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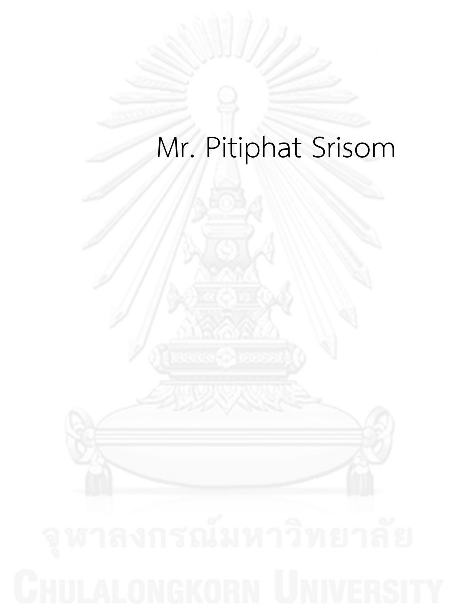
EFFECT OF *ANGELICA DAHURICA* EXTRACT ON PLATELET AGGREGATION IN RAT

Mr. Pitiphat Srisom



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โกฐสอเป็นพืชสมุนไพรที่อยู่ในตำรับยาแพทย์แผนไทย ซึ่งใช้รักษาเกี่ยวกับโรคระบบไหลเวียนโลหิต การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบฤทธิ์ของสารสกัดโกฐสอ (*Angelica dahurica* extract : ADE) ต่อกระบวนการเกาะกลุ่มของเกล็ดเลือด การแข็งตัวของเลือด และผลข้างเคียงต่อกระเพาะอาหารในหนูแรท จากการศึกษานอกกาย พบว่า สารสกัดโกฐสอที่ความเข้มข้น 1 มิลลิกรัมต่อมิลลิลิตรสามารถยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่กระตุ้นด้วยอะดีโนซีนไดฟอสเฟต (ADP) ความเข้มข้น 5 ไมโครโมลาร์ได้อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มที่ให้ตัวทำละลายควบคุมมีค่าเท่ากับ 25.5±3.8% ($p<0.05$) และสามารถยับยั้งเอนไซม์ไซโคลออกซีจีเนส (COX) ชนิด 2 ได้มากกว่าชนิด 1 โดยมีค่า IC_{50} เท่ากับ 0.03 และ 0.76 มิลลิกรัมต่อมิลลิลิตรตามลำดับ ในการศึกษาในกายโดยป้อนสารสกัดโกฐสอแก่หนูแรทขนาด 25, 50, 100 และ 200 มิลลิกรัมต่อกิโลกรัมเป็นเวลา 7 วัน พบว่าสารสกัดโกฐสอขนาด 100 และ 200 มิลลิกรัมต่อกิโลกรัมสามารถยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่กระตุ้นด้วย ADP ความเข้มข้น 5 ไมโครโมลาร์ได้อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มควบคุมมีค่าเท่ากับ 41.1±8.7% ($p<0.001$) และ 45.1±8.9% ($p<0.001$) ตามลำดับ และในกลุ่มได้ที่รับสารสกัดโกฐสอความเข้มข้น 200 มิลลิกรัมต่อกิโลกรัมร่วมกับอะซิทิลซาลิไซลิก (ASA) ขนาด 80 มิลลิกรัมต่อกิโลกรัม พบว่ามีฤทธิ์ยับยั้งการเกาะกลุ่มของเกล็ดเลือดได้มากกว่าการให้สารอะซิทิลซาลิไซลิกเดี่ยวๆอย่างมีนัยสำคัญทางสถิติ มีค่าเท่ากับ 84.7±8.6% เทียบกับ 47.0±11.3% ($p<0.001$) นอกจากนี้ยังศึกษาผลของสารสกัดโกฐสอต่อการแข็งตัวของเลือดในหนูแรทโดยประเมินจากค่า PT aPTT และ TT ซึ่งพบว่ากลุ่มที่ได้รับสารสกัดโกฐสอไม่มีผลต่อค่าการแข็งตัวของเลือด อีกทั้งยังไม่มีผลในการเปลี่ยนแปลงรูปร่างลักษณะทางมหกายวิภาคของกระเพาะอาหาร และทางจุลพยาธิวิทยาซึ่งประกอบด้วยการบวมของชั้นเยื่อเมือก ภาวะเลือดออก การตายของผนังเยื่อบุ และการเกิดการอักเสบของกระเพาะอาหาร สรุปได้ว่าสารสกัดโกฐสอมีฤทธิ์ยับยั้งการเกาะกลุ่มของเกล็ดเลือดหนูแรทเมื่อกระตุ้นด้วยอะดีโนซีนไดฟอสเฟตทั้งนอกกายและในกาย และกลไกการออกฤทธิ์อาจผ่านการยับยั้งเอนไซม์ไซโคลออกซีจีเนสชนิด 1 ร่วมกับการยับยั้งวิถีทางของการกระตุ้นด้วยอะดีโนซีนไดฟอสเฟตในเกล็ดเลือด สารสกัดโกฐสอไม่มีผลต่อค่าการแข็งตัวของเลือดและการเกิดอาการไม่พึงประสงค์ที่กระเพาะอาหาร อีกทั้งการให้สารสกัดโกฐสอร่วมกับแอสไพรินสามารถเพิ่มฤทธิ์การเกาะกลุ่มของเกล็ดเลือดโดยไม่เพิ่มแผลที่กระเพาะอาหาร

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต ....

สาขาวิชา เภสัชวิทยาและพิษวิทยา ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

ปีการศึกษา 2559

5676246633 : MAJOR PHARMACOLOGY AND TOXICOLOGY
KEYWORDS: PLATELETS, ADP, ANGELICA DAHURICA EXTRACT, PLATELET AGGREGATION, CYCLOOXYGENASE, GASTRIC ULCER

PITIPHAT SRISOM: EFFECT OF *ANGELICA DAHURICA* EXTRACT ON PLATELET AGGREGATION IN RAT. ADVISOR: ASST. PROF. RATAYA LUECHAPUDIPORN, Ph.D., 58 pp.

Kot Sor is one of Thai traditional medicine. It is used for improvement of circulatory system. The aims of this study were to investigate the effect of *Angelica dahurica* extract (ADE) on platelet aggregation, coagulation and gastric lesion in rat. From *in vitro* model, ADE at 1 mg/ml significantly inhibited ADP (5 μM)-induced platelet aggregation by 25.5±3.8 % ($p<0.05$) when compare with vehicle control. ADE inhibited COX-2 enzyme more potent than COX-1 enzyme with IC_{50} of 0.03 and 0.76 mg/ml, respectively. *In vivo* model, rats were treated with ADE 25, 50, 100 and 200 mg/kg orally for 7 days. ADE at 100, 200 mg/kg significantly inhibited ADP (5 μM)-induced platelet aggregation by 41.1±8.7% ($p<0.001$) and 45.1±8.9% ($p<0.001$) when compare with control group. The combination of ADE 200 mg/kg and ASA 80 mg/kg significantly inhibited ADP-induced platelet aggregation more than those treated with ASA alone by 84.7±8.6% vs. 47.0±11.3% ($p<0.001$). Moreover, the effects of ADE on coagulation parameters were evaluated by PT, aPTT and TT assays, all group of ADE did not affect to coagulation parameter. Finally, ADE did not change macroscopic morphology and pathohistology including submucosal edema, mucosal hemorrhage, epithelial cell loss and infiltration of inflammatory cell. In conclusion, *Angelica dahurica* extracts can inhibit ADP-induced rat platelet aggregation both *in vitro* and *in vivo* model. The mechanism of action may involve in the inhibition of COX-1 enzyme and ADP-induced pathway in platelets. ADE did not affect to coagulation parameter and gastric adverse effect. Moreover, the combination of ADE and ASA increase antiplatelet aggregation effect without increasing gastric lesion.

Department:	Pharmacology and Physiology	Student's Signature	....
Field of Study:	Pharmacology and Toxicology	Advisor's Signature	....
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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AC	Adenylate cyclase
AD	*Angelica dahurica*
ADE	*Angelica dahurica* extract
ADP	Adenosine 5’ –diphosphate
ASA	Acetylsalicylic acid
cAMP	Cyclic adenosine 3’, 5’-monophosphate
^{o}C	Degree celcius
Cm	Centrimeter
COX	Cyclooxygenase
CVD	Cardiovascular disease
DAG	Diacylglycerol
EDTA	Ethylenediamine tetraacetic acid
GP	Glycoprotein receptor
5-HT	5-Hydroxytryptamine
hr	hour
IP_3	Inositol (1, 4, 5)-triphosphate
µg	Microgram
µl	Microliter
µM	Micromolar
mg	Microgram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
PGI_2	Prostacyclin
PI_3K	Phosphoinositide 3-kinases
PIP_2	Phosphatidylinositol (4, 5)-bisphosphate
PKC	Protein kinase C

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PLA_2	Phospholipase A_2
PLC	Phospholipase C
PPP	Platelet poor plasma
PRP	Platelet rich plasma
TxA_2	Thromboxane A_2
VASP	Vasodilator-stimulated phosphoprotein
vWF	von Willebrand factor
WHO	World Health Organization

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CHAPTER I
INTRODUCTION

Background and rationale

Platelets play an important role in hemostasis and repair at sites of vascular damage by aggregating to form platelet plugs that stop hemorrhaging (1). However, development of excess platelet plugs causes a thrombus which can progress to cardiovascular ailments including coronary artery disease (CAD) strokes and peripheral artery disease (PAD). A World Health Organization (WHO) report in 2014 found that heart diseases and strokes are the main causes of death worldwide affecting 17.3 million people, and this tendency is increasing. In Thailand, the Ministry of Health reported that 54,530 people died from cardiovascular disease in 2013, second only to cancer (2).

Platelets prevent blood loss after vascular injury. Normally platelets circulate in the blood vessels without interacting with the endothelial cells, which counteract coagulation by producing and secreting nitric oxide (NO) and prostacyclin (PGI_2) to inhibit platelet activation. When blood vessel injury occurs, this leads to hemostasis which can be divided into two phases. Primary hemostasis is characterized by vascular contraction, platelet adhesion and formation of a soft aggregate plug. As a consequence of vascular injury, the platelets are exposed to subendothelial matrix, collagen and von Willebrand Factor (vWF), and they bind to specific receptors at the site of injury and slow the bleeding. This is known as platelet adhesion. Platelet activation then leads to increasing cytoplasmic calcium that promotes platelet shape change and releases soluble agonists such as adenosine diphosphate (ADP), serotonin (5-HT), thromboxane A_2 (TxA_2) and adenosine triphosphate (ATP) from dense granules and alpha granules which amplify platelet activation. After that, ADP binds an ADP receptor subtype $P2Y_{12}$ on the platelet membrane. The $P2Y_{12}$ receptor couples to a Gi mediated signaling pathway that inhibits adenylate cyclase and decreases cyclic adenosine 3’, 5’–monophosphate (cAMP) levels. TxA_2 is produced and secreted from the platelets. Arachidonic acid (AA) is a precursor for TxA_2 formation that is cleaved

from the phospholipid membrane by phospholipase A_2 (PLA_2). Then, AA is metabolized by the enzyme activity of cyclooxygenase-1 (COX-1) and thromboxane synthase (TXS) leading to the formation of TxA_2 which binds to TP-**α** and TP-**β** receptors. Both TP receptor subtypes mediate predominantly through Gq protein which activates PLC-**β** to increase intracellular calcium and stimulates protein kinase C (PKC). Thrombin agonist binds to proteinase-activated receptors (PARs) on the platelet membranes. The PAR1 receptor couples to Gi which inhibits adenylate cyclase (AC) and results in decreasing cAMP levels; its signaling is the same as the ADP agonist and increases intracellular calcium. This triggers a conformation change of the glycoprotein IIb/IIIa (GPIIb/IIIa) receptor on the platelet membranes and leads to interaction between fibrinogen cross-linking in the GPIIb/IIIa receptor, called platelet aggregation (3). The last, secondary hemostasis stabilizes the platelet plug by coagulation cascade, including both intrinsic and extrinsic pathways. After the activation of coagulation cascade, prothrombin is cleavage to form thrombin. Finally, the generated thrombin cleave from fibrinogen to fibrin with cross-linking on the platelets. The cross-linked fibrin forms a mesh overlying the platelet plug that completes the clot.

Antiplatelet and anticoagulation agents such as aspirin (ASA), heparin and warfarin are used for the treatment and prevention of cardiovascular disease. The United State Preventive Services Task Force (USPSTF) found that ASA 81 mg per day or 325 mg every other day reduced the risk of myocardial infarction and ischemic stroke by 32% and 17%, respectively (4). In addition, the CAPRIE study (Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events) compared clopidogrel to aspirin in term of effectiveness and safety. The long-term use of clopidogrel prevented peripheral artery disease (PAD) and coronary artery disease (CAD) superior to ASA. However, the main side effects such as bleeding, ulceration and gastric irritation are still the problems (5). Therefore, the development of new antiplatelet agents with superior, low side effects and low cost from Thai traditional medicine should be examined. *Angelica dahurica* is the one of Thai traditional medicine that has a potential to be developed for antiplatelet agent.

Angelica dahurica (AD) (Fisch. Ex Hoffm.) Benth. & Hook.f.ex Franch & Sav. is called Bai Zhi in China and Kot Sor in Thailand. AD is used for the treatment of headaches, toothache, colds and neuralgia in Chinese traditional medicine (6). In Thai traditional medicine, Kot Sor is used for circulatory disorders (7). AD extract (ADE) has the chemical constituents of furanocoumarins such as byakangelicin, byakangelicol, imperatorin, iso- imperatorin, knidilin, oxypeucedanin hydrate, oxypeucedanin methanolate and scopoletin (6). Previous studies found that 1 mg/ml EtOH extract of ADE inhibited platelet aggregation induced by ADP and increased the lag phase in collagen induction, but did not inhibit AA-induced aggregation. ADE increased cAMP level but did not affect the TxA_2 level (8).

Our previous study investigated the *in vitro* effect on antiplatelet aggregation activity of ADE on human platelets. However, effect of ADE is still need to investigate in the *in vivo* model. In the present study, the inhibitory effect of ADE on ADP-induced platelet aggregation in rat platelet was evaluated. So the aims of this study were to examine the *in vivo* effect of ADE on antiplatelet aggregation, coagulation and gastric lesions in rats.

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CHAPTER II

LITERATURE REVIEW

1. Platelets

Platelets, which are also known as thrombocytes, are cells which have no nucleus, and as such are the smallest of all the different blood cell types. They play an important role in the blood by preventing excessive bleeding through the clotting mechanism. They are disc-shaped with a typical size of 2-4 µm in diameter and 0.5 µm in thickness. They occupy a volume of around 7-11 fl. Their production relies upon the cytoplasm derived from megakaryocytes found within the bone marrow. The body will typically generate around 35 x 10^9 new platelets every day, with concentration levels observed to be between 150,000-450,000 cells/mL. The average platelet circulates for around ten days, with two-thirds remaining in circulation while the remainder will be temporarily sequestered in the spleen (9). Eventually, a majority of platelets reach senescence before their removal via macrophages within the liver and spleen.

