ฤทธิ์ต้านออกซิเดชันของสารสกัดเปลือกเมล็ดมะขามต่างสายพันธุ์และยาเตรียมใช้เฉพาะที่

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ANTIOXIDANT ACTIVITY OF SEED COAT EXTRACTS FROM TAMARIND CULTIVARS AND TOPICAL PREPARATION

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วลีวัลย์ เอกนัยน์ : ฤทธิ์ต้านออกซิเดชันของสารสกัดเปลือกเมล็ดมะขามต่างสายพันธุ์และยาเตรียมใช้เฉพาะที่. (ANTIOXIDANT ACTIVITY OF SEED COAT EXTRACTS FROM CERTAIN TAMARIND CULTIVARS AND TOPICAL PREPARATION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ภญ. ดร. สุนันท์ พงษ์สามารถ, 115 หน้า.

ทำการตรวจสอบฤทธิ์ต้านอนุมูลอิสระและศึกษาเบื้องต้นของฤทธิ์ต้านแบคทีเรียของสารสกัดเปลือกเมล็ดมะขาม (TSCEs) ที่สกัดจาก *Tamarindus indica* L. สามสายพันธุ์ คือ พันธุ์ “เปรี้ยว”, “ศรีชมพู” และ “สีทองหนัก” จากจังหวัดนครราชสีมาโดยนำเปลือกเมล็ดมะขามแต่ละสายพันธุ์มาสกัดด้วยตัวทำละลายแตกต่างกัน 2 ระบบ ระบบที่ 1 ใช้ 70% เอทานอล ตามด้วยคลอโรฟอร์มและเอทิลอะซิเตตตามลำดับ ในระบบที่ 2 ใช้ 70% เอทานอล และกำจัดไขมันด้วยคลอโรฟอร์ม นำสารสกัดไประเหยแห้งได้เป็นผง ให้สารสกัดคิดเป็นเปอร์เซนต์ เท่ากับ 54-62% จากการสกัดในระบบ 2 ซึ่งมากกว่าการสกัดระบบที่ 1 ที่ได้ 15-18% ได้ตรวจสอบหาสารสำคัญ total phenolic compounds, tannin และ proanthocyanidin ด้วยวิธีทางเคมี และใช้เทคนิค HPLC พิสูจน์เอกลักษณ์ลายพิมพ์นิ้วมือทางเคมีของ TSCEs ได้ผลที่แสดงให้เห็นรูปแบบของพีคที่คล้ายกัน รวมทั้งพีคที่ตรงกับสารมาตรฐาน (+)-catechin, procyanidin B2 และ (-)-epicatechin ได้ทดสอบฤทธิ์ต้านอนุมูลอิสระของ TSCEs ด้วยวิธีวิเคราะห์ DPPH radical scavenging, reducing power, hydroxyl radical scavenging และ anti-lipid peroxidation assays โดยใช้วิตามินซี และ BHA เป็นสารมาตรฐานควบคุมบวก วิเคราะห์หาค่า EC_{50} ของ TSCEs จากมะขามทั้งสามสายพันธุ์ปลูก จากวิธีวิเคราะห์ดังกล่าวแสดงให้เห็นว่า TSCEs มีฤทธิ์ต้านอนุมูลอิสระสูงเมื่อเทียบกับสารมาตรฐานควบคุมบวก การทดสอบเบื้องต้นของฤทธิ์ต้านแบคทีเรียของ TSCEs ต่อเชื้อ *Staphylococcus aureus* ATCC 6538P และ *Escherichia coli* ATCC 25922 ด้วยวิธี broth microdilution assay ค่า MIC และ MBC ของ TSCEs มีค่าเท่ากับ 0.39-1.5 มก./มล. และ 1.56-3.12 มก./มล. ตามลำดับเมื่อทดสอบกับ *S. aureus* และมีค่า 3.12-6.25 มก./มล. และ ≥25 มก./มล. ตามลำดับเมื่อทดสอบกับ *E. coli* การนำ TSCE จากมะขามพันธุ์ “เปรี้ยว” สกัดด้วยตัวทำละลายในระบบ 2 ไปพัฒนาเป็นผลิตภัณฑ์ครีมสำหรับใช้ภายนอก โดย TSCE creams ที่เตรียมได้สำเร็จประกอบด้วย 100, 300, 500 มก. ของ TSCE/ครีม 100 กรัม ได้ตรวจสอบฤทธิ์ต้านอนุมูลิสระของผลิตภัณฑ์ TSCE cream ที่เตรียมได้ด้วยวิธี DPPH radical scavenging assay พบว่า TSCE cream มีฤทธิ์ scavenging activity สูงเมื่อเทียบกับวิตามินซีและสารสกัด TSCE

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต.....

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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WALEEWAN EAKNAI : ANTIOXIDANT ACTIVITY OF SEED COAT EXTRACTS FROM CERTAIN TAMARIND CULTIVARS AND TOPICAL PREPARATION. THESIS ADVISOR : ASSOC. PROF. SUNANTA PONGSAMART, Ph.D., 115 pp.

Antioxidant and preliminary studied of antibacterial activity of tamarind seed coat extracts (TSCEs) were evaluated. Three tamarind cultivars, *Tamarindus indica* L. "Priao", "Srichomphu" and "Sithong-nak" were collected from Nakhon-Ratchasima Province. The seed coat of each cultivar was extracted by two solvent extraction systems. Solvent in System 1 was 70% ethanol, followed with chloroform and then ethyl acetate, respectively and solvent in System 2 was 70% ethanol and washed out lipid with chloroform. The extracts were dried to powder of TSCE. Higher percent yield of TSCEs at 54-62% in System 2 was obtained compared with TSCEs (15-18%) in System 1. Chemical analyses of the total phenolic compounds, tannin and proanthocyanidin were examined. HPLC technique was used for chemical fingerprint identification of TSCEs. HPLC chromatograms of TSCE of the 3 tamarind cultivars showed the similar pattern of peaks including peaks identical with reference standard (+)-catechin, procyanidin B2 and (-)-epicatechin. Antioxidant activity of TSCEs was evaluated by DPPH radical scavenging, reducing power, hydroxyl radical scavenging and anti-lipid peroxidation assays. Vitamin C and BHA were used as positive control. The EC_{50} values for TSCEs by each assay were determined. The results showed that TSCEs of the three tamarind cultivars possessed high antioxidant activity compared with the positive control. Antibacterial activity of TSCEs was preliminary studied against *Staphylococcus aureus* ATCC 6538P and *Escherichia coli* ATCC 25922 by broth microdilution method. MIC and MBC values against *S. aureus* for TSCEs were 0.39-1.5 mg/ml and 1.56-3.12 mg/ml, respectively; and against *E. coli* were 3.12-6.25 mg/ml and ≥25 mg/ml, respectivel. TSCE of *Tamarindus indica* "Priao" in System 2 was used to develop a preparation of TSCE cream for topical application. TSCE creams with 100, 300, 500 mg TSCE/100 g cream were successfully developed. Antioxidant activity of TSCE cream products was evaluated by DPPH radical scavenging assay. The result showed that TSCE creams possessed high percentage of scavenging activity in comparison with vitamin C and TSCE reference.

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LIST OF ABBREVIATIONS

Abbreviations	Full name
µg	microgram
µL	microliter
µm	micrometer
A°	angstrom
ATCC	American Type Culture of Collection
AU	Absorbance Units
°C	carbon
C	degree Celsius
CFU	Colony-Forming Unit
Co	Company
ed.	edited by
et al.	*et alii*, 'and others'
EC_{50}	The half maximal effective concentration
cm	centimeter
g	gram
h	hour
HPLC	high performance liquid chromatography
Kcal/mol	kilocalorie/mole
Ltd	Limited
m	meter
mg	milligram

min	minute
mL	milliliter
mM	millimolar
mm	millimeter
mPas	millipascal
M	Molarity
Abbreviations	Full name
n.d.	no date
nm	nanometer
N	Normality
NCCLS	The National Committee for Clinical Laboratory Standards
No.	number
OD	Optical Density
pH	Potential of Hydrogen ion
q.s.	quantum satis (latin: a sufficient quantity)
rpm	revolution per minute
rev. ed.	revised edition
RH	Relative Humidity
spp.	(pl.) species, all the individual species within a genus
SE	standard error of measurement or mean
SPSS	Statistic Package for the Social Sciences
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
vols.	volumes
v/v	volume by volume
w/m^2	Watt per Square Meter
w/v	weight by volume

CHAPTER I

INTRODUCTION

A. Background

Tamarind (*Tamarindus indica* L.) is a plant that has long been used as traditional medicine, its several parts are used for treatment of a wide variety of ailment and diseases (Komutarin et al., 2004). Tamarinds belong to Family Fabaceae, Subfamily Ceasalpiniaceae, grow naturally in tropical and subtropical regions worldwide, including in many provinces in Thailand such as Phrae, Nan, Lampang, Phetchabun, Nongkhai, Loei, Nakhonphanom, Ubonratchathani and Nakhon-Ratchasima (Korat) (องค์การตลาดเพื่อการเกษตร : ออนไลน์; Doughri, 2006; Komutarin et al., 2004). Tamarind seeds are the major waste from tamarind food industry, such as tamarind juice, tamarind sauce, tamarind jam and tamarind gum, etc (Kaur, Nagpal and Kaur, 2006; Luengthanaphol et al., 2004). Tamarind seed coat extracts have been recently studied, the phenolic compounds are found to be a major component and the extracts also possess good antioxidant activity (Luengthanaphol et al., 2004; Martinello et al., 2006; Siddhuraju, 2007; Soong and Barlow, 2004; Sudjaroen et al., 2005; Tsuda et al., 1994), however, its antibacterial activity is not widely studied. Tamarind seed coat has been extracted by several organic solvent extraction processes. The general extraction using 70% ethanol to extract the active compounds, followed by chloroform to eliminate lipids and then the principle components are extracted with ethyl acetate, respectively, the resulting ethyl acetate extract exhibits high antioxidant activity (Suksomtip and Pongsamart, 2008). However, low % yield of the extract is obtained. There are correlations of total phenolic compounds and several pharmacological effects such as antioxidant and antibacterial activity have been reported. Therefore, tamarind seed coat extract containing high content of total phenolic compounds may possibly have antibacterial activity. It is an interesting goal to study on antibacterial activity in tamarind seed coat extract as well as to develop the product for topical uses (Almajano et al., 2008; Demirci et al., 2007; Dordevic et al., 2007; Kitzberger et al., 2007; Kukic et al., 2008; Tepe et al., 2005). Skin care products have three main functions: (1) to protect the injured area from the environment and permit the skin to rejuvenate, (2) to

provide skin with hydration or to produce an emollient effect, either, and (3) to convey a medication to the specific effect, either topically or systemically (Allen Jr., 2008). Nowadays, consumers increase demand for compounds from natural plant origin because they concern about safety and side effect of synthetic chemicals. Among the estimated 250,000 plant species existing worldwide, only a small percentage has been investigated phytochemically, and the fraction submitted to biological or pharmacological screening is even smaller (Li, Zhou and Han, 2006). Searching for natural products from plants with bioactive materials for skin care is an interesting goal among researchers.

In the present study, seed coat extracts of three tamarind cultivars were evaluated for their antioxidant and antibacterial activities. Tamarind pods of three tamarind cultivars were collected from Nakhon-Ratchasima Province. Seed coat of each cultivar was extracted by the two processes of solvent extraction system: (1) using 70% ethanol, chloroform and then ethyl acetate, respectively, and (2) using 70% ethanol and chloroform, respectively. Antioxidant and antibacterial activities were determined. Topical preparation containing tamarind seed coat extract was developed.

B. Objective

The main objectives of the present study were as follows:

1. To evaluate antioxidant and antibacterial activities of tamarind seed coat extracts of 3 different Thai-tamarind cultivars.
2. To separate and identify some peaks of phenolic components in tamarind seed coat extracts by using HPLC.
3. To develop topical preparation containing tamarind seed coat extract with antioxidant activity.

CHAPTER II

LITERATURE REVIEWS

A. Tamarind (*Tamarindus indica* Linn.)

1. Origin and production

Tamarind (*Tamarindus indica* Linn.) is medicinal and economically important tree-type of fruit plant, this plant grows naturally in tropical and subtropical countries. It was native to tropical Africa, thought to have originated in Madagascar, and today it has become naturalized in North and South America from Florida to Brazil. Tamarind can be cultivated in many countries: subtropical China, India, Pakistan, Indochina, Philippines, Java, Spain and Thailand (Peter, 2001; Martinello et al., 2006). Tamarind is divided into 2 types of sour and sweet tamarind, there are more than 20 cultivars cultivated in Thailand such as Meunjong, Sithong, Srichomphu, Nampeung, Namduk, Khanti, Jaehom, Kru-in, Piyai, Praroj and Priao. Tamarinds are cultivated through northern, north-eastern, riverbank of Kong river and central parts of the country such as Phrae, Nan, Lampang, Phetchabun, Nongkhai, Loei, Nakhonphanom, Ubonratchathani and Nakhonratchasima (องค์การตลาดเพื่อการเกษตร : ออนไลน์).

Tamarind is worldwide as the fruit which is an important food ingredient in many countries including Thai population. The stem is applied to make furnitures and utensils. The bark extract can be use in coloring and tannin industry. The flower and leaf are eaten as vegetable, curry ingredient, salad and soup and also used as a dying. Leaves are used as fodder (cattle, goats), furnished fodder for silkworms, component of soil and organic fertilizer. The pulp is eaten as fruit and used in spices, seasoning, food component, juice, tamarind syrup, jam, sherbet, ice cream and candy. Moreover, the fruit is used as a fixative with turmeric or annatto in dyeing, served to coagulate rubber latex and cleaned silver, copper and brass by using the mixture solution of tamarind fruit with sea water. The ripe seed is eaten as snack. Tamarind seed is derived to the 3 major products such as Tamarind Kernel Powder (TKP), Seed testa and Kernel Oil. The kernel or TKP is extracted in food manufacturing for using as jelling, thickening and stabilizing that called tamarind gum. The seed testa contains crude fiber (21.6%), fiber

(7.4%) and tannins (20-24%) which tannin is black color, highly polymeric. The seed coat used as a low cost source of antioxidant in lipid containing foods and dyeing. The Kernel Oil like peanut oil or linseed oil and can be used in paints, varnishes and for burning lamps. Interestingly, flour of the seeds may be made into cakes and breads. (Bhattacharya et al., 1997; Kaur et al., 2006; Martinello et al., 2006; Morton, J.F., 1985; Soemardji, 2007; Sudjaroen et al., 2005; Tsuda et al., 1994).

2. Botanical description

Tamarindus indica Linn. is a member of Family Fabaceae in Subfamily Caesalpiniaceae (Doughari, 2006). Tamarind was derived from Arabic which combined Tamar meaning 'date' with Hindi meaning 'of India'. Tamarind has various local name (El-Siddig et al, 2006) as shown in Appendix A, Table A-1 for example tamarinde (South Africa), asam jawa (Malaysia) and tamarindizio (Italian).

Tamarind is a dicotyledon, evergreen or semi-evergreen, long-lived, medium growth bushy tree. It is 20-30 m tall that covered with brownish-grey, rough and scaly bark. It produces a dark red gum that collected at trunk and branches. Tamarind has a deep tap root and extensive lateral root system. The leaves are alternate and even pinnate which compound with 8-18 pairs of leaflet (1-3.5 cm long), up to 15 cm long. Inflorescence is born at the end of branches that combined few to several flower (up to 18). Flowers are bisexual, small (3-5 cm long) and yellow, cream, pink or white, streaked with red. The tamarind fruit is indehiscent pod (dry and hard), brittle, more or less curved and constricted between seeds. It is light grey or brown and scaly (5-10 cm long × 2 cm broad) that contain 1-12 seeds. Pods (3-10 cm long × 1.3 cm broad) ripen about 10 months after flowering and can remain on tree until next flowering period. The seed is hard, flattened, glossy, orbicular to rhomboid, red to purple brown, non arillate and exalbuminous. The seed coat make up 35-40% of the seed, and the kernel 60-65% (Bhatta, Krishnamoorthy and Mohammed, 2001; El-Siddig et al, 2006; Joker, 2000).

3. Herbal medicine and phytochemical

Tamarind has long used as a primitive and modern herbal medicine. Other part of tamarind show pharmacological effects of antihepatotoxic, antidiabetic, antimutagenic, anti-inflammatory, antiatherosclerosis (Martinello et al., 2006) and antibacterial (Doughri, 2006). Especially, seed coat present antihyperlipidemic and antioxidant (Suksomtip et al., 2008) according to its phenolic compounds content. Various parts of tamarind are used in herbal medicine (Table 2.1).

4. Phenolic compounds of tamarind seed coat

The active ingredients from plant extracts are interesting to investigate and many reports concluded that phenolic compounds from various natural plant products exhibited antioxidant and antimicrobial activities and have contributed enormously to the research and development in drug, food and cosmetic (Almajano et al., 2008; Hassan and Fan, 2005; Johnson et al., 1999; Kahkonen et al., 1999; Shan et al., 2008; Siddhuraju, 2007; Soong and Barlow, 2004). The extract of tamarind seed coat has been studied recently for antioxidant activity and compound of phenolic compounds.

Phenolic compounds are plant secondary metabolite (Pyrzynska and Biesaga, 2009) and normally found these bioactive compounds from vegetables, fruits, spices, herbs and medicinal plants. They were synthesized to more than 1,000 forms (Boudet, 2007) and bioactivities about antioxidant, antibacterial, antifungal, antimutagenic, anti-inflammatory, anticarcinogenic and antiatherosclerosis activities have been reported (Hsieh and Yen, 2000; Siddhuraju, 2006; Shan et al., 2008; Viswanath et al., 2009; Yesilyurt et al., 2008). Phytochemicals in various parts of tamarind are shown in Table 2.2. Molecular structures of phenolic compound are divided by hydroxylated aromatic ring, called phenol group (Boudet, 2007). Phenol (carbolic acid) is an organic compound with chemical formula C_6H_5OH that contains a 6 member aromatic ring, bond directly to a hydroxyl group (-OH) (Figure 2.1).

Table 2.1 Application from many parts of tamarind in herbal medicine (Bhattacharya *et al.,* 1997; Doughari, 2006; Komutarin et al, 2004; Martinello et al, 2006; Morton, J.F., 1985; Soemardji, 2007; Sudjaroen et al, 2005)

Part of tamarind	Application in herbal medicine
bark	stomach disorder, general body pain, jaundice, yellow fever, blood tonic, skin cleanser, asthma, eye inflammation, pyretic, amenorrhea, colic, scorbutic and antimicrobial
leaf	cough, stomach disorder, general body pain, jaundice, worm infection, sores, ulcer, yellow fever, blood tonic, skin cleanser, antiseptic, vermifuge, dysentery, conjunctivitis, erysipelas, hemorrhoid, pyretic, rheumatism, insomnia and poultice for swollen joint
flower	cough with blood, locally edema and wound, antiseptic, vermifuge, dysentery, jaundice, conjunctivitis, erysipelas, hemorrhoid, antiseptic, pulmonary tuberculosis, pharinkhitis chronic, rheumatism and poultice for swollen joint
fruit or pulp	digestive, carminative, laxative, expectorant, blood tonic, biliousness, bile disorder, antiscorbutic, breast inflammation urticaria allergic, rheumatism, alleviate sunstroke, Datura poisoning alcoholic intoxication, constipate, pyretic, dysentery, loss of appetite, alcohol toxicity, vomit, worm infection, jaundice, nausea and vomit in pregnant, asthma, morbili and thirsty
seed	snake bite, wound/ulcer, drop off hair, anthelmintic, antidiarrheal, , antidiabetic and antihyperlipidemic
kernel	depression, constipation และ diarrhea
seed coat	treat burn, wound healing, antidysenteric, antioxidant, antibiotic, anti-inflammatory, antihyperlipidemic and antiatherosclerosis

Table 2.2 Phytochemical of tamarind (El-Siddig et al, 2006; Soemardji, 2007)

Part of tamarind	Phytochemical
shoot	vitamin B
bark	phlobatannine, tannins, saponins, flavonoids, tannins, glycosides and peroxidase
leaves	saponins, flavonoids, tannins, sitexin, isovetexin, orientin, isoiorientin,oxalic acid, 1-malic acid, tannin, glycosides, peroxidase and vitamin B
flower	thiamine, riboflavin, niacin, vitamin C and carotenes
fruit	thiamine(B1), riboflavin(B2), niacin(B3), saponins, flavonoids, tannins, carotene, grape acid, apple acid, oxalic acid, citric acid, succinic acid, quinic acid, tartaric acid, pipecolic acid, nicotinic acid, 1-malic acid, phytic acid, trypsin inhibitor, vitexin, isovitexin, orientin, isoorientin, vitamin A, vitamin B3, vitamin C, volatile oils (geranial, geraniol, limonene), cinnamated, serine, beta-alanine, pectin, proline, phenylalanine and leucine
seed	phytic acid, trypsin inhibitor, albuminoid, phytohemaglutinins and flavonoids and polyphenolic

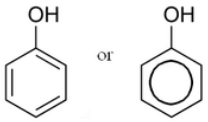


Fig 2.1 Phenol structure

Phenolic compounds are separated into 3 groups (Dewick, 1995).

1. flavanoids
2. lignin
3. tannin (some structures are showed in Appendix A, Fig A-1)

3.1 hydrolyzable tannins : gallic acid and ellagic acid

3.2 non-hydrolyzable tannin, condense tannin, catechin tannins, procyanidin or proanthocyanidin : catechins and epicatechins (Figueiredo et al., 2008)

3.2.1 A-type proanthocyanidins (Wikipedia, 2010 : online)

- proanthocyanidin A1 (epigallocatechin-(2β→7,4β→8)-epicatechin dimer
- proanthocyanidin A2 (dimeric catechin)
- selligueain A
- selligueain B ((-)-4β-carboxymethy epiafzelechin (3'-deoxydryopteric acid), epiafzelechin-(4β→8, 2β→O→7)-epiafzelechin-(4β→8)-3'-deoxydryopteric acid methyl ester)

3.2.2 B-type proanthocyanidins (Wikipedia, 2010 : online)

- proanthocyanidin B1 (epicatechin-(4β→8)-catechin)
- proanthocyanidin B2 ((-)-epicatechin-(4β→8)-(-)epicatechin)
- proanthocyanidin B3 (catechin-(4β→8)-catechin)
- proanthocyanidin B4 (catechin-(4α→8)-epicatechin)
- proanthocyanidin B5 (epicatechin-(4β→6)-epicatechin)
- proanthocyanidin B6 (catechin-(4α→6)-catechin)
- proanthocyanidin B8 (catechin-(4α→6)-epicatechin)
- proanthocyanidin C1 (epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin)

Many researches show that antioxidant activity of tamarind seed coat extract relate to phenolic compound and tannin of the extract for example 2-hydroxy-

3',4'-dihydroxyacetophenone, (-)-epicatechin, methyl 3,4-dihydroxybenzoate and 3,4-dihydroxyphenylacetate (Tsuda et al., 1994), oligomeric proanthocyanidin, anthocyanidin (Komutarin et al., 2004), flavonoids, (+)-catechin, procyanidin B2, olimeric procyanidin (Sudjaroen et al., 2005) and xyloglucan (Pauly, 1999).

B. Principle of phenolic compound analysis by chemical assays

1. Determination of total phenolic compounds

Principle of the determination of total phenolic compounds is used Folin-Ciocalteu reagent that contains phosphomolybdic/phosphotungstic acid complexes in alkaline medium. Electron donation by phenolic compounds and other reducing species (aromatic amines, sulfur dioxide, ascorbic acid, Cu(I), Fe(II), etc.) form blue complexes that can be measure at absorbance 750-765 nm. The increasing of absorbance is direct variation with total phenolic content against gallic acid calibration curve. The value is not a real total phenolic from substance, so this method was preliminary evaluated phenolic compound content (Magalhaes et al., 2008).

2. Determination of tannin content

The principle of tannin contents is the same as the principle of total phenolic compounds that use Foin-Ciocalteu reagent. This method separated data to 3 groups. First value is an absorbance of total phenolic compound (A_1), second value is an absorbance of phenolic that do not absorb by hide powder (A_2) (that compound removed tannin (Hostettmann, Marston and Hostettmann, 1997)), and third value is an absorbance of pyrogallol standard (A_3). Finally all data and weigh of antioxidant substrate (m_1) and pyrogallol in gram (m_2) were calculated by %tannin equation (European pharmacopeia 4^{th}).

$$\%\text{tannin} = \frac{62.5\,(A_1-A_2)m_2}{A_3 x m_1}$$

3. Determination of proanthocyanidin

Proanthocyanidin or condense tannin content can be evaluated by 2 methods as the vanillin assay and acid butanol assay. Acid butanol assay starts from

oxidative cleavage of proanthocyanidin in alcohol under strongly acidic condition. The cleavage products (mainly cyanidin and delphinidin in Fig 2.2) can be measure at absorbance 550 nm. The increasing of absorbance is direct variation with proanthocyanidin content (Graca, Barlocher and Gessner, 2007).

