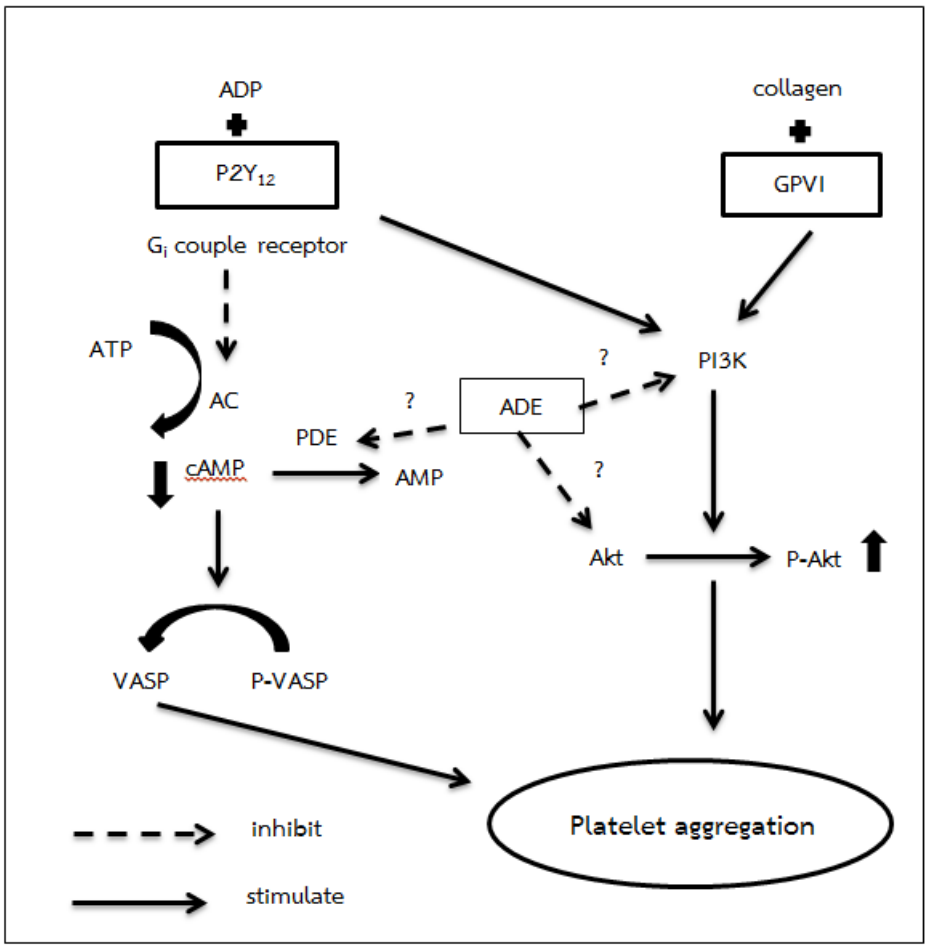
2.7 Objectives

To investigate the molecular mechanism of *Angelica dahurica* extract in antiplatelet aggregation *via* adenylate cyclase – cAMP pathway mediated VASP phosphorylation and/or PI3K/Akt pathways in human platelets.

2.8 Hypothesis

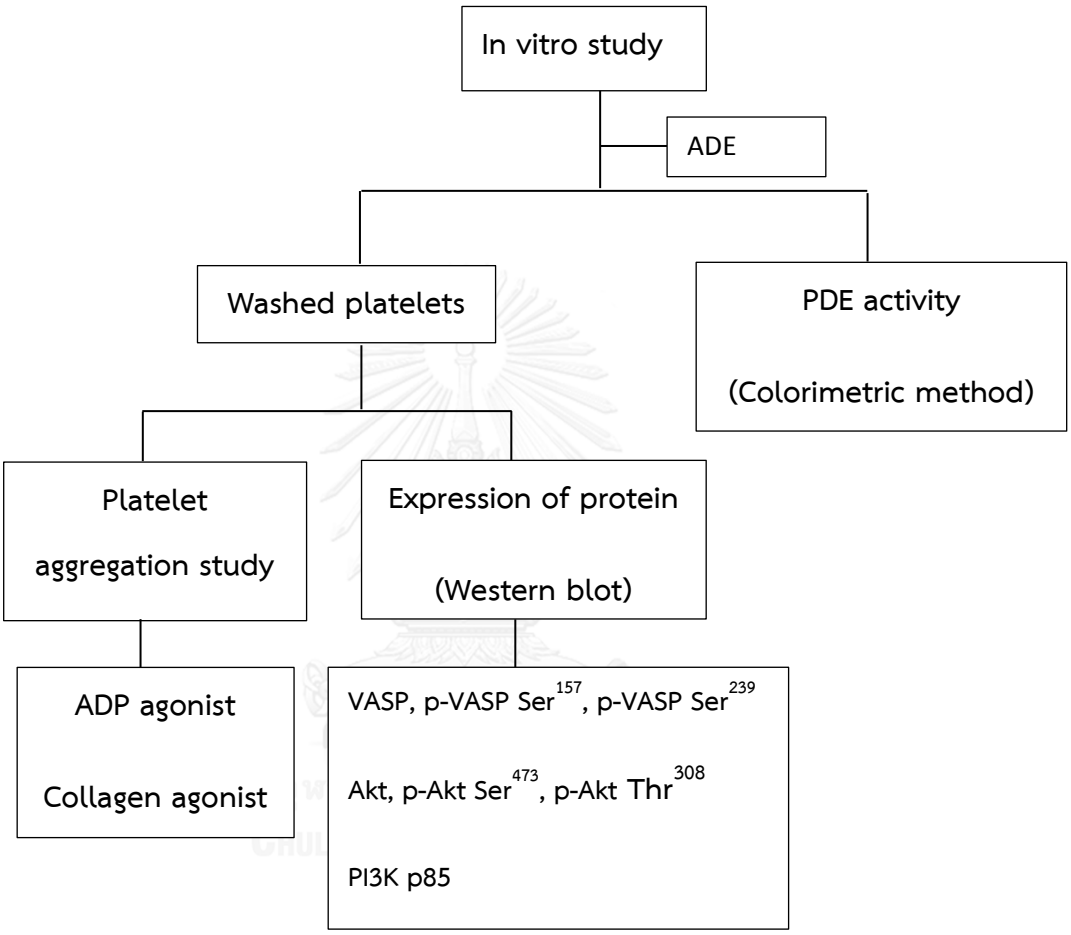
1. ADE can inhibit ADP and collagen-induced platelet aggregation.
2. ADE may decrease platelet aggregation through VASP phosphorylation and PI3K/Akt signaling.
3. ADE may inhibit phosphodiesterase enzyme.

2.9 Conceptual Framework



2.10 Research design

Experimental research



CHAPTER III
MATERIAL AND METHODS

3.1 Materials

The chemicals were purchased from Sigma (St Louis, MO, USA), acetylsalicylic acid (ASA), Adenosine 5' -diphosphate (ADP), apyrase, bovine serum albumin (BSA), calcium chloride ($CaCl_2$), citric acid, collagen solution type I, dimethyl sulfoxide (DMSO), fibrinogen, glucose, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 3-isobutyl-1-methylxanthine (IBMX), magnesium chloride ($MgCl_2$), potassium chloride (KCl), prostacyclin I_2 (PGI_2), sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4), sigma coat, trisodium citrate, Tris base.

