IN VITRO ANTIPLATELET ACTIVITY OF LUSIANTHRIDIN IN HUMAN PLATELETS

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ฤทธิ์ต้านเกล็ดเลือดในหลอดทดลองของลูเซียนทรีดินในเกล็ดเลือดมนุษย์

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ฮลา นู สเว : ฤทธิ์ต้านเกล็ดเลือดในหลอดทดลองของลูเซียนทรีดินในเกล็ดเลือดมนุษย์. (*IN VITRO* ANTIPLATELET ACTIVITY OF LUSIANTHRIDIN IN HUMAN PLATELETS) อ.ที่ปรึกษาหลัก : รศ. ภญ. ดร.รัตยา ลือชาพุฒิพร, อ.ที่ปรึกษาร่วม : ศ. นพ. ดร.พลภัทร โรจน์นครินทร์

ลูเซียนทรีดินเป็นอนุพันธ์ของฟีแนนทรีนแยกได้จาก *Dendrobium venustum* สารประกอบฟีแนนทรีนบางชนิดมีฤทธิ์ต้านการเกาะกลุ่มของเกล็ดเลือดผ่านวิถีทางที่ยังไม่ทราบ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ยับยั้งและกลไกที่เป็นไปได้ของลูเซียนทรีดินต่อการเกาะกลุ่มของเกล็ดเลือด โดยวิธีเทอร์บิโดเมทิก เพื่อวัดการเกาะกลุ่มของเกล็ดเลือด ตรวจวัดระดับไซคลิกอะดีโนซีนโมโนฟอสเฟต (cAMP) โดยชุดตรวจ ELISA และศึกษาผลการยับยั้งเอนไซม์ไซโคลออกซิจีเนส (COX) ของลูเซียนทรีดินโดยชุดตรวจคัดกรองตัวยับยั้ง COX ด้วยฟลูออเรสเซนต์ ผลการศึกษาพบว่าลูเซียนทรีดินมีฤทธิ์แรงที่สุดในการยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่กระตุ้นด้วยกรดอะราคิโดนิก โดยที่ความเข้มข้น 0.025 มิลลิโมลาร์มีค่าร้อยละของการยับยั้งการเกาะกลุ่มของเกล็ดเลือดสูงที่สุดเท่ากับ 89.7 ± 1.2 ในขณะที่ลูเซียนทรีดินที่ 0.4 มิลลิโมลาร์มีค่าร้อยละของยับยั้งการเกาะกลุ่มของเกล็ดเลือดที่กระตุ้นด้วยคอลลาเจนและอะดีโนซีนฟอสเฟต (ADP) เท่ากับ 84.5 ± 2.2 และ 51.9 ± 4.8 ตามลำดับ ลูเซียนทรีดินยังเพิ่มเวลาล่าช้า (delaying time) ของการกระตุ้นด้วยกรดอะราคิโดนิกและเพิ่มเวลาแลค (lag time) ในเกาะกลุ่มของเกล็ดเลือดเมื่อกระตุ้นด้วยคอลลาเจน ลูเซียนทรีดินมีผลยับยั้งการเกาะกลุ่มของเกล็ดเลือดระยะที่สองเมื่อกระตุ้นด้วย ADP นอกจากนี้ลูเซียนทรีดินที่ความเข้มข้น 0.4 มิลลิโมลาร์มีผลยับยั้ง ADP ทำให้มีระดับ cAMP เพิ่มขึ้นในเกล็ดเลือดอย่างมีนัยสำคัญ ($p < 0.05$) ในการวิเคราะห์การจับกันของโมเลกุลพบว่าลูเซียนทรีดินจับบริเวณทางเข้าของเอนไซม์ COX-1 และอาจจับกับบริเวณที่ออกฤทธิ์ของเอนไซม์ COX-2 นอกจากนี้ ลูเซียนทรีดินแสดงผลยับยั้งการทำงานของเอนไซม์ COX-1 และ COX-2 มีค่า IC_{50} เท่ากับ 11.92 ± 0.00 และ 0.21 ± 0.17 ไมโครโมลาร์ ตามลำดับ ผลการศึกษานี้ชี้ให้เห็นว่ากลไกที่เป็นไปได้ของลูเซียนทรีดินในการต้านเกล็ดเลือดอาจออกฤทธิ์ผ่านวิถีทางกรดอะราคิโดนิก-ทรอมบอกแซน และวิถีทาง ADP-อะดีนิวเลทไซเคลส

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KEYWORD: Lusianthridin, antiplatelet aggregation, ADP, arachidonic acid, cyclooxygenase enzymes, cAMP

Hla Nu Swe : *IN VITRO* ANTIPLATELET ACTIVITY OF LUSIANTHRIDIN IN HUMAN PLATELETS. Advisor: Assoc. Prof. RATAYA LUECHAPUDIPORN, Ph.D. Co-advisor: Prof. PONLAPAT ROJNUCKARIN, M.D.,Ph.D.

Lusianthridin is a phenanthrene derivative isolated from *Dendrobium venustum.* Some phenanthrene compounds have antiplatelet aggregation activities *via* undefined pathways. The objective of this study is to determine the inhibitory effects and potential mechanisms of lusianthridin on platelet aggregation. Turbidometric aggregometry method was used to measure the extent of platelet aggregation, then cyclic adenosine monophosphate (cAMP) levels were measured by ELISA kit. Inhibitory effect of lusianthridin on cyclooxygenase enzymes was analyzed by COX fluorescent inhibitor screening assay kit. The results indicated that Lusianthridin most potently inhibited arachidonic acid - induced platelet aggregation by maximum percent inhibition of aggregation of 89.7 ± 1.2 % at 0.025 mM; whereas lusianthridin at 0.4 mM inhibited collagen and ADP-induced platelet aggregation by 84.5 ± 2.2 % and 51.9 ± 4.8 %, respectively. Lusianthridin also increased the delaying time of arachidonic acid-stimulated and the lag time of collagen-stimulated aggregations. Lusianthridin showed the inhibitory effect on the secondary wave of ADP-stimulated aggregation. Furthermore lusianthridin at 0.4 mM inhibited ADP-induced by significantly increase cAMP level in platelets ($p < 0.05$). Molecular docking analysis revealed that lusianthridin bound to the entrance site of cyclooxygenase -1 (COX-1) enzyme and probably the active region of COX-2 enzyme. In addition, lusianthridin showed inhibitory effects on both COX-1 and COX-2 enzymatic activities with the IC_{50} value of 11.92 ± 0.00 μM and 0.21 ± 0.17 μM, respectively. These findings suggested that possible mechanisms of lusianthridin on antiplatelet activity might act *via* arachidonic acid-thromboxane and ADP-adenylate cyclase pathways.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
AA	Arachidonic acid
ADP	Adenosine 5'-diphosphate
AMP	Adenosine monophosphate
ASA	Acetylsalicylic acid (Aspirin)
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine 3', 5'-monophosphate
COX	Cyclooxygenase
DAG	Diacylglycerol
FcRγ	Fc receptor γ-chain
GP	Glycoprotein
IC_{50}	The half maximal inhibitory concentration
IP3	Inositol (1, 4, 5)-triphosphate
µg	Microgram
µl	Microliter
µM	Micromolar
mg	Milligram
min	Minutes

ml	Milliliter
mM	Millimolar
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4, 5)-biphosphate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PGH_2	Prostaglandin H_2
PKC	Protein kinase C
pmol	Picomolar
PPP	Platelet poor plasma
PRP	Platelet rich plasma
TP	Thromboxane receptor
TXA2	Thromboxane A2
vWF	von Willebrand factor

CHAPTER 1

INTRODUCTION

1. Background and rationale

Cardiovascular diseases (CVD) including stroke and coronary artery disease are the global leading cause of morbidity and mortality. According to the World Health Organization (WHO), more than 15.2 million people died from CVD in 2016 (1). The development of these diseases are associated with the formation of atherosclerotic plaque, which is mediated by many factors including lipid peroxidation, foam cell formation, dysfunction of vascular endothelial cell, platelets activation and depletion or deposition of chemical mediators (2).

Platelets activation is also an important factor for thrombus formation. Platelets maintain hemostasis by preventing blood loss and maintaining vascular integrity. When injury occurs, endothelial layer from blood vessel is broken and tough fibers (collagen) are exposed. These collagens adhere to platelets and induce platelet adhesion, activation and aggregation to provide haemostatic plug to prevent bleeding (3). However, the hypersensitive of function of platelets may lead to CVD, such as thrombosis, atherosclerosis, ischaemic stroke and myocardial infarction.

Therefore, antiplatelet agents are used for prevention and treatment of CVD to reduce mortality (2). However, antiplatelet therapies still have some limitations.

Aspirin, a commonly used standard weak antiplatelet agent, has side effects such as gastric ulcers, bleeding and renal injury (4). Another commonly used agent, clopidogrel, causes bleeding with a high cost, although it is effective (3). Therefore, searching the newer antiplatelet agents from medicinal plants is an attractive field. Many of the epiphytic orchids are used as traditional Chinese medicine and pharmacological activities of its chemical constituents have been studied in recent years. The most represented genera of orchids are *Dendrobium* spp. (183 species) (5). There are several bioactive components of *Dendrobium* plants such as alkaloids, bibenzyls, phenanthrenes, dihydrophenanthrenes, phenanthrenequinones, fluorenones, sesquiterpenoids and polysaccharides which showed various biological activities including diuretic, anti-rheumatic, anti-inflammatory, anti-carcinogenic, hypoglycemic activities, antimicrobial, anticonvulsive, neuroprotective, antioxidant and antiplatelet aggregation activities (6-11). In this study, we are interested in lusianthridin, which is a phenanthrene derivative isolated from *Dendrobium venustum*. Chen et al., 2000 showed that the phenanthrene derivatives; 3,7-dihydroxy-2,4-dimethoxyphenanthrene and erianthridin exhibited significant inhibition activities against arachidonic acid induced platelet aggregation with estimated IC_{50} values: 24 μM and 9 μM respectively (12). However, antiplatelet activities of phenanthrene derivative: lusianthridin has not been evaluated yet. This study will focus on evaluating of *in vitro* antiplatelet activities of lusianthridin in a human blood model and on investigating possible modes of actions of this compound.

2. Objectives

1. To evaluate the *in vitro* antiplatelet aggregation activity of lusianthridin in human platelets induced by ADP, collagen or arachidonic acid.
2. To evaluate the potential mechanism of antiplatelet action of this phenolic compound.

3. Hypothesis

- Lusianthridin inhibits platelet aggregation *via* ADP-pathway.
- Lusianthridin inhibits platelet aggregation *via* arachidonic acid pathway.
- Lusianthridin inhibits platelet aggregation induced by collagen.

CHAPTER 2

LITERATURE REVIEW

1. Platelets

Platelets are anucleate and the smallest cells which originate from cytoplasm of megakaryocytes in bone marrow and lung. The production process of platelets is called thrombopoiesis. They have discoid shape and around 100 billion platelets are produced daily. The thickness of platelet is 0.5 μm and approximately 2-5 μm in longitudinal diameter with a mean cell volume of 6-10 femtoliters (13). About 70% of total body platelets stay in the blood circulation while the remaining 30% are sequestered in the spleen. When it releases, platelets circulate in the human body for 7-10 days and then they are cleared by spleen, liver and bone marrow (14).

2. Platelet morphology

In resting state, platelets have a discoid form and a circumferential band of microtubules support this shape. This structure helps their movement in the blood circulation. The anatomy of the platelet has divided into four distinct zones, the peripheral zone, the sol-gel zone, the organelle zone and the membrane zone (3).

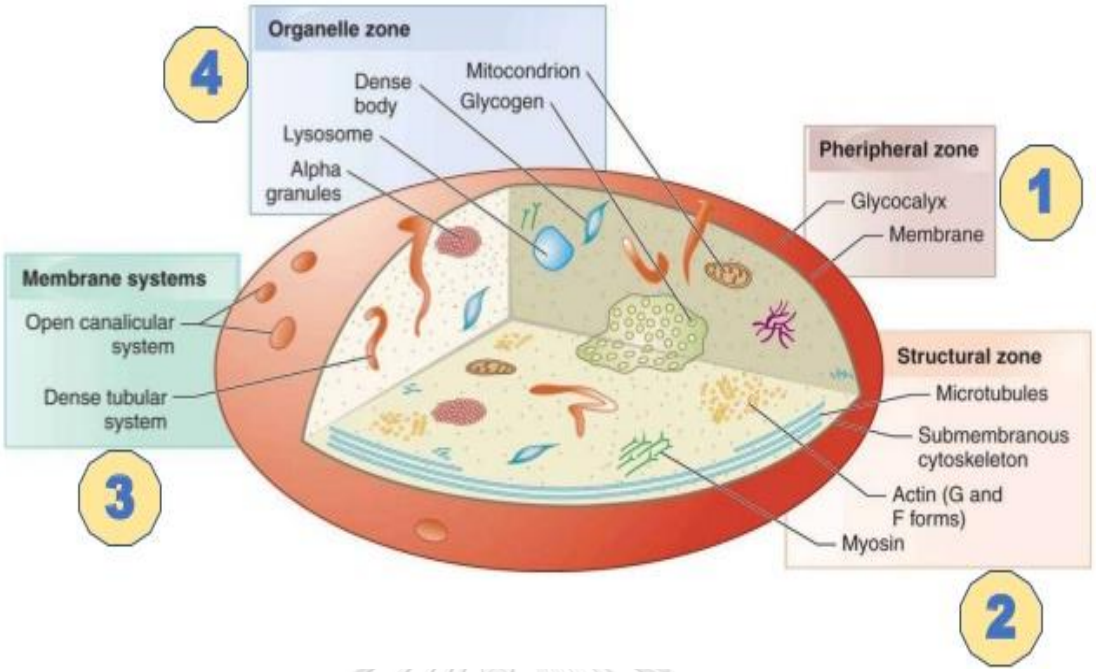


Figure 1. Structure of platelet (15).

2.1. Peripheral Zone

The peripheral zone consists of membranes and closely associated structures including lipid bilayer, cytoskeletal proteins, transmembrane proteins, various glycoprotein-rich interactive domains and transmembrane receptors. These morphological features provide the discoid shape and help the contractile system to facilitate shape changes. Actin is a major portion in the contractile system and other proteins such as myosin, tropomyosin, acting binding protein, α-actinin, gelsolin and profilin are also involved (4).

2.2. Sol-gel Zone

This region is located under the peripheral zone. It contains fibers, filaments and proteins in various states of polymerization. The surface-connected open canalicular system (OCS) provides access to the interior for plasma-bone substances and it serves to release the products during platelet secretion. The changes in the state of polymerization and movement of the fibrous components of the matrix are important for supporting the platelet discoid shape and internal contraction (3).

2.3. Organelle Zone

The organelle zone consists of granules such as **α**-granules and dense granules and peroxisomes, lysosomes, mitochondria and glycogen. This zone serves as the storage site for various enzymes, non-metabolic adenine nucleotides, serotonin, a variety of proteins, calcium and antioxidants such as ascorbic acid, glutathione. The **α**-granules contain several proteins such as fibrinogen, von Willebrand factor (vWF), P-selectin. In dense granules, there are rich in the divalent cations Ca^{2+} and Mg^{2+}, serotonin, adenosine diphosphate (ADP) and adenosine triphosphate (ATP). When platelets activated, these contents are released and cause platelet aggregation **(16)**.

2.4. Membrane Zone

Golgi zones are membrane systems normally decided to the megakaryocyte. This region contains higher amount of calcium which is involved in contractile events. This is also the site for dense tubular system (4).

3. Platelet function

Platelets are involved in prevention of bleeding processes. Platelet activation contains three major processes namely platelet adhesion, platelet secretion and platelet aggregation **(17)**. When injury occurs, endothelial layer from blood vessel is broken and tough fibers (collagen) are exposed. These collagens attach to platelets and induce platelets shape change, granule contents released and platelet activation. Platelets also play roles in several other processes such as fighting microbial infection, causing inflammation and promoting tumor metastasis **(18)**.

3.1. Platelet Adhesion

Adhering to the damage endothelium, the circulating platelet requires sub-endothelial matrix and specialized receptors. When injury occurs, the endothelial layer from blood vessels is broken and tough fibers (collagen) are exposed. Initial adherence is mediated primarily by the platelet vWF receptor GPIb-IX-V. These attached platelets undergo shape changes. Platelets, then, spread out over the endothelial defect and develop firm adherence through their GP IIb-IIIa receptor (19).

