Effects of *Kaempferia parviflora* on Cardiovascular System

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ชื่อวิทยานิพนธ์ ผลของกระชายดำต่อระบบหัวใจและหลอดเลือด

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บทคัดย่อ

การแก่เป็นภาวะที่ซับซ้อนโดยส่วนใหญ่การแก่จะนำไปสู่โรคกลุ่มอาการทางเมแทบอลิก (metabolic syndrome) และโรคระบบหัวใจหลอดเลือด (cardiovascular diseases) เนื่องจากภาวะแก่จะเพิ่มการสะสมของไขมัน เกิดการเปลี่ยนแปลงทางระบบเมแทบอลิก รวมทั้งมีการเปลี่ยนแปลงโครงสร้างและการทำงานอวัยวะในระบบหัวใจและหลอดเลือดส่งผลให้หลอดเลือดมีการคลายตัวน้อยลง โดยการเปลี่ยนแปลงดังกล่าวสามารถตรวจพบได้ตั้งแต่วัยกลางชีวิต ดังนั้นการดูแลสุขภาพโดยการกินสารอาหารที่ช่วยป้องกันหรือชะลอการเกิดโรคกลุ่มอาการทางเมแทบอลิกและโรคระบบหัวใจหลอดเลือดควรเริ่มกินตั้งแต่อายุวัยกลางชีวิต สำหรับทางการแพทย์แผนไทยนั้นได้นำเหง้าจากกระชายดำ *(Kaempferia parviflora)* มาใช้เป็นยาในการควบคุมน้ำหนัก รักษาโรคความดันโลหิต และใช้เป็นยาอายุวัฒนะ เหง้ากระชายดำประกอบด้วยสารบริสุทธิ์กลุ่ม flavonoid อย่างน้อย 11 ชนิด ซึ่งสาร flavonoid ที่เป็นองค์ประกอบหลักมีอยู่ 3 ชนิดคือ 5,7-dimethxyflavone (DMF), 3,5,7,3′,4′-pentamethoxyflavone (PMF) และ 3,5,7-trimethoxyflavone (TMF, Mekjaruskul *et al.*, 2012) โดยมีการรายงานว่า PMF มีฤทธิ์กระตุ้นสมรรถนะทางเพศ โดยเพิ่มการคลายตัวของเนื้อเยื่อ cavernosum มนุษย์ และนอกจากนี้จากการศึกษาของ Mekjaruskul (2012) และคณะพบว่า PMF ยังมีชีวปริมาณออกฤทธิ์สูงกว่า DMF และ TMF (PMF มีค่า 3.32%, DMF มีค่า 2.10% และ TMF มีค่า 1.75 ตามลำดับ) จึงเป็นที่มาของงานวิจัยชิ้นนี้ซึ่งมีวัตถุประสงค์ 4 ข้อคือ (1) เพื่อศึกษาผลของการให้หนูแร็ทเพศผู้วัยกลางชีวิตกินสารสกัดจากเหง้ากระชายดำด้วยไดคลอโรมีเทน (KPD) ขนาด 100 มก/กกวันละ1 หรือ 2 ครั้ง เป็นเวลานาน 6 สัปดาห์ ว่ามีความเป็นพิษต่อร่างกายหรือไม่ โดยดูการเปลี่ยนแปลงทางจุลกายวิภาคศาสตร์ของตับและไต และวัดปริมาณเอนไซม์ในซีรั่ม ได้แก่ ALP, SGOT, SGPT, BUN,

และ creatinine ด้วยวิธีการ enzymatic method (2) ศึกษาผลของการให้กินสาร KPD ขนาดและระยะเวลาดังกล่าวต่อการเปลี่ยนแปลง 1) น้ำหนักสัตว์ทดลอง, อาหารที่กิน, การสะสมของเนื้อเยื่อไขมันและระดับไขมันในเลือด 2) การสะสมของไขมันที่ตับโดยการทำ frozen section แล้วย้อมด้วยสี oil red O 3) การทำงานของหลอดเลือดโดยการตัดแยกหลอดเลือดมาศึกษาใน organ bath ซึ่งงานวิจัยชิ้นนี้เน้นศึกษาผลต่อหลอดเลือด thoracic aorta และ mesenteric artery (3) ศึกษากลไกการออกฤทธิ์ของ PMF ต่อการกระตุ้นการคลายตัวของหลอดเลือด thoracic aorta ที่ตัดแยกมาศึกษาใน organ bath ซึ่งบรรจุสารละลายเครบส์ (4) ศึกษาผลของการให้หนูแร็ทเพศผู้วัยกลางชีวิตกินสาร PMF ขนาด 22 มก/กก วันละ 2 ครั้ง เป็นเวลา 6 สัปดาห์ ต่อความเป็นพิษ การเปลี่ยนแปลงของไขมัน และการทำงานของหลอดเลือดด้วยเทคนิคและวิธีการเช่นเดียวกับวัตถุประสงค์ที่ 1 และ 2 ดังที่ได้กล่าวไว้ข้างต้น จากการทดลองพบว่าการให้กินสาร KPD นาน 6 สัปดาห์ไม่มีผลต่อการเปลี่ยนแปลงทางจุลกายวิภาคศาสตร์ของตับและไต รวมทั้งไม่มีผลต่อระดับของ ALP, SGOT, SGPT, BUN, creatinine และความสมบูรณ์ของเม็ดเลือด โดยการกิน KPD ขนาด 100 mg/kg/1 ครั้งต่อวันไม่มีผลเปลี่ยนแปลงปัจจัยใดๆที่ศึกษา แต่อย่างไรก็ตามหนูที่กิน KPD ขนาด 100 mg/kg/ 2 ครั้งต่อวันมีผลทำให้ลดน้ำหนักตัว น้ำหนักเนื้อเยื่อไขมันในช่องท้องและใต้ผิวหนัง ลดการสะสมของไขมันในตับ รวมทั้งมีผลลดระดับกลูโคสและไตรกลีเซอไรด์ในเลือด อย่างไรก็ตามไม่มีผลต่อความดันโลหิตและอัตราการเต้นของหัวใจพื้นฐานในหนูแร็ทสลบ แต่มีผลต่อการทำงานของหลอดเลือดโดยมีผลลดการหดตัวของหลอดเลือดที่ตอบสนองต่อ phenylephrine (PE) ซึ่งผลดังกล่าวสามารถยับยั้งได้ด้วย N^G-nitro-L-arginine (L-NA, ยับยั้งการสร้าง nitric oxide) หรือโดยการทำลายเซลล์เยื่อบุผนังหลอดเลือด และการคลายตัวที่ตอบสนองต่อ acetylcholine สูงกว่ากลุ่มควบคุม ขณะที่ไม่พบความแตกต่างของการคลายตัวต่อ glyceryl trinitrate และจากการวัดการแสดงออกของโปรตีนพบว่าหลอดเลือดของหนูที่กิน PMF มีการแสดงออกของเอนไซม์ eNOS มากกว่ากลุ่มควบคุม โดยผลดังกล่าวพบทั้งในหลอดเลือด thoracic aorta และ mesenteric artery และจากการศึกษากลไกของ PMF ต่อการกระตุ้นการคลายตัวของหลอดเลือดพบว่า PMF มีฤทธิ์กระตุ้นการคลายตัวของหลอดเลือด ซึ่งฤทธิ์ดังกล่าวสามารถถูกยับยั้งได้ด้วย L-NA, ODQ หรือโดยการทำลายเซลล์เยื่อบุผนัง

หลอดเลือด แต่ในสภาวะที่มี L-NA ร่วมด้วยกับ ODQ หรือ Glybenclamide กลับมีฤทธิ์เสริมการคลายตัวของหลอดเลือด โดยฤทธิ์ดังกล่าวจะถูกยับยั้งได้ด้วย PAG และ SQ 22536 และจากการทดลองให้หนูกินสารบริสุทธิ์ PMF เป็นเวลานานพบว่าช่วยลดการหดตัวของหลอดเลือดที่ตอบสนองต่อ phenylephrine และ ผลดังกล่าวสามารถถูกยับยั้งได้ด้วย L-NA หรือโดยการทำลายเซลล์เยื่อบุผนังหลอดเลือด หลอดเลือดสามารถคลายตัวตอบสนองต่อ acetylcholine ได้ดีกว่ากลุ่มควบคุม แต่อย่างไรก็ตามไม่พบความแตกต่างของการตอบสนองต่อ glyceryl trinitrate นอกจากนี้พบว่า PAG (ยับยั้งการสร้าง H_2S) เพิ่มความตึงพื้นฐาน (baseline tension) ของหลอดเลือดที่ถูกยับยั้งด้วย L-NA ในกลุ่มที่กิน PMF สูงกว่ากลุ่มควบคุม ซึ่งหนูที่กิน PMF มีการแสดงออกของเอนไซม์ eNOS และ CSE มากกว่ากลุ่มควบคุม จึงสามารถสรุปได้ว่าการกินสาร PMF เป็นเวลานาน มีผลเพิ่มการแสดงออกของเอนไซม์ eNOS และ CSE ซึ่งช่วยเพิ่มการสร้างไนตริกออกไซด์และไฮโดรเจนซัลไฟด์ช่วยเพิ่มความยืดหยุ่นให้กับหลอดเลือด

การค้นพบนี้จึงเป็นข้อมูลสำคัญที่จะนำไปสู่การพัฒนาสาร KPD เป็นยาหรือผลิตภัณฑ์เสริมเพื่อป้องกัน หรือชะลอการเกิดโรคทางกลุ่มอาการเมแทบอลิก (metabolic syndrome) และโรคระบบหัวใจและหลอดเลือด (cardiovascular disease) โดยมี PMF เป็นสารบริสุทธ์หลักที่สำคัญตัวหนึ่งที่ออกฤทธิ์เพิ่มประสิทธิภาพการทำงานของหลอดเลือด ป้องกันการเกิดโรคระบบหัวใจหลอดเลือด (cardiovascular disease) ได้

คำสำคัญ: กระชายดำ, ระบบหัวใจและหลอดเลือด, หนูแร็ทวัยกลางชีวิต, การแก่, โรคกลุ่มอาการทางเมแทบอลิก

Thesis Title	Effects of *Kaempferia parviflora* on cardiovascular system
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ABSTRACT

Aging can be complicated. Two of the most common complications that come with aging are metabolic syndrome and cardiovascular diseases. Aging, in some people, leads to an accumulation of fat, alterations in the person's metabolism, as well as structure and function changes of the cardiovascular system. This leads to a decrease in vasodilatation, which starts out in middle-age stage. Therefore, people should begin to take the appropriate micronutrients to prevent or prolong development of the metabolic syndrome and/or cardiovascular disease early during their middle age. In traditional Thai medicine, rhizomes of *Kaempferia parviflora* (KP) have been used for controlling body weight, hypertensive treatment and for prolonging good health and well-being. The active chemical components of KP consist at least of 11 flavonoids with the major components being 5,7-dimethoxyflavone (DMF), 3,5,7,3′,4′-pentamethoxyflavone (PMF) and 3,5,7-trimethoxyflavone (TMF, Mekjaruskul et al., 2012). Among these three, PMF has been reported to act as an aphrodisiac activity as it causes relaxation of isolated human cavernosal strips (Jansakul et al., 2012). Additionally, oral bioavailability of PMF has the greatest bioavailability at 3.32% followed by DMF at 2.10% and TMF at 1.75%, respectively (Mekjaruskul et al., 2012). Recently, there was a study underway aimed to investigate 4 different things. The first experiment was to examine whether a KP dichloromethane (KPD) extracted at 100 mg/kg body weight when fed to middle-aged rats by oral gavage once or twice a day for 6 weeks. It tested for any toxicity effects measured by histology of the liver and kidney using hematoxylin and eosin staining, and liver and kidney functions defining on serum ALP, SGOT, SGPT, BUN and creatinine levels using enzymatic method. The second topic under investigation was if chronic oral administration of KPD once or twice a day had any effects on three different areas. The first was animal body weight and food intake, adipose tissues

accumulation, and lipid profiles. The second was liver lipid accumulation, which was measured by staining cryostat thin sections with oil red O. The third area was tested *in vitro* using isolated thoracic aortic rings incubated in Krebs Heinseleit solution on vascular function specified on thoracic aorta and mesenteric artery functions. The third topic under investigation was mechanisms underlying in vasorelaxation induced by PMF on isolated rat thoracic aortic rings. Studies were performed *in vitro* using isolated thoracic aortic rings in organ baths containing Krebs Henseleit solution. The fourth and final experiment investigated the effects on middle-aged male rat. It attempted to determine whether a 6 week oral administration of PMF at 22 mg/kg body weight twice a day to middle-aged male rats had any toxicity effect, or any effect on lipid profile or blood vessel functions, performed by the same procedure as described in experiments 1 and 2. Results showed that no differences were found between the KPD treated-, and the vehicle-control for histology of the liver and kidney and serum ALP, SGOT, SGPT, BUN and creatinine levels, nor as the total blood cell counts. None of the parameters tested were affected by 100 mg/kg KPD treatment once a day. However the rats were treated with KPD twice a day, which caused a decrease in body weight, visceral and subcutaneous fat and liver lipid accumulation and serum lipids (plasma glucose and triglycerides). Although KPD treatments didn't change basal blood pressure and heart rate in rats, it had an effect on vascular functions as it decreased the maximum contractile response to phenylephrine and this effect was eliminated by N^G-nitro-L-arginine (L-NA) or endothelium denuding. Relaxation of the PE-precontracted thoracic aortic ring to acetylcholine, but not to glyceryl trinitrate, was higher for the rats that were KPD-treated than for the control aortic rings. Western blot analysis showed that the eNOS expression of the PMF treated thoracic aortic ring was higher than the control groups. These effects appeared in both thoracic aorta and mesenteric artery. PMF causes concentration-dependent relaxations in phenylephrine (PE) precontracted aortic rings. This effect was eliminated after pretreatment with L-NA and ODQ (a soluble guanylyl cyclase inhibitor) or endothelium denuding. ODQ and Glybenclamide potentiate the relaxant activity of the PMF and this effect was inhibited by PAG (cystathionine-γ-lyase inhibitor) and SQ 22536 (adenylyl cyclase inhibitor). Also, chronic oral administration

of the PMF to middle-aged male rats caused a decrease in contractions to phenylephrine but in the presence of L-NA or by denudation of the vascular endothelium it was established that the effects disappeared. Moreover, chronic oral administration of the PMF caused enhancements in vasodilatation to acetylcholine. PMF-treated rats showed increases in the baseline tension of the L-NA-pretreated thoracic aortic ring, which was higher than that of the vehicle control group after pretreatment with PAG (a cystathionine-γ-lyase (CSE) inhibitor that utilized L-cysteine to produce H_2S). As previously mentioned, Western blot analysis showed that the eNOS and the CSE expression of the PMF treated thoracic aortic ring was higher than that of the control group. This indicates that PMF caused an upregulation of the expression of blood vessel eNOS and CSE proteins, resulting in an increase of NO and H_2S productions to modulate the blood vessel function. The discovery of these effects is extremely beneficial as KPD can be used to prevent and/or slow down the development of metabolic syndrome and/or cardiovascular disease. Also, these results indicated that PMF is one component of KP rhizomes that can improve the blood vessel function and prevent the development of cardiovascular disorder.

Keywords: *Kaempferia parviflora*, cardiovascular system, middle-aged rat, aging, metabolic syndrome

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LIST OF ABBREVIATIONS AND SYMBOLS

$[Ca^{2+}]_c$	=	cytosolic calcium concentration
AA	=	arachidonic acid
ABP	=	arterial blood pressure
AC	=	adenylate cyclase protein
ACE	=	angiotensin converting enzyme
ACh	=	acetylcholine
ADMA	=	dimethylarginine
Ang 1–7	=	angiotensin 1–7
ALP	=	alkaline phosphatase
ANS	=	the autonomic nervous system
AP	=	arterial pressure
AR	=	adrenergic receptor
ATP	=	adenosine triphosphate
AV	=	arterioventricular node
BAT	=	brown adipose tissue
BH_4	=	tetrahydrobiopterin
BK_{Ca}	=	large conductance Ca^{2+}-activated K^+ channel
BMI	=	body mass index
bpm	=	beat per minute
BUN	=	blood urea nitrogen
Ca^{2+}	=	calcium ion
CaM	=	calmodulin
cAMP	=	cyclic adenosine monophosphate
CAT	=	cysteine aminotransferase
CBS	=	cystathionine β-synthase
CE	=	cholesterol esters
cGMP	=	cyclic gounosine monophosphate
CICR	=	Ca^{2+}-induced Ca^{2+} release
CL	=	cysteine lyase

LIST OF ABBREVIATIONS AND SYMBOLS (CONT.)

CNS	=	central nervous system
COPD	=	chronic obstructive pulmonary
CPI-17	=	protein phosphatase 1 regulatory subunit 14A
cPLA2	=	cytosolic effector molecule phospholipase A2
CREAT	=	creatinine
CSE	=	cystathionine γ-lyase
CVA	=	cerebrovascular disease
CVD	=	cardiovascular diseases
CVS	=	cardiovascular system
C3	=	complement 3
DAG	=	sn-1,2-diacylglyserol
DMSO	=	dimethyl sulfoxide
DP	=	diastolic pressure
DW	=	distilled water
E-C coupling	=	excitation-contraction coupling
ED_{50}	=	median effective dose
EDCFs	=	endothelial-derived constricting factors
EDHFs	=	endothelium-derived hyperpolarizing factors
EDRFs	=	endothelial-derived relaxing factors
EDTA	=	Ethylene diamine *tetra* acetic adic
EDVFs	=	endothelial-derived vasoactive factors
EETs	=	eicosatrienoic acids
E_{max}	=	maximum efficacy
Epi	=	epinephrine
ER	=	endoplasmic reticulum
ET	=	endothelin
FC	=	free cholesterol
FFA	=	free fatty acid
GDI	=	guanine nucleotide dissociation inhibitor

LIST OF ABBREVIATIONS AND SYMBOLS (CONT.)

GEFs	=	guanine nucleotide exchange factors
Gi	=	pertussistoxin-sensitive G inhibitory protein
Glyben	=	glybenclamide
GPCR	=	G-protein couple receptor
Gs	=	heterotrimeric G stimulary protein
GTP	=	guanosine *tri*phosphate
H & E	=	hematoxylin and eosin
H_2O_2	=	hydrogen peroxide
H_2S	=	hydrogen sulfide
HDL-C	=	high density lipoprotein cholesterol
HMPC	=	The Committee for Herbal Medicinal Products
HNO	=	nitroxyl
HPLC	=	high performance liquid chromatography
IAF	=	intra-abdominal fat
I-CAM	=	intercellular adhesion molecule
IICR	=	IP_3-induced Ca^{2+} release
IK_{Ca}	=	intermediate conductance Ca^{2+}-activated K^+ channel
IHD	=	Ischemic heart disease
IL_6	=	Interleukin-6
IP_3	=	inosital (1,4,5)-*tri*phosphate
IR	=	Insulin resistance
IRS	=	Insulin receptor substrate
JAK	=	Janus kinase
K^+	=	potassium ion
K_{ATP}	=	ATP-dependent K^+ channel
K_{ir}	=	inwardly rectifying K^+ channel
K_V	=	voltage-operated K^+ channel
LDL-C	=	low density lipoprotein cholesterol
LG-NSCC	=	ligand- operated non-selective cation channel

LIST OF ABBREVIATIONS AND SYMBOLS (CONT.)

L-NA	=	N^G nitro L-arginine
MAP	=	mean arterial pressure
MAPK	=	mitogen-activated protein kinase
M-ChR	=	muscarinic cholinergic receptor
Mg^{2+}	=	magnesium ion
min	=	minute
MLC	=	myosin light chain
MLCK	=	myosin light chain kinase
MLCP	=	myosin light chain phosphatase
mmHg	=	millimeter of mercury
MMP	=	matrix metalloproteinase
mRNA	=	massager ribonucleic acid
msec	=	millisecond
MS	=	metabolic syndrome
MST	=	3-mercaptopyruvate sulphurtransferase
mtDNA	=	mitochondrial DNA
mV	=	milivolt
MyBP-C	=	myosin-binding protein C
Na^+	=	sodium ion
NCX	=	Na^+/Ca^{2+} exchange
NE	=	norepinephrine
NLAC-MU	=	National Laboratory Animal Centre-Mahidol University
NO	=	nitric oxide
NOS	=	nitric oxide synthase
PAG	=	DL-propargylglycine
PDE	=	phosphodiesterase
PGI_2	=	prostacyclin
PIP_2	=	phosphatidyl inosital (4,5)-*bis*phosphate

LIST OF ABBREVIATIONS AND SYMBOLS (CONT.)

PI3K	=	phosphatidylinositol 3 kinase
PKA	=	cAMP-dependent protein kinase A
PKC	=	protein kinase C
PKG	=	cGMP-dependent protein kinase G
PLB	=	phospholamban
PLA_2	=	phospholipase A_2
PLC	=	phospholipase C
PLD	=	phospholipase D
PP1C	=	protein phosphatase 1C
PT	=	portal triad
PVAT	=	perivascular adipose tissue
RAAS	=	renin-angiotensin-aldosterone system
RMP	=	resisting membrane potential
ROCK	=	Rho-associated, coiled-coil containing protein Kinase
ROS	=	reactive oxygen species
RVLM	=	rostral ventrolateral medulla
RyR	=	ryanodine receptor
S.E.M.	=	standard error of the mean
SA node	=	sinoatrial node
SDS-PAGE	=	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERCA	=	sarco-endoplasmic reticular ATPase pump
SF	=	subcutaneous fat
sGC	=	soluble gaunylate cyclase protein
SGOT	=	serum glutamic oxaloacetic tranaminase
SGPT	=	serum glutamic pyruvic transaminase
SK_{Ca}	=	small conductance Ca^{2+}-activated K^+ channel
SMOCC	=	second messenger- operated Ca^{2+} channel

LIST OF ABBREVIATIONS AND SYMBOLS (CONT.)

SOD	=	superoxide dismutases
SP	=	systolic pressure
SR	=	sarcoplasmic reticulum
SVF	=	stromal vascular fraction
TEA	=	tetraethylammonium
TnC	=	troponin C
TnI	=	troponin I
TNF-α	=	tumour necrosis factor-alpha
TPR	=	total peripheral resistance
Tx	=	Thromboxanes
UCP-1	=	uncoupling protein-1
VF	=	visceral fat
VOCC	=	voltage- operated Ca^{2+} channel
VSMC	=	vascular smooth muscle cell
WAT	=	white adipose tissue
WHO	=	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND AND RATIONALE

Cardiovascular disease (CVD) is an abnormal condition of the heart or the arterial circulation (Labarthe, 1998). It was the result of a large number of deaths in developed and industrialized countries since 1920. In North America alone, more than half of the total deaths were due to CVD. This number began to decrease in the end of the 1960s (Yusuf *et al.*, 2001). Recently, CVD has become the cause of death in many developing countries (Reddy & Yusuf, 1998). In Thailand, disease patterns have changed from infections disease to non-communicable diseases. CVD has become one of the leading causes of death over the period of economic development from the first National Economic Development Plan (1961-1966). It caused approximately 9.9 percent of all deaths in 1999. The two main categories of CVD deaths are cerebrovascular disease (CVA) and Ischemic heart disease (IHD) (59.3 and 27.0 percent of CVD deaths, respectively) (Bundhamcharoen *et al.*, 2002). Cardiovascular disease is caused by several factors, one being metabolic syndrome (MS). Galassi (2006) reported that MS is an important risk factor for CVD and that it has a strong association with disease and mortality. People who have MS are commonly overweight with a high abdominal fat distribution, dyslipidemia, disturbed glucose and insulin metabolism and hypertension. These symptoms are also common with CVD, atherosclerosis, and type-2 diabetes patients (liese *et al.*, 1998). Around 20-25% of the world population suffers from MS (Alberti *et al.*, 2006). It is extremely common in Thai adults over 20 years old. There are a total of 23.2%, 19.5% men and 26.8% women (Aekplakorn *et al.*, 2011). MS is influenced by many factors including age and lifestyle. Elderly MS and type-2 diabetes sufferers are three to five times more at risk for CVD than people without (Tereshina, 2009). For elderly people

obesity is the most prevalent risk factor for MS (Dominguez and Barbagallo, 2007). Inflamed adipokines that alter insulin sensitivities and muscle mass connected with obesity are more specific factors that cause CVD (Wilson and Kannel, 2002). For years it has been estimated that 15 to 20% of persons aged 70 years and over have MS (Banks *et al.*, 2003). Moreover, age directly affects structure and function of the cardiovascular system, which, in turn, leads to CVD. In ways such as, reduced compliance, increased inflammatory response and increased smoothness of vascular muscle cells (Lakatta and Levy, 2003; Marin, 1995; Rivard *et al.*, 1999). Fried *et al.* (1991) also discovered important links between age and deaths and illnesses from CVD. Cases have also come about in the United States of America (USA). 80% of deaths from CVD occurred in adults aged 65 years and over and 50% of people aged 60 years and over experienced CVD illnesses (Lloyd-Jones *et al.*, 2009).

Recently, the global expansion of traditional medicine has gained popularity. Approximately 75 to 80% of the populations of developing countries have used herbal medicines for primary health care., Within the last few years herbal medicine has even made its way into the markets of developed countries (Kamboj, 2000; WHO, 2002). The Committee for Herbal Medicinal Products (HMPC) has provided a definition of traditional medicine stating that,

> "Traditional herbal medicine is the sum total of the knowledge, skills, and practices based on the theories, beliefs and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health, as well as in the prevention, diagnosis, improvement or treatment of physical and mental illnesses."

Humans have used plant materials as herbal remedies since prehistoric times. From as early as 3000 BC ancient Chinese and Egyptian papyrus used plant materials for medicines (Fulder, 1996; Kirby, 1997). Chinese and Indian traditions have had great influence in Thailand (Mashour *et al.*, 1998). The use of traditional medicine decreased and was replaced by chemical drugs in the early 19th century, as scientists were able to access chemical analyses. They were able to extract and modify the active compound from plants in order to make plant compounds (Mashour *et al.*,

1998). The use of natural products and complementary and alternative medicine (CAM) has been on the rise throughout the last decade, especially with adults. Herbal medicine has rapidly developed in the naturceutical industry (Moquin, 2009). More recently, traditional medicine is being brought into medical and pharmaceutical schools. Therefore, studies conducted on the impacts of aging often discover CVD complications in elderly patients and for this reason it is of great interest to begin research in earlier stages in order to prevent CVD. As herbal medical studies have began to make their way back into mainstream medicine studies to suppress symptoms of vascular aging and obesity.

1.2 CARDIOVASCULAR SYSTEM

The circulatory system includes three anatomical components consisting of the heart, the vascular system and the autonomic nervous system. The autonomic nervous system includes the sympathetic and the parasympathetic nervous systems that act via the adrenergic receptors and muscarinic acetylcholine receptors (Hoffman *et al.*, 2001; Opie, 2004). These components interact in a complex manner to contribute blood flow to the organs in order to transport the essential substances to the tissues. The cardiovascular system operates in a closed loop in which blood circulates so that blood pumped out of the heart through one circuit returns to the heart by another circuit (Harvey, 1628). The heart pumps blood through two circuits of the vascular system, one being the pulmonary circuit and the second is the systemic circuit (Rhoades and Pflanzer, 2003). Pulmonary circulation operates as follows; the deoxygenated blood is pumped from the right ventricle through the pulmonary artery to the lungs for re-oxygenation and then the oxygenated blood returns to the left atrium through the pulmonary vein and then blood passes into the left ventricle. Arteries transport essential substances to the tissues. After that, deoxygenated blood that is carried from tissues returns to the heart through vessel veins (Al-Ghazal, 2007).

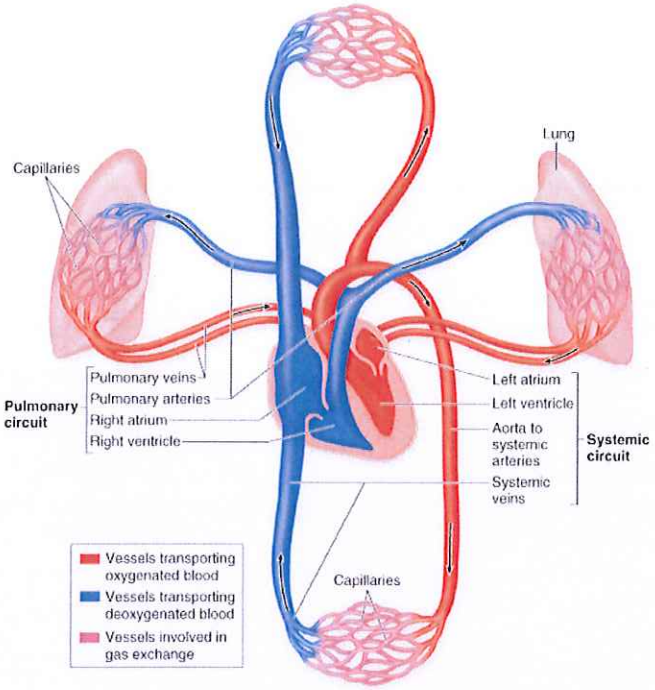

Figure 1 Schematic diagram of the cardiovascular system that consists of two circulation circuits including pulmonary circuit and systemic circuit (http://academic.kellogg.edu)

1.2.1 THE HEART

1.2.1.1 Anatomy

The heart is a very special organ. It is located inside a fibrous sac called the pericardium in the center of the chest above the diaphragm. Another fibrous membrane that is attached to the heart is called the epicardium. The myocardium, the walls of the heart, consists of cardiac muscle cells (Stanfield and Germann, 2008). The myocardium consists of contractile muscle cells known as cardiomyocytes, the pacemaker and conducting cells. The pacemaker and conducting system play a very important role; they initiate and spread the hearts electrical activity from contraction by pumping blood to vessels and the extracellular space (Opie, 2004). The heart is divided into two halves, right and left, and separated by the inter-ventricular septum. Each half is composed of an atrium and a ventricle that is separated by valves called

the atrioventricular (AV) valves. The heart is further divided into four chambers; the right and left atria, that receive deoxygenated blood from the venous system, and the right and left ventricles that pump oxygenated blood into the arterial system (Fox, 2004). The pulmonary and aortic valves consist of the canal of the right ventricle that pumps blood into the pulmonary artery and of the left ventricle that pumps blood into the aorta. These valves protect blood from flowing in the opposite direction during heart contractions and relaxation (Widmaier *et al.*, 2004).

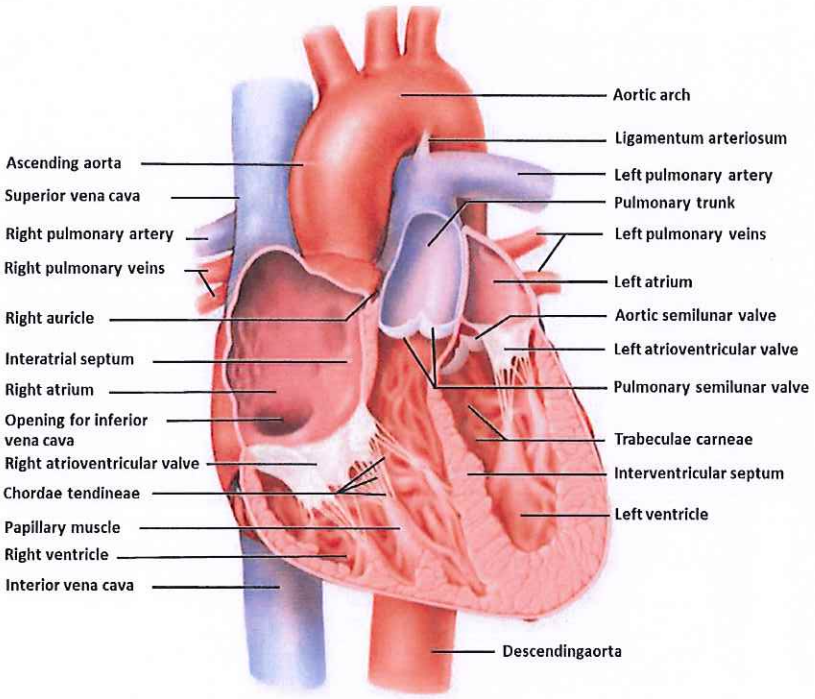

Figure 2 Diagrammatic section of the heart (modified from McKinley and O'Loughlin, 2007)

1.2.1.2 Physiology of cardiac muscle contraction

The anatomical components included in heart contraction include: the pacemaker and conducting tissues, which generate and spread the hearts electrical activity, and cardiomyocytes which function for contraction (Opie, 2004). The contraction of the heart acts as a single cell unit as the cardiac muscle cells are

interconnected in groups by gap function which acts as a unit in response to stimuli (Howland and Mycek, 2006).

The action potential

Cardiac muscle cells have the ability to generate a spontaneous intrinsic electrical impulse using the pacemaker cells located in the sinoatrial (SA), which flows through the right and left atria. After that, the blood flows through the atrioventricular (AV) node and then travels down to the bundle of His into the right bundle branch and the left bundle branch in ventricle for the ventricle contraction (Fig. 3) (Rhoades and Pflanzer, 2003).

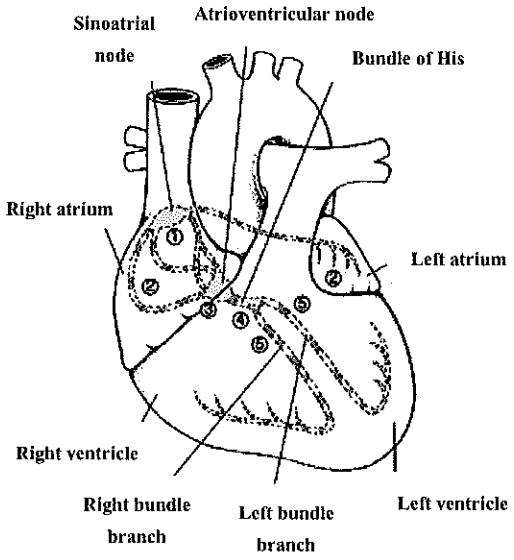

Figure 3 Normal electrical pathways of the heart (modified from Rhoades and Pflanzer, 2003)

The action potential begins during depolarization through the sarcolemma and stimulates the opening of ion-selective channels, which result in alterations across the sarcolemma (Opie, 2004). The functions of action potential can be divided into five distinct phases (0-4) (Howland and Mycek, 2006).

Phase 0: sodium (Na^+) channels open for fast inward Na^+ current to the cells, becoming depolarized.

Phase 1: Na^+ channels closed and potassium (K^+) channels open for outward K^+ current as a result of partial repolarization.

Phase 2: Opening of calcium (Ca^{2+}) channel for the Ca^{2+} entry, while the K^+ exits from the cell. This phenomenon leads to the plateau phase of the action potential.

Phase 3: Closing of Ca^{2+} channel, while the K^+ had opened. This phase is an outward current of K^+ and occurs for repolarization.

Phase 4: The end of the action potential. Spontaneous depolarization occurs by gradual increase in cellular permeability to Na^+.

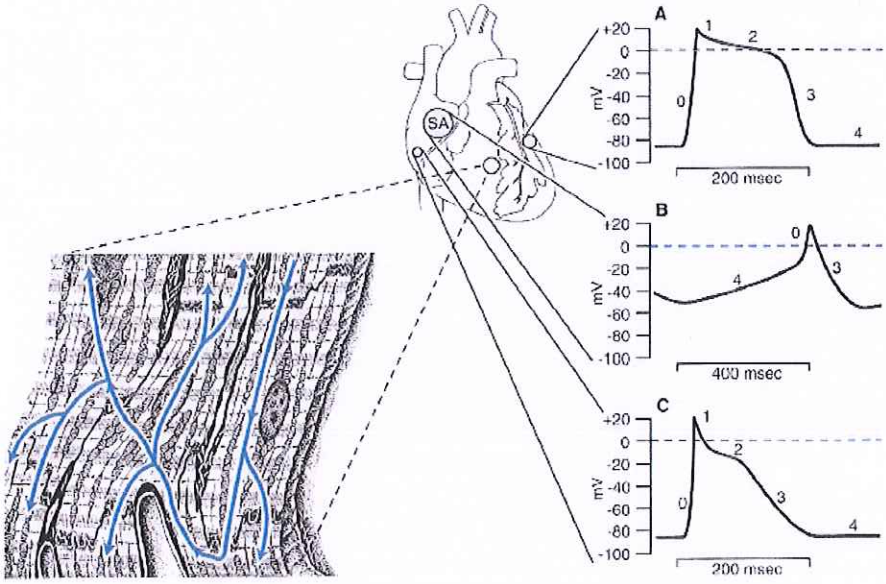

Figure 4 Action potential phases and currents. The four phases of the cardiac action potential (modified from Opie, 2004)

Cardiac excitation-contraction coupling

Cardiac excitation-contraction coupling is the process in which the contracting force of the cardiac muscle is equal to the concentration of free cytosolic Ca^{2+} (Howland and Mycek, 2006). Excitation of cardiac muscle starts when the action potential spreads along the myocardial sarcolemma to the T-tubules for depolarization. The depolarization of the membrane can activate voltage-sensitive Ca^{2+} channels opening for an inward Ca^{2+} current. Then the Ca^{2+} enters and binds to the ryanodine receptor (RyR) to trigger the release of Ca^{2+} from the sarcoplasmic

reticulum (SR) leading to an increase in the cytosolic level of Ca^{2+}, this process is called Ca^{2+}-induced Ca^{2+} release. After that the Ca^{2+} binds to the actin filament on troponin C (TnC) induced by the actin-myosin cross bridge cycle causing contraction, which is also known as the inotropic effect (Howland and Mycek, 2006; Bers, 2000). This contraction finishes by removing the cytosol Ca^{2+} from TnC to induce relaxation. The pathways of Ca^{2+} removal include: 1. Sodium-calcium exchange by exchanger Ca^{2+} ions for Na^{+} ions across the cell membrane, 2. the SERCA pump by reuptake Ca^{2+} ions to sarcoplasmic reticulum (SR) (92% of Ca^{2+} is removed by this pathway), 3. a sarcolemma Ca^{2+}-ATPase by uptake Ca^{2+} to extracellular, and 4. mitochondrial Ca^{2+} uniport by reuptake Ca^{2+} ions to mitochondria (Bers, 2002).

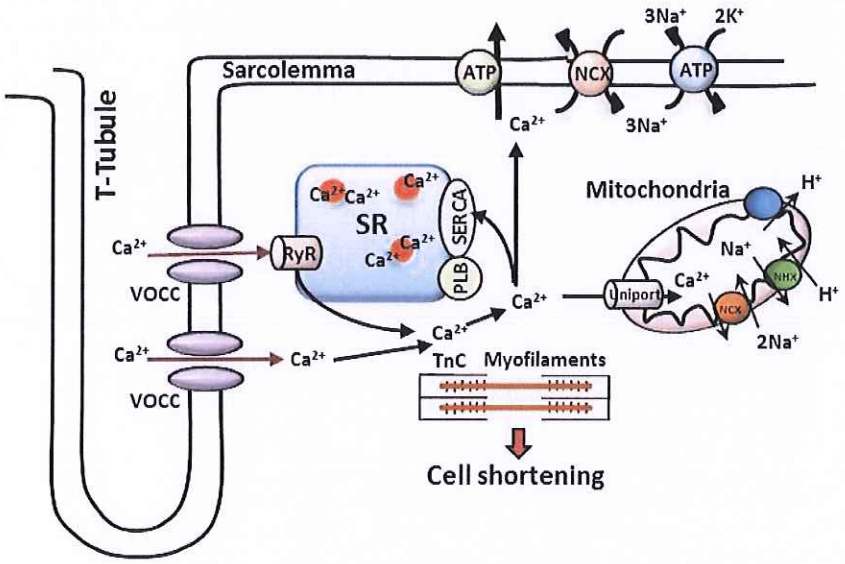

Figure 5 Showing mechanisms that Modulate Ca^{+2} for Excitation-contraction coupling (modified from Bers, 2000).
VOOC= voltage-sensitive Ca^{2+} channels; NCX=Na^{+}/Ca^{2+} exchange; RyR=ryanodine receptor; ATP= a sarcolemmal Ca^{2+}-ATPase; PLB=phospholamban; SR=sarcoplasmic reticulum; TnC=troponin C; SERCA= SR Ca^{2+}-ATPase pump

1.2.1.3 Neuronal and hormonal control

The human nervous system includes the central nervous system (CNS), which consists of the brain and the spinal cord. The spinal cord receives stimulation impulses from the sensory organ by an afferent pathway for translation, and then the information is brought to a vast network of nerves called the peripheral nervous system. The peripheral nervous system is divided into 2 systems consisting of the somatic nervous system, which voluntarily controls organs and the autonomic nervous system (ANS), which regulates individual organ function and homeostasis, and for the most part is not voluntary control. The ANS has components in both the central and peripheral nervous systems that transmit impulses from the CNS to the peripheral organ systems (Mathias, 1992; Sherwood, 2001).

The ANS is further divided into two more systems, the sympathetic and parasympathetic nervous systems. Both of these systems have the preganglionic neuron that has a cell body in the brainstem or spinal cord, and its axon synapses with a postganglionic neuron terminating its axon on the effector organ (Saladin, 2007). The preganglionic neurons in the sympathetic nervous system are very short and begin in the thoracic and lumbar regions of the spinal cord. Its axon synapses with the postganglionic neurons inside a sympathetic ganglion. These release acetylcholine (Ach) as neurotransmitters. Postganglionic fibres are long and terminate on the effector organ (Sherwood, 2001). The postganglionic fibres release norepinephrine and epinephrine to activate the adrenergic receptors of the target organs of the sympathetic nervous system (Johnson *et al.*, 1998). In the parasympathetic nervous system, preganglionic fibres are longer than sympathetic preganglionic fibres and begin in the brain and lower spinal cord. Its axon synapses with postganglionic neurons with release ACh as neurotransmitters similar to sympathetic preganglionic fibers. However, parasympathetic postganglionic fibres are shorter than sympathetic postganglionic fibres (Sherwood, 2001). The postganglionic fibres release ACh to activate the muscarinic receptors of the target organs (Johnson *et al.*, 1998).

The sympathetic nervous system in the heart

In the heart, the postganglionic fibres of the sympathetic nervous system end in the sinoatrial node (SA), atrioventricular node (AV) and the myocardium. The SA, AV nodal and myocardium of right ventricle were stimulated via the right stellate ganglion, while myocardium of left ventricle was stimulated via the left stellate ganglion. Norepinephrine and epinephrine act through two types of adrenergic receptors. The first is the β-adrenergic receptors, which is the predominant receptor in the heart (Bristow *et al.*, 1988; Steinfath *et al.*, 1992). There are three subtypes of β-adrenergic receptors: β_1-, β_2- and β_3-adrenergic receptors. The β_1-adrenergic receptor is the most dominant subtype in the human heart (Brodde, 1991). The second is the α-adrenergic receptor that further divides into the α_1-adrenergic receptor and the α_2-adrenergic receptor. There are three α_1-adrenergic receptor subtypes: α_{1A}-, α_{1B}- and the α_{1D}- adrenergic receptor, and three α_2-adrenergic receptor subtypes: α_{2A}-, α_{2B}- and α_{2C}-adrenergic receptor (Hieble *et al.*, 1995; Michel *et al.*, 1994; Brodde and Michel, 1999). However, the human heart consists mainly of the α_1-adrenergic receptor (Brodde and Michel, 1999). β1-adrenergic receptors are activated by norepinephrine and/or epinephrine via G_S proteins used to activate adenylyl cyclase leading to an increase in the intracellular cyclic adenosine monophosphate (cAMP) activates protein kinase A (PKA) resulting in an increase in intracellular Ca^{2+} (Pavoine *et al.*, 2003; Rozac and Gauthier, 2006). The α_1-adrenergic receptor subtypes are then stimulated via the G_q proteins leading to the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Endoh *et al.*, 1991; Endoh, 2006). IP_3 regulates the intracellular Ca^{2+} responses while DAG activates some of the isomers of protein kinase C (PKC) (Berridge, 1987; Woodcock *et al.*, 2008).

The parasympathetic nervous system in the heart

In the heart, the postganglionic fibres of the parasympathetic nervous system end in the cardiac pacemaker region, the conducting tissue and the myocardium. The postganglionic fibres release Ach to in order to trigger muscarinic receptors resulting in a decreased heart rate and a decrease in cardiac contractility

(Smith and Kampine, 1990). In the heart there are five subtypes of muscarinic receptors; M_1-M_5 receptors, the M_2 receptor is the dominant subtype (Pourageaud *et al.*, 2005; Bonner *et al.*, 1987; Peralta *et al.*, 1987). Ach stimulates the M_2 receptors that combine to produce the pertussis toxin-sensitive protein (G_i/G_0), for suppression adenylyl cyclase results in the decrease of heart rate and cardiac contractility (Felder, 1995).

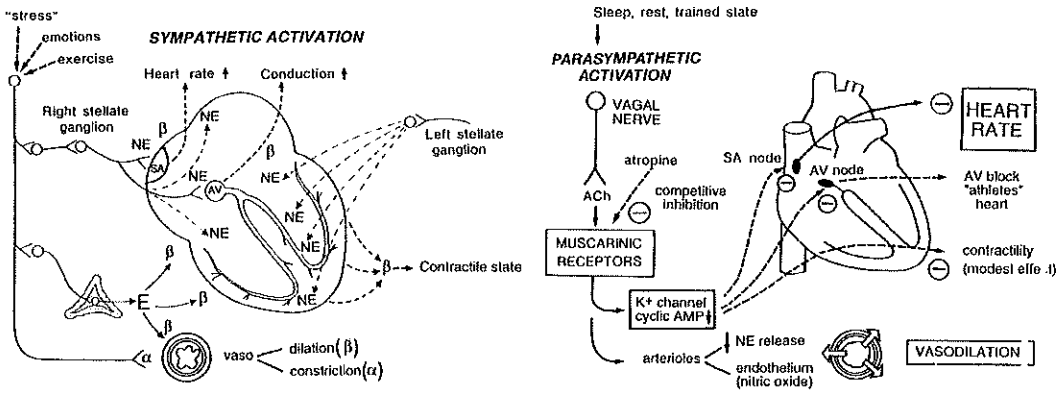

Figure 6 Mechanism of sympathetic and parasympathetic stimulation of heart (Opie, 1998)

1.2.2 THE BLOOD VESSEL

The vascular system's main function is to distribute blood to the organs and to control blood pressure (Levick, 2000). There are three major types of blood vessels, consisting of arteries, capillaries and veins. Arteries carry oxygenates blood and pump it from the left ventricle to several organs. Arteries play an important role in the maintenance of blood pressure. Veins carry deoxygenated blood from the organs back to the right atrium. Thirdly, capillaries exchange gas and nutrients between the blood and tissues (Marieb, 2004). This thesis will only investigate the arteries.

1.2.2.1 Anatomy of an artery

Artery walls consist of three layers (Ganong, 1999; Fox, 2004):

1) The first layer is the tunica intima; it is the innermost layer of the artery. It consists of the endothelium, which is the membrane covered with connective tissue fibre and a layer of elastic fibres.
2) The tunica media is the middle layer consisting of smooth muscle cells, which provide strength and flexibility.
3) The tunica adventitia is the outermost layer of the artery that consists mainly of connective tissue; fibroblasts, collagen, and elastin fibres, which provide stable structures for the vessels. This layer contains the small blood vessels that provide gas and nutrients to the arteries and it is covered with nerves in order to maintain the homeostasis of the vessel.

The arteries are classified into 2 types of functionality, elastic arteries and resistance arteries. Elastic arteries include systemic arteries such as the aorta and the pulmonary artery. These arteries are called elastic arteries due to their flexibility. Their flexibility comes from their tunica media, which contains large amounts of elastic tissues. They are low-resistance and carry blood to the various organs and they are stretched during systole and recoil on the blood during diastole in order to maintain blood flow through the tissues as "pressure reservoir". Resistance arteries consist of medium to small arteries such as mesenteric arteries, coronary arteries and arterioles. Their tunica media have much smoother muscle cells than elastic tissue. Resistance arteries contribute to the resistance to flow and blood flow regulation. During systole and diastole little changes in diameter cause large changes in the total peripheral resistance (TPR) (Ganong, 1999; Widmaier *et al.*, 2006).

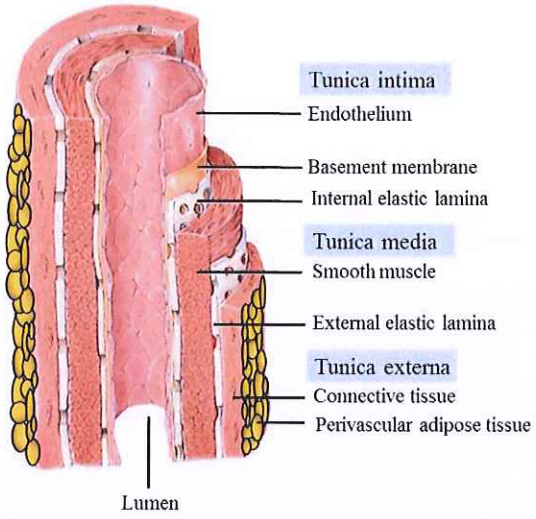

Figure 7 Diagrammatic section of the blood vessel (Adapted from Willey and Sons, 2014)

1.2.1.2 Physiology of contraction and relaxation of vascular smooth muscle cells

The action potential

The action potential induces the contraction of the smooth vascular muscle cells. During depolarization through the sarcolemma voltage-gated Na^+ channels open to create a fast inward Na^+ current leading to a rapid voltage increase of +35 mV. After that, voltage-gated Na^+ close and voltage gated K^+ channels open to create an outward K^+ current causing repolarization, which decreases voltage to the resisting membrane potential (RMP). However, some voltage-gated K^+ channels remain open even after RMP decreases voltage. This causes hyperpolarization and in order for the voltage to maintain voltage at the RMP level there must be a Na^+-K^+ pump.

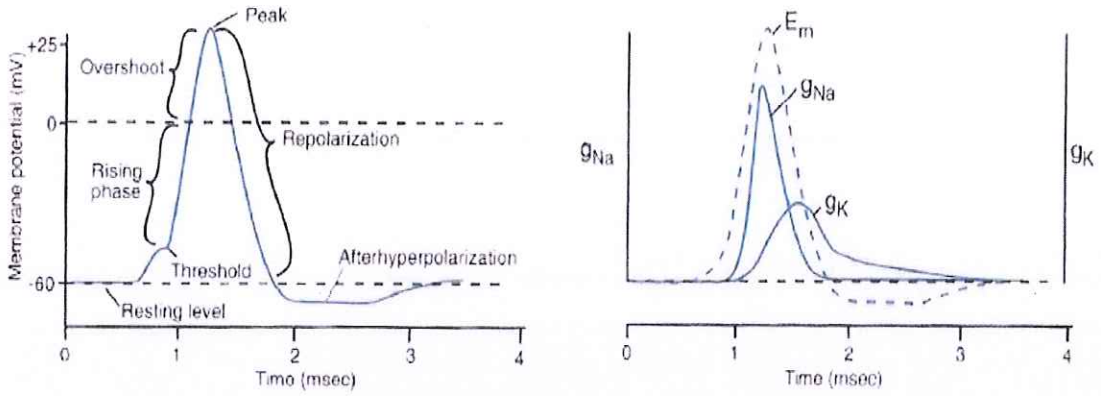

Figure 8 Action potential phases and currents of the smooth muscle cell (modified from Opie, 2004)

Excitation-contraction coupling of vascular smooth muscle cells

The contraction of vascular smooth muscle cells (VSMC) is induced by the action potential born at neurons. The action potential in the sympathetic neuron travels through the axon and activates the secretion of norepinephrine. Norepinephrine arrives in the smooth muscle cell membrane and binds to a G protein coupled with Phospholipase (PLC) receptors and thereby PLC activation leads to hydrolyzes phosphatidylinositol bisphosphate (PIP_2) into two messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). DAG binds the receptors onto the plasma membrane leading to the opening of Ca^{2+} channels which allows Ca^{2+}influx and binds IP_3 receptors on the sarcoplasmic reticulum. Moreover, this opens channels for the release of Ca^{2+} from the sarcoplasmic reticulum (SR) into the cyptosol (Hirasawa and Nishizuka, 1985, Berridge and Irvine, 1989; Akata, 2007). The Ca^{2+} then binds to calmodulin giving rise to the Ca^{2+}-calmodulin complex, which in activates Myosin Light Chain Kinase (MLCK). Next, MLCK phosphorylates myosin ATPase for the myosin to bind to the actin filament and the resultant cross-bridge cycling initiates contraction (Berne and Levy, 1998). Reduction of cytosolic Ca^{2+} activates the relaxation of vascular smooth muscles. The pathways of Ca^{2+} reduction include: (1) SR Ca^{2+}-ATPase pump (SERCA) which transports Ca^{2+} from the cytosol into the reticulum using ATP (2) Na^+/Ca^{2+} exchange across the plasma membrane to

the extracellular (3) passive transport into the mitochondrial lumen. When cytosolic Ca^{2+} concentration is decreased it leads to the release of CaM from MLCK for MLCK inactivation. Under these conditions, myosin light chain phosphatase (MLCP) dephosphorylates MLC20 to release myosin from the actin that results in relaxation (Orallo, 1996; Marin *et al.*, 1999). The relaxation of vascular smooth muscles is activated by several pathways but is mainly activated by cyclic adenosine monophosphate (cAMP) and cyclic guaosine monophosphate (cGMP). Both cyclic nucleotides can foster MLCK reduction of cytosolic Ca^{2+} (Levick, 2000; Opie, 1998).

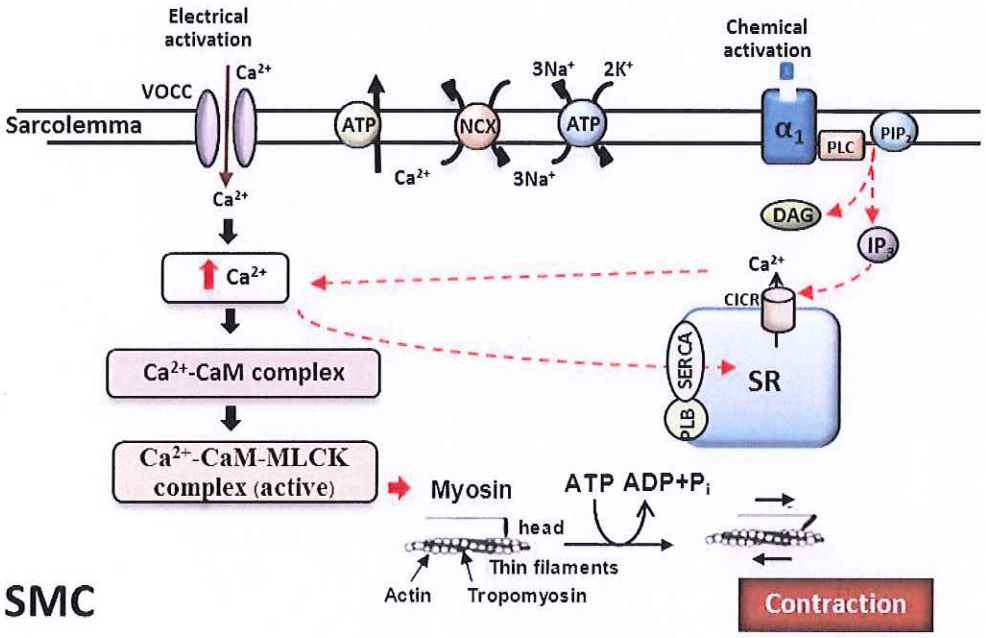

Figure 9 Excitation-contraction coupling of vascular smooth muscle (modified from Berne and Levy, 1998).

Myofilament calcium-sensitization mechanism

The several studies demonstrated that an enhancement of contractile occurs in response to receptor agonists takes place at constant $[Ca^{2+}]_c$ level in membrane-permeabilized VSMCs, activating MLC_{20} phosphorylation by accelerating MLCK phosphorylation and inhibiting myosin light-chain phosphatase (MLCP) (Nishimura *et al.*, 1992; Rembold, 1992). Several Ca^{2+} sensitizing messengers are consist of Rho/Rho-kinase, protein kinase C (PKC), and arachidonic acid (Kandabashi *et al.*, 2003).

- ***Rho/Rho-kinase pathway***

Rho A is a small monomeric G-protein, which is activated by G-protein couple receptor agonist, especially coupling to $G\alpha_{11/12}$. At resting state, inactive form of Rho A-GDP is complexed with guanine nucleotide dissociation inhibitor (GDI) in the cytosol. When stimulated, the GDP of the Rho A- GDI complex is replaces with GTP by guanine-nucleotide exchange factors (GEFs) become to active Rho A-GTP. Rho A-GTP then dissociates from GDI and translocates to the plasma membrane, while GDI is retrained in the cytosol (Fukata *et al.*, 2001). The Rho A-GTP then activates Rho kinase (ROCK) lead to phosphorylates the MYPT of MLCP conducting to inhibits its catalytic activity (Somlyo and Somlyo, 2003). In addition, Rho kinase also activates CPI-17, the phosphorylation-dependent inhibitory MLCP, and thereby inhibits catalytic activity leading to vascular relaxation (Parmentier *et al.*, 2001).

- ***Protein kinase C (PKC)***

PKC is an ubiquitous enzyme, which respond to DAG, causing an increase in myofilament Ca^{2+} sensitivity. Both a constitutively active PKC and PKC activators such as phorbol esters have been shown to increase myofilament Ca^{2+} sensitivity (Horowitz *et al.*, 1996). PKC activates CPI-17, the phosphorylation-dependent inhibitory protein of myosin phosphatase, has been phosphorylated by PKC, and thereby inhibits catalytic activity (Kitazawa *et al.*, 2000).

- ***Arachidonic acid (AA)***

Arachidonic acid is synthesized from arachidonyl phospholipids which is activated by phospholipase A_2 (PLA_2) and/or phospholipase D (PLD) activity.. Arachidonic acid plays a role in enhancement of Ca^{2+} sensitization by direct phosphorylation of Rho kinase leading to inhibit MLCP (Feng *et al.*, 1999; Koyama *et al.*, 2000).

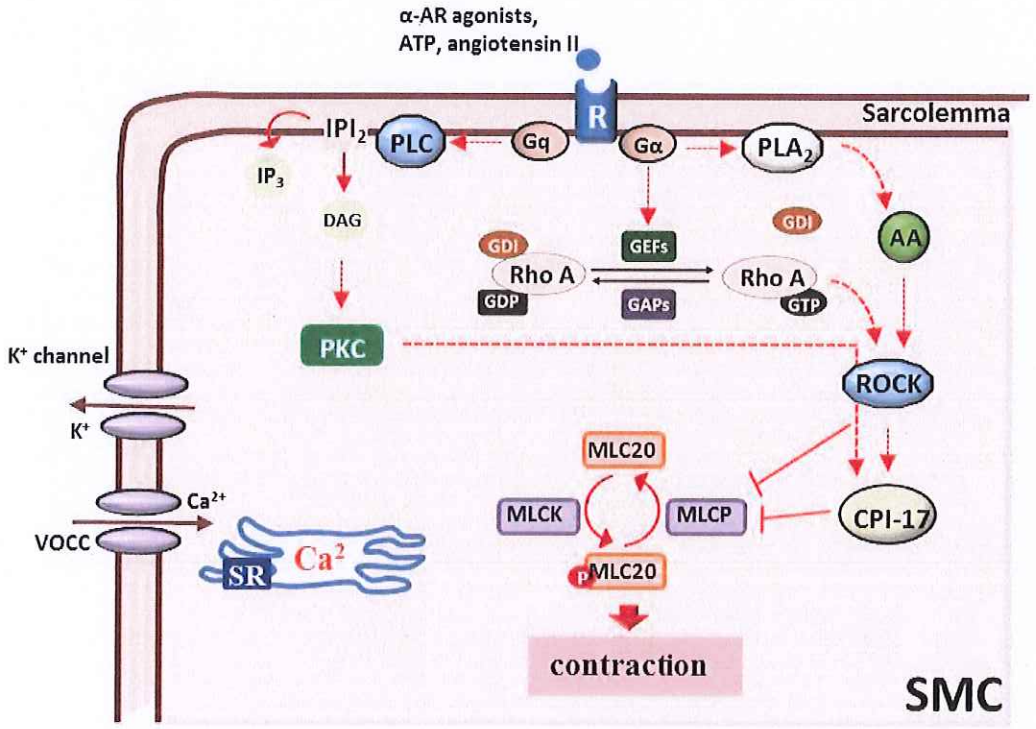

Figure 10 Mechanisms of vascular smooth muscle contraction that indue by myofilament Ca^{2+}-sensitization mechanism including Rho-kinase pathway, PKC pathway, and arachidonic acid pathway (modified from Akata, 2007).