Other chemicals were purchased from commercial sources as follow: Bradford protein determination assay from Merck (Darmstadt, Germany). Methanol blotting buffer was from RCI, Labscan, Ireland. Primary polyclonal antibodies against Akt, phospho-Akt Ser^{473}, Thr^{308}, VASP, phospho-VASP Ser^{157}, Ser^{239} and GAPDH, and secondary antibody anti-rabbit IgG conjugated with HRP were purchased from Cell Signaling Technology (Danvers, MA, USA). Protease inhibitor cocktail was purchased from Sigma (St. Louis, USA). PVDF membranes were from Millipore (Schwalbach, Germany). ECL Prime Western Blotting Detection Reagent was from GE Healthcare UK Limited Little Chalfont (Buckinghamshire, UK). PDE Activity Assay Kit (Colorimetric) (ab139460), Cambridge, United Kingdom.

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

3.2 Instruments

- Aggregometer (Helena laboratory, Texas, USA)
- Microplate reader Wallac model 1420 (Perkin elmer, Victor3, Massachusett, USA)
- Centrifuge Rotina 380 model (Andreas Hettich GmbH & Co. KG, Germany)
- pH meter (CG842 Schott, Scientific Promotion Co.,Ltd., Japan)
- Ultrasonic bath (Bandelin GmbH & Co. KG, Germany)
- Water bath (Memmert, Chatcharee Holding Co., Ltd., Thailand)
- Thermo Fisher Scientific Oy (Microplate Instrumentation, Fin-01621 Vantaa, Finland)
- ImageQuant LAS 4000 (GE Healthcare Bio-Science AB, Uppsala, Sweden)
- Micros 60, Horiba ABX Diagnostics (Thailand) Ltd.
- Electrophoresis apparatus

3.3 Method

3.3.1 Blood collection

Human blood was obtained from healthy volunteers aged from 18 to 55 years who did not take any medicines or vitamin supplements for at least 14 days before participate in the study. All experiments were performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Faculty of

Pharmaceutical Sciences, Chulalongkorn University. Approved date on 15th August 2014 at No.073.1/5.

Preparation of washed platelets

Whole blood sample (30 ml) was collected by venipuncture in a polystyrene tube containing acid/citrate/dextrose (ACD) solution in the ratio of ACD to blood equal to 1:6 to avoid coagulation. Platelet rich plasma (PRP) was prepared by centrifuged 200*g* for 10 min at 21°C. The supernatant was collected and supplemented with prostacyclin I_2 (PGI_2) (0.5μM/ml) and then centrifuged at 1,500g for 15 min at 21°C. Platelet pellets were resuspended in Tyrode-HEPES buffer. PGI_2 (0.5 μM/ml) was added in platelet suspensions and then centrifuged at 1,500*g* for 15 min 21°C. The washed platelets were finally resuspended in Tyrode-HEPES's solution containing 1 mM $CaCl_2$. Washed platelet was adjusted to platelet counts of 3×10^8 platelets/ml (41).

3.3.2 Plant material and preparation of ADE

ADE was prepared by Assoc. Prof. Uthai Sotanaphan; Department of Pharmacognosy, Faculty of Pharmacy, Silpakorn University. Briefly, roots of AD were purchased from local market in Bangkok, Thailand and identified by Assoc. Prof. Uthai Sotanaphan. For preparation of ADE, dried-root powder of AD 100 g was extracted 50% ethanol and evaporated to dryness. The extract yield is 30% of the dried-root powder. ADE was dissolved in DMSO to produce ADE stock solution and diluted with normal saline before uses in the study. Therefore, DMSO at the final concentration at 0.5% was used as a vehicle control through the experiments.

3.3.3 Platelet aggregation study

Platelet aggregation was performed using the turbidimetric method (42). Tyrode-HEPES's solution containing 1 mM $CaCl_2$ was set as 100% of light transmission and washed platelet was set as 0% of light transmission. Washed platelet incubated at 37°C in aggregometer for 1 min with continuous stirring at 1000 rpm speed and measured its turbidity as baseline. Then, incubated ADE, ASA or 0.5% DMSO in washed platelet for 3 min. Aspirin (ASA) at the concentration of 0.5 mM (90 µg/ml) and IBMX at the concentration of 0.5 mM were used as a positive control and 0.5% DMSO was used as a vehicle control. Platelet aggregation was induced by agonists. Then, measurement changes of light transmittance were recorded for 6 min. The maximum aggregation showed in percentage of light transmission. The percentage of inhibition of platelet aggregation was calculated by using the following equation:

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

A is the maximum aggregation rate of vehicle-treated washed platelets, whereas

B is the maximum aggregation of sample-treated washed platelets.

Platelet aggregation induced by ADP

ADE at concentrations of 0.1, 0.25, 0.5 and 1 mg/ml and 0.5%DMSO were pre-incubated with washed platelets for 5 min. For ADP-induced platelet aggregation, 2.5 mg/ml of fibrinogen was added and incubated for 1 min before adding ADP at the concentration of 40 μM (43). Then, measurement changes of light transmittance were recorded for 10 min.

Platelet aggregation induced by collagen

ADE at concentrations of 0.1, 0.25, 0.5 and 1 mg/ml and 0.5%DMSO were pre-incubated with washed platelets for 5 min. Platelet aggregation was induced by adding collagen at the concentration of 20 μg/ml. The measurement changes of light transmittance were recorded for 10 min

3.3.4 Western blot analysis

Washed platelets (3×10^8 platelets/ml) were incubated with ADE for 3 min before the addition of collagen (20 μg/ml) or ADP (10 μM) for 10 min and then centrifuged at 8,700g for 15 minutes. The pellets were dissolved in RIPA lysis buffer (1 M Tris-HCl pH 8.0, 1 M NaCl, 10% SDS, 10% sodium deoxycholate and Triton-X100) with protease inhibitor cocktail (1:100) and phosphatase inhibitor (1:100) for 1 h on ice, and then centrifuged at 8,700*g* for 15 minutes. The total protein concentration was determined using Bradford protein determination assay. Proteins (30 μg/lane) were separated by 12.5% SDS-PAGE with the applied voltage of 90 V for 2 h until bromophenol blue dye reached the lower edge of the gel. The separated proteins on the gels were subsequently electrotransferred onto PVDF membranes by the

applied voltage of 90 V for 45 min. The blots were blocked for 1 h at room temperature with 5% BSA in TBS-T buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.1% Tween 20) and then incubated with rabbit polyclonal primary antibodies for VASP (1:1000), phospho-VASP Ser^{157} (1:1000), phospho-VASP Ser^{239} (1:1000), rabbit polyclonal primary antibodies for Akt (1:1000), phospho-Akt Ser^{473} (1:1000), phospho-Akt Thr^{308} (1:1000) and GAPDH (1:1000) overnight at 4°C with gentle agitation. After washing with TBS-T buffer, the membrane was further incubated with secondary antibody with HRP-conjugated (1:3000) in 5% BSA in TBS-T buffer for 1 h at room temperature. The protein bands were detected by using ECL detection solution. Blot images were acquired using ImageQuant LAS 4000 and quantitative analyzed by ImageJ software. Relative band intensity of each protein was normalized with GAPDH band intensity.

3.3.5 Phosphodiesterase enzyme activity

To investigate the effect of ADE on phosphodiesterase (PDE) activity, 5'AMP release, a product of PDE reaction, were measured. The 5'- AMP released is further cleaved into the nucleoside and phosphate by the enzyme 5'- nucleotidase. The phosphate released due to enzymatic cleavage is quantified using Green Assay Reagent in a modified Malachite Green assay. PDE prepare samples containing PDE enzyme, substrate and test compounds and used IBMX as a positive control and a non-specific PDE inhibitor for screening. Incubate ADE at 1 h. Terminate reaction by adding Green Assay Reagent. The Colorimetric was quantified by measuring the absorbance at 620 nm using microplate reader and 5'AMP release of IBMX was calculated using standard PDE inhibition.

