ฤทธิ์ยับยั้งเชื้อจุลินทรีย์ของสารสกัดเปลือกเมล็ดมะขามต่างสายพันธุ์และฤทธิ์ในยาเตรียมใช้เฉพาะที่

นางสาว ปิยนุช วงศ์ประไพโรจน์

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ANTIMICROBIAL ACTIVITY OF SEED COAT EXTRACTS OF TAMARIND CULTIVARS AND TOPICAL PREPARATION

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biomedicinal Chemistry
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ปิยนุช วงศ์ประไพโรจน์ : ฤทธิ์ยับยั้งเชื้อจุลินทรีย์ของสารสกัดเปลือกเมล็ดมะขามต่างสายพันธุ์ และฤทธิ์ในยาเตรียมใช้เฉพาะที่. (ANTIMICROBIAL ACTIVITY OF SEED COAT EXTRACTS OF TAMARIND CULTIVARS AND TOPICAL PREPARATION) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ภญ. ดร. สุนันท์ พงษ์สามารถ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ภญ. ดร. วิมลมาศ ลิปิพันธ์, 108 หน้า.

มะขาม *Tamarindus indica* L. ได้ใช้เป็นยาแผนโบราณมาเป็นเวลานาน พบว่าเปลือกเมล็ดมะขามประกอบด้วยสารกลุ่มฟลาโวนอยด์ และมีการรายงานฤทธิ์ทางชีวภาพของสารกลุ่มฟลาโวนอยด์เป็นสารต้านอนุมูลอิสระ และยับยั้งเชื้อจุลินทรีย์ งานวิจัยครั้งนี้ออกแบบเพื่อประเมินฤทธิ์การยับยั้งเชื้อจุลินทรีย์ของสารสกัดเปลือกเมล็ดมะขามที่ปลูกในประเทศไทย 8 สายพันธุ์ มีทั้งมะขามชนิดเปรี้ยว และหวาน โดยสกัดเปลือกเมล็ดมะขามด้วย 70% ethanol แล้วนำสารสกัดมาเขย่ากับ chloroform ในอัตราส่วน 1:1 เพื่อสกัดเอาลิปิดออกไป แยกเอาชั้นของ 70% ethanol มาทำให้แห้ง ประเมินฤทธิ์ยับยั้งเชื้อจุลินทรีย์ของสารสกัดเปลือกเมล็ดมะขาม (TSCEs) โดยวิธี agar-well diffusion และ broth microdilution รวมทั้งวิเคราะห์ time-kill analysis ผลการทดลองแสดงให้เห็นว่าสารสกัดเปลือกเมล็ดมะขามจากมะขามไทยทั้ง 8 สายพันธุ์ สามารถยับยั้งเชื้อแบคทีเรียที่ทดสอบทั้งหมด 5 สายพันธุ์ ได้แก่ แบคทีเรียแกรมบวก 3 สายพันธุ์ คือ *Staphylococcus aureus* ATCC 6538P, *S. epidermidis* ATCC 12228 และ *Propionibacterium acnes* แบคทีเรียแกรมลบ 2 สายพันธุ์ คือ *Escherichia coli* ATCC 25922 และ *Pseudomonas aeruginosa* ATCC 27853 แต่อย่างไรก็ตามสารสกัด TSCE ไม่สามารถยับยั้งเชื้อราที่ทดสอบ 1 สายพันธุ์ คือ *Candida albicans* ATCC 10230 พบว่าแบคทีเรียแกรมบวกไวต่อการถูกยับยั้งด้วยสารสกัดเปลือกเมล็ดมะขามได้มากกว่าแบคทีเรียแกรมลบ ส่วนผลของ time-kill analysis พบว่า สารสกัดเปลือกเมล็ดมะขามที่ความเข้มข้น 0.39, 0.78 และ 3.13 mg/ml ตามลำดับ ฆ่าเชื้อ *S. epidermidis*, *S. aureus* และ *P. acnes* โดยสามารถฆ่าเชื้อ ภายใน 10, 14 และ 30 ชั่วโมง ตามลำดับ สารสกัดเปลือกเมล็ดมะขามสามารถลดจำนวนเชื้อเป็นที่นับได้ของ *S. epidermidis* และ *S. aureus* ได้ถึง 99% ใน 4 ชั่วโมง และของ *P. acnes* ใน 24 ชั่วโมง ตามลำดับ นำสารสกัดเปลือกเมล็ดมะขาม สายพันธุ์เปรี้ยวยักษ์ จากจังหวัดเพชรบูรณ์ มาใช้เตรียมเป็นผลิตภัณฑ์ใช้เฉพาะที่เพื่อยับยั้งเชื้อแบคทีเรีย และทำการประเมินลักษณะทางกายภาพของผลิตภัณฑ์ รวมทั้งสี ฟองอากาศ ความหนืด ความเป็นกรด-ด่าง และประสิทธิภาพในการยับยั้งเชื้อแบคทีเรียของผลิตภัณฑ์หลังเตรียมเสร็จ และผลิตภัณฑ์หลังผ่านการทดสอบความคงตัว พบว่าผลิตภัณฑ์เจลเปลือกเมล็ดมะขามมีคุณลักษณะและความคงตัวที่ดี ฤทธิ์การยับยั้งเชื้อแบคทีเรียของผลิตภัณฑ์พบว่าสามารถยับยั้งเชื้อแบคทีเรียที่เป็นสาเหตุของการติดเชื้อที่ผิวหนังคือ *S. aureus*, *S. epidermidis* และ *P. acnes* โดยวิธี agar-well diffusion และ broth microdilution โครมาโทแกรมของสารสกัดเปลือกเมล็ดมะขามสายพันธุ์เปรี้ยวยักษ์ แสดง chemical fingerprint ที่มี peaks ต่างๆ ที่บ่งชี้ตรงกับสารมาตรฐาน (+)-catechin, (-)-epicatechin และ procyanidin B2

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PIYANUCH WONGPRAPAIROJ : ANTIMICROBIAL ACTIVITY OF SEED-COAT EXTRACTS OF TAMARIND CULTIVARS AND TOPICAL PREPARATION. THESIS ADVISOR : ASSOC. PROF. SUNANTA PONGSAMART, Ph.D., THESIS CO-ADVISOR : ASSOC. PROF. VIMOLMAS LIPIPUN, Ph.D., 108 pp.

Tamarindus indica L. has long been used as traditional medicine. Tamarind seed-coats have been found to contain flavonoid compounds. Bioactivities of flavonoids as antioxidant and antimicrobial have also been reported. This study was designed to evaluate antibacterial potential of seed-coat extracts from 8 Thai tamarind cultivars including the sour and sweet types. Tamarind seed-coats were extracted with 70% ethanol and shaken the extracts with chloroform (1:1) to extract lipids, the aqueous-ethanol extract was separated and dried. Antimicrobial activity of tamarind seed-coat extracts (TSCEs) was evaluated by agar-well diffusion and broth microdilution susceptibility tests, as well as the time-kill analysis. The results showed that all of TSCEs extracted from 8 Thai tamarind cultivars inhibited the growth of five tested bacterial strains including three of gram-positive bacteria, *Staphylococcus aureus* ATCC 6538P, *S. epidermidis* ATCC 12228, and *Propionibacterium acnes* (clinical isolate); two of gram-negative bacteria, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. However, one of tested fungus, *Candida albicans* ATCC 10230, was not inhibited by TSCEs. The gram-positive bacteria were more susceptible to TSCEs inhibition than the gram-negative bacteria. Time-kill analysis demonstrated that at 0.39, 0.78 and 3.13 mg/ml of TSCEs, respectively, killed *S. epidermidis*, *S. aureus* and *P. acnes* in 10, 14 and 30 h, respectively. TSCEs exhibited 99% reduction of viable count of *S. epidermidis* and *S. aureus* within 4 h and *P. acnes* within 24 h, respectively. The TSCE from *Tamarindus indica* "Priao-yak" (TI-PY/P) was used as antibacterial agent for the preparation of TSCE gel for topical use. The appearances and physical properties of the finish product including color, air-bubble, viscosity, pH and antibacterial efficacy of TSCE gel were examined after freshly prepared and after stability tested. Good appearances as well as stability of the TSCE gel product were obtained. Antibacterial activity of TSCE gel was also observed against bacteria causing skin infection including *S. aureus*, *S. epidermidis* and *P. acnes*, as evaluated by agar-well diffusion and broth microdilution methods. The HPLC chromatogram of TSCE from the seed coats of *T. indica* "Priao yak" showed the chemical fingerprint of peaks that identical with the standard flavonoids including (+)-catechin, (-)-epicatechin and procyanidin B2.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
BHIA	brain heart infusion agar
BHIB	brain heart infusion broth
ºC	degree Celsius
CFU	colony forming unit
cps	centipoise
DW	distilled water
et al.	et alii, and others
Fig.	figure
g	gram (s)
h	hour
L	liter
LDL	Low-density lipoprotein
MBC	minimal bactericidal concentration
mg	milligram
MHA	mueller hinton agar
MHB	mueller hinton broth
MIC	minimal inhibitory concentration
mm	millimeter
min	minute (s)
ml	milliliter (s)
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCCLS	National Committee for Clinical Laboratory Standard
NSS	normal saline solution
SDA	sabouraud dextrose agar
SDB	sabouraud dextrose broth
SD	standard deviation
Pa	Pascal (s)
pH	The negative logarithm of hydrogen ion concentration
qs.	Make to volume

ABBREVIATIONS (Cont.)

TSCE	tamarind seed coat extract
TEA	triethanolamine
USP	The United States Pharmacopeia
w/w	percent weight in weight
µg	microgram
µl	microliter
%	percentage

CHAPTER I

GENERAL BACKGROUND

Introduction

The use of plant extracts and phytochemicals, both with known antimicrobial properties, can be of great significance in therapeutic treatments. In the last few years, a number of studies have been conducted in different countries to prove such efficiency (Sousa *et al.*, 1991; Shapoval *et al.*, 1994). Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized as the secondary metabolite of the plant. These products are known by their active substances, for example, the phenolic compounds which are part of flavonoid compounds. Flavonoids have been reported many properties including anti-inflammatory, oestrogenic, enzyme inhibitory, antimicrobial, antiallergic, antioxidant, vascular and cytotoxic antitumor activities (Cushnie and Lamb, 2005).

The antimicrobial property of plants have been investigated by a number of researchers worldwide, especially in Latin America. In Argentina, researchers tested 122 known plant species used for therapeutic treatments (Anesini, 1993). It was documented that among the compounds extracted from these plants, inhibited the growth of microorganisms (Martinez *et al.*, 1994, 1996).

Tamarind (*Tamarindus indica* L.) is a tree cultivated in tropical countries through out the world. Tamarind fruits have been used in food industry as seasoning, food component and snack. According to Thai traditional medicine, fruits of tamarind are regarded as a digestive, carminative, laxative, expectorant and tonic. In addition, the seeds of *T. indica* are used as an anthelmintic, antidiarrheal and emetic, and the seed-coat is used to treat burns and aids in wound healing as well as an antidysenteric (Soemardji, 2007). Tamarind seed-coat extracts (TSCEs) have been studied recently, the extracts are composed of flavonoids, polyphenols, including tannins, epicatechin, anthocyanidin and proanthocyanidin (Komutarin, 2004). Antioxidant potential of seed-coat extracts have been studied on lipid peroxidation inhibition (Tsuda *et al.*, 1993, 1994) and also nitric oxide production both in vitro and in vivo (Maiti *et al.*, 2005). Furthermore, aqueous extract of tamarind seeds possess a potent antidiabetic

and antihyperlipidemic activities by reducing blood sugar, total cholesterol and triglycerides, respectively (Maiti *et al*., 2004).

Although there are many studies on bioactivity of tamarind seed-coat extract, there are few studies on the antimicrobial property of tamarind seed-coat extract. Therefore, the purpose of this study was to (1) investigate the antimicrobial activity of the tamarind seed-coat extracts against pathogenic microorganisms that can cause infection to the skin, digestive or respiratory tracts such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Propionibacterium acnes*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*; (2) prepare TSCE gel with antibacterial activity; and (3) separate and identify flavonoid compounds in tamarind seed-coat extracts by High Performance Liquid Chromatography (HPLC).

CHAPTER II

LITERATURE REVIEW

1. Tamarind

Tamarind, *Tamarindus indica* Linn, is a multipurpose tropical fruit tree used primarily for its fruits, which are eaten fresh or processed, as a seasoning or spice, or the fruits and seeds are processed for non-food uses. Several parts of tamarind have long been used in tradition medicine (Soemardji, 2007). The species has a wide geographical distribution in the subtropics and semi-arid tropics and is cultivated in numerous regions.

Tamarind is widely grown as a subsistence crop for meeting local demands. It is also grown commercially. Numerous national programmes have recognized tamarind as an underutilized crop with wider potential since demand for products is substantial and the species can be incorporated into agroforestry systems. There are also well established international trade channels. Further exploitation of tamarind can therefore provide added incomes for poor rural people thereby improving their well-being (El-Siddig *et al*., 2006).

Scientific classification (Soemardji, 2007)

Kingdom : Plantae
Sub Kingdom : Tracheobionta
Division : Spermatophyta
Sub Division : Magniliophyta
Class : Magnoliopsida
Family : Fabaceae
Subfamily : Caesalpiniaceae
Genus : *Tamarindus* L.
Species : *Tamarindus indica* Linn

1.1 Description of tamarind (El-Siddig *et al*., 2006)

1.1.1 Tree

Tamarind is a long-lived, large, evergreen or semi-evergreen tree, 20-30 m tall with a thick trunk up to 1.5-2.0 m across and up to 8 m in circumference. The trunk forks at about 1 m above ground and is often multi-stemmed with branches widely spreading, drooping at the ends and often crooked but forming a spreading, rounded crown. The bark is brownish-grey, rough and scaly.

1.1.2 Root

Tamarind produces a deep tap root and an extensive lateral root system, but the tap root may be stunted in badly drained or compacted soils. The tap root is flexuous and lateral roots are produced from the main root at different levels.

1.1.3 Leaves

Leaves are alternate and even pinnate, in length 5-15 cm, shortly petiolated (up to 1.5 cm long) and petiole glabrous or puberulent as is the leaf rachis. Venation is reticulate and the midrib of each leaflet is conspicuous above and below. Leaflets are in 6-20 pairs/leaf, each narrowly oblong, rounded at the apex and slightly notched and asymmetric with a tuft of yellow hairs.

1.1.4 Flowers

Flowers are borne in lax racemes which are few to several flowered (up to 18), borne at the ends of branches and are shorter than the leaves, the lateral flowers are drooping. Flowers are irregular 1.5 cm long and 2-2.5 cm in diameter each with a pedicel about 5-10 mm long. Bracts are ovate-oblong, and early caducous, each bract almost as long as the flower bud. There are 2 bracteoles, boat shaped, and 8 mm long and reddish.

1.1.5 Fruits and seeds

The fruits are pods 5-16 cm long, oblong, curved or straight, with rounded ends, somewhat compressed and indehiscent although brittle. Within is the firm but soft pulp which is thick and blackish brown. The pulp is traversed by formed seed cavities, which contain the seeds. Seeds are hard, red to purple brown. Seed chambers are lined with a parchment-like membrane (Hong *et al*., 1996; El-Siddig *et al*., 2000).

1.2 Main usage (El-Siddig *et al*., 2006)

1.2.1 Pulp

Tamarind is valued mostly for its fruit especially the pulp, which is used for a wide variety of domestic and industrial purposes. Tamarind is not generally a dessert fruit, although the sweet tamarind is often eaten fresh directly from the pod, almost always so in the West Indies and other parts of MesoAmerica. Fully grown but still unripe fruits are also eaten in the Bahamas; known as swells, they are roasted in coal, the skin is then peeled back and the sizzling pulp is dipped in wood ash and eaten. The pulp is usually removed from the pod and used to prepare juice, jam, syrup and candy. In Asia however, the immature green pods are often eaten by children and adults dipped in salt as a snack. More commonly, the acidic pulp is used as a favorite ingredient in culinary preparations such as curries, chutneys, sauces, ice cream and sherbet in countries where the tree grows naturally.

1.2.2 Seed

Tamarind seed consists of the seed-coat or testa (20-30%) and the kernel or en-dosperm (70-75%). Tamarind seed is a by-product of the commercial utilization of the fruit, however it has several uses. In the past, the seeds have been wasted. In 1942, two Indian scientists, T.P. Ghose and S. Krishna, announced that the decorticated kernels contained 46-48% of a gel-forming substance. It was called jellose, polyose, or pectin, which was found to be superior to other methods of fruit preservation. The name jellose has been suggested for this polysaccharide as it describes both its jelly forming properties and the carbohydrate character. It has been recommended for use as a stabilizer in ice cream, mayonnaise and cheese and as an ingredient or agent in a number of pharmaceutical products. Presently, it is used in food and in industrial applications. Tamarind jellose has not been fully exploited, but due to its abundance and cheapness, seed jellose has great potential for replacing fruit pectins in many industries.

1.2.3 Leaves and Flowers

The leaves, flowers and immature pods of tamarind are also edible. The leaves and flowers are used to make curries, salads, stews and soups in many countries, especially in times of scarcity.

1.2.4 Wood

Tamarind wood has many uses including making furniture, wheels, mallets, rice pounders, mortars, pestles, ploughs, well construction, tent pegs, canoes, side planks for boats, cart shafts and axles, and naves of wheels, toys, oil presses, sugar presses, printing blocks, tools and tool handles, turnery, etc.

1.3 Medicinal usage (Soemardli, 2007)

The taste of tamarind pulps is sour and sweet, cool and astringent, and its ingredients of many parts of tamarind have been used as a traditional medicine for many cases of the human-health.

1.3.1 Leaves

Leaves of tamarind are used to cure cough, pyretic, rheumatism jaundice, worm infection, stomach disorder, general body pain, yellow fewer, blood tonic, skin cleanser, antiseptic, vermifuge, dysentery, conjunctivitis, erysipelas, hemorrhoid, sores ulcer and insomnia. Its leaf is use as a hot juice or decoction.

1.3.2 Flowers

Flowers are used for pulmonary tuberculosis, cough with blood pharinkhitis chronic, rheumatism locally edema and wound, vermifuge, dysentery, jaundice, conjunctivitis, erysipelas, hemorrhoid and antiseptic.

1.3.3 Barks

Barks are used for asthmatic, pyretic amenorrhea, colic and scorbutic, stomach disorder, general body pain, jaundice, yellow fever, blood tonic, skin cleanser, eye inflammation and antimicrobial.

1.3.4 Fruits

Fruits or pulps are used to cure constipate, pyretic, dysentery, loss of appetite, alcohol toxicity, vomit, worm infection, jaundice, nausea, vomit in pregnant, asthmatic breast inflammation urticaria allergic, morbili and thirsty.

1.3.5 Seeds

Seeds are used as an anthelmintic, antidiarrheal, wound/ulcer, drop off hair, antidiabetic, antihyperlipidemic and an emetic.

1.3.6 Kernels

Kernels are used as depressant, treatment of constipation and diarrhea.

1.3.7 Seed-coats

Seed-coats are used to treat burns and aid in wound healing as well as an antidysenteric, antibiotic and anti-inflammatory applications.

2. Research of tamarind seed-coat

Tamarind seed-coat extracts exhibit antioxidant potential by reducing lipid peroxidation in vitro (Tsuda *et al.*, 1993, 1994). The inhibitory effect of seed-coat extract on nitric oxide production in vitro and in vivo was also reported (Komutarin *et al.*, 2004). Furthermore, aqueous extract of tamarind seeds was found to have potent antidiabetic and antihyperlipidemic activities that reduce blood sugar level, total cholesterol and triglycerides, respectively, in streptozotocin (STZ)-induced diabetic male rat (Maiti *et al.*, 2005). Sudjaroen *et al.* (2005) elucidated the polyphenolic compounds with antioxidant capacity in methanolic extracts of seeds and pericarp. The extract is composed of flavonoids including tannin and procyanidin. Siddhuraju (2007) also reported the antioxidant properties of polyphenolic compound extracted from defatted raw and dry-heated tamarind seed-coat. Further, the addition of citric acid with the seed-coat extract resulted in synergistic antioxidant action in the edible oil (Tsuda *et al.*, 1995). Tamarind seed-coats were investigated the possible protective effect of seed-coat extract against LDL (Low-density lipoprotein) oxidation induced by Cu^{2+} using various markers of oxidative stress. Furthermore, the protective effect against super coiled DNA strand scission induced by Fenton-mediated hydroxyl radical was also investigated (Suksomtip and Pongsamart, 2008). Suksomtip *et al.* (2008) reported phenolic compounds content, antioxidant and radical-scavenging properties of methanolic extracts from the seed-coat of certain Thai tamarind cultivars.

Waghmare *et al.* (2010) investigated the antimicrobial activity of the seed-coat extract against certain bacterial species including *Staphylococcus aureus*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* most commonly encountered in food poisoning cases among certain communities which live in remote parts of many

developing nations and has little or no access to modern medicine to control the out beaks of food poisoning.

3. Phytochemical of tamarind seed-coat extract

Tamarind contains antioxidants which are useful in the food, pharmaceutical and cosmetics industries. Tsuda *et al*. (1994) reported four antioxidants in the seed-coat of Indian tamarind: 2-hydroxy-3', 4'-dihydroxyacetophenone; methyl 3, 4-dihydroxybenzoate; 3, 4-dihydroxyphenyl acetate; and (-)-epicatechin. Pumthong (1999) demonstrated the antioxidant activity of seed-coat extract of *Tamarindus indica*. The extract is composed of flavonoid including tannin, anthocyanidin, and oligomeric proanthocyanidins.

Sudlaroen *et al*. (2005) reported the profile (%) of polyphenolics in tamarind seed-coat by analytical high performance liquid chromatography (HPLC) as shown in Table 1. The structures of the monomeric and oligomeric flavonoid are shown Figure 1 and Figure 2.

4. Flavonoid

Flavonoids are ubiquitous in photosynthesizing cells and therefore occur widely in the plant kingdom (Havsteen, 1983). They are found in fruit, vegetables, nuts, seeds, stems and flowers as well as tea, wine (Middleton and Chithan, 1993), propolis and honey (Grange and Davey, 1990), and represent a common constituent of the human diet (Harborne and Baxter, 1999). In the US, the daily dietary intake of mixed flavonoids is estimated to be in the ranges 500–1000 mg, but this figure can be as high as several grams for people supplementing their diets with flavonoids or flavonoid-containing herbal preparations (Skibola and Smith, 2000).

The function of flavonoids in flowers is to provide colors attractive to plant pollinators (Middleton and Chithan, 1993; Harborne and Williams, 2000). In leaves, these compounds are increasingly believed to promote physiological survival of the plant, protecting it from, for example, fungal pathogens and UV-B radiation (Harborne and Baxter, 1999; Harborne and Williams, 2000).