3.2. Platelet Secretion

Platelets release several biologically active substances form their organelles upon activation including the contents of α-granules and dense granules. The released ADP from dense granules causes further platelet stimulation. Activated platelets also release membrane microparticles containing GPIIb-IIIa, thrombospondin, and P-selectin which enhance local thrombin generation **(20)**.

Table 1. Contents of platelet organelles (21).

Organelles		**Contents**
α-granule		Factor V Fibrinogen Fibronectin Plasminogen activator inhibitor – 1 Platelet derived growth factor Platelet factor - 4 P-Selectin β-Thromboglobulin Thrombospondin Vascular endothelial growth factor Vitronectin vWF
Dense granule		ADP ATP Ca2+ Serotonin
Lysosome		Acidic Hydrolase
Cytosol		Factor XIII
Other soluble mediators		Thromboxane A2 Platelet activating factor (PAF) Interlukin-1 β Microparticles

3.4. Platelet Aggregation

Platelet aggregation can also be stimulated by several mediators, including ADP, collagen, epinephrine, arachidonic acid, thrombin, serotonin, thromboxane A_2 (TXA_2) and platelet activating factors. The main receptors and signaling pathways in platelets are as shown in Figure 2 (22).

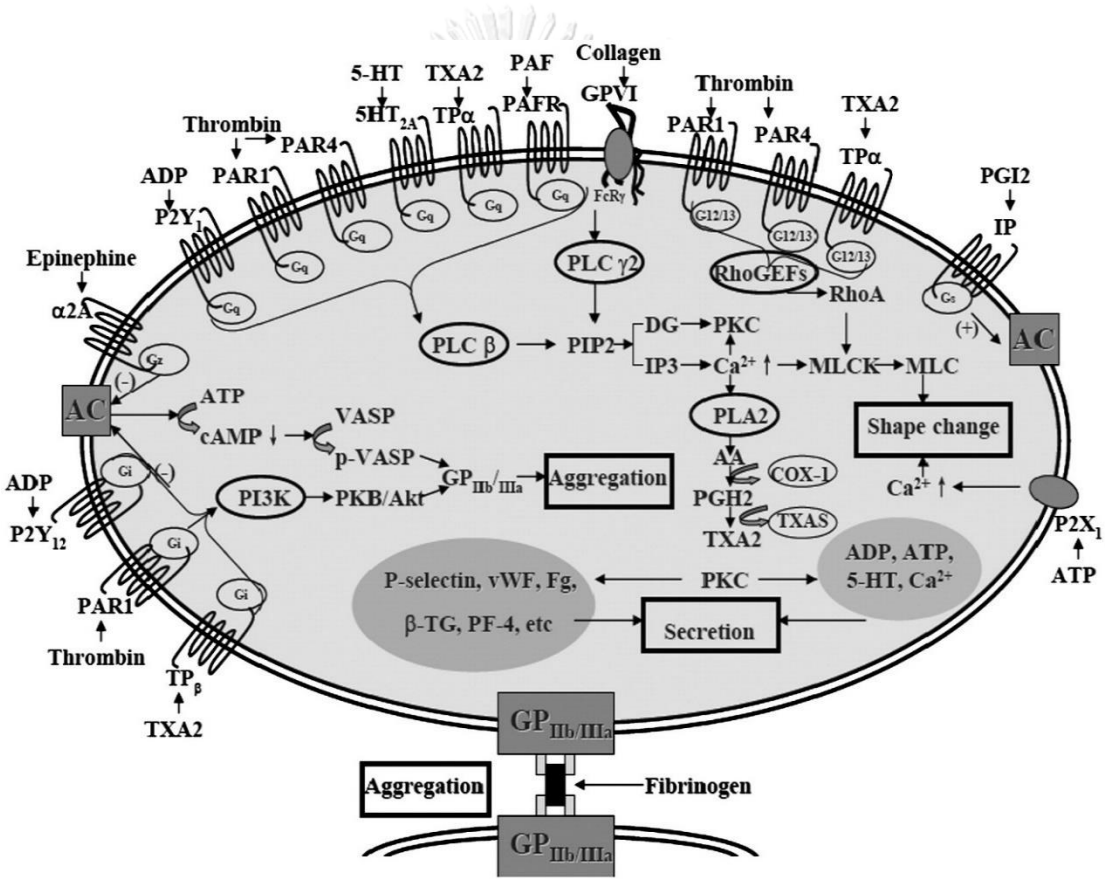


Figure 2. Signaling pathways of platelet activation (22).

3.4.1. Collagen

Collagen is a potent platelet agonist and is exposed when there is damage in endothelium. Platelets have three surface collagen receptors, GPIa-IIa (integrin $\alpha_2\beta_1$), GPVI (a member of the immunoglobulin superfamily), and GPIb-IX-V. For collagen-induced signaling, the platelet immune receptor adaptor Fc receptor γ-chain (FcRγ) is required and it non-covalently associates with GPVI. Platelet activation involves an initial adhesion to collagen *via* GPIa-IIa and subsequent interaction with GPVI/FcRγ. Signaling involves tyrosine phosphorylation of the immunoreceptor tyrosine-based activator motif (ITAM) of the GPIV/ Fc complex by the Src family kinases Lyn and Fyn, leading to Syk binding and activation of phospholipase Cγ2 (PLCγ2). The activation of PLCγ2 hydrolyzes phosphatidylinositol (4, 5)-biphosphate (PIP2) to form two secondary messengers, inositol (1, 4, 5)-triphosphate (IP3) and diacylglycerol (DAG). IP3 is responsible for calcium mobilization from intracellular store, dense tubular system, increasing the intracellular Ca^{2+} concentration and activating Ca^{2+} influx across platelet membrane. DAG is responsible for activation of protein kinase C (PKC) (23). PKC can phosphorylate several proteins such as activating phospholipase A_2 (PLA_2). These signaling molecules trigger the cytoskeletal reorganization that allows a change in platelet shape, secretion of ADP and production of TXA_2. Although higher concentrations of collagen can induce weak platelet aggregation without depending

on ADP release and TXA_2 production, maximal aggregation requires TXA_2 and ADP (20).

3.4.2. Arachidonic Acid

Arachidonic acid (AA) is released from the membrane phospholipid by the action of phospholipase A_2 and it is a substrate for cyclooxygenase (COX) enzyme, which is a bifunctional enzyme exhibiting both COX and peroxidase activities. AA is metabolized to prostaglandin (PG)H_2 by COX-1 enzyme in platelet, and then PGH_2 is converted to TXA_2 by a peroxidase enzyme activity. TXA_2 is labile and has a very short half-life. It is rapidly hydrolyzed to inactive thromboxane B2 (TXB_2). TXA_2 binds to two separate thromboxane receptor (TP); $TP\alpha$ and $TP\beta$ (24). Both TP receptors are related to G-protein coupled receptors which activate Gq and $G_{12/13}$. Gq stimulate phospholipase $C\beta$ ($PLC\beta$) to increase intracellular Ca^{2+} and stimulates the PKC pathway and $G_{12/13}$ mediates the Rho/Rho-kinase pathway. The TXA_2 precursor, PGH_2, also stimulates these G-protein coupled receptors. TXA2 is an important modulator for platelet aggregation (20).

3.4.3. Adenosine Diphosphate

ADP is released from the platelet dense granules by activation of platelets. Release of ADP is also important though it is a weak agonist (25). ADP binds to two G

protein-linked nucleotide receptors: $P2Y_1$, which is linked to activate β-isoform of PLC through Gq and $P2Y_{12}$, which is linked to inhibition of adenylate cyclase through Gi. It also stimulates ligand-gated ion channel: $P2X_1$, which does not involve in main role of ADP-induced platelet aggregation. $P2Y_1$ activated PLC pathway hydrolyzes the membrane PIP2 resulting the production of IP3 and DAG. $P2Y_{12}$ inhibited adenylate cyclase pathway prevent the cyclic AMP (cAMP) formation leading to reduce the phosphorylation of vasodilator-stimulated phosphoprotein (VASP). Inhibition of cAMP formation is also involved in phosphoinositide 3-kinase (PI3K) activation which mediates signals *via* stimulation of Akt phosphorylation in platelet (3). These lead to platelet aggregation.

4. Antiplatelet effect of aspirin

Aspirin is the most commonly used antiplatelet agent, which irreversibly inhibits COX enzyme. This results in inhibition of TXA_2 production from AA, which is an essential modulator for platelet activation (3). Stimulation of the TXA_2 receptor leads to tyrosine phosphorylation of several signaling proteins including Syk, which is involved in collagen-induced signaling pathway. It also serves to amplify the platelet activation signal following stimulation by primary agonists, such as thrombin and ADP. Therefore, subthreshold concentrations of certain agonists such as ADP or thrombin or collagen do not produce full platelet aggregation in the absence of TXA_2 (20).

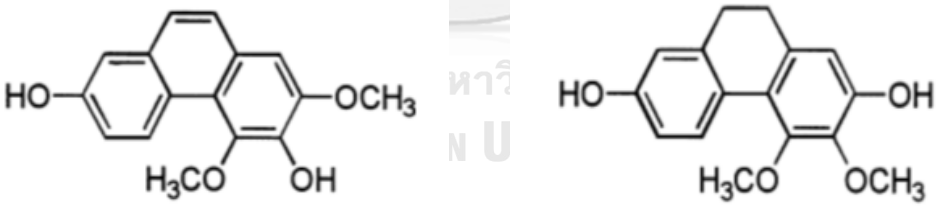
Aspirin is used as a positive control in this study for measurement of platelet aggregation induced by AA, collagen or ADP.

5. Dendrobium species

Dendrobium is one of the largest and most important genera in the family Orchidaceae and have been used in traditional Chinese medicine to reduce fever, hyperglycemia, stomach disease and to promote the production of body fluid (9). More recent ethnopharmacological studies have been shown that *Dendrobium* plants have been used in many parts of the world and in treatment of several diseases including CVD. Several chemical constituents of *Dendrobium* plants have been previously isolated including phenanthrenes, bibenzyls, sterols, alkaloids, fluorenones, coumarins, sesquiterpenes and polysaccharides (26). Some of these compounds have been reported to possess various pharmacological activities including antiangiogenic activity, immunomodulating activity, hepatoprotective activity, antidiabetic activity, antimalarial activity, antiplatelet aggregation and antioxidant activities (9).

Regarding to the antiplatelet aggregation activity of *Dendrobium* plants, methanol extract of the stem of *Dendrobium loddigesii* was found to inhibit aggregation of rabbit platelets induced by arachidonic acid and collagen. Chen et al., 1994 reported that bibenzyl derivatives; moscatilin, moscatilin diacetate and

phenanthrene derivatives; which were isolated from this plant exhibited antiplatelet aggregation activity (7). Fan et al., 2001 reported that scoparone, scopoletin, moscatilin, gigantol and homoeriodictyol from *Dendrobium densifiorum* were found to possess an *in vitro* anti-platelet activity (8). The antiplatelet activities of the phenanthrene derivatives; 3,7-dihydroxy-2,4-dimethoxyphenanthrene and erianthridin were carried out using 4 different agonists: arachidonic acid (AA), thrombin, collagen and platelet activating factor (PAF). Results indicated that both compounds exhibited significant anti-aggregation activities against arachidonic-induced aggregation being most effective with IC_{50} values of 24 μM and 9 μM respectively (12). Recently, lusianthridin is also a phenanthrene derivative of *Dendrobium* species, it is interesting to assess the pharmacological activities.



(a) (b)

Figure 3. Chemical structure of phenanthrene derivatives (a) 3,7-dihydroxy-2,4-dimethoxyphenanthrene (b) erianthridin (12).

6. Dendrobium venustum

Dendrobium venustum Teijsm. & Binn. is widely distributed in Myanmar, Thailand, Cambodia, Laos and Vietnam. In Myanmar, it grows widely in the forest of Mount Popa which is located in Mandalay Division (27). In Thailand, it is known as Ueang dokmakham. The color of the flowers is yellow, and the stems are long about 7-10 cm. The flowers are 10-12 with 2 cm wide (26).

The chemical constituents and biological activities of *Dendrobium venustum* was first reported by Sukphan et al. 2014. Methanol extract from the whole plant of *Dendrobium venustum* showed significant antimalarial and anti-herpetic activities. The structures of the isolated compounds were determined by comparing their spectroscopic data with those reported, and the compounds were identified as flavanthrinin, gigantol, densiflorol B, lusianthridin, batatasin III, phoyunnanin E and phoyunnanin C. Densiflorol B and phoyunnanin E exhibited the strongest antimalarial activity whereas gigantol, batatasin III and phoyunnanin C showed moderate activity. Gigantol and batatasin III also exhibited weak activity against the Herpes simplex virus (26).

7. Lusianthridin

Lusianthridin is a phenanthrene derivative and a plant phenolic include in stilbenoids group. The family for a large number of phenanthrenes has been reported from higher plants, mainly in the Orchidaceae family including *Dendrobium, Bulbophyllum, Eria, Maxillaria, Bletilla, Coelogyna, Cymbidium, Ephemerantha* and *Epidendrum*. A few phenanthrenes have been found in the Hepaticae class and Dioscoreaceae, Combretaceae and Betulaceae families (28). Phenanthrene-containing plants are widely used for the treatment of several disease in Africa, Asia and South America (29). In traditional Chinese medicine, phenanthrene-containing plants have been used to alleviate various diseases such as headache and high blood pressure (30). It is worth noting that almost all of the newly isolated phenanthrenes have been studied for their potential biological activities and several have shown multiple activities (29). Therefore, phenanthrene is a promising group of plant secondary metabolites to explore for its biological activities.

Ibrahim Jantan et al., 2006 showed that phenanthrene compound; 1-(N-acetyl-Nmethylamino)ethyl-3,4,6-trimethoxy-7-hydroxyphenanthrene which was isolated from *Aromadendron elegans* has a strong inhibitory activity on platelet aggregation induced by AA, collagen and ADP in human blood (31). Huey-Ming Lo et al., 2017 reported that a phenanthrene derivative; 5,7-dimethoxy-1,4-phenantherenequinone inhibited collagen and thrombin-induced platelet aggregation

with IC_{50} below 1 μg/ml (32). In 2020, Jeong SY et al., reported that 2,7-dihydroxy-4,6-dimethoxyphenanthrene isolated from *Diocorea batatas* concentration-dependently inhibited the platelet aggregation mediated by ADP, collagen and U46619 in human blood (33).

In this study, a phenanthrene compound; lusianthridin which was isolated from *Dendrobium venustum*, was used for evaluation of antiplatelet activities. It has already proved that lusianthridin has DPPH radical scavenging activity (8.8 – 55.9 μM) and concentration-dependent spasmolytic activity (9). It also has potent antitumor effects against A549 human lung carcinoma and SK-OV-3 human ovary adenocarcinoma (34). In addition, it has potent effect on HL-60 human promyelocytic leukemia with EC_{50} values ranging from 0.11 to 9.8 μg/ml (35). Lusianthridin, which was isolated from *Dendrobium venustum,* also showed dramatically suppression on Src-STAT3-c-Myc pathways in lung cancer cells and the underlying mechanism of lusianthridin is the negatively regulating cancer stem cells-like phenotypes and sensitizing resistant cancer cells to cemetery (36). However, antiplatelet activity of lusianthridin has not been evaluated yet.

