อิทธิพลของสารสกัดทับทิม *Punica granatum* ต่อการต้านออกซิเดชัน การฉายรังสีอัลตราไวโอเลตบีบนผิวหนังหนูแรท และการตายแบบอะพอพโทสิสของเซลล์มะเร็งเต้านมมนุษย์ เอมซีเอฟ-7

นายจิณณวัตร์ มานะเสถียร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2554

THE EFFECTS OF POMEGRANATE *PUNICA GRANATUM* EXTRACTS ON ANTIOXIDATION, ULTRAVIOLET B IRRADIATION ON RAT SKIN AND APOPTOSIS OF MCF-7, HUMAN BREAST CANCER CELLS

Mr. Jinnawat Manasathien

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

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THE EFFECTS OF POMEGRANATE *PUNICA GRANATUM* EXTRACTS ON ANTIOXIDATION, ULTRAVIOLET B IRRADIATION ON RAT SKIN AND APOPTOSIS OF MCF-7, HUMAN BREAST CANCER CELLS

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

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จิณณวัตร์ มานะเสถียร : อิทธิพลของสารสกัดทับทิม *Punica granatum* ต่อการต้านออกซิเดชัน การฉายรังสีอัลตราไวโอเลตบีบนผิวหนังหนูแรท และการตายแบบอะพอพโทสิสของเซลล์มะเร็งเต้านมมนุษย์ เอมซีเอฟ-7 (THE EFFECTS OF POMEGRANATE *PUNICA GRANATUM* EXTRACTS ON ANTIOXIDATION, ULTRAVIOLET B IRRADIATION ON RAT SKIN AND APOPTOSIS OF MCF-7, HUMAN BREAST CANCER CELLS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.กรกช อินทราพิเชฐ, 197 หน้า.

การศึกษาคุณสมบัติของเปลือกทับทิมสกัดด้วยเอทธานอล (PPEE) เปลือกทับทิมสกัดด้วยน้ำ (PPWE) เมล็ดทับทิมสกัดด้วยเอทธานอล (PSEE) เมล็ดทับทิมสกัดด้วยน้ำ (PSWE) และ น้ำทับทิม (PJ) ต่อกิจกรรมการต้านออกซิเดชัน การป้องกันการฉายรังสีอัลตราไวโอเลตบีบนผิวหนังหนูแรท และการตายแบบอะพอพโทสิสของเซลล์มะเร็งเต้านมมนุษย์ (เอมซีเอฟ-7) การทดลองพบว่า ปริมาณสารประกอบฟีโนลิกโดยรวมสามารถเรียงจากมากไปน้อยดังนี้ PPEE > PPWE > PSEE > PSWE > PJ (451.96 ± 4.29 380.54 ± 4.29 77.93 ± 1.62 51.58 ± 0.85 และ 2.55 ± 0.42 µg GAE/mg ตามลำดับ) เช่นเดียวกับปริมาณฟลาโวนอยด์คือ PPEE > PPWE > PSEE > PSWE > PJ (37.61 ± 1.44 26.05 ± 0.93 16.66 ± 0.47 10.55 ± 0.14 และ 0.24 ± 0.03 µg CAE/mg ตามลำดับ) ความเข้มข้นที่ใช้ในการยับยั้ง 50 เปอร์เซ็นต์ (IC_{50}) ซึ่งเกี่ยวข้องถึงความสามารถในการต้านออกซิเดชันสามารถคำนวณได้จากการยับยั้งอนุมูล DPPH การลดเฟอริก และการยับยั้งออกซิเดชันของไลปิด โดยสามารถจัดลำดับค่าความสามารถจากสูงไปถึงต่ำได้ดังต่อไปนี้ PPEE (121.65 ± 2.66 49.07 ± 1.53 และ 15.15 ± 2.46 µg/ml ตามลำดับ) > PPWE (151.78 ± 2.70 64.63 ± 1.23 และ 20.42 ± 2.87 µg/ml ตามลำดับ) > PSEE (1,324.35 ± 16.89 512.54 ± 15.05 และ 166.49 ± 20.38 µg/ml ตามลำดับ) > PSWE (2,577.53 ± 44.06 753.17 ± 17.66 และ 201.82 ± 11.37 µg/ml ตามลำดับ) > PJ (9,925.20 ± 1,116.80 4,615.94 ± 28.90 และ 688.87 ± 44.03 µg/ml ตามลำดับ) และพบว่า ทั้งปริมาณสารประกอบฟีโนลิกโดยรวมหรือปริมาณฟลาโวนอยด์ของสารสกัด มีความสัมพันธ์ต่อกิจกรรมการต้านการออกซิเดชัน ความเข้มข้นของสารที่มีผลต่อการตายร้อยละ 50 ของไรน้ำเค็ม (LC_{50}) จากการวิเคราะห์การตายของไรน้ำเค็ม (BSLA) ความเป็นพิษต่อเซลล์สามารถแสดงผลจากมากไปน้อยคือ PPEE (1,206.98 ± 12.73 µg/ml) > PSWE (1,294.88 ± 61.28 µg/ml) > PPWE (1,743.31 ± 20.17 µg/ml) > PSEE (2,375.28 ± 69.54 µg/ml) > PJ (6,451.46 ± 52.32 µg/ml) และพบว่า ทั้งปริมาณสารประกอบฟีโนลิกโดยรวมและปริมาณฟลาโวนอยด์ ไม่มีความสัมพันธ์กับความเป็นพิษต่อเซลล์ ความสัมพันธ์ในการตอบสนองต่อปริมาณที่ใช้ของสารจากการต้านออกซิเดชันและความเป็นพิษต่อเซลล์ ซึ่งแสดงจากขอบเขตความปลอดภัยของสาร (MOS) และดัชนีการรักษา (TI) สามารถเรียงลำดับจากมากไปน้อยคือ PPWE (125

และ 11.49 ตามลำดับ) > PPEE (75 และ 9.92 ตามลำดับ) > PSEE (5 และ 1.79 ตามลำดับ) > PJ (3 และ 0.65 ตามลำดับ) > PSWE (0.5 และ 0.5 ตามลำดับ) การใช้สารสกัดเปลือกทับทิมต่อการป้องกันแสง 3xMED UVB บนผิวหนังหนูแรท การศึกษาครั้งแรกนี้พบว่า PPEE และ PPWE ลดผื่นแดงของผิวหนัง (2.5 และ 1.8 เท่า) ลดเซลล์เม็ดเลือดในการอักเสบลง (4.7 และ 1.9 เท่า) ลดความหนาของเนื้อเยื่อผิว (1.7 และ 1.6 เท่า) ลดจำนวน sunburn cells (7.4 และ 7.2 เท่า) และยับยั้งการแตกหักของดีเอ็นเอ จากสาเหตุโดยการให้รังสี UVB ในการประเมินการยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งเต้านม (MCF-7) โดยวิธี Trypan blue MTT และ Resazurin เมื่อพิจารณาจากค่า LC_{50} ที่ 24 ชั่วโมง PPEE (347.83 ± 11.85 377.88 ± 13.14 และ 471.50 ± 13.62 µg/ml ตามลำดับ) มีประสิทธิภาพมากกว่า PPWE (365.93 ± 20.00 459.90 ± 15.90 และ 589.45 ± 16.21 µg/ml ตามลำดับ) และพบการแตกหักของดีเอ็นเอและลักษณะการตายแบบอะพอพโทสิสของเซลล์เอมซีเอฟ-7 สารสกัดจากเปลือกทับทิมสามารถชักนำให้เกิด การยับยั้งการเจริญเติบโต ลักษณะกายวิภาคแบบอะพอพโทสิส และการแตกหักของ DNA ซึ่งขึ้นกับปริมาณสารและระยะเวลาที่ใช้ในการทดสอบ สารสกัดเปลือกทับทิมชักนำการตายของเซลล์แบบอะพอพโทสิสสามารถยืนยันผ่านการแสดงออกของระดับโปรตีน Bcl-2 procaspase-9 procaspase-7 และ PARP สรุปแล้วสารสกัดจากเปลือกทับทิมมีศักยภาพสูงในการต้านออกซิเดชัน ช่วยป้องกันผิวหนังจาก UVB ต้านการเกิดมะเร็ง และมีความปลอดภัยในการใช้สูง

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2554 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

JINNAWAT MANASATHIEN : THE EFFECTS OF POMEGRANATE *PUNICA GRANATUM* EXTRACTS ON ANTIOXIDATION, ULTRAVIOLET B IRRADIATION ON RAT SKIN AND APOPTOSIS OF MCF-7, HUMAN BREAST CANCER CELLS THESIS ADVISOR : ASSOC. PROF. KORAKOD INDRAPICHATE, Ph.D. 197 pp.

Pomegranate peel ethanolic extract (PPEE), pomegranate peel water extract (PPWE), pomegranate seed ethanolic extract (PSEE), pomegranate seed water extract (PSWE), and pomegranate juice (PJ) were studied with regards to the properties of antioxidative activity, UVB exposure prevention on rat skin and apoptosis of a human breast cell line (MCF-7). The results found that total phenolic compounds could be ranged by PPEE > PPWE > PSEE > PSWE > PJ (451.96 ± 4.29, 380.54 ± 4.29, 77.93 ± 1.62, 51.58 ± 0.85 and 2.55 ± 0.42 µg GAE/mg, respectively), consistently with the flavonoids content (37.61 ± 1.44, 26.05 ± 0.93, 16.66 ± 0.47, 10.55 ± 0.14 and 0.24 ± 0.03 µg CAE/mg, respectively). The median inhibitory concentration (IC_{50}) which refers to the antioxidative capacity could be calculated by free radical inhibition, ferric reduction, and lipid oxidative inhibition. By which, the range from high to low was PPEE (121.65 ± 2.66, 49.07 ± 1.53 and 15.15 ± 2.46 µg/ml, respectively) > PPWE (151.78 ± 2.70, 64.63 ± 1.23 and 20.42 ± 2.87 µg/ml, respectively) > PSEE (1,324.35 ± 16.89, 512.54 ± 15.05 and 166.49 ± 20.38 µg/ml, respectively) > PSWE (2,577.53 ± 44.06, 753.17 ± 17.66 and 201.82 ± 11.37 µg/ml, respectively) > PJ (9,925.20 ± 1,116.80, 4,615.94 ± 28.90 and 688.87 ± 44.03 µg/ml, respectively). As a result, total phenolic compounds and flavonoids content of the extracts were relative to antioxidative activities. In cytotoxicity, the 50% lethal concentration (LC_{50}) by brine shrimp lethality assay could be arranged by PPEE (1,206.98 ± 12.73 µg/ml) > PSWE (1,294.88 ± 61.28

µg/ml) > PPWE (1,743.31 ± 20.17 µg/ml) > PSEE (2,375.28 ± 69.54 µg/ml) > PJ (6,451.46 ± 52.32 µg/ml). As a result, neither total phenolic compounds nor flavonoids content correlated to cytotoxicity. Dose-response relationship of antioxidant activity and cytotoxicity which is expressed as the margin of safety (MOS) and the therapeutic index (TI) could be ranged by PPWE (125 and 11.49) > PPEE (75 and 9.92) > PSEE (5 and 1.79) > PJ (3 and 0.65) > PSWE (0.5 and 0.5). Topical application of PPEs on hairless rat skin could be against 3xMED UVB damage. This was the first discovery of PPEE and PPWE to reduce erythema (25 and 1.8 fold), to decrease the inflammatory infiltration (4.7 and 1.9 fold), to reduce epidermal thickness (1.7 and 1.6 fold), to mitigate the sunburn cells (7.4 and 7.2 fold), and to inhibit DNA fragmentation causing by UVB irradiation. In MCF-7 antiproliferative investigation via Trypan blue, MTT, and Resazurin assays, PPEE (347.83 ± 11.85, 377.88 ± 13.14 and 471.50 ± 13.62 µg/ml, respectively) was more effective than PPWE (365.93 ± 20.00, 459.90 ± 15.90 and 589.45 ± 16.21 µg/ml, respectively), considering on the LC_{50} at 24 hrs. DNA fragmentation and apoptotic cell morphology of MCF-7 cells were detectable. PPEs could induce antiproliferation, apoptotic cell morphology, and DNA fragmentation with dose- and time-dependent manner. The PPEs-induced apoptosis could confirm via the expression of Bcl-2, procaspase-9, procaspase-7 and PARP protein levels. In conclusion, PPEs showed the high efficacy of antioxidation, skin protection to UVB exposure, anticancer property and safety usage.

School of Biology Student's Signature _____

Academic Year 2011 Advisor's Signature _____

Co-advisor's Signature _____

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CHAPTER I

INTRODUCTION

1.1 Introduction

Plants have long been utilized as traditional medicines in prevention and treatment of a vast variety of diseases. Fruits and vegetables, the major sources of antioxidants in the diet, contain dietary phytochemicals that exhibit antioxidative activity including phenolic compounds, such as flavonoids, as well as tocopherols, carotenoids, and ascorbic acids.

Pomegranate (*Punica granatum*), a shrub, is called "Tab Tim". According to Thai traditional medicine, pomegranate juice is used in dyspepsia and leprosy treatments. In addition, pomegranate bark and root are active against tapeworms, the extracts of pomegranate bark, leaves, young fruit and fruit rind are used as astringents to cure diarrhea, dysentery and haemorrhages. Its dried pulverized flower buds are employed as a remedy for bronchitis (Morton, 1987).

Pomegranate juice and peel possess antioxidant properties, while seed oil has weak estrogenic property. Juice, peel and seed oil also possess anticancer activities by interference with tumor cell proliferation, cell cycle, invasion, and angiogenesis (Lansky and Newman, 2007). Pomegranate juice (PJ) and pomegranate peel extract (PPE) compose of phenol and polyphenols, including aliphatic organic acids, hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavonols, flavones, anthocyanins, anthocyanidins, and ellagitannins (Amakura, Okada, Tsuji, and

Tonogai, 2000b; Artik, 1998; Aslam, Lansky, and Varani, 2006; de Pascual-Teresa, Santos-Buelga, and Rivas-Gonzalo, 2000; Hernandez, Melgarejo, Tomas-Baeberan, and Artes, 1999; Poyrazoglu, Goekmen, and Artik, 2002). Pomegranate seed extract (PSE) consists of hydroxybenzoic acids, conjugated hydroxybenzoic acids, non-conjugated fatty acids, sterols, sex steroids, and phenyl aliphatic glycosides (Schubert, Lansky, and Neeman, 1999; Wahab, Fiki, Mostafa, and Hassan, 1998; Wang *et al.*, 2004).

Numerous studies have revealed that the phytochemical content and antioxidant activity of fruits and vegetables contribute to their protective effects against chronic and degenerative diseases (Heinonen, Meyer, and Frankel, 1998; Record, Dreosti, and McInerney, 2001). PJ can attribute high antioxidant activities as compared to other fruit juices such as red wine and green tea (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, and Kader, 2000). Guo *et al.* (2003) and Li *et al.* (2006) found that PPE had highest antioxidant activity among peel, pulp and seed fractions of 28 kinds of fruits, commonly consumed in China. As well as pomegranate seed oil showed strong antioxidant activity closely to 3-*tert*-butyl-4-hydroxyanisole (BHA) and green tea (Schubert *et al.*, 1999). Seeram *et al.* (2005) demonstrated that PJ showed the antiproliferative activity against oral cancer, colon cancer and prostate cancer cell lines. Flavonoids extracted from PSE showed inhibitions of sheep cyclooxygenase and soybean lipoxygenase (Schubert *et al.*, 1999). PPE exhibited both antioxidant and antimutagenic properties (Negi, Jayaprakasha, and Jena, 2003).

At present, the generation of ROS associates with environmental pollution, dietary habits, and ultraviolet radiation (UVR). UVR reaches the surface of the earth from the sun which is divided into two types, UVB (280-320 nm) and UVA (320-400

nm). UV causes direct biological damage, or indirect damage via the production of ROS. UVA and UVB both cause a large damage. Nonetheless, the concern of UVB is mainly direct damage to cells. Light in the UVB region is absorbed into skin, causing erythema, burns, and eventually skin cancer (Halliday, 2005; Matsumura and Ananthaswamy, 2004). And, cancer is apparently the biggest major medical concerns in terms of morbidity and mortality in many countries, including Thailand. Numerous medicinal plants have been investigated, analyzed, and developed as potential chemopreventive and anticancer agents.

The antioxidant activity of pomegranate is based on the phytochemicals. However, there was no report of pomegranate on UVB-induced skin symptom and anticancer treatment. The purposes of this study were (1) to investigate the pomegranate phytochemicals properties and antioxidant properties of pomegranate extracts of peel and seeds, and juice; (2) to investigate cytotoxic properties of the pomegranate fruit extracts; (3) to examine UV protection on rat skin; and (4) to investigate antiproliferation and apoptotic properties of peel extracts on human cell line (MCF-7). The results of this study were expected to reveal a potential of pomegranate as anticancer agent, which may increase value added to the pomegranate peel.

1.2 Research objectives

1) To observe some phytochemicals of pomegranate peel and seed extracts, and juice.

2) To determine cytotoxicity of pomegranate peel and seed extracts, and juice.

3) To investigate the protective effect of pomegranate peel extracts against ultraviolet B irradiation on rat skin.

4) To examine the potent of pomegranate peel extracts on antiproliferation and apoptosis of a human cell line.

1.3 Research hypothesis

1) Pomegranate peel and seed extracts, and juice contain phenolic compounds and flavonoids, which associate with antioxidant activity.

2) Pomegranate peel and seed extracts, and juice are not toxic to normal higher animals.

3) Pomegranate peel extracts have protective effect against UVB irradiation on rat skin.

4) Pomegranate peel extracts have the antiproliferative and apoptotic activities on a human cell line.

1.4 Scope and limitation of the study

1) Pomegranate was collected at local farms in Saraburee.

2) Pomegranate peel and seed were extracted in ethanolic and water, while juice is evaporated to powder.

3) The extracts and juice were measured for total phenolic compounds and flavonoids content, antioxidant activities, and cytotoxicity effect.

4) UV protective effects of the extracts were observed through rat skin.

5) Antiproliferative and apoptotic effects of the extracts were analyzed using human breast cells, MCF-7.

1.5 Expected results

The anticipanted outcomes from this study are:

1) The determined phytochemicals and antioxidant activity of pomegranate peel and seed extracts, and juice can be used as basic pharmacological data for consideration of their therapeutic potential use in the future.

2) Cytotoxicological information can be benefit for safety evaluation on clinical uses.

3) Understanding UV protective effect of pomegranate peel extracts on rat skin, the data can be used to develop skin cancer therapy.

4) Comprehending antiproliferation and apoptosis of human cell line induced by pomegranate peel extracts, the data of anti-cancer activities can be used in cancer prevention and therapy.

5) Enhancing local economics in growing high potential medicinal pomegranate fruits.

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CHAPTER II

LITERATURE REVIEW

2.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS), derived from the metabolism of molecular oxygen, compose superoxide anion radical ($O_2^{-}\cdot$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($\cdot OH$). In living cells, the major source of endogenous ROS are hydrogen peroxide and superoxide anion, which are generated as by products of cellular metabolism. ROS normally exists in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when the balance between ROS and biochemical antioxidants is shattered because of excess ROS, antioxidants depletion, or both. Therefore, ROS can cause tissue damage by reacting with lipids in cell membranes, sulhydryl groups in proteins, cross-linking/fragmentation of ribonucleoproteins, and nucleotides in DNA (Waris and Ahsan, 2006).

One of the most important contributions to cancer is considered to be oxidative damage to DNA. When a cell containing damaged DNA divides before the damage DNA can be repaired, the result is likely to be a permanent genetic alteration of the steps in carcinogenesis. Somatic cells that rapidly divide are more susceptible to carcinogenesis because there is less opportunity for DNA repair before cell division (Colic and Pavelic, 2000). Singh and Agarwal (2005) explained a standard paradigm of carcinogenesis that transformation of normal cells into the malignant state is

determined by a number of factors that impact to genetic as well as epigenetic molecular events. Mutagenic agents primarily cause oncogenic mutations in tumor suppressor genes that lead to tumor initiation. After initiation, the cells are promoted by non-mutagenic changes such as endogenous/exogenous tumor promoters leading to cell multiplication. At later stages, addition of genetic changes accelerates tumor progression.

2.1.1 Effects of ROS on skin

ROS induce changes of DNA, proteins and unsaturated fatty acids which all cause occurrences of abnormal and dead cells. UVB-exposed skin acquires ROS from transferring energy of melanin pigment. When the energy exceeds antioxidant system, it affects to the oxidative damage on cells and further skin abnormality (Wlaschek *et al.*, 2001).

2.1.2 Effects of ROS on cancer

Multistage process of carcinogenesis can be activated by a various environmental carcinogens (such as cigarette smoke), tumor promoters (such as phorbol ester), and inflammatory agents (such as H_2O_2). ROS induced by carcinogens appear to involve in the modification of genes. It relates to critical genes, such as oncogenes or tumor suppressor genes, the initiation/progression in carcinogenesis can result (Ames, Shigenaga, and Gold, 1993). ROS can play roles at several steps in multistage carcinogenesis (Benhar, Engelberg, and Levitzki, 2002; Waris and Ahsan, 2006). Carcinogen-induced ROS are known to modulate the protein kinase (such as MAPK), transcription factors (such as AP-1), cell cycle proteins (such as cyclin), anti-

apoptotic proteins (such as Bcl-2), cell adhesion molecules, COX-2, and growth factor signaling pathways (Aggarwal and Shishodia, 2006).

Waris and Ahsan (2006) reported that the mitogen-activated protein kinase (MAPK), transducing signals from the cell membrane to the nucleus in response to a wide range of stimuli, consisted of three family members, the extracellular signal-regulated kinase (ERK), the c-Jun NH_2-terminal kinase (JNK), and the p38 MAPK (p38). MAPKs regulate several important processes in carcinogenesis such as proliferation, differentiation, and apoptosis. Several studies reported that ROS-dependent MAPK activation regulated the behavior of the transformed cells. In addition, increased ROS levels stimulate MAPK activity in a mouse keratinocyte cell line that progressed to malignancy (Gupta, Rosenberger, and Bowden, 1999).

2.2 Skin and its functions

The skin, a major integumentary organ of the body, is the largest organ of all mammals, including humans. The skin is an ever-changing organ that contains many specialized cells and structures. Although, there are morphological differences in the skin among species, they have a unique function. The main functions of the skin are protection, sensation, thermoregulation, and metabolism which involve in the work of other organ systems in living organisms.

2.2.1 Anatomy of human skin

The human skin composes of two main layers which are epidermis and dermis (Figure 2.1).

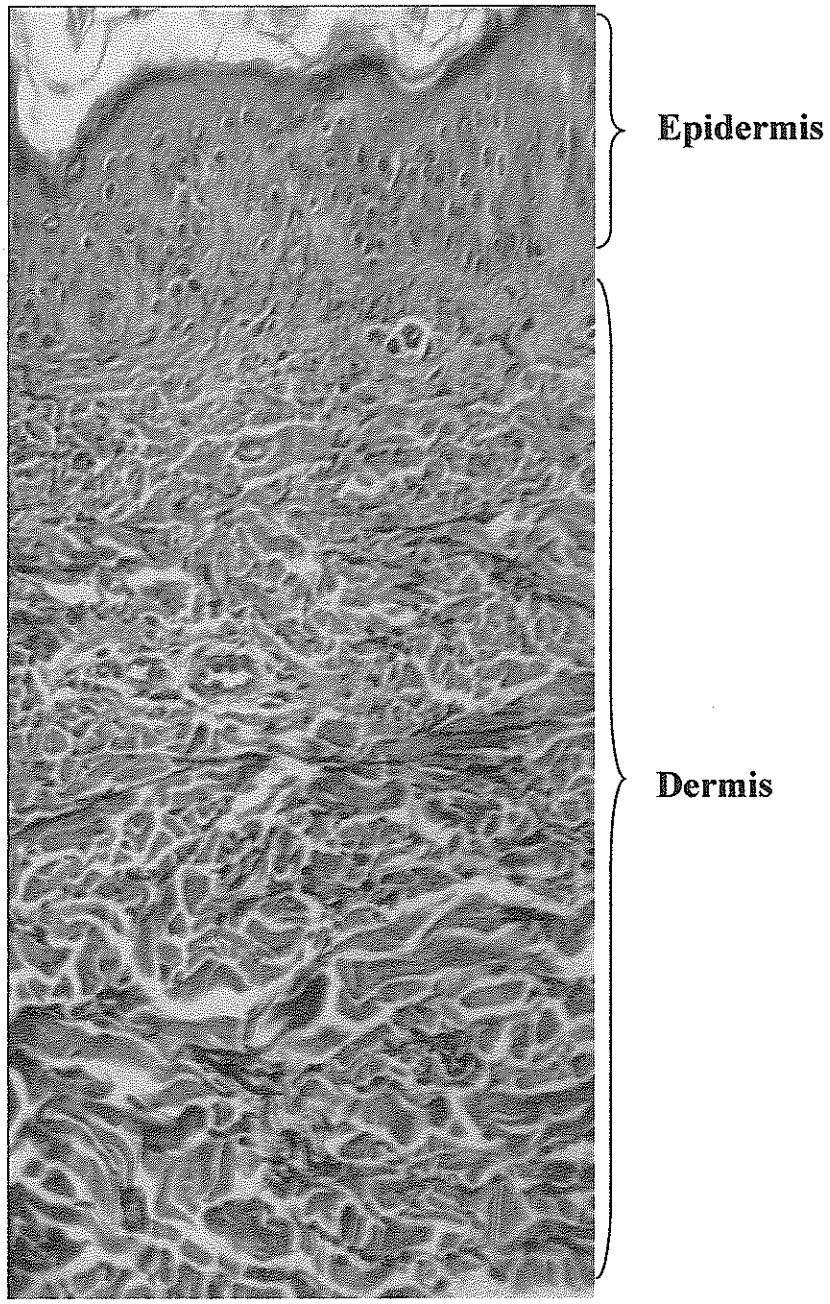

Figure 2.1 Photomicrograph of human skin illustrates two main layers, epidermis and dermis (Burkitt, Young, and Heath, 1993).

2.2.1.1 Epidermis

The epidermis is the outer layer of skin in contact with the external environment. The thickness of epidermis varies in various types of skin. The epidermis, originating from ectoderm, is a stratified squamous epithelium with the surface layer of thightly packed flat plates of protein named keratin. Keratin is produced by the keratinocyte, the main cell type in epidermis. The epidermis is separated from the dermis by the basal layer, the deepest layer of the epidermis. The shapes of basal layer cells are cuboidal attached to the basement membrane. In the epidermis, there are three types of non-keratinizing epidermal cells, melanocytes, Langerhans' cells, and Merkel cells.

2.2.1.2 Dermis

The dermis is the location in which the epidermal appendages, blood supply, nerve supply and lymphatic drainage are situated. The thickness of the dermis varying depends on the location of the skin. The dermis is composed of three types of tissues which are collagen, elastic tissue and reticular fiber. Layer of the dermis is identified to the two layers form reticular layer and papillary layer. The reticular layer contains dense collagen with intervening long thick fibers of elastin, which are arranged parallel to the skin surface. The papillary layer contains less collagen and elastin, but more matrix.

2.2.1.3 Subcutaneous tissue

The subcutaneous tissue, a layer of fat and connective tissue, is the deepest layer. It composes largely of adipose tissue, and houses blood vessels and nerves. The thickness of this layer varies according to a body size of each person.

2.2.2 Skin functions

The skin is an extensive organ covering the exterior of the body. The skin shows specific functions including protection, sensation, thermoregulation, and metabolism.

2.2.2.1 Protection

The interior of the body is protected by the skin from external damaging agents. It is the first defense barrier of body from external physical, chemical, and environmental pollutants, including ultraviolet radiation. The skin reduces the body dehydration.

2.2.2.2 Sensation

The skin, the largest sensation organ of the body, can respond to changing environmental conditions such as touch, pressure, pain and temperature.

2.2.2.3 Thermoregulation

The skin is the best organ for the thermoregulation of the body. The thermoregulation is supported by the several internal parts of the epithelium, which involve sweat glands, hairs, cutaneous plexuses, subpapillary plexuses and subcutaneous adipose tissue.

2.2.2.4 Metabolism

Subcutaneous adipose tissue is the most important energy source. Energy is accumulated into triglyceride form. Moreover, the epidermis can create vitamin D into the body.

2.3 Ultraviolet radiation

UV radiation (UVR) encompasses the wavelengths from 200 to 400 nm, and is divided into three wavelengths, UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). UVA and UVB radiation reach the surface of the Earth and cause direct biological damages or indirect damages via production of ROS. Although UVA is the predominant component of solar UV radiation to which we are exposed, it is supposed to be weak carcinogen, and causes skin aging and wrinkling. UVB is the major wavelength that mainly causes sunburn, inflammation and erythema, post-inflammatory immunosuppression, DNA damage, gene mutation, and skin cancer (Halliday, 2005; Klingman and Klingman, 1986; Matsumura and Ananthaswamy, 2004; Miyachi, 1987; Pathak and Fitzpatrick, 1993).

2.3.1 Acute effects of UVB on skin

Excessive exposure to skin to UV radiation induces skin pathophysiology. Short-term overexposure to UVB causes acute effects, such as erythema, p53 protein, p21 protein, cell cycle arrest, DNA damage, apoptosis, DNA repair, immunological responses, inflammation, cell survival, and skin thickening.

Short-term overexposure to UVB, keratinocytes changes sunburn cells in epidermis layer within 24-48 h. Dead cells are removed to the surface of the skin, becoming parakeratotic stratum corneum. Furthermore, lymphocytes may be found in epidermis layer between 24 to 72 h. The endothelial cells extend at superficial vascular plexus area in dermis. Hypogranulate mast cells and perivenular may be found in deep reticular dermis and subcutaneous fat areas. Neutrophils are suddenly found after short-term overexposure, increasing the highest numbers within 24 h.

Macrophages trend to rise to the highest numbers within 24-48 h (Diffey and Oakely, 1987; Rosario, Mark, Parrish, and Mihm, 1979).

UVB-induced oxidative stress increases blood flow and infiltration, such as macrophages and neutrophils migrate into the skin. This stress also induces the production of prostaglandins (PG), including PGE_2, which in turn cause inflammation in the skin (Hruza and Pentland, 1993). PGE_2 is produced from arachidonic acid by the induction of cyclooxygenase-2 (COX-2). UVB may increase phospholipase activity, which enhance arachidonic acid availability for PG production (Kangrotondo, Miller, Morrison, and Pentland, 1993). There are several experiments supporting a role of inflammation in driving tumor progression. Anti-inflammatory drugs have been shown to reduce the incidence of cancer (Balkwill and Mantovani, 2001). Celecoxib, a COX-2 inhibitor, decreases macrophage and neutrophil infiltration into skin tumors, and inflammation (Liang *et al.*, 2003). Several animal models showed that inhibition of COX-2 prevented skin cancer, including UVR-induced skin carcinogenesis in mice (Fischer, Conti, Viner, Aldaz, and Lubet, 2003; Fischer *et al.*, 1999; Lee, Mukhtar, Bickers, Kopelovich, and Athar, 2003).

Exposure to UVB results in skin thickening, mostly on epidermis. After epithelial cells are exposed by UVB, it is found that several types of mediators (such as ornithine decarboxylase and TGF-α) increase theirs numbers. And those mediators can activate the division of keratinocytes. Consequently, the increased numbers of keratinocytes cuase skin thickening (Murphy, Quinn, Camp, Hawk, and Greaves, 1991).

2.3.2 Chronic effects of UVB on skin

Long-term overexposure to UVB causes chronic effects, which demonstrates important symptoms of solar elastosis, dyspigmentation and photodysplasia. Those symptoms can result in photoaging and skin cancer. The long-term effect of UVB affects mutation in p53 tumor suppressor gene, and deregulation of Fas/FasL signaling, causing skin photoaging, non-melanoma skin cancer, and malignant melanoma (Matsumura and Ananthaswamy, 2004; Melnikova and Ananthaswamy, 2005).

Epidermal pathophysiology of photoaging found in keratinocytes is nuclear atypia and arrange non-regulation. Melanocytes increase in size and numbers in UVB-exposed skin. Langerhans cells trend to reduce in numbers. Epidermis increases the thickness in the first period of change. Nevertheless, the epidermis thickness inclines to abate in the later period. Likewise, normal skin increases the thickening of basement membrane zone (Gilchrest, Szabo, Flynn, and Goldwyn, 1983). In dermis, collagen fibers are substituted by elastic fibers arranging non-regulation, called solar elastosis. Ground substances, composed of proteoglycans, dermatan sulfate, heparin sulfate, glycosamino glycan and hyaluronic acid, increase the vast numbers and aggregate into elastic fiber area. The vast numbers of mast cells surround blood vessels, and there are infiltrations of inflammatory cells such as lymphocyte (Kligman, 1989; Lavker and Kligman, 1988).

UVB can induce the changes in skin that begin with actinic keratosis (AK) and then develop into squamous cell carcinoma (SCC). p53 is the most commonly mutated tumor suppressor gene. Its role in the development of SCC is believed to be central. The mutation of p53 is found more than 90% in SCC, most basal cell carcinoma

(BCC), and most AK (Leffell, 2000). Fukuda, Sakai, Matsunaga, Tokuda, and Tanaka (2006) reported that percentage of mice bearing papillomas and numbers of papillomas per mouse involved in UVB-induced initiation in hairless mice skin exposed to UVB (0.34 J/cm^2) for 15 min (3 times). After initiation, mice were promoted with 12-O-tetradecanoylphorbol-13-acetate (TPA) twice a week for 20 weeks. Kapadia *et al.* (2003) also found that UVB promoted mouse skin carcinogenesis when mice were initiated with 7,12-dimethylbenz(a)anthracene (DMBA), and promoted with UVB (0.34 J/cm^2, 8 min) twice a week for 20 weeks. Thus, UVB act as initiator and promoter in tumorigenesis (Fukuda *et al.*, 2006; Halliday, 2005; Kapadia *et al.*, 2003; Leffell, 2000; van Kranen *et al.*, 2005). In addition, UVB (0.09 J/cm^2/day) induced skin tumors in heterozygous, homozygous p53 deficient hairless mice, and wild type for a chronic UVB exposure experiment. However, tumors develop significantly faster in the p53 knockout mice compared to the wild types.

