NYMPHAEA PUBESCENS EXTRACT AS AN HERBAL INGREDIENT FOR ERECTILE DYSFUNCTION AND VASCULAR DISEASES

TEERAPAP PANKLAI

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Pharmaceutical Sciences
2024

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By Teerapap Panklai

has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Pharmaceutical Sciences of Naresuan University

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ABSTRACT

Background: Due to the increase in the elderly population, the disease caused by degeneration (degenerative diseases) is becoming an important health problem. Cardiovascular disease (CVD) has many common risk factors, including age, hypertension, diabetes, insulin resistance, smoking, increased body mass index (BMI), cholesterol, and lower high-density lipoprotein (HDL). These factors are also linked to erectile dysfunction (ED). ED and CVD are pathophysiological consequences of atherosclerosis and endothelial dysfunction. Endothelial dysfunction also plays a role in the progression of vascular disease in pulmonary arterial hypertension (PAH). From the previous study, we found that the petal of *Nymphaea pubescens* Willd., the member of Nymphaeaceae family, showed high inhibition of phosphodiesterase 5, the enzyme that is involved in the cardiovascular system and erection process. This plant or the plants in the related family might be sources of bioactive compounds for ED and CVD.

The aim of the study: The main aim of this project was exploration of the possibility to apply water lilies and lotuses in families of Nelumbonaceae and Nymphaeaceae for vascular health benefit. The PDE5 and arginase inhibitors from the active extract would be isolated and identified. The underlying mechanisms on

vasodilator action in various blood vessels as well as the cytotoxicity of the extract and its bioactive constituents would be studied. Finally, the methods for quality control and standardization of the water lily extract would be established.

Materials and methods: Thirty-seven ethanolic extracts from different parts of plants in the genus *Nymphaea* and *Victoria* of Nymphaeaceae and genus *Nelumbo* of Nelumbonaceae were screened for PDE5 and arginase inhibitory activities. The bioactive compounds in the active extract were isolated and identified by chromatographic and spectroscopic methods, respectively. The vasorelaxant effects of the extract and its chemical constituents were evaluated on rat PA, aorta and mesenteric arteries. The cytotoxicity of the extract was also tested on the vascular smooth muscle cells (VSMCs) isolated from the rats. The characterization and quantitative analysis of constituents in the active extracts were performed by HPLC.

Results: The ethanolic extracts of the receptacles and pollens of *Nelumbo nucifera* Gaertn., petals of *Nymphaea cyanea* Roxb. ex G.Don, *Nymphaea stellata* Willd., and *Victoria amazonica* (Poepp.) Sowerby and the petals and receptacles of *N. pubescens* showed IC_{50} values on PDE5 of less than 25 µg/mL while none of the extracts showed effects on arginase. The most active extract, *N. pubescens* petal extract (WLE), was fractionated to isolate and identify the PDE5 inhibitors. The six flavonoid constituents including quercetin 3'-O-β-xylopyranoside (1), quercetin 3-methyl ether 3'-O-β-xylopyranoside (2), quercetin (3), 3-*O*-methylquercetin (4), kaempferol (5) and 3-*O*-methylkaempferol (6) inhibited PDE5 with IC_{50} values at the micromolar level. Among these compounds, 2 was the major compound in WLE (17.31±0.05 mg/g) and showed the strongest activity (IC_{50}=5.63±0.20 mM).

The WLE relaxed PA (EC_{50} = 4.96±0.81 µg/ml) more than the aorta (EC_{50} = 27.50±7.61 µg/ml, p<0.001), suggesting its selectivity on the PA vs the aorta. PA vasorelaxation was reduced by endothelial removal or N^G-nitro-L-arginine methyl ester (L-NAME), but was unaffected by indomethacin, apamin plus charybdotoxin, 4-aminopyridine (4-AP), glibenclamide, iberiotoxin, and $BaCl_2$. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin -1- one (ODQ) slightly reduced the relaxation induced by the WLE. Sodium nitroprusside (SNP)-induced relaxation was enhanced by the WLE. WLE cannot inhibit Ca^{2+} channels (extracellular Ca^{2+} influx through

ROCCs/VOCCs and intracellular Ca^{2+} release from the SR), and PE-induced contraction via α_1-receptor. Compounds 2, 3 and 5 non-selectively relaxed the PA and aorta rings with and without endothelium (EC_{50} = 26 - >100 μM). VSMCs incubated in the WLE for 1 hr showed no acute cytotoxicity. The results of mesenteric arteries showed that both WLE and compound 2 induced vasorelaxant effects with EC_{50} of 0.08±0.01 mg/mL and 42.8±6.3 μM, respectively. A significant decrease in these relaxations was observed with L-NAME, but not with apamin-charybdotoxin or indomethacin. In endothelium-denuded condition, WLE-induced relaxation was enhanced by 4-aminopyridine and glibenclamide while iberiotoxin and ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one) had no effect. By contrast, compound 2-induced relaxation was not changed by any of these inhibitors. Both WLE and compound 2 enhanced sodium nitroprusside-induced relaxation and inhibited receptor-operated Ca^{2+} channels. Only WLE was able to reduce PE-induced contraction ($p<0.001$).

Conclusion: Among 37 samples from Nymphaeaceae and Nelumbonaceae families, our research found that *N. pubescens* petals had the strongest PDE5 inhibitory effect. Six flavonoids (1-6) with PDE5 inhibitory activity have been characterized as the constituents in the extract by the HPLC method. The major compound, 2 (17.31±0.05 mg/g of WLE), together with 1 and 6 were found to inhibit PDE5 for the first time in this study. The WLE induced PA relaxation via the endothelial nitric oxide (NO) pathway and mild stimulation of sGC. Importantly, the WLE showed no cytotoxicity on the VSMCs. The vasorelaxant effect of WLE and compound 2 on mesenteric arteries, relying on the potentiation of the NO-cGMP pathway and on calcium inhibitory effects.

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ABBREVATIONS LIST

4-AP	=	4-aminopyridine
°C	=	degree celsius
ACh	=	acetylcholine
AChE	=	acetylcholinesterase
ACN	=	acetonitrile
ATP	=	adenosine triphosphate
BK	=	bradykinin
BSA	=	bovine serum albumin
BW	=	body weight
Ca^{2+}	=	calcium ion
cGMP, cAMP	=	cyclic guanosine (adenosine) monophosphate
cm	=	centrimeter
COX	=	cyclooxygenase
DM	=	dissociation medium
DMSO	=	dimethyl sulfoxide
DTT	=	dithiothreitol
ECs	=	endothelial cells
ED	=	erectile dysfunction
EDHF	=	endothelium-derived hyperpolarizing factor
EDTA	=	ethylenediaminetetraacetic acid
EGTA	=	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
eNOS	=	endothelial nitric oxide synthase
ET-1	=	endothelin-1
FDA	=	food and drug administration
g	=	gram
GTP	=	guanosine triphosphate
HEPES	=	N-[2-hydroxyethyl] piperazine-N′-[2-ethane-sulfonic acid]

ABBREVATIONS LIST (CONT.)

HPLC	=	high performance liquid chromatography
hr	=	hour
IK_{Ca}	=	intermediate conductance Ca^{2+}-activated K^+ channels
i.p.	=	intraperitoneal
IP_3	=	inositol 1, 4, 5 trisphosphate
K^+	=	potassium ion
K_{ATP}	=	ATP-sensitive potassium channel
K_{Ca}	=	Ca^{2+}- activated K^+ channels
Kg	=	kilogram
K_V	=	voltage-gated potassium channel
L-NAME	=	N^G-nitro-L-arginine methyl ester
LOD	=	limit of detection
LOQ	=	limit of quantification
M	=	molar
MA	=	mesenteric artery
Mg	=	milligram
mg/kg	=	milligram per kilogram
mg/ml	=	milligram per milliliter
min	=	minutes
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
mmHg	=	millimeters of mercury
MTT	=	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NANC	=	non-adrenergic noncholinergic
nm	=	nanometer
NO	=	nitric oxide
ODQ	=	1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one

ABBREVATIONS LIST (CONT.)

PA	=	pulmonary artery
PAH	=	pulmonary arterial hypertension
PDE	=	phosphodiesterase
PE	=	phenylephrine
PGI_2	=	prostacyclin
PH	=	pulmonary hypertension
PKA	=	protein kinase A
PKC	=	protein kinase C
PMSF	=	phenylmethylsulfonyl fluoride
ROCCs	=	receptor-operated Ca^{2+} channels
SD	=	standard deviation
SEM	=	standard error of the mean
sGC	=	soluble guanylate cyclase
SNP	=	sodium nitroprusside
SK_{Ca}	=	small conductance Ca^{2+}-activated K^+ channels
SPE	=	solid phase extraction
SR	=	sarcoplasmic reticulum
TNF-α	=	tumor necrosis factor-alpha
TP	=	thromboxane-prostanoid
TX	=	thromboxane
UV	=	ultraviolet
VOCCs	=	voltage-operated Ca^{2+} channels
VSMCs	=	vascular smooth muscle cells
WLE	=	water lily extract
µg/ml	=	microgram per milliliter
µl	=	microliter
µm	=	micrometer
µM	=	micromolar

CHAPTER I

INTRODUCTION

Rationale of the study

Due to the increase in the elderly population, the disease caused by degeneration (degenerative diseases) is becoming an important health problem. Cardiovascular disease (CVD) has many common risk factors, including age, hypertension, diabetes, insulin resistance, smoking, increased body mass index (BMI), cholesterol, and lower high-density lipoprotein (HDL) (Gandaglia et al., 2014). These factors are also linked to erectile dysfunction (ED). ED and CVD are pathophysiological consequences of atherosclerosis and endothelial dysfunction (Raheem et al., 2017). ED is the recurrent or persistent inability to achieve or sustain a penile erection for sexual satisfaction (Mazzilli, 2022). A high prevalence of ED has been reported in eight countries: Brazil, Italy, France, China, Spain, Germany, the United States, and the United Kingdom. Italy has the highest prevalence at 48.6% of the male population, while Brazil has the lowest of these countries at 37.2% (Goldstein et al., 2020). In Thailand, ED prevalence is 37.5% (Kongkanand & Thai Erectile Dysfunction Epidemiological Study Group, 2002).

Moreover, endothelial dysfunction also plays a role in the progression of vascular disease in pulmonary arterial hypertension (PAH) (Santos-Gomes et al., 2022). The PAH is a condition that has a mean pulmonary arterial pressure of more than 20 mmHg at rest together with low capillary pressure as determined by right heart catheterization (Humbert et al., 2022). This causes an increase in right ventricular afterload leading to heart failure and premature death (Humbert et al., 2022; Kherbeck et al., 2013).

One of the most commonly used treatments for ED and PAH is the inhibition of the enzyme phosphodiesterase 5 (PDE5) (Kumar et al., 2022). To date, the FDA-approved oral PDE5 inhibitors that are widely available and used in the market are Sildenafil citrate (Viagra®), Tadalafil (Cialis®), Vardenafil (Levitra®), and Avanafil (STENDRA®) (Burnett et al., 2018). PDE5 is the cyclic guanosine monophosphate (cGMP)-degrading enzyme that is distributed in various tissues including the lungs, platelets, penile corpus cavernosum, smooth muscle cells, and all vascular smooth muscle cells, especially in the pulmonary vessels (Keravis & Lugnier, 2012). Inhibition of PDE5 results in raising the level of cGMP, leading to the relaxation of the vascular smooth muscle of the penile corpus cavernosum (Corbin, 2004). As PDE5 is also found in the lungs, some PDE5 inhibitors are effective to treat PAH. Currently, PDE5 inhibitors are being used to treat PAH given their ability to dilate the pulmonary artery (PA) by inhibiting the destruction of cGMP which is the secondary messenger of nitric oxide (NO) (Archer & Michelakis, 2009). However, any molecules with other mechanisms, that can induce vasorelaxation of pulmonary vasculature are still of interest in PAH treatment (Archer & Michelakis, 2009; Humbert et al., 2022). Indeed, at the vascular level, endothelial cells produce nitric oxide (NO), which stimulates the soluble guanylate cyclase (sGC) that changes guanosine triphosphate (GTP) to cGMP and leads to the relaxation of the vascular smooth muscle (Corbin, 2004). Then, any molecule able to increase either part of

these signaling pathways deserves attention. In recent years, a renewed interest emerged in the role of arginase as a new therapeutic target (Moretto et al., 2021; Pudlo et al., 2017). Arginase is the enzyme implicated in ED that catalyzes the hydrolysis of L-arginine to urea and L-ornithine, while endothelium nitric oxide synthase (eNOS) converts L-arginine to L-citrulline and NO. Therefore, inhibition of arginase leads to L-arginine being more available to use for eNOS and increasing NO availability (Boucher et al., 1999).

Natural products are interesting sources for drug candidates (Atanasov et al., 2021). Several traditional medicinal plants in Thailand have various bioactivities, one of which improves sexual dysfunction by inhibiting the PDE5 enzyme (Chaichamnong et al., 2018; Choonong et al., 2022; Kruangtip et al., 2015; Molee et al., 2018; Sabphon et al., 2015; Temkitthawon et al., 2017; Temkitthawon et al., 2008). From our previous study, the extract from the petals of water lily, *Nymphaea pubescens* Willd., significantly inhibited PDE5 (Panklai et al., 2023). Water lilies belongs to Nymphaeaceae family, members of Thai wetland biota (La-ongsri et al., 2009), which show interesting ethnopharmacology data. Flowers of *Nymphaea* spp. have been traditionally used for the treatment of diabetes, inflammation, jaundice, and eye disorders, and interestingly have been used as aphrodisiacs (Debnath et al., 2013; Singh & Jain, 2017).

Therefore, the main aim of this project was exploration of the possibility to apply water lilies and lotuses in families of Nelumbonaceae and Nymphaeaceae for vascular health benefit. The PDE5 and arginase inhibitors from the active extract would be isolated and identified. The underlying mechanisms on vasodilator action in various blood vessels as well as the cytotoxicity of active extract and its bioactive constituents would be studied. Finally, the methods for quality control and standardization of the active extract would be established.

Objectives of the study

1. To investigate the PDE5 and arginase inhibitory activity of the extracts of water lilies and lotuses in families of Nelumbonaceae and Nymphaeaceae and choose the extract that has a promising activity for further study.

2. To isolate and identify the PDE5 and arginase inhibitors from the chosen active extract.

3. To investigate the vascular effects and mechanisms of action of the active extract and its chemical constituents on rat isolated aorta, pulmonary arteries, and mesenteric arteries.

4. To investigate the cytotoxicity effect of the active extract on vascular smooth muscle cells (VSMCs) isolated from the rats.

5. To develop quality control and standardization methods of the active extract.

CHAPTER II

LITERATURE REVIEW

Cardiovascular system

The cardiovascular system consists of the heart, blood vessels and blood. There is divided into two systems which are systemic circulation and pulmonary circulation. Systemic (or greater) circulation begins with the pumping of blood by the left ventricle into the aorta. This ascends from the top of the heart, bends downward at the aortic arch and descends just anterior to the spinal column. The aorta bifurcates into the left and right iliac arteries, which supply the pelvis and legs. The major arteries supplying the head, the arms and the heart arise from the aortic arch, and the main arteries supplying the visceral organs branch from the descending aorta. The pulmonary (or lesser) circulation begins when blood is pumped by the right ventricle into the main pulmonary artery, which immediately bifurcates into the right and left pulmonary arteries supplying each lung. This 'venous' blood is oxygenated during its passage through the pulmonary capillaries. It then returns to the heart via the pulmonary veins to the left atrium, which pumps it into the left ventricle (Aaronson PI et al., 2020).

In systemic circulation, systolic pressure is the maximum arterial pressure which is the pressure in the left ventricle that increases to around 120 mmHg, and blood is ejected into the aorta. Diastolic pressure is the minimum pressure reached before the next systolic pressure of around 80 mmHg which is the pressure in arterial blood flow that is partly maintained by elastic recoil of the walls of large arteries. The difference between systolic and diastolic pressures is pulse pressure. Whereas pulmonary circulation is shorter than systemic circulation and has a lower resistance to flow. Therefore, pressure is less required to drive blood through the lungs; the pulmonary artery pressure is around 20/15 mmHg (Ward JPT & Linden RWA, 2013).

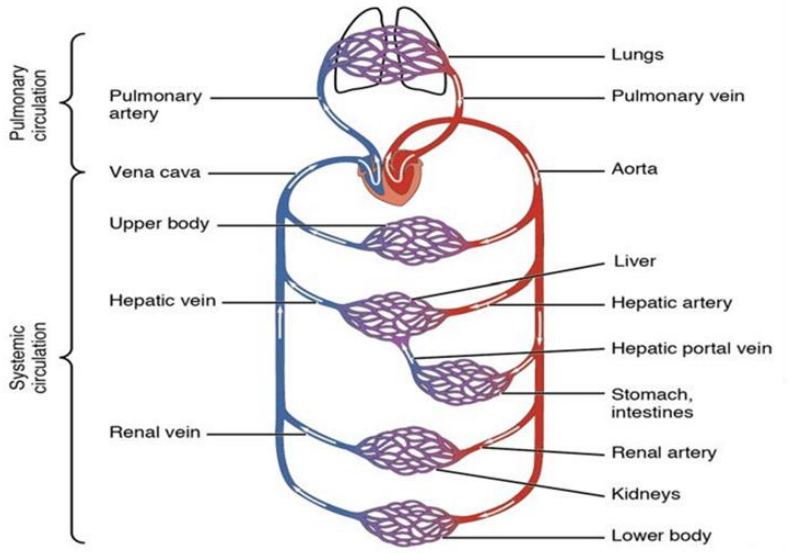


Figure 1 The Cardiovascular circulation
Source: (Betts et al., 2017).

Vascular structure

The walls of blood vessels have the same basic structure. There are comprised of three layers; i) tunica intima (inner layer) consisting of a thin layer of endothelial cells, ii) tunica media (middle layer) containing smooth muscle and elastin filaments that provide elastic properties, and iii) tunica adventitia (outer layer) consisting of fibroblasts and nerves embedded in collagenous tissue (Ward JPT & Linden RWA, 2013). The blood vessels are divided depending on function, location, and size into arteries, capillaries, and veins (Sandoo et al., 2010). Arteries and arterioles have thicker walls than veins and venules because its are closer to the heart and receive blood that has high pressure. Arteries have smaller lumens than veins which this characteristic that helps to maintain the blood pressure moving through the system. Together, their thicker walls and smaller diameters give arterial lumens a more rounded appearance in cross section than the lumens of veins. When blood has passed through capillaries and entered venules, the pressure initially exerted upon it by heart contractions has diminished. Venules and veins withstand a much lower pressure than arteries from the blood that flows through them. Their walls are considerably thinner, and their lumens are correspondingly larger in diameter, allowing more blood to flow with less vessel resistance. Moreover, many veins of the body, particularly those of the limbs, contain valves that assist the unidirectional flow of blood toward the heart. This is critical because blood flow becomes sluggish in the extremities, as a result of the lower pressure and the effects of gravity (Betts et al., 2017).

Table 1 Comparison of Tunics in Arteries and Veins

	Arteries	**Veins**
General appearance	- Thick walls with small lumens - Generally, appear rounded	- Thin walls with large lumens - Generally, appear flattened
Tunica intima	- Endothelium usually appears wavy due to constriction of smooth muscle - Internal elastic membrane present in larger vessels	- Endothelium appears smooth - Internal elastic membrane absent
Tunica media	- Normally the thickest layer in arteries - Smooth muscle cells and elastic fibers predominate (the proportions of these vary with distance from the heart) - External elastic membrane present in larger vessels	- Normally thinner than the tunica externa - Smooth muscle cells and collagenous fibers predominate - Nervi vasorum and vasa vasorum present - External elastic membrane absent
Tunica externa	- Normally thinner than the tunica media in all but the largest arteries - Collagenous and elastic fibers Nervi vasorum and vasa vasorum present	- Normally the thickest layer in veins - Collagenous and smooth fibers predominate - Some smooth muscle fibers Nervi vasorum and vasa vasorum present

Source: (Betts et al., 2017).

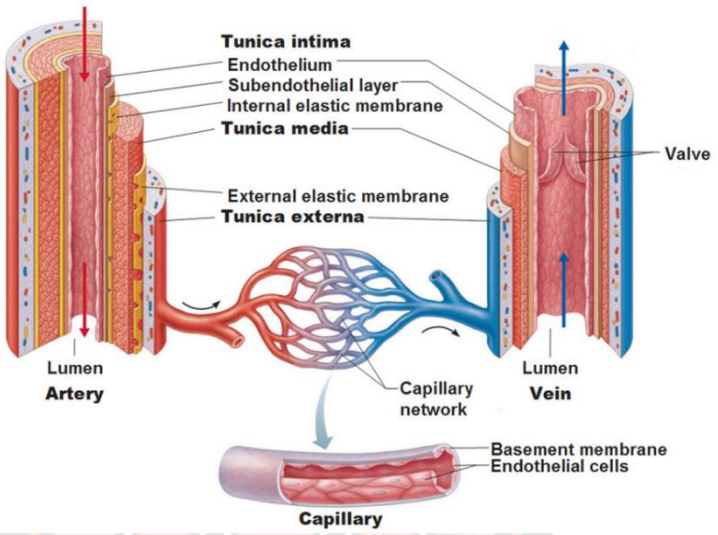


Figure 2 The structure of blood vessel
Source: (Cook, 2019)

Vascular tone

In blood vessels, endothelial cells (ECs) and smooth muscle cells (SMCs) make signaling *via* secreted or diffusible factors an efficient mechanism of communication. Endothelial-derived factors, such as nitric oxide (NO), prostacyclin (PGI_2), and hyperpolarizing agents diffuse to SMCs to cause vascular relaxation. Similarly, endothelial cell-released contracting agents like endothelin and angiotensin II are perceived by SMCs to increase vascular tone (Lilly, 2014; Sumpio et al., 2002).

The vascular-bed-specific characteristics of ECs and SMCs undoubtedly have a substantial impact on how the two cell types communicate in distinct vessels in normal and disease states. Disruption of communication between these two cell types contributes to disease progression (Bacakova L et al., 2018; Lilly, 2014; Sumpio et al., 2002).

The mechanism of vasorelaxation and vasoconstriction

Vasorelaxation

1. Nitric Oxide (NO)

NO is an endothelium-dependent vasodilator of the underlying smooth muscle. NO has been shown to play an important role in the maintenance of basal vasodilator tone of the blood vessels. NO is formed under the influence of the enzyme nitric oxide synthase (NOS), which converts the amino acid L-arginine to NO. Endothelial NOS (eNOS) produces NO in the vasculature and the ability of a blood vessel to dilate is largely dependent upon the activity of eNOS. NO diffuses across the ECs into the adjacent SMCs, where it binds to the enzyme soluble guanylyl cyclase (sGC). Their activated enzyme increases the conversion rate of GTP to cGMP, which decreases smooth muscle tension (Sandoo et al., 2010).

2. Prostanoids

Prostacyclin (PGI_2) production is catalyzed by cyclooxygenase (COX) enzymes. PGI_2 binds to the prostacyclin receptors (IP), which are located on both platelets and vascular SMCs. PGI_2 binding to the SMCs. IP receptor activates AC which induces the synthesis of cAMP. cAMP then activates PKA, which allows relaxation of the smooth muscle in the same way as it does for NO (Sandoo et al., 2010).

3. Endothelium-derived hyperpolarizing factor (EDHF)

EDHF is an unidentified vasodilator substance that hyperpolarizes the underlying SMCs by making the membrane potential of the cell more negative. EDHF is released when ECs are activated by agonists such as bradykinin (BK) and ACh. NO and PGI_2 can also dilate the vessel by hyperpolarizing the SMCs. Activation of endothelial receptors and the subsequent increase in Ca^{2+} levels cause K^+ efflux from the cell. The SMCs respond to changes in the extracellular K^+ levels and also releases K^+ out of the SMCs causing hyperpolarization. The change in the membrane potential of the SMCs reduces intracellular Ca^{2+} levels, resulting in relaxation (Sandoo et al., 2010).

4. K^+ channel

The electrochemical gradient for K^+ ions is such that opening of K^+ channels result in the diffusion of this cation out of the cells and membrane hyperpolarization. Closure of K^+ channels have the opposite effect. K^+ channels play a role in determination and regulation of vascular tone. In vascular muscles, there are identified the functional expression of 4 different types of K^+ channels consisting of ATP-sensitive K^+ (K_{ATP}) channels, large-conductance Ca^{2+}- activated K^+ (BK_{Ca}) channels, voltage-activated K^+ (K_V) channels, and inward rectifier K^+ (K_{IR}) channels (Jackson, 2000).

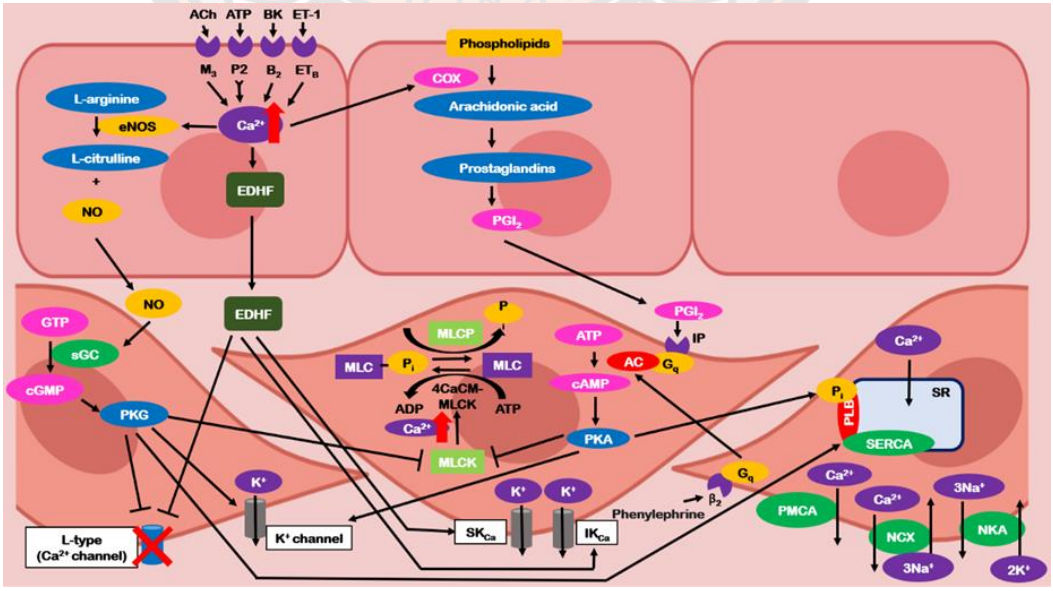


Figure 3 The mechanism of vasorelaxation
Source: Made by Teerapap Panklai (2023)

Abbreviations: Ach, Acetylcholine; ATP, adenosine triphosphate; BK, bradykinin; cAMP, Cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; EDHF, Endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; ET-1, Endothelin-1; GTP, Guanosine triphosphate; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channels; IP, prostacyclin receptors; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NCX, Na^+-Ca^{2+} exchanger; NKA, Na^+, K^+-ATPase; NO, nitric oxide; PKA, protein kinase A; PGI_2, Prostacyclin; PKG, protein kinase G; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; sGC, soluble guanylyl cyclase; SK_{Ca}, small conductance Ca^{2+}-activated K^+ channels.

Vasoconstriction

1. Endothelin-1

ET is a vasoconstrictor that is expressed in three isoforms including ET-1, ET-2, and ET-3. ECs only release ET-1. ET-1 is produced by converting Big ET-1 to ET-1 by endothelin converting enzyme. ET-1 is stimulated by inflammatory cells such as interleukins and TNF (tumor necrosis factor) -α and decreased by NO and PGI_2. ET-1 receptors have been identified both on SMCs (ET_A and ET_B). When ET-1 binds to ET_A or ET_B receptors, smooth muscle Ca^{2+} channels open allowing extracellular Ca^{2+} into the cell. This causes vasoconstriction in a similar way as thromboxane (TXA_2) (Sandoo et al., 2010).

2. Prostanoids

Thromboxane (TXA_2) production is catalyzed by COX enzymes. TXA_2 causes platelet aggregation and vasoconstriction. TXA_2 is synthesized by thromboxane synthase. TXA_2 mediates its effects by its actions on thromboxane-prostanoid (TP) receptors which are located on platelets and their activation causes platelet aggregation. The TP receptor is also found on SMCs and is involved in increasing intracellular Ca^{2+} levels in the smooth muscle, leading to vasoconstriction (Sandoo et al., 2010).

3. Ca^{2+} channel

Voltage-gated Ca^{2+} channels play a role in the regulation of vascular tone by membrane potential. Hyperpolarization closes Ca^{2+} channels and leads to vasodilation, whereas depolarization opens Ca2+ channels, which results in vasoconstriction. Dihydropyridine-sensitive L-type voltage-gated Ca^{2+} channels appear to be dominant in most vascular muscle cells. L-type Ca^{2+} channels play a particularly important role in myogenic reactivity and vasomotion. Voltage-gated Ca^{2+} channels are modulated by several signaling systems. They are activated by vasoconstrictors that activate the protein kinase C pathway. Voltage-gated Ca^{2+} channels are inhibited by increases in intracellular Ca^{2+} and activation of cGMP-dependent protein kinase. Membrane potential not only regulates voltage-gated Ca^{2+} channels, but also appears to influence inositol 1,4,5-trisphosphate–induced release of Ca^{2+} from intracellular stores and the Ca^{2+} sensitivity of the contractile apparatus (Jackson, 2000).

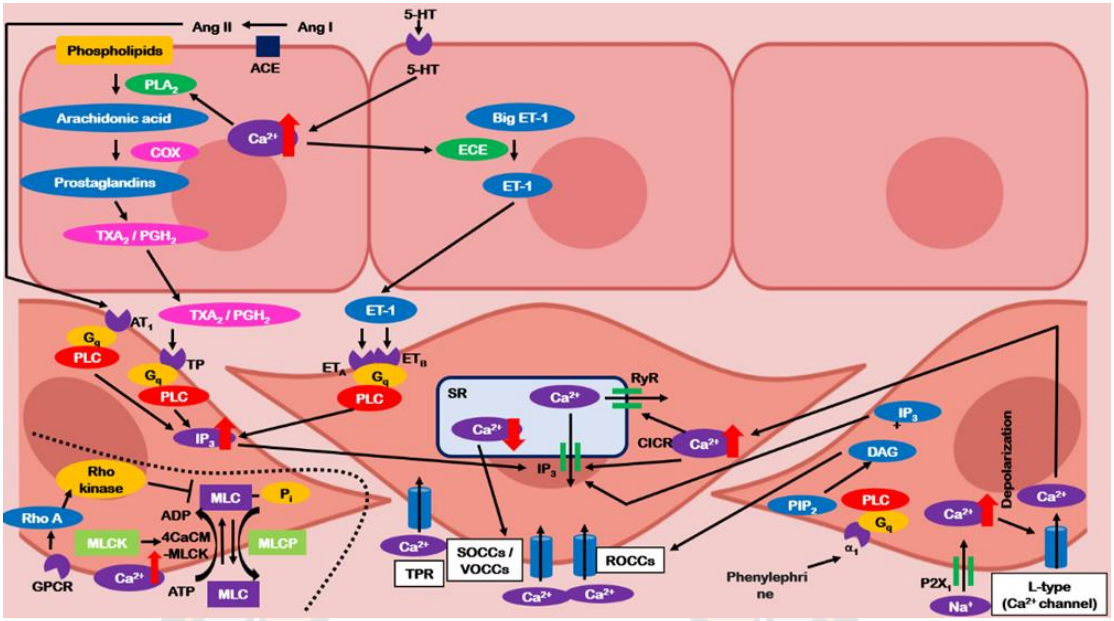


Figure 4 The mechanism of vasoconstriction
Source: Made by Teerapap Panklai (2023)

Abbreviations: ACE, angiotensin-converting enzyme, COX, cyclooxygenase; DAG, diacylglycerol; ET-1, Endothelin-1; GPCR, G-protein-coupled receptor; IP_3, inositol-1,4,5-trisphosphate; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; PGH_2, Prostaglandin H_2; PLA_2, Phospholipase; PLC, phospholipase C; RhoA, Ras homolog family member A; ROCC, receptor-operated Ca^{2+} channels; ROCK, Rho-associated protein kinase; RyR, ryanodine receptor; SOCC, store-operated Ca^{2+} channel; SR, sarcoplasmic reticulum; TXA_2, Thromboxane; VOCC, voltage-operated Ca^{2+} channel.

Endothelial dysfunction

Endothelial dysfunction is a pivotal factor in the pathogenesis of cardiovascular diseases, including hypertension, coronary artery disease, chronic heart failure, peripheral artery disease, diabetes, and chronic renal failure, as it leads to reduced vasodilation, a proinflammatory state, and prothrombotic properties. Mechanisms contributing to this dysfunction involve reduced nitric oxide (NO) generation, oxidative excess, and diminished production of hyperpolarizing factors, along with upregulated adhesion molecules, inflammatory responses, and the accumulation of endogenous NO inhibitors. Endothelial detachment and apoptosis further exacerbate the condition, playing a critical role in atherosclerosis development. Various therapeutic interventions, such as lipid-lowering drugs, ACE inhibitors, physical activity, and antioxidant agents, have demonstrated the potential to ameliorate endothelial function in both coronary and peripheral vessels, suggesting a common mechanism underlying endothelial dysfunction in different vascular territories (Endemann & Schiffrin, 2004; Grover-Páez & Zavalza-Gómez, 2009; Vanhoutte et al., 2009)

Nitric oxide (NO) and cardiovascular system

Nitric oxide (NO) is a multifaceted gas and free radical with crucial physiological roles. Synthesized enzymatically from L-arginine through various isoforms of nitric oxide synthase, NO is integral in regulating endothelium-dependent blood flow and blood pressure, as well as inhibiting platelet activation. It also serves as a neurotransmitter in specific nerve types and contributes to the body's defense mechanisms against microbial threats. NO interacts closely with iron-containing proteins, activating soluble guanylyl cyclase, which, in turn, triggers the production of cyclic GMP, a pivotal signaling molecule. Moreover, NO influences cell activities through alternative pathways involving modifications to enzyme and structural protein structures. These changes are often due to the interactions between NO and other free radicals, such as oxygen and superoxide anions, leading to protein nitrosation and nitration, introducing novel complexities to NO's effects on cellular function. These protein modifications become more evident in inflammatory disorders, potentially contributing to their pathology (Bruckdorfe, 2005). Additionally, NO has a substantial role in various biological processes, acting as a vasodilator, neurotransmitter, and immune system mediator. It contributes to platelet control, cardiac function regulation, and plays a cytotoxic role in host defense. It is also a critical component of inflammation and immune responses (Garcia & Stein, 2006). NO's actions within the cardiovascular system and its involvement in conditions like hypertension, obesity, and diabetes continue to be the subject of extensive research. It participates in oxygen and blood flow sensing, arterial remodeling, and lymphatic pumping, with interventions aimed at improving endothelial function through pharmacological and lifestyle modifications. While NO physiology appears compromised in essential hypertension and diabetes, it may not be the primary cause of increased vascular resistance, but rather a consequence of endothelial damage. Nevertheless, the physiological significance of NO in vascular regulation remains undeniable (Bohlen, 2015). Finally, NO serves as an intricate intra- and inter-signaling molecule, influencing diverse physiological processes, including vessel dilation, neuronal transmission, cardiac contraction, immunomodulation, and stem cell activities. It plays a protective role in the cardiovascular system, inhibiting smooth muscle cell proliferation, enhancing endothelial cell function, and preventing platelet and leukocyte adhesion. NO's inhibitory effects on intimal hyperplasia and its potential therapeutic applications underscore its significance in cardiovascular health (Lei et al., 2013).

This endothelial dysfunction, commonly assimilated to a deficiency on NO availability, is the common hallmark of many cardiovascular diseases. In agreement with this, patients affected by erectile dysfunction or pulmonary arterial hypertension developed these vascular abnormalities (Evans et al., 2021; Konstantinovsky et al., 2019; Musicki et al., 2015). In the next paragraphs, we will address these two pathologies, for their pathogenesis aspects and their therapeutic targets.

Erectile dysfunction (ED)

Definition

Erectile dysfunction is a disability or maintaining an erection that is sufficient for satisfactory sexual performance and affects a considerable proportion of men. The

male erection has two major aspects consisting of i) the reflex erection is accomplished by directly touching the penile shaft and is under the control of the peripheral nerves and the lower parts of the spinal cord, and ii) the psychogenic erection is accomplished by erotic or emotional stimuli and uses the limbic system of the brain, which can be involved in the dysfunction and are subject to therapeutic intervention (Yafi et al., 2016).

Pathophysiology and mechanisms

ED is dependent on a complex interaction of vascular and neural processes. The internal pudendal artery supplies the majority of the blood flow to the penis through the cavernosal branches whereas venous outflow occurs through a network of easily compressible venules. When occurring sexual stimulation, an erection occurs after nitric oxide (NO) is released from non-adrenergic noncholinergic (NANC) nerve fibres and acetylcholine (ACh) is released from parasympathetic cholinergic nerve fibres. The result of the ensuing signaling pathways is increasing cyclic guanosine monophosphate (cGMP) concentrations while intracellular Ca^{2+} levels decreased and leading to smooth muscle cell (SMC) relaxation. As the SMC relaxes, blood is able to fill the lacunar spaces in the corpora cavernosa, leading to compression of the subtunical venules, thereby blocking the venous outflow (veno-occlusion). ED can occur when cGMP is hydrolyzed by phosphodiesterase type 5 (PDE5) (Irwin, 2019; Yafi et al., 2016).