2. Structure of Platelets

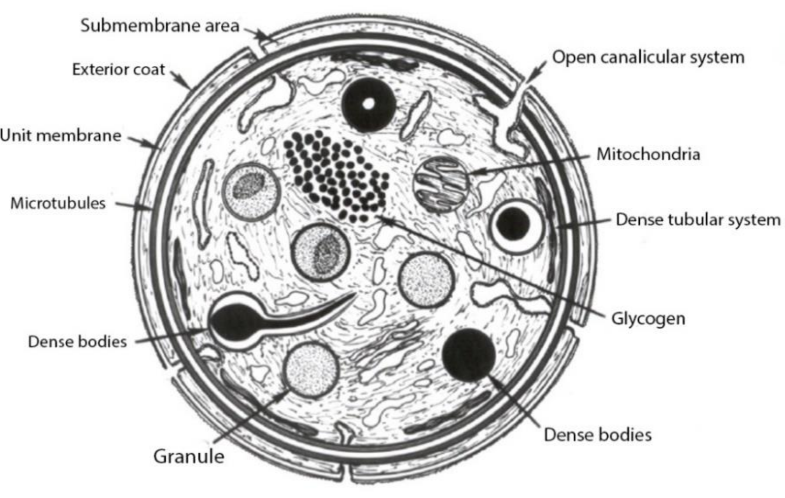

Figure 1. Platelet structure (10)

Platelets are constructed with four principal regions, which can be described as follows:

2.1 Peripheral zone

This region provides the initial hemostasis response of the platelets when injury to a blood vessel is detected. The platelet membranes serve as rich platelet receptors to support the adhesion, activation, and aggregation of the platelets. The platelet receptors remain in a state of continual expression until a conformational change expresses the receptor function as a result of platelet activation. Table 1 presents the various receptor classes along with their respective ligands.

Table 1. Major platelet membrane receptors and their ligand (11).

Receptors	Family	Ligands
Initiation of platelet recruitment		
GPIb-IX-V complex	Leucine-rich repeat family	VWF, thombin, FXI, FXII
Platelet adhesion and aggregation		
GPVI	Ig superfamily	Collagen, laminin
α2β1	Integrins	Collagen
α5β1	Integrins	Fibronectin
α6β1	Integrins	Laminin
Platelet receptors in the amplification phase		
$P2Y_1$	G protein-coupled receptors	ADP
$P2Y_{12}$	G protein-coupled receptors	ADP
$P2X_1$	G protein-coupled receptors	ATP
PAR-1	G protein-coupled receptors	Thrombin (High affinity)
PAR-4	G protein-coupled receptors	Thrombin (Low affinity)
TP-α	G protein-coupled receptors	Thromboxane
β2 adrenergic receptor	G protein-coupled receptors	Epinephrine

2.2 Sol-gel zone

This region is located under the peripheral zone and comprises the cytoskeleton, which provides the structural framework for the platelet. Its role is to maintain the shape of the platelet while simultaneously controlling the contractile system which governs the shape changes, internal contractions, pseudopod extensions, and granular constituent emissions which typically follow activation.

2.3 Organelle zone

This region comprises granules along with cellular components including mitochondria and lysosomes. Organelles play a part in the platelets' metabolic processes while storing various vial substances, such as enzymes, which allow the platelets to function effectively. The zone comprises two granule types: dense granules and alpha granules. Contents of the alpha granules include the adhesive proteins fibronectin, fibrinogen, von Willebrand factor (vWF), vitronectin, and thrombospondin. In addition to the proteins, other substances which promote growth can be found, including platelet factor 4, transforming growth factor, and platelet-derived growth factor (PDGF). The alpha granules also contain coagulation factors such as factor V, high molecular weight kininogen, factor XI, and plasminogen activator inhibitor-1. The dense granules contain serotonin, calcium, non-metabolic adenosine triphosphate (ATP), and adenosine diphosphate (ADP).

2.4 Membrane zone

This region is the site of the dense tubular system and contains high concentrations of calcium, which plays a vital role in triggering contractile events. The enzymatic systems which are required in prostaglandin synthesis are also found in the membrane zone.

3. Process of Hemostasis

Hemostasis is the name given to the process by which blood changes to a solid from its usual liquid form. To clot in this fashion, it is essential that blood vessels remain intact in order to control the process. These intact vessels contain endothelial cells which inhibit thrombus formation through the secretion of tissue plasminogen activator (t-PA) along with the deactivation of thrombin and adenosine diphosphate (ADP). However, if the blood vessels sustain injury, it becomes impossible for these functions to prevent hemostasis, which will follow in two stages: primary hemostasis and secondary hemostasis.

During the process of primary hemostasis, platelets come into contact with the damaged wall of the blood vessel to create what is known as a platelet plug. This occurs as the platelet adheres to the elements of the subendothelium, which is

followed by activation and shape change, before the platelet granular contents are released from the dense and alpha granules. Following this stage, fibrin-stabilized platelet aggregates are formed, alongside clot retraction (12). When the platelets are activated by exposure to negatively charged phospholipids, this enables coagulation factors to accumulate on the platelet membrane, which allows the production of thrombin and the deposition of fibrin. While platelet adhesion can be supported by a heightened shear rate, in order for the platelets to stick to the surface of a damaged blood vessel, it is necessary for both fibrinogen and vWF to be present. The main receptor for the vWF is the platelet glycoprotein (GP) receptor (Ib/IX and V), although it is also possible for vWF to form bonds with GP IIb-IIIa (13), expressing upon activation of the platelets. Fibrinogen and vWF are both able to interact with GP IIb/IIIa. However, vWF is not the only protein to play a part in platelet adhesion, since vitronection, laminin, and thrombospondin can also have some involvement. The activation of platelets is normally triggered when the platelet is exposed either to the damaged blood vessel wall or to the damaged endothelium, although there are several biological agonists which play a part in the process, such as ADP, thrombin, epinephrine, and thromboxane A_2. Upon activation, platelets change their shape from the original disc to a spherical form which exhibits long pseudopodia. At the start of the activation stage, trace amounts of thrombin are generated, which can lead to a strengthening of the coagulation process. This occurs because thrombin is able to activate factor XI within the contact system, along with the other coagulation co-factors V and VIII (14). Although fibrin can form initially at the site of the damage to the blood vessel, it will not yet be stable. Factor XIII, which serves to stabilize fibrin, can be activated by thrombin, which duly permits fibrin strands to be cross-linked, thereby stabilizing the platelet plug.

There are two initial mechanisms by which secondary hemostasis can take place, although both result in the formation of fibrin. The first of these is the extrinsic, or tissue factor pathway, while the other is the intrinsic, or contact activation pathway. In either case, the reactions which are triggered are basically identical and result in fibrin production. While the two pathways were once believed to be equally significant, later research has shown that the extrinsic pathway is the principal means of initiating

blood coagulation. In either case, the pathways represent a sequence of reactions by which the inactive enzyme precursors of a serine protease, known as zymogens, are activated before serving as catalysts for the following reactions which eventually lead to the creation of cross-linked fibrin (15). In this process, the serine proteases typically serve as coagulation factors and operate via the cleaving of downstream proteins, although some exceptions exist. It should be noted that FVIII and FV are glycoproteins, Factor XIII is a transglutaminase, and the coagulation factors tend to circulate in the form of inactive zymogens, resulting in the division of the coagulation reaction sequence into the two different pathways stated above.

The three pathways that makeup the classical blood coagulation pathway

Intrinsic
surface contact
XII → XII_a
XI → XI_a
IX → IX_a
(VIII, PL, Ca^{++})
X → X_a
XII – Hageman factor, a serine protease
XI – Plasma thromboplastin, antecedent serine protease
IX – Christmas factor, serine protease
VII – Stable factor, serine protease
XIII – Fibrin stabilising factor, a transglutaminase
PL – Platelet membrane phospholipid
Ca^{++} – Calcium ions
TF – Tissue Factor
($_a$ =active form)
Extrinsic
$TF:VII_a$ ← tissue damage
Common
X_a ← X
(V, PL, Ca^{++})
prothrombin → thrombin (serine protease)
XIII → $XIII_a$
fibrinogen → fibrin → stable fibrin clot

Figure 2. The classical blood coagulation pathway (15)

The clotting factors VIII, IX, X, XI, and XII are required in the intrinsic, or contact activation pathway, along with high-molecular-weight kininogen (HK or HMWK), prekallikrein (PK), calcium ions, and phospholipids which are secreted from platelets. Each component serves to convert the inactive factor X to become factor Xa. The pathway is initiated when the listed components, prekallikrein, high-molecular-weight kininogen, factor XI, and factor XII come into contact with a negatively charged surface. This contact stage may take place as a consequence of phospholipid interaction. As the components required for the contact phase are accumulated, prekallikrein is duly

converted to kallikrein, which is then able to activate factor XII to create factor XIIa. Factor XIIa achieves two results, in first activating factor XI to factor XIa, and then hydrolyzing further quantities of prekallikrein to become kallikrein. The outcome of this is to construct a sequence of reciprocal activations, whereby kallikrein then impacts HMWK triggering the emission of bradykinin, which works as to dilate the blood vessels. When calcium ions are present, factor XIa is able to activate factor IX creating factor IXa. This active factor IXa subsequently cleaves factor X and results in the production of factor Xa. It is this activated factor Xa which marks the point at which the two pathways, extrinsic and intrinsic, will merge.

The extrinsic, or tissue factor pathway, starts at the point where the damage occurred and is triggered by the release of factor III, which is also known as tissue factor. Tissue factor has a part to play when factor VIIa serves as a catalyst to activate factor X. Factor VIIa is able to cleave factor X to produce factor Xa just as factor IXa is produced in the intrinsic pathway. The resulting factor Xa then induces the conversion of factor II to become factor IIa, or thrombin, and then this thrombin is able to cleave fibrinogen to create the fibrin which is required to reinforce the platelet plug (16).

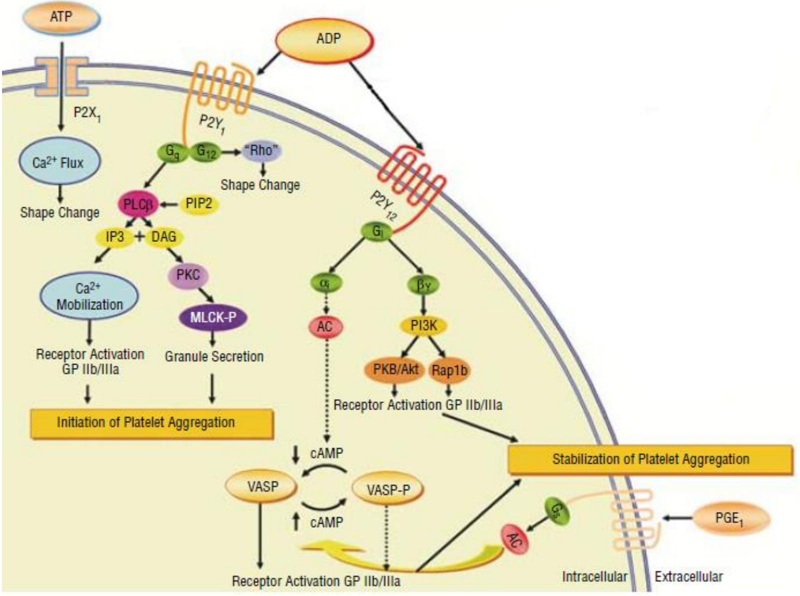

Figure 3. Mechanism of ADP-induce platelet aggregation (16)

4. The ADP-induced Platelet Aggregation Mechanism

ADP, or adenosine 5’–diphosphate is able to interact with the pair of differing purinergic receptors, $P2Y_1$ and $P2Y_{12}$, on platelets. When the $P2Y_1$ receptor is activated, the outcome is a change in shape along with a mild stage of platelet aggregation. ADP binds to the Gq-coupled $P2Y_1$ receptor which then leads to the activation of phospholipase C (PLC), thereby creating both diacylglycerol (DAG) and inositol triphosphate (IP3) from phosphatidylinositol biphosphate (PIP2). The protein kinase C (PKC) is activated by diacylglycerol, causing the phosphorylation of myosin light chain kinase (MLCK-P), while IP3 is able to mobilize intracellular calcium. When the G-protein G_{12} binds to the $P2Y_1$ receptor, this can activate the “Rho” protein, causing a change in the shape of the platelets. When ADP binds to the Gi-coupled $P2Y_{12}$ receptor, this serves to release Gi protein sub-units (ai and bg), allowing the platelet aggregation to become stable. Ai sub-units limit the activity of adenylyl cyclase (AC), thereby cutting the level of cyclic adenosine monophosphate (cAMP) and restricting the cAMP-mediated phosphorylation of vasodilator-stimulated phosphoprotein (VASP-P). The condition of the VASP-P is able to govern the activation of the glycoprotein (GP) IIb/IIIa receptor. Meanwhile, the bg sub-unit initiates a sequence of events whereby phosphatidylinositol 3-kinase (PI3K) is first of all activated, leading to the subsequent activation of the GP IIb/IIIa receptor which is itself triggered by the activation of a serine-threonine protein kinase B (PKB/Akt) along with additional Rap1b GTP binding proteins. In turn, prostaglandin E1 (PGE_1) activates AC, which leads to a rise in cAMP levels and elevates the status of VASP-P (17).

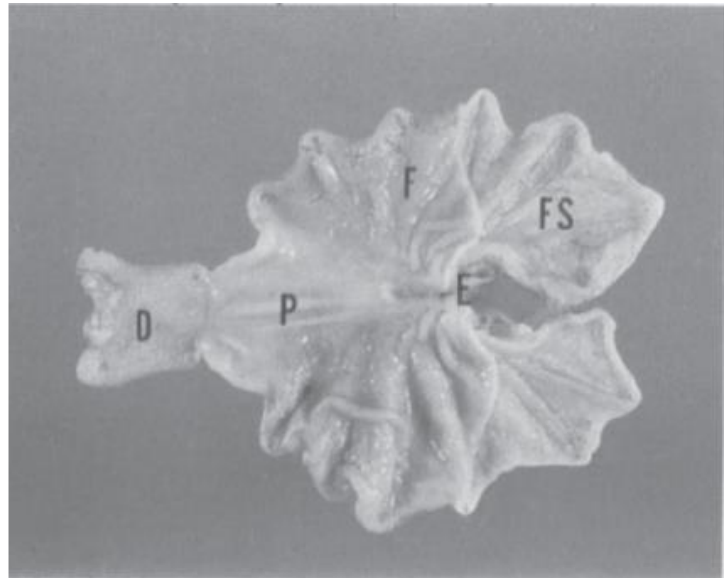

Figure 4. Opened along the greater curvature. FS, forestomach; E, esophagus; F, fundus; P, pylorus (antrum); D, duodenum (18).

5. Factor induce gastric lesion

In general, the stomach can protect itself from various injury by mucus and bicarbonate as well as generate protective prostaglandin (PG) in gastric mucosa. The PG generated by arachidonate metabolism via COX-activity. There are two types of COX-enzyme, COX-1 (constitutive form) are convert arachidonic acid (AA) to Prostaglandins E_2 (PGE_2), prostacyclin (PGI_2) and thromboxane A2 (TxA_2). PGE_2 has cytoprotective effect and enhance mucosal blood flow, TxA_2 involve vasoconstrictor and platelet activity. The COX-2 enzyme (inducible form) important role in inflammatory, COX-2 activated by pro-inflammatory cytokine lead to generate PG that is responsible for the inflammation, pain, swelling, redness, heat, including fever, which the point of action of nonsteriodal anti-inflammatory drug (NSAID) such as aspirin. That used treat inflammation, fever and antiplatelet aggregation. However, the major limitations of clinical application are serious intestinal side effect from decrease PGE_2 level, The PGE_2 activity is strong cytoprotective to enhance epithelial mucus, secretion of bicarbonate, inhibits movement stomach, inhibit free radicals by leukocytes and increased mucosal blood flow (19).

