รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัย

ฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นและการเจริญเติบโตแบคทีเรียของสารสกัดมะตูมนิ่ม

In vitro antioxidation and antibacterial capacity of water extracts of *Aegle marmelos*

โดย

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2553

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ชื่อเรื่อง : ฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นและการเจริญเติบโตของแบคทีเรียของสารสกัดมะตูมนิ่ม
ผู้วิจัย : อาจารย์อุษา สุวรรณสรวล สถาบัน : คณะวิทยาศาสตร์
อาจารย์ ดร. อินทิรา แถมพยัคฆ์
ปีที่พิมพ์ : 2556 สถานที่พิมพ์ : มหาวิทยาลัยรังสิต
แหล่งเก็บรายงานการวิจัยฉบับสมบูรณ์ จำนวนหน้างานวิจัย 41 หน้า
:
คำสำคัญ: ฤทธิ์ต้านปฏิกิริยาออกซิเดชั่น ฤทธิ์ต้านการเจริญเติบโตของแบคทีเรีย มะตูมนิ่ม
ลิขสิทธิ์ :

บทคัดย่อ

สมุนไพรเป็นแหล่งของสารที่มีคุณสมบัติต้านปฏิกิริยาออกซิเดชั่น ผู้วิจัยได้ทำการศึกษาหาฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นและความสามารถในการต้านอนุมูลอิสระของพืชสมุนไพรที่เป็นส่วนประกอบของตำรับยาลูกแปลกแม่ ซึ่งได้แก่ มะตูมนิ่ม กล้วยน้ำไท และพริกไทยดำ วิธีที่ใช้ศึกษามี 5 วิธี โดยใช้สาร 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), Ferric reducing power, superoxide anion radical, hydroxyl radical scavenging และหาปริมาณฟีนอลทั้งหมดด้วย ผลการทดลองพบว่าฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นของสารสกัดน้ำของพืชทั้ง 3 ชนิดแตกต่างกัน แต่ทุกชนิดมีฟีนอลเป็นส่วนประกอบเหมือนกัน สารสกัดพริกไทยดำมีฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นมากที่สุดเมื่อศึกษาด้วยวิธี Ferric reducing power, superoxide anion radical scavenging และ hydroxyl radical scavenging ส่วนสารสกัดมะตูมนิ่มมีฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นเมื่อศึกษาด้วยวิธี scavenging DPPH free radical และ reducing power และยังมีฤทธิ์ต้านการเจริญเติบโตของเชื้อ *Escherichia coli* ในระดับต่ำ แต่ไม่สามารถต้านการเจริญเติบโตของเชื้อ *Staphylococcus aureus* สำหรับสารสกัดกล้วยน้ำไทมีฤทธิ์ reducing power สูงกว่าชุดควบคุม จากผลการศึกษาในครั้งนี้พบว่าสามารถตรวจพบสารฟีนอลในพืชสมุนไพรทั้ง 3 ชนิด มีฤทธิ์ต้านปฏิกิริยาออกซิเดชั่น จึงสามารถนำมาพัฒนาเป็นสารธรรมชาติที่มีฤทธิ์ต้านปฏิกิริยาออกซิเดชั่นได้

มหาวิทยาลัยรังสิต Rangsit University

Title: In vitro antioxidation and antibacterial capacity of water extracts of *Aegle marmelos*

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Intira Thampayak Ph.D. **Publisher:**

Year of Publication: 2013 **No. of page:** 41 pages

Keyword: Antioxidant activities, Antibacterial activity, *Aegle marmelos*,

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ABSTRACT

Herbal plants are source for variety of natural antioxidant. We evaluated the antioxidant activities of some Thai plants which are founded in Thai herbal formulation called as *Luk Plak Mae* (Thai name). It has been used for anti-aging. The water extracts of *Aegle marmelos*, *Musa eumusa* and *Piper nigrum* were investigated for their antioxidant and radical scavenging activities in five different assays. The extracts were evaluated using the 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) free radical scavenging, Ferric reducing power, superoxide anion radical scavenging, and hydroxyl radical scavenging assays. The amount of total phenolics was evaluated. The results showed that the antioxidant activities varied among the different plants used in this study and the total phenolic contents was found in all plants. *Piper nigrum* exhibited strong antioxidant activities by demonstrated in the Ferric reducing power, superoxide anion radical scavenging, and hydroxyl radical scavenging assays. *Aegle marmelos* extract possessed the antioxidant by scavenging DPPH free radical and exploring the reducing power. Moreover, this study showed that the water extract of *A. marmelos* was able to inhibit slightly the growth of *Escherichia coli* but not for *Staphylococcus aureus*. *Musa eumusa* extract showed greater reducing power than that of the standard. These findings show that in these plants provide substantial antioxidant activities. The results suggest the potential of development of useful natural antioxidants.

ACKNOWLEDGEMENTS

It is very great pleasure to thanks many people who made this research possible.

We would like to thank Assist. Prof. Dr. Kantimanee Pradermwong, Department of Zoology, Faculty of Sciences, Kasetsart University and Ms. Saowaluck Ukrisdawithid, Department of Science Service, Ministry of Science and Technology for their guidance, support, and technical advice.

We would like to thank Ms. Khwanruan Phetnoi, Ms. Varumporn Sukkumme and Ms. Thitima Jerajasin for assisting this research.

We would like to thank Ms. Chatwanich Puangmalai, managing director of Greenchat Natural Herb and Ms. Nantawan Puangmlai, managing director of Innovative Pharma Herbs co.,Ltd. for giving the plant crudes

Thanks are also due to Rangsit University for granting financial support to fulfill this research and Department of Zoology, Faculty of Sciences, Kasetsart University for supporting the materials and using the instruments.

Lastly, and most importantly, we wish to thank my family, on whose constant encouragement and love. Their unflinching courage and conviction will always inspire me.

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CHAPTER I

INTRODUCTION

1. Background and rationale

The reactive oxygen species (ROS) and free radical mediated reactions are associated in degenerative or pathological events such as aging, cancer, coronary heart ailments, and Alzheimer's disease. The superoxide anion, hydroxyl radical, and hydrogen peroxide, are generated in specific organelles of the cell under normal physiological conditions Excessive production of these ROS, beyond the antioxidant defense capacity of the body can cause oxidative stress (Sun et al., 2004). There is a large body of evidence to suggest that oxidative stress plays a major role in the intrinsic as well as the extrinsic process of skin aging. While free radical generation is certainly not the only contributor to the aging process, there is ample evidence that oxidative damage to cellular and extracellular components is responsible for many of the degenerative changes observed with age. *Aegle marmelos* or Matoon Nim, *Musa eumusa*, or Kluai Nam Thai and *Piper. nigrum* or Pik Thai Dam are the Thai plants. Three plants are formulated to Thai herbal formulation, called as *Luk Plak Mae* (Thai name), which has administrated by oral route. This traditional herbal formulation has been used for an anti-aging in Thailand for long time ago. Previously experimental studies in India reported that the plants also possessed the antioxidant. The purpose of this study is to determine the antioxidant activities of water extracts of *Aegle marmelos*, *Musa. eumusa*, and *Piper. nigrum* by evaluating the amount of phenolic compounds, the DPPH radical-scavenging assay, the Ferric reducing power assay, the Superoxide radical-scavenging assay, and the Hydroxyl radical ($^{\cdot}OH$)-scavenging assay to provide a pharmacological basis for the potential use of *Luk Plak Mae* for anti-aging. Moreover, the antibacterial properties of the water extracts of *Aegle marmelos* is evaluated in this study

2. Objectives

To study the antioxidation activities of the water extracts of *Aegle marmelos*, *Musa. eumusa*, and *Piper. nigrum.* In addition, antibacterial capacity of the water extracts of *Aegle marmelos* is also considered in this study.