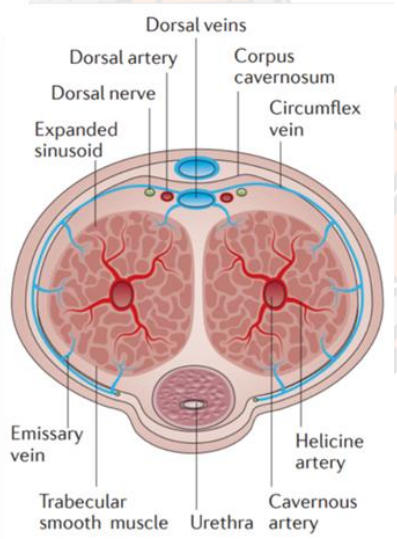


Figure 5 Penile anatomy
Source: (Yafi et al., 2016)

Treatment of ED

There are various treatments available for ED, including: i) PDE5 inhibitor treatment; currently, four PDE5 inhibitors are globally available such as sildenafil, tadalafil, vardenafil, and avanafil. Each PDE5 inhibitor works by inhibiting the PDE5 enzyme action on cGMP. Erection hardness and duration increase with the accumulation of cGMP in the penile cavernosa. ii) Nutraceuticals; previous research

studies on the use of nutraceuticals such as ginseng, saffron, *Tribulus terrestris*, *Pinus pinaster*, and *Lepidium meyenii* or dietary supplements in patients with ED including L-arginine as well as L-citrulline have also been proposed as a nutritional supplement for ED. iii) Intracavernous Drug Administration; the availability of new diagnostic tools such as Doppler made it possible to discover the pro-erectile activity of papaverine and phentolamine administered within the corpora cavernosa, which led to a real revolution in the approach to patients with ED. These two substances were then replaced by Alprostadil (PGE1), which increases cyclic adenosine monophosphate (cAMP) levels through adenylate cyclase stimulation, leading to SMC relaxation, vasodilation, and penile erection. Currently, pharmacoerection with PGE1 is used in situations with endothelial damage characterized by a reduction in the availability of NO. iv) Physical Treatments; ED management has been proposed with the vacuum device which is a cylindrical mechanical device that is placed around the penis and pumped; consequently, it creates a negative pressure vacuum to draw blood into the penis, but this method has side effects. Moreover, over the past decade, low-intensity extracorporeal shockwave therapy (Li-ESWT) has emerged as a treatment modality for ED. v) Surgical Treatment; among the surgical approaches for ED, penile prosthesis is an attractive and effective option for patients unresponsive to medical treatments. vi) Lifestyle changes: Changes of lifestyle, such as losing weight, exercising regularly, quitting smoking, reducing alcohol consumption, and managing stress, can help improve ED (Mazzilli, 2022).

Phosphodiesterase (PDE)

The cyclic nucleotide phosphodiesterases or PDEs are a family of related phosphohydrolases that selectively catalyze the hydrolysis of the 3′ cyclic phosphate bonds of adenosine and/or guanosine 3′,5′ cyclic monophosphate (cAMP and/or cGMP). These enzymes are often referred to as class I cyclic nucleotide PDEs to differentiate them from class II enzymes. Class II enzymes are found in many species including mammals and will also catalyze the hydrolysis of the phosphodiester bond. However, in general, the Class II enzymes do not show the same substrate selectivity as the class I enzymes and much more is known about the class I enzymes (Bender & Beavo, 2006).

The cAMP and cGMP are the secondary messenger that plays a role in signal transduction cascades that regulate many critical physiological and pathophysiological processes, including cellular growth, differentiation, and proliferation; Ca^{2+}-dependent signaling; reproduction; cardiac function; vision; inflammation; and tumor development (Azevedo et al., 2014).

The PDEs are large and complex, and they are derived from separate gene families. Currently, twelve families **(Table 2)** have been identified that catalyze the hydrolysis of cAMP and cGMP (Eskandari et al., 2015).

Table 2 Characteristics of the PDE families

Family	Specificities	Tissue distribution	Inhibitors
PDE1	Ca^{2+}/calmodulin-stimulated	Heart, brain, lung, smooth muscle	Nimodipine, IC86340, IC224, IC295, dioclein
PDE2	cGMP-stimulated	Adrenal gland, heart, lung, liver, platelets, endothelial cells	EHNA, BAY-60–7750, PDP, IC933, oxindole, ND7001
PDE3	cAMP-selective, cGMP-inhibited	Heart, smooth muscle, lung, liver, platelets, adipocytes, immunocytes	Cilostamide, milrinone, siguazodan, cilostazol
PDE4	cAMP-specific,	Brain, Sertolli cells, kidney, liver, heart, smooth muscle, lung, endothelial cells, immunocytes	cGMP-insensitive Rolipram, roflumilast, cilomast, NCS 613
PDE5	cGMP-specific	Lung, platelets, smooth muscle, heart, endothelial cells, brain,	Zaprinast, DMPPO, sildenafil tadalafil, vardenafil
PDE6	cGMP-specific, transducin activated	Photoreceptors, pineal gland, lung	Zaprinast, DMPPO, sildenafil, vardenafil
PDE7	cAMP-specific, high-affinity rolipram-insensitive	Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes	BRL 50481, IC242, ASB16165
PDE8	cAMP-selective, IBMX insensitive rolipram-insensitive	Testes, eye, liver, skeletal muscle, heart, kidney, ovary, brain, T lymphocytes, thyroid	PF-04957325
PDE9	cGMP-specific, IBMX insensitive	Kidney, liver, lung, brain	BAY-73–6691, PF-04447943
PDE10	cGMP-sensitive, cAMP-selective	Testes, brain, thyroid	Papaverine, TP-10, MP-10
PDE11	cGMP-sensitive, dual specificity	Skeletal muscle, prostate, pituitary gland, liver, heart	None selective
PDE12	hydrolyzes 2-5A to ATP and AMP	All tissues/organs	A-74528

Source: (Azevedo et al., 2014; Bender & Beavo, 2006; Eskandari et al., 2015; Keravis & Lugnier, 2012; Poulsen et al., 2012).

Phosphodiesterase 5 (PDE5)

PDE5 is the hydrolyses cyclic guanosine monophosphate (cGMP) degrading enzyme that is distributed on tissue including lung, platelets, penile corpus

cavernosum, smooth muscle cells, and all vascular smooth muscle cells, especially in the pulmonary vessels (Keravis & Lugnier, 2012). PDE5 is characterized by a relative specificity for cGMP hydrolysis at low substrate levels and by the presence of high affinity-binding sites for cGMP. These binding sites are now known to be on the N-terminal regulatory GAF domains of the enzyme (Bender & Beavo, 2006). PDE5 has only one gene which is PDE5A and is expressed as three isoforms (PDE5A1, PDE5A2, and PDE5A3). PDE5A1 and PDE5A2 are widely expressed in a number of tissues, whereas PDE5A3 is explicitly expressed in the VSMCs (Ahmed et al., 2021). PDE5 has been demonstrated to have several therapeutic applications, especially ED and pulmonary arterial hypertension (PAH) (Bender & Beavo, 2006).

PDE5 inhibitors

PDE5 inhibitors inhibited the activity of PDE5 enzymes and consequently raised the level of cGMP in cells of various tissue with several therapeutic benefits. Food and Drug Administration (FDA)-approved PDE5 inhibitors include sildenafil (approved in 1998), tadalafil (approved in 2003), vardenafil (approved in 2003), and avanafil (approved in 2012) (Ahmed et al., 2021). PDE5 inhibitors are known as the molecular target for drugs used to treat ED (Huang & Lie, 2013) and pulmonary arterial hypertension (Barnes et al., 2019). However, there are reported complications of PDE5 inhibitors are headache, flushing, dyspepsia, dizziness, and rhinitis (Mazzilli, 2022). Therefore, the exploration of medicinal plants as new PDE5 inhibitors might decrease the complications. The review suggests and supports that some medicinal plants have effective PDE5 inhibitors such as *Epimedium brevicornum* Maxim, *Ginkgo biloba* L., *Kaempferia parviflora* Wall. ex Baker, *Clerodendrum colebrookianum* Walp., *Eurycoma longifolia* Jack and *Vitis vinifera L*. (Ganapathy et al., 2021).

PDE5 inhibitors and cardiovascular diseases

Cardiovascular disease (CVD) continues to be the largest cause of death globally today despite a number of treatment advancements. Heart and blood vessel disorders like coronary artery diseases (CAD) including angina and myocardial infarction are within the CVD category of illnesses. Many family-selective phosphodiesterase inhibitors (PDEIs) are used clinically or pre-clinically to treat cardiac or vascular-related disorders. The cGMP-specific phosphodiesterase type 5 (PDE5) is expressed in various tissues and functionally controls cGMP-dependent signaling in the aortic smooth muscle cells, heart, placenta, skeletal muscle, pancreas, and, to a much lesser extent, in the brain, liver, and lungs, but most obviously in the corpus cavernosum and the retina (Shafiee-Nick et al., 2017). PDE5 inhibitors, including sildenafil, tadalafil, and vardenafil, are a family of medications intended to extend the physiological effects of nitric oxide/cGMP signaling in tissues by inhibiting cGMP breakdown. Although these drugs were initially intended to treat hypertension and angina, unintended side effects resulted in improvements in the management of erectile dysfunction and, later, pulmonary arterial hypertension. PDE5 inhibitors may offer a wider variety of therapeutic advantages than was previously recognized, according to mounting data over the past ten years. Due to this, there is now more interest than ever in the potential cardiovascular therapeutic benefits of PDE5 inhibitors for treating diseases including hypertension, myocardial infarction,

stroke, peripheral arterial disease, chronic kidney disease, and diabetes mellitus (Reffelmann & Kloner, 2003; Tzoumas et al., 2020).

Arginase

Arginase is an enzyme that requires manganese for the hydrolysis of L-arginine to form L-ornithine and urea. In mammals, arginase enzymes exist in two isozymes, each with distinct tissue distributions and subcellular locations. Arginase I is predominantly found in the liver, catalyzing the final cytosolic step of the urea cycle and playing a crucial role in urea generation. On the other hand, arginase II is a mitochondrial enzyme with a broader distribution across various tissues, including the kidney, brain, skeletal muscle, and liver. Unlike arginase I, arginase II does not to seem to participate directly in the urea cycle. The activity of arginase can effectively inhibit NO-dependent processes by depleting the substrate pool available for NO biosynthesis. This inhibition is particularly relevant which relates to the regulation of vascular tone (Christianson, 2005).

Arginase inhibitor

Arginase inhibition can effectively enhance NO biosynthesis and NO-dependent processes by enhancing L-arginine bioavailability to NO synthase. Moreover, arginase inhibition may enhance the NO-dependent physiological processes required for sexual arousal (Christianson, 2005). Only a limited number of arginase inhibitors have been documented, including modified α-amino acids such as nor-Nω-Hydroxy-Arginine (nor-NOHA), and boronic acids (2(S)-amino-6-boronohexanoic acid (ABH) and R-(2-boronoethyl)-L-cysteine (BEC)). Simultaneously, some arginase inhibitors of natural origin have been identified, primarily belonging to the class of polyphenols in secondary metabolites. The recurring structural feature among these natural arginase inhibitors is the catechol core, with piceatannol, a stilbene derivative, standing out as one of the most efficient options. A certain number of plants have also been identified as possessing arginase inhibitory properties, such as the roots of rhubarbs, roots of a 6-year-old fresh Panax ginseng or Korean Red Ginseng water extract, for example. (From systematic reviews (Girard-Thernier et al., 2015; Minozzo et al., 2018; Moretto et al., 2019)).

Pulmonary hypertension (PH)

Definition and classification

Pulmonary hypertension (PH) is defined by a mean pulmonary arterial pressure (mPAP) of more than 20 mmHg at rest. The general purpose of the clinical classification of PH remains to categorize clinical conditions associated with PH, based on similar pathophysiological mechanisms, clinical presentation, haemodynamic characteristics, and therapeutic management (Humbert et al., 2022).

Table 3 Clinical classification of pulmonary hypertension.

Group 1 Pulmonary arterial hypertension (PAH)
1.1 Idiopathic
1.1.1 Non-responders at vasoreactivity testing
1.1.2 Acute responders at vasoreactivity testing
1.2 Heritable
1.3 Associated with drugs and toxins
1.4 Associated with:
1.4.1 Connective tissue disease
1.4.2 HIV infection
1.4.3 Portal hypertension
1.4.4 Congenital heart disease
1.4.5 Schistosomiasis
1.5 PAH with features of venous/capillary (PVOD/PCH) involvement
1.6 Persistent PH of the newborn
Group 2 PH associated with left heart disease
2.1 heart failure
2.1.1 with preserved ejection fraction
2.1.2 with reduced or mildly reduced ejection fraction
2.2 Valvular heart disease
2.3 Congenital/acquired cardiovascular conditions leading to post-capillary PH
Group 3 PH associated with lung diseases and/or hypoxia
3.1 Obstructive lung disease or emphysema
3.2 Restrictive lung disease
3.3 Lung disease with mixed restrictive/obstructive pattern
3.4 Hypoventilation syndromes
3.5 Hypoxia without lung disease (e.g. high altitude)
3.6 Developmental lung disorders
Group 4 PH associated with pulmonary artery obstructions
4.1 Chronic thrombo-embolic PH
4.2 Other pulmonary artery obstructions
Group 5 PH with unclear and/or multifactorial mechanisms
5.1 Haematological disorders
5.2 Systemic disorders
5.3 Metabolic disorders
5.4 Chronic renal failure with or without haemodialysis
5.5 Pulmonary tumour thrombotic microangiopathy
5.6 Fibrosing mediastinitis

Source: (Humbert et al., 2022)

Pathophysiology

Pathological changes of PAH are enhanced pulmonary arteriole contractility, endothelial dysfunction, remodeling and proliferation of both endothelial and smooth muscle cells, and in situ thrombi. The physiological outcome of

pathological changes is the partial blockage of small pulmonary arteries, leading to increased pulmonary vascular resistance, subsequent right ventricular failure, and death. PAH is caused by impaired vasodilation from reduced PGI_2 production (cyclooxygenase-2 dysregulation) and NO synthase (eNOS) function, with concurrent vasoconstrictive and mitogenic effects of an upregulated ET-1 signaling system, etc. Understanding the mechanism of these pathways has supported rapid development in the quantity and efficacy of targeted pharmacological therapies for PAH (Lan et al., 2018).

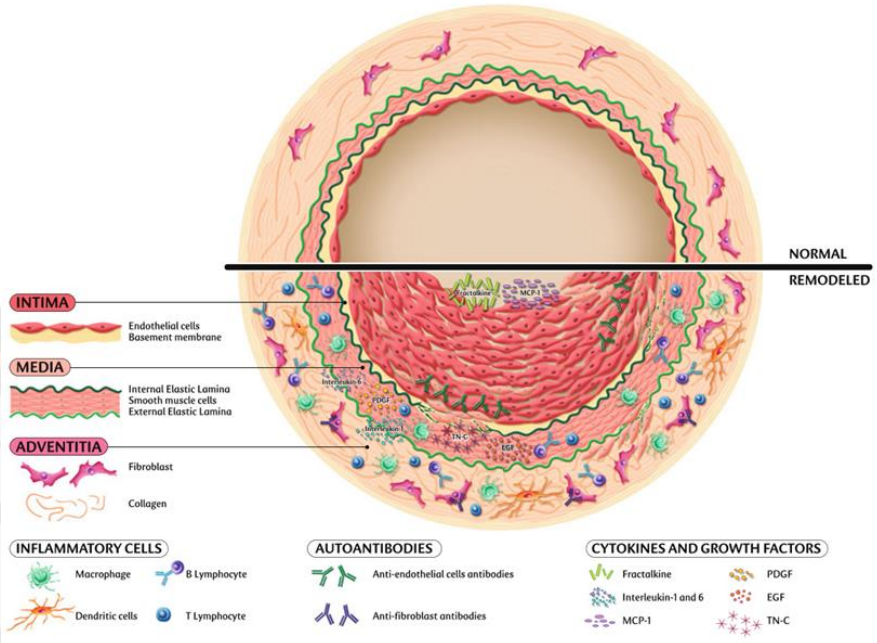


Figure 6 Pulmonary vascular remodeling in pulmonary arterial hypertension (PAH)
Source: (Kherbeck et al., 2013) (Permission from Springer Science Business Media, LLC)

Treatment for PAH (Humbert et al., 2022)

The PAH treatment has been modified, highlighting the importance of cardiopulmonary comorbidities, risk assessment both at diagnosis and follow-up, and the importance of combination therapies. Treatment strategies during follow-up have been based on the four-strata model intended to facilitate more granular decision-making.

1. General measures: in addition to applying PAH drugs, general measures and care in special situations represent integral components of optimized patient care. The recommendations for general measures include 1) Physical activity and supervised rehabilitation, 2) Anticoagulation, 3) Diuretics, 4) Oxygen, 5) Cardiovascular drugs, 6) Anaemia and iron status, 7) Vaccination, 8) Psychosocial support, and Adherence to treatments.

2. Special circumstances: the recommendations for special circumstances include 1) Pregnancy and birth control, 2) Surgical procedures, and 3) Travel and altitude.

3. Pulmonary arterial hypertension therapies: the drug used to treat PAH include 1) Calcium channel blockers, 2) Endothelin receptor antagonists (Ambrisentan, Bosentan, and Macitentan), 3) Phosphodiesterase 5 inhibitors and guanylate cyclase stimulators (Sildenafil, Tadalafil, and Riociguat), 4) . Prostacyclin analogues and prostacyclin receptor agonists (Epoprostenol, Iloprost, Treprostinil, Beraprost, and Selexipag).

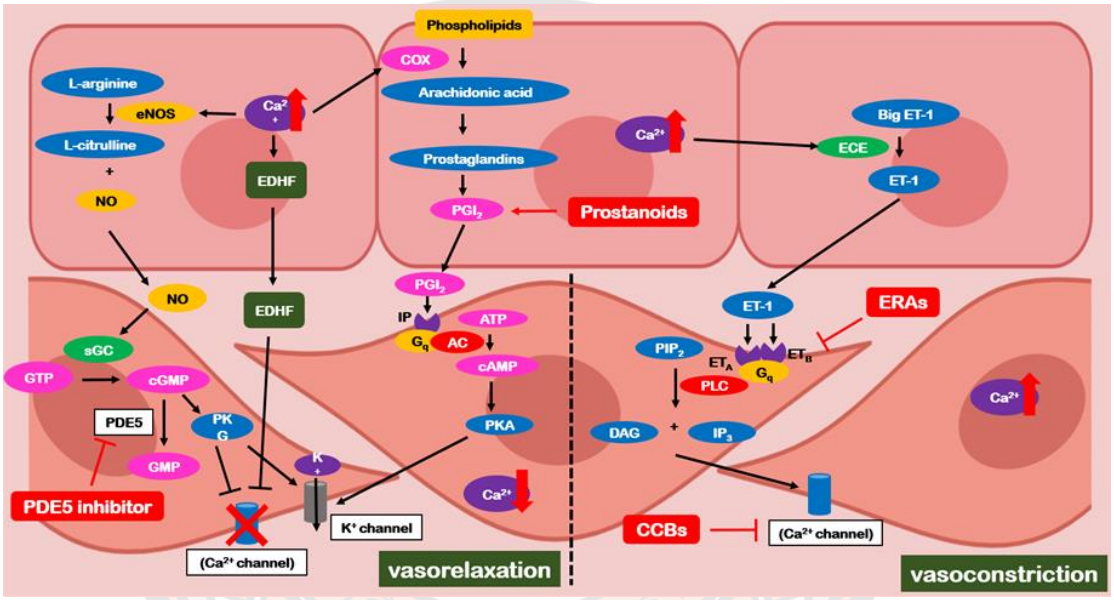


Figure 7 Therapeutic targets in PAH
Source: Made by Teerapap Panklai (2023)

Abbreviations: ATP, adenosine triphosphate; cAMP, Cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; DAG, diacylglycerol; EDHF, Endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; ET-1, Endothelin-1; GTP, Guanosine triphosphate; IP, prostacyclin receptors; PGI_2, Prostacyclin; PKG, protein kinase G; sGC, soluble guanylyl cyclase.

Family Nelumbonaceae and Nymphaeaceae

The genera *Nymphaea* and *Victoria* belong to the family Nymphaeaceae. The *Nymphaea* genus comprises 45–50 species, some of which are consumed as food and utilized in traditional medicine for treating various diseases. These species are found across North America, Africa, Europe and Asia including Thailand (Dkhar et al., 2010; Singh & Jain, 2017). Phytochemical studies have revealed that various *Nymphaea* species contain flavonoids glycosides, alkaloids, glycosides, hydrolysable tannins, lignans, phytosterols and triterpenoid saponins (Selvakumari et al., 2016;

Singh & Jain, 2017). The genus *Victoria* is distributed in the Amazonas and Chaco biogeographical regions of South America (Kite et al., 1991; Smith et al., 2022). It includes species such as *Victoria amazonica* (Poepp.) Sowerby, *V. cruziana* A.D. Orb. and *V. boliviana* Magdalena and L.T.Sm. Previous phytochemical studies have indicated the presence of steroids, benzenoids, chlorophylls, ketone, benzyl esters and methyl esters (Chen et al., 2022; Kite et al., 1991).

Nelumbo Adans. is a genus within the Nelumbonaceae family. These perennial aquatic plants inhabit stagnant water habitats and hold significative value in horticulture, medicine, food, religion, and culture. The genus *Nelumbo* or lotuses consists of two species, i.e., *Nelumbo nucifera* Gaertn. (The Asian lotus) and *N. lutea* Willd. (The American lotus). *N. nucifera* is distributed in various countries of North Australia, and East, South and Southeast Asia including Thailand. On the other hand *N. lutea* is distributed in North America (Hassan et al., 2020; LI et al., 2014). Phytochemical studies have identified flavonoids, alkaloids, polysaccharides, essential oil, triterpenoids, steroids and tannins in *N. nucifera* (Chen et al., 2019; Paudel & Panth, 2015).

***Nymphaea pubescens* Willd. (Water Lily)**

Nymphaea pubescens Willd. Commonly knows as Water Lily is the sole representative of the *Nymphaea* genus. *N. pubescens* is naturally distributed across Myanmar, Laos, Cambodia, Vietnam, Malaysia, Philippines, Indonesia, New Guinea, Sri Lanka, and India, and is widespread throughout all regions in Thailand (La-ongsri et al., 2009).

In traditional medicine, the flower of *N. pubescens* have been used as a blood purifier, and for the treatment of various conditions, including diabetes, cancer, inflammation, jaundice, eye-disorder, and aphrodisiac (Debnath et al., 2013; Singh & Jain, 2017). Additionally, the rhizome of *N. pubescens* have been employed to address diverse such as abortion, blood dysentery, dyspepsia, jaundice, blood purifier, cystitis, nephritis, fever, insomnia, hemorrhoids, leucorrhoea, menorrhagia, and piles) (Arya et al., 2022; Singh & Jain, 2017).

Pharmacologically, *N. pubescens* petals exhibit diverse effects, including antidiabetic, antihepatotoxic, anti-inflammatory, anticancer, antidementia, and antioxidant (Vivekanandarajah et al., 2021), The rhizomes have been reported to possess antioxidant properties (Daffodil & Mohan, 2013) while the tuber (Shajeela .P .S et al., 2012) and leaves (Angadi et al., 2013) have demonstrated antidiabetic, antioxidant, hypolipidemic effects.

Phytochemical, studies have identified alkaloids and tannin (Thiyagarajan et al., 2010) in the flower of *N. pubescens* along with phenolics (Pokhrel et al., 2022) and flavonoid compounds such as isoquercitrin, kaempferol, quercetin, and myricetin (Acharya & De, 2016; Acharya et al., 2018; Debnath et al., 2013). The rhizome contains total phenolic and flavonoids (Arya et al., 2022; Daffodil & Mohan, 2013), while the tuber exhibited the presence of alkaloids, flavonoids, glycosides, terpenoids, tannins, phenols, saponins, and steroids (Shajeela .P .S et al., 2012).

Pharmacological effect and phytochemicals in the flower of plants in Nelumbonaceae and Nymphaeaceae families

Nymphaea stellata Willd.

In the traditional medicine, the flowers of *N. stellata* have been employed for various purposes, including diabetes mellitus, liver disorders, biliousness as an aphrodisiac, vomiting, giddiness, worm infestation, and burning of the skin (Rani et al., 2012).

Pharmacologically, the flowers of *N. stellata* have demonstrated hepatoprotective effects (Rani et al., 2012), as well as antioxidant properties leading to decreased hyperglycemia, cholesterol and triglycerides levels (Pandita et al., 2015). Additional, reported activities anti-nociceptive, immunomodulatory, antipyretic activities (Pandurangan et al., 2013), and antidiabetic (R, 2018).

Phytochemical studies have identified flavonoids, gallic acid, astragalin, quercetin, kaempferol (Rani et al., 2012), and Nymphayol (Pandita et al., 2015; Pandurangan et al., 2013) in the flower of *N. stellata*.

Nelumbo nucifera Gaertn.

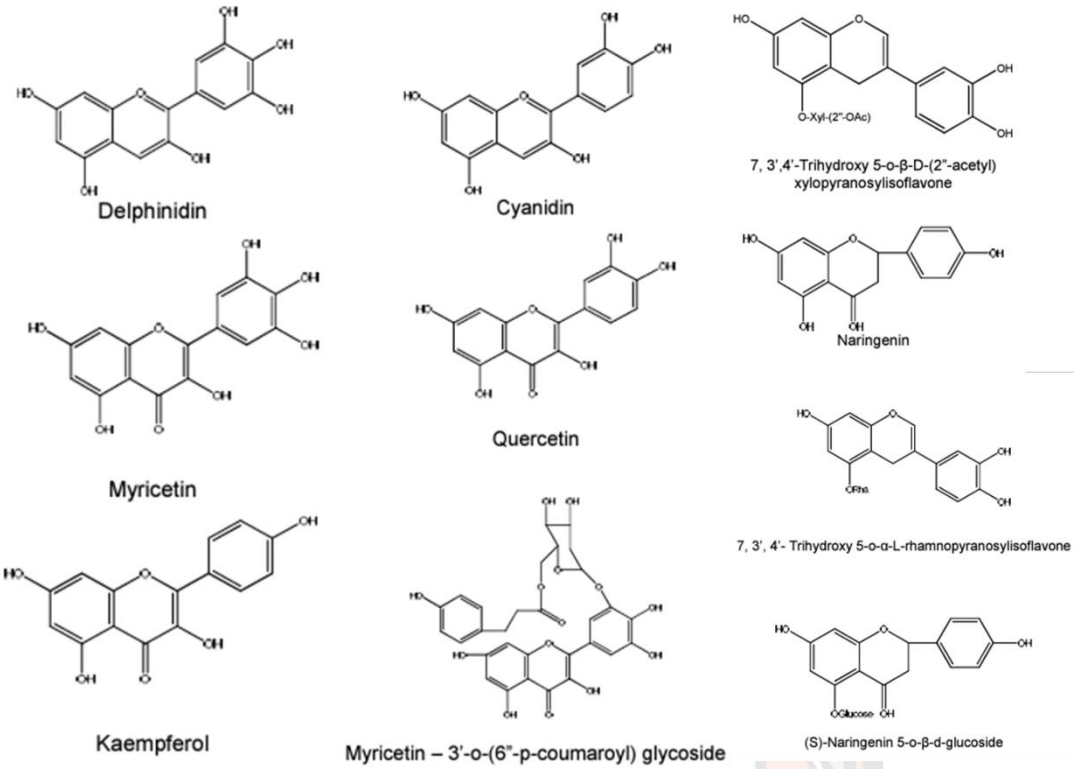
Traditionally, the flowers of *N. nucifera* have been used to address conditions such as diarrhea, cholera, fever and gastric ulcers (Buddhadev & Buddhadev, 2014), as well as hepatopathy and hyperdipsia (Arjun et al., 2012).

Pharmacologically the flower has demonstrated antioxidant properties and has been associated with increased sperm viability (Laoung-on et al., 2021). It serves as a cardiac and hepatic tonics (Showkat et al., 2021) and exhibits neuroprotective and memory-enhancing properties (Sharma et al., 2016). Other reported effects include anti-hyperlipidemic (U.Subasini et al., 2014), antipyretic, aphrodisiac, and hypoglycemic activities (Mehta et al., 2013).

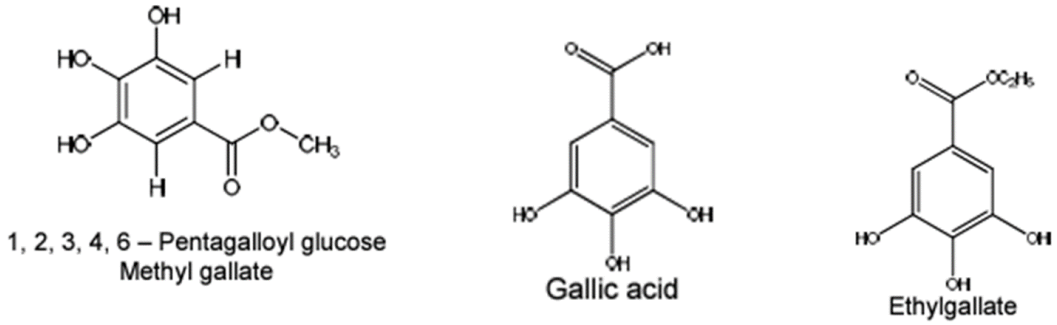
Phytochemical analysis has identified total phenolics, total tannins, and total flavonoids such as quercetin isoquercitrin luteolin and kaempferol in the flower of *N. nucifera* (Arjun et al., 2012; Buddhadev & Buddhadev, 2014; Laoung-on et al., 2021; Mehta et al., 2013; Paudel & Panth, 2015; Sharma et al., 2016; U.Subasini et al., 2014). Additionally, anthocyanins, alkaloids, terpenoids (Arjun et al., 2012; Showkat et al., 2021), and saponins (Dubey & Baghel, 2019) have been reported.

As of now, there are no available reports of pharmacological effects and phytochemical composition of the flowers of *Nymphaea cyanea* Roxb., *Nymphaea* sp. and *Victoria amazonica* (Poepp.) Klotzsch., warranting further study.

Flavonoids



Tannin



Steroids

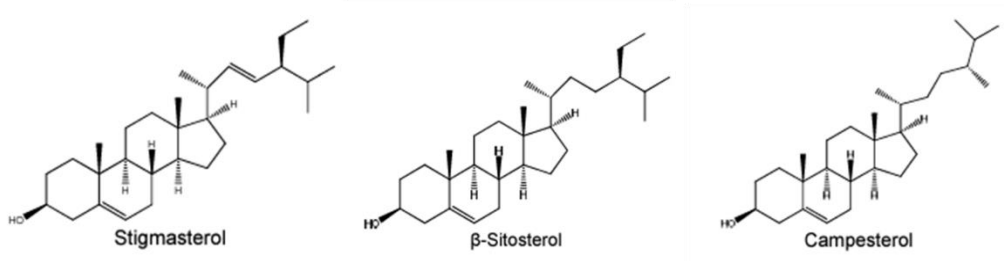


Figure 8 Structures of phytoconstituents from the genus *Nymphaea*.
Source: (Selvakumari et al., 2016)

Triterpenoids

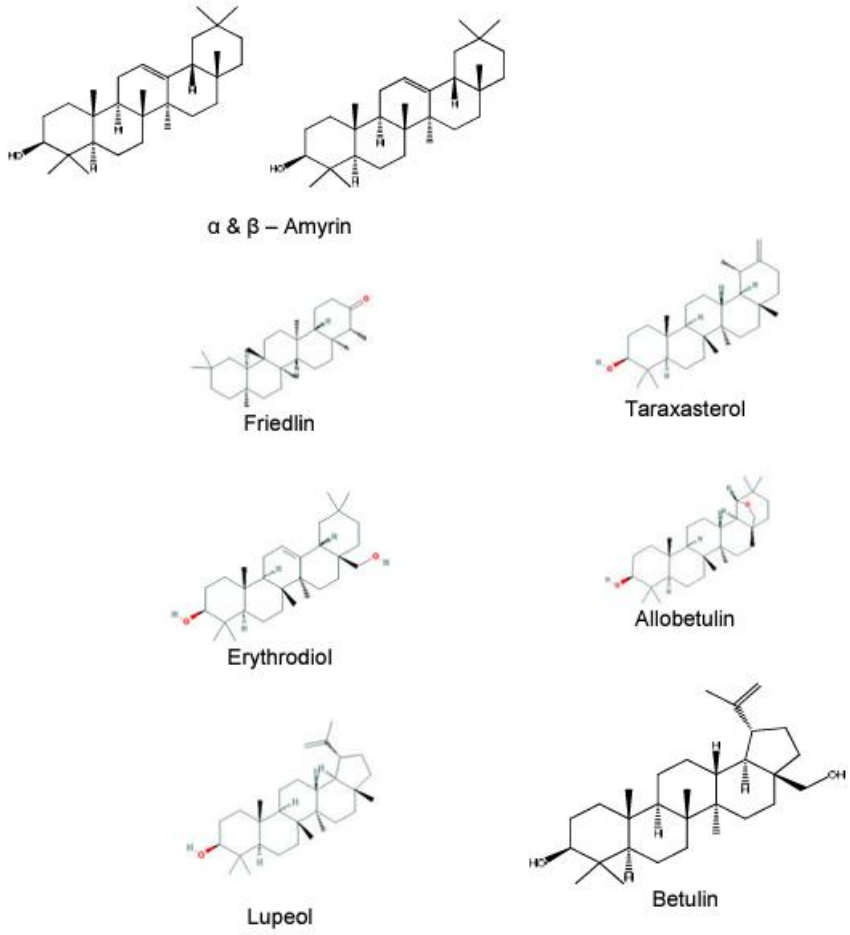


Figure 8 Structures of phytoconstituents from the genus *Nymphaea*. (cont.)

Source: (Selvakumari et al., 2016)

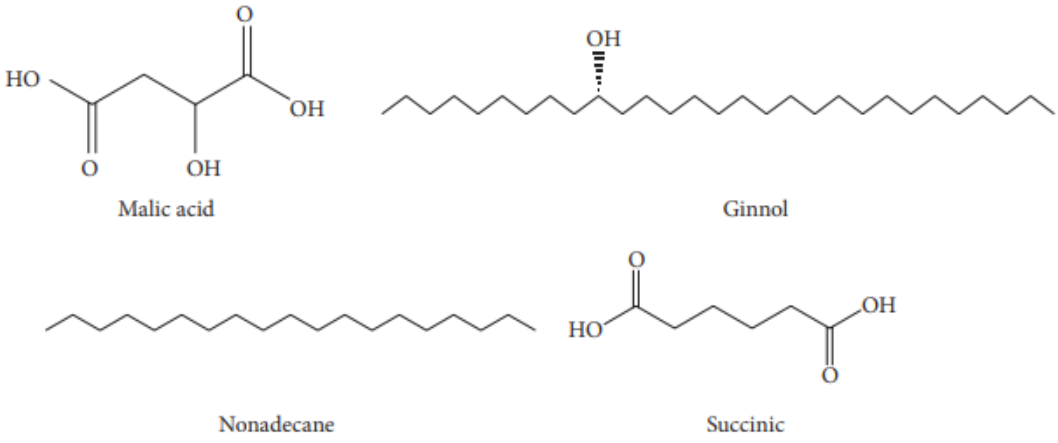
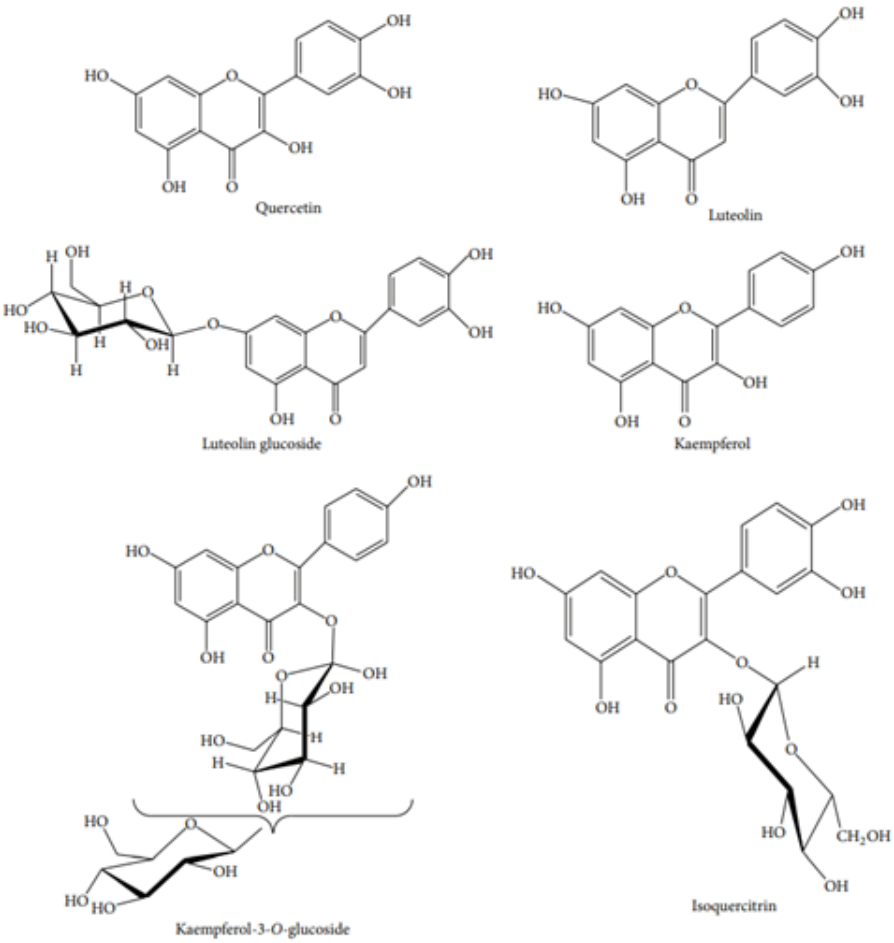


Figure 9 Structures of phytoconstituents from *N. nucifera* flower.
Source: (Paudel & Panth, 2015)

Flavonoids



Alkaloid

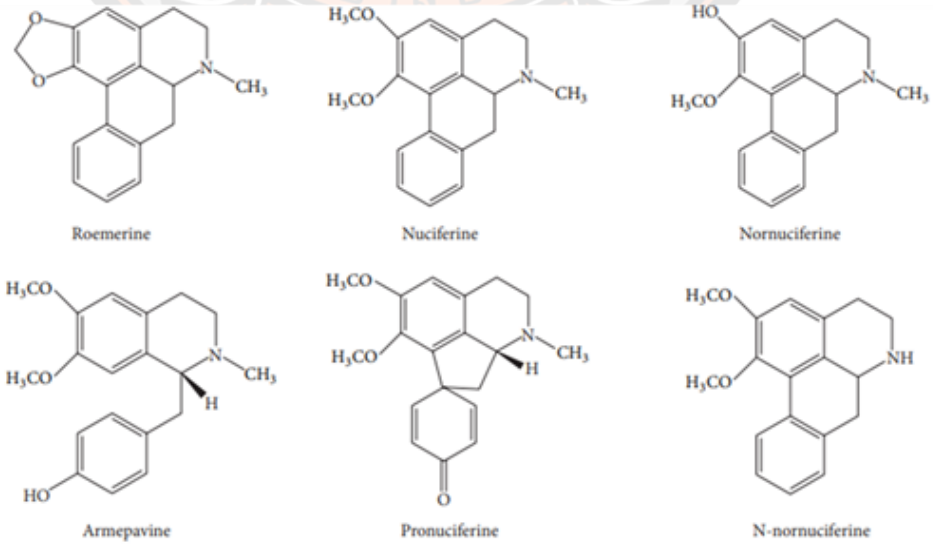
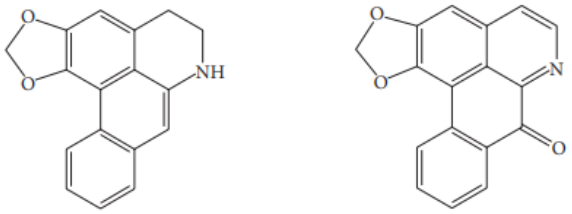


Figure 9 Structures of phytoconstituents from *N. nucifera* flower. (cont.)

