PROTECTIVE EFFECTS OF THAI POMEGRANATE JUICE ON OXIDATIVE STRESS INDUCED BY ISCHEMIA-REPERFUSION IN RAT SKELETAL MUSCLE

Kusuma Ruamthum

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biomedical Sciences

Suranaree University of Technology

Academic Year 2016

ผลการป้องกันของน้ำทับทิมพันธุ์ไทยต่อการเหนี่ยวนำภาวะเครียดออกซิเดชัน โดยอิสคีมิครีเพอฟิวชันในกล้ามเนื้อโครงร่างของหนู

นางสาวกุสุมา รวมธรรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีวเวชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2559

PROTECTIVE EFFECTS OF THAI POMEGRANATE JUICE ON OXIDATIVE STRESS INDUCED BY ISCHEMIA-REPERFUSION IN RAT SKELETAL MUSCLE

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

Thesis Examining Committee

G. Eumkeb

(Assoc. Prof. Dr. Griangsak Eumkeb)

Chairperson

Rungrudee Srisawat

(Asst. Prof. Dr. Rungrudee Srisawat)

Member (Thesis Advisor)

Sajeera Kupittayanant

(Assoc. Prof. Dr. Sajeera Kupittayanant)

Member

Atcharaporn T.

(Dr. Atcharaporn Thaeowmor)

Member

(Prof. Dr. Sukit Limpijumnong)

Vice Rector for Academic Affairs and Innovation

(Prof. Dr. Santi Meansiri)

Dean of Institute of Science

กุสุมา รวมธรรม : ผลการป้องกันของน้ำทับทิมพันธุ์ไทยต่อการเหนี่ยวนำภาวะเครียดออกซิเดชันโดยอิสคีมิครีเพอฟิวชันในกล้ามเนื้อโครงร่างของหนู (PROTECTIVE EFFECTS OF THAI POMEGRANATE JUICE ON OXIDATIVE STRESS INDUCED BY ISCHEMIA-REPERFUSION IN RAT SKELETAL MUSCLE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รุ่งฤดี ศรีสวัสดิ์, 135 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาองค์ประกอบทางเคมีองค์ประกอบสารพฤกษาเคมี ปริมาณสารประกอบโพลีฟีนอล และสมบัติการต้านอนุมูลอิสระของน้ำทับทิมพันธุ์ไทยที่ถูกทำให้เข้มข้นด้วยวิธีการที่แตกต่างกัน รวมทั้งศึกษาผลการป้องกันของน้ำทับทิมพันธุ์ไทยต่อการเหนี่ยวนำภาวะเครียดออกซิเดชันและการเปลี่ยนแปลงทางสัณฐานวิทยาของเซลล์กล้ามเนื้อโครงร่างของหนูขาว พบว่าน้ำทับทิมพันธุ์ไทยสดประกอบด้วยโปรตีน คาร์โบไฮเดรต น้ำตาล (กลูโคสและฟรุกโทส) วิตามิน (ซีและอี) และแร่ธาตุ (แคลเซียม ฟอสฟอรัส โซเดียม โพแทสเซียม และแมกนีเซียม) ฤทธิ์ต้านอนุมูลอิสระและปริมาณสารประกอบโพลีฟีนอลของน้ำทับทิมพันธุ์ไทยที่ทำให้เข้มข้นโดยเครื่องกลั่นระเหยแบบหมุนภายใต้สุญญากาศ และเครื่องไมโครเวฟไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ การให้น้ำทับทิมพันธุ์ไทยทางกระเพาะอาหารในขนาดน้อย กลาง และสูง หนึ่งชั่วโมงก่อนการไหลกลับของเลือดมีผลต้านอนุมูลอิสระในกล้ามเนื้อโครงร่างหนูขาว โดยดูปริมาณของเอนไซม์ต้านอนุมูลอิสระ (ซุปเปอร์ออกไซด์ดิสมิวเทส และคะตะเลส) สารต้านอนุมูลอิสระที่ไม่ใช่เอนไซม์ (กลูตาไธโอน) และสารที่บ่งชี้ถึงสภาวะการทำลายของผนังเซลล์ (มาลอนไดดีไฮด์) ที่อยู่ในกล้ามเนื้อแกสตรอคนีเมียสที่มีการเหนี่ยวนำภาวะเครียดออกซิเดชันโดยอิสคีมิครีเพอฟิวชัน (การเหนี่ยวนำที่หยุดการไหลของเลือดที่ไปเลี้ยงกล้ามเนื้อเป็นเวลา 4 ชั่วโมงและปล่อยให้เลือดกลับไปไหลอีกครั้ง 2 ชั่วโมง) ฤทธิ์ของซุปเปอร์ออกไซด์ดิสมิวเทสและคะตะเลสและระดับของกลูตาไธโอนในกล้ามเนื้อของกลุ่มที่ได้รับน้ำทับทิมพันธุ์ไทย มีค่ามากกว่ากลุ่มที่ควบคุมที่ได้รับน้ำอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) และปริมาณของมาลอนไดดีไฮด์ของกลุ่มที่ได้รับน้ำทับทิมพันธุ์ไทย มีค่าน้อยกว่ากลุ่มควบคุมที่ได้รับน้ำอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) โดยสรุปสารต้านอนุมูลอิสระของน้ำทับทิมพันธุ์ไทยมีส่วนในการลดภาวะเครียดออกซิเดชันดังนั้น

การดื่มน้ำทับทิมพันธุ์ไทยอาจมีประโยชน์สำหรับการป้องกันภาวะเครียดออกซิเดชันที่เกิดขึ้นจากหลายสาเหตุ เช่น การออกกำลังกายจนหมดแรงที่เหนี่ยวนำให้เกิดการสร้างอนุมูลอิสระ

สาขาวิชาปรีคลินิก
ปีการศึกษา 2559

ลายมือชื่อนักศึกษา กุสุมา รวมสาร
ลายมือชื่ออาจารย์ที่ปรึกษา

KUSUMA RUAMTHUM : PROTECTIVE EFFECTS OF THAI POMEGRANATE JUICE ON OXIDATIVE STRESS INDUCED BY ISCHEMIA-REPERFUSION IN RAT SKELETAL MUSCLE. THESIS ADVISOR : ASST.PROF. RUNGRUDEE SRISAWAT, Ph.D. 135 PP.

THAI POMEGRANATE JUICE/ OXIDATIVE STRESS/ ISCHEMIA-REPERFUSION/ SKELETAL MUSCLE

The present study aimed to investigate the chemical composition, phytochemical composition, total phenolic content (TPC) antioxidant activity of Thai pomegranate juice (TPJ), and the protective effects of TPJ on oxidative stress and morphological changes of rat skeletal muscle cell induced by ischemia-reperfusion. Fresh TPJ was found to contain protein, carbohydrate, sugars (glucose and fructose), vitamins (E and C) and minerals (calcium, phosphorus, sodium, potassium, and magnesium). The antioxidant activity and total phenolic content (TPC) of concentrated TPJs by rotary vacuum evaporation and microwave evaporation were not significantly different. Intragastric injection of low, middle, and high doses of TPJ 1 h before reperfusion had an effect on antioxidant activity in rat skeletal muscle by measuring the levels of enzyme antioxidants (superoxide dismutase and catalase), non-enzyme antioxidants (glutathione), and the marker of lipid peroxidation (malondialdehyde, MDA) in gastrocnemius muscle induced by ischemia-reperfusion (the muscle was induced 4 h of ischemia and 2 h of reperfusion). The activities of superoxide dismutase and catalase, and the levels of glutathione in skeletal muscle of TPJ-treated groups were significantly higher than vehicle control group. Moreover,

MDA levels in skeletal muscle TPJ-treated groups were significantly lower than vehicle control group. In conclusion, the antioxidants in TPJ can contribute to the reduction of oxidative stress. Thus, the drinking of TPJ may be useful for protecting oxidative stress occurring from many causes, such as exhaustive exercise-induced free radicals production.

School of Preclinic

Academic Year 2016

Student's Signature Kusuma Ruamthum

Advisor's Signature Rungrudee Srisawat

ACKNOWLEDGEMENTS

I would like to express my deepest and sincere gratitude to my advisor, Assistant Professor Dr. Rungrudee Srisawat for her kindness in providing an opportunity to be her advisee. I am also appreciated for her kind suggestions, contributions, supporting, guidance, encouragement and criticism throughout the course of my study. I would like to express my greatest appreciation and sincere gratitude to all my thesis committees, Associate Professor Dr. Sajeera Kuplttayanant, and Dr. Atcharaporn Thaeomor for giving the comments, and suggestions. I would like to thank to all my teachers in School of Preclinic, Suranaree University of Technology for helpfulness, encouragement, effort and knowledge. In the various laboratories and works, I am grateful to my colleagues and my friends for assistance and encouragement during the studies. I am also grateful to all staff members of the Center for Scientific and Technological Equipment, Suranaree University of Technology for their help and assistance throughout the period of this research work. This study was supported by the National Research Council of Thailand (NRCT) and SUT outstanding academic performance scholarships for providing the financial support and giving me the opportunity to conduct and complete my thesis.

Finally, I would also like to express my sincere gratitude and appreciation to my family for their support, give me a chance to study.

Kusuma Ruamthum

CONTENTS

CONTENTS (Continued)

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

Figure **Page**

CHAPTER I

INTRODUCTION

1.1 Introduction

In recent years, the world health organization (WHO) reported an increase of crude death rate from diabetes mellitus, liver cancer, ischemic heart diseases, stroke, and kidney diseases in Thai population during 2000-2012 (WHO, 2015). Currently, the Thai health promotion foundation is embarking on a campaign to promote stop eating junk food and turn to eat healthy foods such as fruits and vegetables. Vegetables and fruits are sources of vitamins, minerals, fibers, plant protein, carotene, carotenoid, and antioxidants. Eating a diet rich of vegetables and fruits can reduce risk of obesity, heart disease, stroke, diabetes and cancer (WHO, 2016).

Pomegranate juice possesses a healthful antioxidant property. Effects of prolonged pomegranate juice ingestion on systemic oxidative stress protection were demonstrated in mice (Faria *et al.*, 2007). Significant decreases in oxidative biomarkers for the damage of lipids, proteins and DNA were found after prolonged pomegranate juice injection. In term of improving antioxidant function in the elderly subjects, the effect of pomegranate juice was found to be better than the effect of apple juice (Guo *et al.*, 2008). Pomegranate juice, which is high in polyphenols, could improve antioxidant function and reduce oxidative damage to macromolecules significantly in aged rats. In aged rats, pomegranate juice showed significantly higher serum antioxidant capacity than control group. The concentration of serum protein

carbonyl level was significantly decreased in pomegranate juice as compared to apple juice (Xu *et al.*, 2005).

Phenolic compounds, abundantly found in pomegranate juice, are attributed to antioxidant activity that can eliminate free radicals. Pomegranate juice has been considered to be a rich source of antioxidants and total polyphenols content. Pomegranate juice contains many components such as phenolic, tannins, ellagic acid, gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, catechin, phloridzin, quercetin, anthocyanins, punicalagin, phytoestrogens, flavonoids and tannins (Gil *et al.*, 2000). Many previous studies have been reported about the antioxidant activity of pomegranate juice. *In vitro* study, the antioxidant activity of pomegranate juice evaluated by ABTS, DPPH, DMPD, and FRAP has been shown to be better than green tea and red wine (Gil *et al.*, 2000). In human study, the impact of pomegranate juice on biomarker of oxidative stress, muscle damage and inflammation induced by exercise in healthy young men was evaluated. Decrease of malondialdehyde (MDA), a marker for oxidative stress, was found in pomegranate administered group. Lactate dehydrogenase (LDH), C-reactive protein (CRP) and creatine kinase (CK) were not significant in any of the groups (Bayat *et al.*, 2015).

Oxygen free radicals are considered to be important component involving in the physio-pathological tissue alterations observed during ischemia-reperfusion. Ischemia-reperfusion of skeletal muscle can trigger a series of deleterious phenomena in tissue, such as free radical production by the ischemia cells (Choudhury *et al.*, 1991). Free radicals have harmful effects on lipids, proteins, carbohydrates, and nucleic acids that can cause cell injuries. Cell injuries caused by free radicals can be

prevented by antioxidant defense mechanism. Pomegranate juice possesses high antioxidant capacity and antioxidants found in pomegranate juice may prevent the muscles from oxidative stress. Therefore, this study investigated the antioxidant effects of pomegranate juice on oxidative stress induced by ischemia-reperfusion (I/R) in skeletal muscle in the rat model through evaluation of biochemical markers of oxidative stress in the muscle tissue.

1.2 Research objectives

The present study aimed to

1. determine chemical composition, phytochemical composition and total phenolic content (TPC) of Thai pomegranate juice in different concentration methods,

2. determine antioxidant activity of Thai pomegranate juice,

3. determine the best concentration method by comparison of time for evaporation, total phenolic content (TPC) and antioxidant activity of Thai pomegranate juice,

4. examine the protective effects of Thai pomegranate juice on ischemia-reperfusion-induced (I/R-induced) oxidative stress in rat skeletal muscle,

5. examine the effects of Thai pomegranate juice on morphological changes of muscle cell in ischemia-reperfusion-induced (I/R-induced) oxidative stress in rat skeletal muscle.

1.3 Research hypothesis

Thai pomegranate juice can exert beneficial antioxidant effects in reducing and preventing muscle injury during ischemia-reperfusion.

CHAPTER II

LITERATURE REVIEW

2.1 Pomegranate (*Punica granatum* L.)

The pomegranate family has a single genus Punica with two species, *Punica granatum* and *Punica protopunica*. Pomegranate (*Punica granatum* L.) is one of the oldest fruit that widely grown in several regions of the world. Pomegranate is one of the most popular nutrition and healthy fruits. Pomegranate is a native fruit from Iran (Mousavinejad, Emam-Djomeh, Rezaei, and Khodaparast, 2009), the Himalayas in north India (Opara, Al-Ani, and Al-Shuaibi, 2009) and possible to some surrounding area. It is primarily cultivated in Mediterranean countries, Turkey (Ercisli *et al.*, 2007), United States, Afghanistan, Russia, India, China (Li *et al.*, 2015), Japan and Thailand. All parts of the pomegranate have different medicinal benefits. The rind of the pomegranate fruit and bark of the pomegranate tree have been used as a traditional cure against diarrhea, dysentery, and intestinal parasites. The seeds and juice are considered to be a tonic for the heart, throat, eyes, stopping nose bleeds and gum bleeds (Bhowmik *et al.*, 2013). Pomegranate (*Punica granatum* L.) is one of the most popular nutritious and healthy fruits. Pomegranate fruit possesses various biological properties such as antioxidant activity, anti-inflammatory activity, antibacterial, antifungal, anticancer, anti-proliferative, and anti-allergic activity. Pomegranate fruit is an antioxidant-rich food that contains three types of antioxidant polyphenols, including tannins, anthocyanins, and ellagic acid (Qu, Breksa Iii, Pan, and Ma, 2012;

Panichayupakaranant, Tewtrakul, and Yuenyongsawad, 2010; Guo *et al*., 2003; Dahham, Ali, Tabassum, and Khan, 2010; Jeune, Kumi-Diaka, and Brown, 2005; Seeram *et al*., 2005).

Thai pomegranate family is Lythraceae, botanical name is *Punica granatum* L. and the native name is Thap Thim (Figure 1 and 2).

Figure 1 Pomegranate tree and its fruits.

Figure 2 Thai pomegranate (*Punica granatum L.*), or Thap Thim, Fruits from Pakchong, Nakhon Ratchasima, Thailand.

Pomegranate (*Punica granatum* L.) juice

Pomegranate (*Punica granatum*) juice taken from pomegranate fruit is a rich source of antioxidants and total polyphenols content. Pomegranate juice contains many components such as sugar content (fructose, glucose), major acids (citric and

malic acids) (Tezcan, Gültekin-Özgüven, Diken, Özçelik, and Erim, 2009), phenolic, tannins, ellagic acid, gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, catechin, phloridzin, quercetin, anthocyanins, punicalagin, phytoestrogens, flavonoids, and tannins (Gil *et al.*, 2000). Pomegranate juice had showed highly amount of punicalagin. Primary polyphenol in pomegranate juice of pomegranate cultivars in China was punicalagin, punicalagins are tannins. The results were showed punicalagins concentration of the 10 pomegranate juice ranged from 298.99 to1042.93 μg/mL (Li *et al.*, 2015). The study of pomegranate juices (Punica granatum L.) of eight Iranian cultivars were determined components by high performance liquid chromatography coupled to UV–vis detector (HPLC–UV), total phenolic content (determination by Folin–Ciocalteu method), and antioxidant activities (determination by and 2,2-diphenyl-1-picrylhydrazyl (DPPH) method). The significant anthocyanins were delphinidin 3,5-diglucoside (372–5301 mg/l) followed by cyanidin 3,5-diglucoside (242–2361 mg/l), delphinidin 3-glucoside (49–1042 mg/l) and pelargonidin 3,5-diglucoside (7–90 mg/l), respectively. The highest level of total tannins was found in Sweet Alak cultivar (3 mg/l). Saveh Black Leather presented the highest level of ellagic acid (160 mg/l). Antioxidant activity varied among the cultivars (18–42 Trolox equivalents antioxidant capacity) and was directly related to the total phenolics in each type of juice (Mousavinejad *et al.*, 2009). Pomegranate juice from Spain was determined total phenolic content (TPC), total antioxidant activity (TAA), and antioxidant vitamin composition (A, C, and E). Total phenolic content was determined by using Folin-Ciocalteu (FC) method. An antioxidant activity was used Ferric reduction ability power (FRAP assay). Vitamin assessments were conducted by using high performance liquid chromatography detector (HPLC).

An antioxidant vitamin composition in pomegranate juice presented vitamin A was 22.8±0.69 µg/100 g, vitamin C was 57.8±0.59 mg/100 g, and vitamin E was 0.07±0.01 mg/100 g. Total phenolic content in pomegranate juice was showed 2696±49 mgGAE/L, and antioxidant activity was 32±5.1mmol/L (Anahita, Asmah, and Fauziah, 2015). Twenty pomegranate cultivars grown in Iran showed total soluble solids content varied from 11.37 (°Brix) to 15.07 (°Brix), pH values from 3.16 to 4.09, titratable acidity content from 0.33 g 100 g/L to 2.44 g 100 g/L and total sugars content from 13.23 g 100 g/L to 21.72 g 100 g/L. The results also showed that the values of ascorbic acid ranged from 9.91 mg 100 g/L to 20.92 mg 100 g/L. The total anthocyanins content was observed in pomegranate cultivars between 5.56 mg 100 g/L and 30.11 mg 100 g/L. The level of total phenolics was varied from 295.79 mg 100 g/L to 985.37 mg 100 g/L. The antioxidant activity of pomegranate cultivars was presented between 15.59 and 40.72% (Tehranifar *et al.*, 2010). Pomegranate juice has been reported to possess various properties, including anti-proliferative, anti-carcinogenic, anti-microbial, anti-viral, and anti-atherosclerotic activities. Pomegranate juice is a rich source of tannins that possess anti-atherosclerotic properties, anti-aging effect, and potent anti-oxidative characteristics. Moreover, pomegranate juice consumption has been found to have several medical benefits to prevent and treat a wide variety of diseases such as cancer (Wang, Ho, Glackin, and Martins-Green, 2012), cardiovascular disease (Fuhrman, Volkova, and Aviram, 2005), diabetes (Rosenblat, Hayek, and Aviram, 2006), Alzheimer's disease (Hartman *et al.*, 2006) and Parkinson's disease (Tapias, Cannon, and Greenamyre, 2014). The health benefits of pomegranate juice are attributed to its antioxidant activity and high total polyphenol content. Antioxidant activities of pomegranate juice were also

reported in many research studies. Daily consumption of the pomegranate juice during exercise improving antioxidant function was demonstrated in young healthy male. The subjects were randomly divided into two groups, the subjects of first group received one cup of pomegranate juice and second group received water daily for two week in each groups. The subjects were given exhaustive exercise and collected the blood. Glutathione peroxidation, superoxide dismutase, and antioxidant capacity in group received the pomegranate juice was significantly increased. Moreover, it was shown that the pomegranate juice could protect exhaustive exercise inducing in young healthy males (Mazani *et al.*, 2004). The effect of pomegranate on antioxidant was shown against lipid peroxidation of whole plasma in mice. Pomegranate juice consumption could reduce cellular lipid peroxidation and superoxide release in mice (Aviram *et al.*, 2000). All of the participants were separated into two groups by design the treatment group received pomegranate pure juice (500 ml/day containing polyphenol contents of 1685 mg GAE/L) and placebo group received water and exercise for 30 min. The effect of pomegranate juice consumption was attenuated post-exercise oxidative stress and reduced MDA level. In addition, the effect of exercises induced lipid peroxidation (MDA) was significantly decrease in participants received pomegranate pure juice more than placebo received water group (Al-Dujaili, 2016).

Safety of pomegranate fruit

The study of acute toxicities of the pomegranate fruit extracts shown LD 50 of oral administered in rats and mice was greater than 5 g/kg body weight. While, LD 50 of intraperitoneal administered was showed at 217 and 187 mg/kg body weight. Moreover, the investigation of subchronic toxicities of pomegranate extract at dose

600 mg/kg body weight/day shown no observed adverse effect in rats during 90 days (Patel, Dadhaniya, Hingorani, and Soni (2008). In addition, previous research showed that the rats received 6% of punicalagin containing diet for 37 days does not change in any hematology and serum chemistry parameters including antioxidant enzymes glutathione peroxidase and superoxide dismutase. Also, the examination of the liver and kidney in treated group did not change when compared to control group (Cerdá, Cerón, Tomás-Barberán, and Espín, 2003). In atherosclerotic patients with carotid artery stenosis on the progression of the carotid lesion the 3 years of pomegranate juice consumption (121 mg/L EA equivalents, containing tannin and anthocyanins) had no toxic effect on blood chemistry analysis of many organs (kidney, liver and heart function) (Aviram *et al.*, 2004). In the study in 86 overweight human volunteers received pomegranate ellagitannin-enriched polyphenol extract (POMx) capsules in an amount up to 1,420 mg/day (870 mg Gallic acid equivalents) for 28 days, no adverse events reported or adverse change in blood or urine laboratory values observed (Heber *et al.*, 2007).

2.2 Ischemia-reperfusion (I/R)

Ischemia-reperfusion injury is a common and important clinical problem. Ischemia reperfusion injury is a major cause of cell damage to cell death in various organs such as stomach (Yoshikawa *et al.*, 1989), pancreas (Hoffmann, Leiderer, Waldner, Arbogast, and Messmer, 1995), liver (Kitagawa, Yokoyama, Kokuryo, and Nagino, 2013), skeletal muscle (Huk *et al.*, 1997), heart (Cheung *et al.*, 2000), lung (Eppinger, Deeb, Bolling, and Ward, 1997), kidney (Sharples *et al.*, 2004) and brain (Chen, Du, and Zhang, 2000). Cell death from ischemia-reperfusion injury has been

reported to have features of necrosis (Golstein and Kroemer, 2007), apoptosis (Hamacher-Brady, Brady, and Gottlieb, 2006), and autophagy (Takagi, Matsui, and Sadoshima, 2007). The alterations in cell metabolism of ischemia and reperfusion period are shown in Figure 3. Ischemia reperfusion is separated into two phenomena: the first period of ischemia and the second period of reperfusion. Ischemia-reperfusion is a caused of local and systemic damage. The main factors responsible are the reactive oxygen species and activated neutrophils. The common source of reactive oxygen species are the xanthine oxidase and activated neutrophils in ischemia-reperfusion.

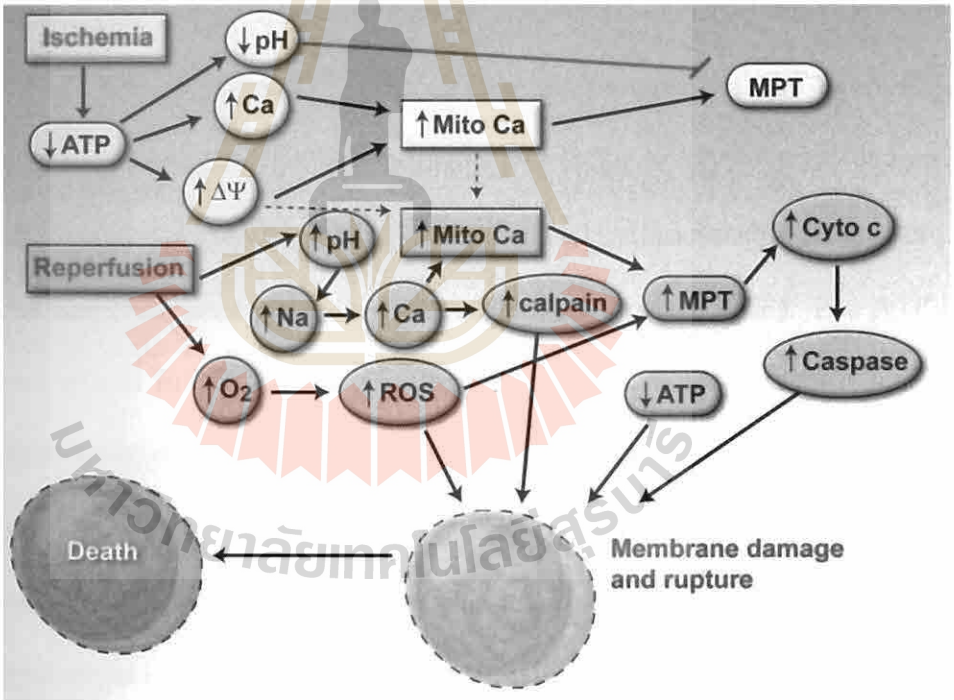

Figure 3 Alterations in cell metabolism of ischemia and reperfusion period. Membrane permeability transition (MPT), Cytochrome C (Cyto C), Reactive oxygen species (ROS), Adenosine triphosphate (ATP). Calcium (Ca), Membrane potential ($\Delta\psi$), Mitochondria (Mito) (Murephy and Steenbergen, 2008).

