ฤทธิ์ทางชีวภาพของเปลือกลำต้นทานาคา *Hesperethusa crenulata*

นางสาวศกุลนา วังทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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BIOLOGICAL ACTIVITIES OF THANAKA *Hesperethusa crenulata* STEM BARK

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ในงานวิจัยนี้ผงเปลือกของลำต้นทานาคาได้ถูกนำมาสกัดด้วยตัวทำละลายอินทรีย์และน้ำ จากนั้นส่วนสกัดทั้งหมดได้ถูกนำมาทำการทดสอบฤทธิ์ทางชีวภาพ ได้แก่ การต้านอนุมูลอิสระ, การยับยั้งการทำงานของเอนไซม์ไทโรซิเนส การกรองรังสีอัลตราไวโอเลต การยับยั้งการเจริญของแบคทีเรียและการยั้บยั้งการอักเสบ ความปลอดภัยของส่วนสกัดต่างๆยังได้ทำการตรวจสอบโดยการตรวจความเป็นพิษต่อเซลล์และความเป็นพิษต่อยีน พบว่าส่วนสกัด เฮกเซน ไดคลอโรมีเทน เอทิลอะซิเตต เมทานอล 85% เอทานอล และน้ำ มีฤทธิ์ทางชีวภาพแตกต่างกันไปตามศักยภาพของส่วนสกัดนั้น ส่วนสกัดตัวทำละลายทั้ง 6 ชนิดและผงทานาคาไม่มีความเป็นพิษต่อยีนจากการทดสอบในที่นี้ นอกจากนี้ส่วนสกัด 85% เอทานอล เมทานอล และน้ำพบความเป็นพิษต่ำมาก โดยมีค่า ค่าการยับยั้งที่ร้อยละ 50 มากกว่า 12 มิลลิกรัมต่อมิลลิลิตร นอกจากนี้ยังได้สาร 2 ชนิดจากงานวิจัยนี้ คือ วานิลิน และ อนุพันธ์ของอะคริเลท อีกด้วย

สาขาวิชา....เทคโนโลยีชีวภาพ....ลายมือชื่อนิสิต....ศกุลนา วังทอง....

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In this work, the stem bark powder of *Hesperethusa crenulata* or Thanaka was extracted with organic solvents and water, and investigated the biological activities including antioxidant, anti-tyrosinase, UV screening, antibacterial and anti-inflammatory. Safety of the extracts and original powder were also done by cytotoxic and genotoxic assay. The hexane, dichloromethane, ethyl acetate, methanol, 85% (v/v) ethanol and water extracts of the bark showed some variation between them in terms of their potency of those biological activities. All six solvent extracts and the original crude bark powder showed non-genotoxicity in the assay carried out here. Nevertheless, the methanol, 85% (v/v) ethanol and water extracts of the bark all exhibited a very low cytotoxicity. Moreover, vanillin and acrylate derivative, were obtained from thanaka stem bark.

Field of Study: Biotechnology .....Student's signature Sakulna Wangthong
Academic year: 2010 .....Advisor's signature .....
Co-advisor's signature .....

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CONTENTS

Page

Page

List of Figures

Figure **Page**

Figure **Page**

List of Tables

Tables **Page**

List of Schemes

Scheme **Page**

List of Abbreviations

^{13}C NMR	Carbon 13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
°C	Degree Celsius
CH_2Cl_2	Dichloromethane, methylene chloride
$CHCl_3$	Chloroform
cm $^{-1}$	Unit of wave number (IR)
COSY	Correlated spectroscopy
d	Doublet
dd	Doublet of doublet
EtOAc	Ethyl acetate
EtOH	Ethanol
g	Gram (s)
h	Hour
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear multiple-quantum coherence
IR	Infrared
J	Coupling constant
Kg	Kilogram (s)
L	Liter (s)
m	Multiplet
MeOH	Methanol
mg	Milligram (s)
mL	Milliliter (s)
mM	Millimolar
MW	Molecular weight
m/z	Mass to charge ration
nm	nanometer (s)
Hz	hertz
min.	minute
mL	milliliter (s)
NMR	nuclear magnetic resonance
%	Percent

λ	Wavelength
ppm	Parts per million
q	Quartet
s	Singlet
t	Triplet
UV	Ultraviolet
δ	Chemical shift
μg	Microgram (s)

CHAPTER I

INTRODUCTION

Natural products have been investigated and utilized to alleviate disease since early human history. In the early 1900s, before the ‘‘Synthetic Era’’, 80% of all medicines were obtained from roots, barks and leaves. At that time, fluid extracts were in trend. Every household had its favorite tea and tonics. Trustful humanity placed its faith in the belief that for every ill there existed a cure in the plants of field and forest. In more recent times, natural products have continued to be significant sources of drugs. Their dominant role is evident in the approximately 60% of anticancer compounds and 75% of drugs for infectious diseases that are either natural products or natural product derivatives [1-2]. After on, a large number of clinical agents have been developed by the pharmaceutical industry, indigenous phytotherapy is still practiced in many rural areas; using treatments were passed from generation to generation. The World Health Organization (WHO) has emphasized the importance of the traditional indigenous medicines, since a large majority of rural people in the developing countries still use these medicines as the first defense in health care [3]. Globally, about 85% of all medications for primary health care are derived from plants [4]. In India, there is long history of herbal medicine, names “Ayurveda” and in the present Ayurveda became to be a well known subject in ethnopharmacology and also Traditional Chinese Medicine (TCM) from china. Thus, Ayurveda and TCM are scripture for ethnopharmacology study. Moreover, South America and Europe have the history of traditional herbs as well. Thus, traditional herbal medicines provide an interesting, largely unexplored source for development of potential new drugs. The potential use of traditional herbal medicines for development of new skin-care cosmetics has been emphasized recently [5]. It is of great interest to know whether preparations used cosmetically in folk medicine have activities that might be useful in modern formulations.

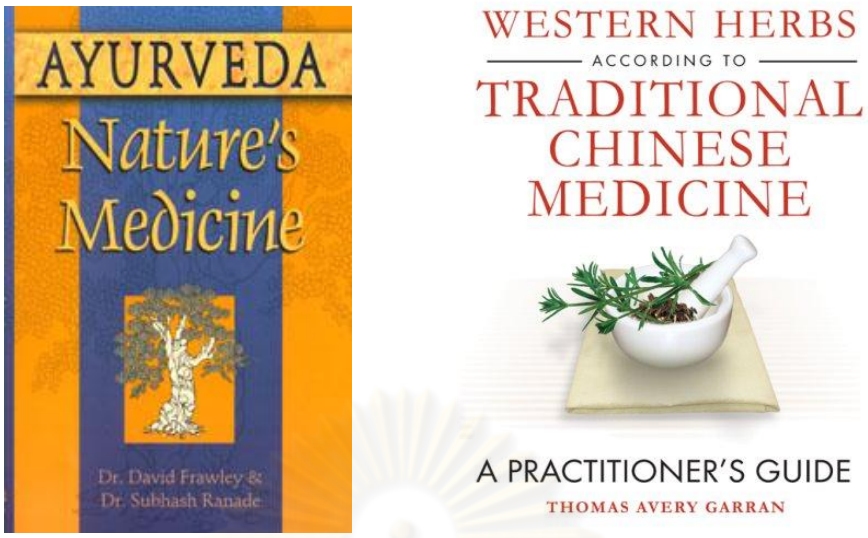


Figure 1.1 The Ayurveda and Traditional Chinese Medicine books

Natural product was used not only for drugs, but also cosmetic. The birth of cosmetics dates back to the dawn of civilization, Egypt and Roman flourish age. Archaeologists estimate that cosmetics existed as long ago as 6000 BC [6]. Ancient Egyptians had a wide extent of make-up utensils. One of them is kohl, which was used as the eyeliner. Indian people painted henna on their hands, hair and fingernails. China people began to stain their fingernails with gum Arabic, beeswax and eggs from around 300 BEC. Japanese geisha wore lipstick made of crushed safflower petals paint the lips and eyebrow. Rice powder was used on their face and back. Moreover, natural beauty is blessing and sign of healthy life. Plants help in preserving and enhancing the beauty and personality of human beings. Natural cosmetics is a general term applied to articles intended to be rubbed, poured, sprinkled, introduced into or otherwise applied to the human body or any part thereof, for cleansing, beautifying, promoting attractiveness, coloring, softening or altering the appearance. There are many parts of plant which have been used as a dermatological medicine and cosmetic. Below is the sample of some plant which has been used as a dermatological medicine and cosmetic [7].

Table 1.1 Samples of medicinal plants for skin diseases and folk cosmetics [8]

Name of Plant	Parts used	Preparation and Application	Healing
Achyranthes aspera L.	Root	Root infusion is taken orally.	Pimples and boils
Adiantum capillus veneris L.	Aerial parts	Decoction of aerial parts is taken orally.	Measles
Ajuga bracteosa Wall.	Aerial parts	Juice of fresh aerial parts is taken orally before breakfast	Gingivitis and boils
Allium cepa L.	Bulb	Slightly warm paste of bulb in mustard oil is tightening over boils and warts for a night.	Boils and warts
Aloe vera auct. non Mill	Leaf pulp	Fresh pulp is layered for a day.	Boils
Amaranthus viridis L.	Leaves	Leaves are smeared with ghee and warmed slightly. These warmed leaves are applied topically	Abscess and boils
Argyrolobium roseum	Leaves	Extract of fresh leaves is taken orally before breakfast	Boils and scabies
Arisaema speciosum	Tuber	Single slice of fresh tuber is tightening over boils, warts ringworms and carbuncle for a night.	Boils, warts, carbuncle
Berberis lycium Royle	Bark	Infusion of fresh or dried bark is taken orally before breakfast.	Pimples, boils
Bergenia ciliata	Rhizom	Powder of dried rhizome is sprinkled on wounds.	Wound healing
Citrus limon (L.)	Fruit	Juice of fruit is applied on face at night.	Face spots and for face freshness

Table 1.1 (Continue)

Name of Plant	Parts used	Preparation and Application	Application
Clematis grata	Leaves	Leaves are crushed and applied directly	Eczema, warts, carbuncles
Colchicum luteum	Tuber	Powder of dried corms is mixed with ghee and this paste is applied topically	Inflammation, muscular pain
Cucumis sativus	Fruit	Thin slices of fruit are placed on face at nigh	Face freshness
Dalbergia sissoo Roxb.	Leaves	Fresh leaves are crushed and mixed with egg. This paste is used to remove dandruff.	Anti dandruff
Ficus virgata	Latex	Latex is mixed with milk. This paste is applied directly.	Pimples, skin burns
Fumaria indica	Aerial parts	Decoction of dried aerial parts is taken orally.	Pimples, skin burns, scabies
Lycopersicon esculentum	Fruit	Paste of fruit is applied topically.	Face freshness, to remove spots
Vitex negundo	Leaves	Leaves are crushed and directly applied.	Skin infection
Zizyphus nummularia	Leaves	Juice of fresh leaves is taken orally.	Boils, pimples

As we know, skin is the largest organ of the body. It serves many important functions, including protection, percutaneous absorption, temperature regulation, fluid maintenance, sensory and disease control. Human skin can be classified into six types according to sensitivity to sunlight as shown in Table 1.2. The most sensitive skin type is type I, which has the least extent of melanin and the lightest skin complexion. The melanin content in the skin increases from type I to VI with a darker skin complexion and less sensitivity to sunlight. Skin color can also be classified according to race or nationality as shown in Table 1.3.

Table1.2 Human skin types [9]

Type	Unexposed skin color	Classification	Reaction to sun	Example
I	White	Sensitive	always burns easily, never tans	Red-haired, freckled
II	White	Sensitive	always burns easily, never tans minimally	Fair-skinned, blue-eyed
III	White	Normal	always burns easily, never tans	Darker whites
IV	light brown	Normal	burns minimally, tans always	Mediterranean
V	brown	Insensitive	rarely burns, tans profusely	Middle eastern, Latin American, light-skinned blacks
VI	dark brown	Insensitive	never burns, deeply pigmented	Dark-skinned blacks

Table1.3 Skin color of various nationalities. [10]

Caucasian	Members of the white race as composed of persons of European, North African or Southwest Asian ancestry.
Mongoloid	Members of a major racial group native to Asia including people of Northern and Eastern Asia, Malaysians, Eskimos and often American Indians.
Negroid	Members of the black race as composed of the majority of the people of Africa, Melanesia and New Guinea.

Thus, the biological activities for the active ingredient which use in cosmetic have been largely interesting such as, anti-oxidant activity, tyrosinase inhibition activity, UV-screening, anti-inflammatory activity and etc. The active ingredients can be combined or separated depended on the application or needed of the consumer.

Biological activity for cosmetic and skin product

Tyrosinase inhibitor

Melanin is one of the most widely distributed pigments and found in bacteria, fungi, plants and animals. It is a heterogeneous polyphenol-like biopolymer with a complex structure and color varying from yellow to black [11]. The color of mammalian skin and hair is determined by a number of factors, the most important of which is the degree and distribution of melanin pigmentation. Melanin is secreted by melanocyte cells distributed in the basal layer of the dermis [12]. The role of melanin is to protect the skin from ultraviolet (UV) damage by absorbing UV sunlight and removing reactive oxygen species (ROS). Various dermatological disorders result in the accumulation of an excessive level of epidermal pigmentation. These hyperpigmented lentigenes include melasma, age spots and sites of actinic damage [13]. Great interest has been shown in the involvement of melanins in malignant melanoma, the most life-threatening skin tumors. The type and amount of melanin synthesized by the melanocyte and its distribution in the surrounding keratinocytes determine the actual color of the skin. The characteristic skin patterns of zebra, giraffes and piebald animals in general are due to this uneven distribution of melanocytes. Melanin is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of tyrosinase.

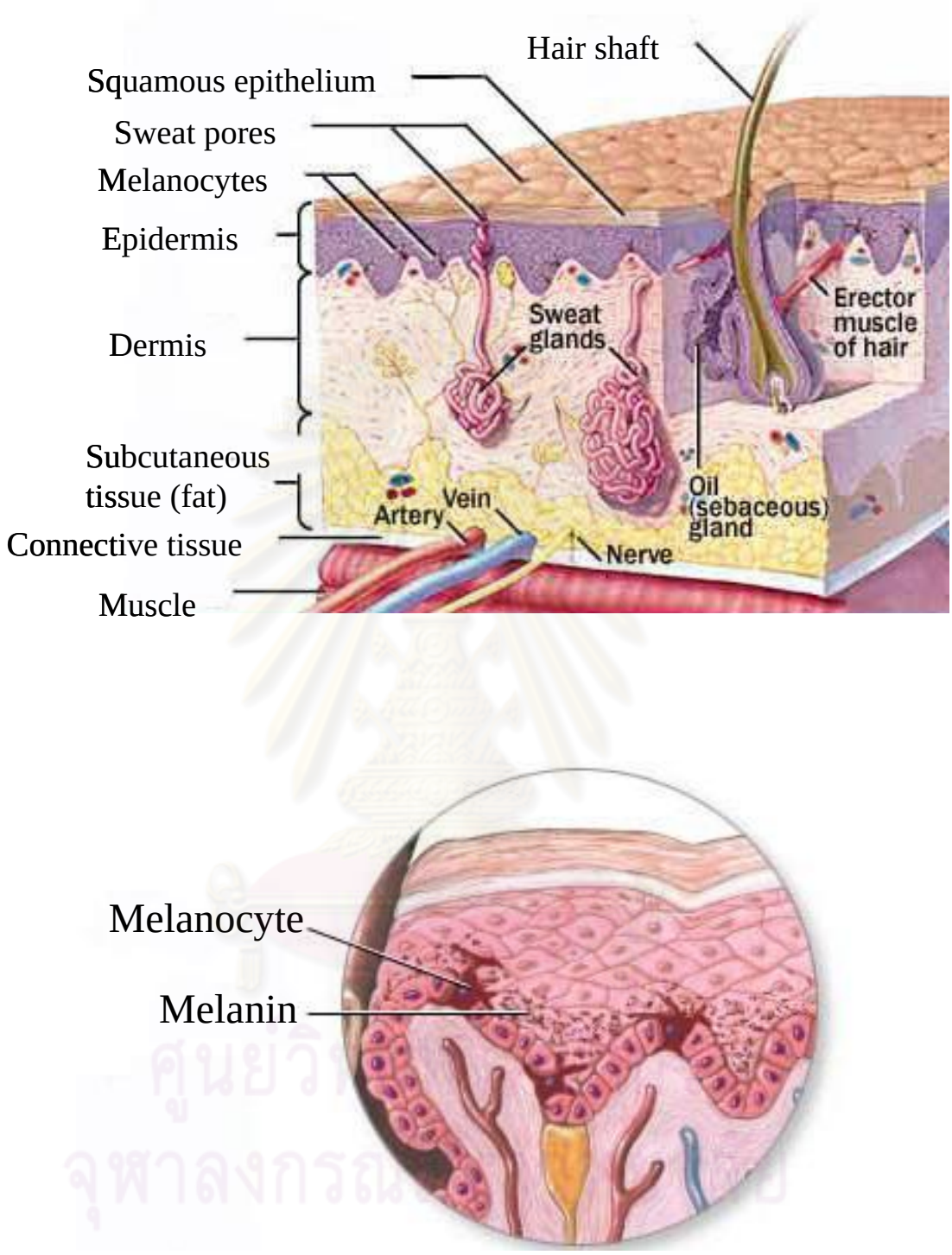


Figure 1.2 Diagram layers of human skin and melanin in melanocyte of the skin.

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions of melanin synthesis: the hydroxylation of tyrosine by monophenolase action and the oxidation of 3,4-dihydroxyphenylalanine (L-DOPA) to *o*-dopaquinone by diphenolase action. However, if L-DOPA is an active cofactor, its

formation as an intermediate during *o*-dopaquinone production is still controversial. *o*-dopaquinone is unstable in aqueous solution and rapidly suffers a non-enzymatic cyclization to leukodopachrome, which is further oxidized non enzymatically by another molecule of *o*-dopaquinone to yield dopachrome and one molecule of regenerated L-DOPA [15–16]. Tyrosinase exists widely in plants and animals, and is involved in the formation of melanin pigments [17–19]. In the food industry, tyrosinase is a very important enzyme in controlling the quality and economics of fruits and vegetables [20–21]. Tyrosinase catalyzes the oxidation of phenolic compounds to the corresponding quinones and is responsible for the enzymatic browning of fruits and vegetables. In addition to the undesirable color and flavor, the quinine compounds produced in the browning reaction may irreversibly react with the amino and sulfhydryl groups of proteins. The quinone-protein reaction decreases the digestibility of the protein and the bioavailability of essential amino acids, including lysine and cysteine. Therefore, development of high-performance tyrosinase inhibitors is much needed in the agricultural and food fields. Tyrosinase is involved in melanogenesis, wound healing, parasite encapsulation and sclerotization in insects [22-24].

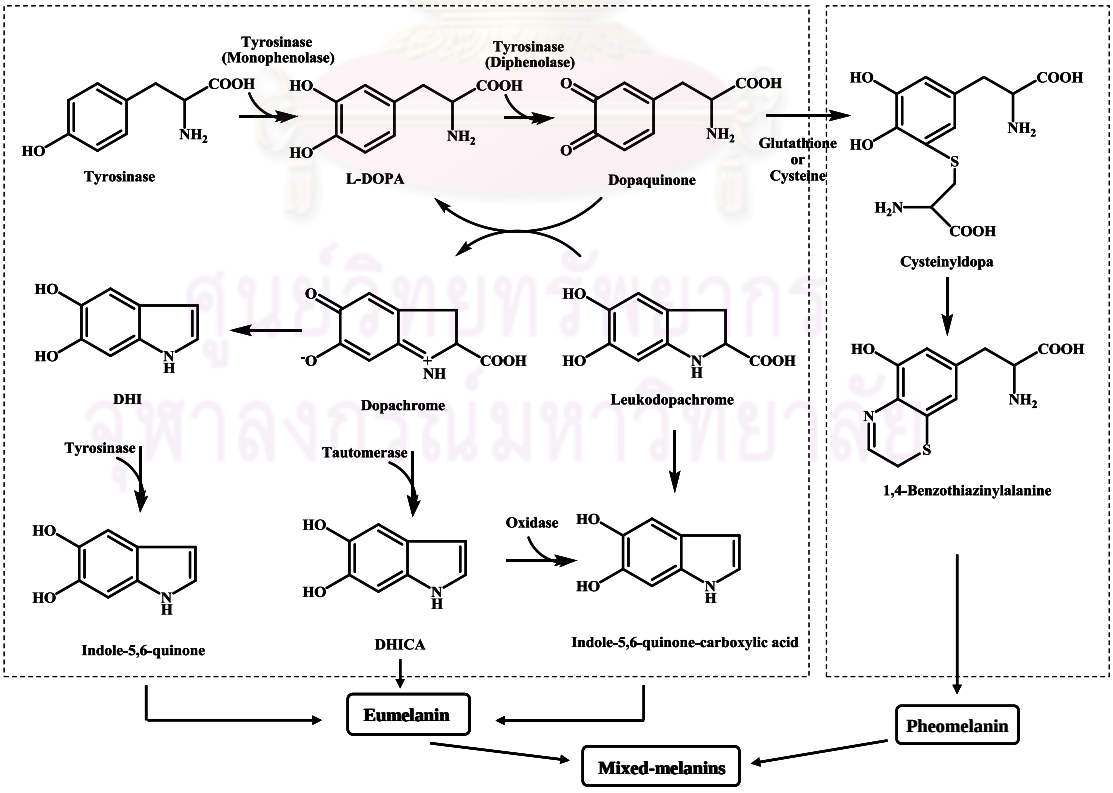


Figure 1.3 Melanin biosynthesis.

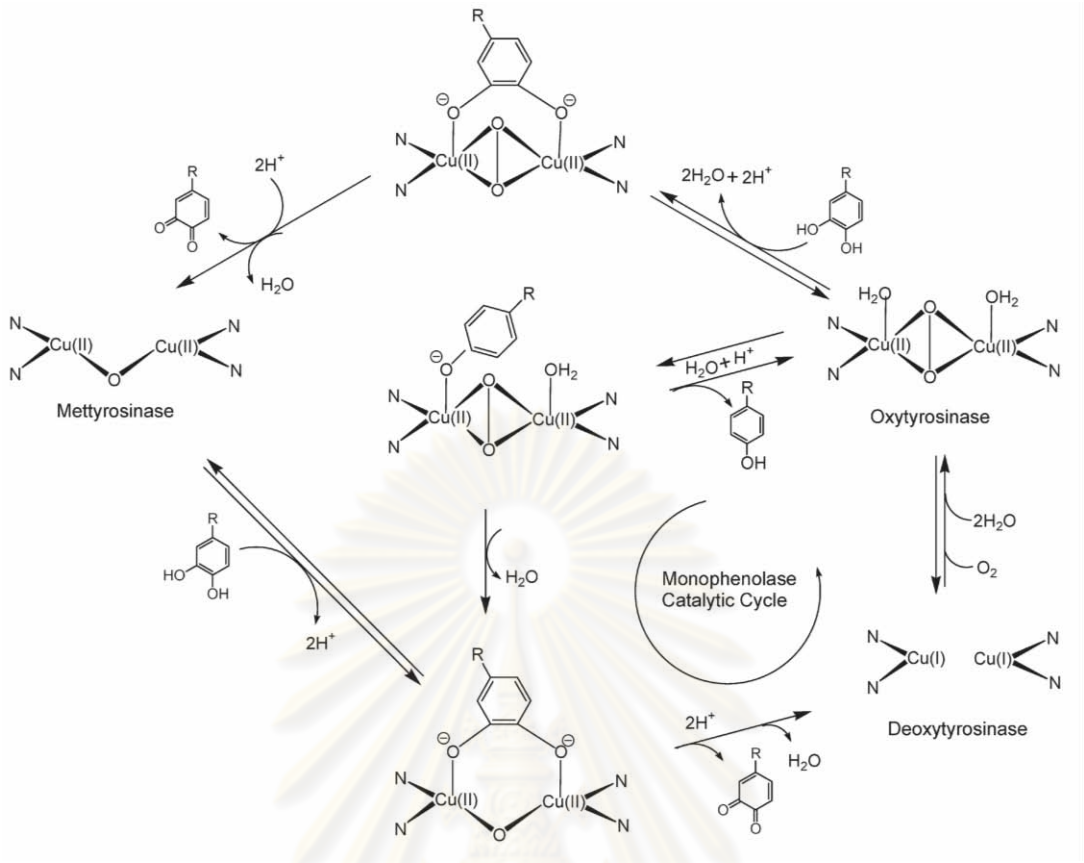


Figure 1.4 Catalytic cycles of the hydroxylation of monophenol and oxidation of *o*-diphenol to *o*-quinone by tyrosinase.

The tyrosinase enzyme, that controls the synthesis of melanin, is a unique product of melanocytes. It is considered to be the rate-limiting enzyme for the biosynthesis of melanin in epidermal melanocytes. Therefore, tyrosinase activity is thought to be a major regulatory step in melanogenesis. Among inhibitors, a distinction could be made between chelatetors (competitive with respect to oxygen) and substrat analogues (competitive towards phenol and/or diphenol substrates). However, such a classification is purely indicative, as many inhibitors cannot be ascribed to a particular group and many of them behave as mixed-type inhibitors (competitive/noncompetitive). Moreover, tyrosinase inhibitors can categorize by source; synthetic and natural. Recently, tyrosinase has become increasingly important for medicinal and cosmetic products that may be used to prevent or treat pigmentation disorders.

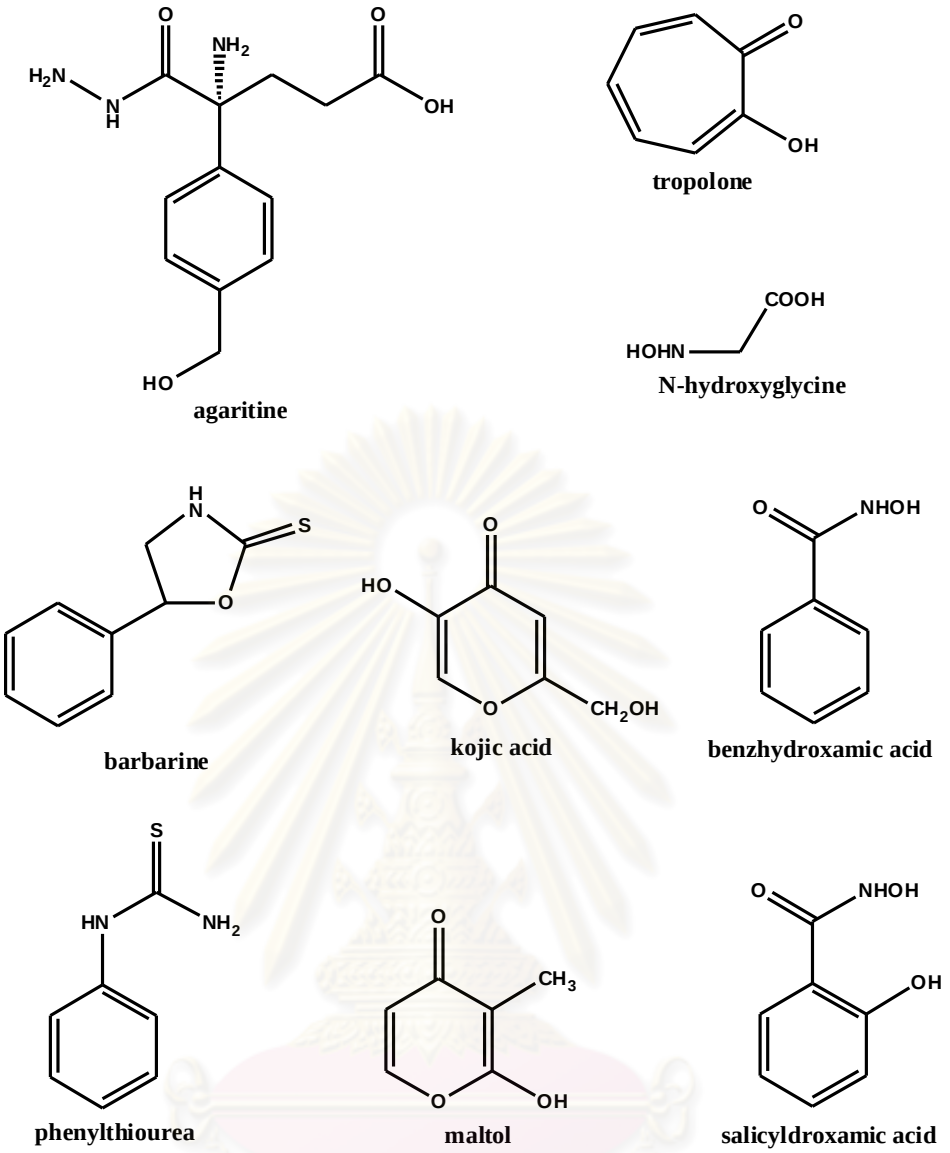


Figure 1.5 The structure of some tyrosinase inhibitors, those presumably act as copper chelators.