Table 1. The percentage of polyphenolic compounds in tamarind seed-coat

Polyphenolic compounds	% of polyphenolic in tamarind seed-coat
(+)-catechin	2
(-)-epicatechin	9.4
taxifolin	7.4
apigenin	2
eriodictyol	6.9
luteolin	5
naringenin	1.4
proanthocyanidins	73.4
procyanidin B2	8.2
procyanidin trimer	11.3
procyanidin tetramer	22.2
procyanidin pentamer	11.6
procyanidin hexamer	12.8

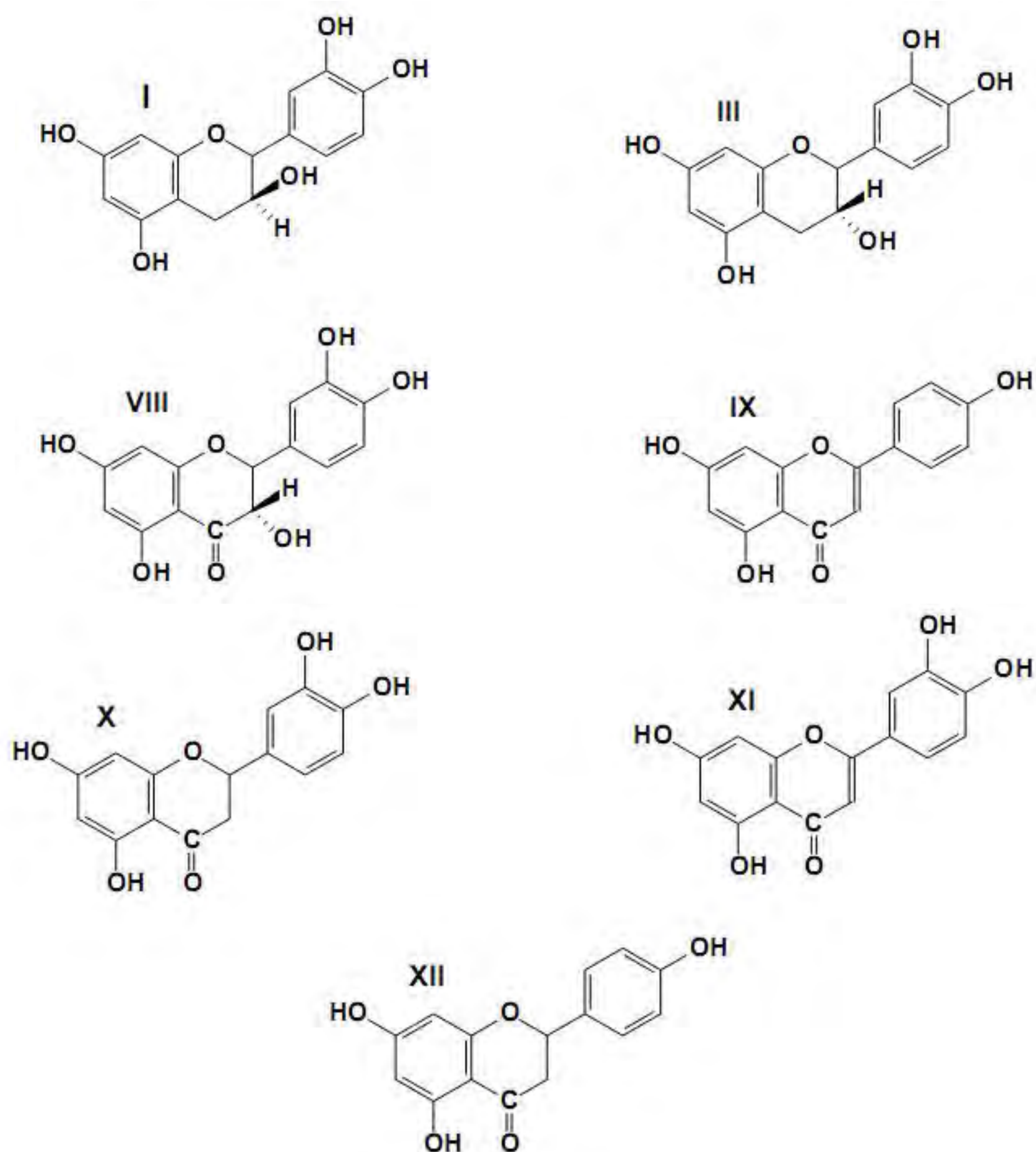


Figure 1. The structures of the monomeric flavonoids are (+)-catechin (I), (-)-epicatechin (III), taxifolin (VIII), apigenin (IX), eriodictyol (X), luteolin (XI) and naringenin (XII).

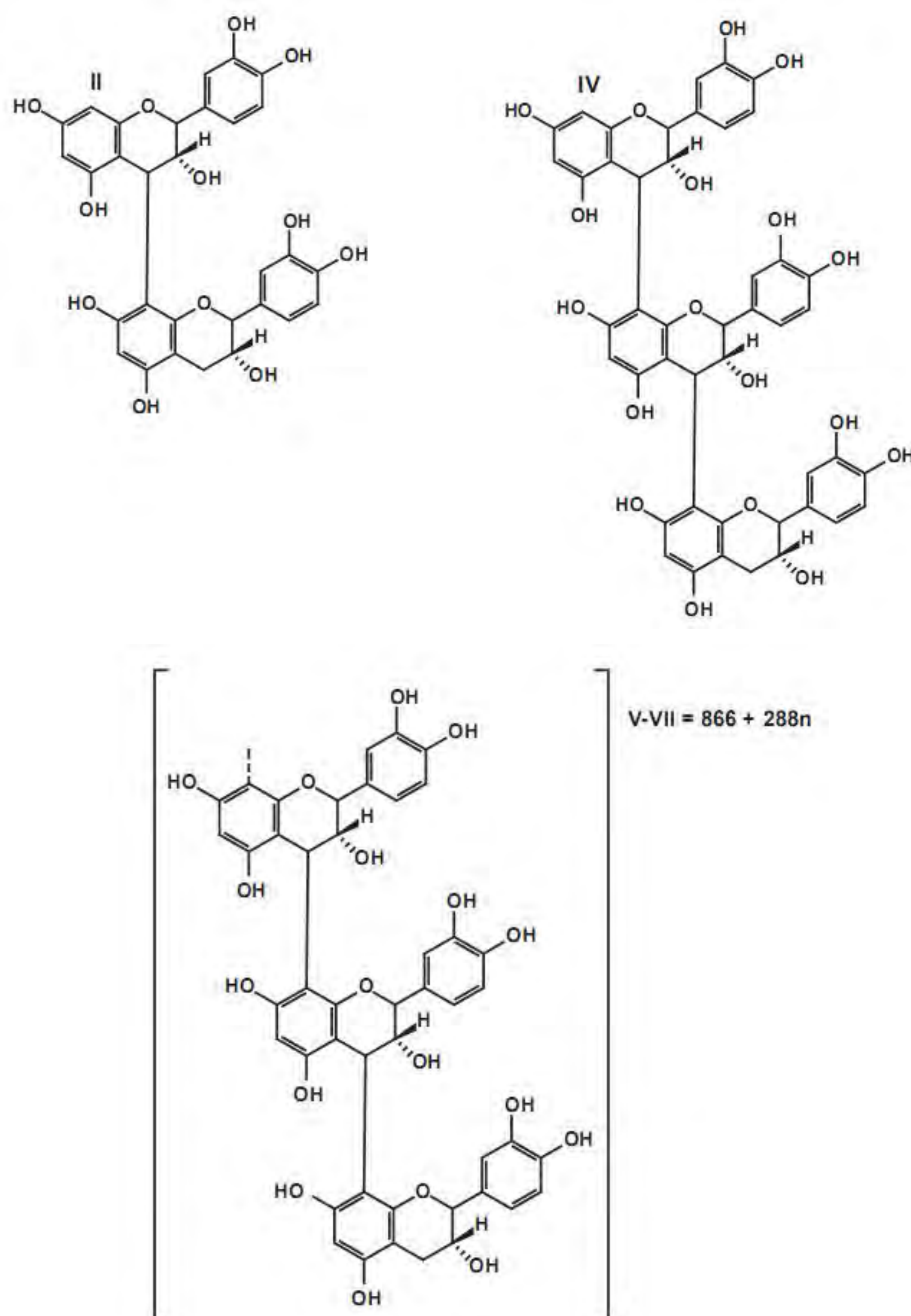


Figure 2. The structures of the oligomeric flavonoids are procyanidin B2 (II), procyanidin trimer (IV), procyanidin tetramer (V), procyanidin pentamer (VI) and procyanidin hexamer (VII).

In addition, flavonoids are involved in photosensitization, energy transfer, the actions of plant growth hormones and growth regulators, control of respiration and photosynthesis, morphogenesis and sex determination (Middleton and Chithan, 1993; Harborne and Williams, 2000).

The basic structural feature of flavonoid compounds is the 2-phenyl-benzo[α]pyrane or flavane nucleus, which consists of two benzene rings (A and B) linked through a heterocyclic pyrane ring (C) as shown in Figure 3. (Brown, 1980). Flavonoids can be classified according to biosynthetic origin. Some classes, for example chalcones, flavanones, flavan-3-ols and flavan-3, 4-diols, are both intermediates in biosynthesis as well as end products that can accumulate in plant tissues. Other classes are only known as end products of biosynthesis, for example anthocyanidins, proanthocyanidins, flavones and flavonols. Two additional classes of flavonoid are those in which the 2-phenyl side chain of flavanone isomerises to the 3 position, giving rise to isoflavones and related isoflavonoids. Structures of the major classes of flavonoids are showed in Figure 4.

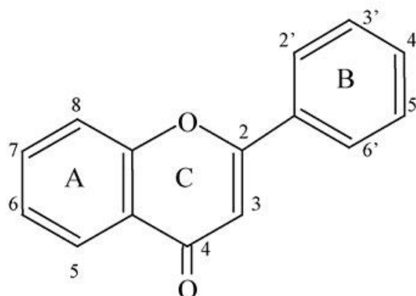


Figure 3. The skeleton structure of the flavones (a class of flavonoids), with rings named and positions numbered.

4.1 Medicinal properties of flavonoids (Havsteen, 1983; Middleton and Chithan, 1993; Harborne and Baxter, 1999; Harborne and Williams, 2000)

Increasingly, flavonoids are becoming the subject of medical research. They have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity, antiallergic activity, antioxidant activity, vascular activity and cytotoxic antitumor activity.

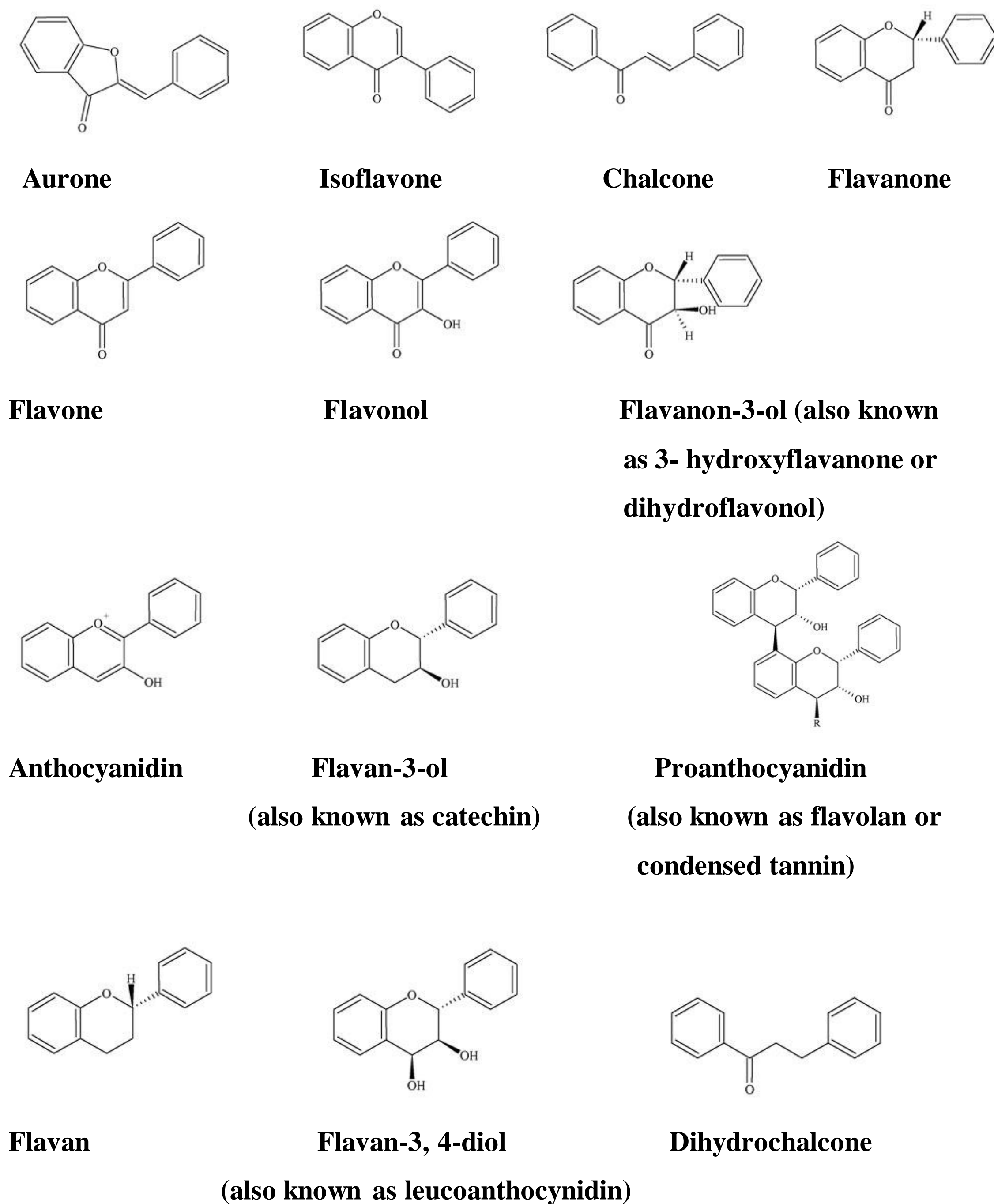


Figure 4. The skeleton structures of the main classes of flavonoids: aurones, isoflavones, chalcones, flavanones, flavones, flavonols, flavanon-3-ols, anthocyanidins, flavan-3-ols, proanthocyanidins (occur as dimers, trimers, tetramers and pentamers; R=0, 1, 2 or 3 flavan-3-olstructures), flavans, flavan-3, 4-diols and dihydrochalcones.

For a group of compounds of relatively homogeneous structure, the flavonoids inhibit a perplexing number and variety of eukaryotic enzymes and have a tremendously wide range of activities. In the case of enzyme inhibition, this has been postulated to be due to the interaction of enzymes with different parts of the flavonoid molecule, e.g. carbohydrate, phenyl ring, phenol and benzopyrone ring.

4.2 History of flavonoid use in antimicrobial treatment

For centuries, preparations that contain flavonoids as the principal physiologically active constituents have been used by physicians in attempts to treat human diseases (Havsteen, 1983). For example, the plant *tagetes minuta* (containing quercetagetin-7-arabinosyl-galactoside) has been used extensively in Argentine folk medicine to treat infectious disease.

The healing properties of propolis are referred to throughout the Old Testament, and this balm was used in Ancient Greece for the treatment of sores and ulcers (Fearnley, 2001).

The antimicrobial properties of propolis have been attributed to its high flavonoid content and particular the presence of the flavonoids galangin and pinocembrin (Cushnie and Andrew, 2005). The herbal medicine has been used systemically and topically for thousands of years in China for the treatment of periodontal abscesses and infected oral wounds.

4.3 Antibacterial activity of flavonoids

4.3.1 Reports of flavonoids possessing antibacterial activity

Crude extracts from plants with a history of use in folk medicine have been screened in vitro for antibacterial activity by many research groups. Flavonoid-rich plant extracts from species of Hypericum, Capsella and Chromolaena (El-Abyad, 1990) have been reported to possess antibacterial activity. Many other phytochemical preparations with high flavonoid content have also been reported to exhibit antibacterial activity (Tereschuk, 1997).

Some researchers have reported synergy between naturally occurring flavonoids and other antibacterial agents against resistant strains of bacteria. Examples of these include epicatechin gallate and sophoraflavanone G. At least one

group has demonstrated synergy between flavonoids with antibacterial activity (Arima *et al*., 2002). Others have synthetically modified natural flavones and analyzed them for antibacterial activity (Stapleton, 2004).

4.3.2 Structure activity relationship for antibacterial activity of flavonoids

Although there have been comparatively few studies on the mechanisms underlying flavonoid antibacterial activity, information from published literature indicates that different compounds within this class of phytochemicals may target different components and functions of the bacterial cell (Mori *et al*., 1987; Haraguchi *et al*., 1998; Tsuchiya and Iinuma, 2000). If this is the case, it is surprising that the small number of groups have investigated the relationship between flavonoid structure and antibacterial activity which have been able to identify common structural features among active compounds. However, it may be that individual antibacterial flavonoids have multiple cellular targets, rather than one specific site of action. Alternatively, these common structural features may simply be necessary for flavonoids to gain proximity to or uptake into the bacterial cell.

Tsuchiya and colleagues (1996) attempt to establish a structure activity relationship for flavanones by isolating a number of differently substituted compounds and determining their MICs against MRSA. Their study indicated that 2', 4' - or 2', 6' -dihydroxylation of the B ring and 5, 7-dihydroxylation of the A ring in the flavanone structure was important for anti-MRSA activity.

A more recent paper (Alcaraz, 2000) also reports the importance of a hydroxyl group at position 5 of flavanones and flavones for activity against MRSA, supporting the earlier findings of Tsuchiya *et al*. (1996). It further states that chalcones are more effective against MRSA than flavanones or flavones, and that hydroxyl groups at the 2' position are important for the anti-staphylococcal activity of these compounds. The importance of hydroxylation at the 2' position for antibacterial activity of chalcones is supported by earlier work from Sato and colleagues, who found that 2, 4, 2'-trihydroxy-5'-methylchalcone and 2, 4, 2' trihydroxy chalcone inhibited the growth of 15 strains of cariogenic streptococci (Sato *et al*., 1997).

4.4 Antibacterial mechanisms of action of various flavonoids

4.4.1 Inhibition of nucleic acid synthesis

Using radio active precursors, Mori and colleagues have shown that DNA synthesis is strongly inhibited by flavonoids in *Proteus vulgaris*, whilst RNA synthesis is most affected in *S. aureus*. Protein and lipid synthesis are also affected but to a lesser extent. The authors suggested that the B ring of the flavonoid may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases and that this may explain the inhibitory action on DNA and RNA synthesis (Mori *et al.*, 1987).

The authors proposed that the observed antibacterial activity of the flavonoids is due in part to their inhibition of DNA gyrase. However, since the level of antibacterial activity and enzyme inhibition do not always correlate, they also suggested that other mechanisms are involved (Ohemeng *et al.*, 1993).

4.4.2 Inhibition of cytoplasmic membrane function

A research team has been found sophoraflavanone G to have intensive antibacterial activity against MRSA and streptococci recently reported attempts to elucidate the mechanism of action of this flavanone. The effect of sophoraflavanone G on membrane fluidity was studied using liposomal model membranes. At concentrations corresponding to the MIC values, sophoraflavanone G has been shown to increase fluorescence polarization of the liposomes significantly. These increases indicate an alteration of membrane fluidity in hydrophilic and hydrophobic regions, suggesting that sophoraflavanone G reduce the fluidity of outer and inner layers of membranes. Naringenin also exhibits a membrane effect but at much higher concentrations. This correlation between antibacterial activity and membrane interference is suggested to support the theory that sophoraflavanone G demonstrates antibacterial activity by reducing membrane fluidity of bacterial cells (Tsuchiya and Iinuma, 2000).

Ikigai and colleagues (1993), carried out research on (-)-epigallocatechin gallate, a strongly antibacterial catechin have found in green tea. In their study, liposomes are used as model bacterial membranes, and it is shown that epigallocatechin gallate induced leakage of small molecules from the intraliposomal space. Aggregation is also noted in liposomes treated with the compound. The

researcher group therefore concludes that catechins primarily act on and damage bacterial membranes. First, catechins may perturb the lipid bilayers by directly penetrating them and disrupting the barrier function. Alternatively, catechins may cause membrane fusion, a process that results in leakage of intramembranous materials and aggregation.

In an investigation into the antimicrobial action of propolis, Mirzoeva and colleague (1997) shows that one of its constituent flavonoids, quercetin, causes an increase in permeability of the inner bacterial membrane and a dissipation of the membrane potential.

4.4.3 Inhibition of energy metabolism

Haraguchi and colleagues (1998) recently carried out an investigation into the antibacterial mode of action of two retrochalcones (licochalcone A and C) from the roots of *Glycyrrhiza inflate*. These flavonoids demonstrate inhibitory activity against *S. aureus* and *M. luteus* but not against *E. coli*, and in preliminary tests licochalcone A inhibits incorporation of radioactive precursors into macromolecules (DNA, RNA and protein). The group hypothesized that the licochalcones may be interfering with energy metabolism in a similar way to respiratory-inhibiting antibiotics, since energy is required for active uptake of various metabolites and for biosynthesis of macromolecules (Mori *et al*., 1987).

Merck Research Laboratories recently reported that the flavanone lonchocarpol A inhibits macromolecular synthesis in *Bacillus megaterium*. Using radioactive precursors, it is demonstrated that RNA, DNA, cell wall and protein synthesis are all inhibited at concentrations similar to the MIC value (Salvatore *et al*., 1998). This may represent another example of a flavonoid that interferes with energy metabolism.

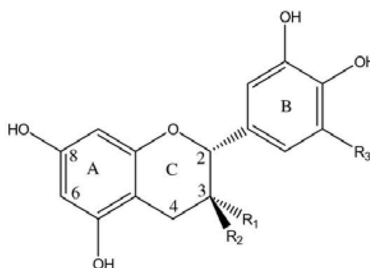
5. Phytochemistry of tamarind seed-coat extract

The tamarind seed-coat extract have been reported containing main flavonoid component such as monomeric flavonoid; (+)-catechin, (-)-epicatechin and oligomeric flavonoid; procyanidin B2, procyanidin trimer, procyanidin tetramer, procyanidin pentamer and procyanidin hexamer (Sudjaroen *et al*., 2005)

Flavonoids are a group of heterocyclic organic compounds that occur widely in the plant kingdom (Havsteen, 1983). They are found in fruits, vegetables, nuts, seeds, stems, flowers as well as tea, wine (Middleton and Chithan, 1993) and honey (Grange and Davey, 1990) and represent a common constituent of the human diet (Harborne and Baxter, 1999). Some of their reported properties include antioxidant, enzyme inhibitory, anti-inflammatory, vascular, oestrogenic, cytotoxic, antitumour and antimicrobial activities.

