ANTIOXIDANT AND ANTI-MELANOGENESIS PROPERTIES OF GUAVA (*Psidium guajava* L.) EXTRACTS *in vitro*

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สมบัติการต้านอนุมูลอิสระและการต้านการสร้างเม็ดสีผิวของสารสกัดจากฝรั่งแบบ *in vitro*

นาย ธนากรณ์ ดำสุด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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Field of Study	Biochemistry
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ธนากรณ์ ดำสุด : สมบัติการต้านอนุมูลอิสระและการต้านการสร้างเม็ดสีผิวของสารสกัดจากฝรั่งแบบ *in vitro*. (ANIOXIDANT AND ANTI-MELANOGENESIS PROPERTIES OF GUAVA (*Psidium guajava* L.) EXTRACTS *in vitro*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. นภา ศิวรังสรรค์ 115 หน้า

ฝรั่ง (*Psidium guajava* L.) เป็นผลไม้เมืองร้อนที่อุดมไปด้วยคุณค่าทางโภชนาการ มีวิตามินซี สารประกอบฟีนอล เพกทิน และใยอาหารที่ไม่สามารถละลายน้ำในปริมาณที่สูง งานวิจัยนี้ศึกษาสารสกัดจากฝรั่งสามสายพันธุ์ที่ปลูกเพื่อการค้าในประเทศไทยคือ พันธุ์พื้นบ้านไทย แป้นสีทอง และกิมจู โดยเปรียบเทียบการสกัดฝรั่งผ่านสามสภาวะได้แก่ การสกัดผ่านน้ำ 40% เอทานอล และสารละลายที่ผ่านการย่อยโดยการจำลองการทำงานของกระเพาะอาหารและลำไส้เล็กของมนุษย์ แล้วศึกษาปริมาณสารประกอบฟีนอลทั้งหมด การต้านอนุมูลอิสระ DPPH, superoxide anion , hydroxyl radical scavenging การต้านการสร้างเม็ดสีผิวโดยศึกษาฤทธิ์ต้านไทโรซิเนส , กรดฟีนอลิก, และปริมาณวิตามินซีทั้งหมด จากการสกัดทั้งสามสภาวะ พบว่า ชนิดของฝรั่ง และสภาวะการสกัด เป็นปัจจัยที่มีอิทธิพลร่วมกัน มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) ปริมาณพอลิฟีนอลทั้งหมด และการต้านอนุมูลอิสระ DPPH ในสายพันธุ์แป้นสีทองมีค่าสูงสุด (IC_{50} = 2.76 mg/mL) เมื่อสกัดผ่าน 40% เอทานอล ในขณะที่คุณสมบัติการต้าน superoxide anion ที่เกิดจาก PMS/NADPH system สายพันธุ์ไทยพื้นบ้านนั้นมีสูงสุด (IC_{50} = 41.47 mg/mL) เมื่อสกัดผ่าน 40% เอทานอล คุณสมบัติการต้าน hydroxyl radical ที่เกิดจาก Fenton system พบว่า สายพันธุ์แป้นสีทองมีค่าสูงสุดเมื่อสกัดผ่านน้ำ สายพันธุ์ไทยพื้นบ้านนั้นมีสูงสุด เมื่อสกัดผ่าน 40% เอทานอล แต่เมื่อผ่านระบบการย่อย (SGD) สายพันธุ์กิมจูมีค่าสูงสุด ในขณะที่คุณสมบัติการต้านการสร้างเม็ดสีผิว ในสายพันธุ์กิมจูมีค่าสูงสุด (IC_{50} = 290.71 mg/mL) เมื่อสกัดผ่านน้ำ การวิเคราะห์ กรดฟีนอลลิก และปริมาณวิตามินซี ด้วย HPLC พบว่า กรดฟีนอลลิกชนิด ferulic acid สูงสุด และมีปริมาณสูงสุดในสายพันธุ์กิมจู (14.00 mg/100g) เมื่อสกัดผ่าน 40% เอทานอล ส่วนปริมาณวิตามิน ซีมีปริมาณสูงสุดในสายพันธุ์กิมจู (159.97 mg/100g) เมื่อสกัดผ่านน้ำ ดังนั้นด้วยคุณสมบัติของสารสกัดฝรั่ง สารสกัดจากฝรั่งจึงเป็นประโยชน์ที่เกี่ยวข้องต่อการป้องกันสุขภาพของมนุษย์

ภาควิชา.....ชีวเคมี.....ลายมือชื่อนิสิต.....

สาขาวิชา.....ชีวเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์หลัก.....

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THANAKORN DAMSUD: ANIOXIDANT AND ANTI-MELANOGENESIS PROPERTIES OF GUAVA (*Psidium guajava* L.*)* EXTRACTS *in vitro*: ASSOC.PROF. NAPA SIWARUNGSON, Ph.D., 115 pp.

Guava (*Psidium guajava* L.) is a tropical fruit, rich in nutrients such as vitamin C, phenolic compounds, pectin and insoluble fiber. We focus on Pansithong, Thai original and Kimju grown locally commercial in Thailand by extracting them with water, 40%ethanol and Simulated Gastrointestinal Digestion (SGD) to analyze total polyphenolic compounds, DPPH scavenging activity, superoxide anion scavenging activity, hydroxyl radical scavenging activity anti-melanogenesis against tyrosinase, total phenolic acid and total vitamin C was found types guava and extracted conditions were the factor that have significant difference ($P<0.05$). The results showed that Pansithong contained the highest of polyphenol compounds and DPPH scavenging activity (IC_{50} = 2.76 mg/mL). Moreover, the superoxide scavenging abilities generated in a PMS/NADPH system Thai original (IC_{50} = 41.47 mg/mL) showed the highest of superoxide anion scavenging activity using 40% ethanol. The hydroxyl radicals were generated by the Fenton system was found Pansithong that used the water for extraction was the highest ability, whereas Thai original that used 40% ethanol for extraction was the highest. Kimju guava was extracted by using SGD demonstrated the highest hydroxyl properties. The anti-melanogenesis against tyrosinase found that Kimju showed the highest extracted (IC_{50} = 290.71 mg/mL) by water. To analyze contained phenolic acid and Vitamin C determine by HPLC. The highest amounts of ferulic acid that was found in Kimju (14.00 mg/100g) by using 40% ethanol. While, the highest amounts of ascorbic acid were found in Kimju (159.97 mg/100g) using water extracts. Therefore, the effect of guava extracts may have a strong potential for use protective effect in human health.

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CONTENTS

PAGE

PAGE

PAGE

PAGE

PAGE

LIST OF TABLES

PAGE

PAGE

LIST OF FIGURES

PAGE

PAGE

LIST OF ABBREVIATIONS

Abs	absorbance
ANOVA	analysis of variance
ATP	adenosine triphosphate
ΔpH	pH gradient or proton gradient
DPPH	2,2-diphenyl-1-picrylhydrazyl
Fe^{3+}	ferric ion
Fe^{2+}	ferrous ion
Fig	Figure
g	gram
H_2O	water
H_2O_2	hydrogen peroxide
°C	degree Celsius
conc.	concentration
h	hour
IC_{50}	median inhibitory concentration
L	liter
min	minute
µl	microliter
ml	milliliter
mg	milligram
mM	millimolor
µM	micromolor
M	Molar

NBT	*p*-Nitro blue tetrazolium chloride
NADH	nicotinamide adenine dinucleotide
PMS	Phenazine methosulfate
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
rmp	round per minute
$^1O_2$	single oxygen
SD	standard deviation
SGD	simulated gastrointestinal digestion
$O_2^{\cdot -}$	superoxide anion
SOD	superoxide distamutase
TBA	Thiobarbituric acid
TCA	Thichoroacetic acid
UV	ultraviolet

CHAPTER I

INTRODUCTION

Health functional properties in fruits and vegetables are of great interest to consumers and researcher because several epidemiological studies have indicated that frequent consumption of fruit and vegetable is associated with lower risk of various chronic diseases such as atherosclerosis, cancer and neurodegenerative diseases (Acheson and Williams, 1983; Steinmetz and Potter, 1991; Block et al., 2000). Free radicals and related species have attracted a great deal of attention in recent years. They are mainly derived from oxygen (reactive oxygen species/ ROS) and nitrogen (reactive nitrogen species/RNS), and are generated in our body by various endogenous system, exposure to different physicochemical conditions or pathological states called pro-oxidant effect (Devasagayam et al,. 2004, Papas, 1999). These free radicals attack biomolecules such as lipid, protein, carbohydrate, and deoxyribonucleic acid (DNA), leading to cell and tissue injury associated with degenerative disease mentioned above. Reactive oxygen species are produced by sunlight, ultraviolet, ionizing radiation, chemical reactions, and normal metabolism (Yen and Chem, 1995; Pietta et al., 1998; Jung et al., 1999).

The skin is always in contact with oxygen and is increasingly exposed to ultraviolet (UV) irradiation. Therefore, the risk of photo-oxidative damage of the skin induced by reactive oxygen species (ROS) has increased substantially. The term reactive oxygen species not only collectively includes oxygen-centered radicals such as the super-oxide anion ($O_2^{\cdot -}$) and the hydroxyl radical ($OH^{\cdot}$), but also some non-radical species, all being produced in the skin upon UV irradiation (Scharffetter-

Kochanek, 1997). Less severe photoaging changes result in wrinkling, scaling, dryness, and uneven pigmentation consisting of hyper-and hypo-pigmentation (Pinnel, 2003). Moreover, exposure to UV irradiation has also shown direct and indirect melanogenic effect on melanocyte to produce pigment melanin (Busca and Ballotti, 2000). Although this pigment in human skin is a major defense mechanism against ultraviolet light from the sun, including ultraviolet A(UVA) and ultraviolet B (UVB) that reach the earth's surface, the production of abnormal pigmentation such as melasma, freckles, senile lentigines, and other form of melanin hyperpigmentation could be a serious aesthetic problem (No et al., 1999). To counteract the harmful effects of ROS and protect the overproduction of melanin, antioxidants and whitening agents have thus been the subject of many studies.

Breeding plant cultivars with high antioxidant content and other nutritional values is an increasingly important objective for plant breeders. Guava fruit is a good source of natural antioxidant compounds such as ascorbic acid (Wilson, 1980; Adsule and Kadam, 1995; Nakasone and Paull, 1998), carotenoids , phenolic compounds such as quercetin, gallic acid, ellagic acid (Seshadri and Vasishta, 1964; Misra and Seshadri, 1968), and flavonoids (Miean and Mohamed, 2001). Its fruit extracts have shown exceptionally high antioxidant capacity (Jimenez-Escrig et al., 2001; Leong and Shui, 2002; Guo et al., 2003, Thaipong et al., 2005, 2006). In addition, guava is very diverse crop species which facilitates the development of guava cultivars with high level of health related functional compounds and excellent taste using traditional breeding methods.

The objectives of this research are to study the antioxidant activity, anti-melanogenesis effect of guava extract.

CHAPTER I I

LITERATURE REVIEW

1. Origin and distribution of guava

More than 80 genera and 3,000 species are members of Myrtaceae. Among these genera, *Psidium* is the most important for cultivation and consists of more than 150 species (Cobley, 1956). Ploidy level ranges from diploid (2n=2x) to octoploid (2n=8x) (Atchison, 1947; Smith-White, 1984). Guava (*Psidium guajava* L.) is the most valuable cultivated species. Its haploid chromosome number is 11(n=x=11) and ploidy level is diploid (2n=2x). Most commercial cultivars are diploid (Atchison, 1947; Smith-White, 1948; Hirano and Nakasone, 1969): although there are a few triploid commercial cultivars (Srisuwan, 2003). Guava varies greatly in grow habit: vegetative and reproductive characters, both within and among species (Jagtiani et al., 1987; Adsule and Kadam, 1995; Nakasone and Paull, 1998).

Guava is native to tropical America (Cobley, 1956) and presently found distributed throughout tropical and subtropical regions. Guava fruit is commercially important in India, Venezuela, New Zealand, the Philippines (Yadava, 1996), Vietnam (Le et al., 1998) and Thailand (Tate, 2000). It was introduced to Thailand in early 1700's Guava plant can grow well in most Thai areas and produce flowers and fruits throughout a year.

1.1 Potential of guava production in Thailand

Although guava is not listed among the most important tropical fruit crops in Thailand, it has a high commercial value. Over 167,000 metric tons from a production area of 73,000 rai (11,680 ha) located mainly in the central and western

parts of the country (Department of Agricultural Extension, 2007) is produced annually. More than 90% of the production is for fresh consumption of which around 3,380 metric tons was exported to 26 countries in 2006 (The Customs Department, 2007). Lower grade fruits are processed to juices, pickled, dried or canned.

Guava is a popular tropical fruit crop for fruit growers because orchard establishment and maintenance are affordable-7,000 Baht/rai (4,754 Baht/ha) in first year and 2,800 Baht/ rai (17,500 Baht/ha) in next years. Guava plant can fruit within 8 to 9 months after planting, and have wide adaptation. Its fruit can be used for fresh consumption and various processing uses. Also, it is highly accepted by consumers and markets due to its years round availability, affordable price, excellent transportation and handling properties and high nutritional value.

2. Free radical and active oxygen species

Free radicals are chemical species, which have unpaired electrons. Electrons are present generally in pairs. However, under certain conditions, molecules have unpaired electrons and become free radicals. Thus, free radicals are in general reactive and attack other molecules, although some radicals are not reactive but stale enough to have long life. Some of them have unpaired electrons and are free radicals but others are not (Halliwell, 1994; Papas, 1999). Table 1 summarizes the active oxygen species, which are relavant to lipid peroxidation and oxidative stress *in vivo*. Nitric oxide and thiyl radical ($RS^{\cdot}$), which do not bear unpaired electrons on oxygen, are also included.

Table 1 Active oxygen and related species

Radicals		Non-radicals	
$O_2^{\cdot -}$	superoxide	H_2O_2	hydrogenperoxide
$HO^{\cdot}$	hydorxyl radical	O_2	singlet oxygen
$HO_2^{\cdot}$	hydroperoxyl radical	LOOH	lipid hyderoxide
$L^{\cdot}$	lipid racical	Fe=O	iron-oxygen complexes
$LO^{\cdot}$	lipid alkoxyl radical	HOCl	hypochlorite
$LO_2^{\cdot -}$	lipid peroxyl radical	O_3	ozone
$NO^{\cdot}$	nitric oxide	N_2O_3	dinitrogen trioxide
$NO_2^{\cdot}$	nitrogen dioxide	N_2O_4	dinitrogen tetroxide
$RS^{\cdot}$	thiyl radical	N_2O_5	dinitrogen pentoxide
$P^{\cdot}$	protein radical	ONO_2	peroxynitrite
$H^{\cdot}$	hydrogen atom	LO_2NO	alkyl peroxynitrite
		O_2NOCO_2	nitrocarbonate
		ONO_2CO_2	nitrosoperoxycarbonate
		RSH	thiol

2.1 Formation of free radical and active oxygen species

2.1.1 Endogenous sources

Free radicals and active oxygen species in the body are by-products of the normal metabolism of mitochondria in aerobic organisms, phagocytic cells during infection and inflammation, and peroxisomes. These are organelles responsible for degrading fatty acids and other molecules (Ames et al,. 1993; Borek, 1997).

2.1.2 Exogenous sources

Free radicals and active oxygen species are formed by various extrinsic sources such as automobile exhaust, cigarette smoke, radiation, alcohol, herbicides, insecticides, dust mold, excessive sunlight, ozone, ultraviolet light, noise, air/water pollution, stress, microorganism, etc. (Papas, 1999; Shilalahi, 2001).

2.2 Antioxidant defense mechanism in biological system

Antioxidant status is the balance between the antioxidant system and pro-oxidants in living organisms. A serious imbalance favoring oxidation is defined as oxidative stress. It may result from excessive production of ROS and free radicals and/or weakening of the antioxidant system due to lower intake or endogenous production of antioxidants or from increased utilization. Major factors affecting the antioxidant status are summarized in Table 2.

Table 2 Determinants of antioxidant status in humans (Papas, 1999)

Antioxidant effect	Pro-oxidant effect
Genetic factors **Diet** - Antioxidant vitamin (A,C,E) - Phytochemicals - Minerals, components of antioxidant enzymes (Se, Zn, Cu, Mn, Fe) - Food antioxidants and supplements **Alcoholic drink (wine and other) containing antioxidants** **Exercise program**	**Genetic factors** **Diet** - Lipids, especially PUFA - Divalent minerals (Cu, Fe) - Prooxidant nutrients and phytochemicals **Environments** - Pollutants - Tobacco smoke - UV radiation **Alcohol** **Injury, disease, and medications** - Trauma, injury/reperfusion - Other diseases - Drugs and medical treatment (radiation therapy, etc.) **Physiological stage or conditions** - Prematurity - Aging - Strenuous exercise **Stress** - Physiological - Emotional

2.3 Reactive species

Free radicals and related species are mainly derived from oxygen (reactive Oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS), and are generated in our body by various endogenous systems, exposure to different physicochemical conditions or pathological states (Devasagayam et al., 2004). Free radicals can be defined as molecules or fragments of molecules, capable of independent existence, containing one or more unpaired electrons in their orbital. They tend to react very easily with various types of biomolecules, to acquire another electron and stabilize the orbital (Andreassi and Andreassi, 2004). Generally, free radicals attack the nearest stable molecules, "stealing" its electrons. The molecule that has been attacked and loses its electron becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, initiating lipid peroxidation which results in destabilization and disintegration of the cell membranes or oxidation of other cellular components such as protein and DNA, finally resulting in the disruption of cells (Kaur and Kapoor, 2001). Free radicals are generally unstable, highly reactive, and energized molecules (Lee et al., 2004). Organisms constantly produce free radicals by different mechanisms. Incomplete reduction of oxygen in the mitochondrial (the major ROS source) electron transport chain releases superoxide anions into the cytoplasm (Andreassi and Andreassi, 2004; Jezed and Jlavata, 2005). Free radicals are formed as a natural consequence of the body's normal metabolic activity and as part of the immune system's strategy for destroying invading microorganisms. For example, $O_2^{\cdot -}$ plays an essential role in the intracellular killing of bacteria by activated phagocytes (for example, neutrophils and macrophages).

Exogenous sources of free radicals include ozone, exposure to ultraviolet radiation in sunlight, and cigarette smoke. Free radicals can adversely alter lipids, protein and DNA and have been implicated in aging and a number of human diseases. In general, free radicals and related species are very short-lived, with half-lives in milli-, micro- or nanoseconds. Details about some of the biologically important reactive species are presented in Table 3.

Table1 3 Reactive oxygen and nitrogen species of biological interest

Reactive species	Symbol	Half life (in sec)	Reactivity/Remarks
Reactive oxygen species			
Superoxide	$O_2^{\bullet-}$	10^{-6}s	Generated in mitochondria, in cardiovascular system and others
Hydroxy radical	$OH^{\bullet}$	10^{-9}s	Very highly reactive, generated during iron overload and such condition in our body
Hydroperoxide	H_2O_2	stable	Formed in our body by large number of reactions and yields potent species like $OH^{\bullet}$
Peroxyl radical	$ROO^{\bullet}$	s	Reactive and formed from lipids, proteins, DNA, sugars etc. during oxidative damage
Organic hydroperoxide	ROOH	stable	Reacts with transient metal ions to yield reactive species
Singlet oxygen	$^1O_2$	10^6s	Highly reactive, formed during photosensitization and chemical reactions
Ozone	O_3	s	Present as an atmospheric pollutant, can react with various molecules, yielding $^1O_2$
Reactive nitrogen species			
Nitric oxide	$NO^{\bullet}$	s	Neurotransmitter and blood pressure regulator, can yield potent oxidants during pathological states
Peroxynitrite	$ONOO^{\bullet}$	10^{-3}s	Formed from NO and superoxide, highly reactive
Peroxynitrous acid	ONOOH	Fairly stable	Protonated form of $ONOO^{\bullet}$
Nitrogen dioxide	NO_2	s	Formed during atmospheric pollution

2.3.1 Superoxide anion radical ($O_2^{\cdot -}$)

Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide anion is an initial free radical formed from mitochondrial electron transport systems (Lee et al., 2004) that is the first reduction of ground state-oxygen, capable of both oxidation and reduction (Cadenas, 1995; Perl-Treves and Perl, 2002). Mitochondria generate energy using 4 electron chain reactions, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anions (Lee et al., 2004). Superoxide formed in vivo is largely –if not completely- converted by SOD-catalysed or non-enzymic dismutation, into H_2O_2 (Halliwell et al., 1995).

$$O_2 \xrightarrow{e^-} HO_2^{\bullet} \xrightarrow{e^-} H_2O_2 \xrightarrow{e^-} OH^{\bullet} \xrightarrow{e^-} H_2O$$

$(O_2^{\bullet})$

The superoxide anion plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living organism. The superoxide anion can react with nitric oxide ($NO^{\cdot}$) and form peroxynitrite ($ONOO^-$), which can generate toxic compounds such as hydroxyl radical and nitric dioxide (Lee et al., 2004).

