ผลของสารสกัดจากบัวบกต่อการลดไขมันและน้ำตาลในเลือด ของหนูขาวเพศผู้พันธุ์วิสตาร์

นายณัฏฐพล ศุภกมลเสนีย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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HYPOLIPIDEMIC AND HYPOGLYCEMIC EFFECTS OF *CENTELLA ASIATICA* EXTRACT IN MALE WISTAR RATS

Nattapon Supkamonseni

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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HYPOLIPIDEMIC AND HYPOGLYCEMIC EFFECTS OF *CENTELLA ASIATICA* EXTRACT IN MALE WISTAR RATS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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การทดลองในครั้งนี้มีจุดประสงค์เพื่อศึกษาผลของสารสกัดบัวบกและรูตินที่มีต่อการลดไขมันและการลดน้ำตาลในเลือด ภายในหลอดทดลอง และในสัตว์ทดลอง ซึ่งประกอบด้วย 4 การทดลอง ดังนี้

ผลของสารสกัดบัวบกจากเอทานอลและรูตินที่มีต่อการทำงานของเอนไซม์ไลเปสจากตับอ่อนในหลอดทดลอง พบว่าบัวบกที่สกัดด้วยร้อยละ 80 ของเอทานอล ให้ผลผลิตเท่ากับร้อยละ 11.81 มีสารประกอบฟีนอลิกทั้งหมดเท่ากับ 97.75 ± 0.01 มิลลิกรัมของกรดแกลิกต่อกรัมของสารสกัดแห้งและมีปริมาณรูตินทั้งหมดเท่ากับ 1.27 ± 5.5 กรัมต่อกิโลกรัมของบัวบกแห้ง การศึกษาในหลอดทดลองพบว่าความเข้มข้นยาออริสแตท (9.52 มิลลิกรัมต่อมิลลิลิตร) มีเปอร์เซ็นต์การยับยั้งเอนไซม์ไลเปสจากตับอ่อนร้อยละ 75.47 สารสกัดบัวบกและรูตินมีค่าความเข้มข้นที่สามารถยับยั้งการทำงานของเอนไซม์ไลเปสร้อยละ 50 (IC_{50}) เท่ากับ 25.03 ± 0.05 และ 48.05 ± 0.08 มิลลิกรัมต่อมิลลิลิตรตามลำดับ

ผลของสารสกัดบัวบกจากเอทานอลและรูตินที่มีต่อการทำงานของเอนไซม์อัลฟาอะไมเลสและเอนไซม์อัลฟากลูโคซิเดสในหลอดทดลอง พบว่าสารสกัดบัวบก รูตินและยาอะคาโบส ซึ่งเป็นยาลดระดับน้ำตาลในเลือด ยาอะคาโบส (0.77 มิลลิกรัมต่อมิลลิลิตร) ยับยั้งเอนไซม์อัลฟาอะไมเลสร้อยละ 96.76 และยาอะคาโบส (0.30 มิลลิกรัมต่อมิลลิลิตร) ยับยั้งเอนไซม์อัลฟากลูโคซิเดสร้อยละ 82.59 สารสกัดบัวบกและรูตินมีค่าความเข้มข้นที่สามารถยับยั้งการทำงานของเอนไซม์อัลฟาอะไมเลสร้อยละ 50 (IC_{50}) เท่ากับ 2.14 ± 0.04 และ 1.53 ± 0.05 มิลลิกรัมต่อมิลลิลิตรตามลำดับ และสารสกัดบัวบกและรูตินมีค่าความเข้มข้นที่สามารถยับยั้งการทำงานของเอนไซม์อัลฟากลูโคซิเดสร้อยละ 50 (IC_{50}) เท่ากับ 0.02 ± 0.009 และ 0.081 ± 0.004 มิลลิกรัมต่อมิลลิลิตรตามลำดับ

ผลของสารสกัดบัวบกจากเอทานอลและรูตินที่มีต่อค่าทางชีวเคมีต่างๆ ของพลาสมาในหนูขาวที่ถูกชักนำให้เกิดภาวะเลือดมีสารไขมันมากด้วย lipid emulsion พบว่าสารสกัดบัวบก (1000 และ 2000 มิลลิกรัมต่อ 4 มิลลิลิตรต่อกิโลกรัม) รูติน (1000 มิลลิกรัมต่อ 4 มิลลิลิตรต่อกิโลกรัม) และยาออริสแตท (45 มิลลิกรัมต่อ 4 มิลลิลิตรต่อกิโลกรัม) ที่ให้ทางปากสามารถยับยั้งการเพิ่มขึ้นของระดับไตรกลีเซอไรด์และคลอเรสเตอรอลรวมในพลาสมาที่ถูกเหนี่ยวนำด้วย lipid emulsion ได้อย่างมีนัยสำคัญทางสถิติ นอกจากนี้สารสกัดบัวบก (1000 และ 2000 มิลลิกรัมต่อ 4 มิลลิลิตรต่อกิโลกรัม) รูตินและยาออริสแตทสามารถลดระดับกลูโคสในพลาสมา

ผลของสารสกัดบัวบกจากเอทานอลและรูตินที่มีต่อการแสดงออกของโปรตีนฟอสในสมองส่วนควบคุมสมดุลพลังงานในหนูขาว พบว่าสารสกัดบัวบก (1000 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) ชักนำให้เกิดการแสดงออกของโปรตีนฟอสใน ARC และ PVN ในขณะที่สารสกัดบัวบก (1000 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) และรูติน (1500 มิลลิกรัมต่อมิลลิลิตรต่อกิโลกรัม) ชักนำให้เกิดการแสดงออกของโปรตีนฟอสในส่วน ARC เท่านั้น ผลของการศึกษานี้ชี้ให้เห็นว่าสารสกัดบัวบกและรูตินอาจมีส่วนเกี่ยวข้องในการควบคุมการกินอาหารโดยไปกระตุ้นการทำงานของเซลล์ประสาทในสมองบริเวณ ARC และ PVN ในไฮโปทามัส

โดยสรุปการศึกษาในครั้งนี้ชี้ให้เห็นถึงสารสกัดบัวบกและรูตินมีฤทธิ์ในการต้านโรคอ้วน ฤทธิ์ในการลดระดับไขมัน ฤทธิ์ในการลดระดับน้ำตาลและการกระตุ้นการทำงานของเซลล์ประสาทในสมองส่วนที่ทำหน้าที่ควบคุมการกินและสมดุลของพลังงาน

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2555 ลายมือชื่ออาจารย์ที่ปรึกษา _____

NATTAPON SUPKAMONSENI : HYPOLIDEMIC AND HYPOGLYCEMIC EFFECTS OF *CENTELLA ASIATICA* EXTRACT IN MALE WISTAR RATS. THESIS ADVISOR : ASST. PROF. RUNGRUDEE SRISAWAT, Ph.D. 202 PP.

CENTELLA ASIATICA/OBESITY/LIPASE ENZYME/ALPHA-AMYLASE ENZYME/ALPHA-GLUCOSIDASE ENZYME/BIOCHEMICAL PARAMETERS/ FOS/ARCUATE NUCLEUS/PARAVENTRICULAR NUCLEUS

The aims of the present study were to investigate the effects of *Centella asiatica* (*C. asiatica*) extract and rutin on hypolipidemic and hypoglycemic effects *in vitro* and *in vivo*. This study consisted of 4 main experiments.

The effects of the aqueous ethanolic extract of *C. asiatica* and rutin on pancreatic lipase activity *in vitro* were performed. The results showed that *C. asiatica* extracted by 80% ethanol had a yield of 11.81%. *C. asiatica* extract contained total phenolic content of 97.75 ± 0.01 mg gallic acid/g dry weight and rutin of 1.27 ± 5.5 g/kg dry plant. In this study, orlistat (9.52 mg/ml) had 75.47% inhibition of pancreatic lipase activity. The concentrations of *C. asiatica* extract and rutin required to inhibit 50% of pancreatic lipase activity (IC_{50}) were 25.03 ± 0.05 and 48.05 ± 0.08 mg/ml, respectively.

The effects of the aqueous ethanolic extract of *C. asiatica* and rutin on alpha-amylase and alpha-glucosidase activities *in vitro* were performed. Acarbose (0.77 mg/ml) had 96.76% inhibition of alpha-amylase activity and inhibitory alpha-glucosidase activity of acarbose (0.30 mg/ml) was 82.59%. The concentrations of

C. asiatica extract and rutin required to inhibit 50% of alpha-amylase activities (IC_{50}) were 2.14 ± 0.04 and 1.53 ± 0.05 mg/ml, respectively. The IC_{50} for inhibition of alpha-glucosidase activities by *C. asiatica* extract and rutin were 0.02 ± 0.009 and 0.081 ± 0.004 mg/ml, respectively.

The effects of the aqueous ethanolic extract of *C. asiatica* and rutin on plasma biochemical parameters in lipid emulsion-induced hyperlipidemic rats were investigated. *C. asiatica* extract (1000 and 2000 mg/4 ml/kg), rutin (1000 mg/ 4 ml/kg), and orlistat (45 mg/4 ml/kg) significantly inhibited the plasma levels of triglyceride (TG) and total cholesterol (TC) induced by lipid emulsion. Moreover, orlistat, rutin, and *C. asiatica* extract (1000 and 2000 mg/4 ml/kg) could reduce the levels of plasma glucose.

The effects of the aqueous ethanolic extract of *C. asiatica* and rutin on Fos expression in the brain areas regulating homeostasis of energy balance in male Wistar rats were investigated. *C. asiatica* extract (1000 mg/ml/kg) induced Fos expression in the ARC and PVN, while *C. asiatica* extract (2000 mg/ml/kg) and rutin (1500 mg/ml/kg) induced Fos expression only in the ARC. The results of this study suggested *C. asiatica* extract and rutin may involve in the regulation of food intake by activating the ARC and PVN in the hypothalamus.

In conclusion, *C. asiatica* extract and its bioactive compound rutin exhibited anti-obesity, hypolipidemic, and hypoglycemic effects, and induced neuronal activation in the brain areas involving in regulation of food intake and energy homeostasis.

School of Biology Student's Signature _____

Academic Year 2012 Advisor's Signature _____

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CHAPTER I

INTRODUCTION

1.1 The global epidemic of obesity

Obesity became a public health problem in developed countries. Worldwide, 2.8 million people die each year as a result of being overweight (including obesity) and an estimated 35.8 million (2.3%) of global populations are caused by overweight or obesity (World Health Organization, 2011). In 2030, about 2.16 billion adults will be overweight and 1.12 billion will be obese (Kelly *et al*., 2008). Historical records from developed countries indicated that height and weight in the population increase during the 19^{th} century. In the 20^{th} century, populations from developed countries began to approach their genetic potential for longitudinal growth. They began to gain proportionally more weight than height, resulting in an increase in average body mass index (BMI) (Caballero, 2007). The prevalence of overweight and obesity in population worldwide (aged 20 years old or over) obtained from the World Health Organization (WHO) in 2010 are shown in Figures 1.1 and 1.2, respectively. Therefore, obesity prevention and treatment are a high public health priority.

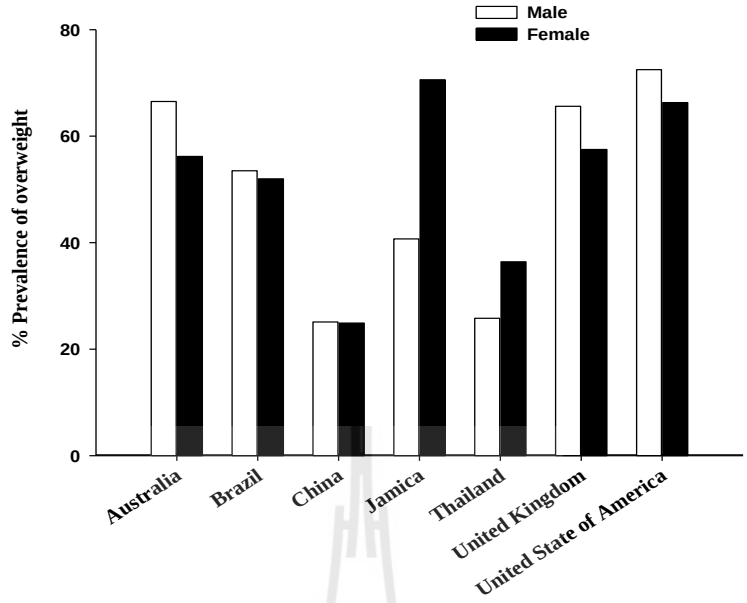

Figure 1.1 Prevalence of overweight males and females (aged 20 years or over) in selected countries in 2010 (WHO, 2011).

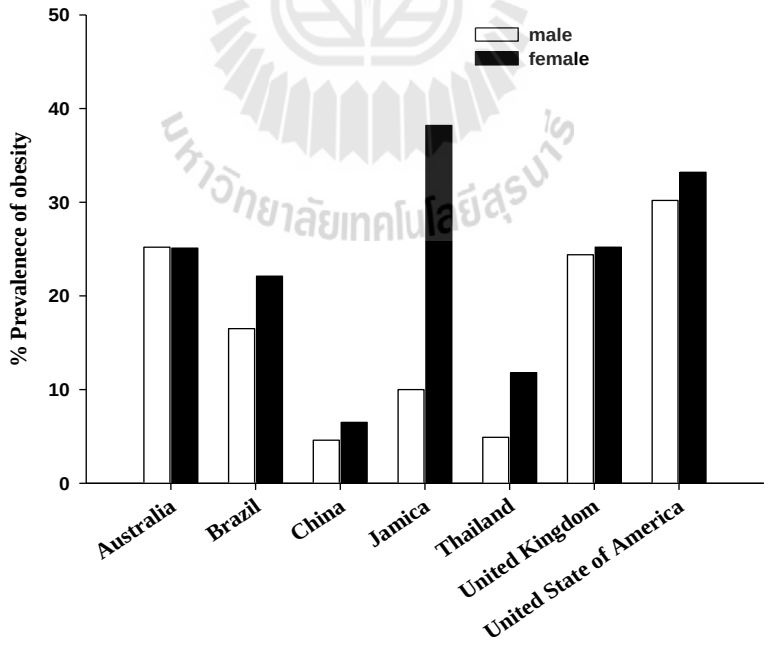

Figure 1.2 Prevalence of obese males and females (aged 20 years or over) in selected countries in 2010 (WHO, 2011).

1.2 Etiology and impact of obesity

Obesity is characterized by excessive generalized deposition of fat in the body (Seidell, 1998). Fat cannot be determined directly, indirect measures are used such as BMI, waist circumference, waist/hip ratio, and skinfold thickness (Knecht, Ellger, and Levine, 2008). Obesity is the prototypical model of a complex genetic condition interacting with lifestyle choices (Flegal, Troiano, and Ballard-Barbash, 2001). The factors contributing to obesity were determined by physiology, environment, and behavior which these factors regulated the balanced of energy expenditure and energy intake that are shown in Figure 1.3 (Knecht *et al*., 2008). Obesity is associated with an increased risk in all cause mortality (Pi-Sunyer, 2002). All adult individuals who are overweight or obese are at risk of morbidity from atherosclerotic diseases associated with type 2 diabetes mellitus, dyslipidemia, hypertension, coronary heart disease, and gallbladder disease (Must *et al*., 1999; Pi-Sunyer, 2002).

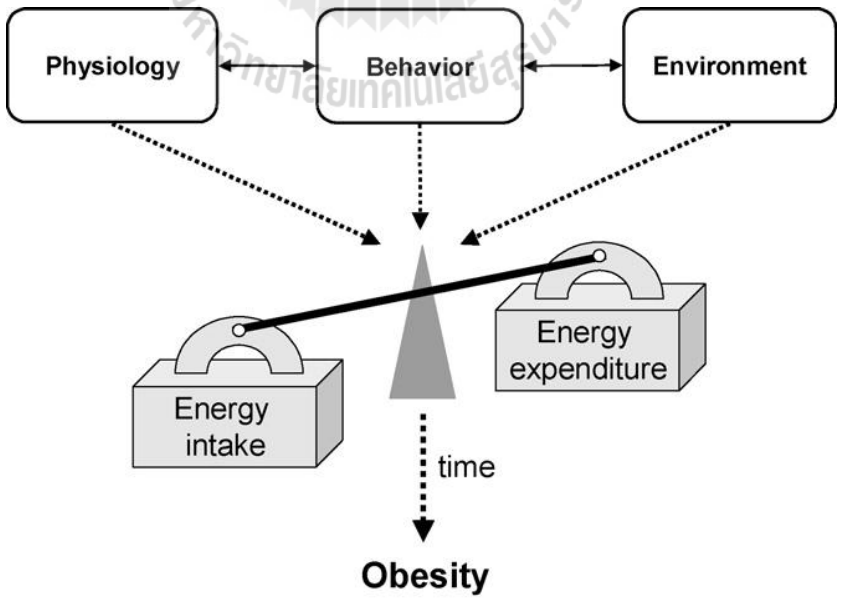

Figure 1.3 Schematic of factors contributing to obesity (Knecht *et al*., 2008).

1.3 The management of obesity

Treatment of obesity will be the most successful if incorporating the use of diet, exercise, and behavior modification with or without pharmacological therapy and/or surgery. Nowadays, many attempts have been made to connect the metabolic disparity of the obesity condition, producing a number of reagents including orlistat (fat absorption inhibitor), sibutramine (appetite suppressant), and rimonabant (cannabiniod CB_1 receptor block) (Padwal and Majumdar, 2007). Some anti-obesity drugs have severe or life-threatening side effects such as hypertension, headaches, insomnia, dry mouth, nausea, vomiting, dizziness, and palpitations (Akbas *et al*., 2009). There was a new alternative for prevention of obesity from medicinal plants such as *Taraxacum officinale* (Zhang *et al*., 2008), *Nelumbo nucifera* (Ono *et al*., 2006), and tea (Han *et al*., 2001; He *et al*., 2007). These medicinal plants could be used for anti-obesity since they inhibited pancreatic lipase activity and suppressed plasma triglyceride.

Fruits and vegetables were particularly rich sources of antioxidant components, including polyphenols (Bagchi *et al*., 2007; Kubola and Siriamornpun, 2008). Polyphenols found in fruits (e.g. apple, raspberry, orange, and grape seed) and vegetables (e.g. *Aesculus turbinata*, Nomame Herba, tea, bean, *Capparis spinosa*, *Chamaemelum nobile*, *and Nelumbo nucifera*) had anti-obesity (Han *et al*., 1999; Hu *et al*., 2008; Lemhadri *et al*., 2007; Morimoto *et al*., 2005; Park, Park, and Cha, 2008; Tanaka *et al*., 2009; Titta *et al*., 2009; Yamamoto *et al*., 2000). Thus, consumption of fruits and vegetables containing high amount of polyphenols may contribute to the prevention of obesity.

Rutin is a flavanol type of flavonoids. Rutin is found in many typical plants and has several pharmacological properties such as anticholesterolaemic (Ziaee *et al*., 2009) and antiglycemic effects (Rauter *et al*., 2010). Furthermore, the addition of rutin or *0*-coumaric acid to the diet could decrease body weight gain, liver weight, adipose tissue weight, triglyceride (TG), phospholipid, total cholesterol (TC), insulin, and leptin in high fat diet-induced obese rats (Hsu *et al*., 2009). Moreover, rutin was reported to inhibit activities of porcine pancreatic lipase activity *in vitro* (Zheng *et al*., 2010).

Centella asiatica (L.) Urban is one of medicinal plants in tropical and subtropical countries, contains a large variety of substances possessing antioxidant activity such as phenolics, tannins, triterpenoids, and numerous flavonoids (catechin, rutin, and quercetin) (Zheng and Qin, 2007). These active compounds have been reported to have anti-obesity effect. Thus, *C. asiatica* could be candidate for treating obesity. Therefore, the effects of aqueous ethanolic extract of *C. asiatica* and rutin on obesity were investigated. Rutin was selected in anti-obesity study since it might be responsible for anti-obesity effects of *C. asiatica*.

1.4 Research objectives

The experiments were designed to clarify the following:

1. To study the effects of the aqueous ethanolic extract of *C. asiatica* on pancreatic lipase, pancreatic alpha-amylase, and alpha-glucosidase activities *in vitro*.

2. To study the effects of the aqueous ethanolic extract of *C. asiatica* on plasma biochemical parameters levels in postprandial hypertriglyceridemic rats.

3. To study the acute effect of the aqueous ethanolic extract of *C. asiatica* on Fos expression in the hypothalamus and the nucleus of the solitary tract in rats.

1.5 Research hypothesis

The aqueous ethanolic extract of *C. asiatica* has anti-obesity effects in rats through the inhibition of fat metabolizing enzyme (pancreatic lipase), carbohydrate hydrolyzing enzymes (alpha-amylase and alpha-glucosidase), and the neuronal activation in the brain areas involved in the regulation of food intake and body energy balance.

1.6 Expected results

The findings will provide the new evidence of the beneficial effects of aqueous ethanolic extract of *C. asiatica* on anti-obesity as it may inhibit pancreatic lipase, alpha-amylase, and alpha-glucosidase activities, decrease triglyceride, total cholesterol, and glucose levels, stimulate neuronal activity in the paraventricular nucleus of the hypothalamus and stimulate neuronal activity in the nucleus of the solitary tract, inhibit neuronal activity in the lateral hypothalamic area and the perifornical area of the hypothalamus, and stimulate neuronal activities in the arcuate nucleus of the hypothalamus.

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CHAPTER II

LITERATURE REVIEW

2.1 *Centella asiatica* (AsiatiC0020Pennywort Linn.)

Centella asiatica (*C. asiatica*) is a genus of the plant family Apiaceae (Umbelliferae). *C. asiatica* is a perennial creeper plant that flowers between August and September. Its flowers are of a light violet color. The leaves have long petioles arising rosette like from a common base (the nodes), and the individual "leaf rosettes" (the nodes) are connected by slender aerial stolons or runners (Brinkhaus *et al*., 2000). The form and shape of the *C. asiatica* plant can differ greatly depend on environmental conditions (James and Dubery, 2009). The leaves of *C. asiatica* has about 1.3-6.3 cm diameter, leaf stalk 2-5 cm long and have a mildly bitter taste which is widely distributed in wet place and warmer regions (Jamil, Nizami, and Salam, 2007).

2.1.1 Chemical constituent of *C. asiatica*

C. asiatica is reported to contain following types of compounds:

Triterpenoids

Triterpene is a major and the most important component of *C. asiatica*. The triterpenes in *C. asiatica* are mainly pentacyclic triterpenic acids and their respective glycosides, belonging to ursane- or oleanane-type, including asiatic acid,

asiaticoside, madecassic acid, madecassoside, brahmoside, brahmic acid, brahminoside, thankuniside, isothankuniside, centelloside, madasiatic acid, centic acid, cenellic acid, betulinic acid, and indocentic acid (Zheng and Qin, 2007). There are some triterpenes such as olean-13-ene triterpene, centellasapogenol A, and its oligoglycoside, ursane-derived saponin (23-O-acetylmadecassoside) and a new oleanane-derived saponin (23-O-acetylasiaticoside B) was found in the leaves of *C. asiatica* (Matsuda *et al*., 2001; Rumalla *et al*., 2010).

Flavonoids

C. asiatica contained numerous flavonoids (quercetin, rutin, catechin, and naringin). Flavonoids are group of the total phenolic compound (Ariffin *et al*, 2011; Hussin *et al*., 2009; Zainol *et al*., 2003). Orhan (2012) showed that *C. asiatica* consisted of kaempferol, patuletin, apigenin, castilliferol, castillicetin and myricetin.

Other components

C. asiatica has also been revealed the presence of polysaccharide, polyene-alkene, amino acids, fatty acids, alkaloids, sterols, carotenoids, tannin, chlorophyll, pectin, and inorganic salts (Brinkhaus *et al*., 2000).

2.2 Mechanisms of actions based on preclinical studies

Wound healing

C. asiatica extract has a potent of wound healing. Sunilkumar *et al*. (1998) reported that the aqueous extract of *C. asiatica* applied to open wounds in rats (3 times daily for 24 days) resulted in the increase of cellular proliferation and

collagen synthesis at the wound site, as shown by an increase in collagen content and tensile strength. Total triterpenoid fraction extracted from *C. asiatica* (TTFCA) increased the percentage of collagen in cell layer fibronectin and thus could help in promoting wound healing (Tenni *et al*., 1998). Moreover, asiaticoside fascilitated wound healing through an increase in peptidic hydroxyproline content, tensile strength, collagen synthesis, angiogenesis, and epithelialization (Bonte *et al*., 1994; Shukla *et al*., 1999).

Treatment for venous insufficiency

C. asiatica acted on the connective tissues of the vascular wall, being effective in hypertensive microangiopathy, venous insufficiency, and decreasing capillary filtration rate by improving microcirculatory parameters (Cesarone *et al*., 1992). TTFCA could improve venous wall alterations in chronic venous hypertension and protect the venous endothelium. TTFCA was active on connective tissue modulation, improved the synthesis of collagen, and other tissue proteins by modulating the action of fibroblasts in the vein wall. TTFCA could stimulate collagen remodeling in the venous wall (Incandela *et al*., 2001).

Gastric ulcer healing

C. asiatica could prevent ethanol-induced gastric mucosal barrier and reduce the damaging effects of free radicals (Cheng and Koo, 2000). The study of Cheng *et al*. (2004) revealed that *C. asiatica* and asiaticoside orally administered to rats with gastric ulcers caused a reduction in the size of the ulcers at day 3 and 7 in a dose-dependent manner, with a concomitant attenuation of myeloperoxidase activity

at the ulcer tissues, and an increase in epithelial cell proliferation and angiogenesis. The expression of basic fibroblast growth factor, an important angiogenic factor, was also upregulated in the ulcer tissues in rats treated with *C. asiatica* and asiaticoside.

Antidepressant properties

The antidepressant effect of total triterpenes from *C. asiatica* has been demonstrated in forced swimming mice (Chen *et al*., 2003). Total tritepenes from *C. asiatica* which reduced the immobility time in forced swimming test and ameliorated the imbalance of amino acid levels in mice brains. Moreover, Chen *et al*. (2005) demonstrated a reduction of the corticosterone level and an increase of the content of norepinephrine and dopamine in rat brain (cortex, hippocampus, and thalamus) following administration of total triterpenes of *C. asiatica*. Total triterpenes of *C. asiatica* play a role in ameliorating the function of hypothalamic pituitary adrenal (HPA) axis and increasing the contents of monoamine neurotransmitter for its antidepressant effects.

Cytotoxic and antitumour activities

Oral administration of *C. asiatica* extract and its partially purified fractions could retard the development of solid and ascites tumours and increased the life span of these tumour bearing mice. Cytotoxic and antitumour effects involved direct action on DNA synthesis (Babu, Kuttan, and Padikkala, 1995). The aquoues extract of *C. asiatica* could inhibit intestinal tumorigenesis. *C. asiatica* extract stimulated modification of cell proliferation and induction of apoptosis in colonic crypts and hence had a chemopreventive effect on colon tumorigenesis (Bunpo *et al*., 2004).

Moreover, the study of Punturee *et al*. (2004) revealed that *C. asiatica* extract could modulate nitric oxide and tumour necrosis factor-α (TNF- α) in mouse macrophages.

Memory improvement

The aqueous extract of *C. asiatica* exhibited significant effect on learning and memory improvement and significant decrease in the levels of norepinepherine, dopamine, and serotonin in the brain (Nalini *et al*., 1992). Moreover, *C. asiatica* extract had the antioxidant property by decreasing the lipid peroxidation and augmenting the antioxidant enzyme activitivity (catalase) in brains which could reduce oxidative stress (Veerendra Kumar, and Gupta, 2002). In addition, aqueous extract of *C. asiatica* could decrease the pentylenetetrazole-kindled seizures and showed improvement in the learning deficit induced by pentylenetetrazole kindling suggesting its potential to be developed as an antiepileptic drug with an added advantage of preventing cognitive impairment (Gupta *et al*., 2000).

Neuroprotection

Asiatic acid derivatives from *C. asiatica* exerted significant neuroprotective effects on cultured cortical cells by potentiating of the cellular oxidative defense mechanism since they could protect neuron from the oxidative damage caused by exposure to excess glutamate (Lee *et al*., 2000).

Antioxidant and scavenging activities

In *in vivo* studies, the crude methanolic extract of *C. asiatica* significantly increased the activities of anti-oxidant enzymes, like superoxide dismutase (SOD),

catalase and glutathione peroxidase (GSHP), and significantly decreased the levels of anti-oxidants like glutathione (GSH) and ascorbic acid in lymphoma-bearing mice (Jayashree *et al*., 2003). Simultaneous supplementation of *C. asiatica* significantly protects rats from arsenic-induced oxidative stress (Gupta and Flora, 2006). There were many studies *in vitro* demonstrated that *C. asiatica* extract possessed antioxidant activities (Table 2.1).

Table 2.1 Antioxidant activities of *C. asiatica* extract determined by 2, 2-diphenyl-1-picrylhydrazyl scavenging activity (DPPH), ferric reducing antioxidant power (FRAP), and inhibition of lipid peroxidation methods.

Extraction method	Solvent	Antioxidant activities			References
		DPPH-scavening activity	FRAP-reducing antioxidant power	Lipid peroxidation	
Maceration	Ethanol	717.68 ± 46.46 (mg ascorbic acid/100 g)	26.58 ± 1.10 (mg ascorbic acid/ g)	-	Lee *et al*., 2011
Maceration	Water	IC_{50} = 31.25 µg/ml	-	-	Pitella *et al*., 2009
No fermentation	Water	32.6 ± 1.4 (µmol trolox/l /g)	42.8 ± 4.1 [a]	-	Ariffin *et al*., 2011
Maceration	Methanol	IC_{50} = 200 µg/ml	-	IC_{50} = 90 µg/ml	Subhasree *et al*., 2009
Maceration	Methanol	IC_{50} = 40.5 ± 10 µg/ml	-	-	Mustafa *et al*., 2010

DPPH = 1, 1-diphenyl-2-picrylhydrazyl, FRAP = Ferric reducing antioxidant power, GAE = gallic acid equivalent, DW = dry weight, a = mg GAE/g DW

Table 2.1 Antioxidant activities of *C. asiatica* extract determined by 2, 2-diphenyl-1-picrylhydrazyl scavenging activity (DPPH), ferric reducing antioxidant power (FRAP), and inhibition of lipid peroxidation methods (Continued).

Extraction method	Solvent	Antioxidant activities			References
		DPPH-scavening activity	FRAP-reducing antioxidant power	Lipid peroxidation	
Maceration	Methanol	IC_{50} = 20 µg/ml	-	-	Pongsathorn *et al*., 2012
Maceration	Methanol	IC_{50} = 19.89 mg/ml	-	-	Gupta *et al*., 2009
Maceration	Acetone	4.0 ± 1.3[a]	6.3 ± 0.5[a]	-	Sulaiman *et al*., 2011
	Ethanol	5.1 ± 2.1[a]	12.7 ± 0.1[a]	-	
	Methanol	8.1 ± 0.2[a]	0.8 ± 0.1[a]	-	
Maceration	Water	-	-	IC_{50} = 9.19 µmol	Mai *et al*., 2007
	Methanol	-	-	IC_{50} = 7.43 µmol	

DPPH = 1, 1-diphenyl-2-picrylhydrazyl, FRAP = Ferric reducing antioxidant power, GAE = gallic acid equivalent, DW = dry weight, a = mg GAE/g DW

2.3 Phenolic compounds possess anti-obesity activity

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate and phenylpropanoid pathways in plants. These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants (Balasundram, Sundram, and Samman, 2006). The two main types of polyphenols are flavonoids and phenolic acids. Flavonoids are themselves distributed among several classes such as flavonols, flavones, isoflavones, flavanones, proanthocyanidins, and anthocyanidins (Scalbert *et al*., 2005). The most common phenolic acids are hydroxycinnamic acid (caffeic acid, ferulic acid, and ρ-coumaric acid) and hydroxybenzoic acid (Bagchi and Preuss, 2007). Phenolic compounds exhibited a wide range of physiological properties such as anti-allergenic, anti-inflammatory, anti-microbial, and antioxidant activities (Balasundram *et al*., 2006). Besides, polyphenols could prevent cardiovascular diseases, cancers, and diabetes mellitus (Scalbert *et al*., 2005). Zainol *et al*. (2003) demonstrated that the antioxidative activity of the different parts of *C. asiatica* containing high phenolic contents was as good as that of α-tocopherol. Total phenolic, total flavonoids, and rutin contents in *C. asiatica* extract were demonstrated in many studies as shown in Table 2.2.

Table 2.2 Yields, total phenolic, total flavonoids, and rutin contents in *C. asiatica* extract.

Extraction method	Solvent	%Yield	Total phenolic content	Total flavonoids content	Rutin	References
Maceration	Ethanol	2.48%	0.32 ± 0.03[a]	-	-	Lee *et al*., 2011
Maceration	Water	-	2.86[b]	0.361 (g /100 g)	-	Pitella *et al*., 2009
Maceration	Water	-	7.3 ± 0.6[a]	6.4 ± 1.4[c]	1196 (µg/g DW)	Ariffin *et al*., 2011
Maceration	Methanol	-	3.23-11.7 (g/100 g DW)	-	-	Zainol *et al*., 2003
Maceration	Methanol	-	> 9 (mg/g wet weight)	> 5 (mg/g)	-	Subhasree *et al*., 2010
Maceration	Methanol	-	193.3 (g cafferic/kg DW)	-	1138.6 (mg/kg)	Hussin *et al*., 2009
Maceration	Methanol	-	183.24 ± 0.68[a]	-	-	Mustafa *et al*., 2010
Maceration	Water	-	24.3[d]	-	-	Mai *et al*., 2007
	Methanol	-	31.5[d]	-	-	

GAE = gallic acid equivalent, DW = dry weight, a = mg GAE/g DW, b = mg tannic acid/100 g, c = mg quercetin/g DW,

d = mg catechin/g DW, e = g/10 g DW

Table 2.2 Yields, total phenolic, total flavonoids, and rutin contents in *C. asiatica* extract (Continued).

Extraction method	Solvent	%Yield	Total phenolic content	Total flavonoids content	Rutin	References
Maceration	Acetone	-	22.4 ± 2.1^{a}	3.5 ± 0.4^{c}	-	Sulaiman *et al*., 2011
	Ethanol	-	52.5 ± 0.7^{a}	1.2 ± 0.3^{c}	-	
	Methanol	-	1.4 ± 0.7^{a}	1.8 ± 0.5^{c}	-	
	Water	-	0.3 ± 0.1^{a}	1.2 ± 0.1^{c}	-	
Maceration	Methanol	-	7.8 ± 1.9^{a}	-	-	Nanasombat *et al*., 2009
Maceration	Ethanol	2.83 ± 0.33^{e}	-	-	-	Hamid *et al*., 2002
	Petrolium	1.13 ± 0.18^{e}	-	-	-	
	Water	2.02 ± 0.11^{e}	-	-	-	
Maceration	Water	-	-	0.52 ± 0.2%	-	Krishnaiah *et al*., 2009

GAE = gallic acid equivalent, DW = dry weight, a = mg GAE/g DW, b = mg tannic acid/100 g, c = mg quercetin/g DW,

d = mg catechin/g DW, e = g/10 g DW

Table 2.2 Yields, total phenolic, total flavonoids, and rutin contents in *C. asiatica* extract (Continued).