Source: (Paudel & Panth, 2015)

Alkaloid



Organic acid

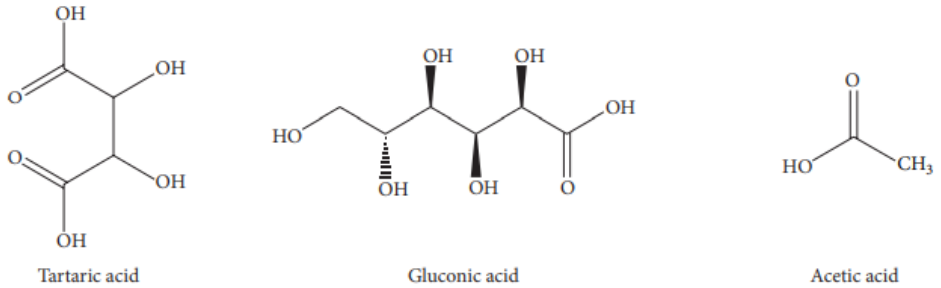


Figure 9 Structures of phytoconstituents from *N. nucifera* flower. (cont.)

Source: (Paudel & Panth, 2015)

Quality control and method validation of herbal drugs

Quality control (Ahmad et al., 2006)

Quality control is a crucial process aimed at preserving the identity, purity, content, and other properties of drugs through various stages. The integration of traditional methods with modern assessment techniques is employed to evaluate the identity and quality of traditional medicines. The aim is to ensure that all medicines, whether synthetic or herbal, meet efficacy and safety requirements through appropriate clinical trials.

Herbal drugs encompass plants or plant parts transformed into phytopharmaceuticals through processes like harvesting, drying, and storage, which can introduce variations in their composition. Quality control for herbal drugs revolves around three key aspects: identity (ensuring the correct plant), purity (free of contaminants), and content/assay (measuring active constituents). These criteria are evaluated using methods such as macroscopic and microscopic examinations, chemical analyses, and modern techniques like chromatography and spectrometry.

The complex nature of herbal drugs poses challenges for quality control. These drugs often consist of mixtures of numerous constituents and their active principles may be unknown. Variability in growth conditions, harvesting methods, and processing further complicates quality control. Good Agricultural Practices (GAP) are pivotal in upholding the quality of raw materials. Standardization involves

adjusting herbal preparations to contain specific known compounds. Manufacturers are obligated to adhere to stringent quality control standards and good manufacturing practices.

Parameters for quality control of herbal drugs

1. Microscopic Evaluation: Microscopic analysis plays a crucial role in identifying herbs, detecting small fragments of powdered herbs, and identifying foreign matter and adulterants. It helps ensure the correct species and plant parts are used, especially when different parts of the same plant have different therapeutic properties.

2. Determination of Foreign Matter: Herbal drugs should be free from foreign matter, including other parts of the same plant or other plants, molds, insects, sand, stones, and chemical residues. Macroscopic examination and microscopy are used to detect foreign matter, while chromatography might be needed for certain cases.

3. Determination of Ash: Ash content is measured to assess the presence of inorganic material. Total ash and acid-insoluble ash are determined, with the latter indicating the presence of silica. This process helps assess the purity of herbal drugs.

4. Determination of Heavy Metals: Contamination by toxic heavy metals is assessed. Techniques such as atomic absorption spectrophotometry (AAS), inductively coupled plasma (ICP), and neutron activation analysis (NAA) are used to measure heavy metal levels, ensuring they're within safe limits.

5. Determination of Microbial Contaminants and Aflatoxins: Herbal drugs may potentially contain microbial contaminants like bacteria, fungi, and viruses. Testing for total aerobic microbial count, total fungal count, and specific pathogens, along with aflatoxins, is conducted to ensure the products are safe for consumption.

6. Determination of Pesticide Residues: Herbal drugs are tested for pesticide residues that might accumulate due to agricultural practices. Techniques like gas chromatography (GC) and mass spectrometry (MS) are employed to detect pesticide residues accurately.

7. Determination of Radioactive Contamination: Radioactive contamination from nuclear accidents is assessed, although naturally occurring radioactive contamination is not a significant concern. Guidelines help evaluate health risks associated with radioactive contamination.

8. Chemical analyses: Pharmacopeias and guidelines provide analytical methods for bioactive chemical constituents or “markers” in herbal drugs. Techniques such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), and mass spectrometry (MS) are used for the precise identification and quantification of these constituents.

9. Method Validation: Method validation is essential to prove that an analytical method is suitable for its intended purpose. Parameters like specificity, linearity, accuracy, and precision are evaluated. Official monographs in pharmacopeias provide validated methods for quality control.

Method validation (Guideline, 2005)

The validation process considers key factors such as specificity, linearity, range, accuracy, precision, limit of detection, limit of quantitation, robustness, and system suitability testing. Specificity is crucial for identification tests, impurity determination, and assays. While achieving absolute discrimination may not always be feasible, combining multiple procedures is advised to enhance accuracy. The approaches to specificity depend on the objective of the procedure.

Linearity must be evaluated across the intended range of the procedure. This can be achieved through dilution of standard solutions or synthetic mixtures of components. A linear relationship can be visually assessed, and statistical methods can validate it. In cases where linearity is not achieved, an appropriate mathematical function describing the relationship may be used.

The specified range is derived from linearity studies and is contingent on the specific application of the procedure. For example, the assay's range is usually 80-120% of the test concentration. Content uniformity, dissolution testing, and impurity determination also have specific ranges.

Accuracy is established across the specified range, determined by known purity or comparison with a second well-characterized procedure. Impurity quantitation requires accuracy assessment on spiked samples. Data from a minimum of 9 determinations over 3 concentration levels should be reported.

Precision is assessed through three parameters: repeatability, intermediate precision, and reproducibility. These measure the impact of random factors on precision, considering variations like days, analysts, and equipment. Reporting should include standard deviation, relative standard deviation, and confidence intervals to provide a comprehensive understanding of precision.

The limit of detection can be determined through visual evaluation, signal-to-noise ratio, or calculation using standard deviation and calibration curve parameters. Similarly, the limit of quantitation considers visual evaluation, signal-to-noise ratio, and standard deviation.

Robustness evaluation is crucial for ensuring reliability despite intentional variations in parameters. This assessment includes system suitability tests to guarantee consistent performance under varying conditions.

CHAPTER III

RESEARCH METHODOLOGY

Plant materials

Nymphaea pubescens Willd was collected in the morning when their flowers were blooming from the Faculty of Pharmaceutical Science, Naresuan University, Phitsanulok, Thailand, and the other plants in the families of Nelumbonaceae and Nymphaeaceae were collected from Lotus Museum, Rajamangala University of Technology Thanyaburi, Pathum Thani, Thailand. The voucher specimens of *N. pubescens* (No. 004664), *Nymphaea* sp. (No. 05744), *Nymphaea stellata* Willd. (No. 05745), *Nymphaea cyanea* Roxb. ex G.Don. (No. 05746), *Nelumbo nucifera* Gaertn. (No. 05747), and *Victoria amazonica* (Poepp.) Sowerby. (No. 05748) were kept at the Faculty of Sciences, Naresuan University, Phitsanulok, Thailand. The plants were identified by Assistant Professor Dr Pranee Nangngam, Department of Biology, Faculty of Sciences, Naresuan University.

Nelumbo nucifera Gaertn. *Nymphaea pubescens* Willd.

Nymphaea stellata Willd. *Nymphaea cyanea* Roxb. ex G.Don.

Victoria amazonica (Poepp.) Klotzsch. *Nymphaea* sp.

Figure 10 Plants in the families of Nelumbonaceae and Nymphaeaceae
Source: Made by Teerapap Panklai (2023)

Extraction and Isolation

The fresh plants were divided into leaves, petals, pollen, seed, receptacle, peduncles, and petioles, which were dried in a hot air oven at 55 °C for two days and then ground into powder. The powder was macerated with 95% ethanol for 3 days/time (two times), and then filtered and evaporated under a vacuum until dry, then stored at −20 °C until used. To isolate the PDE5 inhibitors, 9.67 g *N. pubescens* petals extract was dissolved in 100% MeOH (0.5 mL, concentration 100 mg/mL). The solution was loaded onto a solid-phase extraction (SPE) mini-column Strata C18-E (55 µm, 70 A), washed with 6 mL acetonitrile, and eluted with 0.1% formic acid in 80% water: 20% ACN for fractions 1–3 but fraction 4 was eluted with 100% acetonitrile. Fraction 4 (2.02 g) which contained flavonoids was evaporated to dryness and then purified on preparative HPLC (Gilson PLC 2020) fitted with a Kinetex EVO reverse-phase C18 column (250 × 21.2 mm, 5 µm). The solvent system used was 0.1% formic acid in 70% water (solvent A) and 0.1% formic acid in 30% acetonitrile (solvent B). The elution program (20 mL/min) was 30% B (0–25 min) and followed by a 10 min wash with 100% B and 15 min re-equilibration steps. The injection volume was 300 µL (300 mg/mL), and chromatograms were detected at 366 nm. Four pure compounds; (**1**) quercetin 3′-O-β-xylopyranoside (16.8 mg, Rt 16.5 min), (**2**) quercetin 3-methyl ether 3′-O-β-xylopyranoside (107.7 mg, Rt 17.2 min), (**4**) 3-O-methylquercetin (13.1 mg, Rt 20.2 min), and (**6**) 3-O-methylkaempferol (5.2 mg, Rt 22.9 min) were obtained. The purity of these compounds was checked by HPLC, NMR spectra, and another spectroscopic method.

Chemicals

Acetonitrile and methanol were of HPLC grade (VWR Chemicals, Fontenay-sous-Bois, France). Formic acid was of analytical grade (VWR Chemicals, Fontenay-sous-Bois, France), cGMP, crude snake venom (Crotalus atrox), bovine serum albumin (BSA), imidazole, ethylene glycol tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), magnesium chloride ($MgCl_2$), phenyl methyl sulfonyl fluoride (PMSF), diethylaminoethyl sephadex (DEAE-Sephadex), DTT (Dithiothreitol), and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich. [^{3}H]-cGMP is obtained from Perkin Elmer (Boston, MA, USA). Purified liver bovine arginase 1 (1 U) of bovine arginase corresponding to the amount that was able to convert 1 µmol of L-arginine to urea and L-ornithine per minute at pH 9.5 and 37 °C) was purchase from MP Biomedicals (Illkirch-Graffenstaden, France). The (**3**) quercetin (purity >98%, Sigma-Aldrich, MO, USA), and (**5**) kaempferol (purity >90%, Sigma-Aldrich, MO, USA) were obtained from Sigma Chemical Company (St. Louis, MO, USA). PE, ACh, EGTA, L-NAME, indomethacin, apamin, 4-AP, ODQ, glibenclamide, iberiotoxin, charybdotoxin, SNP, $BaCl_2$, papain, collagenase type IA and taurine were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Glibenclamide and 4-AP were dissolved in DMSO. Indomethacin was dissolved in 0.5% w/v Na_2CO_3 and the pH was adjusted to 7.4 with 1 M NaOH. MTT was dissolved in PBS (at pH 7.4). Other substances are dissolved in distilled water.

Phosphodiesterase 5 Inhibition Assay

Sample Preparation

The extracts were tested at the final concentration of 50 μg/mL and the compounds were tested at 10 μM. All samples were dissolved in 100% DMSO and diluted with distilled water. The final concentration of DMSO was 1%. For extracts or compounds that gave >80% PDE 5 inhibition, the IC_{50} was determined.

Enzyme Preparation

PDE5 enzymes were obtained from the transient PDE5A1 DNA transfection in human embryonic kidney 293 (HEK293) cells. These cells were homogenized using a sonicator probe and sonicate in Buffer A consisting of Tris-HCl (150 mM, pH 7.5), EDTA (6 mM), DTT (3 mM), and phenyl methyl sulfonyl fluoride (100 mM). The homogenate was centrifuged at 14,000 rpm for 20 min at 4 °C and the supernatant was used as a source of PDE5 enzymes (Bhandari et al., 2019).

Experimental Protocols

A PDE5 assay was performed following the method based on a two-step radioactive procedure (Temkitthawon et al., 2011). In the first step of the enzymatic reaction, 25 μL of extracts or solvent (5% DMSO) was added as a control to 25 μL of buffer C consisting of Tris–HCl (100 mM; pH 7.5), imidazole (100 mM), $MgCl_2$ (15 mM), and BSA (1.0 mg/mL), 25 μL of EGTA (10 mM), and 25 μL of PDE5 enzymes were added together with 25 μL of [^{3}H] cGMP (1 μM). This solution was then incubated at 30 °C for 10 min and then the reaction was stopped by placing it in boiling water for 1 min and cooled in ice-cold water. In the second step of the enzymatic reaction, 25 μL of snake venom (2.5 mg/mL) containing 5′-nucleotidase enzymes were added to the reaction mixture which was then incubated at 30 °C for 5 min. Then, 250 μL of Tris–HCl buffer (low salt buffer) (20 mM; pH 6.8) was added to the mixture. The reaction mixture was passed through a DEAE ion exchange resin column, and the uncharged [^{3}H] guanosine was eluted 4 times with 500 μL of a low salt buffer to obtain a hydrolysis product. Finally, the scintillant cocktail was added and the radioactivity was measured using a liquid scintillation analyzer (Tris-Carb 2910 TR, Perkin Elmer). The PDE5 enzymes were standardized to have a hydrolysis activity of 20–30% of the total substrate counts. The % hydrolysis and %PDE5 inhibition were calculated by the following equations.

$$\%\ \text{Hydrolysis}_{\text{sample/control}} = \left(\frac{(\text{CPM}_{\text{sample/control}} - \text{CPM}_{\text{background}})}{(\text{CPM}_{\text{total count}} - \text{CPM}_{\text{background}})} \right) \times 100$$

The $\text{CPM}_{\text{sample}}$ is the radioactive count rate of the assay with an enzyme. $\text{CPM}_{\text{background}}$ is the radioactive count rate of the assay but without enzyme. $\text{CPM}_{\text{control}}$ is the radioactive count rate of the assay with enzyme but without any sample. $\text{CPM}_{\text{total count}}$ is a count rate of 25 μL of substrate plus 2 mL of low salt buffer.

$$\% \text{ PDE inhibition} = \left(1 - \left(\frac{\% \text{ hydrolysis}_{\text{sample}}}{\% \text{ hydrolysis}_{\text{control}}} \right) \right) \times 100$$

The % hydrolysis $_{sample}$ and % hydrolysis $_{control}$ are the enzyme activities of the sample and solvent in the assay.

Arginase Inhibition Assay

Sample Preparation

The extracts were tested at the final concentration of 100 µg/mL. All samples were dissolved in 100% DMSO and diluted with distilled water. The IC_{50} of the extracts giving >70% arginase inhibition was determined.

Experimental Protocols

An arginase assay was performed by a spectrophotometric assay following the method in (Bordage et al., 2017), based on the reaction of urea (product of arginase-catalyzed hydrolysis of L-arginine) and α-isonitrosopropiophenone with the generation of a pink imine monitored at 550 nm. The solutions were added to the 96-well microplate, in the following order: (i) buffer containing Tris-HCl (50 mM, pH 7.5) and 0.1% of bovine serum albumin (TBSA buffer) (10 µL), with or without (control) arginase (0.025 U/µL); (ii) Tris-HCl solution (50 mM, pH 7.5) containing 10 mM $MnCl_2$ as a cofactor (30 µL); (iii) a solution containing an inhibitor or its solvent (as a control) (10 µL); (iv) a solution of L-arginine (pH 9.7, 0.05 M) (20 µL). The microplate was covered with a plastic sealing film and then incubated for 60 min in a 37 °C, then 120 µL of $H_2SO_4/H_3PO_4/H_2O$ (1:3:7) was able to stop the reaction and the microplate was left on ice for 5 min. A 10 µL volume of α-isonitrosopropiophenone (5% in absolute ethanol) was added, and the microplate was covered with an aluminium sealing film and heated in a 100 °C oven for 45 min. The microplate was kept in the dark until reading. After 5 min of centrifugation and cooling for another 10 min, the microplate was shaken for 2 min and the absorbance was read at 550 nm and 25 °C using a spectrophotometer (Synergy HT BioTeck).

Sample Preparation for HPLC Analysis

The concentration of extracts was prepared at 5 mg/mL and the control flavonoids consisting of compounds (**1**) quercetin 3′-O-β-xylopyranoside, (**2**) quercetin 3-methyl ether 3′-O-β-xylopyranoside, (**3**) quercetin (purity >98%, Sigma-Aldrich), (**4**) 3-O-methylquercetin, (**5**) kaempferol (purity >90%, Sigma-Aldrich), and (**6**) 3-O-methylkaempferol were prepared as stock solutions at 1 mg/mL with 100% methanol (HPLC grade). All samples were filtered through a 0.45 µm nylon filter before performing HPLC analysis.

Instrumentation and Chromatographic Conditions

HPLC analysis was performed using a Shimadzu Prominence UFLC system equipped with a Shimadzu SPD-20A UV/Vis detector, a DGU-20A3 degasser, LC-

20AT liquid chromatograph, and CBM-20A communications bus module. The column used was a Phenomenex Luna C18 column (150 mm × 4.6 mm, 5 µm) connected to a Phenomenex C18 (4 mm × 3 mm, 5 µm) guard column that maintained the temperature at 40 °C. The solvent system used was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient system was performed by increasing the ratio of solvent B from 20% to 70% within 25 min. The flow rate was set at 0.5 mL/min, the injection volume was 10 µL, and the UV detector was detected at 366 nm.

Method Validation

The development of the HPLC method was validated for linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, and accuracy which were according to ICH guidelines. The concentration values of each flavonoid consisting of compounds **1**, **3**, **4**, **5**, and **6** were 0.5, 1, 5, 25, 50, 75, and 100 µg/mL, while **2** was 5, 25, 50, 75, 100, 200, and 400 µg/mL. Calibration curves were constructed from each flavo-noid in triplicate (n = 3). The LOD and LOQ were determined using a signal-to-noise ratio of each flavonoid which was 3 for LOD and 10 for LOQ. Intra-day precision was measured in triplicate (n = 3), while inter-day precision was measured in triplicate for three consecutive days (n = 9). Precision was represented by the percentage of relative standard deviation (%RSD). The accuracy was expressed as percentage recovery by using the spiked concentration of each flavonoid in the *N. pubescens* petals. The concentration of compounds **1**, **3**, **4**, **5**, and **6** in a test solution were 3, 25, and 65 µg/mL, while that of **2** was 15, 75, and 300 µg/mL. These experiments were performed in triplicate (n = 3).

Animals

Vascular reactivity study in pulmonary artery and aorta

Male Wistar rats (7 weeks old and 200-250 g) were purchased from Nomura Siam International Co, Ltd., Thailand. Animals were kept under a 12–12 hr light/dark cycle, at 22 ± 1°C, and allowed free access to standard food and water. All experimental protocols were approved by the Naresuan University Animal Care and Use Committee (NUACUC, Naresuan University, Phitsanulok, Thailand, Animal ethics approval number: NU-AE621024).

Vascular reactivity study mesenteric artery

Male Wistar rats (8–12 weeks old) were purchased from Janvier (Le Genest Saint Isle, France). Animals were kept under a 12–12 hr light/dark cycle, at 22 ± 1°C, and allowed free access to standard food and water. The investigation complied with the ARRIVE animal research: reporting in vivo experiments. All protocols were approved by the local committees for ethics in animal experimentation No. 2019/003-PT/5PR of University of Franche-Comté (Besançon, France).

Pulmonary arteries (PA) and aorta preparation

The rats were anesthetized with thiopental sodium (50-70 mg/kg BW; i.p.) (Paracha et al., 2019; Wisutthathum, Kamkaew, et al., 2018). The PA and thoracic aorta were excised, cleaned of connective tissue and cut into lengths of 3 mm which

are referred to as vessel rings. These vessel rings were suspended in a 15 ml organ bath containing Krebs' solution composed of (mM): NaCl 122; KCl 5; N-[2-hydroxyethyl] piperazine-N′-[2-ethane-sulfonic acid] (HEPES) 10; KH_2PO_4 0.5; NaH_2PO_4 0.5; $MgCl_2$ 1; $CaCl_2$ 1.8; and glucose 11; adjust pH 7.4, at 37 °C and bubbled with air. The vessel rings were mounted in an organ bath and the isometric tension was measured via a Mac Lab A/D converter (Chart V5, A.D. Instruments, Castle Hill, NSW, Australia). The resting tension was adjusted to 1 g (Wisutthathum, Demougeot, et al., 2018). Some rings were mechanically denuded of the endothelium. The vessel rings were allowed to equilibrate with Krebs' solution for 60 min with the Krebs' solution exchanged every 15 min. To measure vessel viability, the vessel rings were tested with 80 mM KCl solution. The presence of functional endothelial cells was verified by pre-contracting the vessels with phenylephrine (PE, 10^{-5} M) and adding acetylcholine (ACh, 10^{-5} M) to induce more than 80% relaxation.

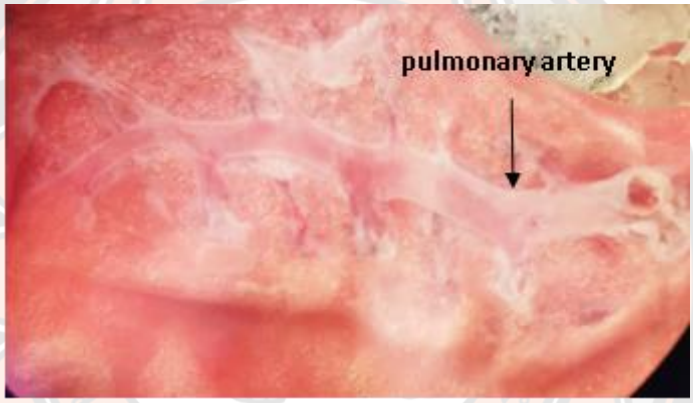


Figure 11 Rat pulmonary artery
Source: Made by Teerapap Panklai (2023)

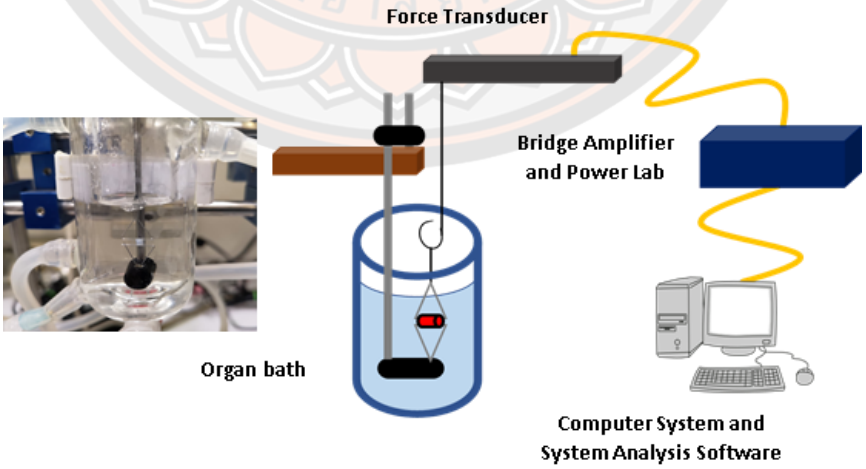


Figure 12 Organ bath setting for measurement of vascular effects.
Source: Made by Teerapap Panklai (2023)

Mesenteric arteries (MA) preparation

The rats were anesthetized with sodium pentobarbital (Ceva Santé Animale, France) (60 mg/kg, i.p.). Second-order branches of mesenteric arteries (MA) were excised, cleaned of connective tissue, and cut into rings ~2 mm in length. The MA rings were suspended in 6 ml organ chambers containing Krebs' solution (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 12, maintained at pH of 7.4, 37°C, and continuously aerated with 95% O_2, 5% CO_2. The MA rings were mounted in organ chambers and threaded on two 40-µm diameter stainless steel wires. To measure isometric force, the artery segments were connected to a Multi Myograph System (Model 610 M v.2.2, DMT A/S, Denmark). The data were recorded using ChartTM Ver.7 (ADInstruments, France). The MA rings were stretched to their optimal lumen diameter for active tension development. The optimal lumen diameter was determined based on the internal circumference/wall tension ratio of the segments by setting the internal circumference to 90% of what the vessels would have if they were exposed to a passive tension equivalent to that produced by a transmural pressure of 100 mmHg. The MA rings were equilibrated with Krebs' solution for 15 min, and for the measurement of vessel viability, the MA rings were tested with 100 mM KCl solution. The presence of functional endothelial cells was verified by pre-contracting with phenylephrine (PE, 10^{-5} M) and adding acetylcholine (ACh, 10^{-5} M) to induce more than 80% relaxation. In some rings, endothelium was mechanically removed (Wisutthathum, Chootip, et al., 2018). The completeness of this endothelial denudation was confirmed by less than 10% relaxation response to ACh (10−5 M).

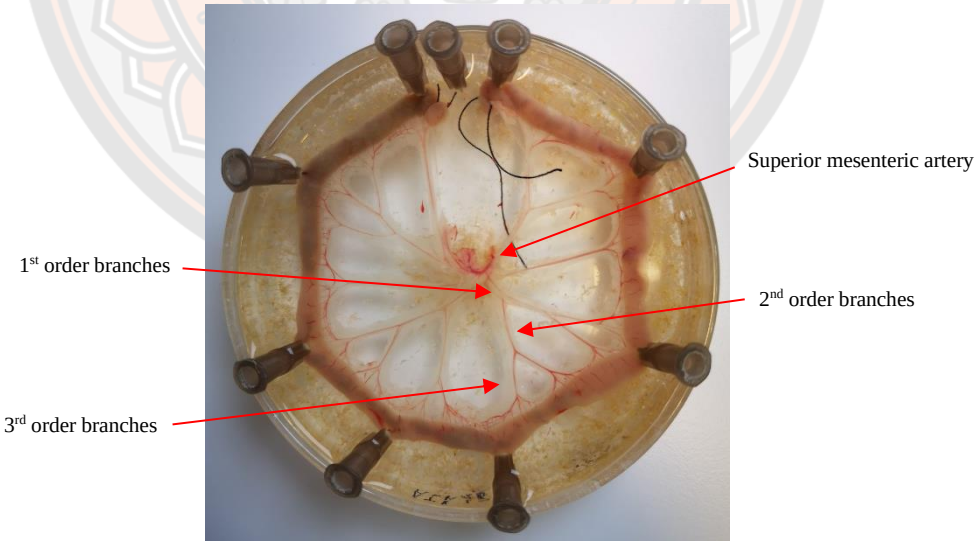


Figure 13 Rat mesenteric artery
Source: Made by Teerapap Panklai (2023)

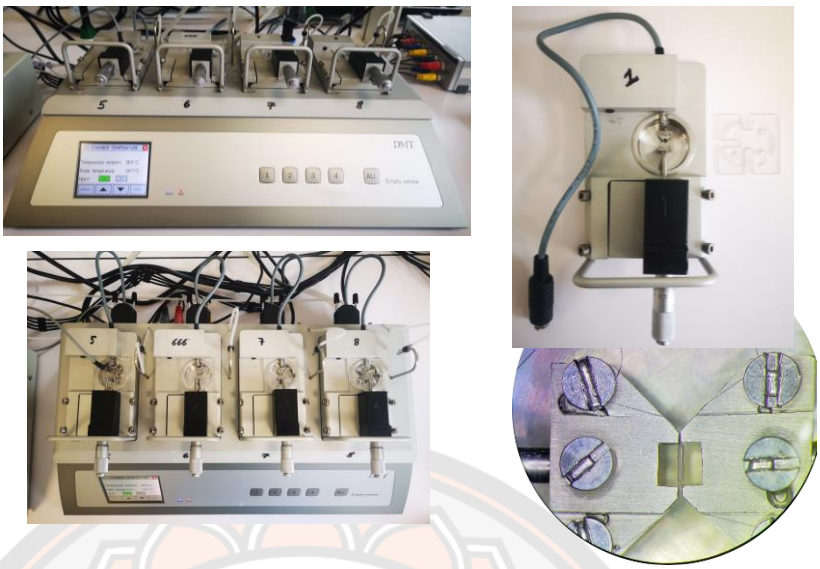


Figure 14 Muti Myograph System for measurement of vascular effects
Source: Made by Teerapap Panklai (2023)

Experimental protocols

1. The vasorelaxant effect of WLE and flavonoids

The vasorelaxation effects of the WLE and flavonoids on the PA, aortic and MA rings were investigated by pre-contracting the rings with PE (10^{-5} M). Cumulative concentrations of WLE (0.001–1000 μg/ml) or compounds **2**, **3**, and **5** (0.1-100 μM) were added to both the endothelium-intact (E+) rings and the endothelium-denuded (E-) rings after the contraction had reached a plateau. The relaxation effect was calculated as the percentage of the contraction in response to the PE. The effect of the solvent, dimethyl sulfoxide (DMSO 0.1% for WLE and flavonoids compounds) was evaluated under the same conditions.

2. Investigation of endothelium-dependent pathways

The role of endothelial nitric oxide synthase (eNOS), cyclo-oxygenase (COX), and endothelium-derived hyperpolarizing factor (EDHF) pathways were investigated using the (E+) PA or MA rings which were incubated with N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M); NOS inhibitor, indomethacin (10^{-5} M); COX inhibitor or apamin (10^{-7} M) plus charybdotoxin (10^{-7} M); small- and intermediate-conductance Ca^{2+}-activated K^+ channel blockers (SK_{Ca} and IK_{Ca}) for 30 min. The PA or MA rings were then pre-contracted with PE (10^{-5} M) before cumulative concentrations of the WLE or **2** were added (Wisutthathum, Chootip, et al., 2018; Wisutthathum, Demougeot, et al., 2018).

3. Investigation of vascular smooth muscle K^+ channels

The role of K^+ channels was investigated using the (E-) PA or MA rings which were incubated with 4-aminopyridine (4-AP, 10^{-3} M); voltage-gated potassium channel (K_V) blocker, glibenclamide (10^{-5} M); ATP-sensitive potassium channel

(K_{ATP}) blocker, iberiotoxin (10^{-7} M); large-conductance Ca^{2+}-activated K^+ channel (K_{Ca}) blocker or $BaCl_2$ ($30x10^{-9}$ M); inward-rectifier K^+ channel (K_{IR}) blocker for 30 min, followed by pre-contraction with PE (10^{-5} M) before cumulative concentrations of the WLE or **2** were added (Wisutthathum, Chootip, et al., 2018; Wisutthathum, Demougeot, et al., 2018).

4. Investigation of sGC/cGMP pathway

The role of the soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway in the VSMCs was investigated using the (E-) PA or MA rings. The PA rings were incubated with the vehicle or with WLE at the EC_{50} for 10 min before being pre-contracted with PE (10^{-5} M), then cumulative concentrations (10^{-17}-10^{-8} M) of sodium nitroprusside (SNP), a NO donor, were added. Whereas The MA rings were performed in 100 mM KCl-preconstricted rings (for the WLE) or PE (10^{-5} M)-preconstricted rings (for the **2**). In addition, to investigate if the WLE can directly activate sGC, the PA or MA rings were incubated with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{-5} M), the selective sGC inhibitor, for 30 min, then pre-contraction with PE (10^{-5} M) before cumulative concentrations of the WLE were added (Wisutthathum, Chootip, et al., 2018; Wisutthathum, Demougeot, et al., 2018).

5. Investigation of Ca^{2+} channels

The role of extracellular calcium (Ca^{2+}) influx was investigated using the (E-) PA rings which were incubated with a Ca^{2+}-free Krebs' solution containing methylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, 1 mM in PA or 2 mM in MA) for 40 min. PE (10^{-5} M) was added to the PA or MA rings to deplete intracellular Ca^{2+} from the sarcoplasmic reticulum (SR), and the PA or MA rings were then washed for 40 min with the Ca^{2+}-free Krebs' solution with the solution changed every 10 min. The PA or MA rings were incubated with the vehicle, the WLE or **2** at the EC_{50} for 10 min then PE (10^{-5} M) (for PA and MA rings) was added and incubated for another 5 min to induce extracellular Ca^{2+} influx via the receptor-operated Ca^{2+} channels (ROCCs) or 80 mM KCl (for MA rings). Alternatively, Ca^{2+}-free 80 mM KCl solution was added to replace the Ca^{2+}-free Krebs' solution, and the PA rings were incubated with the vehicle or the WLE at the EC_{50} for 15 min. Then $CaCl_2$ (10^{-5}-10^{-2} M) (for PA rings) or $CaCl_2$ (10^{-2} M) (for MA rings) were added to evoke contraction via activation of the voltage-operated Ca^{2+} channels (VOCCs) (Wisutthathum, Chootip, et al., 2018; Wisutthathum, Demougeot, et al., 2018).

The role of intracellular calcium (Ca^{2+}) release from SR was investigated using the (E-) PA rings which were incubated with 80 mM KCl solution for 15 min to stimulate initial Ca^{2+} loading of the SR Ca^{2+} stores, and then washed with the Ca^{2+}-free Krebs' solution containing 1 mM EGTA for 15 min. The PA rings were then contracted with PE (10^{-5} M) which triggers the release of stored Ca^{2+} via inositol 1, 4, 5 trisphosphate (IP_3) receptor/channels on the SR membrane. The same protocol was then repeated to ensure that the PA rings had similar contractions with the PE. The PA rings were incubated again with 80 mM KCl solution for 15 min, and washed with the Ca^{2+}-free Krebs' solution containing 1 mM EGTA and the vehicle or the WLE at the EC_{50}, for 15 min. The PA rings were then contracted with PE (10^{-5} M) and the

percentage of PE-induced contractions was compared in the presence or absence of the vehicle or the WLE (Kamkaew et al., 2011).

To assess the effect of WLE and **2** on the intracellular calcium (Ca^{2+}) release, (E-) MA rings were incubated with a Krebs' solution for 40 min, then incubated with the L-type voltage-dependent Ca^{2+} channel inhibitor (Verapamil, 10^{-7} M) for 30 min. Then, MA rings were incubated at its EC_{50} of the WLE, **2**, or the vehicle for 15 min. After that, the PE (10^{-5} M) was added to induce contractions caused by intracellular Ca^{2+} release via the opening of inositol 1,4,5 trisphosphate (IP_3) receptors from SR (Wisutthathum, Chootip, et al., 2018).

6. Investigation of the inhibitory effect of WLE on PE-induced vasocontraction

The (E-) PA or MA rings were incubated with the WLE or **2** at the EC_{50} or vehicle, for 15 min before vasocontraction was performed with PE (10^{-10}-10^{-4} M) and the percentage contraction of the PA or MA rings was compared to the maximum contraction of PE (10^{-5} M) without treatment (Paracha et al., 2019).