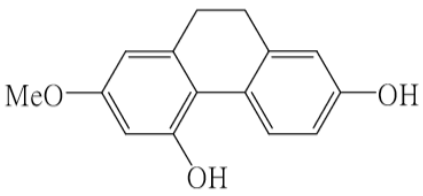
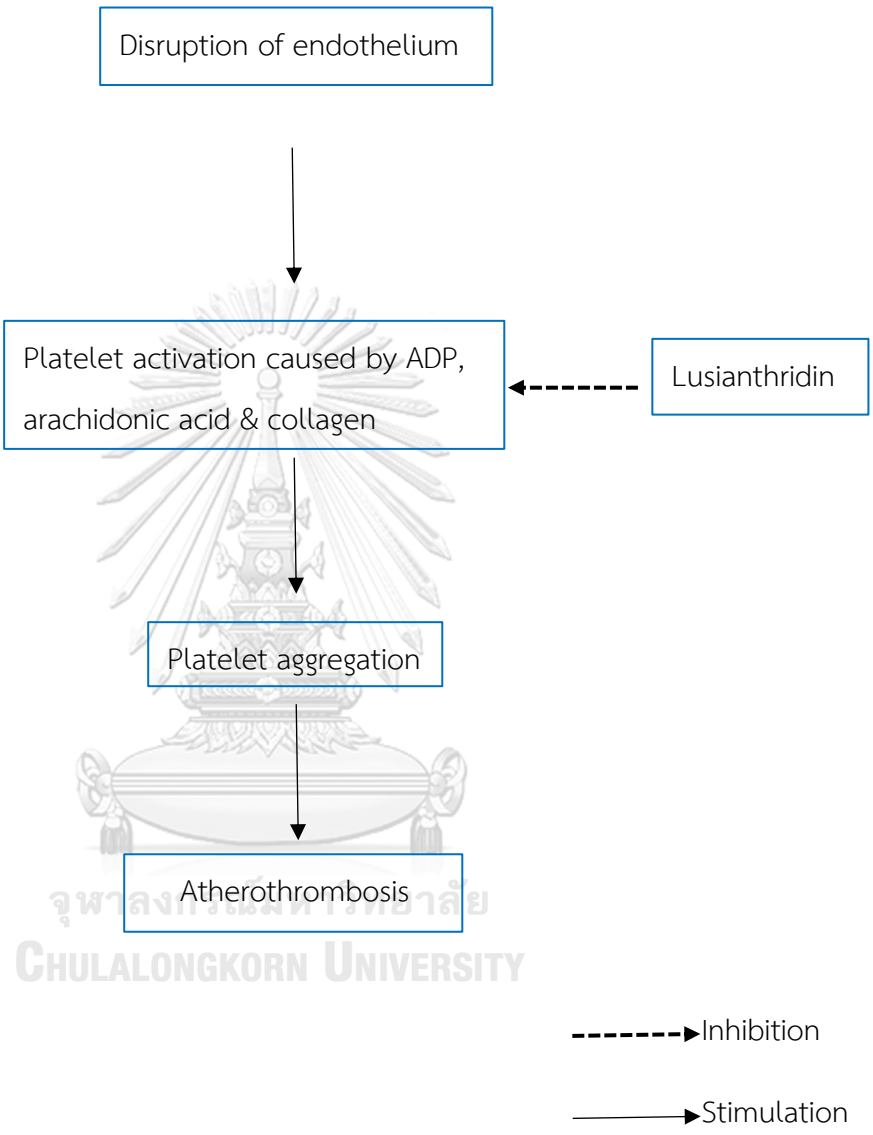


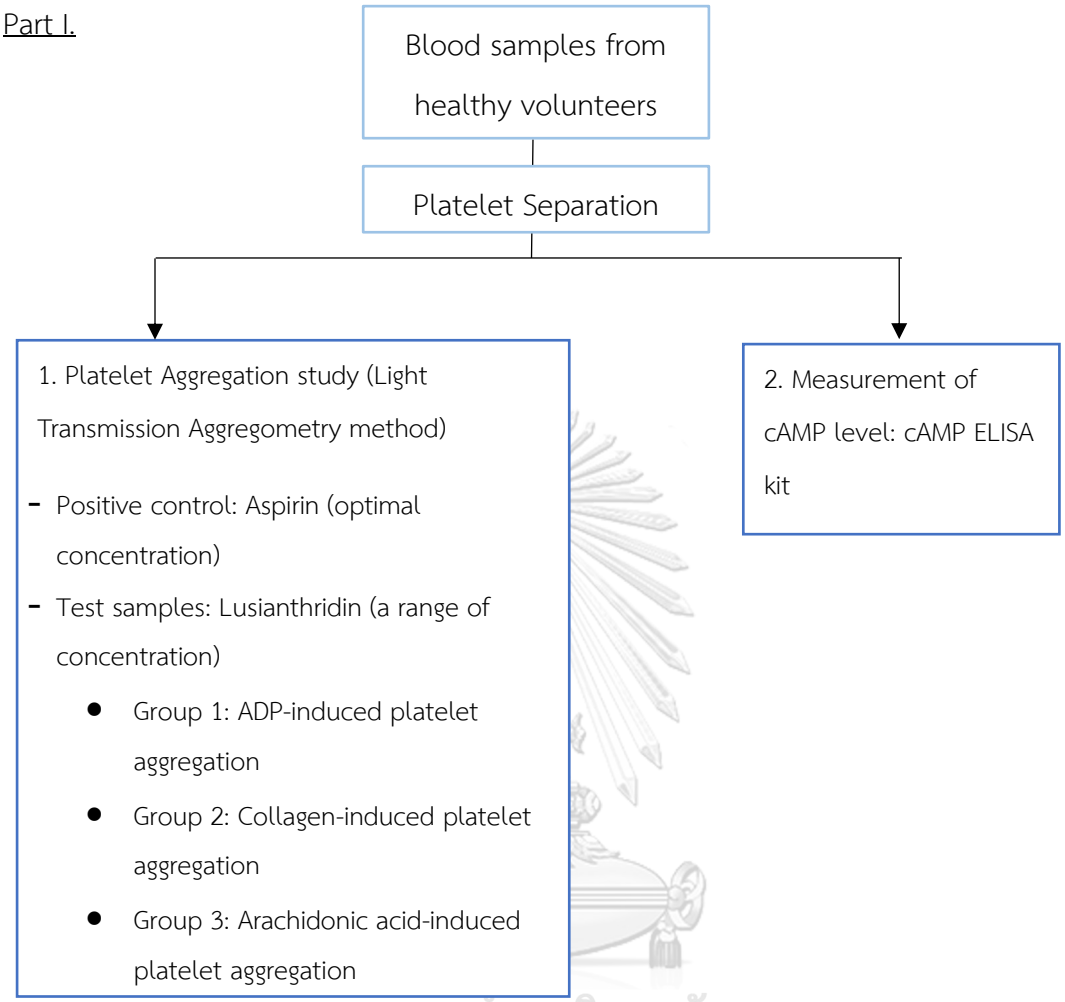
Figure 4. Chemical structure of lusianthridin (26).

8. Conceptual Framework

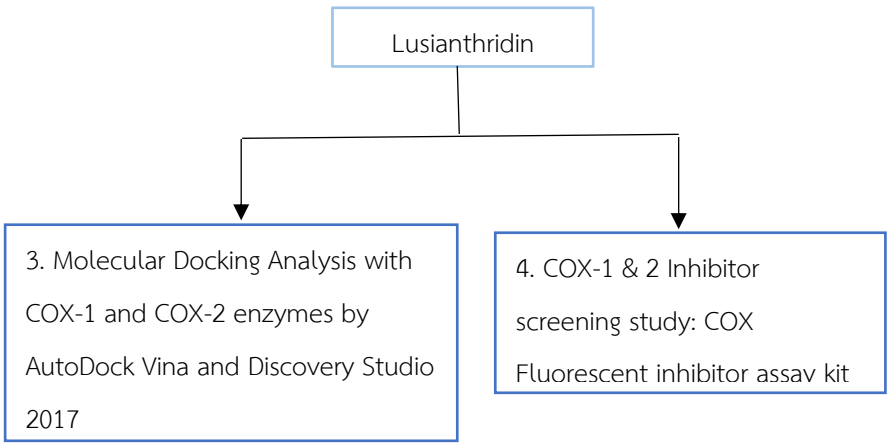


9. Research design: Experimental research

Part I.



Part II.



CHAPTER 3

MATERIALS AND METHODS

1. Materials

1.1 Chemicals

The chemicals: adenosine 5'-diphosphate sodium – ADP, trisodium citrate dihydrate, acetylsalicylic acid – ASA, dimethyl sulfoxide -DMSO, arachidonic acid, sigmacote and other chemicals were purchased from Sigma Chemical Co., St. Louis, U.S.A. Collagen type I was purchased from Chrono-Log, Havertown, PA, USA. Cyclooxygenase (COX) fluorescent inhibitor screening assay kit and cyclic adenosine monophosphate (cAMP) ELISA kit were purchased from Cayman Chemical, Ann Arbor, MI, U.S.A.

Lusianthridin was isolated and purified by Associate Professor Boonchoo Sritularak, Ph.D. Briefly, the methanol extract prepared from the whole plant of *D. venustum* was initially subjected to vacuum liquid chromatography on silica gel, and 8 fractions (A-H) were obtained by eluting with an n-hexane-ethyl acetate gradient. Subsequent chemical investigation of fraction G resulted in the isolation of the phenolic compound, lusianthridin. The structure of the isolate was determined by comparing its spectroscopic data with those reported and the purity was evaluated using NMR spectroscopy (26).

1.2 Instruments

- Aggregometer (Helena laboratory, Texas, USA)
- Refrigerated centrifuge (Falcon:6300, USA)
- Microplate reader (CLARIO Star, Germany)

2. Methods

2.1. Subjects

The study was performed in human blood obtained from healthy volunteers following these inclusion and exclusion criteria:

Inclusion criteria

- Age within 18 to 55 years.
- No prior or ongoing medical conditions
- Non-smokers and non-alcoholics
- Not donating blood in the last one month
- Not taking any medications at least 2 weeks before participated in the study
- Appreciate to participate in this study with sign.

Exclusion criteria

- Female who are pregnant or breast-feeding.
- Female who are during or for 5 days after your period

Blood were collected in the morning at the same time and volunteers were asked to fast at least 8 hours by avoiding lipid-rich food, tomato, turmeric powder and garlic before donating blood. Volunteers need to sign the informed consents before participating in this study. This study was aproved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (COA No. 493/2020).

2.2. Sample Size

- For *in vitro* platelet aggregation test: the sample size for this part was calculated by the resource equation method instead of power analysis method because it is a complex biological experiment with several treatments and multiple endpoints were measured. Follow the equation:

$$E = N - T$$

Where, N is total subjects' degrees of freedom (df) (N = total number of subjects – 1),

T is treatment df (T = total number of treatments – 1)

E is the error degrees of freedom and should be between 10 and 20.

In this research, E = 14 – 2 = 12 with total number of subjects was 15 and 3 treatments for 3 difference agonists (37). Therefore, the number of subjects was 15 with 5 subjects for each agonist.

- For cAMP ELISA assay: 4 subjects were needed for statistical analysis

- Finally, with 20% of dropout rate, the total number of healthy volunteers was 23.

2.3. Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP)

Blood samples (30ml) from overnight fasting healthy volunteers were collected by venipuncture and put into plastic tubes containing 3.2 % sodium citrate (blood: buffer = 9:1 v/v). The platelet-rich plasma (PRP) was prepared by centrifugation of blood samples at 21°C, 200xg for 10 minutes and the top layer was collected as PRP. Isolation of the platelet-poor plasma (PPP) was done by further centrifugation of the rest of blood samples at 21°C, 1500xg for 15minutes. PPP was used as a reference to define the theoretical point of 100% light transmission.

Platelets in plasma were counted using automated cell counter to ensure the platelet count of each subject was in the normal range (150-450 x 10^9 cells/L). Aggregation testing was done at least 15 minutes (min) after PRP was prepared for recovering from refractoriness and completed within 4 hours (hr.) of sample collection to avoid preanalytical artifacts from time-dependent changes in platelet function. PRP and PPP were used for the platelet aggregation test.

2.4. Measurement of platelet aggregation

Platelet aggregation test was performed as our previous study (38) which was modified from Born turbidimetric aggregometry method (39) using an aggregometer (AggramTM, Helena laboratory, USA). There are 3 steps in this study.

Step 1: the submaximal concentration of each agonist for each volunteer was chosen by testing with a range of agonist concentrations which were able to avoid variations and to use in next steps. The submaximal concentration was the concentration generated just submaximal aggregation. ADP at final concentrations of 2-8 μM, collagen at 1-5 μg/mL and arachidonic acid at 0.0625-0.5 mM was tested following the guidline of the clinical and laboratory standards institute (CLSI) 2008 (40).

Step 2: a standard antiplatelet drug- acetylsalicylic acid (aspirin) was used as a positive control. Since the concentration of aspirin to inhibit 50% platelet aggregation *in vitro* with 5 min of incubation was 0.177 mM (41), a range of final concentrations of aspirin (0.025-0.2 mM) was tested to find the optimal concentration. DMSO 0.5% was used as vehicle control to test the activities since both aspirin and the compound were dissolved in DMSO 0.5%.

Step 3: the range of lusianthridin concentrations was used to determine the effective concentrations. The concentration-response curve of lusianthridin was also constructed to determine the relationships between compound concentrations and rate of inhibition of platelet aggregation *in vitro*.

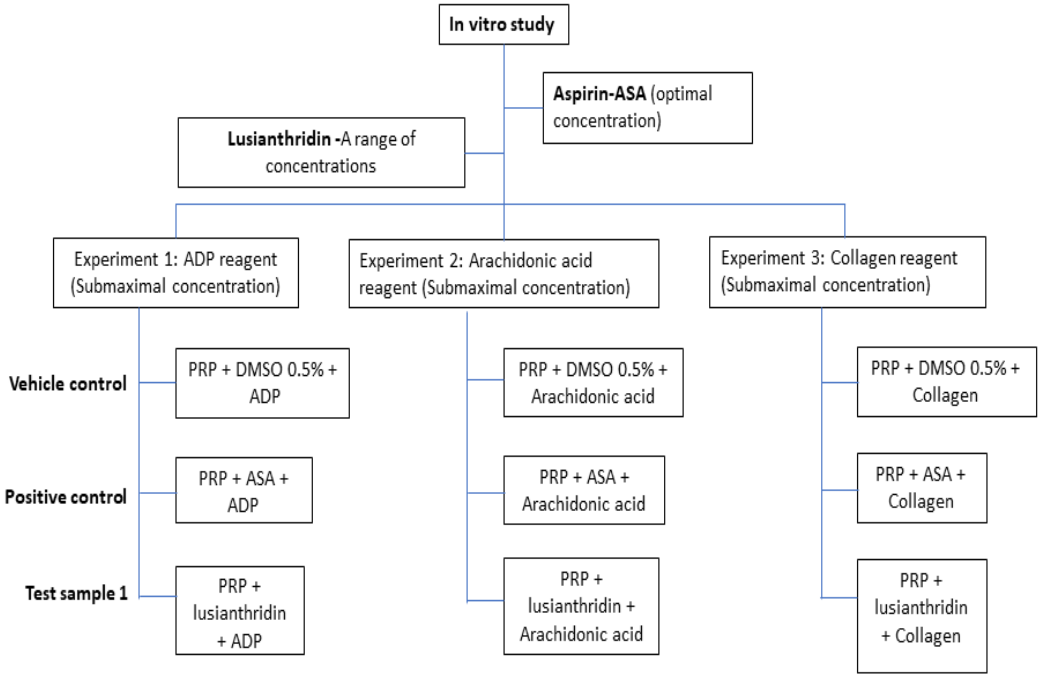


Figure 5. Study design for measurement of platelet aggregation.