CHAPTER II

LITERATURE REVIEW

1. Free radicals

Free radicals and related specie have been interesting to the cause of various disorders in human. They are mainly originated from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS). Free radicals conduct to alter physicochemical conditions. Free radicals attack the endogenous molecules such as lipids, proteins, carbohydrates, and DNA and result in various diseases. (Devasagayam et al., 2004)

Free radicals are chemical species, which have unpaired electrons. Unpaired electrons look for other electrons to become paired. Table 1 summarizes the active oxygen species and related species, which are relevant to lipid peroxidation and oxidative stress *in vivo*. Nitric oxide and thiyl radical which do not bear unpaired electrons on oxygen are also included. (Papas, 1999) Free radicals have been involved with the etiology of several human diseases. The biologically important oxygen reactive species are presented in Table 2. (Devasagayam et al., 2004)

Table 1 Active oxygen and related species

Radicals		**Non-radicals**	
$O_2^{\cdot -}$	superoxide	H_2O_2	hydrogen peroxide
$OH^{\cdot}$	hydroxyl radical	$^1O_2$	singlet oxygen
$OH^{\cdot}{}_2$	hydroperoxyl radical	LOOH	lipid hydroperoxide
$L^{\cdot}$	lipid radical	Fe=O	iron-oxygen complexes
$LO_2^{\cdot}$	lipid peroxyl radical	HOCl	hypochlorite
$LO^{\cdot}$	lipid alkoxyl radical		
$NO_2^{\cdot}$	nitrogen dioxide		
$^{\cdot}NO$	nitric oxide		
$RS^{\cdot}$	thiyl radical		
P	protein radical		

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Table 2 Reactive oxygen species of biological interest

Reactive species	**Reactivity**
Superoxide	Generated in mitochondria, in cardiovascular system and others
Hydroxyl radical	Very highly reactive, generated during iron overload and such conditions in our body
Hydrogen peroxide	Formed in our body by large number of reactions and yields potent species like hydroxyl radical
Organic hydroperoxide	Reacts with transient metal ions to yield reactive species
Singlet oxygen	Highly reactive, formed during photosensitization and chemical reactions
Ozone	Present as an atmospheric pollutant, can react with various molecules, yielding singlet oxygen

2. Molecular damage induced by free radicals

2.1 Lipids and lipoproteins

The oxidation of the lipids and lipoproteins is a lipid peroxidation process in which polyunsaturated fatty acids (PUFAs) contained in the phospholipids are transformed into lipid hydroperoxides and then to some unsaturated aldehydes such as malondialdehyde (MDA), 4-hydroxynonenol (HIVE), and hexenol. Lipid peroxidation (LPO), which generates reactive lipid peroxides ($LOO^{\bullet}$), can be divided into three phases: initial, propagation, and terminal phase. The initial phase is the event of lipoprotein antioxidant depletion, but that of the polyunsaturated fatty acids does not take place significantly. As the PUFAs (mainly linoleic and arachidonic acid) are rapidly oxidized to lipid hydroperoxides and aldehydes, the lipoprotein antioxidants are reduced. The endogenous tocopherol in the LDL, which prevents against the lipid peroxidation, can prolong duration of the lag phase and reduce rate of the propagation phase (Francis, 2000).

Polyunsaturated lipids are essential for the entire supporting system of cells, including cell membranes, endoplasmic reticulum, and mitochondria. Disruption of their

structural properties can therefore have dire consequences for cellular functions. The LPO is implicated in a diverse range of cellular insults and has traditionally been thought to be a major effect of free radicals. Because of this, many of the assay methods to establish free radical-induced injury have measured by-products of the reaction of these molecules with the lipids. However, other cellular components may be as important as, or more important than, lipids in free radical injury. Any assay method that assesses the effects of the free radicals on a particular component of cellular architecture needs to be accompanied by consideration of importance of the type of injury being determined.

The free radicals have high affinity for electron-rich unsaturated covalent bonds, such as those found in the PUFAs. The net result of this reaction is a free radical-mediated abstraction of an electron from the unsaturated covalent bonds of the PUFA, thus generating a PUFA radical ($L^{\bullet}$). Hydroxy, lipoxy, and lipid peroxy radicals have been implicated in initiating this process. Once formed, the lipid radical has several possible fates. It may arrange to a more stable conjugated diene configuration, which enters the self-propagating LPO cascade. Alternatively, it may combine with another molecule, such as another lipid radical or a so-called free radical scavenger, and form a non-reactive complex. In the latter case, the dimer is formed at the expense of a cross-linkage of the PUFAs within the membrane, causing a decrease in membrane fluidity. Because of the reactivity of PUFA radicals, the process is self-propagating. The end result is the chemical alteration of the PUFAs with the disruption of integral cellular components. Whether the LPO is the major site of free radical damage to cells has been questioned. However, even if disruption of cellular lipids is not the final pathway to cell death, it is certainly a key route whereby additional radicals and other toxic substances are produced. Figure 2.2 shows the actual sequence of events in free radical attack of the PUFAs. (Baskin, 1997)

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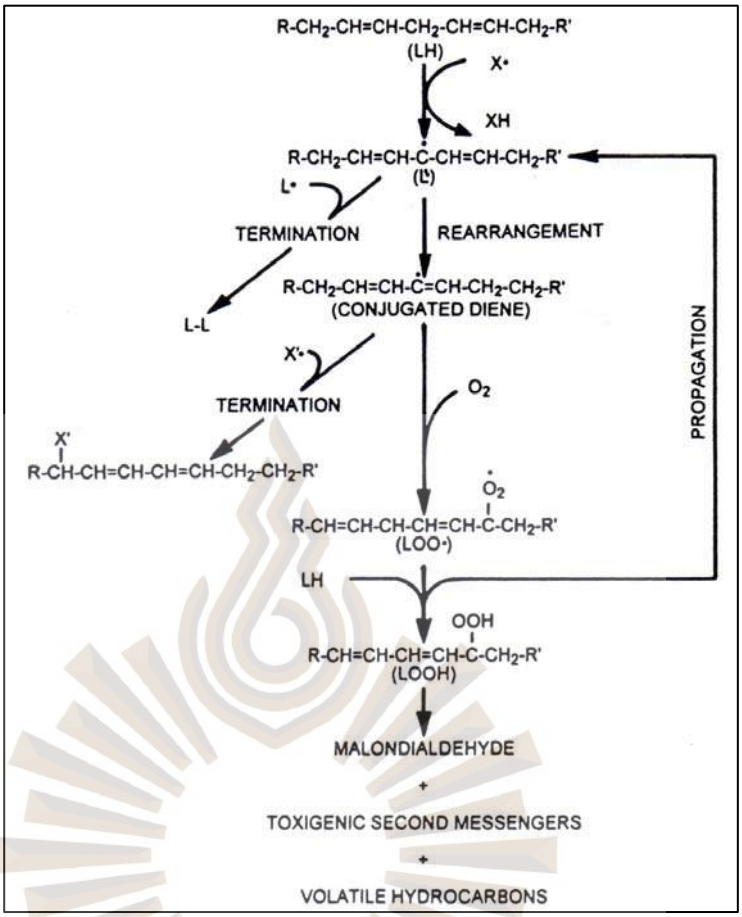

Figure 2.1 The lipid peroxidation cascade

2.2 Carbohydrates

Free radicals such as OH radicals attack and destroy the carbohydrate by randomly abstracting a hydrogen atom from one of the carbon atom, producing a corbon-centered radical. This leads to degradation of hyaluronic acid. In the synovial fluid surrounding joints, an accumulation and activation of neutrophils during inflammation produces significant amounts of oxyradicals, these is also being implicated in rheumatoid arthritis (Devasagayam et al., 2004).

2.3 DNA

Free radicals are produced in cells by cellular metabolism and by exogenous agents. These species attack with biomolecules in cells, including DNA. Free radicals result to damage to DNA, which is also called oxidative damage to DNA, is implicated in mutagenesis, carcinogenesis, and aging. Mechanisms of damage involve abstractions and

addition of reactions by free radicals leading to carbon-centered sugar radicals and OH- or H-adduct radicals of heterocyclic bases (Dizdaroglu et al., 2002).

2.4 Proteins

Proteins are a main target for oxidants which results to change their abundance in biological systems, and generate high rate constants of various reactions. (Davies, 2005) Oxidation of proteins by ROS/RNS can generate the reactive products such as protein hydroperoxides. Some oxidized proteins can gradually accumulate with time and thereby contribute to the damage associated with ageing as well as various diseases (Linton et al., 2001).

3. Free radicals and disorders

3.1 Cardiovascular disease

Cardiovascular is the crucial cause of mortality in Western world. (Fearon and Faux, 2009) Oxidative stress such as reactive oxidative and nitrogen species leads to promotes the pathogenesis of atherothrombotic vascular disease. (Leopold and Loscalzo, 2009). Atherosclerosis is the process which leads to the thickening of the artery walls and gives rise to the many pathology that cause cardio vascular disease as coronary heart disease. Atherosclerosis is progressive, degenerative arterial disease that gradually blocks the affected vessels, thus reducing blood flow through them. Progressive development of the atherosclerosis is in the following:

1. Atherosclerosis is believed to start with injury of the blood vessel wall, which triggers an inflammatory response that sets the stage for the build-up of plaque. Normally, inflammation is a protective response that fights infection and promotes repair of damage tissue. However, when the cause of the injury persists within the vessel wall, the sustained, low grade inflammatory response over a course of decades can insidiously lead to arterial plaque formation and heart disease. Suspected artery-abusing agents that may set off the vascular inflammatory response included oxidized cholesterols, free radicals, high blood pressure, homocysteines, or even bacteria and viruses that damage blood vessel walls. The most common triggering agent appears to be the oxidized cholesterols.

2. Typically, the initial stage of atherosclerosis is characterized by the accumulation beneath the endothelium of excessive amounts of low-density lipoprotein (LDL), the so-called “bad” cholesterol, in combination with a protein carrier. As the LDL accumulates within the vessel wall, this cholesterol product becomes oxidized, primarily by oxidative wastes produced by the blood vessel cells. These wastes are free radicals, very unstable electron-deficient particles that are highly reactive. Antioxidant vitamins that prevent LDL oxidation, such as vitamin E, vitamin C, and beta-carotene, have been shown to slow plaque deposition.

