EFFECTS OF ASIATIC PENNYWORT (*Centella asiatica* Linn.) EXTRACT ON CARDIOVASCULAR SYSTEM IN MALE WISTAR RATS

Thida Intharachatorn

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ผลของสารสกัดจากบัวบกที่มีต่อระบบหัวใจและหลอดเลือด ในหนูขาวเพศผู้พันธุ์วิสตาร์

นางสาวธิดา อินทรชาธร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ธิดา อินทรชาธร : ผลของสารสกัดจากบัวบกที่มีต่อระบบหัวใจและหลอดเลือดในหนูขาวเพศผู้พันธุ์วิสตาร์ (EFFECTS OF ASIATIC PENNYWORT (*Centella asiatica* Linn.) EXTRACT ON CARDIOVASCULAR SYSTEM IN MALE WISTAR RATS). อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รุ่งฤดี ศรีสวัสดิ์, 133 หน้า.

บัวบกเป็นหนึ่งในสมุนไพรที่พบว่ามีการใช้ในการลดความดันโลหิตกันอย่างกว้างขวางในตำรายาอายุรเวช การใช้สารสกัดจากบัวบกที่มีสารกลุ่มฟลาโวนอยด์ ซึ่งมีคุณสมบัติลดความดันโลหิต เช่น รูติน เควอซิทิน และคาทิชิน น่าจะเป็นทางเลือกที่ดีที่จะใช้ทดแทนการใช้ยาลดความดันโลหิตได้ ดังนั้นการทดลองในครั้งนี้มีจุดประสงค์เพื่อศึกษาผลของสารสกัดบัวบกและเควอซิทินที่มีต่อความดันโลหิตและการคลายตัวของกล้ามเนื้อเรียบของเส้นเลือดใหญ่เอออร์ตาในหลอดทดลองและความดันโลหิตในสัตว์ทดลอง โดยประกอบด้วย 3 การทดลองดังนี้

การทดลองที่ 1 ศึกษาผลของสารสกัดบัวบกที่มีต่อกลไกการคลายตัวของกล้ามเนื้อเรียบของเส้นเลือดใหญ่เอออร์ตาในหนูขาวเพศผู้พันธุ์วิสตาร์ในระบบ organ bath พบว่าบัวบกที่สกัดด้วยเอทานอล (80%) ให้ผลผลิตเท่ากับร้อยละ 8.04 มีสารประกอบฟีนอลิกทั้งหมดเท่ากับ 87.47 ± 1.74 mg gallic acid/g dry extract สารสกัดบัวบกและเควอซิทินมีผลทำให้หลอดเลือดคลายตัวได้ การคลายตัวของหลอดเลือดที่เป็นผลจากสารสกัดบัวบกและเควอซิทินไม่ขึ้นกับการทำงานของเซลล์เยื่อบุผนังหลอดเลือดและไม่เกี่ยวข้องกับกลไกของไนตริกออกไซด์ แต่การคลายตัวของกล้ามเนื้อเรียบของหลอดเลือดนั้นเกี่ยวข้องกับการเปิดของช่องสำหรับโพแทสเซียมไอออนและช่องสำหรับแคลเซียมไอออนชนิด L-type

การทดลองที่ 2 ศึกษาผลเฉียบพลันของสารสกัดบัวบกที่มีผลต่อความดันเลือด อัตราการเต้นของหัวใจ และอัตราการหายใจในหนูขาวเพศผู้พันธุ์วิสตาร์ความดันโลหิตปกติและความดันโลหิตสูงเนื่องจากถูกชักนำด้วย N^G-nitro-L-arginine methyl ester (L-NAME) ในภาวะไร้ความรู้สึกจากการวัดความดันโลหิตโดยตรงจากเส้นเลือด พบว่าการให้สารสกัดบัวบกและเควอซิทินทางกระเพาะอาหารมีผลทำให้ความดันโลหิตลดลงในกลุ่มหนูขาวที่ถูกชักนำให้ความดันโลหิตสูงด้วย L-NAME แต่มีผลไปเพิ่มความดันโลหิตให้เพิ่มสูงขึ้นในกลุ่มหนูขาวที่มีความดันปกติ สารสกัดบัวบกและเควอซิทินไม่มีผลต่ออัตราการเต้นของหัวใจและอัตราการหายใจในหนูทั้งสองกลุ่ม

การทดลองที่ 3 ศึกษาผลกึ่งเรื้อรังของสารสกัดบัวบกต่อความดันซิสโตลิกในหนูขาวเพศผู้พันธุ์วิสตาร์กลุ่มความดันโลหิตปกติและกลุ่มที่ถูกชักนำให้เกิดภาวะความดันโลหิตสูงด้วย L-NAME โดยวิธีการวัดความดันที่บริเวณหางหนู จากการศึกษาพบว่าสารสกัดบัวบกและเควอซิทินมีผลในการลดความดันโลหิตสูงในกลุ่มหนูขาวที่ถูกชักนำให้เกิดภาวะความดันโลหิตสูง แต่ไม่มีผลต่อกลุ่มหนูขาวที่มีความดันโลหิตปกติ

โดยสรุปการศึกษาในครั้งนี้ชี้ให้เห็นว่าสารสกัดบัวบกและสารออกฤทธิ์เควอซิทินมีฤทธิ์ในการต้านความดันโลหิตสูง และจากผลที่ได้จึงสนับสนุนการใช้บัวบกในการรักษาโรคทางระบบหัวใจและหลอดเลือดในมนุษย์ที่ใช้ต่อกันมาอย่างยาวนาน

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

THIDA INTHARACHATORN : EFFECTS OF ASIATIC PENNYWORT *(Centella asiatica* Linn.) EXTRACT ON CARDIOVASCULAR SYSTEM IN MALE WISTAR RATS. THESIS ADVISOR : ASST. PROF. RUNGRUDEE SRISAWAT, Ph.D. 133 PP.

Centella asiatica/ASIATIC PENNYWORT/EXTRACT/HYPERTENSION/ RATS/ BLOOD PRESSURE/ CARDIOVASCULAR/ARTERIAL BLOOD PRESSURE/ AORTIC RING

C*entella asiatica* is one of the medicinal herbs that has been used extensively by Ayurvedic Pharmacoeia to alleviate high blood pressure. Therefore, use of this plant containing antihypertensive flavonoids (e.g. rutin, quercetin, and catechin) appeared a natural alternative for antihypertensive drugs replacement. Hence, the aims of the present study were to investigate the effects of *Centella asiatica* extract and quercetin on rat aortic ring vasorelaxation activity *in vitro* and arterial blood pressure in male Wistar rats *in vivo*. This study consisted of 3 main experiments:

Experiment 1 investigated the effects of *Centella asiatica* extract to elucidate the underlying mechanisms on isolated rat aortic rings using organ bath system. The results showed that *Centella asiatica* extract had a yield of 8.04%. Total phenolic content was 87.47 ± 1.74 mg gallic acid/g dry extract. *Centella asiatica* extract and quercetin has a potential ability to regulate vascular tone. Vasorelaxation induced by *Centella asiatica* extract and quercetin were found to be endothelium-independent and unrelated to nitric oxide (NO). Opening of potassium (K^+) channel and L-type calcium (Ca^{2+}) channels was involved in the relaxation process of vascular smooth muscle.

Experiment 2 investigated the acute effects of *Centella asiatica* extract on arterial blood pressure, heart rate, and respiratory rate of anaesthetized normotensive and N^G-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats. The results showed blood pressure lowering effects of intragastric administration of *Centella asiatica* extract and quercetin in L-NAME induced hypertensive rats. Hypertensive effects of *Centella asiatica* extract and quercetin were found in normotensive rats. *Centella asiatica* extract and quercetin did not alter heart rate and respiratory rate in both groups.

Experiment 3 investigated the sub-chronic effects of *Centella asiatica* extract and quercetin on systolic blood pressure (SBP) of normotensive and L-NAME induced hypertensive rats by the tail cuff method. The results showed that *Centella asiatica* extract and quercetin possessed antihypertensive effects on L-NAME induced hypertensive rats, but had no effect on normotensive rats.

In conclusion, the present findings suggest that *Centella asiatica* extract and its bioactive compound quercetin exhibit antihypertensive effects and support traditional use of this plant in human cardiovascular diseases.

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Academic Year 2013

Student's Signature_____
Advisor's Signature_____
Co-advisor's Signature_____

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CHAPTER I

INTRODUCTION

1.1 Rational of the study

Hypertension is one of the most important modifiable risk factors for cardiovascular and cerebrovascular diseases, accompanied by the metabolic abnormalities of hyperinsulinemia, insulin resistance and hypertriglyceridemia (Dimo et al., 2002; Zibadi et al., 2007). The increasing levels of hypertension and its prevalence cannot be ignored as 'an individual's problem'. Uncontrolled hypertension is a major cause of disability and premature death throughout the world (Whelton, 1994).

Cardiovascular diseases (CVD) are the first causes of morbidity and mortality in Western countries, with hypertension affecting about 20-30% of the worldwide adult population. Lifestyle modifications and diet therapy are two of the most important tools in prevention and treatment of hypertension (Grassi, Desideri, and Ferri, 2010). CVD currently accounts for nearly half of noncommunicable diseases (NCDs). NCDs have overtaken communicable disease burden, with CVD remaining the leading global cause of death, accounting for 17.3 million deaths per year, and that number is expected to grow to be more than 23.6 millon by 2030 (Smith et al., 2012; WHO, 2012).

Treatment of hypertension will be most successful if incorporating the use of diet, exercise and behavior modification with or without pharmacological therapy.

Many attempts have been made to correct the metabolic disparity of the hypertension condition, producing a number of reagents including angiotensin converting enzyme (ACE) inhibitor (to reduce angiotensin II formation), beta-blockers, calcium blockers and thiazide diuretics (contain a potassium sparing diuretic) (Morgan, Anderson, and MacInnis, 2001; Singh and Leehey, 2007). However, administration of these drugs is known to often cause numerous side effects, including increased serum lipid level, insulin resistance and edema (Gavras, 2001). Therefore, the use of plant extracts that have hypotensive and antihypertensive effects with less side effects is probably a better way to replace these antihypertensive drugs.

Many medicinal plants (such as *Hisbiscus sabdariffa*, *Rhaptopetalum coriaceum oliv*, garlic, *Musanga cecropioides*, *Cassia occidentalis*, *Vitex dodiana*, *phyllanthus amarus*, *Lepidium latifolium*, hawthorn, celery, ginseng, juniper, *etc*.) have been used in traditional system of medicine for hypertensive treatment (Etuk, 2006). One of the tropical plants that may have antihypertensive effect is *Centella asiatica*. This plant contains high phenolic compounds, triterpenoid saponins and flavonoids, in particular quercetin, cathecin and rutin (Hussin et al., 2007). Quercetin and other flavonoids have been shown to promote relaxation of cardiovascular smooth muscle (antihypertensive effect) (Formica and Regelson, 1995). To date, there is only one study reporting the antihypertensive and bradycardia effects of *Centella asiatica*. Muangnongwa (2004) reported that oral administration of fresh *Centella asiatica* juice (32 g/kg) significantly decreased systolic blood pressure and heart rate in deoxycorticosterone acetate (DOCA)-salt hypertensive rats. However, there is no evidence for the mechanism addressing its antihypertensive effect. Furthermore, the effects of the crude extract from *Centella asiatica* on blood pressure and heart rate

have never been studied before. Therefore, the effects of *Centella asiatica* extract on rat aortic smooth muscle contraction and arterial blood pressure were investigated. It is hope that natural compounds found in *Centella asiatica* could reduce blood pressure. *Centella asiatica* may have pharmaceutical important and useful in hypertensive condition.

1.2 Research objectives

The experiments were designed to clarify the followings:

1. To study the effects of *Centella asiatica* extract and quercetin on the contraction of rat aortic rings *in vitro*.

2. To study the acute and sub-chronic effects of *Centella asiatica* extract and quercetin on blood pressure and heart rate of normotensive and N^G-Nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats.

1.3 Research hypothesis

Centella asiatica extract possesses antihypertensive effects which may involve the direct vasodilation action to vascular smooth muscles.

1.4 Expected results

1. The study will provide a scientific basis for the use of the *Centella asitica* extract for the treatment of hypertension.

2. The findings will provide the first evidence of acute and sub-chronic blood pressure lowering effects of the *Centella asiatica* extract in L-NAME induced hypertensive rats.

3. The findings will provide the first evidence of *in vitro* vasorelaxant activity of the *Centella asiatica* extract on rat aortic rings and the relaxant mechanisms.

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CHAPTER II

LITERATURE REVIEW

2.1 Asiatic Pennywort (*Centella asiatica* Linn.)

Asiatic pennywort (*Centella asiatica*), also known as gotu kola, pegapa, and buabok, is one of the local herb widely grown in Thailand and other Asian countries in both tropical and subtropical regions. *Centella asiatica* is claimed to possess various physiological effects (Jayathirtha and Mishra, 2004; Wongfhun, Gordon, and Apichartsrangkoon, 2010).

Centella asiatica is a perennial herbaceous creeper of the Apiaceae (Umbelliferae) family as shown in Table 2.1 (Brinkhaus et al., 2000), which grows to a length of 50 cm with fan shaped leaves. It is a tropical medicinal plant with a long history of therapeutic used for many conditions such as dermal disorder, vascular diseases (such as hypertension and altherosclerosis), microangiopathy, and inflammatory (De sanctis et al., 2001; Incandela et al., 2001; Suguna et al., 1996). The whole plant can be used for those medicinal purposes. *Centella asiatica* is said to have a direct effect in lowering blood pressure and is often referred to as a rejuvenating medicament in Ayurvedic Pharmacopoeia (Hussin et al., 2007). It is widely used as blood purifier as well as for treating high blood pressure, for memory enhancement and promoting longevity (Gohil, Patel, and Gajjar, 2010). *Centella asiatica* was reported to act on connective tissues of vascular wall, being effective in hypertensive

microangiopathy and venous insufficiency, and decreasing capillary filtration rate by improving microcirculatory parameters (Cesarone, 1992). Moreover, *Centella asiatica* has been reported to have anti-peroxidase and free radical scavenging activities (Katare and Ganachari, 2001; Jayashree et al., 2003).

Table 2.1 Systematic classification of *Centella asiatica* (Brinkhaus et al., 2000).

Classification	**Name**
Kingdom	Eukaryota
Subkingdom	Embryophyta
Division	Spermataphyta
Subdivision	Angiospermae
Class	Dicotyledoneae
Subclass	Rosidae
Superorder	Aralianae
Order	Araliales (Umbelliflorae)
Family	Apiaceae or Umbelliferae
Subfamily	Hydrocotyle
Genus	*Centella*
Species	*Centella asiatica*

This plant is closely related to Hydrocotyle species and produces characteristic essential oil and various type of flavonoid (Yoshinori, Reiko, and Tsunematsu, 1982). The main active compounds of *Centella asiatica* are saponins (also called triterpenoids), which include asiaticosides (in which a trisaccharide moiety is linked to the aglycone asiatic acid), madecassoside, and madasiatic acid (Gohil, Patel, and Gajjar, 2010). All parts of *Centella asiatica* are found to contain high phenolic contents (asiatic acid, asiaticoside, quercetin, rutin, kaempferol, rosmarinic acid,

chicoric acid and chlorogenic acid), which exhibit strong association with its antioxidative activities (Devkota et al., 2010; Gnanapragasam et al., 2004). *Centella asiatica* also contained numerous caffeic acid derivatives and flavonols, in particular quercetin and kaempferol (Castellani, Marai, and Vacchi, 1981), catechin, rutin, and naringin (Zainol et al., 2003). Moreover, the *Centella asiatica* extract is found to contain plant sterols, flavonoids, and other components with no known pharmacological activity namely tannins (20-25%), essential acid (0.1% with beta-chariophylen, trans-beta-pharnesen and germachrene D), phytosterols (campesterol, sitosterol, stigmasterol), mucilages, renins, free amino acids (alanine, serine, aminobutyrate, aspartate, glutamate, lysine, and treonine), flavonoids (derivates of chercetin and kempferol), an alkaloid (hydrochotine), a bitter component (vallerine), and fatty acids (linoleic, linolnelic, oleic, palmitic, and stearic acids) (Srivastava, Shukla, and Kumar, 1997). Asiaticoside is the principal bioactive ingredient in *Centella asiatica* since asiaticoside retains the most profound effect on antibacterial and fungicidal activity against pathogens and fungi (Hausen, 2006). Asiatic acid also exhibits bioactive efficacy (Park et al., 2007). For example, asiatic acid is known to control cell division in human hepatoma, colon cancer, breast cancer, melanoma cells, and cytotoxic activity on fibroblast cells (Coldren et al., 2003). Other components isolated from *Centella asiatica*, such as brahmoside and brahminoside, may be responsible for central nervous system and uterorelaxant actions. Crude extract containing glycosides isothankuniside and thankuniside showed antifertility action in mice (Heidari et al., 2007). Centelloside and its derivatives from *Centella asiatica* are found to be effective in the treatment of venous hypertension (Srivastava, Shukla, and Kumar, 1997).

Triterpenoid-rich extract from bamboo shavings significantly reduced the systolic blood pressure without change in heart rate of spontaneously hypertensive rats (SHR) (Jiao et al., 2007). Friedelin, a main triterpenoid compound isolated from bamboo shavings, were found to decrease vasoconstriction in rat aortic smooth muscle cells. In deoxycorticosterone acetate (DOCA)-salt hypertensive rats, oral administration of fresh *Centella asiatica* leaf juice at the dose of 24 and 32 g/kg significantly decreased systolic blood pressure and heart rate of hypertensive rats (Muangnongwa, 2004).

The triterpenic derivatives (asiaticoside), a major pentacycyclic triterpenoid saponin component of *Centella asiatica*, have been described to have wound healing action, antidepressant-like effect, antiulcer effect, antioxidant, and anti-inflammatory activities. The mechanisms by which asiaticoside exerts its antiulcer and anti-inflammatory effects may be associated with inhibition of nitric oxide (NO) synthesis by its inhibitory effect on inducible nitric oxide synthase (iNOS) (Liang et al., 2008). Healing of gastric ulcers was also accelerated by asiaticoside. Asiaticoside was found to promote angiogenesis, facilitate epithelial proliferation, and suppress myeloperoxidase (MPO) activity during ulcer healing stage (Cheng et al., 2004). Moreover, asiaticoside is transformed into its aglycone asiatic acid *in vivo* by hydrolysis. Several derivatives of asiaticoside and asiatic acid were shown protective effect against beta amyloid-induced neurotoxicity associated with the dementia of Alzheimer's disease (Wijeweera et al., 2006). Total triterpenic fraction of *Centella asiatica* (TTFCA) was found to be active on the microcirculation in venous and diabetic microangiopathy. TTFCA can improve collagen synthesis in and around the venous wall by modulation the action of human embryonic fibroblasts (Incandela et

al., 2001). Moreover, TTFCA was shown to improve microcirculation in venous hypertension and microangiopathy without side effects (Cesarone et al., 2001).

The phenolic compounds, one of the most widely occurring groups of phytochemical, are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants. These compounds are of considerable physiological and morphological importance in plants (Balasundram, Sundram, and Samman, 2006). Phenolic substances, especially polyphenols containing several hydroxyl groups directly associated with a cyclic benzene ring, are widely distributed in plant. There are several hundred plant phenolic compounds including phenolic acid and flavonoids (Stoclet et al., 2004). Zainol et al. (2003) demonstrated that the antioxidative activity of the different parts of *Centella asiatica* containing high phenolic contents was as good as that of α-tocopherol.

Antihypertensive effect of phenolic compounds has been demonstrated by many studies. The total dietary amount of polyphenols is approximately 1 g/day, being an amount that is higher than any other known dietary antioxidant. In fact, it is around 10 times higher than dietary vitamin C and 100 times higher than vitamin E and carotenoid intake (Medina-Remon et al., 2011). On the other hand, epidemiologic data have shown an inverse association between the risk of overall mortality or cardiovascular disease and the consumption of polyphenol rich foods such as fruits, vegetables, tea, olive oil and wine (Medina-Remon et al., 2011). Polyphenol intake, assessed *via* total polyphenol excretion in urine, was negatively associated with blood pressure levels and prevalence of hypertension in an elderly Mediterranean population at high cardiovascular risk. Participants with the highest intake of polyphenol rich foods showed the lowest blood pressure measurements (Medina-Remon et al., 2011).

Polyphenols in cocoa extract caused arterial vasodilation by increasing endothelial production of NO, resulting in the lowering of the blood pressure (Taubert, Roesen, and Schomig, 2007). The blood pressure lowering properties of chokeberry (*Aronia mitchurinii*) have been demonstrated *in vitro* and *in vivo* (Hellström et al., 2010). Chokeberry contains very high contents of polyphenols, namely phenolic acid, proanthocyanidins, anthocyanins, flavonols, and flavanones. In the study of SHR, blood pressure lowering effects of chokeberry juice and its polyphenols extract have been demonstrated (Hellström et al., 2010). The blood pressure lowering effects of juice and polyphenol extract seemed to be short term and were generally highest after 3 h from administration (50 mg/kg/day). Mean reductions in systolic blood pressure were 20.0 ± 8.0 and 15.0 ± 7.0 mmHg in chokeberry juice and polyphenol groups, respectively. Chokeberry polyphenols may possess their antihypertensive effect by enhancing endothelial NO production with an angiotensin converting enzyme (ACE)-independent mechanism, *e.g.* by activation of endothelial nitrix oxide synthase (eNOS) enzyme. Grape seeds extract containing phenolic compounds have been shown to activate eNOS, up-regulate eNOS in cultured endothelial cell and cause an endothelium dependent relaxation of blood vessels (Sivaprakasapillai et al., 2009). In humans, systolic and diastolic blood pressures were lowered after treatment with grape seeds extract (Sivaprakasapillai et al., 2009). In addition, the purple passion fruit peel (PFP) extract containing a mixture of bioflavonoids, phenolic acids, and anthocyanin, showed its antihypertensive effects in SHR (Zibadi et al., 2007). Systolic blood pressure was significantly lowered in SHR received diet supplement with PFP extract at 50 mg/kg for 8 weeks. The antihypertensive effect of PEP extract may be mediated through NO modulation (Zibadi et al., 2007).

Quercetin, a member of the flavonoid family, is one of the most prominent dietary antioxidants. It is ubiquitously present in food including vegetables, fruits, tea, and wine as well as countless food supplements and is claimed to exert beneficial health effects. Quercetin exerts the protective effects against various diseases such as osteoporosis, certain forms of cancer, pulmonary, cardiovascular diseases, and also against aging (Boots, Haenen, and Bast, 2008). The ability of quercetin to scavenge highly reactive species such as peroxynitrite and the hydroxyl radical is suggested to be involved in these possible beneficial health effects (Boots et al., 2008). Several studies had found that chronic oral treatment with quercetin could reduce blood pressure and restore endothelial function in hypertensive animal models. Oral administration of quercetin (1 μmol/L) could reduce blood pressure and prevented endothelial dysfunction (the vascular produced reactive oxygen species (ROS) and inactivate NO) in hypertensive animals (Lodi et al., 2009). In rat models of hypertension, quercetin at oral daily dose of 10 mg/kg for five weeks induced a significant reduction in systolic (-18%), diastolic (-23%), mean (-21%) arterial blood pressure and heart rate (-12%) in SHR, but had no effect in normotensive Wistar Kyoto rats. N_{ω}-nitro-L-arginine methyl ester (L-NAME) induces chronic hypertension in rats was prevented by a single oral daily administration of quercetin, 10 mg/kg fully prevented an increase in blood pressure. Quercetin (10 mg/kg) inhibited the development of DOCA-salt-hypertension in rats and this effect was similar to that of verapamil (20 mg/kg), an L-type calcium channel blocker that has been used in the treatment of hypertension (Perez-Vizcaino et al., 2009). The precise mechanism for quercetin modulation of endothelial function is not fully understood. One mechanism that has been reported is the enhancement in endothelial NO bioavailability achieved

via metabolism of superoxide anions and/or its reactive metabolites (Machha et al., 2007).

2.2 Substances secreted by the endothelium

Endothelium cells, located between the circulating blood and the media and adventitia of the blood vessels, collectively constitute a large and important organ. They respond to flow changes, stretch, and a variety of circulating substances. The vasoactive substances include nitric oxide, prostaglandins, and endothelins.

Nitric oxide (NO)

Important paracrine vasodilator released by endothelial cell is nitric oxide (NO). This endothelium-derived relaxing factor (EDRF) is released continuously in significant amounts by endothelial cells in the arterioles and contributes to arteriolar vasodilation in basal state. NO is synthesized from L-arginine in a reaction catalyzed by nitric oxide synthase (NOS). Three isoforms of NOS have been identified: NOS 1, found in nervous system: NOS 2, found in macrophages and other immune cells: and NOS 3, found in endothelial cells. NOS 1 and NOS 3 are activated by agents that increase intracellular calcium (Ca^{2+}) concentration, including the vasodilators acetylcholine and bradykinin. The NO that is formed in the endothelium diffuses to smooth muscle cells, where it activate soluble guanylyl cyclase (sGC), producing cyclic adenosine monophosphate (cAMP), which in turn mediates the relaxation of vascular smooth muscle. NO is inactive by hemoglobin.

When flow to a tissue is suddenly increased by arteriolar dilation, the large arteries to the tissue also dilate. This flow-induced dilation is probably due to local

release of NO. The products of platelet aggregation also cause release of NO and the resulting vasodilation propably helps keep blood vessels with and intact endothelium patent. This is in contrast to injured blood vessels, where the endothelium is damaged at the site of injury and platelets therefore aggregate and produce vasoconstriction (Ganong, 1995)

Prostacyclin (PGI_2)

Prostacyclin (PGI_2) produces vasodilation by stimulating adenylate cyclase, which increase cAMP levels in vascular smooth muscle cells. PGI_2 release in stimulated by either shear stress or endogenous mediators such as bradykinin, thrombin and serotonin. PGI_2 also prevents platelets from aggregating on the endothelial surface and consequent clot and maintaining blood flow around it (Ganong, 1995; Porth, 1994).

Endothelins

Endothelial cells also produce the most potent vasoconstrictor agent yet isolated, a polypeptide that was initially called endothelin. Subsequent research demonstrated that there is a family of similar 21 amino-acid polypeptides now called endothelin-1 (ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3). They are encoded by at least 3 different gene. ET-1 is produced by endothelial cell, and in humans its gene produces a 38-amino-acid proendothelin-1. Each is encoded by a different gene. Two different endothelin receptor have been cloned, both of which are coupled *via* G proteins to phospholipase C. The ET-A receptor, which is specific for ET-1 is found in many tissue and mediates the vasoconstriction produced by ET-1. The ET-B

receptor responds to all three endothelins. It may mediate vasodilation and it appears to mediate the developmental effects of the endothelins. The biological action of endothelins on hemodynamic are inducing contraction possibly veins more sensitive than arteries on vascular smooth muscle and causing initial depressor response followed by sustained pressor. Endothelins also have cardiac effects which are evoking positive inotropic and chronotropic effects on myocardium and stimulating intense vasoconstriction of coronary arteries (Clancy and Movicar, 1995; Porth, 1994)

2.3 Mechanisms of vascular smooth muscle contraction

The walls of arteries are made up of 3 layers: an outer layer of connective tissue called the adventitia, a middle layer of smooth muscle called the media, and an inner layer called the intima that is made up of the endothelium and underlying connective tissue (Barrett et al., 2010). The vessels are stretched by the force of cardiac ejection during systole, and the elastic tissue permits them to recoil during diastole. This maintains diastolic pressure and aids the forward motion of the blood (Mitrovic, 2010). Contraction and relaxation of vascular smooth muscle cause changes in both the volume of blood vessels and the local blood pressure. Vasoconstriction, refers to the widening of blood vessels, occurs when vascular smooth muscle contracts while vasodilation, refers to the narrowing of blood vessels, occurs when vascular smooth muscle relaxes. Excessive vasoconstriction leads to hypertension (high blood pressure), while excessive vasodilation as in shock leads to hypotension (low blood pressure).

