สารสกัดจากกลีบดอกอัญชันและการเอนแคปซูเลชันสารสกัดจากกลีบดอกอัญชัน: การศึกษาฤทธิ์ทางชีวภาพ ความคงตัวต่อการย่อย และการนำไปใช้ทางชีวภาพเพื่อการประยุกต์ใช้ในอาหาร

นางสาวพรทิพย์ พสุกมลเศรษฐ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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CLITORIA TERNATEA PETAL FLOWER EXTRACT AND ITS ENCAPSULATION: A STUDY OF BIOACTIVITY, DIGESTIVE STABILITY AND BIOACCESSIBILITY FOR FOOD APPLICATIONS

Miss Porntip Pasukamonset

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Food and Nutrition
Department of Nutrition and Dietetics
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Thesis Title	*CLITORIA TERNATEA* PETAL FLOWER EXTRACT AND ITS ENCAPSULATION: A STUDY OF BIOACTIVITY, DIGESTIVE STABILITY AND BIOACCESSIBILITY FOR FOOD APPLICATIONS
By	Miss Porntip Pasukamonset
Field of Study	Food and Nutrition
Thesis Advisor	Associate Professor Sirichai Adisakwattana, Ph.D.
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พรทิพย์ พสุกมลเศรษฐ์ : สารสกัดจากกลีบดอกอัญชันและการเอนแคปซูเลชันสารสกัดจากกลีบดอกอัญชัน: การศึกษาฤทธิ์ทางชีวภาพ ความคงตัวต่อการย่อย และการนำไปใช้ทางชีวภาพเพื่อการประยุกต์ใช้ในอาหาร (*CLITORIA TERNATEA* PETAL FLOWER EXTRACT AND ITS ENCAPSULATION: A STUDY OF BIOACTIVITY, DIGESTIVE STABILITY AND BIOACCESSIBILITY FOR FOOD APPLICATIONS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. สิริชัย อดิศักดิ์วัฒนา, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. Oran Kwon, 178 หน้า.

สารสกัดจากพืชมีแนวโน้มได้รับความสนใจเพิ่มขึ้นเนื่องจากมีประโยชน์ต่อสุขภาพ ดอกอัญชันจัดเป็นหนึ่งในพืชท้องถิ่นที่อุดมไปด้วยสารประกอบโพลีฟีนอล ซึ่งมีผลต่อความหลากหลายทางชีวภาพรวมไปถึงฤทธิ์ต่อการต้านสารอนุมูลอิสระและโรคเบาหวาน ในการตรวจสอบการศึกษาที่ผ่านมาพบว่ายังไม่เคยมีรายงานเกี่ยวกับความคงตัวของสารสกัดจากกลีบดอกอัญชันหลังผ่านระบบย่อยอาหาร รวมทั้งการประยุกต์ใช้ในการต้านออกซิเดชันในอาหาร งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาวิเคราะห์ประสิทธิภาพขององค์ประกอบทางพฤกษเคมีและการออกฤทธิ์ทางชีวภาพของสารสกัดจากกลีบดอกอัญชันที่ผ่านระบบจำลองการย่อยอาหาร การพัฒนาการห่อหุ้มสารสกัดจากกลีบดอกอัญชันด้วยเทคนิคไมโครเอนแคปซูเลชัน พร้อมทั้งตรวจสอบประสิทธิภาพการเอนแคปซูเลชันและความคงตัวหลังผ่านระบบจำลองการย่อยอาหาร และศึกษาความสามารถในการประยุกต์ใช้สารสกัดจากกลีบดอกอัญชันเพื่อใช้เป็นสารต้านอนุมูลอิสระในการยับยั้งการเกิดออกซิเดชันของไขมันและโปรตีนในแบบจำลองอาหารในชิ้นเนื้อหมูบด ผลการศึกษาพบว่าสารสกัดจากกลีบดอกอัญชันที่ถูกย่อยด้วยเอนไซม์ในระบบทางเดินอาหารสามารถเพิ่มประสิทธิภาพการต้านอนุมูลอิสระ รวมทั้งยับยั้งการทำงานของเอนไซม์แอลฟากลูโคซิเดสและแอลฟาอะไมเลส การเพิ่มการทำงานในการจับตัวกับกรดน้ำดี นอกจากนี้สารสกัดจากกลีบดอกอัญชันสามารถชะลอการดูดซึมของน้ำตาลเข้าร่างกายผ่าน sodium-glucose transporter-1: SGLT1 สารประกอบโพลีฟีนอลในสารสกัดจากกลีบดอกอัญชันมีปริมาณลดลงหลังจากผ่านกระบวนการย่อยในกระเพาะอาหาร และเพื่อที่จะป้องกันการลดลงของสารสกัดจากกลีบดอกอัญชันนี้ การเอนแคปซูเลชันสารสกัดจากกลีบดอกอัญชันด้วยอัลจิเนตจึงได้ถูกพัฒนาขึ้น โดยผลการศึกษาสภาวะที่เหมาะสมเพื่อผลิตเอนแคปซูเลชันสารสกัดจากกลีบดอกอัญชันด้วยอัลจิเนต คือความเข้มข้นของสารสกัดจากกลีบดอกอัญชันที่ร้อยละ 10 สารอัลจิเนตร้อยละ 1.5 และ แคลเซียมคลอไรด์ร้อยละ 3 โดยน้ำหนักต่อปริมาตร ผลของลักษณะทางกายภาพพบว่าการเอนแคปซูเลชันมีประสิทธิภาพสูง (ร้อยละ 84.83±0.40%) และมีขนาดอนุภาคขนาดเล็ก 985 ไมโครเมตร แคปซูลที่ได้มีลักษณะกลม ผิวภายนอกมีลักษณะเรียบ การเอนแคปซูเลชันสารสกัดจากกลีบดอกอัญชันสามารถทนความร้อนได้ถึง 188 องศาเซลเซียส และไม่มีการเกิดปฏิกิริยาระหว่างสารห่อหุ้มและสารสกัดจากกลีบดอกอัญชัน นอกจากนี้พบว่าไมโครแคปซูลของสารสกัดจากกลีบดอกอัญชันที่ผ่านการย่อยอาหารสามารถถนอมสารประกอบโพลีฟีนอลและฤทธิ์การต้านอนุมูลอิสระให้คงอยู่ รวมไปถึงเพิ่มประสิทธิภาพการออกฤทธิ์ทางชีวภาพ อันได้แก่ การเพิ่มฤทธิ์การต้านอนุมูลอิสระ เพิ่มประสิทธิภาพการยับยั้งการทำงานของเอนไซม์แอลฟาอะไมเลสที่ใช้ย่อยคาร์โบไฮเดรต และการเพิ่มความสามารถในการจับกับน้ำดี หลังจากผ่านกระบวนการย่อยในระบบจำลองย่อยอาหาร ผลจากการผสมสารสกัดจากกลีบดอกอัญชันในเนื้อหมูพบว่าช่วยเพิ่มประสิทธิภาพในต้านอนุมูลอิสระสูงและสามารถชะลอการเกิดออกซิเดชันของไขมันและโปรตีนในเนื้อหมู และเป็นที่ยอมรับของผู้บริโภคได้ สารสกัดจากกลีบดอกอัญชันที่ความเข้มข้นร้อยละ 0.08-0.16 (โดยน้ำหนักต่อน้ำหนัก) มีความสามารถในการต้านการเกิดออกซิเดชั่นของไขมันและโปรตีนได้เทียบเท่ากับสารต้านอนุมูลอิสระสังเคราะห์ (Butylated hydroxytoluene: BHT) ที่ความเข้มข้นร้อยละ 0.02 (โดยน้ำหนักต่อน้ำหนัก) ดังนั้นสารสกัดจากกลีบดอกอัญชันและการเอนแคปซูเลชันสารสกัดจากกลีบดอกอัญชันนี้อาจจัดว่าเป็นแหล่งของอาหารที่อุดมไปด้วยสารออกฤทธิ์ทางชีวภาพที่มีประสิทธิภาพในอุตสาหกรรมอาหารเพื่อสุขภาพได้

ภาควิชา	โภชนาการและการกำหนดอาหาร	ลายมือชื่อนิสิต	.....
สาขาวิชา	อาหารและโภชนาการ	ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

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KEYWORDS: CLITORIA TERNATEA, ANTHOCYANINS, ENCAPSULATION, ANTIOXIDANT, ALPA-GLUCOSIDASE, ALPHA-AMYLASE, BILE ACID BINDING, GASTROINTESTINAL DIGESTION, LIPID OXIDATION, PROTEIN OXIDATION

PORNTIP PASUKAMONSET: *CLITORIA TERNATEA* PETAL FLOWER EXTRACT AND ITS ENCAPSULATION: A STUDY OF BIOACTIVITY, DIGESTIVE STABILITY AND BIOACCESSIBILITY FOR FOOD APPLICATIONS. ADVISOR: ASSOC. PROF. SIRICHAI ADISAKWATTANA, Ph.D., CO-ADVISOR: PROF. ORAN KWON, Ph.D., 178 pp.

There has been a growing interest in active compounds of edible plants due to their possible health benefits. *Clitoria ternatea* (CT) flowers are one of favorable edible plants enriched phenolic compounds that possess a wide range of biological effects including antioxidants and diabetes. Nevertheless, there are no data available on the stability of CT extract after gastrointestinal (GI) digestion as well as the food additive application. The aim of the study was to investigate the stability and biological activity of CT extract after gastrointestinal digestion together with the development of microencapsulation of CT extract. Finally, the efficacy of CT extract on the protection of curtailing lipid and protein oxidation in cooked pork patties was evaluated. The results showed that CT extract after enzymatic hydrolysis of GI digestion increased total phenolic compounds and antioxidant activity concomitant with an increase in intestinal α-glucosidase (maltase and sucrase) and pancreatic α-amylase inhibitory activities as well as binding to bile acids. Moreover, CT extract delayed glucose absorption by inhibiting glucose uptake through sodium-glucose cotransporter-1 (SGLT1) in Caco-2 cell line. Interestingly, the degradation of total phenolic compounds of CT extract was observed at gastric phase of digestion. In order to protect the degradation, the optimized condition of CT-loaded alginate microencapsulation was developed, comprising of 10% CT, 1.5% alginate, and 3% $CaCl_2$ (w/v). The microencapsulation of CT provides good characteristics including a high percentage of encapsulation efficiency (84.83±0.40%), narrow size distribution (985 μm), spherical shape with smooth surface, desirable thermal stability (188°C) without a chemical interaction between the materials and CT extract. Interestingly, the microencapsulation of CT significantly retained higher amount of polyphenols and improved antioxidant and pancreatic α-amylase inhibitory activity as well as bile acid binding after the GI digestion. In the roles of food additive in meat products, CT extract (0.08–0.16%) exhibited potent radical scavenging activity and retarded lipid and protein oxidation during refrigerated storage together with acceptable sensory characteristics in cooked pork patties. Therefore, CT extract and its microencapsulation could be considered as a potential source of bioactive ingredients for functional food industry.

Department:	Nutrition and Dietetics	Student's Signature	.....
Field of Study:	Food and Nutrition	Advisor's Signature	.....
Academic Year:	2015	Co-Advisor's Signature	.....

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LIST OF ABBREVIATIONS

CT	*Clitoria ternatea* petal flower
CTE	*Clitoria ternatea* petal flower extract
PCTE	Purified *Clitoria ternatea* petal flower extract
DPCTE	Digestion Purified *Clitoria ternatea* petal flower extract
AHCTE	Acid hydrolysis *Clitoria ternatea* petal flower extract
DAHCTE	Digestion Acid hydrolysis *Clitoria ternatea* petal flower extract
GI	Gastrointestinal tract
SGF	Simulated gastric fluid digestion
SIF	Simulated intestinal fluid digestion
A	Sodium alginate
AC	Control beads of calcium alginate
ACT	Calcium alginate beads with *Clitoria ternatea* extract
SEM	Scanning electron microscope
FT-IR	Fourier transform infrared spectroscopy
DSC	Differential scanning calorimetry

CHAPTER I

INTRODUCTION

Background and significance of this study

Plant polyphenols are secondary metabolites that are widely distributed and attracted to scientific research. In general, polyphenols are naturally found all parts of the plant including wood, bark, stems pods, leaves, fruit, roots, flowers, pollen, and seeds. Anthocyanins are known as natural antioxidants and belong to the widespread class of phenolic compounds in a subgroup of the flavonoids (de Pascual-Teresa & Sanchez-Ballesta, 2008; Pietta, 2000). In many countries, polyphenols from edible plants have been used as functional ingredients to fortify foods and beverages because of its ability to prevent or slow the progression of a wide variety of diseases (Gomez & Kalamani, 2003). Recently, the potential health benefits of plant-derived anthocyanin-rich foods have attracted much attention to exert positive effects against obesity, diabetes and cardiovascular diseases (Lila, 2004; Prior & Wu, 2006; Tsuda et al., 2000). These beneficial effects may contribute to the high antioxidant capacity of the anthocyanins through a various number of molecular mechanisms such as scavenging of free radicals, inhibition of radical forming and peroxidative enzymes, upregulation of antioxidant enzymes, regulation of signaling pathways and vasodilatory mechanisms (Kong et al., 2003; Lila, 2004; Prior & Wu, 2006). Plant polyphenol especially anthocyanins have been commonly used in food ingredients. However, there are obvious studies showing the low bioavailability of anthocyanins after digestion (Xiong et al., 2006; Yang et al., 2011) .Accordingly, gastrointestinal digestion process affects the stability of anthocyanins and their biological activity (D'Archivio et al., 2010). Additionally, thermal stress, neutral pH, and the presence of oxygen, metal ions and enzymes are also crucial factors for degradation of bioactive anthocyanins (Arabshahi-D et al., 2007; D'Archivio et al., 2010). From this point of view, it is concerned that plant polyphenol especially anthocyanins might not exhibit a therapeutic effect when

consumed as part of the diet as they are subject to rapid inactivation or degradation. The microencapsulation of bioactive compounds is an alternative technology to solve these problems, which can delay the degradation processes or prevent degradation and maintain stability of the bioactive ingredients until the product is delivered at the sites where adsorption is desired in gastrointestinal tract (Champagne & Fustier, 2007; de Vos et al., 2010).

Clitoria ternatea Linn. (CT), commonly known as butterfly pea, is a plant species belonging to the family Fabaceae. CT is an important plant material for the food industry because its flowers provide distinctive purple color and organoleptic properties. Among various plant polyphenols, *Clitoria ternatea* flowers are rich in phenolic compounds mostly anthocyanin (Terahara et al., 1996; Terahara et al., 1998; Wongs-Aree et al., 2006). Scientific evidences have been reported various pharmacological activities of CT including antioxidant, antimicrobial, antipyretic, anti-inflammatory, analgesic, antidiabetic activities as well as inhibition of blood platelet aggregation and vascular smooth muscle relaxant properties (Mukherjee et al., 2008; Zingare et al., 2013). Experimental studies revealed that CT has multiple protective mechanisms against oxidative stress in human health (Chayaratanasin et al., 2015; Kaisoon et al., 2011; Madhu, 2013; Patil & Patil, 2011). Nowadays, the flower petal of blue pea is recognized as a good source of dietary anthocyanins and is used as a natural blue colorant in a variety of foods especially in desserts and beverages. Nevertheless, the scientific literatures in food additives contain relatively little information of CT on the oxidative prevention of meat products. Moreover, there are no data available on the bioaccessibility of CT. Therefore, it is important for current research to investigate whether the digestion process could degrade bioactive compounds and alter their stability. The findings would lead to develop a novel microencapsulation of CT petal flower in order to increase stabilize and protect it from degradation by gastrointestinal enzymes as well as preserve its biological activity and enhance its bioavailability.

Objectives of the present study

- To evaluate the phytochemical composition of *Clitoria ternatea* petal flower extract and its biological activities including antihyperglycemic and antihyperlipidemic properties before and after gastrointestinal digestion.
- To develop a technique for *Clitoria ternatea* petal flower polyphenols-containing microencapsulation.
- To examine the digestive stability and bioaccessibility of *Clitoria ternatea* petal flower extract and its encapsulation on simulated gastrointestinal conditions.
- To investigate the preventive effect of *Clitoria ternatea* petal flower extract toward protein and lipid oxidations in cooked pork patties during the storage.

Hypotheses of this study

- The gastrointestinal digestion process might affect the degradation of phytochemical content in *Clitoria ternatea* petal flower extract resulting in the increase or decrease in biological activities including antihyperglycemic and antihyperlipidemic properties.
- The microencapsulation process could provide marked characteristics including an increase in % efficiency, the improved thermal stability, particle size distribution, and spherical shape. The microencapsulation process might protect the degradation of the bioactive compounds. Taken together, the microencapsulation could not chemically interact between the materials and *Clitoria ternatea* petal flower extract.
- The encapsulation of *Clitoria ternatea* petal flower extract could retain higher amount of polyphenols and enhance antioxidant property and inhibit pancreatic α-amylase activity as well as improve bile acid binding ability under simulated gastrointestinal conditions.
- The *Clitoria ternatea* petal flower extract might inhibit protein and lipid oxidations without affecting sensorial characteristics in cooked pork patties during the storage.

CHAPTER II

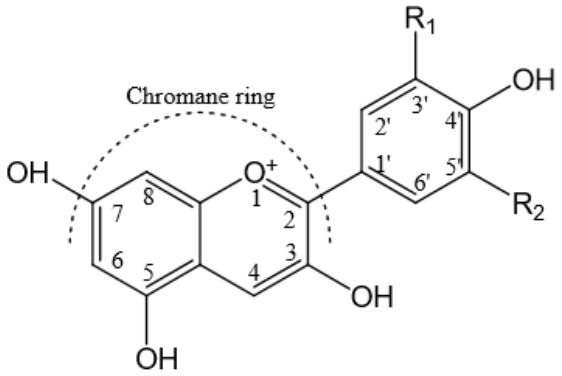
LITERATURE REVIEW

2.1 Anthocyanins

Anthocyanins are members of the flavonoid family of polyphenol phytochemicals found in various plant foods (Tsuda, 2012). Anthocyanins are generally accepted as one of the largest and most important group of water-soluble pigments in nature (He & Giusti, 2010). The word “anthocyanin” is derived from two Greek words: “anthos” (means flower) and “kyanos” (means dark blue). The colorful anthocyanins are the most recognized and is important characteristic as a natural colorant. Its water-soluble natural pigments contributing to the colorful appearance responsible for red, blue, and purple colors of many plant organs such as fruits, flowers, and leaves (Delgado-Vargas & Paredes-López, 2003).

2.1.1 Structure and characteristic of anthocyanins

Anthocyanins belong to the widespread class of polyphenolics named flavonoids, which are secondary metabolites synthesized by higher plants (Tsuda, 2012). The basic structure of anthocyanins are glycosides of hydroxylated and methoxylated derivatives of 2-phenylbenzopyrylium or flavylium salts, which are called aglycones or anthocyanidins (Jing et al., 2008). The differences between individual anthocyanins relate to the patterns of hydroxylation and methylations on the different positions of their flavylium configuration. The basic anthocyanin structure (Figure 1) share a C-6 (A-ring)-C-3 (C-ring)-C-6 (B-ring) the carbon skeleton with conjugated bonds on the C-ring and two positions, R-1 and R-2, which are substituted with hydroxyl, oxymethyl and hydryl groups (Castañeda-Ovando et al., 2009).



Name	Substitution		Visible color	Visible max. (nm) in MeOH-HCl
	R_1	R_2		
Cyanidin (Cy)	OH	H	magenta	535
Peonidin (Pn)	OCH_3	H		532
Pelargonidin (Pg)	H	H	red	520
Malvidin (Mv)	OCH_3	OCH_3	purple	542
Delphinidin (Dp)	OH	OH		546
Petunidin (Pt)	OCH_3	OH		543

Figure 1 *Basic structure of anthocyanins (flavylium cation)*

(He & Giusti, 2010)

Based on different combinations of these side groups results in the six anthocyanidins chromophores most commonly found in nature (Fleschhut et al., 2006; McGhie & Walton, 2007). There are six anthocyanidins (aglycones): cyanidin (Cy), peonidin (Pn), pelargonidin (Pg), malvidin (Mv), delphinidin (Dp), and petunidin (Pt) are commonly found in nature, and approximately 95% of all anthocyanins are derived from these (Kong et al., 2003) (Figure 2).

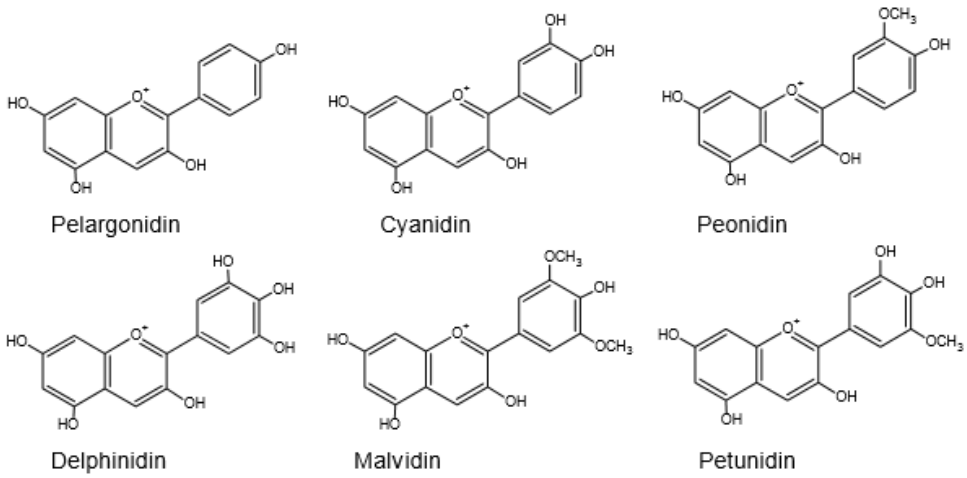


Figure 2 *The most important natural anthocyanidins*

(de Pascual-Teresa & Sanchez-Ballesta, 2008)

Aglycones were reported unstable, therefore they are rarely found in fresh plant material (Prior & Wu, 2006), except in trace quantities. However, the stability of these aglycones can be enhanced by one or more sugar molecules bonded at different hydroxyl positions, with mostly are always glycosylated at C-3 of anthocyanins (Takeoka & Dao, 2002). The most common sugar is glucose, but rhamnose, xylose, galactose, arabinose, and fructose as well as rutinose (6-O--L-rhamnosyl-D-glucose), sophorose (2-O--D-xylosyl-D-glucose), gentobiose (6-O--D-glucosyl-D-glucose), sambubiose (2-O--D-xylosyl-D-glucose), xylosylrutinose (Figure 3) (Delgado-Vargas & Paredes-López, 2003; Takeoka & Dao, 2002).

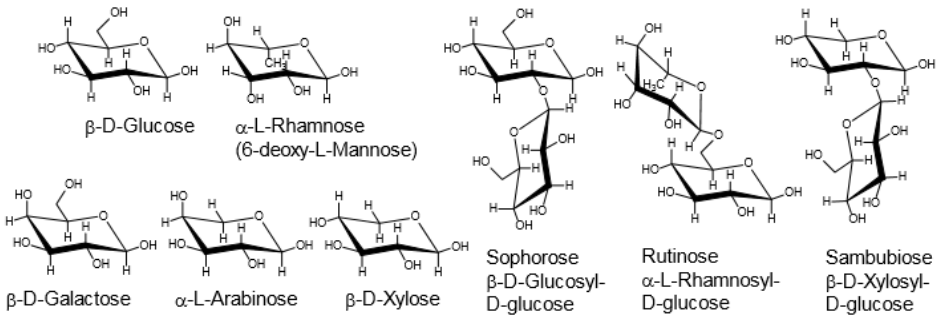


Figure 3 *The most common glycosyl units of anthocyanins*

(Takeoka & Dao, 2002)

These sugar residues may be further acylated with some organic acids such as cinnamic acids (e.g. caffeic, ferulic and sinapic acid) and aliphatic acids (e.g. acetic, malic, malonic, and succinic acid) (Takeoka and Dao 2002). The most common aromatic phenolic acids and aliphatic dicarboxyl acids being part of anthocyanin molecules are shown in Figure 4.

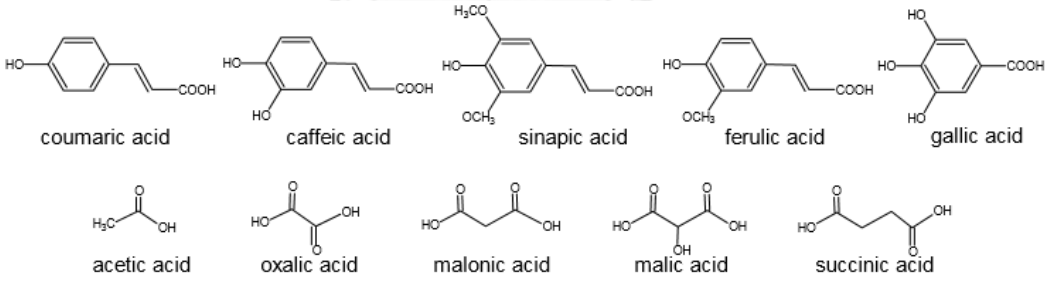


Figure 4 *The most common acyl units of anthocyanins*

(de Pascual-Teresa & Sanchez-Ballesta, 2008)

Therefore, chemical combinations of each anthocyanidin may be glycosylated and acylated by various sugars and acids at different positions (Delgado-Vargas & Paredes-López, 2003). When the aglycone (anthocyanidin) is glycosylated, it is known as anthocyanin. The six anthocyanins differ by three primary aspects: the number of hydroxyl, location of the sugars, and the nature of the aromatic acids attached to the

sugar molecule. The physical and chemical properties of anthocyanins affect by glycosylation and acylation as they modify the molecular size and polarity of the molecule (He & Giusti, 2010).

2.1.2 Putative health-promoting effects of anthocyanins

Anthocyanins have been incorporated into the human diet for several centuries. They were components of traditional herbal medicines that have been used to treat hypertension, pyrexia, liver disorders, dysentery and diarrhea, urinary problems including kidney stones and urinary tract infections, and the common cold (de Pascual-Teresa et al., 2010; de Pascual-Teresa & Sanchez-Ballesta, 2008). Recently, anthocyanins have received increasing attention as they have been shown to exert a number of potential health benefits against many diseases including obesity, diabetes and cardiovascular disease, as well as to have a positive effect on cognitive function (Kong et al., 2003; McGhie & Walton, 2007). Epidemiological studies have proposed that consumption of polyphenols has been associated with reduced incidence of chronic diseases cancer and cardiovascular diseases (Arts & Hollman, 2005). Studies have shown that they can help to prevent tumor growth when induced by chemicals, reduce growth and spreading of tumor that have been injected in mice (Ding et al., 2006). Additionally, anthocyanins have also been shown to exhibit anti-carcinogenic activity against multiple cancer cell types *in vitro* and tumor types *in vivo* studies as reported in Wang and Stoner (Wang & Stoner, 2008). Further studies have shown that they can help preventing cardiovascular diseases like atherosclerosis (Bell & Gochenaur, 2006; de Pascual-Teresa et al., 2010; Wallace, 2011). They have also been proven to have anti-diabetic properties (Grace et al., 2009) and protect against Alzheimer's disease (Shih et al., 2010).

2.1.3 Anthocyanins and antioxidant activity

Anthocyanins are the most abundant polyphenols in fruits and vegetables and possess potent antioxidant activity. Animal study claimed that anthocyanins are potent antioxidants as they can quench free radicals and terminate the chain reaction that is responsible for the oxidative damage (Tsuda et al., 2000). Moreover, *in vitro* studies reported that anthocyanins have been attributed to the strong antioxidant capacity as

they can protect against a variety of oxidants through a various number of mechanisms such as scavenging of free radicals, inhibition of radical forming and peroxidative enzymes, upregulation of antioxidant enzymes, regulation of signaling pathways (Kähkönen & Heinonen, 2003; Kong et al., 2003). Therefore, this could be useful in the treatment of pathologies where free radical production plays a key role.

2.1.4 Anthocyanins and antihyperglycemic activity

A number of recent reports indicate that consumption of fruits and vegetables, especially rich in anthocyanins, have potential effects in reducing the incidence of type 2 diabetes (T2DM). Carbohydrate digestion and glucose absorption are obvious targets for better glycemia control after high-carbohydrate meals (Ludwig, 2002). A variety of polyphenols including flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tannins (proanthocyanidins and ellagitannins) have been shown to inhibit α-amylase and α-glucosidase activities which are the key enzymes responsible for digestion of dietary carbohydrates to glucose (Hanhineva et al., 2010). This glucose is absorbed across the intestinal enterocytes via specific transporters. Therefore, inhibition of the digestive enzymes or glucose transporters would diminish the rate of glucose release and absorption in the small intestine and thus suppress postprandial hyperglycemia (Ludwig, 2002). Moreover, several mechanisms have been proposed by which specific flavonoid constituents can reduce biological pathways related to the development of T2DM. One reviewer about dietary flavonoid intakes and risk of T2DM suggested that flavonoids with particular interest in the flavonol, flavan-3-ol, and anthocyanin subclasses may also decrease glycemia and improve insulin secretion and sensitivity (Hanhineva et al., 2010). Studies in animal models reported that the anthocyanin subclass improved glucose metabolism, insulin resistance, and β-cell dysfunction through GLUT4 regulation (Nizamutdinova et al., 2009; Prior & Wu, 2006). Study in male mice with T2DM found that an anthocyanin-rich bilberry extract ameliorated glycemia and insulin sensitivity via activation of AMP-activated protein kinase and resulted in upregulation of GLUT4 (Takikawa et al., 2010). In human intervention trials using different sources of anthocyanins like blend of bilberries, black currants, cranberries, and strawberries have

been shown that intake of anthocyanin rich foods improve insulin sensitivity (Stull et al., 2010), reduce fasting plasma glucose (Abidov et al., 2006), and reduce the postprandial glucose response to a sucrose load (Törrönen et al., 2010).

2.1.5 Anthocyanins and antihyperlipidemic activity

Hyperlipidemia, also referred to as dyslipidemia which is characterized by increased low-density lipoprotein (LDL)/ very-low-density lipoproteins (VLDL)/ triglycerides (TG) and reduced level of high-density lipoprotein (HDL) (Unnikrishnan et al., 2014). A number of dietary in flavonoids have been reported to lower LDL levels and inhibit oxidative modification and consequently were believed to be potential inhibitors of LDL oxidation and consequent atherogenesis in animal studies (Hirunpanich et al., 2006; Kurowska et al., 2000; Monforte et al., 1995). Bile acids are acidic steroids synthesized in the liver cholesterol and are actively reabsorbed by the terminal ileum and undergo enterohepatic circulation (Kahlon & Smith, 2007). Binding to the bile acids was reported to excrete through the fecal (Ahmed, 2005). This is because binding bile acids in food fractions can prevent reabsorption of bile acid and stimulate plasma and liver cholesterol conversion to additional bile acids in the body. (Kahlon et al., 2007). Plant polyphenols including anthocyanins of various vegetables and fruits such as grapes, peaches, apricots and pears have shown ability in bile acid binding which may relate to their structures natures of the metabolites produced during digestion or their interaction with active binding sites (Kahlon et al., 2007; Kahlon & Smith, 2007). Therefore, *in vitro* bile acid binding is predictor of the cholesterol lowering potential, which is one of the primary factors contributing to atherosclerosis and heart disease.

2.1.6 Anthocyanins in human diet

Anthocyanins are water-soluble and vacuolar pigments found in most species in the plant kingdom. Anthocyanins can be found in all parts of the plants, mostly in flowers and fruits, they also present in leaves, stems, roots, and storage organs (Williams & Grayer, 2004). Several foods that contain anthocyanins are listed in Table 1.

Table 1 *Common fruits, vegetables and other foods containing anthocyanins.*

fruits	vegetables	Other foods
apricot	artichoke	legumes
apples	asparagus	pistachios
blackberry	beans, black	rice
blueberry	beans, small red	sorghum
cherries	carrot	sugar cane
chokeberry	eggplants	Wines (red)
cranberry	garlic	Wines (Port)
currant, black and red	lettuces	
elderberry	olive	
gooseberry	onions	
grapes	parsnip	
loganberry	purple corn	
lychee	red radishes	
mango	red cabbage	
mangosteen	rhubarb	
nectarines	sweet potatoes (red)	
passion fruit	taro	
pomegranate		
peaches		
plums		
raspberry		
strawberry		

Modified from (Wu et al., 2006)

Types of anthocyanins present in each fruit and vegetable are vary. For example, raspberry and strawberry contain cyanidin and pelaragonidin derivatives, red cabbage contains only cyanidin derivatives, interestingly grapes and blueberries have

almost all the anthocyanidin derivatives (Wu et al., 2006). The average total anthocyanin concentration in fruits and vegetables ranges between 0.1 to 1.0 % of dry weight (Wu et al., 2006). The concentration of anthocyanins presents in common dietary sources include a variety of colored fruits and vegetables are shown in Table 2.

Natural anthocyanin colorants are becoming more popular because of the antioxidant and other potential beneficial properties (He & Giusti, 2010). Depending on the nutritional habits, the daily intake of anthocyanins for individuals has been estimated to range from several milligrams to hundreds of milligrams per person. Typical consumption is in the range of 3 – 215 mg anthocyanins /day in the US. Study in the past reported that average daily intakes of anthocyanins estimated at 215 mg during the summer and 180 mg during the winter per day per person (Chun et al., 2007), however the USDA evaluating more than 100 common foods and reported that the estimation consumption was 12.5 mg per day per person in the United States (Wu et al., 2006). Another study reported that anthocyanins consumption in U.S. was 3 mg/day (Chun et al., 2007).

Table 2 *Common sources of anthocyanins*

Food sources	Anthocyanin content (mg litre^{-1} or mg kg^{-1})
Blackberry	1,150
Blueberry	825 – 4,200
Cherry	20 – 4,500
Chokeberry	5,060 – 10,000
Cranberry	600 – 2,000
Cowberry	1,000
Currant (black)	1,300 – 4,000
Elderberry	2,000 – 10,000
Grape (red)	300 – 7,500
Loganberry	774
Plum	20 – 250
Raspberry (black)	1,700 – 4,277
Raspberry (red)	100 – 600
Raspberry (red) single strength juice	4 – 1,101
Strawberry	150 – 350
Cabbage (red)	250
Eggplant	7,500
Onion	Up to 250
Rhubarb	Up to 2,000

Modified from (Wu et al., 2006)

2.2 *Clitoria ternatea* (Blue Pea Flower)

Dietary anthocyanin sources in nature found in many colored fruits and vegetables as well as fruit-based processed foods and beverages such as jelly, juices, and red wine (Delgado-Vargas & Paredes-López, 2003). In North America, blueberry fruit displays their deep blue color, is one of the most popular fruit native to North America and fruits show the most anthocyanins per serving (Routray & Orsat, 2011). Mulberry fruit is well known as a good source of anthocyanins in subtropical regions of Asia that claimed to be blackberry-like fruit (Qin et al., 2010). There are some rich anthocyanins flowers in Southeast Asia are used as a natural food coloring (He & Giusti, 2010). *Clitoria ternatea* or Butterfly pea flowers is an example of a flower that contains a rich history of use in many parts of Asia, especially Southeast Asia. Butterfly pea flowers are claimed to contain a higher anthocyanin index compared to other flowers (Abreu et al., 2014). Butterfly pea flowers are normally squeezed to make herbal flower tea and beverage and also used as a food coloring for Thai desserts in blue and purple colors in Thai cultures (Limsuwan et al., 2014) and used to color rice-cakes in Malaysia (Mohamad et al., 2011).

2.2.1 Origin and distribution

Clitoria ternatea, commonly known as “Butterfly Pea” or “Blue Pea” is a highly nutritious legume used a livestock forage plant in many countries (Figure 5) (Gomez & Kalamani, 2003). It belongs to the family Fabaceae. It most likely originated in tropical Asia South and has been widely distributed to many tropical and subtropical countries such as Central America, East and West Indies, China and India (Mukherjee et al., 2008). *Clitoria Ternatea* is a tropical twining herb, growing wild and also in gardens. It is a very well-known Ayurvedic medicine. *Clitoria ternatea* is also known as a medicinal plant in many parts of the world. This plant has been used in beverage and food to prevent deterioration or as nutraceuticals, as medical products. The roots, seeds and flowers have medicinal value and are used for slightly different purposes to treat various ailments (Gomez & Kalamani, 2003).

Figure 5 *Clitoria ternatea flower*

2.2.2 Bioactive compounds in *Clitoria ternatea*

The blue flower pigment is traditionally utilized as food colorant in Southeast Asia (Mukherjee et al., 2008). *Clitoria ternatea* has several lines with different flower colors: dark blue, light blue, mauve, and white. Blue color in the petals is well-known to accumulate ternatins, a group of (poly)acylated anthocyanins. In a previous work reported that the main anthocyanins in butterfly pea based on delphinidin glycoside called ternatins which attributes to their blue (Terahara et al., 1996; Terahara et al., 1998). The phytochemical analysis of petal of *Clitoria ternatea* reveals that six major anthocyanins ternatins A1, A2, B1, B2, D1, and D2, were isolated (Figure 6), and these structures have been characterized as malonylated delphinidin 3,3′,5′-triglucosides having 3′,5′-side chains with alternating D-glucose and p-coumaric acid units (Terahara et al., 1996). Moreover, previous study found that glycosylation at the 3′- and 5′-positions of anthocyanin is a critical step in producing blue petals in *Clitoria ternatea* and their structures were partly characterized as highly acylated delphinidin derivative. They reported three flavonol glycosides, kaempferol 3-O-(2″-O-**α**-rhamnosyl-6″-O-malonyl)-**β**-glucoside, quercetin 3-O-(2″-O-**α**-rhamnosyl-6″-O-malonyl)-**β**-glucoside, and myricetin 3-O-(2″,6″-di-O-**α**-rhamnosyl)-**β**-glucoside were isolated from the petals of *Clitoria ternatea* (Kazuma et al., 2003; Kazuma et al., 2003).

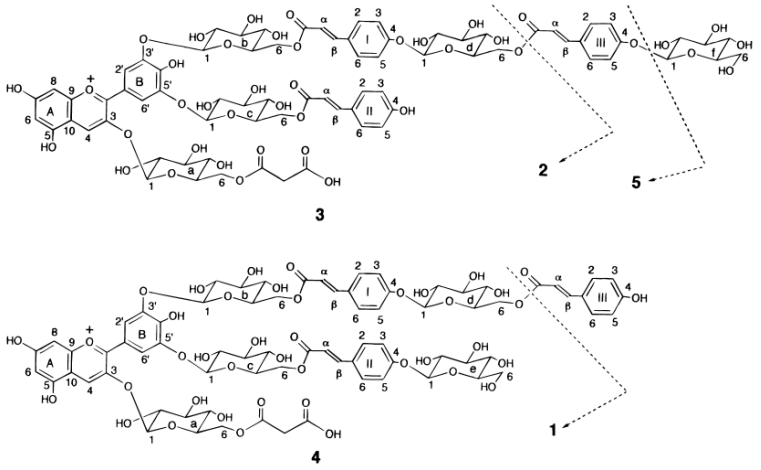


Figure 6 *Structures of ternatins A3 (1), B4 (2), B3 (3), B2 (4), and D2 (5)*
(Terahara et al., 1996)

2.2.3 Pharmacological activities

Clitoria ternatea has been used for centuries as a traditional Ayurvedic medicine. The medicinal properties of this plant have been the subject of several studies. *Clitoria ternatea* is considered as potential sources of antioxidant property as confirmed by Di phenyl picryl hydrazyl (DPPH) free radical, free radical scavengers Ferric reducing power (FRAP), ABTS radical scavenging, super oxide dismutase (SOD) and total poly phenols assays (Chayaratanasin et al., 2015; Madhavarao, 2011; Madhu, 2013; Patil & Patil, 2011). Therefore, *Clitoria ternatea* have the potential to be an alternative source of natural antioxidants. *Clitoria ternatea* possesses nootropic, anxiolytic, antidepressant, anticonvulsant (Jain et al., 2003), enhances memory (Taranalli & Cheeramkuzhy, 2000), and anti-inflammatory (Nair et al., 2015; Shyamkumar & Ishwar, 2012). Animal study also reported the anti-inflammatory effects of *Clitoria ternatea* extract and found that the petroleum ether (60-80°C) flower extract poses significant anti-inflammatory properties at both the dose levels (200 and 400 mg/kg body weight) (Shyamkumar & Ishwar, 2012). *In vitro* studies of antidiabetic activity on carbohydrate enzymes found that *Clitoria ternatea* was able to inhibit the intestinal glucosidase enzymes (IC_{50} of 3.15+/-0.19 mg/ml) against intestinal sucrase (IC_{50} of 4.41+/-0.15 mg/ml) and pancreatic alpha-amylase (IC_{50} 4.05+/-0.32 mg/ml) (Adisakwattana et al.,

2012). Another study evaluated for antihyperglycemic and antioxidative activity of ethanolic extract *Clitoria ternatea* (EECT) (200 and 400 mg/kg) in normal and streptozotocin-induced diabetic rats. The results indicated that EECT has remedial effects on hyperglycemia and oxidative stress in diabetic rats (Talpate et al., 2013). Similar result was reported the effect of aqueous extract of *Clitoria ternatea* leaves and flowers (400 mg/kg body weight) on serum glucose of alloxan-induced diabetic rats. The study clearly indicated that the leaf and flower extract of *Clitoria ternatea* have hypoglycemic effect on alloxan-induced diabetic rats (Daisy & Rajathi, 2009; Daisy et al., 2009). Additionally, the study of *Clitoria ternatea* on antihyperlipidemic in diet-induced hyperlipidemic rats found that *Clitoria ternatea* was able to reduce serum total cholesterol, triglycerides, very low-density lipoprotein cholesterol, and low-density lipoprotein cholesterol levels (at 500 mg/kg) to a similar dose as the statin atorvastatin (50 mg/kg) and Gemfibrozil (50 mg/kg) (Solanki & Jain, 2010). Regarding toxicity, one study assessing oral toxicity and using doses up to 3,000 mg/kg bodyweight failed to notice any salient toxicological signs or deaths with this dose, using concentrated ethanolic extracts of the aerial parts (11:1) and roots (6.4:1) (Taranalli & Cheeramkuzhy, 2000).

