สารพฤกษเคมีและฤทธิ์ทางชีวภาพของสารสกัดกระบองเพชร (*Echinocactus grusonii*)

นางสาวนิภา ชัยเจริญอุดมรุ่ง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีอาหาร
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PHYTOCHEMICAL PROFILE AND BIOACTIVITIES OF *ECHINOCACTUS GRUSONII* EXTRACT

Nipha Chaicharoenaudomrung

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Food Technology

Suranaree University of Technology

Academic Year 2014

PHYTOCHEMICAL PROFILE AND BIOACTIVITIES OF *ECHINOCACTUS GRUSONII* EXTRACT

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

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นิภา ชัยเจริญอุดมรุ่ง : สารพฤกษเคมีและฤทธิ์ทางชีวภาพของสารสกัดกระบองเพชร (*Echinocactus grusonii*) (PHYTOCHEMICAL PROFILE AND BIOACTIVITIES OF *ECHINOCACTUS GRUSONII* EXTRACT) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รัชฎาพร อุ่นศิวิไลย์, 112 หน้า.

วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาและเปรียบเทียบสารพฤกษเคมี คุณสมบัติทางด้านพฤกษเคมีของสารสกัดกระบองเพชรที่อายุ 3 และ 6 ปี จากการตรวจสอบสารพฤกษเคมีของสารสกัดกระบองเพชรทั้งสองช่วงอายุ พบว่าสารสกัดกระบองเพชรมีสารพฤกษเคมีคือ ลูทีน คลอโรฟิลล์เอ คลอโรฟิลล์บี ฟีโอไฟตินเอ ฟีโอไฟตินบี และฟีนอลิกทั้งหมด ผลการตรวจสอบคุณสมบัติการต้านอนุมูลอิสระโดยวิธี DPPH และ ABTS พบว่า สารสกัดกระบองเพชรที่อายุ 3 ปี มีค่า IC_{50} เท่ากับ 112.60 และ 44.62 มิลลิกรัม/มิลลิลิตร ในขณะที่สารสกัดกระบองเพชรที่อายุ 6 ปี มีค่า IC_{50} เท่ากับ 191.90 และ 81.84 มิลลิกรัม/มิลลิลิตร นอกจากนี้การทดสอบด้วยวิธี FRAP สารสกัดกระบองเพชรที่อายุ 3 และ 6 ปี มีค่าเท่ากับ 0.014 และ 0.010 มิลลิโมลเฟอร์รัส/กรัมกระบองเพชรผง ปริมาณของลูทีน คลอโรฟิลล์ทั้งหมด ฟีนอลิกทั้งหมด และกิจกรรมการต้านอนุมูลอิสระของสารสกัดกระบองเพชรที่อายุ 3 ปี มีค่าสูงกว่าอายุ 6 ปี

ส่วนการทดสอบความเป็นพิษของสารสกัดกระบองเพชรก่อนและหลังผ่านแบบจำลองการย่อยอาหารพบว่ามีค่า LC_{50} มากกว่า 200 ไมโครกรัม/มิลลิลิตร ชี้ให้เห็นว่าไม่มีความเป็นพิษในเซลล์ไลน์ Caco-2 และ HepG2 และจากการทดสอบความคงตัวต่อการย่อยของลูทีน คลอโรฟิลล์-ทั้งหมด และฟีนอลิกทั้งหมดในสารสกัดกระบองเพชรที่อายุ 3 ปี พบว่ามีความคงตัวต่อการย่อยเท่ากับ 69.03% 37.64% และ 60.52% ในสารสกัดกระบองเพชรที่อายุ 6 ปี เท่ากับ 58.33% 33.34% และ 56.89% บ่งชี้ว่า ลูทีน คลอโรฟิลล์ทั้งหมด ฟีนอลิกทั้งหมดไม่เสถียรเมื่อผ่านระบบจำลองการย่อยอาหารที่กระเพาะและลำไส้เล็ก จากนั้นทำการศึกษาการดูดซึมเข้าสู่เซลล์ไลน์ Caco-2 ของลูทีน คลอโรฟิลล์ทั้งหมด และฟีนอลิกทั้งหมด พบว่า ลูทีน คลอโรฟิลล์ทั้งหมด และฟีนอลิกทั้งหมดในสารสกัดกระบองเพชรที่อายุ 3 ปี ถูกดูดซึมเข้าไปในเซลล์ไลน์ Caco-2 ที่ระดับร้อยละ 30.63 36.88 และ 28.27 ตามลำดับ ในสารสกัดกระบองเพชรที่อายุ 6 ปี ที่ระดับร้อยละ 26.31 28.10 และ 25.11 ตามลำดับ ในลำดับสุดท้าย การศึกษาการขนส่งผ่านเซลล์พบว่า ลูทีน และฟีนอลิกทั้งหมดถูกขนส่งผ่านเซลล์ไลน์ Caco-2 ที่ระดับร้อยละ 8.05 9.18 และ 7.67 6.95 สำหรับสารสกัดกระบองเพชรที่อายุ 3 และ 6 ปี ตามลำดับ แต่ไม่สามารถตรวจพบการขนส่งผ่านเซลล์ไลน์ Caco-2 ของคลอโรฟิลล์-ทั้งหมด สารสกัดกระบองเพชรที่อายุ 3 ปี ซึ่งมีปริมาณสารพฤกษเคมีและฤทธิ์ทางชีวภาพสูงกว่าอายุ 6 ปี ชี้ให้เห็นความสำคัญของอายุพืช และพบว่า ลูทีน ฟีนอลิกทั้งหมดในสารสกัดกระบองเพชร

สามารถถูกดูดซึมและขนส่งผ่านเซลล์ Caco-2 ได้ แต่ไม่สามารถตรวจพบการขนส่งคลอโรฟิลล์ผ่านเซลล์ลำไส้เล็ก

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา_____

NIPHA CHAICHAROENAUDOMRUNG : PHYTOCHEMICAL PROFILE AND BIOACTIVITIES OF *ECHINOCACTUS GRUSONII* EXTRACT. THESIS ADVISOR : ASST. PROF. RATCHADAPORN OONSIVILAI, Ph.D., 112 PP.

ECHINOCACTUS GRUSONII/PHYTOCHEMICAL/BIOACTIVITIES

The objectives of this study were to investigate the phytochemical properties of 3-year-old golden barrel cactus extracts were compared with 6-year-old cactus extracts. Phytochemical analyses of both cactus extracts revealed the presence of lutein, chlorophyll *a*, chlorophyll *b*, pheophytin *a*, pheophytin *b* and phenolic compounds. The 3-year-old golden barrel cactus extracts showed the IC_{50} values of 112.60 and 44.62 mg raw material (RM)/ml, while the 6-year-old golden barrel cactus extracts showed 191.90 and 81.84 mg RM/ml for DPPH and ABTS assay, respectively. In addition, their antioxidant activity by FRAP assay showed the value at 0.014 and 0.01 mmol Fe^{2+}/g RM for 3- and 6-year-old golden barrel cactus extracts, respectively. Lutein, total chlorophylls, total phenolic and antioxidant activity of the 3-year-old golden barrel cactus extracts were higher than that of 6-year-old extracts.

Cytotoxicity of golden barrel cactus extracts before and after *in vitro* digestion exhibited extremely high value of LC_{50} (>200 µg RM/ml) against Caco-2 and HepG2 cells indicating the non-toxic activity to the cells. The digestive stability of lutein, chlorophylls and phenolic compounds of 3- and 6-year-old golden barrel cactus extracts was 69.03%, 37.64%, 60.52% and 58.33%, 33.34%, 56.89%, respectively. This indicated that the lutein, chlorophylls and phenolics were not stable during simulated gastric and small intestinal digestion. Additionally, the lutein, chlorophylls

and phenolics from 3- and 6-year-old golden barrel cactus extracts were uptaked by Caco-2 cells at the level of 30.63%, 36.88%, 28.27% and 26.31%, 28.10%, 25.11%, respectively. Finally, the investigations of cellular lutein and phenolics transport in Caco-2 cells were 8.05%, 9.18% and 7.67%, 6.95% for 3- and 6-year-old golden barrel cactus extracts, respectively. The chlorophylls transported through Caco-2 cells could not be detected. Phytochemical content and bioactivities of 3-year-old golden barrel cactus extracts being higher than that of 6-year-old cactus extracts indicated the importance of plant maturity. In addition, lutein and total phenolic compounds from golden barrel cactus extracts could be absorbed and transported through Caco-2 cells, but chlorophylls could not be detected in the transport process.

School of Food Technology Student's Signature_____

Academic Year 2014 Advisor's Signature_____

ACKNOWLEDGEMENTS

Completion of this master thesis was possible with the support of several people. I would like to express my sincere gratitude to all of them. I would like to thank Suranaree University of Technology for financial and laboratory facilities supports during my study.

I would like to express my sincere thanks to my thesis advisor, Asst. Prof. Dr. Ratchadaporn Oonsivilai for her invaluable help and constant encouragement throughout the course of this research. I am most grateful for her teaching and advice, not only the research methodologies but also many other methodologies in life. I would not have achieved this far and this thesis would not have been completed without all the support that I have always received from her.

In addition, I am grateful for the lecture at School of Food Technology: Assoc. Prof. Dr. Jirawat Yongsawatdigul, Asst. Prof. Dr. Suwayd Ningsanond, Asst. Prof. Dr. Piyawan Gasaluck, Asst. Prof. Dr. Sunanta Tongta, Asst. Prof. Dr. Siwatt Thaiudom, Assoc. Prof. Dr. Manote Sutheerawattananonda and Dr. Thanawit Kulrattanarak for suggestions and all their help.

I would like to express my gratitude to the dissertation committee, Assoc. Prof. Dr. Jirawat Yongsawatdigul, Asst. Prof. Napron Uengarporn, MD. for their invaluable advice and comments.

Finally, I would like to express my deepest gratitude to my parents, my uncle and my brother for all their support throughout the period of this research..

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LIST OF ABBREVIATIONS

%	=	Percentage
ABTS	=	2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic
BHT	=	Butylated hydroxytoluene
°C	=	Degree Celsius
DPPH	=	1,1-diphenyl-2-picrylhy-drazyl
HPLC	=	High performance liquid chromatography
h	=	Hour
IC_{50}	=	Half maximal "Inhibitory Concentration"
LC_{50}	=	Lethal Concentration 50, the concentration of a given agent which is lethal to 50% of the cells
(m, μ) g	=	(milli, micro) Gram
(m, μ) mol	=	(milli, micro) Mole
(m, μ) l	=	(milli, micro) Liter
M	=	Molar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
min	=	Minute
PBS	=	Phosphate buffered saline
rpm	=	Revolution per minute
RM	=	Raw material

CHAPTER I

INTRODUCTION

1.1 Rational and background

Nowadays, international scientists have studied the cactus extract for different phytochemicals that benefit to human health. It also has applications in medical and a valuable health supporting nutrient (Shetty, Rana, and Preetham, 2012). For example, De Leo, De Abreu, Pawlowska, Cioni, and Braca (2010) studied phytochemicals of *Opuntia ficus-indica* in the *Cactaceae* family which is a native plant in Mexico and also found in widespread throughout Central and South America, Australia, South Africa and the whole Mediterranean area. Moreover, it could grow in all of the semiarid countries mainly cultivated for its fruit (prickly pear) that is sweet, juicy, and rich in nutritional compounds such as ascorbic acid and polyphenols. The fruit has been shown to have several beneficial effects in term of antiulcerogenic (Galati et al., 2003), antioxidant (Galati et al., 2003; Kuti, 2004; Tesoriere, Butera, Pintaudi, Allegra, and Livrea, 2004), anticancer (Zou et al., 2005), and neuroprotective (Dok-Go et al., 2003). Moreover, prickly pear has potential for treatment of gastritis, hyperglycemia, arteriosclerosis, diabetes, and prostate hypertrophy (Agozzino, Avellone, Caraulo, Ferrugia, and Filizzola, 2005). In addition, Galati et al. (2007) found that gel extracted from *Opuntia ficus-indica* could coat stomach and prevent peptic ulcer from alcohol induced in laboratory animals.

Hernández-Urbiola et al. (2010) studied the nutritional composition of Nopal (*Opuntia ficus-indica*, cv. Redonda) at different maturity stages. The results show that older nopal is an important source of calcium and dietary fiber. Nopal can be an economic alternative for use as food supplement mainly at advanced maturation stage i.e. at 135 days and can be ameliorate or prevent the chronic and degenerative disease. Moreover, Peyote (*Lophophora williamsii*) extracts associated with stimulating the central nervous system and regulating blood pressure, sleep, hunger, and thirst (Franco-Molina et al., 2003). A novel food product (NeOpuntia®- a trademark of Bio Serae Laboratories) which is a mixture of both soluble and insoluble fibers made from dehydrated leaves of the cactus *Opuntia ficus-indica* is found to have hypolipaemic properties and hence useful for patients with lipid metabolism disorders. The stems of *Selenicereus grandiflorus* and other species contains a glucoside which, in extract form or tinctures, constitutes a diuretic and also have cardiac properties (Shetty, Rana, and Preetham, 2012).

Several studies have documented about the nutrients and phytochemicals uptake by using Caco-2 intestinal cells. The Caco-2 cells are human colonic adenocarcinoma cells that, when differentiated, exhibit both functional and morphological characteristics similar to enterocytes such as the secretion of brush border enzymes and the presence of microvilli (O'Sullivan, Ryan, and O'Brien, 2007). Its validity as a predictor of intestinal absorption in humans has been established for numerous lipophilic drugs (Ferruzzi, Failla, and Schwartz, 2001). This model has also been utilized to investigate the uptake of nutrients and phytochemicals such as carotenoid (Liu, Glahn and Liu, 2004), lutein (Chitchumroonchokchai, Schwartz, and Failla, 2004) and micellarized carotenoids (Kean, Bordenave, Ejeta, Hamaker, and Ferruzzi, 2011).

As previously described, attention paid to golden barrel cactus (*Echinocactus grusonii*) or "Tang Thong" investigation in Thailand. It is plant in the family of *Cactaceae* originated in Mexico same as *Opuntia ficus-indica*. Now, it is the most popular cacti cultivation in Thailand. Nevertheless, information about phytochemicals and bioactivities of golden barrel cactus are limited. Recently, Huang, Qiu, and Guo (2014) reported that golden barrel cactus spine has highly aligned fiber cells content. However, there are no studies of phytochemical profile, bioaccessibility, and cellular uptake in stems of golden barrel cactus. This research, therefore, focused on assessment the chemical composition, phytochemical, antioxidant activity, cytotoxicity, digestive stability, bioaccessibility, cellular uptake and transport of golden barrel cactus stems crude extracts.

1.2 Research objectives

The objectives of this research were:

1.2.1 To determine chemical composition of 3- and 6-year-old golden barrel cactus powder.

1.2.2 To evaluate phytochemical profile and antioxidant activity of 3- and 6-year-old golden barrel cactus extracts.

1.2.3 To investigate cytotoxicity of golden barrel cactus extracts in Caco-2 and HepG2 cells lines.

1.2.4 To investigate the digestive stability and bioaccessibility of chlorophyll derivatives, lutein and total phenolic contents in golden barrel cactus extracts at the age of 3- and 6-year-old using simulated digestion.

1.2.5 To determine the cellular uptake and transport of chlorophyll derivatives, lutein and total phenolic in golden barrel cactus extracts at the age of 3- and 6-year-old using simulated digestion and Caco-2 human intestinal cell model.

1.2.6 To compare phytochemical, antioxidant activity, digestive stability, bioaccessibility, cellular uptake and cellular transport between 3- with 6-year old golden barrel cactus extracts.

1.3 Research hypotheses

Golden barrel cactus extracts contain phytochemical compounds, antioxidant activity, non-toxic property to Caco-2 and HepG2 cells lines, and phytochemical compounds from golden barrel cactus extracts were stable during simulated gastric and small intestinal digestion. Phytochemical, antioxidant activity, digestive stability, bioaccessibility, cellular uptake and cellular transport of 3-year-old golden barrel cactus extracts is higher than 6-year-old golden barrel cactus extracts.

1.4 Scope of the study

The golden barrel cactus at the age of 3- and 6-year-old collected in Uncle Chorn's Cabin Garden, Pathumthani province, Thailand was used as a raw material. Dried golden barrel cactus powder was analyzed for chemical composition following the AOAC (2005) method. Preparation of golden barrel cactus extracts was done by method of Vermaak, Hamman, and Viljoen (2010). Phytochemical profile (chlorophyll derivatives, lutein, and total phenolic) of golden barrel cactus extracts was investigated. Chlorophyll and lutein were determined by HPLC analysis according to Oonsivilai, Cheng, Bomser, Ferruzzi, and Ningsanond (2007) and total

phenolic was determined by Folin-Cicalteau colorimetric assay. The antioxidant activities of golden barrel cactus extracts were tested based on DPPH, ABTS, and FRAP assay. Cytotoxicity of golden barrel cactus extracts was measured by using the MTT assay against Caco-2 and HepG2 cells lines. The digestive stability of chlorophyll derivatives, lutein, and total phenolic from 3- and 6-year-old golden barrel cactus extracts were investigated after simulated gastric and small intestinal digestion. Moreover, the cellular uptake and cellular transport of chlorophyll derivatives, lutein and total phenolic from 3- and 6-year-old golden barrel cactus extracts were carried out with simulated digestion and Caco-2 human intestinal cell model. Phytochemical, antioxidant activity, digestive stability, cellular uptake and cellular transport of 3-year-old golden barrel cactus extracts were compared with 6-year-old extracts.

CHAPTER II

LITERATURE REVIEWS

2.1 Golden barrel cactus (*Echinocactus grusonii*)

Golden barrel cactus (*Echinocactus grusonii*) or Thailand call “Tang Thong” is plant in the family of *Cactaceae* originated in Mexico. Golden barrel cactus is a well-known species of cactus native to central Mexico from San Luis Potosi to Hidalgo. Described by Heinrich Hildmann in 1891, it is popularly known as the golden barrel cactus or golden ball. The golden barrel cactus is now one of the most popular cacti cultivation in Thailand. Growing as a large roughly spherical globe, it may eventually reach over a meter in height after many years. There may be up to 35 pronounced ribs in mature plants, though they are not evident in young plants, which have a knobby appearance. Younger golden barrel cactus do not look similar to the mature ones. The sharp spines are long, straight or slightly curved, and various shades of yellow or, occasionally, white. Small yellow flowers appear in summer around the crown of the plant, but only after twenty years or so. Widely cultivated in warmer climates around the world, it is considered easy to grow and relatively fast growing. It has been increasingly used as an architectural plant in garden design. While easy to grow, these plants do have some basic requirements; an average minimum winter temperature of 12°C, good drainage with less watering in winter. Excess water in cool periods may lead to rot. Golden barrels cactus are hardy to about -8ºC for brief periods. Outside Mexico, golden barrel cactus specimens may be seen in collections

of desert plants in many botanical gardens (Oonsivilai, Chaijareonudomroung, Huantanom, and Oonsivilai, 2010).

2.2 Physical characteristics of golden barrel cactus

The morphological characteristics of golden barrel cactus in this work are shown in Figure 2.1. Three-year-old golden barrel cactus, its height and diameter around eight and nine centimeters, respectively. While, the 6-year-old golden barrel cactus, its height and diameter around twelve and fourteen centimeters, respectively. The powder of 3-year-old golden barrel cactus shows a green color. While the powder of 6-year-old golden barrel cactus shows a chartreuse color.

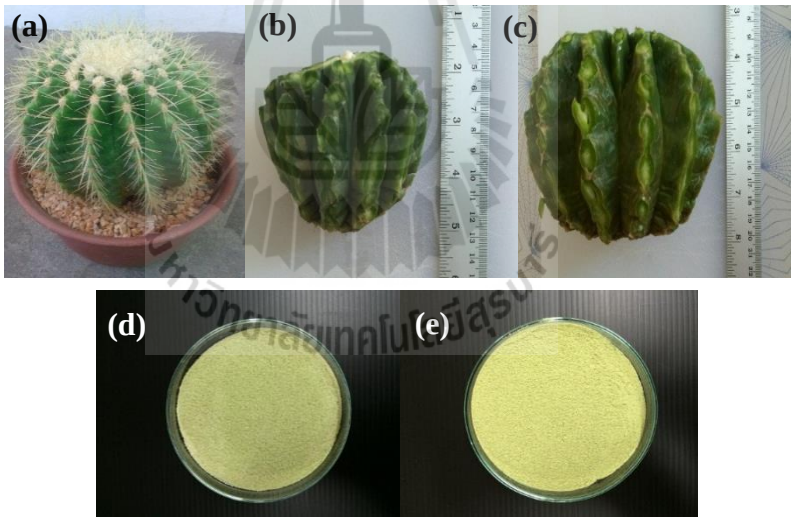


Figure 2.1 Physical characteristics of golden barrel cactus. (a) The golden barrel cactus, (b) Stem of 3-year-old golden barrel cactus, (c) Stem of 6-year-old golden barrel cactus, (d) Powders from stem of 3-year-old golden barrel cactus after drying, (e) Powders from stem of 3-year-old golden barrel cactus after drying.

2.3 Cactus literature reviews

Cactus (plural: cacti, cactuses, or cactus) is a member of the succulent plant family *Cactaceae*. They are often used as ornamental plants, but many are also cultivated as crop plants. Cacti are distinctive and unusual plants, adapted to extremely arid and hot environments, showing a wide range of anatomical and physiological features which conserve water. Their stems have expanded into green succulent structures containing the chlorophyll necessary for life and growth, while the leaves because the spines for which cacti are so well known (Shetty, Rana, and Preetham, 2012).

Currently, international scientists have studied cactus extract for its different phytochemicals and bioactivities that benefit to human health and use as medical and a valuable health supporting nutrient (Shetty, Rana, and Preetham, 2012). For example, De Leo, De Abreu, Pawlowska, Cioni, and Braca (2010) studied *Opuntia ficus-indica* in the family *Cactaceae*, a native plant in Mexico, found in widespread throughout Central and South America, Australia, South Africa and the whole Mediterranean area, including grow in all of the semiarid countries and mainly cultivate for its fruit (prickly pear) that is sweet, juicy, and rich in nutritional compounds such as ascorbic acid and polyphenols. These fruits have shown antiulcerogenic (Galati et al., 2003), antioxidant (Galati et al., 2003; Kuti, 2004; Tesoriere, Butera, Pintaudi, Allegra, and Livrea, 2004), anticancer (Zou et al., 2005), neuroprotective (Dok-Go et al., 2003), and antiproliferative activities (Sreekanth et al., 2007). Moreover, prickly pear may be used for gastritis, hyperglycemia, arteriosclerosis, diabetes, and prostate hypertrophy treatment (Agozzino, Avellone, Caraulo, Ferrugia, and Filizzola, 2005). In addition, Galati et al. (2007) found that gel

from *O. ficus-indica* extract could coat stomach and prevent peptic ulcer from alcohol induced in laboratory animals.