Ischemia period

The skeletal muscle cells are absent of oxygen and nutrient supply as a result of ischemia. Ischemia induced the marked change in energy metabolism in skeletal muscle in a period of 1.5 to 2.5 h but the energy metabolism can be reversible after revascularization and restoration of blood flow (Larsson and Hultman, 2011). The alterations in cell metabolism after ischemia period are shown in Figure 4. In addition, previous study showed prolong ischemia was caused an intracellular adenosine triphosphate (ATP) levels depleting and overloading of calcium in the cells (Nayler *et al.*, 1979). The period of ischemia has arisen a decrease in cellular energy storage causing an abnormality in various systems in the cell such as a loss of energy (decrease in ATP), Ca^{2+} overload in the cytoplasm and mitochondria, Na^{+} overload in the cytoplasm, swelling of mitochondria, and lack of lysosomal enzyme. Mitochondria are essential for cell death in the living cells, mitochondria are center of ATP synthesis and ROS production (Murephy and Steenbergen, 2008). The ATP is the main factor of cell function such as ion exchange in the cell. Ischemia is leading cells to generate a massive of oxygen radical. Mitochondria activation, ischemia was caused of swelling, disruption, superoxide radicals, NADH, flavoprotein and release the component of electron transport system. The utilization of ATP in cells increased during the myocardial ischemia is an indicator of increasing of xanthine concentration (Jennings and Reimer, 1981). The accumulation of xanthine dehydrogenase was converted to xanthine oxidase during the period of canine myocardial ischemia (Chambers et al., 1985). Superoxide and hydrogen peroxide derived from endothelial xanthine oxidase was founded involving in attraction and activation neutrophils in post-ischemia tissue (Juranek and Bezek, 2005). The xanthine oxidase is an enzyme

produced the superoxide radical and hydrogen peroxide in ischemia period. Acute myocardial ischemia have a several potential source of increase oxygen radical production such as dissociation of the intra-mitochondrial electron transport system results in release of ubisemiquinone, flavoprotein, and superoxide radicals. Phospholipase was activated by calcium influx and may enhance arachidonic acid metabolism. The arachidonic acid pathway generating the oxygen free radical (Kontos *et al.*, 1983). Moreover, ischemia can induce nitric oxide (NO) synthase in the cell, and the generated NO can interact with superoxide to form peroxynitrite. The anaerobic glycolysis pathway is a resultant of the absence of oxygen during ischemia period. Oxygen absent causes decreases in pH and ATP from the anaerobic glycolysis pathway. Moreover, ischemia period causes the decrease in phosphocreatine (CrP) levels (40% to 30-60 min and 60% in 60-90 min) and the increase in lactate levels (225% in 30-60 min and 300% after 60-90 min) (Haljamäe and Enger, 1975).

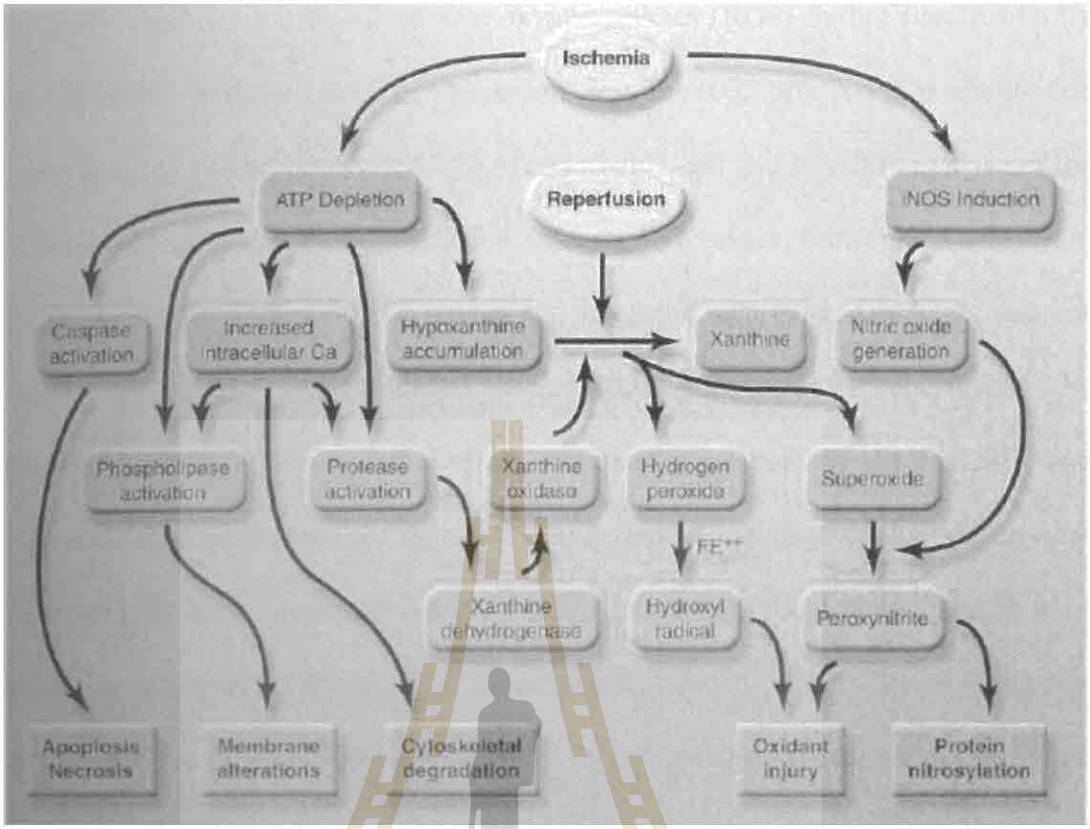

Figure 4 Alterations in cell metabolism after ischemia period (Devarajan, 2006).

Reperfusion period

Restoration of blood flow after a period of ischemia occurs in reperfusion period of ischemia-reperfusion (I/R). Skeletal muscle of the limb is the most susceptible organ to ischemia. The irreversible muscle cell damage starts after 3 h of ischemia and completed about 6 h (Blaisdell, 2002). The reperfusion restores blood flow to muscle again. The free radicals occur when the membrane lipid peroxidation and calcium influx by re-oxygenation and the result of disruption of oxidative re-phosphorylation in the mitochondria. During the reperfusion period, xanthine dehydrogenase was converted into xanthine oxidase by oxygen as a substrate and producing superoxide radical and hydrogen peroxide (Granger *et al.*, 2001).

Neutrophils are one source of reactive oxygen species (ROS) during reperfusion by endothelial cell generates reactive oxygen species. The ROS production is mainly due to activation of xanthine oxidase (XO) in endothelial cell and NADPH oxidase in the inflammatory cell. Ischemia-reperfusion injury (IRI) causes neutrophils activation, increasing plasma TNF-α, IL-1β levels, and releasing free oxygen radicals that are related to the burst of reactive oxygen species (ROS) generated after reperfusion. The increases in plasma TNF-α, IL-1β levels, the antioxidant enzyme (SOD, CAT, and GPx) activities, MDA, nitric oxide (NO), and protein carbonyl content level were shown in ischemia-reperfusion-induced group using 4 h of ischemia and 2 h of reperfusion protocol in rat skeletal muscle model (Avci *et al.*, 2012). Ischemia-reperfusion injury (4 h of ischemia and 24 h of reperfusion) caused necrosis (the average percentage was 18%) and apoptosis (the average percentage was 40%) (Wang *et al.*, 2008).

2.3 Free radical and Reactive oxygen species (ROS)

Free radicals defined as molecules or molecular fragments containing one or more unpaired electrons of an oxygen molecule (Halliwell and Gutteridge, 2015). Reactive oxygen species (ROS) comprise both free radical and non-free radical oxygen intermediates (Suttajit *et al.*, 2012), as shown in Table 1. ROS are chemically reactive molecule containing oxygen including peroxide, superoxide, hydroxyl radical, hydroperoxide, and peroxyl radical. Free radicals are highly reactive and ready to interact with many cell components such as lipids, proteins, carbohydrates and DNA. Destabilization of the cell structure occurs after the electron molecule of the neighbor molecule is stolen by free radical reactions. ROS are formed as a natural

byproduct of normal metabolism of O_2 and plays an important role in cell signaling and homeostasis. Normally, the ROS is an endogenous product of cell metabolism, especially in the mitochondrial oxidative phosphorylation. Increased ROS levels can result from environmental stress such as heat and UV exposures, causing significant cell injury and damage (Devasagayam *et al.*, 2004). Superoxide (O_2^-), a product of molecular oxygen (O_2) reduction, is the precursor of most other reactive oxygen species (Turrens, 2003). Superoxide dismutase catalyzes the dismutation of the superoxide into hydrogen peroxide. Hydrogen peroxide, in turn, may be partially reduced to hydroxyl radical or fully reduced to water (Turrens, 2003). The superoxide is a product of the mitochondria (Cadenas and Sies, 1998). The molecule oxygen is widely available to the aerobic organism. The reduction of oxygen to water in the living cell can proceed by two pathways, including mitochondrial enzyme cytochrome oxidase pathway and the univalent pathway of oxygen reduction. Normally, over 95% of the oxygen consumption of the cell was catalyzed by mitochondrial cytochrome oxidase pathway. Cytochrome oxidase is a mitochondrial enzyme that reduces oxygen to water by tetravalent reduction without producing any intermediates. The remaining 5% of oxygen consumption is reduced by univalent pathway in which several oxygen radical species are produced such as superoxide anion, hydrogen peroxide, and production of hydroxyl radicals. ROS initiate the production of toxic metabolites such as malondialdehyde (MDA), a sensitive marker of lipid peroxidation of cellular membrane in IRI. ROS have a number of functions causing cell damage in IRI, one of which is achieved through the process of lipid peroxidation. The antioxidant enzyme system provides defensive agents against ROS to protect cells from oxidative damage. Oxidative damage is an imbalance between the scavenging capacity of antioxidant

enzyme and ROS. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), these enzymes catalyzed ROS into less reactive substances. Prolonged ischemia reperfusion cause damage to skeletal muscle cells and the imbalance between the radical scavenging capacity of enzyme antioxidant and ROS.

Table 1 Classification of ROS (Rahman, Hosen, Islam, and Shekhar, 2012).

Reactive oxygen species (ROS)			
Radical		**Non-radical**	
$O^{\cdot -}$	Superoxide	H_2O_2	Hydrogen peroxide
$OH^{\cdot}$	Hydroxyl	$HCCl^{-}$	Hypochlorous acid
$RO_2^{\cdot}$	Peroxyl	O_3	Ozone
$RO^{\cdot}$	Alkaxyl	O_2	Siglet oxygen
$HO_2^{\cdot}$	Hydroperoxyl	$ONOO^{-}$	Peroxynitrite .

2.4 Sources of ROS

The main source of ROS in the ischemia reperfusion model is the mitochondrial respiratory electron transport chain and xanthine oxidase (XO or XAO).

Mitochondrial respiratory electron transport chain

Metabolism of the cell, the product of electron-transport chains are ATP, water, and superoxide. Then the superoxide was changed to hydrogen peroxide and hydroxyl radical by 0.15 and 2% of cellular O_2 consumption (Tahara, Navarete, and Kowaltowski, 2009). Mitochondrial electron transport chain is a source of ATP of the cell. Energy transduction, a small number of electrons leak to oxygen prematurely, forming the oxygen free radical superoxide by superoxide are the cause of many diseases (Kovacic, Pozos, Somanathan, Shangari, and O'Brien, 2005; Valko, Izakovic, Mazur, Rhodes, and Telser, 2004). Superoxide is produced from leading electron through the group of protein, including both complexes I and III by electron transport chain mechanism.

Xanthine oxidoreductase (XOR) and xanthine oxidase (XO or XAO)

Xanthine oxidoreductase (XOR) is complex molybdo-flavoenzyme found in milk and many other tissues. XOR is a type of enzyme that generates reactive oxygen species reducing molecular oxygen. Mammalian XOR consists of two interconvertible forms, including xanthine dehydrogenase (XDH), and xanthine oxidase (XO or XAO) (Ardan, Kovačeva, and Čejková, 2004; Martin, Hancock, Salisbury, and Harrison, 2004). Xanthine oxidase is the enzyme that activates a free radical in human. Xanthine oxidase (XO) play an important role in the catabolism of purine by the

accelerating reaction of hypoxanthine to xanthine and xanthine to uric acid together with electron transport chain to superoxide (free radical) as shown in Figure 5 (Harrison, 2002; Hille, 2005). The XO is a superoxide-producing enzyme, with general low specificity (Bonini, Miyamoto, Mascio, and Augusto, 2004). XO can combine with other compounds and enzymes, producing reactive oxidants and oxide substrates.

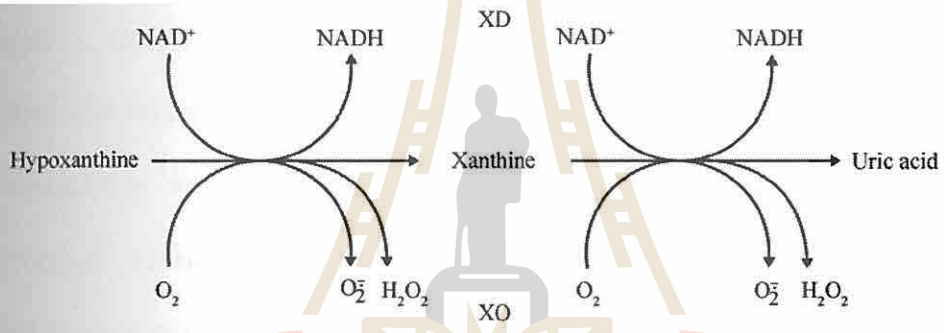

Figure 5 Xanthine oxidase system (Hideharu, 2013).

Xanthine dehydrogenase (XDH) transfers electron to NAD+ and subsequently generates NADH. Xanthine oxidase (XO or XAO) transfers electrons to O_2 and subsequently generates oxidative stress. The period of ischemia can induce cellular calcium overload, enhancing XDH-XO conversion. Calcium-triggered proteolytic attack on XDH causes the release of XO. Reperfusion period, enhanced XO can produce more ROS including superoxide, hydrogen peroxide, and hydroxyl radicals that can exaggerate cellular damage (Tsuda *et al.*, 2012).

2.5 Oxidative stress

The ROS is an important factor to damage of structure cell, lipids, proteins, and nucleic acid (Valko, Rhodes, Moncol, Izakovic, and Mazur, 2006). Oxidative stress results in direct or indirect ROS-mediate damage of macromolecule. Oxidative stress is a state of physiological stress in the body by the imbalance between the generation of reactive oxygen and nitrogen species and the antioxidant defense system. Oxidative stress has been leading to cell damage so that it a cause of oxidative damage and effect to cell structure including lipid, protein, and DNA as shown in Figure 6. Severe damage is causing cell death in the end. The production of free radical and ability of the body to counteract of detoxify their harmful effects through neutralization by antioxidant. Oxidative stress can also result from increased production of reactive oxygen species from activation of endogenous enzymes (NADH oxidase) in chronic inflammatory conditions. The metal-induce generation of ROS results in an attack on other cellular components such as polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation (Siems, Grune, and Esterbauer, 1995). The final product of the peroxidation process being malondialdehyde (MDA) and endoperoxides is a precursor of MDA by peroxyl radicals can be rearranged *via* a cyclization reaction to endoperoxides. 4-hydroxy-2-nonenal (HNE) is aldehyde product of lipid peroxidation (Siems *et al.*, 1995). Hydroxyl radical reacts with all components of DNA molecule such as damaging both the purine (adenine and guanine) and pyrimidine (cytosine and thymine) bases and also the deoxyribose backbone (Halliwell and Gutteridge, 2015). Durable modification of genetic material resulting from these oxidative damage incidents represents the first step involved in mutagenesis, carcinogenesis, and aging.

Oxidative stress is a pathophysiology imbalance between oxidant and antioxidations. Biomarkers of oxidative stress play an important role in understanding the pathogenesis and treatment of many diseases. They can be classified as molecular that are modified by interactions with ROS and molecular of the antioxidant system that change in response to increased redox stress. Type of biomarkers and locations where biomarkers can be found are shown in Table 2.

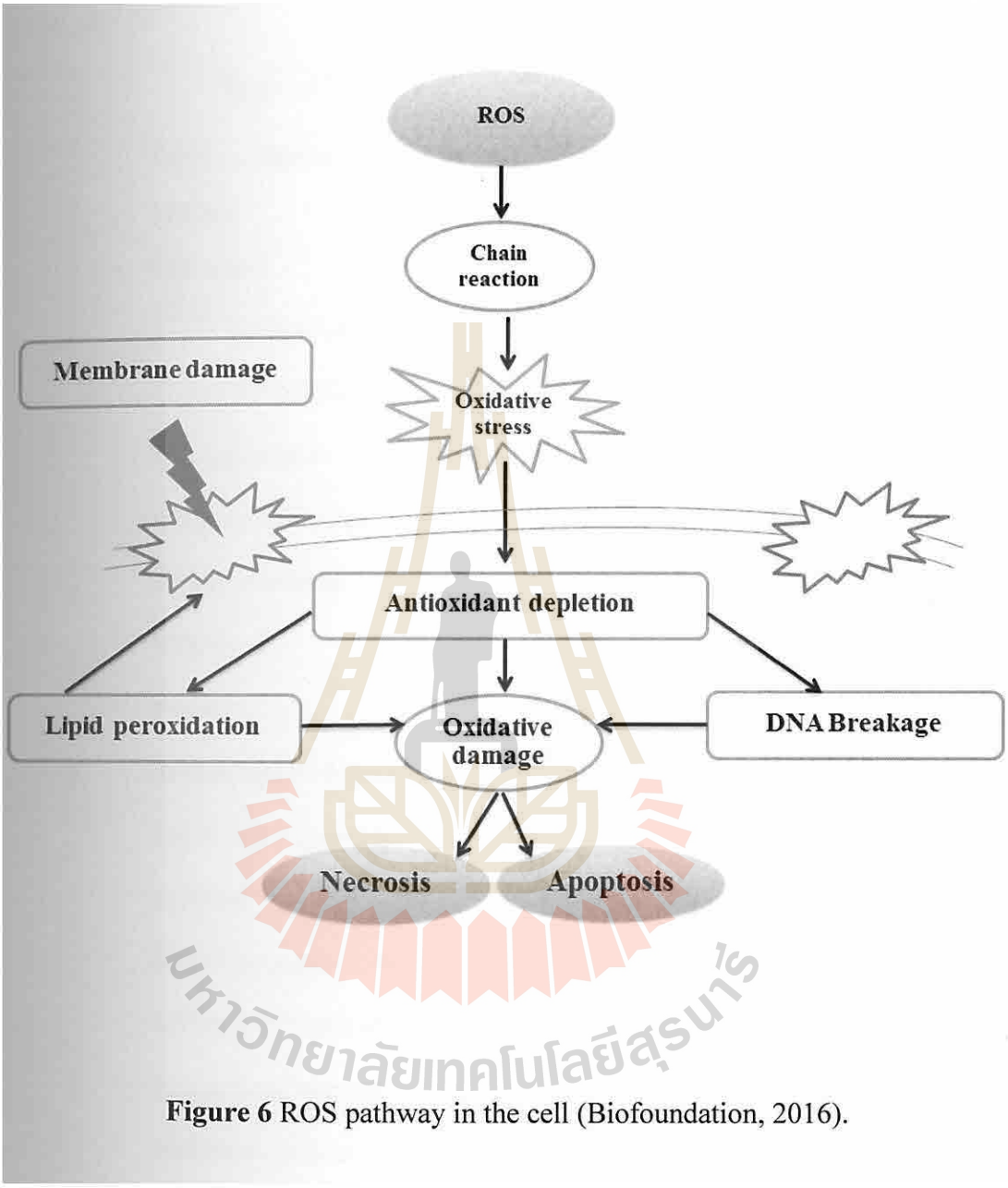

Figure 6 ROS pathway in the cell (Biofoundation, 2016).

Table 2 Biomarkers of oxidative stress (Ho, Karimi Galougahi, Liu, Bhindi, and Figtree, 2013).

	Type of biomarker	Location
1	**Ferric reducing ability of plasma**	Extracellular
2	**Carbonyl**	Extracellular
3	**Lipid peroxidation**	
	Malondialdehyde	Extracellular
	F2-isoprostane	Extracellular
	4-Hydroxynonenal	Extracellular
4	**Plasma vitamin**	
	Vitamin C	Extracellular
	Vitamin E	Extracellular
5	**Antioxidant enzyme**	
	Superoxide dismutase	Intracellular
	Catalase	Intracellular
	Glutathione peroxidase	Intracellular
	GSH/GSSG ratio in erythrocyte	Intracellular
6	**Prooxidant enzyme**	
	Xanthine oxidase	Intracellular
	NADPH oxidase	Intracellular
7	**Other**	
	Endothelial microparticles	Extracellular
	Endothelial microparticles	Extracellular
	Ischemia modified albumin	Extracellular

2.6 Antioxidant

The defense mechanism of free radical-induced oxidative stress is antioxidant defense. Antioxidant means a reaction of inhibiting oxidation with another molecule in a chemical process. The antioxidant can prevent or decrease cell damage. The main function of antioxidant is to counteract the production of free radicals. There are 2 main types of antioxidants which are endogenous antioxidants and exogenous antioxidants. Antioxidants can be separated into two groups: enzymatic antioxidants and non-enzyme antioxidants, as shown in Figure 7. Enzymatic antioxidant defense system includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and non-enzymatic antioxidant defense system includes alpha-tocopherol (Hardie, Fletcher, and Secombes, 1990), glutathione, carotenoids, ascorbic acid, and flavonoids (Lu, Shipton, Khoo, and Wiart, 2014).

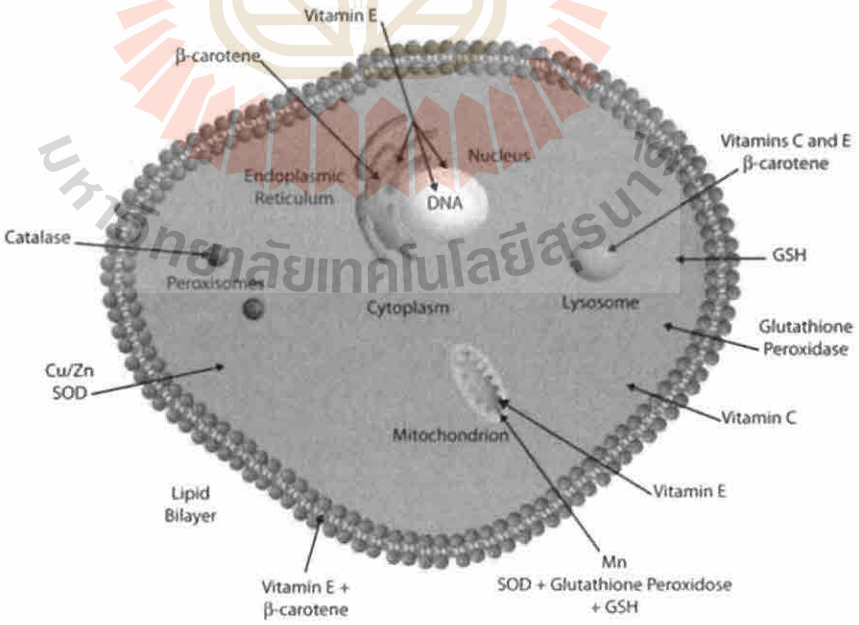

Figure 7 Enzymatic antioxidants and non-enzymatic antioxidants (Ananya, 2012).

2.6.1 Enzymatic antioxidants

Enzymatic antioxidants defenses include several enzymes that catalyze reactions of ROS degradation, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase.

Superoxide dismutase (SOD)

Superoxide dismutase (SOD) is the enzymes that play a vital antioxidant role in human health and presents in almost all aerobic cells and in extracellular fluids (Johnson and Giulivi, 2005). SOD is the enzyme that catalyzes the breakdown of superoxide anion into oxygen and hydrogen peroxide, as shown in figure 8. SOD is a group of the metal-containing enzyme. SOD contains metal ion cofactors at the active site, depending on the isozyme, it can be either copper, zinc, manganese or iron. SOD contains two main forms in the human cell: copper zinc SOD (Cu-Zn-SOD) that can be found primarily in the cytosol, and manganese SOD (MnSOD) that can be found predominantly in the mitochondrion. The mitochondrial SOD is most biologically important (Bannister, Bannister, and Rotilio, 1987). In plants, SOD enzyme is presented in the cytosol and mitochondria, with an iron SOD found in the chloroplast that is absent from vertebrates and yeast (Van Camp, Inzé, and Van Montagu, 1997).

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Mn-SOD: mitochondria
Fe-SOD: chloroplast
CuZn-SOD: chloroplast
CuZn-SOD: cytosol

Figure 8 Reaction of superoxide dismutase (Bryan, 1996).

Catalase (CAT)

Catalase (CAT) is a common enzyme found in all living organisms exposed to oxygen including bacteria, plants, and animals. CAT is found in peroxisome, the organelle found in almost all eukaryotic cells. CAT helps prevent damage to cell and tissue from oxidative damage by reactive oxygen species. CAT is the enzyme catalyze the conversion of hydrogen peroxide to water and oxygen by using ion or manganese cofactor, the only substrate is hydrogen peroxide (Figure 9).

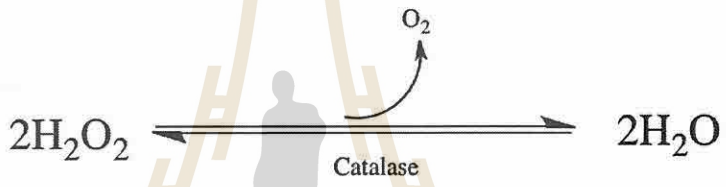

Figure 9 Reaction of catalase (Sellers *et al.*, 2014).

Glutathione peroxidase (GPx)

Glutathione (GSH) system contains the glutathione, glutathione reductase, glutathione peroxidase (GPx) and glutathione S-transferase. GSH is an important antioxidant found in every cells and it neutralizes free radical. Glutathione is a family of the selenium-containing enzyme that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides. GSH is converted to its oxidized form, glutathione disulfide (GSSG). The main biological role of GPx is to protect oxidative damage of cell. GPx reduceds lipid hydroperoxides to their corresponding alcohols, and free hydrogen peroxide to water. Particularly toxic hydroperoxide, GPx is helping to reduce harmless water. GPx has many isozymes encoded by different genes. Eight isoforms of GPx (GPx1-8) have been identified in humans. GPx1 has been found in

the cytoplasm of nearly all mammalian tissues. GPx2 is an intestinal and extracellular enzyme. While GPx3 has been found in extracellular fluid, especially in plasma, GPx4 is expressed in nearly every mammalian cells (Muller, Lustgarten, Jang, Richardson, and Van Remmen, 2007).