2.3 Toxicity and safety of anthocyanins

Anthocyanins are generally considered safe, as they have been consumed by animals and human in the past without apparent adverse effects to health. Previous study investigated the safety and toxicity of anthocyanins in animal model and they concluded that lethal Dose at 50% (LD_{50}) values were over 2000 mg/kg given to mouse and rat without toxic effects. Additionally, single doses of 3000 mg/kg anthocyanins given to dogs did not induce any sign of adverse effects. The oral administration of the extract to rats at doses of 125-150 mg/kg and dogs at 80-320 mg/kg daily for 6 months did not induce mortality or any toxic effects. Another study demonstrated that an observation for 18 weeks of anthocyanin extracts at calculated dose of 225 mg/kg body weight to young rats were associated with a low level of toxicity such that the no-observed-effect-level (NOEL) (Morazzoni & Bombardelli, 1996).

2.4 The effects on the stability of anthocyanins

Anthocyanins are considered as a promising dietary compounds with an important role in human health. In food industries, anthocyanins are of particular interest to the natural food colorant industry due to their ability to their attractive colors (Castañeda-Ovando et al., 2009). However, their usage in the food industry is limited because natural pigments including anthocyanins are generally unstable and easily susceptible to degradation. Anthocyanin degradation during food processing is a major limiting factor when applying anthocyanins to a food matrix. Previous study reported anthocyanins are very sensitive to their surrounding environment and they are instability and tend to degrade when exposed to factors like environmental variations, including temperature, light intensity, oxygen pH and other compounds and environmental factors (Fleschhut et al., 2006; Laleh et al., 2006; Patras et al., 2010; Torskangerpoll & Andersen, 2005). The flavylium structure of anthocyanins is electron deficient and therefore it is readily to react with nucleophilic compounds such as water, peroxide, and sulfur dioxide. Consequently, anthocyanins can be degraded during processing, storage, digestion and absorption of foods and beverages. The stability of anthocyanins depends on several molecular structures. Anthocyanins are more stable than their aglycones which are rare in nature. The stability of anthocyanins is decreased with increasing degree of hydroxylation, however its stability can improve by methylation of anthocyanidin (Prior & Wu, 2006). Glycosylation, especially at C-3, is required for stability and solubility of anthocyanins at pH 3-7. Moreover, glycosylated di-glucosides have a tendency to be more stable to heat and light when compared to mono-glucosides (Fleschhut et al., 2006). The stability of anthocyanins is also affected by pH. The color of anthocyanins shifts from red to blue with increasing pH. Anthocyanins have reported to be more stable at acidic pH, but less stable at alkaline pH at room temperature (Laleh et al., 2006). Additionally, anthocyanins degrade with increasing temperature during food processing and storage. Previous study reported that hydroxylation of anthocyanidin decreases thermal stability, whereas methylation, glycosylation, and acylation can stabilize anthocyanins (Laleh et al., 2006).

2.5The limitation of bioavailability of anthocyanins

Most of the study on the health-promoting properties of anthocyanins has been performed and reported. An understanding of digestion, metabolism and tissue disposition of anthocyanins is needed (Figure 7). The naturally occurring anthocyanin have a range of molecular structures accumulation in plant tissues and can be modified before consumption, particularly during the processing and storage (McGhie & Walton, 2007).

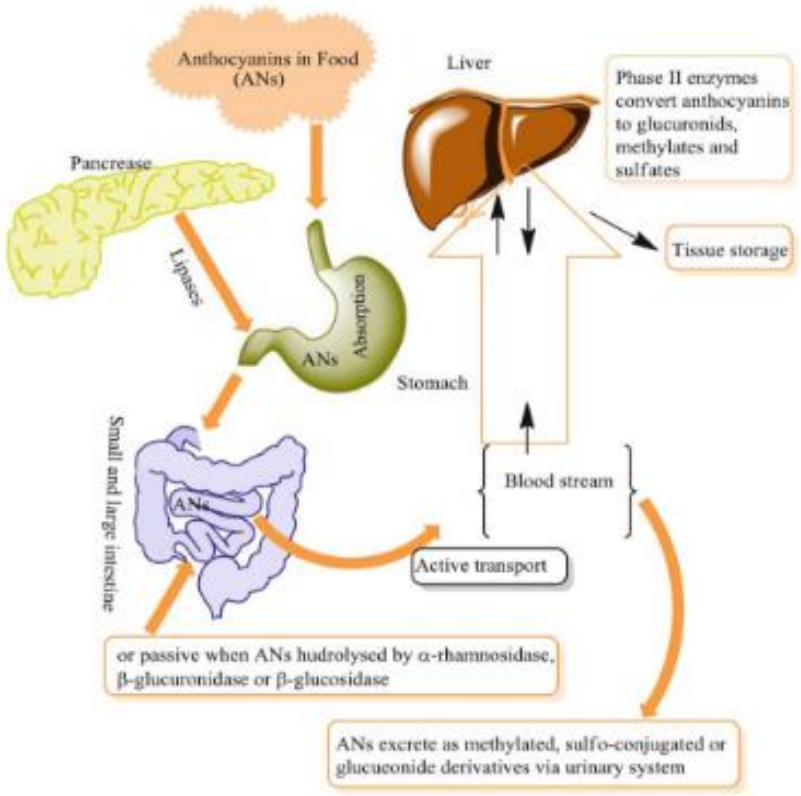


Figure 7 *An overview of anthocyanins absorption and excretion*

(Manach, Williamson et al. 2005)

Once anthocyanins enter to the body, they experience various environments and physiochemical conditions which may also transform them. During the passage of anthocyanins through the gastrointestinal tract (GIT), they explored to different environments including stomach, small intestine, and colon is characterized by different pH and microbial populations; both of which can alter the anthocyanins

structure (McGhie & Walton, 2007). The pH in the stomach is very low around pH 1-2, which preserves the stable form of anthocyanins (the flavylium cation). Previous studies reported that the flavylium cation will exist at low pH of stomach environment and other forms will predominate lower down the GIT (McDougall et al., 2005; McGhie et al., 2003). Previous study assessed the stability of anthocyanins in red cabbage during simulated gastrointestinal digestion. The results showed that the anthocyanins were effectively stable under acidic gastric digestion conditions, the recovery rate after simulated pancreatic digestion was around 25% (McDougall et al., 2007). In contrast, the pH is largely neutral in the small and large intestine, where anthocyanins are much less stable and undergo multiple modifications (Pojer et al., 2013). It has been reported that anthocyanins were significant losses (43%) occur while digestion in the small intestine, however acylated anthocyanins showed higher stability than non-acylated forms during pancreatic digestion (McDougall et al., 2005). Based on previous studies, at neutral pH, the aglycones are unstable and are rapidly degraded to their corresponding phenolic acids, aldehydes through cleavage of the C-ring (Keppler & Humpf, 2005), suggested that anthocyanins are unstable in the neutral to mild alkaline conditions. Furthermore, the microbial populations especially in the colon are likely to modify the molecular structures of anthocyanins. Study in the past reported that the anthocyanins were deglycosylated and demethylated to the corresponding aglycones when exposure to gut microflora (Keppler & Humpf, 2005). Studies on anthocyanins reported that anthocyanins were loss around 50-90% which occurred during *in vitro* digestion (Bermúdez-Soto et al., 2007; Pérez-Vicente et al., 2002). Another study also reported that anthocyanins disappear 60-90% from GIT during ingestion (Prior & Wu, 2006), suggesting that extensive degradation or metabolism within GIT prior to absorption.

Anthocyanins were stated the most poorly absorbed among plant polyphenols (Manach, Williamson et al. 2005). This absorption varies based on the presence or absence of glycosylation on hydroxyl groups, the position of glycosylation, the quality of sugar moiety attached, plant or food matrix, and interactions with proteins, micelles, and emulsifiers (Cermak et al., 2003). *In vitro* study on the transport efficiency of

anthocyanins affirmed that anthocyanin bioavailability is reduced when present more free hydroxyl groups and less OCH_3 groups, and increased by attached glucoses rather than galactoses. Therefore, glucosides was reported higher transport efficiencies when compared to galactosides (Yi et al., 2006). The area of anthocyanins absorption could start from the stomach or the small intestine. Some studies suggested that the stomach is an important site for the bioabsorption of anthocyanins. Additionally, anthocyanins were absorbed in the gastric and intestinal tissues by binding to the protein in these tissues which were detected anthocyanin concentrations greater than in plasma (He et al., 2009). Activity of aglycones from primary flavonoid-glucoside occurs after absorbed by the jejunum or duodenum, because large molecule of anthocyanins are hydrolyzed by endogenous β-glucosidase and hence a lesser extent the galactosides, xylosides and arabinosides (Cermak et al., 2003). Free aglycones are much smaller than their corresponding anthocyanin and more hydrophobic. Hence, they can easily enter the cell membrane passively. Intact glycosides are also absorbed in the small intestine by the sodium-dependent glucose transporter (SGLT1) and by some part by inefficient passive diffusion. Acylated anthocyanins are generally recognized as non-absorbable in the small intestine due to their larger molecular size and lack of a sugar moiety for transporter binding (Cermak et al., 2003; McGhie & Walton, 2007).

Anthocyanin levels detected in the plasma and urine are reported that very low after ingestion of anthocyanin-rich foods. The absorption of ingested anthocyanins is reported very low (ppm), and may only raise human plasma concentrations to the ng/ml. Anthocyanin absorption and excretion studies carried out in animals and humans showed that anthocyanin rutinosides absorbed a larger proportion of than the glucosides in both animals and humans. Maximum level of anthocyanin plasma levels was reached 50 ng/ml at 30-45 minutes after fed with black currant juice. Also, the result showed that urinary excretion of anthocyanins from juice was found only 0.035% represented during the first 4 h after ingestion (Nielsen et al., 2003). The absorbed intact anthocyanins and anthocyanidins are largely excreted in urine whereas the non-absorbed anthocyanins are excreted through feces (Del Rio et al., 2013). These studies

clearly showed that clearance of anthocyanins from plasma is relatively rapid with only small amounts detected in plasma at 6 hours after ingestion. In humans and animals, minimal absorption and urinary excretion (0.004 – 0.2% of dosage), and low fecal excretion amount of anthocyanin-glycosides has been reported (Nielsen et al., 2003; Prior & Wu, 2006).

Moreover, clinical safety was confirmed that most people tolerated the extract well or very well, with only 4% of people complaining of side effects, mainly gastrointestinal, related to the skin and cutaneous annexes and the nervous system when they were given160 mg of the anthocyanin extract twice daily for 1 to 2 months (He & Giusti, 2010). Based on previous results, the Joint FAO/WHO Expert by Committee on Food Additives (JECFA) estimated acceptable daily intake (ADI) of 0-2.5 mg/kg body weight per day (Clifford, 2000). Anthocyanin-containing extracts have concluded to be a very low order of toxicity, based on limited toxicological studies including mutagenicity, reproductive toxicity and teratogenicity (Gupta, 2016).

Microencapsulation of bioactive compounds and its significance to improve the bioavailability

Bioactive compounds produce many health benefits after ingestion; however, these compounds are easily inactivated or degraded during digestion (de Vos et al., 2010). Encapsulation technology has gained great attention from pharmaceutical and food industrial applications in order to protect food against nutritional losses, target delivery of bioactive compounds during digestion or even to add nutritive materials to food after processing (Burey et al., 2008; Han et al., 2008; McClements et al., 2009). Microcapsules or microspheres are multi-particulate delivery systems offering prolonged or controlled drug delivery and improvement of drug bioavailability, stability, and targeting to a specific site (Tiwari et al., 2012).

2.6 Microencapsulation

Microencapsulation has been defined as the technology of entrapping individual particles or droplets of solid, liquid, or gaseous materials (the core) inside one or more polymeric coating (the shell) to produce capsules in the micrometer to millimeter range, known as microcapsules as shown in Figure 8 (Venkatesan et al., 2009). The active compound usually called core material can be a flavor, drug, or pigments. Microencapsulation can release their content at a controlled rate over prolonged periods of time (Đorđević et al., 2015).

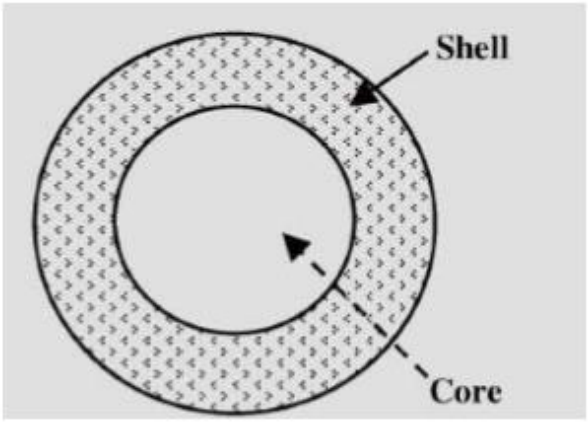


Figure 8 *Microcapsule with core and coat*

(Jyothi, Seethadevi et al. 2012)

The technique of microencapsulation is broadly categorized depending on the physical and chemical properties of the material to be encapsulated (Jyothi et al., 2012). There are various techniques available for the encapsulation of core materials as presented such as spray drying, spray cooling, spray chilling, extrusion, thermal gelation, droplet gelation, coacervation, lypophilization, and cocrystallization. Each one will depend on the composition of the capsule made and the environment it is included in (Desai & Jin Park, 2005). Microcapsules usually have a particle size range between 1 and 1000 μm. Products smaller than 1 μm are referred to as nanocapsules, and larger than 1000 μm are defined as macrocapsules (Jesindha et al., 2013).

Microencapsulation processes with their relative particle size ranges is shown in Table 3.

Table 3 *Microencapsulation processes with their relative particle size ranges*

Physico - chemical processes	Physico - mechanical processes
Coacervation (2 – 1200 um)	Spray-drying (5 – 5000 um)
Polymer-polymer incompatibility (0.5 – 1000 um)	Fluidized- bed technology (20 – 1500 um)
Solvent evaporation (0.5 – 1000 um)	Pan coating (600 – 5000 um)
Encapsulation by Polyelectrolyte multilayer (0.02 – 20 um)	Spinning disc (5 – 1500 um)
Phase Inversion (0.5—5.0 um)	Co-extrusion (250 – 2500 um)
Hot Melt (1—1000 um)	Interfacial polymerization (0.5 – 1000 um)
	In situ polymerization (0.5 – 1100 um)

(Jyothi et al., 2012)

Microencapsulation technique makes sure that these bioactive compounds continue to exist in the high acidity of the gastric environment to release in the small intestine, the site of absorption, to produce maximum nutritional efficacy and absorption (Han et al., 2008; McClements et al., 2009).

Microcapsules based on biodegradable polymer have been extensively investigated as controlled release delivery system over the past three decades. In recent years, a continued interest in microcapsule has been triggered by their application for the controlled release of macromolecular drugs. The microencapsulation technique has been mainly described as a process in which small particles or droplets of a bioactive compound are surrounded by a homogeneous or heterogeneous coating, forming beads or capsules with various applications (Champagne & Fustier, 2007).

In this sense, microparticles, microcapsules or microspheres are defined as the product of the microencapsulation process protecting it from oxygen, water, light or other environmental conditions (Jyothi et al., 2012). So that, these bioactive compounds can be performed to improve stability of active components and hence can be released under specific conditions (Desai & Jin Park, 2005; Laine et al., 2008). Therefore, herbal extracts or antioxidants are encapsulated for the major purposes of controlled release or delivery, extending shelf-life, providing protection to the essence and improving particle qualities.

2.6.1 Microencapsulation techniques

Microencapsulation techniques frequently used for food applications include spray drying, spray chilling/cooling, coacervation, extrusion, fluidized bed coating, and liposome entrapment (de Vos et al., 2010). The advantages and disadvantages of these techniques are summarized in Table 4. Selection of the proper technique is based on the physicochemical properties of the wall and core materials, and the desired functional properties of the microcapsule (Champagne & Fustier, 2007). Cost issues and the lack of available food grade wall materials are mainly limitations on selecting encapsulation techniques (Gibbs et al., 1999).

Table 4 *Comparison of microencapsulation techniques*

Technique	Advantage	Disadvantage
Spray drying	Cost effective and well established	Limited suitable wall materials
Coacervation	High encapsulation capacity	Expensive
Extrusion	High retention and stability	Limited suitable wall materials
Fluidized bed	Wide range of suitable wall materials	Only apply to solid particle coating
Liposome entrapment	Stable at high water activity	Difficult to scale up

(Gouin, 2004)

The most popular technique used in conjunction with external gelation is extrusion because of its ease, simplicity, low cost, gentle formulation conditions, high retention and stability (Gouin, 2004). The method (Figure 9) involves the preparation of a hydrocolloid solution to which the active ingredient is added, and then extruded through a syringe (forming droplets). Then, injected drop-wise into the calcium chloride solution (cross-linking) to induce gelation (McClements et al., 2009). Electrostatic extrusion is one of the most convenient for controlled production of small particles of consistent and desirable size. One advantage of extrusion is the size of the syringe needle gauge determines the size of the alginate bead (de Vos et al., 2010). The diameter of the capsules depends on the diameter of the syringe, hydrocolloid concentration, solution viscosity and the distance between the syringe and the cross linking solution. Additionally, extrusion can easily be scaled up to large scale production within industry (de Vos et al., 2010).

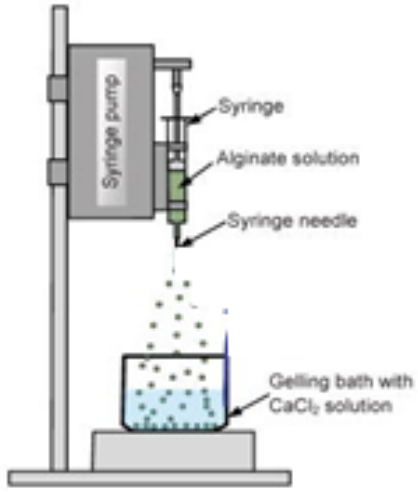


Figure 9 *Schematic representation of the electrostatic droplet generator*

2.6.2 Coating material type

The composition of the coating material is depended on the primary determinant of functional properties of the microcapsule and of how it may be used to control the performance of a particular ingredient. There are different types of encapsulating materials have been used for microencapsulation (Table 5); such as,

polysaccharides (starches, maltodextrins, gum Arabic and corn syrups), lipids (mono and diglycerides) and proteins (casein, milk serum and gelatin) (Gibbs et al., 1999).

Table 5 *Coating materials typically used for microencapsulation of food components*

Category	Coating materials	Widely used methods
Carbohydrate	Starch, maltodextrins, chitosan, corn syrup solids, dextran, modified starch, cyclodextrins	Spray and freeze drying, extrusion, coacervation, inclusion complexation
Cellulose	Carboxymethyl cellulose, methyl cellulose, ethyl cellulose, cellulose acetate-phthalate, cellulose acetatebutylate-phthalate	Coacervation, spray drying and edible films
Gum	Gum Arabic (GA), agar, sodium alginate, carageenan	Spray drying, syringe method (gel beads)
Lipids	Wax, paraffin, beeswax, diacylglycerol, oils, fats	Emulsion, liposomes, film formation
Protein	Gluten, casein, gelatine, albumin, peptides	Emulsion, spray drying

(Desai and Jin Park 2005)

Hydrogel is commonly used as encapsulation material due to its capability of absorbing large amounts of water or biological fluids (Coviello et al., 2007). Hydrogel encapsulants are usually employed as round beads which can produce by any extrusion-based technique (Stojanovic et al., 2012). Among all hydrogel, calcium alginate is employed as the matrix for plant aqueous extract encapsulation as it is the most widely used due to several advantageous features because non-toxicity,

biocompatible, abundant, cheap and its ability to react with divalent cations, mainly calcium, to form stable gels (Peppas et al., 2000).

2.6.3 Formulation of alginate by extrusion technique

Alginate (alginic acid) is a naturally occurring polysaccharide extracted from some types of seaweed and brown (Chan et al., 2010; Han et al., 2008). It is composed of blocks of mannuronic (M) and guluronic (G) acids monomers that are arranged in alternating M and G blocks (Figure 10) that form a linear (de Vos et al., 2010).

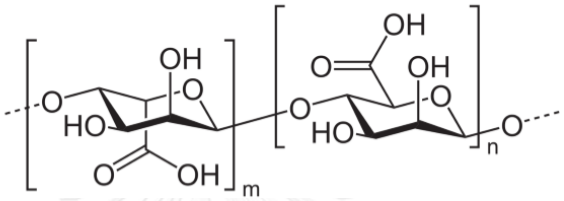


Figure 10 *Structure of alginic acid. m-mannuronic acid n-guluronic acid*

Alginate are soluble in water and can thicken and increase the viscosity when in solution, but require the addition of another substrate to form a gel. The exposure of alginate to polyvalent cations (i.e. $Ca^{2+)}$ induces gelation through the cross-linkage of anionic G-G sequences with the cations as shown in Figure 11. (Chan et al., 2006).

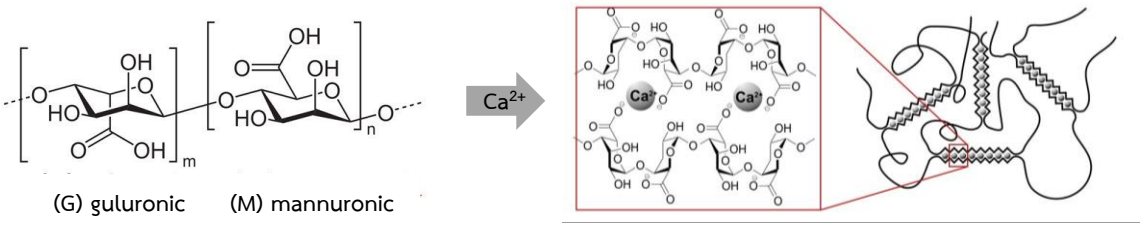


Figure 11 *Biocompatible calcium cross-linked alginate hydrogel*

The preparation of alginate beads can be performed as easily as extruding an alginate solution into a solution containing polyvalent cations (Gåserød et al., 1999). It is used as an encapsulating matrix for bioactive compounds is desirable because it is

not only biocompatible but also easily forms gel beads with chemically inert interiors (Chan et al., 2006). Additionally, alginate beads can withstand acidic pH while degrading under mild basic conditions, making it an attractive encapsulation material for protection against gastric digestion and directed release into the small intestine (de Vos et al., 2010). The mechanical strength, porosity, shrinkage, stability, and other physical properties of alginate beads depend on the composition, sequential structure, and molecular size of the alginate polymer. High ratios of G to M residues have been shown to increase the porosity of alginate beads (Gåserød et al., 1999).

2.6.4 Applications of alginate in food industry

Alginates do not have nutritional value since they are not digestible by enzymes in the gastrointestinal track, but they do have a wide range of applications which are related to their physicochemical properties, and their lack of toxicity. Alginates are notable for providing viscosity, forming gel, stabilizing foam and emulsions, providing adhering properties, and serving as coagulating agents in products such as sauce, gravy, jams, creams, low fat spreads, and ice creams. Alginate coatings are good oxygen barriers that can retard lipid oxidation in foods and also can improve batter adhesion (Krochta JM, 1997). The extensive use of alginate in the food and drink industry can be attributed to its wide spectrum of functional properties (Stephen, 2006). The first application of alginate in the food industry was in ice cream products. The addition of alginate to ice cream helps to reduce the size of ice crystals and provide a smooth texture (Han et al., 2008). Alginate is also widely used in production of restructured food, especially fruit and canned food. Alginates have important applications in pharmaceutical industry. They are not only used as clinically active materials, for example in wound dressing but also they have application in many drug formulations. Alginates beads have also been used increasingly in encapsulation applications due to its pH sensitivity property. They collapse in acidic pH due to the protonation of their carboxyl groups and swell in alkaline pH because of ionization of the carboxyl groups (Yu et al., 2009). It is reported that acid-sensitive drugs incorporated into alginate beads would be protected effectively in the stomach from acid exposure (Kim & Lee, 1992).

2.6.5 Characterization of microcapsules

Microcapsules can be evaluated and characterized using numerous techniques to confirm its ability to meet the requirements for the analysis of microcapsules. These techniques include particle size analysis, Scanning Electron Microscope (SEM), encapsulation efficiency (%EE), Fourier transform infrared (FTIR), and Differential Scanning Calorimeter (DSC) analysis.

2.6.5.1 Particle size analysis of microcapsules

Methods including optical microscopic, ultramicroscopic, polarizing microscopic, electron microscopic light scattering and electrical conductivity methods have all been used to determine the size and also the size distribution of microcapsules. Bead size is an additional important parameter of alginate microcapsules. Microcapsules referred to particle size approximately between 0.2 μm and 5 mm diameter (Jackson & Lee, 1991; Jyothi et al., 2012). This size of the microcapsules products vary depends on the materials and the methods used in preparation of the capsules, as well as application purpose (Jyothi et al., 2012). Basically, the term “microparticle” refers to a particle with a diameter of 1-1000 μm, irrespective of the precise interior or exterior structure (Berkland et al., 2004). However, the usual acceptable for food application of microencapsulation is between 1–1000 μm and can have many morphologies (Desai & Jin Park, 2005; Jackson & Lee, 1991; Schrooyen et al., 2001).

2.6.5.2 Morphology of the microcapsules by Scanning Electron Microscopy

The morphology of microcapsules mostly depends on the core material and the deposition process of the shell (Srivastava et al., 2013). Scanning Electron Microscopy (SEM) has been applied to the morphological examination of the outer structure of microcapsules in various microcapsule systems (Rosenberg et al., 1985). This SEM produces images of sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that can be detected information about the sample’s surface structure and composition (Rosenberg et al., 1985). The outer surface texture is important for evaluating the compactness of the prepared microcapsules as well as detecting the presence of

pores, cracks and irregularities which have a significant impact on the release characteristics of the microcapsules. Microcapsules characteristic should appear spherical with a smooth on surface morphology (Venkatesan et al., 2009).

2.6.5.3 Percentage of encapsulation efficiency (%EE) of the microcapsules

The result of EE% for all microcapsule is used to confirm the amount of total phenolic contents is detected in microcapsules (Jyothi et al., 2012). The higher %EE indicates that this type of microcapsules has the higher encapsulation capacity. The leakage of microcapsules will be investigated by %EE as the encapsulated compounds was achieved low %EE when leakage detected in microcapsules (Belščak-Cvitanović et al., 2011; Champagne & Fustier, 2007; Deladino et al., 2008).

2.6.5.4 The chemical structure of the microcapsules by Fourier transform infrared (FTIR) study

The chemical structure of the microcapsules was inferred from the Fourier transform infrared (FTIR) studies. The FTIR method is widely used to stimulate vibrational levels of known chemical groups in a molecule and induces a variation in chemical reactions (Mooranian et al., 2014). This technique is used to detect pure components, their physical mixture, and the bioactive compounds or drug-loaded microcapsules. There was no change in the functional groups of the different compounds in the microcapsules before and after extract encapsulation. A good characteristic of microcapsules will present no chemical reaction between the extracts and the other materials in the formulation on FTIR spectra (Arhewoh et al., 2016).

2.6.5.5 Thermal analysis of microcapsules by Differential Scanning Calorimeter (DSC)

Differential scanning calorimeter (DSC) is a thermal analysis apparatus measuring how physical properties of a sample change, along with temperature against time (Gill et al., 2010). Thermal analysis determines the temperature and heat flow associated with material transitions since they undergo decomposition at different times and under different thermal conditions (Arhewoh et al., 2016). A change in temperature, DSC measures a heat quantity of the samples on the basis of a

temperature difference between the sample and the reference material (Istenič et al., 2015).

Many studies have given promising results, the successful use of alginate microcapsules as carriers for bioactive compounds to improve its stability and enhance bioavailability (Anbinder et al., 2011; Deladino et al., 2008; Ersus & Yurdagel, 2007; Flores et al., 2016; Istenič et al., 2015). To date, it has been difficult to find an ideal alginate microcapsule that fulfils the multitude of requirements. However, alginate microcapsules should ideally be characterized by high mechanical and chemical stability, controllable release properties on the target site and defined pore size and a narrow pore size distribution (Gibbs et al., 1999; Jackson & Lee, 1991; Jyothi et al., 2012).

2.6.5.6 Advantages of the microencapsulation application

In recent years, extensive research has been carried out to study the health promotion properties of different phytochemicals and to devise novel encapsulation materials and methods, trying to incorporate functional ingredients into foods (Munin & Edwards-Lévy, 2011). Microencapsulation of natural phenolic compounds extracted from different plants has been recently studied by various researchers to trap active components and release them under controlled conditions such as yerba mate (Ilex paraguariensis) (Deladino et al., 2008), piper sarmentosum (Chan et al., 2010), and thyme (*Thymus serpyllum* L.) (Stojanovic et al., 2012). Converting liquids to solids, separating reactive compounds, providing environmental protection, and improved material handling properties have been given as benefits from these microcapsules. Some of these reasons have recently been outlined in previous studies regarding the potential to be very widely used in food industry applications (Desai & Jin Park, 2005; Jyothi et al., 2012).

Microencapsulation can protect sensitive core ingredients from the environmental factors, including oxygen, water and light; undesirable interactions with other ingredients;

- Other roles are to control diffusion or to isolate or control the release of an encapsulated ingredient at the right place and the right time;

- The physical characteristics of the original material can be modified and made easier to handle;
- Evaporation or transfer rate of the core material to the outside environment is decreased or retarded;
- The core material can be diluted when only very small amounts are required and still achieve a uniform dispersion in the host material; and
- It can also be employed to separate components within a mixture that would otherwise react with one another.

Microcapsules offer various significant advantages in medical treatments as drug delivery systems. This will include: (1) an effective protection of the encapsulated active agent against (such as enzymatic) degradation, (2) the possibility to accurately control the release rate of the combined drug over periods from hours to months in the body, (3) an easy administration, and (4) desired target drug release profiles to match the therapeutic needs of the patient (Singh et al., 2010).

2.7 Application of antioxidant in food products

The anthocyanin content, a major contributor to the antioxidant capacity, may be affected by differences in growing season, the location of growth, rainfall, species, and maturity of the fruit. Recently, interest has been growing in finding naturally occurring antioxidants for use in foods to replace synthetic antioxidants. There is increasing interest in the use of antioxidants that can be used as food ingredients to prevent food oxidation. This is because dietary antioxidants can act as free radical scavengers, radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors (Karadag et al., 2009).

The applications of antioxidants have been widespread in the food industry for decades; and are in use in preventing lipids from oxidative degradation. Recently, plant extract products have been shown to improve the quality of food as well as having health benefits. Plant extracts such as rosemary, grape seed, tea and ginkgo biloba extract are used in a variety of food applications to preserve food quality. Previous study reported that rosemary extract compounds have liver protective and anti-tumor

activity, and are also known for its antimicrobial and antioxidant activities. Therefore, rosemary extract has also been used in meat and meat products as herbal spices to enhance food quality (Balentine et al., 2006). Moreover, study conducted on grape seed extract shows that it produces a high antioxidant activity in fish oil and frozen fish, cooked pork patties and cooked turkey (Banon et al., 2007).

2.8 Application of anthocyanin as an antioxidant to prevent oxidation in food products

There have been many recent attempts to control lipid oxidation during the manufacturing process and the storage of meat products that offer the opportunity to minimize rancidity, retard the formation of toxic oxidation products, maintain nutritional quality, and increase the shelf life of food products (Juntachote et al., 2006; Juntachote et al., 2007; Lara et al., 2011). Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroxyquinone (TBHQ) have been used to prevent oxidative reactions in lipid-containing foods (Sasse et al., 2009). However, there is little information on their effects on human health, especially when added to the meat products for the prevention of oxidative processes during refrigerated storage. Much attention has been given to the use of natural antioxidants, mainly those that are derived from edible plants such as rosemary (Estévez & Cava, 2006), lemon balm (Lara et al., 2011), Mediterranean berries (Ganhão et al., 2013), holy basil, galangal (Juntachote et al., 2006; Juntachote et al., 2007), lemongrass (Olorunsanya et al., 2010), and kimchi (Lee et al., 2011).

2.8.1 Oxidation process in food products

Oxidation is one of the most important processes occurring in food systems. Lipids and proteins in foods are very susceptible to oxidation processes which are a major cause of chemical deterioration in foods (Lund et al., 2011; Waraho et al., 2011). The oxidation reaction mostly occurs during manufacturing, storage, distribution and final preparation of foods. Free radical mediated oxidation of lipids and proteins arise from reactive oxygen species (ROS) generated during food processing and storage (Davies, 2003). A free radical contains an unpaired electron in an atomic orbital that is very unstable and react quickly with other compounds to gain stability. These free

radicals attack the nearest stable molecule to steal electron, then the attacked molecule loses its electron becomes a free radical and starts a chain reaction. Free radical reactions play an important role in oxidative degradation of lipids by the production of peroxides and their derivatives (Waraho et al., 2011). Free radicals derived from lipid oxidation reactions are easily transferred to other molecules such as proteins, carbohydrates and vitamins, especially in the presence of metal ions (Lobo et al., 2010). With lifestyle and behavior changes, common ready-to-eat products including precooked and refrigerated foods are becoming even more popular. These products contain meats, which are very susceptible to lipid and protein oxidation because of their high fat content, as well as to odor changes during storage (Estévez et al., 2007; Sasse et al., 2009).

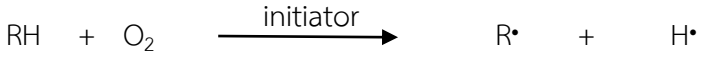
Lipid oxidation, in particular, is the cause of deterioration causing undesirable changes in flavor (volatiles and odor) and color (redness and myoglobin) of meat and meat products (Raharjo & Sofos, 1993). The protein oxidation leads to loss of amino acids and solubility, changes in texture, alterations in protein functionality and may even lead to formation of toxic compounds (Lund et al., 2011; Soladoye et al., 2015). Therefore, the oxidative attacks on macromolecules contribute to deterioration of flavor, aroma, color (unwanted browning reactions), and nutritive value.

2.8.2 Lipid oxidation

Lipids are susceptible to oxidative processes in the presence of light, heat, enzymes, metals, metalloproteins, and micro-organisms. These catalytic systems enhance the development of off-flavors and loss of nutrient values including essential amino acids, fat-soluble vitamins, and other bioactives (Frankel, 1991). lipid oxidation has been a challenge for manufacturers as it is a major cause of food quality deterioration. Lipids can undergo autoxidation, photo-oxidation, thermal oxidation, and enzymatic oxidation under different conditions which involve some type of free radical or oxygen species (Shahidi, 2000). The most common process leading to oxidative deterioration is autoxidation of atmospheric oxygen with lipids. During processing and storage of meat products, oxidation and color deteriorations are the major causes for quality loss generally characterized by flavor deterioration, discoloration, destruction

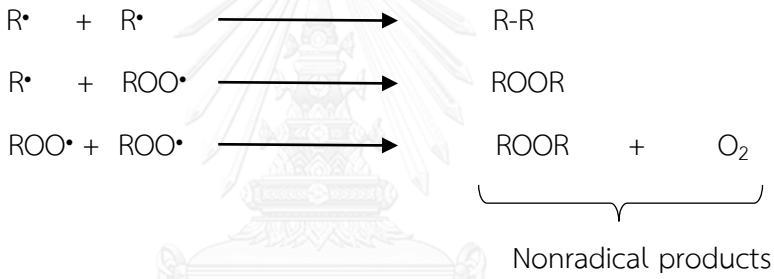
of nutrients, and possible formation of toxic compounds (Wąsowicz et al., 2004). The double bonds of an unsaturated fatty acids can undergo further oxidation via a free radical chain reaction that proceeds through three steps of initiation, propagation, and termination as given below:

Initiation:



Propagation:

Termination:



The oxidation of lipids is commonly presented as an oxidative, oxygen dependent, deterioration of fats, especially the unsaturated fatty acids. This lipids hydroperoxides (ROOH) are the primary lipid oxidation products and once formed. These lipids hydroperoxides when found in muscle foods become susceptible to further free radical chain reaction such as isomerization and decomposition causing secondary products such as pentanal, hexanal, 4-hydro-xynonenal and malondialdehyde (MDA) (Waraho et al., 2011).

Therefore, lipid hydroperoxides have been identified as primary products of autoxidation; decomposition of hydroperoxides yields aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids, and epoxy compounds, known as secondary oxidation products (Figure 12).

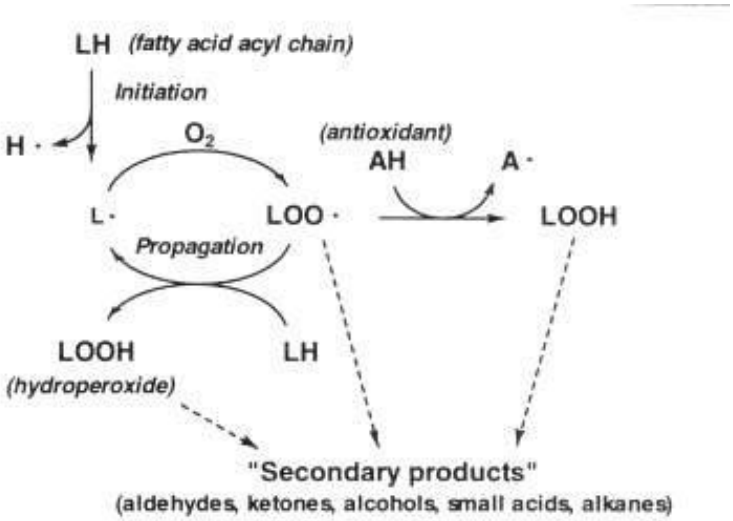


Figure 12 *Autoxidation of polyunsaturated lipid*

(Wąsowicz et al., 2004)

It has been known that lipid in meat easily oxidizes and unsaturated fatty acids form hydroperoxides that are subsequently decomposed to secondary compounds, including malonaldehyde (MDA) and other carbonyl compounds that cause off-flavors (Frankel, 1991). Oxidative compounds of lipid peroxidation in meat products have been implicated in various human diseases such as atherosclerosis, gastrointestinal diseases and cancer (Negre-Salvayre et al., 2010; Negre-Salvayre et al., 2008; Wąsowicz et al., 2004).

2.8.3 Protein oxidation

Oxidation is one of the major causes for quality deterioration of food products during processing and storage. Proteins in food are susceptible to oxidation reactions. Proteins oxidation results in production of various oxidation derivatives. Oxidative modifications of protein induced either by the direct reactions with reactive oxygen species (ROS) or indirect reactions with secondary by-products of oxidative stress (Estévez et al., 2007). It takes place at the side chains of amino acids, which include thiol oxidation, aromatic hydroxylation, and formation of carbonyl groups (Stadtman, 1990). Free radical species can react directly with the protein or other molecules such as lipids and carbohydrates, forming products that subsequently react with the protein (Figure 13).

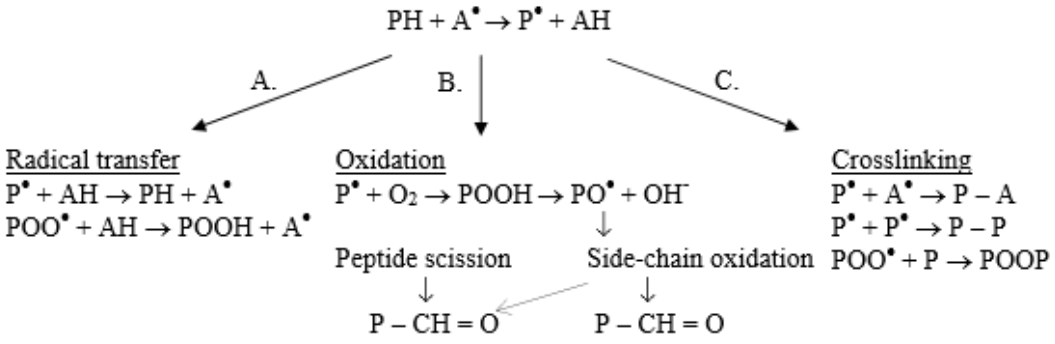


Figure 13 *Protein oxidation pathways via A) free radical transfer, B) oxidation, and C) crosslinking*

(Schaich 2008)

PH = protein, P• = protein radical, AH = any molecule with abstractable hydrogens, A• = non-protein radical, PO• = alkoxyl radical, POO• = peroxyl radical, POOH = hydroperoxide, P-CH=O = secondary products such as aldehydes.