1.2.2.3 Neuronal and hormonal control

The postganglionic fibres of the sympathetic nervous system terminates at the blood vessels to the adventitia layer to release norepinephrine (NE) acts on the smooth muscle cells and endothelial cells to control vascular tone (Kanagy, 2005). NE stimulates via the adrenergic receptor (Smith and Kampine,

1990). β-adrenergic receptors include 3 subytpes; β_1-, β_2- and β_3-adrenergic receptor, the β_2-adrenergic receptor is a predominant subtype (Pourageaud *et al.*, 2005). NE stimulates the β-adrenergic receptors that are coupled with G_S-protein, activating the adenylate cyclase leading to increase in intracellular cAMP to activate protein kinase A (PKA) (Dixon *et al.*, 1986; Guimaraes and Moura, 2001). There are six α-adrenergic receptor subtypes: α_{1A}-, α_{1B}-, α_{1D}-, α_{2A}-, α_{2B}- and α_{2C}-adrenergic receptor, and the α_1-adrenergic receptor is dominant. The α-adrenergic receptor couples to $G_{q/11}$ to activate the phospholipase C (PLC) for production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Hieble *et al.*, 1995).

The postganglionic fibers of parasympathetic nervous system end in the blood vessels in the vascular endothelium by releasing ACh which acts via M_3 muscarinic receptors. This leads to an increase of endothelial [Ca^{2+}] and an increase in the Ca^{2+}-CaM complex activates the enzyme NOS to produce NO resulting in vasorelaxation (Eglen *et al.*, 1996; Caulfield and Birdsall, 1998). Therefore, ACh acts via muscarinic receptors on the sarcolemma causing contractions by activating phosphoinositides (Milnor, 1990).

1.2.2.4 Endothelial cells of blood vessels

Vascular endothelium plays an important role in the regulation of vascular homeostasis via production of several mediators that are associated with the cardiovascular system (Furchgott and Zawadzki, 1980). The endothelium is a single cell layer that lines the interior surface of the blood vessels from the heart to the capillaries, wall of capillaries has only of endothelium. Vascular endothelium acts as a wall between the VSMCs and circulating blood (Levick, 2000). Endothelial cells release many components to control relaxation and contraction of VSMCs to regulate blood flow, coagulation, thrombogenesis, fibrinolysis, platelet activation and immune function. The mediators derived from vascular endothelium are divided to 2 types (Moncada *et al.*, 1976). Type 1 is the endothelium-derived relaxing factors (EDRF) to activate vasodilation and type 2 is the endothelium-derived vasoconstricting factors (EDCFs) to activate vasoconstriction. A pathological state of the endothelium

resulting in an imbalance between EDRFs and EDCFs is called endothelial dysfunction. It leads to cardiovascular diseases like hypertension, heart failure and atherosclerosis.

1.2.2.4.1 Endothelium-derived vasorelaxing factors (EDRFs)

The endothelium-derived vasorelaxing factors (EDRFs) are substances that produce endothelial cells for vasodilation. Main substances of EDRFs consist of prostanoids, nitric oxide (NO), hydrogren sulphide (H_2S) and EDHFs (Furchgott and Zawadzki, 1989; Ress *et al.*, 1986; Jin *et al.*, 2010).

Prostanoids

Prostanoids are arachidonic acid metabolites and the major product of cyclooxygenase in macrovascular endothelium (Bos *et al.*, 2004; Mubarak, 2010). There are many types of prostanoid receptors in vascular systems, composed of DP, EP, FP, and TP. They are contained in the cells plasma or nuclear membrane of VSMCs. However, only DP, EP and IP receptors induce vasodilation, while TP and FP receptors provoke vasoconstrictions (Coleman *et al.*, 1994) . G type proteins are activated, causing an increase in cAMP and PKA activity when the main pathway of prostanoids in arteries shown in $PG1_2$ bind with IP receptors on VSMCs. This causes vasodilation (Fététou and Vanhoutte, 2006, Mubarak, 2010).

Nitric oxide (NO)

Nitric oxide (NO) is a structurally simple molecule that was discovered over 30 years ago. It was first discovered in 1980 by Furchgott and Zawadzki1 as an EDRF (Furchgott and Zawadzki, 1980). NO is produced by nitric oxide synthase (NOS). There are three isoforms of NOS, consisting of endothelial NOS (eNOS, 134 kDa), neuronal NOS (nNOS, 160 kDa) and inducible NOS (iNOS, 130 kDa). They differ in their location, structure and function (Ayajiki *et al.*, 1996). In cardiovascular systems, NO is an important factor for vascular homeostasis. NO effects the regulation of basal vascular tone, exerting antiplatelet and anti-inflammatory and

inhibiting SMC migration and proliferation (Jin and Loscalzo, 2010). However, overproduction of NO leads to septic shock and major neural damage following vascular stroke (Isobe *et al.*, 2001). Many cardiovascular disorders such as hypertension and atherosclerosis have resulted from NO insufficiency (De Caterina *et al.*, 1995). The eNOS is a bidomain structure of an N-terminal oxygenase domain containing binding sites for haem, BH_4 and L-arginine and a C-terminal reductase domain that contains binding sites for FAD, FMN and NADPH (Hemmens and Mayer, 1998). NO was produced by an eNOS by transformation of L-arginine to L-citrulline (Ayajiki *et al.*, 1996). Initially, hydroxylation of L-arginine was hydroxyleted, leading to NG-hydroxyl-L-arginine which acts as a substrate for eNOS. Then, eNOS catalyzed an electron oxidation of the intermediate to form L-citrulline and NO (Bogle *et al.*, 1995). Two major pathways induce production of NO from the cytosol of endothelial cells. The first pathway is biochemical stimuli which includes thrombin, adenosine diphosphate (ADP), serotonin, acetylcholine, and bradykinin which bind at a receptor-mediated agonist to activate the agonist-guanine nucleotide binding protein (G-protein) complex, activating PLC, a membrane bound enzyme. Then, activation IP_3 causes a release of Ca^{2+} from internal stores of endothelial cells. The second pathway is a mechanical stimulus, which includes shear stress, and cyclic strain and can activate stretch-activated cation channels (SAC) leading to an extracellular Ca^{2+} ion influx into the cells. When Ca^{2+} increases in the cytosol of endothelial cells, following the Ca^{2+}-calmodulin complex formation eNOS activates to produce NO (Levick, 2000). NO from endothelial cells maintains vasorelaxation by placing its effects on VSMCs. NO diffuses rapidly into adjacent VSMCs and acts as paracrine effect to activate soluble guanylyl cyclase (sGC) leading to an increase in the synthesis of cGMP from GTP. Then, cGMP activates cGMP-dependent protein kinase (protein kinase G; PKG), which reduces cytosolic Ca^{2+} concentration resulting in inhibition of calcium-calmodulin myosin light chain kinase complex formation in the VSMC causing vascular relaxation (Horowitz *et al.*, 1996). Moreover, NO can activate vasodilation via cGMP-independent pathways by inhibition of voltage-gated Ca^{2+} channels and activation Ca^{2+} -dependent potassium channels such as K_{ATP}, BK_{Ca}, K_{IR}, and/or K_V (Bolotina *et al.*, 1994; Fététou and Vanhoutte, 2006).

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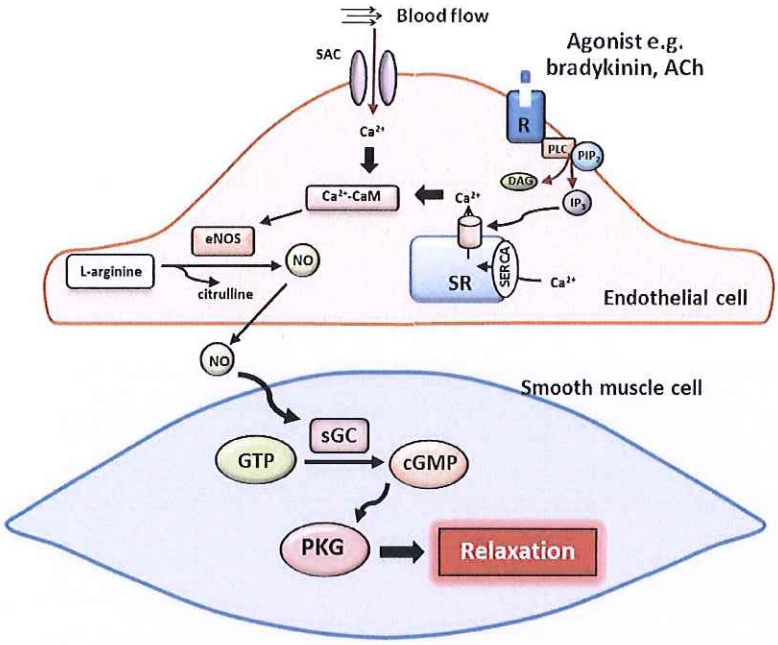

Figure 11 The regulation of nitric oxide production by endothelium (modified from Levick, 2000).

Endothelium-derived hyperpolarizing factor (EDHF)

The Endothelium-derived hyperpolarizing factor (EDHF) is a substance that is created and released from by the endothelial cell. Research shows that with EDHF, the presence of NO synthases and cyclooxygenases inhibitors suggests that there are still other factors causing hyperpolarization for vasorelaxation when activated with agonists such as such as acetylocholine (Ach) and bradykinin. Therefore, there is an additional pathway associated with the hyperpolarization of VSMC known as EDHF (McGuire *et al.*, 2001; Busse *et al.*, 2002). EDHF plays a wide role in the regulation of vascular tone, it is important to hyperpolarize VSMCs in order to produce vasorelaxation (Luksha *et al.*, 2009). EDHFs act by increasing K^+ conductance resulting in hyperpolarization and relaxation (Fleming and Busse, 2006). In almost all blood vessels, EDHF activates small conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) and/or intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}) (Marrelli *et al.*, 2003; Garland and Plane, 1996). This process is initiated through increases in intracellular calcium, which activates phospholipase A_2 (PLC) to produce arachidonic acid, and then generates eicosatrienoic acids (EETs) to activate calcium

dependent potassium (SK_{Ca}) channels in endothelial cells release of K^+ ions to the extracellular space leading to hyperpolarization. Moreover, endothelial hyperpolarization may also directly activate gap junctions on the sarcolemma of smooth muscle cells. When smooth muscles show hyperpolarization it results in close voltage-gated channels leading to a decrease in Ca^{2+} concentration, which induces vasodilation (Feletou and Vanhoutte, 2006). EDHF plays an important role in the resistance vessels by compensating in states of reduced NO availability for the regulation of blood flow, peripheral vascular resistance, and blood pressure (Wu *et al.*, 2001). There are several substances that have been proposed to act as an EDHF, such as K^+ ions (Edwards *et al.*, 1998), hydrogen peroxide (H_2O_2) (Barlow and White, 1998), Epoxyeicosatrienoic acids (EETa's) (Campbell *et al.*, 1996) and arachidonic acid metabolites (Campbell *et al.*, 2007). These substances directly activate calcium-dependent potassium channels or directly contribute to gap junctions leading to hyperpolarization in smooth muscle cells for vasorelaxation.

Hydrogen Sulfide (H_2S)

H_2S is one type of freely permeable endogenous gasotransmitters in vascular system. Initially, researchers believed that only smooth muscle cells activating ATP-sensitive potassium channels synthesized H_2S. However, researchers have recently found that H_2S may be produced in endothelial cells and perivascular adipose tissue (PVAT) as well (Polhemus *et al.*, 2014; Bełtowski and Jamroz-Wiśniewska, 2014). Endothelial H_2S production is actually stimulated by 2 factors. The first factor is biochemical stimuli, including acetylcholine, adipose tissue hormone leptin, and estrogen. The second factor is mechanical stimuli, which is shear stress. H_2S is produced from three enzymes, which includes cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST) (Kimura, 2011). However, in endothelial cells only CSE and 3-MST are expressed, CSE is the predominant enzyme for hydrogen sulfide production in the endothelial cells and smooth muscle cells (Hosoki *et al.*, 1997; Wang 2002; Yang *et al.*, 2008). In the CSE reaction, L-cysteine is metabolized to cysteine. Then CSE may catalyze cystine into pyruvate, thiocystine and NH_3. Catalyze the reaction of thiocystine with other thiol compounds to from H_2S and CysSR. Alternatively,

thiocystine may change to H_2S and cystine by non-enzymatical pathways (Wang, 2002).

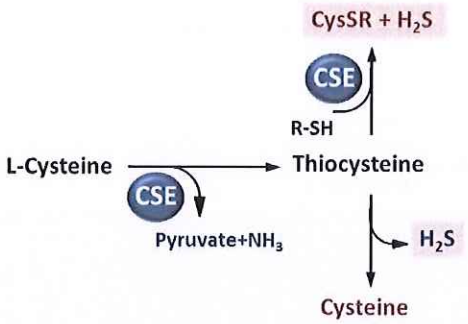

Figure 12 Enzymatic production of hydrogen sulfide by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (modified from Wang, 2002)

Endogenous H_2S shows multiple functions in the cardiovascular system, especially, the regulation of vascular tone (Kimura, 2014). Deficiency of H_2S has been associated with pathological conditions of the cardiovascular system such as hypertension and COPD (chronic obstructive pulmonary) (Chunyu *et al.*, 2003; Hui *et al.*, 2003; Yan *et al.*, 2004). H_2S produced in endothelial cells plays the most important role to endothelium-derived hyperpolarization as it stimulates endothelial SK_{Ca} and IK_{Ca} channels to activate K_{ATP} channels in smooth muscle cells for inducing vasorelaxation (Zhao *et al.*, 2001). H_2S activates endothelial NO synthase by increasing eNOS activity and potentiate the NO-cGMP pathway via inhibiting phosphodiesterase 5 to cGMP degradation (King *et al.*, 2014). In addition, H_2S can directly activate protein kinase G (Stubbert *et al.*, 2014). Moreover, H_2S interacts with NO to form nitroxyl (HNO) that can induce relaxation of adjacent smooth muscle cells (Andrews *et al.*, 2009).

1.2.2.4.2 Endothelium-derived vasoconstricting factors (EDCFs)

The endothelium not only mediates relaxation but it is a source of contracting factors such as endothelin-1, vasoconstrictor prostanoids such as thromboxane A_2 and prostaglandin H_2 and components of the renin-angiotensin system such as angiotensin II. However, the most important vasoconstrictors (endothelium-derived contracting factors) are endothelin-1 (ET-1) (Luscher *et al.*, 1992). Healthy blood vessels show EDHFs more than EDCFs, but in the case of aging and several cardiovascular diseases endothelial dysfunction releases EDCFs, which counteract the effects of EDRFs (Michel *et al.*, 2008; Félétou *et al.*, 2010).

Endothelin

Endothelin (ET) is a vasoconstricting substance produced by endothelial cells and stimulated by shear stress and vasoconstrictor agents such as transforming growth factor-beta, thrombin, interleukin-1, angiotensin II, arginine vasopressin. There are three isoforms of endothelin consisting of ET-1, ET-2 and ET-3, but only ET-1 is produced from endothelium. There are two different endothelin receptors consisting of ETA- and ETB-receptors, these receptors are coupled with seven-transmembrane G-protein coupled receptors (Haynes and Webb, 1994; Marasciulo, 2006). ET-1 can activate vasodilation or contraction depending on the concentration. With high ET-1 concentrations, vasodilation is activated via the ET_A receptor that couples to the pertussis toxin insensitive $G_{\alpha q/11}$ resulting in the activation of phospholipase C, following an increase in IP_3 to activate PKC for vasocontraction (Griendling *et al.*, 1989, Takuwa *et al.*, 1990). Whereas, at low ET-1 concentrations, vasodilation is activated via the ET_B receptor that couples to $G_{\alpha i/o}$ to inhibit the cAMP production for vasodilation (Aramori and Nakanishi, 1992; Aramori *et al.*, 1993; Kawanabe and Nauli, 2011).

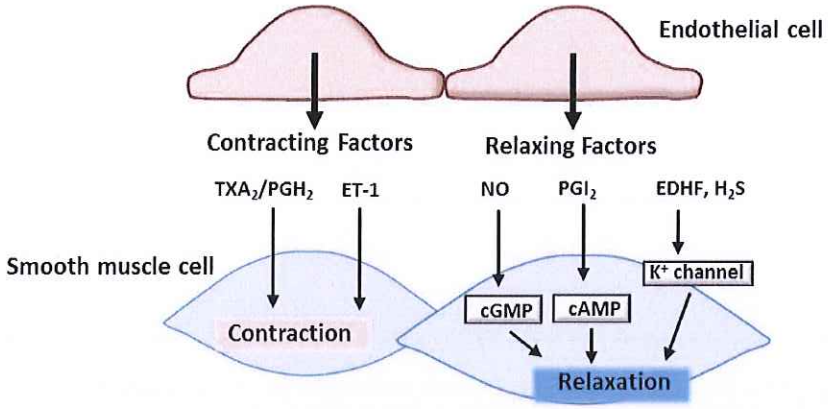

Figure 13 Role of endothelium in vascular regulation (Opie, 1998)

1.2.2.5. Perivascular adipose tissue

The perivascular adipose tissue (PVAT) is an adipose tissue found in the surrounding area of most blood vessels. PVAT is different from morphology and function, which have a direct bearing on vascular cells. PVAT instances of both white adipose tissue (WAT) or brown adipose tissue (BAT) and are clearly not separated from the adventitial layer by a fascial layer or elastic lamina. PVAT is essential to other cells such as the stromal vascular fraction (SVF), including fibroblasts, mesenchymal stem cells and the vasa vasorum, which contains endothelial cells and connects with sympathetic and parasympathetic nervous fibres. These cells can produce and release several adipokines to mediate vascular reactivity. However, the extracellular matrix (ECM) of PVAT is similar to that of adipocytes of other regions containing collagen, laminin, and fibronectin fibers. White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation (Mariman and Wang, 2010). In 1991, the initial description of the anticontractile effect of PVAT was reported to reduce contractions to noradrenaline in rat aorta (Soltis and Cassis, 1991). PVAT is recognized as an active contributor to vascular function through synthesis varied paracrine, including adipokines, cytokines, reactive oxygen species, and gaseous compounds. These secretions can modulate complex processes, including anti-contractile effects on blood vessels, and vascular

inflammation. It also protects against neointimal hyperplasia and smooth muscle cell proliferation and migration (Gu and Xu, 2013; Takaoka *et al.*, 2009). The mechanisms of PVAT include the inhibition of contraction of blood vessels to divide into two pathways. The first pathway is the endothelium dependent pathway and the second is the endothelium independent pathway. The endothelium dependent pathway, mediated by NO derived from NOS and calcium-activated channels cause the activation of K_{Ca} (Gao *et al.*, 2007). PVAT can inhibit contraction of blood vessels via production and release of paracrine in a termed adipocyte-derived relaxing factor (ADRF) that can stimulate the adenosine triphosphate (ATP) channels (K_{ATP}). They have excluded contributions from NOS and from other K^+ channels, such as the voltage-gated (K_V) channels, inwardly rectifying K (K_{IR}) channels and the large conductance calcium-activated (BK_{Ca}) channels (Dubrovska *et al.*, 2004). The endothelium independent pathway is mediated by hydrogen peroxide for sGC activation and H_2S production (Gao *et al.*, 2007). PVAT expresses nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases and can produce reactive oxygen species (ROS), like hydrogen peroxide (H_2O_2), which activate sGC pathways for vasorelaxation (Gao *et al.*, 2007). However, PVAT can stimulate vasoconstriction that is mediated via superoxide production by NADPH oxidase in PVAT adipocytes (Gao *et al.*, 2006). In another pathway, PVAT releases angiotensin II to induce vasocontraction via perivascular nerve stimulation (Lu *et al.*, 2010). In metabolic syndromes conditions can induce PKC_{β}-mediated phosphorylation of eNOS at Thr-495 inhibited sites leading to decreases in endothelium-dependent relaxation (Payne *et al.*, 2010).

PVAT also stimulates the proliferation and migration of VSMCs that are mediated by paracrines called resistin, visfatin and leptin (Barandier *et al.*, 2005). Secretion levels of visfatin by PVAT adipocytes were shown to be higher than subcutaneous or visceral fat depots. Visfatin activates VSMC proliferation via stimulation of nicotinamide mononucleotide production which activates insulin independent NMN-mediated activation of Erk1/2 and p38 MAPKs pathways (Wang *et al.*, 2009). Resistin and leptin are shown to activate both the proliferation and migration of VSMCs via stimulation of MAPK and/or PI3K pathways (Shyu *et al.*, 2011) .

PVAT function in pathophysiological states, in obesity and in metabolic syndromes showed that total PVAT mass and adipocyte size is increased. However, the anticontractile effect-derived from ADRF is decreased in obesity in humans and animals with trigger inflammation, hypoxia and oxidative stress (Verlohren *et al.*, 2004; Achike *et al.*, 2011). Increasing PVAT size leads to impaire adipokine secretion, whereas augment IL-6 and TNF-α secretion (Rittig *et al.*, 2008). Moreover, changes in PVAT mass caused an increase in NADPH oxidase expression resulting in an increase in reactive oxygen species (ROS) production, decreased expression of superoxide dismutases (SOD) and induced eNOS uncoupling (Marchesi *et al.*, 2009). The ROS causes endothellail dysfunction followed by impaired endothelium-dependent relaxation (Ketonen *et al.*, 2010). With obesity, PVAT can release high concentrations of leptin to inhibit VSMC growth via inhibiting the angiotensin II-stimulated VSMC proliferation by NOS-dependent mechanisms (Rodríguez *et al.*, 2007). With hypertension, there is decreased PVAT mass and PVAT adipocyte size, in addition, decreased PVAT anticontractile effects. Decreased PVAT mass causes the reduction of leptin secretion resulting in a decrease in VSMC Ca^{2+} signalling. Angiotensin II-mediated vasoconstriction is also decreased in the hypertension state (Rodriguez, 2006). Moreover, PVAT in hypertensive rat induces to secrete protein was identified as complement 3 (C3) which induced adventitial fibroblast migration and differentiation via JNK activation (Ruan *et al.*, 2010).

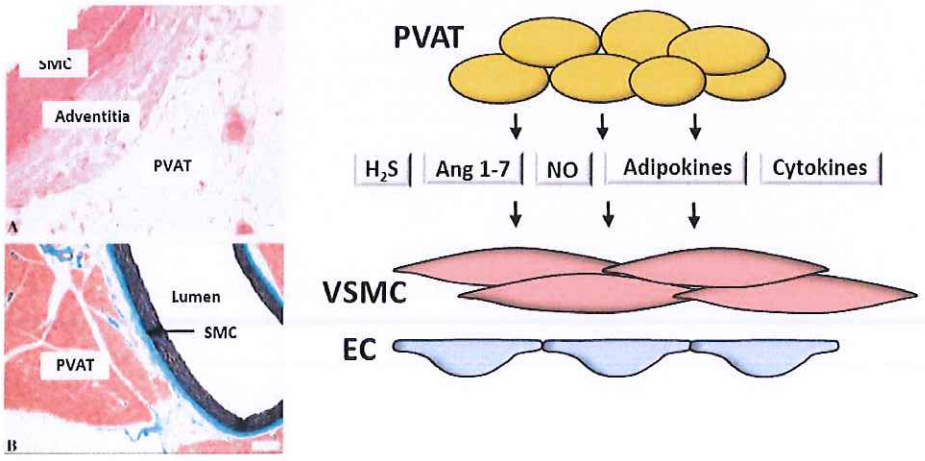

Figure 14 The influence of perivascular adipose tissue on vascular homeostasis (modified from Szasz1 *et al.*, 2013)

Ang 1–7, angiotensin 1–7;; EC, endothelial cell; H_2S, hydrogen sulfide; NO, nitric oxide; PVAT, perivascular adipose tissue VSMC, vascular smooth muscle cell.

1.2.3 ARTERIAL BLOOD PRESSURE

Arterial blood pressure is the force of blood against the walls of the blood vessels. Usually, arterial blood pressure refers to the systemic arterial blood pressure, which is the pressure in the systemic arteries such as the aorta, and the pulmonary artery. The maximum arterial pressure that occurs during peak ventricular ejection is called systolic blood pressure (SBP), while the minimum arterial pressure that occurs just before ventricular ejection is called diastolic blood pressure (DBP) (Martini *et al.*, 2006). The difference in values between SBP and DBP is defined as pulse pressure. Sometimes, arterial blood pressure is shown in mean arterial pressure (MAP), which is nearly equivalent to the DP plus one-third of the pulse pressure (SP-DP), MAP = DP + 1/3 (SP-DP). Mean arterial pressure (MAP) is similar in the large arteries everywhere, because the large arteries have large diameters that offer only little resistance (Vander *et al.*, 1994). The important factors influencing arterial blood pressure are stroke, volume and arterial compliance (Widmaier *et al.*, 2004).

1.3 METABOLIC SYNDROME AND OBESITY

Metabolic syndrome (MS) and obesity are related conditions that show of pathophysiological mechanisms and that act as risk factors for other diseases such as cardiovascular disease and diabetes. MS gives a 2 to 3-fold increased risk for cardiovascular disease and a much greater risk for future diabetes (Alexander *et al.*, 2003). Moreover, MS is an important risk factor for cancer. In the United States (U.S.) it was shown that having MS gives men a 1.78 fold and women a 1.16-fold increased risk for bowel cancer (Giovannucci, 2007). MS is a manner of an abnormal

metabolism with several features; central obesity or abdominal obesity, dyslipidemia (high triglycerides, high LDL and low HDL) blood pressure and insulin resistance (National Cholesterol Educationprogram, 2002). MS is a large problem in the world, especially in developed countries. In the U.S. the total amount of people with MS is highest in the world and was estimated to 23.1% in 1999, and rose to 26.7% in 2000 (Ford *et al.*, 2004). The prevalence of MS depends on age. In the U.S. there are approximately 6.7% adults ages 20 to 29 years old that have MS, while 43.5% of older adults ages 60 to 69 years old have the syndrome. Experts predict that at least half of persons over age 60 will have MS in the U.S. (Ford *et al.*, 2002). In Australia, approximately 12% of people more than 25 years old have MS, similar to countries in Southeast Asia including Singapore at 12%, the Philippines at 14% and Hong Kong at 17% respectively in 1998. The commonness is rose fast to 18, 19 and 22% in 1999 (Nestel *et al.*, 2007). MS is more common in men compared to women (Cameron *et al.*, 2004). As mentioned previously, obesity is an important risk factor for MS. The high prevalence of MS may be a result of the obesity pandemic in modern society. The number of obese individuals who have CVD and diabetes are primarily outcomes of having MS (King *et al.*, 1998). In Thailand, The prevalence of central obesity is estimated at 28.3% and is more common in women than in men (Kaewtrakulpong, 2008). In 2009 approximately 21.1% of people 15 years and older had MS. It appeared more in women than in men and the prevalence of MS rose to 30.4% in persons aged 45 to 49 years and 38.6% in persons age 60-69 year old, respectively (Akparakorn, 2000).

1.3.1 METABOLIC SYNDROME

As mentioned before, metabolic syndrome (MS) is manner of abnormal metabolism with several feature; central obesity or abdominal obesity, dyslipidemia (high triglycerides, high LDL and low HDL) blood pressure and insulin resistance, is most important factors risk to type 2 diabetes mellitus and cardiovascular diseases (CVDs) (Reaven, 1988; Liese *et al.*, 1998). Many international expert groups have established the criteria for the diagnosis of MS. First, the World Health Organization (WHO) introduced a definition for the metabolic syndrome in 1998, after that it was

modified by the National Cholesterol Education Program's Adult Treatment Panel III (NCEP) in 2001. And finally modified by the International Diabetes Federation (IDF) in 2005 (Zimmet *et al.*, 2005).

Table 1 Criteria for development of MS as per ATPIII and IDF guidelines. (adapted from Kassi et al., 2011)

Criterion	NCEP:ATPIII (3 or more criteria)	IDF (2 or more criteria)
Abdominal obesity **Men** **Women**	 > 40 inches > 35 inches	 > 37 inches > 31.5 inches
Hypertriglyceridemia	≥ 150 mg/dL	≥ 150 mg/dL
Low HDL **Men** **Women**	 < 40 mg/dL < 50 mg/dL	 < 40 mg/dL < 50 mg/dL
Hypertension	≥ 130/85 mm Hg Or on antihypertensive medication	≥ 130/85 mm Hg
Fasting glucose	≥ 110 mg/dL Or taking insulin Or hypoglycemic medication	≥ 110 mg/dL

1.3.1.1 Features of the metabolic syndrome

Abdominal adiposity

Obesity is often defined by body mass index (BMI) as obese people showed an excess of mass and size of adiposity. BMI calculated from the weight (kilograms) was divided by the square of the body height (metres) expressed in unit of kg/m^2. Obesity becomes a clinical matter when a person has a body mass index (BMI) of greater than or equal to 30 kg/m^2, whereas an overweight person is defined as a person with a body mass index of 25-29 kg/m^2 (WHO, 2000) . In some cases people with MS need not be clinically obese, they may have a normal-weight BMI that is less

than 30 kg/m^2 but they have an abnormal fat distribution, which accumulated only intraperitoneally (visceral fat) or subcutaneously (Grundy *et al.*, 2005). An increase in visceral adipose tissue induces to rise of fatty acids flux to the liver for promotion and increased secretion of triglyceride, lipoproteins and glucose from the liver. Moreover, an increase in abdominal subcutaneous fat causes a release of lipolytic to the systemic circulation has an effect on hepatic metabolism (Eckel *et al.*, 2005). Visceral fat is strongly associated with MS than other adipose tissue (Carr *et al.*, 2004; Després, 2006). For these reasons, waist circumference, which refers to measure of intra-abdominal fat mass was admired more than BMI for correlation with abdominal obesity and patients at risk of MS. The clinical manner for risk of MS showed waist circumference of greater than or equal to 35 inches for women and 40 inches for men (Canoy *et al.*, 2007).

Insulin resistance

Insulin resistance (IR) is a pathophysiological condition and the clinical component of the MS. Moreover, there is some evidence showing that excess mass adipose tissue causes an increased risk of insulin resistance that showed that half of the obese people have insulin resistance (McLaughlin *et al.*, 2003; Kahn *et al.*, 2006). Insulin resistance is a condition in which cells are supposed to suppress to respond to insulin hormones to mediate glucose uptake to skeletal muscle and adipose tissue (Eckel *et al.*, 2005). Thus, Insulin resistance leading to high blood glucose, and in long time insulin resistance induces high blood insulin (hyperinsulinemia) for compensation, which is normally insulin sensitive (Reaven, 2011). The symptoms of insulin resistance consist of high blood glucose and triglyceride, intestinal bloating, sleepiness after meals, increased hunger, overweight, difficulty losing weight and increased blood pressure (Chiu *et al.*, 2007). IR is caused by dysfunction and reduction in number of insulin receptors and irregularity of intracellular signalling pathways that transduce the function of insulin (Ferrannini, 2006; Kashyap and Defronzo, 2007). Deficiency of phosphatidylinositol 3-kinase, which is crucial for insulin-mediated glucose transportation, leads to IR. On the other hand activation of mitogen-activated protein kinase (MAPK) pathways induces risk of IR that is associated with the development of atherosclerosis (Cusi *et al.*, 2000). The degree of

adipose tissue and physical fitness is approximately 50% of the origin of insulin action and the other 50 % is from genetic origin (Reaven, 2011).

Dyslipidemia

Metabolic syndrome shows several features of lipid serum profiles consisting of high triglycerides, high LDL cholesterol and low HDL cholesterol. HDL cholesterol of less than 40 mg/dL in men or less than 50 mg/dL in women and Triglyceride level of greater than 150 mg/dL (Grundy, 2006). Individuals with obesity tend to have free fatty acid and insulin resistant people have impaired suppression FFA release from adipose tissue. This method induces increased production of apoB containing VLDL particles, especially triglyceride-rich VLDL1 particles, resulting in hypertriglyceridemia (Reynisdottir *et al.*, 1997; Lewis, 1995). Insulin resistance also contributed to failing in lipoprotein lipase activity resulting in reduced HDL-cholesterol that has anti-atherosclerotic by transporting cholesterol in reverse to the liver for removal and rise in LDL-cholesterol can transform to oxidation state increase cardiovascular risk (Ginsberg, 2002; Hulthe *et al.*, 2000; Rader, 2002).

Lipid metabolism

The insulin-resistant state due to obesity or elevated insulin concentrations that indue to dyslipidemia (Howard, 1999). Increasing of mass adipose tissue is excessive concentration of free fatty acids (FFA) in plasma. FFA can induce the accumulation of triglycerides in liver. Moreover, the excess of plasma triglycerides can induces free cholesterol change (FC) to cholesterol esters (CE). Increasing of FFA and CE induce production of triglycerides-enriched VLDL particles led to induce cholesterol ester transfer proteins (CETP) activity, with results in an increased in HDL clearance and decrease HDL concentration. Morover, VLDL can elevate of the LDL particles changes to small-dense LDL (d-LDL). (Zivkovic *et al.*, 2007).

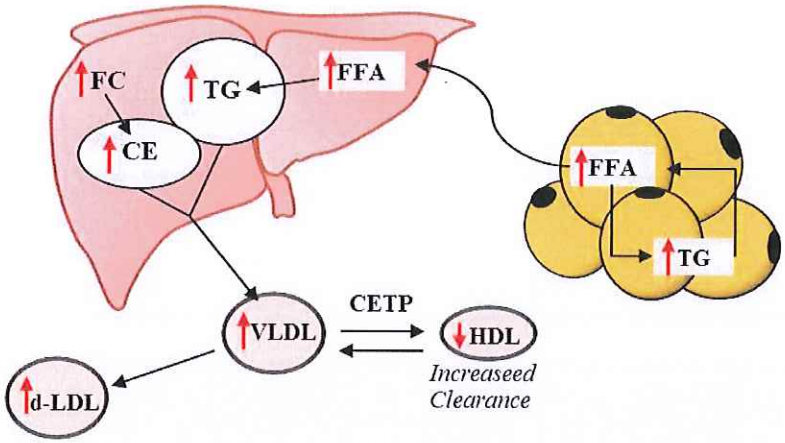

Figure 15 Liver lipid metabolism (Figure adapted from Zivkovic *et al.*, 2007)

Hypertension

Metabolic syndrome shows several feature of lipid serum profiles consisting of high triglycerides, high LDL cholesterol and low HDL cholesterol) HDL cholesterol of less than 40 mg/dL in men or less than 50 mg/dL in women and Triglyceride level of greater than 150 mg/dL (Grundy, 2006). Individuals with obesity tend to have free fatty acid and insulin resistant people have impaired suppression FFA release from adipose tissue. This method induces increased production of apoB containing VLDL particles, especially triglyceride-rich VLDL1 particles, resulting in hypertriglyceridemia (Reynisdottir *et al.*, 1997; Lewis, 1995). Insulin resistance also contributed to failing in lipoprotein lipase activity resulting in reduced HDL-cholesterol that has anti-atherosclerotic by transporting cholesterol in reverse to the liver for removal and rise in LDL-cholesterol can transform to oxidation state increase cardiovascular risk (Ginsberg, 2002; Hulthe *et al.*, 2000; Rader, 2002).

1.3.2 OBESITY

Obesity is defined as an excess of mass and size of adiposity that showed to be a clinical manner when a person body mass index (BMI) is greater than or equal to 30 kg/m^2 (Roche *et al.*, 1981). It is estimated that over 300 million adults are obese individuals (Waxman, 2004). Obesity is a risk factor associated with

diabetes, cardiovascular diseases, and cancer (King *et al.*, 1998). The growing prevalence of obesity is a serious health problem in both industrialized and developing countries around the world. In the US obesity has shown increases from 1990s to 2006. In the 1990s there were only 10 states with <10% obesity and not one state had >15% of people with obesity, but in 2006 there were 22 states with a prevalence of >25% obesity and 2 states showed a prevalence of >30% (Wardlaw *et al.*, 2004).

Obesity is related to increases in adipose tissue mass that is important for MS that is call intra-abdominal fat (IAF) (Phillips *et al.*, 2008). Many researchers showed that an increase in IAF leads to dislipidemia through increases in triglyceride low-density lipoprotein (LDL) cholesterol apolipoprotein B levels with reduced concentration of HDL cholesterol in a plasma profile and elevated blood pressure (Kalkhoff *et al.*, 1983; Hayashi *et al.*, 2003). Obesity leads to increases in adipose tissue mass that increase both size and number of adipocytes. Adipose tissue mass can increase in two ways. The first way is through hyperplasia for increasing in the cell number and the second way is hypertrophyfoe, which increases the size of the cell (Otto and Lane, 2005). Adipocytes develop from preadipocytes, which are fibroblast-like cells and preadipocytes was filled by lipid (triacylglycerol) resulting in an increase in size through accumulation of more lipid droplets. When these adipocytes exhaust their storage capacity induce to increase in cell number for filling more lipid drolets by induce proliferation of adipocytes it causes both an increase in cell number and cell size contributing to adipose mass for regulatory processes developing into obesity (Blumberg *et al.*, 2006). The role of obesity on the metabolic syndrome was mediated by adipokines that release from adipose tissue importantly.

Adipose Tissue

Adipose tissue is made up adipocyte also consists of 80% lipid and 20% of water and protein. Adipose tissue is divided into two types that consist of brown adipose tissue (BAT) and White adipose tissue (WAT). Brown adipose tissue (BAT) is major tissue found in infants and a little in adults. It has a lot of mitochondria and strong blood flow causing it to appear brown. BAT consists of a protein in its inner mitochondrial membrane called uncoupling protein-1 (UCP-1) its

main function is the uncoupling of energy yielding nutrients and ATP production. BAT contributes to thermogenesis by release of energy through physical activities and shivering as response to cold (Wardlaw *et al.*, 2004). White adipose tissue (WAT) functions to store energy as triacylglycerols. WAT is major type of adipose tissue found in our bodies as subcutaneous fat and visceral fat that is important adipose tissue risk to central obesity due to the capacity of growing 50 times in weight. WAT is constituted from adipocytes preadipocytes, macrophages, fibroblasts, and various cell types that can release several adipokine (Rondinone, 2006).

Adipose tissue can produce several protein cytokines called adipokines. These adipokines regulate many mechanisms concerning energy balance, glucose metabolism, insulin action and inflammation. There are several adipokines that can be mediators for metabolic syndrome such as adiponectin, leptin, resistin, visfatin, TNF-α. Adiponectin plays to regulate insulin resistance, atherosclerosis, inflammation and dyslipidemia (Hosch *et al.*, 2006). Leptin act to regulate food intake (Rondinone, 2006). Visfatin also plays a beneficiary role in glucose metabolism, and TNF- α act as pro inflammatory to promote inflammation (Fukuhara *et al.*, 2005).

1.3.2.1 Obesity promotes metabolic syndrome

Metabolic syndrome (MS) is strongly linked to the presence of obesity. In the normal state there is a balance between adipose tissue lipolysis and triglyceride synthesis that was mediated by nutrients and hormones. Adipose tissue act function in the storage of surplus energy and release several adipokines to control metabolic activity (Ahima *et al.*, 2000). Increases in adipose tissue mass lead to decrease in insulin-mediated glucose uptake and increases in insulin resistance (Abbasi *et al.*, 2002; McLaughlin *et al.*, 2003). Especially accumulation of intraperitoneally (visceral fat) or subcutaneously is more strongly associated with insulin resistance and MS than periphral fat (Carr *et al.*, 2004; Després, 2006). On some case have normal-weight body mass index (BMI less than 30 kg/m^2) but these persons have MS need not be clinically obese, but they have an abnormal fat distribution, which accumulated only intraperitoneally (visceral fat) or subcutaneously (Grundy *et al.*, 2005). Increases in visceral adipose tissue induce to raise fatty acids flux to the liver for promotes an

increased secretion of triglyceride, lipoproteins and glucose from the liver. Moreover, increasing in abdominal subcutaneous fat cause a release of lipolytic to the systemic circulation has an effect on hepatic metabolism (Eckel *et al.*, 2005; Despres, 2006).

Obesity and Insulin resistance

There are some evidences showing that excess mass adipose tissue cause increased risk of insulin resistance that showed half of obese persons have insulin resistant (McLaughlin *et al.*, 2003; Kahn *et al.*, 2006). And, the degree of adipose tissue and physical fitness is approximately 50% of the origin of insulin action and the other 50% is from genetic origin (Reaven, 2011). Adipose tissue can release hormones, metabolites, and cytokines contribute toinsulin resistance that compose of Leptin, Adiponectin, Resistin, TNF α, Interleukin-6 and Free Fatty Acids. These substances not only regulate insulin action in adipose tissue but also in liver, skeletal muscle that is the target organs of insulin.

Leptin; Leptin is hormone that is the product of the *ob* gene and binds to OB-R receptors for activates the Janus kinase (JAK) signal transducers. In obesity reduced leptin production or leptin receptor mutations that have crosstalk with suppress phosphatidylinositol 3 kinase (PI3K)/Akt and activate mitogen-activated protein kinase (MAPK) signalling pathway induce risk of Insulin resistance that associated with the development of atherosclerosis (Fruhbeck, 2006). In addition, leptin has direct effect on insulin-sensitizing effects on peripheral tissues (Marcus-Samuels, 2000).

Adiponectin; Adiponectin is adipocyte-derived hormone with two receptors consisting of AdipoR1, which is predominantly expressed in skeletal muscles contributing to increases in glucose utilization by muscle, and AdipoR2 is mainly expressed in the liver acts to suppress hepatic glucose production (Yamashita *et al.*, 2002). In obesity reduce expression of adiponectin in circulating related with insulin sensitivity result insulin resistant (Stumvoll and Haring, 2002).

Resistin; Resistin is a hormone secreted from adipocytes that act to stimulate insulin resistance in obesity. Resistin suppresses insulin-stimulated glucose transported in skeletal muscles and induces glucose production from the liver by inhibiting AMPK (Steppan *et al.*, 2001).

Cytokines; TNF α and IL-6 are cytokines that are derived from abdominal fat skeletal muscle and are increased in obesity (Hotamisligil *et al.*, 1993). TNF α induce insulin resistance by reducing tyrosine phosphorylation of insulin receptor leading to impair insulin signalling (Hotamisligil *et al.*, 1994). IL-6 act as suppressor of cytokine signalling (SOCS) proteins to induce degradation of IRS suppress hepatic insulin signalling to promote insulin resistance (Senn *et al.*, 2002).

Free Fatty Acids; Obesity is related to increases in adipose tissue mass induced that is elevated levels of circulating free fatty acids-derived from adipose tissue. Free fatty acids can induce hepatic glucose (production hepatic gluconeogenesis) and reduce the sensitivity of muscle tissue to insulin stimulation by activate serine/threonine kinases that can phosphorylate insulin receptor following to high blood glucose level that showed of greater than or equal to 6.1 mmol/l (hyperglycemia), finally, results to insulin resistance (Fujimoto, 2000). When high blood glucose levels occur in long term to counterbalance for decreasing blood glucose levels by increasing insulin secretion causing hyperinsulinemia (Liese *et al.*, 1998).

Obesity and Dyslipidemia

Individuals with obesity leading to free fatty acid elevated levels in the circulating system. FFA can stimulated triglyceride accumulation in the liver in direct pathway by passive diffusion or binding to receptors and then metabolized into triglycerides (TG) contribute to dyslipidemia that show hypertriglyceridemia, high level of small low-density lipoprotein (LDL) particles by compositional changes of LDL (small-dense LDL) and low amounts of high density lipoprotein (HDL) cholesterol (Hamilton *et al.*, 2002). FFA release from adipocytes can induce liver to product apoB containing VLDL particles, especially triglyceride-rich VLDL1 particles, result to hypertriglyceridemia. Moreover, in insulin resistance individuals also have suppression in lipoprotein lipase activity in plasma (Lewis, 1995).

Obesity and Hypertension

Obesity can lead to hypertension via many mechanisms; stimulation of the sympathetic nervous system, losing endothelial function and activation the renin-angiotensin-aldosterone system (RAAS). There is evidence of central stimulation of

the SNS by reactive oxygen species in obesity. In Obese individual induce to product reactive oxygen species such as NADPH in the brain mediates arterial pressure elevation that cause to increase sympathetic tone leading to hypertension (Nagae *et al.*, 2009). Effects on the renin-angiotensin-aldosterone system (RAAS) show that in obesity increase expression of angiotensin converting enzyme (ACE), angiotensin type 1 and type 2 receptors, moreover raised secrete angiotensinogen and renin from adipose tissue as well as increased aldosterone levels contribute to hypertension by increased sodium reabsorption lead to increased blood volume (Van Harmelen *et al.*, 2000; Engeli *et al.*, 2000). Moreover, the inflammatory and reactive oxygen species such as the superoxide anion that release from adipose tissue in obesity can promote in obesity. The superoxide anion can induce nitric oxide change to peroxynitrite and decrease endothelial nitric oxide bioavailability. In addition, the superoxide anion cause to endothelial dysfunction leading to reduce eNOS phosphorylation for NO production (Kotsis *et al.*, 2010). These results to deplete the balance between endothelin-1 and NO in endothelial cells leading to vasoconstriction via unopposed endothelin-1 action follow losing vascular compliance (Jin and Loscalzo, 2010). Also there is evidence showed that obesity and Hyperinsulinaemia raise producing leptin which is a contributing factor of impairment of arterial distensibility leading to arterial stiffness and reduce vasodilatory effect on peripheral small arteries (Michel *et al.*, 2006)

1.3.3 The association between abdominal obesity, metabolic syndrome and cardiovascular disease

In recent years, the prevalence of obesity and metabolic syndrome has increased rapidly and is associated with increased cardiovascular morbidity and mortality. According to American Diabetes Association (2005) showed that the incidence rate of CHD with the metabolic syndrome was significantly higher in both men and women (138.4 and 57.5 per 10000 person-years, respectively) than men and women without the metabolic syndrome (92.3 and 22.7 per 10000 person-years, respectively) (Grundy *et al.*, 2005). The cardiovascular risk factors due to metabolic syndrome is associated with the role of adipokines released from visceral adipose

tissue and a pro-imflammatory state contributed to endothelial dysfunction that is main condition to cardiovascular disease (Hansson, 2005; Harrison, 1997). The obesity and metabolic syndrome associated with increasing cardiovascular morbidity and mortality due to several adipocytokines that release from adipose tissue. The plasma levels of inflammatory Mediators, tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are raised production from adipose tissue in obesity. Adipose expression of TNF-α can induce inflammation in obesity by direct. These inflammatory mediators can activate via nuclear factor kappa-beta kinase (NF-kB kinase) and Jun N-terminal kinase (JNK) resulting inflammation that contribute to endothelium dysfunction reduced NO production. Moreover, inflammation is associated with oxidative that reduced NO bioaviability (Aljada *et al.*, 2004). In addition, in obese individual showed to decrease of Adiponectin that release from high mass adipose tissue (Hypoadiponectinaemia) attenuated NO production in endothelial through decreasing of the enzyme AMP-activating kinase (AMPK) (Shimabukuro *et al.*, 2003). Moreover, adiponectin can suppress NF-kB signalling in response to TNF-α for reduced inflammation (Ouchi *et al.*, 2000). Circulation Obesity can induce hyperleptinemia and leptin resistance that contributed to hypertension by stimulation the sympathetic nervous system by mediated effects on both the hypothalamus and local peripheral actions. In addition, leptin can stimulate vasoconstrictive agents such as endothelin-1 and reactive oxygen species impact to NO-derived from endothelial cell (Ouchi *et al.*, 2000). In addition, leptin which is a contributing factor of impairment of arterial distensibility leading to arterial stiffness and reduce vasodilatory effect on peripheral small arteries (Safar *et al.*, 2006). And Obesity is related with increasing in adipose tissue mass induced elevated levels of circulating free fatty acids. This results can induce hepatic glucose production (hepatic gluconeogenesis) and reduce the sensitivity of muscle tissue to insulin stimulation by activate serine/threonine kinases result to decrease of high-density lipoprotein cholesterol (HDL-C) and increase of low-density lipoprotein cholesterol (LDL-C) levels lead to endothelial dysfunction (Boden, 1999). Moreover, elevated blood pressure is the one of component of the metabolic syndrome that due to Obesity. Obesity can lead to hypertension via stimulation of the sympathetic nervous system by increase the production of reactive oxygen species in the brain lead to

increase sympathetic tone mediates arterial pressure elevation. In addition, the obesity increase expression of ACE, angiotensin type 1 and type 2 receptors, angiotensinogen and renin from adipose tissue cause to elevate the activation of renin-angiotensin-aldosterone system (RAAS) increased sodium reabsorption lead to increased blood volume (Van Harmelen *et al.*, 2000; Engeli *et al.*, 2000).

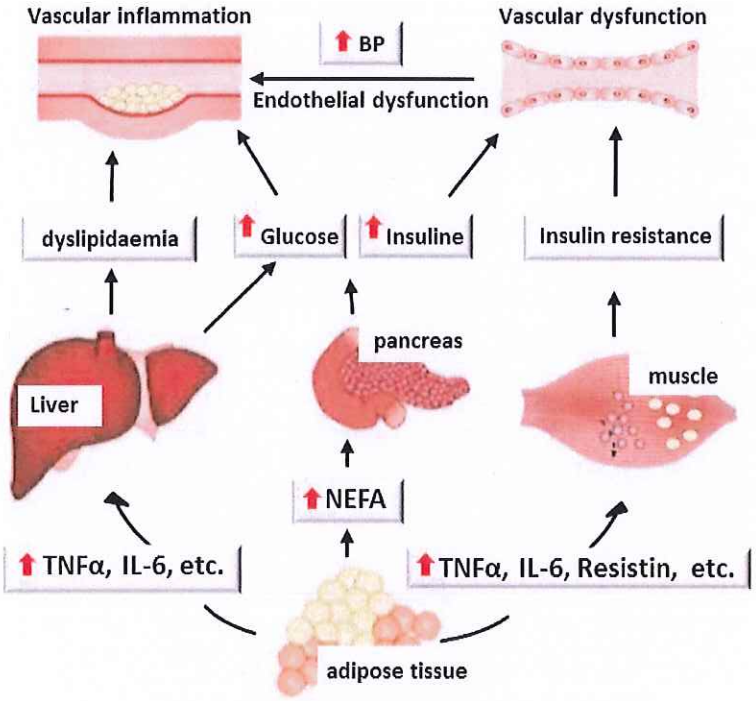

Figure 16 The effects of obesity to metabolic abnormalities. (Figure adapted from Grundy *et al.*, 2005)
tumour necrosis factor-alpha (TNF-α); interleukin-6 (IL-6); fatty acids (NEFA) and blood pressure (BP)

1.4 Aging

Aging is a natural phenomenon beginning at conception and continuing as the organism matures until death. In soon the world will have older people more than children and adult because people have long life due to population aging will continue expand, even accelerate (National Institute on Aging, 2007). The

number of people aged 65 or older is assumed to increase from an estimated 524 million in 2010 to nearly 1.5 billion in 2050 especially in developing countries (Kinsella and He, 2008). The rate of aging depends on genetic and environmental that expect on the rate-of-living and free radical (Harman, 1959). The rate-of-living associated with an organism's metabolic rate is linked to its life expectancy, that this theory showed that the organisms had a higher metabolic rate resulting shorter life spans than the lower metabolic rates (Weinert and Timiras, 2003). The free radical act as oxidative stress also known as reactive oxygen species (ROS) that cause inflammation induce cellular damage to the body (Beckman and Ames, 1998). And the association between the rate-of-living and free radical showed support each other that a high metabolic rate induce to more ROS than in low metabolic rate leading to Ageing (Finkel and Holbrook., 2000)

1.4.1 Aging and metabolic syndrome

Aging is a complicated situation, and one of the most important risk factors for processing a loss in general function to the abnormality, especially metabolic syndrome. The world' s population around 20-25% has metabolic syndrome (Alberti *et al.*, 2006) and Thai adults aged ≥20 years showed around 23.2% for metabolic syndrome (19.5% in men and 26.8% in women) (Aekplakorn *et al.*, 2011). For decade, it has been estimated that 15% to 20% of persons aged over 70 years have the metabolic syndrome (Banks *et al.*, 2007). Several studies suggested that aging induced to increasing of fat accumulation and altered fat metabolism (Tucker and Turcotte, 2002). Indeed, adipose tissue from obese individual is related with production of inflammatory adipokines that caused alter insulin sensitivity and dyslipidemia lead to metabolic syndrome (Wilson and Kannel, 2002). Moreover, Age elevation of the tissue triglyceride concentration and sympathetic activity that may be the compensatory response and the reduced capacity of fatty acid oxidation (Park *et al.*, 2006).

1.4.1.1 Aging and obesity

Aging has been related to the increasing prevalence of obesity. Increased susceptibility to insulin resistance associated with abdominal obesity in aging rats. There are evidence showed that aging lead to increases of subcutaneous fat (SF), visceral fat (VF; fat depots inside the abdominal cavity) and intermuscular adipose tissue (IMAT) with decreasing of total-body skeletal muscle (SM) in both men and women (Folsom *et al.*, 1993). Moreover, the production of adipokines from adipose tissue is regulated by nutrients in aging are overexpression than in adult result sensitive to insulin resistance contributed to metabolic syndrome (Einstein *et al.*, 2008). In addition, in aging has deficient of intracellular fat oxidation via AMPK pathway induces high-glucose and high-fat lead to glucose intolerance and insulin resistance (Qiang *et al.*, 2007). Finally, aging increase body composition (the ratio between fat to muscle) because muscle becomes infiltrated with fat and induce hyperactivation of mTOR signaling contribute to Sarcopenia (Low relative skeletal muscle mass; 0.5–1% loss after the age of 50 year old per year). For these, in aging showed impair in the immune system because to increased production of adipokines and decreased produuction of myokines (Narici and Maffulli, 2010).

1.4.1.2 Aging and inflammation

Aging showed to impaired in immune function due to increase of low-grade inflammation. At above description showed that aging has been related to the increasing prevalence of obesity and adipose tissue can product many low-grade inflammation in circulation system such as C-reactive protein (CRP), interleukin-6 (Greenfield and Campbell, 2006). Moreover, the production of inflammatory cytokines from adipose tissue from aging is high than in young because in old adipocytes can product sphingolipid ceramide higher than in young adipocytes lead to activate NF-κB pathway increasing cytokine production (Wu *et al.*, 2007). In addition, obese individual have higher levels of oxidative stress indue to elevated leptin-to-adiponectin ratio and increased levels of other adipokines, such as tumor necrosis factor and plasminogen activator inhibitor-1 (Wu *et al.*, 2009). Moreover,

aging showed declines in various endocrines have been linked to the aging process. Aging showed increasing of thyroid hormone secretion leading to Hyperthyroidism may elevate metabolic rate, core body temperature, and oxygen consumption, leading to attenuate of ROS and associated oxidative damage. During aging, there is a decreased in stress-induced glucocorticoid sensitivity of pro-inflammatory cytokine production (De *et al.*, 2005). Aging is associated with progressive loss in mitochondrial function. Normally, Mitochondria is the main intracellular source of superoxide anion (O_2^-) due to the mitochondrial respiratory chain and these ROS damage mitochondrial constituents, including proteins, lipids, and mitochondrial DNA (mtDNA) (Bonomini *et al.*, 2015). In aging has to Accumulate of mtDNA damage following mtDNA mutations lead to impairment of the mitochondrial respiratory chain complexes, due to increase in mitochondrial ROS production with increased oxidative damage during aging (Linnane *et al.*, 1989). High concentrations of H_2O_2 promote insulin resistance by stimulates GLUT4 translocation, glucose uptake by adipocytes and muscles, and lipid synthesis in adipocytes. The insulin-like effect of hydrogen peroxide on pathways of lipid synthesis in rat adipocytes Moreover, oxidative stress, have been directlymodify the expression of adiponectin leading to decrease adiponectin levels in circulation system resulting insulin resistence (Pitocco *et al.*, 2010).

1.4.1.3 Aging and hypertension

Aging increase oxidative stress that contributes to hypertension by mediates direct interactions of the central nervous system. Oxidative stress in the rostral ventrolateral medulla (RVLM) can increase glutamatergic excitatory inputs, in contrast reduce GABAergic inhibitory inputs to the RVLM result enhance sympathetic tone from the paraventricular nucleus with mediates arterial pressure elevation that cause to increase sympathetic tone leading to hypertension (Nishihara *et al.*, 2012). Moreover, the inflammatory and reactive oxygen species such as the superoxide anion that elevate when aging can induce nitric oxide change to peroxynitrite and reduced endothelial nitric oxide bioavailability. In addition, the superoxide anion cause to endothelial dysfunction leading to reduce eNOS

phosphorylation for NO production leading to vasoconstriction with hypertension (Jin and Loscalzo, 2010). In addition, cardiovascular anatomy change in anging can contribute to increasing of blood pressure. In aging, the visco-elastic properties of conduit vessels was attenuated and increase stiffening of the arterial vasculature that increase the early return of reflected waves from the peripheral artery each heart beat result to step up of systolic pressure and decrease in DBP (Laurent *et al.*, 2006). Moreover, decrease blood flow to the renal vascular lead to fall in excretion of salt loads efficiently for increasing blood volume with blood pressure levels (Mimran *et al.*, 1992).

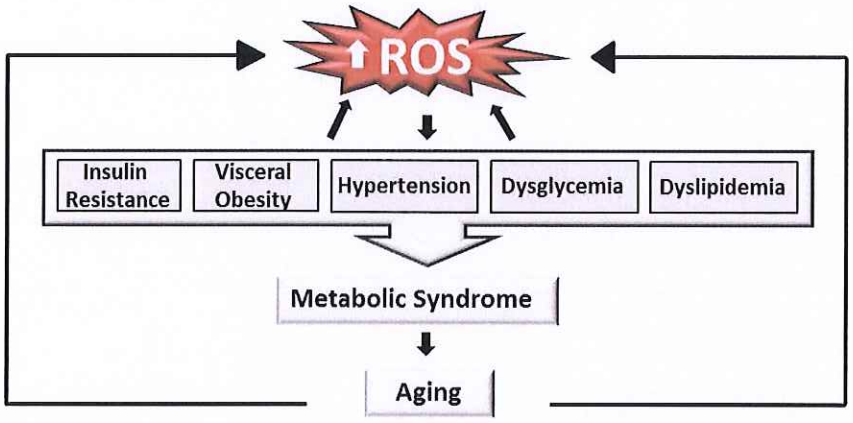

Figure 17 The Relationship of ROS production, accelerated senescence and metabolic syndrome (Figure adapted from Bonomini *et al.*, 2015)

1.4.2 Aging and cardiovascular system

In the U.S. showed deaths attributable to CVD occurred in adults aged ≥65 years estimated 82% of the total number of deaths, and 50% of CVD morbidity occurred in adults aged ≥60 years (Lloyd-Jones *et al.*, 2009). Aging is associated with structure and function changes of cardiovascular system. For the heart structure, aging caused to increase slightly in heart weight lead to hypertrophy (Gerstenblith *et al.*, 1977). On the function, in the resting state, there is no changing of systolic function; however, decreased response to sympathetic stimuli (Rodeheffer *et al.*, 1984; Fleg *et al.*, 1995). Vascular aging is a natural process by alteration of vasculature during aging. Mainly changes consist of endothelial dysfunction lead to endothelial

permeability and reduce nitric oxide-dependent vasodilation. Moreover, aging enhanced oxidative stress to breakdown of NO (Yu and Chung, 2001; Challah *et al.*, 1997). Vascular aging increase migration and proliferation of smooth muscle cell result to large arteries and arterial stiffness is also led to increase arterial pressure (Li *et al.*, 1999; Lakatta and Levy, 2003).

