สารออกฤทธิ์สำหรับเครื่องสำอางจากชานอ้อย (*Saccharum officinarum* L.)

นางสาวพรรณพิไล ชาญชัยศักดิ์

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COSMETIC ACTIVES FROM SUGARCANE (*Saccharum officinarum* L.) BAGASSE

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A Thesis Submitted in Partial Fulfillment of the Requirements
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พรรณพิไล ชาญชัยศักดิ์ : สารออกฤทธิ์สำหรับเครื่องสำอางจากชานอ้อย *Saccharum officinarum* L. (COSMETIC ACTIVES FROM SUGARCANE BAGASSE (*Saccharum officinarum* L.)) อ. ที่ปรึกษา: รศ.ดร.ศุภศร วนิชเวชารุ่งเรือง, 68 หน้า.

ในงานวิจัยนี้ได้นำชานอ้อยมาสกัด และตรวจกรองหาสารออกฤทธิ์ทางชีวภาพต่างๆ จากส่วนสกัดหยาบ โดยใช้วิธีการสกัดแบบไล่ขั้วของตัวทำละลาย การสกัดด้วยการแยกชั้นของตัวทำละลาย และวิธีการสกัดโดยใช้ไดคลอโรมีเทนโดยตรง ฤทธิ์ทางชีวภาพที่มุ่งตรวจสอบครอบคลุมตั้งแต่ฤทธิ์ต้านการทำงานของไทโรซิเนส ฤทธิ์ต้านการออกซิเดชัน และฤทธิ์กรองรังสียูวี พบว่าส่วนสกัดไดคลอโรมีเทน ที่ผ่านกระบวนการสกัดโดยวิธีไล่ขั้วของตัวทำละลาย ให้ฤทธิ์ต้านการออกซิเดชันที่ดีและสามารถยับยั้งการทำงานของไทโรซิเนส นอกจากนี้ยังสามารถดูดกลืนรังสียูวีได้ การแยกส่วนสกัดดังกล่าวด้วยเทคนิคโครมาโตกราฟีให้สารออกฤทธิ์ 6 ชนิด คือ 4-ไฮดรอกซีเบนซัลดีไฮด์ ไซริงจิกแอซิด 3,5-ไดเมททิล-4-ไฮดรอกซีเบนซัลดีไฮด์ 3',5'-ไดเมทอกซี-4'-อะซิโตฟีโนน 4-ไฮดรอกซี-3-เมทอกซีซินนามิกแอซิด และ อูนเดกเคน-4,6-ไดโอน จากการทดสอบฤทธิ์ทางชีวภาพของสารบริสุทธิ์ พบว่า ไซริงจิกแอซิด 3,5-ไดเมททิล-4-ไฮดรอกซีเบนซัลดีไฮด์ และ 4-ไฮดรอกซี-3-เมทอกซีซินนามิกแอซิด แสดงฤทธิ์ต้านอนุมูลอิสระ และ ไซริงจิกแอซิด 4-ไฮดรอกซีเบนซัลดีไฮด์ และ อูนเดกเคน-4,6-ไดโอน แสดงฤทธิ์การยับยั้งการทำงานของเอนไซม์ไทโรซิเนส

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....พรรณพิไล ชาญชัยศักดิ์.....

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PANPILAI CHANCHAISAK: COMETIC ACTIVES FROM SUGARCANE (*Saccharum officinarum* L.) BAGASSE. THESIS ADVISOR: ASSOC. PROF. SUPASON WANICHWECHARUNGRUANG, Ph.D., 68 pp.

In this work, sugarcane (*Saccharum officinarum* L.) bagasse was extracted by sequential solvent extraction, liquid-liquid extraction and single CH_2Cl_2 extraction process. The extracts were tested for antioxidant, tyrosinase inhibition and UV-screening activities. The best process to obtain extract with good antioxidant, tyrosinase inhibition and UV-absorption activities from sugarcane bagasse is CH_2Cl_2 sequential solvent extraction. The high-yielded extracts with good activities were subjected for further isolation by chromatographic techniques and 4-hydroxybenzaldehyde, syringic acid, 3,5-dimethyl-4-hydroxybenzaldehyde, 4-hydroxy-3-methoxycinnamic acid, 3',5'-dimethoxy-4'-hydroxy acetophenone and undecane-4,6-dione could be identified. Free radical scavenging activity of sugarcane extract is partly contributed from the presence of syringic acid, 3,5-dimethyl-4-hydroxybenzaldehyde and 4-hydroxy-3-methoxycinnamic acid. While tyrosinase inhibition activity is partly contributed by the presence of syringic acid, 4-hydroxybenzaldehyde and undecane-4,6-dione.

Field of Study : Biotechnlogy Student's Signature : ภาณุไชย ศักดิ์

Acadamic Year : 2007 Advisor's signature :

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LIST OF ABBREVIATIONS

^{13}C NMR	Carbon 13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
BHT	Butylated hydroxytoluene
CH_2Cl_2	Dichloromethane, methylene chloride
$CHCl_3$	Chlorofrom
COSY	Correlated spectroscopy
DPPH	2,2-diphenyl-1-picryl hydrazyl
EtOAc	Ethylacetate
EtOH	Ethanol
g	Gram (s)
GAE	Gallic acid equivalents
HMBC	Heternuclear multiple bond correlation experiment
HSQC	Heternuclear multiple-quantum coherence experiment
HPLC	High performance liquid chromatography
kg	Kilogram (s)
wt	Weight
L	Liter (s)
MeOH	Methanol
mg	Milligram (s)
ml	Milliliter (s)
mM	Millimolar
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass to charge ratio

nm	Nanometer
ROS	Reactive oxygen species
SiO_2	Silica gel
TLC	Thin layer chromatography
δ	Chemical shift
µg	Microgram (s)
µl	Microliter
w/w	Weight by weight

CHAPTER I
INTRODUCTION

Lignocelluloses are the most abundant organic compounds in nature and represent an important resource for producing valuable products. They are present in agricultural, forestry and fruit and vegetable processing wastes. Thailand is an agricultural country. Each year the country produces not only agricultural products but also a lot of agricultural residues **(Table 1.1)**. It is estimated that there are more than 50 million tons of agricultural residues per year [1]. These forms of wastes accumulate every year in large quantities, causing a deterioration of the environment and loss of potentially valuable resources. Currently, the green house effect is one of the world wide environmental problems, which rises from the rapid increase in the atmospheric CO_2 concentration due to man made causes such as burning of fossil fuel, oil and gas, wood fuel and biomass burning. Agricultural wastes such as sugarcane trash, corn stalk, rice straw and etc. are left in the field after harvesting and burned by the farmers to clear up the field. Moreover, agricultural wastes such as sugarcane bagasse, corn cob, corn hull, rice bran and etc. from the industries especially from food industries are often burned to reduce bulk. Burning of agricultural wastes may have a serious polluting effect. Thus, a significant contribution should be made to the overall problem of wastes recycling and conservation. In any case, the recovery of such bioactive components has to be economically viable. Research must be oriented towards the improvement of extraction techologies, the correct assessment of biological activities and proving the security of the products.

Table 1.1 Resources of agricultural and their residue in Thailand (2004) [2].

Type	Production (1000 ton)	Agricultural residues	Residues (1000 ton)
Sugarcane	70,101	Bagasse Trash	20,399 21,171
Paddy	26,841	Husk Straw	6,173 11,998
Oil palm	4,903	Empty bunches Fiber Shell	1,226 721 240

Thailand is located in the tropical zone which is abundant in natural resources and the country is known as 'agriculture country'. Based on this fertility many resources can be brought to variety of beneficial researches. The by-product of these natural resources is also a serious problem. Application of agro-industrial residues in pulping process and other chemical production on the one hand provides alternative substrates, and on the other hand helps in solving pollution problems, which their disposal may otherwise cause.

