การปรับปรุงเสถียรภาพของ1´-แอซีท็อกซีซาวิคอลแอซีเทตเพื่อประสิทธิภาพในการต้านโรคอ้วน

นางสาวภทรชนก เสียงเพลิน



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

# STABILITY IMPROVEMENT OF 1´- ACETOXYCHAVICOL ACETATE FOR ANTI-OBESITY EFFICACY

Miss Phatharachanok Siangphloen


A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
Faculty of Science
Chulalongkorn University
Academic Year 2014

| | |
|---|---|
| Thesis Title | STABILITY IMPROVEMENT OF 1´-ACETOXYCHAVICOL ACETATE FOR ANTI-OBESITY EFFICACY |
| By | Miss Phatharachanok Siangphloen |
| Field of Study | Biotechnology |
| Thesis Advisor | Associate Professor Supason Wanichwecharuang, Ph.D. |
| Thesis Co-Advisor | Associate Professor Wijit Banlunara, D.V.M.,Ph.D. |

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman
(Associate Professor Vudhichai Parasuk, Ph.D.)

.....Thesis Advisor
(Associate Professor Supason Wanichwecharuang, Ph.D.)

.....Thesis Co-Advisor
(Associate Professor Wijit Banlunara, D.V.M.,Ph.D.)

.....Examiner
(Supawin Watcharamul, Ph.D.)

.....External Examiner
(Prasat Kittakoop, Ph.D.)

ภทรชนก เสียงเพลิน : การปรับปรุงเสถียรภาพของ1´-แอซีท็อกซีชาวิคอลแอซีเทตเพื่อประสิทธิภาพในการต้านโรคอ้วน (STABILITY IMPROVEMENT OF 1´-ACETOXYCHAVICOL ACETATE FOR ANTI-OBESITY EFFICACY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ศุภศร วนิชเวชารุ่งเรือง, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. นสพ. ดร. วิจิตร บรรลุนารา, 62 หน้า.

งานวิจัยนี้ทำการแก้ปัญหาความไม่เสถียรของสารสำคัญหลักที่สกัดได้จากข่าคือหนึ่ง´-อะซิท็อกซี่ชาวิคอลอะซิเตต ด้วยการห่อหุ้มลงในอนุภาคระดับไมโครเมตรที่เตรียมจากพอลิเมอร์ผสมของโพลิไวนิล แอลกอฮอล์และพาราฟิน แวกซ์ ในอัตราส่วน 0.7:1 โดยสามารถเตรียมเป็นอนุภาคระดับไมโครเมตรที่มีลักษณะทรงกลม มีขนาดประมาณ 5-10 ไมโครเมตร กระบวนการเตรียมให้ประสิทธิภาพการห่อหุ้มมากกว่า 60% และอนุภาคที่ได้มีความจุสาร หนึ่ง´-อะซิท็อกซี่ชาวิคอลอะซิเตต ประมาณ 30% อนุภาคที่ได้กระจายตัวในน้ำได้ดี การห่อหุ้มสามารถป้องกันการสลายตัวของสารสกัด หนึ่ง´-อะซิท็อกซี่ชาวิคอลอะซิเตต ที่อุณหภูมิสูงได้ อนุภาคที่ได้แสดงฤทธิ์การยับยั้งการสร้างไขมันในเซลล์ 3T3-L1 adipocyte ได้ดีกว่าสาร หนึ่ง´-อะซิท็อกซี่ชาวิคอลอะซิเตต ที่ไม่ได้ห่อหุ้ม อนุภาคที่เตรียมขึ้นไม่แสดงความเป็นพิษอย่างฉับพลันในเซลล์ และที่น่าสนใจกว่านั้นคือการทดลองในหนูดำ 40 ตัว พบว่าสารสกัดหนึ่ง´-อะซิท็อกซี่ชาวิคอลอะซิเตตที่ถูกห่อหุ้มในอนุภาคระดับไมโครเมตรมีประสิทธิภาพในการลดความอ้วนในหนูทดลองมากกว่าสารสกัด หนึ่ง´-อะซิท็อกซี่ชาวิคอลอะซิเตต ที่ไม่ได้ถูกห่อหุ้ม จะเห็นได้ว่าการห่อหุ้มสารสกัด หนึ่ง´-อะซิท็อกซี่ชาวิคอลอะซิเตต ลงในอนุภาคระดับไมโครเมตรเป็นสารที่สามารถยับยั้งการเพิ่มขึ้นของน้ำหนักและการสะสมไขมันในตับของหนูได้อย่างมีประสิทธิภาพ



สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

# # 5572070623 : MAJOR BIOTECHNOLOGY

KEYWORDS: 1'-ACETOXYCHAVICOL ACETATE, ALPINIA GALANGA, MICROPA RTICLE, MICROPARTICLE/NANOENAPSULATION, ANTI-OBESITY

PHATHARACHANOK SIANGPHLOEN: STABILITY IMPROVEMENT OF 1′-ACETOXYCHAVICOL ACETATE FOR ANTI-OBESITY EFFICACY. ADVISOR: ASSOC. PROF. SUPASON WANICHWECHARUANG, Ph.D., CO-ADVISOR: ASSOC. PROF. WIJIT BANLUNARA, D.V.M.,Ph.D., 62 pp.

This work improves the stability of 1′-acetoxychavicol acetate (ACA), a major active component extracted from the rhizome of *Alpinia galangal* or Galangal by encapsulating the material into microparticles fabricated from a 0.7:1 (w/w) blend of Polyvinyl alcohol (PVA) and paraffin wax. The prepared microparticles showed spherical shape, with the size around 5-10 μm. The process gave encapsulation efficiency of more than 60%, and the water dispersible particles possessed loading capacity of 30%. The ACA-loaded microparticles showed slower degradation of ACA at the high temperature when compared to the non-encapsulated ACA. The ACA-loaded microparticles showed significantly reduced triglyceride (TG) accumulation in 3T3-L1 derived adipocytes and lower proliferation of 3T3-L1 preadipocytes compared to the non-encapsulated ACA, blank microparticle and the degraded ACA. I*n vitro* toxicity test in 3T3-L1 preadipocytes demonstrated that ACA-loaded microparticles were non-toxic to the cells. In vivo test using 40 mice indicated that mice fed high fat diet (HFD) together with ACA-loaded microparticles gained less weight than mice fed only HFD. Thus, microencapsulated ACA is a potential anti-obesity agent.



Field of Study: Biotechnology
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

# ACKNOWLEDGEMENTS

First of all, I would like to express my sincere appreciation and gratitude to my advisor, Assoc. Prof. Dr. Supason Wanichweacharungruang for her helpful guidance, valuable assistance and consolation throughout my thesis. My sincere thanks are extended to Assoc. Prof. Dr. Wijit Banlunara, my co-advisor, for her suggestions and many helps.

I would like to thank all members of the committee Assoc. Prof. Dr. Vudhichai Parasuk, Dr. supawin watcharamul and the invited external examiner Dr. prasat kittakoop for their time, giving helpful comments and suggestions.

My appreciation also goes to Dr. Prasat Kittakoop, Program in Chemical Biology, Chulabhorn Research Institute for extraction of 1'-Acetoxychavicol acetate. I also would like to thank the Department of chemistry and Department of Biotechnology at Faculty of Sciences Chulalongkorn University for providing laboratory facilities.

Finally, I would like to specially thank my parents, my family, my friends for their love, advice, understanding and encouragement throughout my entire education and all the members of Dr. Supason's research group for their companionship, valuable discussion and support.

**CONTENTS**

Page

Page

Page

Page

# LIST OF TABLES

# LIST OF FIGURES

Figure Page

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACA | 1′-Acetoxychavicol Acetate |
| % | Percent |
| °C | Degree Celsius |
| mW | Milliwatt |
| ml | Milliliter |
| µg | Microgram |
| µl | Microliter |
| µM | Micromolar |
| ppm | Parts per million |
| rpm | Revolution per minute |
| min | Minute |
| v/v | Volume by volume |
| w/w | Weight by weight |
| cP | Centipoise |
| DMSO | Dimethyl sulfoxide |
| DSC | Differential scanning calorimetry |
| SEM | Scanning electron microscope |
| TEM | Transmission electron microscope |
| AFM | Atomic force microscopy |
| PVA | Polyvinyl alcohol |

| | |
|---|---|
| HCL | Hydrochloric acid |
| PBS | Phosphate buffer solution |
| ATCC | American Type Culture Collection |
| FBS | Fetal bovine serum |
| DMEM | Dulbecco's modification of Eagle's medium |
| OD | Optical density |
| UV/Vis | Ultraviolet/Visible |
| DPBS | Dulbecco’s phosphate buffer saline |
| %EE | Encapsulation efficiency |
| %loading | Loading capacity |

# CHAPTER I
# INTRODUCTION

Obesity is a growing global concern as it is a common risk factor associated with the various diseases such as diabetes, hypertension, atherosclerosis, coronary heart disease, respiratory disorders, cancer, and osteoarthritis [1]. The international obesity task force is actively working to evaluate the epidemic of obesity worldwide. By their analysis, in the year 2014, they have estimated that approximately 1.9 billion adults are currently falling under the overweight category (body mass index (BMI) 25-29.9 Kg/$m^2$) [1], further they stated that there were 475 million people who are obese. While the Asian-specific BMI cut off points for the obesity (BMI is >30 kg/$m^2$) [1] are taken into consideration, this number of obese people rise to 600 million, which is incredibly high. However, a study by Kopelmen. et al. (2000) showed that obesity development is characterized not only by the increased BMI, but by an increase in the number of fat cells and the accumulation of lipids due to processes such as mitogenesis and differentiation, which are dependent on various factors such as metabolic rate, genetics, environmental factors and nutritional factors Therefore, understanding the role and function of nutrients in cellular processes such as mitogenesis and differentiation can be helpful in deciding preventive strategies for the onset and further progress of obesity in humans [2].

In recent years, several vegetables and fruits have attracted scientist's interest because of their non-toxicity and beneficial health effects. *Alpinia galangal* or galangal, a rhizome closely related to ginger family, is used for cooking, especially in Thai and Indonesia cuisine. In Thai, galangal is used in curry pastes, Tom-Yam (Thai spicy soup) and many curries. Galangal rhizome has also been used widely in many fields such as a traditional medicine [3, 4]. The traditional medicine from galangal can

be used to cure skin diseases, indigestion, colic, dysentery, enlarge spleen, respiratory diseases, cancer of mouth and stomach, and systemic infections and cholera. It can also be used as an expectorant and after childbirth [3]. It has been known that 1′-acetoxychavicol acetate (ACA) is a major component in the extract of a rhizome of *Alpinia galangal* [5]. ACA possesses good aroma and shows antioxidant [6], anti-inflammatory [7] and anti-obesity properties [5]. However, ACA is not stable in aqueous solution and undergoes hydrolysis/isomerization reactions [4]. Thus, this problem of ACA could be solvable by microencapsulation. Microparticulate drug delivery systems can enhance the solubility, bioavailability and permeability of many drugs and bioactive molecules which are difficult to be delivered orally. In addition, microparticles provide ingenious treatment by enabling targeted delivery and controlled release. Several diseases related drugs or bioactive molecules are successfully encapsulated and show improved bioavailability, bioactivity and controlled delivery [8-10]. Thus, the insolubility problem of ACA extract should be solvable by microencapsulation. In this study, the effects of micro-encapsulated ACA on lipid accumulation in adipocytes and proliferation of pre-adipocytes were measured using three independent experiments for each measurement. Micro-encapsulated ACA significantly reduced triglyceride accumulation in 3T3-L1 adipocytes and inhibited preadipocyte proliferation compared to its free ACA, blank PVA/paraffin wax microparticles, degraded ACA. Moreover, acute toxicity and anti-obesity of ACA-loaded microparticles in mice through oral administration of ACA-loaded microparticles was also investigated and is reported.

# CHAPTER II
# LITERATURE REVIEW

## 2.1 Definition of obesity

Obesity, defined as a condition with a body mass index (BMI) at greater than 30 kg/m2, is a major risk factor for many of the diseases. Obesity impairs quality of life and premature mortality [1]. At present, obesity has becoming pandemic [11].The world health organization predicts that by the end of 2015, approximately 2.3 billion adults will be overweight and more than 700 million will be obese. Obesity as well as overweight can lead to number of comorbidities such as atherosclerosis [2], diabetes, hypertension and also cancers [3]. There are various direct and indirect factors involved in development of obesity and overweight. Direct factors include increased consumption of energy dense food, binge eating, inactive lifestyle and low metabolic rate. Indirect factors contributing to obesity and overweight are environment, genetic factors, smoking, psycho-social, including weight gain associated with many psychiatric drugs used to treat depression, anxiety, bipolar disorder, and schizophrenia. Use of too much social networking such as facebook, myspace also influences the lifestyle by making it is sedentary and less physically active [11].



## 2.2 Adipose tissue and obesity

Adipose tissue, which is derived from preadipocytes, is known as an endocrine organ which is highly metabolically active [4]. It contains the stromal vascular fraction (SVF) of cells including preadipocytes, fibroblasts, vascular endothelial cells and a variety of immune cells (i.e., adipose tissue macrophages [ATMs]). A major component of adipose tissue is adipose cells known as adipocyte or fats cells. The main role of adipose tissue is to store energy in the form of lipids, it also cushions and insulates the body. It plays an important role in maintaining the free fatty acid

levels and triglycerides in circulation. Moreover, adipose tissue can affect other organ systems in the body and may lead to diseases [5]. It has been demonstrated that an increased amount of adipose tissue is related to obesity by hyperplasia or hypertrophy of the adipocyte. Hyperplasia is an increase in the number of adipocytes, which occurs by pre-adipocyte differentiating into adipocyte. Adipose tissue mass can also increase by hypertrophic growth, which is an increase of the size of adipocyte [6]. Adipogenesis is a process which encompasses with proliferation of precursor cells and their commitment to adipogenic lineage, It is terminated by differentiation into adipocytes [7]. Differentiation cell is characterized by the definite functions which causes expression of genes leading to increased lipid accumulation. Therefore, adipocytes differentiated from preadipocytes exhibit a high content of lipogenic enzymes, which are responsible in facilitating the synthesis as well as the storage of lipids in the form of triglycerides [4]. Figure 2.1 shows the steps involved preadipocyte to adipocyte differentiation. After determination to adipocyte linage, the preadipocytes increase in numbers and differentiate into mature adipocytes, which can then accumulate triglycerol and increase in size.

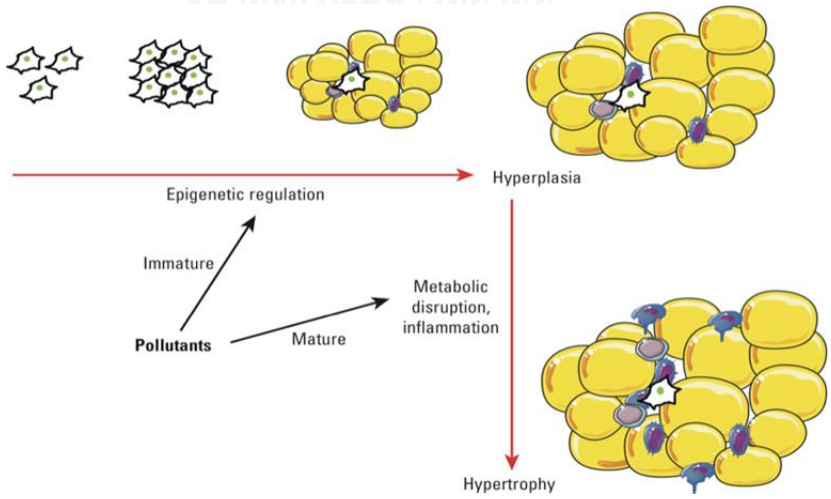



**Figure 2.1** Preadipocyte to adipocyte differentiation: After determination to adipocyte linage, the preadipocytes increase in numbers and differentiate into mature adipocytes [7].