Extraction method	Solvent	%Yield	Total phenolic content	Total flavonoids content	Rutin	References
Maceration	Methanol	164.4 (mg/g)	>15[a]	-	-	Pongsathorn *et al*., 2012
Maceration	Methanol	0.71%	7.79[b]	-	-	Huda-Faujan *et al*., 2009
Maceration	Methanol	-	150[b]	-	-	Gupta *et al*., 2009
Maceration	Hexane	-	17.25 ± 2.06[a]	-	>1000 (µg/g)	Loh *et al*., 2011
	Dichrolo methane	-	1.04 ± 2.06[a]	-	>1000 (µg/g)	

GAE = gallic acid equivalent, DW = dry weight, a = mg GAE/g DW, b = mg tannic acid/100 g, c = mg quercetin/g DW,

d = mg catechin/g DW, e = g/10 g DW

C. asiatica contains numerous active compounds such as triterpenoid saponins including asiaticoside, madecassic acid, centelloside, madecassoside, asiatic acid, volatile oils, flavonoids, tannins, phytosterols, amino acids, and sugars (Thorne Research Incoperation, 2007). Hussin *et al*. (2009) declared that rats treated with either *C. asiatica* powder or extract in normal diet with H_2O_2 treated water could reduce food intake and triacylglycerol (TAG) level and low density lipoprotein (LDL), and increase total cholesterol (TC) and high density lipoprotein (HDL) levels. Several potential mechanisms underlying the reduction of TAG have been suggested; the phenolic compounds may bind protein, decrease the activity of digestive enzymes, and reduce the digestibility and/or absorption of glucose and lipid.

Anti-obesity effect of phenolic compounds has been demonstrated by many studies. Polyphenols found in acacia (Ikarashi *et al*., 2010), tea (Khan and Mukhtar, 2007), grape seed (Park *et al*., 2008), and apple (Nagasako-Akozome *et al*., 2005) could reduce body weight, fat tissue weight, triglyceride (TG), and TC. Apple polyphenols and tea catechins improved lipid metabolism through different manner of action. Apple polyphenols widely inhibited the expression of genes involved in fatty acid synthase (FASN). The regulation of FASN contributed to the reduction of triglycerides and adipose tissue weights in rats fed high-fat diet (Ohta *et al*., 2008). The anti-obesity effect of *Nelumbo nucifera* leaves extract (NNE) containing several flavonoids and alkaloids has been demonstrated. NNE inhibited the activities of α-amylase and lipase, and up-regulated lipid metabolism and the expression of uncoupling protein 3 (UCP3) mRNA in mouse C2C12 myoblasts. In addition, NNE impaired digestion, inhibited absorption of lipids and carbohydrates, accelerated lipid metabolism, and up-regulated energy expenditure (Ono *et al*., 2006). The acacia

polyphenol (AP) that was rich in unique catechin-like flavan-3-ols could increase the expression of energy expenditure-related genes in skeletal muscle and liver which was associated with decreased fatty acid synthesis and fat intake in the liver of obese diabetic mice fed high-fat diet (Ikarashi *et al*., 2010). Moreover, Park *et al*. (2008) demonstrated that polyphenols in grape seed extract (GSE) were the monomeric compounds catechin, epicatechin, gallate, and gallic acid. GSE decreased body weight gain, food intake, TG, and TC in high fat diet (HFD)-induced obese mice. *Hibiscus sabdariffa* extract contained anthocyanins which were one of the major groups of compounds apart from β-carotene, riboflavin, niacin, ascorbic, and hibicic acids. Systemic administration of *Hibiscus sabdariffa* significantly reduced body weight gain and glycemia in monosodium glutamate-induced obese mice. The potential mechanism by which *Hibiscus sabdariffa* causes a reduction of body weight gain include anti-hyperglycemic effect, reduction in plasma cholesterol level, and stimulation of thermogenesis (Alarcon-Aguilar *et al*., 2007). In addition, anthocyanins and ursolic acid were the most abundant bioactive compounds found in Cornelian cherries (*Cornus mas*). Administration of anthocyanin purified from Cornelian cherries could decrease weight gain, lipid accumulation in the liver, and reduce liver triacylglycerol concentration in HFD-induced obese mice (Jayaprakasam *et al*., 2006).

The triterpene saponins are commom secondary plant metabolites containing a hydrophobic triterpenoid structure (aglycone) and sugar chain (glycone) (James and Dubery, 2009). Total triterpenic fraction of *C. asiatica* (TTFCA) was effective in improving venous wall alterations in chronic venous hypertension. TTFCA modulated connective tissue, improved the synthesis of collagen and other tissue proteins by modulating the action of fibroblasts in the vein wall, and stimulated collagen synthesis

around the venous wall (Incandela *et al*., 2001). Crude saponin from Platycodi Radix reduced the increase in plasma TAG after oral administration of lipid emulsion in rats. Furthermore, feeding a HFD containing 3.5, 10 or 30 g/kg crude saponins prevented increases in body and adipose tissue weights associated with decreased hepatic TAG and TC concentrations compared with feeding a HFD alone. The anti-obesity action by crude saponin of Platycodi Radix may be due to the inhibition of pancreatic lipase activity (Han *et al*., 2002). Hu *et al.* (2008) revealed that the extract from the seeds of *Aesculus turbinata* Blume had an inhibitory effect on pancreatic lipase *in vitro* and suppressed the increase in the body weight, adipose tissue weight, and TC levels in rat liver. Plasma TG contents were also reduced at 1, 2, and 3 hour after oral administration of the lipid emulsion with escins. The consumption of HFD containing 2% escins elevated the TG level in mouse feces compared to the HFD consumption alone. The reason for this appears to be due in part to inhibition of the absorption of dietary fat by inhibiting pancreatic lipase in the intestinal mucosa. Another reason was that total escins increased gastrointestinal motility, resulting in a reduction of the absorption of dietary fat in the gastrointestinal tract. Teasaponin inhibited pancreatic lipase activity and decreased plasma TAG level. Teasaponin suppressed the increases in body weight, parametrial adipose tissue weights and diameter in adipose cell size induced by HFD. The anti-obesity effects of teasaponin in high-fat diet treated mice may be partly mediated through delaying the intestinal absorption of dietary fat by inhibiting pancreatic lipase activity (Han *et al*., 2001).

Catechin is one the flavan type of flavonoids which found in red wine, green tea, chocolate and many fruits (Donovan *et al*., 1999). A recent report showed that catechin has anti-obesity effects. Cho *et al*. (2007) revealed that catechin could

stimulate adiponectin protein expression and secretion in adipocytes. Catechin increased insulin-dependent glucose uptake in differentiated adipocytes and augmented the expression of adipogenic marker genes. Green tea catechins could inhibit cholesterol oxidation in LDL which suppressed the generation of hydroxyl radical and superoxide anion (Osada *et al*., 2001). Green tea catechin could promote thermogenic properties and increase fat oxidation (Dulloo *et al*., 1999). Tea catechin had potent inhibitory pancreatic lipase activities *in vitro* and decreased the triacylglyceride levels of after intragastric administration of fat emulsion-induced hypertriacylglycerolemia rats (Ikeda *et al*., 2005).

Kaempferol is natural flavonoids which found in tea, broccoli, strawberries, cranberries, grapefruit, and apple (Park and Kim, 2011). Kaempferol could suppress body weight gain, hepatic triglyceride, cholesterol content, and hepatic lipid accumulation in HFD-fed rats. Kaempferol could reduce the accumulation of visceral fat and improved hyperlipidemia in HFD-fed obese rats by increasing lipid metabolism (Chang *et al*., 2011).

Quercetin (3, 3, 4, 5, 7- pentahydroxyflavone) is one of the most common dietary flavonols with a well characterized *in vitro* antioxidant activity. Quercetin is an integral part of the human diet that can be found in fruits, vegetables, tea, wine, nuts, and seeds (Ahn *et al*., 2008). Yang *et al*. (2008) revealed that treatment with combination of resveratrol and quercetin appeared to significantly suppress lipid accumulation compared to resveratrol or quercetin alone. Moreover, the adipocyte-specific transcription factors peroxisome proliferators-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein (C/EBP_{α}), associated with decreased in viability and increased in apoptosis, were decreased by the combination of

resveratrol and quercetin. A possible mechanism of apoptosis induced by resveratrol and quercetin might be mediated through mitochondria cytochrome C release to the cytosol. Quercetin exerted anti-adipogenesis activity by activating the monophosphate-activated protein kinase (AMPK) signaling pathway in 3T3-L1 preadipocyte cells (Ahn *et al*., 2008).

Rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside), is one the flavanol type of flavonoids. Rutin can be found in many typical plants such as buckwheat and apples (Ziaee *et al*., 2009). Rutin was reported to have pharmacological properties including antioxidant, anticarcinogenic, and antiplatelet activities (Janbaz, Saeed, and Gilani, 2002; Sheu *et al*., 2004). Ziaee *et al*. (2009) revealed that rutin alone or in combination with lovastatin could reduce plasma TC, LDL, and body weight in rats with a high cholesterol diet. Furthermore, the study of Hsu *et al*. (2009) showed that the addition of rutin or *0*-coumaric acid to the diet could decrease body weight gain, liver weight, adipose tissue weight, TG, phospholipid, TC, insulin, and leptin in HFD-induced obese rats. Rutin could inhibit adipogenic development in pre-adipocytes and hepatocytes by down regulating expressions of key adipogenic transcription factors such as PPAR□ and C/EBP□ which activated mature adipocytes in 3T3-L1 cells (Choi *et al*., 2006).

2.4 Disorders associated with obesity

Obesity is defined as an excessive fat accumulation in fat tissue due to imbalance of energy intake and expenditure (Suastika, 2006). It is characterized by enlarged fat mass and elevated lipid concentration in blood (Devlin, Yamovski, and Wilson, 2000; Fujioka, 2002). Excessive fat storage causes exogenous fat and to a

more limited extent by non-fat substrates precursors transformed into body fat, mostly from carbohydrates, a process known as *de novo* lipogenesis (Schutz, 2004).

In human, the widely accepted means of assessing obesity is the body mass index (BMI). A very good correlation has been found between BMI and the percentage of body fat in a population. BMI can be calculated using the equation below.

$$\text{Body Mass Index (BMI)} = (\text{Body weight in kg})/ \text{Height}^2 \text{ in } m^2)$$

Table 2.3 Classification of health risk in adults according to the BMI by the World Health Organization (WHO) (Bagchi *et al*., 2007).

Classification	**BMI**	**Risk of co-morbidities**
Underweight	< 18.50	Low (but risk of other clinical problems increased)
Normal range	18.50 - 24.99	Average
Overweight	25.00 - 29.99	Increased
Obese class I	30.00 - 34.99	Moderate
Obese class II	35.00 - 39.99	Severe
Obese class III	≥ 40.00	Very severe

From Table 2.3, the current value settings are as follows: a BMI lower than 18.5 suggests the person is underweight and may indicate malnutrition, an eating disorder, or other health problems, a BMI of 18.5 to 25 may indicate optimal weight while a number above 25 may indicate the person is overweight; a number above 30 suggests the person is obese (over 40, morbidly obese) (Bagchi *et al*., 2007).

Obesity increases the risk of multiple medical conditions, many of which are associated with high morbidity and mortality, such as type 2 diabetes mellitus, hypertension, and coronary heart diseases. The risks associated with many of these comorbid conditions may be reduced with modest weight loss (Fujioka, 2002). Disorders associated with obesity were shown in Table 2.4.

Table 2.4 Obesity related comorbid conditions (adapted from; Ford *et al*., 2002; Fujioka, 2002; Pi-Sunyer, 2002).

- Coronary heart disease
- Dyslipidemia
- Type 2 diabetes mellitus
- Insulin resistance/ hyperinsulinemia
- Hypertension

Insulin resistance and hyperinsulindemia

One of the possible mechanism of insulin resistance and hyperglycemia is found in the increased levels of free fatty acids (FFA) found in obese individuals contribute to the defects in glucose use and storage. As increased body fat can increase the rate of lipolysis and FFA mobilization which induce an increase the FFA oxidation and declines glucose used, while FFA is used as an alternate energy source in muscles. Glucose production is increased in response to the higher FFA oxidation in the liver. These actions result in hyperglycemia and impair glucose tolerance (Figure 2.1). This mechanism is particularly important among individuals with upper body obesity. The plasma FFA turnover rate was higher among women with upper-

body obesity compared with lower-body obesity or non-obese women (Jensen *et al*., 1989).

On the cellular level, insulin binds to its receptor on the surface of target cells, which activate tyrosine autophosphorylation and consequent intracellular signaling. These events culminate in cellular responses, such as the translocation of glucose transporters to the cell surface to allow glucose uptake for use or glycogen storage. In obesity, insulin signaling is defective. Insulin stimulation protein kinase activity of the insulin receptor, which mediates tyrosine autophosphorylation, is reduced in obese subjects relative to non-obese ones, and it is further reduced in obese type 2 diabetes patients (Caro *et al*., 1989). Furthermore, obesity is associated with other postreceptor binding defects in insulin action, including impaired generation of second messengers, diminished glucose transport, and abnormalities in some critical enzymatic steps involved in glucose use. However, obese subjects with depressed insulin mediated glucose transporter can recover this response after weight loss (Friedman *et al*., 1992).

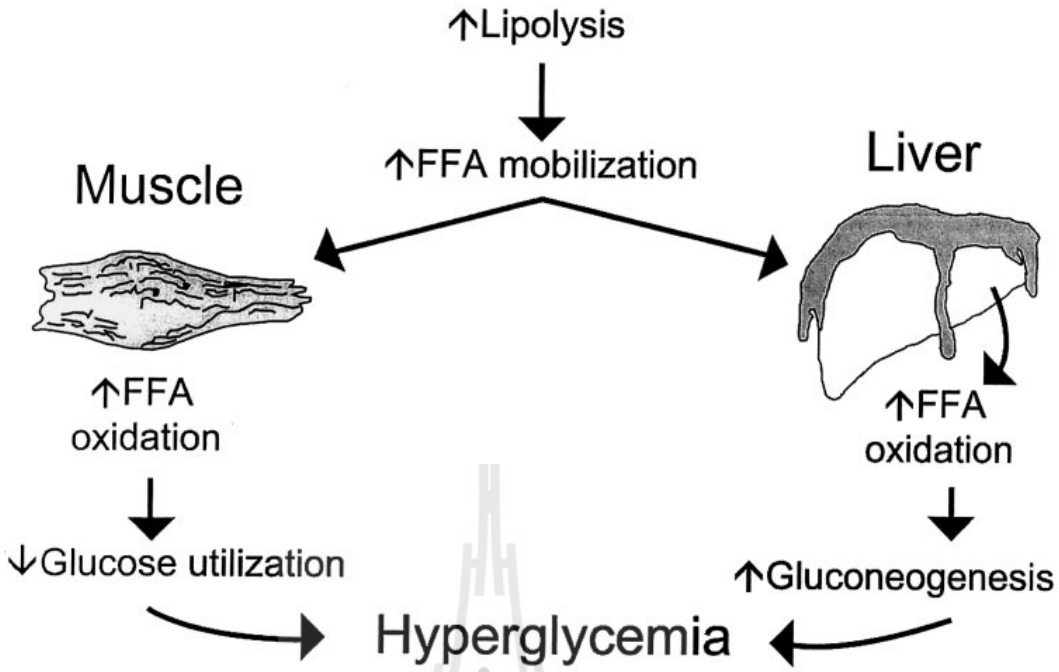

Figure 2.1 Effect of increased lipolysis on glucose use and gluconeogenesis (Pi-Sunyer, 2002). Free fatty acid (FFA).

Diabetes mellitus

Obesity induced type 2 diabetes mellitus that was shown in Figure 2.2. Adipose tissue distribution involves obesity-related diseases and type 2 diabetes mellitus. The visceral adipose tissue is in direct contact with the liver through the portal circulation, considering the alterations of hormonal control of lipolysis in visceral fat (Lafontan and Berlan, 2003). Visceral adipose tissue, through the enhancement of lipolysis as a result of the reduced insulin-induced anti-lipolysis and the enhancement of lipolytic potencies of catecholamines, caused the release of portal non-esterified fatty acids (NEFA) that disturb liver metabolism (Bergman, 2000). NEFA are released by visceral fat and also released by the upper-body subcutaneous fat deposit. An increase of the levels of NEFA causes a decrease of glucose utilization

in skeletal muscle, leading to glucose intolerance and insulin resistance, which can result in type 2 diabetes mellitus related disorders (Bergman and Ader, 2000; Jensen, 2006; Pi-Sunyer, 2002). An increase in the level of NEFA can cause similar consequences by increasing the production of glucose in the liver and increasing the synthesis of very-low-density lipoproteins (VLDL) in the liver (McGarry, 2001). Subcutaneous fat deposits represent a major site of fat storage (reduced lipolytic responsiveness to catecholamines, which is related to a higher α_2-adrenoceptor: β-adrenoceptor ratio and higher insulin responsiveness) (Mauriege *et al*., 1987). Trayhurn and Beattie (2001) demonstrated that subcutaneous fat deposits also played a major role in the production of various adipokines which are involved both in regulation of glucose and lipid metabolism. Adipokines (tumor necrosis factor-α (TNF-α), leptin, and, interleukin-6 (IL-6)) induce insulin resistance, while leptin improves insulin sensitivity (Hussain *et al*., 2010). Pi-Sunyer (2002) revealed that an increase of adipokines (TNF-α, leptin, and, IL-6) and NEFA declines glucose use, while FFA is used as an alternate energy source in muscles which induce glucose intolerance and insulin resistance.

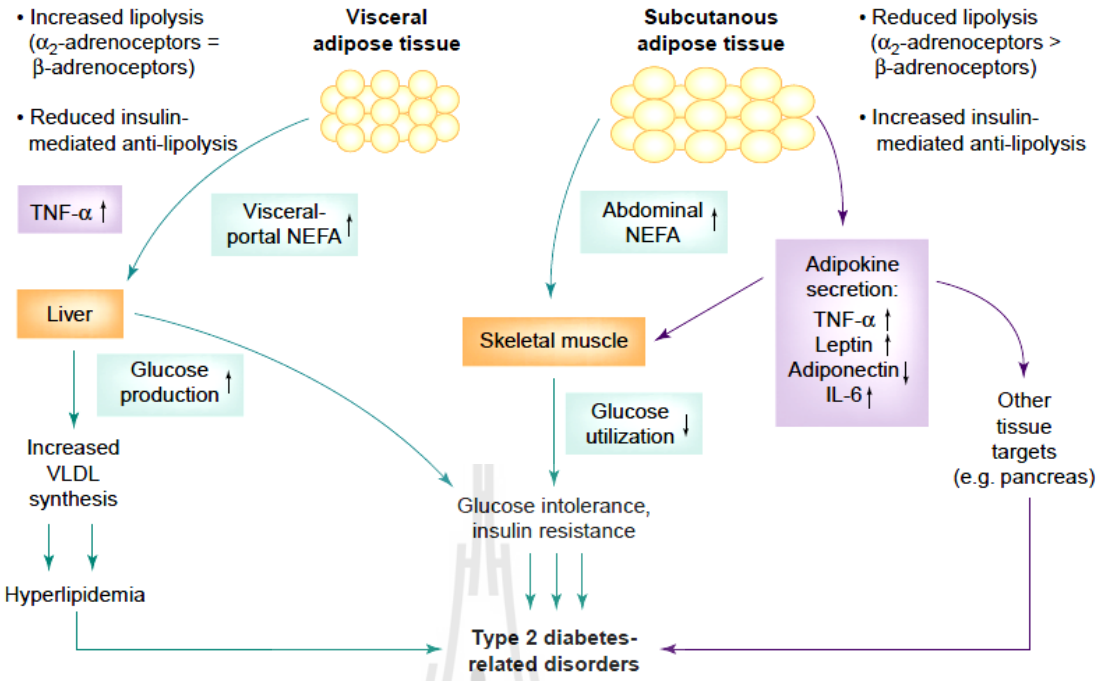

Figure 2.2 Obesity induced type 2 diabetes mellitus (Lafontan *et al*., 2003). Non-esterified fatty acid (NEFA), tumor necrosis factor-α (TNF-α), very low density lipoprotein (VLDL), and interleukin-6 (IL-6).

Hypertension

Several factors may account for the increased sympathetic outflow associated with obesity (Figure 2.3). The increase of FFA, insulin, leptin, aldosterone, and rennin-angiotensin system (RAS) levels induce the increase of sympathetic nerve activity (SNA). Incremental SNA associated with increased leptin, aldosterone, and RAS levels elevate sodium absorption and water retention in kidney which induced hypertension. Another possible mechanism is the elevation of aldosterone, RAS, and endothelin-1 association with the reduction of nitric oxide (NO) production induces hypertension (Rahmouni *et al*., 2005). The increase in SNA develops slowly, and in the long term, it may induce sympathetic-mediated hypertension development through

raised tubular sodium reabsorption and volumn overloading (Kotsis *et al*., 2010). In addition, the chronic pressor effects of leptin are supposed to be simultaneously controlled by endothelial NO production. Deprivation of the endothelium-derived NO markedly promotes blood pressure (Kuo, Jones, and Hall, 2001). Obesity causes endothelial dysfunction, decreased NO release, and hence the expected greater blood pressure outcome. Obesity represents a state of inflammation (vascular and systemic) that can cause endothelial dysfunction (Kotsis *et al*., 2010). In addition, increased tubular absorption and impaired pressure natriuresis in obesity appear to be caused by multiple mechanisms, including activation of the RAS (Hall, Hildebrandt, and Kuo, 2001).

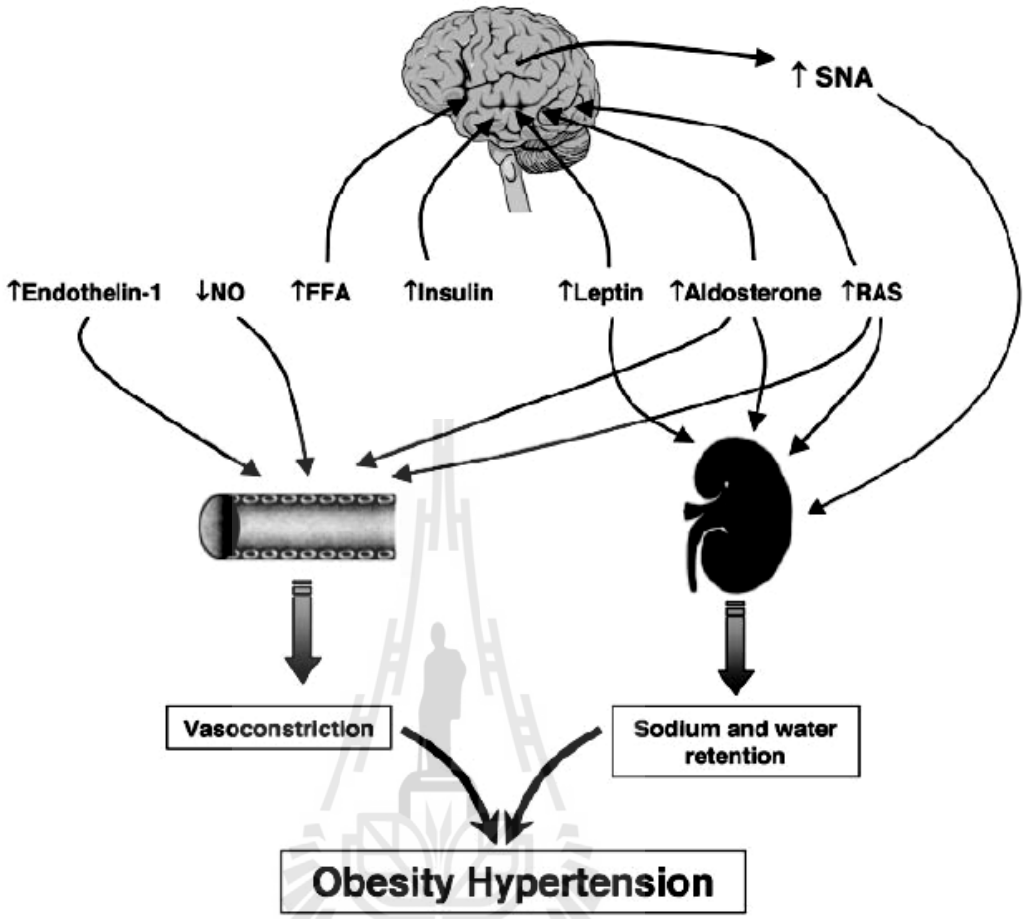

Figure 2.3 Summary of mechanisms and hormonal systems involved in obesity-associated hypertension (Rahmouni *et al*., 2005). Free fatty acid (FFA), nitric oxide (NO), sympathetic nerve activity (SNA), rennin-angiotensin system (RAS).

Dyslipidemia

Dyslipidemia is disorders of lipoprotein metabolism, including lipoprotein overproduction and deficiency which is associated with obesity. They may manifest as one or more of the following: elevated TC, LDL, and TG levels or as decreased HDL level with promotion of insulin resistance causing metabolic syndrome in obesity

(Meisinger *et al*., 2006; Vikram *et al*., 2003). LDL, and high TG levels were most likely to be present when body fat was concentrated in the abdominal area than when body fat was concentrated in the lower body area. Furthermore, overweight or obesity had a lipid irregularity which adversely affected the risk of morbidity and mortality in adults (Sharma, 2003). In addition, increased FFA also affect lipid metabolism by increasing VLDL production by the liver, reducing HDL level, and increasing the number of small dense LDL particles (Bamba and Rader, 2007). Small dense LDL particles were considered to be atherogenic because these particles readily penetrated the arterial wall and had a low affinity for LDL receptor which is also susceptible to oxidation. Even when the LDL level was not change appreciably, atherogenic risk could be higher because of the presence of the smaller LDL particles (Ghassab *et al*., 2010). Taken together, these changed in lipoprotein profile were associated with increased risk of coronary heart disease (CHD) (Bamba *et al*., 2007; Singh *et al*., 2011).

2.5 The physiology of obesity

Energy balance is regulated by homeostatic mechanisms involving humoral signals between ingestive and adipose organs, and integrative cerebral modules, particularly the hypothalamus (Knecht *et al*., 2008). The amount of fat in the body (adiposity) is not, as was once thought, a passive result of bad habits or over-indulgence. It is rather precisely regulated as part of the process of energy homeostasis, a process whereby energy intake (food intake) is matched to energy expenditure (metabolism and exercise) and the size of the body's energy stores (the fat

mass). The major organs regulating this system is the brain, although liver, stomach, and small intestinal participate in the process (Woods and Seeley, 2002).

From Figure 2.4, signals related to the fat mass are integrated with signals from the gastrointestinal system to control all aspects of energy homeostasis. Adiposity signals are connected through central autonomic pathways to centers that process satiety signals. Reduced input from adiposity signals (e.g. after weight loss) increases meal size by reducing brain response to satiety signals (Woods, 2004). Adiposity and satiety signals enter the brain at different levels. Adiposity signals enter the brain at the level of the hypothalamus. Many hypothalamic nuclei are important in the regulation of food intake and energy homeostasis, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), and perifornical area (PeF) (Lawrence, Turnbull, and Rothwell, 1999; Schwartz *et al*., 2000; Valassi *et al*., 2008). The nucleus of the solitary tract (NTS) in the brainstem receives much of the information pertinent to satiety, including vagal afferent information and gustation and integrates and relays this information to the hypothalamus (Wood, 2004). Neural signals from the gastrointestinal system and the liver provide information about the food is being eaten, for example, the taste of the food, how much the stomach is distended, and the chemical content of the food. These satiety signals are sent to the hindbrain. The brain responds to the hormone signals *via* integrated neuropeptide pathways, leading to a number of outputs that are directly related to energy homeostasis. These outputs include neuroendocrine activation from the pituitary gland, motor behaviour (eating, exercise, etc.) and autonomic activity. It has become apparent that the autonomic nervous system has a much greater impact than was once thought upon many fundamental processes of metabolism, including

lipolysis, the secretion of insulin and glucagons from the pancreas, and glucose synthesis and secretion from the liver. It is important to note that while energy expenditure tends to decrease with aging, mainly because of the absence of occupational activity and extreme physical exertion, energy intake does not tend to decrease to the same extent, for a number of reasons, including lifestyle habits. Thus, there is a tendency over time for the body weight to increase (Woods and Seeley, 2002).

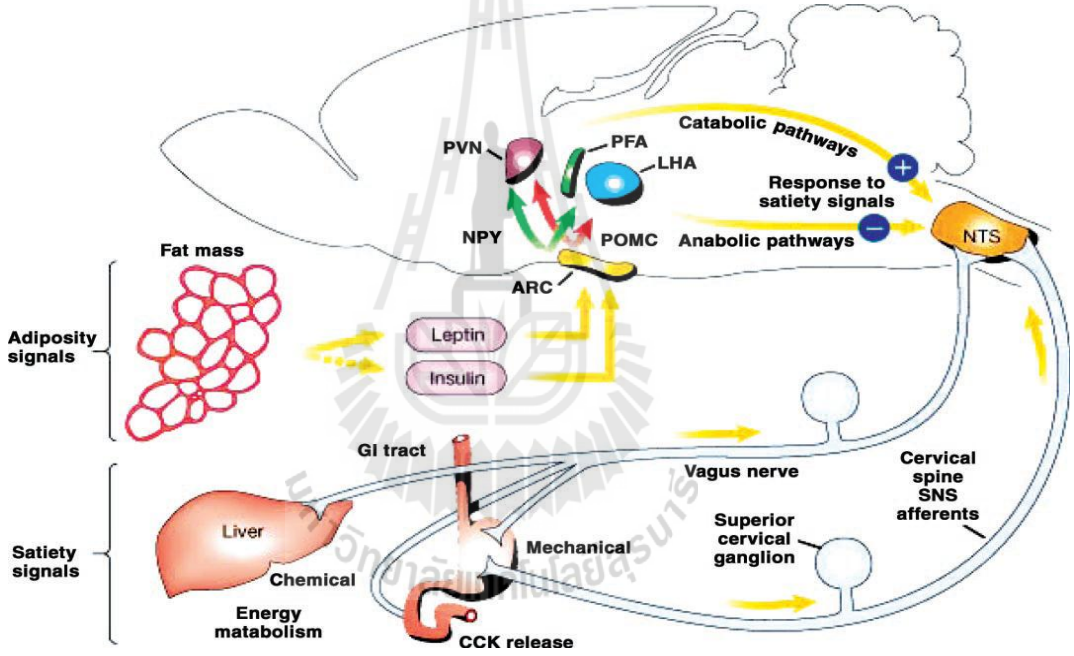

Figure 2.4 Circulating signals related to the size of the fat mass (adiposity signals) are integrated with signals from the gastrointestinal system (satiety signals) to control energy homeostasis (Woods, 2004). Arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamic area (LHA), perifornical area (PeF), the nucleus of the solitary tract (NTS), cholecystokinin (CCK).

2.6 Digestion and absorption of lipids

2.6.1 Activities of lipase enzymes

Lipases are enzymes that digest fats, including triacylglycerol and phospholipids. The human lipases include the pre-duodenal (lingual and gastric) and the extra-duodenal (pancreatic, hepatic, lipoprotein, and endothelial) lipases (Mukherjee, 2003). Pancreatic lipase (PL), the principal lipolytic enzymes synthesized and secreted by the pancreas, play a key role in the efficient digestion of triglycerides. It removes fatty acids from the α and ά position of dietary triglycerides, yielding β-monoglycerides, and long chain saturated and polyunsaturated fatty acids as the lipolytic products (Shi and Burn, 2004; Mukherjee, 2003; Thomson *et al*., 1997).

Lipases mediate the digestion of dietary fats, the uptake of fat into various tissues and the mobilization of fats inside cells. In human, triglyceride lipases are found in the gastrointestinal tract, bound to epithelial surfaces, and inside fat storage cells. The digestion of dietary triglycerides begins in the stomach where gastric lipase releases about 15% of the fatty acids. Lipase secreted by pancreatic acinar cells, complete fat digestion in the proximal small intestine (Lowe, 1997). Lingual lipase is secreted by serous gland, digests approximately one third of ingested fat. Gastric lipase secreted in response to mechanical stimulation, ingestion of food or sympathetic activation, accounts for the hydrolysis of 10-40% of dietary fat. These two enzymes, potentially limit the nutritional impact of the inhibition of lipid absorption that could result from the reduction in the activity of PL alone (Thompson *et al*., 1997).

2.6.2 Dietary fat digestion and absorption

Dietary fats are mainly (90%) comprised mixed triglycerides (TG). TG consists of a single molecule of glycerol, attached by ester bonds to three fatty acids. TG cannot be directly absorbed, TG must be hydrolyzed the ester bonds on the glycerol backbone by intestinal enzymes and are then able to be absorbed. The products of this hydrolysis are mainly free fatty acids (FFA) and 2-monoglycerides (2-MG) which can be absorbed by the duodenum. *In vivo* study, TG hydrolysis is catalyzed by several digestive lipases. There are several human lipases which include the pre-duodenal (lingual lipase, and human gastric lipase (HGL)) and the extra-duodenal (pancreatic, hepatic, lipoprotein, and endothelial) lipases (Mukherjee, 2003)

Lingual lipase is secreted by a serous gland at the back of the tongue and initiates fat digestion (Birari *et al*., 2007). Human gastric lipase is secreted by the chief cells of the fundic mucosa of the stomach, this enzyme is active at a broad pH range (3 to 6) and is stable even at the low pH present in the stomach (Hamosh, 1990). The acinar cells of the pancreas synthesize and secrete several lipolytic enzymes such as colipase-dependent lipase, classical pancreatic lipase or triacylglycerol acyl hydrolase (HPL), pancreatic lipase related-protein 1 and 2 (HPLRP1 and HPLRP2), carboxyl ester hydrolase (also known as bile salt stimulated lipase, carboxyl ester lipase, cholesterol esterase, cholesterol ester lipase, human milk lipase, monoglyceride lipase, and pancreatic non-specific lipase) and phospholipase A2 (Birari *et al*., 2007; van Gaal *et al*., 2004). Cholesterol esters, lipidic vitamin esters, monoglycerides, diglycerides, TG, and phospholipids are hydrolyzed mainly by carboxyl ester hydrolase. The pancreas also secretes colipase, a factor that is necessary to optimize

pancreatic lipase activity. Colipase binds to bile acid micelles and phospholipids covered emulsions. Once bound to these surfaces, colipase facilitates the interaction between pancreatic lipase and the surface of emulsified lipid droplets (Borgstrom and Erlanson-Albertsson, 1982).