1.4.2.3 Structure change of vascular anatomy in aging

For the vascular aging have changes to reduced compliance, increase in vascular smooth muscle cells (smooth muscle cell hypertrophy) lead to elevate in thickening of the intima media and fall in dilation of the lumen (Marin, 1995). There are evident showed that Intimal-Medial Thickness (IMT) in older is a doubling or tripling thicker than younger ones compare between the ages of 20 and 90 years old (Najjar et al., 2005). In vascular aging increase collagen content on the adventitia, the outermost layer of the blood vessel with increase cross-linking of collagen by raise the interaction between collagen and free amino groups and forms advanced glycation end products (Najjar *et al.*, 2005). In contrast, the quality and function of elastin in the adventitia is loss with age as a result of decreasing tensile stress support and elasticity Feldman and Glagov, 1971). The association of increase collagen content and loss of quality of elastin in the adventitia lead to arterial stiffness (London and Pannier, 2010)

1.4.2.4 Functional change of vascular in aging

Vascular aging show ROS mediated protein damage lead to oxidative stress that leading to accelerated senescence the major role to endothelial dysfunction Oliver *et al.*, 1984; Minamino *et al.*, 2002). The endothelial dysfunction caused to decline in endothelium-dependent vasodilatation in resistance and conductance arteries (Matz *et al.*, 2000; Taddei *et al.*, 2000), reduced nitric oxide-derived from endothelial cell induce to reduce response to NO-dependent vasodilator response to acetylcholine (Yu and Chung, 2001; Taddei *et al.*, 1995). Moreover, Aging Decreases the H_2S production that is a signaling molecule leading to relaxation of blood vessels. In cardiovascular system, H_2S is endogenously produced from cystathionine-γ-lyase,

CSE (Geng *et al.*, 2004; Kimura *et al.*, 2005). Vascular aging increase protein expression of cystathionine gamma-lyase (CSE). In contrast, decrease in H_2S concentration (Predmore *et al.*, 2010). Effects of age on the perivascular adipose tissue (PVAT) that is a adipose surrounds the systemic blood vessel and can attenuates vasoconstrictive responses to norepinephrine or phenylephrine via paracrine release of adipokines and other bioactive molecules such as H_2S, NO and angiotensin 1– 7 (Ang 1–7) (Dubrovska *et al.*, 2004). Vascular aging increase PVAT mass, but the anticontractile effect of PVAT is reduced. Some researcher found that PVAT of aged-rats lost the anti-contractile effect due to reduce bioavailability of NO that produced from PVAT (Melrose et al., 2013). In finally, there are impaired of angiogenesis (the growth of small vessels) in aging contribute to impair in an adaptation mechanism after stress and ischemic injury. The impaired of angiogenesis due to aging impairs growth factor production and endothelial dysfuction lead to attenuate endothelial migration resulting reduced tube formation (angiogenesis) (Rivard *et al.*, 1999)

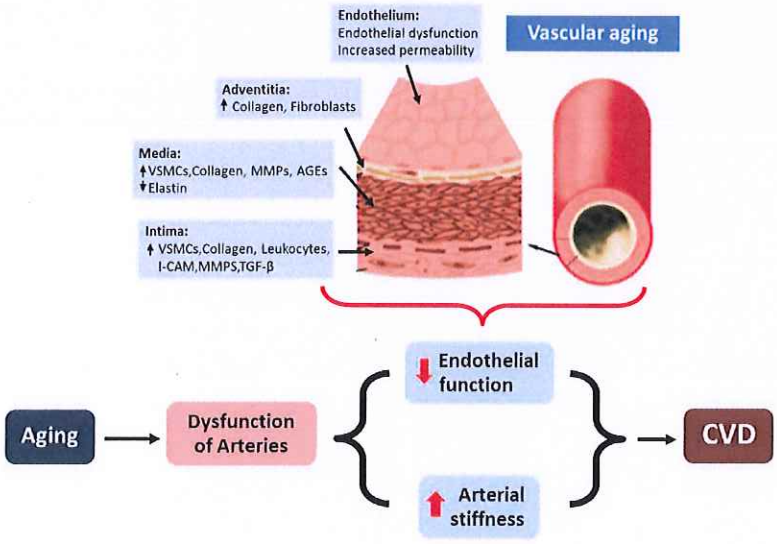

Figure 18 The relationship of aging to cardiovascular disease (Figure adapted from Zieman et al., 2005; Santos-Parker et al., 2014)
I-CAM=intercellular adhesion molecule; MMP=matrix metalloproteinase; TGF-β=transforming growth factor-β and VSMCs=vascular smooth muscle cells

CHAPTER 2

Effects of 6 weeks oral gavage of a dichloromethane extract of *Kaemperia parviflora* rhizomes on lipid profile in middle-aged male rat

2.1 ABSTRACT

Age is one of valuation factors for metabolic syndrome because it cause to changes of the metabolism and function for the body systems. In the present, complementary medicine is one of most matter for prolonging good health. In Thai traditional medicine, rhizomes of *Kaempferia parviflora* have been used for controlling body weight.

The present study investigated the effects of 6 weeks oral gavage of *Kaempferia parviflora* dichloromethane (KPD) extract fed to middle-aged effect on liver and kidney functions, fasting serum lipid profiles, visceral and liver lipid accumulation. The middle-aged male rats were orally gavaged with the KPD extract at the dosage of 100 mg/kg body weight or with the vehicle once or twice a day for 6 weeks. At the end of administration, complete blood count measured by the Automated Hematology Analyzer, blood chemistry was measured by enzymatic methods, liver and kidney morphology measure by Hematoxylin and eosin (H&E) stained sections, and a liver lipid accumulation used Oil Red O to stain neutral lipids in hepatocytes.

There were no differences found for the parameters studied between KPD treated- and vehicle group by the KPD (100 mg/kg) once a day. When the KPD was given twice a day, Treatment group decreased body weight but didn't change in food intake. The morphology of liver and kidney was normal, and the serum liver and kidney enzyme levels (ALP, SGOT, SGPT, BUN and creatinine) were in the normal range. The decreased the serum triglycerides and glucose level was also observed

compared to vehicle treated group with the decreased the visceral and subcutaneous adipose tissue. Liver lipid accumulation was significantly decreased compared to that of the control group.

The researchers concluded that KPD caused to decrease in body weight, visceral and subcutaneous fat, liver lipid accumulation, fasting serum glucose and triglycerides in middle-aged male rat. Therefore, it may also improve health quality of life and decrease risk of metabolic decease in the elderly.

2.2 INTRODUCTION

Aging is a complicated situation, and one of the most important risk factors for processing a loss in general function to the abnormality, especially metabolic syndrome and/or cardiovascular diseases. Several studies showed that aging increase of fat accumulation and altered fat metabolism (Poehlman *et al.*, 1995; Raben *et al.*, 1994; Zurlo *et al.*, 1990). Aging led to increase the visceral fat and the fat of peripheral tissues, such as liver and skeletal muscle (Park *et al.*, 2006). Aging caused increasing the inflammatory response to excess nutrients and vulnerability to FFA-induced insulin resistance, and increasing susceptibility and variability in toluene response (Einstein *et al.*, 2010, MacPhail *et al.*, 2012).

Kaempferia parviflora (KP) Wall Ex. Baker is a perennial Zingiberaceae plant that found in the northern part of Thailand. In Thai traditional medicine it has been used as a folk medicine. Rhizomes of this plant have been used for treating various symptoms including erectile dysfunction, hypertension, inflammation, abdominal pain, anti HIV-1 protease, anti-allergic as well as for the promotion of longevity with good health and well being (Sookkongwaree *et al.*, 2006; Tewtrakool *et al.*, 2008; Wutythamawech, 1997; Yenjai *et al.*, 2004). The active chemical components of KP consist of 11 flavonoids that are 5-hydroxy-7-methoxyflavone, 5-hydroxy-3,7-dimethoxy -flavone, 5,7- dimethoxyflavone, 3,5,7-trimethoxyfla- vone, 5-hydroxy-3,7,4'-trimethoxyflavone, 5-hydroxy-7,4'-dimethoxy-flavone, 5-hydroxy-3,7,3', 4'-tetramethoxyflavone, 5,7,4'-trimethoxyflavone, 3,5,7, 4'-tetramethoxyflavone, 5,7,3',4'-tetra- methoxyflavone, and 3,5,7,3',4'-pentametho-xyflavone (Sutthanut *et al.*, 2007). In the previous, there were many researches about

the toxicity of KP extract. Oral acute toxicity study showed that LD50 value of KP Wall ex Baker was more than 13.33 g/kg. Chronic toxicity study performed orally given KP powder at the doses of 20, 200, 1,000 and 2,000 mg/kg /day for six months respectively. The result suggested that all KP-treated groups had no difference for the parameters. Only 2000 mg/kg KP treated-rats elevated weight of liver ($p<0.05$) which might be due to lower body weight, less eosinophil, increased serum sodium levels; however, the value is in the normal range (Chivapat *et al.*, 2004). Chivapat *et al.* (2010) performed orally given KP ethanolic extract at doses of 5, 50 and 500 mg/kg/day for six months respectively both males and females wistar rats. The results showed that male rats receiving KP ethanolic extract at dose of 500 mg/kg had significantly lower body weight than both control groups. The results showed that male rats were received the highest dose of *Kaempferia parviflora* extract caused to decrease triglyceride level but female rats that received the same dose caused to increase glucose and cholesterol levels than their control groups.

In this decade, there were many researches about the effect of KP extract on lipid metabolism. KP showed suppression on body weight, lipid metabolism, insulin resistance, glucose intolerance, hypertension and hyperinsulinemia in Tsumura, Suzuki, Obese Diabetes TSO mice (a spontaneously obese Type II diabetes model) but no differences in the Non-Obesity mice (the corresponding control mice). These result indicated that KP had antiobesity effects (Akase *et al.*, 2011). Nakao *et al.* (2011) investigated the inhibitory activities of KP against xanthine oxidase (XOD) which expected to improve hyperuricemia (hyperuricemia can promote metabolic syndrome). The methoxyflavones of KP rhizomes showed xanthine oxidase inhibitory activity (38% at 500 μg/ml) that relative with their crystal structures were subjected to the XOD inhibitory test. Among of these, 3,5,7,4',5'-pentamethoxyflavone and 3',4',5,7-tetrame-thoxyflavone showed more potent inhibitory activities than the other methoxyflavone. *Kaempferia parviflora* ethyl acetate extract (KPE) and its Components; 3,5,7,4'-tetramethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone could stimulate differentiation of 3T3-L1 preadipocytes to small adipocytes that could induce adiponectin expression for regulating glucose levels and improving insulin sensitivity. Thus, KPE is involved in maintaining metabolic homeostasis for anti-metabolic disorders effects (Horikawa *et al.*, 2012). Therefore, it was motivation

to investigate of KP rhizome on gross organ abnormality and lipid accumulation in middle-aged rats.

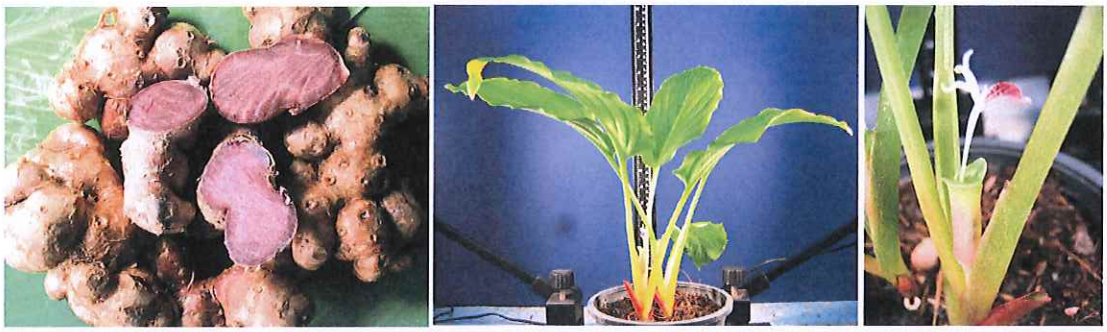

Figure 19 Rhizome, herb, and flower of *Kaempferia parviflora*

2.3 OBJECTIVES

To investigate effects of chronic treatment with the KPD extract on abnormal gross organ developments and fat metabolism in middle-aged male rats. The following parameters were therefore investigated:

(1) animal body weight and food intake

(2) gross abnormalities of internal organs

(3) liver and kidney morphology and functions

(4) complete blood count (CBC) and blood chemistry

(5) visceral and subcutaneous fat, liver lipid accumulation

2.4 METERIALS AND METHODS

2.4.1 Plant material

The study substance, fresh rhizomes of KP were cultivated in Ampur Phurua, Loei Province Thailand and it was purchased in April 2009. Botanical identification of the plant was carried out by comparison with the Herbarium

specimens in the Department of Biology Herbarium, Faculty of Science, Prince of Songkla University, Thailand and the voucher specimen (Collecting no.2548-03) was kept in Herbarium.

2.4.2 Preparation of *Kaempferia parviflora* dichloromethane (KPD) extract

Fresh rhizomes of KP (20 kg) were sliced and pulverized with a Wonder to generate fine powder. The KP powder was extracted of their dark color by macerating twice with 95% ethanol (2x20 L) for two days, then extracting three times with 100% dichloromethane (3X20 L). The dichloromethane soluble part was filtered and evaporated at a temperature not more than 40 °C by rotary evaporation under reduced pressure for removed solvent. After that the dried residue was treated by suction under high pressure with an oil vacuum pump for displacement residual dichloromethane, giving residues of 520 g (2.6 % yield) a yellowish gummy *Kaempferia parviflora* dichloromethane extract (KPD).

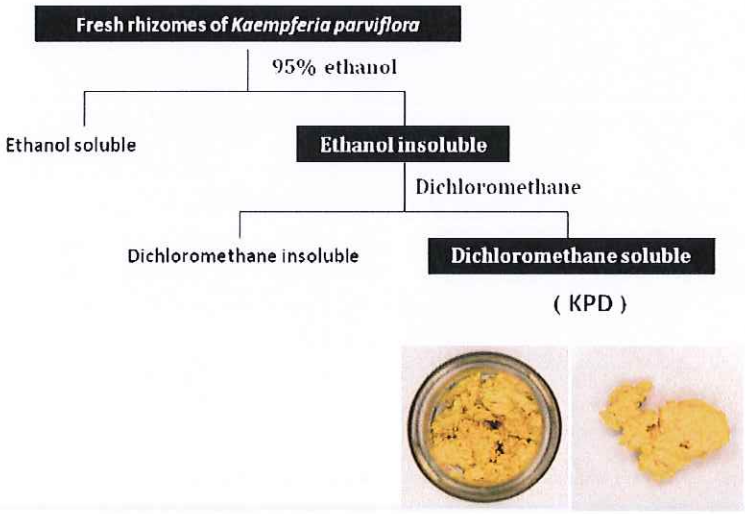

Figure 20 *Kaempferia parviflora* dichloromethane (KPD) extract

The chemical profile of KPD was analyzed by high performance liquid chromatography (HPLC). An aliquot of KPD in methanol was subjected to reversed phase HPLC analysis out on a HP1100 system (Agilent Technologies). Under the following conditions: a Symmetry ® C_{18} column (5μm, 3.9x150 mm i.d.; Waters); gradient elution, solvent A (0.5% trifluoroacetic acid in deionized H_2O) and solvent B (0.5% trifluoroacetic acid in methanol), gradient profile: 0 min, 95% A, 5% B; 30 min, 100% B; 40 min,100% A; 50 min, 5% A, 95% B). The flow rate was 1 ml/min; detection, the UV traces were measured at 210 and 254 nm and the UV spectra (DAD) were recorded between 190 and 500 nm.

2.4.3 Experiment design

Middle-aged and young positive control wistar male rats (12-14 month old and 10 week old, respectively) were purchased from the Animal House, Faculty of Science, Prince of Songkla University. Animals were housed in a hygienic conventional animal room of the Prince of Songkla University Animal Care and Use Committee, where the environment of the room was maintained at 25 °C and 12 hour-light-dark Cycle. The investigation conformed to the Guide for the Care and Use of Laboratory Animals (Ethic No. 22/52). Prior to the effect of KPD chronic oral administation study, all of animals were acclimatized to the environment for 1-2 weeks prior to experimentation with tap water *ad libitum*. The Middle-aged wistar male rats were randomly divided into 6 groups with 6-8 animals each and rats weigh approximately 450-500 g in each group. Two experimental groups were orally administered with KPD at the doses of 100 mg/kg /day (9.00 AM) and the other one was treated twice (9.00 AM and 6.00 PM) a day, for 6 weeks. The other two control groups orally received the vehicle (a mixture of tween 80, 0.2 (g): carboxy-methylcellulose sodium salt, 0.2 (g): distilled water, 10 ml) and two positive control groups received distilled water once or twice a day at the volume of 1 ml/kg in the same period of 6 weeks.

2.4.4 Oral administration and detection of the drug in the blood

For measurement bioavailability of KPD in the blood stream after oral administration. 6 middle-aged wistar male orally administered with KPD at the doses of 100 mg/kg. Rats were anesthetized with sodium pentobarbital (50 mg/kg body weight). After that, 1.0 ml of venous blood was then drawn from the jugular vein into heparinized tubes after 0, 30, 60, 90 min from the first three rats, and at 120, 150 and 180 min from the second three rats. Samples were immediately centrifuged at 4000 rpm for 10 min and the plasma was collected. The methoxyflavones of KP were extracted with 100 % methanol and re-centrifuged for separation methanol soluble part. The supernatant was evaporated to remove methanol under vacuum at a temperature not more than 40 °C until dryness (Speed-Vac AES 1010; Savant Instruments Inc.; Farmindale, NY, USA). Then partition extracted with 100% dichloromethane twice, the soluble part was collected and evaporated to dryness. The residue obtained by sample evaporation was dissolved in 60 µl of 100% methanol (HPLC grade) for injecting into the analytical HPLC. HPLC analysis KPD metabolites was performed With a Symmetry® C_{18} column (5µm, 3.9x150 mm i.d.; Waters), out on a HP1100 system (Agilent Technologies). The mobile phase consisted of a gradient elution, solvent A (0.5% trifluoroacetic acid in deionized H_2O) and solvent B (0.5% trifluoroacetic acid in methanol), gradient profile: 0 min, 95% A, 5% B; 30 min, 100% B; 40 min,100% A; 50 min, 5% A, 95% B). The flow rate was 1 ml/min; detection, the UV traces were measured at 210 and 254 nm. The absorption was measured between 200 and 500 nm using photo-diode array detector.

2.4.5 Body weight change and food intake

Body weight and its 24 h food intake were recorded every week from the start of the experiment to its completion (6 weeks). The mean of food intake was calculated by deducting the weight of the remaining food and the spilled food of the filled feed box. The treated-animals were closely observed for behavior and signs of abnormalities.

2.4.6 Effects of chronic KPD treatment on the hematology and clinical biochemical analysis

At the end of the 6 weeks treatment period, the animals were fasted overnight (13-15 h), the treated or control rats were killed by decapitation with a guillotine. Blood samples were collected from the decapitated rat into a plastic test tube containing EDTA (2 ml, for determining hematological) and a glass test tube (5 ml, for determining serum clinical chemistry). Hematological analysis was performed using the Automated Hematology Analyzer (Celltac E, Model MEK-7222K, Japan). Parameters examined were white blood cell count (WBC), hematocrit (Hct), hemoglobin (HGB), mean corpuscular volume (MCV), mean concentration hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), white blood cell count (WBC) including Neutrophils and lymphocytes, and total platelet count. Blood samples in the glass test tube was placed in room temperature for 30 min before centrifuged at 3200 rpm for 10 min, and the supernatant (serum) was collected to ependops and stored at -70 ºC until measurements for the liver and kiney enzymes (the liver enzymes; alkaline phosphatase (ALP), Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT), the kiney enzymes; blood urea nitrogen (BUN) and creatinine) and lipid levels (lipid profile; Glucose, Triglyceride, Cholesterol, High Density Lipoprotein cholesterol (HDL-C), Low Density Lipoprotein cholesterol (LDL-C)) which were done within 1 month from start collection. Clinical chemistry values were measured by using the Automatic Chemistry Analyzer (Hitachi Modular P800, Germany) routinely operated at the Prince of Songkla University Hospital.

2.4.7 Effects of chronic KPD treatment on internal organs and lipid accumulation

The decapitated rat that already removed of the thoracic aorta and the mesentery was dissected to determine gross pathological alterations of various visceral organs: heart, lung, liver, adrenal gland, kidney, testes. Each organ was cleaned of fat and wash with Krebs Heinseleit solution before plated dry with filter

paper. After that visceral fats was removed carefully from the epididymis, testis and retroperitoneal, mesentery, as well as subcutaneous and weighed by using the Mettler PL2001-L balance (Mettler Toledo International Inc., Switzerland). The visceral organs and visceral fats weights were calculated into relative organ weight (g/100 g body weight). Finally, the livers and kidneys were cut into 1x1 cm for fixed by 10% formaldehyde.

2.4.8 Histopathological study of liver and kidney

Sections of liver and kidney that fixed in 10% formaldehyde, which were done within 2 weeks of fixed sample. The organ sections were processed by dehydration with 80% isopropanol and 100% isopropyl alcohol, dehydrated tissues were cleared xylene and were embedded in paraffin blocks. 5 μm sections were prepared using a microtome and placed on slides. Slides are deparafinized with xylene and rehydrated with 100% isopropyl alcohol, after that they are stained with Hematoxiline-Eosine (H&E) and protected with a coverslip using permount gel mount before review of tissue histology by light microscopic examination. Slides of all groups were studied and photographed. A minimum 12 fields of each section were studied.

2.4.9 Effects of chronic KPD treatment on liver lipid accumulation

The middle lobe of the liver was cut into 2 pieces, were immediately transferred into -20 °C of cryostat Microtome for embedded into a cryostat gel. 20 μm thick sections were prepared using a cryostat microtome and stained with oil red O was prepared by mixing 0.5% Oil Red O into absolute propylene glycol, and cover-slipped using glycerine jelly for observations with a light microscope. After that, each slide of oil red O samples was extracted with 1 ml of 100% DMSO and the oil red O solution was measure the absorption of radiation by Spectrophotometry at 520 nm (Thermo Fisher Scientific, Model G10s UV-VIS, USA), and the concentration of the oil red O was compared with the standard curve that known concentrations (μg/ml).

The duplicate concentration of the oil red O was shown in terms of μg/ml/cm^2, each slide of a section tissue was measured area by using the Auto CAD 2005 program.

2.4.10 Effect on blood pressure

At 6 weeks after administration of the KPD or vehicle control to middle-aged male rats. Rats were anesthetized with sodium pentobarbital by intra-peritoneal injection (60 mg/kg, i.p.). The tracheal tube was cannulated with a polyethylene tube to facilitate spontaneous respiration. A polyethylene catheter was inserted through the right common carotid artery which was connected to a pressure transducer (P23 ID, Gould Statham Instrument, Hato Rey, Puerto Rico) to measure systolic blood pressure (SBP) and diastolic blood pressure (DBP), and the heart rate was recorded using a tachygraphy driven by the blood pressure wave. Both pressure transducer and tachygraphy were connected to a Grass polygraph (model 7D, Grass Instrument, Quincy, MA, U.S.A.) to monitor the blood pressure and heart rate, respectively. The animal was then equilibrated for at least 40 min.

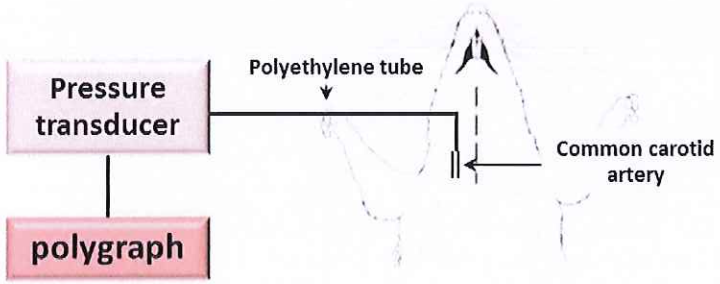

Figure 21 *In vivo* preparation of anesthetized rat

2.4.10 Data analysis

The data were displayed as mean ± S.E.M. (n=6-8), "n" represents the number of rats. Tests of significance between the two measurements were made using the two-tailed Student's unpaired t-test. For multiple comparison tests, ANOVA was used and post-hoc analysis was performed with the Duncan test. In all experiments, a P value ≤ 0.05 was considered statistically significant.

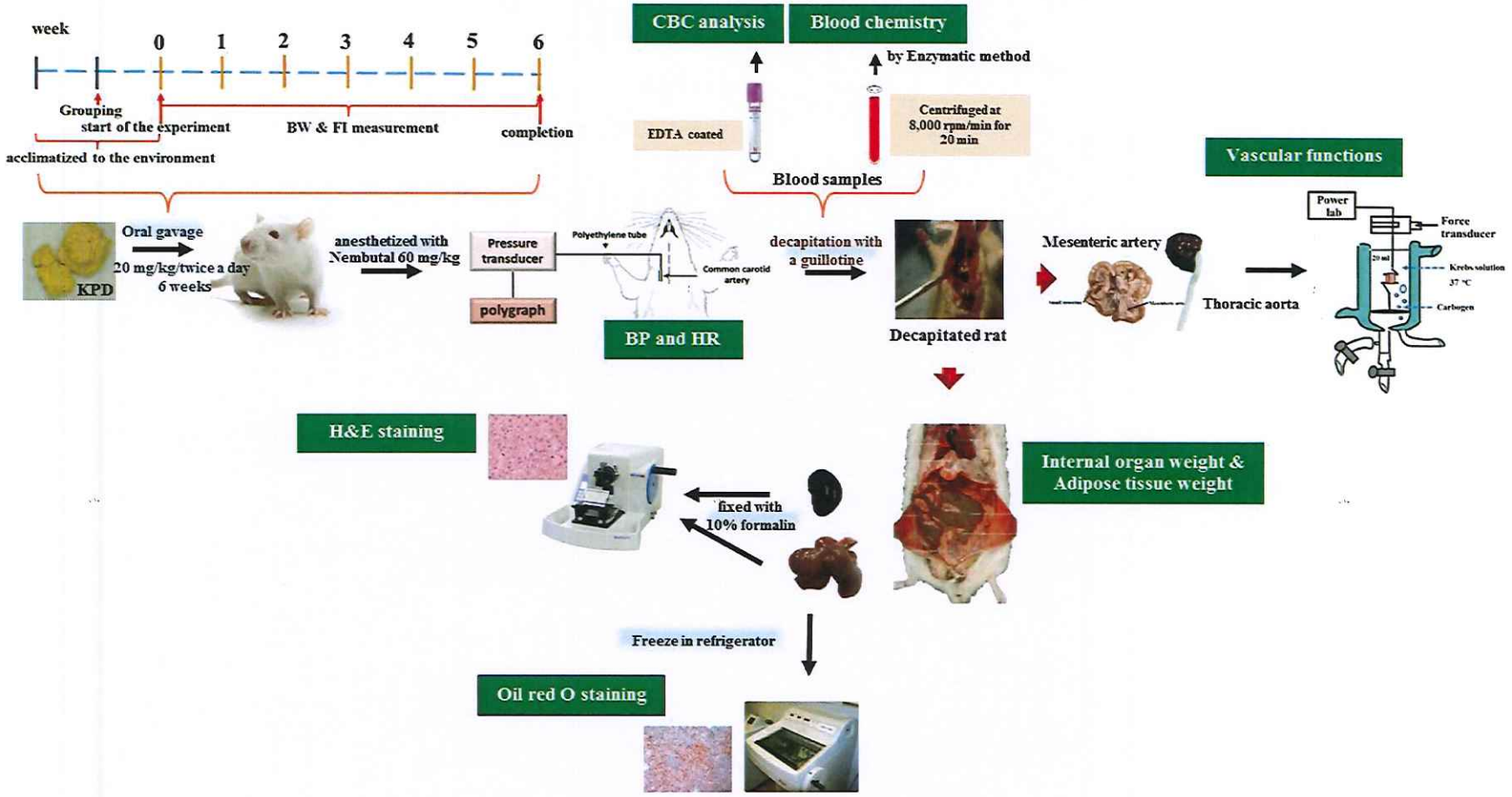

Figure 22 Scope of experimental studies the effects of chronic treatment of KPD on lipid profile

2.5 RESULTS

KPD was a yellowish gummy and was obtained with a 2.6 % yield. HPLC Analysis of KP that was extracted by dichloromethane (KPD) showed high amount of flavonoid amongst that was supported by K. Sutthanut *et al.* (2007). Identification of pure was based on comparisons of retention time and UV-spectra with pure flavonoid standards obtained from Assoc. Prof. Chaweewan Jansakul, Faculty of Traditional Thai Medicine Prince of Songkla University. Chromatograms of the KPD shown its three major pure compound: 3,5,7,3′,4′-pentamethoxyflavone (PMF), 5,7-dimethoxyflavone (DMF) and 5,7,4′-trimethoxyflavone (TMF) which fingerprints using a C18 RP-HPLC method were showed their retention times and corresponding UV spectra are shown in Fig. 23. All pure methoxyflavone showed as turbid white powder. The Quantitative analysis by HPLC chromatography was performed by measuring the area under the peak of each of compounds, as extrapolated by comparison with standard curves prepared with pure standards that known concentrations. Flavonoid content profiles from HPLC analysis of KPD sample were expressed as mg/g of dried powder of total KPD content. Highest level of flavonoid was found in DMF (84.88 mg/g) followed by PMF (70.03 mg/g) and TMF (68.98 mg/g), which together amounted to 223.89 mg/g of dried KPD extract.

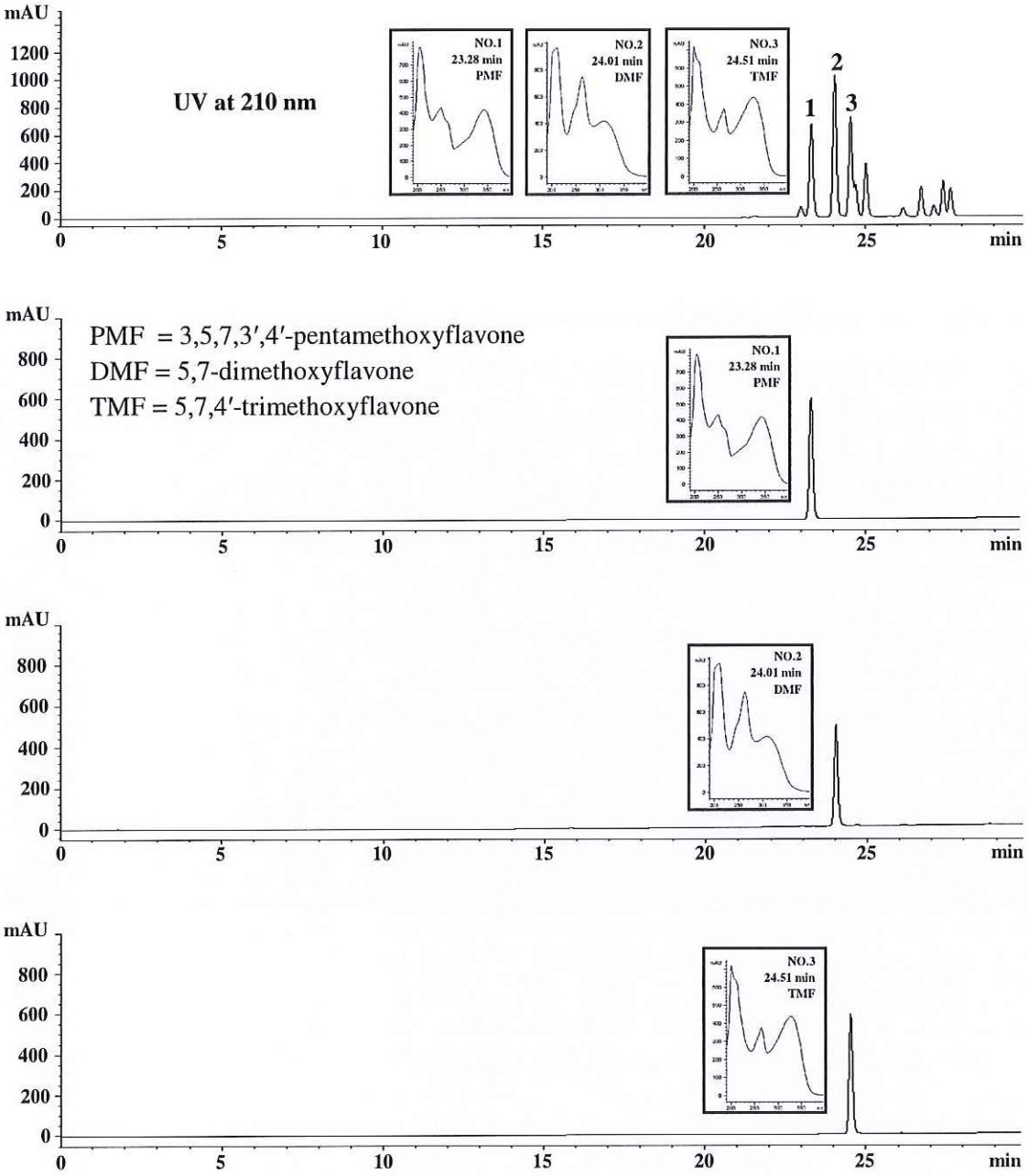

Figure 23 Chromatograms obtained during determination of the major components of the KPD. Detection was at 210 nm, and full scale was 0–1200 mAU. Uppermost was HPLC Finger printing of the KPD followed by their major methoxyflavones: PMF, DMF and TMF.

2.5.1 Absorption, Bioavailability, and Metabolism of the KPD

Flavonoid from KPD was evaluated for pharmacokinetics in rats. Accurately weighed sample of KPD was suspended in a mixture of tween 80, 0.2 (g): carboxy-methylcellulose sodium salt, 0.2 (g): distilled water, 10 ml, at a concentration of 100 mg/ml (equivalent to KPD 100 mg/kg) and was administered to each animal by steel feeding. Blood collection points were decided at 0, 30, 60, 90, 120, 150 and 180 min post administration. The methoxyflavones could not be detected in plasma following oral Administration at 0 and 30 min, it was detected in the blood stream after about 60 min post administration. The three methoxyflavones an extremely rapid fall in the plasma concentration of the drug after 120 min, and none were detected 180 min post administration was observed in all animals tested (n = 3). Fig. 24 showed the HPLC-DAD fingerprint detection was at 210 nm of blank plasma and the three major methoxyflavones are showed in blood sample prior to drug administration, except for the peak 1 that was for an unknown substance that found at the 30 and 180 min time post administration. After 60 min, amounts of three major methoxyflavones were found even at the highest concentration in blood stream. At the 120 min time point, methoxyflavones levels detected were reduced. Absorption and elimination pattern showed that KPD was found to be eliminated from systemic circulation at 3 hour.

Table 2 The concentration of PMF, DMF and TMF in blood samples administration with KPD (100 mg/kg body weight).

Major compound	Concentration of pure compound in blood (μg/ml)						
	0 min	30 min	60 min	90 min	120 min	150 min	180 min
PMF	0.0 ±0.0	0.0 ± 0.0	8.2 ± 0.3	6.1 ± 0.7	1.4 ± 0.3	1.3 ± 0.3	0.0 ± 0.0
DMF	0.0 ±0.0	0.0± 0.0	10.2 ±0.5	7.9 ± 0.9	2.9 ± 0.1	2.3 ± 0.1	0.0 ± 0.0
TMF	0.0 ±0.0	0.0 ± 0.0	7.3 ± 0.6	5.4 ± 0.5	1.2 ± 0.2	1.2 ± 0.2	0.0 ± 0.0

Each point represents a mean ± S.E.M. of 3 rats.

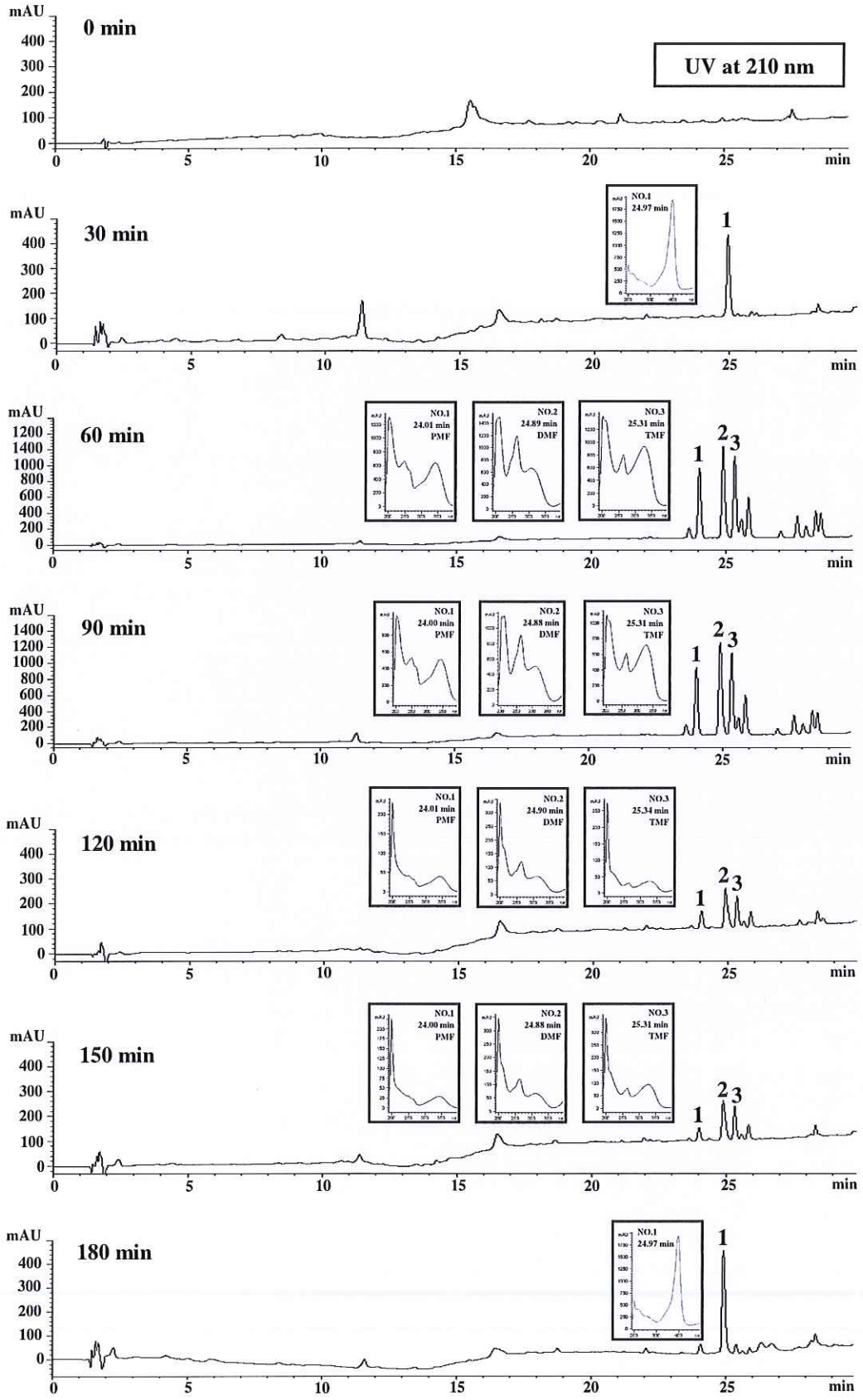
0 min
UV at 210 nm
30 min
NO.1
24.97 min
60 min
NO.1
24.01 min
PMF
NO.2
24.89 min
DMF
NO.3
25.31 min
TMF
90 min
NO.1
24.00 min
PMF
NO.2
24.88 min
DMF
NO.3
25.31 min
TMF
120 min
NO.1
24.01 min
PMF
NO.2
24.90 min
DMF
NO.3
25.34 min
TMF
150 min
NO.1
24.00 min
PMF
NO.2
24.88 min
DMF
NO.3
25.31 min
TMF
180 min
NO.1
24.97 min
mAU
min
1
2
3

Figure 24 The HPLC- DAD fingerprint of PMF, DMF and TMF in blood samples administration with KPD, blood sample prior to KPD administration collected from rat blood 0, 30, 60, 90, 120, 150, and 180 min post oral administration (100 mg/kg body weight).

2.5.2. Effect of the KPD on body weight, food intake and relative organs weight

None of the parameters differences compared between both distilled water and vehicle control. Therefore, the data from the vehicle group were chosen to represent the distilled water control experiment. The KPD (100 mg/kg) once a day treatment groups had no significant differences in body weight compared to vehicle control group (Fig. 25A). However middle aged male rat were received KPD at dose of 100 mg/kg twice a day had significantly lower body weight than the vehicle control groups at week 5 till the end of the administration (Fig. 25C), while the tended of body weight decreased at week 3 of the KPD treatment. All KPD-treated groups had no altered food consumption in during the experiment (Fig. 25B and 25D).

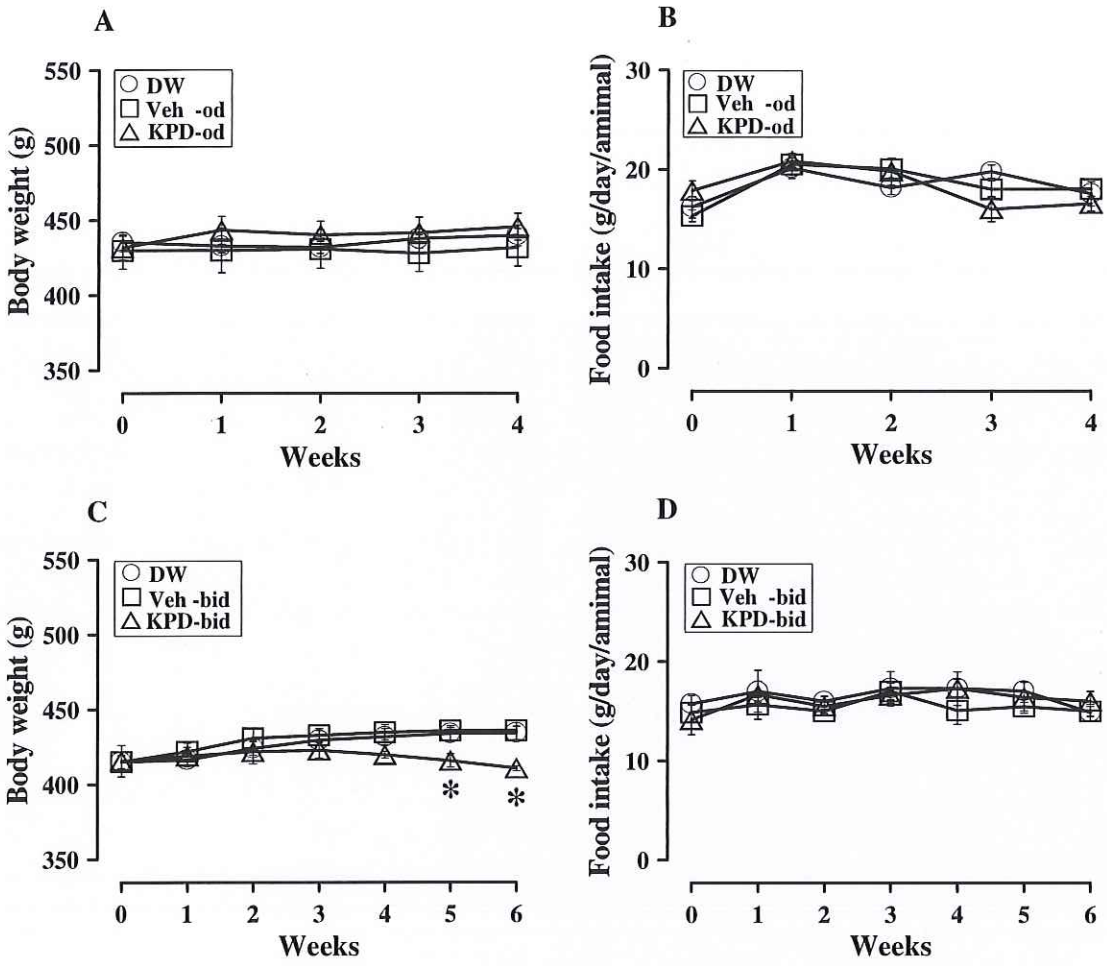

Figure 25 The body weight (left) and food consumption (right) of middle-aged male rats receiving KPD for 6 week. A *Kaempferia parviflora* dichloromethane extract (KPD), distilled water (DW) or vehicle (Veh) once (od), or twice (bid) a day. Data represent a mean ± S.E.M. of 8 animals for each group. * Significantly lower than that of the distilled water and vehicle control group, $P \leq 0.05$.

In addition the chronic effect of KPD on relative weight of some organs showed that both once and twice a day of KPD treatment had no altered relative internal organ weights of adrenal glands, kidneys, liver, lung, spleen, testes, ventricle and atrium, compared to the vehicle control group (Table 3).

Table 3 Body weight (g) and relative organ weight (g/100g body weight) of middle-aged male rats were gavaged KPD for 6 weeks

Treatments	Body weight (g)		n	organs weight (%g)							
	Before	After		Adrenal gl.	Kidneys	Liver	Lung	Spleen	Testis	Ventricles	Atrium
DW	415.25 ± 8.54	434.51 ± 7.82	8	0.016 ± 0.006	0.49 ± 0.02	2.15 ± 0.06	0.49 ± 0.02	0.18 ±0.01	0.88 ± 0.03	0.28 ±0.02	0.012 ±0.002
Veh	430.70 ± 10.29	434.80 ± 13.29	8	0.0145 ± 0.006	0.48 ± 0.01	2.20 ± 0.05	0.50 ± 0.02	0.17 ± 0.01	0.83 ± 0.06	0.27 ± 0.04	0.013 ± 0.001
KPD	431.50 ± 10.05	445.5 ± 11.05	8	0.015 ± 0.004	0.49 ± 0.01	2.22 ± 0.03	0.51 ± 0.02	0.17 ± 0.01	0.91 ± 0.03	0.27 ± 0.08	0.014 ± 0.001
Veh-bid	415.20 ± 6.48	436.20 ± 6.76	8	0.016 ± 0.005	0.49 ± 0.02	2.13 ± 0.05	0.50 ± 0.02	0.18 ± 0.01	0.90 ± 0.02	0.27 ± 0.02	0.013 ± 0.002
KPD-bid	415.86 ± 10.57	410.83 ± 1.57*	8	0.015 ± 0.004	0.51 ± 0.02	2.41 ± 0.06	0.50 ± 0.02	0.17 ± 0.01	0.91 ± 0.05	0.27 ± 0.03	0.014 ± 0.001

Values are expressed as mean ± S.E.M., n=8 for each group. * Significantly lower than control group, $P \leq 0.05$.

2.5.3. Effect of KPD on liver and kidney histopathology

In normally, the liver tissue section shows especially on central vein and portal tracts. The central vein is a conjugation area of sinusoids with practically no connective tissue. On portal tract area consist of portal triad with portal vein, hepatic artery and bile duct. In the normal liver section showed smooth and round portal tract with contains fine elastic fibers up to the portal interface. Histological profile of the normal kidney showed normal glomerulus enclosed by the Bowman's capsule, proximal and distal convoluted tubules without any inflammatory changes (Ragavan and Krishnakumari, 2006). The KPD (100 mg/kg) once or twice a day treatment groups did not alter the structure of both liver and kidney, when compared with distill water control group or vehicle control group. Histological examination of the liver of both KPD (100 mg/kg) once and twice a day treatment groups shown normal histological features (Fig. 26), and no glomerular damage was found in the KPD -treated groups (Fig. 27). There were no remarkables histopathological lesions in KPD- treated groups different from both distill water and vehicle control.

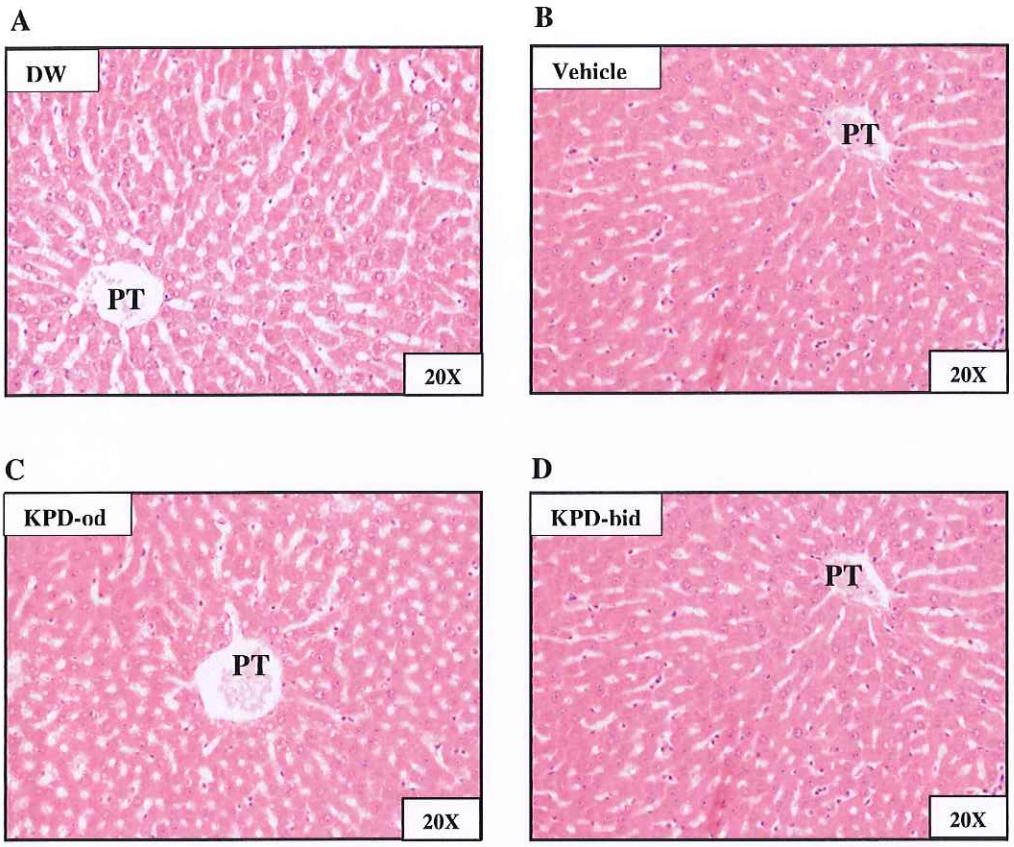

Figure 26 Light micrographs of section in the liver of the 6 weeks KPD treatment rats, showed portal tract (PT) in the portal area and surrounding hepatocytes (X 20). A) normal hepatic tissue, distill water control group (DW); B) vehicle control group (Veh); C) The KPD (100 mg/kg) once a day treatment groups (KPD-od); D) The KPD (100 mg/kg) twice a day treatment groups (KPD-bid).

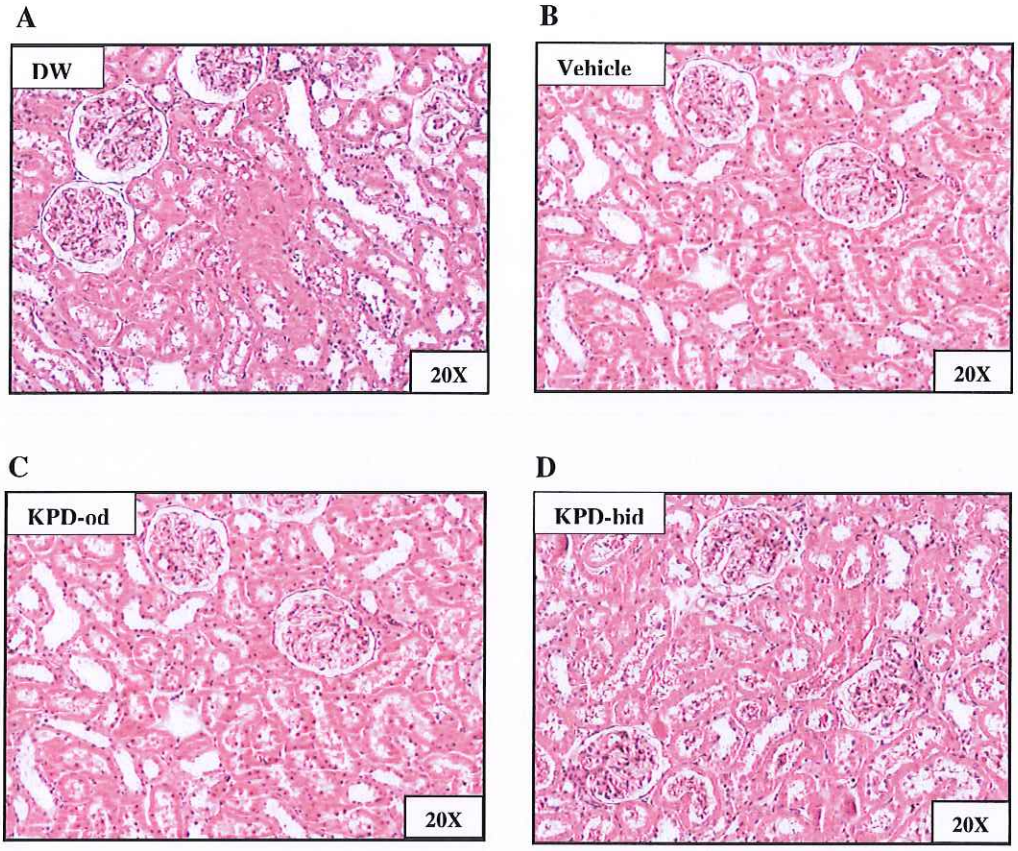

Figure 27 Light micrographs of rat kidney treated with KPD for 6 weeks showed the glomerulus and the Bowman's space well defined, normal renal tubules (X 20). A) normal hepatic tissue, distill water control group (DW); B) vehicle control group (Veh); C) The KPD (100 mg/kg) once a day treatment groups (KPD-od); D) The KPD (100 mg/kg) twice a day treatment groups (KPD-bid).

2.5.4. Effects of KPD on visceral fat and subcutaneous fat

Changing in visceral fat and subcutaneous fat at 6 weeks after KPD administration, the KPD (100 mg/kg) once a day treated- group had no altered on relative adipose tissue weights of the epididymis, retroperitoneal, mesentery and subcutaneous adipose tissues. However, the accumulation of the epididymis,

retroperitoneal and subcutaneous fat was significantly suppressed in the twice a day treatment groups compared to distill water or vehicle control group, but that of mesentery fat was not only tend decreasing. The weight of epididymis, retroperitoneal and subcutaneous fat isolated at dissection in the vehicle control group was about 1.43, 1.59 and 1.31 times that of the KPD twice a day treated-group (Fig. 28-29, Table 4).

Table 4 Effects of 6 weeks oral administration of a KPD on the accumulation of adipose tissue in middle aged male rats; distilled water (DW) or vehicle (Veh), KPD treated-rat once (KPD-od) or twice (KPD-bid) a day.

Treatments	n	Adipose tissue weight (%g)			
		Epididymis	Mesentery	Retroperitoneal	Subcutaneous
DW	8	2.30 ± 0.16	2.08 ± 0.14	2.31 ± 0.21	5.96 ± 0.64
Veh	8	2.31 ± 0.16	2.14 ± 0.12	2.37 ± 0.24	6.09 ± 0.38
KPD	8	2.27 ± 0.09	1.85 ± 0.09	2.07 ± 0.1	5.38 ± 0.30
Veh-bid	8	2.61 ± 0.22	2.07 ± 0.18	2.45 ± 0.31	6.91 ± 0.34
KPD-bid	8	**1.82 ± 0.11***	1.70 ± 0.06	**1.54 ± 0.12***	**5.29 ± 0.30***

Data was show on the relative adipose tissue weight (g/100 g body weight) of middle-aged male rats. Values are mean ± S.E.M for 8 rats in each group.* Significantly lower than control group, $P \leq 0.05$.

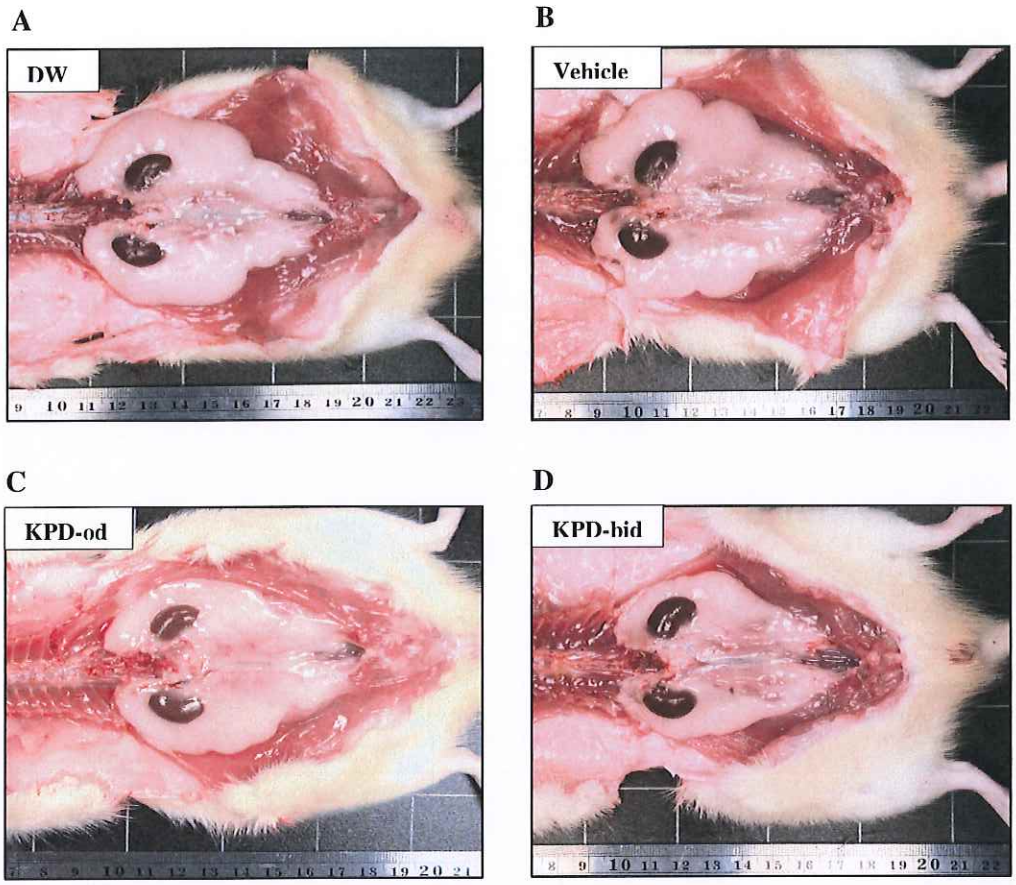

Figure 28 Differences in the accumulation of visceral fat by KPD treatment for 6 weeks; distilled water (DW) or vehicle (Veh), KPD treated-rat once (KPD-od) or twice (KPD-bid) a day.

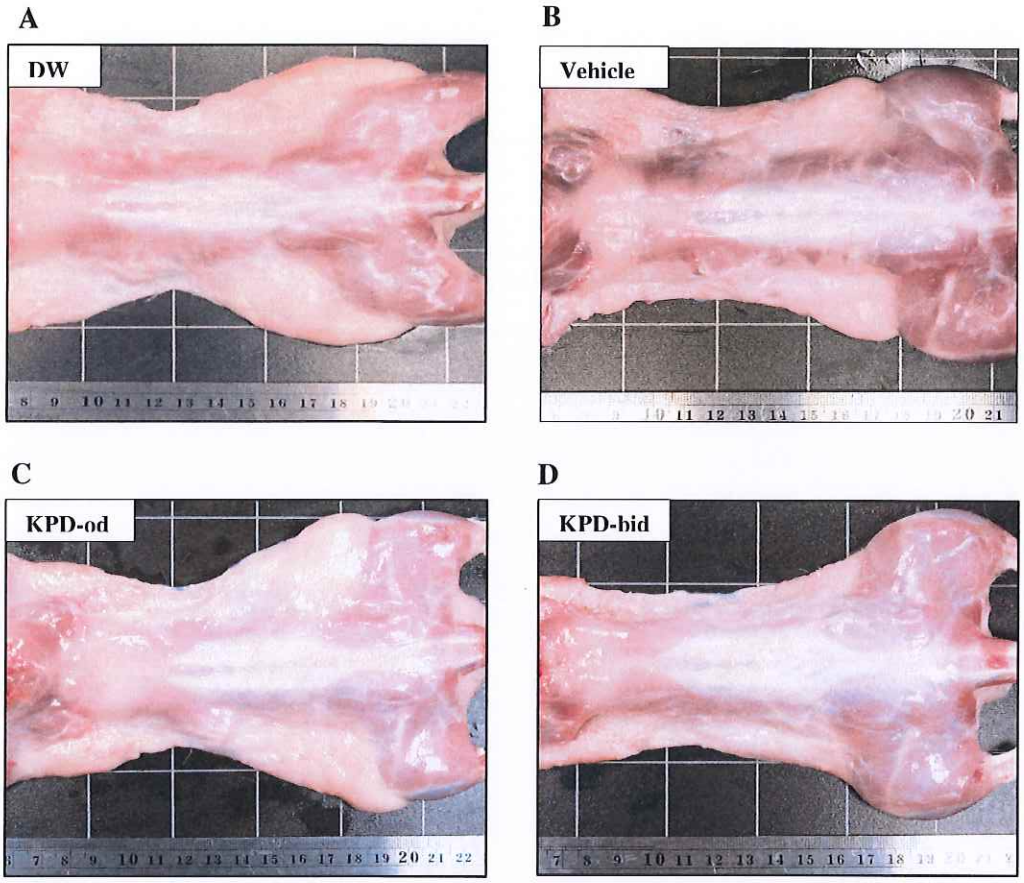

Figure 29 Differences in the accumulation of subcutaneous fat by KPD treatment for 6 weeks; distilled water (DW) or vehicle (Veh), KPD treated-rat once (KPD-od) or twice (KPD-bid) a day.

