ฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและฤทธิ์สลายลิ่มเลือดของสารสกัดโกฐจุฬาลัมพา

นางสาววีรยา แก้วเปรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIPLATELET AGGREGATION AND FIBRINOLYTIC ACTIVITY OF *ARTEMISIA VULGARIS* L. EXTRACT

Miss Weeraya Kaewprem

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biomedicinal Chemistry
Department of Biochemistry and Microbiology
Faculty of Pharmaceutical Sciences
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Thesis Title	ANTIPLATELET AGGREGATION AND FIBRINOLYTIC ACTIVITY OF *ARTEMISIA VULGARIS* L. EXTRACT
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Field of Study	Biomedicinal Chemistry
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วีรยา แก้วเปรม : ฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและฤทธิ์สลายลิ่มเลือดของสารสกัดโกฐจุฬาลัมพา. (ANTIPLATELET AGGREGATION AND FIBRINOLYTIC ACTIVITY OF *ARTEMISIA VULGARIS* L. EXTRACT) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ภญ.ดร. ดวงเดือน เมฆสุริเยนทร์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.นพ.ดร.พลภัทร โรจน์นครินทร์, 127 หน้า.

การเกาะกลุ่มของเกล็ดเลือดมีบทบาทสำคัญในภาวะหลอดเลือดแข็งตัว ซึ่งเป็นหนึ่งในสาเหตุหลักของโรคหลอดเลือดหัวใจ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและฤทธิ์สลายลิ่มเลือดของสารสกัดสมุนไพรจากพิกัดเบญจโกฐ สารสกัดเอทานอลและสารสกัดน้ำของโกฐสอ โกฐเขมา โกฐหัวบัว โกฐเชียง และโกฐจุฬาลัมพา ที่ความเข้มข้น 1 มิลลิกรัมต่อมิลลิลิตร พบว่าไม่เป็นพิษต่อเซลล์และไม่ทำลายดีเอ็นเอของเซลล์บุผิวหลอดเลือดมนุษย์ ECV304 จึงนำสารสกัดที่ความเข้มข้นดังกล่าวมาศึกษาฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดมนุษย์เบื้องต้นด้วยวิธี light transmission aggregometry พบฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดเมื่อกระตุ้นด้วย ADP ของสารสกัดเอทานอลและสารสกัดน้ำของ โกฐจุฬาลัมพา > โกฐสอ > โกฐเชียง ตามลำดับ จึงคัดเลือกเฉพาะสารสกัดโกฐจุฬาลัมพา ซึ่งมีฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดมากที่สุดมาศึกษากลไกการออกฤทธิ์ พบว่าความเข้มข้นของสารสกัดเอทานอลและสารสกัดน้ำของโกฐจุฬาลัมพาที่สามารถต้านการเกาะกลุ่มของเกล็ดเลือด เมื่อกระตุ้นด้วย ADP ได้ 50 เปอร์เซ็นต์คือ 0.43 และ 0.51 มิลลิกรัมต่อมิลลิลิตรตามลำดับ อย่างไรก็ตามสารสกัดทั้งสองไม่สามารถยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่กระตุ้นด้วย arachidonic acid และคอลลาเจน แสดงให้เห็นว่าสารสกัดโกฐจุฬาลัมพายับยั้งการเกาะกลุ่มของเกล็ดเลือดผ่านทางวิถีการส่งสัญญาณของ ADP เพื่อยืนยันว่าฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดไม่ได้เป็นผลจากเกล็ดเลือดถูกทำลายด้วยพิษของสารสกัด จึงศึกษาผลของสารสกัดโกฐจุฬาลัมพาต่อการมีชีวิตของเกล็ดเลือดเมื่อบ่มเป็นเวลา 1 ชั่วโมงด้วยวิธี MTT พบว่าสารสกัดโกฐจุฬาลัมพาที่ความเข้มข้นที่มีฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดไม่เป็นพิษต่อเกล็ดเลือด รวมถึงไม่มีผลต่อการทำงานของเกล็ดเลือดเมื่อศึกษาโดยอาศัยการทำงานของเอนไซม์ acid phosphatase เป็นการพิสูจน์ได้ว่าฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดของสารสกัดโกฐจุฬาลัมพานั้นไม่ได้เกิดจากความเป็นพิษของสารสกัด หรือไปรบกวนการทำงานของเกล็ดเลือด และยังพบว่าไม่มีผลต่อการยึดเกาะของเกล็ดเลือดบนคอลลาเจน เมื่อทิ้งให้ยึดเกาะเป็นเวลา 1 ชั่วโมง เนื่องจากไนตริกออกไซด์ที่หลั่งจากเซลล์บุผิวหลอดเลือดนั้นอาจไปยับยั้งการเกาะกลุ่มของเกล็ดเลือดได้ จึงทำการศึกษาผลของสารสกัดเอทานอลของโกฐจุฬาลัมพาต่อการเติมหมู่ฟอสเฟตของ Akt และ eNOS ในเซลล์ ECV304 ในสภาวะที่ไม่มีชีรัมด้วยวิธี Western blot พบว่าสารสกัดเอทานอลของโกฐจุฬาลัมพาที่ความเข้มข้น 0.5 มิลลิกรัมต่อมิลลิลิตร เมื่อบ่มเป็นเวลา 1 ชั่วโมง สามารถเพิ่มการเติมหมู่ฟอสเฟตของ eNOS ในขณะที่ไม่ส่งผลการเติมหมู่ฟอสเฟตของ Akt ในเซลล์ ECV304 ผลการศึกษาครั้งนี้ได้ชี้ให้เห็นว่าโกฐจุฬาลัมพาซึ่งเป็นหนึ่งในเครื่องยาพิกัดเบญจโกฐนั้นมีศักยภาพที่จะใช้เป็นสมุนไพรรักษาโรคหลอดเลือดที่เกี่ยวข้องกับเกล็ดเลือด

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

5176625333 : MAJOR BIOMEDICINAL CHEMISTRY
KEYWORDS : *ARTEMISIA VULGARIS* / PHIKUD BENJAKOT / ANTIPLATELET AGGREGATION / ADP / HUMAN PLATELETS / GENOTOXICITY / ECV304 CELLS

WEERAYA KAEWPREM : ANTIPLATELET AGGREGATION AND FIBRINOLYTIC ACTIVITY OF *ARTEMISIA VULGARIS* L. EXTRACT. ADVISOR : ASSOC. PROF. DUANGDEUN MEKSURIYEN, Ph.D., CO-ADVISOR : ASSOC. PROF. PONLAPAT ROJNUCKARIN, Ph. D., 127 pp.

Platelet aggregation is involved in atherosclerosis plaque, one of the major causes of cardiovascular disease. The objectives of the present study are to determine antiplatelet and fibrinolytic activities of herbal extracts from Phikud Benjakot. The ethanol and water extracts of each herb in Phikud Benjakot, Kot Soa (*Angelica dahurica*, AD), Kot Kamao (*Atractylodes lancea*, AL), Kot Huabua (*Ligusticum sinense*, LS), Kot Chiang, (*Angelica sinensis*, AS) and Kot Chulalumpa (*Artemisia vulgaris*, AV), at the concentration of 1 mg/mL did not affect both viability and DNA damage of human umbilical vein endothelial ECV304 cells. The effect of the herbal extracts at the concentration of 1 mg/mL on platelet aggregation induced by ADP was performed using light transmission aggregometry method. The result demonstrated that antiplatelet activity of ethanol and water extracts were in descending order of AV > AD > AS. Since AV expressed the highest antiplatelet activity, the ethanol and water extracts of AV were chosen for a concentration-dependent study, resulting in exhibiting IC_{50} values of 0.43 and 0.51 mg/mL, respectively. However, both extracts did not inhibit platelet aggregation induced by arachidonic acid and collagen. The result suggested that the ethanol and water extracts of AV are likely to inhibit platelet aggregation via ADP signaling pathway. To confirm whether the antiplatelet activity of both extracts of AV was not due to platelet damage, platelet viability was then performed using MTT reduction assay. Treatment of platelets with both extracts of AV at the antiplatelet concentration for 1 h did not affect platelet viability. Additionally, AV did not affect platelet function using acid phosphatase assay. The result indicated that antiplatelet activity of AV was not due to their toxicity or interfering platelet function. The effect of both extracts of AV on platelet adhesion to collagen was determined based on acid phosphatase activity. Both extracts of AV did not affect platelet adhesion when allowed to adhere to collagen for 1 h. Since endothelial-derived nitric oxide can inhibit platelet aggregation, the effect of the ethanol extract of AV on phosphorylation of Akt and eNOS in ECV304 cells was studied using Western blot analysis. The result revealed that the ethanol extract of AV at the concentration of 0.5 mg/mL for 1 h treatment could enhance the phosphorylation of eNOS, but not Akt, in ECV304 cells under serum-free condition. Our findings suggested that AV, one of crude drugs in Phikud Benjakot, could be considered as a candidate herb in the treatment of platelet-associated vascular disease.

Department : Biochemistry and Microbiology Student's Signature .....

Field of Study : Biomedicinal Chemistry Advisor's Signature .....

Academic Year : 2011 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

%	percentage
μL	microliter
μM	micromolar
5HT2A	serotonin A2 -receptor
AD	*Angelica dahurica*, Kot Soa
ADES	ethanol extract of *Angelica dahurica* and further spray-dried
ADP	adenosine diphosphate
ADWS	water extract of *Angelica dahurica* and further spray-dried
Akt	Agro-konsult transform, protein kinase B
AL	*Atractylodes lancea*, Kot Kamao
ALES	ethanol extract of *Atractylodes lancea* and further spray-dried
ALWF	water extract of *Atractylodes lancea* and further freeze-dried
AS	*Angelica sinensis*, Kot Chiang
ASA	acetylsalicylic acid; aspirin
ASES	ethanol extract of *Angelica sinensis* and further spray-dried
ASWS	water extract of *Angelica sinensis* and further spray-dried
ATP	adenosine triphosphate
AV	*Artemisia vulgaris*, Kot Chulalumpa
AVES	ethanol extract of *Artemisia vulgaris* and further spray-dried
AVWS	water extract of *Artemisia vulgaris* and further spray-dried
BAECs	bovine aortic endothelial cells
BSA	bovine serum albumin
BSA-PBS	bovine serum albumin-phosphate buffered saline
$CaCl_2$	calcium chloride
Camp	cyclic adenosine monophosphate
CL	compulsory licensing
CO_2	carbon dioxide
COX	cyclooxygenases
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECV304	transformed human umbilical vein endothelial cells
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
GP	glycoprotein
GPCR	G-protein coupled receptor
GPIbβ	glycoprotein Ibβ
GPIb-IX-V	glycoprotein Ib-IX-V complex
GPIbα	glycoprotein Ibα
GPIIb/IIIa	glycoprotein IIb/IIIa
GPIX	glycoprotein IX
GPV	glycoprotein V
GPVI	glycoprotein VI
h	hour
H_2O_2	hydrogen peroxide
HUVEC	human umbilical vein endothelial cells
IC_{10}	10% inhibitory concentration
IC_{25}	25% inhibitory concentration
IC_{50}	50% inhibitory concentration
IU	international unit
kg	kilogram
L	liter
LMA	low melting agarose
LS	*Ligusticum sinense*, Kot Huabua
LSES	ethanol extract of *Ligusticum sinense* and further spray-dried
LSWS	water extract of *Ligusticum sinense* and further spray-dried
mA	milliamp

mg	milligram
min	minute
mL	milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
nm	nanometer
NMA	normal melting agarose
NO	nitric oxide
NP-40	nonyl phenoxylpolyethoxylethanol
NSS	normal saline solution
^{o}C	degree celsius
$P2Y_1$	purinergic $P2Y_1$ receptor
$P2Y_{12}$	purinergic $P2Y_{12}$ receptor
PAF	platelet-activating factor
PAI	plasminogen activator inhibitor
p-Akt	phosphorylated protein kinase B
PBS	phosphate buffered saline
p-eNOS	phosphorylated endothelial nitric oxide synthase
pH	the negative logarithm of hydrogen ion concentration
PKA	protein kinase A
PKB	protein kinase B
PLA2	phospholipase A2
PLC	phospholipase C
PLC_{β}	phospholipase C, β-isoform
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PVDF	polyvinylidene difluoride
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction

SAFO	safrole oxide
SCGE	single cell gel eletrophoresis
SDS–PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SNP	sodium nitroprusside
SSB	single-strand break
TBST	Tris-buffered saline with Tween
TP	thromboxane A_2 receptor
t-PA	tissue-type plasminogen activator
TSP	thrombospondin-1
TXA_2	thromboxane A_2
u-PA	urokinase-like plasminogen activator
V	volt
v/v	volume by volume
vWF	von Willebrand factor

CHAPTER I
INTRODUCTION

A large amount of clopidogrel, an antiplatelet drug, has been imported to Thailand for treatment and prevention of vascular disorders such as coronary artery disease, peripheral artery disease and cerebrovascular disease (stroke). Although, there is Compulsory Licensing for clopidogrel to gain access to medicine, the price is still high. Aspirin, an antiplatelet drug, has a lower price but causes adverse effect. Aspirin sometimes causes gastrointestinal irritation and bleeding, it is usually used in combination with other antiplatelet agents due to lower efficacy. When compared together, clopidogrel and aspirin exhibit antiplatelet effect via different mechanisms. Clopidogrel selectively inhibits the binding of adenosine diphosphate receptors (purinergic $P2Y_{12}$ recepter) of platelets leading to inhibition of a complex glycoprotein (GP) IIb / IIIa activation followed by decreaseing the ability of platelets to aggregate. Meanwhile aspirin irreversibly inactivaties cyclooxygenases (COX), key enzymes in the synthesis of prostaglandins, resulting in a decrease of thromboxane A_2 (TXA_2) production leading to inhibition of platelet aggregation. There are two types of cyclooxygenases, COX-1 and COX-2. Since aspirin is nonselective inhibitor of COX, and COX-1 is responsible for generating protective mucous coating in stomach, aspirin has a negative effect on the stomach (Simon, 2004).

According to the World Health Organization, stroke and other cerebrovascular disease play the major role in the top three causes of death throughout the world in 2008. The death rate in Thailand caused by stroke and other cerebrovascular disease has been increasing due to eating behavior, leading to hypercholesterolemia and obesity. High cholesterol may lead to plague formation and blood vessel lesions. After vessel injury, platelet adhesion and aggregation play a major role in the control of hemostasis. Antiplatelet agents are used in treatment of vascular and circulatory disorders. In addition, thrombophlebitis, vein inflammation, is related to blood clot. Obesity and age are risk factors for thrombophlebitis. Thrombophlebitis is occured by aggregation of platelet in vein and then fibrin becomes crosslinked to form a firm clot which leads to inflammation. Several treatments are used such as surgery, laser treatment, antibiotic and thrombolytic agents but thrombophlebitis still often repeats

occurring. Antiplatelet agents from natural products become an alternative treatment for vascular and circulatory disorders and prevent new clot formation.

Herbal extracts may be an alternative source for the currently used antiplatelet agents, because they constitute a rich source of bioactive chemicals. The antiplatelet activity of *Curcuma longa* L. rhizome-derived materials was reported using a platelet aggregometer and compared with those of aspirin as an antiplatelet agent. Ar-turmerone, an active constituent isolated from *C. longa*, was effective in inhibiting platelet aggregation induced by collagen (IC_{50} 14.4 μM) and induced by arachidonic acid (IC_{50} 43.6 μM) with no inhibitory effect against platelet-activating factor (PAF) or thrombin (Lee, 2006). Aspirin inhibited platelet aggregation induced by arachidonic acid with an IC_{50} value of 35.5 μM without any inhibitory effect on those induced by collagen, PAF, or thrombin. Ar-turmerone may have, at least partly, an effect on cyclooxygenase pathway in platelets like aspirin. Relatively little work has been done on the inhibitory activities of Thai herbs on platelet aggregation, which could lead to the development of an alternative treatment of platelet associated vascular disease.

Yahom, a Thai traditional formulation containing herbs in the Phikud Benjakot, has been widely used for the treatments of circulatory disorders and has been in the “List of Herbal Medicinal Products A.D. 2011” issued by National Drug Committe of Thailand. Phikud Benjakot is consisted of Kot Soa (*Angelica dahurica* Benth., AD), Kot Kamao (*Atractylodes lancea* (Thung) DC, AL), Kot Huabua (*Ligusticum sinense* Oliv. Cv. Chuanxiong, LS), Kot Chiang (*Angelica sinensis* (Oliv.) Diels, AS) and Kot Chulalumpa (*Artemisia vulgaris* L, AV). Quercetin, a constituent in AV, was more effective to inhibit platelet aggregation when combined with α-tocopherol (Kobzar *et al*., 2005).

Long-term use of these herbal extracts may cause DNA damage and increase mutagenic risks. Bergapten, safrole, and isosafrole isolated from AS were previously found to be carcinogenic and quercetin was a well known genotoxic agent (Rueff *et al*., 1992). Therefore, the aim of this study was to determine antiplatelet activities of herbal extracts from Phikud Benjakot and to verify their genotoxicity.

Conceptual framework

According to hemostasis mechanism, the nongenotoxic extracts were screened for antiplatelet activity induced by ADP, arachidonic acid and collagen. The extract exhibiting the highest antiplatelet effect was selected to determine platelet adhesion to subendothelial collagen exposed in injuried vessel. The extract may affect platelet secretion, leading to change in endothelial-derived NO which can inhibit platelet aggregation. Furthermore, the extract may enhance fibrinolysis to increase clot lysis.

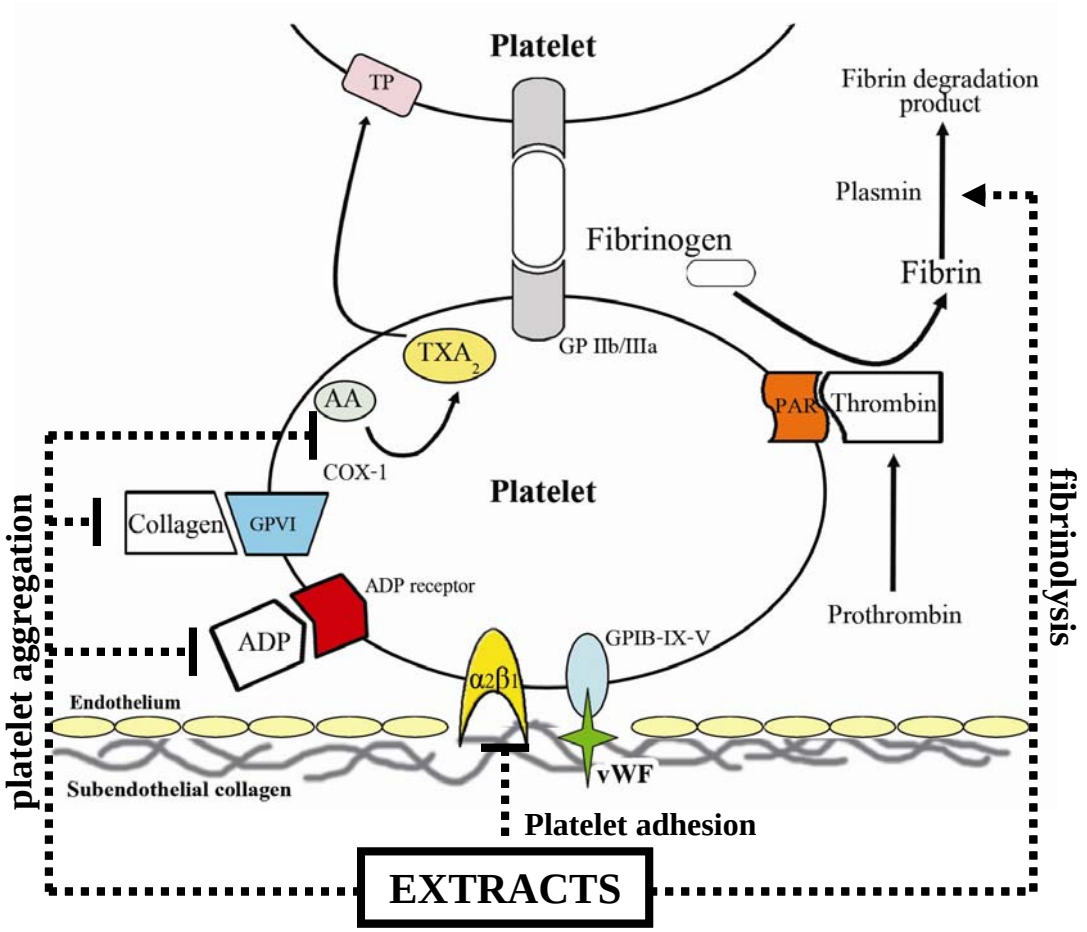


Figure 1 Conceptual framework of the present study

Objectives

1. To determine antiplatelet and fibrinolytic activities of herbal extracts from Phikud Benjakot.
2. To determine mechanisms of antiplatelet activity of herbal extracts from Phikud Benjakot.
3. To evaluate genotoxicity of herbal extracts from Phikud Benjakot.

Scope of study

The present study investigated the effect of of herbal extracts from Phikud Benjakot on platelet aggregation, platelet adhesion and fibrinolysis in human platelet-rich plasma. Cytotoxicity and genotoxicity assessment of the extracts were evaluated in human umbilical vein endothelial ECV304 cells. The phosphorylation of proteins in ECV304 cells was determined using Western blot analysis.

Experimental design

To evaluate genotoxicity assessment of herbal extracts from Phikud Benjakot using alkaline comet assay, cytotoxicity of the IC_{10}, IC_{25} and IC_{50} extracts was performed using MTT reduction assay and genotoxicity of the extracts was evaluated using alkaline comet assay. The antiplatelet activity of the extracts at nontoxic concentration was performed using light transmission aggregametry method. Platelet viability using MTT reduction assay was also performed to confirm nontoxic concentration of the extract. The effect of the extract on platelet adhesion was determined based on acid phosphatase activity. Fibrinolytic activity of the extract was determined by the microtiter plate clot-lysis assay. Effect of the extract on endothelial-derived nitric oxide synthesis was determined using Western blot analysis to determine phosphorylation of proteins on ECV304 cells.

Contributions of the study

1. Information of antiplatelet and fibrinolytic activities of herbal extracts from Phikud Benjakot which might lead to develop antiplatelet agent from herbs.
2. Understanding the mechanism of herbal extracts from Phikud Benjakot on platelet aggregation to maintain hemostasis.

CHAPTER II
LITERATURE REVIEW

Hemostasis

Hemostasis is the process to stop bleeding in the event of vascular injury, consisting of blood vessels, platelets, and the plasma coagulation system including the fibrinolytic factors and their inhibitors (Batty and Smith, 2010). Hemostasis is divided into two stages. Primary hemostasis is temporary plug formation upon injury of the vessel wall, achieved by vasoconstriction, platelet adhesion, and aggregation (Hiller, 2007). Secondary hemostasis, which reinforces the platelet plug by formation of a stable fibrin clot, relies on coagulation factor activation. The process of coagulation can be divided into three distinct parts, namely the intrinsic, extrinsic and common pathways. The intrinsic and extrinsic pathways were independently capable to initiating clot formation (Batty and Smith, 2010). Finally, mechanisms within the fibrinolytic system lead to a dissolution of the fibrin clot and to a restoration of normal blood flow.

Platelet

Platelets, small anucleated cells (~2.5 μm), have highly organized cytoskeleton, unique receptors, and specialized secretory granules. Platelets are derived from the cytoplasm of megakaryocytes in the bone marrow and released into the circulation. The average lifespan of a platelet is normally 8 to 10 days before it is destroyed by phagocytosis in the spleen and Kupffer cells in the liver. The normal platelet count in human is 150-400 $x10^9$/L in blood. Platelets play central roles in the processes of hemostasis and thrombosis by changing shape, secreting granule contents and aggregation (Thon and Italiano, 2010).

Platelets contain four structural regions: peripheral zone, sol–gel zone, organelle zone and platelet membrane systems. **Peripheral zone** is responsible for platelet adhesion and aggregation, consisting of glycocalyx (the exterior surface of the platelet together with other constituents of the peripheral zone) and unit membrane. **Sol-gel zone** is responsible for contraction and support microtubule system containing microtubules, microfilaments, glycogen and smooth and coated vesicles. **Organelle**

zone is a storage site for constituents that stimulate adjacent platelets containing platelet glycosomes, tubular inclusions, mitochondria and three major types of secretory organelles, including α granules, dense bodies (δ granules), and lysosomes. **Platelet membrane system** is responsible for calcium storage and enzymes for prostaglandin synthesis, golgi complexes, the surface-connected open canalicular system, dense tubular system and membrane complexes (White, 2006).

The specific granules (dense granules, α granules, and lysosomes) secrete their constituents during the platelet release reaction in hemostasis (Rendu and Brohard-Bohn, 2001). Dense granules, the smallest platelet granules, contain non-protein small molecules, such as serotonin, ATP, ADP, calcium and pyrophosphate. Dense granules contain high concentrations of adenine nucleotides of approximately 65% of the total platelet content. They also contain serotonin, at a concentration of 65 mM, which is the major osmiophilic factor of dense granules and contain 70% of total calcium content in platelet. Calcium is the predominant ion in platelet granules at a concentration over 100 times higher than that of the whole platelet (McNicol and Israels, 1999). α Granules are large organelles of 200–400 nm diameter with a single membrane. They play a critical role in haemostasis, inflammation, wound healing and cell–matrix interactions and their function derives from contents, including both membrane bound proteins (integrins, immunoglobulin family receptors, leucine-rich repeat family receptors, tetraspanins and other receptors) that express on the platelet surface and soluble proteins that are released during platelet secretion (Blair and Flaumenhaft, 2009).

Platelet adhesion

Platelet adhesion and activation is a multistep process involving multiple platelet receptor-ligand interactions. Platelet adhesion is the first response in vascular injury. Platelets immediately adhere to subendothelial extracellular metrix, such as collagen, laminin and fibronectin. Among these, fibrillar collagens type I and III have a high affinity for von Willebrand factor (vWF) and are effective platelet activators. Collagen is not only the extracellular matrix for platelet adhesion, but also a potent agonist causing platelet activation. Activated platelets secrete TXA_2 and ADP, which play a key role as secondary agonists to recruit more platelets to the injured site (Wu

et al., 1996). Thus, collagen is considered to be the most thrombogenic matrix-mediators of platelet adhesion (Broos *et al.*, 2012). Platelets induce rapid interactions between the glycoprotein (GP) Ib-IX-V complex and vWF bound to subendothelial collagen exposed to the blood vessel. This interaction retains platelets close to the vessel wall and enhances the contact between GPVI and collagen.

The GPIb-IX-V complex, consists of the leucine-rich repeat glycoproteins GPIbα, GPIbβ, GPIX, and GPV, is highly expressed on the platelet surface. GPIb subunit binds many ligands, including vWF, P-selectin, macrophage antigen 1, and the coagulation factors XI, XII and thrombin (Nieswandt *et al.*, 2011). GPIbα is the only receptor on a non-activated platelet with a significant affinity for vWF. Thus, GPIb-IX-V is a major platelet receptor for vWF and other ligands, such as thrombospondin-1 (TSP), the leukocyte integrin αMβ2, and P-selectin expressed on activated endothelial cells or platelets. In addition, GPIb-IX-V acts as a classic signaling receptor to propagate downstream events that activate the platelet integrins, αIIbβ3 and α2β1, leading to stable platelet adhesion and aggregation (Andrews *et al.*, 2006).

GPVI, a platelet-specific member of the immunoglobulin superfamily, is associated with the FcRγ-chain in the platelet membrane. The role of GPVI is considered to enhance strong signaling rather than to establish stable platelet adhesion. GPVI has only low affinity to collagen while GPVI/FcRγ-chain-mediated signaling pathway is crucial for platelet adhesion to collagen (Broos *et al.*, 2012).

Stimulation of platelets by adhesion results in platelet spreading on collagen. The signal transduction during this process leads to activation of platelet resulting in activation of GPIIb/IIIa, which can bind to fibrinogen, and granule secretion. Activated platelets release several components from their granules to recruit other platelets to form aggregates. ADP or serotonin, the important secondary agonists, are released from dense granule of platelets after activation. ADP is considered to amplify the initial platelet activation via ADP receptors on the platelet surface receptors, $P2Y_1$ and $P2Y_{12}$, whereas serotonin amplifies the platelet response via G_q-coupled serotonin A2 -receptor (5HT2A) (Jurk and Kehrel, 2005).

Platelet aggregation

On resting state, GPIIb/IIIa has a low affinity for fibrinogen and vWF. After platelet adhesion and activation, fibrinogen cross-links different platelets by binding to the activated GPIIb/IIIa, resulting in platelet aggregation via α -granule exocytosis. Ligand binding to GPIIb/IIIa induces additional conformational changes, which lead to phosphorylation of tyrosine of the cytoplasmatic GPIIIa-chain leading to disulfide changes in the GPIIb/IIIa complex, which induce high affinity binding sites for fibrinogen (Jurk and Kehrel, 2005). Platelet can be stimulated to activating state by several agonists, such as ADP, arachidonic acid and collagen, which are firstly stimulate during adhesion.

ADP is a weak platelet agonist because it only induces shape change and reversible aggregation in human platelets but still important. Since ADP, secreted from dense granule, can amplify the platelet responses induced by other platelet agonists. Thus, ADP acts as a secondary agonist to enhance secondary aggregation. ADP stimulates platelets by binding to purinergic receptors, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ receptor mediates mobilization of Ca^{2+}, shape change and transient aggregation induced by ADP. The activation of the $P2Y_1$ receptor by ADP leads to the G_q-mediated activation of the β-isoform of phospholipase C (PLC) and an increase in the concentration of intracellular Ca^{2+}, mainly through the release of Ca^{2+} into the cytoplasm from intracellular stores, and partially through influx of Ca^{2+} from external medium. $P2Y_1$ receptor also potentiates the effect of released ADP on platelet aggregation induced by agonists that trigger platelet secretion. In addition, the $P2Y_1$ receptor plays an essential role in platelet shape change induced by collagen when TXA_2 production is prevented. $P2Y_{12}$ receptor is believed to potentiate platelet secretion and involved in sustaining irreversible aggregation. $P2Y_{12}$ receptor mediates inhibition of adenylyl cyclase, whereas ATP is antagonists for both $P2Y_1$ and $P2Y_{12}$ receptors. $P2Y_{12}$ receptor is coupled to inhibition of adenylyl cyclase activity through activation of a G-protein subtype $G_{\alpha i2}$. Although $P2Y_{12}$ receptor mainly activates $G_{\alpha i2}$, it can also couple to other $G_{\alpha i}$ subtypes. The binding of ADP to $P2Y_{12}$ receptor leads to the phosphorylation of PKB/Akt in normal platelets. In addition, ADP by itself can not stimulate the release of dense granules but it can greatly amplify platelet secretion

induced by agonists such as TXA_2 and thrombin receptor-activating peptide (Cattaneo, 2011).

Arachidonic acid is a substrate for COX-1. TXA_2 is produced as a consequence of increased Ca^{2+} levels, which are necessary for the activation of PLA2. Activation of PLA2 results in TXA_2 production via COX-1 and TXA_2 synthase. Released TXA_2 activates additional platelets by acting on its thromboxane A_2 receptor (TP). The TP receptor is a G-protein coupled receptor (GPCR) mediating through G_q. $PLC_{\beta 2}$ is the main effector molecule. Activation of $PLC_{\beta 2}$ resulting in increased cytosolic Ca^{2+} levels (Figure 2) (Broos *et al.*, 2012).

Platelet aggregation testing can be measured by light tranmission to determine the ability of various agonists to platelets to induce *in vitro* activation and platelet-to-platelet activation. Although, various types of blood sample, such as washed platelet, PRP and whole blood can be used in platelet function test, PRP is used in classical light transmission aggregometry (Born, 1962), which is considered the gold standard for platelet function test and is a reliable method for detecting antiplatelet drugs (Velik-Salchner *et al*., 2008). Meanwhile, aggregation in whole blood can be measured using impedance aggregometry, which is based on the measurement of an electrical current between two small wires (Thomas, 1996).

Specific agonists, adrenaline and low dose ADP, give a biphasic aggregation curve (Figure 3) whereas other agonists give only a single wave which is not possible to distinguish the primary wave from the secondary wave. The baseline of light transmission is set before adding agonist. Shape change is rapidly initiated after adding agonist, resulting in a decrease in light transmittance followed by a primary wave of aggregation. The platelets will disaggregate if the stimulus is not sufficiently strong. Meanwhile, the secondary wave of ADP-induced platelet aggregation arises in the strong stimulation. There is a release of platelet granule contents that include fibrinogen, serotonin, thromboxane, and ADP that potentiate the primary aggregation response (Zhou *et al*., 2005).

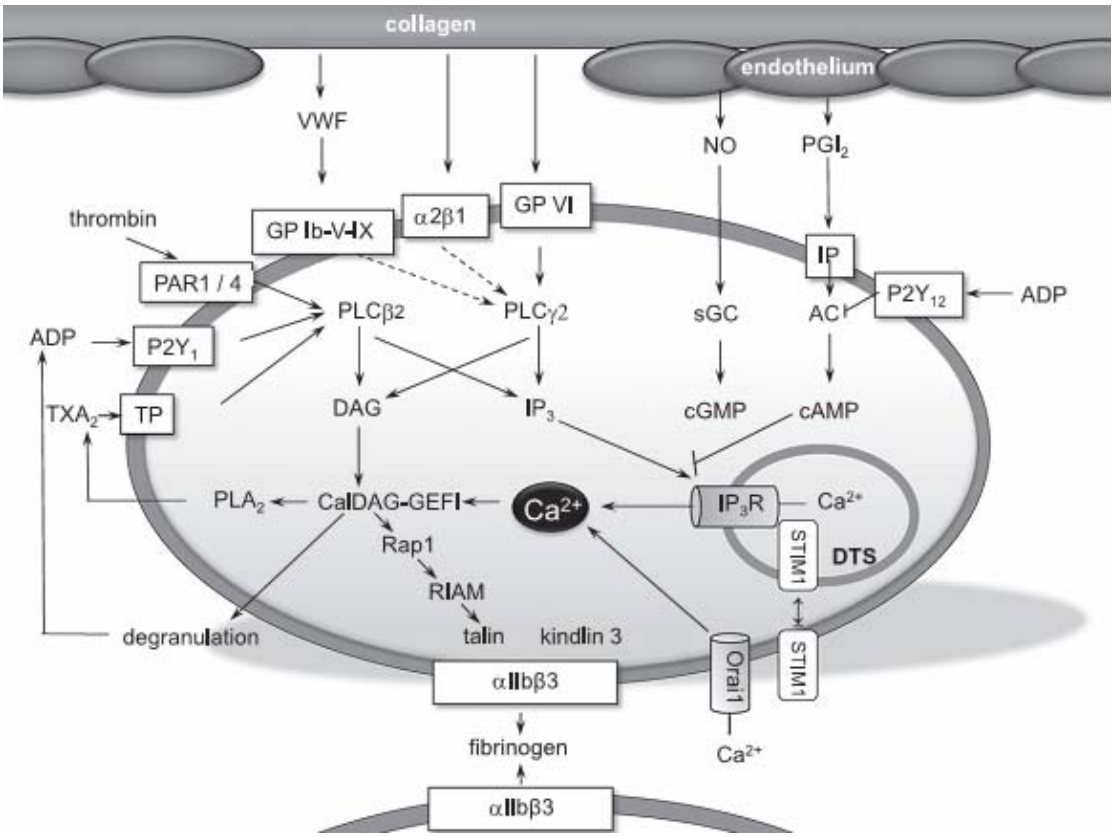


Figure 2 The main platelet receptors and effectors involved in platelet activation, amplification and aggregation (Broos *et al.*, 2012).

