Establishment of standard information of *Punica granatum* fruit peel extract

Acharaporn Issuriya

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Pharmacy in Pharmaceutical Sciences

Prince of Songkla University

2007

Thesis Title	Establishment of standard information of *Punica granatum* fruit peel extract
Author	Miss Acharaporn Issuriya
Major Program	Pharmaceutical Sciences

Major Advisor

.....

(Assoc. Prof. Dr. Pharkphoom Panichayupakaranant)

Co-advisor

.....

(Asst. Prof. Dr. Anusak Sirikatitham)

Examining Committee :

.....Chairperson

(Dr. Chitchamai Ovatlarnporn)

.....Committee

(Assoc. Prof. Dr. Pharkphoom Panichayupakaranant)

.....Committee

(Asst. Prof. Dr. Anusak Sirikatitham)

.....Committee

(Assoc. Prof. Dr. Sompop Prathanthurarug)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Pharmacy Degree in Pharmaceutical Sciences

….....

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

ชื่อวิทยานิพนธ์ การสร้างข้อกำหนดมาตรฐานของสารสกัดเปลือกผลทับทิม
ผู้เขียน นางสาวอัจฉราภรณ์ อิสสุริยะ
สาขาวิชา เภสัชศาสตร์
ปีการศึกษา 2550

บทคัดย่อ

ระบบ HPLC ที่พัฒนาขึ้นเพื่อการวิเคราะห์ปริมาณสาร ellagic acid ในสารสกัดจากเปลือกผลทับทิมสามารถตรวจวัดปริมาณ ellagic acid ได้อย่างถูกต้อง แม่นยำ มีความจำเพาะเจาะจง และรวดเร็ว สามารถเตรียมสารสกัดจากเปลือกผลทับทิม เพื่อให้ได้สารสกัดที่มีปริมาณสาร ellagic acid ในปริมาณสูง ได้โดยการสกัดเปลือกผลทับทิมด้วยวิธีการ reflux กับตัวทำละลายผสมระหว่างน้ำและเมธานอล (10%v/v water in methanol) ทำให้ได้สารสกัดที่มีปริมาณ ellagic acid สูงถึง 7.66 ± 0.006 %w/w สารสกัดที่ได้นำมาผ่านกระบวนการเตรียมสารสกัดให้บริสุทธิ์ขึ้น ด้วยวิธี liquid-liquid extraction โดยใช้เอธิล อะซิเทต และ กรดอะซิติก (2%v/v) เป็นตัวทำละลาย ทำให้ได้สารสกัดที่มีปริมาณสาร ellagic acid สูง โดยพบว่ามีปริมาณ ellagic acid เพิ่มขึ้นเป็น 13.63 ± 0.89% w/w เมื่อทดสอบฤทธิ์ต้านอนุมูลอิสระของสารสกัดที่ได้ด้วยวิธี DPPH radical scavenging assay พบว่าสารสกัดที่ได้มีฤทธิ์ต้านอนุมูลอิสระดี โดยมีค่า ED_{50} เท่ากับ 14.91 ± 0.24 μg/ml (เทียบกับสารมาตรฐาน quercetin, ED_{50} = 3.57 ± 0.64 μg/ml) จึงได้กำหนดเกณฑ์ปริมาณสารสำคัญของสารสกัดเปลือกผลทับทิมไว้ว่า ต้องมีปริมาณสาร ellagic acid ไม่น้อยกว่า 13% w/w เมื่อทำการศึกษาคุณสมบัติทางกายภาพของสารสกัดเปลือกผลทับทิมที่มีปริมาณสาร ellagic acid สูง ทำให้สามารถสร้างข้อกำหนดมาตรฐานของสารสกัดได้ดังนี้ สารสกัดจากแปลือกผลทับทิมควรมีปริมาณความชื้นไม่มากกว่า 3.2 %w/w ไม่พบเถ้า และไม่มีการปนเปื้อนของเชื้อแบคทีเรีย ได้แก่ aerobic bacteria, *Escherichia coli*, ยีสต์และเชื้อรา เมื่อทำการศึกษาค่าการละลาย พบว่าสารสกัดละลายได้ดีในไดเมธิลซัลฟอกไซด์, ละลายได้ในเมธานอล, เอธานอล และ อะซิโตน, ละลายได้บ้างในเอธิล อะซิเทต แต่ไม่ละลายในไดคลอโรมีเธน, คลอโรฟอร์ม, เฮกเซน และน้ำ พบว่าสาร ellagic acid ในสารสกัดเปลือกผลทับทิมนี้ มีค่า partition coefficient (log P_{OW}) เท่ากับ 2.44 ± 0.26 แสดงว่าสาร ellagic acid ในสารสกัดเปลือกผลทับทิมน่าจะถูกดูดซึมผ่านผิวหนังได้ดี สารสกัดมีความคงตัวดีทั้งในสภาวะที่มีแสงและป้องกันแสง และเมื่อเก็บที่อุณหภูมิ 4 ± 2 ºC และ 30 ± 2 ºC รวมถึงเมื่อเก็บในสภาวะเร่งที่ 45 ºC และความชื้นสัมพัทธ์ 75% ตลอดระยะเวลา 4 เดือนของการเก็บรักษา อย่างไรก็ตามการศึกษาอิทธิพลของ pH ต่อความคงตัวของสารสกัดในรูปสารละลาย พบว่าสารสกัด

ไม่คงตัวที่ pH 5.5, 7.0 และ 8.0 จากผลการศึกษานี้แสดงให้เห็นว่าสารสกัดเปลือกผลทับทิมที่มีปริมาณสาร ellagic acid สูง มีความคงตัวดีเมื่อเก็บอยู่ในรูปผงแห้ง แต่ไม่มีความคงตัวเมื่ออยู่ในรูปของสารละลาย

Thesis Title	Establishment of standard information of *Punica granatum* fruit peel extract
Author	Miss Acharaporn Issuriya
Major Program	Pharmaceutical Sciences
Academic Year	2007

ABSTRACT

Study on quantitative HPLC analysis of ellagic acid content in *Punica granatum* fruit peel extract and method validation found that the HPLC system was precise, accurate, specific and rapid. Study on preparation process of *P. granatum* fruit peel extract in order to obtaine the ellagic acid high-yielding *P. granatum* fruit peel extract found that 10%v/v water in methanol under reflux conditions could provide the high-yield ellagic acid extract. This extraction method was capable of improving the ellagic acid content in the crude extract up to 7.66 ± 0.006 %w/w. The extract was subsequently purified using liquid-liquid extraction between ethyl acetate and 2% aqueous acetic acid to produce high-yield ellagic acid extract, which contained the ellagic acid content up to 13.63 ± 0.89% w/w. On the basis of DPPH radical scavenging assay, the ellagic acid high yielding extract exhibited satisfactory antioxidant activity with ED_{50} value of 14.91 ± 0.24 μg/ml (compared to standard quercetin , ED_{50} = 3.57 ± 0.64 μg/ml). These results suggested that standard value of ellagic acid of the extract should not less than 13% w/w. In addition, study on the physical properties of the partially purified extract let to the establishment of the specification of the extract as follows: moisture content (loss on drying) not more than 3.2% w/w, no contained of ash, and no contamination with aerobic bacteria, *Escherichia* coli, yeast and fungi. Solubility evaluation of the extract in various solvents found that the extract was freely soluble in dimethylsulfoxide, sparingly soluble in methanol, ethanol and acetone, slightly soluble in ethyl acetate, and practically insoluble in dichloromethane, chloroform, hexane and water. The partition coefficient (log P_{OW}) value of ellagic acid in the extract was found to be 2.44 ± 0.26. It implies that ellagic acid in the extract has good percutaneous absorption. Stability evaluation of the high-yield ellagic acid extract in the several conditions through the period of 4 months found that the extract was stable either kept under light or protected from light. The

extract was also stable under 4 ± 2 ºC, 30 ± 2 ºC and accelerated conditions 45 ºC with 75% relative humidity. However, study on the effect of pH on stability of the extract in the form of solution found that the extract was not stable at the pH 5.5, 7.0 and 8.0. These results indicated that the high-yield ellagic acid extract was stable when it was kept as dried powder, but it was not stable in aqueous solution.

ACKNOWLEDGEMENTS

I wish to express my deepest and grateful thanks to my advisor, Associate Professor Dr. Pharkphoom Panichayupakaranant for his helpful guidance, suggestion and encouragement throughout the course of this work.

My sincere thanks are expressed to my thesis co-advisor, Assistant Professor Dr. Anusak Sirikatitham, for his kindness and helpful suggestion.

The grant from Graduate School, Prince of Songkla University, was very useful in this study, and I am greatly appreciative.

I would like to extent my sincere thanks to the Department of Pharmacognosy and Pharmaceutical Botany, Department of Pharmaceutical Technology and the Pharmaceutical Laboratory Service Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University for their support in scientific equipment.

I would like to thank all staffs of Faculty of Pharmaceutical Sciences, Prince of Songkla University for their kindness and help.

Finally, I would like to thank my family and friends for their love and encouragement.

Acharaporn Issuriya

CONTENT

CONTENT (Continued)

CONTENT (Continued)

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS AND SYMBOLS

α-	alpha-
β-	beta-
p-	para-
g	gram
kg	kilogram
μg	microgram
mL	milliliter
rpm	round per minute
w	weight
ºC	degree Celcius
MIC	minimum inhibitory concentration
ED_{50}	Effect dose 50 percent
MBC	minimum bactericidal concentration
M	Molar
mmol	millimole
m	meter
nm	nanometer
cm	centimeter
Ca	Calcium
Cs	Cesium
Ce	Cerium
Cl	Chlorine
Co	Cobalt
Cr	Chromium
Cu	Copper
Fe	Ferrous
K	Potassium
Mg	Magnesium

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

Mn	Manganese
Mo	Molybdenum
Na	Sodium
Rb	Rubidium
Sc	Scandium
Se	Selenium
Sr	Strontium
Zn	Zinc
N	Nitrogen
$CuSO_4^-$	Copper sulphate ion

CHAPTER 1

INTRODUCTION

1.1 General introduction

Punica granatum L. is a plant belonging to Punicaceae family. It has a number of common names, including Tab-Tim (Thai), Delima (Malaysia), Pomo Punico (Indonesia), Roma (Brazil) and Pomegranate (English). *P. granatum* is a native plant of Iran to the Himalayas in northern India, and was cultivated and naturalized over the whole Mediterranean region since ancient times. It is widely cultivated throughout India and the drier parts of Southeast Asia, Malaya, the East Indies and tropical Africa (Morton, 1987). The root barks as well as stem barks of the plant are used as astringent and antihelmintic. The dried flowers are used in treatment of haematuria, hemorrhoids, haemoptysis and dysentery. The powdered flower-buds are used to treat bronchitis. The seeds are considered to be stomachic and the pulp are considered to be cardiac and stomachic (Ross *et al.,* 2001). The fruit peel of *P. granatum* has long been utilized as food and used in folk medicine as a diet inconvalescence after diarrhea. Peel of fruit is used as astringent, digestive, cardiotonic, stomachic agent and highly effective in chronic diarrhea and dysentery, dyspepsia, colitis, piles and uterine disorders. (Farnsworth and Bunyapraphatsara, 1992). Several biological activities of *P. granatum* fruit peel, including antibacterial (Alanis *et al.,* 2005), antioxidant (Lansky and Newman, 2007; Ricci *et al.,* 2006), anti-inflammatory and immunomoduratory (Ross *et al.,* 2001) have been reported.

P. granatum extracts are also being investigated for their potential uses as food biopreservatives and nutraceuticals. It has been reported that the methanol extract of the fruit peel that contained total phenolic content of 19.22% w/w exhibited antioxidant activity with Trolox equivalent antioxidant capacity (TEAC) values of 394.66 and 316.29 mmol/100 g dried wight, evaluated by DPPH and ABTS assay, respectively (Surveswaran *et al*., 2007). The major types of phenolic compounds were reported as hydrolysable tannin (punicalin, punicalagin), gallagic acid,

ellagic acid and gallic acid. The aqueous and ethyl acetate extracts of the fruit peel also showed antioxidant activity when evaluated by DPPH, 5-lipoxygenase and chemiluminescence assays (Ricci *et al.,* 2006). On the basis of antioxidant-bioassay guide isolation of *P. granatum* fruit peel extract, ellagic acid was demonstrated as the major potent active constituent (Panichayupakaranant *et al.*, 2005). Although, quantitative HPLC determination of ellagic acid in pomegranate fruit peel extracts has been developed (Seeram *et al.*, 2005), the method is time consuming; a total run time of about 98 minutes is required, and validation of the analytical procedure is not yet established.

Recently, various Thai herbal plants are used widely for preparation herbal products. Unfortunately, vast of commonly used herbal plants and extracts are still lacks of standard information for their quality control. Thus, nowadays many organizations involved in this field have focused on the standardization of the plant extracts to qualify those herbal medicines. In addition, lack of chemical specification, physical properties and stability information of *P. granatum* fruit peel extract is an obstacle of the further development of the herbal products. The chemical specification of *P. granatum* fruit peel extract is necessary for the establishment of the monograph of the extract, which will be useful for its standardization. As a part of our interest in an improvement of ellagic acid content in *P. granatum* fruit peel extracts and its quality control, the extraction method and quantification of ellagic acid in *P. granatum* fruit peel through HPLC were developed. The HPLC method was also validated. Some physical properties as well as stability of the extract were also studied in order to get useful informations for a future studies on development of the herbal medicines from the extract.

1.2 Objectives

1.2.1. To establish and validate the HPLC method for determination of ellagic acid in *P. granatum* fruit peel extract

1.2.2. To optimize the extraction and fractionation methods in order to obtain the high-yield ellagic acid extract

1.2.3. Physical properties and stability determination of the extract in order to get useful informations for a future studies on development of the herbal products from the extract

1.2.4. To establish a monograph of the high-yield ellagic acid *P. granatum* fruit peel extract for further quality control

CHAPTER 2

REVIEW OF LITERATURES

2.1 Botanical description of *Punica granatum*

Figure 2-1 *Punica granatum* Linn.

Scientific name: *Punica granatum* Linn.

Family name: Punicaceae

Synonym: *Punica nana* Linn.

Common name: Pomegranate, Tubtim, Siae lin (Chinese), Phi laa (Nong khai), Philaa khaao (Nan), Ma koh (Northern), Maak-chang (Shan-Mae Hong Son) (นันทวัน บุณยะ-ประภัศร, 2541).

The plant is an erect shrub up to 3 m high, much branched from the base, having branchlets slender, often ending in a spine. Leaves are simple, oblong-lanceolate, 1-9 by 0.5-2.5 cm, and consisting of obtuse or emarginated apex, acute base, shiny and glabrous texture. Flowers are showy, orange red, about 3 cm in diameter, 1-5 borned at branch tips, the others solitary in highest leaf-axils, sessile or subsessile; consisting of calyx 2-3 cm long, tubular, lobes erect or recurved, thick, coriaceous; petals the same numbers as the calyx lobes, rounded or very obtuse, from edge of hypanthium, caduceus; stamen numerous within upper half of hypanthium, filament free; inferior ovary, ovules numerous, style1, stigma capitate. Fruit is globose berry, crowded by persistent calyx-lobes, having pericarp leathery filled with numerous seeds, which are surrounded by pink and red, transparent, juicy, acid, pleasant-tasting pulp (Figure 2-1) (Farnsworth and Bunyapraphatsara, 1992). In each sac there is one angular, soft or hard seed.

Dwarf varieties are also known. It is usually deciduous, but in certain areas the leaves will persist on the tree. The trunk is covered by a red-brown bark which later becomes gray. The branches are stiff, angular and often spiny. There is a strong tendency to sucker from the base. Pomegranates are also long-lived. There are specimens in Europe that are known to be over 200 years of age. The vigor of a pomegranate declines after about 15 years. High temperatures are essential during the fruiting period to get the best flavor. The pomegranate may begin to bear in 1 year after planting out, but 2 ½ to 3 years is more common. Under suitable conditions the fruit should mature in 5 to 7 months after bloom.

2.2 Medicinal properties of *Punica granatum*

The pomegranate has a long history of being herbal medicine dating back more than 3,000 years. All parts of the plant contain unusual alkaloids, known as 'pelletierines', which paralyse tapeworms so that they are easily expelled from the body by using a laxative. The plant is also rich in tannin, which makes it an effective astringent. It is used externally in the treatment of vaginal discharges, mouth sores and throat infections (Bown, 1995). Below, are the list of the uses of pomegranate in disease treatments.

To treat conjunctivitis: Prepare a paste from the green leaves of the plant by washing and grinding on a stone grinder, and then apply to the eyes.

To treat diarrhea: Put about 3 inches of bark into 4 cups of boiling and keep boiling until the volume reduced to 1 cup. *Dosage*: Half a cup in the morning and then again in the evening till cured. Alternatively method is by decoction of fruit peel.