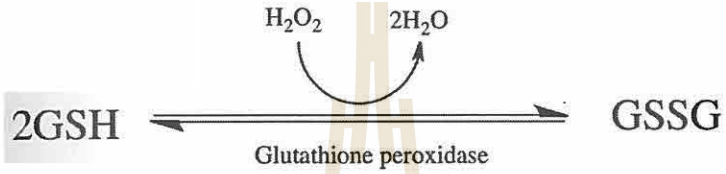

Figure 10 Reaction of glutathione peroxidase (Sellers *et al.*, 2014).

2.6.2 Non-enzymatic antioxidants

None-enzyme antioxidants (such as vitamin C and Vitamin E, shown in Figure 11 and 12) are widely used in dietary supplements. They can be found in the animal cells and various plants. They can help cell protection from damage caused by free radicals and prevention of many diseases.

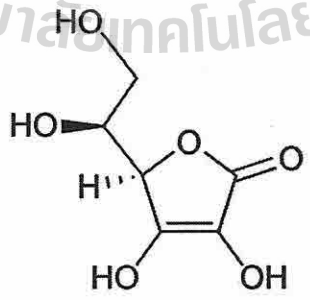

Figure 11 Vitamin C or ascorbic acid structure (Joseph, 2014).

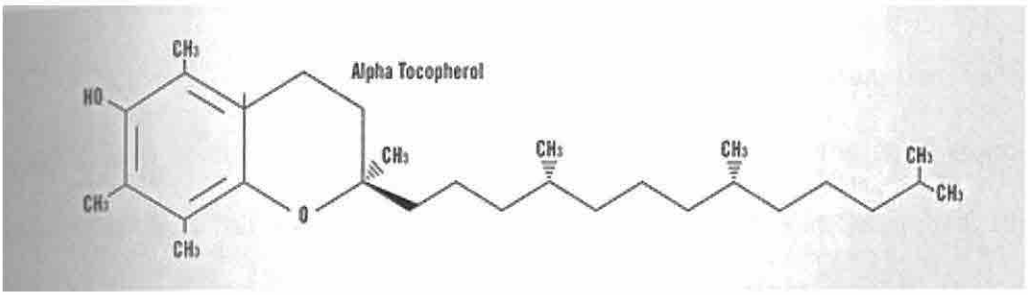

Figure 12 Vitamin E or alpha-tocopherol structure (Lyle, 2006).

Vitamin C or ascorbic acid

Vitamin C or ascorbic acid is a monosaccharide oxidation-reduction catalyst found in many vegetables, fruits, and in the cell. Vitamin C can be found in many fruits such as orange juice, lemon juice, papaya and cashew apple (Assunção and Mercadante, 2003; Kelebek, Selli, Canbas, and Cabaroglu, 2009; Penniston, Nakada, Holmes, and Assimos, 2008; Wall, 2006). Vitamin C is probably best known antioxidant and is important for prevention of many diseases such as prostate cancer (Khassaf *et al.*, 2003; Maramag, Menon, Balaji, Reddy, and Laxmanan, 1997). A lack of vitamin C can cause a disease called scurvy. Previous study vitamin C was used to standard control (positive control) in antioxidant activity models such as antioxidant activity determination of citronellal and crude extracts of cymbopogon citratus by 3 different methods. Three different methods were used to test the antioxidant activity, including ferric reducing antioxidant power (FRAP) assay, 1,1-diphenyl-2-picryl hydrazyl radical reducing power methods (DPPH radical scavenging assay) assay, and β-carotene bleaching assay. The result of plant showed low radical reducing power activity compared to ascorbic acid, gallic acid, and quercetin (Lu, Shipton, Khoo, and Wiart, 2014).

Vitamin E or alpha-tocopherol

Vitamin E or alpha-tocopherol is one of important antioxidant in fruit, vegetable, and animals. Some animals are commonly known as the good source of vitamin E such as salmon (Salmo salar L.) (Hardie, Fletcher, and Secombes, 1990), and rainbow trout (Salmo gairdneri Richardson) (Blazer and Wolke, 1984). Vitamin E can prevent oxidative stress in the body and protect against many diseases such as cancer, heart disease, and ischemia reperfusion injury (Lee *et al.*, 2005; Novelli *et al.*, 1997; Rimm *et al.*, 1993). Previous study vitamin E was used to standard control (positive control) of ischemia-reperfusion injury in rat skeletal muscle models. The results of vitamin E, and curcumin showed increase the biomarker of oxidative stress (antioxidant enzyme) better than ischemia-reperfusion injury group (Avci *et al.*, 2012).

CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of the pomegranate juice

Thai pomegranate fruits (*Punica grantum* L.) were collected in June 2014 from pomegranate garden in Pak Chong, Nakhon Ratchasima Province, Thailand. Thai pomegranate plant was taxonomically identified and authenticated by the office of the forest herbarium, forest and plant conservation research office of ministry of natural resources and environment, Bangkok, Thailand. The voucher specimen was deposited at the Forest Herbarium-BKF Herbarium and the voucher number of BKF No. 188578 was obtained. Fruits were thoroughly washed with tap water to remove surface dirt and manually cut in half. The arils of the fruits were manually separated from the peels, and squeezed to produce the juice. The juice was then filtered through layers of cotton wool laid out over a sieve and 500 ml of pomegranate juice was separated into each plastic bag and stored in the freezer at -20°C and used for further experiments.

3.2 Thai pomegranate juice concentration method

Frozen Thai pomegranate juice (TPJ) was thawed and centrifuged at 4000 RCF for 15 min at 4°C. Soluble solids or degree Brix measured by refractometer of fresh TPJ was 15.53±0.77°Brix. TPJ was concentrated by 2 different heating methods (rotary vacuum evaporation, and microwave evaporation).

The concentration methods were adapted from the studies of Maskan (2006) and Goula and co-workers (2014).

1. Microwave evaporation: The study was carried out at a power level of 450 W by microwave oven (Micro Electrolux Solo 900W, SWEDEN). The juice sample (100 ml) was poured into a beaker and placed at the center of the turntable in the microwave. Samples were taken for measurement of °Brix periodically and replaced again until the final concentration of 60±0.00°Brix was achieved. The percentage yield of concentrated TPJ by this method was measured.

2. Rotary vacuum evaporation: The study was carried out by rotary vacuum evaporator (Buchi Rotavapor R 210, Belgium). The 100 ml juice sample was concentrated in a laboratory rotary vacuum evaporator at 65°C. Samples were taken for measurement of °Brix periodically and replaced again until the final concentration of 60±0.00°Brix was achieved. The percentage yield of concentrated TPJ by this method was measured.

These 2 different evaporation processes were employed for the production of pomegranate juice concentrate from fresh TPJ with an initial total solid content of 15.53±0.77°Brix to a final concentration of 60±0.00°Brix. The final concentration can be achieved within 21 min by using a rotary vacuum evaporator at 65°C, and 11 min by using a microwave evaporator with maximum output of 450 W. The percentage yields of concentrated TPJ by rotary vacuum evaporation was 16.67±0.60% and concentrated TPJ by microwave evaporation was 16.67±0.60% from initial 100 ml of fresh TPJ in both processes. Note: the soluble solids contents of fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum

evaporation were measured by refractometer (Master refractometer, ATAGO® Ltd., JAPAN) expressed in °Brix.

3.3 *In vitro* study

3.3.1 Chemical composition analysis of fresh Thai pomegranate juice

Fresh TPJ sample was analyzed for energy, ash, protein, total fat, total carbohydrate, total sugar, glucose, fructose, sucrose, vitamin E, vitamin C, calcium, phosphorus, sodium, potassium, and magnesium. The analysis was conducted by Food and Nutrition Laboratory, Instituted of Nutrition Mahidol University.

3.3.2 Phytochemical screening analysis

Phytochemical composition of fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation were screened for the presence of protein, carbohydrate, tannins, flavonoids, saponins, glycosides and steroids by using standard methods with some modifications (Harborne, 1973).

Test for protein by using Ninhydrin test

Chemicals:

Ninhydrin ($C_9H_6SO_4$, sigma-Aldrich; St. Louis, USA)

Preparation:

0.2% Ninhydrin solution: 0.004 g of ninhydrin was dissolved in DDD water and made up to 20 ml of final volume and stored at room temperature.

Procedure:

1 ml of fresh TPJ, concentrated TPJ by microwave evaporation, or concentrated TPJ by rotary vacuum evaporation was mixed with 1 ml of 0.2% solution of ninhydrin. A violet color indicates the presence of amino acids or proteins.

Test for carbohydrate by using Iodine test

Chemicals:

Iodine solution (I, sigma-Aldrich; St. Louis, USA)

Procedure:

1 ml of fresh TPJ, concentrated TPJ by microwave evaporation, or concentrated TPJ by rotary vacuum evaporation was mixed with 1 ml of iodine solution. A dark blue or purple color indicates the presence of carbohydrate.

Test for tannins and phenols

Chemicals:

Iron (III) chloride ($FeCl_3$, sigma-Aldrich; St. Louis, USA)

Preparation:

2% Iron (III) chloride solution: 0.4 g of Iron (III) chloride ($FeCl_3$) was dissolved in DDD water and made up to 20 ml of final volume and stored at room temperature.

Procedure:

1 ml of fresh TPJ, concentrated TPJ by microwave evaporation, or concentrated TPJ by rotary vacuum evaporation was mixed with 1 ml of 2% solution of Iron (III) chloride ($FeCl_3$). A blue-green or black color indicates of the presence of phenols or tannins.

Test for flavonoids by using an Alkaline reagent test

Chemicals:

Sodium hydroxide (NaOH, BDH, Ltd., UK)

Preparation:

2% sodium hydroxide solution: 0.4 g of sodium hydroxide was dissolved in DDD water and made up to 20 ml of final volume and stored at room temperature.

Procedure:

1 ml of fresh TPJ, concentrated TPJ by microwave evaporation, or concentrated TPJ by rotary vacuum evaporation was mixed with 1 ml of 2% solution of sodium hydroxide (NaOH). A yellow color that turns colorless on the addition of a few drops of diluted acid indicates the presence of flavonoids.

Test for saponins

Procedure:

2 ml of fresh TPJ, concentrated TPJ by microwave evaporation, or concentrated TPJ by rotary vacuum evaporation was mixed with 4.8 ml of distilled water and shaken vigorously. Formation of stable foam indicates of the presence of saponins.

Test for glycosides by using Salkowski's test

Chemicals:

Sulfuric acid (H_2SO_4, sigma-Aldrich; St. Louis, USA), and Chloroform ($CHCl_3$, BDH, Ltd., UK)

Procedure:

1 ml of fresh TPJ, concentrated TPJ by microwave evaporation, or concentrated TPJ by rotary vacuum evaporation was mixed with 1 ml of chloroform. 1 ml of concentrated sulfuric acid (H_2SO_4) was carefully added to a mixture and then shaken gently. A reddish brown color indicates the presence of glycosides.

Test for steroids

Chemicals:

Sulfuric acid (H_2SO_4, sigma-Aldrich; St. Louis, USA)

Procedure:

1 ml of fresh TPJ, concentrated TPJ by microwave evaporation, or concentrated TPJ by rotary vacuum evaporation was mixed with 1 ml of chloroform. Few drops of concentrated sulfuric acid (H_2SO_4) was carefully added to a mixture and then shaken gently. A red color produced in the lower chloroform layer indicates the presence of steroids.

3.3.3 Total phenolic content by Folin-Ciocalteu method

Chemical:

Sodium carbonate (Na_2CO_3, BDH, Ltd., UK), Folin-Ciocalteu reagent (FCR, Merck Millipore, Germany), Ethanol (C_2H_6OH, Analytical Grade ≥ 99.8%, Carlo Erba Reagents, France), Gallic acid ($C_7H_6O_5$, Grade ≥ 98.0%, sigma-Aldrich; St. Louis, USA).

Preparation:

2% sodium carbonate: 5 g of sodium carbonate was dissolved in double deionized distilled (DDD) water and then adjusted the final volume to 250 ml.

10% ethanol: 2 ml of ethanol was mixed with 18 ml of DDD water.

Folin-Ciocalteu reagent (1:1): 1.5 ml of Folin-Ciocalteu reagent was mixed with 1.5 ml of 10% ethanol to make up total volume of 3 ml.

Gallic acid stock solution (1 mg/ml): 0.01 g of gallic acid was dissolved with 10% ethanol and then adjusted the final volume to 10 ml.

Working standard solutions of gallic acid

Conc. (mg/ml)	Stock (µl)	10% EtOH (µl)
0.00	0	100
0.02	20	80
0.04	40	60
0.06	60	40
0.08	80	20
0.10	100	0

Procedure:

The total phenolic content was determined by Folin-Ciocalteu (FC) method as described by Minussi *et al.* (2003) with some modifications. Briefly, sample (fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation) were diluted with 10% ethanol at the ratio of 1:11. Diluted samples (100 µl) were mixed with 100 µl of Folin–Ciocalteu reagent. Two milliliters of sodium carbonate solution was added to the mixture and then allowed to stand for 30 min at room temperature before the absorbance was measured at 750 nm by spectrophotometer using a (CECIL 1011, England). Gallic acid was used as standard for the calibration curve. Results were expressed as mg of gallic acid equivalents (GAE) in a liter of fruit juice (mg GAE/L of juice). The procedure and preparation were shown in Appendix A. All determinations were performed in triplicate and the mean values were calculated.

3.3.4 Antioxidant assay: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging method

Chemical:

1,1-diphenyl-2-picrylhydrazyl (DPPH) ($C_{18}H_{12}N_5O_6$, sigma-Aldrich; St. Louis, USA), Methanol (CH_3OH, Carlo Erba Reagents, France), Vitamin C ($C_6H_8O_6$, Carlo Erba Reagents, France)

Preparation:

1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (25 mg/ ml): 0.007 g of DPPH was dissolved with methanol and made up to 280 ml of final volume and stored at room temperature.

Vitamin C stock solution (1:1): 0.005 g of vitamin C was dissolved with 5 ml of DDD water and stored at room temperature.

Procedure:

The DPPH radical scavenging method was used to measure the antioxidant activity of fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ rotary vacuum evaporation as described by Villano and colleagues, (2007) with some modifications. Briefly, 50 µl of diluted TPJ samples (fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation) and vitamin C (positive control, 0.001g/ml) were mixed with 1950 µl DPPH solution (25 mg/L) in methanol. The mixture was incubated at room temperature for 30 min in the dark. All solutions were freshly prepared and protected from light. The absorbance of the mixture was then measured at 515 nm using a microplate reader (BioRad-Benchmark Plus microplate reader, USA). All determinations were performed in triplicate. Distilled water (50 µl) mixed with 1950 µl of the DPPH working solution

was used as sample blank. Radical scavenging activity was defined as the inhibition percentage. The procedure and preparation were showed in Appendix B. The scavenging capacity (SC) for DPPH assay was calculated by using the following equation:

% (SC) = [(Abs control – Abs sample) / Abs control]*100

Results were expressed as IC_{50} concentration where 50% inhibition of the DPPH scavenging capacity was obtained. GraphPad Prism software (version 6, GraphPad Software Inc, California, USA) was used to calculate IC_{50} values.

3.3.5 Antioxidant assay: 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical-scavenging method

Chemical:

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) ($C_{18}H_{18}N_4O_6S_4$, sigma-Aldrich; St. Louis, USA), Methanol (CH_3OH, Carlo Erba Reagents, France), Potassium persulfate ($K_2S_2O_8$, sigma-Aldrich; St. Louis, USA), Ethanol (C_2H_6O, Carlo Erba Reagents, France), Vitamin C ($C_6H_8O_6$, Carlo Erba Reagents, France)

Preparation:

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) solution: 0.0179 g of ABTS was dissolved in DDD water and made up to 5 ml and stored at room temperature.

140 mM potassium persulfate solution: 3.784 g of potassium persulfate was dissolved in DDD water and made up to 100 ml and stored at room temperature.

ABTS working solution: 5 ml of ABTS solution was mixed with 88 µl of 140 mM potassium persulfate solution, incubated for 16 hours at room temperature and

protected from light. After incubation, all solutions were diluted with ethanol to an absorbance of 0.70±0.05 at 734 nm.

Vitamin C stock solution (1:1): 0.005 g of vitamin C was dissolved with 5 ml of DDD water and stored at room temperature.

Procedure:

The ABTS radical-scavenging assay was performed as previously described by Re *et al.* (1999) with some modifications. Briefly, 25 µl of diluted TPJ samples (fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation) and vitamin C (positive control, 0.001g/ml) were mixed with 2000 µl ABTS working solution (5 ml of 7 mmol/L ABTS solution reacted with 88 µl of 140 mmol/L potassium persulfate solution and then the reaction solution was diluted with ethanol to make the absorbance of 0.700±0.005 at 734 nm). The mixture was incubated at room temperature for 10 min in the dark. All solutions were freshly prepared and protected from light. The absorbance of the mixture were then measured at 734 nm using a microplate reader (BioRad-Benchmark Plus microplate reader, USA). All determinations were performed in triplicate. Distilled water (25 µl) mixed with 2000 µl of ABTS working solution was used as the control and absolute ethanol was used as sample blank. The procedure and preparation were shown in Appendix C. Radical scavenging activity was defined as the inhibition percentage. The scavenging capacity (SC) for ABTS assay was calculated by using the following equation:

% (SC) = [(Abs control – Abs sample) / Abs control]*100

Results were expressed as IC_{50} concentration where 50% inhibition of the ABTS scavenging capacity was obtained. GraphPad Prism software (version 6, GraphPad Software Inc, California, USA) was used to calculate IC_{50} values.

3.3.6 Antioxidant assay: Ferric reducing antioxidant power (FRAP) method

Chemical:

2,4,6-tripyridyl-s-triazine (TPTZ) (sigma-Aldrich; St. Louis, USA), Hydrochloric acid (HCl, Carlo Erba Reagents, France), Glacial acetic acid (CH_3COOH, sigma-Aldrich; St. Louis, USA), Sodium acetate (CH_3COONa, sigma-Aldrich; St. Louis, USA), Ferric chloride ($FeCl_3.6H_2O$, sigma-Aldrich; St. Louis, USA), Ferrous sulfate ($FeSO_4.7H_2O$, sigma-Aldrich; St. Louis, USA), Vitamin C (Carlo Erba Reagents, France)

Preparation:

Hydrochloric acid (40 M): 0.08 ml of hydrochloric acid was dissolved in DDD water and made up to 20 ml of final volume and stored at room temperature.

2,4,6-tripyridyl-s-triazine (TPTZ) (10 mM): 0.0312 g of 2,4,6-tripyridyl-s-triazine (TPTZ) was dissolved in 40 M hydrochloric acid and made up to 10 ml of final volume at 37°C.

Ferric chloride solution (20 mM): 0.0324 g of ferric chloride was dissolved in DDD water and made up to 10 ml of final volume and stored at room temperature.

Acetate buffer (300 mM): 0.82 g of sodium acetate and 8.11 ml of glacial acetic was dissolved in DDD water and made up to 500 ml of final volume and stored at room temperature at pH 3.6. The solution was adjusted pH with sodium hydroxide.

Ferrous sulfate solution: 0.667 g of ferrous sulfate was dissolved in DDD water and made up to 1 ml of final volume and stored at room temperature.

Procedure:

The FRAP assay was based on the measurement of the iron-reducing capacity of Thai pomegranate juices and performed as previously described by Benzie *et al.* (1996) with some modifications. Briefly, 40 µl of diluted TPJ samples (fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation) and vitamin C (positive control, 0.025g/ml) were mixed with 1800 µl FRAP solution (2.5 ml of 10 mM/L 2,4,6-tripyridyl-s-triazine; TPTZ) solution in 40 mM/L HCl 2.5 ml of 20 mol/L $FeCl_3.6H_2O$, and 25 ml of 0.3 mol/L acetate buffer (pH 3.6), freshly prepared and warmed to 37°C prior to use). The mixture was incubated at 37°C in water bath for 30 min in the dark. All solutions were freshly prepared and protected from light. The absorbance of the mixture was then measured at 593 nm using spectrophotometer (CECIL 1011, England). Distilled water (40 µl) mixed with 1800 µl of FRAP solution was used as the sample blank. Vitamin C was used as positive control. The results were calculated from the standard curve constructed of by different concentrations of Iron (II) sulfate solution. The procedure and preparation were shown in Appendix D. The results were expressed in mmol Fe^{2+}/L. All the measurements were performed in triplicate and the mean values were calculated.

3.4 *In vivo* study: The effects of Thai pomegranate juice on ischemia-reperfusion (I/R)-induced oxidative stress in skeletal muscle of male Wistar rats

3.4.1 Animals

Fifty five male Wistar rats at 8 weeks of age (250-300 g) were randomly divided into 7 groups. The animals were housed in standard housing cages and maintained at 25±1°C on 12/12 h light/dark cycle. The animals were given *ad libitum* free access to food and water. All experimental procedures were reviewed and approved by the animal care and use committee guidelines of the Suranaree University of Technology.

3.4.2 Thai pomegranate juice concentration method

From *in vitro* pilot study, fresh TPJ (15.53±0.77°Brix) was concentrated to 60±0.00°Brix by rotary vacuum evaporation and by microwave evaporation which can be achieved within 21 and 11 mins, respectively. Concentrated TPJs by rotary vacuum evaporation and by microwave evaporation contained similar total phenolic content and showed similar antiradical activity against DPPH and ABTS radicals. Microwave evaporation was the best choice since TPJ was concentrated in a shorter period of time. Thus, concentrated TPJ by microwave evaporation was used in the animal study.

3.4.3 Preparation of concentrated pomegranate juice by microwave evaporation

Fresh TPJ (15.53±0.77°Brix) was concentrated to 60±0.00°Brix by microwave evaporation which was achieved within 11 min. Concentrated TPJ by microwave evaporation was used in the animal study. Dosage of TPJ to be used in the present study is calculated from the total phenolic content of concentrated TPJ by microwave evaporation method and extrapolated from human dose mentioned in the study of Tsang *et al.* (2011), participants received 500 ml/day of pomegranate juice containing 1685 mg total phenolic content/L juice or 14.04 mg total polyphenol content/kg body weight. The total phenolic content of concentrated TPJ by microwave evaporation method was 6675.46 mg/L juice. The human equivalent dose (HED) for Thai pomegranate juice was calculated by using the following equation.

HED (mg/kg) = Animal dose (mg/kg) × Animal K_m ÷ Human K_m)

K_m is a correction factor reflecting the relationship between body weight and body surface area. For a typical adult (body weight 60 kg, body surface area 1.6 m^2), Km is 37. For the most often used laboratory animal species the average K_m are as follows: rat K_m = 6, human K_m = 37 (Reigner and Blesch, 2002; Guidance, 2005).

Calculation:

HED =14.04 × (37/6)

= 86.57 mg TPC/Kg

The present study used 86.57 mg TPC/Kg at volume 20 ml/kg body weight as an initial dose.

3.4.4 Drug solutions

Tween 80 (1%) (100 ml): 1 ml of Tween 80 was dissolved in 99 ml of DDD water and stored at 4°C.

Vitamin E (250 mg/20 ml/kg body weight): 1.052 ml of α-tocopherol was dissolved in 78.948 ml of Tween 80 (10%) and stored at 4°C.

Low dose of TPJ (36.98 mg TPC/20 ml/kg body weight): 22 ml of concentrated TPJ was dissolved in 58 ml of DDD water and stored at 4°C.

Middle dose of TPJ (56.58 mg TPC/20 ml/kg body weight): 34 ml of concentrated TPJ was dissolved in 46 ml of DDD water and stored at 4°C.

High dose of TPJ (86.57 mg TPC/20 ml/kg body weight): 52 ml of concentrated TPJ was dissolved in 28 ml of DDD water and stored at 4°C.

3.4.5 Experimental design

The animals were randomly allocated to one of the 7 groups. Animals were served as an ischemia-reperfusion (I/R) groups (the animals were operated by exposing the femoral artery for inducing I/R) and received different doses of TPJ, α-tocopherol, and double-distilled deionized water (DDD water) *via* intragastric (i.g).

Group 1 served as sham-operated group. Skin incision was made to expose the femoral artery and then closed without inducing I/R, n=7.

Group 2 served as I/R-DDD water group. Rats received single dose of DDD water (20 ml/kg, i.g), n=7.

Group 3 served as I/R-1% Tween 80 group. Rats received single dose of tween 80 (20 ml/kg, i.g), n=7.

Group 4 served as I/R-Positive control group. Rats received single dose of standard vitamin E (250 mg/ 20 ml/kg, i.g), n=7.

Group 5 served as I/R-Low dose TPJ group. Rats received single i.g. administration of low dose of TPJ at a volume of 20 ml/kg body weight, n=7.

Group 6 served as I/R-Middle dose TPJ group. Rats received single i.g. administration of middle dose of TPJ at a volume of 20 ml/kg body weight, n=7.

Group 7 served as I/R-High dose TPJ group. Rats received single i.g. administration of high dose of TPJ at a volume of 20 ml/kg body weight, n=7.

Note: The ischemia reperfusion (I/R) model induced damage to surrounding skeletal muscle tissue and other tissues. Dosage of TPJ to be used in the present study was calculated from the total phenolic content of TPJ and extrapolated from human dose previously mentioned in the study of Tsang *et al.* (2011).

Surgery was performed at the Physiology Laboratory, F9 building, Suranaree University of Technology. All surgical procedures were performed under sodium pentobarbital anesthesia. The rat was injected intraperitoneally with sodium pentobarbital (50 mg/kg body weight). The anesthetized rat were placed on a board and maintained in a dorsal recumbent position. A skin incision was made over the anteromedial surface of the right upper leg, starting at the level of femoral artery, extending upward to the inguinal ligament. The right femoral artery was exposed after isolating of the femoral artery from the surrounding muscle tissues. The rats in the sham-operated group were received exactly the same surgical procedure as I/R groups, without occlusion of the femoral artery (Dong *et al.*, 2014). For intragastric (i.g) administration, a 1 cm abdominal incision was made to expose the stomach and the stomach was cannulated. The body temperature of the rat was maintained at 37±0.5°C by a desk lamp.

For I/R procedure, the right femoral artery was clamped with the microvascular clamp, resulting in ischemia. Ischemia was induced by 4 h of femoral artery occlusion with a microvascular clamp and followed by 2 h of reperfusion as showed in Figure 13. All substances were i.g. injected at 3 h after ischemia (Figure 13). At the end of reperfusion complete, the blood sample from cardiac puncture was collected in the heparinized tube and centrifuged at 2500 x g for 20 min for serum biochemical analysis. The serum levels of creatine phosphokinase (CPK or CK), aspartate aminotransferase (AST), alanine transaminase (ALT) were determined using an autonomic blood analyzer (VITROS 5600 integrated system, USA). The right gastrocnemius muscle was harvested, weighted, frozen on dry ice, and immediately stored at -80°C until further biochemical assessment. The harvested gastrocnemius muscle was assessed for activities of superoxide dismutase (SOD) and catalase (CAT), and the levels of glutathione (GSH), malondialdehyde (MDA) and protein carbonyl. The right soleus muscle was harvested and assessed for the cells damage by histology. The soleus muscle was fixed in 10% formalin, embedded in paraffin and cut on cross section at the muscle mid-section (5 μm thickness). Tissue sections were stained with Hematoxylin and Eosin.