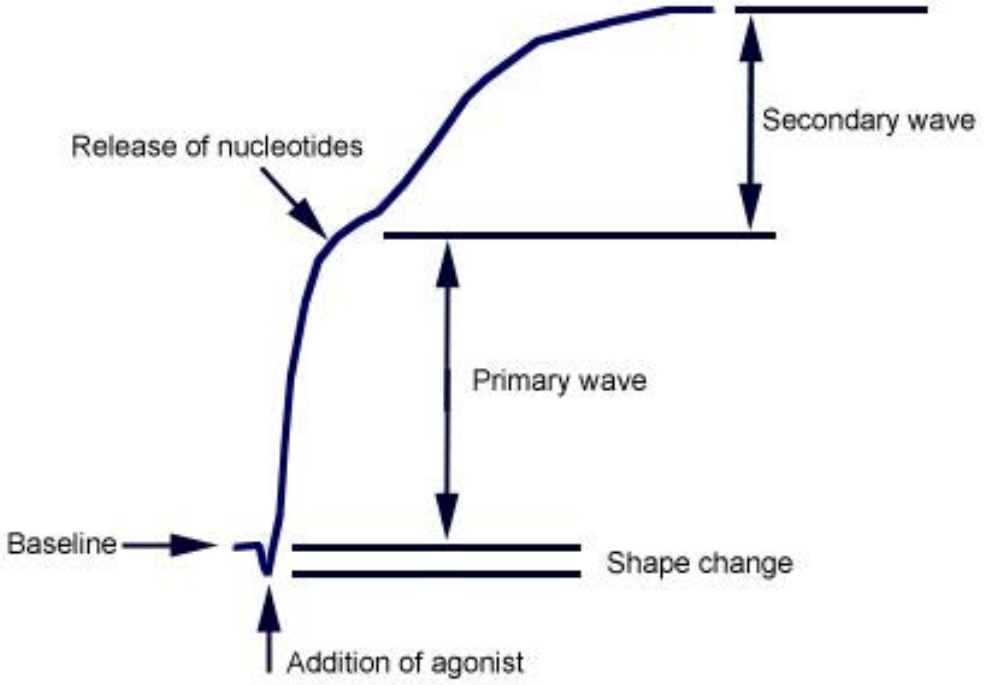


Figure 3 The normal biphasic pattern of aggregation (http://emedicine.medscape.com).

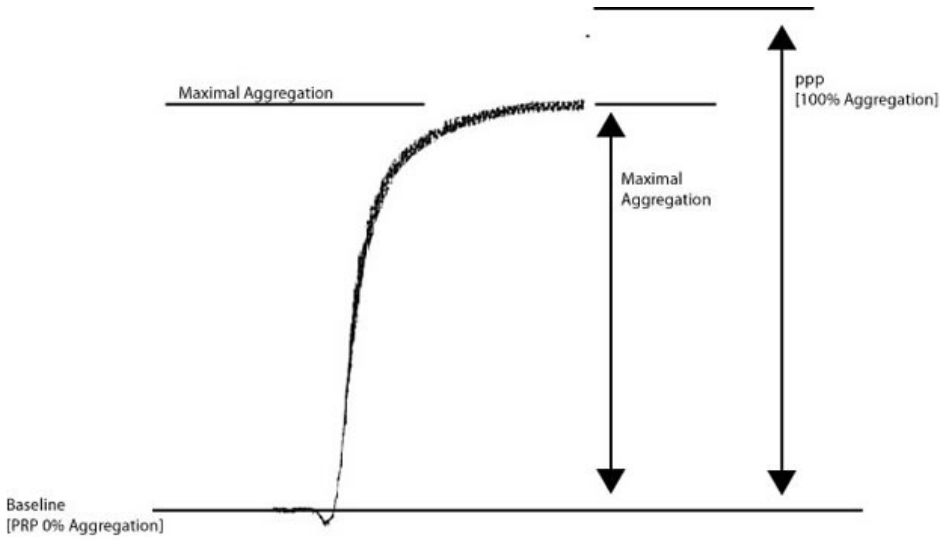
Platelet aggregation response can be determined by measuring maximal aggregation or the angle of the initial aggregation slope. Maximal aggregation is probably the most common parameter used to express platelet aggregation response. The percentage of maximal aggregation is calculated by measuring the distance between the baseline (0% aggregation, PRP) and PPP (100% aggregation), then divided by the maximal aggregation (Figure 4A). Meanwhile, aggregation slope represents the rate of aggregation, reflecting cross-linking of platelets mediated by activation of GPIIb/IIIa (García and Schneider, 2008). The aggregation slope is calculated by drawing a first line at a tangent to the aggregation curve and a second line perpendicular to the baseline from the 1 min point to the intersect point of the tangent. Then, measuring the distance covered from the baseline to the intersect point (X). Derive the maximal height of the aggregation (100% aggregation or maximal aggregation) from the y-axis (Y). Divide X/Y to calculate the slope or rate of aggregation (Figure 4B).

Fibrinolysis

Fibrin is formed during inflammation, tissue repair and hemostasis. The fibrin creates a framework for blood clot to seal a hole or cover a wound. However, fibrin must be removed when normal tissue structure and function are restored (Rijken and Lijnen, 2009).

Fibrinolysis is a process to break down blood clots. Fibrinolytic system depends on the interaction of activators, zymogens, enzymes, and inhibitors to provide local activation at sites of fibrin deposition. In fibrinolysis, an enzyme, plasmin, lyses the fibrin to soluble products (Figure 5) (Francis and Marder, 1986). Plasmin is derived from the cleavage of plasminogen between Arg561 and Val562 by plasminogen activator (Benchenane *et al*., 2005).

A)



B)

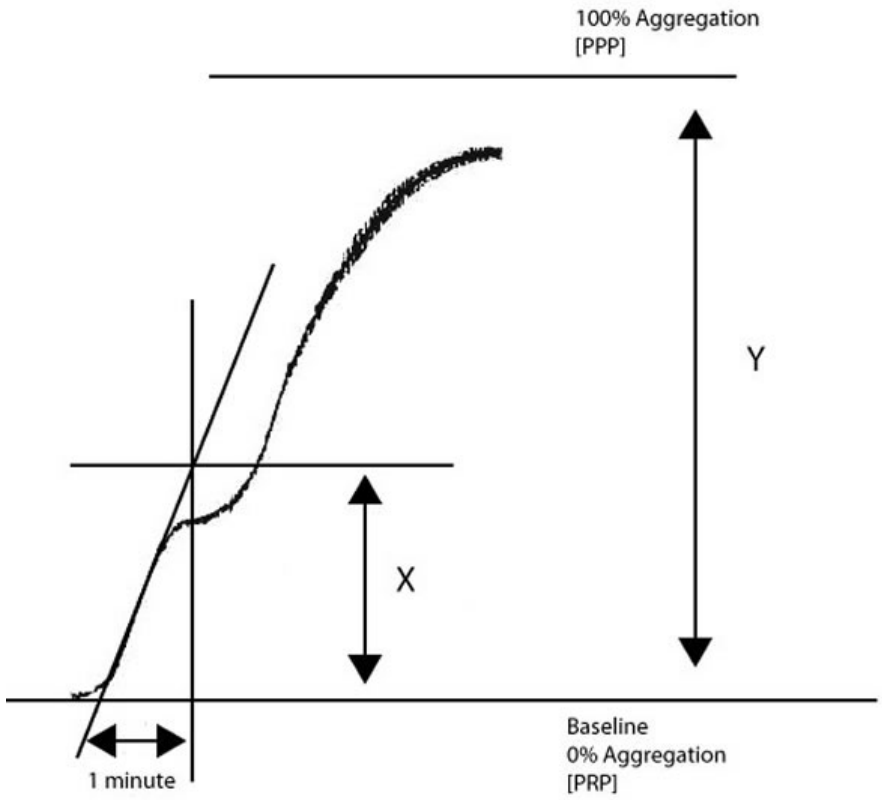


Figure 4 Calculating platelet aggregation parameters from aggregation trace. A) percentage of maximal aggregation and B) aggregation slope (http://emedicine. medscape.com).

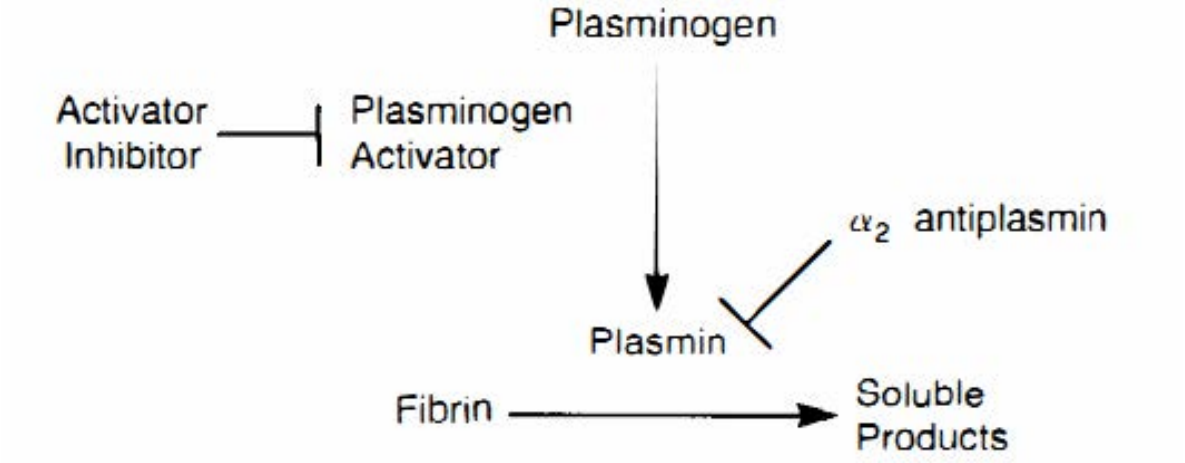


Figure 5 The mechanism of fibrinolytic system (Francis and Marder, 1986).

The plasminogen activators can be divided into two types, tissue-type plasminogen activator (t-PA) and urokinase-like plasminogen activator (u-PA). The t-PA, secreted by endothelial cells, exhibits greater enzymatic activity in the presence of fibrin. Urokinase, the plasminogen activator is originally identified as the protein responsible for the fibrinolytic activity in urine (Cesarman-Maus and Hajjar, 2005).

Streptokinase, the oldest and most widely studied thrombolytic agent, is approved and commercially available for the treatment of thrombotic diseases. Streptokinase alone has no enzymatic activity but acts in two steps, the first of which is formation of an equimolar complex with plasminogen. Streptokinase has low affinity for fibrin, the action is therefore not limited to the site of thrombosis. Therapeutic usage results in systemic plasminemia with proteolytic degradation of plasma fibrinogen and other proteins. This is called a lytic state (Francis and Marder, 1986).

Endothelium-derived nitric oxide

Endothelium-derived NO is produced by endothelial nitric oxide synthase (eNOS), which plays central roles in a vasodilataion and platelet aggregation inhibition (Iwakiri *et al.*, 2002). eNOS is activated via intracellular signal transduction. Two different molecular mechanisms have been uncovered in endothelial cells that independently change inactive eNOS into an active state. In human, there is a dissociation of the enzyme from the cellular membrane into the cytosol, or translocation via an Akt kinase-dependent serine phosphorylation of eNOS in position 1177 (Pott *et al.*, 2006). Akt is activated via phosphorylation at Ser 473 by the activation of receptor tyrosine kinases, G protein-coupled receptors, and mechanical forces, such as shear stress (Iwakiri *et al.*, 2002).

Bioflavonoids were reported to induce activation of eNOS. Hesperidin at a concentration of 0.1 mM induced phosphorylation of eNOS and Akt in human umbilical vein endothelial HUVEC cells within 1 h-incubation (Chiou *et al.*, 2008). Quercetin, isolated from AV, enhanced endothelial NO production by stimulation of eNOS activity in HUVEC (Schmitt and Dirsch, 2009) and concentration-dependently induced eNOS phosphorylation at Ser 1179 (bovine sequence). The peak is at 5 min via an Akt-independent, cAMP/PKA-mediated pathway that enhances the production

of NO in bovine aortic endothelial cells (BEACs) (Li *et al*., 2012). Treatment of quercetin (5 and 10 μM) for 30 or 60 min stimulates phosphorylation of eNOS at Ser 1179 in a concentration-dependent manner in BAECs (Khoo *et al*., 2010).

Genotoxicity

Genotoxicity or DNA damage occurs metabolism is caused by two types of factor, endogenous and exogenous damage. Endogenous damage such as oxidative radical is derived from normal metabolic by products in cells, leading to spontaneous mutation. Exogenous damage is caused radiation, chemical reagent and heavy metal. These factors may lead to toxic to gene in several levels, such as a point mutation resulting in a base pair, DNA strand break or chromosome aberration (Olive *et al*., 2001).

Safety evaluation plays a major role in developing new herbal medicine. Following the Food and Drug Administration (FDA) guideline, various types of toxicity studies including genotoxicity evaluation are needed for new drug approval and product registration (Wu *et al*., 2008). Several herbs were reported to be genotoxic. *Aloe vera* extract produced single-strand break (SSB) in the plasmid DNA by electrophoresis assay (Paes-Leme *et al*., 2005). Curcumin induced micronucleation by blocking DNA synthesis in MCF-7 breast cancer cells (Holy, 2002).

Genotoxicity tests are designed to detect genetic damage such as Ames test, micronuclei chromosomal aberration and comet assay, which may reflect teratogenic and tumorigenic potential of pharmaceuticals and herbal products (Wu *et al*., 2008). Comet assay or single cell gel eletrophoresis (SCGE) is a method for detection of DNA lesions in an individual cell and approved by United States Environmental Protection Agency (U.S. EPA; 2004). Comet assay is a sensitive method for genotoxicity study, requiring less time and number of cell. This method can be used for evaluation of genotoxicity *in vitro* and *in vivo* (Tice *et al*., 2000).

Under alkaline condition, DNA is allowed to unwind and small fragments can migrate through agarose to form the tail of the comet. Alkaline comet assay can detect DNA single-strand break, double-strand break, alkali labile lesion including apoptosis resulting in a long distance between head and tail (Olive *et al*., 2001). Since DNA damage is related with cell death, the highest tested concentration should not induce

excessive cytotoxicity. To avoid a false positive result, cytotoxicity should be evaluated in a preliminary range-finding experiment to find a low cytotoxic concentration. The results can be analyzed using image analysis software to get several parameters, such as tail length (the distance of fragmented DNA migration from comet head to tail that can reflect the size of migrating DNA), %DNA in tail (the intensity of fragmented DNA in comet tail compare to total DNA that can reflect the amount of fragmented DNA) and tail moment (the product of tail length and %DNA in tail that represents both size and the amount of fragmented DNA) (Tice *et al*., 2000)**.**

Antiplatelet and fibrinolytic activities of herbs in Phikud Benjakot

Yahom is Thai traditional formulation for the treatment of circulatory disorders. Effect of Yahom on cardiovascular function in healthy volunteers showed that Yahom powder and its water extract significantly increased diastolic blood pressure and mean arterial blood pressure but decreased pulse pressure when compared with those before taking Yahom (Suvitayavat *et al*., 2005[a]). The dominant effect of Yahom was previously reported to increase blood pressure in rats, even though it caused an initial transient decrease in blood pressure (Suvitayavat *et al*., 2005[b]). Furthermore, Yahom increased the force of isolated rat aortic ring contraction by stimulating the α-receptor on the vascular smooth muscle cells (Suvitayavat *et al*., 2005[c]). Phikud Benjakot is ones of the constituents in Yahom. The part used and antiplatelet activity of crude drugs in Phikud Benjakot was summarized in Table1.

Antiplatelet activity of crude drugs in Phikud Benjakot has been previously reported. Buthanol extract of **AD** can increase 35% survival in the mouse model of thrombosis (Kim *et al*., 1995). *Atractylodis macrocephalae*, the plant in the same genus with **AL**, is a part of Korean traditional prescription namely Danggijakyaksan, which can significantly inhibit arachidonic acid release in collagen-activated platelets. This activity may be due to inhibition of PLC activity, leading to reduced phosphoinositide breakdown and inhibitied TXA_2 (Park *et al.*, 2003). Tetramethylpyrazine, a consituent in *Ligusticum wallichii* Franchat that is in the same genus with **LS**, can inhibit platelet aggregation induced by collagen in human blood (Sheu *et al*., 2000).

Table 1 The part used and antiplatelet activity of crude drugs in Phikud Benjakot

Scientific name	Code	Part used	Family	Antiplatelet activity	Fibrinolytic activity	References
Angelica dahurica Benth.	AD	root	Apiaceae	+	ND	Kim *et al*., 1995
Atractylodes lancea (Thung) DC.	AL	rhizome	Asteraceae	ND	ND	ND
Ligusticum sinense Oliv. Cv. Chuanxiong	LS	root	Apiaceae	ND	ND	ND
Angelica sinensis (Oliv.) Diels	AS	root	Apiaceae	+	ND	Zhang *et al*., 2009
Artemisia vulgaris L.	AV	aerial part	Asteraceae	ND	ND	ND

\+ effective
ND not determine

AS injection can significantly inhibit platelet activation via thromboxane and vWF, leading torelieve vascular endothelial cell injury and improve microcirculation in patients with ulcerative colitis (Dong *et al*., 2004). Z-ligustilide, a constituent of **AS**, was orally administered once daily to rat for 3 days. Blood samples were collected 1 h after final administration for antiplatelet assay. Z-ligustilide at the doses of 10 and 40 mg/kg significantly inhibited ADP-induced platelet aggregation and Z-ligustilide at the dose of 40 mg/kg showed higher inhibitory activity of platelet aggregation than ASA (Zhang *et al*., 2009).

Several plants in the same genus with **AV** were reported for antiplatelet acitivity. Antiplatelet activity of *Artemisia dracunculus* inhibited thrombin-induced platelet aggregation. Pretreatment of 0.2 mg/mL methanol extract and 0.2 mg/mL chloroform fraction of *Artemisia dracunculus* before adding thrombin (0.25 U/mL) obviously decreased aggregated platelets in the uncoated plates relative to the untreated control when evaluated under the inverted microscope (Shahriyary and Yazdanparast, 2007). Essential oil extracted from steam distillation of *Artemisia dracunculus* showed antiplatelet activity against ADP, arachidonic acid and TXA_2 agonist (U46619), exhibiting IC_{50} values ranging from 4 to 132 μg/ml in guinea pig platelet-rich plasma (PRP) (Tognolini *et al*., 2006). Several agents isolated from the methanol extract of *Artemisia capillaris*, such as flavonoids (arcapillin and quercetin), coumarins (artemicapin B, scoparone, scopoletin and aesculetin) and phenylacetylenes (capillaridins A, B and C, capillene, capillin and O-methylcapillene), markedly inhibited rabbit platelet aggregation induced by arachidonic acid and collagen (Wu *et al*., 2001).

CHAPTER III
MATERIALS AND METHODS

Materials

Equipments

- Aggregometer (AggRAM, Helena Laboratories, Texas, USA; Pack-4, Helena Laboratories, Texas, USA)
- Centrifuge (Allegra X-12 R, Beckman Coulter, Inc., California, USA)
- CO_2 Incubator (3121, Forma Scientific Inc, Massachusetts, USA)
- Chemiluminescence imaging system (Gene genome, Syngene, Cambridge, England)
- Electrophoresis chamber (E-C apparatus, Maxicell EC360, USA)
- Hematology analyser (Horiba ABX, Micros 60, Kyoto, Japan)
- Hemocytometer (Bright-line, Hausser Scientific, Pennsylvania, USA)
- Microplate reader (Perkin Elmer, Victor 3, Massachusetts, USA)
- Microscope (CK 30, Olympus, Tokyo, Japan)
- Power supply (Thermo Scientific, EC570-90, USA)

Reagents

- Adenosine 5'-diphosphate sodium salt (ADP) (A6646, Sigma, St. Louis, USA)
- Albumin from bovine serum (A7906, Sigma, St. Louis, USA)
- Arachidonic acid (A4425, Sigma, St. Louis, USA; 5364, Helena Laboratories, Texas, USA)
- Calcium chloride dihydrate (2328.1000, Merck, Darmstadt, Germany)
- Citric acid (1.00244.1000, Merck, Darmstadt, Germany)
- Collagen type I from calf skin (C9791, Sigma, St. Louis, USA)
- Collagen type I from equine tendon (5368, Helena Laboratories, Texas, USA)
- Dimethyl sulfoxide (DMSO) (60153, Merck, Darmstadt, Germany)
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M2128, Sigma, St. Louis, USA)

- Enhanced chemiluminescence (ECL) prime Western blotting detection reagent (PRN2232, Amersham, Buckinghamshire, England)
- Ethylenediaminetetraacetic acid (EDTA) (180, Univar, Sydney, Australia)
- Fetal bovine serum (FBS) (CH30160.02, Hyclone, Utah, USA)
- Goat polyclonal to secondary antibody to rabbit IgG – H&L (HRP) (ab6721, Abcam,.Cambridge, England)
- Hydrogen peroxide (H_2O_2) (H/1750/17, Fisher Scientific, California, USA)
- Low melting agarose (15517-014, Invitrogen, Spain)
- M199 (31100-027, Gibco BRL Life Technologies, New York, USA)
- Mouse monoclonal to ß-actin, horseradish peroxidase conjugated (ab 20272, Abcam,.Cambridge, England)
- p-Nitrophenyl phosphate (71768, Sigma, UK)
- Normal melting agarose (1840000 BDH Chemical, Poole, England)
- Penicillin-streptomycin (P5140, Gibco BRL Life Technologies, New York, USA)
- Protease inhibitor (80-6501-23, Amersham Biosciences, Buckinghamshire, England)
- Rabbit polyclonal to Akt (ab6076, Abcam, Cambridge, England)
- Rabbit polyclonal to Akt (phospho S473) (ab66138 Abcam, Cambridge, England)
- Rabbit polyclonal to eNOS (ab5589, Abcam, Cambridge, England)
- Rabbit polyclonal to eNOS (phospho S1177) (ab75639, Abcam, Cambridge, England)
- Sigmacote (SL2, Sigma, St. Louis, USA)
- Tri-sodium citrate (30128, BDH Chemical, Poole, England)
- Sodium choride (465, Ajax Finechem, Australia)
- Sodium hydroxide (482, Ajax finechem, Australia)
- Sodium nitroprusside (SNP) (71778, Fluka, Spain)
- Streptokinase (S3134, Sigma, St. Louis, USA)
- SYBR Green (S33102, Invitrogen, Paisley, UK)

- Tris hydrochloride (T5941, Sigma, St. Louis, USA)
- Tris (hydroxymethyl)-methylamine (77-86-1, Fisher scientific, California, USA)
- Triton X-100 (93420, Fluka, St. Gallen, Switzerland)
- Trypsin from porcine pancreas (T4799, Sigma, St. Louis, USA)

Methods

Herbal extracts

Herbal extracts from Phikud Benjakot were a generous gift from Dr. Sanya Hokputsa, Phytochemical Research Group, Research and Development Institute, Government Pharmaceutical Organization. Briefly, roots of AD, LS and AS, rhizomes of AL and aerial parts of AV were extracted twice with 50% ethanol or water at the ratio of 1:20 (1 kg of crude drugs: 20 L of solvent) using a reflux apparatus for 3 h. The extraction was repeated twice. Each solvent extract was concentrated and subsequently either spray-dried or freeze-dried. The extracts were kept at room temperature in a desiccator.

The codes of the herbal extracts from Phikud Benjakot were shown in Table 2. The first and second letters are scientific name. The third letter is solvent used for the extraction: ethanol (E) or water (W).The fourth letter is the process of removing solvent either spray-drying (S) or freeze-drying (F).

Table 2 The codes of herbal extracts in Phikud Benjakot.

Scientific name	**Part used**	**Family**	**Code**	
			Ethanol extract	**Water extract**
Angelica dahurica	root	Apiaceae	ADES	ADWS
Atractylodes lancea.	rhizome	Asteraceae	ALES	ALWF
Ligusticum sinense	root	Apiaceae	LSES	LSWS
Angelica sinensis	root	Apiaceae	ASES	ASWS
Artemisia vulgaris	aerial part	Asteraceae	AVES	AVWS

Cell culture

Human umbilical vein endothelial ECV304 cells (Cell Lines Service, Germany) was cultured in M199 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 ºC in a humified atmosphere of 5% CO_2. The culture medium was refreshed every 2 days.

Platelet preparation

The protocol was approved from the Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University as protocol review No. 11-33-012. Volunteers, donating blood at National Blood Centre, Thai Red Cross Society, would be asked to provide blood samples. Before the volunteer decided whether or not to take part in the experiment, the volunteer would be asked to provide health information. The questions were similar to the questions asked of blood donors. The volunteer, deciding to partipate in the experiment, would be asked to sign the consent form.

Inclusion criteria

Volunteer subjects enrolled and treated in this study must meet all of the following criteria:

- aged between 18 years and 55 years
- healthy and provided a personal medical history
- understand and sign the informed consent form.

Exclusion Criteria

Subjects will be excluded from the study if they meet any of the following criteria:

- recent history of alcoholism, smoking or drug addiction
- recieve any medication within 14 days before start of the study
- disease or condition which might compromise the platelet aggregation or hematology, such as thalassemia or Glanzmann's thrombasthenia

The human blood samples from healthy volunteers were collected into plastic conical tube containing 3.2% sodium citrate at ratio 9:1 then the blood tube was mixed gentlely. Platelet-rich plasma (PRP) was obtained by centrifuging citrated

blood at 200 x g for 10 min. The remaining blood was centrifuged at 3,000 x g for 15 min to obtain platelet-poor plasma (PPP) (Chang *et al*., 2005).

To avoid abnormal result, the platelet count in PRP should be in normal range (150,000 – 600,000 cells/μL). PRP were separated to perform platelet count by using Rees and Ecker method. PRP was diluted 200x in Wintrobe's modification Rees-Ecker diluting fluid (Sloan, 1951) and then was counted by using hemocytometer slide.

Cytotoxicity using MTT reduction assay

Viability of ECV304 cells

Cytotoxicity of the extracts from Phikud Benjakot was performed in ECV304 cells using MTT reduction assay. The yellow tetrazolium MTT is reduced by mitocondrial reductase enzymes in living cells, to generate intracellular purple formazan. ECV304 cells were cultured in 96-well plate (1 x 10^4 cells/well) in M199 medium supplement with 10% FBS and 1% penicillin/streptomycin at 37 ºC in a humified atmosphere of 5% CO_2. After 24 h, culture medium was discarded by plate inversion and 200 μL of the extract at various concentrations in complete medium were added into each well. For positive control, cells were treated with H_2O_2 at concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 μM for 24 h. The extracts were exposed on ECV304 cells at various concentrations of 0.01, 0.1, 1, 2 and 5 mg/mL. Cells were incubated at 37 ºC for 24 h and then the extract solutions were discarded by plate inversion. One hundred microliters of 0.4 mg/mL of MTT solution in serum-free medium were added to each well and cells were then incubated at 37 ºC for 4 h. MTT solution was removed by plate inversion. One hundred microliters of DMSO were added to each well to dissolve formazan. Plate was shaken for 15 min before absorbance of 570 nm was measured by microplate reader. The IC_{50} value was calculated from percentage of cell viability by CurveExpert 1.40 software (Daniel Hyams, Tennessee, United States).

% cell viability = [(sample - blank)/(control – blank)] x100

Platelet viability

Platelet concentration in PRP was adjusted to 3 x 10^5 cells/μL with PPP. The extracts at various concentrations (10 μL) were added into 96-well plate. The 90 microliters of PRP were added into 96-well plate. The platelet was exposed to the extracts at 37 ºC for 1 h and then the plate was centrifuged at 3,000 x g for 15 min. The supernate was discarded and 0.4 mg/mL of MTT 100 μL in phosphate buffered saline (PBS) was added. After 4-h incubation at 37 ºC, the plate was centrifuged at 3,000 x g for 15 min. The MTT solution was replaced by 100 μL DMSO. The plate was shaken for 15 min before the optical density was measured with a microplate reader using a wavelength of 570 nm. The IC_{50} value was calculated from percentage of platelet viability by CurveExpert 1.40 software.

% platelet viability = [(sample - blank)/(control – blank)] x100

Genotoxicity test using alkaline Comet assay

Comet assay is a method for detecting the migration of fragmented DNA in an agarose matrix under electrophoretic conditions. Alkaline comet assay was performed with a slight modification from that was described by Bony *et al*. (2006). ECV304 cells at a seeding density of 2 x 10^5 cells/well were cultured in 6-well plate in M199 medium supplement with 10% FBS and 1% penicillin/streptomycin at 37 ºC. After 24 h, culture medium was discarded. The cells were exposed to the 2 mL of the extract at the IC_{10}, IC_{25} and IC_{50} values obtained from the cytotoxicity testing. Treatment of the cells with H_2O_2 at a concentration of 100 μM for 30 min was used as a positive control. After incubation of the treated cells at 37 ºC for 24 h, The cells were washed three times with PBS and then harvested by trypsin/EDTA. Cell suspension was washed twice with PBS by centrifuging at 1200 rpm for 4 min. Cell suspension (1 x 10^5 cells/mL PBS) was mixed with an equal volume of 1% low-melting agarose. Cell mixture was then dropped on a microscope slide which was previously covered with a 0.8% normal-melting agarose layer. After solidification of agarose, the slide was treated with a lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) for 1 h at 4 ºC. The slide was placed in gel-electrophoresis apparatus containing electrophoresis buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 5 min to allow DNA unwinding and was then

electrophoresed at 25 V and 300 mA for 5 min. The slides were washed for 5 min in a neutralizing buffer (0.4 M Tris, pH 7.5) before dehydrating with absolute methanol. The slide was stained with SYBR Green and scored using a fluorescent microscope and image analysis software CometScore 1.5 (TriTek Corporation, Sumerduck, VA, USA). The parameter obtained from CometScore %DNA in tail (the percentage of migrated DNA) was compared to the control group. The result was analyzed using one-way ANOVA at $p < 0.05$.

Platelet aggregation assay using turbudumetry method

Platelet aggregation induced by ADP arachidonic acid and collagen

Antiplatelet activity was determined by light transmission aggregometry method, which measures platelet aggregation as an increase in light transmittance at 650 nm using aggregometer (Born, 1962). The PRP with a normal platelet count was allowed to rest at room temperature for 15 min. Two hundred microliters of PRP were pipetted into siliconized cuvette and PPP was used as blank. Twenty-five microliters of test substances were added into the cuvette and preincubated at 37 ºC for 5 min. PPP was set to be 100% aggregation and PRP was set to be 0% aggregation. Percentage of platelet aggregation was detected under constant stirring at 600 rpm. Baseline was allowed to record for 1 min and then 25 μL of ADP, arachidonic acid and collagen at various concentrations were added. Percentage of platelet aggregation was monitored for 6 min after adding ADP. Minimal concentration of ADP which can induce high percentage of platelet aggregation was selected for the study of antiplatelet activity for each blood sample.

Effect of the extracts on platelet aggregation induced by ADP, arachidonic acid or collagen

Two hundred microliters of PRP with a normal platelet count were pipetted into siliconized cuvette and PPP was used as blank (Chang *et al*., 2005). Twenty-five microliters of the extract were added into the cuvette and preincubated at 37 ºC for 5 min. Aspirin (ASA) was used as positive control. PPP was set to be 100% aggregation and PRP was set to be 0% agggregation. Percentage of platelet aggregation was detected under constant stirring at 600 rpm. Baseline was allowed to record for 1 min

and then 25 μL agonist (ADP at various concentrations or 500 μM arachidonic acid or 3 μg/mL collagen) was added. Percentage of platelet aggregation was monitored for 6 min after adding the agonists. Percent inhibition of platelet aggregation was calculated from percentage of maximal aggregation.

% inhibition = [(A-B)/A] x100

A : maximal aggregation of vehicle control

B : maximal aggregation of sample

Effect of the extracts on platelet adhesion using acid phosphatase assay

Acid phosphatase assay was performed to determine platelet function (Eriksson and Whiss, 2005). Acid phosphatase in platelet catalyzes the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol, a chromogenic product with absorbance at 405 nm. The extract at various concentrations (5 μL) was added into 96-well plate and 45 microliters of PRP (3 x 10^5 cells/μL) were then added. The platelet was exposed to the extract at 37 ºC for 1 h. The 50 microliters of 0.5 mg/mL *p*-nitrophenyl phosphate in citrate buffer (pH 5.4) were added to each well. After 1-h incubation at 37 ºC, the 100 microliters of 2N NaOH were added to each well to stop reaction and develop colour. The plate was shaken for 15 min and then the optical density was measured with a microplate reader at the wavelength of 405 nm. The IC_{50} value was calculated from percentage of relative activity.

% relative activity = [(sample - blank)/(control – blank)] x100

Effect of the extracts on platelet adhesion assay

The 96-well plate was previously coated with collagen type I from calf skin by adding 50 μL of 100 μg/mL of collagen type I in 0.1 M acetic acid to each well. The plate was kept at 4 ºC overnight and then washed twice with 200 μL of NSS by plate inversion before allowing platelet to adhere to collagen (Eriksson and Whiss, 2005). Platelet concentration in PRP was adjusted to 3 x 10^5 cells/μL with PPP. The platelets were incubated with the extract at various concentrations by mixing PRP and the extract at ratio 9:1 in eppendorf and then 100 μL of the mixture were aliquoted to each coated well. SNP was used as positive control. The plate was incubated at 37 ºC

for 1 h without shaking to allow platelet adhesion. The unattached platelets were removed by plate inversion. The plate was then washed twice with 200 μL of NSS by plate inversion. Acid phophatase, a platelet enzyme whose activity is stable independently of platelet stimulation and is not released, was used to determine the amount of platelets adhering to collagen. One hundred microliters of *p*-nitrophenyl phosphate (0.25 mg/mL) in citrate buffer were added to each well. After 1-h incubation at 37 ºC, the 100 microliters of 2N NaOH were added to each well to stop reaction and develop colour. Plate was shaken for 15 min and then the optical density was measured with a microplate reader using a wavelength of 405 nm. Percentage of platelet adhesion was calculated.

% platelet adhesion = [(sample - blank)/(control – blank)] x100

Effect of the extracts on fibrinolysis

A fibrinolysis was performed using a microplate clot-lysis assay (Stief, 2006). Plasma was prepared from citrated blood by centrifugation at 3,000 x g for 15 min. Microclot was generated by plasma recalcification. Two hundred microliters of 1.5 M $CaCl_2$ were added to 20 mL of pooled citrated plasma and then 100 μL of the mixture were immediately pipetted to each well of 96-well plate. The plate was incubated at 37 ºC for 60 min. Twenty five microliters of the extract were added to the fresh microclot in each well. After adding extracts, 100 μL of 6% bovine serum albumin in BSA or plasma were added into each well. Streptokinase was used as a positive control. The absorbance at 405 nm was measured at 0, 0.5, 1, 2, 3, 4, and 5 h. Percentage of clot mass was calculated.

clot mass (%) = (A-B)/(C-B) * 100

A : clot turbidity (absorbance of clot after incubation with sample)

B : 0% clot turbidity (absorbance of completely lysed clot)

C : 100% clot turbidity (absorbance of clot without added sample)

Phosphorylation of eNOS and Akt in endothelial cells affected by the extracts using Western blot analysis.