3. In response to the presence of the oxidized LDL and/or other irritants, the endothelial cells produce chemicals that attract monocytes to the site. These immune cells trigger a local inflammatory response.

4. Once they leave the blood and enter the vessel wall, the monocytes settle down permanently, enlarge, and become large phagocytic cells called the macrophages. They actively phagocytize the oxidized LDL until these cells become so packed with fatty droplets that they appear foamy under a microscope. Now called the foam cells, these greatly engorged macrophages accumulate beneath the vessel lining and form a visible fatty streak, the earliest form of an atherosclerotic plaque.

5. The earliest stage of plaque formation is, therefore, characterized by the accumulation beneath the endothelium of a cholesterol-rich deposit. The disease progresses as smooth muscle cells within the blood vessel wall migrate from the muscular layer of the blood vessel to a position on top of the lipid accumulation, just beneath the endothelium. The migration is triggered by chemical release at the inflammatory site. At their new location, the smooth muscle cells, continue to divide and enlarge, producing atheromas, which are benign (non-cancerous) tumors smooth muscle cells within the blood vessel walls. Together, the lipid-rich core and overlying smooth muscle form a maturing plaque.

6. The plaque progressively bulges into the lumen of the vessel as it continues to develop. The protruding plaque narrows the opening through which blood can flow.

7. Further contributing to vessel narrowing, the oxidized LDL inhibits the release of nitric oxide from the endothelial cells. The nitric oxide is a local chemical messenger that causes relaxation of the underlying layer of normal smooth-muscle cells within the vessel wall. The relaxation of these smooth muscle cells causes the vessel to dilate. Because of

reduced nitric oxide release, vessels damaged by developing plaques cannot dilate as readily as normal.

8. The thickening plaque also interferes with nutrient exchange for the cells located within the involved arterial wall, leading to degeneration of the wall in the vicinity of the plaque. The damaged area is invaded by fibroblasts (scar-forming cells), which form a connective tissue cap over the plaque.

9. In the later stages of the disease, Ca^{2+} often precipitates in the plaque. A vessel so afflicted becomes hard and poorly distensible.

3.2 Cancer

Many types of cancer have been originated from oxidative stress inducing a cellular redox imbalance. The DNA is a crucial target of free radical damage. The free radicals attack the single and double strand breaks including bases yielding products such as 8-hydroxyguanosine (8-OH-G) (fig 2), and thymine glycol. Moreover, the free radical also damage to deoxyribose sugar and DNA protein cross links. These damages affect the DNA mutation in somatic and germ cells. The free radicals associate with tumor suppressor genes and proto-oncogenes. (Halliwell B, 1993) The hydroxyl radical is known to react with all components of the DNA molecule damaging both the purine and pyrimidine base and also the deoxyribose backbone (Dizdaroglu et al., 2002). In addition the hydroxyl radical removes an H-atom from the methyl group of thymine and each of the five carbon atom of 2'deoxyribose. Consequently, damaged DNA leads to the fault of replication and transcription, and genomic instability, all of which are associated with carcinogenesis (Marnett, 2000).

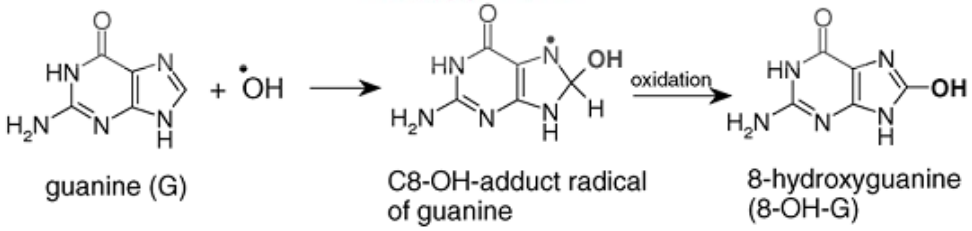

Fig. 2.2 Reaction of guanine with hydroxyl radical.

The carcinogenesis model is three stages as initiation, promotion, and progression. ROS can result to all these stages of carcinogenesis. First, the initiation stage relates a non-lethal mutation in DNA that produces the modified cell followed by at least one round of DNA synthesis to fix the damage (e.g. 8-OH-G) produced during the initiation.

The action of ROS, e.g. hydroxyl radicals including formed through the Fenton-type mechanism can result in DNA damage. Interestingly, the correlation between the tumor size and the amount of 8-OH-G adduct formation is exposed in several studies on benign tumors. Moreover, the transformation from benign to malignant tumors may be determined by the level of 8-OH-G (Loft S, 1996).

The characteristic of promotion stage is explained that the clonal expansion of initiated cells is induced by the cell proliferation and/or inhibition of programmed cell death. (Loft S, 1996) Many tumor promoters can strongly inhibit the cellular antioxidant defense systems such as SOD, catalase, and glutathione. On the other hand, the high oxidative stress level has the cytotoxic effect on the cells as well as stops proliferation by inducing apoptosis or even necrosis, In contrast, a low of oxidative stress can stimulate the cell division in the promotion stage and thus stimulate the promotion of tumour growth (Dreher and Junod, 1996).

Progression is the third and final stage of the carcinogenic process. This stage involves cellular and molecular changes that occur from the preneoplastic to the neoplastic state. This stage is irreversible and is characterized by accumulation of additional genetic damage, leading to the transition of the cell from benign to malignant. This stage is characterized by genetic instability and disruption of chromosome integrity (Valko et al., 2006). These three stages of model and mechanisms of carcinogenesis are shown Fig 3.

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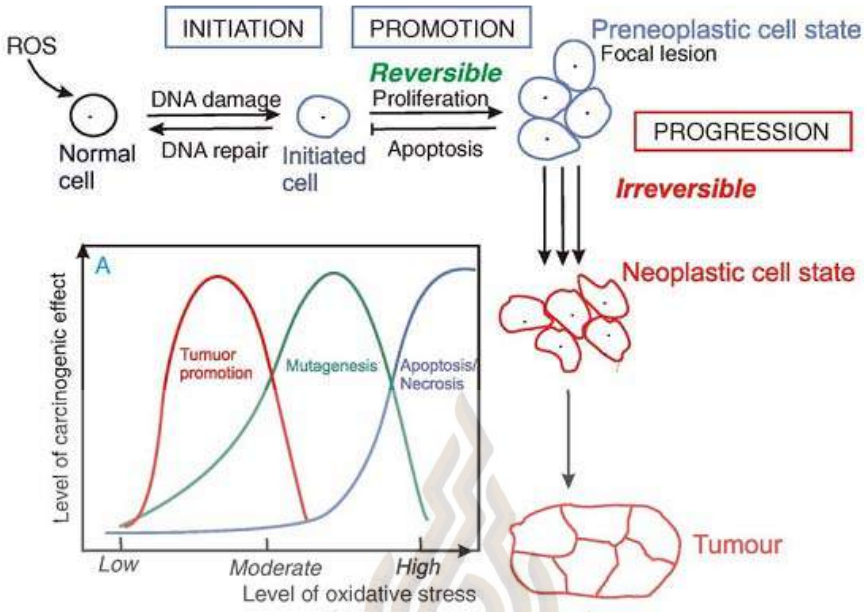

Fig 2.3 Three stages model of carcinogenesis and the level of carcinogenic effect vs. level of free radicals at various stages of carcinogenic process

3.3 Aging

Mitochondrial ROS production and oxidative damage to mitochondrial DNA lead to aging. Further increased lipid peroxidation in cellular membranes due to oxidative stress leads to fatty acid unsaturation. The most recent review on 'free radicals and aging' by Barja (2004) emphasizes that caloric restriction (CR) is the only known experimental manipulation that decreases rate of mammalian ageing, and it has many beneficial effects on the brains of rodents and possibly of humans. Calorie-restricted mitochondria, similar to those of long-lived animal species, avoid generation of ROS efficiently at complex I with pyruvate and malate. The mitochondrial oxygen consumption remains unchanged, but the free radical leak from electron transport chain is decreased in CR. Many investigators realized that increasing the level of defense mechanisms against oxidative stress could extend an organism's health span. Arking's research group's work on artificial selection in flies also produced organisms with a much higher level of oxidative stress resistance and more efficient mitochondria. In fact, the lower level of oxidative damage and delayed onset of senescence in those flies arose from decreased production and increased destruction of ROS. However, using genetic engineering techniques to

insert some extra copies of these oxidative stress-resistance genes into mice has not yet resulted in extending longevity (Devasagayam et al., 2004).

4. Plant and antioxidant

Antioxidant activity is an excellent example of a functional benefit that plant extracts can deliver. Plant is known to contain a variety of natural antioxidants that protect and preserve their physical and metabolic intergrity as well as their heredity by way of their seeds. Many of these extracts and compounds from plants are emerging as candidates for moderating the effects of the aging process on skin by limiting biochemical consequences of oxidation.