6. The mechanism of action of antiplatelet and anticoagulant drug

6.1 Aspirin (acetylsalicylic acid):

Aspirin irreversible COX-inhibition, so inhibited acetylation of cyclooxygenase (COX) enzyme, it will convert arachidonic acid, a substance ecosanoids including leukotrienes, prostaglandins, thromboxane A2 (TXA_2) and prostacyclin (PGI_2), so the effect of aspirin decrease TXA_2 resulting in antiplatelet aggregation. Moreover aspirin low dose (less than 500 mg/day) to inhibit COX-1 enzyme more than COX-2, about 100-160 times therefore have outstanding results in the inhibition of TXA2 and less effective inhibition of PGI_2 (20).

Side effects: Rash, vomiting, GI pain, ulceration, bleeding

6.2 Clopidogrel

The effectiveness of clopidogrel depends upon its ability to inhibit the ADP receptors located upon platelet membranes. Since it is a prodrug, its activation requires the presence of CYP2C19 (21). The immediate effect is to irreversibly inhibit the $P2Y_{12}$ subtype of the ADP receptor in a very specific manner. This process is critical in terms of platelet activation, and the subsequent fibrin cross-linking which eventually results (22). Within just two hours of an oral dose of clopidogrel it is possible to discern clear signs of platelet inhibition, but the effects do begin rather slowly. If a more rapid outcome is necessary, the dose can be increased to up to 600 mg.

Side effects: Nausea, vomiting, rash, agranulocytosis

6.3 Heparin:

Heparin works by binding to antithrombin III (AT), which is an enzyme inhibitor. This leads to its activation as a result of the heightened levels of flexibility at the reactive site loop. Once activated, the antithrombin III will deactivate thrombin along with various other proteases which usually play a role in the blood clotting process. Factor Xa is the most important of these. When heparin is used, its binding effect influences the activity rate of AT in causing the inactivation of the various proteases and can be raised around 1000-fold due (23).

Side effects: Hematoma, hemorrhage, erythema, anaphylaxis, hypersensitivity

6.4 Warfarin: The generally clotting fact such as II, VII, IX and X synthesis in the liver requires vitamin K to cofactor. Vitamin K gives the carboxyl group (COO-) to the glutamate (gamma-carboxylation) in clotting factor, so making active clotting factors. In this process, vitamin K is oxidized to vitamin K epoxide. This can reverse the vitamin K epoxide to vitamin K in normal liver. When warfarin inhibits change vitamin K epoxide to a vitamin K is inhibited gamma-carboxylation of clotting factor II, VII, IX and X makes inactive clotting factors (24).

Side effects: Hemorrhage, rash, anemia, abdominal pain, headache

7. *Angelica dahurica* (Fisch. EX Hoffm.) Benth. & Hook.f. ex Franch & Sav

7.1 Morphology

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A. Aerial parts B. Root part (use)

Figure 5. Morphology of *Angelica dahurica.*

Family : Umbelliferae

Synonym : *Callisace dahurica* Franch & Sav., *Angelica Macrocarpa* H. Wolff, *Angelica porphyrocaulis* Nakai & Kitag., *Amgelica tshiliensis* H. Wolff

Chinese name : Bai Zhi

Common name : Dahurian angelica

Angelica dahurica (AD) is a perennial plant which can reach up to 2 meters in height. It has a ribbed stem which is usually green-purple in color and between 2 and 8 cm in thickness. The root is cylindrical and brown in color, reaching up to 5 cm in diameter. In their first year of growth, the plants form a clump which can reach 75 cm in height, or more in richer soil types. The leaves are complex and divided, reaching a maximum length of 50 cm. They are usually colored a purple-red around the base. When the plant reaches two or three years of age, a strong hollow stalk appears, around 2.5 cm in diameter and 2.4 m in height. The flowers which are produced are green-white in color, reaching 30 cm in diameter, and usually bloom during the summer months of July and August, or occasionally September. The seeds begin to appear from August to October, starting out in green-white before turning brown with a paper-like texture when they are ripe. The flowers contain both male and female reproductive organs, and insects are the means of pollination. Self-pollination is also possible for this species (25).

AD is named as Bai Zhi in China and Kot Sor in Thailand. AD is used for the treatment of headache, toothache, cold and neuralgia in Chinese traditional medicine (6). In Thai traditional medicine Kot Sor is used for treatment of circulatory disorder (7). Moreover, In Thailand the YA-HOM is a mixture of many herbs including the root of AD, is used for treatment of circulatory disorder. In addition, it should be aware of herb-drug interaction between Thai traditional medicines and antiplatelet or anticoagulant.

7.2 Pharmacological activity of *Angelica dahurica*

The previous study showed 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) respectively. While imperatorin, one of the chemical compound in AD, at concentration of 300 μM can inhibit ADP-induced platelet aggregation by 33.96% (p=0.012) compared with vehicle control but not 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). Both ADE and imperatorin could not inhibit AA-induced platelet aggregation but they could delay platelet aggregation. ADE can significantly increase cAMP level (*p*=0.027) (8). In addition, 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 did not decrease phosphorylation of Akt at Thr^{308}. The study suggested that the mechanism of antiplatelet aggregation of ADE may be involved the increase of VASP phosphorylation and mediated through cAMP/PKA pathway (26).

Anti-tumor activity

The gene expression of p53, Bcl, and Bax can be lowered to a significant extent by the ethanolic extract of ADE. Furthermore, apoptosis can be induced via caspase cascade and through cell cycle arrest. Anti-carcinogenic properties have been shown by the ethanol-ethyl acetate fraction when applied in HT-29 cancer cells (27). The ADE extract contains a hexane component which can inhibit human tumor cell proliferation in the case of cancers affecting the ovaries and colon as well as melanomas (28).

Anti-inflammatory activity

The dahuribiethrins A–G, representing a total of seven new dimeric furanocoumarins, were obtained from *Angelica dahurica* via extraction from the roots. Four of the compounds, numbers 2, 3, 4, and 5, were observed to limit the production of nitric oxide within those lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells which had IC_{50} values falling between 8.8 and 9.8 μM (29). ADE was shown to inhibit the expression of both cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), resulting in the suppression of COX-2-derived prostaglandin E_2. Furthermore, production derived from iNOS in lipopolysaccharide (LPS)-stimulated RAW264.7 was also suppressed (30).

Anti-oxidant activity

Moderate DPPH$^{\bullet}$ scavenging activity along with strong ABTS$^{\bullet+}$ scavenging activity was observed in a number of coumarins derived from extracts of *Angelica dahurica* including bergaptol, imperatorin oxypeucedanin hydrate, xanthotoxol, and 5-methoxy-8-hydroxypsoralen. Furthermore, notable inhibition effects upon HepG2 cells were observed with isoimperatorin, phelloptorin, and pabularinone, resulting in respective IC_{50} values of 8.19, 7.49, and 7.46 µM. Meanwhile, pabularinone was also seen to have a moderate inhibitory effect upon HeLa cells, resulting in an IC_{50} value of 13.48 µM (31).

Root extracts of *Angelica dahurica* were observed to exhibit ABTS, DPPH, and hydroxide radical scavenging activity when evaluated at dosages of between 0.12 and 2.0 mg/mL. The respective IC_{50} values for the radical-scavenging activity of ABTS and DPPH were 0.20 and 0.32 mg/ml, while for water extract and ethanol extract the values were 0.24 and 0.13 mg/ml respectively (32).

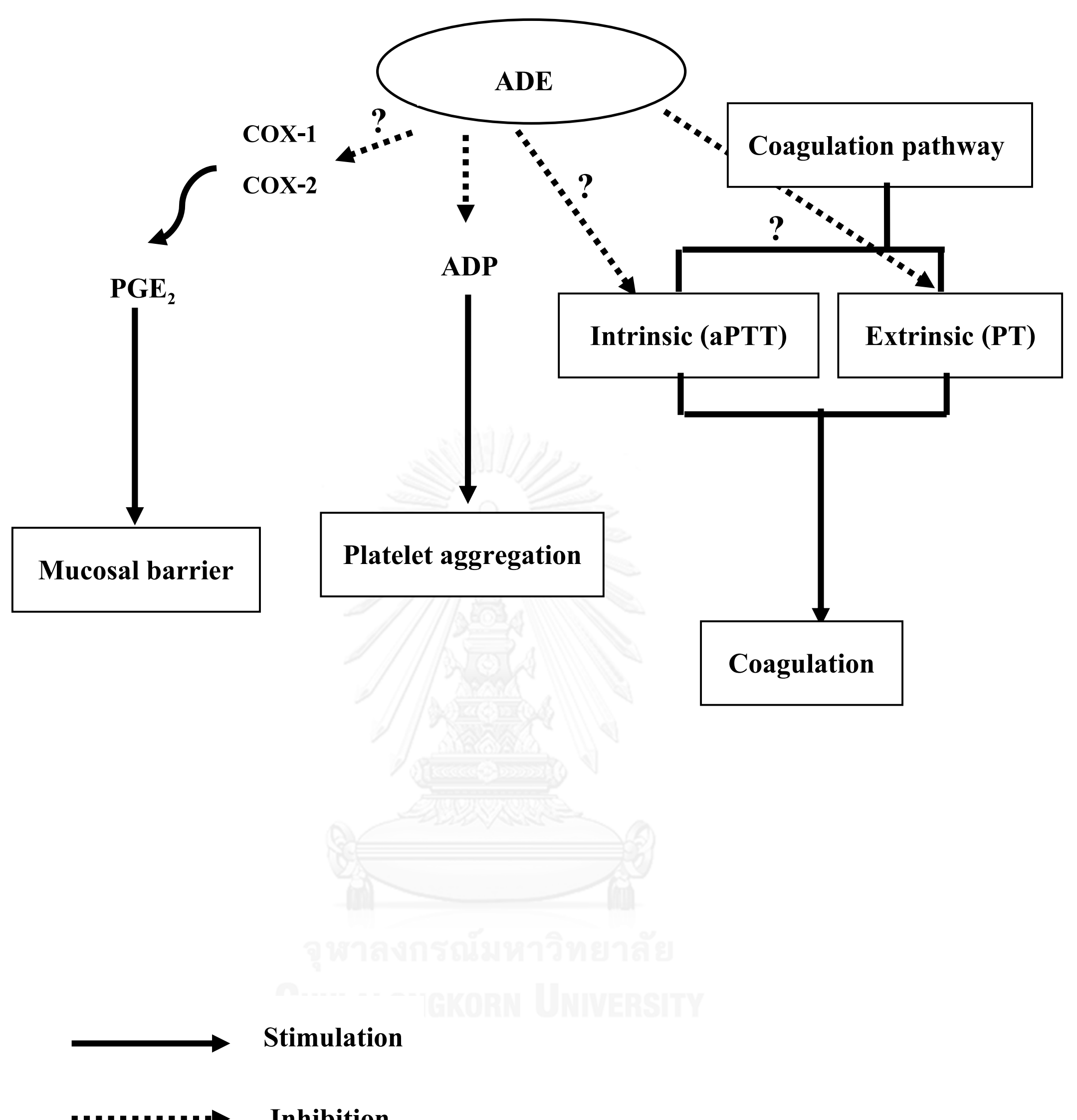
8. Objectives

To investigate the effects of ADE on platelet aggregation, coagulation and gastric lesion in rat.

9. Hypothesis

1. ADE can inhibit ADP-induced platelet aggregation in rat.
2. ADE may inhibit coagulation parameter in rat.
3. ADE do not cause gastric lesion in rat stomach.

10. Conceptual Framework



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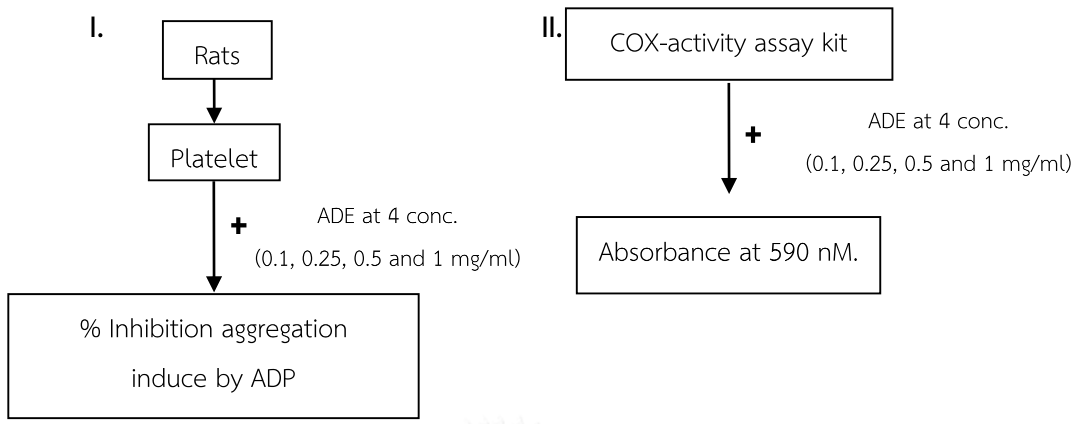
ADE: *Angelica dahurica extract*

11. Research design

11.1 *In vitro* model:

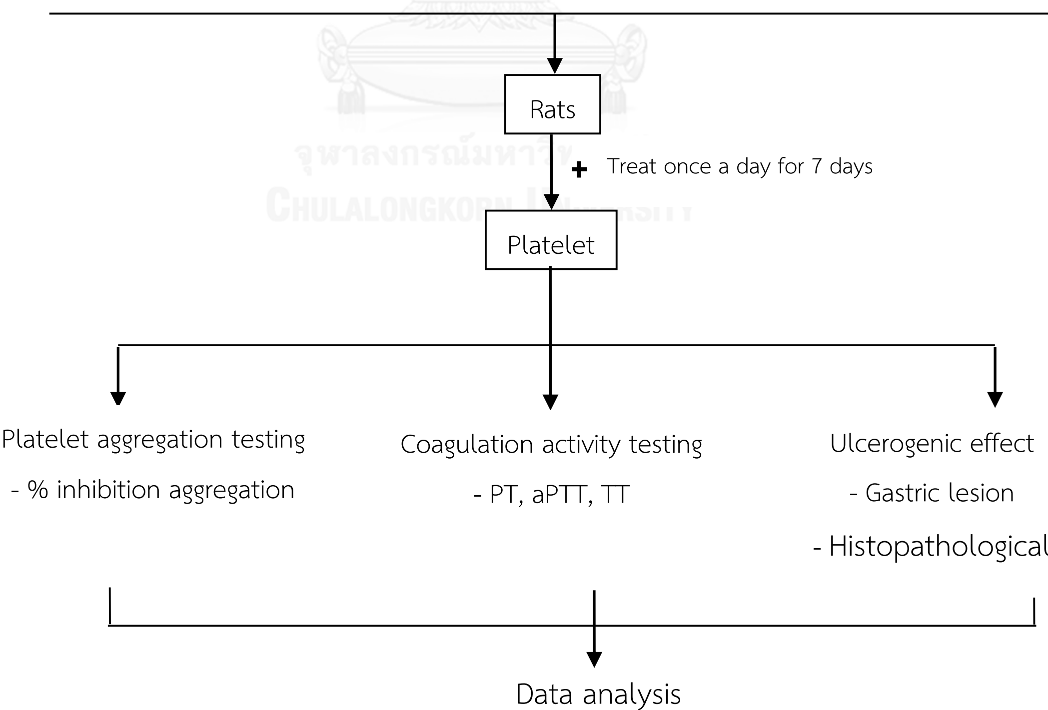


11.2 *In vivo* model:

2% Tween80 10 ml/kg PO (n=8)
ASA 40 mg/kg PO (n=8)
ASA 80 mg/kg PO (n=8)

ADE 25 mg/kg PO (n=8)
ADE 50 mg/kg PO (n=8)
ADE 100 mg/kg PO (n=8)
ADE 200 mg/kg PO (n=8)

ADE 25 + ASA 40 or 80 mg/kg PO (n=8)
ADE 50 + ASA 40 or 80 mg/kg PO (n=8)
ADE 100 + ASA 40 or 80 mg/kg PO (n=8)
ADE 200 + ASA 40 or 80 mg/kg PO (n=8)



ADE: *Angelica dahurica* extract

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 ADE (*Angelica dahurica* extract)

Dried root of AD (figure 5A) was purchased from a traditional drugstore in Bangkok, Thailand during 2015. After that, mash AD root 1 kg by hummer mill to fine powder (figure 5B). Its ground powder was extracted with 50% EtOH (20 L) overnight, 2 time and then filtered by cotton (figure 5C). Remove solvent with rotary vacuum evaporation and water bath until dry (figure 5D-E). The combined extract was evaporated to dryness to obtain ADE for 168.25 g. The extract yield is approximately 17% of the dried-root powder. ADE was dissolved in DMSO for stock solution and diluted to the final concentration of 0.1, 0.25, 0.5 and 1 mg/ml with normal saline. *In vivo* model, ADE was dissolved in 2% Tween 80 to the final concentration of 25, 50, 100 and 200 mg/kg.