Hernández-Urbiola et al. (2010) studied development of the nopal (*Opuntia ficus-indica*, cv. Redonda) composition at advanced maturity stages to evaluate the age relating of changes and nutritional composition for suggestion its human potential use. Chemical proximate analysis, mineral constituents and amino acid profile were carried out at different maturation stages. Insoluble dietary fiber, calcium increased from 17.95% to 34.40% from 40-135 days respectively. The soluble dietary fiber in nopal decreased as age progressed from 40 to 135 age days. The results show that older nopal is an important source of calcium and dietary fiber. Moreover, it there is study in other cactus species such as Peyote (*Lophophora williamsii*) extracts associated with stimulating central nervous system and regulating blood pressure, sleep, hunger and thirst (Franco-Molina et al., 2003). A novel food product (NeOpuntia®- a trademark of Bio Serae Laboratories) which is a mixture of both soluble and insoluble fibers made from dehydrated leaves of cactus *Opuntia ficus-indica* is found to have hypolipaemic properties and hence useful for patients with lipid metabolism disorders. The stems of *Selenicereus grandiflorus* and other species contains a glucoside, in extract form or tinctures, a diuretic and shows cardiac properties (Shetty, Rana, and Preetham, 2012). However, in cases of golden barrel cactus no intensively study has been carried out. Recently, Huang, Qiu, and Guo (2014) reported that golden barrel cactus spine had highly aligned fiber cells content.

2.4 Phytochemicals and bioactivities

Phytochemicals are naturally occurring in the medicinal plants, leaves, vegetables and roots that its mechanism could protect our body from various diseases. Phytochemicals composed of primary and secondary compounds. Chlorophyll, lutein, proteins and common sugars are included in primary constituents and secondary compounds are terpenoid, alkaloids and phenolic compounds (Wadood et al., 2013).

2.4.1 Chlorophylls

Chlorophylls are green compound found in leaves and green parts of plants which play a key role in photosynthesis (Lipke, Trytek, Fiedurek, Majdan, and Janik, 2013). Chlorophylls cannot be synthesized by animal tissues, though animal cells can chemically modify them for assimilation. Thus, these molecules must be obtained from foods (İnanç, 2011). To date, previous studies reported that dietary chlorophyll predominantly composed of lipophilic derivatives including chlorophyll *a* and *b* (fresh fruits and vegetables), metal-free pheophytins and pyropheophytins (thermally processed fruits and vegetables), as well as Zn-pheophytins and Zn-pyropheophytins (thermally processed green vegetables). Water-soluble derivatives including chlorophyllides, pheophorbides, as well as a commercial-grade derivative are known as sodium copper chlorophyllin (SCC) also contribute to the diversity of dietary chlorophyll derivatives (Ferruzzi and Blakeslee, 2007). Structurally, chlorophyll is a substituted tetrapyrrole with a centrally bound magnesium atom are shown in Figure 2.2. In nature, chlorophyll *a* and *b* predominate in higher plants. Chlorophyll *a* differs from chlorophyll *b* have containing a methyl group at the third carbon instead of an aldehyde group.

Several reports have demonstrated that plant pigments play important roles in human health. In fact, the potential health benefit of a diet rich in chlorophylls have been indicated in recent studies that their role as agents could prevent some diseases (İnanç, 2011). Moreover, Ferruzzi, Böhm, Courtney, and Schwartz (2002) reported that dietary chlorophyll derivatives prevalent in both fresh and processed foods and dietary supplements show antioxidant and antimutagenic activities. Chlorophyll and its various derivatives have a long-established history of application in traditional medicine and therapeutic purposes. Chlorophyll has positive effects on inflammation, oxidation, and wound healing (İnanç, 2011) anti-inflammatory properties(Larato and Pfau, 1970), control of calcium oxalate crystals (Tawashi, Cousineau, and Sharkawi, 1980), and anti-carcinogens (Dashwood, 1997).

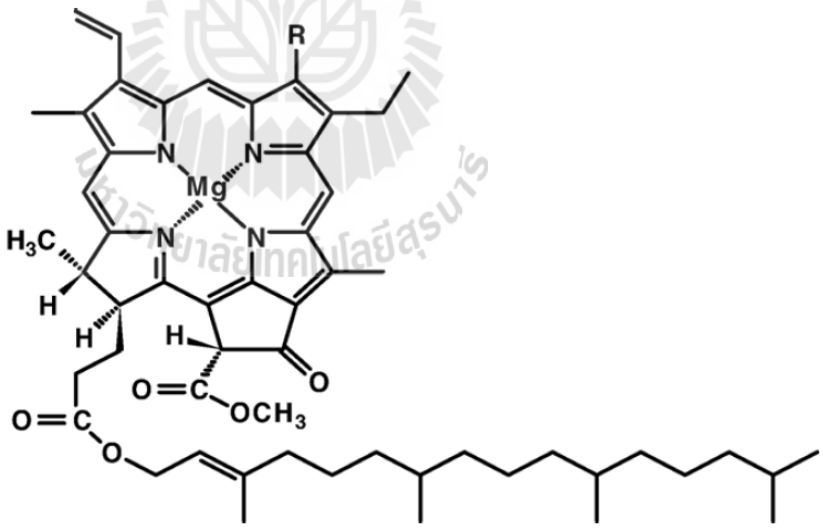


Figure 2.2 Structure of chlorophyll derivatives found in higher plants including chlorophyll a (R=CH_3) and chlorophyll b (R=CHO).

Source: Ferruzzi and Blakeslee (2007).

Ferruzzi and Blakeslee (2007) reported that the sensitivity of natural chlorophylls to extremes pH and temperature allows the formation of several distinct derivatives through processing of vegetable tissue and human digestion. The main degradative reactions are summarized in Figure 2.3. Thermal processing and/or acidification results in a perceivable discoloration of vegetable tissue from green to brown and are known as pheophytinization. This color deterioation is a result of the conversion of natural chlorophylls to Mg^{2+}-free derivatives such as pheophytins and pyropheophytins.

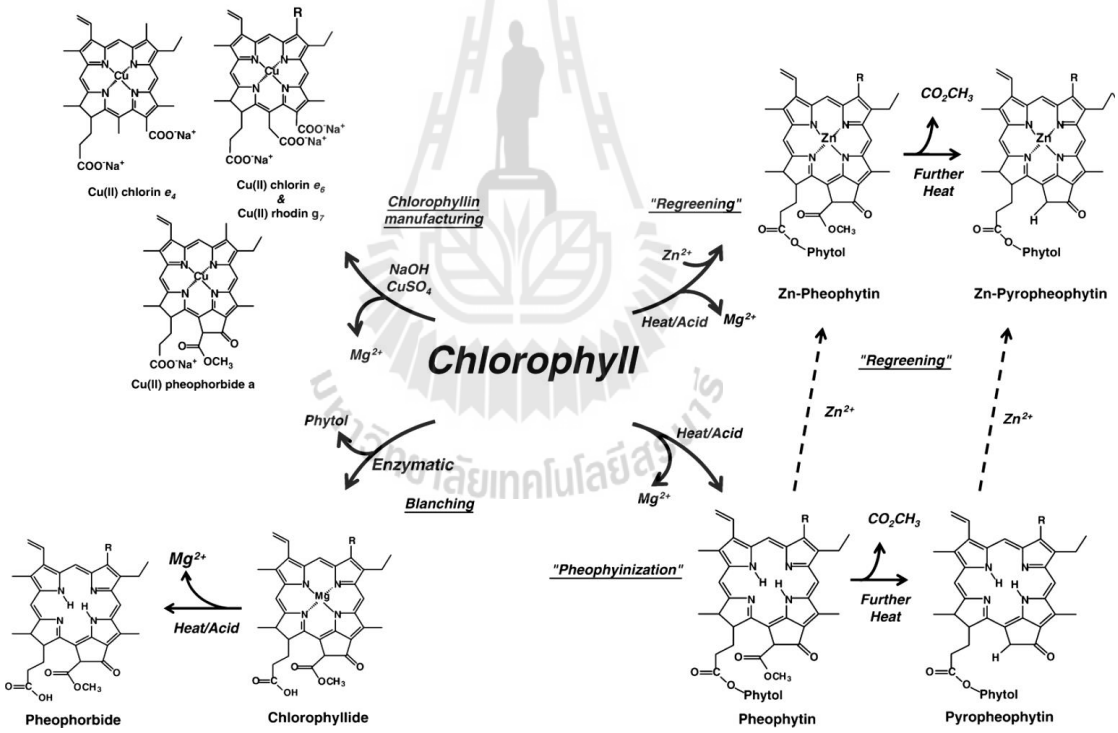


Figure 2.3 Major chlorophyll degradation and derivatization reactions occurring during food processing operations.

Source: Ferruzzi and Blakeslee (2007).

Chlorophyllide derivatives can also be formed through mild thermal processing because of increasing in endogenous chlorophyllase enzyme activity occuring from the mild heating of plant tissues. In a processing known as regreening, metallo-chlorophyll complexes of pheophytin *a* and pyropheophytin *a* are rapidly formed by addition of divalent metal salts of zinc (Zn^{2+}) and copper (Cu^{2+}) in thermally processed vegetables before thermal treatment. Both Cu^{2+} and Zn^{2+} complexes of chlorophylls have been shown to be more significant stable to food processing conditions than their native counterparts and commercially used both in green beans canning for color improvement in finished product and generation of green color additives.

2.4.2 Lutein

Lutein is one of the major xanthophyll family of carotenoids and known selectively accumulate in the macula of human retina. The macula lutea or "yellow spot" in the retina is responsible for central vision and visual acuity. Lutein is the only carotenoids found in both human eye macula and lens, and shows dual functions in both tissues acting as powerful antioxidants and high-energy blue light filter (Landrum and Bone, 2001) for protecting the eyes from such oxidative stresses as cigarette smoking and sunlight exposure, which can lead to age-related macular degeneration and cataracts. Furthermore, a recent study has shown that the visual function of patients with atrophic age-related macular degeneration is improved with lutein supplementation (Richer et al., 2004). In diet, lutein is found at the highest concentrations in dark green, leafy vegetables (spinach, kale, collard greens, and others), corn, and egg yolks (Sommerburg, Keunen, Bird, and van Kuijk, 1998). Chemical structures of lutein are shown in Figure 2.4. Lutein have two hydroxyl

groups, one on each side of the molecule. The various lutein concentrations in fruits, vegetables, and egg products are shown in Table 2.1.

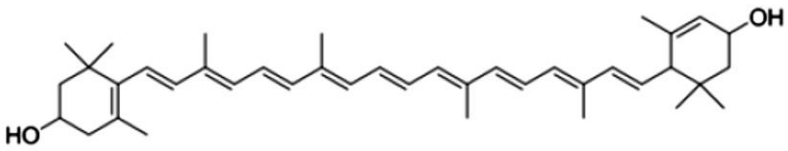


Figure 2.4 Chemical structures of lutein.

Source: Kotake-Nara and Nagao (2011).

Table 2.1 Lutein concentrations in fruits, vegetables, and egg products.

Fruits, vegetables, and egg products	**Lutein content (µg/100 g)**
Apple, red delicious with skin	15
Artichoke heart	95
Asparagus, cooked	991
Broccoli, cooked	772
Brussel sprouts, cooked	155
Cantaloupe, raw	19
Cilantro	7703
Cucumber	361
Endive	399
Grapes, green	53
Grapes, red	24
Green beans, cooked from frozen	306
Kale, cooked	8884
Kiwi	171
Orange juice	33
Parsley	4326

Table 2.1 Lutein concentrations in fruits, vegetables, and egg products (continued).

Fruits, vegetables, and egg products	Lutein content (µg/100 g)
Peach, raw	11
Pepper, green	173
Pepper, orange	208
Pepper, yellow	139
Scallions, raw	782
Spinach, cooked	13504
Spinach, raw	7224
Tomato, raw	32
Watermelon	4
Mango	6
Zucchini, cooked with skin	1355
Egg (yolk + white), cooked	273
Egg yolk, cooked	744
Egg (yolk + white), raw	336
Egg yolk, raw	917

Source: Perry, Rasmussen, and Johnson (2009).

2.4.3 Total phenolic compounds

Plants are a major source of phenolic compounds, and synthesized as secondary metabolites during normal development in response to stress conditions, such as wounding and UV radiation among others (Maisuthisakul, Pasuk, and Ritthiruangdej, 2008). Phenolic compounds provide essential functions in the reproduction and plants growth such as defense mechanisms against pathogens, parasites, and predators and the plants color contribution (Báidez, Gómez, Del Río, and Ortuño, 2007). Natural phenolic compounds play an important role in prevention

and treatment of cancer. In addition, phenolics abundant in vegetables and fruits have been reported for playing an important role as chemopreventive agents, for example, the phenolic components of apples linked with colon cancer inhibition *in vitro* (Veeriah et al., 2006). Many phenolic compounds have been reported to possess potent antioxidant and anticancer or anticarcinogenic/antimutagenic, antiathero-sclerotic, antibacterial, antiviral, and anti-inflammatory activities to a greater or lesser extent (Báidez, Gómez, Del Río, and Ortuño, 2007; Han, Shen, and Lou, 2007; Owen et al., 2000; Veeriah et al., 2006). The recent studies have characterized a large number of natural phenolic compounds from 112 traditional Chinese medicinal herbs associated with anticancer, 133 traditional Indian medicinal herbs, and about 50 dietary plants (such as, spices, cereals, vegetables, and fruits) showed their antioxidant activity and including other bioactivities (Cai, Luo, Sun, and Corke, 2004; Hu, Cai, Li, Corke, and Kitts, 2007; Surveswaran, Cai, Corke, and Sun, 2007). Recently, researchers disclose that the *Opuntia* spp. had the highest total phenolic acid contents, such as gallic acid, p-Coumaric acid, Caffeic acid, 4-hydroxybenzoic acid, Ferulic acid, and salicylic acid (Guevara-Figueroa et al., 2010). Cha et al. (2013) reported that the Korean cactus (*Opuntia humifusa*) fruit extract from 80% ethanol had the total phenolic contents 8.3 μg gallic acid equivalents (GAE)/mg extract. Moreover, Korean cactus fruit contains ferulic acid, protocatechuic acid, p-coumaric acid, isovanilic acid, chlorogenic acid, p-hydroxybenzoic acid, syringic acid, trans-m-coumaric acid, salicylic acid, gallic acid, and trans-cinnamic acid. In addition, Sakong et al. (2011) studied bioactive phytochemical contents of traditional medicinal plants normally cultivated in northeast Thailand and reported that phenolic contents of the plant extracts were highly variable, from the lowest in *Limnocharis flava* (2.39 ± 0.02 mg

GAE/100 g dry weight) to the highest in *Clausena excavata* (1669.81 ± 6.33 mg GAE/100 g dry weight).

2.5 Bioactivities

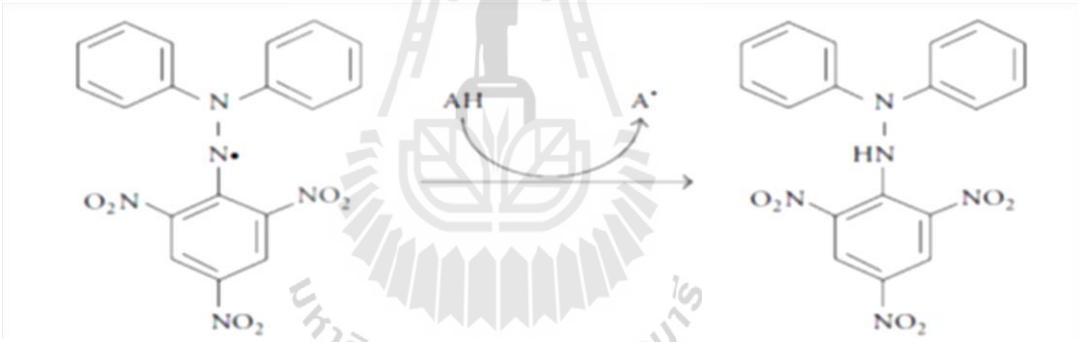
2.5.1 Antioxidant activity

Natural antioxidants, particularly in fruits and vegetables, have gained increasing interest among consumers and the scientific community because of epidemiological studies indicated that frequently consumption of natural antioxidants is associated with a lower risk of cardiovascular cancer disease (Temple, 2000). Recently, a wide range of spectrophotometric assays has been adopted to measure antioxidant capacity of foods, the most popular is 2,2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay including ferric reducing antioxidant power (FRAP) assay (Floegel, Kim, Chung, Koo, and Chun, 2011). These assay are normally applied for measurement of vegetables, fruits, herbs, cactus, traditional medicinal plants, and Thai culinary plants antioxidant content (Guevara-Figueroa et al., 2010; Oonsivilai, Ferruzzi, and Ningsanond, 2008; Sakong et al., 2011; Wangcharoen and Morasuk, 2007).

2.5.1.1 DPPH free radical scavenging assay

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay is routinely practiced for assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compounds (Mishra, Ojha, and Chaudhury, 2012). $DPPH^{\cdot}$ is a stable radical in solution and appears purple colour

absorbing at 515 nm in methanol. This assay is based on the principle that DPPH˙ on accepting a hydrogen (H) atom or electron from the scavenger molecule that is antioxidant, resulting into the reduction of DPPH˙ to DPPH-H, the purple colour changes to yellow (Figure 2.5) with concomitant decrease in absorbance at 515 nm. The colour change is monitored by spectrophotometrically and utilised for the determination of antioxidant properties parameters (Mishra, Ojha, and Chaudhury, 2012). The antioxidant potential is inversely proportional to IC_{50} (mg/ml) value calculated from the linear regression of the percentage inhibition versus the extracts sample concentrations.



AH = antioxidant compound

DPPH = 1, 1-diphenyl-2-picrylhydrazyl

Figure 2.5 Principle of DPPH radical scavenging capacity assay.

Source: Teixeira, Gaspar, Garrido, Garrido, and Borges (2013).

2.5.1.2 Scavenging activity of ABTS radical cation assay

The ABTS (2, 2´-azino-bis-3-ethylbenzthiazoline-6-sulphonic) assay is based on the generation of a blue-green $ABTS^{\bullet+}$ that could be reduced with antioxidants. The generation of $ABTS^{\bullet+}$ involves the direct production of the blue-green $ABTS^{\bullet+}$ chromophore through reaction between ABTS solution and potassium persulfate. The method is a decolorization assay that measures the reduction of absorbance at 734 nm of the $ABTS^{\bullet+}$ solution after addition an antioxidant (Figure 2.6). The antiradical activity, ABTS scavenging ability was expressed as IC_{50} (mg/ml) calculated from the linear regression of the percentage inhibition versus extracts concentrations. The method is applicable to investigation of water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts (Re et al., 1999).

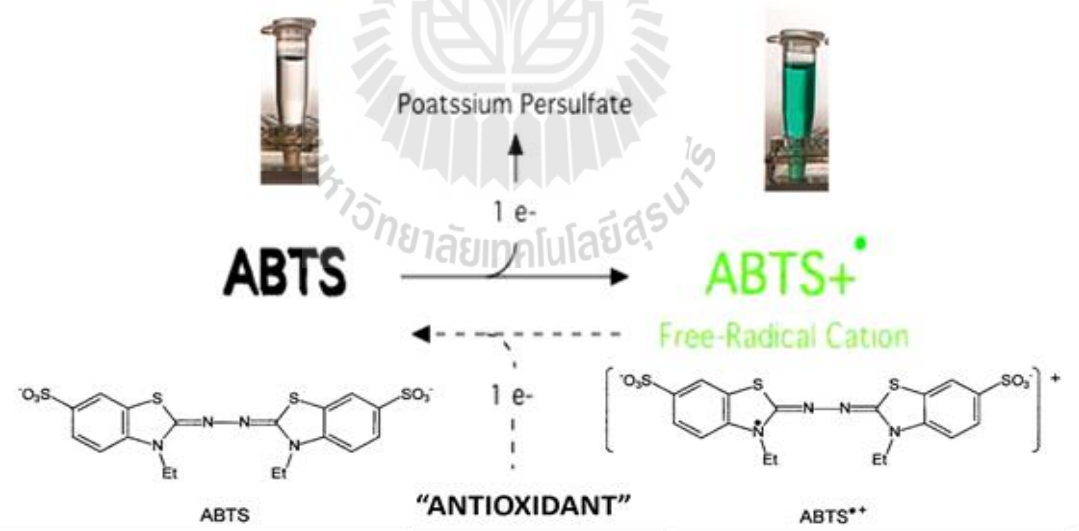


Figure 2.6 Principle of scavenging activity of ABTS radical cation assay.

Source: Pannala, Chan, O'Brien, and Rice-Evans (2001) .

2.5.1.3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay, is presented as method for assessing ‘‘antioxidant power’’ and different from the DPPH and ABTS assay not only there are no free radicals involved but also the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) is monitored. Ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form under acidic (pH 3.6) conditions and gave a blue color ferrous-tripyridyltriazine complex with an absorption maximum at 593 nm (Figure 2.7). FRAP values are obtained by comparing absorbance change at 593 nm in test reaction solution with those containing standard ferrous ions. The FRAP assay is inexpensive and the reagents are simple preparation also the results are highly reproducible including the procedure is straightforward and speedy (Benzie and Strain, 1996).