ECV304 cells (1.5 x 10^6 cells/well) were cultured in 100 mm dish in M199 medium supplement with 10% FBS and 1% penicillin/streptomycin at 37 ºC. After 18

h, the medium was replaced with serum-free medium. After 6 h, the extract at nontoxic concentration of 0.25, 0.5 and 1 mg/mL was added and further incubated for 1 h. The cells were collected by scraping in PBS and centrifuged at 1,500 x g for 10 min. Cells were lysed in lysis buffer [(Tris-HCl pH 7.5 (20 mM), NaCl (150 mM), Triton X-100 0.5%, nonyl phenoxylpolyethoxylethanol (NP-40) 0.5%, $CaCl_2$ (2 mM), NaF (5 mM), sodium orthovanadate (1 mM), pefabloc (1 mM)] at 4 ºC for 30 min. Cell lysates were centrifuged at 12,000xg for 10 min to collect supernatant. The amount of protein in supernatant was determined by Bradford assay. Protein sample was prepared in loading buffer (dH_2O, Tris-HCl, glycerol, SDS, 2-mercaptoethanol, bromophenol blue). Protein (30 μg) was loaded in each well of 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE) and eletrophoresis was performed at 90 V for 90 min. Then protein was transferred from SDS gel to 0.45 μm polyvinylidene difluoride (PVDF) at 100 V for 2 h. To avoid non-specific binding, the PVDF membrane was incubated in 5 % skimmed milk for 1 h and then washed with Tris-buffered saline with Tween (TBST) for 10 min 3 times. The PVDF membrane was incubated with primary antibodies: rabbit polyclonal to eNOS (1:250), rabbit polyclonal to p-eNOS (1:250), rabbit polyclonal to Akt (1:500) and rabbit polyclonal to p-Akt (1:1000) at 4 ºC overnight and then washed with TBST 3 times for 10 min. The PVDF membrane was incubated with mouse monoclonal to ß-actin (1:3000) and goat polyconal to secondary antibody to rabbit IgG (1:3000) at room temperature for 1 h. The protein bands were detected by incubation of enhanced chemiluminescent on PVDF membrane. The intensity of each protein band was analyzed using ImageJ 1.41 software (NIH, Bethesda, MD, USA) and compared with those of untreated cells.

Effect of the extract on phosphorylation of eNOS and Akt in endothelial cells affected by platelet secretion

PRP from citrated plasma was incubated with the extract at the nontoxic concentrations for 5 min and then ADP was added to induce platelet aggregation and secretion. PPP was separated by centrifugation at 3,000xg for 15 min to collect secretory protein from platelet. PPP was incubated for 1 h with the ECV304 cells ($15x10^5$ cells/well) in 100 mm dish (Massberg *et al*., 2003). The cells were collected by scraping in PBS and centrifuged at 1,500 x g for 10 min. Cells were lysed in lysis

buffer [(Tris-HCl pH 7.5 (20 mM), NaCl (150 mM), Triton X-100 0.5%, NP-40 0.5%, $CaCl_2$ (2 mM), NaF (5 mM), sodium orthovanadate (1 mM), pefabloc (1 mM)] at 4ºC for 30 min. Cell lysate was centrifuged at 12,000 g for 10 min to collect supernatant. The amount of protein in supernatant was determined by Bradford assay. Protein sample was prepared in loading buffer (dH_2O, Tris-HCl, glycerol, SDS, 2-mercaptoethanol, bromophenol blue). Protein (30 μg) was loaded in each well of 12% SDS – PAGE and eletrophoresis was performed at 90 V for 90 min. Then protein was transferred from SDS gel to 0.45 μm PVDF at 100 V for 2 h. To avoid non-specific binding, the PVDF membrane was incubated in 5 % skimmed milk for 1 h and then washed with TBST for 10 min 3 times. The PVDF membrane was incubated with primary antibodies: rabbit polyclonal to eNOS (1:250), rabbit polyclonal to p-eNOS (1:250), rabbit polyclonal to Akt (1:500) and rabbit polyclonal to p-Akt (1:1000) at 4ºC overnight and then washed with TBST for 10 min 3 times. The PVDF membrane was incubated with mouse monoclonal to ß-actin (1:3000) and goat polyclonal to secondary antibody to rabbit IgG (1:3000) at room temperature for 1 h. The protein bands were detected by incubation of enhanced chemiluminescent on PVDF membrane. The intensity of protein band was analyzed using ImageJ 1.41 software and compared with untreated cells.

Statistical analysis

All experiments were performed at least 3 times, each performed in triplicate. The results were presented in mean ± standard error of mean. The data was statistically analyzed using SPSS 15.0 software (LEAD Technologies, Inc., Chicago, IL, USA) with one-way analysis of variance (ANOVA) at $p < 0.05$.

CHAPTER IV
RESULTS

Effect of herbal extracts in Phikud Benjakot on viability of ECV304 cells

To define the nontoxic concentration of herbal extracts in Phikud Benjakot, cytotoxicity was performed in ECV304 cells using MTT reduction assay. Treatment of the cells with the extracts for 24 h, the percentage of cell viability decreased in a concentration-depedent manner (Figure 6). Among these extracts, AV (AVES, AVWS) at the concentration of 2 mg/mL exhibited the highest cytotoxicity while AD (ADES, ADWS) and LS (LSES, LSWS) at the concentration of 5 mg/mL exhibited significant cytoxicity. The extracts of AL (ALES, ALWF) and AS (ASES, ASWS) at the concentration of 5 mg/mL did not exhibit significant cytotoxicity, showing the percentage of cell viability at 110.9 ± 19.0, 91.2 ± 16.8, 82.2 ± 3.3 and 84.5 ± 4.0%, respectively (Figure 6). Treatment of ADES, ADWS, LSES, LSWS, AVES and AVWS exhibited IC_{50} values of 3.0 ± 0.3, 4.9 ± 0.6, 3.3 ± 0.5, 3.1 ± 0.4, 0.8 ± 0.1 and 0.9 ± 0.1 mg/mL, respectively, while ALES, ALWF, ASES and ASWS showed IC_{50} values higher than 5 mg/mL (Figure 7).

Effect of the extracts on genotoxicity in ECV304 cells

The IC_{10}, IC_{25} and IC_{50} values of the extracts (Figure 7) were chosen to evaluate genotoxicity using the alkaline comet assay. After SYBR green stained DNA, the images were visualized and analyzed using image analysis CometScore 1.5 software as shown in DNA in tail (%), calculated from the intensity of fragmented DNA in comet tail as compared to total DNA, which can reflect the amount of fragmented DNA. For method validation, the cells were treated with 100 µM H_2O_2, used as a positive control, for 30 min to induce DNA damage. The result showed that fragmented DNA migration significantly increased the percentage of DNA in tail (42.6%) when compare to untreated cells (12.5%) (Figure 8). To avoid false positive results, genotoxicity assessment of AD, LS and AV was studied at the cytotoxic threshold IC_{10}, IC_{25} and IC_{50} (Figure 7). ADES (1, 2 and 3 mg/mL) and ADWS (1.5, 3 and 5 mg/mL) expressed no significant DNA migration, showing less than 20% DNA in tail at all tested concentrations (Figure 9). LSES (1.5, 2 and 3.5 mg/mL) and LSWS

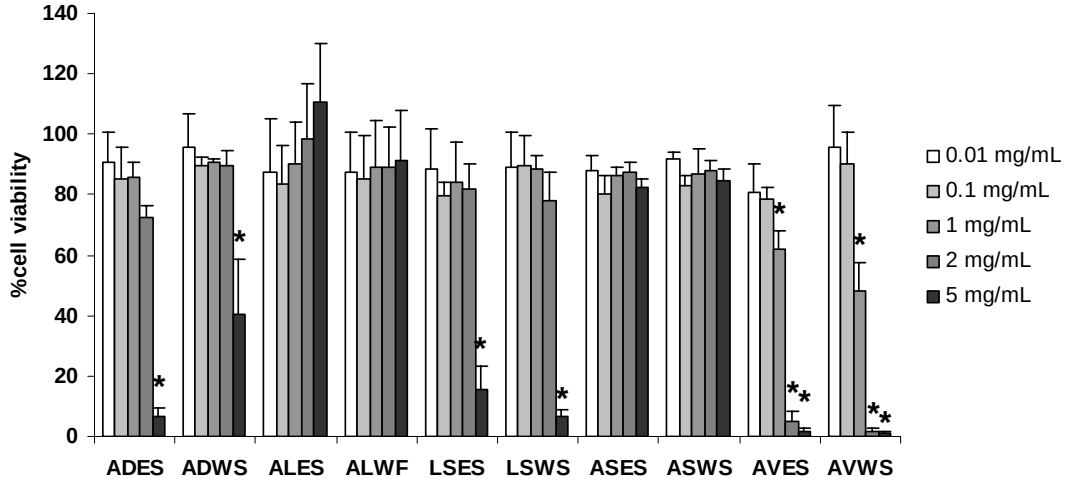


Figure 6 Cytotoxicity of the extracts from Phikud Benjakot on ECV304 cells using MTT reduction assay. The cells were treated with the extract at the concentrations of 0.01, 0.1, 1, 2 and 5 mg/mL for 24 h. The results were expressed as percentage of control observed. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

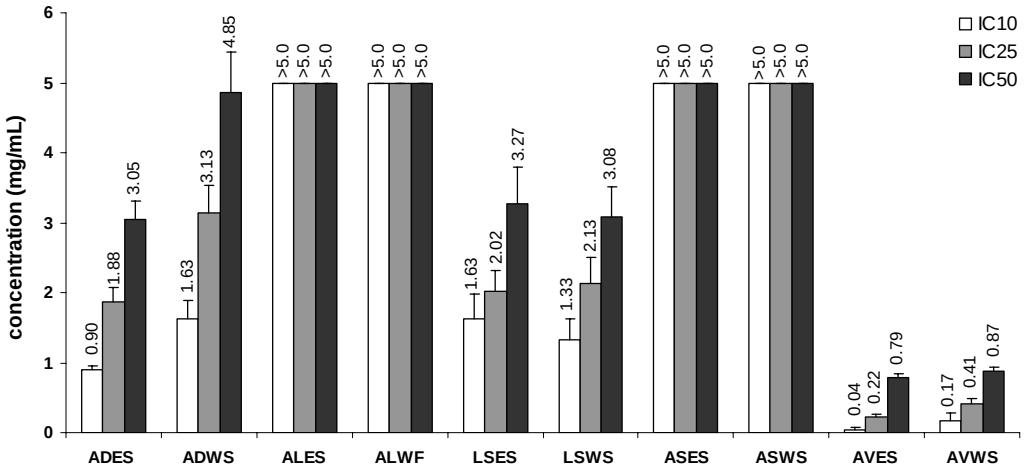


Figure 7 The mean of IC_{10}, IC_{25} and IC_{50} values resulted from cytotoxic testing of the extracts on ECV304 cells. The cells were treated with the extract at concentration of 0.01, 0.1, 1, 2 and 5 mg/mL for 24 h. Cell viability was determined using MTT reduction assay. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

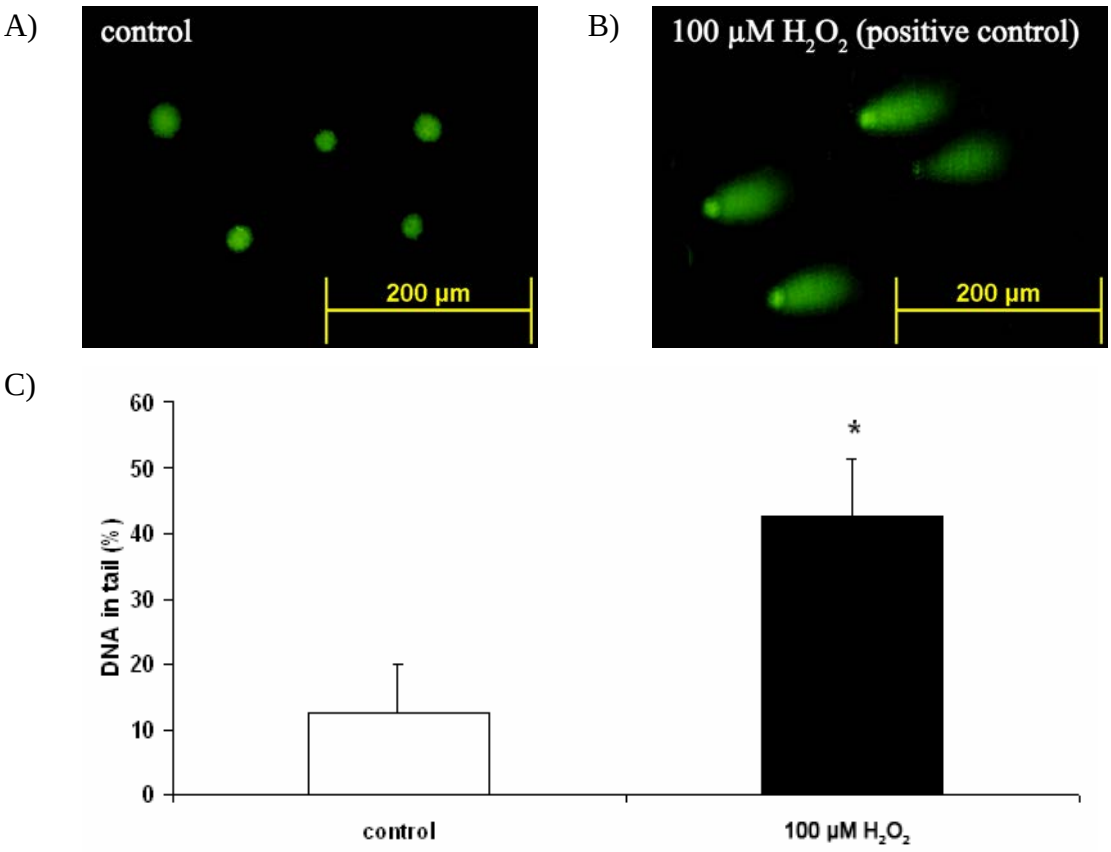


Figure 8 Genotoxic effect of H_2O_2 on ECV304 cells detected by an alkaline comet assay. Representative comet images of cells exposed to (A) untreated as compared to (B) H_2O_2 (100 µM, a positive control) for 0.5 h. DNA was stained with SYBR Green; 10x magnification. Scale bar = 200 µm. (C) % DNA in tail was presented as the mean ± S.E.M. of three different experiments. *$p < 0.05$ compared to the untreated control.

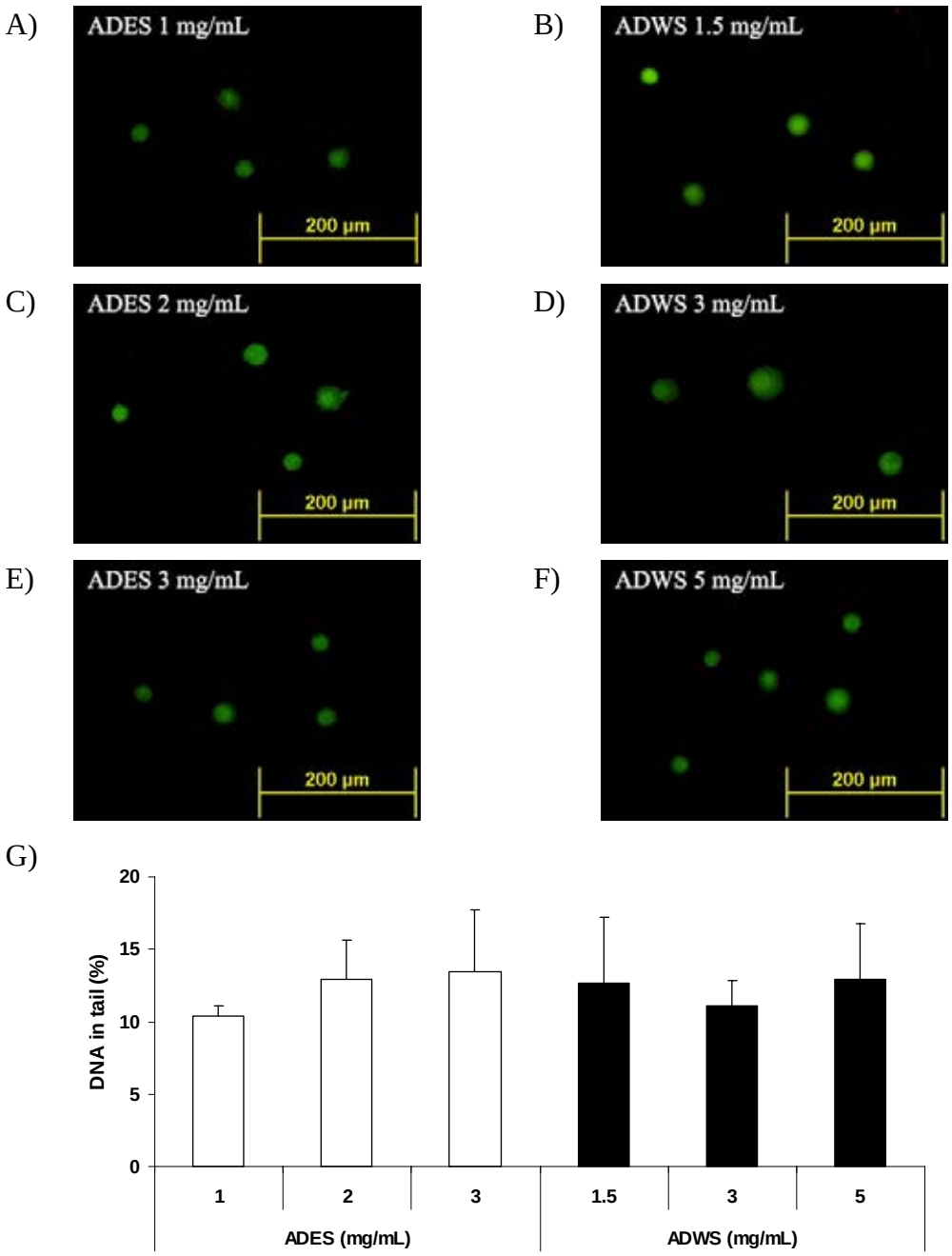


Figure 9 Genotoxic effect of ADES and ADWS on ECV304 cells detected by an alkaline comet assay. Representative comet images of cells exposed to (A) ADES and (B) ADWS at the IC_{10}, (C) ADES and (D) ADWS at the IC_{25}, (E) ADES and (F) ADWS at the IC_{50} for 24 h. DNA was stained with SYBR Green; 10x magnification. Scale bar = 200 µm. (G) % DNA in tail was presented as the mean ± S.E.M. of three different experiments.

(1.5, 2 and 3 mg/mL) induced DNA damage less than 15% DNA in tail but not significant difference from untreated control (Figure 11). AVES (0.05, 0.2 and 0.8 mg/mL) and AVWS (0.25, 0.5 and 1 mg/mL) slightly increased DNA in tail (%) from untreated control (Figure 13). Although AVWS showed the highest values in the percentage of DNA in tail but not significant difference from control groups. For those extracts exhibiting IC_{50} > 5 mg/mL, the treated concentrations of 0.1, 1 and 5 mg/mL were selected for the genotoxicity testing. ALES, ALWF, ASES and ASWS expressed no significant DNA damage, observing from lower percentage of DNA in tail than the untreated control (Figures 10 and 12). All herbal extracts in Phikud Benjakot at the tested concentrations (IC_{10}, IC_{25} and IC_{50}) did not significantly exhibit genotoxicity in comet assay model. Therefore, the nontoxic concentrations of all extracts were further chosen for inhibitory study of human platelet aggregation induced by ADP, arachidonic acid and collagen.

Human platelet aggregation

ADP-induced platelet aggregation

First, ADP at the concentration of 2.5 µM was added to PRP to stimulate platelet aggregation. Aggregation tracing showed platelet shape change at the initial phase after adding ADP resulting in decreased light transmittance followed by aggregation wave which reach maximal aggregation at 85% (Figure 14). Meanwhile, ADP at the concentration of 2.5 µM induced only 41% of maximal aggregation in PRP obtained from another volunteer (Figure 15). Therefore, a concentration-dependent study of ADP was performed to select the appropiate concentration of ADP to activate platelet in each experiment.

ADP concentration-dependently induced platelet aggregation, showing an in increase in maximal aggregation and different pattern of aggregation trace (Figure 15A). Noticeably, ADP at lower concentrations of 1 and 2.5 µM stimulated only a primary wave of aggregation resulting in low percentage of maximal aggregation approximately 40 - 50% (Figure 15B). ADP at higher concentrations of 5 and 10 µM induced higher percentage of maximal aggregation approximately 80% - 90%, resulting from primary and secondary waves of aggregation. ADP at the threshold

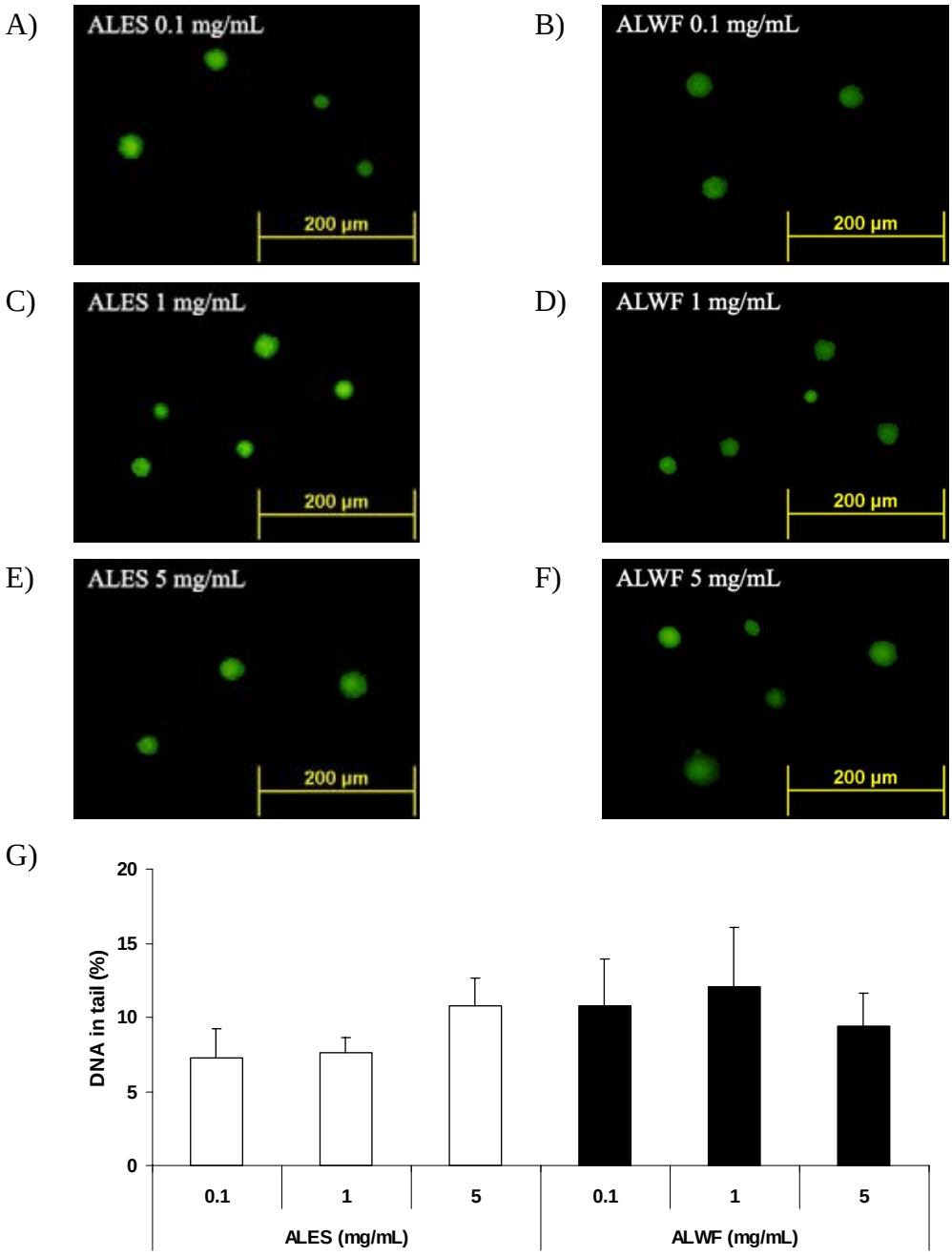


Figure 10 Genotoxic effect of ALES and ALWF on ECV304 cells detected by an alkaline comet assay. Representative comet images of cells exposed to (A) ALES and (B) ALWF at the IC_{10}, (C) ALES and (D) ALWF at the IC_{25}, (E) ALES and (F) ALWF at the IC_{50} for 24 h. DNA was stained with SYBR Green; 10x magnification. Scale bar = 200 µm. (G) % DNA in tail was presented as the mean ± S.E.M. of three different experiments.

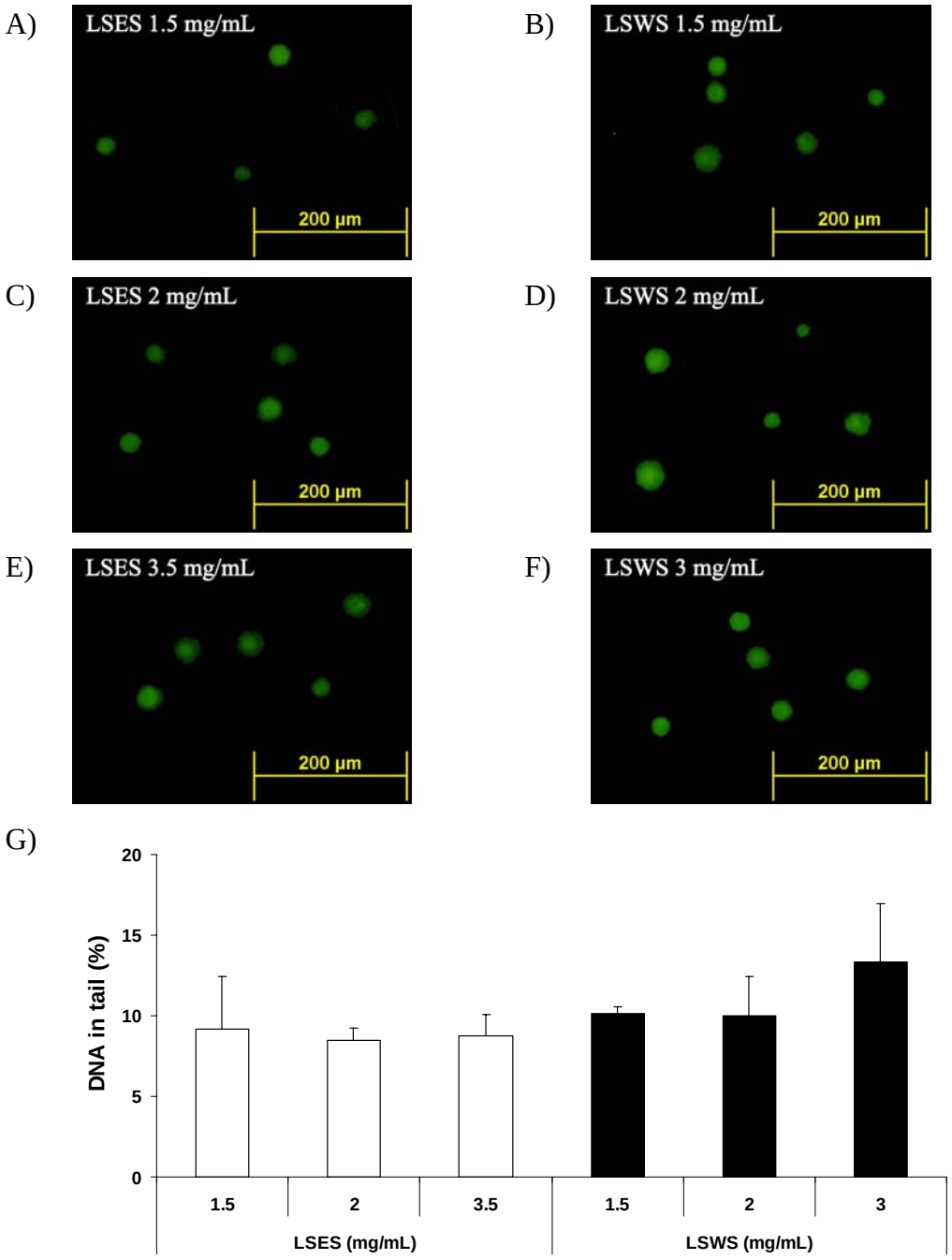


Figure 11 Genotoxic effect of LSES and LSWS on ECV304 cells detected by an alkaline comet assay. Representative comet images of cells exposed to (A) LSES and (B) LSWS at the IC_{10}, (C) LSES and (D) LSWS at the IC_{25}, (E) LSES and (F) LSWS at the IC_{50} for 24 h. DNA was stained with SYBR Green; 10x magnification. Scale bar = 200 µm. (G) % DNA in tail was presented as the mean ± S.E.M. of three different experiments.

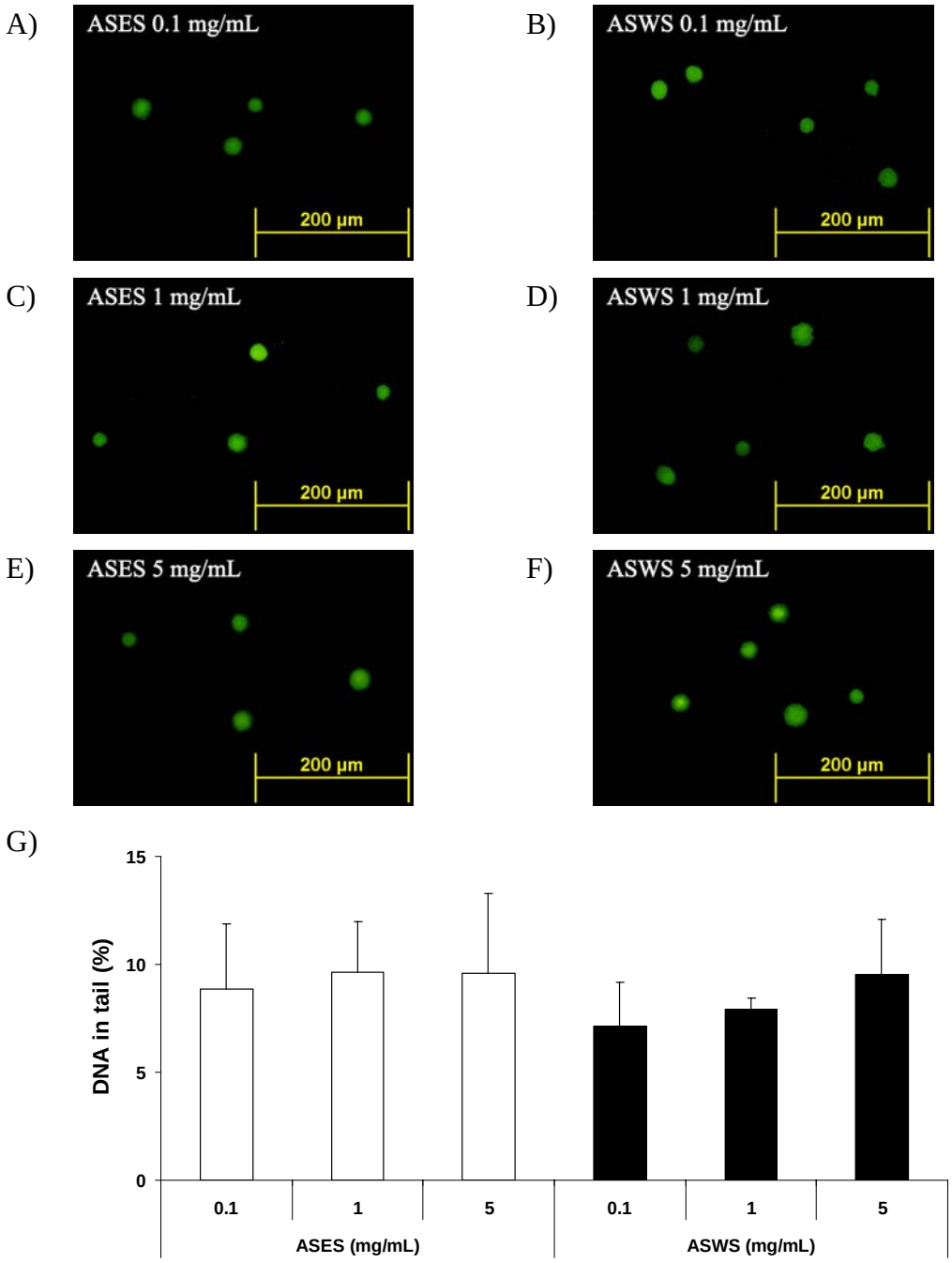


Figure 12 Genotoxic effect of ASES and ASWS on ECV304 cells detected by an alkaline comet assay. Representative comet images of cells exposed to (A) ASES and (B) ASWS at the IC_{10}, (C) ASES and (D) ASWS at the IC_{25}, (E) ASES and (F) ASWS at the IC_{50} for 24 h. DNA was stained with SYBR Green; 10x magnification. Scale bar = 200 µm. (G) % DNA in tail was presented as the mean ± S.E.M. of three different experiments.

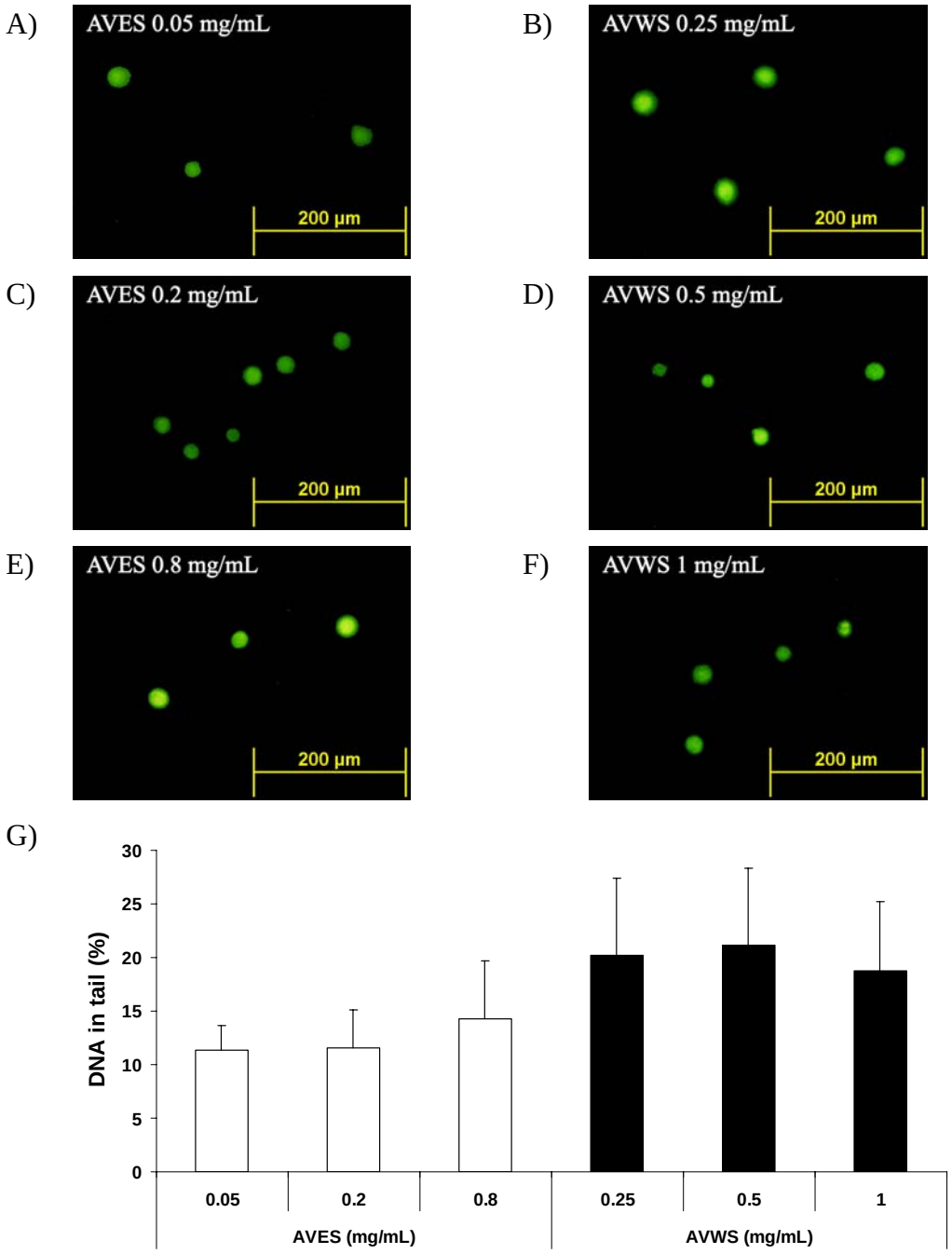


Figure 13 Genotoxic effect of AVES and AVWS on ECV304 cells detected by an alkaline comet assay. Representative comet images of cells exposed to (A) AVES and (B) AVWS at the IC_{10}, (C) AVES and (D) AVWS at the IC_{25}, (E) AVES and (F) AVWS at the IC_{50} for 24 h. DNA was stained with SYBR Green; 10x magnification. Scale bar = 200 µm. (G) % DNA in tail was presented as the mean ± S.E.M. of three different experiments.