The hydrolysis of dietary TG starts in the stomach by the catalytic action of HGL. The secretion of HGL is induced by mechanical stimulation of the stomach, ingestion of food or sympathetic activation (Birari *et al*., 2007). HGL hydrolyzes 5% to 40% of ingested TG (Carriere *et al*., 1993), mainly generating FFA, diglycerides, and a few-2MG molecules (Armand *et al*., 1994). Gastric lipolysis is crucial for the continuation of the digestion process in the duodenum of HPL. Gastric lipolysis ensures: (i) lipid emulsification with creates the lipid-water interface needed for effective lipolysis in the duodenum (Armand *et al*., 1999), (ii), the generation of long-chain FFA in the duodenum will stimulate the release of cholecystokinin (CCK) and HPL secretion, slowing down gastric emptying (Beglinger, 1994), and (iii) the generation of diglycerides, which are hydrolyzed more effectively than TG (Phan and Tso, 2001). The hydrolysis of TG persists in the duodenum by means of the combined actions of HGL (HGL is responsible for further lipolysis contributing 7.5% to total lipolysis in the duodenum), HPL and bile salts. HPL is the principal pancreatic lipolytic enzyme; it hydrolyzes 40% to 70% of TG (Armand *et al*., 1999) yielding 2-MG and long-chain saturated and polyunsaturated FFA as the lipolytic products (Mukherjee, 2003; Shi *et al*., 2004). For full activity under physiological conditions, HPL requires the presence of another pancreatic exocrine protein: colipase. Colipase is secreted as a precursor molecule, in the presence of bile salts, pro-colipase binds, without inducing any conformational change, to the C-terminal domain of the HPL

molecule (Phan and Tso, 2001). In order to be absorbed, bile-derived mixed micelles convert FFA and 2-MG into soluble aggregates. These micelles transport these lipolytic products from the intestinal lumen to the intestinal walls (Ho and Storch, 2001). Once in contact with the enterocyte, the molecules are transported across the cell membrane. The enterocyte re-esterifies 2-MG and FFA into TG, assembles them into chylomicrons and then secretes these into the lymphatic system in order to make them bioavailable (Mu and Hoy, 2004; Phan *et al*., 2001; Van Gaal *et al*., 2004). Lipolysis rates and consequently FFA supply to the body can be affected by several factors such as lipase levels and activity, physiocochemical properties of dietary lipids, and the presence of inhibitors and enhancers (Tucci *et al*., 2010). The physiological role of lipases in lipid digestion and absorption was shown in Figure 2.5.

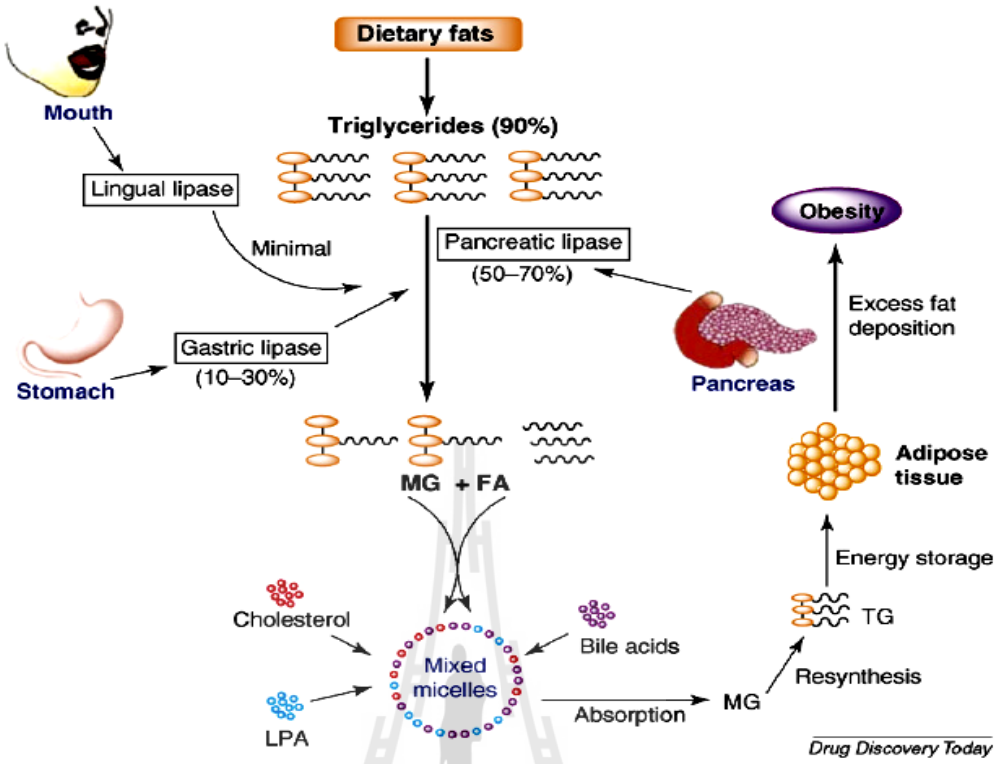

Figure 2.5 Physiological role of pancreatic lipase in lipid absorption (Birari and Bhutani, 2007). Lysophosphatidic acid (LPA); triglyceride (TG); fatty acids (FA); monoglycerides (MG).

2.6.3 Drug that interfere with lipid absorption

Although lipid metabolism is balanced to maintain homeostasis, high-fat diets tend to induce overconsumption and consequencely weight gain. This is mainly due to their high energy content and their low potential for inducing satiety (Hofbauer, 2002). It has been proposed that in the vast majority of cases, overweight, and obesity are the consequences of exaggerated consumption of fat rather than carbohydrates (Seidell, 1998). This does not mean that obesity is not also associated with the consumption of refined sweet carbohydrates, but rather than the intake of this form of carbohydrate is invariably coupled with the intake of dietary fat along with the

consumption of high fat savory food items. Given the central role of dietary fat in weight gain, a rational strategy would be to reduce the proportion of calories derived from fat in the diet. In addition, altering dietary intake, the amount of fat entering the body can be reduced by targeting the enzymes involved in lipid digestion and absorption pathways (Shi *et al*., 2004).

The inhibition of fat digestion and absorption is not without side effect issues. As detailed later, GI distress, and vitamin deficiencies remain a concern with current treatments. However, humans can tolerate a certain degree of inhibition of fat absorption, sufficient to prevent a significant amount of energy entering the body (Aronne, 1998). The benefits of reducing fat absorption are not restricted to weight loss. Specific reduction of lipid levels in subjects with hyperlipidemia would lead to health benefits beyond those expected through reduction in caloric intake and weight loss alone.

Orlistat

Orlistat (Xenical; Roche) is indicated for the management of obesity (including weight loss and weight maintainance) when used in conjunction with a reduced-calorie diet (Henness and Perry, 2006). Orlistat is a more stable analogue of lepstatin, a naturally occurring lipase inhibitor produced by *Streptomyces toxytricini*. It acts locally as a potent, specific, irreversible inhibitor of pancreatic and gastric lipases by covalently binding to a serine residue in the active site, inhibiting the hydrolysis of dietary triglycerides into absorbable FFA and MG. Approximately, one-third of triglyceride intake is subsequently not absorbed by the small intestine, passing through the gastrointestinal tract and resulting in elimination by the faecal route,

thereby contributing to caloric deficit (Cooke and Bloom, 2006). Orlistat is currently the only lipase inhibitor approved for weight loss and a potent inhibitor of pancreatic and gastric lipases, acting locally in the gut lumen with minimal absorption as shown in Figure 2.6. Orlistat inhibits dietary triglycerides hydrolysis by 30%, decreasing proportionally fat absorption (Coutinho, 2009). Some beneficial metabolic impacts of orlistat seem to be independent of weight loss. The observed reduction in TG and LDL levels on orlistat-treated patients was more than 10% higher than the expected for the weight loss. The mechanistic explanation was based on the 25% reduction on the intestinal cholesterol absorption elicited by orlistat (Mittendorfer *et al*., 2001).

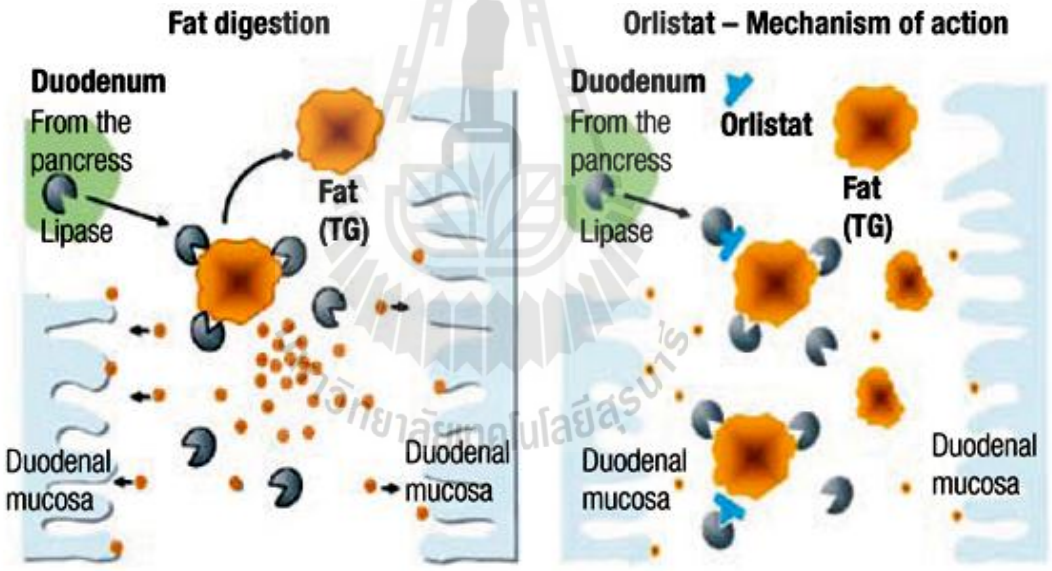

Figure 2.6 Schematic diagram of lipase enzymatic hydrolysis of triglyceride (TG) (left) and mechanism of action for orlistat (anti-obesity drug) in lipase enzyme inhibition (right) (Coutinho, 2009).

2.6.4 Lipase inhibitors from plants

The inhibition of lipase activity is one of the most widely management obesity. Nowadays, orlistat is a drug for obesity treatment which has been shown to inhibit of lipase (Jandacek and Woods, 2004). However, orlistat has certain unpleasant gastrointestinal side effects like oily stools, oily spotting, and flatulence (Birari and Bhutani, 2007). Therefore, there was a new alternative for pancreatic lipase inhibitory activity from medicinal plants and natural products (anti-obesity agents) (Sharma, Sharma, and Seo, 2005; Yamamoto *et al*., 2000; Yoshikawa *et al*., 2002; Zhang *et al*., 2008). The inhibitory effects of orlistat and medicinal plants on lipase enzyme activities *in vitro* and *in vivo* were summarized in Tables 2.6 and 2.7, respectively.

Table 2.5 Effective doses and lipase enzyme inhibitory activities of plant extracts *in vitro*.

Product/plant extract	Dose	Effect	References
Orlistat (anti-obesity drug)	0.1 µg/ml	64% Inhibition of PPL	Kim *et al*., 2010
	0.85 µg/ml	Inhibition of PL	Ikarashi *et al*., 2011
	100 µg/ml	92% Inhibition of PPL	Zheng *et al*., 2010
	250 µg/ml	95.7% Inhibition of PPL	Zhang *et al*., 2008
	1 mg/ml	95% Inhibition of PPL	Kwon *et al*., 2003
	IC_{50} of 32 µM	Inhibition of PPL	Sergent *et al*., 2012
Rutin	100 µg/ml	30.8% Inhibition of PPL	Zheng *et al*., 2010
Taraxacum officinale	250 µg/ml	86.3% Inhibition of PPL	Zhang *et al*., 2008
Quercus infectoria	5 µg/ml	85% Inhibition of PPL	Gholamhoseinian *et al*., 2010
Rosmarinus officinalis	IC_{50} of 13.8 µg/ml	Inhibition of PL	Bustanji *et al*., 2010
Arachis hypogaea nutshell	10 mg/ml	92% Inhibition of HPL	Moreno *et al*., 2006
Eriochloa villosa	0.2 mg/ml	83% Inhibition of PPL	Sharma *et al*., 2005
Dioscorea nipponica	1 mg/ml	85% Inhibition of PPL	Kwon *et al*., 2003

Pancreatic lipase (PL), human pancreatic lipase (HPL), porcine pancreatic lipase (PPL).

Table 2.5 Effective doses and inhibitory lipase enzyme activities of plant extracts *in vitro* (Continued).

Product/plant extract	Dose	Effect	References
Morus bombycis (root)	2.5 µg/ml	85% Inhibition of PPL	Kim *et al*., 2010
Nelumbo nucifera	IC_{50} of 0.46 mg/ml	Inhibition of PL	Ono *et al*., 2006
Mangifera indica (stem bark and leaves)	1 mg/ml	50% Inhibition of HPL 44% Inhibition of HPL	Moreno *et al*., 2006
Saponin from root of *Platycodon gramdiflonon*	1 mg/ml	80% Inhibition of PPL	Han *et al*., 2002
Oolong tea catechins	0.5-2 g/L	Inhibition of PL	Han *et al*., 2001
Acacia polyphenols	IC_{50} of 0.95 mg/ml	Inhibition of PPL	Ikarashi *et al*., 2011
Apple polyphenols and procyanidin fraction	IC_{50} of 5.6 µg/ml	Inhibition of PL	Sugiyama *et al*., 2007
Grape seed extract	1 mg/ml	80% Inhibition of HPL	Moreno *et al*., 2003
Tea polyphenols	0.05 mg/ml	54% Inhibition of PL	He *et al*., 2007

Pancreatic lipase (PL), human pancreatic lipase (HPL), porcine pancreatic lipase (PPL).

Table 2.6 Effective doses and inhibitory lipase enzyme activities of plant extracts *in vivo*.

Product/plant extract	Dose	Effect	References
Orlistat (anti-obesity drug)	15 mg/kg, p.o. (rats)	↓ Plasma TG level	Shim *et al*., 2009
	20 mg/kg, p.o. (rats)	↓ Plasma TG level	Liu *et al*., 2008
	45 mg/kg, p.o. (rats)	↓ Plasma TG level	Han *et al*., 2005
Rutin	1 g in diet, (rats)	↓ Plasma TG and TC levels ↑ Fat excretion	Park *et al*., 2002
	5 mg/ml, i.p. (rats)	↓ Serum TG and TC level	Santos *et al*., 1999
	25 and 50 mg/kg in diet, (mice)	↓ Plasma TC ↓ Body weight and liver weight	Choi *et al*., 2006
	10 and 100 mg/kg in diet, (rats)	↓ Serum TC and LDL-C levels ↓ Plasma TG level	Ziaee *et al*., 2009
	0.8% rutin in diet, (hamsters)	↓ Plasma TG and TC levels ↑ HDL-C	Kanashiro *et al*., 2009
Salix matsudana polyphenols	575 mg/kg, p.o. (rats)	↓ Plasma TG level	Han *et al*., 2003
	5% polyphenols fraction in diet, (mice)	↑ Fat excretion	

Triglyceride (TG), total cholesterol (TC), low-density-lipoprotein cholesterol (LDL-C), high-density-lipoprotein cholesterol HDL-C), oral administration (p.o.), intraperitonial (i.p.).

Table 2.6 Effective doses and lipase enzyme inhibitory activities of plant extracts *in vivo* (Continued).

Product/plant extract	Dose	Effect	References
Chikusetsusaponins from *Panax japonicas*	1000 mg/kg, p.o. (rats) 1% and 3% total chikusetsusaponins in diet, (mice)	↓ Plasma TG level ↓ Body weight, ↓ Parametrial adipose tissue weight ↑ Fat excretion	Han *et al*., 2005
Escins from *Aesculas turbinate* BLUME seed	0.25 and 1g/kg in diet, (rats) 0.35% -1% total escins, (mice) 2% total escins in diet, (mice)	↓ Plasma TG level ↓ Hepatic TG and TC content levels ↓ liver weight ↑ Fat excretion and ↓ Body weight ↓ Parametrial adipose tissue weight ↓ Hepatic TG and TC content ↓ liver weight	Hu *et al*., 2008
Cassia mimosoides L. (Nomame Herba)	2.5% Nomame Herba in diet, (rats) 1.5-3.5% Nomame Herba in diet, (rats)	↓ Body weight and liver weight ↓ Plasma TG level	Yamamoto *et al*., 2000

Triglyceride (TG), total cholesterol (TC), oral administration (p.o.).

Table 2.6 Effective doses and inhibitory lipase enzyme activities of plant extracts *in vivo* (Continued).

Product/plant extract	Dose	Effect	References
Taraxcum officinale	400 mg/kg, p.o. (mice)	↓ Plasma TG level	Zhang *et al*., 2008
Acacia polyphenols	0.25-1 g/kg, p.o. (mice)	↓ Plasma TG level	Ikarashi *et al*., 2011
Panax quinquefolium	Saponin fraction 1g/kg, (rats) 1 and 3% saponin in diet, (mice)	↓ Plasma TG level ↓ Parametrial adipose tissue weight	Liu *et al*., 2008
Diosorea nipponica (DN) (dioscin and diosgenin)	100 mg/kg, p.o.(mice) 5% DN in diet, (rats)	↓ Plasma TG level, ↓ Body weight ↓ Plasma VLDL and LDL-C levels	Kwon *et al*., 2003
Nelumbo nucifera (leaves)	1.5 g/kg, p.o. (rats) 5% in diet, (mice)	↓ Plasma TG level ↓ Body weight ↓ Parametrial adipose tissue weight	Ono *et al*., 2006
Teasaponin	481 mg/kg, (rats) 0.5% teasaponin in diet, (mice)	↓ Plasma TG level ↓ Body weight and Parametrial adipose tissue weight	Han *et al*., 2001

Triglyceride (TG), low-density-lipoprotein cholesterol (LDL-C), high-density-lipoprotein cholesterol (HDL-C), very-low-density-lipoprotein (VLDL), oral administration (p.o.).

2.7 Digestion and absorption of carbohydrates

2.7.1 Activity of alpha-amylase enzyme

Alpha-amylase enzyme (1, 4-α-D-glucan-glucanohydrolase) catalyzes the hydrolysis of starch, maltodextrins, and maltooligosacharides. This enzyme is present in animals, plants, bacteria, and fungi. The digestion of starch involves several stages. Initially, partial digestion by the salivary amylase results in the degradation of the polymetric substrate into shorter oligomers (Truscheit *et al*., 1981). Once this partially digested material reaches the gut, it is then extensively hydrolyzed into smaller oligosaccharides by the alpha-amylase isozyme synthesized in the pancreas and excreted into the lumen. The resultant mixture of oligosaccharides passes through the mucous layer of the brush border membrane, where additional alpha-glucosidase degrade it into glucose, which then enter the blood stream by means of a specific transport system (Tundis *et al*., 2010).

2.7.2 Activity of alpha-glucosidase enzyme

Alpha-glucosidase eznyme catalyzes the hydrolytic reaction to liberate alpha-glucose from the non-reducing end of the substrate (Chiba, 1997). Alpha-glucosidase is mainly classified into two groups, GH-family 13 and 31, based on the sequence homology. The enzyme is widely distributed in microorganisms, plants, and animal tissues and the substrate specificity of alpha-glucosidases are known to differ greatly depending on their source (Kimura *et al*., 2004). These enzymes are membrane-bound enzymes located at the epithelium of the small intestine (Gao *et al*., 2008). Recently, Saqib *et al*. (2008) constructed the three-dimensional model of human alpha-glucosidase and investigated the binding

interactions with competitive inhibitor acarbose (anti-diabetic drug). Besides, the conserved catalytic GH-31 domain (residues 334-779), a variable loop originating from the *N*-terminal domain (residues 227-288) contribute towards the architecture of substrate binding site. Secondary structure elements consist of 10 alpha helices and 28 small beta sheets with intermittent loop regions. Human alpha-glucosidase active is a pocket formed mainly by the GH 31 domain residues specifically Asp398, Asp587, His645, and Arg571. Residues the active site and contribute towards the architecture of the binding site. Additional residues lining the sugar binding site include Asp511, Trp370, Ile435, Trp509, and Met512.

2.7.3 Carbohydrate digestion and absorption

In humans, between 40% and 80% of total caloric intake is accounted for carbohydrates in their various forms, making them the most important energy source. According to their chemical structures, carbohydrates can be classified into absorbable, digestible, fermentable, and non-fermentable forms (Englyst *et al*., 2005). In human diet, the main digestible carbohydrates comprise disaccharides (such as sucrose and lactose), and larger polysaccharides which have to be enzymatically digested into absorbable monosaccharides (Cummings and Stephen, 2007). In contrast, fermentable carbohydrates cannot be digested as enzymes cannot readily break the intersaccharide bonds. However, once in the colon, these carbohydrates are readily metabolized by chronic bacteria through the process of fermentation. Similarly, if digestible carbohydrates such as sucrose and lactose are maldigested or malabsorbed, they will also be fermented in the large intestine. The main end products of carbohydrate fermentation are short-chain fatty acids (acetate, propionate, and

butyrate) and gases (carbon dioxide, methane, and hydrogen). These end products can be absorbed in the large intestine (providing energy) which used as a bacteria substrate, released as flatus, or execreted as biomass in the feces (Cummings, Macfarlane, and Englyst, 2001).

From Figure 2.7, the digestion of carbohydrates begins in the mouth by the action of salivary amylase, which hydrolyzes the 1, 4 bonds in starch, the products of this process are maltose, maltriose, and small dextrins. The starch digestion process continues in the small intestine by the action of pancreatic amylase. The digestion process is completed by enzymes in the brush border of the small intestine (maltase, sucrase, and lactase), also known as disaccharidases or glucosidases) which yields the absorbable monosaccharides glucose, fructose, and galactose. A small proportion of monosaccharides can be absorbed passively; however, a carrier protein is required to absorb the amount ingested in a normal diet (Grabitske and Slavin, 2009).

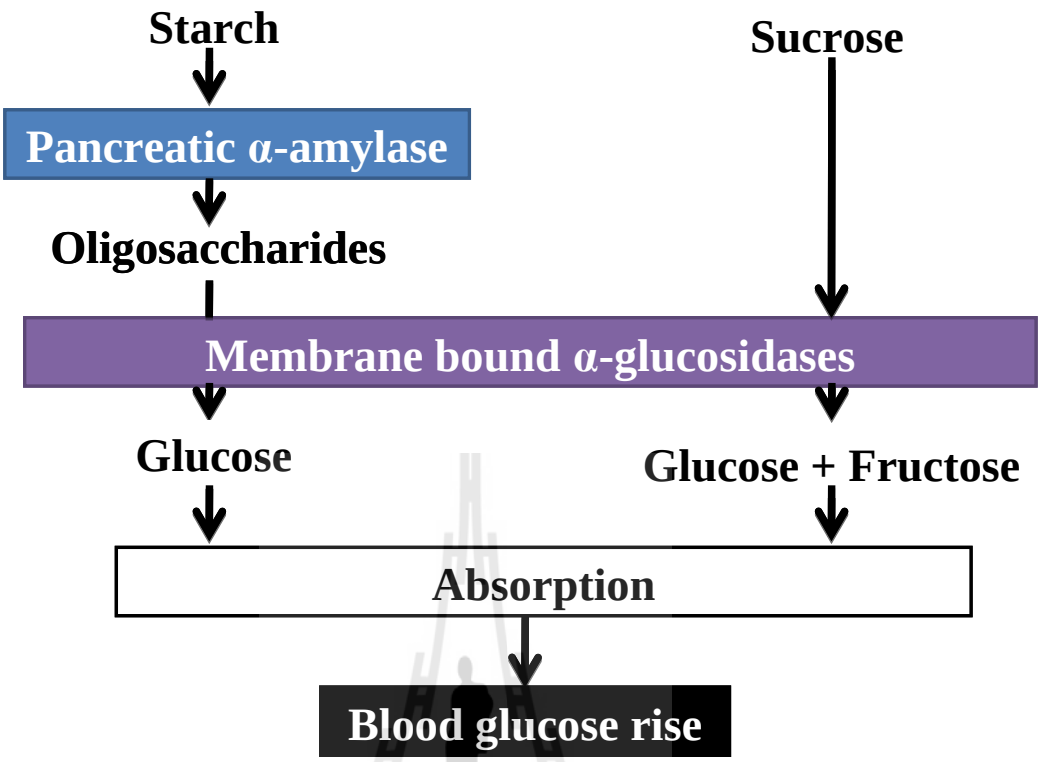

Figure 2.7 Schematic diagram of enzyme degradation of poly- and oligosaccharides and sucrose by intestinal alpha-glucosidases (Bischoff, 1995).

2.7.4 Drug that interfere with carbohydrate absorption

Inhibition of alpha-amylase and alpha-glucosidase could decrease the postprandial increase of blood glucose after a mixed carbohydrate diet which can be an important strategy in the management of type 2 diabetic mellitus (Tundis *et al*., 2010). Blocking these enzymes could decrease the absorption of calories thereby promoting weight loss. The compounds with alpha-amylase and alpha-glucosidase inhibitory activities have pharmacological potential for helping with weight loss and then maintain weight without a dramatic reduction in carbohydrate intake. In addition, since carbohydrate absorption is affected, alpha-amylase and alpha-glucosidase inhibitors could reduce postprandial glucose and insulin responses to dietary

carbohydrates (Tucci, Boyland, and Halford, 2010). Alpha-amylase enzyme inhibitory activity delays gastric emptying by increasing the amount of undigested carbohydrate in the ileum (Jain *et al*., 1991). Therefore, new agents to control postprandial hyperglycemia have been developed. Among them, acarbose and voglibose have received considerable attention in the past decades (Fujisawa *et al*., 2005; Watanaba *et al*., 2004).

Acarbose

Acarbose (Bay g 5421, Glucobay®, Precose®, Prandase®; Bayer) is a psudotetrasaccharide that inhibits intestinal alpha-glucosidase and pancreatic alpha-amylase reversibly at the brush border of intestinal mucosa which was produced from microbial origin (Luo *et al*., 2001). Bischoff (1995) demonstrated that acarbose is structurally comparable to an oligosaccharide derived from starch digestion. Acarbose bind to active site of alpha-glucosidase enzyme that inhibits oligosaccharide digestion (Figure 2.8). Acarbose can retard gastrointestinal absorption of dietary carbohydrates by inhibiting digestion of polysaccharides and disaccharides which suppress postprandial blood glucose levels (Fujisawa *et al*., 2005). Acarbose is poorly absorbed (only 1~2% of active compound) because dose not interact with the Na^+-dependent glucose transporter of the small intestine (Truscheit *et al*., 1981). Acarbose displays alpha-glucosidase inhibitory activity not only in the jejunum but also in the ileum. Consequently, the plasma bioavailability of acarbose is extremely low, but the availability is high at its site of action in the small intestine. This pharmalogical action of acarbose leads to a shift of carbohydrate not digested in the upper parts of the small intestine to the ileum (Bischoff, 1995).

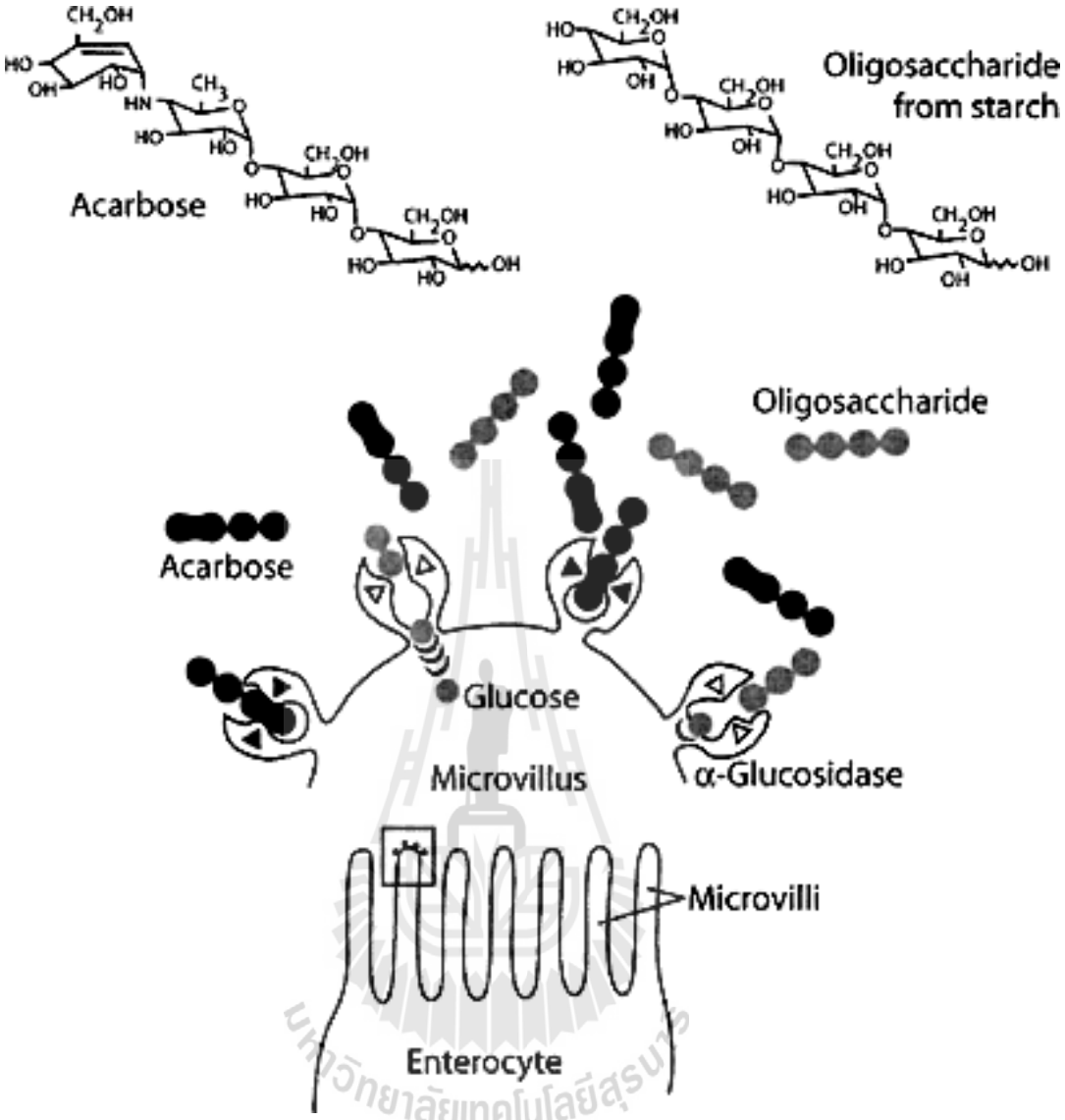

Figure 2.8 Schematic diagrams of enzymatic hydrolysis of oligosaccharides and competitive inhibition of intestinal brush-border alpha-glucosidase by acarbose (Bischoff, 1995).

2.7.5 Alpha-amylase and alpha-glucosidase inhibitors from plants

Nowadays, anti-diabetic drugs (such as acarbose, miglitol, and voglibose) are widely used for treatment diabetes (Kim *et al*., 2005). However, these modern medicines available for management of diabetes mellitus exert serious side effects such as hepatotoxicity, abdominal pain, flatulence, and diarrhea (Fujisawa *et al*., 2005; Singh *et al*., 2008). Drug resistance to these medicines is also reported after prolonged treatment. Therefore, many medicinal plants have been recommended for treatment of diabetes mellitus (Grover, Yadav, and Vats, 2002). Traditional herbal medicines have been used throughout the world for a range of diabetes mellitus (Odhav *et al*., 2010). One therapeutic approach for treating diabetes is to decrease the postprandial hyperglycemia. In particular, inhibition of alpha-amylase and alpha-glucosidase enzyme activities can decrease the postprandial increase of blood glucose after a mixed carbohydrates diet, which is the important strategy in the management postprandial blood glucose level in type 2 diabete mellitus (Ali, Houghton, and Soumyanath, 2006; Lee *et al*., 2007). The inhibitory effects of medicinal plants on alpha-amylase and alpha-glucosidase enzyme activities *in vitro* and *in vivo* were summarized in Tables 2.8 and 2.9, respectively.

Table 2.7 Effective doses and alpha-amylase and alpha-glucosidase enzyme inhibitory activities of plant extracts *in vitro*

Product/plant extract	Dose	Effect	References
Acarbose (anti-diabetic drug)	0.1 µM-10 mM	99.2% Inhibition of HAS	Lo Piparo *et al*., 2008
	0.05-1.0 mM	Inhibition of alpha-glucosidase and alpha-amylase	Jo *et al*., 2010
	1 mg/ml	99% Inhibition of PPA	Odhav *et al*., 2010
	2 mg/ml	42.6% Inhibition of alpha-amylase	Dong *et al*., 2012
		45.8% Inhibition of alpha-glucosidase	
	4 mg/ml	63.8% Inhibition of alpha-glucosidase	Si *et al*., 2010
	IC_{50} of 0.071 µg/ml	Inhibition of alpha-glucosidase	Ikarachi *et al*., 2011
	IC_{50} of 1.75 µg/ml	Inhibition of PPA	Kim *et al*., 2005
	IC_{50} of 2.57 µg/ml	Inhibition of HSA	
	IC_{50} of 35 µg/ml	Inhibition of alpha-glucosidase	
	IC_{50} of 6.2 mg/ml	Inhibition of PPA	Subramanian *et al*., 2008
	IC_{50} of 14.9 mg/ml	Inhibition of alpha-glucosidase	
Rutin	1 mM	41% Inhibition of alpha-glucosidase	Gao *et al*., 2008
	2 mg/ml	25.2% Inhibition of alpha-glucosidase	Dong *et al*., 2012
		23.1% Inhibition of alpha-amylase	
	5 mg/ml	>20% Inhibition of alpha-amylase	Kim *et al*., 2000
	IC_{50} of 0.05-1.0 mM	Inhibition of alpha-glucosidase	Jo *et al*., 2010

Porcine pancreatic alpha-amylase (PPA), human salivary alpha-amylase (HSA)

Table 2.7 Effective doses and alpha-amylase and alpha-glucosidase enzyme inhibitory activities of plant extracts *in vitro*

(Continued)

Product/plant extract	Dose	Effect	References
Morus alba	23 mg/ml	75.6% Inhibition of alpha-amylase	Nickavar *et al*., 2009
Andrographis paniculata	62.5 mg/ml	52.5% Inhibition of PPA 89% Inhibition of yeast alpha-glucosidase	Subramanian *et al*., 2008
Pinus densiflora (bark and needles)	0.1-5 µg/ml	89.6% Inhibition of HSA 72.2% Inhibition of HSA	Kim *et al*., 2004
Acorus calamus L.	1 mg/ml	63.6% Inhibition of alpha-glucosidase	Si *et al*., 2010
Centella asiatica	0.85 mg/ml 5 mg/ml	21% Inhibition of alpha-glucosidase 98.87% Inhibition of alpha-amylase	Mai *et al*., 2007 Odhav *et al*., 2010
Nelumbo nucifera	IC_{50} of 0.82 mg/ml	Inhibition of alpha-amylase	Ono *et al*., 2006
Oolong tea	100 mg/ml	37% Inhibition of alpha-amylase 86% Inhibition of alpha-glucosidase	Kwon *et al*., 2008
Trilobatin of *Lithocarpus polystachyus*	2 mg/ml	21.2% Inhibition of alpha-amylase 48.7% Inhibition of alpha-glucosidase	Dong *et al*., 2012
Punica granatum (flower)	0.5-16 µg/ml	Inhibition of alpha-glucosidase	Li *et al*., 2005

Porcine pancreatic alpha-amylase (PPA); human salivary alpha-amylase (HSA)

Table 2.8 Effective doses and inhibitory alpha-amylase and alpha-glucosidase enzyme activities of plant extracts *in vivo*

Product/plant extract	Dose	Effect	References
Acarbose (anti-diabetic drug)	10 mg/ml, p.o. (rats)	↓ Blood glucose level	Subramanian *et al*., 2008
	20 mg/ml, p.o. (mice)	↓ Serum glucose level	Si *et al*., 2010
	50 mg/ml, p.o. (mice)	↓ Blood glucose level	Kim *et al*., 2005
	300 mg/ml, p.o. (mice)	↓ Plasma glucose level	Li *et al*., 2005
Rutin	4 mg/kg, i.p. (rats)	↓ Blood glucose level	Rauter *et al*., 2010
Pine bark extract	250 mg/kg, p.o. (rats)	↓ Blood glucose level ↓ Body weight	Kim *et al*., 2005
Punica granatum (flower)	250-1000 mg/kg, p.o. (mice)	↓ Plasma glucose level	Li *et al*., 2005
Andrographis paniculata	250-1000 mg/kg, p.o. (rats)	↓ Blood glucose level	Subramanian *et al*., 2008
Acacia polyphenols	0.25-1 g/kg, p.o. (mice)	↓ Plasma glucose level	Ikarachi *et al*., 2011
Acorus calamus	100, 400, and 800 mg/kg, p.o. (mice)	↓ Serum glucose level	Si *et al*., 2010
Centella asiatica	250 mg/ml, p.o. (rats)	↓ Blood glucose level	Chauhan *et al*., 2010

Oral administration (p.o.), intraperitonial (i.p.)