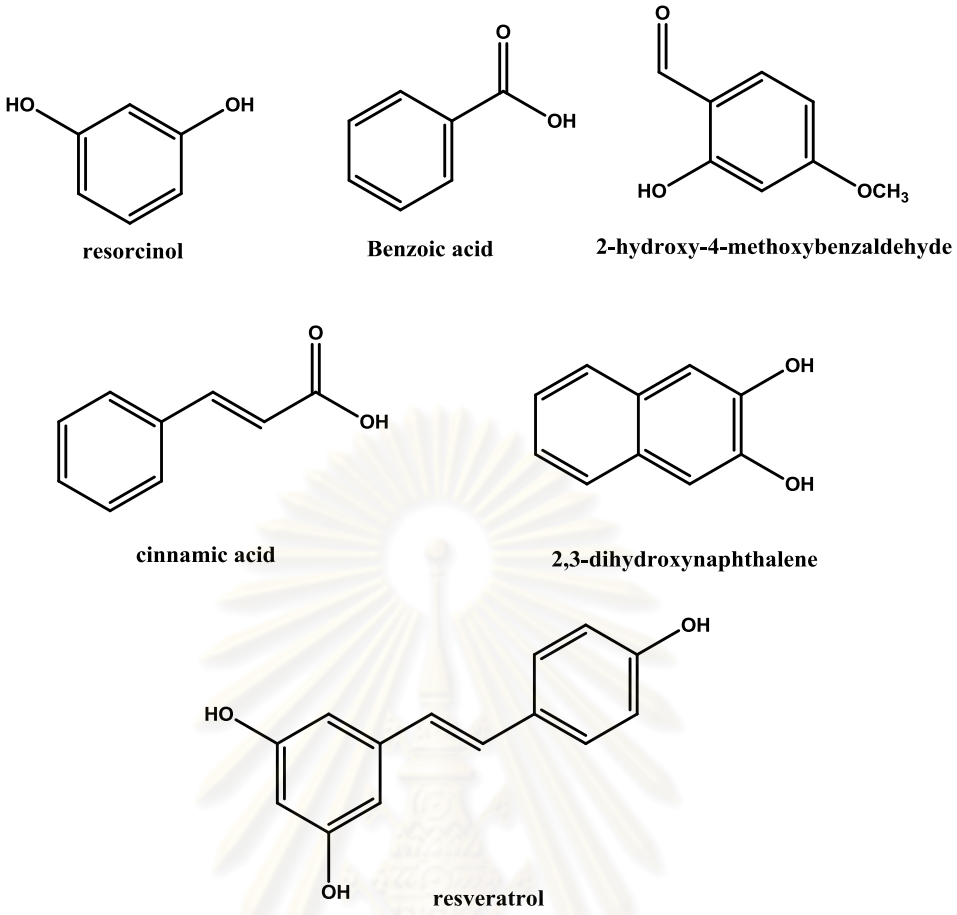


Figure 1.6 The structure of some tyrosinase inhibitors, those acts as substrate analogue.

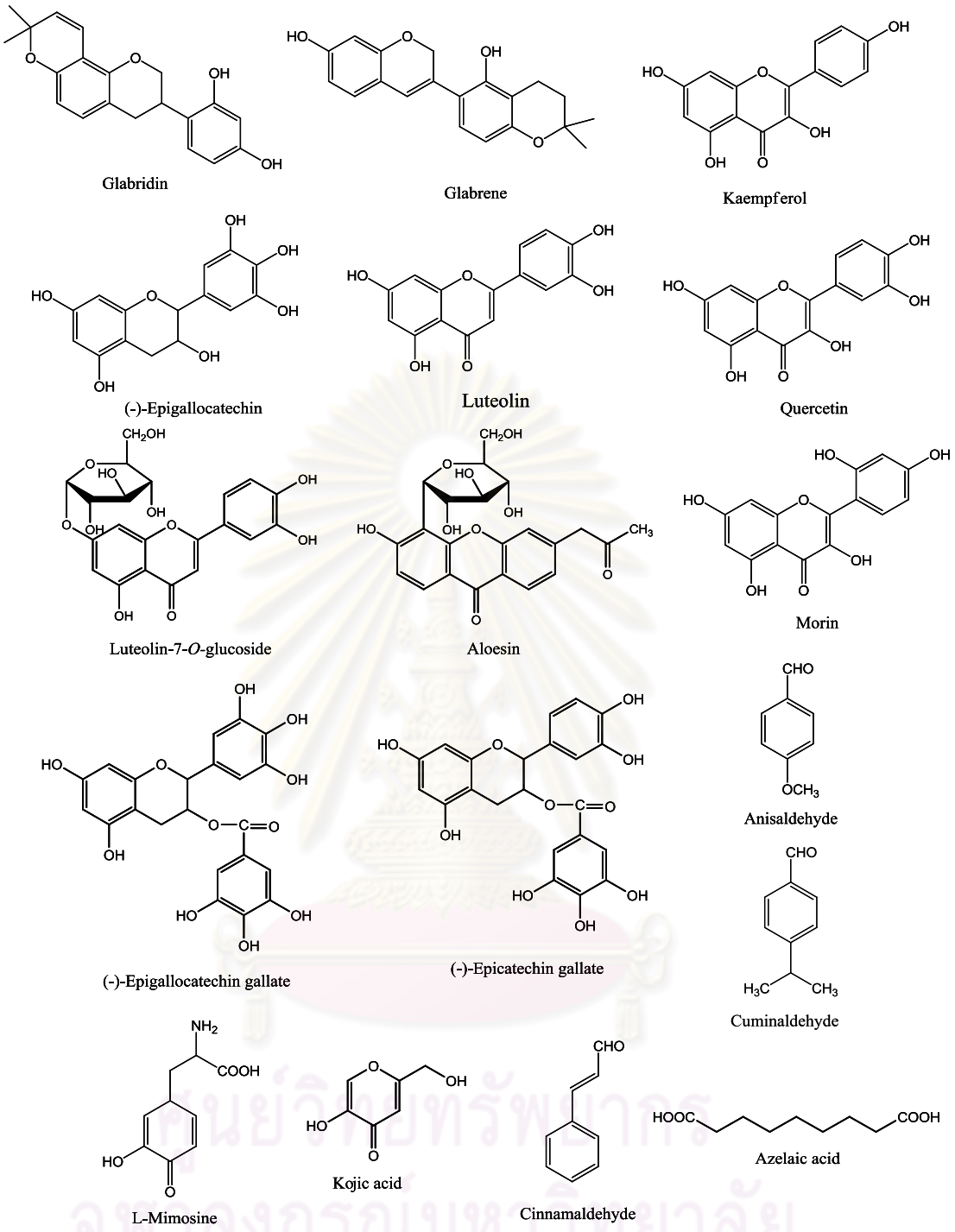


Figure 1.7 Structures of tyrosinase inhibitor from natural sources.

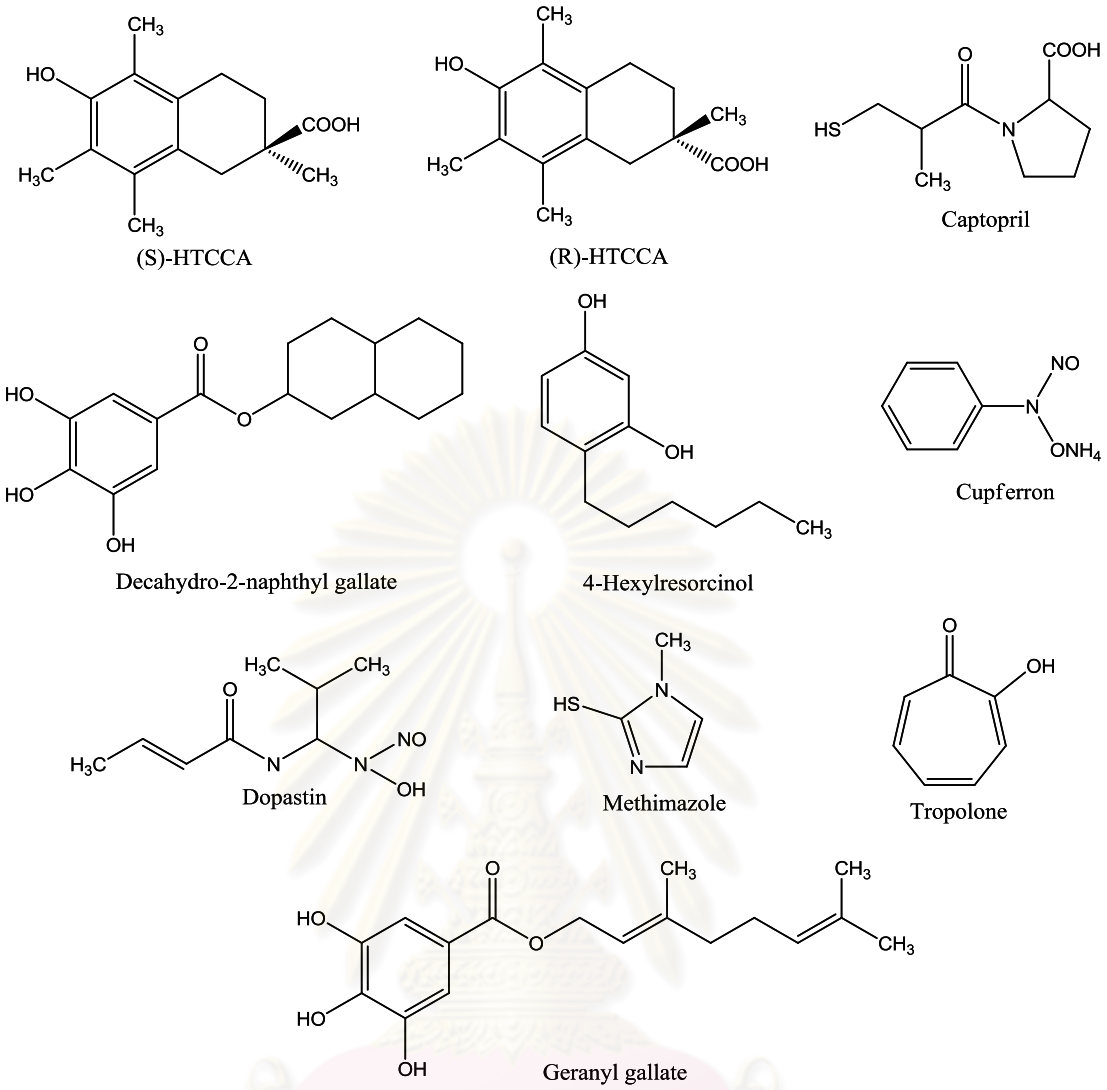


Figure 1.8 Structures of tyrosinase inhibitor from synthetic sources and derivatives of natural compound.

Tyrosinase inhibitor for cosmetics and medicine

Use of tyrosinase inhibitors is becoming increasingly important in the cosmetic and medicinal industries due to their preventive effect on pigmentation disorders. Tyrosinase inhibitors may result in a reduction in melanin biosynthesis and are used in cosmetic products for hyperpigmentation- related concerns, including the formation of freckles. Tyrosinase and its inhibitors may also be targets for developing medicines to treat hypopigmentation-related problems, such as albinism and piebaldism. A number of tyrosinase inhibitors have been reported from both natural and synthetic sources, but only a few of them are used as skin-whitening agents, primarily due to various safety concerns. Among the skin-whitening agents, 1,4-

dihydroquinone is one of the most widely prescribed [27-29]. It causes reversible inhibition of cellular metabolism by affecting both DNA and RNA synthesis. It is also a poor substrate for tyrosinase, thereby competing for tyrosinase oxidation in active melanocytes. Hence, 1,4-dihydroquinone can be considered to be a potent melanocyte cytotoxic agent and has also been reported to induce mutations. As a result of these and other side effects, such as chronosis in African nations [30], the use of 1,4-dihydroquinone has been banned in cosmetics by most countries, and there has been increasing pressure to find alternative herbal and pharmaceutical depigmentation agents. Currently arbutin and aloesin are used in the cosmetic industry as skin-whitening agents because they show strong inhibition toward tyrosinase, which is responsible for pigmentation in human beings. *b*-Arbutin inhibited both tyrosinase activities from mushroom and mouse melanoma noncompetitively, while *a*-arbutin only inhibited tyrosinase from mouse melanoma by mixed-type inhibition [31]. On the other hand, the co-treatment of arbutin and aloesin exhibited the inhibitory effect on tyrosinase in a synergistic manner by acting through different mechanisms: arbutin inhibited competitively, whereas aloesin inhibited noncompetitively [32]. Taken together they inhibit melanin production synergistically by a combined mechanism of competitive and noncompetitive inhibition. This result indicates that it is beneficial to use arbutin and aloesin as a mixture in depigmentation because the co-treatment reduces the doses of these agents for the same inhibitory effect on tyrosinase activity and can diminish adverse side effects. In addition, methimazole has proven effective as a depigmenting agent both in vitro and in vivo, and is noncytotoxic and nonmutagenic. Therefore, methimazole could serve as a lead compound for the discovery of safe and efficient skin depigmenting agents in the future [33].

UV-screening activity

Ultraviolet (UV) radiation is a form of electromagnetic radiation with a wavelength shorter than visible light but longer than soft X-ray. The various forms of energy, or radiation, are classified according to wavelength, measured in nanometer (nm). The effect of UV radiation with shorter the wavelength is more energetic the radiation. In order of decreasing energy, the principal forms of radiation are gamma rays, X rays, ultraviolet (UV) radiation, visible light, infrared (IR) radiation, microwaves and radio waves. Most energy from the sun is in the form of visible and UV radiation. There are three categories of UV radiation; UV-A (wavelength between

320 and 400 nm), UV-B (wavelength between 280 and 320 nm) and UV-C (wavelength between 200 and 280 nm). Generally, UV at the shorter wavelength is more biologically damage UV radiation can be if it reaches the Earth in sufficient quantities. UV-A is the least damaging (longest wavelength) form of UV radiation and reaches the Earth highest quantity. Most UV-A rays pass right through the ozone layer in the stratosphere. UV-B radiation can be very harmful but most of the UV-B radiation is absorbed by ozone in the stratosphere. UV-C radiation is potentially the most damaging because it is very energetic but all of UV-C is absorbed by oxygen and ozone in the stratosphere and never reaches the Earth's surface [34].

The exposure of UV radiation may affect in acute and chronic health of the skin, eyes and immune system [35]. All UVA, UVB and UVC can damage the collagen fibers which can be the cause of wrinkle on the skin. In general, the least harmful is UVA which can penetrate deeply, but it can cause the aging of the skin, DNA damage and possibly skin cancer. UVA dose not contribute sunburn. UVB can penetrate in to the skin lower than UVA, but UVB may cause skin cancer. The radiation excites DNA molecules in skin cells, contributing covalent bonds to form between thymine and thymine base, producing thymidine dimmer (Figure 1.)[36-40]. Thymidine dimers are an abnormal base pair which can cause the DNA helix distortion, stalled replication, gaps and misincorporation. These can lead the mutation which can result the growth of cancer. Such mutations are found in various genes in DNA from human skin cancers and form the molecular basis for the accepted view that inappropriate exposure to UV radiation increases risk of the common skin cancers, malignant melanoma, squamous cell carcinoma and basal cell carcinoma [41-45]. The mutagenicity of UV radiation can be easily observed in bacteria cultures [46]. The cause of cancer by UV radiation is one of the reasons to concern about the ozone opening.

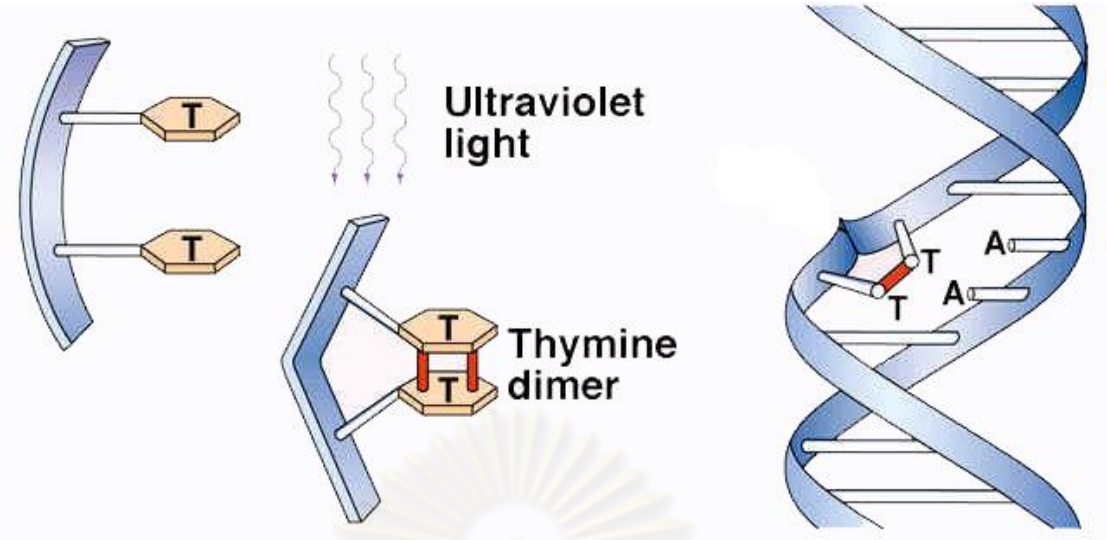


Figure 1.9 The ultraviolet photons harm the DNA molecules of living organism.

Following the cancer, sunburn, aging, and freckle can cause by UV radiation which is explain above, there are many ways to protect the cancer and skin problem, one of the easy ways is to use sunscreen. Sunscreen works by filtering UV radiation with a chemical barrier which absorbs and/or scatters the UV radiation away from the skin. Sunscreens are applied superficially to the skin, and are designed to remain on the uppermost layers with minimal penetration [47-48]. Sunscreens have traditionally been divided into chemical absorbers and physical blockers on the basis of their mechanism of action. Several of chemical have been used as the UV screening agent, in the commercial sunscreen products (Table 1.). The active UV screening are not only from chemical synthesis but also found in natural products such as, Scytonemin from *Scytonema* sp., *Nostodione* A and *Prenostodione* from *Nostoc* sp. Mycosporine-like amino acid from *Heterocapasa* sp., usnic acid from lichen extract, epigallocatechin-3-gallate (EGCG) and silibinin from milk thistle[49-55].

Table 1.4 UV screening agents

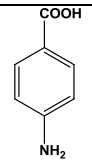
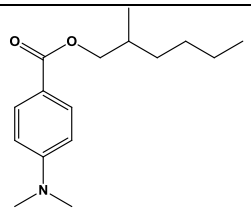
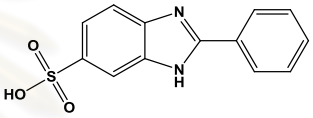
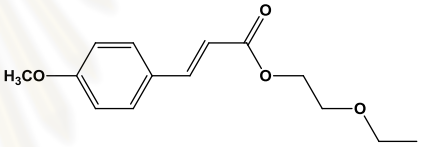
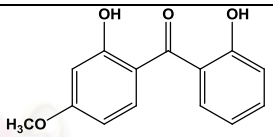
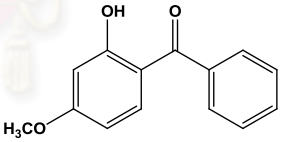
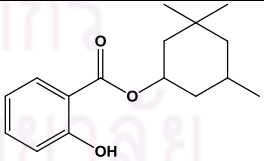
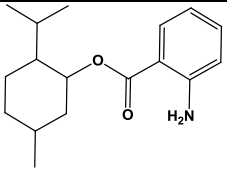
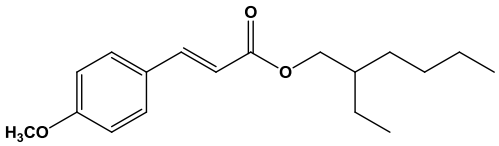
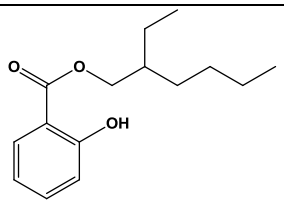
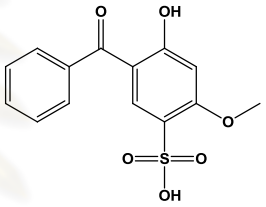
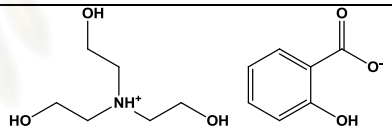
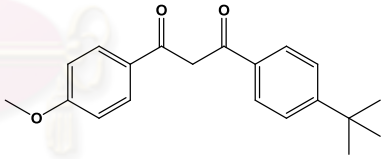
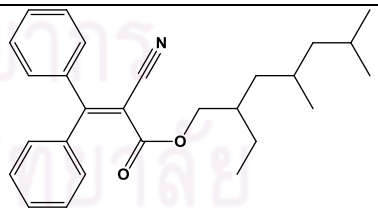
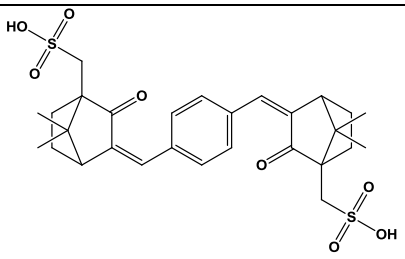
Name	Absorbance range	Structure
4-Aminobenzoic acid, PABA	UVB	
OD-PABA, octyldimethyl-PABA, Padimet O	UVB	
Phenylbenzenimidazole sulfonic acid, Ensulizole, Eusolex 232, PBSA, Parsol HS	UVB	
2-Ethoxyethyl p-methoxycinnamate, Cinoxate	UVB	
Dioxybenzone, Benzophenone-8	UVA,UVB	
Oxybenzone, Benzophenone-3, Eusolex 4360, Escalol 567	UVA, UVB	
Homosalate ,Homomethyl salicylate, HMS	UVB	
Menthyl anthranilate,Meradimate	UVA	

Table 1.4 (Continue)

Octyl methoxycinnamate, Octinoxate, EMC, OMC, Ethylmethoxycinnamate,Escalol 557, 2-ethylhexyl paramethoxy cinnamate, Parsol MCX	UVB	
Octyl salicylate, Octisalate, 2-Ethylhexyl salicylate, Escalol 587	UVB	
Sulisobenzene, 2-Hydroxy-4-Methoxybenzophenone-5-sulfonic acid, 3-benzoyl-4-hydroxy-6-methoxybenzenesulfonic acid, Benzophenone-4, Escalol 577	UVA, UVB	
Trolamine salicylate, Triethanolamine salicylate	UVB	
Avobenzone, 1-(4-methoxy-phenyl)-3-(4-tert-butyl phenyl)propane-1,3-dione, butyl methoxy dibenzoyl-methane,	UVA	
Octocrylene, Eusolex OCR, 2-cyano-3,3diphenyl acrylic acid, 2-ethylhexylester	UVB	
Ecamsule, Mexoryl SX, Terephthalylidene Dicamphor Sulfonic Acid	UVA	
Titanium dioxide	Physical	ZnO
Zinc oxide	Physical	TiO_2

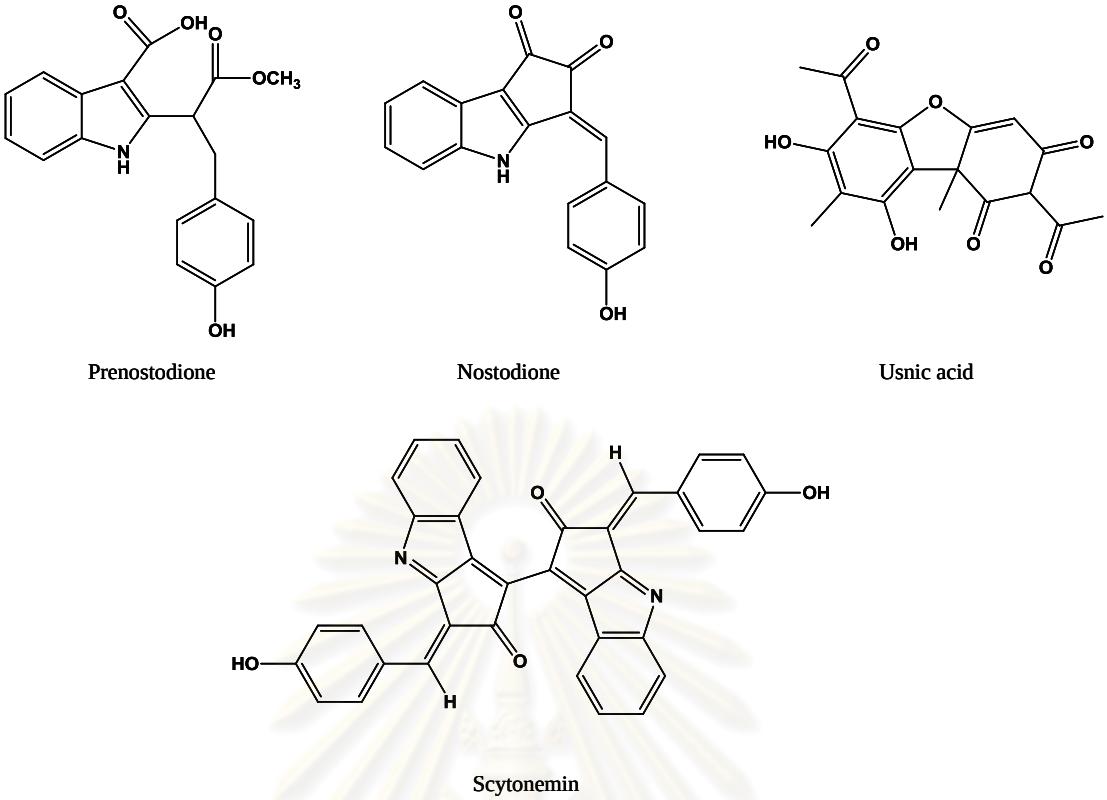


Figure 1.10 Natural compound contained UV screening activity

Anti-oxidant activity

Nowadays, antioxidant became well known for health-conscious consumers [56]. Reactive oxygen species and free radical are generated continuously via normal physiological processes. Free radicals are highly reactive molecules with an unpaired electron that result in damage to surrounding molecules and tissues. The most significant damage by free radicals is to biomembranes and to DNA. Reactive oxygen intermediates are partially reduced forms of atmospheric oxygen. They typically effect from excitation of oxygen to singlet oxygen or from transfer of one, two or three electron to form a superoxide radical, hydrogen peroxide or hydroxyl radical, respectively. Oxidative stress is involved in the pathology of cancer, arteriosclerosis, malaria and rheumatoid arthritis, and could play a role in neurodegenerative diseases and ageing processes [57]. Active oxygen species play an important physiological role and, at the same time, they may also exert toxic effect. The active oxygen species are necessary for energy production, essential biological component synthesis, phagocytosis and critical process of immune system. They also play a vital role in signal transduction which is important for cell function and communication. Thus, various antioxidants, such as vitamin C, vitamin E, α-tocopherol, β-carotene and

polyphenolic compounds, may protect against several diseases which mention above [58]. Biological antioxidants, especially vitamin E, were the first studied [59]. In human body has antioxidant system which generate the antioxidant to remove the free radicals are from the body system. The antioxidants which produce in the human body (endogenous) can divide in to 2 groups; (1) the enzymatic defense, such as superoxide dismutase, glutathione peroxidase and catalase, which metabolite peroxide, hydrogen peroxide and lipid peroxide, and (2) non-enzymatic defense, such as glutathione, histidine-peptide, the iron-binding protein transferin, ferritin, dihydrolipic acid, decreased melatonin and CoQ_{10}. Moreover, there are dietary antioxidants (exogenous) such as α-tocopherol, β-carotene, astaxanthin and ascorbic acid, which can found in fruits and vegetables. However, self-defense is not sufficed by excessive generation of free radicals, which can be operating from external factors (environmental insults, smoking). Hence, dietary antioxidant intake becomes important maintain adequate antioxidant status [60-63]. The dietary antioxidant is classified into 2 categoties; synthetic antioxidant and natural antioxidant. In general, the more frequently used are synthetic antioxidant like; butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ) (Figure 1.) But there are some reports reveal that BHA and BHT could be toxic, and the higher manufacturing costs, together with the increasing consciousness of consumers with regard to food additive safety, created a need for alternative natural and safer sources of food antioxidants [64-65]. The replacement of synthetic antioxidants by natural ones may have benefits due to health implications and functionality in food so the natural antioxidants turn to be a good choice. The natural antioxidants can be phenolic compounds, nitrogen compounds or carotenoids as well as ascorbic acid [66-69].