### 2.2.1 Adipogenesis Transcription factors

The adipogenesis is a process of cell differentiation, which preadipocyte become adipocytes. The preadipocyte undergoes differentiation after induction with the differentiation medium. Expression of some transcription factors are increased which further initiate the expression of adipocyte phenotype [12].

#### 2.2.1.1 CCAAT/enhancer binding proteins *(C/EBPβ/δ)*

C/EBPβ/δ are two major genes which induce differentiation of preadipocytes to adipocytes [13]. Their importance in adipogenesis is proved by the fact that over-expression of the protein in preadipocytes accelerates adipogenesis. The peroxisome proliferator activated receptor gamma (PPARγ) and C/EBPα are two gene expressions inducible by C/EBPβ/δ. After differentiation of preadipocytes with the inducers (isomethyl-butyl-xanthine, dexamethasone, insulin, fatal bovine serum), there is a rapid increase in the process of transcription, leading to the expression of the central gene C/EBPβ (Figure 2.2)

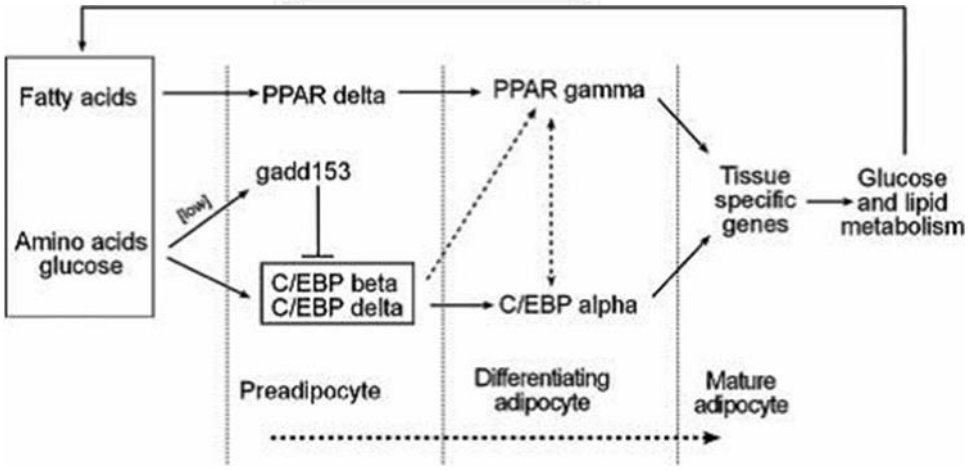



**Figure 2.2** The gene express during the differentiation of pre-adipocytes into mature adipocytes [14].

#### 2.2.1.2 Peroxisome proliferator activated receptor gamma (PPAR)

PPAR is a subfamily of nuclear hormone receptor (NHR) superfamily, which includes retinoic acid receptors, thyroid hormone receptors and so on [10]. Among these members, PPARγ is the gene highly expressed in adipose tissue that is responsible for regulating the lipid accumulation in adipocyte [15]. Once the PPARγ is expressed, it works as transcription factor to other genes, which relates to the differentiation. PPARγ induces the expression of adipocyte-fatty acid binding protein (A-FABP), which is known as the marker of preadipocyte differentiation. The expression of these two genes in 3T3-L1 preadipocyte is very low. Moreover, high gene expression levels are observed when the differentiation progresses. [16]

#### 2.2.1.3 CCAAT/enhancer binding proteins (C/EBPα)

C/EBPα is induced at later stages and thus, active in mature adipocytes .C/EBPα gene is known for its auto-activation phenomenon [17]. Initially, its expression is induced by PPARγ. This gene, however, possesses a regulatory element to which C/EBPα binds and initiates auto-activation. It has anti-mitotic characteristic, which causes cessation of mitotic clonal expansion [18].

#### 2.2.1.4 Adiponectin

Adinopectin is a one importance protein hormone products. It is secreted by adipocyte via C/EBPα [19]. Adiponectin is involved in the functions such as lipid accumulation, preadipocyte differentiation [20]. It's secretion depends on the cell size *in vivo*. Weight loss and decrease in adipocyte size increase the secretion of adiponectin, which can cause regaining the size back through enhancing lipid accumulation.

### 2.2.2 Cell Culture Models

Many researches about adipogenesis or fat cell biology have been studied via in-vitro experimental cell culture model systems. There are two types of cell culture models:

Primary Culture: They are isolated from stromal vascular cells of adipose tissue of humans or animals [18]. There are different methods to isolate these cells. These cells are also expensive and possess limit life span. Furthermore, they are heterogeneous which mean they possess characteristics after each passage [21]. However, model of these cells can be used to confirm the results seen in cell line models.

Cell Lines: They are divided into two types: the first one is multiple fibroblastic cell lines which are not committed and have adipogenic potential such as NIH-3T3, Balb/c3T3, C3H10T1/2 etc [21]. The other is preadipocyte cell lines, which undergo commitment to the adipose lineage such as 3T3-L1, Ob1771, 3T3F442A,TA1, Ob17 etc [21]. They exhibit morphology and biochemical properties similar to in vivo adipocytes, have a long life span in culture and are homogenous [21].



3T3-L1 preadipocytes: 3T3-L1 preadipocyte cell line is a widely used model to investigate the adipocyte differentiation process. They are derived from (mouse) embryo fibroblasts [22]. This model was studied by Horward Green and colleagues [22]. These cells can undergo differentiation in culture and exhibit morphology and biochemical properties similar to adipocytes. Furthermore, the 3T3-L1 preadipocytes can grow in the culture when they are treated with hormone (hormonal cocktail consisting of dexamethasone, insulin and 3-isobutyl-1-methylxanthine(MIX)) Thus, they can differentiate to mature adipocytes [23]. The differentiation period of preadipocytes to mature adipocyte is between 4 to 8 days.

## 2.3 *Alpinia galangal*

### 2.3.1 Botanical description

*Alpinia galangal* (L) Wild is classified in the family of ginger and it is widely cultivated in Sri Lanka, India, Malaysia, Indonesia, and is found abundantly in Thailand (current). The rhizomes of this plant are frequently used for cooking, especially in Thai and Indonesian cuisine [5]. *Alpinia galangal* is a reed–like perennial herb, with 2 m high. Leaves are about 22.5-45 cm long, 3.7-11.2 wide, thick and dark green. The flowers are around 15–30 cm long and are greenish white with a dark-red veined tip. The fruit is round with diameter around 1.25 cm. The pericarp of fruit turns from green to orange-red to black when mature and indehiscent [24]. The branched pieces of rhizome are from 3.8-8 cm in length, and seldom more than 7-10 cm thick. Rhizomes are reddish brown and have an aromatic, spicy and pungent odor and flavor [25]. The rhizome of *Alpinia galangal*, is the pungent component of galangal [4] and has various biological properties, including anti-inflammatory [7], anti-gastric [3], antifungal [26], anti-allergic [27] and antitumor properties [6].

### 2.3.2 Chemical constituents of *Alpinia galangal* extract

The rhizome of *Alpinia galangal* contains a variety of compound. The major bioactive of *Alpinia galangal* was found in its essential oil. The essential oil was isolated from rhizome of *Alpinia galangal*.

**Table 2.1** Essentail oil isolated from rhizome of Alpinia galangal [25].

| Essential oil | Essential oil |
|---|---|
| Methyl cinnamate | (1'S)-l'-acetoxyeugenol acetate |
| Alpinin, kaempferide | (1'S)-l'-acetoxyeugenol acetate |
| 3-dioxy4-methoxy flavone | 1'-acetoxyeugenol acetate |
| Camphor | 1'-acetoxychavicol acetate |
| Galangin | Pinene |
| D-camphor | Pineol |
| Chavicol | 1'-hydroxychavicol acetate |
| Chavicol acetate | *ρ*-hydroxycinammaldehyde |
| Eugenol acetate | α-pinene |
| trans β-faranesene-4-hydoxybenzyldehyde | β-pinene |
| Kaempferol | Camphene |
| Kaempferol-4'- methylether | *ρ*-methane-1,8-epoxy-acethoxychavicol acetate |
| Kaempferol-7'- methylether | 1,8-cineole (13), 3-hydroxy-l,8-cineole glucopyranosides |
| Methylcinnamate | (lS,2S,4R)-trans-2-hydroxy-1,8-cineole -D-glucopyranoside |
| Methyleugenol | (1R,3S,4S)- trans-3-hydroxy-l ,8-cineole-D- glucopyranos |
| 3-carene | Trans coniferyl diacetate |
| 3-carene | Trans -p-coumaryl diace |
| α-thujene | Di-(p-hydroxy-cis-styryl) methane |
| Myrcene | 7-hydroxy-3,5-dimethoxy flavone |
| *ρ*-cymene<br>α-humulene | Borneol<br>α-terpineol |
| α-humulene | Fenchyl acetate |
| Zerumbone | Bornyl acetate |

Essential oils isolated from rhizome of Alpinia galangal contains active components such as 1'-acetoxychavicol acetate, α-Terpineol, *ρ*-cymene, limonene (Figure 2.4)

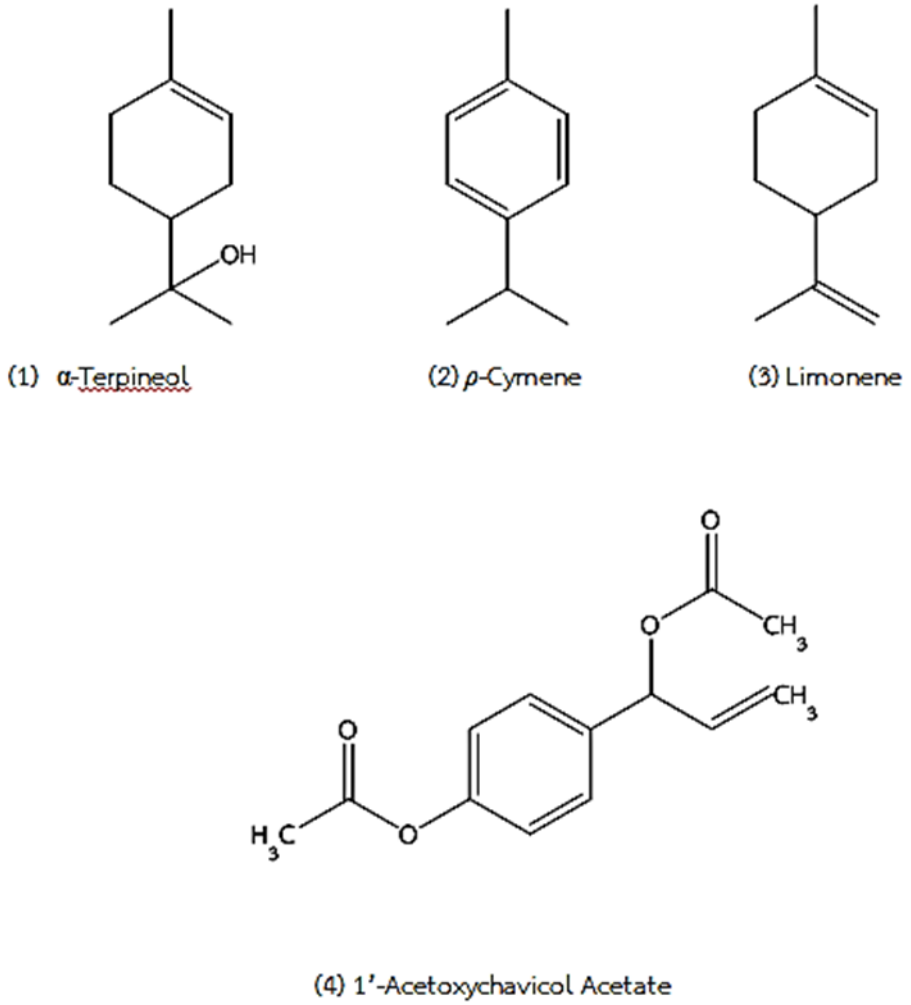



**Figure 2.3** Compound in Rhizome of *Alpinia galangal*.

### 2.3.3 Anti-obesity activity of extracts and pure compounds isolated from rhizome of A. galangal.

Extracts from rhizome of A. galangal have been recognized to have anti-obesity property for a long time. Several studies have demonstrated that extracts from rhizome of A. galangal and its constituent (pure compound like 1'-Acetoxychavicol acetate (ACA) showed board anti-obesity activity.

In 2002, Akhtar et al. [28] showed that extracts from rhizome of A. galangal could decrease blood glucose in rabbits. As compared to alloxan and gliclazide, the extract from rhizome of A. galangal showed higher activity.

In 2011, Duangjai et al. [29] investigated the activity of 12 Thai spices/dietary plant extracts against Hypercholesterolaemia in rats. Among of tested extracts, A. galanga and C. sinensis possessed the most effective inhibition activity against pancreatic lipase with IC50 values of 8.99±3.41 and 12.36±1.23 $\mu gmL^{1}$, respectively.

In 2012, Ohnishi et al. [5] showed that 1' acetoxychavicol acetate (ACA), which extracted from rhizome of A. galangal could inhibit adipogenesis in 3T3-L1 adipocytes with the down-regulation of transcription factors such as PPARγ and C/EBPβ and the upregulation of AMPK. Moreover, ACA also inhibited adipose tissue weight gain in an HFD-induced obesity rat model.

These researches confirmed that extract from rhizome of A. galangal and its constituents particularly 1′- acetoxychavicol acetate (ACA) possesses effective anti-obesity activity, thus further development into a new alternative method for treatment of obesity should be pursued.

### 2.4 Toxicity of extract from rhizome of A. galangal and pure compound

Toxicity is a level to which a substance can harm animals or human. Toxicity can be divided into acute, sub-chronic, and chronic. Toxicity of natural products has to be tested to confirm that the substance is safe for consumption and can be economically marketed. Toxicity of extract from rhizome *of A. galangal* and pure compound has been studied in animal model as summarized in the following reports

In 2012 Subash et al. studied the toxicity of extract from rhizome of *A. galangal* by administering the extracts to mice orally. The extract at the dose of 5, 50, 300, 2000 mg/kg body weight was orally applied to mice weight. However, they reported that the extract showed no mortality in mice at the highest dose of 2000 mg/kg mice weight and extract treated group, observed for over the period of 14 days.

In 2004, Unnisa et al [30] demonstrated the safety use of methanolic extract from rhizome of *A. galangal* by studying the oral toxicity in Wistar rats. Methanol extract at the dose of 50, 300, 2000 and 5000 mg/kg rat weight were administered to rats for 14 days. The results demonstrated that no mortality in rats at the highest dose of 5000 mg/kg rats weight (T3).

In 2000, Qureshi et al.[31] examined acute and subchronic toxicity of *extract from rhizome of A. galangal* in animal model. For acute toxicity study, the extract was given to Swiss albino mice by water and administration orally at a three dose of 0.5 g/kg, 1 g/kg, and 3 g/kg body weight. It did not cause mortality or toxic symptoms in mice after a single dose up to 3 g/kg during 24 h of observation. For the subchronic toxicity study, the extract at 100mg/kg/days body weight was administered by drinking in water during for 3 months. In all case, the results showed that consumption of the extract was non-toxic. It possessed no effect on behavior, food and water intake, growth or health status of tested mice. Their hematology parameter did not change from the control. After the 3 months, no significant dose-related differences in blood biochemical parameters were detected among the male and female groups (T2).

Not only acute and chronic toxicity of *extract from rhizome of A. galangal* in animal model were studied but also cytoxicity in cell was investigated. Ohnishi R. et al. 2012 evaluated ACA extract from *rhizome of A. galangal* in 3T3-L1 adipocyte cell.