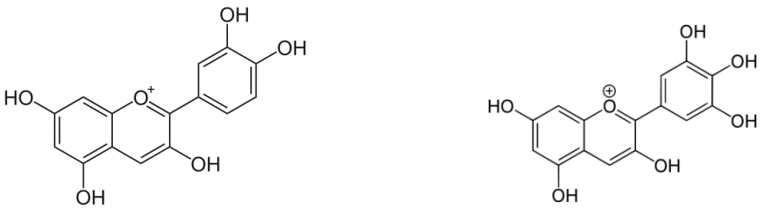


Fig 2.2 Structure of cyanidin (left) and delphinidin (right)

C. High performance liquid chromatography for phenolic compounds analysis

1. **Definition and principle**

High performance liquid chromatography (HPLC) is a liquid-liquid chromatography under high pressure. HPLC provides a specific, sensitive and precise method for analysis of different complicated samples. This technique is specific, accurate, precise, and offers advantage over gas chromatography in analysis of many polar, ionic substance, metabolic products and thermolabile as well non-volatile substances (Kasture et al., 2006). HPLC consist of many parts such as mobile phase reservoir, pump, sample injection system, column and detector (Fig 2.3). When sample compound is injected, the mobile phase that without air bubble is taken sample into column that contain stationary phase and each chemical is eluted, detected and translated to chromatogram, respectively.

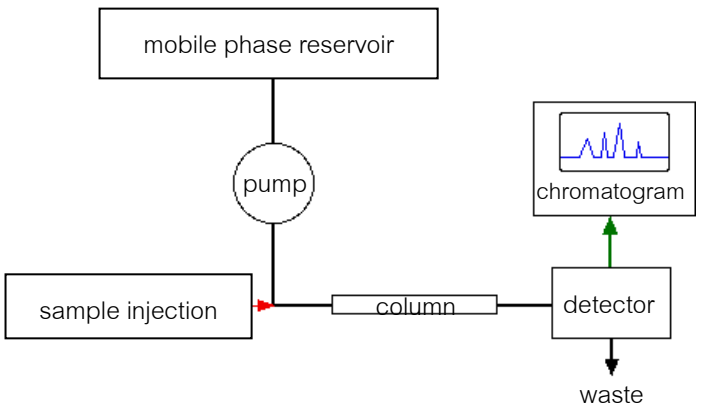


Fig 2.3 Diagram showing the components of HPLC instrument

2. **Some parameters and factors concern with efficacy of isolation** (รัตนา, 2544; Dong, 2006)

   2.1 **retention time (t_R)** is a time that mobile phase take or elute chemical through stationary phase. This value is a time since time at injection a sample into column until time at extreme of peak. It is easily analysis from the chromatogram and easily obtains false data when changing experimental condition such as flow rate.

   2.2 **adjusted retention time (t'_R)** is a time that sample was retained by stationary phase (Fig 2.4). This value calculated through chemical equation.

$$t'_R = t_R - t_m$$

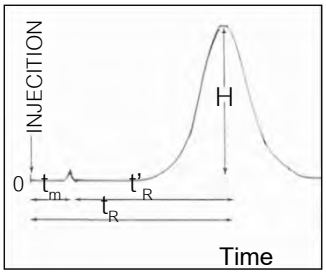


Fig 2.4 Chromatogram showed measurement t_R, t'_R and t_m

3. **Qualitative and quantitative analysis**

   3.1 **Qualitative analysis**

   1. Comparison retention time: The same chemical shows equal retention time at every type of column and/or type of mobile phase and the same result obtains when analyse by HPLC and other chromatography method
   2. Comparison retention volume: The same chemical shows equal retention volume under identical condition.
   3. Comparison relative retention time: Comparison between sample and other standard that present difference retention time. Examination cocaine in sample was added codeine, a secondary standard, into cocaine standard and sample. Then two mixtures were injected to HPLC and calculated relative retention time of cocaine and codeine from two mixtures. If two result show equal value the sample may be cocaine.

4. Spiking: the unknown sample was spiked with a know compound and compared chromatograms of the original and spiked peak increased. Only the height or peak area of the peak of interest with the known compound spiked should be increase, with no change in retention time, peak width, or shape (peak A in Fig 2.5) (เพ็ญพรรณ และคณะ, 2539; Nielsen, 2010).

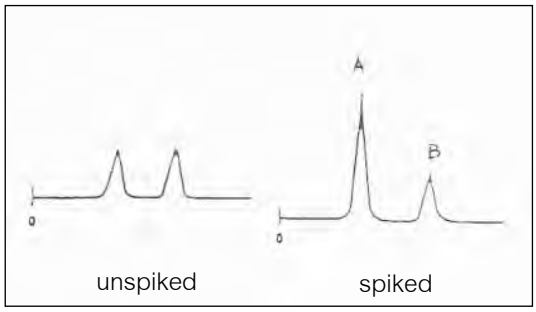


Fig 2.5 Chromatogram show spiking technique

3.2 Quantitative analysis

1. Measurement the peak height
2. Measurement peak area
3. Integrator or computer

D. Free radicals

Free radicals are atoms, molecules or ions with unpaired electrons. In biological processes, free radicals are important role of life such as the intracellular killing of bacteria by phagocytic cell and cell signaling process or redox signaling (Pacher, Beckman and Liaudet, 2007). Free radicals in biological organisms are divided into 2 groups (Table 2.3)

1. Reactive Oxygen Species and Reactive Nitrogen Species (ROS and RNS)
2. Non-radical Reactive Oxygen Species and Non-radical Reactive Nitrogen Species (Non-radical ROS and RNS) (Hsu, Coupar and Ng 2006; Papas, 1998).

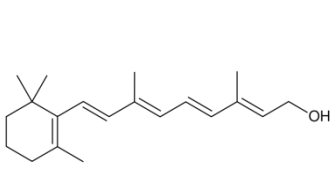
Free radicals are generated in the body by cellular and metabolic activities and also arise from exogenous sources such as light, heat, metals, ionizing radiation, injury, oxidative drug and pollutants. They are formed *in vivo* by various ways

as summarized in Table 2.4 (Hsu et al., 2006; Papas, 1998) and can cause aging and other diseases such as cancer, inflammation, cardiovascular diseases, neurodegenerative diseases and liver injury (Barreira et al., 2008; Yen et al., 2008).

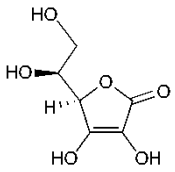
E. Antioxidant substances

In healthy organisms, free radical production is continuously balanced by natural antioxidant defense systems. Unbalance between free radical production and elimination that produce excessive free radical production leads to the process called oxidative stress, biological molecules damage and many clinical diseases such as aging, cardiovascular disease, liver injury, physical injury, cancer, infection, inflammation, acquired immunodeficiency syndrome, immune deficiency diseases and neurodegenerative disease (Gurav et al., 2007; Negi et al., 2003; Siddhuraju, 2007; Yen et al., 2008).

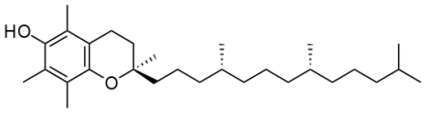
Antioxidant substances have capable of slowing or preventing the cell oxidation by neutralizing free radical and can prolong the shelf-life for food, drug and cosmetic industry. They are separated into 2 groups.



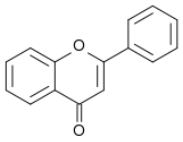
vitamin A



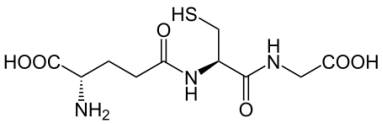
vitamin C



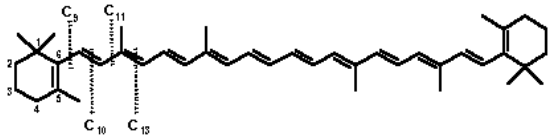
α-tocopherol



flavone backbone



glutathione



β-carotene

Fig 2.6 Natural antioxidant substances

Table 2.3 Active oxygen and related species

Radicals		Non-radicals	
$O_2^{\cdot -}$	superoxide	H_2O_2	hydrogen peroxide
$HO^{\cdot}$	hydroxyl radical	$^1O_2$	singlet oxygen
$HO_2^{\cdot}$	hydroperoxyl radical	LOOH	lipid hydroperoxide
$L^{\cdot}$	lipid radical	Fe=O	iron-oxygen complex
$LO_2^{\cdot}$	lipid peroxyl radical	HOCl	hydrochloride
$LO^{\cdot}$	lipid alkoxyl radical		
$NO_2^{\cdot}$	nitrogen dioxide		
$^{\cdot}NO$	nitric oxide		
$RS^{\cdot}$	thiyl radical		
$P^{\cdot}$	protein radical		

Table 2.4 Production of active oxygen species

Active oxygen species	Formation
Superoxide (Hydoperoxyl radical), $O_2^{\cdot-}$, $HO_2^{\cdot}$	enzymatic and non-enzymatic one electron reduction of oxygen $O_2 + e \rightarrow O_2^{\cdot-} \leftrightarrow HO_2^{\cdot}$ (pK = 4.8)
Hydroxyl radical, $HO^{\cdot}$	radiolysis of water, metal-catalyzed decomposition of hydrogen peroxide, interaction of NO and superoxide $NO + O_2^{\cdot-} \rightarrow ONOO^- \xrightarrow{H^+} HO^{\cdot} + NO_2$
Alkokyl and peroxyl radicals, $LO^{\cdot}$, $LO_2^{\cdot}$	metal-catalyzed decomposition of hydroperoxides
Hydrogen peroxide, H_2O_2	dismutation of superoxide, oxidation of sugar
Iron-oxygen complex, Fe=O, etc	hemoglobin, myoglobin, etc.
Singlet oxygen, $^1O_2$	photosensitized oxidation, biomolecular interaction between peroxyl radicals, reaction of hypochlorite and hydrogen peroxide
Lipid and protein hydroperoxides	oxidation of lipids and proteins
Nitrogen dioxide, $NO_2^{\cdot}$	reaction of peroxyl radical and NO, polluted air and smoking
Nitric oxide, $^{\cdot}NO$	nitric oxide synthase, nitroso thiol, and polluted air
Thiol radical, $RS^{\cdot}$	hydrogen atom transfer from thiols
Protein radical	hydrogen atom transfers from protein

1. Enzymatic system: superoxide dismutase, glutathione peroxidase and catalase
2. Non-enzymatic system
   2.1 Synthetic antioxidant: propyl gallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butyhydro-quinone (TBHQ) (Moure et al., 2001; Scherer and Godoy, 2009)
   2.2 Natural antioxidant: vitamin A (retinol), vitamin B, vitamin C (Ascorbic acid), vitamin E (tocopherols), glutathione, flavonoids, polyphenols, carotenoids, calcium and selenium (Mau et al., 2002; Ozgen et al., 2006; Tsuda et al., 1994). Some natural antioxidants are shown in Fig 2.6.

F. Principle of antioxidant assays

Many kind of assay for determination antioxidant activity can be divided into 2 groups (Magalhaes et al., 2008)

1. Scavenging capacity assays against specific ROS/RNS
   1.1 Peroxyl radical ($ROO^{\cdot}$) scavenging capacity assays
   1.2 Superoxide radical ($O_2^{\cdot-}$) scavenging capacity assays
   1.3 Hydrogen peroxide (H_2O_2) scavenging capacity assays
   1.4 Hydroxyl radical ($HO^{\cdot}$) scavenging capacity assays
   1.5 Hypochlorous acid (HOCl) scavenging capacity assays
   1.6 Singlet oxygen ($^1O_2$) scavenging capacity assays
   1.7 Nitric oxide radical ($NO^{\cdot}$) scavenging capacity assays
   1.8 Peroxynitrite ($ONOO^-$) scavenging capacity assays
2. Scavenging capacity assays against stable, non-biological radicals and evaluation of total reduction capacity
   2.1 Scavenging of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\cdot+}$) or Trolox equivalent antioxidant capacity (TEAC) assay
   2.2 Scavenging of 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$ assay)
   2.3 Ferric reducing antioxidant power (FRAP assay)
   2.4 Folin-Ciocalteu reducing capacity (FC assay)
   2.5 Total reducing capacity estimated by electrochemical method

Moreover some reports were examined antioxidant activity by other antioxidant analysis for example reducing power, inhibition of beta-carotene bleaching, inhibition of erythrocyte hemolysis mediated by peroxyl free radicals, inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS), metal chelating, hypoxanthine/xanthine oxidase และ 2-Deoxyguanosine (Barreira et al., 2008; Geckil et al., 2005; Sudjaroen et al., 2005)

1. DPPH radical scavenging

DPPH radical scavenging method is used worldwide to investigate free radical scavenging activity of various plants and pure compounds. DPPH radical is a stable free radical that odd electron of the nitrogen atom with purple color but it is sensitive to light, oxygen, pH and type of solvent use. This method was measured by direct hydrogen or electron donation from antioxidant agent to DPPH radical in methanol solution. The reaction base on bleaching purple color of DPPH radical and increasing yellow color of DPPH reduced form can be measure at absorbance 515 nm. The decreasing of absorbance was direct variation with H donor ability of antioxidant substance (Hsu et al., 2006; Sanchez-Moreno et al., 1998; Scherer and Godoy, 2009).

2. Reducing power

Reducing power (Fe^{3+} reduction) method is important to examine because some authors said the “total antioxidant power” as the “total reducing power” (Ou et al., 2002). Reducing power method is measured electron donor ability of antioxidant agent to chelate and reduce Fe^{3+}, which cause an increase in oxidative damage in biomolecule. The principle was the reduction of $Fe^{3+}(CN^{-})_6$ to $Fe^{2+}(CN^{-})_6$. The product from the reaction was forming the intense Perl’s Prussian blue that contains blue or green blue color. Fe^{3+}_4 $[Fe^{2+}(CN^{-})_6]_3$ or Prussian blue can be measured at absorbance 700 nm. The increasing of absorbance is direct variation with electron donor ability of antioxidant substance (Hsieh and Yen, 2000; Hsu et al., 2006; Shon, Kim and Sung, 2003).

3. Hydrogen radical scavenging

Hydrogen radical scavenging method is examined the ability of antioxidant substance to eliminate hydroxyl radical, the radical is intermediate product of normal metabolism, and can attacks biological molecules is very high reactivity with a wide range of molecules found in living cell (Hsieh and Yen, 2000; Hsu et al., 2006), by Thiobarbituric Acid-Reactive Substances (TBARS). The reaction uses 2-deoxy-D-ribose as substrate. Hydroxyl radical, product from Fenton reaction or reaction between ascorbic acid, EDTA-Fe^{3+} and H_2O_2 damage the substrate. TBARS chromogen is generated by termination the reaction that adds trichloroacetic acid (TCA) and 2-thiobarbituric acid (TBA) and measured at absorbance 532 nm. The decreasing of absorbance is direct variation with eliminate hydrogen radical ability of antioxidant substance (Hsu et al., 2006; Shon et al., 2006).

4. Anti-lipid peroxidation

In food, cosmetic and pharmaceutical industry lipid peroxidation is oxidation of lipid in products that produces aldehydes and ketones. These molecules can cause loss quality of food such as food dye, color, odor and taste. In organisms, lipid peroxidation can cause DNA damage, ageing, heart disease and cancer. Anti-lipid peroxidation method is examined the inhibition lipid peroxidation by measuring malondialdehyde (MDA), product from this reaction, interacts with TBA at absorbance 532 nm. The decreasing of absorbance is direct variation with inhibition lipid peroxidation ability of antioxidant substance (Jiang et al., 2005; Luengthanaphol et al., 2004).

G. Antibacterial substances

The microorganisms of normal flora (endogenous organism) usually cause no harm or diseases to their host. When the normal balances of microorganisms are disrupted, host defense mechanisms are compromised or endogenous organisms are introduced into other sterile body sites, they become pathogenic. Moreover pathogenic organisms are those from exogenous source that enter the body by ingestion, inhalation and direct penetration (Murray et al., 1999). Pathogen results in

many diseases such as diarrhea, pneumonia, foodborne illnesses and skin diseases including acne (Lertsatitthanakorn, et al., 2006; Shan et al., 2008), and food spoilage that is important problem for food, drug and cosmetic industry production.

Bacteria have several shape and habitat, and can be separated into 6 groups (Murray, 1999) including gram positive cocci (*Staphylococcus, Streptococcus, Enterococcus*), gram positive rods (*Bacillus, Mycobacterium*), gram negative bacteria (*Eschirichia, Salmonella, Pseudomonas*), anaerobic bacteria (*Clostridium*), curved and spiral shaped gram negative rods (*Arcobacter, Helicobactor, Leptospira*), and mycoplasmas and obligate intracellular bacteria (*Mycoplasma, Chalmydia, Rickettsia*).

Preliminary examination of antibacterial agent often chooses representative of gram positive and gram negative bacteria to evaluate the activity. *S. aureus* and *E. coli* are used worldwide to represent gram positive and gram negative bacteria, respectively. Gram negative consists of peptidoglycan and outer membrane (lipopolysaccharide and protein) while gram positive contains peptidoglycan only.

Antibacterial substance can inhibit growth or kill bacteria. They are separated to 3 groups.

1. **Antibiotic**: Amikacin, Ampicilin, Ampicilin/sulbactam, Cefepime, Cefotaxime, Ceftacidime, Ceftriaxone, Cefuroxime, Gentamycin, Levofloxacine, Methicilin, Oxacilin, Piperacilin/tazobactam, Imipenem, Meropenem, Streptomycin, Vancomycin (Arias et al., 2004), Chloramphenicol, Clindamycin, Erythromycin and Tetracycline (Domig et al., 2007).
2. **Preservative**: benzyl alcohol, propylene glycol (Bury et al., 1995), phenoxyethanol, paraben (a para-hydroxybenzoate), imidazolidinyl urea (Mambro and Fonseca, 2005), benzoic acid (Padilla, Palma and Barroso, 2005), sorbic acid (Saad et al., 2007), sodium benzoate and potassium sorbate (Tian et al., 2006)
3. **Natural antibacterial**: phenolic compound (Shan, B., Cai, Y., Brooks, J.D. and Corke, H. 2008) and extracts from garlic, ajowain, black pepper, clove, ginger, cumin, caraway (Arora and Kaur, 1999), essential oils of tea, thyme, sage, coriander, garlic, onion, eucalyptus, melissa, rosemary, mint, rosa moschata, sweet basil, anise, lemon, oregano, lavender (Ponce et al., 2003) and tamarind (stem and bark) (Doughri, 2006).

H. Principle of antibacterial assays

Antimicrobial activity assay often uses can be separated to 4 groups.

1. The disk diffusion method
2. The dilution method
3. The serum killing power
4. Automated method (Black, 2008)

1. Broth Micro/Macrodilution Method

Broth microdilution method is used in the following study and determined minimum inhibitory concentration (MIC) or the lowest concentration of substance that can inhibit growth of bacteria. This method is evaluated by observing growth of bacteria in microtube. The suspension of bacteria are mixed with antibacterial agent and incubated at 37 °C for 24 hours. The lowest concentration of antibacterial agent that shows no growth (clear solution tube) represents the MIC value. Then all clear solution in tubes that show no growth is inoculated on agar plate and incubate at 37 °C for 24 hours. The lowest concentration of antibacterial agent that shows no growth on agar plate represents the MBC value. (Arias, 2004)

I. Emulsion

The term “emulsion” is derived from the word “emulsus.” The verb associated with this word, “emulgere”, means “to milk out” that refer to as original emulsion, the milky liquid extracted from almond (Allen Jr., 2008). In time emulsion is two immiscible liquids (usually oil and water), one of which is encapsulated and dispersed as fine droplet uniformly throughout the other, they are called the dispersed or internal phase and the continuous or external phase, respectively, and emulsifying agent (Chen and Tao, 2005; Swarbrick, 2007; Allen Jr., 2008). Emulsion used in food, drug and cosmetic production and divided into 2 basic from, oil-in-water (O/W or oil droplets-in-matrix water) and water-in-oil (W/O or water droplets-in-matrix oil) emulsion (Chen and Tao, 2005; Pal, 2008). The emulsion model is shown in Fig 2.7.

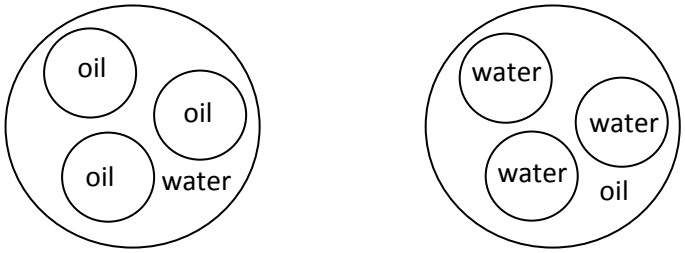


Fig 2.7 Model of oil-in-water (left) and water-in-oil (right)

For cosmetic product, emulsion is prepared to many from such as creams and lotions. Creams are opaque, semisolid emulsion, good spreadility emulsion and easy remove by water. Under gravitation, creams do not flow out through the orifice of reversed containers because of the heavier consistency in comparison with lotions (Barel, Paye and Maibach, 2001; อุบลทิพย์, 2534). The compositions of cream-base are separated to 4 groups of substances (Van de vaart, Hulshoff and Indemen, 1980).

1. water and water-soluble compounds: polymethymethacrylate and glycerol
2. water-insoluble compounds: stearic acid, squalane, capriclic/capric triglyceride, silicone stv-5 and silicone 350
3. emulsifying agents: steareth 2, steareth 21 and cetyl alcohol
4. preservatives and antioxidants: benzoic acid and sodium metabisulfite

Except for emulsifiers, above of these ingredients and other may be divided to 6 types that following to function of cosmetic emulsion (Barel et al, 2001).

1. Emollient: increase sensory properties and spreading ie. stearic acid, cetyl alcohol, squalane and capriclic/capric triglyceride
2. Moisturizers and humectants: increase and control the hydration state of the skin ie. glycerol
3. Viscosity-increasing agent: increase viscosity of external phase ie. xantan gum and cellulose esters.
4. Active substance: ie. antioxidants and vitamins.
5. Preservatives: prevent microorganism growth ie. benzoic acid
6. Perfumes and coloring agent

J. Moisturizing products

Skin is the largest organ (1.5-2 m^2) that normally contain a large number of defense mechanisms to protect the body's internal organ from external agents such as physical, chemical or biological agents, including sunlight, dust, poison, pollution, climate, cleansing, free radical and pathogen as well as control body hydration (พิมพร, 2551; อุบลทิพย์, 2534; Bury et al., 1995; Conno, Ventafridda, and Saita, 1991; Georgetti et al., 2008). In biological organism has a several mechanisms for protect water inside the skin that concern with Rein's barrier (thin keratin layer between stratum lucidum and stratum granulosum), skin fat (waxlike substances), sebum (contained triglyceride, free fatty acid, wax ester, squalene, triglyceride, cholesterol ester and cholesterol) and natural moisturizing factor (NMF).

Three causes to formation dry skin are losing water and lipid or oil from the skin and decreasing sebum that is secreted by sebaceous gland. The moisturizing production can relieve and prevent skin (Fig 2.8) from this problem. Moisturizing products are cream or lotion for normal skin that maintain and save moisture of skin, and prevent all of skin problems such as ageing skin, dry skin, skin rash, cancer and cutaneous autoimmune diseases (พิมพร, 2551; Georgetti et al., 2008). They are separated to many types.

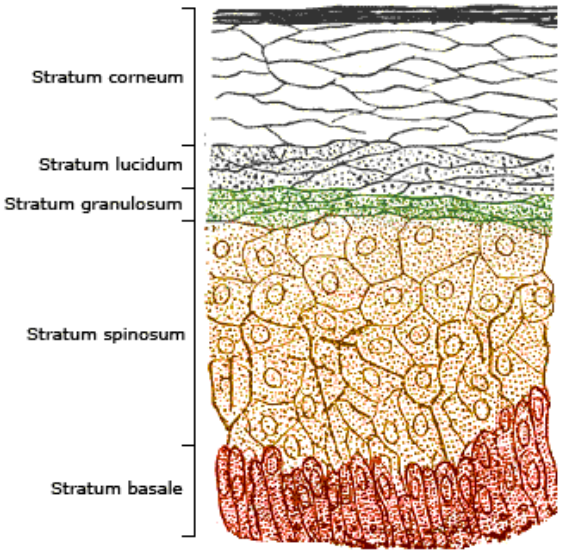


Fig 2.8 Cross section of the upper most layer of skin namely Epidermis (Mahmood : online)

1. Day cream or lotion
2. Nourish cream or night cream and massage cream
3. Hand and body cream or lotion
4. All purpose cream or lotion
5. Barrier cream or lotion

Normally moisturizing creams consist of emollient, humectants and NMF, sometime may be use along skin conditioners. Preparation must take other agents such as emulsifier, thickening agent, preservative, antioxidant, colorant, odor and buffer to the formulation for assist in perform cream or lotion. Production like emulsion preparation under temperature at 75-80 °C, oil phase and water phase were melted and mixed until get smooth cream. Finally cream is added color and odor (พิมพร, 2551).

K. Methods for preparation

Emulsions do not from spontaneously when liquids are mixed. Emulsion preparation must be used energy input such as mechanical agitation, ultrasonic vibration or heat, to break up the liquids and increase the surface area of internal phase. Emulsion can be prepared by both manual and mechanical methods and divided to 6 methods. These methods used including a mortar and pestle, a bottle for shaking, beakers, an electric mixer or a mechanical stirrer, a hand homogenizer and sonifiers (Allen Jr., 2008).

1. The English Method (wet gum method)

This method relies on the use of mucilages or dissolved gums. The ratio of oil:water:emulsifier often ranges from 2 to 4:2:1 for forming the primary emulsion. The small quantity of water is added to the hydrocolloid such as acacia and tragacanth and triturated the mixture until uniform. Then the mixture is added small quantity of oil by using rapid trituration to from thick and viscous mixture. Finally more water is added slowly and triturated rapidly until complete (Allen Jr., 2008).