Contraction of vascular smooth muscle is initiated by the concentration of intracellular Ca^{2+}. To increase intracellular Ca^{2+} can be achieved by mechanism of

extracellular Ca^{2+} transporting through voltage-dependent L-type Ca^{2+} channels (VDCCs) in the plasma membrane which activates Ca^{2+} released from the sarcoplasmic reticulum (SR) (Figure 2.1). In general, when membrane depolarized, VDCCs is opened, which is regulated by energy dependent ion pump (such as the Sodium-potassium adenosine triphosphatase (Na^{+}/K^{+}-ATPase)) and ion channels (such as the Ca^{2+} sensitive K^{+} channel). Local changes in intracellular Ca^{2+} so called Ca^{2+} sparks, resulted from movement of Ca^{2+} moving through VDCCs and thus activates cluster of ryanodine sensitive Ca^{2+} release channels in SR membrane. Ca^{2+} sparks lead to a further direct increase in intracellular Ca^{2+} concentration (Loscalzo, Libby, and Braunwald, 2008).

Biological agonists (norepinephrine (NE), angiotensin II (Ang II), and ET-1) binding to their receptors are able to increase intracellular Ca^{2+}concentration. After binding to the receptor, phospholipase C will be excited. Phospholipase C directly hydrolyzes phosphatidylinositol 4, 5 bisphosphate (PIP_2) to produce diacylglycerol (DAG) and inositol 1, 4, 5 trisphosphate (IP_3). DAG reversely conveys to membrane and trigger protein kinase C to increase the concentration of intracellular Ca^{2+}. In addition, IP_3 binds to its specific receptor found in the SR membrane to increase Ca^{2+} efflux from the SR into the cytoplasm. Thereafter, released Ca^{2+} in cytoplasm binds calmodulin to form Ca^{2+}-calmodulin complex which activate of myosin light chain kinase (MLCK), with phosphorylation of myosin light chain by this kinase, the myosin adenosine triphosphatase (ATPase) activity is increased and contraction sustained. In contrast, myosin light chain phosphatase (MLCP) dephosphorylates myosin light chain, reduction in myosin ATPase activity and contractile force develop and bring about the relaxation of vascular smooth muscle.

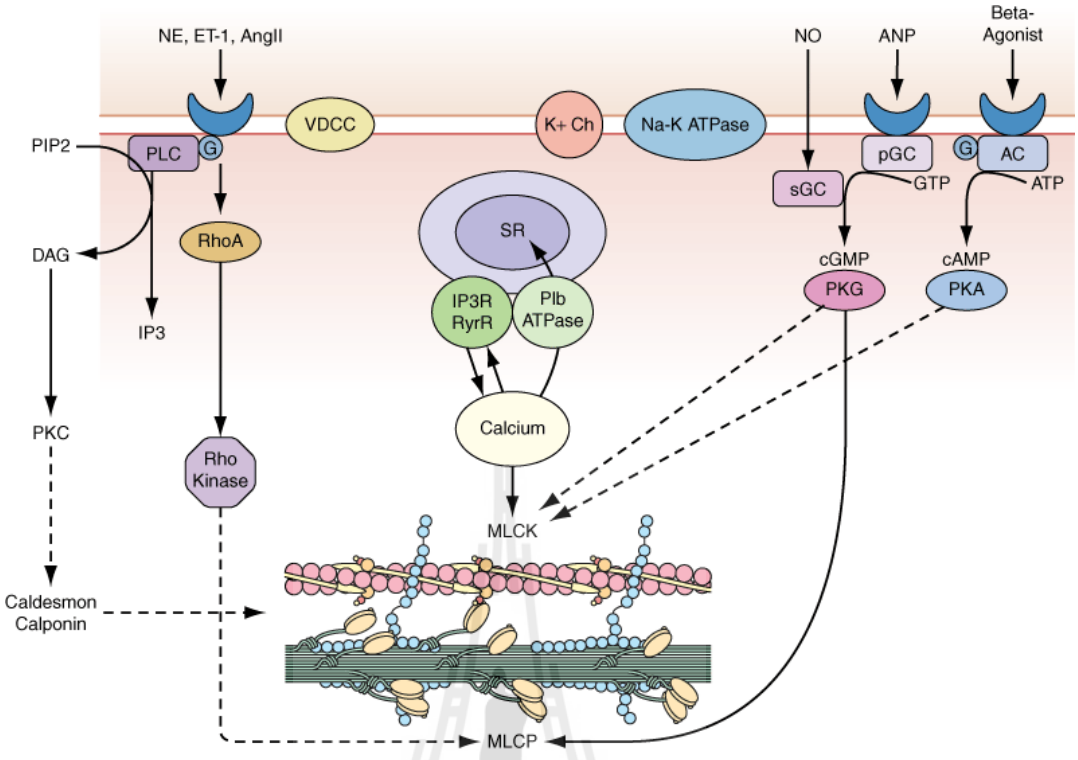

Figure 2.1 Regulation of vascular smooth-muscle cell Ca^{2+} concentration and actomyosin ATPase-dependent contraction. Norepinephrine (NE); endothelin-1(ET-1); angiotensin II (Ang-II); phosphatidylinositol 4,5-biphosphate (PIP_2); phospholipase C (PLC); diacylglycerol (DAG); G-protein (G); voltage-dependent calcium channel (VDCC); inositol 1,4,5-triphosphate (IP_3); protein kinase C (PKC); sarcoplasmic reticulum (SR); nitric oxide (NO); antrial natriuretic peptide (ANP); particular guanylyl cyclase (pGC); adenylyl cyclase (AC); soluble guanylyl cyclase (sGC); protein kinase G (PKG); protein kinase A (PKA); myosin light chain kinase (MLCK); myosin light chain phosphatase (MLCP); inositol 1,4,5-triphosphate receptor (IP3R); ryanadine receptor (RyrR); phospholamban (PIb) (Fauci et al., 2008).

β-Adrenergic agonist binds to G-protein coupled receptor and activates adenylyl cyclase that hydrolyzes adenosine triphosphate (ATP) to cAMP, while another agonist, NO, travels through membrane and activate sGC to convert guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). Like NO, atrial natriuretic peptides (ANPs) acting *via* a G-protein-coupled receptor activate sGC to convert GTP to cGMP. Both cAMP and cGMP activate protein kinase A and protein kinase G, respectively. These enzymes inactivates MLCK and decreases vascular smooth-muscle cell tone. In addition, protein kinase G can directly interact with the myosin-binding substrate subunit of MLCP, which in turn increasing phosphatase activity and decreasing vascular tone. Lastly, several mechanisms drive NO-dependent, protein kinase G mediated reductions in vascular smooth-muscle cell Ca^{2+} concentration, including phosphorylation-dependent inactivation of RhoA; decreased IP_3 formation; phosphorylation of the IP_3 receptor-associated cGMP kinase substrate, with subsequent inhibition of IP_3 receptor function; phosphorylation of phospholamban, which increases Ca^{2+} ATPase activity and sequestration of Ca^{2+} in the SR; and protein kinase G-dependent stimulation of plasma membrane Ca^{2+} ATPase activity, perhaps by activation of the Na^+/K^+-ATPase or hyperpolarization of the cell membrane by activation of Ca^{2+} -dependent K^+ channels (Loscalzo, Libby, and Braunwald, 2008).

2.4 Definition of hypertension

Hypertension is a term used to describe high blood pressure. Hypertension was defined according to the Seventh Report of the Joint National Committee on the Detection, Evaluation and Treatment for High Blood Pressure (JNCVII) as a systolic

blood pressure (SBP) ≥ 140 mmHg or a diastolic blood pressure (DBP) ≥ 90 mmHg. as a shown in Table 2.2 (Carretero and Oparil, 2000). The prevalence of systolic hypertension increase with age, reaching 80% by age 70 years. However, hypertension is more common among men than women in early adulthood, and only becomes more prevalent among women after age 55 years (Roberts et al., 2007).

There are three general types of high blood pressure. Essential hypertension, also known as primary hypertension or idiopathic hypertension, occurs when the condition has no known cause. When high blood pressure is caused by another condition or disease process, it is called secondary high blood pressure or secondary hypertension. When only the SBP number (the top number) is high, it is called isolated systolic hypertension, which is common in older adults (Roberts et al., 2007).

Table 2.2 The classification of hypertension in adults (Carretero and Oparil, 2000).

Category	Systolic, mm Hg		Diastolic, mm Hg
Optimal	<120	and	<80
Normal	<130	and	<85
High normal	130–139	or	85–89
Hypertension			
Stage 1 (mild)	140–159	or	90–99
Subgroup: borderline	140–149	or	90–94
Stage 2 (moderate)	160–179	or	100–109
Stage 3 (severe)	≥180	or	≥110
Isolated systolic hypertension	≥140	and	<90
Subgroup: borderline	140–149	and	<90

Hypertension remains a major modifiable risk factor for cardiovascular disease (CDV) (stroke, myocardial infarction, and heart failure), renal disease, and mortality despite important advances in our understanding of its pathophysiology and the

availability of effective treatment strategies. High blood pressure increases the risk of CDV for millions of people worldwide, and there is evidence that the problem is only getting worse (Carretero and Oparil, 2000). The hallmark of high blood pressure is elevated peripheral resistance, and resistance arteries thus play an important role in the pathogenesis of hypertension (Schiffrin, 1996). A number of factors increase blood pressure, including obesity, insulin resistance, high alcohol intake, high salt intake (in salt-sensitive patients), aging, sedentary lifestyle, stress, low potassium intake, and low calcium intake. Furthermore, many of these factors are additive, such as obesity and alcohol intake (Carretero and Oparil, 2000).

2.5 Rat model of hypertension

Animal models of experimental hypertension have been developed to obtain information on the mechanisms involved in the pathogenesis of hypertension and to test antihypertensive potential of new agents or drugs. There are various types of animal models of hypertension that can be divided into genetic and non-genetic models (secondary hypertension) as shown in Table 2.3 (Lerman et al., 2005). This review will focus on 3 common non-genetic models for hypertensive rats.

DOCA-salt hypertension

DOCA-salt is a common endocrine method to induce hypertension. The generation of hypertension usually requires the partial removal of renal mass in addition with DOCA and a high-salt diet (Monassier, Combe, and Fertak, 2006). DOCA, a mineralocorticoid, leads to an imbalance in renal sodium handling by increasing sodium and water reabsorption in the distal tubules in animals and

exacerbated by excess salt intake and reduced renal mass, leading to an increase in extracellular fluid and plasma volume (Yemane et al., 2009). In DOCA model increase in SBP in a 20-35 mmHg. This hypertension is associated with cardiac and renal hypertrophy, deposition of collagen in the left ventricle and kidney, proteinuria and a major collapse of plasma renin concentration (Monassier et al., 2006).

Fructose-induced hypertension

The fructose-induced hypertensive rat is widely used model to study high blood pressure (Verma, Bhanot, and McNeill, 1999), wherein feeding normal Wistar rats with a fructose enriched diet results in hyperinsulinemia, insulin resistance, hypertriglyceridemia, and consequently hypertension (Mohan, Jaiswal, and Kasture, 2009). In the production of fructose-induced hypertension or insulin resistance/hyperinsulinemia and in the studies of the effects of fructose loading, rats were often given a diet containing 35-72% fructose to eat or 10% or 15% fructose in water to drink *ad libitum* for a period of 2-12 weeks, with fructose treatment at these concentrations and for these durations, the increase in the blood pressure in rats was usually about 20 mmHg above the normal level and was much lower than that in DOCA-treated hypertensive rats (Dai and McNeill, 1995).

L-NAME induced hypertension

L-NAME, non-selective NOS inhibitor, induces hypertension by blocking NO synthesis. This model of hypertension is associated with an increased sympathetic tone, activation of the renin-angiotensin system (RAS), increased vascular resistance, and activation of the arachidonic acid-cyclooxygenase pathway (Santos et al., 2003).

Induction of hypertension by inhibition of eNOS by L-NAME can be amplified by a simultaneous high salt diet. This hypertension is associated with cardiac hypertrophy and fibrosis (Monassier et al., 2006). Santos et al. (2003) demonstrated that the chronic administration of L-NAME reduced the ion transporting function of the cardiac Na^+/K^+-ATPase and it is well established that the Na^+/K^+-ATPase participates in the modulation of the vascular smooth muscle contractility and tone.

Table 2.3 Various experimental models of hypertension (Lerman et al., 2005).

Secondary hypertension		**Genetic models**	
Renovascular	Two-kidneys. one-clip	Phenotype-driven	SHR
	One-kidney. one-clip		SHR-stroke prone
	Aortic coarctation		Dahl salt-sensitive rat
	Total occlusion		Genetically hypertensive rat
	Microembolization		Sabra model
Renal injury	Page-kidney		Lyon hypertensive rat
	Partial or total nephrectomy		Milan SHR
	Ijection-induced inflammation		Obesity-related
	Irradiation		Postmenopause-related
Vasoactive intervention	Renin-angiotensin-aldosterone	Genotype-driven	Renin angiotensin system
	Nitric oxide inhibition		Sympathetic NS
	Noradrenaline		Atrial natriuretic peptide
	Pressor prostaglandins		Nitric oxide
	DOCA-salt		Endothelin
Endocrine and dietary	Glucocoticoids		Neuropeptide Y
	Adrenal regeneration		Vasopressin
	Sex-hormone induced		Prostaglandin
	Dahl salt-sensitive		Kallikrein-kinin
	Pregnancy, pre-eclampsia		Vasopressin
	Psychological		Ion transport systems
Neurogenic	Environmental		
	Central NS stimulation		
	Baroreceptor denervation		

Deoxycorticosterone acetate (DOCA); nervous system (NS); spontaneously hypertensive rat (SHR).

2.6 Mechanisms of blood pressure regulation

Blood pressure is a measurement of the force against the walls of blood vessels as the heart pumps blood through the body. Blood pressure varies between a

maximum pressure (SBP), that occurs when the heart is pumping blood though the body, and a minimum pressure (DBP), that occurs when the blood is flowing between heartbeats. The other term known as mean arterial blood pressure (MABP) is considered an integrated parameter of blood pressure. MABP can be calculated according to the following formula: MABP = DBP + 1/3 (SBP – DBP) (Lee et al., 2006).

Role of NO on blood pressure regulation

NO/EDRF is an endogenous vasodilator substance releasing from endothelial cells in the blood vessels (Raghavan and Dikshit, 2004) that regulates the physiological and pathophysiological functions such as blood pressure regulation, inhibition of platelet aggregation, and neurotransmission (Li et al., 2004). NO that diffuses into smooth muscle cells activates sGC, which catalyzes the formation of cGMP, resulting in smooth muscle relaxation and vasodilation (Chen and Popel, 2006).

Synthesis of NO requires NOS in addition to the substrate L-arginine, and cofactors (nicotinamide adenine dinucleotide phosphate reduced (NADPH), tetrahydrobiopterin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), oxygen (O_2), and protoporphyrin IX) as shown in Figure 2.2. The activation of endothelial cell by acetylcholine, bradykinin, Ca^{2+} ionophore A23187, thrombin, or under shear stress, will increase intracellular Ca^{2+} levels, which leads to eNOS activation. The catalytic reaction incorporates two mono-oxygenation steps: L-arginine is hydroxylated by O_2 and NADPH to form *N*-hydroxy-L-arginine (NOHA) in the first step, and NOHA is oxidatively cleaved by either NOS, or also by

cytochrome P450 (cyt. P450) to yield L-citrulline and NO in the second step (Raghavan and Dikshit, 2004).

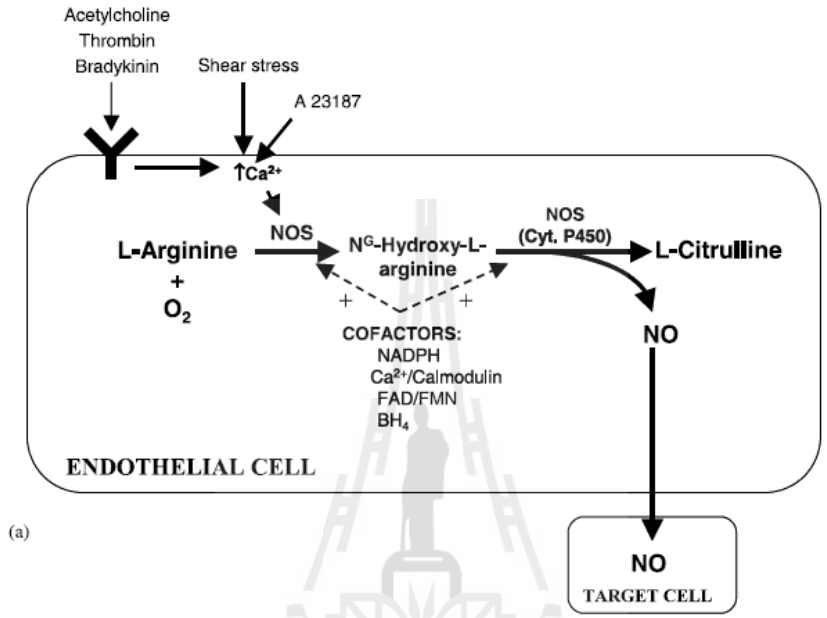

Figure 2.2 Synthesis of NO in the endothelium of the vasculature. Nitric oxide (NO); nitric oxide synthase (NOS); oxygen (O_2); nicotinamide adenine dinucleotide (NADPH); tetrahydrobiopterin (BH_4); flavin adenine dinucleotide (FAD); flavin mononucleotide (FMN); calcium (Ca^{2+}); cytochrome P450 (Cyt. P450) (Raghavan and Dikshit, 2004).

NO metabolism pathway is shown in Figure 2.3. In presence of O_2 and/or alveolar ventilation, NOS are activated and produce NO from L-arginine *via* L-citrulline. The NO activates sGC, which synthesize cGMP, which subsequently activates cGMP-kinase. Activation of K^+ channels is specific for this enzyme and subsequent Ca^{2+} channel inhibition evokes a reduction of intracellular Ca^{2+}

concentration, and finally resulting in vasodilation. The downstream effects of NO are limited by phosphodiesterase (PDE)-induced degradation of cGMP (Ghofrani et al., 2004).

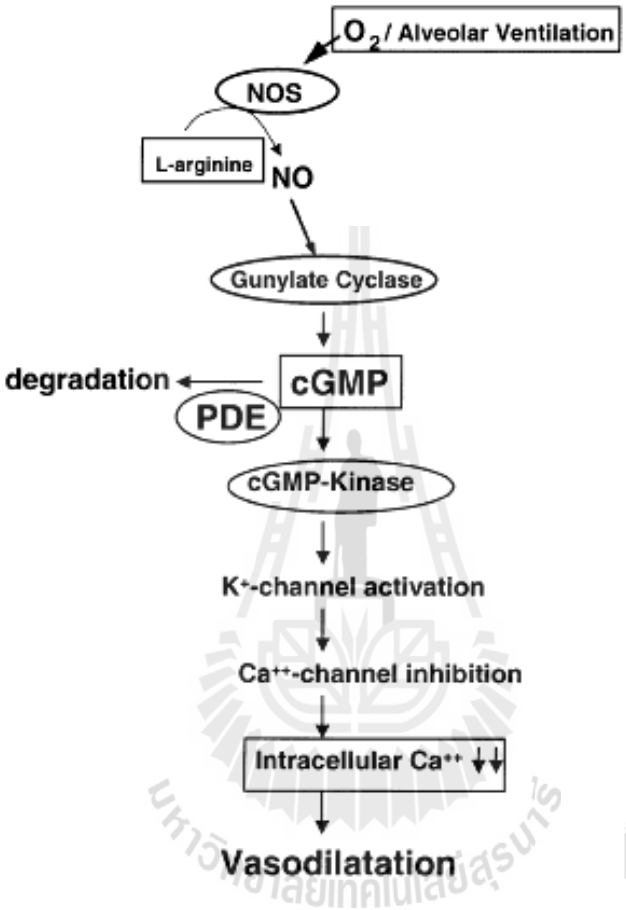

Figure 2.3 NO metabolism pathway. Nitric oxide (NO); nitric oxide synthase (NOS); oxygen (O_2); calcium (Ca^{2+}); Potassium (K^+); phosphodiesterase (PDE); cyclic guanosine monophosphate (cGMP) (Ghofrani et al., 2004).

Role of RAS on blood pressure regulation

RAS is a coordinated hormonal cascade in the control of cardiovascular, renal, and adrenal functions that governs fluid and electrolyte balance and plays a role in the regulation of arterial blood pressure (Pandey et al., 2009). The substrate of the system,

angiotensinogen, an α-glycoprotein, is mainly released from the liver and cleaved in the circulation by the enzyme rennin that is secreted from the juxtaglomerular apparatus of the kidney to form the decapeptide, angiotensin I (Ang-I). Ang-I is then further cleaved by a dipeptidyl carboxypeptidase ACE, which is widely present on the endothelial cells of many vascular beds including the lungs, to produce the octapeptide Angiotensin II (Ang-II). Ang-II is the physiologically active component of the system and can be degraded within seconds by peptidases (collectively termed angiotensinases) at different amino acid sites to form fragments, mainly angiotensin III (Ang-III) and angiotensin IV (Ang-IV) respectively. The action of Ang-II results from its binding to specific receptors, type 1 (AT-1) receptors and type 2 (AT-2) receptors. The AT-1 receptors are found in vascular and many other tissues and are almost certainly the receptors that transduce Ang-II mediated cardiovascular action. Two subtypes of AT-1 receptor, AT-1A and AT-1B, have been identified in mice and the results for their involvement in blood pressure regulation have been mixed. Ang-II is the principle mediator of the pathophysiological actions of RAS *via* the activation of specific Ang-II receptors. Virtually all of the known regulatory actions of Ang-II on blood pressure and osmoregulation have been attributed to the AT-1 receptor. These include potent vasoconstriction, aldosterone and vasopressin release, renal tubular sodium reabsorption, and decreased renal blood flow (Pandey et al., 2009; Paul, Mehr, and Kreutz, 2006). Several classes of blood pressure lowering (antihypertensive) medications may have some effects on this hormonal system. These two classes are ACE inhibitors and angiotensin receptor blockers (especially, Ang-II receptor blockers) that have the most significant effects on the RAS. These classes of drugs have become important clinical tools in the treatment of cardiovascular and renal

diseases such as hypertension, heart failure, and diabetic nephropathy (Paul et al., 2006).

Role of phenylephrine on blood pressure regulation

Phenylephrine is a selective α_1-adrenergic receptor agonist used primarily as a decongestant, as an agent to dilate the pupil, and to increase blood pressure (Okazaki et al., 1994). α_1-Adrenoceptor agonists bind to α-receptors on vascular smooth muscle (Figure 2.4). These receptors are linked to Gq-proteins that activate smooth muscle contraction through the IP_3 signal transduction pathway and induce smooth contraction and vasoconstriction, thus mimicking the effects of sympathetic adrenergic nerve activation to the blood vessels (Klabunde, 2011).

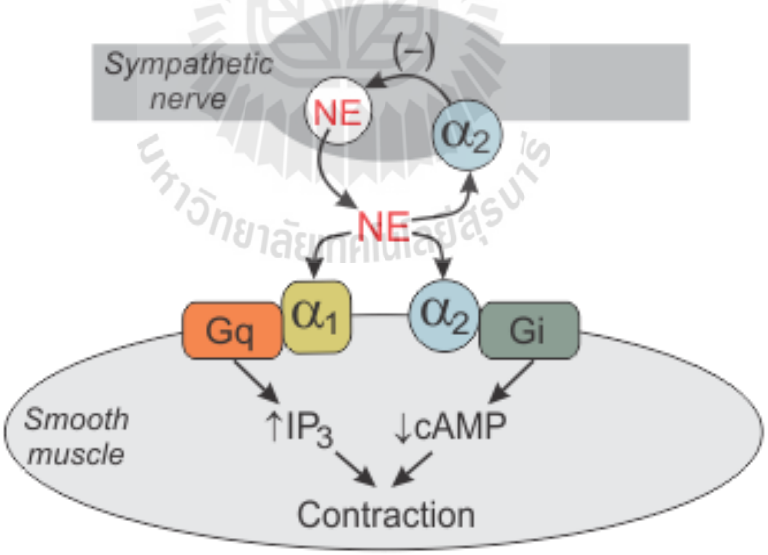

Figure 2.4 Norepinephrine (NE) binding to α-adrenoceptors activates the Gq-protein and inositol triphosphate (IP_3) pathway to cause vascular smooth muscle contraction (Klabunde, 2011a).

Role of L-NAME on blood pressure regulation

L-NAME is a non selective NOS inhibitor (Figure 2.5), inhibited the relaxation, and induces hypertension by blocking NO synthesis (Nakamura, Matsumoto, and Todoki, 2002; Santos et al., 2003).

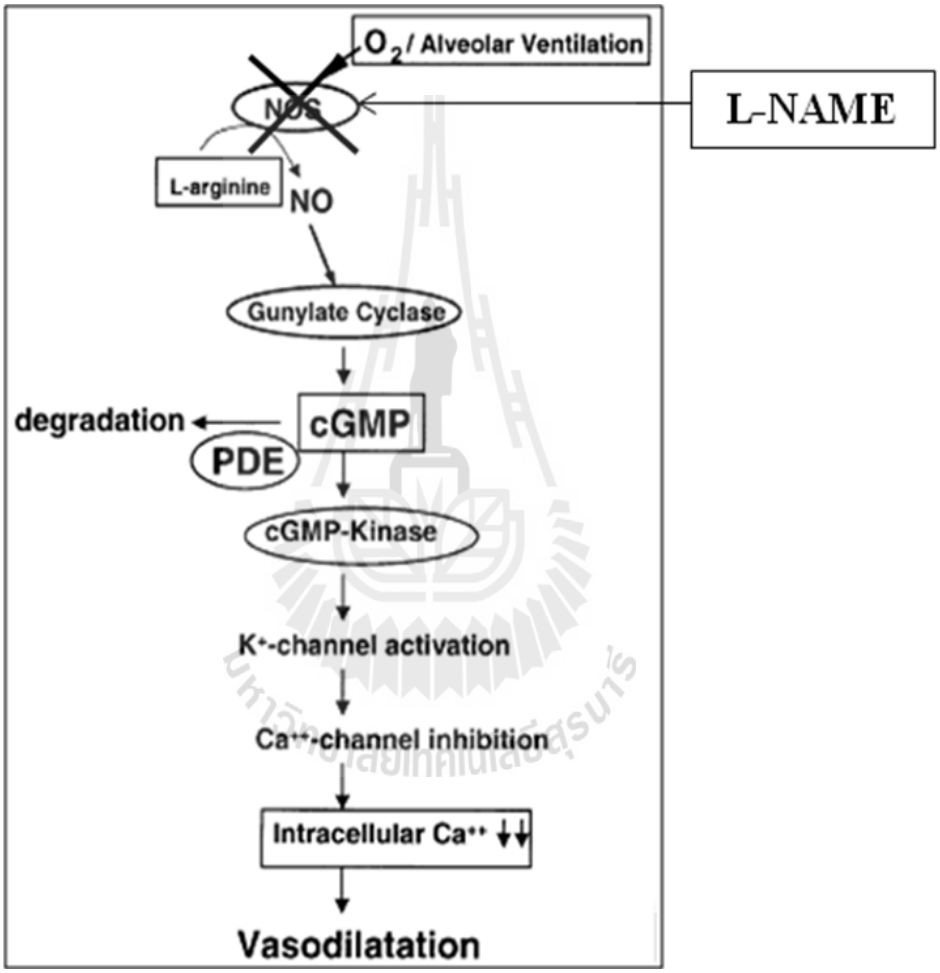

Figure 2.5 L-NAME blocking NO synthesis by inhibited NOS and induces vasoconstriction. Nitric oxide (NO); nitric oxide synthase (NOS); oxygen (O_2); calcium (Ca^{2+}); Potassium (K^+); phosphodiesterase (PDE); cyclic guanosine monophosphate (cGMP); N-nitro-L-arginine methyl ester (L-NAME) (Ghofrani et al., 2004).