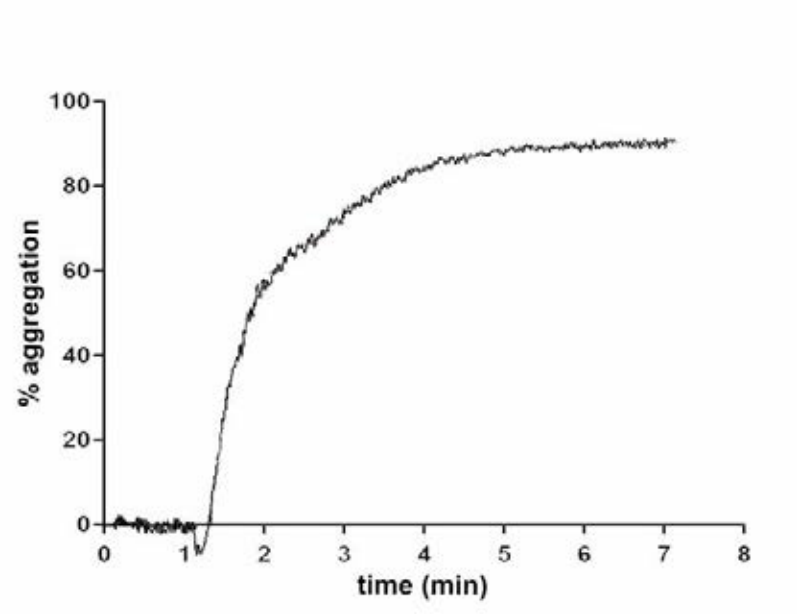
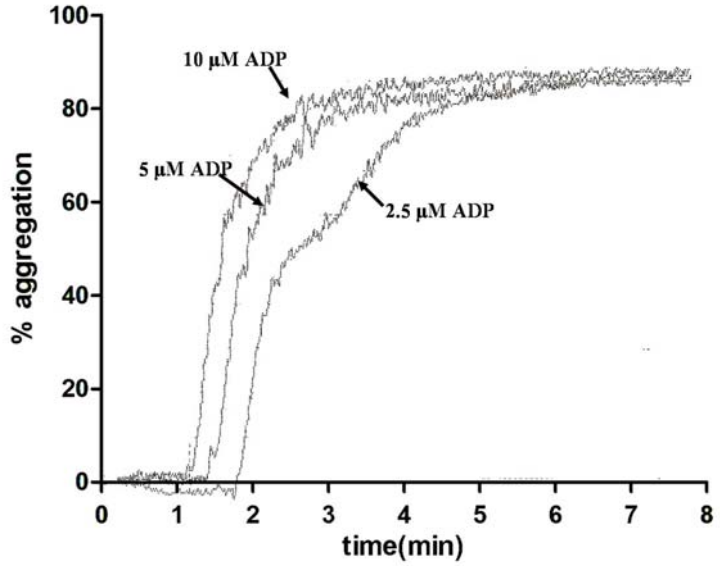


Figure 14 ADP-induced platelet aggregation. PRP was induced by ADP at the concentration of 2.5 μM and monitered platelet aggregation for 6 min. Result was represented as aggregation trace.

A)



B)

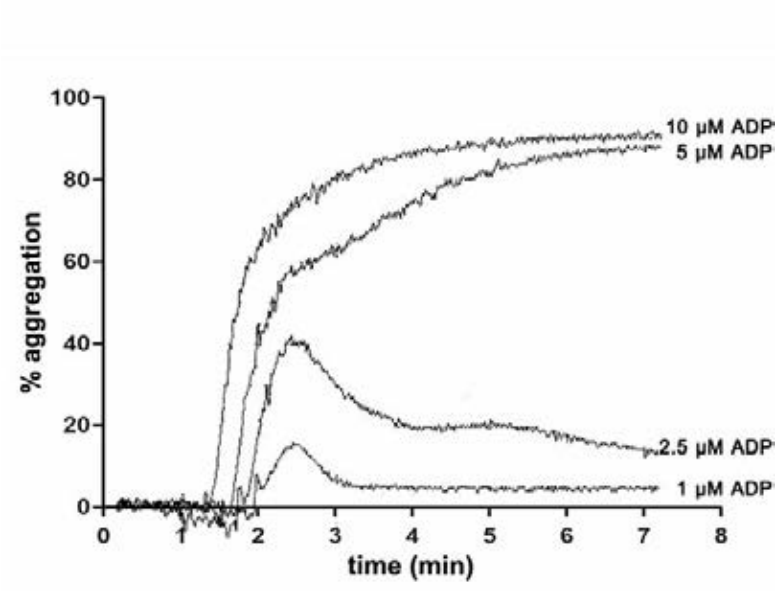


Figure 15 Concentration-dependent study of ADP-induced platelet aggregation. PRP was induced by ADP at the concentrations of 1, 2.5, 5 and 10 µM and monitered platelet aggregation for 6 min in individual blood sample. Result was represented as aggregation trace.

concentrations of 2.5 µM (Figure 15A) and 5 µM (Figure 15B) separately produced primary and secondary wave of aggregation while ADP at higher concentrations of 5, 10 µM (Figure 15A) and 10 µM (Figure 15B) produced a single combined primary and secondary wave of aggregation.

Arachidonic acid-induced platelet aggregation

Arachidonic acid at the concentration of 500 µM was added to PRP to stimulate platelet aggregation. Adding arachidonic acid induced a shape of platelet change from the initial stabilization of the baseline. Arachidonic acid exhibited only a single response wave but not a biphasic change like ADP. Arachidonic acid the concentration of 500 µM induced approximately 80% - 90% of maximal aggregation (Figure 16)**.**

Collagen induced-platelet aggregation

Collagen at the concentration of 3 µg/mL was added to PRP to stimulate platelet aggregation. Collagen did not induced a shape change at the initial after adding like ADP and arachidonic acid but showed longer lag phase (almost 2 min) from the instillation of the agonist to shape change followed by a single response wave in the aggregation trace (Figure 17). Collagen induced approximately 80% - 90% of maximal aggregation**.**

Effect of aspirin on ADP-, arachidonic acid- and collagen-induced platelet aggregation

ASA, a positive control, at the concentration of 500 µM could inhibit platelet agggregation resulting in decreasing percentage of aggregation in all three agonists with different pattern in aggregation trace. ASA inhibited secondary wave of aggregation approximately 30 % when induced by 5 µM ADP (Figure 18A) while completely inhibit aggregation induced 500 µM arachidonic acid (Figure 18B). Treatment of platelet with ASA not only decreased maximal aggregation approximately 70% induced by collagen but also increased lag time with decreasing slope (Figure 18C). The result confirmed that mechanism of action of ASA, a well-known antiplatelet drug, was mediated not only primarily through platelet COX-1 inhibition leading to TXA_2 depletion but also partially through ADP antagonist and collagen signaling pathway.

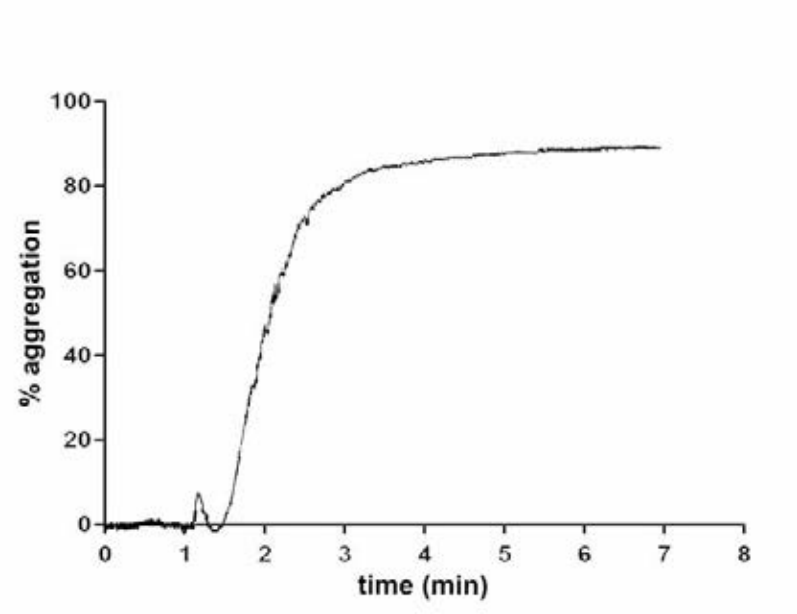


Figure 16 Arachidonic acid-induced platelet aggregation. PRP was induced platelet aggregation by arachidonic acid at the concentration of 500 µM and monitored for 6 min. Result was represented as aggregation trace.

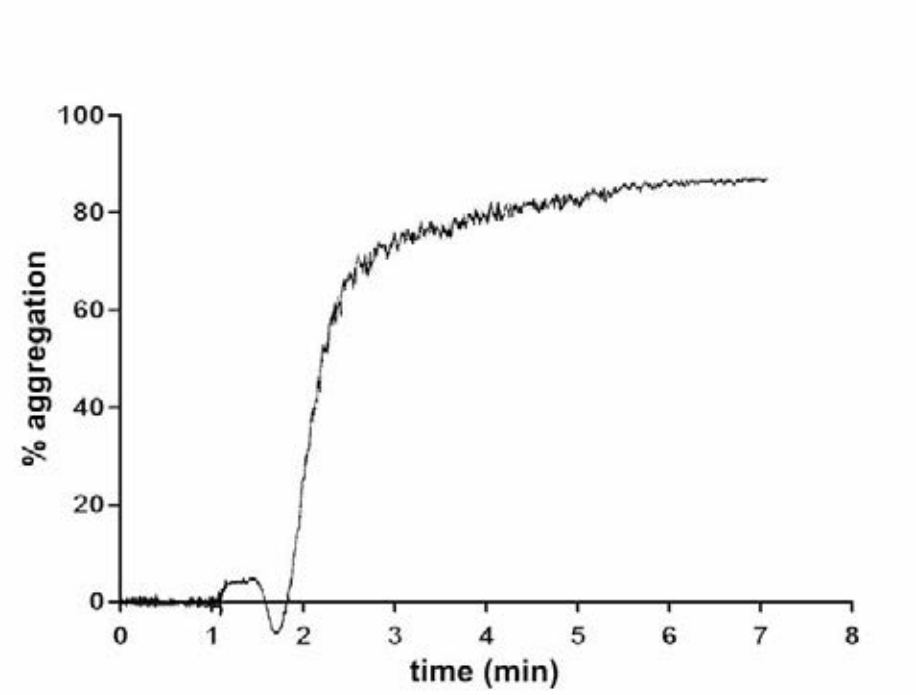
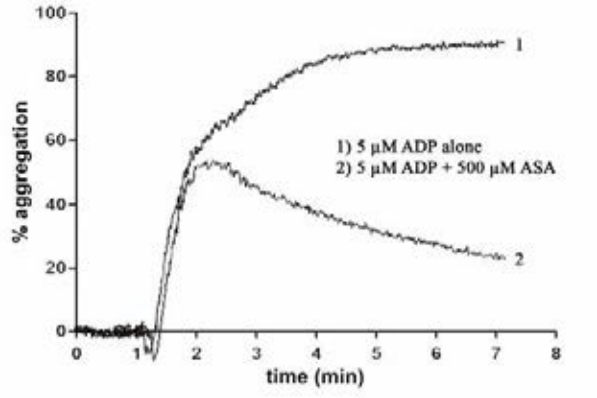
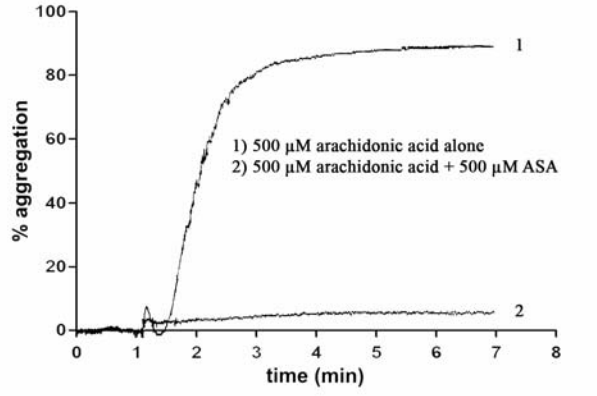


Figure 17 Collagen induced-platelet aggregation. PRP was induced platelet aggregation by collagen at the concentration of 3 µg/mL and monitered for 6 min. Result was represented as aggregation trace.

A)



B)



C)

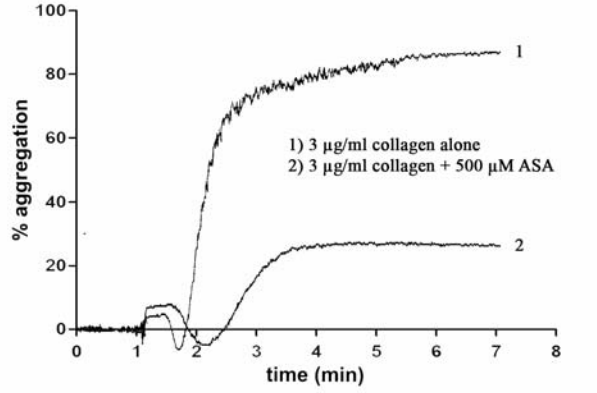


Figure 18 Effect of ASA on platelet aggregation. PRP were preincubated with 500 µM ASA for 5 min before adding A) 5 µM ADP B) 500 µM arachidonic acid and C) 3 µg/mL collagen. The results were represented as aggregation trace.

Effect of the extracts on platelet aggregation induced by ADP, arachidonic acid and collagen

After the selection of the appropiate concentration of ADP, arachidonic acid and collagen for induction of platelet aggregation, antiplatelet effect of the extracts induced by these agonists was performed. Antiplatelet effect of ALES, ALWS, LSES and LSWS at the concentration of 1 mg/mL were slightly different from control (Figure 19C and 19D) while ADES, ADWS, ASES, ASWS, AVES and AVWS showed obviously different from ADP alone (Figure 19B, 19E, 19F). The maximal aggregation was significantly inhibited by AD (ADES, ADWS), AS (ASES, ASWS) and AV (AVES, AVWS) (Figure 20A). The percentage of inhibition was in descending order of the antiplatelet activity of AV > AD > AS (Figure 20B).

Since AV exhibited the highest percentage of antiplatelet induced by ADP, the inhibitory effect of platelet aggregation induced by arachidonic acid and collagen were therefore investigated. The result showed that both AVES and AVWS at the concentration of 1 mg/mL showed no inhibitory effect on platelet aggregation when induced by 500 μM arachidonic acid (Figure 21) and 3 μg/mL collagen (Figure 22). Besides the percentage of maximal aggregation, platelet aggregation induced by collagen can be studied by the observation of the slope of aggregation trace which reflects the rate of aggregation. The slope of aggregation trace of ASA was significantly different from the control (Figures 18C, 22C), revealing that 500 μM ASA could slow down the rate of aggregation induced by collagen. However, AV showed the same pattern of both the percentage of maximal aggregation when induced by collagen (Figure 22B) and the slope of aggregation curve (Figure 22C) as compared to control. Thus, the antiplatelet activity of AVES and AVWS seemed to play a role in the ADP signaling pathway.

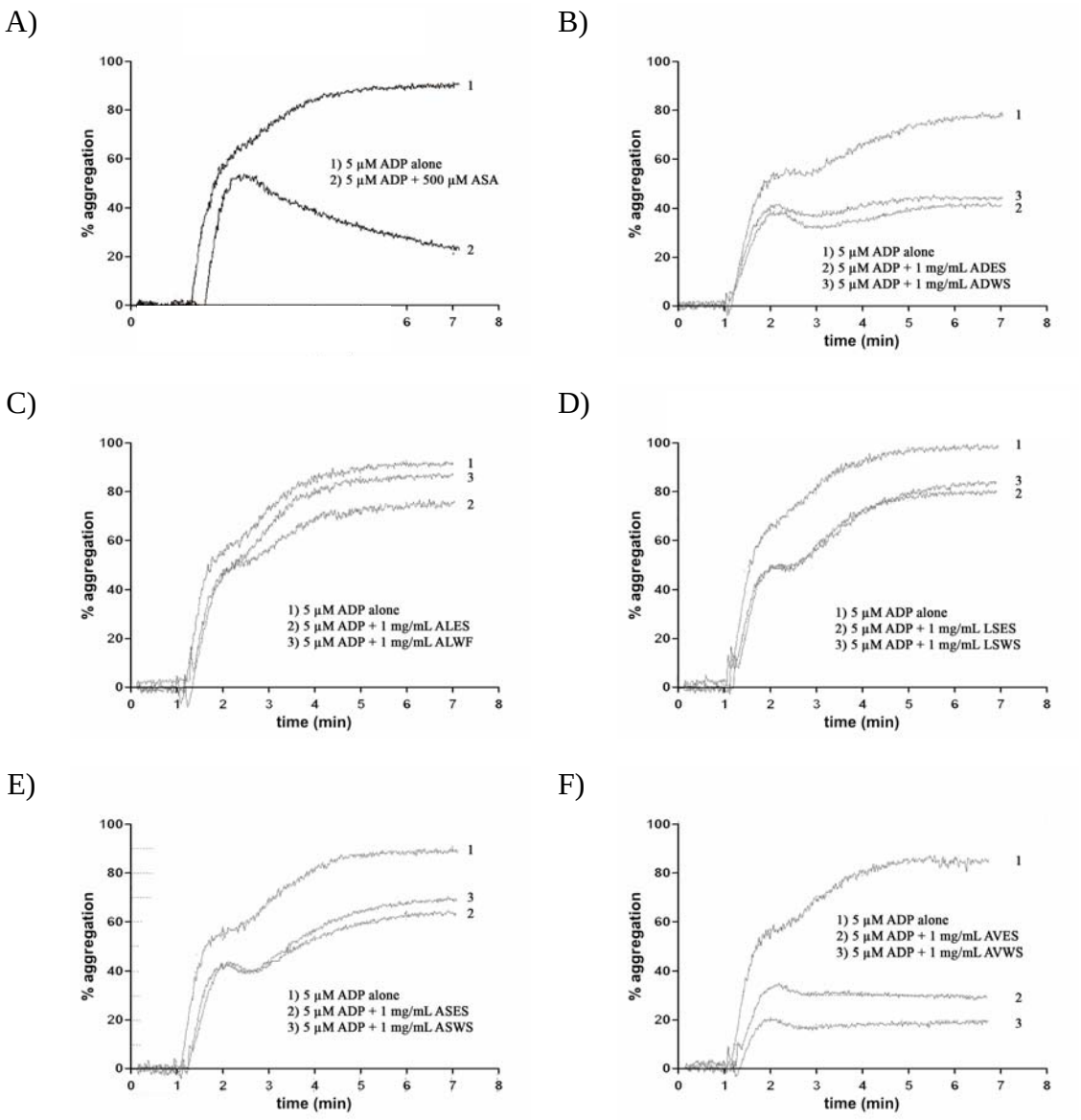


Figure 19 Effect of the extracts from Phikud Benjakot on platelet aggregation induced by ADP. PRP were preincubated with A) 500 µM ASA as a positive control, B) AD (ADES, ADWS), C) AL (ALES, ALWF), D) LS (LSES, LSWS), E) AS (ASES, ASWS) and F) AV (AVES, AVWS) at the concentration of 1 mg/mL for 5 min before adding 5 µM ADP. The results were represented as aggregation trace.

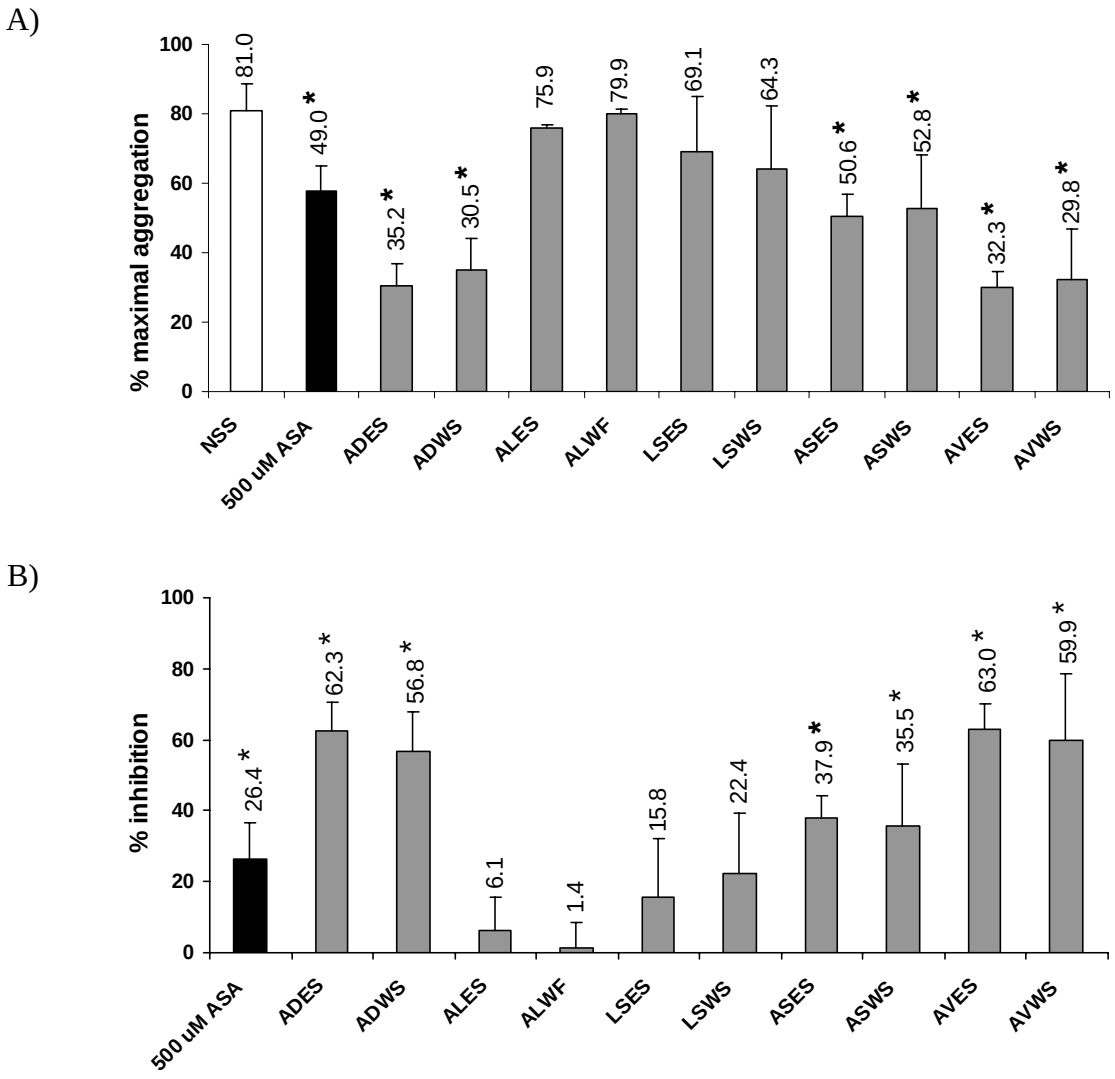
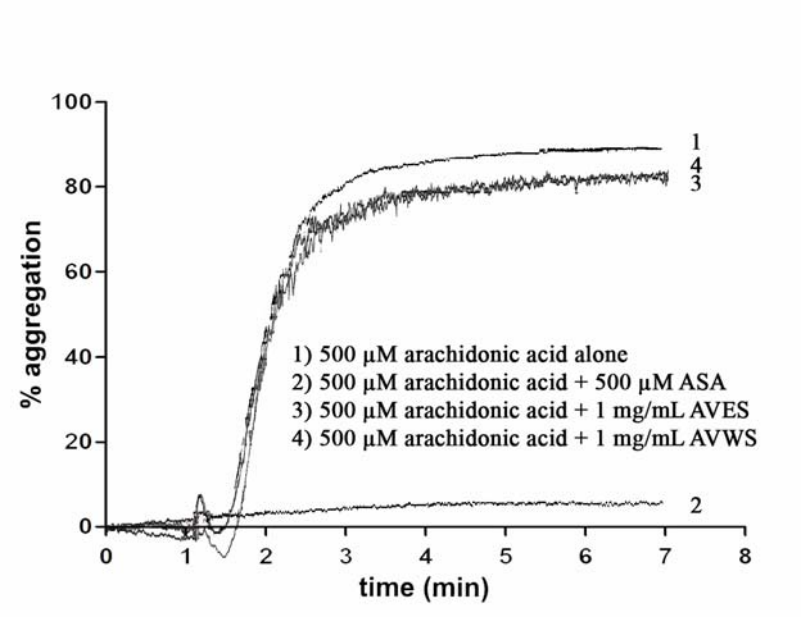


Figure 20 Effect of the extracts from Phikud Benjakot on platelet aggregation induced by ADP. PRP were preincubated with the extracts for 5 min before induced by ADP. The results were represented in A) % maximal aggregation and B) % inhibition of platelet aggregation. Data were expressed as mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

A)



B)

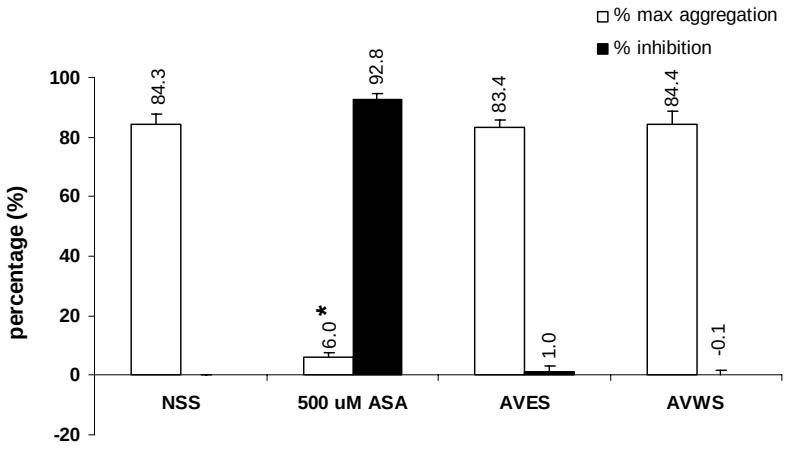
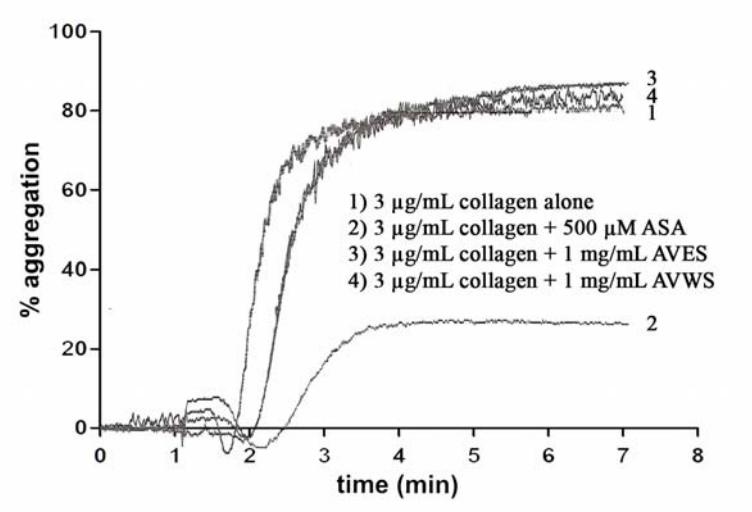


Figure 21 Effect of AV on platelet aggregation induced by arachidonic acid. PRP were preincubated with AVES and AVWS at the concentration of 1 mg/mL for 5 min before adding 500 µM arachidonic acid. The results were represented in A) aggregation trace and B) % maximal aggregation as well as % inhibition of platelet aggregation. Data were expressed as mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

A)



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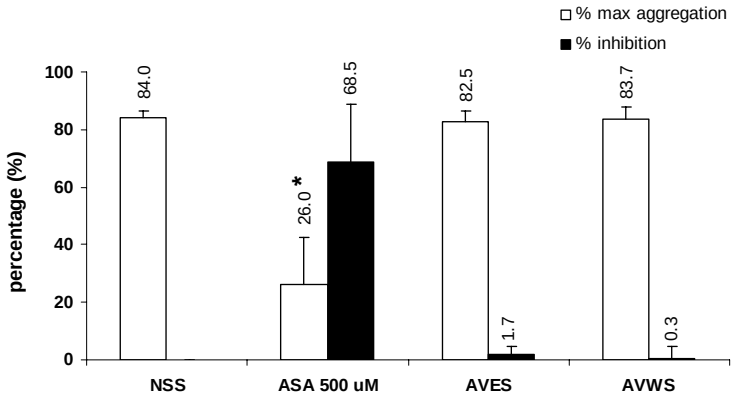


C)

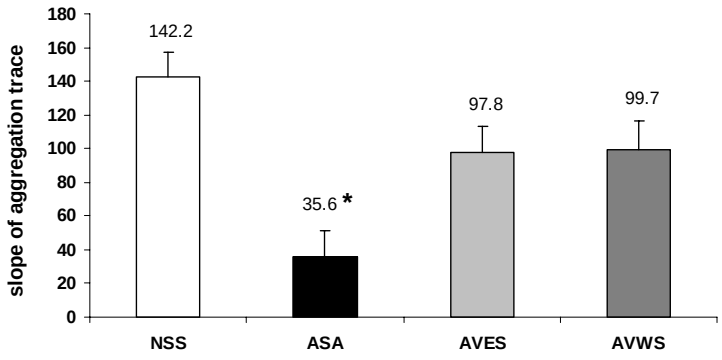


Figure 22 Effect of AV on platelet aggregation induced by collagen. PRP were preincubated with AVES and AVWS at the concentration of 1 mg/mL for 5 min before adding 3 µg/mL collagen. The results were represented in A) aggregation trace B) % maximal aggregation and % inhibition of platelet aggregation and C) slope. Data were expressed as mean ± S.E.M. (N =3, each performed in triplicate). * p < 0.05 compared to the untreated control.

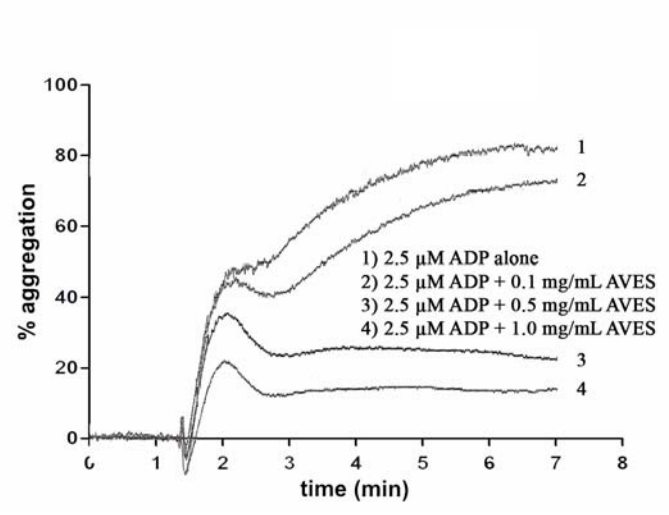
Concentration-dependent study of AV on antiplatelet activity induced by ADP

The above result demonstrated that AVES and AVWS at the concentration of 1 mg/mL exhibited antiplatelet activity induced by ADP but not arachidonic acid or collagen. Concentration-dependent study of AV on platelet aggregation induced by ADP was therefore investigated. The aggregation trace showed that AVES (Figure 23A) and AVWS (Figure 23B) at the concentrations of 0.5 and 1 mg/mL obviously decreased ADP-induced platelet aggregation in both primary and secondary phase of aggregation as compared to untreated control. Both AVES and AVWS significantly decreased the percentage of maximal aggregation as compared to untreated control (Figure 24A), exhibiting the IC_{50} values of 0.43 and 0.51 mg/mL, respectively. Noticeably, AVES (0.5 mg/mL) was slightly more effective than ASA (500 μM or 0.09 mg/mL) and AVWS (0.5 mg/mL) (Figure 24B).

Effect of AV on platelet viability using MTT reduction assay

To confirm that antiplatelet activity of the extracts was not due to the interference caused by the toxicity, platelet viability was performed using MTT reduction assay. Treatment of platelet with AVES or AVWS for 1 h decreased viability and became significantly toxic to platelet at the concentration of 2 mg/mL, exhibiting the IC_{50} values greater than 2 mg/mL (Figure 25A). H_2O_2, used as a positive control, significantly decreased platelet viability, showing an IC_{50} value of 70.17 mM (Figure 25B). However, AVES and AVWS at the antiplatelet concentration of 0.5 and 1 mg/mL (Figure 24) exhibited no significant toxicity to platelet, confirming that the antiplatelet activity of AVES and AVWS was not due to platelet damage.