2. The Continental Method (dry gum method)

The ratio of oil: water: emulsifier is generally about to 4: 2: 1. Oil and hydrocolloid is rapidly mixed for a short time, after which the water is added all at once with rapid trituration until heard a snapping sound that the mixture form the primary emulsion. Finally more water is added slowly with rapid trituration until the emulsion is complete (Allen Jr., 2008).

3. The Bottle Method (shaking)

This method for preparing emulsion that contains volatile oils and nonviscous oil and eliminating the splashing problem that sometimes occurs when two above methods are used. Powder (emulsifier) and oil were added in a bottle and shaken the bottle with short and rapid movement. The required quantity of water is added all at once. The mixture is again shaken rapidly to from the primary emulsion (4:2:1) (Allen Jr., 2008).

4. The Beaker Method

This method is often used with synthetic emulsifying agent. Ingredients are normally divided to oil and water phase. Each phase is heated individually to about 60 °C to 70 °C then the internal phase is stirred into the external phase. Finally the preparation is removed from heated and stirred until it has cooled (congealed) (Allen Jr., 2008).

5. The Mechanical Stirrer (mixer)

The unit's propeller is placed directly into the system to be emulsified. The mixer available commercially and can be found in department stores and gourmet kitchen stores (Allen Jr., 2008).

6. The Hand Homogenizers Function

The mixtures of liquids are forced through a small inlet orifice at a high pressure. The globules are break up by this shearing action (Allen Jr., 2008).

L. Stability test of product

1. Stress condition

Stress condition is used for force drug decomposition. This method is separated to various state, solid, semi-solid and liquid, and type of stability that chemical and physical stability (สุวรรณา, 2547). These methods are showed in Table 2.5.

2. Accelerated condition

This method is examined under higher or lower temperature than ambient temperature or at high relative humidity. Normally this method use 3-4 temperatures for evaluate stability value such as 40, 50, 60 and 70 °C then calculate decay rate constant (k) at this temperature that are used. Finally relation between rate constant and temperature is calculated for predict stability value at room temperature. Stability test by accelerated condition can be use when Activation Energy (E_a), is described by Fig 2.9., of drug in the product is 10-30 kcal/mol. E_a < 10 kcal/mol means drug cannot decomposition by heat and E_a > 30 kcal/mol means drug rapid and strong decomposition (Table 2.6).

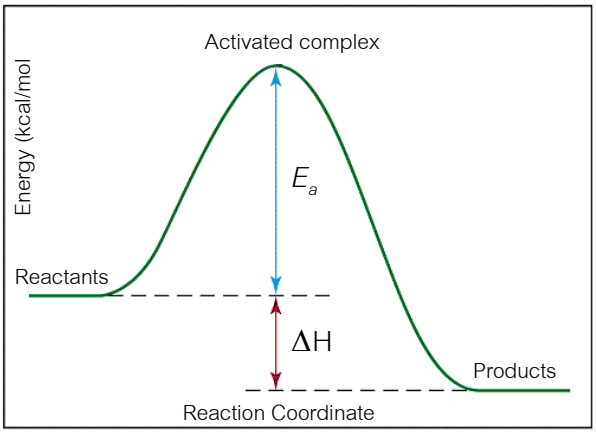


Fig 2.9 Reaction energy (E_a)

3. Normal condition (long term stability)

This method is tested at room temperature and real relative humidity that difference between each country for example 25 °C/60 % RH and 30 °C/70 % RH for long time.

Table 2.5 Stress condition of stability testing for various drugs

Type of stability	State of drug	Storing condition	Time
Physicochemical and Organoleptic stability	Solid	1) open the container until equilibrium at 25 °C/60 % RH, 30 °C/70 % RH, 40 °C/75 % RH	1-2 week
	Semi-solid	1) 5 °C	4 week
		2) ≥ -10 °C	4 week
		3) 5°C and switch to 40 °C every 24 h	2 week
		4) 40 °C (Content Uniformity)	3 month
	Liquid	1) 5 °C	4 week
		2) ≥ -10 °C	4 week
Photostability	All of state	1) xenon light (Atlas Sunset, 250 w/m^2)	48 h
Chemical Stability	Solid	1) 40, 50, 60, 70 °C	3 month
	Semi-solid	1) 30, 40, 50 °C	3 month
	Liquid	1) 40, 50, 60, 70 °C	3 month

Table 2.6 E_a of some reaction

Type of Reaction	E_a (kcal/mol)
Pyrolysis	50-70
Polymorphic Transformation	56
Dehydration	33
Solvolysis	10-30
Oxidation	8-12
Photolysis	2-3

CHAPTER III

MATERIALS AND MATHODS

Materials

A. Chemicals

Chemical name	Grade	Manufacture/Distributor, Country
Acetic acid	HPLC reagent grade	Lab-scan Asia Ltd, Thailand
Ammonium iron (III) sulfate dodecahydrate	analytical reagent grade	Ajax Finechem Pty Ltd, New Zealand
Benzoic acid	analytical reagent grade	BHD Chemicals Ltd, England
n-Butanol	analytical reagent grade	Asia Pacific Specialty Chemicals Ltd, Australia
Butylated hydroxyanisole	analytical reagent grade	Sigma-Aldrich, Germany
Caprylic/capric triglyceride	analytical reagent grade	Numsiang Trading, Thailand
(+)- catechin	HPLC reagent grade	Sigma-Aldrich, Germany
Cetyl alcohol	manufacturing grade	Numsiang Trading, Thailand
Chloroform	analytical reagent grade	Fisher Scientific U.K. Limited, U.K.
D&C YELLOW NO.10	manufacturing grade	International Laboratories Corp., Ltd., Thailand
2-Deoxy-D-ribose	analytical reagent grade	Fluka Chemika, Germany
2,2-Diphenyl-l-picrylhydrazyl	analytical reagent grade	Sigma-Aldrich, Germany
Dipotassium phosphate	analytical reagent grade	Ajax Finechem Pty Ltd, New Zealand
Disodiumethylenediamine-tetracetic acid	analytical reagent grade	analytical reagent grade
Disodium hydrogen phosphate anhydrous	analytical reagent grade	Fluka Chemika, Germany
(-)-Epicatechin	analytical reagent grade	Sigma-Aldrich, Germany

Chemical name	Grade	Manufacture/Distributor, Country
Ethanol	manufacturing grade	Government Pharmaceutical Organization, Thailand
Ethyl acetate	analytical reagent grade	Merk KGaA, Germany
Ferric ammonium sulfate	analytical reagent grade	Schuchardt, Germany
Ferric chloride	analytical reagent grade	Sigma-Aldrich, Germany
Ferrous sulfate heptahydrate	analytical reagent grade	Ajax Finechem Pty Ltd, New Zealand
2N Folin-ciocateu's phenol reagent	analytical reagent grade	Sigma-Aldrich, Germany
Gallic acid	analytical reagent grade	Sigma-Aldrich, Germany
Gentamycin sulfate	analytical reagent grade	Sigma-Aldrich, Germany
Glycerol	manufacturing grade	Numsiang Trading, Thailand
Hide powder	analytical reagent grade	Sigma-Aldrich, Germany
Hydrochloric acid	analytical reagent grade	Merk KGaA, Germany
Hydrogen peroxide	analytical reagent grade	Fisher Scientific, UK
L-ascorbic acid	analytical reagent grade	Fluka Chemika, Germany
Methanol	analytical reagent grade	Merk KGaA, Germany
Methanol	analytical reagent grade	Fisher Scientific, UK
Mueller Hinton Agar	analytical reagent grade	Merk KGaA, Germany
Mueller Hinton Broth	analytical reagent grade	Merk KGaA, Germany
Nitrogen gas	industrial grade	Thai Industrial Gases Co., Ltd., Thailand
Polymethylmethacrylate (MP2700)	manufacturing grade	Adinop, Thailand
Potassium chloride	analytical reagent grade	Merk KGaA, Germany
Potassium dihydrogen phosphate	analytical reagent grade	Merk KGaA, Germany
Potassium hexacyanoferrate (III)	analytical reagent grade	Merk KGaA, Germany

Chemical name	Grade	Manufacture/Distributor, Country
Procyanidin B2	HPLC reagent grade	Sigma-Aldrich, Germany
Silicone 350	manufacturing grade	S. Tong Chemicals Co., Ltd., Thailand
Silicone stv-5	manufacturing grade	Numsiang Trading, Thailand
Sodium carbonate	analytical reagent grade	Ajax Finechem Pty Ltd, New Zealand
Sodium chloride	analytical reagent grade	Ajax Finechem Pty Ltd, New Zealand
Sodium hydroxide	analytical reagent grade	Ajax Finechem Pty Ltd, New Zealand
Sodium metabisulfite	manufacturing grade	Asia Pacific Specialty Chemicals Ltd., Australia
Squalane	manufacturing grade	Numsiang Trading, Thailand
Steareth 2 (Brij 72)	manufacturing grade	The East Asiatic (Thailand) Public Company Ltd., Thailand
Steareth 21 (Brij 721s)	manufacturing grade	The East Asiatic (Thailand) Public Company Ltd., Thailand
Stearic acid	manufacturing grade	The East Asiatic (Thailand) Public Company Ltd., Thailand
2-Thiobarbituric acid	analytical reagent grade	Sigma-Aldrich, Germany
Trichloroacetic acid	analytical reagent grade	Merk KGaA, Germany

B. Equipments

- Analytical balance (Mettler Toledo, Thailand)
- Autoclave (Hiriyama, Japan)
- Blender (Model LB20E* (LB 20 EG), Waring commercial, United State of America)
- Centrifuge (Hitachi, Japan)
- Durham tube (Delta Analyticsl Instrument, United State of America)
- Glassware apparatus (Pyrex, United State of America)
- High Performance Liquid Chromatography system (SCL-10A) with UV-VIS detector (SPD-10AV) and program (class VP 6.14) (Shimadzu, Japan)
- Hot air oven (Memmert, Germany)
- Incubator Chamber (Thelco, United State of America)
- Laboratory Fume Hood (Bioquell, United Kingdom)
- Mechanical stirrers (RW 20.n, Becthai, Thailand)
- Magnetic stirrer (HL Instrument, United State of America)
- Micropipette (Pipetman, France)
- pH meter (Mettler Toledo, Thailand)
- Refrigerator at -20 °C (Sanyo, Japan)
- Rotary evaporator (Buchi R-200, Switzerland)
- Rheometer (Rheowin-RV1 software, HAAKE Rheowin, Germany)
- Spectrophotometer (Spectronic analytical instruments, United Kingdom)
- Stirrer (KMO2, Janke and kenkel GMBC and Co. KG, Germany)
- Suction apparatus (Buchner Funnel, Aspirator, SIBATA circulating aspirator WJ-20, Japan)
- Tube appendox apparatus (Axygen, United State of America)
- Ultrasonic Sonicator chamber (Elma, Germany)
- Vakuum fest (Duran, Germany)
- Vortex mixer (Scientific Industrles, China)
- Water bath (Memmert, German)
- Hebvard Trip Balance (Ohaus, United State of America)

C. Accessories

- Disposable syringe filter nylon 13 mm, 0.45 µm (Chrom Tech, United State of America)
- PEROGATIVE, concentrated fragrance material for manufacturind purposes only (CPL Aromas Ltd., United Kingdom)
- Sartolon polyamide, pore size 0.45 µm (Sartorius, Germany)
- Whatman No. 4 filter paper (Whatman, England)

D. Plant samples

Tamarind (*Tamarindus indica* Linn.) pods of 3 cultivars including sour type "Priao" and sweet types "Srichomphu" and "Sithong-nak" were collected from Nakhon-Ratchasima (Korat) Province on February 2007. Seeds were separated and kept at -20 °C until used. Seeds of *Tamarindus indica* L. of different cultivars are shown in Figure 3.1.

"Priao" or TI-P/K

"Srichomphu" or TI-SP/K

"Sithong-nak" or TI-STN/K

Figure 3.1 Seeds of different tamarind cultivars

F. Microorganisms

Staphylococcus aureus ATCC 6538P and *Escherichia coli* ATCC 25922 were applied as a representative gram positive and gram negative bacteria, respectively. The bacteria were kindly supplied by the Department of Biochemistry and Microbiology Department, Faculty of Pharmaceutical Sciences, Chulalongkorn University

Methods

A. Extraction of tamarind seed coats

Tamarind seeds were heated in acid-washed sand-bath at 100 °C for 5 min. Seed coats were removed from kernels and blended to powder by using blender, seed coat powder was stored in screw cap bottles in desiccators. The yields of tamarind seed coat powder from 3 tamarind cultivars were recorded. Samples of tamarind seed coat powder (TSCP) were extracted by using 2 different solvent extraction systems.

1. Process of extraction 1 (System 1)

TSCP 2 g was extracted with 20 mL 70% ethanol (v/v in H_2O) by placing in ultrasonic sonicator chamber for 30 min and filtered through Whatman No.4 filter paper, repeat extracted the residue with 20 mL 70% ethanol until colorless filtrate was obtained. The filtrates were collected, pooled and defatted by vigorously shaking with an equal volume of chloroform in separating funnel for 20 min. The upper layer was separated and shaken again with ethyl acetate for 20 min in 8:20 ratio, v/v. The upper layer or ethyl acetate was separated and evaporated to almost dry by rotary evaporator at 35 °C. The extracts were dried under nitrogen gas and kept in desiccators at room temperature until used for analysis.

2. Process of extraction 2 (System 2)

TSCP 2 g was extracted with 20 mL 70% ethanol (v/v in H_2O) by placing in ultrasonic sonicator chamber for 30 min and filtered through Whatman No.4 filter paper, repeat extracted the residue until colorless with 20 mL 70% ethanol. The filtrates were collected, pooled and defatted by vigorously shaking with an equal volume of

chloroform in separating funnel for 20 min. The upper layer or aqueous ethanol layer was separated and evaporated to almost dry by rotary evaporator at 50 °C, and then dried under nitrogen gas and kept in desiccators at room temperature until used for analysis.

B. Chemical analysis of TSCEs component

1. Total phenolic compounds

Total phenolic compounds were determined (Spanos and Wrolstad, 1990). Pipetted 0.1 mL of 1 mg/mL TSCEs into each test tube and made up to the volume of 8.4 mL with distilled water, added 0.5 mL of 2N Folin-Ciocalteu's phenol reagent and mixed. After the reaction mixture solutions was incubated at room temperature for 3 min, 1.0 mL of 20% sodium carbonate solution was added into each tube, mixed and incubated at room temperature for 1 h. The absorbance was measured at 765 nm against the reagent blank and the amount of total phenol was calculated as gallic acid equivalents from the calibration curve.

2. Tannin content

Tannin content was determined as described in European pharmacopoeia 4^{th} edition, Council of Europe (2002). Sample of 100 mg/mL TSCEs was diluted with 250 times volume of distilled water and filtered, discarded the first 50 mL of TSCEs filtrate and collected the TSCEs filtrate.

2.1 Determination of total phenol (A_1)

Five milliliter of TSCEs filtrate was diluted to 25 mL with distilled water, then 2 mL of this diluted sample solution was mixed with 1 mL of 2N Folin-ciocateu's phenol reagent and added 10 mL of distilled water. The solution of 29% w/v sodium carbonate was added to make up to the volume of 25 mL and mixed. After standing for 30 min, the absorbance of the reaction mixture was measured at 760 nm against reagent blank. The total phenol was expressed as A_1.

2.2 Determination of phenol not absorbed by hide powder (A_2)

Ten milliliter of TSCEs filtrate was added 0.1 g of hide powder and shaken vigorously for 1 h, then filtered through Whatman No.4 filter paper, 5 mL of the filtrate was diluted to 25 mL with distilled water, 2 mL of this diluted filtrate was mixed with 1mL of 2N Folin-ciocateu's phenol reagent and 10 mL of distilled water. The solution of 29% w/v sodium carbonate was added to make up to 25 mL and mixed. After standing for 30 min, the absorbance of the reaction mixture was measured at 760 nm against reagent blank. Phenol not being absorbed by hide powder was expressed as A_2.

2.3 Determination of standard pyrogallol

Five milliliter of 0.5 mg/mL pyrogallol was diluted to 100 mL with distilled water, 2 mL of this dilute standard solution was mixed with 1 mL of 2N Folin-ciocateu's phenol reagent and 10 mL of distilled water. The solution of 29% w/v sodium carbonate was added to make up to 25 mL and mixed. After standing for 30 min, the absorbance of the reaction mixture was measured at 760 nm against reagent blank. Pyrogallol standard was expressed as A_3.

The percentage content of tannin expressed as pyrogallol was calculated from this equation:

$$\%\text{tannin} = \frac{62.5\,(A_1-A_2)m_2}{A_3 \times m_1}$$

* m_1 = weight of sample in grams

m_2 = weight of pyrogallol in grams

3. Proanthocyanidin

Proanthocyanidin was determined as described by Rathee, Hassarajani and Chattopadhyay (2006). One milliliter of 1 mg/mL of TSCEs was mixed with 6 mL of acid butanol reagent (1 mL conc HCl in 19 mL n-butanol) and added 0.2 mL of iron reagent (1g $FeNH_4(SO_4)12H_2O$ in 50 mL of 2N HCl). The reaction mixtures were incubated at 95 °C for 50 min, cooled and an absorbance was measured at 550 nm against reagent blank.

C. Analysis of TSCEs component by HPLC Technique

1. Stock solution of samples and reference standards

TSCEs in System 1 were dissolved in methanol (HPLC grade) to make 5,000 µg/mL concentration of TSCEs stock solution. Each of the reference standard was dissolved in methanol to make concentration of 100 µg/mL for (-)-epicatechin and procyanidin B2 and (+)-catechin. The standard stock solutions were stored at -20 °C.

2. Samples and reference standard solutions

2.1 TSCEs solution: TSCEs stock solutions were diluted two-fold with methanol to make the final concentration at 2,500 µg/mL.

2.2 Standard solution mixture: The 3 stock standard solutions, each of 250 µL, were mixed with 250 µL of methanol to make the final concentration of each reference standard at 25 µg/mL.

2.3 The mixture of TSCEs with (-)-epicatechin or procyanidin B2: Mixed 500 uL of TSCEs stock solution with 500 µL of (-)-epicatechin or procyanidin B2 stock solution to make the final concentrations of TSCE and (-)-epicatechin or procyanidin B2 at 2,500 and 50 µg/mL, respectively.

2.4 The mixture of TSCEs with (+)-catechin: (+)-catechin stock solution were diluted to make the final concentration at 20 µg/mL. Mixed 500 µL of TSCEs stock solution with 500 µL of 20 µg/mL (+)-catechin to make the final concentration at 2,500 and 10 µg/mL, respectively.

3. Mobile phase

Two mobile phase systems were applied as described by Sudjaroen et al., 2005. Phase A composed of 2% v/v acetic acid in ultrapure water. Phase B was methanol. The mobile phase solutions were prepared and filtered through the Sartolon polyamide (0.45 µm pore size) and degassed in ultrasonic sonicator chamber for 20 min before used.

4. HPLC condition

The phenolic compounds were separated and identified according to the method described by Sudjaroen et al. (2005). The samples and reference standard in 2.1, 2.2, 2.3, and 2.4 were filtered through the disposable syringe filter nylon 13 mm, 0.45 µm before injection into the HPLC column. A volume of 10 µL of sample solutions was injected into the HPLC column (C18 Hypersil GOLD, 5 µm, 250x4.6 mm, Column No. 1251575T), equipped in the HPLC instrument (SCL-10A, Shimadzu) with UV-VIS detector (SPD-10AV), and monitored the HPLC condition by program Class VP 6.14. The following gradient as shown in Table 3.1 was used in a total run time of 50 min. The flow rate was 1.0 mL/min, the column oven was 30 °C (CTO-10AS, Shimadzu, Japan), and UV-VIS detector (SPD-10AV) was set at 278 nm.

Table 3.1 The percentage of mobile phase at difference time

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0-10.00	95	5
10.01-20.00	90	10
20.01-30.00	85	15
30.01-40.00	80	20
40.01-45.00	60	40
45.01-50.00	0	100

D. Antioxidant activity of TSCEs

1. DPPH radical scavenging

DPPH radical scavenging activity was determined by measuring the bleaching of purple color as described by Sanchez-Moreno, Larrauri and Saura-Calixto (1998). Mixed 0.1 mL of 0-500 µg/mL TSCEs with 3.9 mL of methanolic solution containing 0.025 mg/mL DPPH and incubated in the dark at room temperature for 1 h. The absorbance was measured at 515 nm against reagent blank. Ascorbic acid and BHA were used as reference antioxidant positive controls. The EC_{50} values for TSCEs

were examined and the % scavenging values were calculated by using the following equation.

$$\% \text{ scavenging activity} = [(OD_{T=0})-(OD_T)]/ (OD_{T=0}) \times 100$$

$OD_{T=0}$ = Absorbance value at time zero (initial concentration)

OD_T = Absorbance value at the time of 60 min

2. Reducing power

Reducing power was determined by measuring the formation of the intense Prussian blue complex as described by Yen and Chen (1995). One milliliter of 0-160 µg/mL TSCEs were mixed with 2.5 mL of 0.2 M of sodium phosphate buffer pH 6.6 and 2.5 mL of 1% w/v potassium hexaferrocyanate. The reaction mixtures were incubated at 50 °C for 20 min and added 2.5 mL of 10% w/v TCA and then incubated at room temperature for 10 min. Pipetted 2.5 mL of the reaction mixtures into another test tube and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% w/v of $FeCl_3$. The absorbance was measured at 700 nm against reagent blank. Ascorbic acid and BHA were used as reference antioxidant positive controls. The EC_{50} values for TSCEs were examined.

3. Hydroxyl radical scavenging

Hydroxyl radical scavenging assay or the deoxyribose degradation assay (Xiang and Ning, 2008) was evaluated by measuring the formation of thiobarbituric acid-reactive substance (TBARS) using 2-Deoxy-D-ribose as substrate, as described by Hsu et al, 2006. Samples 250 µl of 0-500 µg/mL TSCEs were added and mixed with 250 µL of 0.1 M potassium phosphate buffer pH 7.4, 100 µL of 28 mM of 2-Deoxy-D-ribose, 200 µL of Fe^{3+}EDTA (a mixture of 100µM $FeCl_3$ + 104 µM Na_2EDTA in 1:1 ratio, v/v), 100 µl of 1 mM of H_2O_2 and 100 µL of 1 mM ascorbic acid, respectively. The reaction mixtures were incubated at 37 ºC for 1 h and added 1 mL of 2.8% w/v TCA and 1 mL of 1% w/v TBA, then incubated at 100 ºC for 20 min. The absorbance was measured at 532 nm against reagent blank. Ascorbic acid and BHA were used as

reference antioxidant positive controls. The EC_{50} values for TSCEs were examined and the % scavenging values were calculated by using the following equation.

$$\% \text{ scavenging activity} = [(OD_{T=0})-(OD_T)]/ (OD_{T=0}) \times 100$$

$OD_{T=0}$ = Absorbance value at absence of sample

OD_T = Absorbance value at presence of sample

4. Anti-lipid peroxidation

Anti-lipid peroxidation assay was evaluated by measuring the formation Malondialdehyde (MDA) and Thiobarbituric acid (TBA) to from TBARS, as described by Jiang et al. (2005). Yolk suspension was prepared by diluting egg yolk with 40 times volume of phosphate buffer saline (PBS) pH 7.4, mixed 0.5 mL of yolk suspension, 1 mL of 0-500 µg/mL TSCEs and 24 mM $FeSO_4$ in PBS together. The mixtures were incubated at 37 °C for 15 min and stopped the reaction by adding 0.5 mL of 20% w/v TCA and 1 mL of 0.8% w/v TBA, incubated at 100 °C for 15 min, respectively. Reaction mixtures were centrifuged at 3500 rpm for 20 min and measured the absorbance at 532 nm against reagent blank. Ascorbic acid and BHA were used as reference antioxidant positive controls. The EC_{50} values for TSCEs were examined and the % inhibition values were calculated by using the following equation.

$$\% \text{ inhibition} = [(OD_{T=0})-(OD_T)]/ (OD_{T=0}) \times 100$$

$OD_{T=0}$ = Absorbance value at absence of sample

OD_T = Absorbance value at presence of sample

E. Antibacterial activity of TSCEs

Antibacterial activity of TSCEs was determined by using the broth microdilution method as described by Murray et al. (1999), Oke et al. (2009) and Puma et al. (2009). Two-fold serial dilutions of TSCEs were prepared to make 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78125 and 0.390625 mg/mL concentrations in Mueller Hinton

Broth (MHB). The bacteria were streaked on agar plate and incubated overnight at 37 °C. Then bacteria were inoculated in 5 ml MHB, incubated at 37 °C for 3-4 h. The cells suspension turbidity used was equivalent to 0.5 McFarland standard or OD_{625}=0.08-0.13 (contained 1×10^{8} CFU/mL). The bacterial suspension was diluted by ten-fold serial dilution to make $1x10^{6}$ CFU/ml. Pipetted 100 µL of TSCEs dilution and 100 µL of bacterial suspension into the Durham tube and incubated at 37 ºC for 24 h, the bacterial growth was recorded. Minimum inhibitory concentration (MIC) was the lowest concentration in tube of non-visible growth. Minimum bactericidal concentration (MBC) was the lowest concentration in tube of non-visible growth that shows no growth after streaked on agar plate (Mueller Hinton Agar or MHA) and incubated overnight at 37 ºC. The MIC and MBC values were determined. Gentamycin sulfate was used as a positive control.