The extract was given to the treatment with 2.5 or 5.0 μM for 24 hrs. The results revealed that Cell viability at 24 h was 110:0 ±11:0 and 98:7 ± 11:3% of controls for 2.5 μM ACA-treated cells and 5.0 μM ACA-treated cells, respectively, showing that these concentrations of ACA are not cytotoxic in 3T3-L1 preadipocytes.

The toxicological data obtained from the previous studies indicated that extract from rhizome of *A. galangal* seem to be a safe product [5]. These results agree well with the increased consumption of the extract from rhizome of *A. galangal* for health benefits and remedial purposes worldwide. However, at present, there is no clinical data available on the efficacy of extract from rhizome of *A. galangal* or its compound, in humans. Therefore, further investigation is needed.

## 2.5 Problems of 1' Acetoxychavicol acetate (ACA)

Poor aqueous solubility *in vivo* of *extract from rhizome of A. galangal* and its isolates is the major obstacle to develop the therapeutic application or further the investigation. In a recent study, in 1999, Yang and Eilerman [4] studied pungent principal of ACA and its applications. They found that ACA is not stable in aqueous solution and undergoes hydrolysis/isomerization reactions.

## 2.6 Nanotechnology and nanoencapsulation

Globally, invention is in progress for the development of improved, optimized and advanced drug delivery systems. Nanotechnology is now frequently used for medical therapeutics applications [32-34]. Drug-loaded nanoparticles can affect various characteristics of product including taste, processibility and stability [34-36]. A technique that active molecule like peptide, drug, nucleic acid or natural product extract are being, adsorbed, attached or entrapped to nanoparticles with diameters ranging from 1 to 1000 nm, depending on preparation method which call nanoencapsulation method [37, 38]. Nanoparticle may or may not be biodegradable [39]. Nanoparticle consists of nanosphere and nanocapsule. Nanosphere is a matrix

type particle in which the drug molecules are dissolved or entrapped or attached to the particle matrix. On the other hand, nanocapsules are core-shell structure, in which drug molecules are confined to a cavity surrounded by a polymeric membrane (Figure 2.4) [40, 41]. Normally, active molecules will stay in the core of nanocapsules but in some case it may be adsorbed on the shell surface [41].

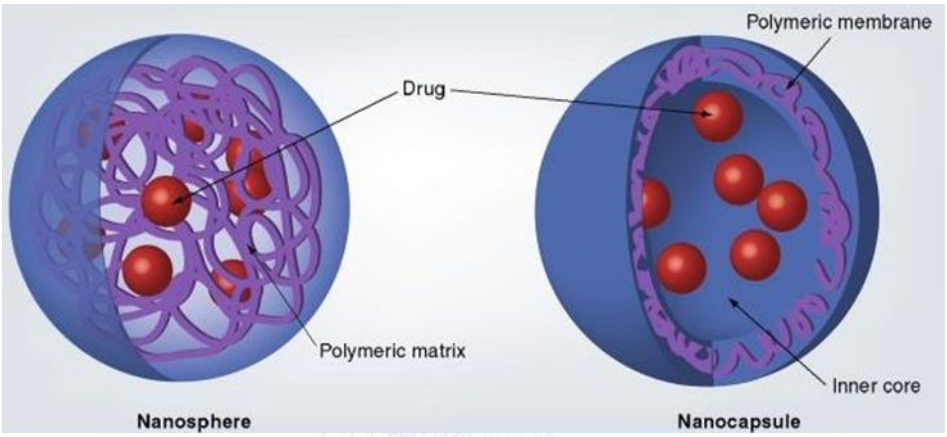



**Figure 2.4** The two main types of polymeric nanoparticles (nanosphere and nanocapsule) [40].

Polymeric nanoparticles (PNPs) are defined as particulate having a size in the range of 10-1000 nm and prepared from polymers (p1). Many polymers have been used to load drug and active molecule, e.g., poly (D,L- lactide), poly(lactic acid) PLA, poly(D,L-glycolide) PLG, chitosan, gelatin etc. Polymeric encapsulation can protect and increase the stability of drug from various environmental threats in body. They can mask the unpleasant tastes such as bitterness. Polymeric nanoparticles are being developed for cancer therapy, vaccine delivery, contraceptives and delivery of targeted antibiotics. Moreover, these drug-loaded polymeric nanoformulations are better than traditional drugs because of their controlled release property, targeted delivery ability and great therapeutic efficiency [41].

## 2.7 Carrier in micro/nano encapsulation systems

The characteristics of prepared particles depend on the nature of particles forming materials. The material should be stable, inert to the loaded drugs, non-hygroscopic, soluble in an aqueous media, and able to control the release of the drug. Many coating/encapsulating materials were have been used in micro/nanoencapsulation nanoencapsulation, e.g., vegetable gums, celluloses and its derivative, condensation polymers, homopolymers, copolymers, proteins etc [42]. Among these, the popular polymer of an encapsulating material is celluloses which widely employed in micro/nanoencapsulation. In this research, polyvinyl alcohol, Beeswax which are molecules, are experimented as shell material for encapsulation. Previous studies proposed various methods for forming the EC microparticles, for example, spray drying [43], emulsion solvent evaporation [44, 45] and self-assemble through solvent displacement.

### 2.7.1 Polyvinyl alcohol (PVA)

Polyvinyl alcohol, (PVA, PV(OH) or PVAL) is known as soluble in water, white powder, tasteless and odorless. It has excellent film forming, emulsifying and adhesive properties. Also, it has high tensile strength and flexibility, as well as high oxygen and aroma barrier properties. Moreover, PVA can be easily biodegraded by a restricted number of aerobic bacteria, among them several species of Pseudomonas. So, PVA is biodegradable of natural polymer.

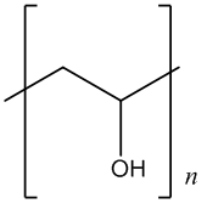



**Figure 2.5** Structure of Polyvinyl alcohol.

### 2.7.2 Beeswax

Beeswax is a natural wax produced by individual honey bees of the genus *Apis*. The three major beeswax products are yellow beeswax, white beeswax, and beeswax absolute. Yellow beeswax is the crude product obtained from the honeycomb. White beeswax is made from yellow beeswax by bleaching, and beeswax absolute is made by treating yellow beeswax with alcohol. Moreover, Beeswax has emollient, soothing and softening properties. The chemical formula for beeswax is [$C_{15}H_{31}COOC_{30}H_{61}$] which consists mainly of esters of higher fatty acids, alcohols and other minor substances produced (Figure 2.6).

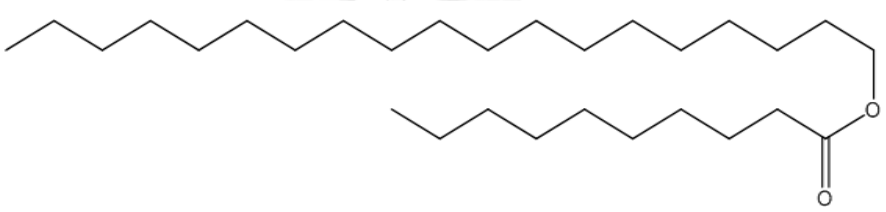



**Figure 2.6** Structure of Beeswax.

### 2.7.3 Applications of polyvinyl alcohol (PVA) and Beeswax

Poor solubilitiy in water of many natural products usually causes difficulty in product formulation. Although surfactants can be used as stabilizing agent, they give turbidity, irritation problems and usually possess limited stability. The encapsulation method can increase solubility of the encapsulated materials and also offer controlled release characteristic.

In 2014, Levic et al [46] studied stability and quality of limonene encapsulated with alginate and poly vinyl alcohol (prepared by freeze-thaw method). It was found that 20% w/w of PVA in polymer blend gave optimal stable gel production. They also found that the loaded limonene in the alginate-poly vinyl alcohol matrix was stable when stored under an elevated temperature.

In 2000, Volkhard and Sven [47] investigated solid lipid nanoparticles (SLN) formulated with either wax or glyceride bulk material. The two were compared with respect to drug encapsulation efficacy, particle size distribution after production and storage, and crystal packing. This research team demonstrated that wax SLN possessed good particle size distribution and physical long-term stability that can be used in drug encapsulation.

## 2.9 Research objectives

The aims of this research can be summarized as follow:

- To load ACA into a blend of PVA and beeswax
- To study stability of ACA from micro/nanoparticles at 30, 45, 75 and 100°C
- To determine the anti-obesity activity of ACA-loaded micro/nanoparticles *in vitro* and *in vivo*
- To evaluate the effect of ACA-loaded micro/nanoparticles on the 3T3-L1 adipocyte
- To investigate the anti-obesity potential of ACA-loaded micro/nanoparticles in mice

# CHAPTER III

# EXPERIMENT

## 3.1 Instruments and Equipments

$^{1}$H Nuclear magnetic resonance (NMR) spectra were obtained in deuterated chloroform (CDCl3-*d1*) with tetramethylsilane (TMS) as an internal reference using ACF 200 spectrometer which operated at 400 MHz for 1H nuclei (Varian Company, Palo Alto, CA, USA). High performance liquid chromatography (HPLC) analysis was performed using C18-AR column (5 µm, 4.6 mm×100 mm, Shandon, UK) at room temperature with UV/VIS detector at 260 nm, using Shimadzu HPLC system (SPD-10A, Shimadzu, Japan). Scanning electron microscopic (SEM) analysis was performed on JSM-6400 scanning electron microscope (JEOL, Tokyo, Japan), SEM samples were prepared by drying in the desiccators at room temperature and coating with gold under vacuum at 15 kV. Transmission electron microscopic (TEM) analysis was performed on a JEM-2100 (JEOL, Tokyo, Japan)

## 3.2 Material and Chemicals

Rhizomes of *Alpinia galangal* was collected from Nakhonsawan, Thailand. Paraffin wax was purchased from Suksapan (Bangkok, Thailand). Poly(vinyl alcohol) (PVA) ($M_w$=146,000-186,000 Da,) was purchased from Sigma-Aldrich (Steinheim, Germany). Acetone methanol and ethanol were purchased from ACI Labscan Limited (Bangkok,Thailand) Other reagents were analytical grade and were used without further purification.

## 3.3 Extraction of 1'-Acetoxychavicol acetate extract

The rhizome of *Alpinia galangal* (20 kg) was extracted with hexane (5000 ml) by macerating the shredded rhizome in the solvent for 24 h at room temperature. The hexane extract was obtained by filtering the suspention through Whatman No. 1

filter paper collecting the filtrate and removing solvent from the liquid filtrate by rotary evaporation. The extract was transferred into tubes and stored at -20℃ for further use.

### 3.3.1 HPLC condition for determining the amount of 1'-Acetoxychavicol acetate extract

The amount of ACA in the extract was quantified by a Shimadzu HPLC system with a C18-AR column (5 µm, 4.6 mm×100 mm, Shandon, UK) at room temperature. The mobile phase was 60:40 (v/v) of methanol: water using a flow rate of 1 mL/min.

## 3.4 Encapsulation of 1'-Acetoxychavicol acetate extract

ACA solid lipid microparticles were prepared. ACA was encapsulated into paraffin wax microparticles using microemulsion homogenization technique, 5 g of paraffin wax were mixed with 5 g ACA at 60°C and the obtained solution was then added to the PVA solution (40 mL. 5 g PVA in water) while homogenizing at 10000 rpm at 60°C for 30 minutes. using the homoginizer. The final product was characterized by scanning electron microscopy (SEM; JSM-6400, JEOL, Tokyo, Japan). Encapsulation efficiency percentage (%EE), and loading capacity (%loading) of the obtained microparticles were evaluated by performing the ethanol extraction of ACA from the microparticles and quantifying ACA in the extract by HPLC (C-18, 4.6 × 100 mm) with the aid of a calibration curve.

### 3.4.1 Determination of the optimum weight ratio of PVA

To adjust release, improve dispersibilty in water and increase mucoadhesive property of the prepared microparticles, PVA was blended with paraffin wax for ACA encapsulation. Varying the weight ratio of PVA and paraffin wax was carried out as shown in Table 3.1.

**Table 3.1** Weight ratio of PVA.

| | Amount of PVA (g) | Amount of paraffin wax (g) | Ratio |
|---|---|---|---|
| 1 | 5 | 5 | 1:1 |
| 2 | 4.5 | 5 | 0.9:1 |
| 3 | 3.5 | 5 | 0.7:1 |
| 4 | 3 | 5 | 0.6:1 |
| 5 | 2.5 | 5 | 0.5:1 |

Encapsulation process was started by dissolve PVA with water (40 ml) at 60 °C. Heating was stopped when PVA solution turned clear. Then, The PVA solution was cooled down to the room temperature. At the same time, Paraffin wax was dissolved with ACA in a beaker at 60°C and the obtained solution was added to the PVA solution while homogenizing at 10000 rpm at 60 °C for 30 minutes. Finally, the %EE and %loading of all preparations were determined. The optimum weight ratio of PVA to paraffin wax was obtained by subjecting the extracts from the particles to HPLC analysis.

### 3.4.2 Determination of the optimum weight ratio of PVA and paraffin wax to ACA

From the previous experiment, the optimum weight ratio of paraffin wax to PVA was 1:1. This weight ratio was fixed while varying the weight ratio of these shell materials to ACA (Table 3.2).

**Table 3.2** Various weight ratios of shell material to ACA.

| | Weight ratio of ACA and Paraffin wax to PVA | Amount of PVA (g) | Amount of paraffin wax (g) | Amount of ACA (g) |
|---|---|---|---|---|
| 1 | 1:1:0.7 | 3.5 | 5 | 5 |

Encapsulation process was carried out similarly to that described in 2.4.1. Briefly, PVA was dissolved in water (40ml) at 60°C until the PVA solution turned clear. Then, PVA solution was added into the liquid mixture of paraffin wax and ACA (heated at 60 °C) while stirring at 10000 rpm until the mixer become emulsion. Finally, the %EE and %loading of all preparations were determined.

## 3.5 Characterization of ACA-loaded microparticles

### 3.5.1 Morphology and size of microparticles

Morphology of ACA-loaded microparticles was characterized using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The size of particles were measured by Atomic force Microscopy (AFM).

#### 3.5.1.1 Scanning electron microscopic analysis (SEM)

SEM was performed by the Center for Analytical Service, Faculty of Science, Chulalongkorn University, Thailand. A drop of the microparticles suspension was placed on a glass slide and dried overnight. The sample was coated with a gold layer under vacuum at 15 mA for 90 s. The coated sample was then mounted on an SEM stud for visualization. The accelerating voltage used was 15 kV. The SEM photographs of ACA-loaded microparticles were obtained using JSM-6400 (JEOL, Ltd., Japan).

#### 3.5.1.2 Transmission electron microscopic analysis (TEM)

TEM was performed by the Scientific and Technological Research Equipment Center, Chulalongkorn University, Thailand. TEM photographs were obtained using JEM-2100 (JEOL, Ltd., Japan) with an accelerating voltage of 120 kV in conjunction with selected area electron diffraction (SAED). A glass slide was dipped into the obtained suspension to gain the dried smooth film of nanoparticles on surface of the glass slide.

#### 3.5.1.3 Atomic force Microscopy (AFM)

AFM was performed by the Scientific and Technological Research Equipment Center, Chulalongkorn University, Thailand. AFM was performed using a PicoSPM instrument (Molecular Imaging Corp. Tempe, AZ). Imaging was carried out using oxide-sharpened silicon nitride AFM probes with a tip radius of ca. 10 nm (cantilever nominal spring constant= 012 N $m^{-1}$; resonance frequency of approximately 26 Hz; Veeco Probes, Camarillo, CA) in the contact mode. The scanning force constant was 50-80 nN and scan rates were approximately 0.2 Hz. Image areas of 5.0 by 5.0 μM were collected. A glass slide was dipped into the obtained suspension to gain the dried smooth film of microparticles on surface of the glass slide.