2.3.2 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) is produced during normal aerobic cell metabolism, and its formation involves a number of enzymatic reaction especially superoxide dismutase enzyme (SOD). H_2O_2 is decomposed to oxygen (O_2) and water (H_2O) by enzyme such as catalase and glutathione peroxidase (Perl-Treves and Perl, 2002). Unlike superoxide radical ($O_2^{\cdot -}$), H_2O_2 is able to cross biological membranes. Both $O_2^{\cdot -}$ and H_2O_2 can find some molecular targets to which they can do direct damage, but on the whole their reactivity is limited. Much like superoxide, H_2O_2 is rather stable and therefore less toxic than other reactive oxygen species (Halliwell et al., 1995; Cadenas, 1995; Perl-Treves and Perl, 2002). Hydrogen peroxide is the least reactive molecule among reactive oxygen species and is stable under physiological pH and temperature in the absence of metal ions (Lee et al., 2004). In the presence of transition metals, H_2O_2 can be reduced by Fenton reaction to form hydroxyl radical ($HO^{\cdot}$), which is highly reactive and can result in lipid peroxidation and many biological effects (Keithahn and Lerchl, 2005).

2.3.3 Hydroxyl radical ($HO^{\cdot}$)

Hydroxyl radical is the most highly reactive free radical and can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as copper or iron. Hydroxyl radicals have the highest l-electron reduction potential (2310 mV) and can react with everything in living organisms at 2^{nd}-order

rate constants of 10^9 to 10^{10} M^{-1} sec^{-1} (Lee et al., 2004; Halliwell et al., 1995). Hydroxyl radicals react with nearly every biomolecule such as nuclear DNA, mitochondrial DNA, proteins and membrane lipids (Keithahn and Lerchl, 2005). The rapid and nonspecific reactivity of $HO^{\cdot}$ renders this free radical particularly dangerous. It may abstract hydrogen from, or hydroxylate, most biomolecular, causing cell injury or death. Hydroxyl radical is believed to be the etiological agent for several diseases and may also be involved in natural aging (Graf et al., 1984). Formation of $HO^{\cdot}$ from $O_2^{\cdot -}$ can be achieved by at least four different mechanisms. One requires traces of catalytic transition metal ions, of which iron and copper seem likely to be the most important *in vivo*. A second mechanism is that background exposure to ionizing radiation causes a steady-state low rate of $HO^{\cdot}$ formation within cells and in food by splitting of water. A third means of forming some $HO^{\cdot}$ is the reaction of $O_2^{\cdot -}$ with the free radical nitric oxide ($NO^{\cdot}$), a reaction that proceeds at a rate comparable to that of $O_2^{\cdot -}$ with SOD. Reaction of HOCl with $O_2^{\cdot -}$ also makes some $OH^{\cdot}$,the rate constant is close to 10^7 M^{-1} sec^{-1}(Halliwell et al., 1995).

$$HOCl + O_2^{\cdot -} \longrightarrow OH^{\cdot} + O_2 + Cl^-$$

The concept of a $O_2^{\cdot -}$-driven Fenton-type reaction requires both $O_2^{\cdot -}$ and H_2O_2 as precursors of $OH^{\cdot}$, it proceeds via an intermediate catalyst, such as a transition metal chelate (eg., Fe or Cu), which is reduced by $O_2^{\cdot -}$ and reacts with H_2O_2

in a "Fenton like" reaction to produce $OH^{\cdot}$ as in the following reaction (Cadenas, 1995).

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{3+} + O_2 \qquad (1)$$

(Cu^{2+}) (Cu^{+})

$$H_2O_2 + Fe^{3+} \longrightarrow OH^{\cdot} + OH^{\cdot} + Fe^{3+} \qquad (2)$$

(Cu^{+}) (Cu^{2+})

These well-known reaction sequences give a net balance (Haber-Weiss reaction) as (Cadenas, 1995):

$$O_2^{\cdot -} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^{\cdot} \qquad (3)$$

The requirements of transition metal chelate for this reaction are two-fold: on the one hand, the chelator alters the redox potential of the transition metal, thereby facilitating electron transfer reaction with $O_2^{\cdot -}$ and H_2O_2 (e.g., the E^{O}values for Fe^{3+}/ Fe^{2+} and Fe-$EDTA^{-}$/Fe-$EDTA^{2-}$ are +770 and +120mV, respectively) and, on the other hand, it maintains the transition metal in solution (Cadenas, 1995). The initial product of reaction may be an oxo-iron complex, possibly ferryl, that then decomposes to form $OH^{\cdot}$. Different ligands to the iron (II) may stabilize this intermediate, so that little $OH^{\cdot}$ is formed. Thus, Fe-EDTA chelates are good sources of $OH^{\cdot}$ to stabilize the intermediate iron (II) in the presence of H_2O_2. Most ferric (Fe^{+3}) complexes react more slowly with H_2O_2 than ferrous (Fe^{+2}) complexes, so that reducing agent stimulate Fenton reaction. $O_2^{\cdot -}$ in reaction (I) can be replaced by a

suitable electron donor(reducing agent), such as ascorbate (reaction 4) (Halliwell et al., 1992; Cadenas, 1995).

$$Fe^{+3} + AH^{-} \longrightarrow Fe^{+2} + AH^{-} \quad (4)$$

(Cu^{2+}) (ascorbate) (Cu^{+})(semidehydroascorbate)

For determination of rate constants for reaction of $OH^{\cdot}$, a deoxyribose method was used which is a simple "test-tube" assay. It was found that deoxyribose reacts with $OH^{\cdot}$ with a rate constant of $3.1*10^{9}$ M^{-1} sec^{-1} (Halliwell et al., 1995; Halliwell et al., 1987).

2.3.4 Singlet oxygen ($^1O_2$)

Singlet oxygen is a nonradical and excited status. Singlet oxygen has been shown to be produced in myeloperoxidase and eosinophil peroxidase-catalysed reactions and has been reported to be generated by some cell type including neutrophils, eosinophils, and macrophages. UV light has been shown to generate $^1O_2$ (Wright, Hawkins and Davies, 2000) by photosensitization reaction (Halliwell et al., 1995). Singlet oxygen itself is not only cytotoxic because it can oxidize DNA directly. Singlet oxygen can be produced by living cells and is capable of causing lipid peroxidation, DNA damage and cell death (Maharaj et al., 2005).

2.3.5 Nitric oxide and nitric dioxide

Nitric oxide ($NO^{\cdot}$) is a free radical with a single unpaired electron. Nitric oxide is formed from L-arginine by NO synthase. Nitric oxide itself is not a

very reactive free radical, but the overproduction of NO is involed in ischemia reperfusion, neurodegenerative and chronic inflammatory disease such as rheumatoid arthritis and inflammatory bowel disease. Nitric oxide exposed in human blood plasma, can deplete the concentration of ascorbic acid and uric acid, and initiate lipid peroxidation (Lee et al., 2004).

Nitric dioxide ($NO^{\cdot}_2$) is formed from the reaction of peroxyl radical and NO, polluted air and smoking. Nitric dioxide adds to double bonds and abstract labile hydrogen atoms initiating lipid peroxidation and production of free radicals, it also oxidizes ascorbic acid (Lee et al., 2004).

3. Skin aging

Aging of the skin is commonly associated with increased wrinkling, sagging and increased laxity. When considering the underlying reasons for these changes, it is important to distinguish between the effects of true biological aging (intrinsic chronologic aging) and environmental factors (extrinsic aging). The first comprises an innate or intrinsic aging mechanism that is similar to internal organs-affect the skin by a slow and partly reversible degeneration of connective tissues. The second process designated as extrinsic or photoaging is mainly due to ultraviolet radiation of sunlight that overwhelmingly contributes to a premature aging phenotype even in young individuals (Scharffetter-Kochanek et. al., 2000). Ultraviolet (UV) irradiation is one of the most ubiquitious environmental hazards that impact every living creature under the sun. Skin is the largest human organ, and is the only organ directly exposed to UV irradiation (Jenkins, 2002; Xu and Fisher, 2005).

Intrinsic chronologic aging is largely genetically determined and clinically associated with increased fragility, loss of elasticity and has a transparent quality. The process of intrinsic skin aging is similar to that occurring in most internal organs, involving slow deterioration in tissue function due to a variety of factors. The stratum coreum remains relatively unchanged, but the epidermis and dermis thins with a flattening of the dermo-epimal junctions. There is also a reduction in the number and biosynthetic capacity of fibroblasts and progressive disappearance of elastic tissue in the papillary dermis (Jenkins, 2002). An age-associated loss in dermal thickness occurs in elderly person. Skin collagen content decreases with age and the fine collagen fibers associated with infancy become increasingly dense and tightly packed and far move randomly orientated. The elastic recovery of aged skin is decreased and this function loss is accompanied by a decrease in the number of elastic fibers. The processes associated with intrinsic skin aging are thought to result from a combination of events including (i) decreased proliferation capacity of skin-derived cells; (ii) decreased matrix synthesis in the dermis; and (iii) increased expression of enzymes that degrade the collageneous matrix. It is proposed that accumulation of non-dividing senescent cells with altered gene expression and subsequent phenotype, eventually leading to a decline in tissue function and integrity that is characteristic of aging. These changes are observed over the entire surface of body (Bernstein andUitto, 1996; Jenkins, 2002). Extrinsic aging is caused by external factors such as smoking, excessive use of alcohol, poor nutrition, and sun exposure (Baumann, 2002). This process refers to premature skin aging. Enhanced generation of reactive oxygen species and induction of matrix metalloproteinases (MMPs) appear to be the most important components of UVA-modulated signal transduction

pathways, ultimate leading to photoaging (Lee et al,. 2003) It has been suggested that as much as 80% of facial aging is attributable to sun exposure. Clinically, photodamaged skin is characterized by loss of elasticity, increased roughness and dryness, irregular pigmentation and deep wrinkling (Jenkins, 2002).

3.1 Theory of hyperpigmentation

3.1.1 Human skin pigmentation

Melanocytes are specialized cells in the skin that are found of their embryonic origin at the neutral crest. During embryonic development, melanoblasts migrate to reach the basal layer of the epidermis where they differentiate to mature melanocytes possessing the complete machinery to ensure melanin synthesis and distribution within the skin. Melanin synthesis takes place within specialized intracellular organelles named melanosomes. Melanin-containing melanosomes then move form perinuclear region to the dendrite tips and are transferred to keratinocytes by a still not well-characterized mechanism. These events, which ensure a uniform distribution of melanin pigment in epidermis, are responsible for skin and hair color in humans and other animals (Busca and ballotti, 2002).

3.1.2 UV-induced pigmentation

Pigmentation of the skin, due to the synthesis and dispersion of melanin in the epidermis, is also the key physiological defense against sun-induced damage. In physiological conditions human skin pigmentation is increased by UV radiation of the solar light. UV radiation gives both direct and indirect melonogenic effect on melanocytes.

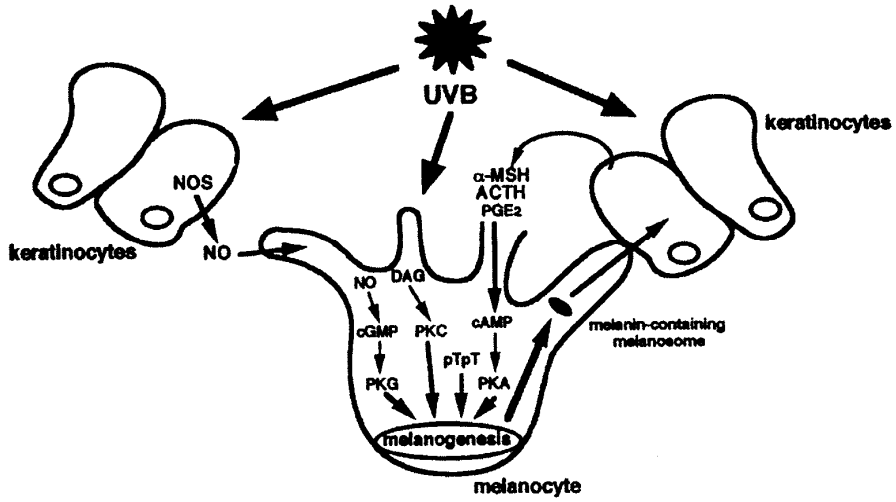


Figure 1 Diagram showing melanogenesis as a result of a complex set of regulatory processes involving direct effects of UV radiation on melanocytes and indirect effects through the release of keratinocyte-derived factors (Busca and Ballotti, 2000).

3.1.3 Melanogenesis

Melanogenesis is a physiological process in the synthesis of melanin pigments. Melanins are synthesized in melanosomes that contain the specific enzyme required for proper melanin production. Among them, the well-characterized is tyrosinase (Busca and Ballotti, 2002).

Synthesis of melanin starts from the conversion of the amino acid L-tyrosine (monophenols) to 3,4-dihydroxyphenylalanin (o-phenols, L-DOPA) by tyrosinase enzyme with hydroxylation reaction and the oxidation of L-DOPA yields dopaquinone (o-quinones) by tyrosinase. These quinines are highly reactive and tend to polymerize spontaneously to from brown pigments of high molecular weight (melamins), which determine the color of mammalian skin and hair. Quinones can also react with amino acids and proteins and thus enhance the development of brown

color (Nerya et al,. 2004). Two types of melanin are eu-melanins (dark-brown) and pheomelanins (yellow-brown). Therefore, the pathway that DOPA quinine transformed to leucoDOPAchrome, DOPAchrome leading to the formation of eu-melanins is interesting. According to melanogenesis pathway, tyrosinase activity is thought to be a major regulatory factor in the initial steps of this pathway.

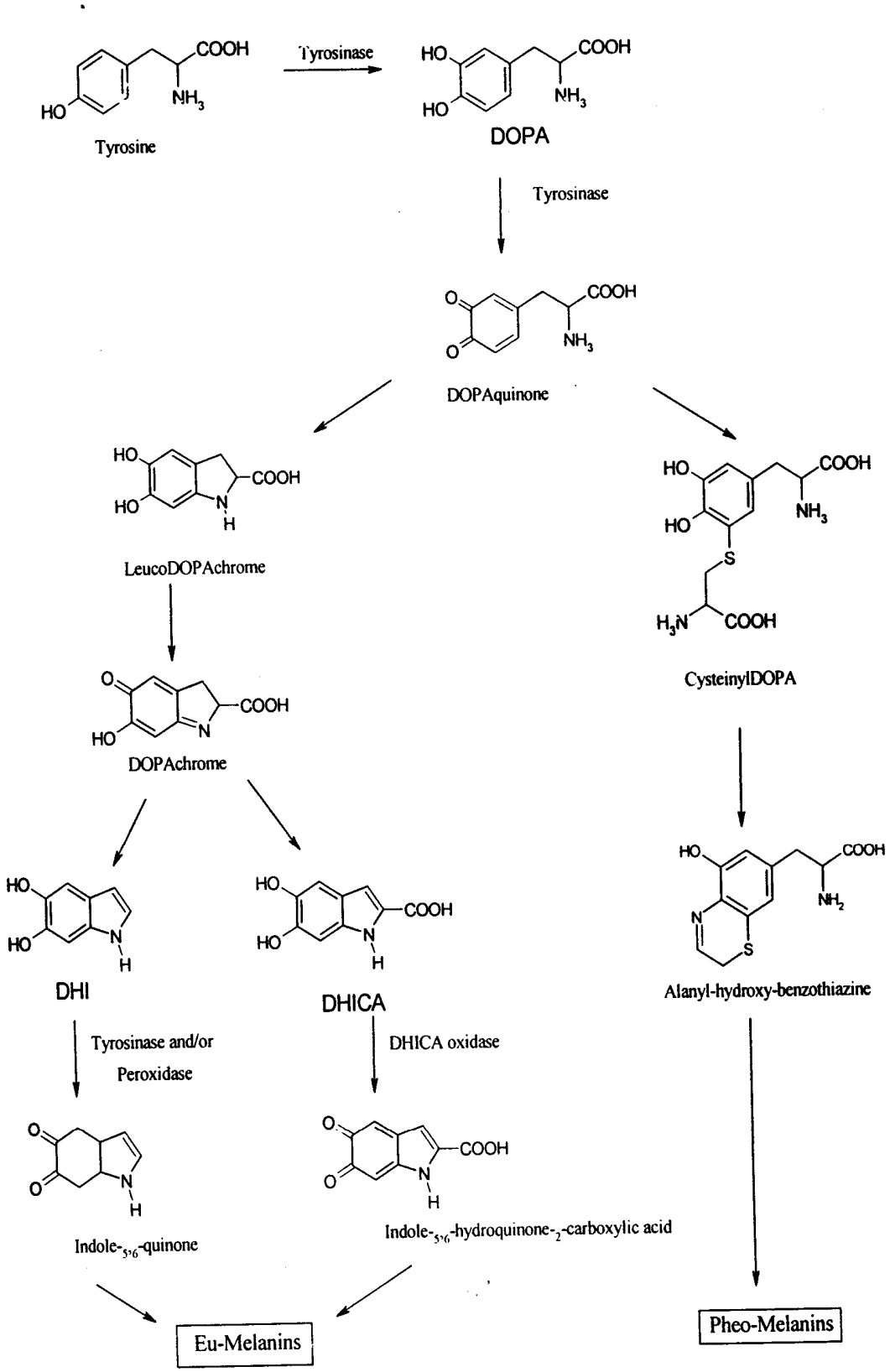


Figure 2 Melanogenesis pathway (Baumann, 2002)

Tyrosinase is a copper-glycoenzyme involved in the biosynthesis of the widespread melanin pigments (Jose, Borrn and Solano, 2002; Nerya et al., 2004). Its structure contains two histidine-based regions named Cu A and Cu B which are the peptide segments involved in binding the two coppers. These are the active site of tyrosinase enzyme. Both regions contain three perfectly conserved histidine resodies. Concerning oxygen, it binds as a side-on peroxide bridge rather than in a u-dioxo disposition. Thus, coppers are penta-coordinated in a distorted square pyramidal geometry (Jose et al., 2002).

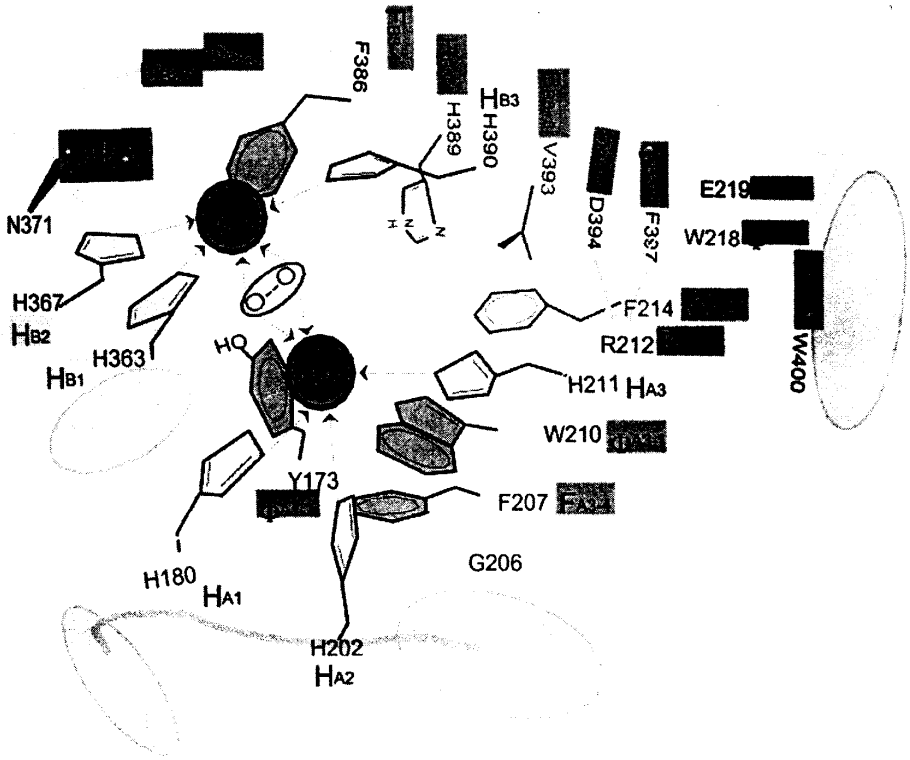


Figure 3 A reasonable model for the active site of mammalian tyrosinase enzyme (Jose et al., 2002).