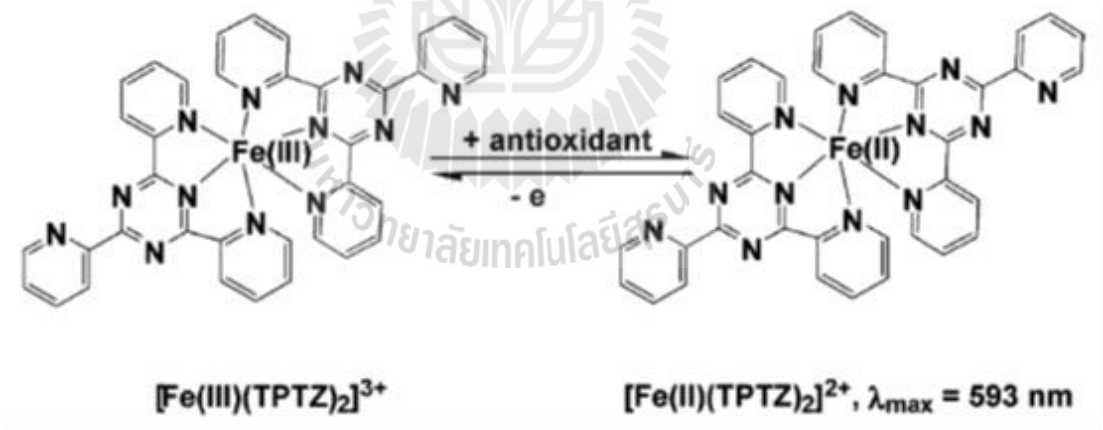


Figure 2.7 Principle of ferric reducing antioxidant power (FRAP) assay.

Source: Huang, Ou, and Prior (2005).

2.5.2 Cytotoxicity

Cell viability and cytotoxicity assays are normally used for drug screening and cytotoxicity tests of chemicals, which widely applied in *in vitro* toxicology studies. Mosmann (1983) have developed a rapid colorimetric assay, based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), that measures only living cells and could investigated on a scanning multiwell spectrophotometer (ELISA reader). The MTT colorimetric assay determines the ability of viable cell to convert a soluble tetrazolium salt MTT into an insoluble formazan precipitate because tetrazolium salt MTT accepted electron from oxidized substrates or appropriate enzyme such as NADH and NADPH (Berridge and Tan, 1993). In particular, MTT is reduced at the ubiquinone and cytochrome *b* and *c* sites of the mitochondrial electron transport system and is the result of succinate dehydrogenase activity within the mitochondria of living cells. This reaction converts the yellow tetrazolium salts to blue-colored formazan crystals that could be dissolved in an organic solvent (Figure 2.8). The formazan product is impermeable to the cell membranes and therefore it accumulates in healthy cells. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density using an ELISA plate reader at the absorbance 570 nm. The MTT assay was tested for its validity in various cell lines (Mosmann, 1983). Owing to the many advantages of the assay, today it is considered a significant advance over traditional techniques. In fact, it is rapid, easy-to-use, safe, versatile, and quantitative, high reproduce and widely applied in both cell viability and cytotoxicity tests. Therefore, this method is suitable for those who are just beginning such experiments (Supino, 1995).

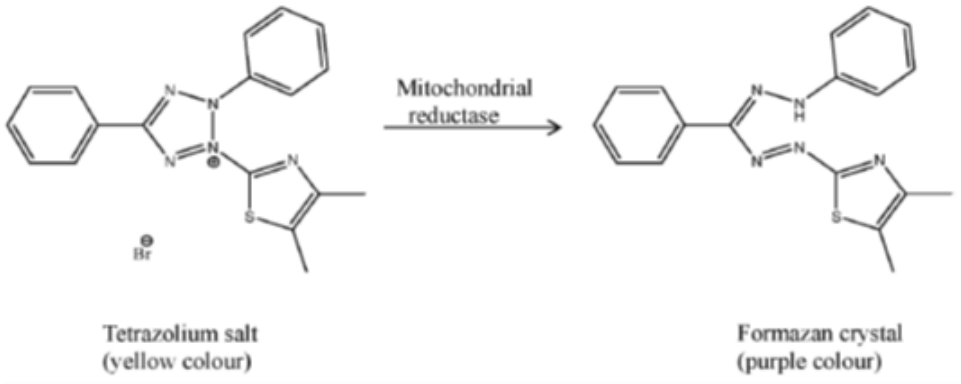


Figure 2.8 The formation of formazan crystals from the tetrazolium salt.

Source: Barahuie, Hussein, Fakurazi, and Zainal (2014).

Cytotoxicity in cell culture is typically expressed as LC_{50}, the concentration of a given agent which is lethal to 50% of the cells (Zhang et al., 2007). Another toxic compounds were expressed as the concentration of sample that is required to achieve 50% growth inhibition as compared to the growth of the control (50% inhibitory concentration, IC_{50}) (Van Meerloo, Kaspers and Cloos, 2011).

Several researches study the *in vitro* cytotoxicity by using the MTT assay. Okonogi, Duangrat, Anuchpreeda, Tachakittirungrod, and Chowwanapoonpohn (2007) studied cytotoxicities of certain fruit peels in Caco-2 cells and peripheral blood mononuclear cells (PBMC) and reported that the rambutan peel extract exhibited extremely high value of IC_{50} (>100 µg/ml) against both cell types indicating non-toxic activity to the cells. Oonsivilai, Ferruzzi, and Ningsanond (2008) investigated cytotoxicities of Rang Chuet (*Thunbergia laurifolia* Lindl.) crude extracts in L929 (mouse connective tissue), BHK(21)C13 (baby hamster Syrian kidney), HepG2 (human liver hepatocarcinoma) and Caco-2 cell lines (a human colon adenocarcinoma). The toxicity indicated at high concentrations over 100 µg/mL for all

extracts, which are no toxicity to the tested cell lines. In addition, Prayong, Barusrux and Weerapreeyakul (2008) studied cytotoxic activity of some indigenous Thai plants against a malignant human hepatoma (HepG2) cell line and a normal African green monkey kidney (Vero) cell line. The extracts of *Polyalthia evecta* and *Erythroxylum cuneatum* showed potent anticancer activity in the HepG2 cell line with IC_{50} of 70 ± 3 µg/ml and 64± 4 µg/ml, respectively.

2.6 Bioaccessibility and bioavailability

Bioaccessibility has been defined as the fraction of a compound that releasing from its matrix in the gastrointestinal tract and might be able to pass through intestinal barrier (Fernández-García, Carvajal-Lérida, and Pérez-Gálvez, 2009; Saura-Calixto, Serrano, and Goñi, 2007). While the term "bioavailability" refers to the fraction of a compound that absorbed from the intestine and becomes available for its use, metabolism, and/or storage by the organism (Ferruzzi and Blakeslee, 2007). Methodologies applied to measure bioaccessibility of nutrients and bioactive compounds could be done by *in vivo* and *in vitro* studies, which studies both present strengths and drawbacks are shown in Table 2.2.

Table 2.2 Strengths and drawbacks of *in vivo* and *in vitro* approaches to assess bioaccessibility of nutrients and bioactive compounds.

Strengths	**Drawbacks**
In vivo	
- *In vivo* situation	- Lower throughput
- Allow enough sampling to perform pharmacokinetic studies	- Overall contribution of factors involved on bioaccessibility

Table 2.2 Strengths and drawbacks of *in vivo* and *in vitro* approaches to assess bioaccessibility of nutrients and bioactive compounds (continued).

Strengths	Drawbacks
In vivo	
- Selection of individuals for specific target population of intended use	- Lack of certified reference standards to compare data among studies/laboratories - Cost and ethical considerations
In vitro	
- High-throughput screening tools - Provide information about efficiency of each digestion/absorption step and transport mechanisms - Validation and standardization with reference material - Cost, automatization, and miniaturization	- Validation against *in vivo* bioavailability data - Dynamic environment of intestine is not fully reproduced with biochemical and cell culture models - Effect of intestinal microflora and hepatic metabolism is not considered

Source: Fernández-García, Carvajal-Lérida, and Pérez-Gálvez (2009).

2.6.1 The *In vitro* simulated digestion and Caco-2 human intestinal cell model for bioavailability study

Simulated digestive processes, isolated intestinal cells and segments, brush-border and basolateral membrane vesicles represent studying models for specific characteristics and regulation of complex processes associated with digestion and absorption. These models have been used for investigation the effects of chemical speciation, food matrix and processing, also dietary components on the digestive stability, accessibility, and intestinal transport including metabolism of bioactive

compounds, phytochemicals, nutrients from foods and supplements (Bhagavan, Chopra, Craft, Chitchumroonchokchai, and Failla, 2007; Garrett, Failla, and Sarama, 1999). However, it is important to note that these relatively simple models are "static" in the sense that they are not influenced by many factors affecting digestive and absorptive process *in vivo*.

2.6.1.1 *In vitro* simulated digestion

In vitro study have been developed for simulation of the physiologic conditions and the sequence of events that occur during digestion in the human gastrointestinal tract. In a first step, an *in vitro* gastrointestinal method is applied to the food, mirroring the physiochemical conditions that take place during human digestion, considering the 3 areas of the human digestive system (mouth, stomach, and intestine). The main features of the *in vitro* gastrointestinal methods are temperature, shaking or agitation, and the chemical and enzymatic composition of saliva, gastric juice, intestinal juice, and bile juice (Fernández-García, Carvajal-Lérida, and Pérez-Gálvez, 2009). Simulated gastric and small intestinal digestion has been widely applied to investigate the digestion of carbohydrates, protein, lipids, and phytonutrients in foods (Englyst, Englyst, Hudson, Cole, and Cummings, 1999; Fouad, Farrell, Marshall, and Van de Voort, 1991; Gil-Izquierdo, Zafrilla, and Tomás-Barberán, 2002; Lindberg, Engberg, Sjöberg, and Lönnerdal, 1998). This method has also been utilized to examine the bioactive compounds stability and partitioning during digestion of foods, meals, and supplements (Ferruzzi, Failla, and Schwartz, 2001; Garrett, Failla and Sarama, 1999). For example, comparison of profiles before and after simulated digestion provides stability information of the components during the gastric and small intestinal phases of digestion. The complete product from digestion is called "digesta". It is centrifuged to collect the aqueous fraction (Figure

2.9). Conditions are optimized for complete digestion of emulsified oil droplets, i.e., micellarization of fat-soluble compounds transferred to the droplets. Components are extracted from the starting material, digesta, and aqueous filtrate and analyzed by HPLC to determine digestive stability and efficiency of micellarization referring to a bioaccessibility (Failla and Chitchumronchokchai, 2005). Additionally, there are many digestive stability and bioaccessibility studies of nutrients and phytochemicals such as carotenoids, chlorophyll derivatives and phenolic pompounds by *in vitro* digestion, which seem to be applicable for the examination of bioavailability of nutrients and phytochemicals in food (Ferruzzi, Failla and Schwartz, 2001; Garrett, Failla and Sarama, 1999; Rodríguez-Roque, Rojas-Graü, Elez-Martínez and Martín-Belloso, 2013; Walsh, Zhang, Vodovotz, Schwartz, and Failla, 2003).

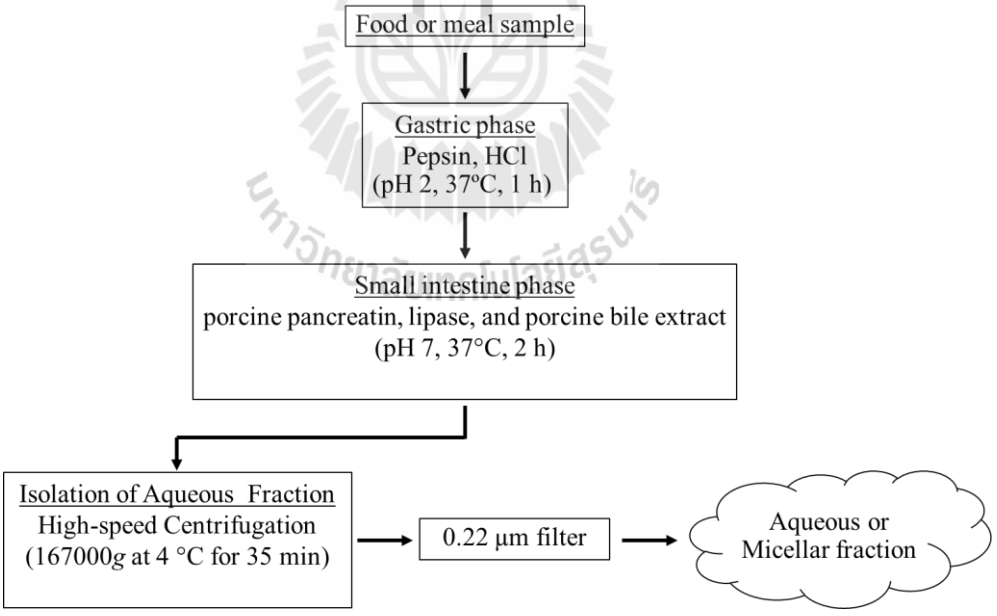


Figure 2.9 General procedure for simulated digestion of food for determination of digestive stability and efficiency of aqueous fraction or micellarization.

Source: Ferruzzi, Failla, and Schwartz (2001).

2.6.1.2 Caco-2 human intestinal cell model

Caco-2 is a cell line originating from human colonic carcinoma that exhibits some morphological and functional characteristics similar to those normal small intestinal enterocytes (Sambruy, Ferruzza, Ranaldi, and De Angelis, 2001). The general characteristics of Caco-2 cells are listed in Table 2.3. Caco-2 cells spontaneously differentiate to an enterocyte-like phenotype when monolayers reach confluency and maintained using conventional culture conditions (Pinto et al., 1983). During the early phases of differentiation, the cells express both colonocyte- and enterocyte-specific proteins. As differentiation proceeds, colonocyte-specific gene expression decreases and morphological including biochemical characteristics of enterocytes develop. After approximately 2 weeks, the monolayer is characterized by highly polarized columnar cells with tight junctions and desmosomes that separate the microvillar (apical) membrane from the basolateral membrane. Moreover, hydrolases such as sucrase-isomaltase, lactase, and dipeptidylpeptidase IV are localized in the apical membrane. These enzymes are normally present in the brush border membrane of enterocytes, but not found in colonocytes.

Table 2.3 Characteristics of Caco-2 human intestinal cells.

Characteristics of Caco-2 human intestinal cells
• Originated from human colon adenocarcinoma
• Differentiate spontaneously into enterocyte-like cells
• Differentiated cells are characterized by:
- Tight junctions between cells
- Basolateral Na^+, K^+-ATPase
- Inducible drug detoxification enzymes

Table 2.3 Characteristics of Caco-2 human intestinal cells.

Characteristics of Caco-2 human intestinal cells
• Differentiated cells are characterized by: - Apical brush border surface enriched with hydrolytic enzymes - Synthesis and vectoral secretion of chylomicrons

Source: Failla and Chitchumronchokchai (2005).

Other biochemical characteristics of differentiated Caco-2 cells that similar to those normal small intestinal enterocytes are included in the following: expression of apical sodium-dependent glucose and amino acid transporters, and the di- and tripeptide transporter (PepT1); synthesis and secretion of chylomicrons and lipoproteins; and the ability to induce phase I and II detoxification enzymes (Failla and Chitchumronchokchai, 2005). The intestinal absorption of compound may occur via passive diffusion (paracellularly or transcellularly), or via carrier-mediated active transport or via carrier-mediated efflux or via vesicular transport (Figure 2.10). The high correlation between the extent of oral drug absorption in humans and transport across the Caco-2 monolayer resulted in the widespread application of Caco-2 cells as a model system for high throughput screening of transport and metabolism of numerous drugs and their derivatives (Failla and Chitchumronchokchai, 2005).

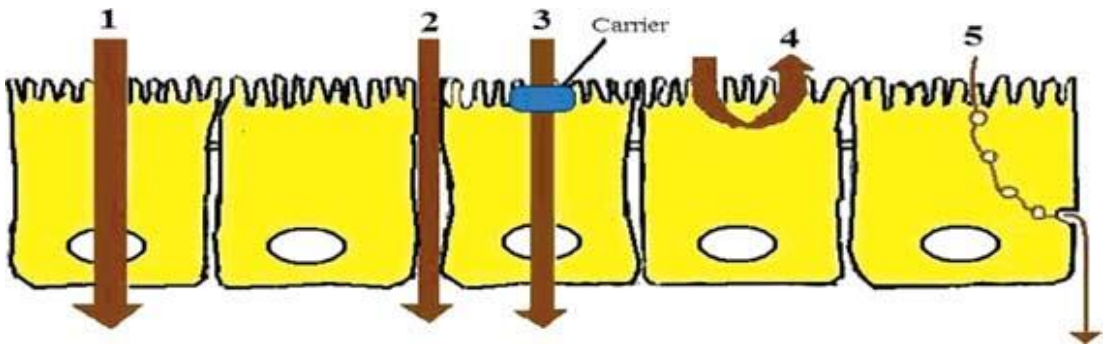


Figure 2.10 Schematic representation of routes and mechanisms of drug transport across the intestinal epithelium: 1 passive transcellular route, 2 passive paracellular route, 3 carrier-mediated transport, 4 carrier-mediated efflux, and 5 vesicular transport.

Source: Shah, Jogani, Bagchi, and Misra (2006).

It is important to note that some characteristics of differentiated Caco-2 cells differ from small intestinal enterocytes. First and most obvious, the cells originate from a human colonic carcinoma rather than normal small intestine. Second, the cell line is genetically and phenotypically heterogeneous. Third, the transepithelial resistance associated with assembly of tight junctions in Caco-2 cells is more characteristic of colonic epithelium than small intestinal epithelium. Finally, Caco-2 cells use the glycerol 3-phosphate pathway for the synthesis of triacylglycerols, whereas the small intestinal epithelium uses the monoacylglycerol pathway. Some of the indicated differences are offset by standardization of procedures associated with the growth and maintenance of Caco-2 cells and the design of studies using this cell line (Failla and Chitchumronchokchai, 2005).

Many investigators have used the Caco-2 model for studying the characteristics and regulation of processes associated with the apical uptake, metabolism, and transepithelial transport of diverse nutrients and other dietary components. These include amino acids (Costa, Huneau, and Tomé, 2000), carotene and xanthophyll carotenoids (O'Sullivan, Ryan, and O'Brien, 2007), carotenoid (Failla, Huo, and Thakkar, 2008; Garrett, Failla, and Sarama, 1999), lutein (Chitchumroonchokchai, Schwartz, and Failla, 2004), chlorophyll derivatives from spinach puree (Ferruzzi, Failla, and Schwartz, 2001), chlorophyll derivatives from pea (Gallardo-Guerrero, Gandul-Rojas, and Mínguez-Mosquera, 2008), flavonoids (Vaidyanathan and Walle, 2003), and retinol (Nayak, Harrison, and Hussain, 2001). Thus, the literature supports the utility of differentiated cultures of Caco-2cells as a model for investigation characteristics and regulation of the dietary compounds apical cellular uptake and transport by absorptive epithelial cells.

The investigation of cellular uptake is different from transepithelial transport (Figure 2.11). Cells for cellular uptake investigation are grown and maintained on a permeable that attached to the base of plastic in a standard cell culture well containing medium. The investigation of transepithelial transport requires the use of a three compartment model. Cells are grown and maintained on a permeable, inert membrane support that attached to the base of a plastic ring suspended in a standard cell culture well containing medium. Thus, the apical and basolateral surfaces of cells face the upper and lower compartments, respectively. (Failla and Chitchumronchokchai, 2005).

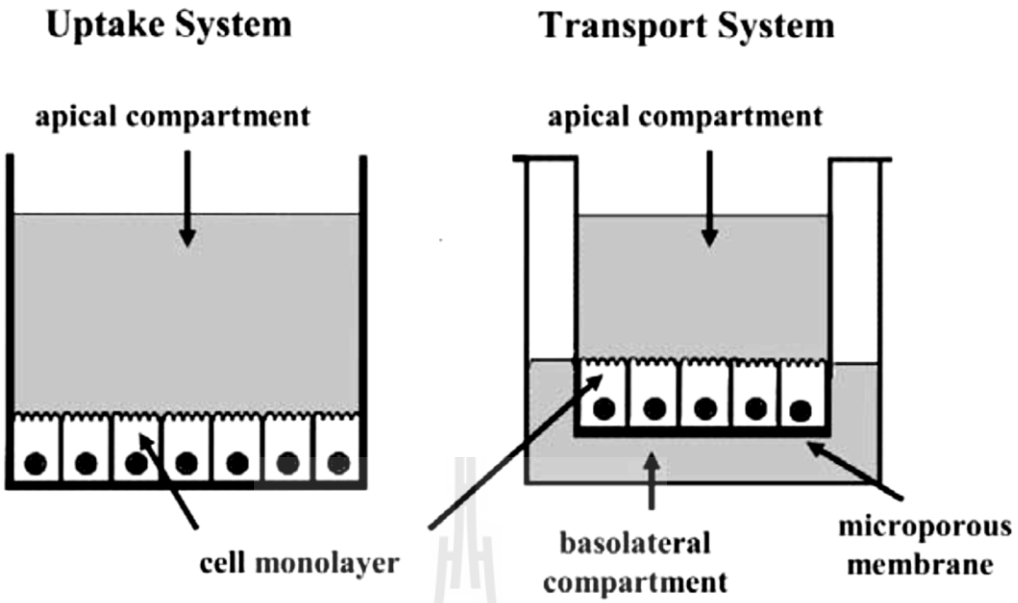


Figure 2.11 Culturing Caco-2 cells monolayers for investigating cellular uptake and transport of bioactive food components.

Source: Failla and Chitchumronchokchai (2005).

2.6.1.3 Coupling *in vitro* digestion with the Caco-2 cell model

Garrett, Failla, and Sarama (1999) developed a two component coupled *in vitro* digestion/Caco-2 cell model to examine the relative bioavailability of carotenoids from digested foods, supplements, and meals. After completing digestion of carotenoid-containing foods and meals *in vitro*, the aqueous (i.e., micellar) fraction is isolated, filtered, and diluted with basal medium. This solution is added to the apical compartment of wells with cells adhered to either the plastic surface of the culture vessel or the membrane insert for investigating the uptake and transport of micellar carotenoids, respectively. Exposure the monolayer of differentiated cells to dilute micellar fraction for 4-6 hours does not adversely affect cellular morphology and metabolic integrity. Chitchumroonchokchai, Schwartz and Failla (2004) studied

bioavailability of lutein from spinach and a commercial supplement. The results showed that lutein and other carotenoids presented in spinach and lutein from a commercial supplement were relatively stable during *in vitro* digestion. Apical uptake of lutein from micelles by Caco-2 human intestinal cells was linear for at least 8 h. Stimulation of chylomicron synthesis in the apical compartment and secretion resulted in transfering of cellular lutein to the basolateral compartment. Whereas, Yonekura and Nagao (2007) reported that the mechanisms of dietary carotenoid absorption by the intestinal cells (Figure 2.12). Carotenoids are released from the food matrix by heat, mechanical and enzymatic treatments during food processing and in the mouth, gastric and small intestinal, by mastication and the action of enzymes. The released carotenoids incorporate into the lipid phase which emulsified into small lipid droplets in the stomach. From lipid droplets, carotenoids are transferred to mixed micelles formation by action of the bile salts, biliary phospholipids, dietary lipids, and their hydrolysis products. However, the less lipophilic xanthophylls can also be solubilized directly in mixed micelles. The mixed micelles migrate to the brush border where carotenoids absorbed by the intestinal cells, packed into chylomicrons and secreted to the lymphatic system. The uptake of carotenoids from the intestinal lumen takes place by simple diffusion down a concentration gradient through the brush border membrane into the cytoplasm of the enterocytes.