The detail procedures were as followed. PPP was set as blank and it was assumed that PPP (250 µl) represented as 100% light transmission and PRP represented as 0% light transmission. 25 µl of tested compounds (positive control-aspirin, vehicle control-DMSO 0.5%, different concentrations of lusianthridin was added to 200 µl of PRP and incubated 4 min at 37°C and then incubated for 1 min under continuous stirring with 600 rpm speed at 37°C. After that, the changes of light transmittance (at 600 nm wavelength) were measure and recorded for 6 min after adding agonists (25 µl). Three different agonists at the final concentrations: ADP 4 µM, collagen 2 µg/ml and arachidonic acid 0.5 µM were used to induce platelet

aggregation. Each sample was independently done in triplicate. The change of light transmission was recorded by using aggregometer and presented in the percentage of light transmission. The outcomes were the percentage of inhibition of platelet aggregation, lag time (the time from addition of agonist till platelet response in shape change), slope and delaying time (the time from the platelet response in shape change or from the addition of agonist till platelet release). The percent inhibition of platelet aggregation was calculated by following equation:

$$\%\ \text{inhibition} = \frac{(\%\ \text{aggregation of vehicle control} - \%\ \text{aggregation of test sample})}{\%\ \text{aggregation of vehicle control}} \times 100$$

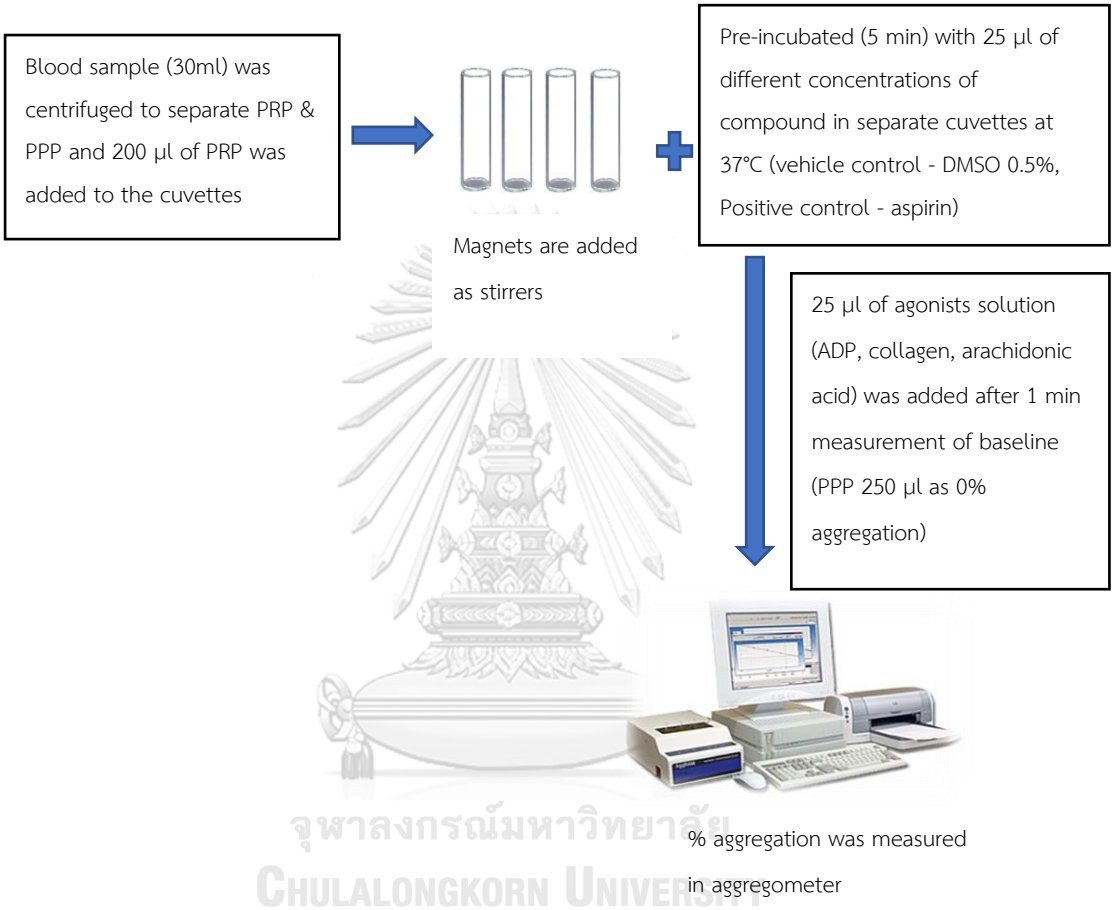


Figure 6. Protocol flow chart for platelet aggregation measurement.

2.5. Molecular Docking Analysis

AutoDock Vina (AutoDockTools 1.5.6.) (42), Discovery studio 2017 (Accerlrys, Inc., San Diego, CA, USA) and ChemBio 3D Ultra 12.0 (Cambridgesoft, USA) were used to perform Molecular docking analysis. From Protein Data Bank (PDB), the crystal structure of COX-1 (PDB ID: 2OYE) and COX-2 (PDB ID: 1CX2) were downloaded. In this study, we also performed docking analysis for two phenanthrenes; 3,7-dihydroxy-2,4-dimethoxyphenanthrene and erianthridin which already showed inhibition of AA-induced platelet aggregation. Therefore, the structures of these two compounds and lusianthridin were constructed by using ChemBioDraw. Before the docking study, ligands and water molecules which associated with enzymes were removed to get free COX-1 and COX-2 proteins by using Discovery studio 2017. After that, hydrogen atoms were added by AutoDock Vina. In the automated docking analysis, the active regions of COX-1 and COX-2 were defined in the grid box with the points, 40 x 40 x 40 (1 Å spacing between grid points) to cover the entire favorable binding site. The X, Y and Z centers for COX-1 protein were 250.516, 108.421 and -40.405, respectively. For COX-2 protein, The X, Y and Z centers were 120.333, 45.083 and 74.917, respectively. The binding sites were determined using the interacted residues of the compound with COX enzymes and their respective binding energy. The lower binding affinity represents a higher inhibitory action. The docking results was visualized and analyzed by using Discovery Studio software (38).

2.6. *In vitro* Cyclooxygenase Activity Assay

Cyclooxygenase (COX) is a bi-functional enzyme exhibit both COX and peroxidase activities (Figure 7). COX-1 is involved in normal cellular homeostasis and also expressed in platelets and COX-2 is responsible for biosynthesis of prostaglandins under acute inflammatory conditions.

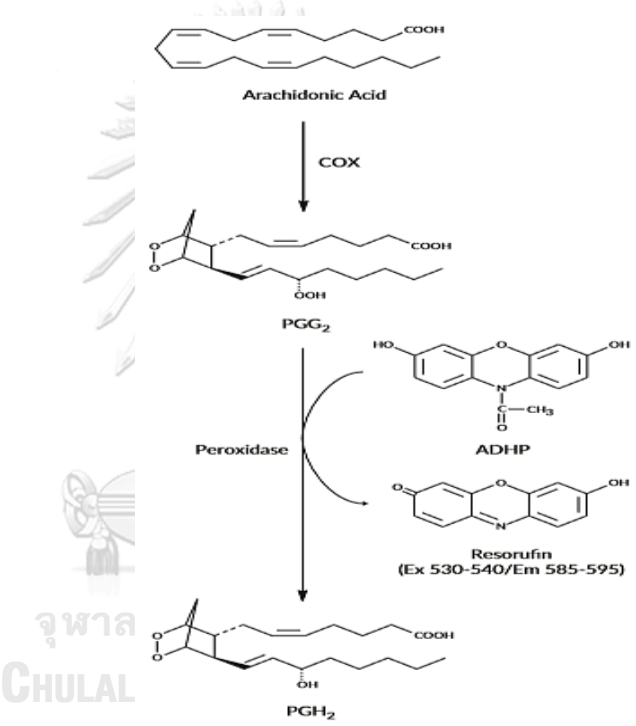


Figure 7. Reaction of COX enzyme in COX fluorescent inhibitor screening assay (43).

The tested compound was incubated directly with COX-1 or COX-2 in assay buffer for 5 min. Then, a substrate, arachidonic acid, and ADHP (10-acety-3,7-dihdroxyphenoxazine) were added and incubated for 2 min. The reaction between prostaglandin G_2 and ADHP produces the highly fluorescent compound resorufin.

Resorufin fluorescence was analyzed with an excitation wavelength of 530-540 nm and an emission wavelength 585-595 nm. This study was carried out by using COX fluorescent inhibitor screening assay kit (Cayman) according to the manufacturer's instructions. The effect of compound on COX activity was evaluated by inhibition rate and calculated as follow:

$$\text{Inhibitory rate (\%)} = \frac{(\%\ \text{inhibition of vehicle control} - \%\ \text{inhibition ofsample})}{\%\ \text{inhibition of vehicle control}} \times 100$$

2.7. Measurement of cAMP level

Adenosine 3', 5' cyclic monophosphate or cAMP was measured by using competitive enzyme immune assay kit. The cAMP is a unique secondary messenger which involve in signaling pathway linking to internal cellular enzymatic activity. The assay based on competition between free cAMP and cAMP-acetylcholinesterase (AChE) conjugate (cAMP tracer) for a cAMP-specific rabbit antibody binding site. The amount of tracer bind to rabbit Ab was inversely proportional to cAMP level in the well. After adding AChE, product was show yellow color and intensity of this color is inversely proportional to free cAMP level in the well. An increase in cAMP level leading to inhibition of platelet aggregation and this inhibition through adenylyl cyclase-pathway inhibition is related with ADP-induced platelet aggregation (20).

PRP ($4x10^8$platelets/ml) was incubated with different concentrations of test compound for 5 min with continuous stirring at 37°C. 3-isobutyl-1-methylxanthine

(IBMX), a phosphodiesterase inhibitor which was used to prevent cAMP metabolism, was used as positive control. Then ADP (4 μmol/L) was added to induce platelet aggregation and the reaction was terminated by 0.5% ice-cold ethanol and samples was vortexed, sonicated and centrifuged to get the supernatant (38). The cAMP level was measured by using cAMP ELISA kit (Cayman) according to the manufacturer's instructions. The developed yellow color was analyzed at the wavelength of 450 nm.

2.8. Data analysis

All the experimental data were expressed as means ± standard errors of means (SEMs). Differences between groups were analyzed using one-way ANOVA followed by the Tukey post-hoc test with SPSS software version 22.0. P-values of less than 0.05 were considered as statistically significance.

CHAPTER 4

RESULTS

1. Antiplatelet Aggregation Activity of Lusianthridin

1.1. Effect of lusianthridin on ADP-induced platelet aggregation

Platelets were pre-incubated with vehicle control (DMSO 0.5%), ASA (positive control) or lusianthridin for 5 minutes at 37°C and ADP (4 μM) was then added to induce platelet aggregation. The results were presented as the maximum percentage of platelet aggregation of ASA at 0.1 mM or lusianthridin at the concentrations of 0.05, 0.1, 0.2 and 0.4 mM (Figure 9).

The representative aggregrams of the effect of lusianthridin on ADP-induced platelet aggregation were shown in Figure 8. Lusianthridin more selectively inhibited the second phase aggregation of ADP-induced platelet aggregation compared with the first phase. Lusianthridin at various concentrations of 0.05, 0.1, 0.2 and 0.4 mM showed a significant decrease in percent platelet aggregation compared with vehicle control as shown in Figure 9 (56.3 ± 4.5 %, 41.9 ± 2.4 %, 35.8 ± 3.1 % and 38.8 ± 3.1 % *vs.* 82.3 ± 1.8 %, respectively, $p < 0.05$). Lusianthridin at 0.1 mM did not significantly difference in the maximal platelet aggregation compared with 0.1 mM ASA (36.9 ± 5.3 %). Lusianthridin showed maximal percent inhibition of ADP-induced platelet aggregation at the concentration of 0.2 mM (55.7 ± 4.4 %) (figure 10).

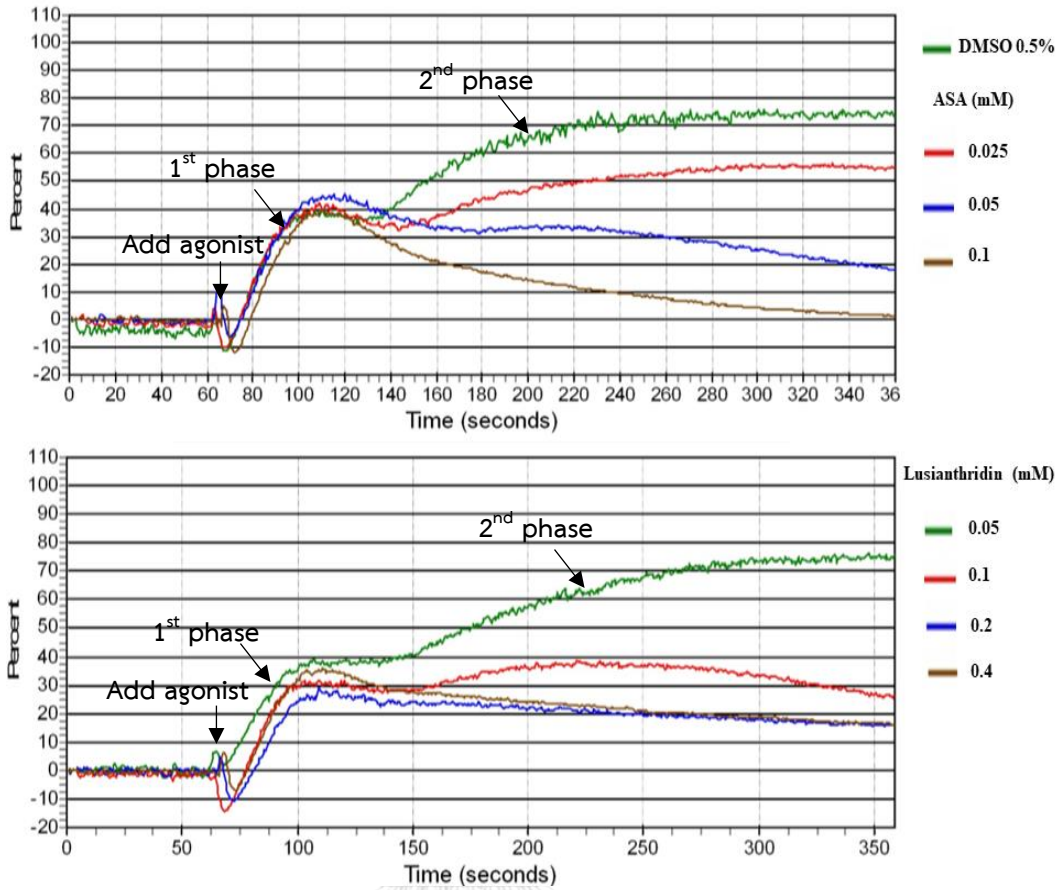


Figure 8. The representative aggregograms of the effect of DMSO 0.5% (vehicle control), ASA at the concentrations of 0.025, 0.05 and 0.1 mM (positive control) and lusianthridin at the concentrations of 0.05, 0.1, 0.2 and 0.4 mM on ADP-induced platelet aggregation.

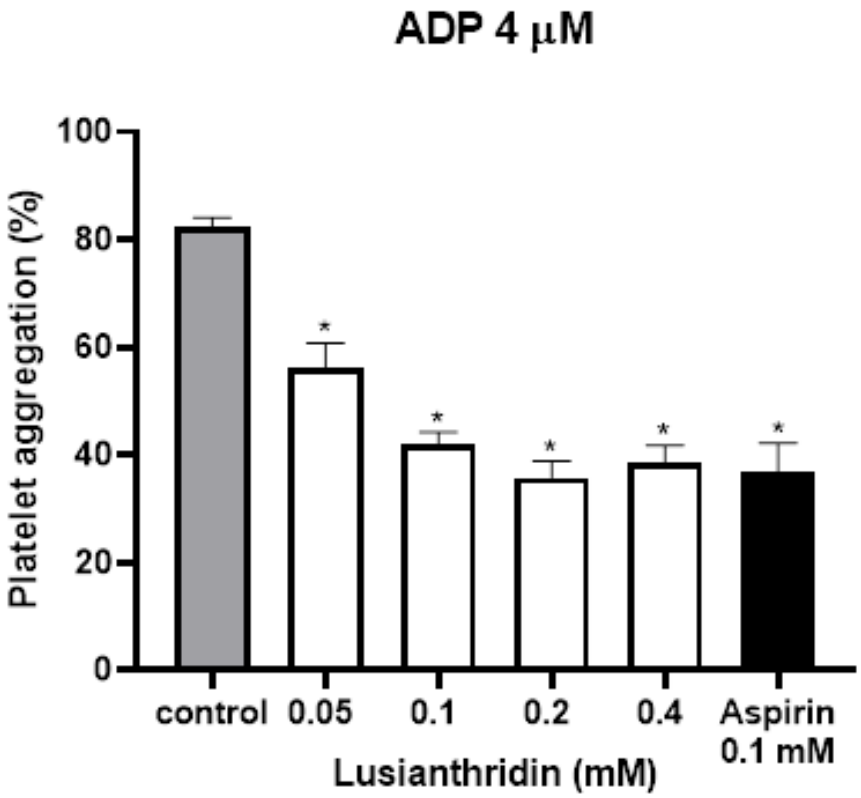


Figure 9. Effects of lusianthridin on ADP-induced platelet aggregation. Platelets were pre-incubated with DMSO 0.5% (vehicle control), lusianthridin or aspirin at 37°C for 5 minutes and then ADP (4 μM) was added to stimulate platelet aggregation. Data are presented as percent aggregation (means ± SEMs; n = 5). *$p < 0.05$ compared with those of vehicle control and analyzed by ANOVA followed by the Tukey post-hoc test.