To treat vomiting: Put half a cup of crushed pomegranate leaves in 1 cup of boiling water for 15 minutes. *Dose:* 1 cup once only.

To treat dysentery: Ground rind and bark into paste and making an infusion. A combination of fruit rind and bark is an efficacious remedy, to be taken internally. Also the juice of the pulp is advised. The resulting solution must be filtered before consuming.

To treat intestinal worms: Ripe fruit skin is dried in the shade and then crushed to prepare a fine powder obtained by straining through a muslin cloth. *Dose*: 1 teaspoon of the powder is taken with water in the morning and evening for 3 days. Or fresh bark, 1 part fresh bark with 20 parts of water, boil until the volume reduce to half, filter, take it 5 mL half hourly four times on an empty stomach and then give castor oil.

To treat weakness: Fruit juice obtained from crushed pulp. To 1 cup of juice, 2 cups of sugar are added. This is boiled to a thick syrup consistency. *Dose*: 2 teaspoons of the syrup are taken in the morning and evening until cured.

To treat abscess: A 3 inch long strip of bark is rubbed on a stone to make a paste. The paste is applied in the morning and evening until cured.

2.3 Chemical constituents of *Punica granatum*

While detailed knowledge of relationships of the chemical contents of pomegranates and their desirable pharmacologic endpoints has yet to be obtained, significant progress has been made toward a much more comprehensive understanding of some of the important pharmacologic components of pomegranate. Chemical studies of pomegranate have reported on many compounds isolated from different parts of the plant. List of the compounds found in pomegranate is shown in Table 2-1. In addition to the more common anthocyanins shown in the table, pentose glycosides of malvidine and pentunidin have been described in the pericarp and juice (Sharma and Seshadri, 1955). Although some limited knowledge of the abundance of selected compounds does exist, e.g., Vitamin C in the juice at 0.47 mg/100 g (Veres, 1976), In general such quantitative knowledge is still lacking, and hence has been left out.

Table 2-1 Chemical constituents of *P. granatum.*

Chemical class	Compound name	Plant part	References
Hydroxybenzoic acids (Figure 2-2)	Gallic acid	Juice, Peel, Fruit	Amakura *et al*. (2000b) Huang *et al*. (2005b)
	Ellagic acid	Juice, Peel, Seed	Amakura *et al*. (2000b) Wang *et al*. (2004)
Hydroxycinnamic acids (phenylpropanoids) (Figure 2-3)	Caffeic acid	Juice, Peel	Artik (1998) Amakura *et al*. (2000b)
	Chlorogenic acid	Juice, Peel	Artik (1998), Amakura *et al*. (2000b)
	p-Coumaric acid	Juice, Peel	Artik (1998), Amakura *et al*. (2000b)
Cyclitol carboxylic acids and their salts (Figure 2-4)	Quinic acid	Juice, Peel	Artik (1998), Amakura *et al*. (2000b)
Flavan-3-ols (Figure 2-5)	Flavan-3-ol	Juice, Peel	de Pascual-Teresa *et al*. (2000)
	Catechin	Juice, Peel	de Pascual-Teresa *et al*. (2000)
	Epicatechin	Juice, Peel	de Pascual-Teresa *et al*. (2000)

Table 2-1 Chemical constituents of pomegranate (*Continued*)

Chemical class	Compound name	Plant part	References
Flavonols (Figure 2-6)	Quercetin	Juice, Peel	Artik (1998)
	Kaempferol	Peel	van Elswijk *et al*. (2004)
Flavonol glycosides (Figure 2-6)	Rutin	Peel, Juice	Artik (1998)
	Kaempferol 3-*O*-glycoside	Peel	van Elswijk *et al*. (2004)
	Kaempferol 3-*O*-rhamnoglycoside	Peel	van Elswijk *et al*. (2004)
Flavones (Figure 2-7)	Luteolin	Peel	van Elswijk *et al*. (2004)
Flavone glycosides (Figure 2-7)	Luteolin 7-*O*-glycoside	Peel	van Elswijk *et al*. (2004)
	Naringin	Peel	van Elswijk *et al*. (2004)
Anthocyanidins (Figure 2-8)	Delphinidin	Peel	Noda *et al*. (2002)
	Cyanidin	Peel	Noda *et al*. (2002)
	Pelargonidin	Peel	Noda *et al*. (2002)

Table 2-1 Chemical constituents of pomegranate (*Continued*)

Chemical class	**Compound name**	**Plant part**	**References**
Ellagitannins (Figure 2-9, 2-10)	Punicalin	Peel, Leaf, Bark, Root	Tanaka *et al*. (1986) Gil *et al*. (2000)
	Punicalagin	Peel, Leaf, Bark, Root	Tanaka *et al*. (1986) Gil *et al*. (2000)
	Corilagin	Peel, Leaf	Satomi *et al*. (1993) Nawwar *et al*. (1994)
	Casuarinin	Peel	Satomi *et al*. (1993)
	Gallagyldilacton	Peel	Satomi *et al*. (1993)
	Pedunculagin	Peel	Satomi *et al*. (1993)
	Tellimagrandin	Peel	Satomi *et al*. (1993)
	Granatin A	Peel	Tanaka *et al*. (1990)
	Granatin B	Peel	Tanaka *et al*. (1990)

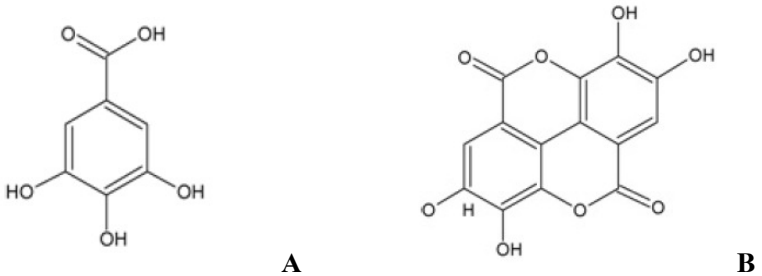

Figure 2-2 Chemical structure of gallic acid (A) and ellagic acid (B)

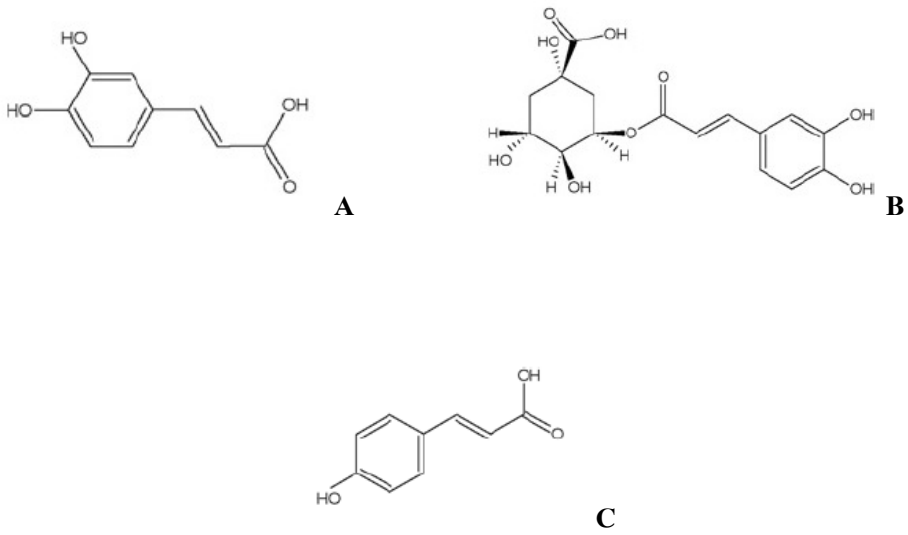

Figure 2-3 Chemical structure of caffeic acid (A), chlorogenic acid (B) and p-coumaric acid (C)

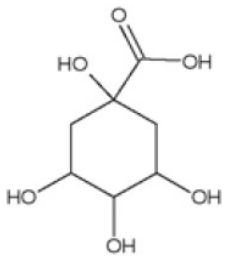

Figure 2-4 Chemical structure of quinic acid

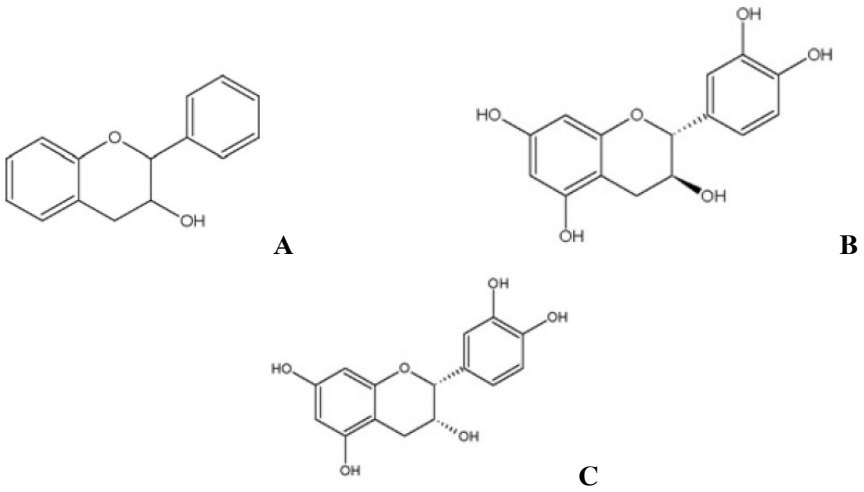

Figure 2-5 Chemical structure of flavan-3-ol (A), catechin (B) and epicatechin (C)

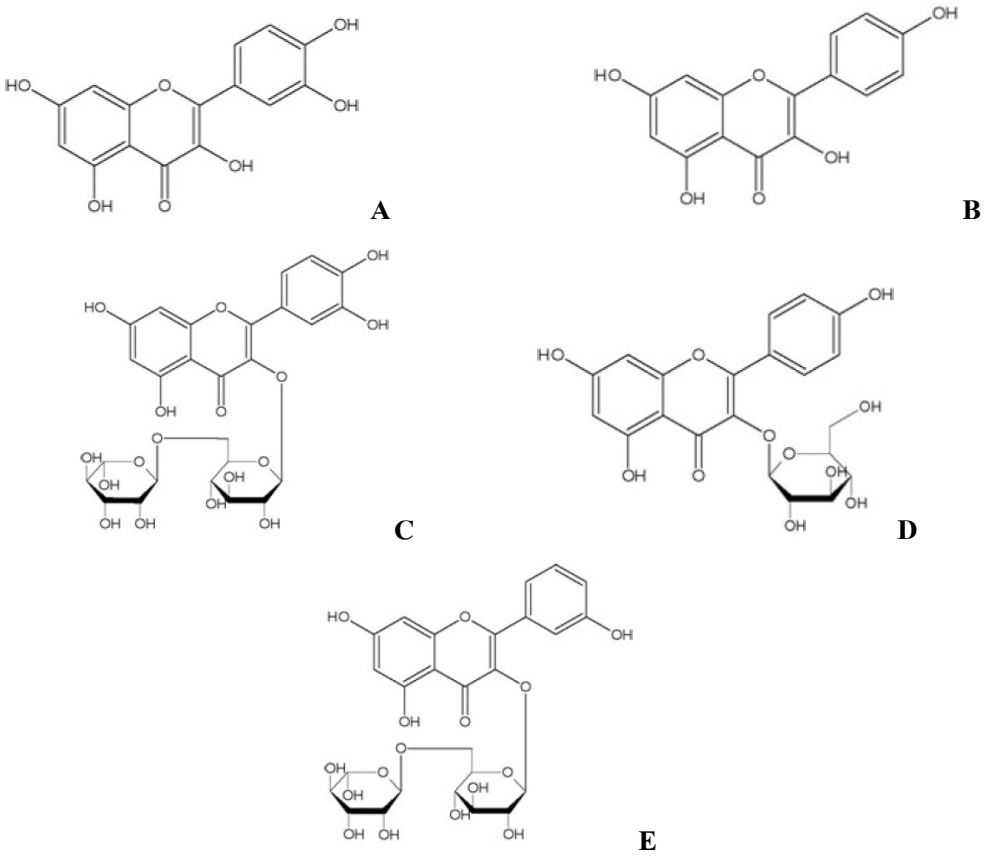

Figure 2-6 Chemical structure of quercetin (A), kaempferol (B), rutin (C), kaempferol 3-*O*-glycoside (D) and kaempferol 3-*O*-rhamnoglycoside (E)

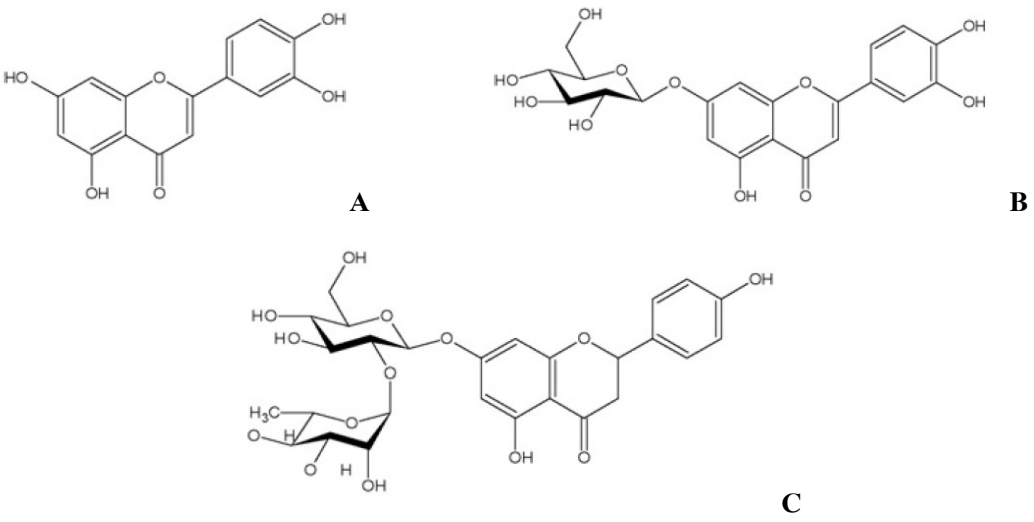

Figure 2-7 Chemical structure of luteolin (A), luteolin 7-*O*-glycoside (B) and naringin (C)

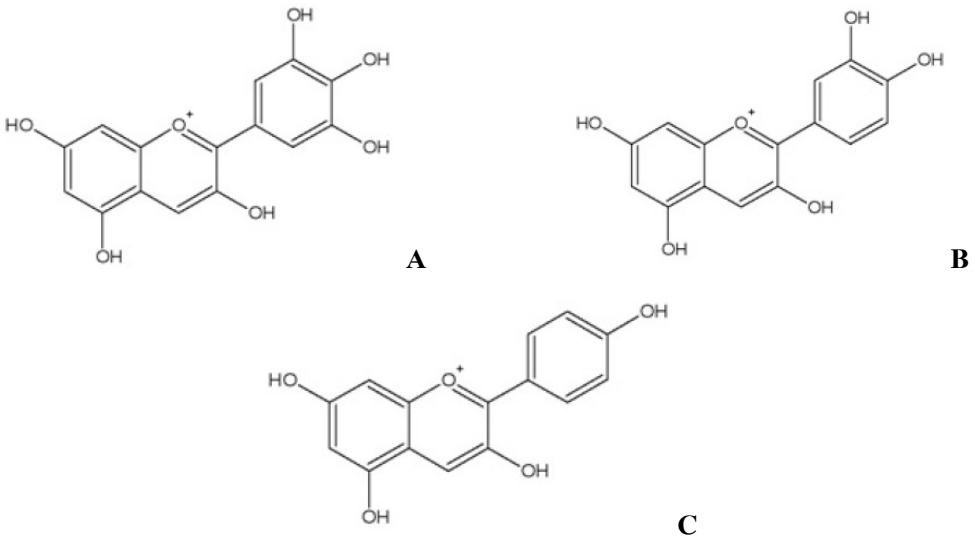

Figure 2-8 Chemical structure of delphinidin (A), cyaniding (B) and pelargonodin (C)