1. Antioxidant activity

A free radical is a very reactive atom with an unpaired electron, which can be in a reduced or oxidized state. The majority of free radicals that damage biological systems are oxygen radicals and other reactive oxygen species (ROS), the main byproducts formed in the cells of aerobic organisms. The amount of free radical production is determined by the balance of many different factors, while the source of ROS formation is mainly constituted by mitochondria during electron transport in the oxidative phosphorylation chain [3]. The examples of free radicals are reactive oxygen species (ROS), various froms of activated oxygen, which include superoxide anion radical (O_2^{-}•), peroxyl radical (ROO•), alkoxyl radical (RO•) hydroxyl radical ($^{\bullet}$OH) and nitric oxide radical (NO•) as well as nonfree-radical species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and hypochlorous acid (HOCl) [4]. In addition, intracellular formation of free radicals can derive from environmental

sources including ultraviolet light, ionizing radiation, and pollutants such as paraquat and ozone [5].

Free radicals activity has also been shown to oxidize and cross-link proteins including enzymes and connective tissue. In particular, the amino acid residues of protein are highly susceptible to oxidative attack and it has been shown that there is a progressive (almost exponential) increase in this form of damage in the cells and tissues of the body as a function of age. The reaction of an oxygen radical with DNA can knock out a base, or cause a strand breakage [6].

Oxidative stress is frequently used in a number of biochemical, physiological and pathophysiological situations [7]. It describes the result of an increased reactive oxygen species production and/or a diminution in their elimination. Based on the fact that reactive oxygen species are dangerous for cells, tissues and organs, it has been inferred that oxidative stress is a cause of a number of pathologies, from atherosclerosis, to neural degenerative disease, Alzheimer's disease [8-9], Parkinson's disease [10], cardiovascular disease, inflammation, cancer, and ageing [11-13]. Antioxidant are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [14]. There are two basic categories of antioxidants, namely, synthetic and natural antioxidants. In general, synthetic antioxidants are compounds with phenolic structures of various degrees of alkyl substitution. Synthetic antioxidants **(Figure 1.1)** such as butylated hydroxynisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroxyquinone (TBHQ) have been used as antioxidants since the beginning of 2008. Restrictions on the use of these compounds, however, are being imposed because of their carcinogenicity [15]. Consequently, the importance of the search for and exploitation of natural antioxidants, particularly on plant origin, has greatly increased. Natural antioxidants can be phenolic compounds (tocopherol, flavonoids, and phenolic acid), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acid, and amines), or carotenoids as well as ascorbic acid [16]**.**

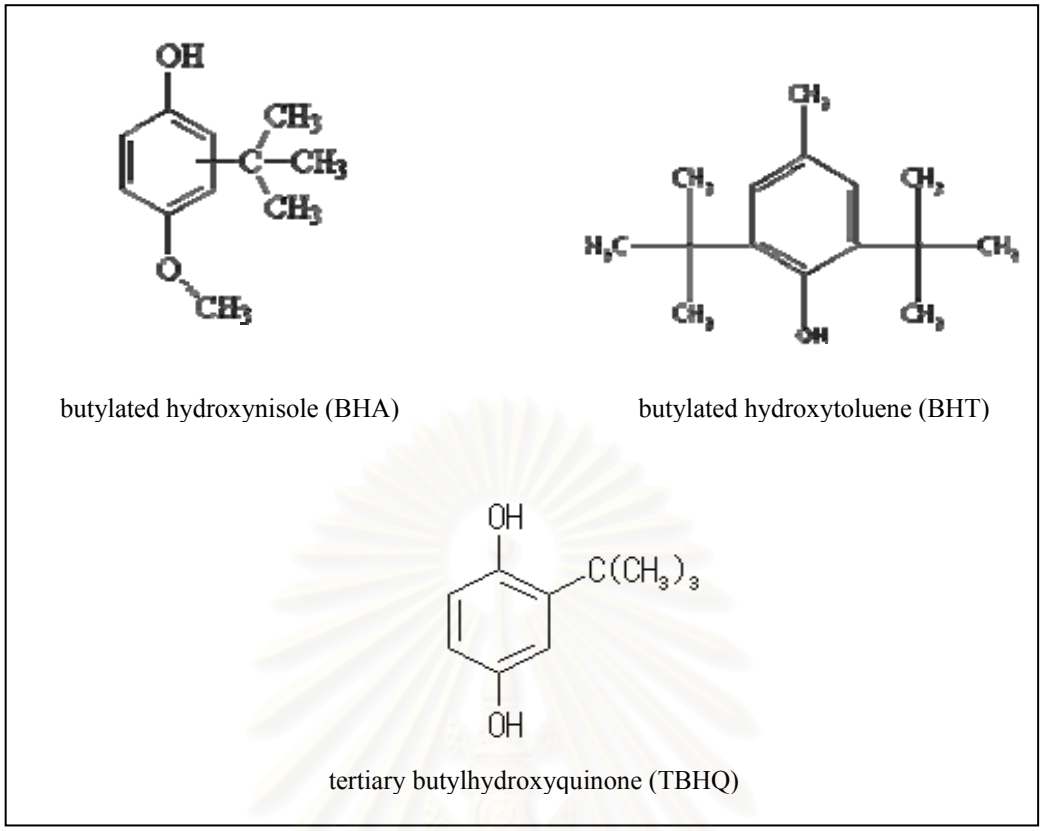


Figure 1.1 Synthetic antioxidants

In this work, antioxidant activity was measured using a free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. DPPH radical scavenging is a measurement of discoloration of DPPH. DPPH **(Figure 1.2)** is a class of nitrogen-centered radical and stable with its resonance system and a radical generating substance to monitor the free radical scavenging abilities (the ability of a compound to donate an electron). The DPPH radical has a deep violet color due to its impaired electron, and radical scavenging can be followed spectrophotometrically by decrease of absorbance at 517 nm, as the pale yellow non-radical form is produced.

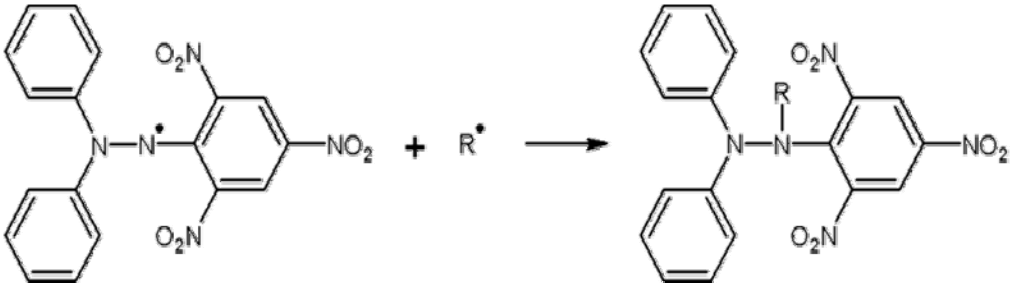


Figure 1.2 Structure of DPPH· and DPPH

2. Tyrosinase inhibition activity

The color in human hair, skin and irises is produced by the pigment melanin, which is produced by the dermal melanocyte cells. The melanocyte cells transform the peptide tyrosinase and phenylalanine into two different forms of melanin, which then is spread throughout the dermal cells and the keratinocytes via melanosomes to darken tissue. Figure 1.3 shows the chemical metabolism that occurs intra-cellular to produce melanin from the precursors phenylalanine **(Figure 1.3)**. There are two different types of melanin, eumelanin and pheomelanin. Eumelanin is metabolized from 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and produces a brown color in hair in its intact form; pheomelanin is metabolized from 5,6-indolequione, which produces a red color in hair in its intact form. From these two slightly different forms of pigment in various degrees of structural integrity come all the differing shades of Caucasian hair [17]. In addition to coloration, melanin pigmentation in the skin also provides photoprotection from UV radiation to the skin [18].