Role of tetraethylammonium on blood pressure regulation

Tetraethylammonium is a non selective K^+ channel blocker (Nui et al., 2008). These drugs block the K^+ channels that are responsible for repolarization. Therefore, blocking these channels slows (delays) repolarization, which leads to an increase in action potential duration and an increase in the effective refractory period (Klabunde, 2011c).

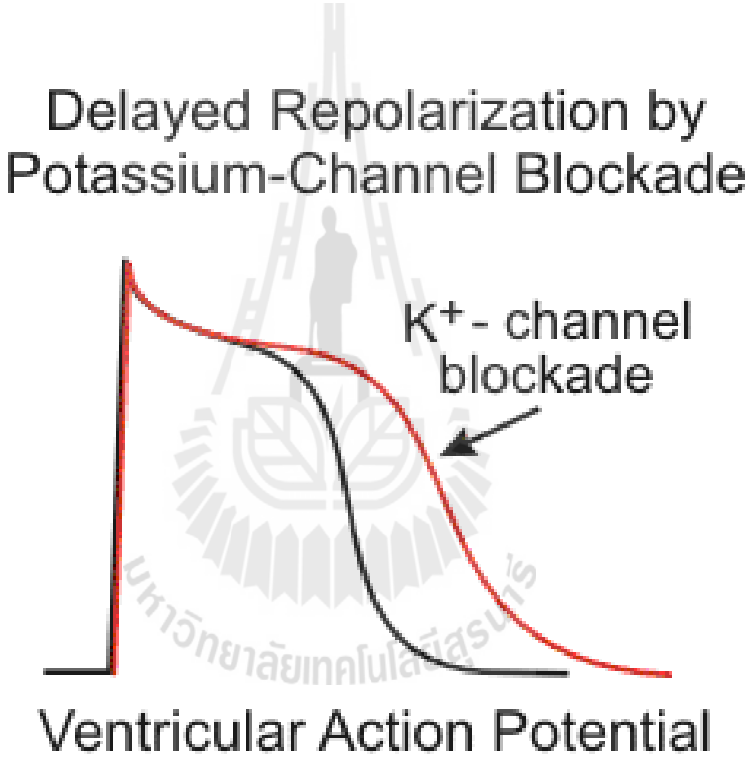

Figure 2.6 Ventricular action potential with delayed repolarization by potassium (K^+) channel blockade (Klabunde, 2011c).

Role of nifedipine on blood pressure regulation

Nifedipine is an L-type Ca^{2+} channel blocker (Figure 2.7) (Zhang and Meng, 2009). These channels are responsible for regulating the influx of Ca^{2+} into muscle

cells, which in turn stimulates smooth muscle contraction and cardiac myocyte contraction. In cardiac nodal tissue, L-type Ca^{2+} channels play an important role in pacemaker currents and in phase 0 of the action potentials. Therefore, by blocking Ca^{2+} entry into the cell, Ca^{2+} channel blocker cause vascular smooth muscle relaxation (vasodilation), decreased myocardial force generation (negative inotropy), decreased heart rate (negative chronotropy), and decreased conduction velocity within the heart (negative dromotropy), particularly at the atrioventricular node (Klabunde, 2011c).

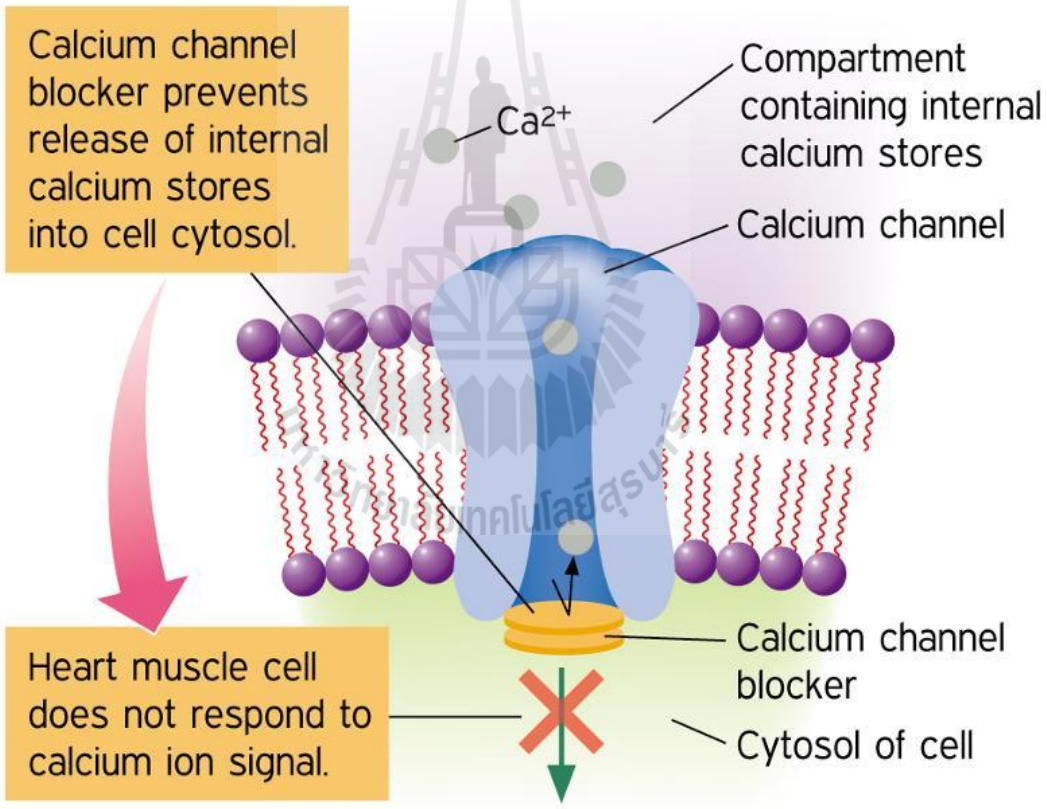

Figure 2.7 Calcium (Ca^{2+}) channel blockers block the entry of Ca^{2+} ions into muscle cells in the artery walls. This channel blocking action decreases blood pressure. (Gelb and Hol, 2002).

2.7 Phenolic compounds and vascular protection

Phenolic compounds are secondary metabolite that are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathway in plants (Randhir, Lin, and Shetty, 2004). These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance inplants. These compounds play an important role in growth and reproduction, providing protection against pathogens and predator (Bravo, 2009). Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic, anti-inflammatory, antimicrobial, antioxidant, anti-thrombotic, cardioprotective, and vasodilatory effects (Benavente-Garcianet et al., 1997; Manach, Mazur, and Scalbert, 2005; Middleton, Kandaswami, and Theoharides, 2000; Puupponen-Pimiä et al., 2001; Samman, Lyons-Wall, and Cook, 1998). The phenolic compounds posses an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to that of complex high-molecular weight polymer. Flavonoids, which bear the C6-C3-C6 structure, account for more than half of the over thousand different phenolic compounds (Balasundram, Sundram, and Samman, 2006).

Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. Over 4000 different flavonoids have been described and they are categorized into flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavonoids (Guardia et al., 2001). Flavonoids have a variety of biological effects in numerous mammalian cell systems, *in vitro* as well as *in vivo*. Flavonoids have been shown to exert antimicrobial, antiviral, antiulcerogenic, cytotoxic, antineoplastic,

mutagenic, anti-inflammatory, antioxidant, antihepatotoxic, hypolipidemic, antiplatelet, and antihypertensive activities (Guardia et al., 2001). Some authors have reported that flavonoids such as rutin (quercetin-3-rutinoside) and quercetin show antioxidant activity (Robinson, Tashjian, and Levine, 1975; Ueno et al., 1984). The bioavailability of flavonols, especially quercetin and glycosylate derivatives, has been widely studied and it is well known that polyphenols present in wines have high solubility and hence the bioavailability of these compounds in wine is high. Flavonoids, and polyphenols in general, have received considerable public, media and scientific interest in the possibility that increased intake of polyphenols may protect against chronic diseases such as cancer and CDV mainly due to their antioxidant properties (Padilla et al., 2005). Other polyphenols, such as resveratrol, a compound that is structurally similar to the phenylpropanoids phenolics, have been found to protect against certain types of cancer (Jang et al., 1997) and also to induce vascular relaxation (Orallo et al., 2002).

Consumption of polyphenol-rich food, such as fruits, vegetables, and beverages derived from plants, such as cocoa, red wine, and tea, may represent a beneficial diet in terms of cardiovascular protection. Stoclet et al. (2004) demonstrated a significant inverse correlation between polyphenol consumption and cardiovascular risk. Among the numerous plausible mechanisms by which polyphenols may confer cardiovascular protection, improvement of the endothelial function and inhibition of angiogenesis and cell migration and proliferation in blood vessels have been the focus. The study of Stoclet et al. (2004) indicated that, in addition to and independently from their antioxidant effects, plant polyphenols (1) enhance the production of vasodilating factors (NO, endothelium-derived hyperpolarizing factor (EDHF), and PGI_2) and

inhibit the synthesis of vasoconstrictor ET-1 in endothelial cell and (2) inhibit the expression of two major pro-angiogenic factors, vascular endothelial grown factor (VEGF) and matrix metalloproteinase-2 (MMP-2) in smooth muscle cells.

2.8 Plant polyphenols induced increase of endothelial nitric oxide synthase activity

Polyphenols have been shown to increase the formation of NO by eNOS. After the pioneer studies of Fitzpatrick et al. (1993), performed in the rat aorta with various grape products, similar conclusions were drawn from studies performed in various animals and human isolated vessels (Aldini et al., 2003; Cishek et al., 1997; Fitzpatrick et al., 2000; Flesch et al., 1998; Mendes et al., 2003; Ndiaye et al., 2003; Moura et al., 2002). Plant polyphenols produced endothelium-dependent vasorelaxation that was generally associated with increased cGMP formation and blunted by inhibitors of NOS, indicating that it was mediated by the NO-cGMP pathway. Fourthermore, the relaxant effect was strongly correlated with the concentration of polyphenols in wine (Burns et al., 2000).

Analysis of structure-activity (endothelial NO release and relaxation of isolated vessels) relationships for polyphenols of different classes has also been extensively examined (Ajay et al., 2003; Chan et al., 2000; Stoclet et al., 2000; Taubert et al., 2002). In red wine, the most active fraction have been found in flavonol-3-ol enriched oligomeric condensed tannins enriched fractions, especially dimmers and trimers, and the most active monomer was the anthocyanin delphinidin (Andriambeloson et al., 1998). Interestingly, other anthocyanins with closely related structures, such as malvidin and cyaniding, were found inactive. Substitution of the

flavan moiety with free hydroxyl residues at precise positions has been found important for induction of endothelial NO release (Taubert et al., 2002).

In endothelium, NO is formed from L-arginine by the constitutive eNOS in response to shear stress, circulating hormones, local autacoids, substance released by platelets, by the coagulation cascade and by the autonomic nervous system (Mombouli and Vanhoutte, 1999). The mechanism of NOS activation in response to the above enumerated stimuli, except shear stress, involve intracellular Ca^{2+}. By contrast, in response to shear strees, activation of the phosphoinositide 3 (PI3)-kinase/Akt pathway by blood flow causes rapid $[Ca^{2+}]_i$- dependent eNOS stimulation through its phosphorylation at Ser1177 (serine at codon 1177) (Dimmeler et al., 1999). Therefore, it can be concluded that plant polyphenols are potent inducers of endothelium-dependent vasorelaxation which involve both NO and EDHF (Figure 2.4). Current evidence suggests that the signaling pathways leading to polyphenol-induced NO and EDHF formation are at least partially common (Stoclet et al., 2004).

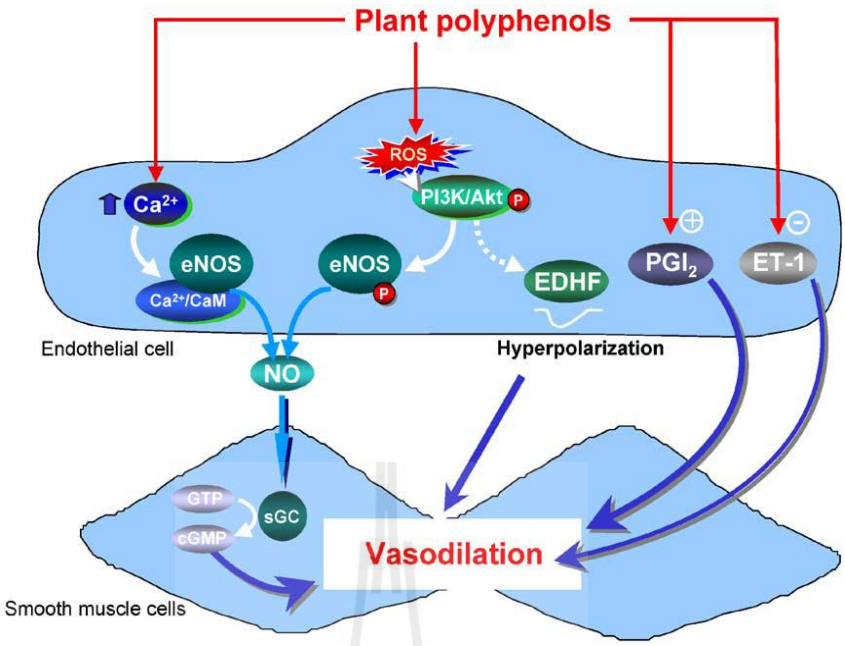

Figure 2.8 Acute endothelium-dependent effects of plant polyphenols. Plant polyphenols induce nitric oxide (NO)-mediated endothelium-dependent relaxations in isolated arteries. The activation of endothelial nitric oxide synthase (eNOS) is due to two distinct mechanisms: (a) an increase in $[Ca^{2+}]_i$ and (b) a phosphorylation of eNOS by the phosphoinositide 3 (PI3)-kinase/Akt pathway. In addition, plant polyphenols cause endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxations of isolated arteries consecutively to a localized and controlled formation of superoxide anions leading to the activation of PI3-kinase/Akt pathway. Polyphenol also increase endothelial prostacyclin release and inhibit the synthesis and the effects of endothelin 1 (ET-1). All these mechanism might contribute to explain the vasodilatory vasoprotective and anti-hypertension effects of polyphenols *in vivo*. Ca^{2+}/calmodulin complex (Ca^{2+}/CAM); soluble guanylyl cyclase (sGC) (Stoclet et al., 2004).

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CHAPTER III

EFFECTS OF *CENTELLA ASIATICA* EXTRACT AND QUERCETIN ON VASCULAR CONTRACTION IN RAT AORTIC RINGS

3.1 Abstract

The present study was designed to investigate the vasorelaxant effects of *Centella asiatica* extract and to elucidate the underlying mechanisms of its action on isolated rat aortic rings. Vasodilative activities of *Centella asiatica* extract, its dependences on endothelium and the involvement of nitric oxide, calcium channel, and potassium channel were investigated using isolated rat aortic rings that mounted in organ bath. Changes in isometric tension were recorded. The endothelium was ascertained by the absence of relaxation induced by acetylcholine (1 μM) in phenylephrine (1 μM) contracted preparations. In first set of experiments, aortic rings with or without functional endothelium precontracted with phenylephrine (1 μM) were exposed to 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mg/ml of *Centella asiatica* extract, quercetin (0.25 mM), double deionized distilled (DDD) water, or propylene glycol. With functional endothelium, the vasorelaxation effects of 2 and 4 mg/ml of *Centella asiatica* extract and quercertin were significantly increased relaxation compared with control (P<0.05). Without functional endothelium, *Centella asiatica* extract concentrations ranging from 0.0625 to 2 mg/ml induced relaxation in aortic

rings. *Centella asiatica* extract at 4 mg/ml and quercetin significantly increased relaxation, compared to control (P<0.05). The another set of experiments used only endothelium-intact aortic ring. Aortic rings were preincubated with N^G-nitro-L-arginine-methyl-ester (L-NAME, 75 mM), tetraethylammonium (10 µM) and nifedipine (1 µM) before adding *Centella asiatica* extract and quercetin (0.25 mM). *Centella asiatica* extract at the concentrations of 2 and 4 mg/ml and quercetin significantly increased relaxation in aortic rings that precontracted with L-NAME (0.75 mM), but did not alter the contraction effect of tetraethylammonium. Pretreatment with nifedipine inhibited *Centella asiatica* extract and quercetin-induced vasodilation. Our findings suggested that *Centella asiatica* extract has a potential ability to regulate vascular tones. Vasorelaxation induced by *Centella asiatica* extract was found to be endothelium-independent and unrelated to nitric oxide. Potassium (K^+) channel and L-type calcium (Ca^{2+}) channel were all involved in the relaxation process. Therefore, *Centella asiatica* extract may be considered as a candidate for drug development for the treatment of hypertensive conditions. All effects and mechanisms of *Centella asitica* extract on blood vessels are needed further investigation.

3.2 Introduction

Asiatic pennywort (*Centella asiatica*), also known as bua bok, is one of the local herbs grown in Thailand and other Asian countries in tropical and subtropical regions that is claimed to possess various physiological effects (Jayathirtha and Mishra, 2004; Wongfhun, Gordon, and Apichartsrangkoon, 2010). The whole plant is used for medicinal purposes. It is widely used as blood purifier as well as for treating

high blood pressure, memory enhancement, and promoting longevity (Gohil, Patel, and Gajjar, 2010). *Centella asiatica* contained numerous caffeic acid derivatives and flavonols (in particular, quercetin, kaempferol, catechin, rutin, and naringin) (Hussin et al., 2007). The phenolic compounds in *Centella asiatica* exhibit strong association with its antioxidative activities (Gnanapragasam et al., 2004). Rutin (quercetin-3-rutinoside) and quercetin show antioxidant activity (Robinson, Tashjian, and Levine, 1975; Ueno et al., 1984). Moreover, *Centella asiatica* was reported to act on connective tissues of vascular wall, being effective in hypertensive microangioparthy, venous insufficiency, and decreasing capillary filtration rate by improving microcirculatory parameters (Cesarone, 1992). Therefore, this study aimed to investigate potential vasorelaxant effects of *Centella asiatica* extract and quercetin as well as to elucidate the underlying mechanism of action in vasodilation using rat aorta.

3.3 Materials and methods

3.3.1 Plant material

Centella asiatica plant was obtained from local market in Nakhon Ratchasima province during June-August 2010. A voucher specimen number of *Centella asiatica* is BFK184894 from BGO Plant Database, The Botanical Garden Organization, Ministry of Environment and Natural Resources, Thailand.

3.3.2 Preparation of plant extract

Edible parts of *Centella asiatica* were washed with copious amounts of water and allowed to air dry at room temperature for 2 to 3 h. The plant was then cut into small thin pieces and dried at 40-50 $^{\circ}$C in hot air oven for 2 to 4 days. The dried

thin pieces of plant were powdered using an electric mill with a 1 mm mesh. The dried powder was extracted by maceration method with 80% aqueous ethanol (100 g dried powder/500 ml of 80% aqueous ethanolic solution) for 7 days in the dark at room temperature. The obtained suspension was filtered though No.1 Whatman filter paper (Whatman Internation Ltd., Maidstone, England). The filtrate was collected, concentrated using a rotary evaporator (Rotavapor® model R-205, Buchi, Switzerland) and then converted into crude extract by freeze dryer (Labconco Corporation Ltd., Missouri, USA). The obtained crude extract was stored at -20 °C until further used and the percentage yield was determined. This stock extract was used in all experiments performed in this thesis. On the day of each experiment, *Centella asiatica* extract was freshly prepared by dissolving in double deionized distilled (DDD) water at the desired concentration.

3.3.3 Animals

Male Wistar rats (245.49 ± 10.36 g) were obtained from Institutional Animal Care, Suranaree University of Technology (SUT). They were maintained under standard laboratory conditions (12:12 h dark-light cycle, ambient temperature 20 ± 1 °C) with free access to food and water. All studies were conducted under permit of the SUT Animal Care and Use Committee.

3.3.4 Drugs and chemicals

Phenylephrine hydrochloride, acetylcholine, tetraethylammonium chloride, nefidipine, propylene glycol, N^G-nitro-L-arginine-methyl-ester (L-NAME), quercetin, and Krebs-Henseleit buffer were all purchased from Sigma Aldrich

Chemical Ltd. (Sigma, St. Louis, MO, USA). Sodium carbonate anhydrous, sodium bicarbonate, and calcium choride dihydate were all purchased from BDH Prolabo® (VWR International Ltd., Lutterworth, UK). All other chemicals were of reagent grade. All the drug solutions were prepared at the time of use. Quercetin was dissolved in propylene glycol. For stock solutions, all other drugs were dissolved in DDD water.

3.3.5 Preparation of the solution

2% Sodium carbonate

2% Sodium carbonate solution was prepared by dissolving 2 g of sodium carbonate (Na_2CO_3; BDH Ltd., UK) to 100 ml of DDD water. This solution was adjusted the volume to 100 ml with DDD water in volumetric flask.

Modified Krebs-Henseleit buffer (pH 7.4)

Modified Krebs-Henseleit buffer, pH 7.4 was (d-glucose 2.0 g/L, magnesium sulfate (anhydrous) 0.141 g/L, potassium phosphate monobasic 0.16 g/L, potassium chloride 0.35 g/L, sodium chloride 6.9 g/L) prepared by adding the powdered medium 9.6 g of Krebs-Henseleit buffer (Sigma, St. Louis, MO, USA) to 90% of final required volume of DDD water (water temperature should be 15-20 $^{\circ}$C) and gently stirred until dissolved. To each liter of the solution, 0.373 g of calcium chloride dihydrate ($CaCl_2.H_2O$; BDH Ltd., UK) were added and stirred until dissolved. After that, 2.1 g sodium bicarbonate ($NaHCO_3$; BDH Ltd., UK) was added to each liter of solution being prepared, and stirred until dissolved. The pH of the

solution was then adjusted to 7.4 with 1 M HCl or 1 M NaOH. This solution was adjusted the volume to 1,000 ml with DDD water in volumetric flask.

1 µM Phenylephrine hydrochloride

Stock solution (10 ml) 0.6 mM phenylephrine hydrochloride was prepared by dissolving 1.222 mg of phenylephrine hydrochloride (Sigma, St. Louis, MO, USA) in 10 ml of DDD water and kept in -20 °C. To prepare 1µM phenylephrine hydrochloride in organ bath chamber, 10 µl of 0.6 mM phenylephrine hydrochloride was added to 5990 µl of modified Krebs-Henseleit buffer (pH 7.4) to make up 6 ml final volume.

1 µM Acetylcholine

Stock solution (10 ml) 0.6 mM acetylcholine was prepared by dissolving 0.0109 g of acetylcholine (Sigma, St. Louis, MO, USA) in 10 ml of DDD water and kept in -20 °C. To prepare 1µM acetylcholine in organ bath chamber, 10 µl of 0.6 mM acetylcholine was added to 5990 µl of modified Krebs-Henseleit buffer (pH 7.4) to make up 6 ml final volume.

0.25 mM Quercetin

Stock solution (10 ml) 0.25 M quercetin was prepared by dissolving 0.755 g of quercetin (Sigma, St. Louis, MO, USA) in 10 ml of propylene glycol (Vidhyasom, Co. Ltd., Thailand). To prepare 0.25 mM quercetin in organ bath chamber, 6 µl of 0.25 M quercetin was added to 5994 µl of modified Krebs-Henseleit buffer (pH 7.4) to make up 6 ml final volume.

75 mM L-NAME

Stock solution (10 ml) 1 M L-NAME was prepared by dissolving 2.69 g of L-NAME (Sigma, St. Louis, MO, USA) in 10 ml of DDD water and kept in -20 °C. To prepare 75 mM L-NAME in organ bath chamber, 450 µl of 1 M L-NAME was added to 5550 µl of modified Krebs-Henseleit buffer (pH 7.4) to make up 6 ml final volume.

10 mM Tetraethylammonium chloride

Stock solution (10 ml) 1 M tetraethylammonium chloride was prepared by dissolving 1.657 g of tetraethylammonium chloride (Sigma, St. Louis, MO, USA) in 10 ml of DDD water and kept in -20 °C. To prepare 10 mM tetraethylammonium chloride in organ bath chamber, 60 µl of 1 M tetraethylammonium chloride was added to 5940 µl of modified Krebs-Henseleit buffer (pH 7.4) to make up 6 ml final volume.

1 µM Nifedipine

Stock solution (10 ml) 25 mM nifedipine was prepared by dissolving 0.0865 g of nifedipine (Sigma, St. Louis, MO, USA) in 10 ml of DDD water and kept in -20 °C. To prepare 1 µM nifedipine in organ bath chamber, 0.24 µl of 25 mM nifedipine was added to 5999.76 µl of modified Krebs-Henseleit buffer (pH 7.4) to make up 6 ml final volume.

3.3.6 Determination of plant extract yield

The yield of evaporated dried extracts based on dry weight basis was calculated from the following equation (Stanojevic et al., 2009):

% Yield (g/100 g of dry plant material) = $(W_1 \times 100) / W_2$

where W_1 was the weight of the extract after the solvent evaporation and W_2 was the weight of the dry plant material.

3.3.7 Determination of total phenolic contents

The total phenolic compounds of *Centella asiatica* extract were measured according to the Folin-Ciocalteu reagent method that was adapted from the method of Minussi et al (2003). Briefly, *Centella asiatica* extract was dissolved in 10% ethanol. The reaction mixtures consisted of *Centella asiatica* extract solution (200 µl) and 4 ml of 2% sodium carbonate (Na_2CO_3, BDH Ltd., UK). Two minutes after mixing, 200 µl of Folin-Ciocalteu reagent was added and incubated at room temperature for 30 min. The mixtures were measured absorbance by using a spectrophotometer (CECIL 1011, England) at 750 nm. The total phenolic compounds were expressed as gallic acid equivalents (GAE) in milligrams per gram of dry extract. All determinations were performed in triplicate.

3.3.8 Preparation of isolated thoracic aortic rings

Rats (n=160) were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg; Cevesanté animale, France) to produce a deep anesthesia. The chest of the rat was opened. The descending thoracic aorta (3-5 cm) was removed out immediately and placed in a petridish containing room temperature

Krebs-Henseleit buffer modified. Fat and connective tissue were dissected out, cut into ring about 3 mm in length (Zhang and Meng, 2009) and placed in organ bath containing 6 ml of Krebs-Henseleit Buffer (pH 7.4) solution. The bath solution was maintained at 37 °C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2 (Rattmann et al., 2008). The thoracic aortic rings, with or without functional endothelium were prepared carefully so that the endothelial cell were not damaged.

3.3.9 Organ bath chamber experiment

Equipment used in this chapter included organ bath system volume 6 ml with constant temperature circulator (Bioer technology Co., Ltd.), isometric transducer (Panlab, Spain), gas tank for delivery of 95% O_2 and 5% CO_2 mixture (Thai special gas Co., Ltd.) and Tension was recorded by PowerLab system equipment with Program Chart (Pty ADInstrument, Ltd., LabChart 5, Australia) connected to computer. Each of these rings was suspended horizontally in the organ bath between two stainless hooks. One of the hooks was fixed to the chamber wall whereas the other was attached to an isometric force transducer. The rings were continuously superfused with prewarmed (37 °C), aerated (95% O_2/5% CO_2) modified Krebs-Henseleit buffer solution. The rings were initially stretched until resting tension reached 1 g and allowed to equilibrate for 45 min (Mascher et al., 2006).

Two aortic rings from each rat were used. The rings were equilibrated for 45 min under 1 g resting tension before exposition to any drug. During the period of stabilization, the bath solution was replaced every 15 min (Machha et al., 2007). Experiments were done using aortic rings with and without functional endothelium. The presence of functional endothelium was confirmed by the ability of acetylcholine

(at a final concentration of 1 μM) to fully relax aortic preparations precontracted for 20 min with phenylephrine (at a final concentration of 1 μM). For the endothelium-denuded preparations, endothelium was removed by gently rubbing the intimal surface with a glass rod. The successful removal of the endothelium was ascertained by the absence of relaxation induced by acetylcholine (1 μM) in aortic preparations precontraced for 20 min with phenylephrine (1 μM) (Rattmann et al., 2008). After that, the organ was washed out several times in intervals of 10 min until it returned to its initial tension (Nguelefack et al., 2009). If a ring failed to contract in response to phenylephrine or failed to relax in response to acetylcholine, it was replaced with a new ring.