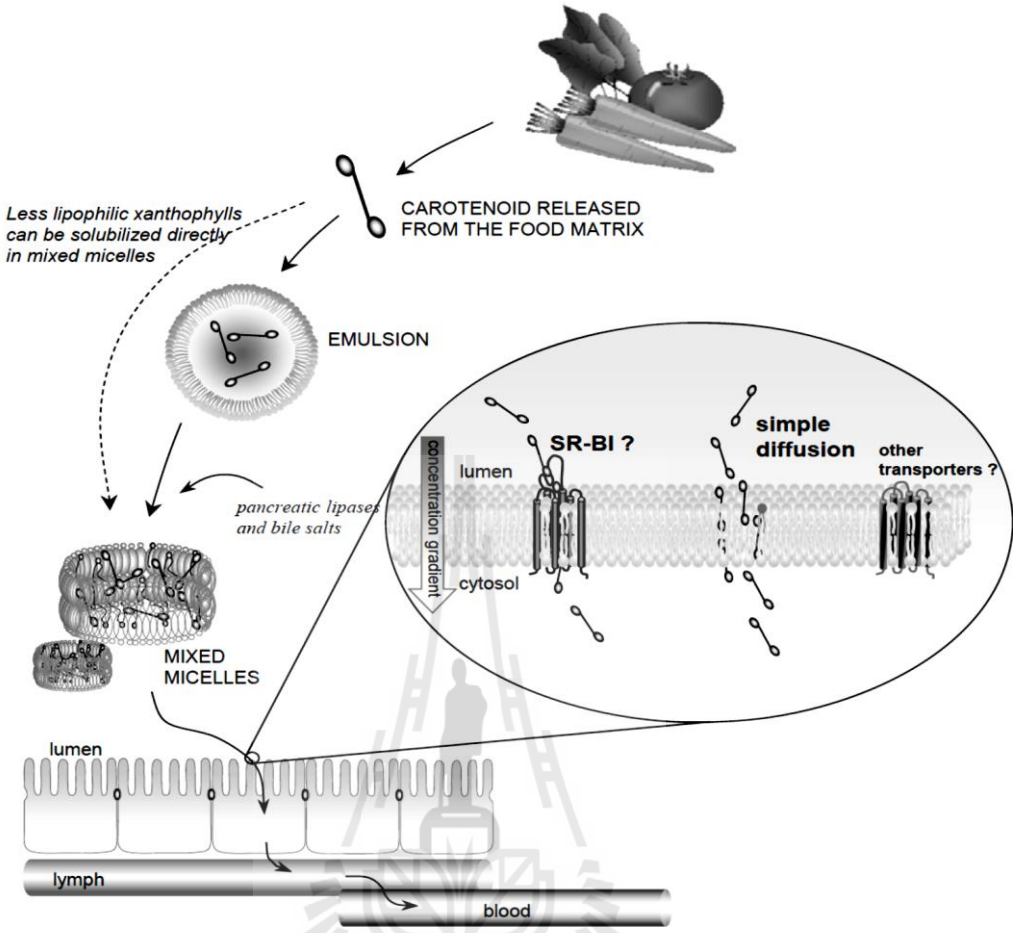


Figure 2.12 Scheme of dietary carotenoid absorption.

Source: Yonekura and Nagao (2007).

Gallardo-Guerrero, Gandul-Rojas, and Mínguez-Mosquera (2008) studied the digestive stability, micellarization, and cellular uptake of the chlorophyll pigments from different preparations of fresh pea (FP), cooked fresh pea (CFP), frozen pea (FZP), cooked frozen pea (CFZP), and canned pea (CP). They found that after the test foods gastric and small intestinal *in vitro* digestion process, the pigment profile was modified for all of the samples, except CP. The acid conditions, which govern in the gastric digestion phase, caused the complete disappearance of chlorophylls *a* and *b* in favor of the respective Mg-free derivatives. In CP, the pigment

profile was unaltered because of the transformations under acid pH already occurred in the raw materials. The transfer of the chlorophyll pigments from the digesta to the micellar fraction was significantly higher efficient in CFZP (57%, < 0.0001), not significantly ($p > 0.05$) different between CFP, FZP, and CP (28-35%), and lowest in FP (20%). The cellular uptake of the chlorophyll pigments in FP was considerably lower whereas no differences among the rest of preparations, which all been subjected to some form of heat treatment. These results demonstrated that the industrial preservation processes of cooking have a positive effect on the bioaccessibility and bioavailability of chlorophyll pigments. In addition, Ferruzzi, Failla and Schwartz (2001) studied the degradation and intestinal cell uptake of chlorophyll derivatives from fresh spinach puree (FSP), heat- and acid-treated spinach puree (HASP), and ZnCl2-treated spinach puree (ZnSP). The results showed that native chlorophylls were converted to Mg-free pheophytin derivatives during digestion. Conversely, Zn-pheophytins were completely stable during the digestive process. Micellarization of total chlorophyll derivatives differed significantly ($p < 0.05$) for FSP (37.6%), HASP (17.2%), and ZnSP (8.7%). Intestinal cell uptake of micellarized pigments were 5-10% of micellarized chlorophyll derivatives. Moreover, Ferruzzi and Blakeslee (2007) reported the digestive and absorptive routes of chlorophyll and sodium copper chlorophyllin (SCC) derivatives (Figure 2.13). After chlorophyll derivatives release from the plant food matrix, natural chlorophyll (CHL) derivatives are exposed to the acidity of gastric digestion resulting in conversion to respective metal-free pheophytins (PHE). Within the upper small intestine, PHE derivatives partition into bile salt lipid micelles resulting in solubilization of PHE and movement across the unstirred water layer and ultimately into the enterocyte presumably by passive diffusion. The extent to which natural chlorophyll derivatives are metabolized and/or

secreted into circulation is currently unknown. While, after digestion, chlorophyllin that release from water-soluble SCC derivatives, significant degrade of Cu-chlorin e6 derivatives occurs, leaving primarily Cu-chlorin e4 and potentially residual amounts of Cu-chlorin e4 ethyl ester. The uptake of SCC derivatives by intestinal cells seems to proceed predominantly by a facilitated process (passive-mediated transport), with absorbed SCC derivatives efficiently effluxed back to the luminal compartment by unidentified efflux system.

Konishi, Kobayashi, and Shimizu (2003) studied the transepithelial transport of such common dietary phenolic acids as p-coumaric acid (CA) and gallic acid (GA) across Caco-2 cell monolayers. They found that CA transport was dependent on pH, and in a vectorial manner in the apical-basolateral direction. The permeation was concentration-dependent. Benzoic acid and acetic acid inhibited the permeation of CA. These results indicate that the transepithelial transport of CA was occured via the monocarboxylic acid transporter (MCT). On the other hand, the permeation of GA was not in a polarized manner, independent of pH and linearly increased with increasing concentration of GA. The transport rate of GA was about 100 times lower than that of CA, suggesting the transepithelial transport of GA occurring via the paracellular pathway.

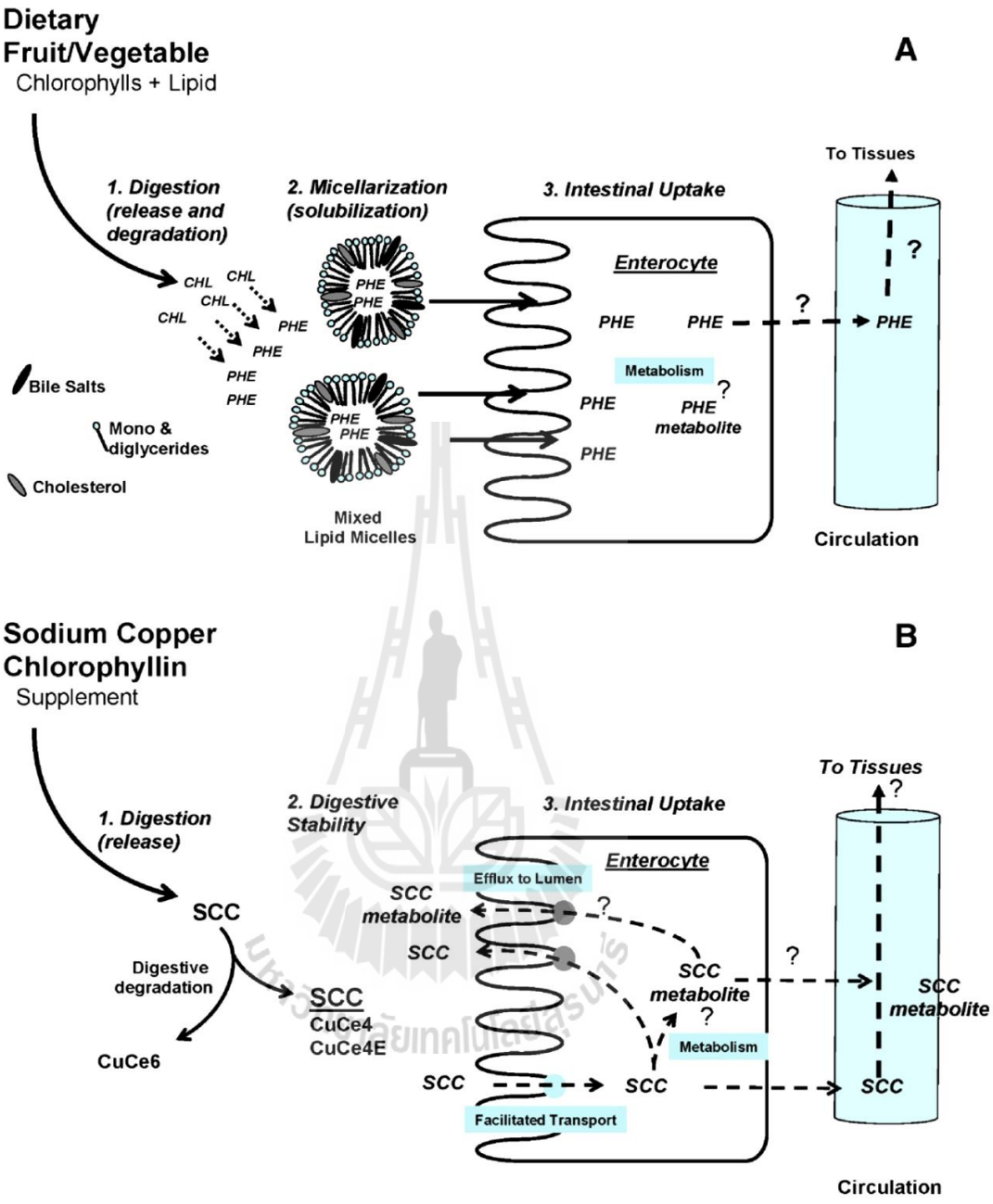


Figure 2.13 Schematic of digestive and absorptive routes for (A) chlorophyll and (B) SCC derivatives.

Source: Ferruzzi and Blakeslee (2007).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Raw material

The 3- and 6-year-old golden barrel cactus collected in Uncle Chorn's Cabin Garden, Pathumthani province, Thailand were used as a raw material. The plants was trimmed and cleaned, chopped and tray drying (Kluay Nam Thai Tow Op, Bangkok, Thailand) at 60°C for 12 h, after which they were ground with Ultra Centrifugal Mill Model ZM-1000 (Retsch, Haan, Germany), sieved with mesh size 0.2 mm and stored in vacuum package at -20ºC until use.

3.1.2 Chemicals

All chemicals including: 1,1-diphenyl-2-picryhydrazyl free radical (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), ferric chloride-6-hydrate, ferrous sulfate 7-hydrate, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium persulfate, gallic acid, BHT , Folin-Ciocalteu phenol reagent, and anhydrous sodium carbonate (Na_2CO_3) were of certified ACS grades and obtained from Sigma-Aldrich Co. (St. Louis, MO). Solvents including acetonitrile, ethanol, hydrochloric acid, sulphuric acid, and petroleum ether (Carlo, Italy).

3.2 Methods

3.2.1 Proximate analysis

Dried golden barrel cactus powder was analyzed for chemical composition following the AOAC (2005) official protocols. Moisture analysis was done by AOAC method 925.10. Ash quantification was done using AOAC method 900.02A. Protein and fat quantification use AOAC method 928.08 and 963.15 respectively. Crude fiber determination was done by AOAC method 978.10. Carbohydrates were determined by difference.

3.2.1.1 Moisture content

Drying to constant weight in an oven (Memmert, Germany) at 105ºC for 24 h was applied to determine moisture content of sample. One gram of sample was placed into a pre-weighed moisture can and dried in an oven at 105ºC for 24 h .After removing the sample from oven, they were placed in a desiccator to cool and reweighted. The moisture content of the sample was calculated as:

$$\text{Moisture (\%)} = \frac{\text{Sample weight (g) - weight of sample after drying (g)}}{\text{Sample weight (g)}} \times 100$$

3.2.1.2 Ash determination

Burning crucible in the muffle furnace at 550ºC for 3h and transfer to the desiccator to cool. Approximately one gram of sample was placed in crucible. The crucible is placed into muffle furnace at 550ºC for 18 h. After burning, the sample were removed from muffle furnace, cooled to room temperature in desiccator and then reweighed. The percentage of ash was calculated according to the formula:

$$\text{Ash (\%)} = \frac{\text{Ash weight (g)}}{\text{Sample weight (g)}} \times 100$$

3.2.1.3 Crude protein determination

Crude Protein determination was by Kjeldahl method of AOAC (2005). One grams of sample was weighed into a Kjedahl digestion flask and 25 ml of concentrated sulphuric acid added and 0.5 g of copper sulphate, 5 g of potassium Sulfate added. The mixture was digested until a clear solution was obtained. The digest was measured into the decomposition chamber of the distillation apparatus. Fifteen millilitres of 40% NaOH was added and the ammonia released was trapped into 25 ml of 4% boric acid solution containing mixed indicator. A colour change from pink to green was observed as the ammonia was trapped. Distillation was continued for 5 minutes and the titration using HCl. Percentage crude protein calculated by using the formula below.

$$\text{Crude protein (\%)} = \frac{N \times V \times 0.014 \times F \times 100}{\text{Sample weight (g)}}$$

N = concentration of HCl (mol)

V = the volume of HCl (ml)

F = 6.25

3.2.1.4 Crude fat determination (Soxhlet Method)

Crude fat was measured by the method of AOAC (2005). One gram of sample was weighed into a cellulose thimble. The extraction was carried out in the extractor (Foss tecator soxtec avanti 2050, Hoganas, Sweden) for 4 h with 80 ml of petroleum ether. The petroleum ether in the can is then heated. As the petroleum ether vapors reaches the condenser through the side arm of the extractor, it condenses to liquid form and drop back into the sample in the thimble, the petroleum ether soluble

substances are dissolved and are carried into solution through the siphon tube back into the can. The thimble is removed and most of the solvent is distilled from the can into the extractor. The can is then disconnected and placed in an oven at 60ºC for 3 h, cool in desiccator and weighed. Perform this experiment in triplicate and the percentage crude fat calculated by using the formula below.

$$\text{Crude fat (\%)} = \frac{\text{After extraction can weight (g) - Before extraction can weight (g)}}{\text{Sample weight (g)}} \times 100$$

3.2.1.5 Crude fiber determination

The organic residue left after sequential extraction of sample with petroleum ether can be used to determine the crude fiber. Weighed the crucible and weighed about 1 g sample into crucible. The crucible is placed into Fibertec 2010 Hot Extractor (Foss tecator, Hoganas, Sweden) and 150 ml of 1.25% sulphuric acid is added and the solution is gently boiled for about 30 min. Filtration and washed several times with boiling water (until the residue is neutral to litmus paper). Then 150 ml of 1.25% NaOH is added and boiled for another 30 min. Filtration and wash thoroughly with hot water and twice with 95% ethanol and dried at 105ºC in the oven for 3 h or until constant weighed. The crucible was cooled in a desiccator and weighed. The crucible is placed into muffle furnace (Gallenkamp muffle furnance, England) at 550ºC for 2 h. The crucible was again cooled and reweighed. The weight difference was recorded and perform this experiment in triplicate and the percentage crude fiber calculated by using the formula below.

$$\text{Crude fiber (\%)} = \frac{\text{Weight of fiber}}{\text{Sample weight (g)}} \times 100$$

3.2.1.6 Available carbohydrate

To calculate available carbohydrate by difference, the amount of moisture, Ash, crude fat, crude protein and dietary fiber are analyzed and subtracted from total carbohydrate, thus:

Available carbohydrate (%) = 100 - ([Moisture (%) + Ash (%) + Crude fat (%) + Crude protein (%) + Crude fiber (%)])

3.3 Preparation of acetonitrile extracts

The golden barrel cactus powder was extracted by the method described by Vermaak et al. (2010) with slightly modification. First 500 mg of golden barrel cactus powder were dissolved in 15 ml of acetonitrile, the solution was vortexed for 1 min and then sonicated in an ultrasonic bath (Elma Ultrasonic, Germany) at 100% power at temperature of 25°C for 20 min, after that the solution was centrifuged (Thermo electron LED GmbH D-37520, Germany) at 3000×g for 10 min and then filtered using Whatman No. 1 paper. The extraction was repeated two times. The supernatant was filtered, combined, and adjust volume to be 50 ml. The solvent was evaporated by rotary evaporator (Buchi Rotavapor R-114, USA) and dried by nitrogen gas at room temperature. The samples were kept at -20°C until use.

3.4 Phytochemical profile

3.4.1 Chemicals and standards

Solvents including acetonitrile, acetone, ethyl acetate, and methanol (Mallinckrodt-Baker, Phillipsburg, NJ) were of certified ACS and HPLC grades. A 1.0 M ammonium acetate buffer solution (Fluka; Ronkonkoma, NY, USA) was

prepared with deionized water and adjusted to pH 4.6 with glacial acetic acid. Standards of chlorophyll *a*, chlorophyll *b*, lutein, and gallic acid were obtained from Sigma-Aldrich (St. Louis, Mo., U.S.A.).

Pheophytin *a* and *b* standards were synthesized from chlorophyll *a* and *b* as described by Ferruzzi et al. (2001). Briefly, 1 mg of chlorophyll *a* or chlorophyll *b* was dissolved in 10 ml of acetone. Then 500 μl of 1.0 N HCl was added into 5 ml of chlorophyll *a* or *b* solution. Conversion was completed in approximately 2 h in the dark presenting a change of color from green to olive brown. Then pheophytins was followed by HPLC analysis as described below. Prior to HPLC calibration, each standard was dissolved in acetone and filtered through a 0.45 μm PTFE membrane.

3.4.2 Chlorophyll and lutein analysis

Chlorophyll and lutein analyses were achieved as described by Oonsivilai et al. (2007) with modification. Briefly, an Agilent 1100 Series HPLC system equipped with a diode array detector was utilized. Separation was achieved using a Grace-Vydac 201TP54 reversed-phase (4.6 mm × 250 mm) polymeric C18 column. A gradient elution parole was used based on a binary mobile phase system consisting of methanol : water : ammonium acetate (73 : 25 : 2, v/v/v) in reservoir A and ethyl acetate in reservoir B. A flow rate of 1.0 ml/min was applied with initial setting at 100% (A) with a linear gradient to 50 : 50 (A/B) over 10 min. The gradient was held for 10 min followed by a 5 min linear gradient back to 100% (A) and equilibration at the initial condition for 5 min for a total run time of 30 min. The injection volume was 25 μL. Detection and tentative identification of all chlorophyll derivatives and lutein accomplished using in-line photodiode array data between 250 and 600 nm. Because of between 250 and 600 nm is a range that maximum for chlorophyll derivatives and lutein absorption.

Identification of peaks was confirmed by comparing retention times obtained from chlorophyll *a*, chlorophyll *b*, lutein (Sigmae Aldrich Co.), pheophytin *a* and *b* standards. Quantification were calculated using the standard calibration curves of all chlorophyll derivatives and lutein. The calibration curves of all chlorophyll derivatives and lutein were linear over the range of 0.1-5 mg/L (correlation coincident = 0.99)

3.4.3 Total phenolic compounds quantification

The total phenolic concentration were determined by the Folin Ciocalteu procedure described by Oonsivilai et al. (2007) and gallic acid (Sigmae Aldrich Co.) were used as a standard. Aliquot of 0.02 ml. gallic acid standard/sample/blank was mixed with 1.58 ml of deionized water. Folin-Ciocalteu reagent of 0.1 ml was added, and the tube was stirred and allowed to stand at room temperature for 5 min. Then, 0.3 ml of Na_2CO_3 (20% w/v) was added to mixture and stored in the absence of light for 2 h at room temperature. Absorbance was measured at 765 nm using spectrophotometer (Biochrom Libra S22 S/N 97765, UK). Gallic acid (0, 50, 100, 250, 500, and 750 mg/L) was used for calibration of a standard curve. The results were expressed as mg gallic acid equivalents (GAE)/100g of raw material. Triplicate measurements were taken and mean values calculated.