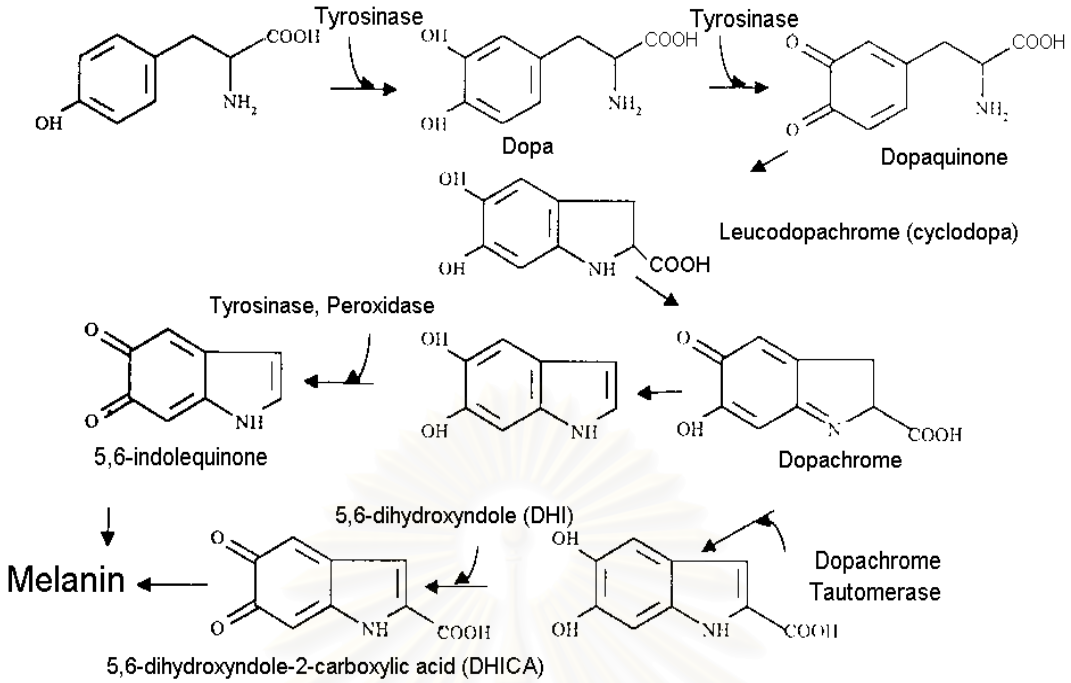


Figure 1.3 Melanin synthesis pathway

Tyrosinase

Tyrosinase (EC 1.14.18.1) is a copper-containing enzyme that catalyzes the ortho-hydroxylation of monophenols to catechols and their subsequent oxidation to ortho-quinones **(Figure 1.4)**.

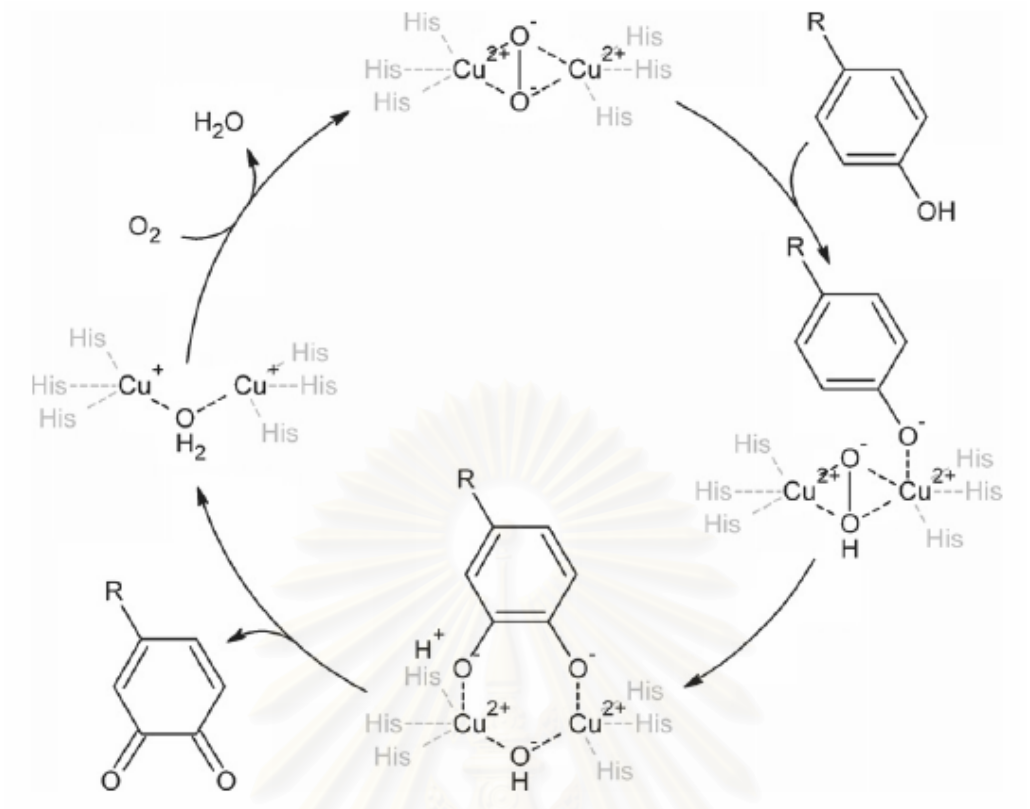


Figure 1.4 Proposed catalytic mechanism of tyrosinase. Tyrosinase catalyzes the ortho-hydroxylation of monophenol and the subsequent conversion of the catechol to the corresponding ortho-quinone [19].

The ubiquitous enzyme initiates the synthesis of melanin and is responsible for the browning that occurs upon bruising or long-term storage of vegetables, fruits, and mushrooms. In mammals, tyrosinase is not only responsible for browning of hair and skin pigmentation [20-21], but also for skin anomalies such as hypo (vitiligo) or hyper (flecks or freckles) pigmentation [22]. Furthermore, tyrosinase may play a role in cancer and neurodegenerative diseases, such as Parkinson's disease [23]. In the food industry, tyrosinase, which is also known as a polyphenol oxidase (PPO) [24], is responsible for enzymatic browning reactions in damaged fruits during post-harvest handling and processing.

Tyrosinase inhibitors

Tyrosinase, the enzyme that controls the synthesis of melanin, is a unique product of melanocytes. It is considered to be the rate-limiting step 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 copper chelators (competitive with respect to oxygen) **(Figure 1.5)** and substrate analogues (competitive towards phenol and/or diphenol substrates) **(Figure 1.6)**. However, such a classification is purely indicative, as many inhibitors can not be ascribed to a particular group and many of them behave as mixed-type inhibitors (competitive/non competitive) [25].