5.1 Proanthocyanidin

Proanthocyanidins are oligomers and polymers of flavan-3-ols monomer units (Figure 5.), which are (+)-catechin and (-)-epicatechin in the case of procyanidins. The simplest procyanidins are dimeric and the most common of these are the four 4-8 linked dimers



R_1 = OH; R_2 = H; R_3 = H; EC(Epicatechin)

R_1 = H; R_2 = OH; R_3 = H; Catechin

Figure 5. Structure of flavan-3-ols unit.

Proanthocyanidin occur widely in the plant kingdom and are considered the second most abundant group of natural phenolics after lignin. In relation to their function in plants, it has been postulated that they accumulate in many different organs and tissues to provide protection against predation. The presence of these compounds in foods (e.g., cereals, fruits, vegetables, wines, etc.) affects their texture, color, and taste (Santos-Buelga and Scalbert, 2000).

The multiple phenolic hydroxyl groups of procyanidins may form complexes with proteins and induce antioxidation properties. The former ability endows procyanidins astringency in taste, antibacterial properties.

The B-type procyanidins include a mixture of oligomers and polymers composed of flavan-3-ol units, (+)-catechin and (-)-epicatechin, linked mainly through C4-C8 and/or C4-C6 bonds, and represent the dominant class of natural proanthocyanidins. Among the dimers, procyanidins B1, B2, B3 and B4 (Figure 7a.) are the most frequently occurring in plant tissues.

Analogues of procyanidin B1 and B2 exhibiting eipicatechin chain extension units (2R, 3R-2,3-*cis* configuration) are very commonly represented in the plant kingdom, whereas many plants also produce analogues of procyanidin B3 to B8 (Figure 7b.).

Procyanidins have been reported various bioactivities in vivo, including cardio vascular protection, low-density lipoprotein oxidation inhibition, anti-inflammation, antitumor proliferation, antibacterial and so forth. Therefore, procyanidins are believed to play an important role in human health.

5.2 Catechin and epicatechin

Catechin and epicatechin belong to the class of a flavan-3-ols which has been isolated from a variety of natural sources including leaf, seed and fruit (Robards and Antolovich, 1997; Manning and Roberts, 2003). So, infection in plants can result in more synthesis of catechin, and epicatechin in plant tissue that might cause to more resistant to plant pathogenic microbe (Hakulinen, 1998; Yamamoto *et al*., 2000). Additional, catechin and epicatechin showed antioxidant, antimicrobial, antitumour, antibacterial and antifungal effects (Nanjo and Goto, 1996; Heinonen, 1998; Menon and Kuttan, 1999; Meyer and Wcyant *et al*., 2001; Hirasawa and Takada, 2004; Baidez *et al*., 2006).

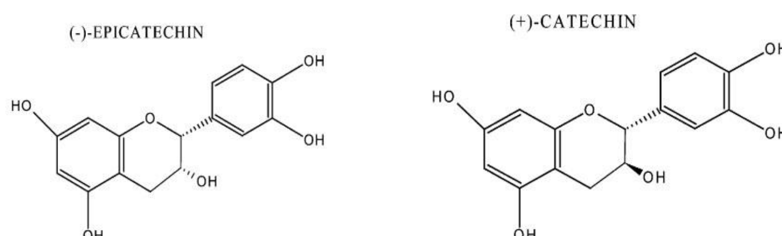
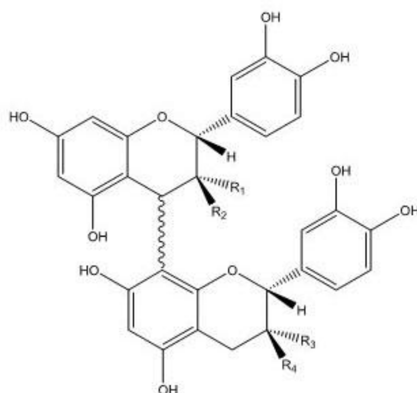


Figure 6. Structures of epicatechin and catechin (Note: the 2, 3-*cis* stereochemistry of (-)-epicatechin and the 2, 3-*trans*-stereochemistry of (+)-catechin)

a.



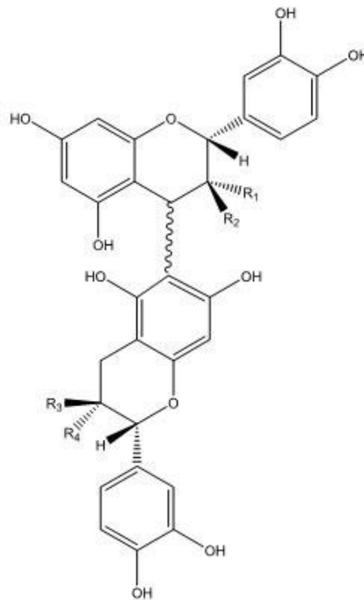
Procyanidin B1: R1=OH; R2=H; R3=H; R4=OH

Procyanidin B2: R1=OH; R2=H; R3=OH; R4=H

Procyanidin B3: R1=H; R2=OH; R3=H; R4=OH

Procyanidin B4: R1=H; R2=OH; R3=OH; R4=H

b.



Procyanidin B5: R1=OH; R2=H; R3=H; R4=OH

Procyanidin B6: R1=H; R2=OH; R3=OH; R4=H

Procyanidin B7: R1=OH; R2=H; R3=OH; R4=H

Figure 7. Structures of some proanthocyanidin dimers of the B-type

6. Antimicrobial of catechin, epicatechin and proanthocyanidin

Extracts of green tea have been shown to inhibit the growth of *S. mutans* in vitro (Sakanaka *et al*., 1989; Yoshino *et al*., 1995) and prevent its attachment to tooth enamel (Sakanaka *et al*., 1989). These activities are probably associated with catechins (Hamilton-Miller, 2001).

Grape seed extract contains 95% phenolic compounds. The product information sheet stated that not less than 80% of the phenols are oligomeric flavonoid whilst monomeric flavonoid (catechin, epicatechin and epigallo-catechin) made up 10-15% of the extract. The results show that polyphenol-containing extracts inhibit *S. mutans*. The flavan-3-ol and procyanidin oligomer content of grapes can vary with the variety, degree of maturity and part of the fruit studied. The skins of red and white grapes contain higher concentrations of catechin and epicatechin whilst grape seeds contain higher concentrations of oligomeric procyanidins (Macheix *et al*., 1990).

The water extracts obtained from *Cocos nucifera* husk fiber (containing catechin, epicatechin and B-type proanthocyanidins) reveal a pronounced antimicrobial activity against strains of the species *S. aureus* (Esquenazi *et al*., 2002).

Chung *et al*. (1998) reported that proanthocyanidins exhibit the growth inhibition of strains of *Aeromonas* spp., *Bacillus* spp., *Clostridium botulinum*, *Clostridium perfringens*, *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Pseudomonas* spp., *Shigella* spp., *S. aureus*, *Streptococcus* spp.,and *Vibrio* spp.

Amarowicz *et al*. (2000) showed that proanthocyanidins of green tea extracts have antibacterial activity against *E. coli*. Puupponen-Pimia *et al*. (2001) studied the antimicrobial activity of eight berry extracts against selected Gram-positive and Gram-negative bacteria, including probiotic bacteria and intestinal pathogens. Puupponen-Pimia *et al*. (2001) suggested that proanthocyanidins might be the class of compounds responsible for antibacterial activity.

The antibacterial activity of proanthocyanidins is also observed with regard to bacterial groups of veterinary interest. Purified proanthocyanidins from three different plants (*Schinopsis balansae*, *Desmodium ovalifolium*, and *Mirtus communis*) are investigated as antimicrobial compounds towards ruminal bacteria of the species *Streptococcus bovis*, *Ruminococcus albus*, *Fibrobacter succinogenes*, and *Prevotella*

ruminicola. Proanthocyanidins from *D. ovalifolium*, and *M. communis* (characterized by a differential polymerization degree) exhibit the highest antimicrobial activity for this study (Nelson *et al*., 1997).

6.1 Antimicrobial mechanisms of catechin, epicatechin and proanthocyanidin

Ikigai *et al*. (1992) investigated the mechanism of the bactericidal properties of catechins, they studied the action of catechins on liposomal and bacterial membranes. To test the interaction of catechins with the liposome membrane, they have determined the fluorescence emission of a lipophilic fluorescence probe, in the preloaded membranes. Tea, more precisely the aqueous extract of tea leaves, had been shown to have antibacterial, antiviral and protein denaturating activities in vitro. These biological activities of tea extracts are attributable to the presence of polyphenol compounds, collectively termed catechins. This study revealed that bactericidal catechin causes leakage of intramembranous materials, and aggregation of the liposomes. Therefore, it is likely that the catechins interact with the membrane and perturb the lipid bilayers possibly by the catechin directly penetrating the lipid bilayer and disrupting the barrier function. It is suggested that catechins may change the membrane fluidity.

Additinal the mechanism of action of catechins, the studies have been carried out on *S. aureus* and *E. coli* cells by Ikigai *et al*. (1993), they reported that their bactericidal effect is primarily involved in the damage of bacterial membranes: catechins induce a rapid leakage of small molecules entrapped in the intraliposomal space, determining the aggregation of the liposomes. These actions cause damage in the membrane lipid bilayer and cell death.

For proanthocyanidins, Jones *et al*. (1994) postulated that their ability to bind bacterial cell coat polymers and their ability to inhibit cell-associated proteolysis might be considered responsible for the observed activity. Accordingly, despite the formation of complexes with cell coat polymers, proanthocyanidins penetrate to the cell wall insufficient concentration to react with one or more ultra structural components and to selectively inhibit cell wall synthesis. Additional hypotheses for their mechanism of action have more recently been proposed. It is well known that proanthocyanidins are able to complex metals through their *ortho*-diphenol groups.

This property is often viewed as imparting negative traits (e.g., reduction of the bioavail ability of essential mineral micro nutrients, especially iron and zinc). Since iron depletion causes severe limitation to microbial growth, their ability to bind iron has been suggested as one of the possible mechanisms explaining the antimicrobial activity of proanthocyanidins (Dixon *et al*., 2005).

Shan *et al*. (2007) studied about barks of *Cinnamomum* plants also contain condensed tannins, that is, dimeric, trimeric, and higher oligomeric proanthocyanidins. This study suggests that the active components of the extracts might bind to the cell surface and then penetrate to the target sites, possibly the phospholipid bilayer of the cytoplasmic membrane and membrane-bound enzymes. The effects might include the inhibition of proton motive force, inhibition of the respiratory chain and electron transfer, and inhibition of substrate oxidation. Uncoupling of oxidative phosphorylation, inhibition of active transport, loss of pool metabolites, and disruption of synthesis of DNA, RNA, protein, lipid, and polysaccharides might follow.

7. Antimicrobial susceptibility tests

The primary purpose of antimicrobial susceptibility test is to guide the clinician the choice of appropriate agents for therapy. The test is also provided accumulating data from which information on the suitable agents for empirical use can be derived. Antimicrobial susceptibility tests are use to evaluate an *in vitro* activity of new agents (Collin *et al*., 1995)

In vitro antimicrobial susceptibility tests are depended on two roles, diffusion and dilution. Laboratory procedures involving diffusion susceptibility tests are commonly performed in agar media called agar diffusion technique.

7.1 Agar diffusion susceptibility test

In general, agar diffusion tests are performed by inoculating a nutrient agar medium in standardized manner and then applying the drug to be studied to the agar surface in some type of reservoir. The drug is allowed to diffuse into the surrounding medium. This exposes the test organism to a continuous gradient of drug concentrations, with concentration diminishing as distance from the reservoir increase. After an appropriate period of incubation, there should be a zone of inhibited

growth around the reservoir. The size of zone may be measured to determine the degree of susceptibility of test organism (Lorian, 1991).

These tests depend on the ability of the antimicrobial agents to diffuse at predictable rates though the agar gel. The concepts have led to a better understanding of variables that influence the formation of a zone inhibition in an agar medium. The more important variable influence diffusion test is described below.

7.1.1 Application of the drug solution to agar medium

Solution of antimicrobial agents may be applied to surface of a seeded agar medium in several different ways; filter paper disks may be saturated with drug and applied to the surface of the agar; metal cylinders may be applied to the agar surface and then filled with the drug solution; wells may be cut from the seeded agar and then filled with drug solution (Lorian, 1991). The convenient method uses filter paper disk that has been moisten with the drug solution and then applied directly on to the agar while still wet. Disks may be prepared more accurately if a micropipette is use to load each disk with a measured volume of drug solution.

Alternatively, glass or cylinders may be applied to the surface of the seeded agar medium to facilitate application of drug solutions. Agar well may be cut from the seeded agar medium by using a hollow tube 4 to 6 mm in diameters and then be filled with the drug solutions.

About diffusion, the drug diffuses in a two-dimensional manner. In the relatively thin layer of agar normally poured into an agar plate, downward diffusion is quite limited and the concentration in the depth of medium soon approached that near the surface. The amount of drug available to diffuse outward is greater and consequently the zone of inhibition is likely to be larger. In a very thick pour plate, the three dimensional diffusion can result in a semicircular zone of inhibition, being small at the bottom and larger at the surface.

7.1.2 Inoculum density

Inocula may be also be prepared directly by inoculating colonies grown overnight or agar plate or slant into broth or saline. This direct inoculum suspension preparation technique, which does not require incubation, but the use of fresh (Mahon and Manuselis, 2000).

The number of organism tested must be standardizing regardless of the method used. The most widely use method of inoculum standardization involves McFarland turbidity standard and 0.5 McFarland standard (10^8 CFU/ml) is the most commonly used (Lorain, 1991). False susceptible results may be occur if too few or too many organisms. Increasing inoculum size reduce the susceptibility to agents in both diffusion and dilution test. In diffusion method, heavy inocula tend to give small zone of inhibition and when inoculum is light, organism often appear to be susceptible and give large zone. Consequently, minor changes in inoculum density result in extreme changes in the result of susceptibility test.

7.1.3 Agar depth

The depth of agar medium in susceptibility testing, the agar medium is usually poured to a depth of about 4 mm (18-25 ml in a 9 cm petri-dish), and at that level fairly major variations in agar depth do not significantly affect the test result. With diffusion tests the size of the zone increases as the depth of the agar decrease but this effect is most marked with very thin plates. When working with very thin agar layers, extreme caution must be taken to avoid minor variations in the depth of the agar medium in different areas of the same plate.

7.1.4 Composition of the agar medium

The agar medium itself profoundly influences the zone sizes in three ways

- It affects the activity of different antimicrobial agents.
- It influences the rate of diffusion of the antimicrobial agents.
- It affects the growth rate of the test organism.

For susceptibility testing, the composition of test medium must be carefully standardized because the activity of antimicrobial agent is markedly influenced by many factors such as cation in the medium, pH of the medium and presence of various antagonistic materials.

The ideal medium should have sufficient nutrients to support growth of organism normally tested. Mueller-Hinton (MH) medium is the most widely recommended for bacterial susceptibility test (Barry *et al.*, 1978; Modugno *et al.*, 1997)

Recently, Mueller-Hinton medium has been shown to be acceptable for a wide variety of antimicrobial susceptibility tests because of the relatively good reproducibility and simplicity being regulated to a reference medium (NCCLS, 1986). MH medium is well suited for standard rapid growing pathogen such as enteric gram-negative bacilli, *Pseudomonas* spp., *Staphylococci* spp. and *Enterococcus* sp. For bacteria that do not grow readily on this medium other supplements or alternative media may be required such as MHA supplemented with 5% defibrinate blood sheep as described in NCCLS (1993) for *S. pneumoniae* and Haemophilus species for rapid grow in the susceptibility test medium.

7.1.5 Preincubation and Prediffusion

In diffusion tests preincubation and prediffusion decrease and increase the sizes of zone, respectively. For susceptibility testing, the agar media is inoculated and then allowed to dry for a defined period of time before the disks are applied. This drying step is essential to prevent leaching of the antimicrobial agent from disk into the layer of moisture that may be left immediately after incubation of the agar medium. However, as the plates are allowed to dry, the microorganism are beginning the growth cycle, and the time allowed for the antimicrobial agent to diffuse through the agar medium will be diminished. Some procedures require a specific period of prediffusion at room temperature after the antimicrobial agent to diffuse through the agar gel before the critical cell mass is reached. This prediffusion period tends to prolong the lag phase of microbial growth and thus increase the critical time during which the position of the zone of inhibition is determined (Lorian, 1996).

7.1.6 Incubation

Susceptibility tests are normally incubated at 35-37 ºC for optimal growth of the common human pathogens. Use alternative conditions only, if essential for growth of the organisms such as, most fungi can grow well at temperature range 30-35 ºC. The rate of growth will be prolonged at lower temperatures and thus the critical concentration of the antimicrobial agent has more time to diffuse further. To further complicate the situation, most antimicrobial agents diffuse more slowly at lower temperatures, partially because of the increased viscosity of the agar medium (Lorian, 1996). Normally, the effect of decrease growth rates predominates and the zone increase with lower temperatures.

Most of in vitro tests with antimicrobial agents have been standardized with "overnight" incubation period, usually defined as 16-18 hr (Isenberg, 1998). With the exception of some organisms, anaerobic bacteria most commonly incubated for 48 hr. (Lorian, 1991). The incubation zone may be come smaller with further incubation because of changes in character of the growth at the edge of the zone. The character of the zone edge will change as a result of (a) the appearance of delayed growth, (b) better visualization of partially inhibition growth or (c) delayed appearance of resistant variants. Occasionally, the zones appear to increase in size because of change in character of the growth at the zone edge or because of actual lysis of the initial growth within the inner ring of the zone.

7.2 Dilution susceptibility test

Dilution antimicrobial susceptibility test method are used to determine quantitatively in term of the minimum inhibitory concentration (MIC), the lowest concentration of antimicrobial agent required to inhibit the growth of an organism isolate or that which kill it, the minimum bactericidal (fungicidal) concentration (MBC, MFC) (Mahon *et al*., 2000). The NCCLS (1997) document described the details of performing MIC and MBC tests by broth macrodilution, broth microdilution and agar dilution.

7.2.1 Broth macrodilution susceptibility test

Broth dilution tests performed in test tube and most suitable for small numbers of tests (Collin *et al*., 1995). Additionally, this method is often used when minimum bactericidal concentration (MBC) endpoints are to be subsequently determined.

Generally, a twofold serial dilution is prepared. Mueller-hinton broth is medium most commonly used for MIC test of fastidious bacteria, blood supplemented may be added to broth for fastidious organisms as for agar media and sabouraud dextrose broth is commonly used for fungi. A standardize suspension of test bacteria and yeast are added to each dilution to obtain final concentration of $5x10^5$ CFU/ml (Mahon *et al*., 2000) and 10^5 CFU/ml (Lorian, 1996), respectively. A growth control tube (broth plus inoculum) and an uninoculated control tube (broth only) are use in

each test. After incubation, the MIC is determined visually as the lowest concentration that inhibits growth, as demonstrate by the absence of turbidity (NCCLS, 1997).

7.2.2 Broth microdilution susceptibility test

Recently, microdilution test commonly used in the susceptibility test as recommended in many studies (Luh *et al*., 2000). The broth microdilution method is an adaptation of the broth dilution method using small volumes for routine testing. It utilizes microtiter plastic plates containing 96 wells. The advantage of the system is that it utilizes small volumes of sample and allows a large number of bacteria to be tested relatively quickly.

Several factors influence the outcome and reproducibility of broth susceptibility results. Most factor, media supplements, pH, incubation and inoculum size are likely affected as described in agar diffusion test.

7.2.3 Agar dilution susceptibility test

The agar dilution method for determining MIC has been accepted as the standard against which other methods are assessed. It has advantages over broth dilution methods in that concentration is more easily seen and reisolation of the required organism is usually not a problem. However, this technique could not determined MBC or MFC likely in dilution techniques.

To determine the MIC for one or more organism isolates, the antimicrobial solution may be incorporated into a liquefied agar medium (45 to 50 ºC), which is then mixed, poured into standard petri-dishes, and allow to solidify. A series of petri-dishes are prepared with increasing concentrations of each antimicrobial agent and growth control plates without antimicrobial agent are prepared. After agar is allowed to solidify, then a standard number of test microbe (10^4 CFU/ml for aerobic bacteria) are spot inoculated into each plate using a replicating device, such as steer's replicator. After overnight incubation the MIC is read as the lowest concentration of antimicrobial agent that inhibits the visible growth of the test microbe (one or two colonies are ignored) (Mahon *et al*., 2000).

However, broth dilution tests are usually preferred for studying the antimicrobial activity of antimicrobial agents because subcultures can be made easily at different time intervals.

8. Types of preparation

8.1 Suspension

Suspensions may be defined as preparations containing finely divided drug particle distributed somewhat uniformly throughout a vehicle in which the drug exhibits a minimum degree of solubility. Some suspensions are available in ready-to-use form, that is, already distributed thought a liquid vehicle with or without stabilizers and other additives.

8.2 Cream

Creams are opaque, soft solids or thick liquids consisting or mediations that are dissolved or suspended in water-removable or emollient base. Creams are usually applied to moist, weeping lesions because they have a somewhat drying effect in that lesions' fluids are miscible with the aqueous external phase of creams (Allen *et al.*, 2004)

8.3 Lotions

Lotions are fluid emulsions or suspensions designed for external application to skin. Most lotions contain finely powdered substances that are insoluble in the dispersion medium and are suspended thought the use of suspending agents and dispersing agent (Allen, 2002).

8.4 Gel (Zatz and Kushla, 1996)

The United States Pharmacopeia (USP) defines gels as semisolids, being either suspension of small inorganic particles or large organic molecules interpenetrate with liquid. Gels are transparent or translucent semi-solid or solid preparations, consisting of solutions or dispersions of one or more active ingredients in suitable hydrophilic or hydrophobic bases. They are made with the aid of a suitable gelling agent. Usually gels exhibit pseudoplastic flow properties and those made with synthetic or semi-synthetic polymers with a high degree of cross-linking have relatively high yield values and low viscosity. Gels are often non-greasy and are generally applied externally. As vehicles for the presentation of water-soluble medicaments, gels are ideal because of their high water content. Products tend to be

smooth, elegant, and produce cooling effects because of evaporation of water; they may also dry out to form films. Ideally, gelling agents for pharmaceutical and cosmetic use should be inert, safe, and nonreactive with other formulation components. A potential incompatibility is illustrated by the combination of a cationic drug, preservative, or surfactant with an anionic gel former. Inactivation or precipitation of the cationic substance is possible.