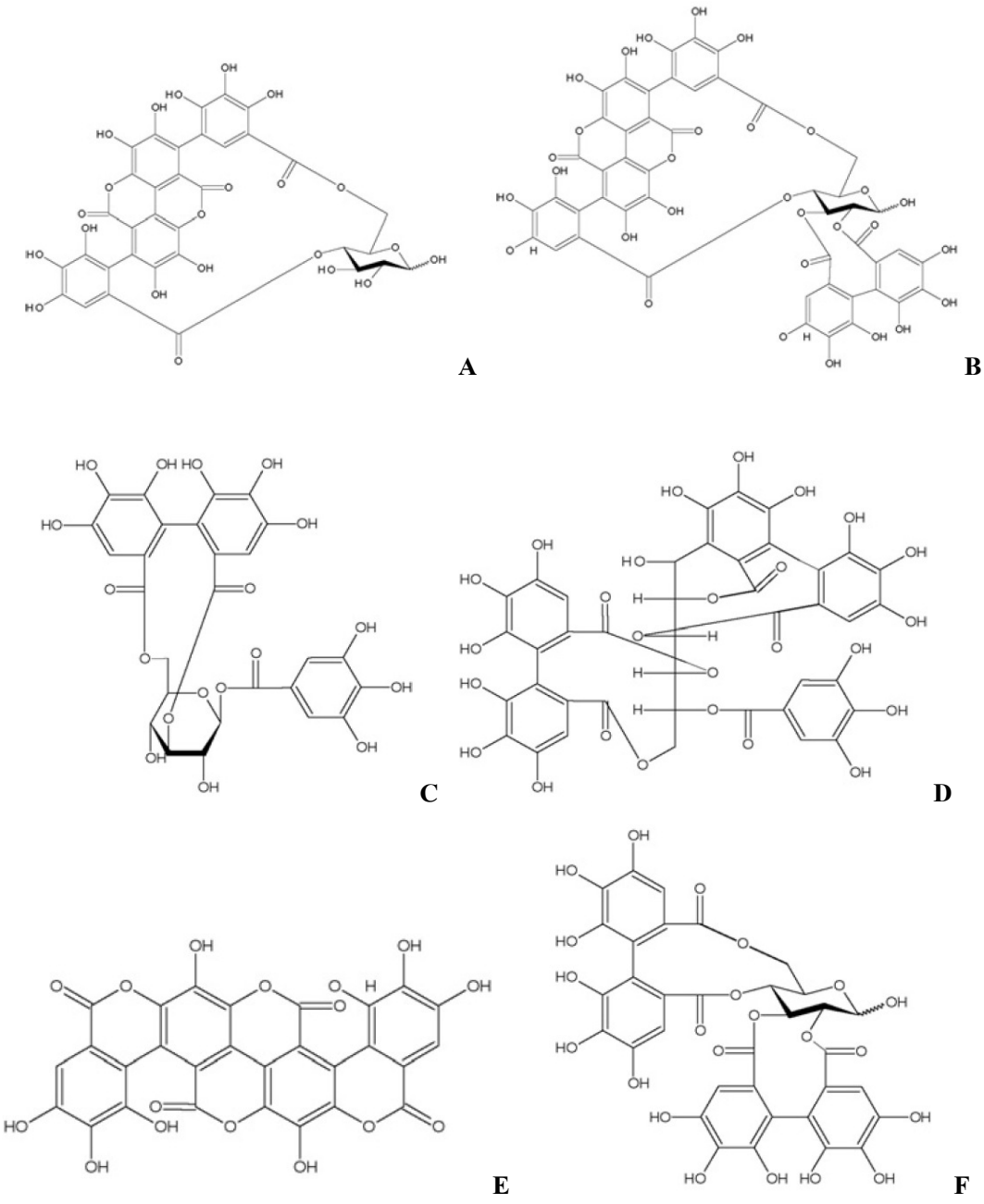

Figure 2-9 Chemical structure of punicalagin (A), punicalagin (B), corilagin (C), casurainin (D), gallagyldilacton (E) and pedunculagin (F)

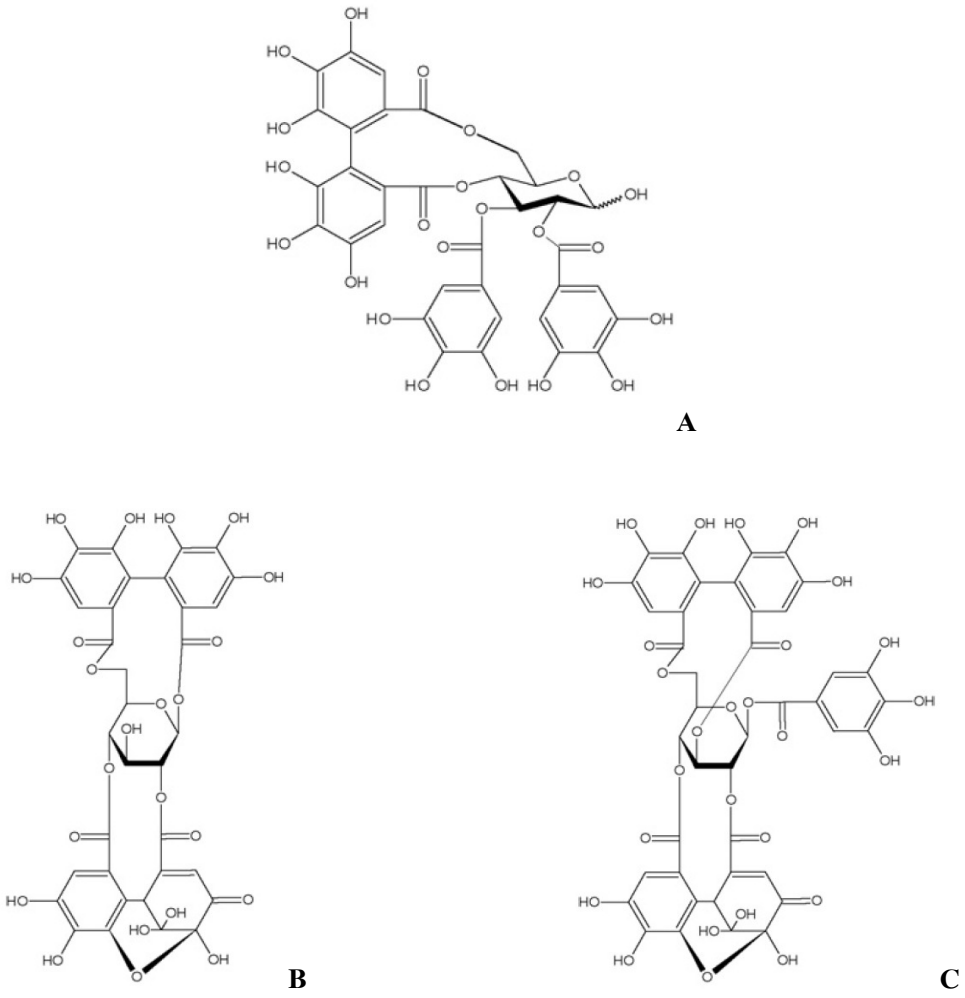

Figure 2-10 Chemical structure of tellimagrandin (A), granatin A (B) and granatin B (C)

Chemical composition of pomegranate seed

Pomegranate seed comprises of oil in 12-20% by weight. The pomegranate seed oil (PSO) consists of approximately 80% of conjugated octadecatrienoic fatty acids, with a high content of *cis* 9, *trans*11, *cis* 13 (i.e. punicic acid), synthesized *in situ* from nonconjugated octadecadienoic fatty acid. PSO contains about 7% of linoleic acid (Hopkins and Chisholm, 1968). The fatty acid content 99% of them are triacyl glycerols. Minor components of the oil include sterols, steroids, and a key component of mammalian myelin sheaths, cerebroside (Tsuyuki *et al*., 1981). Other report bioactive compounds found in seeds of *P. granatum* are lignins. (Dalimov *et al*., 2003), fusion products of cell wall components and hydroxycinnamic acids, and potently antioxidant lignin derivatives (Wang *et al*., 2004).

Chemical composition of pomegranate juice

Anthocyanins, potent antioxidant flavonoids, provide pomegranate juice with its brilliant color, which increases in intensity during ripening (Hernandez *et al*., 1999), and declines after pressing (Perez-Vicente *et al*., 2002; Miguel *et al*., 2004). Minerals in the juice and seed include Fe, relatively prevalent, but not so high in concentrations as in watermelon. Trare element such as Ca, Ce, Cl, Co, Cr, Cs, Cu, K, Mg, Mn, Mo, Na, Rb, Sc, Se, Sr, and Zn are also found in the juice (Waheed *et al*., 2004).

Chemical composition of pomegranate pericarp (peel, rind, hull are synonyms)

Both flavonoids and tannins are found abundantly in the peels of wild-crafted compared to cultivated fruits (Ozcal and Dinc, 1993). Complex polysaccharides from the peels have been studied and partially characterized (Jahfar *et al*., 2003).

Chemical composition of pomegranate leaf

Unique tannins occur in pomegranate leaves, as well in peel. Leaves also contain glycosides of apigenin, a flavone with progestinic (Zand *et al*., 2000) and anxiolytic (Paladini *et al*., 1999) properties. With respect to chemical elements, N is high in medium age, K in young age; Ca and Fe in old leaves. In July and August in the Northern Hemisphere, N and K are both low during flowering and fruit-setting, N further declines during fruit maturity, along with Mg, Fe and Zn (Munde *et al*., 1981).

Chemical composition of pomegranate flower

The flowers contain compounds that also found in peels (e.g. gallic acid) and seed (e.g. ursolic acid).

2.4 Biological activities of *Punica granatum* fruit peel extract

Antioxidant activity

It has been reported that the aqueous extracts of the fruit peel showed antioxidant activity when evaluated by DPPH, 5-lipoxygenase and chemiluminescence assays with IC_{50} values of 0.094 ± 0.001 mg/mL, 0.198 ± 0.013 mg/mL and 0.944 ± 0.031 mg/mL, respectively (Ricci *et al.*, 2006).

Antioxidant activity of ethyl acetate extract of pomegranate fruit peel was determined by DPPH radical scavenging assay, lipid peroxidation and superoxide assay gave IC_{50} values of 8.49 ± 0.042 mg/mL, 0.25 ± 0.008 mg/mL and 6.93 ± 0.189 mg/mL, respectively (Ricci *et al*., 2006).

Extraction pomegranate fruit peel using mixture of ethanol, methanol and acetone displayed markedly higher antioxidant capacity than the pulp extract in scavenging or preventive capacity against superoxide anion, hydroxyl and peroxyl radicals as well as inhibiting $CuSO_4^-$ induced LDL oxidation. Bioassay-guided isolation of the antioxidant active constituent from pomegranate fruit peels found that ellagic acid is the major active compound (Panichayupakaranant *et al*., 2005).

Antibacterial activity

It has been reported that pomegranate fruit peel methanolic extract exhibited percentage of inhibition of bacterial growth against *Escherichia coli, Shigella sonnei, Shigella flexneri* and *Salmonella* sp. of 56.8, 70.0, 100.0 and 51.5 %, respectively (Alanis *et al*., 2005).

Both aqueous and ethanolic extracts of pomegranate fruit peel were highly effective against *Escherichia coli* with the best MIC and MBC values of 0.78, and 0.39 mg/mL, respectively (Voravuthikunchai *et al*., 2004).

It has been reported that methanolic extract of *P. granatum* fruit peel marketed in Puerto Rico possessed strong *in vitro* antibacterial activities against *Escherichia coli* and *Staphylococcus aureus* with MIC values of 2.08, 1.81 mg/mL, respectively (Melendez and Capriles, 2005).

The methanolic extract of *P. granatum* fruit peel exhibited inhibitory effect against *Proteus vulgaris* and *Bacillus subtilis* with MIC values of 1.5 and 6.0 mg/mL, respectively (Prashanth *et al*., 2001).

Cell proliferation promotion

Pomegranate seed oil, juice, peel and seed cake were shown to stimulate keratinocyte proliferation in cell monolayer culture. In parallel, a mind thickening of the epidermis (without the loss of ordered differentiation) was observed in skin cell culture. The same pomegranate seed oil that stimulated keratiocyte proliferation should no effect on fibroblast function. In contrast, pomegranate peel extract fermented juice and seed cake extracts stimulated type I procollagen synthesis and inhibited matrix metalloproteinase-1 (MMP-1; interstitial collagenase) production by dermal fibroblasts, but had no growth-supporting effect on keratinocyte (Aslam *et al*., 2005).

Inhibition of gastric mucosal injury

It has been reported that administration of 70% methanolic extract of pomegranate fruit peel to rat (250 mg/kg and 500 mg/kg) could inhibit aspirin and ethanol induced gastric ulceration 22.37%, 74.21%, 21.95% and 63.41%, respectively when compared to the control (Ranitidine, 96.72 %) (Ajaikumar *et al*., 2005).

Immunomodulatory activity

It has been reported that pomegranate fruit peel powder (PGFRP) at the dose of 100 mg/kg orally as aqueous suspension was found to stimulate the cell-mediated and humoral components of the immune system in rabbits. PGFRP elicited an increase in antibody titer to typhoid-H antigen. It also enhanced the inhibition of leucocyte migration in leucocyte migration inhibition test and induration of skin in delayed hypersensitivity test with purified Pprotein derivative (Ross *et al*., 2001).

Anticancer activity

Flavonols in pomegranate fruit peel were shown to expression of tumor necrosis factor-α, interleukin-1β gene expression in tumor cells and inhibit breast cancer cell proliferation. The compounds inhibited fatty acid synthase activity in human tumor cells and focal adhesive kinase activity, a key regulator of tumor cell invasion. Pomegranate fruit peel exhibited tumor cell apoptosis through up-regulation of death receptor and activation of caspace activities. They also inhibited PC-3 tumor cell invasion and human tumor cell growth *in vitro* and inhibited growth of sarcoma S-180 implanted in mice (Lansky *et al.,* 2007).

Anti-inflammatory activity

Ellagitannin in pomegranate fruit peel were shown to inhibit UV-B mediated activation of NF-kB mitogen-activated protein kinases (MAPK), a protein modifications induce inflammatory cascades. It inhibited inflammatory cell signaling in colon cancer. Patients experiencing this form of oral inflammation received intragingival chips impregnated with pomegranate peel extract, which resulted in reduced inflammatory cykines (IL-1 beta and IL-6) several months post-treatment (Lansky *et al.,* 2007).

Toxicity study on pomegranate

It has been reported that hydroalcoholic extract of whole fruit of pomegranate in chick embryo model, at the doses of the extract less than 0.1 mg per embryo are not toxic. The LD_{50} of the extract, determined in OF-1 mice of both sexes, after intraperitoneal administration, was 731 mg/kg. Confidence limits were 565-945 mg/kg (Vidal *et al*., 2003).

2.5 Biological activity of ellagic acid

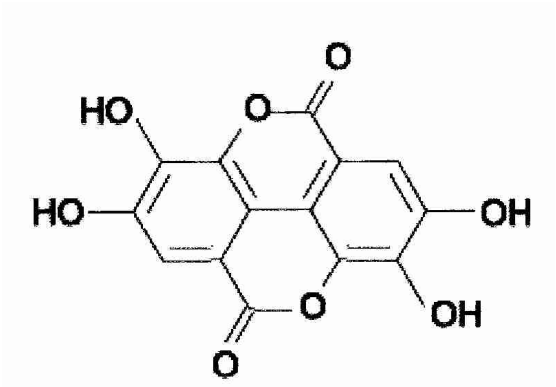

Figure 2-11 Chemical structure of ellagic acid

Ellagic acid (2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3,–cde][1] benzopyran-5,10-dione) (Figure 2-2) has been found in many woody plants and in diverse fruits and nuts in various concentrations. Ellagic acid is believed to be formed by the hydrolytic release from ellagic acid derivatives including ellagic acid glycosides and ellagitannins. The presence of ellagic acid in various fruits and nuts was determined for the purpose of botanical classification, and was identified in strawberries, blackberries, and walnuts. Concentrations of ellagic acid in various fruits and nuts were determined as total ellagic acid, which was hydrolytic ellagic acid from ellagic acid precursors followed by complete hydrolysis with acid (Lee, 2004).

Ellagic acid has a variety of biological activities including anti-oxidant, anti-inflammatory, anti-fibrosis and anti-cancer properties. The anti-cancer properties of ellagic acid include induction of cell cycle arrest and apoptosis, and inhibition of tumor formation and growth *in vivo*. The molecular mechanisms responsible for these effects remain largely unknown. But its potent scavenging action on both superoxide anion and hydroxy anion might be involved (Masamune, 2005).

Ellagic acid has been found to have antimutagenic, antiviral, whitening of the skin and antioxidative properties. Additionally, ellagic acid has now been allowed for use as a food additive in Japan, functioning as an antioxidant. Thus, the development of a simple method for its determination in foodstuffs is required and is important for health and food hygiene (Amakura, 2000a). The phenolic nature of ellagic acid makes itself a powerful antioxidant. It has been reported that ellagic acid can inhibit the mutagenicity and carcinogenicity in a wide range of mutagents especially chemical agents such as aflatoxin B, 1,3-dinitropyrene and nitro compounds *in vivo* and *in vitro*. However, its anti-tumorigenic activity had been evaluated in several *in vivo* model systems often shown conflicting results (Lei, 2003).