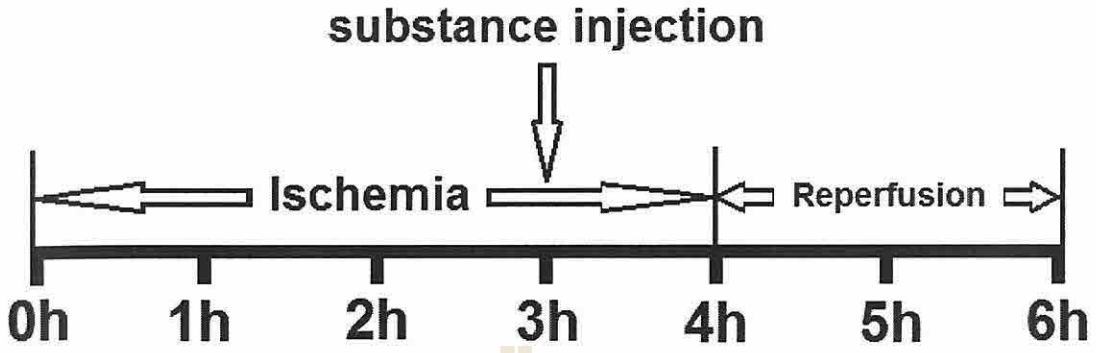

Figure 13 Induction of ischemia-reperfusion (I/R) model.

3.4.6 Determination of biochemical assays

The right gastrocnemius muscles were used to determine antioxidant activities of superoxide dismutase (SOD), and catalase (CAT), and levels of glutathione (GSH), malondialdehyde (MDA), and protein concentration. All the measurements were performed in triplicate and the mean values were calculated.

Preparation of tissue homogenates

The tissue homogenates of right gastrocnemius muscles were prepared by modified methods of Dong *et al.* (2014) and Perrin and Dempsey (1974) as described in Appendix E. Briefly, the frozen gastrocnemius muscle was thawed and homogenized in 50 mmol/L of ice-cold Tris-HCl buffer (pH 7.4) for 5 min, 4°C at 75% power to make a 10% homogenate. Homogenate was centrifuged at 5000 g for 10 min and supernatant was collected.

Determination of protein content

Protein content in right gastrocnemius muscle was determined by the Lowry method (Lowry, Rosebrough, Farr, and Randall, 1951). Solution A: copper sulfate, sodium tartrate, and sodium azide were mixed in 250 ml of DDD water. Solution B: sodium hydroxide, sodium carbonate, and sodium dodecyl sulfate were mixed and DDD water was added upto 250 ml in a volumetric flask and was kept at room temperature. Solution C: 2 N of diluted Folin-Ciocatteau reagent was mixed with with DDD.H_2O at the ratio of 1:20 and was kept at room temperature. Solution D (standard protein): bovine serum albumin was prepared in Tris-HCl buffer solution at different concentrations (0 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, and 2000 µg/ml, respectively) for the standard curve. Procedure: the samples and standards at a volume of 60 µl were added individual wells of a 96-well plate. Solution A was mixed with solution B in the ratio of 1:3, and 100 µl of this mixture was added into all wells, and then incubated for 30 min at room temperature. After that, 150 µl of the solution C was added into all wells. All solutions were mixed and incubated for 30 min at room temperature. The absorbance of the mixture was measured 650 nm using a microplate reader (BioRad-Benchmark Plus microplate reader, USA). The procedure and preparation were shown in Appendix F. The results were expressed the mg protein from the standard curve. All the determinations were performed in triplicate and the mean values were calculated.

Determination of superoxide dismutase

SOD enzyme activity in the right gastrocnemius muscle was determined using the method described by SOD assay kit (Sigma-Aldrich, St. Louis, MO, USA). The samples at the volume of 20 µl were added into wells of a 96-well plate. After that,

200 µl of water-soluble tetrazolium salt (WST) working solution was added into all wells, and mixed well. The mixture was incubated at 37°C for 20 min and the absorbance of the mixtures was measured at 450 nm using a microplate reader (BioRad-Benchmark Plus microplate reader, USA). The procedure and preparation were shown in Appendix G. All determinations were performed in triplicate. The SOD enzyme activity in the sample was calculated from inhibition percentage comparison. Inhibition percentage was calculated by using percent inhibition equation (inhibition rate % = [(Abs control – Abs sample) / Abs control)]*100.

Determination of catalase (CAT) activity

Activity of CAT enzyme in the right gastrocnemius muscle was determined using the method of Goldblith in 1950. The samples and standard enzyme (CAT enzyme) at a volume of 10 µl were mixed with 50 µl of hydrogen Peroxide. After that, 25µl of sulfuric acid and 150 µl of potassium permanganate solution was added and mixed well. Standard enzyme: CAT enzyme was prepared in Triz-HCl buffer solution at different concentrations (0, 40, 60, 80, and 100 units/ml of catalase in buffer) for the standard curve. The absorbance of the mixtures was measured at 490 nm using microplate reader (BioRad-Benchmark Plus microplate reader, USA). The procedure and preparation were shown in Appendix H. The results were expressed the units/mg protein from the standard curve. All determinations were performed in triplicate.

Determination of glutathione (GSH)

Glutathione (GSH) content in right gastrocnemius muscle was determined from the reaction of GSH with the 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB) and glutathione disulfide (GSSG). The method

used in the present study was modified from the study of Akerboom and Sies (1981) and Nair, Singh, and Krishan (1991). Briefly, the samples and glutathione standard at the volume of 10 µl were mixed with 150 µl of the working mixture (the assay buffer of the diluted enzyme solution mixed with DTNB stock solution). The mixed solution was incubated for 5 min at room temperature and then 50 µl of diluted NADPH solution was added to all wells. The yellow color product was obtained and its absorbance was measured at 412 nm using microplate reader (BioRad-Benchmark Plus microplate reader, USA). Glutathione standard solutions were used to determine the standard curve. The procedure and preparation were shown in Appendix I. The results were determined from the standard curve and expressed as nmol/g protein. All determinations were performed in triplicate.

Determination of lipid peroxidation (LPO)

Lipid peroxidation levels in the right gastrocnemius muscle was defined based on malondialdehyde (MDA) levels (Takhtfooladi *et al.*, 2014) and MDA levels were determined by thiobarbituric acid (TBA) reaction (Hussain, Shukla, and Chandra, 1987). The samples and standard (1,1,3,3-tetramethoxypropane (TMP)) (100 µl) were pipetted into a tube and 200 µl of TCA-TBA-HCL reagent was added, mixed, and boiled at 100°C in the water bath for 15 min. After cooling, all mixture was centrifuged at 1000 g for 10 min. The organic layer was transfer to a fresh tube and its absorbance was measured at 535 nm using microplate reader (BioRad-Benchmark Plus microplate reader, USA). The buffer solution (Tris-HCL buffer at ph 7.4) mixed with TCA-TBA-HCL reagent was used as the blank. All determinations were performed in triplicate. The MDA concentration was calculated from the equation generated by the standard curve of TMP. The procedure and preparation were shown

in Appendix J. The LPO level was expressed in nanomole (nm) of MDA per gram of tissue.

3.4.7 Determination of histological change

The skeletal muscle tissue (half soleus muscle tissue) was fixed in 10% formalin and embedded in paraffin. The tissues were dehydrated twice with difference diluted alcohol including 70%, 80%, 95%, and 100% for 60 min each. The tissues were cleared twice with xylene (Carlo Erba) for 60 min each and the infiltrated twice with paraffin at 56 - 58°C for 60 min each. The infiltrated tissues were embedded into paraffin block. The soleus tissue sections were cut into 5 μm slice using rotary microtome (Microm HM 355S, Microm GmbH., Germany) and the sections were floated with distilled water (distilled water was mixed gelatin) in tissue floating bath (Medex Nagel GmbH., Germany) at 45°C, and then mounted on slide. The sections were dried with hotplate (Medex Negel) at 45°C for 24 h after mounted on slide. After that, the sections were stained with hematoxylin-eosin to examine morphology of the skeletal muscle structure. Number of muscle cell and morphology of muscle tissue were examined from three fields of view each muscle sample by randomly screened method under light microscope (ZEISS Microscope Primo Star, USA). The skeletal muscle tissue damage was assessed semi-quantitative. The muscle cell damage was observed number of neutrophils immigrated to surrounding the muscle cells and muscle cells swelling (the muscle cell was bigger than normal cell compared with surrounding muscle cells). Number of neutrophils surrounded the muscle cell or muscle cell swelling that count one muscle cell damage. After that, percentage of cells damage in each field was calculated the mean was accepted as the representative

value of the sample. Histological changes were then scored from 0 to 3, the skeletal muscle tissue damage grade 0 for normal histology (absent, no cells change and damage), grade 1 (slight, less than 15% of skeletal muscle cells change and damage), grade 2 (moderate, 15-35% of cells change and damage), and grade 3 (severe, more than 35% of cells change and damage), (Warzechaa *et al.*, 2004). The results were shown score damage and the value of muscle samples were observed.

3.5 Statistical analysis

Data on total phenolic content, IC_{50} values of DPPH, ABTS, and FRAP assays were presented as mean of triplicates±SEM. Data from *in vivo* study were presented as mean±SEM. All of the experimental data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc (Turkey) testing, using IBM SPSS Statistics software version 20 (System Software Inc., USA). All graphs were created by SigmaPlot software version 10 (System Software Inc., USA). The *P* value less than 0.05 were considered a statistically significant.

CHAPTER IV

RESULTS

4.1 *In vitro* study

4.1.1 The results of yield, time, and volume of concentrate pomegranate juice

The soluble solids on degree brix of fresh TPJ was 15.53±0.77°Brix and was concentrated to the final concentration of 60.00±0.00°Brix by using a rotary vacuum evaporator and a microwave evaporator which were achieved by 21 and 11 min, respectively. The percentage yields of concentrated TPJ by a rotary vacuum evaporation and concentrated TPJ by microwave evaporation were 16.67±0.60% and 18.00±1.26%, respectively. The results showed that concentrated TPJ by microwave evaporation took a shorter period of time to concentrate and give more percentage yield than that of a rotary vacuum evaporation.

4.1.2 Chemical composition of fresh Thai pomegranate juice

Chemical analyses results and quantities of some sugars, vitamins and minerals chemical composition of fresh Thai pomegranate juice were shown in Table 3.

Table 3 The amount of chemical compositions of fresh TPJ.

Components	Amount
Energy (Kcal)/ml	474.00±11.60
Ash (g/L)	6.40±0.00
Protein (g/L)	4.65±0.005
Total Fat (g/L)	-
Total Carbohydrate (g/L)	113.85±2.95
Sugar	
Total sugar (g/L)	112.55±1.05
Glucose (g/L)	62.75±0.35
Fructose (g/L)	49.80±0.70
Sucrose (g/ml)	-
Vitamin	
Vitamin E (mg/100 g)	0.14±0.00
Vitamin C (mg/100 g)	5.33±0.19
Minerals	
Calcium (mg/L)	17.15±0.05
Phosphorus(mg/L)	309.85±8.15
Sodium (mg/L)	90.65±2.05
Potassium (mg/L)	3056.55±63.85
Magnesium (mg/L)	61.50±2.80

Values are expressed as means±SEM. Number values of two independent.

4.1.3 Phytochemical screening analysis.

Phytochemical composition of fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation were screened for the presence of protein, carbohydrate, tannin and phenols, flavonoids, saponins, glycosides and steroids by using standard methods with some modifications (Harborne, 1973). The phytochemical analysis showed the presence of protein, tannin and phenols, flavonoids, saponins, glycosides and steroids in fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation as shown in Table 4. Carbohydrate was not found in all TPJ samples by phytochemical screening methods used in the present study, but total carbohydrate was found in fresh TPJ in chemical composition analysis (Table 3). The phytochemical compounds found in pomegranate juice may be used as potential source of antioxidant in various situations.

Table 4 Phytochemical screening analysis of fresh TPJ, concentrated TPJ by rotary vacuum evaporation, and concentrated TPJ by microwave evaporation.

	Concentration method of TPJ		
	Fresh TPJ	**Rotary vacuum evaporation**	**Microwave evaporation**
Protein	+	+	+
Carbohydrate	-	-	-
Tannins and Phenols	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Glycosides	+	+	+
Steroid	+	+	+

The all results were show symbol of presence (+) and absence (-).

4.1.4 Total phenolic content by Folin-Ciocalteu method

The Folin-Ciocalteu method was used to determine the total phenolic content in pomegranate juice. Fresh TPJ and concentrated TPJs were investigated in triplicate for total phenolic content using Folin-Ciocalteau's reagent. Total phenolic contents of concentrated TPJs by rotary vacuum evaporation and by microwave evaporation were found to be similar (6606.30±432.93 and 6675.46±348.54 mg GAE/L juice, respectively) which were significantly higher than that of fresh TPJ (1938.41±207.86 mg GAE/L juice, $P<0.05$) (as shown in Table 5).

4.1.5 Antioxidant assay: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging method

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging method was used to determine the antioxidant activity of pomegranate juice. Fresh TPJ and concentrated TPJs were investigated in triplicate for DPPH assay. Concentrated TPJs (by rotary vacuum evaporation and by microwave evaporation) and fresh TPJ showed antiradical activity against DPPH radicals with IC_{50} values of 4.34±0.07, 4.82±0.12, and 18.60±0.8 µl/ml, respectively (as shown in Table 5). Antioxidant activities of both concentrated were significantly higher than fresh TPJ ($P<0.05$). Concentrated TPJs (by rotary vacuum evaporation and by microwave evaporation) and vitamin C showed antiradical activity against DPPH radicals with IC_{50} values of 3.50±0.05, 3.65±0.09, and 0.23±0.04 µg/ml, respectively (as shown in Tables 5). Concentrated TPJ by rotary vacuum evaporation and by microwave evaporation exhibited similar radical scavenging activity on DPPH assays, which were significantly higher than that of fresh TPJ ($P<0.05$). Radical scavenging activity on DPPH assay of a positive

control vitamin C was significantly higher than that of both concentrated TPJs (P<0.05) (as shown in Table 5).

4.1.6 Antioxidant assay: 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical-scavenging method

The 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical-scavenging method was used to determine the antioxidant activity of pomegranate juice. Fresh TPJ and concentrated TPJs were investigated in triplicate for ABTS assay. Concentrated TPJs (by rotary vacuum evaporation and by microwave evaporation) and fresh TPJ showed antiradical activity against ABTS radicals with IC_{50} values of 6.33±1.09, 6.86±1.05 and 25.85±2.99 µl/ml, respectively (as shown in Table 5). Antioxidant activities of both concentrated were significantly higher than fresh TPJ (P<0.05). Concentrated TPJs (by rotary vacuum evaporation and by microwave evaporation) and vitamin C expressed IC_{50} values of 5.10±0.88, 5.20±0.80, and 0.14±0.15 µg/ml, respectively (as shown in Table 5). Concentrated TPJs by rotary vacuum evaporation and by microwave evaporation exhibited similar radical scavenging activity on ABTS assays, which were significantly higher than that of fresh TPJ (P<0.05). Radical scavenging activity on DPPH assay of a positive control vitamin C was significantly higher than that of both concentrated TPJs (P<0.05) (as shown in Table 5).

4.1.7 Antioxidant assay: Ferric reducing antioxidant power (FRAP) method

The Ferric reducing antioxidant power (FRAP) method was used to determine the antioxidant activity on the base of the iron-reducing capacity of pomegranate juice. Fresh TPJ and concentrated TPJs were investigated in triplicate for FRAP

assay. Concentrated TPJs by rotary vacuum evaporation and by microwave evaporation exhibited similar iron-reducing capacity of pomegranate juice on FRAP assays, which were significantly higher than that of fresh TPJ (P<0.05). Concentrated TPJs (by rotary vacuum evaporation and by microwave evaporation) and fresh TPJ showed reducing potential (227.14±25.94, 273.74±26.06, and 61.38±6.34 mmol Fe^{2+}/L, respectively), as shown in Table 5.

Table 5 *Total phenolic content and antioxidant activities of concentrated TPJ by rotary vacuum evaporation, and concentrated TPJ by microwave evaporation.*

		Concentration method of TPJ	
Methods	**Vitamin C**	**Rotary vacuum evaporation**	**Microwave evaporation**
Total phenolics (mg GAE/L)	-	6606.30±432.93[a]	6675.46±348.54[a]
DPPH (IC_{50}, µl/ml)	-	4.34±0.07	4.82±0.12
(IC_{50}, mg/ml)	0.23±0.04[b]	3.50±0.05[a]	3.65±0.09[a]
ABTS (IC_{50}, µl/ml)	-	6.33±1.09	6.86 ±1.05
(IC_{50}, mg/ml)	0.14±0.15[b]	5.10±0.88[a]	5.20 ±0.80[a]
FRAP (mmol/l Fe^{+2})	-	227.14±25.94[a]	273.74±26.06[a]

Values are expressed as means±SEM. The different superscript alphabets are significantly different from each other (P<0.05). There was no significant difference between concentrated TPJs by rotary vacuum evaporation and by microwave evaporation. TPC: total phenolic content; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; FRAP: ferric reducing antioxidant power.

4.2 *In vivo* study: The effects of Thai pomegranate juice on ischemia-reperfusion (I/R)-induced oxidative stress in skeletal muscle of male Wistar rats

4.2.1 Serum biochemical assays

The results of serum levels of biochemical parameters (serum aspartate aminotransferase (AST), alanine transaminase (ALT), and creatine phosphokinase (CPK or CK) in rats received concentrated TPJ, DDD water, 1% tween 80, and alpha-tocopherol or vitamin E (positive control) were shown in Table 6. There was no statistically significant difference in all plasma biochemical parameters between all groups.

Table 6 The effects of TPJ and vitamin E on serum biochemical parameters (AST, ALT and CPK).

Group	AST (U/L)	ALT (U/L)	CPK (U/L)
Sham	170.43±20.58	39.14±4.88	216.00±44.51
I/R-DDD water	219.86±41.80	62.43±9.21	246.33±11.89
I/R-Tween 80	197.50±22.51	44.50±6.09	355.67±32.34
I/R-Positive	177.33±23.57	82.43±11.04	200.00±15.14
I/R-Low dose TPJ	186.43±23.57	50.71±3.13	258.00±28.36
I/R-Middle dose TPJ	176.43±29.76	47.17±7.60	239.67±21.67
I/R-High dose TPJ	227.17±33.64	108.14±17.74	254.67±79.10

Values are expressed as means±SEM. TPJ: Thai pomegranate juice; I/R: Ischemia reperfusion; AST: Aspartate aminotransferase, ALT: Alanine transaminase, CPK or CK: Creatine phosphokinase

4.2.2 Muscle biochemical assays

The antioxidant activity of superoxide dismutase (SOD)

The SOD activity of the skeletal muscle tissue were as follows: sham group, 18.39±0.22 U/mg protein; I/R-DDD water group, 13.02±0.55 U/mg protein; I/R-1% Tween 80 group, 14.28±0.70 U/mg protein; I/R-Vitamin E group, 17.06±1.10 U/mg protein; I/R-Low dose TPJ, 16.90±0.57 U/mg protein; I/R-Middle dose TPJ, 17.28±0.55 U/mg protein; and I/R-High dose TPJ, 16.3±0.24 U/mg protein (Figure 14). I/R treatment caused significant reduction of SOD activity, compared to sham group (P<0.05). Low dose TPJ and middle dose of TPJ significantly enhanced SOD activities induced by I/R (P<0.05, Figure 14). The tissue SOD activities in control groups (I/R-DDD water, I/R-1% Tween 80) were statistically lower than those in the sham, I/R-Vitamin E, I/R-Low dose TPJ, and I/R-Middle dose TPJ but not I/R-High dose TPJ. No significant difference was observed between the TPJ treatment groups.

The antioxidant activity of catalase (CAT)

The CAT activity of the skeletal muscle tissue were as follows: sham group, 4.00±0.43 U/mg protein I/R-DDD water group, 1.34±0.44 U/mg protein; I/R-1% Tween 80 group, 2.25±0.07 U/mg protein; I/R-Vitamin E group, 2.87±0.17 U/mg protein; I/R-Low dose TPJ, 3.31±0.70 U/mg protein; I/R-Middle dose TPJ, 3.57±0.39 U/mg protein; and I/R-High dose TPJ, 2.72±0.17 U/mg protein (Figure 15). I/R treatment caused significant reduction of CAT activity, compared to sham group (P<0.05).The tissue CAT activity in control groups (I/R-DDD water) was statistically lower than those in the sham group (P<0.05, Figure 15). Low dose TPJ and middle dose TPJ, but not vitamin E and high dose TPJ, significantly enhanced CAT activities induced by I/R (P<0.05, Figure 15). No significant difference was observed between

the TPJ treatment groups. CAT activity of vitamin E and all groups of TPJ were higher than their control group but no significant difference.

The level of glutathione (GSH)

The GSH levels of the skeletal muscle tissue were as follows: sham group, 0.49±0.02 μmol/mg protein; I/R-DDD water group, 0.42±0.01 μmol/mg protein; I/R-1% Tween 80 group, 0.44±0.01 μmol/mg protein; I/R-Vitamin E group, 0.47±0.00 μmol/mg protein; I/R-Low dose TPJ, 0.49±0.03 μmol/mg; I/R-Middle dose TPJ, 0.48±0.02 μmol/mg protein; and I/R-High dose TPJ, 0.49±0.01 μmol/mg protein (Figure 16). I/R treatment caused significant reduction of GSH level, compared to sham group (P<0.05). The tissue GSH level in control group (I/R-DDD water) was statistically lower than sham group (P<0.05, Figure 16). The GSH level of vitamin E group was similar to its control group (I/R-1% Tween 80). GSH levels of all groups of TPJ were significantly higher than that of their control groups (I/R-DDD water, P<0.05).

The level of malondialdehyde (MDA)

The MDA levels of the skeletal muscle tissue were as follows: sham group, 1.03±0.17 nmol/g; I/R-DDD water group, 2.50±0.38 nmol/g; I/R-1% Tween 80, 2.09±0.31 nmol/g; I/R-Vitamin E group, 1.18±0.11 nmol/g; I/R-Low dose TPJ, 1.22±0.06 nmol/g; I/R-Middle dose TPJ, 1.13±0.08 nmol/g; and I/R-High dose TPJ, 1.44±0.11 nmol/g (Figure 17). I/R treatment caused significant increase in MDA level, compared to sham group (P<0.05). MDA levels of all groups except high dose of TPJ, were significantly lower than that of their control groups (P<0.05). The tissue MDA levels in control groups (I/R-DDD water and I/R-1% Tween 80) were

statistically higher than those in the sham (P<0.05, Figure 17). No significant difference was observed between the TPJ treatment groups.

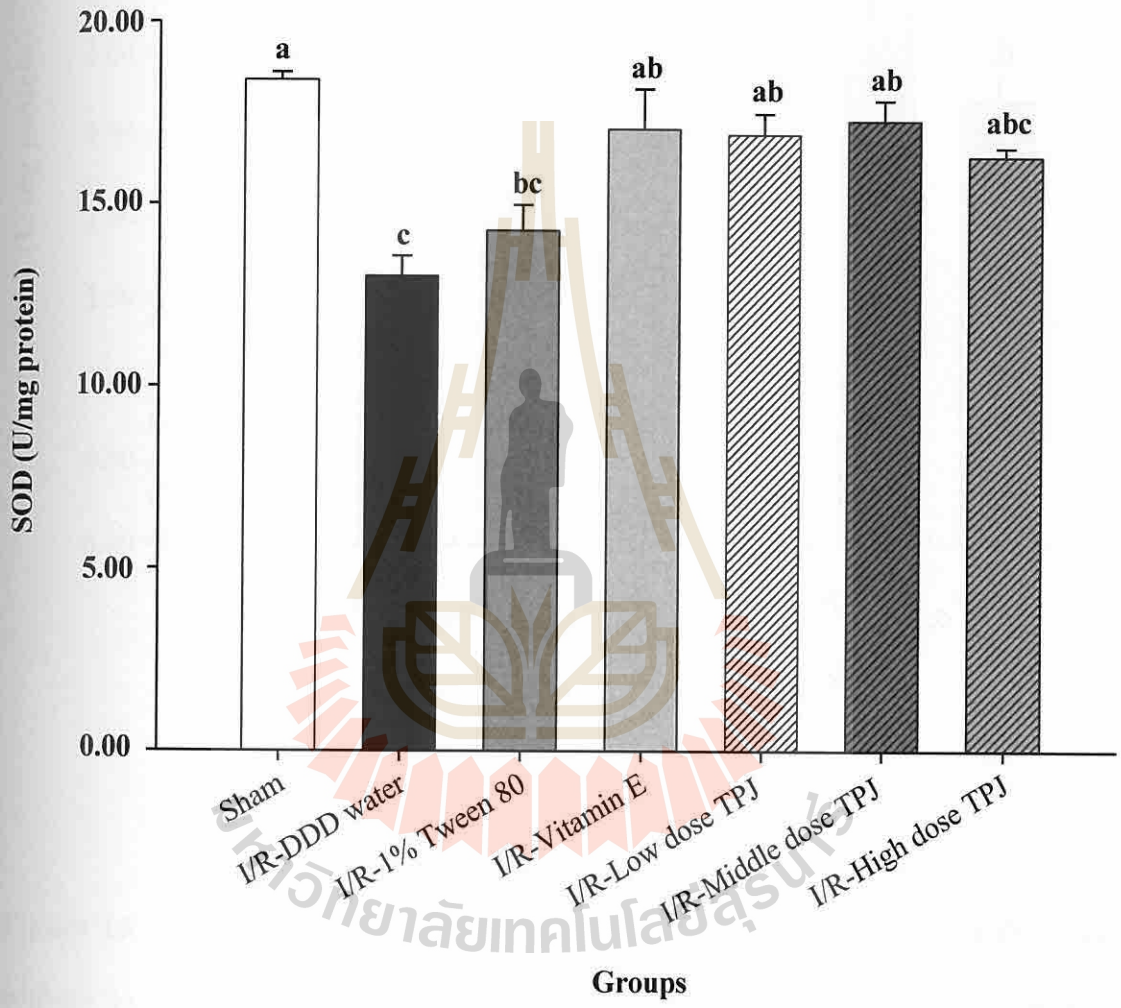

Figure 14 The effects of Thai pomegranate juice (TPJ) and vitamin E on the SOD activity in ischemia/reperfusion-induced oxidative stress in the rat gastrocnemius muscle. The different alphabets are significantly different from each other (P<0.05).