2.8 Adipose tissue

Adipose tissue is specialized connective tissue (Albrignt and Stern, 1998). Adipose tissue functions can be classified into three aspects. First, it is related to lipid metabolism including TG storage and FFA release. Second, it catabolizes TG in order to release glycerol and FFA that participate in glucose metabolism in liver and other tissues. Finally, adipocytes secrete adipokines, which include hormones, cytokines, and other proteins with specific biological functions (Morrison and Farmer, 2000). Adipose tissue has an important influence on physiological processes such as development and growth of the adipocyte and energy homeostasis (Bays *et al*., 2008). There are two types of adipose tissue depending on its cell structure, location, color, vascularization, and function: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the primary site of energy storage in a lipid droplet of the adipocytes in the form of TG, whereas BAT contains multilocular adipocytes or cells with various lipid droplets. WAT has a large number of mitochondria and is specialized in heat production and, therefore, energy expenditure. In humans, BAT is present only in newborns for regulating thermogenic process (Vázquez-Vela, Torres, and Tovar, 2008).

2.8.1 Lipogenesis

Lipogenesis is the synthesis of esterified FFA, which forms TG from carbohydrates or other energy sources acquired in the diet (Figure 2.9). In rats, lipogenesis occurs in liver and WAT. It occurs predominantly in liver and to a lesser extent in adipose tissue, even with high-carbohydrate diets. In rodents, nutritional status and small changes in insulin levels are factors that influence lipogenesis rate

(Vázquez-Vela *et al*., 2008). Lipid synthesis is augmented during postprandial state and after carbohydrate consumption and is inhibited under fasting conditions (Sebokova and Klimes, 1997). In addition, plasma glucose stimulates lipogenesis. Glucose itself is a substrate for lipogenesis. By being glycolytically converted to acetyl-CoA, glucose promotes fatty acid synthesis (Kersten, 2001). Lipid accumulation in adipose tissue depends on circulating FFA uptake (Zechner *et al*., 2000). Vázquez-Vela *et al*. (2008) revealed that fatty acids provided by the enzymatic hydrolysis of TG contains in the chylomicrons by the lipoprotein lipase. After FFA enter the adipocyte, reesterification is necessary for lipid storage in TG form. Several enzymes involved in adipose tissue lipogenesis were induced by insulin such as fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and malic enzymes. Newly synthesized FFA was used as substrates in TG synthesis (Kersten, 2001).

2.8.2 Lipolysis

Triglyceride stored in the lipid droplet is first hydrolyzed by the enzyme adipose triglyceride lipase (ATGL), also known as desnutrin, releasing a diacylglycerol moiety and FFA (Villena *et al*., 2004). After hydrolysis by ATGL, diacylglycerols were then hydrolyzed sequentially by the hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL), producing FFA and glycerol (Holm, 2003). Different lipases gain access to the lipid droplet when proteins that coat the vesicle (perilipins) are phosphorylated. Perilipin normally prevents lipolysis of TG by surrounding the lipid droplet, and preventing the access of lipases (Brasaemle *et al*., 2000). β-adrenergic stimulation of adipocytes and the subsequent protein kinase A-dependent phosphorylation of HSL and perilipin trigger the translocation of HSL

from the cytoplasm to the lipid droplet and induce neutral lipid hydrolysis (Egan *et al*., 1992). During fasting, glucagon and catecholamines stimulate lipolysis in the adipocytes by activating *via* protein kinase A (PKA) several lipases, resulting in a mobilization of FFA from the adipocyte to the circulation, which are then bound to albumin and transported to muscle, liver, heart, and other tissues for its oxidation or reesterification (Lafontan *et al*., 2000). Lipogenesis and lipolysis are illustrated in Figure 2.9.

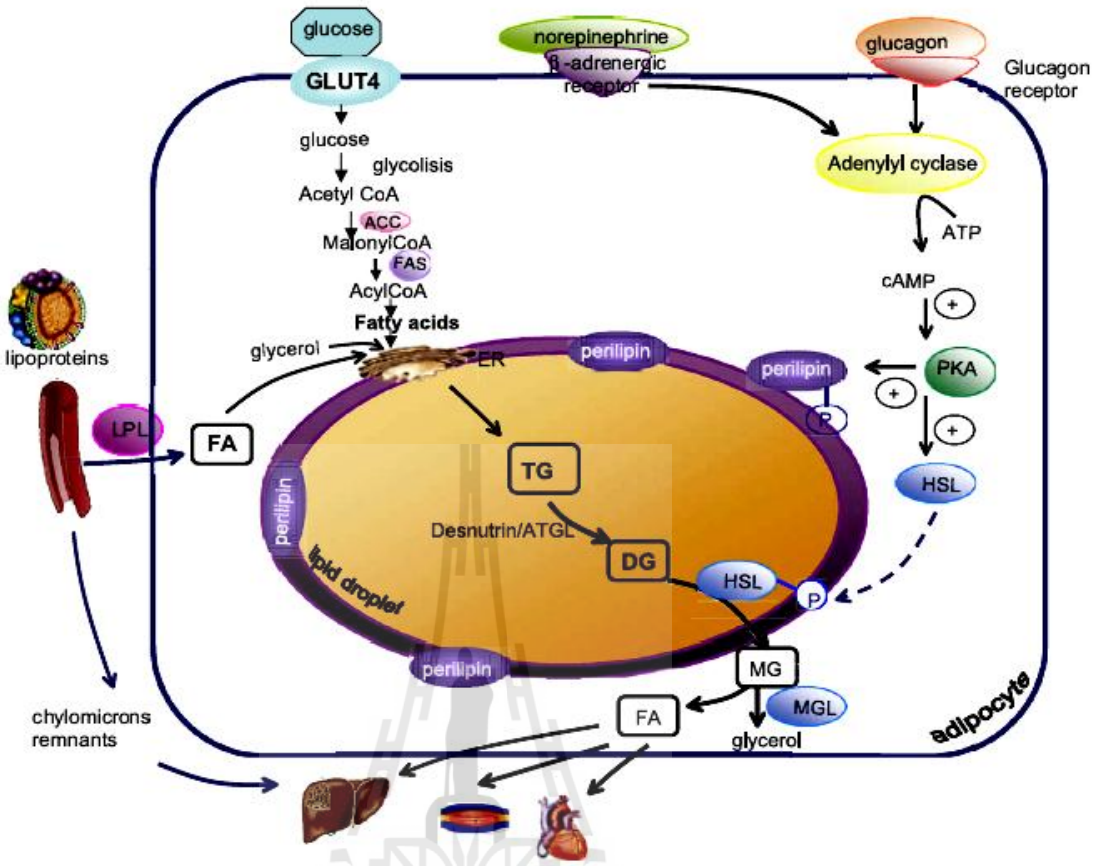

Figure 2.9 Lipogenesis and lipolysis mechanisms (Vázquez-Vela *et al*., 2008). Triglyceride (TG), diglyceride (DG), fatty acid (FA), monoglyceride (MG), monoglyceride lipase(MGL), protein kinase A (PKA), acetyl CoA carboxylase (ACC), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), and fatty acid synthase (FAS).

2.9 Hypothalamic control of food intake and body weight

2.9.1 Overview of hypothalamic organization

The hypothalamic neurons consist of specific neurotransmitters, receptors, and other factors off crucial important in feeding behavior and the development of obesity (Berthoud, 2002). The hypothalamus is a center of convergence and integration of multiple nutrient-related signals, including circulating

adiposity hormones, gastric hormones and nutrients, and received neuroanatomical projections from other nutrient sensors, mainly within the brainstem. The hypothalamus also integrates these signals with various cognitive forebrain-descending information and reward/motivation-related signals coming from the midbrain-dopamine system, to coordinate neuroendocrine, behavioral, and metabolic effectors of energy balance (Blouet and Schwartz, 2010).

Arcuate nucleus (ARC)

The strongest neural inputs to the arcuate nucleus (ARC) are from other periventricular areas including the paraventricular nucleus (PVN) as well as the medial zone nuclei such as the median preoptic nucleus (Horvath, Diano, and van de Pol, 1999). Within the ARC of the hypothalamus, there are two neuronal populations with opposing effects on food intake: neurons which co-expressed neuropeptide Y (NPY) and agouti related peptide (AgRP) stimulate food intake, whereas neurons co-expressed pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) suppress feeding. Both neuronal populations project to the PVN, although the ARC also communicates with other hypothalamic nuclei such as the dorsomedial nucleus (DMN), lateral hypothamic area (LHA), and ventromedial nucleus (VMN) (Simpson, Martin, and Bloom, 2009).

Within POMC neurons, alpha-melanocyte-stimulating hormone (α-MSH) is produced and binds to melanocortin-4 (MC4R) receptors in the PVN to suppress food intake (Schwartz *et al*., 2000). In consistency, MC4R knock-out mice exhibit hyperphagia and obesity (Huszar *et al*., 1997). In humans, MC4R mutations account for approximately 6% of severe early–onset obesity and more than 70

different mutations have been associated with obesity (Tao, 2005). Besides, the role of MC4R on appetite control is still unclear. MC3R deficient mice show increased fat mass and reduced lean body mass (Chen *et al*., 2000), but selective MC3R agonists have no effect on feeding (Abbott *et al*., 2000). The majority of POMC neurons in the ARC also co-expressed *CART* mRNA. In *in vivo* study, intracerebroventricular (ICV) administration of CART inhibited food intake, whereas ICV injection of CART antiserum increased food intake (Kristensen *et al*., 1998). Within the hypothalamus, NPY is an important regulator of body weight through its effects on food intake and energy expenditure. NPY acts at five different receptors (Y1-Y5 receptors), NPY appears to exert its orexigenic effect predominately *via* the Y1 and Y5 receptors (Berthoud, 2002). The majority of neurons expressing NPY in the hypothalamus are found within the ARC and mostly co-expressed AgRP (Stanley *et al*., 2005). NPY/AgRP neurons have extensive projections within the hypothalamus including the PVN, DMN, and LHA which appear to be the main targets for the orexigenic effects of NPY (Simpson *et al*., 2009; Suzuki *et al*., 2010).

Paraventricular nucleus (PVN)

The PVN acts to integrate neuropeptide signals from numerous central nervous system (CNS) regions including the ARC and brain stem (Schwartz, 2000). Microinjection of almost all known orexigenic (e.g. NPY) and anorexigenic (e.g. leptin) signals into the PVN alters appetite (Elmquist *et al*., 1998; Lambert *et al*., 1995). Administration of melanocortin agonists into the PVN potently inhibits food intake (Giraudo, Billington, and Levine, 1998; Kim *et al*., 2000). Conversely, injection of a melanocortin antagonist into the PVN stimulates food intake (Giraudo *et*

al., 1998). The ARC neurons expressing POMC potentiate inhibitory GABAergic (Gamma Aminobutyric Acid) signaling within the PVN, involving in a reduction of food intake. The NPY/AgRP neurons of the ARC inhibit this GABAergic signaling, involving in an increase of food intake (Cowley *et al*., 1999). Several neuropeptides synthesized in the PVN neuron reduce food intake and body weight. The PVN consists of parvocellular (thyrotropin releasing hormone (TRH) and corticotropin releasing hormone (CRH)) and magnocellular (oxytocin) neurons that produce anorexigenic peptides (Remmers and Delemarre-van de Waal, 2011). Moreover, the parvocellular neuron contains NPY (orexigenic peptide) which increase food intake (Schwartz, 2000).

Lateral hypothalamic area (LHA) and perifornical area (PeF)

Other hypothalamic areas including the lateral hypothalamic area (LHA) and the perifornical area (PeF) are involved in downstream signaling. Indeed, the PeF is one of the most sensitive areas for NPY-induced (Stanley *et al*., 1993). The LHA and PeF contain neurons expressing pre-pro-orexin and releasing the peptide products orexin A and B (or hypocretin 1 and 2). The LHA and PeF contain neuropeptides (orexin and melanin-concentrating hormone (MCH)) that increase feeding (Harrold *et al*., 2012; Schwartz *et al*., 2000; Stanley *et al*., 2005). Acute central administration of orexin leads to hyperphagic response in rodents and delays the behavioural onset of satiety (Harrold *et al*., 2012). MCH expression stimulates food intake (Stanley *et al*., 2005).

Ventromedial hypothalamus (VMH)

The VMN is known to play a role in energy homeostasis for many years since the findings that bilateral VMH lesions induce hyperphagia and obesity has been elucidated. The VMH receives NPY, AgRP, and α-MSH immunoreactive projections from the ARC neurons and, in turn, VMH neurons project onto both hypothalamic nuclei (e.g. DMH) and brainstem regions (e.g. the nucleus of the solitary tract or NTS). Expression of neuropeptides in the VMH is modulated by energy status, with altered NPY expression in obese mice (Guan, Yu, and van der Ploeg, 1998) and increased MC4R expression in diet-induced obese rats (Huang *et al*., 2003). Brain-derived neurotrophic factor (BDNF) is highly expressed in the VMN, and its expression is regulated both by food derivation and melanocortin agoinsts. Increases in food intake and body weight occur in mice with reduced BDNF receptor expression or reduced BDNF signaling (Xu *et al*., 2003). Therefore, BDNF neurons in the VMH may act as an additional downstream pathway through which nutritional status and the melanocortin system modulate energy homeostasis.

The chief brain areas and pathways involved in the regulation of food intake are summarized in Figure 2.10. Briefly, the ARC is the chief hypothalamic area involved in the control of food intake and contains two interconnected groups of ‘‘first-order’’ neurons releasing orexigenic substance (NPY and AGRP) that enhance food intake, and the anorexigenic substance (POMC and CART) that reduce food intake. The axons of POMC/CART neurons project to ‘‘second-order’’ neurons located in the areas of the PVN, where the anorexigenic substances TRH, CRH, and oxytocin are secreted. The axons of NPY/AgRP neuron project to “second order” neurons located in the areas of the LHA and PFA where the orexigenic substances

MCH and orexins are produced. When adiposity signals (leptin and insulin) reach the ARC, anorexigenic peptides are released which activate a catabolic circuit. In contrast, the activation of anabolic pathway leads to the release of orexigenic peptides and occurs when adiposity signal levels in the brain are low (Valassi, Scacchi, and Cavagnini, 2008).

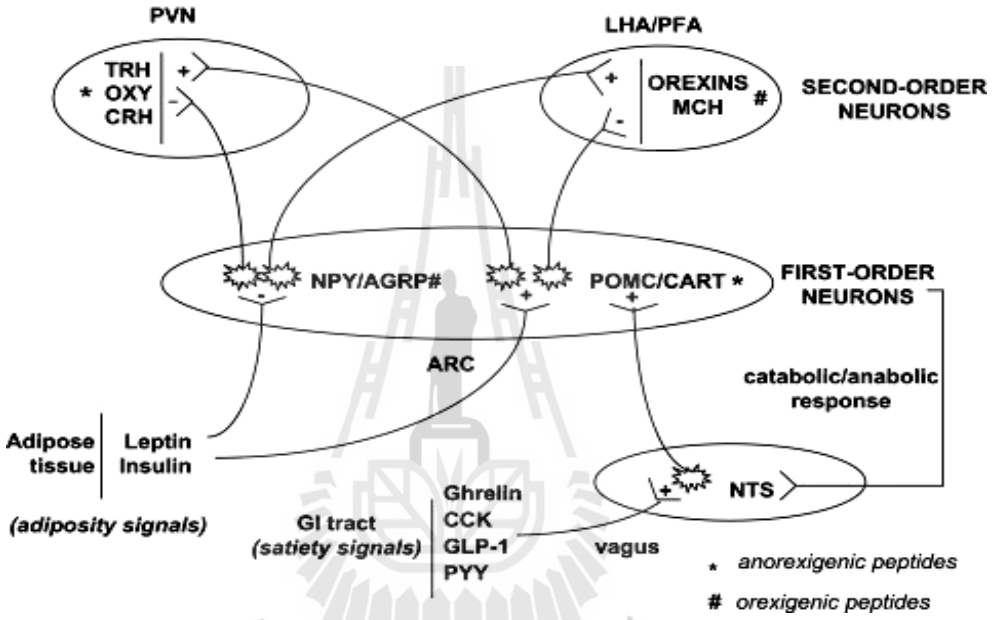

Figure 2.10 A schematic representation of the chief brain pathways involved in the regulation of eating behavior. Arcuate nucleus (ARC); nucleus of the solitary tract (NTS); cholecystokinin (CCK); glucagon-like peptide 1 (GLP-1); peptide YY (PYY); paraventricular nucleus (PVN); lateral hypothalamic area (LHA); perifornical area (PFA); neuropeptide Y (NPY); Agouti-related peptide (AGRP); pro-opiomelanocortin (POMC); cocaine- and amphetamine-regulated transcript (CART); corticotropin-releasing hormone (CRH); thyrotropin-releasing hormone (TRH); oxytocin (OX); melanin-concentrating hormone (MCH) (Valassi *et al*., 2008).

2.10 Fos protein

Fos protein, the translational product of immediate early gene *c-fos*, exerts influence on cellular functions by regulating the induction of its downstream target genes as a transcription factor. Fos protein can be induced rapidly and transiently in neurons after applying a variety of stimuli (Herrera and Robertson, 1996). After translation, Fos protein couples with Jun protein to form a heterodimer nucleoprotein complex that binds with high affinity to a DNA-specific sequence identified as activating protein-1 (AP-1) site (Sasson-Corsi *et al*., 1988). AP-1 is a collective term referring to dimeric transcription factors composed of Jun, Fos or activating transcription factor (ATF) subunits that bind to a common DNA site (Karin, Liu, and Zandi, 1997), as was shown in Figure 2.11. Several *cis* elements mediate *c-fos* induction. Proximal to the *c-fos* TATA box (TGACGTCA) is a cAMP-responsive element (CRE) or ATF proteins, which all mediate *c-fos* induction *via* cAMP- and Ca^{2+}-dependent signaling pathways in response to neurotransmitters and polypeptide hormones (Sheng, Thompson, and Greenberg, 1991). Expression of the immediate early gene *c-fos* and its protein product Fos has been extensively used to map stimulus-evoked functional activity in the brain (Nikolaev *et al*., 2002). Fos act as a marker for neuronal activation that can be identified by immunohistochemistry to be located in the nuclei of neurons (Bullitt, 1990).

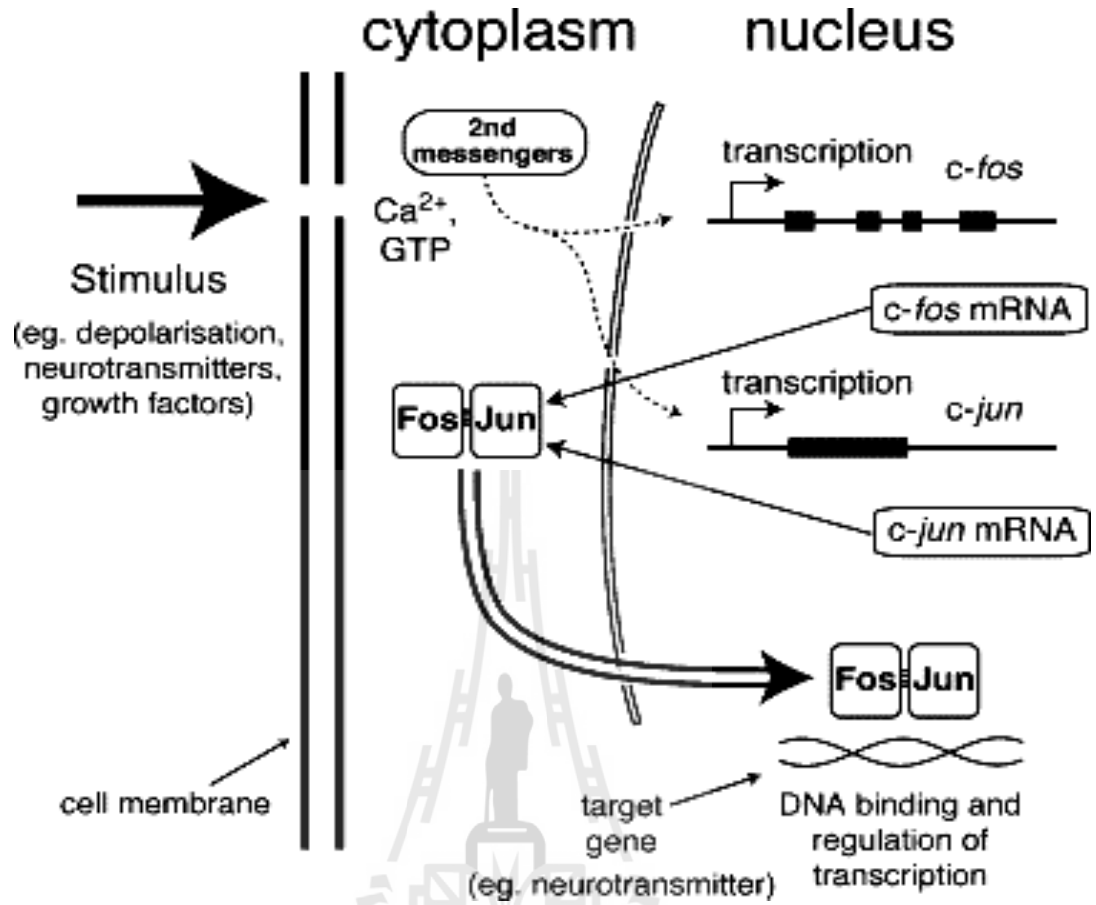

Figure 2.11 Schematic diagram showing intracellular pathways leading to *c-fos* and *c-jun* expression, and the role of their protein products Fos and Jun, respectively, in regulating the expression of other genes (so-called "late-response" genes) (Dampney and Horiuchi, 2003).

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CHAPTER III

EFFECTS OF THE AQUEOUS ETHANOLIC EXTRACT OF *CENTELLA ASIATICA* ON PANCREATIC LIPASE ACTIVITY *IN VITRO*

3.1 Abstract

The aim of this study was to investigate the effect of aqueous ethanolic extract of *C. asiatica* and rutin on porcine pancreatic lipase activity *in vitro*. Orlistat was used as a positive control since orlistat has a strong inhibitory porcine pancreatic lipase activity. Total phenolic and rutin contents were determined by using Folin-Ciocalteu reagent method and high performance liquid chromatography, respectively. *C. asiatica* was extracted by 80% ethanol which had a yield of 11.81%. Total phenolic content of extract was 97.75 ± 0.01 mg gallic acid/g dry extract. The aqueous ethanolic extract of *C. asiatica* contained 1.27 ± 5.5 g/kg dry weight of plant. The inhibition of porcine pancreatic lipase activities of the aqueous ethanolic extract of *C. asiatica* and rutin, at concentrations of 1.19 to 76.16 mg/ml, were measured by titrimetric method using triolein as a substrate. The concentrations of the aqueous ethanolic extract of *C. asiatica* and rutin required to inhibit 50% of pancreatic lipase activity (IC_{50}) were 25.03 ± 0.05 and 48.05 ± 0.08 mg/ml, respectively.

At the concentration of 9.52 mg/ml, orlistat exhibited a strong inhibition on pancreatic lipase activity (75.47%), while the aqueous ethanolic extract of *C. asiatica* and rutin could inhibit 35.63% and 37.24% of porcine pancreatic lipase activity, respectively. At a concentration of 76.16 mg/ml of the aqueous ethanolic extract of *C. asiatica* and rutin had no significant the inhibitory pancreatic lipase activities when compared with orlistat (9.52 mg/ml). The inhibitory pancreatic lipase activities of the aqueous extract of *C. asiatica* was less potent than rutin and orlistat in the present study. Pancreatic lipase inhibitory activity of the aqueous ethanolic extract of *C. asiatica* and rutin could inhibit digestion and absorption of fat. The aqueous ethanolic extract of *C. asiatica* may be replaced orlistat on inhibitory lipase enzyme. Rutin may be responsible for inhibitory pancreatic lipase activity of *C. asiatica*. Further investigation is needed to focus on the effect of the aqueous ethanolic extract of *C. asiatica* on reduction of postprandial triglyceride levels *in vivo*.

3.2 Introduction

Obesity is characterized by the excessive accumulation of body fat that may impair health such as coronary heart disease, dyslipidemia, glucose intolerance, diabetes mellitus, hypertension, and some cancers (Hu *et al*., 2008). The cause of obesity is an energy imbalance between calorie intake and energy expenditure. Thus, the inhibitory of digestion and absorption of dietary fat can be useful in treatment of obesity (Zhang *et al*., 2008). Many studies have been reported that increased intake of foods with high energy and dietary fat content can promote body fat storage which increase a calorie intake and body weight in rats (Hu *et al*., 2008; Uchiyama *et al*., 2011; Yang *et al*., 2010; Yoshikawa *et al*., 2002). Lipases are enzymes that digest fats,

including triacylglycerol, and phospholipids. The human lipases include the pre-duodenal (lingual and gastric) and the extra-duodenal (pancreatic, hepatic, lipoprotein, and the endothelial) lipases (Manjari, 2003). Lipase inhibition is one of the most widely management obesity. Nowadays, orlistat is drug for obesity treatment which has been shown to inhibit activity of lipases (Jandacek and Woods, 2004). However, orlistat has certain unpleasant gastrointestinal side effects like oily stools, oily spotting, and flatulence others (Birari and Bhutani, 2007). Many studies showed pancreatic lipase inhibitory activity natural products, including plant materials, that can be alternative anti-obesity agents (Sharma, Sharma, and Seo, 2005; Yamamoto *et al*., 2000; Yoshikawa *et al*., 2002; Zhang *et al*., 2008; Zheng *et al*., 2010).

Adisakwattana *et al*. (2012) demonstrated the aqueous extract of *C. asiatica* could suppress an increase of postprandial triglyceride and cholesterol levels by inhibitory pancreatic lipase and cholesterol esterase *in vitro*. *C. asiatica* was reported to contain flavonoids such as catechin, quercetin, and rutin (Hussin *et al*., 2009; Zainol *et al*., 2003). Especially, rutin (3, 3', 4', 5, 7-pentahydroxyflavone-3-rhamnoglucoside) is flavonoids of the flavonol type which possess pancreatic lipase inhibitory activity (Zheng *et al*., 2010), and anti-cholesteremic activity (Ziaee *et al*., 2009). Rutin alone or in combination with lovastatin (hypolipidemic agent) mixed in a high-cholesterol diet significantly reduced the level of plasma cholesterol and low density cholesterol, and also markedly decreased liver enzymes and body weight in rats with high cholesterol diet (Ziaee *et al*., 2009). Furthermore, the addition of rutin or *0*-coumaric acid to the diet could decrease body weight gain, liver weight, adipose tissue weight, triglyceride (TG), phospholipid, total cholesterol (TC), insulin, and leptin in high fat diet-induced obese rats (Hsu *et al*., 2009).

However, the effect of the aqueous ethanolic extract of *C. asiatica* on pancreatic lipase activity has not yet been examined. Therefore, the present study was investigated the effects of aqueous ethanolic extract of *C. asiatica* and rutin on pancreatic lipase inhibitory activity *in vitro*.

3.3 Materials and methods

3.3.1 Plant material

C. asiatica (*Centella asiatica*) plant were obtained from local market in Nakhon Ratchasima province during June-August 2010.

3.3.2 Preparation of plant extract

Edible parts of *C. asiatica* (10 kg) 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 in hot air oven at 40 to 45 ^{o}C for 2 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 through 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 (11.81 g) was stored at -20 ^{o}C until further used and the weight percentage yield was determined. This stock extract was used in all experiments performed in this study.

3.3.3 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):

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

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

3.3.4 Determination of total phenolic contents

The total phenolic compounds of the aqueous ethanolic extract of *C. asiatica* were measured according to the Folin-Ciocalteu reagent method that was adapted from the method of Minussi *et al*. (2003). Briefly, the aqueous ethanolic extract of *C. asiatica* was dissolved in 10% ethanol. The reaction mixtures consisted of aqueous ethanolic extract of *C. asiatica* solution (200 µl) and 4 ml of 2% sodium carbonate (Na_2CO_3, BDH Ltd., UK) and were mixed. Two minutes later, 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.

3.3.5 Analysis of rutin by high performance liquid chromotrography (HPLC)

The method was adapted from the method of Zu *et al*. (2006). The aqueous ethanolic extract of *C. asiatica* was dissolved in the mobile phase.

The mobile phase was methanol-acetonitrile-water (40: 15: 45, V/V/V) containing 1.0% of acetic acid. This mobile phase was filtered through a 0.45 µm membrane filter (Millipore, USA). Standard stock solution of rutin was prepared in ethanol at the concentration of 0.280 mg/ml. Standard solution was filtered through a 0.45 µm membrane filter. Flow rate and injection volume were 0.25 ml/min and 10 µl, respectively. Chromatographic analysis was carried out by C18 reversed-phase column (4.0 mm × 100 mm, Agilent Technologies Company, USA). The chromatographic system consisted of system software (Agilent Technology Company, USA). Rutin was quantified by HPLC at 257 nm. The chromatographic peaks of the analysis were confirmed by comparing their retention time and UV spectra with the reference standard. This experiment was carried out at ambient temperature.

3.3.6 Chemicals

Porcine pancreatic lipase enzyme, glyceryl trioleate (grade ≥ 99%), rutin trihydrate were purchased from Sigma-Aldrich (St. Louis, USA) and orlistat (Xenical) was purchased from Roche Ltd. All other chemicals were reagent grade.

3.3.6.1 Materials of porcine pancreatic lipase activity

1 M Phosphate buffer solution (pH 7.4)

1 M Phosphate buffer solution (pH 7.4) was prepared by adding 10.65 g of di-sodium hydrogen phosphate anhydrous (Na_2HPO_4; BDH Ltd., UK) and 3.968 g of sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4.H_2O$; BDH) to 80 ml of double deionized distilled (DDD) water. This solution was stirred on a hot plate magnetic stirrer (VELP Scientifica, Europe) at 50 to 60 ^oC for 1 h, left to be cool

down to room temperature and then adjusted volume to 100 ml with DDD water in a volumetric flask. This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

Porcine pancreatic lipase enzyme solution

Porcine pancreatic lipase enzyme solution (3.33% w/v) was prepared by mixing 0.5 g of porcine pancreatic solution (Sigma, St. Louis, MO, USA) in 15 ml of 50 mM phosphate buffer (pH 8.0). This enzyme solution was prepared in ice-cold 50 mM phosphate buffer (pH 8.0).

1% (v/v) Triolein in Tween 40 suspension

1% (v/v) Triolein in Tween 40 suspension was prepared by adding 1 ml of glyceryl trioleate (Sigma) to 99 ml Tween 40 (Sigma) and then sonicated by using ultrasonic processor (Sonics Vibra Cell™, model VCX 750, Sonics and Materials Inc., New Town, USA) at 40 W for 3 min.

50 mM Phosphate buffer (pH 8.0)

50 mM Phosphate buffer (pH 8.0) was prepared by adding 5 ml of 1 M phosphate buffer (pH 7.4) in 90 ml of DDD water. This solution was adjusted volume to 100 ml with DDD water in volumetric flask. This solution was then adjusted to pH 8.0 with 1 M HCl or 1 M NaOH.

95% Ethanol solution

95% Ethanol solution was prepared by adding 5 ml of ethanol to 95 ml of DDD water.

0.025 N Sodium hydroxide solution

Sodium hydroxide solution (0.025 N) was prepared by mixing 1 g of sodium hydroxide anhydrous pellets (NaOH, Carlo Erba Reagents) in 1000 ml of DDD water.

3.3.7 *In vitro* assay for measuring the inhibition of porcine pancreatic lipase enzyme

Lipase activity was determined by measuring the release rate of oleic acid from triolein using titrimetric method that was adapted from the method of Wrolstad *et al*. (2005). The amount of oleic acid released during the reaction was determined by direct titration with NaOH to a phenolphthalein end point. Briefly, *C. asiatica* extract and rutin were dissolved in DDD water to give concentrations ranging from 1.19 to 76.16 mg/ml. Orlistat (Xenical, Roche Diagnostics GmbH., Germany) dissolved in DDD water (9.52 mg/ml) was used as positive control. The reaction mixtures contained each concentration of 2.5 ml of aqueous ethanolic extract of *C. asiatica*, rutin, or orlistat, 3 ml of 1% Triolein in Tween 40, and 1 ml of 50 mM phosphate buffer (pH 8.0). The mixtures were swirled and incubated in water bath (model WB-22, WiseBath, Korea) at 37 ^{o}C for 30 min. After that, 1 ml of 3.33% (w/v) porcine pancreatic lipase enzyme was added to the mixtures. The mixtures were then incubated in water bath at 37 ^{o}C for 1 h. The reaction was stopped by adding 3 ml of 95% ethanol solution and 2 to 3 drops of phenolphthalein indicator (Sigma, St. Louis, USA). Titration was performed with 0.025 N sodium hydroxide solution using burette and pH meter (model C830, Consort, Belgium) until a light pink color appeared. Individual control (A) and blank (a) were conducted in a similar way by replacing the

sample with 2.5 ml of DDD water, with or without porcine pancreatic lipase enzyme, respectively. Individual test 1 (B) and test 2 (b) were prepared as mentioned above with or without porcine pancreatic lipase enzyme, respectively. Blank (a) and test 2 (b) without porcine pancreatic lipase enzyme solution were replaced by 1 ml of phosphate buffer (50 mM, pH 8.0). All determinations were performed in triplicate. The inhibition percentage of porcine pancreatic lipase enzyme activity was assessed by following formula:

$$\text{Inhibition (\%)} = \left[\frac{\text{(A-a) - (B-b)}}{\text{(A-a)}}\right] \times 100$$

where: A and B were the volume of NaOH used to reach the titration end point of control and sample with porcine pancreatic lipase enzyme, respectively.

a and b were the volume of NaOH used to reach the titration end point of blank and sample without porcine pancreatic lipase enzyme, respectively.

3.4 Statistical analysis

All the analyses were carried out in triplicate and the results were expressed in mean ± standard error of mean (S.E.M). The difference between rutin and the aqueous ethanolic extract of *C. asiatica* within the same concentrations and the difference between orlistat and rutin or the aqueous ethanolic extract of *C. asiatica* were analyzed using one-way ANOVA following by the Tukey test (Sigmastat version 3.5). *P*-value less than 0.05 ($P<0.05$) was considered statistically significant.