Compounds such as vitamin C, vitamin E and rosmarinic acid (RA) are commonly used in foods as well as cosmetics for their potent antioxidant activity that aids in product as well as for skin, and the consumer perception of antioxidants is positive one, making them particularly attractive as cosmetic ingredients. The danger is that the use of a single antioxidant is often positioned as a panacea. The phenomenon belies the scientific understanding that antioxidants work in synergy. The physiological codependence of water soluble vitamin C and lipophilic vitamin E is well accepted. Plant antioxidants differ not only in redox potential and solubility, but also in their mechanism of action. Some quench one or more ROS such as superoxide, hydroxyl radicals, or singlet oxygen. Others inhibit activity or expression of oxidative enzymes like catalase, or chelate oxidizing metal ion, or act by other mechanisms, known and unknown. Given the variety of chemical structures and biological mechanisms of antioxidants described from plants, it is not surprising that not all antioxidants confer the same degree of functional protection to the skin (Cindy K. A., 2008).

5. Assay methods used to estimate antioxidant potential

5.1. DPPH method

The 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging assay is the one of the most extensively used antioxidant assays for plant samples. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species. The antioxidant activity is then measured by the decrease in absorption at 515 nm. The DPPH radical has the violet

color in solution, and it becomes colorless or pale yellow when the sample can trap with the radical. A decrease in the absorbance of the reaction mixture indicates free radical scavenging activity of the compound (Krishnaiah et al., 2011).

5.2. Reducing power assay

The reducing power assay is modified according to the method described by Oyaizu (Oyaizu, 1986). This method is based on the increase of mixture absorbance. The increase in the antioxidant is indicated to the increase in the absorbance. Samples, having reduction potential, reduce potassium ferricyanide to form ferric ferrous complex which is measured at 700 nm. The increase absorbance indicates the increased reducing power.

Potassium ferricyanide + ferric chloride $\xrightarrow{\text{Antioxidant}}$ Potassium ferrocyanide + ferrous chloride

5.3. NBT assay or the superoxide anion scavenging activity assay

The superoxide anion scavenging activity assay is measured NBT (Niro blue tetrazolium) reduction. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT. The superoxide scavenging activity is expressed as percent inhibition compared to the blank, in which buffer is used in place of the sample (Halliwell et al., 1987).

5.4 Folin-Ciocalteu method

The Folin-Ciocalteu reagent assay is used to measure the total phenolic content. The sample is mixed with the Folin-Ciocalteu reagent and the sodium carbonate solution is added. The mixed solution is measured at the 725 nm of absorbance. Gallic acid is used as a standard for the calibration curve (Erkan et al., 2008).

5.5 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging of the sample is directly related with its antioxidant activity. The hydroxyl radical is generated by using Fe^{3+}/ascorbate/EDTA/H_2O_2 system via Fenton reaction. The pentose sugar 2-deoxyribose is used as reactant this method. Hydroxyl radical attacks the pentose sugar 2-deoxyribose to yield a mixture of products. These products are overheated with thiobarbituric acid at low pH to form a pink chromogen which can be measured by it absorbance at 532 nm (Gutteridge, 1982).

6. Some plants and their pharmacological effects

Luk Plak Mae (Thai name) are formulated from three plants, the formulation consists of *Aegle marmelos, Musa Eumusa* and *Piper nigrum.* This traditional herbal formulation has been used for an anti-aging in Thailand for long time ago.

6.1 Bael fruit (*Aegle marmelos* (L.) Correa) or Matoom Nim

Bael fruit or Matoom Nim (Thai name) is an attractive and characteristically sweet aroma fruit, known to be a good source of natural antioxidant and bioactive compounds. It is indigenous in India and used in folk medicines. Therefore, it was though worthwhile to evaluate antioxidant activity of *A. marmelos* fruit pulp to confirm its folk medicinal claim. Many naturally occurring products have been reported to contain large amount of antioxidant compounds other than vitamin C, E and carotenoid (Javanmardia J, 2003). These antioxidants play a vital role in delaying, intercepting or preventing oxidative reactions catalyzed by free radicals (Vilioglu YS, 1998). Antioxidant activity of medicinal plants might be due to the presence of phenolic compounds such as flavonoids (Pietta P, 2010) (Cook NC, 1996), phenolic acids and phenolic diterpine (Sha hidi F, 1992). Synthetic antioxidants like butylated hydroxy anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroxy quinone and gallic acid esters have been suspected to be carcinogenic. Hence, there is a trend to replace them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity (Badam L, 2002). So, search for natural antioxidant has greatly been increase in the recent scenario. In the present investigation, antioxidant activity of alcoholic and aqueous extracts of *A. marmelos* fruit pulp was assessed.

Suvimol and Pranee (2008) reported that fully ripe Thai bael fruit pulps had total, soluble, and insoluble dietary fiber contents of 19.84, 11.22, and 8.62 g/100g dry weight (dw), respectively (Suvimol C, 2008). Determination of antioxidant activities by 2-diphenyl-1-picryhydrazyl (DPPH) free radicals scavenging and ferric reducing antioxidant power (FRAP) assays resulted in 6.21 µg dw/ µg DPPH and 102.74 µM trolox equivalent (TE)/ g dw, respectively. It was also found to have total phenolic, total flavonoid, total carotenoid, and ascorbic acid contents of 87.34 mg gallic acid equivalent (GAE)/g dw, 15.20 mg catechin equivalent (CE)/g dw, 32.98 µg /g dw, and 26.17 mg/ 100 g dw, respectively. Volatile compounds in bael fruit pulp were analyzed using the solid- phase microextraction / gas chromatography, mass spectrometry method. A total of

28 volatile compounds were identified, and the dominant components were monoterpenes and sesquiterpenes. Among these components, limonene was the major constituent producing the characteristic bale fruit flavor.

The bioactive compounds isolated from bael fruits are marmelosin, luvangetin, aurapten, psoralen, marmelide and tannin (Badam L, 2002). Marmelosin has shown antihelmintic as well as antibacterial activitie (Ghosh S, 2003), (Lamba B, 1969), (Shoba FG, 2001). Luvangetin a pyranocoumarin isolated from the seeds of bael fruit protects against gastric ulceration in rodent models (Goel RK, 1997), (Ghosh S, 2003). Aurapten inhibits (IC_{50}=0.6 µg/ml) the chronotrophic effects on cardiac tissue and thus may be useful in treatment of hypertension. Psoralen shows various activities such as antispasmodic activity, artemicide inhibition (LD_{50}=5.93 µg/ml) and cytotoxicity (Saqib QN, 1990). Marmelide is very effective against viruses and is found to influence the early stages of replicative cycle such as absorption, penetration, etc. (Badam L, 2002). Tannin, present in the unripe fruit of this plant, has astringent property and is an excellent remedy for diarrhea (Shoba FG, 2001).

Oxidative stress is produced during normal metabolic processes in the body as well as induced by a variety of environmental and chemical factors, which causes generation of various reactive free radicals and subsequent damage to macromolecules like DNA, proteins and lipids. In artificially induced diabetic animals, the levels of lipid peroxidation, hydroperoxide, conjugated diene, thiobarbituric acid reactive substances, creatine kinase and lactate dehydrogenase increased considerably, and then decreased after treatment with the various extracts of bael leaves and fruits (Kamalakkannan N 2003), (Sabu MC, 2004), (Kamalakkannan N, 2003), Rajadurai N, 2005). On the other hand, antioxidative parameters such as reduced glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase have shown a dose-related increase in their activity and a decrease in lipid peroxidation following the treatment with bael leaf extract (Singh RP, 2000), (Sabu MC, 2004), (Rajadurai N, 2005). The fruit extract at a dose of 250 mg/kg body weight is more effective than glibenclamide (300 µg/kg) (Kamalakkannan N, 2003). Leaf extract (200 mg/kg) is as effective as alpha-tocopherol (60 mg/kg) in isoproterenol (ISO)– treated rats (Rajadurai M, 2005). The antioxidative phytochemicals such as flavonoids, alkaloids, sterols, tannins, phlobatannins and flavonoid glycosides present in the leaf extract posses this free radicals scavenging

activity (Maridonneau-Prini I, 1986), (Uddin S, 1995), (Korkina LG, 1997), (Kar A, 2002), (Jagetia GC, 2003), (Rajadurai M, 2005).

Glutathione is reduced in erythrocyte whereas plasma glutathione-S-transferase and malondialdehyde are increased in male albino rats with diabetes. However, these alterations returned to normal level with bael leaf extract administration, indicating antioxidant potential of bael leaves (Upadhya S, 2004). Eugenol and marmesinin may be responsible for such activity because these compounds have shown their activity against oxidative stress (Vidhya N, 1999), (Ogata M, 2000), (Jagetia GC, 2003), (Vimal V, 2004).