A)



B)

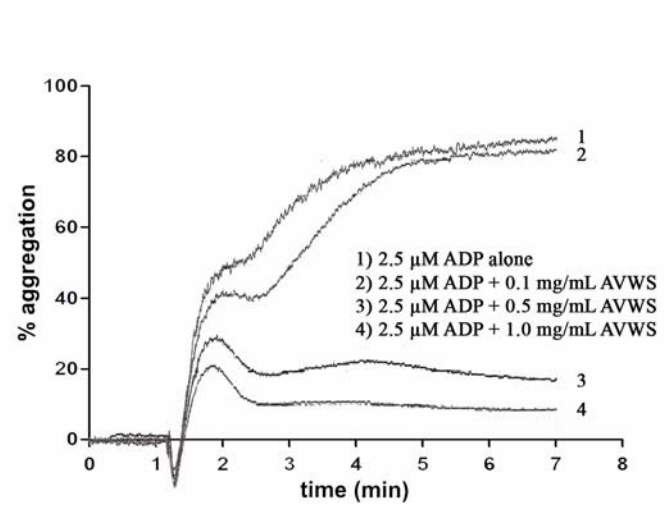
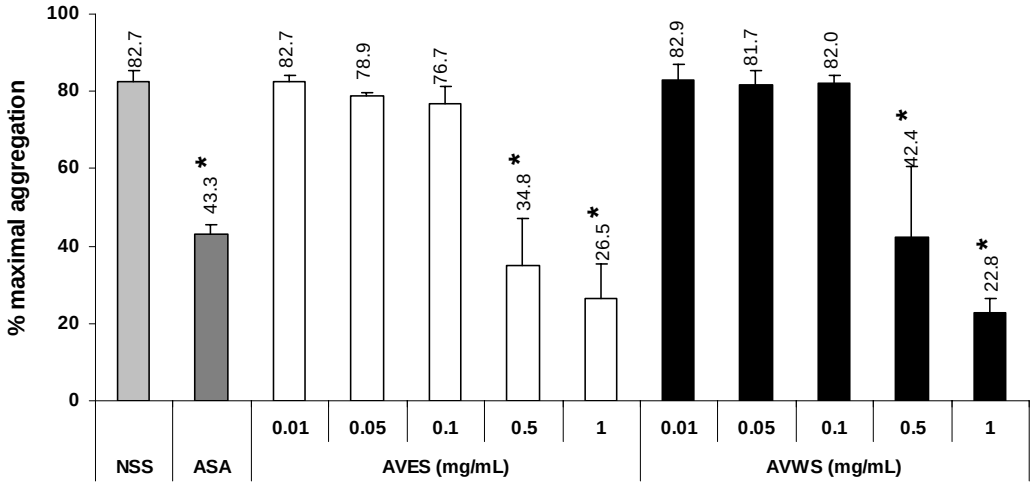


Figure 23 Concentration-dependent study of AV on platelet aggregation in response to ADP. PRP were preincubated with A) AVES and B) AVWS at various concentrations of 0.1, 0.5 and 1 mg/mL for 5 min before adding 2.5 µM ADP. The results were represented as aggregation trace.

A)



B)

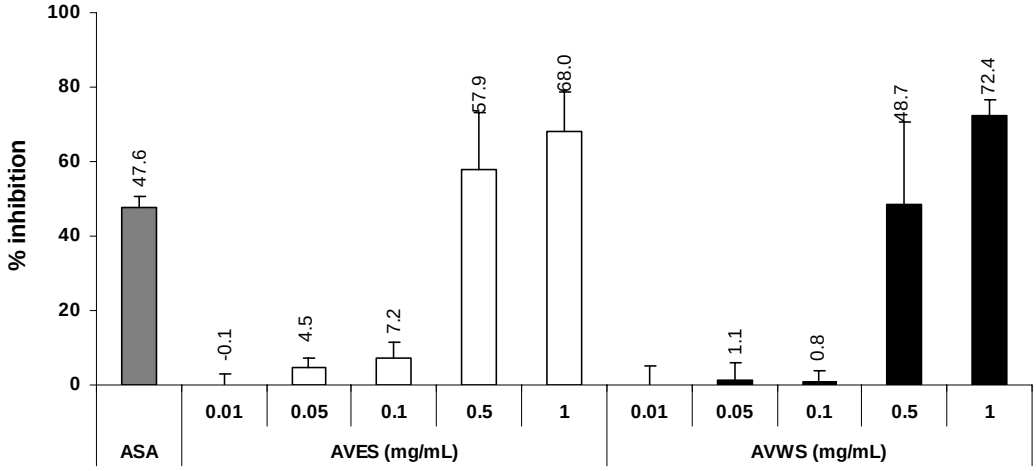
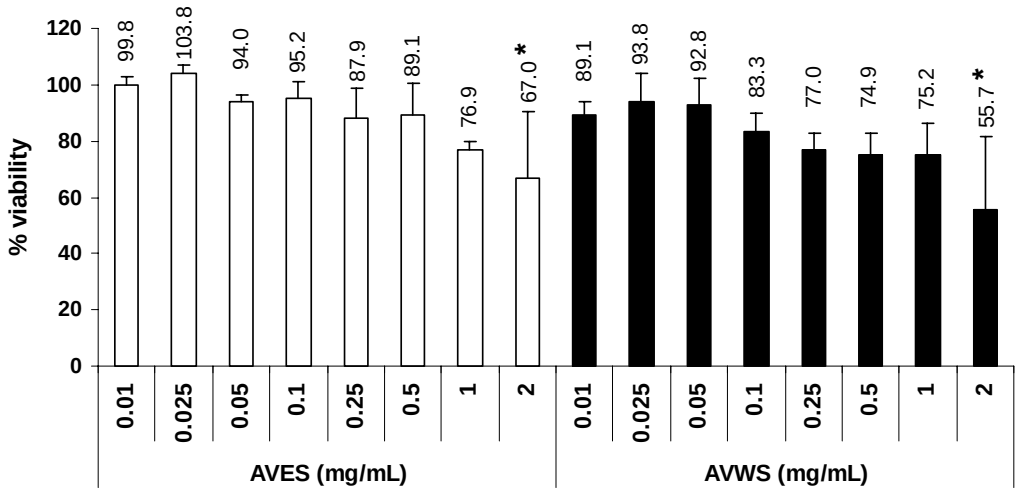


Figure 24 Effect of AV on platelet aggregation induced by ADP. PRP were preincubated with AVES and AVWS at the concentrations of 0.01, 0.05, 0.1, 0.5 and 1 mg/mL for 5 min before adding ADP. The results were represented in A) % maximal aggregation and B) % inhibition of platelet aggregation. Data were expressed as mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

A)



B)

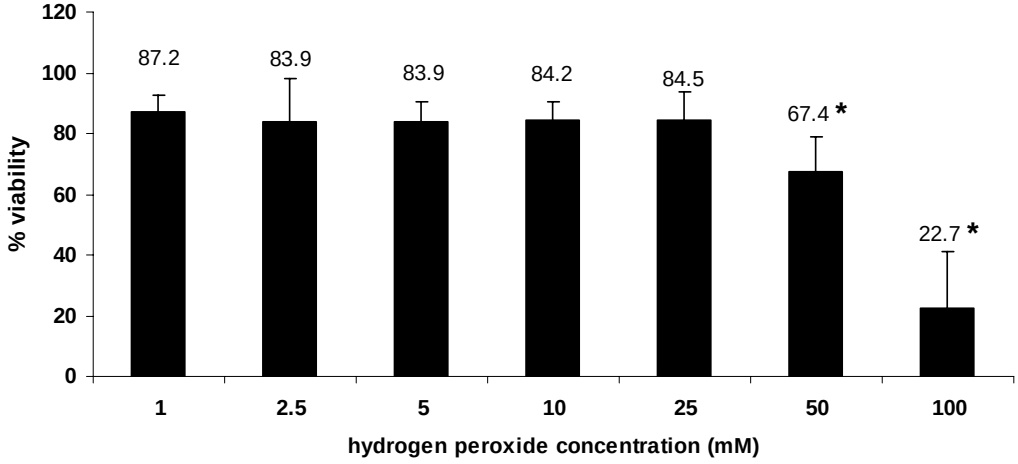


Figure 25 Effect of AV on platelet viability using MTT reduction assay. A) Platelets were treated with AVES and AVWS at the concentrations ranging from 0.01 to 2 mg/mL for 1 h. B) H_2O_2 was used as a positive control. The results were expressed as the percentage of viability as compared to control observed by MTT reduction assay. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate) * $p < 0.05$ compared to the untreated control.

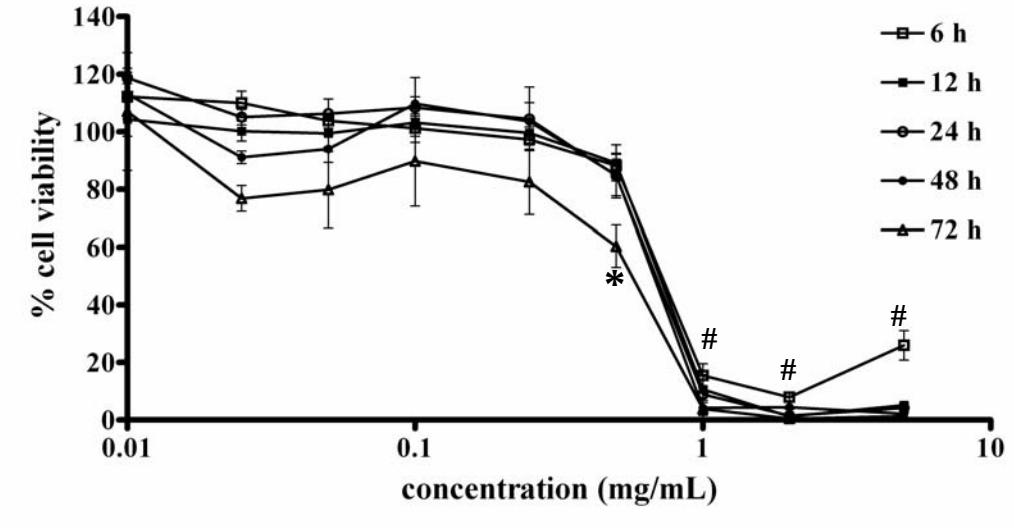
Effect of AV on viability of ECV304 cells

Although platelet viability using MTT reduction assay revealed that AVES and AVWS at the antiplatelet concentration were not toxic to platelet, the effect of AV on viability of ECV304 cells was also studied. Treatment of ECV304 cells with AVES and AVWS obviously decreased viability in a concentration-dependent manner at all exposure time of 6, 12, 24, 48 and 72 h. AVES at the antiplatelet concentration of 0.5 mg/mL for 6, 12, 24, 48 and 72 h showed the percentage of viability of 88.2, 89.1, 84.7, 84.9 and 60.3 (Figure 26A) while AVWS exhibited the percentage of viability of 106.6, 100.9, 101.6, 96.0 and 66.7, respectively (Figure 26B). Exposure of the cells to AVES and AVWS for 72 h became significantly toxic. Additionally, AVES and AVWS at the antiplatelet concentration of 0.5 mg/mL for less than 48 h incubation did not affect viability of ECV304 cells.

Effect of AV on platelet function using acid phosphatase assay

Platelet function was determined based on acid phosphatase activity, which is stable independently of platelet stimulation and is not released from vital platelet, leading to be able to quantify number of viable platelet (Ballabeni *et al*., 2007). To avoid interference of the extract on platelet function, the effect of AV on acid phosphatase activity was determined. Calibration graph demonstrated that acid phosphatase activity was directly proportional to the number of platelet (Figure 27). An appropriate number of platelets were then selected for the determination of acid phosphatase activitiy in platelets treated with the extract. H_2O_2 was used as a positive control. Platelets treated with AVES or AVWS at the concentrations ranging from 0.01 to 2 mg/mL for 1 h showed no significant difference from untreated control (Figure 28A). Meanwhile, treatment of platelets with H_2O_2 at the concentrations of 1, 10 and 100 mM for 1 h significantly decreased acid phosphatase activity as compared to untreated control, exhibiting an IC_{50} value of 3.63 mM (Figure 28B) revealing that H_2O_2 interfered platelet function at 19-fold lower concentration than H_2O_2-induced toxicity (IC_{50} 70.17 mM, Figure 25B). The result revealed that both AVES and AVWS at the antiplatelet concentration did not directly affect platelet function via acid phosphatase activity. The effect of AV on platelet adhesion was further performed based on acid phosphatase assay.

A)



B)

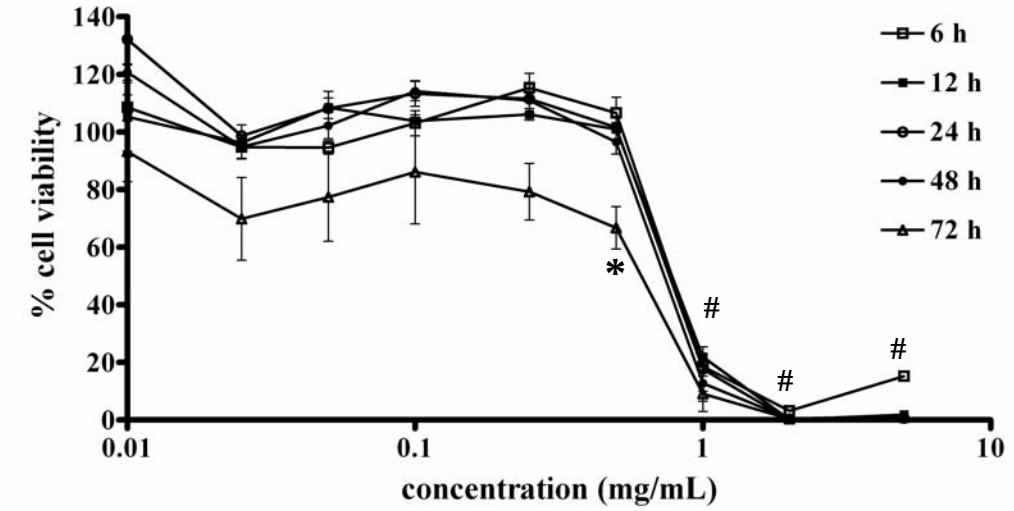


Figure 26 Concentration- and time-dependent studies of AVES and AVWS on viability of ECV304 cells. Cells were treated with A) AVES and B) AVWS at the concentrations ranging from 0.01 to 5 mg/mL for 6, 12, 24, 48 and 72 h. Cell viability was measured by MTT reduction assay. The results were expressed as the percentage of control observed by MTT reduction assay. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control at 72 h. # $p < 0.05$ compared to the untreated control at 6, 12, 24, 48 and 72 h.

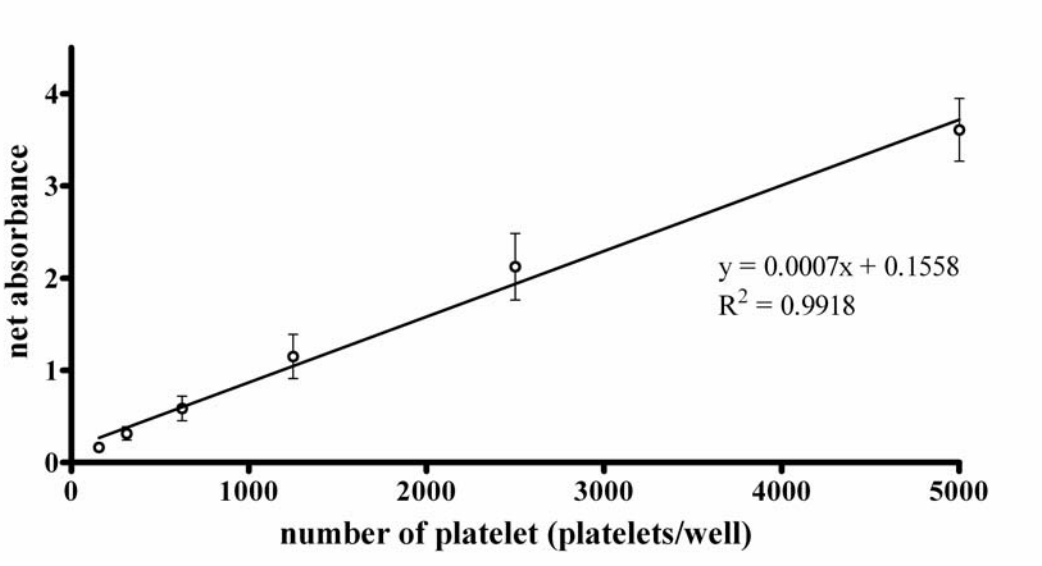
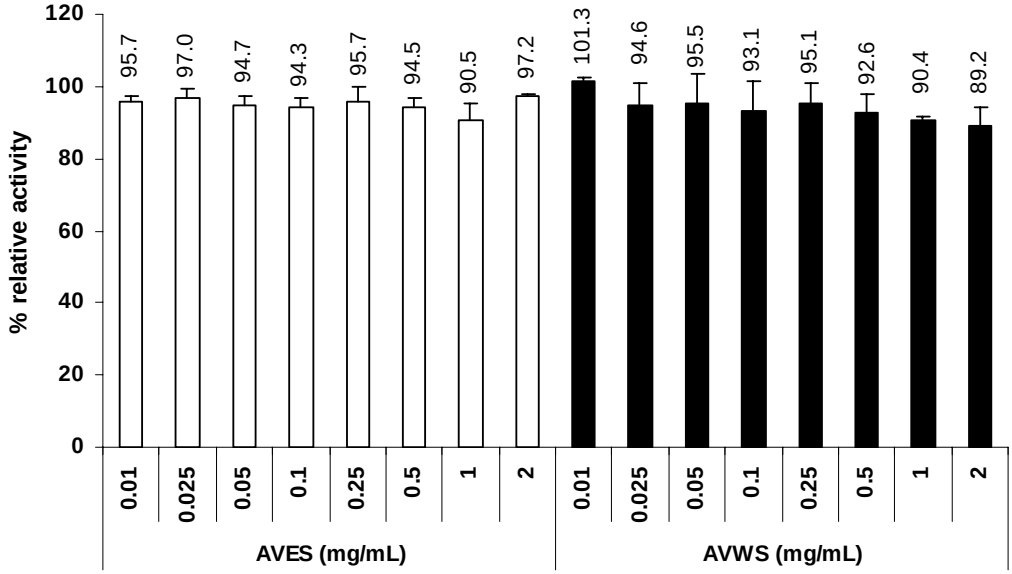


Figure 27 Calibration graph between the number of platelets versus acid phosphatase activity. PRP at various platelet numbers were added to an uncoated 96-well plate before reacted with *p*-nitrophenyl phosphate, as a substrate, for 1 h. NaOH was added to stop reaction. The reaction product, *p*-nitrophenol, was measured by chaging of absorbance at 405 nm. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

A)



B)

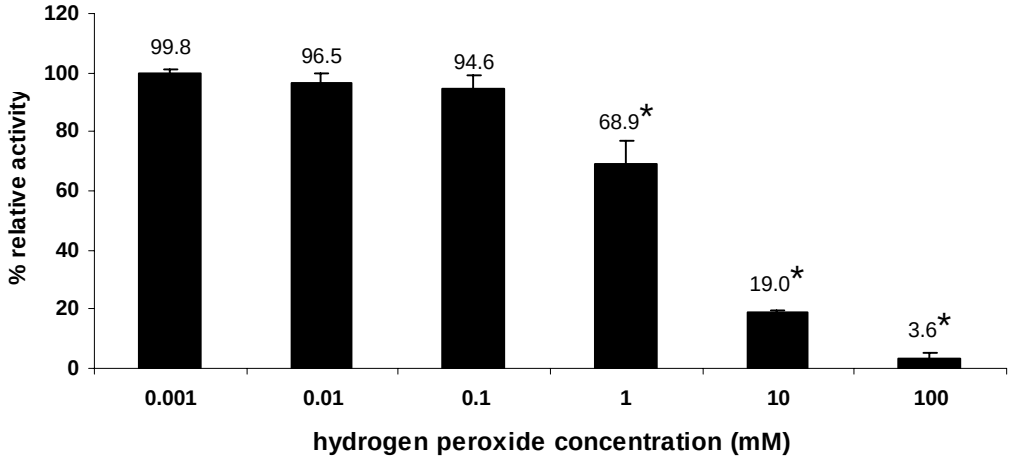


Figure 28 Effect of AV on platelet function using acid phosphatase assay. Platelets were treated with A) AVES and AVWS at the concentrations ranging from 0.01 to 2 mg/mL for 1 h and B) H_2O_2, used as a positive control. The results were expressed as the percentage of control. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

Effect of AV on platelet adhesion using acid phophatase assay

Platelet adhesion to collagen was determined based on acid phosphatase activity. Platelet adhesion was significantly observed when nonactivated platelets were incubated under static condition for 15 min (10 ± 0.5%) or 60 min (30 ± 1%) (Marcondes *et al*., 2006) and platelets were allowed to adhere on collagen for 1 h before detection the amount of attached platelets. The result showed that AVES and AVWS increased platelet adhesion to collagen in a concentration-dependent manner (Figure 29A). Noticeably, the percentage of platelet adhesion was significantly observed in platelets treated with AVES at the concentration of 1 mg/mL and AVWS at the concentrations of 1 and 2 mg/mL. However, AVES and AVWS at the antiplatelet concentration of 0.5 mg/mL did not affect platelet adhesion. Treatment of PRP with SNP, a positive control, at 75 and 100 mM significantly decreased the percentage of adhesion (Figure 29B).

Effect of AV on fibrinolysis using clot lysis assay

To determine the correlation between antiplatelet and fibrinolysis, clot lysis time assay was then performed. The clot lysis was studied in 2 conditions: in BSA to study interaction between extract and fresh microclot and in plasma to study interaction between extract and fresh microclot including nescent microclot, which is formed due to the presence of fibrinogen in added plasma (Stief, 2006).

Streptokinase, a positive control, was incubated with microclot for 0.5, 1, 2, 3, 4 and 5 h. Exposure of streptokinase showed time- and concentration-dependent decrease in clot turbidity of both systems (Figure 30) and reached a stable plateau of clot lysis at 4 h. The percentage of clot mass at 4 h-incubation was therefore selected to determine clot lysis activity. Streptokinase at various concentrations (100, 250, 500, 1,000 and 2,000 IU/mL) significantly decreased clot mass in plasma (Figure 31). Meanwhile, streptokinase induced clot lysis in BSA was more potent than in plasma.

AVES and AVWS slightly increased clot turbidity in plasma at 0.5, 1 and 2 h incubation followed by a decrease in clot turbidity at 3, 4 and 5 h incubation (Figures 32A and 33A). On the other hand, AVES and AVWS exhibited clot lysis in BSA in concentration- and time-dependent manner (Figure 32B and 33B). After 4 h

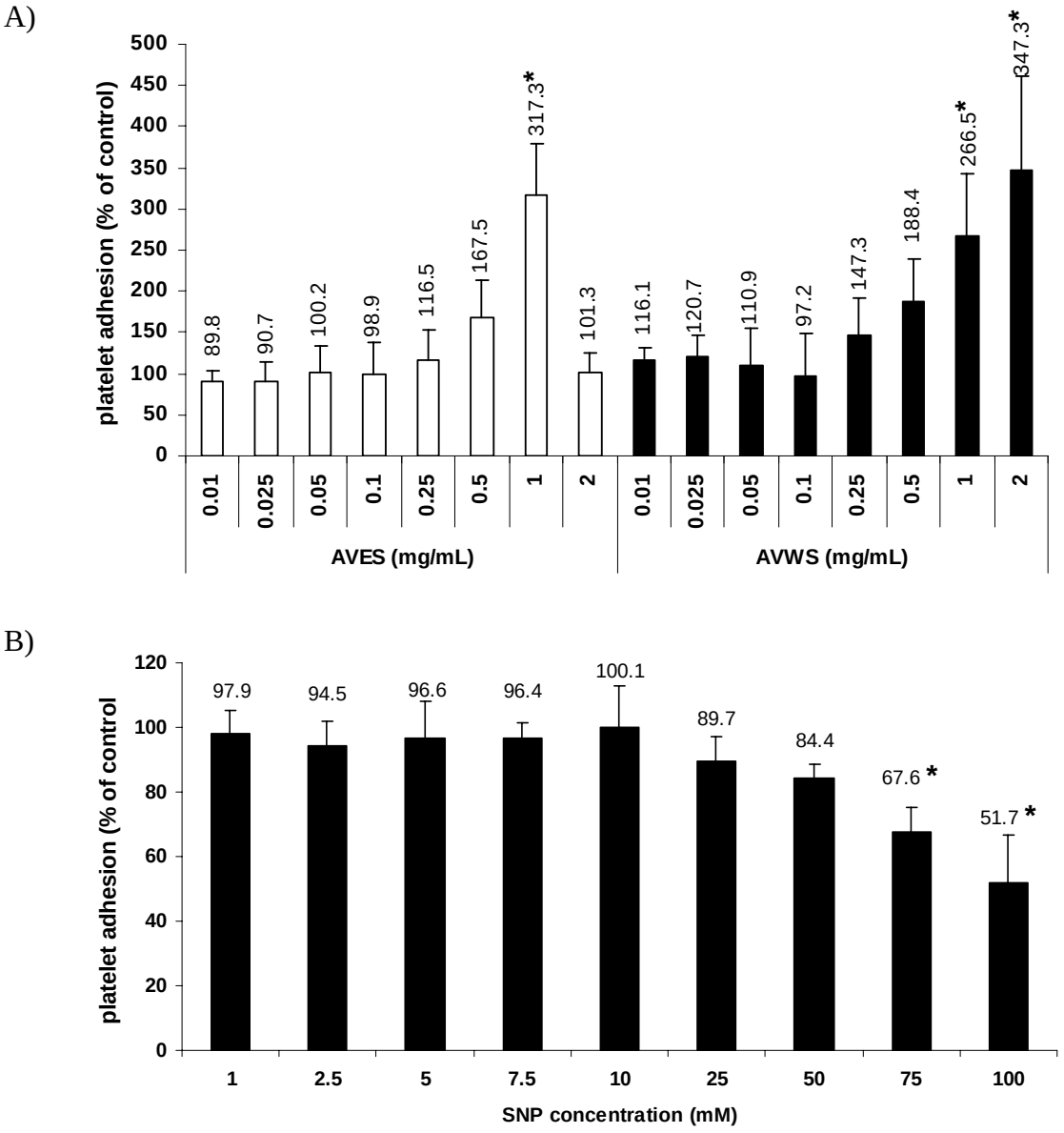
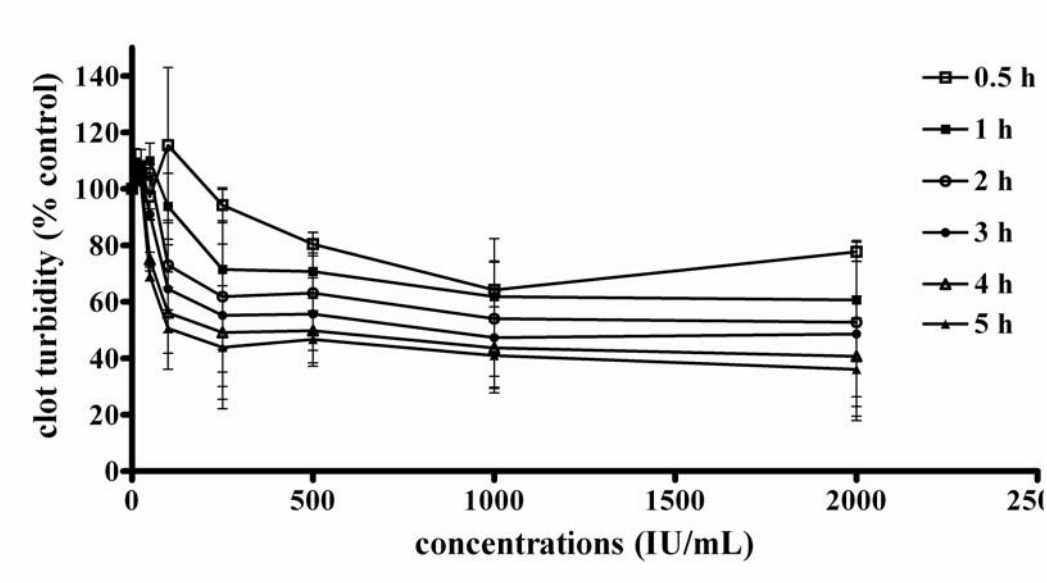


Figure 29 Effect of AV on platelet adhesion using acid phosphatase assay. Platelets were treated with A) AVES and AVWS at the concentrations ranging from 0.01 to 2 mg/mL for 15 min and allowed to adhesion for 1 h and B) SNP, used as a positive control. The results were expressed as the percentage of control. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate) * $p < 0.05$ compared to the untreated control.

A)



B)

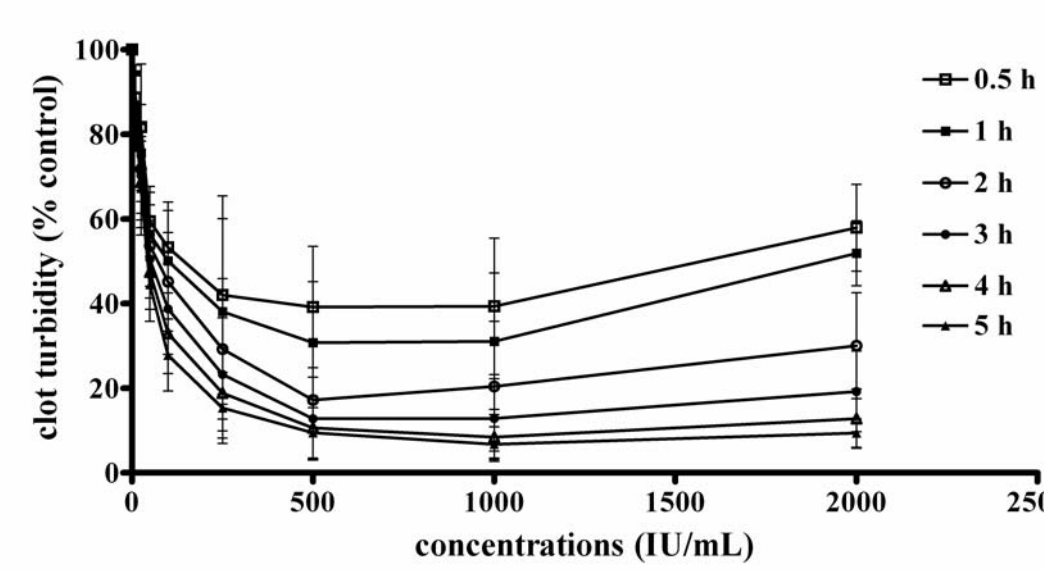


Figure 30 Clot lysis by streptokinase. Streptokinase at the concentrations ranging from 10 to 2,000 IU/mL were incubated with microfresh clot for 0.5, 1, 2, 3, 4 and 5 h in A) plasma and B) BSA. Clot turbidity was detected by measurement of absorbance at 405 nm. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

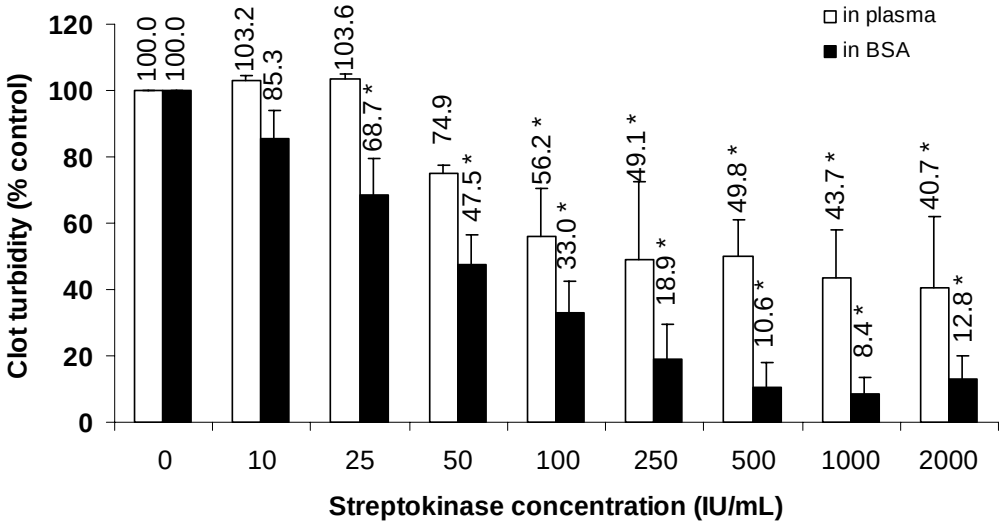
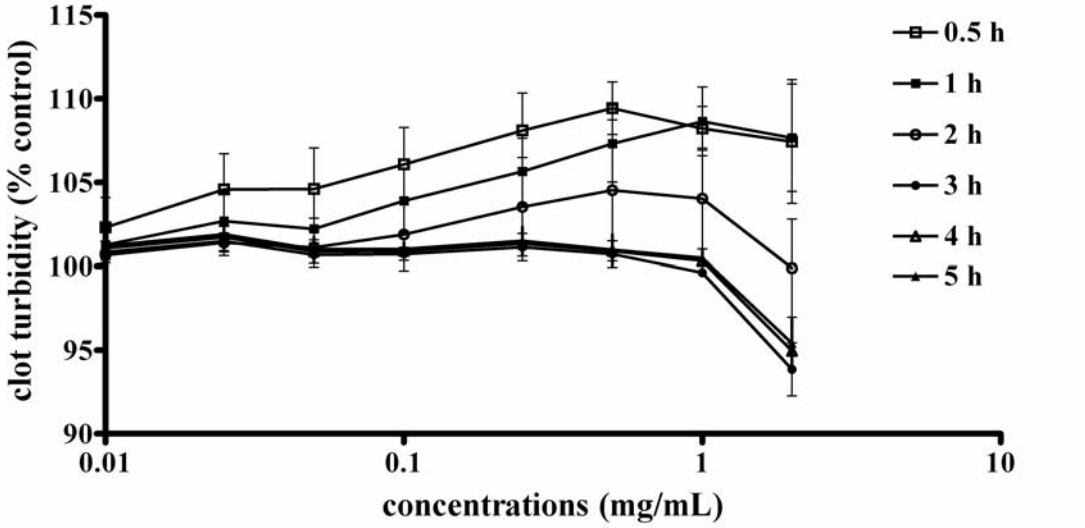


Figure 31 Concentration-dependent study of clot-lysis activity of streptokinase for 4-h incubation. Streptokinase at the concentrations ranging from 10 to 2,000 IU/mL were incubated with microfresh clot in both plasma and BSA. Clot turbidity was detected by measurement of absorbance at 405 nm. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

A)



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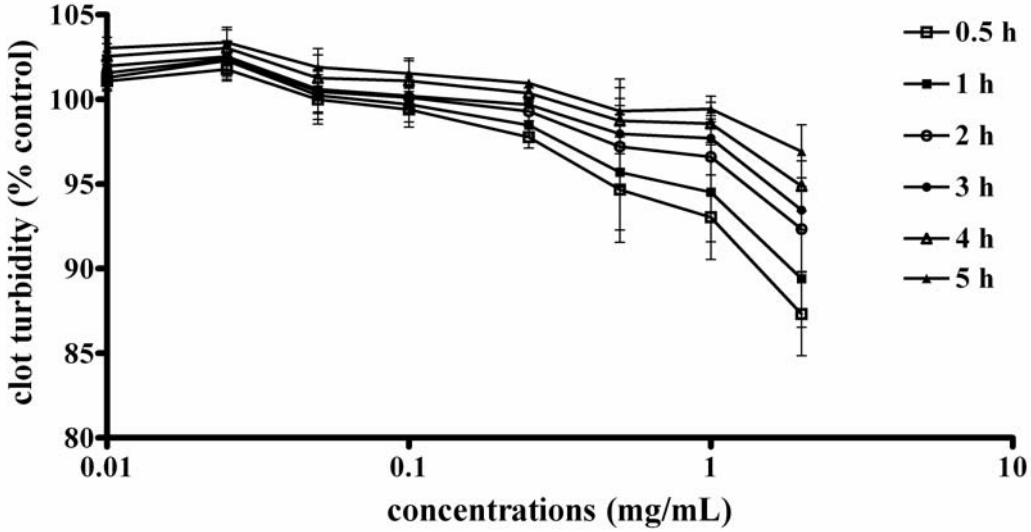
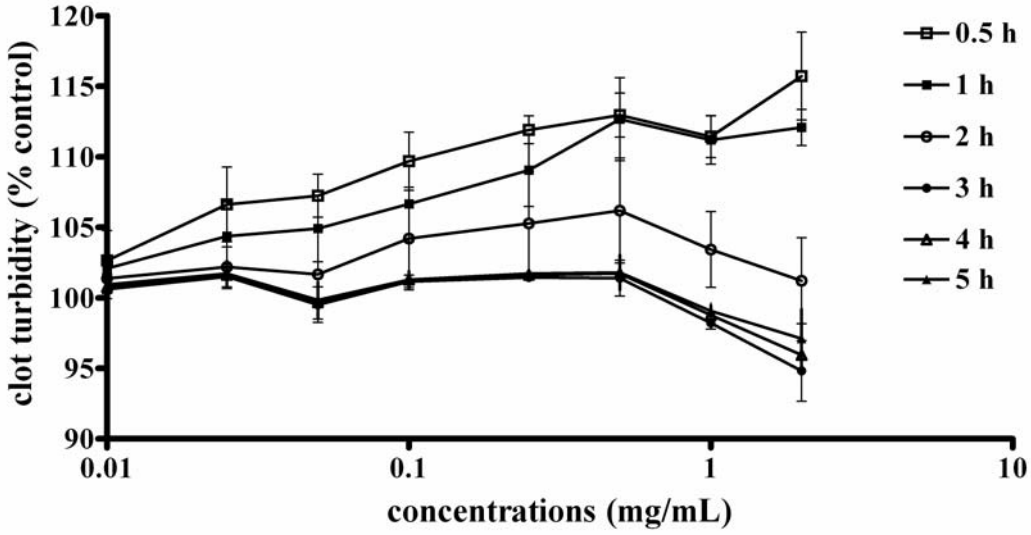


Figure 32 Concentration- and time-dependent study of clot lysis of AVES. AVES at the concentrations ranging from 0.01 to 2 mg/mL were incubated with microclot for 0.5, 1, 2, 3, 4 and 5 h in A) plasma and B) BSA. Clot turbidity was detected by measurement of absorbance at 405 nm. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

A)



B)

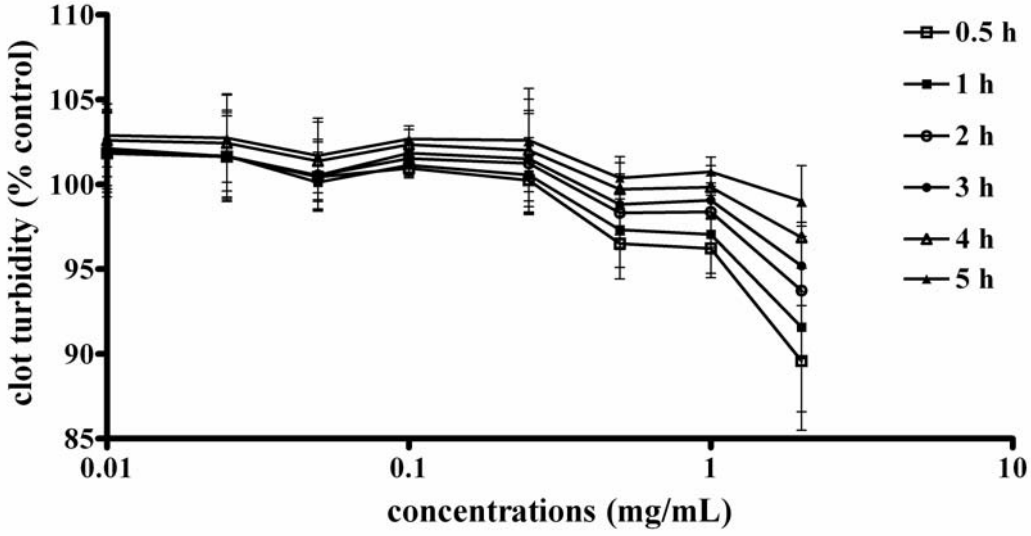


Figure 33 Concentration- and time-dependent study of clot lysis of AVWS. AVWS at the concentrations ranging from 0.01 to 2 mg/mL were incubated with microclot for 0.5, 1, 2, 3, 4 and 5 h in A) plasma and B) BSA. Clot turbidity was detected by measurement of absorbance at 405 nm. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

incubation, AVES and AVWS at the tested concentrations ranging from 0.01 to 2 mg/mL did not showed significant clot-lysis in both plasma and BSA (Figure 34).