CHAPTER IV
RESULTS

4.1 The effect of ADE on agonists-induced platelet aggregation.

4.1.1 The effects of ADE on ADP-induced platelet aggregation

In washed platelets preparations, the level of fibrinogen is not enough to support the aggregation process. So the fibrinogen should be added to support the aggregation process. In this study, washed platelets were pre-incubated with ADE for 5 minutes, and 2.5 mg/ml fibrinogen for 1 minute before adding ADP to initiate aggregation. The results demonstrated that NSS, 0.5% DMSO and ADE at concentration 0.1 mg/ml induced platelet aggregation in the biphasic manner as shown in Figure 4A and 4B. The 0.5% DMSO, the vehicle control, did not affect percent of platelet aggregation in response to ADP as compared to NSS. While ADE at concentration of 0.1, 0.25, 0.5 and 1 mg/ml significantly decreased percent of maximum aggregation compare to vehicle control (Figure 5). Moreover, the percent inhibition of platelet aggregation of ADE at concentration of 0.1, 0.25, 0.5 and 1 mg/ml by 38.2±13.7% (p<0.05), 50.2±11.6% (p=0.01), 73.4±8.5% (p<0.001) and 79.8±7.4% (p<0.001), respectively compare to vehicle control. The results of this study were also shown that 0.5 mM ASA and 0.5 mM IBMX, the positive control, were significantly inhibited ADP-induced platelet aggregation with 67.8±11.7% (p<0.001) and 83.0±8.0% (p<0.001), respectively (Figure 6). All of these results indicated that ADE can inhibit ADP-induced platelet aggregation and 0.5 and 1 mg/ml ADE were potent in comparable to ASA and IBMX.

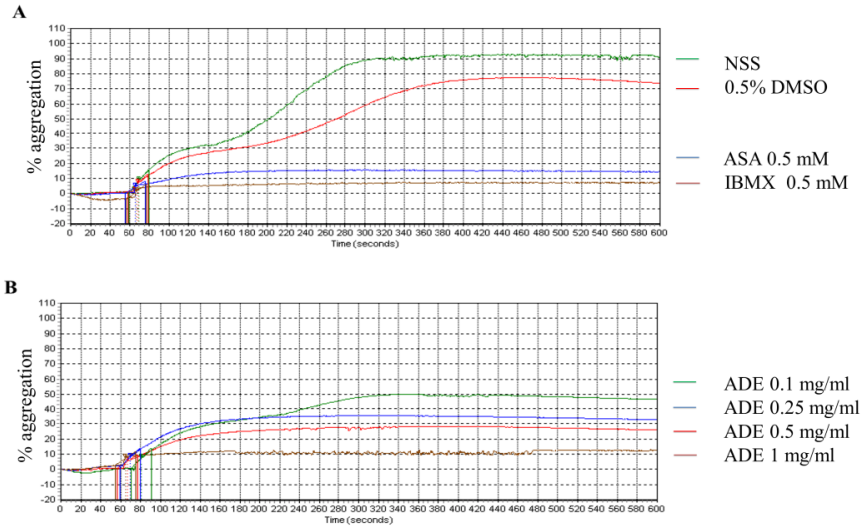


Figure 4. Effect of ADE on platelet aggregation induced by ADP (40 μM). Representative tracing of platelet aggregation of (A) 0.5 mM ASA and 0.5 mM IBMX on inhibition of platelet aggregation when compared to 0.5% DMSO, and (B) ADE at concentration of 0.1, 0.25, 0.5 and 1 mg/ml

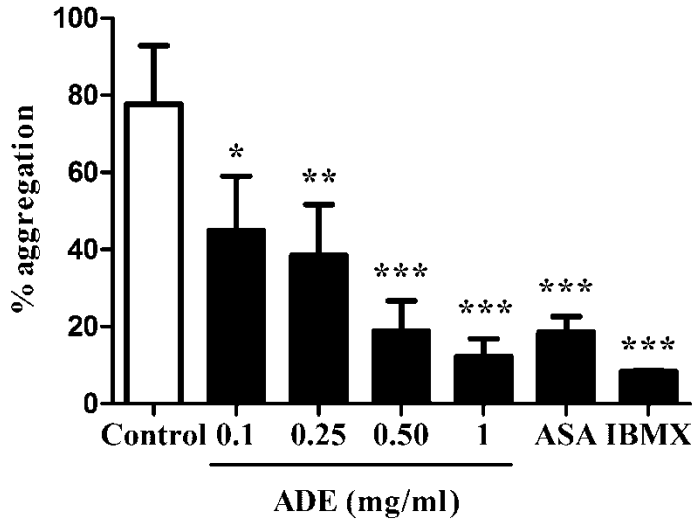


Figure 5. Inhibitory effect of ADE on ADP-induced platelet aggregation. 0.5 mM ASA and 0.5 mM IBMX were used as positive controls. Data were showed as mean ± SEM (n=5). * $p<0.05$, ** $p<0.01$ indicated significant difference when compared to vehicle control (0.5% DMSO).

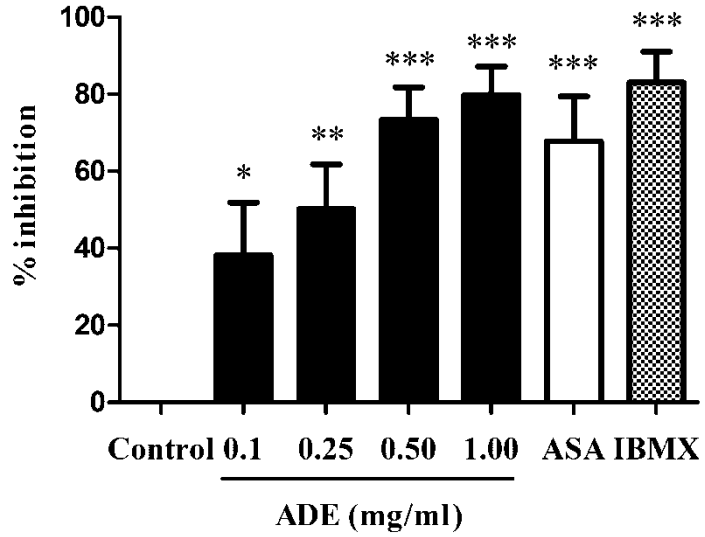


Figure 6. Percent inhibition of platelet aggregation relative to vehicle control. ADE inhibited platelet aggregation induced by ADP. 0.5 mM ASA and 0.5 mM IBMX were used as positive controls. Data were showed as mean ± SEM (n=5). * p<0.05, ** p=0.01 and *** p<0.001 indicated significant difference when compared to vehicle control (0.5% DMSO)

4.1.2 The effects of ADE on collagen-induced platelet aggregation

Washed platelets were pre-incubated with ADE for 5 minutes before adding collagen to initiate aggregation. The results showed that 0.5% DMSO, which was the vehicle control, did not affect percent of platelet aggregation in response to collagen as compared to normal saline (NSS) (Figure 7A). ADE can decrease percent aggregation induced by collagen (Figure 7B and Figure 8). ADE at concentration of 0.1, 0.25, 0.5 and 1 mg/ml significantly inhibited platelet aggregation by 52.8±1%, 60.6±8.9%, 84.3±2% and 85.8±2.3% (p<0.001), respectively, when compared to vehicle control group. In addition, when compared to ASA, a positive control, ADE at 0.5 and 1 mg/ml strongly inhibited platelet aggregation in comparable to ASA 0.5 mM (89.9±0.4%) (Figure 9).