3.5 Antioxidant activity

3.5.1 DPPH free radical scavenging assay

The scavenging activity of the stable 1,1-diphenyl-2-picrylhy-drazyl (DPPH) free radical was determined by the method described by Oonsivilai et al. (2008). Briefly, 100 µM violet solution of 0.1 mM DPPH in methanol was prepared. Aliquots

0.1 ml of standard/sample/blank transferred into test tube. After addition of 1.90 ml DPPH solution, solution was mixed and then let standing for 15 min, the absorbance of solution at 515 nm was read. The percentage of scavenging was calculated as the ratio of the absorption of the sample relative to the control DPPH solution, without the extracts. The BHT and ascorbic acid in methanol solution were used as positive controls. Inhibition of free radical DPPH was calculated according to the formula:

$$\% \text{ Inhibition } = \left[\frac{(Ab - As)}{Ab}\right] \times 100$$

Where A_b is absorbance of sample Blank, A_s is absorbance of sample and from a plot of concentration against % inhibition, a linear regression analysis was performed to determine the IC_{50} or extract concentration resulting in a 50% inhibition value for each sample (Meot-Duros and Magné, 2009).

3.5.2 Scavenging activity of ABTS radical cation assay

ABTS radical scavenging activity of extracts was determined according to Ksouri et al. (2009). The $ABTS^{\cdot+}$ cation radical was produced by the reaction between 5 ml of 14 mM ABTS solution and 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 12-16 h. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734 nm. In a final volume of 2 ml, the reaction mixture comprised 1,900 µl of $ABTS^{\cdot+}$ solution and 100 µl of the sample extracts at various concentrations and distilled water for control. The reaction mixture was homogenized and its absorbance was recorded at 734 nm at 6 min after mixing. Similarly, the mixture of standard group reaction was obtained by mixing 1,900 µl of $ABTS^{\cdot+}$solution with 100 µl of BHT or ascorbic acid for the

antiradical activity, ABTS scavenging ability was expressed as IC_{50} (mg/ml). The inhibition percentage of ABTS radical was calculated using the following formula:

$$\text{ABTS scavenging effect} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

Where A_0 and A_1 are the absorbencies of the control and test sample, respectively. From a plot of concentration against % inhibition, a linear regression analysis was performed to determine the IC_{50} or extract concentration resulting in a 50% inhibition value for each sample (Meot-Duros and Magné, 2009).

3.5.3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure of (Oonsivilai et al., 2008) with slight modification. Briefly, the FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM ferric(III) chloride solution in proportions of 10 : 1 : 1 (v/v) respectively. The FRAP reagent was prepared fresh daily and warmed at 37°C in a water bath prior to use. Fifty microliters of sample were added, along with 150 µl of deionized water to 1.5 ml of the FRAP reagent. The reaction mixture absorbance was then recorded at 593 nm at 4 min. The standard curve was constructed using ferrous sulphate solution (100-1000 µM), and the results were expressed as mM ferrous (II)/g dry weight of plant material. All the measurements were taken in triplicate and the mean values were calculated.

3.6 Cytotoxicity

3.6.1 Cell culture

Caco-2 cells, originating from a human colorectal adenocarcinoma (ATCC Cat. No. HTB-37, ATCC, USA) and HepG2 cell, originating from a human hepatocellular carcinoma (ATCC Cat. No. HB-8065). Cell culture of Caco-2 cells and HepG2 cell performed as described by Garrett et al. (1999); Ferruzzi et al. (2001). The Caco-2 and HepG2 cell were grown in T-75 flask (75 cm^2 cell culture flask) and maintained with complete medium. The complete medium contained with Dulbecco's modified eagle's medium (DMEM, Gibco cat.no. 11965-092), 10.0% (v/v) heat-inactivated fetal bovine serum (FBS, Gibco cat.no. 10270-098), 1% (v/v) non-essential amino acids (MEM NEAA, Gibco cat.no. 11140-050), 1% (v/v) L-glutamine (Gibco cat.no. 25030-081, 1% (v/v) penicillin-streptomycin (Pen-Strep, Gibco cat.no. 15140-122). All cells were incubated at 37ºC in a humidified incubator atmosphere of 95% air 5% carbon dioxide. Fresh complete medium was changer every 2 days.

3.6.2 MTT assay

Caco-2 and HepG2 were seeded in a 96-well plate with 5,000 cells per well in 100 µl media and incubated for 24 h. All the golden barrel cactus dried crude extracts were dissolved in dimethyl sulfoxide (DMSO) to make stock solution 1000 ug RM/ml then diluted to various extracts concentrations in media from 25 ug RM/ml to 500 ug RM /ml. The golden barrel cactus dried crude extracts from simulated digestion or digesta were dissolved and diluted to various digesta concentrations in media from 25 ug/ml to 500 ug/ml. The extracts and digesta were added to the wells and incubated for 24 h and the viability of the cells was measured by using the MTT assay. A modified version of Mosmann (1983) was used. Briefly, a volume of 10 µl of MTT

solution (5 mg/ml in PBS) was added to the medium in each well. The plates were then incubated for 4 h at 37°C. After incubation, a volume of 100 µl of DMSO was added to each well for 10 min at room temperature. Absorbance was measured at 570 nm using microplate reader (Bio-Rad Benmark Plus, UK). The data were analyzed to determine the LC_{50} for each extract sample. Two controls were set up, one with medium as reagent control and the second with the DMSO 0.1% as solvent control. A dose-response curve was derived from 6 concentrations in the test range of 25-500 µg/ml using 5 wells per concentration to determine the mean of each point. Results of toxic compounds were expressed as the concentration of sample required to kill 50% (Median lethal concentration, LC_{50}) of the cells compared with controls. The cell viability was determined by the following formula:

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

Dose-response curves between percentages of cell viability and concentrations of the extracts were constructed. The median lethal concentration (LC_{50}) can be calculated (Okonogi et al., 2007).

3.7 Bioaccessibility and bioavailability

3.7.1 *In vitro* digestion

Simulated digestion of golden barrel cactus crude extracts was carried out according to Garrett et al. (1999); Ferruzzi et al. (2001) with modification. All reagents used in simulated digestion are shown in Appendix A.

3.7.1.1 Gastric phase

The sample, golden barrel cactus crude extracts (2 g of died cactus powder) were homogenized with 20 ml of 120 mM NaCl (5% Tween 80) in 50 ml polypropylene tube by homogenizer (model T25D, Germany), then adjusted pH to 2.0 ± 0.1 with 1 M HCl. Porcine pepsin (40 mg/ml in 100 mM HCl) 2 ml was added. Then the volume was adjusted to 40 ml with 120 mM NaCl, blanked remaining tube with nitrogen gas, tightly cap and sealed with parafilm and incubated in a shaking water bath (JULABO, SW22, USA) for 1 hr at 37ºC, 95 rpm.

3.7.1.2 Small intestinal phase

At the end of gastric phase, the small intestinal phase was followed by bringing up pH to 6.0 ± 0.1 with 1 M sodium bicarbonate ($NaHCO_3$). Crude bile extract (40 mg/ml in 100 mM $NaHCO_3$) 3 ml, pancreatin (12 mg/ml in 100 mM $NaHCO_3$) 2 ml were added to the reaction tube. The pH of sample was increased to 7.0 ± 0.1 with 1 M $NaHCO_3$. Then the tube was filled with nitrogen gas tightly cap and sealed with parafilm before putting in a shaking water bath (JULABO, SW22, USA) for 2 h at 37ºC, 95 rpm to complete the intestinal phase of the in vitro digestion process.

After completion of the gastric and small intestinal phases, which was called “digesta”, 10 ml mixture was transferred into a 15 ml polypropylene tube, blanketed with nitrogen, and stored at -80°C until analysis within two weeks. Chlorophyll and lutein concentrations were determined by HPLC. Total phenolic concentrations were determined with Folin-Cicalteau colorimetric assay. Determination of phytochemical compounds (chlorophyll, lutein, and total phenolic) stability during simulated digestion can be calculated by using following formula:

$$\text{Digestive stability (\%)} = \frac{\text{Amount of phytochemical compounds in digesta}}{\text{Amount of phytochemical compounds in pre-digested sample}} \times 100$$

3.7.1.3 Isolation of the aqueous or micellar fraction from digesta

The digesta was transferred into a 15 ml test tube and centrifuged (Thermo electron LED GmbH D-37520, Germany) at 8,000 rpm at 4°C for 90 min to separate aqueous and residual particulate fractions. The aqueous fraction was filtered through a 0.2 μm PTFE membrane. All procedures were performed in subdued light and samples were blanketed with nitrogen to minimize oxidation. Aliquots of pre-digested, digesta, and aqueous fraction were collected and stored at -80°C until analysis. Chlorophyll and lutein concentrations were determined by HPLC. Total phenolic concentrations were determined by Folin-Cicalteau colorimetric assay. The percentage of bioaccessibility of the chlorophyll, lutein, and total phenolic in the test food present in the filtered aqueous fraction after simulated digestion can be calculated by using the following equation

$$\text{Bioaccessibility (\%)} = \frac{\text{Amount of phytochemical compounds in aqueous fraction}}{\text{Amount of phytochemical compounds in digesta}} \times 100$$

3.7.2 Cell culture

Caco-2 cells, originating from a human colorectal adenocarcinoma (ATCC Cat. No. HTB-37, ATCC, USA). Cultures of Caco-2 cells were used between passage numbers 22 to 32. Cells were grown in T-75 flask (75 cm^2 cell culture flask) and maintained with complete medium. The complete medium contained with Dulbecco's modified eagle's medium (DMEM), 10.0% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) non-essential amino acids, 1% (v/v) L-glutamine, 1% (v/v) penicillin-

streptomycin (antibiotic). All cells were incubated at 37ºC in a humidified incubator atmosphere of 95% air 5% carbon dioxide. Fresh complete medium was changer every 2 days. Cultures were used for experiments 21 days post-confluence, since previous studies have shown that Caco-2 cells exhibit enterocyte characteristics (or maximum differentiation) during this period as assessed by the activities of the marker enzymes alkaline phosphatase and sucrase (Ellwood, Chatzidakis and Failla, 1993). Preparation of media for cell culture are shown in Appendix A.

3.7.3 Cellular uptake of aqueous fraction

Cellular uptake of aqueous fraction was determined according to Ferruzzi et al. (2001) with slightly modification. Caco-2 human intestinal cells (ATCC Cat. No. HTB-37, ATCC, USA; passage 22) were used at seeded density of 5,000 cells/cm^2 in T-25 flask (25 cm^2 cell culture flask) and the complete medium (5 ml/flask) were changed every 2 days. For initial experiments examining the uptake of aqueous fraction, cells directly attached to plastic wells were used 11-14 day after monolayers became confluent as the brush border membrane is fully differentiated at this time (Pinto et al., 1983). Monolayers were washed with DMEM before adding 5 ml of the test medium containing the aqueous fractions that was diluted 1 : 3 (v/v) with complete medium. Monolayers were incubated at 37ºC at indicated time and harvested at 2, 4, and 6 h. The test media was removed and the monolayers were washed twice with 2 ml cold PBS. Preliminary work showed that the aqueous fractions-enriched media was not toxic to the cells (data not shown). All cells were collected by scraping into 1.5 ml of cold PBS and stored at -80°C until analysis. Medium and cells were collected and analyzed by HPLC. Cellular protein concentrations were determined by the Bradford assay using bovine serum albumin as

a standard (Bradford, 1976). Formulas for the calculation of cell uptake was shown as follows:

$$\% \text{ Cell uptake} = \frac{\text{Amount of phytochemical in cell}}{\text{Amount of phytochemical in test medium}} \times 100$$

3.7.4 Transport of chlorophyll derivatives, lutein and total phenolic by Caco-2 cells

Transport of chlorophyll derivatives, lutein and total phenolic by Caco-2 cells was determined according to Chitchumroonchokchai et al. (2004) with slightly modification. To transport experiments, Caco-2 cells (passage 22) were seeded with 3×10^5 cell/well on Transwell inserts (6-well dishes, pore size 0.4 mm, diameter 24 mm, apical volume 1.5 ml, basolateral volume 2.5 ml). Cultures were used for experiments 11-14 day after reaching confluency because lipoprotein synthesis and secretion by Caco-2 cells are maximal at this time (Mehran, Levy, Bendayan and Seidman, 1997). After washing the monolayers with DMEM, the apical side of the transwell plate received 1.5 ml of the test medium containing the aqueous fractions that was diluted 1 : 3 (v/v) with complete medium. The basolateral chamber received 2.5 ml complete medium. Treatment with the test medium was for 6 h after which time the test media was removed and the cell layer was washed with warm PBS. Media (2.5 ml) containing taurocholate (0.5 mM), oleic acid (1.6 mM) and glycerol (45 mM) was added to the apical chamber and incubated for 16 h for the stimulation and secretion of chylomicrons (O'Sullivan et al., 2007). After the incubation time, media from each side of the membrane were removed, the monolayer was washed twice with warm PBS and cells were scraped into 1.0 ml of cold PBS and stored at

-80 °C until analysis. Formulas for the calculation of cell transport was shown as follows:

$$\% \text{ Cell transport} = \frac{\text{Amount of phytochemical in basolateral chamber}}{\text{Amount of phytochemical in test medium}} \times 100$$

3.7.5 Digesta, aqueous fraction, media, and cell pellet extraction

Digesta, aqueous fraction, media and cell pellet extraction by the method described by Ferruzzi et al. (2001) with slightly modification. Thawed samples (2 ml) of digesta, aqueous fraction, and medium from apical and basolateral compartments were extracted by addition of 4.0 ml acetone/petroleum ether (50 : 50) (0.1% BHT) and vortexed for 1 min. The sample was then centrifuged (2000×g) for 5 min to hasten phase separation. The petroleum ether layer was collected and saved. Extraction was repeated a total of 3 times and the combined petroleum ether fractions were dried under a stream of nitrogen gas, redissolved in acetone, and analyzed immediately. Frozen pellets of Caco-2 cells collected were thawed before addition of 0.5 mL of sodium dodecyl sulfate (34.6 mmol/L) in ethanol (0.1% BHT), and the sample was vortexed for 1 min. Following sonicated for 30 s on ice. Chlorophyll derivatives and lutein were extracted by addition of 0.5 mL of acetone/petroleum ether (1 : 2). The samples were vortexed and briefly centrifuged (2000×g) for 5 min to hasten the phase separation. The petroleum ether layer was collected and saved. The extraction was repeated a total of three times and the combined petroleum ether fractions were dried under a stream of nitrogen gas, redissolved in acetone, and analyzed immediately. Chlorophyll and lutein concentrations for all sample were determined by HPLC. Total phenolic concentrations were determined by Folin-Cicalteau colorimetric assay.

3.7.6 Protein determination

Proteins concentration was determined by the Bradford assay using bovine serum albumin as a standard (Bradford, 1976). Dilute the Bradford reagent fivefold in DI water (1 part Bradford: 4 parts DI) and filtered through Whatman No. 1 paper. Add 20 µl of sample/standard to 1 ml of the diluted reagent and mix. Incubate at room temperature for at for 5 min. The absorbance at 595 nm was measured. The calibration curves of bovine serum albumin were linear over the range of 0.125-2 mg/ml.

3.8 Statistical analyses

All of the samples were performed at least in triplicate. All statistical analyzed were perform using SPSS (version 16.0, SPSS Inc., USA). Data are presented as mean ± standard deviation (SD). Means were compared by independent-samples t-test. Differences were considered significantly at $p < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Chemical approximate analysis

The chemical composition of dried golden barrel cactus stems at the age of 3- and 6-year-old powders are presented in Table 4.1. It was found that ash or mineral and crude fiber of 6-year-old golden barrel cactus powder was significant ($p < 0.05$) higher than 3-year-old golden barrel cactus powder. In case of the protein content percentage in both ages was low and similar to protein content percentage in *Opuntia ficus indica* reported by Hernández-Urbiola, Pérez-Torrero, and Rodríguez-García (2011). The fat content of the powders decreased as a function of age and no direct relationship with age was observed. The decreases noticed were perhaps due to physiological changes or differences in climatic condition such as precipitation or irrigation where the plants were grown (Sáenz, 1997). The differences in chemical composition of golden barrel cactus might be due to differences in soils, climatic environment (Rodriguez-Felix, 2000; Rodríguez-García et al., 2007; Stintzing and Carle, 2005) including cactus age different (Hernández-Urbiola et al., 2011). Our result demonstrated that the ash and crude fiber showed a positive relationship related to the age of cactus. The future research may make it possible to apply and develop older golden barrel cactus as a dietary fiber in dietary supplements. It might be better for mineral and fiber source.

Table 4.1 Chemical composition of 3- and 6-year-old golden barrel cactus powders.

Composition	Content (% dry weight)	
	3-year-old cactus powder	**6-year-old cactus powder**
Moisture	0.86 ± 0.06^{a}	0.53 ± 0.25^{a}
Ash	22.83 ± 0.03^{a}	25.33 ± 0.02^{b}
Crude protein	14.48 ± 0.12^{a}	14.82 ± 1.33^{a}
Crude fat	1.39 ± 0.36^{a}	1.02 ± 0.09^{a}
Crude fiber	11.18 ± 0.20^{a}	15.99 ± 0.80^{b}
Available carbohydrate	49.26 ± 0.54^{b}	43.64 ± 1.11^{a}

Note: Data are means ± SD; Data in the same row with different superscripts are significantly different ($p < 0.05$).

4.2 Phytochemical profile of golden barrel cactus extracts

4.2.1 Chlorophyll and lutein profile of golden barrel cactus extracts

Phytochemical analysis revealed the presence of lutein and four chlorophyll derivatives including, chlorophyll *a*, chlorophyll *b*, pheophytin *a* and pheophytin *b* are shown in Figure 4.1. It was found that lutein and total chlorophyll contents of 3-year-old golden barrel cactus extracts was significantly ($p < 0.05$) higher than 6-year-old extracts (Table 4.2). Total chlorophyll contents was 526.29 and 366.37 µg/g RM for 3- and 6-year-old golden barrel cactus extracts, respectively. Chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and pheophytin *b* were 179.41, 97.26, 243.46, 6.16 and 115.15, 91.28, 154.08, 5.87 µg/g RM for 3- and 6-year-old golden barrel cactus extracts, respectively.

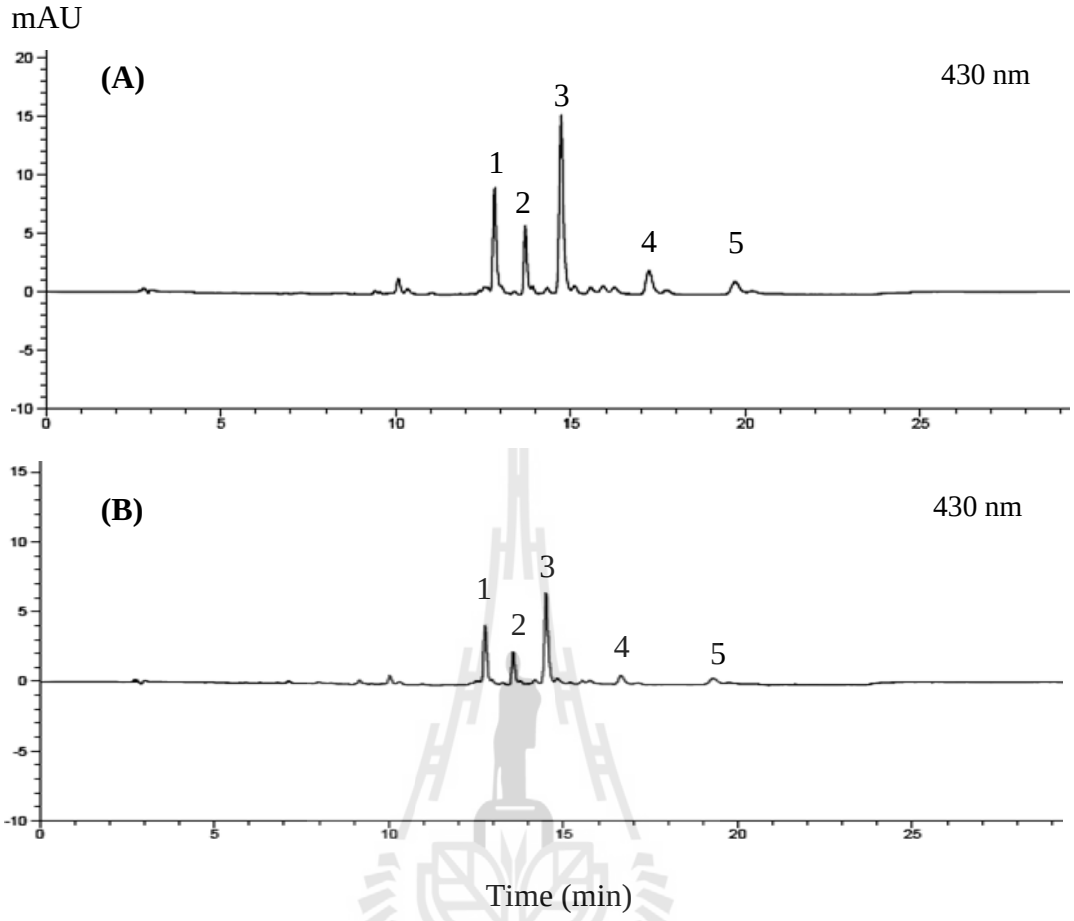


Figure 4.1 Representative HPLC chromatograms of chlorophyll derivatives and lutein present in (A) three-year-old golden barrel cactus extracts, (B) six-year-old golden barrel cactus extracts. Peak identifications: (1) lutein; (2) chlorophyll *b*; (3) chlorophyll *a*; (4) pheophytin *b*; (5) pheophytin *a*.

Table 4.2 Phytochemical content of golden barrel cactus extracts.

Phytochemical content	**Golden barrel cactus extracts**	
	3-year-old	**6-year-old**
Lutein (µg/g RM)	36.14 ± 0.39^{b}	30.44 ± 0.45^{a}
Chlorophyll *a* (µg/g RM)	179.41 ± 2.89^{b}	115.15 ± 5.83^{a}

Table 4.2 Phytochemical content of golden barrel cactus extracts (continued).

Phytochemical content	Golden barrel cactus extracts	
	3-year-old	**6-year-old**
Chlorophyll *b* (µg/g RM)	97.26 ± 0.31^{b}	91.28 ± 0.80^{a}
Pheophytin *a* (µg/g RM)	243.46 ± 10.59^{b}	154.08 ± 5.23^{a}
Pheophytin *b* (µg/g RM)	6.16 ± 1.45^{a}	5.87 ± 1.48^{a}
Total Chlorophylls (µg/g RM)	526.29 ± 10.45^{b}	366.37 ± 1.22^{a}

Note: Data are means ± SD; Data in the same row with different superscripts are significantly different ($p < 0.05$).