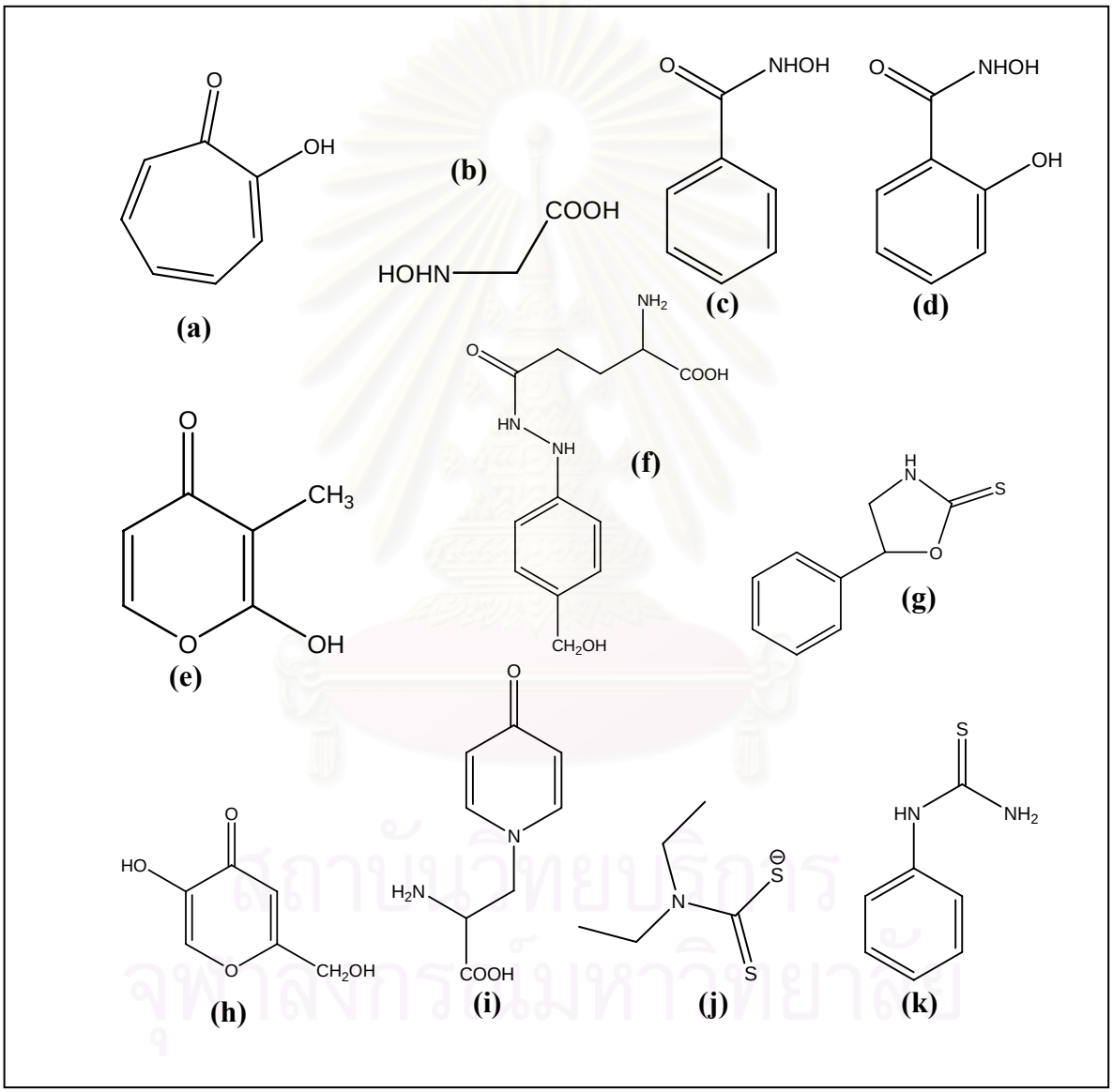


Figure 1.5 The structure of some tyrosinase inhibitors, that presumably act as copper chelators: (a) tropolone; (b) *N*-hydroxyglycine; (c) benzhydroxamic acid; (d) salicylhydroxamic acid; (e) maltol; (f) agaritine; (g) barbarine; (h) kojic acid; (i) minosine; (j) diethyldithiocarbamate; (k) phenylthiourea

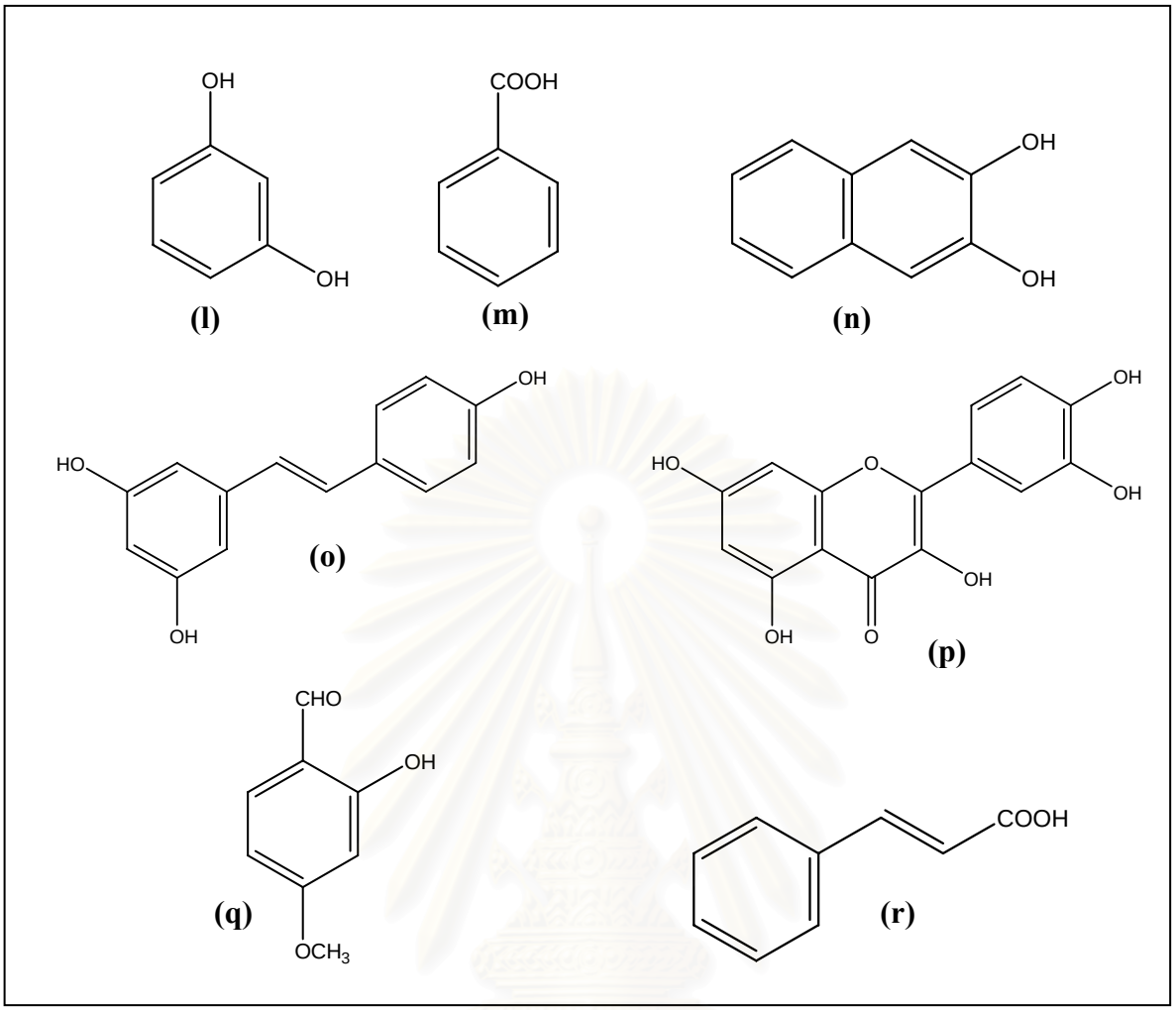


Figure 1.6 Some other tyrosinase inhibitors, that act as substrate analogues: (l) resorcinol; (m) benzoic acid; (n) 2,3-dihydroxynaphthalene; (o) resveratro; (q) 2-hydroxy-4-methoxybenzaldehyde; (f) cinnamic acid

Tyrosinase catalyzes the reaction of melanin biosynthesis in human skin and the epidermal hyperpigmentation results in various dermatological disorders, such as melasma, freckles and age spots [26]. Recently, safe and effective tyrosinase inhibitors have become important for their potential applications in improving food quality and preventing pigmentation disorders and other melanin-related health problems in human beings [27]. Furthermore, tyrosinase inhibitors are also important in cosmetic applications for skin whitening effects [28]. Since plants are a rich source of bioactive chemicals, there is an increasing interest in finding natural tyrosinase inhibitors from them. Some potent tyrosinase inhibitors, such as cuminaldehyde [29],

oxyresveratrol [30], kaempferol [31], quercetin [32] and gallic acid derivatives [33], have been isolated from various plants.