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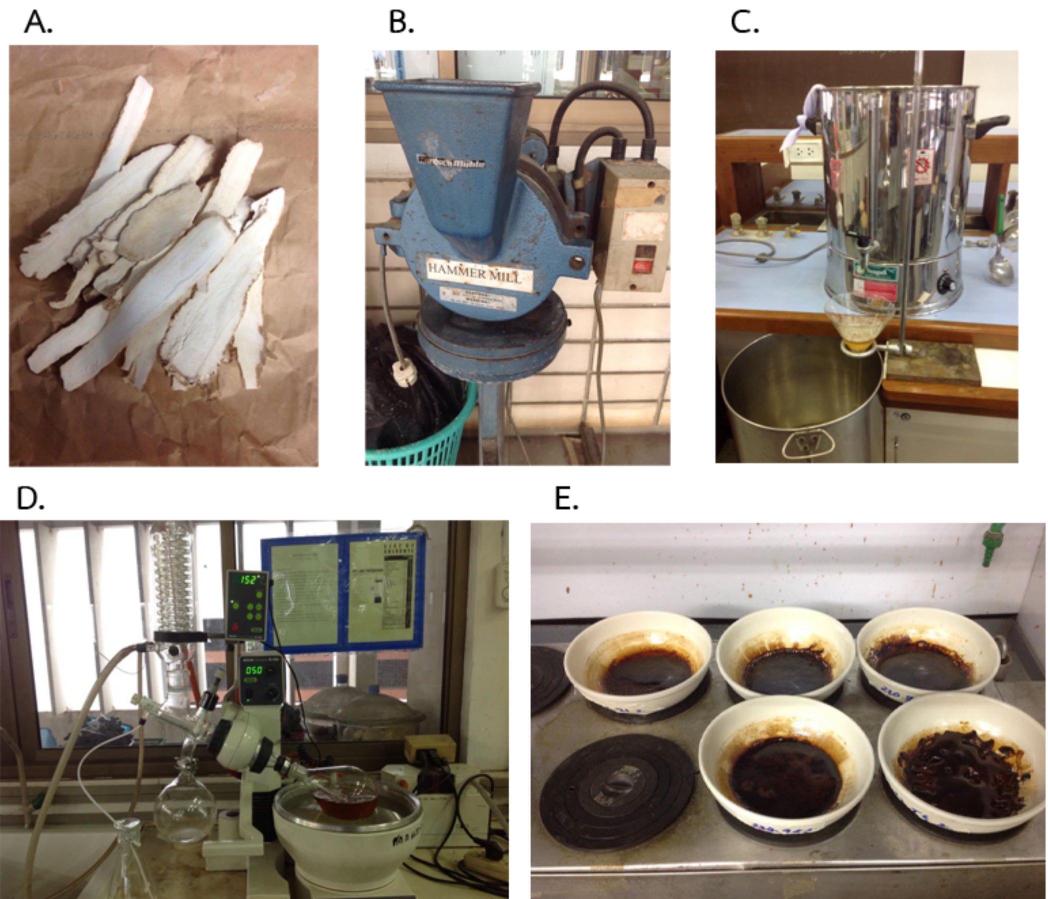

Figure 6. The extraction of *Angelica dahuriga*; mashed AD root (A), Hammer mill to fine powder (B), extracted AD root powder by 50% EtOH overnight, 2 times and then filtered by cotton (C). Remove solvent with rotary vacuum evaporation and water bath until dry (D, E).

1.2 Animals

Male Sprague-Dawley rats, weight 260 - 340 g, were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Thailand. Rats were acclimatized to standard laboratory conditions (25±2 ^{o}C, 55-60% humidity and 12 h light/dark cycle) for 5 days in the animal facility in Faculty of Pharmaceutical Sciences, Chulalongkorn University. Food and water were given ad libitum. All animal use protocol no. 1533006 approval from the Certification of Institutional Animal Care and Use Committee (IACUC).

1.3 Chemicals

The chemicals adenosine diphosphate (ADP), arachidonic acid (AA), acetylsalicylic acid (ASA), COX inhibitor screening assay, sigma coat, trisodium citrate, tween 80 were purchased from Sigma (St Louis, MO, USA).

Other chemicals were purchased from commercial sources as follow: 10% neutral buffered formalin from Bio-optica®, USA. 0.9% normal saline from GHP®, Thailand.

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

1.4 Instruments

- Aggregometer Aggram™, Helena laboratory, USA
- Embedding Center TEC-2800, HESTION, Australia
- Light microscope Nikon Eclipse E200, Nikon, Japan
- Microplate reader Wallac model 1420, PerkinElmer Ltd.,USA
- pH meter CG842 Schott, Scientific Promotion, co., Ltd., Japan
- Rotary evaporator , Eyela, Japan
- Table Top Refrigerated Centrifuge Rotina 380 model, Andreas Hettich GmbH & Co. KG, Germany
- Ultrasonic bath bandelin, Bandelin GmbH & Co. KG, Germany
- Vortex-Mixer, Scientific Industries, USA
- Water bath memmert, Chatcharee Holding co.,Ltd., Thailand

2. Methods

2.1 *In vitro* model

2.1.1 Platelet aggregation assay

Rats were anesthetized using pentobarbital because it does not have platelets aggregation effect (33). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as follows: rat blood collected from heart was anti-coagulated with 3.8% sodium citrated (9:1, v/v), and centrifuged at 170 × g for 15 minutes at 21 °C to get PRP. The precipitate was further centrifuged at 350 × g for 10 minute at 21 °C to yield PPP. PRP was incubated with 0.5% DMSO or various concentrations of aspirin 0.5 and

1 mM (positive control) or ADE 0.1, 0.25, 0.5 and 1 mg/ml at 37 °C for 3 minutes. Then, ADP the aggregation-inducing agent was added. Aggregation was measured using a platelet aggregometer.

2.1.2 COX-screening assay

Various concentration of ADE was incubated directly with COX-1 or COX-2 in assay buffer for 5 min. Then, AA and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) was added as a substrate and incubated for 2 min. The reaction between prostaglandin G_2 (PGG_2) and ADHP produces the highly fluorescent compound resorufin. Resorufin fluorescence can be analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. The effect of ADE on COX activity were evaluated by Inhibition rate and calculated as follow:

Inhibitory rate (%) = (control activity – sample activity) x 100 / control activity.

2.2 *In vivo* model

2.2.1 Treatments

Male Sprague-Dawley rats weighing between 260 – 340 g were divided into eleven groups of 8 animals. Group I received 2% tween80 (10ml/kg); Group II and III received aspirin 40 and 80 mg/kg respectively. Group IV to VII received ADE 25, 50, 100 and 200 mg/kg respectively and Group VIII to XI received ADE 25, 50, 100 and 200 mg/kg plus aspirin 80 mg/kg each group respectively. All drugs were administered orally to rats once daily at 9 AM for 7 days. On last day rat were fasted for 24 hr. with free access to water prior to the experiment. All *in vivo* experiments were conducted 60 min after the treatment on day 7.

2.2.2 Platelet aggregation assay

Platelet aggregation was performed using the turbidimetric method (34). Blood was collected after the last administration. All rats were anesthetized using pentobarbital (i.p., 50 mg/kg body wt.). Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as follows: rat blood collected from heart was anti-coagulated with 3.8% sodium citrated (9:1, v/v), and centrifuged at 170 × g for 10 minutes to get PRP. The precipitate was further centrifuged at 350 × g for 15 minute to yield PPP. Then, 0.2 mL of PRP in a cuvette was placed in the aggregometer and continuous stirred at 1000 rpm at 37°C. After 1 min, ADP in the final concentration of

1, 2.5, 5 or 10 μM was added to induce aggregation, and measured the light transmission. The light transmission change was recorded for 6 min. Data were expressed as the percentage of maximal aggregation. The percentage of inhibition of platelet aggregation activity of ADE or aspirin were calculated by using the following equation:

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

A is the maximum aggregation of control group, whereas

B is the maximum aggregation of treatment group.

2.2.3 Coagulation parameter

The coagulation parameters were measured in 2 ml of blood which anti-coagulated by 3.8% sodium citrated (9:1, v/v) use to measure the coagulation parameter including Prothrombin time (PT), activated partial thromboplastin (aPTT) and Thrombin time (TT). The PT, aPTT and TT were determined according to the Quality lab co. ltd by using a Sysmex CA-530 plasma coagulation analyzer from Sysmex Corporation (Kobe, Japan).

2.2.4 Macroscopic examination

Rats were sacrificed and the stomachs were removed immediately to determine the gastric lesion index after the platelet aggregation assays. The stomach tissues were cut longitudinally, opened along the greater curvature, and fix stomach with tack before measured. The total length (mm) of visible mucosal lesions in each stomach sample was measured and used to establish a lesion area, calculated by $\pi/4 \times a \times b$ mm^2, where a is the long axis and b is the short axis (35). The sum of the total scores was divided by the number of animals to obtain the mean lesion index for each group.

Calculations: Lesion index (UI) = $\sum (mm)^2$ of lesion areas / N

(N = Number of rat used in each group)

2.2.5 Histopathological evaluation

The tissue was fixed with 10% Formalin, embedded in paraffin, and sectioned 4 μm thick (36). The sections were then stained with hematoxylin and eosin and examined under a light microscope. Histological grading was assessed according to the criteria described by (Wang et al.,2016). Briefly, each histological section was assessed

for hemorrhagic damage (score: 0–4), edema in the upper mucosa (score: 0–4), epithelial cell loss (score: 0–3) and the presence of inflammatory cells (score: 0–3). Each section was coded to eliminate observer bias and was evaluated on a cumulative basis to give the histological index, with a maximum score of 14.

2.3 Statistics analysis

All data were expressed as the means ± standard deviation (SD). Differences between groups were assessed by one-way analysis of variance. If there were significant differences among group means, then each group was compared using Tukey method using the SPSS software (Version22). P-values less than 0.05 were accepted as statistical significance.

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CHAPTER IV
RESULTS

1. *In vitro* model

1.1 Platelet aggregation

The representative aggregrams of the effect of ADE on rat platelet aggregation were shown in figure 7. Platelets were pre-incubated with 0.5% DMSO (vehicle control), ASA (positive control) or ADE for 3 minutes and then adding ADP to initiate aggregation. The results showed that 0.5 and 1 mM ASA or ADE at concentration of 0.1, 0.25, 0.5 and 1 mg/ml decrease percent maximum of aggregation.

To assess the effect of ADE on platelet aggregation, we investigated the effect of ADE on maximum platelet aggregation induced by ADP 5 µM. As shown in figure 8, the control group incubated with vehicle has the maximum platelet aggregation of 64.7±1.6%. ADE at 1 mg/ml significantly decrease the maximum platelet aggregation to 49.1±1.3 %.

ADE at concentration of 0.1, 0.25, 0.5 and 1 mg/ml showed % inhibition of ADP-induced platelet aggregation by -3.6±4.7%, -0.2±1.8%, 8.4±2.4% and 25.5±3.8% respectively (figure 9). The results indicated that ADE at 1 mg/ml significantly inhibited platelet aggregation in rat and comparable to 0.5 and 1 mM ASA (14.7±1.9% and 34.5±2.6%, respectively).

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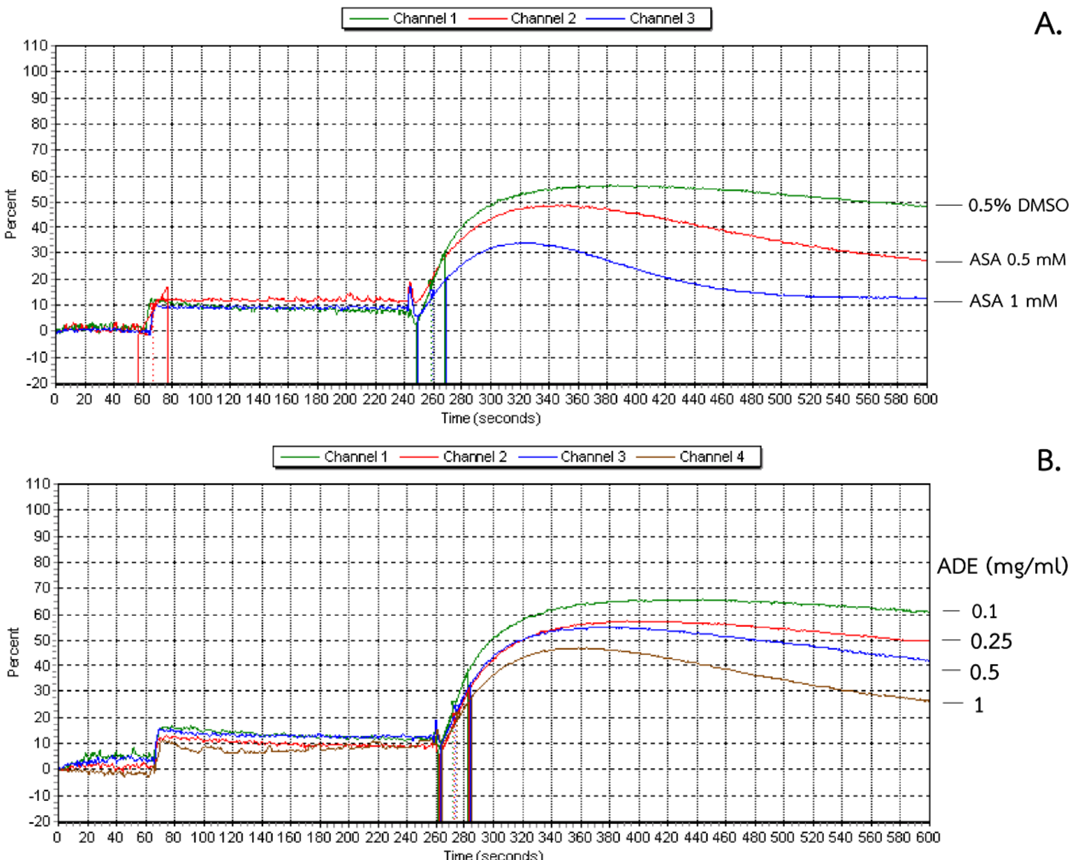

Figure 7. The representive aggregrams of the effect of 0.5% DMSO, ASA at 0.5-1 mM (A) and ADE at 0.1-1 mg/ml (B) on ADP (5 μM)-induced platelet aggregation.