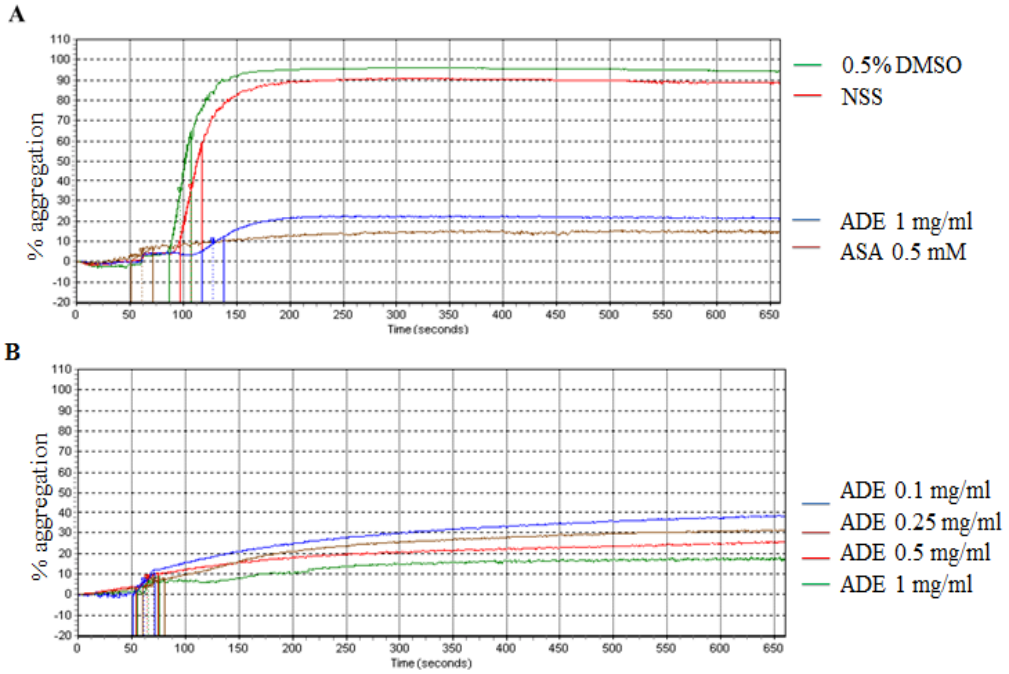


Figure 7. Effect of ADE on platelet aggregation induced by collagen (20 µg/ml). Representative tracing of platelet aggregation of (A) 1 mg/ml ADE and 0.5 mM ASA compared to 0.5% DMSO and NSS, and (B) ADE at concentration of 0.1, 0.25, 0.5 and 1 mg/ml.

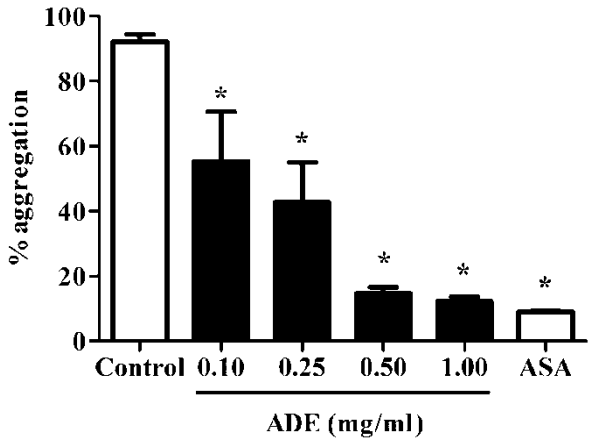


Figure 8. Inhibitory effect of ADE on collagen-induced platelet aggregation. ASA 0.5 mM was used as the positive control. Data were showed as mean ± SEM (n=6). * $p<0.001$ indicated significant difference when compared to vehicle control.

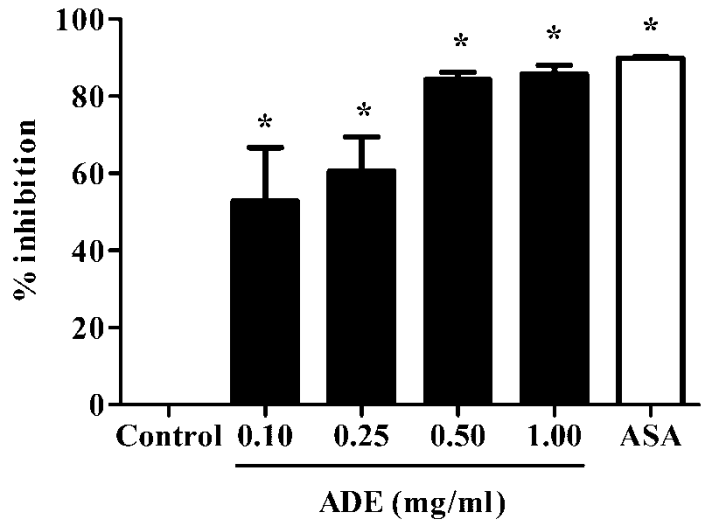


Figure 9. Percent inhibition of platelet aggregation relative to vehicle control. ADE inhibited platelet aggregation induced by collagen. ASA 0.5 mM was used as the positive control. Data were showed as mean ± SEM (n=6). * p<0.001 indicated significant difference when compared to vehicle control (0.5% DMSO).

4.2 The effect of ADE on VASP phosphorylation

To identify the underlying mechanism of inhibitory effect of ADE on ADP-induced platelet aggregation, the effect of ADE on VASP phosphorylation was investigated. Washed platelets were pre-treated with ADE at concentration of 0.5 and 1 mg/ml for 5 min before adding of ADP. IBMX, a phosphodiesterase inhibitor, was used as a positive control. The platelet lysate was subjected to electrophoresis on 12.5% SDS-PAGE and immunoblotting using specific antibodies including VASP, p-VASP Ser^{157}, p-VASP Ser^{239}. GAPDH was used as an internal control. The results showed that ADE at concentration of 0.5 and 1 mg/ml significantly increased the phosphorylation of VASP at Ser^{157} with 6.7±2.3 and 8.3±2.2 folds when compared to control, respectively (p<0.05), which were preferable to those at Ser^{239} with 3.0±1.4 (p=0.054) and 5.1±1.5 fold (p=0.054), respectively (Figure 10).