In this work, the post TLC developing technique [34] was used to detect substances which can inhibit tyrosinase activity. The method involved spraying the TLC plate or chromatographic paper containing sample spot(s) with tyrosinase and L-tyrosine solution successively. The inhibitor molecule situated on the TLC plate can bind to the enzyme molecules around them. Tyrosinase molecule in the spot are, therefore, inactivated. Enzyme molecules in other area, however, are still active and, when in contact with L-tyrosine, will catalyse the formation of dark color product. This process will result in a brownish-purple color all over the plate except the area with tyrosinase inhibitor.

3. UV-screening activity

Ultraviolet (UV) light is electromagnetic radiation with a wavelength shorter than that of visible light, but longer than soft X-rays. UV radiation is subdivided into three categories based on wavelength: the short wavelength UVC (100-290 nm), intermediate wavelength UVB (290-320 nm), and the long wavelength UVA (320-400 nm). UVC, the shortest wavelength in the UV spectrum, is completely absorbed by the gases in the atmosphere and does not reach the skin [35]. UVB and UVA are of much greater concern.

In humans, prolonged exposure to solar UV radiation may result in acute and chronic health effects on the skin, eye, and immune system [36]. UVA, UVB and UVC can all damage collagen fibers and thereby accelerate aging of the skin. In general, UVA is the least harmful, but can contribute to the aging of skin, DNA damage and possibly skin cancer. It penetrates deeply and does not cause sunburn.

The UVB radiation plays essential role in formation of vitamin D and increases skin pigmentation or tanning [37]. In addition, UVB inhibits or interferes DNA, RNA and protein synthesis, induces early and prolonged erythema responses that would lead to photoaging, keratinocyte hyperplasia, immunosuppression and skin cancer [38-39]. UVB light can cause skin cancer. The radiation excites DNA molecules in skin cells, causing covalent bonds to form between adjacent thymine bases, producing thymidine dimers. Thymidine dimers do not base pair normally, which can cause distortion of the DNA helix, stalled replication, gaps, and misincorporation. These can lead to mutations, which can result in cancerous growths

[40]. UVB causes skin cancers by directly damaging cellular DNA, and also by suppressing the skin's immune system. UVB damages DNA by causing base changes in the DNA sequence and inducing the formation of pyrimidine (primarily thymine) dimers [41-42].

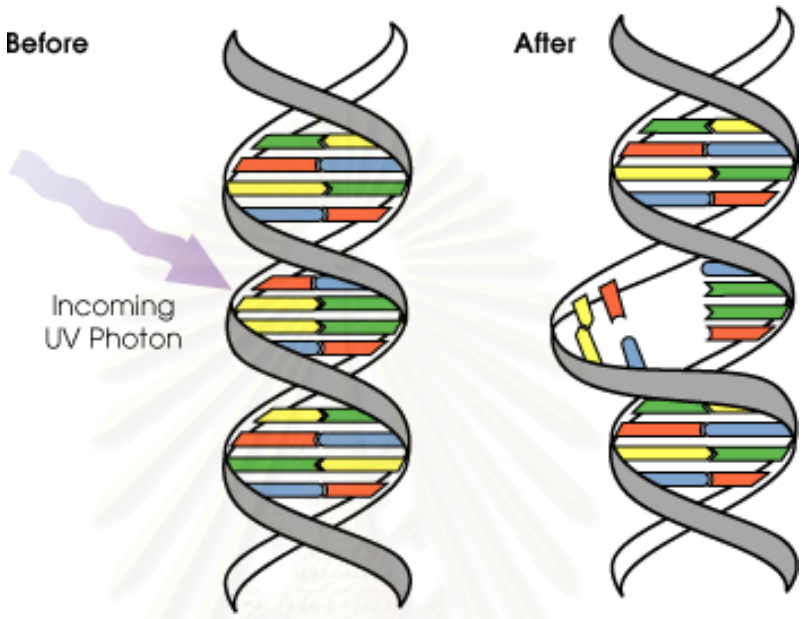


Figure 1.7 The ultraviolet photons harm the DNA molecules of living organisms in different ways.

UVA also causes sunburn, but too much lesser degree than UVB, and many of the potential damaging effects of UVA were overlooked until early in the last decade. UVA is now known to also play a part in melanin pigmentation, photoaging and cancer [43].

The penetration of UV radiation into skin varies with wavelength. UVA is less energetic than UVB but has higher penetration properties into the dermis **(Figure 1.8)**.

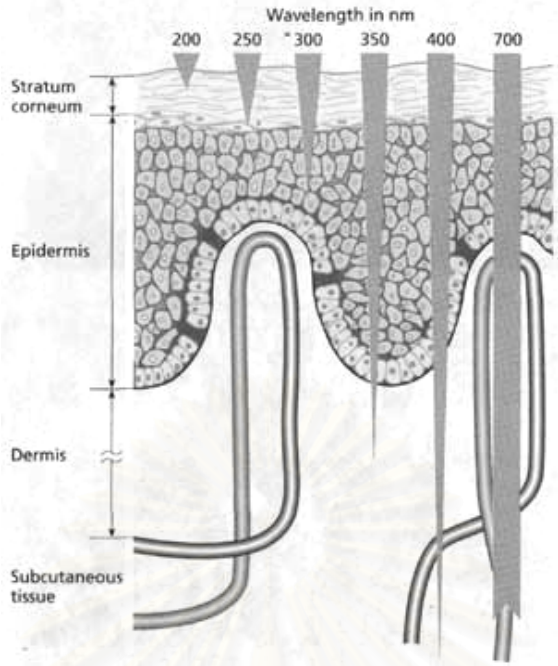


Figure 1.8 Skin penetration of UV radiation.

Protection against photo-degradation can be achieved in various ways including:

- Screen UV absorption and its reduction by substrate, by using some UV absorber.
- Diminishing the initiation reaction rate by using quenchers for excited singlet/triplet states of complex groups.
- Decay of hydro-peroxides into non-radical products.
- Scavenger process of free radicals during their formation stage.

Sunscreen reduces the amount of damaging UV radiation reaching the skin. Sunscreen works by filtering UV radiation with a chemical barrier that absorbs and/or reflects the UV radiations away from skin. Many UV-screening activity have been found in natural product extract such as epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate from green tea extract [44], usinic acid from lichen extract [45] and silibinin from milk thistle *(Slyibrum marianum)* [46]

Literature reviews on agricultural waste and their biological activities.

Researches involving agricultural by-products have demonstrated that active compounds could be obtained from extraction of the waste materials. Active compounds obtained usually includes antioxidants, antimicrobial, anticancer, anti-aging, anti-inflamatory, antimutagenic, tyrosinase inhibition and UV absorption, etc.

In 2002, Jayaprakasha and coworker used the Soxhlet apparatus and high performance liquid chromatography (HPLC) to determine the antibacterial and antioxidant activities of grape (*Vitis vinifera*) seed extracts. The antibacterial activities were tested by pour plate method. It was found that, Gram-positive bacteria were completely inhibited at 850 – 1000 ppm, while Gram-negative bacteria were inhibited at 1250–1500 ppm concentration. The antioxidant activities were determined by the formation of phosphomolybdenum complex method. It was found that acetone:water:acetic acid (90:9.5:0.5) extract was better radical scavenger than methanol:water:acetic acid (90:9.5:0.5) extract [47].

In 2004, Li and coworker evaluated the antioxidant properties of pomegranate peel extract and pomegranate pulp extract. They found that pomegranate peel extract had markedly higher antioxidant capacity than the pulp extract. The contents of total phenolics, flavonoids and proathocyanidins were also higher in peel extract than in pulp extract. The large amount of phenolic compounds contained in the peel extract is accounted for its strong antioxidant ability [48].