The gel should exhibit little viscosity change under the temperature variations of normal use and storage. This minimizes unacceptable changes in the product's characteristics. Many gels, particularly those of a polysaccharide nature are susceptible to microbial degradation. Incorporation of a suitable preservative may prevent contamination and subsequent loss of gel characteristics due to microbial attack. The gel characteristics should match the intended use. A topical gel should not be tacky. Too high a concentration of gel former or the use of an excessive molecular weight may produce a gel difficult to dispense or apply.

8.4.1 The characteristic of gels

Swelling

Gels can swell absorbing liquid with an increase in volume. Swelling can be looked on as the initial phase of dissolution. Solvent penetrates the gel matrix. Gel-gel interactions are replaced by gel-solvent interactions. Limited swelling is usually the result of some degree of crosslinking in the gel matrix that prevent total dissolution. Such gels swell considerably when the solvent mixture possesses a solubility parameter comparable to that of the gallant.

Syneresis

Many gel systems undergo a contraction upon standing. The interstitial liquid is expressed, collecting at the surface of the gel. This process is referred to as syneresis. Syneresis is not limited to organic hydrogels but has been seen in organogels and inorganic hydrogels as well. Typically, syneresis becomes more pronounce as the concentration of polymer decreases.

The mechanism of concentration has been related to the relaxation of elastic stresses developed during the setting of the gel. As these stresses are relieved, the interstitial space available for solvent is reduced, forcing the expression of fluid. Osmotic effects have been implicated, as both pH and electrolyte concentration

influence syneresis from gels composed of the ionic gel formers gelatin or psyllium seed gum.

Viscosity (Rao, 1999, Ramachandran, *et al*., 1999, Tantry, *et al*., 2001)

Viscosity is the measure of the internal friction of a fluid. This friction becomes apparent when a layer of fluid of made to move in relation to another layer. Viscosity is best determined using geometric in which the shear rate can be calculate from experiment data. Shear rate (γ) is the velocity gradient established in a fluid as a result of an applied shear stress (σ). It is expressed in units of reciprocal seconds, s^{-1}. Shear stress is the stress component applied tangentially. It is equal to the force factor (a vector has both magnitude and direction) divided by the area of application and is expressed in force per unit area (Pa). Viscosity is denoted by the symbol η for Newtonian fluids, whose viscosity does not depend on the shear rate, and for non Newtonian fluids to indicate shear rate dependence by η_a. Depending on the flow system and choice of shear rate and shear stress, there are several equations to calculation. Here, it is defined by the equation:

$$\text{Viscosity } (\eta_a) = \frac{\text{shear stress } (\sigma)}{\text{shear rate } (\gamma)}$$

The SI unit (International System of Units) of viscosity is the pascal-second (Pa·s) and the ASTM (American Society for Testing And Materials) standards, as centipoise (cP) (1 cP = 1 mPa·s = 0.001 Pa·s). The fluids flow behavior can be described by mean of shear rate versus shear stress.

9. Preparation of natural product for external use

Phytopreparation are widely used for the treatment of various diseases of skin. The rational combination of phytopreparations with appropriate auxiliary substances and the use of optimum technological schemes in the production of soft medicinal forms for external use (ointment, gels, creams, and liniments) provide for a significant increase in the quality and therapeutic efficacy of phytopreparations intended for the treatment and prophylaxis of various disorders in dermatology as dermatocosmatic, gynecology, dentistry, and cosmetology (Semkina, 2005). Normally, the constituents that have been used in preparation for skin are the following:

9.1 Gelling agent

One of the most versatile delivery systems that can be compounded is the pharmaceutical gel. Gels are an excellent drug delivery system for various routes of administration and are compatible with many different pharmaceutical ingredients drug substances.

Carbomer (Polyacrylic Acid)

Carbomer xxxP is the official name given to one member of a group of acrylic polymers crosslinked with a polyalkenyl ether. Manufactured under the trade name of Carbopol 934P and 940P, it is used as thickening agent in a variety of pharmaceutical and cosmetic products. The suffix "P" identifies a highly purified polymer, suitable for use in orally administered dosage forms, although carbomer 934P, 940P is also used widely in topical preparations.

The acid form of this polymer can be dispersed in water to give a pH of 2.8-3.2, but it does not dissolve. Neutralization of the acid functionality with a base such as sodium, potassium, or ammonium hydroxide produces negatively charged carboxylate groups.

Solution of carbomer is very pseudoplastic. They exhibit a yield value. Due to the extreme shear-thinning, thick gels may be pumped easily. This shear-thinning has made it an excellent choice as a thickener in creams and lotions.

Carbomer forms gels at concentrations as low as 0.5%. In aqueous media, the polymer, which is marketed in the free acid form, is first uniformly dispersed. After entrapped air has been allowed to escape, the gel is produced by neutralization with a suitable base. The introduction of negative charges along the polymer chain causes it to uncoil and expand.

In aqueous systems, a simple inorganic base such as sodium, ammonium or potassium hydroxide, or a basic salt such as sodium carbonate may be employed. The pH should be adjusted to a neutral value; gel character will be adversely affected by either insufficient neutralization or excessive pH. Certain amines, such as triethanolamine, are sometimes used in cosmetic products.

Benefits of carbopol

Carbopol is very well suited to aqueous formulations of the topical dosage forms. Many commercial topical products available today have been

formulated with these polymers, as they provide the following numerous benefits to topical formulations:

- Safe and effective: Carbopol polymers have a long history of safe and effective use in topical gels, creams, lotions, and ointments. They are also supported by extensive toxicology studies.

- Non-sensitizing: Carbopol polymers have been shown to have extremely low irritancy properties and are non-sensitizing with repeat usage.

- No effect on the biological activity of the drug: Carbopol polymers provide an excellent vehicle for drug delivery. Due to their extremely high molecular weight, they cannot penetrate the skin or affect the activity of the drug.

- Excellent thickening, suspending, and emulsification properties for topical formulations

Products with a wide range of viscosities and flow properties have been successfully formulated and commercialized. Carbopol polymers are used to permanently suspend the active ingredients in transdermal reservoirs as well as in topical gels and creams.

Toxicological studies of carbopol

The Carbopol, like other high molecular weight polymers, demonstrate a low toxic and irritation potential based on their physical and chemical properties. Accordingly, such cross-linked, high molecular weight acrylic acid polymers have been found safe for use in a wide variety of cosmetics, detergents and pharmaceuticals by appropriate regulatory and non-regulatory bodies concerned with such products. Acute oral studies with rats, guinea pigs, mice and dogs showed that Carbomers- 910, -934, -940 and –941 have low toxicities when ingested.

9.2 Humectants

Propylene glycol has been used in a wide variety of pharmaceutical formulations and it is generally regarded as a nontoxic material. In topical preparations, propylene glycol is regarded as a nontoxic material. In topical preparations, propylene glycol is regarded as minimally irritant although it is more irritant than glycerin (Paphattarapong, 2005).

Glycerin is used primarily for its humectant and emollient properties, in topical pharmaceutical formulations and cosmetics. Glycerin may also be used be used in topical at concentrations up to 30% (Aoshima *et al*., 2005 and Rowe, 2003).

9.3 Emulsifier

Cremophor RH 40 or PEC-40 dehydrogenates caster oil, a non ionic emulsifier used for solubilizing essential oil in oil-in-water formulas.

Amerchol L-101 or Mineral oil and Lanolin alcohol is an oily liquid used in topical pharmaceutical formulations and cosmetics as an emulsifying agent with emollient properties. The emulsifier used in cosmetic at concentrations up to 20%w/w (Balzer, 1995).

9.4 pH adjustment agent

Triethanolamine (TEA) is an alkalizing agent in cosmetics. It is one of the best recognized alcoholamines. Complexation of triethanolamine with anionic polymers decreased its pH (Musial and Kubis, 2004).

9.5 Preservative

Formulations often contain a number of ingredients which readily support the growth of a variety of microorganisms. As a result, the inclusion of a preservative is necessary part of the formulation process. Several points must be kept in mind in selecting a preservative. Microbial contamination may occur during the development or production of an emulsion or during its use. Frequently, the microbial

contamination can arise from the use of impure raw materials of from poor sanitation during preparation. Prevention of contamination is recommended, and certain cardinal rules must be observed. The most important one is the use of uncontaminated raw materials, including the water. A second precaution is meticulous housekeeping and careful cleaning of equipment. Once a microbiologically uncontaminated product has been prepared, a relatively mild antimicrobial agent suffices to protect the product against chance contamination by microorganisms. It is also desirable that the preservative system be effective against invasion by variety of pathogenic organisms and adequate to protect the product during use by consumer.

Parabens

Parabens, alkyl esters of *p*-hydroxybenzoic acid, are a class of antibacterial agents, particularly useful against molds and yeasts. The compound can have multiple biological effects, but it is generally considered that their inhibitory effects on membrane transport and mitochondrial function processes are key for their actions. The parabens meet several of the criteria of an ideal preservative, in that they have a broad spectrum of antimicrobial activity, are safe to use (i.e. relatively non-irritating, non-sensitizing and non-poisonous), are stable over the pH range, and are sufficiently soluble in water to produce the effective concentration in aqueous phase. The series of parabens (including methyl, ethyl, heptyl and benzyl paraben), used singly or in combination to exert the intended antimicrobial effect.

Cosmetic uses of paraben

Propyl paraben and methyl paraben are the most commonly used preservatives in cosmetic. Parabens formulate well because they have no perceptible odor or taste, are practically neutral, do not produce discoloration, and do not cause hardening or “muddying” of the final product. The popular use of paraben preservatives in cosmetics and toiletries arises from their low toxicity, inertness, broad spectrum of activity, worldwide regulatory acceptance, biodegradability and low cost. Moreover, parabens have excellent chemical stability in relation to pH (effective between pH 4.5 and 7.5). The maximum concentration uses for paraben in pharmaceutical products was not greater than 1% (W/W), which is composed of 2% of propyl paraben and 20% of methyl paraben in propylene glycol (Soni *et al.*, 2001).

10. Stability testing of topical product

Gels should be observed for such physical characteristics as shrinkage, separation of liquid from the gel, discoloration. Many gels will not promote bacteria/mold growth, nor will they prevent it. Consequently, they should be autoclaved or should contain preservatives. The pharmacist should follow standard quality control procedures. These procedures involve checking the appearance, uniformity, weight/volume, viscosity, clarity, pH, and smell of the gels (Zatz and Kushla, 1996).

The Food and Drug Administration's (FDA) guide on stability testing of drug product provides a framework with which to plan a stability test:

Storage condition: Room temperature is considered. Samples remain until failure.

Test intervals: Initially, then every three months for the first year; then every six months for the second year; then annually thereafter.

Freeze-thaw cycling: One cycle of 24 hours of freezer set at 0 or -5 ºC followed by 24 hours at 45 ºC or room temperature.

Products can be placed at ambient, and elevated (e.g. 37 ºC and 45 ºC) temperatures, refrigerated and cycled through freeze/thaw cycles. Two/three months of successful evaluated temperature testing and three or four freeze/thaw cycles will usually indicate that products will have an adequate shelf-life.

11. High-performance liquid chromatography

High-performance liquid chromatography (or high-pressure liquid chromatography, HPLC) is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture.

This chromatography separation is based on the relative solubility's of the solutes in the mobile and stationary phases. There are two types of partition chromatography namely normal-phase and reverse phase chromatography.

Normal phase chromatography: It means the stationary phase is made of polar packing material while the mobile phase is of non-polar or low polarity solvents.

Commonly used polar stationary phase or column is packed with silica. Silica is relatively the most polar compound compared to all other packing materials. Examples of solvents used to make up a normal phase mobile phase are hexane, dichloromethane, chloroform, ethyl ether, and isopropyl alcohol (IPA). In a normal phase application, the non-polar compounds will be eluted faster than the polar compounds.

Reverse phase chromatography: The principle is opposite of normal phase system, where the stationary phase is packed non-polar material and the mobile phase is polar. Commonly used packing material in reverse phase columns are silica linked with carbon-18 (C18). The mobile phase for a reverse phase system usually consists of water or buffer solution, methanol, acetonitrile and isopropyl alcohol (IPA). IPA can be used in both reverse and normal phase as it is miscible with water as well as water immiscible solvents. In a reverse phase, the non-polar compounds are retained in the column longer than the polar compounds. In another words, the polar compounds elute faster than the non-polar compounds.

The main components of an HPLC system are a high-pressure pump, a column and an injector system as well as a detector. The system work as shown in Figure 7: eluent is filtered and pumped through a chromatographic column, the sample is loaded and injected onto the column and the effluent is monitored using a detector and recorded as peaks.

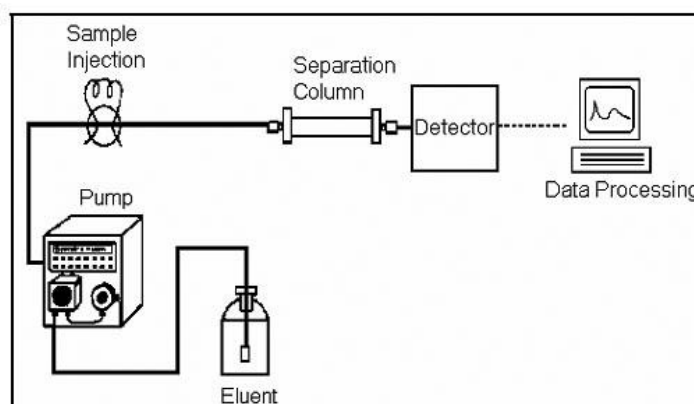


Figure 8. Diagram of HPLC instrument

12. Analysis of phenolic and flavoniod compounds by HPLC technique

In the last twenty years, HPLC has been the analytical technique that has dominated the separation and characterization of phenolic compounds. Several reviews have been published on condition of HPLC methodologies to analysis of phenolic compounds:

12.1 HPLC columns

The introduction of reversed-phase (RP) columns has considerably enhanced HPLC separation of different classes of phenolic compounds. Almost exclusively, RP C_{18} phases ranging from 100 to 250 mm in length and usually with an internal diameter of 3.9 to 4.6 mm are employed. Particle sizes are in the usual range of 3-10 µm. Most HPLC analyses of phenolic compounds are performed at ambient column temperature, but moderately higher temperatures between 30 and 40 ºC have also been recommended.

12.2 HPLC mobile phases

Both isocratic and gradient elution are applied for analyses of phenolic compounds. The choice depends on the number and type of the analytes and the nature of the matrix. Acetonitrile and methanol are the most commonly used organic modifiers. In some cases, acetonitrile leads to better resolution in a shorter analysis time than methanol and, generally, acetonitrile gives sharper peak shapes, resulting in a higher plate number. However, methanol is often preferable to acetonitrile because of its non toxic properties and the possibility of using higher percentages in the mobile phase which could protect the HPLC column. Occasionally, tetrahydrofuran and 2-propanolas less polar solvents with their high elution strength have also been used.

The recommended pH range for the HPLC assay is pH 2-4. The pH value is controlled by adding small amounts of acids to the water organic mixture. Aqueous acidified solvents such as acetic, formic, phosphoric, and most rarely perchloric acid were employed.

Generally, phenolic acids are eluted from RP columns according to decreasing polarities. The loss of polar hydroxyl groups and the presence of the methoxy groups or ethylene side chains could decrease the polarity and increase the retention time.

The derivatives of common phenolic acids with two or more aromatic rings are less polar and are eluted much later than others. So, the gradient program has to be managed in a case dependent manner, according to the number and chemical properties of the analyzed compounds.

12.3 HPLC detection

Phenolics are commonly detected using ultraviolet/visible (UV/VIS), photodiode array (PDA), and UV-fluorescence detectors. Other methods used for the detection of phenolics include electrochemical coulometric array detection, on-line connected PDA and electro array detection, chemical reaction detection techniques, mass spectrometric and NMR detection.

All flavonoid contain at least one aromatic ring and, consequently, efficiently absorb UV light. The first maximum, which is found in the 240-285nm range, is due to the A-ring and the second maximum, which is in the 300-550 nm range, is attributed to the substitution pattern and conjugation of the C-ring.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals

Chemical	Grade	Supplier/manufacturer
Acetic acid	analytical reagent grade	Fisher Scientific, U.K.
Amphotericin B	HPLC grade	Sigma Chemical Co. Ltd., U.S.A.
Anaropack	microbiological grade	Mitsubishi gas chemical Co., Inc., Japan
Anaerobic indicator	microbiological grade	Oxoid Ltd., U.K.
Brain heart infusion agar	microbiological grade	HiMedia, India
Brain heart infusion Broth	microbiological grade	HiMedia, India
Carbopol940	pharmaceutical grade	S. Thong Chemicals Co., Ltd., Thailand
(+)-catechin	HPLC grade	Sigma Chemical Co. Ltd., U.S.A.
Chloroform	analytical reagent grade	Fisher Scientific, U.K.
Clindamycin HCL	HPLC grade	The Department of Medical Sciences, Ministry of Public Health, Thailand.
1% clindamycin gel	pharmaceutical grade	Union Drug Laboratories, Ltd., Thailand
Ethanol	analytical reagent grade	Fisher Scientific, U.K.
(-)-epicatechin	HPLC grade	Sigma Chemical Co. Ltd., U.S.A.
Gentamicin sulfate	pharmaceutical grade	Sigma Chemical Co. Ltd., U.S.A.
Luteolin	HPLC grade	Sigma Chemical Co. Ltd., U.S.A.
Methyl paraben	pharmaceutical grade	S. Thong Chemicals Co., Ltd., Thailand
Methanol	HPLC reagent grade	Fisher Scientific, U.K.
Mueller Hinton Agar	Microbiological grade	Merck, Germany
Mueller Hinton Broth	Microbiological grade	Merck, Germany
%5 benzoyl peroxide gel	pharmaceutical grade	Stiefel Laboratories(Pte) Ltd., Singapore
Propylene glycol	pharmaceutical grade	S. Thong Chemicals Co., Ltd., Thailand
Propyl paraben	pharmaceutical grade	S. Thong Chemicals Co., Ltd., Thailand
Procyanidin B2	HPLC grade	Sigma Chemical Co. Ltd., U.S.A.
Quercetin	HPLC grade	Sigma Chemical Co. Ltd., U.S.A.
Sabouraud dextrose agar	microbiological grade	HiMedia, India
Sabouraud dextrose Broth	microbiological grade	HiMedia, India

2. Equipments

Equipments	Model	Supplier/manufacturer
Analytical balance	PL602-5	Mettler Toledo, Switzerland)
Autoclave	HA-300MD	Hirayama Manufacturing Cooperation, Japan
Blender	LB20E* (LB 20 EG)	Waring commercial., U.S.A.
Hot air oven	Mammert	Becthai Co., Ltd., Thailand
HPLC	Class VP software 6.1	Shimadzu, Japan
HPLC	C18-column Hypersil Gold size 250x4.6 mm	Thermo Scientific, U.K.
Incubator	Model 6	Thelco
Magnetic stirrer	Hermolyne, SP 46920-26	Branstead., U.S.A.
pH meter	SevenMuti	Mettler Toledo Gmbh., Switzerland
Refrigerator	TH-8903	Sharp, Thailand
Rotary evaporator	R-200	Buchi., Switzerland.
Stirrer	KMO2	Jank and Kenkel GMBC and Co. KG, Geramany
Suction apparatus, Buchner Funnel, Aspirator, Circulating aspirator	WJ-20	Sibata., Japan
Rheometer	Rheowin-RV1 software	HAAKE Rheowin., Germany
Ultra sonicator bath	TRASSONIC 890	Becthai Co.Ltd., Thailand

3. Plant Materials

Ripened tamarind pods including sweet and sour types from different cultivars, *Tamarindus indica* L. “Priao” (TI-P/K), “Srichomphu” (TI-SP/K), “Sithong-nak” (TI-STN/K), were collected from Nakhon Ratchasima (Khorat,K) province; where as “Priao-yak” (TI-PY/P), “Srichomphu” (TI-SP/P), “Khuntee” (TI-K/P), “Sithong-bou” (TI-STB/P) and “Sithong-nak” (TI-STN/P) were collected from Chanika farm, Phetchaboon (P) province; Thailand. Tamarind seeds of each cultivar

were separated from their pulps, and the seeds from each cultivar are shown in Figure 9 and 10.

4. Microorganisms

Six strains of microorganisms including 3 of gram-positive bacteria: *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Propionibacterium acnes* (clinical isolate); 2 gram-negative bacteria: *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853; and 1 fungus strain *Candida albican*s ATCC 10230; provided by Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. *Propionibacterium acnes* (clinical isolate) was purchased from Department of Medical Sciences, Ministry of Public Health, Thailand.

Method

1. Extraction of tamarind seed-coats

Tamarind seeds were roasted in sand-bath, cooled and cracked to separate the red-brown seed-coats from the kernels. The seed-coats were ground into a fine powder. Two grams of seed-coats were extracted with 20 ml of 70% ethanol by placing in sonicator chamber and sonicated for 30 minutes, followed by filtration through filter paper (Whatman No. 4) by using Buchner funnel. The seed-coat residues were repeated extraction by the same procedure until the filtrate became colorless. All of the red-brown clear filtrates were pooled and mixed with the equal volume of chloroform and shaken vigorously for 20 min, the upper layer of aqueous-ethanol was separated and concentrated by rotary evaporator at 50 ºC, and then dried under nitrogen gas, respectively. The dried tamarind seed-coat extracts (TSCEs) of each tamarind cultivars were prepared. TSCEs were dissolved in distilled water to make 50 mg/ml concentration and then this TSCE solution was diluted to make two fold serial dilution of TSCEs in sterile distilled water or Muller hinton broth (MHB) for microbiological assays.

“Priao” (TI-P/K)

(Sour type)

“Srichomphu” (TI-SP/K)

(Sweet type)

“Sithong-nak” (TI-STN/K)

(Sweet type)

Figure 9. Seeds of *Tamarindus indica* L. of different cultivars from Nakhon Ratchasima (Khorat,K) province, Thailand

"Priao-yak" (TI-PY/P)
(Sour type)

"Srichomphu" (TI-SP/P)
(Sweet type)

"Khuntee" (TI-K/P)
(Sweet type)

"Sithong-bou" (TI-STB/P)
(Sweet type)

"Sithong-nak" (TI-STN/P)
(Sweet type)

Figure 10. Seeds of *Tamarindus indica* L. of different cultivars from Phetchaboon (P) province, Thailand

2. Preparation of the media for microorganisms

All agar and broth media were dispensed in distilled water and sterilized in autoclave for 15 min at 15 pounds pressure per square inches, 121 ºC.