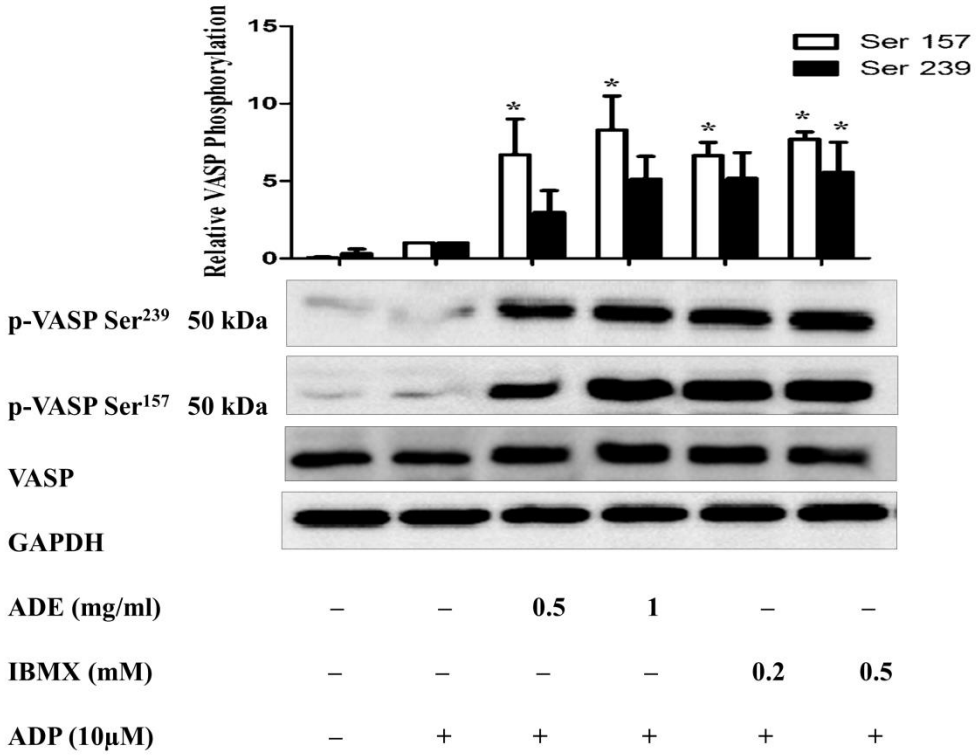


Figure 10. Representative immunoblots and relative band intensity of VASP phosphorylation at Ser^{157} and Ser^{239}. Washed platelets were pre-treated with ADE at the indicated concentration for 5 min before adding ADP. IBMX was used as a positive control. GAPDH was used as an internal control. * $p < 0.05$ indicated statistically significant different from ADP-treated groups (n = 3).

4.3 The effect of ADE on PI3K/Akt phosphorylation

To identify the underlying mechanism of inhibitory effect of ADE on collagen-induced platelet aggregation, the effects of ADE on PI3K/Akt pathway were investigated. Washed platelets were pre-treated with ADE at concentration of 0.25, 0.5 and 1 mg/ml or 0.5% DMSO, a vehicle control, for 5 min before adding collagen. Platelet lysate was subjected to electrophoresis on 12.5% SDS-PAGE and immunoblotting using specific antibodies including PI3K p85, Akt, p-Akt Ser ^{473} and p-Akt Thr ^{308}. GAPDH was used as an internal control. The result showed that the concentration of ADE at 0.25, 0.5 and 1 mg/ml increased PI3K p85 with 0.7±0.3 (p=3.44), 1.3±0.1 (p=2.90) and 1.4±0.4 (p=2.43) folds when compared to control, respectively (Figure 11). ADE concentration of 0.25, 0.5 and 1 mg/ml did not decreased the phosphorylation of Akt at Thr ^{308}, but not Ser ^{473} with 1.5±0.6 (p=0.853), 1.4±0.9 (p=0.595) and 1.2±0.7 (p=0.364) when compared to control, respectively (Figure 12).

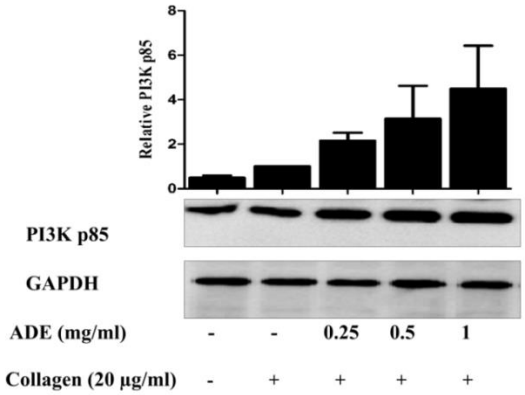


Figure 11. Immunoblots and relative band intensity of PI3K. Washed platelets were pre-treated with ADE at the indicated concentration for 5 min before adding collagen. GAPDH was used as an internal control (n = 3).

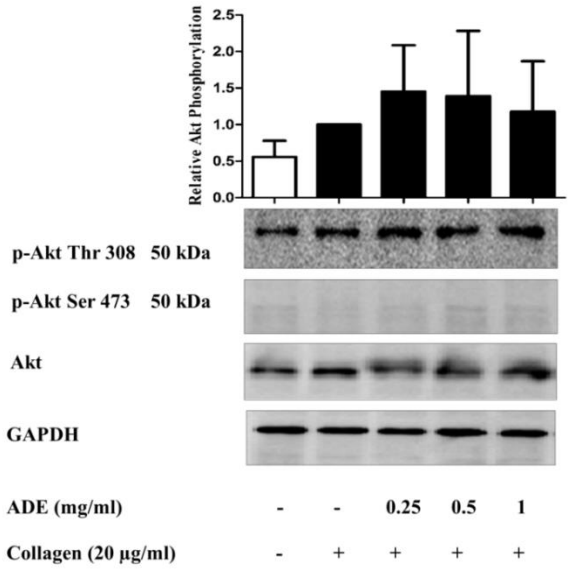


Figure 12. Immunoblots and relative band intensity of Akt phosphorylation at Thr^{308} and Ser^{473}. Washed platelets were pre-treated with ADE at the indicated concentration for 5 min before adding collagen. GAPDH was used as an internal control (n = 3).

4.4 PDE activity

PDE activity was determined from 5'AMP release, a product of PDE reaction after incubation cAMP substrate with PDE enzyme at 60 min. To find the optimum time for incubation with PDE enzyme, IBMX, a non-specific a phosphodiesterase inhibitor was used to test the inhibitory effect on PDE activity. The result showed that 40 μM IBMX successfully inhibited 5'AMP release in the time course manner (Figure 13). Therefore, we selected the incubation time at 60 min which IBMX clearly showed the inhibitory effect different from the control. For the effect of ADE, ADE at the various concentrations were incubated with PDE enzyme for 60 min. The result showed that ADE at concentration of 0.25, 0.5 and 1 mg/ml increased the absorbance at 620 nm about 8, 12 and 15 folds when compared to 0.5% DMSO, a vehicle control group (Figure 14). While IBMX at concentration of 25 and 200 μM showed the inhibitory effect by decreasing 5'AMP release by 96% and 65% different from vehicle control group, respectively (Figure 14).