Cytotoxic effect of WLE on VSMCs

Fresh thoracic aortae and the PA isolated from the rats were cut into small strips and opened out longitudinally. Strips of smooth muscle were immersed in a dissociation medium (DM) solution (mM): NaCl 110; KCl 5; KH_2PO_4 0.5; NaH_2PO_4 0.5; $NaHCO_3$ 10; HEPES 10; taurine 10; EDTA 0.5; $MgCl_2$ 2; glucose 10; and $CaCl_2$ 0.16; adjusted to pH 7.0 with 1 M NaOH, then incubated at 4°C in DM containing 0.8 mg/ml papain, 0.04% bovine serum albumin (BSA) and 0.4 mM DL-Dithiothrcitol (DTT) for 24 hr, and then incubated at 37°C for 10 min. Then 1 mg/ml collagenase was added, and the smooth muscle was further incubated for 5 min. The tissues were transferred into fresh DM and dispersed by gently trituration with a glass Pasteur pipette until some VSMCs appeared in the solution (Chootip et al., 2005; To-On et al., 2022).

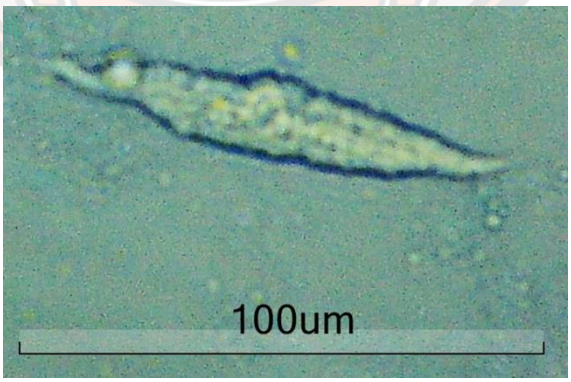


Figure 15 Smooth muscle cells
Source: Made by Teerapap Panklai (2023)

The effect of the WLE on cell viability was quantified (n=6). Cell counting with a hemocytometer using 0.3% trypan blue (GibcoTM, Thermo Scientific, USA)

and the cytotoxicity of the WLE was assessed by 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, VSMCs were seeded at 5 x 104 cells/well in a 96-well plate pre-coated with collagen type I, and were allowed to attach for 24 hr, then treated with WLE (0.1-1,000 μg/ml) dissolved in 0.1% DMSO as the vehicle. After treatment for 1 hr at 37°C, the cells were incubated with MTT solution (0.5 mg/mL) for 4 hr at 37°C. Subsequently, the solutions were removed and 100% DMSO was added and the cells were incubated on a shaker plate for 30 min in the dark. The absorbance was read at 595 nm by spectrophotometry. The viability of the VSMCs was calculated as a percentage of absorbance compared with the control. The cell viability of the untreated control cells was considered to be 100% (Wisutthathum, Kamkaew, et al., 2018).

Acute effect of WLE and its main compound on blood pressure and heart rate

To assess whether the direct *in vitro* vascular effect of WLE and compound **2** on resistance vessels translated into an *in vivo* effect, we studied the acute hypotensive effect of WLE and **-2** in anesthetized rats. Normotensive male Wistar rats were anesthetized with pentobarbital (Ceva Santé Animale, France) (60 mg/kg, i.p.) and supplemented as needed to maintain deep anesthesia. Left carotid artery was cannulated with polyethylene tube (0.279 mm i.d. × 0.609 mm o.d.) filled with heparinized (50 units/mL saline). The arterial catheter was connected to a pre-calibrated pressure transducer (model BP-100 Blood Pressure Transducer, iWorx Systems, Inc., Dover, NH, United States), the output of pressure was recorded by a bridge amplifier coupled to Powerlab® recording system, and an application program (Chart™ Ver.6 ADInstruments, castle Hill, NSW, Australia). After a 15-min stabilization period, systolic arterial blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were recorded before and during intravenous infusion (at 1 mL/min) of 1 mL/kg of saline, vehicle (a saline solution containing 5% DMSO), WLE and **2** (0.01, 0.025, 0.05, 0.1, 0.5, and 1 mg/kg), or the nifedipine (at the same doses). Each subsequent infusion was administered when baseline values were fully recovered and waited for 15 min before the next infusion (Wisutthathum, Demougeot, et al., 2018).

Statistical analysis

Data were expressed as means ± standard error of the mean (SEM) or expressed as means ± standard deviation (SD). The WLE and flavonoids-induced vasorelaxation were calculated as the percentage of contraction to PE (10^{-5} M). The concentration of WLE or flavonoids that induced 50% of the maximal relaxation (EC_{50}) and maximal relaxation induced (E_{max}) or the concentration of extracts or flavonoids that induced 50% of the maximal inhibition (IC_{50}) were determined by fitting the original concentration-response curves using Graph Pad Prism software (version 5.0). Concentration-response relationships were analyzed by using two-way ANOVA for repeated measure followed by Bonferroni's test. Comparison between two values was assessed by unpaired Student t test or Mann-Whitney test when data were not normally distributed. The multiple comparisons were analyzed using one-way ANOVA followed by Tukey's test. The p-value<0.05 was considered to be significantly different.

CHAPTER IV
RESULTS

Part I: Isolation and chemical characterization of phosphodiesterase 5 and arginase inhibitory activities from WLE and its flavonoid constituents

Phosphodiesterase 5 and arginase inhibition of plants in Nelumbonaceae and Nymphaeaceae families

Thirty-seven ethanolic extracts from different parts of six species in the family of Nelumbonaceae and Nymphaeaceae were screened for PDE5 inhibitory activity. Eight extracts showed %PDE5 inhibition of more than 80% at 50 µg/mL, i.e., *N. nucifera* (receptacle and pollen), *Nymphaea* sp. (pollen), *N. cyanea* Roxb. Ex G.Don (petals), *N. stellata* Willd. (petals), *N. pubescens* Willd. (petals and receptacle), and *V. amazonica* (petals) (**Table 5**). These extracts were considered as "active extracts" and were further tested for the concentrations that induced 50% of the maximal inhibition (IC_{50} values) (**Table 6**). The petals of *N. pubescens* extract had the highest IC_{50} and should be investigated for PDE5 inhibitors. This extract underwent fractionation to isolate and identify compounds that contribute to the observed inhibitory activity of PDE. Thirty-seven ethanolic extracts were additionally screened for their arginase inhibitory activity. The results revealed that at the concentration of 100 µg/mL, all extracts exhibited arginase inhibitory activity of less than 50%. (**Table 5**) This level of inhibition is deemed insufficient for envisaging a practical use as an arginase inhibitor.

Table 4 Percent yields of various parts of plants in the family of Nelumbonaceae and Nymphaeaceae were extracted using 95% Ethanol.

No.	Name of the plant	Part of the plant	Quantity (g)	Quantity of extract (g)	Yield (%)
1	*N. nucifera*	petals	20.99	0.71	3.38
		pollen	7.41	0.57	7.69
		seed	3.06	0.04	1.31
		receptacle	10.91	0.31	2.84
		peduncles	35.28	0.65	1.84
		petioles	17.38	2.32	13.35
		leaves	83.35	3.73	4.48
2	*Nymphaea sp*.	petals	2.27	0.62	27.17
		pollen	1.14	0.27	23.56
		receptacle	1.18	0.16	13.45
		peduncles	2.68	0.48	17.90
		petioles	5.41	0.91	16.82
		leaves	10.09	1.18	11.69

Table 4 Percent yields of various parts of plants in the family of Nelumbonaceae and Nymphaeaceae were extracted using 95% Ethanol. (cont.)

No.	Name of the plant	Part of the plant	Quantity (g)	Quantity of extract (g)	Yield (%)
3	*N. cyanea*	petals	8.74	2.16	24.71
		pollen	2.44	0.32	13.05
		receptacle	2.92	0.24	8.21
		peduncles	10.34	2.16	20.89
		petioles	10.43	1.54	14.77
		leaves	33.16	3.42	10.31
4	*N. stellata*	petals	0.31	0.09	30.06
		pollen	0.34	0.24	69.97
		receptacle	0.14	0.03	20.86
		peduncles	1.31	0.32	24.44
		petioles	3.44	0.39	11.34
		leaves	6.70	0.17	2.54
5	*N. pubescens*	petals	181.84	58.79	32.33
		pollen	150.14	21.52	14.33
		receptacle	200.49	10.91	5.44
		peduncles	150.00	14.46	9.64
		petioles	30.06	0.82	2.72
		leaves	12.19	3.28	26.93
6	*V. amazonica*	petals	2.08	0.66	31.73
		pollen	6.19	0.79	12.76
		receptacle	5.48	0.39	7.12
		peduncles	2.19	0.56	25.57
		petioles	13.39	1.52	11.35
		leaves	34.97	2.50	7.15

Table 5 Percentage of PDE5 and arginase inhibitions of ethanolic extracts from some plants in the family of Nelumbonaceae and Nymphaeaceae. The values are the means ± standard deviations (SD) from triplicate experiments.

No.	Samples	Part used	% PDE5 inhibitory activity at 50 µg/ml	% Arginase inhibitory activity at 100 µg/ml
1	*N. nucifera*	petals	75.03 ± 1.77	17.70 ± 0.84
		pollen	85.07 ± 6.08	21.39 ± 3.82
		seed	74.66 ± 4.39	22.25 ± 10.84
		receptacle	94.26 ± 0.74	45.30 ± 5.89
		peduncles	68.37 ± 0.50	19.98 ± 4.70
		petioles	56.71 ± 4.90	2.83 ± 5.08
		leaves	71.46 ± 0.54	21.84 ± 4.92
2	*Nymphaea sp.*	petals	78.28 ± 4.43	31.47 ± 7.71
		pollen	82.04 ± 2.06	36.91 ± 2.51
		receptacle	54.20 ± 3.26	22.07 ± 3.85
		peduncles	34.55 ± 4.37	15.62 ± 4.62

Table 5 Percentage of PDE5 and arginase inhibitions of ethanolic extracts from some plants in the family of Nelumbonaceae and Nymphaeaceae. The values are the means ± standard deviations (SD) from triplicate experiments. (cont.)

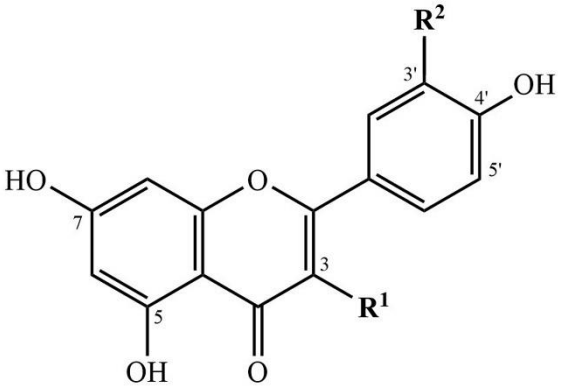
No.	Samples	Part used	% PDE5 inhibitory activity at 50 µg/ml	% Arginase inhibitory activity at 100 µg/ml
		petioles	43.54 ± 3.48	15.03 ± 2.77
		leaves	62.31 ± 4.35	30.19 ± 4.85
3	*N. cyanea*	petals	86.54 ± 1.63	31.28 ± 4.75
		pollen	73.16 ± 2.38	38.73 ± 2.17
		receptacle	62.61 ± 1.26	27.76 ± 3.66
		peduncles	34.40 ± 3.06	10.44 ± 9.32
		petioles	38.26 ± 9.82	12.09 ± 3.85
		leaves	55.53 ± 5.43	23.81 ± 3.87
4	*N. stellata*	petals	84.92 ± 1.86	28.45 ± 5.24
		pollen	38.05 ± 5.83	27.23 ± 14.29
		receptacle	46.07 ± 4.03	23.50 ± 6.07
		peduncles	13.36 ± 6.59	0
		petioles	29.17 ± 7.58	9.86 ± 4.59
		leaves	44.89 ± 7.26	21.13 ± 19.60
5	*N. pubescens*	petals	98.97 ± 0.26	39.49 ± 4.53
		pollen	74.86 ± 2.45	15.20 ± 1.87
		receptacle	82.69 ± 3.36	23.53 ± 3.57
		peduncles	58.90 ± 4.60	23.36 ± 2.52
		petioles	50.23 ± 7.61	11.96 ± 2.65
		leaves	73.26 ± 3.29	37.71 ± 3.23
6	*V. amazonica*	petals	86.67 ± 2.65	30.49 ± 3.22
		pollen	63.35 ± 5.45	27.61 ± 1.47
		receptacle	34.78 ± 6.41	13.30 ± 5.48
		peduncles	8.23 ± 3.92	16.87 ± 4.80
		petioles	9.16 ± 0.73	14.39 ± 5.57
		leaves	43.90 ± 3.78	21.40 ± 4.61

Table 6 IC_{50} values of ethanolic extracts from some plants in the family of Nelumbonaceae and Nymphaeaceae on PDE5 represented as the means ± SD from triplicate experiments.

No.	Samples	Part used	IC_{50} values (µg/ml)
1	*N. nucifera*	Receptacle	10.50 ± 3.65
		Pollen	14.15 ± 3.97
2	*Nymphaea sp.*	Pollen	25.61 ± 2.74
3	*N. cyanea*	Petals	8.07 ± 1.33
4	*N. stellata*	Petals	13.28 ± 0.25
5	*N. pubescens*	Petals	6.37 ± 0.65
		Receptacle	18.61 ± 4.03
6	*V. amazonica*	Petals	21.54 ± 4.23

Isolation and identification of PDE5 inhibitors from *N. pubescens* petals ethanolic extract

From **Table 6**, the ethanolic extract of *N. pubescens* petals showed the highest inhibitory activity on PDE5. The extract was further fractionated by solid-phase extraction (SPE) and preparative HPLC resulting in the finding of 6 flavonoids (**1**–**6**). Four compounds were isolated and identified as quercetin 3′-O-β-xylopyranoside (**1**) (Fossen et al., 1998), quercetin 3-methyl ether 3′-O-β-xylopyranoside (**2**) (Fossen et al., 1998), 3-O-methylquercetin (**4**) (Ganbold et al., 2019) and 3-O-methylkaempferol (**6**) (Csapi et al., 2010) by comparison with their spectroscopic data with the literature. In addition, the presence of quercetin (**3**) and kaempferol (**5**) in the extract was confirmed by comparison with the reference standards using HPLC analysis. The structures of these compounds and their IC_{50} values on PDE5 are shown in **Figure 16**. Sildenafil was used as a reference PDE5 inhibitor (IC_{50} = 1.36 ± 0.21 nM) (Bhandari et al., 2019).



	R¹	R²	PDE5 IC_{50} (μM)
1	OH	O-xyl	4.09 ± 0.76
2	OCH_3	O-xyl	5.63 ± 0.20
3	OH	OH	0.61 ± 0.11
4	OCH_3	OH	5.74 ± 2.11
5	OH	H	0.40 ± 0.09
6	OCH_3	H	4.41 ± 0.46
	Sildenafil		1.36 ± 0.21 nM

Figure 16 Structures of flavonoids isolated from *N. pubescens* petals and their IC_{50} values against PDE5 represented as the means ± SD from triplicate experiments. Sildenafil was used as a positive control in our assay.

The HPLC method for quantitative analysis of flavonoids in plant extracts

The HPLC method for the quantitative determination of **1**–**6** in the extracts of plants in the families Nelumbonaceae and Nymphaeaceae was developed and validated. HPLC chromatograms of **1**–**6** and the ethanolic extract of *N. pubescens* petals are shown in **Figure 17A, B**. The calibration data, LOD, and LOQ values are shown in **Table 7**. The %RSD was less than 3% for intra-day and inter-day precision, and the range of accuracy expressed as percentage recovery is between 85.13 and 109.92% (**Table 8**).

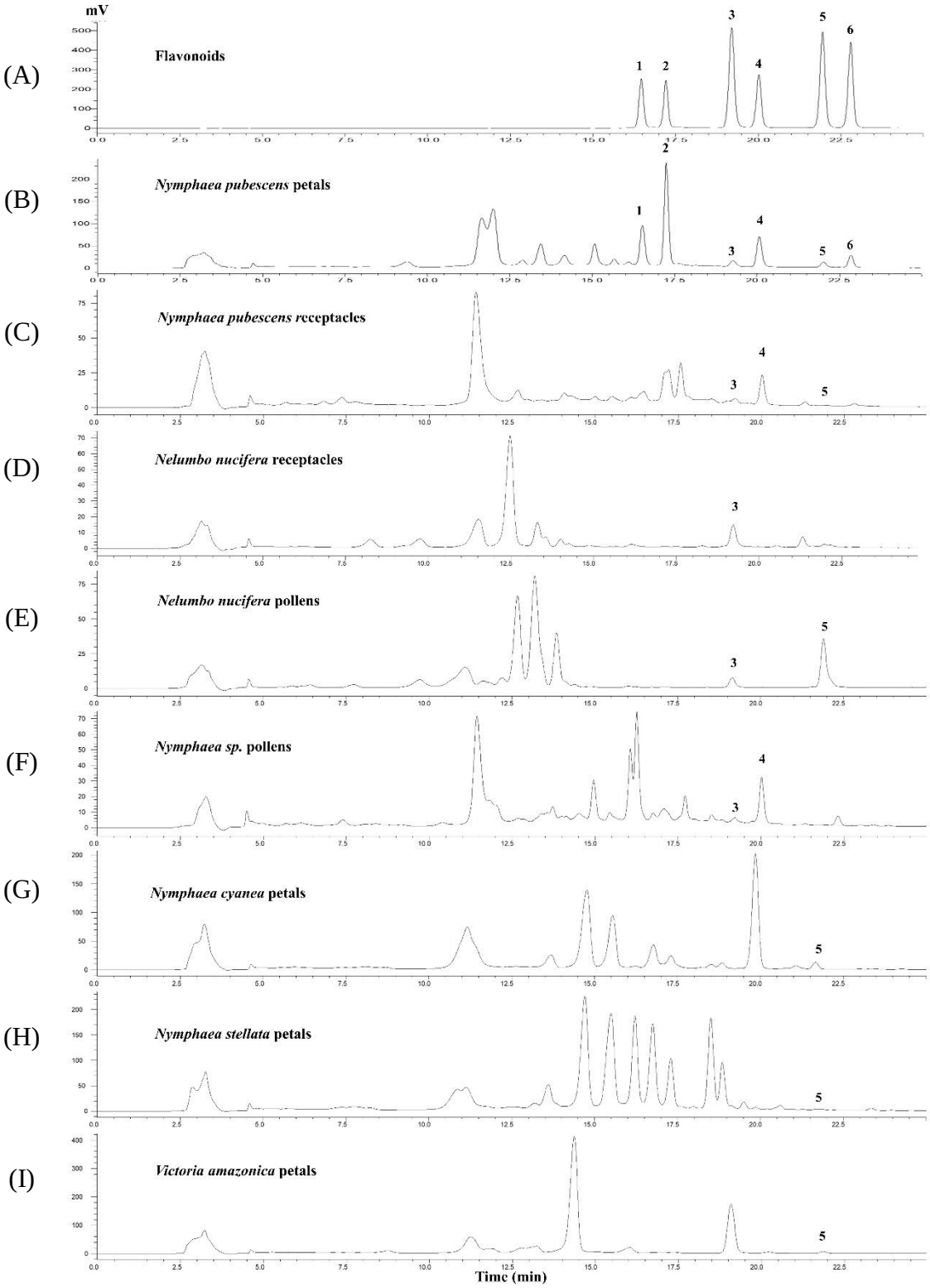


Figure 17 HPLC chromatograms of (A) mixtures of six flavonoids composed of 100 μg/mL (1) quercetin 3′-O-β-xylopyranoside, (2) quercetin 3-methyl ether 3′-O-β-xylopyranoside, (3) quercetin, (4) 3-O-methylquercetin, (5) kaempferol, and (6) 3-O-methylkaempferol, and 5 mg/mL of crude ethanolic extracts of *N. pubescens* petals (B), *N. pubescens* receptacles (C), *N. nucifera* receptacles (D), *N. nucifera* pollens (E), *Nymphaea* sp. pollens (F), *N. cyanea* petals (G), *N. stellata* petals (H) and *V. amazonica* petals (I).

Table 7 Calibration data, LOD, and LOQ of flavonoids analyzed by HPLC.

Flavonoids	Linearity range (μg/ml)	Regression equation	Correlation coefficient (r^2)	LOD (μg/ml)	LOQ (μg/ml)
1	0.5 - 100	y = 23100x - 4376.5	0.9999	0.09	0.5
2	5 - 400	y = 20691x + 69562	0.9997	0.85	5.0
3	0.5 - 100	y = 64186x - 48477	0.9997	0.15	0.5
4	0.5 - 100	y = 27499x - 5199	0.9999	0.05	0.5
5	0.5 - 100	y = 65813x - 10389	0.9999	0.09	0.5
6	0.5 - 100	y = 44580x + 4643.1	0.9999	0.04	0.5

Contents of 6 flavonoids in some plant members of Nelumbonaceae and Nymphaeaceae

HPLC analysis was applied for the detection and quantification of **1**–**6** in eight extracts (5 mg/mL) obtained from plants belonging to Nelumbonaceae and Nymphaeaceae families, which exhibited high to moderate PDE5 inhibitory activity (**Table 9**). A comparison of the six flavonoid constituents found in *N. pubescens* petals with those in other extracts, revealed that compounds **3**, **4** and **5** were also present in the latters whereas **1**, **2**, and **6** were exclusively identified in *N. pubescens* petal extract. The HPLC chromatograms of these compounds are shown in **Figure 17B–I**.

Table 8 Intra- and Inter-day precision and accuracy of flavonoids analyzed by HPLC.

Flavonoids	Concentration levels (µg/ml)	Intra-day (n=3)		Inter-day (n=9)		Accuracy (n=3)	
		Measured concentration (µg/ml) ± SD	Precision (%RSD)	Measured concentration (µg/ml) ± SD	Precision (%RSD)	Recovery (%) Average ± SD	Precision (%RSD)
1	3	2.71 ± 0.00	0.13	2.74 ± 0.05	1.83	90.04 ± 1.87	2.08
	25	23.63 ± 0.56	2.37	25.04 ± 0.73	3.00	86.69 ± 1.47	1.69
	65	62.94 ± 1.01	1.60	64.09 ± 1.04	1.63	90.05 ± 2.54	2.82
2	15	13.08 ± 0.24	1.86	13.01 ± 0.28	2.12	85.13 ± 1.53	1.79
	75	75.90 ± 1.23	1.62	77.15 ± 1.98	2.57	94.82 ± 0.73	0.77
	300	309.05 ± 1.41	0.46	311.04 ± 8.65	2.78	100.97 ± 2.44	2.42
3	3	2.94 ± 0.06	2.06	2.98 ± 0.09	2.85	106.17 ± 1.28	1.20
	25	27.01 ± 0.24	0.89	27.09 ± 0.61	2.25	98.46 ± 1.49	1.51
	65	66.27 ± 1.15	1.74	66.45 ± 1.64	2.47	109.92 ± 0.91	0.82
4	3	2.83 ± 0.07	2.61	2.87 ± 0.06	2.17	86.21 ± 2.40	2.78
	25	23.96 ± 0.56	2.33	24.48 ± 0.60	2.43	86.14 ± 2.06	2.40
	65	63.38 ± 1.26	1.99	64.74 ± 1.57	2.42	90.23 ± 0.57	0.63
5	3	2.58 ± 0.04	1.63	2.65 ± 0.06	2.18	103.43 ± 0.72	0.69
	25	25.47 ± 0.41	1.62	25.50 ± 0.63	2.46	100.67 ± 0.65	0.64
	65	64.63 ± 1.90	2.94	63.16 ± 1.64	2.49	105.74 ± 1.13	1.07
6	3	2.69 ± 0.02	0.90	2.70 ± 0.03	1.28	89.43 ± 1.27	1.42
	25	23.76 ± 0.49	2.06	24.26 ± 0.55	2.26	96.79 ± 2.19	2.27
	65	63.06 ± 1.12	1.77	64.24 ± 1.56	2.43	92.69 ± 0.83	0.89

Table 9 The flavonoid contents in some plants in the family of Nelumbonaceae and Nymphaeaceae determined by the HPLC method (*n* = 3).

Sample	Contents of the Flavonoid Constituents (Mean ± SD) (mg/g Ethanolic Extract)					
	1	**2**	**3**	**4**	**5**	**6**
N. nucifera						
Receptacle			4.55 ± 0.25			
Pollen			2.55 ± 0.18		7.34 ± 0.35	
Nymphaea sp.						
Pollen			3.09 ± 0.13	16.67 ± 056		
N. cyanea						
Petals					2.71 ± 0.18	
N. stellata						
Petals					0.82 ± 0.01	
N. pubescens						
Petals	7.10 ± 0.10	17.31 ± 0.05	0.61 ± 0.00	4.72 ± 0.08	0.40 ± 0.00	1.04 ± 0.01
Receptacle			6.38 ± 035	8.32 ± 0.67	1.40 ± 0.19	
V. amazonica						
Petals					1.77 ± 0.16	

Part II: Cytotoxicity and effect of WLE and its flavonoid constituents on pulmonary artery and aorta

The vasorelaxant effect of WLE and flavonoids

EC_{50} and E_{max} of the WLE and flavonoids-induced relaxation in endothelium-intact and -denuded rat PA or aorta in the absence or presence of various inhibitors are presented in **Table 10**. WLE exhibited a more potent relaxation effect in the PA than the aorta, as indicated by the EC_{50} of 4.96±0.81 µg/ml in the PA and 27.50±7.61 µg/ml in the aorta ($p<0.001$) (**Figure 18A**). However, the E_{max} values in the endothelium-intact PA (E+) and aorta were not different i.e., 99.10±0.45% and 98.82±0.77%, respectively. Removal of the endothelium decreased the relaxant effect of WLE in both PA (**Figure 18B**) and aorta (**Figure 18C**). Flavonoid compounds **2**, **3**, and **5** also relaxed aorta (**Figure 18D, F, H**) and the PA (**Figure 18E, G, I**). In PA, only compounds **2** and **3** showed endothelium-dependent vasorelaxant effect, while in aorta all compounds produced endothelium-dependent effect. Both endothelium-intact and -denuded showed significantly different relaxation in PA rings when comparing **2** with **3** (**Figure 19A, 19B**) and 1 with 3 (**Figures 19C, 19D**), respectively. While, both endothelium-intact and -denuded showed significantly different relaxation in aortic rings when comparing **2** with **3** (**Figure 20A, 20B**). The relaxation between the PA and aortic rings was evaluated with compounds **2**, **3**, and **5** (**Figure 21**), but no statistically significant difference was found. The EC_{50} values of all compounds were not significantly different also. The solvent DMSO had no effects on the vessels.

Role of endothelium-dependent pathways in WLE-induced PA relaxation

The inhibition of eNOS by L-NAME reduced the relaxation induced by WLE **(Figure 22A)** ($p<0.01$), while the inhibition of COX by indomethacin **(Figure 22B)** or the inhibition of EDHF by apamin+charybdotoxin **(Figure 22C)** showed no effect in (E+) PA rings **(Table 10)**.

Table 10 EC_{50} and E_{max} of WLE and flavonoids-induced relaxation in endothelium-intact (E+) and -denuded (E-) rat pulmonary artery (PA) or aorta in the absence or presence of various inhibitors.

	WLE		
	EC_{50} (μg/ml)	**E_{max} (%)**	**n**
Endothelium-intact PA (E+)			
Vehicle	-	9.45 ± 1.91	10
Extract	4.96 ± 0.81	99.10 ± 0.45	12
+ L-NAME	318.63 ± 15.70***	52.78 ± 6.78***	10
+ Indomethacin	1.92 ± 0.23	96.95 ± 1.75	9
+ Apamin + Charybdotoxin	5.22 ± 1.10	97.10 ± 1.53	10
Endothelium-denuded PA (E-)			
Vehicle	-	7.51 ± 2.28	10
Extract	310.18 ± 17.38***	72.00 ± 4.59**	13
+ 4-AP	309.92 ± 17.84	64.86 ± 5.56	12
+ Glibenclamide	323.13 ± 26.18	67.61 ± 6.54	10
+ Iberiotoxin	295.36 ± 27.63	75.55 ± 6.95	5
+ $BaCl_2$	303.10 ± 34.35	77.83 ± 8.78	5
+ ODQ	356.48 ± 12.73	64.30 ± 5.20	12
Endothelium-intact Aorta (E+)			
Vehicle	-	26.96 ± 2.72	6
Extract	27.50 ± 7.61	98.82 ± 0.77	12
Endothelium-denuded Aorta (E-)			
Vehicle	-	12.59 ± 1.60	5
Extract	303.86 ± 20.46	63.85 ± 8.26	8
	Flavonoids		
	EC_{50} (μM)	**E_{max} (%)**	**n**
Endothelium-intact PA (E+)			
Vehicle	-	9.86 ± 3.91	5
2	28.98 ± 4.14	70.28 ± 9.29	6
3	26.35 ± 4.21	96.83 ± 2.90	6
5	30.97 ± 4.06	98.35 ± 0.83	8
Endothelium-denuded PA (E-)			
Vehicle	-	14.76 ± 4.63	5
2	47.18 ± 11.09	53.41 ± 15.82	6
3	40.41 ± 0.27	100.00 ± 0.00	6
5	32.50 ± 3.78	95.15 ± 1.77	7
Endothelium-intact Aorta (E+)			
Vehicle	-	16.36 ± 1.97	5
2	23.43 ± 5.44	71.03 ± 6.78	6
3	39.71 ± 1.79	97.37 ± 2.40	6
5	42.86 ± 6.89	81.16 ± 6.86	6
Endothelium-denuded Aorta (E-)			
Vehicle	-	11.35 ± 2.72	5
2	> 100	28.77 ± 2.62	6
3	> 100	50.36 ± 7.24	6
5	> 100	33.00 ± 3.70	7

Values are means ± SEM of the number n of animals. EC_{50} is the concentration of WLE or flavonoids giving half-maximal relaxation. E_{max} is the maximum response of PA expressed as a relaxation percentage of the contraction induced by PE. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Extract (E+).

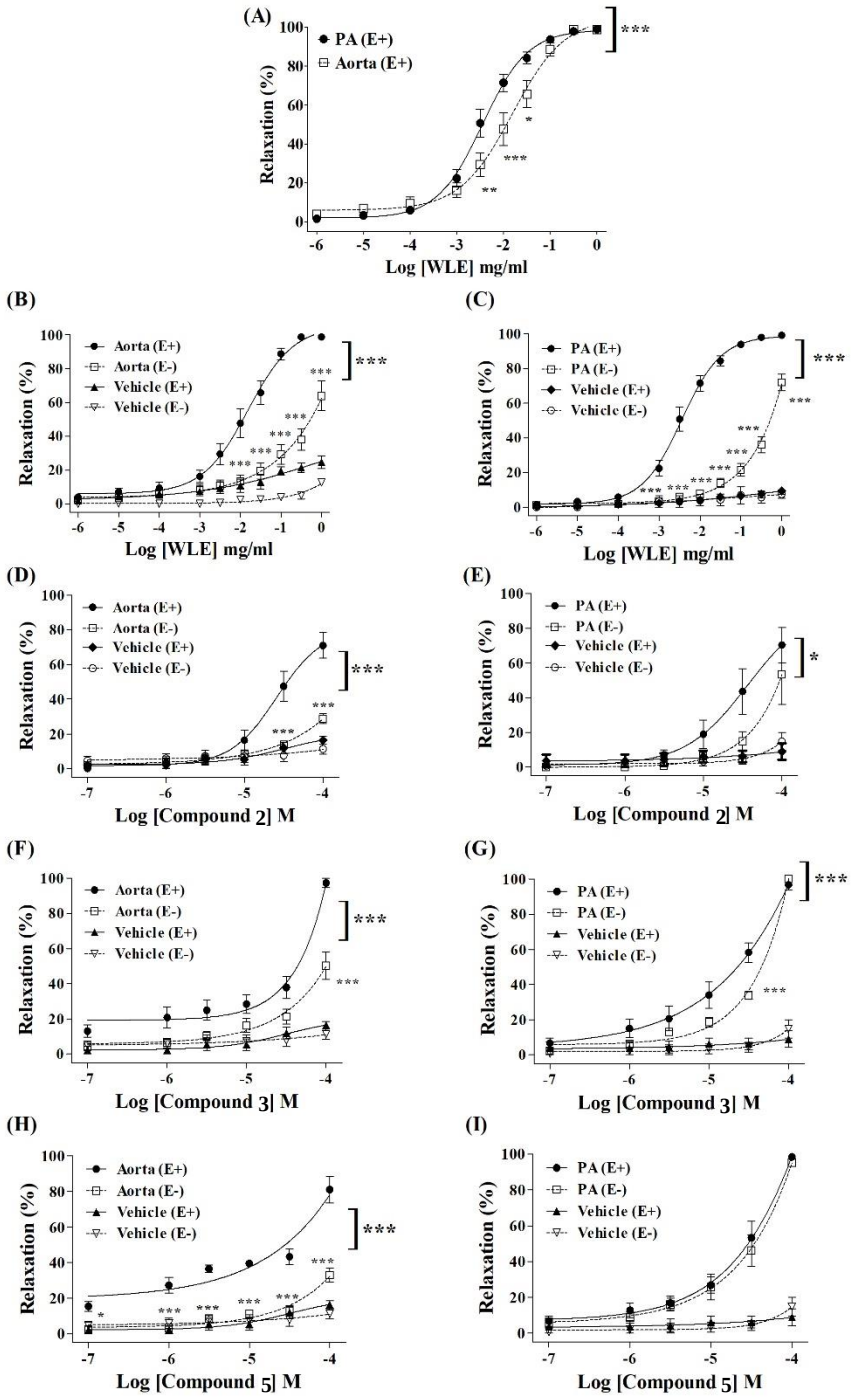


Figure 18 Relaxation of rat pulmonary artery (PA) and aortic rings pre-contracted with PE (10^{-5} M) and treated with accumulating concentrations of WLE (A, B, C) **or quercetin 3-methyl ether 3′-O-β-xylopyranoside (**2) (D, E)**, quercetin (**3) (F, G)**, and kaempferol (**5) (H, I)**. Relaxation to WLE on endothelium-intact (E+) PA and aortic rings** (A)**. Relaxation to WLE on endothelium-intact (E+) and -denuded (E-) aortic rings** (B) **and PA rings** (C)**. Relaxation to** 2 **on (E+) and (E-) aortic rings** (D) **and PA rings** (E)**. Relaxation to** 3 **on (E+) and (E-) aortic rings** (F) **and PA rings** (G)**. Relaxation to** 5 **on (E+) and (E-) aortic rings** (H) **and PA rings** (I)**. Relaxations are expressed as % contractions induced by PE. All values are means ± SEM (n=5-13),** $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

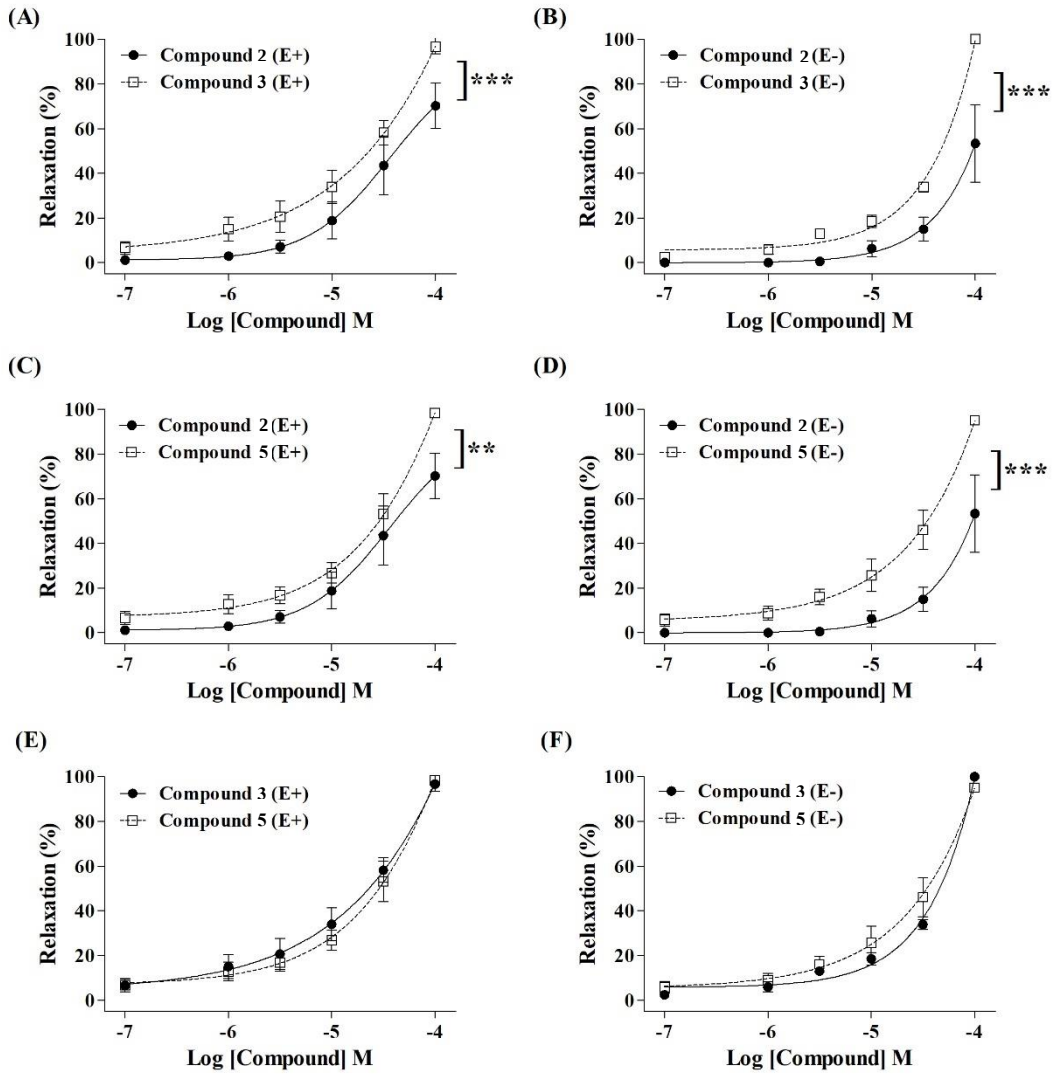


Figure 19 Relaxation of rat pulmonary artery (PA) rings pre-contracted with PE (10^{-5} M) and treated with accumulating concentrations of quercetin 3-methyl ether 3′-O-β-xylopyranoside (2), quercetin (3), and kaempferol (5). Relaxation compared between (2) and (3) on endothelium-intact (E+) (A) and -denuded (E-) (B). Relaxation compared between (2) and (5) on endothelium-intact (E+) (C) -denuded (E-) (D). Relaxation compared between (3) and (5) on endothelium-intact (E+) (E) -denuded (E-) (F). Relaxations are expressed as % contractions induced by PE. All values are means ± SEM (n=5-7), **p<0.01, ***p<0.001.