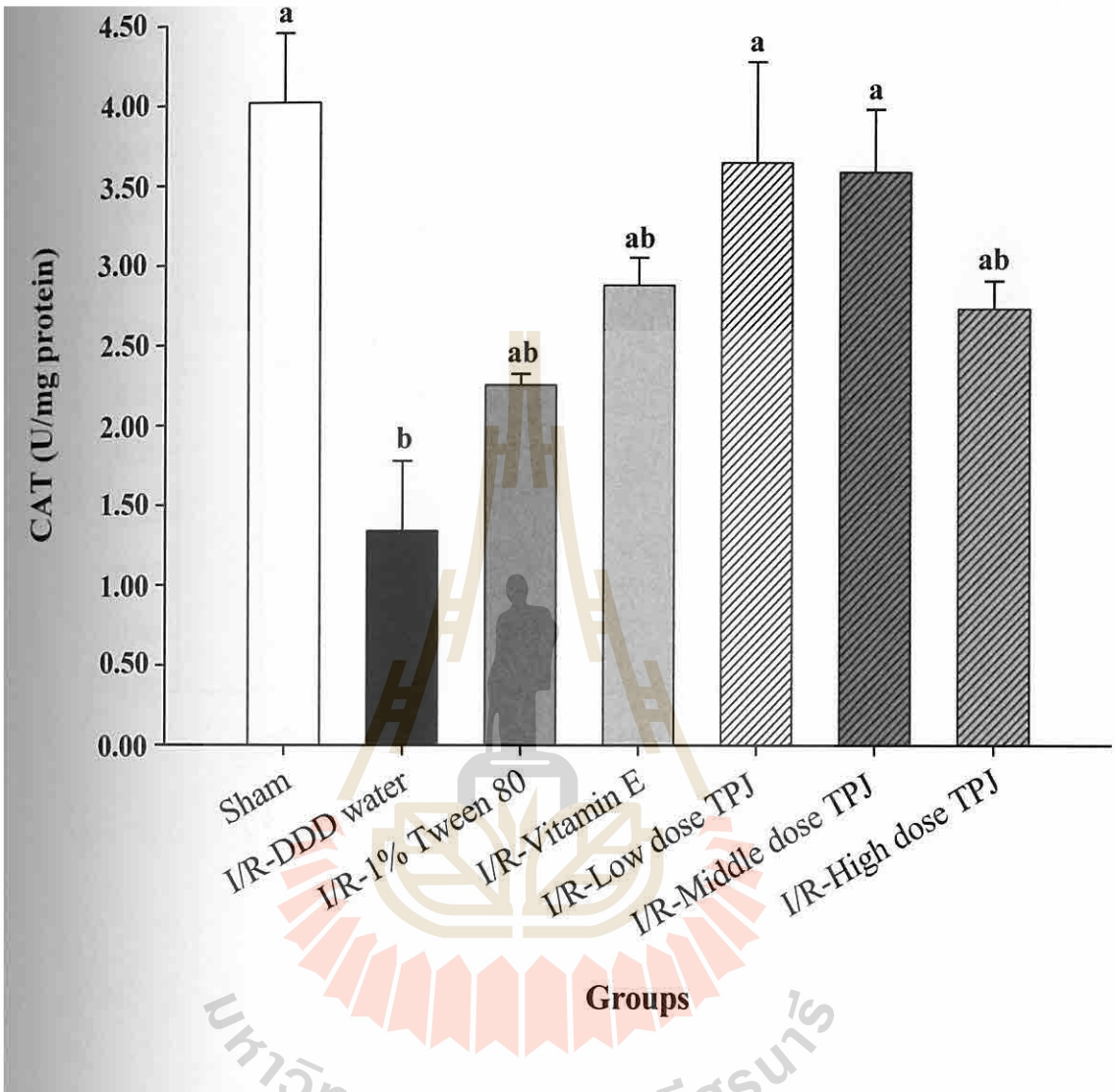

Figure 15 The effects of Thai pomegranate juice (TPJ) and vitamin E on the CAT activity in ischemia/reperfusion-induced oxidative stress in the rat gastrocnemius muscle. The different alphabets are significantly different from each other ($P<0.05$).

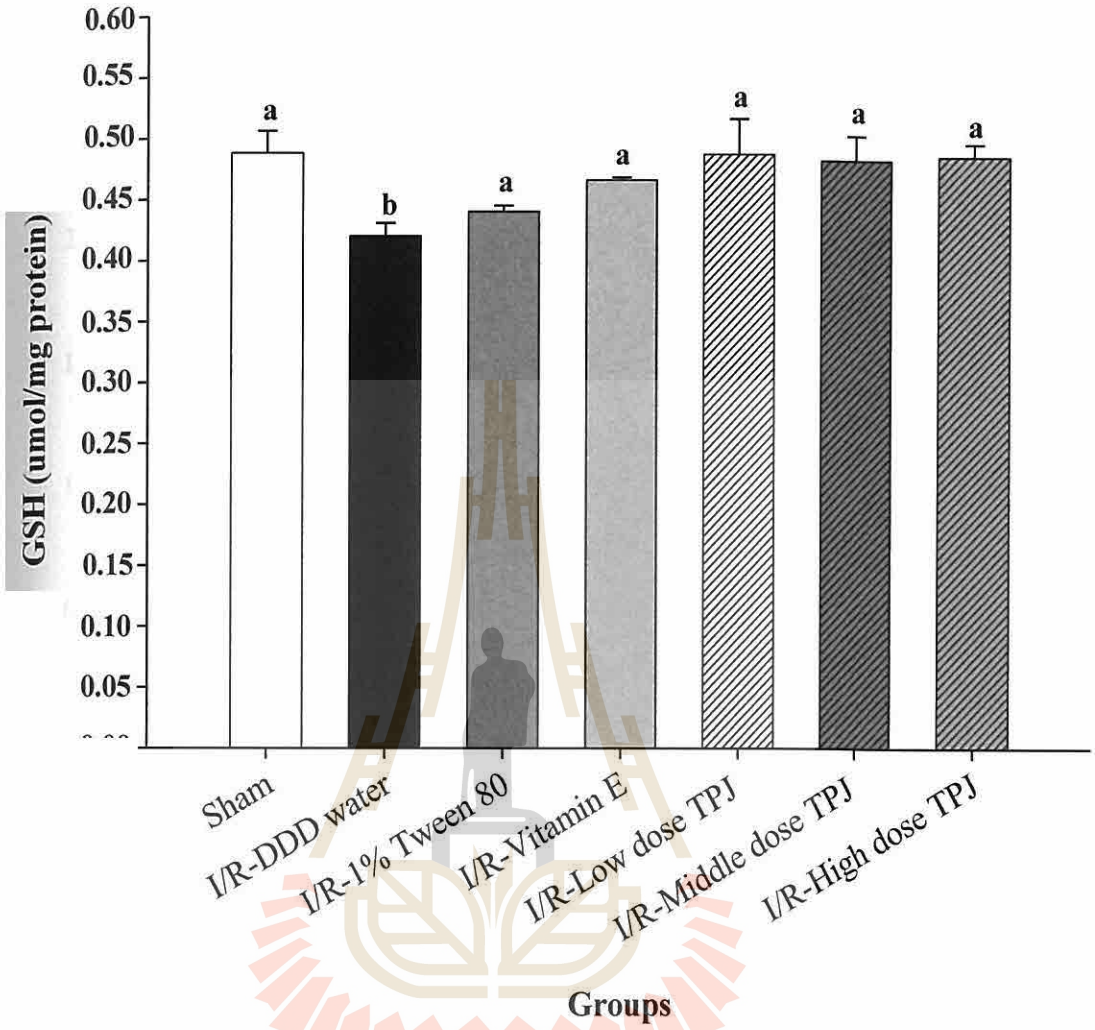

Figure 16 The effects of Thai pomegranate juice (TPJ) and vitamin E on the GSH levels in ischemia/reperfusion-induced oxidative stress in the rat gastrocnemius muscle. The different alphabets are significantly different from each other (P<0.05).

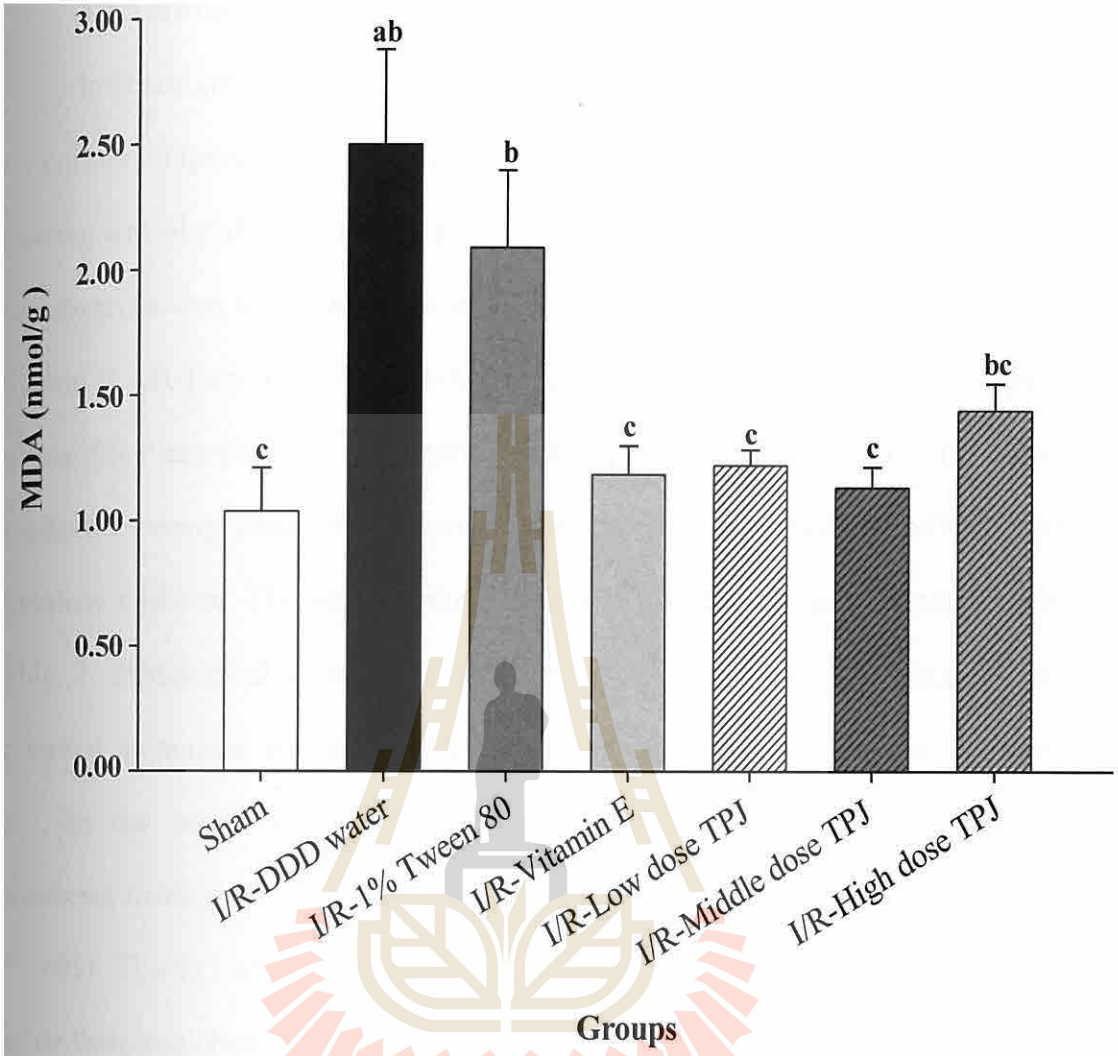

Figure 17 The effects of Thai pomegranate juice (TPJ) and vitamin E on the MDA in ischemia/reperfusion-induced oxidative stress in the rat gastrocnemius muscle. The different alphabets are significantly different from each other ($P<0.05$).

4.2.3 Histological changes in skeletal muscle tissue

In sham group, regular muscle fiber morphology was observed, and staining was uniform (Figure 18). In I/R-DDD water and I/R-1% Tween 80 group muscle fiber structure was disordered, irregular muscle fiber morphology was observed, shrinkage was observed and cell gaps were obviously widened (Figure 19 and 20). In I/R-Vitamin E, I/R-Low dose TPJ, I/R-Middle dose TPJ, and I/R-High dose TPJ groups, muscle fiber morphology was more regular and staining was uniform, irregular muscle fiber morphology was observed, shrinkage was observed and cell gaps were obviously widened. The score of skeletal muscle histological changes was shown in Table 7. Histological changes of the skeletal muscle tissue following I/R were presented as amount of cells, % Cells changes, and histological grade. Amount of cells, in the soleus skeletal muscle of all groups were similar. I/R treatment, significant increase in % cell damage in the soleus muscle, compared to sham group (P<0.05). The % Cells changes in I/R-DDD water and I/R-1% tween 80 groups are higher than that observed in the soleus muscle of other groups. Moderate muscle cell damage was observed in I/R-DDD water group as histological grading for soleus skeletal muscle in this group was scored as 2. Soleus skeletal muscle damage in other groups were mild as histological grading were scored as 1. Vitamin E and all doses of TPJ significantly reduced the increase of % cell damage-induced by I/R (P<0.05, compared to I/R-DDD water).

Table 7 Effects of different doses of TPJ and vitamin E on histological changes of the soleus muscle following ischemia reperfusion.

Groups	Amount of cells	% Cells damage	Histological grading
Sham	81.91±8.89[a]	0.33±0.21[b]	1.0
I/R-DDD water	74.54±6.85[a]	20.38±4.89[a]	2.0
I/R-1% Tween 80	78.49±7.47[a]	7.82±0.854[b]	1.0
I/R-Vitamin E	79.98±7.75[a]	0.80±0.435[b]	1.0
I/R-Low dose TPJ	82.29±5.07[a]	0.36±0.119[b]	1.0
I/R-Middle dose TPJ	91.03±4.72[a]	2.72±0.30[b]	1.0
I/R-High dose TPJ	91.26±6.15[a]	2.00±0.316[b]	1.0

The different superscript alphabets are significantly different from each other ($P<0.05$). Histological changes were scored on a scale from 0 to 3: 0 = absence (no cells change and damage); 1 = mild (less than 15% of skeletal muscle cells change and damage); 2 = moderate (15-35% of cells changes and damage); 3 = severe (more than 35% of cells change and damage).

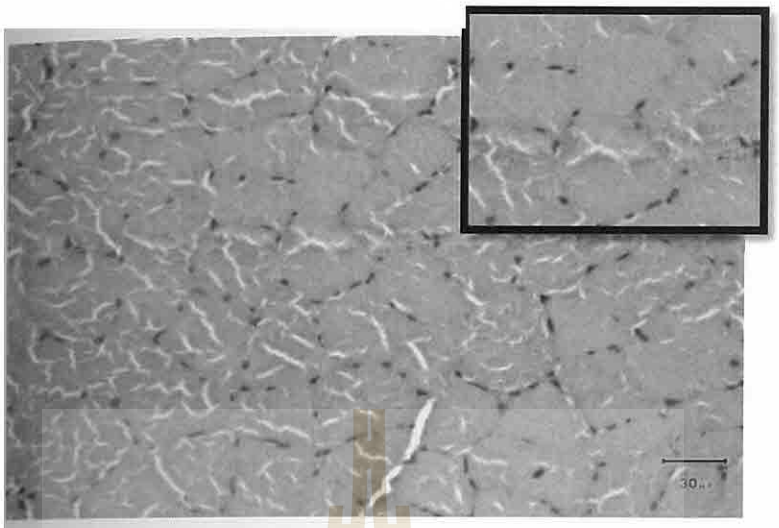

Figure 18 Photomicrograph under light microscopy of normal skeletal muscle tissue in sham group (H&E staining, 40×). Regular muscle fiber morphology and staining was uniform. The sham group was scored as 1.

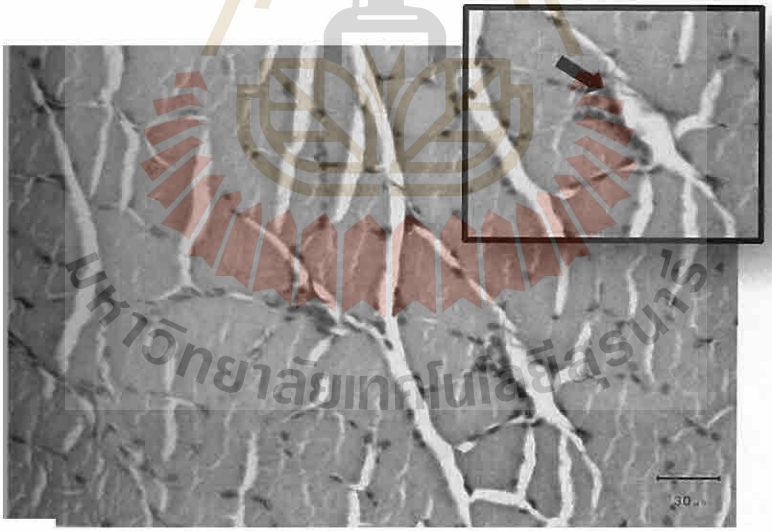

Figure 19 Photomicrograph under light microscopy of skeletal muscle tissue in I/R-DDD water group (H&E staining, 40×). Foci of moderate muscle fiber inflammatory cell infiltration and extensive necrosis (arrow) were shown. The I/R-DDD water group was scored as 2.

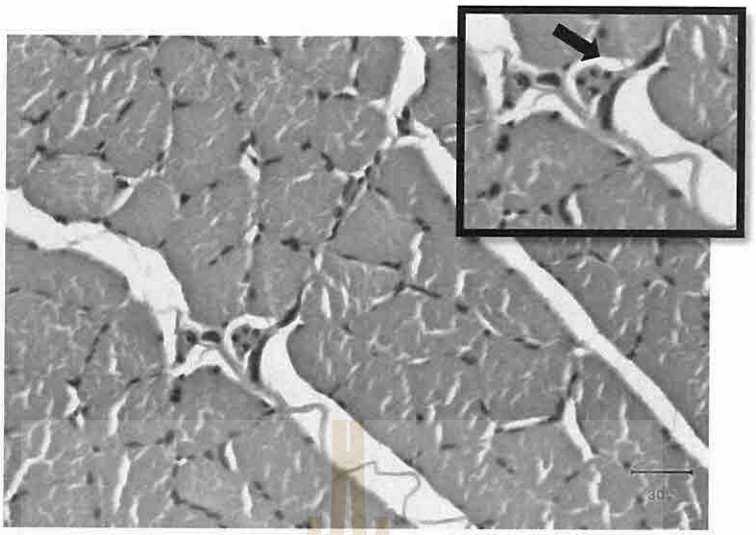

Figure 20 Photomicrograph under light microscopy of skeletal muscle tissue in I/R-1% Tween 80 group (H&E staining, 40×). Foci of mild muscle fiber inflammatory cell infiltration and extensive necrosis (arrow) were shown. The I/R-1% Tween 80 group was scored as 1.

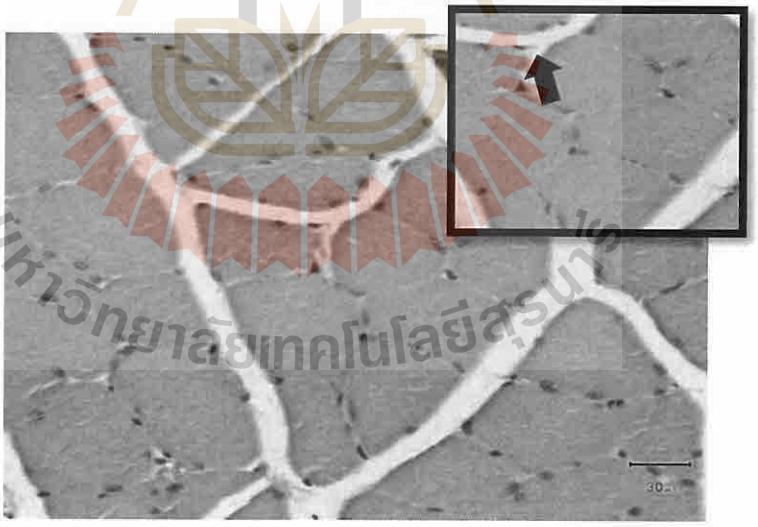

Figure 21 Photomicrograph under light microscopy of skeletal muscle tissue in I/R-Vitamin E group (H&E staining, 40×). Foci of mild muscle fiber inflammatory cell infiltration and extensive necrosis (arrow) were shown. The I/R-Vitamin E group was scored as 1.

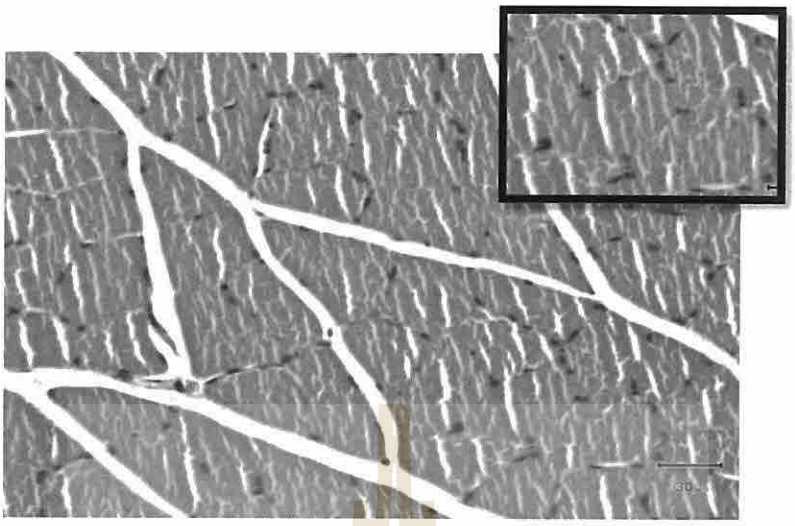

Figure 22 Photomicrograph under light microscopy of skeletal muscle tissue in I/R-Low dose TPJ group (H&E staining, 40×). The I/R-Low dose TPJ group was scored as 1.

Figure 23 Photomicrograph under light microscopy of skeletal muscle tissue in I/R-Middle dose TPJ group (H&E staining, 40×). The I/R-Middle dose TPJ group was scored as 1.

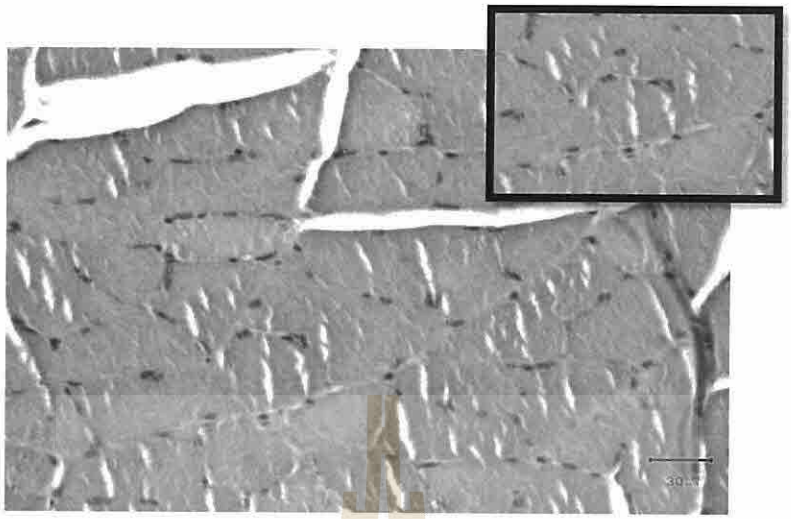

Figure 24 Photomicrograph under light microscopy of skeletal muscle tissue in I/R-High dose TPJ group (H&E staining, 40×). The I/R-High dose TPJ group was scored as 1.

CHAPTER V

DISCUSSION AND CONCLUSION

Polyphenols from many fruits and plants are known to possess various effect properties such as antioxidant activity, cardiovascular disease, and anti-cancer (Wang, Ho, Glackin, and Martins-Green, 2012; Fuhrman, Volkova, and Aviram, 2005). High amount of polyphenolic compound can be found in the juice from the pomegranate fruit (Gil *et al.*, 2000). Pomegranate juice is a source of bioactive compounds with potential health-promoting activity. The pomegranate juice has potent antioxidant profile. Several studies revealed that the pomegranate juice is the rich source of polyphenols (Tezcan, Gültekin-Özgüven, Diken, Özçelik, and Erim, 2009; Gil *et al.*, 2000; Mousavinejad, Emam-Djomeh, Rezaei, and Khodaparast, 2009). Thus, the pomegranate juice may possess biological activities related to polyphenolic compounds. The present study investigated the best concentration method for the assay of total phenolic content and antioxidant activity of Thai pomegranate juice (TPJ), and protective effects of TPJ on oxidative stress induced by ischemia-reperfusion in rat skeletal muscle by measuring changes in important oxidative stress markers in both serum and skeletal muscle tissue. The present study demonstrated the pomegranate juice could enhance antioxidant probably by inhibiting oxidative stress in skeletal muscle of healthy adult male Wistar rats and possibly through an increase antioxidant capacity of ischemic muscle tissue in rats. The results of this study

provided information on chemical composition, phytochemical composition, total polyphenol content, and antioxidant activity of TPJ. Chemical composition of fresh TPJ was investigated in the present study which was similar to pomegranate juice from Tukey and Spain but amount of each components in TPJ were difference from that of pomegranate juice obtained from Turkey and Spain (Table 8). Amounts of ash, vitamin E, phosphorus, sodium, potassium, and magnesium in TPJ were higher than those of pomegranate juice from Turkey and Spain. Amounts of total sugar, glucose, fructose, vitamin C, and calcium were lower than those of pomegranate juice from Turkey and Spain. The differences in amounts of chemical compositions of pomegranate juice may be results of species, landscape, temperature, and environment in each cultivated areas, and also the difference assay methods used in each laboratory.

Table 8 The amount of chemical composition of pomegranate juice from different places.