Bacteria are the most versatile unicellular pathogens, which are normally transmitted through soil, water, air, and food and cause diseases in human and animals. Such types of diseases could be treated with various natural products including bael. Various extracts of bael leaves, roots and fruits have been reported to be active against many bacterial strains. Many *in vitro* studies proved the antibacterial potential of *A. marmelos* extracts towards the pathogenic bacteria. Leaf extracts have shown activity against *Escherichia coli* (George, 1974), (Joshi, 1952). The ethanolic extract of the root has shown activity against *Vibrio cholerae*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* (Pitre, 1987), (Valsaraj et al., 1997). The ethyl acetate of the plant has exhibited activity against *V. cholerae*, *S. typhimurium*, *S. aureus*, *Pseudomonas putida* and *Bacillus anthracis* (Rusia K, 1988). The antibacterial activity of the fruit of *A. marmelos* was reported. The fruit showed activity against *S. aureus* and the MIC result was 1.25 mg/ml (Hamid Reza Gheisari, 2011). Maheshwari et al. (2009) studied on ethanolic extract of dried fruit pulp of *A. marmelos* against various intestinal pathogens i.e. *Shigella boydii, S. sonnei* and *S. flexneri* and proposed that certain phytochemicals including phenols, tannins and flavonoids were effective against all (Joshi, 2009). It was confirmed by Kaur et al. (2009) by getting treat *E. coli* with *A. marmelos* fruit extract (Prabhjit Kaur, 2009). The antibacterial activity of the leaves, fruits and barks of *A. marmelos* was reported. The antibacterial activity of chloroform, methanol and water was performed by disc diffusion method. The antibacterial activity was checked against *B. subtilis, S. aureus*, *K. pneumoniae*, *Proteus mirabilis*, *E. coli*, *Salmonella paratyphi* A and *Salmonella paratyphi* B. The methanol extract showed significantly high activity against above mentioned bacteria than that of the other extracts (Poonkothai M Fau - Saravanan and

Saravanan, 2008). Methanolic and aqueous extract of bael fruit have shown strong action against multi resistant *Salmonella typhi*. The methanolic extract is more potent than the aqueous extract. The minimum inhibitory concentration (MIC) value of methanolic extract is about 256 µg/ml. Thus it is evident that bael fruit has antibacterial activity and the mechanism of action may be the blockade of protein synthesis either at transcription or at translation level and/or peptide-glycan synthesis at membrane level (Perumal Samy et al., 1998), (Rajan S, 2009). Pandey and Mishra (2011) reported that the antibacterial activity was found to best in fruit of the *A. marmelos* against bacterial pathogens compare to all peels and leaves. The ethanolic and ethyl acetate extracts of *A. marmelos* fruit were showing best result while the methanolic and hot water extract were showing minimum inhibition. The antibacterial compounds mainly found in *A. marmelos* were tannins, phlobatannins, saponins, terpenoids, alkaloids and poly phenols (Mishra, 2011).

6.2 Banana (*Musa Eumusa* AA) or Kluai Nam Thai

Banana is the fourth most crucial crop in developing countries, with a worldwide production of about 100 metric tons (Heslop-Harrison and Schwarzacher, 2007). Banana contain various nutrients such as protein, fat, dietary fiber, carbohydrates, calcium, phosphorous, iron magnesium, sodium, potassium, zinc, carotene, vitamin C and vitamin B6. It has a lot of benefits to health such as a caloric dense fruit including an extreme weight loss, a good source of potassium and magnesium which help in balancing normal blood pressure and heart protection, a antacid effect, a cell proliferative effect of stomach mucasa, a source of carotenoid and a soothing effect. (Health Benefits of Banana/Medindia). In addition, Banana also contains dietary fiber that resist hydrolysis by small bowel digestive enzymes. Banana has been proven to be advantage in maintaining remission in human ulcerative colitis, and this protective effect has been related to an increase in the luminal production of short chain fatty acids which are indicated to be an crucial factor in the healthy function of colorectal mucusa (Galvez et al., 2005). Resistant starch is found in banana (*Musa* sp AAA) as 47.3% to 54.2% of the total amount of starch (14%). It is nondigestible polysaccharide used as a dietary fiber that is resistant to digestion in the small intestine and used by colonic microbiota for the anaerobic fermentation production of short-chain fatty acids (DA Mota et al., 2000).

6.3 Black pepper (*Piper nigrum*) or Pik Thai Dam

Black pepper (*Piper nigrum*) is member of family Piperaceae. It is able to be used for different ways such as human dietaries, medicine, preservatives, and biocontrol agents (Hussain, 2011). Black pepper has pharmacological properties that piperine is an active compound. Black pepper helps the digestive process by increasing the production of saliva and gastric secretions (Srinivasan, 2007). The piperine, piperamide, piperamines, and pipene which are active compounds, stimulate the enzyme activation of pancreas, liver and the terminal digestive enzyme of small intestinal mucosa (P Tiwari, 2008). Several studies reported that this plant has the antidiarrhoeal effect. Peppercorn is produced be the anti-diarrhea formula for all ages in the developing counties. The plant reduces the fluid secretion and accumulation by controlling via capsaicin-sensitive neurons (Singh A., 2009). Additionally, antimutagenic and antitumor activity of *P. nigrum* has reported. The active derivatives especially peppercorn extract of *P. nigrum* can inhibit tumor formation in experimental models (Lin Z Fau - Liao et al., 2007). The immunomodulatory, antitumor activity and Dalton's lymphoma have been found efficaciously by the alcoholic extract of peppercorn and piperine (Sunila and Kuttan, 2004). In experimental mice, Piperine is able to reduce the depressant activity and chronic mild stress procedure (Li M Fau - Liu and Liu, 2008), (Xinpeng B, 2011). Furthermore, piperine regulated up the level of brain-derived neurotrophic factor in hippocampus of chronic stressed mice (Singh A., 2009). The piperine acts as the anti-depression and increases the cognitive effect during entire treatment duration in Wister male rats (Wattanathorn et al., 2008). For anti inflammatory activity of *P. nigrum*, piperine inhibits adhesion of endothelial monolayer to neutrophils and the tumor necrosis factor-α induced expression of cell adhesion molecules. Also, piperine strongly decreases the pro-inflammatory cytokines such as GM-CSF, IL6, TNF- α, and IL1 β (Singh A., 2009). When administrated intravenously in normotensive anesthetized rats, piperine reduces the in arterial pressure in dose-dependent manner (Taqvi S I H, 2008). Effect of piperine on rabbit heart, it also decreases the force, the contraction of tissues, and blood flow in coronary vessels in *in vitro* model (Singh A., 2009). In study of *P. nigrum* on hepatoprotective activity, *P. nigrum* prevents the increase of serum GPT and GOT levels in D-galactosamine-induced mice which become to the liver-toxicity mice. *P. nigrum* also possess hepatoprotective effects in experimental animal's models and in humans (Matsuda et al., 2008). Also, Piperine protects the asthma against the production of

histamine, interleukin-5, immunoglobulin E, and interleukin-4 by decreasing the infiltration of eosinophil, hyper responsiveness and inflammation in mice (Kim SH 2009). In antioxidant activity of *P. nigrum*, it is reported that the oxidative stress is inhibited in rats by monitoring the membrane lipid peroxidation, enzymic, and non-enzymic antioxidants. Moreover, *P. nigrum* inhibits the lipid peroxidation and the intestine induced oxidative stress, attacks the various radicals such as hydroxyl and super oxide radicals. It also reduces the lung carcinogenesis (Neha J 2001), (Vijayakumar R.S., 2004), (Naseri MKG, 2007), (Muhtaseb MS, 2008).

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CHAPTER III

METHODS

1. Chemicals

1. The herbal crudes of *Aegle marmelos*, *Musa eumusa,* and *Piper nigrum*
2. Distilled water
3. Sodium carbonate (Na_2CO_3)
4. Folin-Ciocalteau reagent
5. Potassium ferricyanide
6. Trichloroacetic acid (TCA)
7. Thiobarbiburic acid (TBA)
8. Ferric chloride ($FeCl_3$)
9. Ascorbic acid
10. Deoxyribose
11. Ethylene diamine tetraacetic acid (EDTA)
12. Hydrogen peroxide (H_2O_2)
13. Phosphate buffer solution
14. Sodium hydroxide (NaOH)
15. 1,1-Diphenyl-2-picrylhydrazyl (DPPH)

2. Materials and equipments

1. Parafilm
2. Aluminum foil
3. Rubber bulb
4. Test tubes
5. Stopwatch
6. Beaker
7. Dropper
8. Eppendorf Repeater Plus Pipette
9. Microcentrifuge tube
10. Micropipette
11. Rack

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12. Stirring rod
13. Volumetric flask
14. Spectrophotometer
15. Vortex mixer
16. Centrifuge
17. Whatman's filter paper no.1
18. tryptic soy broth
19. Mueller-Hinton agar

3. Methods

3.1 Preparation of the extracts

The powderd plant crudes were given kindly from Ms. Chatwanich Puangmalai, managing director of Greenchat Natural Herb and Ms. Nantawan Puangmlai, managing director of Innovative Pharma Herbs co.,Ltd. Eighty grams of powdered crudes were extracted with distilled water for 2 h at 60°C. The extract was filtered with thin cloth and Whatman no 4, 1, and 5. The extracts were stored in a dry cool place and then were carried out by freeze drying at Department of Zoology, Faculty of Sciences, Kasetsart University.