In the first set of experiments, aortic rings with or without functional endothelium precontracted with phenylephrine (1 μM) for 20 min, were exposed to 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mg/ml (final concentrations) of *Centella asiatica* extract, quercetin (at a final concentration of 0.25 mM, we tested the *in vitro* final concentrations of 0.1-1 mM quercetin and found that quercetin at the final concentration of 0.25 mM was needed to prevent the enhancement of contraction to phenylephrine during three repetitions) DDD water (at the same volume as *Centella asiatica* extract was added), and propylene glycol (at the same volume as quercetin was added) for 15 min.

The another set of experiments was used only endothelium-intact aortic rings in order to study the relaxation of smooth muscle of aortic rings and investigate the mechanism involved. Aortic rings were incubated for 15 min with L-NAME (a non-selective nitric oxide synthase inhibitor) at a final concentration of 75 mM (Kalliovalkama et al., 1999) or tetraethylammonium (a non selective potassium (K^+)

channel blocker) at a final concentration of 10 mM (Nui et al., 2008), before adding the *Centella asiatica* extract (at final concentrations of 0.0625, 0.125, 0.25, 1, 2, and 4 mg/ml), quercetin (at a final concentration of 0.25 mM), DDD water (at the same volume as *Centella asiatica* extract was added), or propylene glycol (at the same volume as quercetin was added) for 15 min. To elucidate whether an L-type calcium (Ca^{2+}) channel was the target of *Centella asiatica* extract for vascular action, aortic rings were preincubated with nifedipine (an L-type Ca^{2+} channel blocker) at a final concentration of 1 μM or without nifedipine (Zhang and Meng, 2009) for 15 min before contracted with phenylephrine (1 μM), then *Centella asiatica* extract (0.0625, 0.125, 0.25, 1, 2, and 4 mg/ml), quercetin (0.25 mM), DDD water (at the same volume as *Centella asiatica* extract), and propylene glycol (at the same volume as quercetin) was added. Each aortic ring was exposed to only one of these drugs. Changes in tension were recorded using Program Chart (ADInstrument pty, Ltd. LabChart 5, Australia) (Chen et al., 2004).

Results were expressed as percentage of relaxation:

$$\% \text{ Relaxation} = \frac{R1-R2}{R1-B} \times 100$$

Where R1 is the contraction response to phenylephrine, L-NAME, and tetraethylammonium; R2 is the response after adding *Centella asiatica* extract, quercetin, and propylene glycol; B is baseline of aortic ring (Wongcome et al., 2007).

3.4 Statistical analysis

All values were expressed as mean ± S.E.M. Statistical analysis was determined by Student's *t*-test for paired comparisons between responses in rings from

the same concentration (SigmaStat software 3.5; St. Louis, MO, USA and and all graphs were created by SigmaPlot software version 10, Systat Software Inc., USA). *P*-values less than 0.05 ($P<0.05$) were considered statistically significant.

3.5 Results

3.5.1 The percent yield and total phenolic content of *Centella asiatica* extract

The percent yield of *Centella asiatica* extracted by 80% aqueous ethanol was 8.04% (Table 3.1). Total phenolic content of extract was 87.47 ± 1.74 mg gallic acid/g dry extract (Table 3.1).

Table 3.1 The percent yield and total phenolic content of *Centella asiatica* extract.

Parameters	Mean ± S.E.M.
Yield (%) dried plant	8.04%
Total phenolic content (mg gallic acid/g dry extract)	87.47 ± 1.74

3.5.2 Effects of *Centella asiatica* extract and quercetin on endothelium-intact aortic rings precontracted with phenylephrine

In endothelium-intact aortic rings precontracted with 1 μM phenylephrine, dose-dependent vasorelaxation effects of *Centella asiatica* extract 0.0625-4 mg/ml was demonstrated, while aortic rings treated with DDD water did not show vasorelaxation effect (Figure 3.1). *Centella asiatica* extract at concentrations of 2 and 4 mg/ml were significantly increased relaxation (80.11 ± 22.90% and 109.93 ± 27.79%) compared to DDD water, respectively ($P<0.05$, one way ANOVA).

In addition, quercetin also induced relaxation effect in phenylephrine precontracted endothelium-intact aortic rings while the propylene glycol had no effect as shown in Figure 3.2. Quercetin at concentration of 0.25 mM significantly relaxed contraction effect of phenylephrine (89.47 ± 6.14%) when compared to control (propylene glycol, 4.58 ± 4.78%, P<0.05). while the propylene glycol had no effect.

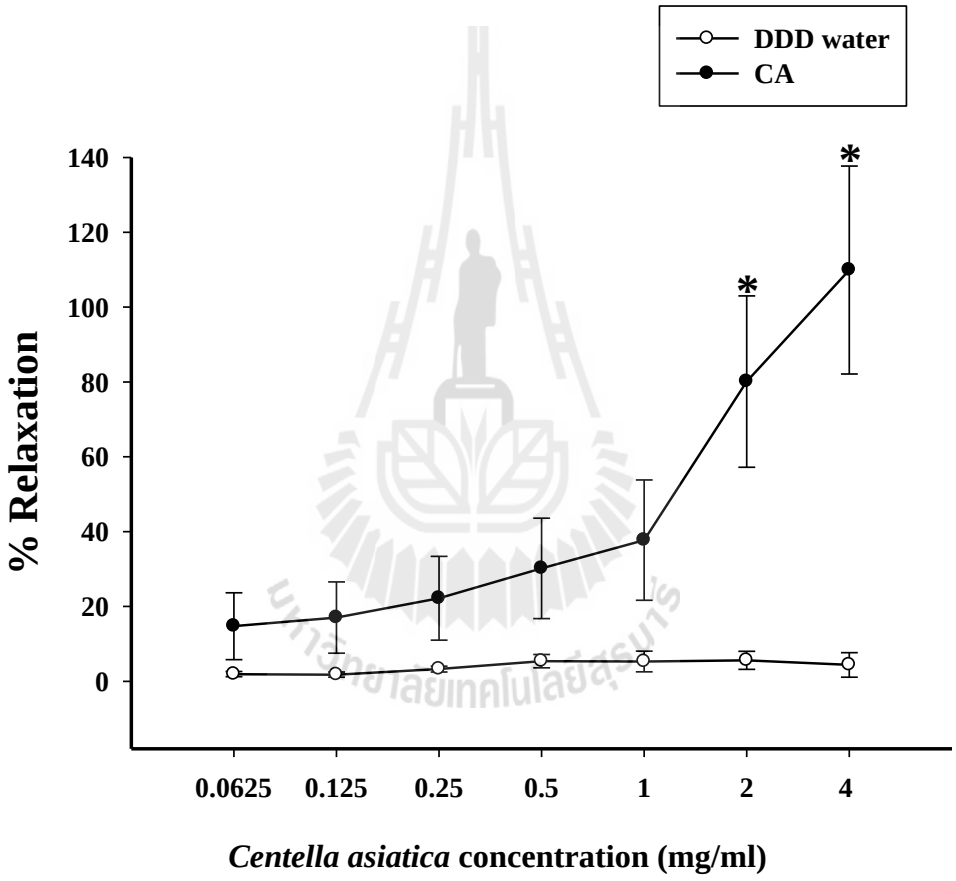


Figure 3.1 Relaxation effects of *Centella asiatica* extract (CA) and double deionized distilled (DDD) water on phenylephrine precontracted endothelium-intact aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). * Significantly different from DDD water, P<0.05 (Student's *t*-test).

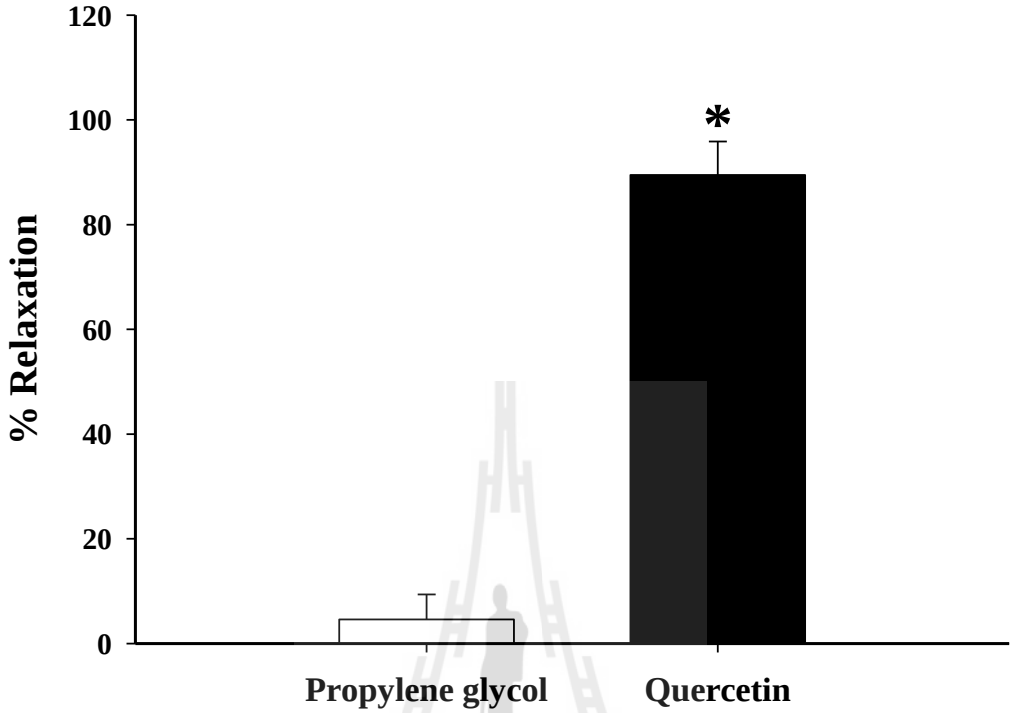


Figure 3.2 Relaxation effects of quercetin and propylene glycol on phenylephrine precontracted endothelium-intact aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). * Significantly different from propylene glycol, $P<0.05$ (Student's *t*-test).

3.5.3 Effects of *Centella asiatica* extract and quercetin on endothelium-denuded aortic rings precontracted with phenylephrine

A concentration response curve for *Centella asiatica* extract (0.0625 - 4 mg/ml) was also established in endothelium-denuded aortic rings precontracted with phenylephrine (1 µM), as shown in Figure 3.3. *Centella asiatica* extract induced a concentration dependent relaxation, while DDD water did not. At the concentration of 4 mg/ml, *Centella asiatica* increased relaxation by 73.90 ± 15.92%, which was significantly higher than control (DDD water, 7.05 ± 11.57%, $P<0.05$).

Vasorelaxation effects of *Centella asiatica* extract on endothelium-intact and -denuded were not significantly different (Figure 3.4), indicating the vasorelaxation caused by *Centella asiatica* extract at high concentration was endothelium-independent.

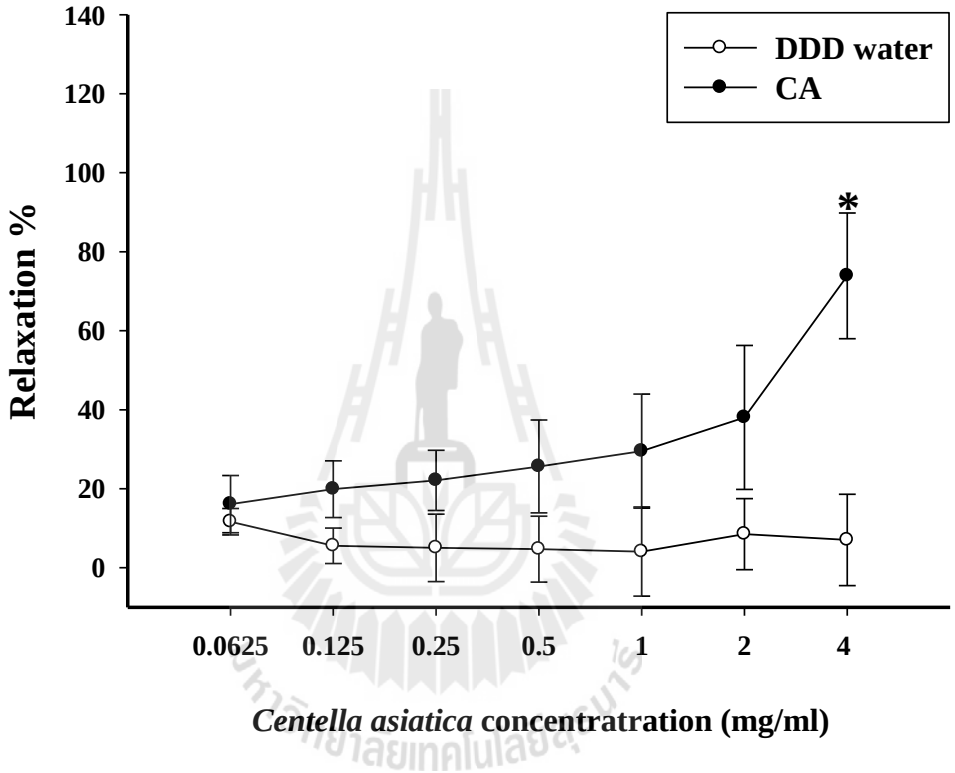


Figure 3.3 Relaxation effects of *Centella asiatica* extract (CA) and double deionized distilled (DDD) water on phenylephrine precontracted endothelium-denuded aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). * Significantly different from DDD water, $P<0.05$ (Student's *t*-test).

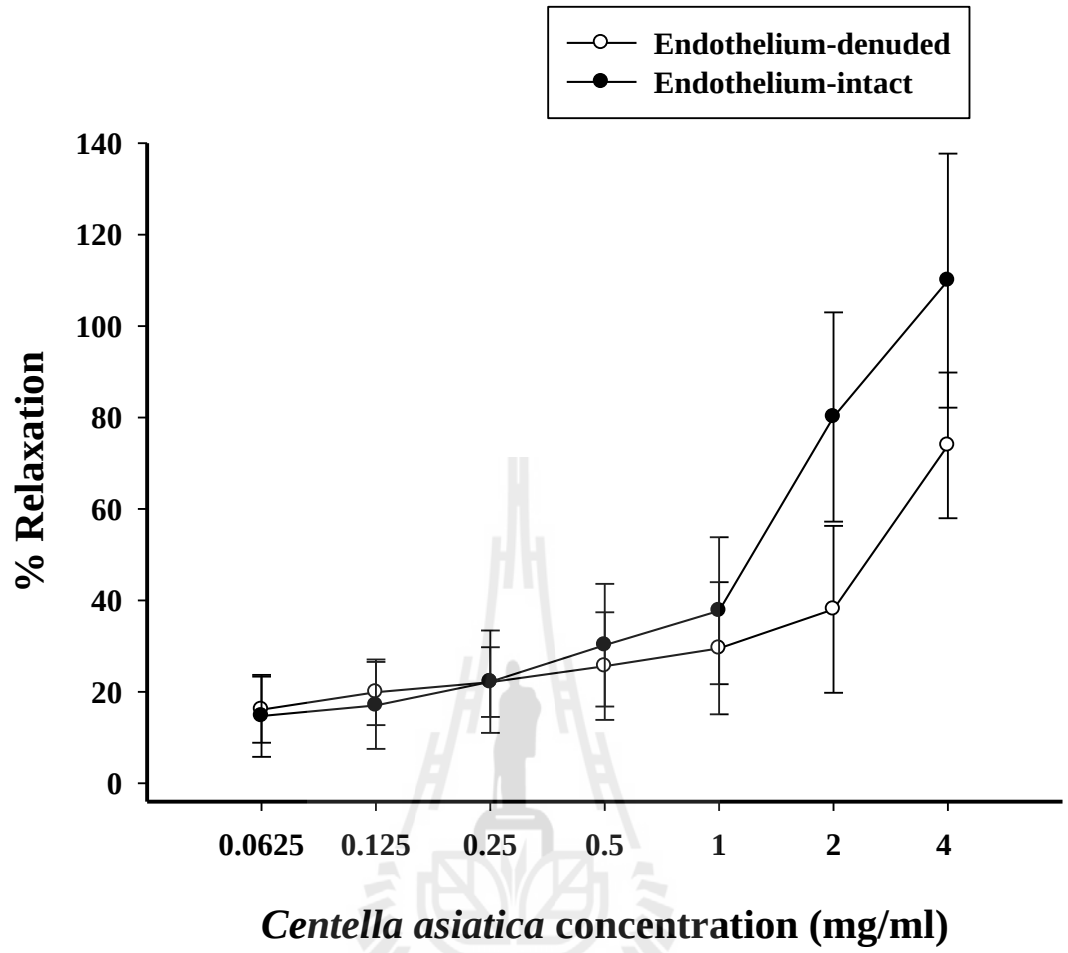


Figure 3.4 Relaxation effects of *Centella asiatica* extract (CA) on phenylephrine precontracted endothelium-intact and -denuded aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). There is no significant difference between endothelium-intact and endothelium-denuded treatment, $P<0.05$ (Student's *t*-test).

As presented in Figure 3.5, quercetin at a concentration of 0.25 mM significantly reduced the contraction induced by 1 μM phenylephrine (81.23 ± 11.42%) in endothelium-denuded aortic rings when compared with propylene glycol (4.30 ± 1.51%, $P<0.05$).

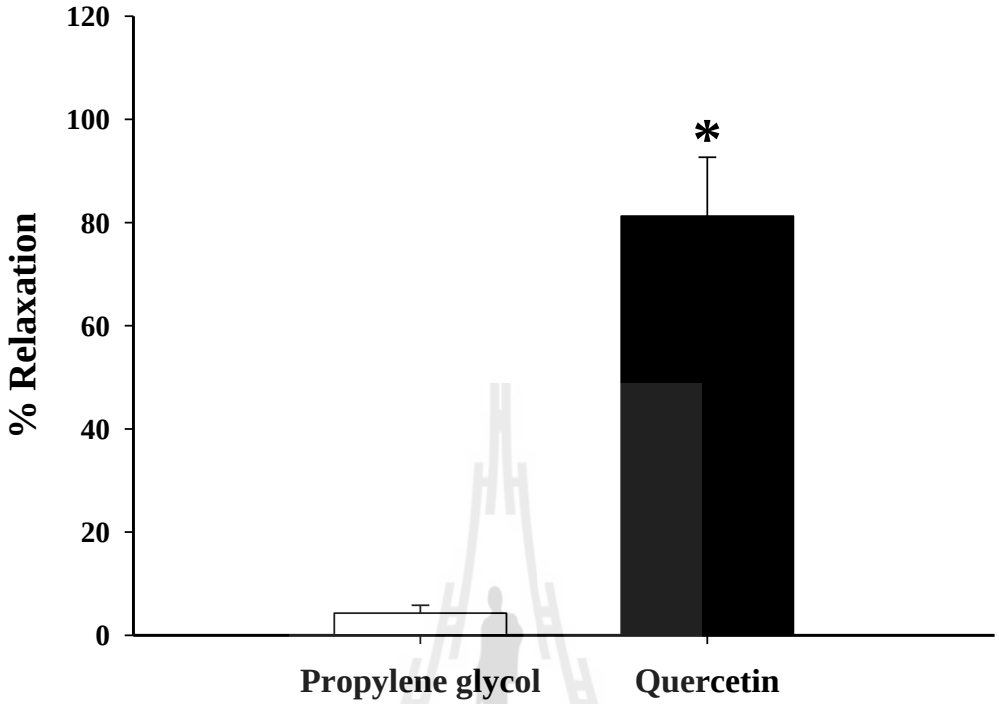


Figure 3.5 Relaxation effects of quercetin and propylene glycol on phenylephrine precontracted endothelium-denuded aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). * Significantly different from propylene glycol, $P<0.05$ (Student's *t*-test).

Vasorelaxation effects of quercetin on endothelium-intact and -denuded were not significant difference (Figure 3.6), indicating the vasorelaxation caused by quercetin was endothelium-independent.

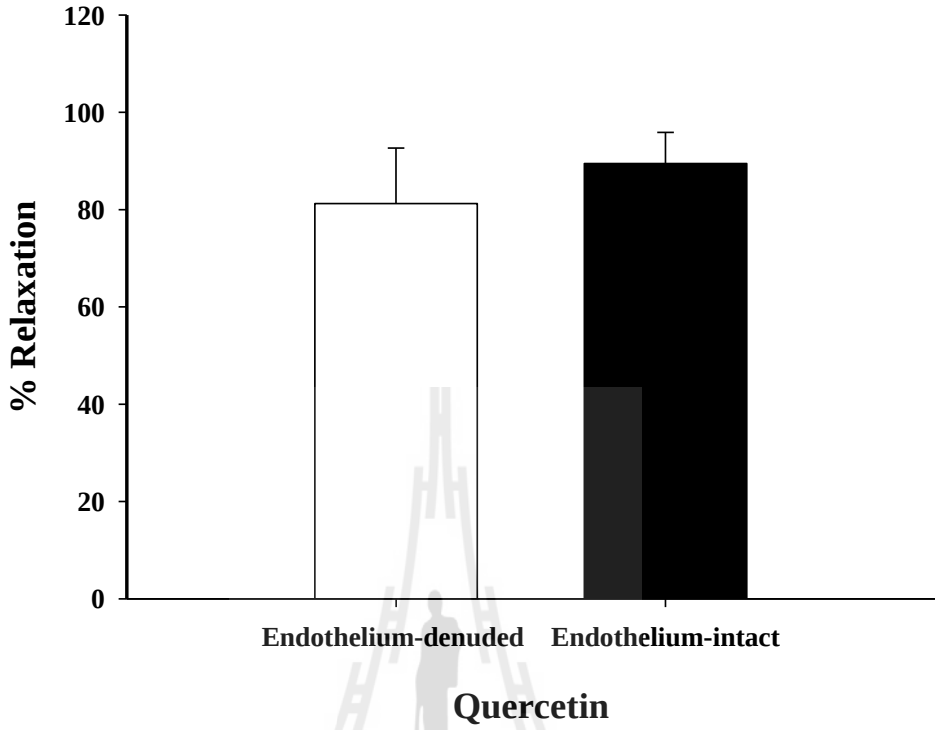


Figure 3.6 Relaxation effects of quercetin on phenylephrine precontracted endothelium-intact and-denuded aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). There is no significant difference between endothelium-intact and endothelium-denuded treatment, $P<0.05$ (Student's *t*-test).

3.5.4 Effects of *Centella asiatica* extract and quercetin on endothelium-intact aortic rings precontracted with L-NAME

In endothelium-intact aortic rings pretreatment with nitric oxide synthase inhibitor L-NAME (75 mM) did not alter the vasodilation effect of *Centella asiatica* extract. Addition of *Centella asiatica* extract induced a concentration-dependent additive relaxation, while DDD water did not induce relaxation (Figure 3.7). *Centella asiatica* extract at the concentrations of 2 and 4 mg/ml were

significantly increased relaxation (104.78 ± 27.04% and 127.89 ± 23.13%, respectively) compared with control (24.97 ± 12.97% and 28.15 ± 7.23%, respectively), $P<0.05$.

In Figure 3.8, quercetin significantly reduced contraction effect of L-NAME in endothelium-intact aortic rings (66.44 ± 4.20%) compared with control (4.78 ± 1.90%), $P<0.05$. The results indicated that nitric oxide (NO) did not contribute to *Centella asiatica* extract and quercetin-induced vasodilation in aorta.

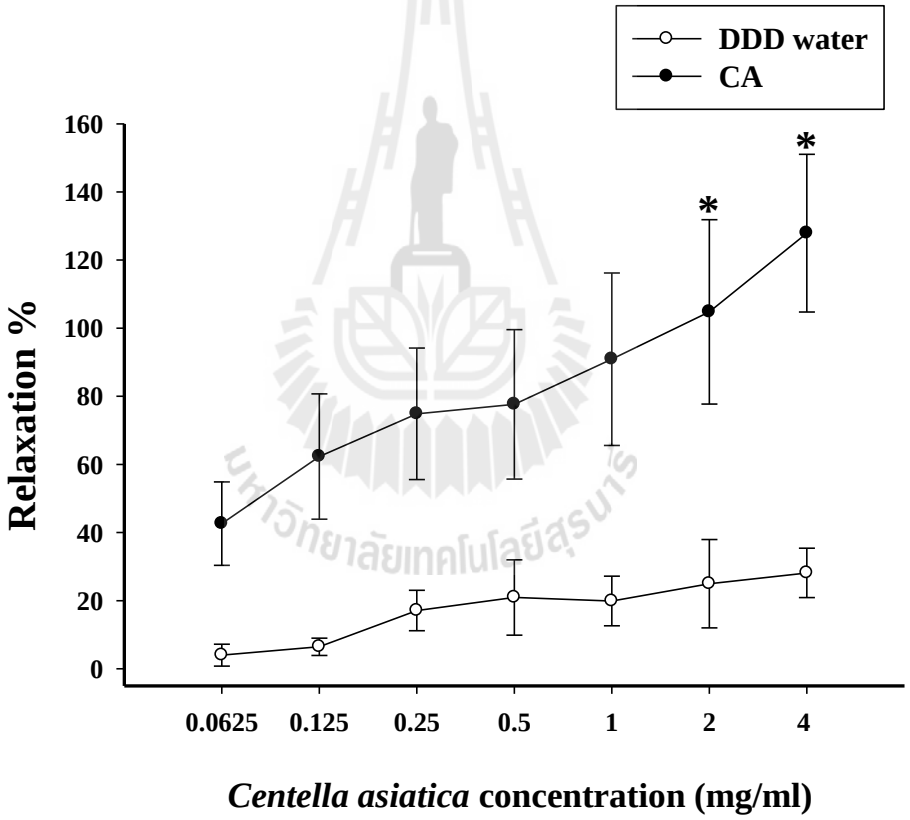


Figure 3.7 Relaxation effects of *Centella asiatica* extract (CA) and double deionized distilled (DDD) water on L-NAME precontracted endothelium-intact aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). * Significantly different from DDD water, $P<0.05$ (Student's *t*-test).

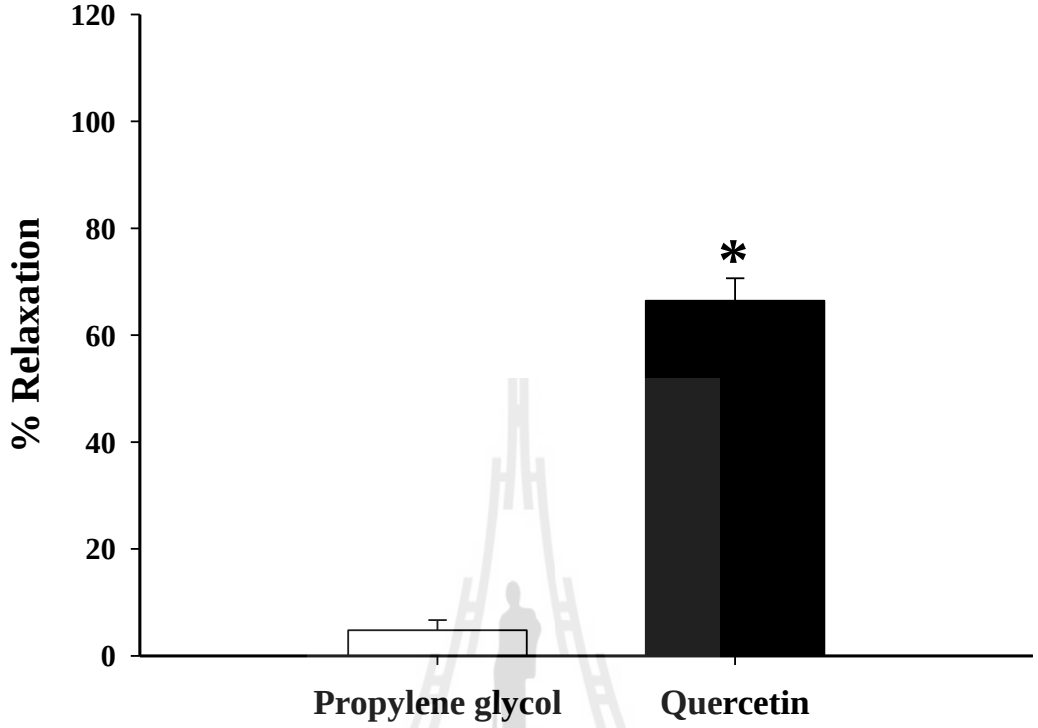


Figure 3.8 Relaxation effects of quercetin and propylene glycol on L-NAME precontracted endothelium-intact aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). * Significantly different from propylene glycol, $P<0.05$ (Student's *t*-test).