Lutein content was 36.14 µg/g RM and 30.44 µg/g RM for 3- and 6-year-old golden barrel cactus extracts, respectively. Lutein is found in many fruits and vegetables including richest and purest plant source such as marigold flower (*Tagetes* spp.). When compared lutein content in golden barrel cactus extracts with marigold flower from previously studied of Piccaglia, Marotti and Grandi (1998), it was found that the lutein content of both golden barrel cactus extracts was less than marigold flower (180 µg/g RM). The lutein and total chlorophyll contents were negative correlation with the age of the golden barrel cactus. The lutein and chlorophyll contents were decreased with the older age of the golden barrel cactus and as a result the lutein/chlorophyll ratio also increased. The reason of differences in phytochemical concentration of golden barrel cactus are physiological changes. The golden barrel cactus stems at age 3-year-old has a green succulent part more than 6-year-old golden barrel cactus. Their stems have green succulent part mainly contain chlorophyll and lutein (Holasova, Dostalova, Fiedlerova, and Horacek, 2009; Shetty, Rana, and

Preetham, 2012). However, low amounts of lutein and total chlorophylls were found in golden barrel cactus extracts. It may be due to application of acetonitrile as a solvent for golden barrel cactus extraction, which might not be appropriate for lutein and chlorophylls extraction. The reason is the polarity of acetonitrile that has high polarity in extraction process. In addition, acetonitrile has been applied in initially for steroidal glycoside extraction.

4.2.2 Total phenolic contents of golden barrel cactus extracts

Total phenolic contents of 3- and 6-year-old golden barrel cactus extracts were tested by Folin-Ciocalteu method. However, the Folin-Ciocalteu method uses molybdotungstophosphoric heteropolyanion reducing reagent which indirectly detects phenolics, but lacks specificity (Prior, Wu, and Schaich, 2005). It has been reported by Prior et al. (2005) that the Folin-Ciocalteu method suffers from a number of interfering substances, in particular, ascorbic acid, sugars, aromatic amines, sulfur dioxide, organic acids, and Fe(II), and correcting for these interfering substances is essential. The list of Folin-Ciocalteu interfering substances not only the aforementioned, but also include at least 50 additional organic compounds naturally found in fruits, vegetables and plant or in the polyphenol extraction media (Prior et al., 2005). The total phenolic contents in each extract are presented in Figure 4.2. It was found that the total phenolic contents of 3-year-old golden barrel cactus extracts was significantly higher than 6-year- old golden barrel cactus extracts ($p < 0.05$). The 3- and 6-year-old golden barrel cactus extracts showed a total phenolic content of 3545.35 and 2557.96 mg gallic acid equivalent / 100 g of RM, respectively. The total phenolic contents of both golden barrel cactus extracts was higher than two commercial and eight wild *Opuntia* spp. (180-2000 mg gallic acid equivalent/100g of

RM), which was reported by Guevara-Figueroa et al. (2010). The phenolic contents of plants are affected by both endogenous such as genetic and environmental factors such as UV light, heavy metal and pathogens (Achakzai, Achakzai, Masood, Kayani, and Tareen, 2009). In this study, acetonitrile was used as a solvent in golden barrel cactus extraction. Some high polarity phenolic compounds may be found in golden barrel cactus extracts such as ferulic acid, sinapic acids, and their esters, or chlorogenic acid that are important phenolic compounds (Oksana, Marian, Mahendra, and Bo, 2012). Recently, researchers unveil that the *Opuntia* spp. had the highest total phenolic acid content such as, *p*-coumaric acid, caffeic acid, 4-hydroxybenzoic acid, ferulic acid, and salicylic acid (Guevara-Figueroa et al., 2010).

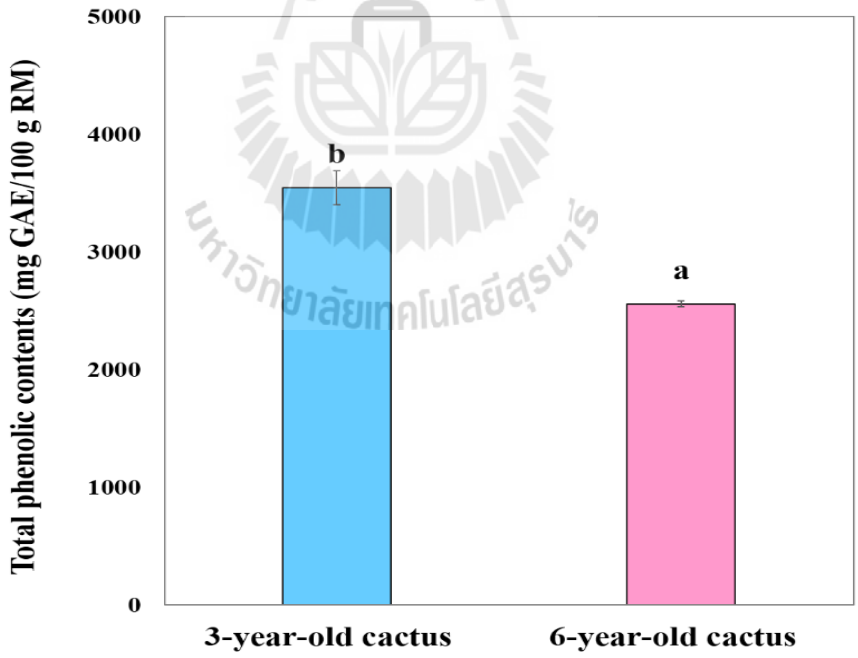


Figure 4.2 Total phenolic contents of 3-year and 6-year-old golden barrel cactus extracts. Data are means ± SD; Different letters over the error bars denote that the means differed significantly ($p < 0.05$).

4.3 Bioactivities

4.3.1 Antioxidant activity of golden barrel cactus extracts

4.3.1.1 DPPH free radical scavenging activity

The antioxidant activity of 3- and 6-year old golden barrel cactus extracts were tested by DPPH radical scavenging activity revealed antioxidant potency based on IC_{50} values when compared with BHT and ascorbic acid as positive control. The antioxidant activity in each sample extracts are presented in Table 4.3. It was found that antioxidant activity of 3-year-old golden barrel cactus extracts was significantly ($p < 0.05$) higher antioxidant activity than 6-year-old golden barrel cactus extracts at IC_{50} values 112.60 mg RM/ml and 191.90 mg RM/ml, respectively. The contents of lutein, total chlorophylls and total phenolic in 3-year-old cactus extracts had more than in 6-year-old cactus extracts, which could be supplied hydrogen atom for DPPH radical.

Table 4.3 Antioxidant activities of 3- and 6-year-old golden barrel cactus extracts by DPPH assay.

Samples	**IC_{50} of DPPH radical scavenging activity (mg RM /ml)**
3-year-old cactus	112.60 ± 1.42^{b}
6-year-old cactus	191.90 ± 0.52^{c}
BHT	0.35 ± 0.04^{a}
Ascorbic acid	0.08 ± 0.01^{a}

Note: Data are means ± SD; Data in the same row with different superscripts are significantly different ($p < 0.05$).

The IC_{50} values was 0.08 mg/ml for ascorbic acid. BHT showed 50% inhibition at 0.35 mg/ml. Moreover, the antioxidant activity of both golden barrel cactus extracts was lower than two species dragon fruits, *Hylocereus undatus* (white dragon fruit) and *Hylocereus polyrhizus* (red dragon fruit) (0.30 and 0.40 mg/mL), which was reported by Ruzlan et al. (2008). However, antioxidant activity of both ages was less than BHT and ascorbic acid, the commercial standards.

4.3.1.2 Scavenging activity of ABTS radical cation

$ABTS^{\cdot+}$ radical scavenging activity of 3- and 6-year-old golden barrel cactus extracts revealed antioxidant potency based on IC_{50} values when compared with BHT and ascorbic acid as positive control are showed in Table 4.4. The antioxidant potential is inversely proportional to IC_{50} value. Low IC_{50} value indicated that radical scavenging activity was increased.

Table 4.4 Antioxidant activities of 3- and 6-year-old golden barrel cactus extracts by ABTS assay.

Samples	**IC_{50} of $ABTS^{\cdot+}$ radical scavenging activity (mg RM/ml)**
3-year-old cactus	44.62 ± 2.43^{b}
6-year-old cactus	81.84 ± 0.42^{c}
BHT	0.09 ± 0.003^{a}
Ascorbic acid	0.04 ± 0.002^{a}

Note: Data are means ± SD; Data in the same row with different superscripts are significantly different ($p < 0.05$).

$ABTS^{\cdot+}$ radical scavenging activity of 3-year-old golden barrel cactus extracts was significantly ($p < 0.05$) higher scavenging activity than in 6-year-old golden barrel cactus extracts at IC_{50} values 44.62 mg RM/ml and 81.84 mg RM/ml, respectively. The trends of antioxidant activity in 3- and 6-year-old cactus extracts was similar to the result obtained by using the DPPH assay. Additionally, the antioxidant activity of both golden barrel cactus extracts was less than the commercial standards. A possible explanation for this might be from the antioxidant activity of golden barrel cactus extracts were calculated based on raw material, while as the BHT and ascorbic acid are a commercial product with high quality and more purity. Moreover, the results of both golden barrel cactus extracts was lower antioxidant activity than medicinal plants such as *Emex spinosus*, *Asphodelus tenuifolius*, *Aizoon canariense* (IC_{50} values were 5.79-19.78 mg/ml) from previously reported by Al-Laith, Alkhuzai, and Freije (2015).

4.3.1.3 Ferric reducing antioxidant power assay (FRAP)

The 3-and 6-year-old golden barrel cactus extracts were tested for antioxidant activity by FRAP assay which is a simple assay that is fast and gives reproducible results (Benzie and Strain, 1996). In this assay, the antioxidant activity is determined based on of the ability to reduce ferric (III) iron to ferrous (II) iron. The results were expressed as mmol ferrous iron (Fe^{2+}) equivalents per gram raw material. The antioxidant activity in each sample extracts are presented in Table 4.5. It was found that the antioxidant activity showed no statistically significant differences between 3- and 6-year-old golden barrel cactus extracts. The FRAP value was 0.014mmol Fe^{2+}/g RM and 0.01 mmol Fe^{2+}/g RM for 3-and 6-year-old golden barrel cactus extracts, respectively. Nevertheless, the antioxidant activity of both golden barrel cactus extracts was less than tested commercial standard. When compared to

Wong, Li, Cheng, and Chen (2006), it was found that the antioxidant activity of both golden barrel cactus extracts was less than Chinese medicinal plants such as *Polygonum multiflorum* Thunb, *Glycyrrhiza uralensis* Fisch, and *Tremella fuciformis* Berk (0.498, 0.115, and 0.049 mmol Fe^{2+}/g of sample).

Table 4.5 Antioxidant activities of 3- and 6-year-old golden barrel cactus extracts by FRAP assay.

Samples	**FRAP values (mmol Fe^{2+}/g RM)**
3-year-old cactus	0.014 ± 0.001^{a}
6-year-old cactus	0.010 ± 0.002^{a}
BHT	2.50 ± 0.21^{b}
Ascorbic acid	8.61 ± 0.14^{d}

Note: Data are means ± SD; Data in the same row with different superscripts are significantly different ($p < 0.05$).

Several reports have shown the positive relationship between antioxidant activity potential and amount of phenolic compounds in fruits, plants, and vegetables (Abdille, Singh, Jayaprakasha, and Jena, 2005; Maisuthisakul, Pasuk, and Ritthiruangdej, 2008; Sellappan, Akoh, and Krewer, 2002). The golden barrel cactus extracts show high total phenolic contents that exhibit strong antioxidant activities. The antioxidant activities of both golden barrel cactus extracts were different. A possible explanation for this might be that multiple factors including concentration of the extracts and phytochemical profile of the extracts. In addition, 3-year-old golden barrel cactus extracts had chlorophyll derivatives, lutein and total phenolic

contents higher than 6-year-old golden barrel cactus extracts relating to their antioxidant activity.

4.3.2 Cytotoxicity

The Caco-2 and HepG2 cell line were used for the cytotoxicity test in this study. The cytotoxicity against the Caco-2 cell line could provide preliminary information for study of toxicity on normal intestinal cell type (Okonogi, Duangrat, Anuchpreeda, Tachakittirungrod, and Chowwanapoonpohn, 2007) and the selection of appropriate concentrations required in the cellular uptake and transport study of golden barrel cactus extracts after simulate *in vitro* digestion. The HepG2 cell line is one of the most widely used for evaluation the toxicity of chemicals and drugs (Knasmüller et al., 2004). Because of the liver is perfectly designed as a drug removal organ. Most xenobiotics enter the body by absorption through the gastrointestinal tract and then venous drainage via the portal vein to the liver. Along with the combination of drug-metabolising enzymes and drug transporters present in the intestinal mucosa, the liver provides an efficient barrier that prevents xenobiotics entering the body's circulation. The Caco-2 and HepG2 cells damage induced by golden barrel cactus extracts were a loss of mitochondria membrane resulting in a decrease uptake of the MTT. An effect on the reduction of mitochondrial succinate dehydrogenase activities was indicated by a decrease in the formazan product of the MTT assay. Cytotoxicity in cell culture is typically expressed as LC_{50}, the concentration of a given agent which is lethal to 50% of the cells. This number is dependent on the incubation time with the agent (Zhang et al., 2007). Cytotoxicity tests of golden barrel cactus extracts before and after *in vitro* digestion at 25-500 μg RM/ml to those cells cultured in complete medium were carried out for 24 hr. The LC_{50} value for both ages of golden barrel cactus extracts before and after *in vitro* digestion are shown in Table 4.6. Our results

indicated that the extremely high value of LC_{50} (> 200 μg RM/ml) against both cell types indicating non-toxic activity to the cells based on MTT assay. This result could provide preliminary information for study of cytotoxicity.

Table 4.6 Cytotoxic activities of 3- and 6-year-old golden barrel cactus extracts against Caco-2 cells and HepG2 cells.

Samples	LC_{50} (μg RM/ml)	
	Caco-2 cells	HepG2 cells
3-year-old cactus	> 200	> 200
6-year-old cactus	> 200	> 200
3-year-old cactus (digesta)	> 200	> 200
6-year-old cactus (digesta)	> 200	> 200

4.4 Bioaccessibility and bioavailability of golden barrel cactus extracts using simulated digestion and Caco-2 human intestinal cells

4.4.1 Digestive stability and percentage of aqueous micellar fraction of chlorophyll derivatives, lutein and total phenolics from 3- and 6-year old golden barrel cactus extracts after simulated digestion

Generally, the bioavailability of a dietary compounds or phytochemicals are dependent upon its digestive stability, accessibility for uptake across the apical surface of intestinal epithelial cells, and the efficiency of its transepithelial passage for delivery to peripheral tissues (Walsh, Zhang, Vodovotz, Schwartz, and Failla, 2003). This study investigated the digestive stability, bioaccessibility and cellular uptake of phytochemical compounds (lutein, chlorophyll derivatives and phenolic compounds)

in 3- and 6-year-old golden barrel cactus extracts by using coupled *in vitro* digestion and Caco-2 intestinal cells model. The quantity of lutein, chlorophyll derivatives and total phenolics in pre-digested, digesta, and aqueous or micellar fraction from golden barrel cactus at age 3- and 6-year-extracts were presented in Table 4.7. Lutein, chlorophyll derivatives and phenolic compounds distribution in the digesta of 3-year-old golden barrel cactus extracts were similar to 6-year-old extracts. Pheophytin *a* showed high values in pre-digested due to in the dried sample process the temperature was 60°C. In the basically, chlorophyll was converted to pheophytin happened above the threshold temperature of 50°C to 60°C (Scheer, 1991). In addition, Ito, Ohtsuka, and Tanaka (1996) reported that chlorophyll *b* released from light-harvesting chlorophyll *a*/*b*-protein complexes of photosystem II and converted to chlorophyll a via 7-hydroxymethyl chlorophyll in the lipid bilayer. Therefore, it is possible that the amount of pheophytin *a* was increased. Chlorophyll *a* and chlorophyll *b* were not detected in digesta and aqueous fraction, the reason may be natural chlorophylls are modified by heat and acid and may be susceptible to degradation when subjected to the harsh digestive environment (Ferruzzi, Failla, and Schwartz, 2001). Since the standard conditions for the gastric phase of *in vitro* digestion are incubation at 37°C in a shaking bath at pH 2 for 1 h resulting in a perceivable discoloration of vegetable tissue from green to brown known as pheophytinization. This color loss is a result of the conversion of natural chlorophylls to Mg^{2+} free derivatives such as pheophytins (Ferruzzi and Blakeslee, 2007). Chlorophyll *a* and chlorophyll *b* were changed to pheophytin *a* and pheophytin *b* by acidification that occurred during simulated digestion in gastric phase (Ferruzzi and Blakeslee, 2007). This results were similar to study by Ferruzzi et al. (2001), which reported that the conversion of chlorophylls *a*

and *b* to their respective pheophytins was complete after incubation at pH 2.0 for 0.5 h.

Digestive stability is defined as the percentage of the chlorophyll derivatives, lutein and total phenolics in 3-years and 6-years-old golden barrel cactus extracts recovered in the digesta. The percentage of digestive stability of lutein, total chlorophylls, and total phenolics from 3-years and 6-years-old golden barrel cactus extracts after simulated digestion were reported in Table 4.8. The 3-year-old golden barrel cactus extracts, lutein showed significantly ($p < 0.05$) better stability than in 6-year-old extracts. It is possible that the 3-year-old golden barrel cactus extracts contain other compounds which could protect lutein degenerate during simulated digestion. Digestive stability of lutein after simulated digestion of 3- and 6-year- old golden barrel cactus extracts were 69.03% and 58.33%, respectively. It implied that the lutein were losses during simulated gastric and small intestinal digestion. The losses were associated with the appearance of an equivalent quantity of 13-*cis*-lutein (Chitchumroonchokchai, Schwartz, and Failla, 2004). Re, Fraser, Long, Bramley, and Rice-Evans (2001) reported that the carotenoids are unstable during the gastric phase of digestion because purified carotenoids are unstable in acidic solutions. Digestive stability of lutein after simulated digestion of golden barrel cactus extracts was not stable and less stable than spinach meal (digestive stability of lutein were 88%) from previously reported by Chitchumroonchokchai et al. (2004). In addition, Courraud, Berger, Cristol, and Avallone (2013) also reported in fresh spinach, lutein that was stability at 76%. The percentage of digestive stability of chlorophylls and phenolic compounds were determined at the value of 37.64, 60.52 and 33.34, 56.89 for 3- and 6-year-old golden barrel cactus extracts, respectively. This results suggested that

chlorophylls and phenolic compounds were not stable during simulated gastric and small intestinal digestion.

The digestive stability of pheophytin *b* increased more than 100% because of chlorophyll *b* was converted to pheophytin *b* by acidification occurring during simulated digestion in gastric phase. The digestive stability of chlorophylls of golden barrel cactus extracts were lower than the study of Gallardo-Guerrero, Gandul-Rojas, and Mínguez-Mosquera (2008) that showed stability of 60% - 87% in fresh pea, cooked fresh pea, frozen pea, cooked frozen pea, and canned pea. In addition, the digestive stability of phenolic compounds result were quite similar to those reported by Vallejo, Gil-Izquierdo, Pérez-Vicente, and García-Viguera (2004). They reported that phenolic compounds were degraded by intestinal conditions because of the remaining phenolic was highly degraded (> 70% additional loss).

Fernández-García, Carvajal-Lérida, and Pérez-Gálvez (2009) have defined bioaccessibility as the fraction of a compound that released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption. Bioaccessibility refers to the quantity of a bioactive compounds or phytochemicals after simulated digestion, which complete for cellular uptake. Therefore, the quantity of chlorophyll derivatives, lutein and total phenolic in aqueous fraction were bioaccessibility.

Table 4.7 Quantity of chlorophyll derivatives, lutein and total phenolics in pre-digested, digesta, and aqueous micellar fraction from 3- and 6-year old golden barrel cactus extracts.

Phytochemical	3-year-old cactus extracts			6-year-old cactus extracts		
	pre-digested	digesta	aqueous micellar fraction	pre-digested	digesta	aqueous micellar fraction
Lutein (µg/g RM)*	36.14 ± 0.39	24.95 ± 1.19	22.35 ± 0.08	30.44 ± 0.45	17.75 ± 0.17	12.12 ± 0.22
Chlorophyll *a* (µg/g RM)*	179.41 ± 2.89	n.d.	n.d.	115.15 ± 5.83	n.d.	n.d.
Chlorophyll *b* (µg/g RM)*	97.26 ± 0.31	n.d.	n.d.	91.28 ± 0.80	n.d.	n.d.
Pheophytin *a* (µg/g RM)*	243.46 ± 10.59	159.50 ± 2.04	118.97 ± 1.97	154.08 ± 5.23	92.17 ± 7.32	62.59 ± 4.91
Pheophytin *b* (µg/g RM)*	6.16 ± 1.45	38.54 ± 1.04	26.34 ± 1.83	5.87 ± 1.48	29.98 ± 1.19	16.40 ± 0.39
Total chlorophylls (µg/g RM)	526.29 ± 10.45	198.04 ± 2.76	145.31 ± 1.12	366.37 ± 1.22	122.15 ± 6.18	78.98 ± 4.67
Total phenolics (mg GAE /g of RM)**	35.45 ± 1.43	21.43 ± 0.45	16.37 ± 0.19	25.58 ± 0.26	14.55 ± 0.16	10.17 ± 0.34

Note: Data are means ± SD; n.d. = not detected, * Determined by HPLC, ** Determined by Folin-Cicalteau colorimetric assay.

Table 4.8 Digestive stability of chlorophyll derivatives, lutein and total phenolics from 3- and 6-year-old golden barrel cactus extracts after simulated digestion.

Phytochemical	% Digestive stability or recovery	
	3-year-old cactus	6-year-old cactus
Lutein	69.03 ± 2.65[b]	58.33 ± 1.38[a]
Pheophytin *a*	65.62 ± 3.63[a]	59.83 ± 4.70[a]
Pheophytin *b*	655.24 ± 187.59[a]	534.84 ± 146.47[a]
Total chlorophylls	37.64 ± 1.09[b]	33.34 ± 1.73[a]
Total phenolics	60.52 ± 2.73[a]	56.89 ± 1.20[a]

Note: Data are means ± SD; Data in the same row with different superscripts are significantly different ($p < 0.05$).