3.2 Total phenolic content (Erkan et al., 2008)

1. Prepared 1,000 µl of the gallic acid solution at the concentrations of 2,4,6,8, and 10 µg/ml for standard curve
2. Prepared 500 µl of the extracts in distilled water at the concentrations of 1,000 mg/ml for *Aegle marmelos*, *Musa eumusa,* and *Piper nigrum*
3. Mixed the extracts at different concentration with the Folin's reagent 2,500 µl for 2 min by vortex mixer
4. The mixtures were added with 2,000 µl of 75 mg/ml Na_2CO_3 solution, and mix for 2 min by vortex mixer
5. Keep the mixture at 50°C for 5 min
6. Measure the absorbance at 725 nm
7. Determined the samples in gallic acid equivalents (GAE).

3.3 DPPH radical-scavenging assay (Badam L, 2002)

1. Prepared the extracts in distilled water at the concentrations of 25, 50, 100, 200, and 400µg/ml

2. Mixed the 10 µl solutions of the extracts at different concentration with 190 µl of 100 µM DPPH in ethanol (start conc.).

3. Shaked the mixtures vigorously and allowed to stand at room temperature in the dark for 25 min

4. Measured the absorbance at 518 nm against each blank and positive control was L-Ascorbic acid.

6. Calculated DPPH radical scavenging activity using the equation:

% scavenging activity = $100 - [(Abs_{sample} - Abs_{blank})/ Abs_{control} \times 100]$

3. 4 Fe^{3+} reducing power assay (Oyaizu, 1986)

1. Prepared 100 µl of the extracts in distilled water at the concentrations of 25, 50, 100, 200, and 400 µg/ml and mixed the solutions of the extracts at different concentration with 2,500 µl of 1% potassium ferricyanide for 30 second by vortex mixer

2. Kept the mixture at 50 °C in water bath for 20 min

3. After cooling, added 2,500 µl of 10% TCA to the mixtures and mixed for 2 min by vortex mixer and centrifuged at 3,100 rpm for 10 min

4. Pipetted 500 µl of the upper layer to a new test tube

5. Mixed the upper layer of solution with 500 µl of 0.1% ferric chloride solution for 2 min by vortex mixer

5. Measured the absorbance at 700 nm and the results was expressed at the value of absorbance

3.5 Superoxide radical-scavenging assay (Ewing and Janero, 1995)

1. Added 60 µl of 43 µM nitro blue tetrazolium (NBT), 60 µl of 2.7 µM phenazine methosulfate , 60 µl of 166 µM NADH and 20 µl of samples in 96 well plate

2. Incubated the mixtures at room temperature

3. Measured the absorbance at 560 nm

4. Identical tubes containing reaction mixture were kept in the dark and served as blanks

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5. The percentage inhibition of superoxide generation was estimated by compared the absorbance of the control and those of the reaction mixture containing test sample as per the equation:

$$\% \text{ Inhibition} = 100\% \times [(A_0 - A_s)/A_0]$$

A_0 = Absorbance of the control

A_s = Absorbance of the tested sample

3.6 Hydroxyl radical ($^{\cdot}$OH)-scavenging assay (Halliwell et al., 1987)

1. Prepared 500 µl of the extracts in distilled water
2. Mixed the solutions of the extracts at different concentration with 200 µl of 12.5 µM 2-Deoxyribose
3. Added 200 µl of 500 µM Ferric chloride to the mixtures and mixed
4. Added 20 µl of 50,000 µM Hydrogen peroxide to the mixtures and mixed
5. Added 380 µl of 268 µM Ascorbic acid to the mixtures and mixed
6. Incubated the mixtures for 1 h at 37 °C
7. Added 1.0 ml of 2.8% trichloroacetic acid (TCA) and 1.0 ml of 1% thiobarbituric acid (TBA) in 0.05 M NaOH, and mixed well
8. Incubated the mixtures for 20 min at 100 °C
9. After cooling on ice, measure the absorbance at 532 nm
10. Calculated the inhibition of 2-Deoxyribose degradation using the following equation:

$$\% \text{ Inhibition} = [A_0 - (A_1 - A_2)] \times 100/A_0$$

A_0 = Absorbance of the control

A_1 = Absorbance of the system which had the extract and 2-Deoxyribose

A_2 = Absorbance of system which had only the extract

3.7 **Antimicrobial activity**

Bacterial isolates used in this study are *Escherichia coli* TISTR 887 and *Staphylococcus aureus* TISTR 517 received from TISTR (Thailand Institute of Scientific and Technological Research) Culture Collection.

Escherichia coli is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some

serotypes can cause serious food poisoning in humans. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K and by preventing the establishment of pathogenic bacteria within the intestine.

Staphylococcus aureus is facultative anaerobic gram-positive cocci which occur singly, in pairs, and irregulular clusters. *S. aureus* is nonmotile, non-spore forming, catalase and coagulase positive. Typical colonies are yellow to golden yellow in color, smooth, entire, slightly raised, and hemolytic on 5% sheep blood agar.

The antimicrobial sensitivity pattern for the extract was studied by disc diffusion method. Sterile discs (6 mm) prepared from Whatman's filter paper no.1 were made to absorb of the test samples. The bacterial cultures were first grown in a tryptic soy broth (TSB) for 18 h before use and standardized to 0.5 Mc Farland standards ($1.5x10^8$ cfu/ml). Mueller-Hinton agar was prepared on the plates as the medium for the test organism. The microbial inoculum was spread onto the surface of agar plate using the sterile cotton bud and then the extract discs, sterile water impregnated discs were positioned on the inoculums agar surface. The antimicrobial activity was interpreted from the size of diameter of zone of inhibition measured to the nearest mm as observed from clear zone surrounding the disc. The extract was assayed in triplicate and the mean of the three values was taken.

3.8 Statistical analysis

Values were expressed as the mean ± S.E.M of three or more separated experiments. One-way analysis of variance (ANOVA) was expressed statistical difference and followed by Tukey's multiple comparisons. Values of $P<0.05$ were considered significant. Statistical analyses were performed using SPSS 15 version software.

CHAPTER IV

RESULTS

1. The antibacterial activity

The water extract of bael fruit (50 mg/ml) has shown little activity against only *E. coli* with diameter of clear zone 8 mm. In the same assay, the concentration of the extract (500 µg/ml) using in antioxidant test did not show antibacterial activity against both in *E. coli*. and *S. aureus*.

2. The amount of phenolic compounds

The content of phenolic compounds (mg acid/g) in plant extracts expressed in gallic acid equivatents (GAE) The highest amounts were found in water extracts of Piper nigrum (0.294 ± 0.0012 mg/g), *Aegle marmelos* (0.033 ± 0.0002 mg/g), and *Musa eumusa* (0.018 ± 0.0014 mg/g) respectively. The result was shown in Table 4.1.

Table 4.1 The amount of phenolic compounds in plant extracts

Extracts	Total phenolic compounds
Musa eumusa	0.018 ± 0.0014
Piper nigrum	0.294 ± 0.0012
Aegle marmelos	0.033 ± 0.0002

mg/g plant extract in gallic acid equivalent was expressed as mean ± SEM. The experiment was carried out in triplicate.

3. DPPH radical-scavenging assay

The water extract of *Aegle marmelos* exhibited a significant dose-dependent inhibition of DPPH radical scavenging activity, with 50% inhibition at a concentration of 178 µg/ml. When compared the DPPH radical scavenging activity *of Musa eumusa* and *Piper nigrum*, the scavenging activity of *Aegle marmelos* is highest significantly at all concentrations (P<0.05). The experiments were carried out as five experiments (Fig. 4.1).