3.5.5 Effects of *Centella asiatica* extract and quercetin on endothelium-intact aortic rings precontracted with tetraethylammonium

In endothelium-intact aortic rings, pretreatment with non selective K^+ channel inhibitor tetraethylammonium (10 mM) reduced the vasodilation effect of *Centella asiatica* extract (Figure 3.9). The vasorelaxation induced by *Centella asiatica* extract was not difference from control.

Figure 3.10 showed that endothelium-intact aortic rings precontacted with tetraethylammonium (10 mM) also reduced the vasodilation effect of quercetin. The vasorelaxation induced by *Centella asiatica* extract was not difference from control. The results indicated that K^+ channel involved in *Centella asiatica* extract and quercetin induced vasodilation in aorta.

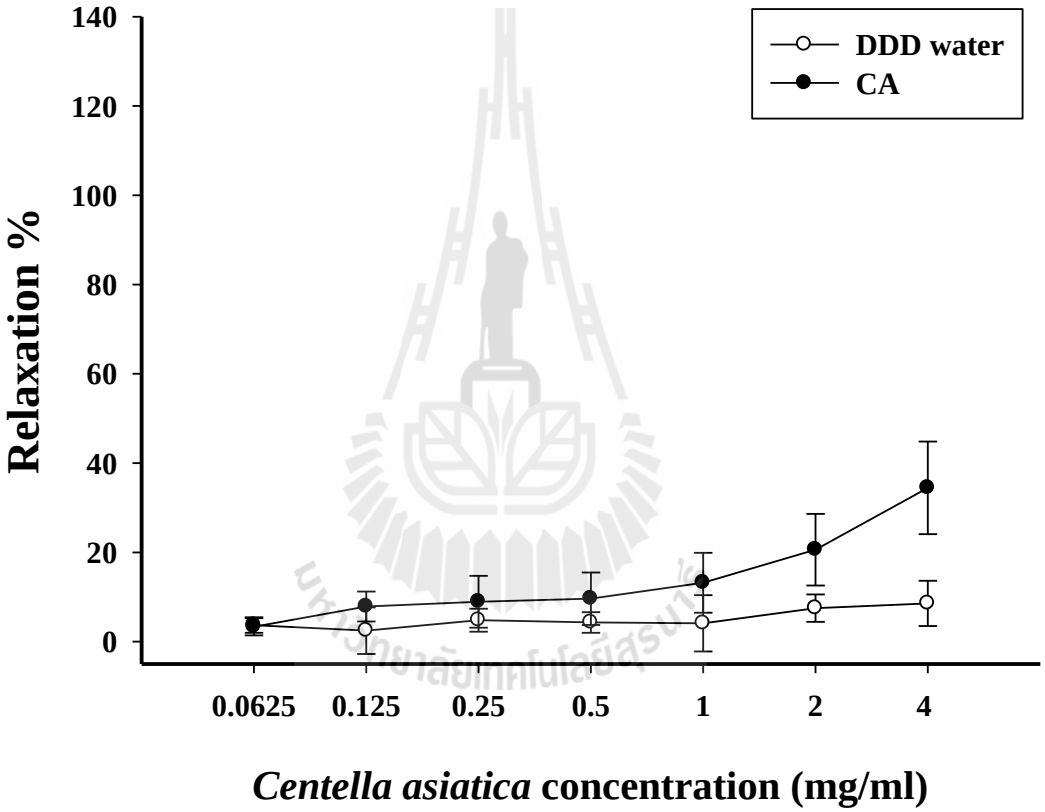


Figure 3.9 Relaxation effects of *Centella asiatica* extract (CA) and double deionized distilled (DDD) water on tetraethylammonium precontracted endothelium-intact aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). This is no significant difference between CA treatment and DDD water treatment, $P<0.05$ (Student's *t*-test).

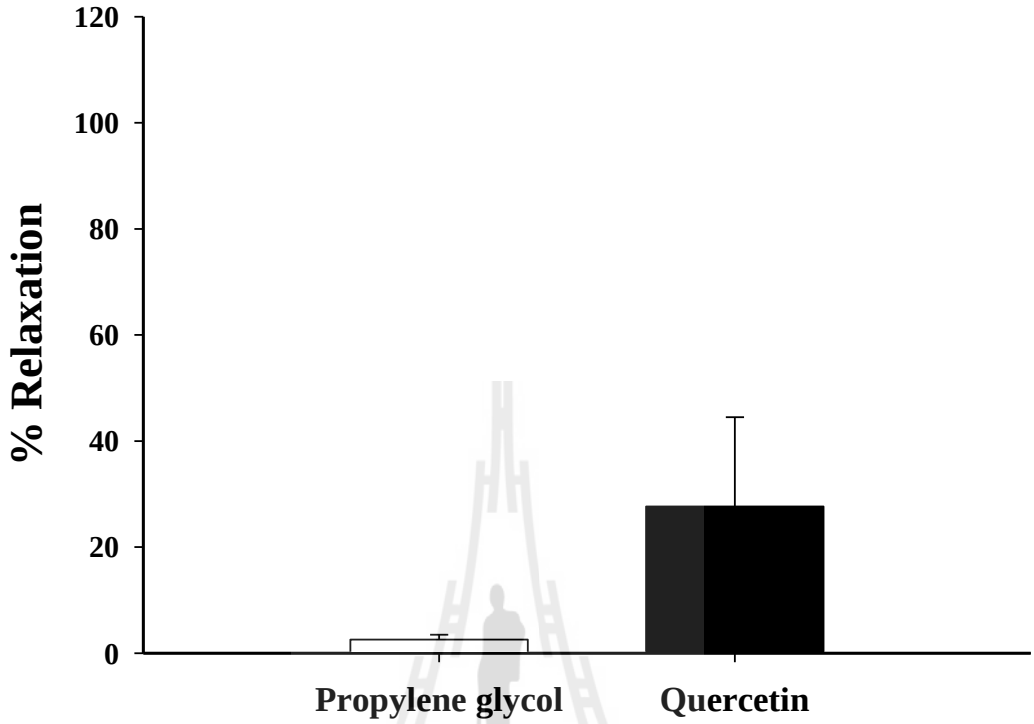


Figure 3.10 Relaxation effects of quercetin and propylene glycol on tetraethylammonium precontracted endothelium intact aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). There is no significant difference between tetraethylammonium treatment and propylene glycol treatment.

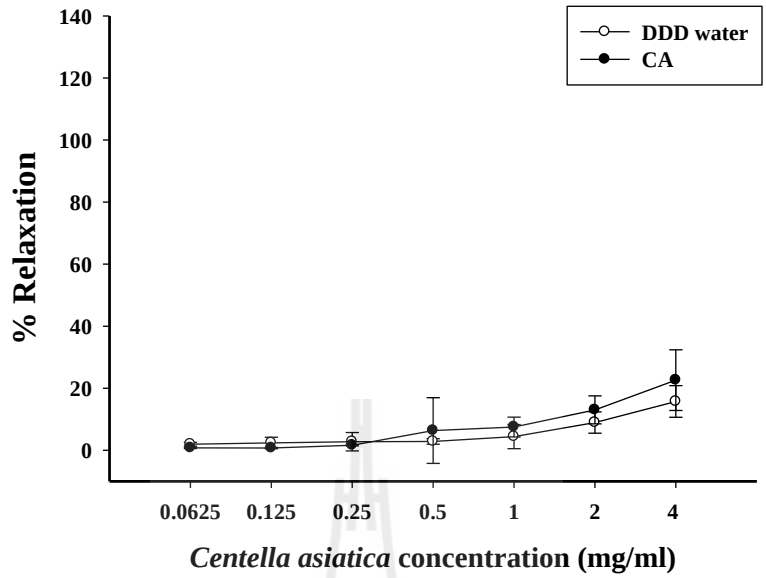
3.5.6 Effects of *Centella asiatica* extract and quercetin on endothelium-intact aortic rings with preincubated with nifedipine before precontracted with phenylephrine

Preincubation with L-type Ca^{2+} channel blocker nifedipine (1 μM) reduced vasorelaxation effect of *Centella asiatica* extract on endothelium-intact aortic rings precontracted with phenylephrine (Figure 3.11A). Endothelium-intact aortic

rings incubated with nifedipine before precontacted with phenylephrine, *Centella asiatica* extract did not show any significant increase in vasorelaxation, while the endothelium that was not incubated with nifedipine showed dose-dependent vasorelaxation effects of *Centella asiatica* extract (Figure 3.11B). *Centella asiatica* extract at the concentrations of 2 and 4 mg/ml were significantly increased vasorelaxation (52.69 ± 9.17 % and 73.44 ± 7.40%, respectively) compared with control (17.73 ± 8.47 % and 24.47 ± 13.96%, respectively), $P<0.05$

In endothelium-intact aortic rings preincubated with nifedipine before precontracted with phenylephrine, addition of quercetin was not affect in relaxation of endothelium, while endothelium that was not preincubated with nifedipine significantly increased vasorelaxation (52.48 ± 2.83%) compared with control (3.08 ± 1.72%), $P<0.05$ (Figure 3.12A, B). The results indicated that L-type Ca^{2+} channel involved in *Centella asiatica* extract and quercetin-induced vasodilation in aorta.

A.



B.

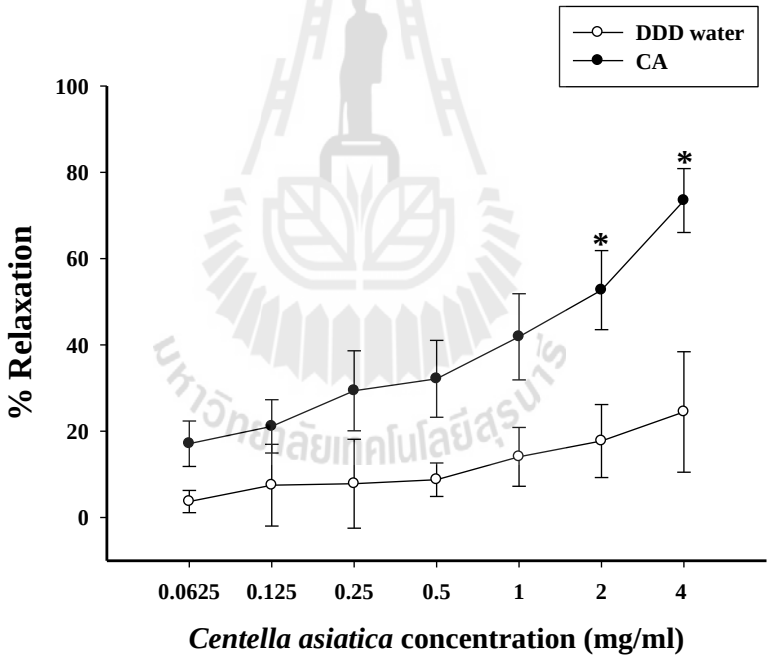


Figure 3.11 Relaxation effects of *Centella asiatica* extract (CA) and double deionized distilled (DDD) water on preincubated with (A) and without (B) nifedipine before phenylephrine precontracted endothelium-intact aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group).

* Significantly different from DDD water, $P<0.05$ (Student's *t*-test).

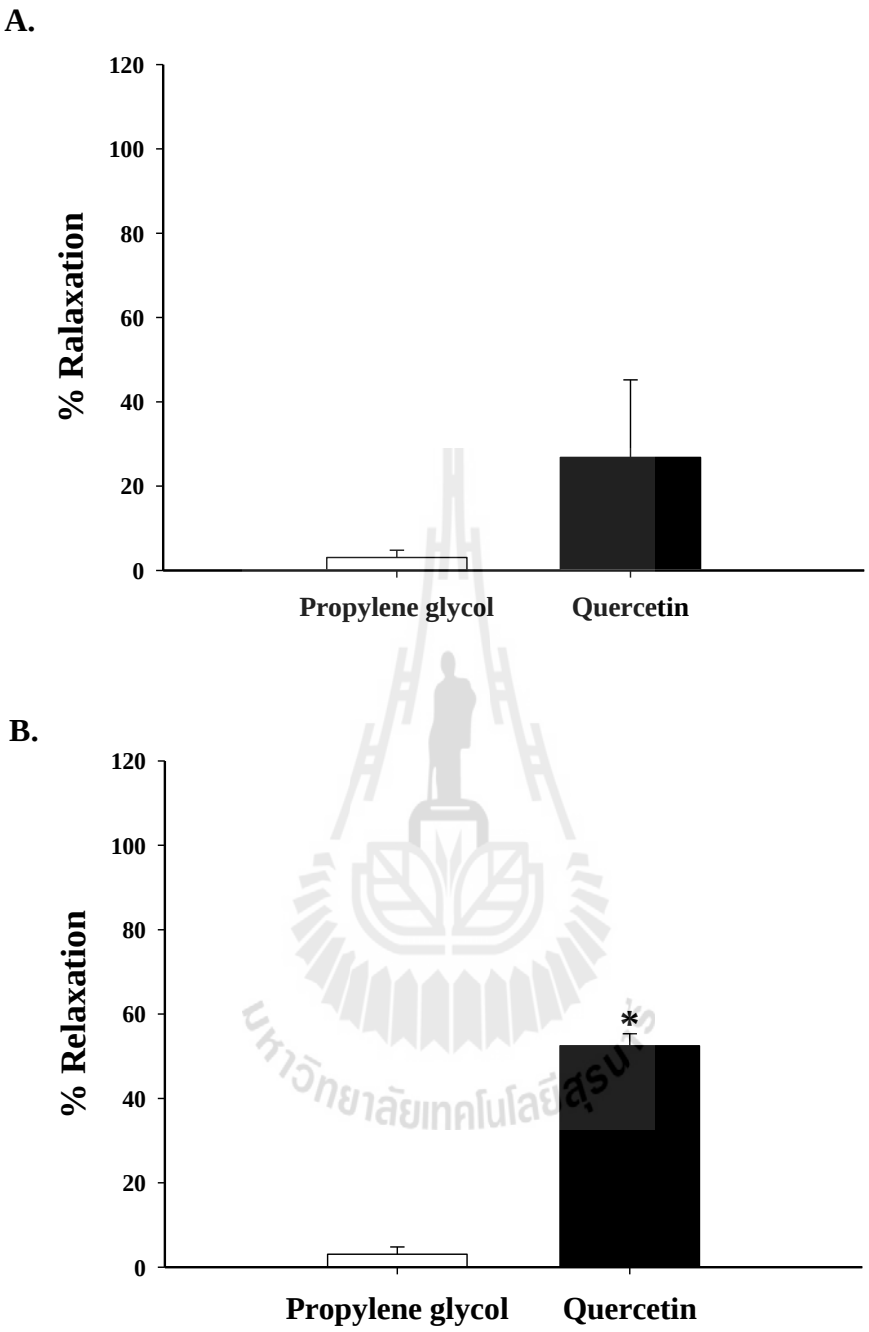


Figure 3.12 Relaxation effects of quercetin and propylene glycol on preincubated with (A) and without (B) nifedipine before phenylephrine precontracted endothelium-intact aortic rings. Data are expressed as mean ± S.E.M. (n=8 per group). * Significantly different from propylene glycol, $P<0.05$ (Student's *t*-test).

3.6 Discussion and conclusion

The present work was undertaken to evaluate *in vitro* cardiovascular effects of *Centella asiatica* extract using endothelium intact and endothelium denude aortic rings. The major findings are: (1) *Centella asiatica* extract and quercetin significantly increased relaxation on phenylephrine precontracted endothelium-intact aortic rings, (2) *Centella asiatica* extract and quercetin significantly increased relaxation on phenylephrine precontracted endothelium-denuded aortic rings, (3) *Centella asiatica* extract and quercetin significantly increased relaxation on L-NAME precontracted endothelium-intact aortic rings in dose dependent manner, (4) The relaxation *Centella asiatica* extract and quercetin did not reduce the contraction of aortic rings induced by tetraethylammonium, and (5) Pretreatment with nifedipine reduced vasorelaxation effect of *Centella asiatica* extract and quercetin in phenylephrine precontracted endothelium-intact aortic rings.

The present results demonstrated that *Centella asiatica* extract caused relaxation of isolated rat aortic rings precontracted with phenylephrine in dose-dependent manner for both endothelium-intact and endothelium-denuded aortic rings. *Centella asiatica* extract at a concentration of 4 mg/ml caused a mark vasorelaxation effect in both endothelium-intact and endothelium-denuded aortic rings, indicating that the vasorelaxation was endothelium-independent. A greater relaxant potency of *Centella asiatica* extract in the endothelium-intact aorta was observed.

Phenylephrine leads to vascular contraction *via* the stimulation of α_1- adrenergic receptors. On smooth muscle and induces the release of intracellular Ca^{2+} and influx of Ca^{2+} from extracellular space (Hudgins and Weiss, 1968). The contraction is achieved by diverse signaling pathways such as suppression of NO

production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase/extracellular signal-regulated kinase 1/2 (ERK1/2) cascade or inositol 1,4,5-triphosphate/protein kinase C (IP_3/PKC) cascade, along with Ca^{2+} channel stimulation (Madamachi et al., 2005). Synthesis of some vasorelaxant prostacyclin (PGI_2) and/or NO, the opening of K^+ channels and facilitating K^+ efflux, and/or reduced Ca^{2+} availability or other mechanisms may be involved in the vasorelaxant effects of a drug (Félétou and Vanhoutte, 2006).

In the second set of experiments, the incubation of the aortic rings with L-NAME. We have examined the effects of *Centella asiatica* extract on vascular endothelium precontacted with L-NAME. These experiments showed that the incubation of the aortic rings with L-NAME before adding *Centella asiatica* extract, which caused the contraction, abolished and induced relaxation in aortic rings. Thus, the mechanism in *Centella asiatica* extract induced vasorelaxation was not related to the functional of endothelium (NO system) because nitric oxide synthase (NOS) is blocked by L-NAME but the relaxation activity still continued. In animals or humans, NO production is inhibited, which lead to vascular smooth muscle contraction and increased blood pressure. Therefore, it is suggested that the decreased level of NO may be important pathophysiological factor in the development and sustenance of hypertension (Kang et al., 2003). Thus, the mechanisms involved in the vasorelaxant effect of *Centella asiatica* extract be unrelated the NO or endothelium-derived relaxing factor (EDRF) in smooth muscle.

The relaxant effect of *Centella asiatica* extract was diminished in the aortic rings precontracted by tetraethylammonium (a non selective potassium channel blocker) (Cook, 1989). Tetraethylammonium nearly abolished the vasorelaxant effect

of *Centella asiatica* extract in aortic rings. *Centella asiatica* extract facilitate K^+ efflux through a mechanism which is blocked by tetraethylammonium. It is strongly suggested that K^+ channel opening may be involved in the vasorelaxation of *Centella acitica* extract. Several types of K^+ channels exist in vascular smooth muscle cells (Cook, 1989). Such as adenosine triphosphate (ATP)-sensitive K^+ channel (K_{ATP}), Ca^{2+} activated K^+ channels (BK_{Ca}), delayed rectifier K^+ channels (K_V), and inward rectifier K^+ channels (K_{IR}) (Bean, 1992; Quayle et al., 1993; Volk et al., 1991; Volk and Shibata, 1993). Activation or opening of these channels increases K^+ efflux, thereby producing hyperpolarization of vascular smooth muscle (Neslson et al., 1990; Standen, 1992), and therefore, the activity of these K^+ channel plays essential roles in regulating membrane potential and vascular tone (Nelson and Quayle, 1995). We observed the effects of these selective K^+ channel blockers on the vasorelaxation of *Centella asiatica* extract in attempt to clarify which K^+ channel *Centella asitica* extract might have selectively acted on. None of these selective K^+ channel blockers exerted any effects on the relaxation induced by *Centella asiatica* extract.

The present study also showed the vasorelaxation effect of *Centella asiatica* extract induced by phenylephrine on endothelium-intact aortic rings that was completely inhibited by the blocker of L-type Ca^{2+} channel nifedipine. The results indicated the possible involvement of L-type Ca^{2+} channel in the vasorelaxant effects of the *Centella asiatica* extract on endothelium intact aortic rings. Generally, Ca^{2+} influx plays an important physiological role in mediating the contraction of vascular smooth muscle cells. During sarcolemmal membrane depolarization, the L-type Ca^{2+} channel will be open to permit Ca^{2+} influx and trigger intracellular Ca^{2+} release, leading to cell contraction (Kruse et al., 1994). It is well known that the

vasoconstriction of vascular smooth muscle is initiated by an increase of intracellular Ca^{2+} influx *via* the voltage-dependent Ca^{2+} channel evoked by depolarization with high K^{+} concentration or *via* the receptor-operating Ca^{2+} channel evoked by norepinephrine and release of intracellular Ca^{2+} (Broekaert and Godfraind, 1979; Saida and Van Breemen, 1983). Nifedipine exerts its clinical effects due to vasodilation of arterial smooth muscle, leading to reduce peripheral resistance, and improve coronary flow. Nifedipine is indicated for the prophylaxis of angina pectoris and in peripheral circulatory disorders such as Raynaud's syndrome (Godfraind, 1994).

In conclusion, our findings demonstated that *Centella asiatica* extract has a potential ability to regulate vascular tones *via* inhibition of Ca^{2+} influx into vascular smooth muscle cells through L-type $Ca2^{+}$ channel, and inhibition of K^{+} effect through K^{+} channel, but not involved in NO system, causing vasodilation. Therefore, *Centella asiatica* extract may be considered as a candidate for drug development for the treatment of hypertensive conditions. Further investigations of all effects and mechanisms of *Centella asitica* extract on blood vessels are needed.

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CHAPTER IV

ACUTE EFFECTS OF *CENTELLA ASIATICA* EXTRACT ON BLOOD PRESSURE AND HEART RATE OF ANAESTHETIZED NORMOTENSIVE AND HYPERTENSIVE RATS

4.1 Abstract

Centella asiatica (L.) Urban, known as Asian pennywort, *guta kola* (Indian) and *bua-bok* (Thai), is one of the medicinal herbs that has been used extensively by Ayurvedic Pharmacoeia to alleviate high blood pressure. This study aimed to assess effects of *Centella asiatica* extract on blood pressure, heart rate, and respiratory rate of anaesthetized normotensive and N^G-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats. Male Wistar rats (n=112) were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). The left carotid artery of the rat was cannulated for invasive blood pressure measurement. Mean arterial blood pressure (MABP), systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and respiratory rate (RR) were recorded continuously throughout the experiment using PowerLab system. Normotensive and hypertensive rats received isotonic saline (1 mg/kg, 0.9% NaCl i.p.) and L-NAME (40 mg/kg, i.p.), respectively. Thirty minutes later, rats were intragastrically administered either double distilled deionized (DDD) water

(20 ml/kg), quercetin (5 mg/20 ml/kg), propylene glycol (20 ml/kg), or *Centella asiatica* extract (4, 8, 16, or 32 g/20 ml/kg), n=8 per group. All recordings were further continued for 90 minutes. In pentobarbital-anaesthetized rats, L-NAME significantly increased MABP, SBP, and DBP, but did not cause any change in HR and RR. *Centella asiatica* extract at the concentration of 16 g/20 ml/kg (i.g.) and quercetin significantly lowered the elevated MABP, SBP, and DBP in L-NAME induced hypertensive rats. In normotensive rats, *Centella asiatica* extract at the concentrations of 4 and 8 g/20 ml/kg significantly increased MABP, SBP, and DBP. Quercetin significantly increased MABP and significantly decreased RR, but had no effect on HR of normotensive rats. The present study demonstrated blood pressure lowering effect of *Centella asiatica* extract in L-NAME induced hypertensive rats and hypertensive effects in normotensive rats. These findings provide scientific support for tradition use of this plant to modify the actions of the human cardiovascular system.

4.2 Introduction

Hypertension is a major risk factor for cardiovascular disease and stroke, thus prevention of hypertension an important in reducing the risk of these debilitating ailments (Lloyd-Jones et al., 2009). Hypertension defined as blood pressure 140/90 mmHg or higher and is the leading cause of chronic renal failure, myocardial infarction, and stroke. There is currently increasing interest in the identification of antihypertensive foods because they are expected to prevent hypertension with lower side-effects than antihypertensive drugs (Chen et al., 2009). Strategies to manage hypertension are considered the key in controlling mortalities from cardiovascular

disease and reducing antihypertensive drug burdens; a reduction in blood pressure of only 3 mmHg may reduce the risk of death by 5% to 8% (Padwal et al., 2005). Researchers are currently looking for antihypertensive compounds derived from various natural products for use in functional foods. Several studies have shown that food ingredients rich in flavonoids and other polyphenols can lower blood pressure (Diebolt et al., 2001; Grassi et al., 2005; Hodgson et al., 2003; Liu et al., 2003).

Centella asiatica contains high total phenolic content which contributed by the flavonoids such as quercetin, kaempherol, catechin, rutin, apigenin, and naringin (Zainol et al., 2004). Evidence has been demonstrated that the *Centella asiatica* is used to promote healthy long life, improve memory, and other cognitive domains. The other probable clinical uses of this plant are in venous insufficiency, wound healing, anxiety, anti-tumor, hypertension, and peptic ulcer (Babu et al., 1995; Belcaro et al., 1990; Chattereje et al., 1992; Shetty, 2006; Somchit et al., 2004; Veerendra and Gupta, 2003; Wollina et al., 2006).

Centella asiatica extract has been shown to exert potent antioxidative activity as indicated by various assay system (Zainol et al., 2003). The herb is said to have a direct effect in lowering blood pressure and is often referred to as a rejuvenating medicament in the Ayurvedic Pharmacopoeia (Jayaweera, 1982). Therefore, the objective of this study was to determine acute effects of *Centella asiatica* extract on blood pressure, heart rate, and respiratory rate of anaesthetized normotensive and hypertensive rats. A flavonoid quercetin found in *Centella asiatica* was used as positive control in this study since it has been shown to promote relaxation of cardiovascular smooth muscle muscle (antihypertensive effects) (Formica and Regelson, 1995).

4.3 Materials and methods

4.3.1 Plant material

The *Centella asiatica* extract obtained from stock extract in chapter 3 was used in the experiments conducted in this chapter. A voucher specimen number of *Centella asiatica* is BFK184894 from BGO Plant Database, The Botanical Garden Organization, Ministry of Environment and Natural Resources, Thailand.

4.3.2 Animals

Male Wistar rats (200-250 g) were obtained from Institutional Animal Care, Suranaree University of Technology (SUT). They were maintained under standard laboratory conditions (12:12 h dark-light cycle, ambient temperature 20 ± 1 $^{\circ}$C) with free access to food and water. This study was conducted under permit of the SUT Animal Care and Use Committee.

4.3.3 Drugs and chemicals

N^{G}-nitro-L-arginine methyl ester (L-NAME), quercetin, and sodium chloride were purchased from Sigma Aldrich Chemical Ltd. (St. Louis, USA). Propylene glycol was purchased from Vidhyasom Co. Ltd., Thailand. All other chemicals were of reagent grade. All the drugs solutions were prepared at the time of use. Quercetin was dissolved in propylene glycol. L-NAME was dissolved in isotonic saline (0.9% NaCl; Thai-Otsuka Co. Ltd., Japan).