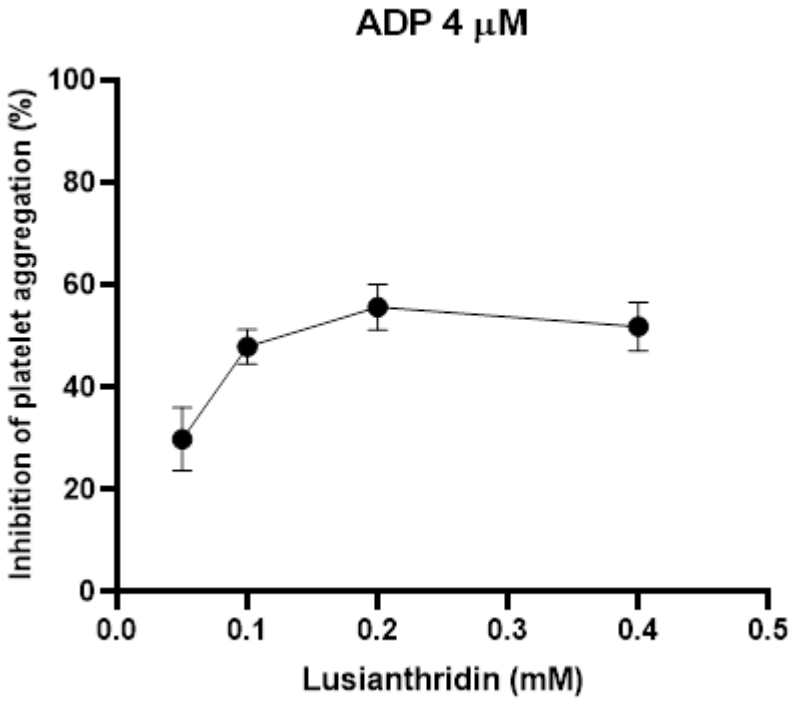


Figure 10. Percent inhibition of Lusianthridin on ADP-induced platelet aggregation. Data are presented as percent inhibition of platelet aggregation (means ± SEMs; n = 5).

1.2. Effect of lusianthridin on AA-induced platelet aggregation

Platelets were incubated for 5 minutes at 37°C and it was pre-treated with vehicle control (DMSO 0.5%), 0.1 mM ASA (positive control) or lusianthridin at the concentration of 0.0125, 0.025, 0.05 and 0.1 mM. Then, AA was added to induce platelet aggregation.

As shown in Figure 11 and Table 2, lusianthridin at 0.0125 mM significantly increased the delaying time compared with the control group (80.0 ± 8.9 *vs* 46.3 ± 5.6 seconds, respectively, $p < 0.05$) although the aggregating inhibition was not observed at this concentration. Lusianthridin at 0.025 mM significantly decreased percent platelet aggregation compared with vehicle control (9.5 ± 1.1 % *vs* 90.8 ± 1.9%, respectively, $p < 0.05$) and this did not significantly different with ASA at 0.1 mM (11.1 ± 0.6 %) (Figure 12). Lusianthridin showed maximal percent inhibition of AA-induced platelet aggregation at the concentration of 0.025 mM (89.7 ± 1.2 %) (Figure 13).

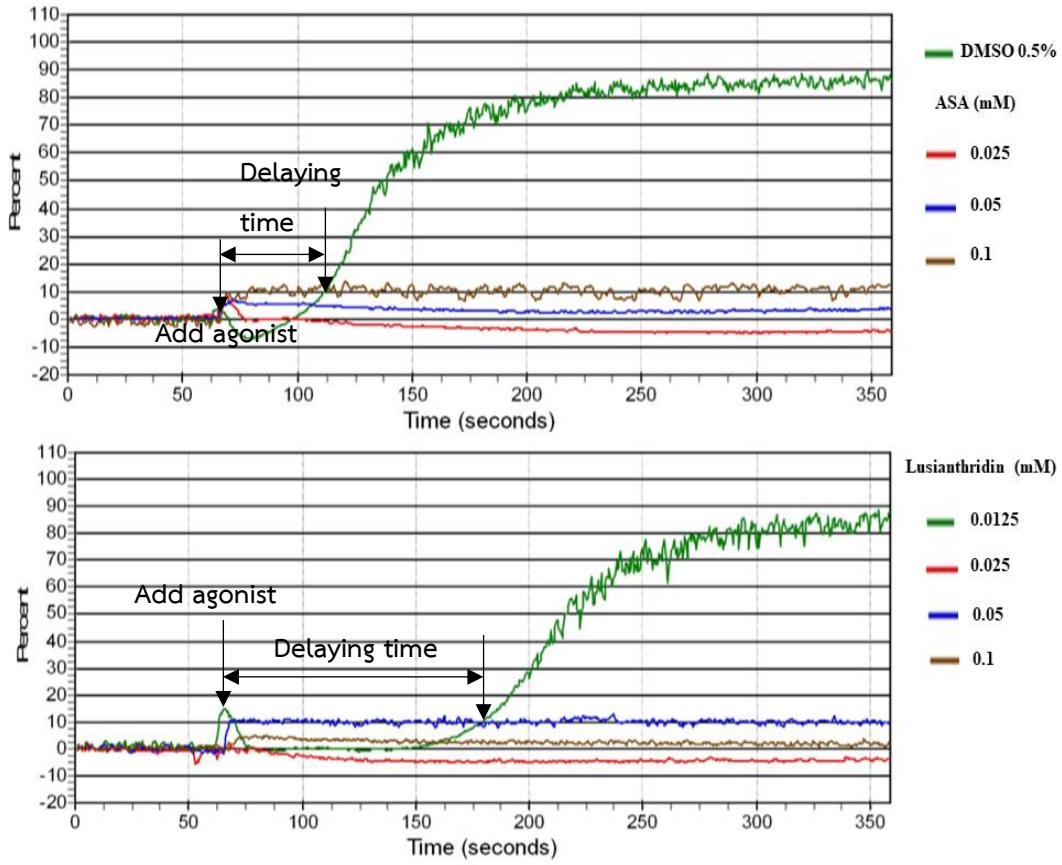


Figure 11. The representative aggregograms of the effect of DMSO 0.5% (vehicle control), ASA at the concentration of 0.025, 0.05 and 0.1 mM (positive control) and lusianthridin at the concentration of 0.0125, 0.025, 0.05 and 0.1 mM on AA-induced platelet aggregation.

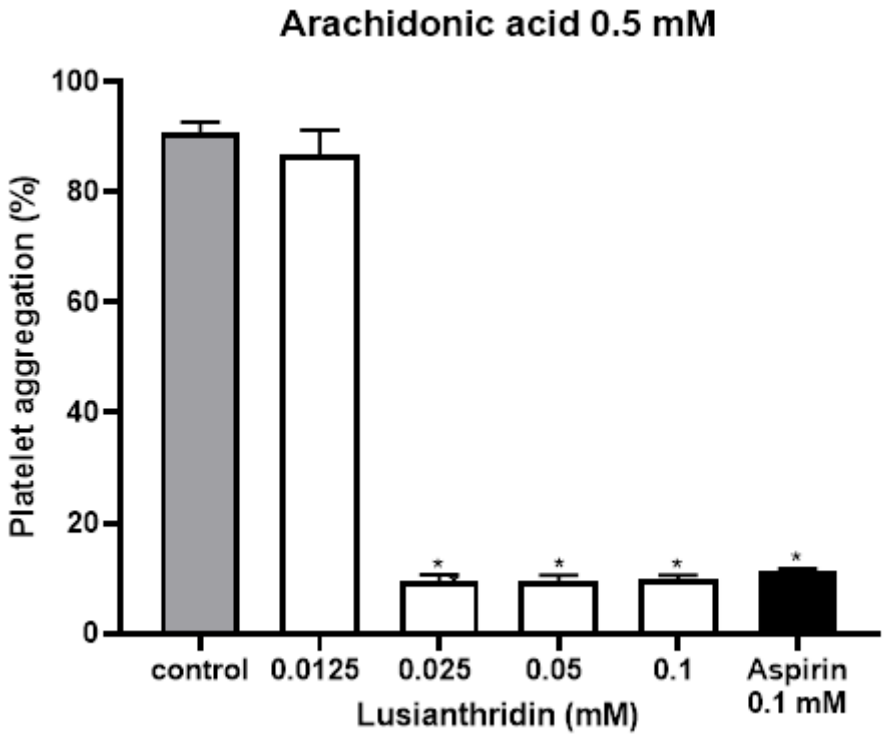


Figure 12. Effects of lusianthridin on AA-induced platelet aggregation. Platelets were pre-incubated with DMSO 0.5% (vehicle control), lusianthridin or aspirin at 37°C for 5 minutes and then AA (0.5 mM) was added to stimulate platelet aggregation. Data are presented as percent aggregation (means ± SEMs; n = 5). *p < 0.05 compared with those of vehicle control and analyzed by ANOVA followed by the Tukey post-hoc test.

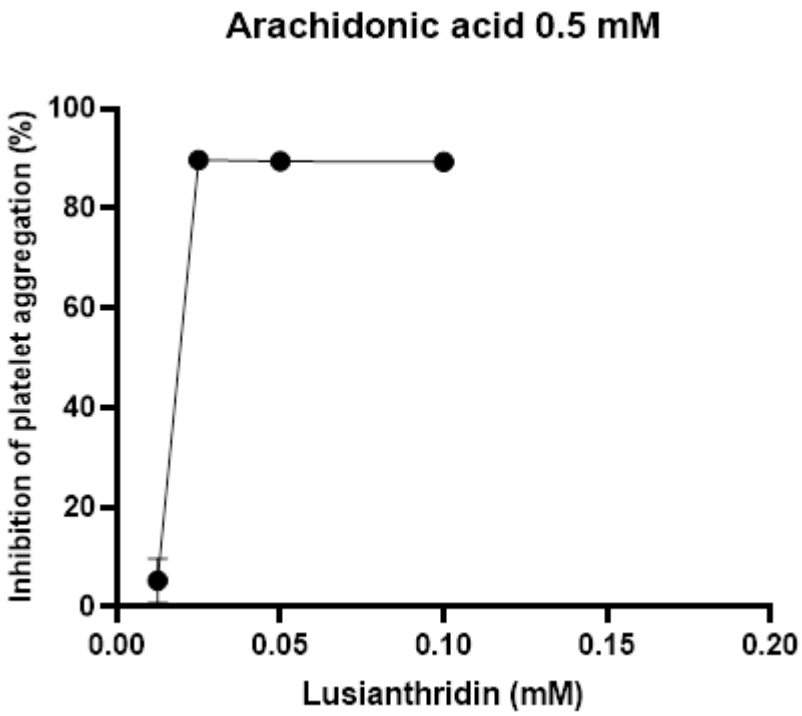


Figure 13. Percent inhibition of Lusianthridin on AA-induced platelet aggregation.

Data are presented as percent inhibition of platelet aggregation (means ± SEMs; n = 5).

1.3. Effect of lusianthridin on collagen-induced platelet aggregation

Collagen (2 μg/ml) was used to induce platelet aggregation in this experiment. Firstly, platelets were pre-incubated with DMSO 0.5%, 0.1 mM ASA or lusianthridin at the concentrations of 0.05, 0.1, 0.2 and 0.4 mM. After that, collagen was added to induce platelet aggregation.

As shown in Figure 14 and Table 2, we found that lusianthridin at 0.4 mM concentration could significantly prolong the lag time of collagen-induced platelet aggregation compared with that of vehicle control (114.5 ± 15.8 *vs* 68.3 ± 5.0 seconds, respectively, $p < 0.05$). Lusianthridin at 0.2 mM significantly inhibited platelet aggregation compared to vehicle control (29.9 ± 7.1 % *vs* 85.6 ± 2.1 %, respectively, $p < 0.05$). The maximal percent platelet aggregation of lusianthridin at 0.4 mM was comparable to that of ASA at 0.1 mM (12.3 ± 1.1 % *vs* 13.24 ± 1.8 %, respectively). Lusianthridin inhibited platelet aggregation induced by collagen with the IC_{50} of 0.14 ± 0.03 mM (Figure 16).

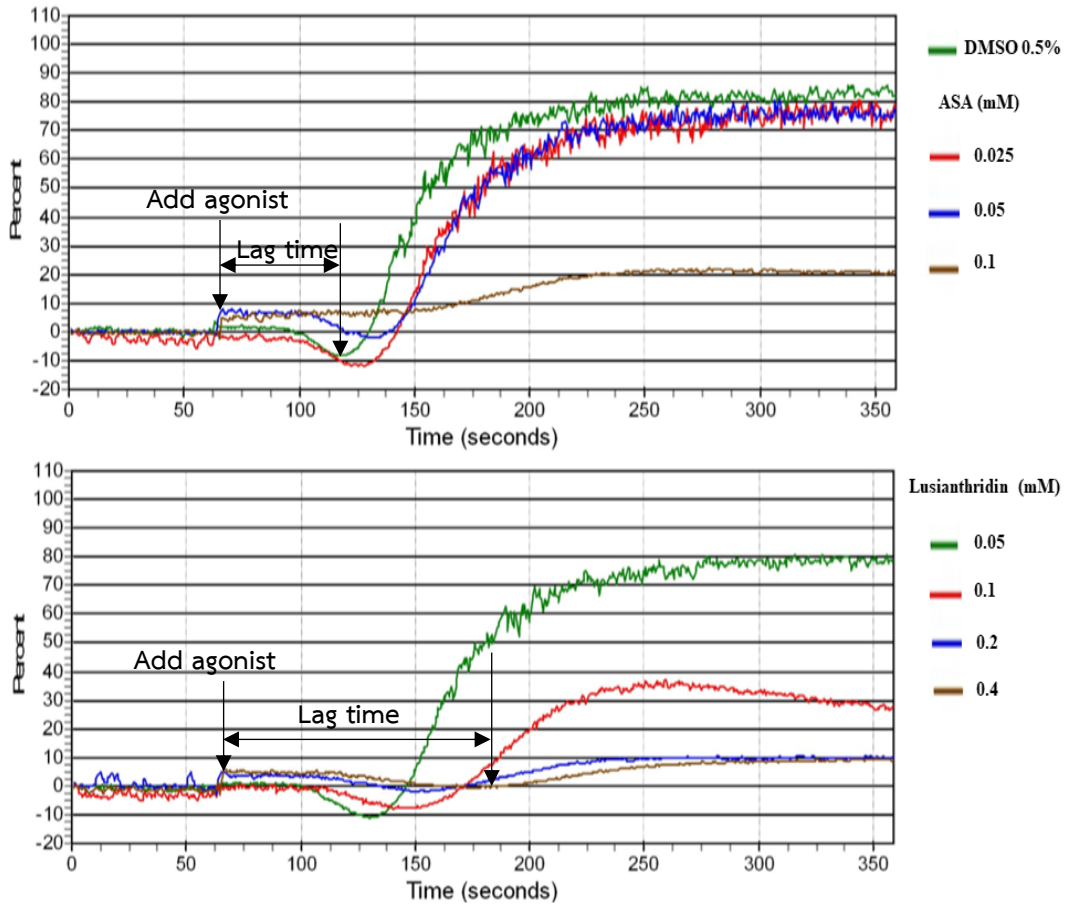


Figure 14. The representative aggregograms of the effect of DMSO 0.5% (vehicle control), ASA at the concentrations of 0.025, 0.05 and 0.1 mM (positive control) and lusianthridin at the concentrations of 0.05, 0.1, 0.2 and 0.4 mM on collagen-induced platelet aggregation.

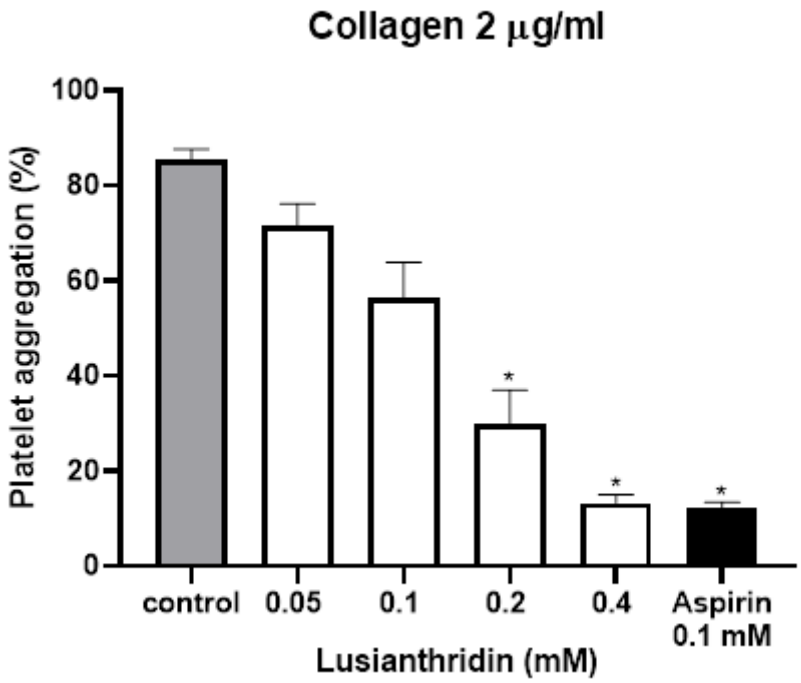


Figure 15. Effects of lusianthridin on collagen-induced platelet aggregation. Platelets were pre-incubated with DMSO 0.5% (vehicle control), lusianthridin or aspirin at 37°C for 5 minutes and then collagen (2 μg/ml) was added to stimulate platelet aggregation. Data are presented as percent aggregation (means ± SEMs; n = 5). *p < 0.05 compared with those of vehicle control and analyzed by ANOVA followed by the Tukey post-hoc test.