2.4 Antioxidant

2.4.1 Antioxidant enzymes

The antioxidant enzymes existing in mammalian cells primarily interfere with the production of free radicals (Combs, 1987).

2.4.1.1 Superoxide dismutase (SOD)

Dismutation of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) by SOD is often called the primary defense, because this enzyme impedes the further generation of free radicals. It exists in virtually all O_2–respiring organisms, and its function catalyzes the

superoxide dismutative reaction (Watcharakup, Boonjhung, Boonyarat, and Autsinthong, 2006).

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

There are two types of SOD inactivating the superoxide anion (Mn SOD located in mitochondria and Cu/Zu localizes in cytoplasm). Its activity differs among organs. The highest levels are found in the liver, kidneys, and spleen. H_2O_2 is continually eradicated by catalase (CAT) and glutathione peroxidase (GPX). Furthermore, SOD assists to prevent the groups of dehydrase enzymes, such as dihydroacid dehydrase and phosphogluconate dehydrase. Induction of SOD also occurs when stimulated by other chemical compounds, such as paraquat (generate $O_2^{\cdot-}$) (Yu, 1994).

2.4.1.2 Catalase (CAT)

Catalase (CAT) is an enzyme composed of ferriprotoporphyrin. It is a major primary antioxidant defense component that primarily works to catalyze the decomposition of H_2O_2 to H_2O and O_2 as well as sharing this function with glutathione peroxidase. At low H_2O_2 concentrations, H_2O_2 are preferentially catalyzed by peroxidase. Nevertheless, at high H_2O_2 levels, they are metabolized by CAT. Like SOD, CAT's activity varies not only between tissues but within the cell itself, and the highest levels are seen in the liver, kidneys, and red blood cells. In hepatocytes, peroxisomes showed an expectedly high CAT activity, although activity was found also in microsomes and in the cytosol (Thomas and Aust, 1985).

$$H_2O_2 + 2H^+ \xrightarrow{CAT} H_2O + O_2$$

2.4.1.3 Glutathione peroxidase (GPX)

Glutathione peroxidase (GPX) consists of selenium as the major component. GPX catalyzes detoxify oxygen reactive radicals by catalyzing the formation of hydroperoxide derived from $O_2^{-\bullet}$, such as lipid peroxide (ROOH) and H_2O_2. In reaction, glutathione co-exists in catalyzing ROOH and H_2O_2 as follows

$$ROOH + 2GSH \xrightarrow{GPX} ROH + GSSH + H_2O$$

or

$$H_2O_2 + 2GSH \xrightarrow{GPX} GSSH + 2H_2O$$

Both types of GPXs, selenium dependent and selenium independent, protects against radical damages by reducing peroxides in mammalian cells. However, they possess different substrate specificities. The selenium dependent peroxidase is found in the cytosol and exhibits a low capacity for reducing H_2O_2. The selenium independent peroxidase uses organic hydroperoxides as preferred substrates over H_2O_2.

Antioxidant enzymes reduce free radicals impacting in mammalian cells to normal level state. However, lack of antioxidant enzymes in extracellular fluids might induce oxidative stress and other diseases. Shindo and Packer (1993) reported that UVR decreased the activity of SOD, CAT, and GPX, and led to loss of skin ascorbate and α-tocopherol in murine epidermis and dermis (Shindo, Witt, Han, and Packer, 1994).

2.4.2 Antioxidant vitamins

Vitamin E and vitamin C directly interfere with propagation stage of free radical generation and scavenge free radicals (Figure 2.2). Vitamin E (α-tocopherol),

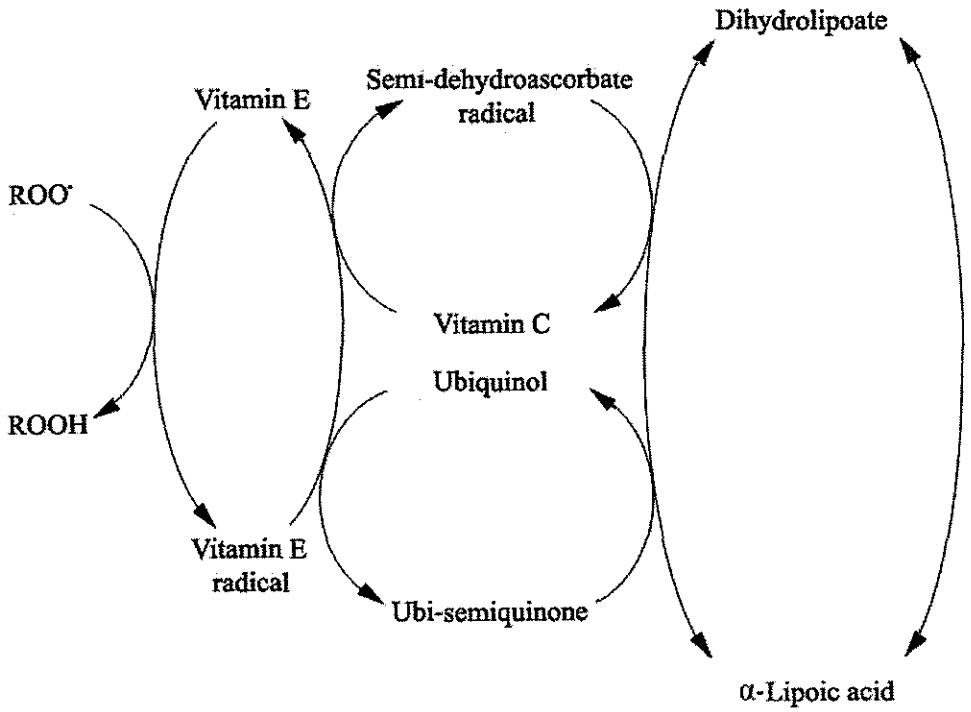

Figure 2.2 Redox reaction cycle of antioxidant vitamins (Watcharakup *et al.*, 2006).

the major lipid soluble antioxidant presents in all cell membranes, protects against lipid peroxidation and prevents loss of membrane fluidity. Vitamin E is characterized as the most critical antioxidant in blood (Burton, Joyce, and Ingold, 1983). Vitamin C (ascorbic acid) quenches free radicals as well as singlet oxygen (Frei, 1991). Vitamin C and ubiquinone regenerate the reduced antioxidant form of vitamin E (Bendich, Machlin, Scandurra, Burton, and Wayner, 1986).

UV-activated molecules oxidize cellular components, inducing a chain reaction of lipid peoxidation in membranes. One main function of vitamin E is to act as an antioxidant during lipid oxidation (Chow, 1991; Fryer, 1993). Vitamin C, a powerful water soluble antioxidant, reduced UVB-induced oxidative damage in mouse keratinocytes *in vitro* (Stewart, Cameron, and Pence, 1996), and protected UVA-induced lipid peroxidation in human keratinocytes *in vitro* (Tebbe *et al.*, 1997). The skin malonaldehyde content was reduced by vitamin C supplement after ultraviolet irradiation on human skin (McArdle *et al.*, 2002). And, vitamin C could regenerate vitamin E radical to reduced antioxidant form in redox reaction cycle. Combinations of antioxidant vitamins might be expected to have greater protective effect against UVR-induced oxidative stress than single antioxidant agents (Lin *et al.*, 2003). Eberlin-Konig, Placzek, and Przybilla (1998) and Fuchs and Kern (1998) reported that combined supplements of vitamins E and C could protect human skin against UVR-induced erythema, and reduce the sunburn reaction in human. In addition, vitamins E and C decreased nuclear binding and activation of NFκB and AP-1 in LNCap prostate cancer cells (Ripple, Henry, Schwarze, Wilding, and Weindruch, 1999). Reddy, Khanna, and Singh (2001) proposed a mechanism by which antioxidants can improve the efficacy of chemotherapy. Vitamin C at nontoxic concentrations increases the

cytotoxic effects against human cervical cancer cells *in vitro* by stabilizing p53 protein.

2.4.3 Antioxidant of plant phenolic compounds

Phytochemicals contain variety chemical groups such as simple phenolic and polyphenolic compounds. Phenolic compounds generally are considered as non-essential by-products called "secondary products" in plant metabolism. Flavonoids, major phenolic compounds, are shown to exhibit flavour and colour of many fruits and vegetables. At present, there is much interest in the biological effects of phenolic compounds since there is abundance of evidence that regular consumption of fruits and vegetables is implicated with reduced risks of cancers and cardiovascular diseases (Block, 1992; Doll, 1990). The variation of antioxidant activity among phenolic compounds is due to their varying levels and types of natural phenolic compounds and the different molecular structures of these compounds (Cai, Sun, Luo, and Corke, 2006).

2.4.3.1 Simple phenolic compounds

Simple phenolic acids, cinnamic acid and its derivatives are found and spread, in plants. They are synthesized via phenylalanine and tyrosine in the shikimate pathway (Figure 2.3). *p*-Coumaric acid (single hydroxyl group), caffeic acid (double hydroxyl groups), and sinapic acid (triple hydroxyl groups) are products from biosynthesis. Oxidation of the side chain induces derivatives of benzoic acids (such as salicylic acid, protocatechuic acid, and gallic acid) or hydroxycinnamic acids (such as coumaric acid, caffeic acid, and ferulic acid).

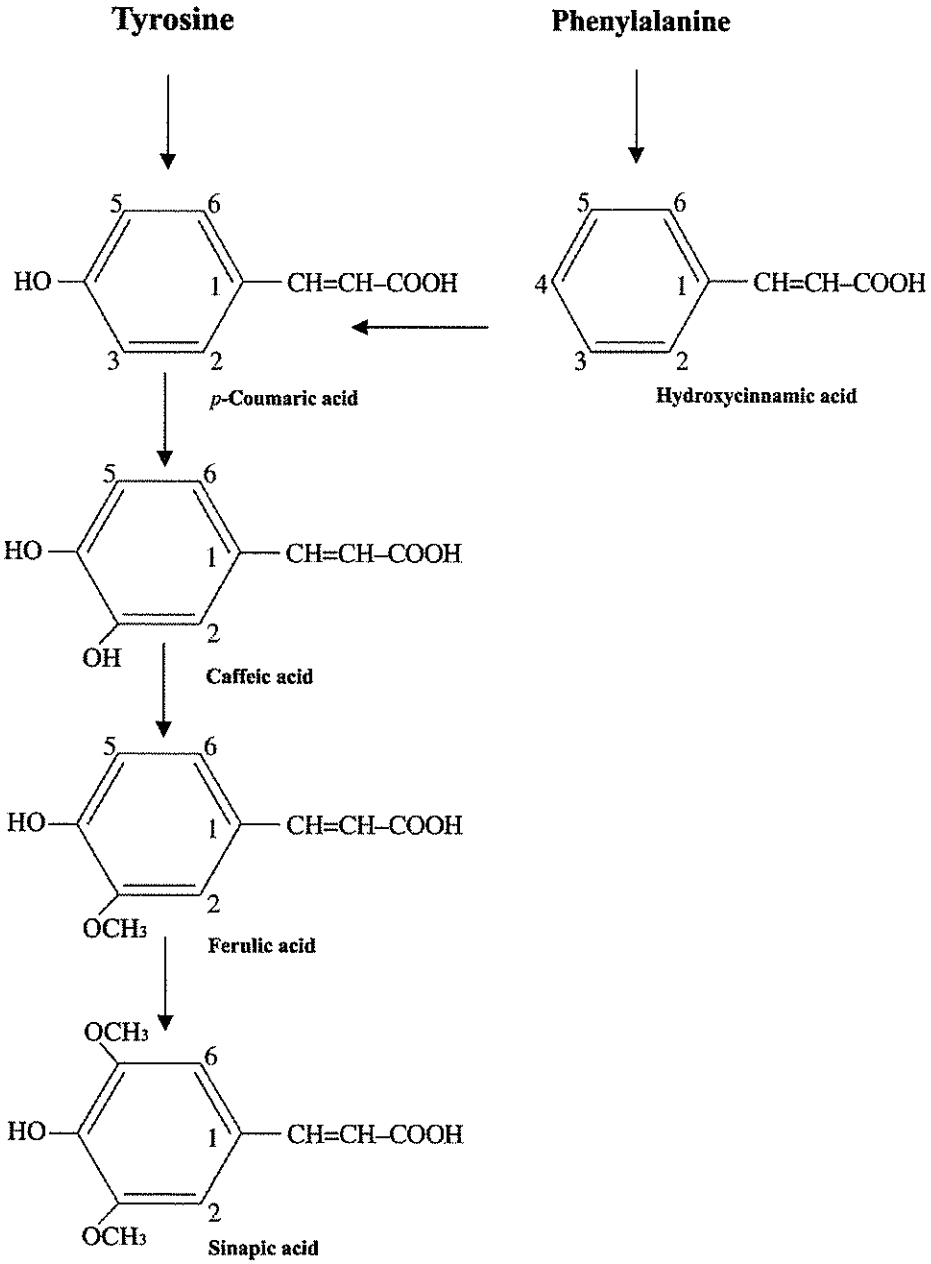

Figure 2.3 Hydroxycinnamic acid and its derivatives (Watcharakup *et al.*, 2006).

Phenolic acids are simple phenolic compounds (such as hydroxybenzoic acid and hydroxycinnamic acid). Radical scavenging activity of phenolic acids depends on the number and position of hydroxyl (-OH) groups and methoxy ($-OCH_3$) substituents in their molecules. In Table 2.1, Cai *et al.* (2006) reported that benzoic acid and cinnamic acid, without hydroxyl group, had no antioxidant activity, and gallic acid. the 3,4,5-trihydroxy-benzoic acid with most hydroxyl groups, had strongest radical scavenging activity, because of pyrogallol structural advantage and potent hydrogen (H)-donating ability (three OH groups). Radical scavenging capacity of hydroxybenzoic acids decreased in the following order: trihydroxy > dihydroxy > monohydroxy. Moreover, Natella, Nardini, Felice, and Scaccini (1999) and Rice-Evans, Miller, and Paganga (1996) suggested that methoxy groups in hydroxybenzoic acid and hydroxycinnamic acid might enhance H-donating capacity and radical scavenging activity. However, in LDL system, the dihydroxy acids had an antioxidant capacity equal to or higher than the hydroxydimethoxy acid. Furthermore, hydroxyl group in the *p*-position (4-OH) conferred the hydroxycinnamic acids (*p*-coumaric acid, 1.96 mM), which was significant enhancement of antiradical capacity as compared to the equivalent *m*- or *o*-coumaric acids. Overall, with the same hydroxyl and methoxy groups, hydroxycinnamic acids tented to be more effective than hydroxybenzoic acids (Cai *et al.*, 2006).

2.4.3.2 Polyphenolic compounds

Polyphenolic compounds consist of thousands of diverse molecules with heterogenous structure and common structure contains one or more phenol rings. Most of the phenolic compounds belong to flavonoids that have main structure of flavan

Table 2.1 Trolox equivalent antioxidant capacity (TEAC) of phenolic compounds (Cai *et al.*, 2006).

Chemical class	Compound names (Phenolic acid)	(-OH) substituents	Number (-OH)	($-OCH_3$) substituents	TEAC (mM)
			OH position	OCH_3 position	
Hydroxy-benzoic acids	Gallic	3,4,5-OH	3		3.52
	Protocatechuic	3,4-OH	2		1.15
	p-hydroxybenzoic	4-OH	1		0.028
	Vanillic	4-OH	1	$3-OCH_3$	0.092
Hydroxy-cinnamic acids	Caffeic	3,4-OH	2		1.31
	Chlorogenic	3,4-OH	2		1.56
	o-Coumaric	2-OH	1		0.93
	m- Coumaric	3-OH	1		0.82
	p- Coumaric	4-OH	1		1.96
	Ferulic	4-OH	1	$3-OCH_3$	1.92

(Figure 2.4). Flavonoids are composed of 15 carbon atoms (C15) and are based on a skeletal structure (C6-C3-C6 system) of two benzene rings joined by a linear C3 chain (chroman structure). Flavonoids are biosynthetically derived from acetate and shinkimate of which the A ring has characteristics of hydroxylation pattern at the 5 and 7 position (Mann, 1978). The B ring is usually 4', 3'4' or 3'4'5' hydroxylated. Flavonoid compounds are classified into five main subgroups: (i) anthocyanidins, anthochlors, and aurones; (ii) minor flavonoids (flavanones, chalcones, dihydroflavonols, and dihydrochalcones) (iii) flavones and flavonols; (iv) isoflavonoids (isoflavones, isoflavanones, isoflavans, and isoflavanols); and (v) flavans, flavanols (flavan-3-ols), and proanthocyanidins (Harborne and Baxter, 1991). Some major dietary sources of phenolic compounds are shown in Table 2.2. The flavanols, particularly catechin, epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG), are found in beverages such as green tea and black tea. The flavanones, such as naringin, taxfolin, and fisetin, are generally found in citrus fruits. Kaemferol and quercetin are discovered in red wine. Anthocyanidins such as cyanidin are mainly found in raspberries (Liu *et al.*, 2002).

Several studies reported that flavonoids structures were relative to antioxidant activity (Bors, Heller, Michel, and Saran, 1990; Cao, Sofic, and Prior, 1997; Chen, Chan, Ho, Fung, and Wang, 1996; van Acker *et al.*, 1996). Table 2.2 is the summary of flavonoids and their radical scavenging activity as followings (Watcharakup *et al.*, 2006).

1. A catechol structure or *ortho*-(3',4')-dihydroxyl group structure in the B ring is the most important structural feature for radical scavenging for electron delocalization;

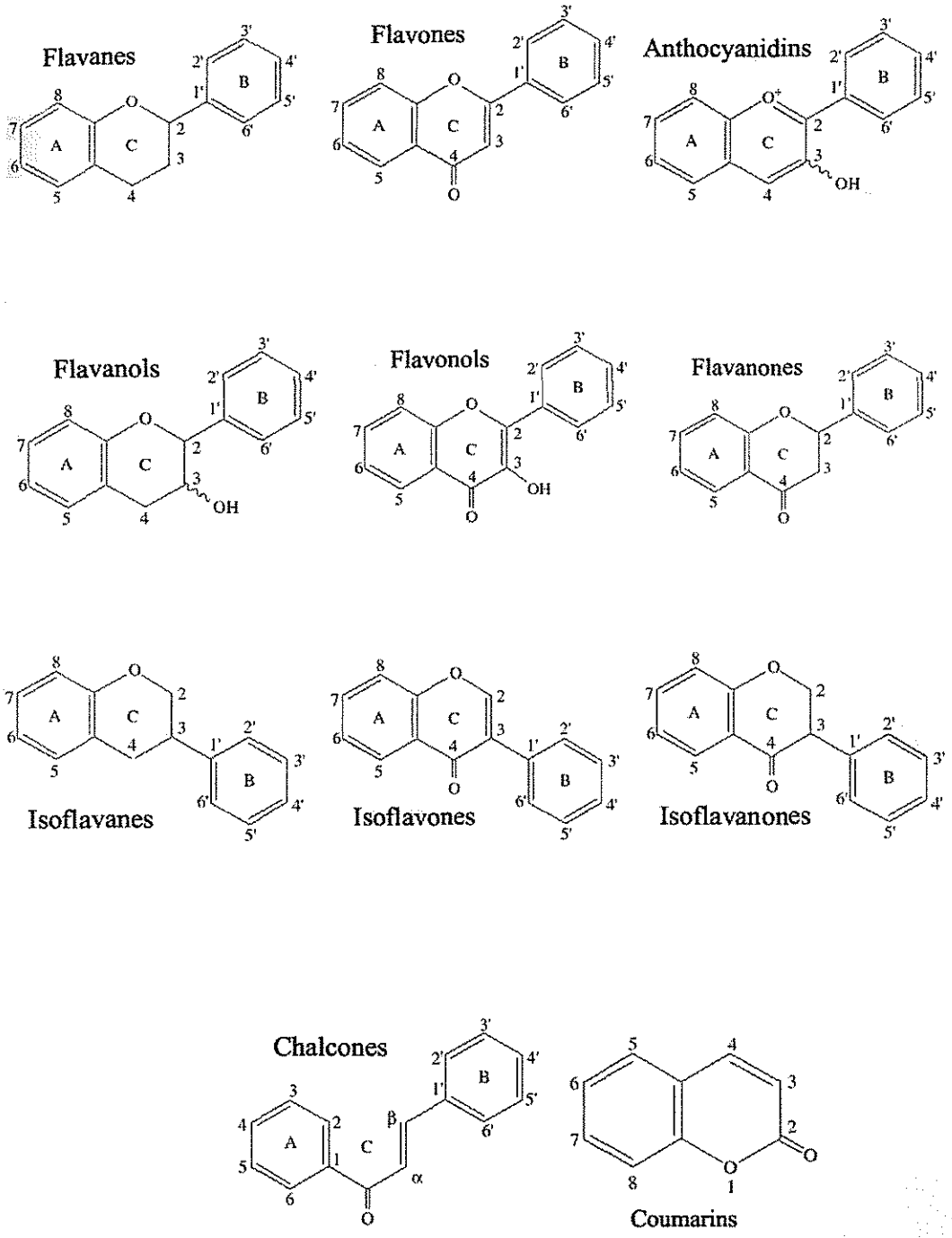

Figure 2.4 The main classes of flavonoids (Cai *et al.*, 2006).

Table 2.2 Trolox equivalent antioxidant capacity (TEAC) of flavonoids (Cai *et al.*, 2006).

Flavonoids type	Compound names	Plant Sources	Number (-OH)	TEAC (mM)
Flavanols	(-)-Epigallocatechin gallate	*Camellaia sinesis* L.	8	5.95
	(-)-Epicatechin gallate	*Camellaia sinesis* L.	7	5.29
	(-)-Epigallocatechin	*Camellaia sinesis* L.	6	3.71
	(-)-Epicatechin	*Acacia catechu* L.	5	3.08
	(+)-Catechin	*Acacia catechu* L.	5	3.04
Flavonols	Myricetin	*Astragalus complanatus* R.	6	1.31
	Quercetin	*Sophora japonica* L.	5	4.42
	Morin	*Morus alba* L.	5	2.68
	Kaempferol	*Alpinia officinarum* Hance.	4	1.59
	Galangin	*Alpinia officinarum* Hance.	3	1.12
Flavones	Luteolin	*Lonicera japonica* Thunb.	4	2.18
	Baicalein	*Scutellaria baicalensis* G.	3	2.56
	Apigenin	*Artemisia argyi*	3	0.08
	Chrysin	*Scutellaria baicalensis* G.	2	0.08
Flavanones	Naringenin	*Citrus aurantium* L.	3	0.22
	Hesperetin	*Citrus aurantium* L.	3	0.40
Isoflavones	Genistein	*Sophora tonkinesis* Gapnep.	3	0.12
	Daidzein	*Pueraria lobata* (Willd.)	2	0.10

Table 2.2 (Continued).

Flavonoids type	Compound names	Plant Sources	Number (-OH)	TEAC (mM)
Chalcones	Butein	*Rhus verniciflua* Stokes.	4	2.42
	Sappanchalcone	*Caesalpinia sappan* L.	3	1.93
	Carthamin	*Caesalpinia sappan* L.	3	1.43
Coumarins	Esculetin	*Fraxinus rhynchophylla*	2	2.38
	Scopolein	*Lycium barbarum* L.	1	0.38
Condensed tannins	Catechin 3-*O*-gallate (monomer)	*Songuisorba officinalis* L.	7	5.25
	Procyanidin C-1 (trimer)	*Vaccinium vitis-idaea* L.	15	8.29

2. A 2, 3 double bond in conjugation with a 4-oxo group provides electron delocalization from the B ring;
3. Hydroxyl groups at C_3 position in the C ring shown induce hydrogen bonding supporting electron delocalization to 4-oxo group.

Flavonoids exhibited variation in the radical scavenging activity. The radical scavenging activities of flavonoids were controlled by the number and configuration of phenolic hydroxyl groups and influenced by glycosylation and configuration of other substituents. The 3-hydroxyl group or the 3-galloyl group in the C-ring associated to high activity of flavonoids. In Table 2.2, the radical scavenging activity of the different classes of flavonoids decreased in the following order: flavanols (flavan-3-ols) > flavones > chalcones > flavones > flavanones > isoflavones. Flavanols exhibited highest radical scavenging activity, because flavanols contain more hydroxyl groups, especially with *ortho*-(3',4')-dihydroxyl groups in the B-ring and 3-hydroxyl group and/or 3-galloyl group in the C-ring (Cai *et al.*, 2006). However, quercetin (flavonol) was more effective than catechin, EC, and EGC (flavan-3-ols). Quercetin possessed 2,3-double bond and 4-oxo function in the C-ring. Therefore, quercetin could support electron delocalization between the A- and B-rings and stabilize the aryloxyl radicals after hydrogen donation (Rice-Evans *et al.*, 1996).

Natural tannins are commonly divided into condensed tannins and hydrolysable tannins (Cai, Luo, Sun, and Corke, 2004; Chung, Wong, Huang, and Lin, 1998; Xiao, Yang, and Hong, 2000). Condensed tannins are mainly the oligomers and polymers of flavan-3-ols (catechin derivatives), known as proanthocyanidins. Several flavan-3-ols mentioned above (EGCG, ECG, EGC, EC, and catechin) are monomers of condensed tannins. Hydrolyzable tannins, including gallotannins and ellagitannins,

possess a central core of polyhydric alcohol such as glucose, and hydroxyl groups, which are esterified either partially or wholly by gallic acid (gallotannins) or by hexahydroxy-diphenic acid and other substituents (Chung *et al.*, 1998; Khanbabaee and van Tee, 2001). Ellagitannins also have monometric, dimeric, trimeric or polymeric forms. Condensed tannins and hydrolysable tannins are powerful antioxidant agents (Bouchet, Laurence, and Fauconneau, 1998; Yokazawa *et al.*, 1998), because they possess a great number of hydroxyl groups, especially many ortho-dihydroxy or galloyl groups. An increase of galloyl group and *ortho*-hydroxyl structure enhanced the activity of the tannins. The activity of both condensed and hydrolysable tannins rose with the increase of their molecular weight in the following order: monomer < dimer < trimer < dimer-digallate. The bigger molecules of tannins possess more galloyl groups and *ortho*-hydroxyl groups and their activities are stronger (Cai *et al.*, 2006).

2.5 Antioxidant effects of plant phenolic compounds

Since reactive oxygen species and lipid peroxidation are indicated harmful molecules and degenerative processes in aging and diseases such as cancer, neurodegenerative disorder and cardiovascular disease. The antioxidant activity of plant phenolic compounds has been investigated (Sami, 1995; Shon, Kim, and Sung, 2003).

The phenolic acids and flavonoids are good antioxidants. A number of studies showed that the numbers and the positions of hydroxyl groups in benzoic acid or cinnamic acid are the main structures of phenolic acids requiring for efficient radical scavenging activity (Cai *et al.*, 2006). Recent studies pointed that phenolic acids

(caffeic acid or gallic acid) inhibited low-density lipoprotein (LDL) and lipid peroxidation in humans (Heinonen *et al.*, 1998; Jang *et al.*, 2008; Meyer, Heinonen, and Frankel, 1998). Chen *et al.* (2004) reported that phenolic acids from oats interacted synergistically with ascorbic acid to protect LDL during oxidation in hamsters. Thus, phenolic compounds, flavonoids and phenolic acids, might interact in radical scavenging activity as synergistic interaction. The interaction may enhance the efficient radical scavenging that may have potential health effects. The synergistic interaction of these antioxidants may be exemplified by the enhancement of the antioxidative and antiproliferative effects of catechin or quercetin by caffeic acid or ascorbic acid, its ability to protect the polyphenol from oxidative degradation (Kandaswami, Perkins, Soloniuk, Drzewiecki, and Middleton, 1993; Peyrat-Maillard, Cuvelier, and Berset, 2003). Mertens-Talcott, Talcott, and Percival (2003) also showed that the interaction of quercetin and ellagic acid exhibited an enhanced anticarcinogenic potential of polyphenol combination in MOLT-4 human leukemia cells.

2.5.1 Effects of antioxidants on skin

Phytochemicals have ability to prevent skin inflammation and cancer. Baliga and Katiyar (2006) and Katiyar (2003) found that some flavonols from green tea, epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate, exhibited antioxidant, anti-inflammatory, and anticarcinogenic activities *in vitro* and *in vivo*. Resveratol was detected in the many plant species, including grapes, peanuts, berries, and pines. Resveratrol involved in cell growth, apoptosis, and inflammation by inhibiting cyclooxygenase, hydroperoxidase, protein kinase C, Bcl-2

administered curcumin reduced the development of tumors after topical application of a carcinogen in mice, and decreased the expression of *ras* and *fos* oncogenes in the skin (Limtrakul, Anuchapreeda, Lipigorngoson, and Dunn, 2001). Quercetin acted as anticancer agents by the inhibition of the expression of mutant p53 in human cell lines (Avila, Cansado, Harter, Velasco, and Notario, 1996; Avila, Velasco, Cansado, and Notario, 1994; Skhlar, 1998).

2.6 *Punica granatum* L. (pomegranate)

2.6.1 Botanical information

Family: Punicaceae (Lythraceae)

Common names: Punica (Roman), Dalim or Dadima (Sanskrit), Dulim or Dulima (Persian), Grenade (French), Granada (Spanish), Granatappel (Dutch), Granatapfel (Germans), Melogranato or Pomogranato (Italians), Romeira or Romazeira (Brazilians), Delima (Malaya), Granato (Indonesia), Tab Tim (Thai)

Botanical description:

Pomegranate is a rounded shrub about 5 meters height. Pomegranate has glossy and leathery leaves that are narrow and lance-shaped. The trunk is covered by a red-brown bark which later becomes gray. The branches are stiff, angular and often spiny. The attractive scarlet, white or variegated flowers have 5 to 8 crumpled petals and a red, fleshy, tubular calyx (Figure 2.5). The flowers may be solitary or grouped in twos and threes at the ends of the branches. The tough, leathery skin or rind is typically yellow overlaid with light or deep pink or red (Figure 2.6). The interior fruit is separated by membranous walls and white, spongy, bitter tissue into compartments packed with sacs filled with red, pink or whitish pulp or aril. In each sac, there is one

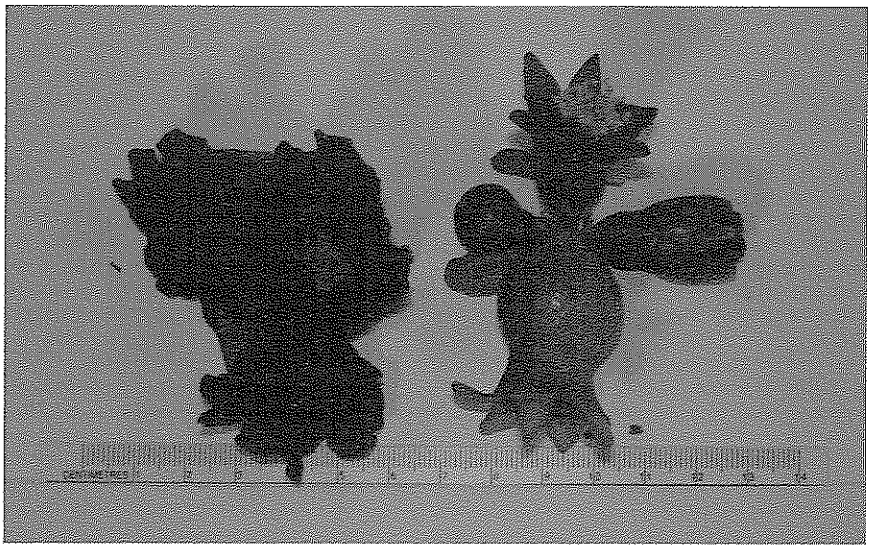

Figure 2.5 The flowers of pomegranate.

Figure 2.6 Pomegranate fruits.

angular, soft or hard seed. High temperatures are essential during the fruiting period to get the best flavor. The pomegranate fruits may begin to bear in 1 year after planting out, but 2 to 3 years is more common. Under suitable conditions, the fruits are mature in 5 to 7 months after flowers bloom.

Ecology and distribution:

Pomegranate is cultivated in all over the tropics for edible fruits, and as ornamental trees. Pomegranate belongs to family Punicaceae and is a native to Iran and Afghanistan. It spreads around the Mediterranean and eastwards to India and China. Pomegranate is now cultivated in Japan, Russia and in Thailand, particularly in Saraburee and Nakhon Ratchasima. It normally grows in tropical and subtropical regions and favors a semi-arid climate. It naturally adapted to regions with cool winters and hot summers. It is one of the most important plant resources as fruit materials in tropical and subtropical regions particularly in China (Morton, 1987).

2.6.2 Chemical constituents of pomegranate

Numerous studies suggested that the phytochemical content and antioxidant activity of vegetables and fruits contribute protective effects against chronic and degenerative diseases (Heinonen *et al.*, 1998; Record *et al.*, 2001). Several studies showed that pomegranate possesses a high level of antioxidant activity, which is associated to the levels of phenolic compounds in the fruit (Sun, Chu, Wu, and Liu, 2002; Vinson, Su, Zubik, and Bose, 2001). Major chemical constituents of pomegranate are shown in Table 2.3.

Table 2.3 Major chemical constituents of pomegranate (Lansky and Newman, 2007).

Chemical class	Compound name	Plant part	References
Enolic furanolactone	Ascorbic acid	J	(Veres, 1976)
	Gallic acid	J, P	(Amakura, Okada, Tsuji, and Tonogai, 2000b; Huang *et al.*, 2005a)
Hydroxybenzoic acids	Ellagic acid	J, P, S	(Amakura *et al.*, 2000b)
	Methylellagic acids	S	(Wang *et al.*, 2004)
Hydroxycinnamic acids	Caffeic acid	J, P	(Amakura, Okada, Tsuji, and Tonogai, 2000a; Artik, 1998)
	Chlorogenic acid	J, P	(Amakura *et al.*, 2000a; Artik, 1998)
	p-Coumaric acid	J, P	(Amakura *et al.*, 2000a; Artik, 1998)
Flavan-3-ols	Flavan-3-ol	J, P	(de Pascual-Teresa, Santos-Buelga, and Rivas-Gonzalo, 2000)
	Catechin	J, P	(de Pascual-Teresa *et al.*, 2000)
	Epicatechin	J, P	(de Pascual-Teresa *et al.*, 2000)
	Epigallocatechin-3-gallate	J, P	(de Pascual-Teresa *et al.*, 2000)
Flavonols	Quercetin	J, P	(Artik, 1998)
	Kaempferol	P	(van Elswijk *et al.*, 2004)

J, Juice; P, peel extract; S, seed extract

Table 2.3 (Continued).