2.5.5. Effects of chronic KPD treatment on the hematology and clinical biochemical analysis

Effects of chronic KPD treatment on hepatic function, no significant changes were observed in serum levels of the alkaline phosphatase (ALP), Serum glutamic-oxaloacetic transaminase (SGOT), Serum glutamic-pyruvic transaminase (SGPT) for all the treated rats (Table 5). Thus, chronic KPD treatment had no effect that related to the toxicity on hepatic function. Effects of chronic KPD treatment on renal function, there were no significant alteration in Blood urea nitrogen (BUN) and Creatinine (CREAT) of all the treated rats in relation to the distill water or vehicle control group (Table 5).

Effects of chronic KPD treatment on serum lipid profile, for KPD treated-rat once a day were not different on the any parameter of serum lipid profile from the control group. In KPD treated-rat twice a day group did not differ significantly on cholesterol, HDL-C and LDL-C compared to distill water or vehicle control group. However, the level of Glucose, triglyceride and LDL-C/HDL-C ratio was decreased significantly in response to the KPD treatment twice a day, but the value is in the normal range (Table 6).

Hematology data are listed in Table 7, no statistic differences were noticed in any parameters of hematology analysis. There were no significant changes in White blood cells (WBC), Hematocrit (HCT), Hemoglobin (HGB), mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MC), Mean corpuscular hemoglobin concentration (MCHC), the polymorphonuclear neutrophils (PMN), lymphocyte (LYMPH) and Platelet count (Plt) between the KPD treated- and that of the vehicle control group.

Table 5 Effects of 6 weeks KPD oral administration on blood biochemistry parameters. Values are mean ± S.E.M for 6 rats in each group, DW, distil water; Veh, Vehicle control; KPD-od, KPD treated-rat once a day; KPD-bid, KPD treated-rat twice a day.

NLAC- MU		ALP (U/L)	SGOT (U/L)	SGPT (U/L)	BUN (mg %)	CREAT (mg %)
normal range	n	46.00 – 92.00	111.00 – 225.00	25.00 – 64.00	10.30 – 23.60	0.54 – 0.69
DW	6	80.54 ± 8.51	258.29 ± 35.74	62.55 ± 4.70	22.58 ± 0.80	0.42 ± 0.01
Veh-od	6	68.20 ± 8.37	240.80 ± 24.15	62.20 ± 7.38	20.35 ± 1.09	0.41 ± 0.01
KPD-od	6	60.67 ± 3.92	242.40 ± 32.80	61.60 ± 2.51	21.37 ± 0.9	0.40 ± 0.01
Veh-bid	6	92.00 ± 11.78	250.80 ± 36.80	65.60 ± 6.38	22.64 ± 1.81	0.40 ± 0.01
KPD-bid	6	89.20 ± 5.64	241.00 ± 13.35	67.20 ± 2.52	24.52 ± 1.74	0.44 ± 0.02

Note: NLAC-MU, National Laboratory Animal Centre, Mahidol University

ALP= Alkaline phosphatase; SGOT= Serum glutamic oxaloacetic tranaminase;

SGPT= Serum glutamic pyruvic transaminase; BUN= Blood urea nitrogen; CREAT= Creatinine.

Table 6 Effects of KPD on serum lipid profiles. Values are mean ± S.E.M for 6 rats in each group; DW, distil water; Veh, Vehicle control; KPD-od; KPD-bid, KPD treated-rat once or twice a day, respectively.

NLAC- MU	mg %	Glucose	Triglyceride	Cholesterol	HDL-C	LDL-C	LDL/HDL ratio
normal range		122.10 –180.80	61.00 –164.00	46.00 –98.00	-	-	-
DW	6	132.96 ± 5.69	80.20 ± 7.34	73.38 ± 1.65	58.22 ± 1.14	12.05 ± 0.63	0.21 ± 0.02
Veh	6	139.00 ± 7.94	72.00 ± 8.00	72.00 ± 0.82	50.55 ± 0.87	10.52 ± 0.96	0.21 ± 0.01
KPD	6	134.33 ± 5.48	77.17 ± 8.01	71.33 ± 3.06	56.21 ± 5.58	10.25 ± 1.00	0.21 ± 0.02
Veh-bid	6	128.00 ± 2.24	85.00 ± 5.27	71.00 ± 3.51	58.18 ± 2.74	13.14 ± 0.89	0.23 ± 0.02
KPD-bid	6	**117.40 ± 3.14***	**64.20 ± 5.62***	77.80 ± 4.45	63.80 ± 3.12	10.42 ± 0.78	**0.16 ± 0.02***

* significantly different from the control group at $P \leq 0.05$.

Note: NLAC-MU, National Laboratory Animal Centre, Mahidol University;

HDL-C= High Density Lipoprotein cholesterol; LDL-C= Low Density Lipoprotein cholesterol.

Table 7 Effects of chronic KPD oral administration on hematology parameters of plasma. Values are mean±S.E.M for 6 rats in each group, DW, distil water; Veh, Vehicle control; KPD-od; KPD-bid, KPD treated-rat once or twice a day, respectively.

		WBC	HCT	HGB	MCV	MCH	MCHC	PMN	LYMPH	Plt
		($x10^3/\mu l$)	(%)	(g/dl)	(fl)	(pg)	(%)	(%)	(%)	($x10^5/\mu l$)
NLAC- MU normal range	n	3.0 – 7.2	33.2 – 46.0	13.5 – 17.6	47.5 – 54.7	17.4 – 26.5	34.7 – 51.8	-	59.0 – 91.0	4.9 – 11.3
DW	6	5.5 ± 0.9	45.2 ± 3.3	15.6 ± 0.2	52.1 ± 0.7	17.9 ± 0.4	33.7 ± 0.3	70.2 ± 6.9	31.9 ± 4.3	7.9 ± 0.3
Veh-od	6	5.4 ± 0.6	40.3 ± 2.9	13.8 ± 0.9	50.0 ± 0.5	17.4 ± 0.2	34.7 ± 0.4	75.6 ± 3.5	26.2 ± 2.1	7.5 ± 1.1
KPD-od	6	5.7 ± 0.6	43.4 ± 0.9	15.3 ± 0.3	51.8 ± 0.4	18.1 ± 0.1	35.5 ± 0.2	81.6 ± 3.9	31.3 ± 3.0	7.9 ± 0.3
Veh-bid	6	5.1 ± 0.6	46.2 ± 1.0	15.8 ± 0.4	52.0 ± 0.3	17.9 ± 0.1	34.5 ± 0.2	61.4 ± 6.6	35.3 ± 1.9	8.3 ± 0.4
KPD-bid	6	6.7 ± 0.6	46.7 ± 0.8	15.9 ± 0.2	54.2 ± 0.7	18.7 ± 0.3	34.3 ±0.4	63.8 ± 1.9	35.5 ± 1.9	8.3 ± 0.2

Note: NLAC-MU = National Laboratory Animal Centre, Mahidol University.

WBC= White blood cell; HCT =Hematocrit; HBG= Hemoglobin; MCV= Mean corpuscle volume; MCH= Mean corpuscle hemoglobin; MCHC= Mean corpuscle hemoglobin concentration; PMN = Polymorphonuclear leukocytes; Plt = Platelet count.

2.5.6 Effects of chronic KPD treatment on liver lipid accumulation

Liver lipid accumulation was examined by Oil Red O to stain neutral lipids in hepatocytes. Lipid accumulation can be quantified using the absorption of radiation by spectrophotometry of the oil red O solution that extracted with 1 ml of 100% DMSO compare to the standard curve that known concentrations. Light micrographs of section in the liver showed the liver lipid accumulation of the chronic KPD treatment rats were lower than the distill water or vehicle control group. Quantitative analysis showed a significant decrease in liver lipid accumulation of the rats were treated twice a day compare to the vehicle control group, However the KPD treated once a day showed decreasing tendency but had no statistical significance compared to distill water or vehicle control group (Fig. 30). Together, these data suggest that chronic KPD treatment could suppress liver lipid accumulation on dose dependent pattern.

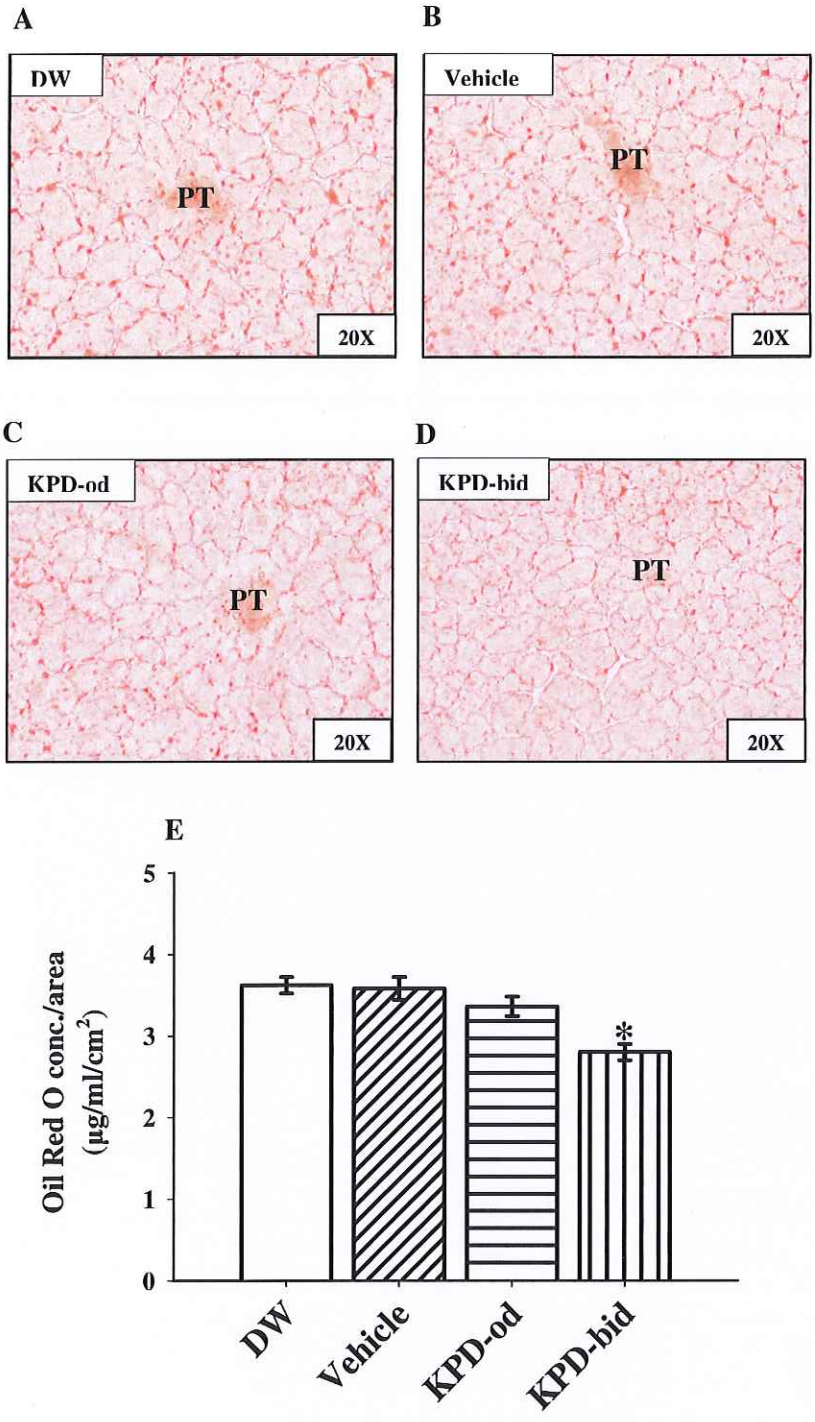

Figure 30 Oil red O-stained neutral lipids in liver tissue frozen section sections from distil water control rat (DW), Vehicle control rat (Veh) or KPD treatment for 6 weeks once (KPD-od) or twice a day (KPD-bid). Values are mean ± S.E.M for 6 rats in each group. * Significantly lower than control group, $P \leq 0.05$. (PT=Portal triad; 20 mm thick, magnification, ×20).

2.5.7 Effect on blood pressure

There were no changes in the basal systolic and diastolic blood pressure and basal heart rate of rats between the chronic KPD treatment rats and the vehicle control group. Table 8 showed basal blood pressure of young rat was significantly lower than the vehicle middle old rat, but not in basal heart rate. Basal blood pressure and basal heart rate of at 6 weeks after administration of KPD no significantly differenced compare to vehicle control rat. In middle aged male rat, KPD did not influence on blood pressure and heart.

Table 8 The effects of chronic oral admistration of KPD on the blood pressure and heart rate of the middle aged male rat.

Treatments	N	Basal systolic BP (mmHg)	Basal diastolic BP (mmHg)	Basal heart rate (bpm)
Young	8	**131.25 ± 0.94***	**100.63 ± 1.75***	386.5 ± 12.45
Veh	8	145.83 ± 4.36	116.71 ± 4.30	393.57 ± 12.22
KPD	8	142.26 ± 3.66	113.15 ± 2.64	397.89 ± 8.61
Veh-bid	8	145.12 ± 3.68	115.41 ± 3.18	396.66 ± 10.11
KPD-bid	8	145.50 ± 4.88	115.33 ± 4.26	397.50 ± 9.10

Values are mean ± S.E.M for 8 rats in each group

* Significantly lower than DW group, $P \leq 0.05$

2.5 DISCUSSION

Chivapat *et al.* (2010) and Mekjaruskul *et al.* (2012) reported that the major components of the KP rhizomes and its ethanolic extract were DMF, TMF and PMF. The initial extracts were very dark in color so fresh rhizomes of the KP were macerated and pre-extracted with 95 % ethanol twice before being macerated and extracted with 100% dichloromethane to produce a clearer extract with a higher content of these three major methoxyflavones, (it is possible that some of the methoxyflavones might also be removed). As showed in Fig. 23, the major components from the HPLC chromatogram of the KPD extract were these three methoxyflavones, that amounted to about 22% of the dried KPD extract. The present study demonstrated that the KPD suspension used could be absorbed via the gastrointestinal tract into the blood stream, (Fig. 24) as the three major methoxyflavones appeared in the blood stream about 60 min after oral administration. However, the three methoxyflavones might have undergone some metabolic changes after absorption (an unknown peak 1 appeared at 30 min after administration), and none of them were detected 180 min after administration except for the unknown peak 1. This is consistent with the report of Mekjaruskul *et al.* (2012). This might explain why, when the 100 mg/kg KPD was given orally to the animal once, but not twice, a day, none of parameters studied changed even though some methoxyflavones did appear in the blood. Since none of the parameters tested were affected by 100 mg/kg KPD treatment once a day, the remaining discussion will be concerned only with the results obtained from animals that were treated with KPD twice a day. The KPD extracts contained the three major methoxyflavones (DMF, TMF, and PMF) as did the dried rhizomes or ethanolic extracts as reported by Mekjaruskul *et al.* (2012). No signs of toxicity was observed in the middle-aged rats after having been chronically oral administration with the KPD extracts at 100 mg/kg twice a day for 6 weeks as there were no differences in the organ weights or abnormal gross observation of the internal organs: heart, testis, epididymis, prostate glands, adrenal glands, liver and kidney between the KPD extracts treated and those of the vehicle control groups. There were no remarkables histopathological lesions of liver and kidney in KPD-treated groups different from both distill water and vehicle control. The finding that

the serum liver and kidney enzyme levels (ALP, SGOT, SGPT, BUN and creatinine) were in the normal range and were not different from the vehicle control, confirmed that chronic KPD treatment in the present study did not affect the liver and kidney functions. These results are similar to those reported by Chivapat *et al.* (2010) who found that an ethanolic extract of KP rhizomes did not significant change the plasma liver and kidney enzymes after 90 days oral gavage of their extract. KPD did exert an anti-obesity property in middle-aged rat, as it was found that after 6 weeks treatment of the KPD there was a significant decrease in the fasting serum triglyceride levels together with a decrease in body weight and a lowering of the visceral and subcutaneous adipose tissues, as well as on liver lipid accumulation compared to that of the vehicle control group. The anti-obesity activity of the KPD was similar to those in the report by Akase *et al.* (2011) and Shimada *et al.* (2011) who found that a KP rhizome powder and an ethylacetate extract from the plant rhizome, that also contained these three major methoxyflavones, had an anti-obesity activity in spontaneously obese type II diabetic mice after 8 weeks of treatment. The authors claimed that the methoxyflavones might be responsible for this activity, although the methoxyflavones have a low potency (290-500 μM/ml) on the inhibitory effect on pancreatic lipase, a key enzyme in the absorption of dietary triglycerides (Shimada *et al.*, 2011). Morover, Horikawa *et al.* (2012) found that polymethoxyflavones from the KP produce a strong induced differentiation of 3T3-L1 preadipocytes to adipocytes. Thus, further studies of the crude extract as well as each pure compound of the three methoxyflavone would be needed to clarify whether its anti-obesity property is a direct or an indirect effect on animal body fat metabolism. In the present study we also found that the KPD also caused a decrease in the fasting serum glucose of normal middle-aged male rats. Thus, it is possible that the active component of the KPD might cause alterations in glucose metabolism: e.g. a reduction in glucose absorption, an increase in tissue glucose expenditure, etc. that caused hypoglycemia, and in this situation it would stimulate the utilization of the liver and adipose tissue fatty acids (Cahill, 2006; Ensling et al., 2011). However, a further study would need to clarify these possibilities. On the other hand, in a report by Chivapat *et al.* (2010) in a study using an ethanolic extract of the KP rhizomes, it was found that at the dosage of 500 mg/kg there was a decrease in body weight and plasma triglycerides, but the plasma

glucose level increased, this was after 3 months of chronic oral gavage in young male rats compared to their vehicle control group. This discrepancy might be due to differences in the chemical composition of the KP extract. In the present study we used a dichloromethane extract that was prepared after most of the dark substances had been removed by pre-extraction with ethanol, and the three major methoxyflavones were about 223.89 mg/g of the extract dry weight, whereas the ethanolic extract prepared by Chivapat contained only 24.138 mg/g of the three major methoxyflavones. The dosage of 500 mg/kg would have about 12.069 mg of the three major methoxyflavones, which is about 3.7 times less than that used for the gavaged group in the present study (100 mg/kg, twice a day, 44.78 mg of the three methoxyflavones). Thus, the reason for the differences might be that the dosage of the three methoxyflavones in the ethanolic extract (12.069 mg) might not be sufficient, and also some other substances in the ethanolic extract might have had an effect on glucose metabolism in the opposite way to result in an increase of the fasting serum glucose levels. Although there were no differences in the basal blood pressure between the KPD treated- and the vehicle control of anesthetized middle-aged male rats.

2.7 CONCLUSION

In conclusion, no signs of gross toxicity were found after 6 weeks of KPD extract treatment in middle-aged male rats. The KPD extract caused a decrease in the visceral and subcutaneous fat, plasma glucose and triglyceridesand liver lipid accumulation each of which is a beneficial parameter to prevent obesity, metabolic syndrome and/or the cardiovascular system.

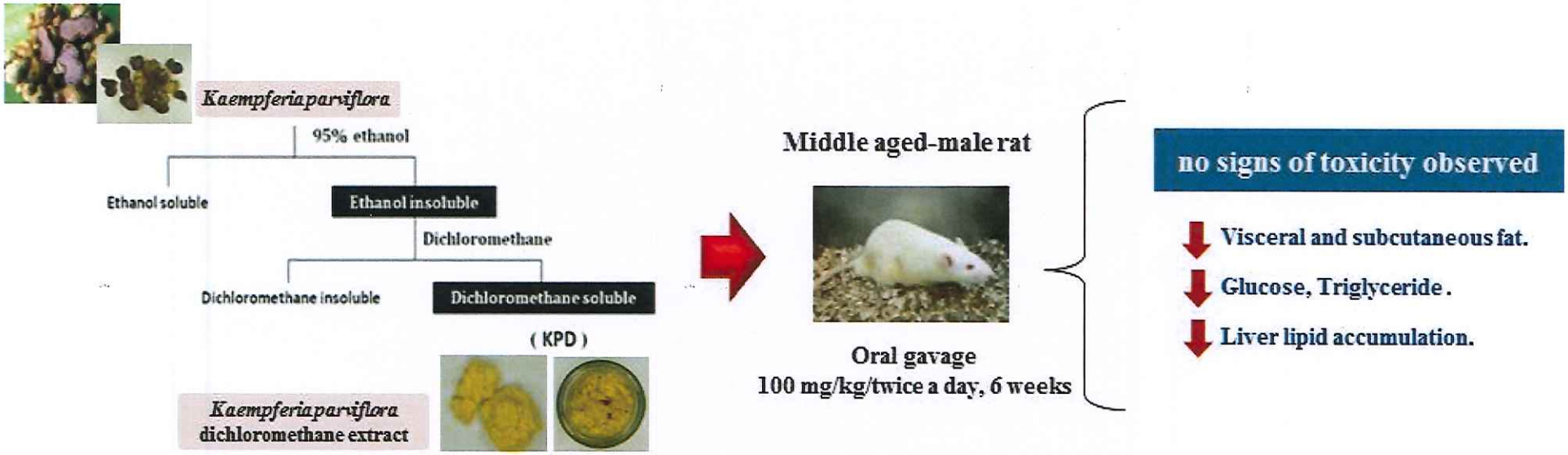

Figure 31 The effects of KPD treatment on body lipid and metabolic profiles

CHAPTER 3

Effects of *Keampferia parviflora* rhizomes dichloramethane extract on vascular functions in middle-aged male rat

3.1 ABSTRACT

Previously, we reported that middle-age male rats were received a *Kaempferia parviflora* dichloromethane extract (KPD) for 6 weeks led to decrease in body weight, visceral and subcutaneous fat, liver lipid accumulation, fasting serum glucose and triglycerides. For these parameters that might decrease risk of metabolic syndrome which had effect on cardiovascular function in the elderly.

The present study investigate the cardiovascular effects of chronic oral gavage of KPD on rat isolated thoracic aorta and mesenteric artery, and its possible mechanism. The tissue was isolated from KPD treated rat and performed *in vitro* in Krebs Heinseleit solution. Vascular functions were examined the concentration-response to phenylephrine (PE), acetylcholine (ACh) or glyceryl trinitrate (GTN) was performed before and after pretreatment with N-nitro-L-arginine (L-NA). The amount of vascular eNOS enzyme was measured by Western blot analysis.

The results showed that the chronic KPD treatment cause decrease the maximal contractile response of the endothelium-intact both thoracic aortic ring and mesenteric artery compare to control group. And this effect was attenuated by removal of the vascular endothelium and addition of L-NA, a nitric oxide synthase inhibitor. Moreover, vasorelaxation response to ACh, but not to GTN, of the chronic KPD treated group was higher than the vehicle control group both in thoracic aortic ring and mesenteric artery. The chronic KPD- treated induced up-regulation of eNOS expression compare to vehicle control group.

In conclusion, the chronic oral administration of KPD extract to middle aged rats were an effective vasodilator by up-regulation of eNOS expression is mainly the activation in nitric oxide from the endothelial cells that decrease in vascular

responsiveness to PE with an increase in the ACh. Therefore KPD may be useful as an adjunct to cardiovascular disease therapy.

3.2 INTRODUCTION

Vascular aging is a natural process by alteration of vasculature during aging. Mainly changes consist of endothelial dysfunction lead to endothelial permeability and reduce nitric oxide-dependent vasodilator; moreover, aging enhance oxidative stress to breakdown of NO (Yu and Chung 2001; Challah *et al.*, 1997; Taddei *et al.*, 1987). Vascular aging increase migration and proliferation of smooth muscle cell result to large arteries and arterial stiffness also lead to increase arterial pressure (Homma *et al.*, 2001; Lakatta and Levy, 2003; Li *et al.*, 1999). Physiological changes during aging contribute to cardiovascular disease. Arterial thickness during aging increase risk of stroke and myocardial infraction (O'Leary *et al.*, 1999). Although these effects can be prevented to by lifestyle habits, such as physical activity, using of suitable micronutrients for preserved vascular health with age, thereby slow down the risk of associated diseases.

Kaempferia parviflora (KP) is belonging to the Zingiberaceae family. KP has been as a various symptoms therapy, such as abdominal pain, erectile dysfunction, inflammation, hypertention, gastrointestinal disorders, abscesses, allergy and aphrodisiac (Wutythamawech, 1997; Yenjai *et al.*, 2004 Pengcharoen, 2002; Rujjinawate *et al.*, 2005; Tewtrakul and Subhadhirasakul, 2008). In respect to cardiovascular effect, KPE induced eNOS expression in human umbilical vein endothelial cells (Wattanapitayakul *et al.*, 2007). The ethanolic extract of KP rhizomes caused relaxation both aortic rings and ileum (Wattanapitayakul *et al.*, 2008). In addition, chronic oral administration of KPE for 5 weeks cause increase in blood flow to the testis for aphrodisiac activity (Chaturapanich *et al.*, 2008). Three compounds isolated from KP are methoxyflavones:5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF) and 3,5,7,3′,4′-pentamethoxyflavone (PMF) showed vasorelaxant activity on aortic ring (DMF and TMF) and human cavevrnosum (PMF). DMF caused endothelium-dependent vasorelaxation that was mediated via NO-cGMP and cyclooxygenase pathways, increasing K^+ efflux, and inhibition of Ca^{2+} influx

from the extracellular space (Tep-areenan *et al.*, 2010). TMF induced vasorelaxant activity via activation nitric oxide (NO) release from endothelial cell, activation K^+ efflux and inhibition Ca^{2+} influx to intracellular (Tep-areenan and Sawasdee, 2010). PMF showed weak effects on the release of nitric oxide (NO), but it activated mainly through voltage-dependent Ca^{2+} channels (VDCC), Ca^{2+} from sarcoplasmicreticulum (SR) or other intracellularstores (Jansakul *et al.*, 2012). However, there were not researchers reported about effects of chronic oral administration of dichloromethane extract from rhizomes of KP on vascular function in middle aged that shows risk of developing to cardiovascular desease (McMurray and Pfeffer, 2005). Thus, this study interested the effects of KPD on vascular function in middle-aged male rats.

3.3 OBJECTIVE

The present studies investigate the effects of chronic treatment of KPD in middle-aged rats on vascular functions as well as to assess the involved mechanism.

3.4 METERIALS AND METHODS

3.4.1 Plant material and preparation of KPD

The protocols used for plant material and preparation of KPD are the same as previous described in Chapter 2.

3.4.2 Experiment design

The Experiment design was described in previous study in Chapter 2.

3.4.3 Preparation of rat thoracic aorta rings and experimental protocol

3.4.3.1 Preparation of thoracic aorta

At the end of the 6 weeks treatment period, the KPD treatment, vehicle or distilled water group, were killed by decapitation with a guillotine, after that the thoracic aorta was removed and dissected free of connective tissue and fat. Five adjacent rings were cut into 4-5 mm long rings. Each ring was mounted between two parallel stainless steel hooks taking extreme care to avoid damage to the endothelium. Then transferred to a 20-ml organ bath filled with Krebs -Henseleit solution (composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 7 H_2O 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, that was maintained at 37 °C and continuously bubbled with 95% O_2 and 5% CO_2 mixture. The Krebs solution in the organ bath was exchanged every 15 min. One of the hooks was fixed to the bottom and another was connected to a force displacement transducer connected to a PowerLab system for the recording of changes in isometric tension. Prior to addition of drugs, all aortic rings were allowed to equilibrate under a resting tension of 1 g for 60 min. In some experiments the endothelium layer was removed mechanically by gently rubbing the intimal surface with a stainless steel rod, using the method of Jansakul *et al.* (1989). The presence of functional endothelium of the thoracic aortic rings was assessed in all preparations by determining the dilatory response to ACh (3 μM) to induce at least a 65% vasodilatation of rings precontracted with PE (3 μM) until the response had plateau (5-8 min). The thoracic aortic rings were screened to be denuded of endothelial function when there was no relaxation response to ACh, after that of the thoracic aortic rings were washed several times with Krebs Heinseleit solution for fully relaxation before the experimental protocol began about 45-60 min.

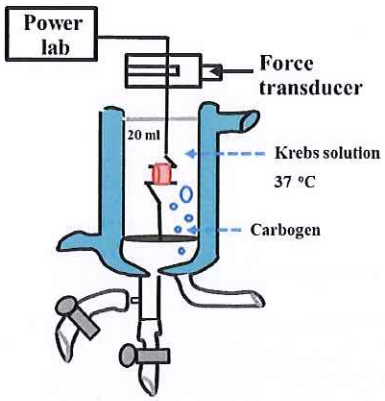

Figure 32 *In vitro* preparation of the thoracic aortic ring in a 20-ml organ bath filled with Krebs -Henseleit solution

3.4.3.2 Experimental protocol

Following a 1 h equilibration period, the endothelium-intact aortic rings aortic rings were divide in to two experiment: (1) one was measured in alone Krebs Heinseleit solution and (2) another one measured the effect of nitric oxide (NO) that synthesis from endothelium nitric oxide synthase (eNOS) by preincubating with 300 μM L-NA, a nitric oxide synthase inhibitor for 60 min before the experiments, and (3) endothelium-denuded thoracic aortic rings were equilibrated under a basal tension of 2 g at least 10 min. After that examined a contractile that response to cumulative concentration of PE (1 nM - 10 μM) and followed by several washing with the buffer solution every 15 min for 60 min. The one of endothelium-intact thoracic aortic ring was examined the effect of chronic oral administration of KPD on a nitric oxide synthase in the relaxant effects of ACh. Aortic rings were first allowed to equilibrate at 2 g tension at least 10 min before were preconstricted with 3 μM PE until the response had plateau, and the relaxant response to cumulative concentration of Ach (1 μM - 10 mM) was recorded. To examine the effect of chronic oral administration of KPD on endothelium independent vasodilatory pathway, measured the response to GTN (an endothelium independent vasodilatory substance). Using the same protocol as above, the one of endothelium-intact thoracic aortic ring were preincubated with 3 mM L-NA for 60 min, then equilibrated under a basal tension of

1 g at least 10 min before were preconstricted with 3 μM PE for 10-15 min (plateau), and the relaxant response to cumulative concentration of GTN (1 nM - 3 μM) was performed.

3.4.4 Preparation of the mesenteric artery and experimental protocol

3.4.4.1 Preparation of the mesenteric artery

At the end of the administration period, the KPD treatment, vehicle or distilled water and positive control young group, were killed by decapitation with a guillotine. Mesenteric arteries were isolated from the abdominal cavity and transferred in a petri dish containing warm oxygenated Krebs Henseleit solution. The third order branch of the mesenteric artery (diameter 200-250 μm) was dissected and the surrounding connective tissue was trimmed off under a binocular microscope. Each artery was cut into one ring segments 2 mm in length. Each vessel segment was mounted on two 40 μm stainless steel in a 10-ml-Dual-Wire Myograph chamber (model 400A, Danish Myo Technology A/S, Denmark) containing Krebs Henseleit solution (pH 7.4), composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4$ 7 H_2O 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, that was maintained at 37 °C and continuously bubbled with 95% O_2 and 5% CO_2 mixture. One wire was connected to a force transducer for the recording of changes in isometric tension and another one was attached to a micrometer. The mesenteric artery was equilibrated under zero tension in Krebs Henseleit solution for 30 min, then the normalization was performed by distending the vessel stepwise and measuring. The stepwise distension was continued until the calculated effective pressure exceeded the target transmural pressure which is normally 100 mmHg (T_{100} = 13.3 kPa), using a LabChart: a DMT Normalization Module (Mulvany and Halpurn, 1977). After the normalization was completed re-equilibration for 30 min with changes of Krebs Henseleit solution every 10-min, then the vessels was tested by stimulating contraction s three times with 10 μM norepinephrine (NE), the vessels were activated for 5 min (plateau) min with each solution followed by washing several time with Krebs Heinseleit solution to allow full relaxation about 20 min. The

presence of functional endothelium of the vessels was determined by measuring the dilatory response to Ach (30 μM) to induce 80% relaxation of vessels preconstricted with with 10 μM PE.

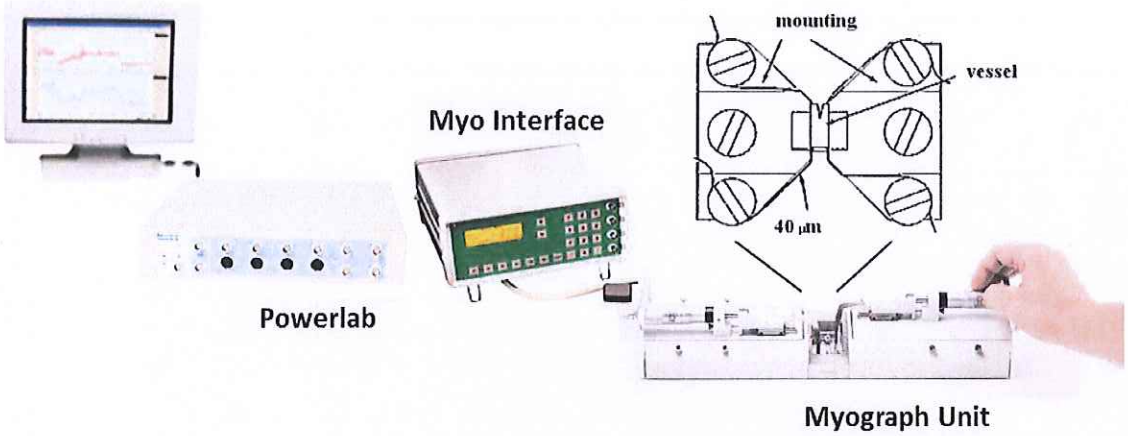

Figure 33 Illustration showing preparation of a vessel segment on two 40 μm stainless steel wires (A), a dual wire myograph chamber (B) and auto dual wire myograph system.

3.4.4.2 Experimental protocol

After equilibration period for 60 min under a basal tension of the target transmural pressure of 100 mmHg, the mesenteric artery examined a contractile that response to cumulative concentration of PE (1 nM - 10 μM) followed by changing of Krebs Heinseleit solution every 15-min for 40 min. After that, the second experiment was performed by the mesenteric artery was precontracted with PE (10 μM) until plateau (10 min) and the relaxant response to cumulative concentration of Ach (1 μM - 10 mM) was recorded, followed by several washing with buffer solution every 15 min for 40 min. The mesenteric artery was then incubated with L-NA (3mM) for 60 min. The second CR-curve to PE was performed in the presence of the L-NA, followed by several washing and re-equilibration in the presence of L-NA for another 60 min. The mesenteric ring was precontracted with 10 μM PE in the presence of -

LNA for 10 min (plateau), and then a cumulative dilatory CR-curve to GTN was performed.

3.4.5 eNOS Western blot analysis

At the end of the 6 weeks treatment period, the KPD treatment, vehicle or distilled water group and 10 weeks old adult (positive control) male rats were killed by decapitation with a guillotine, after that the thoracic aorta (TA) and mesenteric arteries (MA) were dissected and cleaned of adipose tissues and blood with Krebs Heinseleit solution, carefully taken to preserve the endothelium, then the segments were flash-frozen at -70°C until used for protein extraction. For protein extraction, each TA and MA from each rat (n=4) was chopped by small surgical scissor on ice and added the lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM, EDTA and a protease inhibitor cocktail (GE Healthcare)) at ratio 50 mg/300 µl (weight/volume), then homogenized with ultrasonic sonicator until the tissue was fine as a foam. The suspension was centrifuged at 14000 rpm for 20 min and the supernatant was removed for measurement the total protein content using the Biorad protein assay kit compared with BSA standard protein that detect the UV absorption at 595 nm wavelength. The stock protein sample was stored at -80 °C used for Western immunoblotting. Protein samples (50 µg) were run separately on 12% sodium dodecyl sulphatepolyacrylamide gel electrophoresis (SDS-PAGE) and then the separated proteins were transferred to a nitrocellulose membrane in blotting buffer at 4 °C, 100 V for 2 h. Nonspecific-binding sites were blocked for 1 h at room temperature in 5% nonfat dry milk in TBS-T (Tris buffer saline- 0.1% Tween 20). The membranes were then incubated overnight with primary monoclonal antibodies eNOS (diluted 1:250 in 1% nonfat dry milk in TTBS) at 4 °C with gently shaken. Afterwards, membranes were washed three times with 1% nonfat dry milk in TTBS and then incubated with secondary antibody ECL anti-rabbit IgG-horseradish peroxidase (GE Healthcare) for 1 h at room temperature (diluted 1:5000 in 1% nonfat dry milk in TTBS). The membranes were then washed with TTBS for 10 min three times.), after that, ECL chemiluminescent detection kit (Pierce) was added. The membranes were and exposed to a film and intensity bands

were analyzed using Image lab 3 software used the Gel Documentation (BioRad, USA). To ensure equal protein loading, results were normalized to the β-actin protein that probed with anti-β-actin antibody (primary antibody diluted 1:1000 and secondary antibody diluted 1:5000 in 1% nonfat dry milk in TTBS). The data expressed as units relataive to the β-actin.

3.4.6 Drugs

The following drugs were used: acetylcholine chloride (ACh), N^G-nitro-L-arginine (L-NA), norepinephrine (NE), phenylephrine hydrochloride (PE) and pentobarbital sodium, were from Sigma, U.S.A. Glyceryl trinitrate (GTN) was from Mycomed, Denmark. ACh, L-NA, NE and PE were dissolved in a solution containing NaCl 9 g/l, NaH_2PO_4 0.19 g/l and ascorbic acid 0.03 g/l, and GTN was dissolved in distilled water.

3.4.7 Data Analysis

Data are reported as means ± S.E.M. (n=6 animals for each group). Tests of significance were made using the Student's paired or unpaired *t*-test. In all experiments, a P value ≤ 0.05 was considered statistically significant.

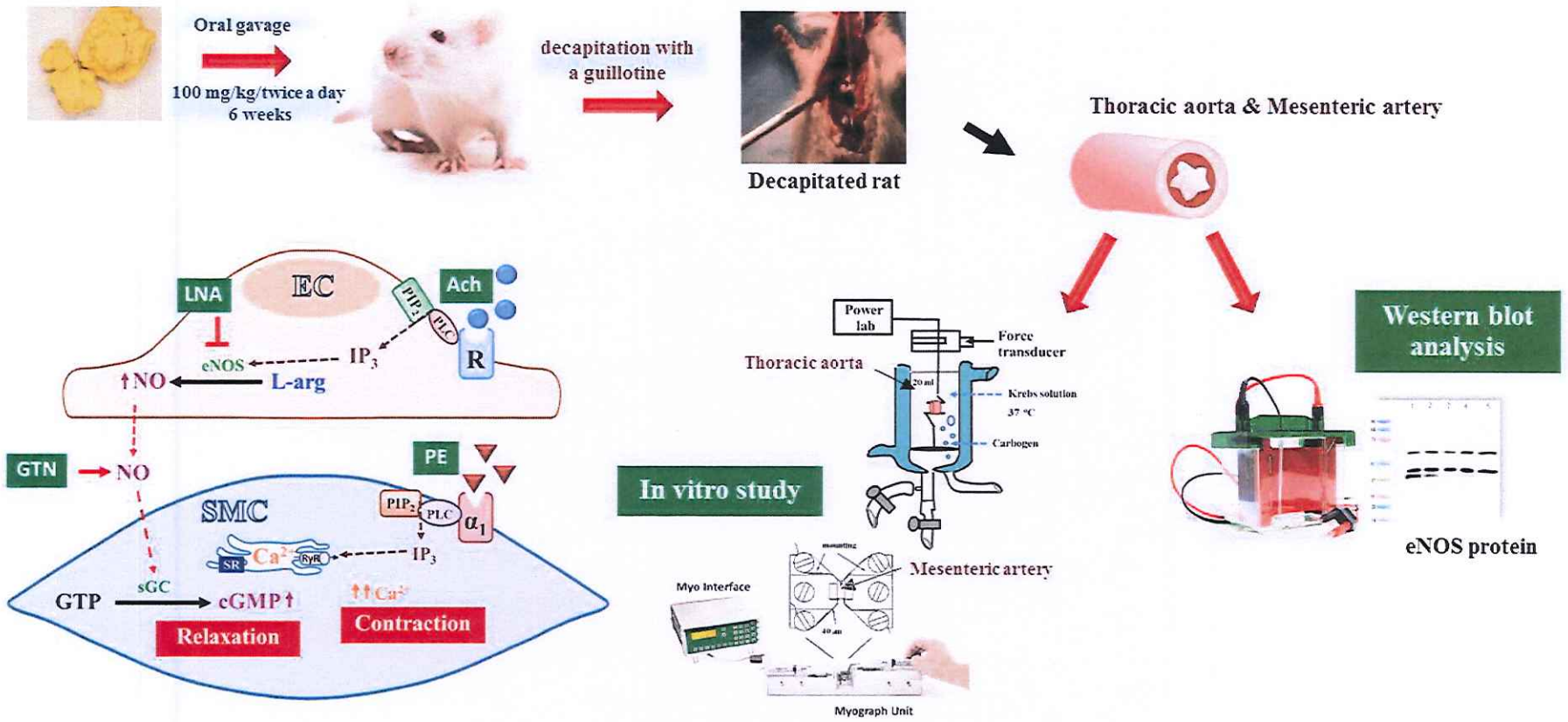

Figure 34 Scope of experimental studies the effects of chronic treatment of KPD on vascular functions

3.5 RESULTS

Firstly for measurement the endothelium dysfunction of the middle-aged male rats (12-14 months) that used in this research, vasorelaxation response to ACh of the thoracic aorta and mesenteric artery obtained from middle-aged male rats precontracted with PE was examined. Thoracic aortas were relaxed maximum and EC_{50} value to 85.4±3.4% and 78.4 nM (42.2 nM - 145.1 nM), respectively in young rat but middle-aged male rats, ACh still produced a maximum relaxation and EC_{50} value to 50.2±2.9% and 287.2 nM (225.4 nM - 410.2 nM), respectively. In the case of the mesenteric artery, the maximum vasorelaxation and EC_{50} value to ACh of young rat were 94.6±0.6% and 13.3 nM (8.4 nM - 21.8 nM), respectively and in middle-aged male rats were 93.4±1.1% and 64.2 nM (32.1 nM - 90.2 nM), respectively (Fig. 35, Table 10). The results showed that in middle-aged male rats aortic rings reduced maximum relaxation to ACh, but the mesenteric artery still decreased sensitivity response to ACh. For these indicated that the middle-aged male rats (12-14 months) have endothelial dysfunction.

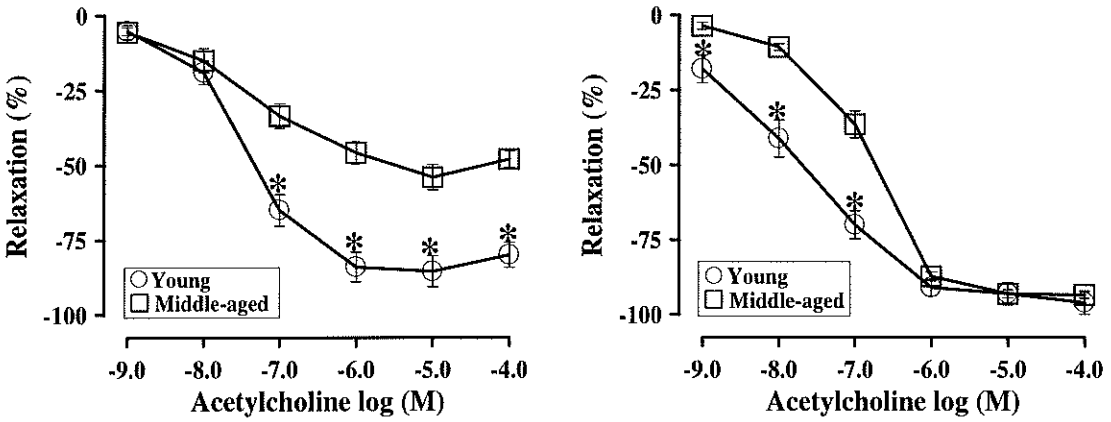

Figure 35 Concentration–relaxation curves for ACh in aortic rings (A) or mesenteric ring (B) precontracted with PE from young rats compare to. Each point represents a mean ± S.E.M. of 6 rats in each group. * Significantly lower than middle aged rats, $P \leq 0.05$.

3.5.1 Effects of chronic KPD treatment on the thoracic aorta reactivity

3.5.1.1 Effects of KPD treatment on the vascular response to PE, ACh and GTN

PE caused decreased contractile responses in KPD treated-group compared to distill water or vehicle control group under a basal tension 2 g (Fig. 36A). Table 9 showed only the maximal contractile response, but not of the EC_{50} values. Treatment with L-NA (0.3 mmol/L) or after removal of the endothelium enhanced PE-induced contractions in the endothelium-intact thoracic aortic ring from all groups, the EC_{50} values were decreased by about 5 fold and increased by about 2 fold in the maximal contractile responses to all groups (Fig. 36B, Table 9). KPD-treated rats showed a greater augmentation of the phenylephrine-induced contraction after removed of NO derived from endothelium compared with distill water or vehicle control group (Figure 36C, Table 9).

Endothelium-dependent relaxation ACh precontracted with PE induced relaxations with EC_{50} value of 145.2 nM (99.1 nM - 235.2 nM) and E_{max} of 72.4±4.1% (n=6) in chronic KPD treatment group that was significantly higher than the vehicle group in maximal response, but not in the EC_{50} values (E_{max}: 51.1±5.0%, EC_{50} values: 277.1 nM (162.2 nM -395.1 nM)) (Fig. 37A, Table 10). The relaxation response to GTN of the endothelium-intact thoracic aortic rings with L-NA precontracted with PE in chronic KPD treatment group were not different both in the EC_{50} values or E_{max} compared with the vehicle control groups (Fig. 37B, Table 10).

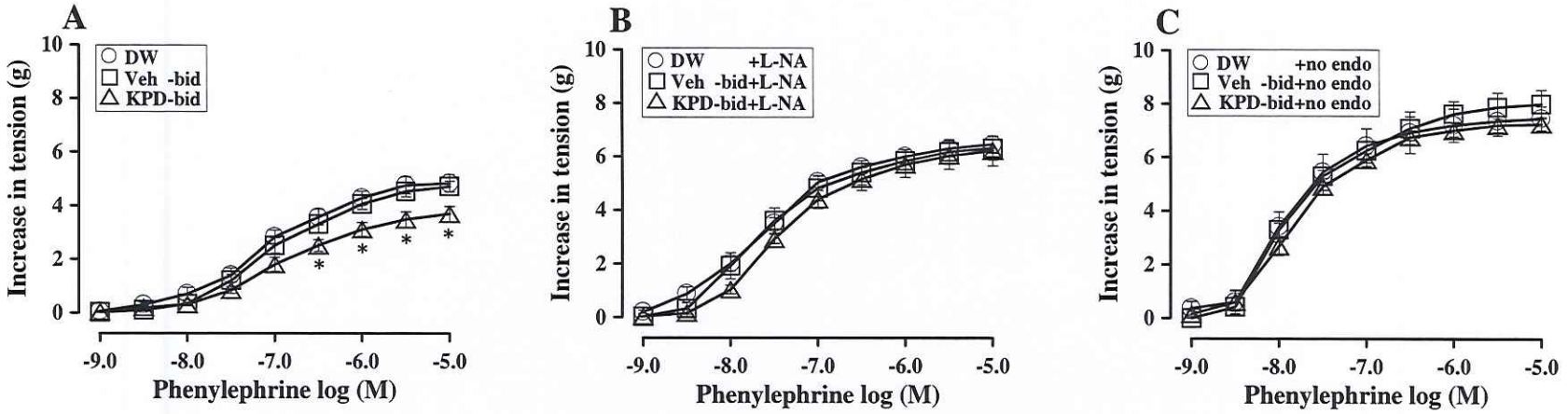

Figure 36 Concentration–response curves to PE in endothelium-intact (endo, A), endothelium-intact with L-NA (+L-NA, B), and without endothelium (Noendo, C) aortic ring of chronic oral KPD treated rats (KPD-bid), distilled water (DW) or vehicle control (Veh-bid) group. Data are showed mean ± S.E.M. of six rats in each group. * Significantly lower than that of the control group (DW, Veh-bid), $P \leq 0.05$.

Table 9 A comparison of the sensitivity (EC_{50}) and maximum response (Emax) of contractile responses to phenylephrine in thoracic aorta from middle-aged male rats with prior 6 weeks treatment of KPD twice a day (KPD-bid) compared to the control group that received distilled water (DW) or vehicle control (Veh-bid).

	EC_{50} (nM):95% confidential limit	E_{max}(g)
Basal tension (g)	2	2
With Endo		
DW	120.3(90.2-168.2)[c]	4.2±0.2[b]
Veh-bid	121.3(93.4-174.1)[c]	4.1±0.2[b]
KPD-bid	182.4(133.8-254.1)[c]	2.7±0.2[a,b]
With Endo+L-NA		
DW	21.8(19.5-27.8)	6.3±0.4
Veh-bid	22.3(0.4-29.1)	6.3±0.2
KPD-bid	25.1(22.4-32.2)	6.2±0.3
Withoutendo		
DW	21.7(17.4-27.2)	6.5±0.6
Veh-bid	22.2(19.4-28.5)	5.9±0.1
KPD-bid	21.3(15.8-28.1)	5.8±0.2

All data are shown as the mean ± SEM for 6 rats in each group. [a]significantly lower than their corresponding control group; [b]significantly lower than the endothelium (Endo) with N-nitro-L-arginine (LNA) and without endothelium (No endo) aortic ring; [c]significantly higher than the endothelium with LNA and the without endothelium aortic ring, $P \leq 0.05$.

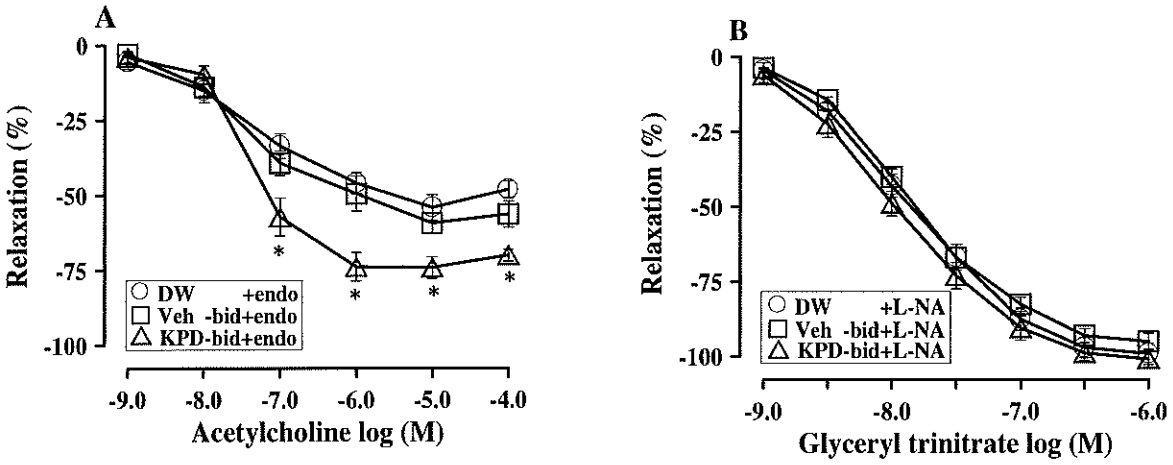

Figure 37 Concentration–relaxation response curves to Ach (A) and GTN (B) of the endothelium-intact thoracic aortic rings with L-NA precontracted with PE in aortic ring from chronic KPD treatment group (KPD-bid) compared with distilled water (DW) or vehicle control (Veh-bid) group. All data represent a mean ± S.E.M. of 6 animals in each group. * Significantly higher than that of the distilled water and the vehicle control group, $P \leq 0.05$.

3.5.2 Effects of chronic KPD treatment on the mesenteric artery

The contractile of the KPD treated-mesenteric artery with EC_{50} value of 4.9 μM (3.3 μM - 6.3 μM) showed decreasing the sensitivity responses to phenylephrine after compared with the vehicle control group with EC_{50} value of 2.6 μM (2.2 μM -3.3 μM), but not in its maximal responses (Veh-bid: 103.7±0.7%, KPD-bid: 98.9±6.9%). After incubation with L-NA (0.3 mM) due to a significant left shift of the dose-response to PE in all group (Fig. 38). The chronic KPD treated rats can induced by phenylephrine in the presence of L-NA greater than the vehicle control group, and the maximal contractile response to PE of the KPD group was significantly higher than that of the vehicle control group (Fig. 38, Table 11).

ACh induced vasorelaxation of the mesenteric ring from the KPD rats that had been precontracted with PE was significantly higher in its sensitivity, but not in its EC_{50} values or maximal relaxation, when compared to that of the vehicle control

groups (Fig. 39A, Table 10). No differences were observed in the EC_{50} values and the maximal responsiveness for the vasorelaxation obtained by GTN on the mesenteric rings precontracted with PE, in the presence of L-NA, between that of the KPD treated rats and the vehicle control groups (Fig. 39B, Table 10).

Table 10 A comparison of the sensitivity (EC_{50}) and maximum response (E_{max}) of vasorelaxation responses to ACh or GTN precontracted with PE from distilled water (DW), vehicle control (Veh-bid) group, KPD-treated group (KPD-bid).

	EC_{50} (nM):95% confidential limit		Emax(%)	
	Thoracic aorta	Mesenteric artery	Thoracic aorta	Mesenteric artery
Response to Ach				
DW	287.2(225.4-410.2)	64.2(32.1-90.2)	50.2±2.9	93.4±1.1
Veh-bid	277.1(162.2-395.1)	58.4(33.1-89.2)	51.1±5.0	94.9±0.3
KPD-bid	145.2(99.1-235.2)	34.7(21.2-45.4)	72.4±4.1[b]	95.9±0.7
Young	78.4 (42.2-145.1)[a]	13.3 (8.4-21.8)[a]	85.4±3.4[b]	94.6±0.6
Response to GTN				
DW	19.4(15.1-22.1)	65.2(46.8-94.2)	100.1±5.2	87.9±1.4
Veh-bid	20.1(18.4-21.9)	63.6(44.3-91.1)	98.85±2.1	89.8±2.6
KPD-bid	18.7(14.9-22.3)	72.4(49.6-106.2)	104.96±2.3	90.1±2.5

Each point represents a mean ± S.E.M. of 6 rats in each group. [a] significantly lower than that of the distilled water and vehicle control, and [b] significantly higher than that of the distilled water and vehicle control groups, $P \leq 0.05$.

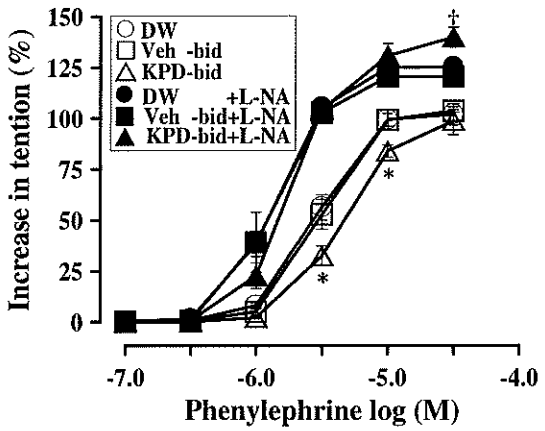

Figure 38 The constriction of the mesenteric ring in chronic oral administration of KPD twice a day (KPD-bid) that response to PE before and after preincubation with L-NA compared to distilled water group (DW) or a vehicle control group (Veh-bid). Each point represents a mean ± S.E.M. of 6 rats in each group. * Significantly lower than that of the distilled water and vehicle control group; † Significantly higher than that of the distilled water and vehicle control group, $P \leq 0.05$.

Table 11 A comparison of the sensitivity (EC_{50}) and maximum response (E_{max}) of contractile responses to PE before and after preincubation with L-NA of the mesenteric rings obtained from middle-aged male rats that had been oral administration for 6 weeks of distilled water (DW), vehicle control (Veh-bid) or *Kaempferia parviflora* dichloromethane extract (KPD-bid) twice a day.

	EC_{50} (μM):95% confidential limit		E_{max}(%)	
Treatments	Control	+ L-NA	Control	+ L-NA
DW	2.5(2.0-3.2)	1.6(1.4-2.1)	102.0 ±5.2	122.4±3.8
Veh-bid	2.6(2.2-3.3)	1.5(1.1-2.0)	103.7±0.7	120.5±2.8
KPD-bid	4.9(3.3-6.3)	2.0(1.6-2.5)	98.9±6.9	139.9±4.9[a]

Each point represents a mean ± S.E.M. of 6 rats (n=6 for each group). [a] significantly higher than that of the distilled water, vehicle control and the ones without L-NA, $P \leq 0.05$.

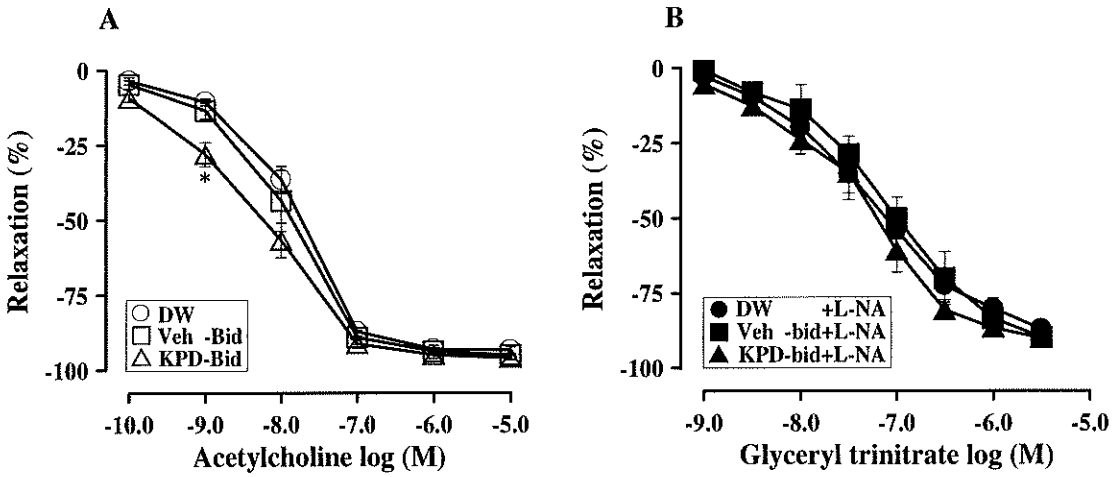

Figure 39 Effects of 6 weeks oral administration of KPD twice a day (KPD-bid), distilled water (DW) or vehicle control (Veh-bid) on the vasorelaxation to ACh (A) or GTN in the presence of L-NA (B) of the mesenteric rings precontracted with PE. Values represent a mean ± S.E.M. of 6 animals. * Significantly higher than that of the distilled water and vehicle control group, $P \leq 0.05$.

3.5.3 eNOS Western blot analysis

Western blot analysis was used to determine the quantitative expression of eNOS protein in the isolated thoracic aorta and the mesenteric artery after chronic oral administration of KPD twice a day compared to that of the vehicle control groups. The ratio of eNOS protein to the β-actin protein increased about 2 fold compared to that of the vehicle control groups (Fig. 40, young group was a positive control).