Mueller hinton agar (MHA), Brain heart infusion agar (BHIA) and Sabouraud dextrose agar (SDA) were the media for agar-well diffusion susceptibility test while Mueller hinton broth (MHB), Brain heart infusion broth (BHIB) and Sabouraud dextrose broth (SDB) were the media for broth microdilution susceptibility test. All test bacteria were inoculated on MHA and MHB except for *P. acnes* was inoculcated on BHIA with 5% sheep blood and BHIB with 5% defibrinated sheep blood. SDA and SDB media were used for *C. albicans*.

3. Preparation of tested microorganisms

MHA and MHB were used for all of tested bacteria except for BHIA and BHIB were used for *P. acnes*, SDA and SDB media were used for *C. albicans*. All tested microorganisms were grown overnight on agar slant at 37 ºC before antimicrobial assay. The microorganisms were collected from surface of an agar slant and suspended in sterile normal saline solution (NSS) or MHB. Cell suspension turbidity was adjusted to match standard McFarland no. 0.5 ($1x10^8$ CFU/ml) or the microbial suspension was measured absorbance at 625 nm to 0.08-0.1 (Lorian, 1991). The microbial suspension was then diluted in series of two fold dilution to make $1x10^6$ CFU/ml of bacterial suspension for the microbiological assay.

4. Antimicrobial susceptibility test of TSCEs

4.1 Agar-well diffusion susceptibility test

Agar-well diffusion susceptibility test was performed (Lorian, 1991). Serial two-fold dilution of TSCEs at concentration of 50, 25, 12.50, 6.25 mg/ml in sterile distilled water were freshly prepared. Sterile agar was melted and inoculated with 1% bacterial suspension (10^6 CFU/ml). A volume of 25 ml of inoculated agar was transferred into 10 cm diameter Petri dishes and allowed to cool and then the solidified inoculated media were cut to make wells on agar plates by using a sterile cork borer (6 mm diameter). Each well was filled with 100 µl of various concentrations of TSCEs. The cultured plates were allowed to stand for prediffusion

at room temperature for 1 h and then incubated at 37 ºC for 24 h, except for *P. acnes*; the blood agar plates were used and incubated at 37 ºC for 48 h under anaerobic conditions. The diameter of inhibition zones surrounding each well was measured. The sterile distilled water was used as negative control, gentamicin sulfate (10 µg/ml), clindamycin HCl (100 µg/ml) and amphotericin B (100 µg/ml) were used as positive control. The experiment was performed in triplicate

4.2 Broth microdilution susceptibility test

A volume of 100 µl of TSCEs in broth media containing various concentrations 0.10-25.00 mg/ml of TSCEs was mixed with 100 µl of bacterial suspension in broth media to make the final concentration of bacteria at $5x10^5$ CFU/ml. The medium without TSCEs was used as a control. The inoculated media with TSCEs were incubated at 37 ºC for 24 h. Minimum inhibitory concentration (MIC) for TSCEs which was the lowest concentration of TSCEs that inhibited visible growth of microorganisms was determined. Minimal bactericidal concentration (MBC) was determined by subculturing from tube showing no visible growth onto agar plates without TSCEs and observed the lowest concentration of TSCEs in tube that showing no growth on agar plate after incubation at 37 ºC for 24 h. Except for *P. acnes* was incubated at 37 ºC for 48 h under anaerobic conditions. The experiment was performed in triplicate.

4.3 Time-kill analysis

The inoculated broth media with each of TSCE at concentration of their MBC values against the tested bacteria were incubated at 37 ºC for 24 h, except for *P. acnes* was incubated at 37 ºC for 48 h under anaerobic conditions. At the indicated time, 20 µl of the bacterial cultures were removed and ten-fold serial dilution were prepared in NSS and the number of viable colonies were counted by drop plate method on agar media (Lorian, 1991). The plates were incubated at 37 ºC overnight. Viable colonies were counted and calculated as colony forming unit (CFU) per milliliter and the percentage of bacterial reduction. Microorganisms in media without TSCEs were used as control to compare microbial survival pattern in the media without TSCEs.

5. Preparation of TSCE gel with antibacterial activity

The formulation of TSCE gel was developed by modifying the formulation as described by Najmuddin *et al*. (2010). The formulation of TSCE gel is shown in Table 2. TSCE dried powder from *Tamarindus indica* "Priao-yak" (TI-PY/P) was dissolved in 50 gram distilled water and stirred with magnetic stirrer. The propylene glycol was slowly added into the TSCE solution and the mixture was stirred continuously. Carbopol 940 powder was slowly dispersed and stirred until uniform. Paraben concentrate was added and stirred until homogeneous. Distilled water was added to make to total weight of 100 g with continuously stirred. The pH of gel was adjusted to pH 4.0-4.5 with triethanolamine (TEA). The TSCE gel was prepared in triplicate. The viscosity and pH of gel were measured. The viscosity of product was measured using cone and plate viscometer at values of rate of shear at 100 rpm. Physical properties, air bubbles, color, phase separation (precipitation) were recorded.

Table 2. Formulation of TSCE gel

Ingredients	Function	Content (%w/w)
TSCE	Antibacterial agent	0.625
Carbopol 940	Gelling agent	0.6
Propylene glycol	Humectants	12
Paraben concentrate	Preservative	1
Triethanolamine (TEA)	adjust pH 4.0-4.5	qs
Distill water	Vehicle to make	100

5.1 Assessment of the stability test (Miner, 2000)

5.1.1 Storage at ambient temperature

Fifty grams of TSCE gel was filled in a wide mouth glass bottle with a tightly closed cap. The product was stored at ambient temperature (28±5) ºC for 2, 4, 8, 12 wks. Any physical changes including pH and viscosity were recorded.

5.1.2 Accelerate stability test

TSCE gel was tested for its stability by the method of freeze-thaw cycle by freezing at -5 ºC for 24 h, followed by incubating at 45 ºC for 24 h (1 cycle),

for 3 cycles. The appearances of the products were observed as described and recorded for any physical changes.

6. Efficacy of antibacterial activity of TSCE gel

Antibacterial efficacy of TSCE gel was evaluated by agar-well diffusion and broth microdilution methods against *S. aureus* ATCC 6538P, *S. epidermidis* ATCC 12228 and *Propionibacterium acnes* (Clinical isolate).

6.1 Agar-well diffusion susceptibility test of TSCE gel

TSCE gel contained 0.625% of TSCE of *T. indica*. "Priao-yak" (TI-PY/P) was evaluated for antibacterial capacity. The gel base without TSCE was used as negative control. Five percent of commercially available benzoyl peroxide gel and 1% clindamycin gel were used as positive control. The diameter of inhibition zones surrounding each well was measured. The experiment was performed in triplicate. Each product was carried out to evaluate bacterial inhibitory activity as described in 4.1

6.2 Broth microdilution susceptibility test of TSCE gel

TSCE gel contained 0.625% of TSCE of *T. indica*. "Priao-yak" (TI-PY/P) was evaluated for antibacterial capacity. The gel base without TSCE was used as negative control. 5% benzoyl peroxide gel and 1% clindamycin gel were used as positive control. Serial two-fold dilutions of TSCE gel products in broth media were freshly prepared to obtain various dilutions from 2 to 11 time or 0.1-50.0% of the finished product in this analysis (microbial suspension was added at volume of 1% of the total volume of gel product dilution to make the final microbial concentration between 1x 10^5 to 1x10^6 cfu/ml). A volume of 198 µl of serial dilution of TSCE gel in broth media containing various concentrations at 0.1-50.0% of the gel product was mixed with 2 µl of microbial suspension in broth media to make the final volume of 200 µl**,** mixed and incubated at 37 °C for 24 h. Except for *P. acnes* was incubated at 37 ºC for 48 h under anaerobic conditions. Minimum bactericidal concentration (MBC) was determined by subculturing from each well onto the medium in agar plates and observed the lowest concentration of the gel product dilution that showed no growth on agar plate after incubation at 37 ºC for 24 h. Except for *P. acnes* was incubated at

37 ºC for 48 h under anaerobic conditions. The experiment was performed in triplicate.

7. Separation and identification of flavonoid compounds in tamarind seed-coat extract by High Performance Liquid Chromatography (HPLC)

7.1 Standard flavonoid compound and tamarind seed-coat extract stock solution

Standard flavonoid compounds including (+)-catechin, (-)-epicatechin, procyanidin B2, quercetin and luteolin were used. Chemical fingerprint of TSCE of *T. indica*. “Priao-yak” (TI-PY/P), which was used to prepare TSCE gel product, was monitored by using HPLC instrument. Each of standard flavonoids or TSCE was dissolved in methanol. The final concentration of the stock standard flavonoids solutions and TSCE were 100 µg/ml and 5,000 µg/ml, respectively. The standard flavonoids and TSCE stock solutions were stored at -20 ºC.

7.2 Chromatographic condition

TSCE and standard flavonoids were identified according to the method described by Sudjaroen *et al*. (2005). The C18 column (Hypersil gold, 5µm, 250x4.6 mm. i.d.) was used, mobile phases were acetic acid (2%) in double distilled water (A) and methanol (B), the following gradient condition as shown in Table 3. was used at the flow rate of 1 ml/min, column temperature was 30 ºC and the UV detector was set at 278 nm.

7.3 Identification of flavonoid compounds

TSCE components were identified according to their retention times of peak identical with those peaks of standards. The identical peaks were also confirmed by spiking method (Sudjaroen *et al*., 2005). The sample solutions were filtered though a 0.45 µm, 13 mm nylon syringe filter before injection into the HPLC under the chromatographic conditions described. Sample volume of 10 µl, at concentration 2,500 µg/ml or standard flavonoids 10 µl, at concentration 25 µg/ml were injected into the column and the compounds were eluted with the mobile phase as described.

Table 3. Gradient condition of HPLC analysis

Run time (min)	2% acetic acid (pump A)	100% methanol (pump B)
0-10	95%	5%
10-20	90%	10%
20-30	85%	15%
30-40	80%	20%
40-45	60%	40%
45-50	0%	100%

CHAPTER IV

RESULTS AND DISCUSSION

1. Extraction of tamarind seed-coat

The dried seed-coat from eight tamarind cultivars including *Tamarindus indica* “Priao” (TI-P/K), “Srichomphu” (TI-SP/K), “Sithong-nak” (TI-STN/K), “Priao-yak” (TI-PY/P), “Srichomphu” (TI-SP/P), “Khuntee” (TI-K/P), “Sithong-bou” (TI-STB/P) and “Sithong-nak” (TI-STN/P) were extracted with 70% ethanol and then shaken with chloroform to wash out contaminated lipids, an upper aqueous phase was collected and dried to obtain tamarind seed-coat extract (TSCE) respectively. Percentage yield of the TSCEs are shown in Table 4. The results showed that sour tamarinds (TI-P/K, TI-PY/P) as well as all of sweet tamarinds gave similar amount of percent yield of TSCEs at about 45-53%.

2. Antimicrobial susceptibility test of TSCEs

The antimicrobial activity of TSCEs of the eight Thai tamarind cultivars was evaluated against 5 bacterial strains and 1 fungus. Three strains of gram-positive bacteria were *S. aureus* ATCC 6538P and *S. epidermidis* ATCC 12228, the bacteria causing skin infection and pus; and *P. acnes*, an anaerobic pathogenic bacterium that plays an important role in the skin disease of acne by inducing certain inflammation. Two strains of gram negative bacteria were *E. coli* ATCC 25922, a bacterium can be found in gastrointestinal tract as normal flora; and *Ps. aeruginosa*, an opportunistic bacterium found in environment that can cause respiratory tract infection. One fungus strain of *C. albicans*, a pathogen that can cause skin infection.

The results show in Figure 11, Table 5 and 6, respectively, indicated that TSCEs of all tested tamarind cultivars possessed inhibitory activity against all of the tested bacteria, except for the fungus *C. albicans*.

Table 4. Percentage of TSCEs yield from 8 cultivars of Thai *Tamarindus indica*

Tamarindus indica cultivars	% yield (mean±SD)
"Priao" (TI-P/K)	45.30 ± 4.42
"Srichomphu" (TI-SP/K)	47.75 ± 6.91
"Sithong-nak" (TI-STN/K)	48.87 ± 9.67
"Priao-yak" (TI-PY/P)	53.01 ± 7.41
"Srichomphu" (TI-SP/P)	49.85 ± 4.49
"Khuntee" (TI-K/P)	53.07 ± 3.49
"Sithong-bou" (TI-STB/P)	48.92 ± 6.65
"Sithong-nak" (TI-STN/P)	47.03 ± 5.34

2.1 Agar-well diffusion susceptibility test

The results of sharp and clear margin inhibition zones are shown in Figure 11. The increasing of inhibition zone diameter was observed with respected to the increasing concentration of TSCEs. The inhibition zone diameters with various concentrations of TSCEs from different tamarind cultivars are summarized in Table 5. Gram positive bacteria, *S. aureus* and *S. epidermidis*, exhibited inhibition zone at the lowest TSCE concentrations of 6.25 mg/ml, while *P. acnes* was inhibited at the lowest TSCE concentration of 3.13 mg/ml. However, TSCEs at 1.56 mg/ml revealed no inhibition zone (data is not shown here). No inhibition zone was found against gram negative bacteria and fungus *C. albicans*.

The agar-well diffusion susceptibility test exhibited inhibition zone only against gram-positive bacteria which was demonstrated in Figure 11 by this test suggested that the gram-positive bacteria were more susceptible to be inhibited by TSCEs than gram-negative bacteria. However, there are several factors that relevant for diffusion capacity of compounds in agar plates must be considered, such as the contact between experimental compounds and agar, molecular weight, size and shape of this antimicrobial agents, load and concentration of the test compounds, agar gel viscosity, and ionic concentration in relation to the medium. Furthermore, control and standardization of inoculation density, evaluation of result, selection of agar medium, selection of microorganisms, depth of agar medium, incubation temperature of the plates, and reading point of inhibition zones are also restricting factors affecting the dynamics and variability of diffusion tests in an agar medium (Leonado *et al*., 2000).

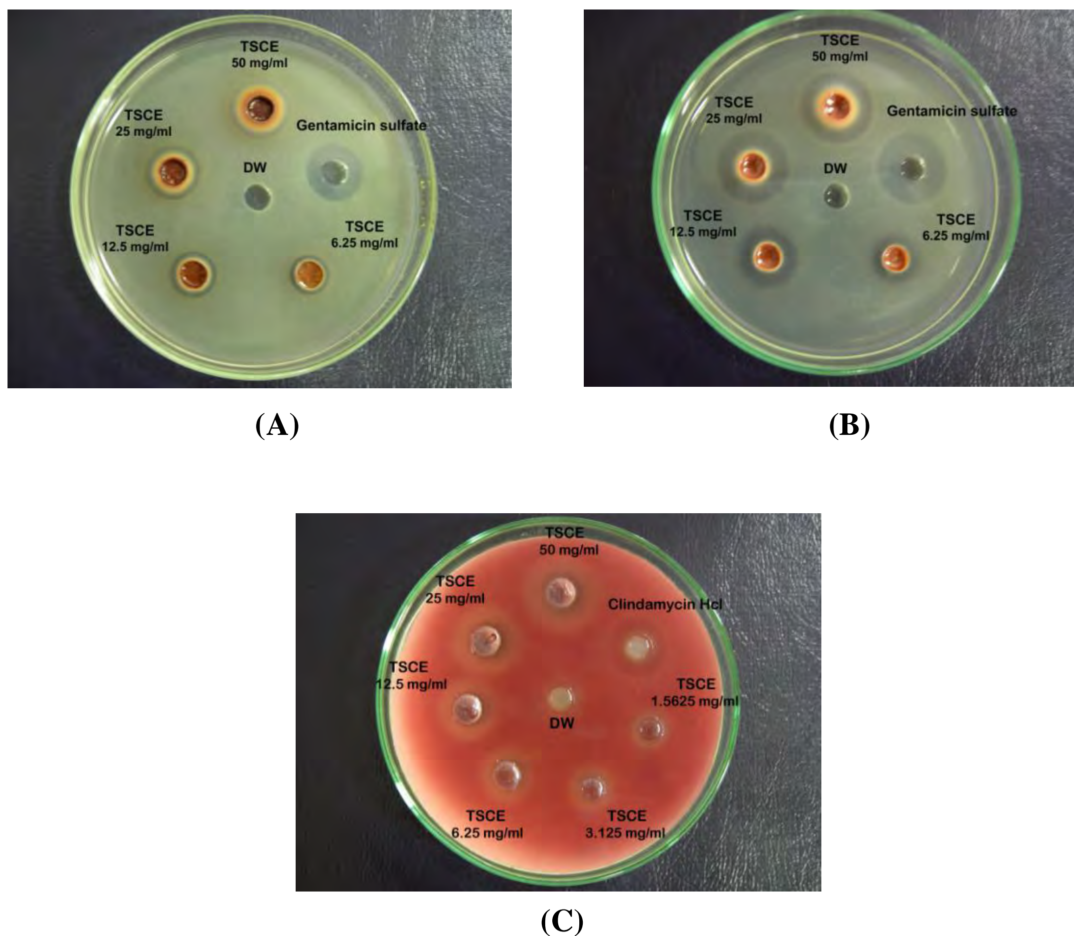


Figure 11. Agar-well diffusion test shows inhibition zones of TSCE of *Tamarindus indica* "Priao-yak" (TI-PY/P) against (A) *S. aureus* ATCC 6538P, (B) *S. epidermidis* ATCC 12228 and (C) *P. acnes* (clinical isolate), respectively. The distilled water (DW) was negative control. Gentamicin sulfate (10 µg/ml) and clindamycin HCL (100 µg/ml) were positive control.

Table 5. Inhibition zone of TSCEs from Thai tamarind cultivars by agar-well diffusion test against 6 strains of microorganisms**.** NZ=No zone, ND= Not determined.

TSCE	Diameter of inhibition zone, mm (mean ± S.D.)					
Concentration (mg/ml)	*S. aureus*	*S. epidermidis*	*P. acnes*	*E. coli*	*Ps. aeruginosa*	*C. albicans*
TI-P/K 50	10.60 ± 0.07	20.21 ± 0.08	20.19 ± 0.07	NZ	NZ	NZ
25	10.42 ± 0.06	20.09 ± 0.08	10.83 ± 0.14	NZ	NZ	NZ
12.5	10.20 ± 0.03	10.80 ± 0.18	10.57 ± 0.12	NZ	NZ	NZ
6.25	9.88 ± 0.01	10.38 ± 0.04	10.25 ± 0.09	NZ	NZ	NZ
TI-SP/K 50	10.60 ± 0.07	20.26 ± 0.10	20.28 ± 0.01	NZ	NZ	NZ
25	10.40 ± 0.09	20.12 ± 0.10	10.83 ± 0.14	NZ	NZ	NZ
12.5	10.15 ± 0.46	10.87 ± 0.25	10.51 ± 0.16	NZ	NZ	NZ
6.25	10.03 ± 0.03	10.33 ± 0.14	10.51 ± 0.16	NZ	NZ	NZ
TI-STN/K 50	10.58 ± 0.09	20.11 ± 0.10	20.21 ± 0.01	NZ	NZ	NZ
25	10.46 ± 0.02	10.99 ± 0.01	13.89 ± 0.35	NZ	NZ	NZ
12.5	10.26 ± 0.11	10.51 ± 0.12	10.60 ± 0.18	NZ	NZ	NZ
6.25	10.06 ± 0.07	10.31 ± 0.10	10.60 ± 0.18	NZ	NZ	NZ
TI-PY/P 50	10.58 ± 0.02	20.14 ± 0.05	20.20 ± 0.01	NZ	NZ	NZ
25	10.40 ± 0.01	20.03 ± 0.07	13.80 ± 0.36	NZ	NZ	NZ
12.5	10.17 ± 0.04	10.71 ± 0.09	10.52 ± 0.15	NZ	NZ	NZ
6.25	10.12 ± 0.10	10.35 ± 0.14	10.31 ± 0.12	NZ	NZ	NZ
TI-SP/P 50	10.55 ± 0.07	20.18 ± 0.06	20.14 ± 0.01	NZ	NZ	NZ
25	10.39 ± 0.09	20.07 ± 0.07	10.80 ± 0.10	NZ	NZ	NZ
12.5	10.17 ± 0.04	10.76 ± 0.10	10.48 ± 0.10	NZ	NZ	NZ
6.25	10.05 ± 0.04	10.35 ± 0.10	10.26 ± 0.12	NZ	NZ	NZ
TI-K/P 50	10.53 ± 0.07	20.23 ± 0.11	20.11 ± 0.09	NZ	NZ	NZ
25	10.34 ± 0.06	20.02 ± 0.12	10.73 ± 0.14	NZ	NZ	NZ
12.5	10.18 ± 0.05	10.63 ± 0.12	10.43 ± 0.15	NZ	NZ	NZ
6.25	9.67 ± 0.03	10.38 ± 0.20	10.24 ± 0.09	NZ	NZ	NZ
TI-STN/P 50	10.45 ± 0.09	20.05 ± 0.05	20.12 ± 0.03	NZ	NZ	NZ
25	10.38 ± 0.07	10.88 ± 0.10	10.70 ± 0.10	NZ	NZ	NZ
12.5	10.16 ± 0.05	10.50 ± 0.04	10.53 ± 0.14	NZ	NZ	NZ
6.25	9.39 ± 0.04	10.38 ± 0.09	10.28 ± 0.17	NZ	NZ	NZ
TI-STB/P 50	10.46 ± 0.01	20.01 ± 0.06	20.13 ± 0.07	NZ	NZ	NZ
25	10.39 ± 0.09	10.78 ± 0.08	10.70 ± 0.10	NZ	NZ	NZ
12.5	10.09 ± 0.04	10.50 ± 0.06	10.51 ± 0.08	NZ	NZ	NZ
6.25	9.33 ± 0.06	10.27 ± 0.13	10.24 ± 0.07	NZ	NZ	NZ
Gentamicin sulfate 10 µg/ml	10.54 ± 0.05	20.15 ± 0.06	ND	10.37±0.07	10.40 ± 0.01	ND
Clindamycin HCL 100 µg/ml	ND	ND	10.56 ± 0.05	ND	ND	ND
Amphotericin B 100 µg/ml	ND	ND	ND	ND	ND	16.50 ± 0.24
Distilled water	NZ	NZ	NZ	NZ	NZ	NZ

2.2 Broth microdilution susceptibility test

The result of antimicrobial activity of TSCEs in Table 6. showed MIC and MBC values for TSCEs against 5 tested bacteria. MBCs for TSCEs against tested bacteria were determined in ranges of 0.10 to 25.00 mg/ml. The values of MICs and MBCs for TSCEs against gram positive bacteria, *S. aureus* ATCC 6538P, *S. epidermidis* ATCC 12228 and *P. acnes*, were 0.10-0.78 and 0.39-3.13 mg/ml, respectively. The values of MICs and MBCs for TSCEs against gram negative bacteria, *E. coli* ATCC 25922 and *Ps. aeruginosa* ATCC 27853, were 3.13-12.50 and 12.50-25.00 mg/ml, respectively. TSCEs did not inhibit growth of fungus *C. albicans* by this assay. The lowest values of MIC and MBC for TSCE were observed against *S. epidermidis* which was 0.10 and 0.39 mg/ml, respectively

2.3 Time-kill analysis

Bactericidal efficacy of TSECs against skin pathogens of gram-positive bacteria was demonstrated by time-kill analysis. TSCEs of the four tamarind cultivars including TI-P/K, TI-PY/P, TI-SP/P and TI-K/P were used in this study. This assay was performed an extension of the MBC and information on the rates at which organisms were killed can be observed. All tested gram-positive bacteria showed the similar survival pattern in time-kill study. All tested TSCEs at the concentration of the MBC value against each the tested bacteria including *S. epidermidis*, *S. aureus*, and *P. acnes*; demonstrated the rapidly decreasing of colony count (cfu/ml) to zero after incubation at 37 ºC for 10, 14, 30 h, respectively (Figure 12,13,14). Percentage of the reduction of viable bacterial count at time indicated are shown in Table 7. TSCEs killed more than 99% of tested bacteria *S. epidermidis* and *S. aureus* within 4 h and *P. acne*s within 24 h, respectively (Table 7.).