Chemical class	Compound name	Plant part	References
Flavonol glycosides	Rutin	J, P	(Artik, 1998)
	Kaempferol glycosides	P	(van Elswijk *et al.*, 2004)
Flavones	Luteolin	P	(van Elswijk *et al.*, 2004)
Flavone glycosides	Luteolin glycoside	P	(van Elswijk *et al.*, 2004)
Flavanone glycoside	Naringin	P	(Kim and Kim, 2002)
Anthocyanidins	Delphinidin	P	(Noda, Kaneyuka, Mori, and Packer, 2002)
	Cyanidin	P	(Noda *et al.*, 2002)
	Pelargonidin	P	(Noda *et al.*, 2002)
Anthocyanins	Cyanidin glucosides	P	Hernandez, Melgarejo, Tomas-Barberan, Artes, 1999)
	Delphinidin glucosides	J	(Hernandez *et al.*, 1999)
	Pelargonidin glucosides	J	(Hernandez *et al.*, 1999)
	Punicalin	J	(Aslam, Lansky, and Varani, 2006)
Ellagitannins	Punicalagin	P	(Aslam *et al.*, 2006)
	Corilagin	P	(Satomi *et al.*, 1993)
	Casuarinin	P	(Satomi *et al.*, 1993)
	Gallagyldilacton	P	(Satomi *et al.*, 1993)
	Pedunculagin	P	(Satomi *et al.*, 1993)

J, Juice; P, peel extract; S, seed extract

Table 2.3 (Continued).

Chemical class	Compound name	Plant part	References
Ellagitannins	Tellimagrandin	P	(Satomi *et al.*, 1993)
Conjugated fatty Acids	Punicic acid	S	(Schubert *et al.*, 1999)
Non-conjugated fatty acid	Linoleic acid	S	(Schubert *et al.*, 1999)
	Oleic acid	S	(Schubert *et al.*, 1999)
	Palmitic acid	S	(Schubert *et al.*, 1999)
	Stearic acid	S	(Schubert *et al.*, 1999)
Sterols	Daucosterol	S	(Wang *et al.*, 2004)
	Camesterol	S	(Wahab, Fiki, Mostafa, and Hassan, 1998)
	Stigmasterol	S	(Wahab *et al.*, 1998)
	β-Sitosterol	S	(Wahab *et al.*, 1998)
	Cholesterol	S	(Wahab *et al.*, 1998)
Sex steroids	17-α-Estradiol	S	(Wahab *et al.*, 1998)
	Estrone	S	(Wahab *et al.*, 1998)
	Testosterone	S	(Wahab *et al.*, 1998)
	Estriol	S	(Wahab *et al.*, 1998)
Tocopherols	γ-Tocopherol	S	(Kim and Kim, 2002)
Triterpenoids	Ursolic acid	S	(Huang, HYang, Harada, Li, and Yamahara, 2005b)

J, Juice; P, peel extract; S, seed extract

2.6.3 Pharmacological activities and its usage

Roots, barks, peels, seeds, and juice of pomegranate have been used as traditional medicine for the treatment of colic, colitis, diarrhea, dysentery, leucorrhea and caked breast (Duke and Ayensu, 1985). Their therapeutic actions were explained as vermifugal, taenicidal, astringent, antispasmodic, antihysteric, diuretic, carminative, sudorific, galactogugue and emmenagogue (Bianchini and Corbetta, 1979). Being extensively used as a folk medicine in many cultures, pomegranate was selected as sign for the Millennium Festival of Medicine (Langley, 2000).

Pomegranate is rich in phytochemicals such as anthocyanidins, flavonoids, hydroxybenzoic acids and ellagitannins (Lansky and Newman, 2007). Heftmann, Ko, and Bennet (1966) reported that pomegranate seeds contain estrone and estradiol compounds. In addition, juice, peel and seed oil also possess weakly estrogenic properties (Lansky and Newman, 2007). Seed- and peel-methanol extracts and juice from pomegranate have antioxidant activity (Gill *et al.*, 2000; Negi *et al.*, 2003; Singh, Murthy, and Jayaprakasha, 2002). Pomegranate juice had potent antiatherogenic effects in healthy humans and atherosclerotic effects in mice (Aviram *et al.*, 2000). The ethyl acetate extract of pomegranate fermented juice inhibited soybean lipooxygenase (LOX) but not sheep cyclooxygenase (COX), while pomegranate seed oil inhibited both LOX and COX (Schubert, Lansky, and Neeman, 1999). Acetone extracts of whole pomegranate fruits inhibited phosphorylation of several cytokines in UV-B irradiated keratinocytes, including mitogen activated protein kinases (MAPK), and reduced activation of nuclear factor kappa B (NF-κB) (Afaq *et al.*, 2005a). Whole pomegranate aqueous extract inhibited NF-κB and MAPK expressions in mouse skin exposed to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Afaq, Saleem, Krueger,

Reed, and Mukhtar, 2005b). Pomegranate seed oil and whole pomegranate aqueous extract reduced skin tumors induced by DMBA and subsequently promote by TPA and multiplicity in female CD-1 mice (Afaq *et al.*, 2005b; Hora, Maydew, Lansky, and Dwivedi, 2003).

Furthermore, juice, peel, and seed extracts of pomegranate inhibited human prostate cancer cell phospholipaseA2 expression (PC-3) *in vitro* (Lansky *et al.*, 2005). Pomegranate seed oil also reduced both the incidence and multiplicity of colon tumors in rats treated with carcinogen azoxymethane (Kohno *et al.*, 2004). Pomegranate juice and purified polyphenols (ellagitannins and ellagic acid) inhibit cancer cell proliferation, and induce cancer cells to undergo apoptosis in HT-29 colon cells (Seeram *et al.*, 2005).

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CHAPTER III

ANTIOXIDANT ACTIVITIES OF POMEGRANATE PEEL AND SEED EXTRACTS, AND JUICE

3.1 Abstract

Pomegranate (*Punica granatum* L.) has long been utilized as Thai traditional medicine in healing of a vast disease. This study aimed to investigate some phytochemical properties of pomegranate fruits. The experiment was designed to use four extracts; peels extracted by ethanol (PPEE) and water (PPWE), and seeds extracted by ethanol (PSEE) and water (PSWE), and juice (PJ). Each sample was screened for their potential as antioxidants measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP) and ferric thiocyanate (FTC) method. The total phenolic compounds was determined by Folin-Ciocalteu's method, and flavonoids content was measured by colorimetric method. Finally, the relationship between phytochemical properties and antioxidants were correlated.

PPEE had highest total phenolic compounds (451.96 ± 4.29 μg/mg sample), followed by PPWE (380.54 ± 5.87 μg/mg sample), PSEE (77.93 ± 1.62 μg/mg sample), PSWE (51.58 ± 0.85 μg/mg sample), and PJ (2.55 ± 0.42 μg/mg sample). Similarly, PPEE contained highest flavonoids (37.61 ± 1.44 μg/mg sample), followed by PPWE (26.05 ± 0.93 μg/mg sample), PSEE (16.66 ± 0.47 μg/mg sample), PSWE

(10.55 ± 0.14 μg/mg sample), and PJ (0.24 ± 0.03 μg/mg sample). PPEE had highest antioxidant activities in all methods, followed by PPWE, PSEE and PSWE, while PJ showed lowest antioxidant activities. Moreover, antioxidant activities of the samples were directly related to the total amount of phenolics and flavonoids in PPEs and PSEs. This data indicated that all of the samples exhibited a pivotal role of the antioxidant activities and free radical inhibitors and lipid peroxidative interceptors.

In conclusion, PPEs have more potential than the other parts of the pomegranate extracts. It would be used as a natural antioxidant supplement.

3.2 Introduction

Traditional, medicinal plants contain high levels of antioxidants, which are associated with a vast disease. Several studies reported that eating fruits and vegetables can decrease cellular stress caused by ROS (Liu *et al.*, 2002; Mayers, Watkins, Pritts, and Liu, 2003). This may due to fruits and vegetables contain dietary phytochemicals which include phenolic compounds, such as flavonoids, tocopherols, carotenoids, and ascorbic acids. These phytochemicals act as natural antioxidants by scavenging free radical species or inhibiting the generation of reactive species during cellular metabolism (Ames, Shigenaga, and Hagen, 1993; Heinonen *et al.*, 1998). Numerous studies revealed that phytochemical contents and their antioxidant activities in fruits and vegetables contribute the protective effect against chronic and degenerative diseases (Heinonen *et al.*, 1998; Record *et al.*, 2001).

Pomegranate juice is used to treat dyspepsia, and leprosy. Pomegranate bark and root are active against tapeworms. Extracts of pomegranate bark, leaves, fruits, and fruit rind are known as astringents to halt diarrhea, dysentery, and haemorrhages.

Dried pulverized flower buds are exploited to remedy for bronchitis (Morton, 1987). Recently, chemical content in pomegranate was reviewed that its peel and juice contain hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavonols, and anthocyanins (Artik, 1998; de Pascual-Teresa *et al.*, 2000; Hernandez *et al.*, 1999; Wang *et al.*, 2004). Pomegranate seed oil contains hydroxybenzoic acids, sterols and sex steroids (Wahab *et al.*, 1998; Wang *et al.*, 2004).

Pomegranate juice (PJ) can attribute high antioxidant activities as compared to other fruit juices, such as red wine and green tea (Gil *et al.*, 2000). Lansky and Newman (2007) reported that juice, peel, and seed oil had weak estrogenic property, and that juice and peel possess potent antioxidant properties. It is interesting that pomegranate seed and peel have been used as traditional medicines since antiquity. Previously, it was found that pomegranate peel had the highest antioxidant activity among different parts of 28 commonly consumed fruits in China (Guo *et al.*, 2003; Li *et al.*, 2006). Pomegranate peel extracted with different solvents, such as ethyl acetate, acetone, methanol and water were compared for the antioxidant activity, which was found that the methanol extract had the highest antioxidant capacity (Negi *et al.*, 2003). Moreover, pomegranate seed oil also showed strong antioxidant activity close to that of 3-*tert*-butyl-4-hydroxyanisole (BHA) and green tea (Schubert *et al.*, 1999).

The objectives of this study were to evaluate phytochemical major groups in pomegranate extracts and juice as followings. (1) Measuring total phenolic compounds and flavonoids content. (2) Determining antioxidant activities by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power (FRAP), and ferric thiocyanate (FTC) assays. (3) Analyzing correlations

between total phenolic compounds, flavonoids content, and antioxidant activities of the extracts.

3.3 Materials and Methods

3.3.1 Materials

Folin-Ciocalteau reagent and gallic acid were obtained from Fluka, Switzerland. Catechin (CA), epigallocatechin-3-gallate (EGCG), ascorbic acid (AA), 3-*tert*-butyl-4-hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and linoleic acid were purchased from Sigma-Aldrich, USA. All other chemicals and solvents were analytical grade and purchased from Sigma-Aldrich, USA.

3.3.2 Preparation for plant extracts

Pomegranate fruits were collected from a farm in Pak Chong district, Thailand, in December 2009. Pomegranate (*Punica granatum* L.) sample was identified and assigned Herbarium No. 080252, and kept in the Royal Forest Department, Bangkok, Thailand. The fruits were cleaned, separated for peel, seed and juice. The peel and the seed were dried in hot air oven, grinded into powder, then passed through a 40 mesh sieve and stored in a -20°C for further use. The powders were extracted with a Soxhlet extractor using 70% ethanol and water. The extract was filtered through Whatman No.1. The filtrate was evaporated, lyophilized and stored at -80°C. The extracts were dissolved in their original for uses in all experiments.

3.3.3 Total phenolic compounds measurement

Total phenolic compounds (TPC) were measured by Folin-Ciocalteu method (Singleton, Orthofer, and Lameula-Raventos, 1999). One hundred microliters of sample was mixed with 2 ml of 2% aqueous sodium carbonate solution containing 100 µl Folin-Ciocalteu reagent, and incubated for 30 min. The optical absorbance was read at 760 nm. Gallic acid was used as a standard. TPC content was expressed as micrograms of gallic acid equivalents (GAE) per milligrams of the sample (µg GAE/mg of sample), obtained from the following formula:

$$Absorbance = (0.0046 \times GA) + 0.0885, \quad R^2 = 0.9964$$

3.3.4 Flavonoids content measurement

Flavonoids content (FC) was quantified using a method described by Jia, Tang, and Wu (1999) and Liu *et al.* (2002). Two hundred and fifty microliters of sample were mixed with 1.25 ml of distilled water and 75 µl of 5% aqueous sodium nitrogen dioxide solution for 6 min. One hundred and fifty microliters of 10% aluminium chloride was added and allowed to stand for 5 min and followed by adding of 0.5 ml of 1M sodium hydroxide. Distilled water was added to adjust the total volume to 2.5 ml. The optical absorbance was read at 510 nm. Catechin was used as the standard. FC was expressed as micrograms of catechin equivalents (CAE) per milligrams of the sample (µg CAE/mg of sample), obtained from the following equation:

$$Absorbance = (0.0039 \times CA) + 0.0388, \quad R^2 = 0.9999$$

3.3.5 Free radical scavenging assay

Free radical scavenging activity was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) inhibition method described by Sanchez-Moreno, Plaza, De Ancos, and Cano (2003). Fifty microliters of sample were mixed with 1.95 ml of DPPH reagent. The mixture was vigorously shaken and allowed to stand in the dark for 45 min. The absorbance was measured at 515 nm. Ascorbic acid (AA), catechin (CA), and epigallocatechin-3-gallate (EGCG) were served as positive control. Radical scavenging activity was calculated using the following formula.

$$\textit{Radical scavenging activity}\,(\%) \quad = \quad \left[1-\frac{(A_1-A_2)}{A_0}\right]\times 100$$

A_0 was the absorbance of control (DPPH radical solution without the extract), A_1 was the absorbance of DPPH radical solution in presence of the treatment group, and A_2 was the absorbance without DPPH radical solution.

3.3.6 Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) was performed as described by Benzie and Strain (1996). Briefly, FRAP reagent containing 5 ml of 10 mM tripyridyltriazine in 40 mM hydrochloric, 5 ml of 20 mM iron (III) chloride and 50 ml of 0.1 M acetate buffer, pH 3.6 was prepared freshly and warmed at 37°C. One hundred microliters of sample were mixed with 2.9 ml of FRAP reagent at 37°C for 30 min. The absorbance was measured at 593 nm. Ascorbic acid (AA) was used as a standard. The percentage of reducing power was presented as AA equivalents using a calibration curve between the absorbance and the percentage of the reducing power ability of AA:

$$OD = [0.0091 \times (percent)] + 0.2679, \quad R^2 = 0.9995$$

The result was expressed as micrograms of AA equivalents per micrograms of sample. CA and EGCG were served as positive controls.

3.3.7 Ferric thiocyanate assay

Ferric thiocyanate (FTC) assay was conducted as described by Huang, Chen, Hou, Lin, and Lin (2006). One milliliter of samples, diluted in 99.5% ethanol, were mixed with 1.5 ml of 2.51% linoleic acid, in 99.5% ethanol, 2.5 ml of 0.05 M phosphate buffer, pH 7.0, and kept at 40°C in the dark. To 0.5 ml aliquot of sample, 4.9 ml of 75% ethanol and 50 μl of 30% ammonium thiocyanate were added, and incubated for 3 min. Fifty microliters of 20 mM iron (II) chloride in 3.5% hydrochloric were added and measured the absorbance at 500 nm every 24 h until one day after the absorbance of control reached its maximum. 3-*tert*-butyl-4-hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), CA, and EGCG were used as positive control. Lipid peroxidative inhibition (LPI) was calculated using the following formula.

$$Lipid\ peroxidative\ inhibition\ (\%) = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \times 100$$

A_0 was the absorbance of control, A_1 was the absorbance in the presence of the treatment group, and A_2 was the absorbance without potassium thiocyanate solution.

3.3.8 Statistical analysis

Statistical analysis was performed by ANOVA, using the least significant test to determine the level of significant at $P < 0.01$ and 0.05. For single comparisons, the significance of difference between means was determined by Student's *t*-test. The criterion for statistical significance was $P < 0.05$. All data expressed are mean ± SE of triplicate. Differences in antioxidant activities and correlations between total phytochemical contents of pomegranate samples and their antioxidant activities were tested by regression analysis.

3.4 Results and discussion

3.4.1 Total phenolic and flavonoid contents

Previously, most investigators focused on vitamin C, vitamin E, and β-carotene isolated from fruits as dietary antioxidants. However, the major chemical components act as antioxidants are phenolic compounds and flavonoids (Pignatelli *et al.*, 2000). Phenolic compounds are commonly found in plant kingdom and have been extensively exploited because of their multiple biological activities, including antioxidant property. Since they contain at least one hydroxyl substituted aromatic ring system, they can form chelate complexes with metal ions and are easily oxidized, as well as serving as important units for donating electrons (Rice-Evans *et al.*, 1997). In this study, pomegranate peel extracts (PPEs) and seed extracts (PSEs), and juice (PJ) were quantified for total phenolic compounds (TPC). Gallic acid was used as equivalent standard. The content of TPC was expressed in Table 3.1. Pomegranate peel ethanolic extract (PPEE) had the highest TPC of 451.96 ± 4.29 μg GAE/mg sample, followed by pomegranate peel water extract (PPWE) of 380.54 ± 5.87 μg GAE/mg sample,

pomegranate seed ethanolic extract (PSEE) of 77.93 ± 1.62 μg GAE/mg sample, and pomegranate seed water extract (PSWE) of 51.58 ± 0.85 μg GAE/mg sample. Pomegranate juice (PJ) had the lowest TPC of 2.55 ± 0.42 μg GAE/mg sample. Pomegranate was found as a rich source of dietary antioxidants (Afaq *et al.*, 2005b). Even though, its TPC and flavonoids content (FC) varied in different parts as shown in Table 3.1. PPEs contained higher TPC than PSEs and PJ. These findings are in agreement with Singh *et al.* (2002). Furthermore, the amounts of TPC and FC varied depending on the different types of extraction solvents. The contents were ranged MeOH > EtOAc > water. Aviram *et al.* (2008) reported that PPE was a rich source of total phenolics including hydroxybenzoic acids (gallic acid and ellagic acid), ellagitannins (punicalin, punicalagin, pedunculagin, gallagic, and ellagic acid ester of glucose), and anthocyanidins (delphinidin, cyaniding, and pelargonidin), while PSE had no phenolics. Schubert *et al.* (1999) and Wahab *et al.* (1998) found that PSE contained conjugated fatty acids (punicic acid), non-conjugated fatty acids (linoleic, oleic, palmitic, and stearic acids), sterols (camesterol, stigmasterol, β-sitosterol, and cholesterol), and sex steroids (estrone, testosterone, and estriol). PJ contained ellagitannins (punicalagin and punicalin) with small amounts of ellagic acid and anthocyanins (delphinidin, cyaniding, and pelargonidin) (Aviram *et al.*, 2008). This study also confirmed that PPEs contained higher TPC than PSEs and PJ.

Flavonoids, one of the most diverse and widespread groups of natural occuring compounds, are probably the most important natural phenolics (Miliauskas, Venskutonis, and Beek, 2004). These compounds possess a board spectrum of chemical and biological activities, including radical scavenging activity. Flavonoids content (FC) of PPEs and PSEs, and PJ were expressed as catechin equivalent (CAE)

and demonstrated in Table 3.1. PPEE contained the highest FC (37.61 ± 1.44 μg CAE/mg sample), followed by PPWE (26.05 ± 0.93 μg CAE/mg sample), PSEE (16.66 ± 0.47 μg CAE/mg sample), PSWE (10.55 ± 0.14 μg CAE/mg sample), and PJ (0.24 ± 0.03 μg CAE/mg sample). Lansky and Newman (2007) reviewed that the pomegranate peel was rich in many phenolic compounds and flavonoids than seed and juice. Aviram *et al.* (2008) identified flavonoids from PPE as anthocyanidins (delphinidin, cyanidin and pelargonidin), while Aslam, Lansky, and Varani (2006) reported that three major flavonoids found in PPE were flavanone (naringenin), flavone (luteolin), and flavonols (kaempferol and quercetin). Furthermore, de Pascual-Teresa *et al.* (2000) found that PPE contained flavan-3-ols (flavan-3-ol, catechin, epicatechin, and epigallocatechin 3-gallate). This experiment was in agreement with the others that flavonoids in PPEs were higher than PSEs and PJ. Ethanol was the solvent that could induce higher flavonoids than that water.

Table 3.1 Total phenolic compounds and flavonoids content of pomegranate peel and seed extracts, and juice.

Extract	Total Phenolics (μg GAE/mg)	Flavonoids (μg CAE/mg)
PPEE	451.96 ± 4.29^{a}	$37.61 \pm 1.44^{a,a}$
PPWE	380.54 ± 5.87^{a}	$26.05 \pm 0.93^{b,b}$
PSEE	77.93 ± 1.62^{b}	$16.66 \pm 0.47^{c,a}$
PSWE	51.58 ± 0.85^{b}	$10.55 \pm 0.14^{d,b}$
PJ	2.55 ± 0.42^{c}	0.24 ± 0.03^{e}

PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract; PSEE, pomegranate seed ethanolic extract; PSWE, pomegranate seed water extract; PJ, pomegranate juice; GAE, gallic acid equivalents; CAE, catechin equivalents. Numbers with different letters within the same column are significantly different ($P < 0.05$).

3.4.2 Free radical scavenging activity

"Antioxidant capacity" means the capacity of antioxidant compounds to scavenge free radicals or the capacity of antioxidative action to inhibit oxidative modification of lipids, proteins, and DNA. Three methods, DPPH, FRAP, and FTC used to evaluate the radical scavenging ability of PE. In DPPH method, hydrogen donating antioxidant dues to the formation of the non-radical form is measured and utilized to preliminarily screen for antioxidant activity of PPEs, PSEs, and PJ. The results expressed as IC_{50} are showed in Table 3.2. The low IC_{50} value the higher DPPH radical scavenging activity. PPEE had the highest activity (121.65 ± 2.66 μg/ml) followed by PPWE (151.78 ± 2.70 μg/ml), PSEE (1,324.35 ± 16.89 μg/ml), and PSWE (2,577.53 ± 44.06 μg/ml), while PJ had the lowest radical scavenging potential (9,925.20 ± 1,116.80 μg/ml).

PPEs, PSEs, and PJ showed the potent proton-donating ability on DPPH° to produce DPPHH, which is an important mechanism of antioxidants. The greater the radical scavenging activity of phenolics and flavonoids were associated largely by number of hydroxyl groups on the aromatic ring (Wang *et al.*, 1998; Wang *et al.*, 2003). Various peel and seed extracts and fruit juice contained phenolic compounds such as gallic acid, ellagic acid, and punicalagin, and flavonoids content such as catechin, epicatechin, and quercetin were directly related to antioxidant activities (Gil *et al.*, 2000; Lansky and Newman, 2007). Singh *et al.* (2002) and Negi *et al.* (2003) demonstrated that the higher TPC and FC in pomegranate peel and seed extracts showed the higher antioxidant activity. Pomegranate peel methanolic extract was reported to give a higher antioxidant activity as compared to seed extract (Ricci, Giamperi, Bucchini, and Fraternale, 2006; Singh *et al.*, 2002). Flavonols (kaempferol

and quercetin), flavan-3-ols (catechin, epicatechin, and epigallocatechin 3-gallate), and ellagitannins (punicalin and punicalagin) containing more hydroxyl groups, had very high ability to scavenge DPPH° radicals. Especially, the galloyl group (catechol structure) could considerably strengthen the activity (Cai, Sun, Xing, Luo, and Corke, 2006).

This study was also confirmed by the relationships between TPC and FC (Table 3.1) and IC_{50} values of free radical scavenging activity (Table 3.2). The relationships were in direct correlation (Figures 3.1 (A) and (B)).

3.4.3 Ferric reducing antioxidant power (FRAP)

The reduction of a ferric-tripyridyltriazine complex to ferrous referred to the reducing power of the antioxidants of PPEs, PSEs, and PJ. This method was selected because it demonstrated the amount in a redox-linked colorimetric reaction and value of the samples which reflected the reducing power of the antioxidants. The results were expressed as IC_{50}, and summarized in Table 3.3. PPEE exhibited highest activity (49.07 ± 1.53 μg/ml) followed by PPWE (64.63 ± 1.23 μg/ml), PSEE (512.54 ± 15.05 μg/ml), and PSWE (753.17 ± 17.66 μg/ml). PJ had lowest radical scavenging potential (4,615.94 ± 28.90 μg/ml).

FRAP capacity of the samples directly correlated between radical scavenging activity and reducing power. The presence of reductants in extracts associated in the reduction of iron (III) to iron (II). Fruits contains high antioxidant activity generally contains more phenolic acids and flavonoids in nature (Guo, Cao, Sofic, and Prior, 1997). The reducing power of PPEs, PSEs, and PJ could be due to hydroxyl (-OH) groups in the structures of the phytochemicals, which donate hydrogen and break the

Table 3.2 Free radical scavenging activity of pomegranate extracts and juice assayed by DPPH.

Extract	Concentration (μg/ml)	Activity (%)	IC_{50} (μg/ml)
PPEE	10	5.06 ± 0.83	
	25	11.75 ± 0.71	
	50	24.48 ± 0.81	
	100	45.66 ± 1.63	
	200	77.88 ± 1.13	121.65 ± 2.66[a]
PPWE	10	4.47 ± 0.39	
	25	9.81 ± 0.64	
	50	18.53 ± 0.78	
	100	35.18 ± 0.75	
	200	64.47 ± 1.02	151.78 ± 2.70[a]
PSEE	200	7.64 ± 1.13	
	400	17.61 ± 0.74	
	1,000	40.73 ± 1.46	
	2,000	73.31 ± 1.01	1,324.35 ± 16.89[a]
PSWE	200	5.85 ± 0.59	
	400	10.75 ± 0.70	
	1,000	24.10 ± 0.65	
	2,000	43.60 ± 1.46	
	4,000	73.02 ± 1.09	2,577.53 ± 44.06[b]
PJ	2,000	14.68 ± 1.49	
	4,000	26.32 ± 3.94	
	8,000	49.82 ± 7.38	
	16,000	72.97 ± 4.59	9,925.20 ± 1,116.80[c]
AA	100	46.00 ± 0.79	113.35 ± 1.95
CA	100	49.27 ± 1.60	111.39 ± 0.73
EGCG	100	73.99 ± 0.76	65.17 ± 0.34

DPPH, 2,2-diphenyl-1-picrylhydrazyl scavenging activity; PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract; PSEE, pomegranate seed ethanolic extract; PSWE, pomegranate seed water extract; PJ, pomegranate juice; AA, ascorbic acid; CA, catechin; EGCG, epigallocatechin-3-gallate; IC_{50}, median inhibition concentration. Numbers with different letters within the same column are significantly different ($P < 0.05$).

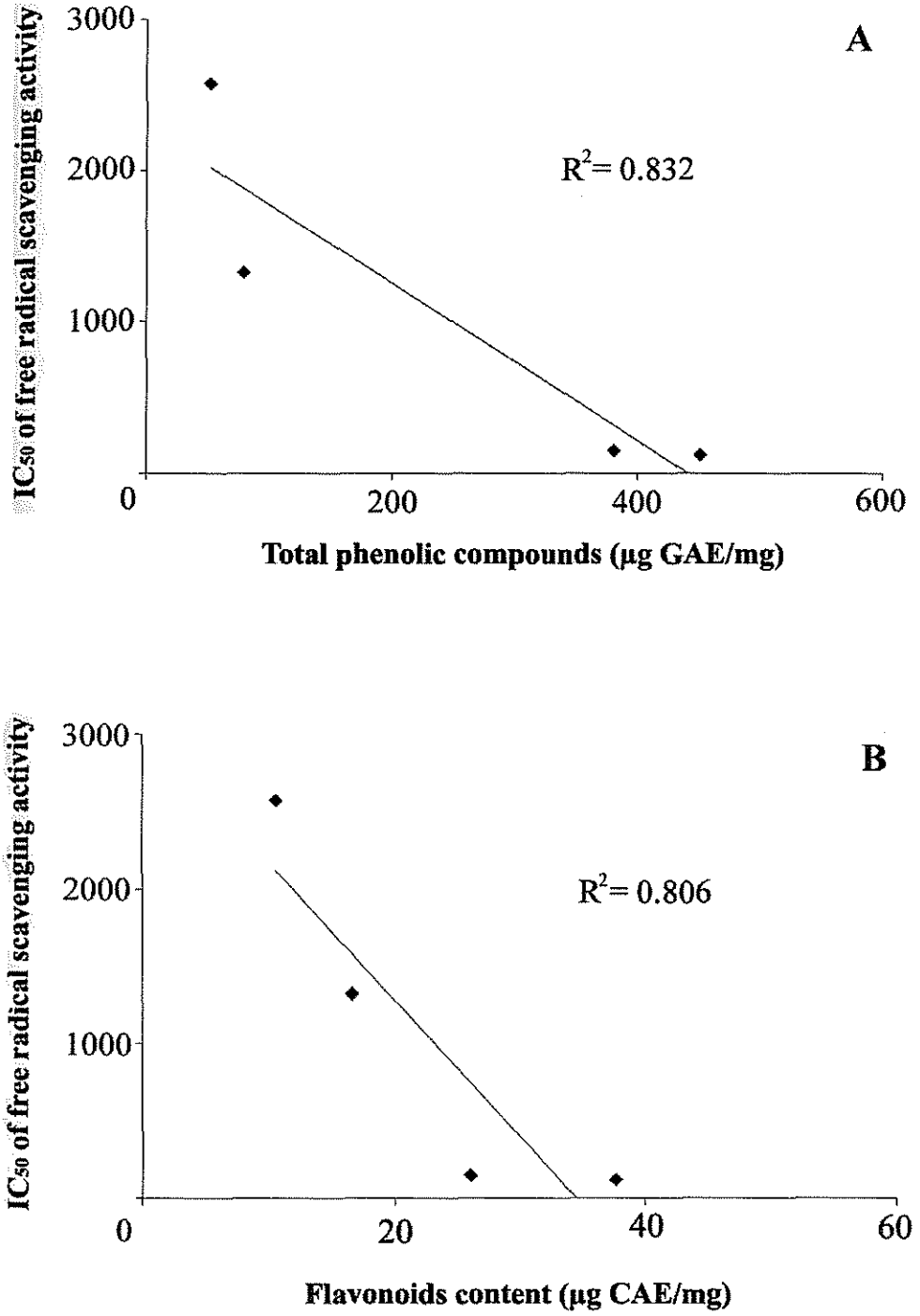

Figure 3.1 Relationship between IC_{50} of free radical scavenging activity, by DPPH assay, and total phenolic compounds (A), and flavonoids content (B) of pomegranate extracts.

free radical chain (Table 3.3). Gil *et al.* (2000) reported that pomegranate fruit squeezed showed stronger reducing power as antioxidants. Singh *et al.* (2002) reported that the extracts of pomegranate peel exhibited higher antioxidant activity *in vitro* as compared to the seed extract. Guo *et al.* (2003) reported that pomegranate peel extract (PPE) had highest FRAP value among all 28 fruit peels and was also stronger than the pulp and seed fractions.

This study demonstrated that reducing power of different parts of pomegranate TPC and FC (Table 3.1). The correlations between IC_{50} value of reducing power and TPC and FC were a directly correlation as in Figures 3.2 (A) and (B).

3.4.4 Ferric thiocyanate (FTC)

Oxidation of unsaturated fatty acid in biological membranes leads to the formation and propagation of lipid radicals, the uptake of oxygen, the rearrangement of the double bonds in unsaturated lipid and the eventual destruction of membrane lipids to breakdown products such as malondialdehyde. Consequently, these effects could be mutagenic and carcinogenic (Miyake and Shibamoto, 1997). Lipid peroxidation has been broadly defined as the oxidative deterioration of polyunsaturated lipids, which could involve in the pathogenesis of various disorders and diseases. Inhibition of lipid peroxidation is one of the important roles for antioxidants. In this experiment, the degree of oxidation due to the formation of alkoxy radicals by the redox reaction with ferrous ions in a linoleic acid emulsion at physiological pH was measured by the thiocyanate method. IC_{50} of lipid peroxidative inhibition (LPI) was shown in Table 3.4. PPEE had highest activity (15.15 ± 2.46 μg/ml) followed by PPWE (20.42 ± 2.87

Table 3.3 Ferric reducing antioxidant power (FRAP) of pomegranate extracts and juice.

Extract	Concentration (μg/ml)	Activity (%)	IC_{50} (μg/ml)
PPEE	25	25.84 ± 0.83	
	50	53.17 ± 1.97	
	100	97.37 ± 0.38	49.07 ± 1.53[a]
PPEW	25	21.72 ± 0.55	
	50	41.41 ± 0.85	
	100	78.86 ± 0.99	64.63 ± 1.23[a]
PSEE	200	21.91 ± 0.78	
	400	43.31 ± 0.90	
	1000	96.99 ± 3.02	512.54 ± 15.05[b]
PSEW	200	14.30 ± 0.16	
	400	29.27 ± 0.29	
	1000	69.32 ± 0.53	753.17 ± 17.66[c]
PJ	2,000	22.06 ± 0.68	
	4,000	44.26 ± 0.57	
	8,000	86.38 ± 0.67	4,615.94 ± 28.90[d]
CA	50	43.53 ± 2.91	58.36 ± 4.02
EGCG	50	75.87 ± 2.62	33.16 ± 1.68

FRAP, ferric reducing antioxidant power; PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract; PSEE, pomegranate seed ethanolic extract; PSWE, pomegranate seed water extract; PJ, pomegranate juice; CA, catechin; EGCG, epigallocatechin-3-gallate; IC_{50}, median inhibition concentration. Numbers with different letters within the same column are significantly different ($P < 0.05$).