Component	Present study (mean±SEM)	Previous study Amount	Area	References
Ash (g/L)	6.40±0.00	3.90	Turkey	Velioglu *et al.*, 1997
Total sugar (g/L)	112.55±1.05	148.75	Turkey	Poyrazoglu *et al.*, 2002
Glucose (g/L)	62.75±0.35	64.80	Turkey	Velioglu *et al.*, 1997
Fructose (g/L)	49.80±0.70	71.50	Turkey	Velioglu *et al.*, 1997
Vitamin E (mg/100 g)	0.14±0.00	0.07	Spain	Anatita *et al.*, 2015
Vitamin C (mg/100 g)	5.33±0.19	57.80	Spain	Anatita *et al.*, 2015
Calcium (mg/L)	17.15±0.05	20.00	Turkey	Velioglu *et al.*, 1997
Phosphorus (mg/L)	309.85±8.15	270.00	Turkey	Velioglu *et al.*, 1997
Sodium (mg/L)	90.65±2.05	9.00	Turkey	Velioglu *et al.*, 1997
Potassium (mg/L)	3056.55±63.85	1209.00	Turkey	Velioglu *et al.*, 1997
Magnesium (mg/L)	61.50±2.80	45.00	Turkey	Velioglu *et al.*, 1997

Screening for phytochemical composition of Fresh TPJ and concentrated TPJs revealed the presence of protein, tannins and phenols, flavonoids, saponins, glycoside, and steroids. Carbohydrate was not found in the present screening method may be due to the screening method is specific for starch, not for all types of carbohydrate. High amounts of polyphenols and antioxidant activity (DPPH, ABTS, and FRAP assays) of fruit TPJ were reported in the present study. Total phenolic content in fresh TPJ (1938.41±207.86 mg GAE/L juice) was higher than previously study (144.00±80.00 mg GAE/L juice) in the study of Tezcan *et al.* (2009). Fresh TPJ expressed 15.53±0.77°Brix which was not different from the juice of pomegranate fruit growing in other regions. Li and co-workers (2015) reported that Brix values of the pomegranate juice ranged from 13.97 to 16.30°Brix of 15 cultivars in China.

The present study provided the best concentration method from the assay for total phenolic content and antioxidant activity of TPJ. Fresh TPJ (15.53±0.77°Brix) was concentrated to 60.00±0.00°Brix by rotary vacuum evaporation, and microwave evaporation (as previously desorbed by Goula and co-workers, 2014) which were achieved within 21 and 11 min, respectively. The total phenolic contents (TPC) of concentrated TPJs by rotary vacuum evaporation, by microwave evaporation, were found to be similar (6606.30±432.93, and 6675.46±348.54 mg GAE/L juice, respectively). Concentrated TPJs by rotary vacuum evaporation by microwave evaporation and fresh TPJ showed antiradical activity against DPPH radicals, antiradical activity against ABTS radicals, and the iron-reducing capacity by FRAP assays. The IC_{50} values of DPPH and ABTS assays for pomegranate juice have never been reported elsewhere. The IC_{50} values of DPPH in other fruit juices were 2.500±2.88 µg/ml (fresh apple juice), 0.156±0.031 µg/ml (fresh guava), and

0.313±0.143 μg/ml (fresh lime) (Beh, 2012). Antioxidant activity of both concentrate TPJs were higher than other fresh fruits juices. The present FRAP value of TPJ was higher than the study of of Tezcan *et al.* (2009). The antioxidant activity of the tested TPJ in DPPH, ABTS, and FRAP methods was correlated with the total phenolic content that was quantified using Folin-ciocalteu test. The similarity in antioxidant activity (by DPPH,ABTS and FRAP assays) and total phenolic quantification of both concentration method for TPJ indicated that both concentration methods can be used for quantitative analysis of total phenolic content and antioxidant activity of TPJ. However, microwave evaporation may be the best choice since TPJ can be concentrated in a short period of time and give more percentage yield. Microwave evaporation has the advantage of heating the TPJ rapidly and uniformly.Total phenolic content in Fresh TPJ was higher than that of Georgia pomegranate aril juice (272.00-849 mg GAE/L juice, reported by Rajasekar and co-workers, 2012), but lower than of Iran pomegranate juice (2376–9.304 mg GAE/L juice reported by Mousavinejad and co-workers (2009). The previous studies showed that total phenolic content levels of pomegranate juice vary greatly among different cultivars and different growing regions in the world. The results of Li and co-workers (2014) showed that polyphenol monomers (punicalagin, gallic acid, catechin, chlorogenic acid, cafeic acid, epicatechine, ferulic acid, ellagic acid, and kaempferol) were found in aril juice of 10 pomegranate cultivars from 4 growing regions of china (Table 9).

Table 9 Polyphenol monomers of aril juice of 10 pomegranate cultivars from 4 growing regions of china (μg/ml) (Li and co-workers, 2014).

Cultivars	Punicalagin	Gallic acid	catechin	Chlorogenic acid	Caffeic acid	Epicatechin	Ferulic acid	Ellagic acid	Kaempferol
XJ-TSL	396.31 ± 0.02 g	2.14 ± 0.03 f	4.88 ± 0.08 j	9.48 ± 0.03 j	2.32 ± 0.06 c	10.04 ± 0.05 i	0.44 ± 0.03 g	1.02 ± 0.03 a	8.12 ± 0.08 f
XJ-SSL	791.81 ± 0.05 b	14.50 ± 0.02 c	5.63 ± 0.05 i	27.11 ± 0.05 d	1.93 ± 0.01 e	14.04 ± 0.02 f	0.46 ± 0.01 g	0.28 ± 0.01 f	10.66 ± 0.09 e
SD-TSL	1042.93 ± 0.01 a	6.23 ± 0.06 d	40.53 ± 0.04 j	25.56 ± 0.01 e	2.56 ± 0.03 a	9.28 ± 0.03 j	1.72 ± 0.02 a	0.73 ± 0.01 b	1.50 ± 0.02 i
SD-SSL	573.31 ± 0.07 c	3.79 ± 0.04 e	9.70 ± 0.05 g	40.83 ± 0.04 d	2.44 ± 0.05 b	35.65 ± 0.01 a	1.27 ± 0.01 b	0.53 ± 0.02 d	1.67 ± 0.02 h
YN-LZ	264.06 ± 0.02 i	0.70 ± 0.03 h	15.46 ± 0.03 e	23.47 ± 0.02 f	1.37 ± 0.04 g	35.02 ± 0.04 b	0.74 ± 0.01 e	0.25 ± 0.01 g	17.30 ± 0.09 b
YN-SZ	149.85 ± 0.02 j	0.74 ± 0.01 gh	5.98 ± 0.02 h	21.49 ± 0.05 g	1.11 ± 0.02 i	21.26 ± 0.02 e	0.23 ± 0.01 h	0.25 ± 0.00 g	17.79 ± 0.09 a
YN-SSL	479.39 ± 0.01 f	2.09 ± 0.07 f	16.55 ± 0.05 d	44.21 ± 0.03 a	2.19 ± 0.03 d	22.61 ± 0.05 d	0.73 ± 0.02 e	0.65 ± 0.01 c	2.65 ± 0.01 g
SX-JPT	298.99 ± 0.03 h	15.93 ± 0.03 b	9.99 ± 0.07 f	13.96 ± 0.07 i	1.62 ± 0.05 f	25.44 ± 0.06 c	0.81 ± 0.02 d	0.27 ± 0.01 fg	16.89 ± 0.08 d
SX-SBT	504.34 ± 0.01 e	0.80 ± 0.02 c	34.44 ± 0.02 c	21.42 ± 0.03 h	1.22 ± 0.04 h	10.66 ± 0.01 h	1.19 ± 0.04 c	0.53 ± 0.02 d	0.25 ± 0.01 j
SX-SSL	560.83 ± 0.01 d	17.19 ± 0.01 a	41.23 ± 0.01 a	32.26 ± 0.01 c	2.20 ± 0.02 d	12.84 ± 0.03 g	0.59 ± 0.01 f	0.44 ± 0.01 e	17.08 ± 0.08 c

Data were expressed as mean ± standard deviation (n=3). Different letters represent significant differences ($P<0.05$). XJ, Xinjiang; SD, Shandong; YN, Yunnan; SX, Shaanxi; TSL, SZ, LZ, JPT, SBT belong to sweet pomegranate, SSL belongs sour pomegranate.

Punicalagin is the most polyphenol monomers found in pomegranate aril juice, suggesting that punicalagin was the primary polyphenol in pomegranate juice in accordant to the findings of Gil *et al.* (2000). Punicalagin and hydrolyable tannins were the richest polyphenol compounds in pomegranate aril juice. High amount of hydrolysable tannins and anthocyanins were found in pomegranate juice indicating high antioxidant activity of pomegranate juice (Li *et al.*, 2015).

Serum levels of biochemical parameters such as aspartate aminotransferase (AST), alanine transaminase (ALT), and creatine phosphokinase (CPK or CK) were determined in I/R rats received concentrated TPJ. AST and ALT, that released into the blood of human and animals, are enzymes moderately sensitive for indicating of liver damage or hepatotoxicity. I/R rats intragastric injected with the 3 doses of TPJ, DDD water, 1% tween 80, and vitamin E (positive control) for 60 min before reperfusion of blood. The present study demonstrated that there was no statistical significant difference in all serum biochemical parameters between all groups of I/R rats treated with different doses of TPJ and vitamin E. Serum AST levels in all groups were higher than normal range (74-143 U/L) of clinical laboratory parameters (Giknis and Clifford, 2008). Serum ALT levels in I/R+DDD water group, IR+vitamin E group, I/R+ Low dose TPJ group, I/R+Middle dose TPJ group, and I/R+High dose TPJ group were higher than normal range (18-45 U/L) of clinical laboratory parameters (Giknis and Clifford, 2008). Normal skeletal muscle cell contains plenty of CPK enzymes. The cytosolic enzyme CPK is also found pre-domainantly in skeletal muscle and is a marker of skeletal muscle tissue damage (Carter *et al.*, 1998). Serum CPK levels were within the normal range (162-1184 U/L) of clinical laboratory parameters (Giknis and Clifford, 2008). Serum CPK level of 1318±208.30

was shown in ischemia-reperfusion group by 2 hours of ischemia and 24 hours reperfusion in study of Takhtfooladi and colleagues (2014) that was statistically higher than to sham and I/R treated groups. The results of serum CPK levels in the present study showed no statistically significant difference between all groups of I/R rats treated with different doses of TPJ and vitamin E. The different results showing in the present study may be a result of the time used ischemia-reperfusion was shorter than the study of Takhtfooladi and colleagues (2014).

Reactive oxygen species (ROS) comprise both free radical and non-free radical oxygen intermediates (Suttajit *et al.*, 2012). ROS are chemically reactive molecule containing oxygen including peroxide, superoxide, hydroxyl radical, hydroperoxide, and peroxyl radical. Free radicals are highly reactive and ready to interact with many cell components such as lipids, proteins, carbohydrates and DNA. Oxidative stress means an alteration in the delicate balance between ROS and the scavenging capacity of antioxidant in the body (Frei, 1994). Oxygen free radicals are considered to be important component involving in the physio-pathological tissue alterations observed during ischemia-reperfusion. The previous studies, the tissues were presented to accumulate free radical within the first few min of reperfusion (Kloner *et al.*, 1989) and post-ischemic endothelium is main source of free radical. The most significant damaging effect of free radicals on tissues is lipid peroxidation (Avci, 2012). Ischemia-reperfusion may lead to excessive free radicals production and increase of lipid peroxidation level. The product of lipid peroxidation is a malondialdehyde (MDA). A significant decrease in MDA level was observed in rats treated with different doses of pomegranate juice (Turk *et al.*, 2008). The study of Novelli and co-workers (1997) presented that MDA level in the skeletal muscle tissue

(gastrocnemius muscle) significantly increased following 2 hours of ischemia and 4 hours of reperfusion. MDA levels in skeletal muscle cell decreased in vitamin E treated patients after a period of ischemia-reperfusion. Treatment of TPJ and alpha-tocopherol (vitamin E) decreased lipid peroxidation level and increased antioxidant activities in skeletal muscle. The results of the present study revealed that ischemia-reperfusion injury led to increase MDA levels in the skeletal muscle tissue. Administration of vitamin E and 3 doses of TPJ were statistically lower than control groups of tissue MDA levels at $P<0.05$. Ischemia-reperfusion caused an increase in MDA level, since MDA levels increase in control groups (I/R-DDD water and I/R-1% tween 80). Decreases in MDA levels were shown following administration of vitamin E and 3 doses of TPJ. This finding suggested that TPJ could protect lipid peroxidation by induced by ischemia reperfusion. The antioxidant SOD enzyme, CAT enzyme, and GSH have complementary activities in the anti-oxidative defense system. The antioxidant SOD enzyme, CAT enzyme, and GSH were assessed to investigate the enzymatic and non-enzymatic antioxidant status in skeletal muscle after ischemia-reperfusion (Xu *et al.*, 2005). A significant increase in GSH and CAT activities were observed in rats treated with different doses of pomegranate juice (Turk *et al.*, 2008). Prolonged pomegranate juice ingestion on general oxidation status showed an increase in SOD activity and a decrease in GSH in hepatic oxidative stress (Faria *et al.*, 2007). Vitamin E is one of the best non-enzymatic antioxidant can prevent oxidative stress, endothelial damage, intramuscular edema, and major muscle fiber damage in the muscle tissue. Alpha-tocopherol has a protective effect on oxidative muscle damage after ischemia-reperfusion in humans (Novelli *et al.*, 1997). The present study demonstrated increases in tissue SOD and CAT activities and GSH

levels in ischemia-reperfusion received DDD water and 1% tween 80 groups compared with sham and treatment (vitamin E and 3 doses of TPJ) groups. Administration of vitamin E and 3 doses of TPJ increased SOD, CAT activities, and GSH levels in skeletal muscle tissue compared with controls group. Increases of SOD, and CAT activities and GSH levels in muscle tissue by TPJ indicated TPJ could protect oxidative stress induced by ischemia reperfusion. Moreover, we found that 3 doses of TPJ and vitamin E possessed similar potency in antioxidant activity in skeletal muscle ischemia-reperfusion. A soleus muscle was commonly used for the histopathology of ischemia reperfusion in skeletal muscle model (Karpati *et al.*, 1974; Carmo-Araujo *et al.*, 2007). A slow twitch muscle fibers with aerobic metabolism (soleus muscle) was selected for histological study because slow twitch muscle fibers are more sensitive to ischemia fast twitch fibers (Jennische, Amundson, and Haljame, 1979; Turoczi *et al.*, 2014). Four hours ischemia, skeletal muscle (slow-twitch fibers) showed 21% necrosis cells. Skeletal muscle cells predominating in fast-twitch fibers had significantly greater necrosis than slow-twitch fibers (Petrasek *et al.*, 1994). The present histological study revealed the presence of necrosis cells of soleus muscles fiber cell in the I/R-DDD water and I/R-1% Tween 80 groups, its incidence being higher when compared to the sham, I/R-Vitamin E, and I/R-Low dose TPJ groups. The amount of % cell changed in I/R-DDD water and I/R-1% Tween 80 groups tended to be higher than I/R-Middle dose TPJ and I/R-High dose TPJ groups. The present findings were similar to those findings in ischemia-reperfusion recovered tramadol (Takhtfooladi, 2014). TPJ could protect cell damage from oxidative stress by induced by ischemia-reperfusion that was inconsistent with other results.

The present study demonstrated protective effects of TPJ against alterations in skeletal muscle cell metabolism in I/R model. Ischemia-reperfusion can cause ROS and cell membrane ruptures damage, and finally cell death. TPJ possesses a protective effect against oxidative stress and skeletal muscle cell damage induced by I/R, as potent as vitamin E. The present, *in vivo* and *in vitro* studies revealed that protective effect is most likely because of the free radical scavenging activity of antioxidants found in TPJ. During the reperfusion periods, antioxidants in TPJ may protect against reactive oxygen species (ROS) induced skeletal cell damage and ameliorate oxidative stress-induced by I/R (Figure 25). The results of 3 doses TPJ-treated groups shown protect oxidative stress but not all is the best doses. The results shown tend to Low and middle dose TPJ is the best doses but High dose presented low potent more than other doses. High dose TPJ contained high pH levels that cause low potent more than other doses.

In conclusion, the present study indicated that TPJ is a rich source of antioxidants and total polyphenols content that have a number of health benefits. TPJ may have protective effects against skeletal muscle tissue injury caused by I/R that are probably through antioxidant defense system against radical damage. Further studies are needed to ensure the protective effects of TPJ on oxidative stress induced by another model such as exhaustive exercise.

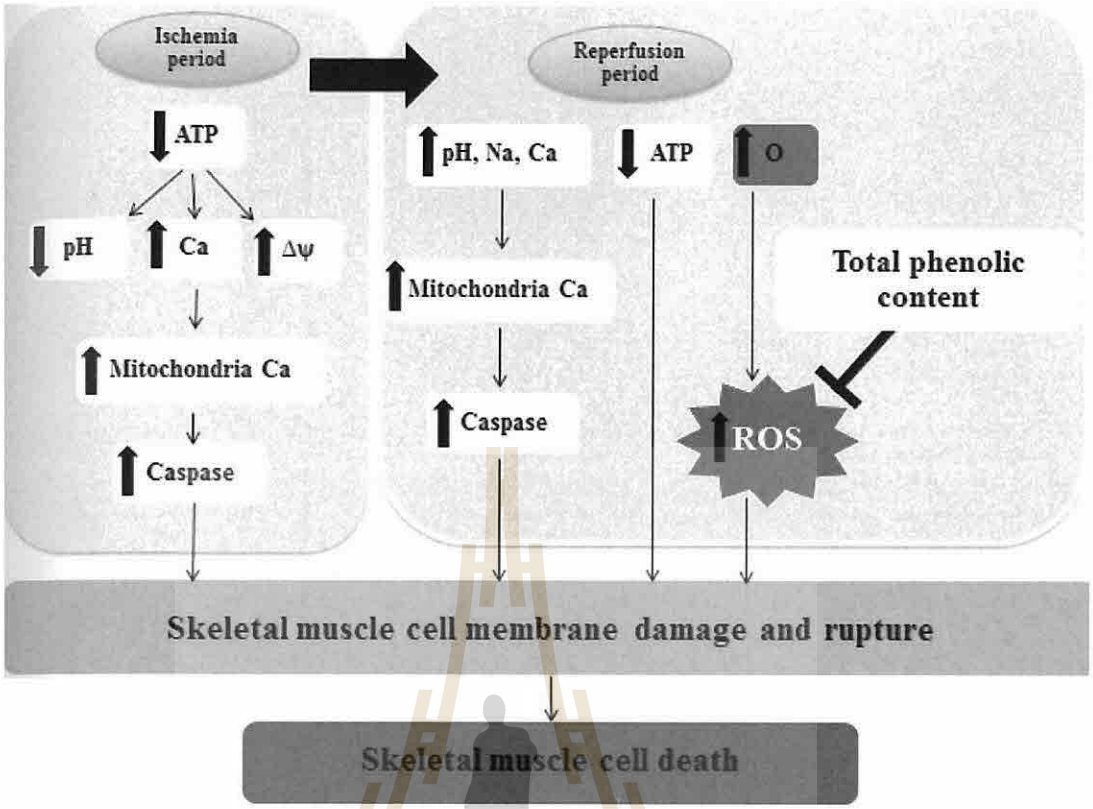

Figure 25 Protective effects of TPJ against alterations in skeletal muscle cell metabolism of ischemia and reperfusion period. During the reperfusion periods, antioxidants in TPJ may protect against reactive oxygen species (ROS) induced skeletal cell damage and ameliorate oxidative stress-induced by I/R. Ca; calcium, Δψ; membrane potential, Mito Ca; mitochondria calcium, Na; sodium, ATP; adenosine triphosphate, O; oxygen, and TPJ; Thai pomegranate juice.

REFERENCES

REFERENCES

Akerboom, T. P., and Sies, H. (1981). Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. **Methods in Enzymology**. 77: 373-382.

Al-Dujaili, E. A. (2016). Consumption of Pomegranate Juice Attenuates Exercise-Induced Oxidative Stress, Blood Pressure and Urinary Cortisol/Cortisone Ratio in Human Adults. **EC Nutrition**. 4(6): 982-995.

Anahita, A., Asmah, R., and Fauziah, O. (2015). Evaluation of total phenolic content, total antioxidant activity, and antioxidant vitamin composition of pomegranate seed and juice. **General Medicine: Open Access**. 3(164): 1-4.

Ananya, M. (2012). **Antioxidant Enzyme Systems** [on-line]. Available: http://www.news-medical.net/health/antioxidant-enzyme-systems.html.

Ardan, T., Kovačeva, J., and Čejková, J. (2004). Comparative histochemical and immunohistochemical study on xanthine oxidoreductase/xanthine oxidase in mammalian corneal epithelium. **Acta Histochemica**. 106(1): 69-75.

Assunção, R. B., and Mercadante, A. Z. (2003). Carotenoids and ascorbic acid composition from commercial products of cashew apple (*Anacardium occidentale* L.). **Journal of Food Composition and Analysis**. 16(6): 647-657.

Avci, G., Kadioglu, H., Sehirli, A. O., Bozkurt, S., Guclu, O., Arslan, E., and Muratli, S. K. (2012). Curcumin Protects Against Ischemia/Reperfusion Injury in Rat Skeletal Muscle. **Journal of Surgical Research**. 172(1): 39-46.

Aviram, M., Dornfeld, L., Rosenblat, M., Volkova, N., Kaplan, M., Coleman, R., Hayek, T., Presser, D., and Fuhrman, B. (2000). Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E–deficient mice. **The American Journal of Clinical Nutrition**. 71(5): 1062-1076.

Aviram, M., Rosenblat, M., Gaitini, D., Nitecki, S., Hoffman, A., Dornfeld, L., Volkova, N., Presser, D., Attias, J., Liker, H., and Hayek, T. (2004). Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. **Clinical Nutrition**. 23(3): 423-433.

Bannister, J. V., Bannister, W. H., and Rotilio, G. (1987). Aspects of the structure, function, and applications of superoxide dismutas. **CRC Critical Reviews in Biochemistry**. 22(2): 111-180.

Bayat, C. E., Fallahzadeh, H., Askari, G., Rahavi, R., Maghsoudi, Z., and Nadjarzadeh, A. (2015). The effect of pomegranate juice supplementation on muscle damage, oxidative stress and inflammation induced by exercise in healthy young men. **Journal of Isfahan Medical School**. 320(32): 2464-2472.

Benzie, I. F., and Strain, J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. **Analytical Biochemistry**. 239(1): 70-76.

Beh, L. K., Zakaria, Z., Beh, B. K., Ho, W. Y., Yeap, S. K., and Alitheen, N. B. M. (2012). Comparison of total phenolic content and antioxidant activities of freeze-dried commercial and fresh fruit juices. **Journal of Medicinal Plants Research**. 6(48): 5857-5862.

Bhowmik, D., Gopinath, H., Kumar, B. P., S.Duraivel, G. A., and Kumar, K. P. S. (2013). Medicinal Uses of *Punica granatum* and Its Health Benefits. **Journal of Pharmacognosy and Phytochemistry**. 1(5): 28-35.

Biofoundation. (2016). **Endogenous Antioxidants are the Potent First Line of Defense against. Reactive Oxygen Species** [On-line]. Available: http://www. biofoundation. org/?p=4432.html.

Blaisdell, F. W. (2002). The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome a review. **Vascular**. 10(6): 620-630.

Blazer, V. S., and Wolke, R. E. (1984). The effects of α-tocopherol on the immune response and non-specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson). **Aquaculture**. 37(1): 1-9.

Bonini, M. G., Miyamoto, S., Di Mascio, P., and Augusto, O. (2004). Production of the carbonate radical anion during xanthine oxidase turnover in the presence of bicarbonate. **Journal of Biological Chemistry**. 279(50): 51836-51843.

Bryan, D. M. (1996). **Oxidative stress** [On-line]. Available: http://www.plantstress. Com/articles/Oxidative%2stress.html.

Cadenas, E., and Sies, H. (1998). The lag phase. **Free Radical Research**. 28(6): 601-609.

Carmo-Araujo, E. M., Dal-Pai-Silva, M., Dal-Pai, V., Cecchini, R., and Anjos Ferreira, A. L. (2007). Ischaemia and reperfusion effects on skeletal muscle

tissue: morphological and histochemical studies. **International Journal of Experimental Pathology**. 88(3): 147-154.

Carter, W. O., Bull, C., Bortolon, E., Yang, L., Jesmok, G. J., and Gundel, R. H. (1998). A murine skeletal muscle ischemia-reperfusion injury model: differential pathology in BALB/c and DBA/2N mice. **Journal of Applied Physiology**. 85(5): 1676-1683.

Cerdá, B., Cerón, J. J., Tomás-Barberán, F. A., and Espín, J. C. (2003). Repeated oral administration of high doses of the pomegranate ellagitannin punicalagin to rats for 37 days is not toxic. **Journal of Agricultural and Food Chemistry**. 51(11): 3493-3501.

Chambers, D. E., Parks, D. A., Patterson, G., Roy, R., McCord, J. M., Yoshida, S., Parmley, L. F., and Downey, J. M. (1985). Xanthine oxidase as a source of free radical damage in myocardial ischemia. **Journal of Molecular and Cellular Cardiology**. 17(2): 145-152.

Chen, Y.-H., Du, G.-H., and Zhang, J.-T. (2000). Salvianolic acid B protects brain against injuries caused by ischemia-reperfusion in rats. **Acta Pharmacologica Sinica**. 21(5): 463-466.

Choudhury, N. A., Sakaguchi, S., Koyano, K., Matin, A. F., and Muro, H. (1991). Free radical injury in skeletal muscle ischemia and reperfusion. **The Journal of Surgical Research**. 51(5): 392.

Dahham, S. S., Ali, M. N., Tabassum, H., and Khan, M. (2010). Studies on antibacterial and antifungal activity of pomegranate (*Punica granatum* L.). **American-Eurasian Journal of Agricultural & Environmental Science**. 9(3): 273-281.

Devarajan, P. (2006). Update on mechanisms of ischemic acute kidney injury. **Journal of the American Society of Nephrology**. 17(6): 1503-1520.

Devasagayam, T., Tilak, J., Boloor, K., Sane, K. S., Ghaskadbi, S. S., and Lele, R. (2004). Free radicals and antioxidants in human health: current status and future prospects. **Journal of the Association of Physicians of India**. 52(794804): 4.