According to Figure 13, Free radical transfer occurs when lipid peroxyl and alkoxy radicals arise from lipid hydroperoxides and protein radicals (P•) are formed and transfer free radicals to proteins by abstracting hydrogens. Additionally, oxidation occurs in the presence of oxygen causing backbone fragmentation of proteins arises

via C-C or β-scission that decarboxylates the target amino acid side-chain during exposure to radicals (radiation, oxidizing lipids). Moreover, free radical crosslinking which is the general reaction generates usually polymers of intact protein monomers, with and without oxygen bridges (Lund et al., 2011). The oxidation of amino acids, peptides and proteins leads to physicochemical and functional property changes which result in formation of toxic compounds (Figure 14) (Rice-Evans & Burdon, 1993). Oxidation of proteins has also been linked to changes occurring during aging, particularly with progression of diseases and disorders in humans (Levine & Stadtman, 2001).

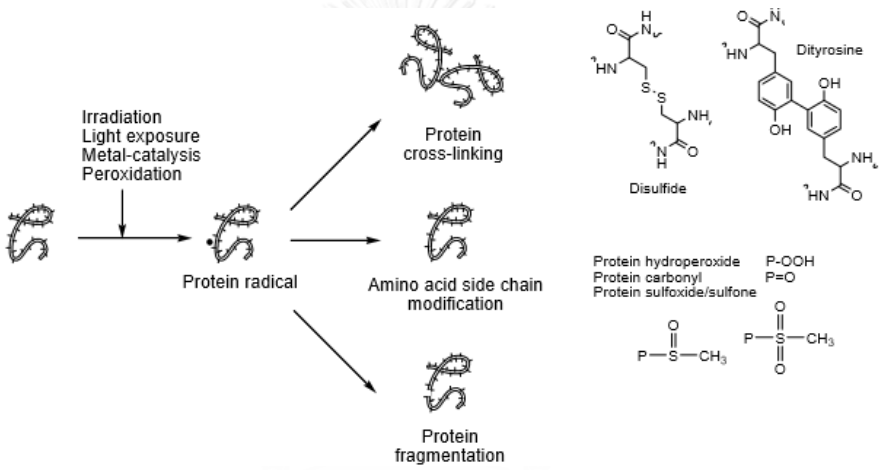


Figure 14 *Different consequences of oxidation of proteins*

(Schaich 2008)

2.9 The nature of color in meat

The color represent in meat product is of the most importance because it has an influence on the first impression of consumers. Light waves for measuring the product color can have varying degrees such as pass through the object, become absorbed by the object, or be reflected by the object. The most important for perceived color wavelengths are reflected back from the object. Furthermore, color that can be visually detected has several attributes. The wavelength of light radiation is hue which is perceived as yellow, green, blue or red color. On the other hand,

chroma is the intensity of the color which it is associated with white light that is mixed with color. The last one is value indicates the brightness that is associated with the reflectance of the color (Mancini & Hunt, 2005).

Pigments of meat color can be absorbed certain wavelengths of light, but there are many factors including the structure and texture of the muscles that influence how eyes perceive these colors (Faustman & Cassens, 1990). Meat color depends on pigment content, ultimate pH, rate of pH decline post mortem, as well as the physical characteristics of the muscle (Zhang et al., 2013). However, myoglobin is the primary pigment that responsible around 80%-90% for meat color and is a water-soluble protein that stores oxygen for aerobic metabolism in the muscle (Soladoye et al., 2015). Myoglobin consists of a protein portion and a non-protein porphyrin ring with a central iron atom that plays an important role in meat color. The oxidation (chemical) state of the iron and compounds (oxygen, water or nitric oxide) are attached to the iron portion of the molecule are the factors that affect meat color. During the cooking process, the cooked pigment is denatured to metmyoglobin which present brown color and is easily recognized in cooked meat products (Faustman & Cassens, 1990).

2. 10Lack of research in *Clitoria ternatea*

Clitoria ternatea is multipurpose forage legumes. Pharmacological studies have confirmed that *Clitoria ternatea* exhibit a broad range of biological effects (Abreu et al., 2014; Gomez & Kalamani, 2003; Gupta et al., 2010). However, the evidence of *Clitoria ternatea* is limited to its bioactive secondary metabolites and bioaccessibility to perform its biological activity such as antihyperglycemic and antihyperlipidemic.

To study about the bioactive secondary metabolites of bioactive compounds in *Clitoria ternatea* may play an important role in improving the bioaccessibility and hence can perform a good potential on biological activity. Many literatures indicated that bioactive compounds are low bioavailability and encapsulation of the bioactive compounds is an alternative way to improve its stability and offer potential advantages over conventional drug delivery systems and also established as unique carrier systems for many pharmaceuticals (targeted drug delivery systems) (Champagne & Fustier, 2007; Jackson & Lee, 1991; Jyothi et al., 2012). The digestive stability and its

encapsulation of CT petal flower is found to lack of literature and research in this field. Therefore, it is important to develop the encapsulation of CT petal flower and investigate its characteristics as well as the digestive stability and bioaccessibility of CT petal flower extract and its encapsulation. This will add value to CT petal flower extract and also provides a novel food-grade encapsulation formulation of plant polyphenols to improve its stability and biological activity.

CT petal flowers are well known to be used as a natural blue colorant in a variety of foods and also herbal beverage. Previous studies reported CT petal flowers are recognized as a good source of dietary anthocyanins and exhibit one of the potent dietary antioxidants (Chayaratanasin et al., 2015; Gomez & Kalamani, 2003; Madhu, 2013; Zingare et al., 2013). However, the application of CT petal flowers extract as an antioxidant on the prevention of lipid and protein oxidation for food preservation in food model have not been investigated. The CT petal flower extract may be used as a natural additive, which can be applied to extend the shelf-life of cooked pork patties in the food industry.

CHAPTER III

MATERIALS AND METHODS

3.1Materials

3.1.1Plant material

Clitoria ternatea petal flowers were collected from local areas of Sisaket Province, Thailand in November of 2013. The plant was authenticated at Division of Plant varieties protection, Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. The herbarium number of the plant is BK 066793.

3.1.2Chemicals

2-Deoxy-D-glucose	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
2,2'-Azino-bis-	Sigma-Aldrich CO.
-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)	(St. Louis, MO, USA)
2,2-diphenyl-1-picrylhydrazyl (DPPH·)	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
2, 4-dinitrophenylhydrazine (DNPH)	Ajax finechem
	(Taren Point, Australia)
2, 4, 6-tri (2-pyridyl)-1, 3, 5-triazine (TPTZ)	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
3-[(3-cholamidopropyl)-dimethylammonio]-	Sigma-Aldrich CO.
-1-propanesulphonate (CHAPS)	Sigma-Aldrich CO.
3-(4,5-dimethylthiazol-2-yl)-2-5	Sigma-Aldrich CO.
diphenyl tetrazolium bromide (MTT)	(St. Louis, MO, USA)
5, 5’-dithiobis-(2-nitrobenzoic acid) (DTNB)	Calbiochem

(Darmstadt, Germany)	
6-hydroxyl-2,5,7,8-tetramethylchromane-	Sigma-Aldrich CO.
-2-carboxylic acid (trolox)	(St. Louis, MO, USA)
Acetonitile	Merck
(Darmstadt, Germany)	
Amyloglucosidase solution	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
Bile extract porcine	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
Bovine Serum Albumin	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
Butylated hydroxytoluene (BHT)	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
Calcium chloride ($CaCl_2$)	Ajax finechem
	(Taren Point, Australia)
Catechin	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
D-(+)-Glucose	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
D-(+)-Maltose monohydrate	Ajax finechem
	(Auckland,New Zealand)
D-[U-^{14}C] glucose	American Radiolabeled
Chemicals	
	(St. Louis, MO, USA)
Dimethyl sulfoxide (99% DMSO)	Sigma-Aldrich CO.
(St. Louis, MO, USA)	
Disodium phosphate (Na_2HPO_4)	Ajax finechem
	(Auckland,New Zealand)

Dulbecco's Modified Eagle Medium (DMEM)	GIBCO Life Technologies (Gaithersburg, MD, USA)
Ethanol	Merck (Darmstadt, Germany)
Ethyl acetate	Fisher (Loughborough, LE, UK)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich CO. (St. Louis, MO, USA)
Fetal bovine serum (FBS)	GIBCO Life Technologies (Gaithersburg, MD, USA)
Folin reagent	Sigma-Aldrich CO. (St. Louis, MO, USA)
Gallic acid	Fluka (St. Louis, MO, USA)
Glycodeoxycholic acid	Sigma-Aldrich CO. (St. Louis, MO, USA)
Guanidine hydrochloride	Calbiochem (Darmstadt, Germany)
Hexane	Fisher (Loughborough, LE, UK)
Hydrochloric acid (HCL) (Mumbai, India)	Loba chemie
Iron(II) sulfate	Ajax finechem (Auckland,New Zealand)
Iron (III) chloride hexahydrate	Ajax finechem (Auckland,New Zealand)
Isopropanol	Merck (Darmstadt, Germany)

L-cysteine	Sigma-Aldrich CO. (St. Louis, MO, USA)
LS 6500 liquid scintillation counter	Beckman Coulter (Brea, CA)
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich CO. (St. Louis, MO, USA)
Methanol	Merck (Darmstadt, Germany)
Monosodium phosphate (NaH_2PO_4)	Qrec chemical co, Ltd. (New Zealand)
N-2-hydroxyethylpiperazine-N'-2--ethanesulfonic acid (HEPES)	Sigma-Aldrich CO. (St. Louis, MO, USA)
Non-essential amino acids (NEAA)	GIBCO Life Technologies (Gaithersburg, MD, USA)
Pancreatin from porcine pancreas	Sigma-Aldrich CO. (St. Louis, MO, USA)
Penicillin-streptomycin (50 units/mL)	GIBCO Life Technologies (Gaithersburg, MD, USA)
Pepsin from porcine gastric mucosa	Sigma-Aldrich CO. (St. Louis, MO, USA)
Phosphate-buffered saline (PBS, pH7.4, for cell culture)	GIBCO Life Technologies (Gaithersburg, MD, USA)
Porcine pancreatic α-amylase	Sigma-Aldrich CO. (St. Louis, MO, USA)
Potassium chloride	Ajax finechem (Auckland,New Zealand)
Potassium persulfate	Ajax finechem

	(Auckland,New Zealand)
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich CO.
	(St. Louis, MO, USA)
Potassium phosphate dibasic (K_2HPO_4)	Ajax finechem
	(Auckland,New Zealand)
Rat intestinal powder	Sigma-Aldrich CO.
	(St. Louis, MO, USA)
Sodium alginate	Sigma-Aldrich CO.
	(St. Louis, MO, USA)
Sodium acetate	Ajax finechem
	(Auckland,New Zealand)
Sodium chloride (NaCl)	Ajax finechem
	(Auckland,New Zealand)
Sodium carbonates (Na_2Co_3)	Ajax finechem
	(Auckland, New Zealand)
Sodium hydrogen carbonates ($NaHCO_3$)	Qrec chemical co, Ltd.
	(New Zealand)
Sodium nitrite ($NaNO_2$)	Qrec chemical co, Ltd.
	(New Zealand)
Sodium hydroxide (NaOH)	Ajax finechem
	(Auckland, New Zealand)
Sucrose	Ajax finechem
	(Auckland, New Zealand)
Taurochenocholic acid	Sigma-Aldrich CO.
	(St. Louis, MO, USA)
Taurodeoxycholic acid	Sigma-Aldrich CO.
	(St. Louis, MO, USA)
Trichloroacetic acid	Merck

	(Darmstadt, Germany)
Trypsin Phosphate Versene Glucose (TPVG)	GIBCO Life Technologies
	(Gaithersburg, MD, USA)
Reagents	
Bio-Rad protein assay reagent	Bio-Rad
	(Richmond, CA)
Glucose liquicolor reagent	Human
	(Wiesbaden, Germany)
Assay kits	
A total bile acid kit	Bio-Quant Co.
	(San Diego, CA, USA)

3.1.3Laboratory equipment company

Autoclave	Hirayama
	(Tokyo, Japan)
CO_2 incubator	Slimcell
	(Europe)
Differential scanning calorimetry (DSC)	Netzsch
	(Selb, Germany)
Fourier-Transform Infrared (ATR-FTIR) spectra	Perkin Elmer
	(Norwalk, CT)
Freezer -20°C	Sanyo
	(Osaka, Japan)
High speed refrigerated micro-centrifuge	Hettich
	(Tuttlingen, Germany)
Hot air oven	Conthem Scientific
	(New Zealand)

High–performance liquid chromatography (HPLC)	Shimadzu Corporation (Kyoto, Japan)
Hunter-Lab MiniScan XE plus spectrocolorimeter (A60-1010-615 Model Colorimeter)	Hunter-Lab (Reston, VA)
Laboratory refrigerator	Sanden intercool (Thailand)
Light microscope	Olympus (Tokyo, Japan)
Orbital shaker	Labnet international, Inc. (Edison, NJ, USA)
pH meter	Thermo Scientific, Inc. (Waltham, MA, USA)
Pipette	Thermo Scientific, Inc. (Waltham, MA USA)
Refrigerator 4°C	Sharp (Kyoto, Japan)
Shaking incubator	Labnet International Inc. (Edison, NJ, USA)
Spectrophotometer	Perkin Elmer (Waltham, MA, USA)
Spray dry machine	Eyela world (Tokyo, Japan)
Ion sputter instrument	Balzers, Bal-Tec GmbH (Germany)
Scanning electron microscope	JEOL (Tokyo, Japan)
Syringe pump	DREMED (Louisville KY, USA)
Vortex	Gemmy industrial corp.

	(Taipei, Taiwan)
Water bath	Memmert
	(Holland, Michigan)

Lab devices

Glassware	Corning
	(New York, USA)
Nitrogen gas	Namheang
	(Bangkok, Thailand)
Nylon filter (0.22 µL)	Corning
	(New York, USA)
Oasis HLB cartridge	Waters Corp.
	(Milford, MA)
Poly- (tetrafluoroethylene) (PTFE)	Corning
membrane syringe filter (0.45 µm pore size)	(New York, USA)
Plasticware	Thermo Scientific
	(Waltham, MA USA)
Plasticware	Axygen
Plasticware (for cell culture)	Corning
	(New York, USA)

3.2 Method

3.2.1 The stability, antioxidant activity and biological activity of *Clitoria ternatea* petal flower extract during simulated *in vitro* gastrointestinal digestion.

3.2.1.1 Preparation of *Clitoria ternatea* flower petal extract

The extract of *Clitoria ternatea* flower petal (CT) was supplied in the form of spray dried powder from Specialty Natural Products Co., Ltd., Bangkok, Thailand. It was vacuum packed and stored at -80°C in an airtight container until used.

3.2.1.2 Purification of sample

Anthocyanin fractions were obtained using an activated Oasis HLB cartridge (Waters Corp., Milford, MA), according to the modified procedure of previous study with minor modification (Wongs-Aree et al., 2006). Briefly, the dried CT was resolubilized in water and applied to an activated Oasis HLB cartridge. The cartridge was washed with 0.01% hydrochloric acid in water, followed by ethyl acetate and then 0.01% hydrochloric acid in methanol to elute the anthocyanins. The anthocyanins were dried with nitrogen gas and used for further analysis.

3.2.1.3 Acid hydrolysis

Acid hydrolysis procedure was adapted from a previous study (Wongs-Aree et al., 2006). CT were hydrolyzed with 3 N HCl and incubated at 100°C for 45 min, then cooled on ice. The hydrolysate was purified using an activated Oasis HLB cartridge by washed with water acidified with 0.01% hydrochloric acid, followed by ethyl acetate and finally methanol acidified with 0.01% hydrochloric acid. The acidified methanol was used to elute the anthocyanins and the fraction was dried with nitrogen gas and used for further analysis.

3.2.1.4 *In vitro* digestion procedure

In vitro gastrointestinal digestion of CT extract, the two-stage *in vitro* digestive model was adapted from a previous study with minor modification (Green et al., 2007). The gastric phase was initiated by addition of 3 mL porcine pepsin solution (40 mg/mL in 0.1 N HCl) and adjustment of the pH to 2.0±0.1 with 1.0 N NaOH or HCl. Samples were incubated at 37°C in a covered shaking water bath for 1 h. The small intestinal phase was initiated by adjusting the pH of the gastric digesta to 4.5 with 1.0 N NaOH or HCl followed by the addition of 0.15 mL of amyloglucosidase solution (120 mg/mL) and incubated for 30 min; adjust pH to 5.3 with combinations of 100 mM $NaHCO_3$ and 1.0 N NaOH and addition of small intestinal enzyme solution (9 mL, pancreatin (3 mg/mL), and bile (12 mg/mL) in 100 mM $NaHCO_3$). The final sample was adjusted to 7.2±0.1 with 1.0 N NaOH or HCl, volume standardized to 20 mL with saline and placed in a 37°C shaking water bath for 2 h. After completion of the small intestinal phase, samples were centrifuged at 12,000 rpm for 1 h at 4°C. Aliquots were collected, filtered

through a 0.22 μm nylon filter, acidified with 2% aqueous acetic acid, and stored frozen at –80°C for further analysis, respectively.

3.2.1.5 The phytochemical analysis

3.2.1.5.1 Total polyphenol content

The total polyphenol content (TPC) was estimated using the Folin-Ciocalteu method with minor modifications (Chayaratanasin et al., 2015). An aliquot of the extracts (10 μL) were mixed with 90 μL of FC reagent (1:9). Then, 100 μL of 10% (w/v) sodium carbonate solution was added to each sample, vortexed, and incubated in the dark for 30 min. Samples were mixed thoroughly after incubation and the absorbance was measured at 750 nm using a spectrophotometer. Gallic acid (0-1000 μg/mL) was used as a standard for the calibration curve.

3.2.1.5.2 Total anthocyanin content

The assessment of total anthocyanin content (TAC) was carried out by the pH differential method according to AOAC as described in previous study (Lee et al., 2005). For the quantification of total anthocyanin content (TAC), aliquots (10 μL) of each sample were diluted with 490 μL of 0.025 M potassium chloride buffer (pH 1) and 490 μL of 0.4 M sodium acetate (pH 4.5) respectively. The absorbance of each dilution was measured at 510 and 700 nm against distilled water as a blank. Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5. The monomeric anthocyanin pigment concentration was expressed as mg cyanidin 3-glucoside equivalents/g dry mass and calculated using the formula:

$$\text{TAC (mg/g)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

where A = (A510 nm – A700 nm) $_{pH\ 1.0}$ – (A510 nm – A700 nm) $_{pH\ 4.5}$; MW (molecular weight) = 449.2 g/mol ; DF = dilution factor; 1 = cuvette pathlength in cm; ε = 26,900 L/mol.cm, molar extinction coefficient for cyanidin 3-O-β-D-glucoside. 10^3: factor to convert g to mg.

3.2.1.5.3 Qualitative analysis of anthocyanins by HPLC

A portion of sample were resolubilized with water to make a final concentration of 2 mg/mL and filtered through a 0.45 μm pore size poly- (tetrafluoroethylene) (PTFE) membrane syringe filter prior to HPLC injection. Quantification of anthocyanin were performed by Shimadzu HPLC system equipped with an auto-sampler, with a photodiode-array detector (DAD 200; Perkin-Elmer, Courtabœuf, France) and an UV-Vis detector and a data acquisition system Shimadzu (Shimadzu, Milan, Italy) at 520 nm. The HPLC analyzes were carried out under these conditions: Shiseido Capcell pak C18 column UG 120 column (4.6 mml.D.x150 mm; 5 μm). The mobile phase consisted of 1% acetic acid in 0.5% formic acid/water (eluent A) and eluent A/acetonitrile (20/80; eluent B) using gradient program. The system was run with a flow rate of 1 mL/min, column temperature, 40 °C; 10 μL injection and the following gradient program: 0 min: 90% A + 10% B, 2 min: 90% A + 10% B, 2-30 min: 55% A + 45% B, 30.1 min: 55% A + 45% B, 30.1–31 min: 90% A + 10% B, and 31–40 min: 90% A + 10% B. Anthocyanins content was expressed as delphinidin 3-glucoside equivalents.

3.2.1.6 *In vitro* Antioxidant capacities

3.2.1.6.1 DPPH scavenging activity

The radical scavenging activity was determined by using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radical using a modification of the previous method (Chayaratanasin et al., 2015). Samples were prepared in deionized water. An aliquot of each solution (10 μL) was mixed with 90 μL of 0.1 mM DPPH solution in methanol. After 30 minutes of incubation in the dark, the absorbance was measured at 515 nm. The antioxidant activity was expressed as % DPPH inhibition and IC_{50} value.

3.2.1.6.2 Ferric Ferric-ion reducing antioxidant power

The FRAP assay was performed according to the previous study (Thaipong et al., 2006) with some modifications (Chayaratanasin et al., 2015). The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 °C before using. Sample (10 μL) were mixed with 90 μL of the FRAP solution for 30 min in the dark condition. Readings of the colored product [ferrous tripyridyltriazine

complex] were then taken at 595 nm. The standard curve ($FeSO_4$) was linear between 0 – 1,000 µg/mL. Results are expressed in µg $FeSO_4$ equivalent/g extract.

3.2.1.6.3 ABTS reducing antioxidant power

Assessment of ABTS radical-scavenging activity was done according to a previously published method (Chayaratanasin et al., 2015). The radical anion ($ABTS^{\circ+}$) was induced by adding potassium persulfate ($K_2S_2O_4$) and ABTS. The mixture was incubated at room temperature for at least 16 hours in the dark. The $ABTS^{\circ+}$ solution was diluted in 0.1 M PBS, pH 7.4 to absorbance at 0.700 ± 0.02 nm. The $ABTS^{\circ+}$ solution (90 µL) was added to aliquots (10 µL) of each sample and incubated for 6 min in the dark condition. The absorbance was measured at 734 nm. The TEAC value was calculated from the standard curve prepared by using a Trolox (0 – 1,000 µg/mL).

3.2.1.7 Bile acid binding

The bile acid binding assay was slightly modified according to a previously described method (Mäkynen et al., 2013). Taurocholic acid, glycodeoxycholic acid, and taurodeoxycholic acid were used as bile acids in this experiment. Briefly, 100 µL of the extract was incubated with 900 µL of 2 mM bile acid at 37°C for 120 min. The mixture was filtered through a 0.22 µL nylon filter to separate the bound from the free bile acids. The bile acid concentration was analyzed using a bile acid analysis kit.

3.2.1.8 Assay for Intestinal α-Glucosidase (maltase and sucrase) activity

The assessment of intestinal α-glucosidase inhibitory activity was based on the modified method previously described (Adisakwattana et al., 2012). Briefly, the crude rat intestinal α-glucosidase enzyme was prepared from rat intestinal acetone powder 100 mg in 3 ml of 0.9% NaCl solution and centrifuged at 12,000 g for 30 min then subjected to assay. The samples (10 µL) at various concentrations were incubated with 86 mM maltose (30 µL) or 400 mM sucrose (40 µL), followed by the addition of the crude enzyme solution (as maltase assay, 10 µL; as sucrase assay, 30 µL), then 0.1 M phosphate buffer, pH 6.9 was added to give a final volume of 100 µL. The reaction was incubated at 37°C for 30 min (maltase assay) or 60 min (sucrase assay). Then, the mixtures were suspended in boiling water for 10 min to stop the reaction. The concentrations of glucose released from the reaction mixtures were determined by

glucose oxidase method with absorbance at a wavelength of 500 nm. Intestinal **α**-glucosidase inhibitory activity was expressed as percentage inhibition using the following formula:

$$\% \text{ Inhibitory} = [(Abs_{Control} - Abs_{Sample}) / Abs_{Control}] * 100$$

Where $Abs_{Control}$ was the absorbance without sample,

$Abs_{Samples}$ was the absorbance of sample extract

3.2.1.9 Assay for pancreatic α-Amylase activity

The pancreatic **α**-amylase inhibition assay was performed according to a previous report (Adisakwattana et al., 2012). Porcine pancreatic **α**-amylase (3 units/ml) was dissolved in 0.1 M phosphate buffer saline, pH 6.9. The various concentrations of the samples (10 µL) were added to a solution containing starch (1 g/L) and phosphate buffer (165 µL). The reaction was initiated by adding enzyme solution (75 µL) to the incubation medium. After 10 min incubation, the reaction was stopped by adding 250 mL dinitrosalicylic (DNS) reagent (1% 3,5-dinitrosalicylic acid, 0.2% phenol, 0.05% Na_2SO_3 and 1% NaOH in aqueous solution) to the reaction mixture. The mixtures were heated at 100°C for 10 min in order to stop the reaction. Thereafter, 250 µL of 40% potassium sodium tartarate solution was added to the mixtures to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance was recorded at 540 nm using a microplate reader. Intestinal **α**-amylase inhibitory activity was expressed as percentage inhibition using the following formula:

$$\% \text{ Inhibitory} = [(Abs_{Control} - Abs_{Sample}) / Abs_{Control}] * 100$$

Where $Abs_{Control}$ was the absorbance without sample,

$Abs_{Samples}$ was the absorbance of sample extract

3.2.1.10 Glucose uptake measurement in Caco-2 cells

3.2.1.10.1 Cell cultures

Human intestinal Caco-2 cells were cultured as previously described (Kim et al., 2013). Caco-2 cells were maintained in 75 cm^2 plastic flasks and cultured in a 95% air/5% CO_2 atmosphere in routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), and 1% penicillin-streptomycin (50 units/mL). Regular media changes were carried out every 3 days. Confluent monolayer cultures were reached every 4-7 days and dissociated with Trypsin Phosphate Versene Glucose (TPVG) solution [0.2% trypsin, 0.02% Ethylenediaminetetraacetic acid (EDTA), 0.05% glucose in PBS]. All experiments were carried out on cells between passage numbers 30 and 40.

3.2.1.10.2 Cell viability

Cytotoxicity of Purified *Clitoria ternatea* extract after simulated gastrointestinal digestion (DPCTE) in Caco-2 cells was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide) assay according to a previous method30 with minor modifications (Kim et al., 2013). Cells were plated in 96-well tissue culture plate at a density of 1.0×10^4 cells/well and incubated for 4 hours at 37°C. Thereafter, the cells were exposed to 20 µL of different concentrations (0 to 2 mg/mL) of samples for 4 hours. Caco-2 cells were incubated with 5 mg/mL of MTT in PBS (20 µL of MTT/well) for 4 hours at 37°C in 96 well plate. At the end of the incubation, blue-violet formazan salt crystals were formed. The supernatant was removed and the insoluble formazan crystals were dissolved in 100 µL dimethyl sulfoxide (99% DMSO); then the plates were shake for 5 minutes in the shaker to ensure complete lysis. The absorbance at 540 nm was measured using a spectrophotometer. Assay was performed in triplicates. The percent relative cell viability was expressed as percent of control cells using the following formula:

%Cell viability = [(absorbance of treated cells) / (absorbance of controls)] * 100

3.2.1.10.3 Effect of CT on Glucose absorption by Caco-2 Cells

The effect of the Purified *Clitoria ternatea* extract after simulated gastrointestinal digestion (DPCTE) on glucose uptake assays were performed according to previous study (Kim et al., 2013) with minor modification. For the experiment, the cells were seeded at a density of 1 x 10^4 cells/cm^2 on 24-well plates (Costar, UK, Buckinghamshire, UK) and grown to confluence and were grown for 14 days. Caco-2 cells were washed twice with phosphate-buffered saline (PBS) and preincubated with Krebs buffer [5 mM glucose, 30 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 130 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$ at pH 7.4]. When a sodium-free buffer was required for investigating facilitative transport, NaCl and Na_2HPO_4 in HBSS were replaced with equimolar amounts of KCl and K_2HPO_4, respectively.

Glucose uptake measurements were initiated by replacing the medium with 300 µL of pre-warmed Krebs buffer without glucose, supplemented with [^{3}H]-2-deoxyglucose and digested PCTE together for 10 min at 37°C. The CT solutions were prepared fresh. Uptake was terminated by adding 1 mL of ice-cold PBS, and the cells were washed 3 times with the same solution before lysis with 300 µL of NaOH (0.1 M)/3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (10 g/L) solution. Aliquots of 100 µL were added to 0.5 mL of scintillation cocktail for radioactivity determination, using a LS 6500 liquid scintillation counter. Glucose transport values were corrected for protein content, as determined by the Bradford method.

3.2.2 Alginate-based encapsulation of polyphenols from *Clitoria ternatea* petal flower extract enhances stability and biological activity under simulated gastrointestinal conditions

3.2.2.1 Preparation of *Clitoria ternatea* petal flower extract (CT)

The fresh butterfly pea flowers (*Clitoria ternatea*) were purchased from the local market at Chiang Mai, Thailand. Briefly, CT petal flower extract was fleshly prepared by pouring 100 mL of distilled water over 5, 10, and 20 g of ground *Clitoria ternatea* petal flower and boiled at 80°C for 40 min with stirring. After this time,

extraction was filtered through paper filter Whatman #1 paper and kept in dark flasks until used.

3.2.2.2 Preparation of alginate beads

The preparation of microbeads was followed according to the previous study with minor modifications (Li et al., 2016). The sodium alginate (1-2 g) were mixed with 100 mL CT solution and heated 80℃ with the aid of magnetic stirring. Once homogenized, the sodium alginate solution was left to stand for about 2 h to remove any air bubbles in the solution. A syringe pump was applied to deliver sodium alginate solution to a calcium chloride solution (1.5-5 % w/v) through a stainless steel needle (0.8*40 mm). The extrusion speed was set at 30 mL/h, and the distance between the tip of the needle and the surface of the collection solution was set at 7 cm. The beads formed in this process were maintained in the gelling bath to harden for 30 min with stirring. Then, they were filtered through Whatman #1 paper. The microbeads were washed out three times, using distilled water. The beads were air dried at 25°C for 24 h and kept until further use in a desiccator at 25℃.

3.2.2.3 Total phenolic content measurement

Total phenolic content (TPC) was done using the Folin–Ciocalteau assay according to the previous study (da Rosa et al., 2014). Briefly, the sample was added to 5 g/100 mL sodium citrate solution to disintegrate the alginate beads. After fully dissolved, 10 µL of the solution along with 90 µL of sodium carbonate (10 % w/v) was pipetted into 100 µL of Folin–Ciocalteau reagent (1:1 diluted). The mixture was left for 30 min before the absorption at 750 nm was recorded on a UV–vis spectrophotometer. The concentration of gallic acid was calculated based on a calibration curve using gallic acid (range 0-1 mg/mL) as the standard reference under the same conditions.

3.2.2.4 Determination of total phenol content and encapsulation efficiency

The encapsulation efficiency was performed according to a previously described study (da Rosa et al., 2014). Aliquots of 10 mg of microbeads were dissolved in 5 mL of sodium citrate (5% w/v), sonicated for 30 min, and centrifuged for 10 min at 3,000 rpm. The encapsulation efficiency was calculated according to the equation below:

EE (%) = TPCe / APCi

where TPCe was the total phenol content encapsulated in beads, while TPCi was the total phenol content in the initial extract solution used for the encapsulation process.

3.2.2.5 Determination of antioxidant activity

To quantify the antioxidant activity, the beads (10 mg) was suspended in 5 mL of sodium citrate (5% w/v), sonicated for 20 min, and centrifuged for 10 min at 3,000 rpm and the supernatant was kept for further analysis.

3.2.2.5.1 DPPH scavenging activity

DPPH assay was carried out according to the previous method with slight modifications (Brand-Williams et al., 1995). The fresh DPPH· (2,2-diphenyl-1-picrylhydrazyl) solution was prepared daily at 0.2 mM in ethanol. After mixing 10 µL of a sample supernatant and 90 µL of the DPPH· solution, the mixture was incubated at room temperature (25°C) in dark for 30 min before measuring absorbance at 515 nm. The results were expressed in percentage of inhibition of DPPH radical.

3.2.2.5.2 Ferric Ferric-ion reducing antioxidant power

The ferric reducing antioxidant power (FRAP) assay was done according to a previous study with minor modifications (Thaipong et al., 2006). The stock solutions included 0.3M acetate buffer, pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution. Sample supernatants (10 µL) were allowed to react with 90 µL of the FRAP solution for 30 min in the dark condition. The absorbance was read at 595 nm. The standard curve was linear between 0 and 500 mg $FeSO_4$. Results are expressed in mg $FeSO_4$ equivalents/g beads.

3.2.2.5.3 ABTS reducing antioxidant power

In the ABTS assay, the procedure was followed according to the previous method with minor modifications (Thaipong et al., 2006). In the assay, the fresh 7 mM ABTS stock solution was mixed with 2.45 mM potassium persulfate and placed at 25°C in dark for 16 h to generate ABTS+·. The ABTS+· stock solution was diluted 10 times in

PBS to get an absorbance between 0.700-0.900 as a working solution. The 10 µL of a sample supernatant was mixed with 90 µL of the ABTS+· working solution. After incubation at 25°C in dark for 10 min, the absorbance was measured at 734 nm. The results are expressed in mg Trolox equivalents (TE)/g beads.

3.2.2.6 Characterization of microbeads

3.2.2.6.1 Scanning electron microscope analysis

The surface microbeads morphology analysis was performed according to previous study (Anbinder et al., 2011). Scanning electron microscope (model JSM-6360LV, JEOL, Tokyo, Japan) was used to examine the morphology and surface appearance of microbeads. The surface morphology was carried out to visualize microstructure. Prior to SEM analysis, beads were attached to stubs using a two-sided adhesive tape, then coated with a layer of gold (40 nm) using an ion sputter instrument (model SCD 040, Balzers, Bal-Tec GmbH, Germany). The coated microcapsules were examined in a SEM at 10 kV with 1.5 nm resolutions.

3.2.2.6.2 Particle size analysis

A laser diffraction-based Malvern particle size analyzer Mastersizer 3000 (Malvern Instruments Inc., UK) was used for determination of particle diameter. Particle characteristics were computed automatically from a compressed range. Particle size measurement tests were replicated three times. Calculation of particle size distribution is based on theoretical models that are well verified for mass median diameter (Dv_{50}) by volume of dispersed particles. A refractive index of 1.52 for calcium chloride, 1.37 for sodium alginate, and 1.38 for *Clitoria ternatea* were chosen to calculate the median of the particle size distribution.

3.2.2.6.3 Attenuated total reflectance Fourier-Transform Infrared (ATR-FTIR) analysis

FT-IR spectra of the samples in KBr discs were recorded by using attenuated total reflectance Fourier-Transform Infrared (ATR-FTIR) spectra (Spectrum one, Perkin Elmer, Norwalk, CT) following the previous study (Pongjanyakul & Rongthong, 2010). Beads were sandwiched between the ATR accessory and the diamond crystal. Spectra

of samples were operated in the transmission mode with 32 scans and a resolution of ±4 cm^{-1} covering a wavenumber range of 400–4,000 cm^{-1}.

3.2.2.6.4 Differential scanning calorimetry analysis

Differential scanning calorimetry (DSC) measurements were performed on a Netzsch DSC 204F1 apparatus (Selb, Germany) following the previous study (Cho et al., 2014). Sample (5.0 mg) was weighted and sealed in an aluminum DSC pan. After holding isothermally at 25 °C for 1 min, DSC scanning was performed from 25 °C to 350 °C at a heating rate of 10 °C min^{-1} under dry nitrogen purge of 50 mL min^{-1}.

3.2.2.7 *In vitro* gastrointestinal digestion release property

In vitro gastrointestinal digestion release study was performed according to a previous report (Green et al., 2007). The gastric phase was initiated by addition of 3 mL porcine pepsin solution (40 mg/mL in 0.1 N HCl) and adjusted the pH to 2.0±0.1. The extract and its microbeads with equal amount of polyphenol contents (0.802 mg GAE) were added into gastric phase solution and incubated at 37°C in a covered shaking water bath for 1 h. The small intestinal phase was initiated by adjusting the pH of the gastric digesta to 4.5 with followed by the addition of 0.15 mL of amyloglucosidase solution (120 mg/ml) and incubated for 30 min; adjust pH to 5.3 with combinations of 100 mM $NaHCO_3$ and 1.0 N NaOH .Then, 9 mL of a small intestinal enzyme solution containing pancreatin (3 mg/mL) and bile acid (12 mg/mL) in 100 mM $NaHCO_3$ was added. The final sample was adjusted to pH 7.2±0.1, volume standardized to 20 mL with saline and placed in a 37°C shaking water bath for 2 h. After completion of the small intestinal phase, the samples were centrifuged at 12,000 rpm for 1 h at 4°C. Aliquots were collected, filtered through a 0.22 μm nylon filter, acidified with 2% aqueous acetic acid, and stored frozen at –80°C for further analysis, respectively. Release of TPC was measured by the amount of total phenol concentration using the Folin–Ciocalteau assay.

3.2.2.8 Bile acid binding ability

The bile acid binding activity was slightly modified according to a previously described method (Mäkynen et al., 2013). Briefly, the digesta fluid was subjected to determine the bile acid concentration using a bile acid analysis kit. The absorbance

was measured at 540 nm using a spectrophotometer. The results were calculated for the percentage of bile acid binding.

3.2.2.9 Pancreatic α-Amylase activity

The pancreatic α-amylase activity was performed according to a previous report (Adisakwattana et al., 2012). The sample solution (10 μL) was added to a solution containing starch (1 g/L) and phosphate buffer (165 μL). After 10 min incubation, the reaction was stopped by adding 250 mL dinitrosalicylic (DNS) reagent (1% 3,5-dinitrosalicylic acid, 0.2% phenol, 0.05% Na_2SO_3 and 1% NaOH in aqueous solution) to the mixture. The mixtures were heated at 100°C for 10 min in order to stop the reaction. Thereafter, 250 μL of 40% potassium sodium tartarate solution was added to the mixtures to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance was recorded at 540 nm using a microplate reader. The concentration of maltose was calculated using the calibration curve of maltose. The results were calculated for the percentage inhibition of pancreatic α-amylase.

3.2.2.10 Release study of microbeads in water

Release study of polyphenols from microbeads were performed at room temperature. One gram of CT beads was placed in a flask containing 10 mL of water under magnetic stirring for 24 hours. The content of polyphenols (TPC) of aliquots at each time point was determined as previously described (Stojanovic et al., 2012).

3.2.3 The effect of *Clitoria ternatea* petal flower extract toward protein and lipid oxidation in cooked pork patties during the storage.

3.2.3.1 Preparation of *Clitoria ternatea* flower petal extract

The *Clitoria ternatea* flower petal extract (CTE) was purchased in the form of spray dried from Specialty Natural Products Co., Ltd., Bangkok, Thailand. It was vacuum packed and stored at -80°C in an airtight container until used.

3.2.3.2 Preparation of pork patty samples

The longissimus dorsi muscle from pigs was obtained from a local meat-producing company (Charoen Pokphand Foods Co., Ltd., Bangkok, Thailand). After

slaughter, it was immediately kept under frozen storage at -20°C and was used for the preparation of pork patties 24 h post-slaughter.

The cooked meat model was performed according t o a previously published method with minor modifications (Ganhão et al., 2010). The experiment for the evaluation of cooked pork patties was carried out using a 6 x 5 factorial design with six treatments (control, CTE 0.02%, 0.04%, 0.08% and 0.16% and BHT 0.02% w/w) and five storage times (day 0, 3, 6, 9 and 12). A randomized design using the addition of CTE and storage time was performed. Pork patties from five different lots of meat packers were done and each lot was analyzed in triplicate. Fresh pork samples were ground twice through an 8-mm plate using a meat mincer. After mincing, the pork meat was divided into six portions for each experiment prior to addition of compounds. Each portion of pork meat (100 g) was added (w/w) according to the following formulation: (1) control (no antioxidant added); (2) CTE 0.02% (w/w); (3) CTE 0.04% (w/w); (4) CTE 0.08% (w/w); (5) CTE 0.16% (w/w) and (6) BHT 0.02% (w/w). After addition of the extract, samples were mixed vigorously and ground pork samples were formed into burger patties by a burger-maker (100 g/patty) to give a diameter of 9 cm and thickness of 1 cm. Patties were placed on trays and cooked in a fan assisted oven at 160°C, until an internal meat temperature of 70°C (measured with a food thermometer) was reached and subsequently held for further 10 min. The cooked pork patties were cooled up to room temperature for 5 min, and then all were packaged in polypropylene trays and wrapped with polyvinylchloride film. The samples were displayed under white fluorescent lighting conditions for 12 days using a refrigerator at 4°C equipped with a glass door, simulating the current conditions at the supermarket. Before and after 3, 6, 9 and 12 days of storage, each sample was separately blended in a blender to obtain a homogeneous sample and then analyzed for antioxidant capacity (TEAC), lipid oxidation (TBARS), conjugated dienes (CD), and protein oxidation (protein carbonyl groups and protein thiol), as well as color parameters and sensorial characteristics.