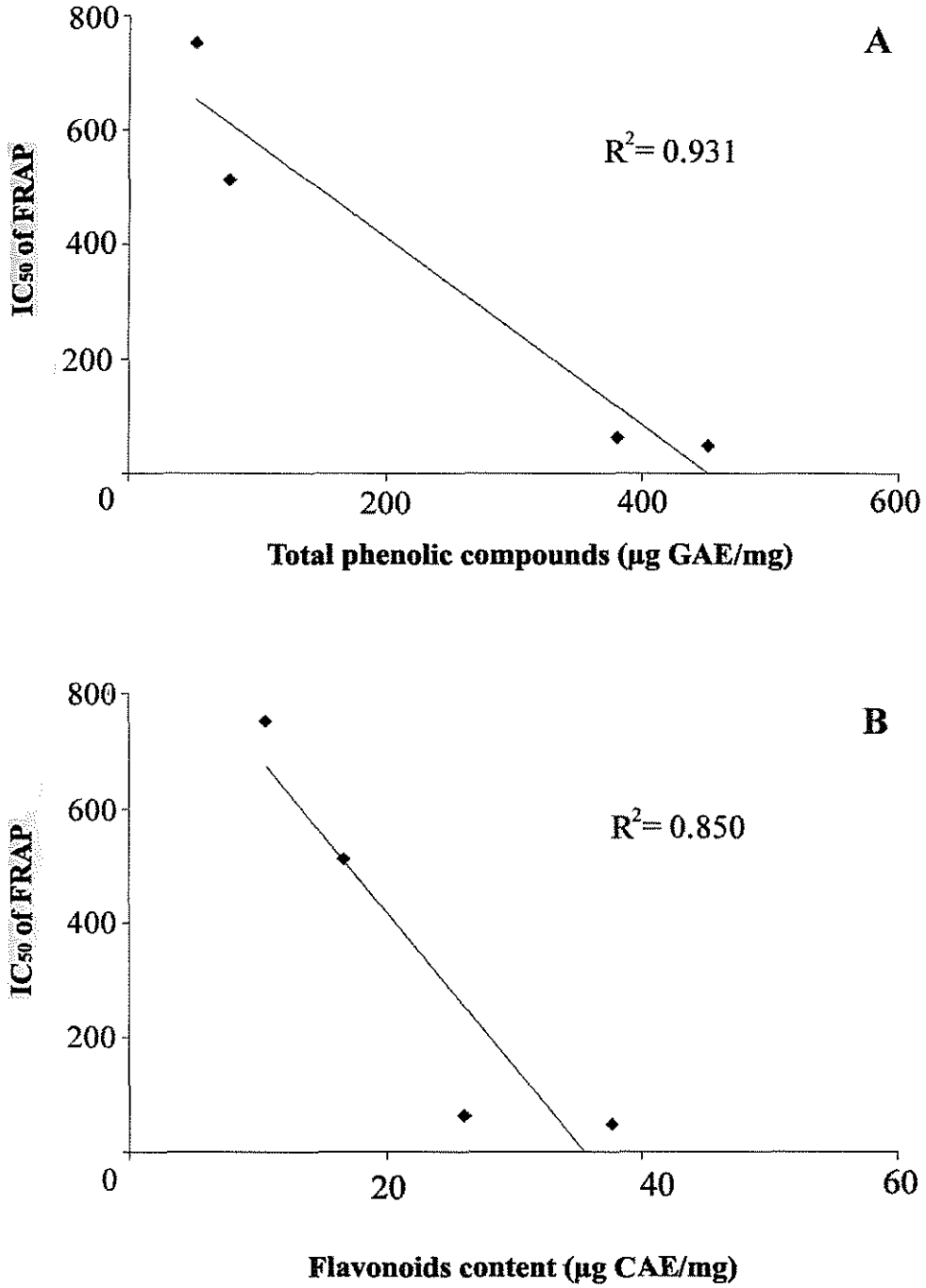

Figure 3.2 Relationship between IC_{50} of ferric reducing antioxidant power (FRAP) and total phenolic compounds (A), and flavonoids content (B) of pomegranate extracts.

μg/ml), PSEE (166.49 ± 20.38 μg/ml), and PSWE (201.82 ± 11.37 μg/ml), while PJ had lowest radical scavenging potential (688.87 ± 0.16 μg/ml).

PPEs, PSEs, and PJ did not only demonstrate excellent ability for radical scavenging activity and reducing power capacity, but also suppressed lipid peroxidation. The FTC expressed the inhibition of lipid peroxidation by transferring hydrogen atom to free radical (Table 3.4), as well as DPPH radical scavenging and FRAP. de Nigris *et al.* (2006); Noda, Kaneyuka, Mori, and Packer (2002) and Singh *et al.* (2002) reported that PPEs, PSEs, PJ, and whole pomegranate fruit (WPF) had ability to suppress lipid peroxidation *in vitro*. The ability to quench hydroxyl radicals seems to directly relate in prevention of propagation of the lipid peroxidation reaction and seems to be a good scavenger of active oxygen species. Thus, it reduces the rate of chain reaction. Moreover, it can be observed that total phenolic compounds and flavonoids contents correlate to their lipid peroxidation inhibition as shown in Figures 3.3 (A) and (B), respectively.

This finding from three antioxidant assays, PPEs possessed high antioxidant activity when compared with commercial antioxidants (Tables 3.2, 3.3, and 3.4). High amounts of TPC and FC associated a large number of hydroxyl groups on the aromatic ring meaning the great activity of radical scavenging (Wang *et al.*, 1998; Wang *et al.*, 2003). The antioxidant activities might directly relate to phenolic compounds and in agreement with this study. Negi *et al.* (2003) and Singh *et al.* (2002) reported that TPC and FC in PPEs assisted a high antioxidant activity.

Table 3.4 Lipid peroxidative inhibition by FTC of pomegranate extracts and juice.

Extract	Concentration (μg/ml)	Activity (%)	IC_{50} (μg/ml)
PPEE	5	7.47 ± 1.99	
	10	40.32 ± 6.02	
	25	57.20 ± 2.68	15.15 ± 2.46[a]
PPEW	5	3.19 ± 1.03	
	10	28.40 ± 3.78	
	25	53.51 ± 3.26	20.42 ± 2.87[a]
PSEE	100	38.32 ± 6.57	
	200	50.93 ± 2.90	
	250	62.44 ± 1.30	166.49 ± 20.38[b,a]
PSEW	100	28.22 ± 4.97	
	200	46.27 ± 2.72	
	250	61.02 ± 2.29	201.82 ± 11.37[b,b]
PJ	100	3.58 ± 0.38	
	250	29.62 ± 4.21	
	1,000	57.55 ± 1.29	688.87 ± 44.03[c]
BHA	10	57.95 ± 1.15	5.97 ± 0.39
BHT	10	59.57 ± 1.43	5.86 ± 0.17
CA	10	58.51 ± 0.19	7.38 ± 0.11
EGCG	10	55.90 ± 0.64	7.41 ± 0.16

LPI, lipid peroxidative inhibition; PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract; PSEE, pomegranate seed ethanolic extract; PSWE, pomegranate seed water extract; PJ, pomegranate juice; BHA; 3-*tert*-butyl-4-hydroxyanisole; BHT, butylated hydroxyl toluene; CA, catechin; EGCG, epigallocatechin-3-gallate; IC_{50}, median inhibition concentration. Numbers with different letters within the same column are significantly different ($P < 0.05$).

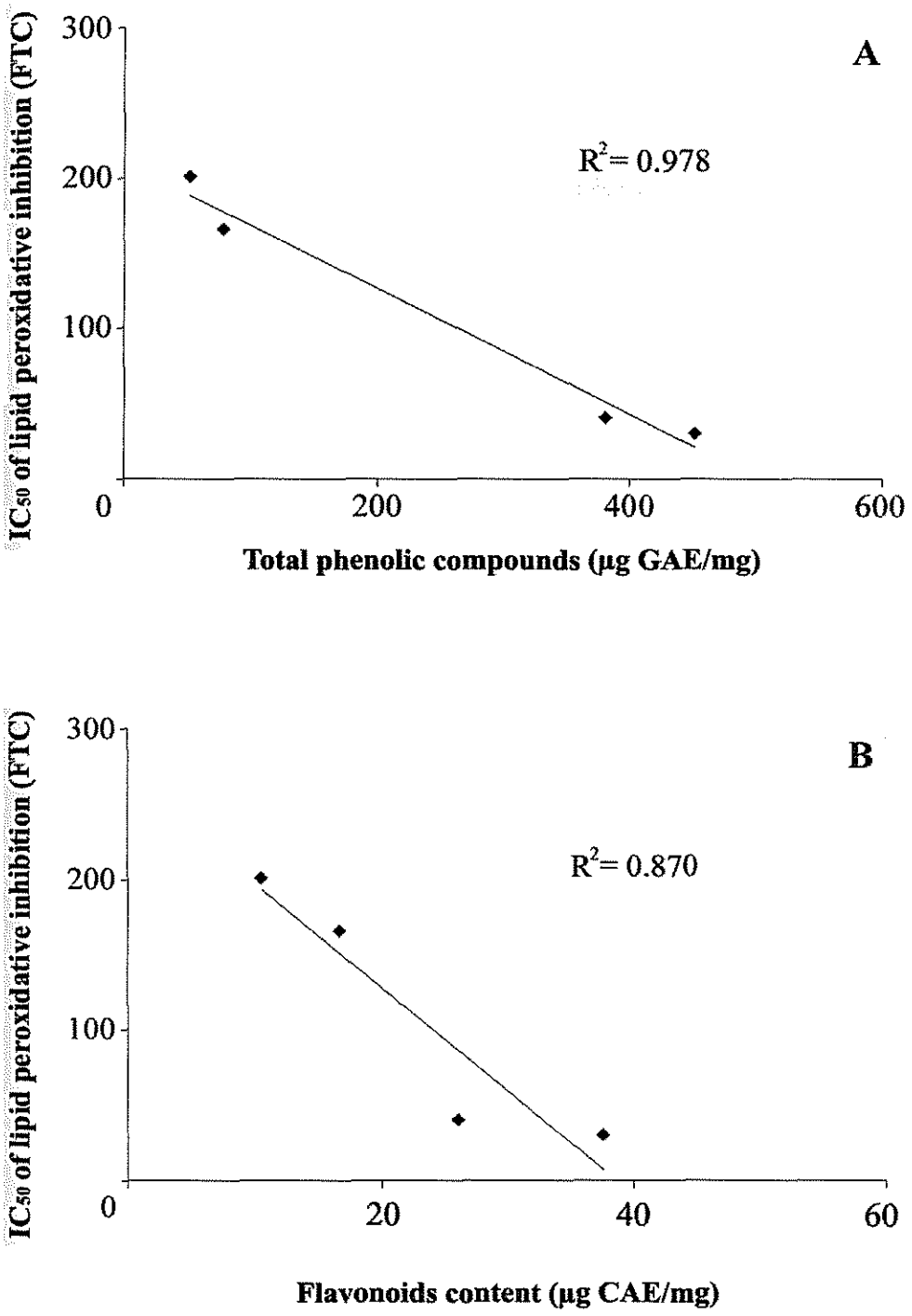

Figure 3.3 Relationship between IC_{50} of lipid peroxidative inhibition assayed by FTC of total phenolic compounds (A), and total flavonoids content (B) of pomegranate extracts.

3.4.5 Relationship between phytochemical contents and IC_{50}

The relationship between TPC (μg GAE/mg sample) and FC (μg CAE/mg sample) and IC_{50} of DPPH, FRAP, and FTC of pomegranate extracts were exhibited in Figures 3.1, 3.2, and 3.3, respectively. They were positive correlations between TPC and DPPH scavenging activity ($R^2 = 0.832$, $p < 0.05$), ferric reducing antioxidant power ($R^2 = 0.931$, $p < 0.05$) and lipid peroxidative inhibition ($R^2 = 0.978$, $p < 0.05$) expressed in Figures 3.1 (A), 3.2 (A), and 3.3 (A), respectively.

Similarly, the positive relationships among FC and DPPH scavenging activity ($R^2 = 0.806$, $p < 0.05$), ferric reducing antioxidant power ($R^2 = 0.850$, $p < 0.05$) and lipid peroxidative inhibition ($R^2 = 0.870$, $p < 0.05$) were obtained, as shown in Figures 3.1 (B), 3.2 (B), and 3.3 (B), respectively.

The overall relationships between the TPC and FC of the pomegranate extracts and the IC_{50} of antioxidant activities (DPPH, FRAP, and LPI) were significant with the R^2 values. These indicate that the TPC and FC significantly contribute to the antioxidant activity of the extracts.

3.5 Conclusion

This research demonstrated PPEs contained higher TPC and FC over than PSEs and PJ. The ethanolic extracts possessed higher phytochemicals than that of water extracts. Base on DPPH, FRAP, and FTC methods, it could be indicated that PPEs were rich in antioxidants. Moreover, antioxidant activities of the pomegranate extracts were directly related to their TPC and FC. These data indicated that all parts of pomegranate fruits were the potential sources of antioxidants.

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CHAPTER IV

CYTOTOXICITY OF POMEGRANATE PEEL AND SEED EXTRACTS, AND JUICE

4.1 Abstract

The toxicity of pomegranate extracts and juice was determined by brine shrimp lethal assay (BSLA). Cytotoxic efficacy was compared by LC_{50}, LC_{95}, and LC_{99} values. The cytotoxicity was correlated with the phytochemical contents and antioxidant activity obtained from chapter III. Toxicological profile, the no-observed-adverse-effect-level (NOAEL), the lowest-observed-adverse-effect-level (LOAEL), the margin of safety (MOS), and the therapeutic index (TI) were estimated.

Pomegranate juice (PJ) had the highest LC_{50} values (6,451.46 ± 52.32 µg/ml), followed by PSEE of 2,375.28 ± 69.54 µg/ml, PPWE 1,743.31 ± 20.17 µg/ml, and PSWE 1,294.88 ± 61.28 µg/ml, while PPEE showed the lowest LC_{50} values of 1,206.98 ± 12.73 µg/ml. LC_{95} and LC_{99} values, PJ had the highest (7,399.81 ± 99.67 and 7,792.74 ± 121.79 µg/ml, respectively), followed by PSEE (4,165.91 ± 100.37 and 4,907.87 ± 147.94 µg/ml, respectively), PSWE (2,693.63 ± 82.26, and 3,272.88 ± 99.48 µg/ml, respectively), and PPWE (2,209.13 ± 44.26 and 2,402.06 ± 54.51 µg/ml, respectively), while PPEE had the lowest LC_{95} and LC_{99} values (2,017 ± 83.15 and 2,352.61 ± 112.75 µg/ml, respectively).

LC_{50} values of extracts did not directly relate to the total phenolic compounds (R^2 = 0.1303, $P > 0.05$) and flavonoids content (R^2 = 0.0836, $P > 0.05$). These

indicated that total phenolic compounds (TPC) and flavonoids content (FC) of all pomegranate extracts were not associated to cytotoxicity.

NOAEL and LOAEL values, PJ had the highest (6,000 and 6,500 µg/ml, respectively), followed by PPWE (1,250 and 1,500 µg/ml, respectively), PSEE (1,000 and 1,500 µg/ml, respectively), and PPEE (750 and 1,000 µg/ml, respectively), while PSWE had the lowest NOAEL and LOAEL values (100 and 500 µg/ml, respectively). MOS and TI values, PPWE had the highest (125 and 11.49, respectively), followed by PPEE (75 and 9.92, respectively), PSEE (5 and 1.79, respectively), and PJ (3 and 0.65, respectively), while PSWE had the lowest MOS and TI values (0.5 and 0.5, respectively).

PPEs, PSEs, and PJ have no cytotoxic activity. The TPC and FC of the extracts had no significant contribution to toxicity PPEs and PSEs. PPEs exhibited the high level of MOS and TI, all of which relate to higher safety dose. This study is a useful tool to predict the pharmacological action and toxicity of pomegranate fruits.

4.2 Introduction

Medicinal plants, called "samunphrai" in Thai, have long been used in traditional healing in addition to western medicine. However, many plants are unknown to be toxic. The reliable bioassays to determine a broad spectrum of pharmacological action and toxicity of medicinal plants are needed.

Brine shrimps play a responsible system to substances similar to mammalian animals such as DNA-dependent RNA polymerase and ouabaine sensitive Na^+ and K^+ dependent ATPase. Brine shrimps can be utilized in a laboratory bioassay to detect cytotoxicity through the estimation of the medium lethal concentration (LC_{50} values)

of toxins and plant extracts (Lewan, Andersson, and Morales-Gomez, 1992; Meyer *et al.*, 1982). Therefore, the convenient general bioassay for active plants constituents favors using brine shrimp cytotoxicity test, which is a rapid, inexpensive and in-house bioassay for screening of physiologically active plant extracts (Fumaral and Garchitorena, 1996; Massele and Nshimo, 1995; Mathews, 1995; Parra, Yhebra, Sardinas, and Buela, 2001; Sanchez-Fortun, Sanz-Barrera, and Barahona-Gomariz, 1995).

The identification of effects in animals that may be predictive of adverse events in humans is the cornerstone of non-clinical safety testing of pharmaceuticals for human therapeutic use. Identification of toxic effects in animals is generally expected to follow a dose-response pattern relative to incidence and severity, allowing the determination of dose levels where substantial, important or relevant effects occur and dose levels where these effects do not occur. The responsibility of the toxicologist is to define, without compromise, where on the dose response curve effects occur that could be considered potentially harmful to humans. This is a simple statement for a complicated and iterative process. The initial opinions on where harmful and non-harmful effects occur may change as additional information related to the molecule is generated. Data from short-term study is an early indicator of a harmful effect (Dorato and Engelhardt, 2005). Dosage levels should be selected to provide information on a dose-response relationship, including a toxic dose, the lowest-observed-adverse-effect-level (LOAEL), the no-observed-adverse-effect-level (NOAEL), the margin of safety (MOS), and the therapeutic index (TI).

In Figures 4.1 and 4.2, the NOAEL is the highest exposure level that there are no statistically or biologically significant increases in the frequency or severity of

adverse effect between exposed and control groups. Some effects may be produced but they are not considered adverse or precursors to adverse effects (Beck *et al.*, 1993; Faustman and Omenn, 2001). IPCS (1999) defined that simple estimate of the highest dose in which the incidence of toxic effect was not significantly different from untreated group (statistically and biologically).

In Figure 4.1, Calabrese and Baldwin (1994) pointed that the LOAEL is a simple estimate of the lowest dose in which the incidence of toxic effect was significantly different from untreated group (statistically or biology).

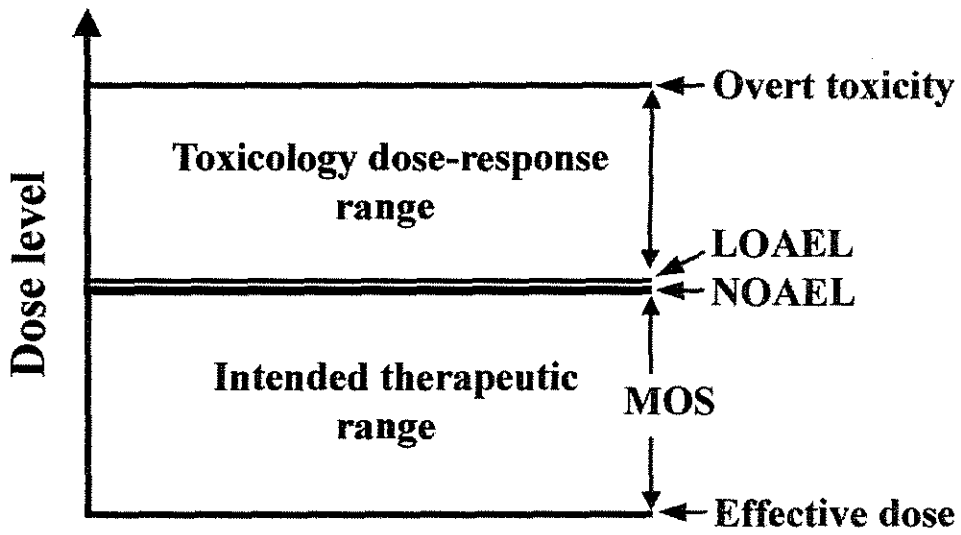

Figure 4.1 Relationship of margin of safety to toxicity profile, showing the lowest-observed-adverse-effect-level (LOAEL), the no-observed-adverse-effect-level (NOAEL), and the margin of safety (MOS) (Dorato and Engelhardt, 2005).

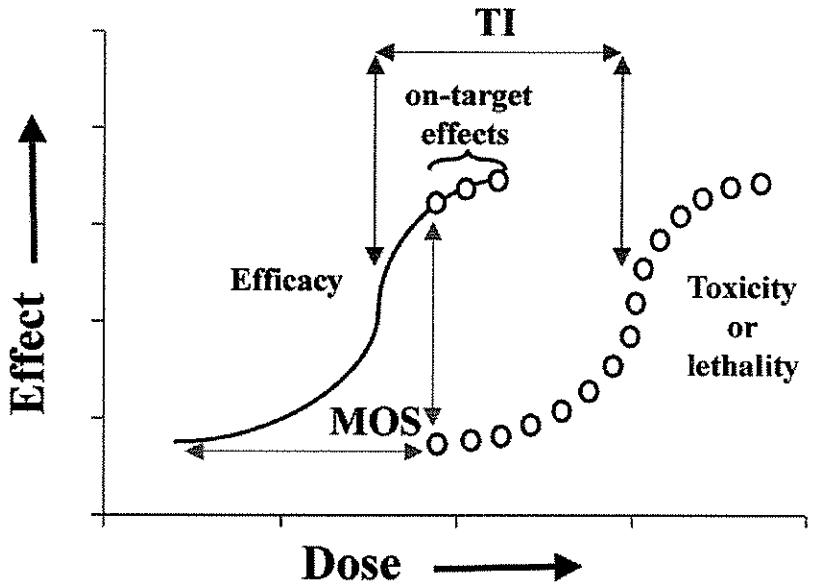

Figure 4.2 Idealized dose-response curves for efficacy and toxicity, showing the relationship of the margin of safety (MOS), the therapeutic index (TI), and the occurrence of high dose on-target effects that may be considered undesirable (Dorato and Engelhardt, 2005).

In general, NOAEL is an important part of the MOS, it is a professional opinion about toxicologically relevant effects and interpretation of expected pharmacology, and it is the subject of iterative interpretation as the toxicology profile develops. The concept of MOS is an important part of the toxicology profile. The rational use of the information is not in the application of the absolute toxicity determination, but in relation to clinical dose/exposure, the clinical indication, available therapy, and overall risk/benefit analysis of the use of a potential new plant extracts (Dorato and Engelhardt, 2005).

Pomeganate has been reported to be used in a vast of disease treatment. Tannins from the pericarp of pomegranate exhibit antiviral activity against the genital herpes virus (Zhang, Zhan, Yao, and Song, 1995). Pomegranate rind extract is also shown to be a potent virucidal agent (Stewart *et al.*, 1998) and has been used as a constituent of antifungal and antiviral preparations (Jassim, 1998). Pomegranate is also used as a part of a fungicidal preparation (Jia and Zia, 1998). Pomegranate peel is reported as a part of a preparation used for treating the infection of male or female sexual organs, mastitis, acne, folliculitis, pile, allergic dermatitis, and scald for curing diarrhea and dysentery (Hu, 1997).

Pomegranate has been widely consumed by people in many different cultures for a long time, largely without untoward incident, and thus is considered generally safe. Squillaci and Di Maggio (1946) reported that consumption of decoction of the tree bark, and to a lesser extent, pericarps of the fruit, may cause severe acute grastic inflammation and even death due to the presence of both tannins and alkaloids. Pomegranate fruit extracts have been shown to cause congestion of internal organs and elevated creatinine *in vivo* (Vidal *et al.*, 2003). Moreover, severe allergic reactions from eating the fruit and esophageal cancer from chronic consumption of roughly ground pomegranate seeds have been reported (Gaig *et al.*, 1999; Ghadirian, 1987; Ghadirian, Ekoe, and Thouez, 1992; Hegde, Mahesh, and Venkatesh, 2002; Igea *et al.*, 1991).

The objectives of this study were to (1) evaluate toxicity of pomegranate extracts, and juice by brine shrimp lethal assay (BSLA); (2) predict correlations between total phenolic compounds and flavonoids content and toxicity of pomegranate extracts, and (3) determine a dose-response relationship between LC_{50} of

toxicity and IC_{50} of free radical scavenging activity, including a toxic dose, the no-observed-adverse-effect-level (NOAEL), the lowest-observed-adverse-effect-level (LOAEL), the margin of safety (MOS), and the therapeutic index (TI) using *in vitro* model.

4.3 Materials and methods

4.3.1 Materials

Pomegranate extracts, juice, and their phytochemicals contents and antioxidant activities were obtained as in Chapter III. Commercial sea salt mixture was obtained from Mariscience Int'I Co., Ltd., Thailand. All other chemicals and solvents were analytical grade and purchased from Sigma-Aldrich, USA.

4.3.2 Brine shrimp

The dried cysts of brine shrimp (*Artimia salina* L.) were incubated in a hatching chamber filled with artificial sea water (Mariscience Int'I Co., Ltd., Thailand). The pH of this water, decreasing of pH during incubation, was adjusted to 9.0 with Na_2CO_3 to shun risk of death to the brine shrimp (Lewan *et al.*, 1992). A plastic divider with several 2 mm holes was clamped in the dish to make two unequal compartments. The eggs (25 mg) were sprunk into the larger compartment, covered with a lid, while the smaller compartment was illuminated. The eggs were incubated at 28°C and allowed nauplii larvae to develop. After 48 hours the larvae were collected.

4.3.3 Brine shrimp lethality assay (BSLA)

Cytotoxicity was performed by brine shrimp lethal assay (BSLA). The procedure for BSLA was modified from the method described by Solis, Wright, Anderson, Gupta, and Phillipson (1993). The various tested concentrations in 800 µl were transferred into 24-well plates, containing 10 brine shrimp larvae, and were filled to 1 ml total volume with artificial sea water. The larvae were not given food to ensure that the mortality observed in bioassay could be attributed to bioactive compounds. The tested 24-well plates were covered and incubated at room temperature. After 24 h, dead (non-motile) and survivor (motile) brine shrimps were counted under a microscope. In any case, hatched brine shrimp nauplii can survive for up to 48 h with out food because they still feed on their yolk-sac (Lewis, 1995). However, in cases where control deaths were detected, the percentage of deaths was calculated as:

$$Deaths\,(\%) = \left[1-\frac{(A_1-A_2)}{A_1}\right]\times 100$$

A_1 was the live control (the medium without the sample) and A_2 was the death in presence of the samples.

4.3.4 Lethal concentration at 50%, 95%, and 99% determinations

The lethal concentration of 50%, 95%, and 99% (LC_{50}, LC_{95}, and LC_{99}) values and 95% confidence intervals were determined from the 24 h counts using the probit analysis method (Finney, 1971). LC_{50}, LC_{95}, and LC_{99} of activity were expressed as micrograms of sample per milliliter (µg/ml) of reaction solution. Six replicates were prepared for each dose level.

4.3.5 Toxicological profile for pharmacological development

Dose-response relationship between LC_{50} of brine shrimp and IC_{50} of DPPH radical scavenging activity, including a toxic dose, the no-observed-adverse-effect-level (NOAEL), the lowest-observed-adverse-effect-level (LOAEL), the margin of safety (MOS), the therapeutic index (TI), and maximal DPPH radical scavenging activity (%), was analyzed as following formula:

$$MOS = \frac{NOAEL}{Effective\ Dose}$$

$$TI = \frac{LC_{50}}{IC_{50}}$$

In Figure 4.4, dose-response data of LC_{50} and IC_{50} were transformed into a straight line by linear regression analysis.

4.3.6 Statistical analysis

Statistical analysis for multiple comparisons was analyzed by one-way ANOVA, using the least significant test to determine the level of significant at $P <$ 0.01 and 0.05. All data were expressed as mean ± SE. The correlations between total phytochemical properties of pomegranate samples and LC_{50} were analyzed by regression analysis. For single comparisons, the significance of difference between means was determined by Student's *t*-test. The criterion for statistical significance was $P < 0.05$.

4.4 Results and discussion

4.4.1 Cytotoxicity

The evaluation of the toxic action of plant extracts is indispensable in order to consider a safe treatment. This literates show the definition of intrinsic toxicity of plants and the effects of acute overdoses. Brine shrimps are sensitive to a variety of substances, the brine shrimp lethal assay can be useful as a quick and simple test for predicting the toxicity of plant extracts and guiding their phytochemical fractionations (Caceres, 1996). Parra *et al.* (2001) reported that LC_{50} value, tested in brine shrimp, and LD_{50} value, tested in mice, were correlated with good correlations between both methods with a correlation coefficient of $R^2 = 0.85$ ($P < 0.05$).

In this study, after testing the different concentrations of the samples of pomegranate fruit products with brine shrimps, the survivors were counted, and percentage of deaths at each dose were calculated. Increase of death is proportional to increase of concentration. The calculated linearity in the dose-effect relationship of every sample and the determination of LC_{50}, LC_{95}, and LC_{99} values as well as corresponding 95% confidence intervals (95% CI), are exhibited in Table 4.1.

PJ had the highest LC_{50} values (6,451.46 ± 52.32 μg/ml), followed by PSEE (2,375.28 ± 69.54 μg/ml), PPWE (1,743.31 ± 20.17 μg/ml), and PSWE (1,294.88 ± 61.28 μg/ml), while PPEE showed the lowest LC_{50} values (1,206.98 ± 12.73 μg/ml) (Table 4.1). LC_{95} and LC_{99} values, PJ had the highest (7,399.81 ± 99.67 and 7,792.74 ± 121.79 μg/ml, respectively), followed by PSEE (4,165.91 ± 100.37 and 4,907.87 ± 147.94 μg/ml, respectively), PSWE (2,693.63 ± 82.26, and 3,272.88 ± 99.48 μg/ml, respectively), and PPWE (2,209.13 ± 44.26 and 2,402.06 ± 54.51 μg/ml, respectively),

Table 4.1 Cytotoxicity of pomegranate extracts and juice.

Sample	Concentration (μg/ml)	Death (%) (24 h)	LC_{50} (μg/ml)	LC_{95} (μg/ml)	LC_{99} (μg/ml)
PPEE	500	2.50 ± 1.02			
	750	5.00 ± 1.02			
	1,000	35.63 ± 2.13			
	1,250	52.50 ± 2.28			
	1,500	73.13 ± 2.13			
	1,750	82.50 ± 2.28	1,206.98 ± 12.73[a]	2,017.07 ± 83.15[a]	2,352.61 ± 112.75[a]
	2,000	95.00 ± 3.54	(1,166.46-1,247.50)	(1,752.45-2,281.68)	(1,993.78-2,711.43)
PPWE	500	1.88 ± 1.20			
	750	3.13 ± 0.63			
	1,000	3.75 ± 0.72			
	1,250	5.63 ± 1.20			
	1,500	18.13 ± 1.20			
	1,750	51.25 ± 1.61	1,743.31 ± 20.17[b]	2,209.13 ± 44.26[a]	2,402.06 ± 54.51[a]
	2,000	81.88 ± 4.83	(1,679.14-1,807.49)	(2,068.28-2,349.97)	(2,228.61-2,575.52)
PSEE	500	5.00 ± 1.77			
	1,000	13.13 ± 4.93			
	1,500	17.50 ± 1.02			
	2,000	38.75 ± 5.15			
	2,500	57.50 ± 2.28			
	3,000	72.50 ± 2.04			
	3,500	85.00 ± 1.02	2,375.28 ± 69.54[c]	4,165.91 ± 100.37[c]	4,907.87 ± 147.94[c]
	4,000	90.63 ± 5.63	(2,153.97-2,596.59)	(3,846.50-4,485.31)	(4,437.05-5,378.68)

PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract; PSEE, pomegranate seed etanolic extract; PSWE, pomegranate seed water extract; PJ, pomegranate juice; LC_{50}, LC_{95}, and LC_{99}, lethal concentrations of 50%, 95%, and 99%. Numbers with different letters within the same column are significantly different ($P < 0.01$).

Table 4.1 (Continued).

Sample	Concentration (μg/ml)	Death (%) (24 h)	LC_{50} (μg/ml)	LC_{95} (μg/ml)	LC_{99} (μg/ml)
PSWE	100	1.88 ± 0.63			
	500	18.13 ± 3.29			
	1,000	45.00 ± 5.10			
	1,500	68.75 ± 1.61			
	2,000	76.25 ± 2.60			
	2,500	86.88 ± 2.13			
	3,000	90.00 ± 1.77			
	3,500	93.13 ± 1.20	1,294.88 ± 61.28[a]	2,693.63 ± 82.26[b]	3,272.88 ± 99.48[b]
	4,000	96.88 ± 1.02	(1,099.86-1,489.89)	(2,431.85-2,955.40)	(2,956.28-3,589.47)
PJ	5,000	2.50 ± 1.02			
	5,500	5.00 ± 1.02			
	6,000	12.50 ± 2.28			
	6,500	42.50 ± 2.28			
	7,000	91.25 ± 3.89	6,451.46 ± 52.32[d]	7,399.81 ± 99.67[d]	7,792.74 ± 121.79[d]
	7,500	96.25 ± 3.89	(6,284.96-6,617.97)	(7,082.62-7,717.01)	(7,405.15-8,180.33)
Vehicle control (0.001% DMSO)		1.25 ± 0.72			

PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract; PSEE, pomegranate seed etanolic extract; PSWE, pomegranate seed water extract; PJ, pomegranate juice; LC_{50}, LC_{95}, and LC_{99}, lethal concentrations of 50%, 95%, and 99%. Numbers with different letters within the same column are significantly different ($P < 0.01$).

while PPEE had the lowest LC_{95} and LC_{99} values of 2,017 ± 83.15 and 2,352.61 ± 112.75 μg/ml, respectively.