Effect of AVES on phosphorylation of Akt and eNOS in ECV304 cells using Western blot analysis

Taken all results together, AVES exhibited the antiplatelet (Figure 24B) and platelet adhesion (Figure 29A) more effective than AVWS. AVES was therefore chosen for Western blot analysis. To rule out whether the antiplatelet activity of AVES came from the endothelial-derived NO production, the effect of AVES in ECV304 cells on the activation of Akt and eNOS was therefore investigated. Hesperidin was used as a positive control to find appropriate time for phosphorylation of Akt and eNOS. However, hesperidin at the concentration of 0.1 mM increased phosphorylation of Akt and eNOS as compared to untreated control during 15 - 60 min exposure time (Figure 35). Treatment with AVES at the concentrations of 0.25, 0.5 and 1 mg/mL exhibited no difference in eNOS level and Akt phosphorylation as compared to untreated control (Figure 36). This might be due to the presence of growth factor in FBS, leading to the interference of the phosphorylation of Akt and eNOS.

The effect of AVES on the phosphorylation of Akt and eNOS was then performed in ECV304 cells under serum-free condition. The cells were starved in serum-free medium for 6 h before adding AVES at various concentrations for 0.5, 1 and 2 h. AVES at the antiplatelet concentration of 0.5 mg/mL for 1-h incubation significantly increased phosphorylation of eNOS but not Akt (Figures 37, 38). The result revealed that phosphorylation of eNOS enhanced by AVES could lead to increase the production of endothelial-derived NO, resulting in the inhibition of platelet aggregation.

Effect of AVES on platelet secretion induced phosphorylation of Akt and eNOS in ECV304 cells was determined. AVES at the concentration of 0.5 mg/mL slightly expressed high ratio in Akt phosphorylation but was not significantly different in both eNOS and Akt phosphorylation ratio for 1 h-incubation (Figure 39).

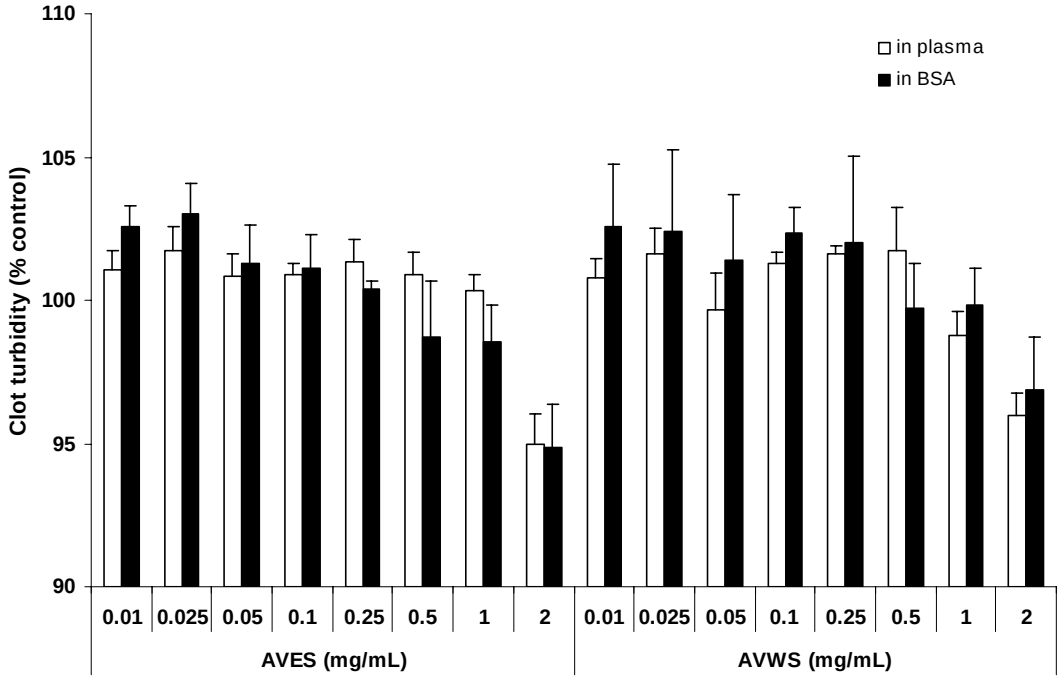
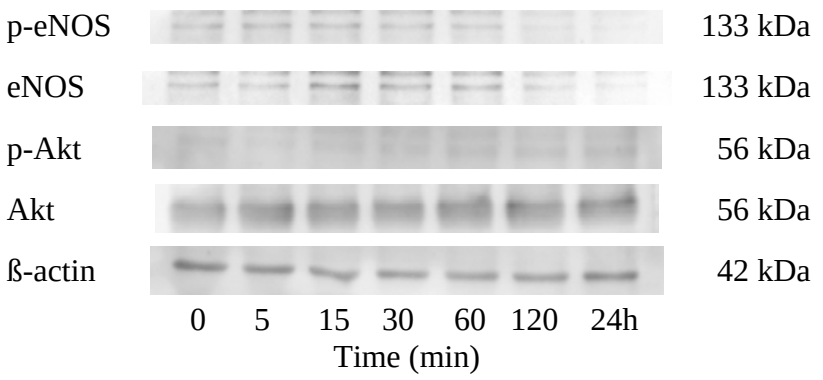
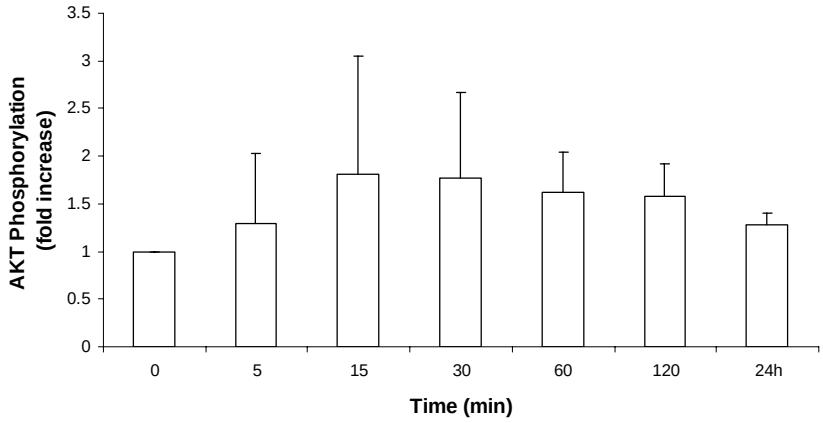


Figure 34 Clot-lysis activity of AVES and AVWS for 4-h incubation. AVES and AVWS at the concentrations ranging from 0.01 to 2 mg/mL were incubated with microfresh clot for 4 h in both plasma and BSA. Clot turbidity was detected by measurement of absorbance at 405 nm. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

A)



B)



C)

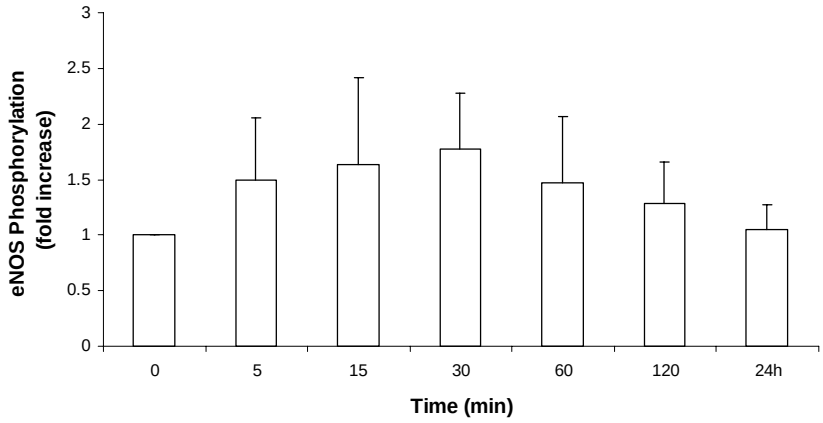
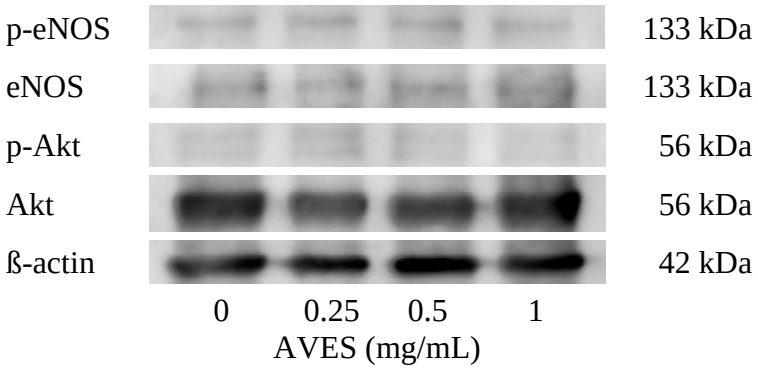


Figure 35 Western blot analysis of hesperidin on phosphorylation of Akt Ser 473 and eNOS Ser 1177 in ECV304 cells. Cells were treated with 0.1 mM hesperidin for various incubation times. The cell lysate was analyzed by Western blot. A) Representative immunoblots of Akt/p-Akt, eNOS/p-eNOS relative to beta-actin Intensity relative to control of phosphorylation of B) Akt and C) eNOS as compared to control. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

A)



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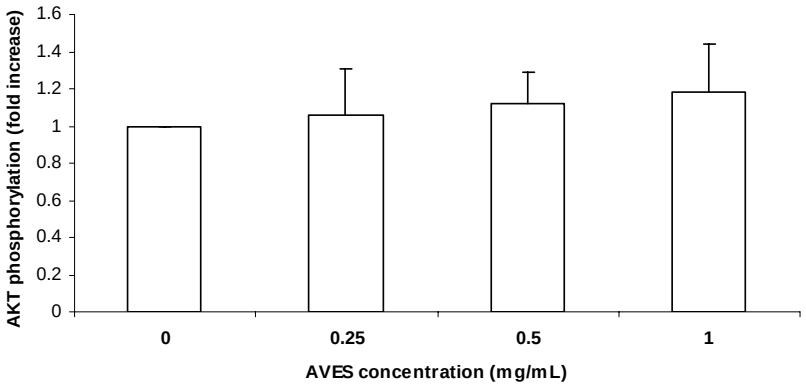

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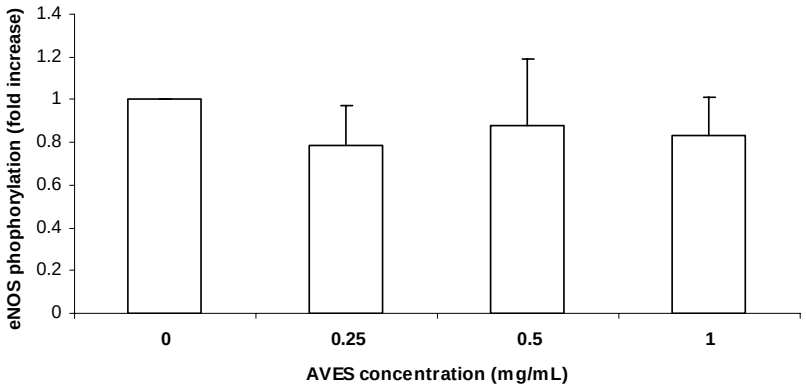
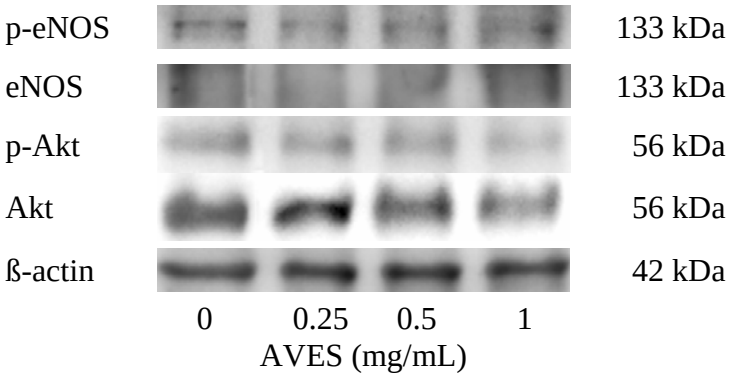


Figure 36 Effect of AVES on phosphorylation of Akt and eNOS in ECV304 cells. Cells were treated with AVES at the indicated concentrations for 1 h. The cell lysate were analyzed by Western blot analysis. A) Representative immunoblots of Akt/p-Akt, eNOS/p-eNOS relative to beta-actin Intensity relative to control of phosphorylation of B) Akt and C) eNOS as compared to control. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate)

A)



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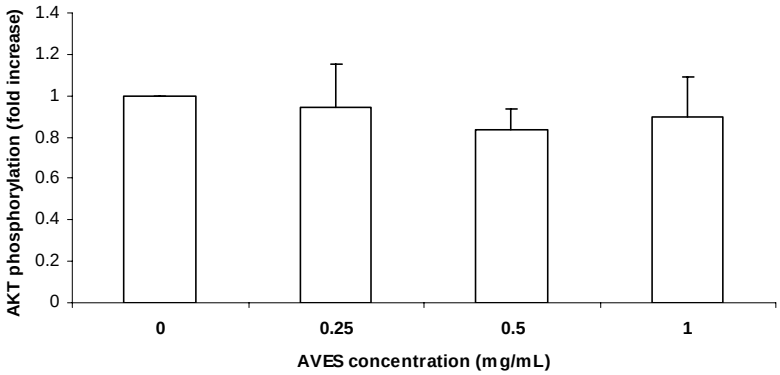


C)

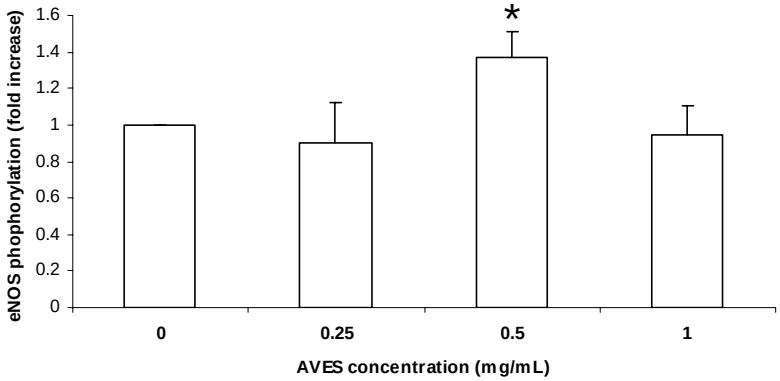
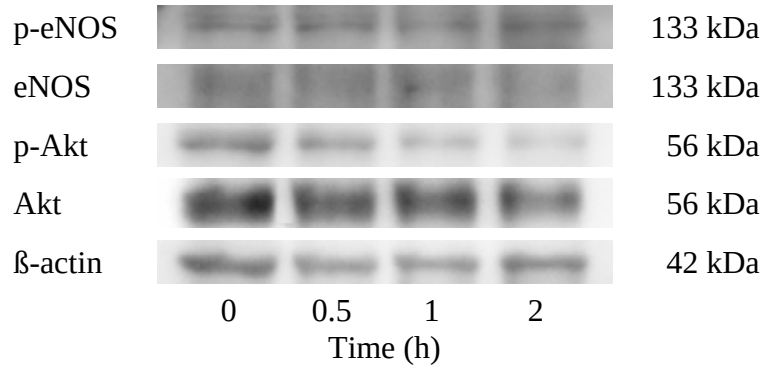


Figure 37 Concentration-dependent studies of AVES on phosphorylation of Akt and eNOS in ECV304 cells. Cells were starved in sereum free medium for 6 h before adding AVES at the concentrations of 0.25, 0.5 and 1 mg/mL for 1 h and were analyzed by Western blot analysis. A) Representative immunoblots of Akt/p-Akt, eNOS/p-eNOS relative to beta-actin Intensity relative to control of phosphorylation of B) Akt and C) eNOS as compared to control. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

A)



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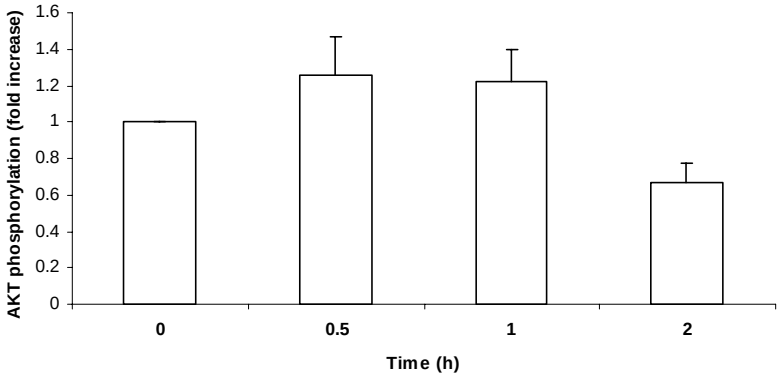


C)

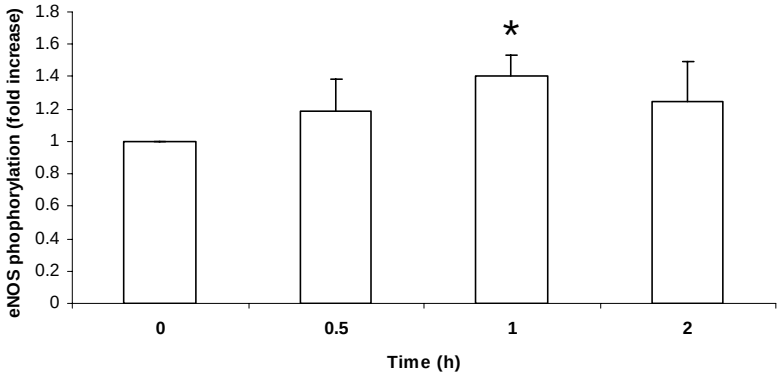
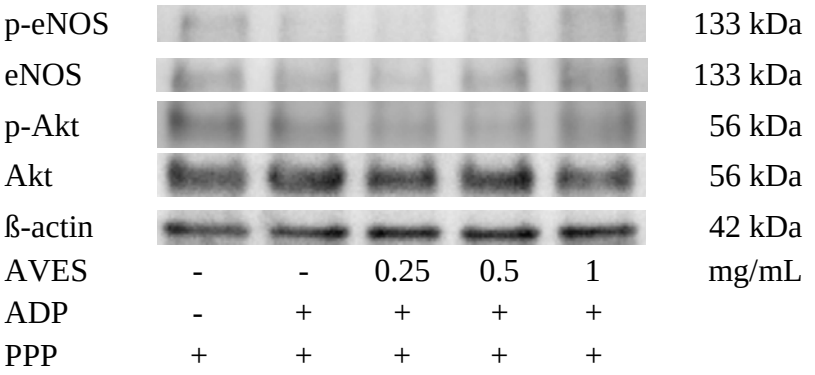


Figure 38 Time-dependent studies of AVES on phosphorylation of Akt and eNOS in ECV304 cells. Cells were starved in serum-free medium for 6 h before adding AVES at the concentration of 0.5 mg/mL for 0, 0.5, 1 and 2 h. The cell lysate was analyzed by Western blot analysis. A) Representative immunoblots of Akt/p-Akt, eNOS/p-eNOS relative to beta-actin Intensity relative to control of phosphorylation of B) Akt and C) eNOS as compared to control. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate)

A)



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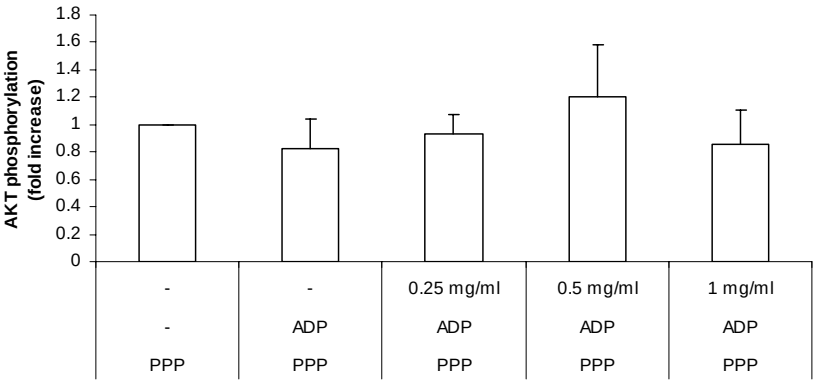

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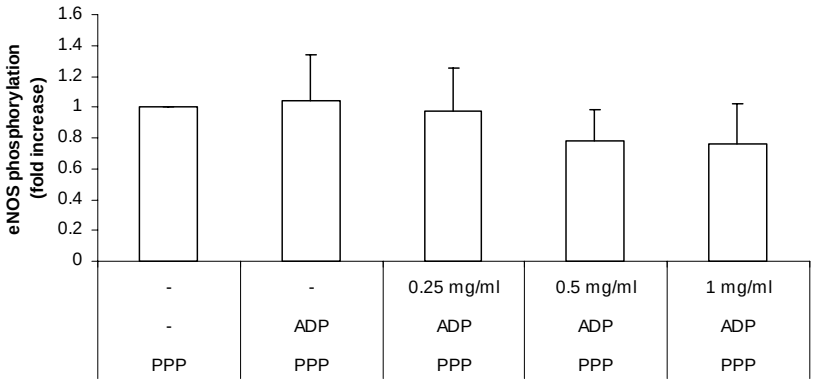


Figure 39 Effect of AVES on platelet secretion induced phosphorylation of Akt and eNOS in ECV304 cells. AVES was incubated with platelets for 5 min before adding ADP to induce platelet activation and secretion. Plasma separated from PRP was treated with ECV304 cells for 1 h. The cell lysate was analyzed by Western blot. A) Representative immunoblots of Akt/p-Akt, eNOS/p-eNOS relative to beta-actin Intensity relative to control of phosphorylation of B) Akt and C) eNOS as compared to control. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate)

Biological activities related to circulatory system of AVES and AVWS was summarized in Table 3. Both AVES and AVWS inhibited ADP-induced platelet aggregation in a concentration-dependent manner, exhibiting the IC_{50} values of 0.43 and 0.51 mg/mL, repectively without affecting viability and function of platelet. AVES and AVWS at the concentration of 1 mg/mL increased platelet adhesion without affecting fibrinolytic activity. Furthermore, treatment of ECV304 cells with AVES at the antiplatelet concentration of 0.5 mg/mL for 1 h under serum-free condition enhanced phosphorylation of eNOS (Ser 1177) but not Akt (Ser 473). The result suggested that AVES might increase endothelial-derived NO production via Akt-independent eNOS phosphorylation, leading to inhibit platelet aggregation. Meanwhile, AVES at the antiplatelet concentration of 0.5 mg/mL did not affect viability (IC_{50} 0.79 mg/mL) and genotoxicity in ECV304 cells.

Table 3 Summary of biological activities of AVES and AVWS at the concentrations ranging from 0.01 to 2 mg/mL.

Biological activity		Effective concentration (mg/mL)	
		AVES	AVWS
Platelets			
Antiplatelet activity induced by	ADP	+	+
		(0.43)	(0.51)
	arachidonic acid	-	-
	collagen	-	-
Viability (1 h)		+	+
		(2.00)	(2.00)
Acid phosphatase		-	-
Platelet adhesion to collagen		+	+
		(1.00)	(1.00)
Fibrinolytic activity		-	-
ECV304 cells			
Phosphorylation	Akt	-	ND
	eNOS	+	ND
		(0.50)	
Viability (24 h)		+	+
		(0.79)	(0.87)
Genotoxicity (24 h)		-	-

- not effective
+ effective
ND not determine

CHAPTER V
DISCUSSION AND CONCLUSION

The herbal extracts in Phikud Benjakot containing AD, AL, LS, AS and AV at the cytotoxic threshold IC_{10}, $IC_{25,}$ and IC_{50} obtained from MTT assay were chosen to evaluate the genotoxicity in ECV304 cells using alkaline comet assay. For those extracts exhibiting IC_{50} > 5 mg/mL, the non-threshold concentrations of 0.1, 1 and 5 mg/mL were selected for the genotoxicity testing. All tested herbal extracts expressed no significant difference in DNA damage between treated and control groups. Earlier work noted that bergapten, safrole and isosafrole, isolated from AS, were carcinogens (Tovar and Petzel, 2009). Safrole oxide (SAFO), metabolite of safrole could induce DNA strand break. Treatment of SAFO at the concentration of 125 µM and higher for 24 h in HepG2 cells induced a 5.1–79.6-fold increase in comet tail moment using alkaline comet assay and a 2.6–7.8-fold increase in the frequency of micronuclei in binucleated cells using cytokinesis-block micronucleus assay (Chiang *et al*., 2011). Genotoxic potential of *Artemisia verlotorum* aqueous extract, which affected the cell cycle of onion (*Allium cepa*), was found at the concentrations of 6, 32 and 48 mg/mL for 24 h treatment by decreasing the mitotic index rate to 7.35%, 3.84% and 3.20%, respectively as compared to control (9.65%) (Bittencourt de Souza *et al*., 2010). Water and ethanol extracts of *Artemisia dracunculus* at the concentration of 3.36 mg/mL expressed strong mutagenic activity using *umu* test system (Caillet *et al*., 2011). In addition, quercetin, previously isolated from AV (Lee *et al*., 1998), at the concentration of 44 µM showed significant genotoxicity in CHO-AT3-2 cells using chromosome aberration test (Carver *et al*., 1983). However, ASES, ASWS, AVES and AVWS did not cause any DNA damage in ECV304 cells in our comet assay model. The results suggested that the amount of genotoxic carcinogens such as SAFO and quercetin in the extracts using our extraction procedure were not high enough to induce DNA damage. All extracts in Phikud Benjakot may be likely not to induce DNA damage. However, the extract could have an influence on even at very low-levels which cannot be detected by low sensitive Comet assay (Speit *et al*., 2008).

High sensitive assay such as micronuclei or chromosome aberrations and/or Ames test should be applied to confirm the absence of genotoxicty (OECD, 2010).

For the study of inhibitory effect of platelet aggregation induced by ADP, ADP at the concentration of 2.5 µM exhibited various response in maximal aggregation in PRP (41 and 85%) depending on volunteers. The appropiate concentration of ADP should be therfore optimized to activate platelet aggregation in each experiment. The threshold concentration for ADP to induce platelet aggregation in most healthy populations is between 1 and 7.5 µM. ADP at lower concentration was not sufficiently strong enough to stimulate platelet aggregation (Zhou and Schmaier, 2005). Therefore, ADP at various concentrations of 1, 2.5, 5 and 10 µM in each blood sample was chosen for the study of antiplatelet effect of the extracts.

For platelet aggregation induced by arachidonic acid**,** arachidonic acid the concentration of 500 µM induced approximately 80 - 90% of maximal aggregation in human PRP**.** Our result was correlated with the previous study, showing that treatment of platelets with arachidonic acid at the concentrations of 1.5 or 2.0 mM induced reversible aggregation even without any drugs (Vinge, 1985). Arachidonic acid at the concentration of 1.64 µM was often used for the study of platelet function (Zhou and Schmaier, 2005), while arachidonic acid at the concentration of 650 µM was used to induced aggregation in guinea pig PRP (ELTahir and Bakheet, 2007). Arachidonic acid at the concentration of 500 µM was used to induce aggregation in human whole blood (Jantan *et al*., 2009). Arachidonic acid at the concentration of 500 µM was therefore chosen for the study of antiplatelet effect of the extracts in our study.

For collagen-induced platelet aggregation, collagen at the concentration of 3 µg/mL induced a full aggregatory response at approximately 80 - 90% of maximal aggregation which was correlated with previous reports (Mori *et al*., 2010)**.** Collagen at high concentration of 5 µg/mL was considered a potent agonist to induce platelet activation in the most seriously impaired platelets, while at lower concentration of 1.25 µg/mL used to induce platelet activation (Zhou and Schmaier, 2005). Collagen at the concentrations ranging from 2 to 3 µg/mL was used to induce aggregation in human whole blood (Jantan *et al*., 2009) and rat blood PRP (Kim *et al*., 2008.). Therefore, collagen at the concentration of 3 µg/mL was used to induce platelet aggregation in this study.

ASA was used as a positive control for the study of antiplatelet induced by all three agonists, ADP, arachidonic acid and collagen. ASA inhibited rat platelet aggregation induced by 20 - 40 μM arachidonic acid and 2 - 3 μg/mL collagen, exhibiting the IC_{50} values of 63.0 and 470.0 μM, respectively (Kim *et al*., 2008). Treatment of 139 μM ASA inhibited 31.3%, 42,9% and 100% when induced aggregation in human whole blood by 10 μM ADP, 500 μM arachidonic acid and 2 μg/mL collagen, respectively (Jantan *et al*., 2009). Our result revealed that ASA (500 μM) inhibited secondary wave of aggregation when induced by ADP while completely inhibited when induced by arachidonic acid through the inhibition of COX-1 leading to a decrease in the TXA_2 production (Awtry and Loscalzo, 2000). Additionally, our result demonstrated that ASA did not inhibit primary wave of aggregation induced by ADP. Primary wave of aggregation allows the release of secondary agonists such TXA_2 and ADP from dense granules in platelets, resulting that inhibition of secondary wave aggregation when induced by ADP was observed in ASA-treated platelets (Woulfe *et al*., 2001).

To determine antiplatelet effect of the extracts, the extracts at the concentration of 1 mg/mL were preliminary studied in ADP-induced platelet aggregation. The extracts of AD (ADES, ADWS), AS (ASES, ASWS) and AV (AVES, AVWS) significantly inhibited ADP-induced platelet aggregation. Previous report showed that Z-ligustilide, isolated from AS, was orally administered once daily to rat for 3 days. Blood samples were colleted 1 h after final administration for antiplatelet assay. Z-ligustilide at the doses of 10 and 40 mg/kg significantly inhibited ADP-induced platelet aggregation while at the dose of 40 mg/kg showed higher inhibitory activity than ASA (Zhang *et al*., 2009). Antiplatelet activity of *Artemisia dracunculus* significantly inhibited thrombin-induced platelet aggregation. Pretreatment of methanol and chloroform extracts of *Artemisia dracunculus* (200 μg/mL) before adding thrombin (0.25 U/mL) obviously decreased aggregated platelets in the uncoated plates relative to the untreated control when evaluated under inverted microscopy (Shahriyary and Yazdanparast, 2007). Essential oil extracted from steam distillation of *Artemisia dracunculus* inhibited guinea pig platelet aggregation induced by ADP, arachidonic acid and TXA_2 agonist (U46619),

exhibiting the IC_{50} values ranging from 4 to 132 μg/ml (Tognolini *et al*., 2006). Several flavonoids, coumarins and phenylacetylenes isolated from the methanol extract of *Artemisia capillaris*, markedly inhibited rabbit platelet aggregation induced by arachidonic acid and collagen (Wu *et al*., 2001). Quercetin and rutin, previously isolated from AV (Lee *et al*., 1998), inhibited ADP-induced platelet aggregation (Kim and Yun-Choi, 2008). Quercetin at the concentration of 20 μM significantly decreased ADP-induced aggregation (24.6% inhibition) using human PRP within 1 min and significantly increased approximately 43% inhibition after 10-min incubation (Kobzar *et al*., 2005). Rutin at the concentration of 300 μM showed mild inhibitory effect on ADP (2-5 μM) induced aggregation in rat PRP (Kim and Yun-Choi, 2008). Several reports suggested that the antiplatelet activity of AD, AS and AV may be due to the presence of active constituents such as Z-ligustilide, quercetin and rutin. Among the extracts of AD, AS and AV, AV at the concentration of 1 mg/mL exhibited the highest percentage of inhibition. Thus AVES and AVWS were further selected for the study of the antiplatelet activity in our study.