3.2.3.3 Determination of total polyphenol content

The total phenolic content of extract was quantified according to a previously published method with minor modifications (Chayaratanasin et al., 2015). The CTE was

dissolved in distilled water. The supernatant (10 µL) was mixed into 90 µL of Folin–Ciocalteu reagent (diluted 10-fold with distilled water). After incubation for 5 min at room temperature, the mixture was incubated with 100 µL of 10% sodium carbonate for 20 min at room temperature in the dark and the absorbance was measured at 750 nm using a spectrophotometer. A standard calibration curve with gallic acid (0.033–1.0 mg/mL) was used for quantification. The total phenolic contents were calculated using a standard curve and expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

3.2.3.4 Determination of antioxidant capacity

Antioxidant activity of the meat was determined using Trolox-equivalent antioxidant capacity (TEAC) assay with minor modifications (Serpen et al., 2012). In brief, the solution of $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS in phosphate buffer saline (PBS) (0.1 M, pH 7.4) with 2.45 mM potassium persulfate ($K_2S_2O_4$) in distilled water. The mixture solution was allowed to stand in the dark at room temperature for 16 h to produce a dark green solution. The $ABTS^{\bullet+}$ solution was diluted with PBS to obtain an absorbance of 0.90±0.04 at 734 nm before experiments. Two grams of minced meat were mixed with 10 mL of a PBS and homogenized for 2 min and centrifuged for 5 min at 3,000 rpm at 4°C. An aliquot of 10 µL of homogenate was mixed to 90 µL of diluted $ABTS^{\bullet+}$ solution and allowed to stand at room temperature in the dark for 6 min. The absorbance was then read at 734 nm. The results were calculated from the standard curve of Trolox and expressed as milligrams of Trolox equivalents per gram of sample.

3.2.3.5 Determination of conjugated dienes

The formation of conjugated dienes (CD) was determined according to a previous method (Juntachote et al., 2007). Meat samples (0.5 g) were suspended in 5 mL of distilled water and homogenized to form a smooth slurry. An aliquot (0.5 mL) of the suspension was then mixed with 5 mL of solution (3:1 hexane: isopropanol) for 1 min. After centrifugation at 2,000 g for 5 min, the absorbance of the supernatant was read at 233 nm. The concentration of CD was calculated using the molar extinction coefficient of 25,200/M/cm and the results were expressed as mmol per mg of sample.

3.2.3.6 Determination of Thiobarbituric Acid Reaction Substances (TBARS)

Lipid peroxidation was measured according to a previously published method with minor modifications (Witte et al., 1970). Briefly, 2 g of sample was homogenized in 6 mL of the mixture solution containing 10% TCA (w/v), 0.1% of ethylenediaminetetraacetic acid (EDTA) (w/v) and 3.3 mM of butylated hydroxytoluene (BHT) and centrifuged at 3,500 rpm for 10 min and filtered through Whatman no.1 filter paper. The filtrate (500 mL) was mixed with an equal volume (500 mL) of 20 mM thiobarbituric acid (TBA) and vigorously vortexed. After that, the test tubes were placed in a boiling water bath (100°C) for 45 min. After cooling, the absorbance was measured at 532 nm using a spectrophotometer. The results were calculated from a standard curve of MDA (0.02–0.39 mg/mL). The TBARS values were expressed as number of milligrams of MDA per kg of sample.

3.2.3.7 Determination of protein thiol groups

Protein thiol groups were determined using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) (Botsoglou et al., 2012). In brief, the meat sample (2 g) was homogenized in 10 mL of 0.1 M phosphate buffer saline (pH 7.4), and 70 µL aliquots was mixed with 130 µL of 2.5 mM DTNB in 0.1 M phosphate buffer saline at pH 7.4. The mixture was then incubated at room temperature for 15 min. The absorbance was measured at 410 nm using a spectrophotometer. The result was expressed as nmol of free thiols per mg of protein.

3.2.3.8 Determination of protein carbonyl groups

Protein oxidation was measured by the formation of total carbonyl content and evaluated by derivatization with 2,4-dinitrophenylhydrazine (DNPH) according to a previously described method with slight modifications (Oliver et al., 1987). Briefly, meat (2 g) was homogenized in 10 mL of 0.1 M phosphate buffer saline (pH 7.4), and two 0.5 mL aliquot of this solution, each mixed with 0.5 mL of 20% trichloroacetic acid and was centrifuged. The precipitated proteins were washed with HCl/acetone (3/100, v/v) twice, followed by washing with 10% trichloroacetic to remove meat chromophores. Then, the first part of precipitation was treated with 0.5 mL 2.5 M HCl for quantifying protein concentration, whereas the second part was treated with 0.5 mL of 10 mM

DNPH in 2.5 M HCl for protein carbonyl measurement. The sample mixtures were incubated in the dark for 1 h at room temperature. After centrifugation, the precipitates were washed once with 0.5 mL 20% trichloroacetic acid and three times with 5 mL of ethanol/ethyl acetate (1/1, v/v) to remove traces of DNPH. The precipitates were then dissolved in 0.5 mL of 6 M guanidine hydrochloride in distilled water. Protein concentration was calculated from the absorption at 280 nm of the sample derived from the precipitate treated with HCl using BSA for the standard curve. Protein carbonyl concentration was calculated from the absorption at 370 nm of the sample derived from the precipitate treated with DNPH and expressed as nmol of carbonyl per mg of protein.

3.2.3.9 Color Measurement of pork patties

The color of cooked pork patties was monitored using Hunter-Lab MiniScan XE plus spectrocolorimeter (A60-1010-615 Model Colorimeter, Hunter-Lab, Reston, VA) with an 8-mm aperture set for illumination of D65/108 standard observer angle. The instrument was calibrated with black and white reference tiles (CIE L^* = 93.56, CIE a^* = -0.91, CIE b^* = +0.89). The values were expressed as L^* (whiteness or brightness/darkness), a* (redness/greenness) and b^* (yellowness/blueness) color values. The values were measured on the surface of each patty in triplicate at three randomly selected locations at days 0, 3, 6, 9 and 12 of storage.

3.2.3.10 Sensory evaluation

3.2.3.10.1 Training of Panelists

Cooked pork patties were evaluated for sensory characteristics according to a previously described method (Kulkarni et al., 2011). Fifteen member descriptive panel were recruited from the students and staff experienced in product evaluation of Kasetsart University at Bang-Khen campus. Selection criteria for panelists were that they were available and motivated to participate on all days of the experiment. The training of panelists was conducted over three open-discussion sessions to evaluate characteristics including rancidity, flavor, juiciness, and surface color of the patties. The training sessions were held prior to the experiment day to familiarize the individual with the attributes and the scale to use. Therefore, each panelist could be able to

thoroughly discuss and clarify each attribute to be evaluated. Sessions 1 and 2 were conducted as round table discussions in order to develop a vocabulary of sensory terms which fulfilled the criteria, relevant to the product, discriminating clearly between samples, non-redundancy, and cognitive clarity to the panel members. In the last training session the panelists were familiarized with the sensory laboratory and the subset of samples was analyzed using the sensory profiling procedure as they were trained. During training, a panel leader facilitated discussions of product characteristics compare with each standard. A Nine-cm line scale anchored on the left by the term 'none' and on the right by the term 'extreme' were used to the indicated preference mark according to previous study (Botsoglou et al., 2012). Ratings were quantified by measuring distance, in cm, from the extreme left of the line scale to the indicated preference mark. A score of 1 represented 'dislike extremely', whereas 5 represented 'neither like nor dislike', and 9 represented 'like extremely'. Panelists were provided mineral water at room temperature to cleanse their palates between samples.

3.2.3.10.2 Descriptive sensory profiling

Sensory analysis was undertaken in the panel booths in accordance with the standard regulations at the university sensory laboratory. Prior to each evaluation the panelists were presented with two standards (a reference of a fresh patty and a reference stored for 0, 3, 6, 9, or 12 days at 4 ℃), which allowed the panel members to distinguish between a fresh and a stored product. Subsequently, the patties were served in a randomized order for individual assessors. At each day of evaluation, a sample set which contained two replicates of both the reference and the six treatments at the given day of storage were presented to the individual panel member. Prior to serving to panelists, pork patties from each treatment were pre-warmed in a conventional microwave oven (Sharp Electronics, Japan) for 30 seconds, cut into 2 cm × 2 cm cubes. During sensory evaluation, the samples were randomized, coded samples with three-digit codes from each of the six treatment groups and served in random order to panelists in individual booths. Panelists were asked to evaluate sample surface color, juiciness, rancidity, and overall acceptability by placing a mark

on a 9 cm, continuous line scale. Water was served for cleansing the mouth between samples.

3.3 Statistical analysis

The results are expressed as means ± standard error of mean (S.E.M.). All data shown are representative of at least three experiments that yielded similar results. Calculation and graph were carried out using Sigma Plot (version 11; Systat Software Inc., San Jose, CA, USA) The data were analyzed by statistical software, SPSS version 18 for windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Duncan's multiple range test were performed to determined differences of means among groups. Differences were considered to be significant when $P<0.05$. Two-way analysis of variance (ANOVA) in order to evaluate the effect of treatment and storage time on the dependent variables. When the effect of factors was significant ($P<0.05$), the means were separated using Duncan's multiple range tests to examine significant differences at each storage interval for individual treatments was employed ($P<0.05$). In case of significant interaction, data obtained at specific storage times were statistically treated by one-way ANOVA and separation of means (both experiments).

CHAPTER IV

RESULTS

4.1 Result 1: The stability phytochemical composition of *Clitoria ternatea* petal flower extract and its biological activities including bile acid binding activity, inhibitory effects of intestinal α-glucosidase and pancreatic α-amylase and glucose uptake through SGLT1 during simulated in vitro gastrointestinal digestion.

4.1.1 Total polyphenol content (TPC) of *Clitoria ternatea* extract (CTE), Purified *Clitoria ternatea* extract (PCTE), and Acid hydrolysis *Clitoria ternatea* extract (AHCTE) and after simulated gastrointestinal digestion

Total polyphenol content in CTE and different process and preparation of *Clitoria ternatea* extract (CTE) was determined by using the Folin-Ciocalteu reagent. The TPC was calculated using the standard curve of gallic acid ($y = 2.2739x + 0.0338$; $R^2 = 0.9993$). The TPC of nondigested CTE (34.55±1.10) < PCTE (52.17±1.20) < AHCTE (52.96±1.08) mg gallic acid equivalents/g dried extract. AHCTE was found to have the highest TPC (52.96±1.08 mg gallic acid equivalents/g dried extract), whereas CTE showed the lowest of TPC (34.55±1.10 mg gallic acid equivalents/g dried extract). Interestingly, the content of TPC in PCTE were increased 12.64%, whereas the TPC in AHCTE was a marginally reduced by 4.60% after simulated gastrointestinal digestion (Figure 15).

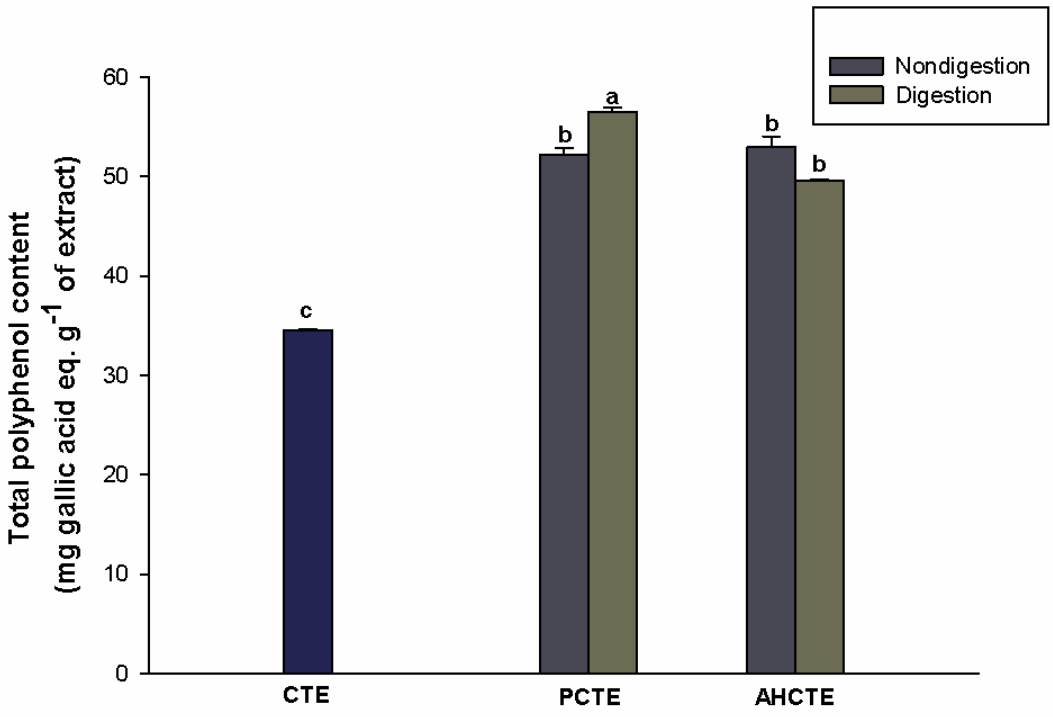


Figure 15 *Phenolic content is expressed as mg gallic acid eq./g extract.*
Data are expressed as mean ± S.E.M. (n = 5). (a-c) in any column are significantly different at *P*<0.05 according to *Duncan's multiple range* test.

4.1.2 Total anthocyanin content of nondigested *Clitoria ternatea* extract (CTE), Purified *Clitoria ternatea* extract (PCTE), and Acid hydrolysis *Clitoria ternatea* extract (AHCTE)

Total anthocyanin content (TAC) of the different process and preparation of *Clitoria ternatea* extract (CTE) was determined by using the AOAC method. Initial total anthocyanin content (TAC) in *Clitoria ternatea* extract (CTE) was 1.49±0.02 mg cyanidin-3-glucoside equivalents/g extract (Figure 16). The highest amount of TAC was found to increase with process and preparation in the following order: AHCTE > PCTE > CTE (*P*<0.05). After the process and preparation of CTE, the TAC of PCTE and AHCTE increased to 2.05±0.01 (37.58%), and 2.48±0.03 (66.44%) mg cyanidin-3-glucoside equivalents/g extract, respectively.

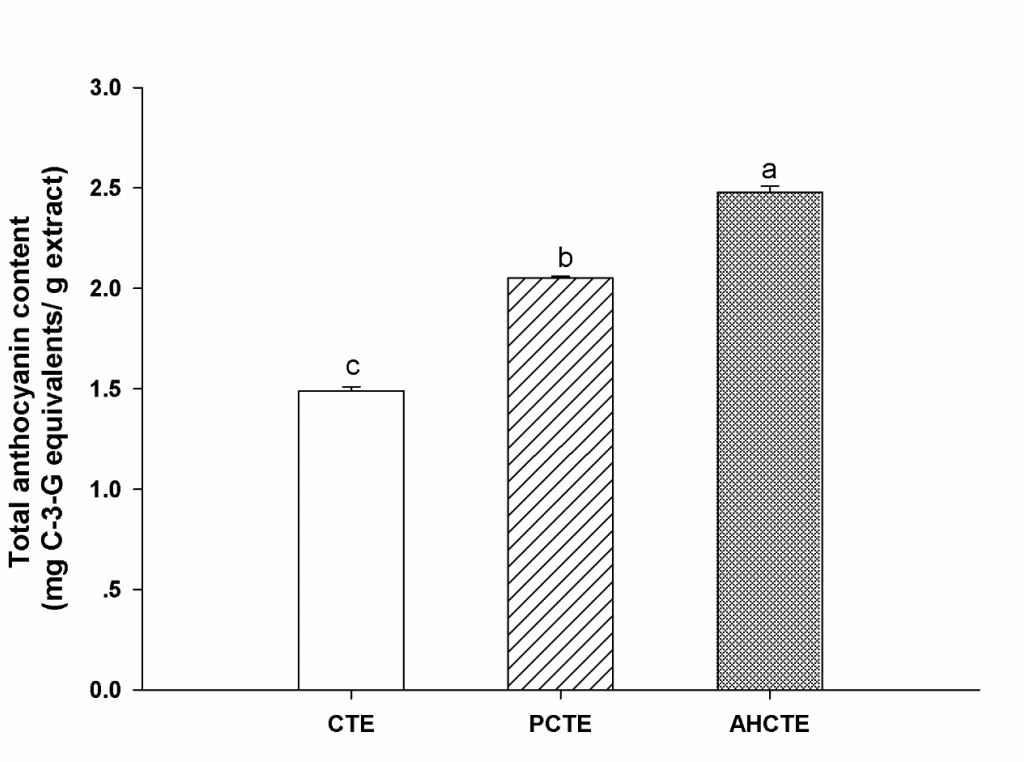


Figure 16 *Total anthocyanin content (mg cyanidin-3-glucoside equivalents/g extract) in Clitoria ternatea extract (CTE), Purified Clitoria ternatea extract (PCTE), and Acid hydrolysis Clitoria ternatea extract (AHCTE).*

The results are expressed as mean ± S.E.M. (n=3). (a-c) in any column are significantly different at $P<0.05$ according to *Duncan's multiple range* test.

4.1.3 Qualitative analysis of anthocyanins in purified *Clitoria ternatea* extract and acid hydrolysis *Clitoria ternatea* extract before and after *in vitro* digestion by HPLC

The chromatogram obtained from non-digested purified *Clitoria ternatea* extract (PCTE) and digested PCTE (DPCTE) are shown in Figure 17a and 17b, respectively. There are 8 tentatively identified shows anthocyanin peaks based on their retention times between 5-25 min on C18 in PDA chromatogram at 520 nm.

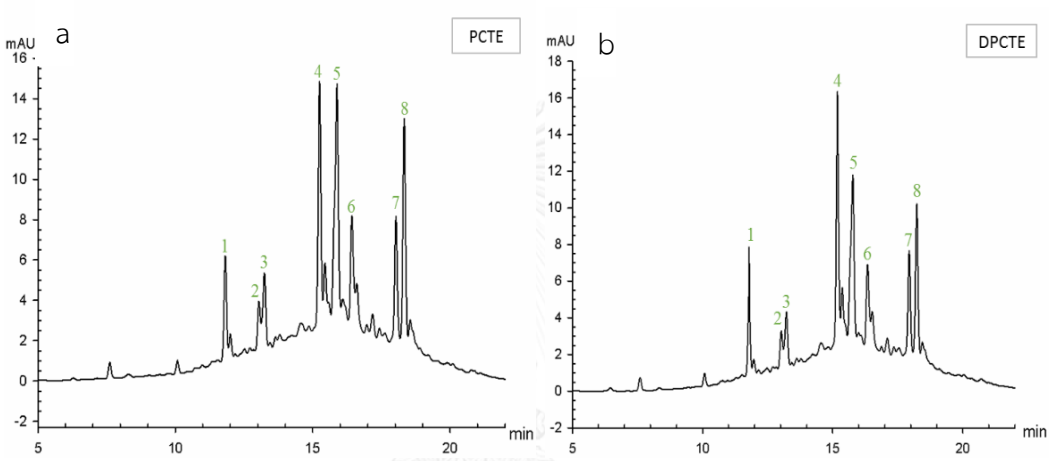


Figure 17 *Chromatographic profile comparison of anthocyanins as a delphinidin-3-glucoside equivalent in non-digested PCTE (a) and digested PCTE (b).*

The contents of each anthocyanin using delphinidin-3-glucoside (D-3-G) equivalent in the purified *Clitoria ternatea* extract before and after digestion are shown in Table 6. The chromatogram profile of anthocyanins from PCTE during simulated digestion presented similar detectable peaks but they have different peak areas. After the *in vitro* digestion procedure in DPCTE, peak 1, 2, 3, 4 and 7 were increased 73.10±2.09%, 25.40±1.76%, 13.29±1.06%, 89.12±3.21%, and 7.27±0.99%, respectively. Whereas, peak area of peak 5, 6, and 8 was decreased 14.75±1.96%, 31.86±1.33%, and 65.74±0.65%, respectively.

Table 6 *Anthocyanin content (μM equi D3G) in purified Clitoria ternatea (PCTE) before and after digestion*

Peak number	Delphinidin-3-glucoside equivalent (μmole/L equi D3G)		
	PCTE	DPCTE	Δ Change (%)
1	2.52±0.26	4.79±0.24	73.10±2.09
2	2.49±0.04	3.12±0.06	25.40±1.76
3	3.66±0.11	4.14±0.09	13.29±1.06
4	6.58±0.23	12.43±0.26	89.12±3.21
5	17.84±0.18	15.21±0.48	-14.75±1.96
6	9.52±0.24	6.49±0.24	-31.86±1.33
7	6.78±0.10	7.27±0.05	7.27±0.99
8	8.59±0.13	2.94±0.07	-65.74±0.65

Figure 18a and 18b shows the chromatogram obtained from non-digested acid hydrolysis *Clitoria ternatea* extract (AHCTE) and digested AHCTE (DAHCTE), respectively. There are 9 anthocyanin peaks identified after acid hydrolysis. The acid hydrolysis CTE (AHCTE) showed clear peaks in the chromatogram with different proportion between peaks.

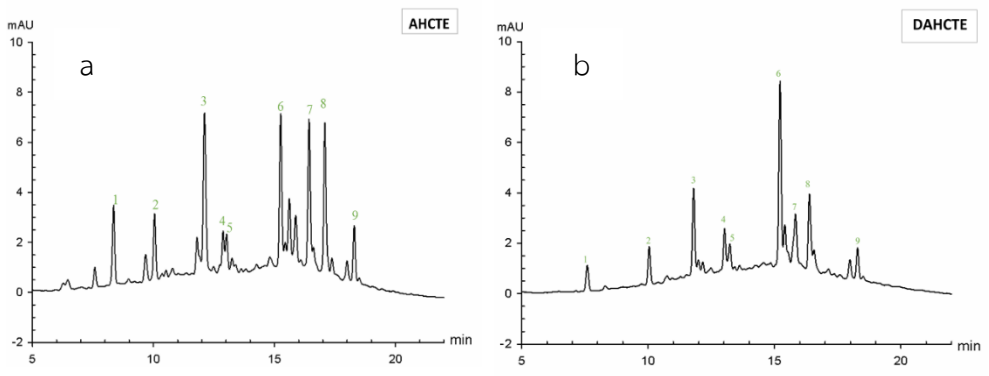


Figure 18 *Chromatographic profile comparison of anthocyanins as a delphinidin-3-glucoside equivalent in non-digested AHCTE (a) and digested AHCTE (b)*

The HPLC chromatograms of DAHCTE revealed the reduction of all peak area, except peak 4 and 6 clearly showed increase 7.40±0.15% and 17.12±0.63% after *in vitro* digestion. (Table 7).

Table 7 *Anthocyanin content (μM equi D3G) in acid hydrolysis Clitoria ternatea (AHCTE) before and after digestion*

Peak number	Delphinidin-3-glucoside equivalent (μmole/L equi D3G)		
	AHCTE	DAHCTE	Δ Change
1	4.06±0.07	2.28±0.07	-43.94±2.36
2	3.95±0.28	2.58±0.09	-34.45±2.33
3	8.04±0.04	4.40±0.09	-45.24±1.26
4	2.37±0.11	2.54±0.12	7.40±0.15
5	2.43±0.12	2.16±0.10	-11.17±0.90
6	7.15±0.07	8.37±0.04	17.12±0.63
7	8.44±0.24	3.21±0.05	-61.87±1.33
8	7.53±0.14	5.17±0.08	-31.24±2.30
9	3.28±0.10	2.36±0.09	-28.16±1.09

4.1.4 Antioxidant activity (DPPH, FRAP and ABTS) in *Clitoria ternatea* extract (CTE), Purified *Clitoria ternatea* extract (PCTE), Acid hydrolysis *Clitoria ternatea* extract (AHCTE), digestion of Purified *Clitoria ternatea* extract (DPCTE), and digestion of Acid hydrolysis *Clitoria ternatea* extract (DAHCTE)

4.1.4.1 DPPH radical scavenging activity

The results of scavenging activity of CTE, PCTE and AHCTE towards DPPH are shown in Table 8. The IC_{50} values of DPPH radical scavenging activity ranged from 2.53±0.14 to 4.53±0.03 mg/mL. For the DPPH radical, AHCTE and PCTE were not significantly different values from each other. However, both PCTE and AHCTE showed a tendency to have lower IC_{50} value of DPPH radical scavenging activity than CTE (P<0.05). After gastrointestinal digestion, PCTE showed the highest radical scavenging activities to 1.89±0.06 mg/mL (P<0.05), whereas AHCTE showed a slightly decrease in DPPH radical scavenging activity to 2.63±0.10 mg/mL.

4.1.4.2 The ferric reducing ability of plasma (FRAP)

The FRAP values of all CTE extracts ranged from 19.14±0.04 to 42.81±0.01 mg $FeSO_4$/g extract. The FRAP value (Table 8) also showed lowest antioxidant activity for the CTE while the highest values were the PCTE and AHCTE. The results demonstrated that antioxidant activity had higher potent after purification or acid hydrolysis. PCTE showed the highest FRAP value (P<0.05) and a slight reduction in FRAP value of AHCTE (6.40%) was also observed after simulated gastrointestinal digestion.

4.1.4.3 ABTS reducing antioxidant power

The results of scavenging activities of the CTE, PCTE and AHCTE towards ABTS radical are shown in Table 8. In agreement with obtained data of the DPPH method, CTE showed lowest ABTS value when compared to PCTE and AHCTE (P<0.05). Apparently, the ABTS values of PCTE and AHCTE are in similar trend activity. After gastrointestinal digestion, PCTE had the higher ABTS values (P<0.05). In contrast, the ABTS value of AHCTE slightly decreased about 2.55%.

Table 8 *Comparison of antioxidant activity (DPPH, FRAP and ABTS) in Clitoria ternatea extract (CTE), purified Clitoria ternatea extract (PCTE), acid hydrolysis Clitoria ternatea extract (AHCTE), digestion of purified Clitoria ternatea extract (DPCTE), and digestion of acid hydrolysis Clitoria ternatea extract (DAHCTE)*

Antioxidant	DPPH (IC_{50} value (mg/mL)	FRAP (mg $FeSO_4$/g extract)	ABTS (mg Trolox/g extract)
CTE	4.53 ± 0.03^{a}	19.14 ± 0.04^{c}	287.14 ± 0.28^{c}
PCTE	2.74 ± 0.08^{b}	42.56 ± 0.02^{b}	327.95 ± 0.05^{b}
DPCTE	1.89 ± 0.06^{c}	54.84 ± 0.05^{a}	342.27 ± 0.34^{a}
AHCTE	2.53 ± 0.14^{b}	42.81 ± 0.01^{b}	337.89 ± 0.94^{ab}
DAHCTE	2.63 ± 0.10^{b}	40.84 ± 0.02^{b}	329.26 ± 0.93^{b}

Data are expressed as mean ± S.E.M (n = 5). DPPH radical scavenging activity is expressed as the IC_{50} value (mg/mL). FRAP and ABTS are expressed as mg $FeSO_4$/g extract and mg trolox/g extract, respectively. (a-c) in any column are significantly different at $P<0.05$ according to *Duncan's multiple range* test.

4.1.5 *In vitro* biological activity of *Clitoria ternatea* extract (CTE), Purified *Clitoria ternatea* extract (PCTE), Acid hydrolysis *Clitoria ternatea* extract (AHCTE), digestion of Purified *Clitoria ternatea* extract (DPCTE), and digestion of Acid hydrolysis *Clitoria ternatea* extract (DAHCTE)

4.1.5.1 Bile acid binding ability

4.1.5.1.1 Glycodeoxycholic acid (GA)

The percentage bile acid binding is shown in Table 9. The results showed that the percentage glycodeoxycholic acid (GDA) binding of CTE, PCTE and AHCTE was 8.06±0.94, 11.48±0.85, and 12.10±1.75, respectively. The lowest percentage of GA binding was CTE as compared to PCTE and AHCTE (P<0.05). After simulated gastrointestinal digestion, the GA binding ability of PCTE increased and it showed the highest binding capacity (P<0.05). However, it was less binding ability than cholestyramine at 1 mg/mL (78.2 ± 0.56%).

4.1.5.1.2 Taurocholic acid (TA)

The percentages of taurocholic acid (TA) binding of CTE, PCTE, and AHCTE are shown in Table 9. When comparing between CTE, PCTE, and AHCTE, the percentage of TA binding was in order to AHCTE (17.92±1.01%) > PCTE (16.10±0.50%) > CTE (8.01± 0.40%). In addition, cholestyramine (1 mg/mL) bound 76.0 ± 0.88% of TA.

4.1.5.1.3 Taurodeoxycholic acid (TDA)

The percentages taurodeoxycholic acid (TDA) binding of CTE, PCTE, and AHCTE is shown in Table 9. PCTE and AHCTE showed higher binding capacity than CTE (P<0.05). After simulated gastrointestinal digestion, PCTE exhibited an increase in binding capacity to 27.85±1.02% (P<0.05), except for AHCTE, which showed a slight reduction to 23.72±1.11% on the percentages of TDA binding. However, cholestyramine (1 mg/mL) showed the most potent binding ability among the extracts (89.3 ± 0.57%).

Table 9 *The effect of Clitoria ternatea extract (CTE), purified Clitoria ternatea extract (PCTE), acid hydrolysis Clitoria ternatea extract (AHCTE), digestion of purified Clitoria ternatea extract (DPCTE), and digestion of acid hydrolysis Clitoria ternatea extract (DAHCTE) on bile acid binding*

Sample (1 mg/mL)	Bile acid binding (%)		
	Glycodeoxycholic acid	Taurocholic acid	Taurodeoxycholic acid
CTE	8.06±0.94^{d}	8.01±0.40^{d}	14.03±1.32^{c}
PCTE	11.48±0.85^{c}	16.10±0.50^{c}	22.46±1.84^{b}
DPCTE	18.14±1.46^{b}	22.02±0.74^{b}	27.85±1.02^{a}
AHCTE	12.10±1.75^{c}	17.92±1.01^{c}	25.14±1.83^{b}
DAHCTE	10.67±1.38^{c}	15.84±1.05^{c}	23.72±1.11^{b}
Cholestyramine	78.20±1.38^{a}	76.00±0.88^{a}	89.30±0.57^{a}

Data are expressed as mean ± S.E.M (n = 5). (a-c) in any column are significantly different at $P<0.05$ according to *Duncan's multiple range* test.

4.1.5.2 Carbohydrate digestive enzymes

4.1.5.2.1 Assay for Intestinal α-Glucosidase (maltase and sucrase) activity

Table 10 shows the inhibitory activity of nondigested CTE, PCTE and AHCTE against intestinal maltase and sucrase and pancreatic α-amylase. AHCTE showed the most effective extract against intestinal α-glucosidase and pancreatic α-amylase ($P<0.05$). When comparison the IC_{50} values of extracts against intestinal maltase and sucrose, it was AHCTE > PCTE > CTE. The findings also showed the consistent potency order for the pancreatic α-amylase inhibitory activity (Table 11). Interestingly, the findings showed that PCTE was the most effective intestinal α-glucosidase inhibitor ($P<0.05$), whereas AHCTE was lower potent inhibitor after digestion. However, the IC_{50} of extracts were less potent than acarbose against intestinal maltase, intestinal sucrase, and pancreatic α-amylase ($P<0.05$).

Table 10 The IC_{50} values of *Clitoria ternatea* extract (CTE), purified *Clitoria ternatea* extract (PCTE), acid hydrolysis *Clitoria ternatea* extract (AHCTE), digestion of purified *Clitoria ternatea* extract (DPCTE), and digestion of acid hydrolysis *Clitoria ternatea* extract (DAHCTE) against intestinal glucosidase (maltase and sucrase) and amylase.

Sample	IC_{50} values (mg/mL)		
	Intestinal Maltase	Intestinal Sucrase	Pancreatic α-amylase
CTE	4.03 ± 0.02^{a}	8.49 ± 0.01^{a}	2.60 ± 0.01^{a}
PCTE	2.48 ± 0.03^{b}	4.87 ± 0.02^{b}	1.17 ± 0.01^{c}
DPCTE	1.51 ± 0.02^{e}	2.46 ± 0.02^{e}	0.74 ± 0.01^{d}
AHCTE	1.73 ± 0.01^{d}	3.31 ± 0.02^{d}	0.93 ± 0.02^{d}
DAHCTE	2.36 ± 0.01^{c}	3.79 ± 0.01^{c}	1.01 ± 0.02^{b}
Acarbose	0.002 ± 0.001^{f}	0.038 ± 0.001^{f}	0.065 ± 0.003^{e}

Data are expressed as mean ± S.E.M (n = 5). IC_{50}, concentration of inhibitor to inhibit 50% of its activity. (a-f) in any column are significantly different at $P<0.05$ according to *Duncan's multiple range* test.

4.1.5.2.2 Glucose uptake measurement in Caco-2 cells

4.1.5.2.2.1 Cell viability

The cytotoxicity of PCTE during simulated gastrointestinal digestion (DPCTE) was examined quantitatively at the range of 0-2 mg/mL by the MTT assay. The activity of succinic dehydrogenase in the absence or presence of DPCTE treatments were not significantly different, indicating that samples at the used concentrations had no effect on the caco-2 cell viability (Figure 19). Based on these results, the maximum concentration for 2 mg/mL was chosen to test the ability of inhibiting glucose uptake.

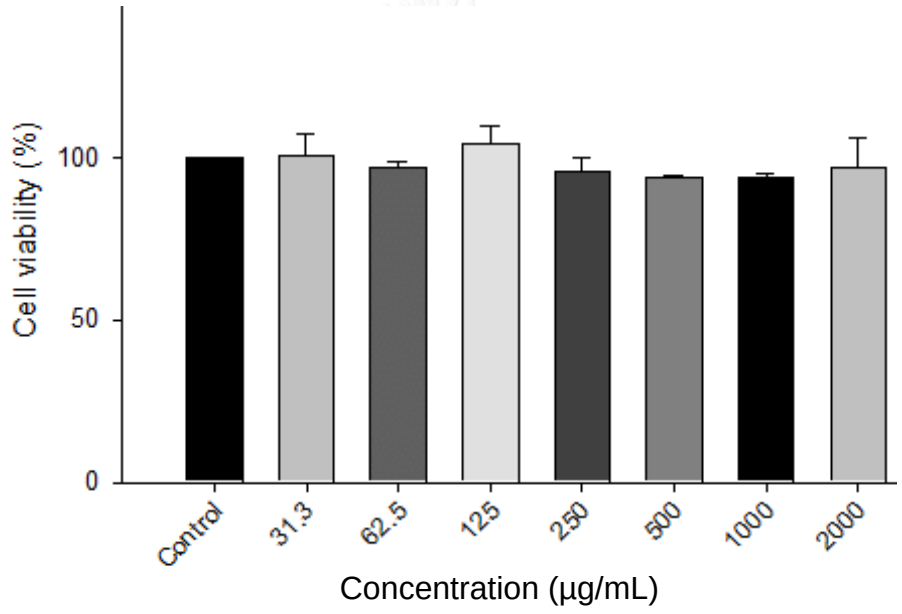


Figure 19 *Caco-2 cells were treated with digested Purified Clitoria ternatea extract (DPCTE) in various concentrations (0.25, 0.5, 1, and 2 mg/mL)*
The results are means ± S.E.M. (n=6 in each group).

4.1.5.2.2.2 Effect of CT on Glucose Absorption by Caco-2 Cells

Based on results from TAC, TPC and antioxidant activity, Purified *Clitoria ternatea* extract digestion (DPCTE) exhibit the highest activity. Therefore, DPCTE were chosen to prove further biological activity for glucose uptake inhibitory activity in the cell culture system. The effect of the Purified *Clitoria ternatea* extract following simulated gastrointestinal digestion (DPCTE) on glucose uptake assays was presented

in Figure 20. In the presence of Na^+ (SGLT1-mediated), the addition of DPCTE to Caco-2 cells led to a concentration dependent inhibition of glucose uptake. Total glucose uptake in sodium-containing buffer was significantly decreased to 35.34±1.31% after exposure to the DPCTE when compared with the control (P<0.05). Under Na+-independent conditions, there were no significant changes observed in inhibitory characteristics of DPCTE when compared with the control.

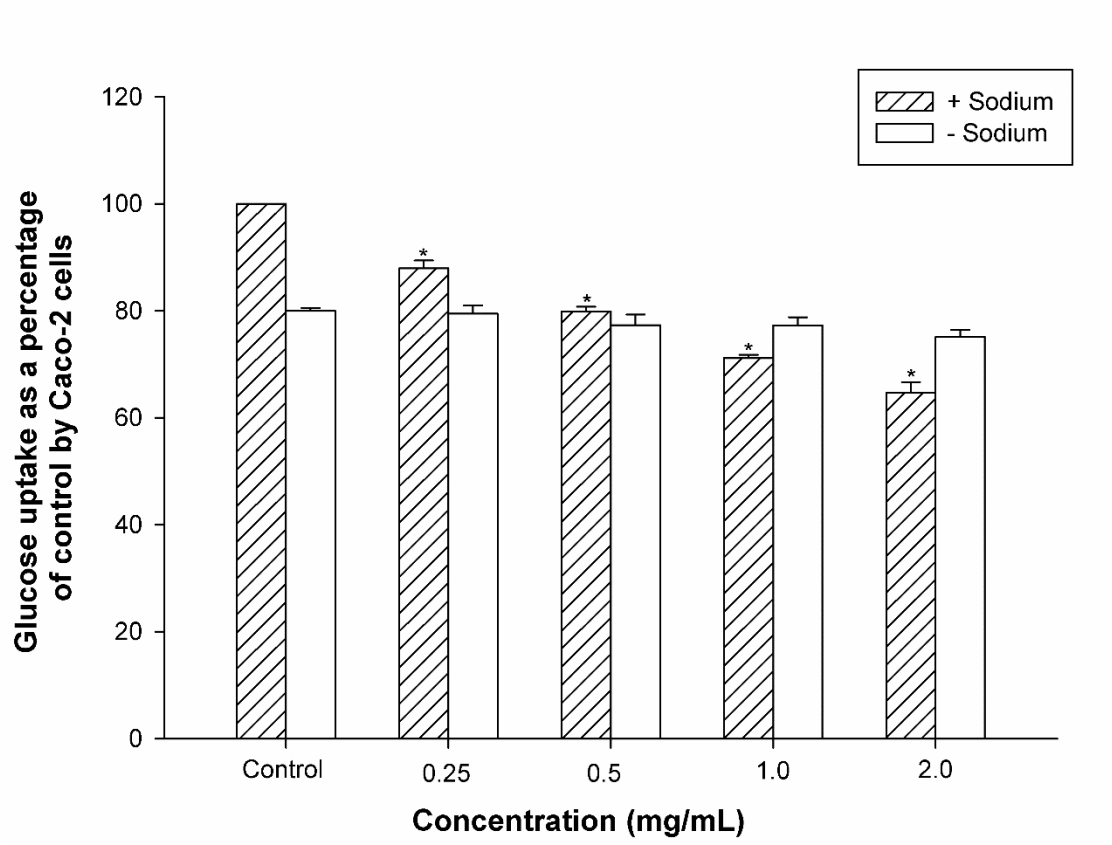


Figure 20 *Caco-2 cells were treated with digested purified Clitoria ternatea extract (DPCTE) in various concentrations (0.25, 0.5, 1, and 2 mg/mL)*

Data are expressed as means ± S.E.M. (n=6 in each group). * P<0.05 (vs. control; all determined by ANOVA followed by the post hoc Duncan's test).

4.2 Result 2: The effect of alginate-based encapsulation of polyphenols from *Clitoria ternatea* petal flower extract enhances stability and functionality under simulated gastrointestinal conditions

4.2.1 Morphology and percentage of encapsulation efficiency of *Clitoria ternatea* (CT) in calcium alginate beads (CT beads) in different formulations

The effects on the morphology were investigated using scanning electron microscopy (SEM). The structure of sodium alginate and *Clitoria ternatea* petal flower are shown in Figure 21 a, b. The morphology of the sodium alginate was characterized by the surface of the membrane appeared to be uneven on the surface structure. On the contrary, *Clitoria ternatea* petal flower showed a different microstructure similar to sponge like structures with interconnected pores on the surface structure.

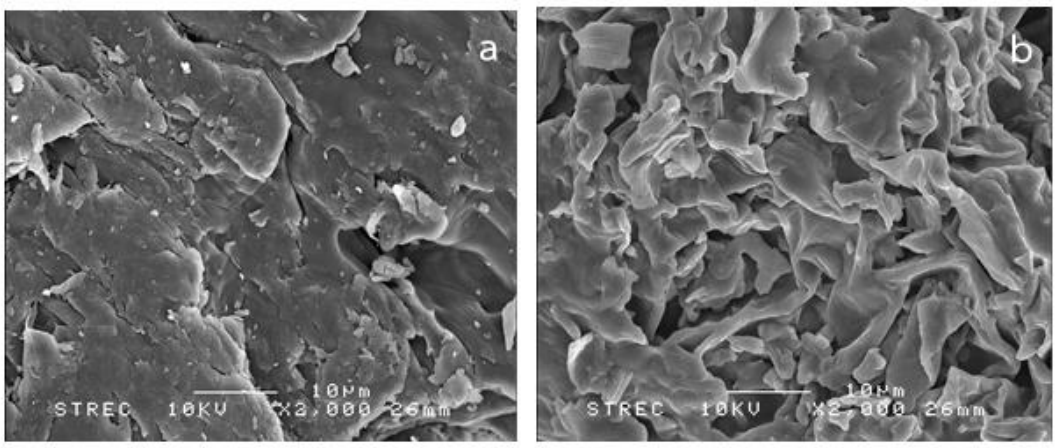


Figure 21 *Scanning electron microscopy (SEM) images of the surface of sodium alginate powder (a) and Clitoria ternatea petal flower (b)*

In the present study using different concentrations of CT (5, 10, and 20% w/v), alginate (1.0, 1.5 and 2.0% w/v), $CaCl_2$ as hardening solution (1.5, 3.0 and 5.0% w/v) were prepared by the extrusion method. The results showed that the spherical shape of the beads was lost completely at concentration of sodium alginate and $CaCl_2$ below 1.0% and 1.5% (w/v), respectively (data not shown). The surface morphologies of the prepared beads are shown in Figure 22. The SEM photographs of the control beads compared with CT beads in different formulations showed the differences in surface morphology. Control beads after drying had a collapsed and shriveled shape as well

as the gaps on the surface. The surface of beads at lower concentration of alginate was rougher than higher concentration.