Brine shrimp lethal assay (BSLA) is a quick, simple, practical, and low-cost method, and allows a great number of samples to be tested (Ohno *et al.*, 1997). Furthermore, it can be useful for the screening of plant extracts in order to predict their toxicity. In toxicity evaluation of plant extracts by BSLA, the LC_{50} value (LC_{50} > 1,000 μg/ml) is not considered to display toxicity (Meyer *et al.*, 1982). In these study, all the tested samples have LC_{50} value > 1,000 μg/ml; therefore, they have no cytotoxic activity. From preliminary results on BSLA, it is possible that PPEs, PSEs, and PJ contained phytochemicals (Chapter III) that have no cytotoxic activity. Meerts *et al.* (2009) found that pomegranate seed oil (PSO) was neither mutagenic nor clastogenic *in vitro*. In oral acute toxicity study, no abnormalities were observed at macroscopic post mortem examination of the animals, and no effects on body weight were observed at a concentration of 2,000 mg/kg body weight. Moreover, in the 28-day dietary toxicity study no effects were observed at 10,000 and 50,000 ppm PSO.

4.4.2 Relationship between phytochemical contents and LC_{50}

It is well known that traditional medicine plants, consisting of phytochemicals, exhibits high levels of antioxidants. In Chapter III, the overall relationship between the total phenolic compounds (TPC) and flavonoids content (FC) of pomegranate extracts and their IC_{50} of antioxidant activities (DPPH, FRAP, and LPI) was statistically significant with the R^2 values. These indicate that TPC and FC significantly contribute to the antioxidant activity of the extracts. Pomegranate phytochemicals could be used in a number of ailments for the treatment (Fengchun,

Liu, and Chen, 1997; Hu, 1997; Singh, Murthy, and Jayaprakasha, 2002). In spite of their beneficially, medicinal uses, many plants are known to be toxic. Therefore, this study to reveals that phytochemicals from pomegranate extracts (Table 3.1) were related to the cytotoxicity effects on BSLA (Table 4.1).

The relationships between TPC (μg GAE/mg sample) and FC (μg CAE/mg sample) (Table 3.1) and the corresponding LC_{50} of pomegranate extracts (Table 4.1) were analyzed. There was no obvious relationship between TPC and LC_{50} values of cytotoxicity ($R^2 = 0.1303$, $P > 0.05$) expressed in Figure 4.3 (A). Similarly, FC did not correlate with LC_{50} value of cytotoxicity ($R^2 = 0.0836$, $P > 0.05$) displayed in Figure 4.3 (B).

The relationship between the TPC and FC of pomegranate extracts and the LC_{50} values of cytotoxicity were not statistically significant with the R^2 values. These indicate that the TPC and FC had no significant contribution to the toxicity of PPEs and PSEs.

4.4.3 Toxicological profile for pharmacological development

The highest dose that is without observed effects in properly designed and executed toxicology studies to determine NOAEL. A high NOAEL renders to a higher safety dose (Table 4.2 and Figure 4.4). These results showed that PJ had the highest NOAEL of 6,000 μg/ml followed by PPWE of 1,250 μg/ml, PSEE of 1,000 μg/ml, and PPEE of 750 μg/ml, while PSWE had the lowest NOAEL of 100 μg/ml.

The lowest dose that is statistically different from control expressed as LOAEL. A high LOAEL renders to higher dose related to adverse effect (Table 4.2 and Figure 4.4). These results showed that PJ had the highest LOAEL of 6,500 μg/ml followed by

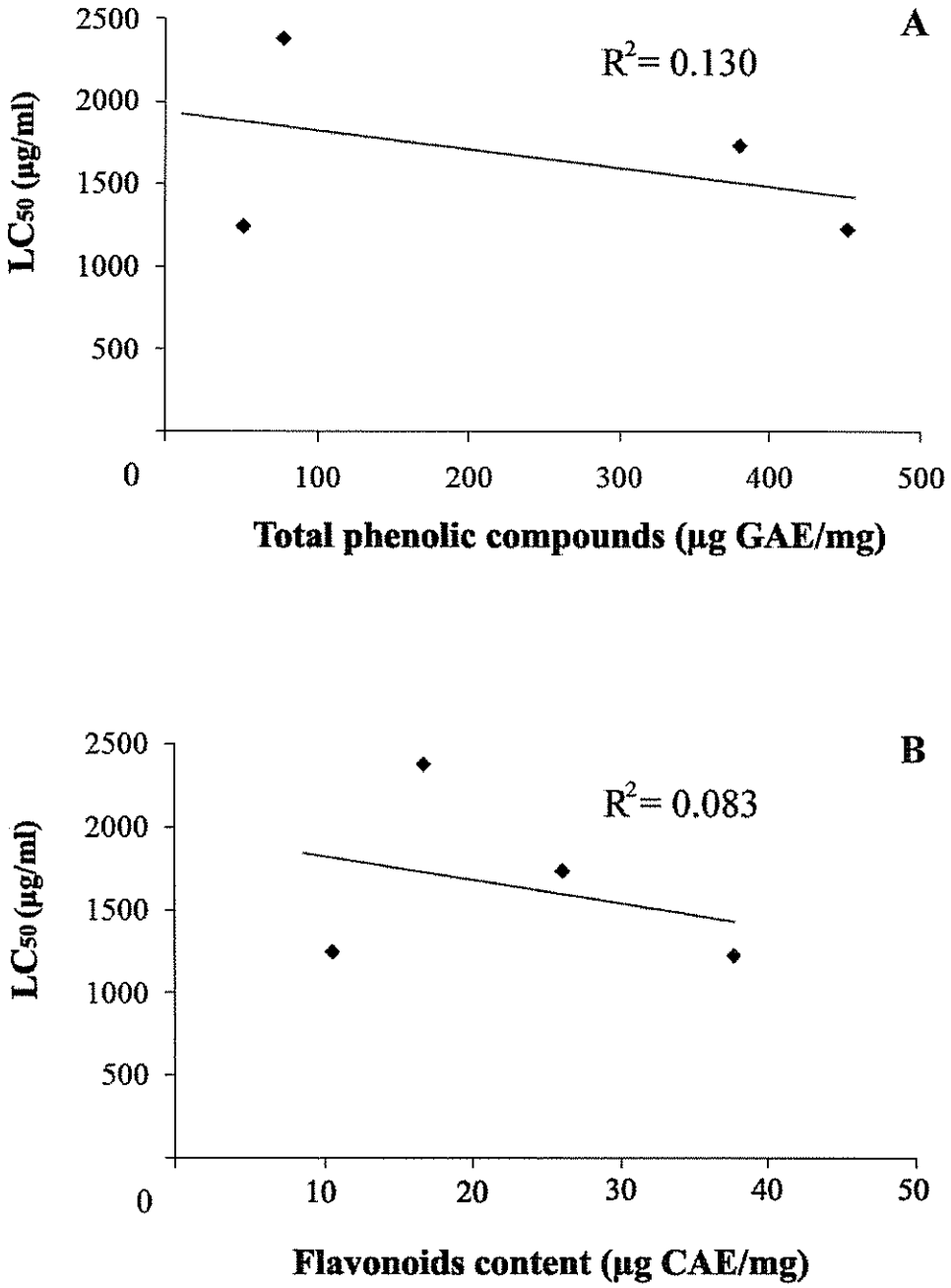

Figure 4.3 Relationships between LC_{50} (%) and total phenolic compounds (A) and flavonoids content (B) of pomegranate extracts.

PPWE of 1,500 μg/ml, PSEE of 1,500 μg/ml, and PPEE of 1,000 μg/ml, while PSWE had the lowest LOAEL of 500 μg/ml.

A safe dose of new drug was measured by calculation for MOS for without adverse effect (Table 4.2). A high MOS associates to higher agent tended to be safe. These results showed that PPWE had the highest MOS (125) followed by PPEE (75), PSEE (5), and PJ (3), while PSWE had the lowest MOS (0.5). TI demonstrated the efficacy of agent utilized for treatment (Table 4.2). A high TI relates to higher agent induced to safety. These results expressed that PPWE had the highest TI (11.40) followed by PPEE (10.07), PSEE (1.80), and PJ (0.60), while PSWE had the lowest TI (0.48).

4.5 Conclusion

Pomegranate peel and seed extracts, and juice possess LC_{50} values higher than 1,000 μg/ml showed no cytotoxic activity. The relationship between the total phenolic compounds and flavonoids content of the extracts and the LC_{50} values of cytotoxicity were not statistically significant with R^2 values. PPEs exhibited high level of MOS and TI, all of which related to higher safety dose of PPEs. Furthermore, in non-clinical studies, PPEs have the property of potential new drugs.

Table 4.2 Toxicological profile for pharmacological development of pomegranate extracts and juice.

Sample	NOAEL (μg/ml)	LOAEL (μg/ml)	MOS	TI
PPEE	750	1,000	75	9.92
PPWE	1,250	1,500	125	11.49
PSEE	1,000	1,500	5	1.79
PSWE	100	500	0.5	0.50
PJ	6,000	6,500	3	0.65

PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract; PSEE, pomegranate seed etanolic extract; PSWE, pomegranate seed water extract; PJ, pomegranate juice; NOAEL, no observed adverse effect level; LOAEL, lowest observed adverse effect level; MOS, margin of safety; TI, therapeutic index.

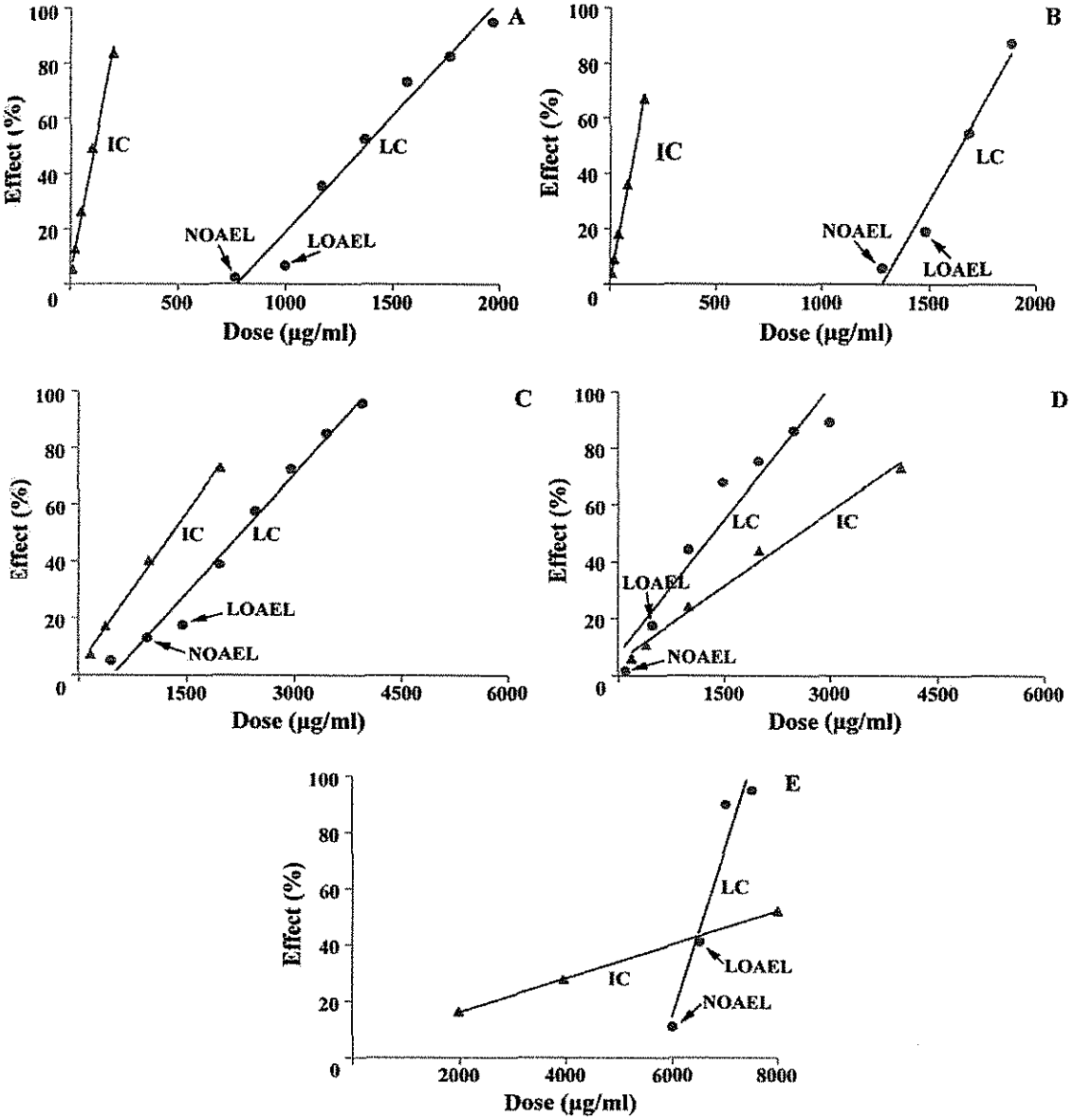

Figure 4.4 Idealized dose-response curves for IC_{50} of free radical scavenging activity and LC_{50} of cytotoxicity of: (A) PPEE; (B) PPWE; (C) PSEE; (D) PSWE, and (E) PJ.

4.6 References

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CHAPTER V

PROTECTIVE EFFICACY OF POMEGRANATE PEEL EXTRACTS AGAINST UVB IRRADIATION ON RAT SKIN

5.1 Abstract

Exposure to UV radiation causes acute and chronic skin damage. Pomegranate peel extract was rich in antioxidants. The objective of this study was to investigate the protective efficacy of pomegranate peel extracts (PPEs) on rat skin against UVB irradiation. Pomegranate peel ethanolic extract (PPEE) and water extract (PPWE) were topically applied on hairless rat skin before 3 minimal erythema dose (MED) twice a week for one month. Erythema, inflammatory infiltration, epidermal thickness, sunburn cells, and DNA fragmentation were observed. Propylene glycol was the vehicle control. PPEs remarkably lessened the UVB-induced lesions on the skin. Topical pretreatments of PPEE and PPWE at 8 mg/cm^2 were able to protect the skin against 3xMED UVB-induced erythema, inflammatory infiltration, epidermal thickness, sunburn cells, and DNA fragmentation. The erythema was reduced (2.5 and 1.8 fold, respectively) and the inflammatory infiltration was decreased (4.7 and 1.9, respectively) of the vehicles. The epidermal thickness was reduced (1.7 and 1.6 fold, respectively) and the sunburn cells was decreased (7.4 and 7.2 fold, respectively) of the vehicles. DNA fragmentation was very slightly occurred. These findings indicate that topical PPEs prior to UVB irradiation

is very effective in prevention the skin lesions and DNA damages. This also makes use of and was value added to the pomegranate peel.

5.2 Introduction

Overexposure to UV radiation is a great concern of inducing sunburn formation and skin cancer. UVA (320-400 nm) and UVB (290-320 nm) cause oxidative damages to skin cells (Halliday, 2005; Matsumura and Ananthaswamy, 2004). UVA induces a variety of reactive oxygen species (ROS) inducing DNA, lipid, and protein damages. UVB is responsible for erythema, inflammation, DNA damage, and skin cancer (Griffiths, Mistry, Herbert, and Lunec, 1997; Pourzand and Tyrrell, 1999). ROS can be generated by UV radiation either directly through interacting with chromphor or indirectly through triggering the inflammatory responses (Floyd, 1990; Frenkel, 1992). UV augments blood flow and infiltration by blood leukocytes, such as macrophages and neutrophil into the skin, leading to inflammation (Cooper *et al.*, 1993; Kang, Hammerberg, Meunier, and Cooper, 1994). In addition, the inflammation changes are considered to be a part of cancer promotion. Malignant transformation of epithelial cells is associated with chronic inflammation whereby the infiltrated and activated leukocytes can generate ROS (H_2O_2) and NO that interact with biomolecules and induce the process of cancer formation in the inflamed tissue (Balkwill and Coussens, 2004; Katiyar and Mukhtar, 2001). The growth of tumor was enhanced only when these inflammatory cells were present at high numbers in the skin. Therefore, UV-induced inflammatory cells promoted skin tumour growth. UV radiation-induced lipid peroxidation increases metabolism of arachidonic acid via lipoxygenase and cyclooxygenase (COX-2) forming hydroxyeicosatetrenoic acid and prostaglandin (PG) metabolites, is associated with skin

inflammation and tumour promotion (Fisher and Slaga, 1985). This is thought to be due to UV increasing phospholipase activity, thus enhancing arachidonic acid availability for PG production (Hruza and Pentland, 1993; Kangrotondo, Miller, Morrison, and Pentland, 1993).

Acute exposure to UVB affects on keratinocytes producing sunburn formation on epidermis and inducing apoptosis (King and Cidlowski, 1995) and hyperproliferation of epidermal cells (Hashimoto, Tsutsui, Matsuo, and Iizuka, 1995). UVB irradiation is known as a cause of cell proliferation and lead to epidermal hyperplasia that is preceded by an initial inhibitory phase in pigs (Hashimoto *et al.*, 1995). UVB irradiation inhibited cell cycle after 48-hours irradiation (Kawagishi *et al.*, 1998). p53 inhibits G1/S transition through p21, a universal inhibitor of CDK (Herzinger *et al.*, 1995; Meikrantz and Schlegel, 1995; Ponten, Berne, Ren, Nister, and Ponten, 1995). p21 directly binds and inhibits proliferating cell nuclear antigen (PCNA), a subunit required by DNA polymerase δ (Flores-Rozas *et al.*, 1994). p21, thus inhibits CDK and PCNA essentially for G_1/S transition, providing times for DNA damage repair. Besides, DNA replication, PCNA is known to be involved in DNA repair (Zeng *et al.*, 1994). p21 dose not interfere with the PCNA-dependent DNA excision repair (Li *et al.*, 2006; Luo, Hurwitz, and Massague, 1995). G_1 arrest was detected at 24 h followed the irradiation, despite a significant induction of PCNA (Kawagishi *et al.*, 1998). It seems that PCNA functions in DNA repair synthesis during the early phase after UVB irradiation, where the p53-dependent induction of p21 plays an essential role for the G_1 check point. The peak of PCNA-positive cells were observed at 72-96 h, monitored by thymidine uptake, suggesting its role in DNA replication (Kawagishi *et al.*, 1998).

Acute effects of UV radiation on DNA damage, lipid peroxidation, and protein crosslinking lead to sunburn. UVB absorbed by DNA induces primarily cyclobutane pyrimidine dimmer (CPD) and pyrimidine (6-4) pyrimidone photoproducts (Mitchell, 1988; Mitchell and Nairn, 1989; Setlow and Carrier, 1966). The DNA lesions occur most frequently in area of tandem pyrimidine residues, which are known as "hot spots" of UV-induced DNA damage (Kanjilal and Ananthaswamy, 1996). UVB can generate ROS. It is more effective in direct interaction with DNA, the formation of DNA photoproducts (thymine dimers) and conversion into single-strand DNA breaks via DNA repair enzymes (Berton, Mitchell, Fisher, and Locniskar, 1997; Green, McGuire, Miska, and Kuewitt, 2001; Katiyar, Matsui, and Mukhtar, 2000; Krutmann, 2001; Mitchell, Byron, Chiarello, and Lowery, 2001). Epidermal cells with sufficient damage can undergo apoptosis, which is most obviously manifested as sunburn cells (SCs). The correlation between SCs and DNA damage was established in many different pathological situations *in vitro* and *in vivo* such as induction by ionizing radiation, by anticancer drugs, and by growth factor deprivation (Haake and Polakowska, 1993). Indeed, after UV irradiation, the accumulation p53 protein arrests the damaged cell in G_1 phase, which allows the repair of DNA damage before its replication in the S phase (Huang, Clarkin, and Wahl, 1996). The *p21/WAF1/CIP1* pathway was discovered as an inhibitor of cyclin-dependent kinase (CDK), whose induction is associated with the expression of p53 (Harper, Adami, Wei, Keyomarsi, and Elledge, 1993). *p21/WAF1/CIP1* inactivates the CDK-cyclin complex by competitively forming complex with CDK, thus leads the cell into G_1 arrest. p53 also contributes the maintenance of genomic stability, promotes proper DNA replication and repairs by *GADD45* and *XPA* genes (Kastan *et al.*, 1992). *p53* gene also plays a leading role in the apoptotic pathway. The amount of DNA damage is too high to

be repaired in due time, so p53-elicited apoptotic cell death becomes visible as small dyskeratotic cells within epidermis sunburn cells. As a transactivator of transcription, p53 protein can induce apoptosis by upregulating the expression of apoptosis-promoting genes such as *Bax, Fas/Apo-1*, or downregulating the expression of apoptosis-suppressing gene such as *Bcl-2* (Muller *et al.*, 1998). Therefore, the amount of UV-induced DNA damage indicates the density of SCs. Long-term and recurrent exposure to UV causes gradual deterioration of skin structure and function. Apparently, accumulation of DNA damages as results of the recurrent and acute DNA injuries and the effect of chronic inflammation (Gilchrest, 1996) could ultimately lead to the development of skin cancers (Melnikova and Ananthaswamy, 2005).

Polyphenols was demonstrated to possess the antioxidant and the anti-inflammatory properties. Green tea polyphenols showed numerous health benefits, including protection from UV carcinogenesis (Katiyar, Elmets, and Katiyar, 2007). Green tea beverage is composed of catechin, principally (-)-epigallocatechin gallate (Graham, 1992). Catechin is a powerful antioxidant, reduce oxidative damage that induced by UVR (Wang *et al.*, 1994). Most of animal studies on skin cancer carried out by other researchers focused on green tea, or else the polyphenolic fractions such as (-)-epigallocatechin-3-gallate. Few studies reported that pomegranate fruit extract was able to suppress UV-induced skin pigmentation, when topically applied (Yoshimura, Watanabe, Kasai, Yamakoshi, and Kota, 2005) or orally administrated (Kasai, Yoshimura, Kota, Aril, and Kawasaki, 2006). It also suppressed human epidermal keratinocyte damage (Syed *et al.*, 2006) and inhibited skin tumorgenesis in CD1 mice (Afaq, Saleem, Krueger, Reed, and Mukhtar, 2005b).

Protective skin actinic damages to UV by using topical sun block produced from plant products is interesting for now. There are few evidences of pomegranate peel on skin protection against UV irradiation. So, in this experiment, topical application of agents was directly anointed on the skin. The study aimed to observe the protective effects of pomegranate peel extracts on rat skin against UVB irradiation. The symptomatic effects on skin and epidermal alls were investigated.

5.3 Materials and methods

Pomegranate peel ethanolic and water extracts preparations were described in Chapter III.

5.3.1 Materials

Epigallocatechin-3-gallate (EGCG) was purchased from Sigma-Aldrich, USA. Agarose was from Promega, Spain. Ethidium bromide was from Bio-Rad, USA. RNase A was purchased from Amresco®, USA. DNA ladder and Genomic DNA Extraction Kit were obtained from RBC Bioscience, USA. All other chemicals and solvents were analytical grade and purchased from Sigma-Aldrich, USA. A UV light source (285-350 nm), Waldmann UV 109B equipped with UV21 lamp and Variocontrol spectroradiometer were from Waldmann (Villingen-Schwenningen, Germany).

5.3.2 Experimental animals

The experiments were carried out on female Wistar rats, 7-8 weeks and 150-200 g, purchased from National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. The animals were housed at 25°C and 50-70% relative humidity in the animal

house of Suranaree University of Technology (SUT) under a control environment and illumination (12 h light, 12 h dark) room. Rats were fed *ad libitum*. Rats were preserved in satisfaction with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, Thailand. The experiments were conducted in accordance with the advice of the Institutional Animal Care and Use Committee, SUT.

5.3.3 UV light source

A Waldmann UV 109 B light booth equipped with UV21 lamp with emission spectrum 285-350 nm (peak at 310-315) was used as UV light source. The strength of UV irradiation at skin surface was measured by Variocontrol spectroradiometer.

5.3.4 Determination for minimal erythema dose (MED)

The minimal erythema dose (MED) energy of UV irradiation required to produce the minimally perceptible erythema reaction of the skin was determined on unprotected skin surface area of 8 cm^2 on a rat (Lowe and Friedlander, 1997). The rats were exposed to increasing doses of 0.04-0.32 J/cm^2 with an increment of 0.04 J/cm^2 of UVB at a distance of 13 cm from the lamps. MED value was determined at 24 h after irradiation ranged between 0.07 and 0.08 J/cm^2.

5.3.5 Topical treatment of extracts and UVB irradiation

For UV exposure, the animals were divided into 6 groups, (I) normal control (-UV); (II) non-vehicle control (-VE); (III) vehicle control (+VE); (IV) positive control (+EGCG, 8 mg/cm^2); (V) PPEE (+PPEE, 8 mg/cm^2), and (VI) PPWE (+PPWE, 8

mg/cm^2). The rat hairs on its back were shaven off about 4 × 2 cm for all experiments. The rat groups IV to VI were topically applied with 8 mg/cm^2 EGCG, PPEE, and PPWE on the hairless skin areas and let stand for 30 min prior to 3xMED UVB irradiation. The experiments were repeatedly performed twice a week (3-4 days at a time) and continued for one month. After 24 hours of the last irradiation, the treated skin feature was examined and photographed. The treated rats were sacrificed, the central exposed area of skin was removed and processed for histological and DNA preparations.

5.3.6 Erythema investigation

Erythema examinations were carried out immediately 24 h after UV irradiation. The UV damages were observed by erythema using photodocumentation and evaluated by the Draize score system (Middlekamp-Hup *et al.*, 2004), on scale of 0 to 4 (0, none; 1, very slight; 2, well define; 3, moderate; 4, severe).

5.3.7 Histological section preparation

The treated skins were fixed in 10% neutral-buffered formalin overnight, embedded in paraffin blocks and sectioned at 3-5 μm thickness and serial sections were mounted onto silane-coated slides and stained with hematoxylin and eosis (H&E) (Hould, 1993). The specimens were observed and quantified for inflammatory cells (histiocytes, mast, and mononuclear cells), epidermal thickness and sunburn cells (SCs). Degree of inflammatory infiltration (Phillips *et al.*, 2000) was graded on a scale of 0 to 3 (0: none, 1: mild, 2: moderate, 3: severe). Epidermal thickness was measured at 400 magnifications. The total of SCs (eosinophilic cells with or without pyknotic nuclei) was manually counted throughout the entire available epidermis. The number of SCs was

divided by the epidermal area, which gave the number of SCs per millimeter of the epidermis. The epidermal area was measured by computerized image analysis.

5.3.8 DNA isolation and electrophoresis

DNA was isolated from the epidermal cells by using a Genomic DNA Extraction Kit. RNase A (10 mg/ml) was added to sample lysate and left to stand at room temperature for 30 minutes. The DNA precipitate was centrifuged at 13,000 rpm for 3 minutes and eluted in elution buffer. The DNA content was quantitated and resuspended in TE buffer containing 10 mM Tris-HCl, pH 7.6, 1 mM EDTA. The DNA at 4 μg/well was electrophoresed in 2% agarose gel containing 0.5 μg/ml ethidium bromide in 45 mM Tris, 45 mM boric acid, 1 mM EDTA at 100 mV for 1.5 h, visualized under UV fluorescence, and photographed.

5.3.9 Statistical analysis

Data were analyzed by ANOVA, using the least significant test to determine the level of significant at $P < 0.01$ and 0.05. All data were expressed as mean ± standard error (SE). For single comparisons, the significance of difference between means was determined by Student's *t*-test at significant level of $P < 0.05$.

5.4 Results and discussion

5.4.1 Erythema reduction

Topical application of PPEs on hairless rat skin prior to UBV irradiation at 3xMED was able to reduce the skin erythema. The UVB irradiation on non-vehicle (-VE)

and vehicle (+VE) pretreated controls induced erythema at 3.69 ± 0.13 and 3.57 ± 0.20 scores, respectively. While, topical application of 8 mg/cm^2 PPEE and 8-mg/cm^2 PPWE significantly lowered erythema at 1.40 ± 0.24 and 2.00 ± 0.00 scores, respectively ($P \leq 0.01$) (Table 5.1 and Figure 5.1). The erythema symptom induced by the UVB irradiation was illustrated in Figure 5.2. Apparently, the erythema reduction by 8-mg/cm^2 PPEE, PPWE and EGCG pretreatment was about 1.8 to 2.5 fold of the vehicle controls. This clearly demonstrated that PPEs well prevented the rat skin from UVB-induced erythema.

UVB induced thymine dimmer formation on DNA (Freeman *et al.*, 1989). DNA may also be the chromophore for erythema (Young *et al.*, 1998). The responsible molecules to light absorption that initiates erythema have not been precisely identified. However, the action spectrum of erythema is consistent with the hypothesis that UV interactions with DNA are of major importance (Parrish, Jaenicke, and Anderson, 1982). This suggested that the principal event would be direct damage to DNA by UVB. Secondary oxidation from endogenous photosensitization reactions could cause indirectly oxidative damage to cells. UV irradiation increases the activity of xanthine oxidase in human keratinocytes and the production of superoxide (Deliconstantinos, Villiotou, and Stavride, 1996). ROS production was increased after UV exposure leading to depletion of antioxidant enzymes, superoxide dismutase, glutathione peroxidase and catalase, for removing ROS from the skin (Podda, Traber, Weber, Yan, and Packer, 1998). Vitamin C reduced UVB-induced oxidative damage in mouse keratinocytes *in vitro* (Steward, Cameron, and Pence, 1996). Polyphenols from green tea effectively reduced erythema response both UVB and UVA radiation (Elmet *et al.*, 2001). (-)-epigallocatechin-3-galate and (-)-epicatechin-3-gallate both contain a galloyl group at the 3 position, are the most efficient in erythema inhibition. In this study, topical pretreatment of PPEs before UVB

Table 5.1 Protective effect of pomegranate peel extracts against erythema, inflammatory infiltration, epidermal thickness, and sunburn cells in rat skin.

Treatment	Erythema (score)	Inflammatory Infiltration (score)	Epidermal thickness (μm)	Sunburn cells (cells/mm^2)
Normal control	None	None	18.47 ± 2.25	None
Non-vehicle control	3.69 ± 0.13	2.92 ± 0.08	70.40 ± 3.78	18.85 ± 3.31
Vehicle control	3.57 ± 0.20 [b]	2.85 ± 0.14 [b]	69.71 ± 1.07 [b]	17.86 ± 3.43 [b]
PPEE	1.40 ± 0.24 [a,a]	0.60 ± 0.24 [a]	40.34 ± 1.12 [a]	2.40 ± 0.24 [a,a]
PPWE	2.00 ± 0.00 [a,b]	1.50 ± 0.29 [a]	42.46 ± 0.62 [a]	4.25 ± 1.11 [a,b]
EGCG	1.17 ± 0.31	0.67 ± 0.21	39.89 ± 0.54	1.67 ± 0.56

PPEE, pomegranate peels ethanolic extract; PPWE, pomegranate peels water extract; EGCG, epigallocatechin-3-gallate. Values are means ± SE for six animals per group. Numbers with different letters within the same column are significantly different ($P < 0.01$).

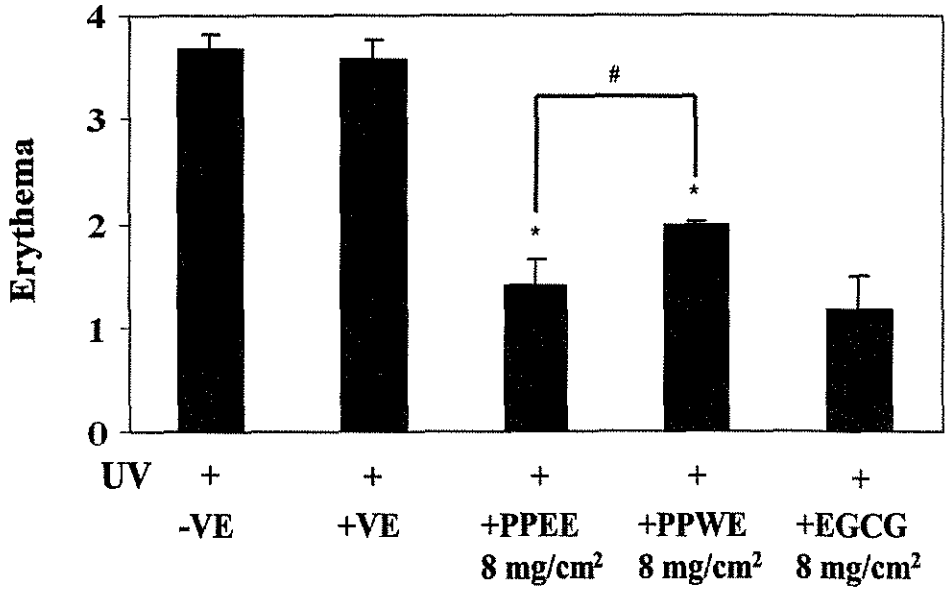

Figure 5.1 The inhibitory effect of pomegranate peel extracts on 3xMED UVB-induced erythema on rat skin. The test skin was pretreated with PPEE, PPWE, epigallocatechin-3-gallate (EGCG), and vehicle control (+UV, +VE) before irradiated with 3xMED, twice a week for a month. Data were shown as the mean score of erythema ± SE, n = 6. * = statistically significant ($P < 0.01$) and # = difference of between means ($P < 0.05$).