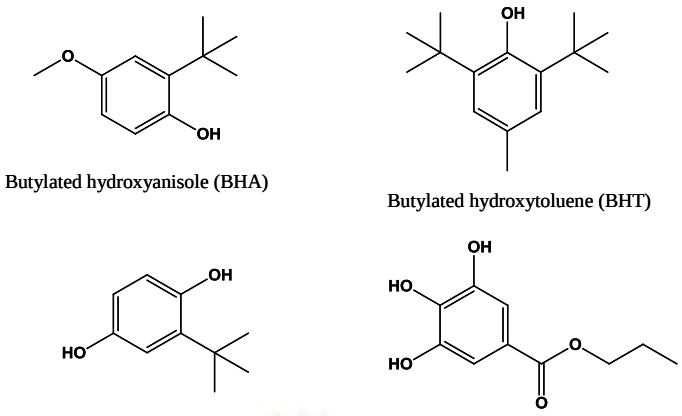


Figure 1.11 The synthetic antioxidants

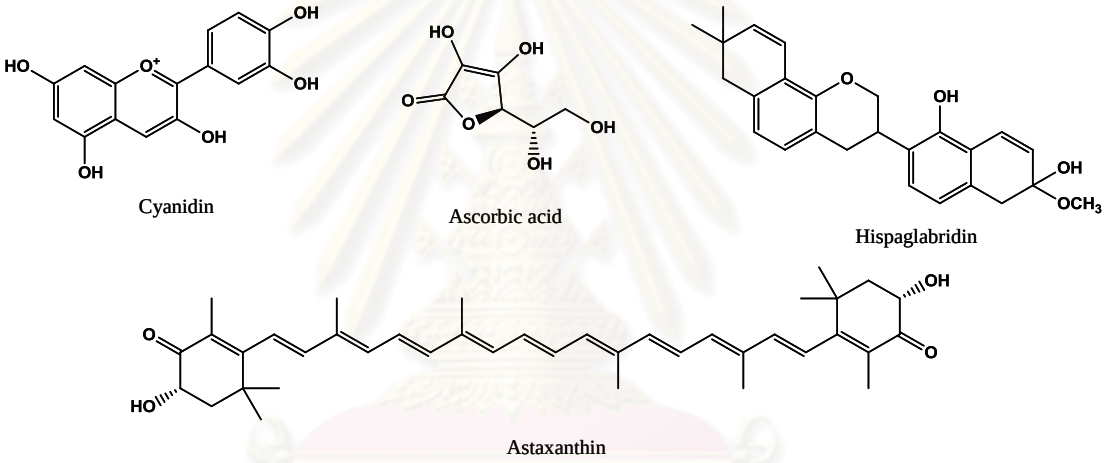


Figure 1.12 The natural antioxidants

Antioxidant in cosmetics

Antioxidants have been proposed, over the last decade, as functional ingredients for anti-aging preparations and to prevent and modulate oxidative skin damages. Cosmetics are commercially available products that are used to improve the appearance of the skin. Since the late 1980s, consumer demand for more effective products that more substantively beautify the appearance has resulted in increased basic science research and product development in the cosmetics industry. The result has been more ingredients that may actually improve not just the appearance of the skin, but the health of the skin as well [70].It is thought that additional, topical use of vitamins and antioxidants in cosmetics can better protect and possibly correct the damage by neutralizing these free radicals. In addition, some vitamins may be

beneficial to the skin because of other actions such as effects of suppression of pigmentation and bruising, stimulation of collagen production, refinement of keratinization, or anti-inflammatory effects [71]. There are many antioxidants which usually use in the cosmetics such as retinol, panthothenic acid and ascorbyl palmitate.

Vitamin A

Vitamin A, its derivatives, and beta-carotene (pro vitamin A) have been accepted additives in cosmetics for years. The popular derivatives of vitamin A that are establish in cosmetics include vitamin A alcohol (retinol), vitamin A esters (retinyl palmitate, retinyl acetate), vitamin A aldehyde (retinal), and tretinoin (retinoic acid). This family of compounds called retinoids has been exposed to have receptor-specific effects on the skin, resulting in decreased roughness and decreased facial wrinkling. Nonetheless, there are data to support some benefit of topical retinyl palmitate on the skin. It has been found to boost epidermal thickness in human skin [72]. Of course the gold standard for retinoid activity on the skin is all-trans-retinoic acid, or tretinoin. Tretinoin was first used for acne, and Kligman first noted and reported its benefits on photoaged skin [73-75]. When used on photodamaged skin, tretinoin's clinical effects included improvement of wrinkling and roughness, and lightening of lentigines and melasma [76–78].

Vitamin C

Vitamin C, ascorbate, a water-soluble vitamin, is important because of its antioxidant actions as well as its function as a cofactor in hydroxylation reactions of collagen production. Humans are unable to synthesize ascorbate, so dietary intake is essential. Interest in vitamin C as a cosmetic ingredient is partly a result of it ability to quench UV induced free radicals directly, and to regenerate vitamin E, another potent antioxidant [79-80]. Vitamin C is also considered an antiaging ingredient because of its potential to stimulate collagen production. There are three forms commonly presented in cosmetics: ascorbyl palmitate, magnesium ascorbyl phosphate, and L-ascorbic acid

Antibacterial from natural products

Microorganisms, including Gram positive and Gram negative bacteria have been recognized as the main causers of various human infections. Thus, antibacterial drugs were discovered. Discovery of penicillin stimulated the search for more antibiotics, including antibacterial. An antibacterial, is a compound or substance that

kills or slows down the growth of bacteria, can injure microbes through several different mechanisms. Antibacterial function specifically in one of the following ways; inhibition of cell wall synthesis, inhibition of nucleic acid synthesis or function, inhibit protein synthesis and interfere the function of cell membrane. These ways are not completely discrete, and some effect can be overlap.

Antibacterial from natural sources become the alternative after the problem of antibacterial resistance has grown. There are thousands of scientific publications describing the antibacterial from natural products. The natural products contained antibacterial activity; such as nigrolineaxanthone F, latisxanthone D and brasilixanthone from *Garcinia nigrolineata*, hyperforin from *Hypericum perforatum* , berbirine from *Berberis vulgaris*, curcumin from *Curcuma longa* and gingerol from *Zingiber officinale*. Moreover, essential oils and plant extracts were also demonstrated antibacterial activity; such as tea tree oil, lavender oil and cinnamon oil []. Antibacterial from plants, which normally use in cosmetic are chamomile oil, eucalyptus oil and hinokitiol [81-85].

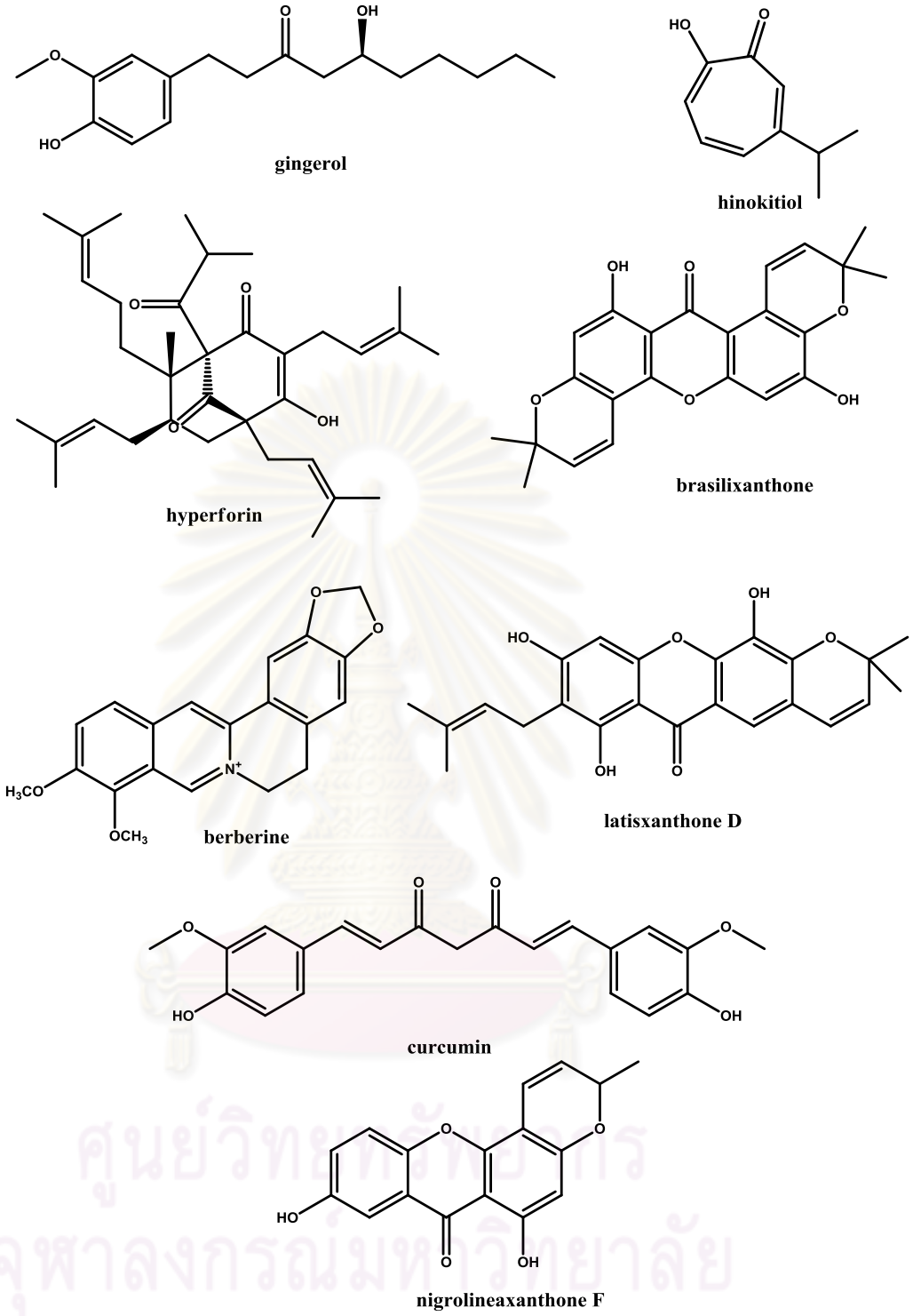


Figure 1.13 Antibaterial from natural products

Anti-inflammatory from natural products

Inflammation is an important nonspecific defense reaction to tissue injury, such as that caused by pathogen or wound. Acute inflammation is the immediate response of the body to death cell or injury cell. Chronic inflammation is a slow

process characterized by the formation of new connective tissue, and it normally causes permanent tissue damage. The different between acute and chronic inflammatory is one of the duration. The signs of inflammation include redness, warmth, pain, swelling and altered function. Anti-inflammatory refers to the ability of compound or substrate which decreases inflammation. The natural products contained anti-inflammatory activity; such as honokiol and magnolol from *Magnolia* sp., retuline and isoretuline from *Strychnos henningsii*. Anti-inflammatory agents in cosmetic are used to prevent localized inflammation of the skin resulting from external stimuli. Typical anti-inflammatory for cosmetics are β-glycyrrhetinic acid, and allantoin [86-90].

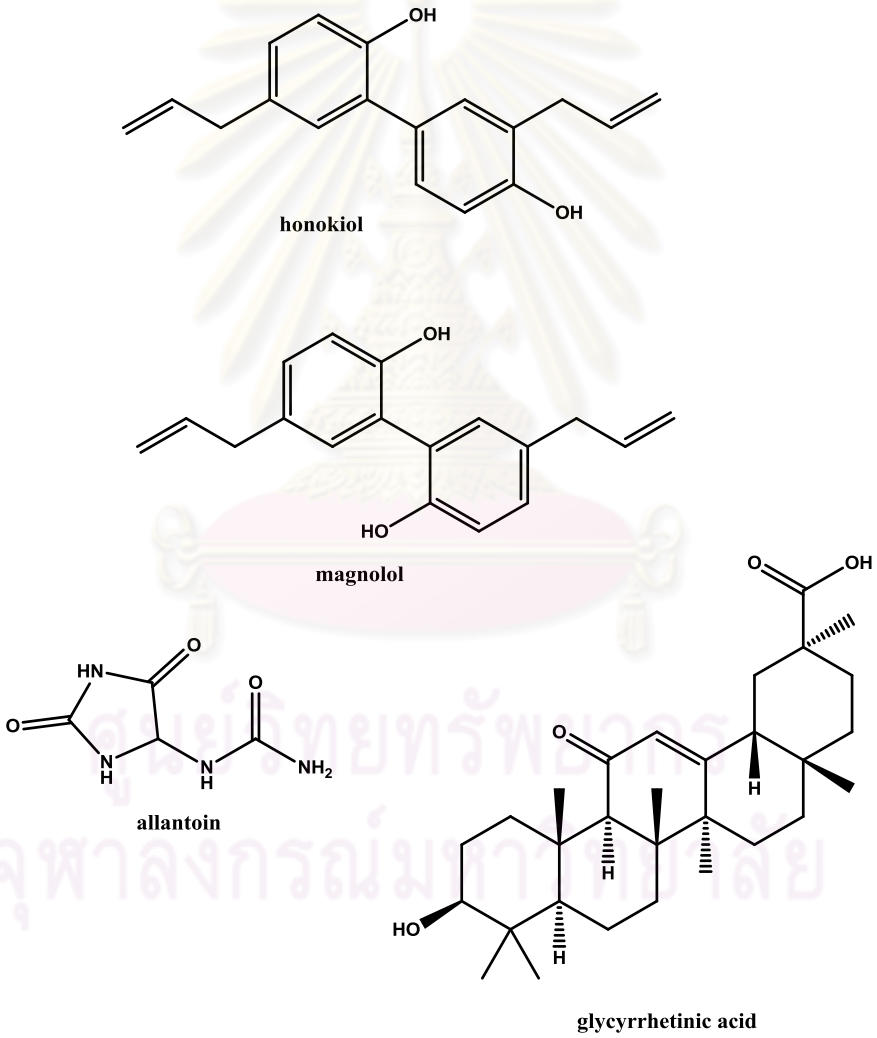


Figure 1.14 Anti-inflammatory from natural products

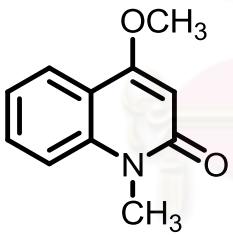
Thanaka stem bark

Hesperethusa crenulata or *Naringi crenulata*, is a common tropical plant species in the Indian subcontinent and south east asia area belonging to the Rutaceae family. In the ancient literature of indigenous system of medicine, various medicinal properties such as purgative, antidote, stomachic and sudorific etc., have been attributed to this plant's preparations [91].

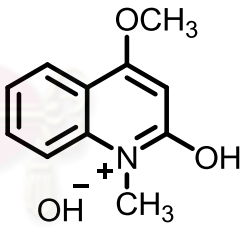
Grounded Thanaka bark, a pale yellow powder, is a traditional skin care commonly applied to faces of Myanmar women. Although there are not many literatures involving researches in chemical constituents and biological activities of *Hesperethusa crenulata* bark, its application in the cosmetic field has increased tremendously. Many Myanmar and Thai cosmetic products have incorporated the thanaka bark powder as their ingredient.

Literature review

In 1971, Nayar *et al.* discovered 2-quinolone and 2-hydroxyquinoline from petroleum extract of *Hesperethusa crenulata* stem bark. However, no biological activity was assigned to these two compounds [92].

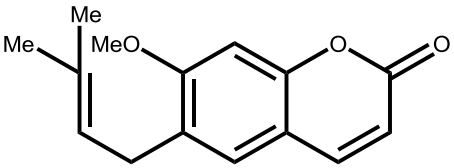


2-quinolone

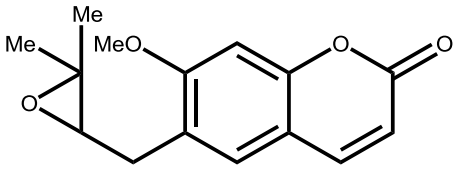


2-hydroxyquinoline

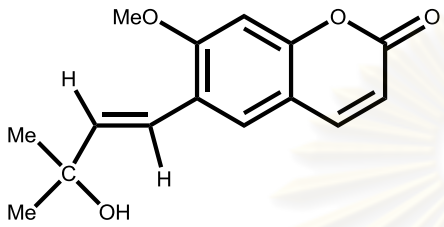
In 1972, Nayar *et al.* purified sitosterol, suberosin, suberenol, 7 methoxy-6-(2,3-epoxy-6-methylbutyl) coumarin**,** 4-methoxy-1-methyl-2-quinolone and marmesin from organic extract of thanaka root bark [93]. Suberosin and marmesin also showed antibacterial and UV absorption property [94-95].



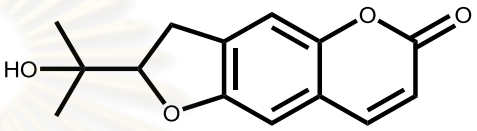
suberosin



7-methoxy-6-(2,3-epoxy-6-methylbutyl) coumarin

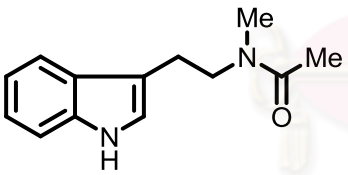


suberenol

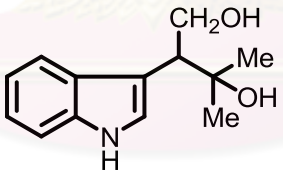


marmesin

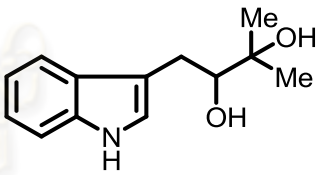
In 1986, Zarga *et al.* found three simple alkaloids including *N*-acetyl-*N*-methyltryptamine, tanakine and tanakamine from stem bark of *Limonia acidissima*. However, no biological activity was reported for these three alkaloids [96].



N-acetyl-*N*-methyltryptamine



tanakamide

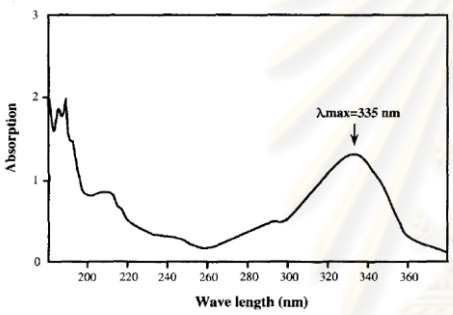


tanakine

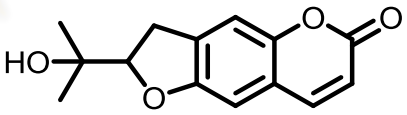
In 1998, Lindsay *et al.* studied the efficiency of mosquito repellent mixture of thanaka root paste and di-methyl benzamide (DEET). The result showed that Karen women preferred using repellent which containing thanaka paste. Apart from a temporary warming sensation at area where repellent thanaka was applied, the repellents were well tolerated. Bioassays using a laboratory strain of *Aedes aegypti* demonstrated that thanaka itself possessed slight mosquito repellent activity at high dosages and the mixture with DEET provided protection for over 10 h [97].

In 2003, Mondal *et al.* purified pectic polysaccharides from fruits of *Naringi crenulata* by extraction with water. The water extract contained large amount of protein. The polymers present in the water extract were fractionated by precipitation with ethanol, anion exchange chromatography, and size exclusion chromatography. Characterization of the subfractions using various chemical and physico-chemical methods revealed pectic polymers [98].

In 2004, Se-Hwan Joo *et al.* purified marmesin from thanaka stem bark and demonstrated that this compound possessed UV-absorbing chromophores, an aromatic ring, a double bond, and a carbonyl group in its structure. The compound absorbs UVA radiation, with λ_{max} at 335 nm [99].

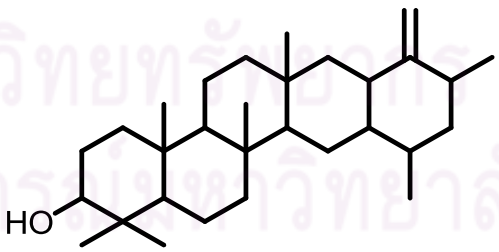


UV absorption of marmesin



marmesin

In 2005, Chitchumnong isolated and purified steroid from hexane extract of the thanaka bark and the extract possessed mild tyrosinase inhibitor activity [100].



1,1,4a,6a,9,11,12b-heptamethyl-8-methylenedocosahydrobenzo[c]tetraphen-2-ol

In 2006, Praiprisankij and Kritsaneepaiboon showed many unpurified fractions of thanaka extract could inhibit tyrosinase enzyme [101].

Objective of this research

The goal of this research is to scientifically verify the basic safety in term of cytotoxicity and genotoxicity of this *Hesperethusa crenulata* stem bark. In addition, other biological activities including antibacterial (*Staphylococcus aureus* and *Escherichia coli*), tyrosinase inhibition activity and anti-inflammatory activity will also be investigated on both extracts of this bark and bark powder. Therefore, objective of this research can be summarized as follows:

1. To identify biological activities and safety in term of cytotoxicity and genotoxicity of thanaka stem bark (*Hesperethusa crenulata*) crude extracts and original powder.

CHAPTER II

EXPERIMENTAL

Materials and Methods

2.1 Materials and Chemicals

Solvents used in spectroscopic techniques, antioxidant activity, UV screening activity and tyrosinase inhibition activity were reagent or analytical grades purchased from Labscan (Bangkok, Thailand). Solvents used in extraction and column chromatography were purified from commercial grade solvents prior use by distillation. Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma Chemical Company (St. Louis, USA). L-Tyrosine was purchased from Fluka Biochemika (Buchs, Switzerland).). Kojic acid used as standard tyrosinase inhibitor was obtained from ACROS Organic (New Jersey, USA). A 1,1-diphenyl-2-picrylhydrazyl (DPPH) used as stable redical in antioxidant activity assay was purchased from Fluka Biochemika (Buchs, Switzerland). Quercetin and Trolox® used as standard antioxidant were purchased from Sigma–Aldrich ChemieGmbH (Steinheim, Germany) and Fluka Biochemika (Buchs, Switzerland), respectively. Agarose and low-melting point agarose used in genotoxic assay were purchased from Research Organics (Cleveland,OH, USA) and Lonza (Basel, Switzerland), respectively.

Media culture for antibacterial study

Tryptic soy broth (TSB) and Tryptic soy agar (TSA) were purchased from Difco laboratories (Detroit, MI, USA). Clindamycin was purchased from Siam Pharmaceutical (Bangkok, Thailand).

Media culture and chemical reagent for cell culture and treatment

Dulbecco's modified minimum essential medium (DMEM) RPMI 1640 media, fetal bovine serum, 100 mM sodium pyruvate, HEPES (4-(2-hydroxyethyl)-1-piperazineethane sulfuric acid) (free acid 1 M solution (238.3 g/L)) and Trypsin-EDTA, were obtained from Hyclone (Utah, USA). MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide) was purchased from USB Corporation

(Ohio, USA). Streptomycin sulphate and Penicillin G (sodium salt) were purchased from M & H manufacturing (Samutprakarn, Thailand). Lipopolysaccharide (LPS; *Samonella minnesota*) used as activator for anti-inflammatory assay was purchased from Sigma–Aldrich ChemieGmbH (Steinheim, Germany). Parthenolide used as standard anti-inflame was purchased from Sigma–Aldrich ChemieGmbH (Steinheim, Germany). Doxorubicin hydrochloride used as cell toxic in cytotoxicity assay was purchased from Sigma–Aldrich ChemieGmbH (Steinheim, Germany).

2.2 Instruments and Equipments

Chemical purification and characterization

^{1}H and ^{13}C NMR spectra were recorded with a Varian Mercury+ 400 (Varian company, CA, USA) which operated at 400 MHz for 1H and 100 MHz for 13C nuclei. The FT-IR spectra were recorded on a Nicolet Fourier Transform Infrared spectrophotometer: Impact 41.0 (Nicolet Instruments Technologies, Inc. WI, USA). Ultraviolet absorption spectra were obtained with the aids of an HP 8453 UV-Vis spectrophotometer (Agilent Technologies, CA, USA), using a quartz cell 1 cm pathlength. ESI-MS analyses were performed with Waters Micromass Quattomicro API ESCi (Waters, MA, USA). The solvent evaporation was operated by BUCHI rotary evaporator R-200 (BUCHI, Flawil, Switzerland). In the experiment high performance liquid chromatography performed with ThermoFinningan spectra SYSTEM (Phenomenex, California, USA) consisting of the UV and diode array detector.

Antibacterial determination

The solutions were sterilized with autoclave SS-352 model (Tomy company, USA). Laminar flow clean V6 used for experiments was supplied from Lab Service Company (Bangkok, Thailand). Bacteria were incubated in incubator shaker SK-737 (Amerex Instruments, CA, USA) and incubator Modell 800 (Memmert company, Schwabach, Germany). Spectrophotometer genesys 20 for experiment was supplied from Thermo spectronic (NY, USA). The pH value of media was obtained with pH meter Mettler Toledo (S-20K, China). Micropipett P10, P20, P100, P1000 and P5000 used for experiment were furnished from Gilson company, Inc. (Ohio, USA). The solutions were mixed with Vortex mixer G-560E (Scientific industries, Inc., NY, USA).