F. Formulation of TSCE cream

Formulation of cream base as modified by Songmek (2007) was used. The cream was prepared by beaker method (Allen Jr., 2008). Ingredients were divided into 2 phases of oil phase and water phase (Table 3.2) and the functions of each ingredient are shown in Table 3.3.

Table 3.2 Ingredients of cream base

Oil phase		Water phase		
Stearic acid	2 g	Polymethymethacrylate		1 g
Cetyl alcohol	1 g	Glycerol		4 g
Steareth 2	3 g	Benzoic acid		0.2 g
Steareth 21	2 g	Sodium metabisulfite		0.5 g
Squalane	1 g	Ultrapure water	q.s. to	100 g
Caprylic/capric triglyceride	1 g			
Silicone stv-5	4 g			
Silicone 350	2 g			

Polymethylmethacrylate was dispersed in water before the other ingredients were added in water phase. The water phase was controlled at 78 °C by using hot steam from water bath. The oil phase including stearic acid, cetyl alcohol, steareth 2 and steareth 21 were respectively melted and mixed with the hot oil, mixture of squalane, caprylic/capric triglyceride, silicone stv-5 and silicone 350. The oil phase was heated to 75 °C over hot steam from water bath and was slowly poured into the water phase while stiring and mixing with speed level 8 for 60 min in the mixer. TSCEs creams were prepared by slowing adding TSCE 0.1, 0.3 or 0.5 g/10mL in 70% ethanol into the cream base at 60-70 °C while stirring, the emulsion was continuously stirring to cool down, color and odor were added into emulsion and stirred until congeal.

Table 3.3 Function of each ingredient in cream formulation

Ingredients	Function
Stearic acid	thickening agent, stability and iridescent effect
Cetyl alcohol	emollient, thickening agent, increase stability and iridescent effect
Steareth 2	emulsifier
Steareth 21	emulsifier
Squalane	emollient and moisturizer
Caprylic/capric triglyceride	emollient
Silicone stv-5	antifoaming and skin condition
Silicone 350	antifoaming and skin condition
Polymethymethacrylate	reduced sticky feeling
Glycerol	humectant
Benzoic acid	preservative
Sodium metabisulfite	antioxidant agent

G. Efficacy of TSCE cream and stability test

1. Stability test

Stability tests were performed as described by สุวรรณา เหลืองชลธาร (2547) and Songmek (2007). The characteristics and physical properties of cream including pH, viscosity at shear rate from 0 to 100 s^{-1} at 25 °C, texture, color, odor, air bubble and phase separation were examined at freshly prepared and after stability test.

1.1 Stress condition

All creams were stood at 5 °C for 24 h and then at 40 °C for 24 h which was 1 cycle. The test was repeated for 3 cycles. An appearance and physical properties of cream were recorded.

1.2 Normal condition or long term stability

All creams were stood at room temperature for 3 months. An appearance and physical properties of cream were recorded after freshly prepared and after 2, 4, 8 and 12 weeks stand at room temperature.

2. Efficacy of free radical scavenging activity of TSCE cream

DPPH radical scavenging activity was determined by hydrogen-donating activity. The 0.1 g of cream was mixed with 9 mL of methanol, added 1 mL of 0.4 mg/mL DPPH in methanolic solution and stood in dark at room temperature for 1 h. The mixture was centrifuged at 1100 rpm for 10 min and the absorbance was measured at 515 nm against reagent blank. The percentages of scavenging activity were calculated. Ascorbic acid and TSCEs solution were used as antioxidant standard reference and control (กรวรรชญ์ มงคล และคณะ, 2006).

H. Statistical analysis

Mean values and standard error of mean (SEM) were calculated from the results (n=3). One way analysis of variance (ANOVA) with Least Significant Difference (LSD) was applied for comparison of the mean values. The p values < 0.05 was regarded as significant. The statistical Package for Social Science (SPSS) program version 15.0 was used for calculation.

CHAPTER IV

RESULTS AND DISCUSSIONS

A. Extraction of TSCEs

Dried Seed coats of different tamarind cultivars including *Tamarindus indica* Linn. “Priao” (TI-P/K), “Srichomphu” (TI-SP/K) and “Sithong-nak” (TI-STN/K) were extracted by the System 1 and System 2 as indicated. Solvents of System 1 were ethanol, chloroform and ethyl acetate, respectively, whereas System 2 were ethanol and chloroform, respectively. TSCEs in System 1 in ethyl acetate fraction and TSCEs in System 2 in ethanol fraction were dried to obtain dried granules of TSCEs. Percentage yield of the TSCEs are shown in Table 4.1. The dried TSCEs were black granules, TSCEs solution in ethanol or methanol were red brown solution. The % yields obtained in System 2 were significantly higher than that of System 1. The % yields of TSCEs of difference tamarind cultivars were not significantly different by using the same process of solvent extraction (Table 4.1).

B. Chemical analysis of TSCEs

Chemical analysis of TSCEs from the 3 tamarind cultivars is shown in Table 4.2. The results showed that the total phenolic compounds in TSCEs in System 2 from the three tamarind cultivars were equivalent to 491-506 mg GAE/g of dry extract. TSCEs in System 1 showed the lower total phenolic compounds than that of TSCEs in System 2. The total phenolic compounds in TSCEs of TI-P/K and TI-SP/K in System 1 was equivalent to 463 and 430 mg GAE/ g of dry extract, respectively, where the total phenolic compounds in TSCEs of TI-STN/K was equivalent to 340 mg GAE/g of dry extract which was significantly lower than that of the TSCEs of TI-P/K and TI-SP/K. The same pattern was also found with the results of tannin and proanthocyanidin contents (Table 4.2). Percentages of tannin and proanthocyanidin contents in TSCEs from all tested tamarind cultivars were not significantly different, except for the TI-STN/K (Table 4.2).

This result showed that the total phenolic compounds that found in TSCEs were higher than those in the extracts of many fruits seed previously studied,

Table 4.1 Percentage yield of TSCEs extracted by two solvent extraction processes. Data are mean±SE (n=3)

Tamarind cultivars	% Yield of TSCEs	
	System 1	System 2
TI-P/K “Priao”	16.52±4.61[a]	58.35±0.69[b]
TI-SP/K “Srichomphu”	15.33±4.27[a]	54.03±3.02[b]
TI-STN/K “Sithong-nak”	18.10±2.58[a]	61.90±4.94[b]

a, b = significantly different between groups

Table 4.2 Chemical analysis of TSCEs component of difference tamarind cultivars and process of extraction

Tamarind cultivars	Total phenolic compounds (mg GAE/g of dry extract)	% tannin (as pyrogallol equivalent)	Proanthocyanidin (OD 550 nm, AU)
TSCEs from System 1			
TI-P/K	462.52±7.59^{b,c}	54.47±5.43^{b}	1.30±0.04^{b}
TI-SP/K	430.21±25.38^{b}	60.98±5.50^{b}	1.26±0.03^{a,b}
TI-STN/K	340.47±8.21^{a}	29.00±2.38^{a}	1.01±0.03^{a}
TSCEs from System 2			
TI-P/K	496.11±22.59^{c}	62.88±9.46^{b}	1.55±0.11^{b,c}
TI-SP/K	506.36±21.91^{c}	56.27±7.61^{b}	1.75±0.12^{c}
TI-STN/K	491.24±22.19^{c}	53.04±7.28^{b}	1.49±0.11^{b}

Data represent mean±SE (n=3).

a, b, c = significantly different ($p<0.05$) between different cultivars in the same column.

according to the report of Soong and Barlow (2006) in fruits of longan, avocado, jack fruit and tamarind seed from Singapore extracted with 50% ethanol (94.5±4.9 mg GAE/g of extract). In addition, Kahkonen et al. (2004) have also reported the lower values in berries seed, rose seed and flax seed. The lower value of total phenolic content of tamarind seed (386.22 mg GAE/g of dry extract) from Pethchaboon province, Thailand was also previously reported (Suksomtip and Pongsamart, 2008).

The total phenolic compounds determined by this method was used as a preliminary evaluation of total phenolic compounds, other reducing species such as aromatic amines, sulfur dioxide, ascorbic acid, Cu(I), Fe(II), etc are also gave positive result (Magalhaes et al., 2008). Then the real value of total phenolic content can be lower than the values observed. TSCEs from the tested tamarind cultivars gave a high value of tannin content (53-63 %tannin), except for the TI-STN/K in System 1 (29 %tannin). The values of tannin content obtained in this study were higher than those found in seed coat extract of tamarind from India (18.23-26.24 %tannin) which was extracted with methanol and 70% acetone (Siddhuraju, 2007).

Tannin is divided into 2 groups including hydrolysable tannin and condense tannin (proanthocyanidin). In this study, proanthocyanidin showed more potent antioxidant activity and free radical scavenging activity than those of simple monomeric phenolic compound (Oszmianski, Wojdylo and Kolniak, 2009; Saito et al., 1998) that was also found in TSCEs. This result also showed that TSCEs in System 2 possesed the higher proanthocyanidin content (1.49-1.75 AU) than that of TSCEs (1.01-1.30 AU) in System 1, proanthocyanidin content in TSCE of TI-STN/K in System 1 showed the lowest value in this study.

C. Analysis of TSCEs component by HPLC Technique

TSCEs were separated and identified their chemical profiles by HPLC technique, column C18 Hypersil GOLD (5 µm, 250x4.6 mm) was used and the mobile phase was 2% acetic acid in ultrapure water (phase A) and methanol (phase B). The following gradient in a total run of 50 min was used: 95% A in 10 min, 90% A in 10 min, 85% A in 10 min, 60% A in 5 min and 0% A in 5 min, respectively. The HPLC chromatograms of TSCE of TI-P/K, TI-SP/K and TI-STN/K in System 1 showed peaks about 20 peaks in Fig 4.1. The HPLC chromatograms of TSCEs of sour type TI-P/K (A) and sweet type TI-SP/K (B) and TI-STN/K (C) showed similar chemical profile (Fig 4.1) including the identical peaks of (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3) reference standards at retention time 18.777, 25.537 and 31.391 min, respectively, (Fig 4.2).

In Fig 4.3, 4.4 and 4.5 showed HPLC chromatograms of the TSCEs of TI-P/K, TI-SP/K and TI-STN/K in System 1 with the spiked peaks of the marker standard (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3), respectively. Their retention time and adjusted retention time are showed in Table 4.3, the adjusted retention time value is more accurate than the retention time which is correlates with condition changing, including flow rate (รัตนา, 2544) to identify peak. The HPLC chromatograms of TSCEs of both sour and sweet tamarinds (Fig 4.3, 4.4 and 4.5) obtained in this study also showed the similar profile of peaks of tamarind seed coat extract previously studied (Sudjaroen et al., 2005). The phenolic compounds that found in TSCEs as well as reference standards, (+)-catechin and (-)-epicatechin exhibited antioxidant activity (Muselík et al., 2007; Othman et al., 2010; Sakano et al., 2005) with DPPH radical scavenging and reducing power assays and the amount of phenolic compounds estimated from peak area was not reduced after stored at -20 °C for 2 months.

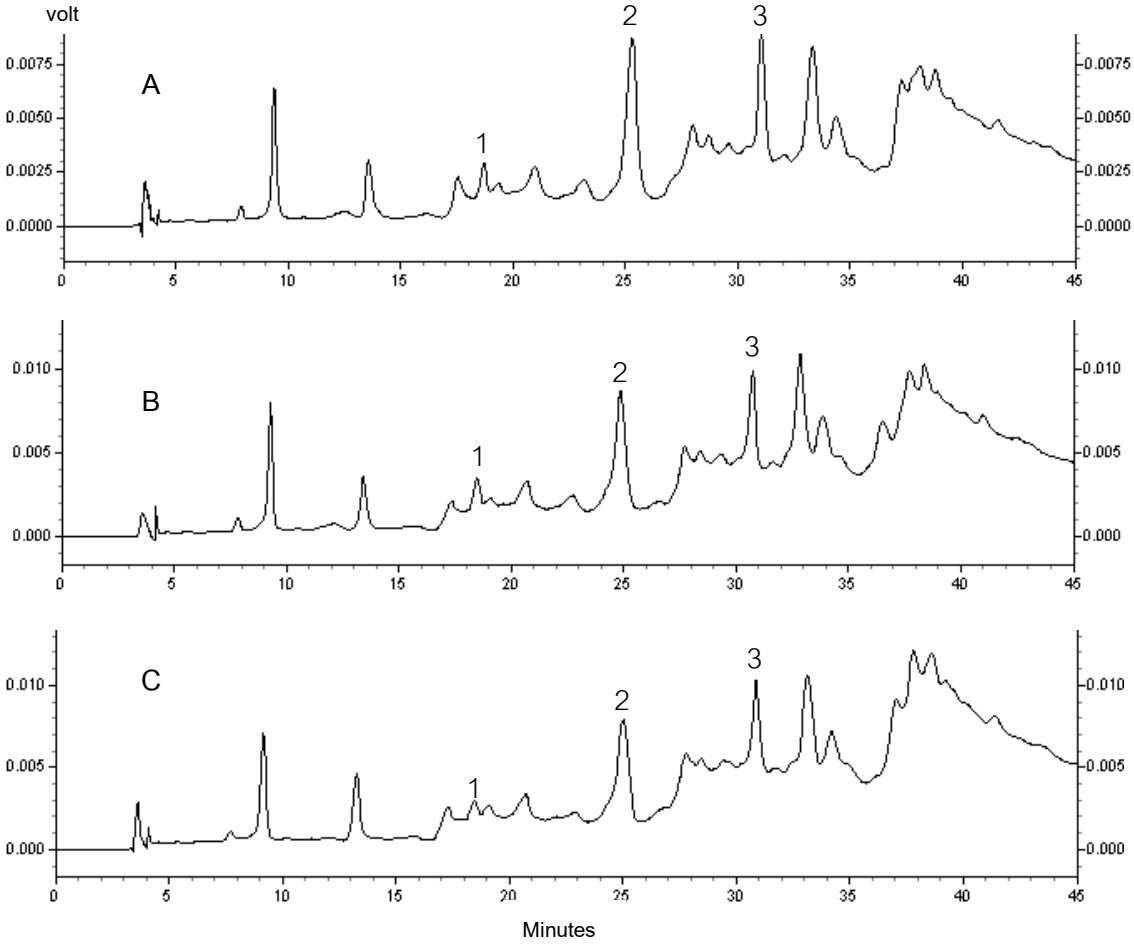


Fig 4.1 HPLC chromatograms of TSCEs of TI-P/K (A), TI-SP/K (B) and TI-STN/K (C) in System 1, peaks showed retention time identical with reference standard (+)- catechin (1), procyanidin B2 (2), and (-)-epicatechin (3).

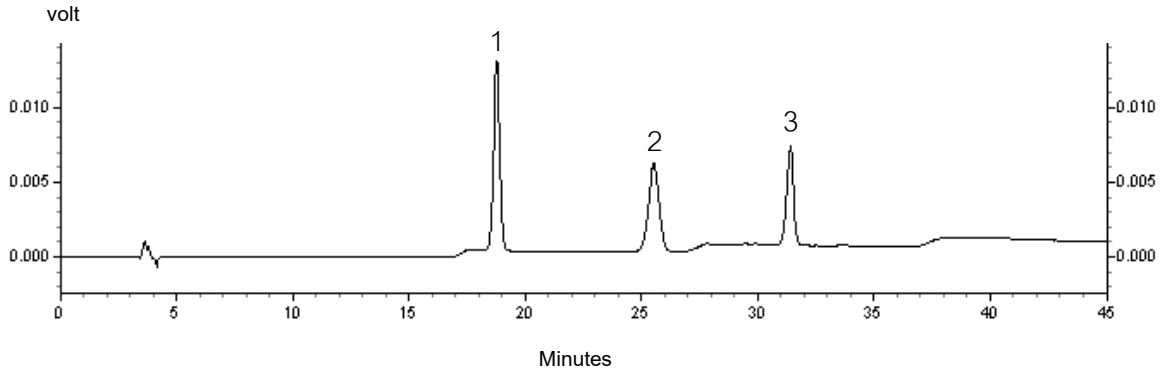


Fig 4.2 HPLC chromatogram of reference standards phenolic compound mixture. The peaks are (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3) at retention time 18.777 min, 25.537 min, and 31.391 min, respectively

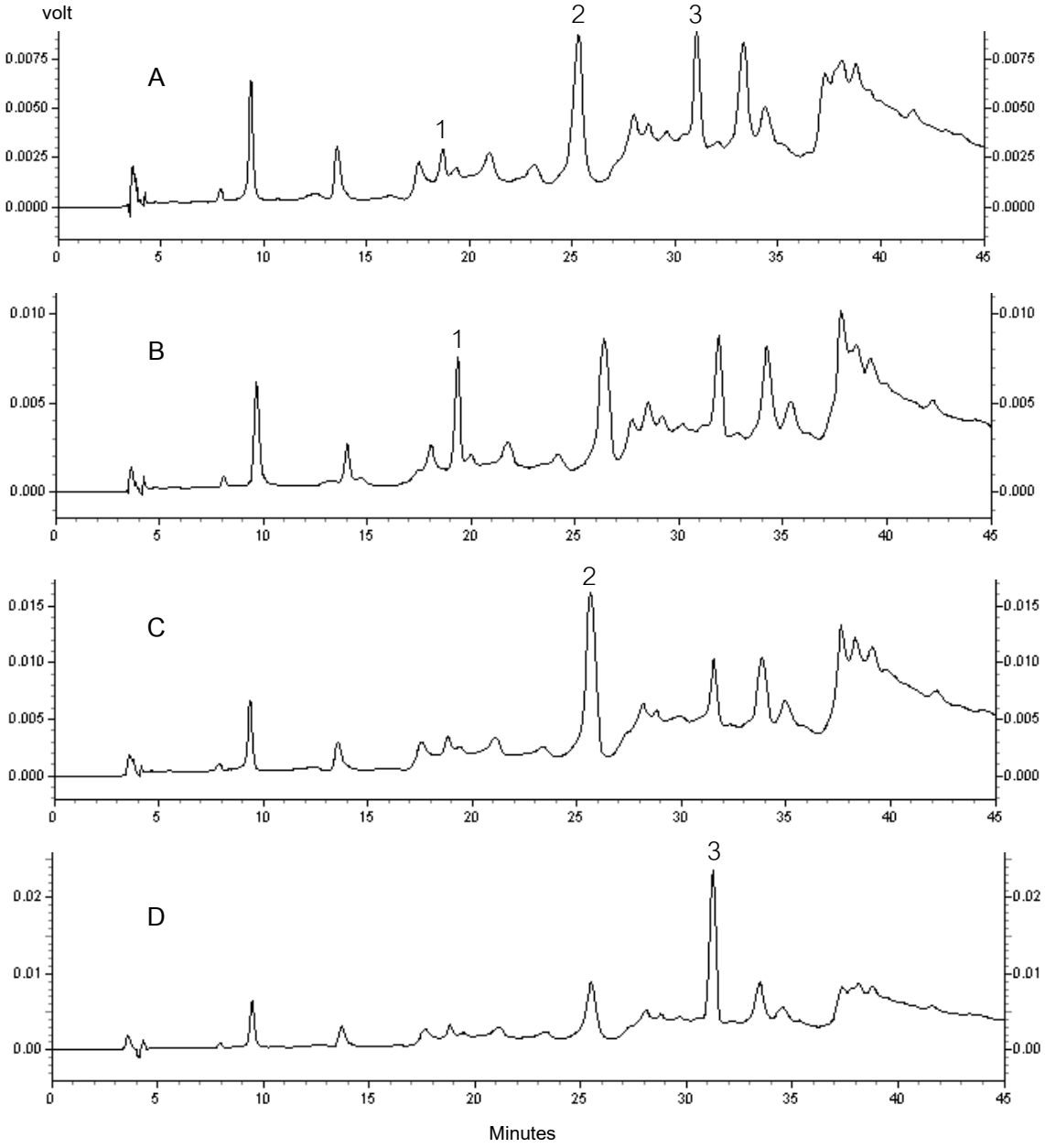


Fig 4.3 HPLC chromatograms of TSEC of TI-P/K in System 1 (A), TI-P/K spiked with (+)-catechin (B), TI-P/K spiked with procyanidin B2 (C) and TI-P/K spiked with (-)-epicatechin (D). TSCE of TI-P/K showed peaks identicle with (+)-catechin (1), procyanidin B2 (2), and (-)-epicatechin (3).

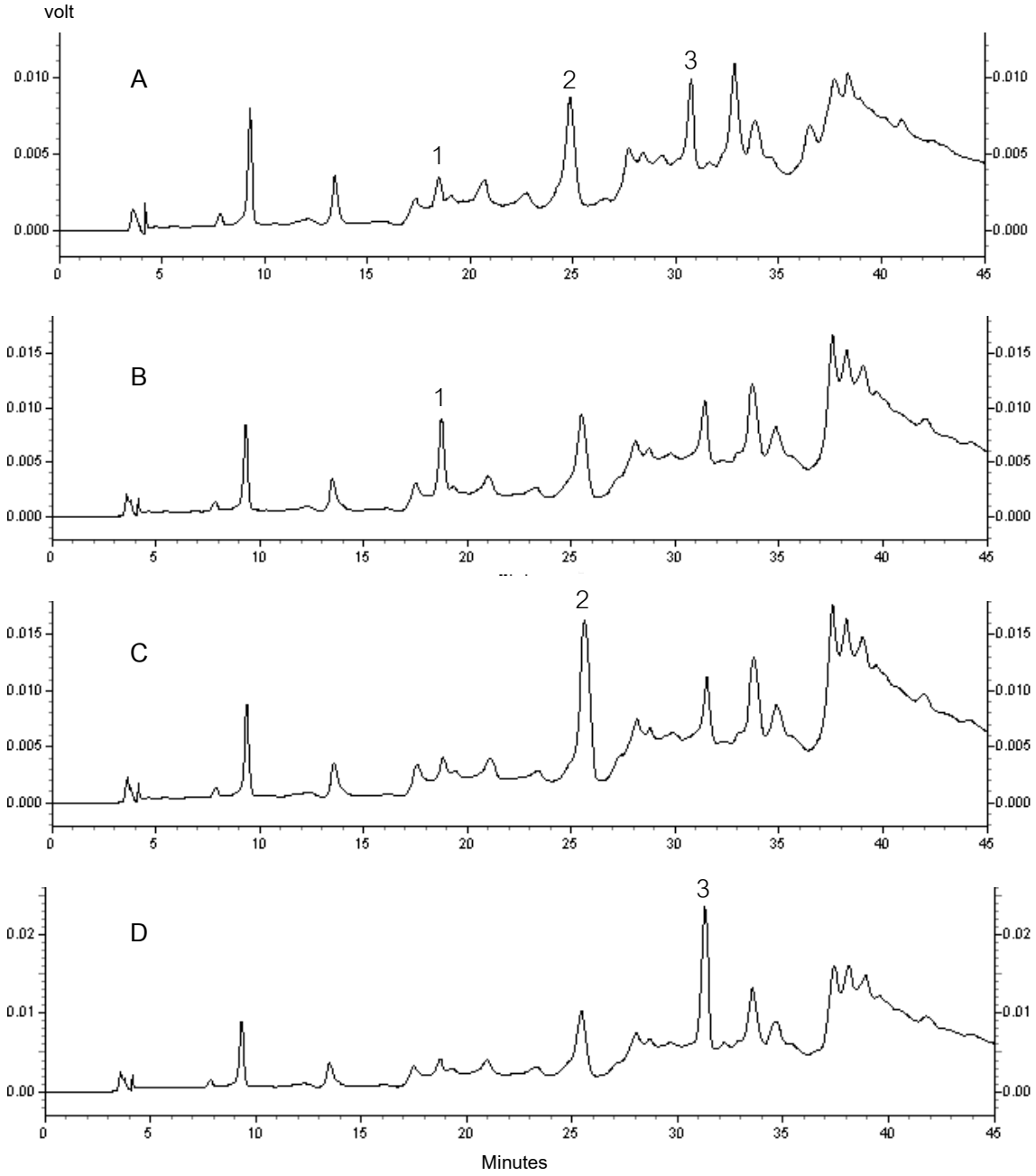


Fig 4.4 HPLC chromatograms of TSEC of TI-SP/K in System 1 (A), TI-SP/K spiked with (+)-catechin (B), TI-SP/K spiked with procyanidin B2 (C) and TI-SP/K spiked with (-)-epicatechin (D). TSCE of TI-SP/K showed peaks identicle with (+)-catechin (1), procyanidin B2 (2), and (-)-epicatechin (3).