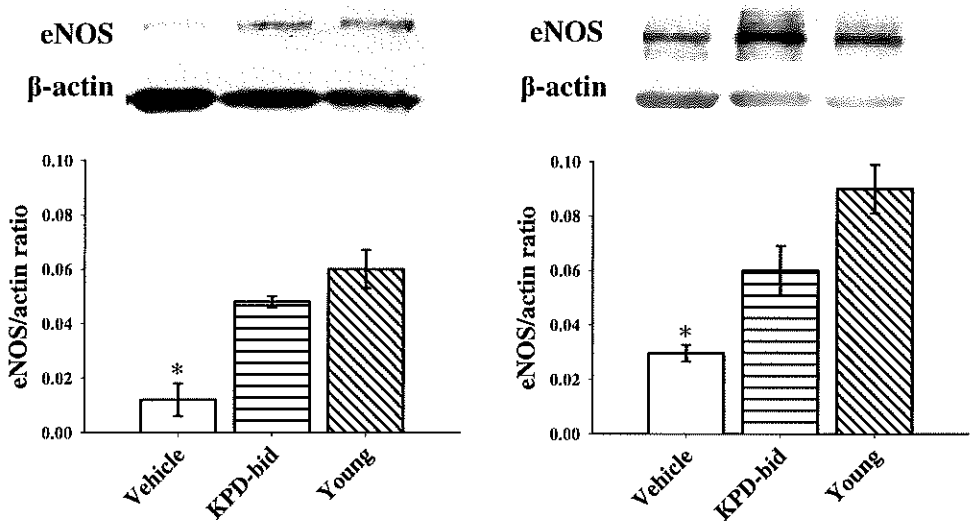

Figure 40 The effect of chronic oral administration of KPD twice a day (KPD-bid) on eNOS protein expression compared to distilled water group (DW) or a vehicle control group (Veh-bid) and untreated-young adult male rats. Each point represents a mean ± S.E.M. of 6 rats in each group. * Significantly lower than the KPD treated middle-aged and young adult male rat, $P \leq 0.05$.

3.6 DISCUSSION

The KPD extracts contained the three major methoxyflavones (DMF, TMF, and PMF) as did the dried rhizomes or ethanolic extracts as reported by Mekjaruskul *et al.* (2012). There have been some reports on the direct effects of an ethanolic extract or the methoxyflavone pure compounds on the vascular responsiveness: an ethanolic extract of the rhizomes caused a relaxation of the isolated thoracic aortic rings precontracted with PE (Wattanapitayakul et al., 2008) and DMF caused an endothelium-dependent relaxation that was partly mediated by the NO-cGMP pathways (Tep-areenan *et al.*, 2010). However, there have been no reports on the effects of the chronic oral administration of the *Kaempferia parviflora* extract on vascular functions especially in the middle-aged animals, the time of risk for developing vascular dysfunction (El Assar *et al.*, 2012; Gong *et al.*, 2014; Hongo

et al., 1988; Laurant *et al.*, 2004; Moritoki *et al.*, 1986). We did experiments using oral administration of the KPD to middle-aged male rats every day for 6 weeks before isolating the animal blood vessels to study their functions in the *in vitro* preparations. As showed in the results section, the middle-aged (12-14 month old) rats in the present study showed endothelium dysfunction as it was found to have a lower maximal and be less sensitive to relaxation from acetylcholine of the middle-aged PE-precontracted thoracic aortic and the mesenteric rings, respectively, together with a decrease in the expression of the blood vessels eNOS (Koga *et al.*, 1989; Matz *et al*, 2000; Shimizu and Toda, 1986; Tanabe *et al.*, 2003). The KPD treatment caused a decrease in the maximal contractile response to PE of the endothelium-intact thoracic aortic rings, and this effect disappeared in the presence of L-NA or after removal of the vascular endothelium. In addition, vasorelaxation of the thoracic aortic ring precontracted with PE was higher for the one obtained from the KPD treated compared to that of the vehicle control rat. A similar result was found for the mesenteric artery, a resistance vessel, with a lower sensitivity to the contractile response to PE, and a higher sensitivity for its vasorelaxation to ACh for the KPD treated artery than that of the vehicle control rat. These results clearly showed that the KPD treatment caused an increase in nitric oxide production from the vascular endothelium to attenuate the vasocontraction caused by PE and potentiated the vasorelaxation to ACh. This effect was confirmed by the finding that expression of the eNOS enzyme, the endothelial nitric oxide synthase, of the thoracic aorta and mesenteric artery were higher for the one obtained from the KPD treated rats than from the vehicle control group. The finding that there were no differences in vasorelaxation to GTN of the thoracic aortic rings of both KPD and vehicle control groups, indicating that the KPD treatment did not affect a nitric oxide independent pathway.

3.7 CONCLUSION

In conclusion, the present study has demonstrated that 6 weeks of KPD extract treatment in middle-aged male rats re-stored endothelium dysfunction of the

middle-aged blood vessels by an increase in nitric oxide production by increasing the eNOS protein at the blood vessels and this resulted in a decrease of the maximal contractile response to phenylephrine and an increase of the dilatation of the blood vessels to acetylcholine. This is the first report to demonstrate that oral administration of the KPD extract has benefits on parameters that prevent disorders of the vascular system.

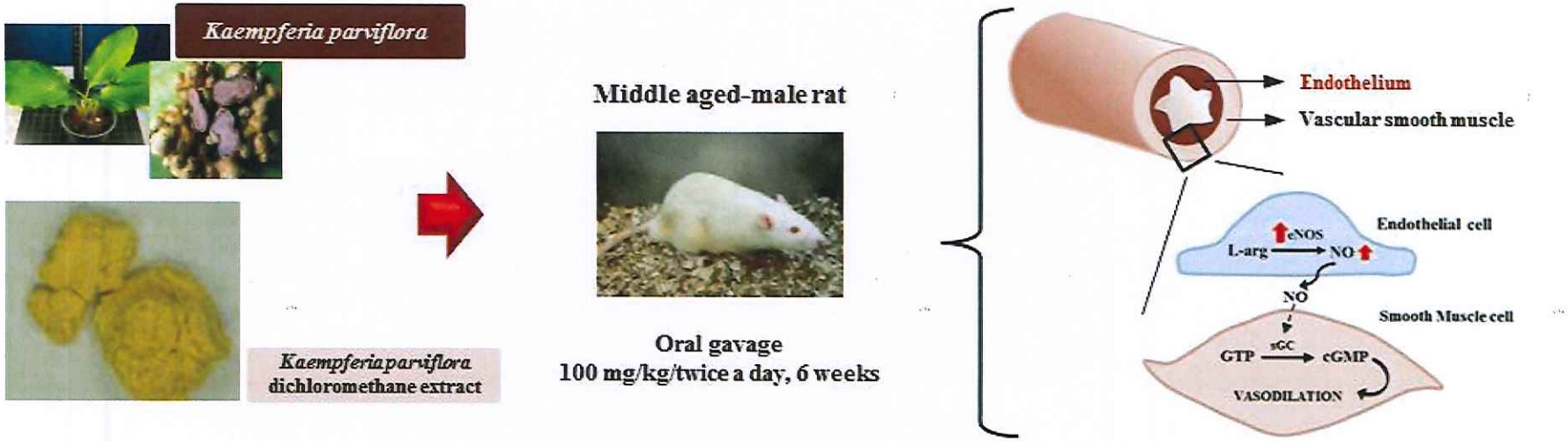

Figure 41 The effects of KPD treatment on vascular function

CHAPTER 4

Vasorelaxant mechanisms of 3, 5, 7, 3', 4'-pentamethoxyflavone isolated from *Keampferia parviflora* rhizomes on isolated rat aorta

4.1 ABSTRACT

3, 5, 7, 3', 4'- Pentamethoxyflavone (PMF) is isolated from rhizomes of *Kaempferia parviflora*, the plant is acts as a vasodilator for treatment of cardiovascular diseases. The present study investigated mechanisms underlying vasorelaxation induced by PMF, one of the major biologically active constituents of the KP rhizomes on isolated rat thoracic aortic ring. Studies were performed *in vitro* using isolated thoracic aortic rings that removed fat and other connective tissue. The mechanisms were measured in organ baths containing Krebs Henseleit solution. PMF caused concentration-dependent relaxations in phenylephrine (PE) precontracted aortic rings, that was significantly reduced by removal of the vascular endothelium and after pretreatment with N^G-nitro-L-arginine (L-NA) and 1*H*-[1,2,4] oxadiazolo [4,3-a]quinoxaline-1-one (ODQ, a soluble guanylyl cyclase inhibitor). ODQ caused to potentiate the relaxant activity of the PMF in the presence of L-NA and this effect was inhibited by DL-propargylglycine (PAG, cystathionine-γ-lyase inhibitor) and 9-(tetrahydro-2-furanyl)-9H-purine-6-amine (SQ22536, adenylyl cyclase inhibitor). Similarly, Glybenclamide, but not tetraethylammonium, potentiated the relaxation activity of the PMF in both LNA was present or not, and this effect was inhibited by PAG and SQ22536. Preincubation with PMF significantly inhibited the contractile responses to the PE of the aortic ring in normal Krebs solution with nifedipine (L-type Ca^{2+} channel blocker), or in Ca^{2+}-free buffer. In the aortic ring treated with thapsigargin (SERCA pump inhibitor), PMF suppressed the PE *C-R* curve and further suppression was found when nifedipine, SKF-96365 (store-operated Ca^{2+} channels

blocker) and/or Y-27632 (Rho-kinase inhibitor) was also added. These results indicate that PMF causes relaxation of thoracic aortic rings mainly due to stimulating release of nitric oxide and H_2S; it was acts as a guanylyl- and adenylyl cyclase stimulator and inhibition of intracellular calcium mobilization, but not as an L-type Ca^{2+} channels inhibitor, a K_{ATP}- or K_{Ca} channel opener or a store-operated Ca^{2+} channel blocker, or as a Rho-kinase inhibitor. For these, the PMF would be a good choice to develop for its potential use as a nutraceutical to prevent any cardiovascular disease.

4.2 INTRODUCTION

Kaempferia parviflora (KP) is a plant belonging to the Zingiberaceae family. It is also known as black galingale, or Kra chai dam. The plant is indigenous to the north-eastern part of Thailand, have been used widely as a health-promoting herb. The rhizomes of KP has been known as health-promoting herbs, and also frequently used for the treatment of an aphrodisiac, an anti-hypertensive to improve blood flow, impotence, allergy, asthma, and diarrhea gastric ulcer healing and antiinflammatory properties (Mekjaruskul *et al.*, 2012; Rujjinawate *et al.*, 2005; Tewtrakul and Subhadhirasakul, 2008; Yenjai *et al.*, 2004). In the cardiovascular field, KP showed enhancing the eNOS mRNA and protein expression in human umbilical vein endothelial cells for vascular endothelial health promotion (Wattanapitayakul *et al.*, 2007). The ethanolic extract of KP rhizomes (KPE) caused concentration-dependent relaxations in aortic rings precontracted with PE and it antagonizes the PE -induced contractile stimuli (Wattanapitayakul *et al.*, 2008; Tep-areenan *et al.*, 2010). 5 weeks of oral administration of KP alcohol extract acted to increase blood flow to the testis (Chaturapanich *et al.*, 2008). The active chemical components of KP consist at least of 11 flavonoids that are 5-hydroxy-7-methoxyflavone, 5-hydroxy-3,7-dimethoxy-flavone, 5,7-dimethoxyflavone, 3,5,7-trimethoxyflavone, 5-hydroxy-3,7,4'-trimethoxyflavone, 5-hydroxy-7,4'-dimethoxy-flavone, 5-hydroxy-3,7,3',4'-tetramethoxyflavone, 5,7,4'-trimethoxyflavone, 3,5,7,4'-tetramethoxyflavone, 5,7,3',4'-tetramethoxyflavone, and 3,5,7,3',4'-pentamethoxy-flavone (Sutthanut *et al.*, 2007). The three major constituents are 5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF) and 3,5,7,3′,4′-

pentamethoxyflavone (PMF). Among these, 5,7,4'-trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone exhibited antiplasmodial activity against *Plasmodium falciparum*. 3,5,7,4'-Tetramethoxyflavone possessed antifungal activity against *Candida albicans*, and also showed mild antimycobacterial activity (Yenjai *et al.*, 2004). 5,7-dimethoxyflavone, 5-hydroxy-7-methoxyflavone and 5-hydroxy-7,4'-dimethoxyflavone, acted as aromatase inhibitors use for the prevention hormone-dependent cancers (Ta and Walle, 2007). 5,7- dimethoxyflavone exhibited an anti-inflammatory agents, chemopreventive agent in human cancer originating from the liver, mouth, esophagus and lung (Panthong *et al.*, 1989; Tsuji *et al.*, 2006). 3,5,7,3',4'-pentamethoxyflavone caused improving the oral bioavailability of anticancer agents by inhibited P-gp-mediated multidrug resistance function (Denpong, 2006). The evidence for vascular effects of these methoxyflavones showed that 5,7-dimethoxyflavone (DMF) acted at least in part on endothelium-dependent vasorelaxation through the NO-cGMP and cyclooxygenase pathways but it acted largely on endothelium-independent through activation of K^+ channels and inhibition of extracellular Ca^{2+} influx (Tep-areenan *et al.*, 2010). Tep-areenan and Sawasdee (2010) reported that 5,7,4'-trimethoxyflavone (TMF) the one of all three major compounds caused dose-dependent vasorelaxation via endothelium dependent through the NO-cGMP and activated K^+ channels together with inhibited Ca^{2+} mobilization. Quercetin 3, 7, 3', 4'-tetramethylether, and quercetin 3, 5, 7, 3', 4'-pentamethylether caused relaxants of isolated guinea-pig cavernosum (Hnatyszyn *et al.*, 2004). In addition, Jansakul *et al.* (2012) showed that 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF) caused relaxant of isolated human cavernosum by inhibition voltage-dependent Ca^{2+} channels and extracellular Ca^{2+} influx. In my previous study reported that PMF is the one of all major three compound (Yorsin, 2014), in addition oral bioavailability of PMF had the greatest bioavailability (3.32%) followed by DMF (2.10%) and TMF (1.75%), respectively (Mekjaruskul *et al.*, 2012). Thus, the 3, 5, 7, 3', 4'-pentamethoxyflavone would be a better choice than another biologically active constituents of the KP rhizomes for the prevention/therapy of cardiovascular disease. Therefore, in the present, we aimed to explore whether PMF has any relaxant activity on the isolated thoracic aorta of rats, and if so what was its mechanism of action.

4.3 OBJECTIVE

The aim of the present study was to investigate whether the PMF have any direct effect on blood vessels functions, as well as to establish the mechanisms that would be involved.

4.4 METERIALS AND METHODS

4.4.1 Plant material

Fresh rhizomes of KP were cultivated in Ampur Phurua, Loei Province Thailand. Botanical identification of the plant was achieved by comparison with the Herbarium specimens in the Department of Biology Herbarium as the same with previous described in Chapter 2. KPD extract was prepared using the same procedure as that described in chapter 2.

4.4.2 Isolation of the PMF from rhizomes of *Kaempferia parviflora*

The KPD extract was chromatographed on a silica gel 100 column and eluted with a CH_2Cl_2-MeOH gradient solvent system to give the fractions. Each fraction tasted on TLC (thin-layer chromatography) patterns for flavonoid pattern. The flavonoid fractions were re- chromatographed on silica gel 60 and eluted with a CH_2Cl_2-MeOH gradient solvent system (from 100% CH_2Cl_2 to 100% MeOH) to give the fractions again. Each fraction was tasted with TLC (thin-layer chromatography) for selection flavonoid active compound. The fraction with flavonoid active compound was further fractionated by silica gel reverse phase C_{18} column using MeOH–H_2O gradient solvent system from 30% MeOH to 100% MeOH to give the fraction of pure compound. The PMF was analyzed by high performance liquid chromatography (HPLC). An aliquot of KPD in methanol was subjected to reversed phase HPLC analysis out on a HP1100 system (Agilent Technologies). Under the following conditions: a Symmetry ® C_{18} column (5μm, 3.9x150 mm i.d.; Waters); gradient elution, solvent A (0.5% trifluoroacetic acid in deionized H_2O) and solvent B

(0.5% trifluoroacetic acid in 100% methanol), gradient profile: 0 min, 95% A, 5% B; 30 min, 100% B; 40 min,100% A; 50 min, 5% A, 95% B). The flow rate was 1 ml/min; detection, the UV traces of the eluants were measured at 210 and 254 nm, absorption between 200 and 500 nm using photo-diode array detector. The structure of each compound was confirmed by comparison of the data with the previous study (Jansakul, 2012).

4.4.3 Pharmacological studies

4.4.3.1 Preparation of thoracic aorta

The procedure for the preparation of aortic rings has been described in chapter 3.

4.4.3.2 Experimental protocol

After a 1 h equilibration period, the aortic rings were precontracted with 3 μM PE for 10 min (plateau reached), PMF (0.03-0.3 mM) were added cumulatively for determination the cumulative concentration-response (*C-R*) relationships of the thoracic aortic ring. After that, followed several washings, and re-equilibration for 45-60 min before determine the mechanisms of PMF-induced vasorelaxation by incubated the aortic rings with each inhibitor added to the bath for 30 min before PE was added to increase tone.

To investigate the involvement on nitric oxide, guanylyl cyclase, adenylyl cyclase, H_2S stimulation, and K^+ channels in vasorelaxation to PMF, effects of PMF on the relaxation response to nitric oxide and guanylyl cyclase pathway were studied by using another two sets of the thoracic aorta: one without endothelium, and the other one with intact endothelium that pre-incubated with a nitric oxide synthase (NOS) inhibitor, L-NA ($3x10^{-4}$ M) and 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 μM, a soluble guanylyl cyclase inhibitor), for 40 and 20 min, respectively. Then the concentration-response relationship of the thoracic aortic rings to PMF was measured after precontracted with 3 μM PE for 10 min. Dilator responses of the

thoracic aorta to PMF via K^+ channels stimulation was investigated using glybenclamide (10 μM, an ATP-sensitive K^+ channel inhibitor), tetraethylammonium (TEA, 1 mM, a voltage activated K^+ channel blocker) were added 20 min before the PMF (0.01-0.1 mM) cumulative *C-R* relationships were determined on the PE-induced contraction.

To investigate the role on adenylyl cyclase and H_2S stimulation pathway, vasorelaxation to PMF was performed in aortic rings preincubated with cystathione-lyase DL-propargylglycine (PAG, a cystathione-lyase inhibitor or H_2S inhibitor) or 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, an adenylyl cyclase inhibitor) after were incubated in 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10 μM, a soluble guanylylcyclase inhibitor) or glybenclamide (10 μM, an ATP-sensitive K^+ channel inhibitor) for 40 min. Then the PMF (0.01-0.1 mM) cumulative *C-R* relationships were determined on the PE-induced contraction in the continuous presence of each drug(s).

To investigate the involvement on voltage-dependent calcium channels, intracellular Ca^{2+} mobilization, store-operated calcium channels, and Rho-kinase, the cumulative dose responses to cumulative concentrations of PE was studied in the normal Krebs. After the re-equilibration period, the *C-R* curve to PE were then determined in presence of nifedipine (3 μM) that pre-incubated for 20 min before experiment. The same procedure was repeated in the presence of nifedipine with PMF (0.03 mM). To examine the effect of PMF on intracellular Ca^{2+} mobilization, cumulative dose responses to cumulative concentrations of PE was studied in Ca^{2+}-free Krebs solution in the absent and present of PMF for 20 min. To determine the role of PMF on the stored-operated Ca^{2+} channel, the cumulative dose responses to cumulative concentrations of PE was studied in the normal Krebs. After the re-equilibration period, the *C-R* curve to PE were then determined in presence of thapsigargin (3 μM), a sarcoplasmic reticulum ATPase inhibitor, for 40 min by which time the small contraction of the thoracic aortic rings had reached a plateau. Then PMF (0.03 mM), nifedipine (3 μM), SKF-96365 (100 μM) and/or Y-27632 (30 μM) were added and incubated for 20 min, at which time the thoracic aortic ring had relaxed to its maximal level. Then a cumulative *C-R* relationship to PE was obtained in the presence of thapsigargin together with their corresponding cocktails.

4.4.4 Drugs

The following drugs were used: phenylephrine hydrochloride (PE), acetylcholine chloride (ACh), N^G-nitro-L-arginine (L-NA), nifedipine, tetraethyl-lammonium (TEA), 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, adenylate cyclase inhibitor) and DL propargylglycine (PAG, cystathione-lyaseinhibitor) and were from Sigma, U.S.A. 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), thapsigargin, SKF-96365 and trans-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinyl cyclohexane carboxamide dihydrochloride (Y-27632) were from Trocis, UK. L-NA, PAG, thapsigargin, SKF-96365 and Y-27632 were dissolved in distilled water, nifedipine and SQ22536 was dissolved in 20% DMSO, and the remainder were dissolved in a solution containing NaCl 9 g/L, NaH_2PO_4 0.19 g/L and ascorbic acid 0.03 g/L. The organic solvents for isolation: methanol and dichoromethane, and the silica gel for column chromatography: silica gel 100 (70-230 mesh ASTH), silica gel 60 (230-400 mesh ASTH) and silica gel 60 RP-18 (40-63 μm), were purchased from Merck, Germany.

4.4.5 Data Analysis

Data are reported as means ± S.E.M. (n=6 animals). Tests of significance were made using the Student's paired or unpaired *t*-test. In all experiments, a P value ≤ 0.05 was considered statistically significant.

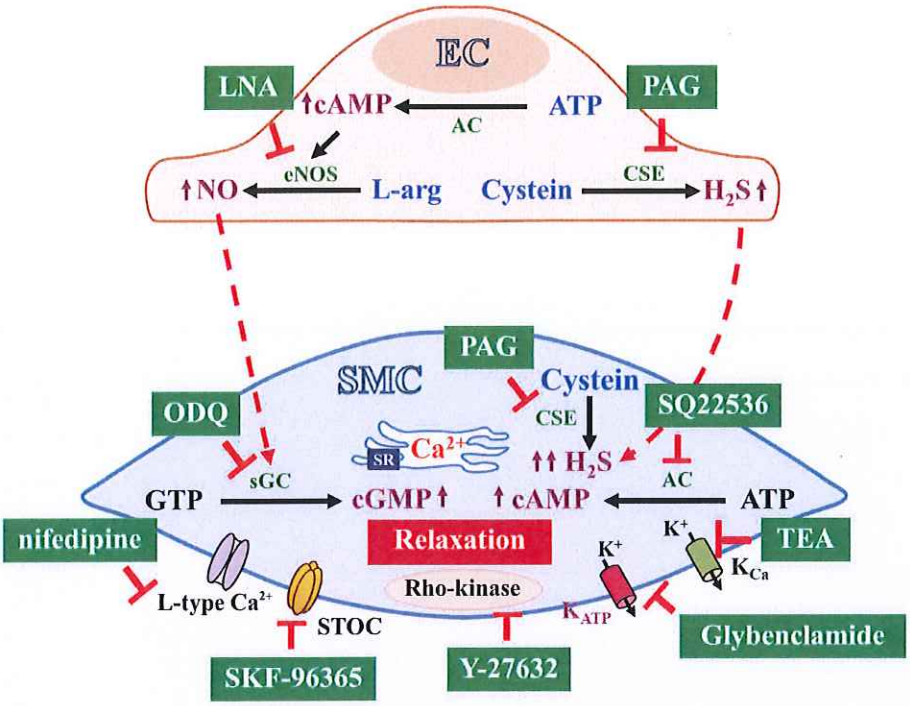

Figure 42 Scope of experimental studies of PMF effect on blood vessels functions, as well as to establish the mechanisms that would be involved.

4.5 RESULTS

PMF was crystallized from methanol as colorless needles and was obtained with a 70.03 mg/g of dried KPD, it was the one of three major pure compound of KPD (in decreasing order): DMF > PMF > TMF that data was supported by Yorsin *et al.* (2014). The chemical structure and HPLC Analysis of PMF are showed in Fig. 43. HPLC Analysis at 210 nm showed retention time (23.28 min) and UV-spectra with pure flavonoid standards obtained from Assoc. Prof. Chaweewan Jansakul, Faculty of Traditional Thai Medicine Prince of Songkla University. According to Chapter 2, The PMF was found at the high concentrations in rat blood after administration of 100 mg/kg KPD after 60 min post administration that showed 8.23 ± 0.31 µg/ml of blood volume. After 120 min, The PMF fall rapidly in the plasma concentration of the drug, and none were detected 180 min post administration.

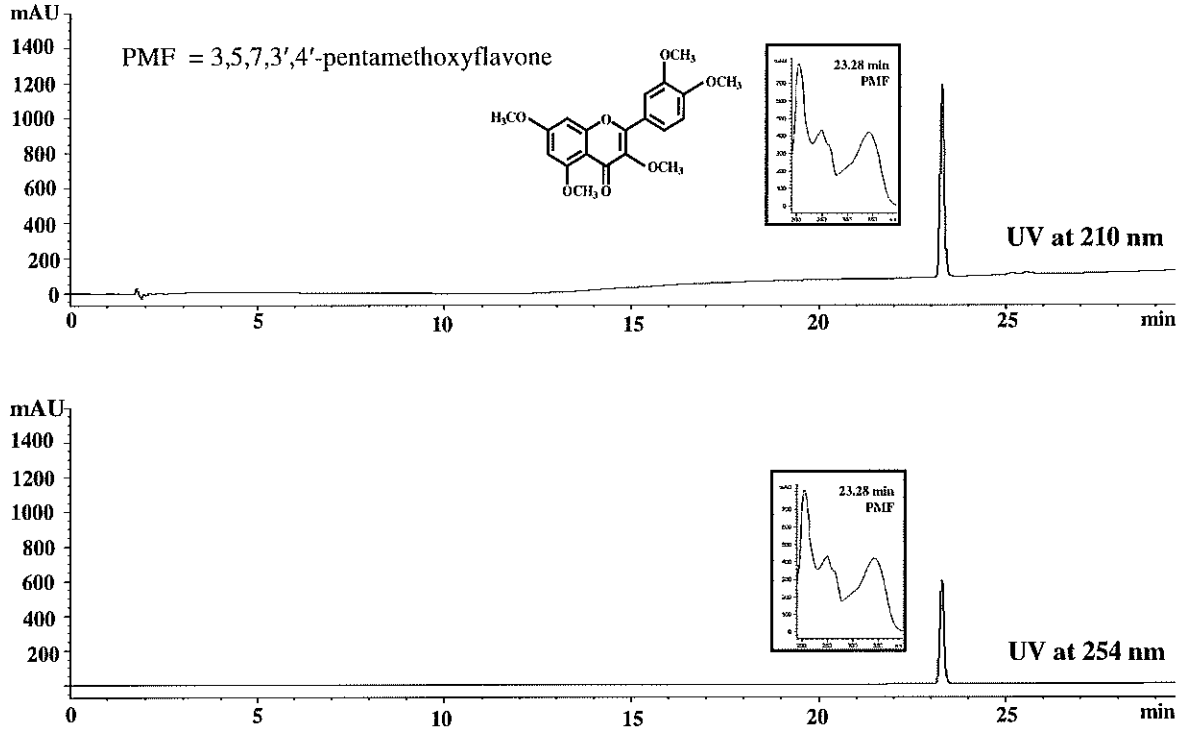

Figure 43 HPLC-UV chromatogram and the chemical structure of PMF (0.1 mg/ml), detection was at 210 nm and 254 nm, and full scale was 0–1600 mAU.

4.5.1 Effects of PMF on Nitric oxide, guanylyl cyclase, adenylyl cyclase, H_2S stimulation, and K^+ channels.

PMF (0.1–30 mg/ml) caused a relaxation of the endothelium-intact thoracic aortic rings preconstricted with 3 μM PE in a concentration-dependent manner. Typical recording of the vasodilator responses to PMF is showed in Fig. 44. L-NA (a nitric oxide synthase inhibitor), ODQ (a soluble guanylylcyclase inhibitor) or removal of the endothelium a significant rightward shift of the concentration-response curves to the PMF (Fig. 45A and B, Table 12). Pre-incubating the vessels with LNA together ODQ caused a potentiation of the vasorelaxation to the PMF. ODQ induced about a 2 fold reduction in the EC_{50} values of the relaxation with no changes the maximal relaxation (Fig. 46C). The potentiating effect of ODQ was significantly attenuate by PAG, but not completely, and when SQ22536 was also added the

potentiating effect of the ODQ was completely inhibited by restored to the same level as their corresponding control groups (Fig. 46D, Table 12).

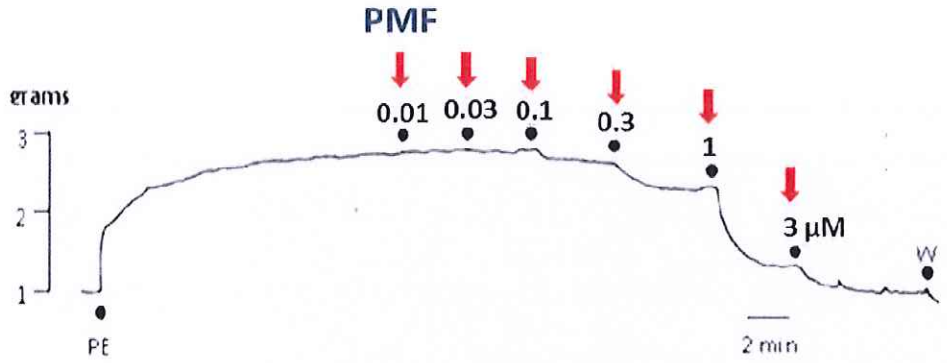

Figure 44 Typical recording showing effect of the 3,5,7,3′,4′-pentamethoxyflavone (PMF) on vasodilatation of thoracic aortic ring.

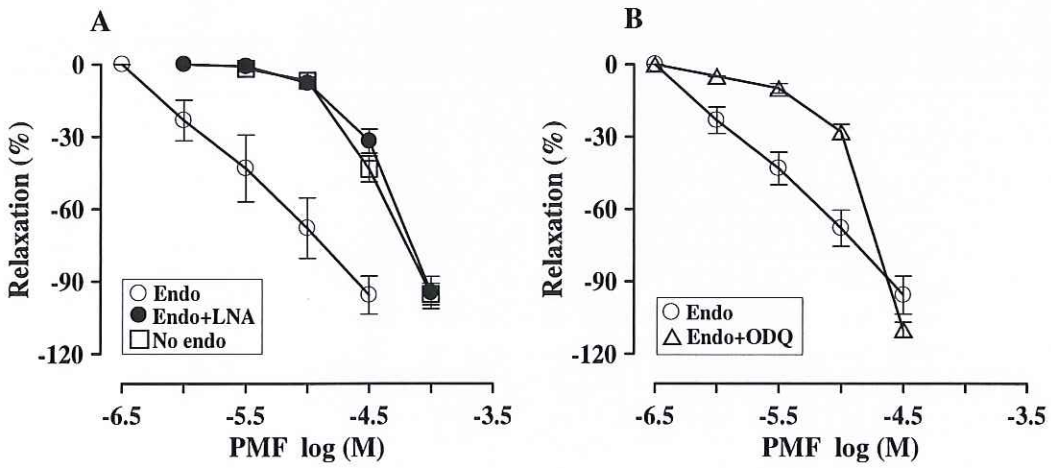

Figure 45 Effect of LNA (A), removal of endothelium (no endo, A) or ODQ (0.01 mM, B) on the dilatation of the thoracic aortic rings preconstricted with 3×10^{-6} M PE to PMF. Each point represents mean ± S.E.M. of 6 experiments for each group (n=6). * Significantly lower than the control group, $P \leq 0.05$.

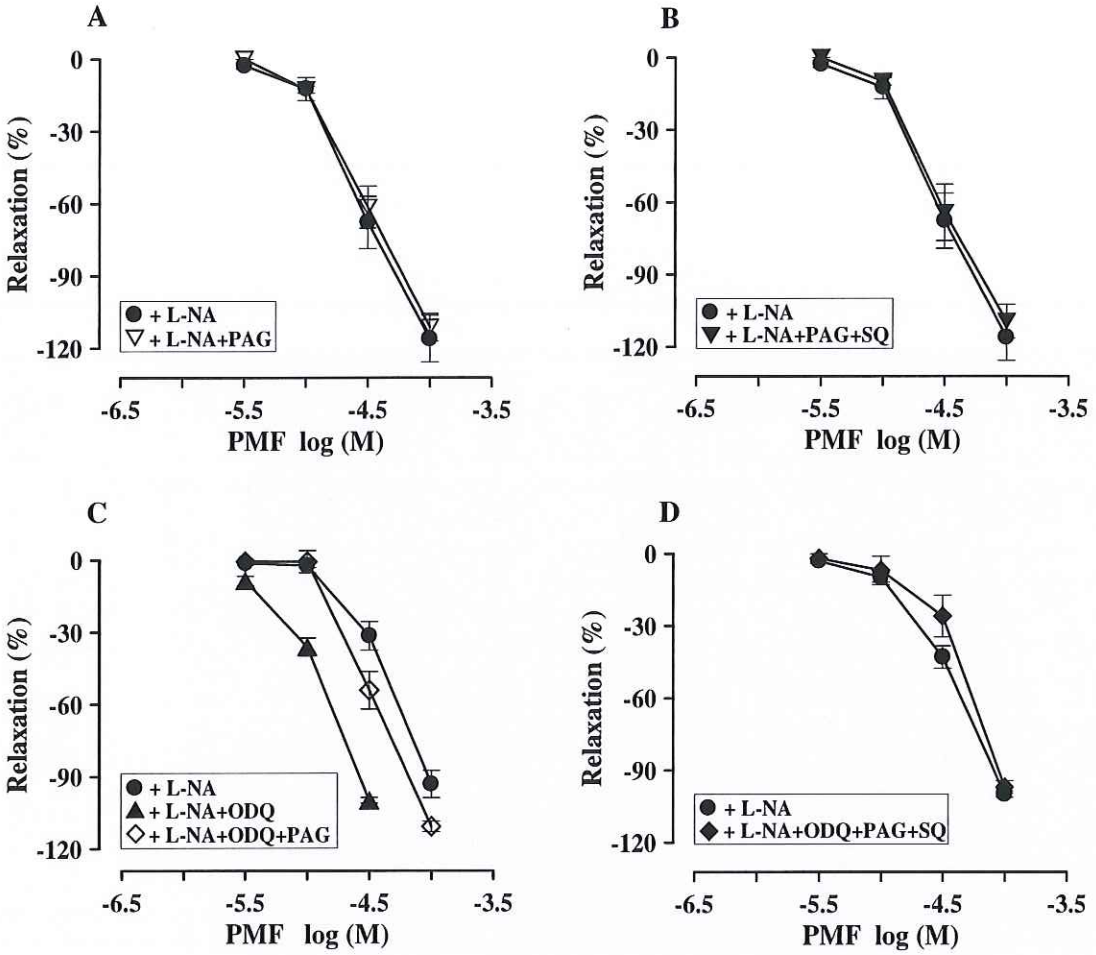

Figure 46 Effects of DL propargylglycine (PAG, 10 mM) and/or -(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, 0.1 mM) on the dilatation response of the thoracic aortic rings to PMF preconstricted with 3 μM PE, and preincubation with L-NA and ODQ (0.01 mM). Each point represents mean ± S.E.M. of 6 experiments. *Significantly higher than those other groups, $P \leq 0.05$.

Table 12 A comparison of the sensitivity (EC_{50}) and maximum response (E_{max}) of relaxation responses to PMF of aortic ring of the endothelium-intact (Endo) or denuded- (No endo) thoracic aortic ring precontracted with phenylephrine under various experimental conditions.

	EC50 (μM) : 95% confidential limit			Emax (%)		
	Endo	Endo+LNA	No endo	Endo	Endo+LNA	No endo
PMF	3.6(2.1-6.2)	28.7(21.4-38.4)	31.5(26.9-35.9)	95.6±9.9	94.6±6.6	95.3±4.4
PMF+ODQ	11.7(9.5-14.5)[b]	11.8(9.04-15.5)[a]	13.5(10.5-17.3)[a]	107.8±4.5	99.8±4.2	99.5±3.8
PMF		29.4(20.2-43.1)			116.1±9.7	
PMF+PAG		32.2(25.4-40.1)			111.3±5.6	
PMF+PAG+SQ		28.6(19.6-39.2)			109.5±7.0	
PMF		33.4(28.3-39.4)	28.6(25.4-32.1)		95.3±3.4	98.5±3.8
PMF+ODQ		10.2(8.9-11.6)[a]	14.5(12.5-17.6)[a]		100.2±2.6	101.4±2.5
PMF+ODQ+PAG		25.1(20.2-28.8)	18.6(16.5-20.6)[a]		112.4±1.8[b]	100.8±1.4
PMF+ODQ+PAG+SQ		36.5(31.5-42.6)	30.5(25.9-3.55)		103.4±1.9	103.5±3.1
PMF	8.1(5.9-11.0)	31.3(27.2-36.1)		102.5±11.6	96.3±5.2	
PMF+Glyben	3.3(2.2-5.1)[a]	13.9(9.9-16.5)[a]		92.1±4.6	101.2±2.9	
PMF+Glyben+PAG		23.6(18.8-27.4)			105.8±3.5	
PMF+Glyben+PAG+SQ		29.7(26.5-35.9)			99.6±3.4	
PMF+TEA	7.8(5.1-10.4)			106.6.±3.1		

Values were obtained from 6 aortic rings each from a different rat (n = 6). [a] Significantly lower than control and [b] significantly higher than control, $P \leq 0.05$.

Preincubation with TEA did not affect PMF-induced responses whereas glybenclamide caused a potentiation of the vasorelaxation to the PMF of the aortic ring with the endothelium when whether L-NA present or absent. The potentiating effect of glybenclamide on the endothelium-intact thoracic aortic ring with L-NA was significantly attenuatted by PAG and when SQ22536 was also added the potentiating effect was inhibited completely with restored to the same level as the corresponding control groups (Fig. 47).

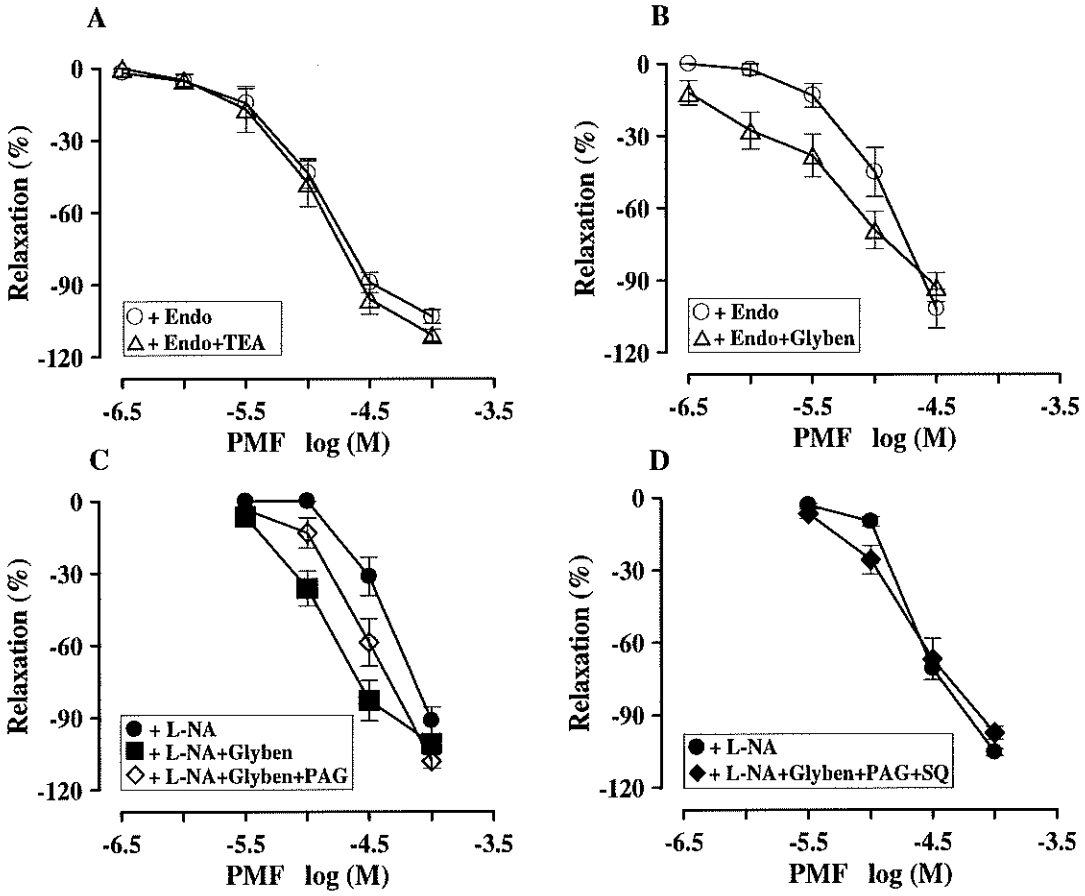

Figure 47 Effects of TEA and glybenclamide on the dilatation of the thoracic aortic rings preconstricted with 3×10^{-6} M PE to PMF; effects of PAG (10 mM) and/or SQ22536 (0.1 mM) on the dilatation response of the thoracic aortic rings to PMF preconstricted with PE, and preincubation with L-NA and glybenclamide. Each point represents mean ± S.E.M. of 6 experiments for each group (n=6). * Significantly lower than the control group, $P \leq 0.05$

4.5.2 Effects of PMF on intracellular calcium mobilization

Pretreatment with nifedipine significantly inhibited the contractile response to PE *C-R* curve when compare with the corresponding control groups and when PMF was also added caused a bigger inhibition (Fig. 48A). Similary, PMF attenuate the contractile response to PE in a concentration-dependent manner in the Ca^{2+} free Krebs medium (Fig. 48B).

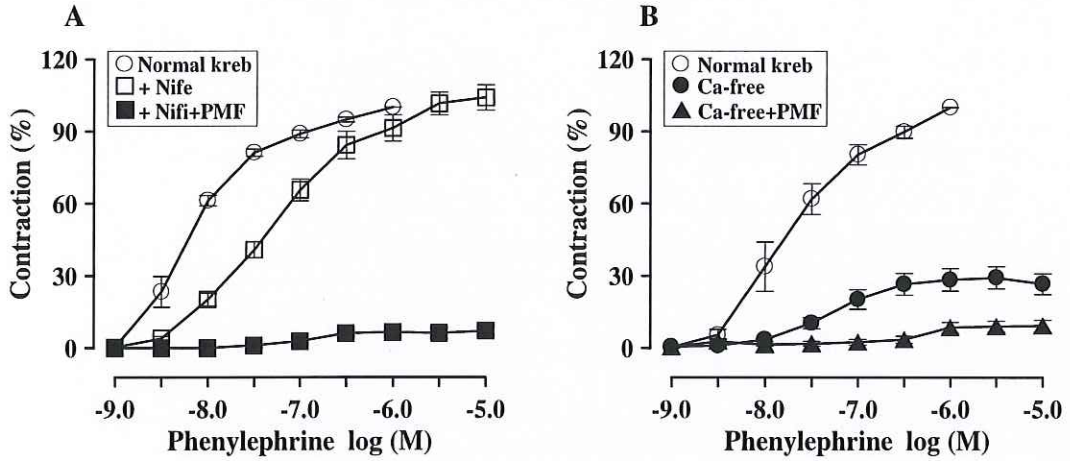

Figure 48 Effects of nifedipine (Nife, 3 mM), Ca^{2+}-free medium (Ca^{2+}-free) and/or PMF (0.03 mM) on contractile responses of the endothelium-intact thoracic aortic ring to phenylephrine C-R curve. Each point represents a mean value ± SEM of 6 aortic rings each from a different rat (n = 6). * Significantly lower than the control group; †Significantly lower than that with nifedipine or in Ca^{2+}-free medium and the control groups, $P \leq 0.05$.

4.5.3 Effects of PMF on Voltage or Store-operated calcium channels and Rho-kinase.

In order to stimulate the opening of the store-operated Ca^{2+} channels, the thoracic aortic rings were incubated with thapsigargin (3 μM), a SERCA pump inhibitor, to reduce the Ca^{2+} in the sarcoplasmic reticulum and this in turn stimulated the opening of the plasma membrane store-operated Ca^{2+} channel (Noguera *et al.*, 1998; Quinn *et al.*, 2004). In the normal Krebs solution in the presence of L-NA, incubating the thoracic aortic ring with thapsigargin (3 μM) resulted in a small contraction of the thoracic aortic ring that developed slowly and reached its maximum of about 0.15 ± 0.02 g and a plateau by 40 min. At the plateau stage when nifedipine (3 μM), SKF-96365 (100 μM), Y-27632 (30 μM) or PMF were also added into the incubation medium, a significant inhibition of PE *C-R* curves on the thoracic aortic ring was found. In this situation, the depression of the PE *C-R* curve by PMF was bigger than the one produced by nifedipine and SKF-96365 at low concentrations of the PE but not at its high concentrations where the maximal responses were the same (Fig. 49A and B). When compared to the effect of Y-27632, the *C-R* curve of the PE in the presence of PMF was significantly lower than the one with Y-27632. When preincubate aortic ring with nifedipine and PMF cause attenuate the PE *C-R* curve than preincubation with only nifedipine. When preincubate with nifedipine, SKF-96365 and PMF cause attenuate the phenylephrine *C-R* curve than preincubation with nifedipine and PMF. Preincubation with combination of nifedipine, SKF-96365, Y-27632 and PMF provide a complete inhibition of the PE *C-R* curve.

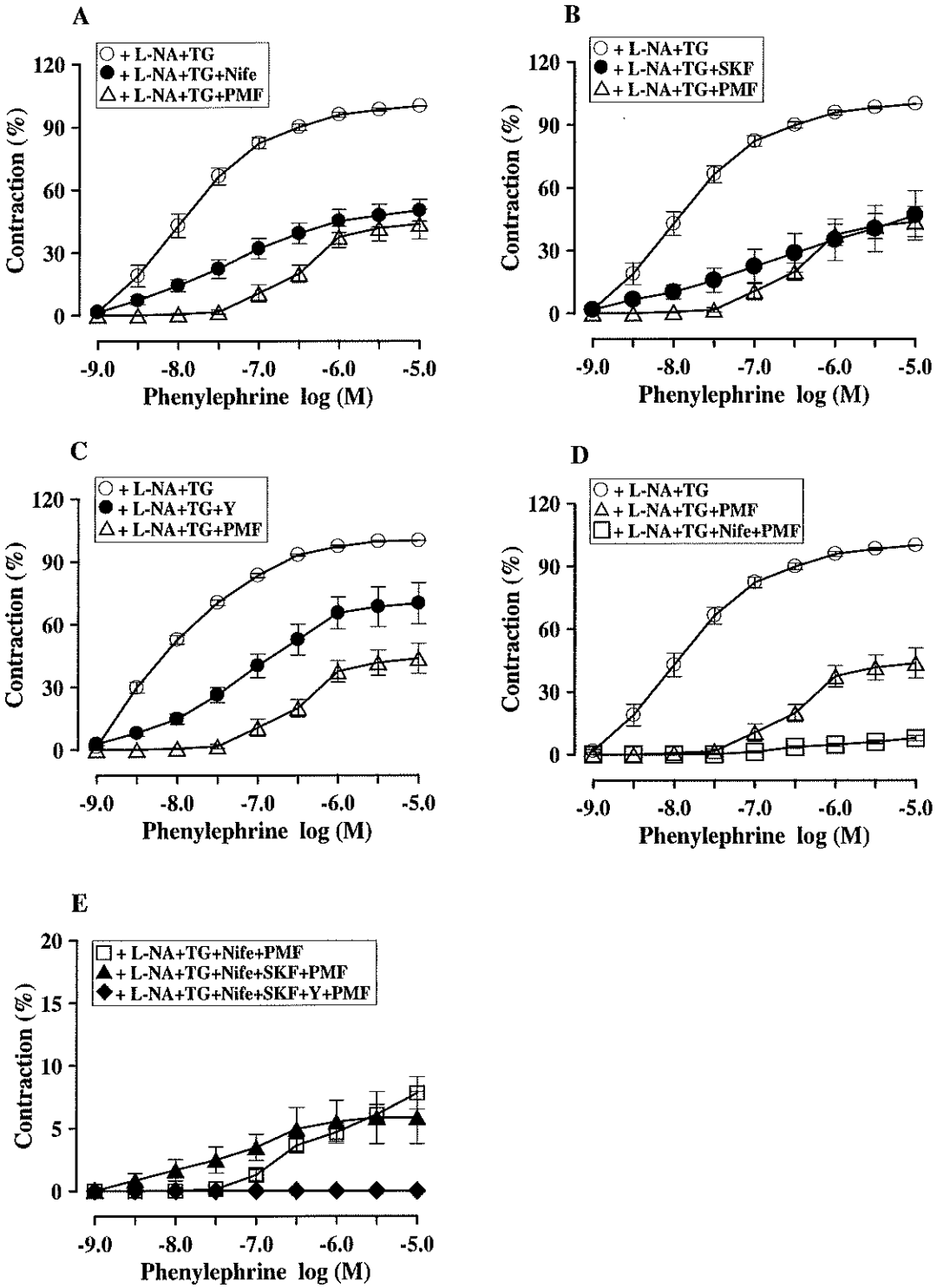
A
○ + L-NA+TG
● + L-NA+TG+Nife
△ + L-NA+TG+PMF
Contraction (%)
120
90
60
30
0
-9.0
-8.0
-7.0
-6.0
-5.0
Phenylephrine log (M)
B
○ + L-NA+TG
● + L-NA+TG+SKF
△ + L-NA+TG+PMF
Contraction (%)
Phenylephrine log (M)
C
○ + L-NA+TG
● + L-NA+TG+Y
△ + L-NA+TG+PMF
Contraction (%)
Phenylephrine log (M)
D
○ + L-NA+TG
△ + L-NA+TG+PMF
□ + L-NA+TG+Nife+PMF
Contraction (%)
Phenylephrine log (M)
E
□ + L-NA+TG+Nife+PMF
▲ + L-NA+TG+Nife+SKF+PMF
◆ + L-NA+TG+Nife+SKF+Y+PMF
Contraction (%)
20
15
10
5
0
-9.0
-8.0
-7.0
-6.0
-5.0
Phenylephrine log (M)

Figure 49 Effects of nifedipine (Nife, 3 mM), Y-27632(Y, 30 mM), SKF-96356 (SKF, 100 mM) or PMF (0.03 mM) on contractile responses of the endothelium-intact thoracic aortic ring to PE C-R curve, preincubate with L-NA. Each point represents a mean value ± SEM of 6 aortic rings each from a different rat (n = 6). * Significantly lower than the control group; † Significantly lower than the control groups, $P \leq 0.05$.

4.6 DISCUSSION

The present study has demonstrated that PMF has a relaxant activity on isolated thoracic aortic rings in an endothelium-independent manner. Mechanisms responsible for the relaxation were explored to determine if PMF acted (1) as a nitric oxide stimulator, a guanylyl cyclase-, an adenylyl cyclase- or H_2S-stimulator and/or by opening of K^+ channels, (2) by blocking voltage-dependent calcium channels, (3) as a Rho-kinase inhibitor, (4) as a store-operated calcium channel inhibitor, or (5) by inhibition of intracellular calcium mobilization.

As a nitric oxide-, a guanylyl cyclase-, an adenylyl cyclase- or H_2S stimulators and/or by opening of K^+ channels, our finding that L-NA inhibited the relaxant activity of the PMF indicated that PMF had an effect on the stimulation of nitric oxide release. Furthermore, the finding that ODQ inhibited the relaxant activity of the PMF indicated that PMF acted as a stimulator of soluble guanylyl cyclase. It was a surprise to see that in the presence of LNA and the endothelium-intact or endothelium-denuded thoracic aortic ring, ODQ potentiated the relaxant activity of the PMF, in a situation when the nitric oxide and soluble guanylyl cyclase was being inhibited. Thus, it is possible that PMF might also activate other signalling pathways: such as the hydrogen sulfide and/or adenylyl cyclase pathways. Recently, it has been accepted that H_2S is the third endogenous gasotransmitter with pivotal roles in regulating vascular homeostasis (Al-Magableh and Hart, 2011; Cheng *et al.*, 2004; Hart, 2011; Martelli, 2013). H_2S is released from the vascular endothelium as well as the vascular smooth muscles (Streeter, 2012). Within the vascular wall, the NO and H_2S pathways coexist and serve a similar function (Bucci, 2010). In the case of the nitric oxide pathway, Li *et al.* (2012) reported that quercetin induced a rapid eNOS

phosphorylation that enhanced the production of NO and promoted vasodilatation of the endothelium-intact thoracic aortic ring via the cAMP/PKA-mediated pathway. They found that the production of the intracellular cAMP was quickly increased by stimulation with quercetin concomitantly with the induction of eNOS phosphorylation at Ser 1179. In addition, the effect of cAMP was also mediated through the allosteric activation of the protein kinase A (PKA), which has been shown to decrease the intracellular Ca^{2+} concentration and lead to a relaxation of the vascular smooth muscle (Jiang *et al.*, 1992a; Jiang *et al*, 1992b; Murthy and Makhlouf, 1998). In a separate finding, Chiwororo and Ojewole (2010) found that quercetin induced relaxation of the rat isolated portal vein, partly via the cAMP-dependent protein kinase pathway. Thus, it is possible that PMF might stimulate the release of the H_2S to compensate for the situation when nitric oxide had been removed and/or when the soluble guanylyl cyclase had been inhibited by ODQ, so the augmentation of the H_2S and/or cAMP by the PMF could also induce vasodilatation. In order to prove these possibilities, the endothelium-intact thoracic aortic rings in the presence of L-NA or endothelium-denuded ones and ODQ were preincubated with PAG, a cystathionine γ-lyase (CSE) inhibitor (inhibits H_2S generation), alone or together with SQ22536, an adenylyl cyclase inhibitor, before performing the *C-R* curve to the PMF. The PAG significantly inhibited the PMF *C-R* curve of the endothelium-intact thoracic aortic ring in the presence of L-NA and ODQ. When SQ22536 was also added with the PAG, the PMF *C-R* curve of both groups was re-stored to the same level as that of their corresponding control groups. These results indicate that the PMF might stimulate the release of H_2S from the vascular endothelium as well as affecting an increase in the generation of cAMP by the adenylyl cyclase from the vascular smooth muscle cells and this resulted in vasodilatation. TEA did not modify the PMF *C-R* curve, whereas glybenclamide potentiated the PMF *C-R* curve of the endothelium-intact thoracic aortic rings whether L-NA was present or not. This indicated that PMF did not open the Ca^{2+}- sensitive K^+ channel (K_{Ca}), nor did it open the ATP sensitive K^+ channel (K_{ATP}) (Brayden, 2002; Ko *et al.*, 2008; Stojnic *et al.*, 2007). The potentiation of the PMF *C-R* curve by glybenclamide would result from a stimulated release of H_2S and/or cAMP by the PMF from the thoracic aorta. This was confirmed when it was found that the potentiating effect of the glybenclamide on the endothelium-intact

thoracic aortic ring with L-NA, but not on the one without endothelium, was significantly inhibited by PAG and the PMF *C-R* curve of these two groups were restored to the original level when SQ22536 was also added. This confirmed the above finding that the PMF stimulated a release of the H_2S and cAMP. The finding that the stimulated release of H_2S by the PMF on the thoracic aortic ring was not inhibited by glybenclamide, an ATP-sensitive K^+ channel blocker, was analogous to that which occurred in the middle cerebral artery where it was found that the exogenous H_2S-mediated relaxation, yet received no contribution from the K_{ATP} channel (Streeter, 2012). Although a few investigators have reported that the mechanism responsible for the vasodilatory effect of the H_2S involved ATP-sensitive K^+channel, because its effect was inhibited by glybenclamide (Moccia *et al.*, 2011; Tang *et al.*, 2005) this was different from our finding. However, in a recent report, an H_2S-mediated relaxation of the endothelium-independent blood vessels did occur by inhibition of the L-type calcium channels with an additional contribution by the K^+ channels, probably K_v7 but not K_{ATP}, K_{Ca}, or K_{ir} subtypes (Martelli *et al.*, 2013 ; Streeter *et al.*, 2012). Therefore, a further study would be needed to confirm the present finding.

Voltage- or store-operative calcium channels, Rho-kinase and intracellular Ca^{2+} mobilization. In vascular smooth muscle, the α_1-adrenoceptor agonist, phenylephrine, induced an initial phasic contraction followed by a tonic contraction. The initial contraction was mediated by the release of intracellular Ca^{2+} from the sarcoplasmic reticulum (Noguera *et al.*, 1998) whereas the sustained tonic contraction resulted from a Ca^{2+} influx via the voltage-dependent Ca^{2+} channels (Abebe *et al.*, 1990; Akata, 2007; Nelson *et al.*, 1988). Our finding that PMF antagonized the phenylephrine-induced phasic contraction of the thoracic aortic ring in the normal Krebs solution with Ca^{2+} as well as in the Ca^{2+}-free Krebs medium indicated that PMF may play a role as a Ca^{2+} channel inhibitor and/or by inhibiting mobilization from the intracellular Ca^{2+} stores. PMF might act as a store-operated Ca^{2+} channel inhibitor, and further experiments were carried out in the presence of thapsigargin, a specific sarcoplasmic-endoplasmic reticulum Ca-ATPase (SERCA) pump inhibitor (Quinn *et al.*, 2006; Thastrup *et al.*, 1990), to deplete the intracellular Ca^{2+} store, which then would stimulate the opening of the plasma membrane store-

operated Ca^{2+} channels to add to the refilling of the intracellular stores (Parekh and Putney, 2005; Putney, 2011). In normal Krebs solution, incubation of the thoracic aortic rings with thapsigargin caused a slow but small increase in the thoracic aortic ring contraction that reached its maximal contraction (20 min) and this result was similar to that reported by Takemura *et al.* (1991). In the presence of nifedipine, SKF-96365 and/or Y-27632, PMF completely inhibited the effect on the phenylephrine *C-R* curve. These results indicated that PMF did not act as an L-type Ca^{2+} channel inhibitor, store-operated Ca^{2+} channel inhibitor or a Rho-kinase inhibitor.

4.6 CONCLUSION

In conclusion, the present study has demonstrated that PMF exerted a relaxant activity on the phenylephrine precontracted isolated thoracic aorta. The results indicated that PMF may act as an inhibitor of the intracellular Ca^{2+} mobilization from the sarcoplasmic reticulum. PMF stimulated the release of nitric oxide and H_2S from the vascular endothelium, and it also acted as a soluble guanylyl cyclase and adenylyl cyclase stimulator, but not as an opener of Ca^{2+}channels or an ATP- sensitive K^+ channel, inhibitor of Rho-kinase or an L-type- or a store-operated Ca^{2+} channel. At the present time, this is the first report to demonstrate that PMF also stimulates release of the H_2S, the third endogenous vasodilatory gasotransmitter, in addition to the nitric oxide from the endothelium of the rat thoracic aorta. Taken together, PMF caused a vasorelaxation of the thoracic aortic ring via concerted reactions with several different pathways that provided significant advantages of having one to compensate if another one undergone defection. Therefore, PMF would be one of the novel vasodilatory compounds to be considered for the development of another treatment for hypertension.