In comparison of the beads prepared with CT, it was found that an increase in percentage of CT resulted in more spherical shape. Smoothness of surface increased when concentration of $CaCl_2$ was increased in the beads. However, increase $CaCl_2$ concentrations led to unstable and weak beads with cracked surfaces.

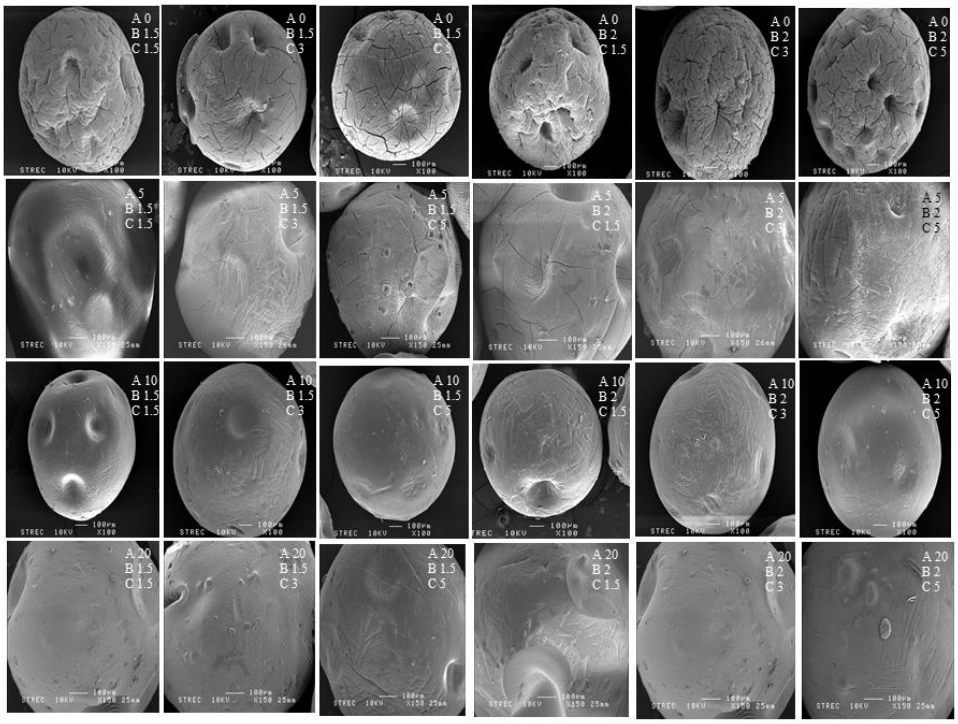


Figure 22 *Scanning electron microscopy (SEM) micrographs of microbeads, Clitoria ternatea petal flower (A); Sodium alginate (B); $CaCl_2$ (C)*

Table 11 shows the effect of process variable on the overall encapsulation efficiency of alginate beads. The results showed that percentage of encapsulation efficiency was ranging from 74.97±0.84 to 84.83±0.40 %. In this study, the encapsulation efficiency of the beads improved when increased the concentration of sodium alginate. Together with SEM photographs, the concentration of $CaCl_2$ was found to have more influence on the encapsulation efficiency, however, it led to form the cracked surfaces and consequently decrease encapsulation efficiency. Based on these

results, the formulation comprising 10% CT, 1.5% alginate, and 3% $CaCl_2$ (w/v) was selected for the further experiments.

Table 11 *Encapsulation efficiency of Clitoria ternatea in calcium alginate beads*

Clitoria ternatea (%w/v)	Sodium alginate (%w/v)	$CaCl_2$ (%w/v)	% Encapsulation Efficiency (%EE)
5	1.5	3	80.17 ± 0.58^{c}
5	1.5	5	$81.53\pm0.89^{b,c}$
5	2.0	3	84.87 ± 0.29^{a}
5	2.0	5	$83.59\pm0.57^{a,b}$
10	1.5	3	84.83 ± 0.40^{a}
10	1.5	5	$83.51\pm0.67^{a,b}$
10	2.0	3	$83.54\pm0.48^{a,b}$
10	2.0	5	$81.37\pm0.66^{b,c}$
20	1.5	3	74.97 ± 0.84^{d}
20	1.5	5	74.17 ± 0.83^{d}
20	2.0	3	$82.72\pm0.72^{b,c}$
20	2.0	5	$82.38\pm0.94^{a,b}$

4.2.2 Quantification of polyphenols and antioxidant activity of *Clitoria ternatea* extract-containing microencapsulation

The content of polyphenol and antioxidant activity of CT and its encapsulation (10% CT, 1.5% alginate, and 3% $CaCl_2$). The initial polyphenol content of CT was 1.08±0.02 mg of gallic acid equivalent per mL. DPPH, ABTS, and FRAP assays were used to evaluate antioxidant power of CT solution (10% w/v). The values of CT obtained by the DPPH, ABTS, and FRAP were 12.65±0.02%, 8.89±0.02 mg of Trolox equivalent mL^{-1} and 4.53±0.01 mg of $FeSO_4$ equivalent mL^{-1}, respectively. Results from this study show that CT exhibited potent radical scavenging activity.

The content of polyphenolic compounds presented in CT beads was 11.76±0.07 mg of gallic acid equivalent g_{beads}^{-1}. Additionally, DPPH, ABTS, and FRAP values in CT beads (1g) were 21.17±0.07%, 13.18±0.03 mg of Trolox equivalent g_{beads}^{-1} and 14.29±0.01 mg of $FeSO_4$ equivalent g_{beads}^{-1}, respectively.

4.2.3 Particle size

The mean sizes of microencapsulation are shown in Figure 23. In this study, the beads loading CT were 985 μm in diameter (Figure. 23a), whereas the mean sizes of the control alginate beads were 1,020 μm (Figure. 23b). When make a comparison, the mean particle size of CT beads was smaller than that of control alginate beads (Figure 23).

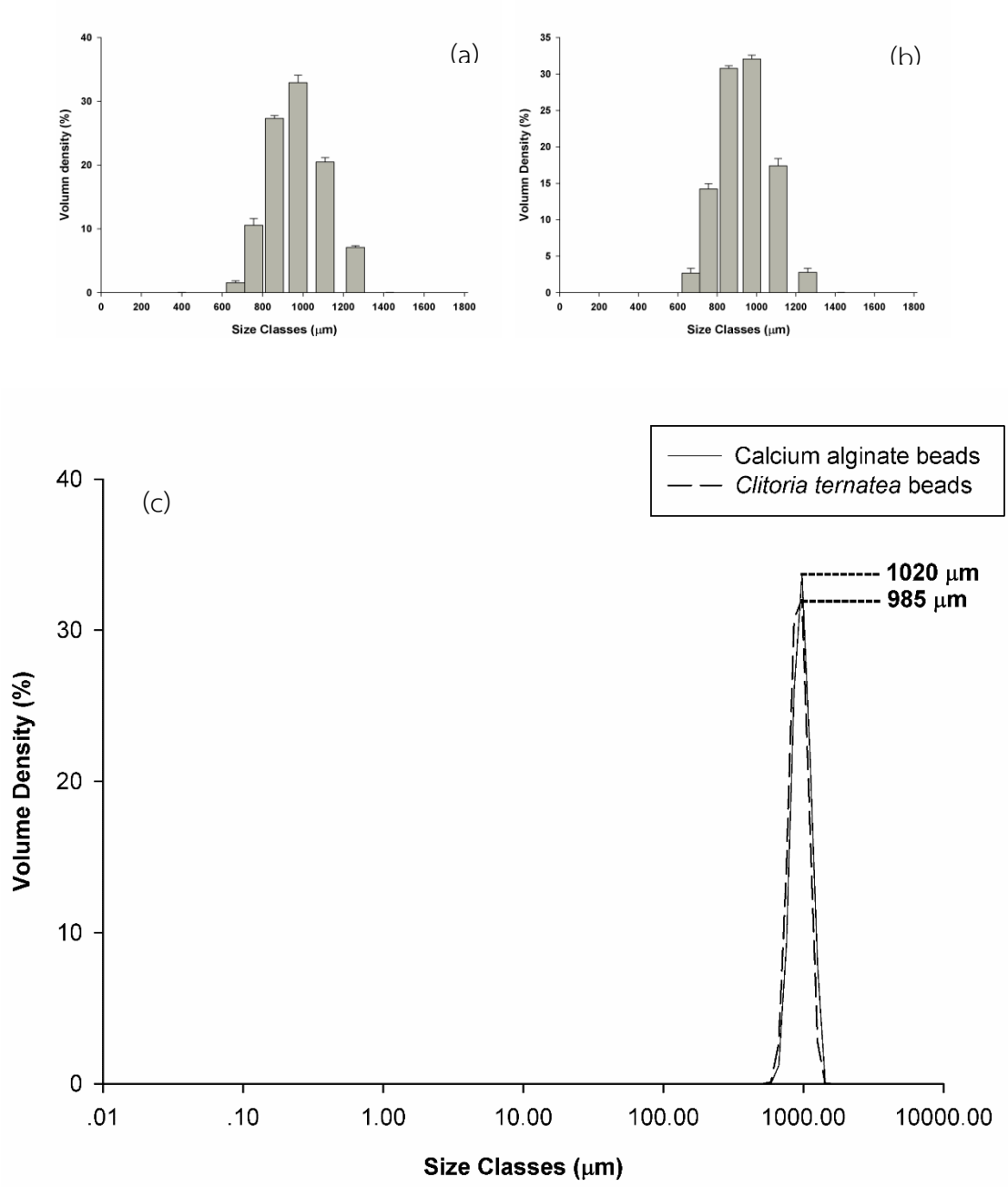


Figure 23 *Size distribution of control beads of calcium alginate (a), calcium alginate beads with Clitoria ternatea (b), and (c) calcium alginate beads compare with calcium alginate beads with Clitoria ternatea*

The results are expressed as mean ± standard deviation (n = 3).

4.2.4 Analysis by Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used to identify functional groups and characterize the relationship between the matrix and CT components. The molecular interactions of sodium alginate powder (A), calcium alginate beads (AC), *Clitoria ternatea* (CT), and CT beads (ACT) were investigated using FTIR spectroscopy. As shown in Figure 24, the FTIR spectra of A powder shows peaks around 3280.79, 1595.40, 1405.06, and 1025.63 cm^{-1}, reflective of O-H, COO^- (asymmetric), COO^- (symmetric), and C-O-C stretching, respectively. The strong and broad absorption band at 3334.05 cm^{-1} has been observed due to O-H stretching. The cross-linking process of AC caused an obvious shift to higher wave number of O-H stretching, which is the stretching and bending mode (Pongjanyakul & Rongthong, 2010). Additionally, the strong asymmetric stretching absorption band at 1598.09 cm^{-1} and weaker symmetric stretching band near 1420.20 cm^{-1} appeared due to the presence of carboxylate anions COO^-. Moreover, the change to lower numbers and a decrease in the intensity of C-O-C stretching peak of AC was also observed Figure 24.

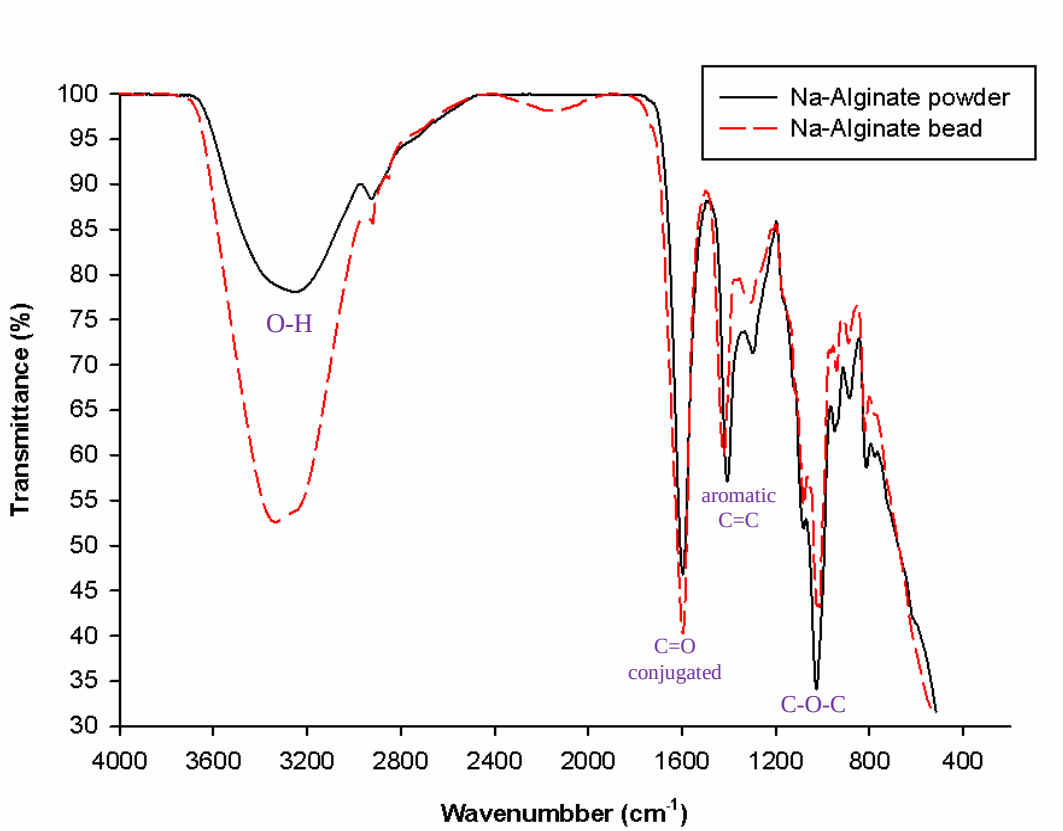


Figure 24 *The FT-IR spectra in the 515-4000 cm^{-1} region of A: sodium alginate and AC: control beads of calcium alginate*

There were several relevant changes in the spectrum of the alginate submicron particles with the encapsulated CT in comparison to the AC. The spectra of the AC and CT encapsulating alginate showed little change in the peak intensity in the range of 800-1600 cm^{-1} (Figure 25).

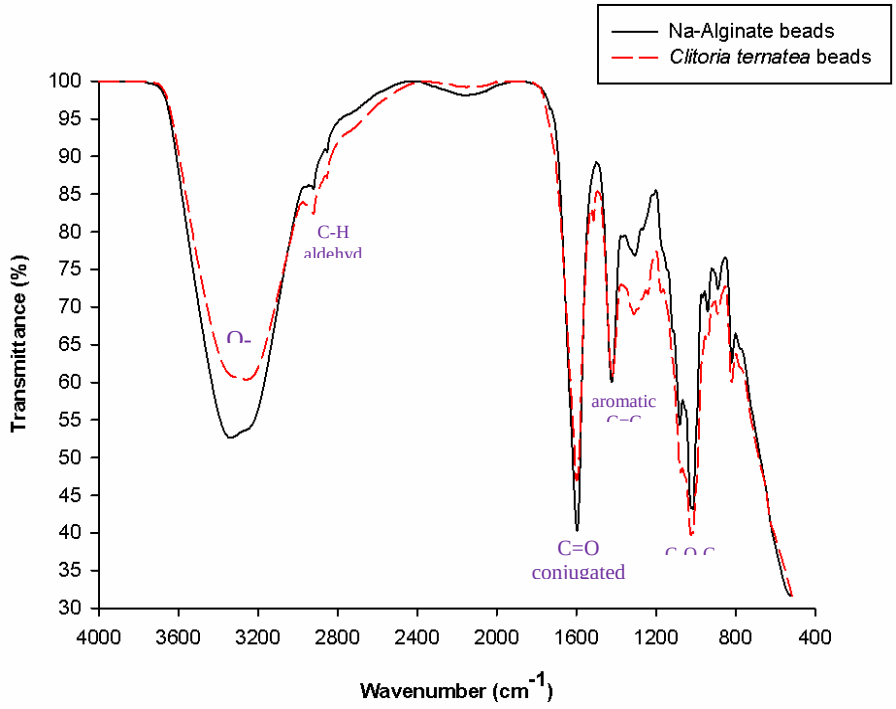


Figure 25 *The FT-IR spectra in the 515-4000 cm–1 region of AC: control beads of calcium alginate and ACT: calcium alginate beads with Clitoria ternatea extract*

The peak of O-H bond at CT (3273.09 cm^{-1}) and A (3280.79 cm^{-1}) was shifted to lower wave number after form CT beads (3255.86 cm^{-1}). Similarly, O-H stretching peak of alginate shifted to lower wavenumber when CT encapsulated into alginate beads, suggesting that a molecular interaction between alginate and CT was formed hydrogen bonding. Furthermore, the intensity of the peaks of 1240.30 cm^{-1} (C-O-C vibration of esters) and 1512.26 cm^{-1} (aromatic ring vibration) in the spectrum of alginate arises after loading with the CT compounds. This formation did not alter the new peaks after formation of the beads. CT peaks might be overlapped with peaks of ions in the thermogram of beads (Figure 26).

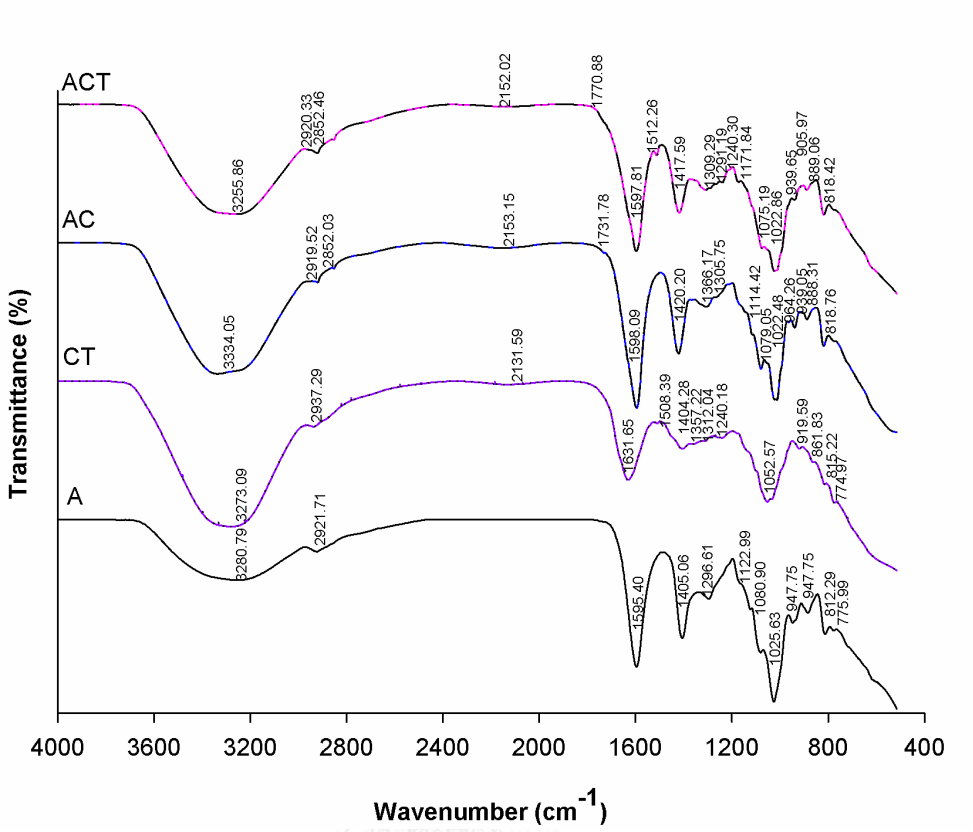


Figure 26 *The FT-IR spectra in the 515-4000 cm–1 region of A: sodium Alginate, CT: Clitoria ternatea extract, AC: control beads of calcium alginate and ACT: calcium alginate beads with Clitoria ternatea extract*

4.2.5 The thermograms by Differential Scanning Calorimeter (DSC) of *Clitoria ternatea* and *Clitoria ternatea* bead

The thermal behavior of microencapsulation is shown in Figure 27. The alginate powder presented a decomposition peak at 245.4°C. In the DSC curves of the alginate beads, the decomposition appeared at the onset temperature of around 197.8°C. The DSC curve of CT showed a sharp endothermic peak, which corresponded to the melting of the CT crystalline structure at the onset temperature of 114.2°C. The interaction of CT and alginate in the beads caused a decrease in the thermal stability indicating from the shift of the exothermic peak to a lower Tm at 188°C. Also, the exothermic peak of the CT beads moved to a higher temperature (188°C). Additionally, the DSC thermograms of the CT beads did not contain the melting peak of CT at 114.2°C.

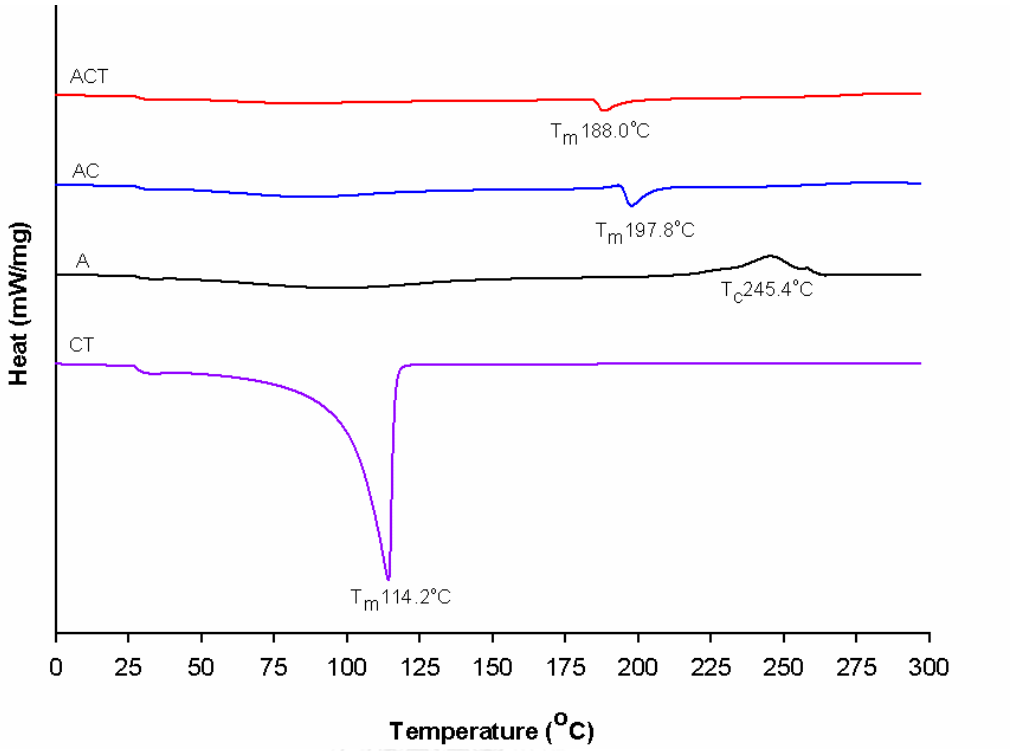


Figure 27 *Thermograms obtained by Differential Scanning Calorimeter (DSC) for pure materials and capsules. CT: Clitoria ternatea, AC: Control beads of calcium alginate and ACT: Calcium alginate beads with Clitoria ternatea extract.*

4.2.6 *In vitro* gastrointestinal digestion release property

4.2.6.1 Phenolic content after *in vitro* gastrointestinal digestion

The polyphenolic content and the biological profiles of the CT and its encapsulation in the simulated gastric and intestinal digestion are demonstrated in Figure 28. The results showed that the release of polyphenol from CT beads occurred within 1 h. The content of polyphenol in CT beads were statistically different from the nonencapsulated CT (P<0.05). Taking into account the total polyphenols mass from CT after simulated gastric fluid (SGF) was higher when compared to CT beads (P<0.05). On the other hand, the total polyphenols of CT after simulated intestinal fluid (SIF) was lower than CT beads (P<0.05).

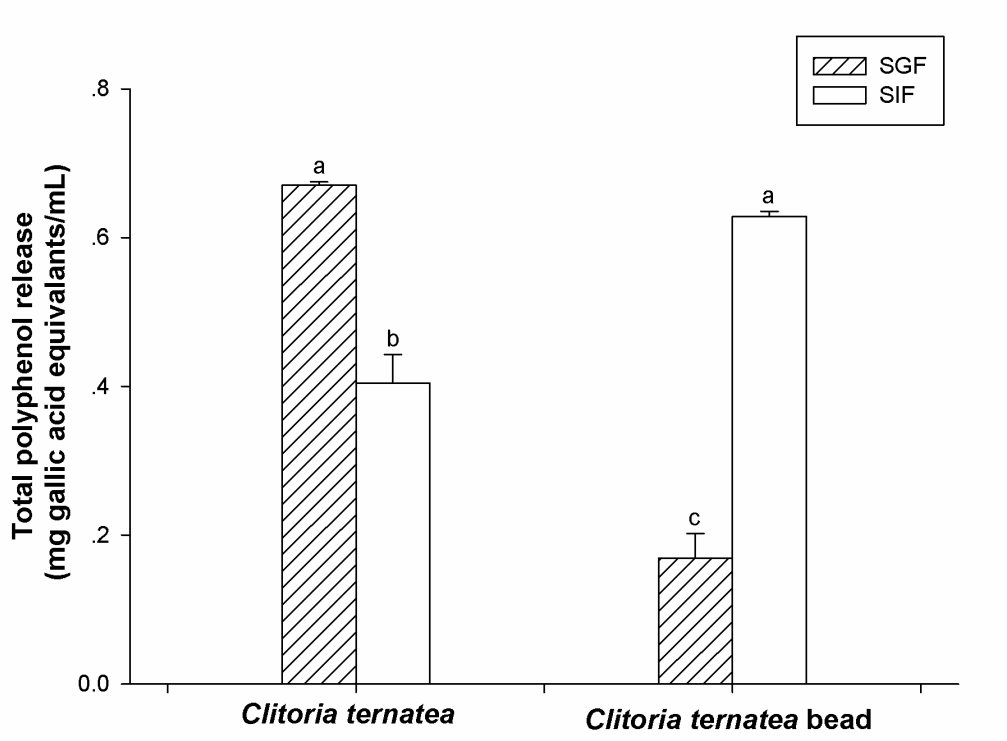


Figure 28 *The total phenolic contents of Clitoria ternatea and Clitoria ternatea bead after simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion*

Data are expressed as mean ± S.E.M. (n = 3). (a-c) Means different between groups according to Duncan's multiple range test ($P<0.05$).

4.2.6.2 Antioxidant capacity after *in vitro* gastrointestinal digestion

The antioxidant capacity (FRAP Figure 29 and ABTS Figure 30) released from CT beads in the SIF was higher than that of SGF. Both ABTS and FRAP values of CT were significant higher than CT beads after SGF ($P<0.05$). Interestingly, the values of antioxidant activity of CT beads were increased and significantly higher after SIF ($P<0.05$).

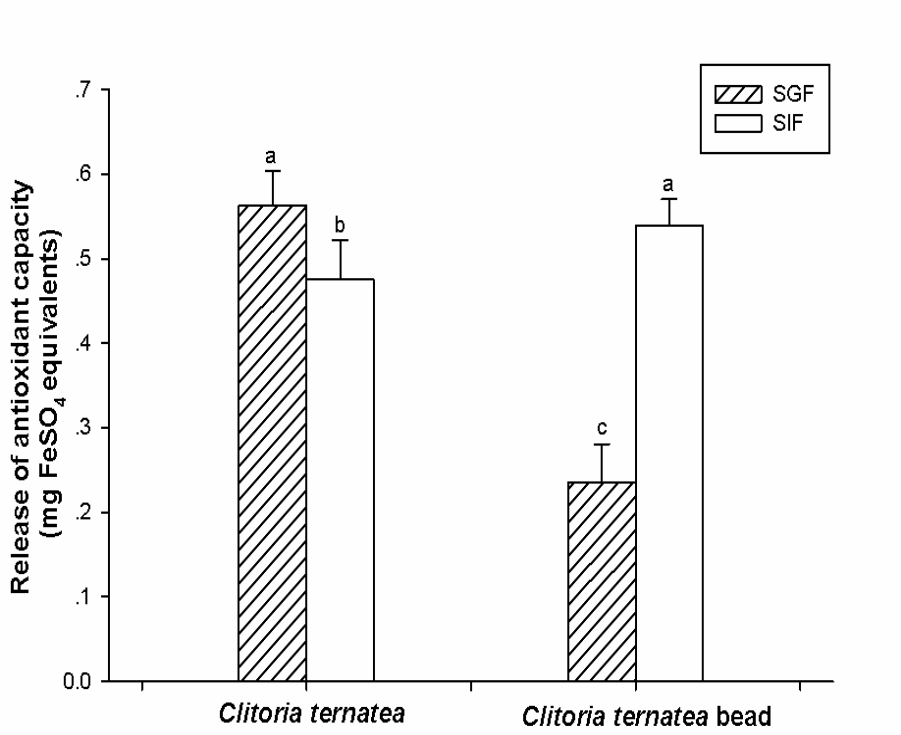


Figure 29 *The Ferric Reducing Ability of Plasma (FRAP) of Clitoria ternatea and Clitoria ternatea bead after simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion*

Data are expressed as mean ± S.E.M. (n = 3). (a-c) Means different between groups according to Duncan's multiple range test ($P<0.05$).

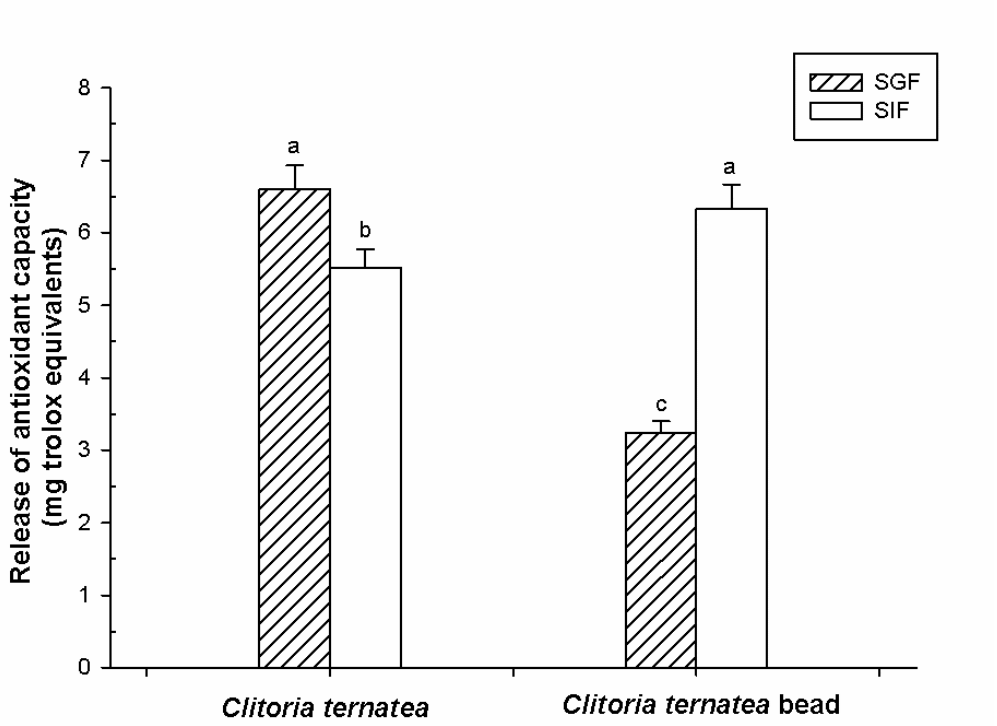


Figure 30 *The ABTS scavenging activity of Clitoria ternatea and Clitoria ternatea bead after simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) digestion* Data are expressed as mean ± S.E.M. (n = 3). (a-c) Means different between groups according to Duncan's multiple range test ($P<0.05$).

4.2.6.3 Inhibition of pancreatic α-amylase

The effects of CT and its encapsulation following simulated gastrointestinal digestion on the inhibition of pancreatic α-amylase are shown in Figure 31. The results showed that CT markedly inhibited pancreatic α-amylase activity (15.12±0.14%) whereas CT beads significantly improved the percentage inhibition of pancreatic α-amylase after simulated gastrointestinal digestion (28.87±0.09%).

4.2.6.4 Bile acid binding

The percentages of total bile acid binding of CT beads and its encapsulation following simulated gastrointestinal digestion are shown in Figure 32. The result

showed that the binding ability of CT and its encapsulation was 8.25±0.19% and 22.01±0.20%, respectively. They indicate that the CT beads increase the ability to bind bile acid when compared to CT after simulated gastrointestinal digestion (P<0.05).

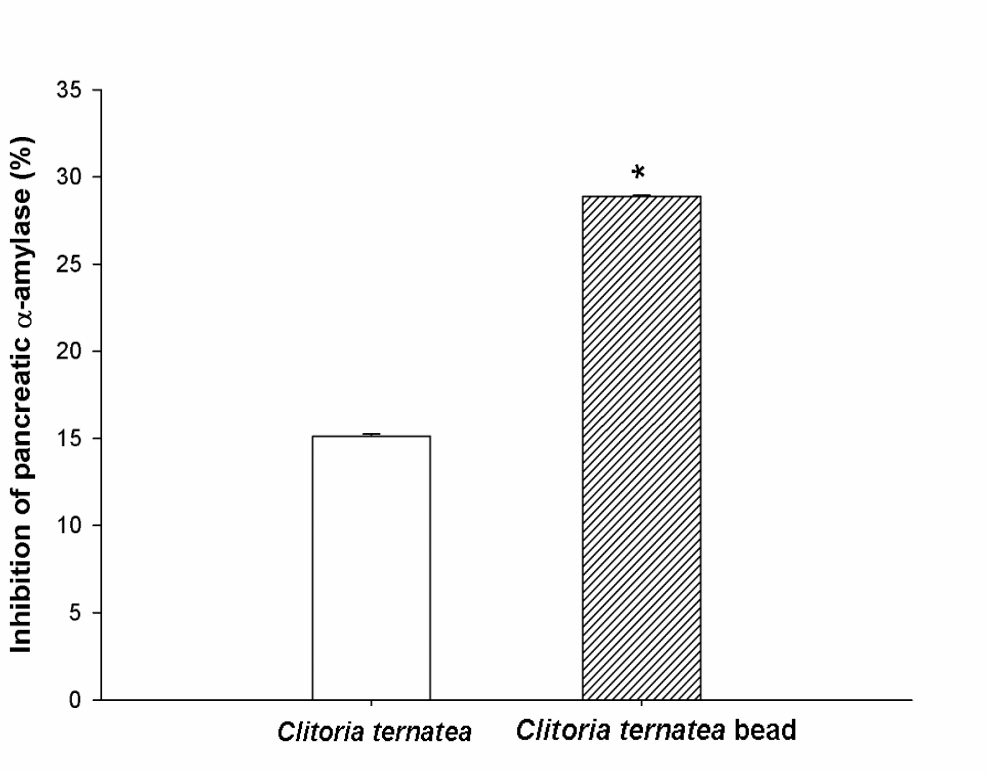


Figure 31 *The effect of Clitoria ternatea and Clitoria ternatea bead following simulated gastrointestinal digestion on the inhibition of maltose released from pancreatic α-amylase enzyme. Total polyphenols in sample 0.802±0.057 mg GAE*

* Means different between *Clitoria ternatea* and *Clitoria ternatea bead* (P<0.05), student *t-test* (P<0.05).

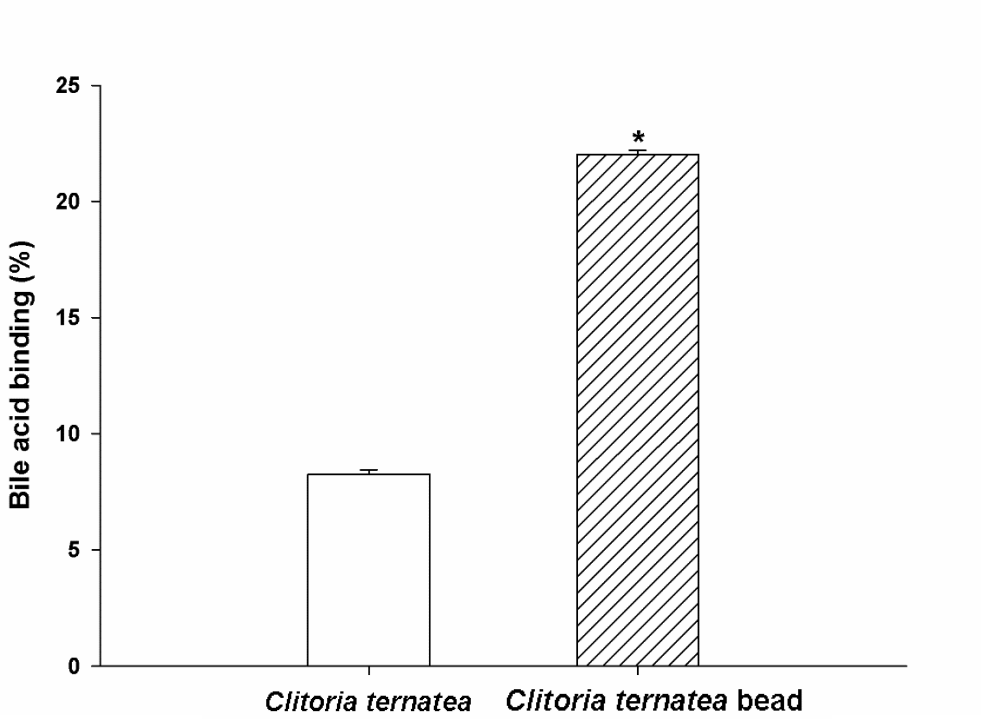


Figure 32 *The percentage of bile acid binding of Clitoria ternatea and Clitoria ternatea bead following simulated gastrointestinal digestion. Total polyphenols in sample 0.802±0.057 mg GAE*

* Means different between *Clitoria ternatea* and *Clitoria ternatea bead* (*P*<0.05), student *t-test* (*P*<0.05).

4.2.6.5 Release study in water

The release profiles for the polyphenols were measured the TPC of the dissolution medium after placing beads in water. The release profiles for native microbeads encapsulating *Clitoria ternatea* extract is shown on Figure 33. The TPC was released relatively rapidly from CT beads after first 30 minutes. Then, the CT beads were continuously released for 24 hours, mainly governed by the passive diffusion without swelling and disintegrating the CT beads.

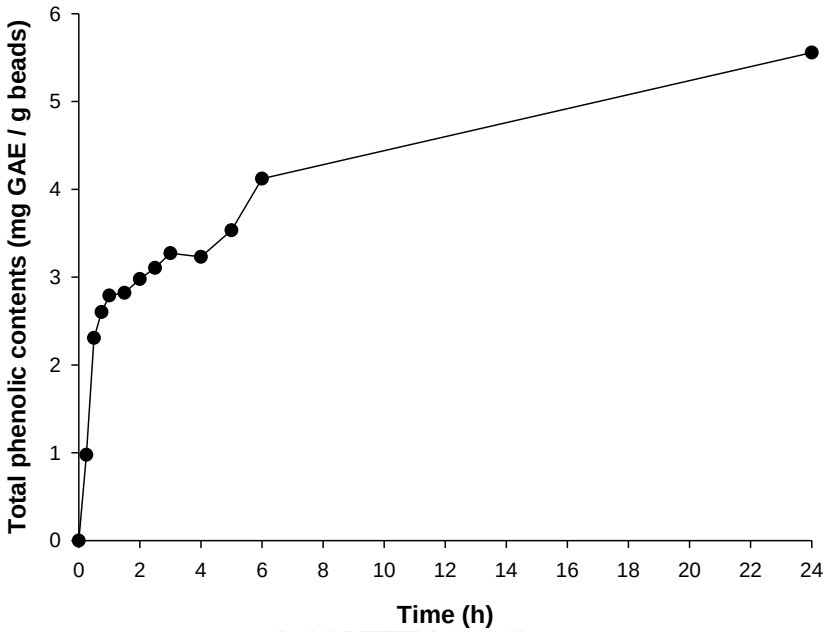


Figure 33 *Release of polyphenols from Clitoria ternatea bead in water*

4.3 Result 3: The effects of *Clitoria ternatea* petal flower extract (CTE) on lipid and protein oxidation in cooked pork patties during refrigerated storage (4±1°c) for 12 days

4.3.1 Total phenolic and anthocyanin contents in CTE

Phenolic compounds constituent in CTE was measured by using Folin's ciocalteu method. As shown in Table 12, the results showed that CTE contained polyphenolic compound about 28.8±0.01 mg gallic acid equivalents/g of CTE using standard curve of gallic acid. The anthocyanin content of CTE was found to be 1.49±0.02 mg cyanidine-3-glucoside equivalents/g extract.