Cytotoxic and anti-inflammatory experiment

The cell lines A-375 used in cytotoxic study and the murine macrophage like cell line, RAW 264.7 for anti-inflammatory assay were harvested with Rotofix 32 centrifuge (Hettich, Kirchlengern, Germany). Pipet-aid used for experiment was furnished from Drummond (USA). Melanoma cell line was maintained in 5% CO_2 incubator 311 (Thermoelectron corporation, USA). Larminar flow carbinet H1 used for experiment was supplied from Lab service LTD part (Bangkok, Thailand). Water bath used for experiment was purchased from Memmert (Schwabach, Germany). Tissue culture 96 well plates for the MTT assay was furnished from NuncTM Brand products (Roskilde, Denmark). Inverted microscope for experiment was supplied from Olympus (London, United Kingdom). Heamotocytometer for cell count was purchased from Boeco (Hamburg, Germany). Tissue culture flask (25 cm^3) for the cell culture was supplied from Corning incorporated (MA, USA).

2.3 Preparation of plant extracts

Thanaka stem bark powder was supplied by Ever Glory Co.,Ltd. (Bangkok, Thailand). Fresh bark was obtained from over 10-year old plants cultivated in the Eastern region of Myanmar. The bark was then grounded into 100–300 micrometer powder. Three kilograms of bark powder were extracted by sequential extraction at room temperature with hexane, dichloromethane, ethyl acetate and methanol, respectively, each at 9 liter volume. In addition, 3 liter of 85% (v/v) aqueous ethanol and distilled water extracts were obtained by the separate maceration of one kilogram of stem bark powder. Each extract was then filtered and evaporated under reduced pressure to yield six different dried crude extracts.

2.4 Determination of UV absorption activity

All thanaka extracts were prepared at 0.1 mg/mL. UV absorption activity was measure using UV-VIS spectrophotometer by wavelength scanned between 200-400 nm.

2.5. Antioxidant activity

2.5.1. Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals

The ability to scavenge DPPH radicals was used as an antioxidant activity assay where the resultant level of reduced DPPH formazan was determined

spectrophotometerically. In this method, fifty microliters of each concentration of sample or 100% DMSO (as a negative control), 0.23, 0.18, 0.14, 0.09 and 0.05mM BHT (as a positive control) or a blank (background subtraction) were allowed to react with 100µl of 25mM DPPH solution in a 96-well microplate in the dark at room temperature for 30 min. The absorbance was measured at 517 nm using a UV–VIS microplate reader. The results are expressed as the concentration of the extracts which scavenged free radicals by 50% (SC_{50}). All tests were run in triplicate. The percent scavenging activity was calculated by the following formula:

$$\% \text{ Scavenging activity} = \left[1 - \frac{\text{Asample}}{\text{Acontrol}} \text{Acontrol}\right] \text{ x } 100$$

Where $A_{control}$ and A_{sample} are the absorbance of the control (DPPH solution without sample) and the test sample (DPPH solution plus test sample or positive control), respectively [102].

2.5.2. Total phenolic content

The amount of total phenolic compounds in each extract was determined with the Folin–Ciocalteu reagent. In this experiment, 10 µl of each sample at the indicated concentration and 50 µl of Folin–Ciocalteu reagent were mixed and allowed to stand at room temperature for 4 min. Then, 50 µl of a 7.5% (w/v) sodium carbonate solution was added and allowed to stand for 2 h at room temperature before the absorbance of reaction mixture was measured at 715 nm. Total phenolic content was standardized against gallic acid and expressed as milligrams per liter of gallic acid equivalents (GAE). All tests were run in triplicate [103].

2.6. Tyrosinase inhibition activity

The tyrosinase inhibition assay was determined spectrophotometerically. Tyrosinase inhibition activity was performed using L-tyrosine as a substrate. Forty microliters of 200 units/ml of mushroom tyrosinase solution (in 20mM phosphate buffer, pH 6.8) and 1µl of the appropriate different concentration of the sample, were mixed. The assay mixture was pre-incubated at room temperature for 10 min and then 40µl of 2mM L-tyrosine in 20mM phosphate buffer (pH 6.8) were added and further

incubated at room temperature for 20 min. The amount of dopachrome formed was measured at 475nm in a microplate reader. Kojic acid, at a final concentration of 0.35, 0.28, 0.21, 0.14, 0.11 and 0.07mM, was used as a standard tyrosinase inhibitor. All tests were run in triplicate and the data are expressed as the percentage (±1 standard error of the mean; SEM) inhibition of tyrosinase activity, obtained as following equation below

$$\% \text{ Inhibition of tyrosinase activity} = \left[\frac{(A-B)-(C-D)}{A-B}\right] \text{x } 100$$

Where A is the absorbance of reaction mixtures without the test compound (tyrosinase + tyrosine), B is the absorbance of the blank of control (tyrosine alone), C is the absorbance of reaction mixture with the test compound (tyrosinase + tyrosine + test compound) and D is the absorbance of the blank sample (tyrosine + test compound).
For blank of control, tyrosinase solution and l-tyrosine solution were replaced with 20 mM phosphate buffer (pH 6.8) [104].

2.7. Antibacterial activity assay

2.7.1. Preparation of bacteria

Staphylococcus aureus (ATCC 25923) and *Escherichia coli* (ATCC 25922) were obtained from the Department of Medical Sciences, Ministry of Public Health. *S. aureus* and *E. coli* were cultured in Tryptic soy broth (TSB) at 37 ◦C for 6 h prior to harvesting and setting the bacterial concentration to approximately 10^7 CFU/ml in TSB [105].

2.7.2. Minimum inhibitory concentration (MIC) assay

The minimum inhibitory concentration (MIC) of various extracts was determined turbidimetrically. All extracts were dissolved in ethanol: water: tween80 (5:93.5:1.5 (v/v/v)) and autoclaved for 15 min at 121 ◦C, while the powder was sterilized using gamma radiation at 2.00–2.27 kGy for 15 min. The sterilized extracts or sterilized powder were adjusted to the desired concentration in a final volume of 20 μl and added into 2 ml of sterile TSB, mixed and serially diluted prior to inoculation with 15 μl of freshly prepared bacteria suspension (10^7 CFU/ml in TSB). The positive

control was performed using 12, 6, 3, 1.5, 0.75, 0.37, 0.18 and 0.09 mM (final concentration) clindamycin, and blank control tubes contained only TSB and the sample solvent as appropriate. After mixing, the tubes were incubated at 37 ◦C for 24 h in an incubator (Mermmet model 800) or shaking incubator (Amerex SK-737) at 200 rpm for the liquid extracts and solid powder samples, respectively. The tubes were then examined after 24 h for visible signs of growth and for turbidity by absorbance at 600 nm. The lowest concentration of each sample that inhibited the growth of bacteria was considered as the minimum inhibitory concentration or MIC. All experiments were run in triplicate [105].

2.7.3. Minimum bactericidal concentration (MBC) assay

The minimum bactericidal concentration (MBC), or the lowest concentration of sample that kills 99.9% of bacteria, was determined by assaying the live organisms of those tubes from the MIC that showed no growth. A loopful of bacterial broth from each of the tubes showing no growth was inoculated onto TSB plates and examined for signs of growth (colonies) after 24 h of incubation at 37 ◦C. All experiments were performed in triplicate [105].

2.8. Cytotoxicity by MTT assay

2.8.1. Cell culture and treatment

The human melanoma A-375 cell line (ATCC CRL-1619) was obtained from the American Type Culture Collection (Manassas, VA, USA), and maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS, 10mM HEPES, 100 mM sodium pyruvate, 0.01% (w/v) penicillin G and 0.05% (w/v) streptomycin sulphate (RPMI-1640 complete medium). All cells were incubated at 37 ◦C in a humidified atmosphere enriched with 5% (v/v) CO_2 (Thermoelectron 311 incubator). Cells were harvested using 0.25% (w/v) trypsin-1mM EDTA with gentle aspiration to form a single cell suspension, washed and then dispersed into RPMI-1640 complete medium to a final cell concentration of $1x10^5$ cells/ml [106].

2.8.2. Cell viability

The evaluation of cytotoxic activity was based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product, which can be measured spectrophotometrically. Ninety-nine microliters of A-375 cells were

seeded per well of a 96-well microplate at a concentration of $1x10^4$cells/well and then cultured for 4–6 h at 37 ◦C to allow for recovery and surface adherence. Then, 1μl of sample (dissolved in DMSO at various concentrations) was mixed into the well and cells were incubated at 37 ◦C for 72 h with the addition of 10 μl of 12 mM MTT reagent in PBS to cells for the last 4 h of incubation. The medium was then carefully removed and replaced with 100 μl of 0.04N HCl–isopropanol and agitated to dissolve the formazan crystals. The absorbance of the dissolved formazan product was measured at 540 nm in microplate reader. Cell viability was calculated using the formula below:

Cell viability (%) = (Abs test cells/Abs control cells) x100

Cell viability of untreated cell was set as 100%, and water was used as negative control. All the experiments were performed in triplicate [107].

2.9. Anti-inflammatory by nitrite assay

2.9.1. Cell culture and treatment

The murine macrophage like cell line, RAW 264.7 (ATCC TIB-71), was obtained from the American Type Culture Collection and cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 10 mM HEPES,

100 mM sodium pyruvate, 0.01% (w/v) penicillin G and 0.05% (w/v) streptomycin sulphate (DMEM complete medium). All cells were incubated at 37 ◦C in a humidified atmosphere enriched with 5% (v/v) CO_2, and were harvested by the addition of 10 mM cold phosphate buffer (pH 7.4) and gently aspirated before washing and dispersion into DMEM complete medium. Ninety-nine microliters of the RAW 264.7 cell suspension were seeded per well of a 96-well microplate at $1x10^4$ cells/well. One microliter of the test sample (in DMSO) was added and the mixture was incubated for 24 h before being subjected to either the cytotoxic assay by MTT method or nitrite measurement. Cells were activated by adding 1 μl of LPS. The potential anti-inflammatory activity of each sample was assessed by evaluating its capacity to inhibit NO production in activated macrophages. Released nitrite in the culture medium, as an indicator of NO production, was measured using the

colorimetric test based on the Griess reaction. Briefly, 50 μl acid, incubated in the dark at room temperature for 10 min. Then 50 μl of a 0.1% (w/v) NED (0.1% N-1-napthylenediamine dihydrochloride in water) solution was added and incubated in the dark at room temperature for a further 10 min. The nitrite concentration was then determined by measuring the absorbance at 540 nm in a microplate reader using a standard calibration curve prepared from $NaNO_2$ standards. The results were expressed as the percentage of NO production compared to the control, as follows:

$$\% \text{ Inhibition} = 100 \text{ x } \left[\frac{NO_{2control} - NO_{2sample}}{NO_{2control}} \right]$$

Where NO_2 control and NO_2 sample are the amount of NO_2 generated from the reaction with and without the test sample addition, respectively [108].

2.9.2. Cell viability through MTT assay

The cell viability was assayed by the MTT assay as described above.

2.10. Genotoxicity by comet assay

2.10.1. Cell culture and treatment

A suspension (990 μl) of A-375 cells in RPMI-1640 complete medium, prepared as described above, was seeded in each well of a 6-well plate at a concentration of

2.5×10^5 cells/well and then cultured for 4–6 h at 37 ◦C to allow recovery and surface adherence. Then 10 μl of sample (in DMSO) was put into the well and incubated for 24 h. The cells were then harvested, washed and redispersed in RPMI-1640 complete medium as described above, prior to evaluation for DNA damage analysis by the COMET assay, outlined below [109-110].

2.10.2. Comet assay

DNA strand breaks were determined using the comet assay. Briefly, 100 μl of the A-375 cell suspension, prepared as above, at a concentration of 10^6 cells/ml was mixed with 10 μl (v/v) of 0.8% (w/v) low-melting temperature agarose in 10 mM PBS at 37 ◦C, and embedded in slides pre-coated with normal agarose. Slides were

immersed in a cold lysis solution (2.5 M NaCl, 0.1 M EDTA and 10 mM Tris–HCl, pH 10) at 4 ˚C for 2 h. To allow unwinding of DNA, the slides were transferred into alkaline denaturizing buffer (300mM NaOH and 1mM EDTA, pH 13) for 20 min. Subsequently, electrophoresis on 7.5 x 2.5 cm conventional glass slide coated agarose using Tris-Boric EDTA (TBE) was performed in a Mupid 2 advance minigel system (Cosmo Bio) at 35 V, 300mA for 20 min. Slides were then stained with 20μg/ml ethidium bromide. A total of 50 cells from each duplicate slide were examined randomly using an inverted fluorescent microscope (Olympus, XI51/XI71, Tokyo, Japan) equipped with Cell^{A} Analysis Software (Olympus soft image solution). The excitation light source was from a mercury lamp screened with a Bp 510–550nm excitation filter and the detection was carried out using a BA 590nm emission filter [109-110].

2.11 Statistical analysis

The results are shown as the mean ±1 S.E.M. A one-tailed test was used to determine if there were any significant differences between the means of the control and the samples, accepting significance at a p-value of ≤ 0.05. Tukey and Duncan tests were used as post hoc of the one-way ANOVA mean comparison.

2.12 Isolation of some compounds from thanaka stem bark

The hexane crude extract and dichloromethane crude extracts were defined as TH1 and TH2 crude extracts, respectively.

The TH1 crude extract (2.8 g) was chromatographed over silica gel column using gradient elution of hexane-dichloromethane-ethyl acetate and obtained 110 fractions. Each fraction was examined by TLC and combined. One pure compound was obtained.

The TH2 crude extract (5.0 g) was chromatographed over silica gel column using gradient elution of hexane-dichloromethane-ethyl acetate-methanol and obtained 72 fractions. Each fraction was examined by TLC and combined.

TH2.3 (brown paste, 1.36 g, combined fraction 32-46) was subject to silica gel open column eluting with gradient hexane-dichloromethane-ethyl acetate-methanol. Fifty fractions were collected and after their examination by TLC, they were combined. The combined fractions were further purified by sephadex-LH20 open

column eluting with isocratic eluent; dichloromethane: methanol (7:3). The pure compounds were obtained.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Yield of thanaka crude extract

The highest yield of thanaka extract was found in 85% ethanol separated extract, followed by methanol, ethyl acetate, dichloromethane, water and the least was hexane extract (Table 3.1)

Table 3.1 Yield of thanaka in the various extracts

Thanaka extracts	% Yield
Hexane extract	0.22%
CH_2Cl_2 extract	0.86%
EtOAc extract	0.20%
MeOH extract	0.82%
85% EtOH extract	4.21%
Water extract	1.52%

3.2 UV screening activity

UV screening activity was measured using UV-VIS spectrophotometer by scanning wavelength from 200 to 400 nm to investigate in the range of UVA (315-400 nm) and UVB (315-280 nm). At the 0.1 mg/mL concentration of all extracts and 0.01 mg/mL of ethylhexyl-4-methoxycinnamate (EHMC) standard, the result showed that dichloromethane, ethyl acetate, methanol and 85% ethanol possessed good UVA and UVB absorption property (Figure 3.1). Comparing the UV absorption property between EHMC, the commercial UV absorption chemical which commonly used in the cosmetic, and thanaka extracts the result showed that UV screening activity of extracts except hexane and water were approximately 10 times lower than the EHMC. Thus, thanaka extract might be a good candidate for use as a UV screening agent in the cosmetic active in the future.

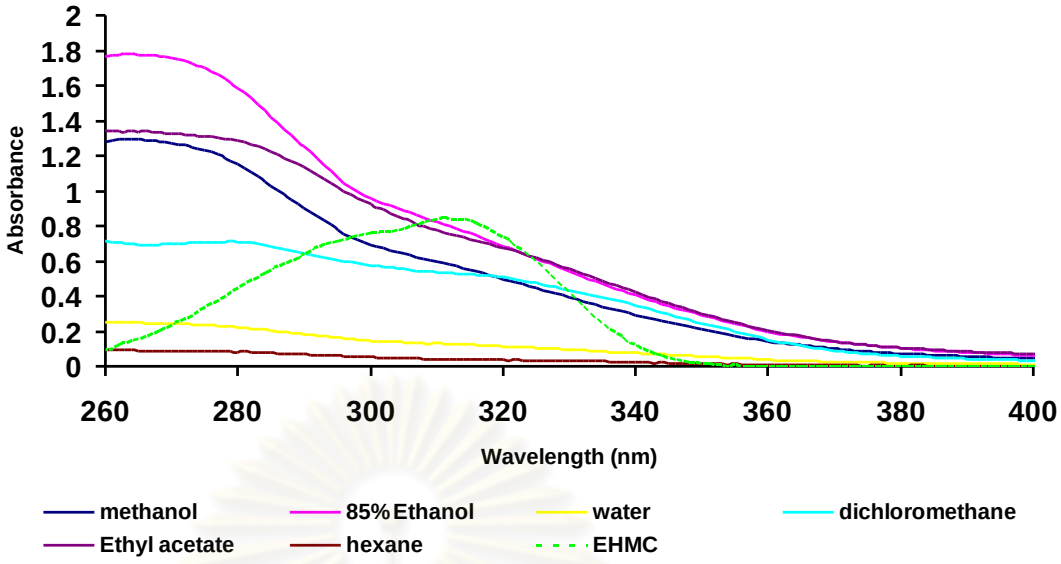


Figure 3.1 UV spectra of Thanaka crude extracts and EHMC standard

3.3 Antioxidant activity study

Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals

The antioxidant activity test was carried out using DPPH assay. The result was reported as the concentration that scavenged fifty percent of DPPH radicals or SC_{50} values. The best scavenging activity was found in 85% ethanol extract followed by ethyl acetate extract, methanol extract, water extract, dichloromethane extract and the least activity was found in hexane extract, respectively (Table 3.2). The thanaka original powder was not tested because of its turbidity which interfered with the detection by spectrophotometer. Good free radical scavenging activity was observed in the 85% (v/v) ethanol, methanol, ethyl acetate, water and dichloromethane extracts, while the hexane extract showed significantly less activity (4-fold less). The SC_{50} of the standard trolox and quercertin was 0.27 and 0.16 mg/mL, respectively. All the thanaka extracts showed less activity than trolox (10 - 20 times lower) and quercertin (18 - 35 times lower). The mean comparison of the SC_{50} of each extracts, standard trolox and standard quercertin by one-way ANOVA with Duncan test indicated that the SC_{50} values of these extracts and standard were significantly different (p = 0.05). The experiments were performed in triplicate.

Table 3.2 Scavenging activity of Thanaka extracts

Sample	SC_{50} of DPPH assay (mg/mL)
Hexane extract	<2
CH_2Cl_2 extract	0.554 ± 0.004^{b}
EtOAc extract	$0.320\pm0.005^{c,d}$
MeOH extract	0.351 ± 0.005^{c}
85% EtOH extract	0.282 ± 0.002^{d}
Water extract	0.425 ± 0.003^{b}
Original powder	-
Trolox®	0.027 ± 0.001^{e}
Quercertin	0.016 ± 0.003^{e}

[a–e] Statistic analysis was performed using ANOVA with Duncan test (N = 3, values with different superscript were significantly different, $p = 0.05$).

Total phenolic content

Total phenolic content was measured by Folin–Ciocalteu method, using the standard curve of gallic acid ($R^2 = 0.99$). The result expressed in milligram gallic acid in crude extract. The amount of total phenolic content in all extracts show in Table 3.3. The less polar hexane and dichloromethane extracts showed less amount of total phenolic content (40.33 and 187.72, respectively). The higher polar extracts such as ethyl acetate, methanol, and 85% ethanol extract except water extract contained more amount of total phenolic content. The hexane extract contained the lowest amount of polyphenols and showed the least free radical scavenging activity, dichloromethane and water extracts possessed moderate amounts of phenolic compounds and also showed moderate free radical scavenging activity, while the ethyl acetate, methanol and ethanol extracts possessed the greatest amounts of polyphenols and showed the best radical scavenging activity. Minor disagreement among ethyl acetate, methanol and ethanol extracts regarding the correlation between radical scavenging activity and phenolic contents, indicated that in addition to the polyphenols, other compounds in the 85% ethanol and ethyl acetate extracts and or different ratios of the same compounds in the mixture (for example differential solubilities and partioning during the sequential solvent extraction) were also radical scavengers. Thus, it can be

concluded that antioxidation activity is one of the biological activities inherited in this bark powder.

Table 3.3 Total phenolic content of Thanaka extract

Sample	Total phenolic content (mg Gallic acid in 100g crude extract)
Hexane extract	40.35 ± 1.75^{a}
CH_2Cl_2 extract	187.72 ± 1.58^{b}
EtOAc extract	288.16 ± 2.01^{c}
MeOH extract	324.56 ± 2.32^{d}
85% EtOH extract	270.18 ± 4.58^{e}
Water extract	142.06 ± 2.41^{f}
Original powder	-

[a–f] Statistic analysis was performed using ANOVA with Duncan test (N = 3, values with different superscript were significantly different, $p = 0.05$).

3.4 Tyrosinase inhibition activity using spectrophotometric method

Thanaka has been used as the traditional cosmetic, which is very famous for Myanmar women from ancient until present; the bark is ground with water and then the wet paste was applied directly to the face. It has been known among Myanmar women that Thanaka bark possesses some skin whitening effect. The tyrosinase inhibition activity of various extractions has been studied using spectrophotometric method. As a result, thanaka all extracts showed a mild tyrosinase inhibition activity (Table 3.4), with expressed as IC_{50} values. It was found that Thanaka's acitivity was approximately 50–150 times lower than that of kojic acid, a standard tyrosinase inhibitor commonly used in cosmetic formulations. The original thanaka powder also showed an obvious color of mild tyrosinase inhibition activity as the reaction between tyrosine and tyrosinase in the presence of the powder showed a less obvious color change to dopachrom (Figure 3.2). However, this activity could not be converted into a meaningful IC_{50} value due to the turbidity of the mixture caused by the powder which interfered the spectroscopic measurement at specific time. The lowest concentration of the original powder needed for an observable positive result (approximately 22% inhibition) was 1 mg/ml.

Thus, in this case the mild tyrosinse inhibition activity detected in Thanaka bark, together with its non-toxic nature, might be able to give a comparable if not better skin whitening activity than many problematic chemicals including kojic acid In other words, its mild tyrosinase inhibition activity and its non-toxicity seems to be more interesting than the other compound with more potent activity but high toxicity.

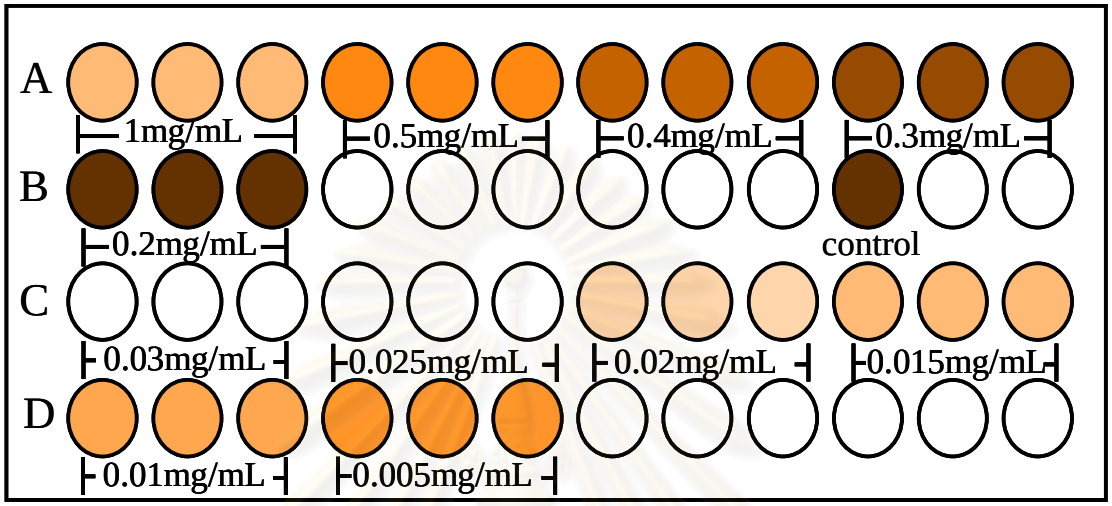


Figure 3.2 Represented picture of tyrosinase inhibition activity of thanaka powder was observed by visualization. Lane A and B are thanaka powder in the various concentration, Lane C and D are kojic acid positive control.

Table 3.4 Tyrosinase inhibitor activity of Thanaka extract

Sample	IC_{50} of tyrosinase inhibition (mg/mL)
Hexane extract	0.623 ± 0.011^{a}
CH_2Cl_2 extract	0.546 ± 0.012^{b}
EtOAc extract	0.697 ± 0.012^{c}
MeOH extract	1.420 ± 0.015^{d}
85% EtOH extract	0.860 ± 0.006^{e}
Water extract	1.089 ± 0.016^{f}
Original powder	Positive
Kojic acid	0.009 ± 0.001^{g}

[a–e] Statistic analysis was performed using ANOVA with Duncan test (N = 3, values with different superscript were significantly different, $p = 0.05$).

3.5 Antibacterial study by turbudimetric method

Determination of MIC and MBC

The antibacterial activity of hexane, dichloromethane, ethyl acetate, methanol, 85% ethanol, water extracts and thanaka powder was carried out compare to standard clindamycin. In this study the turbidimetric method was used against *Staphylococcus aureus* and *Escherichia coli*. The MIC value is the lowest absorbance difference between 0 h and 24 h at 600 nm (A_{600}), when absorbance difference ≤ 0.100. The bacteria inhibition of 6 extracts and powder of thanaka at different concentration were tested against the growth of *S. aureus* and *E. coli*. All extracts and the bark powder showed antibacterial activity against the Gram-positive *S. aureus* and Gram-negative *E. coli*. (Table 3.5)

Table 3.5 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) value of thanaka extracts

Extracts	*Staphylococcus aureus*		*Escherichia coli*	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Hexane extract	5	<10	5	<10
CH_2Cl_2 extract	5	10	5	10
EtOAc extract	5	10	5	10
MeOH extract	10	10	5	10
85% EtOH extract	10	10	5	10
Water extract	<10	<10	<10	<10
Original powder	10	<10	10	<10
Clindamycin	0.016	0.25	0.5	2.0

3.6 Cytotoxicity study

The cytotoxic activities of thanaka powder and associated solvent extracts were determined using MTT assay. MTT is a classical but still the most commonly used colorimetric method for determining cell growth, via measuring metabolic activity in the viable cells. The melanoma A-375 cell line was chosen for this cytotoxicity experiment. Melanoma was incubated for 72 h at the density of 1 x 10^4 cells/well with thanaka samples and doxorubicin control at various concentrations to

determine the IC_{50}. Cell viability was identified using the MTT assay on the third day of culture. The results were expressed in the IC_{50} value. The IC_{50} value were as follows: dichloromethane extract (IC_{50} = 0.30±0.01) < hexane extract (IC_{50} = 0.48±0.01) < ethyl acetate extract (IC_{50} = 0.90±0.01) < 85% ethanol extract (IC_{50} = 12.81±0.16) < methanol extract (IC_{50} = 15.30±0.20) < water extract (IC_{50} = 19.07±0.49) respectively. The thanaka powder suspension at level up to 15 mg/mL performed no detectable cytotoxic effect on melanoma A-375 cells during 72 h assay examined. Moreover, the polar methanol, 85% (v/v) ethanol and water extracts showed greater than 80% cell viability when incubated at concentrations up to 7.5, 5.0 and 7.5 mg/ml, respectively. In contrast, the markedly less polar hexane, dichloromethane and ethyl acetate extracts revealed significantly higher cytotoxic effect with 12- to 62-fold lower IC_{50} values, while the concentrations which showed >80% viability of melanoma A-375 cells, were ~0.1–0.4 mg/ml. Comparing with the doxorubicin positive control which is, known as the anticancer drug that toxic to the melanoma A-375 cell, exhibited the IC_{50} value at 0.0003±0.00 mg/mL, It can be considered that thanaka polar extracts and the original powder are non toxic while less polar extracts are mild toxic. The mean comparison of the IC_{50} of each samples by one-way ANOVA with Duncan test indicated that the IC_{50} values of these extracts were significantly different (p = 0.05).