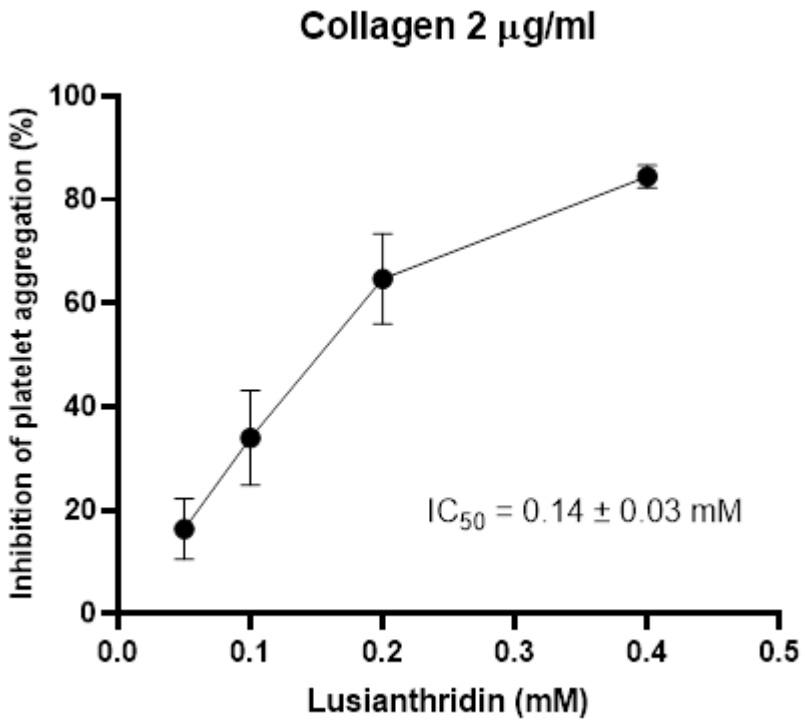


Figure 16. Percent inhibition of Lusianthridin on collagen-induced platelet aggregation. Data are presented as percent inhibition of platelet aggregation (means ± SEMs; n = 5).

Table 2. Summary of antiplatelet aggregation activity of lusianthridin.

Concentration (mM)	ADP (4 μM)	Collagen (2 μg/ml)		Arachidonic acid (0.5 mM)	
	Maximum aggregation (%)	Maximum aggregation (%)	Lag time (seconds)	Maximum Aggregation (%)	Delaying time (seconds)
control	82.3 ± 1.8	85.6 ± 2.1	68.3 ± 5	90.8 ± 1.9	46.25 ± 5.59
Aspirin 0.1 mM	36.9 ± 5.3	12.3 ± 1.1	140 ± 0.0*	11.1 ± 0.6	∞
Lusianthridin 0.0125 mM	ND	ND	ND	86.8 ± 4.5	80 ± 8.91*
Lusianthridin 0.025 mM	ND	ND	ND	9.5 ± 1.1*	∞
Lusianthridin 0.05 mM	56.3 ± 4.5*	71.4 ± 4.8	68.0 ± 4.0	9.5 ± 1.1*	∞
Lusianthridin 0.1 mM	41.9 ± 2.4*	56.4 ± 7.5	83.3 ± 7.9	9.7 ± 0.9*	∞
Lusianthridin 0.2 mM	35.8 ± 3.1*	29.9 ± 7.1*	94.1 ± 9.5	ND	ND
Lusianthridin 0.4 mM	38.8 ± 3.1*	13.2 ± 1.8*	114.5 ± 15.8*	ND	ND

∞ maximum percent inhibition of platelet aggregation occurs (no delaying time). ND not determine this concentration. Data are presented as means ± SEMs (n = 5). *p < 0.05 *vs.* vehicle control (0.5 %), analyzed by ANOVA followed by Tukey post-hoc test.

2. Molecular Docking Analysis

2.1. Docking analysis with COX-1 enzyme

As shown in the Figure 17A, the binding site of lusianthridin was partially the same with the binding site of arachidonic acid on the COX-1 enzyme. Arachidonic acid bound the active site of COX-1 with two hydrogen bonds (Arg-120 and Phe-470), one carbon hydrogen bond (Gly-471) and three hydrophobic bonds (Val-116, Leu-531 and Ala-527) (Figure 17B). Moreover, its binding site existed at the vicinity of amino acid residues Glu-524, Ile-89, Leu-93 and Tyr-355. Lusianthridin bound the COX-1 enzyme by a pi-donor hydrogen bond with Tyr-355, two hydrophobic bonds with Val-116 and Ile-89 and a hydrophobic bond with Leu-93 (Figure 17C). Arg-120 and Glu-524 surrounded the binding site of lusianthridin. Additionally, the binding affinity of lusianthridin was comparable to that of arachidonic acid (-7.2 kcal/mol and -7.9 kcal/mol, respectively).

As shown in Figure 18A, 3,7-dihydroxy-2,4-dimethoxyphenanthrene bound the COX-1 enzyme by a hydrogen bond with Glu-524, a pi-sigma bond with Ile-89 and two pipi-alkyl bonds with Val-116 and Leu-93. Arg-120 surrounded the binding site of this compound and the binding affinity was – 7.5 kcal/mol. Regarding to erianthridin, it bound the COX-1 enzyme by a pi-cation bond with Arg-120, a pi-pi bond with Tyr-355, an alkyl bond with Leu-531, three pi-sigma bonds with Ala-527 and Val-349 (Figure 18B). Moreover, its binding site existed at the vicinity of amino acid residues Ser-530, Tyr-385, Trp-387 and Leu-352 and the binding affinity was – 7.8 kcal/mol.

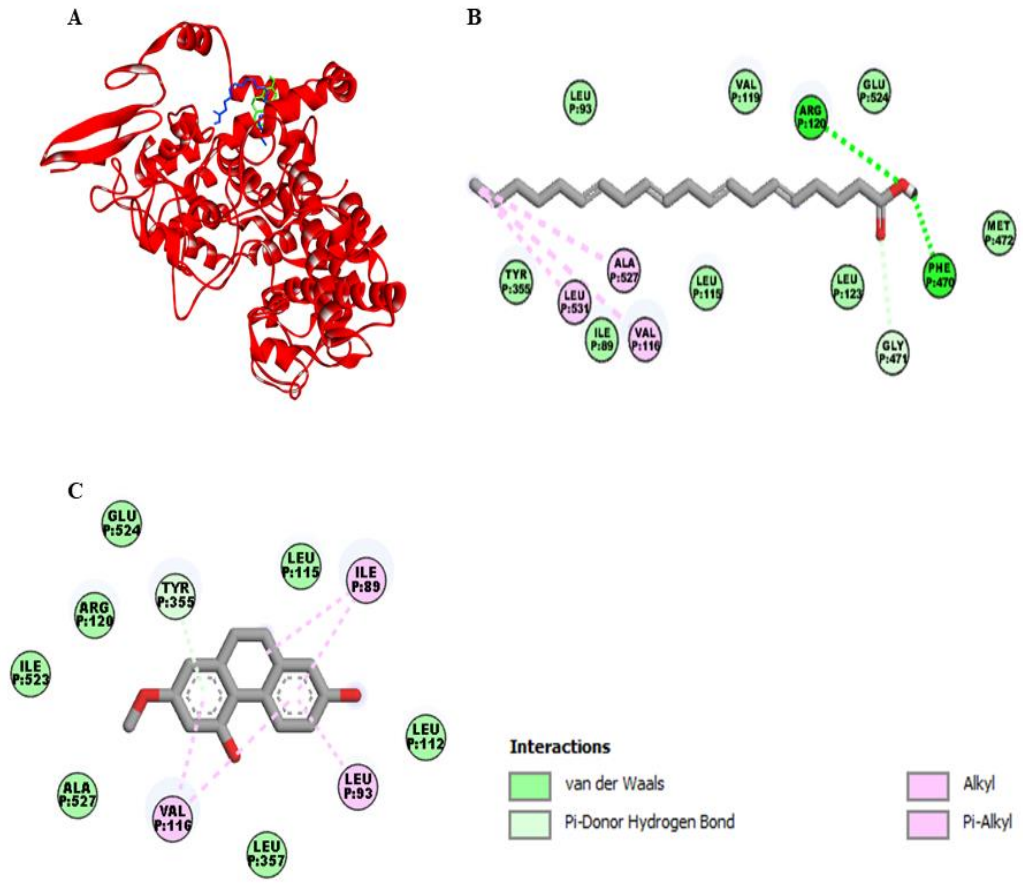
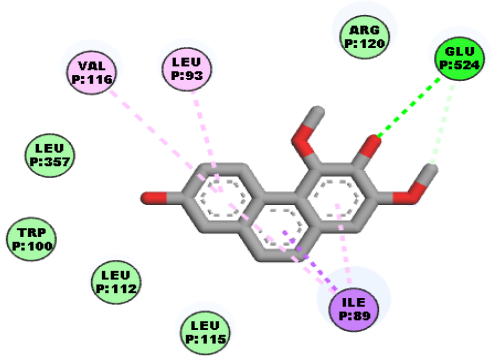


Figure 17. Docking lusianthridin into the COX-1 enzyme. (A) 3D interaction of arachidonic acid (blue) and lusianthridin (green) with the COX-1 enzyme. (B), (C) 2D interaction of binding mode for (B) arachidonic acid (C) lusianthridin inside the COX-1 enzyme.

A



B

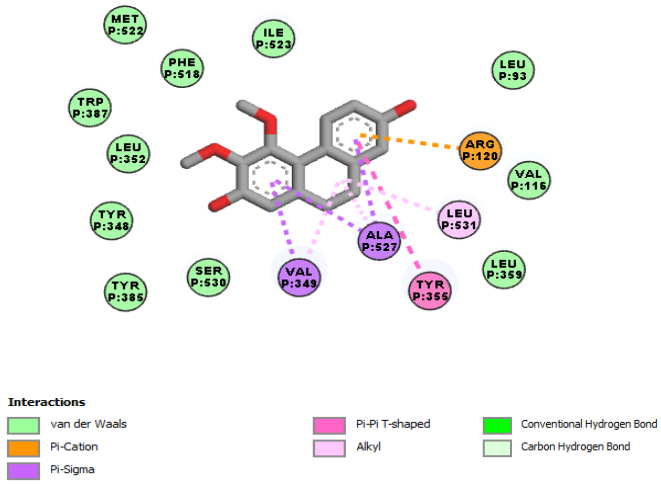


Figure 18. 2D interaction of binding mode for (A) 3,7-dihydroxy-2,4-dimethoxyphenanthrene (B) erianthridin inside the COX-1 enzyme.

2.2. Docking analysis with COX-2 enzyme

Figure 19A represents the interaction of lusianthridin and arachidonic acid with COX-2 enzyme. We found that the binding site of lusianthridin was far from that of arachidonic acid. Arachidonic acid bound with COX-2 enzyme *via* the key amino acid residue, Arg-120 (Figure 19B). When lusianthridin was docked at the same site, it formed two hydrogen bonds with Arg-44 and Asn-39 and three hydrogen bonds with Pro-153, Leu-152 and Cys-47 (Figure 19C). In addition, its binding site existed near the amino acid residues Glu-465 and Arg-469.When we compared the binding affinity of lusianthridin and arachidonic acid, lusianthridin showed lower binding affinity which means higher inhibitory potency on COX-2 enzymatic activity (-9.3 kcal/mol *vs* -7.3 kcal/mol, respectively).

As shown in Figure 20A, 3,7-dihydroxy-2,4-dimethoxyphenanthrene bound the COX-2 enzyme by three carbon hydrogen bonds with Cys-47, Arg-44 and Glu46, five pi-alkyl bonds with Leu-152 and Pro-153. Asn-39, Glu-465 and Arg-469 surrounded the binding site of this compound and the binding affinity was – 9.8 kcal/mol. Regarding to erianthridin, it bound the COX-2 enzyme by a hydrogen bond with Cys-36, four carbon hydrogen bonds with Asn-34, Asp-157, Val-155 and His-133, two pi-pi bonds with Gly-135 and Tyr-136, and two pi-alky bonds with Ala-156 (Figure 20B). Moreover, its binding site existed at the vicinity of amino acid residues Asn-39 and the binding affinity was – 8.4 kcal/mol.

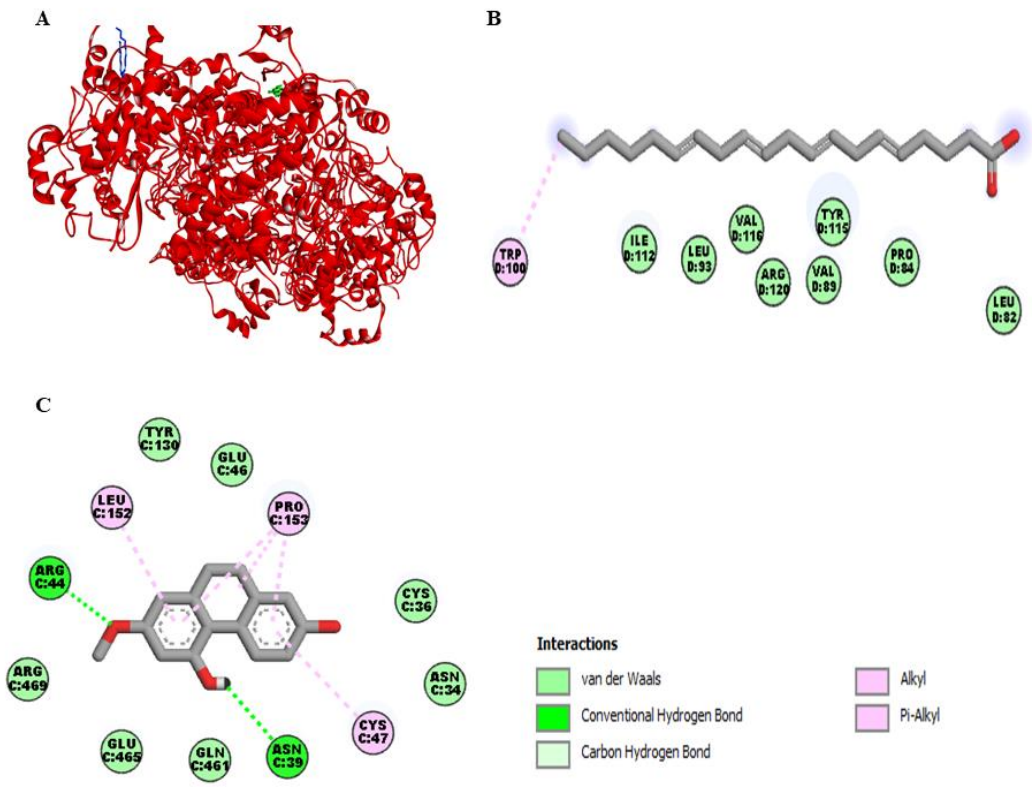


Figure 19. Docking lusianthridin into the COX-2 enzyme. (A) 3D interaction of arachidonic acid (blue) and lusianthridin (green) with the COX-2 enzyme. (B), (C) 2D interaction of binding modes for (B) arachidonic acid (C) lusianthridin inside the COX-2 enzyme.