Although ADP plays an important role in platelet aggregation, platelet can be activated by other agonists such as TXA_2 and collagen, leading to ADP secretion, which further induces platelet aggregation (Tang *et al.*, 2003). ADP induces platelet aggregation via the G protein-coupled purinergic receptors while TXA_2, a metabolite of arachidonic acid formed via the COX-TXA_2 synthase pathway causing granule release (Cattaneo, 2011). Additionally, collagen induces platelet activation through a tyrosine kinase-based signaling pathway, leading to granule release and TXA_2 production (Yu *et al.*, 2011). Besides antiplatelet activity against ADP, quercetin and rutin also inhibited platelet aggregation induced by arachidonic acid and collagen. Rutin (250 and 290 μM) inhibited platelet aggregation in human washed platelet induced by collagen (1 μg/mL) (Sheu *et al*., 2004). Quercetin moderately inhibited platelet aggregation in human whole blood induced by ADP (10 μM), arachidonic acid (500 μM) and collagen (2 μg/mL), exhibiting the IC_{50} values of 188.0, 181.0 and 200.5 μM, respectively (Jantan *et al*., 2009). Quercetin (2500 μM) significantly inhibited ADP-and collagen- induced aggregation in human PRP by 97 ± 4% and 95 ± 4%, respectively (Janssen *et al*., 1998). Therefore, antiplatelet effect of AVES and

AVWS were further investigated when induced by arachidonic acid and collagen. Our result revealed that both AVES and AVWS showed no inhibitory effect on platelet aggregation induced by arachidonic acid and and collagen. The antiplatelet activity of AVES and AVWS seemed to play a role specifically in the ADP signaling pathway. Concentration-dependent study of AVES and AVWS on platelet aggregation induced by ADP was further performed and expressed IC_{50} values of 0.43 and 0.51 mg/mL, respectively. The antiplatelet of AVES was slightly more effective than of AVWS, which might be due to the presence of quercetin and rutin, previously reported by Lee *et al.* (1998).

To ascertain that the antiplatelet activity of AVES and AVWS did not come from the toxicity of the extracts, platelet viability was further performed using MTT reduction assay. Since platelets were found to be temperature-sensitive and MTT reduction assay based on mitochondrial dehydrogenase function in platelets was lost approximately 50%, 80% and 100% at 37 ºC after 24 h, 48 h and 72 h of incubation (Catani *et al*., 2010), platelet viability using MTT assay was then performed at 1 h-incubation according to the previous reports (Morganti *et al*., 2008; Morganti *et al*., 2010). Treatment of platelet with AVES or AVWS at the concentration of 2 mg/mL for 1-h treatment concentration-dependently decreased platelet viability and became significantly toxic to platelet. However, AVES and AVWS at the antiplatelet concentration of 0.5 mg/mL exhibited no significant toxicity to platelet. Our result confirmed that AVES and AVWS at the antiplatelet concentration did not damage platelet. Time-dependent study also demonstrated that AVES and AVWS at the antiplatelet concentration of 0.5 mg/mL for less than 48 h incubation did not affect viability and DNA damage of ECV304 cells. Among flavonoids isolated from AV, eriodictyol and luteolin were the most abundant compounds, more than 40 mg/kg dry weight while apigenin quercetin and rutin were less than 10 mg/kg dry weight (Lee *et al*., 1998). Cytotoxic effect of these flavonoids on viability of HUVEC cells for 24 h-exposure were evaluated using trypan blue dye exclusion assay. Luteolin, quercetin, apigenin and eriodictyol expressed significant toxicity at IC_{50} values of 57, 67, 110 and 112 µM, respectively, while rutin did not show significant toxicity (Matsuo *et al*., 2005).

Since platelet adhesion plays the critical role in the initial process of platelet aggregation, the effect of AV on platelet adhesion was determined based on acid phosphatase activity in platelet. Both AVES and AVWS did not alter platelet acid phosphatase activity, including platelet function. Since platelet adhesion was significantly observed when nonactivated platelets were incubated under static condition for 15 min (10 ± 0.5%) or 60 min (30 ± 1%) (Marcondes *et al*., 2006) and platelets were allowed to adhere on collagen for 1 h before detection the amount of adhered platelets. Our result demonstrated that SNP, used as a positive control, at the concentration of 50 and 100 mM significantly decreased the percentage of adhesion, which was correlated to previous reports (Marcondes *et al*., 2006; Wu *et al*., 1997). SNP (100 μM) inhibited platelet adhesion to collagen approximately 10% from untreated control after 1 h incubation (Wu *et al*., 1997). SNP (0.001–1,000 μM) concentration-dependently inhibited nonactivated platelet adhesion to fibrinogen after 1 h incubation (Marcondes *et al*., 2006). Our result showed that AVES and AVWS at the antiplatelet concentration of 0.5 mg/mL did not affect platelet adhesion, however, AVES and AVWS at higher concentration of 1 mg/mL significantly increased platelet adhesion to collagen. Quercetin, constituent of AV, was determined for platelet spread by imaging at a concentration of 50 μM for 1 h at room temperature decreased 41% and 31% of platelets spread on collagen and fibrinogen, respectively (Navarro-Núñez *et al*., 2010). AV at the antiplatelet concentration induced by ADP did not inhibit both aggregation induced by arachidonic acid and collagen including platelet adhesion to collagen.

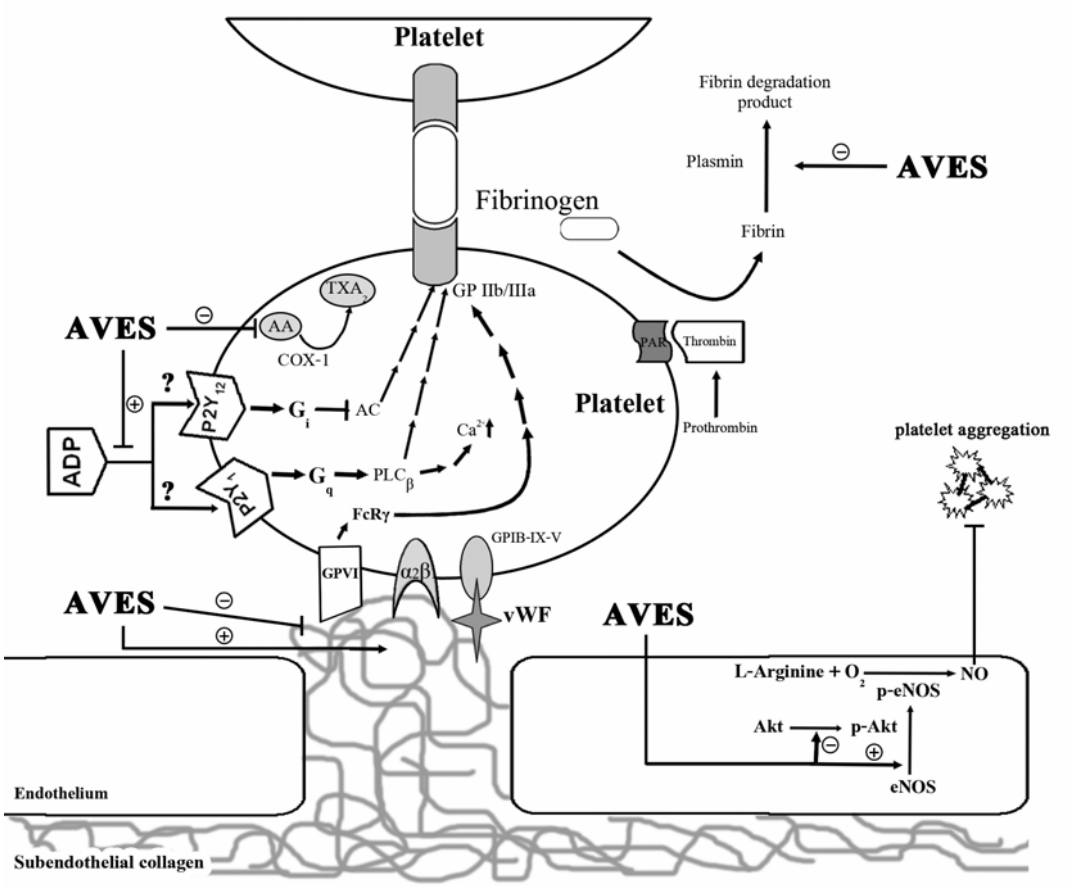
Fibrinolytic dysfunctions cause bleeding or predisposition to thrombosis (Panes *et al*., 2012). Streptokinase, used as a positive control, showed time- and concentration-dependently decreased clot mass and reached a stable plateau of clot lysis at 4 h. Quercetin, a constituents in AV, up-regulates both t-PA and u-PA gene transcription in cultured HUVECs, resulting in an increase in fibrinolytic activity (Abou-Agag *et al*., 2001). Rat administered with quercetin equivalent to that consumed in two glasses of red wine by a 70 kg adult showed 55% reduction in PAI-1 mRNA levels in aortic vascular tissue measured by RT-PCR, leading to enhance endothelial cell-mediated fibrinolytic activity (Grenett *et al*., 2007). However, our result revealed that AVES and AVWS did not exhibit significant fibrinolytic activity

because there is no endothelium in our fibrinolytic model. Although AVES and AVWS had similar biological activity in our experiment, AVES was slightly more effective than AVWS. Therefore, the effect of AVES on endothelial-derived NO production in ECV304 cells was studied.

Endothelial-derived NO, synthesized by eNOS, can also inhibit platelet aggregation (Iwakiri *et al.*, 2002). Phosphorylation and dephosphorylation networks are major post-translational regulatory influences on eNOS activity. Phosphorylation of eNOS at Ser 1177 for human and Ser 1179 for bovine can enhance eNOS activity and catalyzed by numerous kinases, including kinase Akt (Balligand *et al*., 2009). Akt is activated by its phosphorylation at Ser 473 (Motley *et al*., 2007) To determine whether AVES affected on endothelial-derived NO production, the phosphorylation of Akt (Ser 473) and eNOS (Ser 1177) in AV-treated ECV304 cells was performed using Western blot analysis. AVES-treated cells in medium supplement with FBS exhibited no difference in level of phosphorylation of Akt and eNOS as compared to untreated control, which might be due to the presence of FBS providing growth factor, leading to the interference of the phosphorylation of Akt and eNOS. Previous reports about phosphorylation of protein were studied in serum-derived cells (Chiou *et al*., 2008; Connelly *et al*., 2005; Rizza *et al*., 2011). Treatment of ECV304 cells in serum-free medium with AVES at the antiplatelet concentration of 0.5 mg/mL significantly increased phosphorylation of eNOS at Ser 1177 but not Akt, resulting in an increase in endothelial-derived NO production. This result suggested that AVES may stimulate eNOS phosphorylation via Akt independent pathway. Quercetin enhanced endothelial NO production by stimulation of eNOS activity in HUVEC cells (Schmitt and Dirsch, 2009). Quercetin rapidly induced concentration-dependent eNOS phosphorylation at Ser 1179 via an Akt-independent, cAMP/PKA-mediated pathway to enhance the production of NO in bovine aortic endothelial cells (Khoo *et al*., 2010, Li *et al*., 2012). In addition, secretion released from activated platelet contains ADP and serotonin can stimulate the endothelial cells resulting in NO release (Michel and Vanhoutte, 2010). However, our result revealed that AVES did not affect on eNOS phosphorylation induced by platelet secretion. To the best of our knowledge, this is the first report showing that AVES exhibited an inhibitoty effect on

platelet aggregation, which is likely via ADP signaling pathway in platelets and endothelial-derived NO.

In summary, among herbal extracts in Phikud Benjakot, the ethanol and aqueous extracts of AV exhibited the highest inhibitory effect on platelet aggregation induced by ADP, but not arachidonic acid and collagen without interfering viability and function of platelet. The antiplatelet activity of AVES might arise from not only direct action on platelet via the ADP signaling pathway but also indirect action on ECV304 cells through Akt-independent mediated eNOS phosphorylation to enhance the production of NO (Figure 40). Our findings suggest that AV, one of crude druges in Phikud Benjakot, could be considered as a candidate herb in the treatment of platelet-associated vascular diseases. Although AV seem to be less effective than ASA, AV may cause less adverse effect due to specific inhibition of platelet aggregation via ADP pathway. However, the detailed antiplatelet mechanism of AV still remains unclear and need to be clarified.



⊖ not effective

⊕ effective

Figure 40 Proposed antiplatelet mechanism of AVES and eNOS activation in ECV304 cells.

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APPENDICES

APPENDIX A
PREPARATION OF REAGENTS

Acrylamide Gel

1. Preparation of separating gel (main gel)

To make two plates of 8% acrylamide gel, the ingredients of separating gel were

Ultrapure water	5.9	mL
4X separating buffer	2.5	mL
50% acrylamide	1.6	mL
10% APS	50	µL
TEMED	10	µL

The ingredients were thoroughly mixed and immediately poured to the glass plates. Then, DDW was layered on the top of the separating gel (4-5 mm. thick). The gels were left for approximately 60 min to polymerize.

2. Preparation of stacking gel (top gel)

Since the separating gels has completely polymerized, DDW was removed from the top of the gels. To make stacking gel, the ingredients were

Ultrapure water	2.6	mL
4X stacking buffer	1.0	mL
50% acrylamide	0.4	mL
10% APS	30	µL
TEMED	5	µL

The ingredients were thoroughly mixed and immediately poured to the glass plates. Then, the combs were inserted on the top of the gels. The gels were left for approximately 20-30 min to polymerize.

50% acrylamide

49.2 g of acrylamide and 0.8 g of N, N'-methylenebisacrylamide were dissolved in ultrapure water. The solution was stirred until completely solubilized, then adjusted volume to 100 mL and stored in dark bottles at room temperature.

4X separating buffer (100 mL)

1.5 M Tris-HCl (pH 8.8)

0.4% SDS

Adjust volume with ultrapure water to 100 mL

4X stacking buffer (100 mL)

0.5 M Tris-HCl (pH 6.8)

0.4% SDS

Adjust volume with ultrapure water to 100 mL

Ammonium persulfate (APS) 10% APS in ultrapure

N, N, N', N'-tetremethylenediamine (TEMED)

Adenosine diphosphate (ADP)

ADP was dissolved in ultrapure water and then aliquot into 0.1 mL/tube and store at -20 ºC.

Alkaline electrophoresis buffer

Alkaline electrophoresis buffer was freshly prepared before used. To prepare 1 L of alkaline electrophoresis buffer, 0.3M NaOH and 1 mM EDTA were dissolved in ultrapure water. Adjust pH to > 13 and q.s. to 1 L

Arachidonic acid

Arachidonic acid was freshly prepared befored used by dissolving in ultrapure water.

Aspirin (ASA)

ASA was freshly prepared befored used. ASA was dissolved in 0.5 mL DMSO and protected from light.

Bradford reagent

To make 1 L of Bradford reagent, the ingredients including 50 mg Coomasie Brilliant Blue G-250, 25 mL of methanol and 50 mL of 85% phosphoric acid were mixed and adjusted volume to 500 mL with ultrapure water. The solution was filtrated through Whatman No 93 and kept in tight cap can avoid from light at 4ºC.

Citrate buffer pH 5.4

To make 1 L of Citrate buffer, 0.1 M sodium citrate and 0.1 M acetic acid were dissolved in ultrapure water and adjusted the pH to 5.4. The solution was adjusted volume to 1 L. 0.1% (w/v) Triton X-100 was freshly added before use.

Collagen

Collagen was dissolved in ultrapure water. The solution was stirred until completely solubilized and stored at 4 ºC.

1% Low melting agarose (LMA)

To prepare 1% LMA, 10 mg of LMA was dissolved in 1 mL PBS pH 7.4. The solution was heated until complete soluble. Store at 4 ºC and warm at 37 ºC before use.

Lysis buffer for comet assay

To prepare 100 mL of lysis buffer, 2.5 M NaCl, 0.1 M EDTA and 10 mM Tris-base (pH 10) were dissolved and adjusted to 100 mL with ultrapure water. Strored at 4 ºC and 1% v/v Triton X-100 and 10% (v/v) DMSO were freshly added before use.

Lysis buffer for Western blot analysis

To make 30 mL of 2X lysis buffer (40 mM Tris HCl (pH 7.4), 300 mM NaCl, 2% Triton X-100, 2% sodium deoxycholate, 20 mM NaF, 2 mM Pefabloc, 2 mM sodium orthovanadate) for stock solution, the ingredients were dissolved in ultrapure water. The solution was adjusted volume to 30 mL. Before use, 990 µL of the solution were added with 10 µL of protease inhibitor.

Medium of ECV304 cells

M199 powder (1 package) was dissolved with ultrapure water and the 2.2 g sodium hydrogen carbonate was added. The medium was mixed and adjusted pH to 7.2 with HCl. The medium was then adjusted volume to 1 L and further sterilized by filtration with 0.22 µm millipore filter membrane. The medium was added with 10% FBS and 0.1% penicillin-streptomycin.

Phosphate buffered saline (PBS)

The ingredients for 1 L of PBS, 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.15 g Na_2HPO_4, were dissolved in ultrapure water and adjusted the pH to 7.4 with NaOH. The solution was adjusted volume to 1 L.

Running and transfer solution

To make 1 L of 10X running and transfer solution (250 mM Tris (pH 8.3), 1.92 M glycine) for stock solution, the ingredients were dissolved in ultrapure water and adjusted volume to 1 L.

1. Running buffer for Western blot analysis

To make 1 L of 1X running buffer, 100 mL of 10X running and transfer solution was mixed with 10 mL of 10% SDS. The solution was adjusted volume to 1 L with ultrapure water.

2. Transfer buffer for Western blot analysis

To make 1 L of 1X transfer buffer, 80 mL of 10X running and transfer solution was mixed with 220 mL of methanol. The solution was adjusted volume to 1 L with ultrapure water.

Sample buffer for Western blot analysis

To make 50 mL of 5X sample buffer (60 mM Tris-HCl (pH 6.8), 2% SDS, 25% glycerol, 14.4 mM β-mercaptoethanol and 0.1% bromophemol blue) for stock solution, the ingredients were dissolved in ultrapure water. The solution was adjusted volume to 50 mL. 5X sample buffer was aliquot into 1 mL/tube and store at -20 ºC.

Sodium citrate (3.2%)

3.2 g of trisodium citrate was dissolved in ultrapure water. The solution was stirred until completely solubilized, then adjusted volume to 100 mL and stored at room temperature.

Sodium nitropusside (SNP)

SNP was freshly prepared befored used. SNP was dissolved in ultrapure water and protected from light.

Streptokinase

Streptokinase was dissolved in ultrapure water and then aliquot into 0.1 mL/tube and store at -20 ºC.

0.4 M Tris buffer solution pH 7.5

0.4 M Tris was dissolved in 800 mL ultrapure water. Adjust pH to 7.5 and adjust volume with ultrapure water to 1 L.

Tris-buffered saline (TBS) for Western blot analysis

To make 1 L of 10X TBS (100 mM Tris (pH 7.5), 1 M NaCl) for stock solution, 50 mL of 2 M Tris and 87.6 g of NaCl were dissolved in ultrapure water. The solution was adjusted volume to 1 L.

Tris-buffered saline, 0.05% Tween 20 (TBST)

To make 1 L of 1X TBST, 100 mL of 10X TBS was mixed with 0.5 mL of Tween 20. The solution was adjusted volume to 1 L with ultrapure water.

APPENDIX B
TABLES OF EXPERIMENTAL RESULTS

Table 4 The percentage of cell viability of H_2O_2 at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Concentration (uM)	% Cell viability
3.125	106.21 ± 12.06
6.25	107.20 ± 11.05
12.5	96.07 ± 6.45
25	89.23 ± 3.52
50	50.91 ± 13.68
100	1.59 ± 2.33

Table 5 The percentage of cell viability of AD at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Concentration (mg/mL)	% Cell viability	
	ADES	ADWS
0.01	90.53 ± 10.16	95.79 ± 10.75
0.1	85.25 ± 10.67	89.56 ± 2.87
1	85.92 ± 4.59	90.86 ± 0.73
2	72.55 ± 3.83	89.45 ± 5.08
5	6.66 ± 2.89*	40.28 ± 18.12*

Table 6 The percentage of cell viability of AL at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Concentration (mg/mL)	% Cell viability	
	ALES	ALWF
0.01	87.65 ± 17.74	87.19 ± 13.60
0.1	83.70 ± 12.63	85.43 ± 14.19
1	90.43 ± 13.68	89.05 ± 15.80
2	98.30 ± 18.43	88.82 ± 13.70
5	110.90 ± 18.97	91.18 ± 16.80

Table 7 The percentage of cell viability of LS at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Concentration (mg/mL)	% Cell viability	
	LSES	LSWS
0.01	88.81 ± 13.12	89.27 ± 11.23
0.1	79.48 ± 4.82	89.88 ± 9.78
1	84.18 ± 13.39	88.35 ± 4.67
2	82.17 ± 7.92	77.94 ± 9.35
5	15.63 ± 7.40*	6.62 ± 2.08*

Table 8 The percentage of cell viability of AS at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Concentration (mg/mL)	% Cell viability	
	ASES	ASWS
0.01	87.97 ± 5.05	91.59 ± 2.32
0.1	80.26 ± 5.98	82.96 ± 3.21
1	86.56 ± 2.58	86.81 ± 8.15
2	87.68 ± 3.23	88.13 ± 2.91
5	82.19 ± 3.26	84.46 ± 4.00

Table 9 The percentage of cell viability of AV at various concentrations for 24 h measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Concentration (mg/mL)	% Cell viability	
	AVES	AVWS
0.01	80.88 ± 9.42	95.85 ± 13.45
0.1	78.36 ± 4.14	90.38 ± 10.48
1	61.79 ± 6.48*	47.98 ± 9.48*
2	5.08 ± 3.12*	1.47 ± 1.33*
5	1.67 ± 1.00*	0.88 ± 0.60*

Table 10 The IC_{10}, IC_{25} and IC_{50} values of herbal extracts from Phikud Benjakot on ECV304 cells. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate

Samples	IC_{10}	IC_{25}	IC_{50}
ADES	0.90 ± 0.06	1.88 ± 0.21	3.05 ± 0.27
ADWS	1.63 ± 0.26	3.13 ± 0.40	4.85 ± 0.58
ALES	>5	>5	>5
ALWF	>5	>5	>5
LSES	1.63 ± 0.35	2.02 ± 0.30	3.27 ± 0.52
LSWS	1.33 ± 0.29	2.13 ± 0.37	3.08 ± 0.43
ASES	>5	>5	>5
ASWS	>5	>5	>5
AVES	0.04 ± 0.04	0.22 ± 0.05	0.79 ± 0.05
AVWS	0.17 ± 0.11	0.41 ± 0.07	0.87 ± 0.06

Table 11 The percentage of DNA in tail of ECV304 cells of herbal extracts from Phikud Benjakot. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Samples	Concentration (mg/mL)	%DNA in tail
control	0	12.49 ± 7.53
H_2O_2	100 uM	42.58 ± 8.63*
ADES	1	10.39 ± 0.72
	2	12.90 ± 2.72
	3	13.43 ± 4.34
ADWS	1.5	12.71 ± 4.51
	3	11.10 ± 1.76
	5	12.96 ± 3.84
ALES	0.1	7.22 ± 1.97
	1	7.61 ± 0.98
	5	10.80 ± 1.81
ALWF	0.1	10.74 ± 3.17
	1	12.05 ± 4.04
	5	9.36 ± 2.29
LSES	1.5	9.16 ± 3.27
	2	8.44 ± 0.80
	3.5	8.75 ± 1.30
LSWS	1.5	10.13 ± 0.42
	2	10.01 ± 2.42
	3	13.34 ± 3.64
ASES	0.1	8.88 ± 2.99
	1	9.62 ± 2.36
	5	9.57 ± 3.69
ASWS	0.1	7.15 ± 2.01
	1	7.93 ± 0.53
	5	9.53 ± 2.54
AVES	0.05	11.38 ± 2.27
	0.2	11.61 ± 3.48
	0.8	14.29 ± 5.35
AVWS	0.25	20.22 ± 7.16
	0.5	21.19 ± 7.10
	1	18.74 ± 6.50

Table 12 Effect of herbal extracts from Phikud Benjakot on platelet aggregation induced by ADP. PRP were preincubated with the extracts for 5 min before induced by ADP. The results were represented in % maximal aggregation and % inhibition of platelet aggregation, data were expressed as mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

Samples	% max aggregation	% inhibition
NSS	81.0 ± 7.6	-
500 uM ASA	57.8 ± 7.4*	26.4 ± 10.3*
ADES	30.5 ± 6.3*	62.3 ± 8.3*
ADWS	35.2 ± 8.8*	56.8 ± 10.9*
ALES	75.9 ± 0.9	6.1 ± 9.6
ALWF	79.9 ± 1.4	1.4 ± 7.2
LSES	69.1 ± 15.8	15.8 ± 16.4
LSWS	64.3 ± 18.1	22.4 ± 17.1
ASES	50.6 ± 6.3*	37.9 ± 6.4*
ASWS	52.8 ± 15.2*	35.5 ± 17.7*
AVES	29.8 ± 4.8*	63.0 ± 7.2*
AVWS	32.3 ± 14.5*	59.9 ± 59.9*

Table 13 Effect of AV on platelet aggregation induced by arachidonic acid. The results were represented in % maximal aggregation as well as % inhibition of platelet aggregation. Data were expressed as mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

Samples	% Max aggregation	% Inhibition
NSS	84.28 ± 3.56	-
500 uM ASA	6.01 ± 1.53*	92.82 ± 1.96*
AVES	83.41 ± 2.29	0.97 ± 2.15
AVWS	84.36 ± 4.12	-0.07 ± 1.49

Table 14 Effect of AV on platelet aggregation induced by collagen. The results were represented in % maximal aggregation, % inhibition of platelet aggregation and slope. Data were expressed as mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

Sample	% Max aggregation	% Inhibition	Slope
NSS	83.96 ± 2.27	-	142.23 ± 15.15
500 uM ASA	26.04 ± 16.47*	68.46 ± 20.34*	35.63 ± 15.60*
AVES	82.51 ± 3.73	1.75 ± 2.83	97.82 ± 15.40
AVWS	83.69 ± 3.94	0.30 ± 4.189	99.7 ± 16.65

Table 15 Concentration-dependent study of AV on platelet aggregation induced by ADP**.** The results were represented in % maximal aggregation as well as % inhibition of platelet aggregation. Data were expressed as mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

Samples	Concentration (mg/mL)	% Max aggregation	% Inhibition
NSS		2.5 ± 2.5	-
ASA	500 uM	43.3 ± 2.1*	47.6 ± 3.0*
AVES	0.01	82.7 ± 1.5	-0.1 ± 2.9
	0.05	78.9 ± 0.8	4.5 ± 2.9
	0.1	76.7 ± 4.7	7.2 ± 4.0
	0.5	34.8 ± 12.5*	57.9 ± 15.3*
	1	26.5 ± 8.7*	68.0 ± 10.5*
AVWS	0.01	82.9 ± 4.1	-0.4 ± 5.5
	0.05	81.7 ± 3.8	1.1 ± 4.9
	0.1	82.0 ± 2.2	0.8 ± 3.2
	0.5	42.4 ± 18.0*	48.7 ± 22.2*
	1	22.8 ± 3.8	72.4 ± 4.3

Table 16 The percentage of platelet viability of H_2O_2 at various concentrations for 1 h measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Concentration (uM)	% Cell viability
0	100.0 ± 0.0
1000	87.2 ± 5.7
2500	83.9 ± 14.2
5000	83.9 ± 6.3
10000	84.2 ± 6.4
25000	84.5 ± 8.9
50000	67.4 ± 11.3*
100000	22.7 ± 18.4*

Table 17 The percentage of platelet viability of AV at various concentrations for 1 h measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *p < 0.05 compared to the untreated control.

Concentration (mg/mL)	% Cell viability	
	AVES	AVWS
0.01	99.8 ± 3.2	89.1 ± 4.8
0.025	103.8 ± 3.1	93.8 ± 10.0
0.05	94.0 ± 2.5	92.8 ± 9.4
0.1	95.2 ± 6.1	83.3 ± 6.7
0.25	87.9 ± 11.1	77.0 ± 6.0
0.5	89.1 ± 11.7	74.9 ± 8.0
1	76.9 ± 3.1	75.2 ± 11.3
2	67.0 ± 23.5*	55.7 ± 26.1*

Table 18 The percentage of cell viability of H_2O_2 in concentration- and time- dependent manner measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Concentration (mg/mL)	% Cell viability				
	6 h	12 h	24 h	48 h	72 h
6.25	116.4 ± 13.8	103.3 ± 12.1	116.4 ± 10.8	105.2 ± 1.3	94.2 ± 11.6
12.5	112.3 ± 19.1	99.9 ± 12.3	120.0 ± 16.7	105.0 ± 4.6	94.0 ± 16.8
25	99.4 ± 8.2	78.3 ± 3.0*	78.1 ± 8.8*	69.1 ± 1.9*	56.7 ± 10.0*
50	21.2 ± 2.6*	14.2 ± 4.5*	12.8 ± 5.2*	14.5 ± 3.1*	5.5 ± 1.2*
100	-0.8 ± 2.3*	-2.0 ± 1.7*	-0.7 ± 1.8*	1.0 ± 0.7*	0.8 ±0.4*

Table 19 The percentage of cell viability of AVES in concentration- and time- dependent manner measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Concentration (mg/mL)	% Cell viability				
	6 h	12 h	24 h	48 h	72 h
0.01	112.2 ± 1.8	104.3 ± 5.9	118.7 ± 2.0	113.1 ± 8.9	107.0 ± 20.5
0.025	110.0 ± 4.0	100.1 ± 3.4	105.0 ± 2.7	91.1 ± 2.2	76.8 ±
0.05	103.8 ± 1.3	99.4 ± 4.7	106.2 ± 5.1	94.0 ± 4.7	79.8 ± 13.3
0.1	101.1 ± 4.9	103.1 ± 4.7	108.3 ± 3.8	109.7 ± 9.0	89.7 ± 15.6
0.25	97.3 ±1.9	99.6 ± 2.0	104.4 ± 11.1	103.6 ± 6.4	82.6 ± 11.3
0.5	88.2 ± 1.0	89.1 ± 6.3	84.7 ± 7.7	84.9 ± 7.2	60.3 ± 7.3*
1	15.5 ± 4.1*	10.6 ± 4.6*	8.9 ± 6.9*	4.2 ± 2.7*	3.8 ± 3.9*
2	7.9 ± 0.5*	1.3 ± 1.4*	1.5 ± 2.2*	4.4 ± 5.0*	0.5 ± 0.2*
5	25.9 ± 5.1*	5.1 ± 0.7*	4.2 ± 1.6*	2.2 ± 0.7*	1.3 ± 0.5*

Table 20 The percentage of cell viability of AVWS in concentration- and time- dependent manner measured by MTT assay. Each value represented the mean ± S.E.M. of three experiments. Each performed in triplicate. *$p < 0.05$ compared to the untreated control.

Concentration (mg/mL)	% Cell viability				
	6 h	12 h	24 h	48 h	72 h
0.01	108.5 ± 4.4	105.2 ± 11.9	132.2 ± 8.9	120.7 ± 2.8	93.2 ± 10.5
0.025	94.8 ± 3.9	96.3 ± 1.6	98.6 ± 3.8	94.8 ± 4.2	69.8 ± 14.3
0.05	94.6 ± 2.3	108.4 ± 5.6	108.2 ± 3.6	102.2 ± 4.5	77.3 ± 15.3
0.1	103.0 ± 4.3	103.8 ± 2.1	113.3 ± 4.4	114.2 ± 3.4	86.1 ± 18.0
0.25	115.3 ± 5.0	106.1 ± 2.0	111.5 ± 4.8	108.0 ± 6.6	79.2 ± 9.8
0.5	106.6 ± 5.4	100.9 ± 0.8	101.6 ± 1.6	96.0 ± 3.6	66.7 ± 7.4*
1	18.6 ± 0.6*	21.9 ± 0.9*	17.6 ± 7.7*	12.7 ± 6.3*	9.1 ± 6.1*
2	3.2 ± 1.0*	0.1 ± 1.1*	-0.1 ± 1.7*	0.5 ± 0.2*	0.3 ± 0.1*
5	15.2 ± 0.5*	1.8 ± 0.7*	0.7 ± 1.4*	1.3 ± 0.3*	0.9 ± 0.2*

Table 21 Net absorbance of PRP at various concentrations by acid phosphatase assay. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

Number of platelets (cells/well)	Net absorbance
156.25	0.164 ± 0.042
312.5	0.315 ± 0.072
625	0.588 ± 0.133
1250	1.151 ± 0.240
2500	2.124 ± 0.361
5000	3.609 ± 0.340

Table 22 Effect of H_2O_2 on platelet function using acid phosphatase assay. The results were expressed as the percentage of control observed by acid phosphatase activity assay. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

Concentration (mM)	% Relative activity
0	100.00 ± 0.00
0.001	99.77 ± 1.38
0.01	96.48 ± 3.13
0.1	94.61 ± 4.36
1	68.87 ± 8.39*
10	18.97 ± 0.53*
100	3.59 ± 1.60*

Table 23 Effect of AV on platelet function using acid phosphatase assay. The results were expressed as the percentage of control observed by acid phosphatase activity assay. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate). * $p < 0.05$ compared to the untreated control.

Concentration (mg/mL)	% Relative activity	
	AVES	AVWS
0	100 ± 0	100 ± 0
0.01	95.74 ± 1.46	101.32 ± 1.01
0.025	97.00 ± 2.19	94.56 ± 6.22
0.05	94.71 ± 2.64	95.49 ± 8.03
0.1	94.34 ± 2.35	93.05 ± 8.52
0.25	95.69 ± 4.24	95.11 ± 5.67
0.5	94.50 ± 2.44	92.64 ± 5.35
1	90.51 ± 4.69	90.45 ± 1.43
2	97.21 ± 0.81	89.18 ± 5.32

Table 24 Effect of SNP on platelet adhesion using acid phosphatase assay. The results were expressed as the percentage of control. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate) * $p < 0.05$ compared to the untreated control.

Concentration (mM)	% Platelet adhesion
0	100 ± 0
1	97.92 ± 7.30
2.5	94.45 ± 7.58
5	96.58 ± 11.66
7.5	96.45 ± 5.04
10	100.08 ± 12.58
25	89.69 ± 7.57
50	84.39 ± 4.26
75	67.60 ± 7.84*
100	51.72 ± 15.17*

Table 25 Effect of AV on platelet adhesion using acid phosphatase assay. The results were expressed as the percentage of control. Each values represents the mean ± S.E.M. (N =3, each performed in triplicate) * $p < 0.05$ compared to the untreated control.