#### 3.5.1.4 Differential scanning calorimetry analysis (DSC)

DSC was performed by the Scientific and Technological Research Equipment Center, Chulalongkorn University, Thailand using a Netzsch DSC 204 Phoenix. Ten milligrams of the dry samples were precisely weighed into aluminum cups and sealed. A small hole was done at the top of the cup in order to allow the release of water. An empty cup was used as reference. The experiment was scanned from -4 to 300°C. The experiments were performed under nitrogen with a scanning rate of 10 °C min−1

### 3.6 Determination of loading capacity and encapsulation efficiency of ACA-loaded microparticles

All of the obtained suspension of ACA-loaded microparticles (polymer:ACA 1.7:1 w/w) (5 ml) were obtained solid on filter that was soaked and mixed in 5 ml ethyl acetate for 1 hour at room temperature to ensure that the entire extract was dissolved. Then, the ethyl acetate was evaporated using evaporator. After that, filter was soaked and mixed in 5 ml ethanol for 1 hour at room temperature to ensure

that the entire extract was dissolved. Then, ethanol solution was filtered through filtering centrifugal tube (MWCO 10,000 (Amicon Ultra-15)). Centrifugation was carried out on an Allegra 64R Avanti 30 (Beckman Coulter, Inc, Brea, USA). Finally, the ethanol solution was quantified for ACA using High Performance Liquid Chromatography at 267 nm with the aid of a calibration standard curve. Standard solutions were freshly prepared in ethanol with the final volume of 5 ml at concentration of 0, 3,000, 5,000, 7,000, 15,000 and 20,000 ppm (see calibration curve in appendix A) using Shimadzu HPLC system (SPD-10A, Shimadzu, Japan). The encapsulation efficiency (%EE), loading capacity (%loading) were calculated using equation (1) and (2), respectively.

$$\%EE = \frac{\text{Weight of encapsulated ACA in the particles}}{\text{Weight of ACA initially used}} \times 100 \quad (1)$$

$$\%loading = \frac{\text{Weight of encapsulated ACA in the particles}}{\text{Weight of encapsulated ACA + polymer}} \times 100 \quad (2)$$

### 3.7 Determination of anti-obesity activity

#### 3.7.1 Cell culture and culture condition

3T3-L1 preadipocyte (fibroblast) were ordered from American Type Culture Collection (ATCC) and cells were received in the frozen state. 3T3-L1 preadipocytes are fibroblasts therefore they are adherent in nature. The 3T3-L1 were grown on DMEM (ATCC 30-2002™) supplemented with 10% bovine calf serum (ATCC 30-2030™) for 48 h.and incubated at 37°C under microaerophilic conditions (5% $O_2$, 10% $CO_2$, and 85% $N_2$) in a jar enclosed with a AnaeroPack-MicroAero (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) Next, the identical volume of medium was replaced with differentiation medium (DM) (Day 0) and incubated for 48 h at 37°C in humidified atmosphere containing 5% $CO_2$. After that, the differentiation medium was replaced with adipocyte maintenance medium (AMM); AMM was changed every 48h.

Finally, the cells were fully differentiated at day 10 after induction. To examine the effect of free ACA, ACA-loaded nanoparticles, Blank PVA/paraffin wax microparticles and Degraded ACA on adipocyte differentiation, 3T3-L1 cells were treated with pre-adipocyte expansion medium PEM until day 8 in the absence or presence of 5 concentrations from 0 μM to 100 μM of free ACA, ACA-loaded microparticles, Blank PVA/paraffin wax microparticles and Degraded ACA. Also, a flask of cells containing PEM (non-differentiated cells) was maintained until day 8.

**Table 3.3** Description of media formulations.

| MEDIUM | FORMULATION |
|---|---|
| Pre-adipocyte Expansion | 90% Dubelcco's Modified Eagle's Medium (DMEM), and 10% Bovine Calf Serum |
| Differentiation | 90% DMEM, 10% Fetal Bovine Serum (FBS), 1.0 µM Dexamethasone, 0.5 mM Isobutylmethylxanthine (IBMX), and 1.0 µg/ml Insulin |
| Adipocyte Maintenance | 90% DMEM, 10% FBS, and 1.0 µg/ml Insulin |

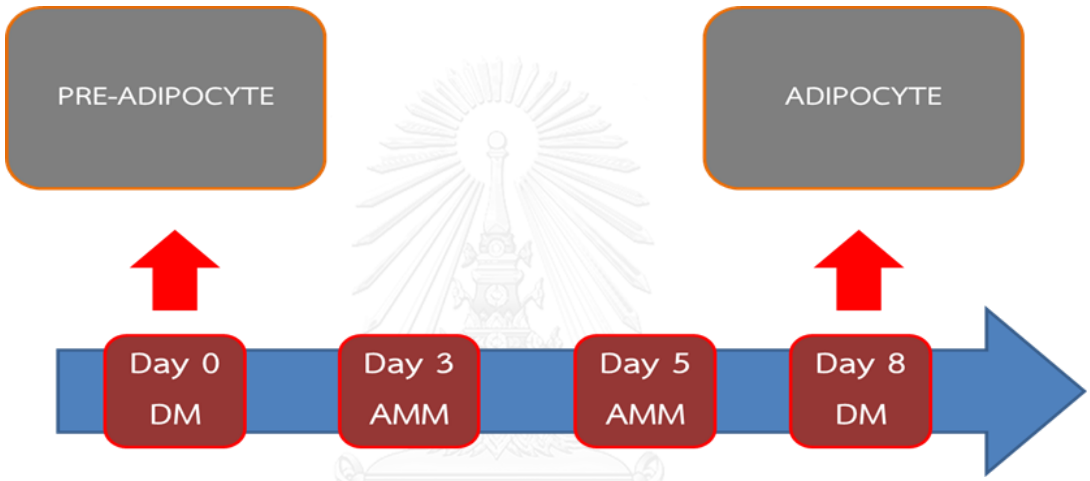



**Figure 3.1** Protocol for chemical-induction of differentiation. Differentiation Medium (DM), Adipocyte Maintenance Medium (AMM).

3.7.2 MTT Cell Viability Assay

3T3-L1 cells were seeded in 96-well plates at a density of 1.0x104 cells per well. After post-confluence for 2 days, cells were differentiated with various concentrations of free ACA, ACA-loaded microparticles, Blank PVA/paraffin wax microparticles and degraded ACA (0-100 µM). At the end of the treatment period, the cell viability was detected by MTT assay. In brief, the MTT assay was performed by incubating cells in 100 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) dissolved in serum-free DMEM for 4h at 37°C incubator. All unreacted dye in each well was aspirated, and then the insoluble formazan crystals were dissolved in 100 µL acidified isopropanol (0.04N HCl). The

MTT assay was measurement of absorbance a 540 nm by a microplate reader (Model 340, Germany).

### 3.7.3 Oil Red O Staining for lipid accumulation

The 3T3-L1 cells were seeded in 48-well plates at a density of $1.05x10^4$ cells per well. Cells were subjected to differentiation following the protocol described before including non-differentiated, control, and different concentrations of free ACA, ACA-loaded nanoparticles, Blank PVA/paraffin wax microparticles and Degraded ACA (0-100 μM). At day 8 of differentiation, 3T3-L1 adipocytes were washed with PBS and fixed with 10% formalin for 30 min. Then they were washed with distilled water twice, the cells were stained for 45 minutes in a 37°C incubator with diluted oil red O solution. Treatments were photographed with a microscope OLYMPUS Model DP72 (Center Valley, PA). Finally, the dye retained in 3T3-L1 cells was eluted with isopropanol and pipetted in a 48 well-plate to measure the absorbance by a microplate reader (Titertek Multiskan MCC/340 (Dusseldorf, Germany) at 510 nm.

### 3.7.4 Statistical analysis

All data were presented as means ± standard deviation (SD). Statistical significant difference between experiment and control groups was determined by one-way analysis of variance (ANOVA) and pos hoc Dunnett test at the 95% confidence level using SPSS Statistic Base 17.0 (SPSS Co., Ltd., Thailand, Server IP Address; dc1.win.chula.ac.th). Statistical significance was accepted at $p < 0.05$.

## 3.8 *In vivo* anti-obesity of ACA in mice

### 3.8.1 Sample preparation

Three samples, unencapsulated ACA (40 mg/ml), ACA –loaded microparticles, (70.2 mg/ml containing ACA 40 mg/ml) and blank polymer blend of

PVA and paraffin wax microparticles (70.2 mg/ml), in emulsion form, were resuspended in sterile water.

### 3.8.2 Animals and animal care

In this experiment, forty healthy C57BL/6 male mice weigh between 25 to 30 g were used. All animals were purchased from National Laboratory Animal Centre, Mahidol University (Nakhonpathom, Thailand). These mice were randomly divided into 8 groups and 5 mice per group. The procedures were approved by the ethics committee and the institutional animal care and use committee (IACUC) of Chulalongkorn University (no. 1431047).

The animals were kept for at least 7 days before starting the experiment at Faculty of Veterinary Science, Chulalongkorn University, Bangkok Thailand, for their acclimatization to the laboratory conditions. The animal room was 24-25°C with 12:12 light dark cycle. All animals had free access to water and food, throughout the study, except for the short fasting period before the oral administration of ACA suspension. The selected individual animals were weighed and identified the number of animals. Five mice of a group were housed per cage. Additionally, all cages were labeled with details of sample and dose administered.

### 3.8.3 Preparation of diet

The animals were fed a pelletized chow diet for a week. They were then randomly divided into 8 dietary groups (n=5). At the second week, 20 mice were fed with normal-diet (ND), and 20 mice were fed with the HFD containing 240 g fat/kg (170 g lard plus 70 g soybean oil in normal diet) for 5 weeks.

### 3.8.4 Oral administration

Mice were administration of unencapsulated ACA, ACA –loaded microparticles, blank polymer blend of PVA and paraffin wax microparticles (5-10

μM). All mice were fed. After that, each of group was assigned to receive dose of free ACA 300 mg/kg bodyweight and ACA –loaded microparticles (Paraffin wax 860.8 mg/kg and PVA 423.9 mg/kg) of mice. ACA –loaded microparticles and free ACA. Control group received sterile water at the same volume of sample via oral route using feeding needle.

### 3.8.5 Anti-obesity test in mice

#### 3.8.5.1 Monitoring of animal after samples administration

After the administration of the samples suspension via oral route, the general behavior and toxic symptoms of the mice were observed at 1, 2, 4 and 6 hours after administration and the number of survival mice was recorded after 24 hours of administration. Then, daily behavior and unusual symptoms were observed. Weights of mice were measured at day 1, 4, 7, 14 and 35. At Day 35, all survival mice were anesthetized to collect blood, organ weight and liver samples.

#### 3.8.5.2 Serum biochemistry analysis

All survival mice were anesthetized at 35 days of administration. Blood were collected and put into the microcentrifuge tube. After clotted, serum was separated by centrifugation. Then, the serum were analyzed for blood urea nitrogen (BUN), creatinine, cholesterol, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatases (ALP), triglyceride and low density lipoprotein (LDL) by spectrophotometry (Vitalab Flexor XL, Holliston, USA).

#### 3.8.5.3 Liver lipid evaluation

The liver samples were immediately freezed in dry ice in the cryopreserve medium ( O.C.T. Compound; Tissue-Tek®, Sakura, Japan) and transfer to -80°C until cut. The frozen liver were cut with Cryostat (Leica 1800, Leica Biosystems, Germany) into 6 microns thickness placing in the microscopic slides and

stained with oil red O staining. The stained slide were photographed for 5 randomized filed at 20x magnification with the central vein as a land marker using light microscope, then the positive oil red O stained area was evaluated by the Image J analytical program (http://imagej.nih.gov/ij/). The percentages of positive-oil red O staining were recorded.

**3.8.5.4 Statistical analysis**

All data were presented as means ± standard deviation (SD). Statistical significant difference between experiment and control groups was determined by one-way analysis of variance (ANOVA) and pos hoc Dunnett test at the 95% confidence level using SPSS Statistic Base 17.0 (SPSS Co., Ltd., Thailand, Server IP Address; dc1.win.chula.ac.th). Statistical significance was accepted at $p < 0.05$.

# CHAPTER IV
# RESULTS AND DISCUSSION

Obesity is a growing global concern since it can induce various diseases such as diabetes, hypertension, atherosclerosis, coronary heart disease, respiratory disorders, cancer, and osteoarthritis [1]. Prevalence of obesity worldwide is approximately 39%. The obesity drugs are limited for long term success because the weight is regained when treatment is discontinued [5]. These factors indicate the need to find new anti-obesity agent from natural products. *Alpinia galangal* or *Galangal*, a rhizome closely related to ginger family, is usually used for cooking especially for Thai and Indonesia foods such as curry pastes, Tom-Yam (Thai soup) and many other curries. The rhizome of *Alpinia galangal* has been used as a medicinal agent by Southeast Asians for centuries, for the treatment of many diseases including skin diseases, indigestion, cancer of mouth and stomach [8-10]. Recently, antiobesity activity has also been identified for the *Alpinia galangal* extract. There is a report showing that 1′-acetoxychavicol acetate (ACA) is a major component in the extract of *Alpinia galangal*'s rhizome [6]. Although ACA possesses many medicinal and pharmaceutical activities such as antioxidant [7], anti-inflammatory [8] and anti-obesity properties [5], therapeutic efficiency and application of ACA are limited because of its instability, poor aqueous solubility and low bioavailability [4]. Here, microencapsulation was used to overcome such problems.

## 4.1 Extraction and stability of ACA

ACA was extracted from the fresh of *Alpinia galangal* by macerating the shredded rhizome in the hexane for 24 h at room temperature. The hexane extract was obtained by filtering the mixture through Whatman No. 1 filter paper and

collecting the filtrate and after that, solvent was removed from the liquid filtrate by rotary evaporation. The extraction process gave the final yield of 0.7% (wt extract to weight of the fresh rhizomes). NMR analysis of the extract (Figure 4.1(A)) indicated acceptable purity of ACA.

We investigated the stability of ACA which is the major compound in the obtained extract. The obtained ACA was subjected to refluxed in 5% (v/v) ethanol (ethanol was needed to make ACA miscible with water) in water for 2 hours and the structural change of ACA was analyzed by $^{1}$H NMR. The $^{1}$H NMR analysis of the product indicated degradation of ACA as shown in Figure 4.1. It was obvious that isomerization reaction took place and cinnamic alcohol was identified as the major breakdown product. This result agrees well with previous report [6].Therefore, we conclude that ACA can undergo hydrolysis under a two-h reflux in water and the degraded products were *p*-acetoxycinnamic alcohol. Thus we conclude that ACA is unstable in hot water.