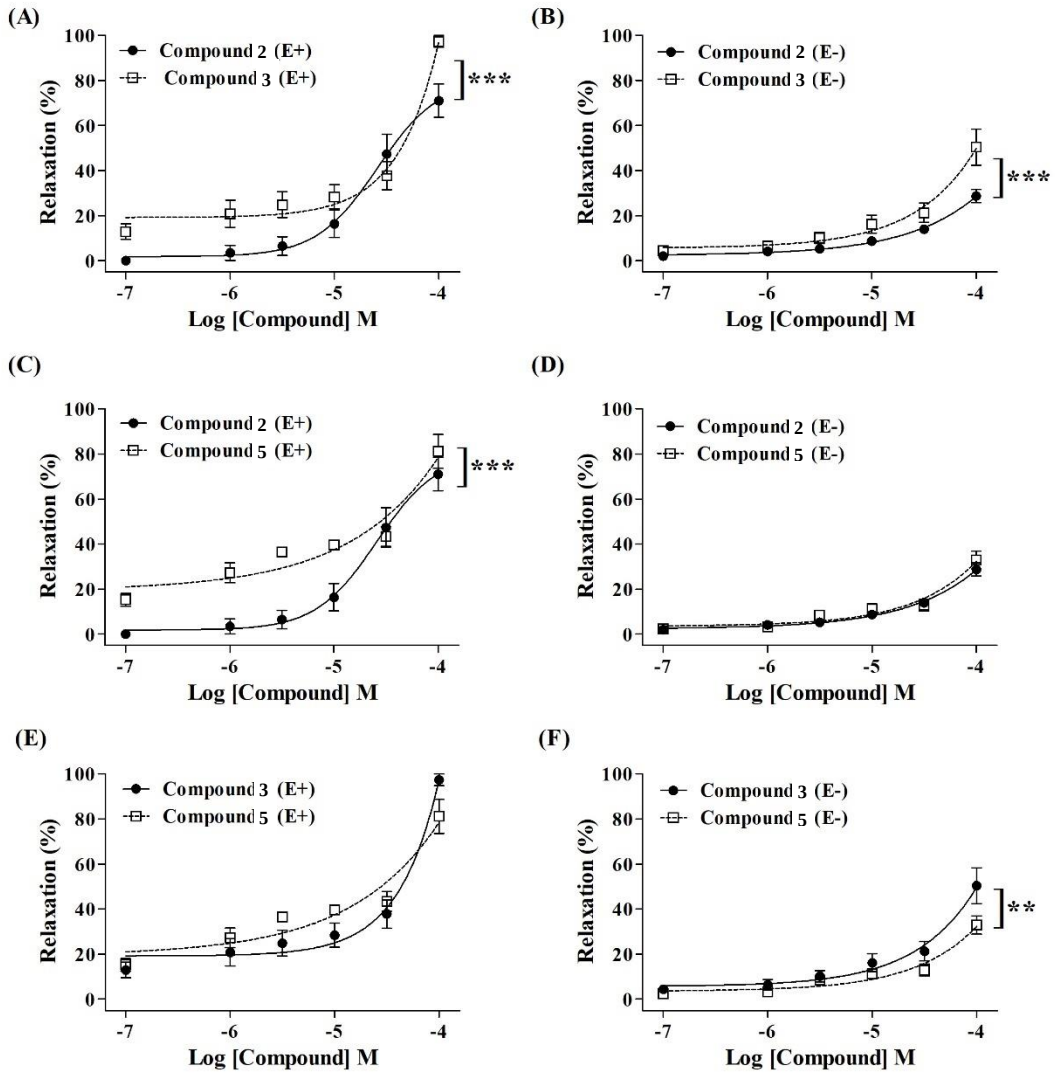


Figure 20 Relaxation of rat aortic rings pre-contracted with PE (10^{-5} M) and treated with accumulating concentrations of quercetin 3-methyl ether 3′-O-β-xylopyranoside (2), quercetin (3), and kaempferol (5). Relaxation compared between (2) and (3) on endothelium-intact (E+) (A) and -denuded (E-) (B). Relaxation compared between (2) and (5) on endothelium-intact (E+) (C) - denuded (E-) (D). Relaxation compared between (3) and (5) on endothelium-intact (E+) (E) - denuded (E-) (F). Relaxations are expressed as % contractions induced by PE. All values are means ± SEM (n=5-7), **p<0.01, ***p<0.001.

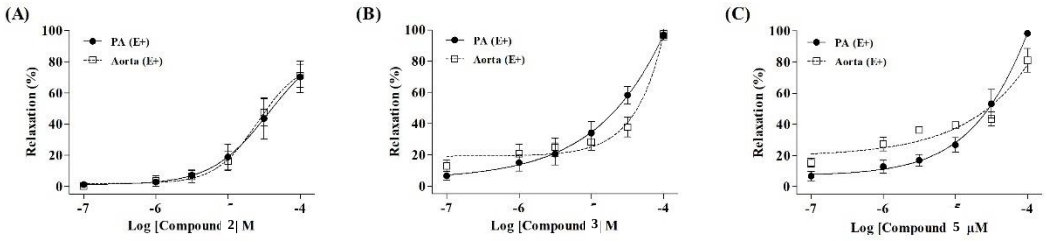


Figure 21 Relaxation of rat pulmonary artery (PA) and aortic rings pre-contracted with PE (10^{-5} M) and treated with accumulating concentrations of quercetin 3-methyl ether 3′-O-β-xylopyranoside (2), quercetin (3), and kaempferol (5). Relaxation to (2) on endothelium-intact (E+) PA and aortic rings (A). Relaxation to (3) on endothelium-intact (E+) PA and aortic rings (B). Relaxation to (5) on endothelium-intact (E+) PA and aortic rings (C). Relaxations are expressed as % contractions induced by PE. All values are means ± SEM (n=5-7).

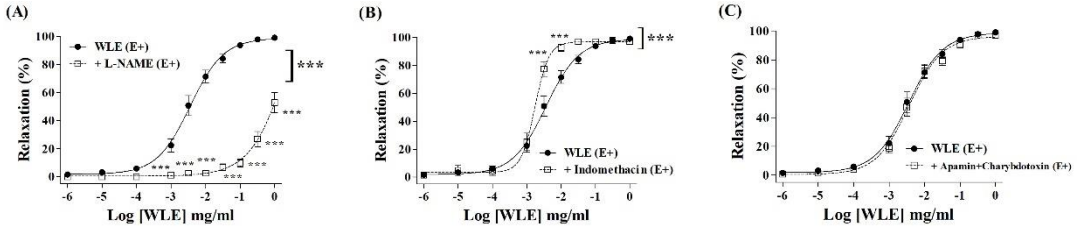


Figure 22 WLE-induced relaxations of endothelium-intact (E+) PA rings pre-contracted with PE and pretreated with inhibitors of various endothelial signaling pathways including 10^{-4} M L-NAME (A), 10^{-5} M indomethacin (B), or 10^{-7} M apamin plus 10^{-7} M charybdotoxin (C). All values are means ± SEM (n = 9–12), $^{**}p<0.01$, $^{***}p<0.001$.

Role of vascular smooth muscle K^+ channels in WLE-induced PA relaxation

The K^+-channel blockers, 4-AP (K_V) **(Figure 23A)**, glibenclamide (K_{ATP}) **(Figure 23B)**, iberiotoxin (K_{Ca}) **(Figure 23C)** or $BaCl_2$ (K_{IR}) **(Figure 23D)** did not reduce the relaxation in (E-) PA rings **(Table 10)**.

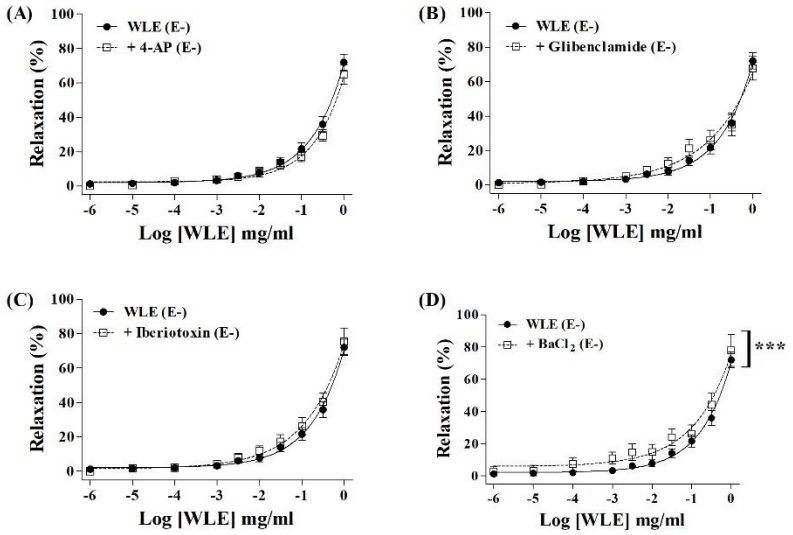


Figure 23 WLE-induced relaxations of endothelium-denuded (E-) PA rings pre-contracted with PE (10^{-5} M) and pretreated with various K^+ channel inhibitors including 10^{-3} M 4-AP (A)**, 10^{-5} M glibenclamide** (B)**, 10^{-7} M iberiotoxin** (C) **or 30 x 10^{-9} M $BaCl_2$** (D)**. All values are means ± SEM (n = 5-13).**

Role of sGC/cGMP pathway in WLE-induced PA relaxation

The relaxation induced by SNP in the presence of 5 µg/ml of WLE (EC_{50} = 1.24±0.66 nM, E_{max} = 93.86±2.55%) was increased compared with vehicle (EC_{50} = 10.87±6.75 nM, E_{max} = 82.97±5.44%) ($p<0.05$, **Figure 24A**), suggesting that the WLE could possibly induce vasorelaxation through sGC and/or cGMP activation. Thus, to determine whether WLE directly activates sGC, the effect of ODQ (a sGC inhibitor) was investigate and the results showed that PA relaxation induced by the WLE was reduced by ODQ ($p<0.05$) (**Figure 24B**).

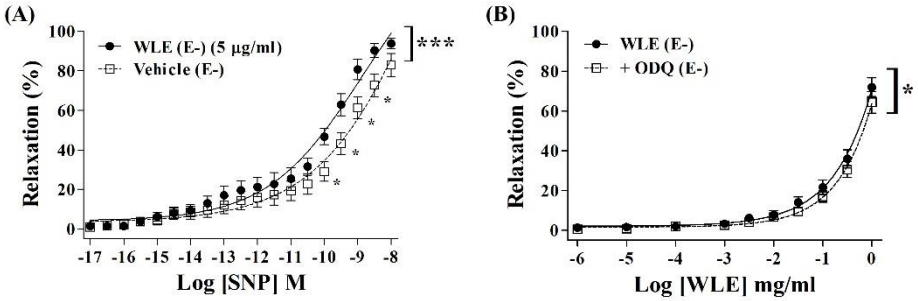


Figure 24 Effect of WLE on sGC/cGMP-induced relaxation. Endothelium-denuded (E−) PA rings pre-incubated with WLE or vehicle and pre-contracted with PE (10^{-5} M) (A) **followed by relaxations with cumulative concentrations of SNP, a nitric oxide donor. WLE** (B) **-induced relaxation of denuded (E−) PA rings pre-contracted with PE (10^{-5} M) and pretreated with 10^{-5} M ODQ, sGC inhibitor. All values are means ± SEM (n = 5-10), *p< 0.05.**

Role of Ca^{2+} influx and Ca^{2+} release in WLE-induced PA relaxation

WLE at EC_{50} (5 μg/ml) did not inhibit extracellular $CaCl_2$-induced PA vasoconstriction via neither ROCCs (**Figure 25A**) nor VOCCs (**Figure 25B**).

Regarding the effect of the WLE on intracellular Ca^{2+} release induced by PE leading to vasoconstriction, WLE at EC_{50} (5 μg/ml) showed no effect on the vasoconstriction evoked by PE when compared to the vehicle (**Figure 25C**).

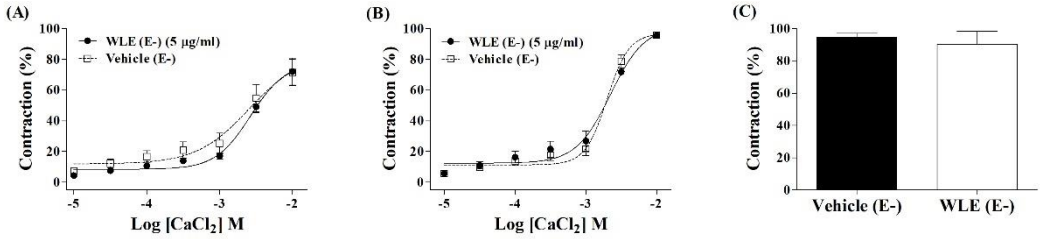


Figure 25 Effect of WLE on extracellular Ca^{2+} induced vasoconstriction, endothelium-denuded (E-) PA rings were incubated in Ca^{2+}-free Krebs solution, then pre-incubated with vehicle or WLE plus either PE (10^{-5} M) (A) **or Ca^{2+}-free 80 mM K^+ solution** (B)**, followed by the addition of cumulative concentration of $CaCl_2$. Effect of WLE on intracellular Ca^{2+} induced vasoconstriction, endothelium-denuded (E-) PA rings pre-contracted with 80 mM K^+ solution and washed with Ca^{2+}-free Krebs' solution containing 1 mM EGTA and then added the vehicle or the WLE. The PA rings were contracted with PE (10^{-5} M)** (C)**. The percentage of PE-induced contractions was compared in the presence or absence of the vehicle or the WLE. All values means ± SEM (n = 5).**

Inhibitory effect of WLE on PE-induced vasoconstriction

PE-induced vasoconstriction in the (E-) PA rings was unaffected by WLE-preincubation. **(Figure 26).**

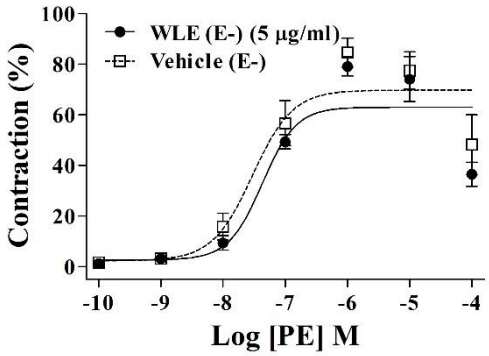


Figure 26 Concentration-response curves for PE-induced contraction (10^{-10}-10^{-4} M) on endothelium-denuded (E−) PA rings pre-incubated with WLE or vehicle. All values are means ± SEM (n = 7-8).

Cytotoxicity of WLE on VSMCs

Incubation of the WLE for 1 hr did not affect cell viability of aorta **(Figure 27A)** and PA **(Figure 27B)** VSMCs.

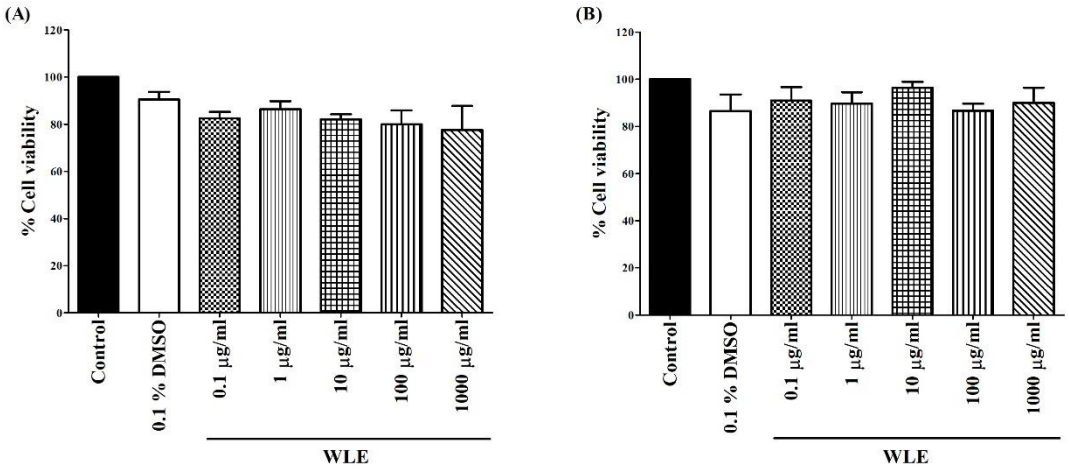


Figure 27 Effect of WLE (0.1-1,000 µg/ml) on cell viability of rat isolated aortic (A) **and pulmonary arterial** (B) **smooth muscle cells using MTT assay. All values are means ± SEM (n = 6).**

Part III: Effect of WLE and its main active compound (Compound 2; Quercetin 3-methyl ether 3′-O-β-xylopyranoside) on mesenteric artery

WLE and its main compound induced vasorelaxant effect relying on both endothelium-dependent and endothelium-independent mechanisms.

As compared to vehicle, WLE (E_{max}= 97.90 ± 1.17 %, EC_{50}= 0.08±0.01 mg/mL) and compound **2** (E_{max}= 64.14 ± 5.08 %, EC_{50}= 42.76±6.32 µM) induced a dose-dependent vasorelaxation in (E+) MA rings (**Figure 28, Table 11**). Removal of the endothelium significantly decreased the relaxant effect in both WLE and compound **2** as confirmed by the increase in the EC_{50} values to 0.25±0.03 mg/mL and 91.42±18.45 µM, respectively ($p<0.001$).

Table 11 EC_{50} and E_{max} of WLE or quercetin 3-methyl ether 3′-O-β-xylopyranoside (2)**-induced relaxation in endothelium-intact (E+) and -denuded (E-) mesenteric rings in the absence or presence of various inhibitors.**

	WLE		
	EC_{50} (mg/ml)	**E_{max} (%)**	**n**
Endothelium-intact (E+)			
Vehicle	-	31.66 ± 2.08	5
Extract	0.08 ± 0.01	97.90 ± 1.17	11
+ L-NAME	0.18 ± 0.02	95.85 ± 1.08	7
+ Indomethacin	0.04 ± 0.01	95.49 ± 1.03	7
+ Apamin + Charybdotoxin	0.03 ± 0.01	97.69 ± 0.36	7
Endothelium-denuded (E-)			
Vehicle	-	17.17 ± 1.27	5
Extract	0.25 ± 0.03***	96.43 ± 0.73	11
+ 4-AP	0.06 ± 0.02††	98.09 ± 0.66	7
+ Glibenclamide	0.14 ± 0.04	98.00 ±0.56	7
+ Iberiotoxin	0.26 ± 0.05	95.01 ± 0.71	7
+ ODQ	0.14 ± 0.03	89.45 ± 4.60	7
	Quercetin 3-methyl ether 3′-O-β-xylopyranoside (2)		
	EC_{50} (µM)	**E_{max} (%)**	**n**
Endothelium-intact (E+)			
Vehicle	-	17.10 ± 1.17	5
2	42.76 ± 6.32	64.14 ± 5.08	10
+ L-NAME	> 100	43.16 ± 7.71	7
+ Indomethacin	38.67 ± 8.35	78.41 ± 7.14	7
+ Apamin + Charybdotoxin	4.42 ± 0.78**	79.56 ± 3.59	7
Endothelium-denuded (E-)			
Vehicle	-	8.58 ± 2.29	5
2	> 100	27.71 ± 2.66***	10
+ 4-AP	> 100	36.40 ± 7.80	6
+ Glibenclamide	> 100	40.04 ± 7.67	6
+ Iberiotoxin	> 100	19.08 ± 2.34	6
+ ODQ	> 100	40.19 ± 7.95	7

Values are means ± SEM of the number n of animals. EC_{50} is the concentration of WLE or **2** giving half-maximal relaxation. E_{max} is the maximum response of MA expressed as a relaxation percentage of the contraction induced by PE. $^{**}p< 0.01$, $^{***}p< 0.001$ vs. Extract (E+) or **2** (E+). $^{\dagger\dagger}p< 0.01$ vs. Extract (E-)

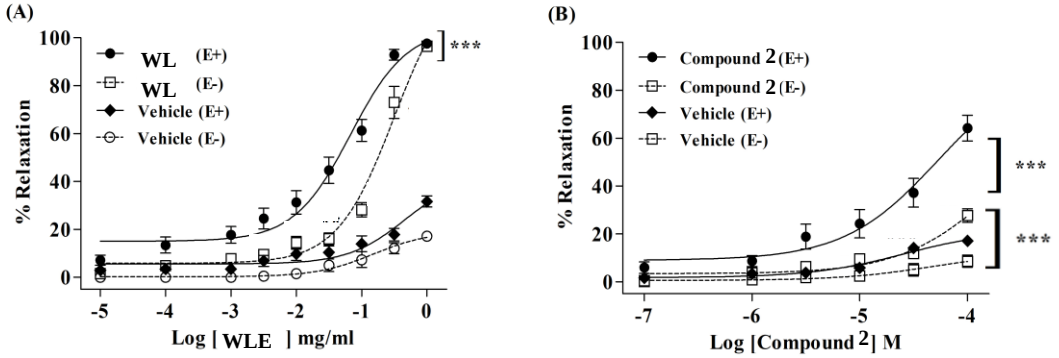


Figure 28 Relaxation of rat mesenteric artery (MA) rings pre-contracted with PE (10^{-5} M) and treated with accumulating concentrations of WLE (A) or quercetin 3-methyl ether 3′-O-β-xylopyranoside (2) (B) in endothelium-intact (E+) and -denuded (E-). Relaxations are expressed as % contractions induced by PE. All values are means ± SEM (n=5-11), $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Endothelium-dependent vasodilation of WLE and compound 2 is mainly NOS-dependent

L-NAME significantly reduced the relaxation induced by WLE (**Figure 29A**) and by compound **2** (**Figure 29D**) ($p<0.001$), whereas indomethacin had no effect (**Figure 29B and E**). Incubation of mesenteric arteries with apamin plus charybdotoxin did not modify the WLE-induced relaxation (**Figure 29C**), while it enhanced the compound **2**-induced relaxation (**Figure 29F**) ($p<0.001$).

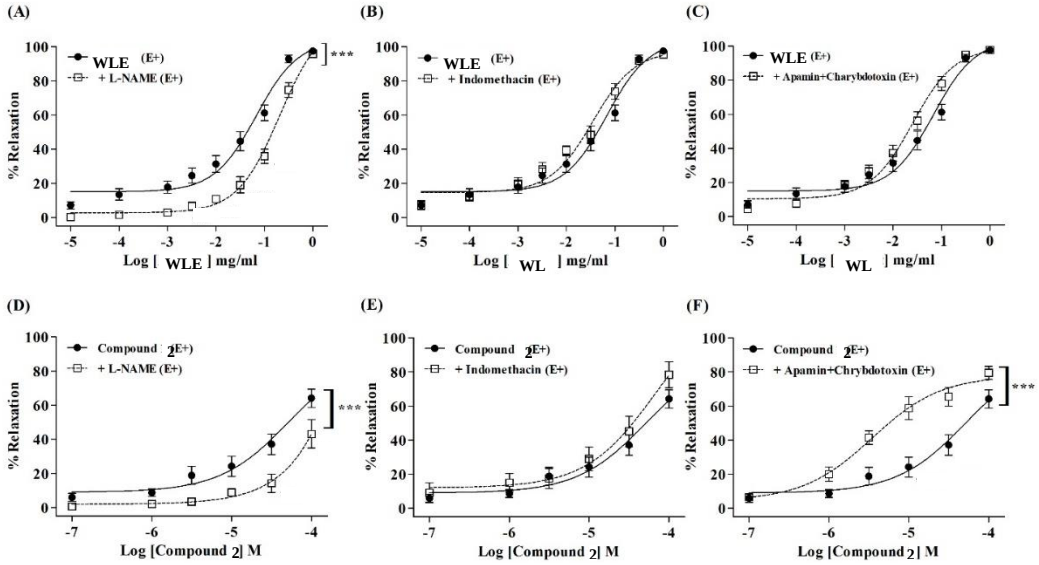


Figure 29 Relaxations of WLE or quercetin 3-methyl ether 3′-O-β-xylopyranoside (2) on endothelium-intact (E+) MA rings pre-contracted with PE and pretreated with inhibitors of various endothelial signaling pathways including 10^{-4} M L-NAME (A, D), 10^{-5} M indomethacin (B, E), or 10^{-7} M apamin plus 10^{-7} M charybdotoxin (C, F). All values are means ± SEM (n = 7-11), $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

WLE and its main compound increased cGMP pathway

As endothelial NO production is strongly involved in WLE and compound-**2**-induced relaxations, we further explore if WLE or compound **2** might modulate signaling pathways downstream of NO in smooth muscle cells (VSMCs). As shown in **Figure 30A and C**, the NO donor SNP-induced relaxation was significantly enhanced in the presence of WLE and compound **2** ($p<0.001$). This data indicated that WLE and compound **2** induced vasorelaxation through either direct sGC activation and/or downstream modulation of cGMP concentration. To confirm if a direct activation of sGC might be involved, the effect of ODQ was investigated. We found that ODQ did not modify the WLE nor compound **2**-induced relaxations (**Figure 30B and D**).

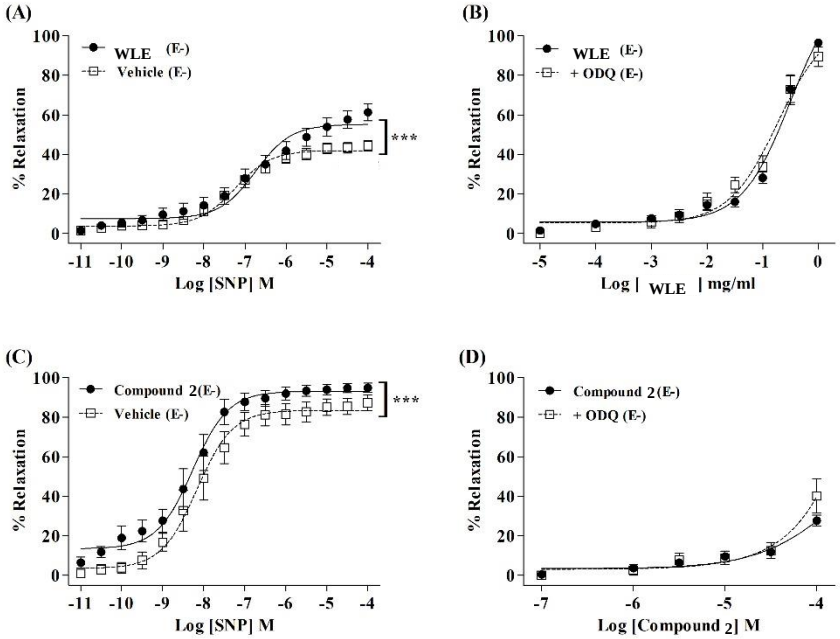


Figure 30 Effect of WLE or quercetin 3-methyl ether 3′-O-β-xylopyranoside (2) on sGC/cGMP-induced relaxation. Endothelium-denuded (E-) MA rings pre-incubated with WLE or 2 or vehicle and pre-contracted with 100 mM KCl solution (A) or PE (10^{-5} M) (C) followed by relaxations with cumulative concentrations of SNP (10^{-11}-10^{-4}). WLE (B) or 2 (D)-induced relaxation of endothelium-denuded (E-) MA rings pre-contracted with PE (10^{-5} M) and pretreated with ODQ (10^{-5} M). All values are means ± SEM (n = 7-11), $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Endothelium-independent effect was not reduced by K^+ channel blockers

As shown in **Figure 31**, none of the K^+ channel blockers were able to reduce the relaxation induced by the WLE or by compound **2**. On the contrary, WLE-induced relaxation was significantly enhanced in the presence of 4-AP and glibenclamide (**Figure 31A and B**).

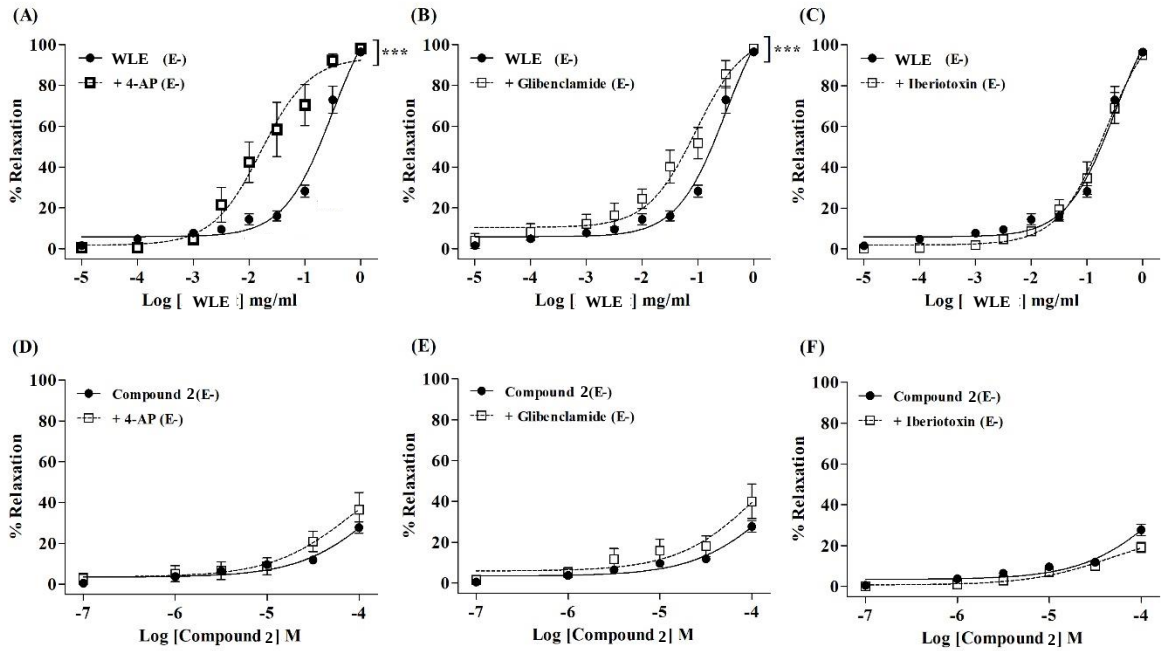


Figure 31 Relaxations of WLE or quercetin 3-methyl ether 3′-O-β-xylopyranoside (2) on endothelium-denuded (E-) MA rings pre-contracted with PE (10^{-5} M) and pretreated with various K^+ channel inhibitors including 10^{-3} M 4-AP (A, D), 10^{-5} M glibenclamide (B, E), or 10^{-7} M iberiotoxin (C, F). All values are means ± SEM (n = 6-10), $^{*}p<0.05$, $^{***}p<0.001$.

The vasorelaxant effect of WLE and compound 2 relied on receptor-operated Ca^{2+} channels inhibition.

The contributions of extracellular or intracellular Ca^{2+} fluxes were presented in **Figure 32**. As compared to vehicle, WLE and compound **2** slightly but significantly reduced the contraction elicited by extracellular Ca^{2+} influx in PE-exposed rings (opening of receptor-operated Ca^{2+} channels: ROCCs, $p<0.05$, **Figure 32A and D**) whereas they did change neither the extracellular Ca^{2+} influx in high KCl-exposed rings (opening of voltage-operated Ca^{2+} channel: VOCC) nor the intracellular Ca^{2+} release from SR.

WLE blocked α_1 receptor

As shown in **Figure 32G**, WLE inhibited PE-induced contraction suggesting an α_1 receptor antagonism activity ($p<0.001$) whereas compound **2** had no effect (**Figure 32H**).

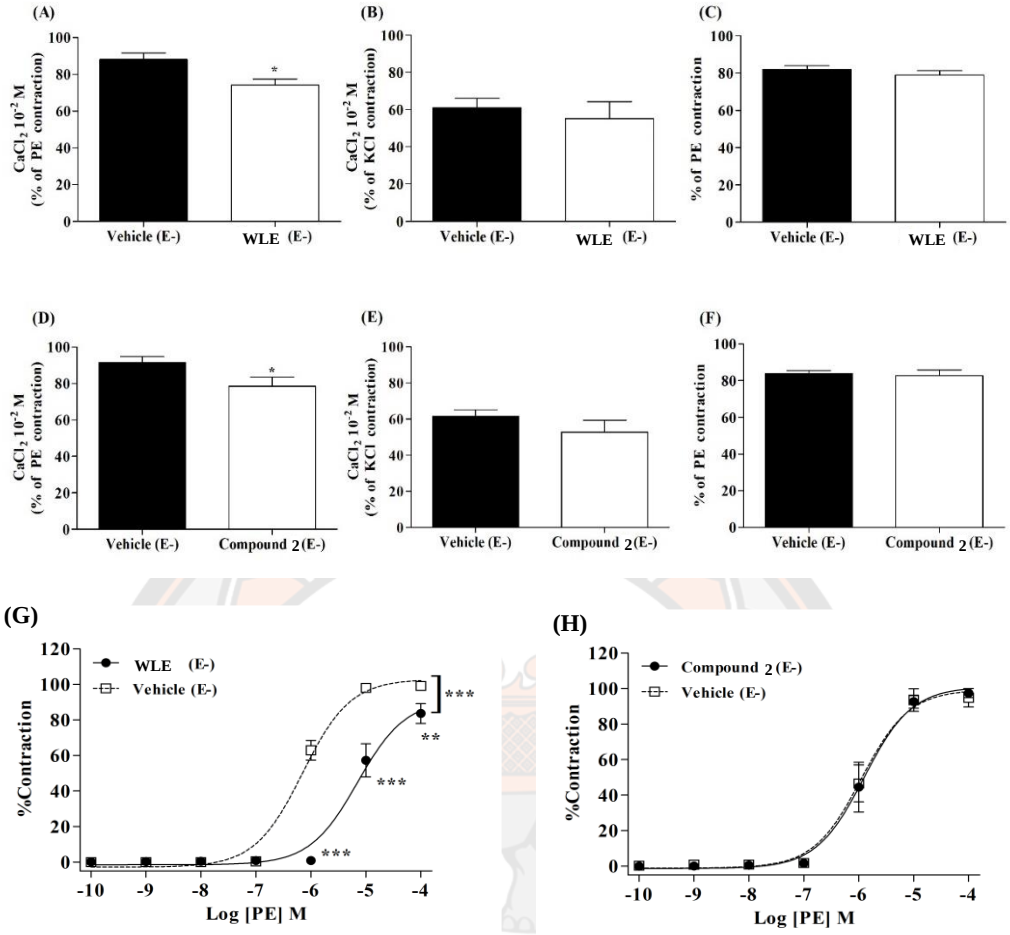


Figure 32 Effect of WLE or quercetin 3-methyl ether 3′-O-β-xylopyranoside (2) on extracellular/intracellular Ca^{2+} fluxes and on α_1-adrenergic receptors. Experiments were performed in endothelium-denuded (E-) MA rings. $CaCl_2$ (10^{-2} M) was added in the presence of vehicle or WLE or compound 2 in Ca^{2+}-free Krebs solution, after pre-constriction with PE (10^{-5} M) (A, D) or 80 mM KCl (B, E). In separate experiments, rings were pre-incubated with verapamil (10^{-7} M) for 30 min, then pre-incubated with vehicle or WLE or 2 before adding PE (10^{-5} M) (C, F). Finally, rings were pre-incubated with WLE or 2 or vehicle, followed by contraction with cumulative concentrations of PE (10^{-10}-10^{-4} M) (G, H). Values are means ± SEM (n = 7-10), $^{*}p<0.05$, $^{***}p<0.001$.

WLE and its main compound reduced blood pressure

As compared to vehicle, infusion of WLE significantly decreased systolic (SBP) and diastolic blood pressure (DBP) at doses ranking from 0.025 to 1 mg/kg (**Figure 33A and B**). These hypotensive effects were lower compared to those of nifedipine. Interestingly, the effect of compound **2** in reducing blood pressure was similar to that of nifedipine (**Fig. 33D and E**). Moreover, the rebound tachycardia found at the highest dose of nifedipine was not shown with WLE or compound **2** ($p<0.05$, **Fig 33C and F**).