4. Antioxidants

Antioxidants are a group of substances which present at low concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being oxidized themselves. In recent years there has been an increased interest in the application of antioxidants to medical treatment as information is constantly gathered linking the development of human diseases to oxidative stress (Vaya and Aviram, 2001).

4.1 Antioxidant mechanisms

4.1.1 Antioxidant as reducing agents

Many compounds with antioxidant activity are, as might be expected, readily oxidizable materials. This property allows them both to intercept primary oxidants such as transition metal ions, molecular oxygen, hydroxyl radical (OH), or hydrogen peroxide (H_2O_2), and also to compete with chain-carrying free radicals to terminate autooxidation processes. In principle, it should be possible to correlate the antoxidative effectiveness of a compound toward and oxidizing species such as an electrophilic free radical by an electron transfer process *in vitro (*Larson, 1995).

4.1.2 Antioxidants as radical quenchers

A general ability to enter into rapid reactions with free radicals is a great advantage for a potential antioxidant compound. Many synthetic antioxidants are specifically designed to react with oxygen radical and to form sterically hindered or otherwise inactive radical products that effectively terminate radical chains. This property stop the free radical chain of oxidative reactions by contributing hydrogen from the phenolic hydroxyly groups by

themselves and forming stable free that do not initiate or propagate further oxidation of lipids (Kaur and Kapoor, 2001). Many naturally occurring compounds could have mechanisms of action that are similar to those that have been established for synthetic materials. Their effectiveness appears to depend on their capacity to form stable radicals, which then react further with polymer free radicals to stop the chain reaction (Larson, 1995).

4.1.3 Antioxidants as metal ion complexing agents

Several oxidized transition metal ions such as iron (III) and copper (II) have readily accessible reduced states and, furthermore, are present in high enough concentrations in many tissues to make them plausible reactants for one- electron oxidations or reductions that could generate reactive free radicals. For example, a well-know route to hydroxyl radical (OH°) and other radical generation is Fenton reaction. Agents that could bind reactive transition metal cations by complexation could decrease their biological effects dramatically. A second effect of complexed metal ions could be to change the redox properties of their ligands, and therefore either promote or inhibit their antioxidant capacities (Larson, 1995).

4.1.4 Synergistic effects

It has often been noted that combinations of antioxidants are more effective than one another acting independently. For example, ascorbic acid (vitamin C) and tocopherol (vitamin E) have long been known to be highly effective in combination, although vitamin C is significantly less effective when used alone. Ascorbate regenerates tocopherol from the tocopheryl radical, and this recycling action presumably contributes to the synergistic effect of combined ascorbate and tocopherol supplementation (Larson, 1995; Fuchs, 1998).

4.2 Available antioxidant product

A wide range of antioxidants, both natural and synthetic, has been proposed for use in treatment of human disease (Hilliwell et al., 1992). A compound might exert antioxidant actions *in vitro* in food by inhibiting generation of ROS, or by directly scavenging free radicals. Additionally, *in vivo* an antioxidant might act by raising the levels of endogenous antioxidant defenses (e.g. by up regulating expression of the genes encoding SOD, catalase or glutathione peroxidase). Some quite simple experiments can be performed to examine direct antioxidant ability *in vivo* and to test for possible pro-oxidant effects on different molecular targets (Halliwell et al., 1995).

4.2.1 Synthetic antioxidants

Some of the important synthetic antioxidants are butylated hydroxyanisole (BHA), butyated hydroxyl toluene (BHT), *tert*-butylhydroquinone (TBHQ), propyl gallate (PG) and tocopherols. In general, synthetic antioxidants are compounds with phenolic structures and various degree of alkyl substitutions (Kaur and Kapoor, 2001). Trolox® is a cell-permeable, water-soluble derivative of α-tocopherol with the hydrophobic side chain replaced by hydrophilic-COOH group. It is a chain-breaking antioxidant that acts as a scavenger of radicals via the H-donating group in its chromanol nucleus. Its protective effects against oxidative damages, particularly against lipid peroxidation, have been demonstratated *in vitro* and *in vivo.* It is a very efficient antioxidant, good scavenger of peroxyl and alkoxyl radicals, prevents peroxynitrite-mediated oxidative stress and prevents apoptosis in cell culture. Trolox® is commercially available for experimentation especially in an aqueous system (Suarna, Dean and Southwell-Keely,

1997; Feugnag et al., 2004). However, Trolox® may be harmful if it swallowed and may cause respiratory tract, eye and skin irritation.

4.2.2 Polyphenols

Phenolic compounds or polyphenols are ubiquitous in plants with more than 8000 structure. Natural polyphenols can range form simple molecules (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group. Polyphenolics exhibit a wide range of biological effects including antibacterial, anti-inflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions. Many of these biological functions have been attributed to their free radical scavenging and antioxidant activity (Soobrattee et al,. 2005). Antioxidant mechanisms of polyphenolic compounds are based on reducing properties, hydrogen donating abilities and chelating metal ions. After donating a hydrogen atom, phenolic compounds become resonance-stabilized radicals, which do not easily participate in other radical reactions (Kaur and Kapoor, 2001; Lee et al., 2004). It is known that the degree of glycosylation significantly affects the antioxidant properties of the compound, for example, glycones of quercentin and myricetin were more active than their glycosides. The molecular scavenging mechanism of these molecules is closely related to their stereo chemical structure (Kaur and Kapoor, 2001). Both the number and configuration of H-donating hydroxyl group are the main structural features influencing the antioxidant capacity of phenolics (Soobrattee et al., 2005). Catechins and their epimers serve as powerful antioxidant for directly eliminating super oxide anion radicals. These are basically flavonoids and some related compounds (Kaur and Kapoor, 2001).

More recently, attention has been focused on the antioxidant properties of plant polyphenols found in green tea and red wines. But before their relative contribution to preventing oxidative damage can be assessed, considerably more information on the absorption, metabolism and excretion of these compounds in human is needed (Hughes, 2000).

-EGCG or (-)-epigallocatechin-3-gallate is the main polyphenolic component of catechins in green tea, which has been consumed as popular beverage in Asian countries for many centuries (Baumann, 2002). Topical EGCG reduced UVB-induced inflammatory responses and infiltration of leukocytes in human skin. Moreover, topical application of EGCG also inhibited carcinogenesis and selectively increased apoptosis in UVB-induced skin tumors in mice (Pinnell, 2003). EGCG enhance the activity of superoxide dismutase (SOD) and catalase in mouse striatum thus suggesting that flavan-3-ols can also exhibit their neuroprotective effect via regulation of gene expression. The high antioxidant activity of EGCG is explained by the presence of galloyl moiety attached to flavan-3-ol at the 3 position, adding three more hydroxyl groups (Soobrattee et al., 2005). EGCG has been represented as a powerful radical scavenger, as investigated by many *in vivo* and *in vitro* techniques (Geetha et al., 2004; Hsu, 2005; Soobrattee et al., 2005). However, a low concentration of EGCG increased amounts of double base lesions of DNA, especially 8-oxodG in HL-60 cells, further supporting the involvement of H_2O_2 in cellular DNA damage. These results suggested that EGCG can act not only as an antioxidant, but also as a pro-oxidant in the presence of metal ion (Furukawa et al., 2003). Green tea has caused so much excitement in the media that many pharmaceutical and cosmetic companies are supplementing their skin care products with green tea extracts. It will

be interesting to see the long-term results of green tea therapy for photoprtection (Baumann, 2002).

- Red wine has been shown to inhibit *in vitro* oxidation of human LDL. The phenolics substances in wine mainly originate from grapes and include nonflavonoids such as hydroxycinnamates, hydroxybenzoates and stilbene in addition to flavonoids such as flavan-3-ol (catechin), anthocyanins, flavonols and polyphenol tannins. Red wine has great antioxidant potential, because of phenolic compounds (tannins and anthocyanins) which are present in sufficient quantities to ensure optimum free radical scavenging activity of compounds and even combined actions between them leading to synergistic effect of these polyphenols (Kaur and Kapoor, 2001).

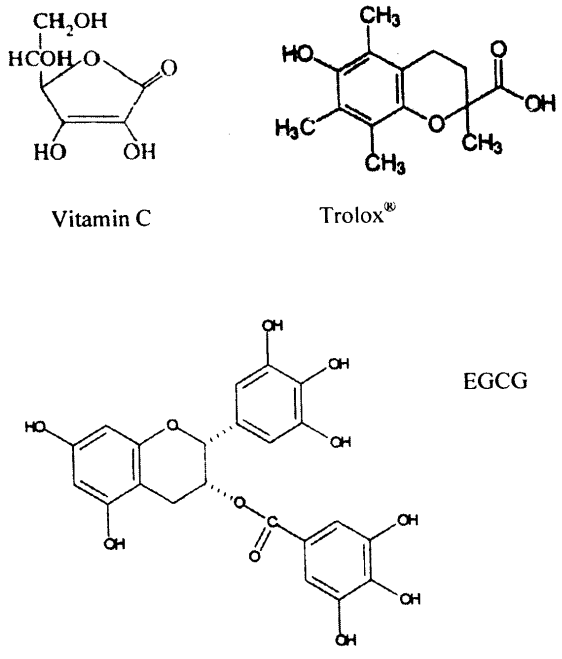


Figure 4 Structures of vitamin C, Trolox® and EGCG

However, plant phenolics have sometimes been found to shown pro-oxidant properties toward non lipid under certain circumstances. Several flavonoids have been shown to auto-oxidize and generate reactive oxygen species, such as hydrogen peroxide. They are also capable of reducing Fe^{3+} to Fe^{2+}, resulting in the formation of hydroxyl radicals by interaction of $Fe^{2+}$with H_2O_2 (Laughton et al., 1989; Halliwell et al., 1992; Li and Xie, 2000). Aruoma et al., (1993) reported that several phenolic antioxidants can accelerate oxidative damage of DNA, protein, and carbohydrate *in vitro*. Tea polyphenol (TP) was also reported to have pro-oxidant effects at lower dosages in the aqueous phase. The phenolic compounds act as pro-oxidants under certain conditions, dependent on parameters such as concentration of phenolic compounds and transition metal ions (Yen, Chen and Peng, 1997). Hagerman et al., (1998) reported that high molecular weight plant polyphnols such as tannins were excellent biological antioxidants and that polygalloyl glucose (polyGG) has no pro-oxidant effect in the deoxyribose assay. Many investigations of the antioxidant and pro-oxidant effects of simple phenols have done, and Li and Xie (2000) reported that the scavenging affects of tea catechin oxypolymers (TCOP) to both the hydroxyl radical and superoxide radical was stronger than that of tea catechin (TC), and also they had no pro-oxidant effect.

5. Anti-agents

Many modalities of treatment for acquired skin hyperpigmentation are available including chemical agents or physical therapies, but none are completely satisfactory. As a result of the key role played by tyrosinase in melanin biosynthesis, most whitening agents act specifically to reduce the function of this enzyme by means of several mechanisms: interference with its transcription and/or glycosylation, inhibition by different modalities, reduction of by-products and post-transcriptional control. The classification of tyrosinase inhibitors is difficult because of the capacity of several compounds to work in different ways, interacting with both catalytic and regulatory sites of the enzyme or being metabolized to a product that, in turn, can act as either a non-competitive or a competitive inhibitor. Most of the inhibitors are phenol/catechol derivatives, structurally similar to tyrosine or DOPA, which act as alternative substrates of tyrosinase without producing pigment (Briganti, Camera and Picardo, 2003). A number of naturally occurring tyrosinase inhibitors have been described, the majority of which consisting of a phenol structure or of metal chelating agents. Antioxidants or compounds with redox properties can prevent or delay pigmentation by different mechanisms: by scavenging ROS and RNS, know to induce melanin synthesis, or by reducing *o*-quinones or other intermediates in the melanin biosysnthesis, and thus delaying oxidative polymerization. Chalcones have been reported to have the potency of tyrosinase inhibition and the antioxidant behavior by presenting phenolic hydroxyls and exhibiting the ability to donate electrons (Nerya et al., 2004).

6. Antioxidants in guava

Guava is a fresh fruit crop, which contains high levels of antioxidant compounds and other nutritional values. Its fruits contain high ascorbic acid content (50-300 mg/100g fresh weight) (Thaipong and Boonparakob, 2005), which is 3 to 6times higher than the content in orange. Carotenoids in a red flesh Brazillian guava identified were as phytofluene, β-carotene, γ-carotene, lycopene, β-cryptosanthin, rubixanthin, cryptoflavin, lutein and neochrome (Mercadante et al., 1999), whereas only β-carotene and lycopene were identified from ripe pink-fleshed guava (Wilberg and Rodriguez-Amaya, 1995). Setiwas et al,. (2001) reported that guava was found to be excellent sources of provitamin A carotenoids among 18 selected Indonesia fruits. Phenolic compounds such as myricetin and apigenin (Miean and Mohamed, 2001), ellagic acid, and anthocyanin (Misra and Seshadri, 1968) were also found high in guava fruits. Guava juice showed high antioxidant activity (Jimenez-Escrig et al., 2001; Leong and Shui, 2002; Guo et al., 2003; Thaipong et al., 2005, 2006). Guava fruit showed a 2-fold higher antioxidant activity level in the skin than in the flesh (Suwanphong, 2007). When compared with 27 other selected fruits in Singapore market, guava was in the high antioxidant activity group along with plum, strawberry, seedless grape, starfruit and salak. Forty-eight percent of the antioxidant activity was contributed by ascorbic acid (Leong and Shui, 2003).

7. *In vitro* digestion and metabolism of phenolic compound

The human gastrointestinal physiology, the digestion of carbohydrates and the metabolism of phenolic compounds are described in the following sections in order to elucidate the choices regarding the reagents, enzymes, operating pH values and incubation times of each stage on the *in vitro* digestion moles.

7.1 Mouth

The digestion of food starts in the mouth, where teeth grind the plant food with a force of 300-1000 N. The disintegrated plant matrix contains cell-wall tissue pieces with a large proportion of intact cells (McDougall et al., 1996). The hydrolysis of starch in the mouth, where maltose, maltotriose and α-limit dextrins are formed. However, only 5 % of all the starch eaten is hydrolysed by the time the food swallowed. During mastication the principal salivary glands start their secretion of saliva, which ranges daily between 800 and 1500 ml. Saliva contains two major types of proteins: ptyalin, an α-amylase for starch digestion, and mucin for lubrication and surface protection purposes. Saliva also contains a high contains a high concentration of potassium and bicarbonate and some sodium and chloride.

7.2 Stomach

In the stomach, gastric glands secrete pepsin as pepsinogen, gastrin hormone and hydrochloric acid for protein digestion. Hydrochloric acid is secreted as a 160 mM solution at pH 0.8. Total daily gastric secretion is about 1500 ml. The hydroysis of starch continues in the stomach for 1 hour and 30-40% of starch in hydrolysed to maltose. Salivary amylase activity is inhibited by the acid when the pH of the chime falls below 4.0. Many plant cells burst in the acidic conditions of the

stomach due to breakage of acid-labile bonds, and polysaccharide residues may be released. The solubility of carbohydrates is enhanced and the viscosity of the stomach contents is increased causing the felling of satiety. The movements of the gastric muscles mix and disintegrate food further to produce a semifluid chime with gastric secretions. The stomach stores food and regulates the chime at suitable rate for digestion and absorption.

7.3 Duodenum and ileum

In the human body the amount and the characteristics of pancreatic juice are determined by the presence of chime and its food components in the upper portions of the small intestine. Pancreatic juice contains proteolytic enzyme (trypsin, chymotrypsin, carboxypolypeptidase, elastase and nuclease), amylase for starch and glycogen hydrolysis, lipase for neutral and cholesterol esterase. The daily pancreatic secretion is approximately 1000 ml. Bicarbonate ions are also secreted in order to increase the duodenal pH. Proteolytic enzymes are in an inactive form and are activated enzymatically by enterokinase or by active trypsin. Pancreatic juice also contains a trypsin inhibitor. When phenolic compound are consumed in the diet, they are released from the matrix after mastication, the released part is absorbed through the ileal epithelium and the rest is released, metabolised and absorbed in the colon. Absorption is affected by the structure of phenolic compound (glycosylation, molecular weight and esterification) (Scalbert et al., 2002). Phenolic compounds can be transported through the epithelium as glycosides by sugar transporters. In the epithelial cells, cytosolic β-glucosidase hydrolyzes these glycosides, and aglycones are formed after absorption (Figure 5). Aglycones can also be formed in the lumen by

the action of membrane-bound lactase phlorizin hydrolase (LPH) and they are absorbed passively through the epithelium (Scalbert and Williamson 2000).

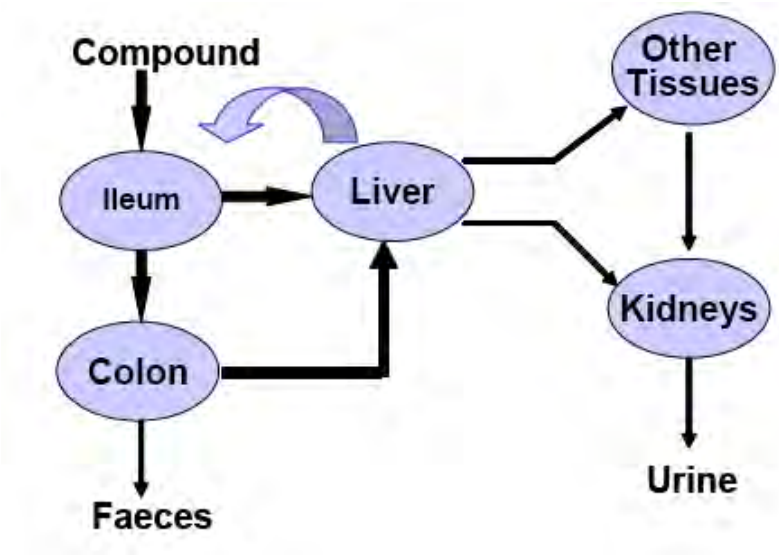


Figure 5 General metabolism of phenolic compounds, modified from Scalbert and Williamson (2001).

Once absorbed, aglycones undergo a conjugation in the ileal epithelium or in the liver. Hepatic metabolites (methylated, sulphated or glucuronidated conjugates) are returned to the luminal side via bile (enterohepepatic circulation) (Figure 6)

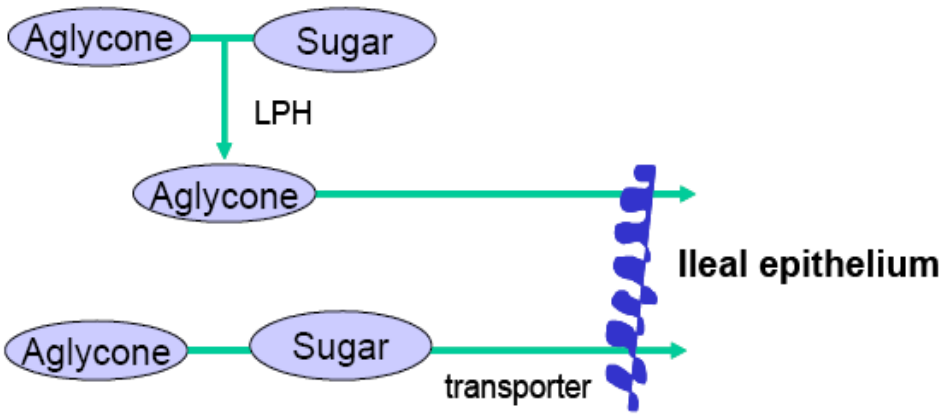


Figure 6 The deglycosylation of phenolic compounds, modified from Day et al., 2002

7.4 Colon

The colon is divided into the caecum, ascending colon, transverse colon, descending colon, sigmoid and rectum. Due to the absorption only about 1500 ml of chime is emptied from the ileum to the caecum daily. The only secretory function in the colon is the mucus secretion with bicarbonate. The epithelial cells contain enzymes only in small amounts. Mucus holds the faecal matter together, protects the colonic wall from the microbial activity and bicarbonate provides a barrier against the acidic metabolites formed by the microbial action in the colon. Radiotelemetric measurements have shown that the pH at the ileo-caecal valve is 7.9, but decrease rapidly to 5.4 in the transverse colon, indicating a rapid formation of acidic metabolites. Study of these conversion reactions is the main interest *in vitro* colon model and these aspects are discussed in detail in the following sections.