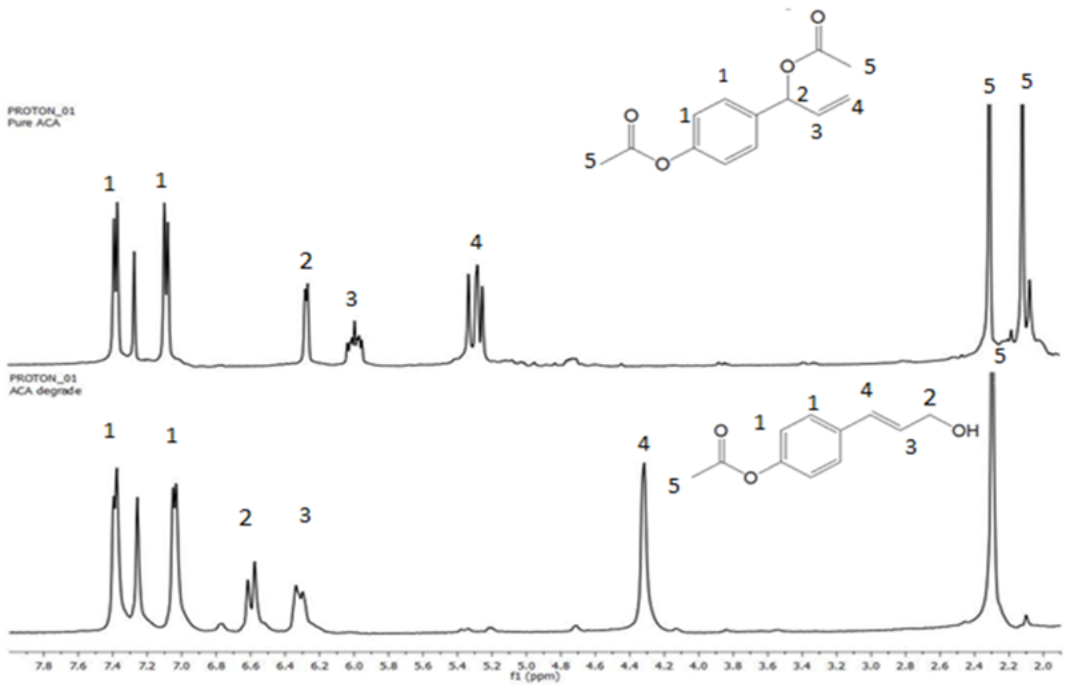



**Figure 4.1** $^{1}$H NMR spectrum of free ACA (A) and degraded ACA (B) in CDCl3.

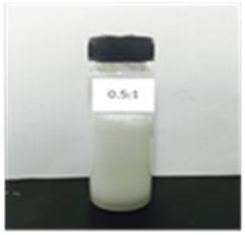
## 4.2 Encapsulation of ACA

To increase stability of the ACA, we prepared ACA-loaded microparticles using solid lipid microparticle formation method via homogenization. ACA was loaded into wax-polymeric microparticles via self-assembly process. Here, polyvinyl alcohol (PVA) and paraffin wax were used as a particle matrix. This method was adapted from the method of solid-lipid particle formation. Here the solid paraffin wax was melted and the liquid wax was homogenize with water to form microdroplets, and poly(vinyl alcohol) or PVA was used to surround the particles so that they could be dispersible in water.

### 4.2.1 Determination of the optimum weight ratio of PVA and paraffin wax

The optimal weight ratio between paraffin wax and PVA was investigated. Five ratios which were 0.5:1, 0.6:1, 0.7:1, 0.9:1 and 1:1, of PVA to paraffin wax were compared by observing the precipitation and viscosity of the obtained suspension (Table 4.2). The ratios of PVA to paraffin wax of 1:1 and 0.9:1 gave too sticky suspension. The ratios of PVA to paraffin wax of 0.5:1and 0.6:1 gave obvious precipitates. Therefore, the best ratio between paraffin wax and PVA was 0.7:1, the suspention obtained at this ratio appeared as white suspension with no precipitate. Thus, the optimum weight ratio of paraffin wax: PVA for ACA encapsulation was 0.7:1. This ratio was used to encapsulated ACA in the further experiments.

**Table 4.1** Appearance of prepared microparticles at different weight ratios of PVA of different.to paraffin wax.

| | Ratio<br>Paraffin wax:PVA | Appearance of particle suspension | |
|---|---|---|---|
| 1 | 1:1 |  |  |
| 2 | 0.9:1 |  |  |
| 3 | 0.7:1 |  |  |
| 4 | 0.6:1 |  |  |
| 5 | 0.5:1 |  |  |

### 4.2.3 ACA encapsulation and determination of encapsulation efficiency (%EE) and loading capacity (%loading)

Here ACA was loaded into the PVA-wax particle (1:0.7 (w/w) PVA to paraffin wax) using similar process to that used to form the unloaded particles. Here ACA was mixed into the ethanolic solution of wax, and the obtained liquid oil phase was homogenized with water in the presence of PVA. The %EE and %loading of ACA-loaded PVA-wax microparticles prepared at the weight ratio of ACA: PVA: wax of 1:1:0.7, was 63.15 ± 0.50% and 28.30 ± 0.30%, respectively (Table 4.3). The obtained suspension showed no precipitate and gave high amount of ACA loading. Therefore, we conclude that the ACA microcapsules can be prepared at the weight ratios of ACA and paraffin wax to PVA for 1:1:0.7.

## 4.3 Characterization of ACA-loaded microparticles

We observed that the ACA particle dispersed well in water with no prescipitation, even after being kept for more than 6 months at room temperature (Figure 4.2). Characteristics of ACA-loaded microparticles were analyzed using confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and atomic force microscopy (AFM).

CLSM, SEM and AFM photographs showed that the ACA-loaded microparticles were spherical (Figure 4.2 and 4.3) and particle size was around 5-10 μm.

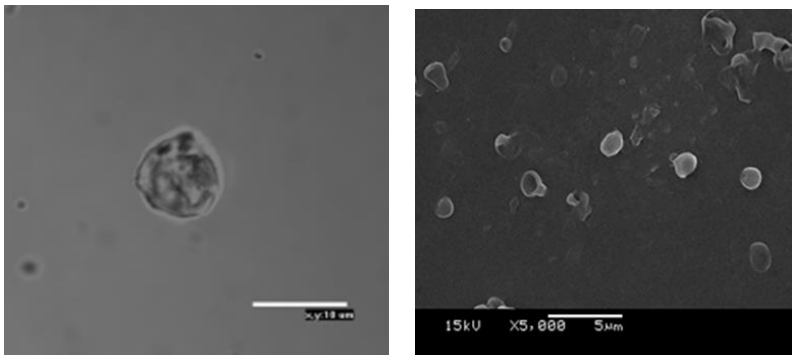



**Figure 4.2** CLSM (left) and SEM (right) photographs of ACA-loaded microparticles.

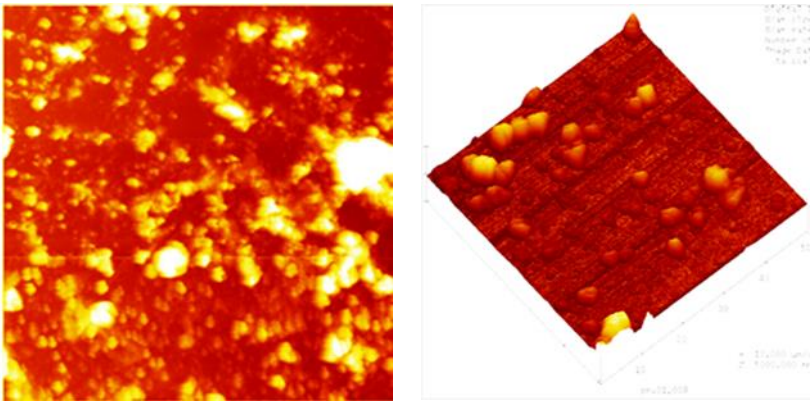


**Figure 4.3** AFM photographs of ACA-loaded microparticles.

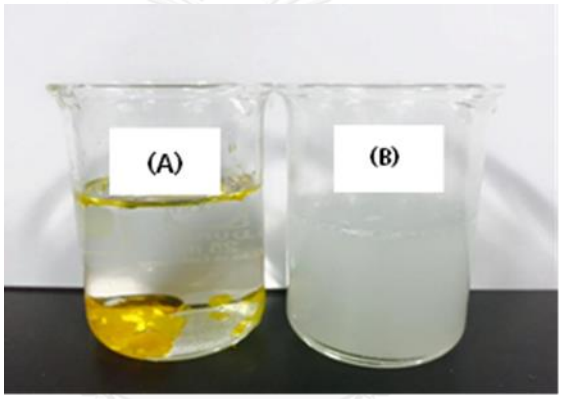
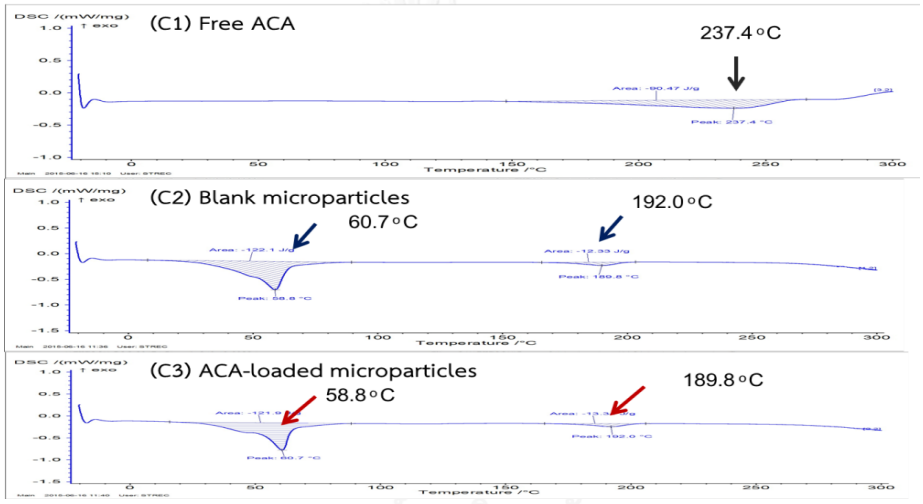



**Figure 4.4** Appearance of unencapsulated ACA (a) and ACA-loaded microparticles (ACA and paraffin wax to PVA for 1:1:0.7) in water (b).

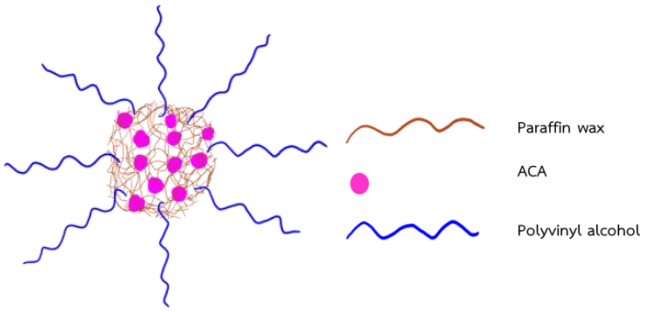
### 4.3.1 Form of ACA within polymeric microparticles

The interaction of ACA and polymer in the microparticles was investigated using differential scanning calorimetry (DSC). The endothermic peak of free ACA was observed at 237.4°C (Figure 4.5 a, and C1) which corresponded to the melting temperatures of ACA while the endothermic peak at 237.4°C of ACA-loaded microparticles was absent (Figure 4.12 and C2). This indicates that there is no crystalline phase of ACA inside the particles. Moreover, the endothermic peaks of ACA-loaded microparticles were observed at 58.8°C and 189.8°C (Figure 4.5 a, and C3). These peaks were different from the endothermic peaks of blank microparticles

(60.7°C and 192.0°C) (Figure 4.5 a, C2), which represented the melting peak of paraffin wax and PVA polymerchains. The shift of all endothermic peaks of ACA-loaded microparticles comparing to the endothermic peaks of free ACA and blank microparticles indicated good interaction between ACA and wax molecules and polymer chains in the particles. Thus, it could be concluded that in the microparticles, ACA was in an amorphous or disordered-crystalline phase, it was likely to be in the solid solution state with good interaction between ACA and sphere matrix components (Figure 4.5 b).




(a)




(b)

**Figure 4.5** Differential scanning calorimetric thermogram of free ACA, blank microparticles, ACA-loaded microparticles (a). The model of ACA-loaded microparticles (b).

### 4.4 *In vitro* ACA stability study

Previous research showed that ACA was not stable in aqueous solution [4]. So, we encapsulated ACA into the wax microparticles to improve its stability. We examined the stability of free ACA and ACA –loaded microparticles using 1H NMR. After refluxing with water at 30, 45, 75, 100°C, the amounts of remained ACA was observed for the encapsulated and non-encapsulated ACA. The result showed that the free ACA markedly degraded after 2 h incubation at 45, 75, 100 °C. On contrary, the encapsulated ACA showed no significant degradation when kept at 45 and 75 °C. ACA –loaded microparticles showed approximately30 % degradation when kept at 100 °C (Figure 4.7). At room temperature, both encapsulated and non-encapsulated ACA were stable because microparticle shell which made form PVA polymer and paraffin wax could protect the ACA from moisture.

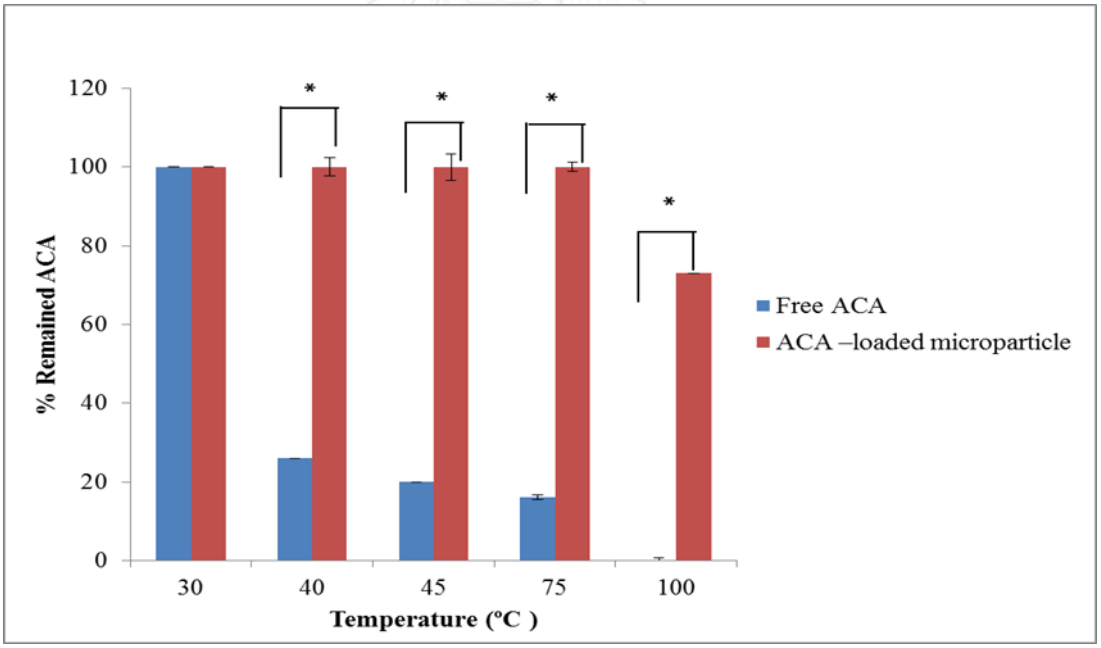



**Figure 4.6** Effect of temperature on degradations of free ACA and ACA-loaded microparticles. The samples were exposed to the tested temperatures for 2 h. Data are shown as the mean ± 1 SD and are derived from 3 independent repeats. Means with a different lower case letter (above the bar) are significantly different (* $p < 0.01$).

## 4.5 Cytoxicity

The free ACA and the ACA-loaded microparticle were tested for their toxicity on a 3T3- L1 preadipocyte cell line using the MTT assay. Unloaded microparticles and degraded ACA (obtained by subjecting ACA to heat, characterized by NMR) were also analysed.

As shown in Figure 4.6, the free ACA (23 µg/mL (100 µM)), ACA–loaded microparticle (23 µg/mL ACA, 55 µg/mL wax, 38.3 µg/mL PVA), blank microparticles (55µg/mL wax, 38.3 µg/mL PVA) and degraded ACA (23 µg/mL) did not showed any toxicity to 3T3-L1 preadipocyte cell. The samples were incubated with the cells for 24 h. Concentration of ACA was 23 µg/mL. For the particles, the wax and PVA concentration were 55 and 38.3 µg/mL,respectively.