3.5 Results

The percent yield of *C. asiatica* extracted by 80% aqueous ethanol was 11.81%. Total phenolic content of extract was 97.75 ± 0.01 mg gallic acid/g dry extract. The aqueous ethanolic extract of *C. asiatica* contained 1.27 ± 5.5 g/kg dry weight of plants (Table 3.1). The inhibitory activities of the aqueous ethanolic extract of *C. asiatica* and rutin against porcine pancreatic lipase were shown in Figure 3.1. The results in Table 3.2 showed the IC_{50} values of rutin and the aqueous ethanolic extract of *C. asiatica* on pancreatic lipase activities (25.33 ± 0.05 and 48.05 ± 0.08 mg/ml, respectively). The concentrations ranging from 1.19 to 76.16 mg/ml, the aqueous ethanolic extract of *C. asiatica* and rutin had dose-dependent on inhibitory porcine pancreatic lipase activities. No significant different between the inhibitory pancreatic lipase activities of the aqueous ethanolic extract of *C. asiatica* and rutin showed at the concentrations ranging from 1.19 to 19.04 mg/ml. At a concentration of 9.52 mg/ml, orlistat was significantly higher the inhibition on porcine pancreatic lipase activity (75.47%), while rutin and the aqueous ethanolic extract of *C. asiatica* had 37.24% and 35.63% of the inhibition porcine pancreatic lipase activities, respectively. At the concentration of 38.08 mg/ml, the percent inhibitory pancreatic lipase activity of rutin was significantly higher than that of the aqueous ethanolic extract of *C. asiatica*. At a concentration of 76.16 mg/ml, there was no significant difference between rutin and the aqueous ethanolic extract of *C. asiatica* on the inhibitory pancreatic lipase activities. At this concentration, both the aqueous ethanolic extract of *C. asiatica* and rutin had no significant difference on inhibitory pancreatic lipase activities when compared with orlistat (9.52 mg/ml).

Table 3.1 The percent yield, total phenolic content, and rutin levels of the aqueous ethanolic extract of *C. asiatica*.

Parameters	**Mean ± S.E.M.**
Yield (%) dried plant	11.81%
Total phenolic content (mg gallic acid/g dry extract)	97.75 ± 0.01
Rutin (g/kg dry weight of dry plant)	1.27 ± 5.5

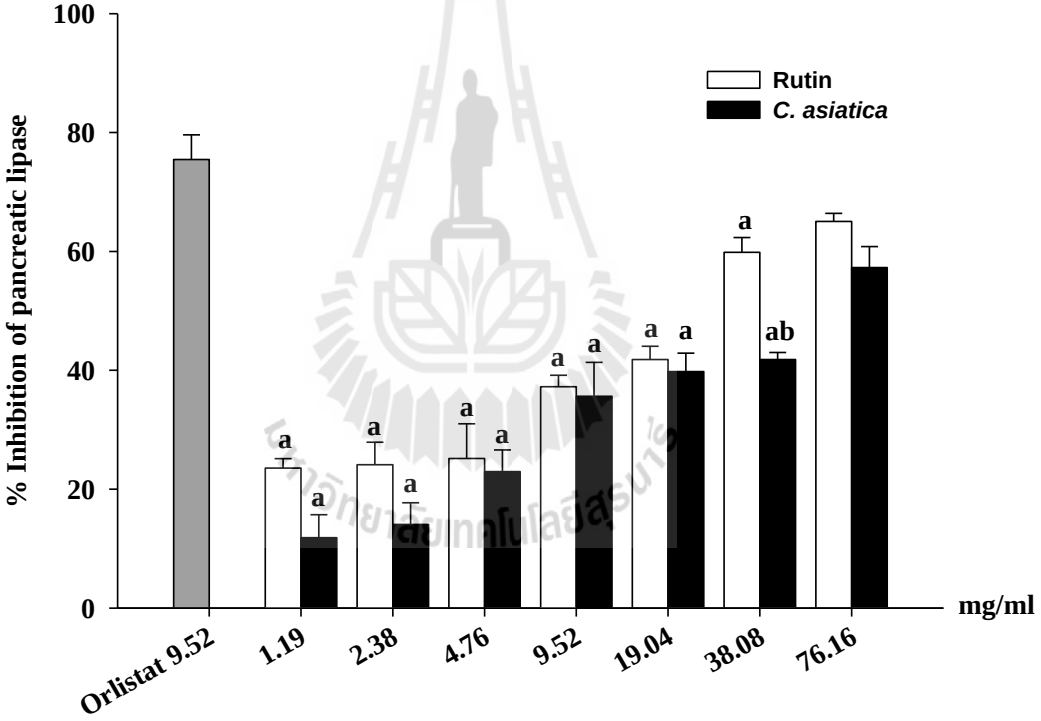

Figure 3.1 Inhibitory effects of rutin and the aqueous ethanolic extract of *C. asiatica* on porcine pancreatic lipase activity *in vitro*. The results were expressed as mean ± S.E.M. of three independent experiments. a, $P<0.01$ compared to orlistat on inhibition of pancreatic lipase enzyme. b, $P<0.05$ compared to rutin on inhibition of pancreatic lipase enzyme at the same concentration.

Table 3.2 The IC_{50} values for rutin and the aqueous ethanolic extract of *C. asiatica* on pancreatic lipase enzyme activities.

Samples	**IC_{50} (mg/ml)**
Rutin	25.03 ± 0.05
The aqueous ethanolic extract of *C. asiatica*	48.05 ± 0.08

Concentration of samples to inhibit 50% of its activity (IC_{50})

3.6 Discussion and conclusion

One of the most important strategies for prevention of obesity is inhibition of fat digestive enzyme (lipase) and absorption of dietary fat (Zhang *et al*., 2008). Pancreatic lipase is the key enzyme for dietary fat digestion and the inhibition of this enzyme could be an effective way to alter fat absorption (Tucci, Boyland, and Halford, 2010). Orlistat, the anti-obesity drug, as expected, had a strong inhibitory effects against lipase enzyme activity since a potent inhibitor of gastric, pancreatic, and carboxylester lipase (Sharma *et al*., 2005) that inhibited the absorption of fat and promote excretion of ingested fat leading to weight loss (Shi *et al*., 2005). In fact, orlistat has undesirable side effects such as fecal incontinence, flatulence, and steatorrhea (Birari and Bhutani, 2007; Jandacek *et al*., 2007). There was safer alternative to orlistat for anti-obesity from medicinal plants that could restrict pancreatic lipase activity such as grape seed extract (Moreno *et al*., 2003), *Nelumbo nucifera* extract (Ono *et al*., 2006), peanut shell extract (Moreno *et al*., 2006), oolong tea (Han *et al*., 1999), and *Dioscorea nipponica* Makino (Kwon *et al*., 2003).

This present study demonstrated that the percentage yield and total phenolic content of the aqueous ethanolic extract of *C. asiatica* was 11.81% and 97.75 ± 0.01

mg gallic acid/g dry extract, respectively. Lee *et al*. (2011) showed that the ethanolic extract of *C. asiatica* gave 2.48% yield and total phenolic content of the extract was 31.58 ± 3.08 mg gallic acid/g dry extract. The aqueous ethanolic extract of *C. asiatica* contained 1.27 ± 5.5 g/kg dry of rutin in the present study while the previous study exhibited that the methanolic extract of *C. asiatica* contained 1.14 ± 7.7 g/kg dry plant (Hussin *et al*., 2009). Phenolic compounds (flavonoids, terpenoids, and phenolic acids) are secondary metabolites in plants which have beneficial effects in preventing obesity since the inhibition of lipase activity (Birari and Bhutani, 2007; Sergent *et al*., 2012; Zheng *et al*., 2010) and the reduction of TG and TC levels (Santos *et al*., 1999). Especially, rutin has anti-obesity effects due to reduce the levels of TG, TC, and LDL (Santos *et al*., 1999; Ziaee *et al*., 2009), inhibit of lipase activity (Zheng *et al*., 2010), and reduce lipid accumulation in hepatic cells (Wu *et al*., 2011).

Adisakwattana *et al*. (2012) showed that the aqueous extract of *C. asiatica* could inhibit pancreatic lipase activity (IC_{50} = 0.12 ± 0.01 mg/ml) using colorimetric method *in vitro*. The present study revealed provide the first evidence for the inhibition of the aqueous ethanolic extract of *C. asiatica* on porcine pancreatic lipase activity (IC_{50} = 48.05 ± 0.08 mg/ml) using the titrimetric method *in vitro*. The present study found that the aqueous ethanolic extract of *C. asiatica* was less potent inhibition of pancreatic lipase than the study of Adisakwattana *et al*. (2012). Orlistat was reported to inhibit porcine pancreatic lipase activity by 95.7% at a concentration of 250 µg/ml using the colorimetric method (Zhang *et al*., 2008). In the present study, orlistat at a concentration of 40 mg/ml could inhibit pancreatic lipase activity by 75.45% using titrimetric method. At the same concentration, the aqueous ethanolic extract of *C. asiatica* and rutin were less potent than orlistat on inhibitory porcine

pancreatic lipase activity. The application of rutin at a concentration of 100 µg/ml could suppress porcine pancreatic lipase activity (30.8%) using colorimetric method (Zheng *et al*., 2010). The results from this study suggested that the aqueous ethanolic extract of *C. asiatica* may be an alternative medicinal plant to orlistat which has side effects such as oily stools, oily spotting, and flatulence others (Birari and Bhutani, 2007). The present findings suggested that the aqueous ethanolic extract of *C. asiatica* and rutin could supress fat digestion and absorption by inhibitory pancreatic lipase activity.

In conclusion, the aqueous ethanolic extract of *C. asiatica* had a potential to be used as lipase inhibitor agent due to its ability to inhibit pancreatic lipase activity. Rutin might be the active compound that was responsible for the inhibitory activity against pancreatic lipase of the aqueous ethanolic extract of *C. asiatica*. Further investigation is needed to study mechanisms of the aqueous ethanolic extract of *C. asiatica* on hypertriglyceridemia *in vivo*.

3.7 References

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CHAPTER IV

EFFECTS OF THE AQUEOUS ETHANOLIC EXTRACT OF *CENTELLA ASIATICA* ON PANCREATIC ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE ACTIVITIES *IN VITRO*

4.1 Abstract

Obesity increases risk for many disorders that are associated with high mortality and morbidity, especially diabetes that have abnormal high blood sugar level (hyperglycemia). The one possibility of lowering postprandial glucose level is the inhibition of alpha-amylase and alpha-glucosidase activities which leads to decrease carbohydrate digestion and absorption. There has been an enormous interest in the development of alternative medicines for type 2 diabetes mellitus, specifically screening for phytochemicals with the ability to delay or prevent glucose absorption. *Centella asiatica* (*C. asiatica*) is a medicinal plant and consists of active compounds such as rutin and quercetin which can suppress the level of blood glucose. Therefore, the aim of study was to investigate the inhibitory effects of the aqueous ethanolic extract from *C. asiatica* on pancreatic alpha-amylase and alpha-glucosidase activities *in vitro*. The present study revealed that *C. asiatica* extract and rutin could inhibit pancreatic alpha-amylase and alpha-glucosidase activities. The concentrations of the

aqueous ethanolic extract of *C. asiatica* and rutin required to inhibit 50% of alpha-amylase activity (IC_{50}) were 2.14 and 1.53 mg/ml, respectively. The concentrations of the aqueous ethanolic extract of *C. asiatica* and rutin required to inhibit 50% of alpha-glucosidase activity (IC_{50}) were 0.02 and 0.081 mg/ml, respectively. Inhibition of pancreatic alpha-amylase and alpha-glucosidase activities delay carbohydrate digestion and glucose absorption causing reduction of blood glucose levels (hypoglycemia). Hence, the aqueous ethanolic of *C. asiatica* extract and rutin may exert their hypoglycemic effect by pancreatic alpha-amylase and alpha-glucosidase inhibition activities. Rutin may be responsible for their anti-diabetic action the aqueous ethanolic extract of *C. asiatica*. Further investigation is needed to clarify the effect of aqueous ethanolic extract of *C. asiatica* on reduction of postprandial hyperglycemia *in vivo*.

4.2 Introduction

Obesity is associated with insulin resistance and hyperinsulinemia which can develop type 2 diabetes mellitus (Weyer *et al*., 2001). Impairment multiple biochemical mechanisms are associated with micro- and macrovascular complications which are major causes of morbidity and death in diabetes mellitus (Berger, Stenstrom, and Sundkvist, 1999). Anti-diabetic drugs that can control blood sugar level such as acarbose, miglitol, and voglibose are commonly used for treatment of diabetes mellitus (Kim *et al*., 2005). However, the modern medicines available for management of diabetes have serious side effects such as hepatotoxicity, abdominal pain, flatulence, and diarrhea (Fujisawa *et al*., 2005; Singh *et al*., 2008). After prolonged treatment, drug resistance to these medicines has been reported. Many

medicinal plants have been recommended for diabetic treatment (Grover, Yadav, and Vats, 2002). Traditional herbal medicines have been widely used throughout the world for a range of diabetes (Odhav *et al*., 2010).

Diabetes mellitus is a metabolic disorder characterized by high blood glucose level resulting from defects in insulin production, insulin action, or both. One therapeutic approach for treating diabetes mellitus is to decrease the postprandial hyperglycemia. In particular, inhibition of alpha-amylase and alpha-glucosidase emzyme activities involved in the digestion of carbohydrate can decrease the postprandial increase of blood glucose after a mixed carbohydrates diet which is the important strategy in the management of postprandial blood glucose level in type 2 diabetes mellitus (Ali, Houghton, and Soumyanath, 2006; Lee *et al*., 2007). An anti-diabetic drug, acarbose is a pseudotetrasaccharide that inhibits intestinal alpha-glucosidase reversibly at the brush border of intestinal mucosa. Consequently, the transformation of disaccharides to monosaccharides is prevented. The uptake of monosaccharides is retarded and thus postprandial insulin and glucose levels are reduced (Hillebrand *et al*., 1979). Currently, there are renewed interest in functional foods and medicinal plants modulating physiological effects in the prevention and treatment of diabetes and obesity. Numerous studies demonstrated alpha-amylase and alpha-glucosidase inhibitory activities of many plant extracts such as cranberry (Apostolidis, Kwon, and Shetty, 2006), *Andrographis paniculata* (Subramanian, Asmawi, and Sadikun, 2008), *Carica papaya*, *Manihot esculenta*, *Cosmos caudatus*, and *Centella asiatica* (Loh and Hadira, 2011) *in vitro*. Natural alpha-amylase and alpha-glucosidase inhibitors from plant sources offer an attractive strategy for the control of postprandial hyperglycemia such as *Gnidia glauca* and *Dioscorea bulbifera*

(Ghosh *et al*., 2012), *Punica granatum* (Li *et al*., 2005), *Acorus calamus* (Si *et al*., 2010), and pine bark extract (Kim *et al*., 2005).

Centella asiatica (*C. asiatica*) is a medicinal plant in tropical and subtropical countries that possess antioxidant, anticancer, hypotensive effects, wound healing, antigastric ulcer activities, and memory improvement (Zheng and Qin, 2007). *C. asiatica* contains active compounds such as triterpenoids (James and Dubery, 2009), and flavonoids (rutin, quercetin, catechin, naringin, luteolin, and keampherol) (Zainol *et al*., 2009). Rutin is a flavonoid glycoside that is synthesized in plants (Vogrincic *et al*., 2010). Rutin is capable of inhibition of alpha-amylase (Kim, Kwon, and Son, 2000) and alpha-glucosidase activities (Li *et al*., 2009). In *in vitro* study, rutin could inhibit pancreatic alpha-amylase activity but could not suppress alpha-glucosidase activity (Kim *et al*., 2000). Rutin could inhibit alpha-glucosidase activity but could not suppress on alpha-amylase activity (Jo *et al*., 2009). Rutin isolated from *Tribulus terrestris* and tartary buckwheat had alpha-amylase and alpha-glucosidase inhibitory activities (Li *et al*., 2009; Ye *et al*., 2010). In *in vivo* study, glucose tolerance was also significantly improved in streptozotocin-induced diabetic rats treated with rutin (Rauter *et al*., 2010).

There were many reports of alpha-amylase and alpha-glucosidase inhibitory activities of *C. asiatica* extract *in vitro*. The hexane and dichloromethane extract of *C. asiatica* could inhibit alpha-amylase and alpha-glucosidase activities *in vitro* (Loh *et al*., 2011). The aqueous extract of *C. asiatica* could inhibit alpha-amylase activitiy (Odhav *et al*., 2010). Moreover, the aqueous and methanolic extracts of *C. asiatica* had alpha-glucosidase inhibitory activity (Mai *et al*., 2007). However, the aqueous ethanolic extract of *C. asiatica* on alpha-amylase and alpha-glucosidase inhibitory

activities has never been demonstrated. Therefore, the present study aimed to investigate the effects of the aqueous ethanolic extract of *C. asiatica* and rutin on pancreatic alpha-amylase and alpha-glucosidase activities *in vitro*.

4.3 Materials and methods

4.3.1 Plant material

The aqueous ethanolic extract of *C. asiatica* obtained from stock extract in chapter III was used in the experiments conducted in this chapter.

4.3.2 Chemicals

Porcine pancreatic alpha-amylase (500 KU), yeast alpha-glucosidase (28 U/mg protein), dinitrosalicylic acid (DNS), L-glutathione, 4-nitrophenyl-alpha-D-glucopyranoside (PNPG) and rutin trihydrate were purchased from Sigma Aldrich Chemical Ltd. (St. Louis, USA). Potato starch was purchased from Carlo Erba Ltd. (Italy). Acarbose (Glucobay) was purchased from Bayer Ltd. (USA). All other chemicals were of reagent grade.

4.3.3 Materials for determination of pancreatic alpha-amylase enzyme activity

1 M Phosphate buffer solution (pH 7.4)

1 M Phosphate buffer solution (pH 7.4) was prepared by adding 10.65 g of di-sodium hydrogen phosphate anhydrous (Na_2HPO_4; BDH Ltd., UK) and 3.968 g of sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4.H_2O$; BDH) to 80 ml of double deionized distilled (DDD) water. This solution was stirred on a hot plate

magnetic stirrer (VELP Scientifica, Europe) at 50-60 °C for 1 h, left to be cool down to room temperature and then adjusted volume to 100 ml with DDD water in a volumetric flask. This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

20 mM Sodium phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride

Sodium phosphate buffer (20 mM) was prepared by adding 0.0392 g of sodium chloride (Sigma) to 2 ml of 1 M PB (pH 7.4) and then added 98 ml of DDD water. This solution was then adjusted volume to 100 ml with DDD water in a volumetric flask and then adjusted to pH 6.9 with 1 M HCl or 1 M NaOH.

Alpha-amylase enzyme solution

Alpha-amylase enzyme solution (0.01% w/v) was prepared by adding 0.01 g of porcine pancreatic alpha-amylase (Type VI-B, 500 KU; Sigma) to 100 ml of ice-cold 20 mM sodium phosphate buffer (pH 6.9) containing 6.7 mM sodium chloride.

Starch solution

Starch solution (1% w/v) was prepared by boiling and stirring 1 g of potato starch (Carlo Erba Reagents) in 60 ml of DDD water for 15 min. This solution was adjusted volume to 100 ml with DDD water in volumetric flask.

2 M Sodium hydroxide

Sodium hydroxide (2 M) was prepared by mixing 8 g of sodium hydroxide (NaOH; Carlo Erba Reagents) with 90 ml of DDD water. This solution was adjusted volume to 100 ml with DDD water in a volumetric flask.

96 mM 3, 5-Dinitrosalicylic acid (DNS)

3, 5-Dinitrosalicylic acid (96 mM) was prepared by mixing 0.438 g of DNS ($C_7H_4N_2O_7$; Sigma) with 20 ml of DDD water.

Color reagent solution

Color reagent solution was prepared by mixing 12 g of potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6$. $4H_2O$; Sigma) with 8 ml of sodium hydroxide (2 M) and then added 20 ml of 96 mM DNS. This solution was prepared by using hot plate magnetic stirrer (VELP Scientifica, Europe) at 45-50 °C until this solution was melted. After then, this solution was added by 12 ml of DDD water and was kept in amber bottle.

4.3.4 Assay for pancreatic alpha-amylase inhibitory activity

Alpha-amylase enzyme activity was determined by colorimetric method using starch as a substrate. The current method was adapted from the method of Ghosh *et al*. (2012). The aqueous ethanolic extract *C. asiatica* and rutin were dissolved in DDD water to give concentrations ranging from 0.38 to 24.62 mg/ml. Fifty microliters of each *C. asiatica* extract and rutin concentrations and 50 µl of alpha-amylase enzyme solutions were mixed and incubated at 25 °C for 30 min. After

that, 100 µl of starch solution was added to 100 µl of this mixture and incubated at 25 ^{o}C for 3 min. Then, 100 µl of color reagent solution was added. The mixture was then placed into an 85 ^{o}C water bath. Fifteen minutes later, the mixture was removed from the water bath and cooled to room temperature. Thereafter, 900 µl of DDD water was added. Maltose released from PNPG was detected at 540 nm using microplate spectrophotometer (Benchmark plus, Japan). Individual blanks were prepared as mentioned above without porcine pancreatic alpha-amylase. Acarbose was used as positive control at a concentration of 10 mg/ml (Subramanian *et al.*, 2008). The inhibition percentage of alpha-amylase activity was assessed by the following formula:

$$\text{Inhibition (\%)} = 100 \times (\Delta A_{Control} - \Delta A_{Sample})/\Delta A_{Control}$$

$$\Delta A_{Control} = A_{Test1} - A_{Blank1}$$

$$\Delta A_{Sample} = A_{Test2} - A_{Blank2}$$

where A_{Test1} and A_{Test2} were defined as the absorbance of DDD water and sample with alpha-amylase enzyme. A_{Blank1} and A_{Blank2} were defined as the absorbance of DDD water and sample without alpha-amylase enzyme.

4.3.5 Materials for determination of alpha-glucosidase enzyme activity

Alpha-glucosidase enzyme solution

Alpha-glucosidase enzyme solution (3 U/ml) was prepared by adding 4.89 mg solid of alpha-glucosidase (Type I) (Sigma) from Baker's yeast to 34.23 ml of ice-cold DDD water. This enzyme was aliquoted into separate microtubes and stored at -80 ^{o}C. To make 5 ml of 0.3 U/ml alpha-glucosidase enzyme solution, 0.5 ml of 3 Unit/ml alpha-glucosidase enzyme was added to 4.5 ml of DDD water.

1 M Potassium phosphate buffer (pH 6.8)

Potassium phosphate buffer (1 M) was prepared by adding 1.361 g of potassium phosphate monobasic (KH_2PO_4; Sigma) to 90 ml of DDD water. This solution was adjusted volume to 100 ml with DDD water in a volumetric flask. This solution was then adjusted to pH 6.8 with 1 M HCl or 1 M NaOH.

67 mM Potassium phosphate buffer (pH 6.8)

Potassium phosphate buffer (67 mM) was prepared by 6.7 ml of 1 M potassium phosphate buffer was added to 90 ml of DDD water. This solution was adjusted volume to 100 ml with DDD water in a volumetric flask. This solution was then adjusted to pH 6.8 with 1 M HCl or 1 M NaOH.

10 mM 4-Nitrophenyl-alpha-D-glucopyranoside (PNPG) solution

4-Nitrophenyl-alpha-D-glucopyranoside solution (10 mM) was prepared by mixing 0.0046 g of 4-Nitrophenyl-alpha-D-glucopyranoside (Sigma) with 5 ml of DDD water.

3 mM L-glutathione (GSH) solution

L-glutathione solution (3 mM) was prepared by mixing 0.151 g of L-glutathione (Sigma) with 5 ml of DDD water.

0.1 M Sodium carbonate solution

Sodium carbonate solution (0.1 mM) was prepared by mixing 1.06 g of sodium carbonate ($NaCO_3$; BDH) with 100 ml of DDD water.

4.3.6 Assay for alpha-glucosidase inhibitory activity

A colorimetric method for the determination of alpha-glucosidase activity using PNPG as a substrate was modified from Si *et al*. (2010). The mixture consisted of 25 µl of 3 mM reduced glutathione solution, 250 µl of 0.067 M potassium phosphate buffer (pH 6.8), and 25 µl of alpha-glucosidase solution (0.3 U/ml). The mixture was then added with 25 µl of each concentration of *C. asiatica* extract and rutin which dissolved in DDD water to give concentrations ranging from 0.01 to 0.30 mg/ml. The mixture was incubated at 37 ^{o}C for 10 min. After that, 25 µl of 10 mM PNPG was added to initiate the enzyme reaction and incubated at 37 ^{o}C for 10 min. The reaction was stopped by 400 µl of 0.1 M sodium carbonate solution. Individual blanks were prepared as mentioned above without alpha-glucosidase. Acarbose was used as positive control at a concentration of 10 mg/ml (Subramanian *et al*., 2008). *p*-Nitrophenol released from PNPG was detected at 400 nm using microplate spectrophotometer (Benchmark plus, Japan). The inhibition percentage of alpha-glucosidase activity was assessed by the following formula:

$$\text{Inhibition (\%)} = (1\text{-}A_x/A_0) \times 100$$

where A_0 represented the absorbance of DDD water and sample with alpha-glucosidase enzyme. A_x represented the absorbance of DDD water and sample without alpha-glucosidase enzyme.

4.4 Statistical analysis

All the analyses were carried out in triplicate and the results were expressed in mean ± standard error of mean (S.E.M). The difference between rutin and the aqueous ethanolic extract of *C. asiatica* within the same concentrations and the difference between orlistat and rutin or the aqueous ethanolic extract of *C. asiatica* were analyzed using one-way ANOVA following by the Tukey test (Sigmastat version 3.5). *P*-value less than 0.05 ($P<0.05$) was considered statistically significant.

4.5 Results

The present study demonstrated that both the aqueous ethanolic extract of *C. asiatica* and rutin had alpha-amylase and alpha-glucosidase inhibitory activities. Inhibitory effects of rutin and the aqueous ethanolic extract of *C. asiatica* on pancreatic alpha-amylase and alpha-glucosidase activities *in vitro* were shown in Figures 4.1 and 4.2. In this study, IC_{50} value of rutin and the aqueous ethanolic extract of *C. asiatica* on inhibition of alpha-amylase activities were 1.53 and 2.14 mg/ml, respectively. Significant different between the inhibitory pancreatic alpha-amylase activities of the aqueous ethanolic extract of *C. asiatica* and rutin at the concentrations ranging from 0.38 to 6.16 mg/ml compared acarbose (0.77 mg/ml). Rutin (24.62 mg/ml) and the aqueous ethanolic extract of *C. asiatica* (12.31 and 24.62 mg/ml) had no significant inhibitory pancreatic alpha-amylase activities compared with acarbose. No significant different between the inhibitory pancreatic alpha-amylase activities of the aqueous ethanolic extract of *C. asiatica* and rutin showed at the concentrations ranging from 0.38 to 24.62 mg/ml, while the aqueous ethanolic extract of *C. asiatica*

was less potent than rutin on inhibitory pancreatic alpha-amylase at a dose of 3.08 mg/ml.

At the concentration ranging from 0.01 to 0.30 mg/ml, both the aqueous ethanolic extract of *C. asiatica* and rutin exhibited alpha-glucosidase inhibitory activity in a dose-dependent manner. The IC_{50} value of alpha-glucosidase activities from rutin and the aqueous ethanolic extract of *C. asiatica* were 0.081 and 0.02 mg/ml, respectively. Within the studied concentration range, the aqueous ethanolic extract of *C. asiatica* exhibited stronger inhibition on alpha-glucosidase activities than rutin at a dose of 0.02 to 0.15 mg/ml. At the concentration of rutin (0.01 to 0.15 mg/ml) and the aqueous ethanolic extract of *C. asiatica* (0.01 to 0.02 mg/ml) were less potent than acarbose on alpha-glucosidase. Inhibitory alpha-glucosidase at a dose of 0.30 mg/ml, rutin and the aqueous ethanolic extract of *C. asiatica* had no significant when compared with acarbose.

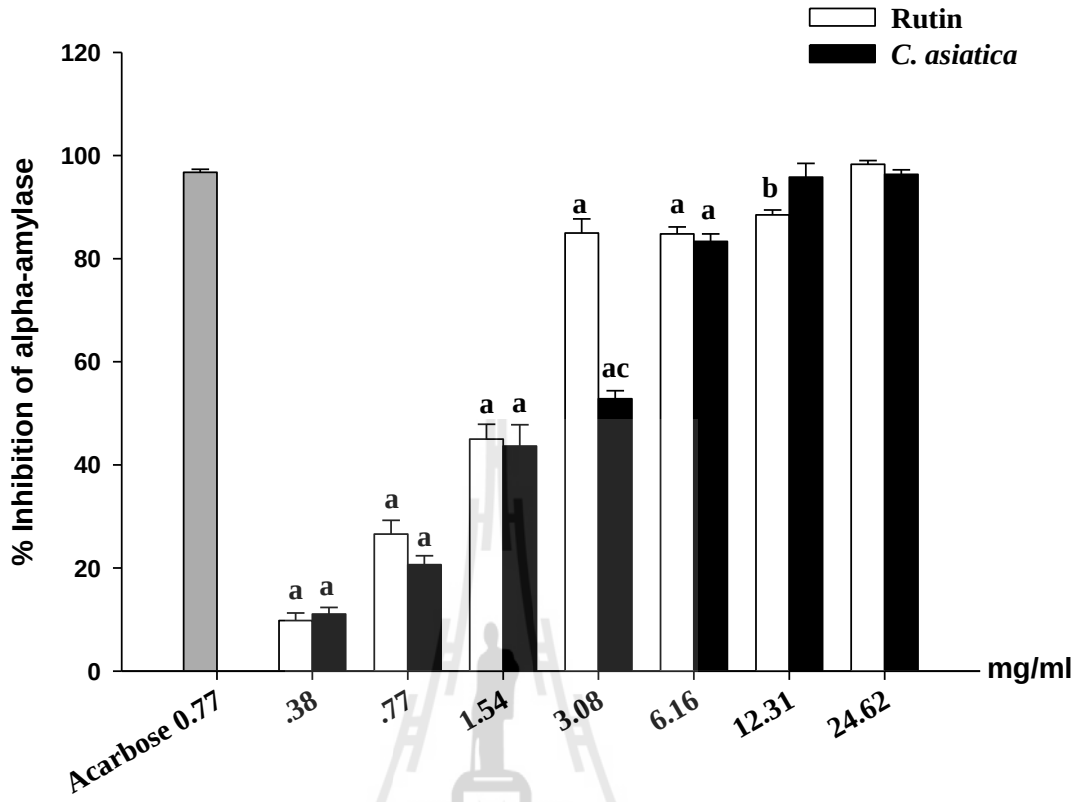

Figure 4.1 Inhibitory effects of the aqueous ethanolic of *C. asiatica* extract and rutin on pancreatic alpha-amylase activity *in vitro*. The results were expressed as mean ± S.E.M. of three independent experiments. a and b compared to acarbose (P<0.001 and P<0.01, rspectively). c, P<0.001 compared to rutin at the same concentration.

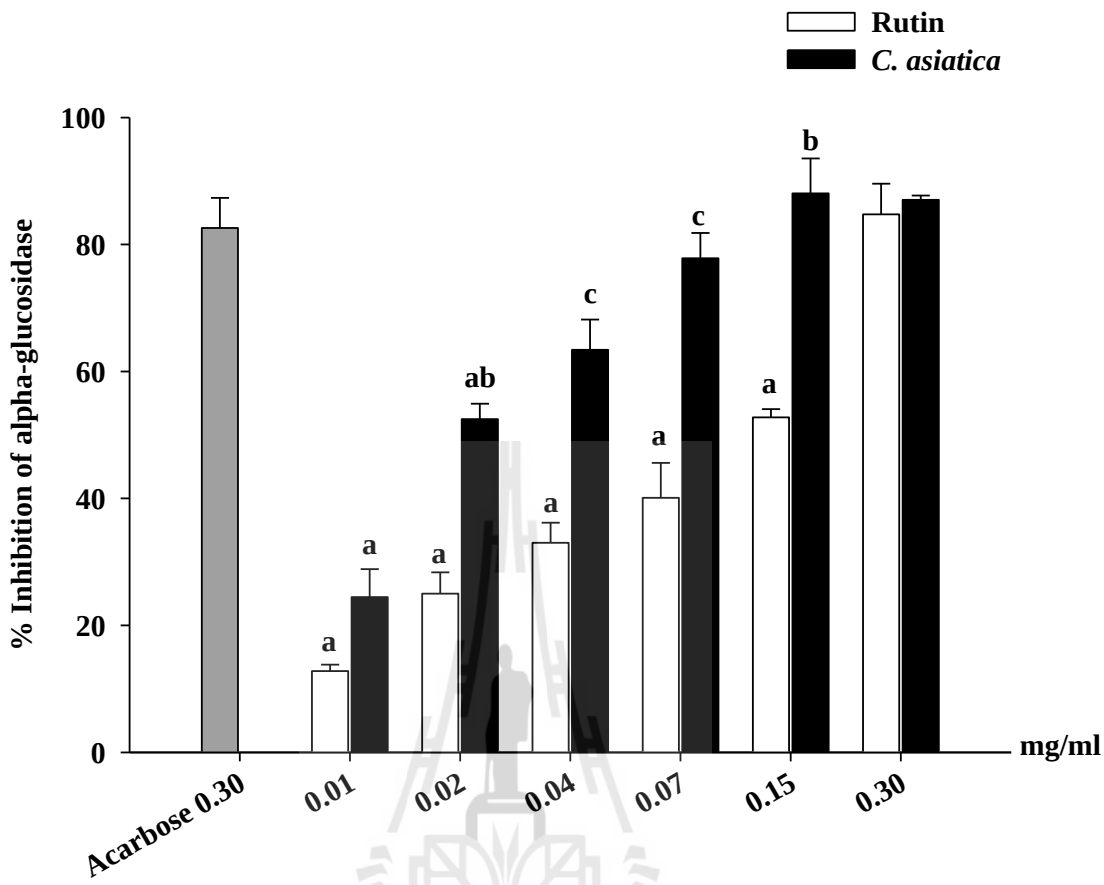

Figure 4.2 Inhibitory effects of the aqueous ethanolic of *C. asiatica* extract and rutin on alpha-glucosidase activity *in vitro*. The results were expressed as mean ± S.E.M. of three independent experiments. a, $P<0.001$ compared to acarbose. b and c compared to rutin at same concentration ($P<0.01$ and $P<0.001$, respectively).

Table 4.1 The IC_{50} values for rutin and the aqueous ethanolic extract of *C. asiatica* on alpha-amylase and alpha-glucosidase enzyme activities.