2.6 Biosynthesis of ellagic acid (Copy from Hakkinen, 2000)

It is likely that there are several pathways for the biosynthesis of individual hydroxybenzoic acids, depending on the plant. They can be derived directly from the shikimate pathway, especially from dehydroshikimic acid; this reaction is the main route to gallic acid. However, they can also be produced by the degradation of hydroxycinnamic acids, in a similar manner to the β-oxidation of fatty acids; the main intermediates are cinnamoyl-CoA esters. Hydroxybenzoates are also produced occasionally by the degradation of flavonoids. Moreover, hydroxylations and methylations of hydroxybenzoic acids are known to occur in an analogous way to the phenylalanine/hydroxycinnamate pathway. Knowledge of the mechanisms and, particularly, the enzymes involved in the biosynthesis of hydroxybenzoic acids and their derivatives is rather limited, especially regarding fruits, although gallic acid and its derivatives play an important role in the formation of hydrolysable tannins.

The biogenesis of hexahydroxydiphenoyl esters and their hydrolysis to give ellagic acid. The existence of isozymes in the biosynthesis of ellagic acid have not been determined. Ellagic acid is formed by oxidation and dimerization of gallic acid. Oxidation is hastened by alkaline conditions, whereas hydrolysis and lactonization are favored by acidic conditions. Gallic acid and its dimeric form ellagic acid can react with hydroxyl-containing compounds to form esters. Gallic acid and ellagic acid are the main components of hydrolysable tannins. Tannins which yield only gallic acid are defined as gallotannins, and those which give hexahydroxydiphenic acid normally as ellagic acid, the dilactone form of hexahydroxydiphenic acid are called ellagitannins.

2.7 Standardized of herbal extract (Tierra, 1999)

Standardized extracts arised out of the need to create a uniform product. There are two types of standardization. One is based on identifying and quantifying an extract to a characteristic chemical marker compound. The second identifies and concentrates one or more as active constituents, making it closer to the level of a chemical isolate. This means that other naturally occurring constituents are displaced at the expense of one or a number of compounds.

Standardization of herbal extract will provide a more consistent, stronger and more effective product by chemical analysis to confirm the presence and ratio quantity of one or a number of characteristic plant constituents. This will increase consumer confidence and good for greater acceptance of herbs by the medical establishment and the mainstream.

Quality and effectiveness of herbal extracts may be compromised in a number of ways:

1. For the extracts based on active constituents, the high degree of concentration causes a corresponding displacement and lack of other constituents, which in a few cases have been subsequently shown to be even more effective than the originally presumed active constituent.

2. For active constituent extracts, given that there may be only a partial representation of the herb's normally occurring constituents, this limits the broad range of traditionally known properties and uses of an herb in favor of a single use.

3. The use of chemical constituents as active or marker compounds creates misinformation encouraging the misuse of herbs as a substitute for drugs. This demeans in popular understanding the broader context of their use for the treatment of underlying imbalances as the cause of disease.

4. Not all herbs branded as standardized are manufactured the same. Some involve methods that refined tincture or a concentrated dried extract while others employ the use of toxic solvents that may go against the sensibilities and ethics of individuals who are attracted to the use herbal remedies as an alternative to drugs. Furthermore, different methods of standardization produce significant differences in the finished product of which the consumer is not aware.

5. The need to extract high isolates of a single biochemical constituent fosters poor harvesting and wild crafting (ecologically sound harvesting of wild herbs) where quantity is sacrificed for quality.

6. The promotion of standardized extracts for the treatment of a named pathology encourages marketing opportunism. This tends to distract from other herbs and products such as the use of Chinese red sage (*Salvia milthiorrhiza*) or Tienchi ginseng (*Panax pseudoginseng*) instead of hawthorn for heart disease, chrysanthemum flowers (*Chrysanthemum morifolium*) instead of feverfew for migraine headaches and honeysuckle blossoms (*Lonicera japonica*) instead of echinacea for the common cold.

7. The technology necessary to produce truly standardized extracts as espoused by some of their leading exponents greatly changes the way herbs are handled at all stages from growth to final product. Some proposed trends such as the exclusive use of cultivated herbs over wild harvested ones, is counter to the traditional time-honored principles and practices of herbal medicine. Herbalists have always felt that herbs grown in the wild are superior to those under cultivation and by definition, wild herbs cannot be standardized. To manipulate herbs to conform to an artificial process of standardization makes them more like 'phytopharmaceutical' drugs. This in turn means that they can only be manufactured into products by well-vested pharmaceutical companies to be distributed and sold in pharmacies under prescription by medical doctors. Herbal medicine is a rigorous study and medical practice unto itself. It is presumptuous to assume that the majority of medical doctors are or ever will be qualified in their proper use. With profit as the primary motive, there is good reason to distrust pharmaceutical companies considering that it was from this sector that one may largely attribute the nearly complete

suppression of herbal medicine from the mid-1920's to the late 60's. Up to recently, pharmaceutical companies were unable to cash on the sale of herbs because they were unpatentable. With the advent of standardization, there is a pattern established where a company that is able to spend huge amounts of money on research is entitled to develop an exclusive patent for the process of extraction and standardization of an herbal product accompanied with a license to sell them on the international market.

As described, by definition, a standardized herbal extract involves predetermining one or a number of biochemical constituents as either active or as marker compounds. The result involves two very distinct types of extracts.

1. Marker extract

This type establishes a specified amount of a marker compound is present in the finished product. It must be noted that a marker does not represent as the active constituents but is selected as a biochemical constituent characteristic of the plant. In general, the insoluble compounds, such as cellulose and fiber, are excluded. The concentrated extracts may present in the form of dried and powdered or mixed with a neutral material such as corn starch, and in still others. The most important distinction is that marker extracts are used for positive identification or to create a higher degree of uniform potency but not proven as active constituent. For the latter, it as yet remains to be proven whether this is consistent with the potency of the whole herb.

Table 2-2 Examples of marker extracts (Tierra, 1999)

Herb	Marker constituent	% content
Artichoke	Cynarin	2-5
Chamomile	Apigenin	1.2
	Essential oil	0.5
Devil's claw	Harpogosides	5
Echinacea	Echinacosides	4
Ephedra	Ephedrine/Pseudoephedrine	6-8
Feverfew	Parthenolides	2.6
ginseng	Ginsenosides	5-15
Goldenseal	Hydrastine	5
Horsechestnut	Aescin	20
Ursi	Arbutin	20
Gotu Kola	Asiaticosides	10
Green tea	Polyphenols	20-50
Licorice	Glycyrrhizin	12
St Johnswort	Hypericin	0.3-0.5
Schisandra	Schisandrins	2.6-4
Valerian	Valerenic acid	0.8-1
Willow	Salicin	8

2. An active constituent extract

This regulates a specific biochemical constituent to a level that may not be naturally found in the plant. Concentrating 95% curcuminoids, for instance, in a standardized turmeric extract creates a product that while derived from the crude herb, is not expected to be naturally found concentrated at that level. The other turmeric constituents in leaves, which the curcumin is combined only 5%. The active constituent may be found to be not primarily responsible for the therapeutic action of the herb, i.e. other constituents may be discovered to be more biologically active.

Table 2-3 Examples of active constituents extracts (Tierra, 1999)

Herb	Active constituents	% content
Gingko	flavoglycosides	24
Milk thistle	silymarin	80
Grape seed	polyphenols	95
turmeric	curcumin	95
Saw palmetto	free fatty acids	90
Green tea	catechins	60
Cascara sagrada	anthraquinones	20-30
Bilberry	anthocyanosides	25
Pygeum	phytosterols	12
Kava	kavalactones	30-40

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

Punica granatum fruits were collected from Mengzhi pomegranate garden, Yunnan, China. The fruit peels were dried at 50-60 ^{o}C in hot air oven for 24 hours, and were reduced to coarse powder using a grinder.

3.1.2 Chemicals and reagents

3.1.2.1 For quantitative determination of ellagic acid in the extract

Ellagic acid standard grade (Fluka, Switzerland)

Purified water (Milli-Q system)

Methanol, HPLC grade (Lab scan Asia, Thailand)

Acetic acid, glacial AR grade (Lab scan Asia, Thailand)

3.1.2.2 For extraction and pre-purification

Methanol, AR grade (Lab scan Asia, Thailand)

Ethyl acetate, AR grade (Lab scan Asia, Thailand)

Acetic acid, glacial AR grade (Lab scan Asia, Thailand)

3.1.2.3 For antioxidant activity assay

Ethanol, AR grade (Lab scan Asia, Thailand)

1, 1-diphenyl-2-picrylhydrazyl (DPPH) AR grade (Fluka, Switzerland)

Quercetin AR grade (Sigma, Switzerland)

3.1.2.4 For establishment of the standard specification of the extract

Methanol, AR grade (Lab scan Asia, Thailand)

Ethyl acetate, AR grade (Lab scan Asia., Thailand)

Ethanol, AR grade (Lab scan Asia, Thailand)

Hexane, AR grade (Lab scan Asia, Thailand)

Chloroform, AR grade (Lab scan Asia, Thailand)

Dimethyl sulfoxide (DMSO) (Riedel-de Haen, Germany)

Acetic acid, glacial AR grade (Lab scan Asia, Thailand)

n-Octanol AR grade (Riedel-de Haen, Germany)

Eosin methylene blue agar (EMB) (Becton, Dickinson, France)

Sabouraud Dextrose Agar (SDA) (Becton, Dickinson, France)

Melted plate count agar (PCA) (Becton, Dickinson, France)

Peptone (Becton, Dickinson, France)

Anhydrous *di*-sodium hydrogen orthophosphate AR grade (Anala, England)

Potassium *di*-hydrogen orthophosphate AR grade (M & B, England)

di-Potassium hydrogen orthophosphate AR grade (M & B, England)

Ellagic acid standard grade (Fluka, Switzerland)

3.1.3 Instruments

Table 3-1 General information of equipments

Instrument	Model	Company
UV-visible spectrophotometer	Genesis 5	Miltonroy, USA
Hot air over	DIN 12880-KI	Memmert, Germany
HPLC	Agilent 1100 series	Palo Alto, USA
HPLC column	TSK-gel ODS-80Tm	Bioscience, Japan
pH meter	PHM 82	Radiometer, Denmark
Rotary evaporator	N-N Series	EYELA, Japan
Milli-Q system	Millipore	Bedford, USA
Satorious moisture analyzer	WDS 400	Itin Scale, USA

3.2 Methods

3.2.1 Quantitative analysis of ellagic acid in *P. granatum* fruit peel extract

3.2.1.1 Preparation of standard solution

A stock solution (concentration 1 mg/mL) of the reference standard, ellagic acid was made in methanol, the solution was stable for 7 days in temperature 4 ºC and subsequently diluted to provide a series of the standard solution ranging from 3 - 50 µg/mL for use in constructing calibration curve of ellagic acid.

3.2.1.2 Sample extraction

P. granatum fruit peel powder (100 mg) was extracted with methanol (20 mL) under reflux conditions for an hour. The extract was then filtered and concentrated under reduced pressure. The residue was reconstituted and adjusted to 10 mL with methanol. Samples were analyzed immediately after extraction in order to avoid possible chemical degradation. The experiments were carried out in triplicate.

3.2.1.3 HPLC Conditions

HPLC analysis was carried out using Agilent 1100 series equipped with a Agilent 1100 series phodiode-array detector (PDA) and autosampler. Data analysis was performed using Agilent software (Agilent, USA.). Separation was achieved at 25 ºC on a TSK-gel ODS-80Tm (4.6 mm X 15 cm) column. The mobile phase consisted of methanol and 2% aqueous acetic acid, gradient elution from 40% to 60% v/v methanol within 15 min and maintained at 60% v/v for 5 minutes and reduced to 40% after 5 minutes for each run at a flow rate of 1 mL/min. The injection volume was 20 µL. The quantitation wavelength was set at 254 nm.

3.2.1.4 Method validation (Association of Official Analytical Chemists, 2002)

Calibration curve

Calibration curves were constructed on three consecutive days by analysis of the standard compound (ellagic acid) at five concentrations (3.125, 6.25, 12.50, 25.00 and 50.00 µg/mL) using the HPLC conditions as described in section 3.2.1.3. The average peak area of each concentration was plotted against the concentration of each standard. The linearity of the detector response for the standards was assessed by means of linear regression.

Accuracy

The *P. granatum* fruit peel extract from section 3.2.1.2 (1 mL) was fortified with 25, 50 100 µg/mL of standard ellagic acid (1 mL) in order to assay accuracy data. Prior to standard ellagic acid fortification, the background levels of ellagic acid in pomegranate fruit peel extracts were determined to calculate actual recoveries. The amount of ellagic acid was determined in triplicate and percentage recoveries were then calculated.

Precision

Precision experiments were conducted for intraday and interday. The solution of *P. granatum* fruit peel extract from section 3.2.1.2 concentration 0.5 mg/mL was used to achieve repeatability testing. The data of repeatability was the content of six injections separately in the same day. The data used to calculate %R.S.D. of interday precision was the content of three samples of *P. granatum* fruit peel extract from section 3.2.1.2 concentration 0.5 mg/mL analyzed in three days (three injections in succession each day).

Specificity

Peak identification was carried out using the standards ellagic acid and diode-array detector. The UV spectra were taken at various points of the peaks obtained from the standard ellagic acid solution concentration 12.5 µg/mL in methanol to check peak homogeneity.

Limit of detection (LOD) and Limit of quantification (LOQ)

The limits of detection and quantification were determined by means of serial dilution concentration 0.5-5 µg/mL based on signal-to-noise ratios of 3:1 and 10:1, respectively.

3.2.2 Determination of solvent for extraction and fractionation

The dried powder (100 mg) was extracted with various solvents (20 mL x2), including ethyl acetate, methanol, 5, 10, 15 and 20% v/v water in methanol under reflux conditions for an hour. The pooled extracts of the same solvent were concentrated under reduced pressure, adjusted to 10 mL with methanol and subjected to HPLC analysis. The experiments were performed in triplicate.

Fractionation of the extract was performed using liquid-liquid extraction. The *P. granatum* fruit peel extract from reflux condition, was weight and dissolved in water and fractionated by organic solvents including ethyl acetate, *n*-butanol and a mixture of ethyl acetate and *n*-butanol (1:1). The organic phase was concentrated under reduced pressure the residue was reconstituted and adjusted to 10 mL with methanol and subjected to HPLC analysis. The extracts were also evaluated for antioxidant activity by DPPH radical scavenging assay. The experiments were carried out in triplicate.

The *P. granatum* fruit peel extract from reflux condition, was weight and dissolved in 2% aqueous acetic acid and fractionated by using between organic solvent were give high ellagic acid content and 2% aqueous acetic acid. The organic phase was concentrated under reduced pressure the residue was reconstituted and adjusted to 10 mL with methanol and subjected to HPLC analysis. The extracts were also evaluated for antioxidant activity by DPPH radical scavenging assay. The experiments were carried out in triplicate.

3.2.3 Determination of antioxidant activity

The antioxidant activity of the extracts was evaluated by DPPH radical scavenging assay which was originally described by Blois (1958).

DPPH (1,1-diphenyl-2-picrylhydrazyl) is considered as a stable radical because of the paramagnetism conferred by its odd electron (delocalization of the spare electron over the molecule as a whole). The solution (in absolute ethanol) appears as a deep violet colour and shows a strong absorption band at 520 nm. DPPH radical can accept an electron or hydrogen radical to become a stable diamagnetic molecule and has pale violet. If substance for testing antioxidant activity is mixed with DPPH solution and gives rise to pale violet, it suggests that this substance has antioxidant effect by mechanism of free radical scavenging activity.

To determine antioxidant activity of *P. granatum* extract, the dried residue obtained from extraction was dissolve in absolute ethanol (1 mg/ml) as stock solution. Diluted each sample for at least 5 concentrations in absolute ethanol (two-fold dilutions). Transferred 500 µL of each sample solution into an eppendorf tube.

DPPH solution ($6x10^{-5}$ M) was prepared in absolute ethanol and transfered (500 µL) to mix with sample solution. The mixture was shaken and stood at the room temperature for 30 minutes in the dark. The mixture was then measured the absorbance at 520 nm, using a mixture of 500 µL sample solution and 500 µL absolute ethanol as blank. The control solution in each experiment was prepared as follows:

- **Control ethanol:** mix of absolute ethanol (500 µL) with $6x10^{-5}$ M DPPH in absolute ethanol (500 µL); **blank:** absolute ethanol.

- **Control water:** mix distilled water (500 µL) with $6x10^{-5}$ M DPPH in absolute ethanol (500 µL); **blank:** mixture of distilled water (500 µL) and absolute ethanol (500 µL).

Calculation of % inhibition with equation as follows :

$$\% \text{ inhibition} = \frac{\text{OD control-OD sample}}{\text{OD control}} \times 100$$

Dose-response curve was plotted between % inhibition and concentrations. Linear regression analysis was carried out for calculating the effective concentration of sample required to scavenge DPPH radical by 50 % (ED_{50} value). In each experiment standard quercetin and standard ellagic acid used as the positive control. Dissolve the standard compounds with absolute ethanol. Dilute each standard compound for at least 5 concentrations in absolute ethanol (two-fold dilutions).