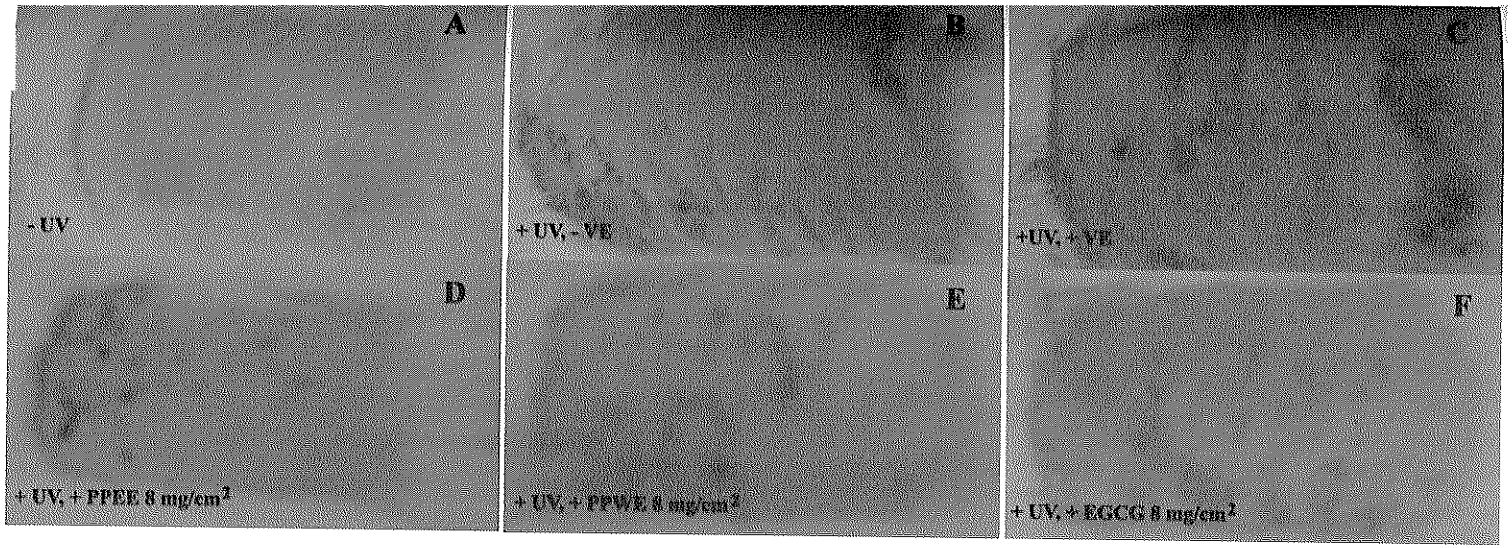

Figure 5.2 Photographs of the hairless rat skin topically protected with pomegranate peel extracts before 3xMED UVB irradiation. 3xMED UVB-induced erythema is compared. A, normal control; B, UVB and non-vehicle control; C, UVB and vehicle control; D, UVB and 8 mg/cm^2 PPEE; E, UVB and 8 mg/cm^2 PPWE and F, UVB and 8 mg/cm^2 EGCG.

exposure showed less erythema. As a result, PPEs could prevent UV adverse effects on rat skin it could imply that the antioxidant property of PPEs inducing the UV photoprotection. PPEs were reported that they were the rich source of many phenolic compounds including flavanoids (flavan-3-ols, flavonols, flavones, anthocyanidins, and other complex flavanoids), hydroxybenzoic acids (gallic acid and ellagic acid), and ellagitannins (punicalin, punicalagin, pedunculagin, gallagic and ellagic acid ester of glucose), which could account for almost of the antioxidant activities (Lansky and Newman, 2007). The combined supplements of vitamins E and C could protect against UVR-induced erythema, and reduced the sunburn reaction in human (Eberlin-Konig, Placzek, and Przybilla, 1998; Fuchs and Kern, 1998; Lin *et al.*, 2003). Therefore, the synergistic photoprotection may be achieved when many substances exist in phenolic compounds as in crude extract of pomegranate peel.

5.4.2 Inflammatory infiltration

UVB induced a significant amount of inflammatory cells in the vehicle-pretreated group. PPEs could decrease the inflammatory cells. The skin samples of vehicle control had the highest inflammatory infiltration (2.85 ± 0.14), which was on the severe score. PPEE and PPWE pretreated groups showed mild and moderated inflammatory infiltration 0.60 ± 0.24 and 1.50 ± 0.29, respectively (Table 5.1 and Figure 5.3). Histological examination revealed an epidermis with inflammatory infiltration in the dermal connective tissue (Figures 5.4 (B) and (C)).

There are studies reported that the beneficial effects of PPEs against early markers of inflammatory cells induced by ROS, lipid peroxidation and inflammation (Floyd, 1990; Frenkel, 1992). It appears that PPEs inhibited UVB-induced ROS

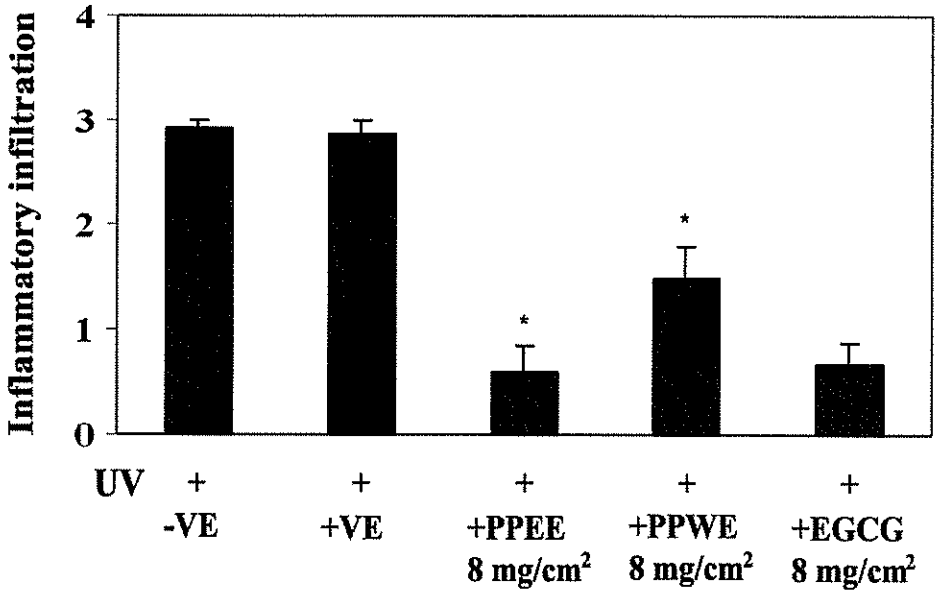

Figure 5.3 The inhibitory effect of pomegranate peel extracts on 3xMED UVB-induced inflammatory infiltration on rat skin. The test skin was pretreated with PPEE, PPWE, epigallocatechin-3-gallate (EGCG), and vehicle control (+UV, +VE) before irradiated with 3xMED, twice a week for a month. Data were shown as the mean score of inflammatory infiltration ± SE, n = 6. * = statistically significant ($P < 0.01$).

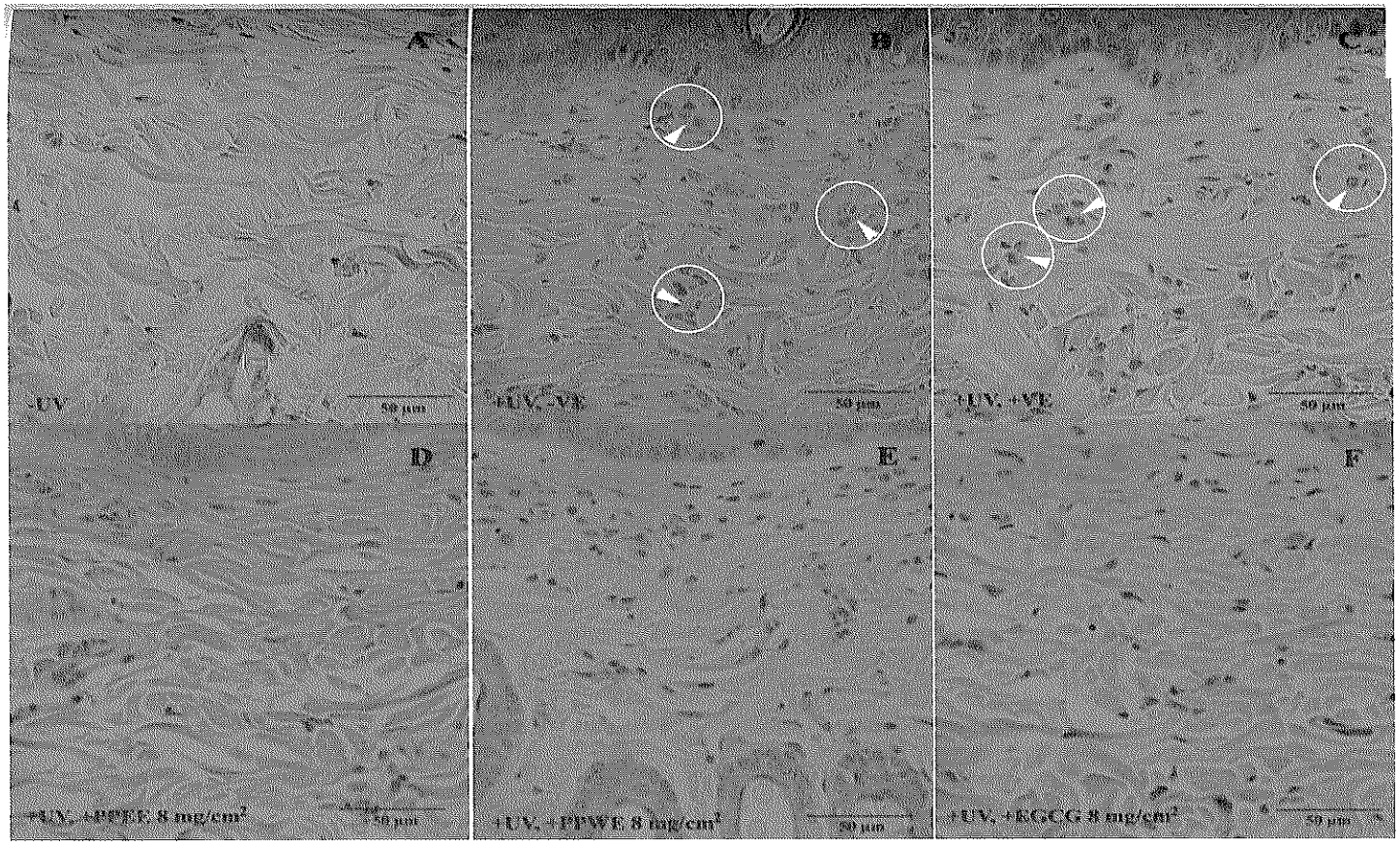

Figure 5.4 Photomicrographs of 3xMED UVB irradiated rat skin demonstrated inflammatory infiltration in dermis (400 magnifications). The inflammatory infiltration indicated by circles. A, normal control; B, UVB and non-vehicle control; C, UVB and vehicle control; D, UVB and 8 mg/cm^2 PPEE; E, UVB and 8 mg/cm^2 PPWE and F, UVB and 8 mg/cm^2 EGCG.

production through either directly scavenging effect or indirectly inhibitory effect of neutrophil recruitment. Pomegranate fruit extract (PFE) inhibited UVB-induced generation of ROS, and reduced NF-κB in human skin fibroblast (Pacheco-Palencia, Noratto, Hingorani, Talcott, and Mertens-Talcott, 2008). Polyphenolic compounds in PPEs had antioxidant activity and inhibited pro-inflammatory enzymes including the COX-2 and LPO *in vivo* (Murthy, Jayaprakasha, and Singh, 2002; Singh, Murthy, and Jayaprakasha, 2002), and inhibited phospholipase A_2 expression of PC-3 prostate cancer cells (Lansky *et al.*, 2005). Anthocyanidins showed inhibition of COX-2 activity and NO production in leukemia cells (Hou *et al.*, 2003). Ellagitannins (punicalagin and punicalin) reduced inflammatory cell signaling in colon cancer cells (Adams *et al.*, 2006; Seeram *et al.*, 2005). In normal human epidermal keratinocytes, PFE inhibited UVB-mediated phosphorylation of ERK, JNK, and p38 and PFE inhibited UVB-induced phosphorylation of IKKα and IκBα and activated nuclear translocation of NF-κB/p65 (Afaq *et al.*, 2005a). Inhibition of NF-κB, MAPK, and cytokines by PFE occurred in mouse skin when exposed to 12-*O*-tetradecanoylphorbol-13-acetate (Afaq *et al.*, 2005b). These findings demonstrated that pomegranate had the potential to inhibit both UVB and TPA-induced oxidative stress-mediated activation of signaling pathways both *in vitro* and *in vivo*.

5.4.3 Epidermal thickness

PPEs could lead to a significant decrease in UV-induced hyperproliferation in rat skin. PPEE- and PPWE-topical pretreatments could decrease epidermal thickness 40.34 ± 1.12 μm and 42.46 ± 0.62 μm, respectively as compared to the vehicle pretreatment of 69.71 ± 1.07 μm (Figures 5.5 and 5.6). Epidermal thickness of PPEE and PPWE rat

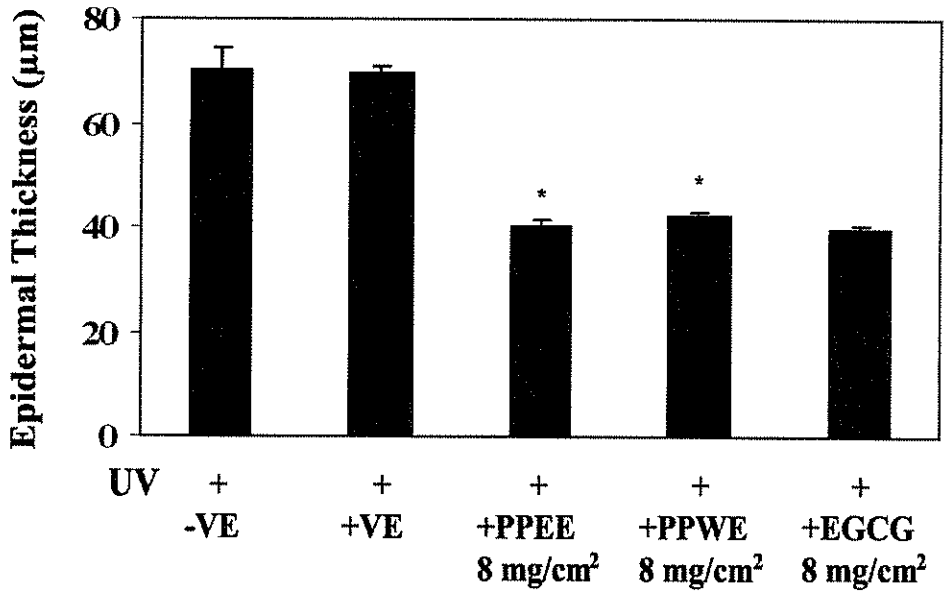

Figure 5.5 The inhibitory effect of pomegranate peel extracts on 3xMED UVB-induced epidermal thickness on rat skin. The test skin was pretreated with PPEE, PPWE, epigallocatechin-3-gallate (EGCG), and vehicle control (+UV, +VE) before irradiated with 3xMED, twice a week for a month. Data were expressed as the mean ± SE, n = 6. * = statistically significant ($P < 0.01$).

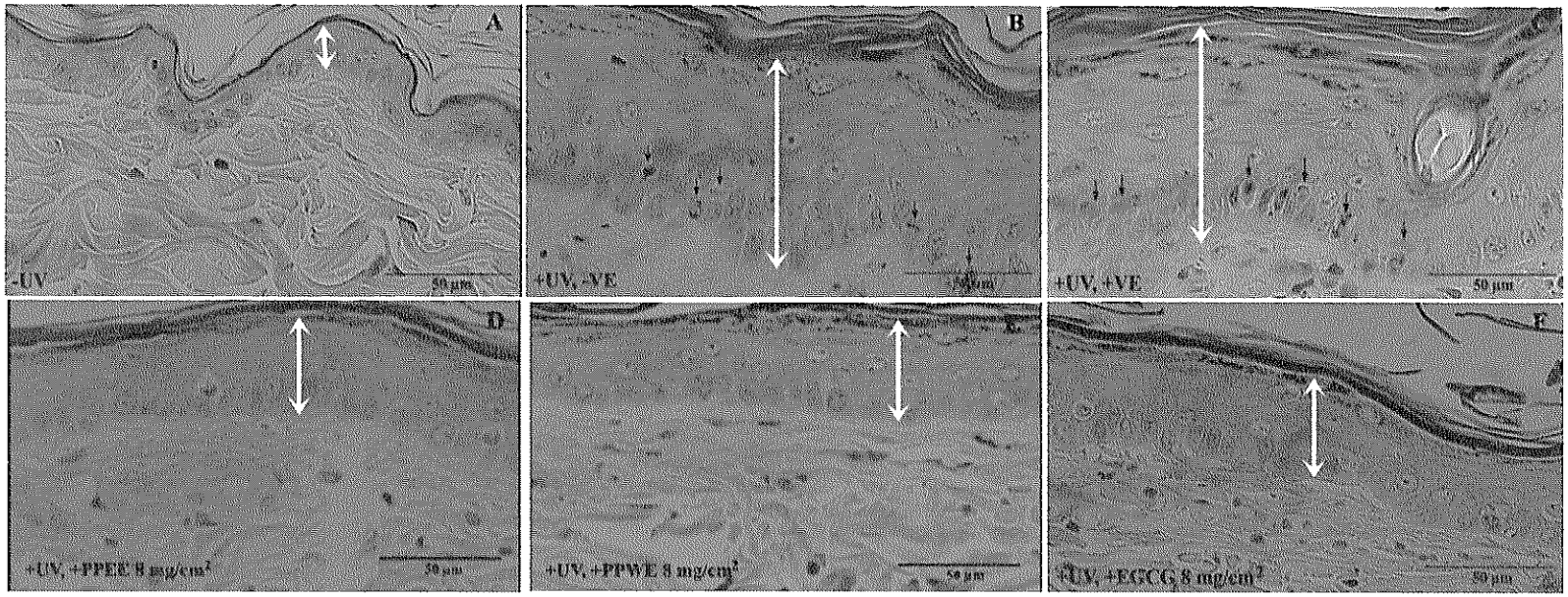

Figure 5.6 Photomicrographs of 3xMED UVB irradiated skin illustrated the epidermal thickness (double head arrows) and sunburn cells (arrows) at 400 magnifications. A, normal control; B, UVB and non-vehicle control; C, UVB and vehicle control; D, UVB and 8 mg/cm^2 PPEE; E, UVB and 8 mg/cm^2 PPWE and F, UVB and 8 mg/cm^2 EGCG.

treated groups was significantly thiner than that of the vehicle control ($P < 0.01$). Pomegranate fruit extract (PFE) inhibited UV-induced cell death by reducing cell cycle, arrested at G_1 as well as S and G_2/M phases of SKU-1064 human skin fibroblasts (Pacheco-Palencia *et al.*, 2008). UVB-induced increase p21 expression and decrease PCNA expression in pomegranate treated cells indicated that inhibition of cell proliferation could be one of the mechanisms in protecting damaged cells from entering the cell cycle of human skin EpiDerm (Afaq *et al.*, 2010; Zaid *et al.*, 2007). Similarly, PFE treatment resulted in the downregulation of PCNA and further increased the p53 and p21 protein levels in the murine skin (Afaq *et al.*, 2010) and in SKH-1 mice (Quhtit *et al.*, 2000). Interestingly, PCNA, which is initially expressed only in the basal layer, becomes dispersed throughout the basal and suprabasal layers of the skin at 48-96 h. This was characteristics of marked hyperplasia as well. The recent finding that cell proliferative activity was closely associated with apoptotic response (Borner, 1996). These definitely supported this study that topical pretreatment of PPEs attenuated the skin symptom, inhibited epidermal cells proliferation and reduced epidermal cell apoptosis caused by UVB irradiation.

5.4.4 Sunburn cells and DNA damage

This study found that UVB induced an increasing in SCs in the pretreated vehicle, which could cause by PPEs treatment (Figures 5.6 and 5.7). Skin of the vehicle control expressed the highest SCs of 17.86 ± 3.43 cells/mm. PPEE and PPWE significantly reduced the SCs to 2.40 ± 0.24 cells/mm and 4.25 ± 1.11 cells/mm, respectively ($P < 0.01$). Furthermore, after UVB exposure DNA fragmentation was more intense in the vehicle-pretreated skin, but it was slight in the PPEs-pretreated skin (Figure 5.8). High

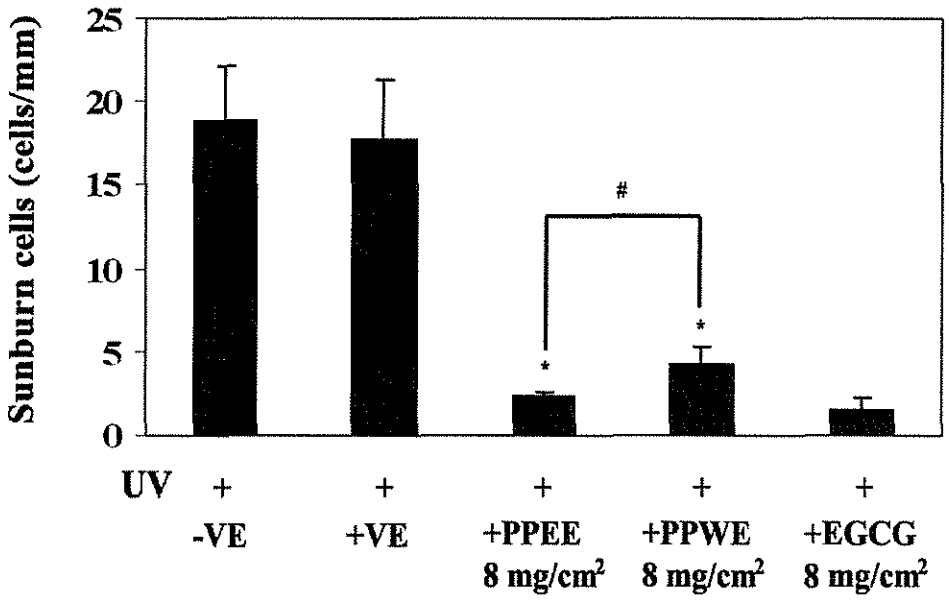

Figure 5.7 The inhibitory effect of pomegranate peel extracts on 3xMED UVB-induced sunburn cells on rat skin. The test skin was pretreated with PPEE, PPWE, epigallocatechin-3-gallate (EGCG), and vehicle control (+UV, +VE) before irradiated with 3xMED, twice a week for a month. Data were expressed as mean ± SE, n = 6. * = statistically significant ($P < 0.01$) and # = difference of between means ($P < 0.05$).

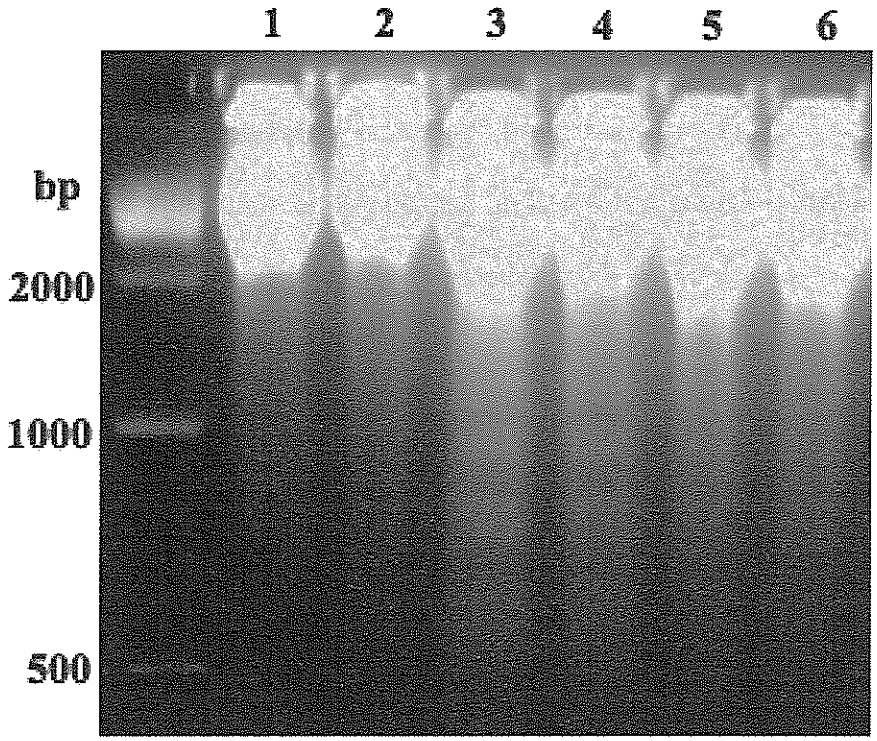

Figure 5.8 Effects of 3xMED UVB irradiation on DNA fragmentation of rat epidermal cells: DNA, 4 μg, was electrophoresed on 2% agarose gel. Lane 1, normal control; Lane 2, 8 mg/cm^2 EGCG; Lane 3, non-vehicle control (-VE); Lane 4, vehicle control (+VE); Lane 5, 8 mg/cm^2 PPEE and Lane 6, 8 mg/cm^2 PPWE.

dose of UV irradiation caused DNA damage in the skin by producing pyrimidine dimmers and increasing the level of ROS (Krutmann, 2001). Nucleosomes from DNA fragmentation induced by 3xMED UVB irradiation was significantly observed in vehicle control (Figure 5.8). PPEE and PPWE-pretreated prior irradiation induced no detectable DNA fragmentation. Apoptotic cells appeared in 12-24 h after UVB irradiation, and DNA isolated from the cells showed fragmentation both *in vitro* and *in vivo* (Baba, Hanada, and Hashimoto, 1996; Iwasaki, Izawa, and Mihara, 1996). Similarly, SCs were induced in 24 h after UVB irradiation, which was consistent with the time course of the induction of SCs in rat epidermal keratinocytes (Iwasaki *et al.*, 1996). The findings of DNA fragmentation, SCs formation could suggest that apoptotic process actively happend in the UV-irradiated skin. The reductions in the number of SCs and in DNA fragmentation could be used as an indication of a protective effect of antioxidant. Exposure of epidermal cells to antioxidants was demonstrated to reduce the number of SCs, presumably because some of the free radicals generated by UV could be removed by antioxidants before they caused excessive damage (Miyachi, Horio, and Imamura, 1983). Topical vitamin C or E alone, and their combination could reduce the number of SCs in Yorkshire pigs (Lin *et al.*, 2003).

PFE was effective in protecting human skin fibroblasts from cell death following UV exposure, achieved to reduce UV-induced ROS levels, increase intracellular antioxidant capacity, and increase G_0/G_1 phase, associated with DNA repair (Pacheco-Palencia *et al.*, 2008). PFE was provided in drinking water for 14 days before irradiating mice with a single dose exposure of UVB. PFE protected against UVB-induced DNA damage by inhibiting CPDs and 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation in the mouse skin and further increase the p53 and cyclin kinase inhibitor p21 protein levels

(Afaq *et al.*, 2010). Up-regulation of the enzyme ornithine decarboxylase (ODC) with subsequent increases in the levels of the polyamines is an obligatory step in skin tumor development. PFE treatment results in the downregulation of PCNA and ODC expressions in the murine skin and further increases the p53 and cyclin kinase inhibitor p21 protein levels (Afaq *et al.*, 2010). Similarly, pretreatment of EpiDerm (morphological and metabolic properties similar to human skin) with juice, oil or byproduct of pomegranate resulted in marked inhibition in the number of CPDs and 8-OHdG positive cells, reflecting a protective effect of these compounds against UVB-mediated DNA damage and SCs (Zaid, Afaq, Khan, and Mukhtar, 2007). Moreover, the primary biological response to DNA damage in living cells is DNA repair (Friedberg, 2001). When the DNA repair system is faulty or inefficient, the mutation frequency increases leading to an enhanced susceptibility of cancer risk (Kunkel and Bebenek, 2000).

The decrease in DNA damage kinetics by pomegranate extract (PE) suggested the possible involvement of the DNA repair system in photoprotective efficacy of PE against photocarcinogenesis. However, more studies are needed to investigate the effect of PE on DNA repair caused by UVB radiation. From this study, it could be concluded that PPEs could affect substantial protection from UV-induced DNA damage via modulation in early biomarkers of photocarcinogenesis.

5.5 Conclusion

In conclusion, pomegranate peel was rich in antioxidants, played remarkable roles in prevention skin lesion from UVB irradiation by alleviation of erythema, inflammatory infiltration, sunburn cells, and DNA damages. These findings provide useful knowledge

of pomegranate peel for further researches on its potential in chemoprevention of skin cancer induced by UVB radiation and in agro-industry. This could make use of the pomegranate by-products. Further study, I suggest that pharmacologically safe of pomegranate peel is needed in validate study in confirming for human use.

5.6 References

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CHAPTER VI

THE EFFECT OF POMEGRANATE PEEL EXTRACTS ON CELL PROLIFERATION, DNA FRAGMENTATION, AND APOPTOSIS DETERMINATION

6.1 Abstract

The pomegranate peel possess major sources of total phenolic compounds and represent potent antioxidant properties. The use of peel has also been shown to possess anticancer activities. The present study demonstrates that peel extracted by ethanol (PPEE) and water (PPWE) induces antiproliferative effect, morphological change, DNA fragmentation, and proteins-induced apoptosis in MCF-7 cells, which lack caspase-3. In three assays (Trypan blue, MTT, and Resazurin) of antiproliferative effect determined the lethal concentrations at 50% (LC_{50}) values in μg/ml at 6-24 h. LC_{50} values of PPEE were more effect compared to PPWE at the same concentration of treatments. Therefore, the antiproliferation by PPEs was depended on dose- and time-manner. As well as degree of DNA fragmentation and apoptotic cell morphology were detected. Apoptosis was accompanied by Bcl-2, caspase-9, caspase-7, and poly (ADP-ribose) polymerase (PARP) levels. By the reduction of Bcl-2 activity and activation of caspase-9, caspase-7, and PARP activities involved with the mechanism of PPEs-induced apoptosis. These protein levels associated with morphological change and

DNA fragmentation in MCF-7 cells. These results suggest that a deficit in caspase-3 is not sufficient to block PPEs-induced apoptosis. Interestingly, PPEs, a by-product from pomegranate juice making, might expect to be added-value by-products to be a potential natural source as anticancer treatment.

6.2 Introduction

Breast cancer is the most common disease in most Asian countries, and it tends to increase at a more rapid rate than in Western countries. It may due to some changes of ones lifestyles and dietary (Yip, 2009). Presently, phytochemicals have received attention for cancer chemopreventative and therapeutic is one agents. Pomegranate composes of phenol and polyphenols, including aliphatic organic acids, hydroxybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavonols, flavones, anthocyanins, anthocyanidins, and ellagitannins (Lansky and Newman, 2007). Pomegranate extracts inhibited proliferation and invasion of LnCaP, DU-145, and PC-3 cells human prostate cancer (Lansky, Harrison, Froom, and Jiang, 2005b; Lansky *et al.*, 2005a), and pomegranate fruit extract induced apoptosis and increased pro-apoptotic protein in PC-3 cells (Malik *et al.*, 2005). Anthocyanins and ellagitannins, the major phytochemicals of pomegranate peel extract, inhibited invasion of HT115 colon cancer cell *in vitro* (Coates *et al.*, 2007). Larrosa, Tomas-Barberan, and Espin (2006) suggested that anticarcinogenic effect of ellagic acid and ellagitannins induced apoptosis via mitochondrial pathway by releasing of cytochrome c into the cytosol, activating of initiator caspase-9 and effector caspase-3, in colon cancer Caco-2 cells.

Apoptosis is a genetically regulated mechanism of cellular suicide which demonstrates a crucial role in homeostasis and in development. The apoptotic mode is

programmed to cause by the coordination of the caspase protein family. Caspases are the cystein-asparagine proteases. It is well established that initiator caspases (caspase-2, -8, -9, -10, -11, and -12) are coupled to pro-apoptotic signals. Once activated, these caspases cleave and activate downstream effector caspases (caspase-3, -6, and -7), which in turn execute apoptosis by cleaving cellular proteins (Danial and Korsmeyer, 2004; Degterev, Boyce, and Yuan, 2003).

One of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomals. Caspase-3 is the primary executioner in apoptosis, since it is activated by many death signals leading to chromatin condensation, DNA fragmentation, and cleavage of the DNA repair enzyme poly (ADP-ribose) polymerase (PARP). (Kothakota *et al.*, 1997). Lamins are intra-nuclear proteins that maintain the shape of the nucleus and mediate interactions between chromatin and the nuclear membrane. Degradation of lamins by caspase-6 results in the chromatin condensation and nuclear fragmentation (Orth, Chinnaiyan, Garg, Froelich, and Dixit, 1996; Takahashi *et al.*, 1996). DNA fragmentation into nucleosomals is caused by caspase-activated DNase (CAD). Normally, CAD exists as an inactive complex with inhibitor CAD (ICAD) that is cleaved by caspases (such as caspase-3) to release CAD during apoptosis (Enari *et al.*, 1998; Liu, Zou, Slaughter, and Wang, 1997).

MCF-7, the breast carcinoma cells, is lack of caspase-3 owing to the functional deletion on the *CASP-3* gene, which can be killed by apoptotic stimuli, such as tumour necrosis factor (TNF) and staurosporine, without DNA fragmentation and many of the hallmarks of apoptosis. Caspase-3 is likely to be essential for apoptotic cell death (Janicke, Sprengart, Wati, and Porter, 1998).

This study focused on the effects of pomegranate peel extracts (PPEs) on the proliferation and the cell apoptosis of a cancer cell line using breast cancer cells (MCF-7) as a model. The study emphasized on the followings. (1) To investigate the alteration of cell proliferation induced by PPEs, Trypan blue, MTT, and Resazurin assays. (2) To observe apoptotic cell morphology and DNA fragmentation. (3) To determine some apoptotic factors involved with emphasis on Bcl-2, procaspase-7, procaspase-9, and PARP. It was expected to elucidate a possible apoptotic pathway of pomegranate peel extracts in inducing MCF-7 cell growth and death. The knowledge obtained would be benefitial to the use of pomegranate by-products.

6.3 Materials and methods

Pomegranate fruit collection and pomegranate peel extracts were pre-described in Chapter III.