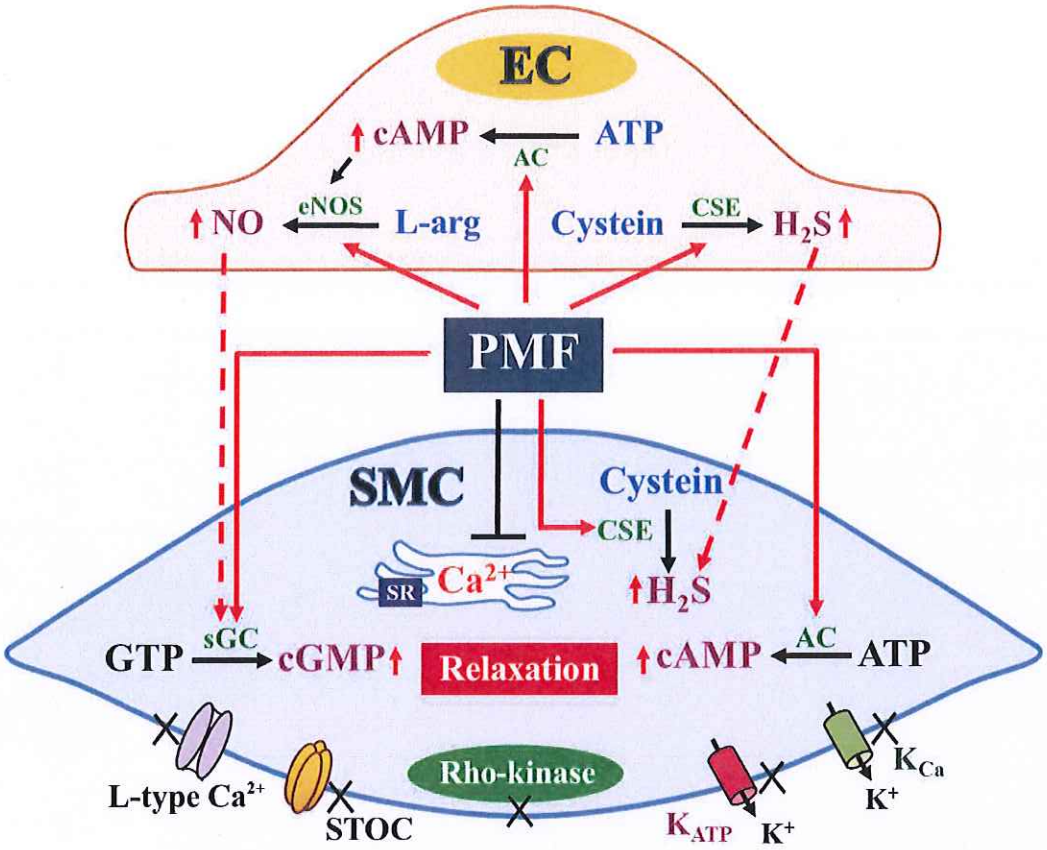

Figure 50 The effects of PMF on vascular functions

CHAPTER 5

Effects of oral administration of 3, 5, 7, 3', 4'-pentamethoxyflavone isolated from Keamperia parviflora rhizomes on cardiovascular profile in middle-aged male

5.1 ABSTRACT

3, 5, 7, 3', 4'- pentamethoxyflavone (PMF) is isolated from rhizomes of *Kaempferia parviflora*, the plant is acts as a vasodilator for treatment of cardiovascular diseases. The present study investigated the effects of 6 weeks oral administration of PMF, isolated from the rhizomes of KP on lipid accumulation and cardiovascular system and its possible mechanisms in middle-aged male rats. The treatment groups were orally gavaged PMF extract at the dosage of 22 mg/kg twice a day for 6 weeks compared with control group that received the vehicle. At the end of period, lipid accumulation concluding blood chemistry, visceral and adipose tissue accumulation and liver lipid accumulation were measured. For the effect on cardiovascular function were performed *in vitro* using isolated thoracic aortic rings with or without perivascular adipose tissue and mesenteric artery incubated in chamber perfused with Krebs Heinseleit solution. The amount of eNOS and CSE expression were measured by Western blot analysis. There were no changes in the liver and kidney functions between KPD treated- and vehicle group. The PMF-treated rats had lower serum glucose, higher HDL-C levels, but no change in other parameters when compared to the vehicle control group. Moreover, no significant changes were observed in the basal systolic and diastolic blood pressure and the basal heart rate between the PMF treated- and the vehicle control group. Thoracic aortic and mesenteric rings of PMF treated rats produced lower maximal contraction to phenylephrine (PE) that was eliminated by N^G-nitro-L-arginine (L-NA) or endothelial removal. The aortic- and mesenteric rings of the PMF treated rats showed higher

relaxation to acetylcholine (ACh), but not to glyceryl trinitrate (GTN), and had higher eNOS protein. DL-propargylglycine (PAG) caused greater increase in the baseline tension of the PMF-treated aortic ring, and higher contraction to low concentrations of PE. PVAT lowered the contractile response of the L-NA pretreated aortic rings to PE for both groups, but PAG had no effect. The cystathionine-γ-lyase (CSE) protein of the thoracic rings, but not of the PVAT, was also higher after PMF treatment. Overall, PMF treatment of middle aged rats, increased production of NO and H_2S from the blood vessels by upregulating the expression of eNOS and CSE. Treatment also lowered vascular contraction to PE with an increased relaxation to ACh. PMF also decreased fasting serum glucose and increased HDL-C levels, with no toxicity to liver and kidney functions. For these, the PMF would be a good choice to develop for its potential use as a nutraceutical to prevent the development of diabetes type II and/or cardiovascular disease or improve health quality of life in the elderly.

5.2 INTRODUCTION

In soon the world will have older people more than children and adult because people have long life due to population aging will continue expand, even accelerate. The number of people aged 65 or older is assumed to increase from an estimated 524 million in 2010 to nearly 1.5 billion in 2050 especially in developing countries (Kinsella and He, 2009). The major causes of death at the dawn of the 20th century were predominantly from infectious diseases. In recent decades, the major causes of death transition to non-communicable diseases, most notably cardiovascular disease (CVD) especially in developed countries (Howson *et al.*, 1998). Recently, CVD has been a shift from high socioeconomic groups to the poor groups (Yusuf *et al.*, 2001). Aging relate with increasing in CVD both morbidity and mortality (Fried *et al.*, 1991). In the U.S. showed deaths attributable to CVD occurred in adults aged ≥65 years estimated 82% of the total number of deaths, and 50% of CVD morbidity occurred in adults aged ≥60 years (Lloyd-Jones *et al.*, 2009). Aging is associated with structure and function changes of cardiovascular system. Vascular aging reduced compliance, increase in vascular smooth muscle cells led to elevated in thickening of the intima media and fall in dilation of the lumen (Marin, 1995; Najjar *et al.*, 2005).

Moreover, vascular aging show increasing of inflammatory response and impair angiogenesis (Rivard *et al.*, 1999). Blood pressure in older adults show increase in systolic blood pressure (SBP) and decrease in diastolic blood pressure (DBP) because aging blood pressure is driven by central artery stiffness (Franklin *et al.*, 1997). Vascular aging increase ROS mediated protein damage lead to oxidative stress that accelerate senescence that the major role to endothelial dysfunction (Oliver *et al.*, 1984). The endothelial dysfunction cause to decline in endothelium-dependent vasodilatation in both resistance and conductance arteries. Reduction of nitric oxide-derived from endothelial cell leads to reduce response to NO-dependent vasodilator response to ACh (Egashira *et al.*, 1993; Matz *et al.*, 2000; Taddei *et al.*, 2000). It is now well established that the perivascular adipose tissue (PVAT) that is a adipose surrounds the systemic blood vessel and can attenuates vasoconstrictive responses to NE or PE via paracrine release of adipokines and other bioactive molecules such as hydrogen sulfide (H_2S), nitric oxide (NO), angiotensin 1– 7 and other unknown relaxing factors called PVAT-derived relaxing factors (Dubrovska *et al.*, 2004; Gil-Ortega *et al.*, 2010). However, the anti-contractile effect of PVAT is impaired in hypertension and aging (Galvez-Prieto *et al.*, 2012; Lu *et al.*, 2011; Szasz *et al.*, 2012). Vascular aging increase PVAT mass, in contrast the anticontractile effect of PVAT was reduced. Some researcher found that in PVAT of aged-rats lost the anti-contractile effect caused by a reduced bioavailability of NO that produced from PVAT (Melrose *et al.*, 2013). Moreover, aging decreases the H_2S production that is a signaling molecule leading to relaxation of blood vessels. In cardiovascular system H_2S is endogenously produced from cystathionine-γ-lyase, CSE (Ebrahimkhani *et al.* 2005; Kimura *et al.* 2005).Vascular aging increase protein expression of cystathionine gamma-lyase (CSE), in contrast, showed decrease in H_2S concentration (Predmore *et al.*, 2010).

Kaempferia parviflora (KP) Wall Ex. Baker, black ginger, belongs to the family Zingiberaceae. In Thai traditional medicine, rhizomes of this plant have been used for many purposes (see more details in Yorsin *et al.*, 2014), but especially for the treatment of hypertension and as a male aphrodisiac (Wutythamawech, 1997). A number of scientific investigations have been reported on the activities of ethanol extracts from the KP rhizome that include: promoting male sexual desire

(Chaturapanich *et al.*, 2011; Sudwan *et al.*, 2006), anti-inflammation (Sae-wong *et al.*, 2009; Horigome *et al.*, 2014), and increased blood flow (Chaturapanich *et al.*, 2008; Malakul *et al*, 2011; Murata *et al.*, 2013). An ethyl acetate extract of KP rhizome reduced obesity in spontaneously obese type II diabetic mice by suppressing accumulation of visceral and subcutaneous fat and plasma triglyceride levels (Akase *et al.*, 2011; Shimada *et al.*, 2011). Recently, Yorsin *et al.* (2014) also found that a dichloromethane extract of the KP rhizomes (KPD) caused a lowering in the accumulation of body and liver lipid, as well as on plasma levels of triglycerides and glucose in middle-aged male rats after receiving an oral dose of KPD for 6 weeks. In addition, the KPD treatment caused an increase in expression of eNOS in blood vessels that sequentially enhanced the nitric oxide production to oppose vasocontraction to PE and to facilitate vasodilation to ACh of the blood vessel of the middle-aged male rat. These parameters were beneficial for preventing or prolonging the development of cardiovascular diseases. However, KPD is a mixture of several polyphenolic compounds with the major constituents being 5,7-dimethoxyflavone (DMF), 3,5,7-trimethoxyflavone (TMF) and 3,5,7,3′,4′-pentamethoxyflavone (PMF, Yorsin *et al.*, 2014). Among these three, PMF has been reported to possess an aphrodisiac activity as it caused relaxation of isolated human cavernosal strips by acting partly via the NO-cGMP pathway and inhibition of intracellular Ca^{2+} mobilization (Jansakul *et al.*, 2012). Yorsin *et al.* (2015) also found that PMF caused relaxation of isolated thoracic aortic rings both in an endothelium-independent and -dependent fashion, partly by stimulating the release of NO and H_2S. Thus, in the present study, PMF, isolated from KP rhizomes, was chosen for further study to obtain more information on the action of this compound from KPD on lowering the sugar and lipid levels as well as on the improvement of vascular functions in middle-aged male rats by using the same protocol as was previously used for the KPD extracts but with additional experiments on the role of PVAT and H_2S on the vascular functions.

5.3 OBJECTIVE

The aim of the present study was to investigate the effects of chronic treatment of PMF on its toxicity, lipid accumulation and vascular functions. The following parameters were therefore investigated:

1) animal body weight and food intake
2) toxicity on liver and kidney
3) lipid accumulation profile
4) vascular functions of isolated thoracic aortic, mesenteric rings and perivascular adipose tissue on their constrictor and dilatory responses to phenylephrine and/or acetylcholine, and measurement the eNOS and CSE protein expression in these tissue.

5.4 METERIALS AND METHODS

5.4.1 Plant material

Fresh rhizomes of KP were cultivated in Ampur Phurua, Loei Province Thailand. Botanical identification of the plant was achieved by comparison with the Herbarium specimens in the Department of Biology Herbarium as the same with previous described in Chapter 2. KP dichloromethane (KPD) extract was prepared using the same procedure as that described in chapter 2.

5.4.2 Isolation of the PMF from rhizomes of KP

The method used to measure body weight change and food intake had been previously fully described in chapter 2.

5.4.3 Experiment design

Middle-aged male rats (12-14 month old; the Animal House, Faculty of Science, Prince of Songkla University) were housed 6-8 animals per cage in the

room was maintained temperature at 25°C and 12 hour-light-dark Cycle. All experiments complied with guidelines from the standards for the ethical use of animals in research (Ethic No. 22/52). All animals were acclimatized to the environment for 1-2 weeks prior to experimentation with feeding distilled water by feeding tubes about 1.5 inches in length. PMF were prepared fresh each experimental day in a mixture of tween 80, 0.2 (g): carboxy-methylcellulose sodium salt, 0.2 (g): distilled water, 10 ml (the vehicle). The PMF-treated group were feeding by feeding tubes with PMF twice a day at the doses of 22 mg/kg/day (9.00 AM and 6.00 PM), for 6 weeks. The control group orally received the vehicle twice a day at the volume of 1 ml/kg in the same period.

5.4.4 Pharmacological studies

5.4.4.1 Body weight change and food intake

The method used to measure body weight change and food intake had been previously fully described in chapter 2.

5.4.4.2 Effects of chronic PMF treatment on the hematology and clinical biochemical analysis

The protocols used for measurement the hematology and clinical biochemical analysis, are the same as those described in Chapter 2 for studying the effect of chronic PMF treatment.

5.4.4.3 Effects of chronic PMF treatment on internal organs and lipid accumulation

Internal organs and lipid accumulation was performed by the same procedure as that described in chapter 2.

5.4.4.4 Effects of chronic PMF treatment on blood pressure and heart rate *in vivo* study

The method used to measure blood pressure and heart rate had been previously fully described in chapter 2.

5.4.4.5 Pharmacological studies of chronic PMF treatment on isolated thoracic aorta function

Preparation of thoracic aorta

The procedure for the preparation of aortic rings has been described in chapter 3. However this experiment prepared the thoracic aorta with adhering perivascular adipose tissue (PVAT) was removed from the decapitated rat and placed in oxygenated 37 °C Krebs-Henseleit solution. Six adjacent rings of 4-5 mm in length were cut, two rings were left with an intact PVAT, and the other 4 rings had their PVAT removed together with adhering connective tissue and for one ring the endothelium layer was removed by a small cotton bud. Each aortic ring was mounted with two stainless steel hooks in a 20-ml organ bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4 \cdot 7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at 37 °C and bubbled with a carbogen (95% O_2 and 5% CO_2 gas mixture). One of the hooks was fixed at the bottom and the other was connected to a transducer for recording the isometric tension by a Polygraph. The tissues were equilibrated for 60 min under a resting tension of 1 g and the bath solution was replaced with pre-warmed oxygenated Krebs-Henseleit solution every 15 min. At the end of equilibration period, each aortic ring was tested for its functional endothelium by precontraction with PE (3 μM) until the response reached a plateau (5-8 min), and then ACh was added (30 μM). Endothelial viability was judged by a > 65% vasorelaxation back to the tension generated by the ring before adding the phenylephrine. Denudation was confirmed by the complete absence of vasorelaxation following the response to the addition of acetylcholine. The preparations were then

washed several times with Krebs Henseleit solution, and allowed to fully relax for 45 min before the experimental protocol began.

Experimental protocol

After being at equilibrium for 60 min, to study the effects of chronic gavage PMF on vascular function. The aortic rings were divided in to several experiments: (1) one was measured in alone Krebs Heinseleit solution and (2) another one measured the effect of endothelium nitric oxide synthase (eNOS) by preincubating with 300 μM L-NA (a nitric oxide synthase inhibitor) and (3) endothelium-denuded thoracic aortic rings. The method used to measure the contraction response to cumulative CR-curves of phenylephrine had been previously fully described in chapter 3. Role of PVAT and H_2S After equilibration, the endothelium-intact thoracic aortic rings with and without PVAT were incubated with L-NA for 40 min under a basal tension of 2 g. Then a cumulative *C-R* curve to phenylephrine was performed in the presence of L-NA, followed by several washings and re-equilibration for 60 min in the presence of L-NA to allow full relaxation of the blood vessels to their original baseline of 2 g. After that, DL-propargylglycine (PAG, a H_2S inhibitor) was added into the incubation and left for 10-15 min until the aortic contraction reached a plateau, and the cumulative *C-R* curve to phenylephrine was performed in the presence of L-NA and PAG. Using two other sets of endothelium-intact thoracic aortic rings, The one of endothelium-intact thoracic aortic ring was examined the effect of chronic oral administration of KPD on nitric oxide –derived from eNOS via the relaxant effects response to ACh, and to study the effect on endothelium independent vasodilatory pathway by measurement the response to GTN (an endothelium independent vasodilatory substance; NO dornor). The method used to measure the relaxation of PE-precontracted thoracic aortic rings to ACh and GTN had been previously fully described in chapter 3.

5.4.4.6 Pharmacological studies of chronic PMF treatment on isolated mesenteric arteries function

Preparation of mesenteric artery

The protocols used for Preparation of mesenteric artery are the same as those described in Chapter 3.

Experimental protocol

After equilibration period for 60 min under a basal tension of the target transmural pressure of 100 mmHg, the mesenteric artery examined on the vascular reactivity to PE or/and preincubating with 300 μM L-NA, a nitric oxide synthase inhibitor, ACh and GTN with L-NA. The Experimental protocol to measure the mesenteric artery reactivity had been previously fully described in chapter 3.

5.4.4 eNOS and CSE Western blot analysis

To analyze the expression of the eNOS and CSE enzyme, the thoracic aortae, mesenteric arteries and PVAT of the PMF treatment and vehicle control groups (n=4) were harvested and kept at -70 °C until used. Protein extraction from the tissues and Western blot analysis were carried out as previously described (Yorsin *et al.*, 2014). Briefly, each tissue from each animal was chopped on ice and homogenized in lysis buffer with 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM EDTA and the protease inhibitor cocktail (GE Healthcare), centrifuged, and the total protein measured in the supernatants by the Biorad protein assay method. 50 μg of protein was electrophoretically separated on 12% polyacrylamide-SDS gels and the proteins were transferred onto a nitrocellulose membrane. Nonspecific-binding sites were blocked with 5% low fat dry milk in TBS-T (Tris buffer saline- 0.1% Tween 20) and then incubated with primary antibodies against eNOS (1:250), CSE (1:1,000) and β-actin (1:1,000) antibodies dissolved in 1% low fat dry milk in TBS-T overnight at 4 °C [eNOS and β-actin antibodies were from Cell Signaling (U.S.A); CSE was from Abnova (U.S.A)]. Membranes were incubated with horseradish- peroxidase anti-rabbit IgG antibody diluted to 1:5,000 (eNOS and β-actin) and horseradish-peroxidase anti-mouse IgG antibody diluted to 1:5,000 (CSE) in 1% low fat dry milk in TBS-T for 1 h. After being washed with TBS-T three times, the proteins were detected by an ECL chemiluminescent detection kit (Pierce) and the reaction was

visualized by the chemiluminescence imaging instrument (Vilber Lourmat, France). The intensity of the bands was analyzed using the Fusion Capt Advance quantitation analysis program. To ensure equal protein loading, results were normalized to the β-actin protein expression and expressed as units relative to the β-actin densitometry.

5.4.5 Drugs

The following drugs used: acetylcholine chloride (ACh), N^G-nitro-L-arginine (L-NA), norepinephrine (NE), phenylephrine hydrochloride (PE), pentobarbital sodium, DL propargylglycine (PAG, cystathione γ -lyase inhibitor) and the DMSO and oil red O were from Sigma, U.S.A. Glyceryl trinitrate (GTN) was from Mycomed, Denmark. ACh, NE, and PE were dissolved in a solution containing NaCl 9 g/L, NaH_2P0_4 0.19 g/L and ascorbic acid 0.03 g/L, and GTN, PAG (cystathione γ -lyase inhibitor) was dissolved in distilled water. PMF was dissolved in 20% DMSO. The organic solvents for isolation: methanol and dichoromethane, and the silica gel for column chromatography: silica gel 100 (70-230 mesh ASTH), silica gel 60 (230-400 mesh ASTH) and silica gel 60 RP-18 (40-63 μm), were purchased from Merck, Germany.

5.4.6 Data Analysis

Data are reported as means ± S.E.M. (n=6 animals). Tests of significance were made using the Student's paired or unpaired *t*-test. In all experiments, a P value ≤ 0.05 was considered statistically significant.

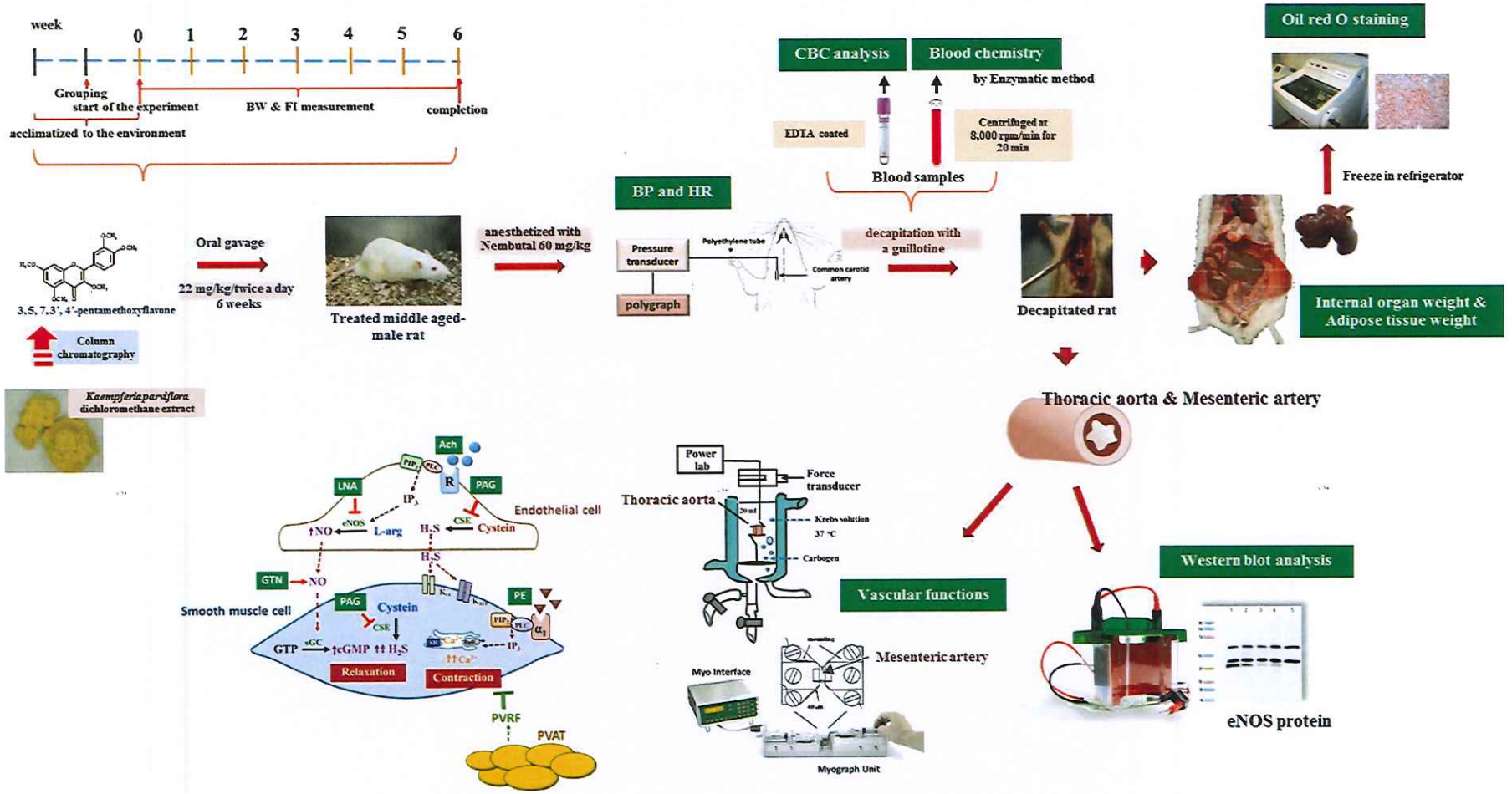

Figure 51 Scope of experimental studies

5.5 RESULTS

PMF was crystallized from methanol as colorless needles and was obtained with a 70.03 mg/g of dried KPD. The chemical structure and HPLC Analysis of PMF are shown in Fig. 43 in Chapter 4. We have previously showed that the oral administration of a KPD to middle-aged male rats caused a lowering in the accumulation of body fat, plasma glucose and triglycerides levels, and improved the functional compliance of the blood vessel in middle-aged male rats (Yorsin *et al.*, 2014). However, KPD is a mixture of many substances with the three major compounds of DMF, TMF and PMF that accounted for about 22% of the dried KPD (Yorsin *et al.*, 2014). Thus, in the present study PMF at a dosage of 22 mg/kg was used to treat the rats, twice a day, in the same manner as was previously obtained for the KPD.

5.5.1. Effects of KPD on body weight, food intake and relative organs weight

The PMF (100 mg/kg) twice a day treatment groups had no significant differences in body weight compared to vehicle control group (Fig. 52). Moreover, all PMF-treated groups had no altered food consumption in during the experiment and had no significant differences compared to vehicle control group.

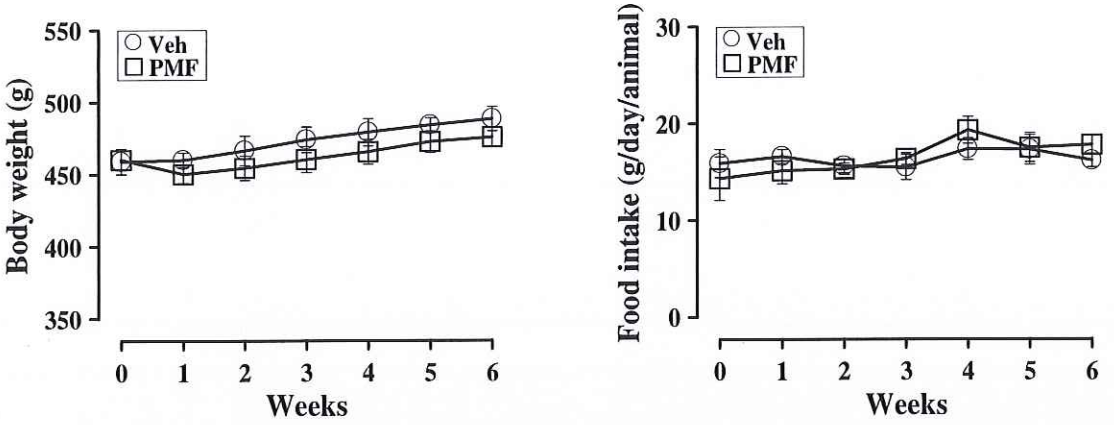

Figure 52 The body weight (left) and food consumption (right) of middle age male rats receiving PMF for 6 week. PMF treated-rat (PMF) or vehicle (Veh). Data represent a mean ± S.E.M. of 6 animals in each group.

In addition, none of the relative internal organ weights of adrenal glands, kidneys, liver, lung, spleen, testes, ventricle and atrium differences compared between the PMF (100 mg/kg) twice a day treatment groups and vehicle control (Table 13).

Table 13 Body weight (g) and relative organ weight (g/100g body weight) of middle-aged male rats were gavaged PMF for 6 weeks; vehicle control group, PMF treated-rat (PMF). Data was shown on the relative adipose tissue weight (g/100 g body weight) of middle-aged male rats.

Treatments	Body weight (g)		n	organs weight (%g)							
	Before	After		Adrenal gl.	Kidneys	Liver	Lung	Spleen	Testis	Ventricles	Atrium
Vehicle	459.00 ± 8.73	488.25 ± 4.63	8	0.015 ± 0.00	0.47 ± 0.01	2.39 ± 0.05	0.41 ± 0.01	0.20 ± 0.01	0.86 ± 0.02	0.26 ± 0.01	0.014 ± 0.001
PMF	460.14 ± 7.13	475.57 ± 6.56	8	0.016 ± 0.00	0.49 ±0.01	2.46 ±0.03	0.41 ± 0.02	0.19 ± 0.01	0.90 ± 0.01	0.27 ± 0.01	0.015 ± 0.001

Data represent a mean ± S.E.M. of 6 rats in each group.

5.5.2. Changes in visceral fat and subcutaneous fat

The PMF (100 mg/kg) twice a day treated- group had no altered on relative adipose tissue weights of the epididymis, retroperitoneal, mesentery and subcutaneous tissues when compared to vehicle control group (Table 14).

Table 14 Effects of 6 weeks oral administration of 3PMF on the accumulation of adipose tissue in middle aged male rats; vehicle control group, PMF treated-rat (PMF). Data was shown on the relative adipose tissue weight (g/100 g body weight) of middle-aged male rats.

Treatments	n	Adipose tissue weight (% g body weight)			
		Epididymis	Mesentery	Retroperitoneal	Subcutaneous
Vehicle	8	2.56 ± 0.08	2.06 ± 0.10	2.79 ± 0.18	7.24 ± 0.26
PMF	8	2.41 ± 0.10	2.08 ± 0.11	2.64 ± 0.20	7.20 ± 0.32

Values are mean ± S.E.M for 8 rats in each group

5.5.3. Effects of chronic KPD treatment on the clinical biochemical and hematology analysis.

Effects of chronic PMF treatment on clinical biochemical analysis showed that there no significant changes were observed in serum levels of the alkaline phosphatase (ALP), serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), blood urea nitrogen (BUN) and Creatinine (CREAT) for the PMF (100 mg/kg) twice a day treatment groups compared to vehicle control (Table 15). Effects of chronic PMF treatment on Serum Lipid Profile, in PMF

treated-rat group did not different significantly on triglyceride, cholesterol, LDL-C and LDL-C/HDL-C ratio compared to vehicle control group. However, In PMF treatment group, the level of Glucose, was decreased significantly, in other hand HDL-C was increased significantly when compared to vehicle control group, but the value is in the normal range (Table 16). There were no significant changes in White blood cells (WBC), Hematocrit (HCT), Hemoglobin (HGB), mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MC), Mean corpuscular hemoglobin concentration (MCHC), the polymorphonuclear neutrophils (PMN), and lymphocyte (LYMPH) between the PMF treated- and that of the vehicle control group and the value is in the normal range (Table 17).

Table 16 The effects of chronic oral administration of PMF on Serum Lipid Profile; vehicle control group, PMF treated-rat (PMF).

NLAC- MU normal range (mg %)	n	Glucose 122.10 –180.80	Triglyceride 61.00 –164.00	Cholesterol 46.00 –98.00	HDL-C -	LDL-C -	LDL/HDL ratio -
Vehicle	6	**125.67 ± 4.45**	87.00 ± 6.06	76.67 ± 4.54	**58.55 ± 1.98**	13.14 ± 1.96	0.22 ± 0.03
PMF	6	**110.67 ± 3.77** [a]	79.40 ± 5.52	83.40 ± 4.98	**71.15 ± 3.10** [b]	13.92 ± 1.59	0.20 ± 0.02

Values are mean±S.E.M for 6 rats in each group

Note: NLAC-MU normal range = National Laboratory Animal Centre-Mahidol University normal range.

HDL-C= High Density Lipoprotein cholesterol and LDL-C= Low Density lipoprotein cholesterol.

[a] Significantly lower than control group, $P \leq 0.05$

[b] Significantly lower than control group, $P \leq 0.05$

Table 17 The effect of chronic PMF oral administration on hematology parameters of plasma; vehicle control group, PMF treated-rat.

		WBC ($x10^3/\mu l$)	HCT (%)	HGB (g/dl)	MCV (fl)	MCH (pg)	MCHC (%)	PMN (%)	LYMPH (%)
NLAC- MU normal range		3.0 – 7.2	33.2 – 46.0	13.5 – 17.6	47.5 – 54.7	17.4 – 26.5	34.7 – 51.8	-	59.0 – 91.0
Vehicle	6	5.1 ± 0.3	45.0 ± 2.0	16.0 ± 0.4	56.5 ± 0.5	19.5 ± 0.5	35.5 ± 0.5	61.0 ± 6.1	33.0 ± 5.5
PMF	6	5.0 ± 0.7	44.2 ± 0.4	16.4 ± 0.2	52.5 ± 1.5	19.5 ± 0.7	37.3 ± 0.3	62.8 ± 8.3	33.0 ± 6.2

Values are mean ± S.E.M for 6 rats in each group,

Note: NLAC-MU = National Laboratory Animal Centre, Mahidol University.

WBC= White blood cell; HCT =Hematocrit; HBG= Hemoglobin; MCV= Mean corpuscle volume; MCH= Mean corpuscle hemoglobin; MCHC= Mean corpuscle hemoglobin concentration; PMN = Polymorphonuclear leukocytes; Plt = Platelet count.

5.5.4 Effect of chronic PMF treatment on liver lipid accumulation

The PMF (22 mg/kg) twice a day treated- group had no altered on liver lipid accumulation when compared to vehicle control group (Fig. 53). These data suggest that chronic PMF treatment had no any effects on liver lipid accumulation.

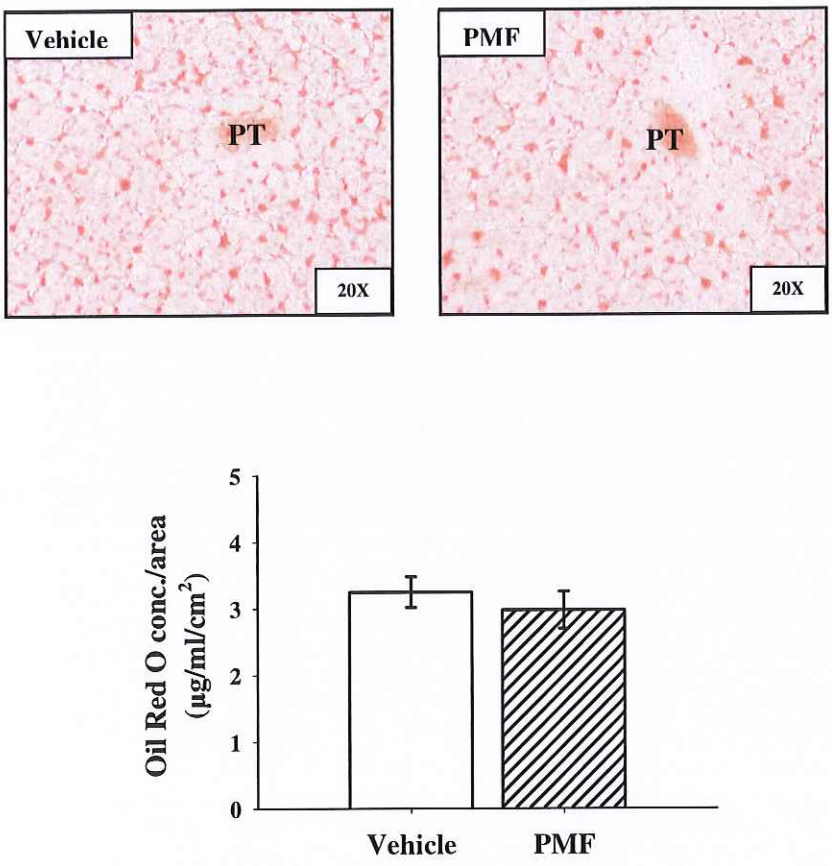

Figure 53 Effect of 6 weeks oral administration of PMF on liver lipid accumulation, Vehicle control rat (Veh) or PMF treatment for 6 weeks twice a day (PMF). Values are mean ± S.E.M for 6 rats in each group. (PT=Portal triad; 20 mm thick, magnification, ×20.).

5.5.5 Effect of chronic PMF treatment on blood pressure

There were no differences in the basal systolic and diastolic blood pressure and basal heart rate of rats between the chronic PMF treatment rats and the vehicle control group.

Table 18 Effects of chronic oral gavage of 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF) on basal blood pressure (BP) and basal heart rate of anesthetized middle-aged male rats in vivo; vehicle control group, PMF treated-rat (PMF).

Treatments	N	Basal systolic BP (mmHg)	Basal diastolic BP (mmHg)	Basal heart rate (bpm)
Vehicle	6	145.53 ± 4.06	119.50 ± 3.20	442.00 ± 10.20
PMF	6	142.00 ± 1.13	115.00 ± 4.47	429.00 ± 12.49

Values are mean ± S.E.M for 6 rats in each group

5.5.6 Effects of chronic PMF treatment on the thoracic aorta reactivity

In presence of endothelium, PMF treated-rat exerted significantly lower the contractile response to PE than the Vehicle control rat (Fig.54A) that related with the maximal contractile response (PMF, Emax values: 2.6 ± 0.1; Veh, Emax values: 4.0 ± 0.5), but not different of the EC_{50} values (PMF, EC_{50} values: 45.4 (10.8 - 99.2); Veh, EC_{50} values: 50.6 (28.4 - 90.8) (Table 19). The anticontractile effect to PE of PMF treated-rat was attenuated in pre-treatment with L-NA (0.3 mmol/L) or the absence of endothelium (Fig. 54B and C). For these suggested that effect of chronic oral administration of PMF was lost by inhibition of NO derived from eNOS or removed of NO derived from endothelium. Addition of PAG into the incubation

medium caused a spontaneous contraction of the thoracic aortic rings, that was significantly greater for the aortic rings of the PMF-treated rats than that for the vehicle control groups (Endo+L-NA+PAG; Veh 0.55±0.30 g; PMF 2.2±0.51 g). Subsequently there was a greater contraction for low concentrations of the phenylephrine *C-R* curve of the PMF-treated rats than that of the vehicle control group (Fig. 54D). Pre-contracted with PE (3 μmol/L), ACh caused a concentration-dependent vasorelaxation. The results showed endothelium-dependent relaxation ACh precontracted with PE induced relaxations with E_{max} of 87.4 ± 3.7 in chronic PMF treatment group that was significantly higher than the vehicle group with E_{max} of 65.4 ± 4.8, but not different in the EC_{50} values (PMF, EC_{50} values: 45.4 nM (10.8 - 99.2 nM); Veh, EC_{50} values: 50.6 nM (28.4 - 90.8 nM) (Fig. 55A, Table 19). Role of a nitric oxide independent endothelium pathway, the relaxation response to GTN (the nitric oxide donor) of the endothelium-intact thoracic aortic rings with L-NA precontracted with phenylephrine in chronic PMF treatment group had on any significant response when compare with the vehicle control groups (Figure 55B, Table 20).

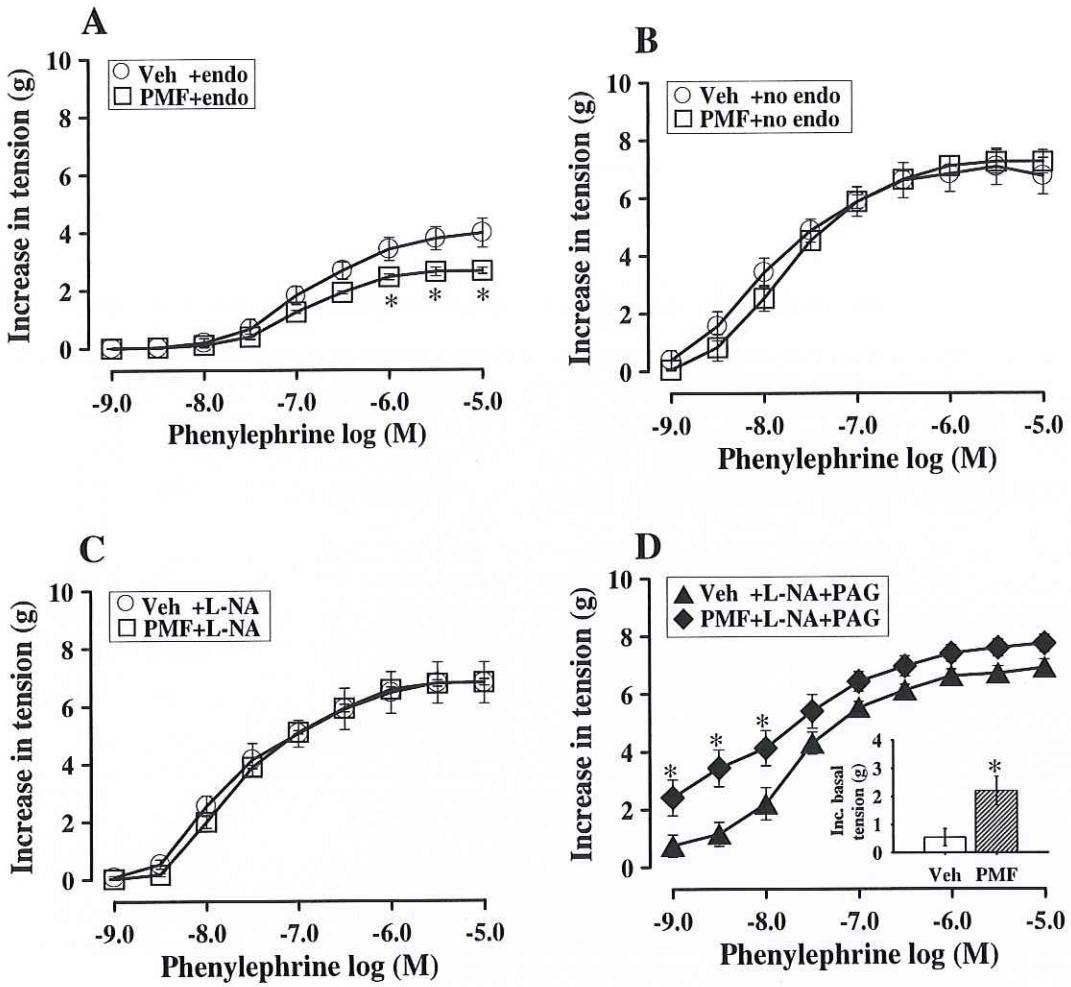

Figure 54 Cumulative concentration–response curve to PE in endothelium-intact (endo, A), without endothelium (Noendo, B), endothelium-intact with L-NA (+L-NA, C), endothelium-intact with L-NA and PAG (+L-NA +PAG, D) in aortic rings from chronic oral PMF treated rats (PMF) compared with the vehicle control group (Veh). Data was shown mean ± S.E.M. of six rats in each group. * Significantly lower than that of the control group (Veh-bid), $P \leq 0.05$.

Table 19 Comparison of the sensitivity (EC_{50}) and maximum response (E_{max}) of contractile responses to PE in thoracic aorta from middle-aged male rats with prior 6 weeks treatment with oral PMF twice a day (PMF) compared to the vehicle control.

	EC_{50} (nM) : 95% confidential limit		Emax (g)	
Treatments	Vehicle	PMF	Vehicle	PMF
+Endo	101.4 (73.2 - 149.2)	116.8 (87.7 - 173.7)	4.0 ± 0.5	2.6 ± 0.1[a]
Endo+L-NA	21.1 (15.7 - 30.9)[b]	22.4 (15.4 - 32.5)[b]	6.8 ± 0.7[c]	6.7 ± 0.2[c]
Endo+L-NA+PAG	21.7 (15.6 – 30.7)[b]	3.6 (1.3 – 9.4)[a,b]	6.9 ± 0.3[c]	7.7 ± 0.3[c]
No endo	18.9 (15.7 - 25.5)[b]	17.6 (14.7 - 21.1)[b]	7.1 ± 0.6[c]	7.2 ± 0.4[c]
PVAT+no endo	86.6 (44.2 - 143.1)	76.7 (37.8- 104.2)	3.0 ± 0.2	3.0 ± 0.1
PVAT +L-NA	79.5 (42.2 - 128.7)	70.6 (39.5 – 102.4)	3.7 ± 0.3	3.5 ± 0.3
PVAT +L-NA+TEA	49.8 (29.7- 83.8)	40.5 (24.2 – 57.3)[b]	4.3 ± 0.3	4.7 ± 0.6[c]
PVAT +L-NA+PAG	46.1 (29.9 - 72.9)	52.2 (39.8 – 78.5)[b]	4.6 ± 0.4	5.2 ± 0.3[c]

Values were obtained from 6 experiments (n = 6) for each group.

[a] Significantly lower than their corresponding control group, [b] Significantly lower than the ones with endothelium and [c] Significantly higher than the ones with endothelium, $P \leq 0.05$.

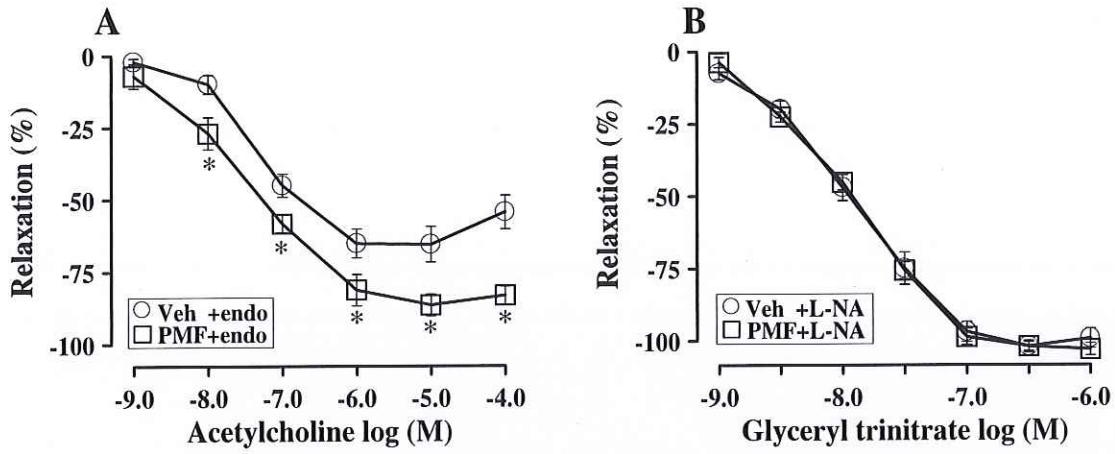

Figure 55 Concentration–relaxation response curves to ACh (A) and GTN of the endothelium-intact thoracic aortic rings with L-NA precontracted with PE (GTN, B) in aortic ring from chronic PMF treatment group (PMF) compared with vehicle control group (Veh). All data represent a mean ± S.E.M. of 6 rats in each group. * Significantly higher than that of the vehicle control group, $P \leq 0.05$.

Table 20 A comparison of the sensitivity (EC_{50}) and maximum response (E_{max}) of vasorelaxation responses to ACh or GTN of the thoracic aortic precontracted with PE of middle-aged male rats with prior 6 weeks treatment with oral PMF twice a day (PMF) compared to the control group that vehicle control.

Treatments	Thoracic aorta	
	EC_{50} (nM) : 95% confidential limit	Emax (%)
Response to Ach		
Vehicle	50.6 (28.4 - 90.8)	65.4 ± 4.8
PMF	45.4 (10.8 - 99.2)	87.4 ± 3.7[a]
Response to GTN		
Vehicle	19.7 (14.6 - 26.5)	105.9 ± 1.5
PMF	25.1 (18.1 - 35.0)	108.6 ± 2.9

Values were obtained from 6 experiments (n = 6) for each group.

[a] Significantly higher than that of the vehicle control groups, $P \leq 0.05$

5.5.7. Effects of PMF treatment on the aortic perivascular adipose tissue (PVAT)

The maximal contraction to phenylephrine of aortic rings with PVAT was about 2-fold lower with a 2-fold greater increase in the EC_{50} compared to the one without PVAT (Fig. 56 and Table 19). However, The *C-R* curves to phenylephrine of aortic rings with PVAT from the PMF-treated rats were similar to that of the vehicle control rat in both endothelium removed and after preincubation with the nitric oxide synthase by L-NA. When PVAT aortas with endothelium were incubated with L-NA and PAG, There were no differences in contractile responses to PE between aortas from the vehicle control group and the chronic PMF treatment group (Fig. 55D). A small spontaneous contraction of the aortic ring with intact PVAT sometimes seen after adding the PAG for the PMF-treated rats was not a statistically significant.

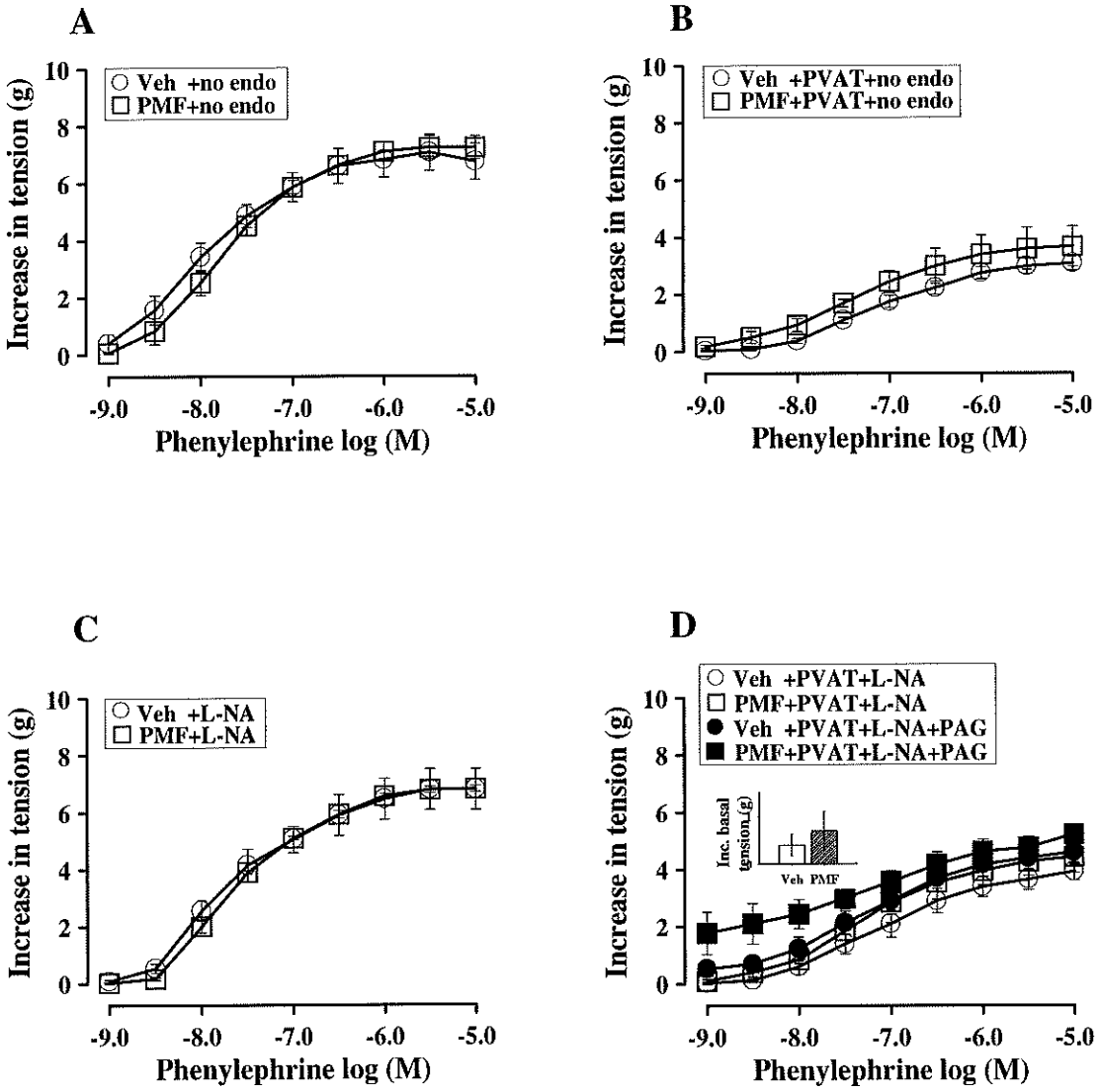

Figure 56 Cumulative concentration–response curve to PE in endothelium-removed aorta (no endo, A), a perivascular adipose tissue with endothelium removed aortas (PVAT+ no endo, B), endothelium-intact with L-NA (+L-NA, C), a perivascular adipose tissue with endothelium -intact with L-NA (PVAT+ +L-NA), and a perivascular adipose tissue with endothelium -intact with L-NA and PAG (PVAT+L-NA+PAG, D) in aortic rings from chronic oral PMF treated rats (PMF) compared with the vehicle control group (Veh).

5.5.8. Effects of chronic PMF treatment on the Mesenteric artery reactivity

The Mesenteric artery that presence of endothelium, PMF treated-rat exerted significantly lower the contractile response to PE than the vehicle control rat (Fig. 57) that related with the maximal contractile response of PMF treated-rat significantly lower than the vehicle control rat (PMF, E_{max} values: 56.6 ± 9.1%; Veh, E_{max} values: 94.8 ± 3.4 %, Table 21), but not different of the EC_{50} values (PMF, EC_{50} values: 6.6 μM (4.8 - 9.3 μM); Veh, EC_{50} values: 6.5 μM (4.2 - 8.9 μM), Table 21). After incubated with L-NA (0.3 mmol/L) due to a significant left shift of the dose-response to PE in all group. L-NA can attenuate the anticontractile effect to PE of PMF treated-rat (Fig. 57, Table 21). The effect of the ACh induced vasorelaxation of the mesenteric ring that precontracted with PE in PMF treated-rat had higher sensitivity than the vehicle control rat (Fig. 58A). However did not any effect on EC_{50} and E_{max} values (Table 22). To determine the role of a nitric oxide independent endothelium pathway showed that there were no differences found in the vasorelaxation reponsed to GTN (the nitric oxide donor) on the mesenteric rings precontracted with phenylephrine, in the presence of L-NA, compared between the chronic PMF treatment group and the vehicle control (Fig. 58B, Table 22).

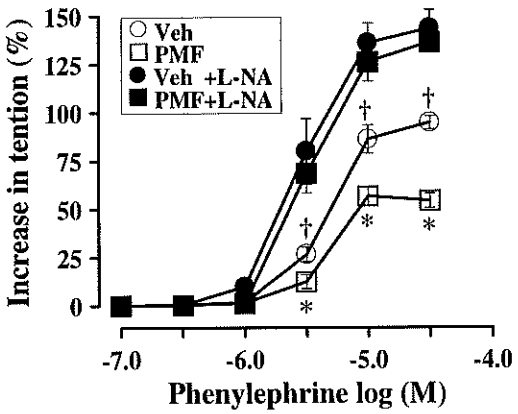

Figure 57 The constriction response to PE before and after preincubation with L-NA (L-NA). Each point represents a mean ± S.E.M. of 6 animals in each

group. * Significantly lower than that of the vehicle control group, $P \leq 0.05$. † Significantly higher than that of the corresponding control group, $P \leq 0.05$.

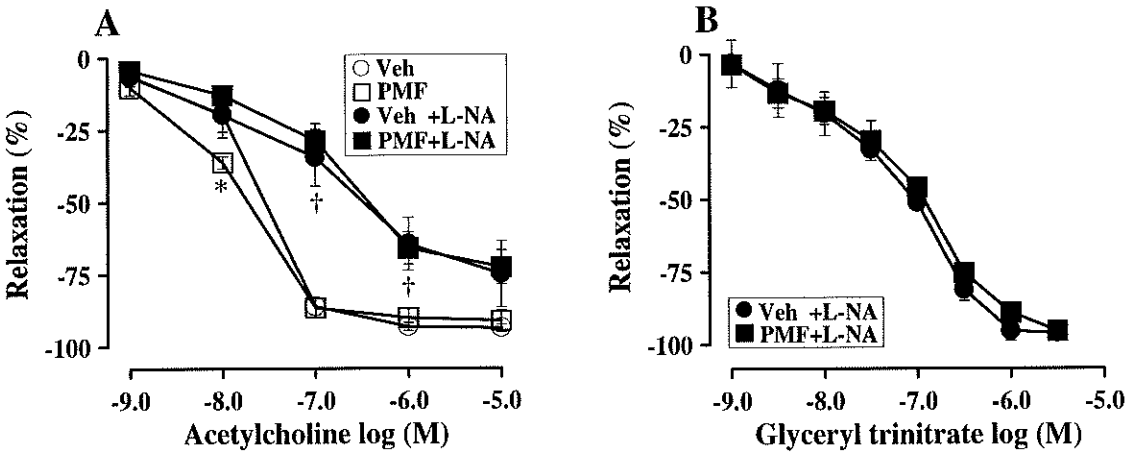

Figure 58 The vasorelaxation response to ACh precontracted with PE (A), The vasodilatory responses to GTN in the presence of L-NA precontracted with PE (B) of the mesenteric ring after chronic oral administration of PMF twice a day compared to that of the vehicle control groups. Each point represents a mean ± S.E.M. of 6 animals in each group. * Significantly lower than that of the vehicle control group; † Significantly higher than that of the corresponding control group, $P \leq 0.05$.

Table 21 A comparison of the sensitivity (EC_{50}) and maximum response (E_{max}) of contractile responses to PE of the mesenteric rings obtained from middle-aged male rats that had been oral administration for 6 weeks with vehicle or 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF) before and after preincubation with L-NA.

Treatments	EC_{50} (μM) : 95% confidential limit		E_{max} (%)	
	Control	+ L-NA	Control	+ L-NA
Vehicle	6.5 (4.2 - 8.9)	2.8 (1.9 - 3.9)[a]	94.8 ± 3.4	149.6 ± 9.8[c]
PMF	6.6 (4.8 - 9.3)	2.9 (1.8 - 4.1)[a]	56.6 ± 9.1[b]	136.5 ± 9.7[c]

Values were obtained from 6 experiments (n = 6) in each group.

[a] Significantly lower than that of their corresponding control group, [b] Significantly higher than that the control group (Veh), [c] Significantly higher than that of their corresponding control group, $P \leq 0.05$.

Table 22 A comparison of the sensitivity (EC_{50}) and maximum response (E_{max}) of vasorelaxation responses to ACh or GTN of the mesenteric artery precontracted with PE from PMF-treated group (PMF) with vehicle control group.

Treatments	Mesenteric artery	
	EC_{50} (nM) : 95% confidential limit	Emax (%)
Response to Ach		
Vehicle	25.3 (15.4 - 46.2)	93.3 ± 2.0
PMF	15.5 (6.3 - 35.4)	89.3 ± 4.8
Response to GTN		
Vehicle	93.8 (57.4 - 153.3)	94.2 ± 1.1
PMF	128.8 (69.7 - 238.0)	96.0 ± 0.8

Values were obtained from 6 experiments (n = 6) for each group.

[a] Significantly higher than that of the vehicle control groups, $P \leq 0.05$.

5.5.9 eNOS and CSE Western blot analysis

The expression of eNOS was examined in via SDS-PAGE and Western blotting. eNOS protein (140 kDa) was detected in thoracic aorta and mesenteric artery after chronic oral administration of KPD twice a day for comparison with vehicle control groups. To determine whether increased eNOS protein expression was indicated for the augmented NO production from endothelial cell. The quantitative expression of the eNOS protein as determined by Western blotting from the isolated thoracic aorta and the mesenteric artery was significantly higher for those obtained from the PMF-treated rat compared to that of the vehicle control groups (Fig. 59). Another one, to determine whether increased CSE protein expression was indicated for the augmented H_2S production from endothelial cell. CSE protein expression (43 kDa) in both aorta and the aortic perivascular adipose tissue (PVAT) was significantly affected by age (Watchara *et al.*, 2015). The expression of the CSE protein in the thoracic aorta and mesenteric artery of the PMF-treated rats was also greater than that of the vehicle control group (Fig. 59).

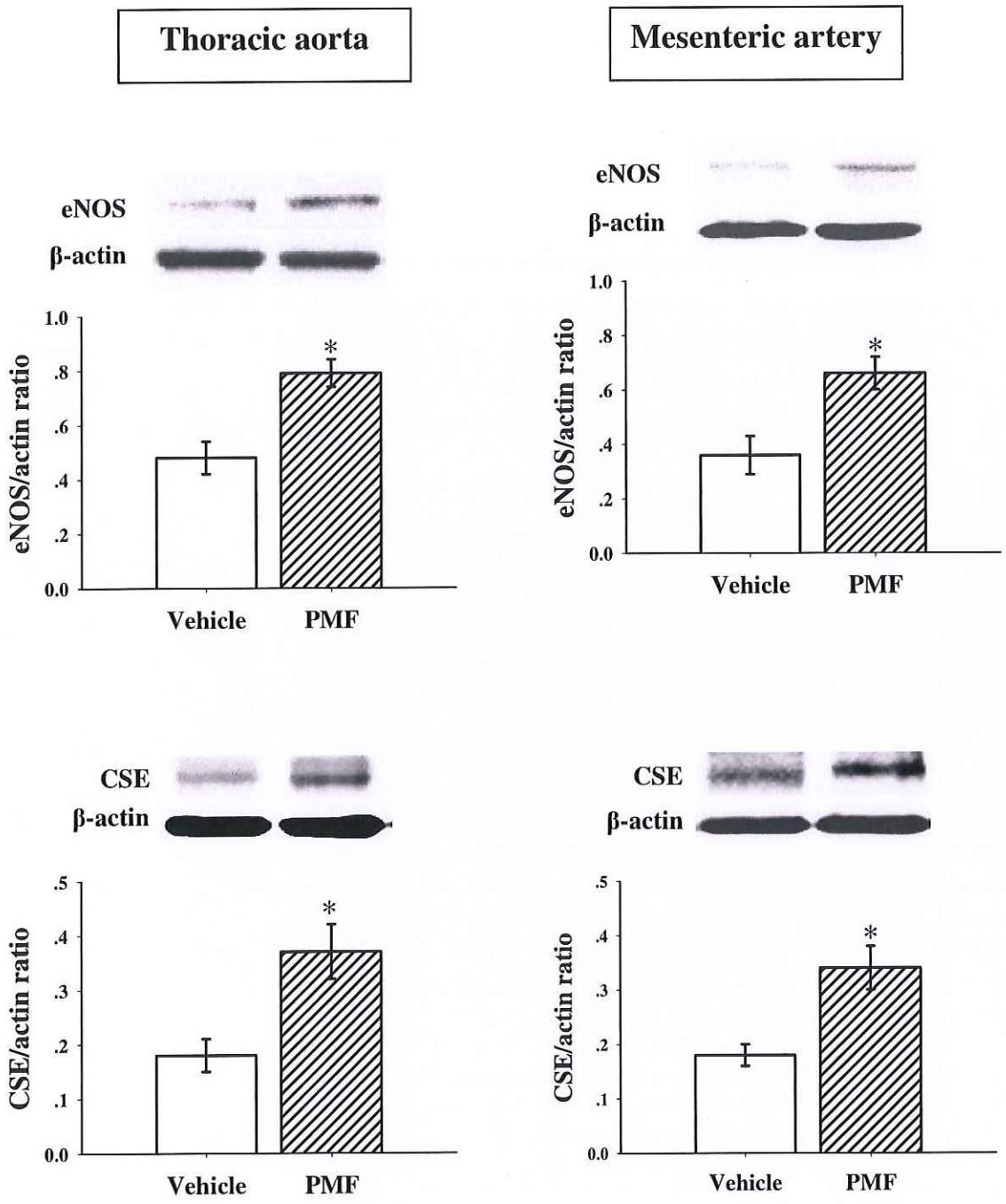

Figure 59 Typical Western blots showing the expression of eNOS and CSE protein expression in thoracic aorta and mesenteric artery from chronic oral PMF treated rats (PMF) compared with the vehicle control group (Veh). For each blot, β-actin expression is shown as a loading control. Values were obtained from 6 experiments (n = 6) in each group. * Significantly lower than the KPD treated middle-aged and young adult male rat, $P \leq 0.05$.