3.2.4 Preparation of ellagic acid high yielding extract

The dried powder of *P. granatum* fruit peels (0.5 kg) were refluxed for 1 hour with 10% v/v water in methanol (2 L x2) and the extract then was evaporated to dryness *in vaccuo*. The resulting residue was weight and dissolved in water and fractionated by using partitioned between ethyl acetate and 2% aqueous acetic acid the organic fraction was then separated and evaporated to dryness *in vaccuo* to produce the residue which will be called “high-yield ellagic acid extract”. The high-yield ellagic acid extract was kept in well-closed containers, protected from light at room temperature until use.

3.2.5 Determination of the moisture content

Moisture content of the high-yield ellagic acid *P. granatum* fruit peel extract was performed using loss on drying method. The extract was accurately weighed about 0.5 g in the pan of the Sartorius Moisture Analyzer and dried at 105 ºC for 4-8 minutes until the weight was constant. The percentage loss on drying of the test sample was automatically recorded. The analyses were performed in triplicate.

3.2.6 Ash content (Subcommittee on the establishment of the Thai Herbal Pharmacopoeia, 1998).

The high-yield ellagic acid *P. granatum* fruit peel extract was accurately weighed about 0.5 g and placed in a tarred crucible, which was previously ignited, cooled and weighed. The sample was incinerated by gradually increasing the temperature not exceeding 450 ºC in muffle furnace until free from carbon, then cooled and weighed. The percentage of the total ash was calculated with reference to the weight of the original extract. The analyses were performed in triplicate.

3.2.7 Determination of the microbial contamination (British Pharmacopoeia Commission, 2001)

3.2.7.1 Sample preparation

The high-yield ellagic acid *P. granatum* fruit peel extract was accurately weighed about 1 g and suspened in 9 ml of 0.1% (v/v) peptone water, then adjusted to 10 ml with 0.1% (v/v) peptone water. The stock solution was diluted with 0.1% (v/v) peptone water to provide five concentrations ranging from 10^{-1}-10^{-5} g/mL.

3.2.7.2 Determination of aerobic bacteria contamination

Determination of aerobic bacteria contamination was performed by pour plate method. The high-yield ellagic acid *P. granatum* fruit peel extract solution in DMSO (1 mL) and melted Plate Count Agar (about 20 mL) were added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 37 ºC for 3 days. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.2.7.3 Determination of *Escherichia coli* contamination

Determination of *E. coli* contamination was performed by pour plate method. The high-yield ellagic acid *P. granatum* fruit peel extract solution in DMSO (1 mL) and Eosin Methylene Blue Agar (about 20 mL) were added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 37 ºC for 1 day. Growth of red, non- mucoid colonies of gram-negative rods indicates the possible presence of *E. coli*. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.2.7.4 Determination of fungal contamination

Determination of fungi contamination was performed by pour plate method. The high-yield ellagic acid *P. granatum* fruit peel extract solution in DMSO (1 mL) and Sabouraud Dextrose Agar (and 20 mL) were added into Petri dishes (9 cm in diameter), and mixed until homogenously. At least two Petri dishes were prepared for each level of dilution. The plates were incubated at 28 ºC for 5 days. The plates corresponding to one dilution and showing the highest number of colonies less than 300 were selected. The number of colony-forming units per gram was calculated.

3.2.8 Determination of solubility (British Pharmacopoeia Commission, 2001)

The high-yield ellagic acid *P. granatum* fruit peel extract was accurately weighed about 10 mg and placed in a vessel of at least 100 mL capacity. The vessel was placed in a constant temperature device, maintained at a temperature of 25 ± 0.2 ºC. Various solvents (water, ethanol, methanol, ethyl acetate, chloroform and hexane) were examined by adding of the strength prescribed in the monograph by increments of 10 µL, shaking frequently and vigorously for 10 minutes. Record the volume of solvent added when a clear solution was obtained. If the solution was becomes cloudy or undissolved. The sample was continuously added until 10 mL. After addition of 10 mL of solvents, the sample or parts of it remained undissolved, the experiment had to be repeated in a 100 ml volumetric flask. At lower solubility, the time required to dissolve a substance can be considerably longer, at least 24 hours was allowed.

Descriptive term of solubility and approximate volume of solvents required to completely dissolve a solute (in milliliters per gram of solute) are drawn as follow (Table 3-2).

Table 3-2 Solubility criteria of the extract in various solvent

Solubility term	Volume of solvent required to dissolve 1 g of solute (ml)
Very soluble	less than 1
Freely soluble	from 1 to 10
Soluble	from 10 to 30
Sparingly soluble	from 30 to 100
Slightly soluble	from 100 to 1000
Very slightly soluble	From 1000 to 10,000
Practically insoluble	more than 10,000

The term partly soluble is used to describe a mixture of which only some of the components dissolve.

3.2.9 Partition coefficient (Organization for Economic Cooperation and Development, 1995)

3.2.9.1 Preparation of *n*-octanol pre-saturated with water

Before a partition coefficient was determined, *n*-octanol (250 mL) and water (250 mL) was mixed and shaked at 25 °C for 24 hours and let them stand long enough to allow the phases to separate and to achieve a saturation state.

3.2.9.2 Test substance

The stock solution of the high-yield ellagic acid *P. granatum* fruit peel extract was prepared in *n*-octanol that pre-saturated with water to produce 3 concentrations (0.2, 0.1 and 0.05 mg/mL) of the extract. To the stock solution of the high-yield ellagic acid *P. granatum* fruit peel extract each concentration in *n*-octanol (5 mL), 5 ml of water was added in test vessels. The test vessels were placed in a reciprocator and rotating quickly through 360° at 25 °C, at 1200 rpm for 24 hours. After that, the concentrations of the ellagic acid in both phases by an aliquot of each of the two phases was taken and analyzed by validated HPLC. The ellagic acid content in both phases was calculated and compared with the ellagic acid content originally introduced by this equation.

$$K = \frac{C_{n-octanol}}{C_{water}}$$

K = Partition coefficient = Log P_{ow}

$C_{n\text{-}octanol}$ = Concentration of ellagic acid in *n*-octanol phase (µg/ml)

C_{water} = Concentration of ellagic acid in water phase (µg/ml)

3.2.10 Stability test

3.2.10.1 Effect of light on stability of the extract

The high-yield ellagic acid *P. granatum* fruit peel extract was weighed about 100 mg and kept in well-closed containers. The extract was then stored at room temperature (30 ± 2 ºC) and under exposed to light (36-watt fluorescent lamp and 40 cm distance from the containers). An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the ellagic acid using validated HPLC. The experiments were performed in triplicate.

3.2.10.2 Effect of temperature on stability of the extract

The high-yield ellagic acid *P. granatum* fruit peel extract was weighed about 100 mg and kept in well-closed containers, protected from light. The extract was then stored at 4 ± 2 ºC and room temperature (30 ± 2 ºC). An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the ellagic acid using validated HPLC. The experiments were performed in triplicate.

3.2.10.3 Effect of accelerated conditions for stability of the extract

The high-yield ellagic acid *P. granatum* fruit peel extract was weighed about 100 mg and kept in well-closed containers, protected from light. The extract was then stored in a stability chamber at 45 ºC, 75% humidity for 4 month. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the ellagic acid using validated HPLC. The experiments were performed in triplicate.

3.2.10.4 Effect of pH on stability of the extract

The high-yield ellagic acid *P. granatum* fruit peel extract was accurately weighed to 100 mg and dissolved in 1 mL methanol and then phosphate buffer solution was add to achieve pH value of 5.5, 7.0, and 8.0. The sample solutions were kept in well-closed containers, protected from light and stored at room temperature (30 ± 2 ºC) for 4 month. An aliquot of each sample was taken at 0, 1, 2, 3, 4, 6, 8, 12, and 17 weeks and subjected to quantitative analysis of the ellagic acid using validated HPLC. The experiments were performed in triplicate.

3.2.11 Statistic analysis

All values are expressed as mean ± S.D. Data were analyzed by Student t-test. The level of statistical significance was taken at $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantitative determination of ellagic acid in *P. granatum* fruit peel extract

The optimal conditions for quantitative determination of ellagic acid in *P. granatum* fruit peel extract was performed using gradient reverse phase HPLC system. As the compound has maximum absorption at 254 nm, this wavelength was then used for quantification. Mixtures of methanol and 2% aqueous acetic acid were examined as the mobile phase, and the ratios as well as gradient elution system were optimized. The amount of methanol in 2% aqueous acetic acid was increased from 40% to 60% v/v within 15 minutes and maintained at 60% v/v for 5 minutes and reduced to 40% after 5 minutes for each run (Figure 4-1). Ellagic acid was eluted with the retention time of 6-7 minutes (Figure 4-2) with satisfactory resolution. This HPLC method is sample and faster than the previously reported HPLC method which takes a longer analytical time; with a total run time of about 98 minutes is required, and validation of the analytical procedure is not yet established (Seeram *et al.*, 2005). On the basis of the HPLC analysis, ellagic acid was found as the major constituent in *P. granatum* fruit peel extract with the content was 5.8 %w/w (Table 4-1).

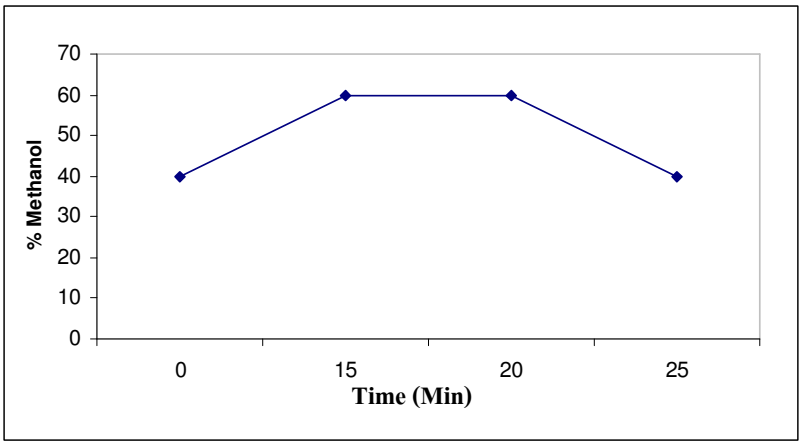

Figure 4-1 Gradient profile of the mobile phase (2% aqueous acetic acid: methanol)

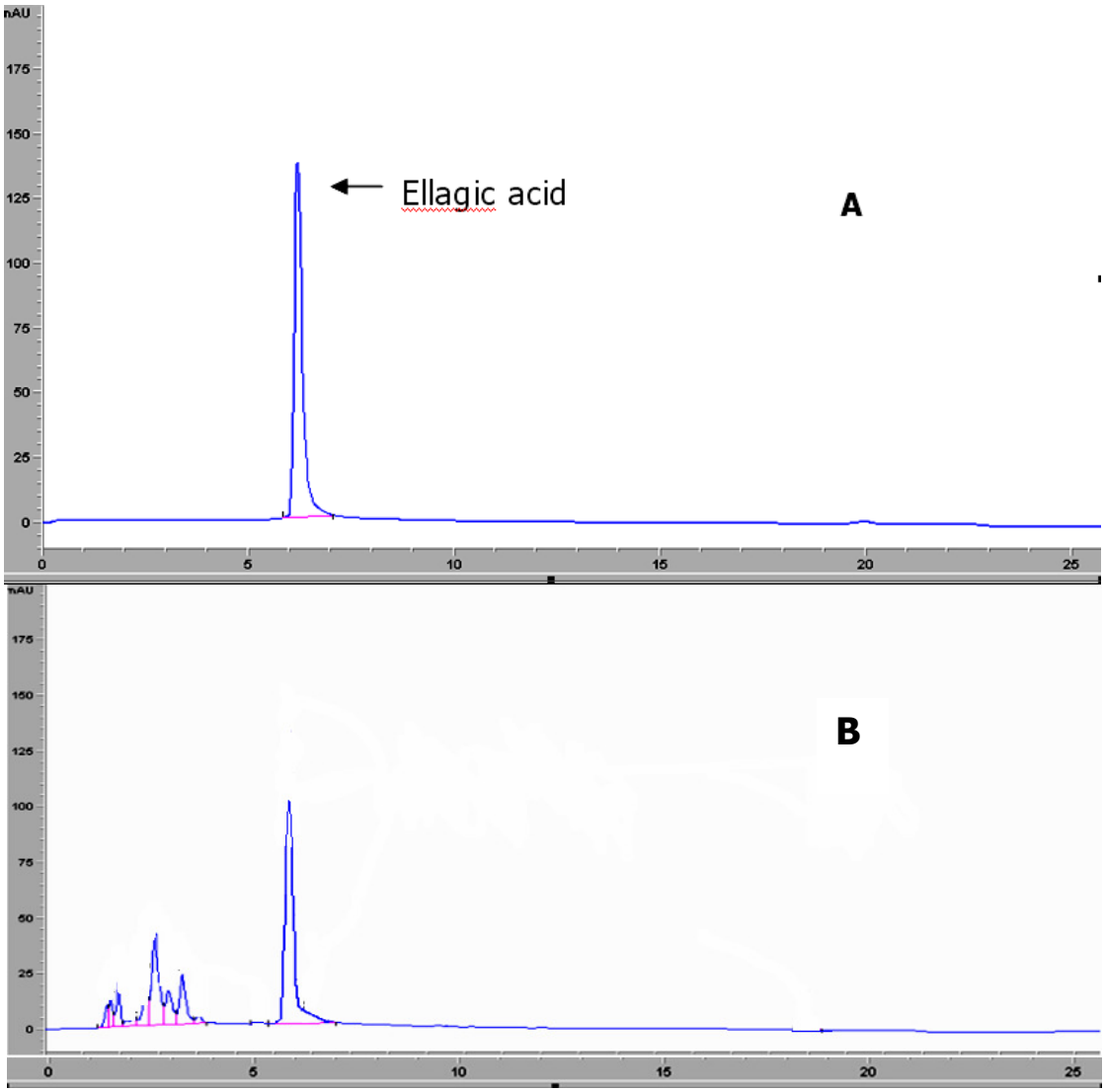

Figure 4-2 HPLC-chromatograms of (A) standard ellagic acid (concentration 25.0 µg/mL) (B) *P. granatum* fruit peel extract (concentration 5 mg/mL), separation was achieved at 25 ºC on a TSK-gel ODS-80Tm (4.6 mm X 15 cm) column. The mobile phase consisted of methanol and 2% aqueous acetic acid, gradient elution from 40% to 60% v/v methanol within 15 min and maintained at 60% v/v for 5 minutes and reduced to 40% after 5 minutes for each run at a flow rate of 1 mL/min. The injection volume was 20 µL. The quantitation wavelength was set at 254 nm.

Table 4-1 Ellagic acid content of *P. granatum* fruit peel extract

Compound	Content (% w/w ± S.D.)
Ellagic acid	5.83 ± 0.031

Defining the linearity, accuracy, intraday- and interday-precision, specificity and limits of detection and quantitation validated the HPLC method. Linearity was evaluated using standard ellagic acid over five calibration points (from 3.12 - 50.0 μg/mL) with six measurements for each calibration point. Ellagic acid exhibited linearity over the evaluated ranges with correlation coefficient of 0.9995 (Table 4-2).

Table 4-2 Linear ranges and correlation coefficients (r^2) of calibration curve

Constituent	Y = aX + b linear model[a]	r^2	Concentration (μg/mL)
Ellagic acid	Y = 139158X + 26.146	0.9995	3.12 - 50.0

[a] Y = peak area; X = concentration (μg/mL)

Intraday-precision was estimated by the relative standard deviation of six measurements for ellagic acid. Analysis of three independently prepared samples of pomegranate fruit peel extracts determined the interday-precision. The relative standard deviation values for both intraday and interday analysis of ellagic acid were less than 5% (Table 4-3).

Table 4-3 Precision for determination of ellagic acid in *P. granatum* fruit peel extract

Constituent	R.S.D. (%)	
	Intraday (n = 6)	Interday (n = 3)
Ellagic acid	1.36	4.40

Table 4-4 Accuracy for determination of ellagic acid in *P. granatum* fruit peel extract

Concentration of standard ellagic acid (µg/mL)	% Recovery (Mean ± S.D.)
25	97.42 ± 1.812
50	98. 91 ± 1.619
100	99. 20 ± 0.647
Mean of % Recovery	98.51 ± 1.359

Method accuracy was determined by analyzing *P. granatum* fruit peel extracts fortified with known quantities of each standard analyte. Recoveries in the range of 98.51 ± 1.36% were observed for ellagic acid (Table 4-4).