In 2005, Yu and coworker reported total phenolics and total antioxidant activities (TAA) of peanut skin extracts. The compounds found in peanut skin are considered potent antioxidants, particularly, flavonoids and resveratrol. The authors demonstrated that peanut skin extracts had higher antioxidant potential than green tea infusions [49].

In 2005, Wu and coworker reported a present of five flavonoids (hesperidin, naringin, hesperedin, narigenin and rutin) and ascorbic acid in grapefruit juice. They also reported two flavonoids (hesperidin, naringin) and ascorbic acid in grapefruit peel [50].

In 2006, Ajila and coworker determined polyphenol, anthocyanin and carotenoid contents in acetone extract of mango peels **(Figure 1.9)**. Ripe peels contained higher amount of anthocyanins and carotenoids compared to raw peels while raw mango peel had high polyphenol content [51].

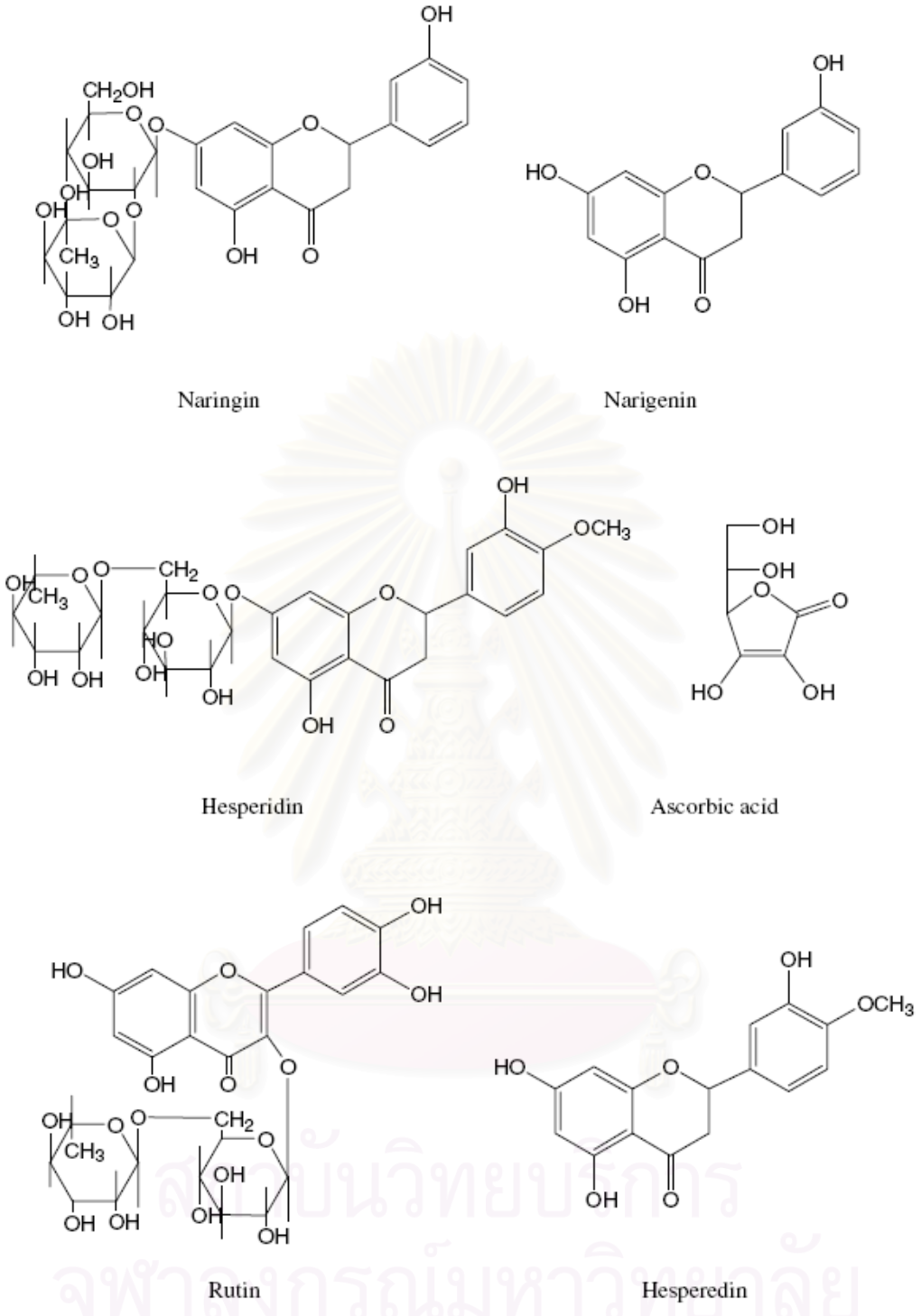
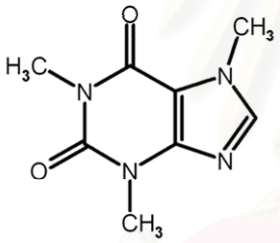


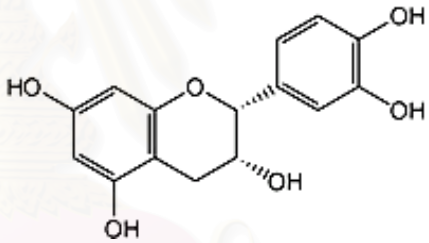
Figure 1.9 anthocyanins and carotenoids in acetone extract of mango peels.

In 2006, Anagnostopoulou and coworker evaluated radical scavenging activity and determine the total phenolic content in seven different extracts of Navel sweet orange (*Citrus sinensis*) peel. High phenolic content and radical scavenging activities were found in the ethyl acetate fraction. The antioxidant activity was compared with reference compounds, Trolox, ascorbic acid and quercetin, which are already known for their good antioxidant activity. The radical scavenging activity of the ethyl acetate fraction approached the activity of the standards [52].

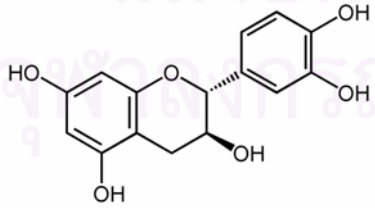
In 2006, Majhenič and coworker determined antioxidant and antibacterial activities of guarana (*Paullinia cupana*) seed extracts. The seeds were extracted with water, methanol, 35% acetone and 60% ethanol at room (TR) and at boiling (TB) temperature of solvent. Extracts were analyzed for the contents of caffeine and catechins, epicatechin (EC), catechin (C) and epicatechin gallate (ECG) **(Figure 1.10)**, by HPLC. The guarana seed water extract obtained at room temperature contained higher amounts of caffeine and catechins than did guarana seed extracts. Seed extracts of guarana possess strong antimicrobial and antioxidant properties [53].



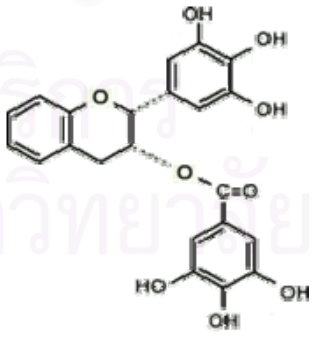
caffeine



epicatechin(EC)



catechin (C)



epicatechin gallate (ECG)

Figure 1.10 Chemical structures of caffeine, catechins, catechin and epicatechin gallate of guarana (*Paullinia cupana*) seed extracts.

In 2006, Tachakittirungrod and coworker investigated the ethanol extracts of plant species commonly found in Thailand. The leaves of guava (*Psidium guajava*) showed the highest antioxidant capacity followed by the fruit peels of rambutan (*Nephelium lappaceum*) and mangosteen (*Garcinia mangostana*). Further evaluation of guava leaves, using different solvents has demonstrated that the highest antioxidant activity was in the methanol fraction [54].