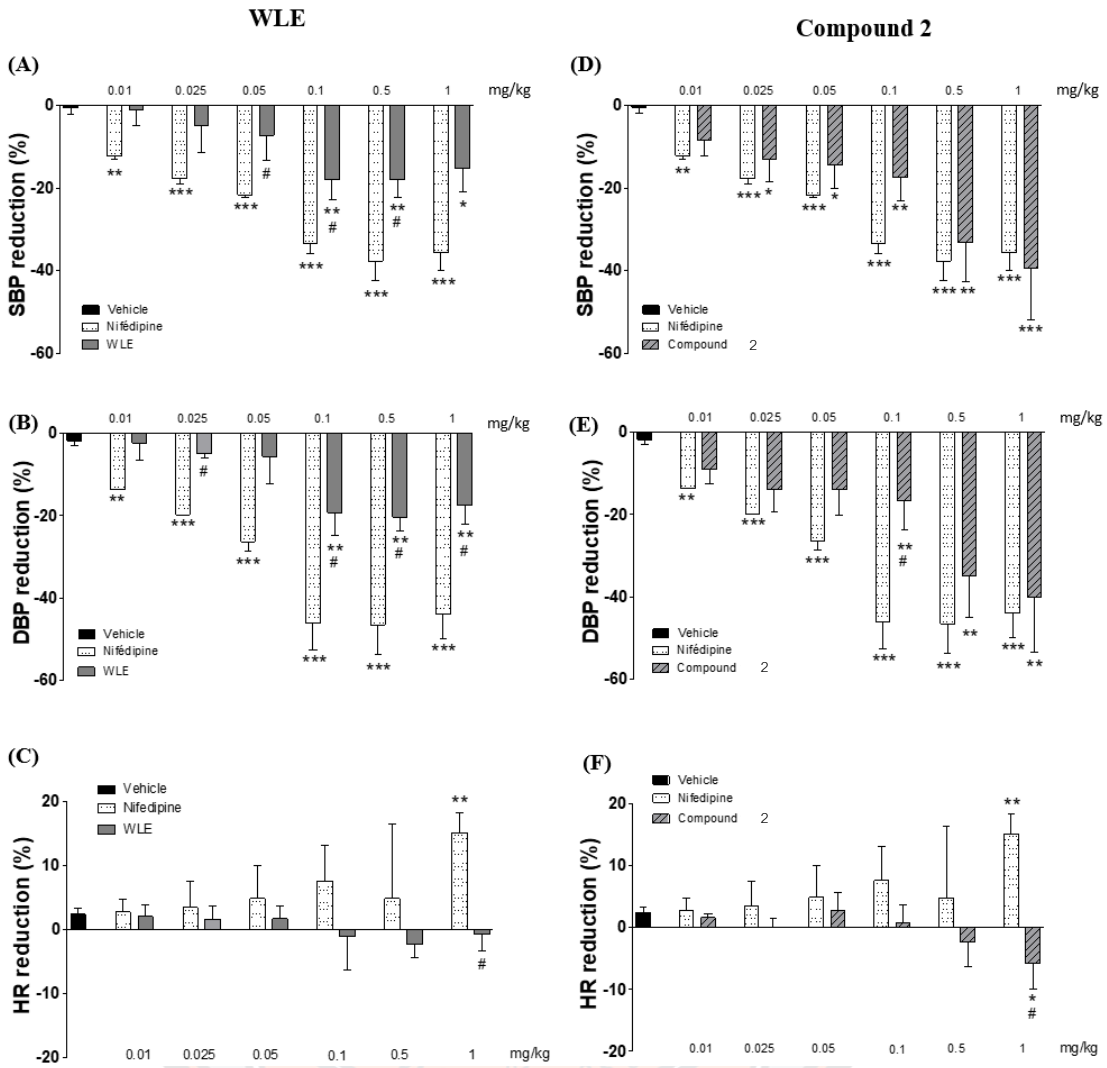


Figure 33 Acute hypotensive effect of WLE and its main compound 2 (quercetin 3-methyl ether 3′-O-β-xylopyranoside) in rats. Percentage of reduction of SBP: systolic blood pressure (A, D), DBP: diastolic blood pressure (B, E), and HR: heart rate (C, F). Values are means ± SEM (n=4-5 rats per group). *p<0.05, **p<0.01, ***p<0.001 vs vehicle. # p<0.05 vs nifedipine at the same dose.

CHAPTER V
DISCUSSIONS

Part I: Isolation and chemical characterization of phosphodiesterase 5 and arginase inhibitory activities from WLE and its flavonoid constituents

In this study, thirty-seven ethanolic extracts from the plants of the Nelumbonaceae and Nymphaeaceae family were screened for their PDE5 and arginase inhibitory activities. This is the first time that this screening has been performed on these plants. No extract showed any significant inhibitory activity on arginase, meaning that none of the extracts was able to enhance the production of NO. This means that there was insufficient availability of NO resulting from L-arginine (Boucher et al., 1999). Conversely eight extracts showed PDE5 inhibitory activity, i.e., extracts from *N. nucifera* receptacle and pollen, *Nymphaea* sp. pollen, *N. cyanea* petals, *N. stellata* petals, *N. pubescens* petals and receptacle and *V. amazonica* petals. The petals of *N. pubescens* had the lowest IC_{50} values of 6.37 ± 0.65 µg/mL whereas the pollen of *Nymphaea* sp. had the highest IC_{50} values of 25.61 ± 2.74 µg/mL. The effect of PDE5 inhibition of these extracts have not been previously reported. The ethanolic extract of *N. stellata* leaves has shown aphrodisiac activity in male rats (MK et al., 2012). Neferine, which is a compound isolated from the green seed embryo of *N. nucifera*, was also reported to have a relaxant effect on rabbit corpus cavernosum tissues (Chen et al., 2007). However, there is still no evidence that such activities related to PDE5 inhibitory effects.

Six flavonoid constituents of the *N. pubescens* petals ethanolic extract showed micromolar levels of IC_{50} against PDE5 activities. Compounds **1**, **2**, **4** and **6** were isolated and identified as components in *N. pubescens* petals for the first time. Previous studies reported that **4** was found in the N. stellata flowers (Raja et al., 2010), **3** and **5** were found in the *N. pubescens* petals (Acharya et al., 2018; Debnath et al., 2013; Pokhrel et al., 2022), while **6** was found both in the *N. stellata* flowers (Verma et al., 2012) and the *N. Alba* flowers (Shakya, 2020) and leaves (Bakr et al., 2017). Compound **4** had been previously isolated from *Rhamnus nakaharai* Hayata (Rhamnaceae) and reported to inhibit PDE5 enzymes isolated from the lungs and hearts of male guinea pigs with an IC_{50} value of 86.9 µM (Ko et al., 2003), **3** and **5** have been previously isolated from *Anaxagorea luzonensis* and reported to inhibit PDE5 enzymes isolated from the mice lungs (Sabphon et al., 2015), while **1**, **2**, and **6** has not previously been reported for their effects on PDE5. Moreover, quercetin, kaempferol, and their derivatives have shown other biological activities such as antibacterial, antifungal, and anti-oxidative properties including reducing LDL oxidation, platelet aggregation, and cardiovascular complications (Jan et al., 2022).

The structure–activity relationship (SAR) on the PDE5 inhibitory activity of quercetin (**3**) and kaempferol (**5**) is depicted in **Figure 16**. The PDE5 inhibitory activities of **3** were 7–9 times stronger than those of **1**, **2**, and **4**, which indicated that the methyl group at position C-3 and the sugar at position C-3’ of quercetin reduced the activities. The same results were seen between **5** and **6**. The inhibitory action

against PDE5 is decreased by a methyl group substitution at position C-3 of kaempferol. The PDE5 inhibitory activity of quercetin, kaempferol, and their derivatives have been reported in previous studies (Ganapathy et al., 2021; Ko et al., 2004; Sabphon et al., 2015). These compounds not only inhibit the PDE5 enzyme but also have antioxidant activity (Ganapathy et al., 2021). According to the review article, using PDE5 inhibitors and antioxidants in combination led to better ED without a rise in side effects (Mykoniatis et al., 2021). However, we demonstrated the SAR of quercetin and kaempferol for the first time, which involves the substitution of methyl groups and sugars at positions C-3 and C-3'.

The HPLC method was used for the chemical characterization of the extracts in our studies, especially for the *N. pubescens* petal extract. The HPLC fingerprints of the "active" extracts were established and the quantitative determination of the six flavonoids in the extracts was validated. The HPLC chromatogram exhibited good separation (**Figure 17**). The calibration curves provided a linear range of 0.5–400 µg/mL for **1**–**6** with satisfactory correlation coefficient values (r^2 = 0.9997–0.9999) (**Table 7**). The precisions expressed by %RSD, were less than 10% and the percentage recovery was in the range of 80–120% (**Table 8**), which was in the acceptable range according to ICH guideline. Our results showed that this method exhibits satisfactory sensitivity, precision and accuracy to detect and determine the flavonoids **1**–**6** in plant extracts. From the HPLC analysis, **2** was the major flavonoid compound in the *N. pubescens* petal extract with a concentration of 17.31 ± 0.05 mg/g. Compounds **1**, **3**, **4**, **5**, and **6** were present at much lower concentrations than **2** (2, 28, 4, 43, and 17 folds, respectively). This HPLC method can be used in a quality control process of *N. pubescens* petal extract if the extract is to be used for its health benefits relating to its PDE5 inhibitory activity. Although some HPLC systems for flavonoid analyses in other plant extracts have been reported with other plant extracts (Kim et al., 2010; Wang et al., 2010), our HPLC method was suitable for analysis of **1–6** in *N. pubescens* samples and other related spp. in our studies.

HPLC analyses of flavonoid constituents in the ethanolic extract of *N. pubescens* petals when compared with other ethanolic extracts, showed the presence of **3** in *N. nucifera* receptacle and pollen, *Nymphaea* sp. pollen and *N. pubescens* receptacle, **4** in *Nymphaea* sp. pollen and *N. pubescens* receptacle, and **5** in *N. nucifera* pollen, *N. cyanea* petals, *N. stellata* petals and *V. amazonica* petals. Paudel and Panth (Paudel & Panth, 2015) reported that **3** and **5** were found in *N. nucifera* flower and stamen, which was similar to our results. Raja et al. (Raja et al., 2010) and Verma et al. (Verma et al., 2012) have reported that **3**, **4**, **5**, and **6** were found in *N. stellata* flower while we could observe only **5** in *N. stellata* petals. In addition, we identified the presence of flavonoids **5** in *N. cyanea* petals and *V. amazonica* petals for the first time.

Part II: Cytotoxicity and effect of WLE and its flavonoid constituents on pulmonary artery and aorta

WLE showed endothelium-dependent relaxation in the PA, as indicated by the 60-fold increase in the EC_{50} value after endothelium removal. The endothelium-

dependent vasorelaxant effect was then performed on various blockers including L-NAME (eNOS inhibitor), indomethacin (COX inhibitor), and apamin plus charybdotoxin (SK_{Ca} and IK_{Ca} blocker). Only L-NAME reduced the relaxation induced by the WLE, suggesting that WLE acts *via* the NO pathway. The WLE also produced the endothelium-independent vasorelaxant effect which was unaffected by various K^+ channel (K_V, K_{ATP}, K_{Ca}, and K_{IR}) blockers suggesting that it does not act *via* K^+ channels. Furthermore, the WLE had no effect on neither extracellular Ca^{2+} influx through the ROCCs/VOCCs nor intracellular Ca^{2+} release from the SR and did not affect PE-induced contraction *via* α_1-receptor, indicating that the WLE mechanism of vasorelaxant action does not involve Ca^{2+} channel and α_1-adrenergic receptor inhibition. Other mechanisms, such as the RhoA/Rho-kinase pathway, might affect the contraction and relaxation of PA (Baek, Jeon, Kim, et al., 2009; Nossaman et al., 2010; Yu et al., 2014). PE stimulates vasoconstriction through this pathway (Qiao et al., 2014), and the flavonoid constituents in WLE might inhibit PE by stimulating cAMP/PKA-dependent effects (Baek, Jeon, Song, et al., 2009; Seok et al., 2008; Sharma et al., 2020). Thus, WLE may influence these mechanisms. The efficacy (E_{max}) of PA and the aorta are similar, but the potency (EC_{50}) of PA is lower than that of the aorta (**Figure 18A**). The relaxation to the WLE was apparently more selective to the PA than the aorta, thus WLE might be useful for the selective treatment of PAH. Since PDE5 is highly expressed in PA (Corbin et al., 2005), it is possible that the WLE could act via PDE5 resulting in the modulation of the sGC/cGMP pathway. Indeed, the present study showed that the WLE enhanced the relaxation by SNP and also directly activated sGC in the PA. WLE could inhibit PDE5 resulting in increasing cGMP availability and enhancing SNP effect (Panklai et al., 2023). Nevertheless, previous studies reported that the WLE components, flavonoid compounds **2**, **3** and **5**, exhibited the PDE inhibitory activity (Panklai et al., 2023; Sabphon et al., 2015) and also relaxed the PA isolated from rats (Mahobiya et al., 2018) and goats (Palai & Parija, 2017). Investigating the effects of WLE in a hypoxia or monocrotaline-induced pulmonary arterial hypertension rat model (Inci & Serdar, 2010; Nakazawa et al., 1999), and confirming biomarker expression levels through western blotting or qRT-PCR is noted to be interesting and could be considered for a future study.

We found that the EC_{50} values of flavonoid constituents **2**, **3**, and **5** on PA relaxation, with and without endothelium, were in the same range (26 - 47 µM), which is in agreement with the study of Mahobiya et al., (Mahobiya et al., 2018) which reported that the effect of **5** on rat PA intact and denuded rings, was not significantly different. In our study, it is noted that value of E_{max} of **2** was lower than that of **3** and **5**, and all three flavonoids showed less vasodilating effects on denuded the aorta than on the intact ones indicating that endothelium plays a role in this effect. Our results are different to the study of Perez-Vizcaino et al., (Pérez-Vizcaíno et al., 2002) which stated that the vasodilating effects of **3** and **5** on rat aorta did not significantly differ when removing endothelial cells. It is interesting that the effect of **3** on rat PA has never been reported before while this is the first time that the vasorelaxation effects of **2** on both PA and aorta has been reported. Compound **2** is a derivative of **3** where the C3 hydroxyl group was replaced by the methoxy group and the C3′ hydroxyl group was replaced by xylose (**Figure 16**). The study of Loh et al., (Loh et al., 2020) reported that compound **3** exhibited the highest potency of the vasorelaxant effects of all the 64 types of flavonoids, and they also suggested that the

type of substituents in positions C3′ of the B-ring could modify the vasorelaxant effect depending on the structure of the compound. Therefore, it is possible that derivatives of **3** and **5** also have the same pharmacological activity.

WLE had no acute cytotoxicity to VSMCs isolated from aortae and PA, a similar result as the study by Debnath et al., (Debnath et al., 2013) which showed that water extract of *N. pubescens* flowers was safe up to a maximum concentration of 500 µg/ml.

Part III: Effect of WLE and its main active compound (Compound 2; Quercetin 3-methyl ether 3′-*O*-β-xylopyranoside) on mesenteric artery

The new findings of our study are that: 1) an ethanolic extract of *N. pubescens* petals induced a vasorelaxant effect on resistance vessels through mechanisms combining endothelium-dependent and -independent pathways, 2) this direct vascular effect translated into an acute hypotensive effect in vivo, 3) the main component of the extract, quercetin 3-methyl ether 3′-O-β-xylopyranoside, induced vasorelaxant effects that can, at least partly, explain the relaxing actions of the extract.

In line with the traditional uses of extracts from *Nymphaea* petals to promote erectile function through increase in endothelial NO production (Mireille et al., 2016), the present study showed that the *N. pubescens* extract induced a potent vasorelaxant effect relying on endothelium-dependent mechanisms. However, the relaxant effect of the extract was not abolished by endothelium removal as illustrated by the switch to the right of the relaxation curves without a decrease in the E_{max} value of the extract, thus indicating that endothelium-independent mechanisms are also important contributors. Our data demonstrated that the effects of the extract involved the activation of the NO-GCs-cGMP pathway. Indeed, the relaxant effect was inhibited by a NOS inhibitor indicating the capacity of the extract to stimulate endothelial NO production, but the results revealed that the extract also promoted the NO signaling at the level of VSMCs. Moreover, the extract enhanced the effect of NO donor but did not act itself as a direct sGC activator. Altogether, these results suggested that the extract might have phosphodiesterase inhibitory properties that deserve to be investigated in future studies. Regarding the effect of the extract on calcium fluxes, only extracellular calcium entry through ROCCs was, weakly but significantly, inhibited by the extract, without any effect on VOCC or intracellular calcium release from SR. In addition, the extract exhibited a weak inhibition of the PE-induced contracting effect, suggesting a α_1-receptor antagonism. Regarding this latter mechanism, as ROCCs are activated by agonists acting on G-protein-coupled receptors, such as phenylephrine, we cannot exclude that the inhibitory effect of the extract on ROCC contribute to, or its responsible for, the reduction of the PE constricting action. Relaxation of VSMCs is also mediated by hyperpolarization secondary to the opening of different types of K^+ channels, including K_v, K_{ATP} and K_{Ca} (Bosnjak, 1993). Here, we showed that the effect of *N. pubescens* extract was not reduced by any of the K^+ channels blockers. In contrast, 4-aminopyridine and glibenclamide enhanced extract-induced relaxation.

The main compound of the extract is the quercetin derivative, quercetin 3-methyl ether 3′-O-β-xylopyranoside with a contain of 1.73% (w/w) (Panklai et al., 2023). The present study reported for the first time that this quercetin derivative had a vasodilatory effect, albeit not very potent as illustrated by its E_{max} that did not reach 100% of relaxation. This data suggests that this compound is probably involved in the relaxant effect of the extract, but that other compounds, that remain to be identified, are also involved. Mechanistically, this compound shared with the extract the same effects on the NO-sGC-cGMP pathway and ROCCs but did not present a α_1-receptor antagonistic effect. As compared to quercetin which is a known vasodilator of MA (Nishida & Satoh, 2013; Satoh & Nishida, 2014), our data indicate that methyl ester group in the 3' position and the presence of the xylopyranoside derivative did not alter the vasorelaxant properties. Nevertheless, a few mechanistic differences exist as the relaxing effects of quercetin on MA was mainly related to EDHF and slightly through the endothelial NO pathway (Nishida & Satoh, 2013).

To determine if the vasorelaxant effects observed *ex vivo* were still present after *in vivo* administration, the acute hypotensive effects of WLE and quercetin 3-methyl ether 3′-O-*β*-xylopyranoside were investigated. The results showed that the extract acutely reduced blood pressure, with an effect however lower than nifedipine used as a comparator. The limitation of our experiment is that it was done in normotensive rats, i.e. rats with a normal endothelial function, thus probably hampering the endothelial component of the relaxant effect of WLE. Nevertheless, our results sufficiently support the conclusion that WLE can be beneficial in the treatment of vascular diseases. Further studies exploring the effect of the extract on animal models of hypertension are now required. However, this hypotensive action is in agreement with the anti-hypertensive effect previously reported with an aqueous extract from the flowers of another *Nymphaea (lotus)* L. (Kameni et al., 2019). The hypotensive effect of quercetin 3-methyl ether 3′-O-*β*-xylopyranoside was found similar from that of nifedipine. Again, this data indicates that the methyl ester group in the 3' position and the presence of the xylopyranoside derivative did not reduce the hypotensive effect of quercetin (Brüll et al., 2015; Duarte et al., 2001). It is noteworthy that the *in vivo* hypotensive effect of quercetin 3-methyl ether 3′-O-*β*-xylopyranoside is more pronounced than the effect of WLE, while its efficacy to induce mesenteric artery relaxation is less. Moreover, as compared to nifedipine, this quercetin derivative did not induce rebound tachycardia at the high concentrations but on the contrary reduced heart rate. Altogether, these results suggested that a direct cardiac effect is possible with this compound. Further studies will be needed to better characterize this putative mechanism.

CHAPTER VI

CONCLUSIONS

Among 37 samples from Nymphaeaceae and Nelumbonaceae families in our research, we identified that *N. pubescens* petals exhibited the strongest PDE5 inhibitory effect, a finding reported for the first time. Using an HPLC method, we characterized six flavonoids (**1**-**6**) with PDE5 inhibitory activity in the extract. Notably, compounds **1**, **2**, and **6** were found to inhibit PDE5 for the first time in this study.

The ethanolic extract of WLE demonstrated a vasorelaxant effect both in the pulmonary and systemic arterial systems. WLE, the major flavonoid compound **2**, as well as flavonoid aglycones, compounds **3**, and **5** exhibited relaxation effects on rat pulmonary arteries and aortae. Our study revealed that WLE induced relaxation of the rat pulmonary arteries (PA) through the endothelial NO pathway and slightly activated the sGC, with no observed cytotoxicity on vascular smooth muscle cells (VSMCs). These findings hold for the further development of herbal products from WLE for pulmonary arterial hypertension (PAH). Furthermore, WLE also demonstrated a vasorelaxant effect on the systemic resistance vessels, through the potentialization of nitric oxide (NO) production by the endothelium. Additionally, it relied on endothelium-independent mechanisms such as the activation of the sGC-cGMP pathway and inhibition of ROOCs. Our data provide pharmacological evidence supporting the traditional use of this plant in diseases associated with hampered endothelial function. While the main compound, quercetin 3-methyl ether 3′-O-β-xylopyranoside, is a potential contributor of these effects, other molecules are likely involved and await identification.

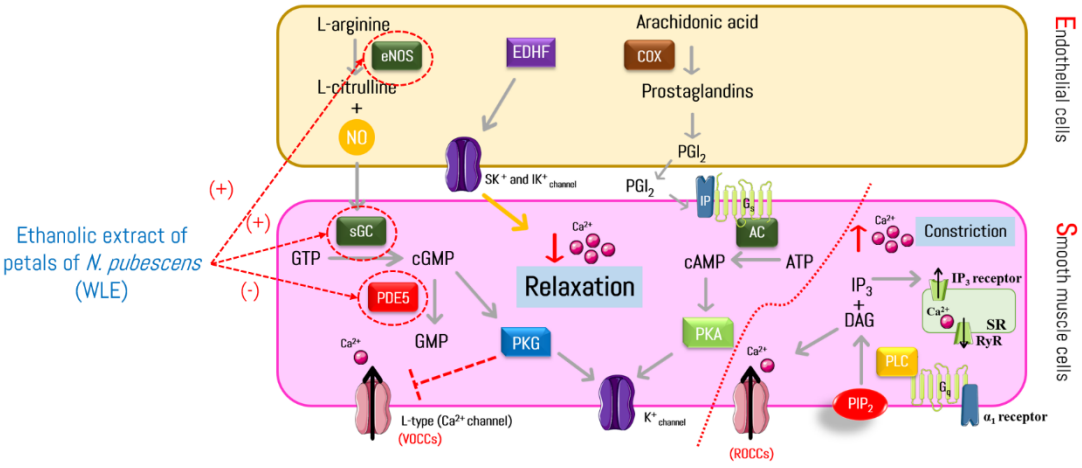


Figure 34 Summary of the effect of WLE on rat pulmonary circulation
Source: Made by Teerapap Panklai (2023)

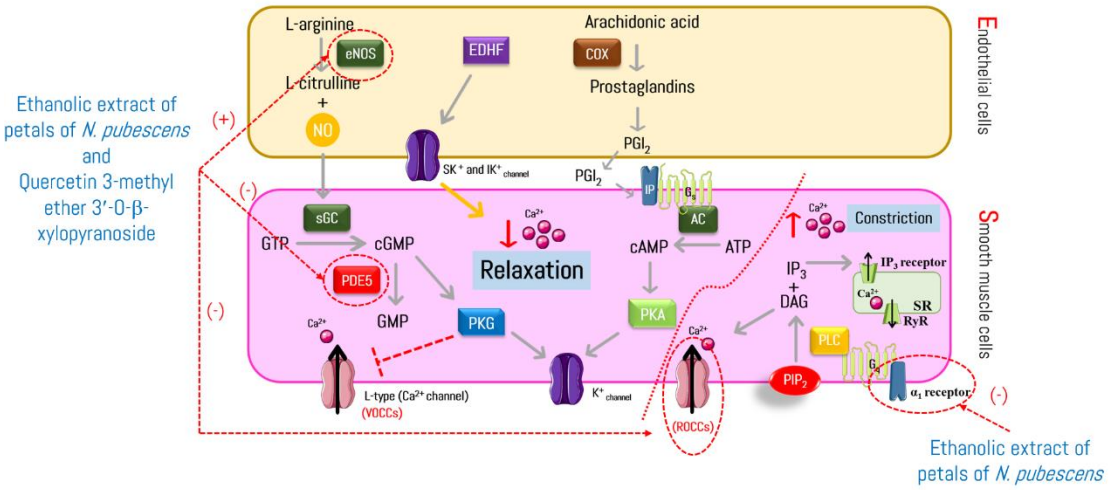


Figure 35 Summary of the effect of WLE and quercetin 3-methyl ether 3′-O-β-xylopyranoside on rat systemic circulation
Source: Made by Teerapap Panklai (2023)

Further studies

1. Investigated the toxic effects of the WLE and its main active compound.

2. Investigated the metabolism, drug interactions, bioavailability, and pharmacokinetics of both the WLE and its main active compound.

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APPENDIXES

APPENDIX A: Ethic

Animal ethic

เอกสารรับรองโครงการ
คณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์
มหาวิทยาลัยนเรศวร (คกส.)

ชื่อโครงการ	ฤทธิ์การคลายตัวของหลอดเลือดแดงปอดที่แยกจากหนูและกลไกการออกฤทธิ์ของสารสกัดบัว Vasorelaxant effect on rat isolated pulmonary artery and mechanism of action of water lilies extraction
เลขที่โครงการ	NU-AE621024
เลขที่เอกสารรับรอง	62 01 021
ประเภทการรับรอง	เต็มรูปแบบ
ชื่อหัวหน้าโครงการ/ผู้ยื่นขอฯ	รศ.ดร.กรองกาญจน์ ชูทิพย์
สังกัดหน่วยงาน /คณะ	วิทยาศาสตร์การแพทย์
วันที่รับรอง	27 มกราคม 2563
วันสิ้นสุดการรับรอง	27 มกราคม 2565

ขอรับรองว่าโครงการวิจัยนี้ ได้รับการรับรองด้านจรรยาบรรณการใช้สัตว์
จากคณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์ มหาวิทยาลัยนเรศวร (คกส.)

(รองศาสตราจารย์ ดร.รัตติมา จีนาพงษ์)
ประธานคณะกรรมการกำกับดูแลการดำเนินการต่อสัตว์เพื่องานทางวิทยาศาสตร์ (คกส.)
มหาวิทยาลัยนเรศวร

หมายเหตุ เอกสารรับรองฉบับนี้ใช้ควบคู่กับหนังสือราชการ
เลขที่ อว. 0603.01/4454 ลงวันที่ 13 กุมภาพันธ์ 2563

Human ethic

AF 04-09/5.0

COE No. 010/2021

IRB No. P10002/64

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร
99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000 เบอร์โทรศัพท์ 05596-8752

หนังสือรับรองการยกเว้นพิจารณาจริยธรรมโครงการวิจัย

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ มหาวิทยาลัยนเรศวร ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากล ได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ	: การวิจัยและพัฒนาบัวสายเพื่อเป็นผลิตภัณฑ์สมุนไพรสำหรับภาวการณ์เสื่อมสมรรถภาพทางเพศและโรคที่เกี่ยวข้องกับหลอดเลือด (in vitro)
ผู้วิจัยหลัก	: นายธีรภาพ ปานคล้าย
สังกัดหน่วยงาน	: คณะเภสัชศาสตร์
วิธีทบทวน	: แบบยกเว้น (Exemption Review)
รายงานความก้าวหน้า	: ไม่ต้องส่ง รายงานความก้าวหน้า (Progress Report)

ลงนาม:.....

(นายแพทย์สมบูรณ์ ตันสุภสวัสดิกุล)
ประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์
มหาวิทยาลัยนเรศวร

วันที่รับรองการยกเว้นพิจารณาจริยธรรม : 13 มกราคม 2564

หมายเหตุ

1. ไม่ต้องส่ง รายงานความก้าวหน้า (Progress Report) และรายงานสรุปผลการวิจัย (Final Report)
2. หากมีการแก้ไขโครงการวิจัยภายหลังการรับรอง ให้ผู้วิจัยดำเนินการส่งส่วนแก้ไขเพิ่มเติมโครงการวิจัย (Amendment) หรือจัดทำเป็นโครงการวิจัยใหม่

APPENDIX B: Publications

First publication

Teerapap Panklai, Nungruthai Suphrom, Prapapan Temkitthawon, Perle Totoson, Krongkarn Chootip, Xiao-Liang Yang, Hui Ming Ge, Zhu-Jun Yao, Nattiya Chaichamnong, Kornkanok Ingkaninan* and Corine Girard. Phosphodiesterase 5 and Arginase Inhibitory Activities of the Extracts from Some Members of Nelumbonaceae and Nymphaeaceae Families. *Molecules* 2023, 28, 5821. (Q1, impact factor 4.6).

molecules MDPI

Article

Phosphodiesterase 5 and Arginase Inhibitory Activities of the Extracts from Some Members of Nelumbonaceae and Nymphaeaceae Families

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Abstract: The objectives of this study were (1) to investigate the effect of extracts from some plants in the families Nelumbonaceae and Nymphaeaceae on phosphodiesterase 5 (PDE5) and arginase, which have been used in erectile dysfunction treatment, and (2) to isolate and identify the compounds responsible for such activities. The characterization and quantitative analysis of flavonoid constituents in the active extracts were performed by HPLC. Thirty-seven ethanolic extracts from different parts of plants in the genus *Nymphaea* and *Victoria* of Nymphaeaceae and genus *Nelumbo* of Nelumbonaceae were screened for PDE5 and arginase inhibitory activities. The ethanolic extracts of the receptacles and pollens of *Nelumbo nucifera* Gaertn., petals of *Nymphaea cyanea* Roxb. ex G.Don, *Nymphaea stellata* Willd., and *Victoria amazonica* (Poepp.) Sowerby and the petals and receptacles of *Nymphaea pubescens* Willd. showed IC_{50} values on PDE5 of less than 25 μg/mL while none of the extracts showed effects on arginase. The most active extract, *N. pubescens* petal extract, was fractionated to isolate and identify the PDE5 inhibitors. The results showed that six flavonoid constituents including quercetin 3′-*O*-*β*-xylopyranoside (**1**), quercetin 3-methyl ether 3′-*O*-*β*-xylopyranoside (**2**), quercetin (**3**), 3-*O*-methylquercetin (**4**), kaempferol (**5**) and 3-*O*-methylkaempferol (**6**) inhibited PDE5 with IC_{50} values at the micromolar level.

Keywords: *Nymphaea pubescens*; phosphodiesterase 5; arginase; erectile dysfunction; flavonoids; lotus; water lily

1. Introduction

Erectile dysfunction (ED) is the recurrent or persistent inability to achieve or sustain a penile erection for sexual satisfaction [1]. A high prevalence of ED has been reported in eight countries: Brazil, Italy, France, China, Spain, Germany, the United States, and the United Kingdom. Italy has the highest prevalence at 48.6% of the male population,

while Brazil has the lowest of these countries at 37.2% [2]. In Thailand, ED prevalence is 37.5% [3]. One of the most commonly used treatments for ED is the inhibition of the enzyme phosphodiesterase 5 (PDE5) [4]. To date, the FDA-approved oral PDE5 inhibitors that are widely available and used in the market are Sildenafil citrate (Viagra®), Tadalafil (Cialis®), Vardenafil (Levitra®), and Avanafil (STENDRA®) [5]. PDE5 is the cyclic guanosine monophosphate (cGMP)-degrading enzyme that is distributed in various tissues including the lungs, platelets, penile corpus cavernosum, smooth muscle cells, and all vascular smooth muscle cells, especially in the pulmonary vessels [6]. Inhibition of PDE5 results in raising the level of cGMP, leading to the relaxation of the vascular smooth muscle of the penile corpus cavernosum [7]. Additionally, the endothelial cells produce nitric oxide (NO), which stimulates the soluble guanylate cyclase (sGC) that changes guanosine triphosphate (GTP) to cGMP. Arginase is the enzyme implicated in ED that catalyzes the hydrolysis of L-arginine to urea and L-ornithine, while endothelium nitric oxide synthase (eNOS) converts L-arginine to L-citrulline and NO. Therefore, inhibition of arginase leads to L-arginine being more available to use for eNOS and increasing NO availability [8].

Natural products are interesting sources for drug candidates [9]. Several traditional medicinal plants in Thailand have various bioactivities, one of which improves sexual dysfunction by inhibiting the PDE5 enzyme [10–16]. Water lilies (*Nymphaea* spp.) are members of Thai wetland biota [17] that show interesting ethnopharmacology data. Flowers of *Nymphaea* spp. have been traditionally used for the treatment of diabetes, inflammation, jaundice, and eye disorders, and interestingly have been used as aphrodisiacs [18,19]. In this study, we explored the possible roles of some *Nymphaea* spp. and other aquatic flowering plants commonly found in Thailand in the treatment of ED by the inhibition of PDE5 and arginase enzymes. *Nymphaea* and *Victoria* genera belong to the family Nymphaeaceae. Genus *Nymphaea* contains 45–50 species which can be consumed as food and are used in traditional medicine for the treatment of various diseases. They are distributed throughout North America, Africa, Europe and Asia including Thailand [19,20]. Phytochemical studies have reported that various species of the genus *Nymphaea* contain flavonoids glycosides, alkaloids, glycosides, hydrolysable tannins, lignans, phytosterols and triterpenoid saponins [19,21]. Genus *Victoria* is distributed in the Amazonas and Chaco biogeographical regions of South America [22,23]. This genus is composed of *Victoria amazonica* (Poepp.) Sowerby, *V. cruziana* A.D. Orb. and *V. boliviana* Magdalena and L.T.Sm. Previous phytochemical studies have reported that plants in this genus contain steroids, benzenoids, chlorophylls, ketone, benzyl esters and methyl esters [22,24].

Nelumbo Adans. is a genus belonging to the family Nelumbonaceae. They are perennial aquatic plants of stagnant water habitats with important value in horticulture, medicine, food, religion, and culture. *Nelumbo* or lotuses contains two species, i.e., *Nelumbo nucifera* Gaertn. (The Asian lotus) and *N. lutea* Willd. (The American lotus). *N. nucifera* is distributed in many countries of North Australia, and East, South and Southeast Asia including Thailand, while *N. lutea* is distributed in North America [25,26]. Phytochemical studies have reported that the *N. nucifera* contains flavonoids, alkaloids, polysaccharides, essential oil, triterpenoids, steroids and tannins [27,28].

Some alkaloids, phenolics, and polycyclic aromatics can inhibit PDE5 activity [10–16,29], or arginase activity [30]. Related types of such compounds can be found in the 3 genera of aquatic plants mentioned above and therefore, the plant extracts might be good sources of the PDE5 or arginase inhibitors. The objectives of this study were to (i) investigate PDE5 and the arginase inhibitory activities of ethanolic extracts from some plants belonging to *Nelumbo*, *Nymphaea* and *Victoria* genera, (ii) isolate and identify the PDE5 and/or arginase inhibitors of the most active extract, and (iii) determine and quantitatively analyze the constituents in the various extracts.

2. Results

2.1. Phosphodiesterase-5 Inhibition of the Extracts

Thirty-seven ethanolic extracts from different parts of six species in the family of Nelumbonaceae and Nymphaeaceae were screened for PDE5 inhibitory activity. Eight extracts showed %PDE5 inhibition of more than 80% at 50 µg/mL, i.e., *N. nucifera* (receptacle and pollen), *Nymphaea* sp. (pollen), *N. cyanea* Roxb. Ex G.Don (petals), *N. stellata* Willd. (petals), *N. pubescens* Willd. (petals and receptacle), and *V. amazonica* (petals) (Table 1). These extracts were considered "active extracts" and were further tested for the concentrations that induced 50% of the maximal inhibition (IC_{50} values) (Table 2). The petals of *N. pubescens* extract had the highest IC_{50} and should be investigated for PDE5 inhibitors. This extract was fractionated to isolate and identify compounds supporting observed inhibitory activity of PDE5.

Table 1. Percentage of PDE5 and arginase inhibitions of ethanolic extracts from some plants in the family of Nelumbonaceae and Nymphaeaceae. The values are the means ± standard deviations (SD) from triplicate experiments.