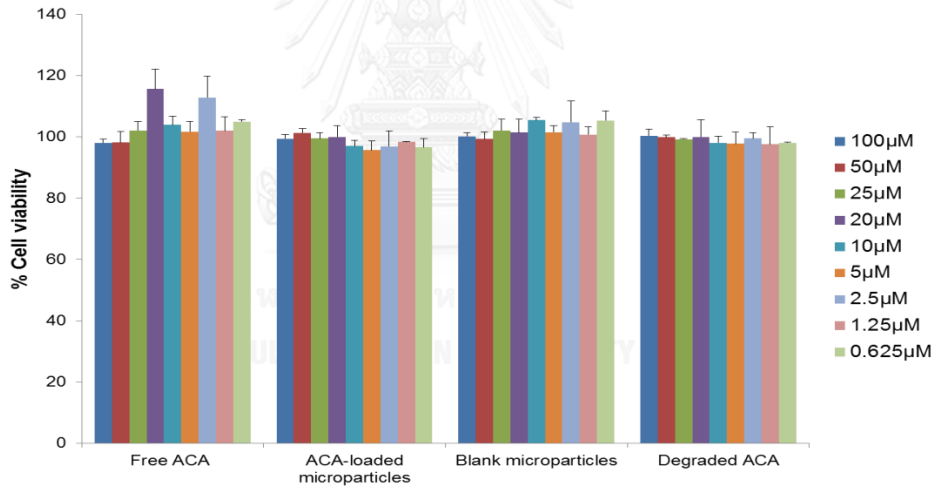


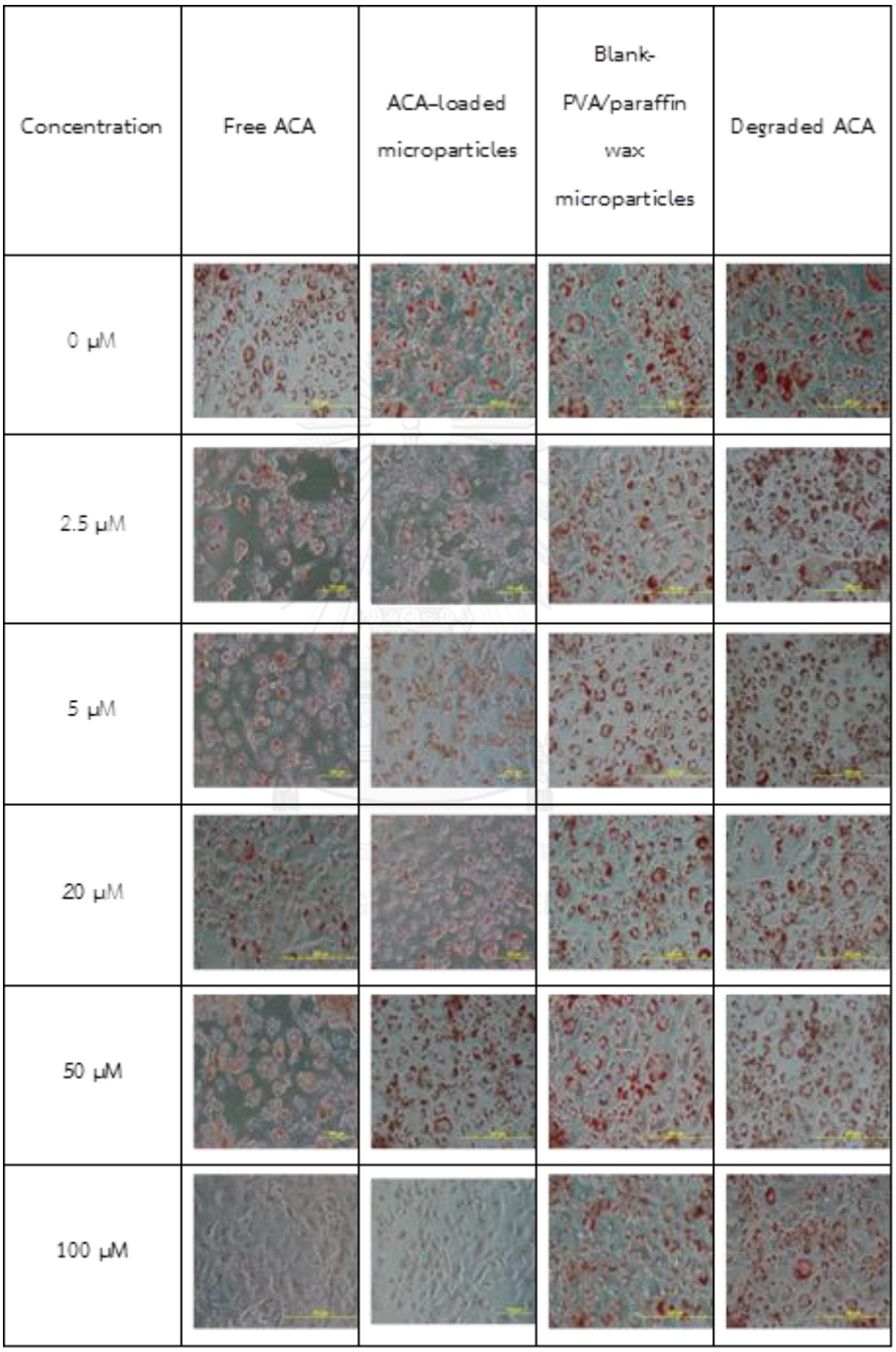

**Figure 4.7** Effect of free ACA, ACA–loaded microparticle, unloadedPVA-wax microparticles and degraded ACA, on the proliferation of preadipocytes.

## 4.6 In vitro anti-obesity activity

Ability to inhibit fat accumulation in 3T3-L1 adipocyte cells was investigated by incubating various concentrations of tested samples with the cells and evaluating the fat content in the cells using oil red O staining. The oil red O usually can bind to fats and give red color appearance [20]. As shown in Figure 4.8, the results indicated

that free ACA and ACA-loaded microparticle could inhibit the lipid accumulation in 3T3-L1 cells more effectively than either the blank PVA wax microparticles or the degraded ACA. This result indicated that the ACA-loaded microparticles could inhibited lipid accumulation up to 43% while free ACA could inhibited lipid accumulation up to 38.5% at high condition (100μM) (Figure 4.9). The result indicated that ACA-loaded microparticles could improve anti-obesity activity of ACA. We speculated that the improvement was a result of better stability of ACA in the aqueous medium.

**Figure 4.8** The effect of free ACA, ACA –loaded microparticle, unloaded PVA-wax microparticles and degraded ACA, on lipid accumulation in cultured 3T3-L1 adipocytes. Scale bars, 200 μm.



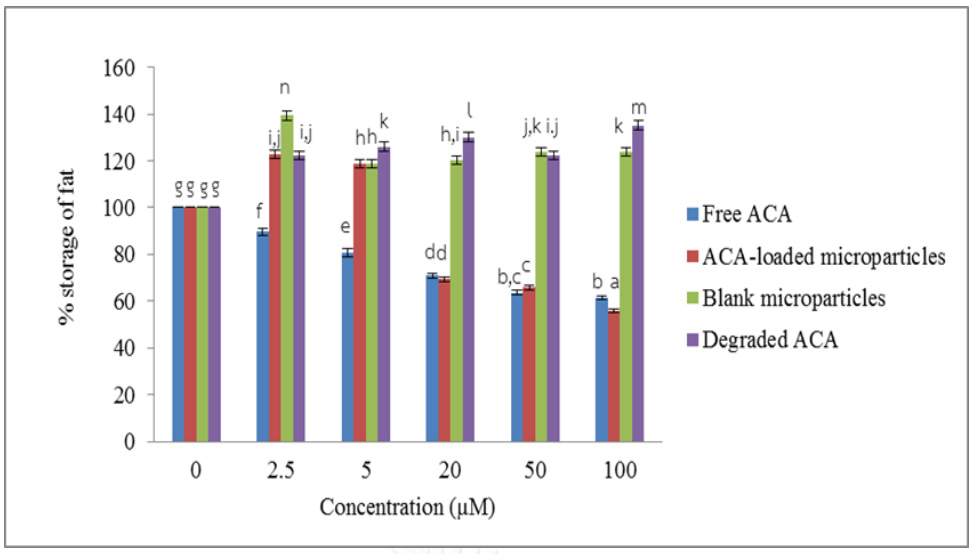



**Figure 4.9** Effect of free ACA, encapsulated ACA, unloaded particle and degraded ACA, on lipid accumulation in 3T3-L1 adipocytes cell. The results are presented as the mean±S.D. of six experiments and are derived from 3 independent repeats. Means with a different lower case letter (above the bar) are significantly different p < 0:05 from control cells (no sample).

## 4.7 *In vivo* test in mice

Previous research showed that free ACA could decrease fat accumulation in mice [5]. Moreover, the cytotoxicity of ACA and their encapsulated were observed at 0-100 µM and ACA-loaded microparticles also possessed good **anti-obesity** activity *in vitro*. These properties were very interesting, therefore evaluated for the material was further **anti-obesity** activity *in vivo*.

Here, a preliminary *in vivo* obesity clearance study of ACA-loaded microparticles was carried out in forty C57BL/6 mice fed with the normal diet (ND) and high fat diet (HFD) groups. All experimental animals were separated into 4 groups to feed with distilled water (negative group), blank PVA/paraffin wax microparticles (blank carrier group), ACA-loaded microparticles (encapsulated ACA group) and free ACA (unencapsulated ACA group) at a ACA dose of 300 mg/kg (or corresponding

PVA/paraffin wax particle dose for the empty PVA/paraffin wax microparticle control, respectively) for thirty five consecutive days using a feeding needle. This experiment observed changes in at the end point measured including body weight, adiposity, and liver histopathology. The HFD increased body weight (Fig. 4.10). No significant difference was observed in the mean food intake (g) of HF groups and ND groups over the 35 days period. However, the mean body weight of the HFD group was more weight than did ND groups, although this trend was not significant. The amount of food intake was similar in mice fed an ND with free ACA and mice fed an ND with ACA-loaded microparticles and between mice fed an HFD with free ACA and mice fed an HFD with ACA-loaded microparticles. To examine whether the body weight in the ACA-treated group was related to decrease the weight of organs and visceral fat were examined (Table 4.3).

The most observed lipid accumulation in liver was observed by oil red O stained. Liver samples of animal group which was fed ND with ACA-loaded microparticles (encapsulated ACA group), showed less lipid accumulated on tissue while the liver samples from the other groups (unencapsulated ACA group, negative control group and blank carrier group) showed higher lipid accumulation. This implied good inhibit lipid accumulation in the liver of the material. As shown in Figure 4.11 and 4.12, ACA-loaded microparticles possessed the highest anti-obesity activity in mice.

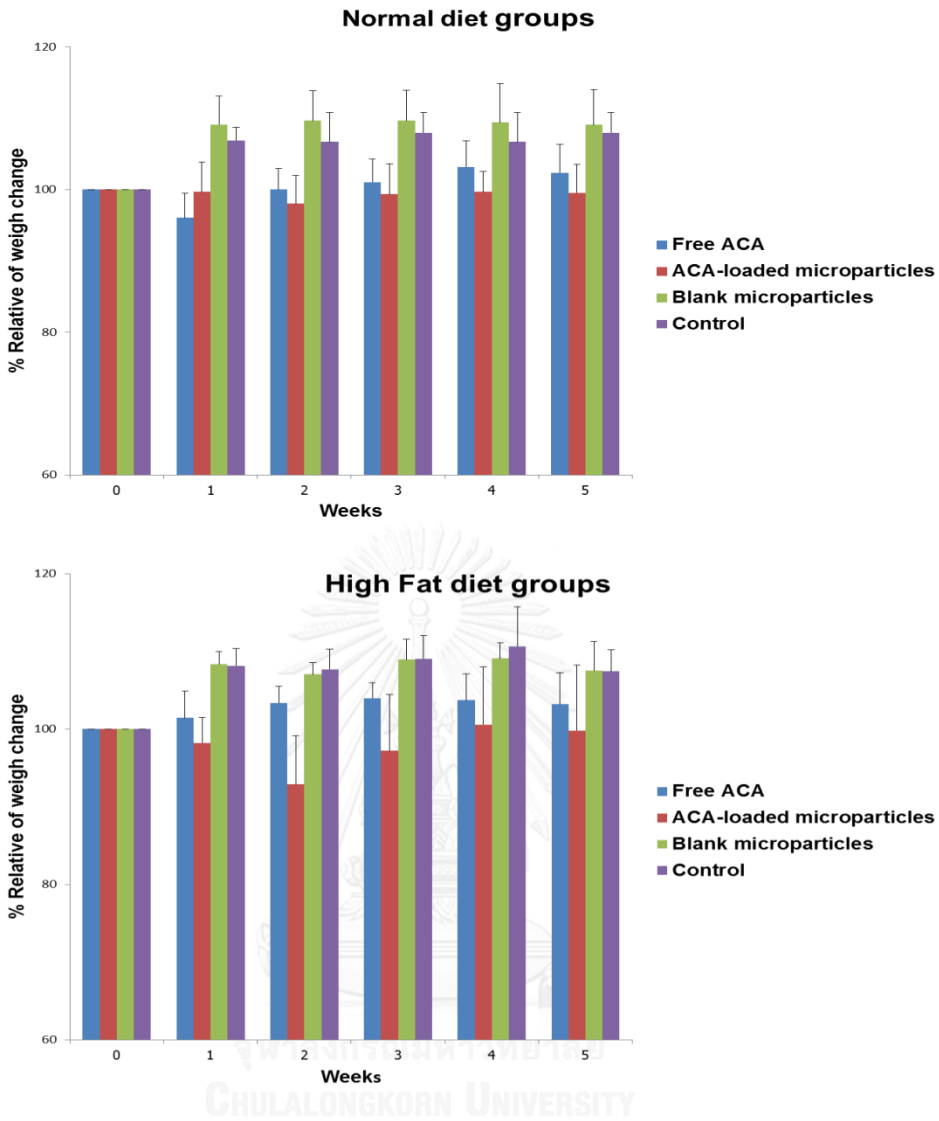



**Figure 4.10** Changes in body weight of mice; Normal diet and high fat diet fed with; Free ACA; ACA–loaded microparticles and Blank-PVA/paraffin wax microparticles.