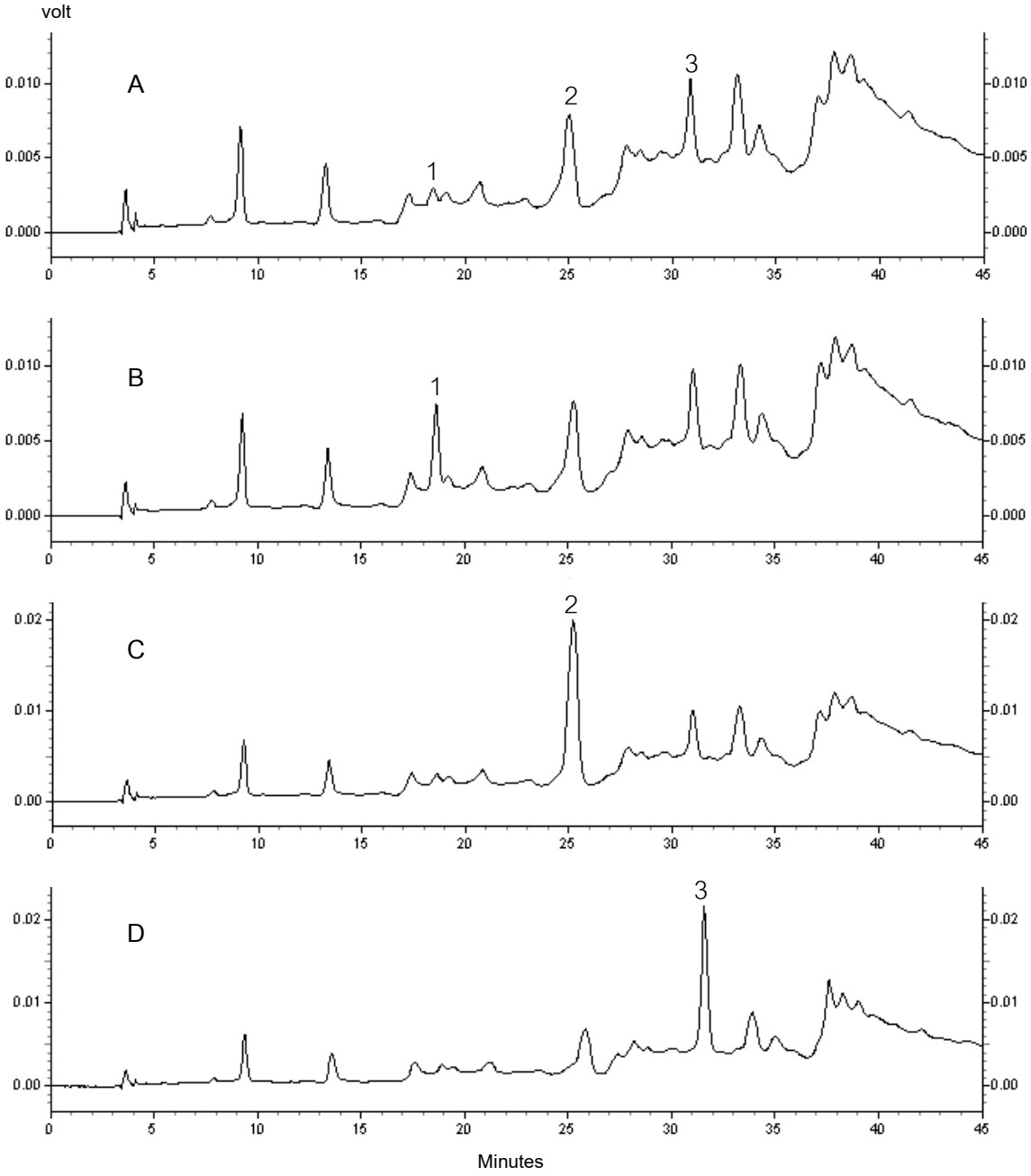


Fig 4.5 HPLC chromatograms of TSEC of TI-STN/K in System 1 (A), TI-STN/K spiked with (+)-catechin (B), TI-STN/K spiked with procyanidin B2 (C) and TI-STN/K spiked with (-)-epicatechin (D). TSCE of TI-SP/K showed peaks identicle with (+)-catechin (1), procyanidin B2 (2), and (-)-epicatechin (3).

Table4.3 Retention time and adjust retention time of (+)-catechin, procyanidin B2 and (-)- epicatechin standard reference and TSCEs in System 1

Sample	Peak identification	Retention time	Adjusted retention time
Standard	(+)-Catechin	18.777	15.119
reference	Procyanidin B2	25.537	21.87
	(-)-Epicatechin	31.391	27.733
TI-P/K	(+)-Catechin	18.699	15.072
	Procyanidin B2	25.298	21.671
	(-)-Epicatechin	31.060	27.433
TI-SP/K	(+)-Catechin	18.472	14.872
	Procyanidin B2	24.868	21.268
	(-)-Epicatechin	30.725	27.125
TI-STN/K	(+)-Catechin	18.448	14.816
	Procyanidin B2	25.022	21.390
	(-)-Epicatechin	30.866	27.234

D. Antioxidant activity of TSCEs

1. DPPH radical scavenging

The percentage of scavenging activity of TSCEs of the three tamarind cultivars are showed in Fig 4.6 and Appendix B, Table B-2. Increasing the percentage of scavenging activity was observed with the increasing concentration of TSCEs. The TSCEs of the 3 tamarind cultivars in System 1 and System 2 showed a comparable scavenging activity as assay by DPPH radical scavenging method (Fig 4.6), however the extracts in System 2 possessed DPPH radical scavenging activity as good as the standard positive control vitamin C and BHA (Fig 4.6, B). TSCEs at 5-7.5 µg/mL in System 1 and System 2 showed high DPPH scavenging activity of 87.04±1.50 - 88.75±0.04 % and 91.34±0.55 - 92.33±0.52 %, respectively, whereas at 10-12.5 µg/mL of the standard positive control vitamin C and BHA showed 96.36±0.09 and 93.23±0.07% DPPH scavenging activity, respectively. The 5 µg/mL of TSCE of TI-P/K in System 2 possessed the highest the % scavenging activity (92.33±0.52 %) with DPPH radical scavenging assay. TSCEs in System 1 showed slow increasing % scavenging activity in comparison with TSCEs in System 2 (Fig 4.6). These results are consistent with the report of Rathee et al. (2006) about DPPH radical scavenging activity revealed most polar fraction. Moreover, TSCE of TI-STN/K in System 1 showed a slowly increase activity compared with TSCEs of TI-P/K and TI-SP/K in System 1. The high % scavenging activity showed that TSCEs possess antioxidant substance as hydrogen atom donated to nitrogen atom of DPPH radical in methanol solution.

The effective concentration at which 50% scavenging for DPPH radical scavenging assay are represent as EC_{50} values, obtained by linear regression analysis. The lower value of EC_{50} indicating the higher antioxidant activity. In the Table 4.4 shows EC_{50} values of TSCEs in System 2 were 2.15±0.07 - 2.23±0.06 µg/ml and these values were lower than that of the standard positive control vitamin C (2.66±0.01 µg/ml), but were not significantly different with the standard positive control BHA (2.21±0.02 µg/ml). EC_{50} values of TSCEs in System 1 showed the higher value (2.98 – 4.00 µg/ml) than that of the standard positive control vitamin C and BHA (2.66±0.01 and 2.21±0.02 µg/ml, respectively). However, these values showed that TSCEs were good hydrogen donor

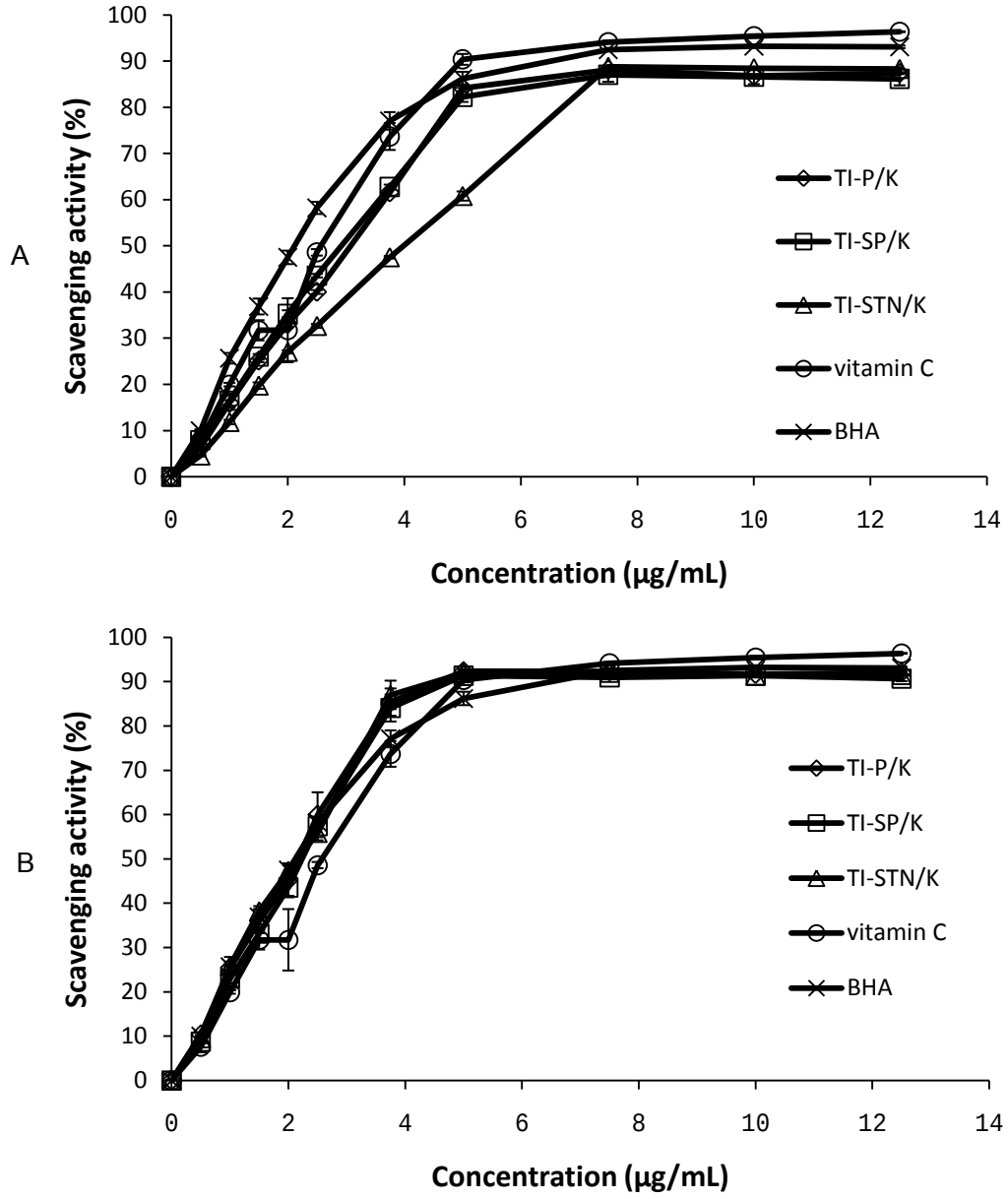


Fig 4.6 Scavenging activity (%) as determined by DPPH radical assay against concentrations of TSCEs of the 3 cultivars extract by different extraction processes; A = System 1 and B = System 2. The values are mean±SE (n=3). Vitamin C and BHA were used as a positive control.

and gave high radical scavenging activity compared with some natural antioxidant such as Chlorogenic acid from South-China honeysuckle (EC_{50} 112.3±2.3 µg/mL) and Chlorogenic laurate, formed by acylation of Chologenic acid with laurate chloride (EC_{50} 70.5±0.7 µg/mL), as previously determined by DPPH radical scavenging assay (Xiang and Ning, 2008).

2. Reducing power

Reducing power of TSCEs of the 3 tamarind cultivars is represent as the increasing absorbance at 700 nm, the increasing of reducing power was observed with increasing the concentration of TSCEs. The TSCEs of the 3 tamarind cultivars in System 2 showed higher reducing power than that of the extract in System 1 (Fig 4.7), the extracts in System 2 possessed reducing power as good as the standard positive control BHA (Fig 4.7, B). TSCEs at 18.82 µg/mL in System 1 and System 2 showed the high absorbance values at 0.594±0.005 - 0.729±0.018 AU and 1.171±0.023 - 1.304±0.049 AU, respectively, whereas the standard positive control vitamin C at 18.82 µg/mL concentration showed a higher value of absorbance (1.639±0.070 AU), however, the extracts in System 2 gave high reducing power comparable with the standard positive control BHA (1.211±0.026 AU). TSCE of TI-P/K in System 2 showed reducing power activity of 1.171±0.023 AU at concentration 18.82 µg/mL which was lower than that of the standard positive control vitamin C (1.639±0.070 AU) and BHA (1.211±0.026 AU) at concentration 18.82 µg/mL. This result showed that TSCEs in System 2 possessed a good reducing agent and gave high electron donor by reducing power assay compared with some plant extracts such as various tea extracts (0.25-0.5 AU at 100 µg/mL approximately), as previously determined by reducing power assay (Yen and Chen, 1995).

Reducing power activity represented by EC_{50} values showed that the lower value of EC_{50} indicating the higher reducing power. In the Table 4.4 shows EC_{50} values of TSCEs in System 1 and System 2 were 12.74±0.12 - 15.37±0.09 µg/ml and 6.76±0.30 - 7.63±0.15 µg/ml, respectively and these values were significantly higher than the standard positive control vitamin C (4.82±0.10 µg/ml). However, reducing power of TSCEs in System 2 was comparable with the standard positive control BHA

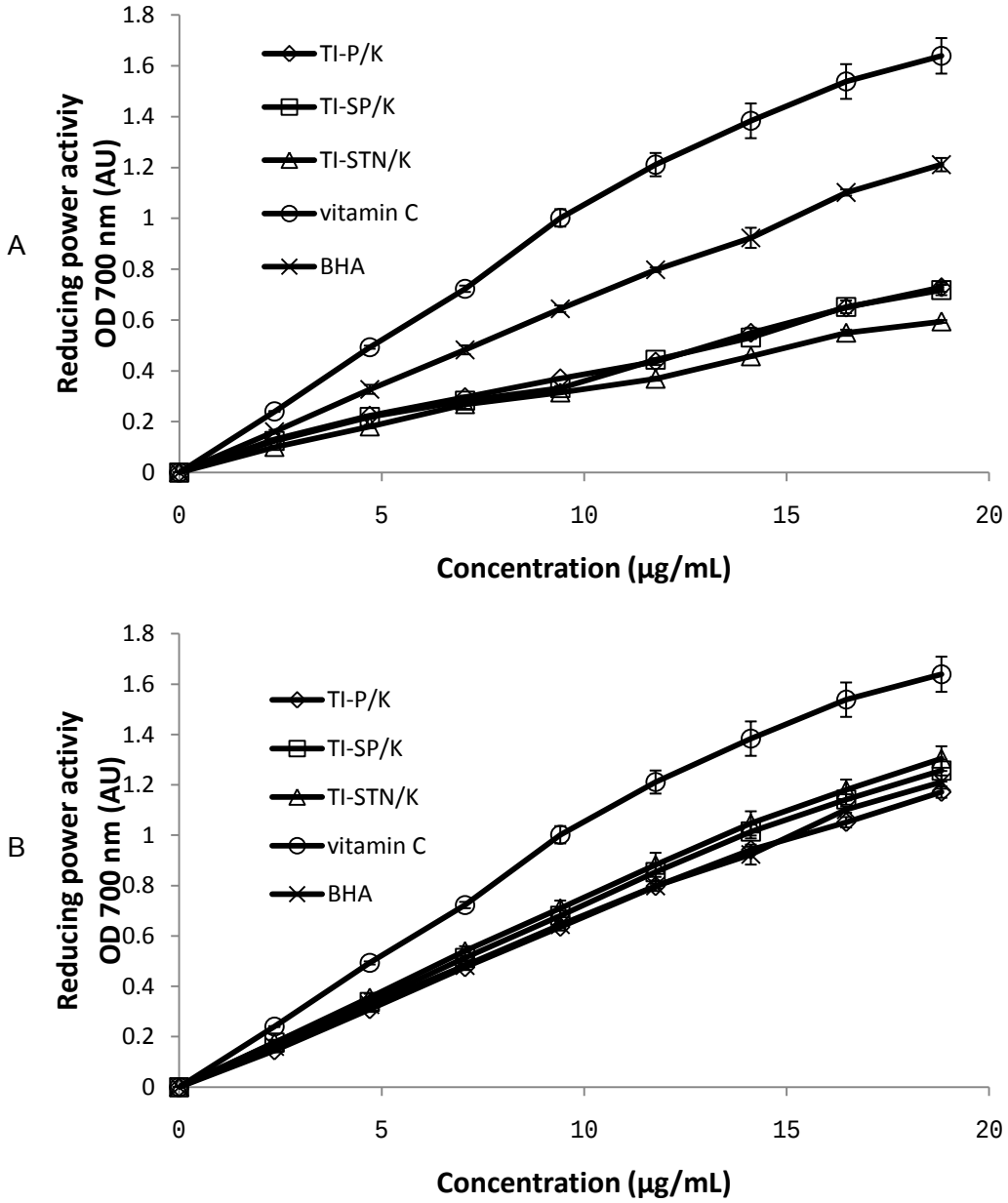


Fig 4.7 Reducing power activity represent as OD 700 nm determined by Reducing power assay against concentrations of TSCEs of the 3 cultivars extract by different extraction processes; A = System 1 and B = System 2. The values are mean±SE (n=3). Vitamin C and BHA were used as a positive control.

(7.45±0.19 µg/ml). Reducing power activity in TSCE of TI-STN/K in System 2 showed the lowest EC_{50} values that represent the highest reducing power of the extract of this tamarind cultivar.

3. Hydroxyl radical scavenging

Increasing the percentage of scavenging activity of TSCEs was observed with the increasing the concentration of TSCEs. The TSCEs of the 3 tamarind cultivars in System 1 and System 2 showed a comparable scavenging activity as assay by hydroxyl radical scavenging method (Fig 4.8 and Appendix B, Table B-4).

TSCEs at 41.67 µg/mL in System 1 and System 2 showed the high hydroxyl radical scavenging of 78.95±1.98 - 81.83±1.59 % and 81.08±0.63 - 84.39±0.90 %, respectively, whereas the standard positive control BHA at 41.67 µg/mL showed 88.29±1.51 % hydroxyl radical scavenging activity. This result showed that the % scavenging hydroxyl radical of TSCEs were higher than those found in the extracts of tamarind seed from India (35.4-56.6 % at 25 µg/mL) previously observed by the same assay (Siddhuraju, 2007). Moreover Siddhuraju, 2007 has also reported that the extract from dry-heated seed (135 °C± for 25 min) contained the highest hydroxyl radical scavenging activity. In this assay, the hydroxyl radical is generated by H_2O_2, Fe^{3+}EDTA and ascorbic acid and interacted with deoxyribose. Vitamin C cannot provide this action then it cannot be used as the standard positive control in this assay (Hou et al., 2003; Hsu et al., 2006).

The lower values of EC_{50} of hydroxyl radical scavenging for TSCEs indicates the higher antioxidant activity, in the Table 4.4 shows EC_{50} values for TSCEs in System 1 and System 2 were 2.79±0.06 – 3.31±0.32 µg/ml and 5.23±0.21 - 5.69±0.68 µg/ml, respectively, and these values were significantly higher than that of the standard positive control BHA (1.30±0.03 µg/ml). Hydroxyl radical scavenging activity of TSCEs was not as good as the standard positive control BHA. TSCEs of TI-SP/K in System 1 showed the highest hydroxyl radical scavenging activity. These results showed that TSCEs of TI-SP/K which contain the highest hydroxyl radical ($^{\cdot}OH$) scavenging activity is a good antioxidant natural source. Because TSCEs can eliminate the main free radical

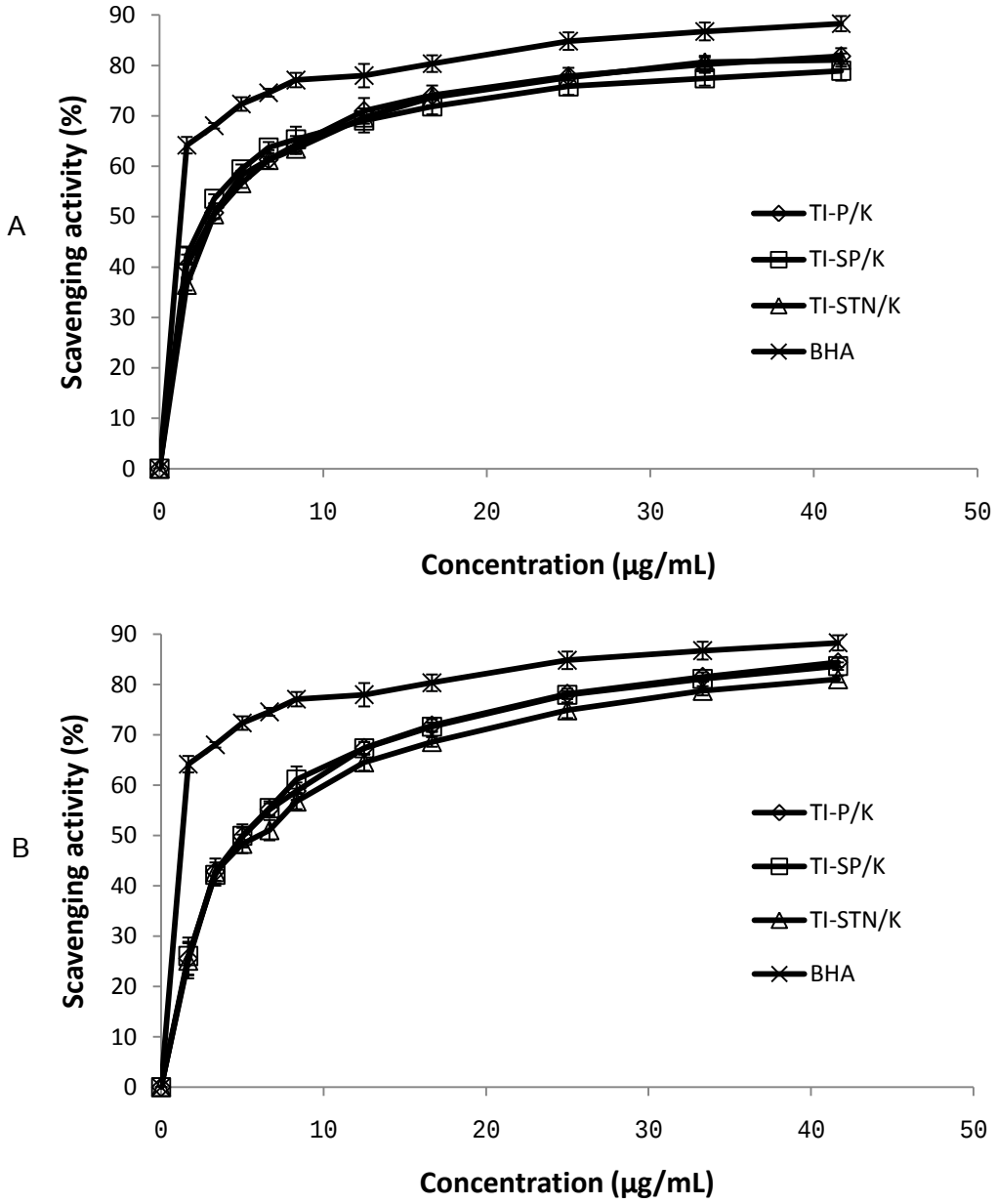


Fig 4.8 Scavenging activity (%) as determined by Hydroxyl radical assay against concentrations of TSCEs of the 3 cultivars extract by different extraction processes; A = System 1 and B = System 2. The values are mean±SE (n=3). BHA was used as a positive control.

present *in vivo* that may cause peroxidation of cell membrane lipids, liberation of toxic, and cell or tissue injury (Caillet et al., 2007; Yen and Chen, 1995). EC_{50} values for TSCEs by hydroxyl radical assay from this study showed high hydroxyl radical scavenging activity compared with Black tea (EC_{50}= 8 µg/ml), as previously studied by Hsu et al. (2006). Moreover, the % yield of Black tea (29.4%) was less than % yield of TSCEs obtained in System 2 (54-62%). TSCEs may be applied as a new source of food additive in beverage and skin care industries.

4. Anti-lipid peroxidation

The result in Fig 4.9 and Appendix B, Table B-5 shows that the increasing the percentage of lipid peroxidation inhibition was obtained with the increasing the concentration of TSCEs. This assay applied hydroxyl radicals to initiate the lipid peroxidation process (Rathee et al., 2006). The TSCEs of the 3 tamarind cultivars in System 1 and System 2 showed a comparable % inhibition of lipid peroxidation as assay by anti-lipid peroxidation method, however, TSCEs at higher concentration in System 1 and System 2 possessed anti-lipid peroxidation as good as standard positive control vitamin C and BHA (Fig 4.9). TSCEs in System 1 and System 2 at 114.29 - 142.86 µg/mL showed high percentage of lipid peroxidation inhibition at 72.01±1.94 – 80.05±2.45 % and 76.12±4.97 – 76.18±5.59 %, respectively, which was higher than the standard positive control vitamin C (showed 69.26±6.35 % inhibition at 142.86 µg/mL) but its activity was comparable with the standard positive control BHA at 17.14 µg/mL (79.51±6.84 µg/mL).