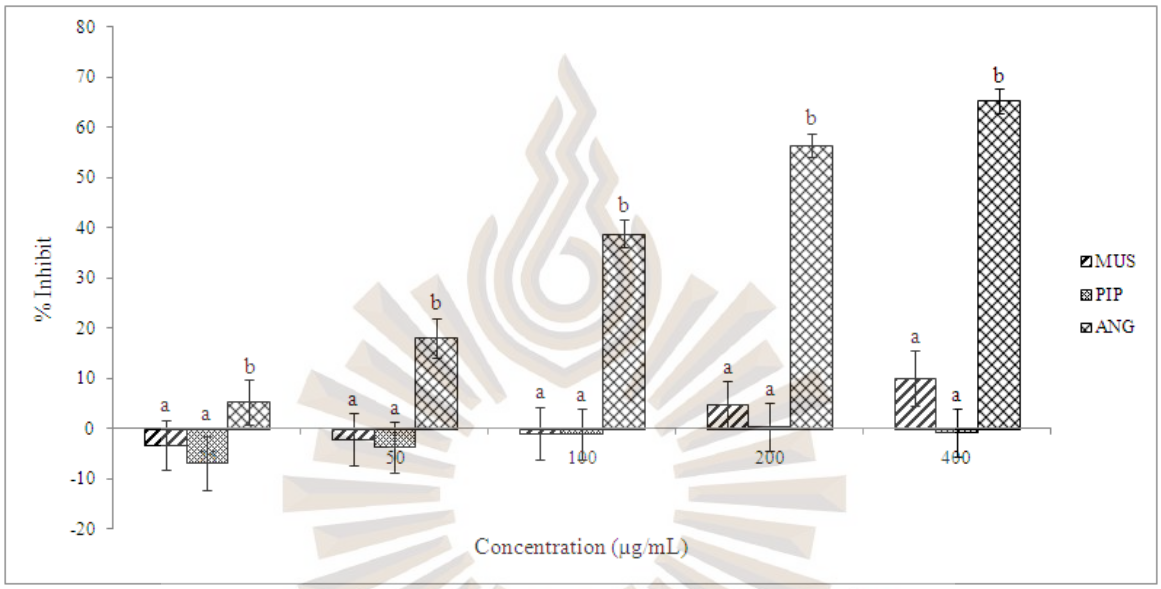

Fig. 4.1 Radical scavenging activity in the DPPH assay

a, b Significant at p<0.05 level when compare in each concentrations

MUS *Musa eumusa*
ANG *Aegle marmelos*
PIP *Piper nigrum*

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4. Fe^{3+} reducing power assay

Fig. 4.2 showed the reductive ability of the plant extracts. This indicated that, water extracts of *Musa Eumusa*, *Aegle marmelos* and *Piper nigrum* at 400 µg/ml could reduce the most Fe^{3+} to Fe^{2+} ions significantly when compared with solvent control ($p<0.05$). When compared among the three extracts at 400 µg/ml, the increased the absorbance of *Piper nigrum* extract was the highest significantly ($p<0.05$).

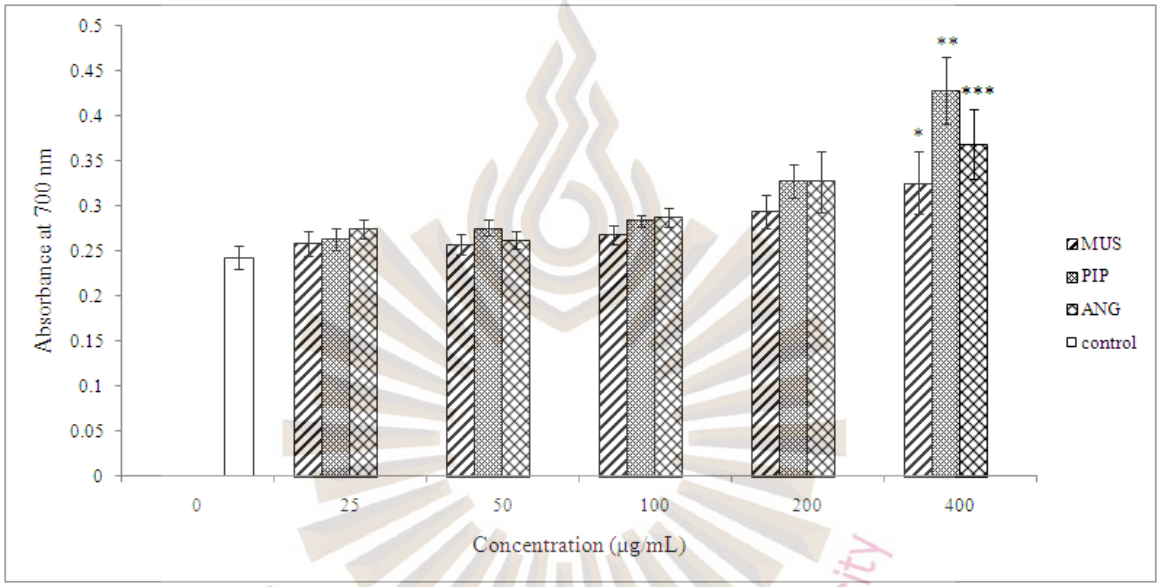

Fig. 4.2 Reducing power assay

Results were mean of five parallel OD measurements.

*, **, *** Significant at $p<0.05$ level when compared control

MUS *Musa eumusa*
ANG *Aegle marmelos*
PIP *Piper nigrum*

5. Superoxide radical-scavenging assay

The superoxide anion derived from dissolve oxygen by phenazine methosulphate/NADH coupling reaction reduces nitro blue tetrazolium. The decrease in the absorbance at 560 nm with the plant extract thus indicates the consumption of superoxide anion in the reaction mixture. As shown as Fig 4.4, the water extract of *Piper nigrum* possessed the scavenging activity of 50% inhibition at 1,330 µg/ml. The *Piper nigrum* extract was a dose-dependent increase in inhibition. At 400 µg/ml of the extracts, *Piper nigrum* extract was able to scavenge superoxide highest of all significantly ($p<0.05$).

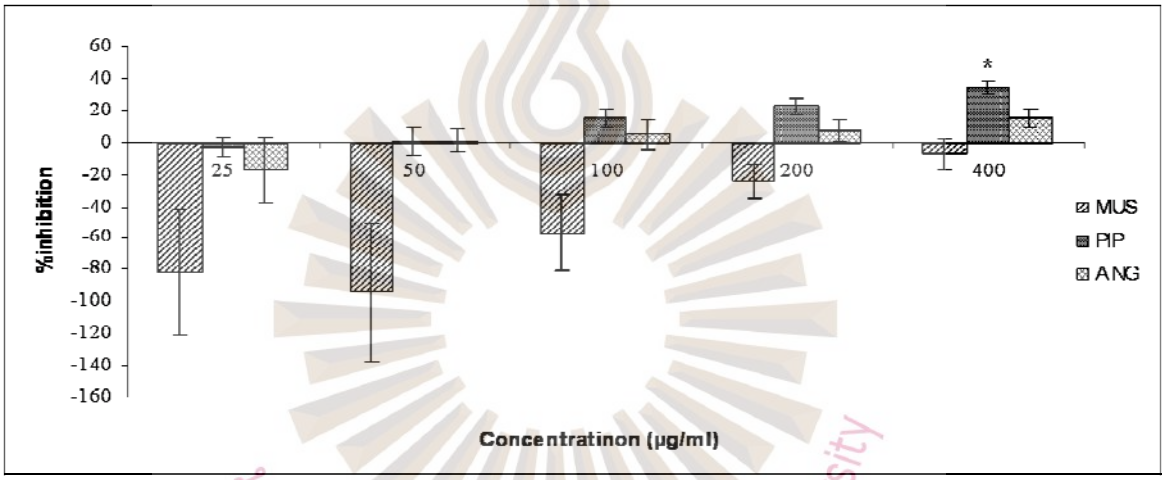

Fig. 4.3 Superoxide radical-scavenging assay

Results were mean of five independent experiments.

*, Significant at $p<0.05$ level

MUS *Musa eumusa*
ANG *Aegle marmelos*
PIP *Piper nigrum*

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6. Hydroxyl radical (·OH)-scavenging assay

To attack the substrate deoxyribose, hydroxyl radicals were generated by the reaction of Fe^{3+} -EDTA together with H_2O_2 and ascorbic acid. When the *Piper nigrum* extract was incubated with the above reaction mixture, it could protect the damage against the sugar. The result was expressed in Fig 4.4, the extract acted as the OH^- scavenger in dose-dependent manner. When compared at 400 µg/ml of all extracts, the inhibiting activity of *Piper nigrum* extract was highest significantly ($p<0.05$).

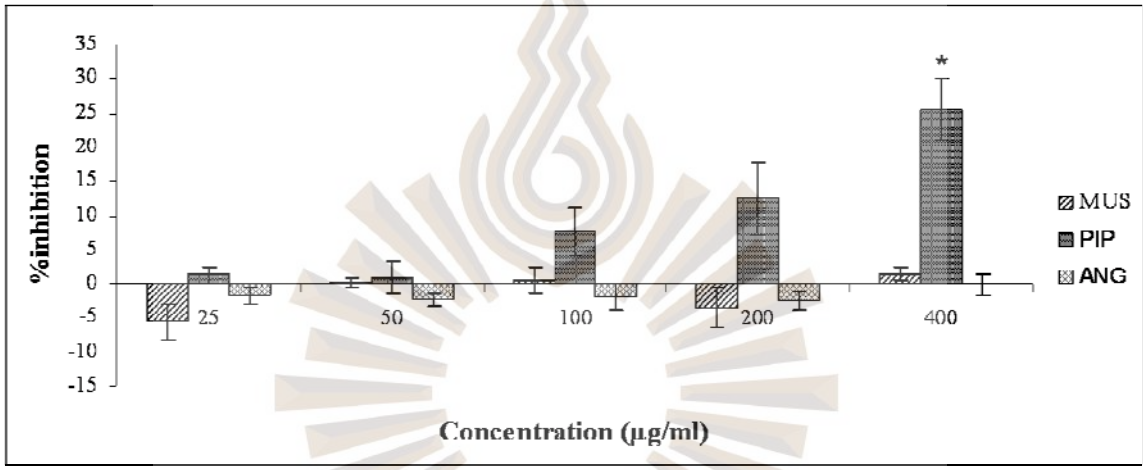

Fig. 4.4 Hydroxyl radical (·OH)-scavenging assay

Results were mean of five independent experiments.