**Table 4.2** Changes in the Relative Organ Weight of mice of normal diet and high fat diet- and ACA-Treated mice.

| Groups | Body weight | relative weight of organ | | | |
|---|---|---|---|---|---|
| | | Liver | Kidney | Spleen | Visceral Fat |
| ND-Control | 27.88±1.52 | 4.35±0.03[a] | 0.63±0.02 | 0.24±0.03 | 1.60±0.20 |
| ND-Free ACA | 26.08±0.74 | 5.52±1.12[a] | 0.56±0.07 | 0.29±0.07 | 1.30±0.06 |
| ND-ACA –loaded microparticles | 25.74±1.16 | 6.58±0.32[b] | 0.61±0.03 | 0.24±0.02 | 1.34±0.18 |
| ND- Blank-PVA/paraffin wax microparticles | 27.59±1.65 | 4.26±0.46[a] | 0.56±0.14 | 0.44±0.43 | 1.65±0.40 |
| HFD- Control | 28.05±1.64 | 4.07±0.37[b] | 0.60±0.06 | 0.27±0.02 | 1.56±0.17 |
| HFD- Free ACA | 25.98±1.32 | 6.22±0.44[b] | 0.61±0.03 | 0.28±0.01 | 1.49±0.17 |
| HFD- ACA –loaded microparticles | 25.84±1.05 | 7.34±0.63[c] | 0.66±0.03 | 0.27±0.04 | 1.35±0.11 |
| HFD- Blank-PVA/paraffin wax microparticles | 28.57±1.36 | 4.13±0.23[a] | 0.64±0.03 | 0.32±0.07 | 1.45±0.22 |

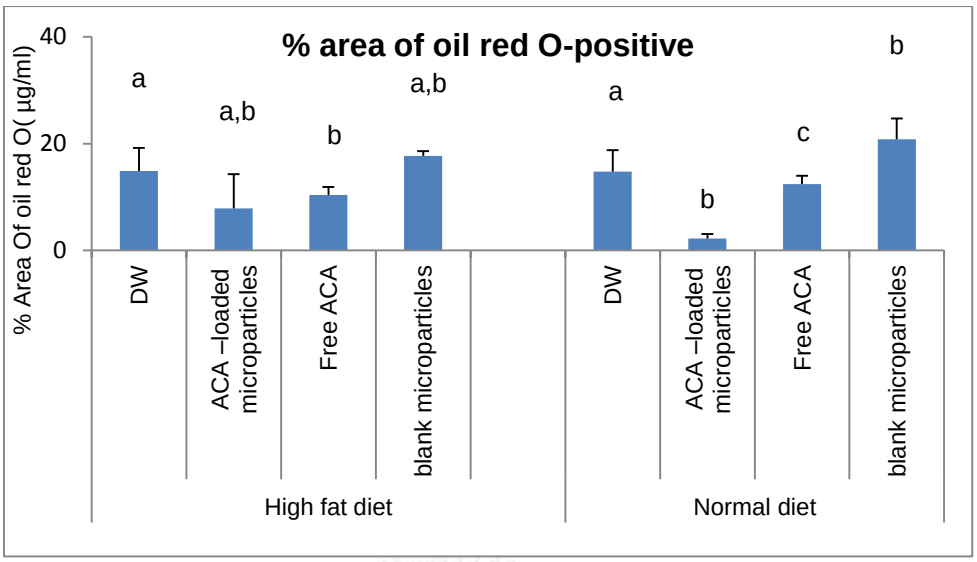
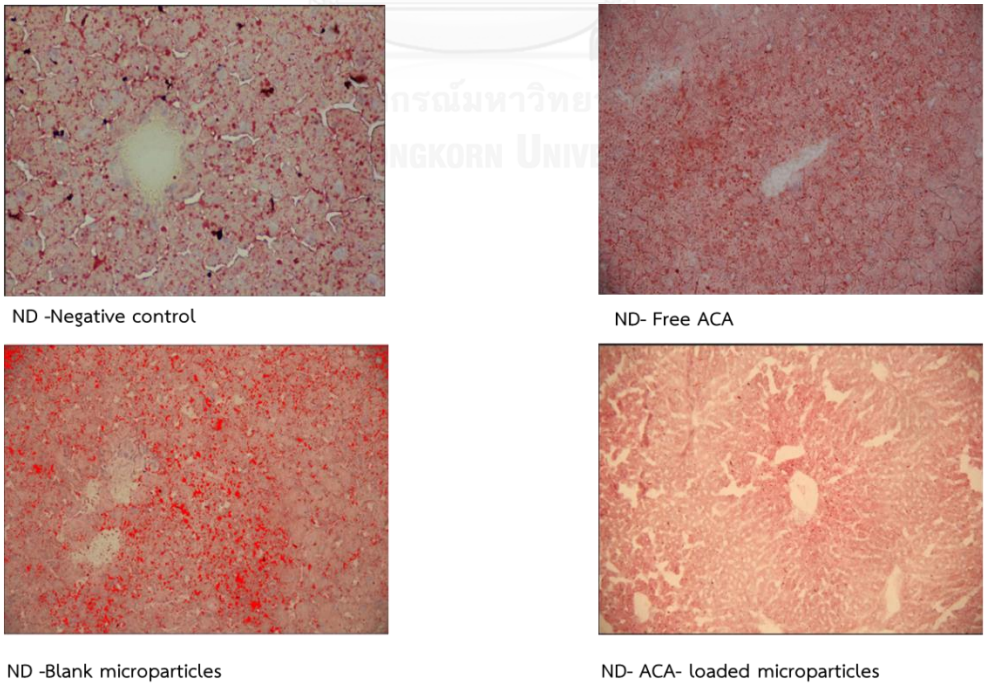
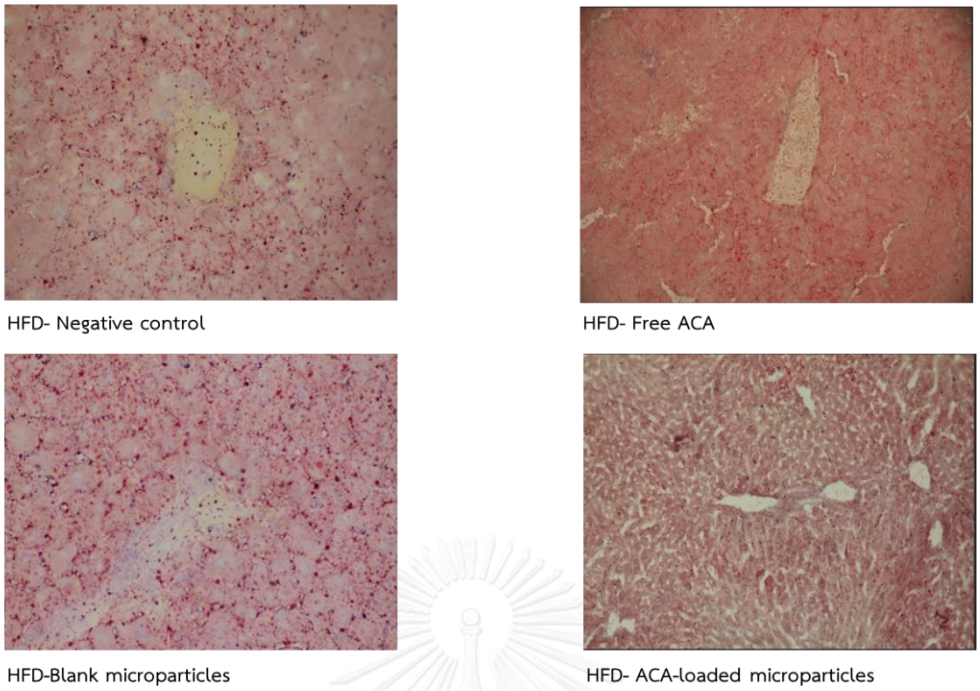



**Figure 4.11** Effect of free ACA and their encapsulated on lipid accumulation in the liver show at the bottom of the bar graph in µg/ml. Data show as the mean ± 1 SD. Means with a different lower case letter (above the bar) are significantly different ($p < 0.05$).




(A)




**(B)**

**Figure 4.12** The effect of ACA on morphological changes in liver sections were stained by oil red O. ; Normal diet (A) and high fat diet (B) fed with; Control diet (DW); ACA–loaded microparticles and Blank-PVA/paraffin wax microparticles

.

### 4.7.3. The anti-obesity effects of free ACA, ACA –loaded microparticle, Blank-PVA/paraffin wax microparticles on serum lipid level

Blood chemistry analysis could indicate the effect of free ACA, ACA –loaded microparticle, Blank-PVA/paraffin wax microparticles on internal organs likes liver and kidney. As shown in Table 4.4. The triglyceride of the free ACA and ACA –loaded microparticles was reduced, but the cholesterol was significantly increased. However, the liver enzymes (AST, ALT) were reduced in the free ACA and ACA –loaded microparticles group compared to the others. The reducing of liver enzymes in the free ACA and ACA–loaded microparticles related to the lower fat accumulation in liver by the oil red O stain and increasing of the relative weight of the liver indicate the reducing of fat accumulation from the tissue and body weight reducing.

**Table 4.3** Changes in the serum chemistry of mice of normal diet and high fat diet- and ACA-Treated mice.

| Groups | Serum chemitry parameters[a] | Groups | | | |
|---|---|---|---|---|---|
| | | Control (DW) | Free ACA | ACA-loaded microparticles | Blank-PVA/paraffin wax microparticles |
| Normal diet fed mice | Triglyceride (mg/dl) | 78.2±10.8 | 61.3±10.6 | 63.2 ± 15.2 | 94 ± 22.7 |
| | Cholesterol (mg/dl) | 83.4±3.9 | 110.8±29.6 | 132.8 ± 14.3 | 94 ± 22.20 |
| | AST (IU/L) | 175 ± 51.2 | 98 ± 34 | 51.16 ± 15.3 | 110.6 ± 40 |
| | ALT (IU/L) | 34±7.1 | 33.5±7.9 | 23.8 ± 6.30 | 27.2 ± 4 |
| | ALP (IU/L) | 57.4± 3.5 | 121.3±18.5 | 115.8 ± 19.4 | 52.4 ± 7.8 |
| | LDL-direct (mg/dl) | 5.8±0.5 | 8±1.6 | 10.6±2.2 | 10±2.6 |
| High fat diet fed mice | Triglyceride (mg/dl) | 62.3±12.5 | 55.6 ± 7.9 | 43 ± 5.7 | 73.2 ± 18 |
| | Cholesterol (mg/dl) | 98.3±8.3 | 138 ± 11 | 165 ± 2.8* | 108 ± 7.5* |
| | AST (IU/L) | 130.7±19.2 | 69.2 ± 35* | 45 ± 4.2 | 87.8 ± 62.7 |
| | ALT (IU/L) | 30.7± 5 | 29.4 ± 18 | 27 ± 7.1 | 21.4 ± 7.1 |
| | ALP (IU/L) | 48.7± 2.5 | 108.8 ±23* | 98.5 ± 23 | 49.8 ± 6.5 |
| | LDL-direct (mg/dl) | 8.67±1.2 | 11±1.2 | 15±4.2 | 10±1.2 |

(* $p < 0.05$).

# CHAPTER V
# CONCLUSION

Here we show that the problems of low aqueous solubility and instability of ACA, could be successfully tackled by encapsulated the ACA into the microparticles constructed from the blend of PVA and paraffin wax at the weight ratio of ACA: PVA: wax of 1: 1: 0.7. The process of homogenization at elevated temperature gave high encapsulation efficiency (63.033± 0.492%(w/w)) and high loading capacity (28.262± 0.307%(w/w)). The size of ACA-loaded microparticles was around 5-10 μm. The ACA-loaded microparticles showed slower degradation of ACA at the high temperature than free ACA. The ACA –loaded microparticles exhibited effective prevention of intracellular lipid accumulation in 3T3-L1 adipocytes and showed no cytotoxic against preadipocytes. Preliminary *in vivo* anti-obesity clearance test in mice clearly indicated that the body weight and the lipid accumulate in liver of the mice fed high-fat diet and treated with encapsulated ACA were significantly less than those treated with free ACA. Therefore, the ACA-loaded microparticles were a fairly anti-obesity agent.

## REFERENCES


[1] Kopelman, P.G. Obesity as a medical problem. Nature 404(6778) (2000): 635-643.

[2] Moon, H.S., Chung, C.S., Lee, H.G., Kim, T.G., Choi, Y.J., and Cho, C.S. Inhibitory Effect of (−)-Epigallocatechin-3-Gallate on Lipid Accumulation of 3T3-L1 Cells. Obesity 15(11) (2007): 2571-2582.

[3] Janssen, A. and Scheffer, J. Acetoxychavicol Acetate, an Antifungal Component of Alpinia galanga1. Planta Medica 51(6) (1985): 507-511.

[4] Yang, X. and Eilerman, R.G. Pungent principal of Alpinia galangal (L.) Swartz and its applications. J Agric Food Chem 47(4) (1999): 1657-1662.

[5] Ohnishi, R., et al. 1′-Acetoxychavicol acetate inhibits adipogenesis in 3T3-L1 adipocytes and in high fat-fed rats. The American Journal of Chinese Medicine 40(06) (2012): 1189-1204.

[6] Moffatt, J., et al. Apoptosis induced by 1′-acetoxychavicol acetate in Ehrlich ascites tumor cells is associated with modulation of polyamine metabolism and caspase-3 activation. Carcinogenesis 21(12) (2000): 2151-2157.

[7] Nakamura, Y., Murakami, A., Ohto, Y., Torikai, K., Tanaka, T., and Ohigashi, H. Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by a superoxide generation inhibitor 1′-acetoxychavicol acetate. Cancer Research 58(21) (1998): 4832-4839.

[8] Anand, P., et al. Design of curcumin-loaded PLGA nanoparticles formulation with enhanced cellular uptake, and increased bioactivity in vitro and superior bioavailability in vivo. Biochemical pharmacology 79(3) (2010): 330-338.

[9] Huang, Q., Yu, H., and Ru, Q. Bioavailability and delivery of nutraceuticals using nanotechnology. Journal of Food Science 75(1) (2010): R50-R57.

[10] Kumari, A., Yadav, S.K., and Yadav, S.C. Biodegradable polymeric nanoparticles based drug delivery systems. Colloids and Surfaces B: Biointerfaces 75(1) (2010): 1-18.

[11] Popkin, B.M., Adair, L.S., and Ng, S.W. Global nutrition transition and the pandemic of obesity in developing countries. Nutrition Reviews 70(1) (2012): 3-21.

[12] Ntambi, J.M. and Young-Cheul, K. Adipocyte differentiation and gene expression. The Journal of Nutrition 130(12) (2000): 3122S-3126S.

[13] Cao, Z., Umek, R.M., and McKnight, S.L. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes & Development 5(9) (1991): 1538-1552.

[14] Wu, Z., Puigserver, P., and Spiegelman, B.M. Transcriptional activation of adipogenesis. Current Opinion in Cell Biology 11(6) (1999): 689-694.

[15] Auboeuf, D., et al. Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator–activated receptors and liver X receptor-α in humans: no alteration in adipose tissue of obese and NIDDM patients. Diabetes 46(8) (1997): 1319-1327.

[16] Bezy, O., Vernochet, C., Gesta, S., Farmer, S.R., and Kahn, C.R. TRB3 blocks adipocyte differentiation through the inhibition of C/EBPβ transcriptional activity. Molecular and Cellular Biology 27(19) (2007): 6818-6831.

[17] Christy, R.J., Kaestner, K.H., Geiman, D.E., and Lane, M.D. CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. Proceedings of the National Academy of Sciences 88(6) (1991): 2593-2597.

[18] Otto, T.C. and Lane, M.D. Adipose development: from stem cell to adipocyte. Critical Reviews in Biochemistry and Molecular Biology 40(4) (2005): 229-242.

[19] Kern, P.A., Di Gregorio, G.B., Lu, T., Rassouli, N., and Ranganathan, G. Adiponectin expression from human adipose tissue relation to obesity, insulin resistance, and tumor necrosis factor-α expression. Diabetes 52(7) (2003): 1779-1785.

[20] Fukuhara, A., et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science 307(5708) (2005): 426-430.

[21] Hwang, C.-S., Loftus, T.M., Mandrup, S., and Lane, M.D. Adipocyte differentiation and leptin expression. Annual Review of Cell and Developmental Biology 13(1) (1997): 231-259.

[22] Todaro, G.J. and Green, H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. The Journal of Cell Biology 17(2) (1963): 299-313.

[23] Thomson, B., Ahrens, J.M., Ntambi, J.M., DeLuca, H.F., and Clagett-Dame, M. 2-Methylene-19-nor-1α-hydroxyvitamin D 3 analogs inhibit adipocyte differentiation and PPARγ2 gene transcription. Archives of Biochemistry and Biophysics 460(2) (2007): 192-201.

[24] Staples, G. and Kristiansen, M.S. Ethnic Culinary Herbs: A Guide to Identification and Cultivation in Hawai'i. University of Hawaii Press (1999): 44-60.

[25] Arambewela, L. and Wijesinghe, A. Sri Lankan Medicinal plant Monograph and Analysis: Alpinia galanga. National Science Foundation 10 (2006): 4-36.

[26] Mitsui, S., Kobayashi, S., Nagahori, H., and Ogiso, A. Constituents from seeds of Alpinia galanga Wild, and their anti-ulcer activities. Chemical & Pharmaceutical Bulletin 24(10) (1976): 2377-2382.