The antibacterial activity of tamarind seed-coat extracts were determined by the antimicrobial susceptibility test including agar-well diffusion, broth microdilution and time-kill analysis. The result showed that all tested tamarind seed-coat extracts (TSCEs) exhibited inhibitory activity against both gram-positive and gram negative bacteria, except for fungus *C. albicans*. It was noted in this study that the bacteria gram-positive were more susceptible to be inhibited by TSCEs than the gram-negative bacteria.

Table 6. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for TSCEs from 8 cultivars of Thai *Tamarindus indica* by broth microdilution susceptibility test against 5 strains of bacteria. ND = not determined

TSCEs of *Tamarindus indica* cultivars	*S. aureus*		*S. epidermidis*		*P. acnes*		*E. coli*		*Ps. aeruginosa*	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
“Priao” (TI-P/K)	0.20	0.78	0.10	0.39	0.78	3.13	3.13	12.50	6.25	12.50
“Srichomphu” (TI-SP/K)	0.20	0.78	0.10	0.39	0.78	3.13	3.13	12.50	3.13	12.50
“Sithong-nak” (TI-STN/K)	0.20	0.78	0.39	0.78	0.78	3.13	3.13	12.50	12.50	25.00
“Priao-yak” (TI-PY/P)	0.20	0.78	0.10	0.39	0.78	3.13	3.13	12.50	3.13	12.50
“Srichomphu” (TI-SP/P)	0.20	0.78	0.10	0.39	0.78	3.13	3.13	12.50	6.25	12.50
“Khuntee” (TI-K/P)	0.20	0.78	0.10	0.39	0.78	3.13	3.13	12.50	6.25	12.50
“Sithong-bou” (TI-STB/P)	0.20	0.78	0.10	0.39	0.78	3.13	3.13	12.50	6.25	25.00
“Sithong-nak” (TI-STN/P)	0.20	0.78	0.20	0.39	0.78	3.13	3.13	12.50	6.25	25.00
Gentamicin sulfate (µg/ml)	1.56	6.25	0.20	1.56	ND	ND	0.78	1.56	0.39	3.13
Clindamycin HCL(µg/ml)	ND	ND	ND	ND	0.78	1.5625	ND	ND	ND	ND

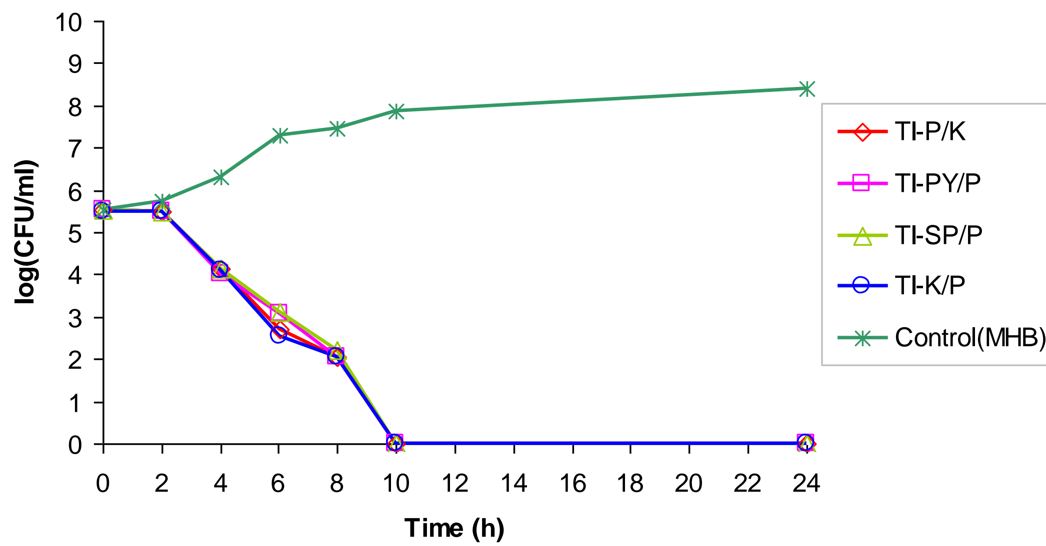


Figure 12. Time kill analysis of tamarind seed-coat extracts (TSCEs) at the concentration of their MBC value (0.39 mg/ml) for each TSCE tested against *Staphylococcus epidermidis* ATCC 12228

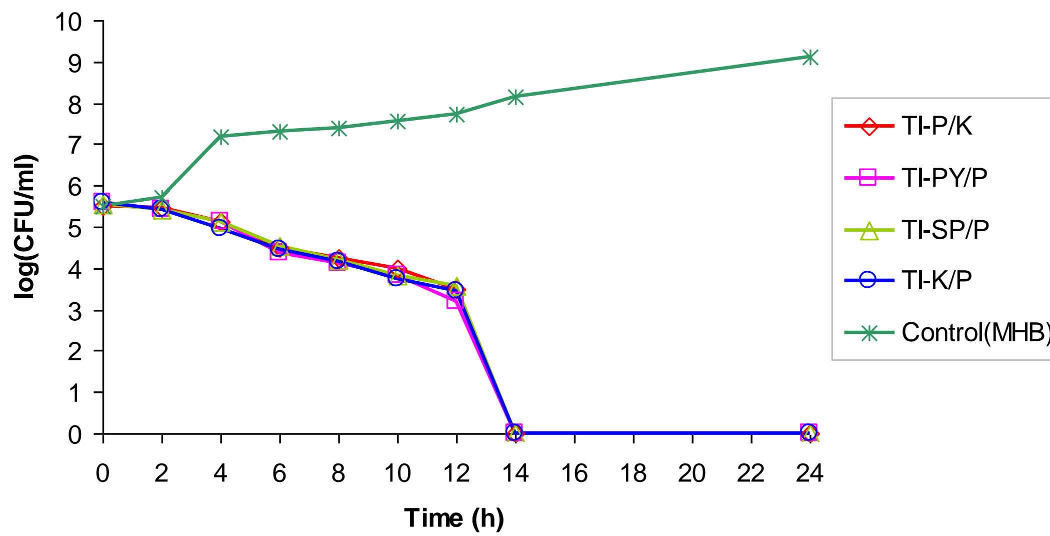


Figure 13. Time kill analysis of tamarind seed-coat extracts (TSCEs) at the concentration of their MBC value (0.78 mg/ml) for each TSCE tested against *Staphylococcus aureus* ATCC 6538P

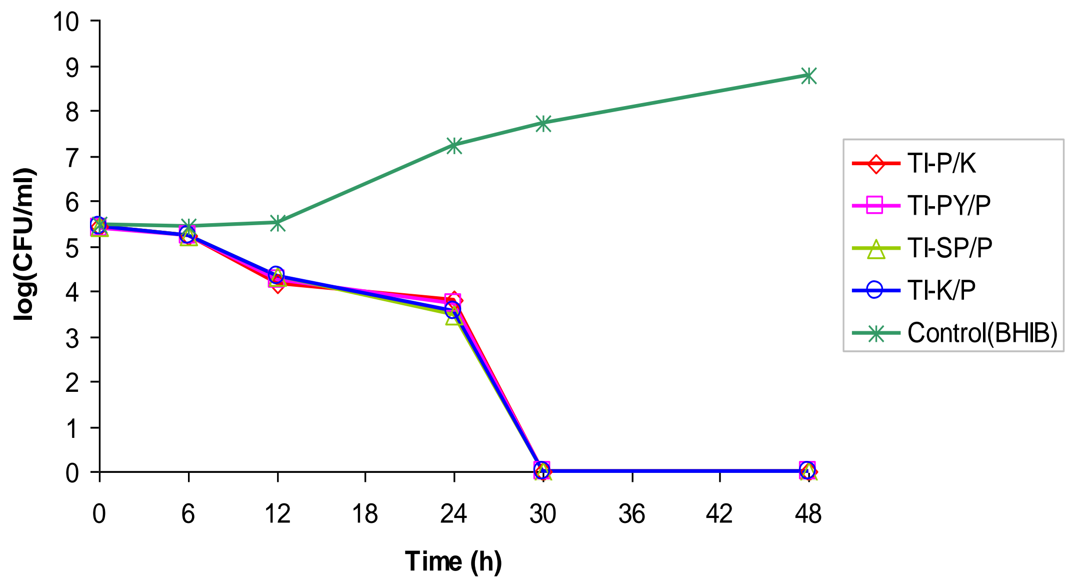


Figure 14. Time kill analysis of tamarind seed-coat extracts (TSCEs) at the concentration of their MBC value (3.13 mg/ml) for each TSCE tested against *Propionibacterium acnes* (Clinical isolate).

Table 7. Time kill analysis of tamarind seed-coat extracts (TSCEs) against *S. epidermidis*, *S. aureus* and *P. acne*s. ND = not determined

Time (h)	*S. epidermidis* % colony count reduction with 0.39 mg/ml TSCEs				*S. aureus* % colony count reduction with 0.78 mg/ml TSCEs				*P. acnes* % colony count reduction with 3.13 mg/ml TSCEs			
	TI-P/K	TI-PY/P	TI-SP/P	TI-K/P	TI-P/K	TI-PY/P	TI-SP/P	TI-K/P	TI-P/K	TI-PY/P	TI-SP/P	TI-K/P
2	46.041	47.360	43.990	45.450	43.328	45.634	46.458	45.799	ND	ND	ND	ND
4	99.325	99.480	99.310	99.360	99.370	99.333	99.320	99.572	ND	ND	ND	ND
6	99.992	99.993	99.992	99.993	99.808	99.845	99.778	99.824	36.760	37.695	36.449	39.563
8	99.999	99.999	99.999	99.999	99.934	99.950	99.937	99.946	ND	ND	ND	ND
10	100	100	100	100	99.974	99.965	99.982	99.984	ND	ND	ND	ND
12	ND	ND	ND	ND	99.994	99.997	99.993	99.995	95.493	94.213	93.255	92.907
14	ND	ND	ND	ND	100	100	100	100	ND	ND	ND	ND
24	ND	ND	ND	ND	ND	ND	ND	ND	99.963	99.969	99.983	99.980
30	ND	ND	ND	ND	ND	ND	ND	ND	100	100	100	100

This result was similary to the recently studies of Waghmare *et al.* (2010), they have reported antimicrobial activity of the tamarind seed-coat extract against certain bacterial species including *Staphylococcus aureus*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*.

Furthermore, Tewtrakul *et al.* (2008) studied antimicrobial activity of thirteen Thai crops including banana, okra, jackfruit, germinated rice, rambutan, durian, jampadah, hausa potato, tamarind, coconut, mango, fan palm fruit and dioscorea tuber. These 13 crops, some of which included different parts such as skin, flesh, and seed, were extracted with four solvents separately (95% ethanol, 50% ethanol, water and hot water). Antimicrobial activity of tamarind seed-coat is shown by the inhibition of *Staphylococcus aureus* and *Bacillus subtilis*, with MIC values ranging from 0.500-1.000 mg/ml, but no inhibitory effect towards *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Their result indicates that TSCE inhibits growth of bacteria, especially gram-positive bacteria.

Antibacterial activity of TSCEs probably associated with phytochemistry of TSCEs with high flavonoid content, the extracts compose of monomeric flavonoid, (+)-catechin, (-)-epicatechin; and oligomeric flavonoid, procyanidin B2; procyanidin trimer, procyanidin tetramer, procyanidin pentamer and procyanidin hexamer (Sudjaroen *et al*., 2005).

Phytochemistry of TSCEs have been reported of compounds with antimicrobial activity such as catechins and epicatechin are extracted from teas, they are screened for their antimicrobial activity against gram-positive and gram-negative bacteria of the species *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Salmonella* sp. and *Staphylococcus aureus.* (Wiseman *et al*., 1997).

Shan *et al.* (2007) studied the antimicrobial activity from plants, *Cinnamomum burmannii* extracts. The extracts inhibit five common foodborne pathogenic bacteria (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella anatum*). Major compounds in *Cinnamomum burmannii* extracts are tentatively identified by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC-MS), the extracts contain predominant volatile oil component and several polyphenols (mainly proanthocyanidins and epicatechins).

Moussaoui *et al*. (2010) have identificated flavonoid compounds in *Launaea resedifolia*, the *n*-BuOH extract of the aerial parts, four flavonoids are identified as apigenin, luteolin, apigenin 7-O-β-glucoside and apigenin 7-O-β-glucuronide. Moreover the antibacterial and antifungal activities of the crude extract against several microorganisms are under studied.

The ethanolic extracts of *Mentha longifolia* inhibit the growth of *S. aureus*, *B. cereus* and *B. subtilis*. However, it do not show any inhibitory activity against *E. coli* and *P. aeruginosa*. The phytochemical screening is studied for the ethanolic extract and flavonoids are found in this extract. The phytochemical screening of this extract indicates that it contains mainly five flavonoids identified as luteolin-7-O-glycoside, luteolin-7,3'-O-diglycoside, apigenin, quercetin-3-O-glycoside and kaempferol-3-O-glycoside (Akroum,2009).

The extracts of roots and stems of *Bauhinia sirindhornia* exhibit inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus*. The extracts from roots and stems are purified as pure compounds. Results have shown that five compounds are found to be active. Eriodictyol, isoliquiritigenin, isoliquiritigenin 4-methyl ether, naringenin and luteolin are found to be active against *B. subtilis* (Athikomkulchai *et al*., 2005).

Furthermore, there are many medicinal plants with high flavonoid content have also been reported of antibacterial activity (Bosio, 2000) and crude extracts from plants with a history use in folk medicine have been screened in vitro for antibacterial activity by several research groups. Therefore, polyphenolic compounds and flavonoids are believed to play an important role in antibacterial activity. The flavonoid compounds that are active against different organisms are shown in Table B-1(appendix B).

There are many natural plants exhibit antimicrobial properties. Antibacterial effect of natural plant may be due to phytochemicals in natural plant such as phenolics and phenolic acids, quinines, flavonoids and alkaloids. Antimicrobial phytochemicals of natural plants are summarized in Table B-2 (appendix B) (Cowan., 1999).

Gram-positive bacteria in this study were more susceptible to be inhibited by TSCEs than gram-negative bacteria. These results are agreeable with the previous studies by Hajima *et al*. (1993), they studied the antibacterial activity of the green tea

extracts, their results show that gram-negative bacteria are more resistant to bactericidal effect of catechins than the gram-positive bacteria.

Raybaudi-Massilia *et al*. (2006) have determined antimicrobial activity of essential oils against *Salmonella enteritidis*, *Escherichia coli*, and *Listeria innocua* in fruit juices. The results indicate that the extracts are more active against gram-positive bacteria than gram-negative bacteria. This may be attributed to the functional barrier present on the outer membrane of these bacteria. Gram-negative bacteria has the outer membrane exterior to the cytoplasmic membrane, thus forming a tight diffusion barrier against hydrophobic compounds and hydrophilic compounds with the large size.

Furthermore, the outer membrane serves as a barrier for the bacterium against the destructive effects of various antibiotics (e.g. erythromycin, penicillin, amoxicillin), digestive enzymes like lysosomal enzymes, heavy metals, detergent substances, bile salts, and several dyes (Shagam, 2006; Wheelis, 2007).

3. Preparation of TSCE gel with antibacterial activity

TSCE gel was formulated and prepared successfully. TSCE of *T. indica*. “Priao-yak” (TI-PY/P) was applied as antibacterial agent (0.625 g/100g gel) and carbopol 940 was used as a gelling agent. TSCEs gel was adjusted to pH 4-4.5 with triethanolamine. The product descriptions and its appearance are shown in Table 8 and Figure 15. The product showed homogeneous texture of the clear gel, brown color and no air bubbles.

The viscosity and pH values of the finish products were measured at ambient temperature after fleshly prepared and after stored for 2, 4, 8 and 12 wks and its appearances were recorded. The viscosity and pH values after freshly prepared were 1,111.28 $\pm$ 123 cps and 4.32 $\pm$ 0.05, respectively (Table 8).

TSCE gel showed good appearance and stability of physical properties. The finished product also possessed good stability after stored at ambient temperature for 12 wks as well as after stability tested by freeze-thaw cycle for 3 cycles as indicated in Table 8. and Figure 16.

The formulation of TSCE gel showed good appearance, no physical changes after stability test. This may be attributed to the formulation consists of suitable

ingredients. In the formulation of TSCE gel, Carbopol 940 was used as a gelling agent, carbopol is very well suitable for aqueous formulations of the topical preparation and it provide the benefits to topical formulations, this compound is safe and effective use in topical gels, creams, lotions, and ointments (Gumma, 1971; Ruiz *et al*., 1994). The concentration of the gelling agents is typically less than 10%, usually in the ranges of 0.5% to 2.0%, with some exceptions (Allen, 2002). Carbopol 940 at 0.6% w/w concentration was used for this study.

Propylene glycol has been used in a wide variety of pharmaceutical formulations. In topical preparations, propylene glycol is a moisturizing agent or humectants and regarded as a nontoxic material (Paphattarapong, 2005). In the formulation of TSCE gel, 12% w/w of propylene glycol was used.

Triethanolamine is a counteragent to control pH level. In the cosmetic products, it neutralizes cabomer to give cohesive power. Complexation of triethanolamine with anionic polymers decreased its pH (Musial and Kubis, 2004). TSCEs gel was adjusted to pH 4.0-4.5 with triethanolamine.

Paraben concentrates are the most commonly used preservatives in cosmetic. Parabens can be used to formulate well because they have no perceptible odor or taste and practically neutral, do not produce discoloration of the final product. The maximum concentration use of paraben in pharmaceutical products is not greater than 1% (w/w) (M.G. Soni *et al*., 2001). Paraben concentrates (2% of propyl paraben and 20% of methyl paraben in propylene glycol) at 1% w/w concentration was used for this study.

4. Efficacy of antibacterial activity of TSCE gel

Antibacterial activity of TSCE gel was evaluated by agar well diffusion method against skin pathogens, including *S. aureus* ATCC 6538P, *S. epidermidis* ATCC 12228 and *Propionibacterium acnes* (Clinical isolate).

The TSCE gel product was evaluated for their antibacterial activity after the product was freshly prepared and after the stability test (storage at room temperature for 12 wks).

Table 8. Description and appearance of TSCE gel (0.625 g TSCE/100 g gel) after freshly prepared and stability tested.

Description of TSCE gel	Freshly prepared	Stand at ambient temperature				After freeze-thaw cycles for 3 cycles
		2 wks	4 wks	8 wks	12 wks	
Appearance	clear, homogenous	clear, homogenous	clear, homogenous	clear, homogenous	clear, homogenous	clear, homogenous
Air bubbles	none	none	none	none	none	little
Color	brown	brown	brown	brown	brown	brown
Phase separation (Precipitation)	none	none	none	none	none	none
pH	4.32 $\pm$ 0.05	4.31 $\pm$ 0.07	4.33 $\pm$ 0.08	4.31 $\pm$ 0.06	4.32 $\pm$ 0.06	4.32 $\pm$ 0.03
Viscosity (cps)	1,111.28 $\pm$ 123.00	1,148.67 $\pm$ 125.40	1,136.78 $\pm$ 79.56	1,060.99 $\pm$ 87.91	1,130.32 $\pm$ 136.47	1,186.11 $\pm$ 49.02

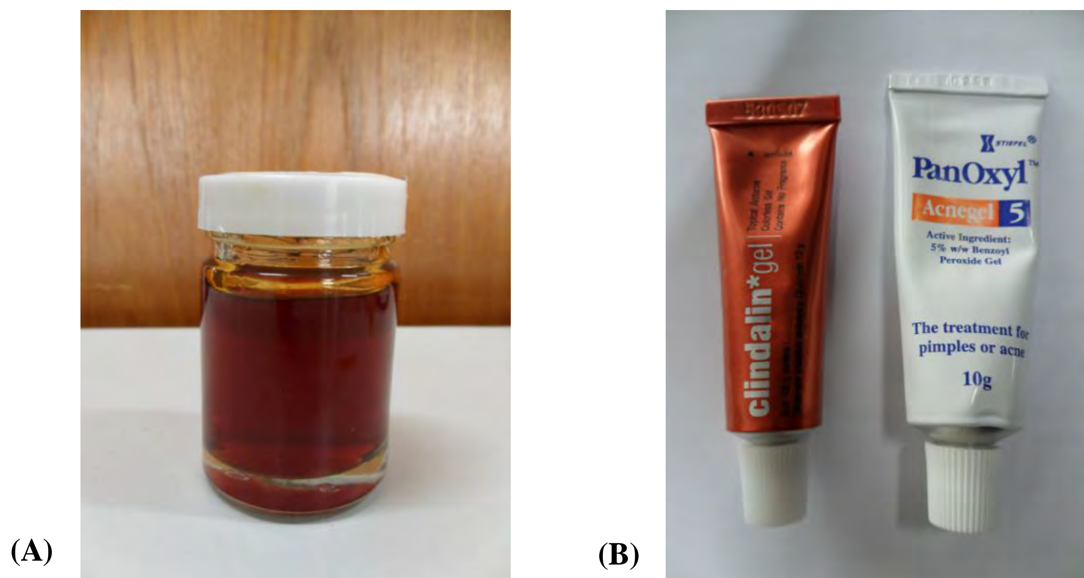


Figure 15. TSCE gel formulation contained 0.625 g TSCE: The finish product showed clear homogeneous texture, without air-bubble and brown color (A). One percent of commercially available clindamycin gel and 5% benzoyl peroxide gel were used as positive control (B).