Vitamin C at very low concentration (<20 µg/ml) in Fig 4.9 as wel as TSCEs in System 2 (Fig 4.9, B) showed minus % inhibition, this result was explained by Kongkachuay (2001) that the low concentration of vitamin C is a prooxidant that support to generate more hydroxyl radical, initiate the lipid peroxidation process and has described this reaction by the following equation:

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^\cdot$ Fenton´s reaction

$Fe^{3+} + Asc \longrightarrow Fe^{2+} + Asc^\cdot$ (1)

$O_2^- + Asc^\cdot + H^+ \longrightarrow H_2O_2 + Asc$ (2)

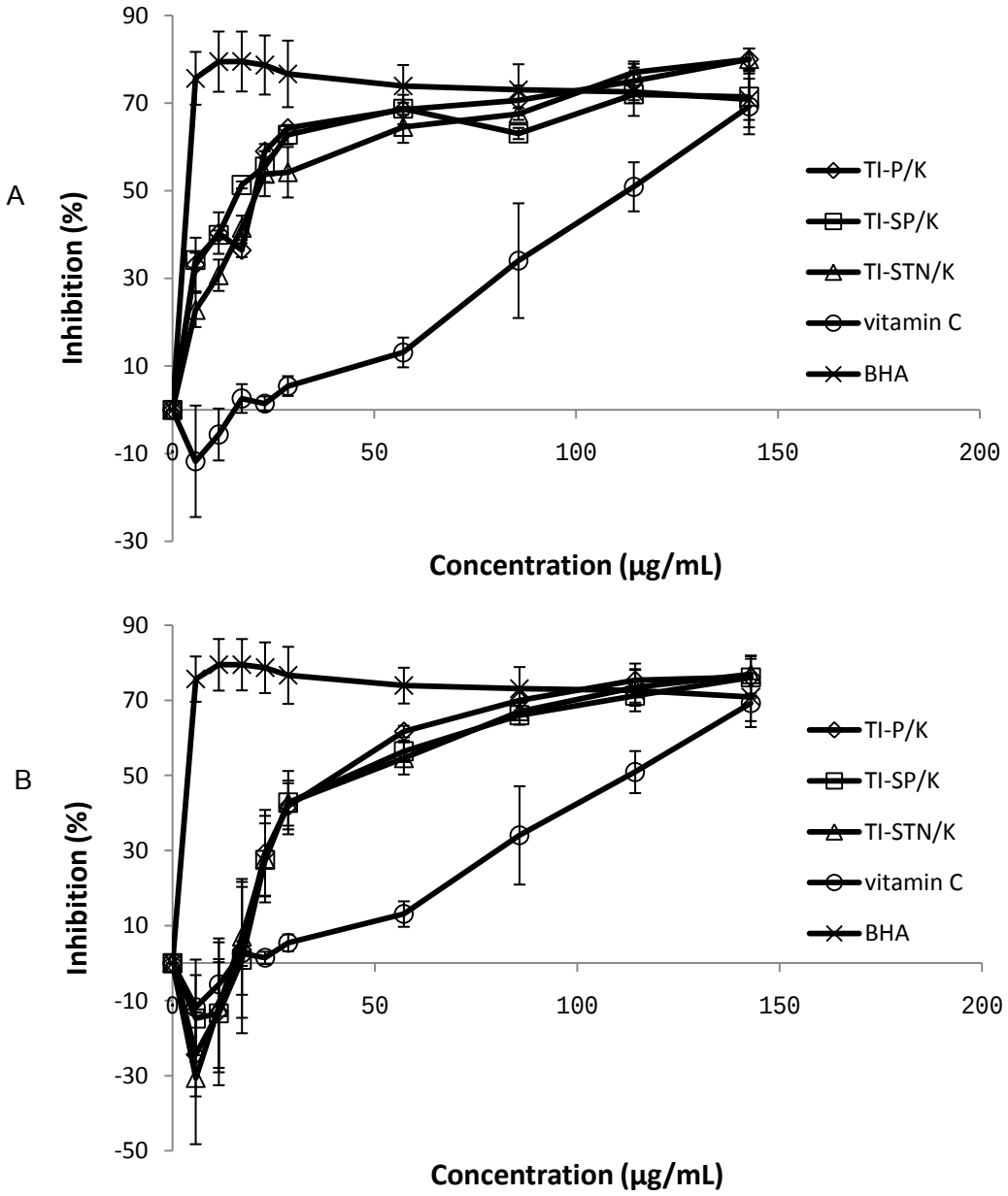


Fig 4.9 Lipid peroxidation inhibitory activity (%) as determined by anti-lipid peroxidation assay against concentrations of TSCEs of the 3 cultivars extract by different extraction processes; A = System 1 and B = System 2. The values are mean±SE (n=3). Vitamin C and BHA were used as a positive control.

Table 4.4 The EC_{50} values for TSCEs of 3 tamarind cultivars tested as examined by 4 different assay, the TSCEs were extracted by 2 different extraction processes as System 1 and System 2

Tamarind cultivars	EC_{50} (µg/mL)			
	DPPH radical scavenging	Reducing power	Hydroxyl radical scavenging	Anti-lipid peroxidation
TSCEs from System 1				
TI-P/K	3.02±0.01^{c}	12.74±0.12^{d}	3.19±0.32^{b}	19.90±0.69^{a,b}
TI-SP/K	2.98±0.01^{c}	13.00±0.22^{d}	2.79±0.06^{b}	15.24±0.34^{a,b}
TI-STN/K	4.00±0.04^{d}	15.37±0.09^{e}	3.31±0.32^{b}	26.06±4.72^{b,c}
TSCEs from System 2				
TI-P/K	2.18±0.11^{a}	7.63±0.15^{c}	5.23±0.21^{c}	40.08±8.72^{c}
TI-SP/K	2.23±0.06^{a}	7.02±0.23^{b,c}	5.40±0.31^{c}	43.44±8.93^{c}
TI-STN/K	2.15±0.07^{a}	6.76±0.30^{b}	5.69±0.68^{c}	44.66±11.45^{c}
vitamin C	2.66±0.01^{b}	4.82±0.10^{a}	nd*	113.66±6.28^{d}
BHA	2.21±0.02^{a}	7.45±0.19^{c}	1.30±0.03^{a}	3.83±0.33^{a}

Data represent mean±SE (n=3).

a, b, c, d, e = significantly different ($p<0.05$) between cultivars.

* nd = not determined

On the Fenton´s reaction equations, ferrous iron reduced H_2O_2 to generate $OH^{\cdot}$ (hydroxyl radical) and it becomes ferric iron. In low concentration of vitamin C condition, vitamin C (Asc) converts ferric iron back to ferrous (1), and become to an oxidized ascorbic acid ($Asc^{\cdot}$). $Asc^{\cdot}$ then reacts with O_2^{-} and H^{+} to generate H_2O_2 (2), the substrate from Fenton's reaction and increase the amount of hydroxyl radical and lipid peroxidation process in condition (Herbert, Shaw and Jayatileke, 1996; Kongkachuay, 2001).

However, vitamin C at high concentration can reduced Fe^{3+} to Fe^{2+}, and inhibited LDL oxidation by electron donation. TSCEs in System 1 did not show this property. TSCEs in System 2 may be contained other unknown chemicals as well as vitamin C that having prooxidant property. The lower value of EC_{50} of percentage lipid peroxidation inhibition for TSCEs indicates the higher anti-lipid peroxidation activity. In the Table 4.4 showed EC_{50} values for TSCEs in System 1 and System 2 of 15.24±0.34 - 26.06±4.72 µg/ml and 40.08±8.72- 44.66±11.45 µg/ml, respectively, these values were significantly lower than that of the standard positive control vitamin C (113.66±6.28 µg/ml), but higher than that of the standard positive control BHA (3.83±0.33 µg/ml). TSCE of TI-P/K and TI-SP/K in System 1 showed the lowest value of anti-lipid peroxidation. EC_{50} values for TSCEs of the 3 tamarind cultivars in System 1 were lower than that of TSCEs in System 2.

E. Antibacterial activity of TSCEs

Antibacterial activity of TSCEs from the 3 tested tamarind cultivars was preliminary studied. The broth microdilution susceptibility test as recommended by The National Committee for Clinical Laboratory Standards (NCCLS) was used in the determination of MIC and MBC values (Arias et al., 2004) for TSCEs by observing turbidity of bacterial growth and no bacteria growth on agar plate, respectively (Tepe et al., 2005). Gentamycin was used as a positive control. After incubated the bacterial suspension with TSCEs for 24 h, the bacterial growth was observed and the TSCEs at the concentrations showed no visible growth were carried out to determine the minimum concentration of TSCEs that killed all of bacteria. The result in Table 4.5 showed MIC and MBC values against 2 tested bacteria, *Staphylococcus aureus* ATCC 6538P and

Table 4.5 MIC and MBC values of TSCEs of 3 tested tamarind cultivars against *S. aureus* and *E. coli*

Type of extracts	*Staphylococcus aureus*		*Escherichia coli*	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
TSCEs from System 1				
TI-P/K	1.5625	3.125	6.25	25
TI-SP/K	1.5625	1.5625	6.25	25
TI-STN/K	0.39625	1.5625	6.25	25
TSCEs from System 2				
TI-P/K	0.39625	1.5625	3.125	>25
TI-SP/K	0.39625	1.5625	6.25	>25
TI-STN/K	0.39625	1.5625	6.25	>25
Gentamycin (µg/ml)	1.5625	6.25	0.78125	1.5625

Escherichia coli ATCC 25922, the represented of gram positive and gram negative bacteria, respectively.

MIC values for TSCEs of TI-P/K and TI-SP/K in System 1 against *S. aureus* (1.5625 mg/mL) were higher than that of TSCE of TI-STN/K in System 1 and TSCEs of the 3 tested tamarind cultivars in System 2 (0.39625 mg/mL). MBC for the TSCEs of TI-P/K in System 1 against *S. aureus* (3.125 mg/mL) showed the highest value compared with all of the tested TSCEs (1.5625 mg/mL). MIC values for all tested TSCEs in System 1 and System 2 against *E. coli* were 6.25 mg/mL excepted for the TSCE of TI-P/K in System 2 showed the lowest MIC value at 3.125 mg/mL. The MBC value for TSCEs in System 1 against *E. coli* was 25 mg/ml where TSCEs in System 2 show no bactericidal activity at concentration 25 mg/ml. The result in Table 4.5 demonstrated that TSCEs of the tested tamarind cultivars possessed inhibitory activity against the 2 tested bacteria, and the gram positive bacteria was more susceptible to TSCEs compared with the gram negative bacteria.

This is in agreement with the previous studied (Arias et al., 2004). The reasons for different sensitivity could be the morphological differences of the cell membrane between these microorganisms. Gram negative bacteria like *E. coli* have an outer phospholipid membrane carrying the structural lipopolysaccharide components which is an impermeable membrane to the lipophilic solutes and selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da. Whereas gram positive bacteria like *S. aureus* are more susceptible bacteria because they have only an outer peptidoglycan layer which is not an effective permeability barrier (Arias et al., 2004). Moreover the TSCEs in the bacterial suspension produced red brown precipitates after incubated for 24 h, these precipitates may be formed by proanthocyanidin-protein interaction, the proteins in the media can be bound and precipitated due to proanthocyanidin in the TSCEs (Hagerman and Butler, 1980).

Gentamycin sulfate, a positive control in this study, showed the lowest MIC and MBC values against the two bacteria (Table 4.5). Many studies reported the increasing number of bacteria may be develop resistance to classical antibiotic and became a widespread medical problem and undesirable side effect (Holowachuk, Bal and Buddington, 2003; Rabanal et al., 2002; Zampini et al., 2009). The investigation for

new bactericidal compounds especially from natural sources is important. Generally good antibacterial from natural sources showed lower values of MIC and MBC than that found in this study, Rabanal et al. (2002) reported the inhibitory activity of the aerial parts extract of *Hypericum* spp. against *S. aureus* (MIC= 0.011-0.22 mg/mL) except *E. coli*. The leaf and flower extracts of *Acacia aroma* as previously studied also showed inhibitory activity against *E. coli*, MIC = 0.246±0.046 mg/mL and MBC = 0.214±0.030 mg/mL (Arias et al., 2004). The extract from other parts of tamarind previously studied in Nigeria against *S. aureus* and *E. coli* (Doughari, 2006) showed that stem bark (MBC = 20 and 15 mg/ml, respectively) and leaf (MBC = 20 and 18 mg/ml, respectively) showed lower inhibitory activity than TSCEs in the present study.

F. Formulation of TSCE cream

According to the result of high antioxidant activity and high percentage yield of TSCE of TI-P/K in System 2 was obtained. The TSCE of TI-P/K in System 2 was selected for using in the formulation of TSCEs cream and the cream preparation was prepared by beaker method (Allen Jr., 2008). In addition, TI-P/K is a sour type tamarind which is highly used in tamarind industry and a lot of tamarind seeds are the waste product (สำนักส่งเสริมการค้าสินค้าเกษตร: ออนไลน์). The seed coat of TI-P/K can be a good source of natural material with antioxidant activity among these tamarind cultivars. TSCE creams were successfully prepared by using the three concentrations of TSCE at 100 mg, 300 mg, and 500 mg in 100 g of TSCE cream, the TSCE cream products were described in Table 4.6 and the pictures are shown in Fig 4.10.

Sodium metabisulfite was a suitable antioxidant for using in this preparation due to it is dissolved in water and stable in the low pH preparation where sodium bisulfate and sodium sulfite are suitable with neutral and alkali pH preparation, respectively (พิมพร, 2534; วราภรณ์, 2527).

1. Stability tests

The characteristics and physical properties of TSCE creams in Table 4.6 indicated that after freshly prepared, cream base was a white cream where TSCE creams (without color added) were pink to purple color, both of cream base and TSCE

Table 4.6 Product description of TSCE creams show physical properties and appearences of the products after freshly prepared and after stability tests

Cream products	Description of cream products		
	Freshly prepared	After 12 weeks	After heating-cooling cycle for 3 cycles
Cream base			
Texture	smooth and soft	smooth and soft	smooth and soft
Color	white	white	white
Odor	slightly	slightly	slightly
Air bubble	no	no	no
Phase separate	no	no	no
pH	4.77±0.02[b]	4.51±0.01[a]	4.44±0.04[a]
Viscosity (mPas)	7509±621.01[a]	11981±946.09[b]	10310±534.20[b]
TCSE cream$_1$ (TSCE 100 mg in 100 g TSCE cream)			
Texture	smooth and soft	smooth and soft	smooth and soft
Color	pink	upper: yellow lower: light pink	upper: yellow lower: light pink
Odor	slightly	slightly	slightly
Air bubble	no	no	no
Phase separate	no	no	no
pH	4.80±0.02[b]	4.60±0.05[a]	4.57±0.05[a]
Viscosity (mPas)	6780 ±167.67[a]	14111±719.17[c]	10174±746.60[b]
TCSE cream$_2$ (TSCE 300 mg in 100 g TSCE cream)			
Texture	smooth and soft	smooth and soft	smooth and soft
Color	light purple	upper: yellow lower: pink	upper: yellow lower: pink
Odor	slightly	slightly	slightly
Air bubble	no	no	no
Phase separate	no	no	no
pH	4.89±0.04[b]	4.75±0.03[a]	4.69±0.03[a]
Viscosity (mPas)	8204±932.59[a]	20534±3676.12[b]	11747.78±1066.77[a]

Table 4.6 Product description of TSCE creams show physical properties and appearences of the products after freshly prepared and after stability tests (continue)

Cream products	Description of cream products		
	Freshly prepared	After 12 weeks	After heating-cooling cycle for 3 cycles
TCSE cream$_3$ (TSCE 500 mg in 100 g TSCE cream)			
Texture	smooth and soft	smooth and soft	smooth and soft
Color	purple	upper: yellow lower: light purple	upper: yellow lower: light purple
Odor	slightly	slightly	slightly
Air bubble	no	no	no
Phase separate	no	no	no
pH	4.80±0.06[b]	4.56±0.03[a]	4.56±0.06[a]
Viscosity (mPas)	7816.45±789.54[a]	13308±1394.68[b]	9826.44±905.23[a, b]

Data show mean±SEM (n=3).

a, b = significantly different ($p<0.05$) between different time in the same sample

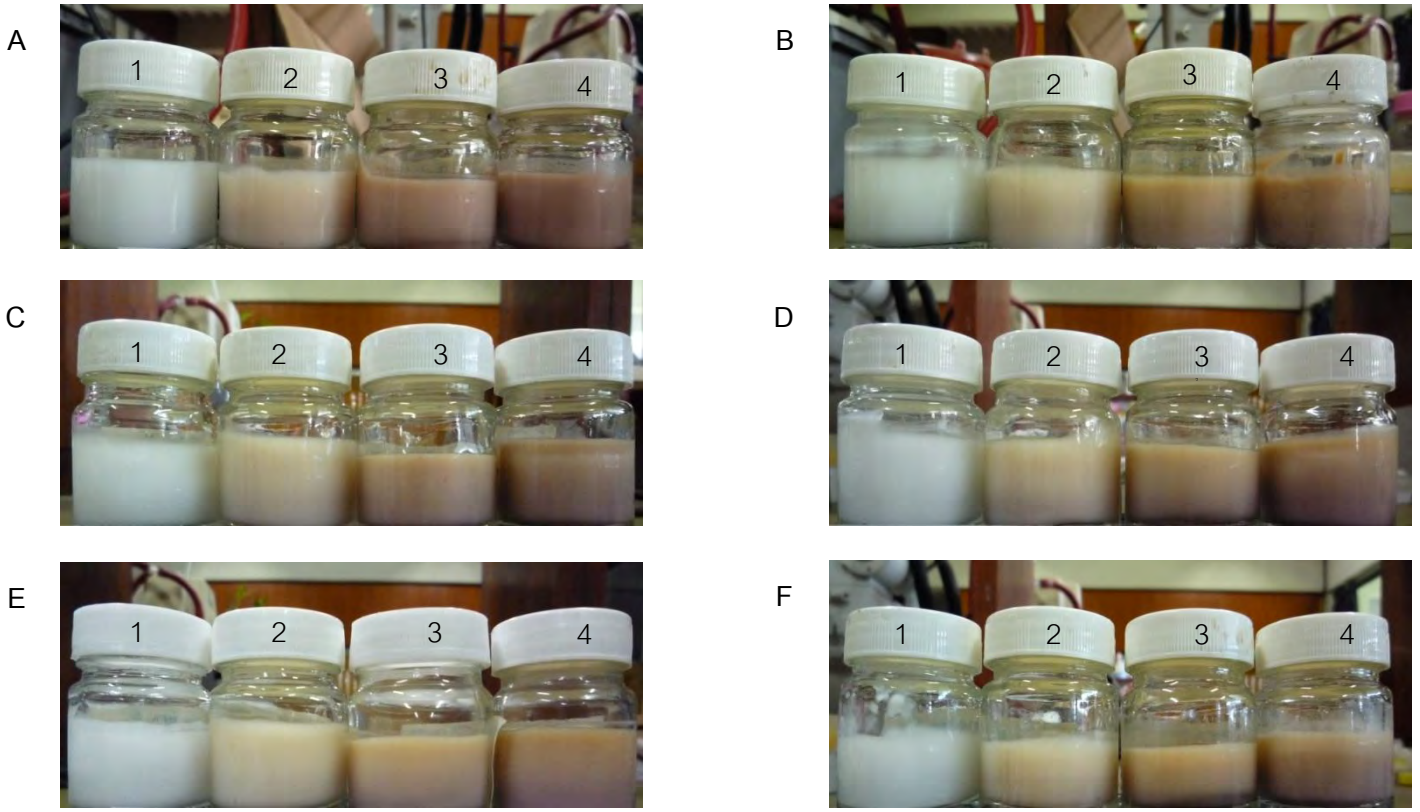


Fig 4.10 The picture of TSCE creams show (1) cream base, (2) TSCE cream$_1$, composed of 100 mg TSCE, (3) TSCE cream$_2$, composed of 300 mg TSCE, and (4) TSCE cream$_3$, composed of 500 mg TSCE; The cream product at freshly prepare (A), after stand at room temperature for 2 weeks (B), after stand at room temperature for 4 weeks (C), after stand at room temperature for 8 weeks (D), after stand at room temperature for 12 weeks (E) and after heating-cooling cycle test for 3 cycles

creams were smooth and soft creams, slightly odor, no air bubble and no phase separated. All of cream base and TSCE creams of different concentrations of TSCE were not change after stability tested (Table 4.6). After freshly prepared, the TSCE cream$_1$ (100 mg TSCE/ 100g cream) showed a pink color cream, TSCE cream$_2$ (300 mg TSCE/ 100g cream) showed a light purple color cream and TSCE cream$_3$ (500 mg TSCE/ 100g cream) showed a purple color cream. The color of all TSCE cream were not stable (Table 4.6), the color was changed from pink or purple to yellow on the top surface after TSCE creams were stored at ambient temperature for 12 weeks and after stability test by heating-cooling cycle for 3 cycles. The yellow color may be formed by the condensation or polymerization or oxidation of proanthocyanidin (Zoecklein, 1995) that normally occurs with the phenolic reaction in red wine as demonstrated in Fig 4.11. Then TSCE cream$_1$ was formulated by using the color D&C YELLOW No.10, the color was added at the last step of preparation during the TSCE cream was congeal to make a permanent yellow color cream as showed in Fig 4.12. This cream showed yellow color appearance and were stable after stability test.

The values of pH and viscosity of the TSCE cream product are shown in Table 4.6. The pH values of TSCE creams were significantly decrease whereas the viscosity values were increase, after stability tested by storage at ambient temperature for 12 weeks and by heating-cooling cycle for 3 cycles. The pH value in TSCE creams product decrease as the H^+ concentration in this product increase (Zumdahl, 2007), this may be occurred due to the phenolic compound contents which is a H^+ donor atom in TSCE. The increasing viscosity value of oil-in water emulsion such as TSCE cream has been found to be very effective in reducing the drop size (Hayati et al., 2007; Salager, Perez-Sanchez and Garcia, 1996) and can be increased beyond 1000 cP, when the emulsion contains less than 30% water and stirring situation changes to the so-called high internal phase ratio emulsification (Salager, Perez-Sanchez and Garcia, 1996).

2. Efficacy of free radical scavenging activity of TSCE cream

Antioxidation activity of the product TSCE creams containing various concentrations of TSCE was evaluated the free radical scavenging activity by DPPH radical scavenging assay and the results are shown the percentage scavenging activity

of each cream product in Table 4.7. Trace of scavenging activity (4.33-6.49%) was also observed in the cream base without TSCE may be due to an effect of sodium metabisulfite, a widely used antioxidant substance (Maia et al., 2006), in the cream formulation. Increasing TSCE concentration in TSCE $cream_2$ resulted in increasing % scavenging activity compared to TSEC $cream_1$, however, TSCE $cream_3$ did not followed this pattern and the higher concentration of TSCE in the TSCE $cream_3$ did not effected to increase % scavenging activity in the TSCE $cream_3$ product compared with TSCE $cream_2$ product (Table 4.7). The % scavenging activity in the TSCE $cream_2$ and TSCE $cream_3$ were rather stable after stability tested and antioxidation activity in the TSCE cream products did not significantly decreased.

After freshly prepared TSCE $cream_1$, TSCE $cream_2$ and TSCE $cream_3$ showed 67.53±5.20%, 93.08±2.26% and 94.64±1.49% scavenging activity, respectively, and after stability test these products still showed activity as good as the standard positive control of vitamin C and reference standard TSCE (92.60-96.73% and 90.53-91.17%, respectively). Increasing of % scavenging activity in the TSCE $creams_1$ may be correlated with the increasing the degree of polymerization, the size of procyanidin (Gu et al., 2006), for (-)-epicatechin as the monomer of procyanidin in TSCEs. Increasing the number of free hydroxyls, potential donors of hydrogen atoms was observed with the increasing the antioxidant potential (da Silva Porto, Laranjinha, and de Freitas, 2003). The TSCE cream has been developed from the extract of tamarind seed coat (ethyl acetate fraction) of tamarind cultivar from Chiang Rai, Thailand with milky base lotion formulation which contained different ingredients compound to the formulation in this study (Lourith, Kanlayavattanakul and Chanpirom, 2009). This anti-wrinkle cosmetic was added tamarind seed coat extract at 0.17, 0.13 and 0.51 mg in 100 g lotion and showed chemically and physically stables, moreover their viscosity observed (9090-9270 mPas) is similarly to TSCE cream in this study.