Those phenolic compounds which enter the colon are unabsorbed glycosides, such as quercetin-3-rhamnoglucoside, and conjugates after ilea and hepatic metabolism via enterohepatic circulation (Scalbert and Williamson 2000). Compound can also be enclosed in the food matrix and thus absorption can be prevented (Nielsen et al., 2003). When compounds reach the caecum, they are subjected to the microbial metabolism. Many flavonoids under ring-fission, in which the B-ring is degraded and phenolic acids are formed (Figure 7). *Butyrivibrio* sp. C_3, *Clostridium orbiscindens* sp. nov. and *Eubacterium ramulus* were capable of the ring-fission of quercetin, whereas only *Enterococcus casseliflavus* was able to utilize glucose from quercetin-3-glucoside and it did not attack the C-ring. Microbial metabolites are absorbed from the colon and are again subjected to the metabolism of

the liver resulting in glucuronidated and sulphated conjugates. Microbial metabolites also appear in plasma and urine. Microbial metabolites of several flovonoids have been identified either from *in vivo* animal trials or from clinical trials involving human subjects. Furthermore, phenolic compounds have been incubated with animal caecal or human faecal microbiota.

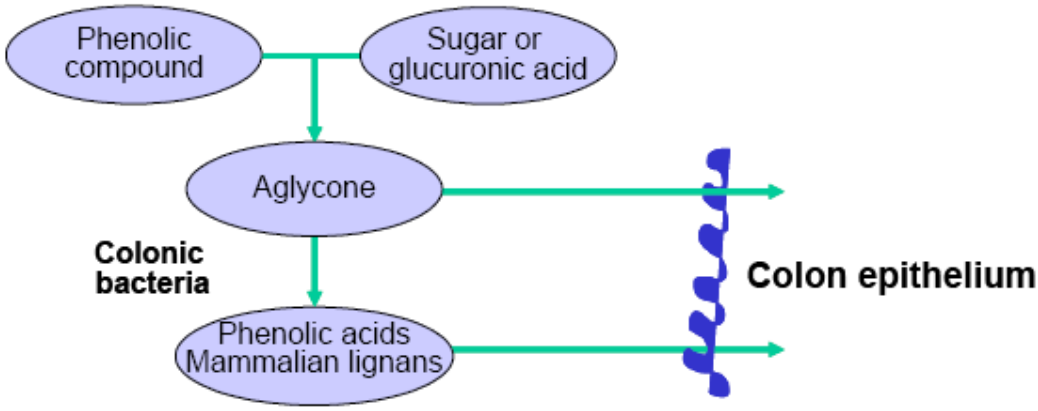


Figure 7 The microbial metabolism of phenolic compounds in the colon.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Guava (*Psidium guajava* L.) samples

1.2 Chemicals

1.3 Equipments

2. Methods

2.1 Sample preparation and extraction

2.2 Determination of total polyphenolic contents

2.3 Determination of free radical scavenging activity using DPPH method

2.4 Determination of superoxide anion scavenging activity

2.5 Determination of hydroxyl radical scavenging activity

2.6 Determination of anti-tyrosinase activity

2.7 Determination of vitamin C contents

2.8 Determination of phenolic compounds

2.9 Statistical analysis

1. Materials

1.1 Guava (*Psidium guajava* L.) samples

- Pansithong
- Thai original
- Kimju

The guava was obtained from Sampran Nakornpathom in Thailand.

1.2 Chemicals

- (-)-Epigallocatechin gallate (EGCG) (Sigma USA)
- 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma USA)
- 2-Deoxy-D-Ribose($C_5H_{10}O_4$) (Fluka, Switzerland)
- 2-Thiobarbituric acid anhydrous (TBA) (**S**igma USA)
- 95% Ethanol (C_2H_5OH), commercial grade (Merck, Germany)
- Acetic acid (CH_3COOH), analytical grade, Carlo Erba reagent)
- Acetonitrile (CH_3CN) (HPLC grad), (Lab scan)
- Bile extract porcine (Sigma USA)
- Di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) (Carlo Erba reagent)
- Ferulic acid, (HPLC) grade (Fluka, Switzerland)
- Folin-Ciocalteu's phenol reagent (Carlo Erba reagent)
- Gallic acid monohydrate ($C_7H_6O_5.H_2O$) (Sigma USA)
- Hydrogen peroxide 30% (H_2O_2) (Merck, Germany)
- Iron (III) chloride anhydrous ($FeCl_3$) (Merck, Germany)
- L-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma USA)

- L(+)-Ascorbic acid ($C_6H_8O_6$), Vitamin C), analytical grade (Fluka, Switzerland)
- Lipase (Sigma USA)
- Methanol (CH_3OH) (HPLC grad), (Lab scan)
- Mushroom tyrosinase (Sigma USA)
- Nitro Blue Tetrazolium ($C_{40}H_{30}C_{l2}N_{10}O_6$, NBT), analytical grade (Sigma USA)
- Pancreatin (Sigma, USA)
- Pepsin (Sigma, USA)
- Phenazine methosulfate ($C_{13}H_{11}N_2CH_3SO_4$), (PMS), (Acros Organics)
- Sodium bicarbonate ($NaHCO_3$),analytical grade (Carlo Erba reagent)
- Sodium carbonate (Na_2CO_3), analytical grade (Carlo Erba reagent)
- Sodium chloride (NaCl), analytical grade (Carlo Erba reagent)
- Sodium dihydrogen phosphate-2-hydrate ($NaH_2PO_4.2H_2O$) (Carlo Erba reagent)
- Sodium hydroxide (NaOH), analytical grade (Lab Scan)
- Titriplex®EDTA, analytical grade (Merck, Germany)
- Trichloroacetic acid (TCA), analytical grade (Sigma, USA)
- β-nicotinamide adenine dinucleotide anhydrous, tetrasodium salt type I, reduced from (β-NADH) (Sigma, USA)

1.3 Equipments

- Autopipette: Multipette plus, Eppendorf, Germany
- Centrifuge, refrigerated: Model HARRIER 18/80, Sanyo Gellenkamp PLC, UK.
- Homoginizer, Turbora, Thailand
- High Performance Liquid Chromatography (HPLC) : Model LC-3A Shimadzu, Japan
- Intertsil® ODS-3, GL Science Inc.,Japan
- Incubator shaker: Innova 4000, New Brunswick Scientific, USA
- Magnetic stirrer: Model Fisherbrand, Fisher Scientific, USA
- pH meter: pH900, Precisa, Switzerland
- Shaking water bath, NEW BRUNSWICK SCIENIFIC EDISON, N.J, USA
- UV-VIS spectrophotometer
- Vortex-Mixer: Model K-550-GE, Science Industries, USA
- Water bath, Memmert, Germany

Materials

- Parafilm, American National Can TM, USA
- Whatman, International Ltd Maidstone England

2. Methods

2.1 Sample preparation and extraction

2.1.1 Preparation

Fresh guava Pansitong, Thai original and Kimju were obtained from Sampran Nakornpathom in Thailand. All fresh guavas were washed in water and then rinsed with water.

2.1.2 Water extraction

Three grams of guava were mixed with 25 ml water (Deionized water) was homogenized (ultra turrax T25) at 13,500 rpm for 1 min. After that homogenates were kept at 4°C for how long and then centrifuged at 15,000 rpm for 20 min. The supernatants were recovered and stored at 4°C for further analysis.

2.1.2 Ethanol extraction

Three grams of guava were with 25 ml 40%ethanol. The homogenates were kept 4°C for 12 h and then centrifuged at 15,000 rpm for 20 min. The supernatants were recovered and stored at -20 °C for further analysis.

2.1.3 Simulated gastrointestinal digestion

Three grams of guava were mixed with 60 ml NaCl (120mM). After that homogenizer (ultra turrax T25) was used at 13,500 rpm for 1 min. The pH was decreased to 2.1±0.1 by drop-wise addition of 1M HCl. and pepsin was added to a final concentration of 40 mg/ml in 100 mM HCl (4 ml) adjust volume to 80 ml by NaCl (120 mM). The digestate was incubated in a shaking water bath 85 rpm at 37°C for 1 hour. The pH of the digestate was then increased to 6.0±0.1 with the drop-wise addition of 1M $NaHCO_3$. Porcine bile extract at a final concentration of 40 mg/ml in 100 mM $NaHCO_3$ (6 ml) and pancreatin-lipase were added to a final concentration of 10 mg/ml and 5 mg/ml in 100 mM $NaHCO_3$ (4 ml). The pH was increased to 6.9±0.1 by drop-wise addition of 1M $NaHCO_3$. After that the solution was adjusted volume to100 ml by adding salt solution (NaCl 120mM) and the samples were incubated for 2 hour in a shaking water bath 85 rpm at 37°C. The samples were centrifuged at 6,000 rpm, 4°C for 30 min and filtration by Whatman No 541. The supernatants were stopped reaction of enzyme in water bath 100°C for 20 min and stored at 4°C for further analysis.

2.2 Determination of total polyphenolic contents

Total polyphenolic contents in fruit extracts were determined according to Folin-Cioclateu's procedures (Parejo et al., 2002). Briefly, the reaction mixture contained 100 μl of each Guava extracts were added to 2 ml of 2% Na_2CO_3. After 2 min, 50% Folin-Cioclateu's reagent (100 μl) was added to the mixture which was then left to stand for 30 min. Absorbance was measured at 750 nm on a spectrophotometer. Gallic acid was used as a standard and the total polyphenolic contents were expressed as total gallic acid equivalent (GAE) (mg/g wet weight).

2.3 Determination of free radical scavenging activity using DPPH method

The H-donor activity of fruit extracts was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Abe et al., 2002). The DPPH is a stable radical; its ethanolic solution showed an absorbency maximum at 517 nm. The H-donor molecules reduced DPPH radical and thus decreased its absorbance at 517 nm.

Briefly, the reaction mixture contained 0.5 ml of dilutions of the ethanolic solution of Guava extracts, 0.5 ml of 0.1mM DPPH (in methanol). After standing for 30 minutes, the absorbance of the mixture was measured at 517 nm. The antioxidant compounds in the presence of DPPH radical scavenging activity was calculated by the following formula.

$$\% \text{ Radical scavenging activity} = \frac{[A_{\text{control}} - A_{\text{guava extracts}}]}{A_{\text{control}}} \times 100$$

$A_{\text{guava extract}}$ is the absorbance value of DPPH containing guava extract.

A_{control} is the absorbance value of DPPH

The DPPH scavenging activity was plotted against the concentration of the guava extract. A logarithmic regression curve was established in order to calculate the IC_{50}, which is the amount of concentration of guava extract necessary to decrease by 50% the absorbance value of the control.

2.4 Determination of superoxide anion scavenging activity

Antiradical activity in guava extracts was determined spectrophotometrically reader by minitoring the effect of guava extracts on the reduction of Nitro Blue Tetrazolium (NBT) to the blue chromogen formazan by superoxide radical ($O_2^{\bullet -}$), at 570 nm. Superoxide anions were generated according to the method of Valentao et al., (2001), except NADPH was used instead of NADH. The reaction mixture in the sample wells consisted of β-nicotinamide adenine dinucleotide ($NADH+H^+$) (468 μM), NBT (150 μM), phenazine methosulfate (PMS) (60 μM), and various concentration of guava extracts, in final volume of 1 ml then adjusted volume with 19 mM phosphate buffer, pH 7.4. The reaction conducted at room temperature for 15 minutes and initiated by the addition of PMS. The percentage of superoxide anion scavenging activity was calculated according to the formula below,

$$\% \text{ Superoxide anion scavenging activity} = \frac{[A_{\text{control}} - A_{\text{guava extracts}}] \times 100}{A_{\text{control}}}$$

$A_{\text{guava extract}}$ is the absorbance value of the reaction mixture containing guava extract.

A_{control} is the absorbance value of the reaction mixture without guava extract.

The superoxide anion scavenging activity was plotted against the concentration of guava extract. A logarithmic regression curve was established in order to calculate the $IC_{50,}$ which is the amount of concentration of guava extract necessary to decrease by 50% the absorbance value of the control.

2.5 Determination of hydroxyl radical scavenging activity

The deoxyribose method was used for determining the scavenging effect of the guava extract on hydroxyl radicals (Valentao et al., 2002). Briefly, the reaction mixture contained, in final volume of 1 ml, ascorbic acid (1mM), $FeCl_3$ (1mM), EDTA (1mM), H_2O_2 (10mM), deoxyribose (37.5mM), and various concentrations of guava extracts then adjusted volume with 20mM phosphate buffer pH 7.4. After incubation at 37°C in the water bath for 1 hour, 1 ml of 2.8%Trichloroacetic acid (w/v) and 1 ml of 1% Thiobarbituric acid (w/v) were added, and the mixture was heated in a water bath at 100°C for 15 minutes. The absorbance of the resulting solution was measured at 532 nm. This assay was also performed in the same manner but without ascorbic acid or EDTA, to evaluate the pro-oxidant and metal chelation potential of the guava extracts, respectively.

2.6 Determination of anti-tyrosinase activity

The guava extracts were screened for *o*-diphenolase inhibitor activity of tyrosinase using L-DOPA as the substrate (Sritularak, 2002; Nerya et al., 2004). Briefly, the reaction mixture contained, in final volume of 1 ml, L-DOPA (0.85mM), Mushroom tyrosinase (480units/ml) and various concentrations of guava extracts then adjusted volume with 20mM phosphate buffer pH 6.8. After the solution was mixed and preincubated at room temperature for 10 min, L-DOPA was added, and the mixture was further incubated at room temperature for 10 min. The absorbance was measured at 492 nm. The percentage of tyrosinase inhibition was calculated according to the formula below.

$$\% \text{ Tyrosinase inhibition} = \frac{[A-B]-[C-D]}{[A-B]}$$

A = the absorbance after incubation at 492 nm without test guava extracts

B = the absorbance after incubation at 492 nm without guava extracts and enzyme

C = the absorbance after incubation at 492 nm with guava extracts

D = the absorbance after incubation at 492 nm with guava extracts, but without enzyme

2.7 Determination of phenolic compounds

The contents of phenolic compounds in extracts from guava were determined by HPLC, UV-Vis detector set at 285 nm. The analyses were carried out on Inertsil® ODS-3 column. The elution solvents were (A) 0.2 M NaH_2PO_4 adjusted to pH 3.0 by phosphoric acid, and then diluted with distilled water to 50 mM NaH_2PO_4 and (B) 50 mM NaH_2PO_4/methanol/acetonitrile (30/20/50, v/v/v). The solvent gradient elution programmed used was as follows: initial 100% A, hold for 6 min; linear gradient to 92% A in 8 min and hold for 6 min; linear gradient to 82% A in 35 min; linear gradient to 62% A in 10 min and hold for 15 min; linear gradient to 0% A in 10 min and hold for 10 min. The flow rate was 1 ml/min (Hui-Yin chen et al., 2007). Phenolic compounds were identified by comparison of their retention time (Rt) values and UV spectra with those of known standards and determined by peak areas from the chromatograms. All analyses were run in triplicate and mean values were calculated.

2.8 Determination of vitamin C contents

The contents of vitamin C in extracts from guava were determined by HPLC, UV-Vis detector set at 243 nm. The analyses were carried out on Inertsil® ODS-3 column using 2% acetic acid (v/v) solution in water pH 2.5 as the mobile phase at flow-rate of 1.0 ml/min at ambient temperature. The injection volume was 20 μl (Naoto Furusawa 2001). Vitamin C compounds were identified by comparison of

their retention time (Rt) values and UV spectra with those of known standards and determined by peak areas from the chromatograms. All analyses were run in triplicate and mean values were calculated.

2.9 Statistical analysis

Data were presented as mean ± standard deviation (S.D.) of triplicate examination. Two-way ANOVA using SPSS version 17 software was used for multiple comparisons. A value of $p < 0.05$ was considered significant.

CHAPTER IV

RESULTS

1. Determination of total polyphenolic contents
2. Determination of free radical scavenging activity using DPPH method
3. Determination of superoxide anion scavenging activity
4. Determination of hydroxyl radical scavenging activity
5. Determination of anti-tyrosinase activity
6. Determination of phenolic compounds
7. Determination of vitamin C contents

1. Determination of total polyphenolic contents

By using gallic acid as a standard in Folin-Ciocalteu method, the data showed that the amount of total polyphenolic contents in guava extracts was shown in Table 4. The contents of total polyphenolic compounds in every condition were in the order: Pansithong>Kimju>Thai original ($P<0.05$).

Table 4 The contents of total polyphenolic compounds of extracts from water (direct), 40%ethanol, SGD of guava.

Sample	**Total polyphenolic contents (mg gallic acid/100g)**		
	Thai original	**Pansithong**	**Kimju**
Water	$71.75\pm2.56^{A, a}$	$105.81\pm2.22^{B, a}$	$75.09\pm3.03^{A,a}$
40% Ethanol	$143.46\pm7.32^{A, b}$	$175.33\pm4.416^{B, b}$	$128.16\pm5.09^{A,b}$
SGD	$69.97\pm3.45^{A, c}$	$84.47\pm16.05^{B, c}$	$68.81\pm2.43^{A, c}$

A, B The number in vertical significant difference ($P<0.05$)

a, b, c The number in horizontal significant difference ($P<0.05$)

In the present work, the guava extracts by 40%ethanol contained higher amount of polyphenolic than of water and SGD in the each extraction ($P<0.05$).

2. Determination of free radical scavenging activity using DPPH method

DPPH is a free radical that has been widely used to measure the free radical scavenging activity. This method is based on the reduction of DPPH, stable free radical. The odd electron of DPPH shows a strong absorption maximum at 517 nm and its solution is deep purple color. As the odd electron of the radical becomes paired off, the absorption strength is decreased. The resulting decolorization is stoichiometric with respect to the number of electrons captured. The mechanism of the DPPH radical and various oxidizable groups were shown in Fig 8.

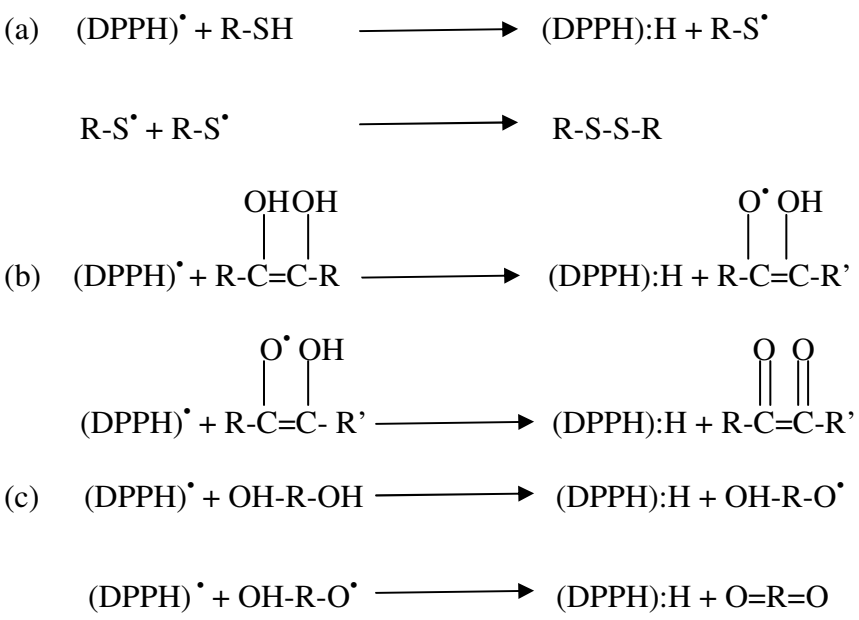


Figure 8 Schema of the reactions between the DPPH radical and various oxidizable groups. (a) Sulphydryl groups interacting in the ration of (1:1); (b) oxidation of the conjugated of ascorbic acid to the dehydrol form (2:1); (c) oxidation of a hydroquinone (2:1).