Samples	**IC_{50} (mg/ml)**	
	alpha-amylase	**alpha-glucosidase**
Rutin	1.53 ± 0.05	0.081 ± 0.004
The aqueous ethanolic extract of *C. asiatica*	2.14 ± 0.04	0.02 ± 0.009

Concentration of samples to inhibit 50% of its activity (IC_{50})

4.6 Discussion and conclusion

Obesity associated with type 2 diabete mellitus (Pi-Sunyer, 2002). Diabetes mellitus has been defined as a chronic disease with elevated blood glucose concentration which leads to chronic complications (Balamurugan and Ignacimuthu, 2011). The one possibility of lowering postprandial glucose level is the inhibition of alpha-amylase and alpha-glucosidase activities which retard the digestion of carbohydrates (Tundis, Loizzo, and Menichini, 2010). The inhibition of alpha-amylase and alpha-glucosidase by pharmaceutical agents such as acarbose and voglibose are accepted clinical strategy in the management of blood glucose level in the diabetic patients (Lo Piparo *et al*., 2008). However, these drugs have undesirable side effects, causing hypoglycemia at higher doses, liver problems, and diarrhea. Currently, there is renewed interest in medicinal plants with less side effects and effectiveness for the treatment of diabetes mellitus which has less side effects and effectiveness (Tundis *et al*., 2010). The effect of *C. asiatica* extract by different extraction solvents on the activities of alpha-amylase and alpha-glucosidase were previously demonstrated. The hexane and dichloromethane extract of *C. asiatica* could inhibit the alpha-amylase and

alpha-glucosidase activities (Loh *et al*., 2011). The aqueous and methanolic extracts from *C. asiatica* showed that restricted on alpha-glucosidase activity (15% and 21%, respectively) (Mai *et al*., 2007). The aqueous extract of *C. asiatica* had 98.87% inhibition alpha-amylase activity at the concentration of 5 mg/ml (Odhav *et al*., 2010). The present study demonstrated the first evidence of pancreatic alpha-amylase and alpha-glucosidase inhibitory activities of the aqueous ethanolic extract from *C. asiatica* extract *in vitro*. The aqueous ethanolic extract of *C. asiatica* was more potent than rutin on inhibitory alpha-glucosidase activities, while the aqueous ethanolic extract of *C. asiatica* was less potent than acarbose on inhibitory alpha-amylase activities. Both the aqueous ethanolic extract from *C. asiatica* extract and rutin exerted pancreatic alpha-amylase and alpha-glucosidase inhibitory activities in a dose-dependent manner. These findings suggested that the aqueous ethanolic extract of *C. asiatica* may possess anti-diabetic activity by inhibiting pancreatic alpha-amyalse and alpha-glucosidase activities. However, the reaction mechanism involved in inhibition of pancreatic alpha-amylase and alpha-glucosidase enzymes by *C. asiatica* are not clearly understood.

C. asiatica contains active compounds such as flavonoid (rutin, catechin, and quercetin) and triterpenoid (Hussin *et al*., 2009; Zainol *et al*., 2003; Zheng *et al*., 2007) that have potential to prevent hyperglycemia. Rutin could inhibit the activities of alpha-amylase and alpha-glucosidase inhibitory *in vitro* (Jo *et al*., 2010; Kim *et al*., 2000; Li *et al*., 2009). Ye *et al*. (2010) revealed that rutin isolated from *Tribulus terrestris* had alpha-amylase and alpha-glucosidase inhibitory activities *in vitro*. In *in vivo* study, rutin could reduce the blood glucose level in streptozotocin-induced diabetic rats (Rauter *et al*., 2010). The possible mechanism for hypoglycemic of rutin

is the inhibition of alpha-amylase and alpha-glucosidase enzymes which can reduce the postprandial increase of blood glucose after a mixed carbohydrate diet (Tucci, Boyland, and Halford, 2010). Inhibition these enzymes causes delayed digestion prolonged digestion, thus the rate of glucose absorption is reduced. As a consequence, the postprandial rise in blood glucose levels and insulin resistane is reduced (Creutzfeldt, 1999). Rutin (100 mg/kg, p.o.) could decrease plasma glucose and increase insulin secretion levels in streptozotocin-induced diabetic rats (Stanley Mainzen Prince and Kamalakkannan, 2006). Polyphenols are important phytochemicals and flavonoids are member of the polyphenol group. Polyphenol could reduce postprandial blood glucose levels and improve insulin secretion and insulin sensitivity. The possible mechanisms of polyphenols on hypoglycemic effects include inhibition of carbohydrate digestion and glucose absorption in the intestine, stimulation of insulin secretion from the pancreatic β-cells, modulation of glucose release from the liver, and stimulation of insulin receptor and glucose uptake insulin-sensitive tissues (Hanhineva *et al*., 2010).

In conclusion, the aqueous ethanolic extract of *C. asiatica* has potential to be used as hypoglycemic agent due to strong inhibitory effects of the aqueous extract of *C. asiatica* on pancreatic alpha-amylase and alpha-glucosidase activities *in vitro*. Rutin may be an active compound found in the aqueous ethanolic of *C. asiatica* extract that responsible for inhibitory activities against pancreatic alpha-amylase and alpha- glucosidase. Further studies are needed to elucidate hypoglycemic effects of the aqueous ethanolic of *C. asiatica* extract *in vivo*.

4.7 References

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CHAPTER V

EFFECTS OF THE AQUEOUS ETHANOLIC EXTRACT OF *CENTELLA ASIATICA* ON PLASMA BIOCHEMICAL PARAMETERS IN LIPID EMULSION-INDUCED HYPERLIPIDEMIC RATS

5.1 Abstract

This study was performed to clarify the effects of the aqueous ethanolic extract of *C. asiatica* and rutin on plasma levels of triglyceride (TG), total cholesterol (TC), glucose, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in lipid emulsion-induced hyperlipidemic rats. Male Wistar rats were administered an oral lipid emulsion alone (4 ml/kg), lipid emulsion with orlistat (45 mg/4 ml/kg), lipid emulsion with the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/4 ml/kg) or lipid emulsion with rutin (1000 mg/4 ml/kg). The plasma TG, TC, glucose, AST, and ALT level were measured at 0, 1, 2, 3, and 4 h later. The aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/4 ml/kg) and rutin (1000 mg/4 ml/kg) appeared to inhibit an increase levels of plasma TG and TC at 3 h after lipid emulsion administration. Plasma glucose levels were decreased after administration of rutin and the aqueous ethanolic extract of *C. asiatica*. Furthermore, the plasma AST and ALT levels had no change after oral administration lipid emulsion alone, lipid emulsion with orlistat or the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/

4 ml/kg) at 1, 2, 3, and 4 h. In present study, no significant decrease in the plasma TG, TC, and glucose levels following the aqueous ethanolic extract of *C. asiatica* compared with either orlistat or rutin. Both the aqueous ethanolic extract of *C. asiatica* and rutin had anti-obesity and anti-diabetic effects which could suppress the increase plasma TG and TC levels in lipid emulsion-induced hyperlipidemic rats. The aqueous ethanolic extract of *C. asiatica* had no toxicity on liver. Therefore, the aqueous ethanolic extract of *C. asiatica* is a beneficial for lipid-lowering and glucose-lowering effects. Rutin may be the key active compound responsible for hypolipidemic and hypoglycemic effects of *C. asiatica*.

5.2 Introduction

Obesity is commonly associated with insulin resistance and hyperinsulinemia which is a major risk factor for the development of type 2 diabetes (Weyer *et al*., 2001). Obesity is associated with an important risk factor for liver inflammation (Rodriguez-Hernandez *et al*., 2011). In order to manage obesity and diabetic conditions, anti-obesity drug (orlistat) is widely used which can inhibit the absorption of fat and excrete of ingested fat leading to weight loss (Shi *et al*., 2005) and anti-diabetic drug (acarbose) is a pseudotetrasaccharide that inhibits intestinal alpha-glucosidase activity at the brush border of intestinal mucosa which reduce the uptake of monosaccharides (Tucci, Boyland, and Halford, 2010). However, these drugs (orlistat and acarbose) have undesirable side effects such as flatulence and diarrhoea which its use may be limited (Chiasson *et al*., 2002; Kang and Park, 2012). Therefore, there were new alternatives for obesity therapy. Certain medicinal plants for anti-obesity properties such as jasmine green tea (Chan *et al*., 1999), grape seed (Moreno *et*

al., 2003), and longan flower (Yang *et al*., 2010) which consisted of flavonoids. Flavonoids are naturally occurring substances in plants which have been exhibited a wide range of biological effects such as antioxidant (Peng *et al*., 2003), anticancer (Li *et al*., 2007), hypoglycemic (Rauter *et al*., 2010), and hypolipidemic (Feng *et al*., 2011) activities.

Flavonoids, especially rutin is a flavanol type of flavonoids (Kuntic *et al*., 2007) which is found in many typical plants and has several pharmacological properties such as hypolipidemic (Santos *et al*., 1999; Ziaee *et al*., 2009) and hypoglycemic effects (Rauter *et al*., 2010). In *in vitro* studies, rutin was reported to have pancreatic lipase inhibitory activity (Zheng *et al*., 2010), alpha-amylase inhibitory activity (Kim, Kwon, and Son, 2000) and alpha-glucosidase inhibitory activity (Gao *et al*., 2008). In *in vivo* study, rutin alone or in combination with lovastatin (hypolipidemic agent) mixed in a high-cholesterol diet significantly reduced plasma levels of total cholesterol (TC) and low density lipoprotein (LDL), and also markedly decreased liver enzymes and body weight in rats (Ziaee *et al*., 2009). Furthermore, the addition of rutin or *0*-coumaric acid to the diet could decrease body weight gain, liver weight, adipose tissue weight, triglyceride (TG), phospholipid, TC, insulin, and leptin in high fat diet (HFD)-induced obese rats (Hsu *et al*., 2009). Rutin suppressed the elevation of serum TC and TG levels and increased high density lipoprotein (HDL) level after oral administration triton-induced hyperlipidemic rats (Santos *et al*., 1999). In streptozotocin induced diabetic rats, 7 days treatment of rutin (4 mg/kg, i.p.) caused a reduction in blood glucose levels, and an increase in alanine aminotransferase (ALT) levels, but did not cause any change in aspartate aminotransferase (AST) levels (Rauter *et al*., 2010).

C. asiatica contains flavonoid such as rutin (Ariffin *et al*., 2011; Hussin *et al*., 2009; Mohd Zainol *et al*., 2009). Hussin *et al*. (2009) demonstrated that the leaves of methanolic extract of *C. asiatica* had 1138.6 ± 7.7 mg/kg of rutin. *C. asiatica* has been used for the treatment of life-style related diseases such as hypertension, tumor, and memory disorders (Zheng and Qin, 2007). The results from chapter III and chapter IV demonstrated that 80% ethanolic extract of *C. asiatica* could inhibit porcine pancreatic lipase, pancreatic alpha-amylase, and alpha-glucosidase activities *in vitro* (Figures 3.1, 4.1, and 4.2). It has been demonstrated the dicholomethane and hexane extracts of *C. asiatica* on alpha-amylase and alpha-glucosidase enzyme inhibitory activities *in vitro* (Loh *et al*., 2011). The aqueous extract of *C. asiatica* also had alpha-amylase inhibitory activities *in vitro* (Odhav *et al*., 2010). Furthermore, both aqueous and methanolic extracts of *C. asiatica* inhibited alpha-glucosidase activity *in vitro* (Mai *et al*., 2007). The previous study reported that the maximum reduction in blood glucose was observed after 3 h oral administration the methanolic and ethanolic extracts of the leaves from *C. asiatica* in alloxan induced diabetic rats (Chauhan *et al*., 2010). Thus, *C. asiatica* extract may process anti-obesity and anti-diabetic activities *in vivo*. The effects of aqueous ethanolic extract from *C. asiatica* and rutin on plasma TG, TC, glucose, AST, and ALT levels were to investigate in lipid emulsion-induced hyperlipidemic rats. Rutin was selected to study since it might be responsible for anti-obesity and anti-diabetic effects of *C. asiatica*.

5.3 Materials and methods

5.3.1 Plant material

C. asiatica extract obtained from stock extract in chapter III was used in the experiments conducted in this chapter.

5.3.2 Animals

Male Wistar rats (250 ± 4.13 g) were obtained from Institutional Animal Care, SUT. They were maintained under standard laboratory conditions (12:12 h dark-light cycle, ambient temperature 20 ± 1 ^{o}C) with free access to food and water. This study was conducted under of the SUT Animal Care and Use Committee.

5.3.3 Measurement of plasma triglyceride, total cholesterol, glucose, alanine animotransferase, and aspartate amino transferase levels after oral administration of lipid emulsion to rats.

The rats were orally administered by either lipid emulsion (20% Intralipid, Sino-Swed, China, 4 ml/kg, n=8) served as control, lipid emulsion with orlistat (45 mg/4 ml/kg, n=8), lipid emulsion with rutin (1000 mg/4 ml/kg, n=8), lipid emulsion with *C. asiatica* extract (1000 mg/4 ml/kg, n=8) or lipid emulsion with of *C. asiatica* extract (2000 mg/4 ml/kg, n=8). The lipid emulsion composed of purified soybean oil (200 g), purified egg phospholipids (12 g), and glycerol anhydrous (22 g). Blood samples were collected from the tail vein at 0, 1, 2, 3, and 4 h after the oral administration of each treatment and centrifuged at 5000 × g for 5 min. Plasma triglyceride (TG), total cholesterol (TC), glucose, aspatate aminotransferase, and

alanine aminotransferase (ALT) were determined using an automatic blood analyzer (Hitachi 911, Japan).

5.4 Statistical analysis

Data were expressed as the mean ± standard error of mean (S.E.M.). To analyse plasma levels of TG, TC, glucose, AST, and ALT differences between groups and times were analyzed using a two-way repeated measures analysis of variance (ANOVA) following by the Tukey test. $P<0.05$ and $P<0.01$ were considered statistically significant, respectively.

5.5 Results

To clarify the effects of the aqueous ethanolic extract of *C. asiatica* and rutin were orally administered to rats, and the serial changes in plasma TG, TC, glucose, AST, and ALT levels were measured. Plasma TG response to a single oral dose of lipid emulsion (4 ml/kg) alone or lipid emulsion with orlistat (45 mg/4 ml/kg), lipid emulsion with either rutin (1000 mg/4 ml/kg), or aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/4 ml/kg) were shown in Figure 5.1. Significant increases in plasma TG levels were found at 1, 2, and 3 h after oral administration of the lipid emulsion alone (88.00 ± 15.12, 97.17 ± 13.71, and 118.80 ± 8.83 mg/dl, respectively) compared with 0 h (65.06 ± 5.5 mg/dl). The incremental TG levels following lipid emulsion alone reached a peak at 3 h. At 3 h, the elevation of the plasma TG levels induced by lipid emulsion were significantly reduced by orlistat, rutin, and the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/4 ml/kg). In comparison to the rats treated lipid emulsion with orlistat, there was no significant

difference in the TG levels of the rats treated lipid emulsion either rutin or the aqueous ethanolic extract of *C. asiatica* at 3 h.

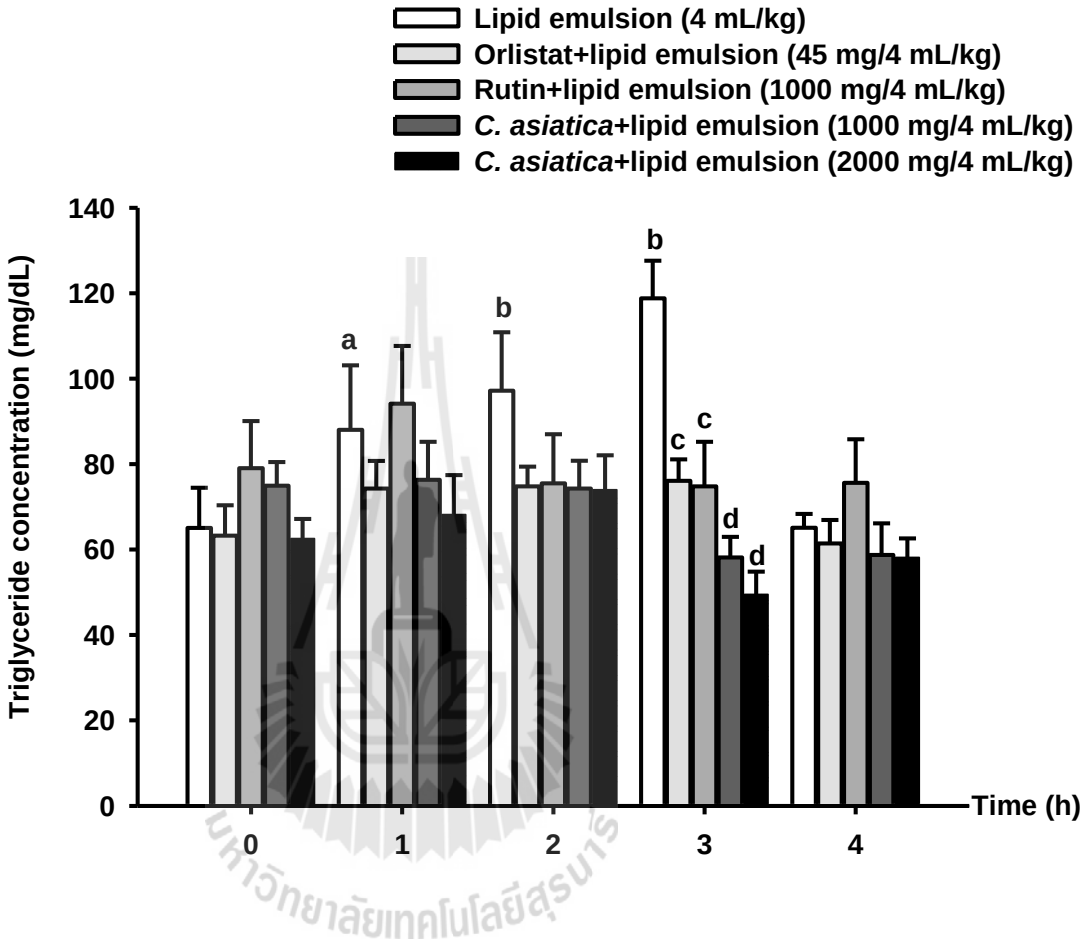

Figure 5.1 Plasma levels of TG after oral administration of lipid emulsion alone, lipid emulsion with either orlistat, rutin or the aqueous ethanolic extract of *C. asiatica*. The results were expressed as mean ± S.E.M of 8 rats. a and b compared to 0 h within the group ($P<0.05$ and $P<0.001$, respectively). c and d compared to lipid emulsion treated group within time ($P<0.05$ and $P<0.001$, respectively).

Changes in plasma TC levels following single oral dose of lipid emulsion alone (4 ml/kg) or lipid emulsion with either orlistat (45 mg/4 ml/kg), rutin (1000 mg/4 ml/kg) or the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/ 4 ml/kg) were shown in Figure 5.2. An increase in plasma TC levels with a peak at 3 h was found in lipid emulsion alone group. In lipid emulsion orally administered group, plasma TC levels 3 h (90.6 ± 1.82 mg/dl) were significantly higher than 0 h (65.74 ± 2.78 mg/dl). At 3 h, increased plasma TC levels including lipid emulsion were significantly decreased by orlistat, rutin, and *C. asiatica* extract (1000 and 2000 mg/ 4 ml/kg). There was no significant difference in the TC levels of the rats treated lipid emulsion either rutin or the aqueous ethanolic extract of *C. asiatica* when compared to the rats treated lipid emulsion with orlistat at 3 h.

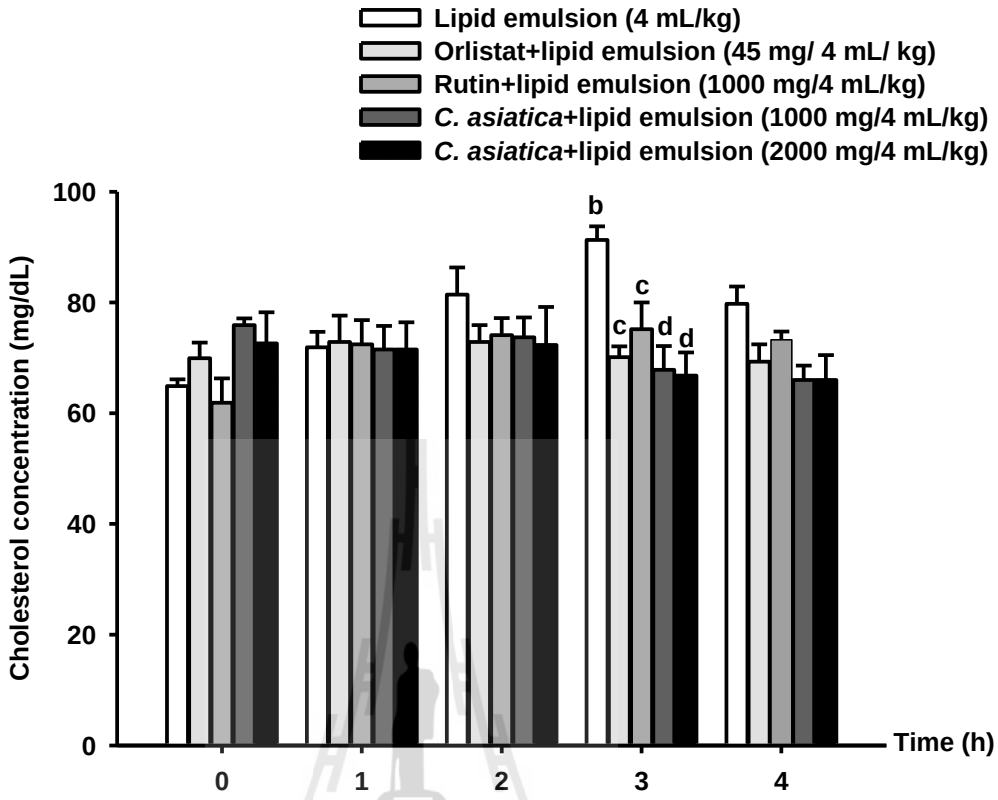

Figure 5.2 Plasma levels of TC after oral administration of lipid emulsion alone, lipid emulsion with either orlistat, rutin or the aqueous ethanolic extract of *C. asiatica*. The results were expressed as mean ± S.E.M of 8 rats. b compared to 0 h with in the group (P<0.001). c and d compared to lipid emulsion within group within time (P<0.05 and P<0.001, respectively).

Plasma glucose levels after oral administration of lipid emulsion alone or lipid emulsion with either orlistat, rutin or *C. asiatica* extract (1000 and 2000 mg/kg b.w.) were shown in Figure 3. There was no change in plasma glucose levels following lipid emulsion alone compared to 0, 1, 2, 3, and 4 h. Significant decreases in plasma glucose levels were found at 3 h after oral administration of lipid emulsion with *C. asiatica* extract (1000 and 2000 mg/kg b.w.) compared to 0 h within group (p<0.05 and p<0.001). Plasma glucose level was significantly lower in the rat groups that were

orally administered by lipid emulsion with rutin and *C. asiatica* extract (2000 mg/kg b.w.) compared with the lipid emulsion alone at 3 h. Significant decreases in plasma glucose levels were found at 4 h after lipid emulsion with either orlistat or *C. asiatica* extract (1000 mg/kg b.w.) compared to 0 h within the group ($p<0.05$). However, there was no significant difference in plasma glucose levels of the rats treated lipid emulsion with *C. asiatica* extract (1000 and 2000 mg/kg b.w.) when compared to the rats treated lipid emulsion with either orlistat or rutin at 3 and 4 h.

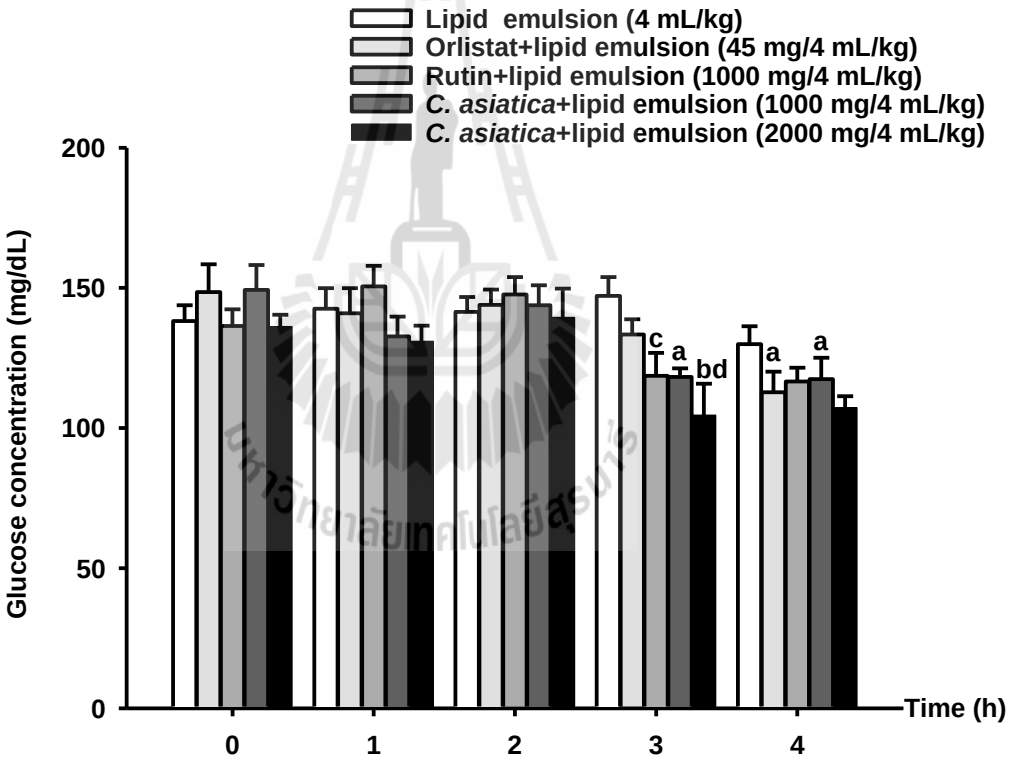

Figure 5.3 Plasma levels of glucose after oral administration of lipid emulsion alone, lipid emulsion with either orlistat, rutin or the aqueous ethanolic extract of *C. asiatica*. The results were expressed as mean ± S.E.M of 8 rats. a and b compared to lipid emulsion treated group within time ($P<0.05$ and $P<0.001$), respectively. c and d compared to 0 h within the group ($P<0.05$ and $P<0.001$, respectively).

Plasma AST and ALT levels were determined as an evaluation of hepatic function. Plasma levels of AST after oral administration of lipid emulsion alone or lipid emulsion with either orlistat, rutin or the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/4 ml/kg) were shown in Figure 5.4. Within 4 h, the plasma levels of AST were not change after oral administration of lipid emulsion alone, lipid emulsion with either orlistat, rutin or the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/4 ml/kg).

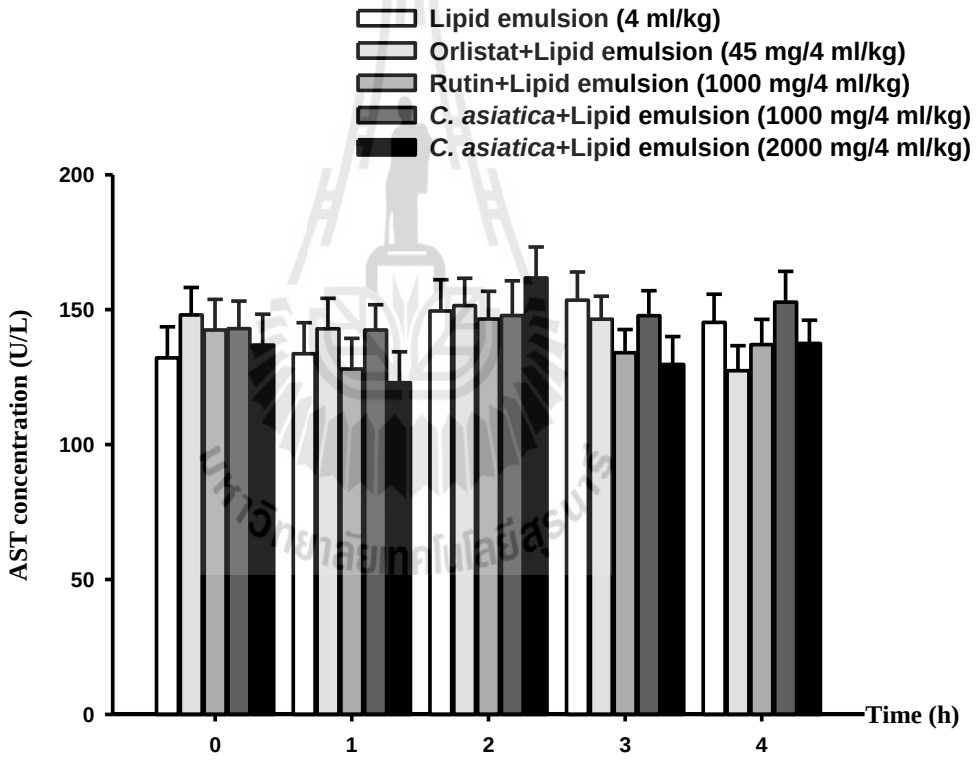

Figure 5.4 Plasma levels of AST after oral administration of lipid emulsion alone, lipid emulsion with either orlistat, rutin or the aqueous ethanolic extract of *C. asiatica*. The results were expressed as mean ± S.E.M of 8 rats.

The effects of the aqueous ethanolic extract of *C. asiatica* on plasma ALT after oral administration of lipid emulsion alone or lipid emulsion with either orlistat, rutin or the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/4 ml/kg) were shown in Figure 5.5. No significant change was noted in ALT levels at 1, 2, 3, and 4 h when compared with 0 h after oral administration of lipid emulsion alone or lipid emulsion with either orlistat, rutin or aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/4 ml/kg).

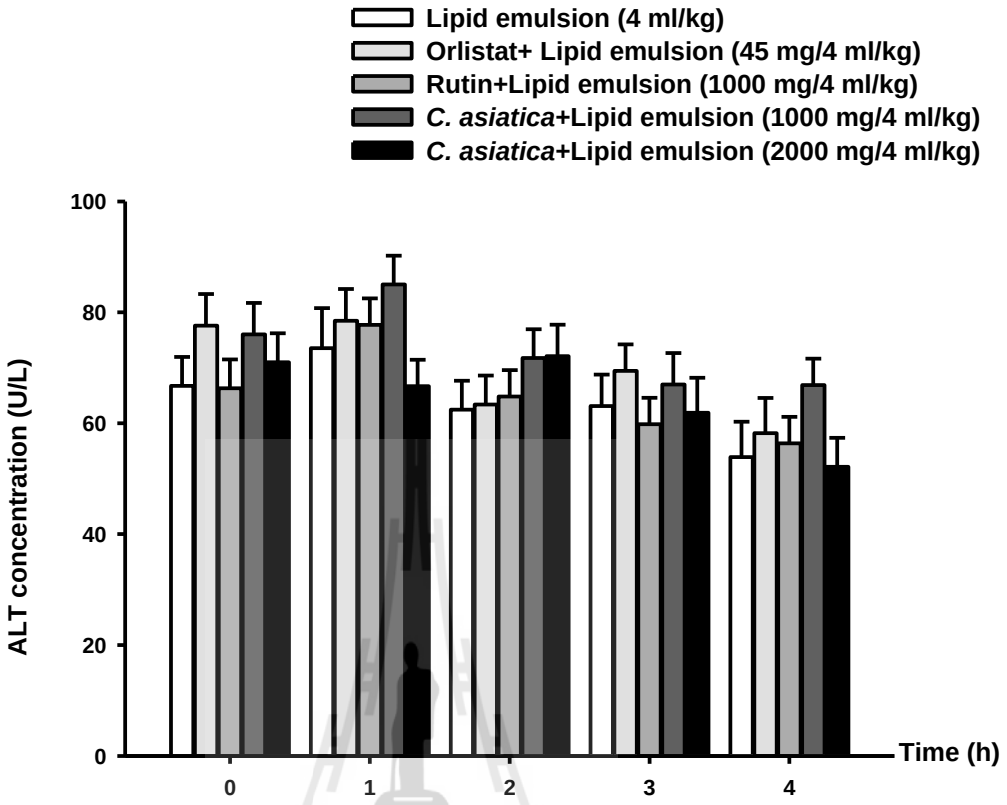

Figure 5.5 Plasma levels of ALT after oral administration of lipid emulsion alone, lipid emulsion with either orlistat, rutin or the aqueous ethanolic extract *C. asiatica*. The results were expressed as mean ± S.E.M of 8 rats.

5.6 Discussion and conclusion

Flavonoids, especially rutin, had potent antihyperlipidemic and antihyperglycemic effects (Rauter *et al*., 2010; Santos *et al*., 1999). The result of this study showed that the aqueous ethanolic extract of *C. asiatica* contained rutin. Therefore, the present study was carried out in order to determine the effects of aqueous ethanolic extract of *C. asiatica* and rutin on postprandial plasma TG, TC, glucose, AST, and ALT levels in the lipid emulsion-induced hyperlipidemic rats. The present study provided the first evidence the TG and TC levels induced by lipid

emulsion were significantly decreased by orlistat (positive control) rutin and the aqueous ethanolic extract of *C. asiatica* when compared with the control group at 3 h (Figures 5.1 and 5.2). There was no significant difference in the TG and TC levels of the rats treated lipid emulsion either rutin or the aqueous ethanolic extract of *C. asiatica* when compared with lipid emulsion with orlistat group at 3 h. The present findings were consistent with the previous study of Han *et al*., 2005. The plasma TG level was significantly reduced by orlistat (the final concentration 45 mg/ml/kg) when compared with the control group at 2 and 3 h (Han *et al*., 2005). The results of both *in vitro* studies in chapter III and *in vivo* study in this chapter supported the view that the aqueous ethanolic extract of *C. asiatica* and rutin could prevent lipid emulsion and lipase interactions. The inhibition of pancreatic lipase by the aqueous ethanolic extract of *C. asiatica* and rutin could restrict fat digestion by suppression the hydrolysis of TG contained in lipid emulsion and could inhibit absorption of these TG which reduce the plasma levels of TG in lipid emulsion-induced hyperlipidemic rats. The supplementation of rutin (1000 mg/kg) in diet containing cholesterol could reduce plasma and hepatic cholesterol, and promote the excretion of fecal sterols in rats (Park *et al*., 2002). The supplementation of rutin (100 mg/kg) in high-cholesterol diet could reduce the plasma TC and LDL levels in rats (Ziaee *et al*., 2009). Moreover, mixing high fat diet with rutin which was isolated from *Dimorphandra mollis* caused a lowering effect on plasma TG levels, but did not cause any change in plasma TC and HDL levels in Golden Syrian hamsters (Kanashiro *et al*., 2009). Santos *et al*. (1999) demonstrated that rutin could reduce the serum TC and TG levels in triton-induced hypolipidemic rats. The most likely mechanism of action of rutin may be the conversion of rutin to quercetin. Quercetin was one of the metabolite of rutin that

decreased HMG-CoA reductase activity, decreased absorption of dietary cholesterol, and increased in fecal sterols (Bok *et al*., 2002).