Table 3.6 Cytotoxicity of thanaka crude extracts

Sample	IC_{50} of melanoma A-375 (mg/mL)
Hexane extract	0.48 ± 0.01^{b}
CH_2Cl_2 extract	0.30 ± 0.01^{b}
EtOAc extract	$0.90\pm0.01^{c,d}$
MeOH extract	15.30 ± 0.20^{c}
85% EtOH extract	12.81 ± 0.16^{d}
Water extract	19.07 ± 0.49^{b}
Original powder	>15
Doxorubicin	0.0003±0.00

[a–d] Statistic analysis was performed using ANOVA with Duncan test (N = 3, values with different superscript were significantly different, p = 0.05).

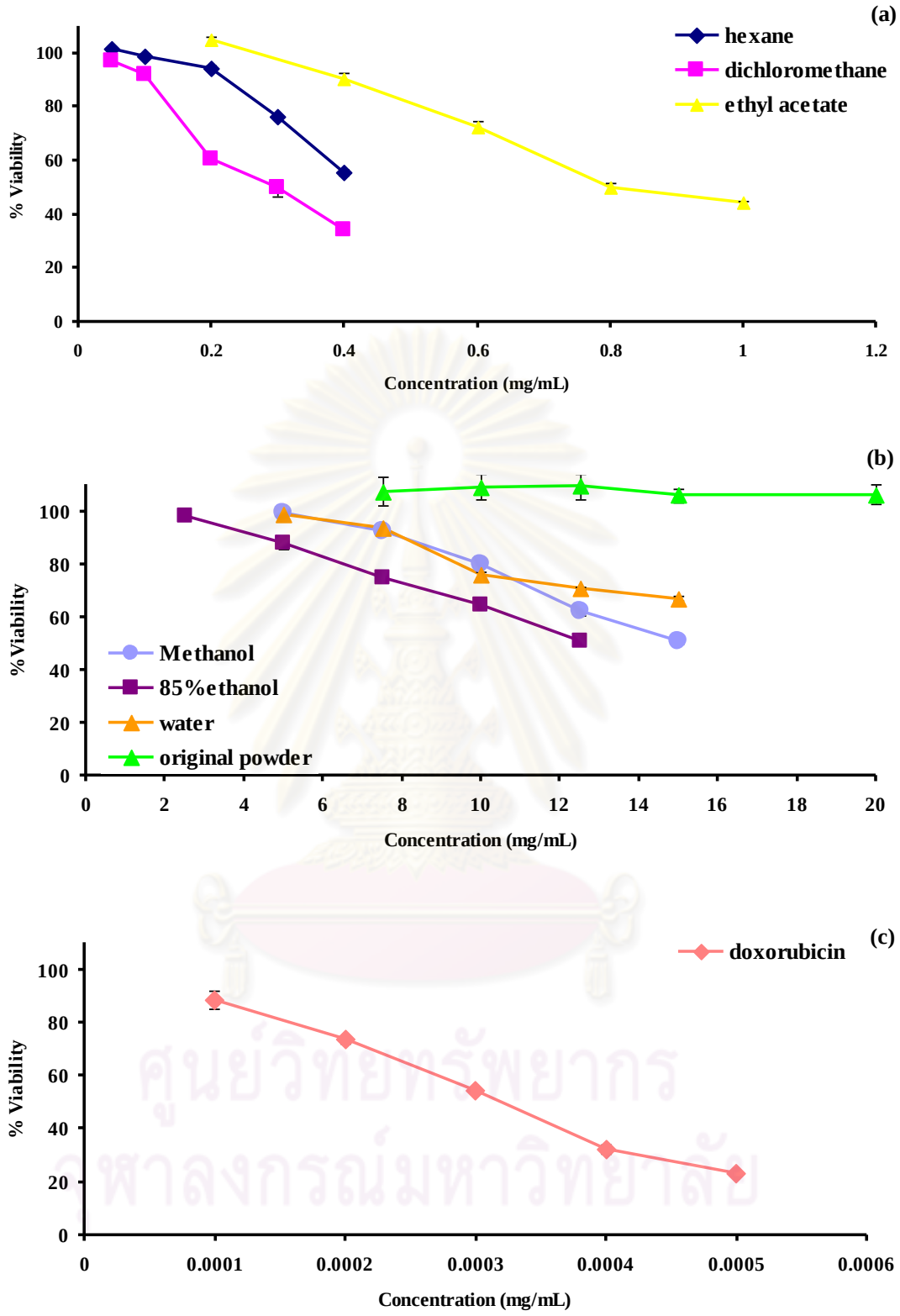


Figure 3.3 Cytotoxicity of Thanaka bark: (a) the almost non-polar (ethyl acetate, dichloromethane

(CH_2Cl_2) and hexane) bark extracts, (b) the polar (water, methanol and 85% (v/v) ethanol) bark extracts and the original bark powder and (c) doxorubicin standard, against melanoma A-375 cells. Data are expressed as the mean (±1 S.E.M.)
% viability of cells, derived from triplications.

3.7 Anti-inflammatory study

The anti-inflammatory activity of thanaka extract was studied *in vitro* for their inhibitory effects on chemical mediators release (LPS-induced NO production) from macrophages. Activated macrophages produce large amounts of chemical mediators that indicate inflammation. Nitric oxide (NO), a bioactive free radical, is one of these critical mediators which is produced by inducible NO synthase in inflammatory macrophages when stimulated with LPS. Excessive production of NO has been confirmed both in chronic and acute inflammation. In fact, NO production induced by LPS through inducible NO synthase induction may reflect the degree of inflammation and may provide a measure for assessing the effect of the extract on the inflammatory process. As shown in Figure 3.4, the 6 extracts of thanaka at different concentrations showed the reduction of NO production in a dose-dependent manner. More than 80% viability of macrophage with the hexane extract exhibited ~80% inhibition of NO synthase at 0.3 mg/mL, dichloromethane extract exhibited ~75% inhibition at 0.4 mg/mL, ethyl acetate extract exhibited ~70% inhibition at 0.6 mg/mL, methanol extract exhibited ~80% inhibition at 2 mg/mL, 85% ethanol extract exhibited 90% inhibition at 2.5 mg/mL, water extract exhibited 80% inhibition at 3 mg/mL and original powder showed non toxic to the macropharge with slightly inhibition of NO synthase at 10 mg/mL, compare to the pathenolide, anti-inflammatory drug, which show the inhibiton of 80% at 0.003 mg/mL. These extracts significantly reduced the release of NO in a dose-dependent and saturateable manner (Fig. 3.4). The anti-inflammatory activity of the samples decreased in the following order: hexane extract > CH_2Cl_2 extract > ethyl acetate extract > 85% ethanol extract >methanol extract >water extract. Surprisingly, at the concentrations that showed more than 80% cell survival (non-toxic concentrations), all six solvent extracts showed an anti-inflammatory activity as high as 80–90%. The result suggested a presence of various potent anti-inflammatory agents with different polarity in *Hesperethusa crenulata* bark.

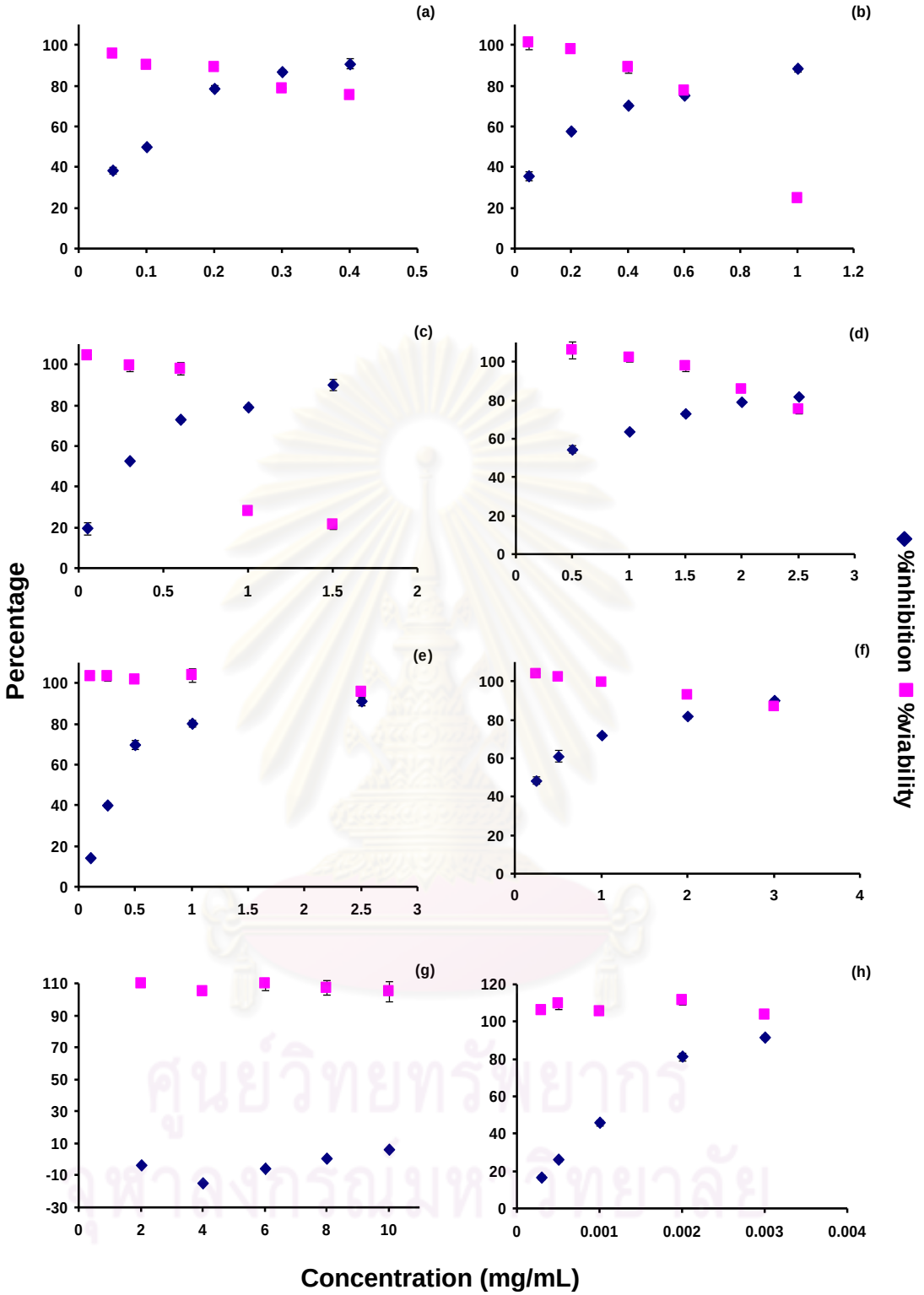
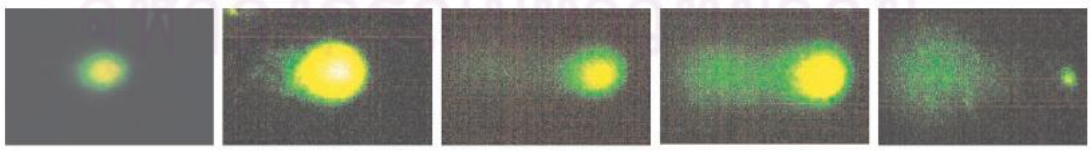


Figure 3.4 Anti-inflammatory activity and cytotoxicity of (a) hexane, (b) dichloromethane, (c) ethyl acetate, (d) methanol, (e) 85% (v/v) ethanol extract and (f) water extracts of Thanaka bark, (g) the original bark powder and (h) parthenolide standard. Data are shown as the mean (±1 S.E.M.) % anti-inflammatory activity, derived from three replications

3.8 Genotoxic study using Comet assay

Total DNA strand breaks in the A-375 cells were analyzed using a Comet assay. Comet assay is commonly used to identify the individual cell DNA migration patterns produced. Cells was cultured in the tissue culture flask then seeded in each well of a 6-well plate at a concentration of 2.5 $x10^5$ cells/well and then cultured for 4–6 h at 37 °C to allow recovery and surface adherence. After that 10 μl of sample (in DMSO) was put into the well and incubated for 24 h. The cells were then harvested, washed and redispersed in RPMI-1640 complete medium, $1x10^4$ cells were dispersed in 0.8% with low-melting temperature agarose and embedded in to the slice coated with 1% agarose. The treated cells was lysed and denatured by lysis solution and denaturizing buffer. The denatured cells was migrated the damaged DNA by electrophoresis. Then all treated slides were dyed with ethidium bromide and evaluated the single cell with inverted fluorescence electron microscope. Each cell was measure the tail distance to collect the data to calculate the tail length. Moreover, one hundred randomly selected non-overlapping cells were visually assigned a score on an arbitrary scale of 0–4 as in figure 3.5 Scoring for DNA damage cell, score 2-4 were counted as a DNA damage cell and calculated in to percentage of DNA damage cell. The experiments were performed after exposure of the cells to the six extracts and the original powder of *Hesperethusa crenulata* for 24 h. It was evident that all six extracts and the original powder were not genotoxic, while obvious DNA strand breaks could be observed in the cells treated with very low concentrations of hydrogen peroxide (figure 3.6 and table 3.7). These results, and the cytotoxic results, concur with the apparent safety of the long term indigenous use of Thanaka bark in the form of a wet powdery paste.



0 1 2 3 4

Figure 3.5 The represent of comet images from cells, cell classes 0–4.

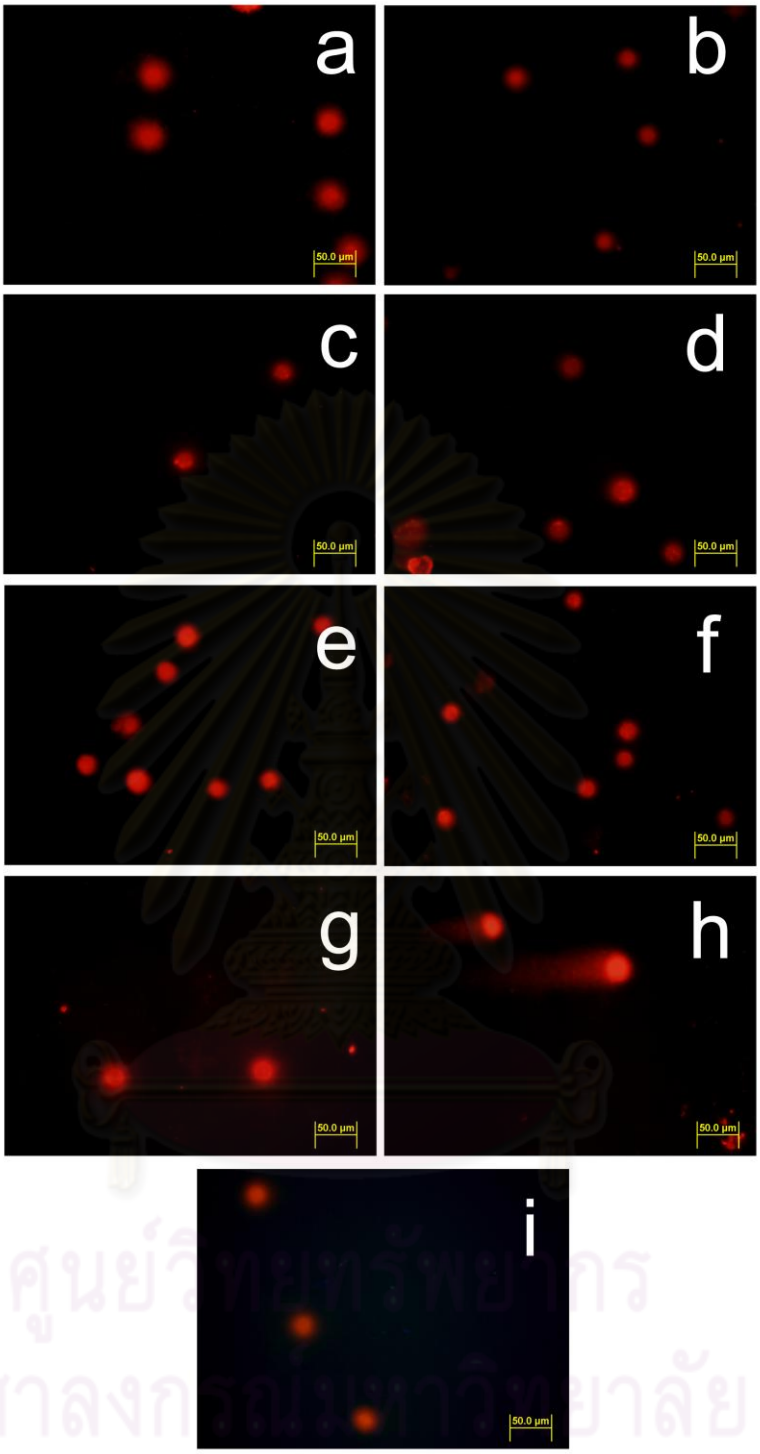


Figure 3.6 Comet assay photographs of the A-375 melanoma cells treated with (a) hexane, (b) dichloromethane, (c) ethyl acetate, (d) methanol, (e) 85% (v/v) ethanol and (f) water extracts of the Thanaka bark; plus (g) the original bark powder (h) hydrogen peroxide and (i) untreated melanoma A-375 cells (control). All photographs are representative of at least 50 such events per sample and three independent samples.

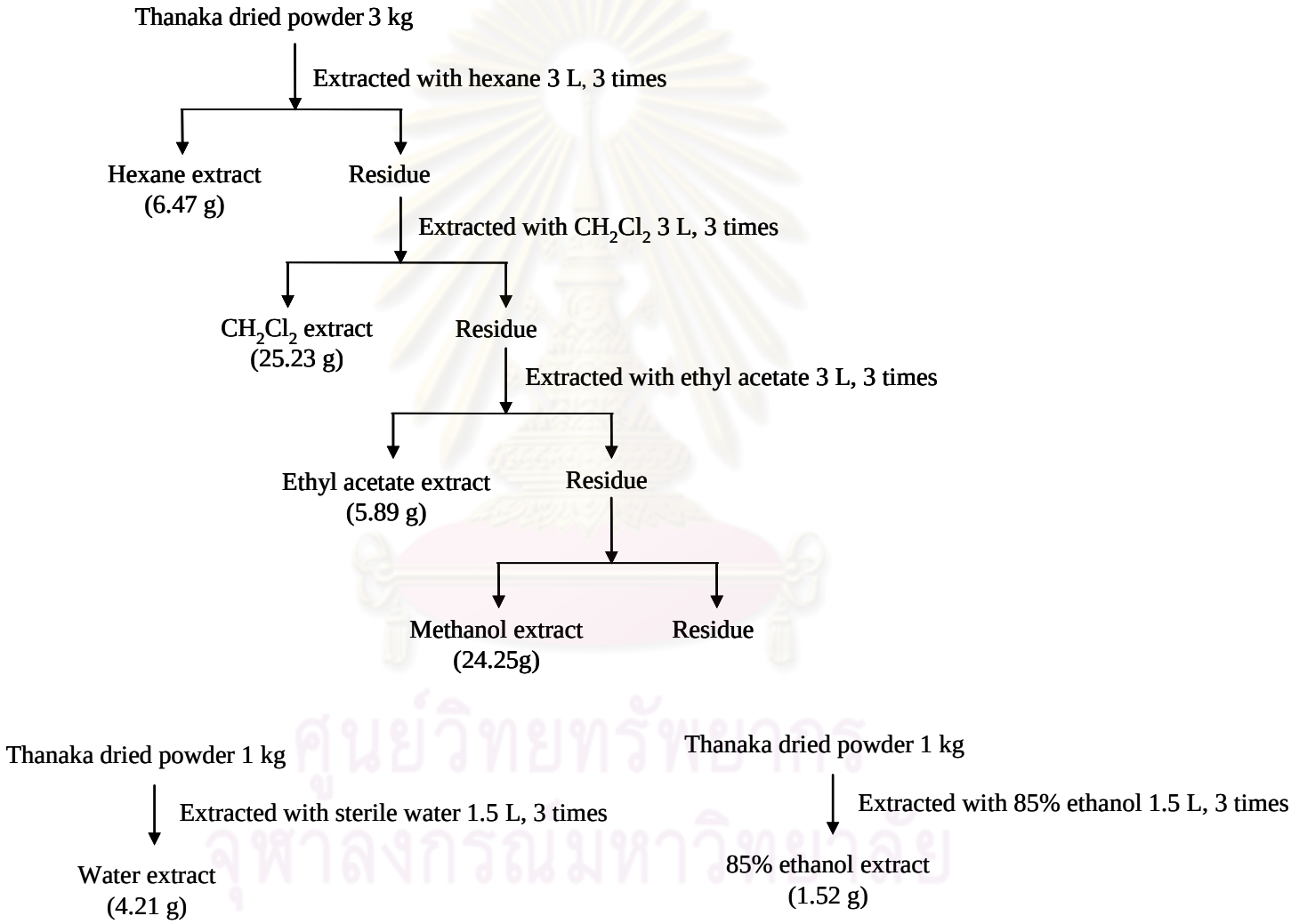
Table3.7 DNA damage induced in melanoma A-375 celld after culturing with *Hesperethusa crenulata* bark extracts or the original powder

Samples	Concentration (mg/mL)	Tail length (μm)	DNA damage cell (%)
Control (DMSO)	0.0	7.53±0.28[a]	22.08±0.65[c]
Hexane extract	0.3	7.54±0.43[a]	21.42±0.58[c]
Dichloromethane extract	0.3	6.53±0.29[a]	21.45±0.64[c]
Ethyl acetate extract	0.7	6.10±0.31[a]	22.68±0.80[c]
Methanol extract	1.5	6.44±0.21[a]	21.08±0.46[c]
85%(v/v) ethanol extract	5.0	6.05±0.26[a]	21.09±0.40[c]
Water extract	10.0	6.44±0.26[a]	21.08±0.53[c]
Original powder	10.0	5.90±0.21[a]	20.98±0.46[c]
Hydrogen peroxide	10 μM	154.18±6.95[b]	85.67±0.64[d]

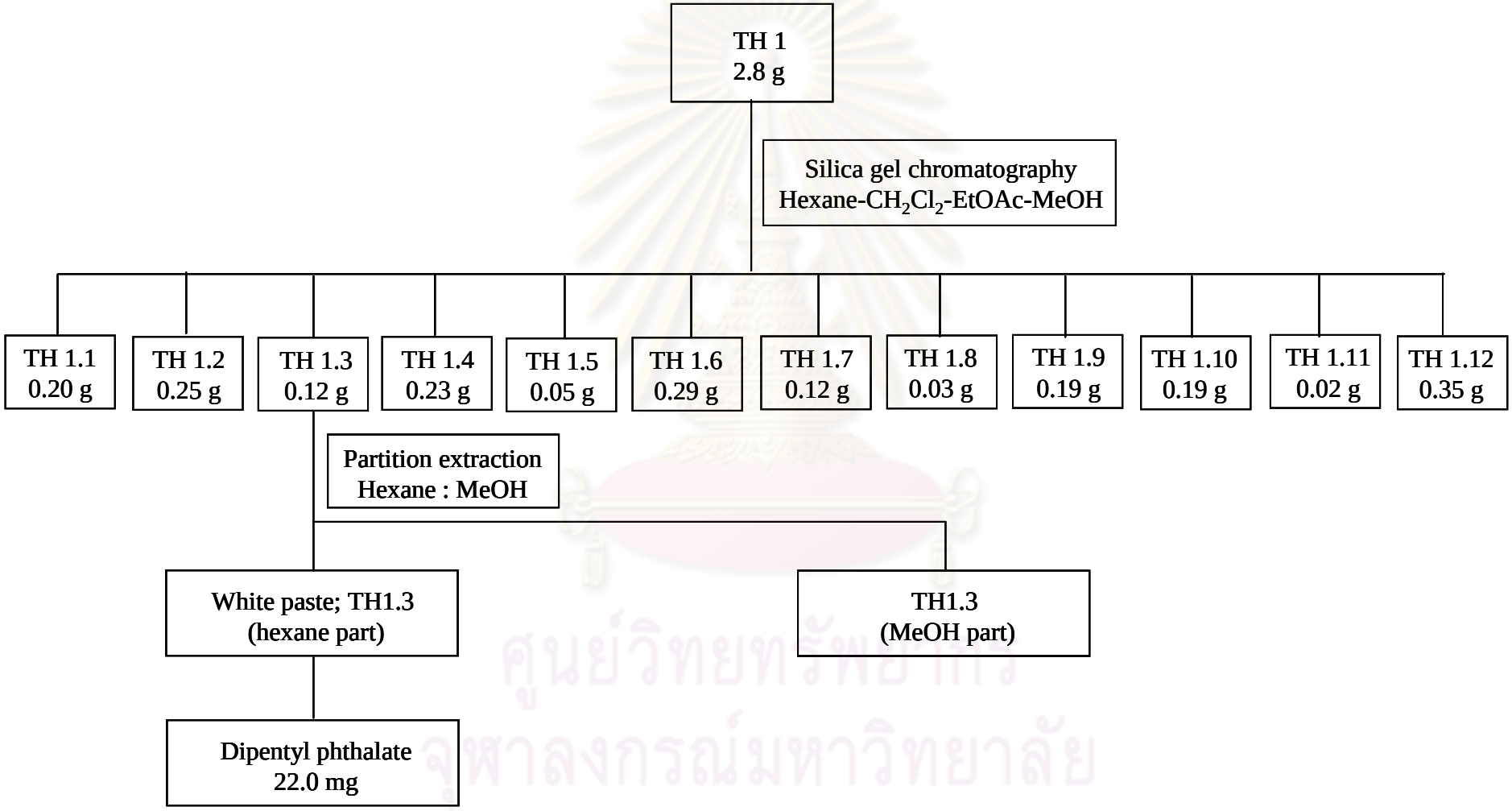
[a–d] Statistic analysis was performed using ANOVA with Duncan test (N = 3, values with different superscript were significantly different, $p = 0.05$).