The plots of percentages of DPPH scavenging activity (% inhibition of free radical) at various concentrations (0, 1.5, 2.5, 3.5, 4.5, 5.5 and 6.5 mg/mL) of the sample shown in Fig 9. It was found of Pansithong the highest DPPH scavenging activity in water and 40% ethanol conditions extracts. The SGD extract showed highest in Kimju and the lowest scavenging activity on DPPH.

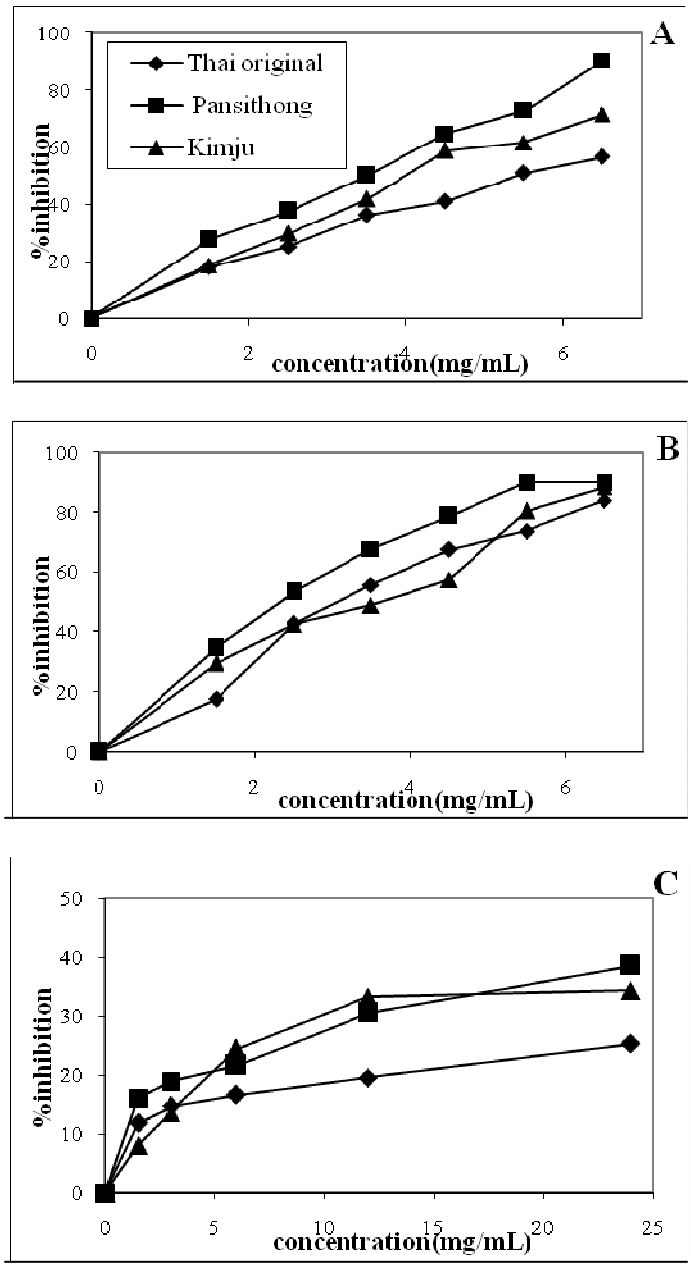


Figure 9 Relationship between percent DPPH radical inhibitions of guava fruit its various concentrations. A= water, B= 40%ethanol and C= SGD (Mean ± SD, n=3).

The concentration at 50% inhibition (IC_{50}) of each sample was calculated form equation of the logarithmic regression curve for determine and give in Table 5.

Table 5 IC_{50} values of guava extracts from water, 40%ethanol, SGD on DPPH radical scavenging activities.

Sample	**DPPH scavenging activity IC_{50} (mg/mL)**		
	Thai original	**Pansithong**	**Kimju**
Water	$5.46\pm0.35^{A,a}$	$3.51\pm0.07^{B,a}$	$4.30\pm0.01^{B,a}$
40% Ethanol	$3.52\pm0.06^{A,a}$	$2.76\pm0.08^{B,a}$	$3.47\pm0.02^{B,a}$
SGD	$61.63\pm7.77^{A,b}$	$30.15\pm0.53^{B,b}$	$27.83\pm1.93^{B,b}$

A, B The number in vertical significant difference ($P<0.05$)

a, b The number in horizontal significant difference ($P<0.05$)

For the comparison with the guava extracts by water direct and 40%ethanol obtained here gave higher DPPH scavenging activity (lower IC_{50} values) more than SGD extract as seen from the significantly lower IC_{50} value ($P <0.05$). This chemical structure of phenolic compound was digested by the *in vitro* digestion models.

3. Determination of superoxide anion scavenging activity

Nitro Blue Tetrazolium (NBT) is often used as an indicating scavenger for $O_2^{\bullet-}$ In which role it offers several advantages. Thus, it is reduced by $O_2^{\bullet-}$ and produces a water-insoluble formazan. The superoxide radicals were generated in a PMS/NADH system and assayed by the reduction of NBT according to the method of Valentao et al., (2001), except NADPH was used instead of NADH.

$$NADPH + H^+ + PMS \longrightarrow NADP^+ + PMSH_2 \quad (1)$$

When NBT was present with PMS+NADPH, it was reduced (reaction (2) and (3)). The NBT radical ($NBTH^{\bullet}$) causes the univalent reduction of dioxygen (reaction (4)). We therefore write the following reactions.

$$PMSH_2 + NBT \longrightarrow PMSH^{\bullet} + NBTH^{\bullet} \quad (2)$$

$$PMSH^{\bullet} + NBT \longrightarrow PMS + NBTH^{\bullet} \quad (3)$$

$$NBTH^{\bullet} + O_2 \longleftrightarrow NBT + H^+ + O_2^{\bullet-} \quad (4)$$

$$2NBTH^{\bullet} \longrightarrow NBT + NBTH_2 \quad (5)$$

Reaction (4) is an equilibrium which can be displaced to the right by the removal of $O_2^{\bullet-}$. Antioxidant compound would lower the steady-state concentration of $NBTH^{\bullet}$ and there decreased the rate of production of the formazan by reaction (5)

The plots of percentages of superoxide anion scavenging activity (% inhibition of free radical) at various concentrations (0, 40, 60, 80, 100, and 120 mg/mL) of the sample shown in Fig 10. It was found of Thai original extracted using 40%ethanol demonstrated the highest superoxide anion scavenging properties. The SGD extract of three samples presented the highest inhibition activity at the concentration of 60 mg/mL.

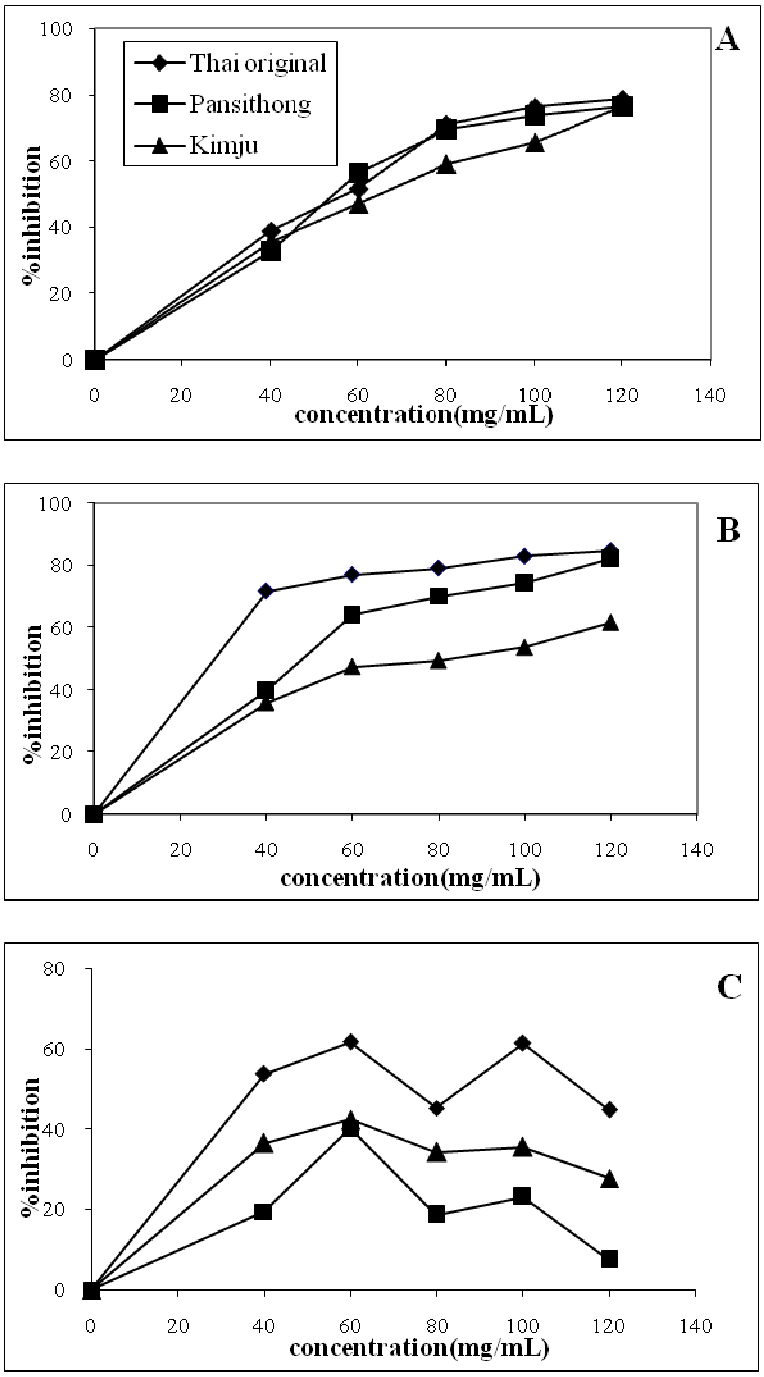


Figure 10 Plot of percent inhibition of guava extracts against superoxide anion scavenging activity. A= water, B= 40%ethanol and C= SGD (Mean ± SD, n=3).

IC_{50} is the concentration of each guava extracts which necessary to decrease by 50% the absorbance value of the control. The result was shown in Table 6.

Table 6 IC_{50} values of guava extracts from water, 40%ethanol on superoxide anion scavenging activities.

Sample	**Superoxide scavenging activity IC_{50} (mg/mL)**		
	Thai original	**Pansithong**	**Kimju**
Water	62.54 ± 0.88^{A}	64.54 ± 1.88^{B}	71.13 ± 5.70^{C}
40% Ethanol	41.47 ± 2.04^{A}	59.11 ± 0.02^{B}	85.03 ± 2.30^{C}

A, B, C The number in vertical significant difference ($P<0.05$)

SGD = not available

4. Determination of hydroxyl radical scavenging activity

Deoxyribose (2-deoxy-D-ribose) is degraded into malonaldehyde on exposure to hydroxyl radicals that generated by the Fenton system. The reaction mixture is heated under acid conditions and may be detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen (Halliwell et al., 1987; Aruoma, 1994). Including a reducing agent, such as ascorbic acid, in the reaction mixture may increase the rate deoxyribose degradation and maintaining a supply of Fe^{2+}.

$$Fe^{3+}\text{ - EDTA + ascorbate} \longrightarrow Fe^{2+}\text{ - EDTA + oxidized ascorbate} \quad (1)$$

The oxidation of deoxyribose proceeds by the following mechanis

$$Fe^{2+}\text{ - EDTA + } H_2O_2 \longrightarrow Fe^{3+}\text{ - EDTA + } OH^- + {}^{\bullet}OH \quad (2)$$

$${}^{\bullet}OH\text{ + deoxyribose} \longrightarrow \text{fragments} \xrightarrow[\text{Heat with TBA}]{\text{Plus acid}} \text{MDA} \quad (3)$$

$$2\text{TBA + MDA} \longrightarrow \text{pink chromogen} \quad (4)$$

The hydroxyl radicals were generated by the Fenton system. Hydroxyl radicals detected by their to degrade the sugar deoxyribose into fragments, on heating with thiobarbituric acid at low pH, which generated a pink chromogen.

Table 7 Scavenging effect of Thai original (water) on hydroxyl radical

Conc. (mg/mL)	**A_{532}**	**A_{532}(-AA)**	**A_{532}(-EDTA)**
0	0.072±0.002	0.088±0.009	0.123±0.006
20	0.056±0.001*	0.099±0.007	0.107±0.008
40	0.051±0.002*	0.099±0.015	0.099±0.013*
60	0.045±0.003*	0.096±0.008	0.084±0.011*
80	0.041±0.005*	0.105±0.010	0.091±0.009*
100	0.045±0.001*	0.116±0.003*	0.105±0.006
120	0.051±0.005*	0.134±0.017*	0.140±0.027

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control (0 mg/mL of Thai original (water)) values:*P <0.05.

Table 8 Scavenging effect of Pansithong (water) on hydroxyl radical

Conc. (mg/mL)	**A_{532}**	**A_{532} (-AA)**	**A_{532} (-EDTA)**
0	0.138±0.005	0.094±0.002	0.144±0.009
20	0.057±0.007*	0.064±0.001*	0.073±0.060*
40	0.060±0.003*	0.069±0.006*	0.070±0.017*
60	0.054±0.007*	0.062±0.009*	0.092±0.008*
80	0.072±0.005*	0.068±0.003*	0.091±0.006*
100	0.081±0.006*	0.084±0.012	0.102±0.009*
120	0.097±0.006*	0.084±0.003	0.114±0.009*

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control (0 mg/mL of Pansithong (water)) values:*P <0.05.

Table 9 Scavenging effect of Kimju (water) on hydroxyl radical

Conc. (mg/mL)	**A_{532}**	**A_{532} (-AA)**	**A_{532} (-EDTA)**
0	0.130±0.003	0.095±0.004	0.126±0.006
20	0.090±0.003*	0.083±0.003*	0.090±0.000*
40	0.094±0.005*	0.089±0.002	0.081±0.007*
60	0.094±0.005*	0.091±0.002	0.087±0.003*
80	0.105±0.005*	0.108±0.012*	0.088±0.004*
100	0.120±0.009*	0.112±0.004*	0.095±0.008*
120	0.137±0.006	0.115±0.007*	0.099±0.007*

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control (0 mg/mL of Kimju (water)) values:*P <0.05.

Table 10 Scavenging effect of Thai original (40%ethanol) on hydroxyl radical

Conc. (mg/mL)	**A_{532}**	**A_{532} (-AA)**	**A_{532} (-EDTA)**
0	0.112±0.001	0.107±0.003	0.139±0.002
20	0.041±0.011*	0.080±0.009	0.098±0.012*
40	0.087±0.007*	0.098±0.007	0.113±0.001*
60	0.095±0.003	0.107±0.003	0.109±0.007*
80	0.116±0.010	0.109±0.017	0.134±0.017
100	0.144±0.008*	0.137±0.026*	0.177±0.006*
120	0.181±0.022*	0.173±0.019*	0.155±0.022

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control (0 mg/mL of Thai original (40%ethanol)) values:*P <0.05.

Table 11 Scavenging effect of Pansitong (40%ethanol) on hydroxyl radical

Conc. (mg/mL)	**A_{532}**	**A_{532} (-AA)**	**A_{532} (-EDTA)**
0	0.128±0.008	0.121±0.003	0.101±0.002
20	0.091±0.014	0.094±0.013	0.082±0.004*
40	0.114±0.011	0.128±0.005	0.085±0.002*
60	0.110±0.026	0.135±0.003	0.099±0.001
80	0.131±0.012	0.145±0.004	0.118±0.001*
100	0.154±0.024	0.160±0.023*	0.121±0.002*
120	0.149±0.031	0.141±0.026	0.133±0.003*

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control (0 mg/mL of Pansithong (40%ethanol)) values: *P <0.05.

Table 12 Scavenging effect of Kimju (40%ethanol) on hydroxyl radical

Conc. (mg/mL)	**A_{532}**	**A_{532} (-AA)**	**A_{532} (-EDTA)**
0	0.111±0.005	0.114±0.002	0.134±0.007
20	0.075±0.019*	0.087±0.001*	0.139±0.016
40	0.103±0.011	0.090±0.005*	0.119±0.008
60	0.116±0.005	0.098±0.019	0.101±0.007*
80	0.135±0.007*	0.112±0.010	0.147±0.009
100	0.136±0.011*	0.100±0.014	0.157±0.009
120	0.157±0.022*	0.147±0.022*	0.157±0.026

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control (0 mg/mL of Kimju (40%ethanol)) values: *P<0.05.

Table 13 Scavenging effect of Thai original (SGD) on hydroxyl radical

Conc. (mg/mL)	Control	A_{532}	Control	A_{532} (-AA)	Control	A_{532} (-EDTA)
20	0.054±0.011	0.079±0.004*	0.032±0.002	0.050±0.002*	0.084±0.000	0.067±0.007*
40	0.056±0.006	0.082±0.001*	0.028±0.005	0.050±0.003*	0.074±0.011	0.065±0.004*
60	0.057±0.006	0.057±0.003	0.029±0.006	0.055±0.001*	0.085±0.007	0.046±0.010*
80	0.058±0.011	0.049±0.006*	0.072±0.008	0.028±0.009*	0.060±0.021	0.038±0.007*
100	0.049±0.012	0.055±0.018*	0.028±0.012	0.054±0.009*	0.080±0.018	0.037±0.005*
120	0.016±0.008	0.049±0.012*	0.003±0.016	0.036±0.023*	0.068±0.011	0.001±0.025*

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control of Thai original (SGD) values:*P <0.05.

Table 14 Scavenging effect of Pansithong (SGD) on hydroxyl radical

Conc. (mg/mL)	Control	A_{532}	Control	A_{532} (-AA)	Control	A_{532} (-EDTA)
20	0.054±0.011	0.099±0.007*	0.032±0.002	0.065±0.003*	0.084±0.000	0.095±0.009
40	0.056±0.006	0.115±0.018*	0.028±0.005	0.099±0.006*	0.074±0.011	0.088±0.004*
60	0.057±0.006	0.125±0.008*	0.029±0.006	0.095±0.005*	0.085±0.007	0.089±0.021
80	0.058±0.011	0.120±0.009*	0.072±0.008	0.101±0.007*	0.060±0.021	0.088±0.004*
100	0.049±0.012	0.120±0.012*	0.028±0.012	0.137±0.013*	0.080±0.018	0.072±0.025
120	0.016±0.008	0.101±0.020*	0.003±0.016	0.102±0.013*	0.068±0.011	0.108±0.014*

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control of Pansithong (SGD) values:*$P<0.05$.

Table 15 Scavenging effect of Kimju (SGD) on hydroxyl radical

Conc. (mg/mL)	Control	A_{532}	Control	A_{532} (-AA)	Control	A_{532} (-EDTA)
20	0.054±0.011	0.063±0.003*	0.032±0.002	0.066±0.002*	0.084±0.000	0.077±0.006
40	0.056±0.006	0.069±0.005*	0.028±0.005	0.072±0.007*	0.074±0.011	0.056±0.004*
60	0.057±0.006	0.066±0.012	0.029±0.006	0.046±0.006*	0.085±0.007	0.057±0.006*
80	0.058±0.011	0.073±0.002*	0.072±0.008	0.043±0.004*	0.060±0.021	0.056±0.007*
100	0.049±0.012	0.084±0.004*	0.028±0.012	0.066±0.006*	0.080±0.018	0.054±0.004*
120	0.016±0.008	0.004±0.001	0.003±0.016	0.088±0.015*	0.068±0.011	0.064±0.006

Each value is the mean ± S.D. of three replicates; A_{532} (-AA) is the absorbance value of the reaction mixture without ascorbic acid; A_{532} (-EDTA) is the absorbance value of the reaction mixture without EDTA. Significance of the mean differences from each set of the control of Kimju (SGD) values:*P <0.05.

The abilities of the hydroxyl scavenger are followed: Pansithong> Thai original>Kimju when used water extracted of guava in Table 7-9, while Thai original> Kimju> Pansithong when used 40% ethanol in Table 10-12. Kimju guava extracted using SGD demonstrated the highest hydroxyl scavenging properties in Table13-15.

To evaluate to pro-oxidant potential of guava extracts, this assay was conducted in absence of ascorbic acid (Velaentao et al., 2002). As show in Table 8-14 every guavas and extracted condition showed pro-oxidant properties

Deoxyribose was also damaged by the reaction mixture that absence of EDTA, because omission of the chelator allows iron ions to bind directly to the sugar. Compounds which can inhibit deoxyribose degradation in the absence of EDTA are those with iron ion-binding capacity and which can withdraw the iron ions and render them inactive or poorly active in Fenton reaction (Paya et al., 1992). The assay performed in the absence of EDTA has showed weak metal chelation potential in the every guavas and extracted condition.