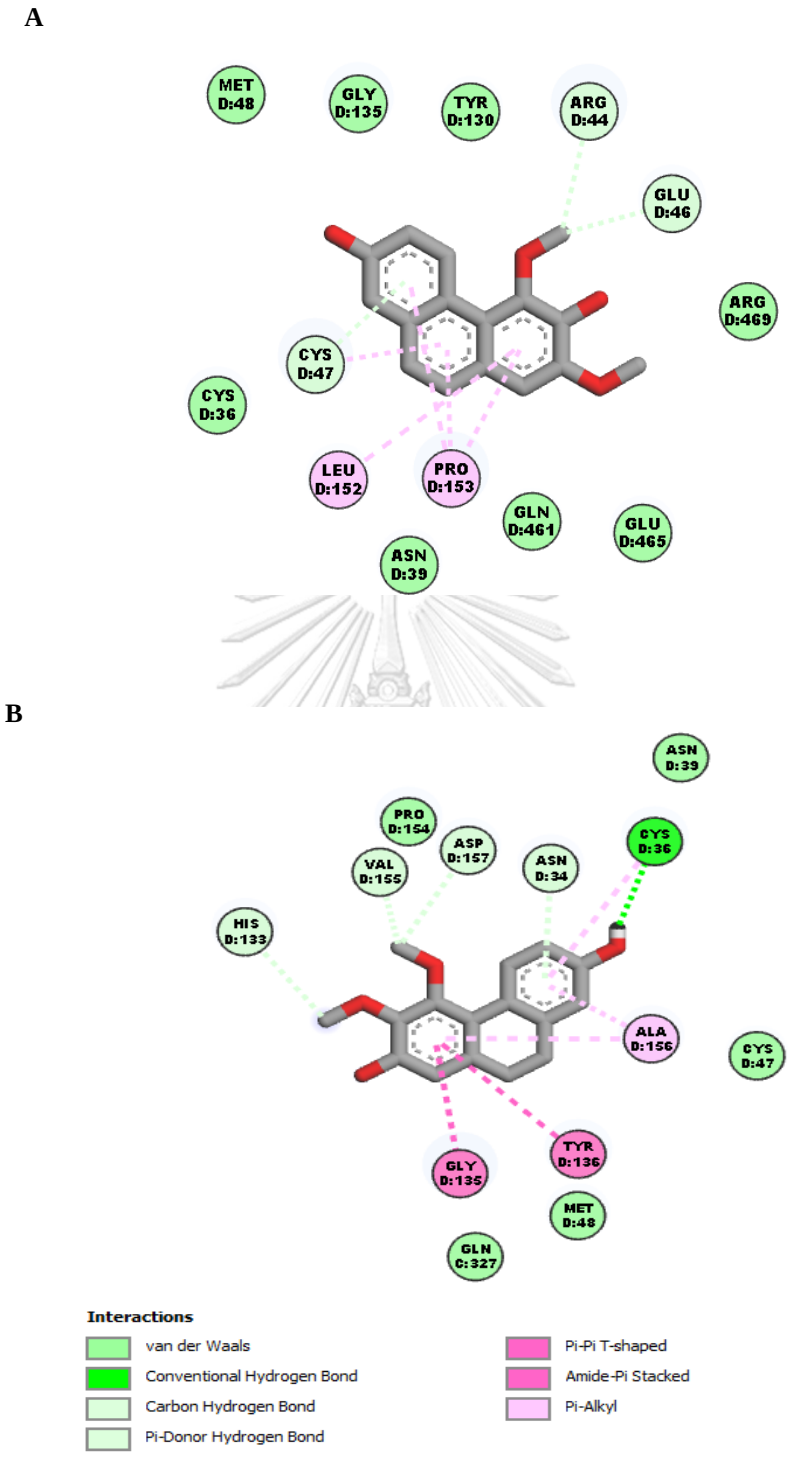


Figure 20. 2D interaction of binding mode for (A) 3,7-dihydroxy-2,4-dimethoxyphenanthrene (B) erianthridin inside the COX-2 enzyme.

3. Effect of Lusianthridin on cyclooxygenase enzymes activity

The effect of lusianthridin on cyclooxygenase activity was determined using COX fluorescent inhibitor screening assay kit. Lusianthridin showed concentration-dependent inhibitory effects on both COX-1 and COX-2 enzymatic activities with the IC_{50} value of 11.9 ± 0.0 μM and 0.2 ± 0.2 μM and, respectively (Figure 21A and 21B).

When we compared the results of platelet aggregation study with the results of COX-1 enzyme activity assay (Figure 22), the effect of lusianthridin on AA-induced platelet aggregation showed strong negative correlation with its inhibitory effect on COX-1 enzymatic activity (r = - 0.85).

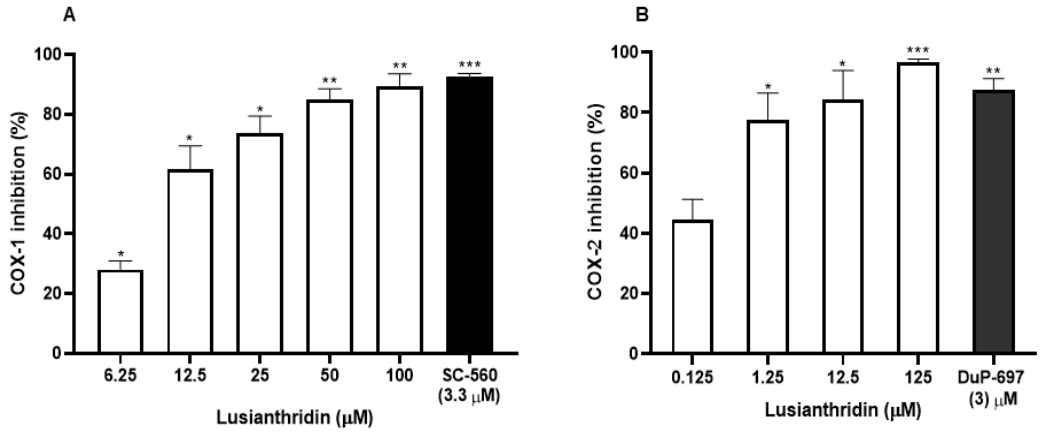


Figure 21. The effects of various concentrations of lusianthridin on the activities of (A) COX-1 and (B) COX-2 enzymes. Tested compounds were incubated directly with COX-1 or COX-2 in the assay buffer for 5 min and then the activities were measured by a COX fluorescent inhibitor screening assay kit. SC-560, a COX-1 selective inhibitor, and DuP-697, a COX-2 selective inhibitor, were used as positive controls. Data are presented as means ± SEMs (n = 3). *p < 0.05, **p<0.001, ***p<0.0001 compared with control and analyzed by ANOVA followed by the Tukey post-hoc test.

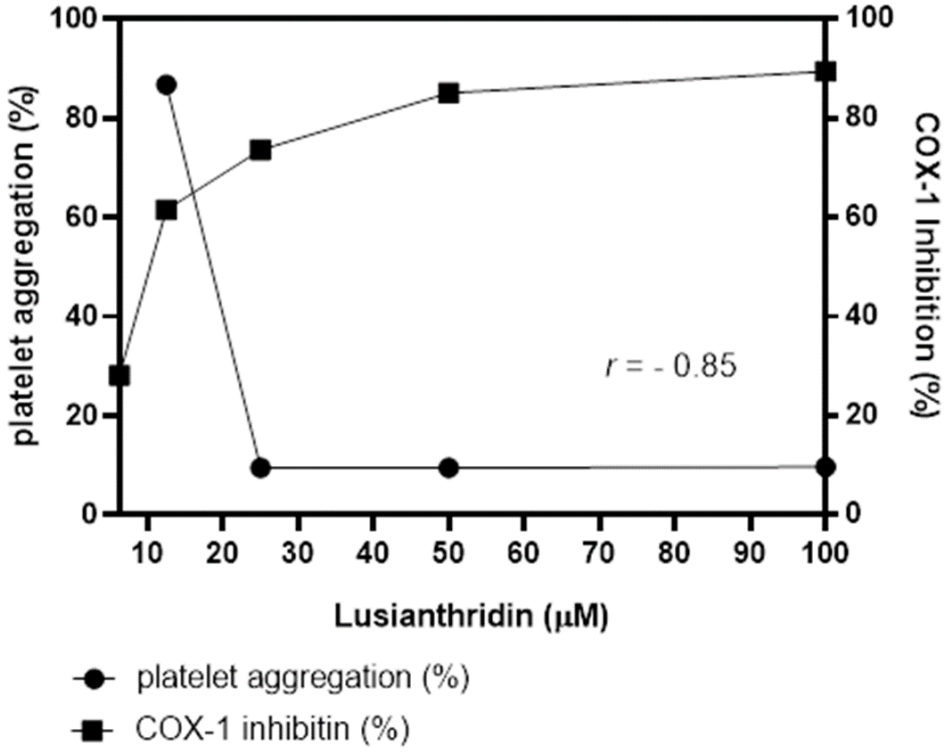


Figure 22. Correlation between platelet aggregation study and COX-1 enzyme activity assay.

4. Effect of Lusianthridin on cAMP level

To assess the level of platelet cAMP, the experiment was carried out by using cAMP ELISA kit. As shown in Figure 23, ADP 4 μM significantly decreased the cAMP level in platelets (7.0 ± 0.2 pmol/10^8 platelets) compared to the basal cAMP level (11.0 ± 0.4 pmol/10^8 platelets). When platelets were pre-incubated with lusianthridin at the concentration of 0.4 mM, the ADP-induced cAMP level decrease in platelets was prevented (12.2 ± 1.4 pmol/10^8 platelets, $p < 0.05$). The 3-isobutyl-1-methylxanthine (IBMX) was used as a positive control and the results of lusianthridin were very similar to those of IBMX (11.7 ± 1.9 pmol/10^8 platelets).

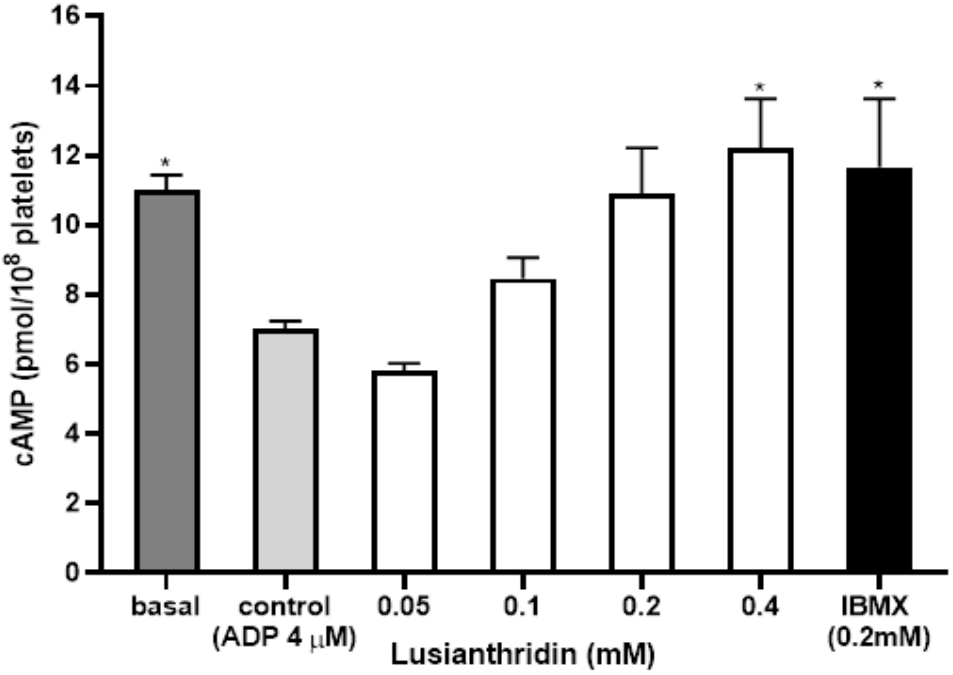


Figure 23. The effects of various concentrations of lusianthridin on platelet cAMP levels. Platelets were pre-incubated with different concentrations of compounds for 5 min with continuous stirring at 37°C and ADP (4 μM) was then added to induce platelet aggregation. Basal means not adding ADP to induced platelet aggregation. Control means only ADP 4 μM was added. IBMX (3-isobutyl-1-methylxanthine), a phosphodiesterase inhibitor, was used as a positive control. cAMP levels were measured by an ELISA kit. Data are presented as means ± SEMs (n = 4). *p < 0.05 compared with control and analyzed by ANOVA followed by the Tukey post-hoc test.

CHAPTER 5

DISCUSSION AND CONCLUSION

Arterial thrombosis is a complication which is derived from the chronic atherosclerosis leading to cardiovascular disease (CVD). CVD is the most common causes of mortality in developed countries nowadays. Although the normal function of platelets is to prevent bleeding, platelets are the most important components of thrombi which occlude the blood vessel and involve in development of atherosclerotic plaque and atherothrombosis (44). Therefore, antiplatelet agents are intensively used for prevention and treatment of CVD. Many antiplatelet drugs inhibit aggregation of platelets *via* their specific pathways, but they also have some problems such us side effects and a high cost (45). Thus, searching the newer antiplatelet agents from natural products as an alternative therapy is an attractive field.

Phenanthrene-containing plants have been used in traditional medicines and phenanthrenes are promising candidates to explore their biological activities (29). Orchidaceae are the most abundant source of this compound and orchids are also widely used as traditional medicines in China. *Dendrobium* spp. is the largest and most important genera among orchids (28). Phenanthrene derivatives from *Dendrobium* such as moscatin, 3,7-dihydroxy-2,4-dimethoxyphenanthrene and

erianthridin showed antiplatelet aggregation activity (7, 12). According to the present study, lusianthridin, a phenanthrene compound from *Dendrobium*, is also a promising candidate for antiplatelet activity. Lusianthridin showed inhibitory activity on ADP, collagen and AA-induced platelet aggregation and these data showed that this compound could be an antiplatelet candidate for the development of new antiplatelet agent. In the present study, we evaluated the possible mode of actions of lusianthridin, which was isolated from *Dendrobium venustum*, in human platelets.

Lusianthridin inhibited platelet aggregation stimulated by arachidonic acid, ADP and collagen. These three agonists have their specific signaling pathways leading to platelet aggregation and they also share the common arachidonic acid-thromboxane pathway (46). The ADP-induced platelet aggregation pathway includes two G protein-linked nucleotide receptors namely $P2Y_1$ and $P2Y_{12.}$ The $P2Y_1$ receptor is linked to G_q which leads to the activation of the β-isoform of phospholipase C (47). This receptor is responsible for changing in platelet shape and mobilization of intracellular calcium which causes initial aggregation (primary phase). $P2Y_{12}$ is coupled to G_i for inhibition of the adenylate cyclase to reduce cAMP levels. This receptor is responsible for secretion of thromboxane A_2 (TXA_2) and other mediators which act on their specific receptors and produce more platelet aggregation (secondary phase) (46). These two phases of ADP-stimulated platelet aggregation can be detected by turbidimetric aggregometry method (48). $P2Y_1$ receptor antagonists inhibit both primary and secondary phases of ADP-stimulated platelet aggregation and $P2Y_{12}$

receptor antagonists inhibit the secondary phase without an effect on shape changes (49). According to the current study, lusianthridin more selectively inhibited the second phase aggregation of ADP-stimulated platelet aggregation compared with the first phase. This result indicated that lusianthridin might not directly inhibit the pathway of $P2Y_1$ but inhibit the $P2Y_{12}$ pathway (adenylate cyclase pathway). In addition, maximum percent inhibition of lusianthridin after ADP induction was only around 55 %. This may also be due to ADP binds with $P2Y_{12}$ receptor and undergoes other signaling pathways to induce platelet aggregation. Therefore, it is possible that lusianthridin might partially inhibit the $P2Y_{12}$ signaling.

Moreover, lusianthridin increased cAMP levels in ADP-stimulated platelet aggregation. These results confirm that the inhibitory effect of lusianthridin on platelet aggregation might come from adenylate cyclase pathway. Prostaglandin (PGE_1) is a potent inhibitor of platelet aggregation. It directly stimulates adenylate cyclase causing increased intracellular cAMP. The phosphodiesterase inhibitors inhibit the conversion of cAMP to AMP and increased the level of cAMP, leading to less calcium influx and secretion by suppressing DAG, IP_3 and TXA_2 (50). Therefore, the inhibitory effect of lusianthridin on ADP-induced suppression of cAMP levels might come from the stimulation of adenylate cyclase activity or inhibition of phosphodiesterase enzyme.

Collagen causes the activation of $PLC\gamma_2$ producing inositol 1,4,5 triphosphate (IP_3), which increases the release of calcium from dense tubular system and 1,2-

diacylglycerol (DAG), which activates the PKC (51). Calcium release activates phospholipase A_2 (PLA_2) which causes further increase in intracellular calcium levels, secretion of granules and production of TXA_2. This collagen-stimulated signaling pathway depends on TXA_2 secretion to some extent (20). Aspirin, a COX inhibitor, could inhibit collagen-stimulated platelet aggregation by inhibition of the TXA_2 production (52). In the present study, lusianthridin prolonged the lag time which means that it prolonged the time to induce changes in platelet shape and inhibited collagen-stimulated platelet aggregation. Therefore, lusianthridin might act on calcium mobilization or TXA_2 secretion to inhibit platelet aggregation. Further studies are needed in this area.

Another agonist that we used in our study to induce platelet aggregation was arachidonic acid. Arachidonic acid is metabolized to prostaglandin $(PG)H_2$ by the COX-1 enzyme in platelets. Then, PGH_2 is converted to TXA_2 by the peroxidase enzyme. TXA_2 binds to two separate isoforms of TXA_2 receptor: TPα, which is linked to Gq and TPβ, which is linked to Gi (24). This study showed that lusianthridin very potently inhibited platelet aggregation induced by arachidonic acid. Although lusianthridin could not inhibit AA-induced platelet aggregation at the concentration of 0.0125 mM, lusianthridin at 0.0125 mM prolonged the delaying time which means that it delayed the production of TXA_2. Therefore, the antiplatelet activity of lusianthridin might be involved in the arachidonic acid metabolic pathway.