Table 12 *Phenolic and flavonoid quantification in Clitoria ternatea petal flower extract (CTE)*

	Total phenolic compound (mg gallic acid equivalent/ g dry extract)	Total anthocyanin content (mg catechin/ g dry extract)
CTE	28.8±0.01	1.49±0.02

Data were expressed as mean ± S.E.M. (n = 3).

4.3.2 Total phenolic content in raw and cooked pork patties

The total phenolic (TP) compound in raw and cooked pork patties were measured by using Folin's ciocalteu method. The changes of TP in the raw and cooked pork patties during refrigerator storage for 12 days are shown in Figure 34. At day 0, the TP contents of raw pork patties were 0.148, 0.155, 0.157, and 0.162 mg gallic acid equivalent per g sample in patties with 0.02%, 0.04%, 0.08%, and 0.16% CTE, respectively. Moreover, no significant difference in TC is observed between raw and cooked pork patties at day 0. The TC contents in cooked pork patties with CTE (0.02–0.16%) declined continuously and significantly until the end of storage (day 12).

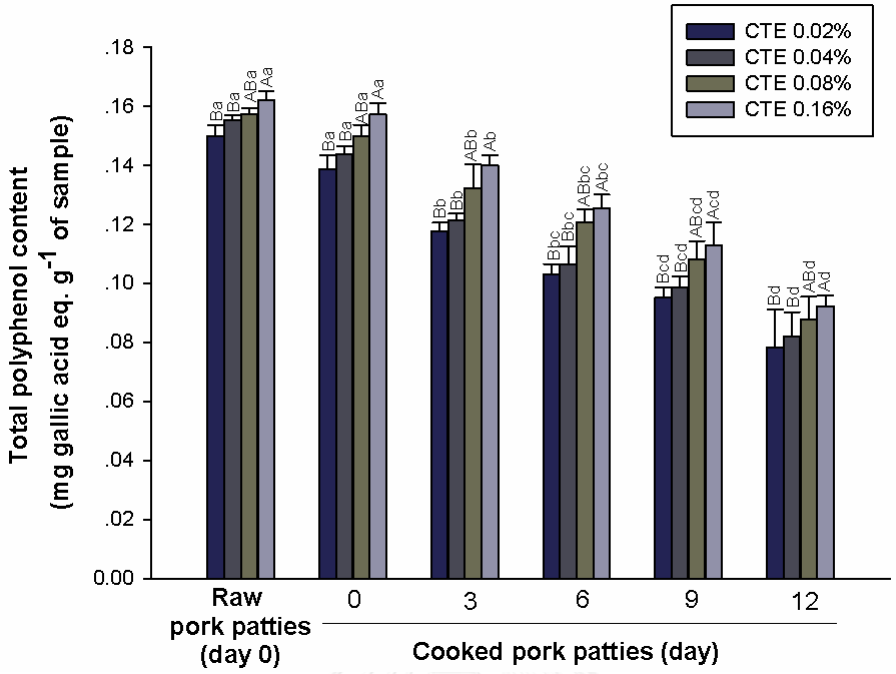


Figure 34 *Total phenolic contents (mg gallic acid eq. g^{-1} of sample) in raw and cooked pork patties during refrigerated storage for 12 days*

Control: without added CTE; CTE 0.02%, CTE 0.04%, CTE 0.08%, and CTE 0.16%: with added *Clitoria ternatea* extract 0.02%, 0.04%, 0.08%, and CTE 0.16%, respectively; BHT 0.02%: with added butylated hydroxytoluene 0.02%. The results are expressed as mean ± S.E.M. (n=5). Means with different superscripts differ significantly ($P<0.05$); a–d: day effects, A–C: treatment effects ($P<0.05$).

4.3.3 The antioxidant capacity (ABTS of CTE in cooked pork patties during refrigerated storage for 12 days

By the ABTS assay, the antioxidant activity of CTE was estimated at day 0, 3, 6, 9, and 12 during refrigerated storage. The TEAC values of the cooked pork patties are summarized in Figure 35. The level of antioxidant capacity in cooked pork patties increased when the concentration of CTE added in cooked pork patties increased. At day 0, the total antioxidant scavenging capacity (ABTS) values of the cooked pork patties were in the range 0.103–0.166 mg trolox equivalent/g sample. The results showed that the TEAC values of cooked meat samples gradually declined in all patties during day 12 of storage. However, no significant differences were observed in the

levels of the TEAC values of cooked pork patties between day 9 and day 12 of storage. The cooked pork patties with 0.16% CTE and 0.02% BHT showed significantly higher TEAC values than the control group ($P<0.05$).

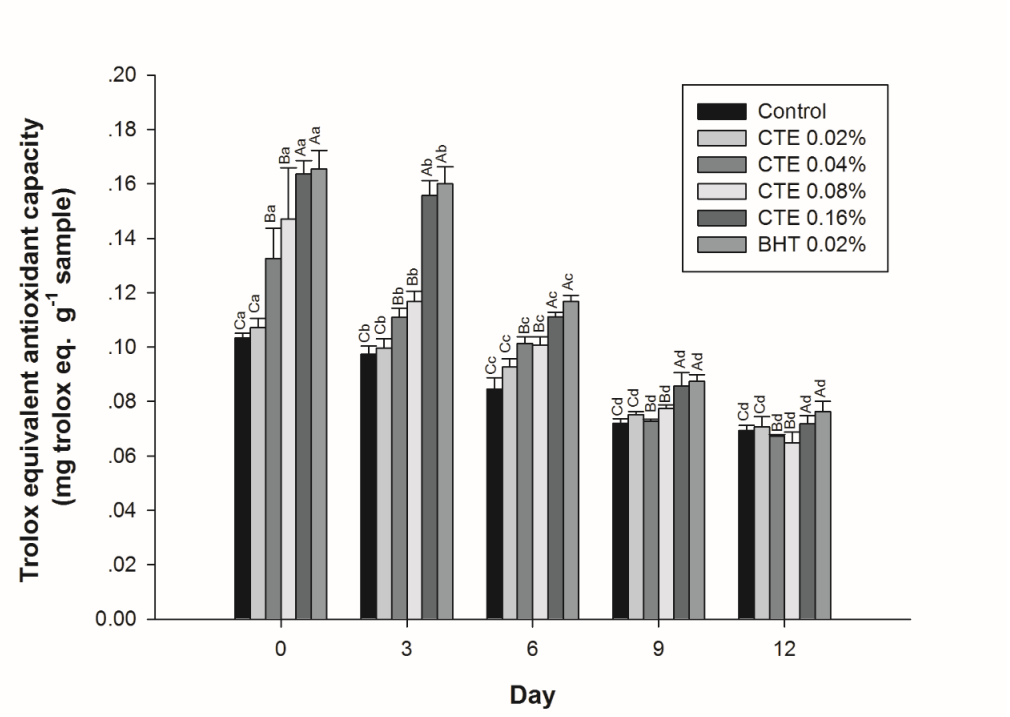


Figure 35 *Trolox equivalent antioxidant capacity (mg trolox eq. /g of sample) in cooked pork patties during refrigerated storage for 12 days*

Control: without added CTE; CTE 0.02%, CTE 0.04%, CTE 0.08%, and CTE 0.16%: with added *Clitoria ternatea* extract 0.02%, 0.04%, 0.08%, and CTE 0.16%, respectively; BHT 0.02%: with added butylated hydroxytoluene 0.02%. The results are expressed as mean ± S.E.M. (n=5). Means with different superscripts differ significantly ($P<0.05$); a–d: day effects, A–C: treatment effects ($P<0.05$).

4.3.4 Lipid oxidation in cooked pork patties during refrigerated storage for 12 days

4.3.4.1 Conjugated dienes

The generation of CD was examined on the basis of the hydroperoxides formed in extracted lipids of the cooked pork patties (Table 13). The treatments had a significant influence ($P<0.05$) on the change of conjugated diene value in the pork patties and also impact on the change over time as evidenced by statistically significant interaction ($P<0.05$) between treatments and time. Initially (day 0), the concentration of CD significantly increased for all groups. The results showed that the highest concentration of CD was observed in cooked pork patties at day 6 of storage and then decreased until the end of the storage. After the cooking process, cooked pork treated with CTE (0.16%) had lower concentrations of CD compared with control cooked pork patties. On day 0 of storage, the CTE 0.04%, 0.08%, and 0.16% groups showed decreased ($P<0.05$) concentrations of conjugated dienes compared to groups CTE 0.02% and control, which did not differ ($P>0.05$) among each other. On day 3, CTE (0.02–0.16%) significantly inhibited the formation of CD, whereas it reduced the concentration of CD in pork patties at the end of the storage period. On day 12 of storage, the control, CTE 0.02% group showed the higher ($P<0.05$) concentrations of CD, whereas the CTE 0.04%, 0.08% and 0.16% groups significantly inhibited the production of CD compared to control group. The findings also indicate that CTE (0.04-0.16%) was equally effective to 0.02% BHT in decreasing the concentration of CD in cooked pork patties.

Table 13 Conjugated dienes (μmol/mg sample) in cooked pork patties during refrigerated storage (4±1°c) for 12 days.

	Conjugated dienes (μmol/mg sample)				
Experiments	Day 0	Day 3	Day 6	Day 9	Day 12
Control	0.34 ± 0.01^{Ab}	0.46 ± 0.01^{Aa}	0.47 ± 0.03^{Aa}	0.38 ± 0.01^{Ab}	0.37 ± 0.01^{Ab}
CTE 0.02%	0.33 ± 0.01^{Ac}	0.37 ± 0.01^{Bb}	0.46 ± 0.01^{ABa}	0.36 ± 0.02^{ABb}	0.35 ± 0.01^{ABbc}
CTE 0.04%	0.31 ± 0.04^{ABb}	0.37 ± 0.02^{Bb}	0.43 ± 0.01^{ABa}	0.36 ± 0.01^{ABb}	0.33 ± 0.01^{BCb}
CTE 0.08%	0.31 ± 0.02^{ABb}	0.33 ± 0.02^{Bb}	0.36 ± 0.01^{Ca}	0.30 ± 0.09^{Cb}	0.32 ± 0.01^{Cb}
CTE 0.16%	0.28 ± 0.07^{Bc}	0.36 ± 0.02^{Bb}	0.41 ± 0.01^{BCa}	0.35 ± 0.02^{ABb}	0.31 ± 0.01^{Cbc}
BHT 0.02%	0.27 ± 0.04^{Bc}	0.33 ± 0.03^{Bb}	0.37 ± 0.02^{Ca}	0.29 ± 0.01^{Cc}	0.28 ± 0.01^{Cc}

The results are expressed as mean ±S.E.M. (n=5). Means with different superscripts with the same row (a–c: day effects) are significantly different ($P<0.05$). Means with different superscripts with the same column (A–C: treatment effects) are significantly different ($P<0.05$).

4.3.4.2 Thiobarbituric acid reaction substance (TBARS)

Figure 36. shows the effect of CTE on the TBARS values in cooked pork patties during 12 days of refrigerated storage. The significant difference of TBARS values was observed between pork patties with CTE treatments (0.02–0.16%) and control pork patties on any of the storage days ($P<0.05$). The respective treatments significantly ($P<0.05$) influenced TBARS values and also impacted the change over time as evidenced by statistically significant interaction ($P<0.05$) between treatments and time. TBARS concentrations increased ($P<0.05$) gradually as storage time progressed to 3, 6, 9 and 12 days. However, the concentrations of TBARS in all treated groups continued to be lower ($P<0.05$) than those of the control group at all time points. On the initial day, the TBARS values of CTE containing samples were significantly lower than those for the control. At day 3, CTE (0.02–0.16%) significantly inhibited the formation of TBARS in pork patties when compared with control cooked pork patties. At the end of storage day, CTE (0.02–0.16%) significantly protected lipids against oxidation in pork patties. Furthermore, the addition of CTE at 0.16% was equally effective to 0.02% BHT to protect cooked pork patties from oxidative changes over 12 days of storage.

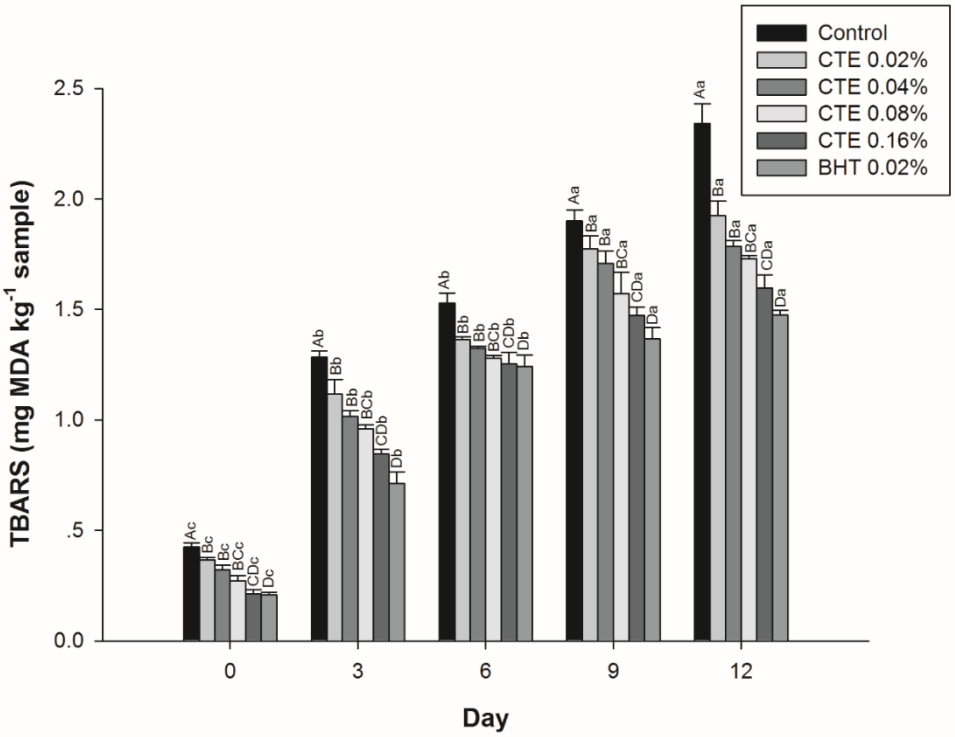


Figure 36 *Thiobarbituric acid reaction substance (TBARS) (mg MDA/mg of sample) in cooked pork patties during refrigerated storage for 12 days*

Control: without added *Clitoria ternatea*; CTE 0.02%, CTE 0.04%, CTE 0.08%, and CTE 0.16%: with added *Clitoria ternatea* extract 0.02%, 0.04%, 0.08%, and CTE 0.16%, respectively; BHT 0.02%: with added butylated hydroxytoluene 0.02%. The results are expressed as mean ± S.E.M. (n=5). Means with different superscripts differ significantly (P<0.05); a–c: day effects, A–D: treatment effects (P<0.05).

4.3.5 Protein oxidation in cooked pork patties during refrigerated storage for 12 days

4.3.5.1 Protein thiol

In the present study the oxidation of protein thiols was investigated in pork patties added CTE (0.02–0.16%) during 12 days refrigerated storage. The effects of CTE on the level of protein thiol in cooked pork patties are shown in Table 14. The respective treatments significantly (P<0.05) influenced protein thiol content and also impacted the change over time as evidenced by statistically significant interaction (P<0.05) between treatments and time. At day 0, the initial thiol concentration was found to be higher in pork patties added CTE than the control pork patties without added CTE. After that the levels of protein thiol groups in all cooked pork patties rapidly decreased during 12 days of refrigerated storage. However, more rapid thiol loss was observed for the control samples compared to samples added CTE, resulting in significantly (P<0.05) different thiol levels for pork patties stored for 12 days in the refrigerator. The cooked pork patties containing 0.08 and 0.16% CTE displayed significantly higher thiol group levels compared with the control throughout the refrigerated storage.

Table 14 Protein thiol levels (nmol/mg protein) in cooked pork patties during refrigerated storage (4±1°c) for 12 days

	Protein thiol levels (nmol/mg protein)				
Experiments	Day 0	Day 3	Day 6	Day 9	Day 12
Control	55.47 ± 4.89^{Ca}	40.96 ± 2.97^{Cb}	41.81 ± 1.08^{Cb}	25.78 ± 0.51^{Cc}	24.85 ± 2.33^{Cc}
CTE 0.02%	61.39 ± 2.60^{CBa}	44.91 ± 2.92^{CBb}	47.04 ± 2.29^{BCb}	32.02 ± 0.91^{BCc}	29.74 ± 2.32^{BCc}
CTE 0.04%	62.42 ± 2.33^{ACBa}	46.28 ± 2.26^{ABCb}	49.52 ± 2.79^{BCb}	34.51 ± 2.18^{BCc}	31.25 ± 2.99^{BCc}
CTE 0.08%	63.99 ± 7.80^{ABa}	51.12 ± 3.46^{ABb}	52.15 ± 1.79^{Bb}	35.63 ± 1.42^{Bc}	32.89 ± 3.54^{Bc}
CTE 0.16%	64.94 ± 3.53^{ABa}	58.29 ± 1.53^{ABb}	53.87 ± 1.01^{ABb}	36.31 ± 0.64^{ABc}	34.52 ± 1.85^{ABc}
BHT 0.02%	65.78 ± 3.26^{Aa}	64.23 ± 3.33^{Ab}	61.25 ± 3.29^{Ab}	37.73 ± 1.81^{Ac}	35.21 ± 1.97^{Ac}

The results are expressed as mean ± S.E.M. (n=5). Means with different superscripts with the same row (a–c: day effects) are significantly different ($P<0.05$). Means with different superscripts with the same column (A–C: treatment effects) are significantly different ($P<0.05$).

4.3.5.2 Protein carbonyl

The effects of CTE on the levels of protein carbonyl in cooked pork patties are shown in Table 15. The control cooked patties showed the highest levels on each of the sampling day. In the control group the initial concentration of protein carbonyls in cooked pork patties was approximately 1.57±0.29 nmol mg^{-1} of protein. The protein carbonyl content was significant (P<0.05) increase over time, and the treatments had a significant impact (P<0.05) on the change of protein carbonyl content in the pork patties. The content of protein carbonyls in the control group increased (P<0.05) during refrigerated storage. The increase reached its maximum (9.69±1.61 $nmolmg^{-1}$) on day 12 of storage, which was more than three-fold the initial level of the control group. The concentrations of carbonyls in all treated groups, except the addition of 0.02% CTE were considerably lower (P<0.05) than those of the control group at all time points. Compared with the control group, the addition of 0.08% and 0.16% CTE significantly inhibited the formation of protein carbonyls in pork patties throughout the storage. The findings suggest that 0.16% CTE was equally effective to 0.02% BHT to protect the loss of thiol group and reduce the protein carbonyl in cooked pork patties.

Table 15 Protein carbonyl levels (nmol/mg protein) in cooked pork patties during refrigerated storage (4±1°c) for 12 days

	Protein carbonyl levels (nmol/mg protein)				
Experiments	Day 0	Day 3	Day 6	Day 9	Day 12
Control	1.57 ± 0.29^{Ad}	3.00 ± 0.25^{Ac}	3.24 ± 0.19^{Ac}	6.12 ± 0.71^{Ab}	9.69 ± 1.61^{Aa}
CTE 0.02%	1.33 ± 0.25^{ABd}	2.55 ± 0.60^{ABc}	3.35 ± 0.19^{ABc}	4.47 ± 0.52^{ABb}	7.78 ± 1.04^{ABa}
CTE 0.04%	0.78 ± 0.09^{BCd}	2.42 ± 0.27^{BCc}	3.07 ± 0.14^{BCc}	3.75 ± 0.36^{BCb}	5.36 ± 1.39^{BCa}
CTE 0.08%	0.62 ± 0.22^{CDd}	1.24 ± 0.19^{CDc}	1.95 ± 0.34^{CDc}	3.46 ± 0.3^{CDb}	4.53 ± 0.65^{CDa}
CTE 0.16%	0.57 ± 0.1^{CDd}	1.03 ± 0.18^{CDc}	1.26 ± 0.14^{CDc}	2.24 ± 0.22^{CDb}	3.41 ± 0.68^{CDb}
BHT 0.02%	0.92 ± 0.12^{Dd}	1.13 ± 0.17^{Dc}	2.38 ± 0.19^{Dc}	3.43 ± 0.77^{Db}	3.46 ± 0.56^{Db}

The results are expressed as mean ± S.E.M. (n=5). Means with different superscripts with the same row (a–c: day effects) are significantly different ($P<0.05$). Means with different superscripts with the same column (A–C: treatment effects) are significantly different ($P<0.05$).

4.3.6 The color values in cooked pork patties during refrigerated storage (4±1°c) for 12 days

Table 16. shows the effect of CTE on the color values of cooked pork patties during the storage. The changes observed in parameters of color were mainly related to the additional concentration of CTE. The color for lightness (L*) and redness (a*) values was significant (P<0.05) color loss (decrease in redness) over time, and the treatments had a significant impact (P<0.05) on redness of the pork patties. Pork patties mixed with TP showed lower values (P<0.05) for lightness (L*) and for redness (a*), but higher values (P<0.05) yellowness (b*) than the patties without CTE during 12 days storage. At day 0, the control pork patties exhibited the highest color values for L^* (lightness), a^* (redness) and b^* (yellowness) values. Comparing with the control patties, the L^* and a^* values of cooked pork patties were lower in the samples containing CTE. After 12 days of the storage, the control patties had significantly higher L^* values compared with the control at day 0, meanwhile, the L^* values of pork patties containing CTE (0.02–0.16%) and BHT (0.02%) were lower than the control pork patties. As the storage period increased, the a^* values of cooked pork patties decreased. When compared on the initial day, the a^* values of cooked pork patties containing CTE was lower than the control sample. However, the redness of samples with CTE (0.08 and 0.16%) was higher than that of control samples after 12 days of refrigerated storage.

Table 16 The color values in cooked pork patties during refrigerated storage (4±1°c) for 12 days

Trait	Experiments	Day 0	Day 3	Day 6	Day 9	Day 12
CIE L* value (lightness)	Control	45.47 ± 0.57^{Ae}	46.97 ± 0.75^{Ad}	48.94 ± 0.41^{Ac}	51.27 ± 0.59^{Ab}	54.39 ± 0.37^{Aa}
	CTE 0.02%	44.33 ± 0.49^{Be}	45.62 ± 1.31^{Bd}	46.52 ± 0.57^{Bc}	48.82 ± 0.48^{Bb}	53.60 ± 0.41^{Ba}
	CTE 0.04%	$44.20 \pm .79^{BCe}$	45.13 ± 1.68^{BCd}	47.11 ± 0.32^{BCc}	48.39 ± 0.10^{BCb}	53.05 ± 0.56^{BCa}
	CTE 0.08%	$43.37 \pm .28^{BCDe}$	$44.95 \pm .69^{BCDd}$	$45.98 \pm .21^{BCDc}$	$46.77 \pm .21^{BCDb}$	$49.25 \pm .34^{BCDa}$
	CTE 0.16%	$42.94 \pm .42^{CDe}$	44.68 ± 0.73^{CDd}	45.62 ± 0.22^{CDc}	46.40 ± 1.10^{CDb}	$48.45 \pm .44^{CDa}$
	BHT 0.02%	45.19 ± 0.49^{De}	45.81 ± 0.28^{Dd}	46.19 ± 0.55^{Dc}	47.83 ± 0.31^{Db}	49.27 ± 0.26^{Da}
CIE a* value (redness)	Control	5.74 ± 0.27^{Ba}	4.73 ± 4.44^{Bb}	4.44 ± 0.29^{Bc}	4.23 ± 0.16^{Bc}	2.70 ± 0.13^{Bd}
	CTE 0.02%	5.71 ± 0.19^{Ba}	4.64 ± 0.21^{Bb}	4.18 ± 0.24^{Bc}	3.68 ± 0.09^{Bc}	3.25 ± 0.08^{Bd}
	CTE 0.04%	5.08 ± 0.27^{Ba}	5.54 ± 0.17^{Bb}	3.46 ± 0.19^{Bc}	3.38 ± 0.19^{Bc}	3.04 ± 0.17^{Bd}
	CTE 0.08%	4.41 ± 0.28^{Ca}	3.70 ± 0.11^{Cb}	3.37 ± 0.13^{Cc}	3.19 ± 0.08^{Cc}	2.95 ± 0.07^{Cd}
	CTE 0.16%	3.96 ± 0.33^{Ca}	3.44 ± 0.13^{Cb}	3.20 ± 0.25^{Cc}	3.07 ± 0.19^{Cc}	2.90 ± 0.10^{Cd}
	BHT 0.02%	6.73 ± 0.18^{Aa}	6.54 ± 0.24^{Ab}	5.36 ± 0.22^{Ac}	4.96 ± 0.30^{Ac}	3.77 ± 0.10^{Ad}
CIE b* value (yellowness)	Control	14.28 ± 0.42^{Aa}	14.92 ± 0.21^{Aa}	15.12 ± 0.16^{Aa}	15.83 ± 0.21^{Aa}	15.63 ± 0.16^{Aa}
	CTE 0.02%	14.35 ± 0.44^{Aa}	15.02 ± 0.17^{Aa}	15.26 ± 0.25^{Aa}	15.44 ± 0.20^{Aa}	15.75 ± 0.67^{Aa}
	CTE 0.04%	14.62 ± 0.31^{Aa}	14.75 ± 0.29^{Aa}	15.17 ± 0.29^{Aa}	15.95 ± 0.36^{Aa}	16.15 ± 0.29^{Aa}
	CTE 0.08%	14.65 ± 0.19^{Aa}	14.90 ± 0.19^{Aa}	15.43 ± 0.35^{Aa}	16.03 ± 0.25^{Aa}	16.25 ± 0.34^{Aa}
	CTE 0.16%	14.75 ± 0.12^{Aa}	14.82 ± 0.25^{Aa}	15.71 ± 0.19^{Aa}	16.14 ± 0.17^{Aa}	16.41 ± 0.23^{Aa}
	BHT 0.02%	14.13 ± 0.33^{Aa}	14.50 ± 0.34^{Aa}	15.20 ± 0.28^{Aa}	16.15 ± 0.16^{Aa}	16.37 ± 0.37^{Aa}

The results are expressed as mean ± S.E.M. (n=5). Means with different superscripts with the same row (a–d: day effects) are significantly different ($P<0.05$). Means with different superscripts with the same column (A–D: treatment effects) are significantly different ($P<0.05$).

4.3.7 Sensory evaluation in cooked pork patties during refrigerated storage (4±1°c) for 12 days

The sensory evaluation data for cooked ground pork samples are summarized in Table 17. At the initial day, there were no significant differences in the surface color score between control and treatments with the exception of CTE 0.16% ($P<0.05$). On day 12, cooked pork patties treated with CTE (0.04–0.16%) significantly improved acceptance of the color score. Unlike the control group, patties of the control group received the unacceptable surface color score of 3.03 ± 0.20 ($P<0.05$). There were no significant differences in juiciness and rancidity between control and treatments at day 0. After 3 and 6 days of storage, the scores of pork patties treated with CTE patties had an overall acceptability in juiciness that statistical significance was higher than those of control patties ($P>0.05$). Moreover, addition of CTE (0.04–0.16%) in cooked pork patties resulted in improved juiciness and rancidity after 12 days of the storage ($P<0.05$). Patties of the CTE 0.04-0.16% groups received the overall acceptability score after 12 days of the storage ($P<0.05$). Increasing storage time decreased the sensory scores of color, juiciness, rancidity, and overall acceptability in all samples except for CTE treated pork patties at concentration between 0.04-0.16%.

Table 17 The sensory evaluation in cooked pork patties during refrigerated storage (4±1°c) for 12 days

Sensory Attributes	Experiments	Storage days				
		Day 0	Day 3	Day 6	Day 9	Day 12
Surface color	Control	6.33 ± 0.12^{Aa}	5.56 ± 0.41^{Aab}	5.70 ± 0.45^{Aab}	4.35 ± 0.25^{Bc}	3.03 ± 0.20^{Bd}
	CTE 0.02%	6.41 ± 0.33^{Aa}	5.74 ± 0.36^{Aab}	5.75 ± 0.36^{Aab}	5.14 ± 0.29^{Ab}	3.72 ± 0.32^{Bc}
	CTE 0.04%	6.23 ± 0.25^{Aa}	5.62 ± 0.27^{Aab}	5.60 ± 0.25^{Bab}	5.28 ± 0.21^{Ab}	3.88 ± 0.49^{Ac}
	CTE 0.08%	6.13 ± 0.25^{Aa}	5.32 ± 0.31^{ABa}	5.58 ± 0.16^{Ba}	5.35 ± 0.11^{Aa}	3.91 ± 0.31^{Ab}
	CTE 0.16%	5.88 ± 0.15^{Ba}	5.58 ± 0.45^{Aa}	5.55 ± 0.23^{Ba}	5.33 ± 0.45^{Aa}	3.95 ± 0.11^{Ab}
	BHT 0.02%	6.37 ± 0.19^{Aa}	5.56 ± 0.32^{Aa}	5.52 ± 0.41^{Ba}	5.35 ± 0.52^{Aa}	3.92 ± 0.28^{Ab}
Juiciness	Control	6.53 ± 0.27^{Aa}	5.66 ± 0.11^{Bb}	5.54 ± 0.26^{Bb}	3.57 ± 0.21^{Bc}	3.03 ± 0.12^{Bcd}
	CTE 0.02%	6.33 ± 0.12^{Aa}	5.74 ± 0.36^{Aab}	5.46 ± 0.22^{Ab}	4.37 ± 0.46^{Bc}	3.17 ± 0.32^{Bd}
	CTE 0.04%	6.56 ± 0.38^{Aa}	5.85 ± 0.15^{Aab}	5.45 ± 0.32^{Ab}	4.13 ± 0.27^{Ac}	3.67 ± 0.27^{Ad}
	CTE 0.08%	6.64 ± 0.21^{Aa}	5.82 ± 0.31^{Aab}	5.42 ± 0.14^{Ab}	4.34 ± 0.32^{Ac}	3.75 ± 0.26^{Ac}
	CTE 0.16%	6.67 ± 0.14^{Aa}	5.85 ± 0.38^{Aab}	5.48 ± 0.34^{Ab}	4.53 ± 0.28^{Ac}	3.79 ± 0.42^{Ac}
	BHT 0.02%	6.88 ± 0.11^{Aa}	5.83 ± 0.25^{Aab}	5.48 ± 0.19^{Ab}	4.59 ± 0.52^{Ac}	3.77 ± 0.34^{Ac}
Rancidity	Control	6.37 ± 0.45^{Aa}	6.06 ± 0.27^{Aa}	5.58 ± 0.27^{Ab}	3.58 ± 0.46^{Bc}	2.89 ± 0.32^{Bd}
	CTE 0.02%	6.76 ± 0.34^{Aa}	6.14 ± 0.36^{Aab}	5.62 ± 0.36^{Ab}	4.65 ± 0.43^{Ac}	4.12 ± 0.15^{Ac}
	CTE 0.04%	6.33 ± 0.12^{Aa}	6.25 ± 0.22^{Aa}	5.53 ± 0.15^{Aa}	4.67 ± 0.29^{Ab}	4.06 ± 0.38^{Ab}
	CTE 0.08%	6.41 ± 0.33^{Aa}	6.34 ± 0.28^{Aa}	5.55 ± 0.21^{Ab}	4.59 ± 0.26^{Ac}	4.11 ± 0.10^{Ac}
	CTE 0.16%	6.53 ± 0.28^{Aa}	6.38 ± 0.34^{Aa}	5.58 ± 0.24^{Ab}	4.64 ± 0.11^{Ac}	4.12 ± 0.32^{Ac}
	BHT 0.02%	6.61 ± 0.15^{Aa}	6.38 ± 0.26^{Aa}	5.67 ± 0.13^{Ab}	4.56 ± 0.32^{Ac}	4.23 ± 0.27^{Ac}
Overall acceptability	Control	6.68 ± 0.15^{Aa}	6.14 ± 0.29^{Ba}	6.14 ± 0.29^{Ba}	3.52 ± 0.46^{Cb}	2.89 ± 0.23^{Bc}
	CTE 0.02%	6.57 ± 0.19^{Ba}	6.36 ± 0.24^{Aa}	6.16 ± 0.24^{Ba}	4.65 ± 0.43^{Bb}	3.17 ± 0.32^{Bc}
	CTE 0.04%	6.53 ± 0.27^{Ba}	6.27 ± 0.19^{Aa}	6.24 ± 0.11^{Aa}	5.45 ± 0.18^{Ab}	4.27 ± 0.34^{Ac}
	CTE 0.08%	6.57 ± 0.35^{Ba}	6.31 ± 0.43^{Aa}	6.22 ± 0.29^{Aa}	5.42 ± 0.32^{Ab}	4.29 ± 0.16^{Ac}
	CTE 0.16%	6.54 ± 0.23^{Ba}	6.28 ± 0.15^{Aa}	6.25 ± 0.13^{Aa}	5.44 ± 0.25^{Ab}	4.22 ± 0.41^{Ac}
	BHT 0.02%	6.66 ± 0.32^{Aa}	6.29 ± 0.26^{Aa}	6.25 ± 0.49^{Aa}	5.53 ± 0.16^{Ab}	4.29 ± 0.22^{Ac}

Scale from 9 to 1. For liking attributes, 9=like extremely, 5=neither like nor dislike, and 1=dislike extremely. The results are expressed as mean ± S.E.M. (n=5). Means with different superscripts with the same row (a–d: day effects) are significantly different ($P<0.05$). Means with different superscripts with the same column (A–B: treatment effects) are significantly different ($P<0.05$).

CHAPTER V

DISSCUSSION

5.1 The stability, antioxidant activity and biological activity of *Clitoria ternatea* petal flower extract during simulated *in vitro* gastrointestinal digestion.

Clitoria ternatea flower petal is well known to have a bright blue color due to the different chemical structures of anthocyanins or anthocyanidins in the flower (Terahara et al., 1996; Terahara et al., 1998). Over the years, CT has been used as potential medicines with interesting biological activity such an effective antioxidant against free radicals and other reactive oxygen species (Gomez & Kalamani, 2003; Gupta et al., 2010; Zingare et al., 2013). Antioxidant activity of CT was reported due to the presence of ternatin anthocyanins, namely, A1–A3, B1–B4, C1–C4, and D1–D3, and phenolic flavonols such as quercetin and kaempferol derivatives in CTE (Kazuma et al., 2003; Kazuma et al., 2003; Nair et al., 2015; Terahara et al., 1996; Terahara et al., 1998). However, these ternatins derivatives in CTE exhibited the different chemical structures at the number and position of the hydroxyl groups on the aromatic ring (Kazuma et al., 2003; Kazuma et al., 2003). The major concern in the development of bioactive polyphenols and food ingredients has risen in the limitation of bioavailability. The different chemical structures of polyphenols may either not be absorbed or experience metabolic degradation from gastrointestinal digestion (D'Archivio et al., 2010; Del Rio et al., 2013; Manach et al., 2004). According to results from this study, the total polyphenol content (TPC) and total anthocyanin content (TAC) in CTE were significantly higher than initial concentrations after purification. This indicated that purification can be a great desirable for improved purity and concentration by removal of undesirable compounds such as sugars, amino acids, and metals which may accelerate degradation of anthocyanins in the extracts (He & Giusti, 2011). This result was in agreement with previous study that the purification procedure improve a purity and concentrate the

bioactive small molecules in the crude extract which result in enhancing the bioactive activity of certain plants (Koh et al., 2009).

In comparison on TAC and TPC, acid hydrolysis (AHCTE) were relatively higher than that of the CTE. The findings indicate that the amount of phenolic compounds increased after acid hydrolysis. Previous study reported that acid hydrolysis process was suitable for the extraction of phenols from plant extracts as it can enhance the extraction concentration of total phenolic compound (Yang et al., 2013). The reason for explanation might be the release of linked sugar residues from the structures of polyphenol after acid hydrolysis (Rivelli et al., 2011). After gastrointestinal digestion, TPC was a significant increase in PCTE, whereas AHCTE slightly decreased but did not show significant difference. The findings also indicate that antioxidant activity of PCTE was significantly higher after enzymatic digestion. Similar result was consistent with the current findings (Bouayed et al., 2011). It is possible that chemical compounds may be metabolized, oxidized, or degraded into other chemicals which thus produced more phenolic hydroxyl (Liang et al., 2012). Moreover, the presence of the amyloglucosidase enzyme in the simulated digestion produces the deglycosylation by hydrolyzing 1,4-linked **α**-D-glucose and 1,6-**α**-D-glucosidic bonds and consequently removes the sugars from the structures (Kasprzak et al., 2012). Structural changes to the aglycones directly contribute to increase antioxidant capacity after gastrointestinal digestion (Li et al., 2009; Rivelli et al., 2011). Therefore, natural breakdown of products to small molecular phenolic compounds by the intestinal enzymes may increase the TPC content and bioactivity of CTE. The antioxidant activity of AHCTE was not significantly different after gastrointestinal digestion. It is notation that the acidic solution of AHCTE may help increase the stability of anthocyanins under simulated gastric digestion. Consistent with other studies, when introduction of AHCTE into the higher pH environment during intestinal digestion, it resulted in decreased overall stability of anthocyanins (He et al., 2009; Prior & Wu, 2006).

Bile acid biosynthesis in the intestinal tract plays an important role in cholesterol homeostasis. The possible mechanism has been hypothesized for lowering plasma cholesterol levels in the body through the binding of bile acids and increasing

of their fecal excretion. Binding bile acids and preventing their recirculation results in reduced fat absorption and cholesterol utilization to synthesize more bile acids (Insull, 2006). In this present study, the bile acid binding of CTE and its hydrolysis during simulated gastrointestinal digestion was investigated. In this study, the binding capacity was not significantly different between PCTE and AHCTE, however the binding effect of PCTE significantly increased after simulated gastrointestinal digestion. This may due to the chemical structure or active binding sites of plant bioactive compounds which are able to interact with bile acids under the conditions of the small intestine (Kahlon et al., 2007; Kahlon & Smith, 2007; Ngamukote et al., 2011). The ability of bile acid-binding capacity was also reported in anthocyanin enriched grape seed extract (Ngamukote et al., 2011) and polyphenol enriched pomelo pulp extract (Mäkynen et al., 2013)

Anthocyanins-enriched edible plants (berry extracts and sweet potato extract) have been implicated in the inhibition of intestinal maltase and sucrase and pancreatic α-amylase (Benalla et al., 2010; Grussu et al., 2011) (Matsui et al., 2004). The results demonstrate that anthocyanins act as a competitive α-glucosidase inhibitor which is similar to that of acarbose (Grussu et al., 2011). Alpha-glucosidase are important enzymes for the digestion of carbohydrate by hydrolyzing alpha (1⟶4) bonds in carbohydrate compounds. The inhibition of these enzymes could reduce postprandial blood glucose (Wang, Liu, et al., 2013). Our present investigation affirmed that CTE showed moderated α-glucosidase inhibitory activity in agreement with previous results (Adisakwattana et al., 2012). The mechanism of inhibition action on α-glucosidase and α-amylase may be due to the presence of anthocyanins in CTE. The hydroxyl groups of anthocyanins form hydrogen bonds with the polar groups in the allosteric sites, where the molecular characteristics are important for inhibition (Li et al., 2009). These interactions would change the enzyme's molecular configuration and hydrophilic and hydrophobic properties, resulting in a decrease in enzyme activities (Adisakwattana et al., 2012). There were significant differences between nondigested and digested PCTE and AHCTE. After simulated gastrointestinal digestion, PCTE was the most effective

inhibitor against intestinal maltase and sucrase and pancreatic α-amylase. Moreover, AHCTE appeared to be lower inhibitory effects after digestion.

The intestinal absorption of glucose from dietary carbohydrates is principally mediated by two transporters, sodium-dependent glucose transporter 1 (SGLT1) in the apical membrane (AM) and glucose transporter 2 (GLUT2) in the basolateral membrane of enterocytes (Takata, 1996). In the presence of PCTE, the inhibition of glucose uptake across the epithelial cells under sodium-dependent conditions was observed. However, PCTE exerted no effect on the inhibition of glucose transport under sodium-free conditions.

Sodium is essentially required for inhibiting glucose transportation of CT suggesting that the underlying mechanism for the inhibition of glucose uptake may involve in SGLT-1. In this study, the inhibition of glucose uptake by CT extract is consistent with other studies demonstrating that the anthocyanins enriched berry extract inhibit the intestinal absorption of glucose through SGLT1 (Alzaid et al., 2013) . Additionally, bioactive compounds of pomegranate are able to reduce glucose absorption by interfering with SGLT1 protein expression (Kim et al., 2013). Further study is required to investigate the effect of CT extract on SGLT1 protein expression.