The percentage of bioaccessibility or efficiency of micellarization of chlorophyll derivatives, lutein and total phenolic from 3- and 6-year-old golden barrel cactus after simulated digestion were presented in Figure 4.3. This study found that the percentage of bioaccessibility of lutein, total chlorophylls and total phenolic from 3- year-old golden barrel cactus extracts was significant ($p < 0.05$) higher than in 6-year-old extracts. Because lutein, total chlorophylls and total phenolic from 3-year-old golden barrel cactus extracts showed digestive stability better than 6-year-old extracts. Therefore, it could form micellar lutein, total chlorophylls and total phenolic more than 6-year-old extracts. The bioaccessibility of total chlorophylls was 73.38% and 64.65% for 3- and 6-year-old golden barrel cactus extracts, respectively. These results were higher than fresh spinach puree in the study of Ferruzzi et al. (2001) at level of 37.6%. Percentage of bioaccessibility of lutein from golden barrel cactus extracts

were lower than that in the study of Ryan, O'Connell, O'Sullivan, Aherne, and O'Brien (2008) (82.8%), which used tomato as a sample. On the other hand, it was higher than the study of Courraud et al. (2013) (22.5%) which used carrot juice as a sample. Additionally, Failla and Chitchumronchokchai (2005) proposed that the efficiency of micellization of carotenoids (quantities transferred into the aqueous micellar fraction), during simulated digestion of plant foods, is an effective tool for the initial screening of the relative bioavailabilitiy of carotenoids.

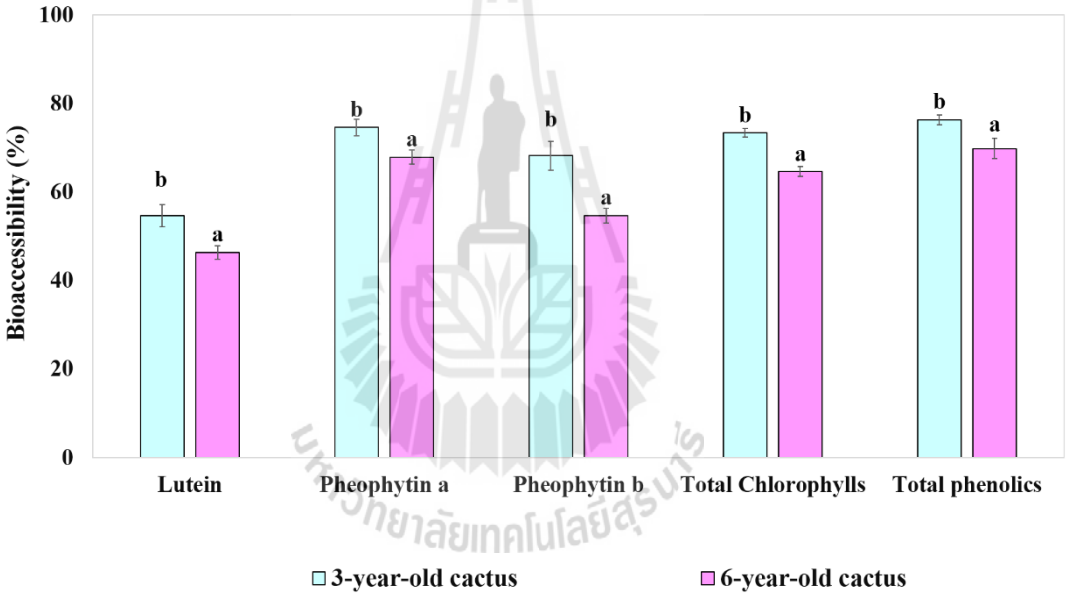


Figure 4.3 Bioaccessibility of chlorophyll derivatives, lutein and total phenolics from 3- and 6-year-old golden barrel cactus extracts after simulated digestion. Data are means ± SD. Different letters over the error bars denote that the means differed significantly ($p < 0.05$).

4.4.2 Bioavailability of chlorophyll derivatives, lutein and total phenolics from 3- and 6-year-old golden barrel cactus extracts by Caco-2 human intestinal cells

Cellular uptake of chlorophyll derivatives, lutein and phenolic compounds from the apical compartment were examined by incubating monolayers of Caco-2 cells in DMEM with the aqueous fraction from either generated during simulated digestion of 3- and 6-year-old golden barrel cactus extracts. The uptake of micelles chlorophyll derivatives, lutein and phenolic compounds from 3- and 6-year-old golden barrel cactus extracts by Caco-2 monolayers after different incubation time periods are shown in Figure 4.4 and Figure 4.5, respectively. The amounts of chlorophyll derivatives, lutein and phenolic compounds from 3-year-old golden barrel cactus extracts uptake did not show a significant ($p > 0.05$) difference between 4 and 6 h of incubation. These results were quite similar to that previously reported by Liu, Glahn and Liu (2004). They reported that the amounts of lutein, zeaxanthin, and β-carotene uptake did not show a significant ($p > 0.05$) change after 4 h incubation. These results suggested that chlorophyll derivatives, lutein and phenolic compounds from 3-year-old golden barrel cactus extracts reached a saturated level after 4 h. While, the amounts of chlorophyll derivatives, lutein and phenolic compounds from 6-years-old golden barrel cactus extracts uptake showed a significantly change ($p < 0.05$) between 4 and 6 h incubation. These results were proposed that chlorophyll derivatives, lutein and phenolic compounds from 6-year-old golden barrel cactus extracts reached a saturated level after or more than 6 h. Generally speaking, the patterns of time of 3- and 6-year-old golden barrel cactus extracts uptake by Caco-2 cells were different. A possible explanation for this might be that the concentration of chlorophyll derivatives (pheophytin *a* and pheophytin *b*), lutein and total phenolic contents of 3-year-old

golden barrel cactus extracts were different or higher than 6-year-old extracts. The uptake of chlorophyll derivatives, lutein and phenolic compounds from the intestinal lumen takes place by simple diffusion down a concentration gradient through the brush border membrane into the cytoplasm of the enterocytes. The high amount of chlorophyll derivatives, lutein and total phenolics added to cells causes diffusion rate into cell fast occurred and fast reached a saturated level. These results were quite similar to that previously studied of Garrett, Failla, Sarama and Craft (1999). They studied the effect of micellar concentration of lutein in medium on the uptake of lutein by Caco-2 cells. They found that cellular level of lutein also increased proportionally as the concentration of lutein in micellar medium was elevated and reached a level of 126.50 pmol/mg cell protein when the initial medium content was 1.9 mmol/L. The cellular uptake of lutein, pheophytin *a*, pheophytin *b* and phenolic compounds from 3-year-old golden barrel cactus extracts after 6 h incubation were 795.43, 3221.50, 1241.73 pmol/mg cell protein and 3169.18 nmol/mg cell protein, respectively. While, the cellular uptake of lutein, pheophytin *a*, pheophytin *b* and phenolic compounds from 6-year-old golden barrel cactus extracts after 6 h incubation were 426.28, 1365.04, 596.74 pmol/mg cell protein and 3168.91 nmol/mg cell protein, respectively. Since the concentrations of lutein, pheophytin *a*, pheophytin *b* and total phenolics in the test medium differed, then the results presented in Figure 4.6 were shown the percentage of lutein, pheophytin *a*, pheophytin *b* and phenolic compounds present within Caco-2 cells after 6 h of incubation. The percentage of cellular uptake of lutein and chlorophyll derivatives from 3 years old golden barrel cactus extracts was significantly ($p < 0.05$) higher than 6-year-old extracts. Because lutein, total chlorophylls and from 3-year-old golden barrel cactus extracts showed bioaccessibility or efficiency of micellarization more than 6-year-old extracts. Hence,

at high percentage of micellarization showed high percentage of cellular uptake for lutein and chlorophylls. Therefore, the results supported the possibility that differences in the efficiency of micellarization of lutein, pheophytin *a*, pheophytin *b* and total phenolics have a marked impact on the extent to which the compounds are absorbed. The percentage of cellular uptake of phenolic compounds did not show a significant ($p > 0.05$) difference between 3- and 6-year-old golden barrel cactus extracts. The extent of cellular uptake of lutein from golden barrel cactus extracts (30.63 and 26.31% for 3-and 6-year-old golden barrel cactus extracts, respectively) were quite similar to those reported by Garrett, Failla and Sarama (1999) (29.0%). The cellular accumulation efficiency of chlorophyll derivatives may be a result of specific physiochemical properties of chlorophyll. Parameters such as hydrophobicity, ionization, and molecular size have been related with differential intestinal absorption (Chan and Stewart, 1996). In addition, Ferruzzi et al. (2001) proposed that spinach puree established the amount of pigments absorbed by intestinal cells which is proportional to the pigments content of the micellar fraction and emphasized the importance of an efficient micellarization for the maximization of cellular concentration of chlorophylls and carotenoids. As a high percentage of micellarization, the cellular uptake for lutein and chlorophylls would be high level. However, during the *in vitro* digestion process are present other compounds of the test foods, which may also affect the chlorophyll pigment and lutein absorption (Gallardo-Guerrero et al., 2008). Reboul et al. (2007) showed that the main dietary antioxidants, such as carotenoids and polyphenols and these authors suggested that the intestinal absorption of those compounds can be impaired by the presence of other dietary antioxidants, such as the carotenoids lycopene and β-carotene and the polyphenol naringenin.

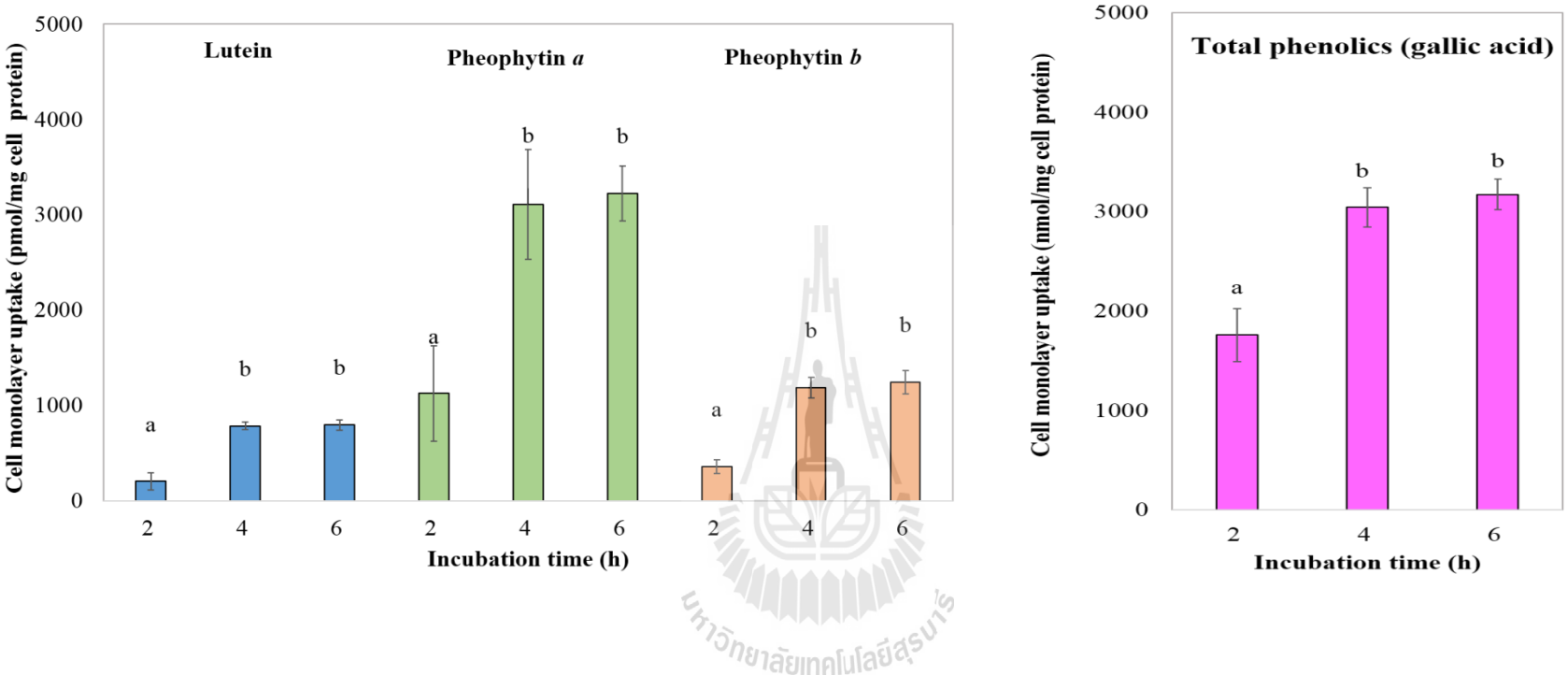


Figure 4.4 Effect of incubation time on cell monolayer uptake of chlorophyll derivatives, lutein and total phenolics from digested of 3-year-old golden barrel cactus extracts by Caco-2 human intestinal cells. Cultures of Caco-2 cells were incubated with test media containing micelle fraction generated during simulated digestion of the 3-year-old golden barrel cactus extracts for 2, 4 and 6 h. Data are means ± SD; n = 3. Different letters over the error bars denote that the means differed significantly ($p < 0.05$).

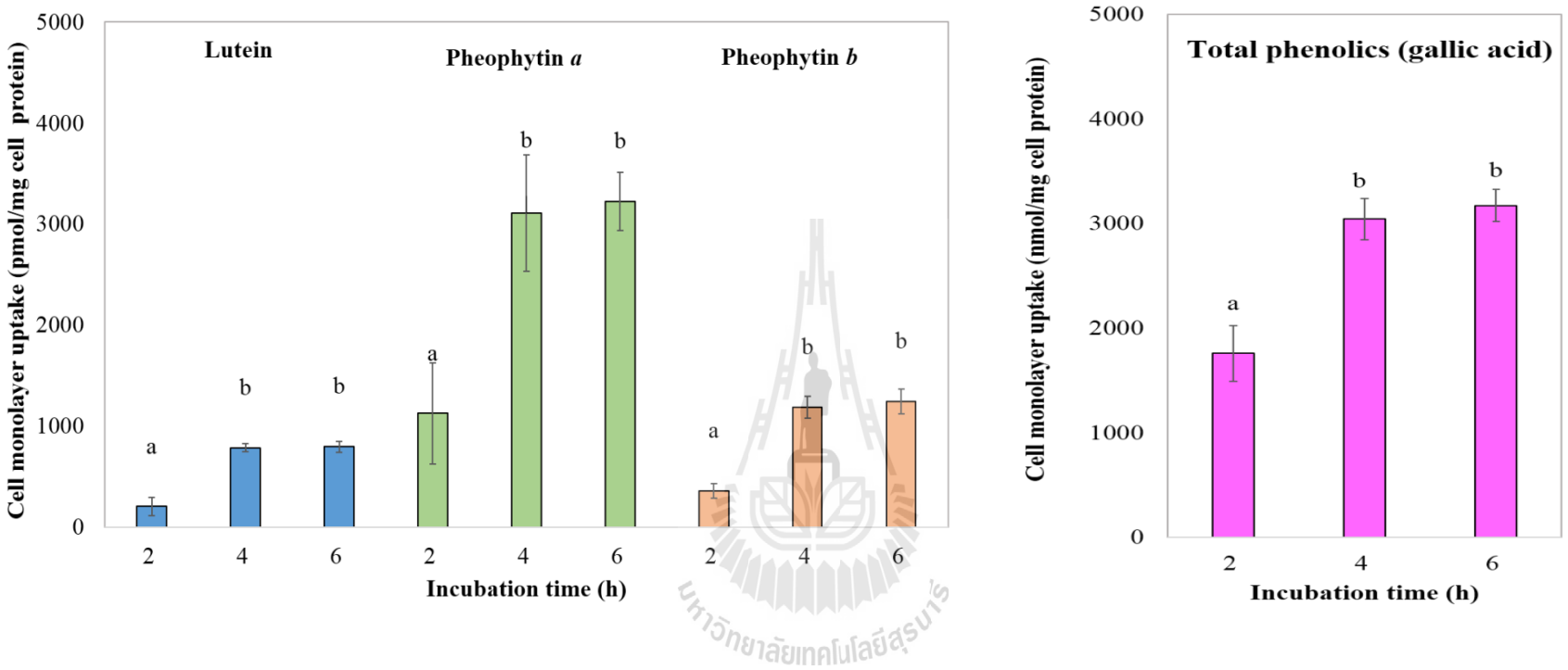


Figure 4.5 Effect of incubation time on cell monolayer uptake of chlorophyll derivatives, lutein and total phenolics from digested of 6-year-old golden barrel cactus extracts by Caco-2 human intestinal cells. Cultures of Caco-2 cells were incubated with test media containing micelle fraction generated during simulated digestion of the 6-year-old golden barrel cactus extracts for 2, 4 and 6 h. Data are means ± SD; n = 3. Different letters over the error bars denote that the means differed significantly ($p < 0.05$).

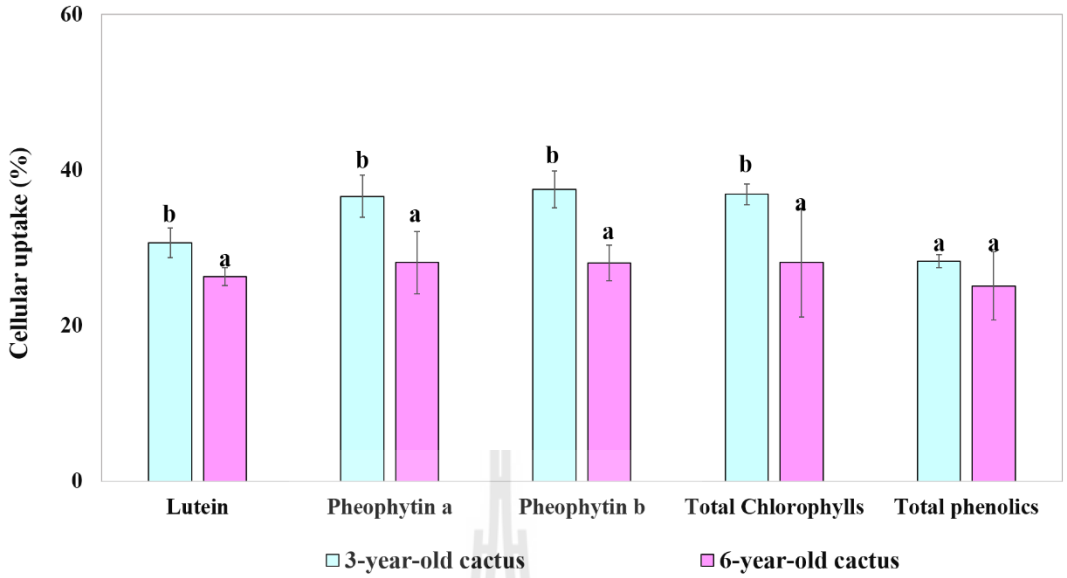


Figure 4.6 The percentage of cellular uptake of chlorophyll derivatives, lutein and total phenolics from digested of 3 and 6-year-old golden barrel cactus extracts by Caco-2 human intestinal cells.

Moreover, Gallardo-Guerrero et al. (2008) reported that the micellarization degree of the pigments was not the only factor intervening in the amount of pigments absorbed and but there were other factors associated with the food matrix which could inhibit or mediate in their absorption. These results demonstrated that the industrial preservation processes of pea-freezing and canning as well as the cooking have a positive effect on the bioaccessibility and bioavailability of the chlorophyll pigments. Furthermore, Zaripheh, and Erdman (2002) also reviewed factors affecting bioavailability of carotenoids, which they reported that lutein, zeaxanthin and canthaxanthin are present predominantly in green leafy vegetables and in fruit. Carotenoids are particularly concentrated in chromoplasts or chloroplasts of plant foods and are noncovalently bound to protein or fiber, dissolved in oil or existed in

crystalline form, making optimal absorption difficult to achieve. Some major factors are limiting the availability of xanthophylls include physical disposition in food sources (food matrix), structure of the xanthophyll molecule, interaction of xanthophylls with other nutrients (mainly dietary fat) and malnutrition. Besides, the efficacy of dispersion and solubilization significantly affect bioavailability, because solubilization is a prerequisite for absorption by intestinal epithelia (Nagao, 2014).

4.4.3 Transport of chlorophyll derivatives, lutein and total phenolics by Caco-2 cells

The Caco-2 human intestinal cell line is a potentially useful model for studying the transport and metabolism of dietary phytochemicals (Liu et al., 2004). However, in this study, an *in vitro* cell culture system mimicking the *in vivo* intestinal absorption of dietary phytochemicals was established and involves several crucial steps: release of dietary phytochemicals from the food matrix, solubilization of dietary phytochemicals into mixed micelles formed in the lumen, cellular uptake of dietary phytochemicals by small intestinal absorptive epithelial cells, and transport of dietary phytochemicals and their metabolites into the lymphatic circulation (During, Hussain, Morel, and Harrison, 2002). The Caco-2 cell model was previously used to mimic the intestinal absorption of carotenoids (Chitchumroonchokchai et al., 2004; Courraud et al., 2013; Garrett, Failla, and Sarama, 1999; Liu et al., 2004; Ryan et al., 2008), chlorophyll derivatives (Ferruzzi et al., 2001; Gallardo-Guerrero et al., 2008) and total phenolic (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, and Martín-Belloso, 2013; Vallejo et al., 2004). There were some reports on uptake and transport of carotenoids through Caco-2 cell monolayers (Aherne, Daly, Jiwan, O'Sullivan, and O'Brien, 2010; O'Sullivan, Ryan, and O'Brien, 2007).