*, Significant at $p<0.05$ level

MUS *Musa eumusa*
ANG *Aegle marmelos*
PIP *Piper nigrum*

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CHAPTER V

DISCUSSION AND CONCLUSION

Reactive Oxygen Species (ROS) is free radical such as the hydroxyl radical, the super oxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and lipid eposides. These radicals attack the macro-molecules including membrane lipids, nucleic acids, proteins and enzymes and other small molecules, resulting in cellular damage (Shivaprasad H.N., 2005). There is increasing evidence indicating that ROS and free radical mediated reactions are involved in degenerative of pathological events such aging, cancer, coronary heart diseases, and Alzheimer's disease (Sun et al., 2004). Antioxidant activity is an excellent example of a functional benefit that plant extracts can deliver. Plant is known to contain a variety of natural antioxidants that protect and preserve their physical and metabolic intergrity as well as their heredity by way of their seeds. Many of these extracts and compounds from plants are emerging as candidates for moderating the effects of the aging process on skin by limiting biochemical consequences of oxidation.

For the antibacterial activity, this study showed that the water extract of *A. marmelos* was able to inhibit slightly the growth of *Escherichia coli* but not for *Staphylococcus aureus*. Previous study showed that the ethanolic and ethyl acetate extracts of *A. marmelos* fruit were showing best result while the methanolic and hot water extract were showing minimum inhibition. The antibacterial compounds mainly found in *A. marmelos* were tannins, phlobatannins, saponins, terpenoids, alkaloids and poly phenols (Mishra, 2011). The methanolic extract is more potent than the aqueous extract. The minimum inhibitory concentration (MIC) value of methanolic extract is about 256 µg/ml. Thus it is evident that bael fruit has antibacterial activity and the mechanism of action may be the blockade of protein synthesis either at transcription or at translation level and/or peptide-glycan synthesis at membrane level (Perumal Samy et al., 1998), (Rajan S, 2009).

For the antioxidant activity, there are some reports concerning the presence of phenolic compound in *A. marmelos*. Also, flavonoid are found in this plant (Suvimol C, 2008). This result found that phenolic compound was measured in the water extract of *A. marmelos*. In DPPH assay, DPPH is a stable free radical that reacts with compounds that

can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species. The antioxidant activity is then measured by the decrease in absorption at 515 nm. In this experiment, *A. marmelos* could scavenge the DPPH radical highly when compared with others. Abullakasim (2007) reported that the beal fruit drink possess high level of phenolic compound and was also a good antioxidant in DPPH assay (Abdullakasim P, 2007). Moreover, the hydroalcoholic extract of bael pulp can scavenge the nitric oxide *in vitro* (Jagetia GC, 2004). Rajan (2011) reported that the water extract of bael fruits in India found the amount of phenolic compounds as 147.66 mg/g and IC50 of DPPH anion scavenging power with the value of 93 µg/ml (Rajan S, 2010). Although, our result for the phenolic compounds in bael fruits were expressed only 0.033 mg/g but the IC50 for DPPH scavenging power was calculated as the value of 178 µg/ml. Not only the flavonoids were presented the bael fruit but also the caroteniods, coumarins (marmin, aurapten, psoralen, luvangetin, umbeliferone) and marmelosin were founded (Baliga et al., 2011). Previously reported literature indicated in umbelliferone and psoralen was found in the methanol extract of bael fruit pulp that these active compounds possessed the antioxidant activity. The extract was found to be a scavenger of DPPH radical and superoxide anion with IC50 values of 1.015 and 1.102 mg/ml (Dhalwal IK., 2008).

Reducing power is related with antioxidant activity. The reducing power of compounds indicates they can donate the electron and reduce the oxidized intermediates of lipid peroxidation process. In this assay, the reducing power of each compounds are associated with the change color of solution from yellow to green and blue. As presented with reducer, Fe^{3+}/ferricyanide complex is converted to the ferrous form by measuring at 700 nm. This result showed that water extracts of *M. eumusa*, *A.marmelos* and *P. nigrum* possessed the reducing power. Previously studying have reported the antioxidant activity of banana (*Musa* sp AAA), showed by decrease in lipid peroxides and an increase in GSH level in the rat liver (Kaimal S Fau - Sujatha et al.). In addition, the antioxidant activity of flavonoids from banana (*Musa paradisiaca*) could decrease the concentrations peroxidation products namely malondialdehyde, hydroperoxides and conjugated dienes in rats fed normal as well as high fat diets and also increase catalase and superoxide dismutase. Level of glutathione were also enhanced in the treated animals (Vijayakumar et al., 2008). Rajan S. (2010) reported that the aqueous extract of *A. marmelos* fruit pulp

showed the potent ferric reducing power. The increased concentration of extract led to increase the reducing power (Rajan S, 2010).

This study reported that the water extract of *P. nigrum* possessed the antioxidant activity in several assays. The phenolic content was presented a value of 0.294 mg/g. Another study could detect the total phenlic conent in the water extract of *P. nigrum* at a level of 54.3 mg/g by determining Folin-Ciocalteu procedure (Gulcin, 2005). Whereas, the extract of *P. nigrum* in this study showed the low level of phenolic content but the extract exhibited the various antioxidant activities. In reducing power assay, *P. nigrum* demonstrated the potent of reducing power. Gulcin (2005) studied that water crude extracts from black pepper (*Piper nigrum*) were presented for reducing power activity (Gulcin, 2005). This study reported the effect of the *P. nigrum* extract on superoxide scavenging that the extract was able to scavenge superoxide. Also, the *P. nigrum* extract protected the degradation of deoxyribose against the hydroxyl radicals. Many studies of antioxidant activity of *P. nigrum*, it is reported that the oxidative stress is inhibited in rats by monitoring the membrane lipid peroxidation, enzymic, and non-enzymic antioxidants. Moreover, *P. nigrum* inhibits the lipid peroxidation and the intestine induced oxidative stress, attacks the various radicals such as hydroxyl and super oxide radicals. It also reduces the lung carcinogenesis (Neha J 2001), (Vijayakumar R.S., 2004), (Naseri MKG, 2007), (Muhtaseb MS, 2008). In high-fat treated rat, piperine, an active compound in black pepper (*P. nigrum*), reduced the elevated levels of thiobarbituric acid reactive substances and conjugated dieneds contrastly, maintained superoxide dismutase, glutathione, glutathione peroxidase, glutathione-S-transferase and glutnthione (Vijayakumar R.S., 2004).

Based on the result in the study, it was concluded that water extracts from *M. eumusa*, *A.marmelos* and *P. nigrum* were explored to be a good source of antioxidant. These plants used in Thai herbal formulation or *Luk Plak Mae* (Thai name). On the other hand, the water extract of *A.marmelos* did not show the antibacterial activity in this study. Further studies are required to identify specific active compounds of these plants for the significant antioxidant effect.

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Antioxidant

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Nusuetrong, P., **Suwannasual, U**., and Meksuriyen, D. 2010. *Spermidine-Induced Apoptosis via Reactive Oxygen Species Generation and Caspase 3 Activation in Mouse P19 Embryonal Carcinoma Cells.* **International Journal of Pharmacology**. 6 : 903-909.

PRESENTATIONS

2009 **Suwannasual, U**., Pengsuparp, T., and Leuchapudiporn, R., *Protective effect of deferiprone on hemin-induced oxidation of low and high density lipoprotein*s presented in Graduate Research Conference, 23-24 April 2009, King Mongkut's Institute of Technology Ladkrabang, Thailand (oral presentation and proceeding)

2008 **Suwannasual, U.**, Pengsuparp, T., and Leuchapudiporn, R., *Protective effect of deferiprone on hemin-induced oxidation of low and high density lipoproteins* presented in the TRF-Master Research Congress II, 4-6 April 2008, Chonburi, Thailand (oral presentation)

2004 **Suwannasual, U.**, Kovivadhi, S., and Kovivadhi, U., *Using of freshwater mussel, Hyriopsis (Limnoscapha) myersina and giant hygrophilla, Hydrilla verticillata for removal of turbidity from fish pound*, Presented in Industrial and Research Projects for Undergraduate Students exhibition II, 30 Apirl-2 May 2004, Bangkok, Thailand (Poster presentation)

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PUBLICATION

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