Utilizing the PDA makes it possible to obtain the UV spectra. Specificity of the method was evaluated using UV-absorption spectra produced by diode-array detector. The spectra were taken at three points of the peak of ellagic acid, compared with the standard ellagic acid, homogeneity for spectra of the peak was observed (Figure 4-3). Therefore, it was found that the developed HPLC method was very sensitive for analysis of ellagic acid in *P. granatum* fruit peel extracts with LOQ and LOD were 2.50 and 1.0 µg/mL, respectively.

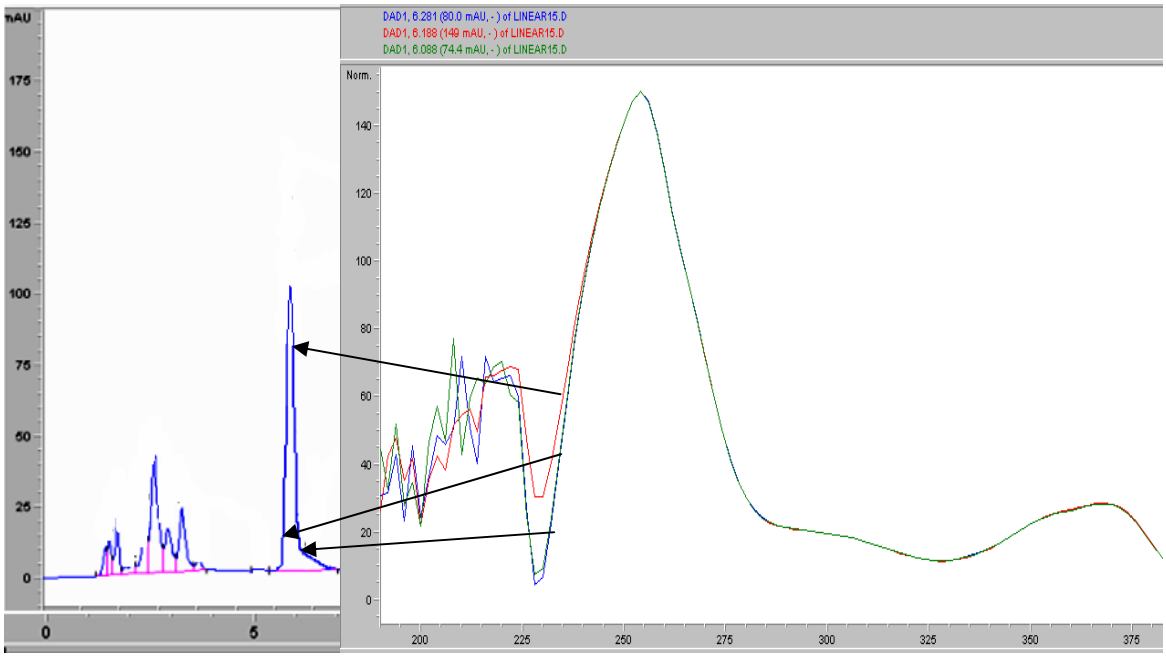

Figure 4-3 UV-absorption spectra of the chromatographic of ellagic acid peak of *P. granatum* fruit peel extract solution (concentration 5 mg/mL)

4.2 Optimization of the solvent for extraction

Two types of organic solvents (Ethyl acetate and methanol) were examined to maximize the ellagic acid content in *P. granatum* fruit peel extract. The result showed that methanol was more suitable than ethyl acetate for ellagic acid extraction (Table 4-5). With regards to the polarity of ellagic acid, combinations of water and methanol were examined as the extraction solvent. The results showed that 10% v/v water in methanol gave significantly higher ellagic acid content than those of the other extraction solvents. The extract was then further purified by partitioning between water and a few organic solvents including ethyl acetate, n-butanol and a mixture of n-butanol and ethyl acetate (1:1). It was found that the ethyl acetate fraction showed the highest ellagic acid content as well as the antioxidant activity (Table 4-6). Increasing ellagic acid content resulted in an increase in the antioxidant activity of the extract. Partitioning between water and ethyl acetate was therefore an appropriate method for the preparation of the high antioxidant potency extract of pomegranate fruit peel. Further study on fractionation the extract

between 2% aqueous acetic acid and ethyl acetate found that the ellagic acid content as well as antioxidant activity of the ethyl acetate fraction was significantly increased (Table 4-7). Suppression of ellagic acid ionization by aqueous acidic conditions was capable of decreasing water solubility of ellagic acid. Thus, an increasing of ellagic acid content in the ethyl acetate fraction was observed. Therefore, the suitable solvents for liquid-liquid extraction of *P. granatum* fruit peel extract in order to improve ellagic acid content and antioxidant activity of the extract are using mixtures of ethyl acetate and 2% aqueous acetic acid.

Table 4-5 Ellagic acid content in *P. granatum* fruit peel extracts extracted under reflux with various solvents

Solvent for extraction	Ellagic acid content (%w/w of extract) Mean ± S.D.
Ethyl acetate	5.21 ± 0.004*
Methanol	5.84 ± 0.031*
5% v/v Water in methanol	7.11 ± 0.171*
10% v/v Water in methanol	7.66 ± 0.008
15% v/v Water in methanol	6.82 ± 0.372*
20% v/v Water in methanol	6.18 ± 0.613*

*significant difference (P<0.05) when compared with 10% v/v water

Table 4-6 Ellagic acid content and antioxidant activity of *P. granatum* fruit peel extracts, which were partitioned between water and various organic solvents

Extract/fraction	Ellagic acid content (% w/w of extract) Mean ± S.D.	Antioxidant activity† ED_{50} (μg/mL)
Methanol extract	7.06 ± 0.025*	38.21 ± 0.138*
Ethyl acetate fraction	9.48 ± 0.065	15.17 ± 0.402
n-Butanol fraction	8.16 ± 0.014*	22.16 ± 0.514*
Ethyl acetate and *n*-butanol fraction	8.57 ± 0.063*	18.11 ± 0.502*
Standard quercetin	-	3.57 ± 0.637
Standard ellagic acid	-	3.12 ± 0.349

† Evaluated by DPPH radical scavenging assay

*significant difference (P<0.05) when compared with ethyl acetate fraction within the same column

Table 4-7 Ellagic acid content and antioxidant activity of the ethyl acetate fractions, which were partitioned between ethyl acetate and water or 2% aqueous acetic acid

Solvents for partition	Ellagic acid content (%w/w of extract) Mean ± S.D.	Antioxidant activity† ED_{50} (μg/mL)
Ethyl acetate and water	9.24 ± 0.511	16.31 ± 0.722
Ethyl acetate and 2% aqueous acetic acid	10.12 ± 0.374*	13.87 ± 0.610

† Evaluated by DPPH radical scavenging assay

* significant difference (P<0.05)

4.3 Establishment of the standard information (monograph) of *P. granatum* fruit peel extract

4.3.1 Preparation of high-yield ellagic acid extract

After the extraction and fractionation methods for preparation of the high-yield ellagic acid extract of *P. granatum* fruit peels were optimized, the established methods were applied to prepare the standard extract of *P. granatum* fruit peels used for further studies as followed. Dried powder of pomegranate fruit peels (0.5 kg) were refluxed twice with 10% v/v water in methanol (2 L) for 1 hour then the combined extract was evaporated to dryness *in vaccuo*. The residue (2 g) was redissolved in ethyl acetate 200 mL and transfer to separatory funnel, 2% aqueous acetic acid 200 mL was then added. The mixture was then shaken at room temperature for 1 min. (4 times). The ethyl acetate fraction was separated and combined extract was evaporated to dryness *in vaccuo*. Brown powder was obtained after evaporated to dryness (Figure 4-4) and the yield of the extract was 16.56 ± 1.13% w/w when compared to the weight of the dried powder (Table 4-8). Only ellagic acid was found as the major compound in the extract with the content of 13.63 ± 0.89% w/w compared to the weight of the extract (Table 4-8). The extract was then subjected to evaluation of antioxidant activity again. The results showed that the extract still exhibited a satisfactory antioxidant activity with the ED_{50} value was 14.91 µg/mL.

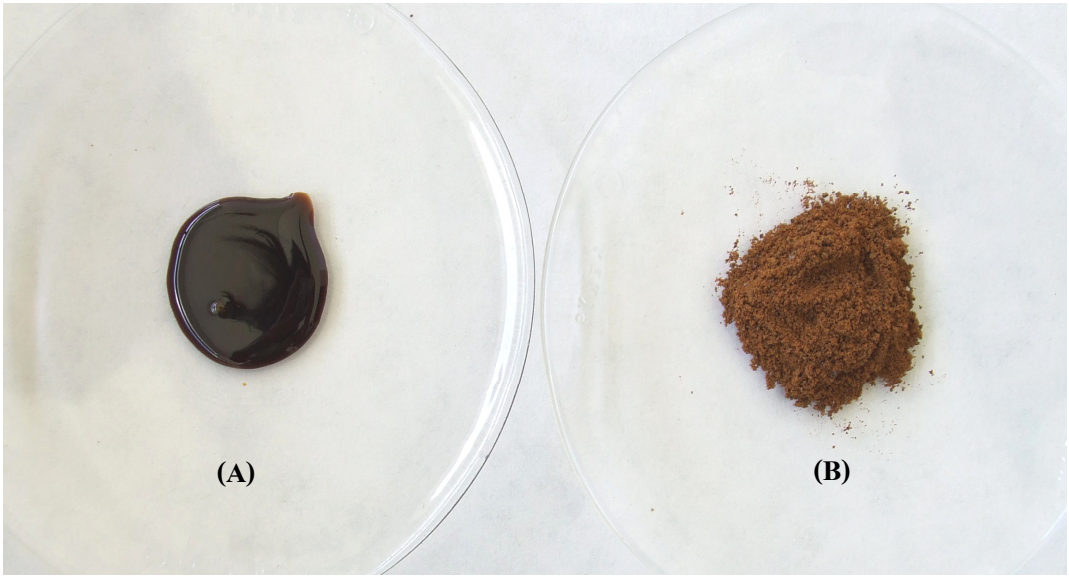

Figure 4-4 *P. granatum* fruit peel extracts: (A) crude extract (B) high yield ellagic acid extract

Table 4-8 Extraction yield ellagic acid content and antioxidant activity of of the high-yield ellagic acid extract

Lot No.	Extraction yield % w/w of dried powder	Ellagic acid content %w/w of extract (Mean ± S.D.)	Antioxidant activity[†] ED_{50} (µg/mL)
1	17.75	12.72 ± 0.213	15.27 ± 0.913
2	15.02	14.12 ± 0.182	14.88 ± 0.424
3	16.67	13.06 ± 0.328	15.44 ± 0.608
4	16.81	14.61 ± 0.457	14.65 ± 0.314
Mean ± S.D.	16.56 ± 1.132	13.63 ± 0.891	14.91 ± 0.239

[†] Evaluated by DPPH radical scavenging assay

4.3.2 Determination of the moisture content

Moisture content of the *P. granatum* fruit peel extract was performed by gravimetric method (loss on drying). The gravimetric method is easier to use and applicable for sample containing non-volatile substances. The presence of excess water in herbal raw materials or extracts can promote the growth of microbes and the hydrolysis of the constituents leading to deterioration of herbal raw materials or extracts (Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998). Generally, the upper limit of the moisture content of an herbal drug is 8 -14 %w/w, with a few exceptions (Dechatiwongse Na Ayudhya *et al*., 1993). In this study, it was found that the moisture contents of high-yield ellagic acid *P. granatum* fruit peel extract were in range of 2.82-3.25% w/w (Table 4-9). The variation of the moisture content of ellagic acid high-yielding *P. granatum* fruit peel extract may be due to the process of partitioning with water and drying. The results suggested that the upper limitation of the moisture content of the ellagic acid high-yielding extract should be less than 3.2% w/w.

Table 4-9 Moisture content of the high-yield ellagic acid *P. granatum* fruit peel extract

Lot No.	Moisture content (% w/w; Mean ± S.D.)
1	2.82 ± 0.313
2	3.25 ± 0.141
3	2.97 ± 0.283
Mean ± S.D.	3.01 ± 0.246

4.3.3 Determination of the total ash content

The total ash value is of importance and indicates to some extent the amount of care taken in the preparation of the extract (Trease and Evans, 1983). Determination of the total ash content of the high-yield ellagic acid extract demonstrated that the extract contained no ash after ignition (Table 4-10). This implies that inorganic salts in the fruit peels was eliminated by the extraction and fractionation processes. The result suggests that the high-yield ellagic acid extract should contain no ash.

Table 4-10 Total ash content of the high-yield ellagic acid *P. granatum* fruit peel extract

Samples	Weight before incinerated (g)	Weight after incinerated (g)
1	0.5597	0.0000
2	0.5189	0.0000
3	0.5329	0.0000

4.3.4 Determination of the microbial contamination

The limits for microbial contamination are suggested for non-sterile herbal preparations by the Thai herbal pharmacopoeia 1998 (Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998). The herbal preparations should be tested regularly to provide an indicator whether the microbiological requirements for good manufacturing practices are reached or not. In this study the limit for microbial contamination of topical preparations for intact skin are used for the quality control of high-yield ellagic acid g *P. granatum* fruit peel extract. The limit for microbial contamination of topical preparations for intact skin such as creams, lotions, ointments, solutions, and powders are as follow; total aerobic microbial count does not exceed 500 colonies/g (mL), and a one-g (mL) sample is free from Enterobacteria, *Pseudomonas aeruginosa*, *Staphylococus aureus*, yeast, and moulds (Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998). The results showed that neither bacteria nor fungi contamination was found in the extracts (Table 4-11). Thus, the extracts are reached the microbiological requirement for good manufacturing practices.

Table 4-11 Microbial contamination in the high-yield ellagic acid *P. granatum* fruit peel extract

Microbes	Microbial contamination	Result interpretation
Aerobic bacteria	not found	not contaminated with aerobic bacteria
Escherichia coli	not found	not contaminated with *Escherichia coli*
Yeasts and moulds	not found	not contaminated with fungi

4.4 Determination of solubility

Solubility is commonly expressed as a maximum equilibrium amount of solute that can normally dissolve per amount of solvent or a maximum concentration of a saturated solution. These maximum concentrations are often expressed as grams of solute per 100 mL of solvent. The solubility test of the high-yield ellagic acid *P. granatum* fruit peel extract is used to estimate the dissolution of the extract in various solvents. The results showed that high-yield ellagic acid *P. granatum* fruit peel extract is freely soluble in dimethylsulfoxide (DMSO), sparingly soluble in ethanol, methanol and acetone, slightly soluble in ethyl acetate. It is practically insoluble in dichloromethane, chloroform, hexane and water (Table 4-12). The high-yield ellagic acid *P. granatum* fruit peel extract contains most likely moderate polar compounds therefore the suitable solvents for the high-yield ellagic acid *P. granatum* fruit peel extract should be a moderate polar solvents.

Table 4-12 Solubility property of the high-yield ellagic acid *P. granatum* fruit peel extract

Solvent	Volume of solvent in mL/g of solute	Level of solubility
DMSO	2	Freely soluble
Methanol	35	Sparingly soluble
Ethanol	40	Sparingly soluble
Acetone	75	Sparingly soluble
Ethyl acetate	250	Slightly soluble
Dichloromethane	>10,000	Practically insoluble
Chloroform	>10,000	Practically insoluble
Hexane	>10,000	Practically insoluble
Water	>10,000	Practically insoluble

4.5 Partition coefficient

The partition coefficient (K) is defined as the ratio of the equilibrium concentrations of a dissolved substance in a two-phase system consisting of two largely immiscible solvents, such as *n*-octanol and water. The result is usually given in the form of its logarithm to base ten.

The *n*-octanol–water partition coefficient of ellagic acid in high-yield ellagic acid *P. granatum* fruit peel extract were determined from the ratio of the molar concentration of the ellagic acid in the *n*-octanol phase to that in the water phase after equilibration at room temperature (30 ± 2 ºC). The log P_{OW} value of ellagic acid in high-yield ellagic acid *P. granatum* fruit peel extract was determined by traditional shake-flask method. Due to a very small concentration of the ellagic acid in the water phase, their concentration in the *n*-octanol phase was taken as the initial concentration, prior to equilibration with the water phase. The result showed that the hydrophobic parameter (log P_{OW} value) of the ellagic acid in the extract was 2.44 ± 0.26 (Table 4-13). Some general guidelines of the optimum log P_{OW} values for certain classes of drugs have been reported (Earll, 1999). The optimum log P_{OW} values of the compounds suggested for CNS penetration, oral absorption, intestinal absorption, colonic absorption, sublingual absorption and percutaneous absorption are 2.0, 1.8, 1.35, 1.32, 5.5, and 2.6, respectively. The result in this study implies that the ellagic acid in the high-yield ellagic acid *P. granatum* fruit peel extract have good percutanous absorption.