4.3.4 Preparation of solution

40 mg/ml L-NAME

40 mg/ml L-NAME stock solution was prepared by dissolving 0.64 g of L-NAME (Sigma, St. Louis, MO, USA) in 16 ml of isotonic saline (0.9% NaCl; Thai-Otsuka Co. Ltd., Japan) and kept in -20 ◦C (for n=40).

5 mg/20 ml Quercetin

5 mg/20 ml Quercetin was prepared by dissolving 8 mg of quercetin (Sigma, St. Louis, MO, USA) in 32 ml of propylene glycol (Vidhyasom Co. Ltd., Thailand) (for n=4).

4.3.5 L-NAME induced hypertension

For hypertensive rats, hypertension was induced experimentally in male Wistar rats by L-NAME, a nitric oxide synthase inhibitor (40 mg/ml/kg, i.p.) (Bernatova et al., 1999). L-NAME was prepared everyday by dissolving in isotonic saline (0.9%, NaCl). For normotensive rats, male Wistar rats received isotonic saline (1 ml/kg, 0.9% NaCl, i.p.).

4.3.6 Acute effects of *Centella asiatica* extract on blood pressure by invasive (direct) method

The rats were anaesthetized by intraperitoneal injection with sodium pentobarbital (50 mg/kg; Cevesanté animale, France) (Chu et al., 2003). The trachea was exposed and cannulated to facilitate spontaneous respiration (Wongcome et al., 2007) and the left carotid artery was cannulated. The blood pressure was measured

directly from the cannula using a pressure transducer PowerLab system with Chart program (Pty ADInstrument Ltd., LabChart 5, Australia) (Chen et al., 2004) and the chart recorder was calibrated in mmHg to measure the blood pressure. After a 30 min period of equilibration (Abdul-Ghani and Amin, 1997), mean arterial blood pressure (MABP), systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), and respiratory rate (RR) were recorded 5 min before the rats were injected with isotonic saline (1 ml/kg, 0.9% NaCl, i.p.) for normotensive groups (n=56) or L-NAME (40 mg/ml/kg, i.p.) (Bernatova et al., 1999) for hypertensive groups (n=56). For intragastric administration of extract, a 1 cm abdominal incision was made to expose the stomach and the stomach was cannulated. After a stabilization period of 30 min, the *Centella asiatica* extract (4, 8, 16, 32 g/20 ml/kg), double deionized distilled (DDD) water, quercetin (5 mg/20 ml/kg, Perez-Vizcaino, 2009), and propylene glycol (20 ml/kg) were intragastrically injected. The blood pressure and heart rate were recorded for a further period of 90 min. At the end of experiment, rats were terminated with an overdose of sodium pentobarbital.

4.4 Statistical analysis

All values were expressed as mean ± S.E.M. Statistical analysis was determined by two-way repeated measured (ANOVA) followed by Fisher LSD method using the SigmaStat software (version 3.5, Systat Software Inc., USA) and all graphs were created by SigmaPlot software (version 10, Systat Software Inc., USA). *P*-values less than 0.05 ($P<0.05$) were considered statistically significant.

4.5 Results

4.5.1 Effects of *Centella asiatica* extract and quercetin on arterial blood pressure, HR, and RR of anaesthetized normotensive rats

In pentobarbital-anaesthetized normotensive rats, a single intragastric administration of quercetin did not cause changes in SBP and HR (Figure 4.2 and 4.4). During the period of 20-90 min after administration, *Centella asiatica* extract at concentrations of 4 and 8 mg/20 ml/kg significantly increased MABP when compared to control (DDD water), as shown in Figure 4.1. *Centella asiatica* extract at concentration of 16 and 32 g/20 ml/kg significantly increased MABP at 35 min, and 10, 35 min after administration, respectively. During the period of 65-90 min after administration, quercetin (5 mg/20 ml/kg) significantly decreased MABP when compared to control (propylene glycol). Significant increases in SBP were found in anaesthetized normotensive rats received *Centella asiatica* extract (4 and 8 g/ 20 ml/kg) during the period of 25-70 min and 30-50 min after administration, respectively (Figure 4.2). Significant increases in DBP were found in anaesthetized normotensive rats received *Centella asiatica* extract (4 and 8 g/20 ml/kg) during the period of 20-80 min. *Centella asiatica* extract at a concentration of 16 g/20 ml/kg significantly increased DBP at 40 and 45 min after administration, compared to control (Figure 4.3). Significant decrease in DBP was found at 65 min after administration of quercetin, compared to control (propylene glycol) (Figure 4.3). All doses of *Centella asiatica* extract and quercetin had no effect on HR of anaesthetized normotensive rats (Figure 4.4). During the period of 55-90 min (except 75 min) after administration, quercetin significantly increased RR when compared to control (propylene glycol) (Figure 4.5).

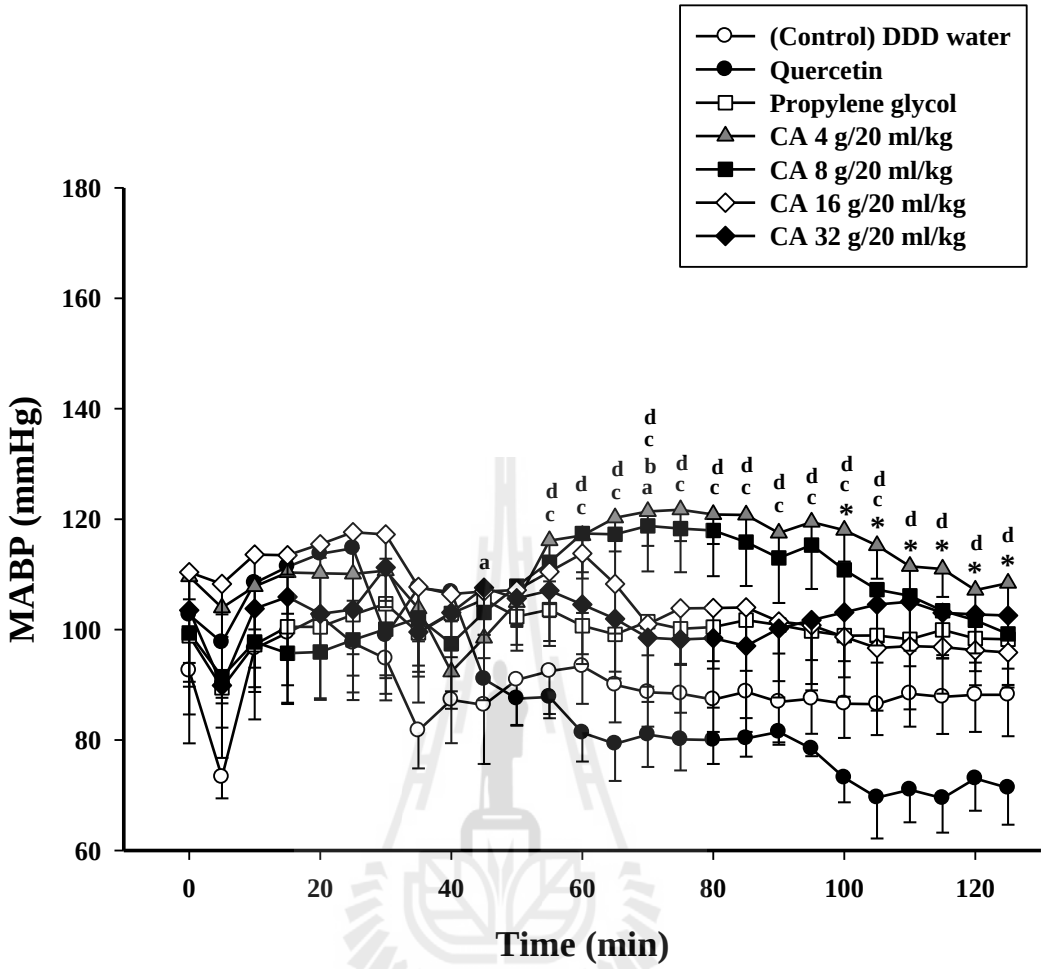

Figure 4.1 Effects of *Centella asiatica* extract (CA) on MABP of anaesthetized normotensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [a], [b], [c], and [d] denote significantly different between CA at concentrations of 32 g/20 ml/kg, 16 g/20 ml/kg, 8 g/20 ml/kg, and 4 g/20 ml/kg compared to control (DDD water), $P<0.05$, respectively. * denotes significantly different between quercetin compared to control (propylene glycol), $P<0.05$.

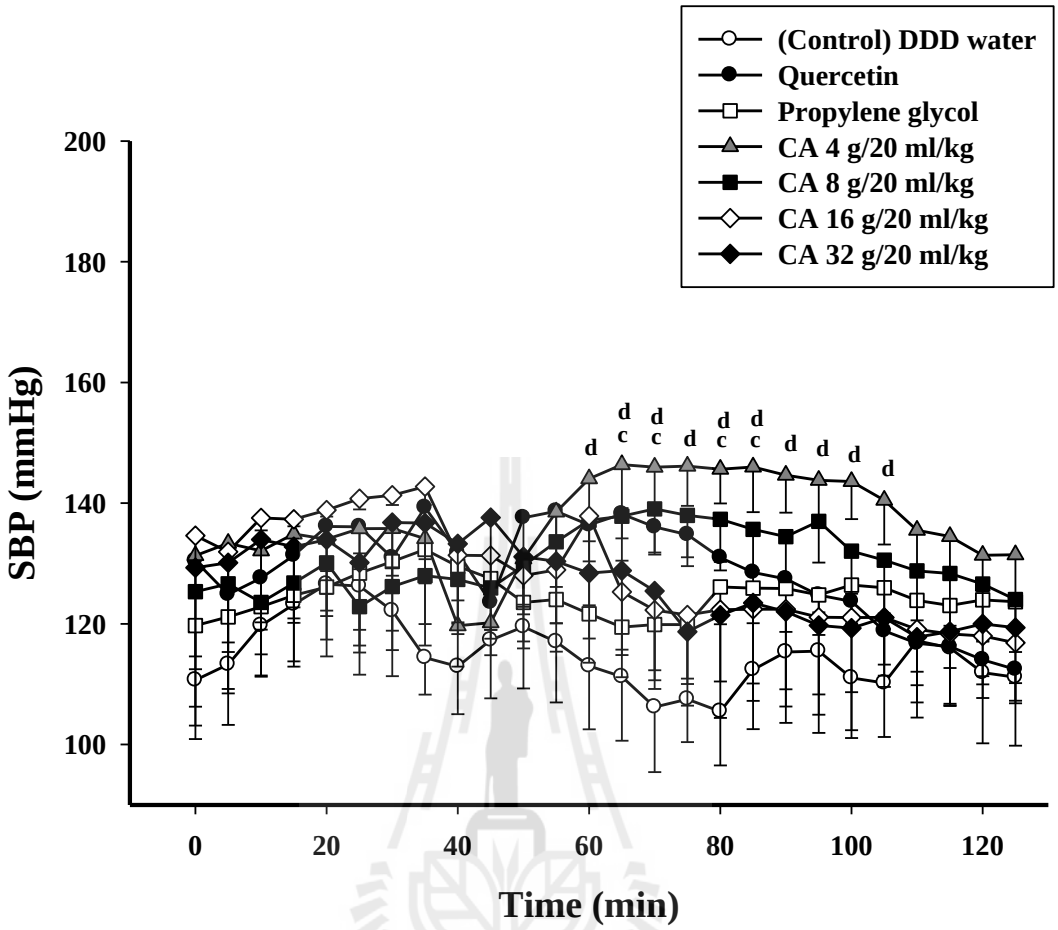

Figure 4.2 Effects of *Centella asiatica* extract (CA) on SBP of anaesthetized normotensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [c] and [d] denote significantly different between CA at concentrations of 8 g/20 ml/kg and 4 g/20 ml/kg compared to control (DDD water), $P < 0.05$, respectively.

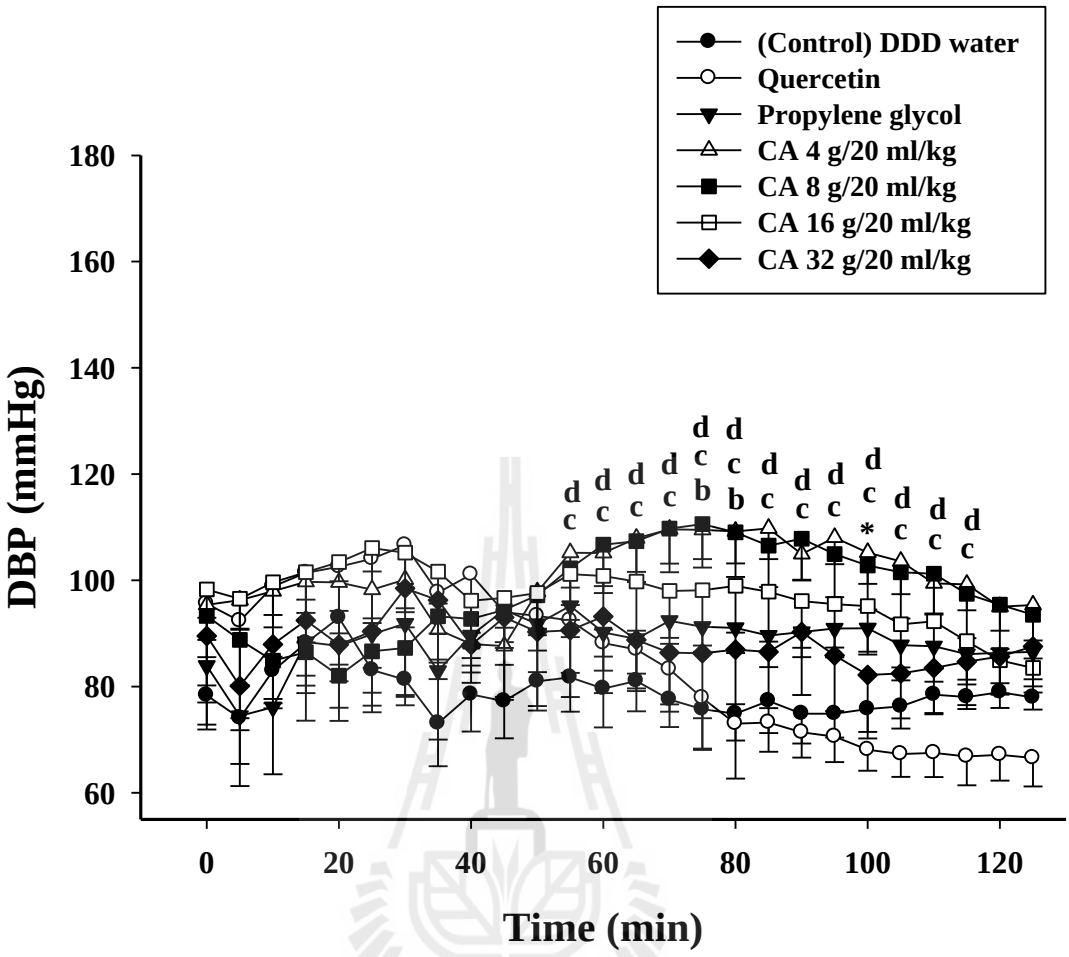

Figure 4.3 Effects of *Centella asiatica* extract (CA) on DBP of anaesthetized normotensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [b], [c], and [d] denote significantly different between CA at concentrations of 16 g/20 ml/kg, 8 g/20 ml/kg, and 4 g/20 ml/kg compared to control (DDD water), $P< 0.05$, respectively. * denotes significantly different between quercetin compared to control (propylene glycol), $P<0.05$.

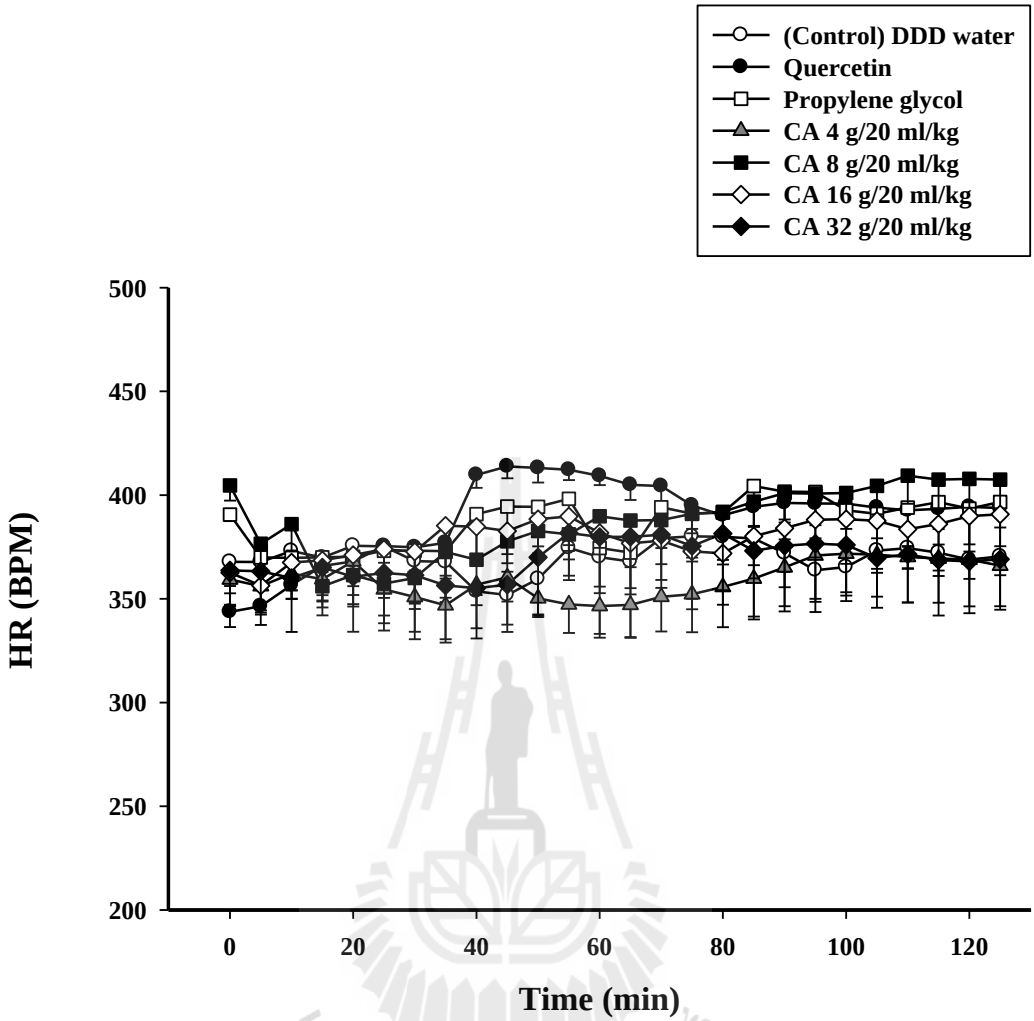

Figure 4.4 Effects of *Centella asiatica* extract (CA) on HR of anaesthetized normotensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). These is no significant difference between CA at concentrations of 32 g/20 ml/kg, 16 g/20 ml/kg, 8 g/20 ml/kg, and 4 g/20 ml/kg compared to control (DDD water) and there is no significant difference between quercetin compared to control (propylene glycol).

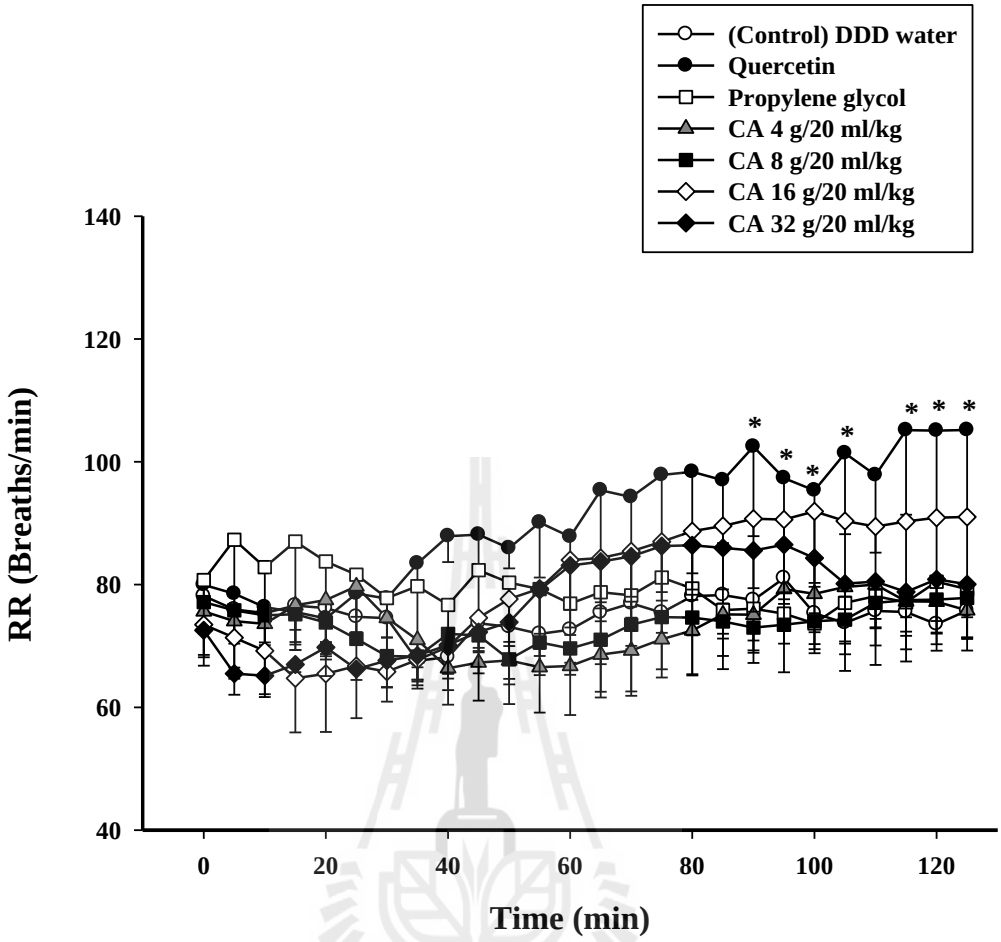

Figure 4.5 Effects of *Centella asiatica* extract (CA) on RR of anaesthetized normotensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). * denotes significantly different between quercetin compared to control (propylene glycol), $P<0.05$.

4.5.2 Effects of *Centella asiatica* extract and quercetin on arterial blood pressure, HR, and RR of anaesthetized hypertensive rats

In pentobarbital-anaesthetized rats, L-NAME significantly increased MABP, SBP, and DBP, but not HR and RR. A single intragastric administration of *Centella asiatica* extract (4, 8, and 32 g/ 20 ml/kg) did not cause changes in MABP, SBP, and DBP (Figure 4.6-4.8) of pentobarbital-anaesthetized L-NAME induced

hypertensive rats. At 90 min after administration, *Centella asiatica* extract (16 g/20 ml/kg) significantly decreased the elevated MABP and DBP compared to control (DDD water) as shown in Figure 4.6 and 4.8. During the period of 75-90 min, *Centella asiatica* extract (16 g/20 ml/kg) significantly decreased the elevated SBP compared to control (DDD water) as shown in Figure 4.7. From the period of 5-90 min after administration, quercetin (5 mg/20 ml/kg) significantly decreased the elevated MABP when compared to control (propylene glycol) as shown in Figure 4.6. During the period of 45-90 min after administration, quercetin significantly decreased the elevated SBP and DBP (Figure 4.7 and 4.8). All doses of *Centella asiatica* extract and quercetin had no effect on HR and RR of anaesthetized hypertensive rats as shown in Figure 4.9 and 4.10.

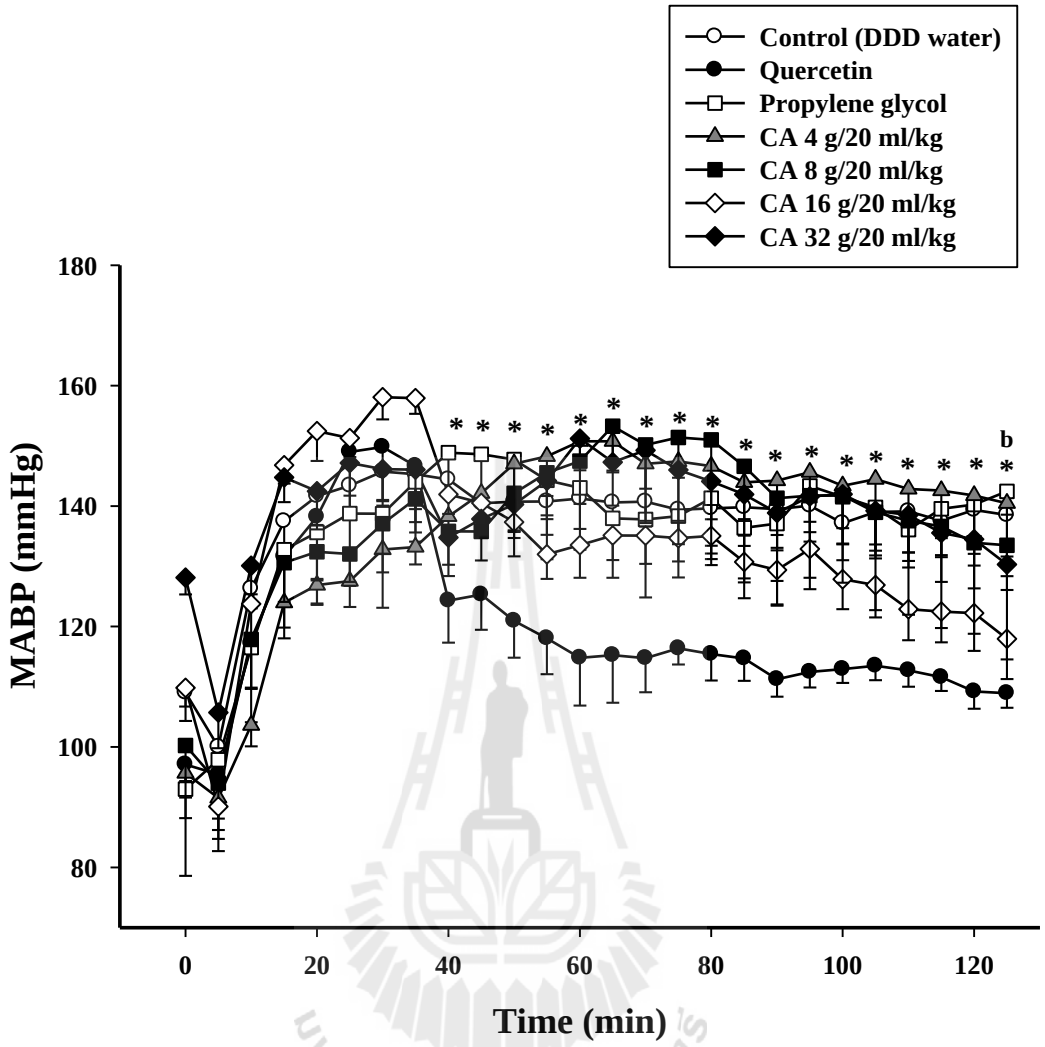

Figure 4.6 Effects of *Centella asiatica* extract (CA) on MABP of anaesthetized hypertensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [b] denotes significantly different between CA at a concentration of 16 g/20 ml/kg compared to control (DDD water), $P< 0.05$. * denotes significantly different between quercetin compared to control (propylene glycol), $P<0.05$.