No.	Samples	Part Used	% PDE5 Inhibitory Activity at 50 µg/mL	% Arginase Inhibitory Activity at 100 µg/mL
1	*Nelumbo nucifera*	petals	75.03 ± 1.77	17.70 ± 0.84
		pollens	85.07 ± 6.08	21.39 ± 3.82
		seeds	74.66 ± 4.39	22.25 ± 10.84
		receptacles	94.26 ± 0.74	45.30 ± 5.89
		peduncles	68.37 ± 0.50	19.98 ± 4.70
		petioles	56.71 ± 4.90	2.83 ± 5.08
		leaves	71.46 ± 0.54	21.84 ± 4.92
2	*Nymphaea* sp.	petals	78.28 ± 4.43	31.47 ± 7.71
		pollens	82.04 ± 2.06	36.91 ± 2.51
		receptacles	54.20 ± 3.26	22.07 ± 3.85
		peduncles	34.55 ± 4.37	15.62 ± 4.62
		petioles	43.54 ± 3.48	15.03 ± 2.77
		leaves	62.31 ± 4.35	30.19 ± 4.85
3	*Nymphaea cyanea*	petals	86.54 ± 1.63	31.28 ± 4.75
		pollen	73.16 ± 2.38	38.73 ± 2.17
		receptacle	62.61 ± 1.26	27.76 ± 3.66
		peduncles	34.40 ± 3.06	10.44 ± 9.32
		petioles	38.26 ± 9.82	12.09 ± 3.85
		leaves	55.53 ± 5.43	23.81 ± 3.87
4	*Nymphaea stellata*	petals	84.92 ± 1.86	28.45 ± 5.24
		pollens	38.05 ± 5.83	27.23 ± 14.29
		receptacles	46.07 ± 4.03	23.50 ± 6.07
		peduncles	13.36 ± 6.59	0
		petioles	29.17 ± 7.58	9.86 ± 4.59
		leaves	44.89 ± 7.26	21.13 ± 19.60
5	*Nymphaea pubescens*	petals	98.97 ± 0.26	39.49 ± 4.53
		pollens	74.86 ± 2.45	15.20 ± 1.87
		receptacles	82.69 ± 3.36	23.53 ± 3.57
		peduncles	58.90 ± 4.60	23.36 ± 2.52
		petioles	50.23 ± 7.61	11.96 ± 2.65
		leaves	73.26 ± 3.29	37.71 ± 3.23
6	*Victoria amazonica*	petals	86.67 ± 2.65	30.49 ± 3.22
		pollens	63.35 ± 5.45	27.61 ± 1.47
		receptacles	34.78 ± 6.41	13.30 ± 5.48
		peduncles	8.23 ± 3.92	16.87 ± 4.80
		petioles	9.16 ± 0.73	14.39 ± 5.57
		leaves	43.90 ± 3.78	21.40 ± 4.61

Table 2. IC_{50} values of ethanolic extracts from some plants in the family of Nelumbonaceae and Nymphaeaceae on PDE5 represented as the means ± SD from triplicate experiments.

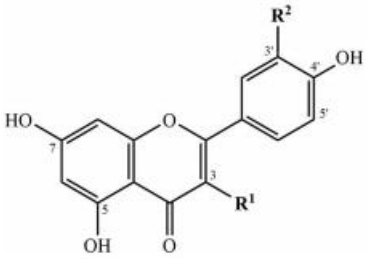
No.	Samples	Part Used	IC_{50} Values (μg/mL)
1	*Nelumbo nucifera*	Receptacles	10.50 ± 3.65
		Pollens	14.15 ± 3.97
2	*Nymphaea* sp.	Pollens	25.61 ± 2.74
3	*Nymphaea cyanea*	Petals	8.07 ± 1.33
4	*Nymphaea stellata*	Petals	13.28 ± 0.25
5	*Nymphaea pubescens*	Petals	6.37 ± 0.65
		Receptacles	18.61 ± 4.03
6	*Victoria amazonica*	Petals	21.54 ± 4.23

2.2. Arginase Inhibition of the Extracts

Thirty-seven ethanolic extracts were also screened for their arginase inhibitory activity. The results showed that all extracts had an inhibitory activity on arginase of less than 50% at 100 μg/mL, (Table 1) which is not sufficiently effective to be used as an arginase inhibitor.

2.3. Isolation and Identification of PDE5 Inhibitors from N. pubescens Petal Ethanolic Extract

From Table 2, the ethanolic extract of *N. pubescens* petals showed the highest inhibitory activity on PDE5. The extract was further fractionated by solid-phase extraction (SPE) and preparative HPLC resulting in the finding of 6 flavonoids (**1–6**). Four compounds were isolated and identified as quercetin 3′-*O*-*β*-xylopyranoside (**1**) [31], quercetin 3-methyl ether 3′-*O*-*β*-xylopyranoside (**2**) [31], 3-*O*-methylquercetin (**4**) [32] and 3-*O*-methylkaempferol (**6**) [33] by comparison with their spectroscopic data with the literature. In addition, the presence of quercetin (**3**) and kaempferol (**5**) in the extract was confirmed by comparison with the reference standards using HPLC analysis. The structures of these compounds and their IC_{50} values on PDE5 are shown in Figure 1. Sildenafil was used as a reference PDE5 inhibitor (IC_{50} = 1.36 ± 0.21 nM) [34].



	R^1	R^2	PDE5 IC_{50} (μM)
1	OH	O-xyl	4.09 ± 0.76
2	OCH_3	O-xyl	5.63 ± 0.20
3	OH	OH	0.61 ± 0.11
4	OCH_3	OH	5.74 ± 2.11
5	OH	H	0.40 ± 0.09
6	OCH_3	H	4.41 ± 0.46
	Sildenafil		1.36 ± 0.21 nM

Figure 1. Structures of flavonoids isolated from *N. pubescens* petals and their IC_{50} values against PDE5 represented as the means ± SD from triplicate experiments. Sildenafil was used as a positive control in our assay.

2.4. The HPLC Method for Quantitative Analysis of Flavonoids in Plant Extracts

The HPLC method for the quantitative determination of **1–6** in the extracts of plants in the families Nelumbonaceae and Nymphaeaceae was developed and validated. HPLC chromatograms of **1–6** and the ethanolic extract of *N. pubescens* petals are shown in Figure 2A,B. The calibration data, LOD, and LOQ values are shown in Table S1. The %RSD was less than 3% for intra-day and inter-day precision, and the range of accuracy expressed as percentage recovery is between 85.13 and 109.92% (Table S2).

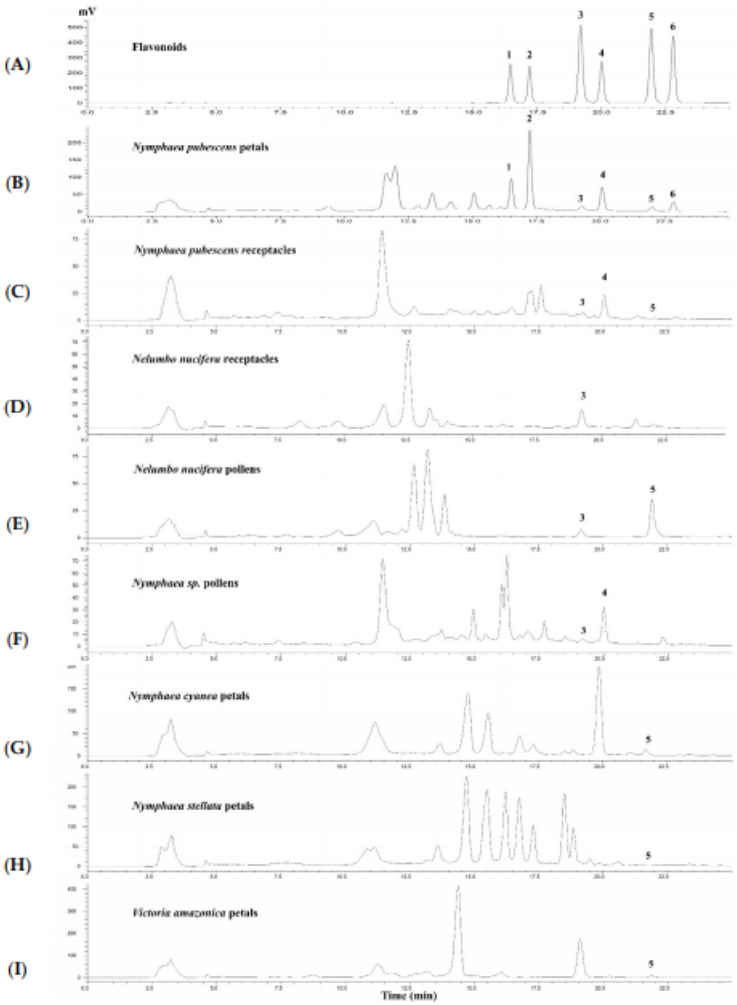


Figure 2. HPLC chromatograms of (**A**) mixtures of six flavonoids composed of 100 μg/mL (**1**) quercetin 3′-*O*-*β*-xylopyranoside, (**2**) quercetin 3-methyl ether 3′-*O*-*β*-xylopyranoside, (**3**) quercetin, (**4**) 3-*O*-methylquercetin, (**5**) kaempferol, and (**6**) 3-*O*-methylkaempferol, and 5 mg/mL of crude ethanolic extracts of *N. pubescens* petals (**B**), *N. pubescens* receptacles (**C**), *N. nucifera* receptacles (**D**), *N. nucifera* pollens (**E**), *Nymphaea* sp. pollens (**F**), *N. cyanea* petals (**G**), *N. stellata* petals (**H**) and *V. amazonica* petals (**I**).

2.5. Contents of 6 Flavonoids in Some Plant Members of Nelumbonaceae and Nymphaeaceae

HPLC analysis was applied for the detection and quantification of **1–6** in eight extracts (5 mg/mL) from plants belonging to the family of Nelumbonaceae and Nymphaeaceae which showed high to moderate PDE5 inhibitory activity (Table 3). From a comparison of six flavonoid constituents found in *N. pubescens* petals with other extracts, it was found that **3**, **4** and **5** were also present in other extracts whereas **1**, **2**, and **6** were only found in *N. pubescens* petal extract. Their HPLC chromatograms are shown in Figure 2B–I.

Table 3. The flavonoid contents in some plants in the family of Nelumbonaceae and Nymphaeaceae determined by the HPLC method (n = 3).

Sample	Contents of the Flavonoid Constituents (Mean ± SD) (mg/g Ethanolic Extract)					
	1	2	3	4	5	6
Nelumbo nucifera						
Receptacles	-	-	4.55 ± 0.25	-	-	-
Pollens	-	-	2.55 ± 0.18	-	7.34 ± 0.35	-
Nymphaea sp.						
Pollens	-	-	3.09 ± 0.13	16.67 ± 056	-	-
Nymphaea cyanea						
Petals	-	-	-	-	2.71 ± 0.18	-
Nymphaea stellata						
Petals	-	-	-	-	0.82 ± 0.01	-
Nymphaea pubescens						
Petals	7.10 ± 0.10	17.31 ± 0.05	0.61 ± 0.00	4.72 ± 0.08	0.40 ± 0.00	1.04 ± 0.01
Receptacles	-	-	6.38 ± 035	8.32 ± 0.67	1.40 ± 0.19	-
Victoria amazonica						
Petals	-	-	-	-	1.77 ± 0.16	-

3. Discussion

In this study, thirty-seven ethanolic extracts from the plants of the Nelumbonaceae and Nymphaeaceae family were screened for their PDE5 and arginase inhibitory activities. This is the first time that this screening has been performed on these plants. No extract showed any significant inhibitory activity on arginase, meaning that none of the extracts was able to enhance the production of NO. This means that there was insufficient availability of NO resulting from L-arginine [8]. Conversely eight extracts showed PDE5 inhibitory activity, i.e., extracts from *N. nucifera* receptacle and pollen, *Nymphaea* sp. pollen, *N. cyanea* petals, *N. stellata* petals, *N. pubescens* petals and receptacle and *V. amazonica* petals. The petals of *N. pubescens* had the highest IC_{50} values of 6.37 ± 0.65 µg/mL whereas the pollen of *Nymphaea* sp. had the lowest IC_{50} values of 25.61 ± 2.74 µg/mL. The effect of PDE5 inhibition of these extracts have not been previously reported. The ethanolic extract of *N. stellata* leaves has shown aphrodisiac activity in male rats [35]. Neferine, which is a compound isolated from the green seed embryo of *N. nucifera*, was also reported to have a relaxant effect on rabbit corpus cavernosum tissues [36]. However, there is still no evidence that such activities related to PDE5 inhibitory effects.

Six flavonoid constituents of the *N. pubescens* petals ethanolic extract showed micromolar levels of IC_{50} against PDE5 activities. Compounds **1**, **2**, **4** and **6** were isolated and identified as components in *N. pubescens* petals for the first time. Previous studies reported that **4** was found in the *N. stellata* flowers [37], **3** and **5** were found in the *N. pubescens* petals [18,38,39], while **6** was found in the *N. stellata* flowers [40] and the *N. Alba* flowers [41] and leaves [42]. Compound **4** has been previously isolated from *Rhamnus nakaharai* Hayata (Rhamnaceae) and reported to inhibit PDE5 enzymes isolated from the lungs and hearts of male guinea pigs with an IC_{50} value of 86.9 µM [43], **3** and **5** have been previously isolated from *Anaxagorea luzonensis* and reported to inhibit PDE5 enzymes isolated from the mice lungs [14], while **1**, **2**, and **6** has not previously been reported for their effects on PDE5. Moreover, quercetin, kaempferol, and their derivatives have shown other biological

activities such as antibacterial, antifungal, and anti-oxidative properties including reducing LDL oxidation, platelet aggregation, and cardiovascular complications [44].

The structure–activity relationship (SAR) on the PDE5 inhibitory activity of quercetin (**3**) and kaempferol (**5**) is depicted in Figure 1. The PDE5 inhibitory activities of **3** were 7–9 times stronger than those of **1**, **2**, and **4**, which indicated that the methyl group at position C-3 and the sugar at position C-3′ of quercetin reduced the activities. The same results were seen between **5** and **6**. The inhibitory action against PDE5 is decreased by a methyl group substitution at position C-3 of kaempferol. The PDE5 inhibitory activity of quercetin, kaempferol, and their derivatives have been reported in previous studies [14,29,45]. These compounds not only inhibit the PDE5 enzyme but also have antioxidant activity [29]. According to the review article, using PDE5 inhibitors and antioxidants in combination led to better ED without a rise in side effects [46]. However, we demonstrated the SAR of quercetin and kaempferol for the first time, which involves the substitution of methyl groups and sugars at positions C-3 and C-3′.

The HPLC method was used for the chemical characterization of the extracts in our studies, especially for the *N. pubescens* petal extract. The HPLC fingerprints of the "active" extracts were established and the quantitative determination of the six flavonoids in the extracts was validated. The HPLC chromatogram showed good separation (Figure 2). The calibration curves provided linearity range of 0.5–400 µg/mL for **1–6** with satisfactory correlation coefficient values (r^2 = 0.9997–0.9999) (Table S1). The precisions expressed by %RSD were less than 10% and the percentage recovery was in the range of 80–120% (Table S2), which was in the acceptable range according to ICH guideline. Our results showed that this method had satisfactory sensitivity, precision and accuracy to detect and determine the flavonoids **1–6** in plant extracts. From the HPLC analysis, **2** was the major flavonoid compound in the *N. pubescens* petal extract with a concentration of 17.31 ± 0.05 mg/g. Compounds **1**, **3**, **4**, **5**, and **6** were present at much lower concentrations than **2** (2, 28, 4, 43, and 17 folds, respectively). This HPLC method can be used in a quality control process of *N. pubescens* petal extract if the extract is to be used for its health benefits relating to its PDE5 inhibitory activity. Although some HPLC systems for flavonoid analyses in other plant extracts have been reported with other plant extracts [47,48], our HPLC method was suitable for analysis of **1–6** in *N. pubescens* samples and other related spp. in our studies.

HPLC analyses of flavonoid constituents in the ethanolic extract of *N. pubescens* petals when compared with other ethanolic extracts, showed the presence of **3** in *N. nucifera* receptacle and pollen, *Nymphaea* sp. pollen and *N. pubescens* receptacle, **4** in *Nymphaea* sp. pollen and *N. pubescens* receptacle, and **5** in *N. nucifera* pollen, *N. cyanea* petals, *N. stellata* petals and *V. amazonica* petals. Paudel and Panth [28] reported that **3** and **5** were found in *N. nucifera* flower and stamen, which was similar to our results. Raja et al. [37] and Verma et al. [40] have reported that **3**, **4**, **5**, and **6** were found in *N. stellata* flower while we could observe only **5** in *N. stellata* petals. In addition, we identified the presence of flavonoids **5** in *N. cyanea* petals and *V. amazonica* petals for the first time.

4. Materials and Methods

4.1. Plant Materials

N. pubescens was collected from the Faculty of Pharmaceutical Science, Naresuan University, Phitsanulok, Thailand, and the other plants in the families of Nelumbonaceae and Nymphaeaceae were collected from Lotus Museum, Rajamangala University of Technology Thanyaburi, Pathum Thani, Thailand. The voucher specimens of *N. pubescens* (No. 004664), *Nymphaea* sp. (No. 05744), *N. stellata* (No. 05745), *N. cyanea* (No. 05746), *N. nucifera* (No. 05747), and *V. amazonica* (No. 05748) were kept at the Faculty of Sciences, Naresuan University, Phitsanulok, Thailand. The plants were identified by Assistant Professor Dr Pranee Nangngam, Department of Biology, Faculty of Sciences, Naresuan University.

4.2. Extraction and Isolation

The fresh plants were divided into leaves, petals, pollen, seed, receptacle, peduncles, and petioles, which were dried in a hot air oven at 55 °C for two days and then ground into powder. The powder was macerated with 95% ethanol for 3 days/time (two times), and then filtered and evaporated under a vacuum until dry, then stored at −20 °C until used. To isolate the PDE5 inhibitors, 9.67 g *N. pubescens* petals extract was dissolved in 100% MeOH (0.5 mL, concentration 100 mg/mL). The solution was loaded onto a solid-phase extraction (SPE) mini-column Strata C18-E (55 µm, 70 A), washed with 6 mL acetonitrile, and eluted with 0.1% formic acid in 80% water: 20% ACN for fractions 1–3 but fraction 4 was eluted with 100% acetonitrile. Fraction 4 (2.02 g) which contained flavonoids was evaporated to dryness and then purified on preparative HPLC (Gilson PLC 2020) fitted with a Kinetex EVO reverse-phase C18 column (250 × 21.2 mm, 5 µm). The solvent system used was 0.1% formic acid in 70% water (solvent A) and 0.1% formic acid in 30% acetonitrile (solvent B). The elution program (20 mL/min) was 30% B (0–25 min) and followed by a 10 min wash with 100% B and 15 min re-equilibration steps. The injection volume was 300 µL (300 mg/mL), and chromatograms were detected at 366 nm. Four pure compounds; (**1**) quercetin 3′-*O*-*β*-xylopyranoside (16.8 mg, Rt 16.5 min), (**2**) quercetin 3-methyl ether 3′-*O*-*β*-xylopyranoside (107.7 mg, Rt 17.2 min), (**4**) 3-*O*-methylquercetin (13.1 mg, Rt 20.2 min), and (**6**) 3-*O*-methylkaempferol (5.2 mg, Rt 22.9 min) were obtained. The purity of these compounds was checked by HPLC, NMR spectra, and another spectroscopic method.

4.3. Chemicals

Acetonitrile and methanol were of HPLC grade (VWR Chemicals, Fontenay-sous-Bois, France). Formic acid was of analytical grade (VWR Chemicals, Fontenay-sous-Bois, France). cGMP, crude snake venom (Crotalus atrox), bovine serum albumin (BSA), imidazole, ethylene glycol tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), magnesium chloride ($MgCl_2$), phenyl methyl sulfonyl fluoride (PMSF), diethylaminoethyl sephadex (DEAE-Sephadex), DTT (Dithiothreitol), and tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma-Aldrich. [^{3}H]-cGMP is obtained from Perkin Elmer (Boston, MA, USA). Purified liver bovine arginase 1 (1 U) of bovine arginase corresponding to the amount that was able to convert 1 µmol of L-arginine to urea and L-ornithine per minute at pH 9.5 and 37 °C) was purchase from MP Biomedicals (Illkirch-Graffenstaden, France). The (**3**) quercetin (purity > 98%, Sigma-Aldrich, St. Louis, MO, USA), and (**5**) kaempferol (purity > 90%, Sigma-Aldrich, St. Louis, MO, USA) were obtained from Sigma Chemical Company (St. Louis, MO, USA).

4.4. Phosphodiesterase 5 Inhibition Assay

4.4.1. Sample Preparation

The extracts were tested at the final concentration of 50 µg/mL and the compounds were tested at 10 µM. All samples were dissolved in 100% DMSO and diluted with distilled water. The final concentration of DMSO was 1%. For extracts or compounds that gave >80% PDE 5 inhibition, the IC_{50} was determined.

4.4.2. Enzyme Preparation

PDE5 enzymes were obtained from the transient PDE5A1 DNA transfection in human embryonic kidney 293 (HEK293) cells. These cells were homogenized using a sonicator probe and sonicate in Buffer A consisting of Tris-HCl (150 mM, pH 7.5), EDTA (6 mM), DTT (3 mM), and phenyl methyl sulfonyl fluoride (100 mM). The homogenate was centrifuged at 14,000 rpm for 20 min at 4 °C and the supernatant was used as a source of PDE5 enzymes [34].

4.4.3. Experimental Protocols

A PDE5 assay was performed following the method based on a two-step radioactive procedure [49]. In the first step of the enzymatic reaction, 25 µL of extracts or solvent

(5% DMSO) was added as a control to 25 µL of buffer C consisting of Tris–HCl (100 mM; pH 7.5), imidazole (100 mM), $MgCl_2$ (15 mM), and BSA (1.0 mg/mL), 25 µL of EGTA (10 mM), and 25 µL of PDE5 enzymes were added together with 25 µL of [^{3}H] cGMP (1 µM). This solution was then incubated at 30 °C for 10 min and then the reaction was stopped by placing it in boiling water for 1 min and cooled in ice-cold water. In the second step of the enzymatic reaction, 25 µL of snake venom (2.5 mg/mL) containing 5′-nucleotidase enzymes were added to the reaction mixture which was then incubated at 30 °C for 5 min. Then, 250 µL of Tris–HCl buffer (low salt buffer) (20 mM; pH 6.8) was added in the mixture. The reaction mixture was passed through a DEAE ion exchange resin column, and the uncharged [^{3}H] guanosine was eluted 4 times with 500 µL of a low salt buffer to obtain a hydrolysis product. Finally, the scintillant cocktail was added and the radioactivity was measured using a liquid scintillation analyzer (Tris-Carb 2910 TR, Perkin Elmer). The PDE5 enzymes were standardized to have a hydrolysis activity of 20–30% of the total substrate counts. The % hydrolysis and %PDE5 inhibition were calculated by the following Equations (1) and (2).

$$\%\ \text{Hydrolysis}_{\text{sample/control}} = \left[\frac{\left(\text{CPM}_{\text{sample/control}} - \text{CPM}_{\text{background}}\right)}{\left(\text{CPM}_{\text{total count}} - \text{CPM}_{\text{background}}\right)} \right] \times 100 \quad (1)$$

The $\text{CPM}_{\text{sample}}$ is the radioactive count rate of the assay with an enzyme. $\text{CPM}_{\text{background}}$ is the radioactive count rate of the assay but without enzyme. $\text{CPM}_{\text{control}}$ is the radioactive count rate of the assay with enzyme but without any sample. $\text{CPM}_{\text{total count}}$ is a count rate of 25 µL of substrate plus 2 mL of low salt buffer.

$$\%\ \text{PDE inhibition} = \left[1 - \left[\frac{\%\ \text{hydrolysis}_{\text{sample}}}{\%\ \text{hydrolysis}_{\text{control}}} \right] \right] \times 100 \quad (2)$$

The $\%\ \text{hydrolysis}_{\text{sample}}$ and $\%\ \text{hydrolysis}_{\text{control}}$ are the enzyme activities of the sample and solvent in the assay.

4.5. Arginase Inhibition Assay

4.5.1. Sample Preparation

The extracts were tested at the final concentration of 100 µg/mL. All samples were dissolved in 100% DMSO and diluted with distilled water. The IC_{50} of the extracts giving >70% arginase inhibition was determined.

4.5.2. Experimental Protocols

An arginase assay was performed by a spectrophotometric assay following the method in [50], based on the reaction of urea (product of arginase-catalyzed hydrolysis of L-arginine) and α-isonitrosopropiophenone with the generation of a pink imine monitored at 550 nm. The solutions were added to the 96-well microplate, in the following order: (i) buffer containing Tris-HCl (50 mM, pH 7.5) and 0.1% of bovine serum albumin (TBSA buffer) (10 µL), with or without (control) arginase (0.025 U/µL); (ii) Tris-HCl solution (50 mM, pH 7.5) containing 10 mM $MnCl_2$ as a cofactor (30 µL); (iii) a solution containing an inhibitor or its solvent (as a control) (10 µL); (iv) a solution of L-arginine (pH 9.7, 0.05 M) (20 µL). The microplate was covered with a plastic sealing film and then incubated for 60 min in a 37 °C, then 120 µL of $H_2SO_4/H_3PO_4/H_2O$ (1:3:7) was able to stop the reaction and the microplate was left on ice for 5 min. A 10 µL volume of α-isonitrosopropiophenone (5% in absolute ethanol) was added, and the microplate was covered with an aluminium sealing film and heated in a 100 °C oven for 45 min. The microplate was kept in the dark until reading. After 5 min of centrifugation and cooling for another 10 min, the microplate was shaken for 2 min and the absorbance was read at 550 nm and 25 °C using a spectrophotometer (Synergy HT BioTeck).

4.6. Sample Preparation for HPLC Analysis

The concentration of extracts was prepared at 5 mg/mL and the control flavonoids consisting of compounds (**1**) quercetin 3′-*O*-*β*-xylopyranoside, (**2**) quercetin 3-methyl ether 3′-*O*-*β*-xylopyranoside, (**3**) quercetin (purity > 98%, Sigma-Aldrich), (**4**) 3-*O*-methylquercetin, (**5**) kaempferol (purity > 90%, Sigma-Aldrich), and (**6**) 3-*O*-methylkaempferol were prepared as stock solutions at 1 mg/mL with 100% methanol (HPLC grade). All samples were filtered through a 0.45 µm nylon filter before performing HPLC analysis.

4.7. Instrumentation and Chromatographic Conditions

HPLC analysis was performed using a Shimadzu Prominence UFLC system equipped with a Shimadzu SPD-20A UV/Vis detector, a DGU-20A3 degasser, LC-20AT liquid chromatograph, and CBM-20A communications bus module. The column used was a Phenomenex Luna C18 column (150 mm × 4.6 mm, 5 µm) connected to a Phenomenex C18 (4 mm × 3 mm, 5 µm) guard column that maintained the temperature at 40 °C. The solvent system used was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient system was performed by increasing the ratio of solvent B from 20% to 70% within 25 min. The flow rate was set at 0.5 mL/min, the injection volume was 10 µL, and the UV detector was detected at 366 nm.

4.8. Method Validation

The development of the HPLC method was validated for linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, and accuracy which were according to ICH guidelines. The concentration values of each flavonoid consisting of compounds **1**, **3**, **4**, **5**, and **6** were 0.5, 1, 5, 25, 50, 75, and 100 µg/mL, while **2** was 5, 25, 50, 75, 100, 200, and 400 µg/mL. Calibration curves were constructed from each flavonoid in triplicate (n = 3). The LOD and LOQ were determined using a signal-to-noise ratio of each flavonoid which was 3 for LOD and 10 for LOQ. Intra-day precision was measured in triplicate (n = 3), while inter-day precision was measured in triplicate for three consecutive days (n = 9). Precision was represented by the percentage of relative standard deviation (%RSD). The accuracy was expressed as percentage recovery by using the spiked concentration of each flavonoid in the *N. pubescens* petals. The concentration of compounds **1**, **3**, **4**, **5**, and **6** in a test solution were 3, 25, and 65 µg/mL, while that of **2** was 15, 75, and 300 µg/mL. These experiments were performed in triplicate (n = 3).

4.9. Statistical Analyses

Data were expressed as the means ± standard deviation (SD). The concentration of extracts or compounds that induced 50% of the maximal inhibition (IC_{50}) was determined by fitting the original concentration–response curves using Graph Pad Prism software (version 5.0).

5. Conclusions

Among 37 samples from Nymphaeaceae and Nelumbonaceae families, our research found that *N. pubescens* petals had the strongest PDE5 inhibitory effect and have been re-ported for the first time. Six flavonoids with PDE5 inhibitory activity have been characterized as the constituents in the extract by the HPLC method. Compounds **1**, **2**, and **6** were found to inhibit PDE5 for the first time in this study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules28155821/s1, Table S1: Calibration data, LOD, and LOQ of the flavonoids **1–6** analyzed by HPLC. Table S2: Intra- and inter-day precision and accuracy of flavonoids **1–6** analyzed by HPLC.

Author Contributions: Conceptualization, T.P., C.G. and K.I.; methodology, T.P., K.I., N.S., P.T. (Prapapan Temkitthawon), C.G., N.C., X.-L.Y. and H.-M.G.; software, T.P.; validation, T.P., C.G. and K.I.; formal analysis, T.P., C.G. and K.I.; investigation, T.P.; resources, K.I., P.T. (Prapapan Temkitthawon), N.S. and C.G.; data curation, T.P., C.G. and K.I.; writing—original draft, T.P., C.G. and K.I.; writing—review and editing, T.P., C.G. and K.I.; visualization, T.P., K.I., N.S., P.T. (Prapapan Temkitthawon), K.C., C.G. and P.T. (Perle Totoson); supervision, K.I., C.G., H.-M.G. and Z.-J.Y.; project administration, K.I.; funding acquisition, K.I. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Naresuan University for human embryonic kidney 293 (HEK293) cells (approval number 010/2021; approval date 13 January 2021).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Sample Availability: Samples of compounds **1–6** are available from the authors.

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APPENDIX B: Publications

Second publication

Teerapap Panklai, Kornkanok Ingkaninan, Krongkarn Chootip, Prapapan Temkitthawon, Nungruthai Suphrom, Maude Tournier-Nappey, Corine Girard, Céline Demougeot, Perle Totoson*. Vasorelaxant and hypotensive effects of an ethanolic extract of *Nymphaea pubescens* and its main compound quercetin 3-methyl ether 3′-O-β-xylopyranoside. *Front. Pharmacol.* 2024. 15:1379752. (Q1, impact factor 5.6).

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Vasorelaxant and hypotensive effects of an ethanolic extract of *Nymphaea pubescens* and its main compound quercetin 3-methyl ether 3′-O-β-xylopyranoside

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Aim: *Nymphaea* plants were traditionally used to treat diseases associated with endothelial dysfunction. The present study investigated the effects of an ethanolic extract of *Nymphaea pubescens* Willd. (commonly named water lily, WL) and its main compound **1** (quercetin 3-methyl ether 3′-O-β-xylopyranoside) on vascular function in rats.

Materials and methods: The vasorelaxant effects of the WL extract and its main compound **1** and their underlying mechanisms of action were evaluated on isolated mesenteric arteries from Wistar rats. Blood pressure and heart rate were measured in anesthetized rats after infusion (i.v) of vehicle, WL extract, and compound **1** (at 0.01, 0.025, 0.05, 0.1, 0.5, and 1 mg/kg). Nifedipine was used as a positive control.

Results: Both WL extract and compound **1** induced vasorelaxant effects (with EC_{50} of 0.08 ± 0.01 mg/mL and 42.8 ± 6.3 μM, respectively) that were reduced by endothelium removal. A significant decrease in these relaxations was observed with L-NAME but not with apamin–charybdotoxin or indomethacin. In the endothelium-denuded condition, WL extract-induced relaxation was enhanced by 4-aminopyridine and glibenclamide, while iberiotoxin and ODQ (1H-[1,2,4] oxadiazolo[4,3-a]quinoxaline-1-one) had no effect. In contrast, compound **1**-induced relaxation was not changed by any of these inhibitors. Both WL extract

Abbreviations: 4-AP, 4-aminopyridine; ACh, acetylcholine; cGMP, cyclic guanosine monophosphate; COX, cyclooxygenase; DBP, diastolic blood pressure; DMSO, dimethyl sulfoxide; E, endothelium; EDHF, endothelium-derived hyperpolarizing factor; EGTA, ethylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid; HR, heart rate; IP_3, inositol 1,4,5 trisphosphate; L-NAME, N^G-nitro-L-arginine methyl ester; MA, mesenteric artery; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one; PE, phenylephrine; ROCCs, receptor-operated Ca^{2+} channels; SBP, systolic arterial blood pressure; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; SR, sarcoplasmic reticulum; VOCCs, voltage-operated Ca^{2+} channels; WL, water lily.

and compound **1** enhanced sodium nitroprusside-induced relaxation and inhibited receptor-operated Ca^{2+} channels. Only the WL extract was able to reduce PE-induced contraction ($p < 0.001$). As compared to the vehicle, the infusion of WL extract and compound **1** lowered systolic and diastolic blood pressure. Interestingly, the hypotensive effect of the compound was similar to that of nifedipine. The rebound tachycardia found at the highest dose of nifedipine was not observed with the WL extract or compound **1** ($p < 0.05$).

Conclusion and discussion: Our study demonstrated a vasorelaxant effect of the WL extract and its main compound quercetin 3-methyl ether 3′-O-β-xylopyranoside, relying on the potentiation of the NO-cGMP pathway and calcium inhibitory effects. These vasorelaxant effects were associated with a potent hypotensive effect, providing pharmacological evidence for the traditional use of this plant.

KEYWORDS

Nymphaea pubescens, vasorelaxation, mesenteric artery, hypotensive effect, compound

1 Introduction

The family Nymphaeaceae includes aquatic plants commonly named water lilies (WLs) and is classified into six genera, namely, *Barclaya*, *Euryale*, *Ondinea*, *Victoria*, *Nuphar*, and *Nymphaea*. *Nymphaea* is the most diverse and widespread, almost worldwide, and comprises approximately 45–50 species (Selvakumari et al., 2016). In many rural areas, young flowers and peduncles of *Nymphaea* are consumed fresh, cooked as vegetables, or used for the treatment of diseases such as erectile dysfunction or cardiometabolic diseases. Thus, the flowers of *Nymphaea pubescens* Willd. are widely used in many Asian countries as enhancers of erection (La-ongsri et al., 2009). In addition, ethnopharmacology studies demonstrated that extracts from *N. pubescens* petals had anti-diabetic, hypolipidemic, and antioxidant properties (Sathasivampillai and Rajamanoharan, 2021; Pokhrel et al., 2022). As the common hallmark of these diseases is the presence of endothelial dysfunction (Musicki et al., 2015; Konstantinovsky A et al., 2019; Evans CE et al., 2021), the above data suggest that extracts from *N. pubescens* petals have direct endothelial actions. Consistent with this hypothesis, previous studies using extracts from *Nymphaea lotus* Linn reported their capacity to enhance nitric oxide (NO) production from the endothelium of the aorta and corpora cavernosa (Mireille et al., 2016; Kameni et al., 2019). However, whether *N. pubescens* extract induces such a favorable effect on endothelial NO production has never been investigated. Of note, our previous work revealed that the main compound of an ethanolic extract of flowers from *N. pubescens* is a quercetin derivative, quercetin 3-methyl ether 3′-O-β-xylopyranoside (Panklai et al., 2022; Panklai et al., 2023). As quercetin is well-known to induce vasorelaxant effects through NO-dependent effects (Maaliki et al., 2019; Ciumărnean et al., 2020; Chen and Zhang, 2021; Grosso et al., 2022), this main compound might contribute to a positive endothelial effect in *N. pubescens*.

In the present study, the effects of an ethanolic extract of *N. pubescens* petals and its main compound (quercetin 3-methyl ether 3′-O-β-xylopyranoside, named compound **1**) were studied in an isolated rat mesenteric artery, with a focus on their endothelium-dependent and endothelium-independent mechanisms. To assess whether the results obtained *ex vivo* translated into a pharmacological effect *in vivo*, their acute hypotensive effect was determined in anesthetized rats.

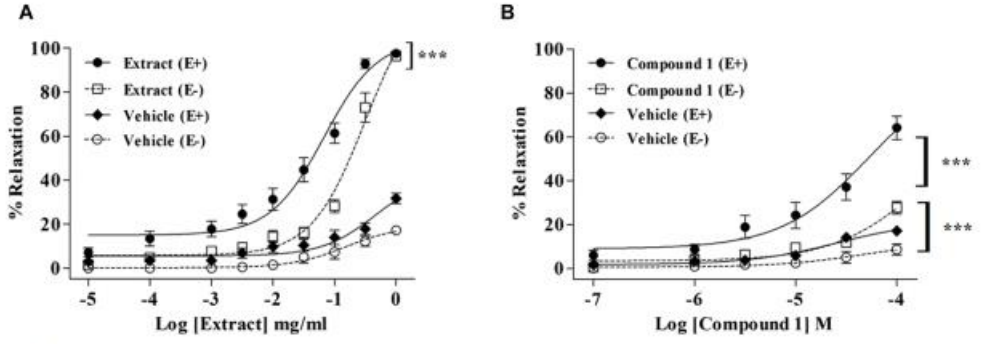

FIGURE 1
Relaxation of rat mesenteric artery rings pre-contracted with PE (10^{-5} M) and treated with accumulating concentrations of WL extract **(A)** or quercetin 3-methyl ether 3′-O-β-xylopyranoside **(1) (B)** in endothelium-intact (E+) and -denuded (E−). Relaxations are expressed as % contractions induced by PE. The values are presented as the means ± SEM (n = 5–11); ***$p < 0.001$.

TABLE 1 EC_{50} and E_{max} of WL extract- or quercetin 3-methyl ether 3′-O-β-xylopyranoside (1)-induced relaxations in mesenteric artery rings in the absence or presence of various inhibitors.