Dong, X., Xing, Q., Li, Y., Han, X., and Sun, L. (2014). Dexmedetomidine protects against ischemia–reperfusion injury in rat skeletal muscle. **Journal of Surgical Research**. 186(1): 240-245.

Ercisli, S., Agar, G., Orhan, E., Yildirim, N., and Hizarci, Y. (2007). Interspecific variability of RAPD and fatty acid composition of some pomegranate cultivars (*Punica granatum* L.) growing in Southern Anatolia Region in Turkey. **Biochemical Systematics and Ecology**. 35(11): 764-769.

Faria, A., Monteiro, R., Mateus, N., Azevedo, I., and Calhau, C. (2007). Effect of pomegranate (*Punica granatum*) juice intake on hepatic oxidative stress. **European Journal of Nutrition**. 46(5): 271-278.

Frei, B. (1994). Reactive oxygen species and antioxidant vitamins: mechanisms of action. **The American Journal of Medicine**. 97(3): S5-S13.

Fuhrman, B., Volkova, N., and Aviram, M. (2005). Pomegranate juice inhibits oxidized LDL uptake and cholesterol biosynthesis in macrophages. **The Journal of Nutritional Biochemistry**. 16(9): 570-576.

Giknis, M. L. A., and Clifford, C. B. (2008). **Clinical laboratory parameters for Crl: WI (Han) rats**. Massachusetts: Charles River Laboratories International.

Gil, M. I., Tomas-Barberan, F. A., Hess-Pierce, B., Holcroft, D. M., and Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. **Journal of Agricultural and Food Chemistry**. 48(10): 4581-4589.

Golstein, P., and Kroemer, G. (2007). Cell death by necrosis: towards a molecular definition. **Trends in Biochemical Sciences**. 32(1): 37-43.

Goula, A. M., Tzika, A., and Adamopoulos, K. G. (2014). Kinetic Models of Evaporation and Total Phenolics Degradation during Pomegranate Juice Concentration. **International Journal of Food Engineering**. 10(3): 383-392.

Granger, D. N., Stokes, K. Y., Shigematsu, T., Cerwinka, W. H., Tailor, A., and Krieglstein, C. F. (2001). Splanchnic ischaemia–reperfusion injury: mechanistic insights provided by mutant mice. **Acta Physiologica Scandinavica**. 173(1): 83-91.

Guidance, F. D. A. (2005). **Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers**. Maryland.

Guo, C., Wei, J., Yang, J., Xu, J., Pang, W., and Jiang, Y. (2008). Pomegranate juice is potentially better than apple juice in improving antioxidant function in elderly subjects. **Nutrition Research**. 28(2): 72-77.

Guo, C., Yang, J., Wei, J., Li, Y., Xu, J., and Jiang, Y. (2003). Antioxidant activities of peel, pulp and seed fractions of common fruits as determined by FRAP assay. **Nutrition Research**. 23(12): 1719-1726.

Haljamäe, H., and Enger, E. (1975). Human skeletal muscle energy metabolism during and after complete tourniquet ischemia. **Annals of Surgery**. 182(1): 9.

Halliwell, B., and Gutteridge, J. M. (2015). **Free Radicals in Biology and Medicine**.

Oxford University Press, USA.

Hamacher-Brady, A., Brady, N. R., and Gottlieb, R. A. (2006). The interplay between pro-death and pro-survival signaling pathways in myocardial ischemia/reperfusion injury: apoptosis meets autophagy. **Cardiovascular Drugs and Therapy**. 20(6): 445-462.

Harborne, A. J. (1973). **Phytochemical methods. In: A Guide to Modern Techniques of Plant Analysis**. London: Springer Science & Business Media.

Hardie, L. J., Fletcher, T. C., and Secombes, C. J. (1990). The effect of vitamin E on the immune response of the Atlantic salmon (*Salmo salar* L.). **Aquaculture**. 87(1): 1-13.

Harrison, R. (2002). Structure and function of xanthine oxidoreductase: where are we now. **Free Radical Biology and Medicine**. 33(6): 774-797.

Hartman, R. E., Shah, A., Fagan, A. M., Schwetye, K. E., Parsadanian, M., Schulman, R. N., Finn, M. B., and Holtzman, D. M. (2006). Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease. **Neurobiology of Disease**. 24(3): 506-515.

Heber, D., Seeram, N. P., Wyatt, H., Henning, S. M., Zhang, Y., Ogden, L. G., Dreher, M., and Hill, J. O. (2007). Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size. **Journal of Agricultural and Food Chemistry**. 55(24): 10050-10054.

Hideharu, S. (2013). **Determination of Xanthine Oxidase** [On-line]. Available: http://www.omicsonline.org/determination-of-xanthine-oxidase-2153-2435.S7-004.php?aid=15442.html.

Hille, R. (2005). Molybdenum-containing hydroxylases. **Archives of Biochemistry and Biophysics**. 433(1): 107-116.

Ho, E., Karimi Galougahi, K., Liu, C. C., Bhindi, R., and Figtree, G. A. (2013). Biological markers of oxidative stress: Applications to cardiovascular research and practice. **Redox Biology**. 1: 483-491.

Hoffmann, T., Leiderer, R., Waldner, H., Arbogast, S., and Messmer, K. (1995). Ischemia reperfusion of the pancreas: a new *in vivo* model for acute pancreatitis in rats. **Research in Experimental Medicine**. 195(1): 125-144.

Huk, I., Nanobashvili, J., Neumayer, C., Punz, A., Mueller, M., Afkhampour, K., Mittlboeck, M., Losert, U., Polterauer, P., Roth, E., Patton, S., and Malinski, T. (1997). L-arginine treatment alters the kinetics of nitric oxide and superoxide release and reduces ischemia/reperfusion injury in skeletal muscle. **Circulation**. 96(2): 667-675.

Hussain, T., Shukla, G. S., and Chandra, S. (1987). Effects of cadmium on superoxide dismutase and lipid peroxidation in liver and kidney of growing rats: *in vivo* and *in vitro* studies. **Pharmacology and Toxicology**. 60(5): 355-358.

Jennings, R. B., and Reimer, K. A. (1981). Lethal myocardial ischemic injury. **The American Journal of Pathology**. 102(2): 241.

Jennische, E., Amundson, B., and Haljame, H. (1979). Metabolic responses in feline "red" and "white" skeletal muscle to shock and ischemia. **Acta Physiologica Scandinavica**. 106(1): 39-45.

Jeune, M. L., Kumi-Diaka, J., and Brown, J. (2005). Anticancer activities of pomegranate extracts and genistein in human breast cancer cells. **Journal of Medicinal Food**. 8(4): 469-475.

Johnson, F., and Giulivi, C. (2005). Superoxide dismutases and their impact upon human health. **Molecular Aspects of Medicine**. 26(4): 340-352.

Joseph, N. (2014). **What is vitamin C How much vitamin C do you need** [On-line]. Available: http://www.medicalnewstoday.com/articles/219352.html.

Juranek, I., and Bezek, S. (2005). Controversy of free radical hypothesis: reactive oxygen species–cause or consequence of tissue injury. **General Physiology and Biophysics**. 24(3): 263-78.

Karpati, G., Carpenter, S., Melmed, C., and Eisen, A. A. (1974). Experimental - ischemic myopathy. **Journal of the Neurological Sciences**. 23(1): 129-161.

Kelebek, H., Selli, S., Canbas, A., and Cabaroglu, T. (2009). HPLC determination of organic acids, sugars, phenolic compositions and antioxidant capacity of orange juice and orange wine made from a Turkish cv. Kozan. **Microchemical Journal**. 91(2): 187-192.

Khassaf, M., McArdle, A., Esanu, C., Vasilaki, A., McArdle, F., Griffiths, R., Brodie, D., and Jackson, M. (2003). Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. **The Journal of Physiology**. 549(2): 645-652.

Kitagawa, T., Yokoyama, Y., Kokuryo, T., and Nagino, M. (2013). Protective effects of branched-chain amino acids on hepatic ischemia-reperfusion-induced liver injury in rats: a direct attenuation of Kupffer cell activation. **American Journal of Physiology-Gastrointestinal and Liver Physiology**. 304(4): G346-G355.

Kloner, R. A., Przyklenk, K., and Whittaker, P. (1989). Deleterious effects of oxygen radicals in ischemia/reperfusion. **Resolved and Unresolved Issues, Circulation**. 80(5): 1115-1127.

Kontos, H. A., Wei, E. P., Christman, C. W., Levasseur, J. E., Povlishock, J. T., and Ellis, E. F. (1983). Free oxygen radicals in cerebral vascular responses. **Physiologist**. 26(3): 165-169.

Kovacic, P., Pozos, R. S., Somanathan, R., Shangari, N., and O'Brien, P. J. (2005). Mechanism of mitochondrial uncouplers, inhibitors, and toxins: focus on electron transfer, free radicals, and structure-activity relationships. **Current Medicinal Chemistry**. 12(22): 2601-2623.

Larsson, J., and Hultman, E. (2011). The effect of long-term arterial occlusion on energy metabolism of the human quadriceps muscle. **Scandinavian Journal of Clinical and Laboratory Investigation**.

Lee, I.-M., Cook, N. R., Gaziano, J. M., Gordon, D., Ridker, P. M., Manson, J. E., Hennekens, C. H., Buring, J. E. (2005). Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. **The Journal of the American Medical Association**. 294(1): 56-65.

Li, X., Wasila, H., Liu, L., Yuan, T., Gao, Z., Zhao, B., and Ahmad, I. (2014). Physicochemical characteristics, polyphenol compositions and antioxidant potential of pomegranate juices from 10 Chinese cultivars and the environmental factors analysis. **Food Chemistry**. 175(1): 575-584.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. **Journal of Biological Chemistry**. 193(1): 265-275.

Lu, Y., Shipton, F. N., Khoo, T. J., and Wiart, C. (2014). Antioxidant activity determination of Citronellal and crude extracts of *Cymbopogon citratus* by 3 different methods. **Pharmacology & Pharmacy**. 5(04): 395.

Lyle, M. (2006). **What Makes Gamma Tocopherol Superior to Alpha Tocopherol** [On-line]. Available: http://www.lifeextension.com/magazine/2006/4/report-_gramma/Page-01.html.

Maramag, C., Menon, M., Balaji, K., Reddy, P. G., and Laxmanan, S. (1997). Effect of vitamin C on prostate cancer cells *in vitro*: effect on cell number, viability, and DNA synthesis. **The Prostate**. 32(3): 188-195.

Martin, H. M., Hancock, J. T., Salisbury, V., and Harrison, R. (2004). Role of xanthine oxidoreductase as an antimicrobial agent. **Infection and Immunity**. 72(9): 4933-4939.

Maskan, M. (2006). Production of pomegranate (*Punica granatum* L.) juice concentrate by various heating methods: colour degradation and kinetics. **Journal of Food Engineering**. 72(3): 218-224.

Mazani, M., Fard, A. S., Baghi, A. N., Nemati, A., and Mogadam, R. A. (2014). Effect of pomegranate juice supplementation on matrix metalloproteinases 2 and 9 following exhaustive exercise in young healthy males. **Journal of the Pakistan Medical Association**. 64: 785-790.

Minussi, R. C., Rossi, M., Bologna, L., Cordi, L. v., Rotilio, D., Pastore, G. M., and Durán, N. (2003). Phenolic compounds and total antioxidant potential of commercial wines. **Food Chemistry**. 82(3): 409-416.

Mousavinejad, G., Emam-Djomeh, Z., Rezaei, K., and Khodaparast, M. H. H. (2009). Identification and quantification of phenolic compounds and their effects on antioxidant activity in pomegranate juices of eight Iranian cultivars. **Food Chemistry**. 115(4): 1274-1278.

Muller, F. L., Lustgarten, M. S., Jang, Y., Richardson, A., and Van Remmen, H. (2007). Trends in oxidative aging theories. **Free Radical Biology and Medicine**. 43(4): 477-503.

Murephy, E., and Steenbergen, C. (2008). Mechanisms Underlying Acute Protection from Cardiac Ischemia-Reperfusion Injury. **Physiological Review**. 88(2): 581-609.

Nair, S., Singh, S. V., and Krishan, A. (1991). Flow cytometric monitoring of glutathione content and anthracycline retention in tumor cells. **Cytometry**. 12(4): 336-342.

Nayler, W. G., Poole-Wilson, P. A., and Williams, A. (1979). Hypoxia and calcium. **Journal of Molecular and Cellular Cardiology**. 11(7): 683-706.

Novelli, G. P., Adembri, C., Gandini, E., Orlandini, S. Z., Papucci, L., Formigli, L., Manneschi, L. I., Quattrone, A., Pratesi, C., and Capaccioli, S. (1997). Vitamin E protects human skeletal muscle from damage during surgical ischemia-reperfusion. **The American Journal of Surgery**. 173(3): 206-209.

Opara, L. U., Al-Ani, M. R., and Al-Shuaibi, Y. S. (2009). Physico-chemical properties, vitamin C content, and antimicrobial properties of pomegranate fruit (*Punica granatum* L.). **Food and Bioprocess Technology**. 2(3): 315-321.

Panichayupakaranant, P., Tewtrakul, S., and Yuenyongsawad, S. (2010). Antibacterial, anti-inflammatory and anti-allergic activities of standardised pomegranate rind extract. **Food Chemistry**. 123(2): 400-403.

Patel, C., Dadhaniya, P., Hingorani, L., and Soni, M. G. (2008). Safety assessment of pomegranate fruit extract: acute and subchronic toxicity studies. **Food and Chemical Toxicology**. 46(8): 2728-2735.

Penniston, K. L., Nakada, S. Y., Holmes, R. P., and Assimos, D. G. (2008). Quantitative assessment of citric acid in lemon juice, lime juice, and commercially-available fruit juice products. **Journal of Endourology**. 22(3): 567-570.

Perrin, D. D., and Dempsey, B. (1974). **Buffers for pH and Metal Ion Control**. New York: Halsted.

Petrasek, P. F., Homer-Vanniasinkam, S., and Walker, P. M. (1994). Determinants of ischemic injury to skeletal muscle. **Journal of Vascular Surgery**. 19(4): 623-631.

Poyrazoglu, E., Gökmen, V., and Artık, N. (2002). Organic acids and phenolic compounds in pomegranates (*Punica granatum* L.) grown in Turkey. **Journal of Food Composition and Analysis**. 15(5): 567-575.

Qu, W., Breksa Iii, A. P., Pan, Z., and Ma, H. (2012). Quantitative determination of major polyphenol constituents in pomegranate products. **Food Chemistry**. 132(3): 1585-1591.

Rahman, T., Hosen, I., Islam, M. T., and Shekhar, H. U. (2012). Oxidative stress and human health. **Advances in Bioscience and Biotechnology**. 3(7A): 997.

Rajasekar, D., Akoh, C. C., Martino, K. G., and MacLean, D. D. (2012). Physico-chemical characteristics of juice extracted by blender and mechanical press from pomegranate cultivars grown in Georgia. **Food Chemistry**. 133(4): 1383-1393.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., and Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology and Medicine**. 26(9): 1231-1237.

Reigner, B. G., and Blesch, K. (2002). Estimating the starting dose for entry into humans: principles and practice. **European Journal of Clinical Pharmacology**. 57(12): 835-845.

Rimm, E. B., Stampfer, M. J., Ascherio, A., Giovannucci, E., Colditz, G. A., and Willett, W. C. (1993). Vitamin E consumption and the risk of coronary heart disease in men. **New England Journal of Medicine**. 328(20): 1450-1456.

Rosenblat, M., Hayek, T., and Aviram, M. (2006). Anti-oxidative effects of pomegranate juice (PJ) consumption by diabetic patients on serum and on macrophages. **Atherosclerosis**. 187(2): 363-371.

Samuel A., and Goldblith, B. E. P. (1950). Photometric determination of catalase activity. **The Journal of Biological Chemistry**. 1: 705-709.

Seeram, N. P., Adams, L. S., Henning, S. M., Niu, Y., Zhang, Y., Nair, M. G., and Heber, D. (2005). *In vitro* antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are

enhanced in combination with other polyphenols as found in pomegranate juice. **The Journal of Nutritional Biochemistry**. 16(6): 360-367.

Sellers, Z. P., Williams, R. A., Overbay, J. W., Cho, J., Henderson, M., and Reed, T. T. (2014). **Current Therapeutic Modalities, Enzyme Kinetics, and Redox Pro - teomics in Traumatic Brain Injury** [On-line]. Available: http://www.intech open.com/books/trumatic-brain-injury/current-therapeutic-modalities-enzyme-kinetics-and-redox-proteomics-in-traumatic-brain-injury.html.

Sharples, E. J., Patel, N., Brown, P., Stewart, K., Mota-Philipe, H., Sheaff, M., Kieswich, J., Allen, D., Harwood, S., Raftery, M., Thiemermann, C., and Yaqoob, M., (2004). Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. **Journal of the American Society of Nephrology**. 15(8): 2115-2124.

Siems, W. G., Grune, T., and Esterbauer, H. (1995). 4-Hydroxynonenal formation during ischemia and reperfusion of rat small intestine. **Life Sciences**. 57(8): 785-789.

Suttajit, M., Banjerdpongchai, R., Kukongviriyapan, V., Siriamornpun, S., Chaiyasut, C., Porasuphatana, S., Panich, U., Thephinlap, C., Aengwanich, W., and Charoeansin, S. (2012). **Free radicals and Antioxidants**. Changmai: Health Innovation publisher.

Tahara, E. B., Navarete, F. D. T., and Kowaltowski, A. J. (2009). Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. **Free Radical Biology and Medicine**. 46(9): 1283-1297.

Takagi, H., Matsui, Y., and Sadoshima, J. (2007). The role of autophagy in mediating cell survival and death during ischemia and reperfusion in the heart. **Antioxidants and Redox Signaling**. 9(9): 1373-1382.

Takhtfooladi, H. A., Takhtfooladi, M. A., Karimi, P., Asl, H. A., and Mobarakeh, S. Z. (2014). Influence of tramadol on ischemia-reperfusion injury of rats' skeletal muscle. **International Journal of Surgery**. 12(9): 963-968.

Tapias, V., Cannon, J. R., and Greenamyre, J. T. (2014). Pomegranate juice exacerbates oxidative stress and nigrostriatal degeneration in Parkinson's disease. **Neurobiology of Aging**. 35(5): 1162-1176.

Tehranifar, A., Zarei, M., Nemati, Z., Esfandiyari, B., and Vazifeshenas, M. R. (2010). Investigation of physico-chemical properties and antioxidant activity of twenty Iranian pomegranate (*Punica granatum* L.) cultivars. **Scientia Horticulturae**. 126(2): 180-185.

Tezcan, F., Gültekin-Özgüven, M., Diken, T., Özçelik, B., and Erim, F. B. (2009). Antioxidant activity and total phenolic, organic acid and sugar content in commercial pomegranate juices. **Food Chemistry**. 115(3): 873-877.

Tsang, C., Wood, G., and Al-Dujaili, E. (2011). Pomegranate juice consumption influences urinary glucocorticoids, attenuates blood pressure and exercise-induced oxidative stress in healthy volunteers. **Bioscientifica**. 25: P139.

Tsuda, H., Kawada, N., Kaimori, J.-y., Kitamura, H., Moriyama, T., Rakugi, H.,Takahara, S., and Isaka, Y. (2012). Febuxostat suppressed renal ischemia–reperfusion injury via reduced oxidative stress. **Biochemical and Biophysical Research Communications**. 427(2): 266-272.

Turk, G., Sonmez, M., Aydin, M., Yuce, A., Gur, S., Yuksel, M., and Aksoy, H. (2008). Effects of pomegranate juice consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats. **Clinical Nutrition**. 27(2): 289-296.

Turoczi, Z., Aranyi, P., Lukats, A., Garbaisz, D., Lotz, G., Harsanyi, L., and Szijarto, A. (2014). Muscle fiber viability, a novel method for the fast detection of ischemic muscle injury in rats. **Plos One**. 9(1): 84783.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. **The Journal of Physiology**. 552(2): 335-344.

Velioglu, S., Unal, C., and Cemeroglu, B. (1997). Chemical characterization of pomegranate juice. **Fruit Processing**. 8: 307-310 .

Valko, M., Izakovic, M., Mazur, M., Rhodes, C. J., and Telser, J. (2004). Role of oxygen radicals in DNA damage and cancer incidence. **Molecular and Cellular Biochemistry**. 266(1-2): 37-56.

Valko, M., Rhodes, C., Moncol, J., Izakovic, M., and Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. **Chemico-Biological Interactions**. 160(1): 1-40.

Van Camp, W., Inzé, D., and Van Montagu, M. (1997). The regulation and function of tobacco superoxide dismutases. **Free Radical Biology and Medicine**. 23(3): 515-520.

Wall, M. M. (2006). Ascorbic acid, vitamin A, and mineral composition of banana (*Musa* sp.) and papaya (*Carica papaya*) cultivars grown in Hawaii. **Journal of Food Composition and Analysis**. 19(5): 434-445.

Wang, L., Ho, J., Glackin, C., and Martins-Green, M. (2012). Specific Pomegranate Juice Components as Potential Inhibitors of Prostate Cancer Metastasis. **Translational Oncology**. 5(5): 344-IN345.

Wang, W. Z., Fang, X. H., Stephenson, L. L., Khiabani, K. T., and Zamboni, W. A. (2008). Ischemia/reperfusion-induced necrosis and apoptosis in the cells isolated from rat skeletal muscle. **Journal of Orthopaedic Research**. 26(3): 351-356.

Warzechaa, Z., Dembinskia, A., Ceranowicza, P., Dembinskia, M., Kownackia, P., Kontureka, S. J., Tomaszewskab, R., Stachurab, J., Hladkic, W., and Pawlika, W. W. (2004). Immunohistochemical expression of FGF-2, PDGF-A. **Journal of Physiology and Pharmacology**. 55(4): 791-810.

Xu, J., Guo, C. J., Yang, J. J., Wei, J. Y., Li, Y. F., Pang, W., and Cheng, S. (2005). Intervention of antioxidant system function of aged rats by giving fruit juices with different antioxidant capacities. **Chinese Journal of Preventive Medicine**. 39(2): 80-83.

Yoshikawa, T., Ueda, S., Naito, Y., Takahashi, S., Oyamada, H., Morita, Y., Yoneta, T., and Kondo, M. (1989). Role of oxygen-derived free radicals in gastric mucosal injury induced by ischemia or ischemia-reperfusion in rats. **Free Radical Research Communications**. 7(3-6): 285-291.

APPENDICES

APPENDIX A

ASSAY FOR TOTAL PHENOLIC CONTENT

Chemicals:

- Sodium carbonate (Na_2CO_3, BDH, Ltd., UK)

- Folin-Ciocalteu reagent (FCR, Merck Millipore, Germany)

- Ethanol (C_2H_6O, Carlo Erba Reagents, France)

- Gallic acid ($C_7H_6O_5$, sigma-Aldrich; St. Louis, USA)

Reagents:

1. Solution A: 2% Sodium carbonate solution

Dissolved 5 g of sodium carbonate in DDD water and made up to 250 ml of final volume and stored at room temperature.

2. Solution B: 10% Ethanol solution

Dissolved 2 ml of ethanol in DDD water and made up to 20 ml of final volume and stored at room temperature.

3. Solution C: Folin-Ciocalteu (FC) solution

Dissolved 1.5 ml of Folin-Ciocalteu in solution B and made up to 3 ml of final volume and stored at room temperature.

4. Solution D: Gallic acid solution

Dissolved 0.01 g of gallic acid in DDD water and made up to 10 ml of final volume and stored at room temperature.

5. Solution E: Diluted fresh Thai pomegranate juice (1:11)

Dissolved 100 ml of fresh Thai pomegranate juice in 10% ethanol and made up to 1100 µl of final volume and stored at 4°C.

6. Solution F: Diluted concentrated Thai pomegranate juice by rotary vacuum evaporation (1:11)

Dissolved 100 ml of concentrated Thai pomegranate juice in DDD water and made up to 1100 µl of final volume and stored at 4°C.

7. Solution G: Diluted concentrated Thai pomegranate juice by microwave evaporation (1:11)

Dissolved 100 ml of concentrated Thai pomegranate juice in DDD water and made up to 1100 µl of final volume and stored at 4°C.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	**Standard (µl)**	**Blank (µl)**
DDD water	-	-	100
Standard	-	100	-
Sample	100	-	-
Folin–Ciocalteu reagent	100	100	100
Sodium carbonate solution	2000	2000	2000

- Pipetted 100 µl of diluted sample (fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation) and 100 ml of Folin–Ciocalteu in the tube.

- 2 ml of sodium carbonate solution was added to the mixture and then allowed to stand for 30 min at room temperature.

- The absorbance was measured at 750 nm using spectrophotometer (CECIL 1011, England).

- Gallic acid was used as standard for the calibration curve. Results were expressed as mg of gallic acid equivalents (GAE) in a liter of fruit juice (mg GAE/L of juice).

- Determinations were performed in triplicate and the mean values were calculated.

APPENDIX B

ASSAY FOR ANTIOXIDANT: 1,1-DIPHENYL-2-PICRYLHYDRAZYL (DPPH) RADICAL-SCAVENGING

Chemicals:

- 1,1-diphenyl-2-picrylhydrazyl (DPPH)($C_{18}H_{12}N_5O_6$, Sigma-Aldrich; St. Louis, USA)

- Methanol (CH_3OH, Carlo Erba Reagents, France)

- Vitamin C ($C_6H_8O_6$, Carlo Erba Reagents, France)

Reagents:

1. Solution A: 25 mg/L 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution

Dissolved 0.007 g of 1,1-diphenyl-2-picrylhydrazyl in absolute methanol and made up to 280 ml of final volume and stored at room temperature.

2. Solution B: Vitamin C solution

- Dissolved 0.005 g of vitamin C with 5 ml of DDD water and stored at room temperature.

- Vitamin C solution was then diluted with DDD water to make serial concentration of 0.001 g/ml, 0.003 g/ml, 0.005 g/ml, 0.010 g/ml, 0.020 g/ml, 0.030 g/ml, 0.040 g/ml, and 0.050 g/ml, respectively.

3. Solution E: Fresh Thai pomegranate juice solution

- Dissolved 2,000 µl of fresh Thai pomegranate juice in DDD water and made up to 1,000 µl of final volume and stored at 4°C.

- Fresh Thai pomegranate juice was then diluted with DDD water to make serial concentrations of 0.00 µl/ml, 0.20 µl/ml, 0.40 µl/ml, 0.78 µl/ml, 1.56 µl/ml, 3.13 µl/ml, 6.25 µl/ml, 12.50 µl/ml, and 25.00 µl/ml, respectively.