The HPLC-MS analyses of CTE have recently shown the presence of ternatins derivatives, namely, A1–A3, B1–B4, C1–C4, and D1–D3, and phenolic flavonols such as quercetin and kaempferol derivatives (Terahara et al., 1996; Terahara et al., 1998). Recent study has reported that twelve phenolic metabolites (nine ternatin anthocyanins and three glycosylated quercetins) were identified from the blue flowers of *Clitoria ternatea* by HPLC-DAD-ESI/MS (Nair et al., 2015). Moreover, the chromatogram patterns obtained in the current study were similar to the chromatogram patterns in the past (Nair et al., 2015) which indicated the most observed peaks are delphinidin and ternatin derivatives in the CT extract. Additionally, previous study indicated that both acid and enzymatic hydrolysis could terminate the bond between the anthocyanins and the glycoside moieties, transforming all of the anthocyanin derivatives to their aglycone forms (Nuutila et al., 2002; Rivelli et al., 2011). Rutin (quercetin-3-rutinoside) is easily released the sugar moieties under hydrolysis

conditions (Wang, Zhao, et al., 2013). The HPLC chromatograms of CTE both acid hydrolysis and the simulated digestion may be the similar compounds with previous studies, ternatin derivatives. Especially, there are the obvious change of intensity of chromatograms indicating the increased or decreased concentration of compounds. It is possible that they occur the release of sugars from the ternatin derivatives under acid and enzymatic hydrolysis in the simulated digestion. Further investigation on HPLC-MS/MS is needed to confirm for the chemical structures of compounds in CTE and its hydrolysis.

Therefore, antioxidant and biological activity of CTE may predominantly contribute from phenolic compounds, especially anthocyanins. The hydrolysis of CTE can enhance stability, antioxidant, and biological activity of CTE. Interestingly, PCTE exhibited the effective antioxidant and the increased biological activities including the inhibition of intestinal **α**-glucosidase (maltase and sucrase) and pancreatic **α**-amylase activities and glucose uptake via SGLT1 as well as increased binding to bile acids after gastrointestinal digestion. However, the acid hydrolysis of CTE could not enhance to increase the antioxidant and biological activities. Therefore, *Clitoria ternatea* exhibited great potential to be developed into functional foods for the management of chronic diseases.

5.2 Alginate-based encapsulation of polyphenols from *Clitoria ternatea* petal flower extract enhances stability and biological activity under simulated gastrointestinal conditions

Stability of polyphenols mostly depends on the acidity, pH, light, temperature and storage (Arabshahi-D et al., 2007). Moreover, during processing, polyphenols were disintegrated when heated at high temperatures and longer heating times caused a reduction in the antioxidant capability (Arabshahi-D et al., 2007; Friedman & Jürgens, 2000; Ross et al., 2011). Therefore, the administration of polyphenols may require the formulation to increase its bioavailability and maintain the bioactive compounds in gastrointestinal tract. One technology that may assist in reducing losses of nutrients in foods and improving delivery systems, offering prolonged or controlled release of food ingredients and improve bioavailability of bioactive food compounds, stability, and

targeting to a specific site is microencapsulation (Champagne & Fustier, 2007; Desai & Jin Park, 2005; Tiwari et al., 2012). This is designed to protect sensitive materials and enhance stability while facilitating controlled release under appropriate conditions. Encapsulation has been used for a number of years in various industries, particularly for pharmaceutical applications. In this context, the encapsulation of CT petal flower into different matrices or delivery systems has become a great challenge for stabilizing and protecting it from degradation in order to preserve its biological activity and enhance its bioavailability. Accordingly, the broad aim of this work has been to provide practical approaches to develop a calcium alginate method for preparation of CT petal flower polyphenols-containing microencapsulation then compare the digestive stability and biological activity of CT and its encapsulation in simulated digestion fluid.

The encapsulating agent used in this study was sodium alginate. Calcium alginate beads containing CT polyphenol extract were encapsulated by ionic gelation method. Formulation factors such as sodium alginate concentration, calcium chloride concentration, calcium chloride exposure time, gelling bath time maintaining, and extract concentration had a great influence on microbead characteristics. Sodium alginate is an anionic polymer which can be easily cross-linked with calcium chloride, this is because the calcium ions are bound to carboxylate residues of both mannuronic acid and glucournic acid which are components of sodium alginate (Zam et al., 2014). The results showed that there was no formation of spherical shape of the beads at concentration of sodium alginate and $CaCl_2$ below 1.0% and 1.5% (w/v), respectively. The formulation containing an extract of 10% w/v of CT encapsulated with 1.5 % of sodium alginate cured in 3% w/v of calcium chloride for 30 minutes and kept in a gelling bath for 20 minutes was chosen as the optimum formula regarding the encapsulation morphology and efficiency. This may because of binding parameters between sodium alginate and calcium chloride at low $CaCl_2$ concentrations might contribute to the lack of enough carboxyl groups and calcium ions mass for gelation (Blandino, Macías, & Cantero, 1999; Liu et al., 2003).

The SEM photographs of the control beads compared with CT in calcium alginate beads showed the differences in surface morphology. Control beads after drying had a collapsed and shriveled shape as well as the gaps on the surface. The

results showed that the CT beads represented a high degree of surface smoothness and more spherical shape. The finding suggest that the CT fulfilled cavities of the matrix, therefore causing reduction in porosity and presenting more smoothness surface (Trifkovi´c et al., 2014). Moreover, the smoothness of surface increased when concentration of $CaCl_2$ was increased in the beads. This suggests that the binding sites on the gel formation process influence by mass of Ca^{2+} ions in the alginate chains which act as cross-linkers that stabilize alginate chains forming a surface structure. Therefore, this phenomenon may be a result of increasing the interaction between Ca2+ and alginate at the surface and restrict further entry to the core, leading to stabilize the structure and increase the bead strength (Blandino et al., 1999; Liu et al., 2003).

In this study, the encapsulation efficiency of the beads improved when increased the concentration of sodium alginate. Together with SEM photographs, the concentration of $CaCl_2$ was found to have more influence on the encapsulation efficiency, however, it led to form the cracked surfaces and consequently decrease encapsulation efficiency. The concentration of alginate utilized in this study was consistent with the previous study for the encapsulation of yerba mate polyphenols (Deladino et al., 2008). The extrusion method is an effective technique for producing beads of desired diameter and appears as regular spheres of a uniform size (Manojlovic et al., 2008).

The mean particle size of CT beads was smaller than that of control alginate beads. The extrusion method is an effective technique for producing beads of desired diameter and appears as regular spheres of a uniform size (Manojlovic et al., 2008). The size and shape of beads are controlled by many factors such as needle diameter, distance between the needle and the collecting solution, and surface tension (Lee, Ravindra, & Chan, 2013). The size of a Ca-alginate bead produced by this technique is commonly greater than 1,000 μm with narrow size distribution. Regarding size of the beads, the polyphenols in CT can establish extensive hydrogen bonding (via hydrophilic phenolic hydroxyl groups) with alginate polyelectrolyte (El-Kamel, Al-Gohary, & Hosny, 2010), which allows an increase in interfacial tension between

aqueous and organic phase, leading to decrease size of the beads (Goh, Heng, & Chan, 2012; Mane, Ponrathnam, & Chavan, 2015).

The content of polyphenolic compounds was presented in CT beads. Additionally, CT beads also showed antioxidant ability (DPPH, ABTS, and FRAP values). It can be clearly seen that after encapsulation, CT beads preserved the polyphenolic antioxidants and exhibit a potent antioxidant capacity determined by the ABTS, FRAP and DPPH assays. The currents findings are in agreement without previous reports that demonstrated antioxidant activity of CT petal flower extract in various in vitro models (Rabeta & An Nabil, 2013; Phrueksanan, Yibchok-anun, & Adisakwattana, 2014; Chayaratanasin, Barbieri, Suanpairintr, & Adisakwattana, 2015). In the phytochemical analysis, ternatins and its derivatives are mainly blue anthocyanins found in the petals of CT suggesting that they may be responsible for antioxidant (Chayaratanasin, Barbieri, Suanpairintr, & Adisakwattana, 2015).

FT-IR was used to identify functional groups and characterize the relationship between the matrix and CT components. The molecular interactions of sodium alginate powder (A), calcium alginate beads (AC), Clitoria ternatea (CT), and CT beads (ACT) were investigated using FT-IR spectroscopy. The FT-IR spectra of A powder shows peaks around 3280.79, 1595.40, 1405.06, and 1025.63 cm^{-1}, reflective of O-H, COO- (asymmetric), COO- (symmetric), and C-O-C stretching, respectively (Pongjanyakul & Puttipipatkhachorn, 2007; Pongjanyakul & Rongthong, 2010). These characteristic bands were in agreement with previous reported results (Istenič et al., 2015). The strong and broad absorption band at 3334.05 cm^{-1} has been observed due to O-H stretching. The cross-linking process of AC caused an obvious shift to higher wave number of O-H stretching, which is the stretching and bending mode (Pongjanyakul & Rongthong, 2010). In agreement with a previous study, the beads prepared with $CaCl_2$ was observed a new peak at 2131.59 – 2153.15 cm^{-1} which did not appear in the pure alginate, indicating that the cross-linked reaction occurred between pure alginate and $CaCl_2$ (Cho et al., 2014). Additionally, the strong asymmetric stretching absorption band at 1598.09 cm^{-1} and weaker symmetric stretching band near 1420.20 cm^{-1} appeared due to the presence of carboxylate anions COO- (Stojanovic et al., 2012). Moreover,

the change to lower numbers and a decrease in the intensity of C-O-C stretching peak of AC was also observed. Consistent with a previous study, it indicates the presence of an ionic bond between the calcium ion and the carboxyl groups of A (Pongjanyakul & Rongthong, 2010).

FT-IR was used to identify functional groups and characterize the relationship between the matrix and CT components. The molecular interactions of sodium alginate powder (A), calcium alginate beads (AC), Clitoria ternatea (CT), and CT beads (ACT) were investigated using FT-IR spectroscopy. The FT-IR spectra of A powder shows peaks around 3280.79, 1595.40, 1405.06, and 1025.63 cm^{-1}, reflective of O H, COO– (asymmetric), COO– (symmetric), and C-O-C stretching, respectively (Pongjanyakul & Puttipipatkhachorn, 2007; Pongjanyakul & Rongthong, 2010). These characteristic bands were in agreement with previous reported results (Istenič et al., 2015). The strong and broad absorption band at 3334.05 cm^{-1} has been observed due to O-H stretching. The cross-linking process of AC caused an obvious shift to higher wave number of O-H stretching, which is the stretching and bending mode (Pongjanyakul & Rongthong, 2010). In agreement with a previous study, the beads prepared with CaCl2 was observed a new peak at 2131.59–2153.15 cm^{-1} which did not appear in the pure alginate, indicating that the cross-linked reaction occurred between pure alginate and $CaCl_2$ (Cho et al., 2014). Additionally, the strong asymmetric stretching absorption band at 1598.09 cm−1 and weaker symmetric stretching band near 1420.20 cm^{-1} appeared due to the presence of carboxylate anions COO– (Stojanovic et al., 2012). Moreover, the change to lower numbers and a decrease in the intensity of C-O-C stretching peak of AC was also observed. Consistent with a previous study, it indicates the presence of an ionic bond between the calcium ion and the carboxyl groups of A (Pongjanyakul & Rongthong, 2010). There were several relevant changes in the spectrum of the alginate submicron particles with the encapsulated CT in comparison to the AC suggesting that the CT was successfully incorporated into the submicron particles. The spectra of the AC and CT encapsulating alginate showed little change in the peak intensity in the range of 800–1600 cm^{-1}. The peak of O H bond at CT (3273.09 cm^{-1}) and A (3280.79 cm^{-1}) was shifted to lower wave number after the formation of CT beads (3255.86

cm^{-1}). Similarly, O-H stretching peak of alginate shifted to lower wavenumber when CT encapsulated into alginate beads, suggesting that a molecular interaction between alginate and CT was formed via hydrogen bonding. Furthermore, the intensity of the peaks of 1240.30 cm^{-1} (C-O-C vibration of esters) and 1512.26 cm^{-1} (aromatic ring vibration) in the spectrum of alginate arises after loading with the CT compounds. This formation did not alter the new peaks after formation of the beads suggesting that no chemical interactions were found between plant extract and alginate beads. CT peaks might be overlapped with peaks of ions in the thermogram of beads. This could be ascribed to the amorphous state of the CT in the beads. It could be seen that the peaks of the complexes were shifted from those of the physical mixture (Jelvehgari, Barghi, & Barghi, 2014). The absence of chemical interactions between the phytochemical compounds of CT and alginate was in agreement with other studies (Cho et al., 2014; Pongjanyakul & Rongthong, 2010; Stojanovic et al., 2012). Therefore, it can be assumed that the calcium alginate bead is a compatible material for encapsulating biochemical active compounds of CT.

This confirms the results obtained from thermal behavior of microencapsulation of DSC experiments. The interaction of CT and alginate in the beads caused a decrease in the thermal stability indicating from the shift of the exothermic peak to a lower temperature. Shifts on endothermic and exothermic peaks and shifts on maximum infrared peaks observed between individual polyanion complexes and final microcapsule carriers were understood as ionic interactions which led to the formation of new chemical entities with different thermal and absorption properties. The results suggest that the CT maintained its chemical instability during the encapsulation process (Jelvehgari et al., 2014). This thermal behavior has also been observed in the previous study (Pongjanyakul & Rongthong, 2010). Also, the exothermic peak of the CT beads moved to a higher temperature, indicating that the interaction of A and CT could enhance the thermal stability of CT. These data suggest that alginate could improve the thermal stability of the CT and maintained its chemical instability during the encapsulation process which led to the formation of new chemical entities with different thermal and absorption properties (Jelvehgari et al., 2014). Similar data

were reported in the resveratrol beads indicating that the absence of the resveratrol peak was due to the resveratrol encapsulation within the microparticles (Cho et al., 2014).

The results of polyphenolic content and the biological profiles of the CT and its encapsulation in the simulated gastric and intestinal digestion are demonstrated that CT had higher the content of total polyphenolic compounds when compared to CT beads after simulated gastric fluid (SGF). On the other hand, it was lower than CT beads after simulated intestinal fluid (SIF). The results are consistent with antioxidant activity after stimulated gastric and intestinal digestion that the antioxidant activity (ABTS and FRAP) in the SIF was higher than that of SGF. The findings suggest that the alginate might act as a barrier for CT release and protect polyphenols from the SGF, allowing them to reach the SIF at higher quantity. Alginate beads are stable in acidic solution and degrade under higher pH value (Li et al., 2016). This system would be useful for microbeads with specific delivery purposes. In agreement with the previous reports (Lucinda-Silva et al., 2010), a higher swelling degree and faster drug release were observed in a SIF environment (pH 7.5), as compared to a SGF environment (pH 1.2).

The result of effects of CT and its encapsulation following simulated gastrointestinal digestion on the inhibition of pancreatic α-amylase shows that CT markedly inhibited pancreatic α-amylase activity whereas CT beads significantly improved the percentage inhibition of pancreatic α-amylase after simulated gastrointestinal digestion. In this regard, the inhibition of pancreatic α-amylase could delay the breakdown of carbohydrates in the small intestine and decreases the postprandial blood glucose excursion (Kwon et al., 2007).

The result showed that the binding ability of CT beads was higher than CT. They indicate that the CT beads increase the ability to bind bile acid when compared to CT after simulated gastrointestinal digestion. The ability to bind bile acid of compounds is the underlying mechanisms for the reduction of cholesterol in the blood circulation. When the compounds bind bile acid, they disrupt the enterohepatic circulation of bile acids by preventing their reabsorption from the gut and increasing

of their fecal excretion. The liver requires more cholesterol synthesis to replace the lost of bile acids by LDL cholesterol uptake from the blood circulation (Insull, 2006).

5.3 The effects of *Clitoria ternatea* petal flower extract (CTE) on lipid and protein oxidation in cooked pork patties during refrigerated storage (4±1°c) for 12 days

The total phenolic content of CTE used in this study was 28.8 mg gallic acid equivalents/g of CTE. Previous studies reported that the amount of phenolic content and antioxidant activity of CTE were 53 mg gallic acid equivalents/g dried extract, and 0.17 ± 0.01 mg trolox equivalents/mg dried extract respectively (Chayaratanasin et al., 2015). The antioxidant capacity of the cooked pork patties with CTE showed significantly higher TEAC values than the control group ($P<0.05$). It is well documented in the literature that the phytochemical analysis of *Clitoria ternatea* flower petal extract consisted of bioactive compounds such as delphinidin-3,5-glucoside, delphinidin-3β-glucoside, malvidin-3β-glucoside, kaempferol, p-coumaric acid and six major ternatins (ternatins A1, A2, B1, B2, D1 and D2) (Terahara et al., 1996; Terahara et al., 1998). These phytochemical compounds have demonstrated antioxidant activity in various models (Madhavarao, 2011; Rabeta & An Nabil, 2013). The findings suggest that antioxidant capacity in the cooked pork patties might be related to phytochemical compounds in CTE.

The respective treatments significantly ($P<0.05$) influenced lipid and protein oxidations and also impacted the change over time as evidenced by statistically significant interaction ($P<0.05$) between treatments and time. The TBARS values of control pork patties significantly increased throughout 12 days of storage, which was consistent with previous reports (Botsoglou et al., 2012; Botsoglou et al., 2014; Choe et al., 2011; Jia et al., 2012; Jiang et al., 2013; Kong et al., 2010; Olorunsanya et al., 2010). Malonaldehyde is a common product of autoxidation of polyunsaturated fatty acids with three or more double bonds estimated by the TBARS reaction. Especially, cooked meat is more susceptible to lipid oxidation than raw meat because the heating process during cooking accelerates the oxidative process and increases the formation of free radicals and secondary products of oxidation (Ganhão et al., 2013; Olorunsanya et al., 2010). In the meantime, iron is released from heme pigments during the cooking

process, leading to the formation of non-heme iron and stimulating the autoxidation process and further development of off-flavor in meat products (Ganhão et al., 2013; Min & Ahn, 2005; Olorunsanya et al., 2010). Besides cooking, storage period has a significant influence on the induction of lipid oxidation in meat, resulting in extensive increase in the TBARS values during refrigerated storage (Ganhão et al., 2013; Min & Ahn, 2005; Olorunsanya et al., 2010; Sampaio et al., 2012). The TBARS values of CTE-containing samples were significantly lower than those for the control on the initial day. The findings suggest that CTE retarded lipid oxidation during and immediately after cooking.

The generation of conjugated dienes (CD) on initial day was significantly increased for all groups. The results showed that the highest concentration of CD was observed in cooked pork patties at day 6 of storage and then decreased until the end of the storage. Our findings were in agreement with previous studies indicating that the concentration of CD dramatically increased on the first day, followed by a decrease during the refrigerated storage (Botsoglou et al., 2014; Choe et al., 2011; Juntachote et al., 2006; Pena-Ramos & Xiong, 2003). It is noteworthy that the formation of CD occurs in early stages of lipid peroxidation and also generates conjugated hydroperoxides that can decompose to the secondary lipid peroxidation products (Pena-Ramos & Xiong, 2003). The present results demonstrated that a decrease in concentration of CD was associated with an increase in TBARS in cooked pork patties. The oxidative stability effect of CTE on the ability to reduce the concentration of CD and the formation of lipid peroxidation apparently may be related to their polyphenol content. Generally, phenolic compounds that possess antioxidant activity have been characterized as phenolic acids and flavonoids (Rabeta & An Nabil, 2013). Polyphenolic compounds including flavonoids can stabilize free radicals by donation of hydrogen from the phenolic groups, thereby forming a stable end product and hence terminating lipid oxidation reaction (Flora, 2009). According to the findings, the ability of CTE to reduce the concentration of CD and the formation of lipid peroxidation must be related to antioxidant activity of polyphenolic compounds to scavenge free radicals.

Protein oxidation is associated with depleting protein thiol and increasing protein carbonyl. The products from lipid oxidation including radicals, hydroperoxides and secondary compounds easily oxidize with protein constituents including thiols and amino acids. The reaction leads to protein oxidation and the loss of its functionality (Shi et al., 2014). Previous studies have shown that cooking and refrigerated storage promote protein oxidation in cooked pork patties (Ganhão et al., 2010; Kristensen & Purslow, 2001). Our findings indicate that CTE can inhibit protein oxidation and prevent the loss of thiol group during the heating process and the refrigerated storage. Phenolic compounds prevent protein oxidations by acting as antioxidants (Estévez & Cava, 2006; Salminen et al., 2006). Previous studies have reported the protective effect of phenolic compounds in edible plants against protein oxidation in several meat systems (Jia et al., 2012; Salminen et al., 2006; Shi et al., 2014). It is known that phenolic compounds protect protein oxidation by acting as metal chelating agents and free radical scavengers (Estévez & Cava, 2006). Our previous findings reported that CTE also markedly reduced the oxidation of protein by decreasing protein carbonyl content and preventing free thiol depletion against fructose-induced glycation in albumin (Chayaratanasin et al., 2015). The probable mechanism by which CTE exerts anti-glycation activity may be in association with antioxidant activity of phenolic compounds in the extract. Antioxidant activity of CTE in the present study may relate to phenolic compounds in the extract, which act as free radicals scavengers and thereby protect the depletion of thiol group and protein carbonyl formation.

Recent findings indicate that an increased oxidation of hemoglobin was associated with the discoloration of pork patties during the storage (Sánchez-Escalante et al., 2003). This might be a reason for the loss of red color in cooked pork patties. When compared on the initial day, the a* values of cooked pork patties containing CTE was lower than the control sample. However, the redness of samples with CTE (0.08% and 0.16%) was higher than that of control samples after 12 days of refrigerated storage. This is possibly due to the typical purple color of phytochemicals. These trends are similar to those observed in the studies of blackberry (Ganhão et al., 2010),

mustard leaf kimchi (Lee et al., 2011) and herbal medicines including clove, rosemary, cassia bark, liquorice, nutmeg, and ground cardamom (Kong et al., 2010).

Descriptive attribute sensory evaluation is a method of quantifying specific sensory attributes of meat sample. This use of structured line scaling has been the common used method of sensory evaluation in meat characteristic studies because it does not restrict panelists to categories, allows them to use the scale freely and different scale use by panelists (Kulkarni et al., 2011). Treatment and time had significant effects on sensory profiling ($P< 0.05$). Surface color of consumer preference were unaffected ($P>0.05$) by addition of CTE. These results indicate that these natural functional ingredients can be incorporated into pork patties without having a detrimental effect on product quality producing a healthy meat product. Previous study found that the addition of functional ingredients such as grape seed and green tea extracts to cooked beef patties also had no effect on sensory traits (Bañón et al., 2007). Juiciness, rancidity, and overall acceptability scores improved when compared to the control group during the storage. Furthermore, it was further observed that addition of CTE (0.02-0.16%) did not have any negative effect on sensory attributes of cooked pork patties. Thus, all the products were equally acceptable. However, the panelists indicated a relatively low incidence of scores in all cases (scores of 2.89–4.29 on a 9-point scale), and it is likely to be low acceptability especially at day 6 and 12 of storage. This is likely due to the change of meat quality that occurs at day 6 and 12 of storage. From this study, the decrease of meat perception was suggested to be caused partly by degradation of sulphur-containing amino acids as well as the increase in lipid oxidation products as reported by previous study (Kulkarni, et al., 2011).

Therefore, the findings of the inhibitory effects of CTE on lipid and protein oxidation in ready-to-eat meat product indicated that CTE has great potential as a natural antioxidant for producing functionally improved meat products. These results demonstrate the potential combined use of blue pea extract to the meat industry in the development of novel healthy meat products with improved shelf life of stored products without affecting other qualities and hence making the consumer healthier.

Although the scientific research on CT suggests a huge biological potential of this plant but the evidence is limited in respect to its bioactive secondary metabolites and bioaccessibility. This is because, many bioactive compounds must be released from the food matrix and modified in the gastrointestinal tract and absorbed in the intestinal tract to exert important of these beneficial effect (Wu et al., 2006). However, the studies indicate that the apparent bioavailability and the pH-dependent degradation of anthocyanins after oral consumption is very low compared with other polyphenolics and flavonoids may prevent sufficient concentrations of bioactive anthocyanins reaching target sites in the body (Manach et al., 2005; McGhie et al., 2003). The anthocyanins metabolism and bioavailability in foods and their biological activities have recently attracted scientific interest to assess their biological function and to know whether they reach the tissues where they could exert their activities. It is important to determine how this digestion process effects anthocyanins and their stability which will affect their biological activity.

CHAPTER VI

CONCLUSION

Antioxidant and biological activity of *Clitoria ternatea* (CT) petal flower may predominantly contribute from phenolic compositions, particularly anthocyanins. The results showed that CT extract after enzymatic hydrolysis of GI digestion increased total phenolic compounds and antioxidant activity concomitant with an increase in intestinal α-glucosidase (maltase and sucrase) and pancreatic α-amylase inhibitory activities as well as binding to bile acids. Moreover, CT extract delayed glucose absorption by inhibiting glucose uptake through sodium-glucose cotransporter-1 (SGLT1).

The present study demonstrates that the microencapsulation of CT provides good characteristics including a high percentage of encapsulation efficiency (84.83±0.40%), narrow size distribution (985 μM), spherical shape with smooth surface, desirable thermal stability (188°C) without a chemical interaction between the materials and CT extract. Interestingly, the microencapsulation of CT significantly retained higher amount of polyphenols and improved antioxidant and pancreatic α-amylase inhibitory activity as well as bile acid binding after the GI digestion.

The present results highlight the potential effect of application of CT extract to inhibit oxidative reactions in cooked pork patties during storage at 4°C for 12 days. In the roles of food additive in meat products, CT extract (0.08–0.16%) exhibited potent radical scavenging activity and retarded lipid and protein oxidations together with acceptable sensory characteristics in cooked pork patties during refrigerated storage. Therefore, the findings of this study suggest that CT extract, as a natural additive, can be applied to extend the shelf-life of cooked pork patties.

Therefore, CT extract and its microencapsulation could be considered as a potential source of bioactive ingredients for functional food industry.

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APPENDIX

1. Chemical preparations

1.1 Preparation of Phosphate buffer saline (0.1 M PBS)

Chemical (10x (0.1 M) PBS, 500 mL)

NaCl	43	g
Na_2HPO_4	11.62	g
NaH_2PO_4	2.71	g
Distilled water		

Method

Step1. Together weighted the Na_2HPO_4 and NaH_2PO_4 in the same beaker as well as dissolved the solution with distilled water.

Step2. NaCl was dissolved in another beaker with distilled water.

Step3. Pooled the solution in the beaker and added distilled water approximately adjusting at 400 mL.

Step4. Magnetic stirrer was used for dissolved it well.

Step5. The buffer was adjusted pH at 7.4.

Step6. Volumetric flask was used to adjusted the volume at 500 mL

Step7. The solution was diluted 10-folds before used. In order to prepare 1x PBS (0.1 M PBS) (1000 mL), mixed the solution in this ratio distilled water: 10x PBS = 900: 100 mL. After homogeneous, kept the solution at the room temperature in Duran bottle.

1.2 Preparation of Folin-Ciocalteu reagent

Chemical

10x Folin-Ciocalteu reagent	1	mL
Distilled water	9	mL

Method

Step1. 10 x Folin-Ciocalteu reagent (1 mL) was diluted by 9 mL distilled water adjusting the volume in volumetric flask 10 mL.

Step2. The solution was inversed 2-3 times for well dissolving.

*The reagent should keep in darkness at room temperature before used

1.3 Preparation of 10% Na_2CO_3

Chemical

Na_2CO_3	2.5	g
Distilled water	25	mL

Method

Step1. Na_2CO_3 (2.5 g) was weighted on the weighting paper and dissolved with 25 mL distilled water in volumetric flask reach to 25 mL.

Step2. Magnetic stirrer was used for dissolved the solution well.

1.4 Preparation of gallic acid as a standard

Chemical (stock concentration)

Gallic acid	1	mg
Distilled water	1	mL

Method

Step1. Gallic acid (1 mg) was weighted on the weighting paper and dissolved with 1 mL distilled water in micro centrifuge tube 1 mL.

Step2. Vortex was used to mix the solution clearly.

1.5 Preparation of 0.3 M Sodium acetate buffer (pH 3.6)

Chemical

Sodium acetate trihydrate (MW=136.08)	0.3	g
Acetic acid	1.6	mL
Distilled water	100	mL

Method

Step1. Sodium acetate trihydrate (0.3 g) was weighted on the weighting paper and add 1.6 mL acetic acid and distilled water adjusting the volume in volumetric flask 100 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

1.6 Preparation of 10 mM TPTZ ใน 40 mM HCl

Chemical

2,4,6-Tripyridyl-s-triazine or TPTZ (MW=312.33)	31.233	mg
40 mM HCl	10	mL

Method

Step1. TPTZ (31.233 mg) was weight on the weighting paper and dissolved with 40 mM HCl in volumetric flask reach to 10 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

1.7 Preparation of 20 mM $FeCl_3$

Chemical

$FeCl_3$ (MW=162.2)	32.44	mg
Distilled water	10	mL

Method

Step1. $FeCl_3$ (32.44 mg) was weight on the weighting paper and dissolved with distilled water in volumetric flask reach to 10 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

1.8 Preparation of 2 mM $FeSO_4$ as a standard

Chemical (stock concentration)

$FeSO_4$ (MW=278.01)	5.56	mg
Distilled water	10	mL

Method

Step1. $FeSO_4$ (5.56 mg) was weight on the weighting paper and dissolved with distilled water in volumetric flask reach to 10 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

1.9 Preparation of 7 mM ABTS

Chemical

ABTS (diammonium salt; M_w 514.62)	38.407	mg
PBS	10	mL

Method

Step1. ABTS (38.407 mg) was weight on the weighting paper and dissolved with PBS pH 7.4 in volumetric flask reach to 10 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

1.10 Preparation of 2.45mM $K_2S_2O_4$

Chemical

$K_2S_2O_4$ (Potassium persulfate; M_w 220.322)	6.62	mg
Distilled water	10	mL

Method

Step1. $K_2S_2O_4$ (6.62 mg) was weight on the weighting paper and dissolved with distilled water in volumetric flask reach to 10 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

1.11 Preparation of trolox as a standard

Chemical (stock concentration)

Trolox	1	mg
PBS	1	mL

Method

Step1. Trolox (1 mg) was weighted on the weighting paper and dissolved with 1 mL PBS pH 7.4 in micro centrifuge tube 1 mL.

Step2. Vortex was used to mix the solution clearly.

1.12 Preparation of 6 M DTNB reagent

Chemical

DTNB	118.84	mg
0.1 M PBS pH 7.4	50	mL

Method

Step1. DTNB (118.84 mg) was weight on the weighting paper and dissolved with PBS pH 7.4 in volumetric flask reach to 50 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

1.13 Preparation of L-cysteine

Chemical (1,000,000x L-cysteine)

L-cysteine	12	mg
0.1 M PBS pH 7.4	10	mL

Method

Step1. L-cysteine (12 mg) was weight on the weighting paper and dissolved with PBS pH 7.4 in volumetric flask reach to 10 mL.

Step2. The solution was diluted from 1,000,000X to 10,000x.

Step3. Then, 2-fold dilution was started from

10,000x → 5,000x → 2,500x →1,250x → 625x→ 0

The final concentration was in the range from 0 -10 µM.

1.14 Preparation of 2.5 M HCl

Chemical

11.45 M Conc. HCl	218.32	mL
Distilled water		

Method

Step1. Distilled water was poured in the cylinder 1,000 mL

Step2. HCl (218.32 mL) was added into the water and did in the laboratory cabinet.

Step3. The volumetric flask was used for adjust the volume at 1,000 mL

1.15 Preparation of 10 mM DNPH reagent

Chemical

10 mM DNPH	495.3	mg
2.5 M HCl	250	mL

Method

Step1. DNPH (495.3 mg) was weight on the weighting paper and dissolved with 2.5 M HCl in volumetric flask reach to 250 mL.

Step2 Magnetic stirrer was used to clearly dissolve.

*The powder of DNPH was quite difficultly dissolved. Thus, DNPH were pulverized before used.

1.16 Preparation of 20% (w/v) TCA

Chemical

TCA	50	g
Distilled water		

Method

Step1. TCA (50 g) was weighted on the weighting paper and dissolved with distilled water in volumetric flask reach to 250 mL.

Step2. Magnetic stirrer was used to clearly dissolve.

1.17 Preparation of *Clitoria ternatea* solution for HPLC

Chemical

***Clitoria ternatea* petal flower extracts (CTE)**	**2**	**mg**
Methanol (HPLC grade)	**1**	**mL**

Method

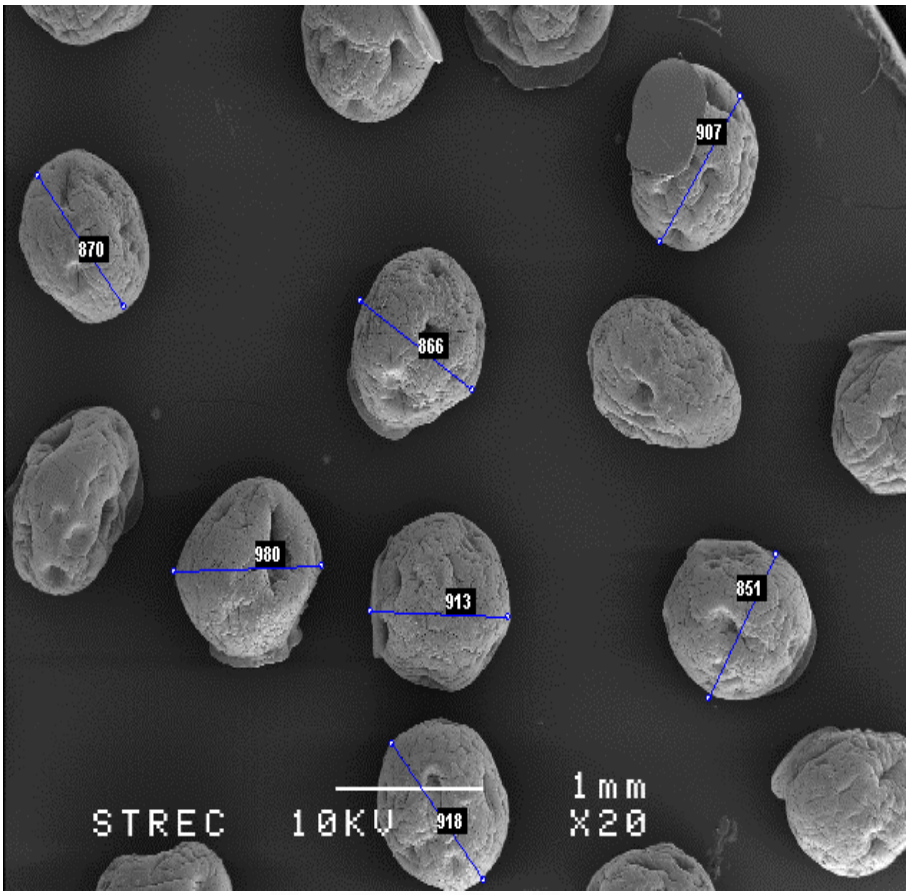
Step1. CTE (2 mg) was weighted in micro centrifuge tube and dissolved with 1 mL of methanol.

Step2. Mixed the solution by using vortex.

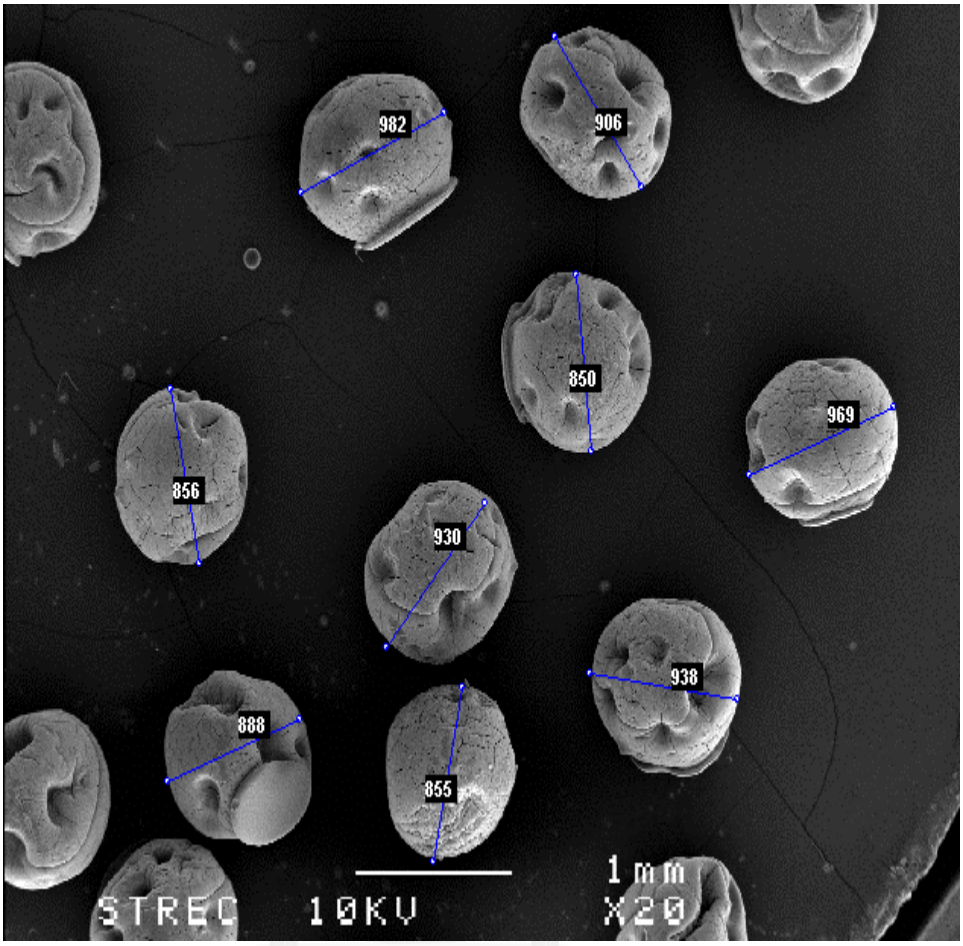
Step3. PES syringe filter was used to filter the extract solution into the amber.