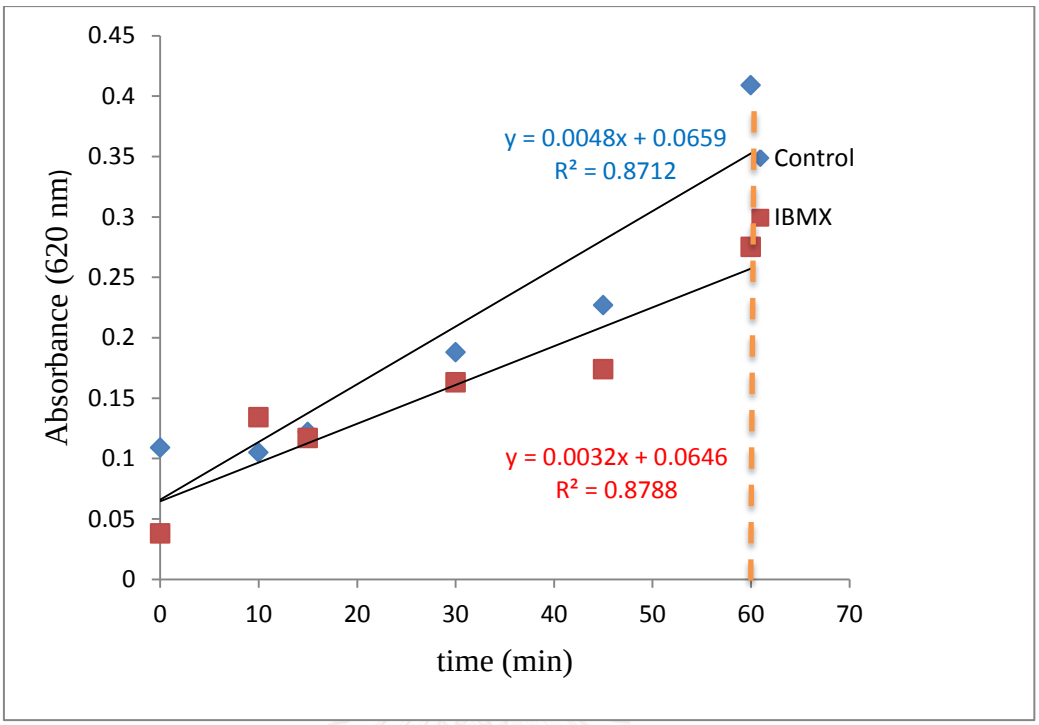


Figure 13. Comparison of time course of cAMP hydrolysis by PDE reaction between control and the PDE inhibitor, IBMX. PDE enzyme (20 mU/well) was incubated with cAMP (200 µM) and 5'- nucleotidase (50 kU/well) with or without the inhibitor IBMX (40 µM) at 30°C for indicated times. Reactions were terminated by addition of 100 µL of Green Assay Reagent and absorbance values were measured at wavelength 620 nm.

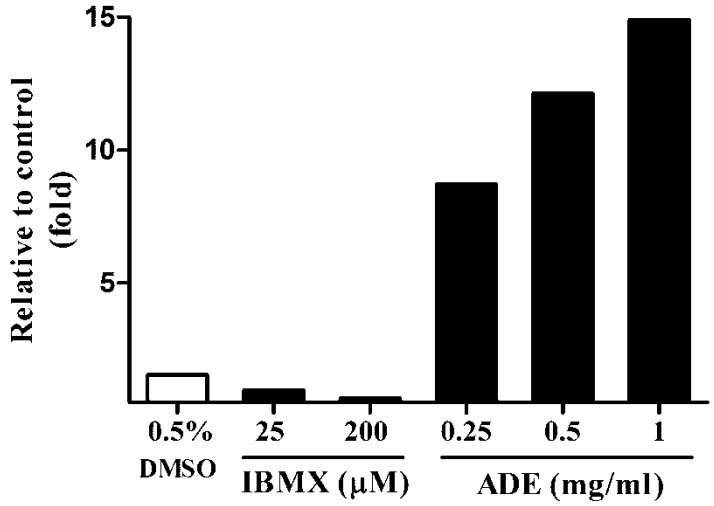


Figure 14. PDE activity was measured from 5'-nucleotidase by incubation of ADE at indicated concentrations containing PDE enzyme for 60 minutes and measured the absorbance at 620 nm.

CHAPTHER V
DISCUSSION AND CONCLUSION

Platelets have an essential role in the maintenance of vascular hemostasis by preventing blood loss and maintain vascular integrity. Antiplatelet agents are intensively used for prevention and treatment of cardiovascular diseases. However, there are some problems in using antiplatelet drugs such as drug resistance, side effects and drug cost. Alternative therapies such as natural products which exhibited the antiplatelet aggregation activity are in focus nowadays. The root of *Angelica dahurica* is widely used in the traditional medicine as treatment for various symptoms such as antipyretic, analgesic or cold, toothache and headache. In Thailand, this herb is also used as an ingredient in the polyherbal traditional medicine such as YA-HOM formulation, which has been listed in the Thailand's List of Herbal Medicinal Products for treatment of circulatory disorder. From the previous study, the ethanolic extract of *Angelica dahurica* (ADE) exhibited the inhibitory effect of platelet aggregation in human platelet rich plasma (11). However, the underlying mechanism is still unclear. Therefore, the present study was aimed to elucidate the mechanism of ADE on inhibition of platelet aggregation induced by collagen and ADP in human washed platelets. According to, a variety of plasma proteins and enzymes including coagulation factors in plasma which might interfere the effect of ADE, washed platelet preparations were used in aggregometry studies and western blot analysis (43). Although both ADP and collagen are the common aggregating agents that strongly induced platelet aggregation but the responsible signaling pathways are different. Our results were found that ADE at the concentration ranging from 0.1 to 1 mg/ml significantly inhibited platelet aggregation induced by both collagen and ADP, suggesting that ADE might act via one or more signaling mediated by ADP and collagen.

ADP is the platelet agonist, which is secreted from platelet dense granules and also be released from damaged red blood cells at sites of vascular injury. The activation of ADP-induced platelet aggregation involves two purinergic receptors on platelet membrane, $P2Y_1$ and $P2Y_{12}$. Our result demonstrated that ADP induced platelet aggregation in the biphasic manner, which are primary and secondary wave of aggregation (44, 45). The primary wave is thought to be responsible for mediating platelet shape change, intracellular calcium mobilization, and initiates aggregation involving with $P2Y_1$ receptor coupled to $G\alpha_q$ (46). Meanwhile the secondary wave involves $P2Y_{12}$ receptor coupled to $G\alpha_i$ by inhibition of adenylate cyclase, which is responsible for secretion and production of TxA_2 in order to amplify and activate platelet (47). The aggregation in washed platelet was different from platelet rich plasma by the unclear secondary phase of platelet aggregation and platelet insensitivity to ADP due to inadequate fibrinogen to activate integrin $\alpha IIb\beta 3$. So the addition of exogenous fibrinogen must be considered for aggregation response (43). ADE 0.5 and 1 mg/ml strongly inhibited platelet aggregation with 80% inhibition, which were as potent as 0.5 mM of ASA and IBMX. ADE was able to inhibit both primary and secondary waves, implied that ADE can inhibit platelet shape change, platelet activation and platelet aggregation.

In the activation of platelet by ADP, the co-activation of both $P2Y_1$ and $P2Y_{12}$ is required for produce full response of platelet aggregation (48). ADP binds to $P2Y_1$ receptor to induce platelet shape change and a transient platelet aggregation. While, binding to $P2Y_{12}$ receptor, which is G_i-coupled receptor, results in activation of $G_{i\alpha}$ subunit leading to the inhibition of adenylyl cyclase. Hence the cAMP levels are decreased, which inhibits the cAMP/PKA-mediated VASP phosphorylation at Ser^{157} site (27). Non-phosphorylated VASP could further activate GP IIb/IIIa receptor, leading to platelet aggregation. This informed that VASP phosphorylation is crucial for GP

IIb/IIIa receptor inhibition that required in anti-platelet aggregation. Our study showed that the levels of VASP phosphorylation at both Ser^{157} and Ser^{239} sites did not change in ADP-induced platelet when compared to resting stage. While ADE increased the level of VASP phosphorylation at both sites, in particular, Ser^{157} is preferable to Ser^{239}. The phosphorylation of VASP at Ser^{157} is the major PKA phosphorylation site while Ser^{239} is the major PKG phosphorylation site (49). This result suggested that ADE might act mainly on the activation of cAMP/PKA and in a lesser extent on the cGMP/PKG signaling pathway. The inhibitory effect of ADE on platelet aggregation induced by ADP might be mainly via the elevation of cAMP level mediated VASP phosphorylation, resulting reduced activation of integrin $\alpha_{IIb}\beta_3$ and inhibited platelet aggregation.