In the present study, the aqueous ethanolic extract of *C. asiatica* and rutin could suppress the elevation of plasma glucose level. The result of this study showed that orlistat could reduce the plasma glucose level. Orlistat (120 mg) mixed in a high-cholesterol fat diet could decrease plasma LDL, TC, and glucose, and could increase plasma HDL in hypercholesterolemic patients (Lucas, Boldrin, and Reaven, 2003). The possible mechanisms for the effect of orlistat on hypoglycemic may involve in a reduction of plasma non-esterified fatty acid concentration, improvement of insulin sensitivity, and stimulation of glucagon-like peptide-1 (GLP-1) secretion in the lower small intestine (Jabob *et al*., 2009). A reduction in fat absorption and an increase in intestinal fat content by orlistat may lead to increase secretion of gut hormones, including GLP-1 which stimulates insulin secretion (Mancini and Halpern, 2008). Glucose-lowering effects of *C. asiatica* extract have been demonstrated in alloxan-induced diabetic rats models in 2010 (Chunchan *et al*., 2010). A significant reduction of the blood glucose level at 1, 3, and 5 h following the ethanolic and methanolic extract of *C. asiatica* (250 mg/kg) administration were demonstrated in alloxan-induced diabetic rats compared with untreated group (Chunchan *et al*., 2010). Hypoglycemic effects of the aqueous ethanolic extract of *C. asiatica* and rutin reported in this chapter may be due to their inhibition effects on alpha-amylase and alpha-glucosidase enzyme activities. Inhibition of these enzymes can retard the rate of carbohydrate digestion and glucose absorption in the intestine and reduce the postprandial increase of blood glucose after a mixed carbohydrate diet (Tundis *et al*., 2010). Polyphenols attenuated the levels of blood glucose which could inhibit of

carbohydrate digestion and glucose absorption in the intestine, stimulate insulin secretion from the pancreatic β-cells, modulate glucose release from liver, activate insulin receptors and glucose uptake in the insulin-sensitive tissues, and modulation of hepatic glucose output (Hanhineva *et al*., 2010). Moreover, rutin could decrease hyperglycemia since inhibit the pancreatic islets, increase the insulin secretion, decrease gluconeogenesis, and increase glycolysis after treatment with rutin in the diabetic rats (Stanley Mainzen Prince and Kamalakkannan, 2006). The result from chapter IV suggested that the aqueous ethanolic extract of *C. asiatica* and rutin were potent inhibitory of alpha-amylase and alpha-glucosidase *in vitro* which lead to a reduction in the intestinal absorption of glucose in hypoglycemia *in vivo*, resulting in a reduction of plasma glucose as shown in this chapter. Natural alpha-amylase and alpha-glucosidase inhibitors in the aqueous ethanolic extract of *C. asiatica* have been demonstrated to be beneficial in reducing postprandial hyperglycemia by slowing down the digestion of carbohydrates and the absorption of glucose.

Reduction of postprandial hyperglycemia by alpha-amylase and alpha-glucosidase inhibitors could prevent glucose uptake into adipose tissue which turn in inhibit synthesis and accumulation of TG (Maury *et al*., 1993). The inhibition of digestion and absorption of dietary fat and carbohydrate can prevent obesity (Tucci *et al*., 2010). The aqueous ethanolic extract of *C. asiatica* and rutin are ideal natural sources to induce anti-obesity effects. Anti-obesity effects of the aqueous ethanolic extract of *C. asiatica* are mainly attributed to its polyphenol content especially for rutin. Rutin might be the active compound responsible for hypoglycemic and hypolipidemic effects of the aqueous ethanolic extract of *C. asiatica*. However, the underlying mechanisms are needed to investigate.

Both AST and ALT are enzymes produced by liver cells which were released when liver cell damaged (Patil, Somashekarappa, and Rajashekhar, 2011). The level of plasma glucose was reduced by lipid emulsion with either orlistat or the aqueous ethanolic extract of *C. asiatica*. Rauter *et al*. (2010) demonstrated that rutin increased ALT levels after 7 days administration of rutin (4 mg/kg, i.p.) in streptozotocin–induced diabetic rats. The possible mechanism of the effect of orlistat on the reduction of ALT levels may be related to a reduction in plasma free fatty acid (FFA) levels, a reduction in FFA flux into the liver, and an increase in hepatic insulin sensitivity (Zelber-Sagi *et al*., 2006). The result of this study demonstrated that orlistat, rutin and the aqueous ethanlic extract of *C. asiatica* had no changed the levels of plasma AST and ALT. The previous studies declared that orlistat could suppress the increase of FFA levels which reduced a characteristic response of liver to the proinflammatory cytokine tumor necrosis factor-α (TNF-α) which protected hepatocyte injury and affecting the integrity of liver cells. (Amin and Nagy, 2009; Rodriguez-Hernandez *et al*., 2011; Vozarova *et al*., 2002). Zhang *et al*. (2010) demonstrated that asiaticoside, a triterpenoid product isolated from *C. asiatica*, could protect lipopolysaccharide/D-galactosamine-induced liver injury in mice since asiaticoside could decrease serum TNF-α and suppress the increases of AST and ALT levels.

In addition, the present findings showed that increased levels of TG, TC, and glucose in rats received lipid emulsion were reduced by the aqueous ethanolic extract of *C. asiatica* (2000 mg/4 ml/kg). Therefore, the aqueous ethanolic extract of *C. asiatica* is a beneficial for lipid-lowering and glucose-lowering effects and the aqueous ethanolic extract of *C. asiatica* is not toxicity on liver which could reduce

ALT levels. Rutin may be the key active compound responsible for hypolipidemic and hypoglycemic effects of *C. asiatica*.

5.7 References

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CHAPTER VI

EFFECTS OF THE AQUEOUS ETHANOLIC EXTRACT OF *CENTELLA ASIATICA* ON FOS EXPRESSION IN THE BRAIN AREAS REGULATING HOMEOSTASIS OF ENERGY BALANCE IN MALE WISTAR RATS

6.1 Abstract

There is no evidence to suggest anti-obesity effects of aqueous ethanolic extract of *C. asiatica* and rutin through central effects, especially for the hypothalamus and brainstem. The hypothalamus and brainstem are major brain regions that play an importance role in regulation of energy balance *via* autonomic nervous control of intake and energy expenditure. Hence, this study investigated the effects of the aqueous ethanolic extract of *C. asiatica* and rutin on Fos expression in the brain areas involving a regulation of energy homeostasis which are the paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), arcuate nucleus (ARC), lateral hypothalamic area (LHA), and perifornical area (PeF) that are neuronal nuclei in the hypothalamus and the nucleus of solitary tract in the brainstem. Forty rats were divided into five groups of eight rats. Rats were fasted overnight and then orally given single dose of the aqueous ethanolic extract of *C. asiatica* (1000 or 2000 mg/ml/kg), rutin (1000 or 1500 mg/ml/kg) or double deionized distilled (DDD) water (1 ml/kg). Two hours later, rats were decapitated. Brains were removed and

hypothalamus was assessed for expression of Fos, a marker for neuronal activation, using immunocytochemistry. The present findings demonstrated the first evidence that the aqueous ethanolic extract of *C. asiatica* and rutin could induce Fos expression in the PVN and ARC of hypothalamus but could not induce Fos expression in other studied areas. The aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/ml/kg) and rutin (1500 mg/ml/kg) significantly increased the number of Fos-positive neurons in the ARC compared to the control group ($P<0.05$). Fos expression in the ARC was predominantly in the lateralposterior region. In the PVN, the aqueous ethanolic extract of *C. asiatica* (1000 mg/ml/kg) significantly increased the number of Fos-positive neurons when compared with the control group ($P<0.05$). Neuronal activation in the PVN and ARC of the hypothalamus by *C. asiatica* and rutin suggested that the aqueous ethanolic extract of *C. asiatica* and rutin may involve in regulation of food intake and energy homeostasis through neuronal activity in the ARC and PVN of the hypothalamus. Further studies are required to clarify which neuronal types of the PVN are activated by the aqueous ethanolic extract of *C. asiatica*.

6.2 Introduction

Understanding the neural mechanisms underlying the regulation of appetite and the control of energy expenditure by the central nervous system is becoming important in obesity research because the neuronal system originating from various hypothalamic nuclei regulates feeding behavior and metabolic processes (Wilding, 2002). Many hypothalamic nuclei and brainstem are important in the regulation of food intake and energy homeostasis, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), lateral

hypothalamic area (LHA), and perifonical area (PeF) of the hypothalamus and the nucleus of the solitary tract (NTS) of the brainstem (Lawrence, Turnbull, and Rothwell, 1999; Schwartz *et al*., 2000; Valassi *et al*., 2008). The ARC consists of neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons which increase feeding (orexigenic action) and pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons which reduce feeding (anorexigenic action) (Schwartz, 2000; Valassi *et al*., 2008). These neurons project to the PVN, PeF, and LHA (Simpson, Martin, and Bloom, 2009; Stanley *et al*., 2005). The PVN consists of parvocellular (thyrotropin releasing hormone (TRH) and corticotropin releasing hormone (CRH)) and magnocellular (oxytocin) neurons that produce anorexigenic peptides (Remmers and Delemarre-van de Waal, 2011). Moreover, the parvocellular neuron contains NPY (orexigenic peptide) (Schwartz, 2000). The LHA and PeF contain neuropeptides (orexin and melanin-concentrating hormone (MCH)) that increase feeding (Harrold *et al*., 2012; Schwartz *et al*., 2000; Stanley *et al*., 2005). Apart from central of food intake, peripheral control can also be achieved by integration between satiety signals and adiposity signals. Satiety signals, including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), originate from the gastrointestinal tract during a meal and, through the vagus nerve, reach the NTS in the caudal brainstem (Schwartz *et al*., 2000; Valassi *et al*., 2008). When adiposity signals, leptin and insulin, reach the ARC, alpha-melanocyte-stimulating hormone (α-MSH) are released by anorexigenic neurons (POMC/CART neuron) which activates a catabolic circuit. In contrast, NPY and AgRP are released by orexigenic neurons (anabolic pathway) which occur when adiposity signal concentrations in the brain are low (Baskin *et al*., 1999; Schwartz *et al*., 2000).

Long term HFD could decrease the number of neurons carrying α-MSH and CART peptide in the ARC of the rat hypothalamus (Tian *et al*., 2004). The effect of dietary fats on c-Fos-like immunoreactivity in mouse hypothalamus was demonstrated. Expression of the immediate early gene *c-fos* and its protein product Fos has been extensively used to map stimulus-evoked functional activity in the brain (Nikolaev *et al*., 2002). Therefore, Fos has been used as neuronal activation marker that can be identified by immunohistochemistry to be located in the nuclei of neurons (Bullitt, 1990). The c-Fos immunoreactivity neurons were markedly increased in the dorsal lateral hypothalamus area, while in the VMH was decreased by saturated fat feeding for 1 week. The c-Fos immunoreactivity neurons in the PVN were also increased in high saturated fat fed mice at week 7 and week 11 (Wang, Storlien, and Huang, 1999). Some plants extract (such as adlay seed and Korean red ginseng) with anti-obesity activity in periphery could exhibit their actions centrally. The study of Kim *et al* (2007) demonstrated that NPY expression in the PVN, levels of leptin receptor expression, and levels of TG and TC in HFD-induced obese rats treated with adlay seed extract were lower than in control HFD-induced obese rats. The hypothalamic NPY expression and serum leptin level, and weight of perirenal and peritoneal fat in HFD-induced obese rats treated crude saponin of Korean red ginseng (CS) were lower than in control HFD-induced obese rats (Kim *et al*., 2005). *C. asiatica* is a medicinal plant which consists of flavonoids (such as rutin, quercetin, and catechin) which has potential to prevent obesity (Hussin *et al*., 2009; Zainol *et al*., 2003). However, there is no evidence about the aqueous ethanolic extract of *C. asiatica* and rutin effects on the brain areas which involve energy homeostasis. Therefore, this study was focused on the effect of the aqueous ethanolic extract of

C. asiatica and rutin on Fos expression, a marker for neuronal activation, in the PVN, VMH, ARC, PeF, and LHA which are a neuronal nucleus in the hypothalamus, and the nucleus of the solitary tract of the brainstem. These areas involve in a regulation of food intake and energy homeostasis.

6.3 Materials and methods

6.3.1 Plant material

The aqueous ethanolic extract of *C. asiatica* obtained from stock extract in chapter III was used in the experiments conducted in this chapter.

6.3.2 Animals

Male Wistar rats (247.88 ± 2.76 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 ^{o}C) with free access to food and water. This study was conducted under permit of the SUT Animal Care and Use Committee.

6.3.3 Chemicals

0.9% Normal saline solution

0.9% Normal saline solution was prepared by adding 9 g of sodium chloride (NaCl; Sigma Chemical Co., St. Louis, MO, USA) to 900 ml of deionized distilled (DI) water. This solution was adjusted volume to 1000 ml with DI water in a volumetric flask.

Heparinised saline solution

Heparinised saline (5 IU/ml) solution was prepared by adding 1 ml of a 5000 units/ml heparin solution (LEO Phamaceutical product, Ballerup, Denmark) to 999 ml of 0.9% normal saline solution.

1 M Phosphate buffer solution (pH 7.4)

1 M Phosphate buffer solution (pH 7.4) was prepared by adding 106.5 g of di-sodium hydrogen phosphate anhydrous (Na_2HPO_4; BDH Ltd., UK) and 39.68 g of sodium dihydrogen orthophosphate 1-hydrate ($NaH_2PO_4.H_2O$; BDH) to 800 ml of double deionized distilled (DDD) water. This solution was stirred on a hot plate magnetic stirrer (VELP Scientifica, Europe) at 50-60 ^oC for 1 h, left to be cool down to room temperature and then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH. This solution was adjusted volume to 1000 ml with DDD water in a volumetric flask.

0.1 M Phosphate buffer solution (pH 7.4)

0.1 M Phosphate buffer solution (pH 7.4) was prepared by adding 100 ml of 1 M phosphate buffer solution (pH 7.4) to 900 ml of DI water. This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

5 M Sodium chloride solution

5 M Sodium chloride solution was prepared by adding 292.2 g of sodium chloride (NaCl; Sigma) to 600 ml of DDD water. This solution was then adjusted volume to 1000 ml with DDD water in volumetric flask.

0.1 M Phosphate buffer saline (PBS) solution (pH 7.4)

0.1 M Phosphate buffered saline solution was prepared by adding 30 ml of 5 M sodium chloride to 970 ml of 0.1 M phosphate buffer (pH 7.4). This solution was then adjusted volume to 1000 ml with DDD water in volumetric flask. This solution was then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

4% Paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4)

4% Paraformaldehyde solution was prepared by adding 40 g of paraformaldehyde (Acros Organics, New Jersey, USA) to 900 ml of 0.1 M phosphate buffer solution (pH 7.4). This solution was stirred on a hot plate magnetic stirrer (VELP Scientifica, Europe) at 150-200 ºC for 1-2 h, left to be cool down to room temperature and then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH. This solution was adjusted volume to 1000 ml with DI water in a volumetric flask.

30% Sucrose in 4% PFA in 0.1 M phosphate buffer (pH 7.4)

30% Sucrose in 4% PFA in 0.1 M phosphate buffer solution was prepared by adding 30 g of sucrose ($C_{12}H_{22}O_{11}$; Ajax Finechem Pty Ltd., Australia) to 60 ml of 4% PFA in 0.1 M phosphate buffer. This solution was then adjusted volume to 100 ml with 4% PFA in 0.1 M phosphate buffer in volumetric flask (pH 7.4). This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

30% Sucrose in 0.1 M phosphate buffer (pH 7.4)

30% Sucrose in 0.1 M phosphate buffer solution was prepared by adding 30 g of sucrose to 60 ml of 0.1 M phosphate buffer. This solution was then adjusted

volume to 100 ml with 0.1 M phosphate buffer in volumetric flask (pH 7.4). This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

Anti-freeze cryoprotectant solution

Anti-freeze cryoprotectant solution was prepared by mixing 30 ml of 0.1 M phosphate buffer (pH 7.4), 30 g of sucrose, and 30 ml of ethanediol ($C_2H_6O_2$; Ajax Finechem) and then 1 g of polyvinylprrolidone (C_6H_9NO, PVP-40; Sigma) was added. This solution was adjusted volume to 100 ml with 0.1 M phosphate buffer (pH 7.4) water in volumetric flask. This solution was adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

0.1 M Washing solution (PBS-T) solution

0.1 M washing solution was prepared by adding 3 ml of 0.3% v/v Triton X-100 (Panreac Analytical Reagent and Fine Chemical, Spain) to 95 ml of 0.1 M phosphate buffer saline (pH 7.4). This solution was adjusted volume to 100 ml with 0.1 M phosphate buffer saline (pH 7.4) water in volumetric flask. This solution was then adjusted to pH 7.4 with 1 M HCl or 1 M NaOH.

3% Hydrogen peroxide solution

3% Hydrogen peroxide solution was prepared by 79 ml of 0.1 M phosphate buffer saline (pH 7.4) adding 1 ml of hydrogen peroxide (H_2O_2; Merck Schuchardt OHG., Hohenbrunn, Germany) and 20 ml of methanol (CH_4O; BDH). This solution was prepared immediately prior to use.

Preincubation buffer

Preincubation buffer was a prepared by adding 5 ml of normal goat serum (Millipore Corperation, USA) to 95 ml of washing solution (0.1 M PBS-T).

Primary antibody

Rabbit polyclonal anti-Fos (*c-fos* Ab-5, Calbiochem; USA) was diluted 1:5,000 in preincubation buffer.

Secondary antibody

Secondary antibody was a washing solution containing 1% v/v biotinylated anti-rabbit immunoglobulin and 3% v/v normal goat serum (Vectastain Elite ABC Kit rabbit IgG's, Vector Laboratories, Burlingame, USA).

Avidin-biotinylated horseradish peroxidase complex (ABC)

ABC solution was a washing solution containing 2% v/v Avidin and 2% v/v biotinylated horseradish peroxidase (Vectastain Elite ABC Kit rabbit IgG's). This solution was left to incubate for at least 30 minutes prior to use.

0.1 M Acetate buffer (stop solution)

0.1 M Acetate buffer was prepared by adding 0.82 g of sodium acetate (CH_3COONa; BDH Ltd, UK) to 100 ml of DDD water. This solution was then adjusted to pH 6.0 with acetic acid.

Ni-DAB-H_2O_2 Solution

Ni-DAB-H_2O_2 solution was prepared by mixing 98 ml of 0.1 M of PBS solution (pH 7.4), 0.05 g of ammonium nickel (II) sulfate ($NiN_2H_8S_2O_8.6H_2O$; Sigma), 2 ml of diaminobenzidine (DAB) (3,3´-Diaminobenzidine tetrahydrochloride hydrate, $C_{12}H_{14}N_4.4HCl.H_2O$; Sigma), and 50 µl of hydrogen peroxide (H_2O_2; Merck). This solution was then filtered through No.1 Whatman filter paper (Whatman) prior to use.

Chrome alum gelatin

Chrome alum gelatin solution was prepared by adding 1.3 g of gelatine pellets (Ajax, Australia) and 0.1 g of chromium (II) potassium sulfate dodecahydrate ($CrKO_8S_2.12H_2O$; Fluka) to 1000 ml of DI water.

6.3.4 Methods

Forty rats were selected by stratified randomization and then divided into five groups of eight rats. Rats were fasted overnight and then orally given single dose of aqueous ethanolic extract of *C. asiatica* dissolved in DDD water (1000 or 2000 mg/ml/kg) or single dose of rutin (1000 or 1500 mg/ml/kg) dissolved in DDD water at a dosing volume of 1 ml/kg, while the control group received DDD water (1 ml/kg). Two hours after each treatment, all rats were anesthetized with pentobarbital sodium (Nembutal, Ceva Sante Amimale, Libourne, France) at a dose of 60 mg/kg (i.p.).

The rats were perfused through the left ventricle of the heart with 200-250 ml of ice-cold heparinized saline at a flow rate of 40 ml/min using peristaltic pump (model SP 311, VELP Scientifica, Europe). Immediately after starting the

pump, the right atrium was cut to allow an escape route for the blood and perfusion fluid. After the atrium effluent was clear, the rats were perfused with 300-350 ml of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at a flow rate of 40 ml/min to fix the brain. After that, the brain were removed and soaked in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 ^{o}C. Brains were then transferred into 30% sucrose in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight or until the brain sank at 4 ºC. The brains were then soaked in 30% sucrose in 0.1 M phosphate buffer (pH 7.4) at 4 ^{o}C for further 24 h. After that, the brains were covered with powdered dry ice until frozen and then stored at -20 ºC until sectioned.

The brains regions of the hypothalamus (PVN, LHA, VMH, PeF, and ARC and the nucleus of the solitary tract in the brainstem; Figure 6.1) were identified according to the rat brain stereotaxic atlas of Paxinos and Watson (2009). Frozen brains were coronally sectioned (30 µm thickness) through the levels of the PVN (-1.08 to -1.92 mm from bregma), LHA (-2.16 to -3.84 mm from bregma), PeF (-2.64 to -3.60 mm from bregma), ARC (-1.80 to -4.36 mm from bregma), and VMH (-1.72 to -3.36 mm from bregma) using a cryostat (Microm HM 525, Microm International GmbH., Germany). Free floating sections were then stored in an anti-freeze cryoprotectant solution at -20 ºC until further use. Frozen brainstem were coronally sectioned (40 µm thickness) through the level of the NTS (-11.96 to -15.96 mm from bregma).

Table 6.1 Abbreviations of the hypothalamic brain regions involving energy balance. Nomenclature and abbreviations are from stereotaxic atlas of the rat brain (Paxinos and Watson, 2009).

Abbreviation	**Full name**
ArcD	Arcuate hypothalamic nucleus, dorsal part
ArcL	Arcuate hypothalamic nucleus, lateral part
ArcLP	Arcuate hypothalamic nucleus, lateroposterior part
ArcM	Arcuate hypothalamic nucleus, medial part
ArcMP	Arcuate hypothalamic nucleus, medialposterior part
F	Fornix
MCLH	Magnocellular nucleus of the lateral hypothalamus
MRe 3V	Mammillary recess of the 3rd ventricle
PaAP	Paraventricular hypothalamic nucleus, anterior parvicellular part
PaDC	Paraventricular hypothalamic nucleus, dorsal cap
PaLM	Paraventricular hypothalamic nucleus, lateral magnocellular part
PaMM	Paraventricular hypothalamic nucleus, medial magnocellular part
PaMP	Paraventricular hypothalamic nucleus, medial parvicellular part
PaV	Paraventricular hypothalamic nucleus, ventral part
PeFLH	Perifornical part of the lateral hypothalamus
PeF	Perifornical nucleus
PLH	Peduncular part of the lateral hypothalamus
TuLH	Tuberal part of the lateral hypothalamus
VMHVL	Ventromedial hypothalamus, ventral part
3V	3rd ventricle

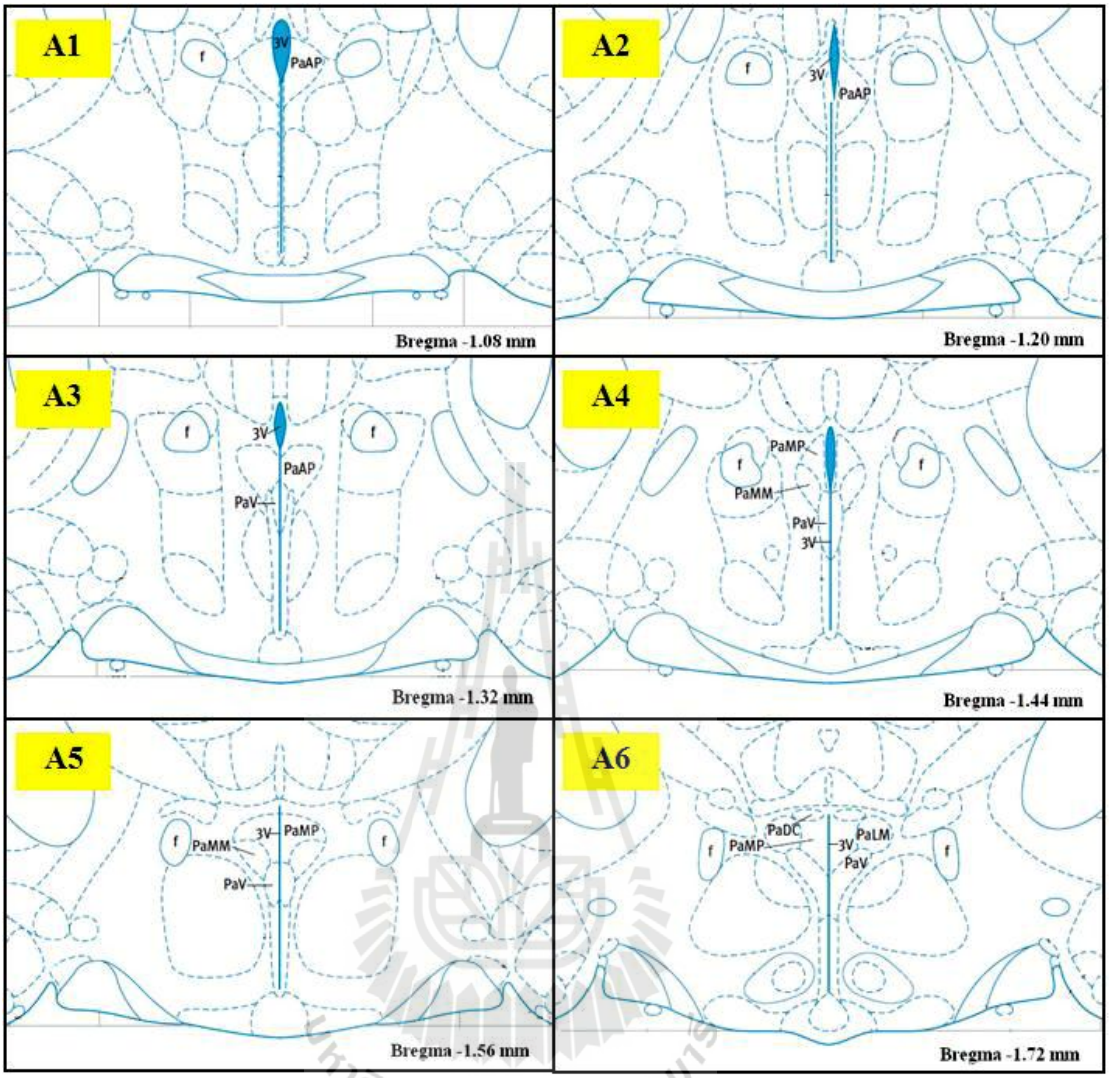

Figure 6.1 Schematic diagrams of coronal sections illustrating the brains regions of the hypothalamus. The PVN (-1.08 to -1.92 mm from bregma) from A1-A8, the ARC (-1.80 to -4.36 mm from bregma) from A7-A24, the LHA (-2.16 to -3.84 mm from bregma) from A9-A20, the PeF (-2.16 to -3.84 mm from bregma) from A9-A20, and the VMH (-1.72 to -3.36 mm from bregma) from A9-A16. Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2009). For abbreviations, see Table 6.1.

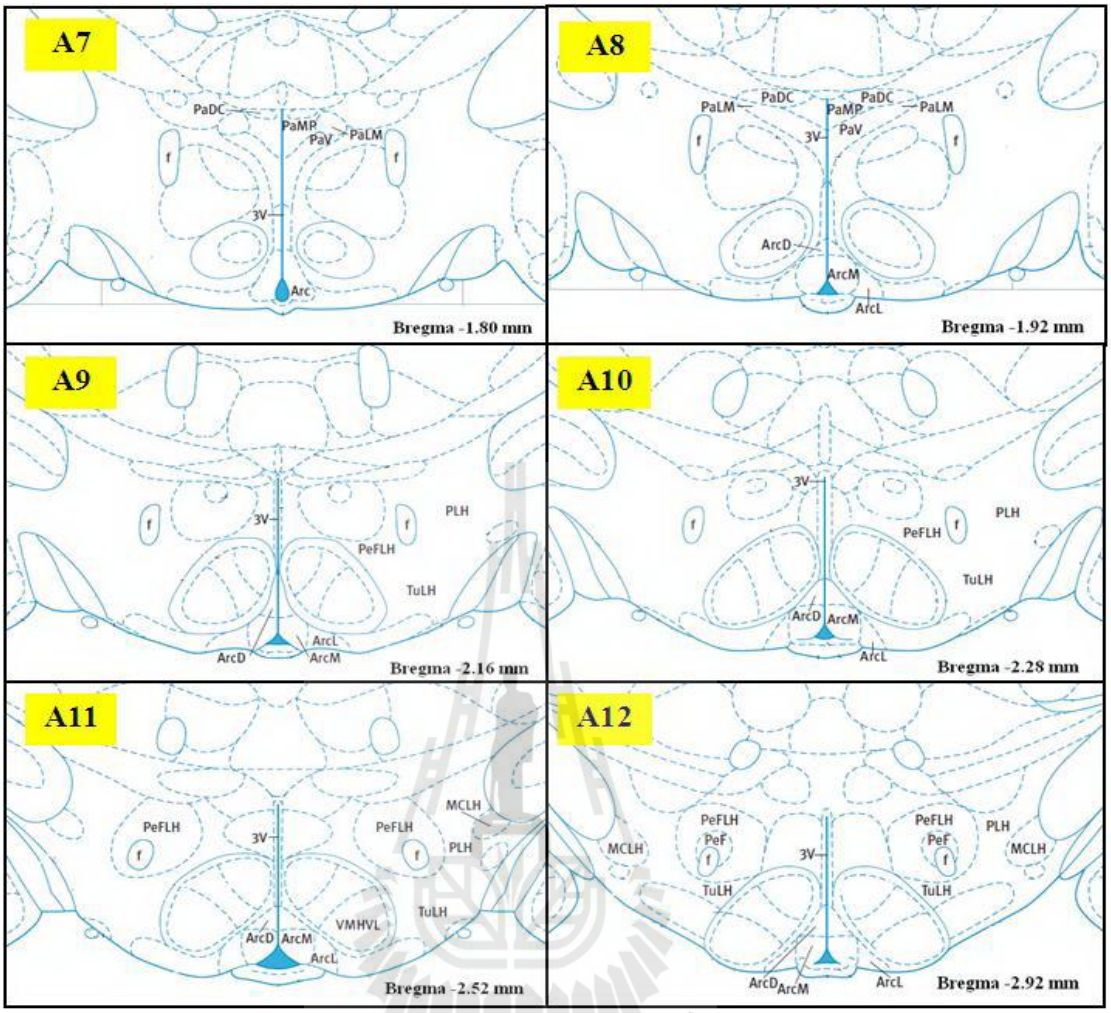

Figure 6.1 Schematic diagrams of coronal sections illustrating the brains regions of the hypothalamus (Continued). The PVN (-1.08 to -1.92 mm from bregma) from A1-A8, the ARC (-1.80 to -4.36 mm from bregma) from A7-A24, the LHA (-2.16 to -3.84 mm from bregma) from A9-A20, the PeF (-2.16 to -3.84 mm from bregma) from A9-A20, and the VMH (-1.72 to -3.36 mm from bregma) from A9-A16. Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2009). For abbreviations, see Table 6.1.

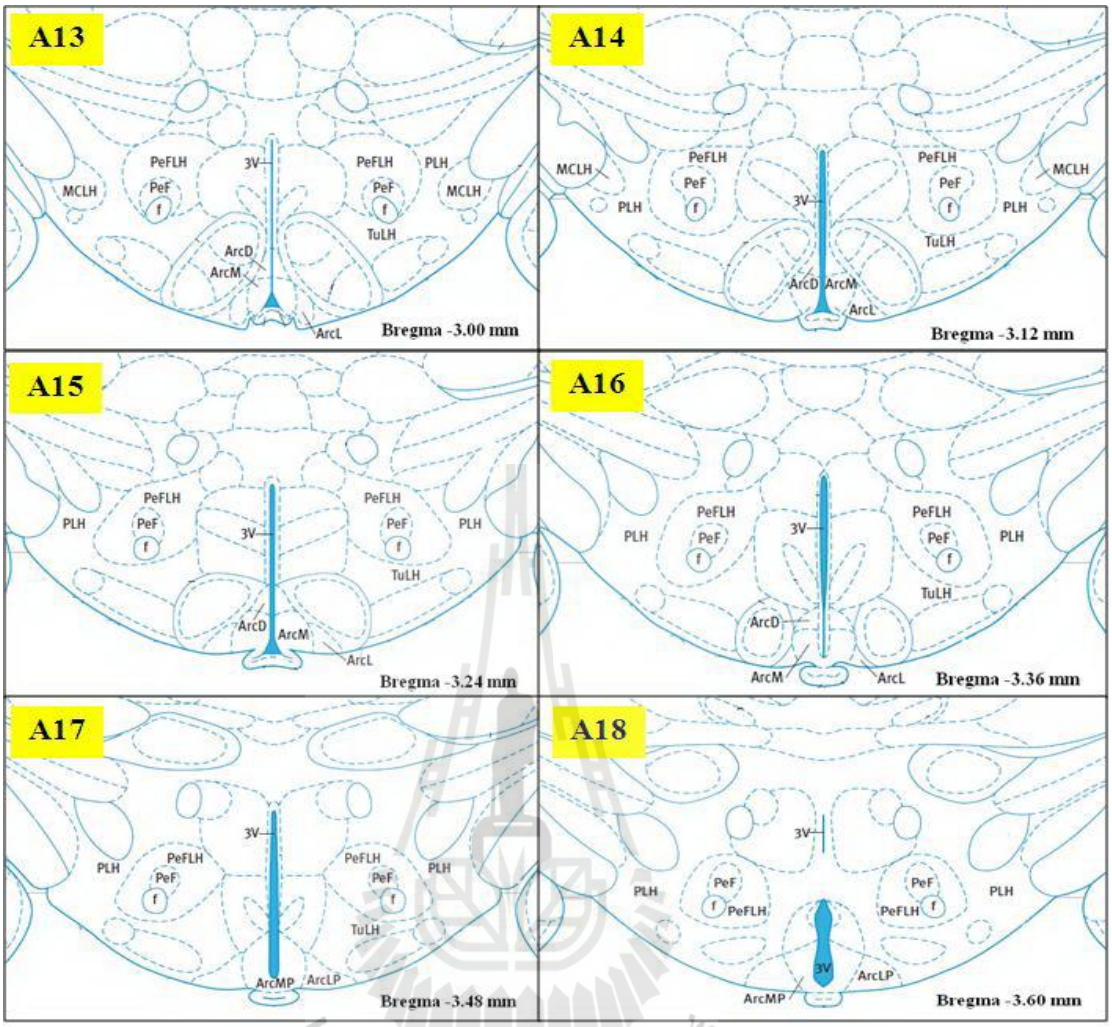

Figure 6.1 Schematic diagrams of coronal sections illustrating the brains regions of the hypothalamus (Continued). The PVN (-1.08 to -1.92 mm from bregma) from A1-A8, the ARC (-1.80 to -4.36 mm from bregma) from A7-A24, the LHA (-2.16 to -3.84 mm from bregma) from A9-A20, the PeF (-2.16 to -3.84 mm from bregma) from A9-A20, and the VMH (-1.72 to -3.36 mm from bregma) from A9-A16. Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2009). For abbreviations, see Table 6.1.