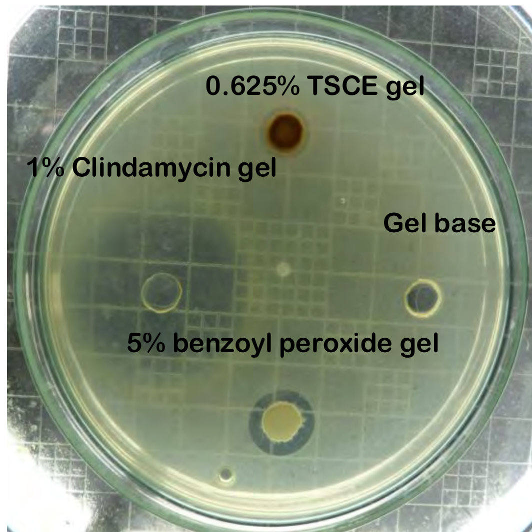
Figure 16. An appearance of TSCE gel: After freshly prepared (1); After stored at room temperature for 12 wks (2); and After freeze-thaw cycle for 3 cycles (3).

4.1 Agar-well diffusion susceptibility test

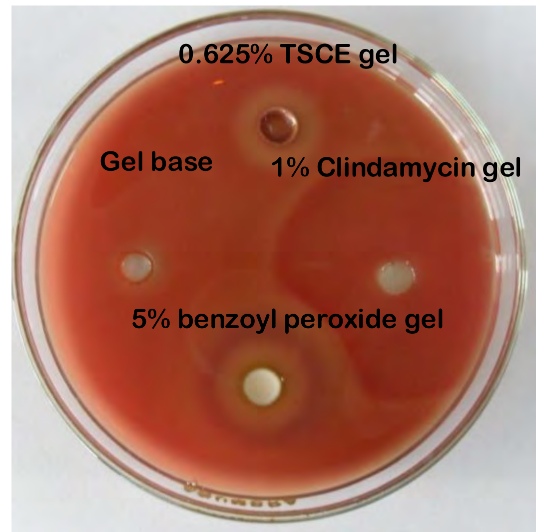
The antibacterial activity of TSCE gel product was evaluated by observing the inhibition zones of TSCE gel contained 0.625% TSCE of *T. indica* “Priao-yak” (TI-PY/P), gel base preparation without TSCE was used as negative control, 1% clindamycin gel and 5% benzoyl peroxide gel were used as positive control (Figure 15). The inhibition zones of antibacterial TSCE gel preparation against *S. aureus* and *P. acnes* showed sharp and clear margin inhibition zones as good as 5% benzyl peroxide gel but *S. epidermidis* showed turbid inhibition zone, whereas 1% clindamycin gel exhibited wide inhibition zones against tested bacteria. The negative control gel base preparation exhibited no inhibition zone. The results of inhibition zones of TSCE gel against *S. aureus*, *S. epidermidis* and *P. acnes* are shown in Figure 17. and Table 9, 10, 11

4.2 Broth microdilution susceptibility test

The MBC values for TSCE gel was evaluated against *S. aureus* ATCC 6538P, *S. epidermidis* ATCC 12228 and *Propionibacterium acnes* (Clinical isolate). The results are shown in Table 12, 13 and 14. The MBC values for TSCE gel contain 0.625% TSCE were observed against *S. epidermidis*, *S. aureus* and *P. acnes* with the 8 time dilution of TSCE gel product or at 12.50% of TSCE gel product, the 4 time dilution of TSCE gel product or at 25% of TSCE gel product and the 2 time dilution of TSCE gel product or at 50% of TSCE gel product, respectively. The MBC values of the positive control, 5% benzoyl peroxide gel, against *S. epidermidis*, *S. aureus*, and *P. acnes* were observed at 0.39, 0.78 and 0.10% of the product, respectively. In addition, the MBC values of the positive control for 1% clindamycin gel against bacteria *S. epidermidis*, *S. aureus*, and *P. acnes*, observed at 1.56, 50 and 0.20% of this product, respectively. The positive control (5% benzoyl peroxide gel) exhibited the strongest antibacterial activity, inhibition zone was observed with its lowest dilutions as shown in Table 12-14. The results indicate that TSCE gel may be used as an antiseptic or antibacterial preparation for treatment of skin infection.



(A)



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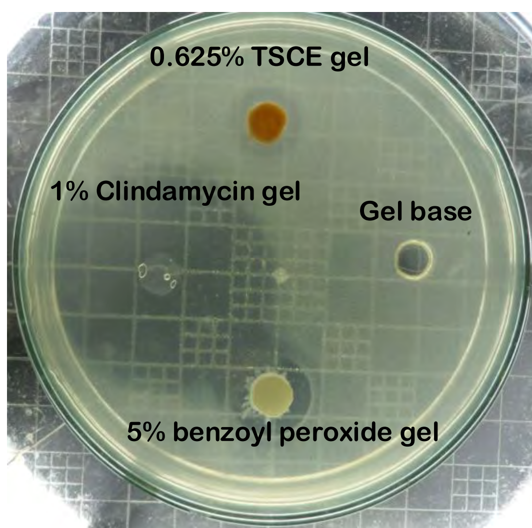

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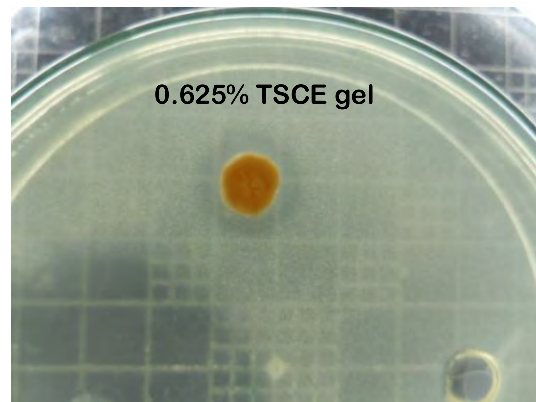


(D)

Figure 17. Agar-well diffusion test shows inhibition zone of 0.625%TSCE gel against *S. aureus* ATCC 6538P (A); *P. acnes* (clinical isolate) (B); and *S. epidermidis* ATCC 12228 (C), (D). The gel base was negative control. 5% benzoyl peroxide gel and 1% clindamycin gel were positive control.

Table 9. Efficacy of antibacterial TSCE gel by agar-well diffusion test against *S. aureus* ATCC 6538P. Data shows inhibition zone as mean±SD. TSCE gel product was tested after freshly prepared and after store at room temperature. NZ= No zone

Samples	Diameter of inhibition zone, mm ± SD					
	After freshly prepared	After storage at ambient temperature for				After freeze-thaw cycles for 3 cycles
		2 wks	4 wks	8 wks	12 wks	
0.625% TSCE gel	9.65±0.07	9.66±0.07	9.52±0.05	9.31±0.04	8.71±0.06	9.23±0.02
5% benzoyl peroxide gel	10.22±0.07	10.24±0.05	10.22±0.03	10.25±0.05	10.22±0.03	10.23±0.06
1% clindamycin gel	20.93±0.02	20.80±0.06	20.78±0.03	20.79±0.09	20.78±0.08	20.73±0.005
Gel base	NZ	NZ	NZ	NZ	NZ	NZ

Table 10. Efficacy of antibacterial TSCE gel by agar-well diffusion test against *S. epidermidis* ATCC 12228. Data shows turbid inhibition zone as mean± SD. TSCE gel product was tested after freshly prepared and after store at room temperature. NZ= No zone

Samples	Diameter of inhibition zone, mm ± SD					
	After freshly prepared	After storage at ambient temperature for				After freeze-thaw cycles for 3 cycles
		2 wks	4 wks	8 wks	12 wks	
0.625% TSCE gel	10.18±0.04	10.16±0.03	10.17±0.04	10.15±0.04	10.12±0.05	10.15±0.04
5% benzoyl peroxide gel	10.35±0.06	10.32±0.07	10.35±0.05	10.34±0.04	10.31±0.01	10.30±0.04
1% clindamycin gel	30.43±0.15	30.30±0.10	30.23±0.15	30.39±0.02	30.36±0.05	30.40±0.08
Gel base	NZ	NZ	NZ	NZ	NZ	NZ

Table 11. Efficacy of antibacterial TSCE gel by agar-well diffusion test against *P. acnes* (clinical isolate). Data shows inhibition zone as mean±SD. TSCE gel product was tested after freshly prepared and after store at room temperature. NZ= No zone

Samples	Diameter of inhibition zone, mm ± SD					
	After freshly prepared	After storage at ambient temperature for				After freeze-thaw cycles for 3 cycles
		2 wks	4 wks	8 wks	12 wks	
0.625% TSCE gel	10.60±0.10	10.58±0.08	10.52±0.08	10.51±0.04	10.40±0.08	10.48±0.05
5% benzoyl peroxide gel	10.78±0.08	10.62±0.08	10.58±0.03	10.55±0.05	10.60±0.10	10.53±0.07
1% clindamycin gel	40.55±0.07	40.45±0.05	40.38±0.08	40.46±0.06	40.34±0.05	40.44±0.05
Gel base	NZ	NZ	NZ	NZ	NZ	NZ

Table 12. Minimum bactericidal concentration (MBC) of TSCEs gel by broth microdilution susceptibility test against *S. aureus* ATCC 6538P. TSCE gel product was tested after freshly prepared and after store at room temperature. NA= No activity

Samples	Minimum bactericidal concentration (MBC) Concentration(%) of the product					
	After freshly prepared	After storage at ambient temperature for				After freeze-thaw cycles for 3 cycles
		2 wks	4 wks	8 wks	12 wks	
0.625% TSCE gel	25	25	25	25	25	25
5% benzoyl peroxide gel	0.78	0.78	0.78	0.78	0.78	0.78
1% clindamycin gel	50	50	50	50	50	50
Gel base	NA	NA	NA	NA	NA	NA

Table 13. Minimum bactericidal concentration (MBC) of TSCEs gel by broth microdilution susceptibility test against *S. epidermidis* ATCC 12228. TSCE gel product was tested after freshly prepared and after store at room temperature. NA= No activity

Samples	Minimum bactericidal concentration (MBC) Concentration(%) of the product					
	After freshly prepared	After storage at ambient temperature for				After freeze-thaw cycles for 3 cycles
		2 wks	4 wks	8 wks	12 wks	
0.625% TSCE gel	12.50	12.50	12.50	12.50	12.50	12.50
5% benzoyl peroxide gel	0.39	0.39	0.39	0.39	0.39	0.39
1% clindamycin gel	1.56	1.56	1.56	1.56	1.56	1.56
Gel base	NA	NA	NA	NA	NA	NA

Table 14. Minimum bactericidal concentration (MBC) of TSCEs gel by broth microdilution susceptibility test against *P. acnes* (clinical isolate). TSCE gel product was tested after freshly prepared and after store at room temperature. NA= No activity

Samples	Minimum bactericidal concentration (MBC) Concentration(%) of the product					
	After freshly prepared	After storage at ambient temperature for				After freeze-thaw cycles for 3 cycles
		2 wks	4 wks	8 wks	12 wks	
0.625% TSCE gel	50	50	50	50	50	50
5% benzoyl peroxide gel	0.10	0.10	0.10	0.10	0.10	0.10
1% clindamycin gel	0.20	0.20	0.20	0.20	0.20	0.20
Gel base	NA	NA	NA	NA	NA	NA

5. Separation and identification of flavonoid compounds in tamarind seed-coat extracts by High Performance Liquid Chromatography (HPLC)

The flavonoid compound in Tamarind seed-coat extract of *T. indica*. "Priao-yak" (TI-PY/P) was separated and identified peaks of flavonoid compounds including (+)-catechin, (-)-epicatechin and procyanidin B2 by using the HPLC method as described by Sudjaroen *et al.* (2005). Standrad flavonoids were (+)-catechin, (-)-epicatechin, procyanidin B2, quercetin and luteolin. The final concentration of TSCE was 2,500 µg/ml and the concentration each of standard was 25 µg/ml, respectively. The result showed that the chromatogram of TSCE showed peaks identical with the peaks of three flavonoid such as (+)-catechin, (-)-epicatechin and procyanidin B2, however the peaks of luteolin and quercetin standards were not observed in this condition. Flavonoid compounds in TSCE were determined from the retention times of the peaks of flavonoid standard. Identification of each peak was confirmed by the spiking method (Sudjaroen *et al.*, 2005). The chromatograms of the flavonoids standard mixture, TSCE solution and, TSCE the spiked with (+)-catechin standard, TSCE spiked with (-)-epicatechin standard and TSCE spiked with procyanidin B2 standard are showed in Figure 18-22, respectively. The retention time of the flavonoid standards and TSCE spikeds with each of standard flavonoid are showed in Table 15.

The peaks of three flavoniods including (+)-catechin, procyanidin B2 and (-)-epicatechin of TSCEs were well separated. This may be according to suitable chromatographic condition such as type of HPLC column, mobile phase, temperature, ultraviolet detector.

The reversed-phase (RP) is suitable for separation of phenolic compounds. The polar compounds were eluted faster than the non-polar compounds. The mobile phase for a reverse phase system is polar-solvent such as of water or buffer solution, methanol, acetonitrile and isopropyl alcohol (IPA). Most HPLC analyses of phenolic compounds are performed at ambient column temperature, but moderately higher temperatures between 30 and 40 ºC have also been recommended. The maximum absorption, which is found in the 240-285 nm range, is due to the A-ring of flavoniod structure (Stalikas, 2007).

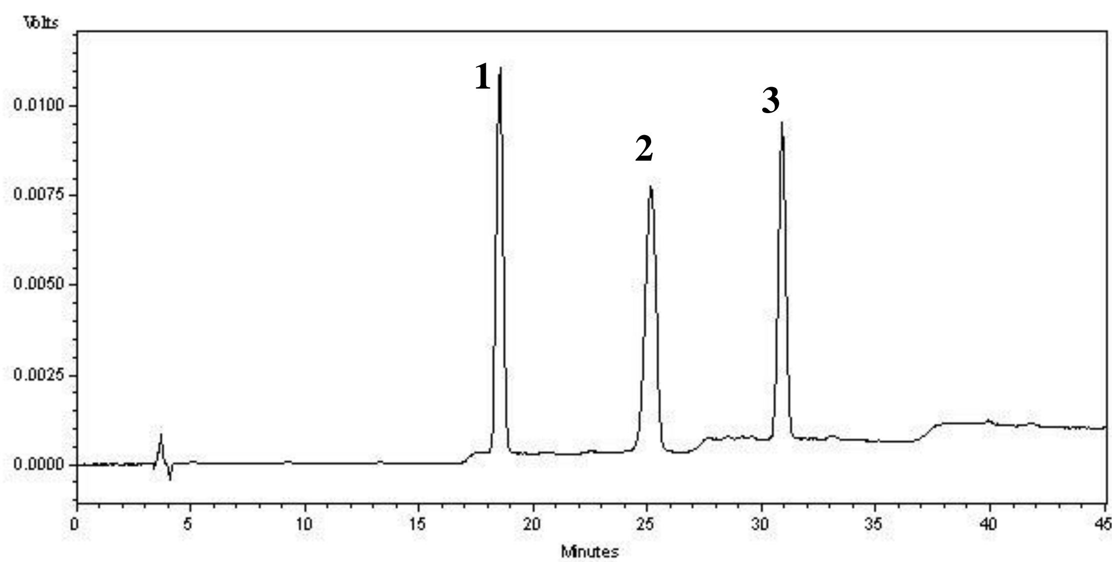


Figure 18. Chromatogram of standard mixture flavonoids, the 3 peaks are the standard (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3), respectively.

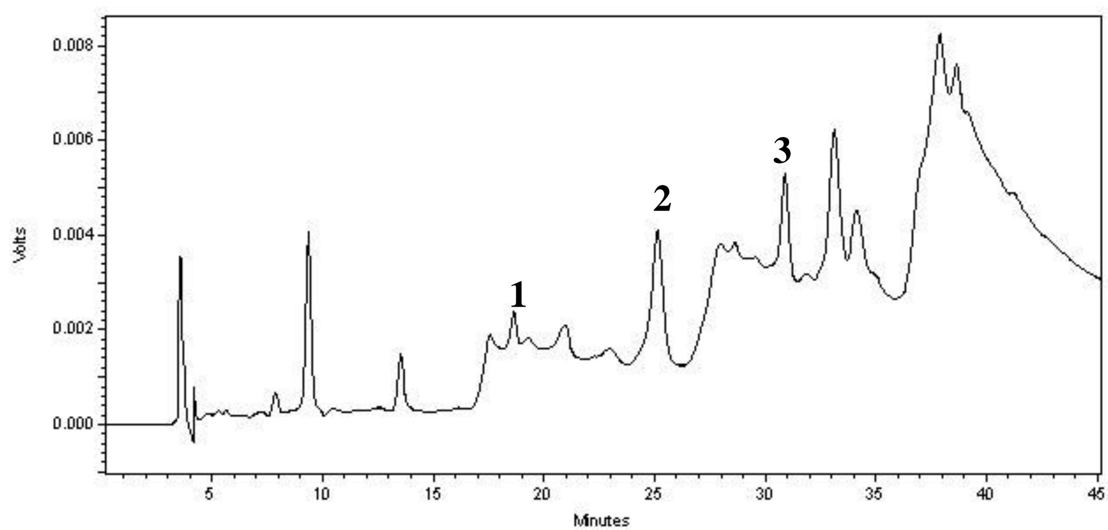
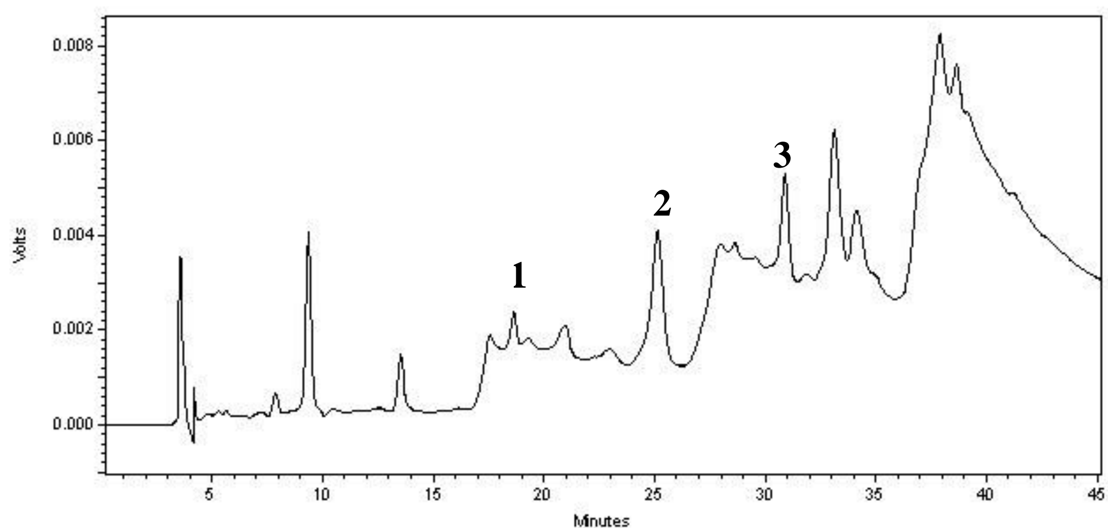


Figure 19. Chromatogram of TSCE of *T. indica*. "Priao-yak" (TI-PY/P), the 3 peaks are identical with the standard (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3), respectively.

A



B

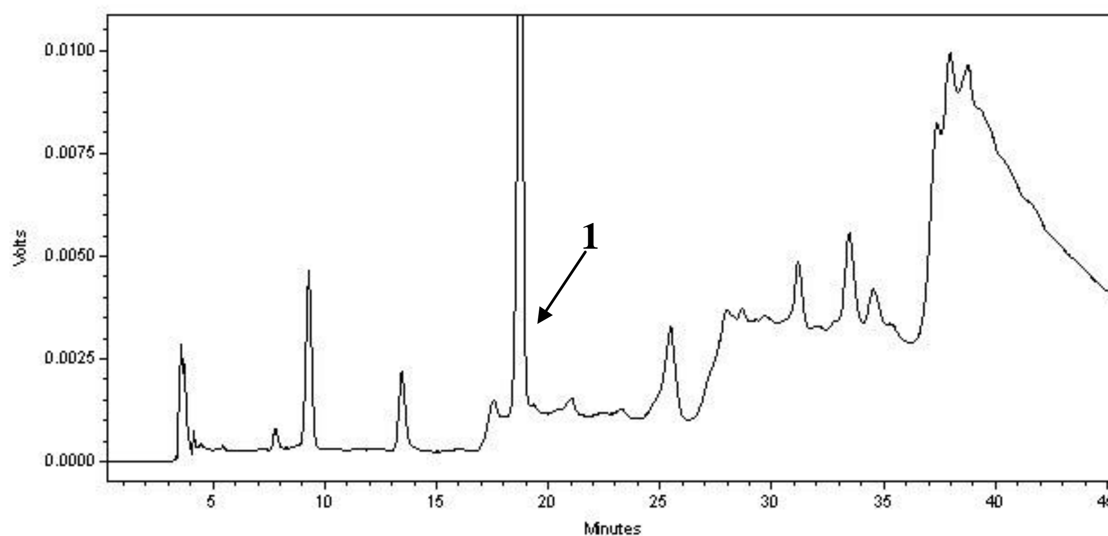
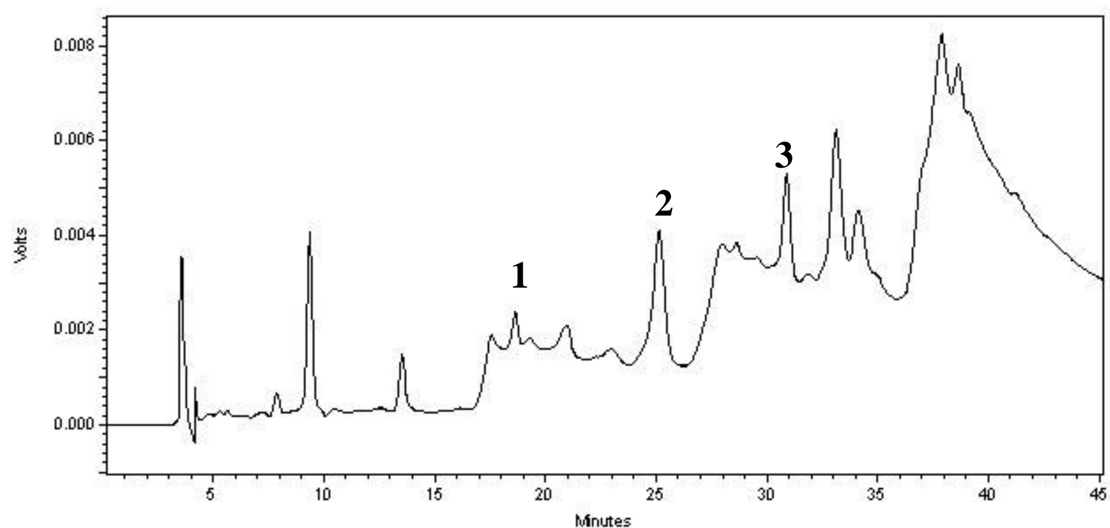


Figure 20. Chromatograms of flavoniod compounds in TSCE of *T. indica* “Priao-yak” (TI-PY/P), (A) chromatogram of TSCE, the 3 peaks are identical with the standard (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3), respectively. (B) chromatogram of TSCE spiked with (+)-catechin standard (1).