5.6 DISCUSSION

We have previously shown that the oral administration of a KPD to middle-aged male rats caused a lowering in the accumulation of body fat, plasma glucose and triglycerides levels, and improved the functional compliance of the blood vessel in middle-aged male rats (Yorsin *et al.*, 2014). However, KPD is a mixture of many substances with the three major compounds of DMF, TMF and PMF that accounted for about 22% of the dried KPD (Yorsin *et al.*, 2014). Thus, in the present study PMF at a dosage of 22 mg/kg was used to treat the rats, twice a day, in the same manner as was previously obtained for the KPD. As showed in the result section, PMF had no effect on body weight, body fat or accumulation of liver lipid. This indicated that the anti-obesity effects of the KPD are unlikely be caused by PMF. However, PMF treatment did cause a lowering in the plasma glucose levels with an increase in the HDL-C level, both of which are beneficial parameters for preventing the development of Type II-diabetes and cardiovascular disease. Matsuda *et al.* (2014) reported that PMF significantly increased the mRNA levels of the glucose transporter 4 (GLUT-4) of 3T3-L1 in a cell culture experiment. Thus, it was possible that the lowering of plasma glucose activity by PMF shown in the present study might be due to the enhancement of glucose uptake via the GLUT-4 of the adipose tissue and other GLUT-4-dependent cells. However further studies will be needed to confirm this possibility. The finding that the serum liver and kidney enzyme levels (ALP, SGOT, SGPT, BUN and creatinine) together with the hematological parameters were in the normal range and were not different from the vehicle control, as well as there being no signs of gross toxicity observed. This confirmed that the PMF treatment in the present study had no effect on the liver and kidney functions and had no gross toxicities. Although PMF treatment did not alter the basal systolic, diastolic and basal heart rate in anesthetized rats, it did cause an increase in the functional compliance of the blood vessels. Thus it was found that the thoracic aortic- and mesenteric ring of the PMF treated group had a lower contractile response to PE with an increase in the dilatation to ACh (precontracted with PE) than that of the vehicle control group. The underlying mechanism for this could be due to PMF, a very poor antioxidant compound (Jakhar *et al.*, 2014), that caused an increase in nitric oxide production

from the vascular endothelial cell that opposed the vasocontraction to PE and enhanced vasodilation to acetylcholine. To support this, other experiments were performed in the presence of L-NA or by denudation of the vascular endothelium and it was established that the effects disappeared. In addition this, up-regulation of the expression of eNOS protein by the PMF-treated thoracic and mesenteric artery in comparison to the vehicle control group also supported this conclusion. It is unlikely that PMF treatment caused a change in the vascular smooth muscle functions, as the endothelium-denuded aortic ring showed a similar contractile response to PE by the thoracic- and mesenteric rings obtained from the PMF treated- and the vehicle control rats. This was supported by the finding that there was no difference in the vasodilatation to GTN of the endothelium-intact aortic- and mesenteric ring that had been pretreated with L-NA between the PMF-treated and the vehicle control groups. PMF treatment caused an increase in the basal release of H_2S from the thoracic aortic ring, as it was found that PAG, a cystathionine-γ-lyase (CSE) inhibitor that utilized L-cysteine to produce H_2S (Hosoki *et al.*, 1997), caused an increase in the baseline tension of the L-NA- pretreated thoracic aortic ring that was higher than that of the vehicle control group. By this effect, a sequentially higher vasoconstrictory response to lower concentrations, but not the higher concentrations, of the PE *C-R* curve compared to that of the vehicle control group was observed. This was supported by the finding that the amount of the CSE protein expression by the thoracic aorta was higher for the PMF treated- than for the vehicle-treated control group. It is unlikely that PMF affected the PVAT functions of the middle-aged male rat, as PVAT attenuated the contractile response of the thoracic aortic ring with L-NA to PE that was similar to that of the PMF treated- and the vehicle control. Furthermore, there was no change in the PVAT-H_2S production after PMF treatment, although PAG did cause a rise of the baseline tension of the L-NA pretreated PVAT-intact thoracic aortic of some PMF treated rats compared to that of the vehicle control group, but the difference did not reach statistical significance. This was also supported by the finding that the amount of PVAT-CSE protein expression was similar for the PMF treated and the vehicle control group.

5.7 CONCLUSION

In conclusion, oral administration of the PMF to middle-aged male rats caused an up-regulation of the expression of blood vessel eNOS and CSE proteins, that resulted in an increase of NO and H_2S productions to modulate the blood vessel function by: opposing contraction to phenylephrine and enhancing vasodilatation to acetylcholine. PMF also caused a decrease in plasma glucose, and an increase in plasma HDL cholesterol levels of the rats. These parameters are beneficial factors for preventing and/or delaying the development of Type II diabetes and cardiovascular disorder. Thus, these animal data suggest that PMF is a novel compound that might be developed as a food supplement for preventing or delaying the development of diabetes and/or cardiovascular diseases in ageing human beings.

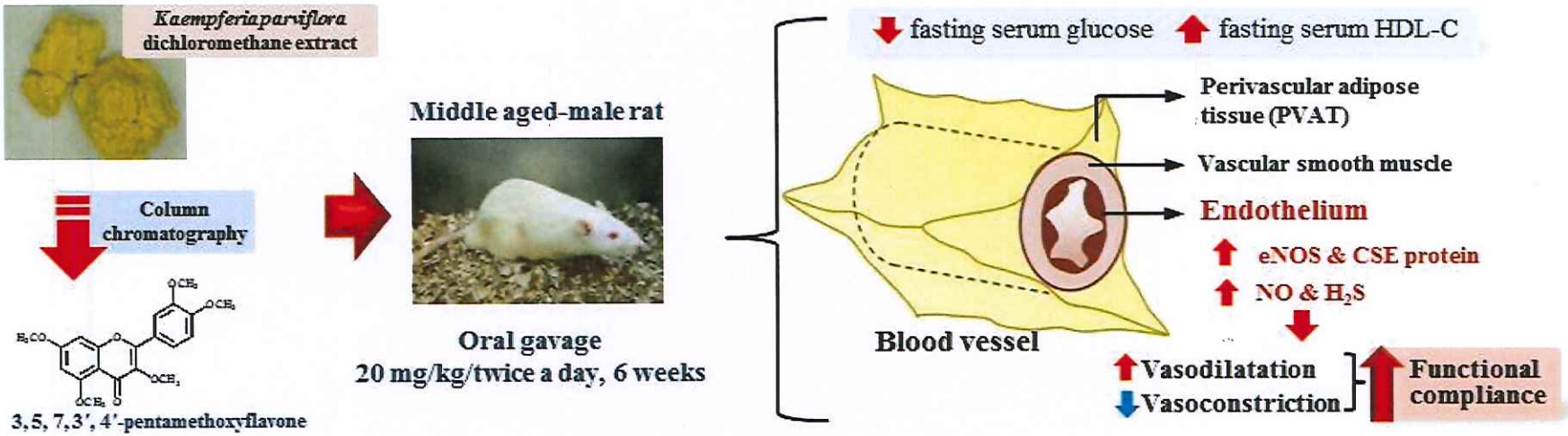

Figure 60 The effects of PMF on metabolic profile and vascular function

CONCLUSION

This study has demonstrated that chronic oral administration of KPD to middle-aged male rats for 6 weeks caused alterations in lipid metabolism and led to endothelium dysfunction of the blood vessels through an increase in nitric oxide production by increasing the eNOS protein at the blood vessels. KPD consists of three major components; 5,7-dimethoxyflavone (DMF), 3,5,7,3′,4′-pentamethoxyflavone (PMF) and 3,5,7-trimethoxyflavone (TMF). Among these three, this study showed that PMF has been reported to cause upregulation of the expression of blood vessel eNOS and CSE proteins, resulting in an increase of NO and H_2S productions to modulate the blood vessel function. Also, PMF treatment did cause a decrease in the plasma glucose levels and an increase in the HDL-C level. This data from the animals suggest that PMF can improve the blood vessel function and prevent the development of Type II diabetes and cardiovascular disorder. Therefore, these findings are an innovative way to use traditional medicine for preventing or prolonging development of the metabolic syndrome and cardiovascular disease.

Table 23 A comparison of the effects of KPD treatment and PMF treatment on lipid profiles and vascular functions.

Treatment	Pharmacological effects	
	Lipid profiles/Glucose	**Vascular functions**
KPD	↓ Visceral and subcutaneous fat ↓ Fasting serum Glucose and triglyceride ↓ Liver lipid accumulation	↑ eNOS protein expression ↑ NO production ↑ Vasodilatation ↓ Vasoconstriction
PMF	↓ Fasting serum glucose; ↑ serum HDL-C	↑ eNOS and CSE protein ↑ NO and H_2S production ↑ Vasodilatation ↓ Vasoconstriction

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APPENDIX

Definition

Haft maximal effective concentration (EC_{50}) is the concentration of a drug that gives half-maximal response. It commonly used as a response halfway between the baseline (Bottom) and maximum response (Top) for measure of drug's potency.

E_{max} is the maximum responsible effect for the agonist.

Sample size is calculated using the following formula (Kadam and Bhalerao, 2010):

$$n = \frac{2(Z_{\alpha}+Z_{1-\beta})^{2}(\sigma)^{2}}{\Delta^{2}}$$

n is the required sample size.

Z_{α} is a constant (set by convention according to the accepted α error) as shown below:

α-error	**5%**	**1%**	**0.1%**
2-sided	1.96	2.58	3.29
1-sided	1.65	2.33	

$Z_{1-\beta}$ is a constant set by convention according to power of the study as shown below:

Power	80%	85%	90%	95%
Value	0.84	1.04	1.28	1.64

σ is the standard deviation.

Δ is the difference to be detected of two interventions which is required.

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Research Paper

Effects of *Kaempferia parviflora* rhizomes dichloromethane extract on vascular functions in middle-aged male rat

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ABSTRACT

Ethnopharmacological relevance: In Thai traditional medicine, rhizomes of *Kaempferia parviflora* (KP) have been used for treating hypertension and for the promotion of longevity with good health and well being. Ageing is one of the most important risk factors for development of cardiovascular disease. To investigate whether a 6 weeks oral administration of a dichloromethane extract of fresh rhizomes of *Kaempferia parviflora* (KPD) had any effects on vascular functions, on the accumulation of lipid, as well as on any signs of gross organ toxicity in middle-aged rats.

Materials and methods: Fresh rhizomes of *Kaempferia parviflora* were first macerated twice with 95% ethanol to remove the dark color before extracting three times with 100% dichloromethane. The dichloromethane extract was evaporated under reduced pressure to obtain the dried *Kaempferia parviflora* dichloromethane extract (KPD). The rats were orally administered with the KPD at a dosage of 100 mg/kg body weight, or with the same volume of the vehicle (tween 80, 0.2 g: carboxymethylcellulose sodium, 0.2 g: distilled water 10 ml) once or twice a day for 6 weeks. Vascular functions were studied on isolated thoracic aorta and the mesenteric artery. The vascular eNOS enzyme was measured by Western blot analysis. Blood chemistry was measured by enzymatic methods. Liver cell lipid accumulation was measured using oil red O staining.

Results: A 6 weeks treatment of KPD once a day had no significant effects on any of the studied parameters. When the KPD was given twice a day, the contractile responses to phenylephrine of the thoracic aorta and mesenteric artery were lower than the vehicle control group, and this effect was abolished by N^G-nitro-L-arginine or by removal of the vascular endothelium. Vasorelaxation to acetylcholine, but not to glyceryl trinitrate, by the thoracic aortic and mesenteric ring precontracted with phenylephrine was higher from the KPD treated rats than those from the vehicle control groups. Western blot analysis showed a higher quantity of thoracic- and mesenteric-eNOS protein obtained from the KPD treated rats. In addition, the body weight, serum glucose and triglycerides levels, visceral and subcutaneous fat, as well as liver lipid accumulation were all significantly decreased in the KPD treated rats compared to those of the vehicle control. No differences were found between the KPD treated-, and the vehicle-control for animal food intake, internal organ weight, serum ALP, SGOT, SGPT, BUN and creatinine levels, serum cholesterol, HDL-C and LDL-C levels, nor total blood cell counts.

Conclusions: The chronic oral administration of KPD extract, to middle aged rats, caused a decrease in vascular responsiveness to phenylephrine with an increase in the acetylcholine induced vasorelaxation, due to an increase in nitric oxide production from their blood vessels. The extract also caused a decrease in visceral and subcutaneous fat, fasting serum glucose and triglyceride levels and liver lipid accumulation, with no changes to liver and kidney functions or to total blood cell counts. It is possible that these KPD extracts could be developed as a health product for mid-aged humans to reduce obesity, diabetes type II and cardiovascular disease.

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1. Introduction

Age is one of the most important risk factors for the development of metabolic syndrome and/or cardiovascular diseases.

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Advancing age is associated with increased body fat especially the accumulation of lipid in the viscera and liver (Park et al., 2006), and this can be responsible for the age-related risks of diabetes type II, hypertension, thrombosis and atherosclerosis (Barbagallo et al., 2001; Okosun et al., 2001; Blokhin and Lentz, 2013). It has been shown that reduction of visceral fat even with a modest loss in weight (18%) could prevent the insulin resistance and glucose intolerance of aging in F344/Brown Norway and Zucker Diabetic Fatty rats (Gabriely et al., 2002). In addition, there was an increase in plasma insulin level in Sprague-Dawley rats that led to a reduced weight and also decreased the risk factors associated with diabetes type II and cardiovascular disease (Barzilai and Gupta, 1999; Resnick et al., 2000; Wing et al., 2011). For humans the careful use of suitable micronutrients that prevent lipid accumulation is likely to prevent or reduce disease and prolong our healthy vascular functions.

Kaempferia parviflora (KP) Wall. Ex Baker belongs to the family Zingiberaceae and is found in the northern part of Thailand. In Thai traditional medicine, rhizomes of this plant have been used for treating various symptoms including erectile dysfunction, hypertension, inflammation, abdominal pain, as well as for the promotion of longevity with good health and well being (Wutythamawech, 1997; Yenjai et al., 2004). In Laos folk medicine, it has been used for lowering blood glucose levels, improving blood flow and increasing vitality (Akase et al., 2011). In Japan, KP extract is commercially available as a food supplement for the treatment of metabolic syndrome (Nakao et al., 2011). A number of scientific investigators have reported on the activities of an ethanolic extract from KP that included: acting as an aphrodisiac in male rats (Sudwan et al., 2006; Chaturapanich et al., 2011), as an agent to prevent gastric ulcers (Rujjanawate et al., 2005), as an anti-inflammatory agent (Tewtrakul and Subhadhirasakul, 2008; Sae-wong et al., 2009), and to prevent myocardial ischemic-reperfusion injury in an isolated rat heart (Malakul et al., 2011). In an *in vivo* experiment, a *Kaempferia parviflora* ethanolic extract caused an increase in testicular blood flow and an alteration in the blood clotting mechanisms by activation of fibrinolysis after chronic oral administration to young male rats (Chaturapanich et al., 2008; Murata et al., 2013). When a less polar organic solvent was used for extraction of a *Kaempferia parviflora*, plant rhizome powder with ethyl acetate, Akase et al. (2011) and Shimada et al. (2011) reported that it had an anti-obesity activity in spontaneously obese type II diabetic mice (Tsumura, Suzuki, Obese Diabetes TSO mice), but not in the Non-Obesity (TSNO) mice, by suppressing accumulation of visceral and subcutaneous fat and plasma triglycerides. On the other hand, Horikawa et al. (2012) found that an ethyl acetate extract of the *Kaempferia parviflora* rhizomes and its purified compounds: 3,5,7,4′-tetramethoxyflavone and 3,5,7,3′,4′-pentamethoxyflavone caused enhancement of adipogenesis. However, there has been no evidence for any effect of extracts from *Kaempferia parviflora* rhizomes on vascular functions and lipid accumulation especially in middle-age animals. Middle-aged rats do tend to accumulate body and liver lipid, and have an advanced risk of developing metabolic syndrome and cardiovascular disease, and become more susceptible to certain chemical substances (Park et al., 2006; Einstein et al., 2010; MacPhail et al., 2012). Thus, it was of interest to investigate whether extracts from the *Kaempferia parviflora* rhizome had any effect on vascular functions, as well as on lipid accumulation and gross organ abnormality in middle-aged rats. Therefore the first aim of the present study was to investigate the effects of chronic treatment of middle-aged rats with a dichloromethane extract of *Kaempferia parviflora* rhizomes (KPD) on vascular functions. The second aim of the study was to determine whether this chronic treatment with the KPD extract had any effects on the accumulation of lipid, the clinical chemical profiles and observations of abnormal gross organ developments. The following parameters were therefore investigated: (1) a study of the vascular functions of isolated thoracic aortic and mesenteric rings on their constrictor and dilatory responses to phenylephrine and acetylcholine, respectively. In addition, vascular eNOS protein expression was studied by Western blot analysis, (2) animal body weight and food intake, (3) visceral and subcutaneous fat, liver lipid accumulation, (4) gross abnormalities of internal organs, (5) liver and kidney functions, and (6) fasting serum glucose and serum lipid profiles. We would therefore expect that if the KPD extract led to an improvement of vascular functions and/or a lipid lowering activity in middle-aged rats with no signs of toxicity, the KPD extract would be a good choice for further development for use as a health product for preventing or delaying the development of obesity, metabolic syndrome and/or cardiovascular disease.

2. Materials and methods

2.1. Plant material

Fresh Rhizomes of *Kaempferia parviflora* were collected in Ampur Phurua, Loei Province, Thailand in April 2009. Authentication was achieved by comparison with the herbarium specimens in the Department of Biology Herbarium, Faculty of Science, Prince of Songkla University, Thailand, where a voucher specimen (Collecting no. 2548-03) of the plant material used has been deposited.

2.2. Extraction, drug preparation for oral administration and detection of the drug in the blood

Fresh rhizomes of *Kaempferia parviflora* (20 kg) were blended and extracted successively by macerating twice with 95% ethanol (2 × 20 L) for two days to extract most of their dark color, followed by extracting three times with 100% dichloromethane (3 × 20 L). The dichloromethane soluble part was filtered and evaporated under reduced pressure. The dried residue from the dichloromethane extract was further treated by suction under high pressure with an oil vacuum pump in order to remove residual dichloromethane and a yellowish gummy dichloromethane *Kaempferia parviflora* extract (KPD) was obtained with a 2.6% yield.

The KPD was analyzed by high performance liquid chromatography (HPLC) in order to obtain a chemical profile. Analytical HPLC was carried out on a HP1100 system equipped with a photodiode array detector (Agilent Technologies). The extract was analyzed on a Symmetry® C_{18} column (5 μm, 3.9 × 150 mm i.d.; Waters), with a gradient of CH_3OH:H_2O+0.05% of trifluoroacetic acid (10:90→100:0). The flow rate was 1 ml/min; the UV traces of the eluants were measured at 210 and 254 nm and the UV spectra (DAD) were recorded between 200 and 500 nm. The HPLC chromatograms of the KPD and its three major pure compounds: 5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF) and 3,5,7,3′,4′-pentamethoxyflavone (PMF) together with their retention times and corresponding UV spectra are shown in Fig. 1. Quantitative analysis by HPLC chromatography was carried out by measuring the area under the peak of each of the three major compounds, as determined by comparison with the standard curve of known concentrations of the pure compounds and showed 84.88 mg/g for DMF, 68.98 mg/g for TMF, and 70.03 mg/g for PMF, which together amounted to 223.89 mg/g of dried KPD extract.

For oral administration, the KPD was suspended in a mixture of tween 80, 0.2 (g): carboxy-methylcellulose sodium salt, 0.2 (g): distilled water, 10 ml, at a concentration of 100 mg/ml. The control rat was orally administered with this vehicle using the same volume as that for the KPD treated rat.

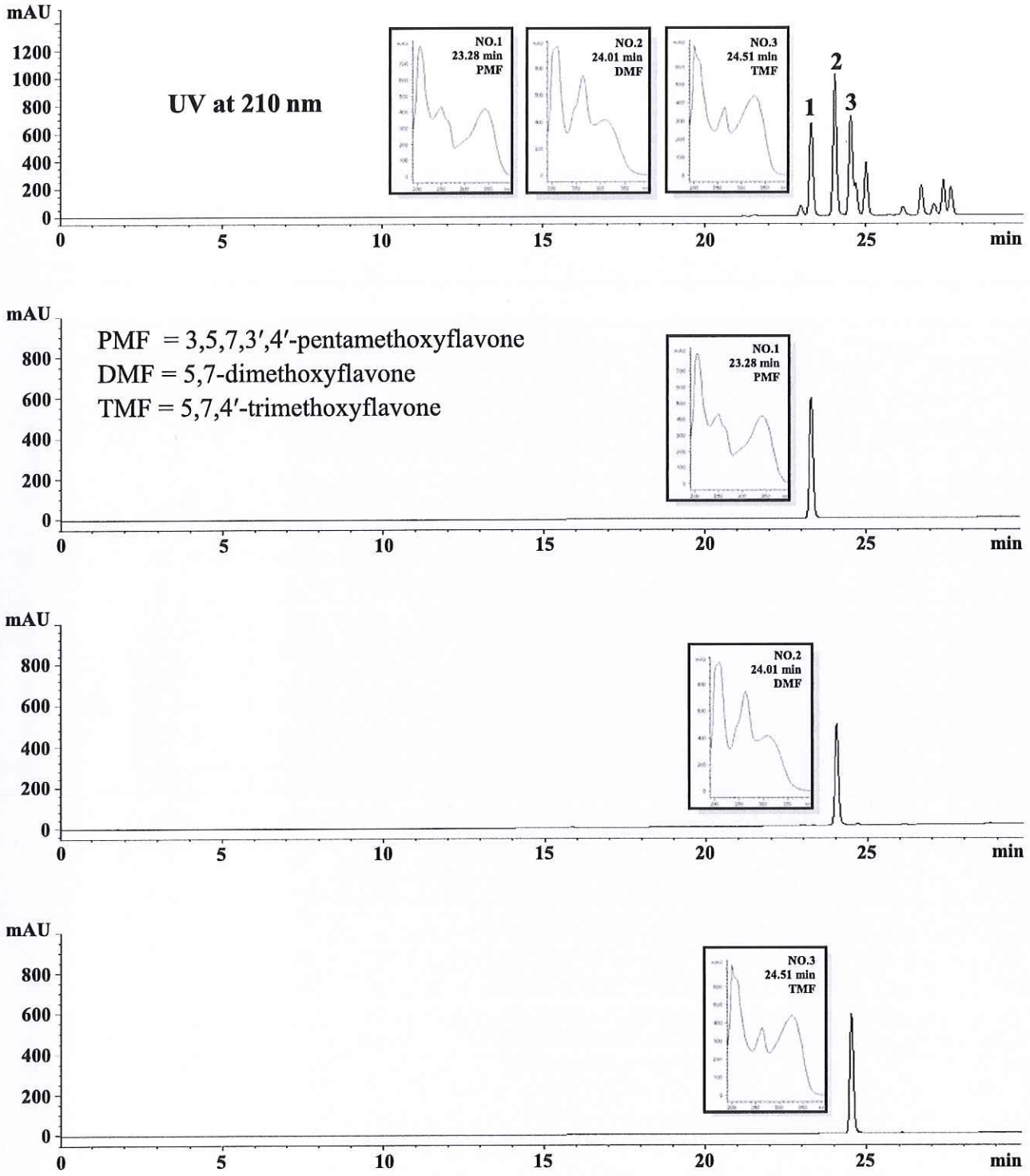

Fig. 1. HPLC chromatograms of the *Kaempferia parviflora* dichloromethane extract, and their major methoxyflavones: 5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF) and 3,5,7,3′,4′-pentamethoxyflavone (PMF). The column eluant from each extract was scanned at the wavelength 210 nm.

In order to demonstrate that the KPD suspension was being absorbed into the blood stream after oral administration, the KPD constituents in the blood were analyzed by HPLC chromatography after administration of the KPD. To do this, 6 middle-aged male rats were orally administered with 100 mg/kg of KPD, 1.0 ml of venous blood was withdrawn from each animal at the jugular vein after the animals were anesthetized (diethyl ether inhalation) after 0, 30, 60, 90 min from the first three rats, and at 120, 150 and 180 min from the second three rats, and each placed into a heparinized tube. The collected blood was centrifuged at 4000 rpm for 10 min, and the plasma was collected and proteins were precipitated using 100% methanol and re-centrifuged. The methanol soluble part was collected, evaporated to remove methanol, and then partition extracted with 100% dichloromethane twice. The dichloromethane soluble part was collected, evaporated to dryness and dissolved in 60 μl of 100% methanol

(HPLC grade) before injecting (30 μl) into the analytical HPLC by the method described above. The HPLC chromatograms, the retention times of the compounds and the UV spectrum of the three major methoxyflavones are shown in Fig. 2. The three methoxyflavones were detected in the blood stream after about 60 min, and declined after 120 min, and none were detected 180 min after oral administration of the KPD extract, except for the peak 1 that was for an unknown substance that was similar to that found at the 30 min time after oral administration of the KPD.

2.3. Pharmacological studies

Middle-aged (12–14 month old) and young adult (10 week old, positive control for vascular endothelium function test) Wistar male rats were supplied from the Animal House, Faculty of Science, Prince of Songkla University. The animals were housed in controlled environmental conditions at 25 °C on a 12 h dark and 12 h light cycle and allowed access to standard food and tap water *ad libitum*. The animal methods employed in this study were approved by the Prince of Songkla University Animal Care and Use Committee. The investigation conformed to the Guide for the Care and Use of Laboratory Animals (Ethic no. 22/52). The rats were acclimatized at their new environment at the drug treatment room at the Southern Laboratory Animal Facility for 1–2 weeks. The rats were then randomly divided into 6 groups, each with 6–8 animals. There were two experimental groups, the first one was treated by oral administration with 100 mg/kg KPD extract once (9.00 AM) a day, and the other one was treated twice (9.00 AM and 6.00 PM) a day, for 6 weeks. Two vehicle control groups received the vehicle and two positive control groups received distilled water once or twice a day using the same volume as that for the KPD extract (1 ml/kg animal body weight) in the same period of 6 weeks. Each rat had its body weight recorded and its 24 h food intake at day 0 (one day before receiving oral administration of the vehicle or KPD suspension), and then every consecutive 7 days over a 6 week period.

2.3.1. Effects of chronic KPD treatment on the hematology and clinical biochemical analysis

At the end of the 6 weeks oral administration with the KPD extracts, vehicle or distilled water, the rats (13–15 h fasting) were killed by decapitation with a guillotine, and at the same time 5 ml and 2 ml of blood samples were separately immediately collected from the decapitated rat into a glass test tube and a plastic test tube containing EDTA, respectively. The tube with the 5 ml blood was left in room temperature for 30 min and then centrifuged at 1200 rpm for 10 min, and the serum was then collected and kept in −70 °C until measurements were made for the kidney and liver enzymes, for glucose and lipid levels by enzymatic methods using the Automatic Chemistry Analyzer (Hitachi Modular P800, Germany) routinely operated at the Prince of Songkla University Hospital, which were done within 1 month of sample collection. The EDTA blood was sent to the hematology laboratory for a total blood count procedure measured by the Automated Hematology Analyzer (Celltac E, Model MEK-7222K, Japan).

2.3.2. Effects of chronic KPD treatment on internal organs and lipid accumulation

The decapitated rat (after removal of the thoracic aorta and the mesentery) was dissected to collect internal organs: heart, lung, liver, adrenal gland, kidney, and testes. Each organ was cleared of adipose tissue, any excess blood was blotted away with filter paper and the organ weighed. Visceral fats from the epididymis, testis and retroperitoneal, as well as subcutaneous fats were removed and weighed using the Mettler PL2001-L balance (Mettler Toledo International Inc., Switzerland).

The middle lobe of the liver was cut into 2 pieces, embedded into a cryostat gel for cryostat sectioning (20 μm thick) and stained with oil red O (0.5% in absolute propylene glycol), then mounted with glycerin jelly for observations with a light microscope. The oil red O of each slide was extracted with 1 ml of 100% DMSO and the oil red O solution was read spectrophotometrically (Thermo Fisher Scientific, Model G10s UV–vis, USA) at 520 nm, and the concentration of the oil red O was read from the standard curve of known concentrations of the oil red O in 100% DMSO (μg/ml). The area of a whole thin section of liver tissue from each slide was measured using the Auto CAD 2005 program, a duplicate slide was done for each section of the liver obtained from each rat. The amount of the liver lipid accumulation was expressed in terms of μg/ml/cm^2 of the liver tissue thin section area.

2.3.3. Preparation of the thoracic aortic rings and mesenteric artery

The thoracic aorta was removed from the decapitated rat and carefully cleaned of adhering fat and connective tissue. Five adjacent rings, 4–5 mm in length were cut. In one ring the endothelium layer was removed mechanically by gently rubbing the intimal surface with a stainless steel rod, using the method of Jansakul et al. (1989). The aortic rings with or without a functional endothelium were mounted horizontally between two parallel stainless steel hooks taking extremely care not to damage the endothelium of the endothelium-intact aortic rings, and suspended in a 20-ml organ bath containing Krebs Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4 \cdot 7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at ,37 °C and continuously bubbled with a 95% O_2 and 5% CO_2 gas mixture. One of the hooks was fixed at the bottom and the other was connected to a force displacement transducer that was connected to a Grass polygraph for recording the changes in the isometric tension. Prior to addition of the drugs, the tissues were equilibrated for 60 min under a resting tension of 1 g and the bath solution was replaced with pre-warmed oxygenated Krebs Henseleit solution every 15 min. After equilibration, the presence of a functional endothelium of the thoracic aortic rings was assessed in all preparations as follows: the thoracic aortic ring was precontracted with 3 μM phenylephrine until the response had reached a plateau (5–8 min), and the dilatory response to 30 μM acetylcholine was then recorded. The experiment was then continued only when there was at least a 65% vasodilatation to acetylcholine by the endothelium-intact thoracic aortic rings, or no vasodilatation in response to acetylcholine for the denuded-thoracic aortic rings. The preparations were then washed several times with Krebs Henseleit solution, and allowed to fully relax for 45 min before the experimental protocol began.

The mesenteric artery was removed from the abdominal cavity and placed in a petri dish containing warm oxygenated Krebs Henseleit solution. The adipose tissue of the third order branch of the mesenteric artery (diameter 200–250 μm) was viewed by a binocular microscope and removed. Segments of the clean mesenteric artery each of about 2 mm long were cut and mounted on two 40 μm stainless wires in the jaws of a10-ml-Dual-Wire Myograph chamber (model 400 A, Danish Myo Technology A/S, Denmark) in Krebs Henseleit solution (pH 7.4) that was being bubbled with carbogen and warmed at 37 °C. The mesenteric artery was equilibrated for 60 min, and then normalization of the blood vessel was performed using a LabChart: a DMT Normalization Module in order to determine the internal circumference for setting the pretension of the vessels rings from a relaxed status to the target transmural pressure of 100 mmHg (Mulvany and

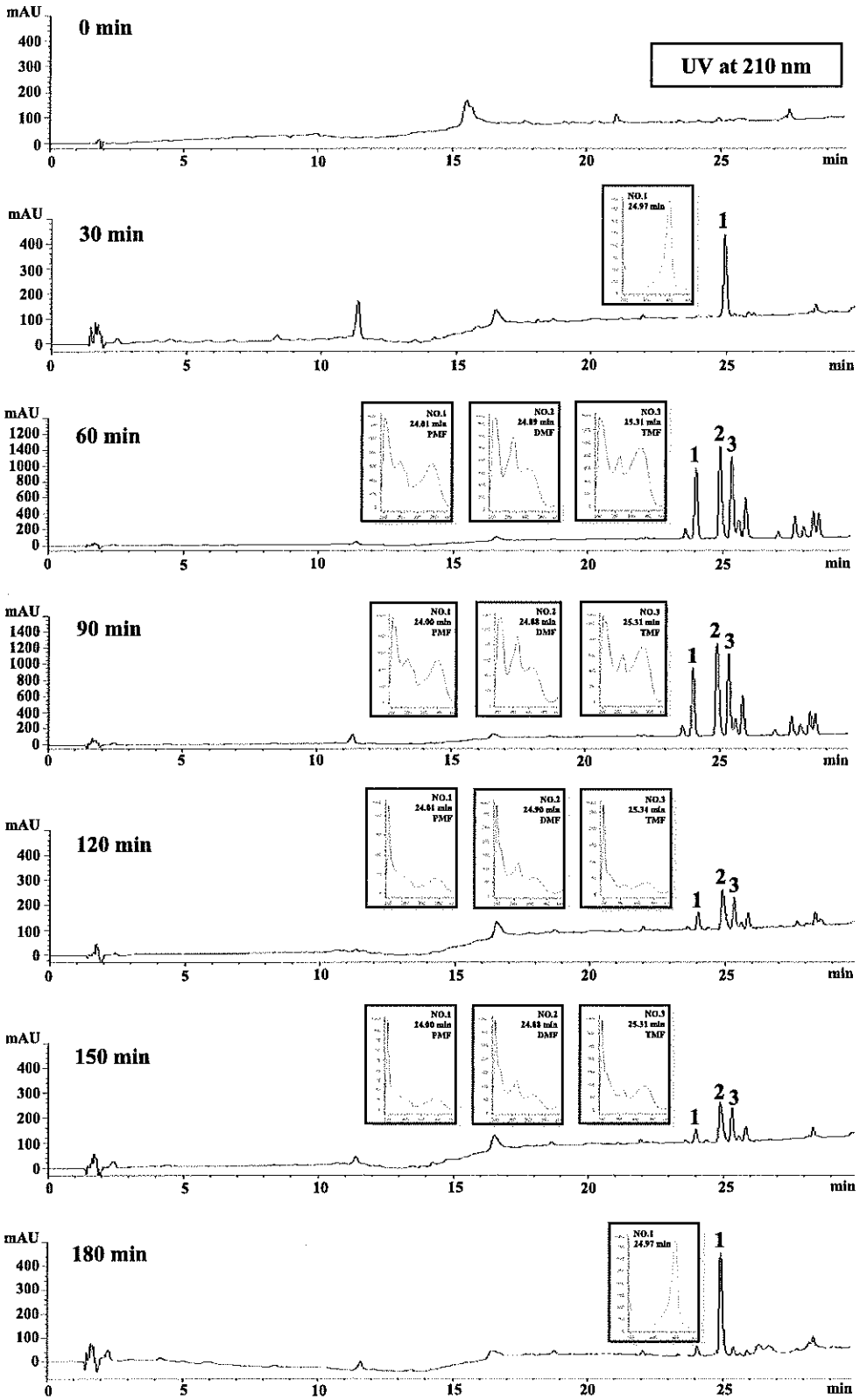

Fig. 2. HPLC chromatogram for detecting methoxyflavones: 5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF) and 3,5,7,3′,4′-pentamethoxyflavone (PMF) in blood samples from middle-aged male rats after oral administration with a dichloromethane extract from *Kaempferia parviflora* after 0, 30, 60, 90, 120, 150, and 180 min. The column eluant from each extract was scanned at the wavelength 210 nm.

Halpern, 1977). The vessel rings were re-equilibrated for 30 min, then the contractile function of each vessel rings was tested with 10 μM norepinephrine for 10–15 min (plateau), followed by washing several time with Krebs Henseleit solution and equilibration for another 20 min. This procedure was repeated three times. The function of the intact endothelium was determined by precontracting the mesenteric artery with 10 μM noradrenaline (NA) until the response had reached a plateau (10–15 min), and the dilatory response to acetylcholine (10 μM) was then recorded. The preparations were then washed several times with Krebs Henseleit solution, and allowed to fully relax for 45 min before the experimental protocol began.

2.3.4. Effects of the KPD extract on the vascular reactivity to phenylephrine, acetylcholine and glyceryl trinitrate

2.3.4.1. Effect on the thoracic aorta. Two endothelium-intact thoracic aortic rings: one in Kreb's solution alone and the other that had been preincubated with 3 mM N^G-nitro-L-arginine (LNA, a nitric oxide synthase inhibitor) for 40 min were prepared. Also rings with their endothelium removed were all equilibrated under a basal tension of 3 g for 10 min. A contractile response to a cumulative concentration of phenylephrine was then carried out.

A different set of endothelium-intact thoracic aortic rings under a basal tension of 1 g for both groups at the same time as rings from a young positive control group were precontracted with phenylephrine (3 μM) for 10–15 min (plateau) and a cumulative concentration C-R curve to acetylcholine was commenced.

Using the same protocol as above, another set of endothelium-intact thoracic aortic rings of both groups were incubated with 3 mM LNA for 40 min, and then precontracted with phenylephrine (0.3 μM) for 10–15 min (plateau) followed by a cumulative dilatory C-R curve to glyceryl trinitrate, (an endothelium independent vasodilatory substance).

2.3.4.2. Effect on the mesenteric artery. After equilibration, a cumulative contractile response to phenylephrine was performed, followed by several washings with Krebs Henseleit solution and equilibration of the mesenteric artery for another 40 min with washing every 10–15 min. The mesenteric artery was precontracted with phenylephrine (10 μM) for 10 min (plateau), a cumulative dilator C-R curve to acetylcholine was performed, followed by several washing with Krebs Henseleit solution and equilibration of the mesenteric artery for 40 min. The mesenteric artery was then incubated with LNA (3 mM) for 60 min. The second C-R curve to phenylephrine was performed in the presence of the LNA, followed by several washing and re-equilibration in the presence of LNA for another 60 min. The mesenteric ring was precontracted with 10 μM phenylephrine in the presence of LNA for 10 min (plateau), and then a cumulative dilatory C-R curve to glyceryl trinitrate was performed.

2.3.4.3. eNOS Western blot analysis

The thoracic aortae and mesenteric arteries of KPD, treated with the vehicle as control for middle-aged and from young adult (10 weeks old, positive control) male rats were dissected from decapitated rats and the surrounding adipose tissue was removed, then kept at −70 °C until used. On the day of the experiment, each blood vessel from each animal was chopped on ice and homogenized with lysis buffer [25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 0.5 mM EDTA and a protease inhibitor cocktail (GE Healthcare)]. The total protein content of the extracted sample was measured using the Biorad protein assay kit. 50 μg of each sample was run separately on 12% polyacrylamide-SDS gels and then proteins were transferred to a nitrocellulose membrane in glycine–methanol buffer at 4 °C and 100 V for 120 min. The membranes were blocked by shaking in 5% nonfat dry milk in TBS-T (Tris buffer saline −0.1% Tween 20) for 60 min at room temperature. The membranes were incubated overnight, while being gently shaken, with primary antibodies against eNOS (1:250) and actin (1:1000) as an internal control in 5% nonfat dry milk in TBS-T, all antibodies were from Cell Signaling. The membranes were washed 3 times (5 min/wash) and then incubated with ECL anti-rabbit IgG-horseradish peroxidase (GE Healthcare) diluted to 1:1000 (eNOS) and 1:5000 (actin) in 1% nonfat dry milk in TBS-T for 60 min at room temperature and washed 3 times (5 min/wash). The proteins were visualized using an ECL chemiluminescent detection kit (Pierce) and exposed to a film. Bands were quantified densitometrically using the Gel Documentation Bio-Rad (Image lab 3). Four replicates of each tissue obtained from different animals of each group were studied. Results were normalized to the β-actin expression and expressed as units relative to the β-actin.

2.4. Effect on blood pressure

At the end of 6 weeks oral administration (twice a day) of the KPD or vehicle control to middle-aged male rats, each rat was anesthetized by intra-peritoneal injection of pentobarbital sodium (60 mg/kg, i.p.). The tracheal tube was cannulated with a polyethylene tube to maintain airway potency and the animals breathed room air spontaneously. A polyethylene catheter was inserted through the right common carotid artery which was connected to a pressure transducer (P23 ID, Gould Statham Instrument, Hato Rey, Puerto Rico) and connected to a Grass polygraph (model 7D, Grass Instrument, Quincy, MA, U.S.A.) to monitor the systemic blood pressure. The animal was equilibrated for 60 min. The basal systolic- and diastolic blood pressure was measured.

2.5. Drugs

The following drugs used: acetylcholine chloride, N^G-nitro-L-arginine (LNA), norepinephrine, phenylephrine hydrochloride, pentobarbital sodium, and the DMSO and oil red O were from Sigma, U.S.A. Glyceryl trinitrate was from Mycomed, Denmark. 5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF) and 3,5,7,3′,4′-pentamethoxyflavone (PMF) were a gift from Prof. Chawee Yenjai, Khonken University, Thailand. Acetylcholine chloride, norepinephrine and phenylephrine were dissolved in a solution containing NaCl 9 g/L, NaH_2P0_4 0.19 g/L and ascorbic acid 0.03 g/L, and glyceryl trinitrate was dissolved in distilled water.

2.6. Statistical analysis

Organ and adipose tissue weights are expressed in 100 g of animal body weight. All data are expressed as mean value ± S.E.M. (n=6–8). "n" represents the number of organs, tissues or blood vessels, each one from a different animal. Absolute tension developed in g was measured for the thoracic aortic ring contractile response, and as the percentage contraction to norepinephrine (10 μM) for the mesenteric ring. Vasorelaxation was expressed as the percentage relaxation from phenylephrine precontraction levels. Comparisons were made of both the sensitivity and the maximal responsiveness among the groups. The drug concentration that produced 50% of the maximal response for the drug (EC_{50}) was derived from regression analysis over the linear portion of the concentration-response curve. Statistical differences between the two measurements were determined by the two-tailed unpaired Student's t-test and among groups were determined by one way ANOVA and post-hoc analysis was performed with the Duncan test. A P value < 0.05 was considered to be significantly different in all experiments.

3. Results

3.1. Effects of chronic KPD treatment on body weight, food intake, blood chemistry, organ and adipose tissue weight

There were no differences found for any of the parameters between the vehicle control and the positive control group that had been given distilled water. Thus, the data from the vehicle control group were chosen to represent the control experiment. None of the parameters studied were found to be altered after 6 weeks of treatment by the KPD (100 mg/kg) once a day. When the KPD was given twice a day, however, a number of parameters were altered. The animal body weight tended to decrease from the third week of the KPD treatment and was statistically significantly different from the 5th week onwards, whereas no change in food intake was found (Fig. 1, Supplementary material). There were no differences in the internal organ weights: adrenal glands, kidneys, liver, lung, spleen, testes, ventricle and atrium, after 6 weeks of treatment with the KPD extract twice a day compared to the vehicle control group (Table 1, Supplementary material). Relative adipose tissue weights of the epididymis, retroperitoneal and subcutaneous tissues were lower in the KPD treated animals compared to the vehicle control group (Table 1). The fasting serum levels for glucose and triglycerides, as well as the liver lipid accumulation of the KPD treated rats were significantly lower than those of the vehicle control group (Table 2 and Fig. 3). However the levels of cholesterol, HDL-C and LDL-C were not different from the control group. Neither were there any differences in serum levels of the alkaline phosphatase, SGOT, SGPT, BUN and creatinine, nor were the determined hematological values between the KPD treated group and that of the vehicle control group (Tables 2 and 3, Supplementary material).

3.2. Vasorelaxation of the blood vessel to acetylcholine of middle-aged and young male rats

For thoracic aortic rings obtained from middle-aged male rats precontracted with phenylephrine the maximal relaxation to acetylcholine was about 1.6 fold lower and the EC_{50} value was about a 3.5 fold higher compared to those obtained from the young male rats. In the case of the mesenteric artery, the vasorelaxation to the acetylcholine of the mesenteric rings precontracted with phenylephrine of the middle-aged rats was also found to have a higher EC_{50} value (about 4.3 fold) compared to those obtained from young male rats but the maximal relaxation was similar (Fig. 4, Table 4).

Table 1
Effects of chronic oral administration of a *Kaempferia parviflora* dichloromethane extract (KPD), distilled water (DW) or vehicle (Veh), once (od) or twice (bid) a day, on the relative visceral and subcutaneous adipose tissue weight (g/100 g body weight) of middle-aged male rats.

Treatments	n	Adipose tissue weight (% g body weight)			
		Epididymis	Mesentery	Retroperitoneal	Subcutaneous
DW	8	2.30 ± 0.16	2.08 ± 0.14	2.31 ± 0.21	6.52 ± 0.37
Veh-od	8	2.31 ± 0.16	2.14 ± 0.12	2.37 ± 0.24	6.09 ± 0.38
KPD-od	8	2.27 ± 0.09	1.85 ± 0.09	2.07 ± 0.1	5.38 ± 0.30
Veh-bid	8	2.61 ± 0.22	2.07 ± 0.18	2.45 ± 0.31	6.91 ± 0.34
KPD-bid	8	1.82 ± 0.11[a]	1.70 ± 0.06	1.54 ± 0.12[a]	5.29 ± 0.30[a]

Values are expressed as a mean ± S.E.M.
[a] Significantly lower than their vehicle control groups, $P < 0.05$.

3.3. Effects of KPD treatment on the vascular response to phenylephrine, acetylcholine and glyceryl trinitrate

3.3.1. Effect on the thoracic aorta

The maximal contractile response to phenylephrine on the endothelium-intact thoracic aortic ring obtained from a KPD treated rat was significantly lower than that of the vehicle control group, but the EC_{50} values were similar (Fig. 5A, Table 3). However, this effect disappeared in the presence of LNA or after removal of the vascular endothelium, although the EC_{50} values were decreased by about 5 fold and increased by about 2 fold in the maximal contractile responses to all groups (Fig. 5B and C).

Vasorelaxation due to acetylcholine of the endothelium-intact thoracic aortic ring precontracted with phenylephrine of the KPD treated rats was significantly higher in its maximal response, but not in the EC_{50} values, compared to that of the vehicle control group (Fig. 6A). For the endothelium-intact thoracic aortic rings with LNA precontracted with phenylephrine, the vasorelaxation to glyceryl trinitrate were not significantly different either in the EC_{50} values or the maximal relaxation between the ones obtained from the KPD treated rats and the vehicle control groups (Fig. 6B, Table 4).

3.3.2. Effect on the mesenteric artery

The contractile response of the KPD treated-mesenteric artery to phenylephrine was higher in EC_{50} values, but not in the maximal responses, when compared to that of the vehicle control groups. LNA caused a significant shift of the phenylephrine C-R curve to the left with a decrease in the EC_{50} of both groups to the same extent. However, in the presence of LNA, the maximal contractile response to phenylephrine of the KPD group was significantly higher than that of the vehicle control group (Fig. 7, Table 5).

Acetylcholine induced vasorelaxation of the mesenteric ring from the KPD rats that had been precontracted with phenylephrine was significantly higher in its sensitivity, but not in its EC_{50} values or maximal relaxation, when compared to that of the vehicle control groups (Fig. 8A). No differences were observed in the EC_{50} values and the maximal responsiveness for the vasorelaxation obtained by glyceryl trinitrate on the mesenteric rings precontracted with phenylephrine, in the presence of LNA, between that of the KPD treated rats and the vehicle control groups (Fig. 8B, Table 4).

3.3.3. eNOS Western blot analysis

The quantitative expression of the eNOS protein in the isolated thoracic aorta and the mesenteric artery was significantly higher for those obtained from the young adult male rat and the KPD treated rat compared to that of the vehicle control groups (Fig. 9).

3.4. Effect of chronic KPD treatment (twice a day) on blood pressure

There were no differences in the basal systolic and diastolic blood pressure of the anesthetized rats between the KPD extract treated rats (systolic: 145.50 ± 4.88 mmHg; diastolic: 115.30 ± 4.26 mmHg) and the vehicle control group (systolic: 145.12 ± 3.68 mmHg; diastolic: 115.41 ± 3.18 mmHg).

4. Discussion

Chivapat et al. (2010) and Mekjaruskul et al. (2012) reported that the major components of the *Kaempferia parviflora* rhizomes and its ethanolic extract were 5,7-dimethoxyflavone (DMF), 5,7,4′-trimethoxyflavone (TMF) and 3,5,7,3′,4′-pentamethoxyflavone

Table 2
Effects of chronic oral administration of a *Kaempferia parviflora* dichloromethane extract (KPD), distilled water (DW) or vehicle (Veh), once (od) or twice (bid) a day, on the fasting serum glucose and lipids levels of middle-aged male rats.

NLAC-MU (2014)	mg %	Glucose	Triglyceride	Cholesterol	HDL-C	LDL-C	LDL/HDL ratio
Normal range	*n*	122.10–180.80	61.00–164.00	46.00–98.00	–	–	–
DW	6	132.96 ± 5.69	80.20 ± 7.34	73.38 ± 1.65	58.22 ± 1.14	12.05 ± 0.63	0.21 ± 0.02
Veh-od	6	139.00 ± 7.94	72.00 ± 8.00	72.00 ± 0.82	50.55 ± 0.87	10.52 ± 0.96	0.21 ± 0.01
KPD-od	6	134.33 ± 5.48	77.17 ± 8.01	71.33 ± 3.06	56.21 ± 5.58	10.25 ± 1.00	0.21 ± 0.02
Veh-bid	6	128.00 ± 2.24	85.00 ± 5.27	71.00 ± 3.51	58.18 ± 2.74	13.14 ± 0.89	0.23 ± 0.02
KPD-bid	6	117.40 ± 3.14[a]	64.20 ± 5.62[a]	77.80 ± 4.45	63.80 ± 3.12	10.42 ± 0.78	0.16 ± 0.02[a]

Values are expressed as a mean ± S.E.M.
Note: NLAC-MU = National Laboratory Animal Centre, Mahidol University; HDL-C = High Density Lipoprotein cholesterol; LDL-C = Low Density Lipoprotein cholesterol.
[a] Significantly lower than the vehicle control group, $P < 0.05$.

(PMF). The initial extracts were very dark in color so fresh rhizomes of the *Kaempferia parviflora* were macerated and pre-extracted with 95% ethanol twice before being macerated and extracted with 100% dichloromethane to produce a clearer extract with a higher content of these three major methoxyflavones, (it is possible that some of the methoxyflavones might also be removed). As shown in Fig. 1, the major components from the HPLC chromatogram of the KPD extract were these three methoxyflavones that amounted to about 22% of the dried KPD extract.

The present study demonstrated that the KPD suspension used could be absorbed via the gastrointestinal tract into the blood stream (Fig. 2), as the three major methoxyflavones appeared in the blood stream about 60 min after oral administration. However, the three methoxyflavones might have undergone some metabolic changes after absorption (an unknown peak 1 appeared at 30 min after administration), and none of them were detected 180 min after administration except for the unknown peak 1 (Fig. 2). This is consistent with the report of Mekjaruskul et al. (2012). This might explain why, when the 100 mg/kg KPD was given orally to the animal once, but not twice, a day, none of parameters studied changed even though some methoxyflavones did appear in the blood. Since none of the parameters tested were affected by 100 mg/kg KPD treatment once a day, the remaining discussion will be concerned only with the results obtained from animals that were treated with KPD twice a day.

The KPD extracts contained the three major methoxyflavones (DMF, TMF, and PMF) as did the dried rhizomes or ethanolic extracts as reported by Mekjaruskul et al. (2012). There have been some reports on the direct effects of an ethanolic extract or the methoxyflavone pure compounds on the vascular responsiveness: an ethanolic extract of the rhizomes caused a relaxation of the isolated thoracic aortic rings precontracted with phenylephrine (Wattanapitayakul et al., 2008) and DMF caused an endothelium-dependent relaxation that was partly mediated by the NO-cGMP pathways (Tep-Areenan et al., 2010). However, there have been no reports on the effects of the chronic oral administration of the *Kaempferia parviflora* extract on vascular functions especially in the middle-aged animals, the time of risk for developing vascular dysfunction (Moritoki et al., 1986; Hongo et al., 1988; Laurant et al., 2004; El Assar et al., 2012; Gong et al., 2014). We did experiments using oral administration of the KPD to middle-aged male rats everyday for 6 weeks before isolating the animal blood vessels to study their functions in the *in vitro* preparations. Blood vessels of the middle-aged (12–14 month old) rats in the present study would be expected to have impairment of the vascular endothelium, as suggested by the finding of less vasodilator responsiveness to acetylcholine as well as a decrease of eNOS expression (Shimizu and Toda, 1986; Koga et al., 1989; Matz et al., 2000; Tanabe et al., 2003).

Although there were no differences in the basal blood pressure between the KPD treated- and the vehicle control of anesthetized middle-aged male rats, the KPD treatment caused a decrease in the maximal contractile response to phenylephrine of the endothelium-intact thoracic aortic rings, and this effect disappeared in the presence of LNA or after removal of the vascular endothelium. In addition, vasorelaxation of the thoracic aortic ring precontracted with phenylephrine was higher for the one obtained from the KPD treated compared to that of the vehicle control rat. A similar result was found for the mesenteric artery, a resistance vessel, with a lower sensitivity to the contractile response to phenylephrine, and a higher sensitivity for its vasorelaxation to acetylcholine for the KPD treated artery than that of the vehicle control rat. These results clearly showed that the KPD treatment caused an increase in nitric oxide production from the vascular endothelium to attenuate the vasocontraction caused by phenylephrine and potentiated the vasorelaxation to acetylcholine. This effect was confirmed by the finding that expression of the eNOS enzyme, the endothelial nitric oxide synthase, of the thoracic aorta and mesenteric artery were higher for the one obtained from the KPD treated rats than from the vehicle control group.

The finding that there were no differences in vasorelaxation to glyceryl trinitrate of the thoracic aortic rings of both KPD and vehicle control groups, indicating that the KPD treatment did not affect a nitric oxide independent pathway.

No signs of toxicity was observed in the middle-aged rats after having been chronically oral administration with the KPD extracts at 100 mg/kg twice a day for 6 weeks as there were no differences in the organ weights or abnormal gross observation of the internal organs: heart, testis, epididymis, prostate glands, adrenal glands, liver and kidney between the KPD extracts treated and those of the vehicle control groups. The finding that the serum liver and kidney enzyme levels (ALP, SGOT, SGPT, BUN and creatinine) were in the normal range and were not different from the vehicle control, confirmed that chronic KPD treatment in the present study did not affect the liver and kidney functions. These results are similar to those reported by Chivapat et al. (2010) who found that an ethanolic extract of *Kaempferia parviflora* rhizomes did not significant change the plasma liver and kidney enzymes after 90 days oral adminstration of their extract.

KPD did exert an anti-obesity property in middle-aged rat, as it was found that after 6 weeks treatment of the KPD there was a significant decrease in the fasting serum triglyceride levels together with a decrease in body weight and a lowering of the visceral and subcutaneous adipose tissues, as well as on liver lipid accumulation compared to that of the vehicle control group. The anti-obesity activity of the KPD was similar to those in the report by Akase et al. (2011) and Shimada et al. (2011) who found that a *Kaempferia parviflora* rhizome powder and an ethyl acetate extract from the plant rhizome, that also contained these three major

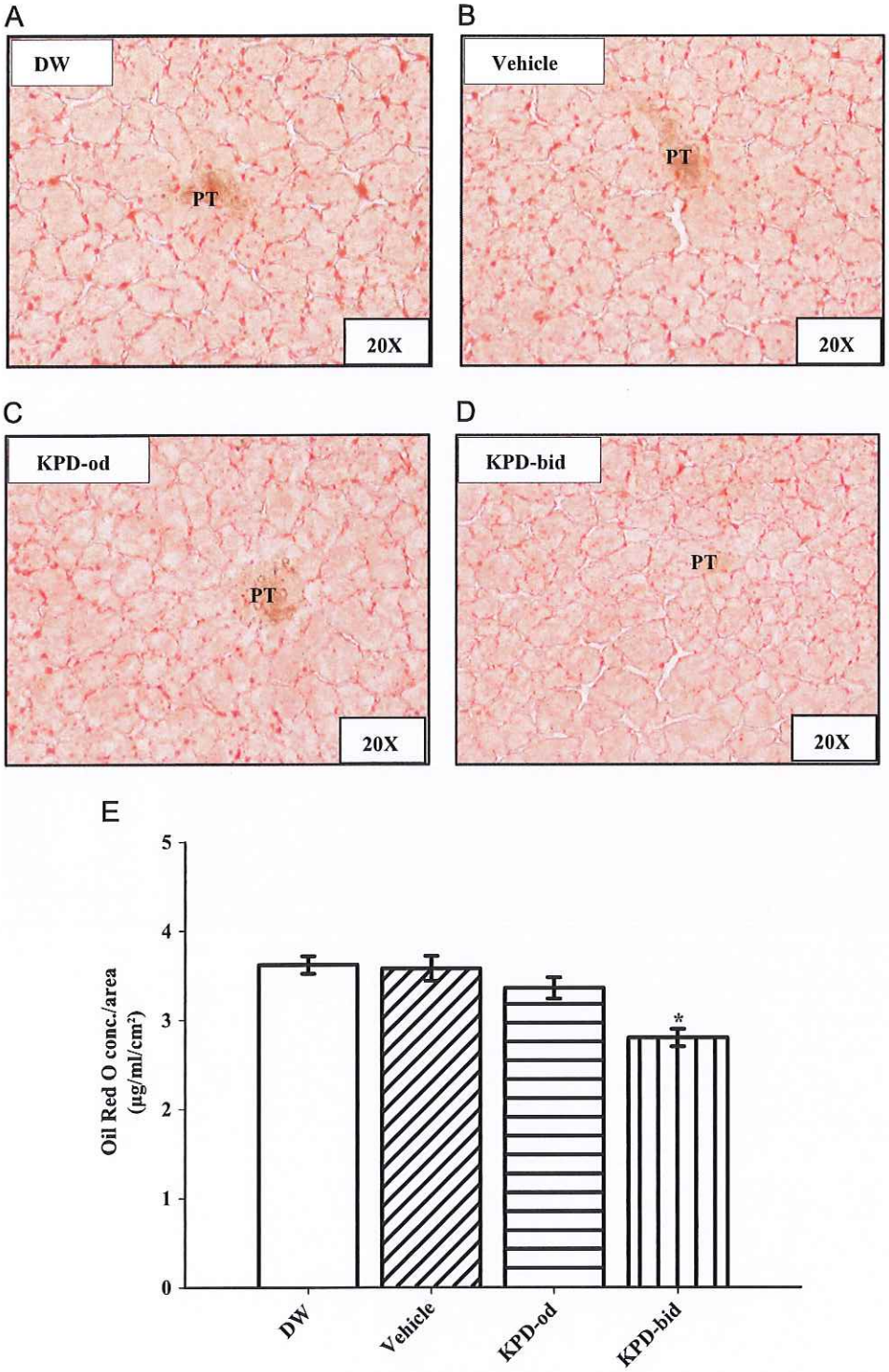

Fig. 3. Effect of 6 weeks oral administration of a *Kaempferia parviflora* dichloromethane extract on liver lipid accumulation. (A) distilled water (DW), (B) vehicle control, (C) once a day (KPD-od), (D) twice a day (KPD-bid), and (E) oil red O concentration. Values represent a mean ± S.E.M. of 6 animals. *Significantly lower than the control group, $P < 0.05$. (PT=Portal triad; oil red O staining of liver tissue frozen section, 20 μm thick, 20 × magnification).

methoxyflavones, had an anti-obesity activity in spontaneously obese type II diabetic mice after 8 weeks of treatment. The authors claimed that the methoxyflavones might be responsible for this activity, although the methoxyflavones have a low potency (290–500 μM/ml) on the inhibitory effect on pancreatic lipase, a key enzyme in the absorption of dietary triglycerides (Shimada et al., 2011). On the other hand, Horikawa et al. (2012) found that polymethoxyflavones from the *Kaempferia parviflora* produce a strong induced differentiation of 3T3-L1 preadipocytes to adipocytes. Thus, further studies of the crude extract as well as each pure compound of the three methoxyflavone would be needed to clarify whether its anti-obesity property is a direct or an indirect effect on animal body fat metabolism. In the present study we also found that the KPD also caused a decrease in the fasting serum

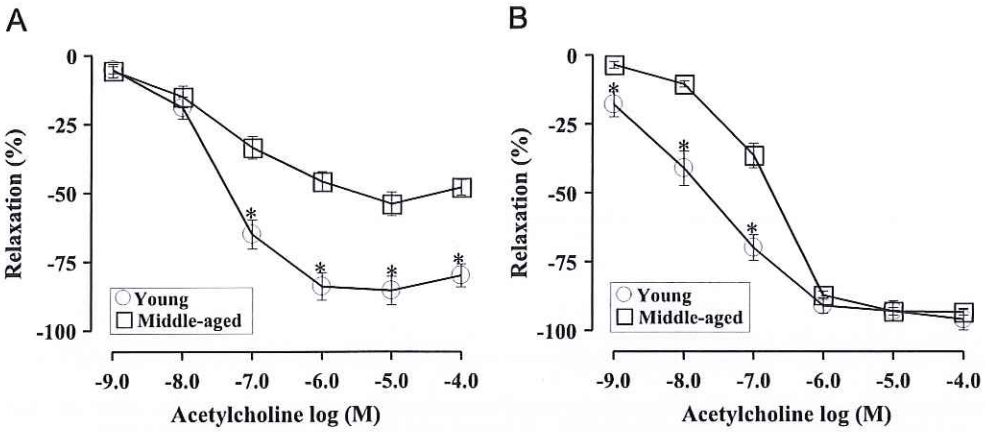

Fig. 4. Vasorelaxation to acetylcholine by the thoracic aortic ring (A) or mesenteric ring (B) precontracted with phenylephrine by rings obtained from young and middle-aged male rats. Values represent a mean ± S.E.M. of 6 animals. *Significantly higher than that of the middle-aged group, $P < 0.05$.