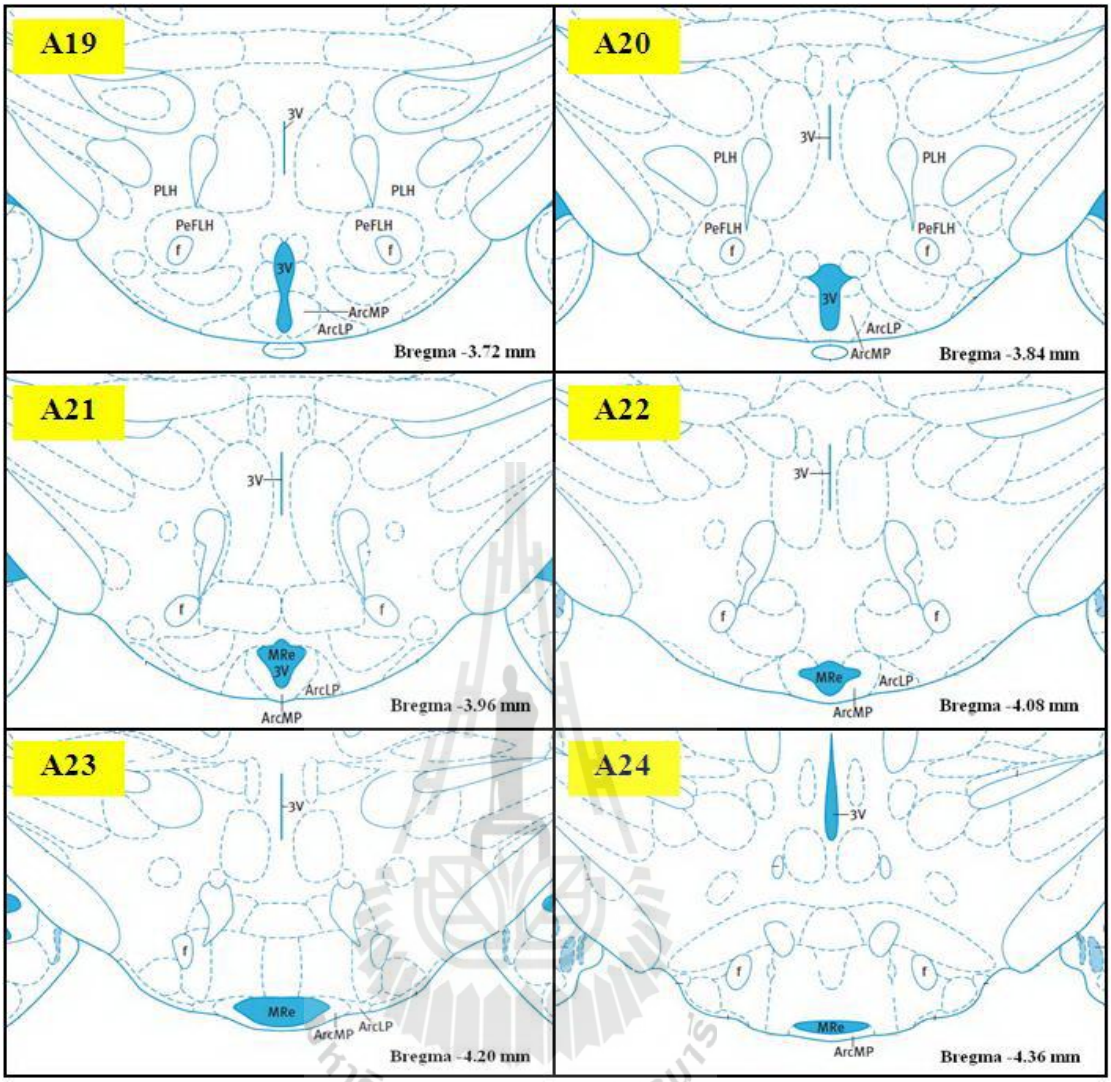

Figure 6.1 Schematic diagrams of coronal sections illustrating the brains regions of the hypothalamus (Continued). The PVN (-1.08 to -1.92 mm from bregma) from A1-A8, the ARC (-1.80 to -4.36 mm from bregma) from A7-A24, the LHA (-2.16 to -3.84 mm from bregma) from A9-A20, the PeF (-2.16 to -3.84 mm from bregma) from A9-A20, and the VMH (-1.72 to -3.36 mm from bregma) from A9-A16. Coronal illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the rat brain (Paxinos and Watson, 2009). For abbreviations, see Table 6.1.

6.3.4.1 Fos immunohistochemistry

Free-floating sections were immunostained for Fos according to the avidin-biotin complex (ABC) methods. Briefly, these sections were washed three times (×10 min) with 0.1 M phosphate buffer (pH 7.4), fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, washed three times (×10 min) with 0.1 M phosphate buffer (pH 7.4) and then washed six times (×15 min) with washing solution. Endogenous peroxidase was then deactivated with 3% hydrogen peroxide solution for 15 min, washed three times (×10 min) with washing solution and then blocked background staining with preincubation buffer for 30 min. After that, the sections were incubated with c-Fos Ab-5 primary antibody (1: 5,000 diluted in preincubation buffer) for 48 h in 500 µl labelled plastic bottles (Sterilin®, Sterilin Ltd., Aberbargoed, UK) at 4 ºC. Sections were then washed eight times (×5 min) with washing solution, incubated in secondary antibody solution for 1 h at room temperature and then washed three times (×10 min) with washing solution. After that, the sections were incubated in ABC complex solution for 1 h at room temperature and then washed two times (×10 min) with washing solution. The sections were rinsed with 0.1 M acetate buffer for 5 min and then incubated with Ni-DAB-H_2O_2 solution for approximately 10 min. The reaction was stopped by stop solution for 5 min. The sections were then washed in phosphate buffer saline solution (pH 7.4) for 5 min, rinsed three times (×5 min) with 0.1 M phosphate buffer (pH 7.4) and mounted onto the chrome alum gelatin subbed slides. Slices were then dried at 45 ^{o}C on the slide warmer (Medex Nagel GmbH., Germany). After that, the sections were dehydrated with serial dilution alcohol (70%, 90%, 95%, 100%, and 100%, 5 min each) followed by xylene (2×5 min). Slides were finally coverslipped using DPX mountant (BDH).

This method was performed with gentle agitation on an orbital shaker VRN-360 (Gemmy Industrial Corp., Taiwan).

6.3.4.2 Quantitative analysis

Quantitative assessment of Fos-positive neuron was achieved by counting the number of Fos-positive cells in the PVN, LHA, PeF, VMH, ARC, and NTS areas. These areas were counted the number of Fos-positive neurons in 6 sections/rat. Cells with distinct blue-black nuclear Fos staining in the PVN, LHA, PFA, VMH, and ARC areas were manually counted under light microscopy (×10 objective, Nikon ECLIPSE 80i, Nikon Corporation Ltd., Japan) and images were captured and stored by DP72 software (Olympus, Tokyo, Japan). Number of cells for each sampled area was transformed to cells per square millimeter.

6.4 Statistical analysis

Results were expressed as mean ± S.E.M. and analyzed by one-way ANOVA (SigmaStat version 3.5). Differences between groups were analyzed using one way ANOVA following by the Tukey test. *P*-values less than 0.05 ($P<0.05$) were considered statistically significant.

6.5 Results

The present study demonstrated for the first time that the aqueous ethanolic extract of *C. asiatica* and rutin could activate neuron in the brain areas regulating food intake. The aqueous ethanolic extract of *C. asiatica* and rutin could induce Fos expression in the ARC and PVN of the hypothalamus (Figures 6.2 and 6.4) but no

Fos-positive neurons in the VMH, LHA, and PeF of the hypothalamus and the nucleus of solitary tract in the brainstem. Prominent Fos expression were found in the ARC of the rat treated with the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/ml/kg, p.o.) and rutin (1500 mg/ml/kg, p.o.) and in the PVN of the rat treated with the aqueous ethanolic extract of *C. asiatica* (1000 mg/ml/kg, p.o.). In comparison to the control (79.31± 33.09 neuron/area), aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/ml/kg) induced significant increases in the number of Fos-positive neurons in the ARC (182.31 ± 38.87 and 225.15 ± 43.25 neurons/area, $P<0.05$) (Figure 6.3).

There was no significant difference in the number of Fos-positive neurons in the ARC of the rats treated with 1000 and 2000 mg/ml/kg of the aqueous ethanolic extract of *C. asiatica*. The number of Fos-positive neurons in the ARC of the rat treated with 1500 mg/ml/kg rutin was significant higher than the control group (195.6 ± 26.85 neurons/area, $P<0.05$, Figure 6.3). Significant difference in the number of Fos-positive neurons in the ARC were found between 1000 mg/ml/kg rutin treated group and 1500 mg/ml/kg rutin treated group (98.31 ± 12.52 and 195.6 ± 26.85 neurons/area, $P<0.05$, Figure 6.3).

In the PVN, the aqueous ethanolic extract of *C. asiatica* at the concentration of 1000 mg/ml/kg induced significantly higher in the number of Fos-positive neurons when compared with the control (157.25 ± 48.42 and 16.09 ± 11.34 neurons/area, $P<0.05$, Figure 6.5). There was no significant difference in the number of Fos-positive neurons in the PVN of the rats treated with the aqueous ethanolic extract of *C. asiatica* (2000 mg/ml/kg) and rutin (1000 and 1500 mg/ml/kg) when compared with the control (Figure 6.5).

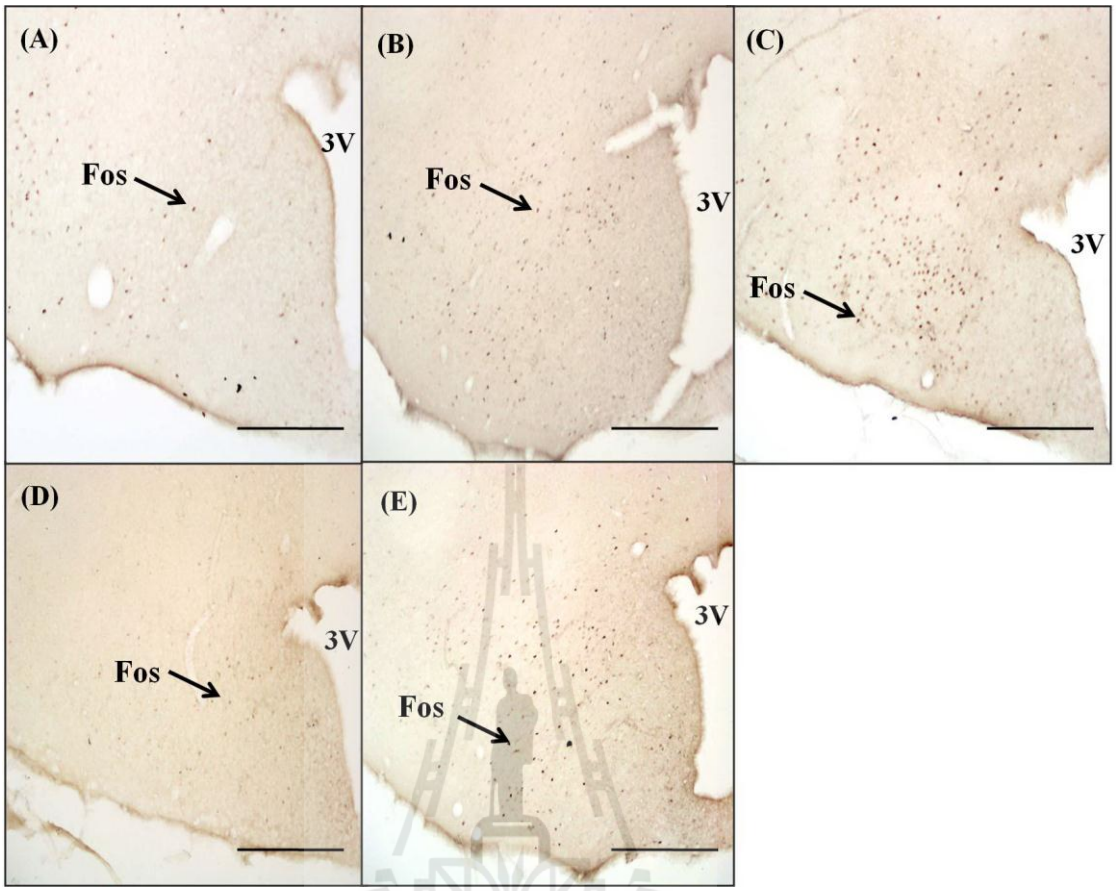

Figure 6.2 Effects of oral administration of aqueous ethanolic extract of *C. asiatica* and rutin on Fos expression in the ARC. Photomicrographs illustrate Fos expression in the ARC after oral administration of either 1 ml/kg of DDD water (A), 1000 mg/ml/kg of aqueous ethanolic extract of *C. asiatica* (B), 2000 mg/ml/kg of aqueous ethanolic extract of *C. asiatica* (C), 1000 mg/ml/kg of rutin (D), and 1500 mg/ml/kg of rutin (E). Prominent Fos expression was observed in the ARC of the rat treated with the aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/ml/kg) and rutin (1500 mg/ml/kg). The results were expressed as mean ± S.E.M. of 8 rats. Abbreviations: 3V, third ventricle. Arrow indicates Fos-positive neuron. Scale bar = 100 µm.

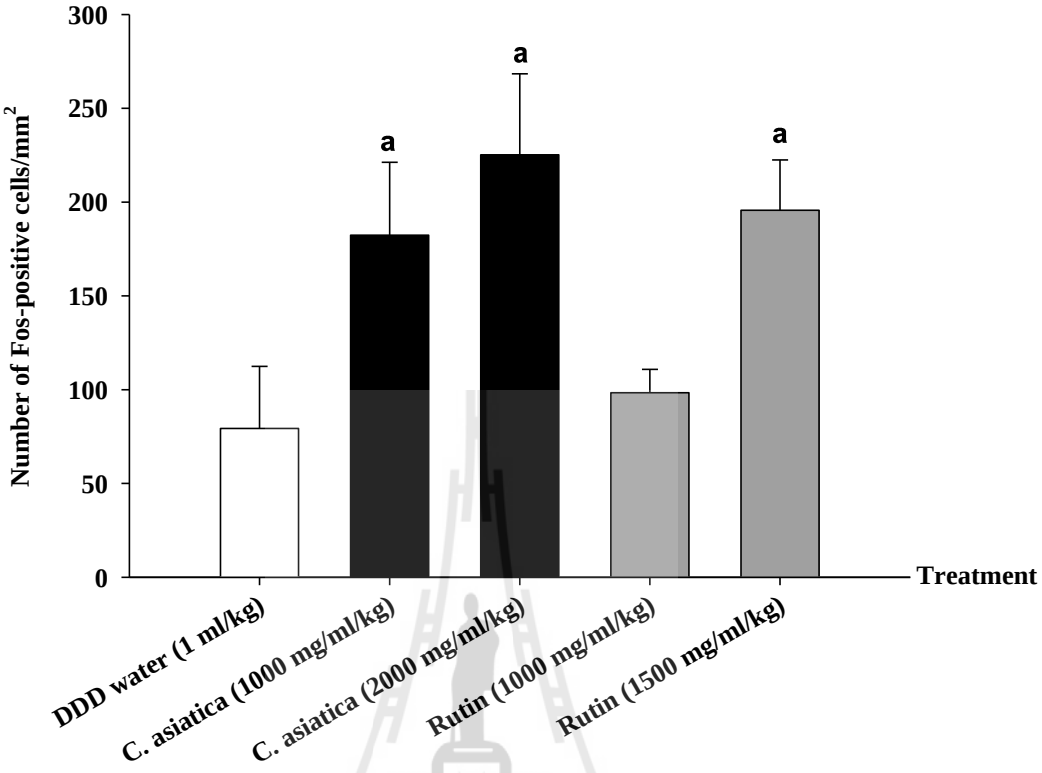

Figure 6.3 Effects of the aqueous ethanolic extract of *C. asiatica* and rutin on the number of Fos-positive neurons per area of the ARC ranging from -1.80 to -4.36 mm from bregma. Values are expressed as mean ± S.E.M. of average number of cells/area in the ARC. a, $P<0.05$ compared to the control group (1 ml/kg DDD water).

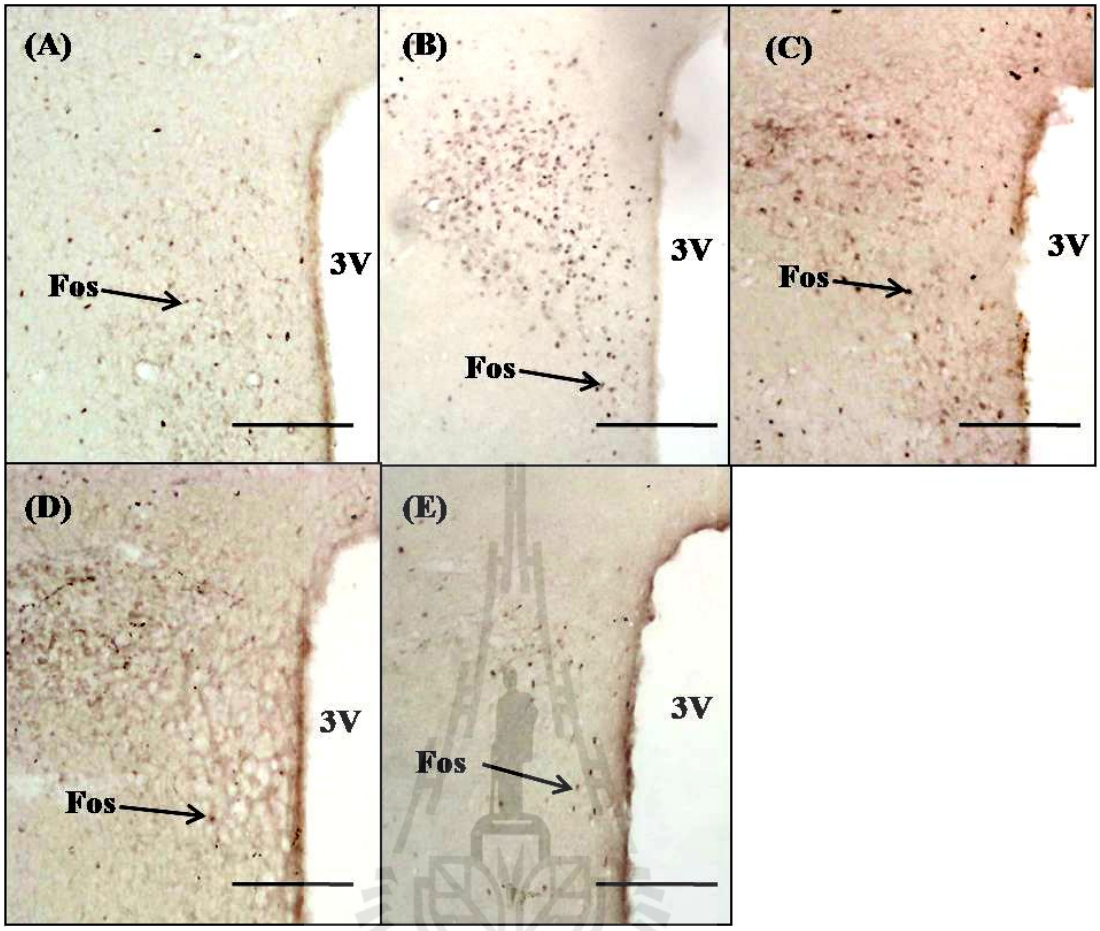

Figure 6.4 Effects of oral administration of the aqueous ethanolic extract of *C. asiatica* and rutin on Fos expression in the PVN. Photomicrographs illustrate Fos expression in the PVN after oral administration of either 1 ml/kg of DDD water (A), 1000 mg/ml/kg of aqueous ethanolic extract of *C. asiatica* (B), 2000 mg/ml/kg of aqueous ethanolic extract of *C. asiatica* (C), 1000 mg/ml/kg of rutin (D), and 1500 mg/ml/kg of rutin (E). Prominent Fos expression was observed in the PVN of the rat treated with the aqueous ethanolic extract of *C. asiatica* (1000 mg/ml/kg). The results were expressed as mean ± S.E.M. of 8 rats. Abbreviations: 3V, third ventricle. Arrow indicates Fos-positive neurons. Scale bar = 100 µm.

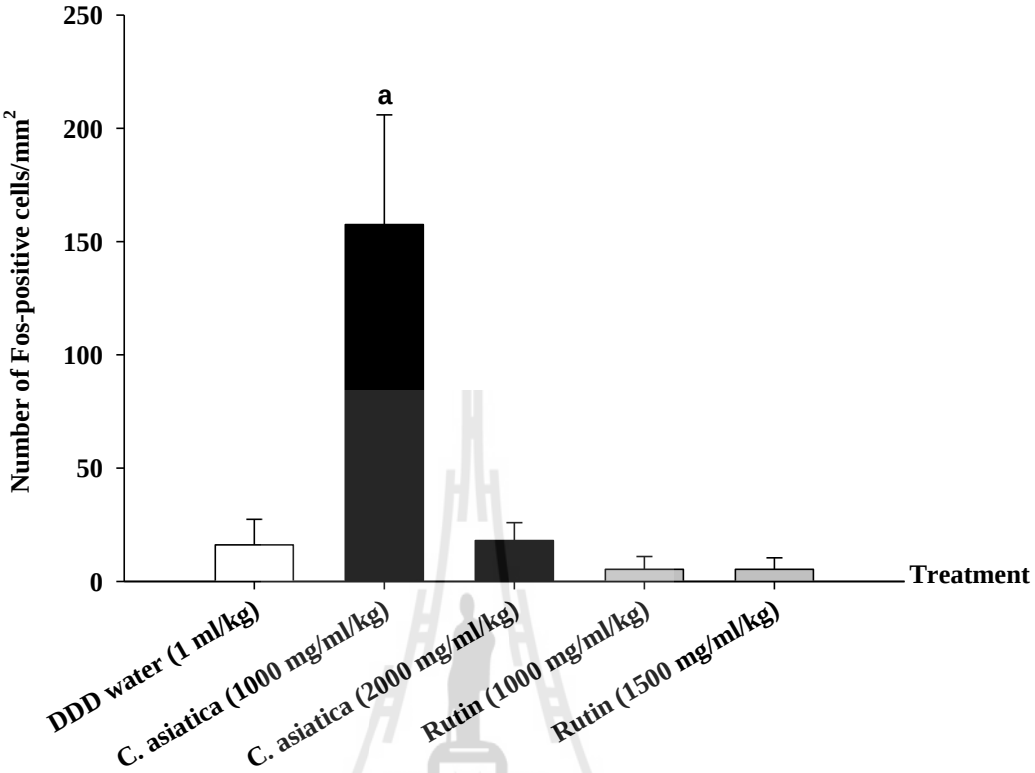

Figure 6.5 Effects of aqueous ethanolic extract of *C. asiatica* and rutin on the number of Fos-positive neurons per area of the PVN ranging from -1.08 to -1.92 mm from bregma. Values are expressed as mean ± S.E.M. of average number of cells/area in the PVN. a, $P<0.05$ compared to the control group (1 ml/kg DDD water).

6.6 Discussion and conclusion

Obesity reflects an imbalance between energy uptake and expenditure (Knecht, Ellger, and Levine, 2008). Food intake and energy expenditure is regulated by hypothalamic area (ARC, PVN, LHA, VMH, and PeF) and the NTS in the caudal medulla brainstem (Berthoud, 2002; Harrold *et al*., 2012; Schwartz *et al*., 2000). Several studies have demonstrated that medicinal plant extracts have a neuronal activity effects in the hypothalamus that involve the regulation of food intake and energy homeostasis (Kim *et al*., 2005; Kim *et al*., 2007; Xiong *et al*., 2010). Fos expression has been used as an index of neuronal activities in rats (Chen, Dong, and Li, 2003; Kim *et al*., 2005). The present findings revealed the first evidence of aqueous ethanolic extract of *C. asiatica* (1000 and 2000 mg/ml/kg; p.o.) and rutin (1500 mg/ml/kg; p.o.) significantly increased Fos-positive neurons in the ARC when compared with the control group. In the PVN, Fos-positive neurons induced by the aqueous ethanolic extract of *C. asiatica* (1000 mg/ml/kg) was significantly higher than the control group, 2000 mg/ml/kg of aqueous ethanolic extract of *C. asiatica,* and rutin (1000 and 1500 mg/ml/kg) treated groups. The increases in the number of Fos-positive neurons by the aqueous ethanolic extract of *C. asiatica* were not dose dependent.

Two primary neuronal populations within the ARC integrate signals of nutritional status and influence energy homeostasis (Schwartz *et al*., 2000; Simpson *et al*., 2009; Woods and Seeley, 2002). A subpopulation of neurons in the medial ARC express the orexigenic neuropeptides (NPY and AgRP) which produce in the NPY and AgRP neuron and the lateral ARC express the anorexigenic neuropeptides (α-MSH and CART) that produce in the POMC and CART neurons (Morton *et al*.,

2006; Remmers *et al*., 2011; Stanley *et al*., 2005). Surprisingly, the ARC neurons activated by the aqueous ethanolic extract of *C. asiatica* and rutin were predominently in lateroposterior parts of the ARC. Hence, aqueous ethanolic extract of *C. asiatica* may reduce food intake and body weight by activating neurons in the lateroposterior ARC neurons. Fos expression in the ARC may be induced by the aqueous ethanolic extract of *C. asiatica* and rutin through median eminence (ME) since the ARC is able to respond to active compound from aqueous ethanolic extract of *C. asiatica* and rutin *via* the ME, which lack a blood-brain barrier (Norsted, Gömüç, and Meister, 2008). Active of neurons in the PVN by the aqueous ethanolic extract of *C. asiatica* and rutin was shown in this study. The aqueous ethanolic extract of *C. asiatica* may activate anorexigenic neurons (TRH and CRH neurons) in the parvocellular PVN and oxytocin neurons in the magnocellular neurons or orexigenic neurons (NPY neurons in the parvocellular PVN) (Schwartz *et al*., 2000). The present study demonstrated that the aqueous ethanolic extract of *C. asiatica* and rutin did not induce Fos expression in the PeF, VMH, and LHA areas of the hypothalamus. The aqueous ethanolic extract of *C. asiatica* may inhibit NPY, orexigenic peptide, release or transport in the LHA, PeF, and VMH. The evidence that no Fos expression in the NTS following the aqueous ethanolic extract of *C.asiatica* and rutin treatment suggested that the aqueous ethanolic extract of *C.asiatica* and rutin may possess central, but not peripheral, control of food intake. The results of the present study suggested that the aqueous ethanolic extract of *C. asiatica* may involve in the regulation of food intake and energy homeostasis by activate neurons in the PVN and ARC of the hypothalamus. Rutin may be an active compound responsible for anti-obesity effects of *C. asiatica*. The aqueous ethanolic extract of *C. asiatica* and rutin may exert anti-obesity effect by

inhibiting NPY/AGRP neurons in the medial ARC and activate POMC/CART neurons in the lateral ARC, and activating magnocellular (oxytocin) and parvocellular (CRH, TRH or NPY) neurons in the PVN or inhibiting parvocellular neurons (NPY) in the PVN. However, further studies are required to clarify which neuronal types of the PVN and ARC of the hypothalamus that are activated by the aqueous ethanolic extract of *C. asiatica* and rutin.

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CHAPTER VII

CONCLUSION

C. asiatica is a medicinal plant which is a good source of phenolic compounds including flavonoids which possess potent antioxidant activity and anti-obesity effects. In the present study, the percent yield of 80% ethanolic extract of *C. asiatica* was 11.81%. Total phenolic content and rutin were 97.75 ± 0.01 mg gallic acid/g dry extract and 1267.69 ± 5.5 mg/kg dry weight, respectively. The present study provided the first evidence of the potent anti-obesity effect of the aqueous ethanolic extract of *C. asiatica* and rutin both *in vitro* and *in vivo*. The aqueous ethanolic extract of *C. asiatica* and rutin could suppress porcine pancreatic lipase, pancreatic alpha-amylase, and alpha-glucosidase enzyme activities *in vitro*. Inhibition of pancreatic lipase activity by the aqueous ethanolic extract of *C. asiatica* and rutin could suppress lipid digestion and absorption, resulting in a reduction of plasma triglyceride and total cholesterol levels in lipid emulsion-induced hyperlipidemic rats. Inhibition of alpha-amylase and alpha-glucosidase activities by the aqueous ethanolic extract of *C. asiatica* and rutin could suppress carbohydrate digestion and absorption, resulting in a reduction of plasma glucose levels. Inhibition of these digestive enzymes is beneficial for the aqueous ethanolic extract of *C. asiatica* and rutin in prevention and treatment of obesity. Both the aqueous ethanolic extract of *C. asiatica* and rutin did not cause liver damage since the increase levels of aspartate aminotransferase and

alanine aminotransferase were not shown. The mechanisms of anti-obesity actions of the aqueous ethanolic extract of *C. asiatica* and rutin are shown in Figure 7.1.

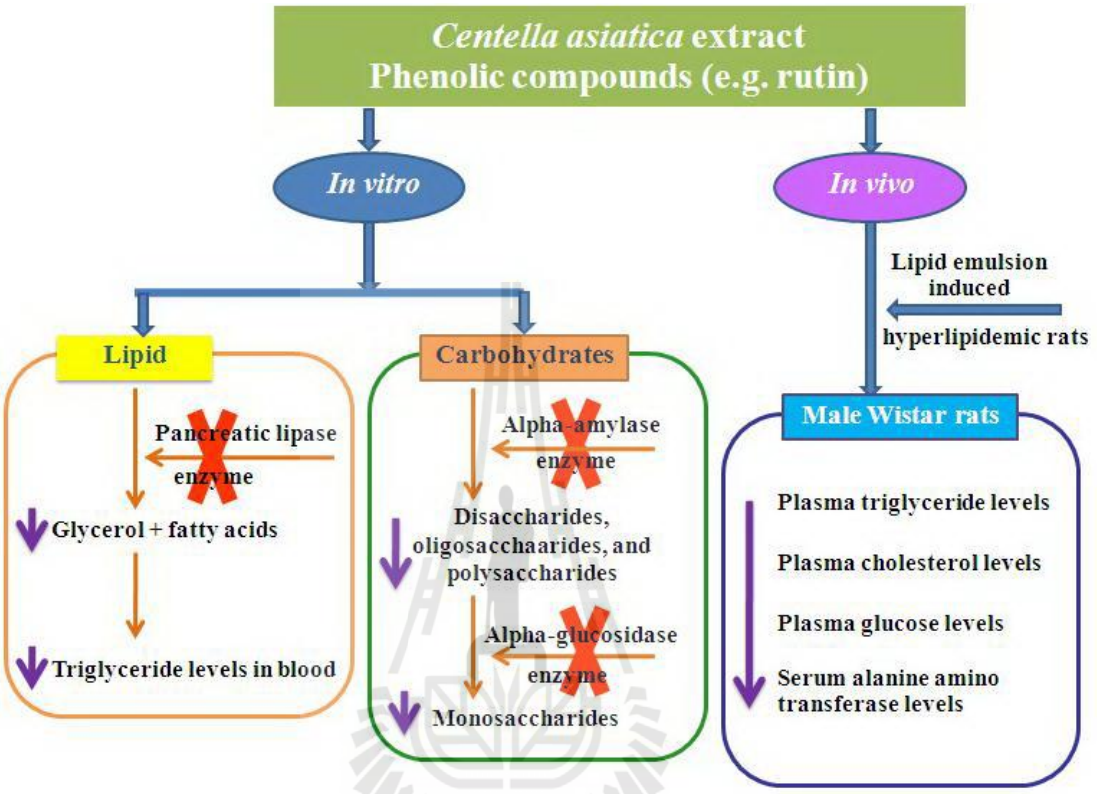

Figure 7.1 Mechanisms of hypolipidemic and hypoglycemic actions of the aqueous ethanolic extract from *C. asiatica* and rutin *in vitro* and *in vivo*.

Additionally, central effects of the aqueous ethanolic extract of *C. asiatica* and rutin were demonstrated (Figure 7.2). The aqueous ethanolic extract of *C. asiatica* and rutin had a modulatory effect on the expression of a neuronal activation marker Fos in the arcuate nucleus (ARC) and the aqueous ethanolic extract of *C. asiatica* induced Fos expression in the paraventricular nucleus (PVN). The aqueous ethanolic extract of *C. asiatica* and rutin did not induce Fos expression in other areas of hypothalamus and the nucleus of the solitary tract. The aqueous ethanolic extract of *C. asiatica* and rutin

markedly increased Fos-positive neurons in the lateralposterior region of the ARC which involved the reduction of feeding. The aqueous ethanolic extract of *C. asiatica* and rutin markedly increased Fos-positive neurons in both parvocellular and magnocellular regions of the PVN. Thus, the aqueous ethanolic extract of *C. asiatica* and rutin may involve in the regulation of feeding. However, further study is required to clarify which anorexigenic neuropeptides neurons (TRH, CRH, and oxytocin) and orexigenic neuropeptide neurons (NPY) in the PVN are activated by the aqueous ethanolic extract of *C. asiatica* and rutin.

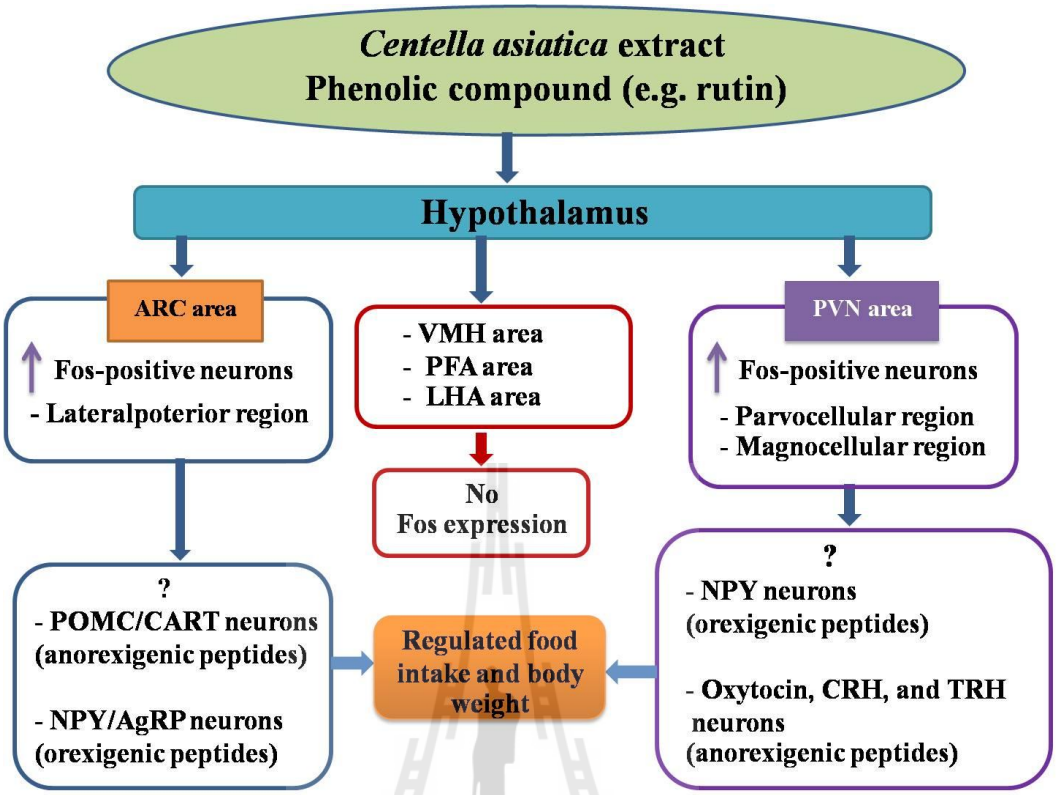

Figure 7.2 Central effects of the aqueous ethanolic extract of *C. asiatica* and rutin in male Wistar rats. ARC: arcuate nucleus; PVN: paraventricular nucleus; LH: lateral hypothalamic area; PFA: perifornical area; NPY: neuropeptide Y; AGRP: Agouti-related peptide; POMC: pro-opiomelanocortin; CART: cocaine- and amphetamine-regulated transcript); CRH: Corticotropin-releasing hormone; TRH: Thyrotropin-releasing hormone.

In conclusion, this study demonstrated potent anti-obesity of the aqueous ethanolic extract of *C. asiatica* and rutin *in vitro* and *in vivo*. Central effects of the aqueous ethanolic extract of *C. asiatica* and rutin on the arcuate nucleus and the paraventricular nucleus in the hypothalamus suggested the role of the aqueous ethanolic extract of *C. asiatica* and rutin in regulation of body weight. Phenolic

compounds, notably rutin, may be bioactive compounds responsible for the anti-obesity activity of the aqueous ethanolic extract from *C. asiatica*. Further studies are needed to clarify the underlying mechanisms involving anti-obesity effects of the aqueous ethanolic extract of *C. asiatica*.

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