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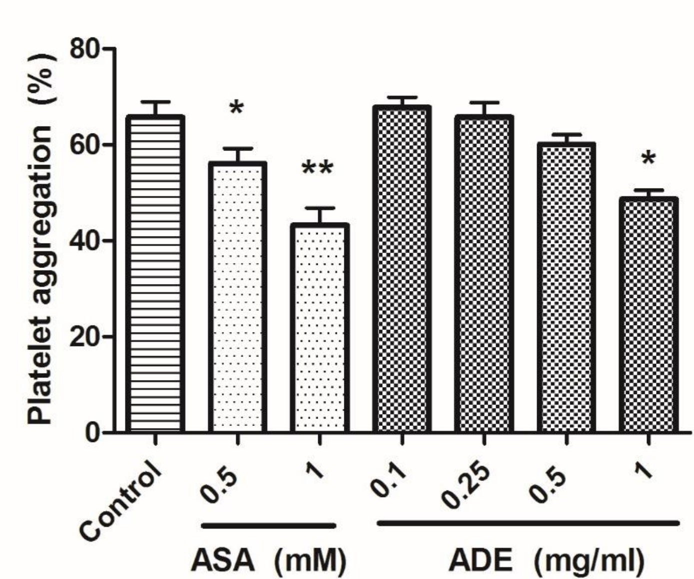

Figure 8. Percent maximum aggregation of ADP-induced platelet aggregation. The graph showed the effect of 0.5% DMSO, ASA at 0.5-1 mM and ADE at 0.1-1 mg/ml on ADP (5 μM)-induced platelet aggregation. Results showed as mean±SD (n=4). * $p<0.05$ indicated significant difference when compared to vehicle control, ** $p<0.01$ indicated significant difference when compared to vehicle control.

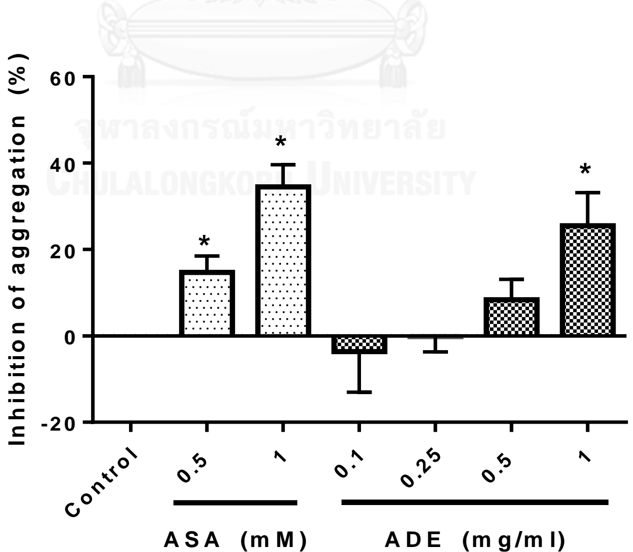

Figure 9. Inhibitory effect of ADE on ADP (5 μM)-induced platelet aggregation. Results showed as mean±SD (n=4). * $p<0.05$ indicated significant difference when compared to vehicle control.

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1.2 COX-screening assay

ADE had an Inhibitory effect against both isoforms of cyclooxygenase (COX). While ADE showed inhibitory effect on COX-1 activity with IC_{50} 0.76 mg/ml and on COX-2 activity with IC_{50} 0.03 mg/ml as shown in figure 10. the results demonstrated that ADE selectively inhibits COX-2 over COX-1 by approximately 25-fold.

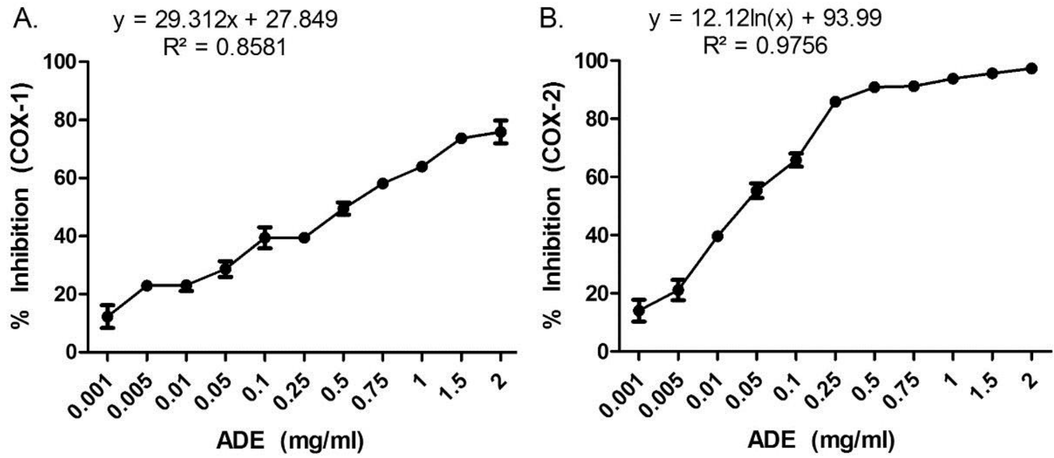

Figure 10. Inhibitory effect of ADE on the enzymatic activities of COX-1 (A) and COX-2 (B). Results showed as mean±SD (n=3).

2. *In vivo* model

2.1 Platelet aggregation

Figure 11 presents the effect of ADE on platelet aggregation induced by various concentration of ADP in *ex vivo* experiment. When platelets were induced by ADP 1 μM, all groups of rat treated with ADE alone or combination of 80 mg of ASA plus ADE did not show the inhibition of platelet aggregation when compare with control group. When platelets were induced by ADP 2.5 μM, the percent inhibition of platelet aggregation were significant different from control by 44.3±19.1% ($p<0.001$), 36.3±14.7% ($p<0.01$), 44.9±13.2% ($p<0.001$) and 60.7±10.7% ($p<0.001$) for ADE at the dose of 25, 50, 100 and 200 mg/kg, respectively. The % inhibition of aggregation in rat treated with 80 mg of ASA plus ADE for each dose were 42.7±18.7% ($p<0.001$), 65.7±14.0% ($p<0.001$) and 78.4±4.7% ($p<0.001$) for 50, 100 and 200 mg/kg, respectively. When platelets were induced by ADP 5 μM, the % inhibition of platelet aggregation were significant different from control by 22.7±13.6% ($p<0.05$), 22.6±9.3% ($p<0.05$), 41.1±8.7% ($p<0.001$) and 45.1±8.9% ($p<0.001$) for ADE at the dose of 25, 50, 100 and

200 mg/kg, respectively. The % inhibition of aggregation in rat treated with 80 mg of ASA plus ADE for each dose were 21.2±8.5% ($p<0.05$), 41.2±18.1% ($p<0.001$), 58.6±17.4% ($p<0.001$) and 84.7±8.6% ($p<0.001$) for 25, 50, 100 and 200 mg/kg respectively. In addition, when compared with ASA, rat treated with ADE at 200 mg/kg plus ASA strongly inhibited platelet aggregation more than those treated with ASA 40 or 80 mg/kg (31.7±14.3% and 47.0±11.3%). Finally, when platelets were induced by ADP 10 μM, the % inhibition of platelet aggregation were significant different from control by 29.6±5.6% ($p<0.001$) and 31.5±12.4% ($p<0.001$) for ADE at dose of 100 and 200 mg/kg, respectively. The % inhibition of aggregation in rat treated with 80 mg of ASA plus ADE for each dose were 24.9±8.8% ($p<0.001$), 40.4±13.0% ($p<0.001$) and 79.0±11.9% ($p<0.001$) for 50, 100 and 200 mg/kg, respectively. In addition, when compared with ASA, rat treated with ADE at 200 mg/kg plus ASA strongly inhibited platelet aggregation more than those treated with ASA 40 or 80 mg/kg (21.9±16.1% and 34.2±6.2%).

Figure 12 shows the % inhibition of ADP-induced platelet aggregation in ADE treated rat and ADE plus ASA treated rat. We found that at ADP 1 μM, all groups of ADE plus ASA were not significant different when compared with ADE alone. At ADP 2.5, 5 and 10 μM, we found that ADE 200 mg/kg treated group were significant different form ADE plus ASA treated group. The % inhibition of platelet aggregation were increased in the group that treated with ADE 200 mg/kg plus ASA when compared with those treated with ADE 200 mg/kg from 60.7±10.7% to 78.4±4.7%, from 45.1±8.9% to 84.7±8.6% and from 31.5±12.4% to 79.0±11.9% when induced aggregation by ADP 2.5, 5 and 10 μM, respectively.

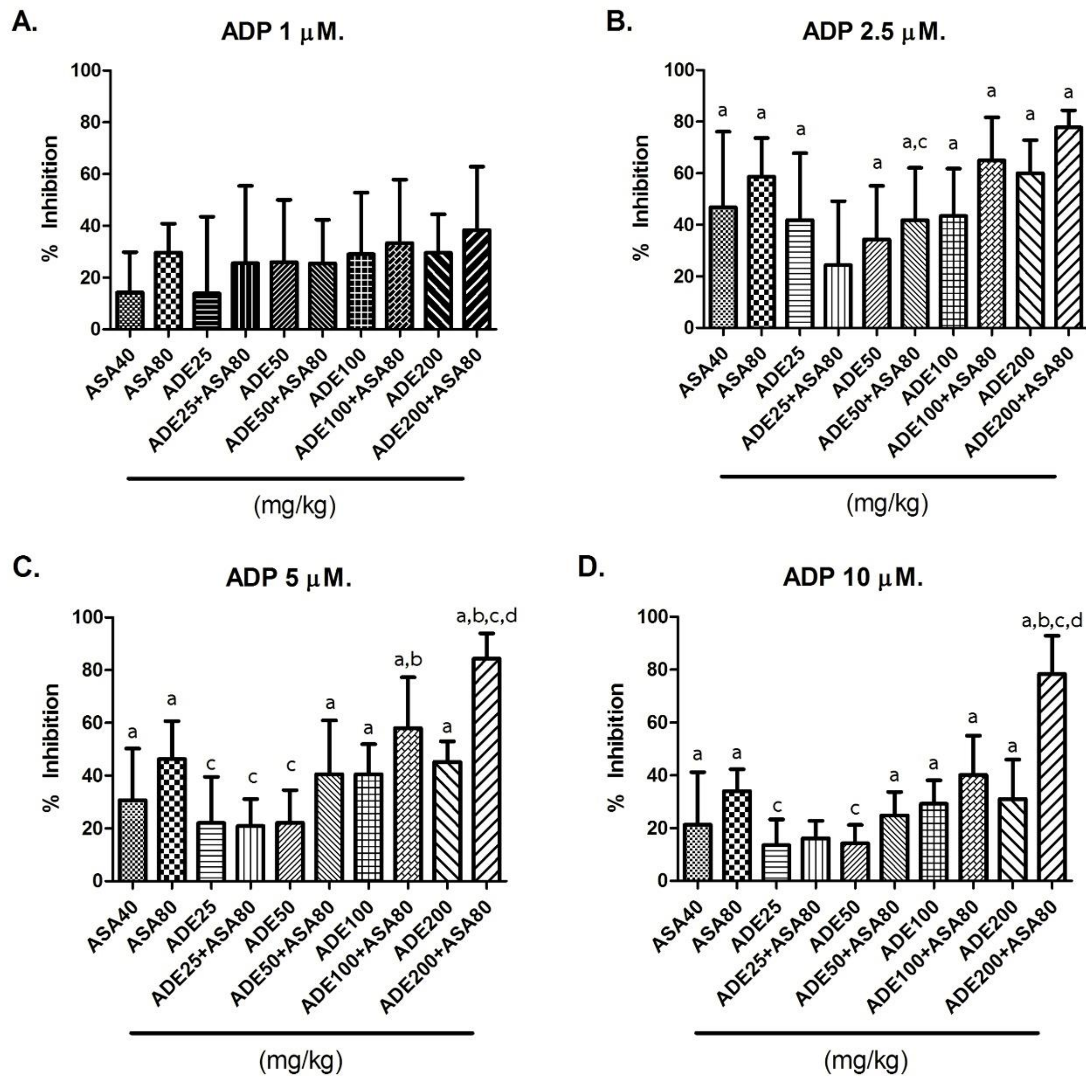

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Figure 11. Percent inhibitory effect of ADP-induced platelet aggregation in rat treated with ADE or ADE plus ASA. ASA 40 and 80 mg/kg was used as the positive control. Data are expressed as mean ± SD (each group, n=8).

a significant difference ($p<0.05$) when compared to 2% Tween80.

b significant difference ($p<0.05$) when compared to ASA 40 mg/kg.

c significant difference ($p<0.05$) when compared to ASA 80 mg/kg.

d significant difference ($p<0.05$) when compared to ADE alone at the same dose.

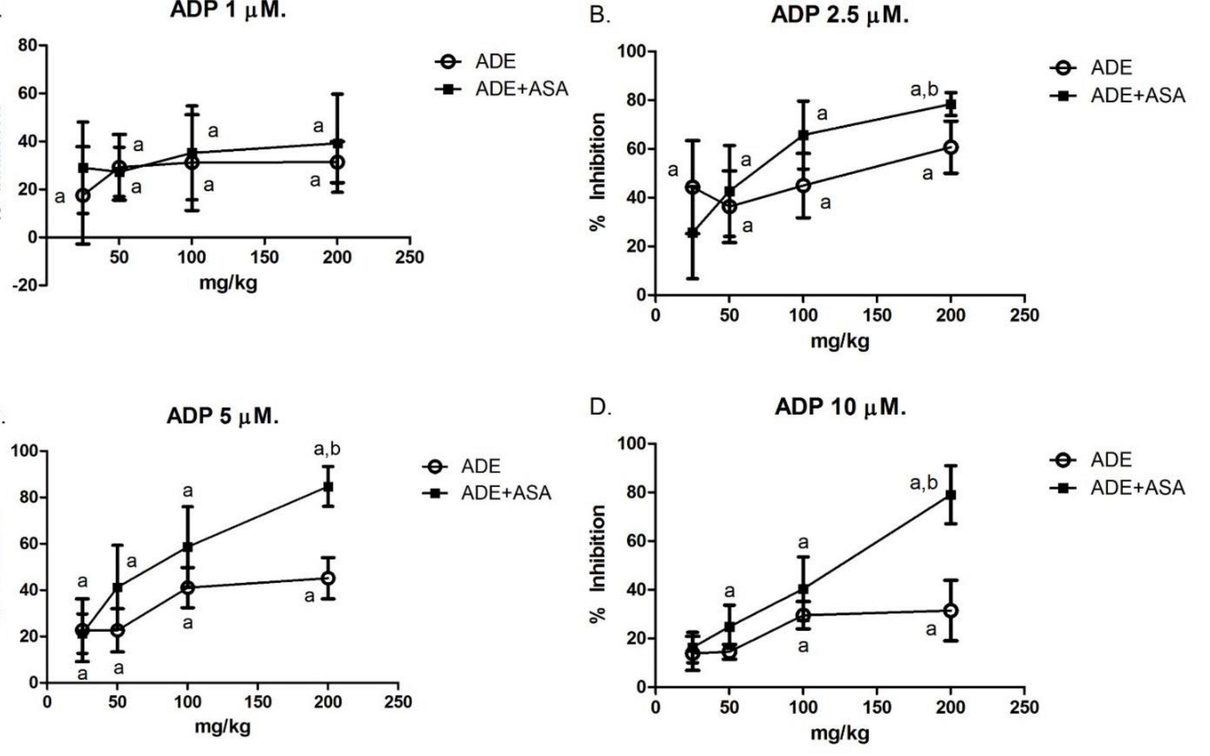

Figure 12. Percent inhibition of platelet aggregation. Data are expressed as mean ± SD (each group, n=8).

a significant difference ($p<0.05$) when compared to 2% Tween80.

b significant difference ($p<0.05$) when compared to ADE alone at the same dose

2.2 Coagulation parameter

The effects of ADE on coagulation parameters were evaluated by PT, aPTT and TT assays in rat blood. The PT of control rats was 12.1±0.3 second and the PT of rats treated with 25, 50, 100 and 200 mg/kg of ADE were 12.1±0.3, 12.3±0.4, 12.3±0.4 and 12.3±0.4 sec respectively. The aPTT of control rats was 47.1±11.8 second and the aPTT of rats treated with 25, 50, 100 and 200 mg/kg of ADE were 66.3±22.5, 71.6±22.4, 68.9±26.7 and 84.4±21.9 second respectively. In addition, the TT of control rats was 39.6±7.7 sec and the TT of rats treated with 25, 50, 100 and 200 mg/kg of ADE were 40.1±11.9, 39.9±10.0, 36.9±4.8 and 40.4±10.8 second. All of coagulation parameter in the rats treated with ADE were not significantly different compared with the control rats ($p>0.05$).