3.9 Isolation and purification of Thanaka crude extract

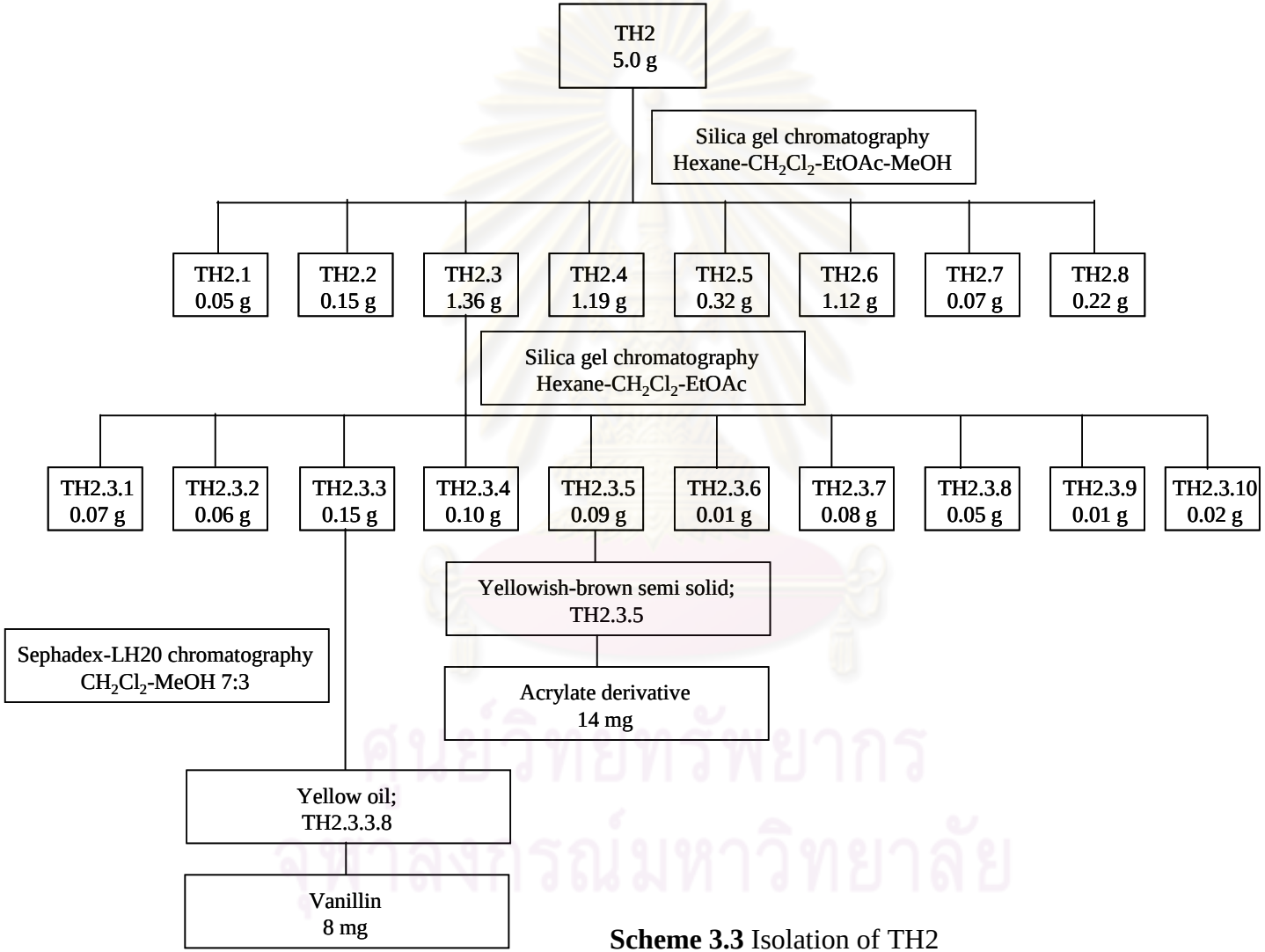
All 6 extracts of thanaka stem bark were done the biological activities which were previously explained, after that the isolation and purification of some component was investigated.



Scheme 3.1 Extraction thanaka stem bark powder



Scheme 3.2 Isolation of TH1



Scheme 3.3 Isolation of TH2

3.10 Characterization of thanaka isolated compound

3.10.1 Dipentyl phthalate

TH1.3 (22 mg) was obtained as a white semi-solid from hexane crude extract. IR spectrum showed absorption band of C=O stretching at 1725 cm^{-1} and C=C stretching at 1460 cm^{-1} (Figure A-6, Appendix, Page 83).

The ^{13}C NMR spectrum of TH1.3 contained nine singals which are one methyl, four methylene, two methane and one quaternary carbon (a carbonyl of ester) (Table 3.8). The ^{1}H NMR of TH 1.3 show two aromatic signals at δ_H 7.7 and 7.5 (H-3 and H-4), one methyl proton at δ_H 0.9 ppm (H-5') and four methylene proton at δ_H 4.2 ppm (H-1'), 1.6 ppm (H-2'), 1.3 ppm (H-3') and 1.17 ppm (H-4') (Table 3.8). The direct connectivity (one bond) of proton and carbon atoms were deduced from HSQC spectrum (Figure A-3, Appendix, Page 80).

Table 3.8 1D and 2D NMR spectroscopic data for dipentyl phthalate in $CDCl_3$

position	δ_H, mult. (*J* in Hz)	δ_C	COSY	HMBC
1		167		
2		132		
3	7.7 (1H, d, 5.06)	128	H-4	C-1,C-3,C-4
4	7.5 (1H, d, 5.06)	130	H-3	C-3
1'	4.2 (2H, t, 6.18)	68	H-2'	C-1,C-2',C-3', C-4,
2'	1.6 (2H, M)	38	H-4'	
3'	1.3 (2H, M)	29		C-5'
4'	1.17 (2H, M)	23	H-2', H-5'	
5'	0.89 (3H, t, 14.76)	14	H-2', H-4'	C-4'

The ^{1}H -^{1}H cosy spectrum (Figure A-5, Appendix, Page 82) revealed the presence of the following connectivity as shown in Figure 3.8

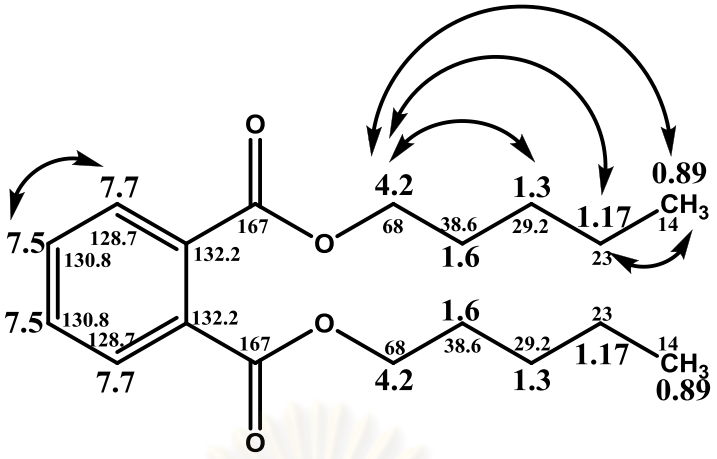


Figure 3.7 The COSY correlation of dipentylphthalate

The HMBC correlation (Figure A-4, Appendix, Page81) were observed between the proton signals at δ_H 7.7 (H-3) to carbon signal at δ_C 132.2 ppm (C-2), 128.7 ppm (C-3) and 130.8 ppm (C-4), between the proton signal of H-4 to carbon signal at δ_C 128.7 ppm (C-3), H-3 (δ_H 7.7 ppm) to carbonyl carbon of ester (C-1), which led to the aromatic ester part of TH1.3 structure. The HMBC correlation of the proton signal of H-1' (δ_H 4.2 ppm) to C-2', C-3' and C-4', H-3' to C-5' and H-5' to C-4' led to the aliphatic part of the TH1.3 structure. HMBC correlation of H-1' (4.2 ppm) to C-1 (δ_C 167 ppm) and H-3 (δ_H 7.7 ppm) to C-1 indicated that the two partial structures of TH1.3 were connected the ester. This agrees with the C=O stretching in the IR spectrum. Consequently, the structure of TH1.3 was determined as dipentyl phthalate.

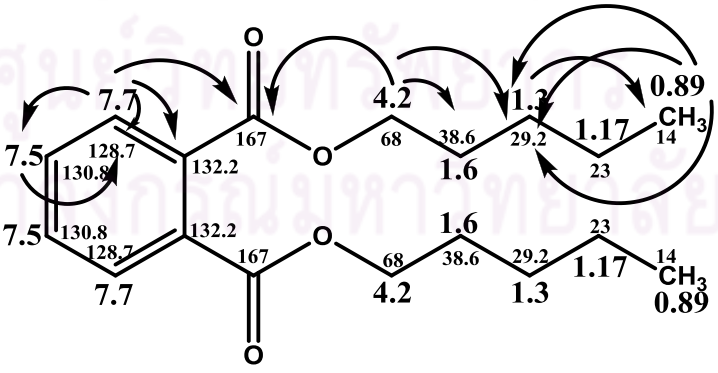


Figure 3.8 HMBC correlation of dipentyl phthalate

Moreover, the positive ESI mass spectrum (Figure A-7, Appendix, Page 84). showed a $[M+2H_2O+H]^+$ ion at /m/z 343, thus confirming the dipentyl phthalate.

There are many phthalates such as butyl benzyl phthalate, di 2-ethylhexyl phthalate and diisodecyl phthalate (DIDP), are used as plasticizer to make vinyl soft and flexible [111]. Some phthalates such as dibutyl phthalate (DBP), dimethyl phthalate (DMP) and diethyl phthalate (DEP) have been a key ingredient in fragrances and nail polish. They are used primarily at concentrations of less than 10% as plasticizers in products such as nail polished (to reduce cracking by making them less brittle) and hair sprays (to help avoid stiffness by allowing them from flexible film in the hair) and as solvents and perfume fixatives in various other products [112].

3.10.2 Vanillin

TH 2.3.3.8 (8 mg) was obtained as yellowish oil from dichloromethane crude extract.

The ^{1}H NMR of TH 2.3.3.8 show two aromatic proton signals at δ_H 7.3 and 6.9 ppm (H-3, H-4 and H-5), one methoxy proton at δ_H 3.9 ppm (H-1) and one hydroxy proton at δ_H 6.2 ppm (H-2) and carboxy proton at δ_H 9.8 ppm (H-6) (Table 3.9). When Compare the ^{1}H NMR spectrum of standard vanillin and TH2.3.56.8 showed similar chemical shift almost all but H-2 (6.2 ppm) of TH2.3.3.8 was absence so TH2.3.3.8 was confirmed using HPLC technique. The confirmation of vanillin using HPLC technique, the result showed that fraction TH2.3.3.8 showed major peak at retention time 1.75 min and tiny minor peak at 2.8 min (Figure 3.12 a) when compared to vanillin standard which showed only single peak at retention time 1.75 min (Figure 3.12 b) after that fraction TH 2.3.3.8 was mixed with standard vanillin in the half quantity of each, the result showed the intensity of major peak at 1.75 min increase from 1100 to 2000 more over minor peak still appear at 2.8 min with the same intensity as when inject only fraction TH 2.3.3.8. From the result can conclude that this fraction is vanillin.

Table 3.9 ^{1}H-NMR spectroscopic data for dipentyl phthalate in $CDCl_3$

position	δ_H, mult. (*J* in Hz)
1	3.9 (3H, s)
2	5.2 (1H, s)
3	6.9 (1H, d, 8.5)
4,5	7.4 (1H, m)
6	9.8 (1H, s)

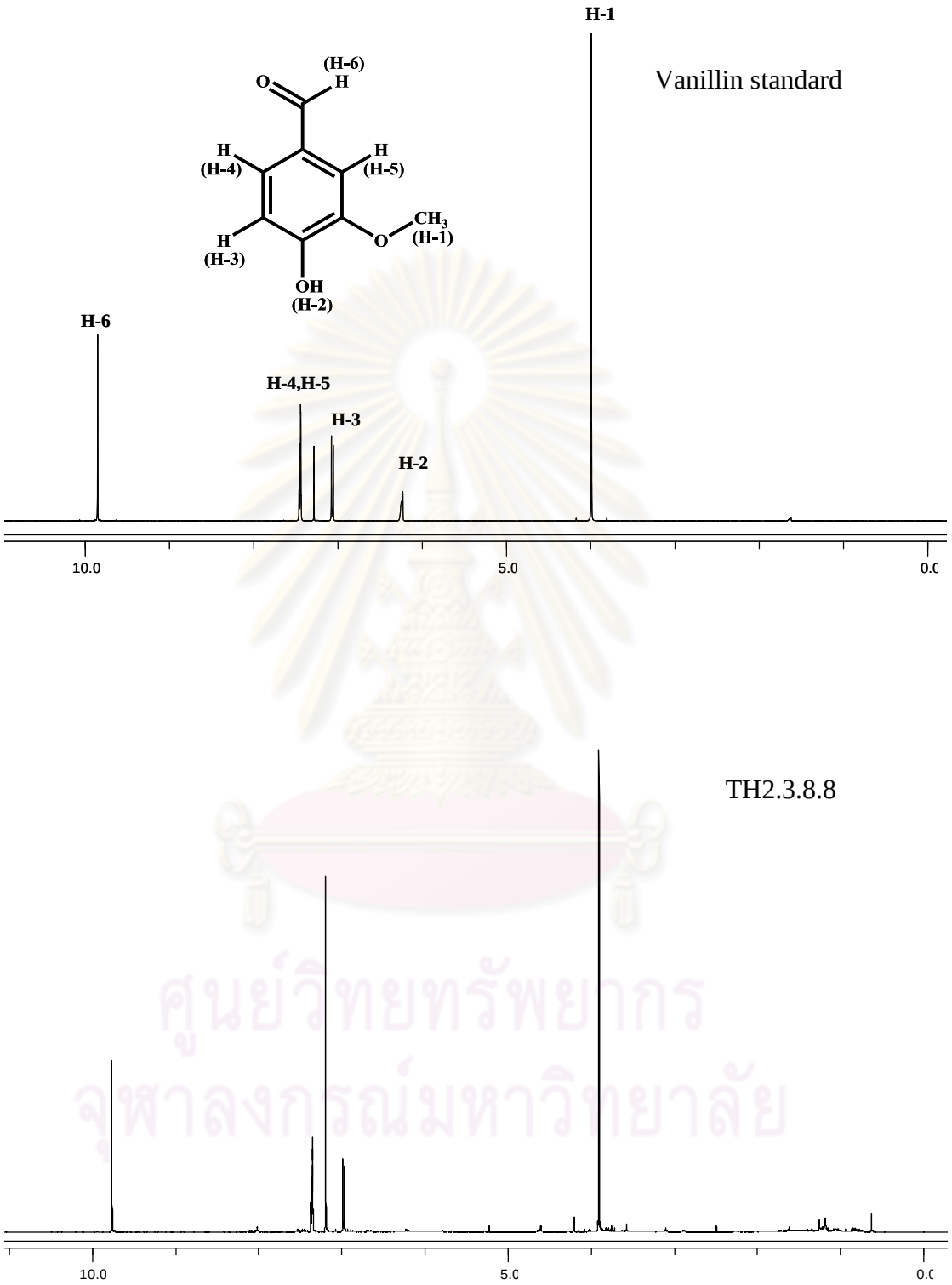


Figure 3.9 The 400 MHz ^{1}H NMR of standard vanillin and TH2.3.3.8 in $CDCl_3$

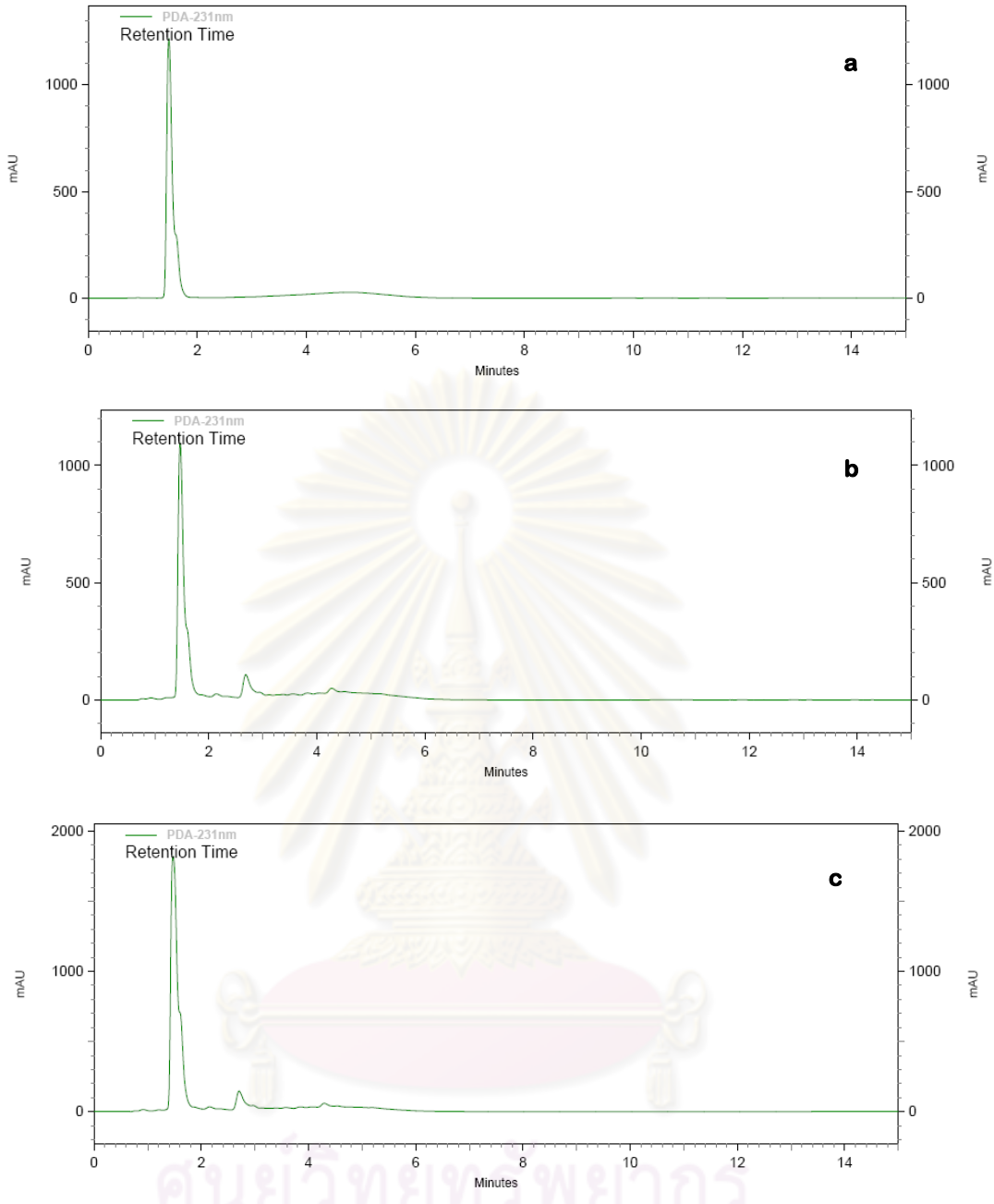


Figure 3.10 HPLC chromatograpm of standard vanillin and TH2.3.3.8

3.10.3 Acrylic acid derivative

TH 2.3.5 (14 mg) was obtained as a yellowish brown semi-solid from fraction 110 of TH 2.3.110 of dichloromethane crude extract. The IR spectrum showed absorption band for hydroxyl group (3430 cm^{-1}), carbonyl group of ester (1710 cm^{-1}) and C=C stretching of aromatic (1625 cm^{-1}) (Figure A-13, Appendix, Page 90)

The ^{13}C NMR spectrum of TH2.3.5 contained six resonances resulting from one quaternary carbon of ester, one quaternary carbon on aromatic ring, three carbons on aromatic ring connect to hydroxyl group and two methylene (Table 3.10). The ^{1}H

NMR of TH 2.3.5 show aromatic proton at δ_H 7.0 ppm (H-2 and H-6), three hydroxyl proton at δ_H 5.1 ppm (H-3, H-4 and H-5) and two methylene proton at δ_H 7.4 and 6.5 ppm (H-7 and H-8) (Table 3.10). The direct connectivity (one bond) of proton and carbon atoms were deduced from HSQC spectrum (Figure A-10, Appendix, Page 87).

Table 3.10 1D and 2D NMR spectroscopic data for acrylate part of TH 2.3.5 in $CDCl_3$

position	δ_H, mult. (J in Hz)	δ_C	COSY	HMBC
1		125		
2,6	7.0 (1H, s)	123		C-6, C-9
3,4,5	5.1 (1H, s)	130		
7	7.4 (1H,d, 9.5)	144	H-8	C-7, C-9
8	6.5 (2H, d, 9.5)	98	H-7	C-1, C-9
9		163		

The ^1H-^1H cosy spectrum (Figure A-12, Appendix, Page 89) revealed the presence of the following connectivity as shown in Figure 3.13.

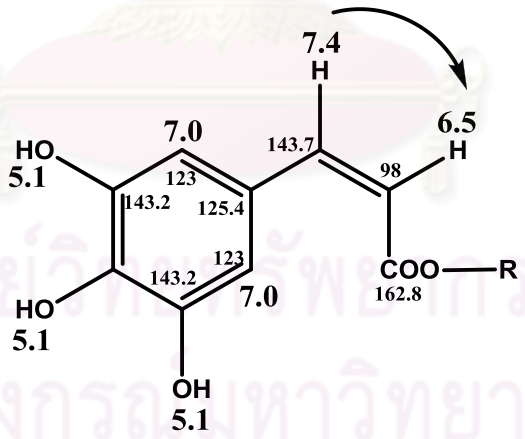


Figure 3.11 The COSY correlation of acrylate derivative.

The HMBC correlations (Figure A-11, Appendix, Page 88) were observed between the proton signal at δ_H 7.4 (H-7) to carbon signal at δ_C 163 ppm (C-9) and 123 ppm (C-6), between the proton signal of δ_H 7.0 (H-2 and H-6) to carbon signal at δ_C 144 ppm (C-7) and δ_C 163 ppm (C-9), H-8 (δ_H 6.5 ppm) to carbonyl carbon of ester

(C-9). This agrees with the C=O stretching in the IR spectrum. Consequently, the structure of TH2.3.5 was determined as acrylate derivative.

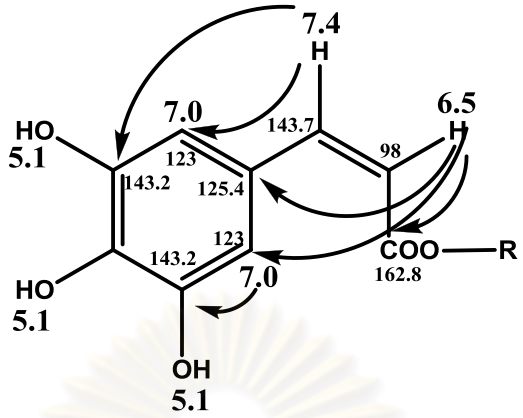


Figure 3.12 HMBC correlation of acrylate derivative

Furthermore, the negative ESI mass spectrum (Figure A-14, Appendix, Page 91) showed a $[M]^-$ ion m/z 195, thus confirming the acrylate group.

CHAPTER IV

CONCLUSION

Thanaka stem bark, the traditional beauty regiment of Myanmar women, was investigated the UV screening, antioxidation, tyrosinase inhibition, antibacterial and anti-inflammatory activities, including cytotoxic and genotoxic. Although the hexane, dichloromethane, ethyl acetate, methanol, 85% (v/v) ethanol and water extracts of the bark showed some variation between them in terms of their potency of those biological activities. It can be concluded that the 85% ethanol extract of this bark possesses significant antioxidation and anti-inflammatory activities and mild tyrosinase inhibition activity and anti-bacterial activity. The crude bark powder also demonstrated all the above-mentioned activities. All six solvent extracts and the original crude bark powder showed no detectable genotoxicity in the assay performed here. However, the methanol, 85% (v/v) ethanol and water extracts showed a very low cytotoxicity with IC_{50} values of more than 12 mg/ml. This study thus not only scientifically supports the pharmaceutical activities of *Hesperethusa crenulata* bark, the indigenous beauty regiment of Myanmar women, but also indicates that *Hesperethusa crenulata* extracts could be a good candidate source for cosmetic ingredients. Moreover, vanillin and acrylate derivative were purified and characterized in this study.

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APPENDIX

APPENDIX

1. Stock solution preparation

Phosphate buffer pH 6.8 stock solution

Preparation of phosphate buffer stock solution 500 mM 1000 ml with K_2HPO_4 (MW 174.18, 42.5521 g) and KH_2PO_4 (MW 136.09, 34.7982 g) in deionizers water. K_2HPO_4 and KH_2PO_4 were dissolved in 900 mL deionizers water and measured pH with pH meter (pH 211 microprocessor pH meters, HANNA Instrument) then adjust pH to 6.8 with 0.1 M HCl and 0.1 M NaOH next adjust volume to 1000 mL.

$$pH = pKa + \log \frac{[HPO_4^{2-}]}{[H_2PO_4^-]}$$

$$6.8 = 6.82 + \log \frac{[HPO_4^{2-}]}{[H_2PO_4^-]}$$

$$0.02 = \log \frac{[H_2PO_4^-]}{[HPO_4^{2-}]}$$

$$\frac{1.0471}{1} = \frac{[H_2PO_4^-]}{[HPO_4^{2-}]}$$

$[H_2PO_4^-] = [CA]$

$[CA] = (1.0471/2.0471) \times 0.5$

$[CA] = 0.2557$

KH_2PO_4 was used 0.2557 moles, 34.7982 g

$[HPO_4^{2-}] = [CB]$

$[CB] = (1/2.0471) \times 0.5$

$[CB] = 0.2443$

K_2HPO_4 was used 0.2443 moles, 42.5521 g

2. Preparation of base slides for comet assay

1. Prepare 1% (500 mg per 50ml PBS) and 0.5% LMPA (250 mg per 50 ml PBS) and 1.0%NMA (500 mg per 50 ml in Milli Q water). Microwave or heat until near boiling and the agarose dissolves. For LMPA, aliquot 5 mL samples into scintillation vials (or other suitable containers) and refrigerate until needed. When needed, briefly melt agarose in microwave or by another appropriate method. Place LMPA vial in a 37ºC dry/water bath to cool and stabilize the temperature.

2. Dip the slides in methanol and burn them over a blue flame to remove the machine oil and dust.

3. While NMA agarose is hot, dip conventional slides up to one-third the frosted area and gently remove (2). Wipe underside of slide to remove agarose and lay the slide in a tray on a flat surface to dry. The slides may be air dried or warmed at 50 °C for quicker drying. Store the slides at room temperature until needed; avoid high humidity conditions.

3. Preparation of solution for comet assay

Lysing solution

Lysing Solution: Ingredients per 1000 mL: NaCl 146.1 gm, EDTA 37.2 gm, Trizma base 1.2 gm Add ingredients to about 700 mL dH_2O and begin stirring the mixture. Add ~8 gm NaOH and allow the mixture to dissolve (about 20 min). Adjust the pH to 10.0 using concentrated HCl or NaOH. q.s. to 890 mL with dH2O (the Triton X-100 and DMSO will increase the volume to the correct amount), store at room temperature. Final lysing solution: add fresh 1% Triton X-100 and 10% DMSO, and then refrigerate for at least 30 minutes prior to slide addition

Electrophoresis buffer

Electrophoresis buffer (300 mM NaOH / 1 mM EDTA):

Prepare from stock solutions: NaOH (200 g/500 mL dH_2O), EDTA (14.89 g/200 mL dH_2O, pH 10). Store both at room temperature.

For 1X Buffer (made fresh before each electrophoresis run): per liter, add 30 mL NaOH and 5.0 mL EDTA adjust volume to 1000 mL, mix well. The total volume depends on the gel box capacity.

Neutralization Buffer

Neutralization Buffer: 0.4 M Tris - 48.5 gm added to ~800 mL dH_2O, adjust pH to 7.5 with concentrated (>10 M) HCl: adjust volume to 1000 mL with dH_2O, store at room temperature.

4. Electrophoresis of microgel slides

The procedure described is for electrophoresis under pH>13 alkaline conditions.

1. After at least 2hour at ~4ºC, gently remove slides from the Lysing Solution. Place slides side by side on the horizontal gel box near one end, sliding them as close together as possible.

2. Fill the buffer reservoirs with freshly made pH>13 Electrophoresis Buffer until the liquid level completely covers the slides (avoid bubbles over the agarose).

3. Let slides sit in the alkaline buffer for 20 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage.

NOTE: The longer the exposure to alkali, the greater the expression of alkali-labile damage.