A



B

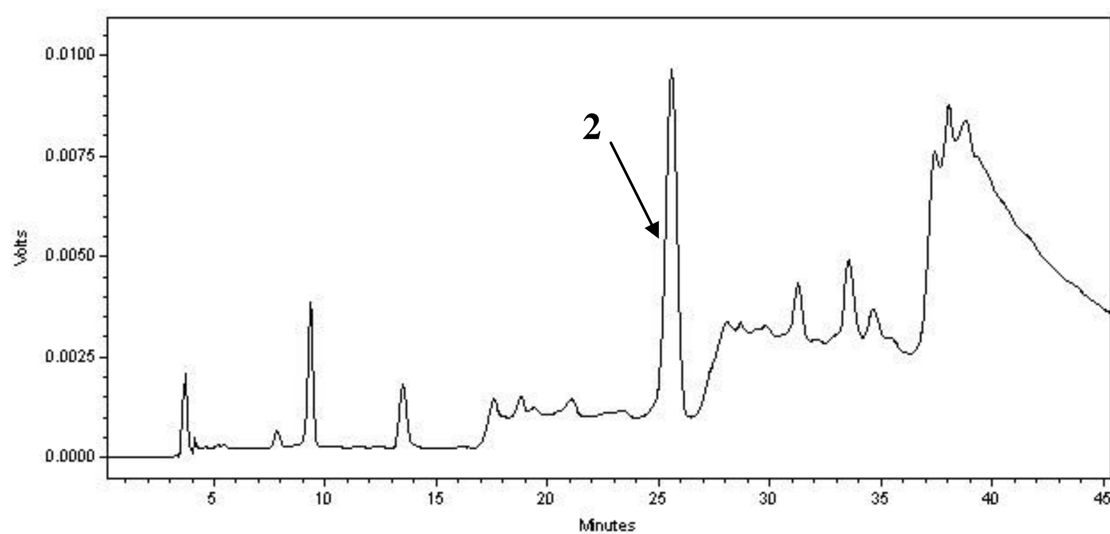
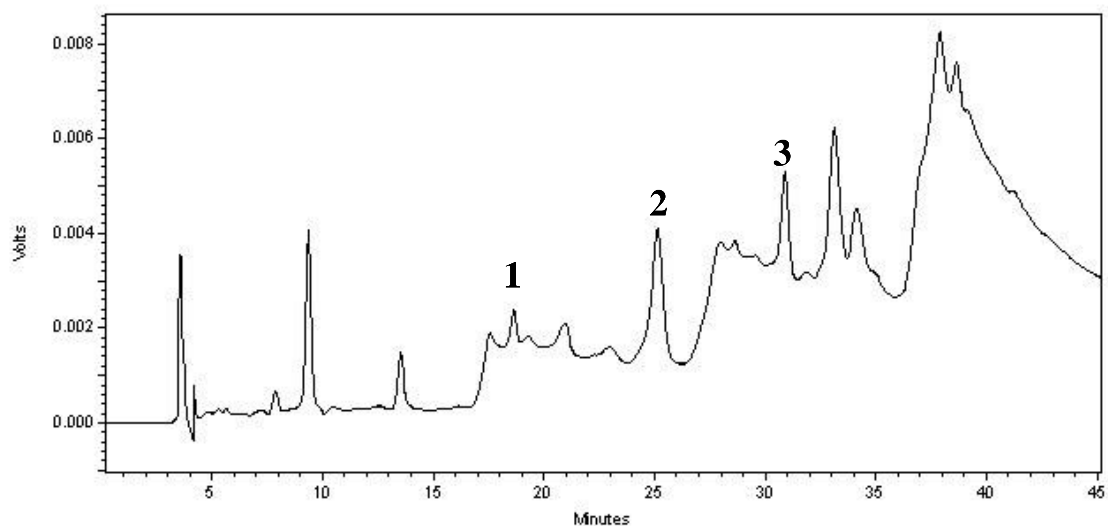


Figure 21. Chromatograms of flavoniod compounds in TSCE of *T. indica* "Priao-yak" (TI-PY/P), (A) chromatogram of TSCE, the 3 peaks are identical with the standard (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3), respectively. (B) chromatogram of TSCE spiked with procyanidin B2 standard (2).

A



B

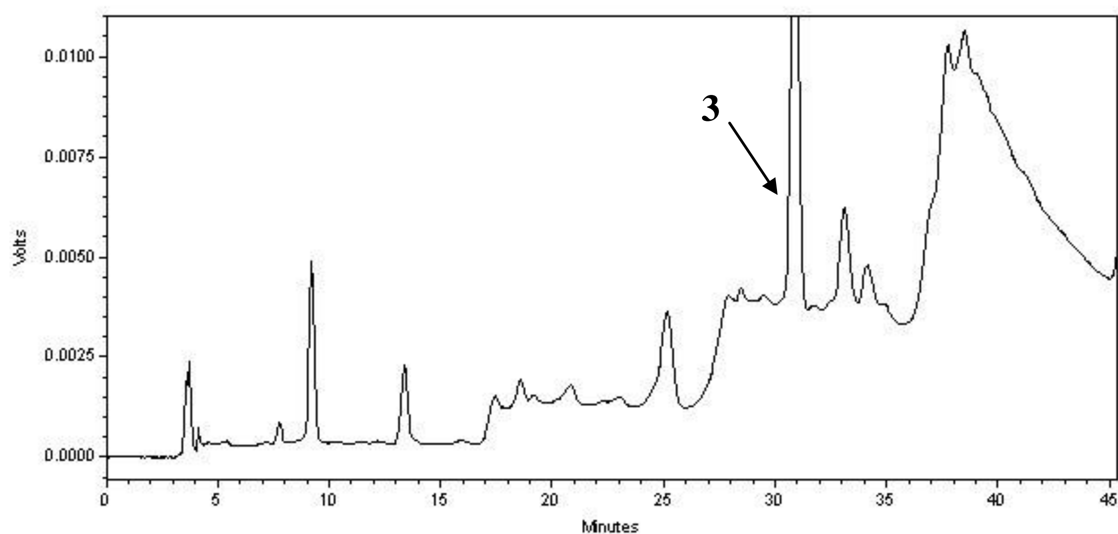


Figure 22. Chromatograms of flavoniod compounds in TSCE of *T. indica* "Priao-yak" (TI-PY/P), (A) chromatogram of TSCE, the 3 peaks are identical with the standard (+)-catechin (1), procyanidin B2 (2) and (-)-epicatechin (3), respectively. (B) chromatogram of TSCE spiked with (-)-epicatechin standard (3).

Table 15. Retention time (min) of flavonoid standard and TSCE spiked with each flavonoid standard

Sample	Retention time (min)
(+)-catechin standard	18.551
procyanidin B2 standard	25.144
(-)-epicatechin standard	30.906
TSCE spiked with (+)-catechin standard	18.726
TSCE spiked with procyanidin B2 standard	25.575
TSCE spiked with (-)-epicatechin standard	30.900

The condition for separation and identification of flavonoid compounds in tamarind TSCE according to information was previously described. This condition is agreeable with the studies by Chen et al (2001), they analyzed phenolic and flavonoid compounds such as gallic, caffeic, sinnapic, *p*-coumaric, chlorogrnic, 3,4,5-trimethoxycinnamic acids, myricetin, quercetin in cranberry juice. The mobile phases were acetic acid (3%) in double distilled water (A) and methanol (B), the flow rate 1 ml/min and the UV detector at 280 nm are used for their study.

CHAPTER V

CONCLUSION

Antimicrobial activity of tamarind seed coat extracts (TSCEs) from 8 Thai tamarind cultivars was evaluated by agar-well diffusion and broth microdilution susceptibility test, as well as time-kill analysis. Tamarind seed coat extracts (TSCEs) inhibited growth of both gram-positive and gram negative bacteria, except for fungus *C. albicans*. It was noted that bacteria gram-positive were more susceptible to be inhibited by TSCEs than gram-negative bacteria.

TSCE gel was formulated and TSCE gel product was prepared successfully. TSCE of *T. indica*. "Priao-yak" (TI-PY/P) was applied as antibacterial agent (0.625 g/100g gel). The product of TSCE gel was evaluated for its stability and antibacterial efficacy. Stable product of TSCE gel with antibacterial activity was obtained.

In this study, three flavoniod compounds including (+)-catechin, (-)-epicatechin and procyanidin B2 in the TSCE of *T. indica*. "Priao-yak" (TI-PY/P) were separated and identified by HPLC technique. The chemical profile of TSCE showed peaks that identical with the (+)-catechin, (-)-epicatechin and procyanidin B2 standard.

TSCEs seemed to be economically important as a good source containing compounds with antioxidants and antibacterial activities that may be useful for nutraceutical and cosmetic application in the future.

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APPENDICES

APPENDIX A

Media

1. Mueller hinton agar (MHA)

Component	Grams/liter
Infusion from meat	2.0
Casein hydrolysate	17.5
Starch	1.5
Agar	13.0

pH 7.4±0.2 at 25 ºC

2. Mueller hinton broth (MHB)

Component	Grams/liter
Infusion from meat	2.0
Casein hydrolysate	17.5
Starch	1.5

pH 7.4±0.2 at 25 ºC

3. Sabouraud dextrose agar (SDA)

Component	Grams/liter
Mycological peptone	10
Dextrose	40
Agar	15

pH 5.6±0.2 at 25 ºC

4. Sabouraud dextrose broth (SDB)

Component	Grams/liter
Mycological peptone	10
Dextrose	40

pH 5.6±0.2 at 25 ºC

5. Brain heart infusion agar (BHIA)

Component	Grams/liter
Calf brain, infusion from	200
Beef heart, infusion from	250
Proteose peptone	10
Dextrose	2
Sodium chloride	5
Disodium phosphate	2.5
Agar	15

pH 7.4$\pm$0.2 at 25 ºC

6. Brain heart infusion broth (BHIB)

Component	Grams/liter
Calf brain, infusion from	200
Beef heart, infusion from	250
Proteose peptone	10
Dextrose	2
Sodium chloride	5
Disodium phosphate	2.5

pH 7.4$\pm$0.2 at 25 ºC

APPENDIX B

Table B-1. Antimicrobial activity of various flavonoid compounds (Chung *et al*., 1998; Taddese, 2004; Athikomkulchai *et al*, 2005; Mandalari *et al*., 2007; Akroum *et al*.,2009,)

Flavonoid compounds	Organisms tested
epicatechin	*Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas fluorescens*, *Salmonella* sp. and *Staphylococcus aureus*
catechin	*Staphylococcus aureus*, *Escherichia coli*, *Streptococcus Mutans*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Salmonella* sp.
proanthocyanidin	*Aeromonas* spp., *Bacillus* spp., *Clostridium botulinum*, *Clostridium perfringens*, *Enterobacter* spp., *Klebsiella* spp., *Proteus* spp., *Pseudomonas* spp., *Shigella* spp., *Staphylococcus aureus*, *Streptococcus* spp.,and *Vibrio* spp.
Quercetin	*Staphylococcus aureus*, *Escherichia coli, Candida. tropicalis*
Naringin	*Staphylococcus aureus*, *Shigella boydii*, *Pseudomonas aeroginosa*
Apigenin	*Streptococcus pyogens*, *Streptococcus viridans*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeroginosa*, *Bacillus subtilis*
Rutin	*Staphylococcus aureus*, *Shigella boydii*, *Pseudomonas aeroginosa*, *Bacilus anthracis*
Hesperetin	*Staphylococcus aureus*, *Shigella boydii*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas putida*
Baicalin	*Staphylococcus aureus*, *Pseudomonas aeroginosa*
Chrysin	*Streptococcus jaccalis*, *Streptococcus baris*, *Streptococcus pneumoniae*

Flavonoid compounds	Organisms tested
Eriodictyol	*Bacillus subtilis*, *Staphylococcus aureus*, *Shigella boydii*, *Escherichia coli*, *Salmonella enterica*, *Pseudomonas putida*, *Listeria innocua*, *Saccharomyces cerevisiae*
Naringenin	*Escherichia coli*, *Salmonella enterica*, *Pseudomonas putida*, *Bacillus subtilis*
luteolin	*Bacillus subtilis*, *Staphylococcus aureus*

Table B-2. Antimicrobial phytochemicals of natural plants (Cowan., 1999).

Common name	Compound	Class	Activity
Allspice	Eugenol	Essential oil	Gram-positive organisms
Bael tree	Essential oil	Terpenoid	Fungi
Barberry	Berberine	Alkaloid	Bacteria, protozoa
Basil	Essential oils	Terpenoids	*Salmonella*, bacteria
Black pepper	Piperine	Alkaloid	Fungi, *Lactobacillus*, *Micrococcus*, *E. coli*, *E. faecalis*
Chamomile	Anthemic acid	Phenolic acid	*M. tuberculosis*, *S. typhimurium*, *S. aureus*, helminthes
Coca	Cocaine	Alkaloid	Gram-negative and Gram-positive cocci
Eucalyptus	Tannin	Polyphenol	Bacteria, viruses
Turmeric	Turmeric oil	Terpenoids	Bacteria, protozoa
Onion	Allicin	Sulfoxide	Bacteria, *Candida albicans*
Oregon grape	Berberine	Alkaloid	*Plasmodium Trypansomes*
Senna	Rhein	Anthraquinons	*S. aureus*
Tansy	Essentialoils	Terpenoid	Helminths, bacteria
Treebard	Totarol	Flavonol	*P. acnes*, other gram-positive bacteria

APPENDIX C

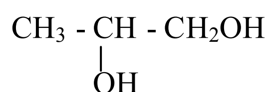
Physicochemical properties of substances

1. Humectent

1.1 Propylene Glycol (American Pharmaceutical Association and the Pharmaceutical Society of Great Britain, 1986; John, 1990; Reynolds, 1993)

Chemical name: (±) – propane – 1, 2 – diol

Structure formula of propylene glycol



Molecular formulation: $CH_3.CHOH.CH_2OH$

Molecular weight: 76.10

Propylene glycol is clear, colorless, viscous and practically odorless liquid having a sweet, slight acrid taste. It has boiling point at 188 ºC and flash point at 99 ºC. It is miscible with water, acetone, alcohol, glycerin and chloroform, and immiscible with light mineral oil and fixed oils.

Application: Propylene glycol is a solvent or co-solvent used in solutions, parenterals, topical preparations and aerosol solution and used as humectant in topical preparation.

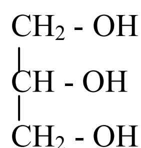
Incompatibility: It is incompatible with oxidizing reagents such as potassium permanganate.

Stability and storage condition: It is stable in well closed containers, but at high temperature in the open it trends to oxidize, giving the products such as propionaldehyde, lactic acid, pyruvic acid and acetic acid. It absorbs moisture when is expose to moist air. The material should be stored in well closed container and protected from light.

1.2 Glycerin (John, 1990; Reynolds, 1993)

Chemical name: Propane - 1,2,3 – triol

Structure formula of glycerin



Molecular formulation: $C_3H_8O_3$

Molecular weight: 92.09

Glycerin is a clear, colorless, odorless, viscous, hygroscopic liquid; it has a sweet taste approximately 0.6 times as sweet as sucrose. It has melting point at 17.8 ºC. It is miscible with ethanol, methanol, water and practically insoluble with benzene, chloroform and oils.

Application: Glycerin is used in wide variety of pharmaceutical formulations including oral, ophthalmic, topical and parenteral. It is also used in cosmetic, food additive and as a plasticizer of gelatin in the production of soft gelatin capsules and gelatin suppositories.

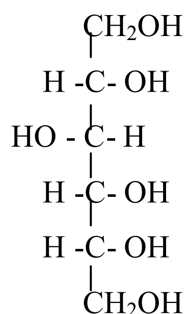
Incompatibility: Glycerin may explode if mixed with strong oxidizing agents, such as chromium trioxide, potassium chlorate or potassium permanganate.

Stability and storage condition: It is hygroscopic. Pure glycerin is not prone to oxidation by the atmosphere under ordinary storage conditions, but decomposes on heating, with the evaluation of toxic acrolence

1.3 Sorbitol

Chemical name: D-Glucitol, Sorbitolum

Structure formula of sorbitol



Molecular formulation: $C_6H_{14}O_6$

Molecular weight: 182.17

Sorbitol is a hexahydric alcohol with the empirical structure is $C_6H_{14}O_6$ with molecular weight 182.17 which occurs naturally in fruits, but is prepared synthetically from glucose by high pressure hydrogenation. High % sorbitol solution is much more viscous than correspond glycerin solution. It is quite soluble in hot alcohol, sparingly solution in cold alcohol and also soluble in methanol, isopropanol, butanol, cyclohexanol, phenol, acetone and acetic acid. Practically insoluble in most other organic solvents.

Application: Sorbitol is a polyhydric alcohol with half the sweetening power of sucrose. Sorbitol also acts as a bulk sweetening agent. It is used in limited quantities either as a sweetening agent or as a source of carbohydrate in diabetic food product. It is also used as a sweetening agent instead of sucrose in much sugar-free oral liquid preparation and in sugar-free preparations for the prevention of dental caries. Sorbitol also has humectant and stabilizing properties and is used in various pharmaceutical and cosmetic products including toothpaste. Sorbitol may also be used analytically as a marker for assessing liver blood flow.

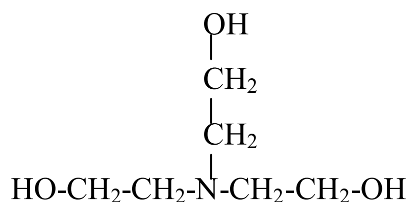
Incompatibility: Sorbitol will form water-soluble chelates with many divalent and trivalent metal ions in strongly acidic and alkaline conditions. Addition of liquid polyethylene glycols to sorbitol solution, with vigorous agitation, produces a waxy, water-soluble gel with melting point of 35-40 ºC

2. pH adjuster

Triethanolamine

Chemical name: 2,2',2"- Nitrilotriethanol; trihydroxytriethylamine; tris (hydroxyethyl) amine; triethlolamine.

Structure formula of Triethanolamine



Molecular formulation: $N(CH_2CH_2OH)_3$

Molecular weight: 149.19

Triethanolamine is Produced along with mono- and diethanolamine by ammonolysis of ethylene oxide, Very hygroscopic, viscous liquid. Slight ammoniacal odor. Turns brown on exposure to air and light

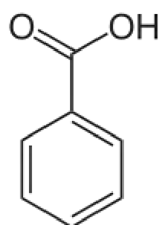
Application: Pharmaceutic aid (alkalizing agent).

3. Preservative

3.1 Benzoic Acid

Chemical Name: Benzenecarboxylic acid; phenylformic acid; dracylic acid

Structure formula of benzoic acid



Molecular Formulation: $C_7H_6O_2$

Molecular weight: 122.12

Benzoic acid is monoclinic tablets, plates, leaflets. White crystalline powder or colorless crystals.

Application: Preserving foods, fats, fruit juices, alkaloidal solns, etc. Pharmaceutic aid (antifungal agent, antimicrobial agent).

Solubility: Slightly soluble in water, free soluble in alcohol and methanol.

Toxicity: Mild irritant to skin, eyes, mucous membranes.

Stability and storage condition: Store in a well-closed container protected from light.

3.2 Parabens

Structure of Parabens

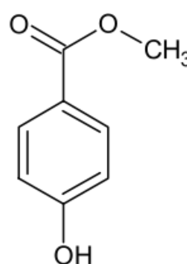
Parabens ester of p-hydroxybenzoic acid, have been used as preservatives of cosmetics for 60 years. The methyl and propyl parabens are the most commonly used preservatives for cosmetics and they are widely used for pharmaceuticals as well. As has been demonstrated over their long history, the parabens are very safe.

The utility of parabens is often limited by their low water solubility. They are readily extracted into organic solvents and oils and can also be lost from a product by absorption into rubber closures.

3.2.1 Methyl paraben

Chemical name: Methyl 4-Hydroxybenzoate, 4-Hydroxybenzoic acid methyl ester, methyl p-hydroxybenzoate

Structural formula of Methyl paraben



Molecular Formulation: $C_8H_8O_3$

Molecular weight: 152.15

Description: Colorless crystals or white needles crystalline powder.

Application: Pharmaceutical aid (antimicrobial preservative). Preservative in foods, beverages and cosmetics. (0.1-0.3%)

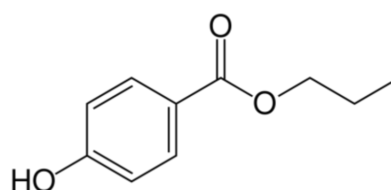
Solubility: Free soluble in Ethanol (95%), in ether and in methanol, very slightly soluble in water.

Stability and storage condition: Store in well-closed containers.

3.2.2 Propyl paraben

Chemical name: Propyl 4-Hydroxybenzoate, 4-Hydroxybenzoic acid propyl ester; propyl p-hydroxybenzoate

Structural formula of Propyl paraben



Molecular Formulation: $C_{10}H_{12}O_3$

Molecular weight: 180.20

Description: Small, colorless crystals or white powder.

Application: Pharmaceutical aid (antimicrobial preservative).

Solubility: Miscible with alcohol and with fatty and essential oils, very slightly soluble in water.

Stability and storage condition: Store in a well-closed containers and protected from light.

3.3 Sorbic Acid

Chemical name: 2,4- Hexadienoic acid; 2-propenylacrylic acid.

Structural formula of Sorbic Acid: $CH_3CH{=}CHCH{=}CHCOOH$

Molecular Formulation: $C_6H_8O_2$

Molecular weight: 112.12

Description: Needles from water.

Solubility: Solubility in water slightly

Application: Mold and yeast inhibitor. Fungistatic agent for foods

Incompatibility

Obvious sources of pharmaceutical instability include the incompatibility of various ingredients within a formulation. It has been defined as a physical or chemical interaction between two or more ingredients that leads to a visibly recognizable change. The latter may be in the form of gross precipitate, haze, or color change. On the other hand, a chemical incompatibility is classified as a reaction in which a visible change does not occur. Since there is no visible evidence of deterioration, this type of incompatibility requires trained, knowledgeable personnel to recognize it, should it occur.

VIVA

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