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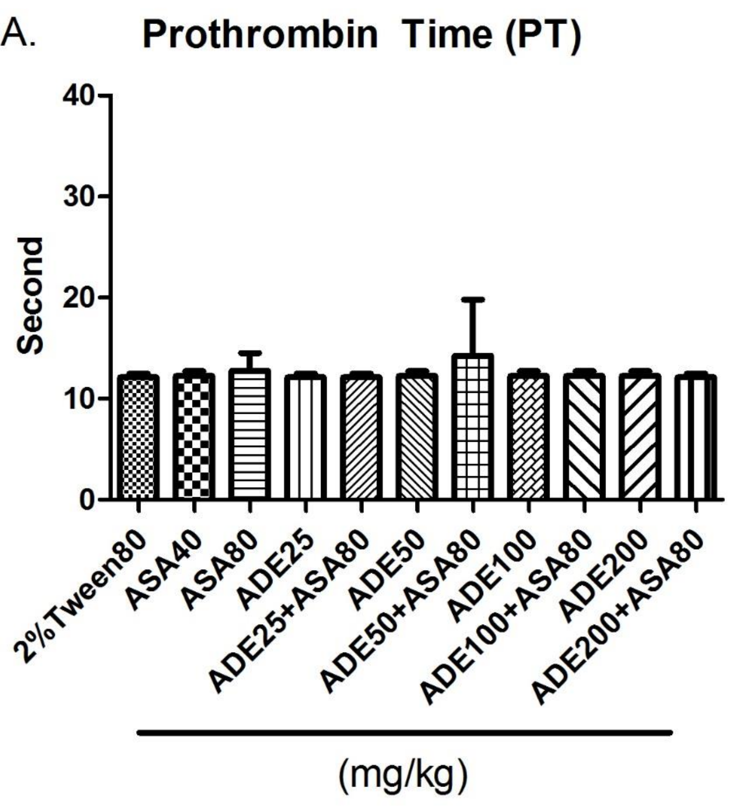
A.



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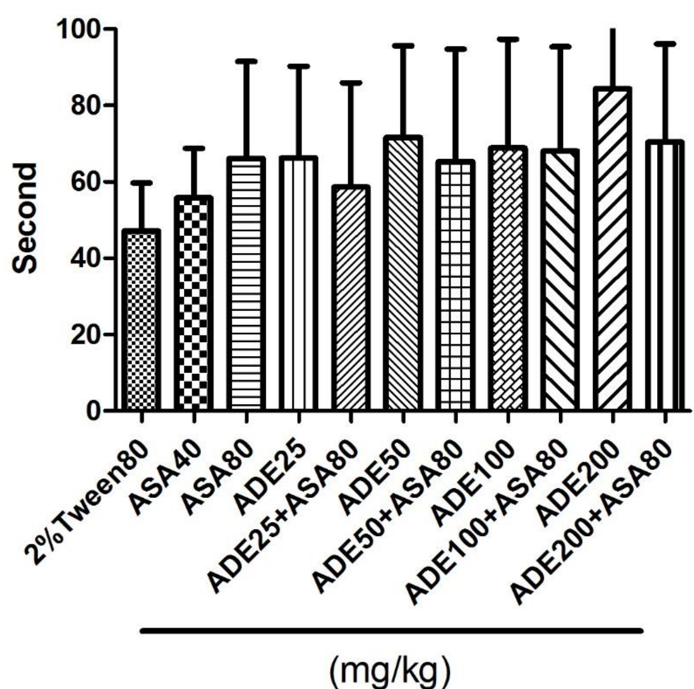

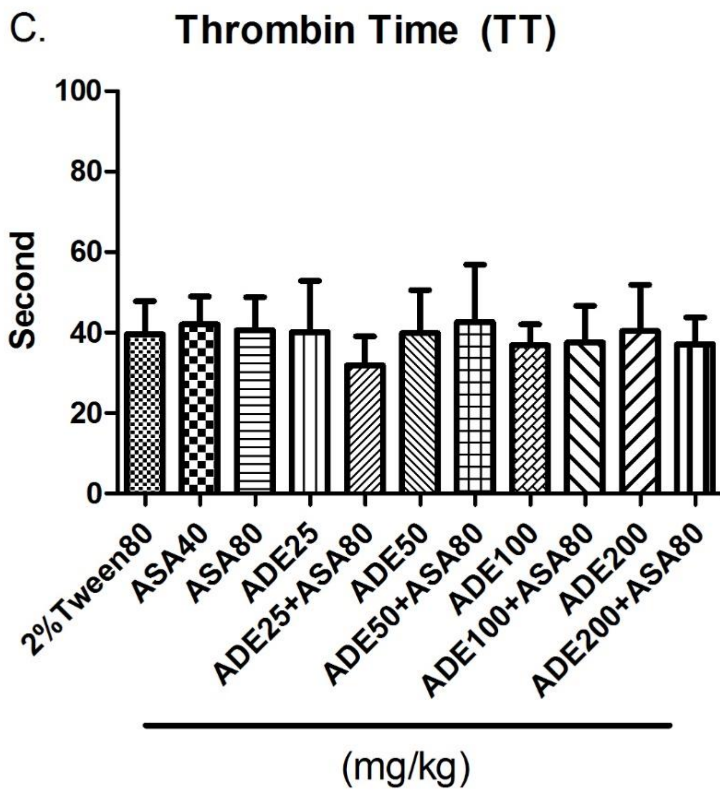

Figure 13. Effect of ADE on coagulation time in rats. (A; prothrombin time, B; activated partial thromboplastin time, C; thrombin time). Data are expressed as mean ± SD (each group, n=8).

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2.3 Gastric lesion

The effect of ADE on ASA-induced gastric lesions were also investigated The macroscopic findings of open stomachs are shown in Figure 14. We found that oral administration of aspirin 80 mg/kg induced mucosal damage which was evidenced by presence fields of hyperemia as shown in figure 14C.All dose of ADE did not show any macroscopic toxicity, preserving the morphological integrity of the gastric mucosa are shown in figure 14D-14G compared to control group. Furthermore, the animals treated with ADE 25, 50, 100 and 200 mg/kg was able to prevent the damage induced by ASA 80 mg/kg as shown in figure 14H-14K.

In addition, the administration of ASA 80 mg/kg induced a significant increase of ulcer index in relation to control animals. However, the group treated with ADE at 25, 50, 100 and 200 mg/kg plus ASA 80 mg/kg showed a significant decrease of lesion index compared to animal treated with ASA 80 mg/kg. Moreover, the animals that received ADE alone did not show any significantly different of the lesion index compared to control group as shown in figure 15. These data suggest that ADE has protective activities and it also prevents ASA-induced gastric lesion formation.

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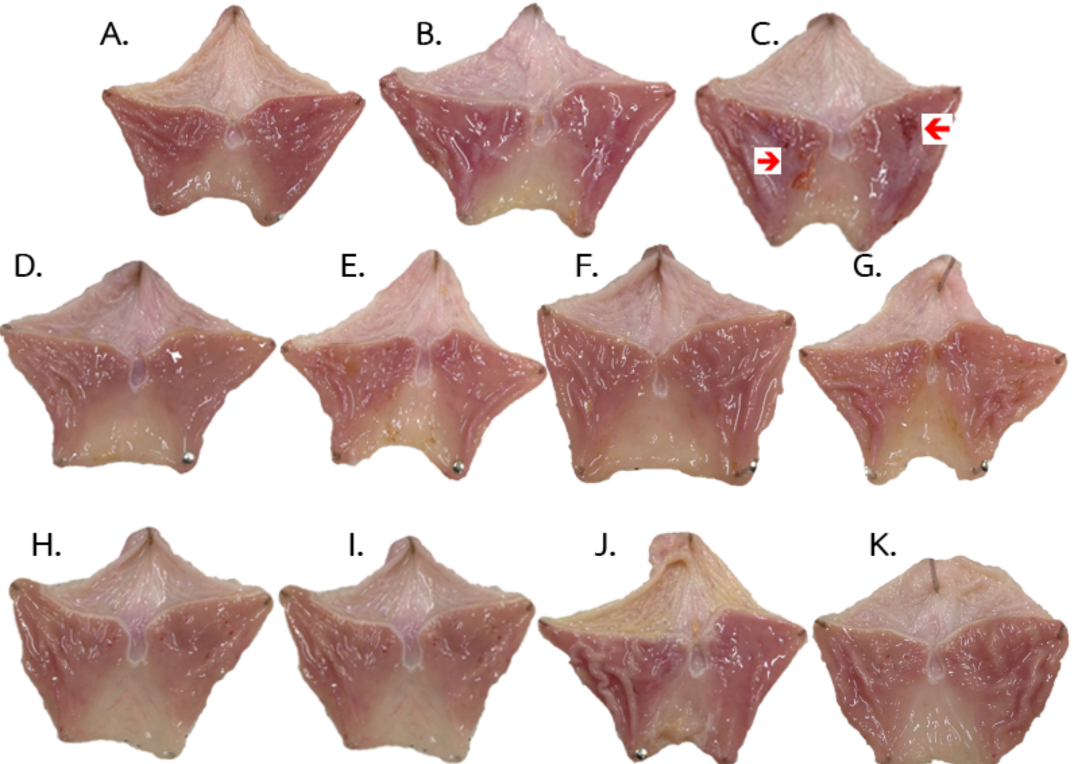

Figure 14. Effects of ADE on the macroscopic morphology of the gastric mucosa in rats. Macroscopic appearances of the gastric mucosa of rats treated with ADE and ADE plus ASA. (A) Control group; (B) ASA 40 mg/kg; (C) ASA 80 mg/kg; (D) ADE 25 mg/kg; (E) ADE 50 mg/kg; (F) ADE 100 mg/kg; (G) ADE 200 mg/kg; (H) ADE 25 mg/kg plus ASA 80 mg/kg; (I) ADE 50 mg/kg plus ASA 80 mg/kg; (J) ADE 100 mg/kg plus ASA 80 mg/kg; (K) ADE 200 mg/kg plus ASA 80 mg/kg group. The arrows show the damage in the gastric mucosa with hyperemic region.

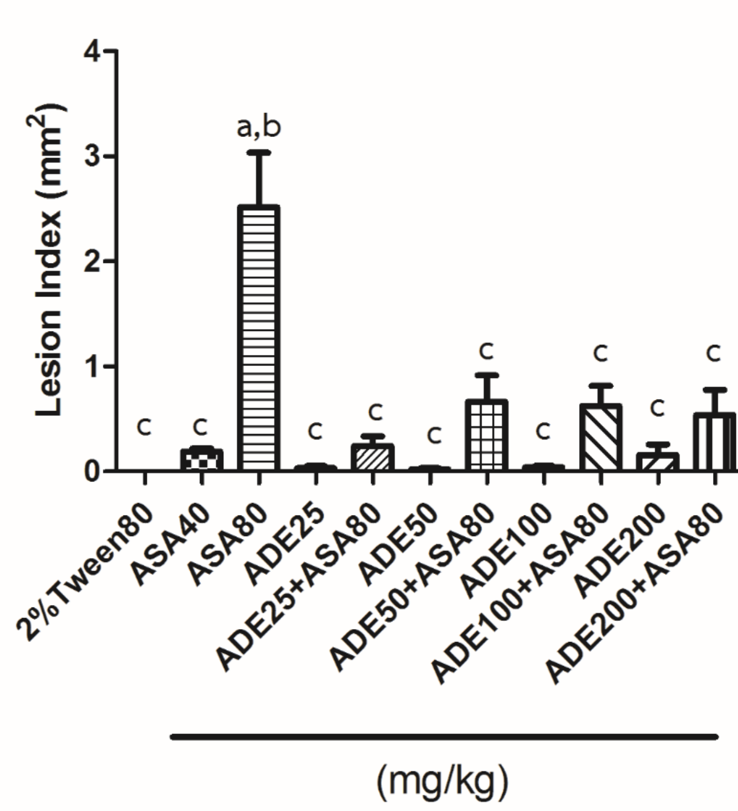

Figure 15. Effect of ADE and ADE plus ASA in rat on lesion index. Data are expressed as mean ± SD (each group, n=8).

a significant difference (p<0.05) when compared to 2% Tween80.

b significant difference (p<0.05) when compared to ASA 40 mg/kg.

c significant difference (p<0.05) when compared to ASA 80 mg/kg.

d significant difference (p<0.05) when compared to ADE alone at the same dose.

2.4 Histopathology studies

Histopathological alterations in stomach specimens of the ADE, ASA and ADE plus ASA treated groups are shown in figure16-19. No histological changes were observed in gastric mucosal, submucosal and muscularis layers from control group (figure 16; A1-A3). In contrast, exposure of rats to ASA caused moderate to severe mucosal hemorrhage (figure 17; A-20X), edema (figure 17; B-4X), epithelial cell loss (figure 17; C-20X) and inflammation characterized by neutrophil infiltration in gastric mucosal and submucosal layers (figure 17; D-10X). Pre-treatment with ADE showed no histological change compare with control group (figure 18).

Moreover, rats which received ADE plus ASA can improve these alterations, and showed less mucosal and submucosal hemorrhage and mild degree of submucosal edema, epithelial cell loss and inflammatory cell infiltration compared to the ASA 80 mg/kg. In histological score assessment of gastric lesion, the ASA 80 mg/kg demonstrates higher microscopic histopathological score than that control group, but pre-treatment with ADE caused a significant decrease in pathological score in ASA-induced gastric lesion in rats (table 2).