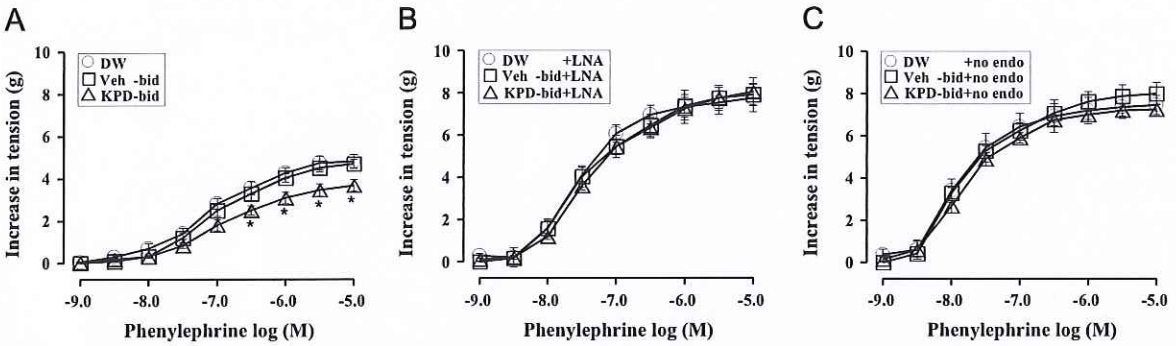

Fig. 5. Effect of 6 weeks oral administration of a *Kaempferia parviflora* dichloromethane extract twice a day (KPD-bid), distilled water (DW) or vehicle control (Veh-bid) on the contractile response of endothelium-intact (A), endothelium-intact with N^G-nitro-L-arginine (LNA, B), and endothelium-denuded thoracic (No endo, C) aortic ring to phenylephrine. Values represent a mean ± S.E.M. of 6 animals. *Significantly lower than that of the distilled water and vehicle control group, $P < 0.05$.

Table 3
EC_{50} and E_{max} values of contractile responses to phenylephrine of the thoracic aortic rings obtained from middle-aged male rats that had been oral administration for 6 weeks with distilled water (DW), vehicle control (Veh-bid) or a *Kaempferia parviflora* dichloromethane extract (KPD-bid) twice a day.

Treatments	EC_{50} (nM):95% confidential limit	E_{max} (g)
With endothelium		
DW	85.6(68.4–126.2)	4.8 ± 0.3
Veh-bid	83.4(62.7–124.1)	4.7 ± 0.2
KPD-bid	142.4(104.7–194.1)	3.5 ± 0.2[a]
With endothelium + LNA		
DW	19.9(17.9–25.8)[b]	7.8 ± 0.5[c]
Veh-bid	20.4(18.7–26.6)[b]	7.9 ± 0.2[c]
KPD-bid	22.3(19.4–29.1)[b]	8.1 ± 0.4[c]
Without endothelium		
DW	18.8(15.9–25.5)[b]	7.9 ± 0.5[c]
Veh-bid	18.2(15.4–24.9)[b]	7.9 ± 0.3[c]
KPD-bid	19.4(16.3–22.8)[b]	7.4 ± 0.3[c]

Values were obtained from 6 experiments ($n = 6$) for each group.
[a] Significantly lower than the control groups.
[b] Significantly lower than the ones with endothelium.
[c] Significantly higher than the ones with endothelium, $P < 0.05$.

glucose of normal middle-aged male rats. Thus, it is possible that the active component of the KPD might cause alterations in glucose metabolism: *e.g.* a reduction in glucose absorption, an increase in tissue glucose expenditure, *etc.* that caused hypoglycemia, and in this situation it would stimulate the utilization of the liver and adipose tissue fatty acids (Cahill, 2006; Ensling et al., 2011). However, a further study would need to clarify these possibilities. On the other hand, in a report by Chivapat et al. (2010) in a study using an ethanolic extract of the *Kaempferia parviflora* rhizomes, it was found that at the dosage of 500 mg/kg there was a decrease in body weight and plasma triglycerides, but the plasma glucose level increased, this was after 3 months of chronic oral administration in young male rats compared to their vehicle control group. This discrepancy might be due to differences in the chemical composition of the *Kaempferia parviflora* extract. In the present study we used a dichloromethane extract that was prepared after most of the dark substances had been removed by pre-extraction with ethanol, and the three major methoxyflavones were about 223.89 mg/g of the extract dry weight, whereas the ethanolic extract prepared by Chivapat contained only 24.138 mg/g of the three major methoxyflavones. The dosage of 500 mg/kg would have about 12.069 mg of the three major methoxyflavones, which is about 3.7 times less than that used for the treatment group in the present study (100 mg/kg, twice a day, 44.78 mg of the three methoxyflavones). Thus, the reason for the differences might be that the dosage of the three methoxyflavones in the ethanolic extract (12.069 mg) might not be sufficient, and also some other substances in the ethanolic extract might have had an effect on glucose metabolism in the opposite way to result in an increase of the fasting serum glucose levels.

In conclusion, the present study has demonstrated that 6 weeks of KPD extract treatment in middle-aged male rats re-stored endothelium dysfunction of the middle-aged blood vessels by an increase in nitric oxide production by increasing the eNOS protein at the blood vessels and this resulted in a decrease of the maximal contractile response to phenylephrine and an increase of the dilatation of the blood vessels to acetylcholine. There were no

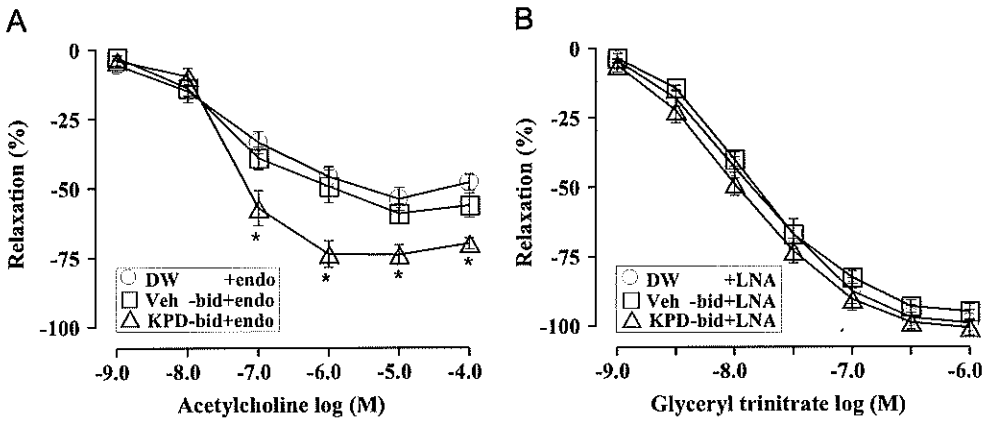

Fig. 6. Effect of 6 weeks oral administration of a *Kaempferia parviflora* dichloromethane extract twice a day (KPD-bid), distilled water (DW) or vehicle control (Veh-bid) on the vasorelaxation of the endothelium-intact thoracic aortic ring precontracted with phenylephrine to acetylcholine (A), or to glyceryl trinitrate in the presence of N^G-nitro-L-arginine (B). Values represent a mean ± S.E.M. of 6 animals. *Significantly higher than that of the distilled water and the vehicle control group, $P < 0.05$.

Table 4
EC_{50} and E_{max} values of the vasodilatory responses to acetylcholine (ACh) or glyceryl trinitrate (GTN) of the thoracic aortic and mesenteric rings precontracted with phenylephrine obtained from middle-aged male rats that had been oral administration for 6 weeks with distilled water (DW), vehicle control (Veh-bid) or a *Kaempferia parviflora* dichloromethane extract (KPD-bid) twice a day and in young rats (positive control).

Treatments	EC_{50} (nM) : 95% confidential limit		E_{max} (%)	
	Thoracic aorta	Mesenteric artery	Thoracic aorta	Mesenteric artery
Response to ACh				
DW	287.2(225.4–410.2)	64.2(32.1–90.2)	50.2 ± 2.9	93.4 ± 1.1
Veh-bid	277.1(162.2–395.1)	58.4(33.1–89.2)	51.1 ± 5.0	94.9 ± 0.3
KPD-bid	145.2(99.1–235.2)	34.7(21.2–45.4)	72.4 ± 4.1[b]	95.9 ± 0.7
Young	78.4 (42.2–145.1)[a]	13.3 (8.4–21.8)[a]	85.4 ± 3.4[b]	94.6 ± 0.6
Response to GTN				
DW	19.4(15.1–22.1)	65.2(46.8–94.2)	100.1 ± 5.2	87.9 ± 1.4
Veh-bid	20.1(18.4–21.9)	63.6(44.3–91.1)	98.9 ± 2.1	89.8 ± 2.6
KPD-bid	18.7(14.9–22.3)	72.4(49.6–106.2)	105.0 ± 2.4	90.1 ± 2.5

Values were obtained from 6 experiments ($n=6$) for each group.
[a] Significantly lower than that of the distilled water and vehicle control.
[b] Significantly higher than that of the distilled water and vehicle control groups, $P < 0.05$.

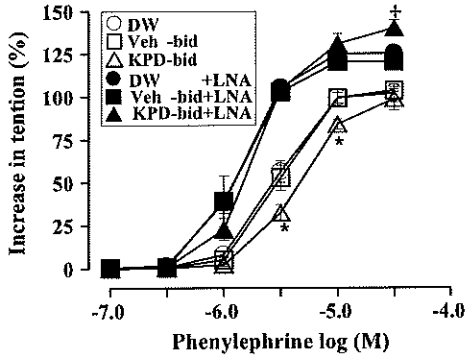

Fig. 7. Effect of 6 weeks oral administration of a *Kaempferia parviflora* dichloromethane extract twice a day (KPD-bid), distilled water (DW) or a vehicle control (Veh-bid) on the contractile response of the mesenteric ring to phenylephrine before and after pre-incubation with N^G-nitro-L-arginine (LNA). Values represent a mean ± S.E.M. of 6 animals. *Significantly lower than that of the distilled water and vehicle control group, $P < 0.05$. †Significantly higher than that of the distilled water and vehicle control group, $P < 0.05$.

Table 5
EC_{50} and E_{max} values of contractile responses to phenylephrine of the mesenteric rings obtained from middle-aged male rats that had been oral administration for 6 weeks with distilled water (DW), vehicle control (Veh-bid) or a *Kaempferia parviflora* dichloromethane extract (KPD-bid) twice a day before (control) and after preincubation with N^G-nitro-L-arginine (LNA).

Treatments	EC_{50} (μM) : 95% confidential limit		E_{max} (%)	
	Control	+LNA	Control	+LNA
DW	2.5(2.0–3.2)	1.6(1.4–2.1)[b]	102.0 ± 5.2	122.4 ± 3.8
Veh-bid	2.6(2.2–3.3)	1.5(1.1–2.0)[b]	103.7 ± 0.7	120.5 ± 2.8
KPD-bid	4.9(3.3–6.3)[a]	2.0(1.6–2.5)[b]	98.9 ± 6.9	139.9 ± 4.9[c]

Values were obtained from 6 experiments ($n=6$) for each group.
[a] Significantly higher than that of the control (DW and Veh-bid) groups.
[b] Significantly lower than their corresponding control groups.
[c] Significantly higher than the other groups, $P < 0.05$.

signs of gross toxicity observed. In addition, the KPD extract also caused a decrease in the visceral and subcutaneous fat, plasma glucose and triglycerides and liver lipid accumulation. This is the first report to demonstrate that oral administration of the KPD extract has benefits on parameters that prevent obesity, metabolic syndrome and disorders of the vascular system. However, further studies would be needed to clarify which of the bioactive compound(s) of the KPD extract was responsible for the change in vascular functions: a direct effect of that bioactive compound(s) on the blood vessels or a consequence of its action on the reduction of the accumulation of body lipid. Further work is also required to confirm the most effective way to prepare standard material from the rhizomes to ensure its effectiveness in humans.

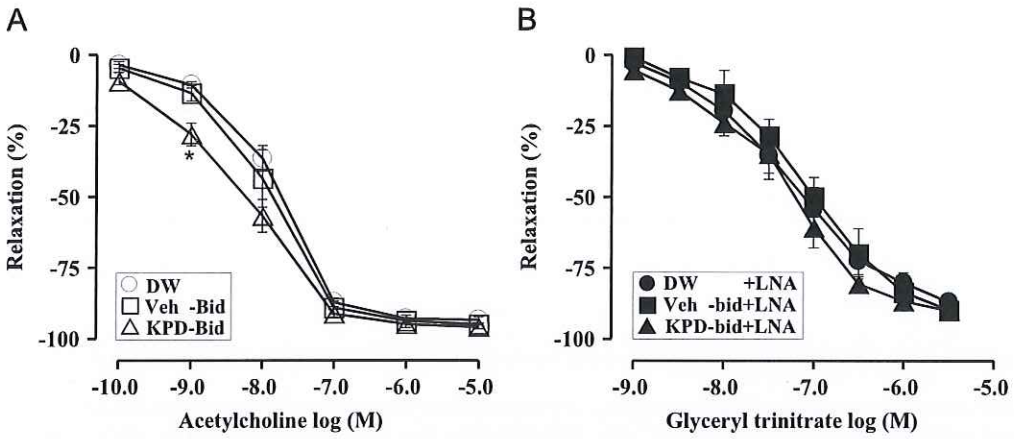

Fig. 8. Effect of 6 weeks oral administration of a *Kaempferia parviflora* dichloromethane extract twice a day (KPD-bid), distilled water (DW) or vehicle control (Veh-bid) on the vasorelaxation to acetylcholine (A) or glyceryl trinitrate in the presence of N^G-nitro-L-arginine (LNA) (B) of the mesenteric rings precontracted with phenylephrine. Values represent a mean ± S.E.M. of 6 animals. *Significantly higher than that of the distilled water and vehicle control group, $P < 0.05$.

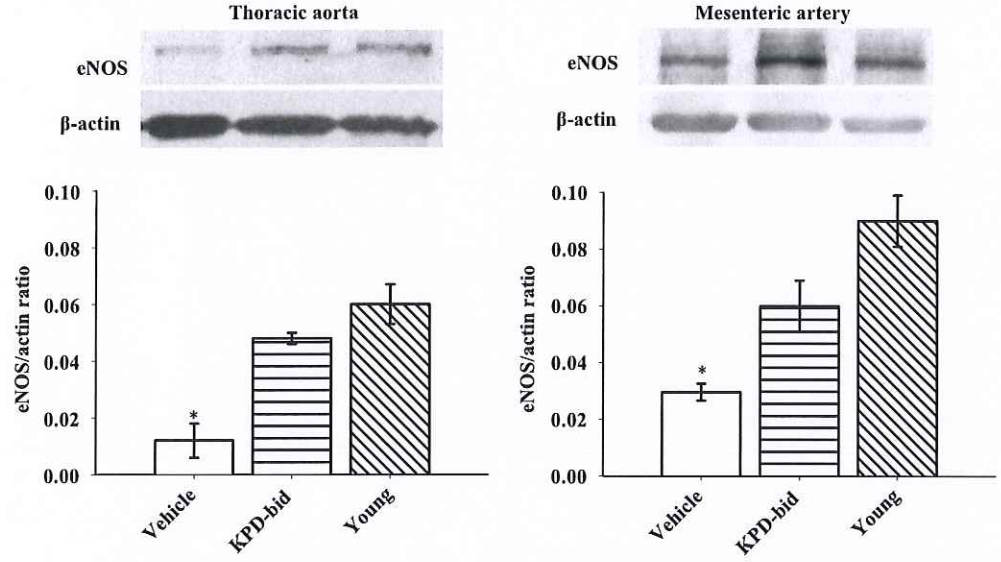

Fig. 9. Western blot analysis of the expression of the eNOS protein of the thoracic aorta and mesenteric artery obtained from a *Kaempferia parviflora* dichloromethane extract administered twice a day (KPD-bid) and the vehicle control of the middle-aged and untreated- young adult male rats. Values represent a mean ± S.E.M. of 4 animals. *Significantly lower than the KPD treated middle-aged and young adult male rat, $P < 0.05$.

Acknowledgments

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Appendix A. Supporting material

Supplementary material associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.08.020.

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Vasorelaxant effects of 3,5,7,3',4'-pentamethoxyflavone isolated from *Kaempferia parviflora*: partly stimulating the release of NO and H_2S by rat thoracic aorta

Somruedee Yorsin, Yauwapa Sukpondma, Chaweewan Jansakul

Abstract

Pentamethoxyflavone (PMF) was isolated from the rhizomes of *Kaempferia parviflora*, a plant that is claimed to have anti-hypertensive effects. We have investigated the activity of the PMF on isolated rat thoracic aortic rings. PMF caused a relaxation of phenylephrine precontracted aortic rings, and this effect was inhibited by N^G-nitro-L-arginine (LNA), ODQ (guanylyl cyclase inhibitor), or by removal of the vascular endothelium. In the presence of LNA or removal of the endothelium, ODQ potentiated the relaxant activity of the PMF, and this effect was inhibited by DL-propargylglycine (PAG, a cystathionine-γ-lyase inhibitor) and SQ22536 (an adenylyl cyclase inhibitor). Glybenclamide, but not tetraethylammonium, potentiated the relaxation of the PMF whether LNA was present or not, and the potentiation was inhibited by PAG and SQ22536. In normal Krebs solution with nifedipine, or in a Ca^{2+}-free Krebs solution, PMF caused a further inhibition of the phenylephrine concentration- response (*C-R*) curve of the aortic ring. In the aortic ring treated with thapsigargin, PMF suppressed the phenylephrine *C-R* curve and a further suppression was found when nifedipine, SKF-96365 (store-operated Ca^{2+} channel inhibitor) and/or Y-27632 (Rho-kinase inhibitor) was also added. These results revealed that PMF caused a relaxation of thoracic aortic rings by stimulating the release of nitric oxide and H_2S, that act as an adenylyl cyclase stimulator, and an inhibitor of intracellular calcium mobilization.

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Quercetin has attracted numerous beneficial health claims[1] and is the most abundant flavonoid polyphenolic compound present in human dietary vegetables and fruits.[2] In the cardiovascular system, it has been shown to be antihypertensive in various rat models of hypertension.[1,3] *In vitro*, quercetin is a vasorelaxant independent of the endothelium[4] yet in addition also increases endothelial NO production.[5] On the other hand, quercetin itself is poorly absorbed, has a high susceptibility to metabolic conjugation, and exists mostly in a conjugated form in the systemic circulation, that results in its low bioavailability.[6-7] Thus quercetin is largely ineffective *in vivo*. This low bioavailability is mainly due to an efficient system for conjugation of its polyhydroxylated groups with glucuronic acid and sulfate in normal situations. However, it has been reported that the intestinal transportation of this flavonoid could be much improved through methylation. In an *in vivo* experiment in the rat, oral administration of one methylated flavone resulted in a higher bioavailability and tissue distribution with no detectable levels of its unmethylated analogue. Thus, methoxylation appears to be a simple and effective way to increase both the metabolic resistance and the transport of the flavonoid into the circulatory system.[8-9] Some methoxylated flavonoids occur naturally including methoxylated quercetins, quercetin 3,7,3',4'-tetramethylether and quercetin 3,5,7,3',4'-pentamethylether (3,5,7,3',4'-pentamethoxyflavone) found in some medicinal plants such as *Achyrocline satureioide*[10] and the rhizomes of *Kaempferia parviflora*.[11-12] A further ten other polymethoxyflavones are found in the peel of *Citrus* fruits that are used in several traditional medicines from Eastern China.[13-15] At least three of these i.e., tangeretin, nobiletin (5,6,7,8,3,4-hexamethoxyflavone) and sinensetin, have biological activity on G1 cell cycle arrest in human breast and colon cancer cells, on cardiovascular protection via its antihypertensive actions, and show attenuation of atherosclerosis, are anti-inflammatory, have antiangiogenesis activity, inhibit platelet adhesion, and have anti-adipogenic properties on mature 3T3-L1 adipocytes.[16-21] To date, there seem to have been no reports on the effects of polymethoxyflavone on the endothelial-vascular smooth muscle unit of isolated blood vessels.

Rhizomes of *Kaempferia parviflora* (Zingiberaceae family) have been used in Thai traditional

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medicine for many purposes, including its use as an aphrodisiac, as an anti-hypertensive compound, to improve blood flow, and to promote longevity with good health and well being.[11-12] There is some limited scientific support for these therapeutic claims. Chaturapanich *et al.*[22] demonstrated that a *Kaempferia parviflora* alcohol extract produced a significant increase in blood flow to the testis in male rats. Later Murata *et al.*[23] showed that a methanolic extract of *Kaempferia parviflora* caused an improvement to the blood clotting mechanism where the active principles were methoxyflavones. One of these compounds, 3,5,7,3',4'-pentamethoxyflavone (PMF), relaxed isolated human cavernosum by inhibiting both voltage-dependent Ca^{2+} channels and intracellular calcium mobilization.[24] Hnatyszyn *et al.*[10] showed that quercetin 3,7,3',4'-tetramethylether, quercetin 3,5,7,3',4'-pentamethylether and quercetin itself were all equally effective relaxants of isolated guinea-pig cavernosum. For isolated blood vessels, quercetin molecules with an increasing number of methoxylations showed different activity profiles for potency and mechanism of action on vasorelaxation.[25] Thus in rat thoracic rings, 5,7-dimethoxyflavone (DMF) produced an endothelium-dependent relaxation via an increased K^+ efflux and by inhibition of extracellular Ca^{2+}entry through the NO-cGMP and cyclooxygenase pathways. Trimethoxyflavone had similar effects except that it failed to activate the cyclooxygenase pathway.[26]

Thus, polymethoxylated flavonols promise to offer a quantal leap forward in the efficacious cardiovascular treatment regimens hitherto unrealised by quercetin and other polyphenols in the clinic. The simplest starting point would be 3,5,7,3',4'-pentamethoxyflavone since it works in other tissues and complete methoxylation improves absorption, allows for greater systemic stability and improves cellular uptake.[8-9,11,27] Although we have already studied corpus cavernosum, the greatest clinical impact is likely to be on vascular function. Therefore, in the present work we aim to analyze the action of PMF on isolated rat aorta with such an application in mind.

Materials and Methods

Plant material

Fresh rhizomes of *Kaempferia parviflora* (black ginger or kra-chai-dum in Thai) were collected in Phurua District, Loei Province, Thailand. Authentication was achieved by comparison with the herbarium specimen in the Department of Biology Herbarium, Faculty of Science, Prince of Songkla University, Thailand, where a voucher specimen (Collecting No. 2548-03) of the plant material has been deposited.

Extraction and isolation of the 3,5,7,3',4'-pentamethoxyflavone (PMF) from rhizomes of *Kaempferia parviflora*

The method used to isolate the pure PMF and the HPLC method used for its assay has been previously fully described by Jansakul *et al.*[24]

Preparation of thoracic aortic rings

Adult female Wistar rats were supplied by the Southern Laboratory Animal Facility, Faculty of Science, Prince of Songkla University. The animals were housed in controlled environmental conditions at 24-26 °C on a 12 h dark and 12 h light cycle with access to standard rat food and tap water *ad libitum*. The methods employed and the experimental protocols were approved by the Prince of Songkla University Ethical Committee (Ethic No. 166/1724) and the investigation conformed to the Guide for the Care and Use of Laboratory Animals.

Adult female Wistar rat in estrous weighing 220-250 g were killed by cervical dislocation. The thoracic aorta was removed and carefully cleaned of adhering fat and connective tissue. Two adjacent rings of 4-5 mm in length were cut. In one ring the endothelium layer was removed mechanically by gently rubbing the intimal surface with a stainless steel rod, using the method of Jansakul *et al.*[28] The aortic rings with or without a functional endothelium were mounted horizontally between two parallel stainless steel hooks, with extreme care not to damage the endothelium of the endothelium-intact aortic rings, and resuspended in a 20-ml organ bath containing Krebs-Henseleit solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 1.9, $MgSO_4 \cdot 7H_2O$ 0.45, KH_2PO_4 1.18, $NaHCO_3$ 25.0, glucose 11.66, Na_2EDTA 0.024 and ascorbic acid 0.09, maintained at 37 °C and continuously bubbled with a 95% O_2 and 5% CO_2 gas mixture. One hook was fixed at the bottom and the other was connected to a force displacement transducer that was connected to a Grass polygraph for the recording of changes in the isometric tension. Prior to testing, tissues were equilibrated for 60 min under a resting tension of 1 g and the bath solution was replaced with pre-warmed oxygenated Krebs-Henseleit solution every 15 min.

As a prelude to testing, a functional endothelium of the intact thoracic aortic rings was verified in every preparation as follows: The thoracic aortic ring was precontracted with 3 μM phenylephrine until the response reached a plateau (5-8 min), and then acetylcholine was added (30 μM). Ring viability was judged by a > 80% vasorelaxation back to the tension generated by the ring before adding phenylephrine. Denudation was confirmed by the complete absence of vasorelaxation following the response to the addition of acetylcholine. The preparations were then washed several times with Krebs-Henseleit solution, and allowed to fully relax for 45 min before the experimental protocol began.

Experimental protocol

Effects on nitric oxide, guanylyl cyclase, adenylyl cyclase, H_2S stimulation, and K^+ channels

After equilibration, the thoracic aortic rings were

precontracted with 3 μM phenylephrine for 10 min (plateau reached), and the cumulative concentration-response (*C-R*) relationships of the thoracic aortic ring to PMF (0.0003-0.1 mM) was determined. Following several washings and re-equilibration of the thoracic aortic ring for another 60 min with changes to the Krebs-Henseleit solution every 15 min, the thoracic aortic rings were incubated with N^G-nitro-L-arginine (LNA, 0.3 mM, inhibitor of nitric oxide synthase); 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 0.01 mM, a soluble guanylyl cyclase inhibitor); glybenclamide (0.01 mM, an ATP sensitive K^+ channel inhibitor); tetraethylammonium (TEA, 1 mM, a voltage activated K^+ channel blocker) for 40 min; and/or by an addition of DL-propargylglycine (PAG, 10 mM, a cystathionine-γ-lyase inhibitor or an H_2S inhibitor); or 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, 0.1mM, an adenylyl cyclase inhibitor) as required. Then the PMF (0.003-0.1 mM) cumulative *C-R* relationships were determined on the phenylephrine-induced contraction in the continuous presence of each drug.

Inhibition of voltage-dependent calcium channels, intracellular Ca^{2+} mobilization, store-operated calcium channels, and Rho-kinase

In all the experiments described in this section, the thoracic aortic rings were first equilibrated for 60 min in Krebs solution, followed by a functional assessment of the endothelium as above, after which they were incubated with Krebs solution in the presence of LNA (0.3 mM) for 60 min with a replacement of the incubation medium every 20 min. Thereafter LNA was present throughout.

To determine whether PMF played a role as a voltage-dependent Ca^{2+} channel blocker, the *C-R* curve to phenylephrine was studied in the normal Krebs medium. This was followed by several washings and re-equilibration for 40 min, then the thoracic aortic rings were incubated with nifedipine (3 μM) for 20 min and again the *C-R* curve to phenylephrine was determined in the presence of nifedipine. The same procedure was repeated in the presence of nifedipine with PMF (0.03 mM).

To determine whether PMF inhibited intracellular Ca^{2+} mobilization, a similar protocol was carried out with another set of thoracic aortic rings in Ca^{2+}-free Krebs solution before and after incubating the thoracic aortic ring with PMF.

To determine the role of PMF on the store-operated Ca^{2+} channel and/or the Rho-kinase inhibitor, eight sets of thoracic aortic rings were used. Each set of the thoracic aortic rings was first constructed with the *C-R* curve to phenylephrine in the presence of LNA, followed by several washings and re-equilibration for another 40 min. The thoracic aortic rings were then incubated with thapsigargin (3 μM), a sarcoplasmic reticulum ATPase inhibitor, for 40 min by which time the small contraction (~ 0.1-0.2 g) of the thoracic aortic rings had reached a plateau, in one set of these the *C-R* relationship to phenylephrine was determined in the presence of thapsigargin. For the others, PMF (0.03 mM), nifedipine (3 μM), SKF-96365 (100 μM, a store-operated Ca^{2+} channel blocker) and/or Y-27632 (30 μM, a Rho-kinase inhibitor) were added sequentially as required and incubated for 20 min, at which time the thoracic aortic ring had relaxed to its original basal level, then the cumulative *C-R* relationship to phenylephrine was obtained in the presence of thapsigargin together with their corresponding cocktails.

Drugs

The following drugs were used: acetylcholine chloride, nifedipine, N^G-nitro-L-arginine (LNA), phenylephrine hydrochloride, tetraethylammonium (TEA), DL-propargylglycine (PAG, cystathionine-γ-

Table 1 E_{Max} and EC_{50} values of the relaxation of the endothelium-intact (Endo) or denuded- (No endo) thoracic aortic ring precontracted with phenylephrine to 3,5,7,3′,4′-pentamethoxyflavone (PMF) either in the presence of N^G-nitro-L-arginine (LNA), ODQ, Glybenclamide (Glyben), TEA, DL-propargylglycine (PAG) and/or SQ22536 (SQ).

	EC_{50} in μM (95% confidence limit)			E_{Max} (%)		
	Endo	Endo+LNA	No endo	Endo	Endo+LNA	No endo
PMF	3.6 (2.1-6.2)	28.7 (21.4-38.4)	31.5 (26.9-35.9)	95.6±9.9	94.6±6.6	95.3±4.4
PMF+ODQ	11.7 (9.5-14.5)[b]	11.8 (9.0-15.5)[a]	13.5 (10.5-17.3)[a]	107.8±4.5	99.8±4.2	99.5±3.8
PMF		29.4 (20.2-43.1)			116.1±9.7	
PMF+PAG		32.2 (25.4-40.1)			111.3±5.6	
PMF+PAG+SQ		28.6 (19.6-39.2)			109.5±7.0	
PMF		33.4 (28.3-39.4)			95.3±3.4	98.5±3.8
PMF+ODQ		10.2 (8.9-11.6)[a]			100.2±2.6	101.4±2.5
PMF+ODQ+PAG		25.1 (20.2-28.8)			112.4±1.8[b]	100.8±1.4
PMF+ODQ+PAG+SQ		36.5 (31.5-42.6)			103.4±1.9	103.5±3.1
PMF	8.1 (5.9-11.0)	31.3 (27.2-36.1)		102.5±11.6	96.3±5.2	
PMF+Glyben	3.3 (2.2-5.1)[a]	13.9 (9.9-16.5)[a]		92.1±4.6	101.2±2.9	
PMF+Glyben+PAG		23.6 (18.8-27.4)			105.8±3.5	
PMF+Glyben+PAG+SQ		29.7 (26.5-35.9)			99.6±3.4	
PMF+TEA	7.8(5.1-10.4)			106.6±3.1		

Values were obtained from 6 aortic rings each from a different rat (n = 6). [a] Significantly lower than control and [b] significantly higher than control.

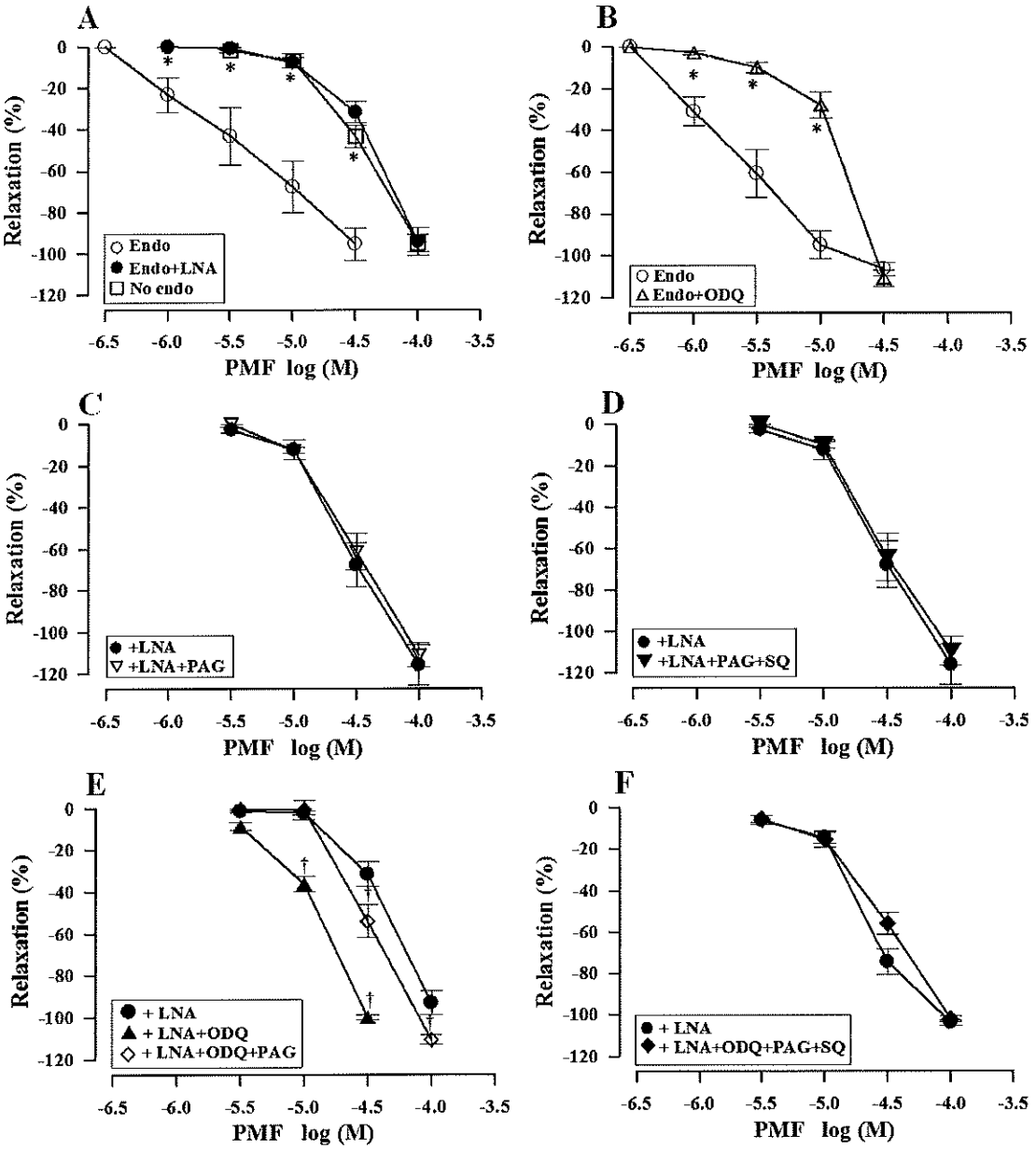

Figure 1 Effects of the removal of endothelium (no endo), N^G-nitro-L-arginine (LNA, 0.3 mM), ODQ (0.01 mM), DL-propargylglycine (PAG,10 mM) and/or 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536: SQ, 0.1 mM) on the relaxation of the thoracic aortic rings that had been precontracted with phenylephrine (3 μM for endothelium-intact, 0.3 μM for that with LNA, ODQ and/or -denuded vessel) to 3,5,7,3',4'-pentamethoxyflavone (PMF). Drug-relaxation is expressed as the percentage inhibition of the maximum phenylephrine tension. Each point represents a mean value ± SEM of 6 aortic rings each from a different animal (n = 6). *Significantly higher than the control (○) group, $P < 0.05$ and †significantly lower than the one with LNA (●), $P < 0.05$.

lyase inhibitor) and 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536, adenylyl cyclase inhibitor) were all from Sigma, U.S.A. 1*H*-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), thapsigargin, SKF-96365 and trans-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecarboxamide dihydrochloride (Y-27632) were from Trocis, UK. LNA, thapsigargin, SKF-96365, Y-27632 and PAG were dissolved in distilled water, nifedipine and SQ22536 were dissolved in 20% DMSO, and the remainder were dissolved in a solution (1 liter) containing NaCl 9 g, NaH_2PO_4 0.19 g and ascorbic acid 0.03 g.

Statistical analysis

Results are expressed as mean ± SEM where n indicates the number of thoracic aortic rings. Each thoracic aortic ring was obtained from a different rat. Drug-induced relaxation was measured as the decline from the maximal steady tension produced by phenylephrine. The steady decline achieved at each drug concentration was expressed as a percentage of the initial maximum produced by phenylephrine. The

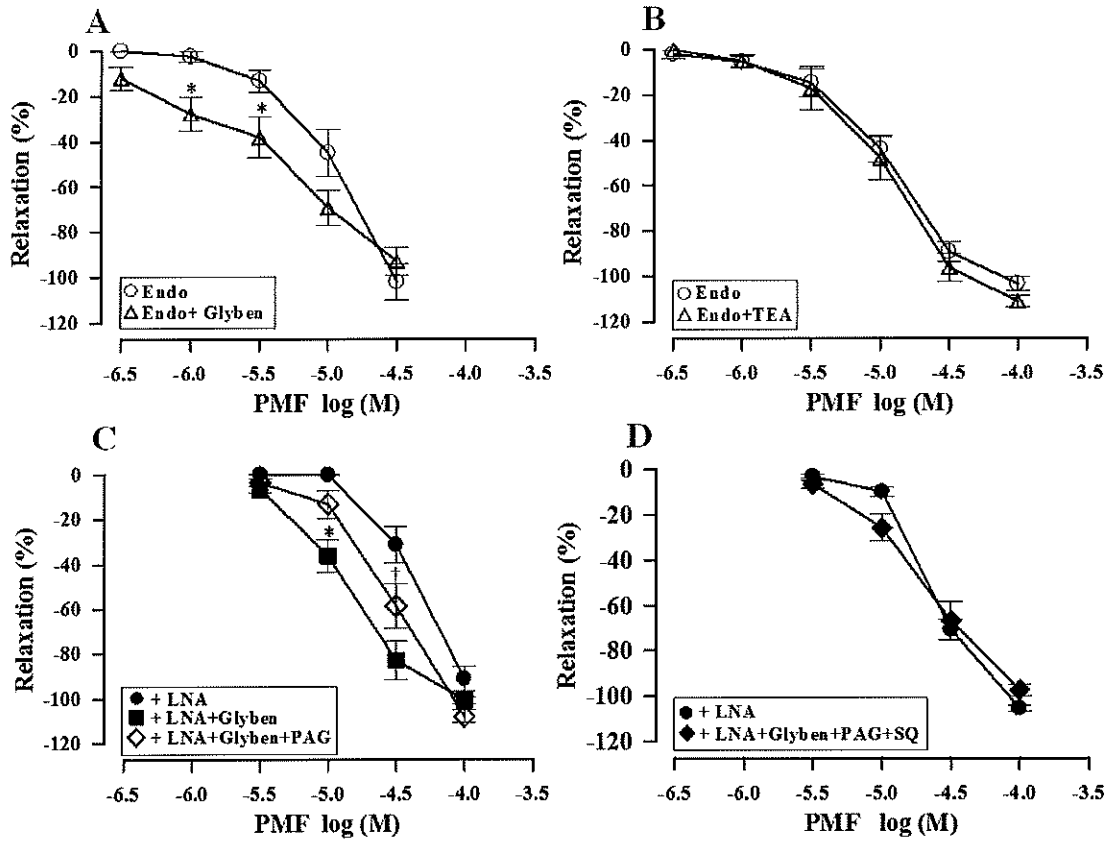

Figure 2 Effects of N^G-nitro-L-arginine (LNA, 0.3 mM), glybenclamide (Glyben, 0.01 mM), TEA (1 mM), DL-propargylglycine (PAG, 10 mM) and/or 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536:SQ, 0.1 mM) on the relaxation of thoracic aortic ring precontracted with phenylephrine (3 μM for the control, 0.3 μM for that with LNA) to 3,5,7,3',4'-pentamethoxyflavone (PMF). Drug-relaxation is expressed as the percentage inhibition of the maximum phenylephrine tension. Each point represents a mean ± SEM of 6 aortic rings each from a different animal (n = 6). *Significantly lower than their control (○) groups and †significantly lower than the one with LNA (●), $P < 0.05$.

contractile cumulative *C-R* curve to phenylephrine, and the steady increase achieved at each phenylephrine concentration was expressed as a percentage of the E_{max} obtained from their control group. The drug concentration that produced 50% of the maximal response for the drug (EC_{50}) was derived from regression analysis over the linear portion of the concentration-response curve. Statistical differences between two measurements was determined by two-tailed unpaired Student's *t*-test; differences among groups was determined by one way ANOVA and post hoc analysis was performed with a Duncan test. A P value ≤ 0.05 was considered to be a significant difference in all experiments.

Results

Effects on nitric oxide, guanylyl cyclase, adenylyl cyclase, H_2S stimulation, and K^+ channels

PMF caused a relaxation of the thoracic aortic ring precontracted with phenylephrine in a concentration-dependent manner. LNA, ODQ or removal of the vascular endothelium significantly shifted the *C-R* curve of the PMF to the right (Figure 1A and B) and increased the EC_{50} values 3-8 fold (Table 1). In the endothelium-intact aortic ring in the presence of LNA, PAG alone or together with SQ22536 there was no change in the relaxant activity of the PMF (Figure 1C and D), whereas ODQ caused a potentiation of the relaxant activity of the PMF with a decrease in its EC_{50} values (Table 1). The potentiating effect of ODQ on the PMF of these blood vessels was significantly inhibited by PAG and when SQ22536 was also added the potentiating effect of the ODQ was restored to the same level as their control groups (Figure 1E-H).

TEA did not modulate the PMF *C-R* curve (Figure 2B) whereas glybenclamide potentiated the vasorelaxant *C-R* curve of the PMF with a decrease in the EC_{50} values of the endothelium-intact thoracic aortic rings no matter whether LNA was present or not (Figure 2A and C and Table 1). The potentiating effect of glybenclamide on the PMF *C-R* curve of the endothelium-intact thoracic aortic ring with LNA (Figure 2C) was significantly inhibited by PAG and when SQ22536 was also added the *C-R* curve of PMF was restored to the same level as that of the control groups (Figure 2D).

Block of voltage-dependent calcium channels, and/or intracellular calcium mobilization

Nifedipine significantly inhibited the PMF *C-R* curve

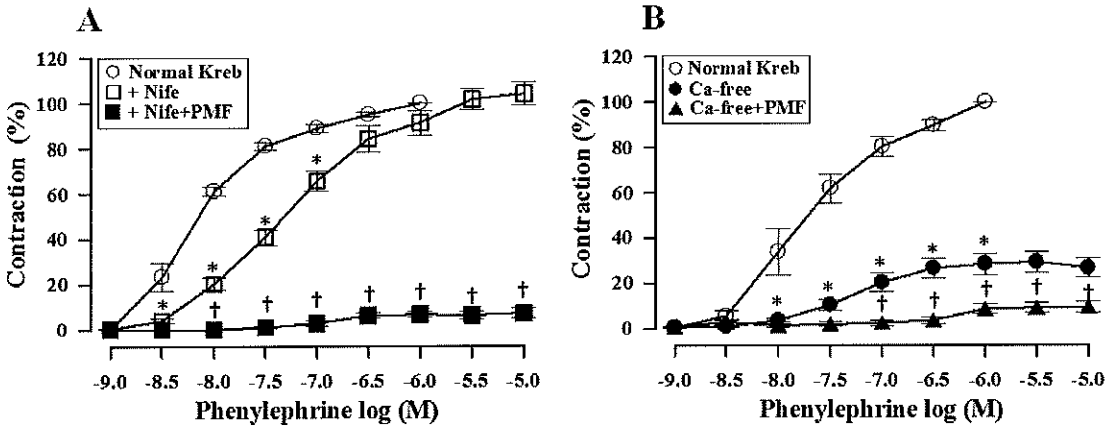

Figure 3 Contractile responses of the endothelium-intact thoracic aortic ring in the presence of LNA to phenylephrine in normal Krebs solution before and after blocking the L-type Ca^{2+} channel with nifedipine (3 μM, left) for 20 min or in a Ca^{2+}-free medium (right) and with 3,5,7,3',4'-pentamethoxyflavone (PMF, 0.03 mM). Each point represents a mean value ± SEM of 6 aortic rings each from a different rat (n = 6). * Significantly lower than the control (○) group, $P < 0.05$. †Significantly lower than that with nifedipine (□) or in Ca^{2+}-free medium (●) and the control groups, $P < 0.05$.

and a further inhibition was found when PMF was also added (Figure 3A). A similar result was found in the Ca^{2+} free Krebs medium (Figure 3B).

Store-operated calcium channels, Rho-kinase

In the normal Krebs solution in the presence of LNA and thapsigargin, nifedipine (3 μM), SKF-96365 (100 μM), Y-27632 (30 μM) or PMF (0.03 mM) caused a significant inhibition of the phenylephrine *C-R* curves on the thoracic aortic ring. In this situation, the depression of the phenylephrine *C-R* curve by PMF was bigger than the one produced by nifedipine and SKF-96365 at a low concentration of the phenylephrine but not at its high concentrations where the maximal responses were the same (Figure 4A and B). When compared to the effect of Y-27632, the *C-R* curve of the phenylephrine in the presence of PMF was significantly lower than the one with Y-27632 throughout (Figure 4C). When nifedipine and/or SKF-96365 were added together with PMF, a further depression of the phenylephrine *C-R* curve was obtained (Figure 4D). A further significant inhibition of the phenylephrine *C-R* curve was found when Y-27632 was also added together with nifedipine, SKF-96365 and PMF (Figure 4E).

Discussion

The present study has clearly demonstrated that PMF has a relaxant activity on the isolated thoracic aortic rings in an endothelium-dependent and -independent manner. Possible mechanisms responsible for the relaxation were explored to determine if PMF acted 1) as a nitric oxide stimulator, a guanylyl cyclase-, an adenylyl cyclase- or H_2S stimulator and/or by opening of K^+ channels, 2) by blocking voltage-dependent calcium channels, 3) by inhibition of intracellular calcium mobilization, 4) as a store-operated calcium channel inhibitor, or 5) as a Rho-kinase inhibitor.

Possible actions as a nitric oxide stimulator, a guanylyl cyclase-, an adenylyl cyclase- or H_2S stimulator and/or by the opening of K^+ channels

Our finding that LNA inhibited the relaxant activity of the PMF indicated that PMF stimulated nitric oxide release. ODQ alone was also found to inhibit the relaxant activity of the PMF on the endothelium-intact thoracic aortic rings, however no further additional inhibition was obtained when LNA was also present. These results indicated that PMF would not act directly via a stimulation of the guanylyl cyclase in the vascular smooth muscle. The inhibitory effect of the ODQ could be indirect via the soluble guanylyl cyclase that was activated by the endothelial NO that resulted from the PMF stimulation. These results are different from our previous reports on the human cavernosum, in which it was found that PMF showed very little effect on the nitric oxide stimulation with no effect on the soluble guanylyl cyclase stimulation.[24] The reason for this is most likely due to the different types of blood vessels, and human cavernosum might have a different underlying mechanism for its relaxant activity to the PMF: the relaxation of the cavernosum is predominantly a response as the result of neuronal NO transmission.[29-30] However, a further study would be needed to clarify this possibility. In addition, it was a surprise to see that in the presence of LNA with the endothelium-intact thoracic aortic ring, ODQ potentiated the relaxant activity of the PMF, in a situation when the nitric oxide and soluble guanylyl cyclase was being inhibited. Thus, it is possible that PMF might also activate other signalling pathways: such as the H_2S and/or adenylyl cyclase pathways.

It has now been accepted that H_2S is the third endogenous gasotransmitter with pivotal roles in regulating vascular homeostasis.[31-34] H_2S is released from the vascular endothelium as well as from the vascular smooth muscles by cystathionine-γ-lyase (CSE), the key enzyme that utilizes L-cysteine as a

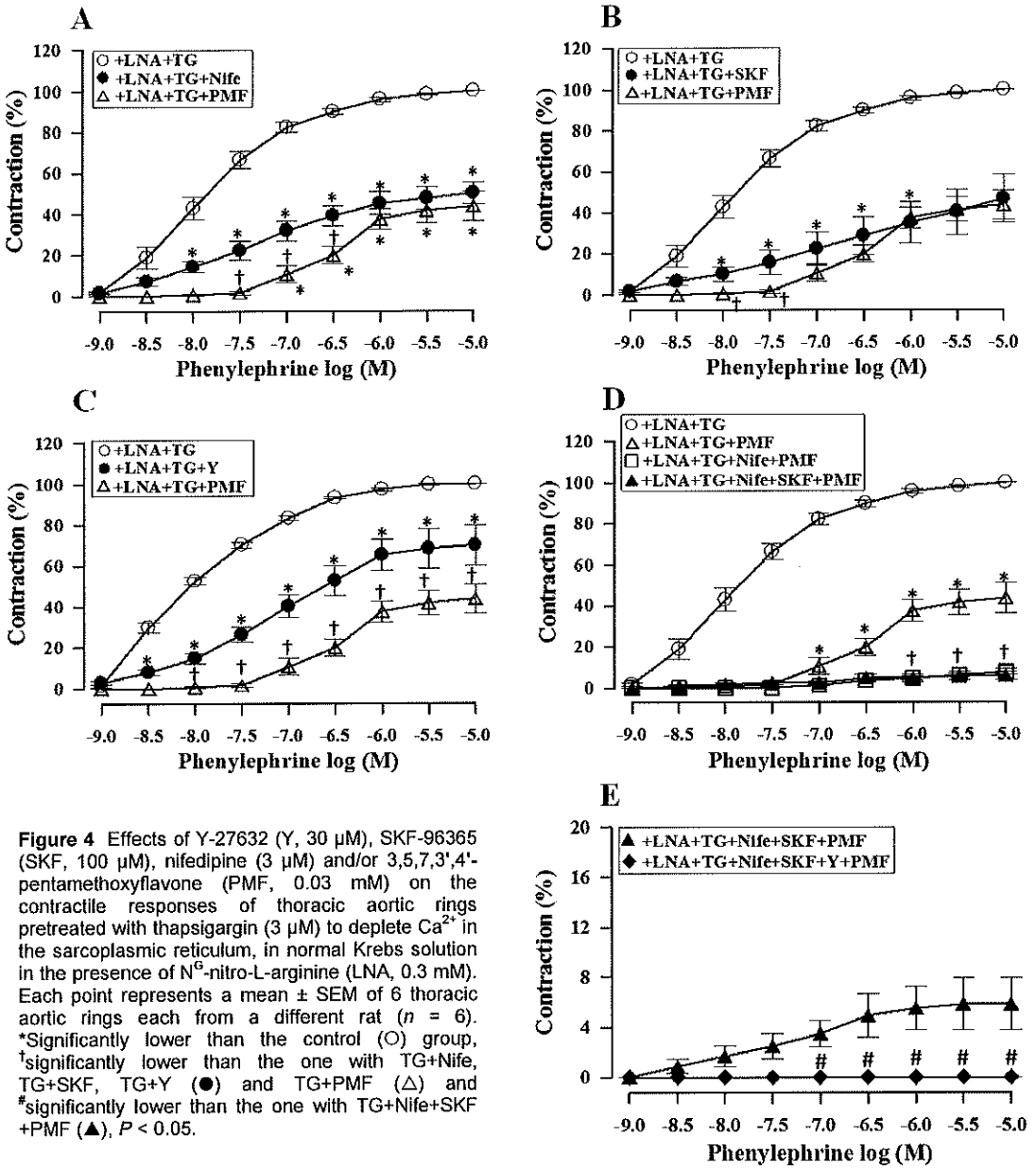

Figure 4 Effects of Y-27632 (Y, 30 μM), SKF-96365 (SKF, 100 μM), nifedipine (3 μM) and/or 3,5,7,3',4'-pentamethoxyflavone (PMF, 0.03 mM) on the contractile responses of thoracic aortic rings pretreated with thapsigargin (3 μM) to deplete Ca^{2+} in the sarcoplasmic reticulum, in normal Krebs solution in the presence of N^G-nitro-L-arginine (LNA, 0.3 mM). Each point represents a mean ± SEM of 6 thoracic aortic rings each from a different rat (n = 6). *Significantly lower than the control (○) group, †significantly lower than the one with TG+Nife, TG+SKF, TG+Y (●) and TG+PMF (△) and #significantly lower than the one with TG+Nife+SKF +PMF (▲), $P < 0.05$.

substrate to form H_2S.[35-37] Within the vascular wall, the NO and H_2S pathways coexist and serve similar functions.[38] In the case of the nitric oxide pathway, Li *et al.*[39] reported that quercetin induced a rapid eNOS phosphorylation that enhanced the production of NO and promoted vasodilatation of the endothelium-intact thoracic aortic ring via the cAMP/PKA-mediated pathway. They found that the production of the intracellular cAMP was quickly increased by stimulation with quercetin concomitantly with the induction of eNOS phosphorylation at Ser 1179. In addition, the effect of cAMP was also mediated through the allosteric activation of the protein kinase A (PKA), which has been shown to decrease the intracellular Ca^{2+} concentration and lead to a relaxation of the vascular smooth muscle.[40-42] In a separate finding, Chiwororo and Ojewole[43] found that quercetin-induced relaxation of the rat isolated portal vein, partly via the cAMP-dependent protein kinase pathway. Thus, it is possible that PMF might stimulate the release of H_2S when nitric oxide had been removed and when the soluble guanylyl cyclase had been inhibited by ODQ, so the augmentation of the H_2S and/or cAMP by the PMF could also induce vasodilatation. In order to prove these possibilities, the endothelium-intact thoracic aortic rings in the presence of LNA and/or ODQ were preincubated with PAG, a cystathionine- γ-lyase inhibitor (inhibits H_2S generation), alone or together with SQ22536 (an adenylyl cyclase inhibitor) before performing the *C-R* curve to the PMF. As shown in Figure 1C and D, in the presence of LNA, PAG alone or together with SQ22536 did not modify the PMF *C-R* curve when compared to that of the one with LNA. However,

when ODQ was added together with LNA, the PAG significantly inhibited the PMF *C-R* curve, and when the SQ22536 was also added with the PAG, the PMF *C-R* curve was restored to the same level as that of the control groups (Figure 1E and F). These results indicated that the PMF might stimulate the release of H_2S as well as affecting an increase in the generation of cAMP by the adenylyl cyclase from the vascular smooth muscle and this resulted in vasodilatation.

TEA did not modify the PMF *C-R* curve, whereas glybenclamide potentiated the PMF *C-R* curve of the endothelium-intact thoracic aortic rings whether LNA was present or not. This indicated that PMF did not open the Ca^{2+} sensitive K^+ channel (K_{Ca}), nor did it open the ATP sensitive K^+ channel (K_{ATP}).[44-46] These results are similar to those found in the human cavernosum.[24] The potentiation of the PMF *C-R* curve by glybenclamide would result from a stimulated release of H_2S and/or cAMP by the PMF from the thoracic aorta (Figure 2C and D). This confirmed the above finding that the PMF stimulated H_2S release and cAMP formation. The finding that the stimulated release of H_2S by the PMF on the thoracic aortic ring was not inhibited by glybenclamide, a K_{ATP} channel blocker, was analogous to what occurred in the middle cerebral artery where it was found that the exogenous H_2S mediated relaxation, yet received no contribution from the K_{ATP} channel.[36] Although a few investigators have reported that the mechanism responsible for the vasodilatory effect of the H_2S, involved K_{ATP} channel, because its effect was inhibited by glybenclamide,[32,47-48] this was different from our finding. However, in a recent report, an H_2S-mediated relaxation of the endothelium-denuded blood vessels did occur by inhibition of the L-type calcium channels with an additional contribution by the K^+ channels, probably K_v7 but not K_{ATP}, K_{Ca}, or K_{ir} subtypes.[31, 34, 36] Therefore, a further study would be needed to confirm the present finding.

Possible reactions via voltage- or store-operative calcium channels, Rho-kinase and intracellular Ca^{2+} mobilization

Our finding that PMF antagonized the phenylephrine-induced contraction of the thoracic aortic ring in the normal Krebs solution with Ca^{2+}channel blocker, nifedipine, as well as in the Ca^{2+} free Krebs medium indicated that PMF might inhibit Ca^{2+} mobilization from the intracellular stores.[49-52] These results were also similar to those found in the isolated human cavernosum.[24]

PMF might act as a store-operated Ca^{2+} channel inhibitor, and further experiments were carried out in the presence of thapsigargin, a specific sarcoplasmic-endoplasmic reticulum Ca-ATPase (SERCA) pump inhibitor,[53-54] to deplete the intracellular Ca^{2+} store, which then would stimulate the opening of the plasma membrane store-operated Ca^{2+} channels to add to the refilling of the intracellular stores.[55-56] Thus it would be expected that if PMF played a role as a voltage-, store-operated Ca^{2+}-channel blocker, or a Rho-kinase inhibitor, the phenylephrine *C-R* curve of the thapsigargin that induced the intracellular Ca^{2+} depleted-thoracic aorta could not be further inhibited by addition of the PMF after having been pre-incubated with nifedipine, SKF-96365, or Y-27632 respectively. As shown in the result section, when PMF was added together with nifedipine, SKF-96365 and/or Y-27632, a complete inhibition of the phenylephrine *C-R* curve was obtained. These results indicated that PMF did not act as an L-type Ca^{2+} channel inhibitor, a store-operated Ca^{2+} channel inhibitor or a Rho-kinase inhibitor. These results are consistent with those for human cavernosum except for the blockade of the voltage operated Ca^{2+} channel that was found in the isolated human cavernosum.[24] This also confirmed that different types of smooth muscle from different animals can have different mechanism of reaction to PMF.

In conclusion, the present study has demonstrated that PMF exerted a relaxant activity on the isolated thoracic aorta by stimulating the release of nitric oxide and H_2S from the blood vessel. In addition, it also acted as an adenylyl cyclase stimulator, and might act as an inhibitor of the intracellular Ca^{2+} mobilization from the sarcoplasmic reticulum. It does not appear to act as an opener of K_{ATP}- or K_{Ca} channel, an inhibitor of Rho-kinase or an L-type- or a store-operated Ca^{2+}channel. At the present time, this is the first report to demonstrate that PMF also stimulates the release of H_2S, the third endogenous vasodilatory gasotransmitter, in addition to the nitric oxide of the rat thoracic aorta. Taken together PMF caused a vasorelaxation of the thoracic aortic ring via concerted reactions with several different pathways that provided significant advantages in that one could compensate if another signalling pathway had undergone defection. Therefore, PMF is a novel vasodilatory compound to be considered for the development of an alternative treatment for hypertension.

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Conflict of interest

None to declare.

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List of Publication and Proceeding

1) **Yorsin, S.**, Kanokwiroon, K., Radenamad, N. and Jansakul, C. Effects of *Kaempferia parviflora* rhizomes dichloromethane extract on vascular functions in middle-aged male rat. Journal of Ethnopharmacology; 2014 (156), 162-174.

2) **Yorsin, S.**, Sukpondma, Y. and Jansakul, C. Vasorelaxant effects of 3, 5, 7, 3', 4'-pentamethoxyflavone isolated from *Kaempferia parviflora*: partly stimulating the release of NO and H_2S from rat thoracic aorta. Journal of Physiological and Biomedical Sciences; 2015 (28).