Concentration (mg/mL)	% Platelet adhesion	
	AVES	AVWS
0	100 ± 0	100 ± 0
0.01	89.79 ± 12.65	116.11 ± 15.43
0.025	90.67 ± 24.55	120.69 ± 24.80
0.05	100.23 ± 33.42	110.90 ± 44.50
0.1	98.85 ± 38.56	97.22 ± 51.32
0.25	116.49 ± 37.40	147.31 ± 45.54
0.5	167.51 ± 46.49	188.36 ± 50.66
1	317.31 ± 62.60*	266.50 ± 75.72*
2	101.30 ± 24.58	347.25 ± 114.43*

Table 26 The percentage of clot turbidity by streptokinase in plasma. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

Concentration (IU/mL)	% Clot turbildity						
	0 h	0.5 h	1 h	2 h	3 h	4 h	5 h
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
10	100.0 ± 0.0	105.0 ± 1.3	109.4 ± 4.8	105.2 ± 1.7	103.9 ± 1.7	103.2 ± 1.4	102.5 ± 1.3
25	100.0 ± 0.0	105.7 ± 3.0	108.6 ± 5.3	105.3 ± 1.6	104.8 ± 1.7	103.6 ± 1.6	102.5 ± 1.2
50	100.0 ± 0.0	97.3 ± 8.4	109.9 ± 6.3	105.8 ± 2.8	91.0 ± 1.9	74.9 ± 2.6	69.2 ± 1.8*
100	100.0 ± 0.0	115.4 ± 27.5	93.8 ± 11.7	73.0 ± 15.8*	64.6 ± 15.6*	56.2 ± 14.4*	50.6 ± 4.6*
250	100.0 ± 0.0	94.2 ± 6.2	71.4 ± 28.3	61.9 ± 26.8*	55.2 ± 25.2*	49.1 ± 23.6*	43.9 ± 21.8*
500	100.0 ± 0.0	80.4 ± 4.1	70.8 ± 13.9*	63.1 ± 14.2*	55.6 ± 12.8*	49.8 ± 11.5*	46.7 ± 9.5*
1000	100.0 ± 0.0	64.2 ± 9.8*	61.8 ± 20.5*	54.0 ± 20.4*	47.3 ± 17.6*	43.7 ± 14.5*	41.0 ± 13.2*
2000	100.0 ± 0.0	77.7 ± 3.4*	60.7 ± 21.0*	52.7 ± 26.3*	48.6 ± 25.7*	40.7 ± 21.3*	36.1 ± 18.2*

Table 27 The percentage of clot turbidity by streptokinase in BSA. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

Concentration (IU/mL)	% Clot turbildity						
	0 h	0.5 h	1 h	2 h	3 h	4 h	5 h
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
10	100.0 ± 0.0	88.6 ± 7.7	86.6 ± 8.4	85.9 ± 8.7	85.6 ± 8.7	85.3 ± 8.8	84.9 ± 8.9
25	100.0 ± 0.0	81.7 ± 14.9	75.6 ± 11.5	71.8 ± 10.5*	70.2 ± 10.5*	68.7 ± 10.8*	67.3 ± 11.1*
50	100.0 ± 0.0	59.4 ± 8.3*	56.3 ± 9.9*	53.7 ± 9.6*	50.5 ± 9.2*	47.5 ± 8.8*	44.5 ± 8.7*
100	100.0 ± 0.0	53.2 ± 10.7*	50.1 ± 11.9*	45.1 ± 11.7*	38.8 ± 10.8*	33.0 ± 9.5*	27.8 ± 8.5*
250	100.0 ± 0.0	42.0 ± 23.4*	38.1 ± 21.9*	29.3 ± 16.6*	23.3 ± 13.3*	18.9 ± 10.7*	15.4 ± 8.4*
500	100.0 ± 0.0	39.2 ± 14.3*	30.8 ± 14.4*	17.2 ± 14.0*	12.8 ± 9.8*	10.6 ± 7.5*	9.4 ± 5.9*
1000	100.0 ± 0.0	39.3 ± 16.1*	31.1 ± 16.1*	20.4 ± 15.3*	12.9 ± 9.4*	8.4 ± 5.3*	6.8 ± 4.1*
2000	100.0 ± 0.0	57.9 ± 10.2*	51.9 ± 7.7*	30.0 ± 12.5*	19.2 ± 9.5*	12.8 ± 7.0*	9.4 ± 3.3*

Table 28 The percentage of clot turbidity by AV in plasma. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

Samples	Concentration (mg/mL)	% Clot turbildity						
		0 h	0.5 h	1 h	2 h	3 h	4 h	5 h
AVES	0.01	100.0 ± 0.0	102.3 ± 1.8	101.3 ± 1.1	100.7 ± 0.3	100.8 ± 0.6	101.1 ± 0.7	101.9 ± 0.9
	0.025	100.0 ± 0.0	104.6 ± 2.1	102.7 ± 1.8	101.4 ± 0.6	101.5 ± 0.9	101.7 ± 0.9	101.9 ± 0.9
	0.05	100.0 ± 0.0	104.6 ± 2.5	102.2 ± 0.6	101.1 ± 0.9	100.7 ± 0.7	100.9 ± 0.7	101.0 ± 0.8
	0.1	100.0 ± 0.0	106.1 ± 2.2	103.9 ± 1.9	101.9 ± 2.2	100.7 ± 0.4	100.9 ± 0.4	101.0 ± 0.4
	0.25	100.0 ± 0.0	108.1 ± 2.2	105.6 ± 2.0	103.5 ± 2.9	101.1 ± 0.8	101.3 ± 0.8	101.5 ± 0.8
	0.5	100.0 ± 0.0	109.4 ± 1.6	107.3 ± 2.3	104.5 ± 4.2	100.7 ± 0.8	100.9 ± 0.8	101.0 ± 0.9
	1	100.0 ± 0.0	108.2 ± 1.3	108.6 ± 2.1	104.0 ± 3.0	99.6 ± 0.1	100.3 ± 0.6	100.5 ± 0.5
	2	100.0 ± 0.0	107.4 ± 3.7	107.7 ± 3.2	99.9 ± 2.9	93.9 ± 1.6	95.0 ± 1.1	95.4 ± 1.5
AVWS	0.01	100.0 ± 0.0	102.6 ± 2.1	102.1 ± 0.4	101.4 ± 1.1	100.6 ± 0.7	100.8 ± 0.7	100.9 ± 0.7
	0.025	100.0 ± 0.0	106.6 ± 2.6	104.4 ± 2.4	102.2 ± 1.4	101.5 ± 0.9	101.6 ± 0.9	101.7 ± 0.9
	0.05	100.0 ± 0.0	107.2 ± 1.5	104.9 ± 2.4	101.7 ± 3.2	99.5 ± 1.3	99.7 ± 1.2	99.8 ± 1.3
	0.1	100.0 ± 0.0	109.7 ± 2.1	106.7 ± 2.7	104.2 ± 3.6	101.2 ± 0.4	101.3 ± 0.4	101.3 ± 0.6
	0.25	100.0 ± 0.0	111.9 ± 1.0	109.0 ± 2.6	105.3 ± 3.7	101.4 ± 0.2	101.6 ± 0.2	101.7 ± 0.3
	0.5	100.0 ± 0.0	113.0 ± 1.6	112.7 ± 2.9	106.2 ± 3.7	101.4 ± 1.3	101.7 ± 1.5	101.8 ± 1.2
	1	100.0 ± 0.0	111.4 ± 1.5	111.2 ± 1.7	103.4 ± 2.7	98.2 ± 0.4	98.8 ± 0.9	99.1 ± 0.4
	2	100.0 ± 0.0	115.7 ± 3.1	112.1 ± 1.3	101.2 ± 3.1	94.8 ± 2.1	96.0 ± 0.8	97.1 ± 2.1

Table 29 The percentage of clot turbidity by AV in BSA. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate).

Sample	Concentration (mg/mL)	% Clot turbildity						
		0 h	0.5 h	1 h	2 h	3 h	4 h	5 h
AVES	0.01	100.0 ± 0.0	101.1 ± 0.	101.3 ±0.8	101.6 ± 1.0	102.0 ± 0.7	102.5 ± 0.7	103.0 ± 0.6
	0.025	100.0 ± 0.0	101.8 ± 0.7	102.3 ± 1.1	102.4 ± 1.1	102.5 ± 0.9	103.0 ± 1.1	103.4 ± 0.9
	0.05	100.0 ± 0.0	100.0 ± 1.5	100.2 ± 1.4	100.5 ± 1.3	100.6 ± 1.4	101.3 ± 1.4	101.9 ± 1.1
	0.1	100.0 ± 0.0.	99.4 ± 1.1	99.7 ± 1.0	100.1 ± 0.9	100.2 ± 1.0	101.1 ± 1.2	101.5 ± 0.9
	0.25	100.0 ± 0.0	97.8 ± 0.6	98.5 ± 0.6	99.3 ± 0.6	99.7 ± 0.	100.4 ± 0.3	100.9 ± 0.2
	0.5	100.0 ± 0.0	94.7 ± 3.1	95.7 ± 3.4	97.2 ± 2.4	98.0 ± 2.1	98.7 ± 2.0	99.3 ± 1.9
	1	100.0 ± 0.0	93.0 ± 2.5	94.5 ± 2.9	96.6 ± 2.2	97.7 ± 1.3	98.6 ± 1.3	99.4 ± 0.8
	2	100.0 ± 0.0	87.3 ± 2.5	89.4 ± 2.9	92.3 ± 2.5	93.5 ± 1.1	94.9 ± 1.5	96.9 ± 1.6
AVWS	0.01	100.0 ± 0.0	101.8 ± 2.6	101.9 ± 2.4	102.0 ±2.2	102.1 ± 2.2	102.6 ± 2.1	102.9 ± 1.9
	0.025	100.0 ± 0.0	101.6 ± 2.4	101.7 ± 2.7	101.7 ± 2.6	101.6 ± 2.	102.4 ± 2.8	102.7 ± 2.6
	0.05	100.0 ± 0.0	100.4 ± 1.4	100.1 ± 1.6	100.5 ± 2.0	100.5 ± 2.1	101.4 ± 2.3	101.7 ± 2.2
	0.1	100.0 ± 0.0	101.0 ± 0.6	101.1 ± 0.7	101.5 ± 0.9	101.8 ± 0.8	102.3 ± 0.9	102.7 ± 0.8
	0.25	100.0 ± 0.0	100.2 ± 2.0	100.6 ± 2.2	101.2 ± 2.9	101.5 ± 2.8	102.0 ± 3.0	102.6 ± 3.1
	0.5	100.0 ± 0.0	96.5 ± 2.1	97.3 ± 2.2	98.3 ± 2.2	98.8 ± 1.8	99.7 ± 1.6	100.4 ± 1.3
	1	100.0 ± 0.0	96.2 ± 1.7	97.1 ± 2.3	98.4 ± 2.1	99.1 ± 1.0	99.8 ± 1.3	100.7 ± 0.9
	2	100.0 ± 0.0	89.6 ± 4.1	91.6 ± 5.0	93.7 ± 4.0	95.2 ± 2.3	96.9 ± 1.8	99.0 ± 2.1

Table 30 Effect of AVES on phosphorylation of Akt and eNOS in ECV304 cells. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate)

Concentration (mg/mL)	eNOS Phosphorylation (fold increase)	AKT Phosphorylation (fold increase)
0	1.00 ± 0.00	1.00 ± 0.00
0.25	0.79 ± 0.18	1.06 ± 0.25
0.5	0.88 ± 0.32	1.12 ± 0.17
1	0.83 ± 0.18	1.18 ± 0.26

Table 31 Concentration-dependent study of AVES on phosphorylation of Akt and eNOS in ECV304 cells. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate)

Concentration (mg/mL)	eNOS Phosphorylation (fold increase)	AKT Phosphorylation (fold increase)
0	1.00 ± 0.00	1.00 ± 0.00
0.25	0.90 ± 0.23	0.95 ± 0.21
0.5	1.37 ± 0.15*	0.84 ± 0.10
1	0.95 ± 0.16	0.90 ± 0.19

Table 32 Time-dependent study of AVES on phosphorylation of Akt and eNOS in ECV304 cells. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate)

Time (h)	eNOS Phosphorylation (fold increase)	AKT Phosphorylation (fold increase)
0	1.00 ± 0.00	1.00 ± 0.00
0.5	1.19 ± 0.20	1.26 ± 0.21
1	1.40 ± 0.13*	1.22 ± 0.18
2	1.25 ± 0.25	0.67 ± 0.10

Table 33 Effect of AVES on platelet secreted protein induced phosphorylation of Akt and eNOS in ECV304 cells. Results were represented as mean ± S.E.M. (N =3, each performed in triplicate)

Samples	eNOS Phosphorylation (fold increase)	AKT Phosphorylation (fold increase)
PPP	1.00 ± 0.00	1.00 ± 0.00
PPP+ADP	1.04 ± 0.30	0.82 ± 0.22
PPP+ADP+AVES 0.25 mg/mL	0.97 ± 0.28	0.94 ± 0.14
PPP+ADP+AVES 0.5 mg/mL	0.78 ± 0.20	1.21 ± 0.37
PPP+ADP+AVES 1 mg/mL	0.76 ± 0.26	0.85 ± 0.25

APPENDIX C
HUMAN RESEARCH ETHICS

Protocol Review No. 11-33-012

Study Protocol Approval

The Ethics Committee of The Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol dated and/ or amended as follows:

Study Title: Antiplatelet aggregation and fibrinolytic activity of herbal extracts from Phikud Kot

Study Code: -

Centre: CHULALONGKORN UNIVERSITY

Principal Investigator : Weeraya Kaewprem

A list of the Ethics Committee members and positions present at the Ethics Committee meeting on the date of approval of this study has been attached.

This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Chairman of Ethics Committee: .....Parkpoom Tengamnuay.....

(Parkpoom Tengamnuay, Ph.D.)

Secretary of Ethics Committee: .....Suyanee Pongthananikorn.....

(Suyanee Pongthananikorn, Ph.D.)

Date of Approval: May 20, 2011

Date of Approval Expiration: May 20, 2012

เอกสารแนบ 1

หนังสือแสดงความยินยอม

(Consent Form)

การศึกษาวิจัยเรื่อง (ภาษาไทย) ฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและฤทธิ์สลายลิ่มเลือดของสารสกัดสมุนไพรจากพิกัดโกฐ

(ภาษาอังกฤษ) ANTIPLATELET AGGREGATION AND FIBRINOLYTIC ACTIVITY OF HERBAL EXTRACTS FROM PHIKUD KOT

วันให้คำยินยอม วันที่..... เดือน..... พ.ศ. .....

ข้าพเจ้า (นาย/ นาง/ นางสาว) ..... **นามสกุล** .....

อยู่บ้านเลขที่ ..... **ซอย** ..... **ถนน** ..... **แขวง/ ตำบล** .....

เขต/ อำเภอ ..... **จังหวัด** ..... **รหัสไปรษณีย์** .....

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ **ข้าพเจ้าได้รับเอกสารข้อมูลคำอธิบาย/ คำชี้แจงสำหรับอาสาสมัครที่เข้าร่วมการวิจัย** จากผู้วิจัยให้ทราบถึงวัตถุประสงค์ของการวิจัย วิธีวิจัย อันตรายหรืออาการข้างเคียงที่อาจเกิดขึ้นจากการวิจัย รวมทั้งประโยชน์ที่เกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว

ผู้วิจัยได้ตอบคำถามต่าง ๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจ ไม่ปิดบัง ซ้อนเร้น จนข้าพเจ้าพอใจ

ข้าพเจ้าเข้าร่วมโครงการนี้โดยความสมัครใจและมีสิทธิที่จะบอกเลิกการเข้าร่วมโครงการวิจัยนี้เมื่อใดก็ได้ โดยไม่มีผลกระทบใด ๆ ทั้งสิ้นต่ออาสาสมัคร

ผู้วิจัยรับรองว่า “จะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าไว้เป็นความลับ และจะเปิดเผยได้เฉพาะในรูปที่เป็นสรุปผลการวิจัย” เท่านั้น

ในการวิจัยครั้งนี้ ข้าพเจ้ายอมให้ผู้วิจัยเจาะเลือดเพียงครั้งเดียวซึ่งจะทำการเจาะจากหลอดเลือดดำที่แขน 30 มิลลิลิตร (2 ช้อนโต๊ะ)

ผู้วิจัยได้อธิบายให้ข้าพเจ้าทราบและเข้าใจแล้วว่าการเจาะเลือดเพียงเล็กน้อย โดยทั่วไปจะไม่เกิดอันตรายใด ๆ แก่ข้าพเจ้าเลย นอกจากอาจมีรอยช้ำบริเวณที่เจาะเพียงเล็กน้อย ซึ่งอาจหายได้เองภายใน 7 วัน

ผู้วิจัยรับรองว่า หากเกิดอันตรายใด ๆ จากการวิจัยดังกล่าว ข้าพเจ้าจะได้รับการรักษาพยาบาลโดยไม่คิดมูลค่า และจะได้รับการชดเชยรายได้ที่สูญเสียไประหว่างการรักษาพยาบาลดังกล่าว ตลอดจนเงินทดแทนความพิการที่อาจจะเกิดขึ้น และรายละเอียดเกี่ยวกับการรักษาพยาบาลหรือเงินชดเชยดังกล่าว โดยข้าพเจ้าสามารถติดต่อได้ที่ ภาควิชาชีวเคมีและจุลชีววิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย โดยบุคคลที่รับผิดชอบเรื่องนี้คือ นางสาววีรยา แก้วเปรม หมายเลขโทรศัพท์ 086-733-4217

ข้าพเจ้าได้อ่านข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ จึงได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจ

ลงนาม ..... ผู้ให้ความยินยอม

(.....) ชื่อตัวบรรจง

วันที่ …… เดือน …….. พ.ศ. ……

ลงนาม ..... ผู้รับผิดชอบการวิจัย

(.....) ชื่อตัวบรรจง

วันที่ …… เดือน …….. พ.ศ. ……

ลงนาม ..... พยาน

(.....) ชื่อตัวบรรจง

วันที่ …… เดือน …….. พ.ศ. ……

ลงนาม ..... พยาน

(.....) ชื่อตัวบรรจง

วันที่ …… เดือน …….. พ.ศ. ……

เอกสารแนบ 2

เอกสารข้อมูลคำอธิบาย/ คำชี้แจงสำหรับอาสาสมัครที่เข้าร่วมการวิจัย

(Patient or Participant Information Sheet)

ชื่อโครงการศึกษาวิจัยเรื่อง	ฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและฤทธิ์สลายลิ่มเลือดของสารสกัดสมุนไพรจากพิกัดโกฐ
ชื่อผู้วิจัยหลัก	นางสาววีรยา แก้วเปรม
หน่วยงานที่ทำการศึกษาวิจัย	ภาควิชาชีวเคมีและจุลชีววิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
โทรศัพท์ (สามารถติดต่อได้ 24 ชั่วโมง)	086-733-4217

บุคคลและวิธีการติดต่อเมื่อมีเหตุฉุกเฉินหรือความผิดปกติที่เกี่ยวข้องกับการวิจัย (ติดต่อได้ 24 ชั่วโมง)

นางสาววีรยา แก้วเปรม โทร 086-733-4217 (ผู้ดำเนินโครงการวิจัย)
รองศาสตราจารย์ นายแพทย์ ดร. พลภัทร โรจน์นครินทร์ โทรศัพท์ 085-911-5211 (แพทย์ผู้ดูแล)

ท่านได้รับเชิญให้เข้าร่วมการศึกษาวิจัยเรื่อง “ฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและฤทธิ์สลายลิ่มเลือดของสารสกัดสมุนไพรจากพิกัดโกฐ” ก่อนที่ท่านจะตัดสินใจให้ความยินยอมเข้าร่วมการศึกษาวิจัยนี้ ผู้วิจัยใคร่ขอชี้แจงรายละเอียดของโครงการวิจัยให้ท่านทราบ และขอให้ท่านทำความเข้าใจขั้นตอนที่ผู้วิจัยจะขอให้ท่านปฏิบัติ ขั้นตอนนี้เป็น “กระบวนการให้คำยินยอม” ก่อนเริ่มดำเนินการวิจัย ผู้วิจัยจะขอทำการสอบถามข้อมูล เพื่อดูว่าท่านมีสุขภาพดีตามเกณฑ์คัดเลือกเข้าร่วมการวิจัย หากท่านผ่านการตรวจคัดกรองแล้วพบว่าท่านมีความเหมาะสมที่จะเข้าร่วมการวิจัย ผู้วิจัยจะดำเนินการเป็นขั้นตอนดังจะชี้แจงให้ทราบต่อไป

กรุณาอ่านข้อมูลต่อไปนี้ด้วยความรอบคอบ และสอบถามถึงข้อสงสัยต่าง ๆ โดยไม่ลังเล

1. บทนำ

ปัจจุบันประเทศไทยได้นำเข้ายาต้านการเกาะกลุ่มของเกล็ดเลือด clopidogrel เป็นจำนวนมาก เพื่อใช้ในการรักษาภาวะการอุดตันของหลอดเลือด ได้แก่ โรคเส้นเลือดหัวใจอุดตัน (coronary artery disease) โรคหลอดเลือดแดงส่วนปลายตีบ (peripheral arterial disease) และโรคหลอดเลือด

สมองอุดตัน (cerebrovascular disease เช่น stroke) แม้ว่าจะมีการต่อรองราคายานำเข้าโดยการใช้มาตรการการใช้สิทธิต่อยาที่มีสิทธิบัตร แต่ยาที่นำเข้าก็ยังมีราคาค่อนข้างสูง ซึ่งมีผลให้ผู้ป่วยต้องรับภาระค่าใช้จ่ายในการรักษามากขึ้น และถึงแม้ว่าจะมียาต้านการเกาะกลุ่มของเกล็ดเลือดชนิดอื่นที่มีราคาต่ำกว่า อย่างเช่น aspirin แต่พบว่าเป็นยาที่ก่อให้เกิดอาการแพ้และมีผลข้างเคียงสูง เช่น ระคายเคืองและทำให้เกิดแผลในกระเพาะอาหาร ทำให้มีความจำเป็นที่จะต้องใช้ยาต้านการเกาะกลุ่มของเกล็ดเลือดกลุ่มอื่น เพื่อใช้ในผู้ป่วยมีอาการแพ้หรือไม่สามารถทนต่อผลข้างเคียงของ aspirin เมื่อพิจารณากลไกการออกฤทธิ์พบว่ายาทั้งสองชนิดมีกลไกการออกฤทธิ์แตกต่างกันโดย clopidogrel นั้นออกฤทธิ์ต้านการจับตัวของ ADP กับ receptor $P2Y_{12}$ ที่เซลล์เกล็ดเลือด ส่งผลไปยับยั้งการเกาะกลุ่มของเกล็ดเลือดผ่านทาง glycoprotein IIb/IIIa pathway ส่วน aspirin จะไปยับยั้งการทำงานของเอนไซม์ cyclooxygenase ส่งผลไปลดการสร้าง thromboxane A2 ทำให้เกิดการยับยั้งการเกาะกลุ่มของเกล็ดเลือด และรักษาภาวะการอุดตันของหลอดเลือด

รายงานองค์การอนามัยโลกพบว่าโรคระบบไหลเวียนโลหิตเป็นสาเหตุการตายหลักของประชากรโลก โดยติด 1 ใน 5 อันดับแรกของสาเหตุการตายในปี 2005 ทั้งโรคหลอดเลือดหัวใจและโรคหลอดเลือดสมอง (WHO, 2007) และยังเป็นปัญหาสาธารณสุขของประเทศไทยเช่นกัน เนื่องจากอัตราการเสียชีวิตด้วยโรคหัวใจและหลอดเลือดในประเทศไทยเพิ่มสูงขึ้น อันเนื่องมาจากพฤติกรรมการบริโภคอาหารที่มีไขมันสูง ทำให้เกิดภาวะไขมันในเลือดสูงและโรคอ้วนตามมา เพิ่มอัตราเสี่ยงต่อผนังหลอดเลือดโดยเสียสภาพขาดการยืดหยุ่น หลอดเลือดฉีกขาดง่าย ซึ่งจะส่งผลกระตุ้นเกล็ดเลือดมายึดเกาะกับผนังหลอดเลือดและเกาะกลุ่มกัน จึงทำให้ต้องใช้ยาที่มีฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือด เพื่อใช้ในการรักษาโรคระบบไหลเวียนโลหิตเพิ่มสูงขึ้นตามไปด้วย นอกจากนี้พยาธิสภาพภายนอกที่บ่งชี้ว่าเริ่มเข้าสู่ปัจจัยเสี่ยงของโรคระบบไหลเวียนโลหิตอีกประการหนึ่งคือ ภาวะเส้นเลือดขอดตามข้อพับเข่า (thrombophlebitis) ที่มีอุบัติการณ์เพิ่มขึ้นตามอายุและน้ำหนักตัวที่เพิ่มขึ้น ภาวะเส้นเลือดขอดเกิดจากการเกาะกลุ่มของเกล็ดเลือดที่บริเวณเส้นเลือดที่ข้อพับเข่า และมีไฟบรินเข้ามาทำให้เกล็ดเลือดเกาะกลุ่มแน่นยิ่งขึ้นและสลายได้ยาก ก่อให้เกิดการอักเสบของเส้นเลือดบริเวณนั้น การรักษาภาวะเส้นเลือดขอดสามารถทำได้โดยการผ่าตัดหรือให้ยาสลายลิ่มเลือด แต่ปัญหาที่พบบ่อยคือการกลับมาเป็นซ้ำอีก ปัจจุบันได้มีการพัฒนานำเลเซอร์เข้ามาใช้ในการรักษาพบว่าโอกาสกลับมาเป็นซ้ำต่ำแต่ก็มีค่าใช้จ่ายสูง จึงเกิดแนวคิดในการศึกษาฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและฤทธิ์สลายลิ่มเลือดของสมุนไพร ที่อาจพัฒนาเป็นผลิตภัณฑ์ที่ใช้ในการรักษาและป้องกันการกลับมาเป็นซ้ำของภาวะเส้นเลือดขอดรวมทั้งโรคระบบไหลเวียนโลหิต ซึ่งจะช่วยลดค่าใช้จ่ายในการรักษาของผู้ป่วยได้ ปัจจุบันมีการศึกษาวิจัยพบว่าสารสกัดจากสมุนไพรหลายชนิดสามารถลดการเกาะกลุ่มของเกล็ดเลือดได้ เช่น ขมิ้นชัน

(Lee, 2006) ชิง (Nurtjahja-Tjendraputra *et al.*, 2003) และชาเขียว (Yen *et al*., 2004) โครงการวิจัยนี้จึงสนใจที่จะคัดเลือกสมุนไพรในตำรับยาสามัญประจำบ้านแผนโบราณ (ประกาศกระทรวงสาธารณสุข เรื่อง ยาสามัญประจำบ้านแผนโบราณ พ.ศ. 2542) ซึ่งน่าจะมีความปลอดภัยระดับหนึ่ง เนื่องจากมีประวัติการใช้เป็นระยะเวลานานตามภูมิปัญญายาแผนโบราณ ตัวอย่างเช่น สมุนไพรในพิกัดโกฐ (โกฐสอ โกฐเขมา โกฐหัวบัว โกฐเชียง และโกฐจุฬาลัมพา) ซึ่งเป็นตัวยาในตำรับยาหอมเป็นยาแก้ลมวิงเวียน จากกรอบแนวคิดดังกล่าวข้างต้นโครงการวิจัยนี้จึงมีวัตถุประสงค์ที่จะนำสมุนไพรในตำรับยาสามัญประจำบ้านแผนโบราณมาพิสูจน์ทางวิทยาศาสตร์โดยจะศึกษาฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและฤทธิ์สลายลิ่มเลือดของสารสกัดสมุนไพร และจะคัดกรองเฉพาะสารสกัดที่มีฤทธิ์ต้านการเกาะกลุ่มเลือดอย่างมีนัยสำคัญมาศึกษากลไกการออกฤทธิ์ โครงการวิจัยนี้คาดว่าจะได้ข้อพิสูจน์ทางวิทยาศาสตร์นำมาอธิบายองค์ความรู้ภูมิปัญญาของสมุนไพรในตำรับยาแผนโบราณแล้ว ยังนำมาสนับสนุนผลิตภัณฑ์ยาสมุนไพรที่มีจำหน่ายในท้องตลาดในปัจจุบัน ให้มีศักยภาพมากพอก็จะพัฒนาเป็นผลิตภัณฑ์สุขภาพรูปแบบใหม่ ๆ เพื่อลดอุบัติการณ์ของโรคหลอดเลือดและประชาชนสามารถเข้าถึงยาได้ง่าย อีกทั้งช่วยลดการนำเข้าเวชภัณฑ์จากต่างประเทศ รวมทั้งได้ข้อมูลปัญหาอาการข้างเคียงที่อาจเกิดขึ้นหากบริโภคสมุนไพรเป็นประจำ

2. วัตถุประสงค์ของการวิจัย

เพื่อศึกษาฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดและกลไกการออกฤทธิ์รวมถึงฤทธิ์สลายลิ่มเลือดของสารสกัดสมุนไพรจากพิกัดโกฐ

3. วิธีการศึกษาวิจัย

ผู้วิจัยจะทำการสอบถามข้อมูลจากท่านเพื่อคัดกรองว่าท่านมีคุณสมบัติที่เหมาะสมตามเกณฑ์หรือไม่ หากท่านมีคุณสมบัติเหมาะสมตามเกณฑ์ของโครงการวิจัยในครั้งนี้ คือเป็นผู้ที่มีสุขภาพดี ไม่เป็นโรคที่มีผลต่อการเกาะกลุ่มของเกล็ดเลือดและโรคที่เกี่ยวกับเม็ดเลือด เช่น โรคธาลัสซีเมีย ไม่ดื่มสุราเป็นประจำหรือสูบบุหรี่ และไม่ได้ใช้ยาใด ๆ ก่อนการเจาะเลือดเป็นเวลาอย่างน้อย 14 วัน ผู้วิจัยจะดำเนินการตามขั้นตอนดังนี้

ผู้วิจัยจะขอเจาะเลือดจากท่านซึ่งจะทำการเจาะจากหลอดเลือดดำที่แขนเพียง 1 ครั้ง ปริมาณ 30 มิลลิลิตร (2 ช้อนโต๊ะ) โดยพยาบาลวิชาชีพ ซึ่งตัวอย่างเลือดของท่านจะถูกนำไปใช้ในการศึกษาวิจัยในโครงการนี้เท่านั้น

4. ความเสี่ยง ความไม่สบาย และผลข้างเคียงที่อาจเกิดขึ้น

ท่านอาจมีอาการเจ็บในขณะเจาะเลือด และอาจเกิดการเขียวช้ำในบริเวณที่เจาะเพียงเล็กน้อย ซึ่งจะเป็นอยู่เพียงไม่กี่วันก็จะหายไปเอง (โปรดสอบถามแพทย์ผู้ดูแลหากท่านมีข้อสงสัย) ผู้วิจัยขอให้ท่านรายงานผลข้างเคียงทุกอย่างที่เกิดขึ้นให้กับพยาบาลผู้เจาะเลือดทราบทันที

5. ผลประโยชน์ที่อาจจะได้รับ

ท่านจะไม่ได้รับผลประโยชน์โดยตรงจากการเข้าร่วมการวิจัย แต่องค์ความรู้ที่ได้จากการวิจัยนี้จะถูกนำไปใช้ในการค้นคว้าและพัฒนายาสมุนไพรที่มีประสิทธิภาพในการรักษาความผิดปกติเกี่ยวกับเกล็ดเลือดให้ทัดเทียมกับยาแผนปัจจุบัน อันจะนำมาซึ่งการพัฒนาทางการแพทย์และทางเศรษฐกิจตามมา

6. ทางเลือกอื่นในการรักษา

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7. ค่าใช้จ่ายและค่าชดเชย

ท่านจะไม่ได้รับค่าตอบแทนในการเข้าร่วมงานวิจัยนี้

8. เงินชดเชยสำหรับการบาดเจ็บหรืออันตรายที่อาจเกิดขึ้น

หากเกิดอันตรายใด ๆ จากการเข้าร่วมการศึกษาวิจัยนี้ ขณะกำลังเจาะเลือด และ/หรือภายใน 1 สัปดาห์นับจากวันเจาะเลือด ท่านจะได้รับการรักษาพยาบาลและจะได้รับการชดเชยรายได้ที่ได้สูญเสียไประหว่างการรักษาพยาบาล โดยบุคคลผู้รับผิดชอบในการออกค่าใช้จ่าย คือ นางสาววีรยา แก้วเปรม ภาควิชาชีวเคมีและจุลชีววิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย โทร. 086-733-4217 และแพทย์ผู้ดูแลคือ รองศาสตราจารย์ นายแพทย์ ดร. พลภัทร โรจน์นครินทร์ ภาควิชาอายุรศาสตร์ สาขาโลหิตวิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย โทร. 02-256-4564 ต่อ 104

9. สิทธิในการถอนตัวออกจากการศึกษาวิจัย

ท่านมีสิทธิ์ในการถอนตัวออกจากการเข้าร่วมโครงการวิจัยนี้เมื่อใดก็ได้ โดยไม่มีผลกระทบใด ๆ ทั้งสิ้น

10. การรักษาความลับของบันทึกทางการแพทย์ และข้อมูลการศึกษาวิจัย

ข้อมูลเกี่ยวกับตัวท่านจะถูกเก็บไว้เป็นความลับ จะไม่มีชื่อท่าน หรือข้อมูลที่สามารถบ่งชี้ถึงตัวท่านในเอกสารบันทึกข้อมูล ชื่อของท่านจะถูกระบุเป็นอักษรย่อและหมายเลขประจำตัววิจัยและจะไม่ระบุถึงตัวท่านในรายงานหรือสิ่งพิมพ์ใด ๆ ที่เป็นผลจากการวิจัยนี้

11. การเปิดเผยข้อมูลการศึกษาวิจัย

จะเปิดเผยข้อมูลเฉพาะในรูปที่เป็นการสรุปผลวิจัยเท่านั้น

12. การสอบถามข้อสงสัย

หากท่านมีคำถามเกี่ยวกับการวิจัยโปรดติดต่อนางสาววีรยา แก้วเปรม ภาควิชาชีวเคมีและจุลชีววิทยา คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ผู้ดำเนินงานวิจัยได้ตลอดเวลา 24 ชั่วโมงได้ที่หมายเลขโทรศัพท์ 086-733-4217

โครงการวิจัยนี้ได้รับการรับรองทางด้านจริยธรรมการทำวิจัยในคนจากคณะกรรมการพิจารณาจริยธรรมคณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย หากผู้วิจัยไม่ปฏิบัติตามที่ชี้แจงในเอกสารข้อมูลคำอธิบาย ท่านสามารถร้องเรียนมายังคณะกรรมการพิจารณาจริยธรรมการศึกษาวิจัยในมนุษย์ คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย โทร.02-218-8256

การขอให้ท่านลงนามในหนังสือแสดงความยินยอม ไม่มีผลทำให้ท่านสูญเสียสิทธิทางกฎหมายใด ๆ ที่ท่านพึงได้รับจากการเข้าร่วมการวิจัยนี้

VITA

Miss Weeraya Kaewprem was born on May 3, 1986 in Samutprakan Thailand. In 2008, she recieved Bachelor of Science in Botany (Honours program) from the Faculty of Science, Chulalongkorn University. After graduation, she entered the Master's degree program in Biomedicinal Chemistry at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.