Table 4-13 Partition coefficient values of ellagic acid in high-yield ellagic acid *P. granatum* fruit peel extract determined by shake-flask method

No.	Concentration in water phase (Mean, µg/mL ± S.D.)	Concentration in octanol phase (Mean, µg/mL ± S.D.)	Log P_{ow} (Mean ± S.D.)
1	0.41 ± 0.012	0.95 ± 0.008	2.31 ± 0.632
2	1.53 ± 0.136	3.47 ± 0.045	2.27 ± 0.331
3	1.87 ± 0.142	5.12 ± 0.023	2.74 ± 0.158
	Mean ± S.D.		2.44 ± 0.374

4.6 Stability test

4.6.1 Effect of light on the stability of the extract

The effect of light on the stability of the high-yield ellagic acid *P. granatum* fruit peel extract was examined. The extracts were kept in the well-closed containers and stored either under fluorescent light or protected from light for a period of 4 months. Physical appearance of the extract was observed as well as the content of ellagic acid was analyzed at the initial times and every week (or month) in four months period. Physical appearance of the extracts that kept in both conditions was not changed through the period of 4 months. In addition, the ellagic acid contents of the extracts kept in both conditions were not significantly decreased through period of 4 months (Table 4-14). Normally, antioxidant compounds can degrade in light condition, because antioxidant compounds were oxidized to other form in light condition. From the result, light have no effect to high-yield ellagic acid *P. granatum* fruit peel extract. d Other constituents can degrade after ellagic acid, because they have high antioxidant activity potency such as flavonoid and other polyphenol (punicalagin, punicalin, quercetin, luteolin, kaempferol and naringennin) were present in previous study of constituents were found in *Punica granatum* fruit peel (Ricci *et al*., 2006)

Table 4-14 Ellagic acid content of the high-yield ellagic acid *P. granatum* fruit peel extracts stored under light and protected from light conditions

Week	Ellagic acid content (%w/w; Mean ± S.D.)			
	Protected from light	% Remaining	Light	% Remaining
0	13.24 ± 0.482	100	13.24 ± 0.508	100
1	13.31 ± 0.148	100.53	13.15 ± 0.273	99.32
2	13.37 ± 0.472	100.98	13.39 ± 0.193	101.13
3	13.12 ± 0.361	99.09	13.21 ± 0.345	99.77
4	13.32 ± 0.144	100.60	13.19 ± 0.233	99.62
6	12.36 ± 0.214	93.35	13.27 ± 0.381	100.23
8	13.22 ± 0.217	99.85	13.33 ± 0.176	100.68
10	13.17 ± 0.456	99.47	13.18 ± 0.739	99.55
12	13.18 ± 0.369	99.55	13.31 ± 0.382	100.53
14	13.11 ± 0.171	99.02	13.25 ± 0.313	100.08
17	13.21 ± 0.614	99.77	13.18 ± 0.436	99.55

4.6.2 Effect of temperature on the stability of the extract

The effect of temperature on the stability of the high-yield ellagic acid *P. granatum* fruit peel extract was examined under two temperatures, 4 ºC and 30 ºC for 4 months. The extracts were kept in well-closed container protect from light. Physical appearance of the extracts was observed as well as the content of ellagic acid was analyzed at the initial times and every week (or month) in four-month period. The result showed that both tested temperatures did not affect either the physical appearance of the extracts or ellagic acid content through the four-month period (Table 4-15). It implies that the high-yield ellagic acid *P. granatum* fruit peel extract is stable under temperatures of 4 ºC and 30 ºC at least in the period of 4 months.

Table 4-15 Ellagic acid content of high-yield ellagic acid *P. granatum* fruit peel extracts stored under 4 ºC and 30 ºC

Weeks	Ellagic acid content (%w/w; Mean ± S.D.)			
	4 ºC	% Remaining	30 ºC	% Remaining
0	13.24 ± 0.482	100	13.24 ± 0.209	100
1	13.35 ± 0.212	100.83	13.51 ± 0.191	102.04
2	13.28 ± 0.183	100.30	13.23 ± 0.371	99.92
3	13.62 ± 0.449	102.87	13.42 ± 0.224	101.34
4	13.34 ± 0.343	100.76	13.31 ± 0.358	100.53
6	13.51 ± 0.184	102.04	13.29 ± 0.411	100.38
8	13.14 ± 0.201	99.24	13.37 ± 0.231	100.98
10	13.27 ± 0.311	100.22	13.18 ± 0.414	99.55
12	13.31 ± 0.338	100.53	13.25 ± 0.506	100.08
14	13.21 ± 0.147	99.77	13.39 ± 0.117	101.13
17	13.41 ± 0.418	101.28	13.50 ± 0.092	101.96

4.6.3 Effect of accelerated conditions on the stability of the extract

The accelerated stability test of the high-yield ellagic acid *P. granatum* fruit peel extract was carried out using a stability chamber. The extracts were kept in well-closed containers protected from light and stored in the chamber at 45 ºC with 75% relative humidity. Physical appearance of the extract was observed as well as the content of ellagic acid was analyzed at the initial times and every week (or month) in four-month period. The result demonstrated that the physical appearances as well as the ellagic acid content of the extract were not changed even stored under accelerated conditions in the period of four months (Table 4-16). This result implies that the high-yield ellagic acid *P. granatum* fruit peel extract should be stable when kept in the closed container and stored at the room temperature.

Table 4-16 Ellagic acid content of high-yield ellagic acid *P. granatum* fruit peel extract stored under accelerated conditions

Weeks	Ellagic acid content (%w/w; Mean ± S.D.)	% Remaining
0	13.24 ± 0.482	100
1	13.11 ± 0.311	99.018
2	13.38 ± 0.181	101.06
3	13.19 ± 0.714	99.62
4	13.44 ± 0.515	101.51
6	13.18 ± 0.338	99.55
8	13.38 ± 0.178	101.06
10	13.50 ± 0.181	101.96
12	13.28 ± 0.252	100.30
14	13.29 ± 0.381	100.38
17	13.25 ± 0.414	100.08

4.6.4 Effect of pH on the stability of the extract

The acid-base stability study of the high-yield ellagic acid *P. granatum* fruit peel extract in the solution was determined at three different pH including 5.5, 7.0, and 8.0. The solution of high-yield ellagic acid *P. granatum* fruit peel extract at pH 5.5 was yellow, at pH 7.0 was brownish-yellow and at pH 8.0 was brown (Figure 4-5). The extracts were kept in well-closed containers, protected from light and stored at the room temperature (30 ± 2 ºC) for 4 months. An adequate sample was taken at the initial time and every week (or month) for the analysis of the ellagic acid content. It was found that, in all tested pH, the ellagic acid content of the extract was significantly decreased after kept for four weeks (Table 4-17). The percent remaining of ellagic acid in the extract after 4 weeks of storage was significantly lower than the initial time. In these conditions, HPLC chromatograms showed peak of degraded product with the retention time about 1.6 minute (Figure 4-6). The degraded product has higher polarity than ellagic acid, resulting in lower retention time. The instability of ellagic acid in the solution should be considered as their hydrolysis of the ellagic acid (Figure 4-7). The ester group of ellgic acid was hydrolyzed to hexahydroxyphenic acid in aqueous, acid and base solution (Gayon, 1972). Thus, preparation of the *P. granatum* fruit peel extract in an aqueous solution should be performed carefully.

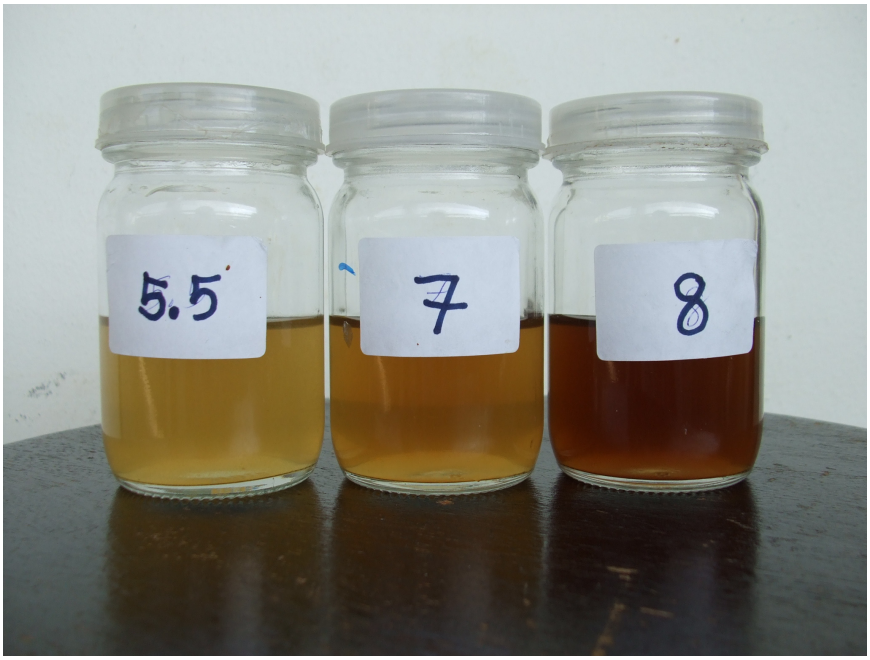

Figure 4-5 High-yield ellagic acid *P. granatum* fruit peel extracts in the solution at pH 5.5, 7.0, and 8.0 (freshly prepared)

Table 4-17 Ellagic acid content of high-yield ellagic acid *P. granatum* fruit peel extracts in the solution at pH 5.5, 7.0 and 8.0 at room temperature for 4 months

Weeks	Ellagic aid content (%w/w; Mean ± S.D.)					
	pH 5.5	% Remaining	pH 7.0	% Remaining	pH 8.0	% Remaining
0	12.81 ± 0.669	100	12.81 ± 0.673	100	12.81 ± 0.671	100
1	12.82 ± 0.408	100.08	12.68 ± 0.533	98.99	12.59 ± 0.809	98.28
2	12.71 ± 0.189	99.22	12.76 ± 0.614	99.61	12.68 ± 0.513	98.98
3	12.59 ± 0.808	98.28	12.62 ± 0.523	98.52	12.76 ± 0.582	99.61
4	11.02 ± 0.321*	86.03	12.02 ± 0.838*	93.83	11.92 ± 0.367*	93.05
6	11.89 ± 0.512*	92.82	11.15 ± 0.789*	87.04	10.03 ± 0.177*	78.30
8	10.41 ± 0.484*	81.26	9.77 ± 0.4111*	76.27	9.31 ± 0.432*	72.68
10	7.01 ± 0.314*	54.72	8.21 ± 0.573*	64.09	7.29 ± 0.514*	56.91
12	4.21 ± 0.332*	32.86	7.66 ± 0.728*	59.80	3.93 ± 0.831*	30.68
14	4.09 ± 0.708*	31.93	7.65 ± 0.424*	59.72	3.17 ± 0.518*	24.75
17	2.69 ± 0.361*	21.00	3.29 ± 0.253*	25.68	2.57 ± 0.447*	20.06

* Significance at $P < 0.05$ compared with the content at initial time

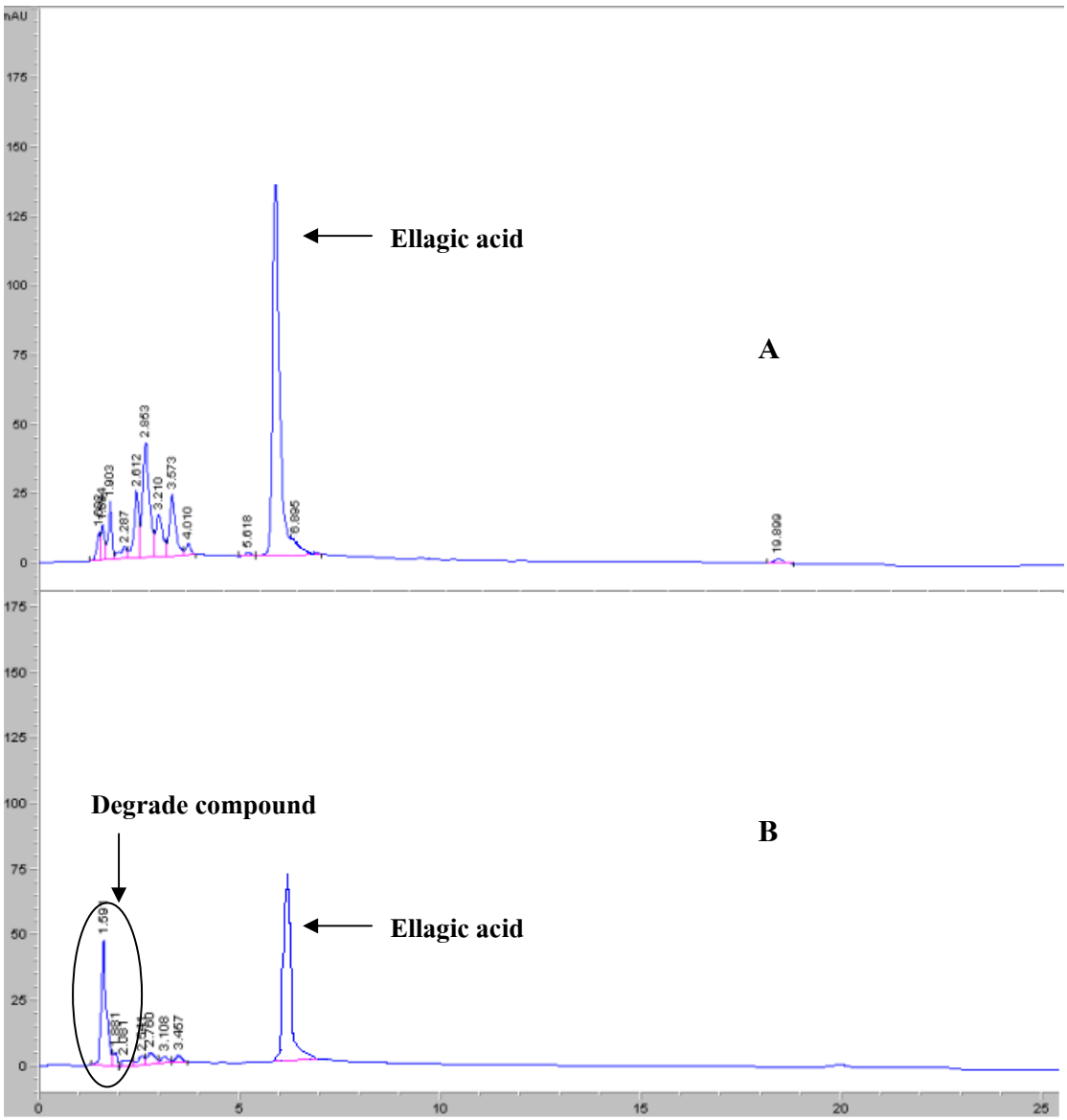

Figure 4-6 HPLC-chromatogram of (A) high-yield ellagic acid *P. granatum* fruit peel extract at initial time and (B) high-yield ellagic acid *P. granatum* fruit peel extract after 6 weeks storage in pH 8.0

Figure 4-7 Hydrolysis of ellagic acid to hexahydroxydiphenic acid

The results from the stability tests indicated that the high-yield ellagic acid *P. granatum* fruit peel extract possesses satisfactory stability for further development of the herbalmedicines. However, preparation of the *P. granatum* fruit peel extract in an aqueous solution should be performed carefully due to hydrolysis of ellagic acid during storage.

CHAPTER 5

CONCLUSIONS

The quantitative HPLC analysis of ellagic acid, preparation of the high-yield ellagic acid *P. granatum* fruit peel extract, establishment of standard information of the extract, and stability study of the extract were performed and the following conclusions can be drawn:

Mobile phase	: methanol and 2% aqueous acetic acid
Flow rate	: 1 mL/min
Gradient	: elution from 40% to 60% v/v methanol within 15 minutes and maintained at 60% v/v for 5 minutes and reduced to 40% after 5 minutes for each run
Column	: TSK-gel ODS-80Tm (4.6 mm X 15 cm)
Injection volumn	: 20 μL
Detector	: Phodiode-array detector at 254 nm

1. A simple, specific, precise, accurate, rapid and reproducible HPLC method has been developed and validated to quantify the ellagic acid in *P. granatum* fruit peel extract. The HPLC quantitative determination of ellagic acid provides useful marker information for the quality control of *P. granatum* fruit peel extract.

2. The fruit peels of *P. granatum* were successively extracted under reflux conditions using the solution of 10% water in methanol as the solvent for 1 hour. This method was capable of increasing the ellagic acid content in the fruit peel extract.