[27] Matsuda, H., Morikawa, T., Managi, H., and Yoshikawa, M. Antiallergic principles from Alpinia galanga: structural requirements of phenylpropanoids for inhibition of degranulation and release of TNF-α and IL-4 in RBL-2H3 cells. Bioorganic & Medicinal Chemistry Letters 13(19) (2003): 3197-3202.

[28] Akhtar, M., Khan, M., and Malik, M. Hypoglycaemic activity of Alpinia galanga rhizome and its extracts in rabbits. Fitoterapia 73(7) (2002): 623-628.

[29] Duangjai, A., Ingkaninan, K., and Limpeanchob, N. Potential mechanisms of hypocholesterolaemic effect of Thai spices/dietary extracts. Natural Product Research 25(4) (2011): 341-352.

[30] Unnisa, A. and Parven, T. Anti-inflammatory and acute toxicity studies of the extract from the rhizomes of Alpinia galangal Willd. Der Pharmacia Sinica 2(2) (2011): 361-367.

[31] Quadri, S.A., Qadri, A.N., Hahn, M.E., Mann, K.K., and Sherr, D.H. The bioflavonoid galangin blocks aryl hydrocarbon receptor activation and polycyclic aromatic hydrocarbon-induced pre-B cell apoptosis. Molecular Pharmacology 58(3) (2000): 515-525.

[32] Chakraborty, M., Jain, S., and Rani, V. Nanotechnology: emerging tool for diagnostics and therapeutics. Applied Biochemistry and Biotechnology 165(5-6) (2011): 1178-1187.

[33] Vamvakas, S., Martinalbo, J., Pita, R., and Isaac, M. On the edge of new technologies (advanced therapies, nanomedicines). Drug Discovery Today: Technologies 8(1) (2011): e21-e28.

[34] des Rieux, A., Fievez, V., Garinot, M., Schneider, Y.-J., and Préat, V. Nanoparticles as potential oral delivery systems of proteins and vaccines: a mechanistic approach. Journal of Controlled Release 116(1) (2006): 1-27.

[35] Brannon-Peppas, L. and Blanchette, J.O. Nanoparticle and targeted systems for cancer therapy. Advanced Drug Delivery Reviews 64 (2012): 206-212.

[36] Sun, M., et al. Advances in nanotechnology-based delivery systems for curcumin. Nanomedicine 7(7) (2012): 1085-1100.

[37] Couvreur, P. Polyalkylcyanoacrylates as colloidal drug carriers. Critical Reviews in Therapeutic Drug Carrier Systems 5(1) (1987): 1-20.

[38] Couvreur, P., Dubernet, C., and Puisieux, F. Controlled drug delivery with nanoparticles: current possibilities and future trends. European Journal of Pharmaceutics and Biopharmaceutics 41(1) (1995): 2-13.

[39] Cassano, D., Martir, D.R., Signore, G., Piazza, V., and Voliani, V. Biodegradable hollow silica nanospheres containing gold nanoparticle arrays. Chemical Communications 51 (2015): 9939-9941.

[40] Bei, D., Meng, J., and Youan, B.-B.C. Engineering nanomedicines for improved melanoma therapy: progress and promises. Nanomedicine 5(9) (2010): 1385-1399.

[41] Allemann, E., Gurny, R., and Doelker, E. Drug-loaded nanoparticles: preparation methods and drug targeting issues. European Journal of Pharmaceutics and Biopharmaceutics 39(5) (1993): 173-191.

[42] Bansode, S., Banarjee, S., Gaikwad, D., Jadhav, S., and Thorat, R. Microencapsulation: a review. International Journal of Pharmaceutical Sciences Review and Research 1(2) (2010): 38-43.

[43] Sivam, G.P., Lampe, J.W., Ulness, B., Swanzy, S.R., and Potter, J.D. Helicobacter pylori—in vitro susceptibility to garlic (Allium sativum) extract. Nutrition and Cancer 27(2) (1997): 118-121.

[44] Cellini, L., Campli, E., Masulli, M., Bartolomeo, S., and Allocati, N. Inhibition of Helicobacter pylori by garlic extract (Allium sativum). FEMS Immunology & Medical Microbiology 13(4) (1996): 273-277.

[45] Iimuro, M., et al. Suppressive effects of garlic extract on Helicobacter pylori-induced gastritis in Mongolian gerbils. Cancer Letters 187(1) (2002): 61-68.

[46] Lević, S., et al. Characterization of sodium alginate/d-limonene emulsions and respective calcium alginate/d-limonene beads produced by electrostatic extrusion. Food Hydrocolloids 45 (2015): 111-123.

[47] Jenning, V. and Gohla, S. Comparison of wax and glyceride solid lipid nanoparticles (SLN®). International Journal of Pharmaceutics 196(2) (2000): 219-222.

# APPENDIX

# APPENDIX I

## Calculation of percent yield of extraction

% yield = (amount extracted / starting amount) x 100

## Calculation of encapsulation efficiency (% EE) and loading capacity (% loading) of ACA encapsulation

The calibration curve of ACA was prepared using ACA solution in ethanol at concentration 0, 3000, 5000, 7000, 15000 and 20000 ppm. A calibration curve was plotted between each ACA concentration and its corresponding absorbance value as shown in Table A1 and Figure A1 The concentration of ACA in sample was determined by comparison to this calibration curve.

**Table A1** ACA concentration and its corresponding absorbance value at 264 nm

| Concentration (ppm) | 0 | 3,000 | 5,000 | 7,000 | 15,000 | 20,000 |
|---|---|---|---|---|---|---|
| Absorbance at 264 nm | 0 | 1,129,374 | 3,888,809 | 5,455,005 | 11,599,261 | 14,630,173 |



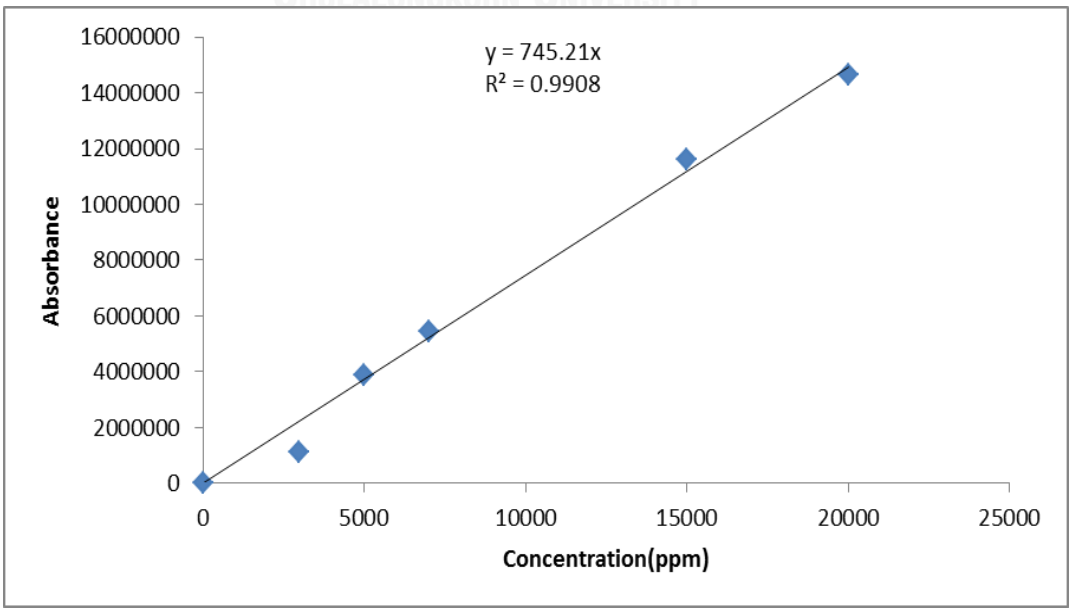


**Figure A1** Calibration curve of ACA in ethanol solution at 264 nm

From the equation of calibration curve

$$Y = 745.21x$$

Y = absorbance of ACA in ethanol at 264 nm

X = concentration of ACA in ethanol (ppm)

$$\text{Concentration (ppm)} = \frac{\text{Weight (mg)}}{\text{Volume (L)}}$$

So, Weight (mg) = Concentration (ppm) x Volume (L)

By plotting a graph between absorbance and concentration of standard ACA solutions, a linear relationship was obtained and used for calculation of concentration of ACA.

**Encapsulation efficiency (% EE) calculation**

$$\%EE = \frac{\text{Weight of encapsulated ACA in the particles}}{\text{Weight of ACA initially used}} \times 100 \quad (1)$$



ACA initial used = 1250 mg

ACA found in particles = 791 mg

% EE = 791 x 100/1250

= 63.36%

**Loading capacity (% loading) calculation**

$$\%\text{loading} = \frac{\text{Weight of encapsulated ACA in the particles}}{\text{Weight of encapsulated ACA + polymer}} \times 100 \quad (2)$$

Weight of encapsulated ACA in the filtered particles = 2791.95 mg

ACA found in particles = 791.54 mg

% loading = 791.54x 100/2791.95

= 28.37 %

# APPENDIX II

## Chemicals and culture medium preparation

### 1. Cell Culture and adipocyte Differentiation

The mouse embryo 3T3-L1 cells were obtained from American Type Culture Collection (ATCC). The 3T3-L1 preadipocytes were incubated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 100 U/ml penicillin-streptomycin. Adipocytic differentiation was induced by incubating 105 cells in DMEM containing 10% FBS, 0.5mM IBMX, 0.25 µM DEX and 1.2 µM INS for two days.The cells were then incubated for an additional two days in DMEM containing 10% FBS and 1.2 µM INS. The medium was then changed to a normal culture medium, which was freshly replaced every two days. The cells were harvested eight days after the initiation of differentiation. Cell culture conditions were 37°C in a humidified 5% $CO_2$ incubator.

### 2. MTT assay

The MTT assay is based on the protocol described for the first time by Mossmann (1983). The assay was optimized for the cell lines used in the experiments.Briefly, for the purposes of the experiments at the end of the incubation time, cells were incubated for 4 h with 0.8 mg/ml of MTT, dissolved in serum free medium (MEM or DMEM for 3t3-l1 adipocytes cells respectively). Washing with PBS (1 ml) was followed by the addition of DMSO (1 ml), gentle shaking for 10 min so that complete dissolution was achieved. Absorbance values at each dose were calculated by subtracting absorbance of blank well which contained no cells from the treated wells. The cell viability percentage (%) was expressed as the percentage of viable cells vesus the control as a 100% reference

### 3. Oil Red O staining

Intercellular lipid accumulation was measured by Oil Red O staining. The Oil Red O working solution was prepared as described previously (Ramirez-Zacarias et al., 1992; Fotakis and Timbrell, 2006). Undifferentiated 3T3-L1 preadipocytes were induced to differentiate into adipocytes as described above. Cells were incubated with 0–100 μM ACA during the differentiation periods. Cells were washed twice with phosphate buffered saline(PBS) and then fixed for 30 s with 70% ethanol. The cultures were then incubated for 2 h with a saturated solution of Oil Red O in 99% isopropyl alcohol, rinsed for 3 s in 50% ethanol, and washed twice with deionized water.

# APPENDIX III

## HPLC chromatograms

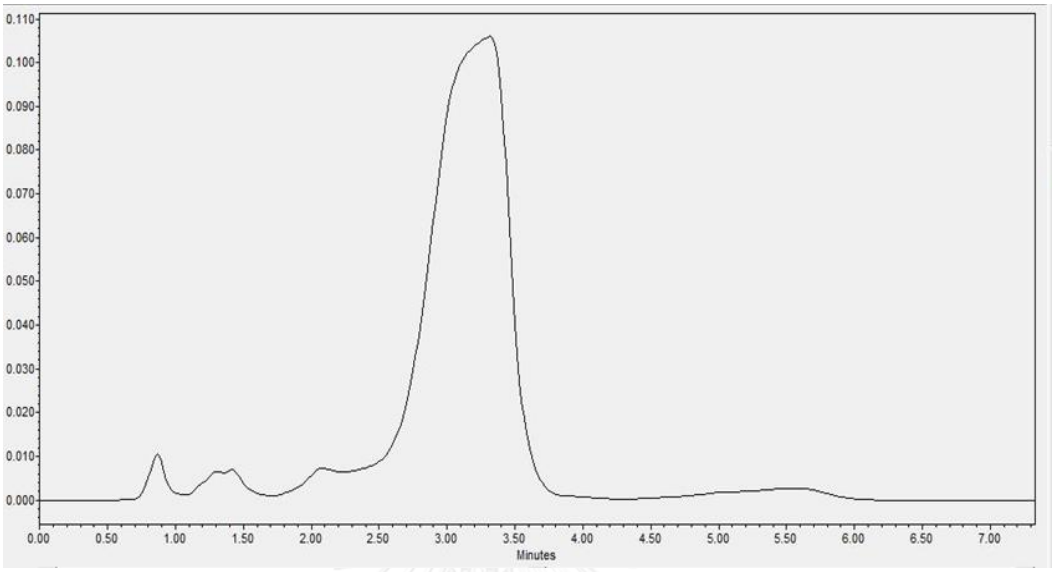



**Figure A2** HPLC chromatogram of free ACA

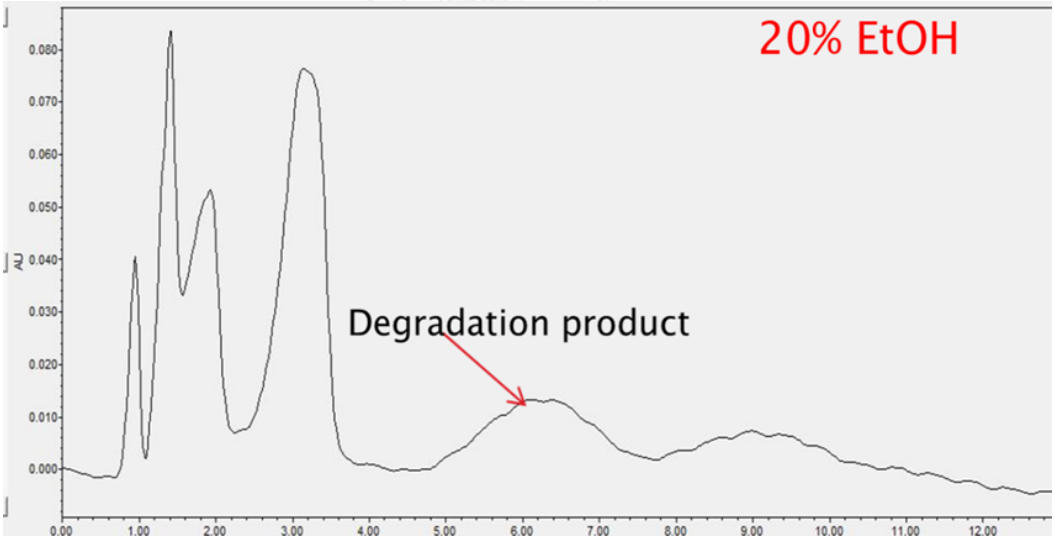



**Figure A3** HPLC chromatogram of degraded ACA

# VITA

Miss Phatharachanok Siangphloen was born on December 05, 1988 in Chiangmai, Thailand. I got a Bachelor's Degree of Science in Cosmetic Science from Mae Fah Luang University in 2010. Then she started her graduate study on Master's degree in the Program of Biotechnology, Faculty of Science, Chulalongkorn University. During master study, she had the great opportunity to present her work in poster session in the topic of "Stability Improvement of 1´-Acetoxychavicol Acetate by Microencapsulation" at the IUPAC World Polymer Congress 2014 (MACRO 2014), Chiangmai, Thailand.

Her address is 513/2 Phetburi 9 Road Ratchathewi Bangkok 10400.