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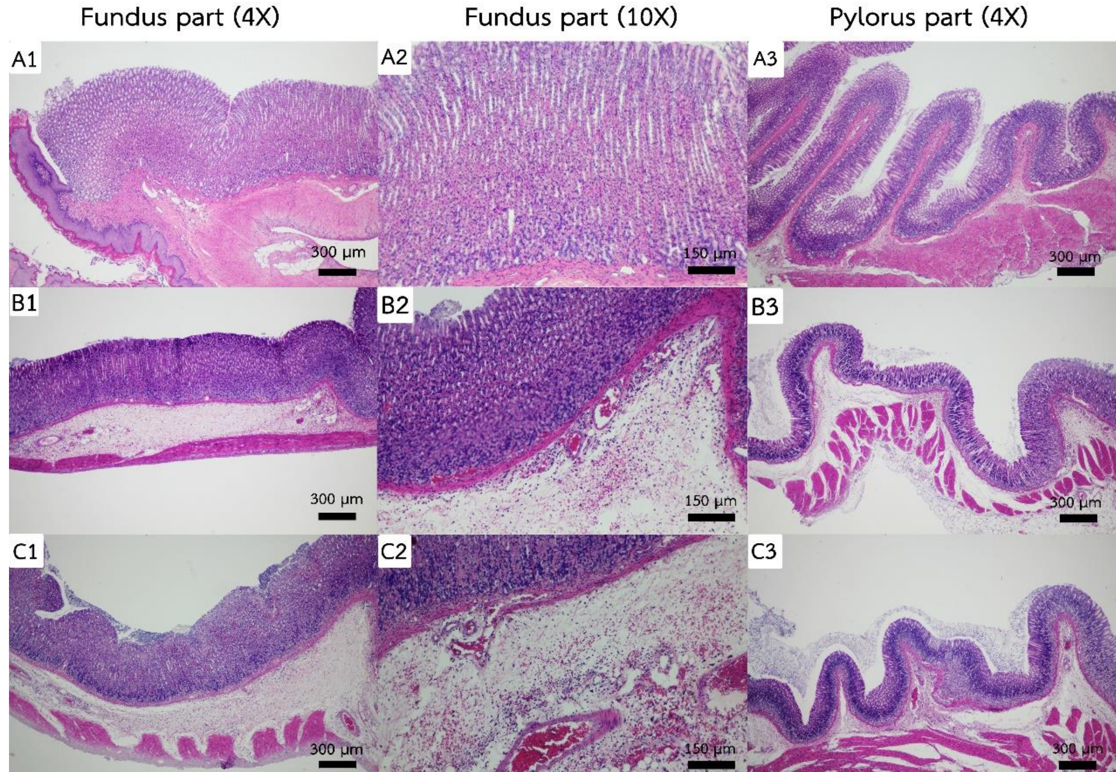

Figure 16. Histopathology analysis of rat stomach from control (fundus part, A1 and A2, pylorus part, A3), ASA 40 mg/kg (fundus part, B1 and B2, pylorus part B3), ASA 80 mg/kg (fundus part, C1 and C2, pylorus part, C3).

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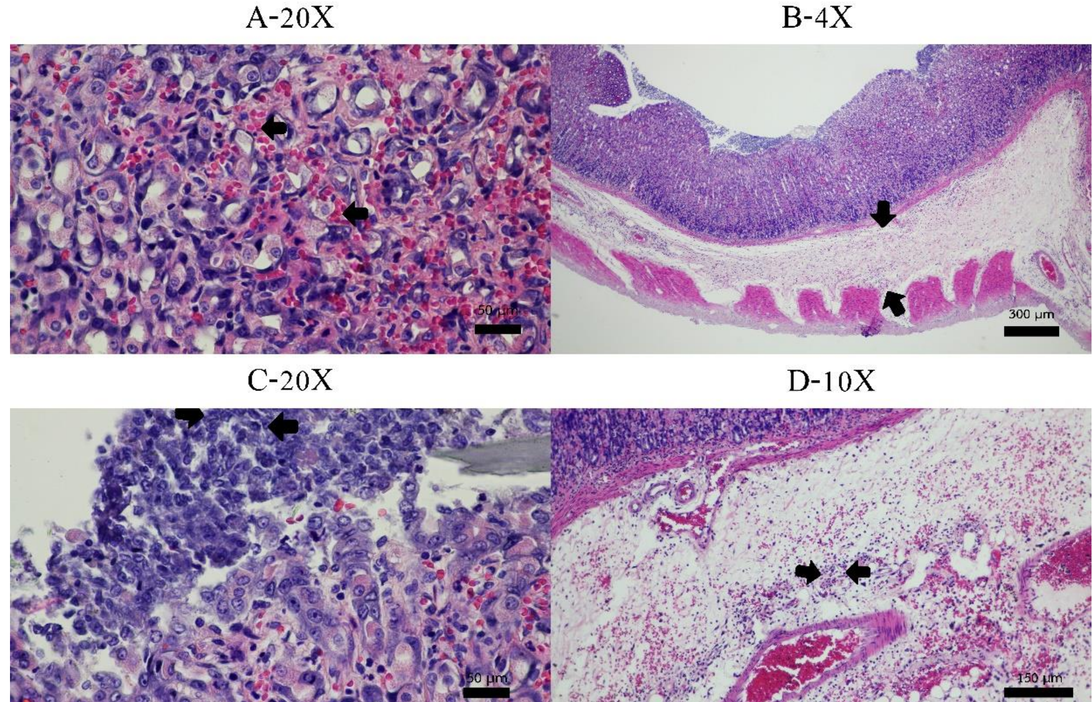

Figure 17. Histopathological lesion of rat stomach treated with ASA 80 mg/kg. (A= severe mucosal hemorrhage, B = severe submucosal edema, C = severe epithelial cell loss and D = infiltration of inflammatory cells in submucosal area)

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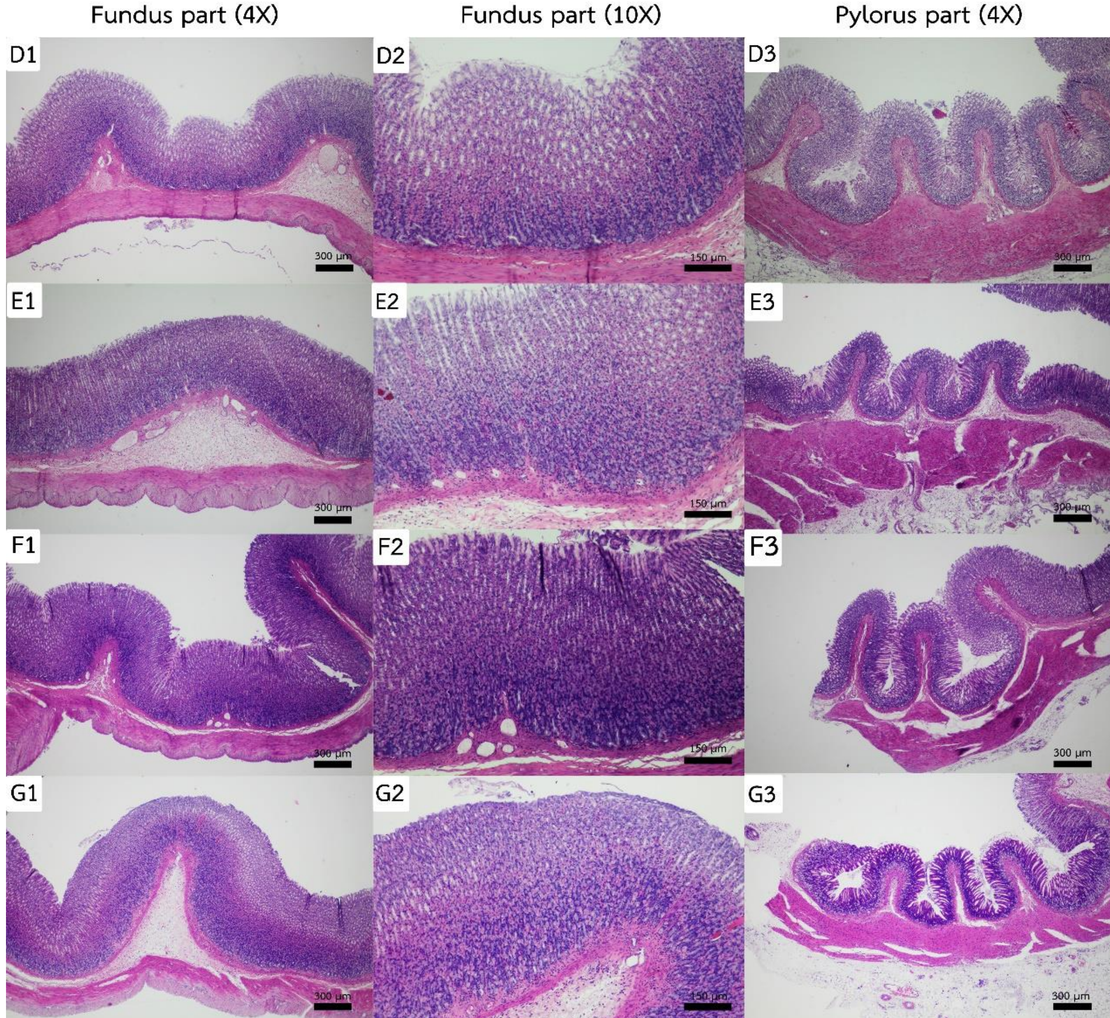

Figure 18. Histopathology analysis of rat stomach treated with ADE 25 mg/kg (fundus part D1, D2, pylorus part D3), ADE 50 mg/kg (fundus part E1, E2, pylorus part E3), ADE 100 mg/kg (fundus part F1, F2, pylorus part F3), ADE 200 mg/kg (fundus part G1, G2, pylorus part G3).

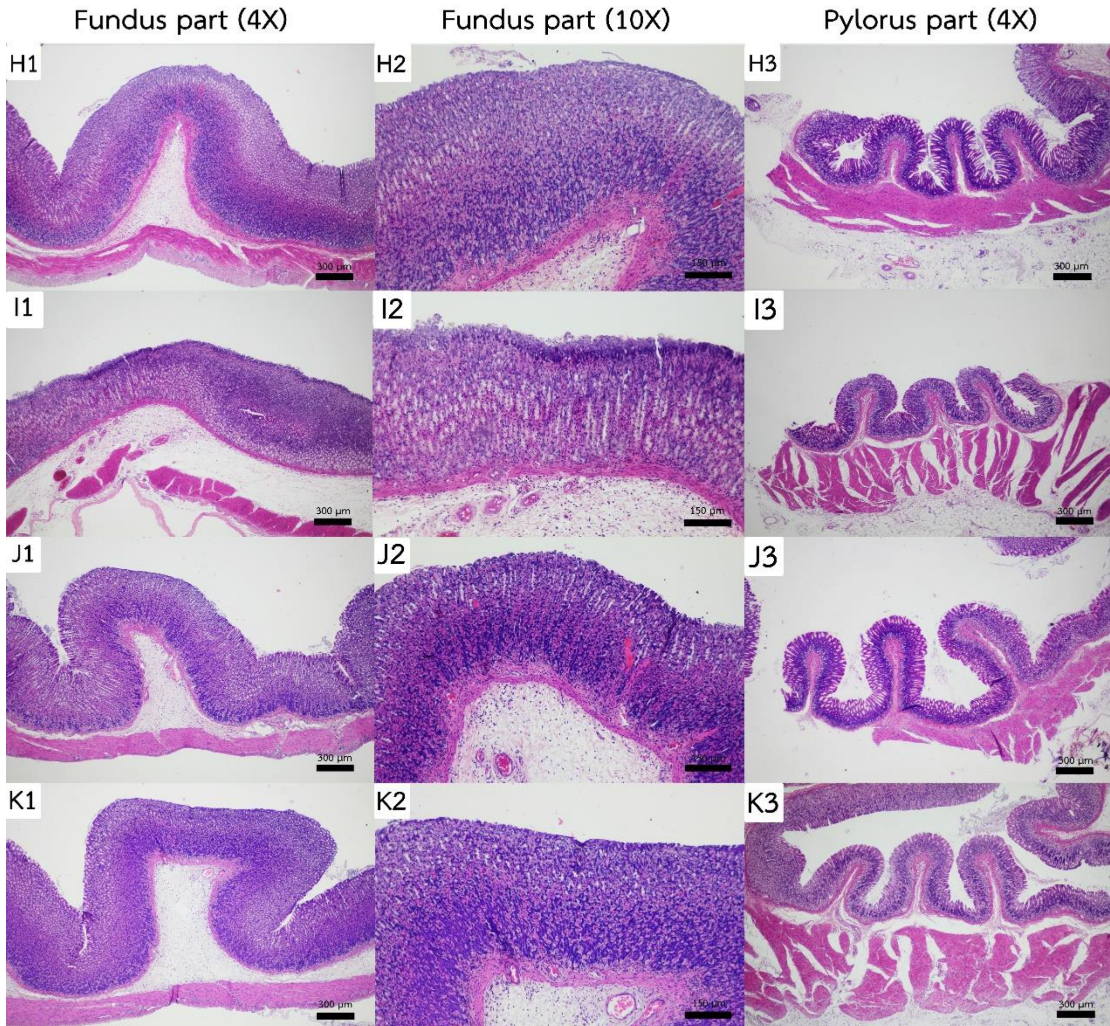

Figure 19. Histopathology analysis of rat stomach treated with ADE 25 mg/kg plus ASA (fundus part H1, H2, pylorus part H3), ADE 50 mg/kg plus ASA (fundus part I1, I2, pylorus part I3), ADE 100 mg/kg plus ASA (fundus part J1, J2, pylorus part J3), ADE 200 plus ASA mg/kg (fundus part K1, K2, pylorus part K3).

Table 2. Effects of ADE on histopathological score.

Experimental group (n=8)	Hemorrhagic score (0-4)		Edema (0-4)		Epithelial cell loss (0-3)		Inflammatory cell (0-3)		Total (14)	
	fundus	pylorus	fundus	pylorus	fundus	pylorus	fundus	pylorus	fundus	pylorus
2% Tween80 (control)	0	0	0.75 (0-2)	0.29 (0-1)	0	0	0.50 (0-1)	0.14 (0-1)	1.25	0.43
ASA 40 mg/kg	1.71 (0-4)	0.14 (0-1)	2.71 (1-4) [a,e]	1.14 (0-3)	1.29 (0-2) [a,e]	0	2.29 (2-3) [a,e]	1.00 (1-1)	8.33 [a,e]	2.29
ASA 80 mg/kg	2.43 (0-4) [a,e]	0	2.57 (1-3) [a]	1.67 (0-4)	1.71 (1-3) [a,e]	0.33 (0-2)	2.14 (1-3) [a,e]	1.33 (0-3)	8.86 [a,e]	3.33 [a]
ADE 25 mg/kg	0.29 (0-1) [c]	0	1.57 (1-3) [e]	0.20 (0-1)	0.14 (0-1) [c]	0	1.29 (0-2) [e]	0.40 (0-3)	3.29 [c]	0.60 [c]
ADE 50 mg/kg	0 [c]	0	1.43 (0-3) [e]	0.60 (0-1)	0 [b,c]	0	1.00 (1-1) [b]	0.60 (0-1)	2.43 [b,c]	1.20
ADE 100 mg/kg	0 [c]	0	1.43 (1-3) [e]	0.20 (0-1)	0.29 (0-1) [c]	0.20 (0-1)	0.86 (0-1) [b,c]	0.40 (0-1)	2.57 [b,c]	0.80
ADE 200 mg/kg	0.14 (0-1) [c]	0	1.43 (0-3) [e]	0.29 (0-1)	0.43 (0-2) [c]	0.14 (0-1)	1.00 (0-2) [b]	0.57 (0-1)	3.00 [c]	1.00
ADE 25 mg/kg plus ASA	0.43 (0-1) [c]	0	1.71 (0-3) [e]	0.50 (0-2)	0.86 (0-2) [e]	0	1.86 (1-3) [a]	1.17 (0-3)	4.86	1.67
ADE 50 mg/kg plus ASA	0.63 (0-4) [c]	0	2.00 (0-4) [e]	0.43 (0-2)	1.25 (0-2) [a,d,e]	0.14 (0-1)	2.00 (1-3) [a,e]	0.57 (0-1)	5.88 [e]	1.14
ADE 100 mg/kg plus ASA	0.63 (0-3) [c]	0	2.00 (1-4) [e]	0	0.75 (0-2) [e]	0	2.00 (1-3) [a,e]	0.50 (0-2)	5.38 [e]	0.50 [c]
ADE 200 mg/kg plus ASA	0.50 (0-2) [c]	0	2.25 (1-4) [e]	0.38 (0-1)	0.88 (0-2) [e]	0	2.00 (1-3) [a,e]	0.25 (0-1)	5.63 [e]	0.63

The table shows median value followed by minimum and maximum score (in brackets). Kruskal–Wallis nonparametric test followed by Dunn's test.

a significant difference ($p<0.05$) when compared to 2% Tween80.

b significant difference ($p<0.05$) when compared to ASA 40 mg/kg.

c significant difference ($p<0.05$) when compared to ASA 80 mg/kg.

d significant difference ($p<0.05$) when compared to ADE alone at the same dose.

e significant difference ($p<0.05$) when compared to pylorus part of stomach.