According to the results of platelet aggregation study, lusianthridin inhibited all three agonists induced platelet aggregation. Lusianthridin most potently inhibited AA-induced platelet aggregation by reaching maximum percent aggregation to 9.5 % at 0.025 mM whereas lusianthridin at 0.4 mM decreased collagen and ADP-induced platelet aggregation by 13.2 % and 38.8 %, respectively. From these findings, we could estimate that the antiplatelet activity of lusianthridin might come from the arachidonic acid-thromboxane pathway. Moreover, lusianthridin showed inhibition of the secondary phase of ADP-induced platelet aggregation and increased cAMP levels in ADP-induced platelet aggregation. Therefore, this activity might come from activation of adenylate cyclase or inhibition of phosphodiesterase enzyme.

Molecular docking and COX activity assays showed that lusianthridin probably inhibited COX-1 by binding to the entrance site (lobby region) and COX-2 enzymes by binding to the active sites of these enzymes. Lusianthridin seemed to bind the entrance site of the COX-1 enzyme (lobby region). Most conformations belonging to this site were characterized by the presence of a bond between Arg-120 and Glu-524. Aromatic residues including Pro-86, Ile-89, Leu-93 and Val-116 are assumed to be involved in the interaction of inhibitors with lobby region (53). In the present study, lusianthridin interacted with Tyr-355 by a pi-donor hydrogen bond and three aromatic residues (Ile-89, Leu-93 and Val-116) by hydrophobic bonds. Moreover, the key residues (Arg-120 and Glu-524) were surrounded the binding site of lusianthridin. Therefore, lusianthridin probably inhibited the activity of COX-1 by blocking the

lobby region of the enzyme preventing its substrate, arachidonic acid, from reaching the catalytic site of the enzyme. In the current study, we also performed the docking analysis of two phenanthrenes which have been known about their antiplatelet activity. 3,7-dihydroxy-2,4-dimethoxyphenanthrene bound the COX-1 enzyme by a hydrogen bond with Glu-524, a pi-sigma bond with Ile-89, two pipi-alkyl bonds with Val-116 and Leu-93 and Arg-120 surrounded it binding site. Therefore, it also inhibited COX-2 enzyme by blocking to the lobby region of the enzyme which was similar with lusianthridin. However, the binding site of erianthridin to COX-1 enzyme was different from lusianthridin and 3,7-dihydroxy-2,4-dimethoxyphenanthrene. Erianthridin bound the COX-1 enzyme by a pi-cation bond with Arg-120, a pi-pi bond with Tyr-355, an alkyl bond with Leu-531, three pi-sigma bonds with Ala-527 and Val-349. Moreover, its binding site existed at the vicinity of amino acid residues Ser-530, Tyr-385, Trp-387 and Leu-352. Val-349, Ala-527 and Leu-531 are the key amino acid residues for indomethacin binding pocket and Ser-530 and Tyr-385 are the key residues of catalytic site of COX-1 enzyme (53). Therefore, erianthridin inhibited COX-1 enzyme by binding to the catalytic active site of COX-1 enzyme. When we compared the binding affinity on COX-1 enzyme, lusianthridin, 3,7-dihydroxy-2,4-dimethoxyphenanthrene and erianthridin showed comparable binding affinity with -7.2, -7.5 and -7.8 kcal/mol, respectively.

For interaction with the COX-2 enzyme, lusianthridin did not interact closely with the binding site of arachidonic acid. However, lusianthridin formed hydrogen

bonds with three amino acid residues (Pro-153, Arg-44 and Asn-39) which were probably the active region of the COX-2 enzyme interacted with isoindolines (54), caprolactam-salicylic ionic liquid (55), rofecoxib (56), naproxen modified derivatives (57) and aryl/heteroaryl substituted celecoxib derivatives(58). For 3,7-dihydroxy-2,4-dimethoxyphenanthrene, it bound the COX-2 enzyme by three carbon hydrogen bonds with Cys-47, Arg-44 and Glu46, five pi-alkyl bonds with Leu-152 and Pro-153. Asn-39, Glu-465 and Arg-469 surrounded the binding site of this compound. Therefore, this compound also bound to the similar binding site of lusianthridin on COX-2 enzyme. Moreover, Asn-34 surrounded the vicinity of the binding site of erianthridin. Therefore, all these three compounds had the similar binding site on COX-2 enzyme. When we compared the binding affinity, lusianthridin and 3,7-dihydroxy-2,4-dimethoxyphenanthrene showed lower binding affinity than erianthridin (-9.3 kcal/mol, -9.8 kcal/mol and -8.4 kcal/mol, respectively). According to these results, we can conclude that lusianthridin and 3,7-dihydroxy-2,4-dimethoxyphenanthrene had stronger inhibitory potency to COX-2 enzyme than that of erianthridin. This inhibitory effect of the COX-2 enzyme might not be evident in platelet study as a small amount of COX-2 is expressed inside the platelets (59). In the previous findings, a COX-2 selective inhibitor reduced the production of thromboxane B_2 although to a lesser amount compared to a COX-1 selective inhibitor (60).

When we correlated the platelet aggregation study and COX-1 enzymatic activity assay, lusianthridin showed a strong negative correlation between its effect on AA-induced platelet aggregation and its inhibitory effect on COX-1 enzymatic activity. These results confirmed that the inhibitory effect of lusianthridin on AA-induced platelet aggregation might come from the inhibition of COX-1 enzyme. According to all of these findings, we can conclude that the possible mechanisms of antiplatelet activity of lusianthridin probably act *via* arachidonic acid-thromboxane and adenylate cyclase pathways. Lusianthridin might stimulate the adenylate cyclase or inhibited phosphodiesterase enzyme to increase cAMP levels in the platelets. In addition, Lusianthridin inhibited the production of TXA_2 production by blocking to the entrance site of COX-1 enzyme (Figure 24).

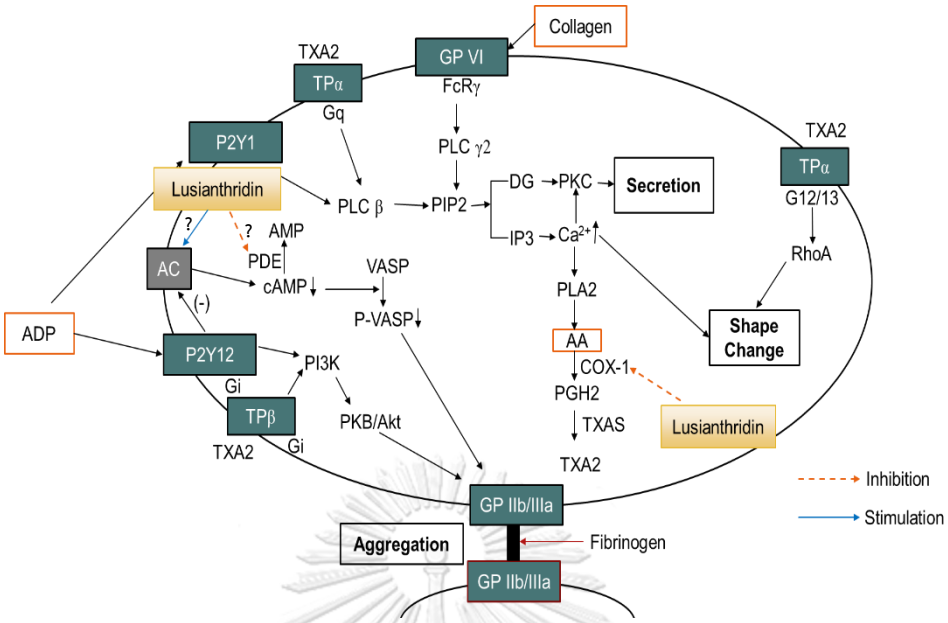


Figure 24. Possible mode of antiplatelet activity of lusianthridin.

When the IC_{50} values of lusianthridin on COX1 and COX-2 enzymatic activities were compared, lusianthridin showed a stronger effect on the COX-2 enzyme than on the COX-1 enzyme. This result was related with the binding affinity of lusianthridin on COX-1 and COX-2 enzymes (-7.2 and -9.3 kcal/mol, respectively). To evaluate COX selectivity of compounds, cyclooxygenase selectivity was expressed as a ratio of concentration of a compound that the IC_{50} of COX-1 to the IC_{50} COX-2. A ratio of less than 1 indicated selectivity for COX-1, whereas a ratio of more than 1 indicated selectively for COX-2 (61). Lusianthridin inhibited COX-2 enzyme about 50 times higher than COX-1 enzyme. Therefore, lusianthridin might have selective inhibitory effect on COX-2 enzyme. However, aspirin covalently modifies both COX-1 and COX-2, although it is 10-100 times more potent against COX-1 than against COX-

2 (53, 62, 63). Therefore, further studies are required to evaluate the selectivity of lusianthridin on COX-1 and COX-2 enzymatic activities.

This study revealed that lusianthridin inhibited platelet aggregation induced by arachidonic acid, collagen and ADP and its antiplatelet aggregation activities might act *via* arachidonic acid-thromboxane and adenylate cyclase pathways. Therefore, lusianthridin might be beneficial for the protection of atherosclerotic cardiovascular diseases. Lusianthridin also showed an inhibitory effect on the COX-2 enzyme and further studies on its anti-inflammatory effects are warranted. Further studies for detail analysis of its antiplatelet activities and the antiplatelet activities in animal models to explore the potentials in prevention of atherothrombosis are required.

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APPENDIX

1. Extraction, isolation and identification of lusianthrindin

(A) Plant material

Samples of D. venustum were purchased from Jatujak market, Bangkok, in May 2012. Authentication was performed by comparison with herbarium specimens at the Department of National Park, Wildlife and Plant Conservation, Ministry of National Resources and Environment.

(B) Extraction and isolation

The dried and powdered whole plant (2 kg) was macerated with MeOH (3 x 10 L) to afford a methanol extract (164 g) after removal of the solvent. This material was subjected to vacuum-liquid chromatography (VLC) on silica gel (n-hexane-ethyl acetate gradient) to give 8 fractions (A-H). Fraction G was selected for further investigation as it showed antimalarial and antiherpetic effects. Fraction G (16.3 g) was fractionated by CC over silica gel eluting with a CH_2Cl_2-ethyl acetate gradient to give 10 fractions (GI-GX). Fraction GV (1.0 g) was separated by CC (silica gel; CH_2Cl_2-ethyl acetate gradient) and then further purified on Sephadex LH 20 (acetone) to afford lusianthridin (618 mg).

(C) Identification and purity

The structure of the isolate was determined by comparing its spectroscopic data with those reported and the purity was evaluated using NMR spectroscopy.

2. Preliminary data for antiplatelet aggregation activity of lusianthridin

Compound	ADP (n=2)		Collagen (n=2)	
	Aggregation (%)	Inhibition (%)	Aggregation (%)	Inhibition (%)
Control (DMSO 0.5%)	81.40 ± 0.10		86.1 ± 5.94	
Lusianthridin 0.025 mM	77.40 ± 0.03	4.91 ± 0.16	70.3 ± 4.83	18.35 ± 3.24
Lusianthridin 0.05 mM	63.14 ± 0.79	22.43 ± 1.07	48.25 ± 19.37	43.96 ± 2.50
Lusianthridin 0.1 mM	41.22 ± 0.05	49.37 ± 0.00	19.37 ± 49	77.51 ± 4.35

3. The effect of lusianthridin on ADP-induced platelet aggregation

(A) The percent inhibition of lusianthridin compared with vehicle control

Compound	Inhibition (%)	
	mean	SEMs
Lusianthridin 0.05 mM	29.84	6.23
Lusianthridin 0.1 mM	47.96	3.37
Lusianthridin 0.2 mM	55.71	4.42
Lusianthridin 0.4 mM	51.88	4.77

4. The effect of lusianthridin on AA-induced platelet aggregation

(A) The percent inhibition of lusianthridin compared with vehicle control

Compound	Inhibition (%)	
	mean	SEMs
Lusianthridin 0.0125 mM	5.30	4.45
Lusianthridin 0.025 mM	89.71	1.24
Lusianthridin 0.05 mM	89.48	1.37
Lusianthridin 0.1 mM	89.31	1.11

5. The effect of lusianthridin on collagen-induced platelet aggregation

(A) The percent inhibition of lusianthridin compared with vehicle control

Compound	Inhibition (%)	
	mean	SEMs
Lusianthridin 0.05 mM	16.37	5.91
Lusianthridin 0.1 mM	33.96	9.16
Lusianthridin 0.2 mM	64.74	8.73
Lusianthridin 0.4 mM	84.49	2.17

6. The effect of lusianthridin on COX-1 and COX-2 enzymatic activity

(A) The percent inhibition of lusianthridin on COX-1 enzymatic activity

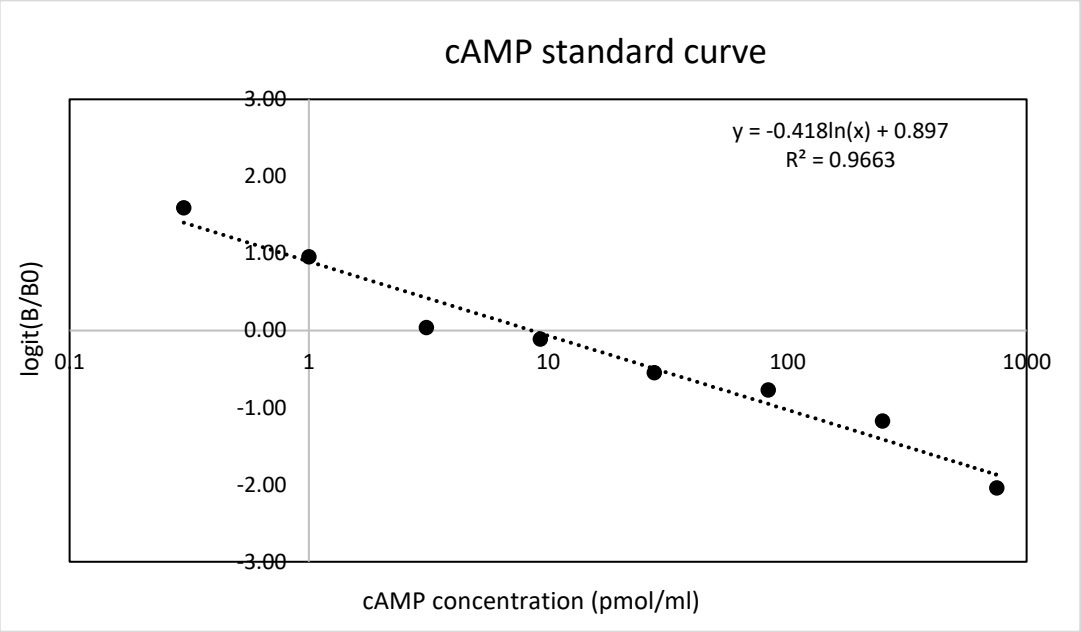
Compound	Inhibition (%)	
	mean	SEMs
SC-560 (3.3) μM	93.23	1.40
Lusianthridin 6.25 μM	28.11	2.89
Lusianthridin 12.5 μM	61.55	7.95
Lusianthridin 25 μM	73.64	5.85
Lusianthridin 50 μM	85.12	3.47
Lusianthridin 100 μM	89.42	4.29
IC_{50}	11.92	0.00

(B) The percent inhibition of lusianthridin on COX-2 enzymatic activity

Compound	Inhibition (%)	
	mean	SEMs
DuP-697 (3) μM	87.44	2.78
Lusianthridin 0.125 μM	44.42	5.50
Lusianthridin 1.25 μM	77.74	8.70
Lusianthridin 12.5 μM	84.34	9.59
Lusianthridin 125 μM	96.67	1.07
IC_{50}	0.21	0.17

7. The effect of lusianthridin on cAMP level in platelets

(A) Standard curve of cAMP measurement for lusianthridin



(B) effect of lusianthridin on cAMP level

Compound	cAMP level (pmol/10^8 platelets)	
	mean	SEMs
Basal cAMP	11.01	0.43035
DMSO 0.5 %	7.04	0.21
IBMX (0.2mM)	11.66	1.98
Lusianthridin 0.05 mM	5.82	0.22
Lusianthridin 0.1 mM	8.47	0.60
Lusianthridin 0.2 mM	10.91	1.31
Lusianthridin 0.4 mM	12.21	1.42

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