In 2007, Pereira and coworker separate phenolic compounds from Walnut (*Juglans regia* L.) leaves by reversed-phase HPLC/ photo diode array detector (DAD). Ten compounds were identified as 3- and 5-caffeoylquinic acids, 3- and 4-pcoumaroylquinic acids, *p*-coumaric acid, quercetin 3-galactoside, quercetin 3-pentoside derivative, quercetin 3-arabinoside, quercetin 3-xyloside and quercetin 3-rhamnoside (**Figure 1.11)** [55].

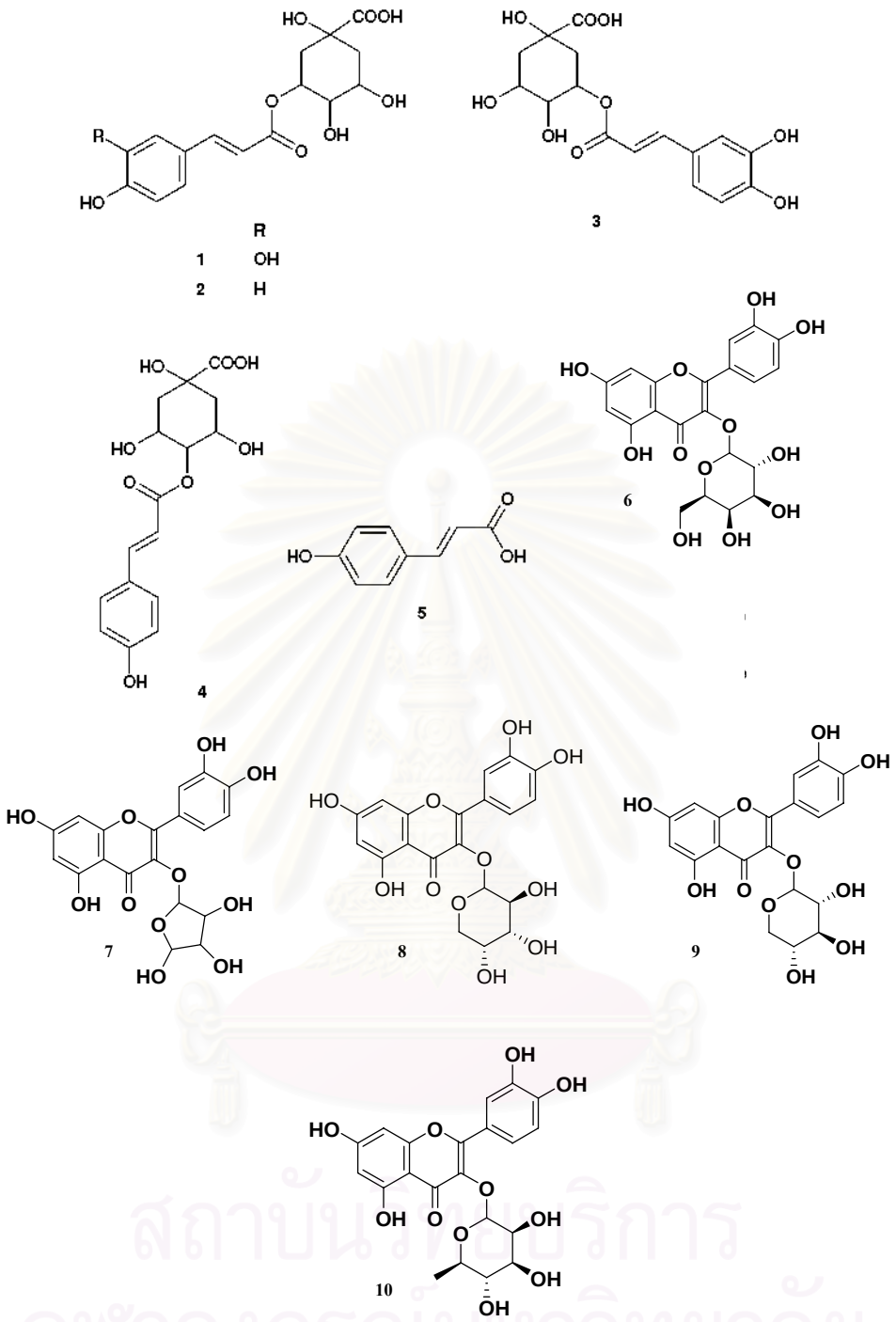


Figure 1.11 Chemical structures of the phenolic compounds identified in walnut leaves. (1) 3-caffeoylquinic acid; (2) 3-p-coumaroylquinic acid; (3) 5-caffeoylquinic acid; (4) 4-*p*-coumaroylquinic acid; (5) p-coumaric acid; (6) quercetin 3-galactoside; (7) quercetin 3-pentoside derivative; (8) quercetin 3-arabinoside; (9) quercetin 3-xyloside; (10) quercetin 3-rhamnoside.

In 2007, Pan and coworker studied the longan (*Dimocarpus Longan* Lour.) peel that was extracted with 95% ethanol employing microwave-assisted extraction (MEL) and Soxhlet extraction method (SEL). The total phenolic content of the product reached 96.78 mg/g and 90.35 mg/g dry weight, for the MEL and the SEL respectively. MEL and SEL showed excellent antioxidant in all test systems compared with synthetic antioxidant 2,6-di-ter-butyl-4-methylphenol (BHT) and the antioxidant activities of MEL were all superior to those of SEL [56].

In 2007, Ajila and coworker examined the antioxidant activity of mango peel and ripe peels extracts. The mango peel extracts contained polyphenol, anthocyanin and carotenoid. The ripe peel extract contained higher amount of anthocyanins and carotenoids compared to raw peels. The raw mango peel had high polyphenol content. The IC50 values were found to be in the range of 1.39–5.24 g of gallic acid equivalents [57].

In 2007, S. Rout and R. Banerjee reported free radical scavenging, anti-glycation and tyrosinase inhibition properties of a polysaccharide fraction isolated from pomegranate rind (*Punica granatum*) (PFP). The results strongly suggested possible use of PEP as skin whitener [58].

Sugarcane bagasse

Sugarcane (*Saccharum officinarum* L.) is one of the major economic crop in Thailand, total planted area of sugarcane in Thailand is the 4th biggest in the world following Brazil, India and China **(Table 1.2)** Total planted area of sugarcane in Thailand accounted for 60% of the total cultivation area of the country. The main cultivation area is in Karnjanaburi, Supanburi, Udonthani, Chaiyaphoom, Nakornratchasima, Konkaen, Kampangpeth and Nakornsawan province. Production over period from 1997/98 to 2003/04 has an average value of about 57.7 million tons per year [59]. In the year 2005, Thailand was the 2nd exporter of sugarcane; total export value was 29,541 million Baht. The main export market is in Japan, South Korea, Indonesia and Malaysia [60].

Table 1.2 Sugarcane in the world: area, production and productivity.

Country	Area (million ha)	Production (million tons)	Productivity (Tons/ha)
Brazil	5.343	386.2	72.3
India	4.608	289.6	62.8
China	1.328	92.3	65.5
Thailand	0.970	64.4	66.4
Pakistan	1.086	52.0	47.9
Mexico	0.639	45.1	70.6
Colombia	0.435	36.6	84.1
Australia	0.423	36.0	85.1
USA	0.404	31.3	77.5
Philippines	0.385	25.8	67.1
Indonesia	0.350	25.6	73.1
Cuba	0.654	22.9	35.0
South Africa	0.325	20.6	63.4
Argentina	0.295	19.2	65.2
Myanmar	0.165	7.5	45.4
Bangladesh	0.166	6.8	41.2
WORLD	20.42	1333.2	65.2

Sugarcane bagasse is a residue produced in large quantities by sugar and alcohol industries. In general, one ton of sugarcane generates 280 kg of bagasse, the fibrous byproduct remaining after sugar extraction from sugarcane. About 54 million dry tons of bagasse are produced annually throughout the world [61]. This material represents a great morphological heterogeneity. It consists of fiber bundles and other structural elements like vessels, parenchyma, and epithelial cells [62]. Chemically, about 40–50% of the dry residue is the glucose polymer cellulose, much of which is in a crystalline structure. Another 25–35% are hemicelluloses, an amorphous polymer usually composed of xylose, arabinose, galactose, glucose, and mannose. The

remainder is mostly lignin plus lesser amounts of minerals, waxes, and other compounds [63]. Efforts on using these agricultural residues include either in an energy aspect and extraction technology aspect.