CHAPTER V
DISCUSSION AND CONCLUSION

Platelets contribute to the hemostatic process in two different ways. First, through their adhesive and cohesive functions that lead to the formation of a hemostatic plug. Second, they can activate coagulation mechanisms. Platelets have pivotal roles in the pathogenesis of thrombotic complications including atherosclerosis, myocardial infarction, thromboembolism and other vascular diseases (37). Many available antiplatelet agents interfere with platelet function at various levels of activation which results in several clinical gastrointestinal side effects and hemorrhage (5).Therefore, a search was conducted for safer and more effective antiplatelet agents without these adverse effects. The root of *Angelica dahurica* (Kot Sor) has been widely used in many countries for the treatment of headaches, toothache, colds, and neuralgia. In Thai traditional medicine, Kot Sor is used for the treatment of circulatory disorders (7). Previous studies have reported that a 1 mg/kg EtOH extract of ADE inhibited human platelet aggregation induced by ADP (8). Moreover, ADE activated the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (26). However, the effect of ADE still requires investigation in the *in vivo* model. In this study, the inhibitory effect of ADE on ADP-induced platelet aggregation in rat platelets was evaluated.

In the resting state, platelets are distributed throughout the blood vessels as free floating cells. When vascular damage occurs, the platelets adhere to damaged vessels and platelet activation and aggregation commences. This process is stimulated by endogenous agonists including ADP, thrombin, collagen, serotonin and TxA_2. Although ADP is a weak agonist, it is the most important mediator among these, as it causes alterations in platelet shape, aggregation and TxA_2 secretion (11). ADP, along with adenosine triphosphate and serotonin, is stored in dense granules inside the platelets and secreted at sites of injury by platelet activation.

The released ADP binds to the purinergic receptor $P2Y_1$ or $P2Y_{12}$. Activation of the $P2Y_1$ receptor pathway triggers intracellular calcium mobilization and alters platelet shape, while $P2Y_{12}$ receptor activation inhibits adenylyl cyclase-dependent

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cyclic adenosine monophosphate generation and prolongs platelet aggregation (17). The antiplatelet agents currently in use (e.g. clopidogrel, ticagrelor and prasugrel) effectively reduce platelet aggregation by inhibiting ADP-$P2Y_{12}$ receptor binding (21). In this *in vitro* study, we examined the antiplatelet effect of ADE on platelet function by measuring ADP-induced platelet aggregation in rat platelets. Results showed that ADE 1 mg/ml inhibited ADP-induced platelet aggregation by 25.5±3.8%. From our previous study, ADE 1 mg/ml inhibited platelet aggregation by 62.8% in human platelet rich plasma (26), and 79.8±7.4% in human washed platelets (8). Some studies reported that sensitivity to ADP was similar for human and rat platelets (38); however, our results indicated that rat platelets were less sensitive to ADE than human platelets. These sensitivity differences in the antiplatelet effect of ADE between humans and rats indicated that the action mechanism might involve not only the ADP – $P2Y_{12}$- pathway but also the other signaling pathways and this requires further study.

Results indicated that ADE inhibited COX-1 less than COX-2 enzyme by 25-fold. ADE showed inhibitory effects on COX-1 and COX-2 enzyme activity with IC_{50} values of 0.76 and 0.03 mg/ml, respectively. The COX-1 enzyme is responsible for the synthesis of thromboxane A_2 (TXA_2) which is a potent platelet aggregating substance (39). COX-1 is constitutively expressed in most tissues including platelet and gastrointestinal mucosa, and it is thought to exert homeostatic properties that are crucial for vascular and gastric physiologic functions. COX-2 is absent from most healthy tissue but it is induced by inflammatory stimuli, hormones and growth factors. ADE has anti-inflammatory activity by suppressed COX-2 expression (40). Therefore, the antiplatelet aggregation effect of ADE may be involved in COX-1 inhibitory activity.

This study also evaluated the anti-platelet aggregation of ADE in the *ex vivo* model. The rats were divided into 3 set. The first set was treated with 2% tween80 (control) and ASA (positive control). The second set was treated with ADE alone and the last set was treated with a combination of ADE and ASA. Rats in the second set were treated with ADE at 25, 50, 100 or 200 mg/kg. These doses of ADE were equivalent to those used in Thai traditional medicine at 3-9 g/day. The LC_{50} of ADE is equal to 12.796 g/kg (41). Thus, the doses of 25-200 mg/kg used in the experiment by oral administration were safe and well justified.

Results determined that all doses of ADE did not significantly inhibit platelet aggregation induced by ADP at 1 μM. Because of the low concentration, ADP at 1 μM-induced aggregation by only 20%. At ADP 2.5 μM, the 25-200 mg/kg of ADE inhibited platelet aggregation, and when combined with ASA the inhibitory effect on platelet aggregation at 50-200 mg/kg increased. At ADP 5 and 10 μM, we found that ADE 200 mg/kg plus ASA significantly increased the inhibitory effect on platelet aggregation when compared to ASA 80 mg/kg, in addition, the dose of ADE 200 mg/kg alone did not significant different when compare with ASA 80 mg/kg. This result determined that ADE treated can be used in animal models with antiplatelet aggregation effect. Moreover, the higher dose of ADE 200 mg/kg combined with ASA exerted an increasing antiplatelet aggregation effect. This may be a synergistic effect because ADE treated with ASA can increase anti-platelet aggregation effect more than ADE or ASA alone at ADP at all concentration especially at ADP 10 μM. In contrast, ADE at 25-50 mg/kg plus ASA decreased antiplatelet aggregation compared to ASA 80 mg/kg. The mechanism for this is still unclear and requires further investigated.

To assess the effect of ADE on secondary hemostasis, we evaluated coagulation parameters in rat blood after oral administration for 7 days. Coagulation is a complicated process that is highly related to platelet interaction with coagulation factors and blood vessels. PT, aPTT and TT assays are generally used to predict the risk of bleeding and other adverse effects (42). The PT assay indicates the extrinsic coagulation pathway, whereas the aPTT assay is used to evaluate the intrinsic coagulation pathway (43) and the TT assay measures an abnormality in the conversion of fibrinogen (a soluble protein) to fibrin, an insoluble protein (44). These tests are recognized as useful methods to confirm the bleeding risk and the potential for thrombotic adverse effect. In our experiment, PT, aPTT and TT were measured to evaluate the effect of ADE on coagulation time. The results suggested that ADE had no effect on the coagulation system.

The side effects of ADE on the stomach were evaluated after oral administration once daily for 7 days. ASA 80 mg/kg was used as the positive control because this is the standard treatment to prevent platelet aggregation for cardiovascular disease. However, the major problems caused by ASA are GI side effects

with incidences of 10-40% (45). Our results determined that the stomachs of rats in the ASA group underwent morphological changes including mucosal erosion, hemorrhage and mucosal erythema. The mucosal layer of the fundus part of the stomach had more severe inflammation than the pylorus because the fundus contains glandular areas such as parietal cells, chief cells, and mucous neck cells (46). Furthermore, the lesion index in the ASA-treated group increased significantly compared with the control group. There are many mechanisms for gastric ulcer formation including ASA contact directly with the stomach (47). ASA destroys the gastric mucosal barrier by decreasing gastric mucosa and bicarbonate secretion which increases stomach acidity (48), and it also destroys capillaries and increases histamine secretion. Histamine binding to H_1-receptors plays a major role in allergic reactions. Vasodilation increases vascular permeability and binding to H_2-receptors at parietal cells and increases HCl secretion which can cause gastric ulcers (49). In addition, ASA decreases prostaglandin especially PGE_2 and PGI_2 from COX-1 enzyme inhibition which leads to reduced blood flow in the stomach and promotes mucosal ischemia (50). This can result in neutrophils adhering to endothelial cells and induce ROS generation (51) which causes mucosal damage related to histopathological findings. We discovered that the ASA group had a higher degree of inflammatory cell accumulation than the control group, especially for neutrophils. The ADE treated group did not show any significant lesions in the stomach by gross appearance and histopathology compared with the control group. This indicated that ADE did not affect the gastric lesions in the stomach. This may be because ADE selectively inhibits COX-2 more than the COX-1 enzyme. Moreover, ADE significantly reduced gastric lesions combined with ASA when compared with the ASA treated group. The possible mechanism might be a decrease of the local effect as a COX-2 inhibitor.

In conclusion, Thai traditional medicine Kot Sor is useful for circulatory disorders, since ADE promotes antiplatelet aggregation. The mechanism for this may involve the inhibition of the COX-1 enzyme and the ADP-induced pathway in platelets. ADE did not affect the coagulation system and GI adverse events. Moreover, treatments of ADE plus ASA increased the antiplatelet aggregation effect without increasing gastric lesions.

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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.

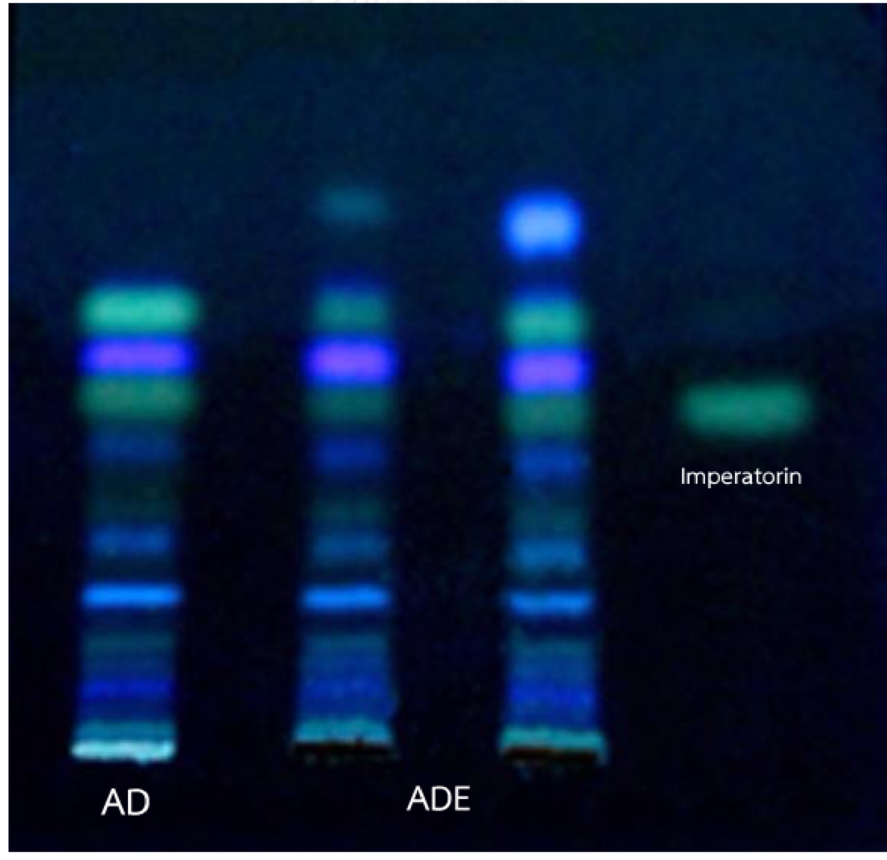

Figure 20. TLC chromatogram of the 50% ethanolic extract of Angelica dahurica

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

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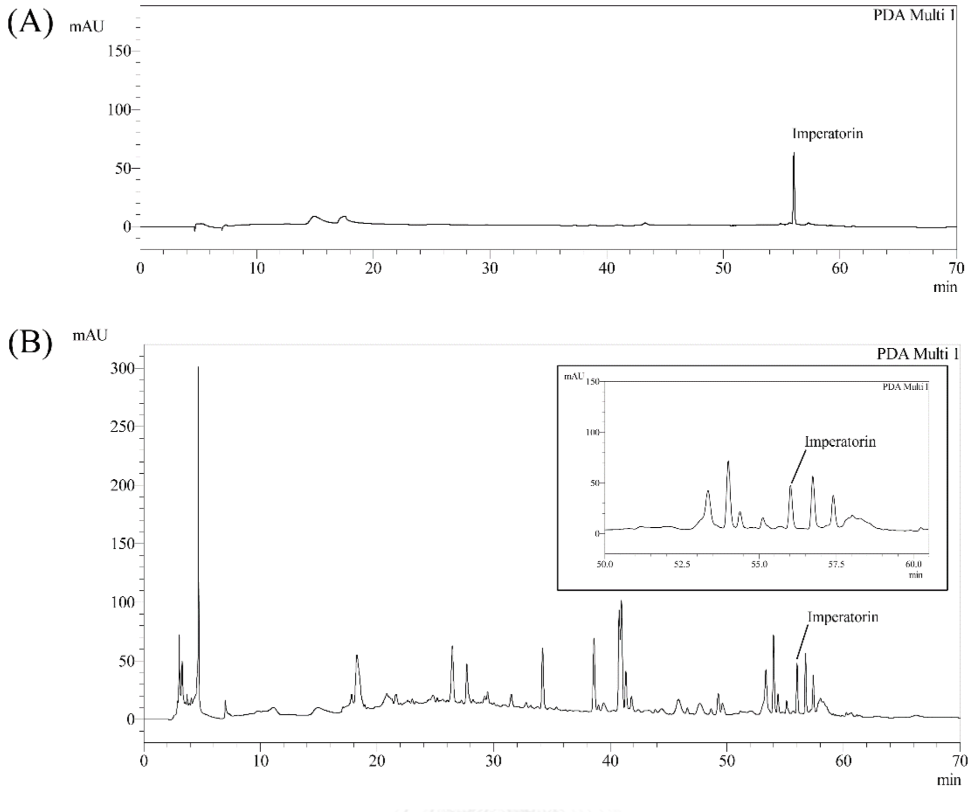

Figure 21. HPLC chromatogram of the 50% ethanolic extract of *Angelica dahurica*

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Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☑ Original ☐ Renew
Animal Use Protocol No. 15-33-006	**Approval No. 15-33-006**

Protocol Title
Effect of *Angelica dahurica* extract on platelet aggregation in rat

Principal Investigator

Rataya LUECHAPUDIPORN, PhD

Certification of Institutional Animal Care and Use Committee (IACUC)

This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Date of Approval	Date of Expiration
December 22, 2015	December 21, 2017

Applicant Faculty/Institution
Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Road., Pathumwan
BKK-THAILAND. 10330

Signature of Chairperson	Signature of Authorized Official
Name and Title	Name and Title
THONGCHAI SOOKSAWATE, Ph.D.	PORNCHAI ROJSITTHISAK, **Ph.D.**
Chairman	**Associate Dean (Research and Academic Service)**

The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals.

This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.

VITA

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Poster Presentation

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