4. Solution F: Concentrated Thai pomegranate juice by rotary vacuum evaporation

- Dissolved 640 µl of concentrated Thai pomegranate juice in DDD water and made up to 2,000 µl of final volume and stored at 4°C.

- Concentrated Thai pomegranate juice was then diluted with DDD water to make serial concentrations of 0.00 µl/ml, 0.06 µl/ml, 0.13 µl/ml, 0.25 µl/ml, 0.50 µl/ml, 1.00 µl/ml, 2.00 µl/ml, 4.00 µl/ml, and 8.00 µl/ml, respectively.

7. Solution G: Concentrated Thai pomegranate juice by microwave evaporation

- Dissolved 640 µl of concentrated Thai pomegranate juice in DDD water and made up to 2,000 µl of final volume and stored at 4°C.

- Concentrated Thai pomegranate juice was then diluted with DDD water to make serial concentrations of 0.00 µl/ml, 0.06 µl/ml, 0.13 µl/ml, 0.25 µl/ml, 0.50 µl/ml, 1.00 µl/ml, 2.00 µl/ml, 4.00 µl/ml, and 8.00 µl/ml, respectively.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	Standard (µl)	Blank (µl)
DDD water	-	-	50
Standard	-	50	-
Sample	50	-	-
DPPH working solution	1950	1950	1950

- Pipetted 50 µl of diluted TPJ samples (fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation) and vitamin C (positive control, 0.001 g/ml) into test tube.

- Pipetted 1950 µl of DPPH solution (25 mg/L) in methanol in to test tube.

- The mixture was incubated at room temperature for 30 min in the dark.

- All solutions were freshly prepared and protected from light.

- The absorbance of the mixture was then measured at 515 nm using a microplate reader (BioRad-Benchmark Plus microplate reader, USA).

- All determinations were performed in triplicate.

- Distilled water (50 µl) mixed with 1950 µl of the DPPH working solution was used as the control and absolute methanol was used as sample blank.

- Radical scavenging activity was defined as the inhibition percentage. The scavenging capacity (SC) for DPPH assay was calculated by using the following equation:

% (SC) = [(Abs control – Abs sample) / Abs control]*100

- Results were expressed as IC_{50} concentration where 50% inhibition of the DPPH scavenging capacity is obtained. GraphPad Prism software (version 6, GraphPad Software Inc, California, USA) was used to calculate IC_{50} values.

APPENDIX C

ASSAY FOR ANTIOXIDANT: 2,2'-AZINO-BIS-3-ETHYLBENZTHIAZOLINE-6-SULPHONIC ACID (ABTS) RADICAL-SCAVENGING

Chemicals:

- 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) ($C_{18}H_{18}N_4O_6S_4$, Sigma-Aldrich; St. Louis, USA)
- Methanol (CH_3OH, Carlo Erba Reagents, France)
- Potassium persulfate ($K_2S_2O_8$, Sigma-Aldrich; St. Louis, USA)
- Ethanol (C_2H_6O, Carlo Erba Reagents, France)
- Vitamin C ($C_6H_8O_6$, Carlo Erba Reagents, France)

Reagents:

1. Solution A: 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid solution

Dissolved 0.0179 g of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid in DDD water and made up to 5 ml and stored at room temperature.

2. Solution B: 140 mM potassium persulfate solution

Dissolved 3.7849 of potassium persulfate in DDD water and made up to 100 ml and stored at room temperature.

3. Solution C: 7 mmol/L 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) working solution

- Solution A	5	ml
- Solution C	88	µl

Solution A was mixed with solution B and incubated at room temperature. The solution was protected from light for 16 h.

4. Solution D: Vitamin C solution

- Vitamin C (0.005 g) was dissolved in 5 ml of DDD water and stored at room temperature.

- Vitamin C solution was then diluted with DDD water to make serial concentrations of 0.001 g/ml, 0.003 001 g/ml, 0.005 g/ml, 0.010 g/ml, 0.020 g/ml, 0.030 g/ml, 0.040 g/ml, and 0.050 g/ml, respectively.

5. Solution E: Diluted fresh Thai pomegranate juice

- Dissolved 2000 µl of fresh Thai pomegranate juice in DDD water and made up to 1,000 µl of final volume and stored at 4°C.

- Fresh Thai pomegranate juice was then diluted with DDD water to make serial concentrations of 0.00 µl/ml, 0.20 µl/ml, 0.40 µl/ml, 0.78 µl/ml, 1.56 µl/ml, 3.13 µl/ml, 6.25 µl/ml, 12.50 µl/ml, and 25.00 µl/ml, respectively.

6. Solution F: Diluted concentrated Thai pomegranate juice by rotary vacuum evaporation.

- Dissolved 640 µl of concentrated Thai pomegranate juice in DDD water and made up to 2,000 µl of final volume and stored at 4°C.

- Concentrated Thai pomegranate juice was then diluted with DDD water to make serial concentrations of 0.00 µl/ml, 0.06 µl/ml, 0.13 µl/ml, 0.25 µl/ml, 0.50 µl/ml, 1.00 µl/ml, 2.00 µl/ml, 4.00 µl/ml, and 8.00 µl/ml, respectively.

7. Solution G: Diluted concentrated Thai pomegranate juice by microwave evaporation.

- Dissolved 640 µl of concentrated Thai pomegranate juice in DDD water and made up to 2,000 µl of final volume and stored at 4°C.

- Concentrated Thai pomegranate juice was then diluted with DDD water to make serial concentrations of 0.00 µl/ml, 0.06 µl/ml, 0.13 µl/ml, 0.25 µl/ml, 0.50 µl/ml, 1.00 µl/ml, 2.00 µl/ml, 4.00 µl/ml, and 8.00 µl/ml, respectively.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	**Standard (µl)**	**Blank (µl)**
DDD water	-	-	25
Standard	-	25	-
Sample	25	-	-
ABTS working solution	2,000	2,000	2,000

- Pipetted 25 µl of diluted TPJ samples (fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation) and vitamin C (positive control) into microplate.

- Pipetted 2,000 µl ABTS working solution (5 ml of 7 mmol/L ABTS solution reacted with 88 µl of 140 mmol/L potassium persulfate solution and then the reaction

solution was diluted with ethanol to make the absorbance of 0.700±0.005 at 734 nm) into microplate. All solutions were freshly prepared and protected from light.

- The mixture was then incubated at room temperature for 10 min in the dark.

- The absorbance of the mixture was then measured at 734 nm using a microplate reader (BioRad-Benchmark Plus microplate reader, USA).

- All determinations were performed in triplicate. Distilled water (25 µl) mixed with 2,000 µl of ABTS working solution was used as the control and absolute ethanol was used as sample blank.

- Radical scavenging activity was defined as the inhibition percentage.

- The scavenging capacity (SC) for ABTS assay was calculated by using the following equation:

% (SC) = [(Abs control – Abs sample) / Abs control]*100

- Results were expressed as IC_{50} concentration where 50% inhibition of the ABTS scavenging capacity is obtained. GraphPad Prism software (version 6, GraphPad Software Inc, California, USA) was used to calculate IC_{50} values.

APPENDIX D

ASSAY FOR ANTIOXIDANT: FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

Chemicals:

- 2,4,6-tripyridyl-s-triazine (TPTZ) (Sigma-Aldrich; St. Louis, USA)
- Hydrochloric acid (HCl, Sigma-Aldrich; St. Louis, USA)
- Glacial acetic acid (CH_3COOH, Sigma-Aldrich; St. Louis, USA)
- Sodium acetate (CH_3COONa, Sigma-Aldrich; St. Louis, USA)
- Ferric chloride ($FeCl_3.6H_2O$, Sigma-Aldrich; St. Louis, USA)
- Ferrous sulfate ($FeSO_4.7H_2O$, Sigma-Aldrich; St. Louis, USA)
- Vitamin C (Carlo Erba Reagents, France)

Reagents:

1. Solution A: 40 mM hydrochloric acid (HCl) solution

Dissolved 0.08 ml of hydrochloric acid in DDD water and made up to 20 ml of final volume and stored at room temperature.

2. Solution B: 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution

Dissolved 0.0324 g of 2,4,6-tripyridyl-s-triazine (TPTZ) in solution A and made up to 10 ml of final volume at 37°C.

3. Solution C: 20 mM ferric chloride solution

Dissolved 0.0324 g of ferric chloride in DDD water and made up to 10 ml of final volume and stored at room temperature.

4. Solution D: 300 mM acetate buffer at pH 3.6

- Sodium acetate	0.82	g
- Glacial acetic acid	8.11	ml

Dissolved all chemicals in DDD water and made up to 500 ml of final volume and stored at room temperature. The solution was adjusted pH with sodium hydroxide.

5. Solution E: Ferrous sulfate solution

Dissolved 0.667 g of ferrous sulfate in DDD water and made up to 1 ml of final volume and stored at room temperature.

- Ferrous sulfate was diluted with DDD water to make serial concentrations of 0 µmol/ml, 58.59 µmol/ml, 117.19 µmol/ml, 234.38 µmol/ml, 468.75 µmol/ml, and 937.50 µmol/ml, respectively.

6. Solution F: Diluted fresh Thai pomegranate juice (1:11)

Dissolved 100 µl of fresh Thai pomegranate juice in 10% ethanol and made up to 1,100 µl of final volume and stored at 4°C.

7. Solution G: Diluted concentrated Thai pomegranate juice by rotary vacuum evaporation (1:11)

Dissolved 100 µl of concentrated Thai pomegranate juice in DDD water and made up to 1100 µl of final volume and stored at 4°C.

8. Solution H: Dissolved concentrated Thai pomegranate juice by microwave evaporation (1:11).

Dissolved 100 µl of concentrated Thai pomegranate juice in DDD water and made up to 1,100 µl of final volume and stored at 4°C.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	Standard (µl)	Blank (µl)
DDD water	-	-	40
Standard	-	40	-
Sample	40	-	-
DDD water	200	200	200
FRAP solution	1,800	1,800	1,800

- Pipetted 40 µl of diluted TPJ samples (fresh TPJ, concentrated TPJ by microwave evaporation, and concentrated TPJ by rotary vacuum evaporation) and quercetin (positive control, 0.025 g/ml) into test tube.

- Pipetted 200 µl of DDD water into test tube.

- Pipetted 1,800 µl FRAP solution into test tube. FRAP solution containing 2.5 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl 2.5 ml of 20 mM $FeCl_3.6H_2O$, and 25 ml of 0.3 mM acetate buffer (pH 3.6), was freshly prepared and warmed to 37°C prior to use.

- The mixture was then incubated at 37°C in water bath for 30 min in the dark.

- All solutions were freshly prepared and protected from light.

- The absorbance of the mixture was then measured at 593 nm using spectrophotometer (CECIL 1011, England).

- Distilled water (40 µl) mixed with 1,800 µl of FRAP solution was used as the sample blank.

- Quercetin was used as positive control.

- The results were calculated from the standard curve constructed of by different concentrations of Iron (II) sulfate solution.

- The results were expressed in mmol Fe^{2+}/L. All the measurements were taken in triplicate and the mean values were calculated.

APPENDIX E

PREPARATION OF TISSUE HOMOGENATE

Chemicals:

- Triz-base acid (Sigma-Aldrich; St. Louis, USA)

- Hydrochloric acid (HCl, Carlo Erba Reagents, France)

Reagents:

1. Solution A: 50 mM Triz-base solution

Dissolved 12.12 g of triz-base in DDD water and made up to 2,000 ml of final volume and stored at room temperature.

2. Solution B: 50 mM hydrochloric acid (HCl) solution

Dissolved 2.475 ml of hydrochloric acid in DDD water and made up to 250 ml of final volume and storage at room temperature.

3. Solution C: 50 mM Triz-HCl solution

- Solution A	500	ml
- Solution B	403	ml

Solution A was mixed with solution B was kept at 2-4°C. This solution was used as homogenization buffer and should be ice cold at the time of use.

APPENDIX F

ASSAY FOR PROTEIN

Chemicals:

- Copper sulfate ($CuSO_4.5H_2O$, Sigma-Aldrich; St. Louis, USA)
- Sodium tartate ($Na_2H_4H_4O_6.H_2O$, Sigma-Aldrich; St. Louis, USA)
- Sodium azide (BD, Ltd., UK)
- Sodium hydroxide ($NaOH.5H_2O$, Sigma-Aldrich; St. Louis, USA)
- Sodium carbonate (Na_2CO_3, Sigma-Aldrich; St. Louis, USA)
- Sodium dodecyl sulfate (SDS: $C_{12}H_{25}NaO_4S$, BDH, Ltd., UK)
- Folin-Ciocatteau reagent (Merck Millipore, Germany)
- Bovine Serum albumin (Sigma-Aldrich; St. Louis, USA)
- Hydrochoric acid (HCl, Carlo Erba Reagents, France)
- Trizma base (Sigma-Aldrich; St. Louis, USA)

Reagents:

1. Solution A

- Copper sulfate	0.15	g
- Sodium tartate	0.30	g
- Sodium azide	0.05	g

All chemicals were dissolved in DDD water and made up to 250 ml of final volume stored at room temperature.

2. Solution B

- Sodium hydroxide 2.0 g
- Sodium carbonate 8.0 g
- Sodium dodecyl sulfate 1.0 g

All chemicals were dissolved in DDD water and made up to 250 ml of final volume stored at room temperature.

3. Solution C

Freshly mixed solution A and solution B (1:3)

4. Solution D

2 N of diluted Folin-Ciocatteau reagent was mixed with DDD water (1:20) and stored at room temperature.

5. Solution E (Standard protein)

Dissolved bovine serum albumin (BSA) in DDD water to make serial concentrations of 0 µg/ml, 62.5 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 1,000 µg/ml, 2,000 µg/ml, respectively.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	**Standard (µl)**	**Blank (µl)**
DDD water	-	-	30
Standard	-	30	-
Sample	30	-	-
Solution C	100	100	100
Solution D	150	150	150

- Pipette 30 µl of the sample and standard protein into 96 well plates.

- Pipetted 100 µl of solution C into 96 well plates, and then incubated for 60 min at room temperature.

- Pipette 150 µl of the solution D into 96 well plates.

- All solutions were mixed and incubated for 30 min at room temperature.

- The absorbance of the mixture was then measured at 650 nm using a microplate reader (BioRad-Benchmark Plus microplate reader, USA).

Calculation:

$$\Delta Abs_{standard} = Abs_{standard} - Abs_{blank}$$

Plotted the $\Delta Abs_{standard}$ againt protein concentration on the standard graph.

$$\Delta Abs_{sample} = Abs_{sample} - Abs_{blank}$$

The results determined the mg protein from the standard curve.

APPENDIX G

ASSAY FOR SUPEROXIDE DISMUTASE ENZYME ACTIVITY

Chemicals:

- Thiobabituric acid (TBA, Sigma-Aldrich; St. Louis, USA)
- Thiochloroacetic acid (TCA, Sigma-Aldrich; St. Louis, USA)
- Hydrochoric acid (HCl, Carlo Erba Reagents, France)
- 1,1,3,3-tetramethoxy propane (TMP, Sigma-Aldrich; St. Louis, USA)
- Trizma base (Sigma-Aldrich; St. Louis, USA)

Reagents:

1. Water-soluble tetrazolium salt (WST) working solution

   Diluted 1 ml of WST solution with 19 ml of buffer solution.

2. Enzyme working solution

   Pipetted 15 µl of enzyme solution with 2.5 ml of dilution buffer.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	Control (µl)
Sample	20	-
Tris-HCL	-	20
WST working solution	200	200
Enzyme working solution	20	20

- Added 20 µl of sample solution into each well.

- Added 200 µl of WST working solution into each well, and mixed.

- Added 20 µl of enzyme working solution into each well, mixed thoroughly, and the mixture was then incubated at 37°C for 20 min.

- The absorbance of the mixture was measured at 450 nm using a microplate reader (BioRad-Benchmark Plus microplate reader, USA).

- The SOD activity (inhibition rate %) was calculated using the following equation:

SOD activity (inhibition rate % = [(Abs control – Abs sample) / Abs control)]*100

APPENDIX H

ASSAY FOR CATALASE ENZYME ACTIVITY

Chemicals:

- Hydrogen Peroxide (H_2O_2, Merck Millipore, Germany)

- Potassium permanganate ($KMnO_4$, BDH, Ltd., UK)

- Sulfulic acid (H_2SO_4, Sigma-Aldrich; St. Louis, USA)

- Disodium phosphate or Disodium hydrogen phosphate (Na_2HPO_4, Sigma-Aldrich; St. Louis, USA)

- Standard Catalase enzyme solution (Sigma-Aldrich; St. Louis, USA)

- Hydrochoric acid (HCl, Carlo Erba Reagents, France)

- Trizma base (Sigma-Aldrich; St. Louis, USA)

Reagents:

1. 0.01 N of Hydrogen Peroxide (H_2O_2) solution

Dissolved 0.0568 ml of hydrogen peroxide 30% (w/w) in 50 mM potassium phosphate buffer, pH 7 at 25°C, made up the final volume to 100 ml.

2. 0.005 N Potassium permanganate ($KMnO_4$) solution

Dissolved 0.016 g of potassium permanganate in 100 ml of DDD water at room temperature.

3. 5 N of sulfulic acid (H_2SO_4)solution

Dissolved 3.507 ml of sulfulic acid in DDD water and made up to 25 ml of final volume and stored at room temperature.

4. 50 mM Potassium phosphate buffer solution, pH 7 at 25°C

Dissolved 6.80 g of potassium phosphate in 1,000 ml DDD water and stored at 25°C temperature.

5. Standard catalase enzyme solution

Immediately before use, serial concentrations of standard catalase enzyme solution were prepared in Triz-HCl buffer solution: 0 unit/ml, 1.56 unit/ml, 3.13 unit/ml, 6.25 unit/ml, 12.50 unit/ml, 25.00 unit/ml, respectively.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	Standard (µl)	Blank (µl)
Tris-HCL	-	-	10
Standard CAT enzyme	-	10	-
Sample	10	-	-
H_2O_2	50	50	50
H_2SO_4	25	25	25
$KMnO_4$	150	150	150

- Pipetted 10 µl of the sample and standard enzyme into 96 well plates.

- Pipetted 50 µl of hydrogen peroxide solution into 96 well plates.

- Added 25 µl of the sulfulic acid solution (5 N) into 96 well plates.

- Pipetted 150 µl of potassium permanganate solution into 96 well plate and all solutions was mixed.

- The absorbance was then measured at 490 nm using a microplate reader (BioRad- Benchmark Plus microplate reader, USA).

- The distilled water mixed of hydrogen peroxide solution, sulfulic acid solution and potassium permanganate solution was used as the control.

- Hydrogen peroxide solution, sulfulic acid solution and potassium permanganate solution was used as blank.

- All determinations were performed in triplicate.

- Catalase enzyme activity was expressed as unit/mg protein.

Calculation:

$$\Delta Abs_{standard} = Abs_{standard} - Abs_{blank}$$

Plotted the ΔAbs_{490} standard againt catalase enzyme concentration on the standard graph.

$$\Delta Abs_{sample} = Abs_{sample} - Abs_{blank}$$

Determined the catalase enzyme activity from the standard curve.

APPENDIX I

ASSAY FOR GLUTATHIONE

Chemicals:

- Potassium phosphate (K_3O_4P, Sigma-Aldrich; St. Louis, USA)
- Ethylene diamine tetraacetic acid (EDTA, Sigma-Aldrich; St. Louis, USA)
- Glutathione Reductase (Sigma-Aldrich; St. Louis, USA)
- Glutathione Reduced (Sigma-Aldrich; St. Louis, USA)
- Ammonium sulfate ($(NH_4)_2SO_4$, Sigma-Aldrich; St. Louis, USA)
- Dithiothreitol ($C_4H_{10}O_2S_2$, Sigma-Aldrich; St. Louis, USA)
- 5,5'-Dithiobis(2-nitrobenzoic acid) ($C_{14}H_8N_2O_8S_2$DTNB, Sigma-Aldrich; St. Louis, USA)
- Nicotinamide adenine dinucleotide phosphate ($C_{21}H_{29}N_7O_{17}P_3$, Sigma-Aldrich; St. Louis, USA)
- Dimethyl Sulfoxide ($(CH_3)_2SO$, Sigma-Aldrich; St. Louis, USA)
- Triz-base (Sigma-Aldrich; St. Louis, USA)
- Hydrochloric acid (HCl, Sigma-Aldrich; St. Louis, USA)

Reagents:

1. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) solution

Dissolved 8 mg of 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) in 5.33 ml of dimethyl sulfoxide (DMSO) to make a 1.5 mg/ml solution.

2. Nicotinamide adenine dinucleotide phosphate (NADPH) solution (0.16 mg/ml)

Diluted 42 µl of NADPH stock solution (40 mg/ml) in dilution assay buffer and made up to 10,500 µl of final volume.

3. Assay buffer solution

- Assay buffer included 500 mM of potassium phosphate and 5 mM of EDTA at 30 ml of total volume.

- Diluted 8.58 ml of assay buffer in 34.32 ml of water and stored at 2-4°C temperature.

4. Enzyme solution (6 unit /ml)

Diluted 13.68 µl of glutathione reductase (400 unit /ml) in dilution assay buffer and made up to 900 µl of final volume.

5. Working mixture

- Enzyme solution	897.75 µl
- DTNB solution	897.75 µl

Dissolved all solutions in 31.5 ml of assay buffer solution and stored at 2-4°C.

6. Glutathione (GSH) standard solution (10 mM)

Dissolved 0.3 mg of GSH in 0.1 ml of DDD water and stored at 2 to 4°C. GSH standard solution were diluted with Triz-HCl solution to make serial concentrations of 250 µmol/10µl, 125.00 µmol/10µl, 62.50 µmol/10µl, 31.25 µmol/10µl, 15.63 µmol/10µl, 7.81 µmol/10µl, and 0.00 µmol/10µl, respectively.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	Standard (µl)	Blank (µl)
Tris-HCL	-	-	10
Standard	-	10	-
Sample	10	-	-
Working mixture	150	150	150
	Mix and incubate 5 min		
NADPH	50	50	50

1. Added 10 µl samples and standard into each well of microplate.

2. Added 150 µl of the working mixture into each well with a multichannel pipette. Mixed by pipetting up and down.

3. The mixture was then incubate 5 min at room temperature.

4. Added 50 µl of the diluted NADPH solution into each well with a multichannel pipette and mixed by pipetting up and down.

5. The absorbance of the mixture was then measured at 412 nm using a microplate reader (BioRad- Benchmark Plus microplate reader, USA).

6. The GSH levels were calculated using the following equation:

Calculation:

$$\Delta Abs_{standard} = Abs_{standard} - Abs_{blank}$$

Plotted the $\Delta Abs_{standard}$ standard against GSH concentration on the standard graph.

$$\Delta Abs_{sample} = Abs_{sample} - Abs_{blank}$$

The GSH levels were determined as µmol/mg protein from the standard curve.

APPENDIX J

ASSAY FOR MALONDIALDEHYDE

Chemicals:

- Thiobabituric acid ($C_2H_4N_2O_2S$, Sigma-Aldrich; St. Louis, USA)

- Triochloroacetic acid (Cl_3CCOOH, Sigma-Aldrich; St. Louis, USA)

- Hydrochoric acid (HCl, Carlo Erba Reagents, France)

- 1,1,3,3-tetramethoxypropane ($(CH_3O)_2CHCH_2CH(OCH_3)_2$, Sigma-Aldrich; St. Louis, USA)

Solution:

1. Solution A: TCA-TBA-HCl solution

- 0.375 % (w/v) Thiobabituric acid (TBA)	0.375	g
- 15% (w/v) Triochloroacetic acid (TCA)	15	g
- 0.25 N Hydrochoric acid (HCl)	2.475	ml

All chemicals were dissolved in 100 ml DDD water and made up to 100 ml of final volume.

2. Solution B: 1,1,3,3-tetramethoxy propane (TMP)

Dissolved 10 µl of 1,1,3,3-tetramethoxy propane (TMP) in DDD water and made up to 990 ml of final volume and stored at room temperature. TMP was diluted with DDD water to make serial concentrations of 0 nmol/ml, 0.46 nmol/ml,0.93 nmol/ml, 1.85 nmol/ml, 3.71 nmol/ml, 7.41 nmol/ml, 14.82 nmol/ml, 29.65 nmol/ml, and 59.30 nmol/ml, respectively.

Procedure:

Pipetted the solution into each well as followed:

	Sample (µl)	Standard (µl)	Blank (µl)
Tris-HCL		-	100
Standard TMP	-	100	-
Sample	100	-	-
TCA-TBA-HCl solution	200	200	200

- Pipetted 100 µl of the sample and standard enzyme into tube.

- Pipetted 200 µl of TCA-TBA-HCL reagent into each tube, mixed well.

- The mixture was heated with a boiling water bath (100°C) for 15 min.

- After cooling, the mixture was centrifuged at 1,000 g for 10 min.

- The absorbance of the mixture (supernatant) was then measured at 535 nm using microplate reader (BioRad- Benchmark Plus microplate reader, USA).

- DDD water mixed with TCA-TBA-HCL reagent was used as the control and TCA-TBA-HCL reagent was used blank. All determinations were performed in triplicate. The TMP concentrations were calculated using following equation:

Calculation:

$$\Delta Abs_{standard} = Abs_{standard} - Abs_{blank}$$

Plotted the $\Delta Abs_{standard}$ standard against TMP concentration on the standard graph.

$$\Delta Abs_{sample} = Abs_{sample} - Abs_{blank}$$

The TMP (equivalence with MDA) level was determined as nmol/mg tissue weight from the standard curve.

CURRICULUM VITAE

Name	**Miss KUSUMA RUAMTHUM**
Date of birth	**3 September 1990**
Place of birth	**Buriram, Thailand**
Education	
2013-Present	M.Sc. (Biomedical Sciences) Suranaree University of Technology, Nakhon Ratchasima, Thailand.
2009-2012	B.Sc. (Sports Science) Suranaree University of Technology, Nakhon Ratchasima, Thailand.
Work Experience	
2013-Present	Teaching Assistant in School of Preclinic, Suranaree University of Technology, Nakon Ratchasima, Thailand.
Publications	
2016	Ruamthum, K., and Srisawat, R. (2016). Comparative evaluation of different concentration methods for the assays of total phenolic content and antioxidant activity of Thai pomegranate juice. Proceedings of the 6th International Conference on Natural Products for Health and Beauty (NATPRO 6). January 21-23, 2016 Pullman Raja Orchid Hotel, Khon Kean, Thailand.