6.3.1 Materials

Human breast cancer cell, MCF-7, was obtained from American Type Culture Collection; ATCC, USA. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F-12), fetal bovine serum (FBS), and penicillin/streptomycin were from GIBCO, USA. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and Hoechst 33342 were purchased from Molecular Probes, USA. Trypan blue, tripsin-EDTA, and Resazurin were from Invitrogen, USA. Agarose was from Promega, Spain. Ethidium bromide was from Bio-Rad, USA. RNase A was purchased from Amresco®, USA. DNA ladder and Genomic DNA Extraction Kit were obtained from RBC Bioscience, USA. Monoclonal antibodies (PARP, Bcl-2, caspase-7, caspase-9, and

Actin), secondary antibody, chemiluminescent substrate, and CL-XPosure film were from Santa Cruz Biotechnology, USA. All other chemicals and solvents were analytical grade and purchased from Sigma-Aldrich, USA.

6.3.2 MCF-7 culture

Human breast cancer cell (MCF-7) was grown in D-MEM/F-12 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated in a 5% CO_2 atmosphere at 37°C. MCF-7 was incubated for 2 to 3 days before experiments.

6.3.3 Cell viability measurement by Trypan blue exclusion method

Trypan blue is the staining dye used to discriminate viable and dead cells. Dead cells absorb the dye and appear blue in cytoplasm of cells when viewed under a microscope, while viable cells were not stained. The cultured cells were trypsinized by tripsin-EDTA 100 µl of the cells, was transferred into a 1.5 ml microcentrifuge tube, 100 µl of 0.4% (w/v) Trypan blue was added to the tube and mixed thoroughly, and stained for 2 min. The stained cells were transferred onto hemocytometer. The viable cells were counted under inverted microscope. The percent viable cells were calculated according to the following formula:

$$Viable\ cells\ (\%) = \left[\frac{Total\ viable\ cell\ per\ ml}{Total\ cells\ per\ ml}\right] \times 100$$

6.3.4 Cell viability test by MTT assay

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was used for the cell proliferation measurement. This is colorimetric assay based on the

conversion of the yellow MTT solution to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells (Hansen, Nielsen, and Berg, 1989). MCF-7 cells were plated out at 10,000 cells/well of 96-well plate in 100 µl of complete medium for 24 h. The cultured cells were treated with pomegranate peel extracts at various concentrations (1-2000 µg/ml) diluted in complete medium without phenol red, and incubated for 24 h. In addition, other concentration levels were used when required. The final diluted DMSO concentration (0.01% DMSO) was used as a control. The treated cells were cultured in 5% CO_2, 37°C incubator for 6-24 h. One hundred microliters of MTT solution (5 mg/ml in phosphate-buffered saline, pH 7.4) was added and incubated for 4 h, after which the plate was centrifuged at 1,800 x g for 5 min at 4°C. The cultured medium was discarded. One hundred and fifty microliters of DMSO (100% DMSO) were added. The plate was gently agitated until the formazan precipitate was dissolved. The optical absorbance of the color change was measured under a microplate reader at the wavelength of 570 nm with the reference wavelength at 630 nm. Decreasing in absorbance indicated a reduction in cell viability (Babu, Shylesh, and Padikkala, 2002; Okonogi, Duangrat, Anuchpreeda, Tachakittirungrod, and Chowwanapoonpohn, 2007; Perillo, Sasso, Abbondanza, and Palumbo, 2000; Zhou, Yan, Sun, and Zhu, 2003). Experiments for each sample were carried out in four replicate. The results were expressed as the percentage of viable cells with respect to the control.

$$Antiproliferative\ activity\ (\%) = \left[1-\frac{(A_1-A_2)}{(A_0-A_2)}\right]\times 100$$

A_0 was the absorbance of control, A_1 was the absorbance of the treated, and A_2 was the absorbance of treated sample without cells. Antiproliferative activity (%) was plotted against the concentrations of samples, and the median lethal concentration of 50% (LC_{50}) was derived from the best fit line obtained by linear regression analysis.

6.3.5 Cell viability test by Resazurin assay

Resazurin assay is a colorimetric method, based on the conversion of the purple resazurin solution to the red resorufin solution via reduction reactions of metabolically active cells. The MCF-7 cells were plated at 10,000 cells/well in 96-well plate. The cultured cells were treated with the extracts at various concentrations in complete medium, and incubated for 24 h. The final diluted DMSO concentration (0.01% DMSO) was used as a control. One hundred microliters of resazurin solution (100 µl/ml in DMEM/F-12 without fetal bovine serum) was added to each well and incubated for 2 h. The optical absorbance of the color change was measured under a microplate reader at the wavelength of 570 nm with the reference wavelength at 600 nm. Decreasing in absorbance indicated a reduction in cell viability (Goegan, Johnson, and Vincent, 1995; Lancaster and Fields, 1996; Page, Page, and Noel, 1993). The percentage of cell viability was calculated according to the following formula:

$$Antiproliferative\ activity\ (\%) = \left[1-\frac{(A_1-A_2)}{(A_0-A_2)}\right]\times 100$$

A_0 was the absorbance of control, A_1 was the absorbance of the treated cells, and A_2 was the absorbance of sample without cells. Cell viability (%) and LC_{50} were calculated.

6.3.6 Observation of apoptotic cell morphology

MCF-7 cells were cultured in 75 cm^2 cell culture flask until log-phase. The cells were transferred onto 24-well plate 10^6 cells/well, and cultured for 24 h. The cultured cells were treated with various concentrations of extracts and incubated for 24 h. The cells were washed twice with ice-cold PBS, pH 7.4. The cells were fixed with 500 µl formaldehyde (10%, v/v) for 5 min, washed with PBS, and then stained with 1 µg/µl Hoechst 33342 in PBS for 15 min. Cell morphology was observed and photographed under a microscope with Digital Camera DP50, Olympus.

6.3.7 DNA isolation and electrophoresis

DNA was isolated from the PPEs treated MCF-7 cells by using a Genomic DNA Extraction kit. RNase A (10 mg/ml) was added to sample lysate and left to stand at room temperature for 30 minutes. The precipitated DNA was dried under centrifuge at 13,000 rpm for 3 minutes and eluted in elution buffer to eluted purified DNA. The DNA sample was quantitated spectrophotometrically and resuspended in TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA). DNA (2 µg/well) was electrophoresed in 1% agarose gel containing ethidium bromide (0.5 µg/ml) in 45 mM Tris, 45 mM boric acid, 1 mM EDTA at 100 mV for 1.5 h. The gel was visualized UV fluorescence, and then photographed.

6.3.8 Western blotting

MCF-7 cells were cultured in 5% CO_2 incubator at 37°C for 24 h treated with various concentrations of pomegranate peel extracts and cultured in 5% CO_2 incubator at 37°C for 24 h. The treated cells were trypsinized and lysed with ice-cold radio

immuno precipitation assay (RIPA) lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM sodium orthovanadate, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1% Triton X, and protease inhibitor cocktail, for 60 min, and then centrifuged at 13,000 rpm for 10 min. The lysate were kept in -80°C for further experiments.

The lysate protein was quantitated spectrophotometrically by Bradford assay (Bradford, 1976), and separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Bollag, Rozycki and Edelstein (1996). The lysate protein (30 µg/well) was mixed with 4 parts of 5x loading buffer containing 60 mM Tris-HCl, 14.4 mM 2-mercapthoethanol, 25% (v/v) glycerol, 2% (w/v) SDS and 0.1% (w/v) bromophenol blue, and loaded onto 12% SDS-PAGE. The gel was run at 100 Volts, 35 mAmps for 90 min. The SDS-PAGE gel was transferred onto poly vinylidene difluoride membrane (PVDF) at 100 Volts, 400 mAmps for 90 min at 4°C. The blotted PVDF was blocked with 2% (w/v) bovine serum albumin (BSA) in Tween20-Tris buffered saline (TTBS) containing 0.1% Tween20, 100 mM Tris-HCl and 150 mM NaCl for 60 min at room temperature. The blocked PVDF was incubated with the first antibodies of anti-mouse monoclonal antibodies, Bcl-2 (1:1,000 dilution), procaspase-7 (1:1,000 dilution), procaspase-9 (1:500 dilution), PARP (1:500 dilution), and Actin (1:500) for 24 h at 4°C. Actin protein was used as protein amount control in SDS-PAGE. The incubated PVDF was washed in TTBS (3 × 10 min), and incubated with the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse antibody (1:20,000 dilution) for 1 h and then washed in TTBS (3 × 10 min). Super Signal West Pico chemiluminescent substrate was

added for 5 min at room temperature. The developed protein was photographed onto CL-XPosure film in dark room (Choudhuri *et al.*, 2002).

6.3.9 Statistical analysis

Statistical analysis was performed by ANOVA, using the least significant test to determine the level of significant at $P < 0.01$ and 0.05. All data were expressed as mean ± standard error (SE). For single comparisons, the significance of difference between means was determined by Student's *t*-test. The criterion for statistical significance was $P < 0.05$.

6.4 Results and discussion

6.4.1 Cell proliferation

Trypan blue exclusion, MTT, and Resazurin methods were used to measure the cell proliferation. According to Trypan blue exclusion method, the LC_{50} values at 6, 12, 18, and 24 h of PPEE (648.40 ± 37.54, 381.35 ± 13.72, 362.50 ± 13.82, and 347.83 ± 11.85 µg/ml, respectively) were more effective than PPWE (993.38 ± 65.35, 418.78 ± 15.47, 373.45 ± 9.48, and 365.93 ± 20.00 µg/ml, respectively) as shown in Table 6.1. Consistent with the MTT method, the LC_{50} values at 6, 12, 18, and 24 h of PPEE (737.13 ± 35.96, 415.48 ± 24.92, 395.50 ± 15.53, and 377.88 ± 13.14 µg/ml, respectively) were more effective than PPWE (1004.35 ± 66.70, 513.73 ± 23.19, 481.33 ± 21.79, and 459.90 ± 15.90 µg/ml, respectively) (Table 6.2). Similarly, under Resazurin methods, the LC_{50} values at 6, 12, 18, and 24 h of PPEE (960.15 ± 43.56, 816.73 ± 31.63, 526.05 ± 17.15, and 471.50 ± 13.62 µg/ml, respectively) were lower

than that of PPWE (1175.80 ± 55.39, 1044.10 ± 42.69, 660.60 ± 20.51, and 589.45 ± 16.21 μg/ml, respectively) (Table 6.3).

Based on these methods, it was observed that pomegranate peel extracts (PPEs) reduced cell proliferation in a dose- and time-dependent manner (Figures 6.1 and 6.2). The cell antiproliferation was significantly increased as the concentration of PPEs increased. Therefore, PPEs had the antiproliferative effect on MCF-7 cancer cells.

Pomegranate peel extract (PPE) could delay cell proliferation in many different human cancer cell lines (Kawaii and Lansky, 2004; Mavlyanov, Islambekov, Karimdzhanov, and Ismailov, 1997; Settheetham and Ishida, 1995). In human prostate cancer cells, DU-145, LNCaP, and PC-3 cells were more sensitive to PPE (Lansky *et al.*, 2005b; Lansky *et al.*, 2005a). Punicic acid, luteolin, ellagic acid, and caffeic acid, the phytochemicals in PPE, showed suppression of PC-3 prostate cancer cells invasion (Lansky *et al.*, 2005b; Lansky *et al.*, 2005a). Punicalagin, ellagic acid, and total pomegranate tannins showed greatest antiproliferative activity against HCT116 and HT-29 colon cancer cells (Seeram *et al.*, 2005). PPE were most pronounced against estrogen responsive MCF-7 cells, less pronounced against estrogen negative MDA-MB-231 cells, and least pronounced against immortalized normal breast epithelial cells MCF-10A (Toi *et al.*, 2003). Some chemicals in PPEs such as gallic acid, ellagic acid, tannic acid, quercetin, kaempferol, luteolin, and tannins synergistically played to inhibit the proliferation of CaCo-2 and HT-29 colon, MCF-7 and Hs578T breast, and DU145, LNCaP, and PC-3 prostatic cancer cells by inhibiting cell cycle progression, suppressing angiogenic factors, and inducing apoptosis (Ackland, van de Waarsenburg, and Jones, 2005; Bawadi, Bansode, Trappey II, Truax, and Losso, 2005; Fernandes *et al.*, 2009; Loizzo *et al.*, 2009; Saleem, Husheem, Harkonen, and Pihlaja, 2002). This

Table 6.1 *Effect of pomegranate peel extracts on the viability of MCF-7 cells by trypan blue exclusion assay.*

Extract	Concentration (μg/ml)	Cell viability (%)			
		6 h	12 h	18 h	24 h
PPEE	300	72.08 ± 5.53	70.36 ± 1.40	73.75 ± 2.23	80.70 ± 1.89
	400	57.06 ± 2.41	39.42 ± 7.19	20.68 ± 5.64	20.49 ± 3.21
	500	54.06 ± 1.38	9.23 ± 0.15	6.92 ± 0.28	7.39 ± 0.60
	LC_{50} (μg/ml)	648.40 ± 37.54[a]	381.35 ± 13.72	362.50 ± 13.82	347.83 ± 11.85
PPWE	400	71.22 ± 2.55	49.81 ± 1.87	45.30 ± 9.13	65.47 ± 1.54
	500	66.03 ± 2.96	28.81 ± 3.02	19.29 ± 1.24	16.17 ± 2.80
	600	65.56 ± 5.48	14.81 ± 3.36	10.21 ± 0.36	10.07 ± 0.82
	LC_{50} (μg/ml)	993.38 ± 65.35[b]	418.78 ± 15.47	373.45 ± 9.48	365.93 ± 20.00

PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract. The data are expressed as the mean ± SE, n = 4. Numbers with different letters within the same column are significantly different ($P < 0.05$).

Table 6.2 Effect of pomegranate peel extracts on the viability of MCF-7 cells by MTT assay.

Extract	Concentration (μg/ml)	Cell viability (%)			
		6 h	12 h	18 h	24 h
PPEE	300	93.93 ± 4.89	83.46 ± 1.12	85.49 ± 1.77	80.37 ± 1.32
	400	80.63 ± 2.13	58.73 ± 5.75	42.12 ± 4.97	31.49 ± 0.97
	500	77.97 ± 1.22	22.11 ± 0.27	17.87 ± 0.50	16.52 ± 0.93
	LC_{50} (μg/ml)	737.13 ± 35.96[a]	415.48 ± 24.92[a]	395.50 ± 15.53[a]	377.88 ± 13.14[a]
PPWE	400	93.17 ± 2.26	67.03 ± 1.50	62.93 ± 7.24	69.71 ± 1.08
	500	88.57 ± 2.62	50.25 ± 2.42	39.67 ± 2.19	30.18 ± 4.36
	600	83.30 ± 3.00	32.02 ± 5.97	23.66 ± 0.64	20.68 ± 1.28
	LC_{50} (μg/ml)	1,004.35 ± 66.70[b]	513.73 ± 23.19[b]	481.33 ± 21.79[b]	459.90 ± 15.90[b]

PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract. The data are expressed as the mean ± SE, n = 4. Numbers with different letters within the same column are significantly different ($P < 0.05$).

Table 6.3 Effect of pomegranate peel extracts on the viability of MCF-7 cells by Resazurin assay.

Extract	Concentration (μg/ml)	Cell viability (%)			
		6 h	12 h	18 h	24 h
PPEE	400	85.75 ± 1.05	88.64 ± 1.07	85.19 ± 4.70	72.18 ± 1.13
	500	82.62 ± 0.32	86.05 ± 2.17	71.41 ± 7.05	52.40 ± 1.81
	600	78.95 ± 3.45	65.30 ± 2.75	20.77 ± 2.09	7.85 ± 0.27
	LC_{50} (μg/ml)	960.15 ± 43.56[a]	816.73 ± 31.63[a]	526.05 ± 17.15[a]	471.50 ± 13.62[a]
PPWE	400	90.08 ± 4.14	92.96 ± 1.03	94.58 ± 4.24	93.07 ± 0.77
	500	82.17 ± 4.96	90.24 ± 1.26	81.03 ± 1.66	87.58 ± 1.22
	600	78.30 ± 4.99	81.37 ± 1.50	61.12 ± 2.09	54.73 ± 2.17
	LC_{50} (μg/ml)	1,175.80 ± 55.39[b]	1,044.10 ± 42.69[b]	660.60 ± 20.51[b]	589.45 ± 16.21[b]

PPEE, pomegranate peel ethanolic extract; PPWE, pomegranate peel water extract. The data are expressed as the mean ± SE, n = 4. Numbers with different letters within the same column are significantly different ($P < 0.05$).

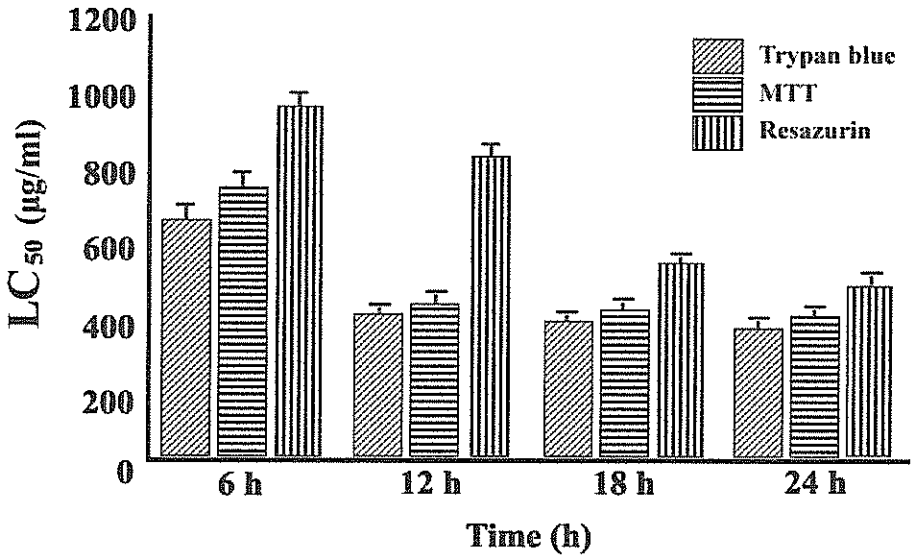

Figure 6.1 LC_{50} of antiproliferative activity of the PPEE-treated MCF-7 cells during 6-24 h. Data are expressed as means ± SE, n = 4.

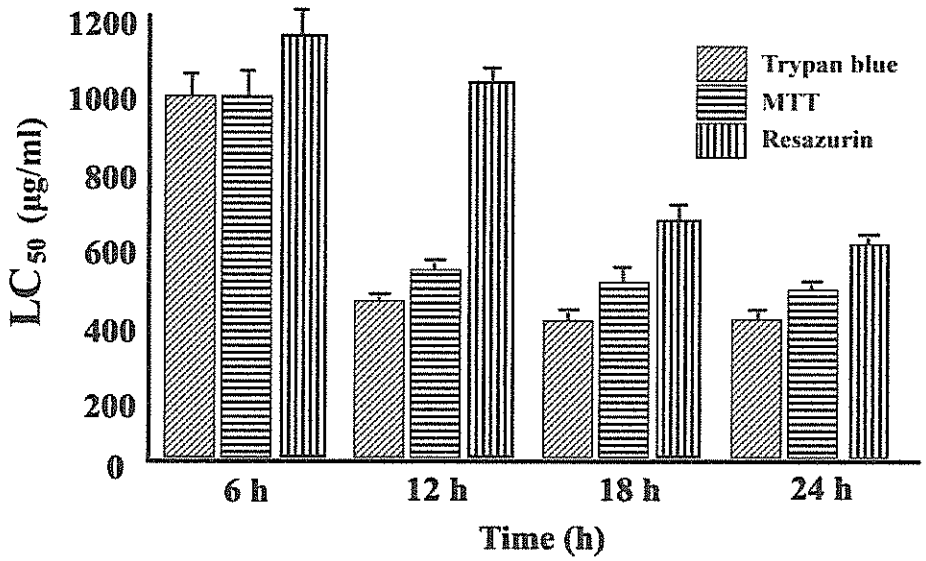

Figure 6.2 LC_{50} of antiproliferative activity of the PPWE-treated MCF-7 cells during 6-24 h. Data are expressed as means ± SE, n = 4.

study suggests that PPEs could be agents that induced MCF-7 cells death by apoptotic pathway.

6.4.2 Apoptotic cells and DNA fragmentation

PPEs could induce apoptosis of MCF-7 cell. There was a constant loss of cell viability after 24 h PPEs treatments (300-500 μg/ml). This study found that treated with 400 μg/ml PPEs nearly reduced 50% MCF-7 cell viability (Figures 6.3 and 6.4). On the second day, there was no living cells observed (data not shown). The apoptotic cell morphology of PPEs treated MCF-7 cells was distinguishable at 24 h of all treatments. Hoechst 33342 stained cells slightly exhibited condense nuclei and DNA fragmentation as represented by 400 μg/ml PPEs treated cells in Figure 6.5. This study conducted two additional experiments on the morphological and biochemical feature of the PPEs-treated cells to determine whether or not this cell death was apoptosis. Morphological change is a well-known biochemical characteristic of apoptosis and dose- and time-dependent condense nuclei and DNA fragmentation was observed (Wyllie, 1988). MCF-7 cells were treated with various concentrations of PPEs and incubated for 12-24 h. DNA of treated MCF-7 cells appeared that following dose- and time- dependent DNA fragmentation (Figures 6.6 and 6.7).

PPEs induced DNA fragmentation as dose- and time- dependent fashion. MCF-7 cells lack of caspase-3 of apoptosis which is the crucial protease and known as the executioner. Activation of caspase-3 leads to chromatin condensation, PARP cleavage, and DNA fragmentation. Coates *et al.* (2007); Larrosa *et al.* (2006) and Seeram *et al.* (2005) reported that anthocyanins, ellagic acid, punicalagin, and ellagitannins of pomegranate peel extract induced apoptosis in HT-29, HT115, HCT116, and Caco-2

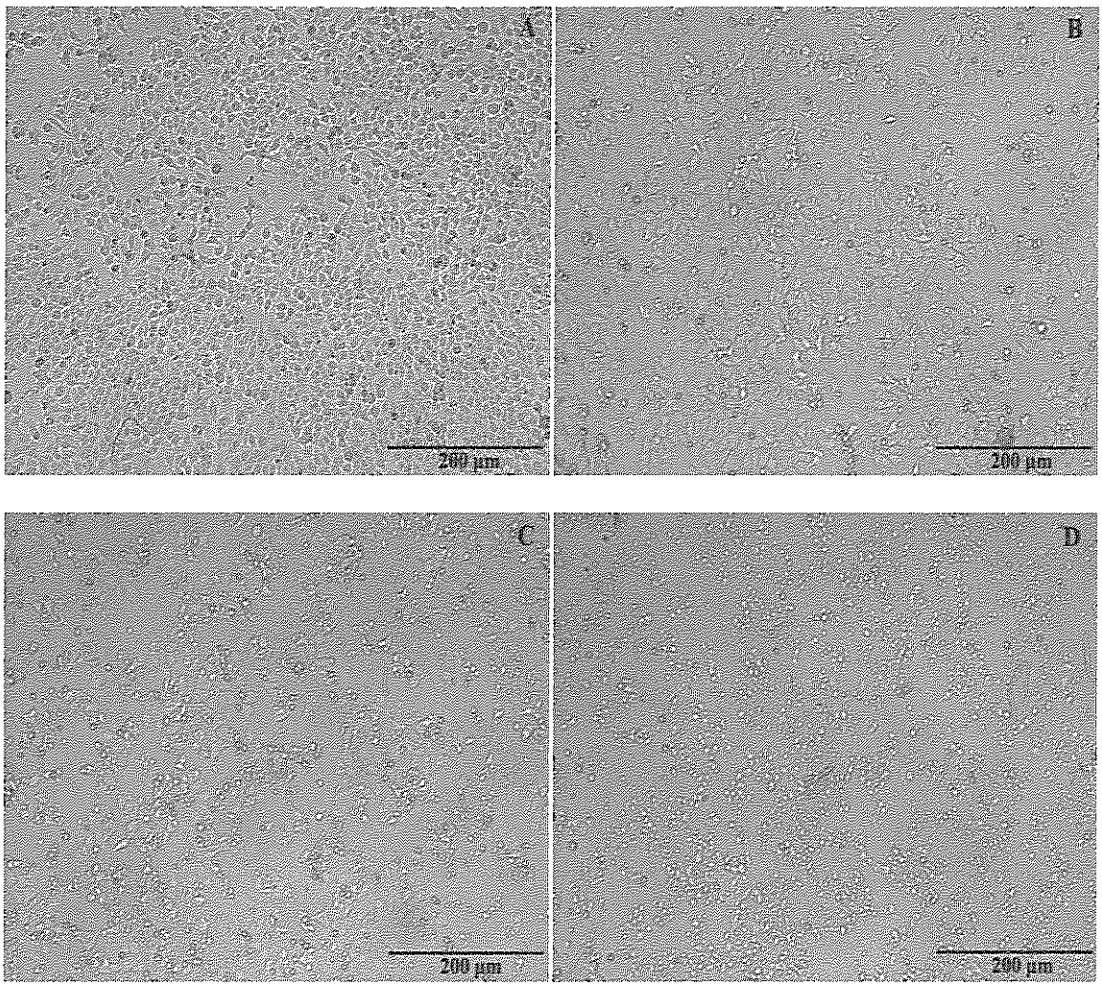

Figure 6.3 Viability and morphological changes in MCF-7 cells were observed under inverted microscopy (40 magnifications). The viability of MCF-7 cells was examined for 24 h after treatment with PPEE. A: untreated cells, B: PPEE 300 µg/ml, C: PPEE 400 µg/ml, D: PPEE 500 µg/ml. Bar = 200 µm.

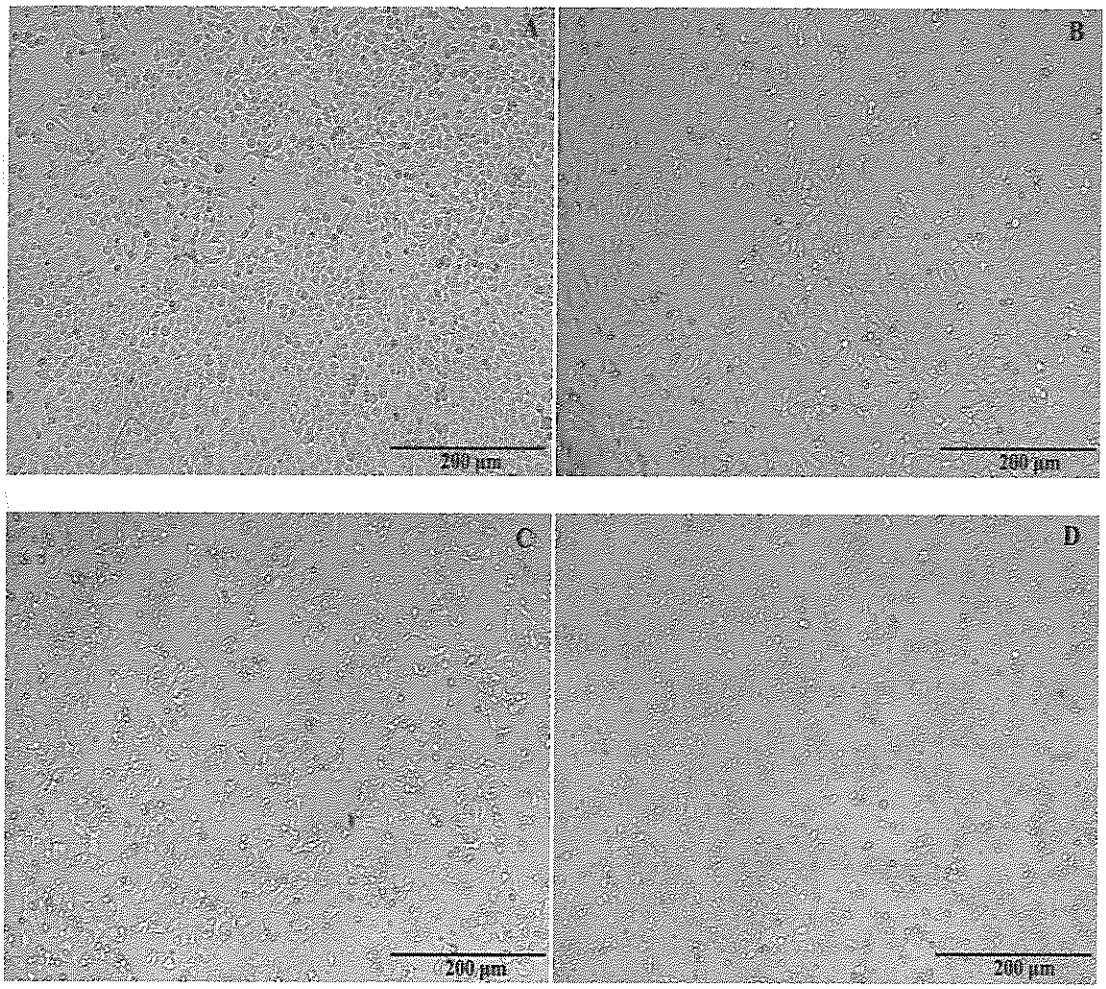

Figure 6.4 Viability and morphological changes in MCF-7 cells were observed under inverted microscopy (40 magnifications). The viability of MCF-7 cells was examined for 24 h after treatment with PPWE. A: untreated cells, B: PPWE 300 µg/ml, C: PPWE 400 µg/ml, D: PPWE 500 µg/ml. Bar = 200 µm.

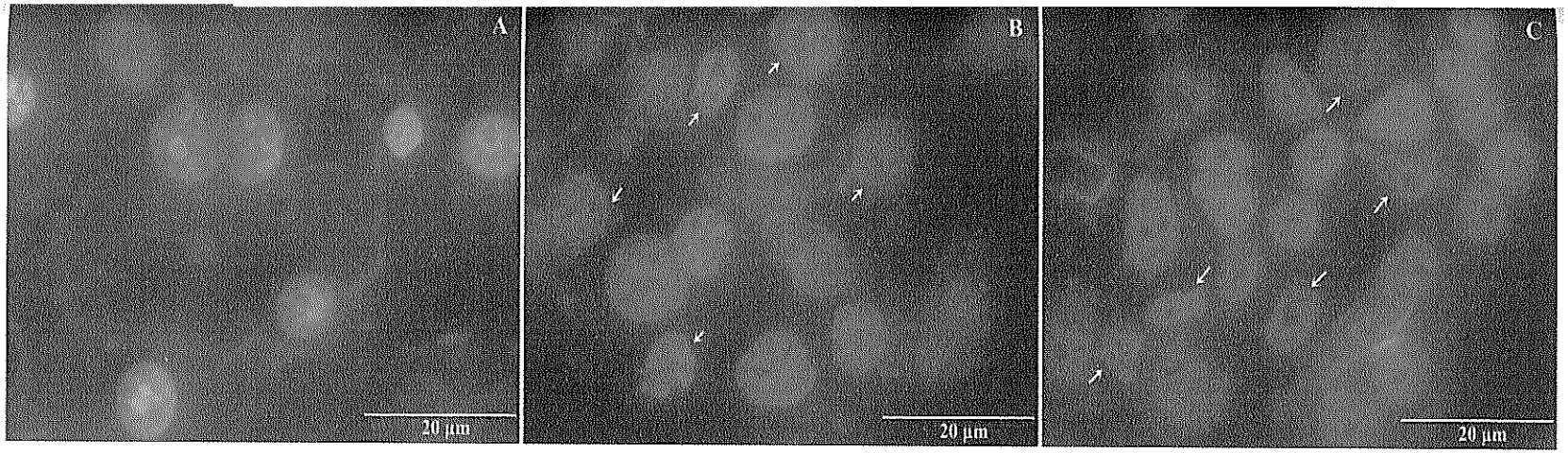

Figure 6.5 Morphological changes of PPEs-induced apoptotic MCF-7 cells (400 magnifications). The cells were incubated with pomegranate peel extracts for 24 h, fixed and stained with Hoechst 33342. A: untreated cells, B: 400 µg/ml PPEE, C: 400 µg/ml PPWE. Bar = 20 µm. Apoptotic cells (arrows).

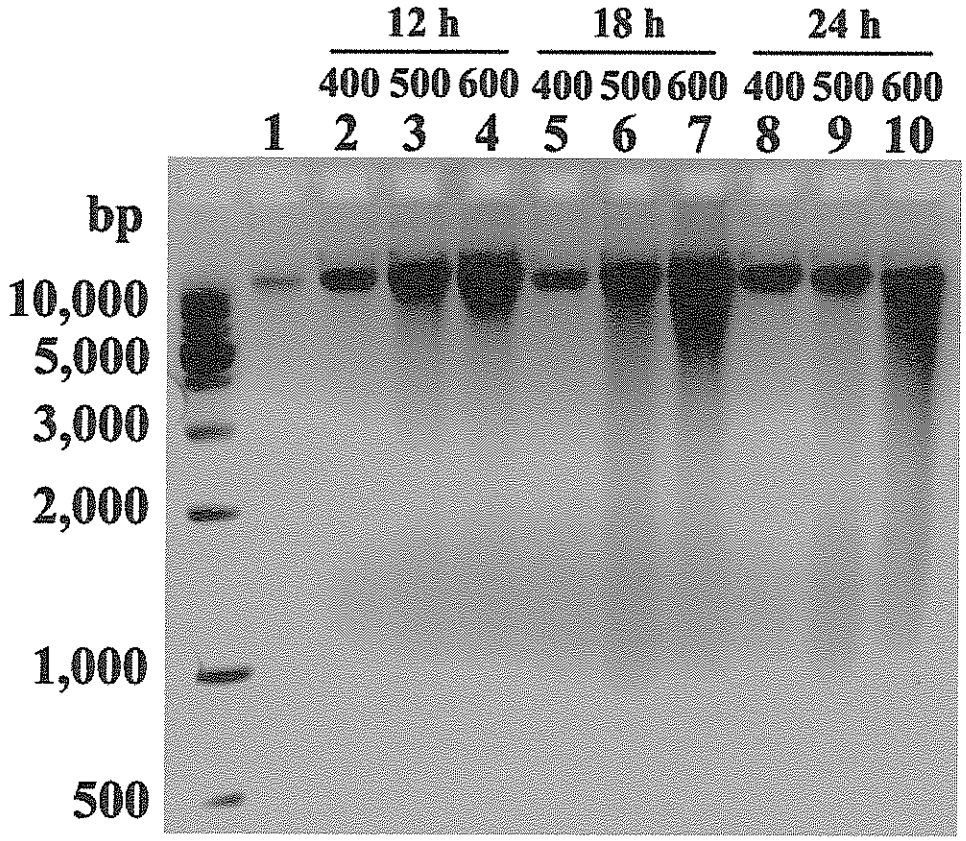

Figure 6.6 DNA fragmentation in MCF-7 cells treated with PPEE at designated concentrations and times. Lane 1: control. Lane 2-4: 400-600 μg/ml PPEE, 12 h. Lane 5-7: 400-600 μg/ml PPEE, 18 h. Lane 8-10: 400-600 μg/ml PPEE, 24 h.