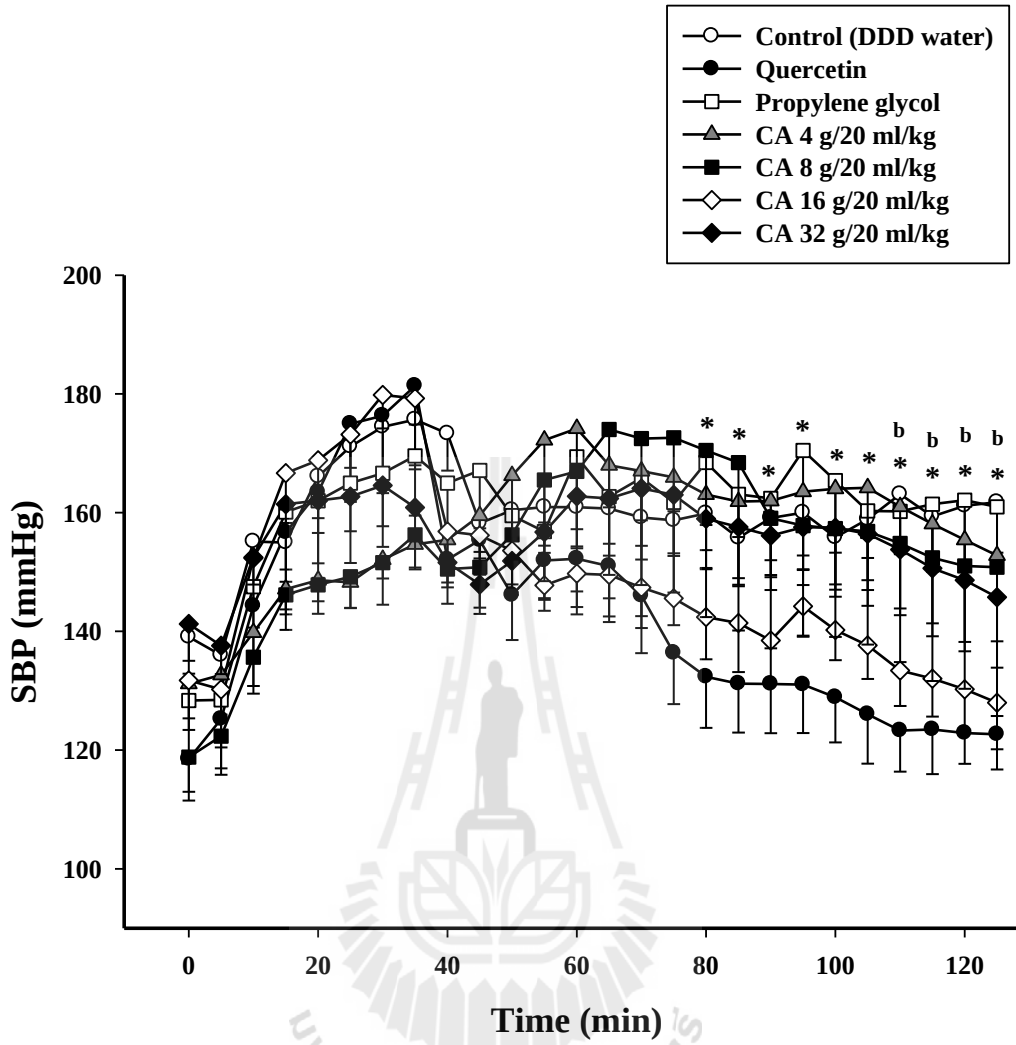

Figure 4.7 Effects of *Centella asiatica* extract (CA) on SBP of anaesthetized hypertensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [b] denotes significantly different between CA at a concentration of 16 g/20 ml/kg compared to control (DDD water), $P< 0.05$. * denotes significantly different between quercetin compared to control (propylene glycol), $P<0.05$.

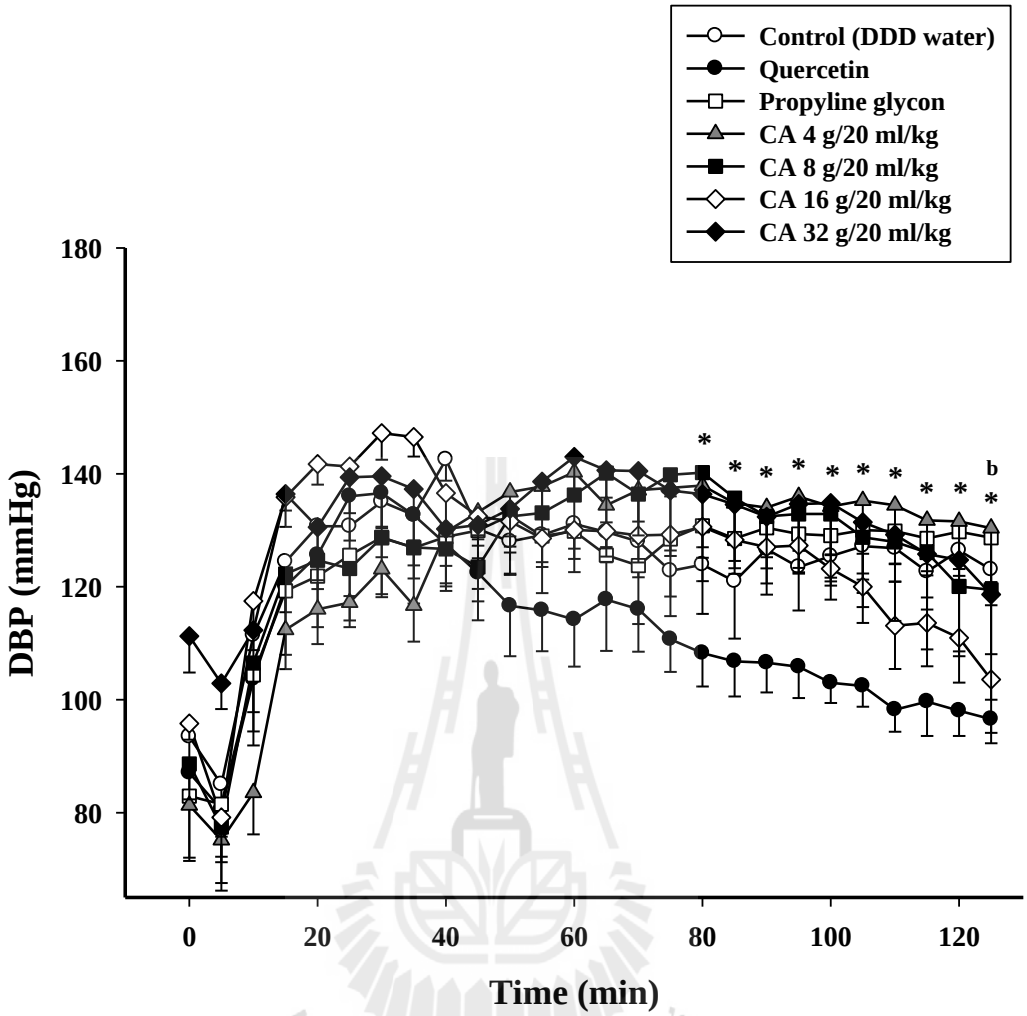

Figure 4.8 Effects of *Centella asiatica* extract (CA) on DBP of anaesthetized hypertensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [b] denotes significantly different between CA at a concentration of 16 g/20 ml/kg compared to control (DDD water), $P< 0.05$. * denotes significantly different between quercetin compared to control (propylene glycol), $P<0.05$.

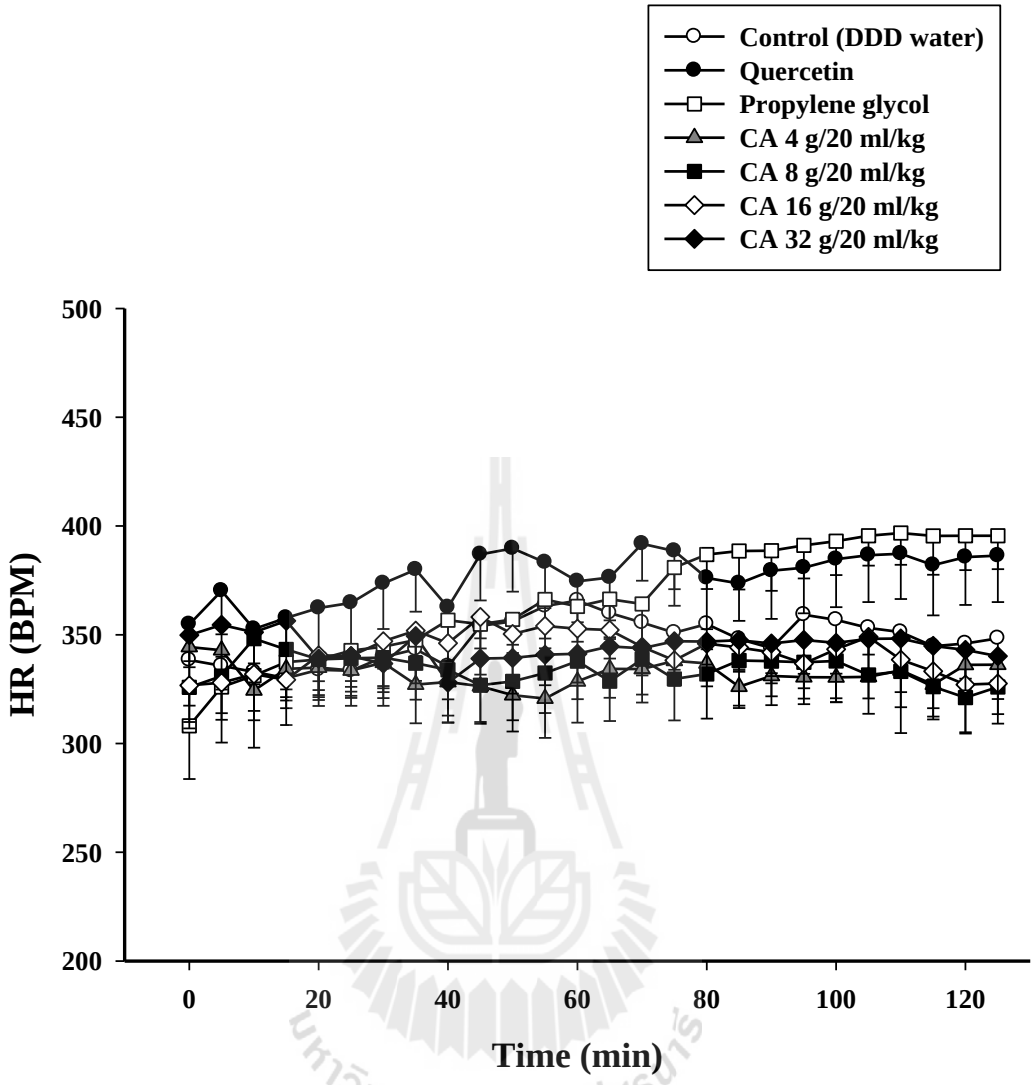

Figure 4.9 Effects of *Centella asiatica* extract (CA) on HR of anaesthetized hypertensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). These is no significant difference between CA at concentrations of 32 g/20 ml kg, 16 g/20 ml/kg, 8 g/20 ml/kg, and 4 g/20 ml/kg compared to control (DDD water) and these is no significant difference between quercetin compared to control (propylene glycol).

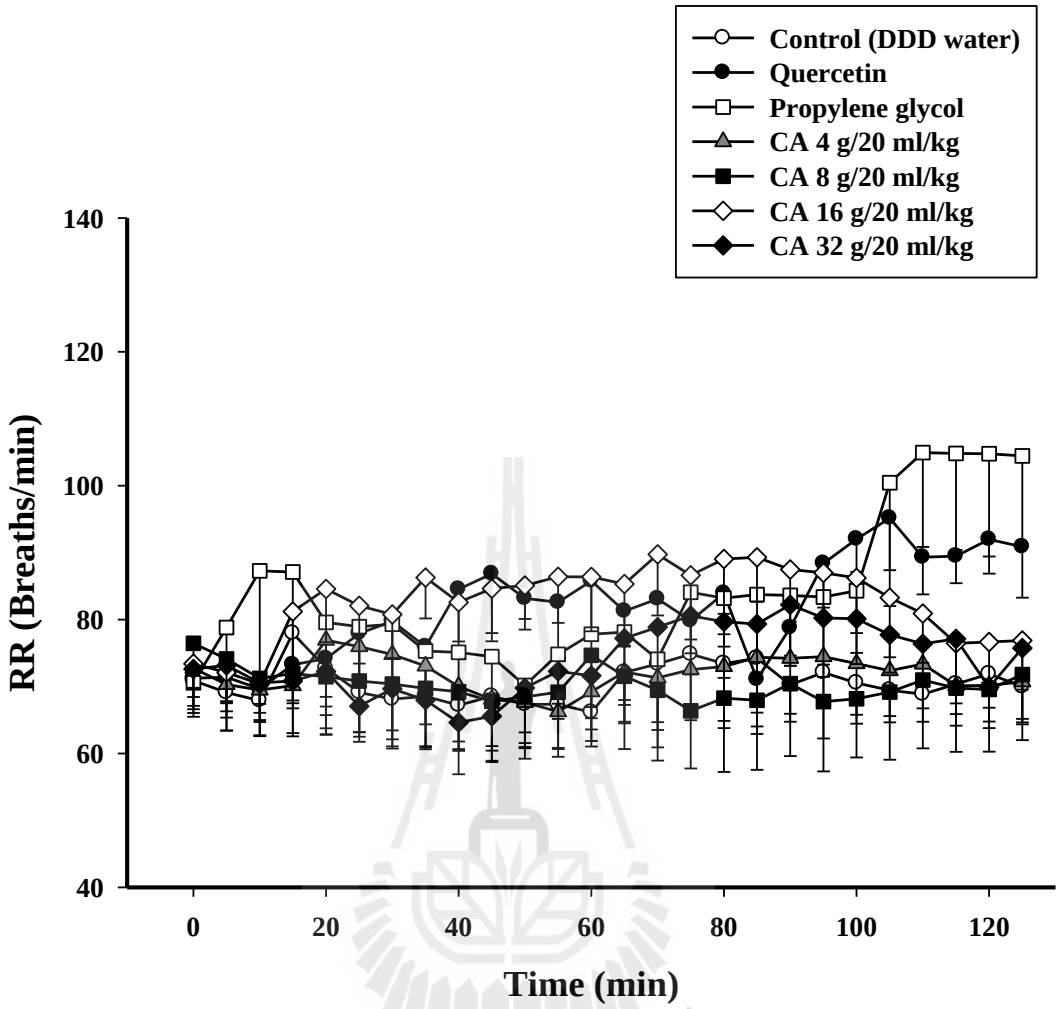

Figure 4.10 Effects of *Centella asiatica* extract (CA) on RR of anaesthetized hypertensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). These is no significant difference between CA at concentrations of 32 g/20 ml kg, 16 g/20 ml/kg, 8 g/20 ml/kg, and 4 g/20 ml/kg compared to control (DDD water) and these is no significant difference between quercetin compared to control (propylene glycol).

4.6 Discussion and conclusion

The present results demonstrated antihypertensive effect of *Centella asiatica* extract at the concentration of 16 g/20 ml/kg (i.g.) and it flavonoid quercetin at the concentration of 5 mg/20 ml/kg (i.g.) in L-NAME induced hypertensive rats, but not in normotensive rats. Significant decreases in the elevated MABP, SBP, and DBP in L-NAME induced-hypertensive rats were found after administration of *Centella asiatica* extract. In normotensive rats, *Centella asiatica* extract at the concentrations of 4 and 8 g/20 ml/kg significantly increased MABP, SBP, and DBP. These findings could be implied that *Cenella asiatica* extract possessed hypotensive effects on L-NAME induced-hypertensive rats and hypertensive effects on normotensive rats. The findings of the current study are consistent with those of Muangnongwa (2004) who found that fresh juice of *Centella asiatica* (32 g/kg, p.o.) could decrease SBP in deoxycorticosterone acetate (DOCA)-salt induced hypertensive rats, but had no effect in normotensive rats.

Quercetin, a flavonoid found in *Centella asiatica*, was found to possess hypotensive effects in both normotensive and L-NAME induced hypertensive rats. Quercetin significantly decreased the elevated MABP, SBP, and DBP in L-NAME induced-hypertensive rats and significantly decreased MABP and DBP, but not SBP in normotensive rats. These findings are an important extension of previous studies showing that quercetin can lower blood pressure in hypertensive animals (Duarte et al., 2001; Duarte et al., 2002; Garcia-Saura et al., 2005; Jalili et al., 2006; Payne et al., 2003). *Centella asiatica* extract and quercetin administration did not caused alterations in heart rate in normotensive rats. In L-NAME induced hypertensive rats,

Centella asiatica extract and quercetin administration did not cause alteration in HR and RR.

In conclusion, *Centella asiatica* extract supplement resulted in antihypertensive effects in hypertensive rats and hypertensive effects in normotensive rats. Quercetin may be in part responsible for the antihypertensive effect, but not hypertensive effect, of *Centella asiatica* extract. More detailed studies are required to evaluate the hypotensive and hypertensive effects of purified flavonoids from *Centella asiatica.*

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CHAPTER V

SUB-CHRONIC EFFECTS OF *CENTELLA ASIATICA* EXTRACT ON BLOOD PRESSURE OF NORMOTENSIVE AND HYPERTENSIVE RATS

5.1 Abstract

Hypertension is one of the most important modifiable risk factors for cardiovascular diseases. The sub-chronic effects of *Centella asiatica* extract on systolic blood pressure (SBP) of normotensive and N^G-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats were investigated. SBP was recorded from caudal artery in 64 male Wistar rats by the non-invasive tail cuff method using single stand tail cuff plethysmography blood pressure system (IITC Life Science Inc., USA) connected to PowerLab system. The rats were divided into eight groups: the first to the fourth groups were freely allowed access to reversed osmosis (RO) water and daily orally administered with double deionized distilled (DDD) water (10 ml/kg), *Centella asiatica* extract (16 g/10 ml/kg), quercetin (5 mg/5 ml/kg), and propylene glycol (5 ml/kg), respectively, the fifth to the eighth groups were freely allowed to drinking water containing L-NAME (40 mg/kg/day) and daily orally administered with DDD water (10 ml/kg), *Centella asiatica* extract (16 g/10 ml/kg), quercetin (5 mg/5 ml/kg), and propylene glycol (5 ml/kg), respectively, for 28 days SBP was recorded at day 0, 7, 14, 21, and 28 after drinking RO water containing L-NAME.

Body weight was recorded every weeks and daily water intake was measured. The SBP in all groups was 126.27 ± 0.63 mmHg. The groups that consumed drinking water containing L-NAME significantly increased SBP to 173.87 ± 1.73 mmHg at day 7 and 185.57 ± 1.52 mmHg at day 28 of experiment, compared to their respective control group. *Centella asiatica* extract significantly dropped the increased SBP after drinking RO water containing L-NAME to 146.65 ± 4.05, 146.60 ± 2.55, 141.78 ± 2.16 mmHg on day 14, 21, and 28, respectively, when compared to L-NAME with DDD water treated group (180.51 ± 3.68, 180.12 ± 2.38, and 184.50 ± 4.40 mmHg). Quercetin significantly dropped the increased SBP after drinking RO water containing L-NAME to 156.91 ± 5.34, 147.77 ± 4.48, 140.89 ± 2.37 mmHg on day 14, 21, and 28, respectively, when compared to L-NAME with propylene glycol treated group (176.70 ± 2.55, 180.15 ± 3.05, and 186.64 ± 3.27 mmHg). Four weeks administration of quercetin significantly increased body weight compared to propylene glycol in normotensive rats. Water intake at day 21 and day 28 was significantly decreased by quercetin in L-NAME induced hypertensive rats. Water intake at day 7 and day 28 was significantly decreased by *Centella asiatica* extract in normotensive rats. In conclusion, this sub-chronic study demonstrated antihypertensive effects of *Centella asiatica* extract. *Centella asiatica* extract could ameliorate SBP in L-NAME induced hypertensive rats. These data support the use of this plant for hypertension treatment in traditional medicine.

5.2 Introduction

Hypertension is a major global health challenge because of its high prevalence and concomitant risks of cardiovascular disease which is the principal cause of death

in all developed countries accounting for 50% of all deaths (Chockalingam, 2008). Several epidemiological studies revealed the inverse association between the flavonoid intake and reduction in the occurrence of cardiovascular disease including myocardial infarction (Hertog et al., 1993a). Among dietary flavonols, quercetin is by far the most abundant representing approximately 60% of the total intake (Hertog et al., 1993b). It has also been shown that quercetin causes endothelium-dependent relaxation in rat aorta (Fitzpatrick et al., 1993; Duarte et al., 1993). In addition, its cardiovascular protective effects have been well-documented (Duarte et al., 2001).

Despite the large number of antihypertensive drugs and the progresses made in the efficacy and tolerability of these agents, it is acknowledged that less than 25% treated individuals achieve target blood pressure (i.e. <140/90 mmHg) (Evans et al., 2005). Trial outcomes have shown that each agent is associated with relative benefits and drawbacks, often within the context of various patient characteristics such as age, co-morbidities and risk status (Germino, 2009). The current trend towards lower blood pressure goals suggest that more effective and better tolerated antihypertensive therapies will be needed, of which natural products can be considered as one of the potential sources. Indeed, various plant preparations have been used and claimed to have antihypertensive effects. The antihypertensive effects of some of these plants have been validated and others disproved. One of the plants used for the treatment of hypertension is *Centella asiatica*. It is a tropical medicinal plant with a long history of therapeutic used for conditions such as dermal disorder, vascular diseases (such as hypertension and altherosclerosis), microangioparthy and anti-infammatory, (De sanctis et al., 2001; Incandela et al., 2001; Suguna et al., 1996). It is widely used as blood purifier as well as for treating high blood pressure, for memory enhancement

and promoting longevity (Gohil, Patel, and Gajjar, 2010). *Centella asiatica* has been reported to have anti-peroxidase and free radical scavenging activities (Katare and Ganachari, 2001; Jayashree et al., 2003). The herb is said to have a direct effect in lowering blood pressure and is often referred to as a rejuvenating medicament in Ayurvedic Pharmacopoeia (Hussin, 2007). Thus, the present study attempted to evaluate the chronic antihypertensive effects of *Centella asiatica* extract in normotensive and L-NAME-induced hypertensive rats.

5.3 Materials and methods

5.3.1 Plant material

Centella asiatica extract obtained from the stock extract in chapter 3 was used in the experiments conducted in this chapter. A voucher specimen number of *Centella asiatica* is BFK184894 from BGO Plant Database, The Botanical Garden Organization, Ministry of Environment and Natural Resources, Thailand.

5.3.2 Animals

Male Wistar rats (240-250 g) were obtained from Institutional Animal Care, Suranaree University of Technology (SUT). They were maintained under standard laboratory conditions (12:12 h dark-light cycle, ambient temperature 20 ± 1°C) with free access to food and water. This study was conducted under permit of the SUT Animal Care and Use Committee.

5.3.3 Drugs and chemicals

N^G-nitro-L-arginine methyl ester (L-NAME) and quercetin were purchased from Sigma Aldrich Chemical Ltd. (St. Louis, USA). Propylene glycol was purchased from Vidhyasom Co. Ltd., Thailand. All other chemicals were of reagent grade. All the drug solutions were prepared at the time of use. All the drug solutions was dissolved in double deionized distilled (DDD) water.

5.3.4 Preparation of drug solutions

40 mg/kg L-NAME

40 mg/kg L-NAME solution was prepared by dissolving 400 mg of L-NAME (Sigma, St. Louis, MO, USA) with 1200 ml of reverse osmosis (RO) water (for n=32 per day, 75 ml for 2 rats).

5 mg/ml Quercetin

5 mg/ml Quercetin was prepared by dissolving 20 mg of quercetin (Sigma, St. Louis, MO, USA) with 4 ml of propylene glycol (Vidhyasom, Co. Ltd., Thailand) (for n=16 per day).

16 g/ 10 ml *Centella asiatica* extract

16 g/ 10 ml *Centella asiatica* extract was prepared by dissolving 64 g of *Centella asiatica* extract with 40 ml of DDD water (for n=16 per day).

5.3.5 Traditional restrained tail cuff pressure measurement

Restrained tail cuff pressure measurement is a non invasive technique to measure SBP in rats that is accurate, reliable simple and economical. It is an ideal instrument for cardiovascular research in physiology laboratories to measure parameters like blood pressure in rats (Pauline, Avadhany, and Maruthy, 2011). Sixty four male Wistar rats (240-250 g) were randomly divided into 8 experimental groups, n=8 each. For a period of 28 days, the first group (control group) was allowed access to reverse osmosis (RO) water and daily received DDD water (10 ml/kg, p.o.); the second group was allowed access to RO water and daily received *Centella asiatica* extract (16 g/10 ml/kg, p.o.) (effective dose from invasive blood pressure experiment in chapter IV); the third group was allowed access to RO water and daily received quercetin (5 mg/5 ml/kg, p.o.) (Perez-Vizcaino, 2009); the fourth group (control of quercetin group) was allowed access to RO water and daily received propylene glycol (5 ml/kg, p.o.); the fifth group was allowed to drink RO water containing L-NAME (40 mg/kg/day) (Kumar, Prahalathan, and Raja, 2012) and daily received DDD water (1 ml/kg, p.o.); the sixth group was allowed to drink RO water containing L-NAME (40 mg/kg/day) and daily received *Centella asiatica* extract (16 g/10 ml/kg, p.o.); the seventh group was allowed to drink RO water containing L-NAME (40 mg/kg/day) and daily received quercetin (5 mg/5 ml/kg, p.o.); the eighth group was allowed to drink RO water containing L-NAME (40 mg/kg/day) and daily received propylene glycol (5 ml/kg, p.o.).

5.3.6 Experimental protocol

The rats were accustomed to tail cuff blood pressure measurement for 7 days before the experiment started. Caudal artery blood pressure was measured by non-invasive tail cuff method using the single stand IITC Life Science tail cuff plethysmography blood pressure system (IITC Life Science Inc., USA). For blood pressure measurement, the conscious rats were placed into restrainer (IITC Life Science Inc., USA) in a posture which give a comfortable condition and fixed the rat tail in a tail cuff plethysmography (IITC Life Science Inc., USA). The rats were then prewarmed in an incubator at 37°C with temperature adjustment (IITC Life Science Inc., USA) for 10 min before the experiment start and kept at all times of recording (Lee et al., 2002). The tail cuff plethysmography inflated when start the single stand and set max cuff pressure is 200 mmHg. When systolic blood pressure (SBP) was detected, the tail cuff prethylmography was deflated automatic. The tail cuff pressure was monitored every 2 min, 3-4 times, until a stable reading was obtained. SBP was recorded before oral administration of DDD water, *Centella asiatica* extract, quercetin, and propylene glycol at day 0, 7, 14, 21, and 28. Water intake was measured everyday and body weight was recorded every weeks.

5.4 Statistical analysis

All values were expressed as mean ± S.E.M. Statistical analysis was determined by two-way repeated measured ANOVA followed by Tukey's test method using the Sigmastat software (version 3.5, Systat software Inc., USA). All graphs were created using the SigmaPlot software (version 10, Systat software Inc., USA). *P*-values less than 0.05 ($P<0.05$) were considered statistically significant.

5.5 Results

5.5.1 Sub-chronic effects of *Centella asiatica* extract and quercetin on SBP of normotensive and L-NAME induced hypertensive rats

The effects of *Centella asitica* extract on SBP during 28 days periods of normotensive and L-NAME induced hypertensive rats were shown in Figure 5.1. At the beginning of the study (day 0), the SBP of normotensive rats (122.80 ± 1.43, 126.56 ± 1.19, 127.91 ± 2.16, and 125.72 ± 2.18 mmHg for group 1, 2, 3, and 4) and L-NAME induced hypertensive rats (125.44 ± 2.50, 127.85 ± 4.43, 127.51 ± 2.66, and 126.27 ± 3.14 mmHg for group 5, 6, 7, and 8) were not significant difference. In normotensive rats, *Centella asiatica* extract, DDD water, quercetin, and propylene glycol did not cause changes in SBP. Drinking RO water containing L-NAME with DDD water and propylene glycol significantly increased SBP to 175.09 ± 3.98 mmHg and 172.64 ± 4.99 mmHg, respectively, at day 7 and significantly increased SBP to 184.50 ± 4.40 and 186.64 ± 3.27 mmHg, respectively, at day 28, when compared to their baseline values. When compared to L-NAME with DDD water treated group, *Centella asiatica* extract significantly dropped the SBP to 158.55 ± 4.45, 146.65 ± 4.05, 146.60 ± 2.55, 141.78 ± 2.16 mmHg on day 7, 14, 21, and 28, respectively. Quercetin significantly dropped the SBP to 155.30 ± 5.88, 156.91 ± 5.34, 147.77 ± 4.48, 140.89 ± 2.37 mmHg on day 7, 14, 21, and 28, respectively, when compared to L-NAME with propylene glycol treated group.