5. Determination of anti-tyrosinase activity

Tyrosinase is a copper-containing enzyme involved in producing high molecular weight brown pigment (melanins), which is the key physiological defense against sun-induced damage. Tyrosinase inhibitors have become increasingly important in medication and in cosmetics to prevent hyperpigmentation by inhibiting tyrosinase enzyme (Nerya et al., 2004). The inhibition effect on tyrosinase activity was examined by the DOPAchrome enzymatic method using L-DOPA as the substrate. Dopachrome is one of the intermediate substances in the melanin biosynthesis in which the red color can be detected by visible light. In the present study was used to measure the changes in absorbance at 492 nm due to the formation of DOPAchrome.

The plots of percentages of anti-tyrosinase activity at various concentrations (0, 40, 60, 80, 100, and 120 mg/mL) of the sample shown in Fig 11. It was found of Kimju extracted using water demonstrated the highest anti-tyrosinase activity properties. The SGD extract of three samples presented the highest inhibition activity at the concentration of 40 mg/mL.

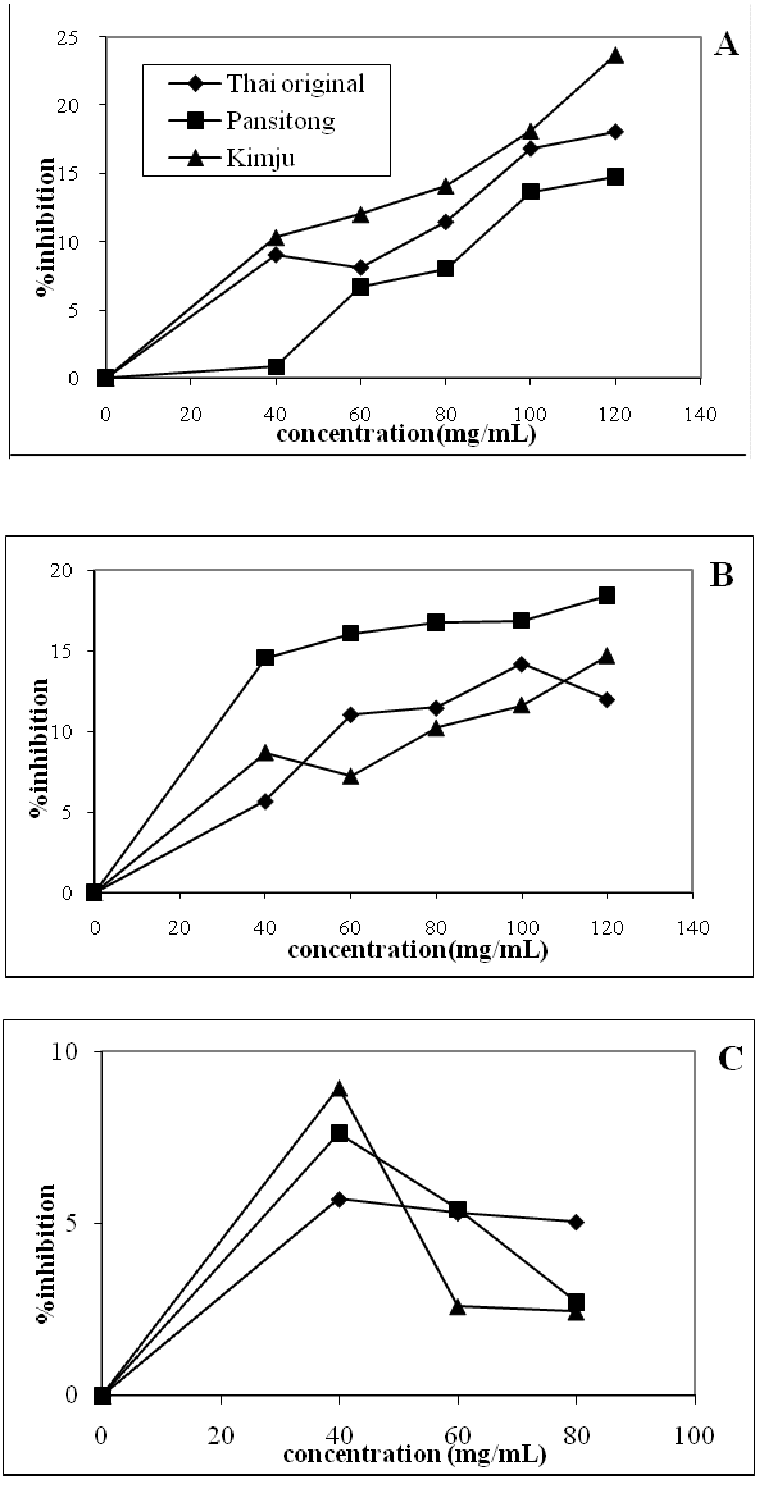


Figure 11 Plot of percent inhibition of guava extracts against tyrosinase activity. A= water, B= 40%ethanol and C= SGD (Mean ± SD, n=3).

The anit-tyrosinase activity was plotted against the concentration of guava extracts. A logarithmic regression curve was established. The results are shown in Table 16.

Table 16 IC_{50} values of guava extracts from water, 40%ethanol, of guava on tyrosinase.

Sample	**Tyrosinase inhibition IC_{50} (mg/mL)**		
	Thai original	**Pansithong**	**Kimju**
Water	364.52 ± 17.46^{A}	389.49 ± 17.01^{A}	290.71 ± 29.48^{A}
40% Ethanol	404.93 ± 53.98^{A}	319.20 ± 31.35^{A}	392.97 ± 66.83^{A}

A, B The number in vertical significant difference ($P<0.05$)

SGD = not available

The ani-tyrosinse contained modulate in every condition and the inhibition activity was decreased every condition.

6. Determination of phenolic compounds

The contents of phenolic compounds in extracts from guava were determined by HPLC, UV-Vis detector set at 285 nm. The analyses were carried out on Inertsil® ODS-3 column. Phenolic compound, such as quercetin, rutin, narigin, catechins, caffeic acid, gallic acid and chlorognic acid, are very important plant constituents because of their antioxidant activities. However, the result from the chromatogram indicated that guava extracts contained phenolic acid. Fig 12 shows the choromatogram of standards. A good resolution, with sharp peaks, was achieved for all phenolic compound standards within 90 min. The results of HPLC analyses show that three main peaks were identified by comparison of their retention time value and UV spectra with those of known standards. The contents of phenolic acids in guava extracts are shown in Table 17.

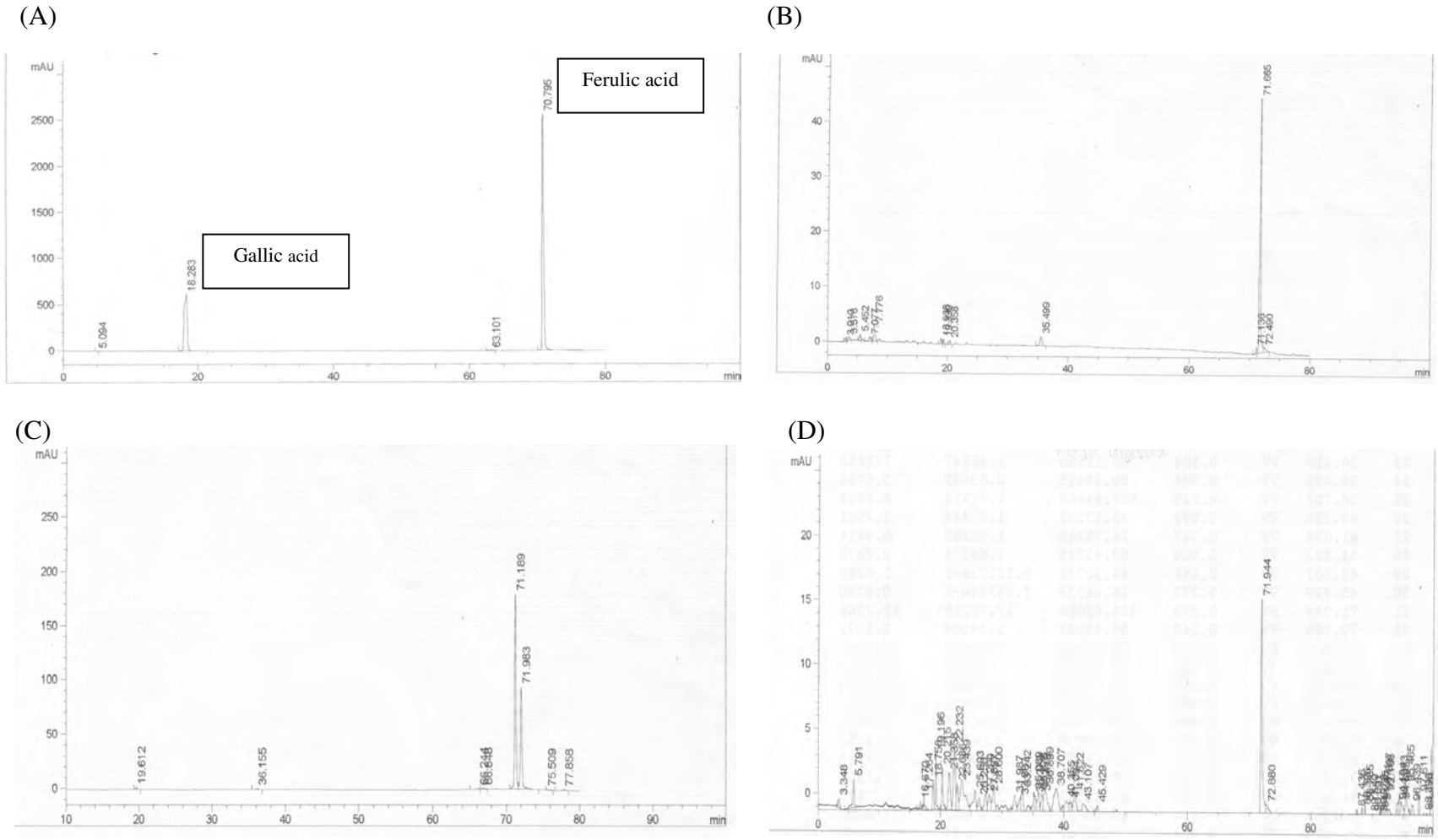


Figure 12 HPLC chromatograph of phenolic compounds (A) phenolic compound standards (B) water extract from Kimju (C) 40%ethanol extract from Kimju (D) SGD extract from Kimju.

Table 17 The contents of total phenolic acids of extracts from guava.

Sample	Ferulic acid (mg/100g)		
	Thai original	**Pansithong**	**Kimju**
Water	$7.00\pm2.00^{A,a}$	$5.29\pm2.47^{B,a}$	$5.28\pm4.59^{C,a}$
40% Ethanol	$7.07\pm5.92^{A,b}$	$6.58\pm5.37^{B,b}$	$14.00\pm6.32^{C,b}$
SGD	$3.96\pm3.76^{A,c}$	$3.31\pm2.17^{B,c}$	$3.67\pm3.76^{C,c}$

A,B,C The number in vertical significant difference ($P<0.05$)

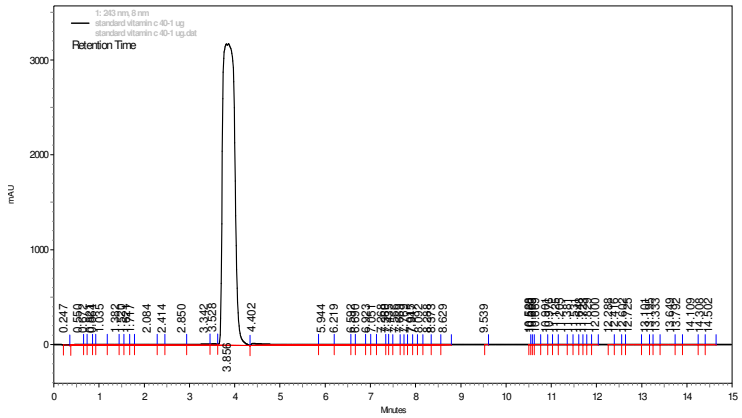
a,b.c The number in horizontal significant difference ($P<0.05$)

The highest amounts of ferulic acid were found in Kimju using 40% ethanol extracts ($P<0.05$) when in SGD the structure of phenolic acid in guava digested by stimulated gastrointestinal digestion see in Fig 12.

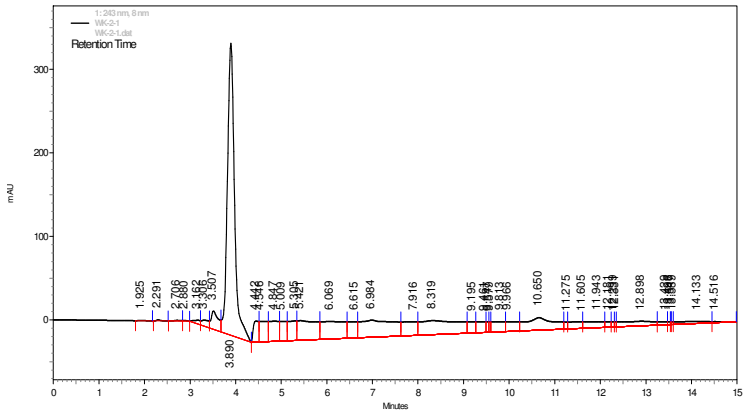
1. Determination of vitamin C contents

The presents a fast and simple method for identification/quantification of vitamin C in guava by HPLC using UV-Vis detector set at 243 nm. The analyses were carried out on Inertsil® ODS-3 column. The result from the chromatogram indicated that guava extracts contained vitamin C. Fig 13 show the choromatogram of standards. A good resolution, with sharp peaks, was achieved for vitamin C standards within 15 min. The results of HPLC analyses show that three main peaks were identified by comparison of their retention time value and UV spectra with those of known standards. The contents of vitamin C in guava extracts are shown in Table 18.

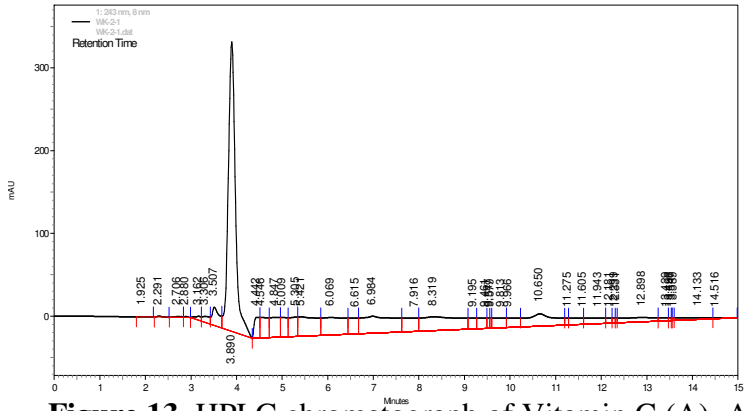
(A)



(B)



(C)



(D)

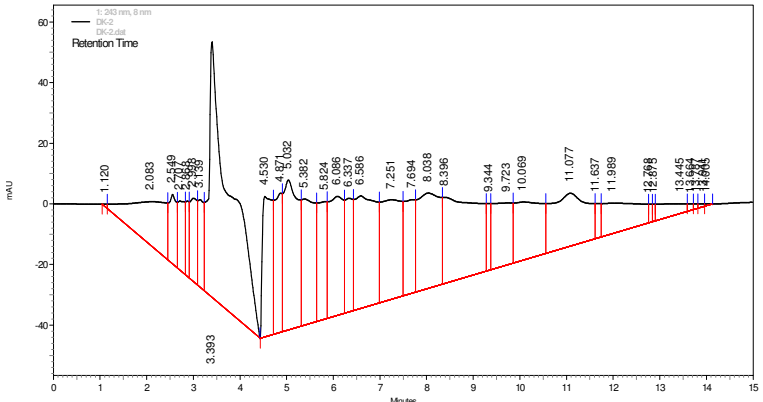


Figure 13 HPLC chromatograph of Vitamin C (A) Ascorbic acid standards (B) water extract from Kimju (C) 40%ethanol extract from Kimju (D) SGD extract from Kimju.

Table 18 The contents of total ascorbic acids of extracts from guava.

Sample	Ascorbic acid (mg/100g)		
	Thai original	**Pansithong**	**Kimju**
Water	$150.54\pm0.23^{A,a}$	$131.65\pm0.77^{B,a}$	$159.97\pm2.33^{C,a}$
40% Ethanol	$121.83\pm0.35^{A,b}$	$121.93\pm0.53^{B,b}$	$130.59\pm0.93^{C,b}$
SGD	$111.96\pm1.31^{A,c}$	$110.61\pm0.17^{B,c}$	$111.75\pm0.12^{C,c}$

A, B, C The number in vertical significant difference ($P<0.05$)

a,b,c The number in horizontal significant difference ($P<0.05$)

The highest amounts of ascorbic acid were found in Kimju using water extracts ($P<0.05$). Vitamin C was showed the decrement after SGD condition.

CHAPTER V

DISCUSSION

1. Determination of total polyphenolic contents
2. Determination of free radical scavenging activity using DPPH method
3. Determination of superoxide anion scavenging activity
4. Determination of hydroxyl radical scavenging activity
5. Determination of anti-tyrosinase activity
6. Determination of phenolic compounds
7. Determination of vitamin C contents

1. Determination of total polyphenolic contents

The polyphenolic contents in guava extracts was found types of guava and conditions were the factors that have significant difference ($P<0.05$) interaction for total polyphenolic contents. The Pansithong is showed the highest using 40% ethanol of total polyphnolic compounds in every condition. The recovery of polyphenols from plant materials is influenced by the solubility of the phenolic compound in the solvent used for the extraction process. Furthermore, the solvent polarity will play a key role in increasing phenolic solubility (Naczk & Shahidi, 2006). Ethanol and water mixtures are commonly used for the extraction of phenols from plant materials (Durling et al., 2007). The total phenolic content in guava was relatively low compared to berry fruits, but it was relatively high compared to other fruit crops (Connor et al., 2002). The phenolic contents (TPC) of ripe and unripe guava fruits (Kampuchean variety) were decreased in polyphenol content (unpripe = 270, ripe = 138 (mg GAE/100g) of guava fruit causes a loss in astringency during ripening of the fruit (Yan et al., 2006). The four guava genotypes, Allahabad Safeda, Fan Retief, Ruby Supreme, Advanced selection, varied from 344-170 mg/100g fresh mass were determined by the Folin-Ciocalteu reagent using gallic acid standard (Thaipong et al., 2006). The total phenolics contents (mg GAE/100g) was 278 in 'Keynok Rayong', a pink-fleshed germplasm, and ranged from 162 for 'Keynok Daeng' to 274 for 'Daeng Siam'(Thaipong 2007). The peel portion of guava (*Psidium guajava* L.) contained higher amount of polyphenols than its pulp (Jime´nez-Escrig et al., 2001).

2. Determination of free radical scavenging activity using DPPH method

The effect of guava extracts on the DPPH radical scavenge that was measured IC_{50} was found types of guava and condition. These factors have significant difference ($P<0.05$) interaction for IC_{50} in the DPPH radical scavenging properties. The data indicated that 40% ethanol extract of Pansithong showed the highest DPPH radical scavenging properties. Leong and Shui (2002) and Mahattanatawee et al., (2006) reported similar results, showing that guava had high DPPH radical scavenging properties. The DPPH scavenging activity of the unripe guava was measured by the Ascorbic acid Equivalent Antioxidant Capacity (AEAC) value (310±170 mgAA/100g) and the decrement of AEAC during ripening suggested that the antioxidant activities of guava declined during fruit ripening (Yan et al., 2006). The comparison of the peel and pulp, guava peel presented lower Efficient Concentration 50 (EC_{50}) (1.92±0.08) more than pulp EC_{50} (3.70±0.06) on the basis of dry weight of the fruit portion. (Jime´nez-Escrig et al., 2001).