Increasing of intracellular cAMP and cGMP is the one of major signaling pathway in platelet aggregation, which their levels have been regulated by PDE enzymes by catalyzing the hydrolysis of cAMP and cGMP. IBMX also increased VASP phosphorylation at both Ser^{157} and Ser^{239} sites in the similar manner. IBMX might act as both nonselective PDE inhibitor and nonselective adenosine receptor antagonist (50), resulting in activation of VASP phosphorylation and inhibition of platelet aggregation. Therefore, the compounds that could inhibit the PDE activity might lead to increase of cAMP levels and further activated VASP phosphorylation, resulting in inhibition of platelet aggregation. In our study on PDE activity by measurement of 5'AMP release, we found that IBMX successfully inhibited PDE activity when compared to control. Concernedly, ADE increased the amount of 5'AMP release, suggesting that ADE increased PDE activity. This result might be interfered by the color of ADE, a brown solution, resulting affect to the absorbance detection by green assay reagent. Due to the difference in tested system in the study of PDE activity, ADE directly exposed to PDE enzymes in the system that other signaling molecules

were absent. While in the platelet aggregation study, we used the short pre-incubation of ADE with washed platelets. It was still unclear whether ADE and its components acted by interaction with some molecules on the platelet membrane or crossing the platelet membrane to interact directly with signaling molecules inside platelets. Unlike ADE, IBMX, which is a membrane-permeable PDE inhibitor, it was clearly that IBMX could cross the platelet membrane and directly inhibited PDE enzymes (51), resulting in correlation between the PDE activity and inhibition of platelet aggregation by VASP phosphorylation. To clarify this point, the study of the effect on PDE activity should further investigate in the washed platelet instead of pure PDE enzyme, and measured the intracellular cAMP using HPLC (52), fluorescent cAMP analogue (53) or radiolabeled-cAMP (54).

Collagen, a potent inducer of platelet aggregation, interacts with two receptors on platelets, integrin $\alpha 2\beta 1$ and GPVI which are the major signaling receptors on platelet membrane. GPVI is associated with the Fc receptor γ chain which is mediated via tyrosine kinase phosphorylation by Src family kinases, leading to activation of several proteins including PI3K (55). Activation of PI3K resulted in phosphorylation of Akt by phosphatidylinositol-dependent kinases 1 and 2 (56) which further triggered the degranulation and fibrinogen binding in platelet aggregation process (57). From our result, platelet aggregation induced by collagen showed only primary wave in light-transmission platelet aggregometry, since collagen is the strong agonist that directly induced platelet aggregation and granule secretion. Our result revealed that ADE successfully inhibited collagen-induced platelet aggregation in the concentration-dependent manner especially at high concentrations (0.5 and 1 mg/ml) which showed inhibition as strong as ASA, a classical antiplatelet drug. The activation of PI3K plays an important role in platelet function including activation, and aggregation. The consequence of PI3K signaling pathway downstream of collagen

receptor is the phosphorylated Akt, a mechanism that is important for stabilizing platelet aggregates and thrombus formation. ADE seemed to increase PI3K p 85 and did not decrease Akt phosphorylation at Thr^{308}. Interestingly, the rising of PI3K which has many subunits, ADE might be decrease PI3K other subunits. PI3K p 55-p 85 subunits are in the class IA (α, β and δ) isoforms and are regulated by tyrosine kinase, while PI3K p101 subunit is in the class IB PI3K (γ) isoform and is activated by GPCR (31). Therefore, the activation of other PI3K isoform would be need to further investigation. However, this result suggested that ADE can inhibit collagen-induced platelet aggregation, but did not reduce the activation of PI3K p85 subunit. The mechanism is still unclear and need to further clarify whether it act through the downstream of Akt phosphorylation or the other pathways.

In our study, ADE successfully inhibition platelet aggregation by activation of VASP phosphorylation. The results were in accordance with previous studies which ethanolic extract of AD also increased cAMP level and inhibited platelet aggregation in platelet rich plasma (11). In addition, butanolic extract enhanced increase survival rate in the mouse model of thrombosis (36). There were also reports the antiplatelet activity in other *Angelica spp*. such as *Angelica keiskei* and *Angelica sinensis. Angelica keiskei* and its components such as chalcone 4-hydroxyderricin and xanthoangelol have been reported to inhibit platelet aggregation induced by collagen but could not inhibit in thrombin induction (58). *Angelica sinensis* or Danggui in Chinese is also one of the herbs which have been widely reported the effects about blood homeostasis. Several studies revealed that *Angelica sinensis* and its active components such as Z-ligustilide exhibited the inhibitory effects of platelet aggregation induced by various agonists such as ADP or collagen and other effects on blood homeostasis both *in vitro* and *in vivo* (59-63). These studies suggested the potential of *Angelica spp.* in modulation of blood homeostasis.

For the components found in AD which are mainly furanocoumarins such as byakangelicin, imperatorin, isoimperatorin, oxypeucedanin, methanolate and phellopterin, has also been reported the effects on blood homeostasis and anti-platelet aggregation (64-66). In the characterization of ADE, we found that ADE contained imperatorin which has been used as a chemical marker in characterization step (see appendix). Imperatorin has been reported to show the effects on blood homeostasis such as vasodilatation by inhibiting voltage-dependent calcium channel and receptor-mediated Ca^{2+} influx and release, and opening calcium-activated potassium channel and competitive antagonism of 5-HT receptors (40). Previous study also revealed the inhibitory effect on platelet aggregation in platelet-rich plasma (11). Therefore, imperatorin might be a one of active components accounting for anti-platelet activity in ADE.

In conclusion, this study has revealed that the mechanism of the inhibitory effect of ADE on platelet aggregation induced by ADP in human platelets is mediated by the activation of cAMP/PKA-mediated VASP phosphorylation at Ser^{157}. While the mechanism of the inhibitory on collagen-induced platelet aggregation is still unclear and need to further investigation. This finding might provide the strong evidence for antiplatelet activity of *Angelica dahurica* extract which has the potential for development into alternative therapy for circulatory disorder.

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APPENDIX

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

AD was identified by Associate Professor Dr. Uthai Sotanaphun. Voucher specimens MUS 1122 have been deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand. Dried-root powder of *Angelica dahurica* 200 mg were extracted with 50% ethanol and ultra-sonication for 30 min. To characterize, the 15 mL of supernatant was applied to TLC plate, using silica gel $60F_{254}$ as the coating substance. Solvent system is toluene-ethyl acetate-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.