	WL extract		
	EC_{50} (mg/mL)	E_{max} (%)	n
Endothelium intact (E+)			
Vehicle	-	31.66 ± 2.08	5
Extract	0.08 ± 0.01	97.90 ± 1.17	11
+ L-NAME	0.18 ± 0.02	95.85 ± 1.08	7
+ Indomethacin	0.04 ± 0.01	95.49 ± 1.03	7
+ Apamin + charybdotoxin	0.03 ± 0.01	97.69 ± 0.36	7
Endothelium denuded (E−)			
Vehicle	-	17.17 ± 1.27	5
Extract	0.25 ± 0.03***	96.43 ± 0.73	11
+4-AP	0.06 ± 0.02††	98.09 ± 0.66	7
+ Glibenclamide	0.14 ± 0.04	98.00 ± 0.56	7
+ Iberiotoxin	0.26 ± 0.05	95.01 ± 0.71	7
+ ODQ	0.14 ± 0.03	89.45 ± 4.60	7
	Quercetin 3-methyl ether 3′-O-β-xylopyranoside (1)		
	EC_{50} (μM)	E_{max} (%)	n
Endothelium intact (E+)			
Vehicle	-	17.10 ± 1.17	5
1	42.76 ± 6.32	64.14 ± 5.08	10
+ L-NAME	>100	43.16 ± 7.71	7
+ Indomethacin	38.67 ± 8.35	78.41 ± 7.14	7
+ Apamin + charybdotoxin	4.42 ± 0.78**	79.56 ± 3.59	7
Endothelium denuded (E−)			
Vehicle	-	8.58 ± 2.29	5
1	>100	27.71 ± 2.66***	10
+4-AP	>100	36.40 ± 7.80	6
+ Glibenclamide	>100	40.04 ± 7.67	6
+ Iberiotoxin	>100	19.08 ± 2.34	6
+ ODQ	>100	40.19 ± 7.95	7

Values are presented as the means ± SEM. EC_{50} indicates the concentration of WL extract or compound **1** giving half-maximal relaxation. E_{max} is the maximum response of MA and is expressed as a relaxation percentage of the PE-induced contraction.
**$p < 0.01$.
***$p < 0.001$ vs Extract (E+) or 1 (E+).
††$p < 0.01$ vs Extract (E−).

2 Materials and methods

2.1 Plant names and parts used

The ptals of *N. pubescens* (WL) were collected from Phitsanulok at the Faculty of Pharmaceutical Science of Naresuan University, Thailand. The voucher specimen was identified by Assistant Professor Dr. Pranee Nangngam, Department of Biology, and kept at the Faculty of Sciences, Naresuan University, Phitsanulok, under No. 004664. The petals were first dried in a hot air oven at 55°C for 2 days. This dried material (182 g) was ground into powder and macerated with 95% ethanol (1.5 L) for 3 days (two times). Then, it was filtered and evaporated until dryness to give a yield of 32.33% (w/w) of crude ethanolic extract. In brief, compound **1** was isolated and purified using a solid phase extraction (SPE) mini-column, Strata C18-E (55 μm, 70 A), and a preparative PLC (Gilson

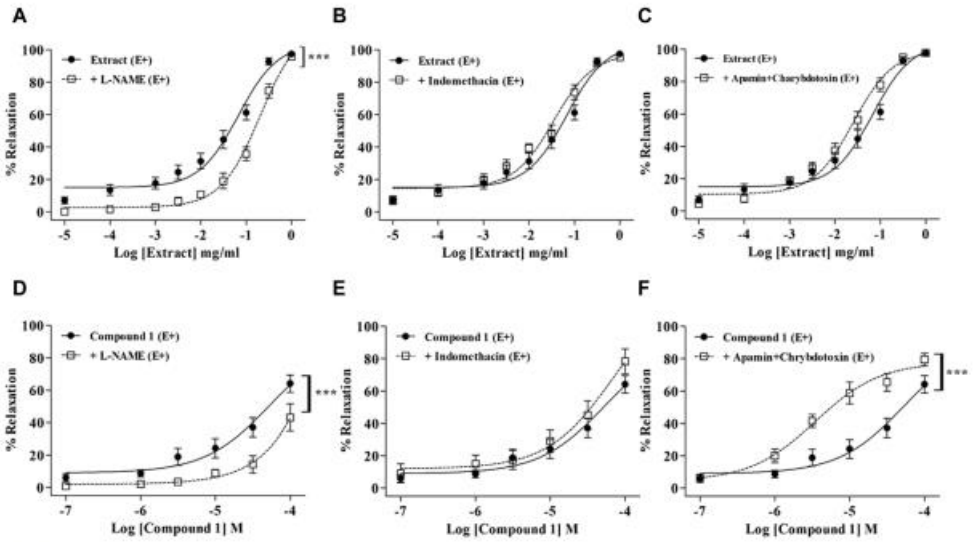

FIGURE 2
Relaxations of WL extract or quercetin 3′-methyl ether 3′-O-β-xylopyranoside (**1**) on endothelium-intact (E+) MA rings pre-contracted with PE and pretreated with inhibitors of various endothelial signaling pathways including 10^{-4} M L-NAME (**A, D**), 10^{-5} M indomethacin (**B, E**), or 10^{-7} M apamin plus 10^{-7} M charybdotoxin (**C, F**). The values are presented as the means ± SEM (n = 7–11); ***$p < 0.001$.

PLC 2020) fitted with a Kinetex EVO Reverse-Phase C18 Column (250 × 21.2 mm, 5 µm). Spectroscopic analysis was used to elucidate the structure of the isolated compound. The purity of compound **1** was greater than 98%, as measured by HPLC. The WL extract and compound **1** were stored at −20 °C until used.

2.2 Animal studies

Male Wistar rats (8–12 weeks old) were purchased from Janvier (Le Genest-Saint-Isle, France). Animals were kept at 22°C ± 1°C, under a 12–12 h light/dark cycle, with free access to drinking water and food pellets. The experimental design of the study was approved by the local committees for ethics in animal experimentation at the University of Franche-Comté (Besançon, France) under the number 2019/003-PT/5PR. All the investigation conforms to the Guiding Principles for Research Involving Animals: ARRIVE animal research.

2.3 Preparation of mesenteric arteries

The rats were anesthetized by intraperitoneal administration of sodium pentobarbital (60 mg/kg) and exsanguinated. Then, we excised the second-order branches of mesenteric arteries (MAs), cleaned them of connective tissue, and cut them into rings ~2 mm in length. The MA rings were loaded into organ chambers containing 6 mL of Krebs buffer (composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, and 12 mM glucose) at 37°C and continuously aerated with 95% O_2 and 5% CO_2. The MA rings were threaded on two stainless steel wires of 40 µm diameter. The contractile response (isometric force in mN) was measured by a force transducer connected to a multi-myograph system (Model 610 M v.2.2, DMT A/S, Denmark) and coupled to a data acquisition system: ChartTM Ver.7 software (ADInstruments, France). Resting tension was fixed for an initial equilibration period by stretching to their optimal lumen diameter. This optimal lumen diameter was chosen by setting the internal circumference to 90% of what the vessels would have if they were exposed to a transmural pressure of 100 mmHg. The MA rings were equilibrated for 15 min with normal Krebs. Then, they were routinely challenged with a 100 mM KCl solution to measure the vessel viability. The presence of functional endothelial cells was verified by pre-contracting with phenylephrine (PE, 10^{-5} M) and adding acetylcholine (ACh, 10^{-5} M) to induce more than 80% relaxation. In some rings, the endothelium was mechanically removed by gently rubbing inside the vessel with small mouse whiskers (Wisutthathum et al., 2018). The relaxation response to ACh (10^{-5} M) of less than 10% attested to the completeness of this endothelial denudation.

2.3.1 Study of the vasorelaxant effect of WL extract and compound 1

Endothelium-intact (E+) MA rings and endothelium-denuded (E−) MA rings were sub-maximally pre-contracted with PE (10^{-5} M), and then the response to cumulative concentrations of

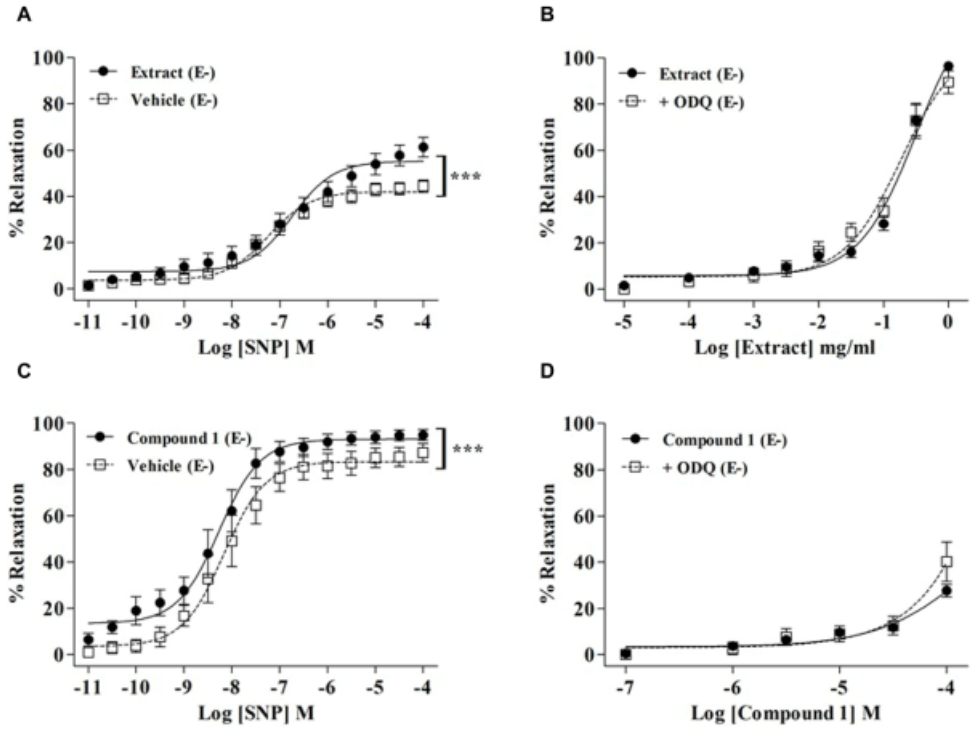

FIGURE 3
Effect of WL extract or quercetin 3-methyl ether 3′-O-β-xylopyranoside **(1)** on the sGC/CMP pathway. Endothelium-denuded (E−) MA rings were pretreated with WL extract, compound **1**, or vehicle and then pre-contracted with a 100 mM KCl solution **(A)** or PE (10^{-5} M) **(C)** prior to obtaining cumulative concentration–response for SNP (10^{-11}–10^{-4} M). WL extract- **(B)** or compound **1 (D)**-induced relaxation on endothelium-denuded (E−) MA rings pre-contracted with PE (10^{-5} M) and ODQ (10–5 M). The values are presented as the means ± SEM (n = 7–11); ***$p < 0.001$.

WL extract (10^{-5}-1 mg/mL) or compound **1** (10^{-7}–10^{-4} M) was determined to obtain concentration–response curves. The relaxation effect was calculated as the percentage of the contraction in response to PE. The effect of the vehicle (DMSO 0.09% for the WL extract and 0.1% for the compound **1**) was evaluated under the same conditions.

2.3.2 Role of endothelium-dependent pathways

The role of three endothelium-dependent pathways consisting of i) nitric oxide synthase (NOS), ii) cyclooxygenase (COX), and iii) endothelium-derived hyperpolarizing factor (EDHF) in the vasorelaxant activities of WL extract and compound **1** was investigated. For this purpose, the vasorelaxant effects of WL extract or compound **1** were studied in (E+) MA rings in the presence of N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M); an NOS inhibitor, indomethacin (10^{-5} M); a COX inhibitor or apamin (10^{-7} M) and charybdotoxin (10^{-7} M); and small- and intermediate-conductance Ca^{2+}-activated K^+ channel blockers (SK_{Ca} and IK_{Ca}) (Wisutthathum et al., 2018).

2.3.3 Role of vascular smooth muscle K^+ channels

To investigate the contribution of three types of K^+ channels to the relaxant effect of WL extract and compound **1**, (E−) MA rings were pre-incubated with i) glibenclamide (10^{-5} M), an ATP-sensitive potassium channel (K_{ATP}) blocker; ii) 4-aminopyridine (4-AP, 10^{-3} M), a voltage-gated potassium channel (K_V) blocker; or iii) iberiotoxin (10^{-7} M), a large-conductance Ca^{2+}-activated K^+ channel (K_{Ca}) blocker for 30 min. Then, PE (10^{-5} M) was added, and the cumulative concentration effects of WL extract or compound **1** were studied (Wisutthathum et al., 2018).

2.3.4 Role of the soluble guanylyl cyclase and cyclic guanosine monophosphate pathway

We investigated the involvement of the soluble guanylyl cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway in the relaxant effects induced by WL extract and compound **1**. To explore whether WL extract and compound **1** might modulate cGMP levels, the (E−) MA rings were incubated for 10 min with WL extract (at EC_{50} of 0.25 mg/mL), compound **1** (at EC_{50} of 100 μM), or the vehicle (0.03% for the WL extract and 0.1% for the **1**). Then, cumulative concentration

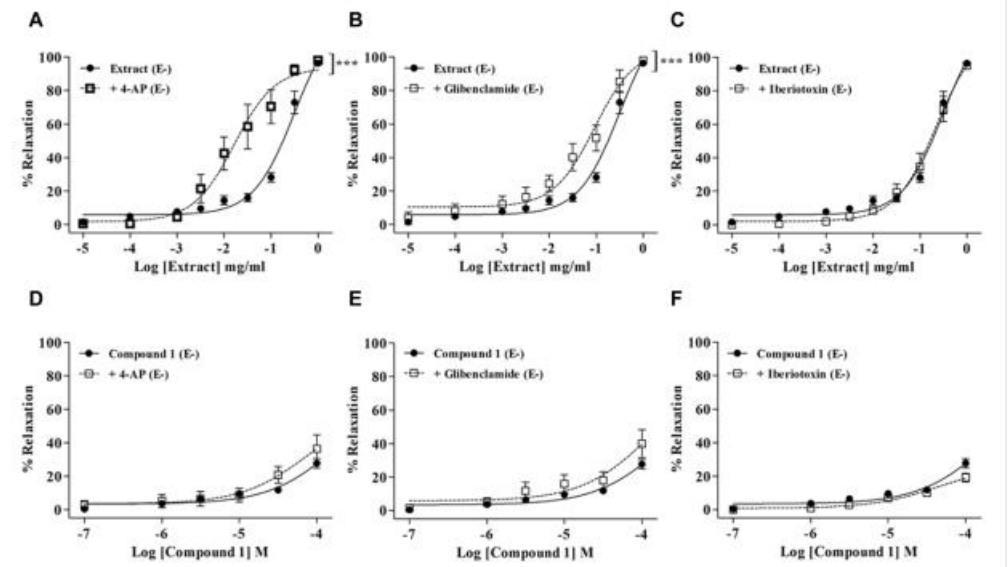

FIGURE 4
Relaxations of WL extract or quercetin 3-methyl ether 3′-O-β-xylopyranoside (**1**) on endothelium-denuded (E−) MA rings pre-contracted with PE (10^{-5} M) and pretreated with various K^+ channel inhibitors including 10^{-3} M 4-AP (**A, D**), 10^{-5} M glibenclamide (**B, E**), or 10^{-7} M iberiotoxin (**C, F**). The values are presented as the means ± SEM (n = 6–10); ***$p < 0.001$.

effects (10^{-11}–10^{-4} M) of sodium nitroprusside (SNP), a NO donor, were studied in 100 mM KCl-preconstricted rings (for the WL extract) or PE (10^{-5} M)-preconstricted rings (for compound **1**). Second, to determine whether a direct activation of sGC was involved in the WL extract- or compound **1**-induced relaxations, (E−) MA rings were incubated for 30 min with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{-5} M), a selective inhibitor of sGC, before adding 10^{-5} M of PE and subsequent cumulative concentrations of WL extract or compound **1**.

2.3.5 Possible action of WL extract or compound 1 on extracellular Ca^{2+}-induced contraction and sarcoplasmic reticulum Ca^{2+} release

To investigate the role of extracellular calcium (Ca^{2+}) influx in the WL extract- or compound **1**-induced relaxations, (E−) MA were incubated with a Ca^{2+}-free Krebs' solution containing methylene glycol-bis (2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, 2 mM) for 40 min. Then, PE (10^{-5} M) was added, and rings were washed with the Ca^{2+}-free Krebs' solution for 30 min (washed three times every 10 min) to deplete intracellular Ca^{2+} stores from the sarcoplasmic reticulum (SR). Then, MAs were incubated at EC_{50} of the WL extract (0.25 mg/mL), compound **1** (100 μM), or the vehicle, respectively, for 10 min, before adding PE (10^{-5} M) or 80 mM KCl for the opening of receptor-operated Ca^{2+} channels (ROCCs) or voltage-operated Ca^{2+} channels (VOCCs). $CaCl_2$ (10^{-2} M) was added to induce a contractile response.

To assess the effect of WL extract and compound **1** on the intracellular calcium (Ca^{2+}) release, (E−) MA rings were incubated with a Krebs' solution for 40 min, then incubated with the L-type voltage-dependent Ca^{2+} channel inhibitor (verapamil, 10^{-7} M) for 30 min. Then, MA rings were incubated at EC_{50} of the WL extract (0.25 mg/mL), compound **1** (100 μM), or the vehicle for 15 min. After that, PE (10^{-5} M) was added to the bath, and the transient contraction was determined to estimate the amount of Ca^{2+} release from the sarcoplasmic reticulum.

2.3.6 Role of α_1 receptor in WL extract and its main compound-induced relaxations

For this purpose, (E−) MA rings were incubated at EC_{50} of the WL extract (0.25 mg/mL, compound **1** (100 μM), or the vehicle for 15 min before cumulative vasocontraction was performed with PE (10^{-10}–10^{-4} M). The results were obtained in percentage contraction by comparison of the maximum contraction of PE (10^{-5} M) without treatment (Paracha et al., 2019).

2.4 Blood pressure and heart rate measurements

To assess whether the direct *in vitro* vascular effect of WL extract and compound **1** on resistance vessels translated into an *in vivo* effect, we studied the acute hypotensive effect of WL extract and compound **1** in anesthetized rats. Normotensive male Wistar rats were anesthetized with pentobarbital (60 mg/kg, i.p.); then, the left carotid artery was catheterized using a polyethylene tube (0.279 mm i.d. × 0.609 mm o.d.) filled with heparinized saline(50 units/mL saline) and connected to a pressure transducer (model BP-100 Blood Pressure Transducer, iWorx Systems, Inc., Dover, NH, United States). The output pressure was recorded by using a bridge amplifier coupled to PowerLab® Recording

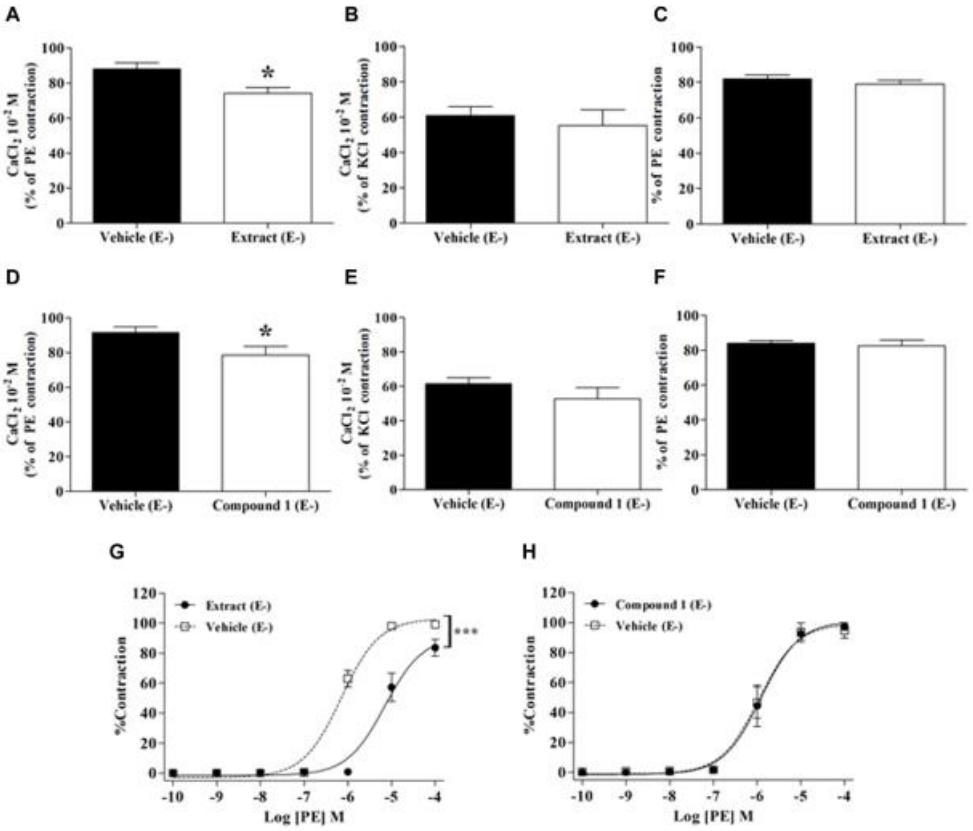

FIGURE 5
Effect of WL extract or quercetin 3-methyl ether 3′-O-β-xylopyranoside (**1**) on extracellular/intracellular Ca^{2+} fluxes and α1-adrenergic receptors. Experiments were performed in endothelium-denuded (E−) MA rings. $CaCl_2$ (10^{-2} M) was added in the presence of vehicle, WL extract, or compound 1 in Ca^{2+}-free Krebs solution, after pre-constriction with PE (10^{-5} M) (**A**, **D**) or 80 mM KCl (**B**, **E**). In separate experiments, rings were pre-incubated with verapamil (10^{-7} M) for 30 min, then pre-incubated with vehicle, WL extract, or compound **1** before adding PE (10^{-5} M) (**C**, **F**). Finally, rings were pre-incubated with WL extract, compound **1**, or vehicle, followed by contraction with cumulative concentrations of PE (10^{-10}–10^{-4} M) (**G**, **H**). The values are presented as the means ± SEM (n = 7–10); *$p < 0.05$ and ***$p < 0.001$.

System and a Chart™ application program (Ver.6 ADInstruments, Castle Hill, NSW, Australia). In addition, 15 min of stabilization period was applied, and then systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were recorded before and during intravenous injection (at 1 mL/min) of vehicle (a saline solution containing 5% DMSO, 1 mL/kg) or cumulative and increasing doses (0.01, 0.025, 0.05, 0.1, 0.5, and 1 mg/kg) of WL extract, compound **1**, or nifedipine. Each drug was administered to a different animal.

2.5 Drugs

4-AP, ACh, apamin, EGTA, glibenclamide, iberiotoxin, indomethacin, L-NAME, nifedipine, ODQ, PE, SNP, and verapamil were purchased from Sigma ChemicalCompany (St. Louis, MO, U.S.A.). Charybdotoxin was obtained from Enzo Life Sciences Company (France), and DMSO was obtained from VWR International Ltd. (Prolabo Chemicals, United Kingdom). All substances were dissolved in distilled water except 4-AP, compound **1**, glibenclamide, nifedipine, and WL extract, which were dissolved in DMSO and indomethacin in 0.5% w/v Na_2CO_3.

2.6 Statistical analysis

Values represent the means ± standard error of the mean (SEM). The WL extract- and compound **1**-induced

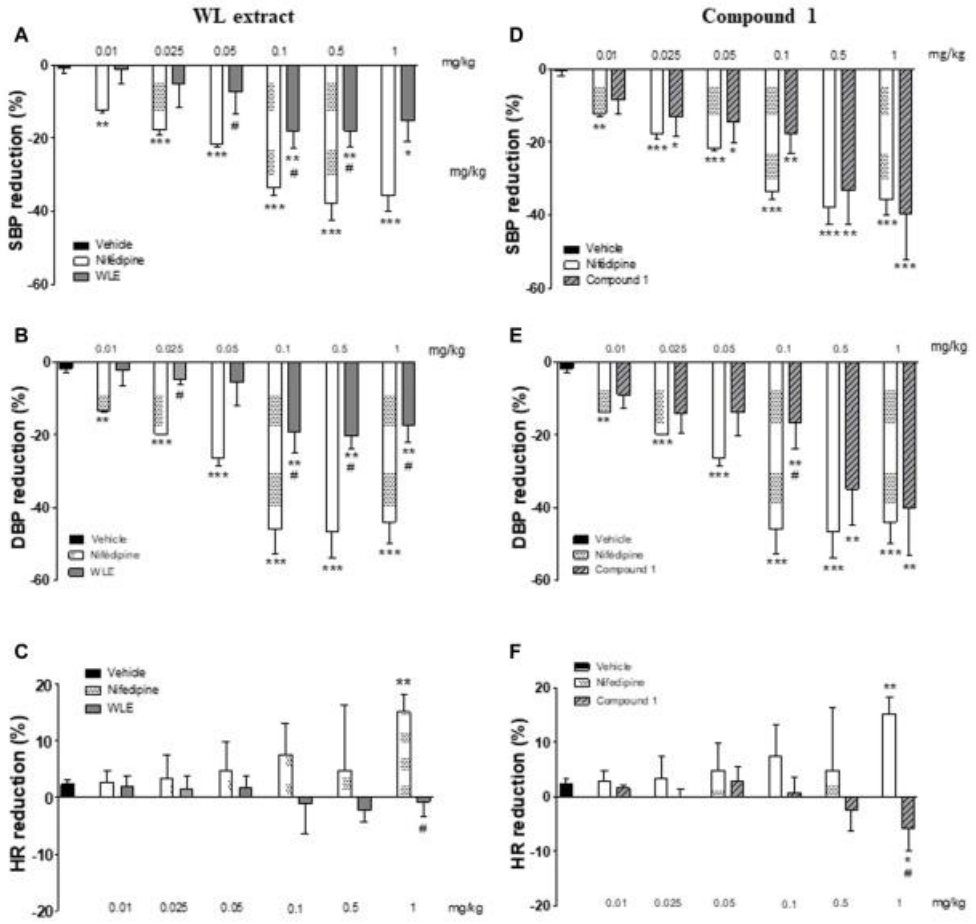

FIGURE 6
Acute hypotensive effect of WL extract and its main compound **1** (quercetin 3-methyl ether 3'-O-β-xylopyranoside) in rats. Percentage of reduction in SBP: systolic blood pressure **(A, D)**, DBP: diastolic blood pressure **(B, E)**, and HR: heart rate **(C, F)**. The values are presented as the means ± SEM (n = 4–5 rats per group). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. vehicle, and #$p < 0.05$ vs. nifedipine at the same dose.

vasorelaxations were calculated as the percentage of contraction to PE (10^{-5} M). The concentration of WL extract or compound **1** that induced 50% of the maximal relaxation (EC_{50}) and maximal relaxation induced (E_{max}) was determined by logit transformation of the normalized concentration–response curves using the 5.0 version of GraphPad Prism software. The concentration–response curves were compared using a two-way ANOVA for repeated measures, followed by a Bonferroni's test. A comparison between two values was assessed using an unpaired Student's t-test or Mann–Whitney *U* test when the data were not normally distributed. The *p*-value < 0.05 was considered to be significantly different.

3 Results

3.1 WL extract and its main compound induced vasorelaxant effect, relying on both endothelium-dependent and endothelium-independent mechanisms

As compared to the vehicle, WL extract (E_{max} = 97.90 ± 1.17%; EC_{50} = 0.08 ± 0.01 mg/mL) and compound **1** (E_{max} = 64.14 ± 5.08%; EC_{50} = 42.76 ± 6.32 μM) induced a concentration-dependent vasorelaxation in (E+) MA rings (Figure 1; Table 1). The removal of the endothelium significantly decreased the relaxant

effect in both WL extract and compound **1**, as confirmed by the increase in the EC_{50} values to 0.25 ± 0.03 mg/mL and 91.42 ± 18.45 μM, respectively ($p < 0.001$).

3.2 The endothelium-dependent vasodilation of WL extract and compound 1 is mainly NOS-dependent

L-NAME significantly reduced the relaxation induced by WL extract (Figure 2A) and compound **1** (Figure 2D) ($p < 0.001$), whereas indomethacin had no effect (Figures 2B,E). The incubation of mesenteric arteries with apamin and charybdotoxin did not modify the WL extract-induced relaxation (Figure 2C), while it enhanced the compound **1**-induced relaxation (Figure 2F) ($p < 0.001$).

3.3 The WL extract and its main compound increased the cGMP pathway

As endothelial NO production is strongly involved in WL extract- and compound-1-induced relaxations, we further explore whether WL extract or compound 1 might modulate signaling pathways downstream of NO in smooth muscle cells (VSMCs). As shown in Figures 3A,C, the NO donor SNP-induced relaxation was significantly enhanced in the presence of WL extract and compound **1** ($p < 0.001$). These data indicated that WL extract and compound **1** induced vasorelaxation through direct activation of sGC and/or by a downstream modulation of cGMP. To confirm whether a direct activation of sGC might be involved, the effect of ODQ was investigated. We found that ODQ did not modify the WL extract- or compound **1**-induced relaxations (Figures 3B,D).

3.4 The endothelium-independent effect was not reduced by K^+ channel blockers

As shown in Figure 4, none of the K^+ channel blockers were able to reduce the relaxation induced by the WL extract or compound **1**. On the contrary, WL extract-induced relaxation was significantly enhanced in the presence of 4-AP and glibenclamide (Figures 4A,B).

3.5 The vasorelaxant effect of WL extract and compound 1 relied on receptor-operated Ca^{2+} channel inhibition

The contributions of extracellular or intracellular Ca^{2+} fluxes are presented in Figure 5. As compared to the vehicle, WL extract and compound 1 slightly but significantly reduced the contraction elicited by extracellular Ca^{2+} influx in PE-exposed rings (opening of receptor-operated Ca^{2+} channels: ROCCs, $p < 0.05$, Figures 5A,D), whereas they did not change either the extracellular Ca^{2+} influx in high KCl-exposed rings (opening of voltage-operated Ca^{2+} channel: VOCC) or the intracellular Ca^{2+} release from the SR (Figures 5B,C,E,F).

3.6 The WL extract blocked α_1 receptor

As shown in Figure 5G, WL extract inhibited PE-induced contraction, suggesting an α1 receptor antagonism activity ($p < 0.001$), whereas compound **1** had no effect (Figure 5H).

3.7 WL extract and its main compound reduced blood pressure

As compared to the vehicle, the infusion of WL extract significantly decreased SBP and DBP at doses ranging from 0.025 to 1 mg/kg (Figures 6A,B). These hypotensive effects were lower compared to those of nifedipine. Interestingly, the effect of compound **1** in reducing blood pressure was similar to that of nifedipine (Figures 6D,E). Moreover, the rebound tachycardia found at the highest dose of nifedipine was not shown with WL extract or compound **1** ($p < 0.05$, Figures 6C,F).

4 Discussion

The new findings of our study are that 1) an ethanolic extract of *N. pubescens* petals induced a vasorelaxant effect on resistance vessels through mechanisms combining endothelium-dependent and -independent pathways; 2) this direct vascular effect translated into an acute hypotensive effect *in vivo*; and 3) the main component of the extract, quercetin 3-methyl ether 3′-O-β-xylopyranoside, induced vasorelaxant effects that can, at least partly, explain the relaxing actions of the extract.

In line with the traditional uses of extracts from *Nymphaea* petals to promote erectile function through an increase in endothelial NO production (Mireille et al., 2016), the present study showed that the *N. pubescens* extract induced a potent vasorelaxant effect relying on endothelium-dependent mechanisms. However, the relaxant effect of the extract was not abolished by endothelium removal, as illustrated by the switch to the right of the relaxation curves without a decrease in the E_{max} value of the extract, thus indicating that endothelium-independent mechanisms are also important contributors. Our data demonstrated that the effects of the extract involved the activation of the NO-GCs-cGMP pathway. Indeed, the relaxant effect was inhibited by a NOS inhibitor, indicating the capacity of the extract to stimulate endothelial NO production, but the results revealed that the extract also promoted NO signaling at the level of VSMCs. Moreover, the extract enhanced the effect of the NO donor but did not act itself as a direct sGC activator. Altogether, these results suggest that the phosphodiesterase inhibitory properties exhibited by the extract (Panklai et al., 2023) may contribute to its relaxant effect, but the causal relationship has to be investigated in future studies. Regarding the effect of the extract on calcium fluxes, only extracellular calcium entry through ROCCs was weakly but significantly inhibited by the extract, without any effect on VOCC or intracellular calcium release from SR. In addition, the extract exhibited a weak inhibition of the PE-induced contracting effect, suggesting α_1-receptor antagonism. Regarding this latter mechanism, as ROCCs are activated by agonists acting on G-protein-coupled receptors, such as phenylephrine, we cannot

exclude that the inhibitory effect of the extract on ROCC contributes to, or is responsible for, the reduction of the PE constricting action. The relaxation of VSMCs is also mediated by hyperpolarization secondary to the opening of different types of K^+ channels, including Kv, K_{ATP}, and K_{Ca} (Bosnjak, 1993). Here, we showed that the effect of *N. pubescens* extract was not reduced by any of the K^+ channel blockers. In contrast, 4-aminopyridine and glibenclamide enhanced extract-induced relaxation. These unexpected results could be explained by the interaction of several active molecules on different isoforms of these K^+ channels, the specificity of which deserves further investigation.

The main compound of the extract is the quercetin derivative, quercetin 3-methyl ether 3′-O-*β*-xylopyranoside, with a content of 1.73% (w/w) (Panklai et al., 2022). The present study reported for the first time that this quercetin derivative had a vasodilatory effect, albeit not very potent, as illustrated by its E_{max}, which did not reach 100% relaxation. These data suggest that this compound is probably involved in the relaxant effect of the extract, but other compounds that remain to be identified are also involved. Of interest, our previous work demonstrated that the WL extract also contains, to a lesser extent, quercetin 3′-O-β-xylopyranoside, quercetin, 3-O-methylquercetin, kaempferol, and 3-O-methylkaempferol (Panklai et al., 2023). Mechanistically, the main compound quercetin 3-methyl ether 3′-O-*β*-xylopyranoside shared with the extract the same effects on the NO-sGC-cGMP pathway and ROCCs but did not present an α_1-receptor antagonistic effect. As compared to quercetin, which is a known vasodilator of MA (Nishida and Satoh, 2013; Satoh and Nishida, 2014), our data indicate that the methyl ester group in the 3′ position and the presence of the xylopyranoside derivative did not alter the vasorelaxant properties. Nevertheless, a few mechanistic differences exist, as the relaxing effects of quercetin on MA were mainly related to EDHF and slightly through the endothelial NO pathway (Nishida and Satoh, 2013).

To determine whether the vasorelaxant effects observed *ex vivo* were still present after *in vivo* administration, the acute hypotensive effects of WL extract and quercetin 3-methyl ether 3′-O-*β*-xylopyranoside were investigated. The results showed that the extract acutely reduced blood pressure, with an effect that was, however, lower than that of nifedipine, used as a comparator. The limitation of our experiment is that it was conducted in normotensive rats, i.e., rats with a normal endothelial function, thus probably hampering the endothelial component of the relaxant effect of the WL extract. Further studies exploring the effect of the extract on animal models of hypertension are now required. However, this hypotensive action is in agreement with the anti-hypertensive effect previously reported with an aqueous extract from the flowers of another *Nymphaea (lotus)* L. (Kameni et al., 2019). The hypotensive effect of quercetin 3-methyl ether 3′-O-*β*-xylopyranoside was found to be similar to that of nifedipine. Again, these data indicate that the methyl ester group in the 3′position and the presence of the xylopyranoside derivative did not reduce the hypotensive effect of quercetin (Duarte et al., 2001; Brüll et al., 2015). It is noteworthy that the *in vivo* hypotensive effect of quercetin 3-methyl ether 3′-O-*β*-xylopyranoside is more pronounced than the effect of the WL extract, while its efficacy to induce mesenteric artery relaxation is less. The pharmacokinetic parameters of compound **1** deserve to be further explored to understand this potent hypotensive activity. Moreover, as compared to nifedipine, this quercetin derivative did not induce rebound tachycardia at high concentrations but, on the contrary, reduced heart rate. Altogether, these results suggest that a direct cardiac effect is possible with this compound. Further studies will be needed to better characterize this putative mechanism.

5 Conclusion

The present study showed that the ethanolic extract of *N. pubescens* petals induced vascular relaxing effects on the rat mesenteric artery, relying not only on the potentiation of NO production by the endothelium but also on endothelium-independent mechanisms such as the activation of the sGC-cGMP pathway and inhibition of ROOCs. Our data provide pharmacological evidence for the traditional use of this plant in diseases associated with hampered endothelial function. The main compound, quercetin 3-methyl ether 3′-O-*β*-xylopyranoside, is a possible contributor to these effects, but other molecules are likely involved that remain to be identified.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the local committees for ethics in animal experimentation of the University of Franche-Comté, under reference 2019/003-PT/5PR. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

TP: conceptualization, data curation, formal analysis, investigation, and writing–original draft. KI: conceptualization, funding acquisition, project administration, resources, supervision, and writing–review and editing. KC: conceptualization, funding acquisition, project administration, resources, and writing–review and editing. PT: conceptualization, data curation, investigation, methodology, and writing–review and editing. NS: data curation, formal analysis, methodology, software, and writing–review and editing. MT-N: data curation, formal analysis, methodology, software, and writing–review and editing. CG: conceptualization, funding acquisition, project administration, resources, supervision, writing–original draft, and writing–review and editing. DC : conceptualization, funding acquisition, project administration, resources, validation, writing–original draft, and writing–review and editing. PT: conceptualization, data curation, project administration, supervision, validation, and writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX C: Communications

1. Oral presentation at the congress of the Forum des Jeunes Chercheurs 2022, École Doctorale Environnement & Santé, Bâtiment Gabriel, Université de Bourgogne, Dijon, France, between 16-17 June 2022. The title is “Phosphodiesterase 5 inhibition and vasorelaxant effects of petals extract and its main compound”.