3. Determination of superoxide anion scavenging activity

The ability of superoxide anion scavenging activity that was measured by IC_{50} was found types of guava and extracted condition was the factor that have significant difference ($P<0.05$) interaction for IC_{50} in the superoxide anion scavenging properties. The data indicated that 40% ethanol extract of Thai original showed the highest superoxide anion scavenging activity. The SGD extracts of three samples were presented the highest inhibition activity at the concentration of 60 mg/mL. Noda et al., (2002) reported the Anthocyanidens: delphinidin, cyanidin, and pelargonidin in pomegranate fruit extract scavenged $O_2^{\cdot -}$ in dose-dependent manner, while Lim and colleagues (2002) reported the six compounds in tea that were showed superoxide scavenging abilities in the following order: (-)-epigallocatechin-3-gallate (EGCG) > theaflavin-3-gallate (TF_2) > theaflavin (TF_1) > gallic acid (GA) > theaflavin-3,3'-digallate (TF_3) > propyl gallate (PG).

4. Determination of hydroxyl radical scavenging activity

The ability of hydroxyl radical scavenging was found Pansithong that used the water for extraction was the highest ability, whereas Thai original that used 40% ethanol for extraction was the highest. The last, Kimju guava was extracted by using SGD demonstrated the highest hydroxyl properties. All types of guava and extracted conditions have the pro-oxidant ability and weak metal chelation potential. Moreover, All of these have significant difference ($P<0.05$) of hydroxyl radical scavenging properties.

The pro-oxidation activity in copper-mediated oxidation was observed in elderberry (*Sumbucus nigra*) (Abuja et al., 1998) and vegetables but tea was showed a pro-oxidant activity (Cao et al., 1996). Anthocyanidins: delphinidin, cyaniding, and pelargonidin in pomegranate fruit extract inhibited a Fenton reagent $^{\bullet}OH$ generating system possibly by chelating with ferrous ion (Noda et al., 2002). The tea extracts, especially the green, pouching, and oolong tea extracts markedly stimulated the oxidation of deoxyribose in the presence of Fe^{+3} and H_2O_2. However, the oxidation was decreased by a high dosage of tea extract (Yen et al., 1997). Tea polyphenols from both black and green teas inhibited the $^{\bullet}OH$ fluxes in a concentration-dependent manner (Grinberg et al., 1997).

5. Determination of anti-tyrosinase activity

The effect of guava extracts on the anti- tyrosinase that was measured IC_{50} was found types of guava and condition. These factors have significant difference ($P<0.05$) interaction for IC_{50} in the anti-tyrosinase properties. The data indicated that water extract of Kimju showed the highest anti-tyrosinase properties. The SGD extracts of three samples were presented the highest inhibition activity at the concentration of 40 mg/mL. It is interesting to note polarity of the solvent played an important role in the anti-tyrosinase activity of the extract. The less polar solvent used, the higher the anti-tyrosinase activity. Kubo et al., 2002 reported quercetin was shown to inhibit the enzymatic oxidation of L-DOPA by chelating copper in the enzyme. Three major components of green tea, ECG, GCG, and EGCG showed the most inhibition of tyrosinase activity (No, J.K., et al., 1999).

6. Determination of phenolic compounds

The content of phenolic compounds in guava that was determined by HPLC was found type of guava and extracted condition were the factor that have significant difference ($P<0.05$). The highest amounts of ferulic acid (Fig 14) that was found in Kimju by using 40% ethanol and phenolic compounds was showed the decrement after SGD condition. The gastric digestion in Broccoli presented stability of phenolic compounds under this condition was high, whereas the phenolics compounds were degraded by intestinal condition (Vallejo et al., 2004). P´erez-Vicente et al., (2002) reported those Pomegranate juice showed phenolic compounds are available during the digestion.

In previously study Yen et al., (2009) reported found ferulic acid, which appeared in guava leaves. The precursors, *p*-coumaric acid and caffeic acid of Ferulic acid is synthesized in plants, especially in cereals and vegetables, such as rice, wheat, oats, tomatoes, asparagus, olives and many plants. The inhibition of lipid oxidation of the phenolic compound and antioxidant standards followed the order: rutin, ferulic acid > tannic acid, gallic acid, resveratrol > BHA, quercetin > tocopherol > caffeic acid, in a linoleic acid system. The free radical-scavenging activity was in the order: gallic acid > tannic acid, caffeic acid, querecetin, BHA, rutin > ferulic acid, tocopherol, resveratrol (Larrauri and Saura-Calixto 1999). In contraction, several researches were indicated that Ferulic acid was ineffective, and even promoted the oxidation of low-density lipoprotein that induced by copper (Chalas et al., 2001).

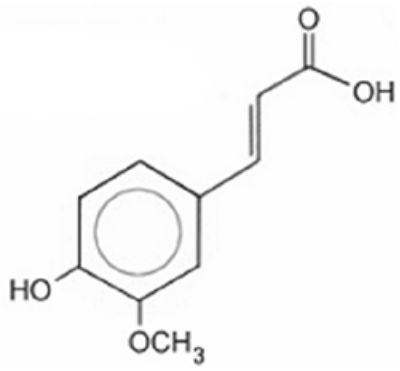


Figure 14 Structure of Ferulic acid

7. Determination of vitamin C contents

The content of vitamin C in the guava that was determined by HPLC was found types guava and extracted conditions were the factor that have significant difference ($P<0.05$). The highest amounts of ascorbic acid were found in Kimju using water extracts. Vitamin C was showed the decrease after SGD condition. Vallejo et al., (2004) reported similar result, showing the greater decrease after intestinal digestion (91%loss) in the Broccoli, while P´erez-Vicente et al., (2002) reported of Pomegranate juice during gastrointestinal digestion demonstrated of Vitamin C degradation more than 95%. Nutritive values of Thai foods were showed Vitamin C content in guava (187mg/100g) (Nutrition division, Department of Health, Ministry of Public Heath). Thaipong et al., (2007) reported the ascorbic content (mg/100g) was 216 in 'Khoa Um-porn', a white-fleshed cultivar, and ranged from 74 for 'Keynk Daeng' to 205 for 'Keynok Rayong'. This indicated that ascorbic acid content in guava was very high compared to other fruit crops.

CHAPTER VI

CONCLUSIONS

Guava fruit contained relatively high ascorbic acid and total phonolic contents which are two major natural antioxidants and are the two major contributors to the antioxidant activity in guava. In this study, the antioxidants, and anti-melanogenesis effect was extracted using three kind, water, 40%ethanol and after the simulated gastrointestinal digestion (SGD). The guavas extracted were further evaluated for their antioxidant, anti-melanogenesis activities using several *in vitro* systems. The results obtained in this work can be summarized as follows:

1. Total polyphnolic contents in guava extracts were screened by using gallic acid as a standard in Folin-Ciocalteu method. The data showed that the polyphenolic content in guava showed Pansithong the highest in every condition.
2. The effect of guava fruit extracts on the scavenging DPPH radical that 40%ethanol extract of Pansithong showed the highest DPPH radical scavenging properties.
3. The ability of guava extracts on superoxide anion scavenging activity was showed Thai original guava extracted using 40%ethanol demonstrated the highest superoxide anion scavenging properties. The SGD extract of three samples presented the highest inhibition activity at the concentration of 60 mg/ mL.

4. The ability of hydroxyl radical scavenging was found Pansithong that used the water for extraction was the highest ability, whereas Thai original that used 40% ethanol for extraction was the highest. The last, Kimju guava was extracted by using SGD demonstrated the highest hydroxyl properties.
5. The anti-tyrosinase activity was showed Kimju extracted by water exhibited the highest inhibition. The SGD extract of three samples showed the highest inhibition activity at the concentration of 40 mg/mL.
6. The contents of phenolic compounds in extracts from guava were determined by HPLC. The highest amounts of ferulic acid were found in Kimju using 40% ethanol extracts when in SGD the structure of phenolic acid in guava digested by stimulated gastrointestinal digestion.
7. The content of vitamin C in the guava that was determined by HPLC. The highest amounts of ascorbic acid were found in Kimju using water extracts. Vitamin C was showed the decrement after SGD condition.

The obtained results indicated that phenolic compounds, and vitamin C, all of which demonstrated antioxidant capacity, would be bioavailable after digestion and might contribute to protect humans from several diseases. Nevertheless, further work has to be carried out to determine the bioavailability of guava and other phenolic compounds and their protective effect in human health, because, as it has been said above release of a compound during digestion does not necessarily signify an antioxidant action *in vivo*.

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APPENDICES

APPENDIX A

GUAVA

Figure A.1 Thai original

Figure A.2 Pansithong

Figure A.3 Kimju

APPENDIX B

DPPH radical scavenging activity

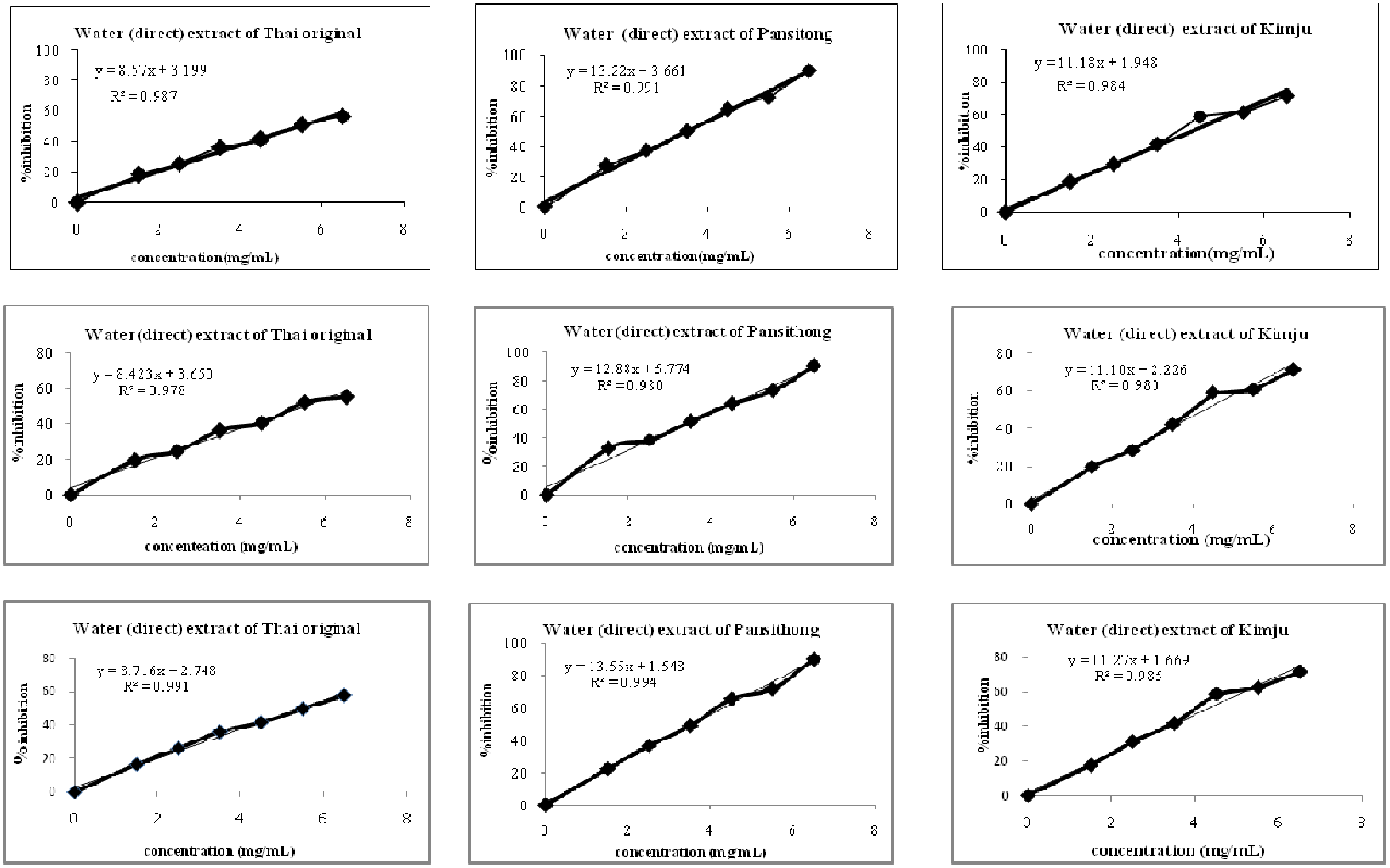

Figure B.1 The relation of the %DPPH inhibition-concentration profile of water (direct) extracts in the individual antioxidant. The logarithmic regression curve for determine the IC_{50}.

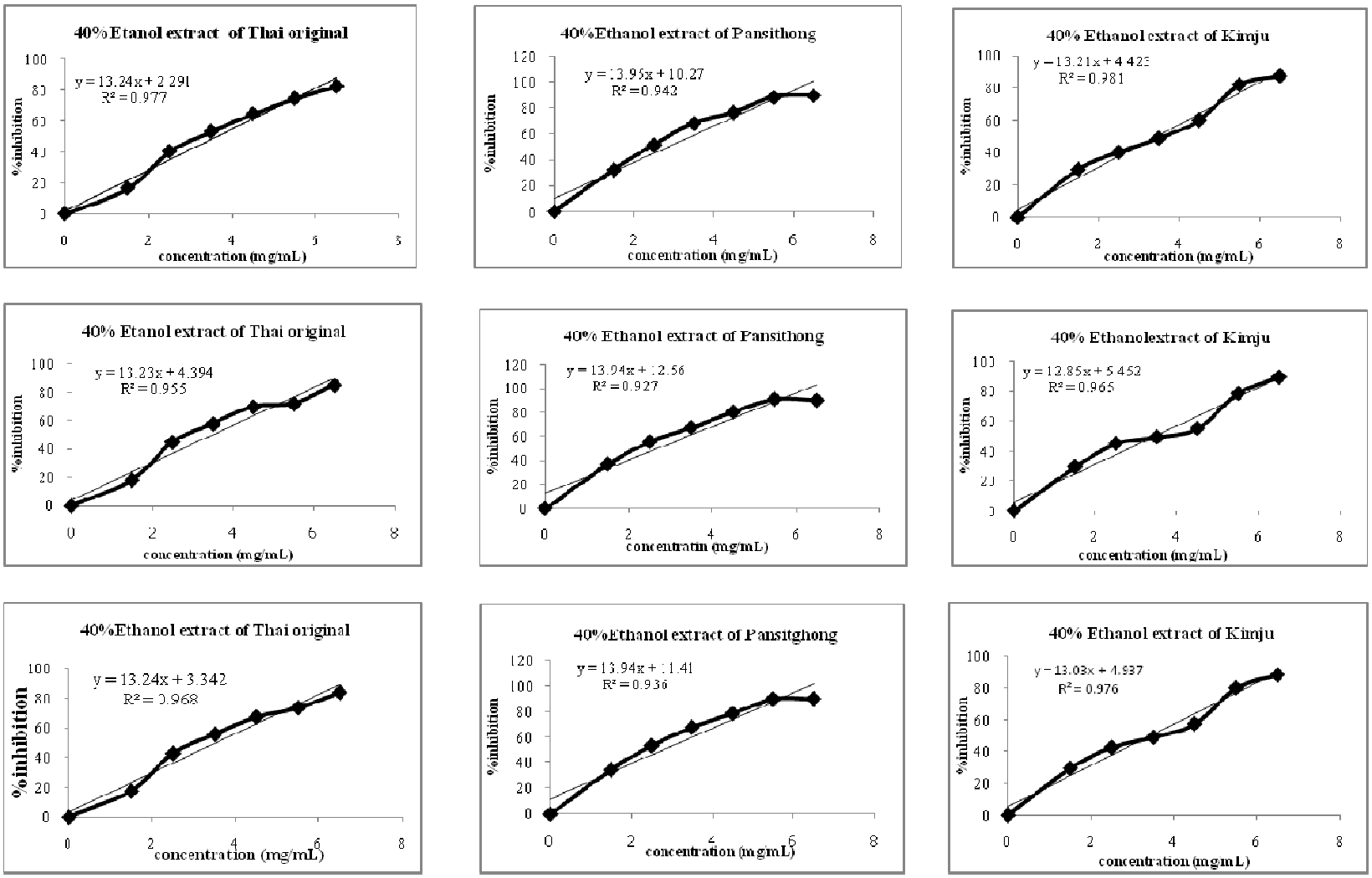

Figure B.2 The relation of the %DPPH inhibition-concentration profile of 40%ethanol extracts in the individual antioxidant. The logarithmic regression curve for were determine the IC_{50}.

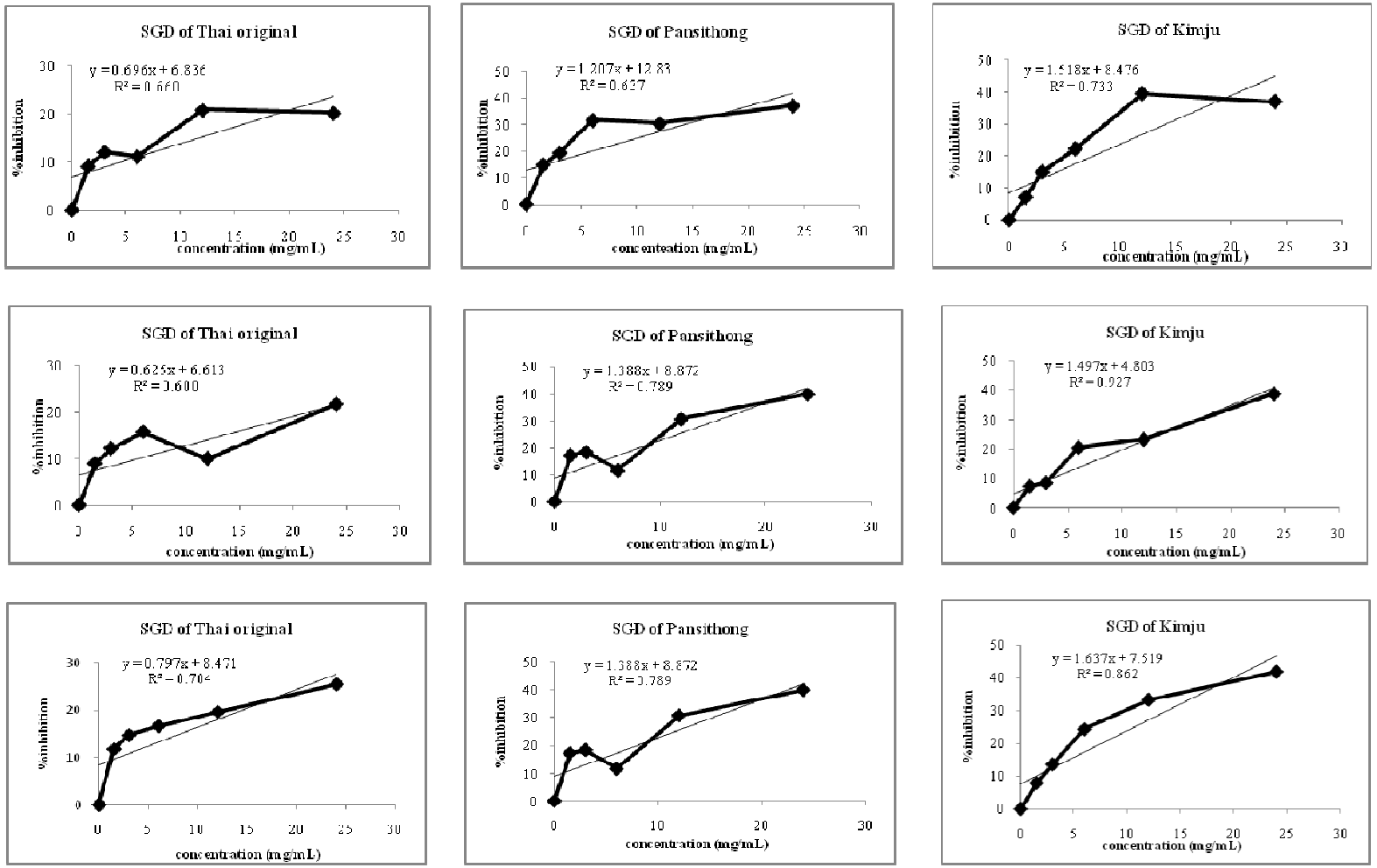

Figure B.3 The relation of the %DPPH inhibition-concentration profile of Simulated Gastrointestinal Digestion (SGD) in the individual antioxidant. The logarithmic regression curve for determine the IC_{50}.