3. Fractionation of the crude extract of *P. granatum* fruit peels using liquid-liquid extraction between ethyl acetate and 2% aqueous acetic acid was capable of improving ellagic acid content in the extract up to 13.63 ± 0.89% w/w. This method was also capable of producing the strong antioxidant fraction with ED_{50} value of 24.91 ± 1.24 μg/ml compare to standard quercetin and standard ellagic acid of 3.57 ± 0.64 and 3.12 ± 0.35 μg/ml respectively.

4. The standard specification of the high-yield ellagic acid *P. granatum* fruit peel extract was established as follow;

- ellagic acid is not less than 13% w/w.
- The moisture content (loss on drying) is not more than 3.2% w/w.
- No ash
- Microbial contamination: No contamination with aerobic bacteria, *E. coli* and fungi.

5. The high-yield ellagic acid *P. granatum* fruit peel extract contains most likely moderate polar compounds therefore the suitable solvents to dissolve the fruit peel extract should be a moderate polar solvent such as DMSO, methanol, ethanol, acetone and ethyl acetate.

6. The hydrophobic parameter (log P_{OW} value) of the ellagic acid in the high-yield ellagic acid *P. granatum* fruit peel extract was 2.44 ± 0.26. It implies that ellagic acid in the high-yield ellagic acid *P. granatum* fruit peel extract have good percutanous absorption.

7. Stability evaluations of the high-yield ellagic acid *P. granatum* fruit peel extract in several conditions in the period of 4 months found that the extract possessed a satisfactory stability. However, the extract should be stored in well-closed container, protected from light. The aqueous solutions of the extract are not stable either in acid or base conditions.

MONOGRAPH

สารสกัดเปลือกผลทับทิม (*Punica granatum* fruit peel extract)

Synonym Pomegranate fruit peel extract, Pomegranate fruit rind extract

Category Astringent in diarrhea and dysentery and Antioxidant

Constituents : contain ellagic acid not less than 13%w/w

Description of the extract : Brown powder, odorless

Preparation of the extract

The extraction and fractionation methods for preparation of the high yield ellagic acid extract of *P. granatum* fruit peels: Dried powder of pomegranate fruit peels (0.5 kg) were refluxed twice with 10% v/v water in methanol (2 L) for 1 hour then the combined extract was evaporated to dryness *in vaccuo*. The residue (2 g) was redissolved in ethyl acetate 200 mL and transfer to separatory funnel, 2% aqueous acetic acid 200 mL was then added. The mixture was then shaken at room temperature for 1 minute (x 4). The pooled ethyl acetate fraction was was evaporated to dryness *in vaccuo*. Brown powder was obtained after evaporated to dryness.

Quantitative analysis of ellagic acid

A. *P. granatum* fruit peel powder (100 mg) was extracted with methanol (20 mL) under reflux conditions for an hour. The extract was then filtered and concentrated under reduced pressure$. The residue was reconstituted and adjusted to 10 mL with methanol.

B. HPLC analysis : Separation was achieved at 25 ºC on the mobile phase consisted of methanol and 2% aqueous acetic acid, gradient elution from 40% to 60% v/v methanol within 15 min and maintained at 60% v/v for 5 minutes and reduced to 40% after 5 minutes for each run at a flow rate of 1 mL/min. The injection volume was 20 µL. The quantitation wavelength was set at 254 nm.

Loss on drying Not more than 3.2% w/w.

Total ash No ash

Microbial contamination No contamination with aerobic bacteria, *E. coli* and fungi.

Packaging and storage *P. granatum* fruit peel powder kept in well-closed containers and dry place. Stored at room temperature.

BIBLIOGRAPHY

นันทวัน บุนยะประภัศร. 2541. *สมุนไพรไม้พื้นบ้าน เล่ม 2*. กรุงเทพฯ: โรงพิมพ์ประชาชน.

Ajaikumar, K.B., Asheef, M., Babu, B.H. and Padikkala, J. 2005. The inhibition of gastric mucosal injury by *Punica granatum* L. (pomegranate) methanolic extract. *Journal of Ethnopharmacology.* 96:171-176.

Alanis, A.D., Calzada, F., Cervantes, J.A., Torres, J. and Ceballos, G.M. 2005. Antibacterial properties of some plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. *Journal of Ethnopharmacology.* 100: 153-157.

Amakura, Y., Okada, M., Tsuji, S. and Tonogai, Y. 2000a. High-performance liquid chromatography determination with photodiode array detection of ellagic acid in fresh and processed fruits. *Journal of Chromatography A*. 896: 87-93.

Amakura, Y., Okada, M., Tsuji, S. and Tonogai, Y. 2000b. Determination of phenolic acids in fruit juices by isocratic column liquid chromatography. *Journal of Chromatography A*. 891: 183-188.

Artik, N. 1998. Determination of phenolic compounds in pomegranate juice by using HPLC. *Fruit Processing*. 8: 492-499.

Aslam, M.N., Lansky, E.P. and Varani, J. 2005. Pomegranate as a cosmeceutical source: Pomegranate fractions promote proliferation and procollagen synthesis and inhibit matrix metalloproteinase-1 production in human skin cells. *Journal of Ethnopharmacology* 103: 311-318.

Association of Official Analytical Chemists. 2002. Validation of chemical methods. *AOAC requirements for single laboratory.* Gaithersburg: United State of America.

Blois, M.S. 1958. Antioxidant determinations by the use of a stable free radical. *Nature*. 181: 1199-1200.

British Pharmacopoeia Commission. 2001. *British Pharmacopoeia*. Vol.1. London: Her Majesty's Stationery Office.

Bown, D. 1995. *Encyclopaedia of Herbs and their Uses*. London: Dorling Kindersley.

Dalimov, D.N., Dalimova, G.N. and Bhatt, M., 2003. Chemical composition and lignins of tomato and pomegranate seeds. *Chemistry of Natural Compounds (Translation of Khimiya Prirodnykh Soedinenii)*. 39: 37-40.

Dechatiwongse na Ayudhya, T., Techadamrongsin, Y. and Jirawattanapong, W. 1993. *Chemical Specification of Thai Herbal Drugs*. Vol 1. Bangkok: Ministry of Public Health.

de Pascual, T.S., Santos, B.C. and Rivas, G.J.C. 2000. Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *Journal of Agricultural and Food Chemistry.* 48: 5331-5337.

Earll, M. 1999. A guide to Log *P* and pKa measurements and their use [online]. Available, http://www.raell.demon.co.uk/chem/logppka.htm [cited 2007 April 19].

Farnsworth, N.R. and Bunyapraphatsara, N. 1992. *Thai medicinal plant, recommended for primary health care system*. Bangkok: Prachacon.

Gil, M.J., Tomas B.F.A., Hess, P.B., Holcroft, D.M. and Kader, A.A. 2000. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry.* 48: 4581-4589.

Gayon, P.R. 1972. *Plant Phenolics*. T. and A. Britain: Constable.

Hakkinen, S. 2000. *Flavonols and Phenolic Acids in Berries and Berry Products*. Doctoral dissertation. Kuopio: The Faculty of Medicine of the University of Kuopio.

Hernandez, F., Melgarejo, P., Tomas-Barberan, F.A. and Artes, F. 1999. Evolution of juice anthocyanins during ripening of new selected pomegranate (*Punica granatum*) clones. *European Food Research and Technology*. 210: 39-42.

Hopkins, C.Y. and Chisholm, M.J. 1968. A survey of the conjugated fatty acids of seed oils. *Journal of the American Oil Chemists Society*. 45: 176-182.

Huang, T.H., Hyang, Q., Harada, M., Li, G.Q., Yamahara, J., Roufogalis, B.D. and Li, Y. 2005a. Pomegranate flower extract diminishes cardiac fibrosis in Zucker diabetic fatty rats: modulation of cardiac endothelin-1 and nuclear factor-kappaB pathways. *Journal of Cardiovascular Pharmacology*. 46: 856-862.

Huang, T.H.W., Penga, G., Kota, B.P., Li, G.Q., Yamahara, J., Roufogalis, B.D. and Li, Y. 2005b. Anti-diabetic action of *Punica granatum* flower extract: Activation of PPAR-γ and identification of an active component. *Toxicology and Applied Pharmacology*. 207: 160-169.

Jahfar,M., Vijayan, K.K. and Azadi, P. 2003. Studies on a polysaccharide from the fruit rind of *Punica granatum*. *Research Journal of Chemistry and Environment*. 7: 43-50.

Lansky, E.P. and Newman, R.A. 2007. *Punica granatum* (pomegranate) and its potential for prevention and treatment of inflammation and cancer. *Journal of Ethnopharmacology*. 109: 177-206.

Lee, J. 2004. *Hydrolytic and antioxidant properties of ellagic acid and its precursors present in muscadine grape*. A dissertation presented to the graduate school of The University of Florida in partial fulfillment of the requirements for the degree of doctor of philosophy. Florida: University of Florida.

Lei, F., Xing, D.M., Xiang, L., Zhao, Y.N., Wang, W., Zhang, L.J. and Du, L.J. 2003. Pharmacokinetic study of ellagic acid in rat after oral administration of pomegranate leaf extract. *Journal of Chromatography*. 796: 189–194.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J. and Cheng, S. 2006. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Journal of Agriculture and Food Chemistry*. 96: 254-260.

Masamune, A., Satoh, M. and Kikuta, K. 2005. Ellagic acid blocks activation of pancreatic stellate cells. *Biochemical Pharmacology*. 70: 869–878

Melendez, P.A. and Capriles, V.A. 2005. Antibacterial properties of tropical plants from Puerto Rico. *Journal of Ethnopharmacology*. 100: 153-157.

Miguel, G., Fontes, C., Antunes, D., Neves, A. and Martins, D. 2004. Anthocyanin concentration of "Assaria" pomegranate fruits during different cold storage conditions. *Journal of Biomedicine and Biotechnology*. 2004: 338-342.

Morton, J. F. 1987. *Fruits of Warm Climates*. London:Creative Resources Systems.

Munde, S.S., Patil, V.K. and Chavan, S.D. 1981. Chemical composition of pomegranate (*Punica granatum* Linn.) leaves sampled during different stages of crop. *Food Farming Agriculture*. 13: 177-181.

Nawwar, M.A.M., Hussein, S.A.H. and Merfort, I. 1994. NMR spectral analysis of polyphenols from *Punica granatum*. *Phytochemistry.* 36: 793-798.

Neuhofer, H., Witte, L., Gorunovic, M. and Czygan, F.C. 1993. Alkaloids in the bark of *Punica granatum* L. (pomegranate) from Yugoslavia. *Pharmazie*. 48: 389-391.

Noda, Y., Kaneyuka, T., Mori, A. and Packer, L. 2002. Antioxidant activity of pomegranate fruit extract and it anthocyanidins: delphinidin, cyaniding and pelargonidin. *Journal of Agricutural and Food Chemistry*. 50: 166-171.

Organization for Economic Cooperation and Delevopment. 1995. *Guidelines for the testing of chemicals. OECD 107, Partition coefficient (n-octanol/water) (Shake flask method)*, Paris. France.

Ozcal, N. and Dinc, S. 1993. Evaluation of the pomegranate (*Punica granatum* L.) peels from the standpoint of pharmacy. *Eczacilik Fakultesi Dergisi*. 22: 21-29.

Paladini, A.C., Marder, M., Viola, H., Wolfman, C., Wasowski, C. and Medina, J.H. 1999. Flavonoids and the central nervous system: from forgotten factors to potent anxiolytic compounds. *The Journal of Pharmacy and Pharmacology*. 51: 519-526.

Panichayupakaranant, P., Panyokaew, T., Tewtrakul, S. and Yuenyoungsawad, S. 2005. Antioxidant and HIV-1 integrase inhibitory activities of ellagic acid isolated from *Punica granatum* fruit peel. *Pharma Indochina IV Pharmacy in cooperation for development and integration*. Ho Chi Min City, Vietnam, November 10-13: 261-263.

Perez-Vicente, A., Gil-Izquierdo, A. and Garcia-Viguera, C. 2002. In vitro gastrointestinal study of pomegranate juice phenolic compounds, anthocyanins and Vitamim C. *Journal of Agricultural and Food Chemistry*. 50: 2308-2312.

Prashanth, D., Asha, M.K. and Amit, A. 2001. Antibacterial activity of *Punica granatum*. *Fitoterapia*. 72: 171-173.

Ricci, D., Giamperi, L., Bucchini, A. and Fraternale, D. 2006. Antioxidant activity of *Punica granatum* fruits. *Fitoterapia*. 77: 310-312.

Ross, R.G., Selvasubramanian, S. and Jayasundar, S. 2001. Immunomodulatory activity of *Punica granatum* in rabbits a preliminary study. *Journal of Ethnopharmacology*. 78: 85-87.

Satomi, H., Umemura, K., Ueno, A., Hatano, T., Okuda, T. and Noro, T. 1993. Carbonic anhydrase inhibitors from the pericarps of *Punica granatum* L. *Biological and Pharmaceutical Bulletin.* 16: 786-790.

Seeram, N., Lee, R., Hardy, M. and Heber, D. 2005. Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry. *Separation and Purification Technology*. 41: 49–55.

Sharma, J.N. and Seshadri, T.R. 1955. Survey of anthocyanins from Indian sources II. *Journal of Scientific and Industrial Research Section B*. 14: 211.

Subcommittee on the establishment of the Thai herbal pharmacopoeia. 1998. *Thai Herbal Pharmacopoeia*. Vol.1. Bangkok: Prachachon.

Surveswaran, S., Cai, Y.Z., Corke, H. and Sun, M. 2007. Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. *Journal of Food Chemistry*. 102: 938-953.

Tanaka, T., Nonaka, G.and Nishioka, I. 1986. Tannin and related compounds. XL. Revision of the structures of punicalin and punicalagin, and isolation and characterization of 2-*O*-galloylpunicalin from the bark of *Punica granatum* L. *Chemical and Pharmaceutical Bulletin.* 34:477-482.

Tanaka, T., Nonaka, G.and Nishioka, I. 1990. Tannin and related compounds. Reaction of the dehydrohexahydroxydiphenic acid ester with bases, and its application to structures determination of pomegranate tannins, granatins a and b. *Chemical and Pharmaceutical Bulletin.* 38:9424-9428.

Tierra, M. 1999. Why standardized herbal extracts? An herbalist's perspective [Online]. Available, http://www.planethebs.com/articles/standardized%20extracts.htm [cited 2007 April 21].

Trease, G.E. and Evans, I.C. 1983. *Pharmacognosy. 12th ed*. Bailliere Tindall. London.

Tsuyuki, H., Ito, S. and Nakatsukasa, Y. 1981. Lipids in pomegranate seeds. *Nihon Daigaku No-Juigakubu Gakujutsu Kenkyu Hokoku*. 38: 141-148.

van Elswijk, D.A., Schobel, U.P., Lansky, E.P., Irth, H. and van der Greef, J. 2004. Rapid dereplication of estrogenic compounds in pomegranate (*Punica granatum*) using on-line biochemical detection coupled to mass spectrometry. *Phytochemistry*. 65: 233-241.

Veres, M. 1976. Study of the mechanical and chemical composition of cultivated pomegranate. *Hrana Ishrana*. 17: 426-432.

Vidal, A., Fallarero, A., Pena, B.R., Medina, M.E., Gra, B., Rivera, F., Gutierrez, Y. and Vuorela, P.M. 2003. Studies on the toxicity of *Punica granatum* L. (Punicaceae) whole fruit extracts. *Journal of Ethnopharmacology*. 89: 295-300.

Voravuthikunchai, S., Lortheeranuwat, A., Jeeju, W., Sririrak, T., Phongpaichait, S. and Supawita, T. 2004. Effective medicinal plants against enterohaemorrhagic *Escherichia coli* O157:H7. *Journal of Ethnopharmacology*. 94: 49-54.

Waheed, S., Siddique, N., Rahman, A., Zaidi, J.H. and Ahmad, S. 2004. INAA for dietary assessment of essential and other trace elements in 14 fruits harvested and consumed in Pakistan. *Journal of Radioanalytical and Nuclear Chemistry*. 260: 523-531.

Wang, R.F., Xie, W.D., Zhang, Z., Xing, D.M., Ding, Y., Wang, W., Ma, C. and Du, L.J. 2004. Bioactive compounds from the seeds of *Punica granatum* (Pomegranate). *Journal of Natural Products*. 67: 2096-2098.

Zand, R.S., Jenkins, D.J. and Diamandis, E.P. 2000. Steroid hormone activity of flavonoids and related compounds. *Breast Cancer Research and Treatment*. 62: 35-49.

VITAE

Name Miss Acharaporn Issuriya

Student ID 4852037

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biology)	Prince of Songkla University	2004

List of Publication and Proceeding

Acharaporn Issuriya, Pharkphoom Pnichayupakaranant, Anusak Sirikatitham and Wei Wang . 2007. Quantitative HPLC Determination and Extraction of Ellagic Acid in *Punica granatum* Fruit Peel. Proceeding of 8th National Grad Reserch Conference. p. 208.