Table 4.7 Efficacy of scavenging activity (%) determined by DPPH radical assay of TSCE cream products 0.1 g after freshly prepared and after stability tests

Cream products	% scavenging activity							
	Freshly prepared	After 2 weeks	After 4 weeks	After 8 weeks	After 12 weeks	After 3 Heating-cooling cycle	TSCE (TI-P/K)	Vitamin C
Cream base	4.33±0.68	5.32±1.12	5.71±0.84	4.50±0.41	6.49±0.06	5.12±0.72	0±0	0±0
TCSE cream$_1$	67.53±5.20^{a}	66.75±1.85^{a}	72.00±1.80^{a}	80.96±1.95^{b}	88.16±1.17^{c}	71.61±1.51^{a}	91.12±0.64^{c}	92.60±0.82^{c}
TCSE cream$_2$	93.08±2.26$^{a, b}$	93.47±2.07$^{a, b}$	94.28±0.62$^{a, b, c}$	97.73±0.07^{c}	96.02±0.16$^{b, c}$	91.86±0.28^{a}	91.17±0.54^{a}	96.54±0.76$^{b, c}$
TCSE cream$_3$	94.64±1.49$^{c, d}$	94.14±2.00$^{b, c}$	94.93±0.49$^{c, d}$	97.56±0.13^{d}	95.95±0.15$^{c, d}$	91.56±0.49$^{a, b}$	90.53±0.66^{a}	96.73±0.67$^{c, d}$

Data show mean±SE (n=3).

a, b, c, d = significantly different ($p<0.05$) between different time in the same sample

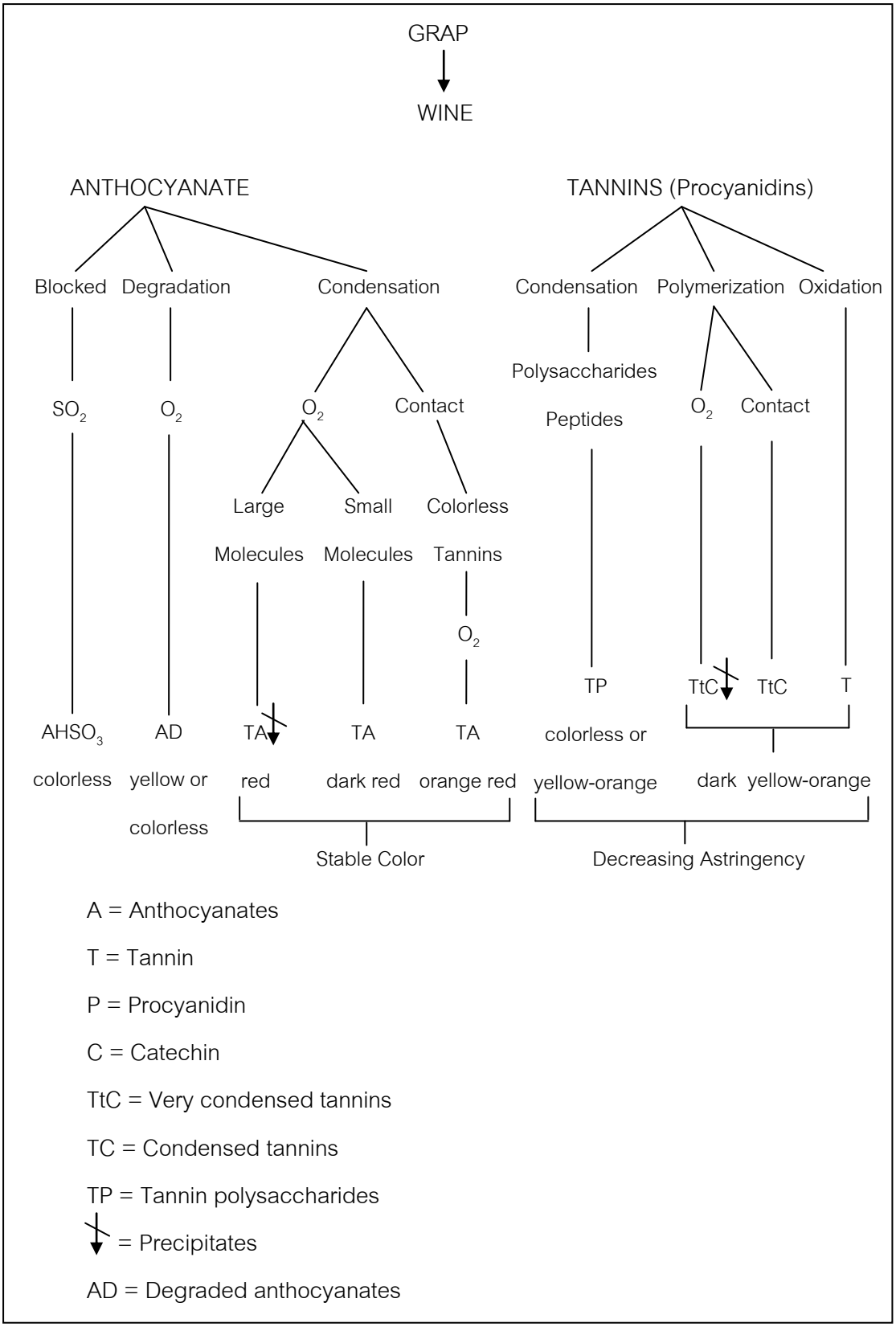


Fig 4.11 Phenolic reactions occurring in red wines (Zoecklein, 1995).

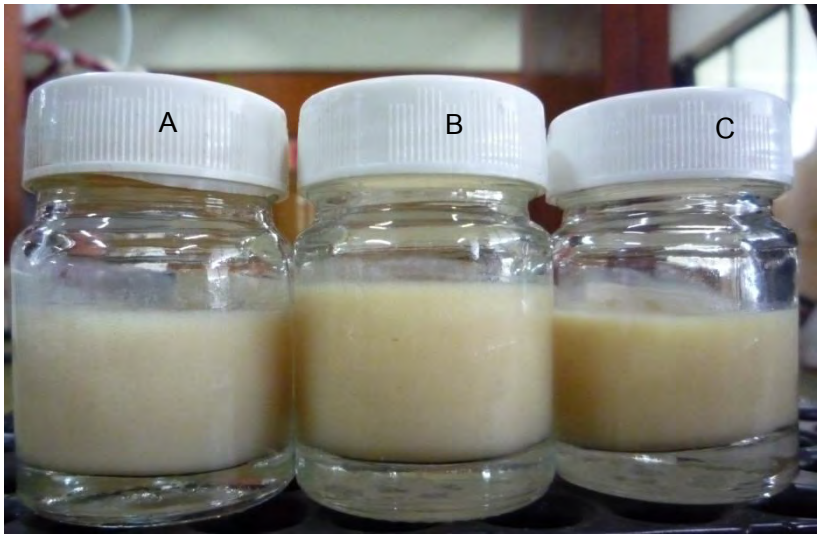


Fig 4.12 An appearance of TSCE cream$_1$ with color D&C yellow No. 10 added, in 100 g of cream added 50 µl (A), 100 µl (B), 150 µl (C) of color

CHAPTER V

CONCLUSION

TSCEs of different 3 tamarind cultivars were extracted by the 2 different systems of solvent extraction. The TSCEs in System 2 showed higher % yield of TSCEs than that of System 1, however, all of the TSCEs tested contained high phenolic compounds especially proanthocyanidin or condense tannin. The identification of chemical peaks by HPLC technique with spiked peaks, the HPLC chromatograms showed peak identical with (+)-catechin, procyanidin B2 and (-)-epicatechin in the TSCEs from both of sour and sweet types of tamarind. HPLC chromatograms of TSCEs tested showed similar chemical finger print profile.

For several years, many studies have long been searching for powerful antioxidants together with antibacterial from natural sources, especially from edible or medicinal plants, and TSCEs showed this capability. Antioxidant activity of TSCEs are expressed as EC_{50} values, the results showed that TSCEs possessed as good activity as the standard positive control. Antibacterial activity of TSCEs against gram positive and gram negative bacteria are demonstrated that *Staphylococcus aureus* ATCC 6538P was more susceptible to be inhibited by TSCEs, more detail for antibacterial studies are interested.

TSCE cream products were developed and successfully prepared by using TSCE of TI-P/K in System 2 as an active ingredient for antioxidant. TSCE creams with smooth and soft texture was obtained, good skin penetration without sticky feeling when application on skin. The products of TSCE showed high antioxidant as good as the standard positive control vitamin C and reference standard TSCE by DPPH radical scavenging assay. Antioxidant activity of TSCE cream products were not reduced after stability test.

According to the results in this study, TSCEs may be applied as new natural source of antioxidant with antibacterial potential for application in skin care product with high antioxidant activity.

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APPENDICES

APPENDIX A

Table A-1 Botanical description: shows the local name of *Tamarindus indica* L.

Country	Language	Name(s)
Africa		
	Bemba	mushishi
	Fula	dabe, jammeth, jammi
	Jola	budahar
	Mandinka	timbimb, timbingo, tombi, tomi
	Tigrina	humer
	Wolof	daharg, dakah, dakhar, nclakhar
Ethiopia	Amharic	hemor, homor, humar, komar, tommar
	Tigrina	arabeb
	Gamo/Oromo	b/roka, racahu, dereho, dindie, ghroma, gianko, omar
Kenya	Swahili	mkwaju
	Masai	ol-masambural
	Turkana	Eopduran
	Borana	roka
	Luo	chwa, waa
	Meru	muthithi
	Pokot	oran
Malawi	Chewa	ukwaju, bwemba
	Yao	mkwesu
	Nkande	nkewesu
Nigeria		tsamiya
Somalia	Somali	hamar
South Africa	Afrikaans	tamarinde
Sudan	Arabic	aradeib, tamarihindi
	Nuba	sheker, kuashi, danufi
Tanzania	Swahili	Ukwaju

Table A-1 Botanical description: shows the local name of *Tamarindus indica* L.

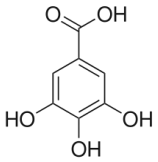
(continue)

Country	Language	Name(s)
Uganda	Teso	esukuru, esuguguru (leaves)
	Teso/Karamojong	e/apedura (fruit)
	Bari/Ma'di	iti
	Acholi/lango	chwa/o
	Kakwa/Acholi	pitei
	Luganda	mukoge
Zambia	Bemba	mushishi
	Nyanja	mwemba
	Tonga	musika
Asia		
Cambodia	Khmer	'am' pul, ampil, khoua me
China	Sino-Tibetan	khaam, mak kham
India	Hindi	ambli, amli, imli
	Sanskrit	amalika
	Bengali	tintiri,tintul, tetul
	Marathi	chinch, chitz, amli
(Hyderabad)		chis, hunchi
(Mysore)		karanji, kamal, asam, hunse
	Kannada	hunse, unsi, hulimara
	Coorg	pulinje
	Uriya	koya, trntuli
	Gondi	chita, hitta, sitta
	Telegu	chinta
	Tamil	puli, pulian
	Assamese	tetili
	Gujarati	amali, ambali
Indonesia		asam jawa, asam, tambaring
Malaysia		asam jawa

Table A-1 Botanical description: shows the local name of *Tamarindus indica* L.

(continue)

Country	Language	Name(s)
Myanmar		magyi, magyee majee-pen
Nepal	Nepali	ttri, imli
	Newari	titis, paum
Philippines	Tagalog	sampalok
	Bisaya	kalamagi
	Ilokano	salomagi
Sri Lanka	Sinhala	siyambala, maha siyambala
	Tamil	puli
Thailand	general	makham
	northern	bakham
	peninsular	somkham
Vietnam		me, trail me
Elsewhere		
Virgin		tanan
Islands	Arabic	ardeib
	Creole	tamarenn
	Dutch	tamarinde
	English	Madeira mahogany, tamarind, Indian date
	French	tamarin, tamarainer, tamarindier
	German	tamarinde
	Italian	tamarindizio
	Portuguese	tamarindo
	Spanish	tamarin, tamarindo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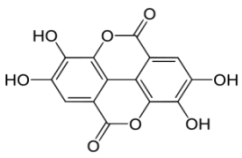
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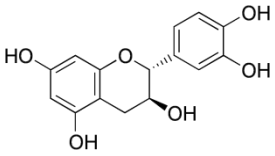
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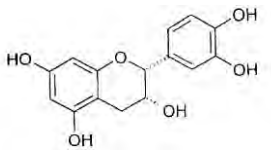
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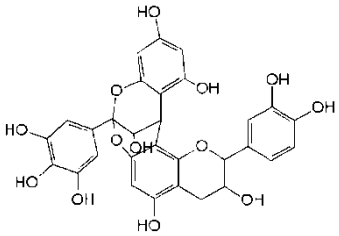
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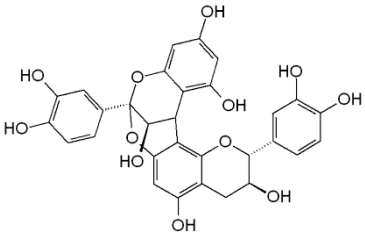
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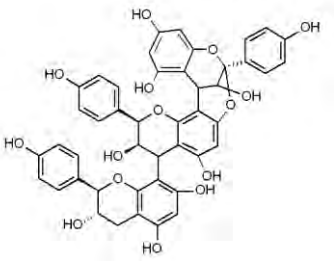
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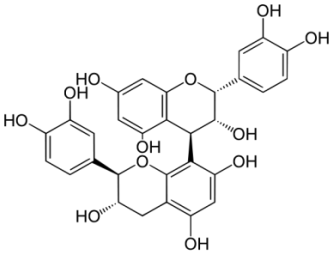
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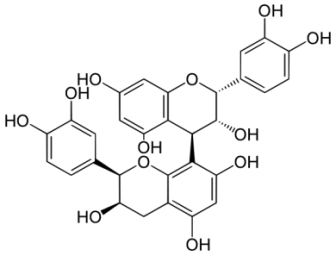
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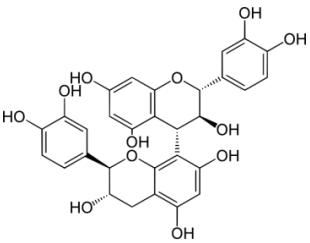
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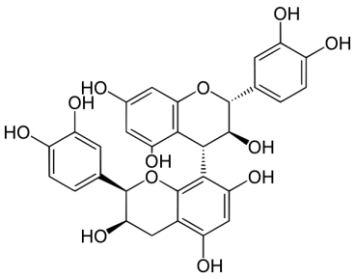
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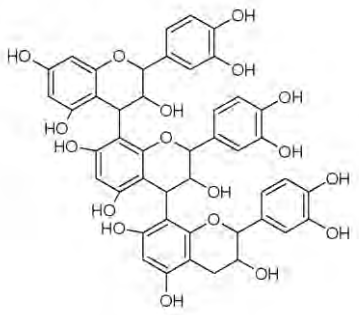
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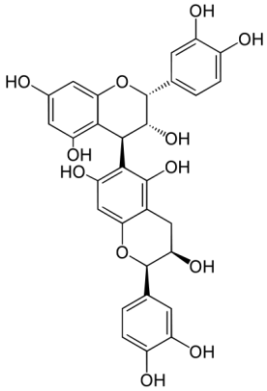
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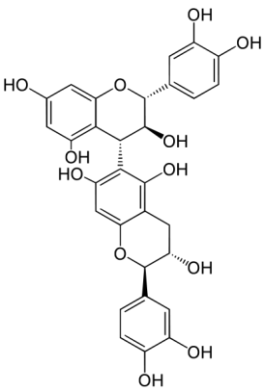
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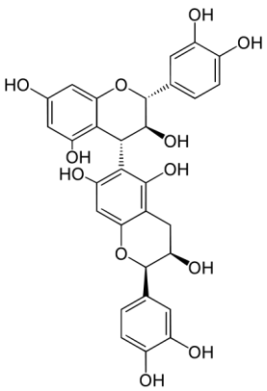
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Fig A-1 Phenolic compounds: shows some structures of flavanoid and tannin

APPENDIX B

Table B-1 Total phenolic compound analysis: calibration of gallic acid concentration VS absorbance at 765 nm

Concentration of gallic acid (mg/L)	Absorbance at 765 nm (mean±SEM)
0	0±0
100	0.268±0.003
200	0.476±0.002
300	0.780±0.006
400	1.021±0.003
500	1.266±0.003

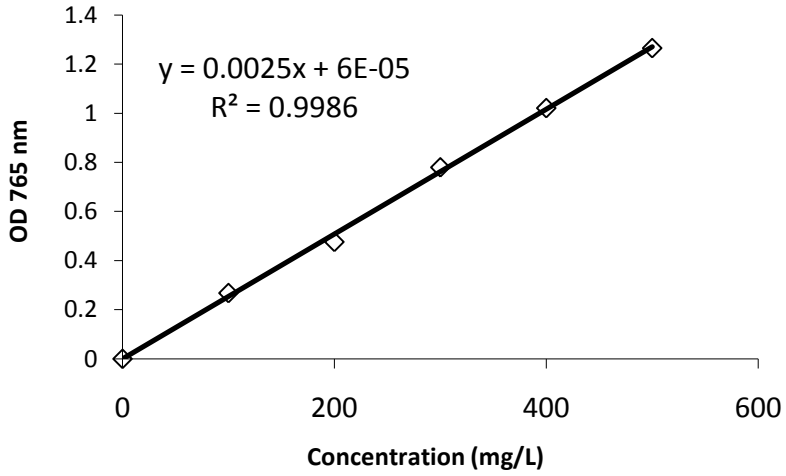

Fig B-1 Total phenolic compound analysis: shows calibration graph of gallic acid concentration VS absorbance 765 nm

Table B-2 DPPH radical scavenging assay: shows % scavenging activity with various concentration of TSCEs

Concentration (µg/mL)	%Scavenging (Mean±SE)							
	System 1			System 2			Positive control	
	TI-P/K	TI-SP/K	TI-STN/K	TI-P/K	TI-SP/K	TI-STN/K	vitamin C	BHA
0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
0.50	6.36±0.22	7.91±1.76	4.59±0.19	10.36±0.53	8.76±0.36	9.52±0.52	7.62±1.27	9.99±0.27
1.00	15.99±0.61	16.44±1.49	11.84±0.47	25.42±0.48	22.97±2.61	25.86±2.00	19.95±0.39	25.66±1.14
1.50	25.10±0.28	26.01±0.56	19.74±0.69	35.94±2.72	33.13±1.35	38.17±1.16	31.71±2.19	36.87±1.72
2.00	33.12±0.68	35.27±0.77	27.04±0.35	45.18±3.55	43.55±2.24	47.30±1.15	31.72±6.93	47.50±1.46
2.50	40.01±0.42	43.54±0.38	32.64±0.41	59.94±5.07	57.39±0.42	55.86±1.70	48.59±0.70	58.21±1.32
3.75	61.56±0.68	62.74±0.51	47.52±0.27	85.34±3.09	84.02±3.05	86.97±3.21	73.67±2.90	77.06±1.90
5.00	84.15±0.84	82.24±1.08	60.79±0.99	92.33±0.52	91.34±0.55	91.94±0.57	90.38±1.17	86.18±1.51
7.50	88.13±0.10	87.04±1.50	88.75±0.04	92.28±0.43	90.93±0.17	91.99±0.51	94.11±0.32	92.49±0.08
10.0	86.81±0.18	86.63±1.62	88.48±0.10	91.69±0.59	91.34±0.36	91.34±0.39	95.42±0.38	93.23±0.07
12.50	87.37±0.18	86.15±1.40	88.29±0.02	92.03±0.23	90.64±0.58	91.48±0.06	96.36±0.09	93.08±0.28

Table B-3 Reducing power assay: shows % scavenging activity with various concentration of TSCEs

Concentration (µg/mL)	Absorbance (Mean±SE)							
	System 1			System 2			Positive control	
	TI-P/K	TI-SP/K	TI-STN/K	TI-P/K	TI-SP/K	TI-STN/K	vitamin C	BHA
0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
2.35	0.130±0.003	0.124±0.001	0.100±0	0.145±0.007	0.177±0.011	0.176±0.006	0.240±0.002	0.161±0.007
4.71	0.223±0.005	0.219±0.007	0.181±0.006	0.309±0.010	0.338±0.018	0.356±0.015	0.493±0.006	0.327±0.018
7.06	0.296±0.007	0.283±0.004	0.269±0.018	0.475±0.010	0.513±0.023	0.540±0.020	0.723±0.012	0.483±0.018
9.41	0.370±0.004	0.332±0.009	0.315±0.001	0.636±0.014	0.680±0.021	0.710±0.030	1.002±0.034	0.644±0.013
11.76	0.436±0.013	0.444±0.005	0.369±0.007	0.798±0.019	0.854±0.034	0.883±0.048	1.211±0.046	0.798±0.009
14.12	0.549±0.021	0.531±0.006	0.457±0.005	0.942±0.012	1.015±0.027	1.047±0.048	1.384±0.068	0.923±0.040
16.47	0.649±0.004	0.652±0.025	0.550±0.012	1.052±0.021	1.142±0.021	1.180±0.041	1.538±0.068	1.102±0.012
18.82	0.729±0.018	0.718±0.020	0.594±0.005	1.171±0.023	1.257±0.044	1.304±0.049	1.639±0.070	1.211±0.026

Table B-4 Hydroxyl radical scavenging assay: shows % scavenging activity with various concentration of TSCEs

Concentration (µg/mL)	%Scavenging (Mean±SE)						
	System 1			System 2			Positive control
	TI-P/K	TI-SP/K	TI-STN/K	TI-P/K	TI-SP/K	TI-STN/K	BHA
0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
1.67	40.20±2.28	42.26±1.61	36.47±1.12	25.56±3.33	26.05±3.68	25.11±3.49	64.17±1.64
3.33	50.79±1.10	53.54±0.90	50.46±0.96	42.82±1.83	42.18±1.69	42.73±2.70	68.03±0.55
5.00	58.22±0.88	59.39±0.96	56.16±0.93	49.78±1.76	49.93±2.29	48.35±1.84	72.34±1.34
6.67	61.34±1.85	63.71±1.09	61.22±0.33	55.19±1.19	55.44±1.35	51.06±2.02	74.57±0.79
8.33	64.20±1.79	65.34±2.50	63.47±0.64	58.81±1.73	61.12±2.61	56.75±1.52	77.10±1.43
12.50	70.99±2.55	69.08±2.39	69.66±0.45	67.39±1.18	67.34±1.26	64.55±1.67	77.98±2.32
16.67	74.05±1.96	71.84±1.55	73.66±0.53	71.86±1.28	71.61±0.84	68.60±0.96	80.35±1.65
25.00	77.86±1.66	75.85±1.70	77.61±0.62	78.16±1.01	77.95±0.73	74.89±1.55	84.82±1.74
33.33	80.22±1.69	77.44±1.45	80.64±0.97	81.52±0.91	81.13±0.55	78.76±0.92	86.74±1.77
41.67	81.83±1.59	78.95±1.98	81.07±1.30	84.39±0.90	83.66±0.75	81.08±0.63	88.29±1.51

Table B-5 Anti-lipid peroxidation assay: shows % scavenging activity with various concentration of TSCEs

Concentration (µg/mL)	%Scavenging (Mean±SE)							
	System 1			System 2			Positive control	
	TI-P/K	TI-SP/K	TI-STN/K	TI-P/K	TI-SP/K	TI-STN/K	vitamin C	BHA
0	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
5.71	33.16±6.13	34.17±1.69	22.79±3.90	-24.34±11.15	-14.73±11.57	-30.61±17.60	-11.76±12.73	75.68±6.03
11.43	40.35±4.74	39.93±1.00	30.71±3.58	-12.94±19.56	-13.38±14.49	-11.74±17.31	-5.65±5.92	79.49±6.88
17.14	36.44±1.58	51.34±0.80	41.54±2.77	3.56±18.10	0.84±19.49	7.02±15.43	2.58±3.29	79.51±6.84
22.86	59.04±1.64	55.54±3.53	53.87±5.11	29.41±11.43	27.51±9.73	27.73±11.52	1.42±1.63	78.71±6.75
28.57	64.34±0.42	62.81±2.23	54.23±5.76	41.80±6.15	42.77±8.43	42.66±6.01	5.42±2.26	76.69±7.60
57.14	68.55±3.36	68.74±1.37	64.58±3.63	61.72±1.53	56.44±2.87	54.57±4.30	13.09±3.40	73.94±4.77
85.71	70.64±3.42	63.08±1.37	67.56±1.36	69.98±3.22	66.04±2.51	67.04±2.33	34.05±13.10	73.09±5.80
114.29	75.10±4.42	72.01±1.94	77.08±1.90	75.32±4.50	71.23±1.83	73.45±4.88	50.91±5.60	72.61±5.51
142.86	80.05±2.45	71.47±5.30	80.05±2.43	76.18±5.59	76.12±4.97	76.89±5.06	69.26±6.35	70.88±6.39

Table B-6 Formulation of TSCE cream: shows physical properties description of freshly prepared and after stability test

Cream products	Description of cream products					
	Freshly prepared	After 2 weeks	After 4 weeks	After 8 weeks	After 12 weeks	After 3 heating-cooling cycle
Cream base						
Texture	smooth and soft	smooth and soft	smooth and soft	smooth and soft	smooth and soft	smooth and soft
Color	white	white	white	white	white	white
Odor	slightly	slightly	slightly	slightly	slightly	slightly
Air bubble	no	no	no	no	no	no
Phase separate	no	no	no	no	no	no
TCSE cream$_1$						
Texture	smooth and soft	smooth and soft	smooth and soft	smooth and soft	smooth and soft	smooth and soft
Color	pink	upper: yellow lower: light pink	upper: yellow lower: light pink	upper: yellow lower: light pink	upper: yellow lower: light pink	upper: yellow lower: light pink
Odor	slightly	slightly	slightly	slightly	slightly	slightly
Air bubble	no	no	no	no	no	no
Phase separate	no	no	no	no	no	no

Table B-6 Formulation of TSCE cream: shows physical properties description of freshly prepared and after stability test (continue)

Cream products	Description of cream products					
	Freshly prepared	After 2 weeks	After 4 weeks	After 8 weeks	After 12 weeks	After 3 heating-cooling cycle
TCSE cream$_2$						
Texture	smooth and soft	smooth and soft	smooth and soft	smooth and soft	smooth and soft	smooth and soft
Color	light purple	upper: yellow	upper: yellow	upper: yellow	upper: yellow	upper: yellow
		lower: pink	lower: pink	lower: pink	lower: pink	lower: pink
Odor	slightly	slightly	slightly	slightly	slightly	slightly
Air bubble	no	no	no	no	no	no
Phase separate	no	no	no	no	no	no
TCSE cream$_3$						
Texture	smooth and soft	smooth and soft	smooth and soft	smooth and soft	smooth and soft	smooth and soft
Color	purple	upper: yellow	upper: yellow	upper: yellow	upper: yellow	upper: yellow
		lower: light purple	lower: light purple	lower: light purple	lower: light purple	lower: light purple
Odor	slightly	slightly	slightly	slightly	slightly	slightly
Air bubble	no	no	no	no	no	no
Phase separate	no	no	no	no	no	no

Table B-7 Formulation of TSCE cream: shows pH and viscosity values of TSCE cream products of freshly prepared and after stability test

Cream products	Description of cream products					
	Freshly prepared	After 2 weeks	After 4 weeks	After 8 weeks	After 12 weeks	After 3 heating-cooling cycle
Cream base						
pH	4.77±0.02^{C}	4.63±0.02^{B}	4.60±0.03^{B}	4.54±0.08$^{A, B}$	4.51±0.01$^{A, B}$	4.44±0.04^{A}
Viscosity (mPas)	7509±621.01^{a}	8941±627.86$^{a, b}$	10351±897.62$^{a, b}$	12105±1537.59^{b}	11981±946.09^{b}	10310±534.20$^{a, b}$
TCSE cream$_1$						
pH	4.80±0.02^{C}	4.69±0.01$^{B, C}$	4.61±0.04$^{A, B}$	4.59±0.01^{A}	4.60±0.05$^{A, B}$	4.57±0.05^{A}
Viscosity (mPas)	6780±167.67^{a}	10042±357.22^{b}	11634±405.72$^{b, c}$	12887±577.70$^{c, d}$	14111±719.17^{d}	10174±746.60^{b}
TCSE cream$_2$						
pH	4.89±0.04^{B}	4.84±0.06$^{A, B}$	4.70±0.09^{A}	4.71±0.02^{A}	4.75±0.03$^{A, B}$	4.69±0.03^{A}
Viscosity (mPas)	8205±932.59^{a}	11600±1333.69$^{a, b}$	13017±1612.80$^{a, b}$	16147±1157.32$^{b, c}$	20534±3676.12^{c}	11748±1066.77$^{a, b}$
TCSE cream$_3$						
pH	4.80±0.06^{B}	4.73±0.06$^{A, B}$	4.65±0.08$^{A, B}$	4.54±0.07^{A}	4.56±0.03^{A}	4.56±0.06^{A}
Viscosity (mPas)	7816±789.54^{a}	10056±110.20$^{a, b}$	10988±118.91$^{b, c}$	12167±674.79$^{b, c}$	13308±1394.68^{c}	9826±905.23$^{a, b}$

Data represent mean±SEM (n=3).