4. Turn on power supply to 50 volts, electrophorese the slides for 15 minutes.

NOTE: The goal is to obtain migration among the control cells without it being excessive. The optimal electrophoresis duration differs for different cell types. If crosslinking is one of the endpoints being assessed then having controls with about 25% migrated DNA is useful. A lower voltage, amperage and a longer electrophoresis time may allow for increased sensitivity. Different gel boxes will require different voltage settings to correct for the distance between the anode and the cathode.

5. Turn off the power. Gently lift the slides from the buffer and place on a drain tray. Drop wise coat the slides with Neutralization Buffer, let sit for at least 5 minutes. Drain slides and repeat two more times.

6. Slides may be stained Ethidium Bromide, leave for 5 min and then dipped in chilled distilled water to remove excess stain. The coverslip is then placed over it and the slides are scored immediately.

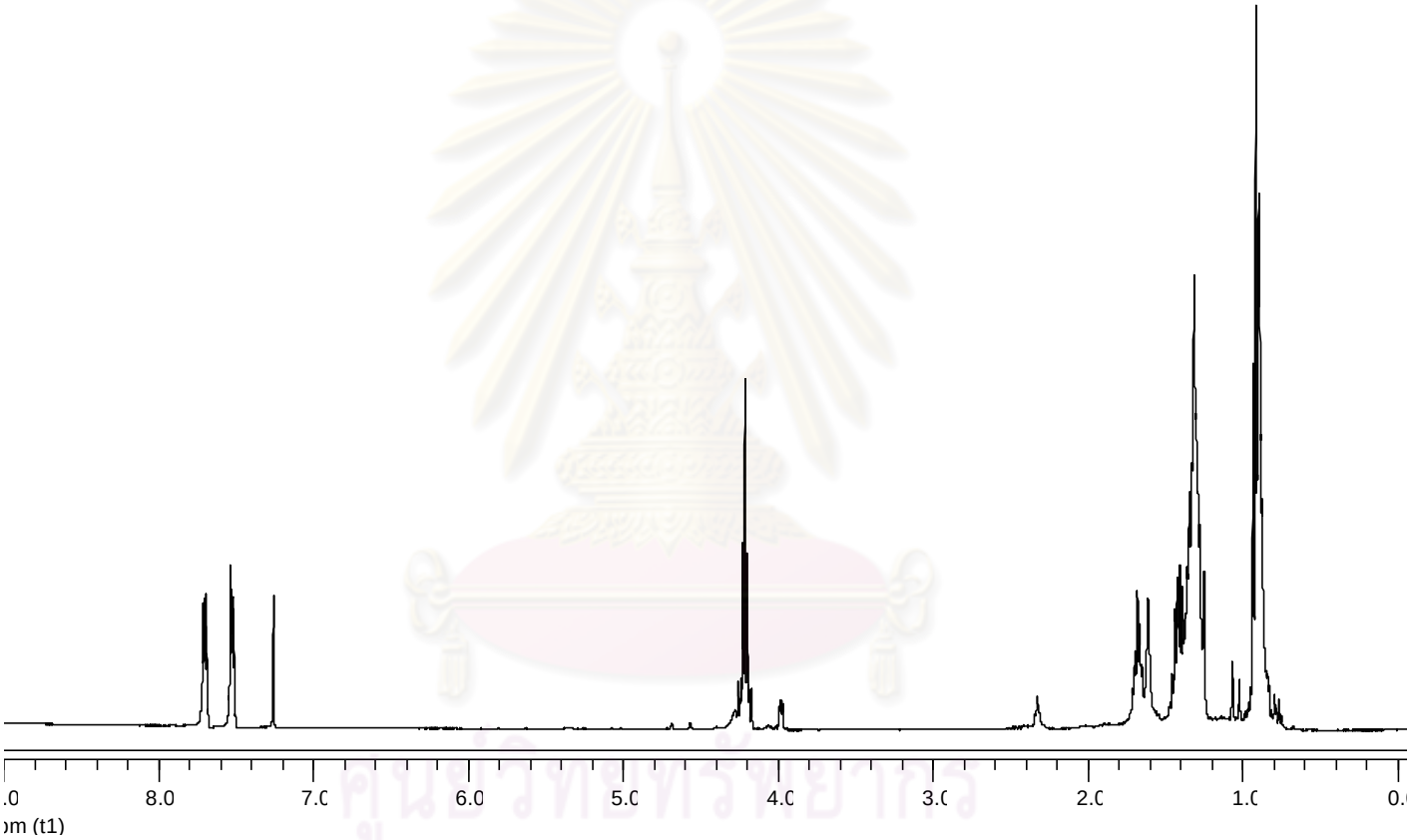

Figure A-1 ^{1}H NMR spectrum ($CDCl_3$) of dipentyl phthalate

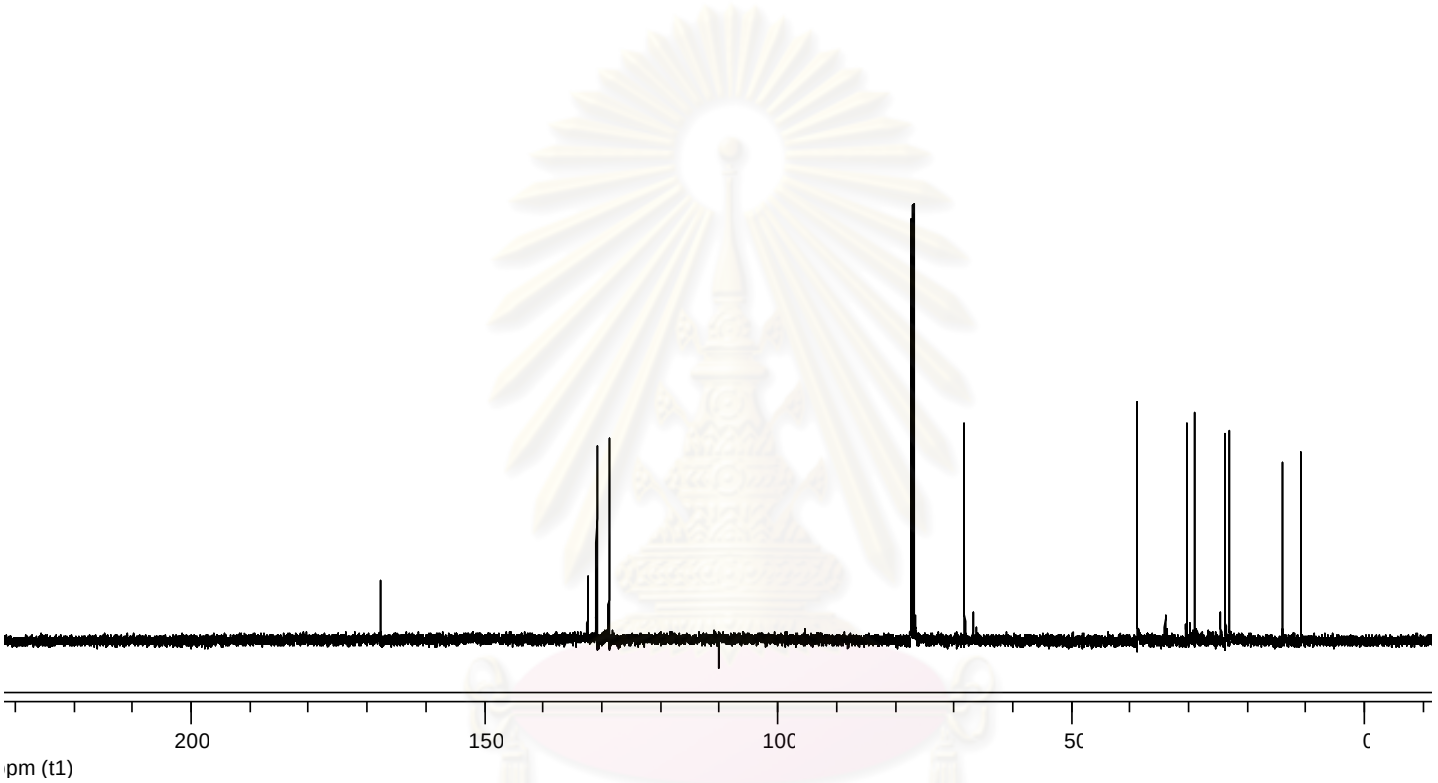

Figure A-2 ^{13}C NMR spectrum ($CDCl_3$) of dipentyl phthalate

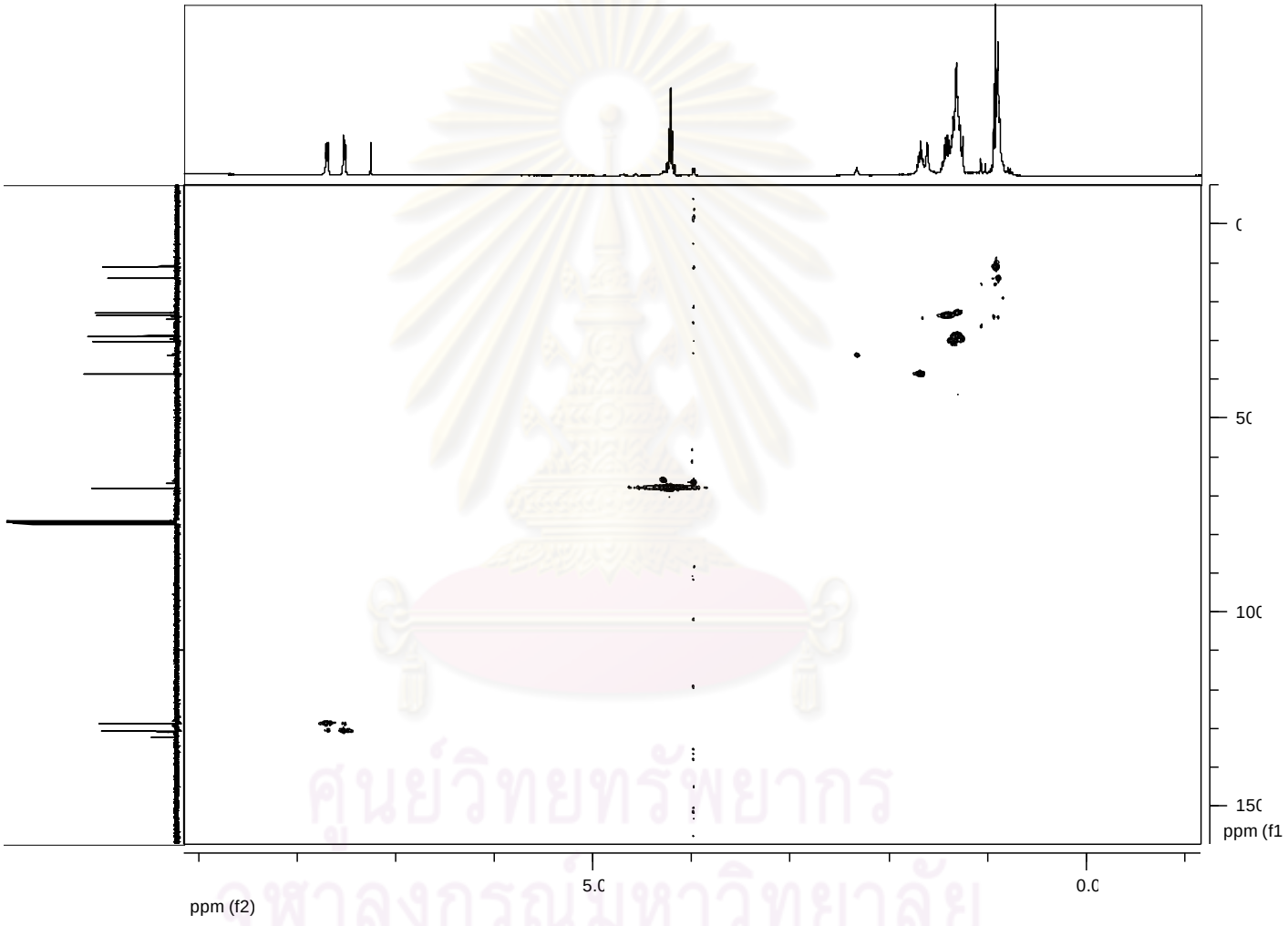

Figure A-3 The HSQC spectrum ($CDCl_3$) of dipentyl phthalate

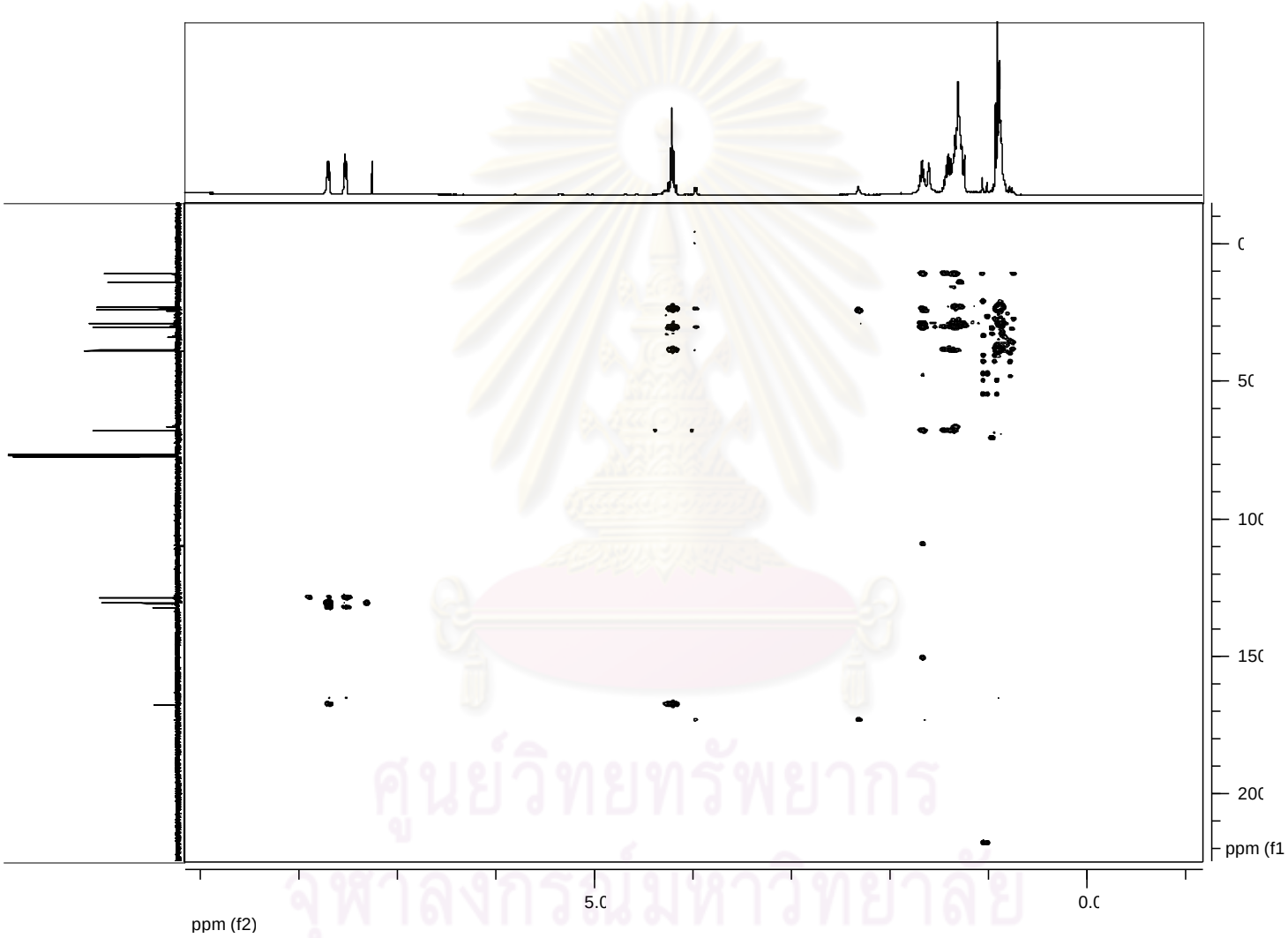

Figure A-4 The HMBC spectrum ($CDCl_3$) of dipentyl phthalate

81

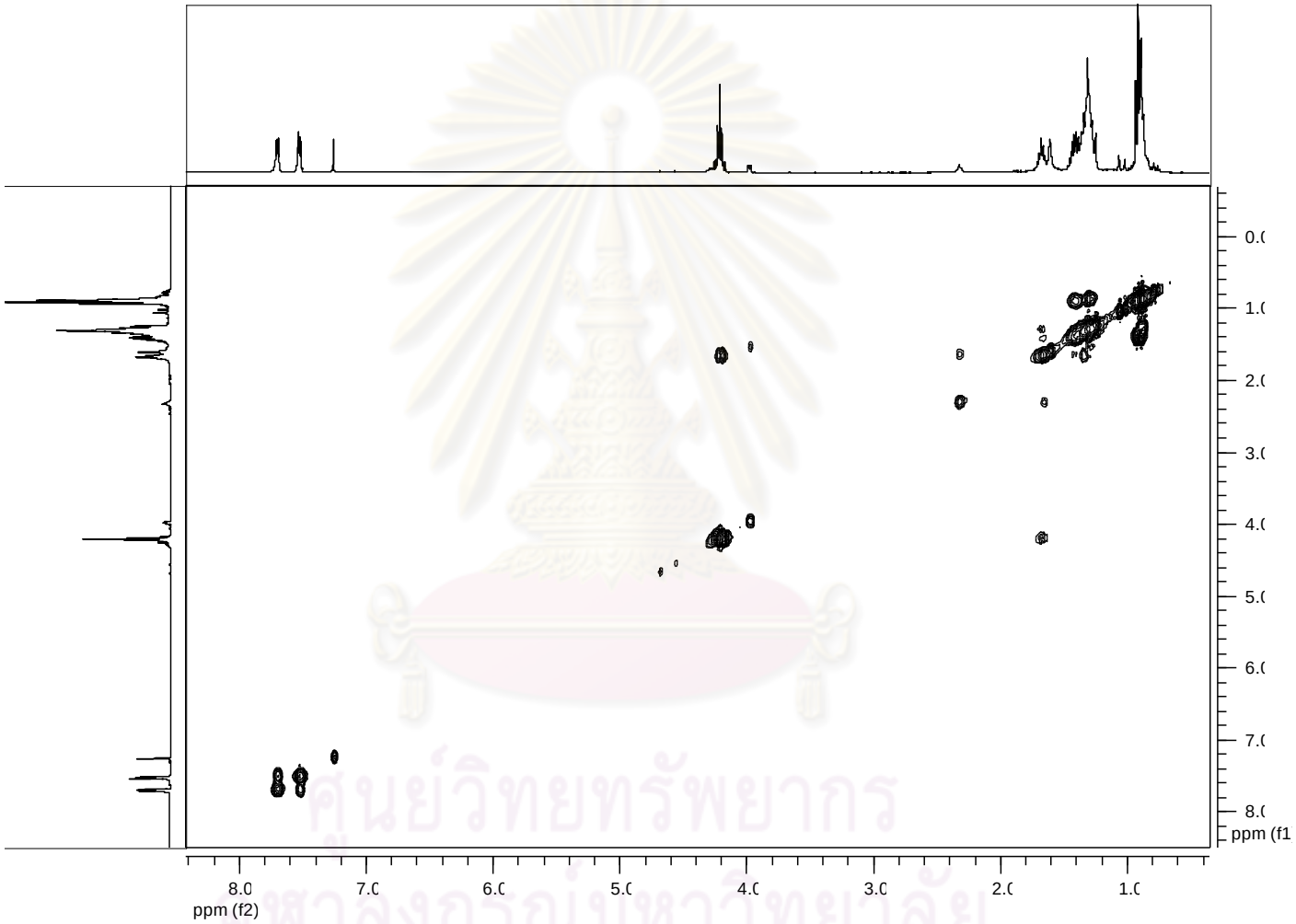

Figure A-5 The COSY spectrum ($CDCl_3$) of dipentyl phthalate