APPENDIX C

Superoxide anion scavenging activity

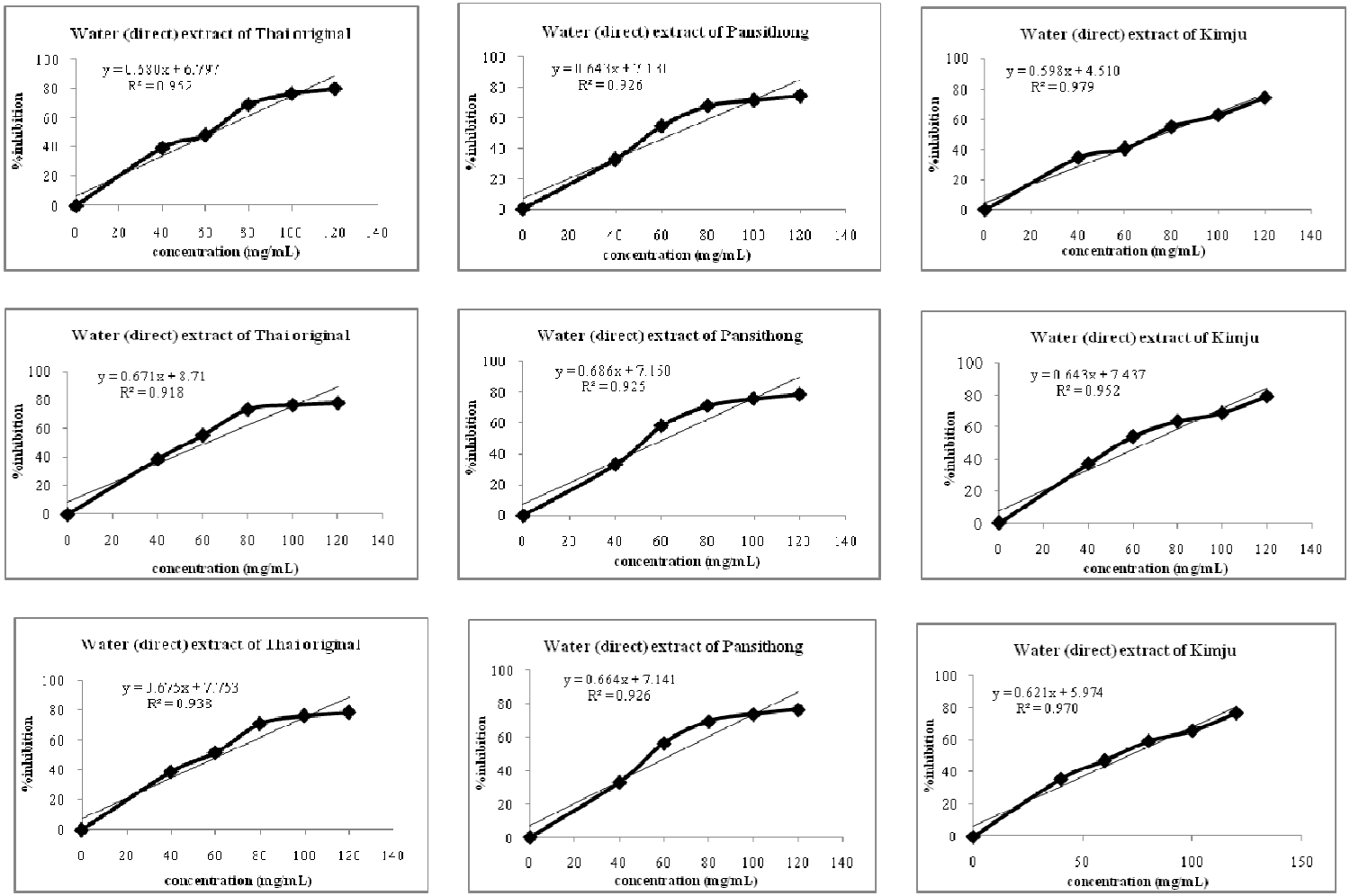

Figure C.1 The relation of the superoxide anion scavenging activity profile of water (direct) extracts in the individual antioxidant. The logarithmic regression curve for were determine the IC_{50}.

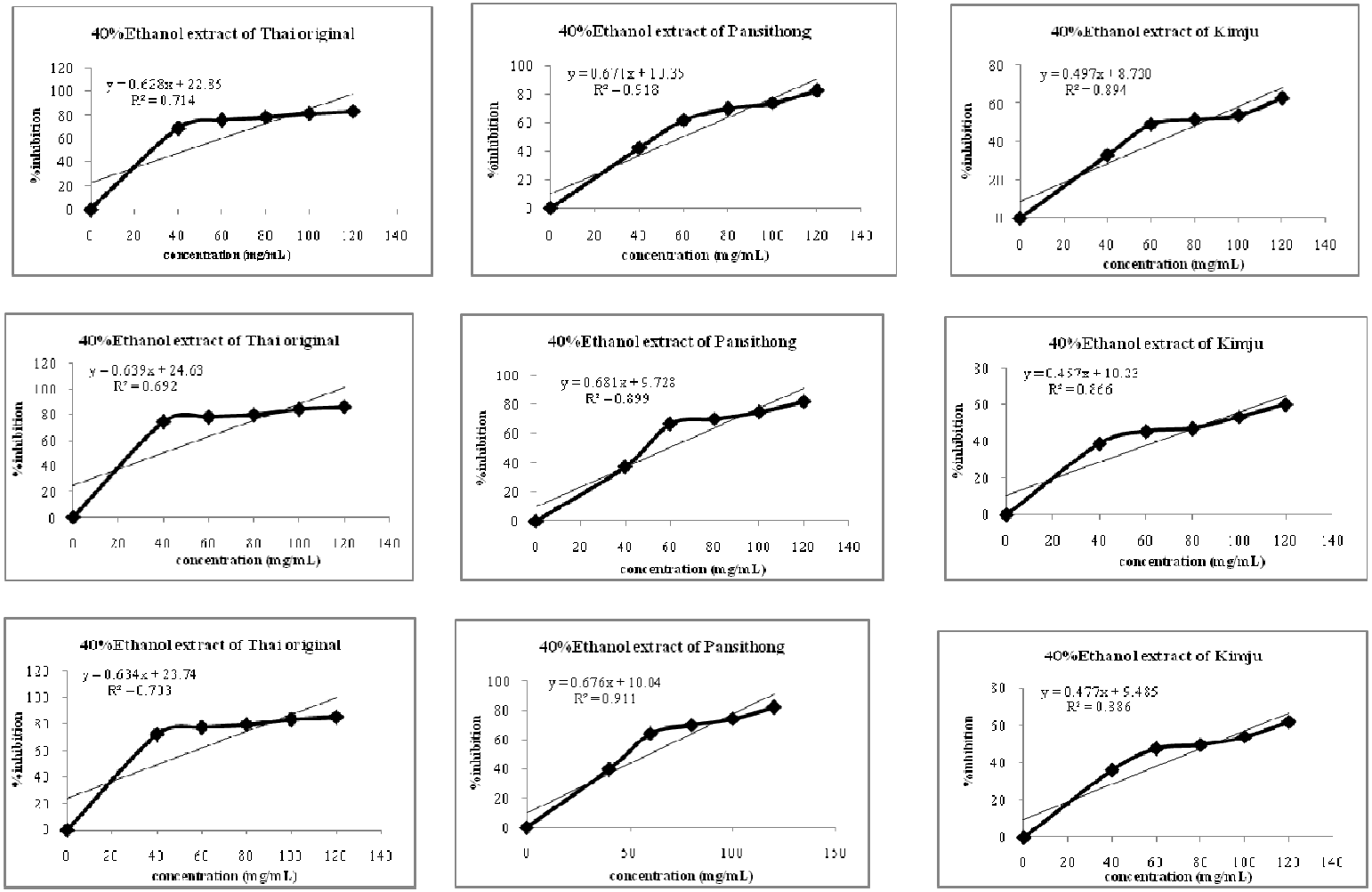

Figure C.2 The relation of the superoxide anion scavenging activity profile of 40%ethanol extracts in the individual antioxidant. The logarithmic regression curve for were determine the IC_{50}.

APPENDIX D

Anti-tyrosinase activity

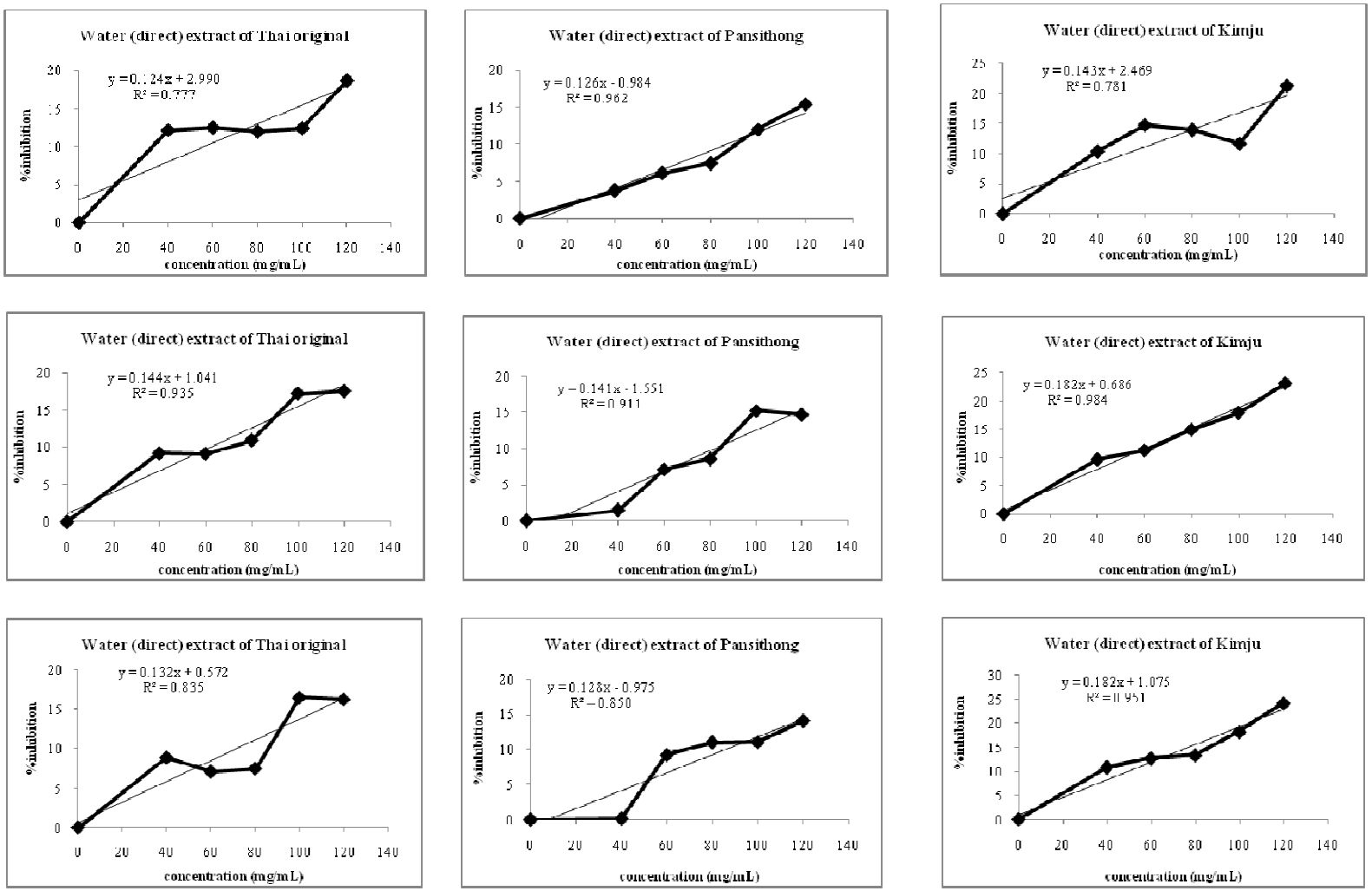

Figure D.1 The relation of the anti-tyrosinase activity profile of water (direct) extracts in the individual antioxidant. The logarithmic regression curve for were determine the IC_{50}.

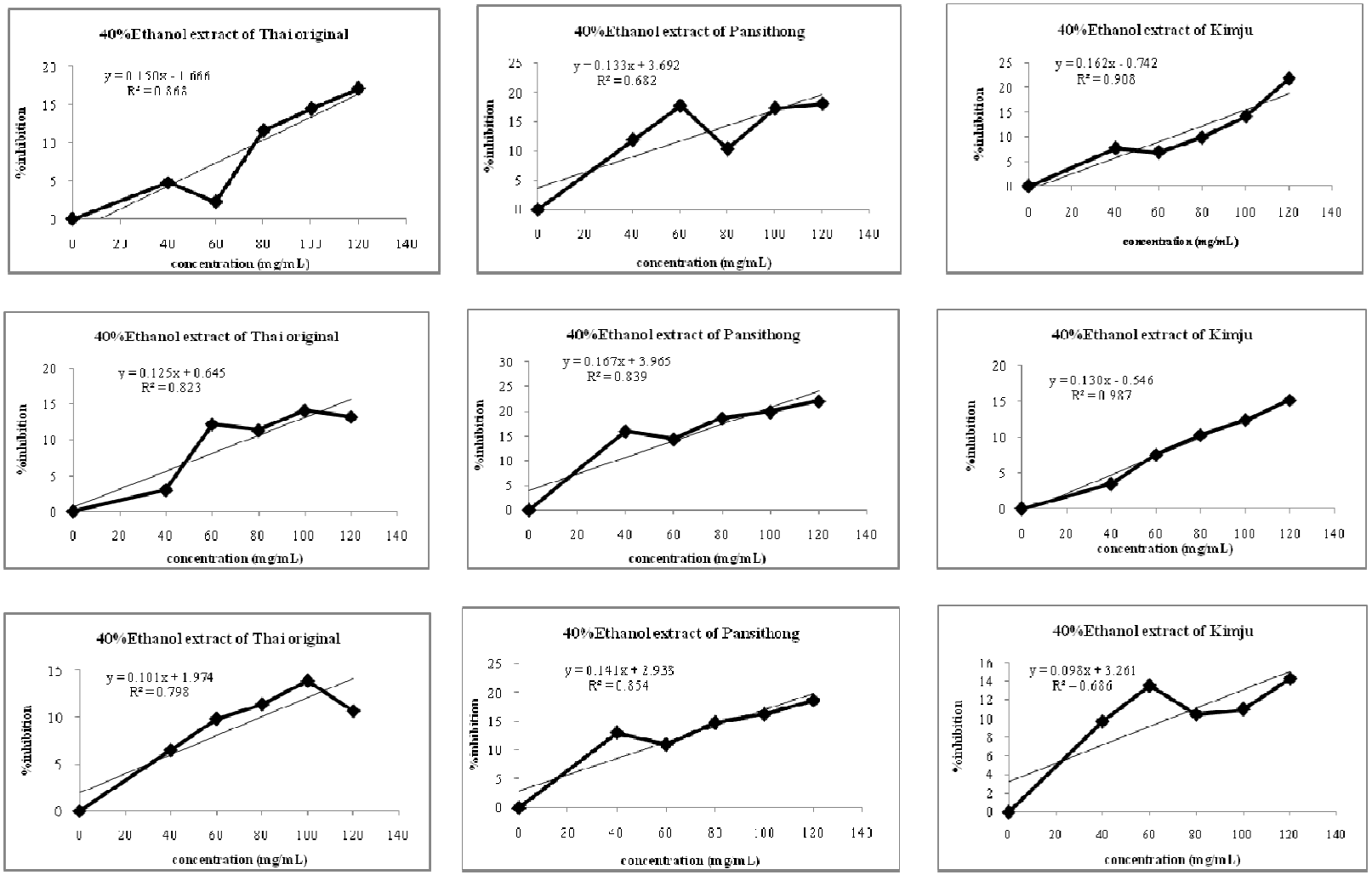

Figure D.2 The relation of the anti-tyrosinase activity profile of 40%ethanol extracts in the individual antioxidant. The logarithmic regression curve for were determine the IC_{50}.

APPENDIX E

Standard of Gallic acid, Ferulic acid and Vitamin C

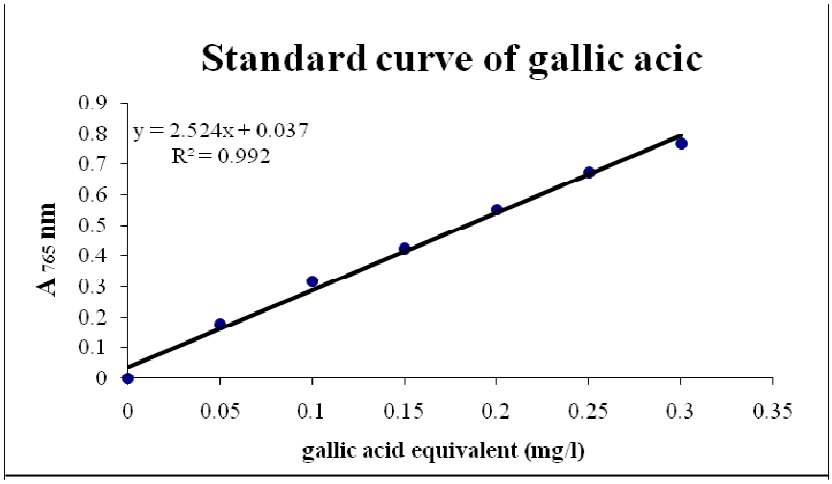

Figure E.1 Standard of gallic acid

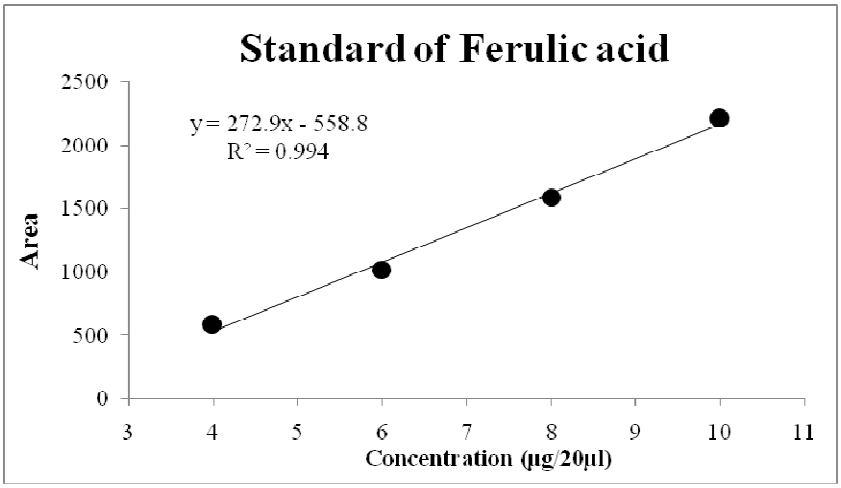

Figure E.2 Standard of ferulic acid

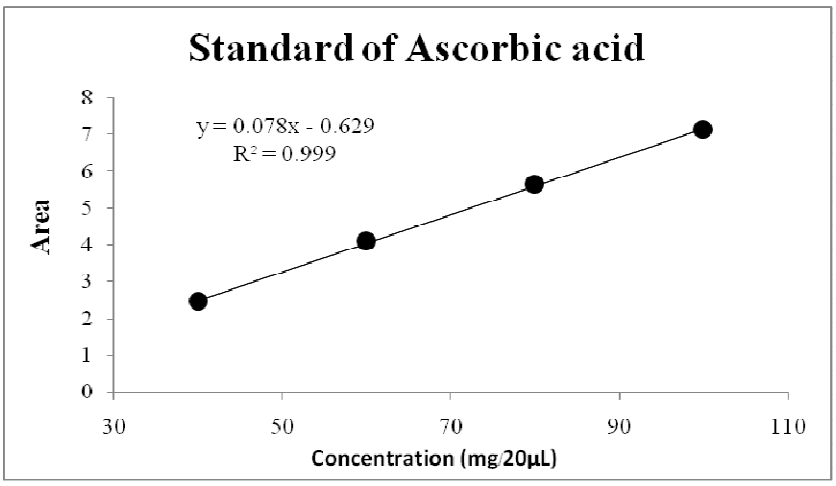

Figure E.3 Standard of Ascorbic acid

BIOGRAPHY

Mr. Thanakorn Damsud was born on May 29, 1983 in Trang province. He graduated with the degree of Bachelor degree of Science (Public Health) from the Department Nutrition, Faculty of Public Health at Mahidol University in 2006. In 2007, he has studied to Master degree of Science in Biochemistry at Chulalongkorn University since 2008.