A, B, C = significantly different ($p<0.05$) between different time in the same sample of pH value.

a, b, c = significantly different ($p<0.05$) between different time in the same sample of viscosity value.

APPENDIX C

HPLC chromatograms of HPLC analysis

Chromatograms of standard marker (+)-catechin, procyanidin B2 and (-)-epicatechin

Shimadzu CLASS–VP V 6.13 SP1 *Internal Standard*

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\catechin-procyanidinB2-epicatechin-each25ug.dat

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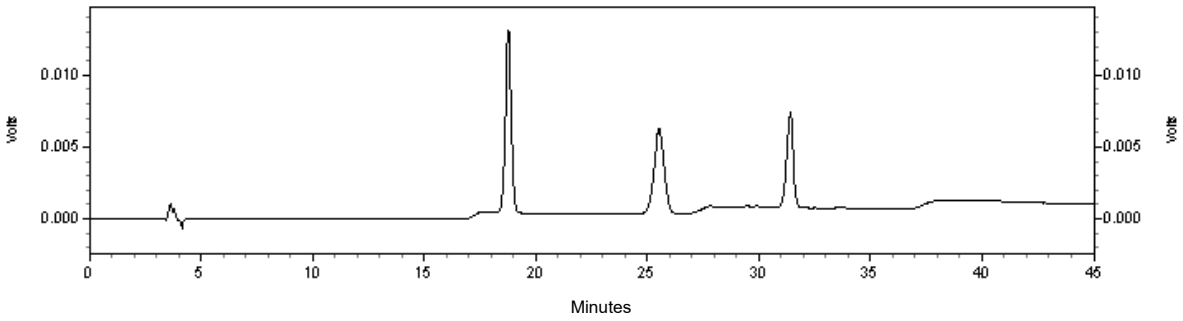

Detector A (278 nm)

Pk #	Name	Retention Time	Area
2	T_0	3.658	13698
12	(+)-catechin	18.777	247205
15	procyanidin B2	25.537	186360
20	(-)-epicatechin	31.391	139042
Total			969354

Chromatograms of TSCEs of TI-P/K

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\TI-PK-2500ug.dat

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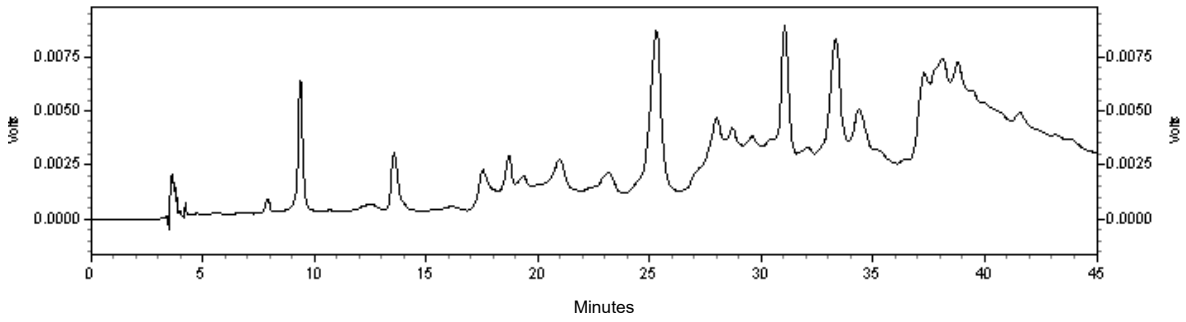

Detector A (278 nm)

Pk #	Name	Retention Time	Area
3	T_0	3.627	20322
30	(+)-catechin	18.699	66435
36	procyanidin B2	25.298	283128
42	(-)-epicatechin	31.060	204437
Total			5842454

Chromatograms of TSCEs of TI-P/K spiked with (+)-catechin

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\TI-PK-2500ug-catechin-10ug.dat

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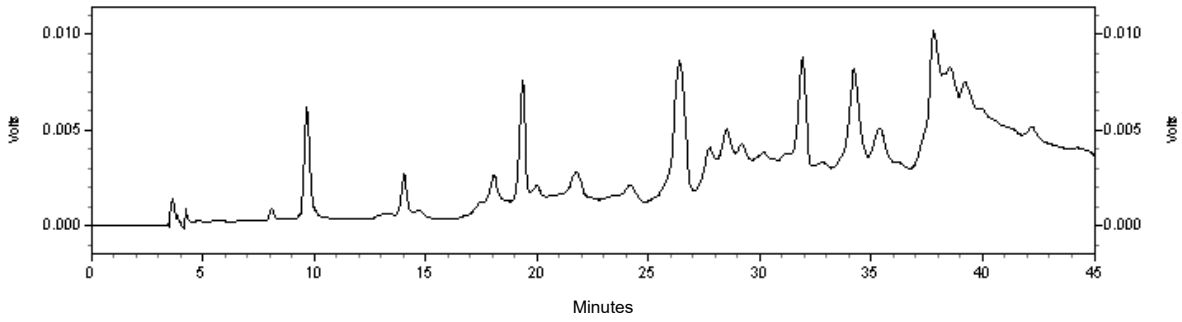

Detector A (278 nm)

Pk #	Name	Retention Time	Area
4	T_0	3.651	19073
28	(+)-catechin	19.344	152184
34	procyanidin B2	26.412	277189
41	(-)-epicatechin	31.905	210330
Total			7049779

Chromatograms of TSCEs of TI-P/K spiked with procyanidin B2

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\ TI-PK-2500ug-procyanidinB2-50ug.dat

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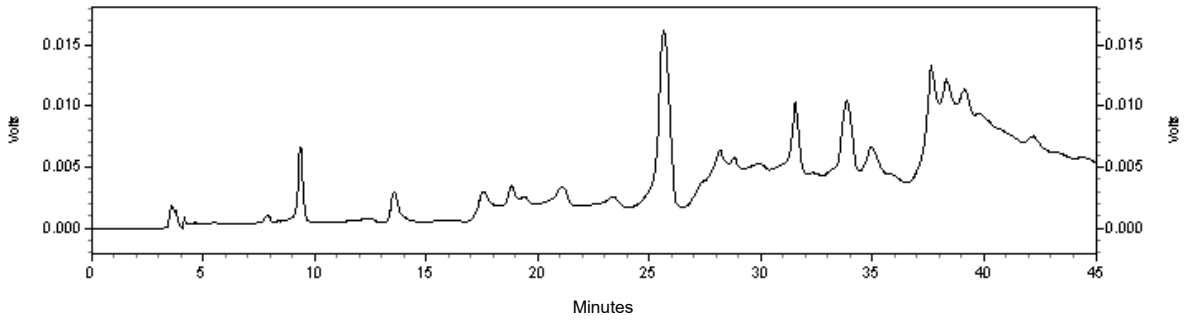

Detector A (278 nm)

Pk #	Name	Retention Time	Area
1	T_0	3.625	23801
24	(+)-catechin	18.849	54510
28	procyanidin B2	25.668	510753
33	(-)-epicatechin	31.551	363999
Total			11846241

Chromatograms of TSCEs of TI-P/K spiked with (-)-epicatechin

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\ TI-PK-2500ug-epicatechin-50ug.dat

User: System

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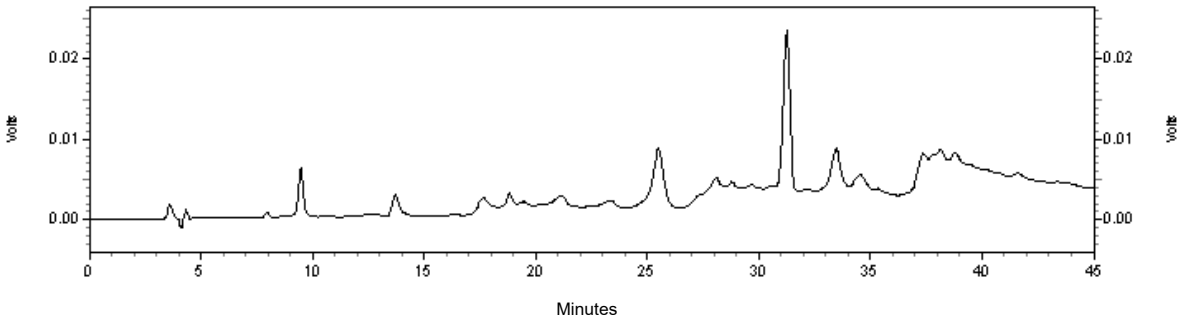

Detector A (278 nm)

Pk #	Name	Retention Time	Area
2	T_0	3.595	45278
30	(+)-catechin	18.853	74858
36	procyanidin B2	25.505	286418
41	(-)-epicatechin	31.237	456397
Total			6496306

Chromatograms of TSCEs of TI-SP/K

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

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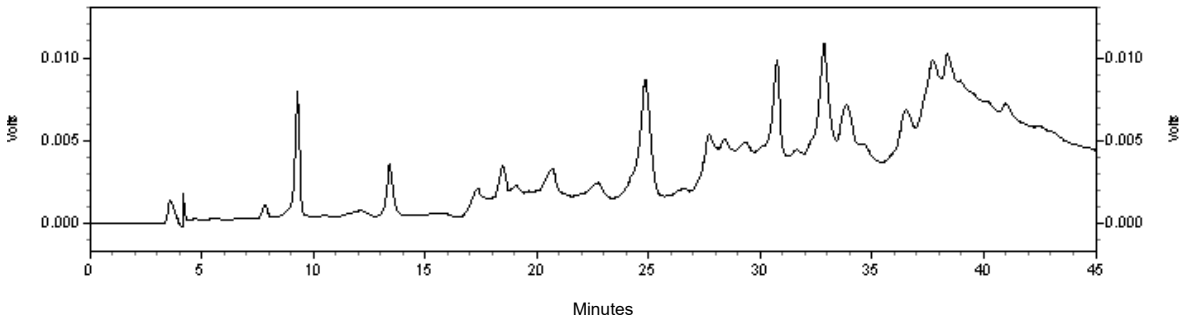

Detector A (278 nm)

Pk #	Name	Retention Time	Area
2	T_0	3.600	28457
29	(+)-catechin	18.472	50572
33	procyanidin B2	24.868	264336
39	(-)-epicatechin	30.725	176400
Total			6940951

Chromatograms of TSCEs of TI-SP/K spiked with (+)-catechin

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\TI-SPK-2500ug-catechin-10ug.dat

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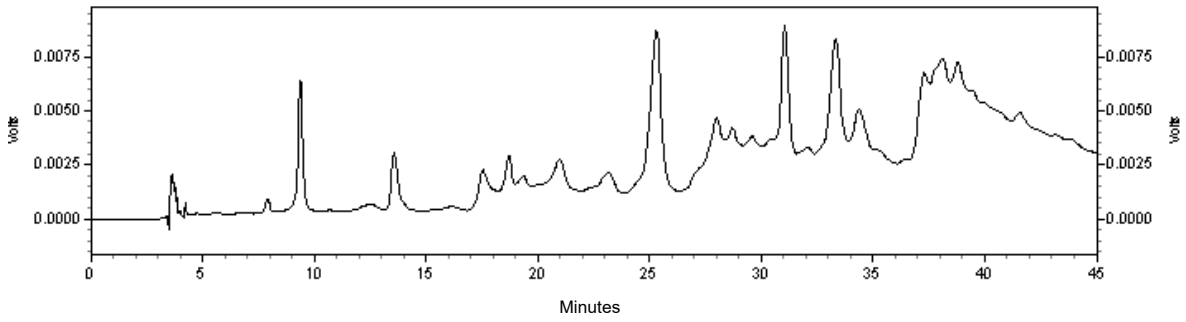

Detector A (278 nm)

Pk #	Name	Retention Time	Area
2	T_0	3.616	21012
25	(+)-catechin	18.758	204008
30	procyanidin B2	25.515	309591
34	(-)-epicatechin	31.420	396877
Total			13686091

Chromatograms of TSCEs of TI-SP/K spiked with procyanidin B2

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\TI-SPK-2500ug-procyanidinB2-50ug.dat

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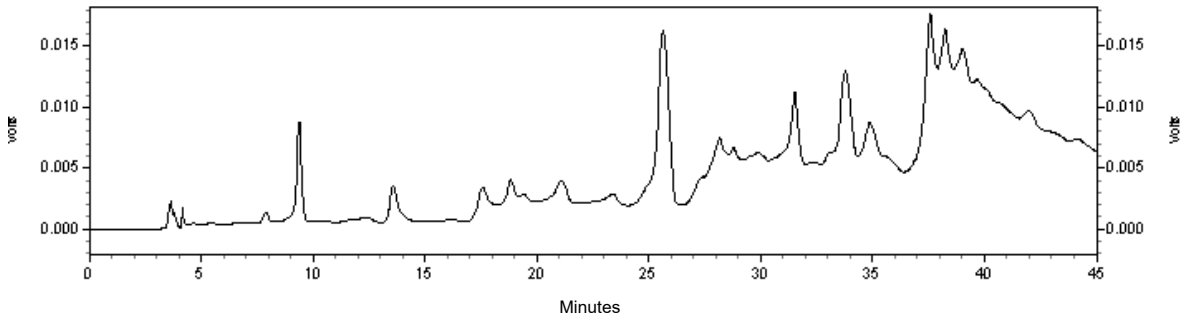

Detector A (278 nm)

Pk #	Name	Retention Time	Area
2	T_0	3.642	22600
29	(+)-catechin	18.848	105085
35	procyanidin B2	25.659	546501
41	(-)-epicatechin	31.525	298253
Total			13472085

Chromatograms of TSCEs of TI-SP/K spiked with (-)-epicatechin

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\ TI-SPK-2500ug-epicatechin-50ug.dat

User: System

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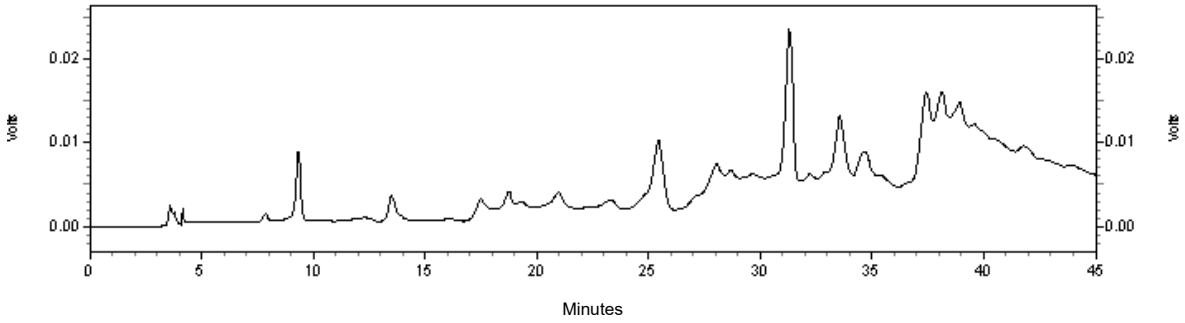

Detector A (278 nm)

Pk #	Name	Retention Time	Area
2	T_0	3.615	23751
23	(+)-catechin	18.736	46999
30	procyanidin B2	25.443	320277
34	(-)-epicatechin	31.296	665103
Total			13981885

Chromatograms of TSCEs of TI-STN/K

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\TI-STNK-2500ug.dat

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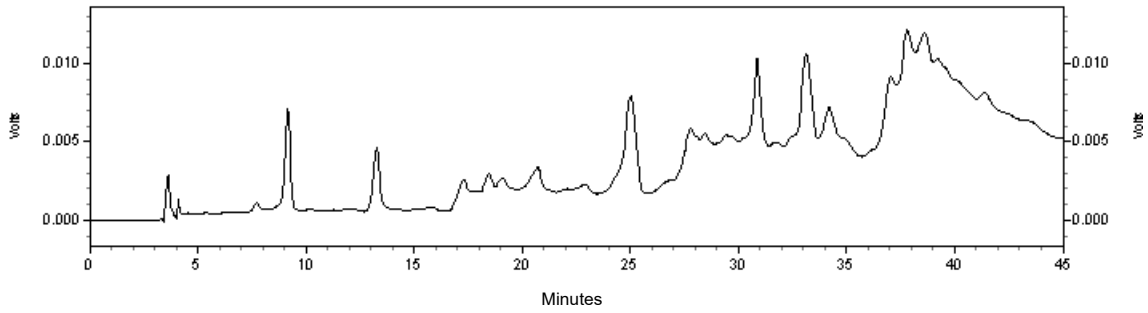


Detector A (278 nm)

Pk #	Name	Retention Time	Area
2	T_0	3.632	36528
24	(+)-catechin	18.448	66939
30	procyanidin B2	25.022	245602
36	(-)-epicatechin	30.866	342618
Total			12364805

Chromatograms of TSCEs of TI-STN/K spiked with (+)-catechin

Shimadzu CLASS–VP V 6.13 SP1 *Internal Standard*

Report Page 1 of 1

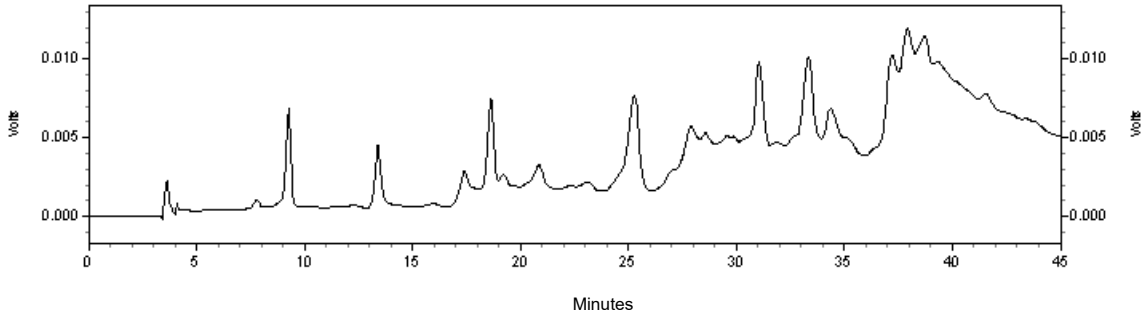
Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\TI-STNK-2500ug-catechin-10ug.dat

User: System

Acquired: 9/24/2010 1:30:12 AM

Printed: 9/24/2010 2:26:10 AM



Detector A (278 nm)

Pk #	Name	Retention Time	Area
3	T_0	3.639	29186
25	(+)-catechin	18.624	165649
32	procyanidin B2	25.264	257913
37	(-)-epicatechin	31.056	330815
Total			12250266

Chromatograms of TSCEs of TI-STN/K spiked with procyanidin B2

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

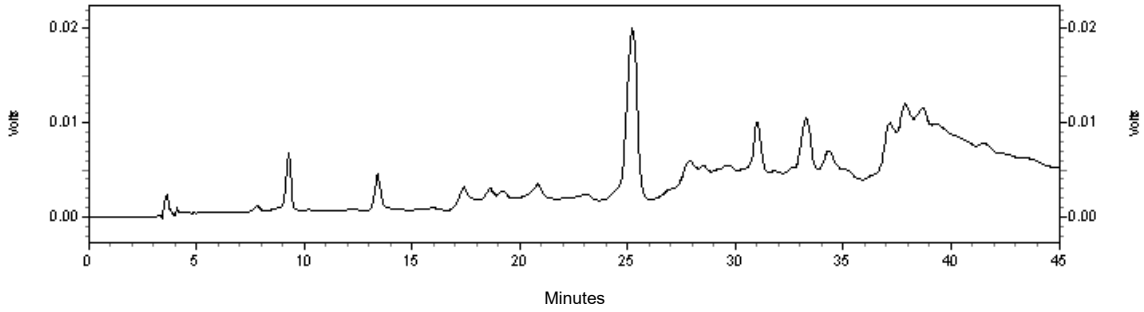
Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\ TI-STNK-2500ug-procyanidinB2-50ug.dat

User: System

Acquired: 8/8/2010 11:46:04 AM

Printed: 8/8/2010 11:46:04 AM



Detector A (278 nm)

Pk #	Name	Retention Time	Area
7	T_0	3.630	30093
25	(+)-catechin	18.621	38630
29	procyanidin B2	25.219	628202
33	(-)-epicatechin	31.019	323630
Total			12253889

Chromatograms of TSCEs of TI-STN/K spiked with (-)-epicatechin

Shimadzu CLASS–VP V 6.13 SP1 *Internal*

Standard

Report Page 1 of 1

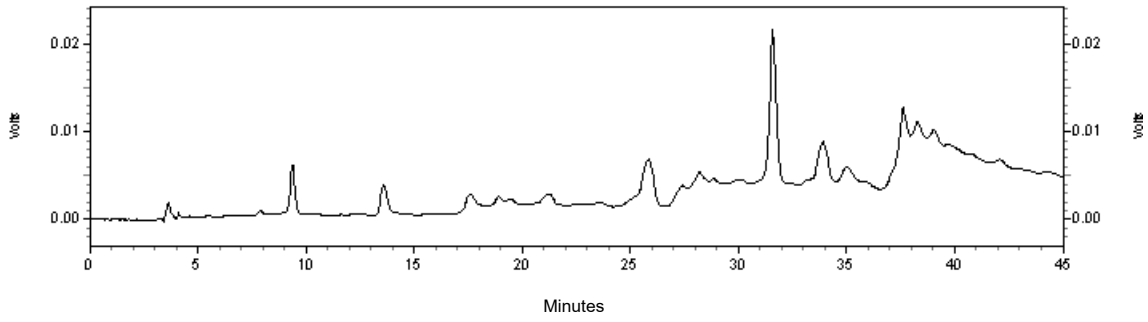
Method Name: D:\joy\Method\WashcolumnTSCEs-test1-30C.met

Data Name: D:\joy\New Folder\ TI-STNK-2500ug-epicatechin-50ug.dat

User: System

Acquired: 5/12/2010 10:38:25 AM

Printed: 5/12/2010 11:30:58 AM



Detector A (278 nm)

Pk #	Name	Retention Time	Area
3	T_0	3.649	25802
26	(+)-catechin	18.929	48720
31	procyanidin B2	25.830	218313
38	(-)-epicatechin	31.592	579911
Total			9729736

BIOGRAPHY

Miss Waleewan Eaknai was born on December 4, 1985 in Bangkok, Thailand. She graduated in high school level from Thammasat Klongluang Wittayakom School, Phathumtani province in 2003. In 2007, she received her Bachelor's degree of Science in Botany program from the Faculty of Science, Chulalongkorn University and majoring Botany. Then she continues studied in Master Degree of Science in Biomedicinal Chemistry program from the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Publications

1. Eaknai, W., Wongprapairoj, P., Suksomtip, M., and Pongsamart, S. 2009. Antioxidant of seed coat extracts of Thai tamarind cultivars from Nakornratchasima province, at The 3rd Asian Pacific Regional ISSX Meeting, May, 10-12, 2009, Bangkok, Thailand, page 96.
2. Wongprapairoj, P., Eaknai, W., Lipipun, V., and Pongsamart, S. 2009. Antibacterial activity of seed coat of certain Thai tamarind cultivars, at The 3rd Asian Pacific Regional ISSX Meeting, May, 10-12, 2009, Bangkok, Thailand, page 95.
3. Eaknai, W., and Pongsamart, S. 2009. Antioxidant and antibacterial acitivities in seed-coat extracts of certain tamarind cultivars, The 2nd International Symposium on Medicinal and Nutraceutical Plants, November, 25-27, 2009, New Delhi, India, page 97.
4. Eaknai, W., and Pongsamart, S. 2010. Development of tamarind seed coat extract with antioxidant activity as a topical cream, Proceeding of The 36th Congress on Science and Technology of Thailand, October, 26-28, 2010, Bangkok, Thailand, page 90.