82

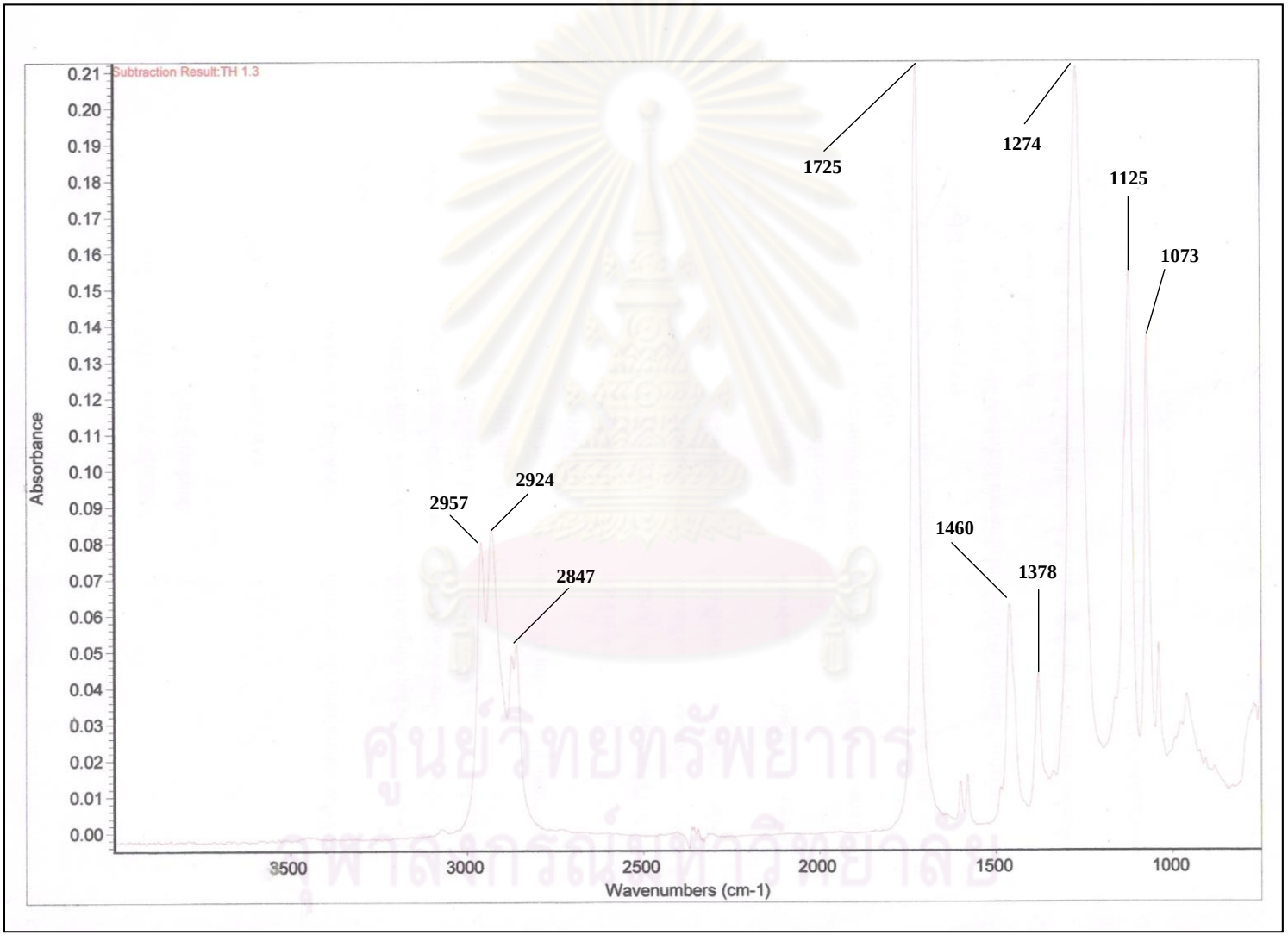

Figure A-6 The IR spectrum of dipentyl phthalate

83

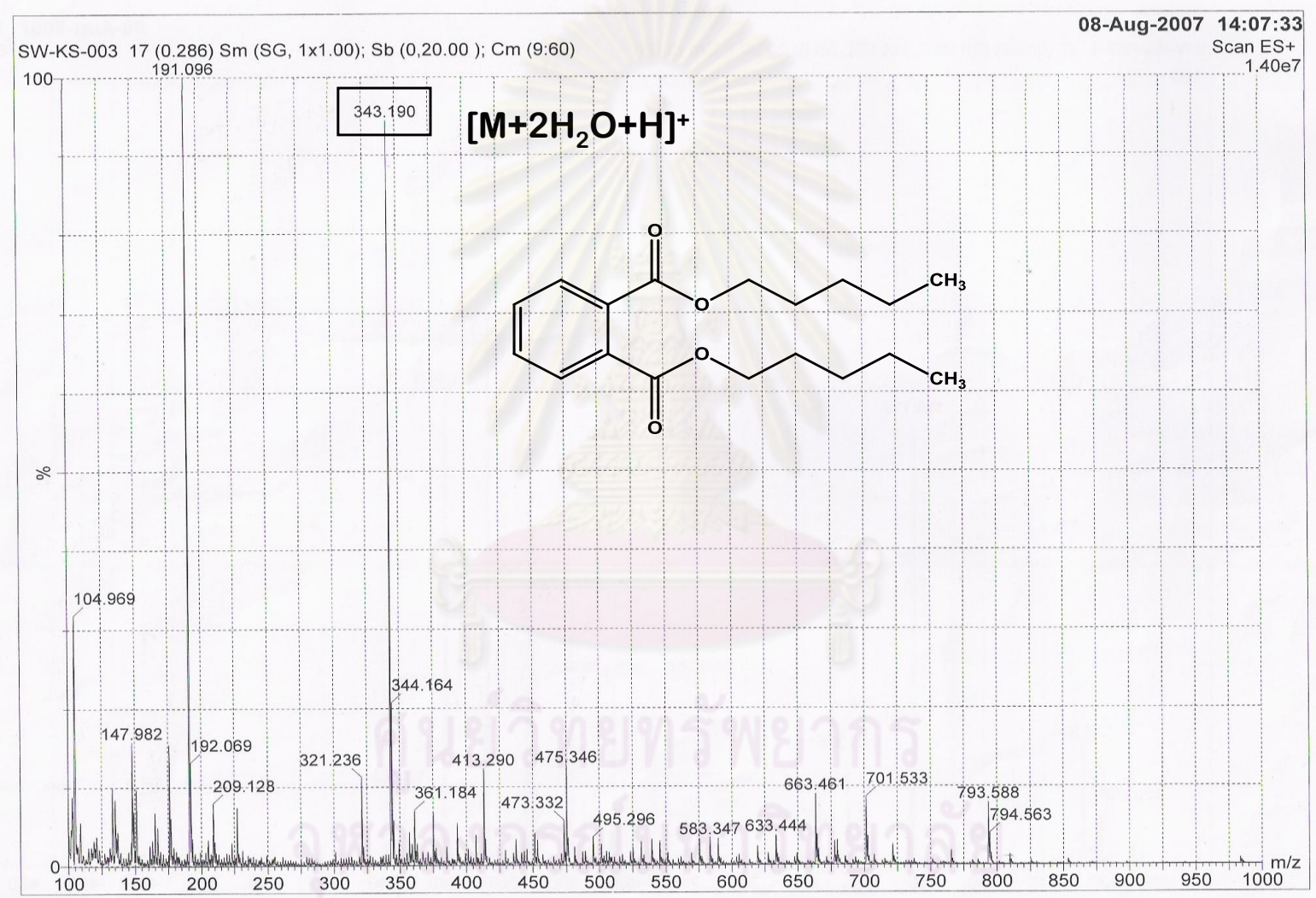

Figure A-7 The negative ESI-MS spectrum of dipentyl phthalate

84

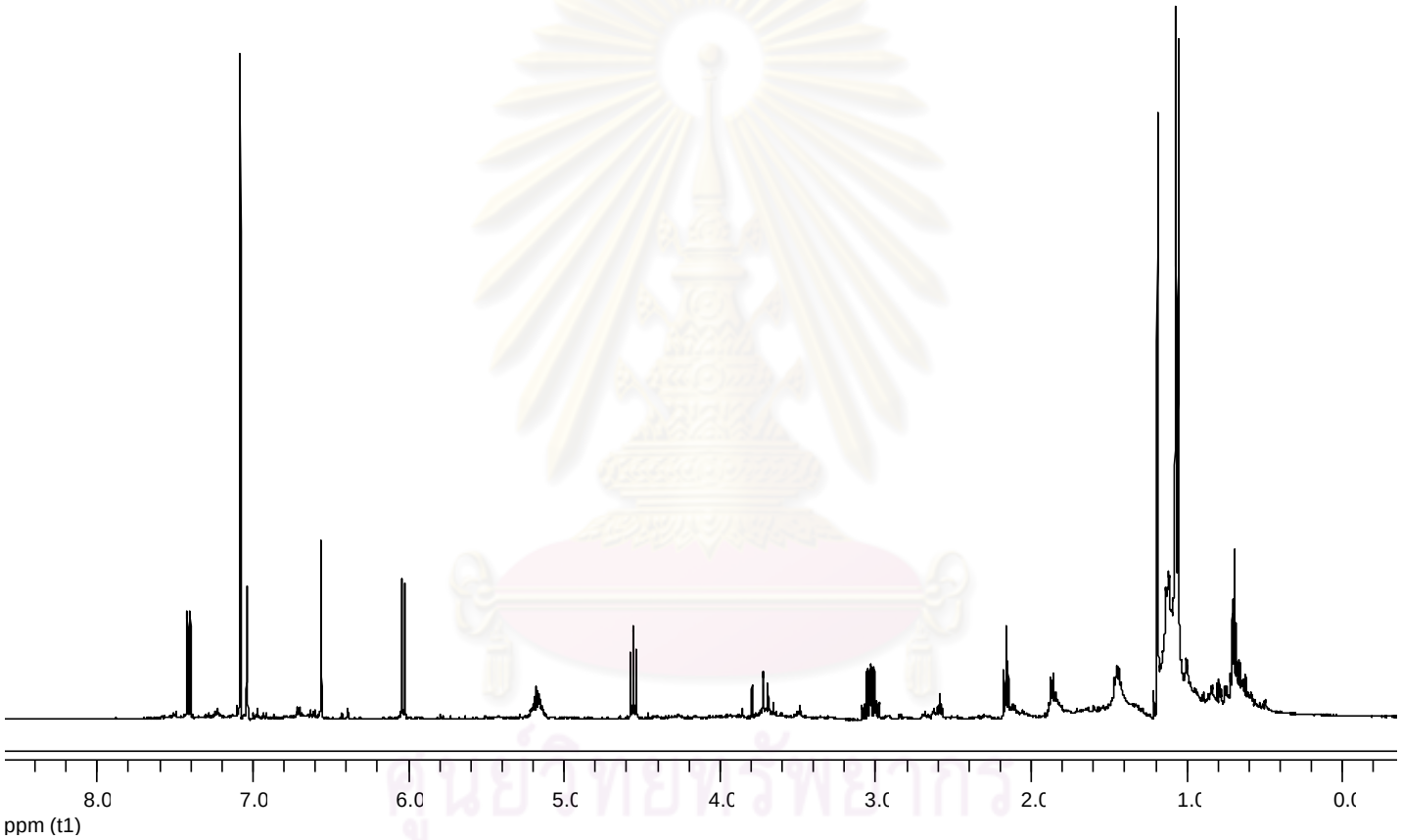

Figure A-8 ^{1}H NMR spectrum ($CDCl_3$) of acrylate derivative

85

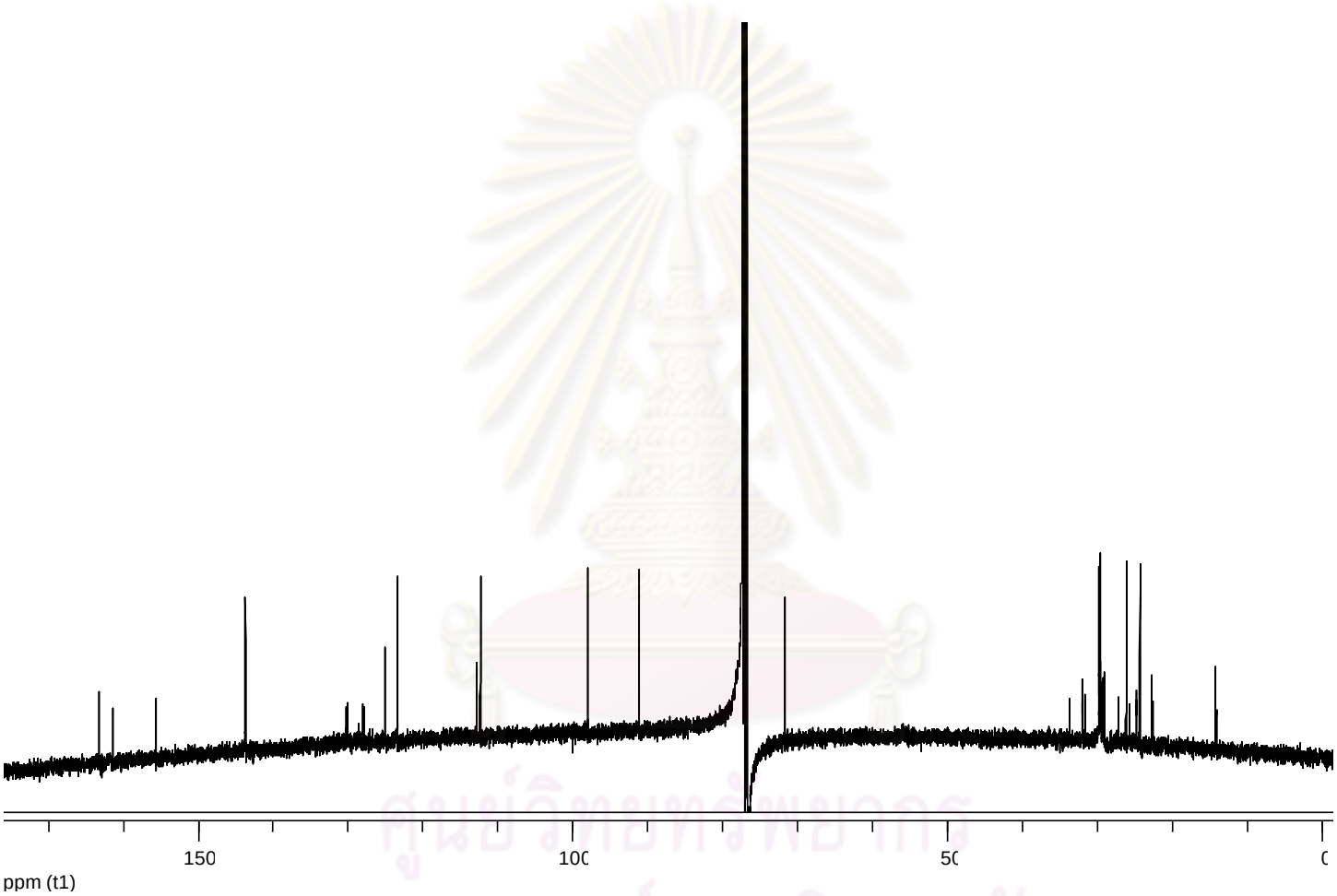

Figure A-9 ^{13}C NMR spectrum ($CDCl_3$) of of acrylate derivative

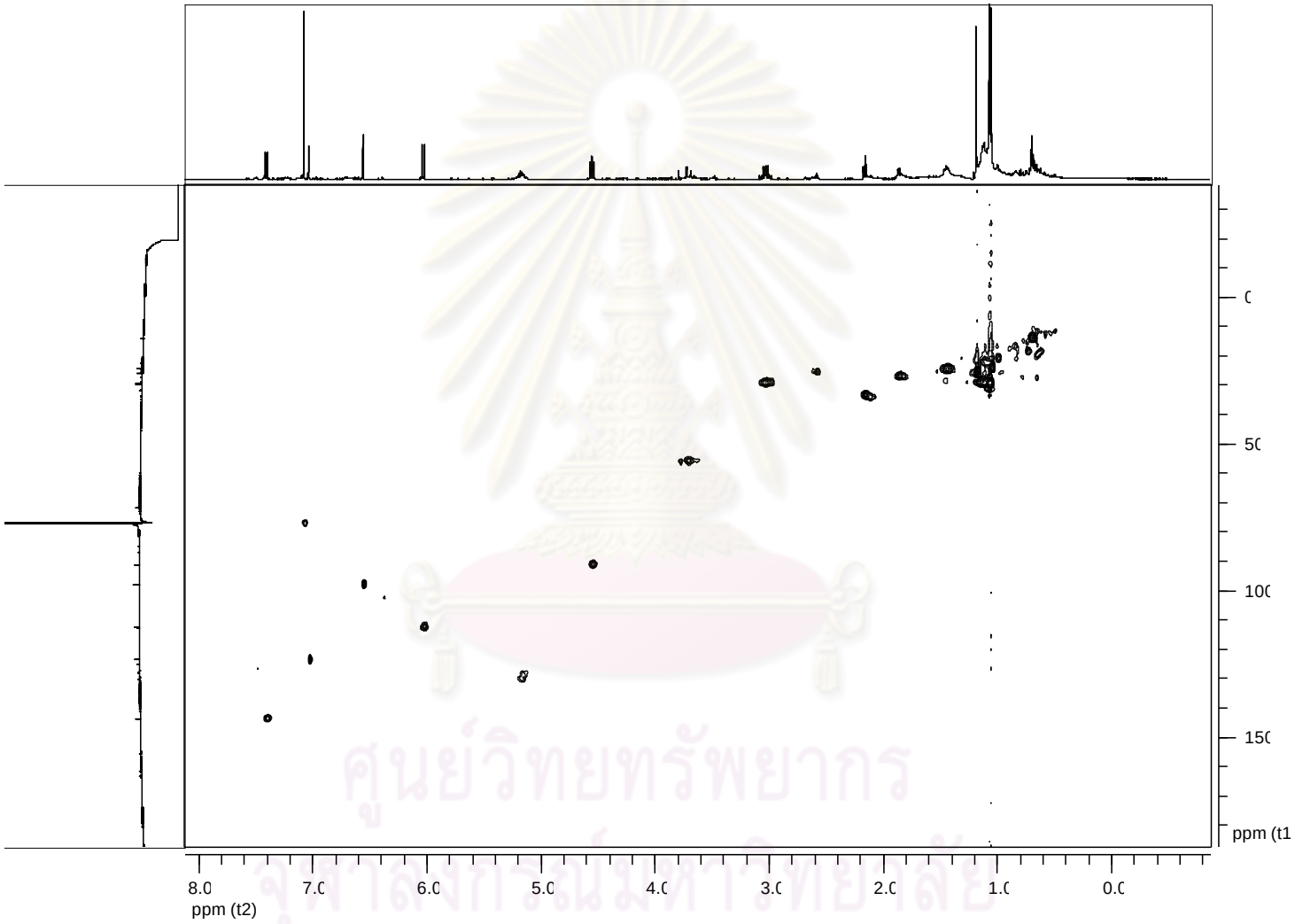

Figure A-10 The HSQC spectrum ($CDCl_3$) of of acrylate derivative

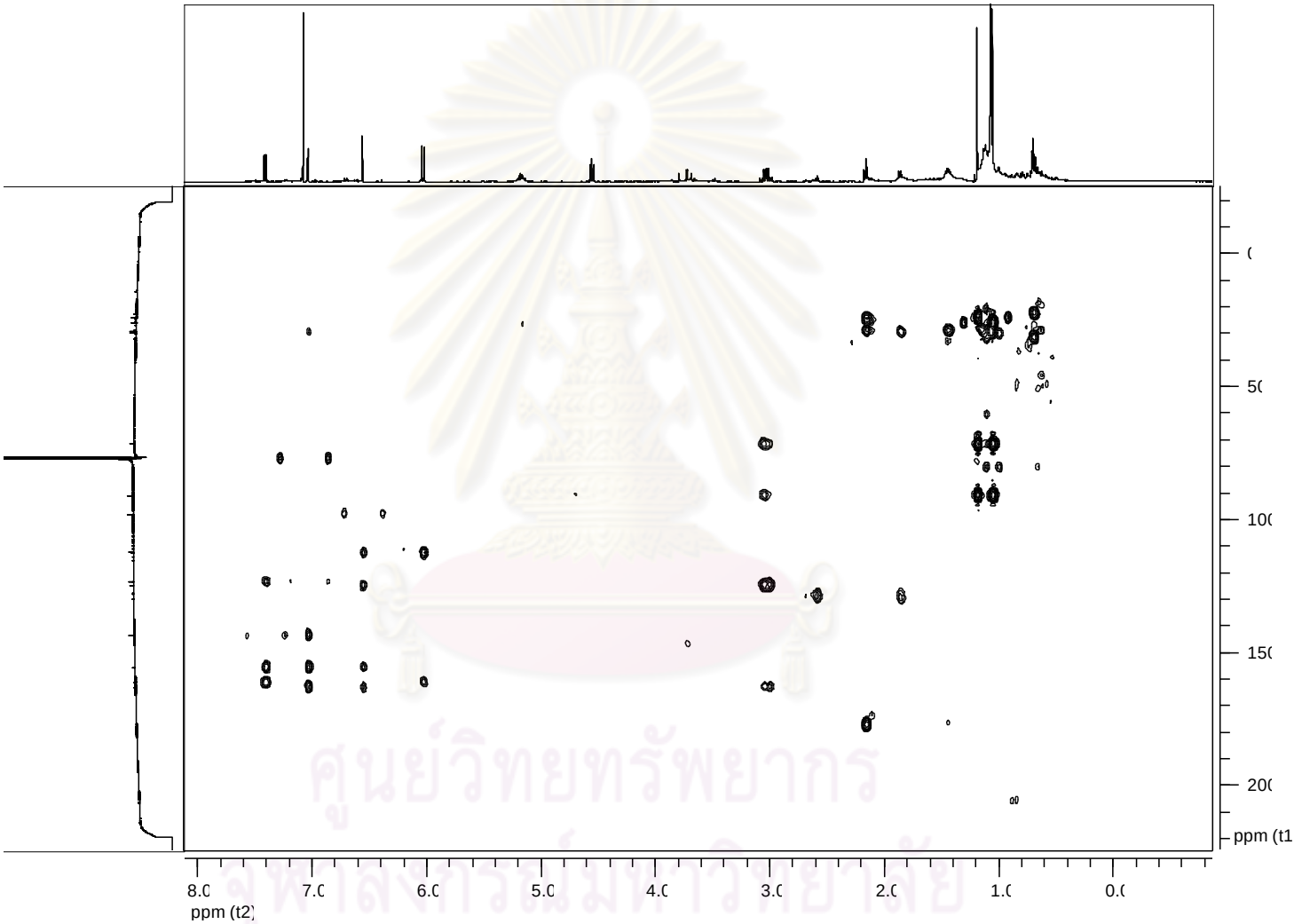

Figure A-11 The HMBC spectrum ($CDCl_3$) of of acrylate derivative

88

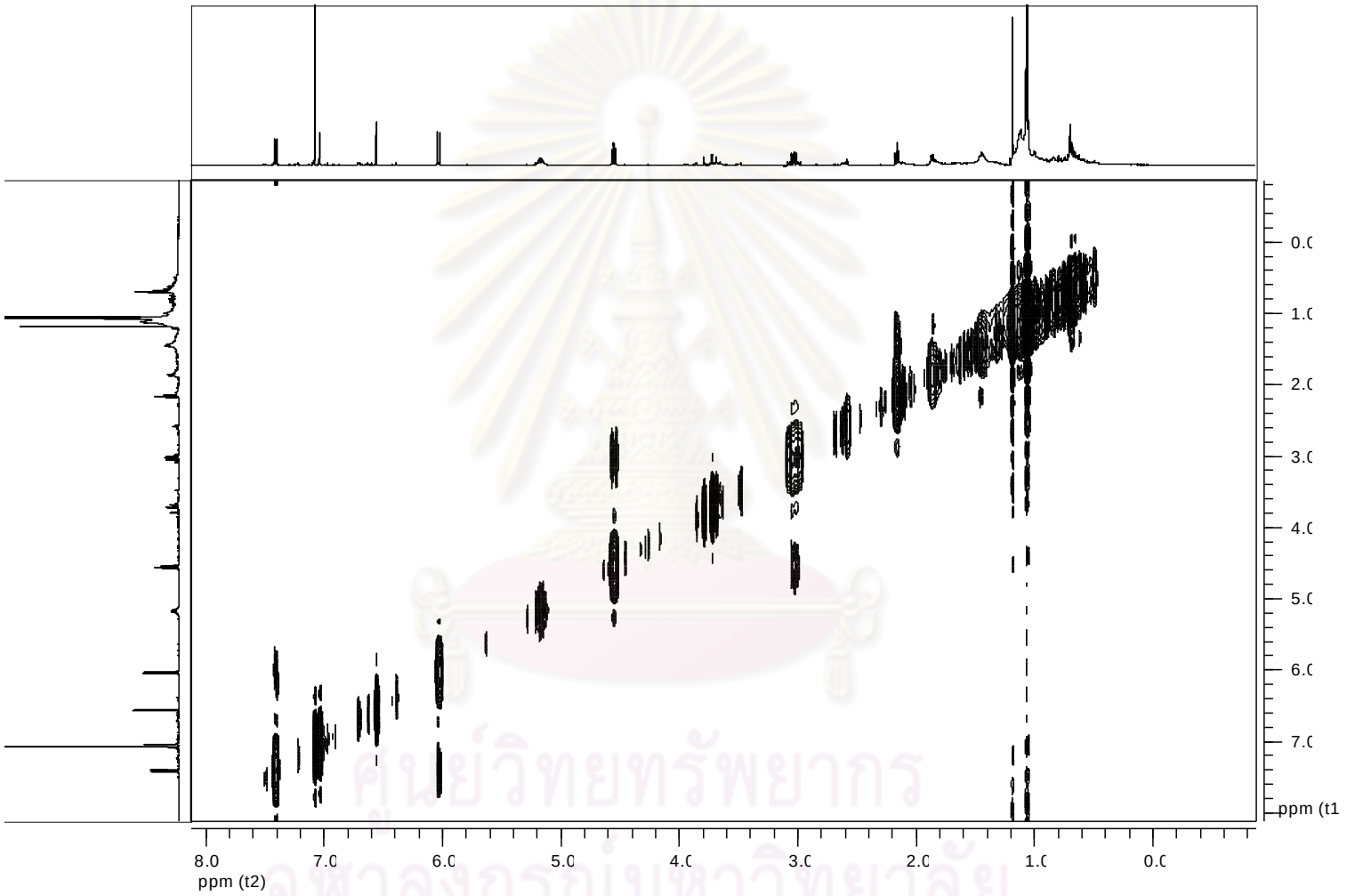

Figure A-12 The COSY spectrum ($CDCl_3$) of acrylate derivative

89

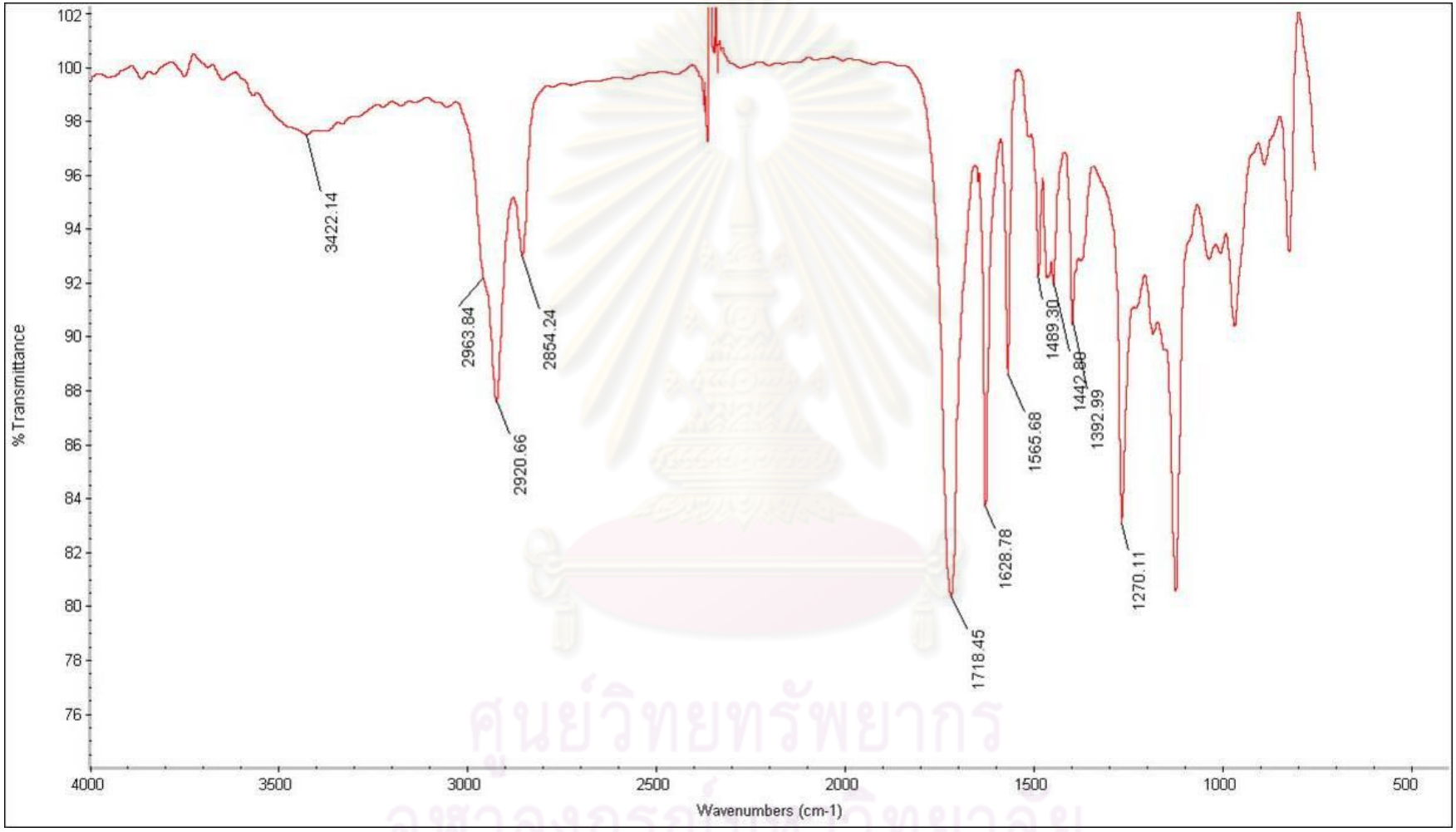

Figure A-13 The IR spectrum of acrylate derivative

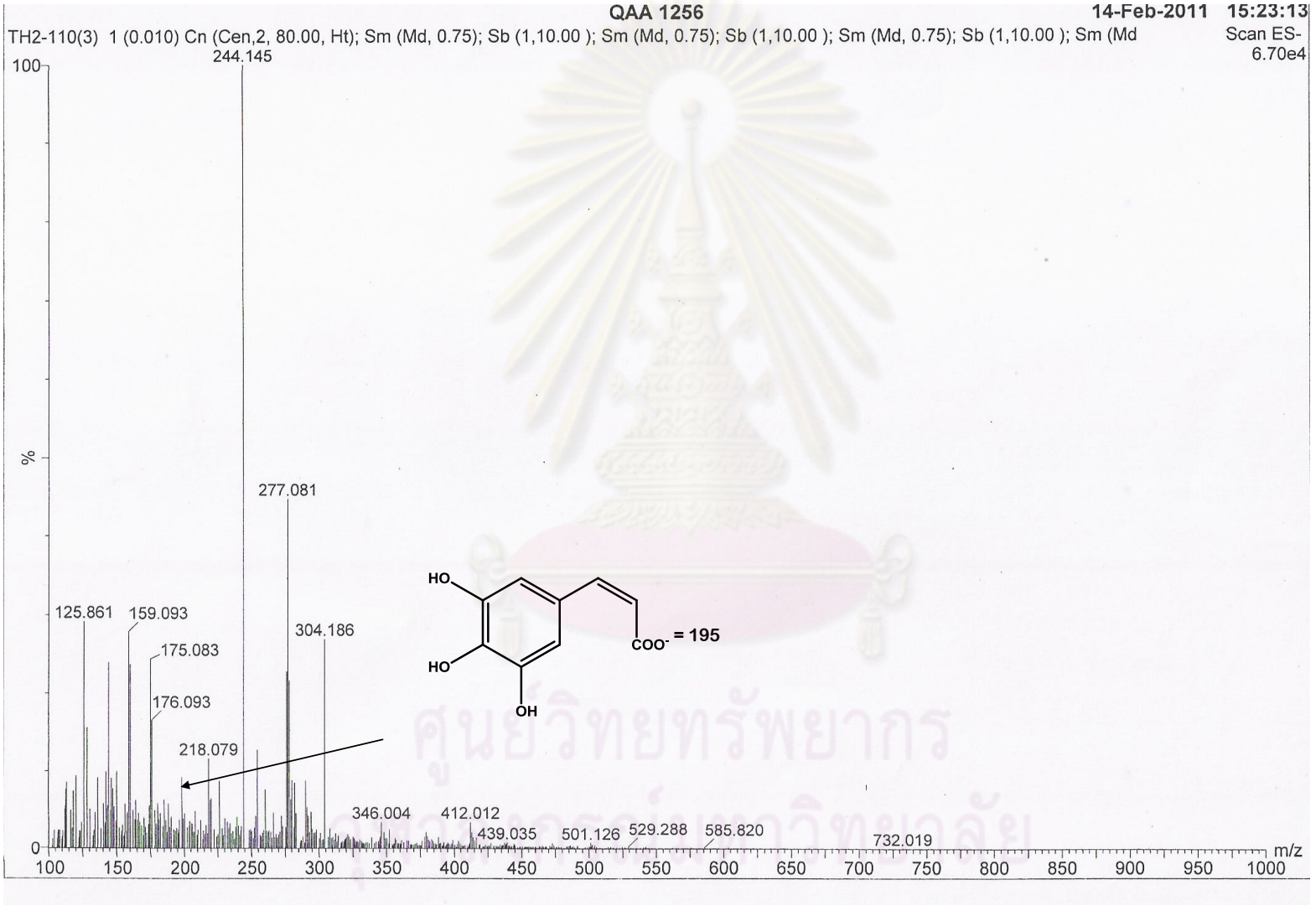

Figure A-14 The negative ESI-MS spectrum of acrylate group

91

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INTERNATIONAL PUBLICATIONS

Wangthong, S., Tonsiripakdee, I., Monhaphol, T., Nonthabenjawan, R., and Wanichwecharungruang, S.P. Post TLC developing technique for tyrosinase inhibitor detection. *Biomedical Chromatography*. 2007, 21, 94-100.

Wangthong, S., Palaga, T., Rengpipat, S., Wanichwecharungruang, S.P., Chanchaisak, P., and Heinrich, M. Biological activities and safety of thanaka (Hesperethusa crenulata) stem bark. *Journal of Ethnopharmacology*. 2010, 132, 466-472.