5.5.2 Effects of *Centella asiatica* extract and quercetin on the body weight of normotensive and L-NAME induced hypertensive rats

Increases of body weight in normotensive and hypertensive rats following rat's age were shown in Figure 5.2. In normotensive rats, body weight of *Centella asiatica* extract treated group was not different from DDD water treated group. Four weeks after treatment, quercetin significantly increased body weight of normotensive rats, compared to propylene glycol treated group in normotensive rats. In L-NAME induced hypertensive rats, there was no significant difference in all treatment groups.

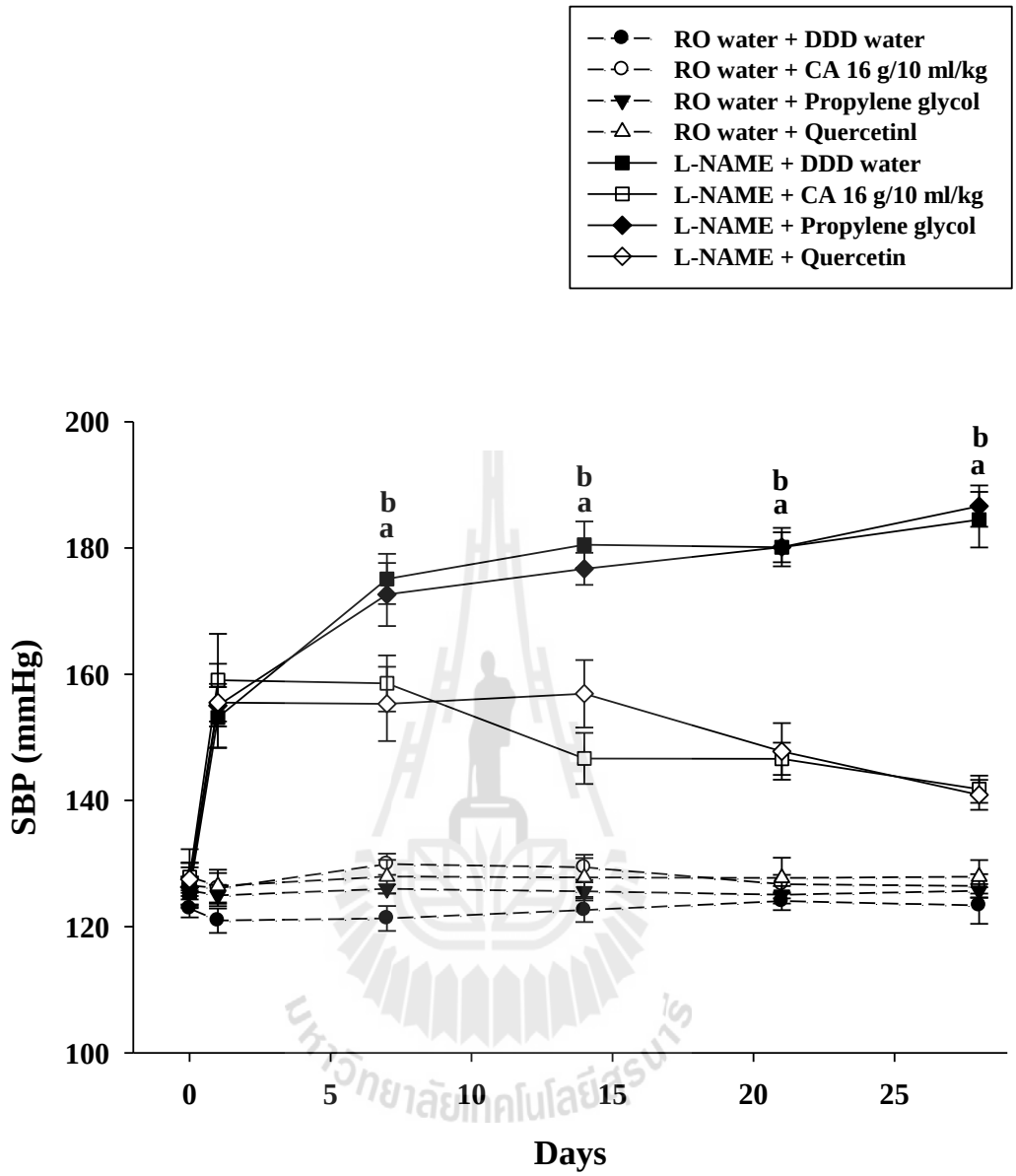

Figure 5.1 Effects of sub-chronic oral administration of *Centella asiatica* (CA) extract on SBP of normotensive and L-NAME induced hypertensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [a] denote significantly different between L-NAME with CA group compared to L-NAME with DDD water group (control group of CA), [b] denote significantly different between L-NAME with quercetin group compared to L-NAME with propylene glycol group (control group of quercetin), $P<0.05$ (Two-way repeated ANOVA; Tukey's test).

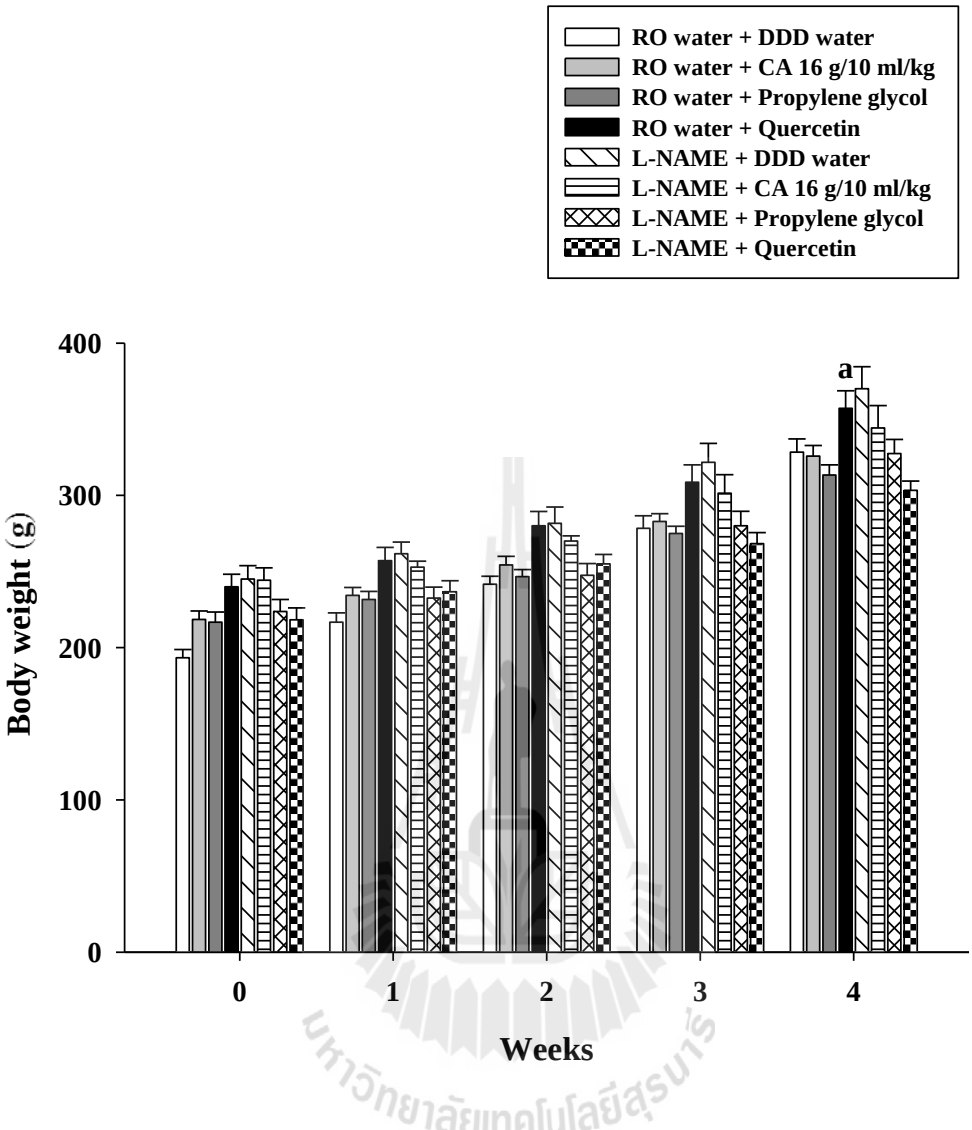

Figure 5.2 Effects of sub-chronic administration of *Centella asiatica* extract (CA) and quercetin on body weight of normotensive and L-NAME induced hypertensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [a] denotes significantly different between RO water with quercetin group compared to RO water with propylene glycol group (control group of quercetin), $P<0.05$ (Two-way repeated ANOVA; Tukey's test).

5.5.3 Effects of *Centella asiatica* extract and quercetin on water intake in normotensive and L-NAME induced hypertensive rats

Water intake at day 0, 7, 14, 21, and 28 of normotensive and hypertensive was shown in Figure 5.3. At day 7 after treatment, water intake of RO water with *Centella asiatica* extract treated group was significantly lower than RO water with DDD water treated group. At day 21 after treatment, water intake of L-NAME with quercetin treated group was significantly lower than L-NAME with propylene glycol treated group, At day 28 after treatment, water intake of RO water with *Centella asiatica* extract was significantly lower than RO water with DDD water treated group, and of L-NAME with quercetin treated group was significantly lower than L-NAME with propylene glycol treated group.

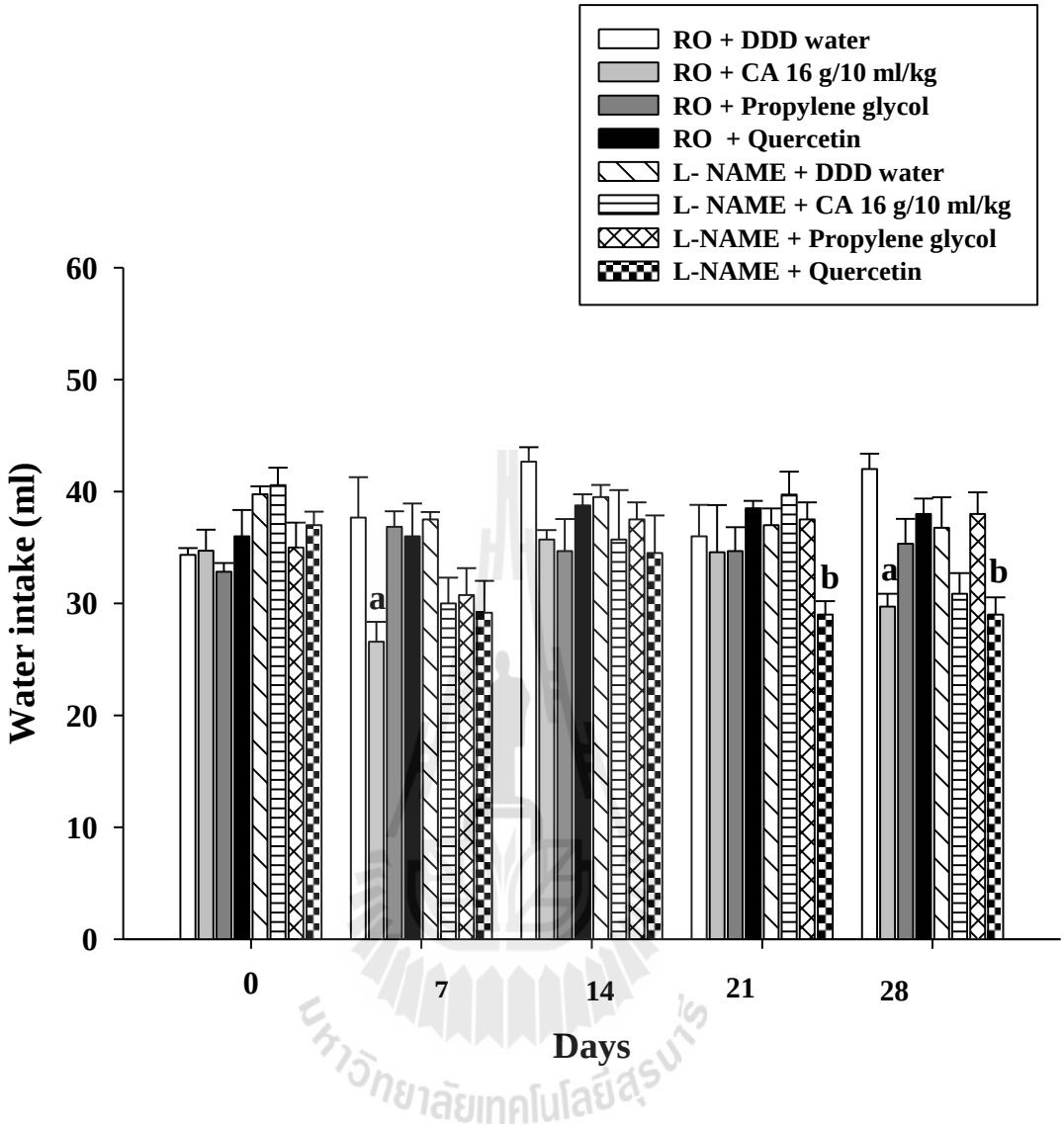

Figure 5.3 Effects of sub-chronic administration of *Centella asiatica* extract (CA) and quercetin on water intake of normotensive and L-NAME induced hypertensive rats. Data are expressed as mean ± S.E.M. (n=8 per group). [a] denotes significantly different between RO water with CA group compared to RO water with DDD water group (control group of CA), [b] denotes significantly different between L-NAME with quercetin group compared to L-NAME with propylene glycol group, $P<0.05$ (Two-way repeated ANOVA; Tukey's test).

5.6 Discussion and conclusion

The present study provides the first evidence of sub-chronic antihypertensive effect of *Centella asiatica* extract in L-NAME induced hypertensive rats. Oral administration of *Centella asiatica* extract and quercetin for 28 days could attenuate the increase of SBP in L-NAME induced hypertensive rats. The reduction of SBP caused by *Centella asiatica* extract and quercetin within 28 days could not return to their original baselines.

The inhibition of NO production by L-NAME leads to vascular smooth muscle contraction, renal dysfunction, and increased blood pressure (Siragy et al., 1992; Chen et al., 1998). Various models of hypertension have been associated with a decreased release of NO. For example, in hypertension mediated by volume overload, such as deoxycorticosterone acetate (DOCA)-salt and Dahl-salt sensitive hypertensive models, the renal and aortic NO levels are decreased (Chen et al., 1998; Hirata et al., 1995). Therefore, it is suggested that the decreased levels of NO may be important pathophysiological factor in the development and sustenance of hypertension (Kang et al., 2004). Pharmacological long-term blockade of NO synthesis by the chronic administration of L-NAME produces systemic arterial hypertension, vascular structural change, and renal dysfunction (Siragy et al., 1992; Chen et al., 1998).

Oral administration of *Centella asiatica* extract for 28 days could ameliorate elevated SBP in L-NAME induced hypertensive rats which is in accordance to the studies conducted by Muangnogwa (2004) and Leuadnakrob (1999). In DOCA-salt hypertensive rats, *Centella asiatica* juice at the doses of 24 and 32 g/kg significantly decreased SBP at two hours after single oral administration (Muangnogwa, 2004). Repeated oral administration of *Centella asiatica* extract from hexane fraction

(1 g/kg/day) for 7 days significantly decreased the rise in SBP in L-NAME induced hypertensive rats (Leuadnakrob, 1999). A reduction of SBP may be a result of relaxation of vascular smooth muscle caused by *Centella asiatica* extract. This postulate is supported by our findings from *in vitro* and acute *in vivo* studies (chapters III and IV). *Centella asiatica* extract could relax the precontraction of smooth muscle in endothelium-intact aortic rings induced by L-NAME and a single intragastric administration of *Centella asiatica* extract (16 g/20 ml/kg) could attenuate the elevated MABP, SBP, and DBP caused by L-NAME.

The present findings found that oral administration of quercetin for 28 days could drop the increased SBP induced by L-NAME which was resemble with the study of Zibadi et al. (2007). Dietary quercetin supplementation (10 mg/kg/day) reduced the elevated blood pressure in salt-sensitive rats fed with high salt intake (Aoi et al., 2004). A reduction of SBP caused by quercetin may be a result of relaxation of vascular smooth muscle. Quercetin and other flavonoids have been shown to promote relaxation of cardiovascular smooth muscle (antihypertensive effect) (Formica and Regelson, 1995). From our *in vitro* and *in vivo* studies (chapters III and IV) also showed that quercetin (0.25 mM) could possess the vasorelaxation effect on endothelium-intact aortic rings precontracted with L-NAME and single oral administration of quercetin (5 mg/20 ml/kg) could attenuate the elevated MABP, SBP, and DBP in L-NAME induced hypertensive rats.

During 28 days of treatment, body weight and water intake of the normotensive and hypertensive rats treated with *Centella asiatica* extract and quercetin are within normal values. Body weight in both normotensive and hypertensive rats was increased following their ages. Notably, the national laboratory

animal center (Mahidol, University) indicates that the average standard of water intake of adult Wistar rat (250-300 g) is 25-45 ml/day. Although significant different in water intake were demonstrated in this study, but the amount of water intake is within the average amount.

In conclusion, *Centella asiatica* extract and quercetin possessed sub-chronic antihypertensive effects. Quercetin may be in part responsible for hypertensive effect of *Centella asiatica*. Further investigations are needed to elucidate the potential of *Centella asiatica* in chronic hypertension.

5.7 References

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CHAPTER VI

CONCLUSION

Centella asiatica is a medicinal plant which is a good source of phenolic compounds including flavonoids that possess potent antihypertensive effects. In the present study, the percent yield of 80% ethanolic extract of *Centella asiatica* was 8.04%. Total phenolic content was 87.47 ± 1.74 mg gallic acid/g dry extract. In the present study, we have described the potent antihypertensive effects of *Centella asiatica* extract *in vitro* and *in vivo*. The *in vitro* study provided the first evidence of *Centella asiatica* extract and quercetin in potential ability to regulate vascular tones. Vasorelaxation induced by *Centella asiatica* extract and quercetin were found to be endothelium-independent and unrelated to nitric oxide (NO). Potassium (K^+) channel and L-type calcium (Ca^{2+}) channel were all involved in relaxation process of vascular smooth muscle (Figure 6.1).

Acute and sub-chronic effects of *Centella asiatica* extract were demonstrated in male Wistra rats. Data from acute effects indicated that *Centella asiatica* extract at the concentration of 16 g/20 ml/kg (i.g.) and quercetin (5 mg/20 ml/kg, i.g.) significantly lowered the elevated arterial blood pressure (mean arterial blood pressure (MABP), systolic blood pressure (SBP), and diastolic blood pressure (DBP) in N^G-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats. *Centella asiatica* extract and quercetin did not cause any change in heart rate (HR) and respiratory rate (RR) of rats receiving L-NAME. In normotensive rats, the rat that

received *Centella asiatica* extract at the concentration of 4 and 8 g/20 ml/kg significantly increased arterial blood pressure. In normotensive rats, quercetin significantly decreased MABP and significantly increased RR, but had no effect in HR. These findings indicated that *Centella asiatica* extract possess antihypertensive effects in L-NAME induced hypertensive rats and hypertensive effects in normotensive rats (Figure 6.2).

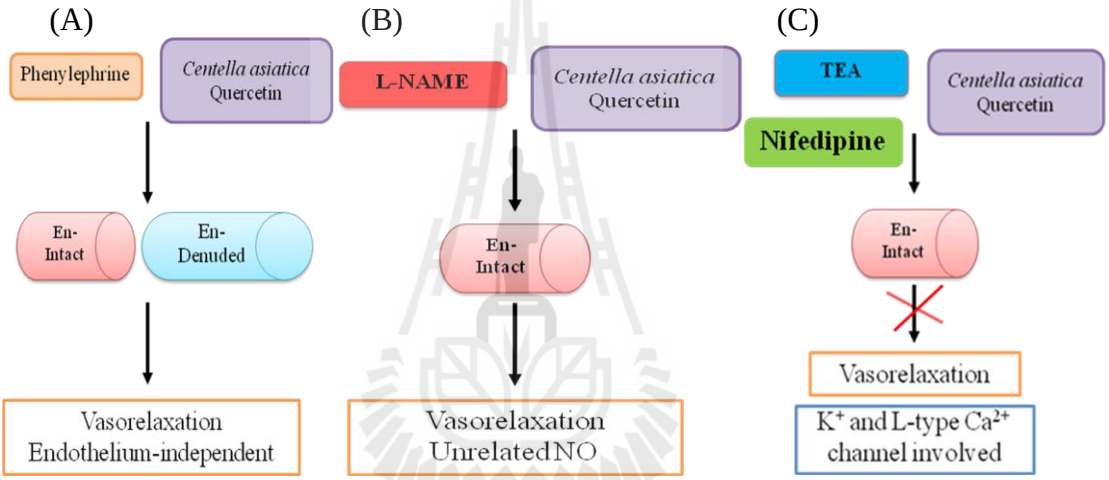

Figure 6.1 Mechanism of vasorelaxation activity of *Centella asiatica* extract and quercetin *in vitro*. (CA: *Centella asiatica*; En-Intact: endothelium-intact aortic ring; En-Denuded: endothelium-denuded aortic ring; TEA: tetraethylammonium; NO: nitric oxide; K^+: potassium channel; Ca^{2+}: calcium channel).

Additionally, data from sub-chronic effects indicated that *Centella asiatica* extract (16 g/ 20 ml/kg, p.o.) and quercetin (5 mg/5 ml/kg, p.o.) significantly lowered the increased SBP following L-NAME in hypertensive rats. In normotensive rats that

reverse osmosis (RO) water, sub-chronic administration of *Centella asiatica* extract and quercetin had no effects in SBP (Figure 6.3).

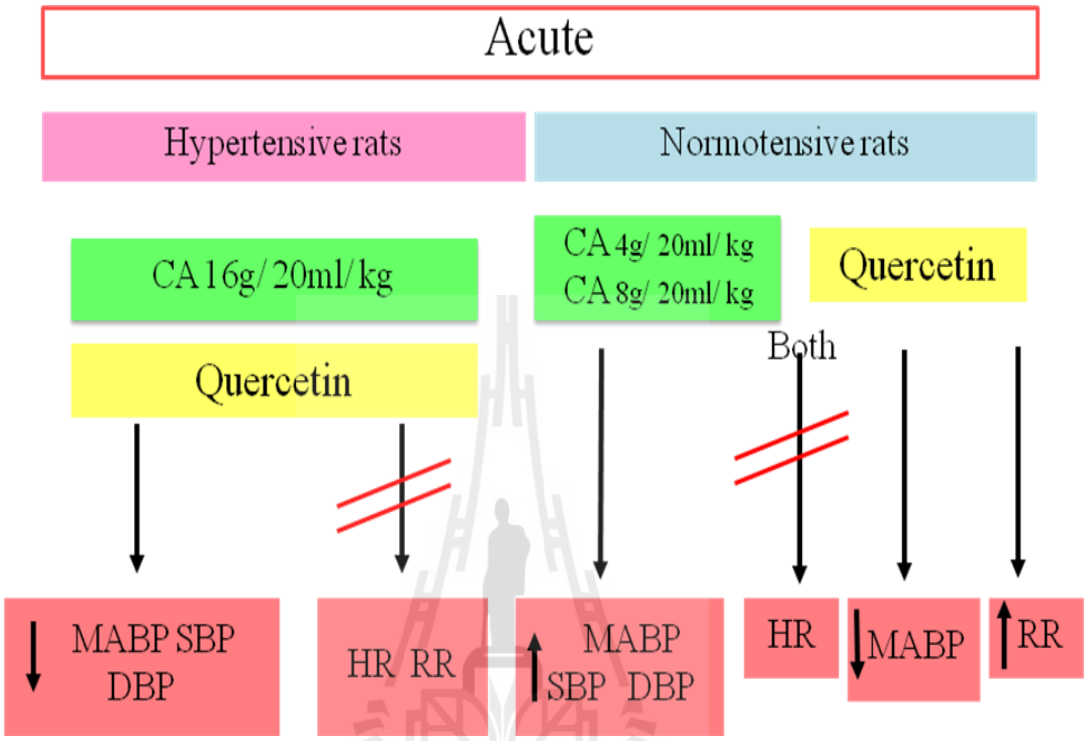

Figure 6.2 Acute effects of *Centella asiatica* extract and quercetin in male Wistar rats. (CA: *Centella asiatica* extract; MABP: mean arterial blood pressure; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; RR: respiratory rate).

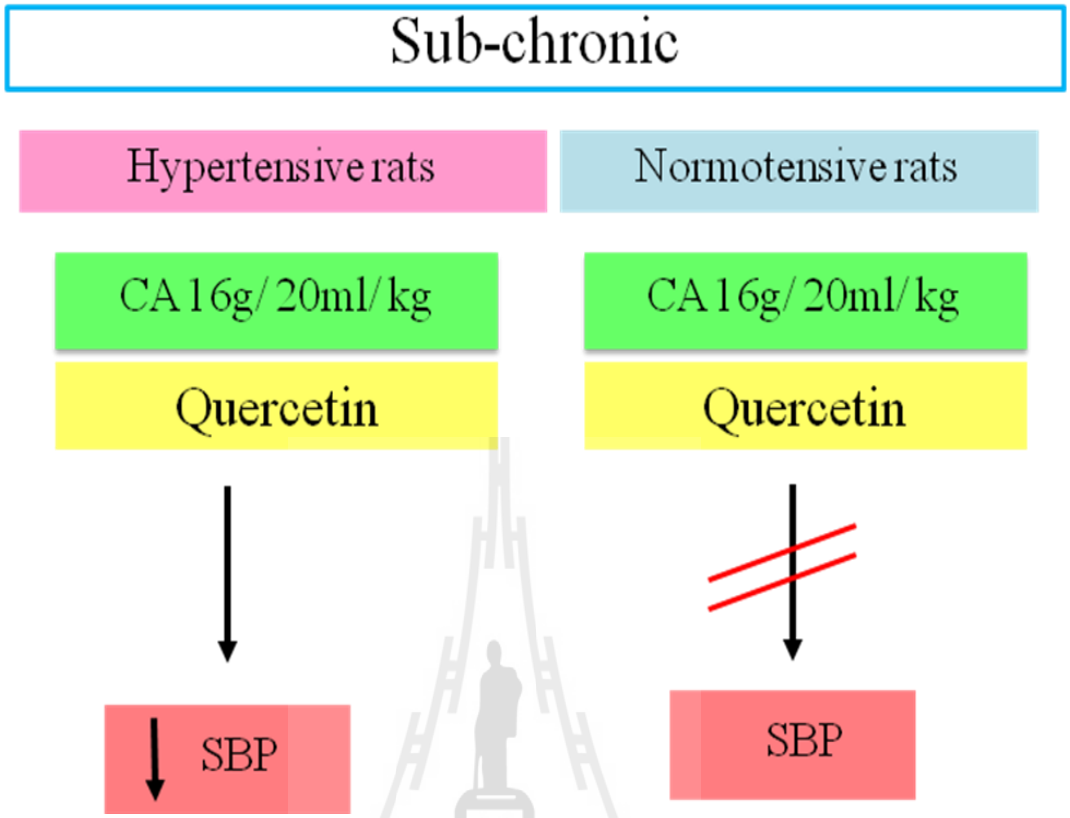

Figure 6.3 Sub-chronic effects of *Centella asiatica* extract and quercetin in male Wistar rats. (CA: *Centella asiatica* extract; SBP: systolic blood pressure).

In conclusion, this study demonstrated potent vasorelaxation activity and antihypertensive effects of *Centella asiatica* extract *in vitro* and *in vivo*. Phenolic compounds, notably quercetin, may be bioactive compounds responsible for the antihypertensive activity of *Centella asiatica* extract. Further studies are needed to clarify the underlying mechanisms involving antihypertensive effects of *Centella asiatica* extract.

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