2. Bead size screening by SEM study

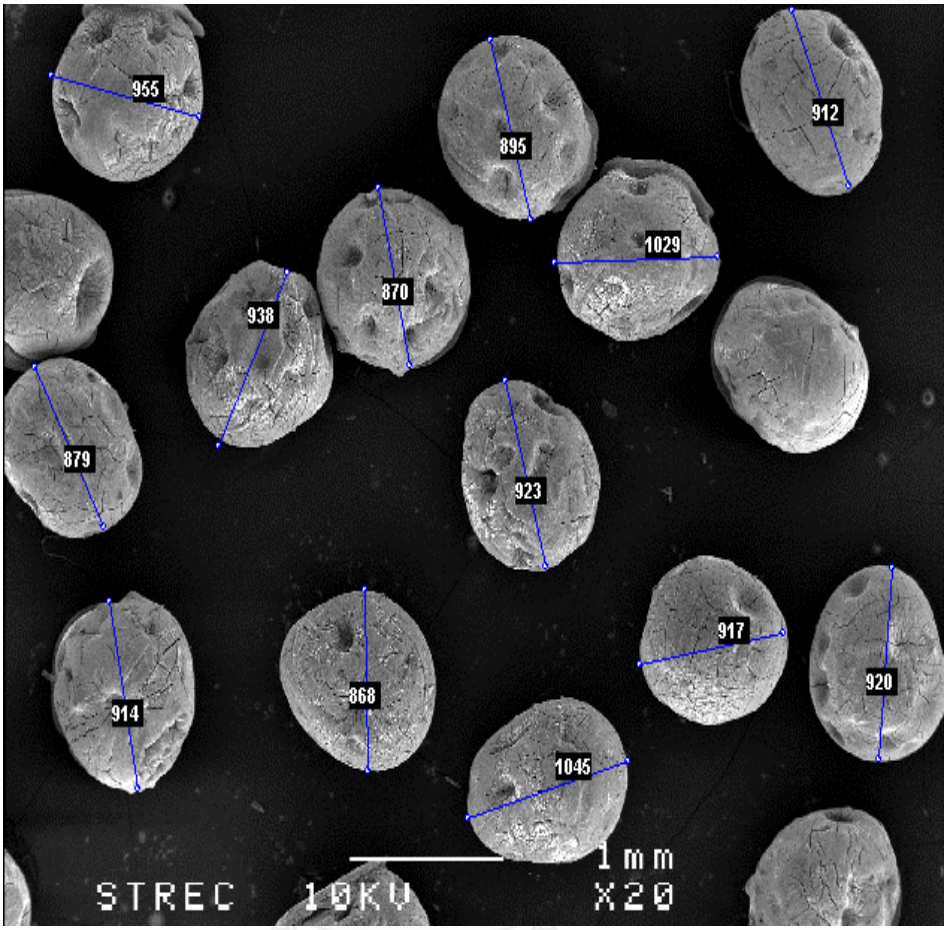
- 1.5% (w/v) Alginate: 1.5% (w/v) $CaCl_2$



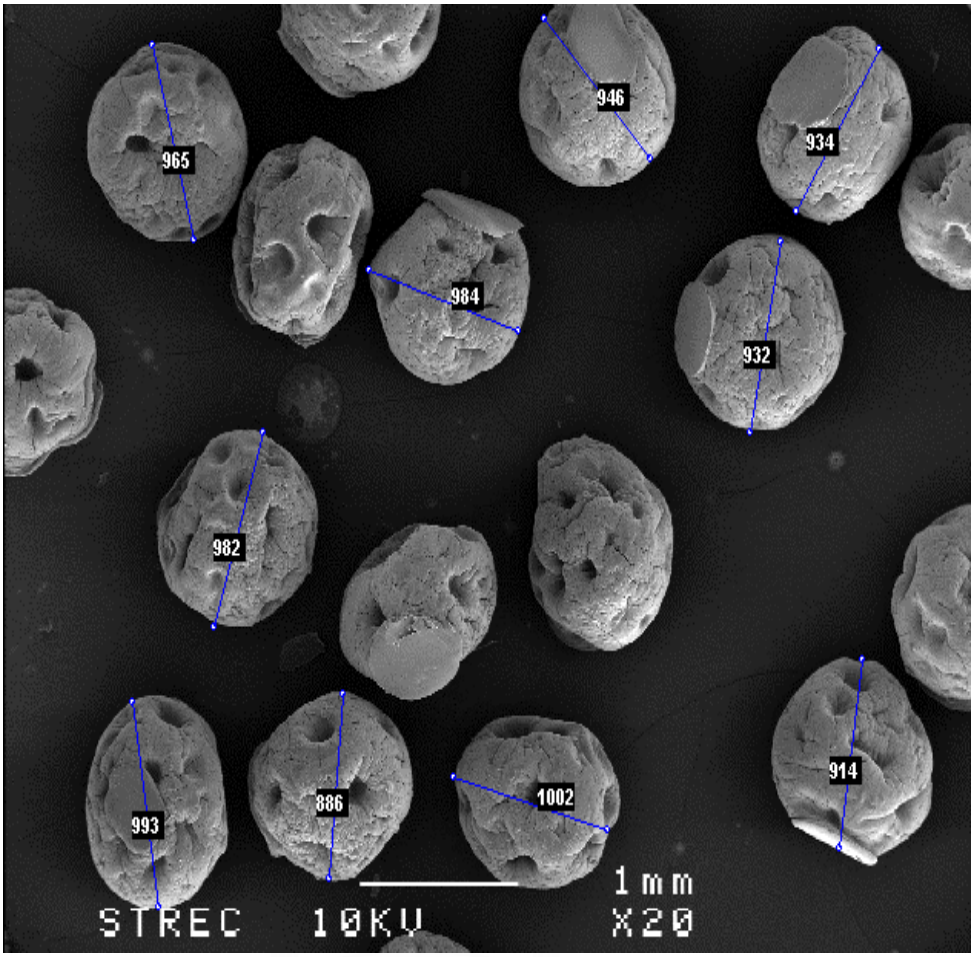
- CT: 1.5% (w/v) Alginate: 3% (w/v) $CaCl_2$



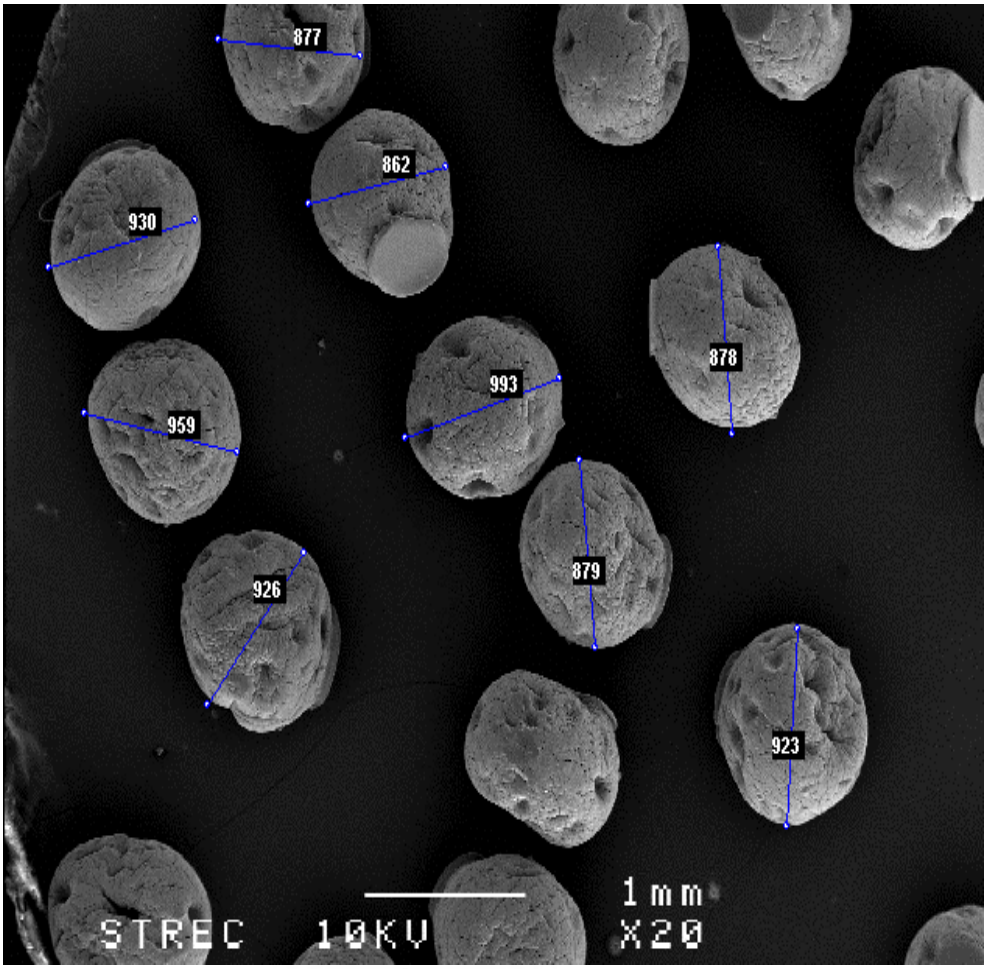
- 2% (w/v) Alginate: 1.5% (w/v) $CaCl_2$



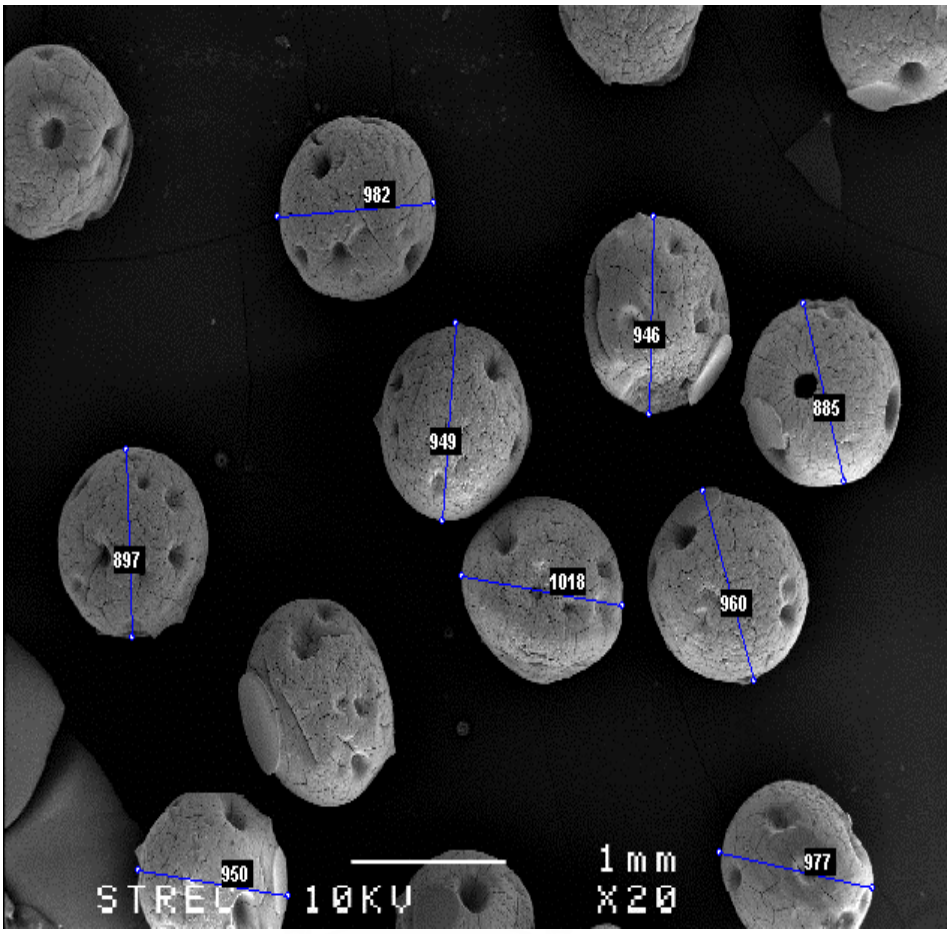
- 2% (w/v) Alginate: 3% (w/v) $CaCl_2$



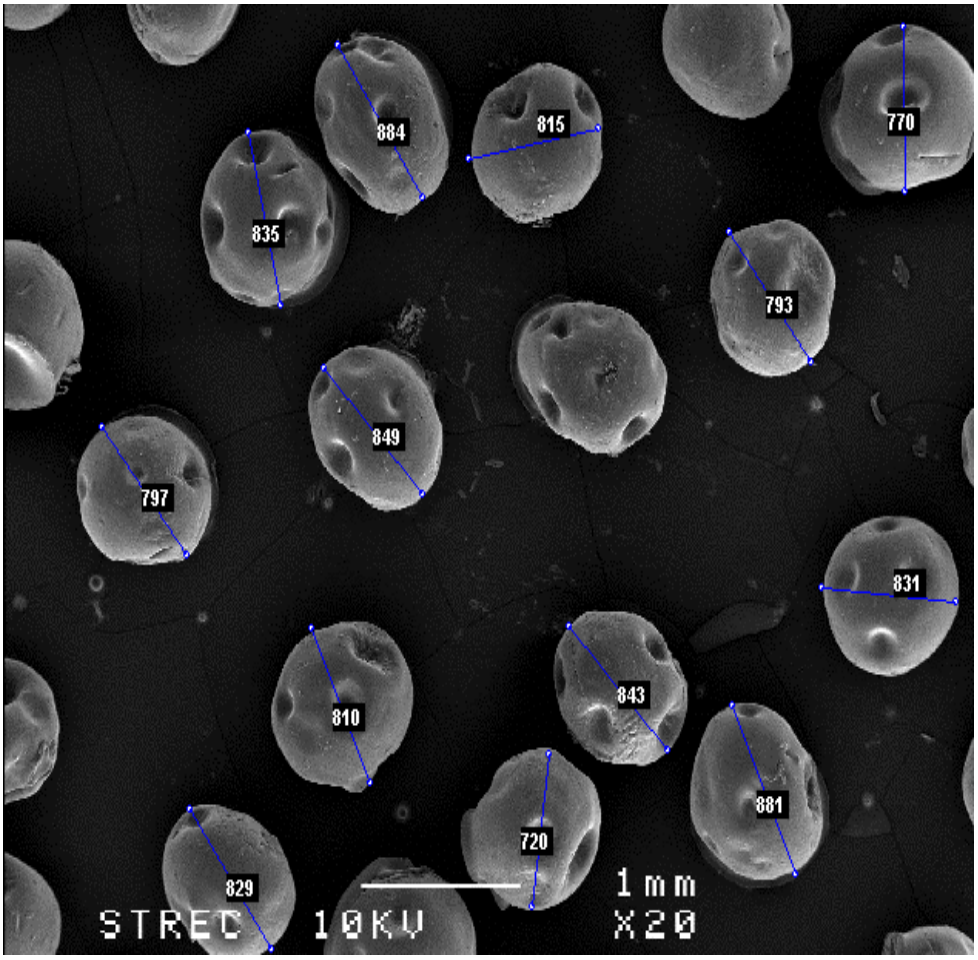
- 5% (w/v) CT: 1.5% (w/v) Alginate: 1.5% (w/v) $CaCl_2$



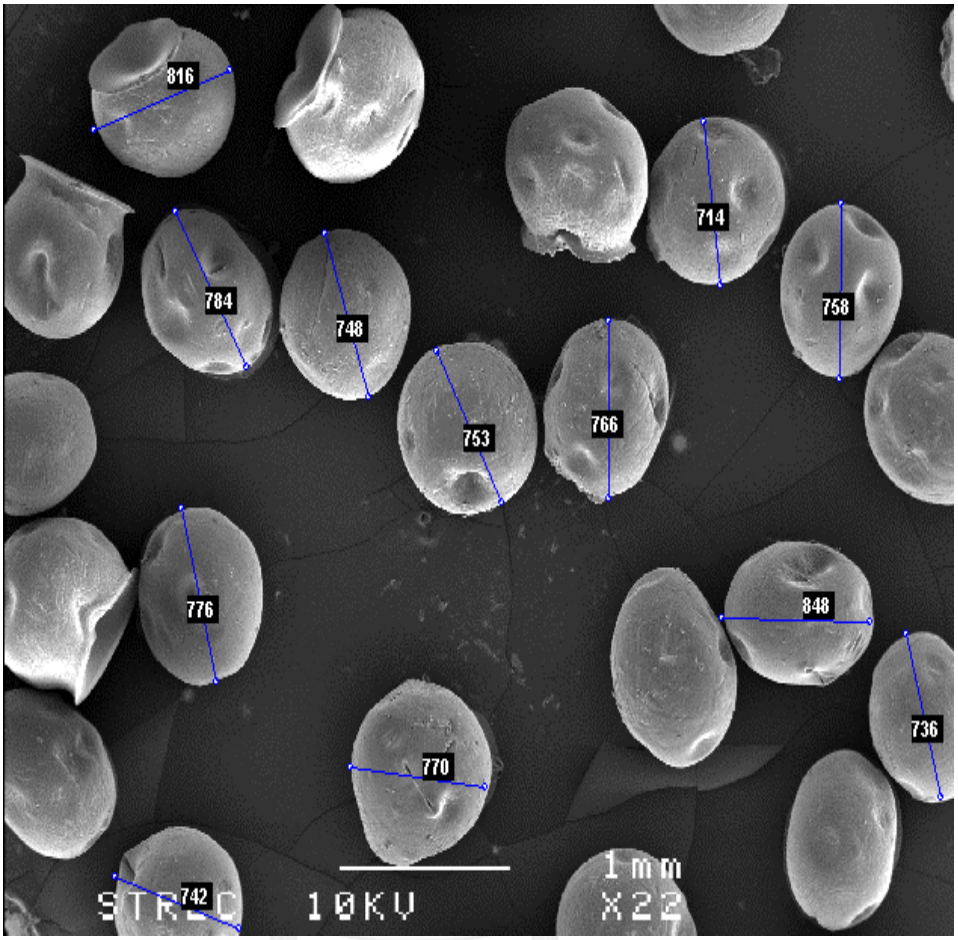
- 5% (w/v) CT: 1.5% (w/v) Alginate: 3% (w/v) $CaCl_2$



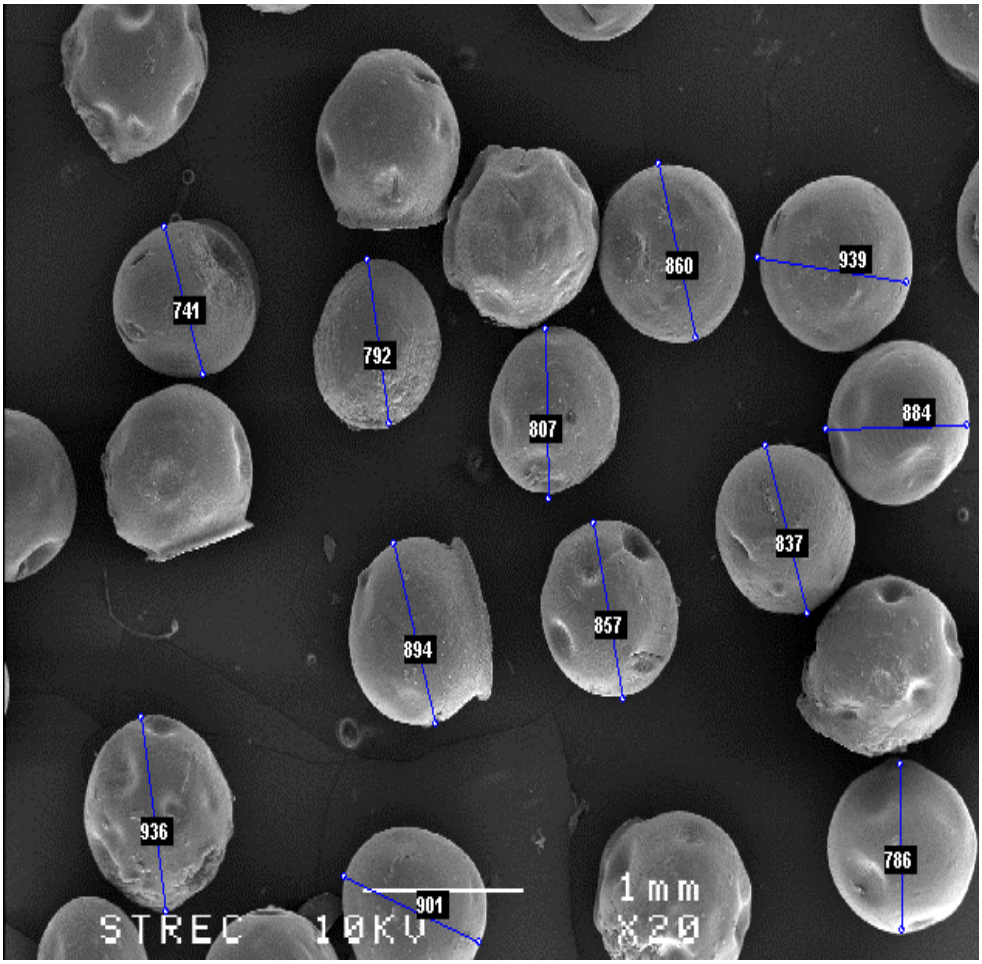
- 5% (w/v) CT: 2% (w/v) Alginate: 1.5% (w/v) $CaCl_2$



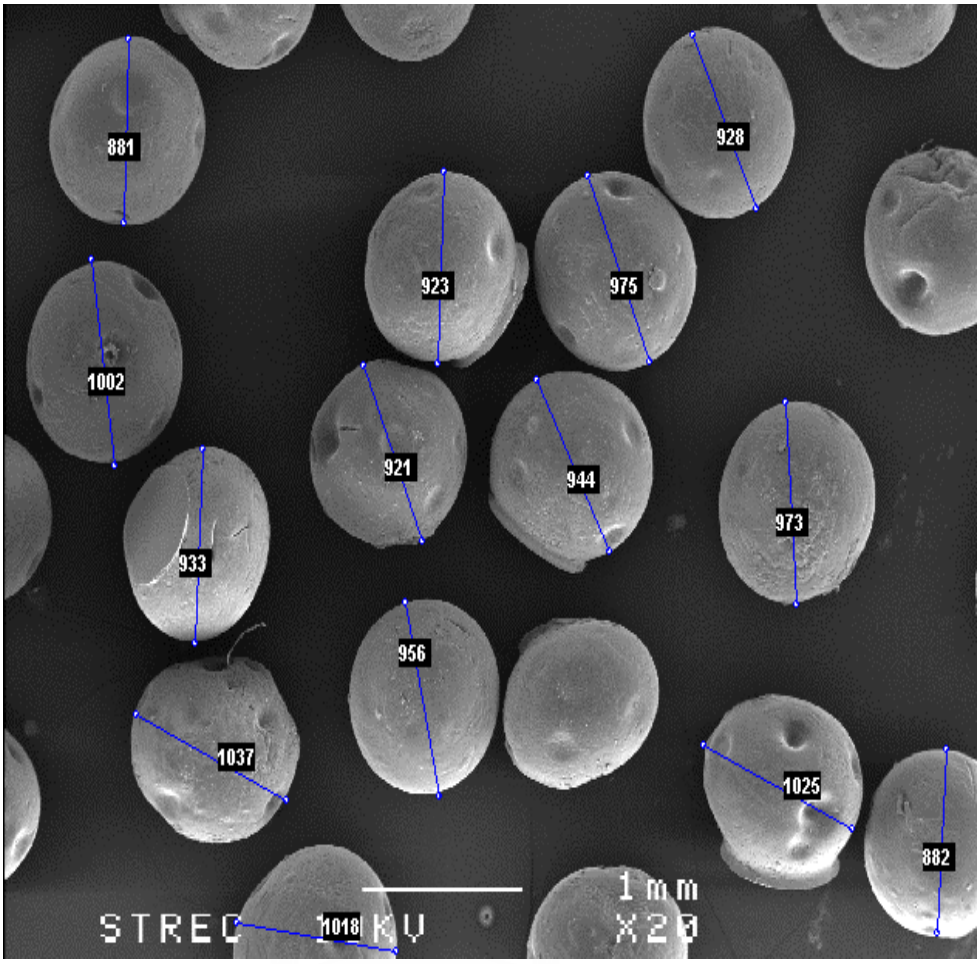
- 5% (w/v) CT: 2% (w/v) Alginate: 3% (w/v) $CaCl_2$



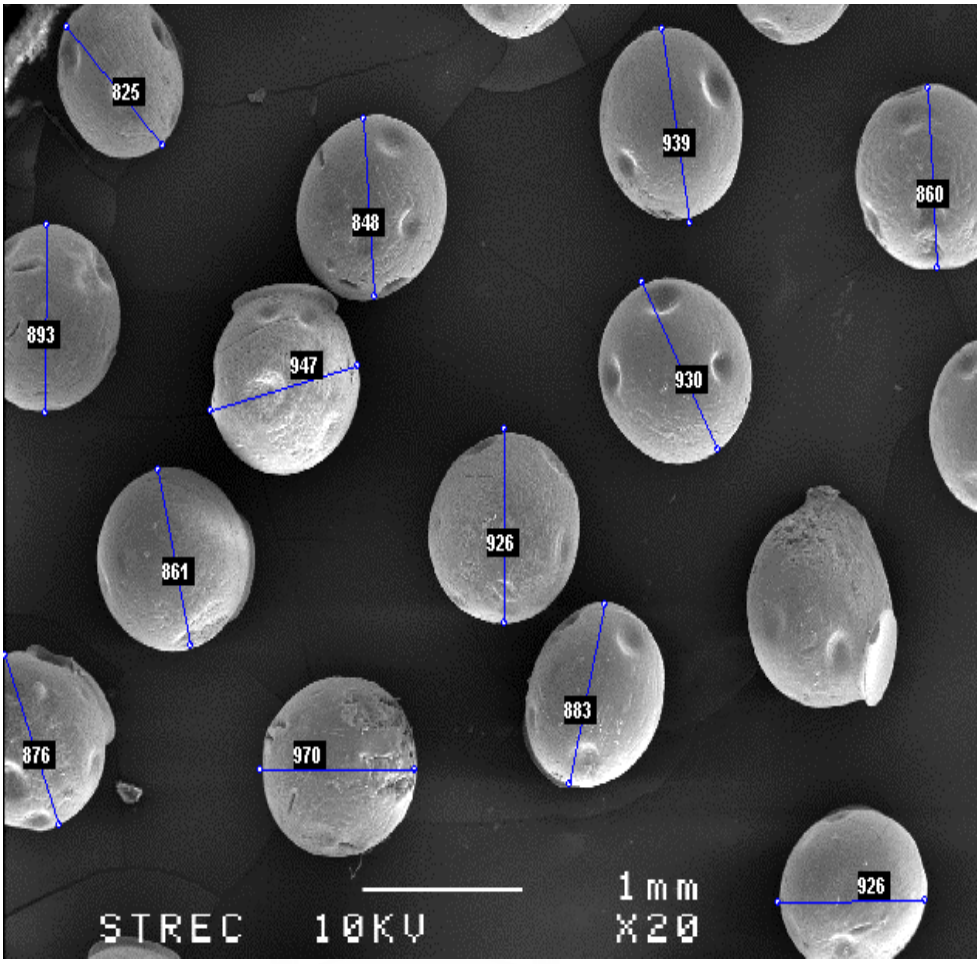
- 10% (w/v) CT: 1.5% (w/v) Alginate: 1.5% (w/v) $CaCl_2$



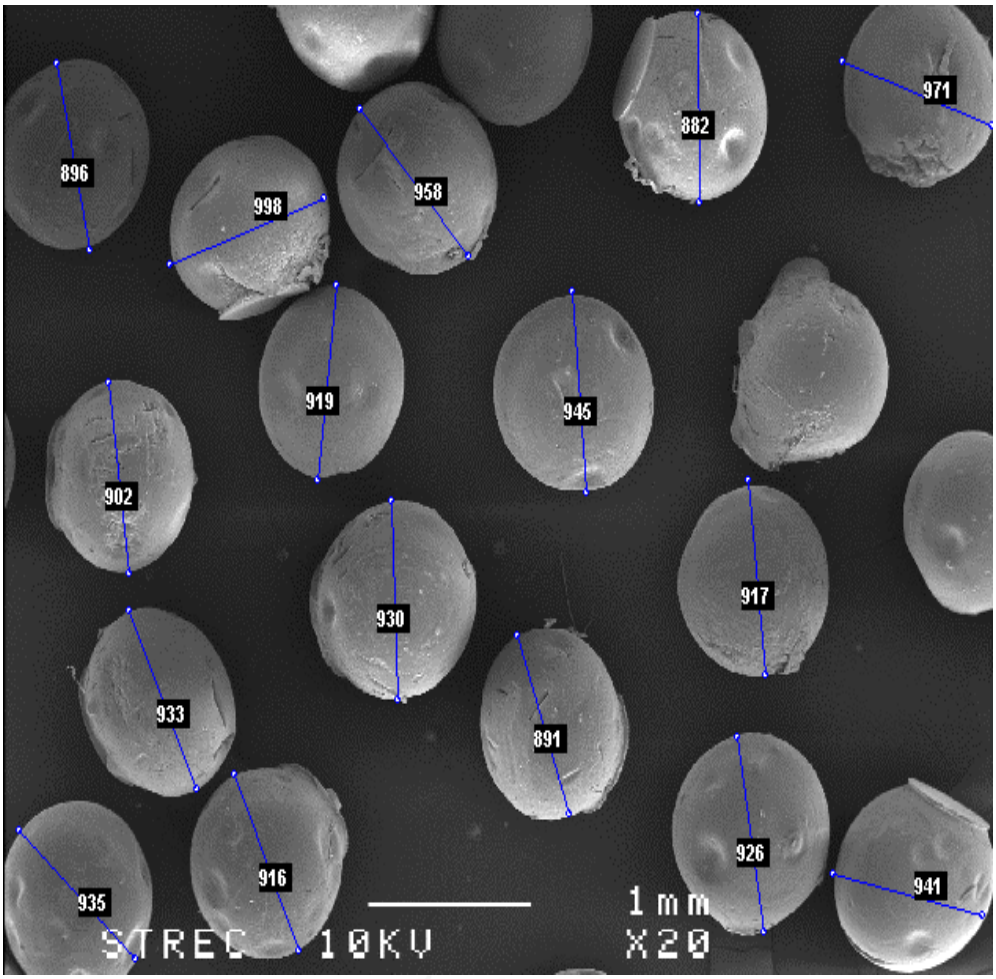
- 10% (w/v) CT: 1.5% (w/v) Alginate: 3% (w/v) $CaCl_2$



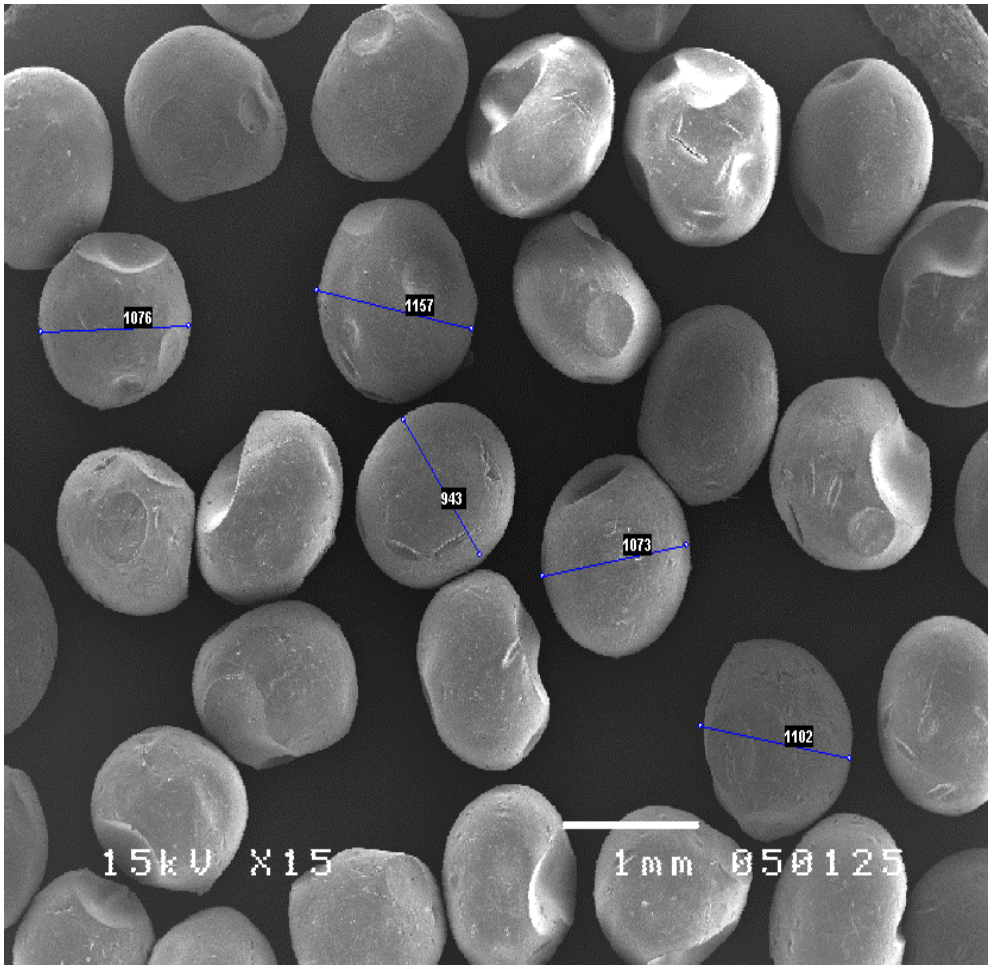
- 10% (w/v) CT: 2% (w/v) Alginate: 1.5% (w/v) $CaCl_2$



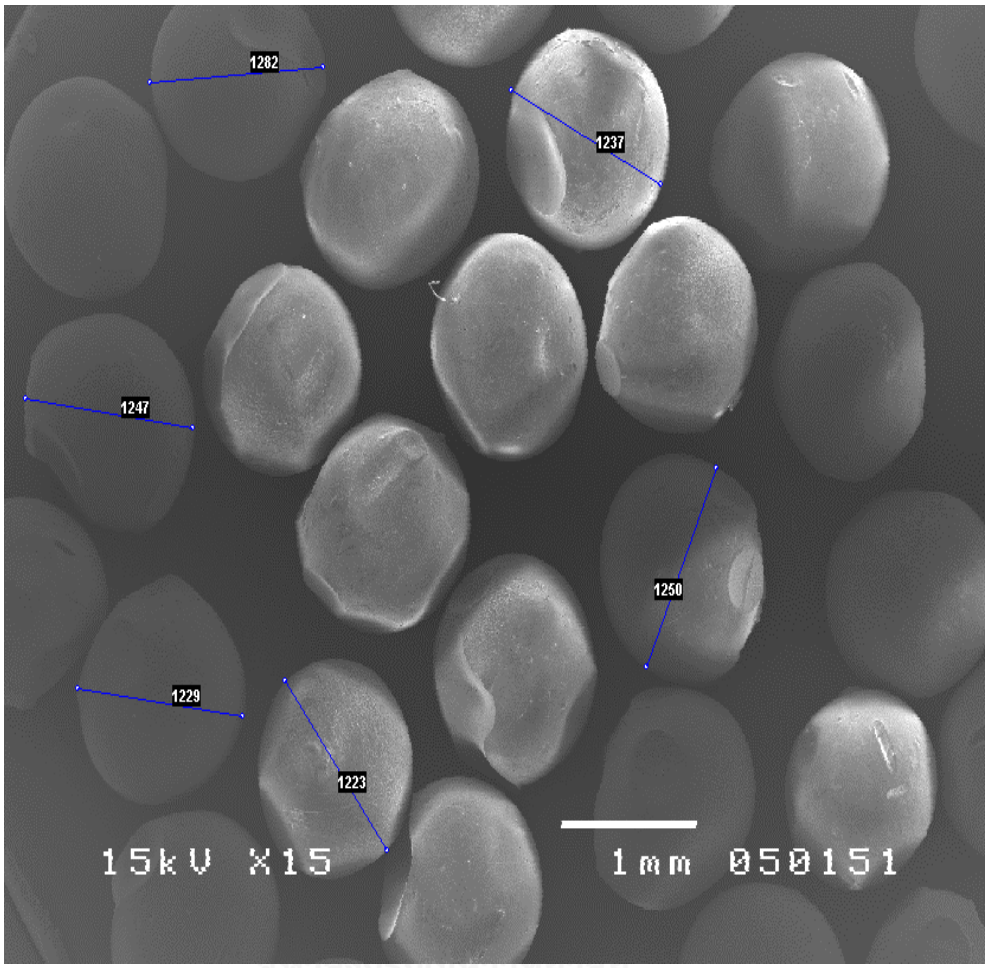
- 10% (w/v) CT: 2% (w/v) Alginate: 3% (w/v) $CaCl_2$



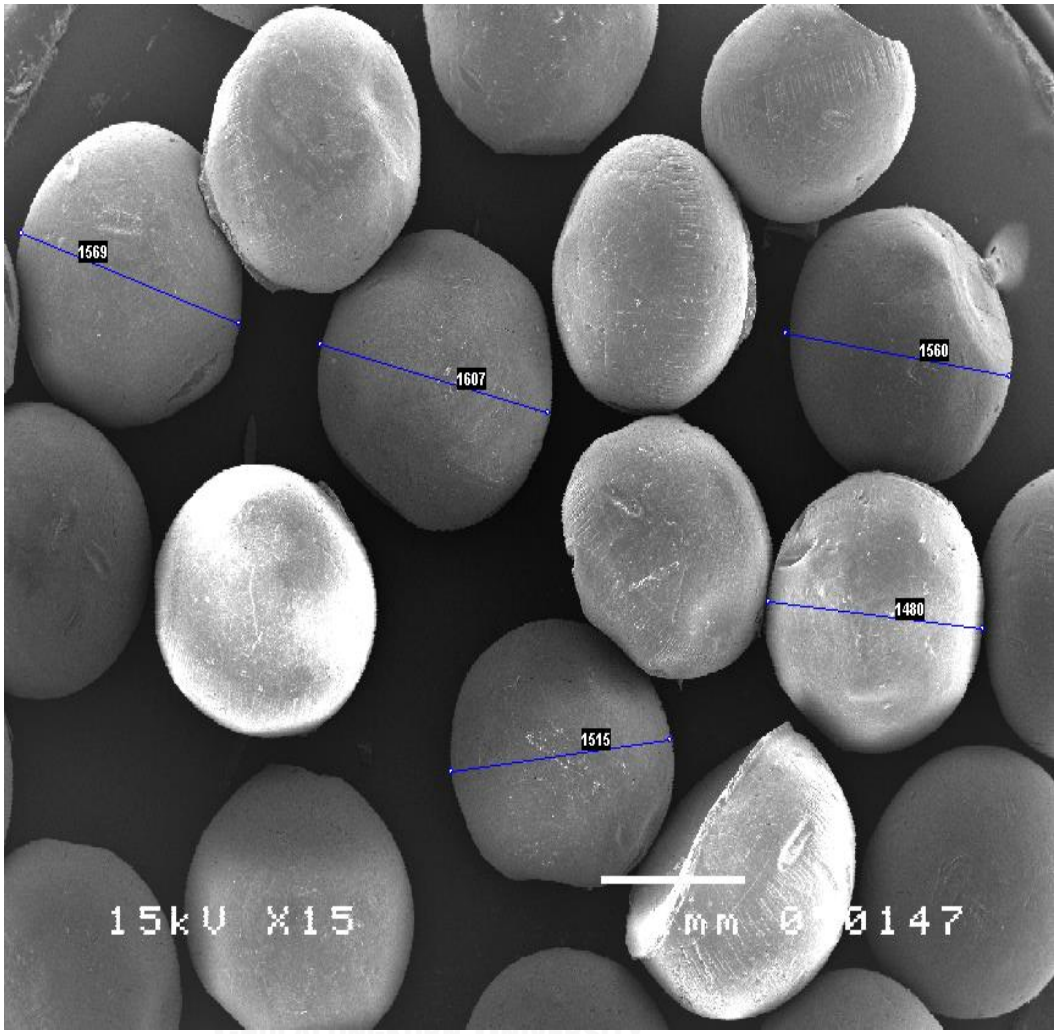
- 20% (w/v) CT: 1.5% (w/v) Alginate: 1.5% (w/v) $CaCl_2$



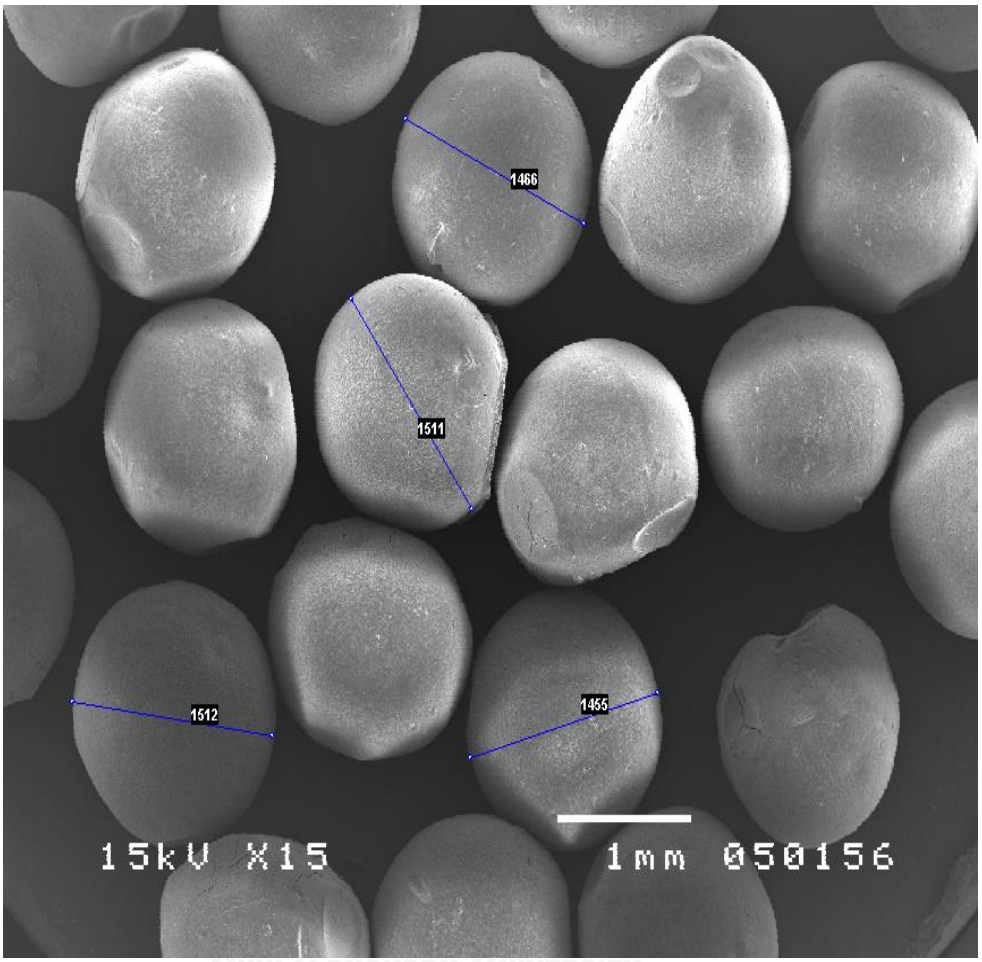
- 20% (w/v) CT: 1.5% (w/v) Alginate: 3% (w/v) $CaCl_2$



- 20% (w/v) CT: 2% (w/v) Alginate: 1.5% (w/v) $CaCl_2$



- 20% (w/v) CT: 2% (w/v) Alginate: 3% (w/v) $CaCl_2$



VITA

Porntip Pasukamonset was born in December 9th, 1982 in Songkla, Thailand. After graduating undergraduate degree in 2006 in Food and Nutrition, she attended Postgraduate Course in Nutrition and Dietetics, Faculty of Science, Medicine and Health, University of Wollongong, Australia. She achieved a master's degree in Nutrition and Dietetics in year 2009. After that she desired to continue her study. Thus, she enrolled in a doctoral course in the field of Food and Nutrition at Faculty of Allied Health Sciences, Chulalongkorn University in the department of Nutrition and dietetics. Her research focused on bioactive compounds in Clitoria ternatea and bioactivity, digestive stability and bioaccessibility for food applications. Her thesis advisor was Associate Professor Sirichai Adisakwattana.