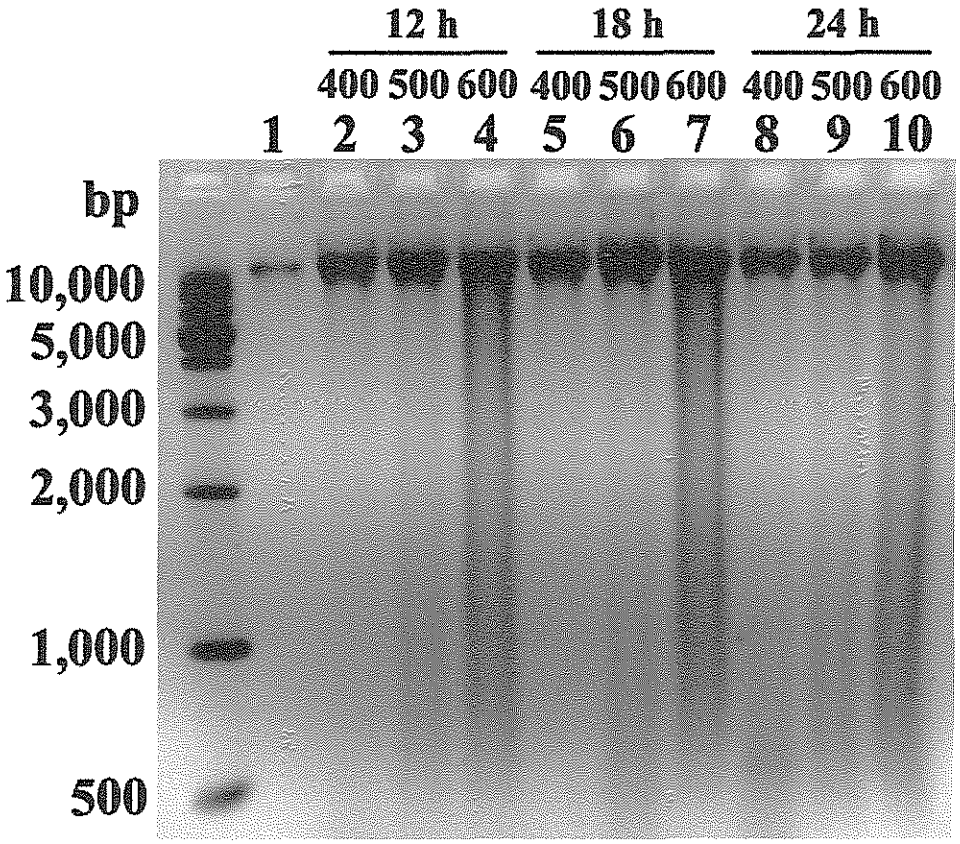

Figure 6.7 DNA fragmentation in MCF-7 cells treated with PPWE at designated concentrations and times. Lane 1: control. Lane 2-4: 400-600 μg/ml PPWE, 12 h. Lane 5-7: 400-600 μg/ml PPWE, 18 h. Lane 8-10: 400-600 μg/ml PPWE, 24 h.

colon cancer cells. Ellagic acid induced cell detachment, decreased cell viability, and induced apoptosis, as measured by DNA strand breaks and alterations of the cell cycle, in SH-SY5Y human neuroblastoma cells (Fjaeraa and Nanberg, 2009). The MCF-7 cell line is known to undergo cell death in response to stimulation of TNF/cycloheximide (TNF-c), staurosporine and other agents (Tewari *et al.*, 1995; Janicke *et al.*, 1998). TNF/cycloheximide-treated MCF-7 cells resulted in cell death but their ladder nuclear fragmentation was not observed (Mc Gee *et al.*, 2002). Less than 10% of photodynamic therapy (PDT)-treated MCF-7 cells were found to have condensed chromatin by 4 or 6 h post-PDT; however, 60% of the PDT-treated MCF-7c3 cells (*Casp3*-transfected MCF-7 cells) displayed these apoptotic features (Xue, Chiu, and Oleinick, 2001). These data supporting that caspase-3 was required for ladder DNA fragmentation and apoptotic body in apoptosis. There were at least two phases for DNA fragmentation in apoptotic cells (Walker, Kokileva, LeBlanc, and Sikorska, 1993). The first phase involved the generation of large DNA fragments of 50 and 300 kb and the second phase resulted in oligonucleosome-size fragments. PDT-treated MCF-7c3 cells demonstrated a strong DNA band of 50 kb, and the intensity of this band increased with time post-PDT. For PDT-treated MCF-7 cells, the 50 kb DNA band was slightly apparent at 20 h post-PDT, and its intensity was much weaker than that of PDT-treated MCF-7c3 cells at the same time point (Xue *et al.*, 2001). The results indicated that the rate and extent of apoptosis was much reduced in PDT-treated MCF-7 cells than in MCF-7c3 cells due to the deletion of caspase-3.

A weak *Bax* expression could be one of the mechanisms by which MCF-7 cells confered resistance against chemotherapy induced apoptosis, and that these agents could induce cell death by a common inhibiting pathway of *Bcl-2* (Sakakura *et al.*,

1997). Kagawa *et al.* (2001) showed that Bax overexpression produced more DNA fragmentation in *Casp3* (caspase-3)-transfected MCF-7 cells (24.2%) than it did in untransfected-MCF-7 (7.5%). Caspase-3-deficient MCF-7 cells failed to undergo morphological nuclear and DNA fragmentation, whereas *Casp3*-transfected MCF-7 displayed intact nuclear dismantling and DNA fragmentation (Kagawa *et al.*, 2001). Wolf, Schuler, Escheverri, and Green (1999) reported that only caspase-3 and caspase-7 participated to induce DNA fragmentation by inactivating ICAD. However, *in vitro*, caspase-3 inactivated ICAD and promoted DNA fragmentation more effectively than caspase-7. Perhaps in that particular instance, caspase-7 activation did not reach the level to initiate DNA fragmentation. Therefore, it could be assumed that PPEs was able to induce apoptosis in MCF-7 cells with untypically apoptotic features. The cells slightly showed cytoskeletal rearrangement, nuclei condensation, and DNA fragmentation. The cells were not blebbed. The death of MCF-7 cells would definitely processe as the results of PPEs induction.

6.4.3 Apoptosis modulation

In this experiment it is found that PPEs changed apoptotic proteins in MCF-7 cells at 12-24 h cultures as compared with control (Figures 6.8 (A) and (B)). The decreases Bcl-2, procaspase-9, and procaspase-7 were a time-dependent manner. In this study was especially focused on caspase-7-cleaved PARP induced by PPEs-induced MCF-7 apoptosis after PPEs signal transmission into the cells that caspase-9 was activated followed by the reaction of caspase-7, the cleavage of PARP (Figures 6.8 (A) and (B)).

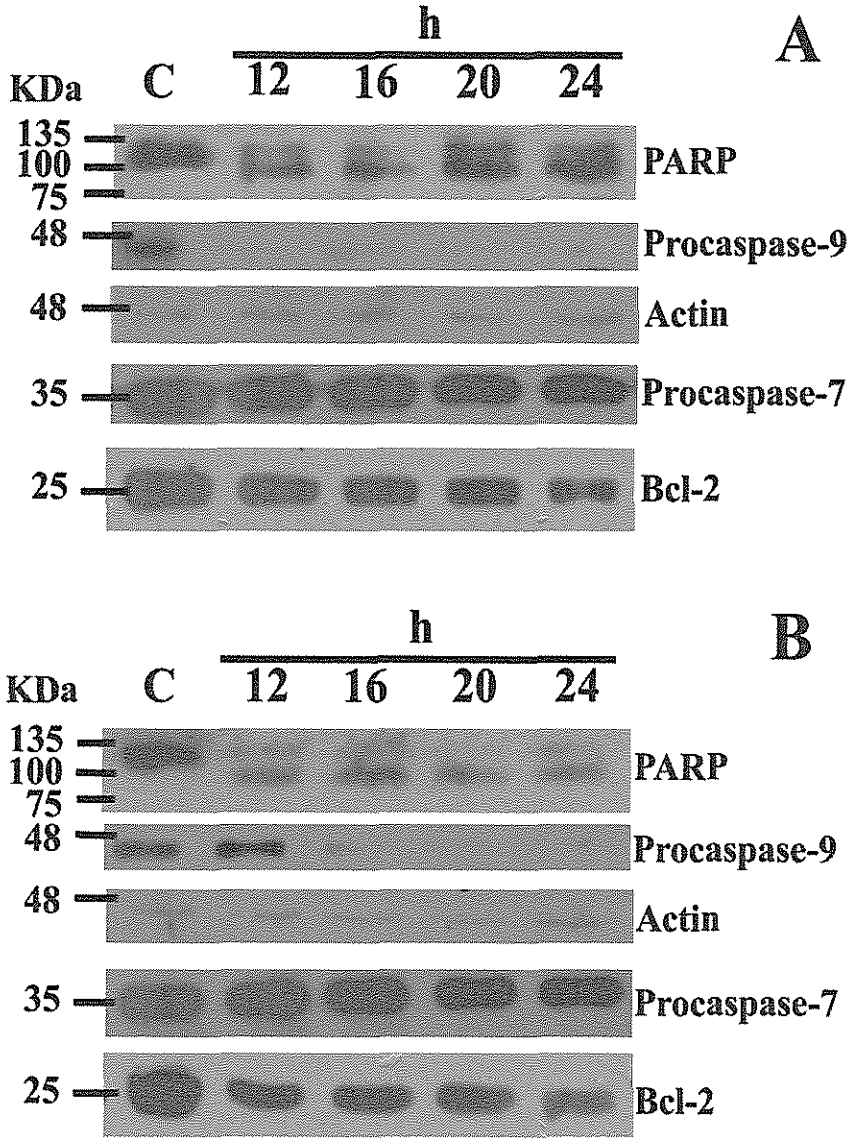

Figure 6.8 Effect of pomegranate peel ethanolic extracts (PPEs) on the expression of PARP, procaspase-9, procaspase-7 and Bcl-2 proteins. MCF-7 cells were treated with 500 μg/ml PPEs at different time intervals. Proteins of cell lysates (30 μg/well) were subjected to SDS-PAGE and western blot analysis. PARP, procaspase-9, procaspase-7 and Bcl-2 proteins were detected by using specific the monoclonal antibodies. Lane C: control. Panel A: 500 μg/ml PPEE treatment. Panel B: 500 μg/ml PPWE treatment.

It is well known that apoptosis can occur by two major pathways; the extrinsic pathway (Fas/FasL or TNF-αR/TNF-α) and the intrinsic pathway (mitochondrial pathway). The product of the *Bcl-2* gene is well known to play a role in promoting cell survival and inhibiting apoptosis in a variety of cancer cells, while the related protein Bax accelerates apoptosis and counters the cell survival function of *Bcl-2* (Korsmeyer, 1992). *Bcl-2* gene plays a role in breast cancer development since it is overexpressed in 70% of breast cancer specimens (Silvestrini *et al.*, 1994). *Bax* gene, another apoptosis-promoting element, is downregulated in breast cancer cells leading to cell resistant to apoptosis (Bargou *et al.*, 1995). *Bcl-2* gene is known in an association with the development of breast cancer by inhibiting apoptosis mediated by chemotherapy (Dole *et al.*, 1994), and blocking both p53-dependent and independent cell death pathway (Kamesaki *et al.*, 1993). Studies of the mechanism of apoptosis in response to other agents are consistent with a role for mitochondria. During the development of a cell-free model of apoptosis, a mitochondrially-enriched cell fraction was found to be an essential component (Newmeyer, Farschon, and Reed, 1994). Buprenorphine hydrochloride (Bph) induced very rapid apoptosis that related to overexpression of Bcl-2 protein causing inhibition of the Bph-induced apoptosis, in NG108-15 cell line (Kugawa, Ueno, and Aoki, 2000). Tumor necrosis factor-α (TNF) and epigalocatechin-3-gallate (EGCG) induced to a much greater increase in the level of reactive oxygen species (ROS). These results indicated that increased ROS generation and decreased mitochondrial membrane potential maybe involved in TNF and EGCG-induced apoptosis in MCF-7 cells (Hsuuw and Chan, 2007; Siemankowski, Morreale, and Briehl, 1999). TNF and EGCG induced apoptosis by modulating the expression of Bcl-2 family members. Bcl-2 functions as a suppressor of apoptosis while Bax normally

acts as a sensor of cellular damage and stress (Hsuuw and Chan, 2007; Siemankowski *et al.*, 1999). In response to significant damage or stress, Bax relocates to the mitochondrial surface and disrupts the normal function of the anti-apoptotic Bcl-2 protein. This process leads to loss of membrane potential and efflux of cytochrome c (Green and Reed, 1998). This findings that MCF-7 cells treated with PPEs could decrease Bcl-2 expression (Figures 6.8 (A) and (B)) and corresponding with the decline of cell proliferation (Tables 6.1, 6.2, and 6.3). It could be suggested that Bcl-2 responsed to PPEs-induced apoptosis of MCF-7 cells. Tannins could induce apoptosis of MCF-7 cells via intrinsic pathway (Soyocak, Cosan, Basaran, Gunes, and Degirmenci, 2011). Consistently, treatment of pomegranate fruit extract on CaCo-2 colon, A549 lung, PC-3 prostate, and MDA-MB-435 breast cancer cells showed on induction of Bax (proapoptotic) and down-regulation of Bcl-2 (antiapoptotic) (Khan *et al.*, 2007; Kim *et al.*, 2002; Larrosa *et al.*, 2006; Malik *et al.*, 2005). Therefore, it is strongly supported that PPEs-induced apoptosis in MCF-7 cells through the intrinsic pathway.

Except the previous study, the apoptosis of MCF-7 by PPEs could be possibly via the extrinsic pathway. FasL and TNF-α might activate either JNK leading to the inhibition of Bcl-2 or caspase-8 cleaving of Bid and truncated Bid (tBid). Cleaved Bid and tBid translocates into mitochondria leading to cytochrome c release (Debatin and Krammer, 2004; Wajant, 2002). Similarly, EGCG and catechin could activate extrinsic death pathway as demonstrated through an increase of caspase-8 expression levels (Ahmeda *et al.*, 2010; Alshatwi, 2010).

The released, cytochrome c forms a complex with Apaf-1 (apoptosis protease activating factor) and procaspase-9 producing apoptosome. Apoptosome is a key event

that triggers the activation of the caspase cascade, including caspases-3, -7 and -6, which execute the programme cell death (Debatin and Krammer, 2004; Wajant, 2002). Treatment of Caco-2 colon, MDA-MB-231 breast, and PC-3 prostatic cancer cells with ellagitannins, ellagic acid, EGCG, and proanthocyanidins could induce apoptosis via caspase-9 activation (Kim, Lee, Moon, and Choe, 2009; Larrosa *et al.*, 2006; Malik *et al.*, 2005). EGCG and ellagitannins could lead apoptosis of MCF-7 via initiator caspase-9 (Hsuuw and Chan, 2007; Losso, Bansode, Trappey II, Bawadi, and Truax, 2004). This study indicated that procaspase-9 declined after PPEs treatment in a time-dependent manner. These data suggested that PPEs induced apoptosis by regulating initiator caspase-9.

Caspases contributed to the drastic morphological changes associated with apoptosis by proteolyzing a number of key substrates, including the structural proteins such as c-fodrin, gelsolin, PARP, p21-activated kinase, focal adhesion kinase and DFF45 (Nicholson *et al.*, 1995; Rudel and Bokoch, 1997; Walsh *et al.*, 2008; Wen *et al.*, 1997). It is now well established that certain the upstream or initiator caspase-9 activates the downstream or effector caspases (caspases-3, -6, and -7). Caspase-3 is believed to be the primary executioner of apoptosis, since it is activated by many death signals and the major characteristics of apoptosis, such as cytoskeletal rearrangement, chromatin condensation, DNA fragmentation, and cleavage of PARP, depend on its activation. Nevertheless, caspase-3 deficiency, on the B6 background in mice, had little discernable impact on cell numbers, and survival (Houde *et al.*, 2004; Lakhani *et al.*, 2006; Leonard, Klocke, D'Sa, Flavell, and Roth, 2002). However, mice double deficient for *CASP-3* and *CASP-7* on the B6 background died immediately after birth because of defective heart development (Lakhani *et al.*, 2006). Caspase-3 and -7

preferentially cleaved DEVD-based peptide substrate with equal efficiency (Mc Gee *et al.*, 2002; Walsh *et al.*, 2008). Furthermore, caspase-3 and -7 both cleaved proteins such as ROCK I, RhoGDI, c-fodrin, PARP, and ICAD equally well (Walsh *et al.*, 2008). However, the majority of substrates such as Bid, gelsolin, XIAP, and caspase-6 were more efficiently cleaved by caspase-3 than caspase-7 (Walsh *et al.*, 2008). Mc Gee *et al.* (2002) suggested that caspase-7 is involved in the mechanism by which pyrrolo-1,5-benzoxazepine (PBOX-6) induces apoptosis in MCF-7 cells. Caspase-7 activity could be increased from 3 to 5 fold over than the basal levels, signifying an activation of this caspase in MCF-7 cells treated with *Phyllanthus* extracts (Lee, Jaganath, Wang, and Sekaran, 2011). So, caspase-7 is indeed responsible for the proteolysis of these proteins during the terminal phase of apoptosis in caspase-3-deficient MCF-7 cells. Consistently with the previous study, caspase-9 cleaved procaspase-7 which declined after PPEs treatment (Figures 6.8 (A) and (B)). Here, this study showed that caspase-9 as an initiator caspase activated procaspase-7 to caspase-7 and evaluated its activation after MCF-7 cells-treated PPEs. Therefore, procaspase-9 was activated first, and then followed by procaspase-7. Both were clearly as a time-dependent manner, 12-24 h after PPEs treatment.

The enzyme poly (ADP-ribose) polymerase or PARP, is an important DNA repair enzyme and was one first proteins identified as a substrate for caspases. The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3 and caspase-7 (Walsh *et al.*, 2008). Treatment of MCF-7 cells with PPEs showed cleavage PARP, a biochemical hallmark of apoptosis and processing of caspase-7 into a small active fragment (Figures 6.8 (A) and (B)). Similarly, PARP was excellent substrate for caspase-7 in treated caspase-3-deficient MCF-7 cells (Walsh *et*

al., 2008). Therefore, it could suggested that certain cleavage of PARP occurred by compensation of caspase-7 in MCF-7 cells.

6.5 Conclusion

This study is the frist report showing that pomegranate peel extracts possess anticancer property. PPEs could induce antiproliferative effect, apoptotic morphology and DNA fragmentation in caspase-3-deficient MCF-7 cells. Moreover, PPEs could reduce of Bcl-2 and activate procasepase-9 to casepase-9. After, caspase-9 could activate procaspase-7 to caspase-7, which induced to be capable of cleaving certain substrates such as PARP. Degradation of PARP reduced its ability in DNA damage repairing. Therefore, the apoptosis of MCF-7 by PPEs could possible via the intrinsic pathway. Interestingly, PPEs, a by-product from pomegranate juice making, might expect to be added-value by-products to be a potential natural source as anticancer treatment.

6.6 References

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APPENDICES

APPENDIX A

STANDARD CURVES

1. Total phenolic compounds

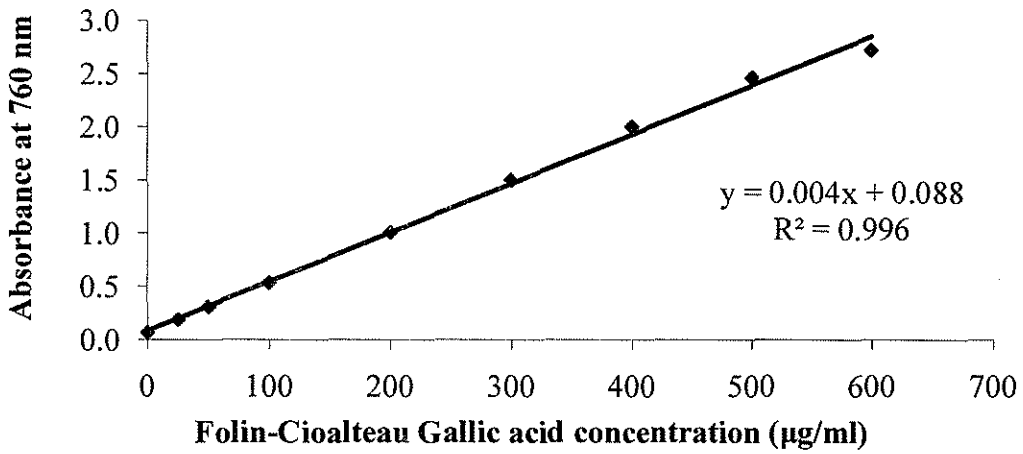

Figure A1 Calibration curve of gallic acid standard (µg/ml) measured by Folin-Cioalteau's method with slightly modification. Each value represents the mean ± SE (n=4).

2. Flavonoids content

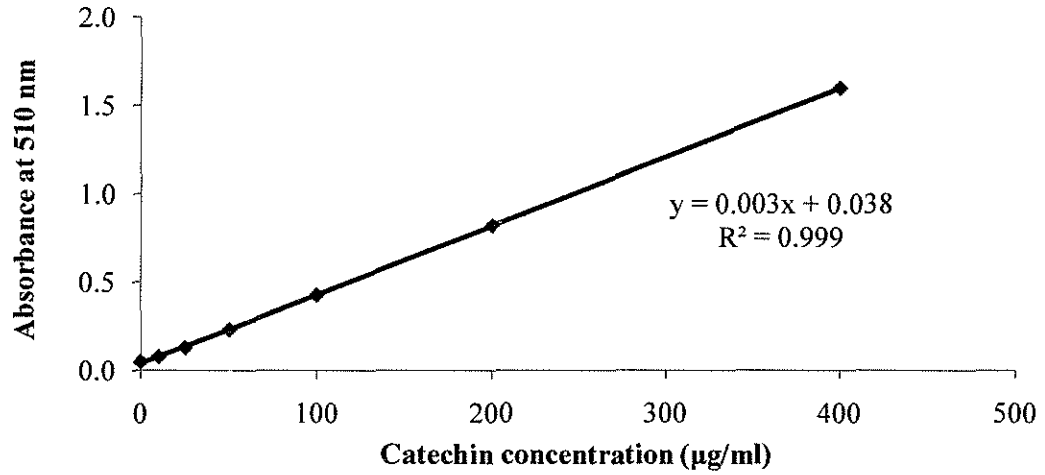

Figure A2 Calibration curve of catechin standard (µg/ml). Each value represents mean ± SE (n=4).

3. Ferric reducing antioxidative power

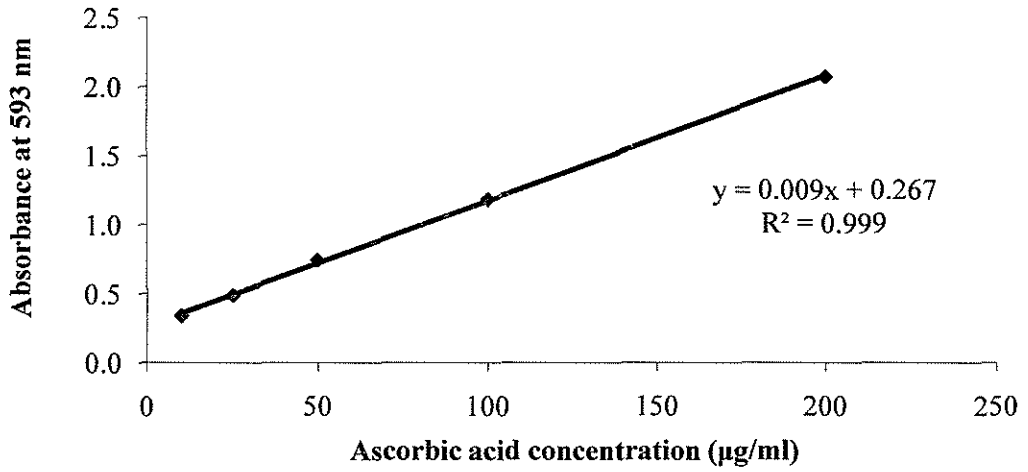

Figure A3 Representative ascorbic acid standard reference curve (µg/ml). Each value represents mean ± SE (n=4).

APPENDIX B

CYTOTOXICITY

1. Sample preparation for cytotoxicity test

Sixty four milligrams of pomegranate peel extracts (PPEs), pomegranate seed extracts (PSEs) and pomegranate juice (PJ) were dissolved in 80 µl of 0.08% (v/v) DMSO and mixed with 6,320 µl of artificial sea water to make 10,000 µg/ml of the final concentration. Different concentrations of PPEs, PSEs, and PJ were prepared from stock solution. Serial stock solution of the extracts and juice varied concentrations was further diluted using artificial sea water to attain the final concentration of each. The serial stock solution (800 µl) was transferred into each well of 24-well plates. Each well contained 10 larvae of brine shrimp, include the control group, and were filled to 1 ml total volume with artificial sea water. In each well, tested concentrations of PPEs were 500, 750, 1000, 1250, 1500, 1750, and 2000 µg/ml, and PSEs were 500, 1000, 1500, 2000, 2500, 3000, 3500, and 4000 µg/ml. Finally, PJ were 5000, 5500, 6000, 6500, and 7000 µg/ml. In addition, other levels were used when required. The final diluted DMSO concentration was set as a control group.

APPENDIX C

IN VIVO ASSAY IN RAT

1. Determination for minimal erythema doses (MED)

Minimal erythema doses (MED) means the minimum amount of UVB that produces redness 24 hours after exposure. MED can be calculated as following the fomular:

$$Fluence\left(\frac{J}{cm^2}\right) = Irradiance\left(\frac{W}{cm^2}\right) \times Time\ (sec)$$

Distance between rat skin and a UVB lamp is fixed at 13 cm, but time for irradiation is varied from 180 to 480 sec. Time will be increase every 60 sec. For this experiment, "1xMED" is between 0.07 and 0.08 J/cm^2 from time of 300 to 360 sec.

2. Sample preparation for UVB-irradiated protection test

Pomegranate peel extracts (PPEs) or EGCG	64	mg
70% Ethanol	85	µl
Sterile ultra purified water	85	µl
Propylene glycol	30	µl

PROCEDURE:

For ethanolic extract, firstly dissolve with 85 µl of 70% ethanol and then mix with 85 µl of distilled water. For water extract, do similary of the ethanolic extract but start with distilled water first and follow by 70% ethanol. Finally, for each, add 30 µl of propylene glycol to prepare sample (8 mg/cm^2/rat).

APPENDIX D

IN VITRO ASSAY IN MCF-7

1. D-MEM/F-12 (1X Culture media)

Commercial D-MEM/F-12 powder (GIBCO)	15.6	g
$NaHCO_3$ (GIBCO)	1.2	g
FBS (GIBCO)	100	ml
Streptomycin (GIBCO)	100	µg
Penicillin (GIBCO)	100	unit
Sterile ultra purified water add to	1,000	ml

PROCEDURE:

Mix the solution together and adjusted pH to 7.25. Then, sterilize the solution by the filtration using a sterile filter with a pore size of 0.4 µm. After that, the solution is stored at 8-12°C.

2. Phosphate buffer saline

NaCl (Sigma-Aldrich)	8	g
KCl (Sigma-Aldrich)	0.2	g
Na_2HPO_4 (Sigma-Aldrich)	1.25	g
KH_2PO_4 (Sigma-Aldrich)	0.2	g
Sterile ultra purified water add to	1,000	ml

PROCEDURE:

Mix the solution together and adjusted pH to 7.4. After, autoclave at 121°C and store at room temperature.

3. Freezing Medium

DMSO (Amresco)	5	ml
FBS (GIBCO)	15	ml
D-MEM/F-12 (GIBCO)	30	ml

PROCEDURE:

Mix the solution together under the sterile hood. The solution is aliquoted for now and stored at -20°C.

4. MTT stock

MTT (Molecular Probes)	100	mg
Phosphate buffer saline	20	ml

PROCEDURE:

Dissolve and sterilize powder by the filtration using a sterile filter with a pore size of 0.2 μm. The solution is aliquoted and stored at 4°C. After, dilute solution for cell proliferation assay at the ratio of 1: 10 (MTT stock: media).

5. Hoechst 33342 stock

Hoechst 33342 (Molecular Probes)	10	mg
Sterile ultra purified water	10	ml

PROCEDURE:

Dissolve and sterilize powder by the filtration using a sterile filter with a pore size of 0.2 μm. The solution is aliqouted and stored at -20°C. This solution is used for cellular staining at the ratio of 1: 100 (Hoechst stock: cell suspension).

6. Tris borate, EDTA (TBE)

Tris-base	5.4	g
Boric acid	2.75	g
Na_2EDTA	0.47	g
Sterile ultra purified water	500	ml

PROCEDURE:

Mix the solution together and store at room temperature.

7. Agarose gel electrophoresis

Agarose (Bio-Rad)	1	g
TBE	100	ml

PROCEDURE:

Mix the solution, heat it using microwave, and then stand at room temperature. After that, add 10 µl ethidium bromide, swirl gently and transfer to the cassette. Insert comb carefully into gel by without bubbles.

8. Radio immuno precipitation assay (RIPA) buffer

Tris-HCl 50 mM	5	ml
NaCl 150 mM	5	ml
Sodium orthovanadate 100 mM (Sigma-Aldrich)	0.5	ml
0.5% sodium deoxycholate (Sigma-Aldrich)	2.5	ml
0.1% sodium dodecyl sulphate (Amresco)	2.5	ml
1% Triton X-100 (Amresco)	0.5	ml
Sterile ultra purified water add to	45	ml
Protease inhibitor cocktail (10X) (Roche Applied Science)	5	ml

PROCEDURE:

Firstly, prepare "Incompleted RIPA buffer" and then store at -20°C. Dissolve and aliqoute "Protease inhibitor cocktail (10X)" and then store at -80°C. For cell lysis, "Completed RIPA buffer" is used at the ratio of 1: 9 (protease inhibitor cocktail (10X): Incompleted RIPA buffer).

9. Bradford reagent

Coomassie blue G 250 (Bio-Rad)	40	mg
95% ethanol	20	ml
85% phosphoric acid	40	ml
Sterile ultra purified water add to	400	ml

PROCEDURE:

Dissolve and filtrate powder by using Whatman no.1 filter paper. Then, store the solution at room temperature.

10. Loading buffer (5X)

Tris-HCl (pH 6.8) 1 M	0.6	ml
50% glycerol	5	ml
10% SDS	2	ml
1% β-mercaptoethanol	0.5	ml
1% bromophenol blue	1	ml
Sterile ultra purified water	0.9	ml

PROCEDURE:

Mix the solution together and adjuste pH to 6.8. The solution is filtrated using Whatman no.1 filter paper. After that, the solution was aliqouted and stored at -20°C.

11. Stock solution for gel electrophoresis of protein

Solution A:

Acrylamide	29.2	g
Bis-acrylamide	0.8	g
Sterile ultra purified water add to	100	ml

Solution B:

Tris-HCl (pH 8.8)	75	ml
10% SDS	4	ml
Sterile ultra purified water add to	100	ml

Solution C:

Tris-HCl (pH 6.8)	12.5	ml
10% SDS	1	ml
Sterile ultra purified water add to	25	ml

PROCEDURE:

Mix each solution and stored at 4°C.

12. Gel preparation

12% Separating gel for two mini gels

Solution A	4	ml
Solution B	2.5	ml
Sterile ultra purified water	3.5	ml
10% Ammonium persulfate	150	µl
TEMED	15	µl

5% Stacking gel for two mini gel

Solution A	0.67	ml
Solution C	1	ml
Sterile ultra purified water	2.3	ml
10% Ammonium persulfate	30	µl
TEMED	7	µl

PROCEDURE:

Swirl the solution gently and transfer it to the cassettes using a pipette and bulb. Insert the well-forming combs between the gel plates of the cassettes and tilt them at a slight angle to provide a way for air bubbles to escape.

13. Gel Electrophoresis buffer (1X)

Tris-base	3	g
Glycine	14.4	g
SDS	1	g
Sterile ultra purified water add to	1,000	ml

PROCEDURE:

Dissolve and mix powders together, and then adjust pH to 8.3.

14. Immunoblotting transfer buffer (1X)

Tris-base	3	g
Glycine	14.4	g
SDS	1	g
Sterile ultra purified water add to	1,800	ml
Methanol	200	ml

PROCEDURE:

Dissolve and mix powders together, and then adjust pH to 8.3.

CURRICULUM VITAE

Jinnawat Manasathien was born on January 16th, 1977, in Bangkok. He graduated with a Bachelor of Science degree in Animal Production Technology from Suranaree University of Technology in 1997. He received his Master of Science degree in Environmental Biology from Suranaree University of Technology in 2001. During his master's degree, he was supported by the Graduate Research Fund of Suranaree University of Technology. He received Outstanding Academic Performance Fund at Suranaree University of Technology during 2007 to 2008. And, he received Strategic Consortia for Capacity Building of University Faculties and Staff Ph.D. Scholarship from the Office of the Higher Education Commission, Ministry of Education, Thailand at Suranaree University of Technology during 2008 to 2011.