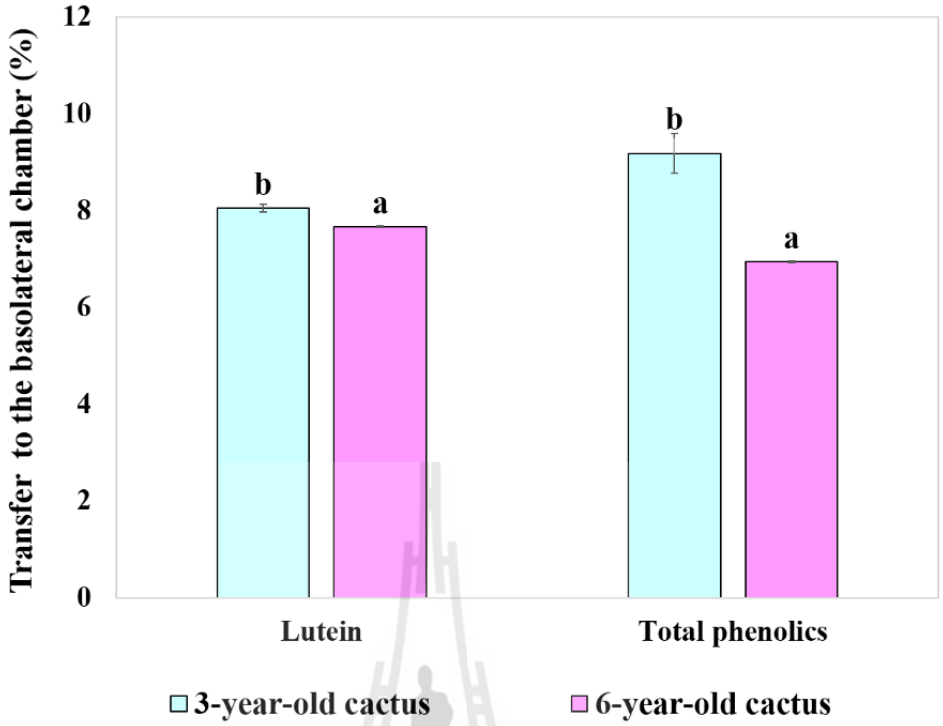


Figure 4.7 The percentage transport of lutein and total phenolics through differentiated Caco-2 cell monolayers to the basolateral chamber. Data are means ± SD. Different letters over the error bars denote that the means differed significantly ($p < 0.05$).

The amounts of chlorophyll derivatives (pheophytin *a* and pheophytin *b*), lutein and total phenolics were added to cells (~ 1.05, ~ 0.42, ~ 1.48 µmol/L, ~ 5.32 mmol/L and ~ 0.88, ~ 0.32, ~ 1.34 µmol/L, ~ 3.89 mmol/L for 3- and 6-year-old golden barrel cactus extracts, respectively). The amount of chlorophyll derivatives, lutein and total phenolics that is taken up by the Caco-2 cells, packaged into chylomicrons, transported through the monolayer and secreted into the basolateral chamber of the transwell plate is recorded (O'Sullivan et al., 2007). Normally, this value is reported as percentage transfer of cellular transport. The percentage transfer

of lutein and phenolic compounds to the basolateral chamber were 8.05, 9.18 and 7.67, 6.95 for 3- and 6-year-old golden barrel cactus extracts, respectively (Figure 4.7). The percentage transfer of lutein was pretty similar to those reported by Chitchumroonchokchai et al. (2004) (7.60) and O'Sullivan et al. (2007) (9.70). Konishi, Kobayashi and Shimizu (2003) reported that the permeation of gallic acid was independent of pH and linearly increased with increasing concentration of gallic acid, suggesting the transepithelial transport of gallic acid to be via the paracellular pathway. However, Chlorophyll derivatives were not detected in the basolateral chamber. It is difficult to explain the reasons, but it might be related to concentration of chlorophyll derivatives lower than detection limit of chlorophyll by HPLC. Moreover, chlorophylls could be lost or damaged during simulated gastric and small intestinal digestion. In addition, chlorophylls is also easily damaged by light. Also, chlorophylls might be metabolized by the Caco-2 cells. Thus, chlorophylls could not be detected transport through Caco-2 cell into basolateral chamber of the trans-well plate. However, this result are not clearly explain about transport through Caco-2 cell into the blood circulation of chlorophyll derivatives. Ferruzzi, Failla and Schwartz (2002) reported transfer of cellular sodium copper chlorophyllin (SCC) (a mixture of water-soluble chlorophyll derivatives) to the basolateral compartment was only 2-5%.

CHAPTER V

CONCLUSIONS

The ash or mineral and crude fiber of 6-years-old golden barrel cactus powder was significantly ($p < 0.05$) higher than 3-years-old golden barrel cactus powder. The phytochemicals content and antioxidant activity had a difference between 3-years and 6-years-old golden barrel cactus extracts. Phytochemicals content and antioxidant activity of 3-years-old golden barrel cactus extracts was higher than 6-years-old golden barrel cactus extracts. Both golden barrel cactus extracts were analyzed for phytochemicals. Phytochemical analyses revealed the presence of lutein and four chlorophyll derivatives, including chlorophyll *a*, chlorophyll *b*, pheophytin *a* and pheophytin *b*. In addition, these golden barrel cactus extracts showed potential as a source of extracts rich in phenolic constituents and the major antioxidants in plants. The 3-years and 6-years-old golden barrel cactus extracts showed a total phenolic content of 3545.35 and 2557.96 mg gallic acid equivalent/100g of RM, respectively. The extracts of golden barrel cactus were evaluated for their antioxidant activity by DPPH and ABTS assay. The 3-years-old golden barrel cactus extracts showed the IC_{50} values of 112.60 and 44.62 mg RM/ml, while the 6-years-old golden barrel cactus extracts showed 191.90 and 81.84 mg RM/ml for each assay, respectively. In addition, their antioxidant activity by FRAP assay showed the value at 0.014 and 0.01mmol Fe^{2+}/g RM for 3-years and 6-years old golden barrel cactus extracts, respectively. Moreover, the extracts of 3-years and 6-years-old golden barrel cactus

before and after *in vitro* digestion indicated toxicity to Caco-2 and HepG2 cells within the tested concentration ranging 25 ug/ml to 500 ug/ml. Our results indicated that the extremely high value of LC_{50} (> 200 μg RM/ml) against both cell types indicating non-toxic activity to the cells based on MTT assay. This result could provide preliminary information for study of cytotoxicity. Furthermore, the digestive stability of lutein and total chlorophylls of 3-years-old golden barrel cactus extracts (69.03%, 37.64%) showed significantly ($p < 0.05$) better stability than 6-years-old golden barrel cactus extracts (58.33%, 33.34%). The percentage of digestive stability of phenolic compounds was not significantly different ($p > 0.05$) between 3-years and 6-years-old golden barrel cactus extracts (60.52, 56.89). These results suggested that the lutein, total chlorophylls and total phenolic were not stable during simulated gastric and small intestinal digestion. The percentage of bioaccessibility of lutein, total chlorophylls and total phenolic from 3-years-old golden barrel cactus extracts was significantly ($p < 0.05$) higher than 6-years-old extract. Bioaccessibility of lutein, total chlorophylls and total phenolic were 54.72%, 73.38%, 76.39% and 46.39%, 64.65%, 69.88% for 3-years and 6-years-old golden barrel cactus extracts, respectively. Additionally, the percentage of cellular uptake of lutein and chlorophyll derivatives from 3-years-old golden barrel cactus extracts (30.63, 36.88) was significantly ($p < 0.05$) higher than 6-years-old-extract (26.31, 28.10). The percentage of cellular uptake of total phenolic has no statistically significant differences between 3-years and 6-years-old golden barrel cactus extracts (28.27, 25.11). Finally, the percentage transfer of lutein and total phenolic to the basolateral chamber were 8.05, 9.18 and 7.67, 6.95 for 3-years and 6-years-old golden barrel cactus extracts, respectively. Chlorophyll derivatives were not detected in basolateral chamber. It is difficult to

explain this result, but it might be related to concentration chlorophyll derivatives lower than detection limit of chlorophyll by HPLC. Therefore, the phytochemical and bioactivities showed a negative relationship related to the age of cactus. The development of younger golden barrel cactus as dietary supplement ingredients with better qualification and quantification for lutein, chlorophylls, phenolic compounds and antioxidant source should be carried out in the future research.

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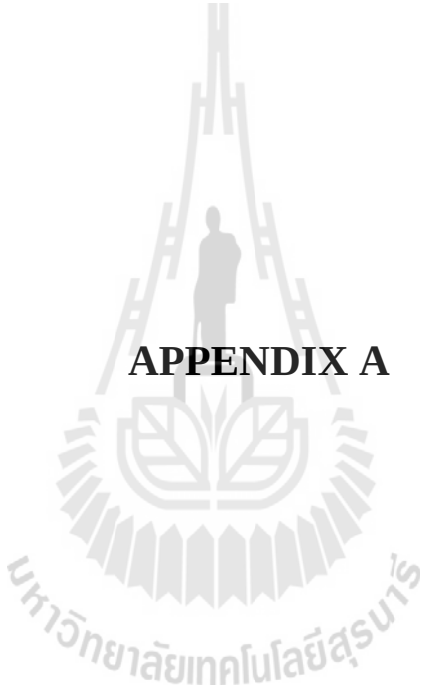
APPENDIX

APPENDIX A



APPENDIX A

Reagent preparations and cell culture media

1. Sodium carbonate (Na_2CO_3) (20%w/v)

Sodium carbonate	20.0	g
Add distilled water to bring volume up to	100.0	ml

2. Standard gallic acid (5 mg/ml)

Gallic acid	125.0	mg
95% Ethanol	2.5	ml
Add distilled water to bring volume up to	25.0	ml

3. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (0.1 mM)

2, 2-diphenyl-1-picrylhydrazyl (DPPH)	3.9432	mg
Add methanol to bring volume up to	100.0	ml

4. Standard BHT (1 mg/ml)

BHT	50	mg
Add 95% ethanol to bring volume up to	50.0	ml

5. Standard ascorbic acid (1 mg/ml)

Ascorbic acid	50	mg
Add 95% ethanol to bring volume up to	50.0	ml

6. ABTS (14 mM)

ABTS	38.4	mg
Add distilled water to bring volume up to	5.0	ml

7. Potassium persulfate ($K_2S_2O_8$) (4.9 mM)

Potassium persulfate	66.2250	mg
Add distilled water to bring volume up to	50.0	ml

8. Acetate buffer pH 3.6 (300 mM)

Sodium acetate	4.0824	g
Glacial acetic acid	1.6	ml
Add distilled water to bring volume up to	100.0	ml

9. HCl (40 mM)

Hydrochloric acid (37% w/w)	333.0	µl
Add distilled water to bring volume up to	100.0	ml

10. TPTZ (10 mM)

TPTZ	31.23	mg
Add 40 mM hydrochloric acid to bring volume up to	100.0	ml

11. Ferric (III) chloride (20 mM)

Ferric chloride	54.06	mg
Add distilled water to bring volume up to	10.0	ml

12. Standard ferrous (II) sulphate (1 mM)

Ferrous sulphate	27.80	mg
Add distilled water to bring volume up to	100.0	ml

13. MTT solution (5 mg/ml in PBS)

MTT	125.0	mg
Add PBS to bring volume up to	25.0	ml

14. Complete cell culture media

DMEM	40.0	ml
FBS	4.5	ml
Non-essential amino acids	500	µl

L-glutamine	500	µl
Penicillin-streptomycin (antibiotic)	500	µl

15. NaCl (120 mM) (5% Tween 80)

Sodium chloride	3.51	g
Tween80	25.0	ml
Add distilled water to bring volume up to	500.0	ml

16. HCl (1 M)

Hydrochloric acid (37% w/w)	8.33	ml
Add distilled water to bring volume up to	100.0	ml

17. HCl (100 mM)

Hydrochloric acid (37% w/w)	833	µl
Add distilled water to bring volume up to	100.0	ml

18. Porcine pepsin (40 mg/ml in 100 mM HCl)

Porcine pepsin	400.0	mg
Add 100 mM HCl to bring volume up to	10.0	ml

19. $NaHCO_3$ (1 M)

Sodium bicarbonate	8.4	g
Add distilled water to bring volume up to	100.0	ml

20. $NaHCO_3$ (100 mM)

Sodium bicarbonate	840.0	mg
Add distilled water to bring volume up to	100.0	ml

21. Crude bile extract (40 mg/ml in 100 mM $NaHCO_3$)

Crude bile extract	400.0	mg

Add 100 mM $NaHCO_3$ to bring volume up to	10.0	ml

22. Pancreatin (12 mg/ml in 100 mM $NaHCO_3$)

Pancreatin	120.0	mg
Add 100 mM $NaHCO_3$ to bring volume up to	10.0	ml

23. DMEM containing taurocholate (0.5 mM), oleic acid (1.6 mM) and glycerol (45 mM)

Taurocholate	13.4	mg
Oleic acid	23.0	µl
Glycerol	207.0	µl
Add DMEM to bring volume up to	50.0	ml

24. Sodium dodecyl sulfate (34.6 mmol/L) in ethanol (0.1% BHT)

Sodium dodecyl sulfate	249.5	mg
BHT	25	mg
Add 95% ethanol to bring volume up to	25.0	ml

25. Bovine serum albumin (10 mg/ml)

BSA	250.0	mg
Add distilled water to bring volume up to	25.0	ml

APPENDIX B

APPENDIX B

1. Gallic acid standard calibration curve

The standard curve of gallic acid was prepared stock solution 5 mg/ml of gallic acid by dissolving 125.0 mg of gallic acid in 2.5 ml of 95% ethanol and add distilled water to bring volume up to 25.0 ml. Prepared concentrations 0, 50, 100, 250, 500, and 750 mg/ml standard gallic acid solutions. Aliquot of 0.02 mL. gallic acid standard was mixed with 1.58 mL of deionized water. Folin-Ciocalteu reagent of 0.1 mL was added, and the tube was stirred and allowed to stand at room temperature for 5 min. Then, 0.3 mL of Na2CO3 (20% w/v) was added to mixture and stored in the absence of light for 2h at room temperature. Absorbance was measured at 765 nm using spectrophotometer (Biochrom Libra S22 S/N 97765, UK).

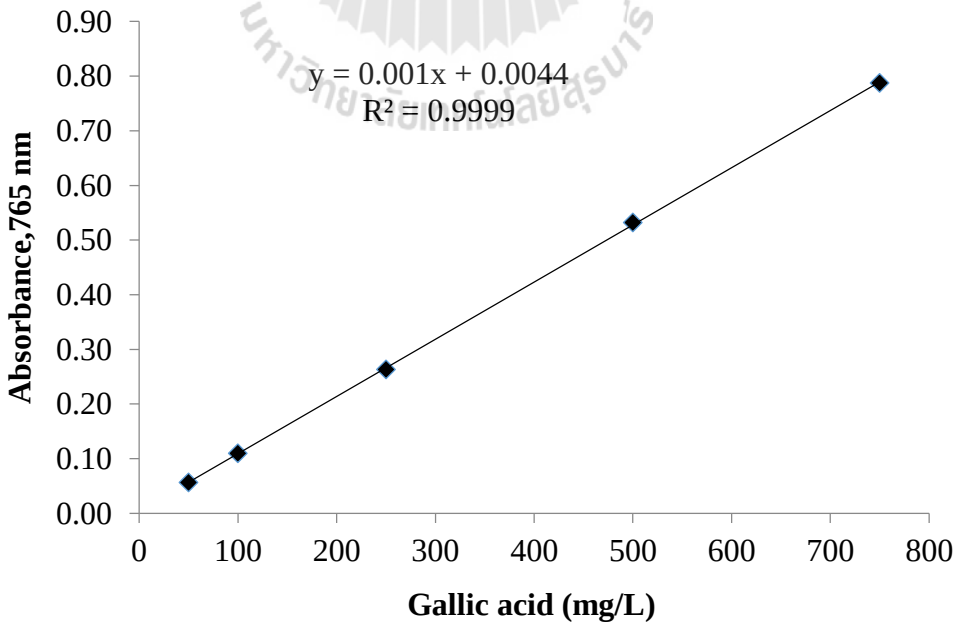


Figure 1B Calibration curve with gallic acid as standard.

2. Bovine serum albumin (BSA) standard calibration curve

Proteins concentration was determined by the Bradford assay using bovine serum albumin as a standard (Bradford, 1976). Prepared stock solution 10 mg/ml of BSA by dissolving 250.0 mg of BSA and add distilled water to bring volume up to 25.0 ml and diluted to obtain various concentrations 125, 250, 500, 750, and 1000 mg/L. Dilute the Bradford reagent fivefold in DI water (1 part Bradford: 4 parts DI) and filtered through Whatman No.1 paper. Add 20 µl of standard to 1 ml of the diluted reagent and mixed. Incubate at room temperature for at for 5 min. Absorbance was measured at 595 nm using spectrophotometer (Biochrom Libra S22 S/N 97765, UK).

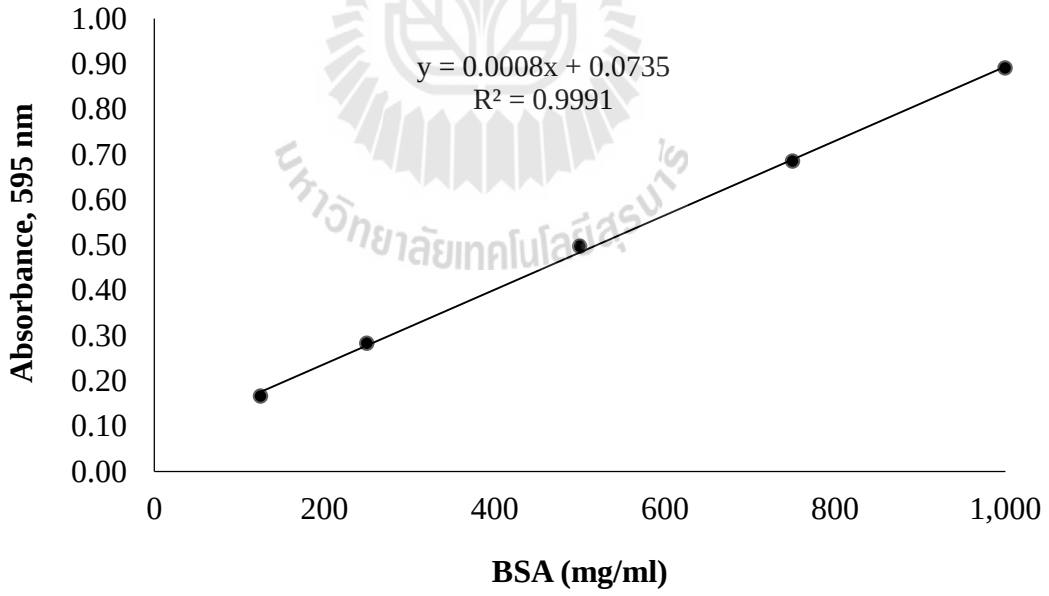


Figure 2B Calibration curve with bovine serum albumin as standard.

3. Morphology of Caco-2 cells

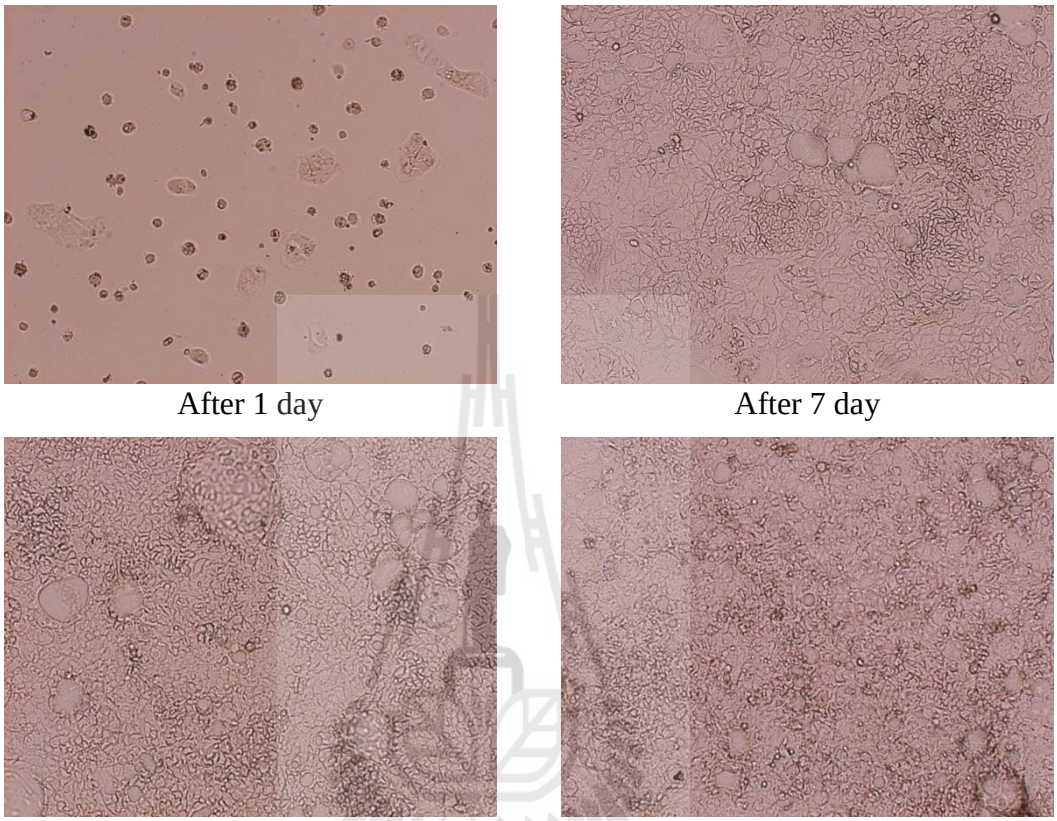


Figure 3B Morphology of Caco-2 cells after 1, 7, 14, 21 days in culture (4x Objectives).

BIOGRAPHY

Ms. Nipha Chaicharoenaudomrung was born in August 27, 1988 in Suphanburi Province, Thailand. She received Bachelor's Degree in B.Sc. (Food Technology) from Institute of Agricultural Technology, Suranaree University of Technology, Thailand in 2010. In 2011, she enrolled in a Food Technology Master program, Suranaree University of Technology, Thailand. Her research topic was phytochemical profile and bioactivities of *Echinocactus grusonii* extract. The results from some part of this research have been presented as a poster presentation at The 15th Food Innovation Asia Conference, June 13-14, 2013, BITEC, Bangkok, Thailand. The results from part of this research have been presented and published Chaicharoenaudomrung, N., Oonsivilai, A, and Oonsivilai, R. (2014). Chlorophylls contents in *Echinocactus grusonii* extract. In **The 5th KKU international engineering conference.** (pp1507-1511). Pullman KhonKaen raja orchid hotel, Khonkaen, Thailand.