Figure 15. TLC chromatogram of the 50% ethanolic extract of *Angelica dahurica*

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

Sample preparation: Dried-root powder of Angelica dahurica

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

Volume of injection:	20 µl
Flow rate:	1.0 ml/min
Detector:	Diode-array detector
Wavelength:	254 and 270 nm
Temperature:	25°C
Run time:	70 min

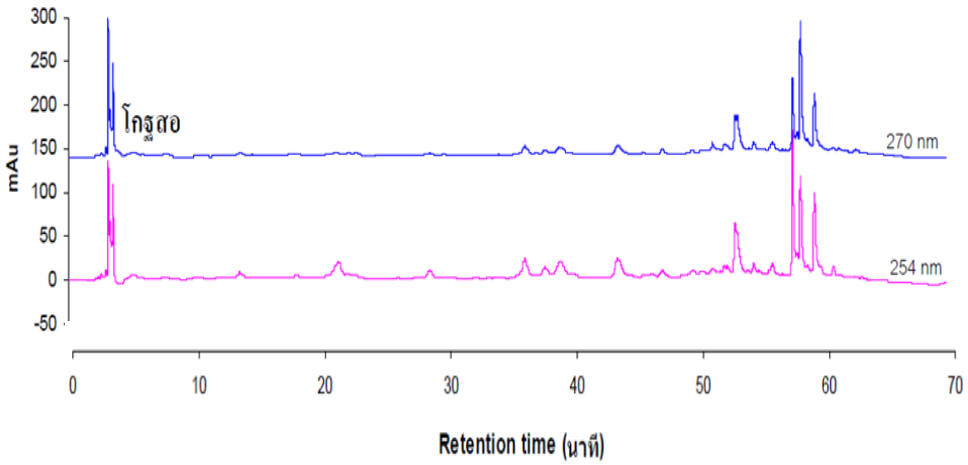

Figure 16. HPLC chromatogram of the 50% ethanolic extract of *Angelica dahurica*

PREPARATION OF REAGENTS

Tyrode's buffer preparation

Stock 1 :	2.73M NaCl	16 g
	53.6 mM KCl	0.4 g
	238 mM $NaHCO_3$	2 g
	8.6 mM NaH_2PO_4	0.116 g
	H_2O qs	100 ml
Stock 2 :	0.1M $MgCl_2.6H_2O$	2.03 g
	H2O qs	100 ml
Stock 3 :	0.1M $CaCl_2.6H_2O$	2.1 g

HEPES stock : 0.5M (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] sodium salt made up to 100 ml

Tyrode's buffer	Volume
Stock 1	5 ml
Stock 2	1 ml
Stock 3	2 ml
HEPES	1 ml
Glucose	0.1 g

Adjust to pH 7.4 and finally, adjust the total volume to 100 ml.

Acrylamide solution

To make 100 mL of 30% acrylamide solution, 30.0 g of acrylamide and 0.8 g of N'N'-bis-methyleneacrylamide were dissolved in 30 mL of ultrapure water. The solution was stirred until completely solubilized, then adjusted volume to 100 ml. Store the solution in the dark at 4° C.

10% APS solution

To make 300 µl of 10% APS solution, 30 mg of APS was dissolved in 300 µl of ultrapure water. The solution was mixed until completely solubilized. Prepare freshly before use.

5× blotting buffer (125 mM Tris-base, 960 mM glycine)

To make 1 L of 5× blotting buffer, the ingredients were:

Tris-base	15.1	g
Glycine	72	g

All ingredients were dissolved in ultrapure water. The solution was stirred until completely solubilized. Finally, adjust the total volume to 1 L. Store the buffer at room temperature. Dilute to 1× before use.

1× blotting buffer (25 mM Tris-base, 192 mM glycine, 20% MeOH)

To make 1 L of 1× blotting buffer, the ingredients were:

5× blotting buffer	200	ml
MeOH	200	ml
Adjust volume with ultrapure to	1	L

RIPA lysis buffer for immunoblotting

1 M Tris-HCl pH 8.0	50	µl
1 M NaCl	150	µl
10% SDS	10	µl
10% Na deoxycholate	50	µl
Triton-X100	10	µl
R.O. water	630	µl

Freshly add the 100× cocktail protease inhibitor in ratio of 1 µl per 100 µl of RIPA buffer before use.

10× running buffer for SDS-PAGE (250 mM Tris-base, 1.92 M glycine, 1% SDS)

To make 1 L of 10× Tris-glycine running buffer, the ingredients were:

Tris-base	30.2	g
Glycine1	44	g
SDS	10	g

All ingredients were dissolved in ultrapure water. The solution was stirred until completely solubilized. Finally, adjust the total volume to 1 L. Store the buffer at room temperature. Dilute to 1× before use.

1× running buffer for SDS-PAGE (25 mM Tris-base, 192 mM glycine, 0.1% SDS)

To make 1 L of 1× Tris-glycine running buffer, the ingredients were:

10× running buffer	100	ml
Adjust volume with ultrapure to		1 L

10% SDS solution

To make 100 mL of 10% SDS solution, 10 g of SDS was dissolved in 80 mL of ultrapure water. The solution was stirred until completely solubilized. Carefully adjust the total volume to 100 ml. Store the solution at room temperature.

Separating gel

To prepare 2 separating gels for Mighty small II SE250/SE260, the ingredients of separating gel were:

	12.5%	
Ultrapure water	14.464	ml
1.5 M Tris-HCl pH 8.8	11	ml
10% SDS	0.44	ml
30% acrylamide solution	17.856	ml
10% APS	220	µl
TEMED 13.2	22	µl

All ingredients were thoroughly mixed and immediately pour gel between the glass plates. Ultrapure water was immediately layered the top of the gel. The gels were leaved overnight for complete polymerization.

Stacking gel

Once the separating gel has completely polymerized, ultrapure was removed from the top of the polymerized gel. To prepare 2 stacking gels for Mighty small II SE250/SE260, the ingredients of stacking gel were:

Ultrapure water	3.06	ml
0.5 M Tris-HCl pH 6.8	1.26	ml
10% SDS	50	µl

30% acrylamide solution	0.66	ml
10% APS	31.2	µl
TEMED	5	µl

All ingredients were thoroughly mixed and immediately pour gel between the glass plates. Combs were inserted between the glass plates to make sample loading wells. The gels were leaved at least 30 min to polymerize.

10× TBS

To make 1 L of 10× TBS buffer, the ingredients were:

Tris-base	24.23	g
NaCl	80.06	g

All ingredients were dissolved in ultrapure water. The solution was stirred until completely solubilized. Finally, adjust the total volume to 1 L. Store the buffer at room temperature. Dilute to 1× before use.

1× TBS-T

To make 1 L of 1× TBS-T buffer, the ingredients were:

10× TBS	100	ml
Tween 20	1	ml
Adjust volume with ultrapure to	1	L

0.5 M Tris-HCl pH 6.8

To make 100 mL of 0.5 M Tris-HCl pH 6.8 buffer, 6.1 g of Tris-base was dissolved in 80 mL of ultrapure water. The solution was stirred until completely solubilized. Adjust to pH 6.8 with 6N HCl. Finally, adjust the total volume to 100 ml. Store the buffer at 4°C.

1.5 M Tris-HCl pH 8.8

To make 100 mL of 1.5 M Tris-HCl pH 8.8 buffer, 18.15 g of Tris-base was dissolved in 80 ml of ultrapure water. The solution was stirred until completely solubilized. Adjust to pH 8.8 with 6N HCl. Finally, adjust the total volume to 100 ml. Store the buffer at 4°C.

VITA

Miss Vivan Prompradith was born on January 6, 1984 in Bangkok, Thailand. She received her Bachelor's degree of Pharmaceutical Science from the Faculty of Pharmacy, Silpakorn University, Nakornpathom, Thailand in 2007. She worked at Bureau of Drug Control, Food and Drug Administration, Ministry of Public Health, Tiwanon Road, Muang, Nonthaburi 11000

Poster Presentation

- Prompradith V., Sotanaphan U., and Luechapudiporn R. 2015. Effect of Angelica dahurica extracts on platelet aggregation induced by collagen. The 5th National and International Graduate Study Conference 2015 "Creative Education: Intellectual Capital toward ASEAN", Bangkok, Thailand.