In this research, the sugarcane bagasse was extracted and screened for their biological activities in cosmetics, including antioxidant, tyrosinase inhibition and UV screening activities. Some of bioactive compounds were also isolated and characterized.

Sugarcane or Sugar cane (*Saccharum*)

Sugarcane or Sugar cane (*Saccharum*) is a genus that contains 6 to 37 species (depending on taxonomic interpretation) of tall perennial grasses (family Poaceae, tribe Andropogoneae). This plant is native to warm temperate to tropical regions. They have stout, jointed, fibrous stalks that are rich in sugar and measure 2 to 6 meters tall.

Scientific classification

Kingdom : Plantae
Division : Magnoliophyta
Class : Liliopsida
Order : Poales
Family : Poaceae
Genus : Saccharum L.

Literature reviews of sugarcane extract and their biological activities.

In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues, including sugarcane bagasse. Several processes and products have been reported that utilize sugarcane bagasse as a raw material. These include electricity generation, pulp and paper production, and products based on fermentation. The various products, which have been obtained from the processes involving bagasse also include chemicals and metabolites.

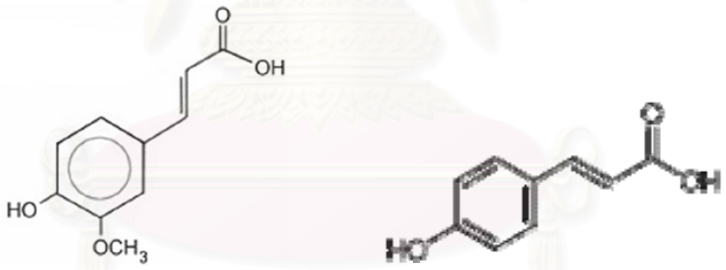
The researches on chemical constituents of the Sugarcane bagasse were widely investigated. Numerous types of organic compounds have been isolated.

In 2002, Nuissier and coworker studied the chemical composition of sugarcane waxes in rum factory wastes by GC–mass spectrometry. Series of linear alkanes (C19–C33), and wax esters constitute the main components. In addition, phytosterols,

triterpene methyl ethers, ethyl and methyl esters of fatty acids, and free fatty acids were found as minor components [64].

In 2005, Colombo and coworker investigated sugarcane (*Saccharum officinarum* L., Gramineae) bagasse and leaves for their flavonoid content. Transgenic sugarcane ("Bowman-Birk" and "Kunitz") was compared with non-modified ("control") plants. Analyses were carried out on HPLC coupled to diode array UV detection (LC/UV) with post-column addition of shift reagents, and tandem MS. Three naturally occurring flavones glycosides and two unusual *erythro*- and *threo*-diastereoisomeric flavolignan 7-*O*-glucosides were identified together with their aglycones [65].

In 2005, Xu and coworker determined ferulic acid (FA) and *p*-coumaric acids (*p*-CA) **(Figure 1.12)** and related phenolic compounds in the sugarcane bagasse cell wall (SCB) by mild alkaline hydrolysis of the alkali-soluble lignin preparations and acid hydrolysis of the 90% acidic dioxane-soluble lignin fractions. The hydrolysis by alkaline released 48.8% of the total ester-linked *p*-CA and 43.8% of the total esterified FA while the hydrolysis by 90% aidic dioxane released 38.8% of the total ether-linked *p*-CA and 38.5% of the total ether-linked FA [66].



ferulic acid *p*-cumaric Acid

Figure 1.12 Chemical structure of ferulic acid and *p*-Cumaric Acid

In 2006, Colombo and coworker used HPLC/DAD to separate and quantify flavonoids in sugarcane leaves and bagasses. The result showed that sugarcane flavonoid consist of a complex mixture of aglycones and glycosides including flavonolignan glycosides [67].

In 2007, Mauricio and coworker investigated sugarcane juice. A flavone was identified by spectroscopic methods as tricin-7-o-β-(6"-methoxycinnamic)-glucoside

and orientin. This tricin derivative was shown to have higher antioxidant activity than Trolox. Sugarcane juice showed *in vitro* antiproliferative activity against several human cancer cell lines, with higher selectivity toward cells of the breast resistant NIC/ADR line [68].

In 2007, Ou and coworker tried to separate *trans*-ferulic acid form sugarcane bagasse by alkaline-hydrolysis and purification through combination of activated charcoal adsorption and anion macroporous resin exchange chromatography. The results showed that powdered activated charcoal had much higher adsorption capacity for ferulic acid than that of granular activated charcoal. High purity of ferulic acid was obtained by further treatment with anion macroporous resin exchange chromatography [69].

In 2008, Kadam and coworker studied antioxidant action in sugarcane (*Saccharum officinarum* L.) juice using various methodology including oxygen radical absorbance capacity (ORAC), DPPH radical scavenging abilities, ferric reducing antioxidant power (FRAP); and protection of membranes examined by inhibition of lipid peroxidation. In addition, the content of phenols and total flavonoids were measured. The aqueous extracts of three varieties of sugarcane (DSEM Co-C-671, Co-C-86032 and Co-C-419) were studied. They all showed good antioxidant properties [70].

Objective of research

1. Screening for antioxidant, tyrosinase inhibition and UV- absorption activities in extracts from sugarcane bagasse.
2. Finding the most simple processes for obtaining extracts with cosmetic value from sugarcane bagasse.
3. Isolating and characterizing some active components in the extracts.

CHAPTER II
EXPERIMENTAL

2.1 Instuments and Equipments

^{1}H and ^{13}C NMR spectra were recorded with a Varian model Mercury+ 400 (Varian company, CA, USA) which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C nucleus. The chemical shift in δ (ppm) was assigned with reference to the signal from the residual proton in deuterated solvent or signal from TMS internal standard. MS spectra were acquired with Waters Micromass Quattomicro API ESCi (Waters, MA, USA). UV-Visible absorption spectra were obtained with the aid of UV 2500 UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan) using a quartz cell with 1 cm path lenght. Semi-preparative High Performance Liquid Chromatography (HPLC) was performed on a Thermo Finnigan spectra SYSTEM (Phenomenex, California, USA) connected to UV6000LP detector (Thermo Finnigan spectra SYSTEM, Phenomenex, California, USA). Thin layer chromatography (TLC) was performed on aluminum sheets precoated with silica gel (Merck Kieselgel 60 F254) (Merck KgaA, Darmstadt, Germany). Column chromatography was performed on silica gel (Merck Kieselgel 60 G, Merck KgaA, Darmstadt, Germany). Microtiter plate spectrophotometer was performed on a Microtiter plate reader, Model Sunrise (TECAN, Salzburg, Austria).

2.2 Chemicals

Solvents used in spectroscopic technique during the antioxidant and tyrosinase inhibition assays were reagent or analytical grade purchased from Labscan (Bangkok, Thailand). Solvents used in extraction and column chromatography were purified from commercial grade solvents prior to use by distillation. Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma Chemical Company (St Loius, MO, USA). L-tyrosine and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Fluka (Buchs, Switzerland). Kojic acid used as standard tyrosinase inhibitor was purchased from Acros Organics (Geel Belgium). A 2,6-di-tert-butyl-p-hydroxytoluene (BHT) was purchased from Panreac Sintesis (Bacelona, Spain).

2.3 Sample materials

Sugarcane bagasse (*Saccharum officinarum* L.) was obtained from Chon Buri, Thailand. It was first sun dried for a week. The dry bagasse was then cut into small pieces and put in a cabinet oven with air circulation for 16 h at 60 □C before being grounded into powder.

2.4 Extraction and Isolation

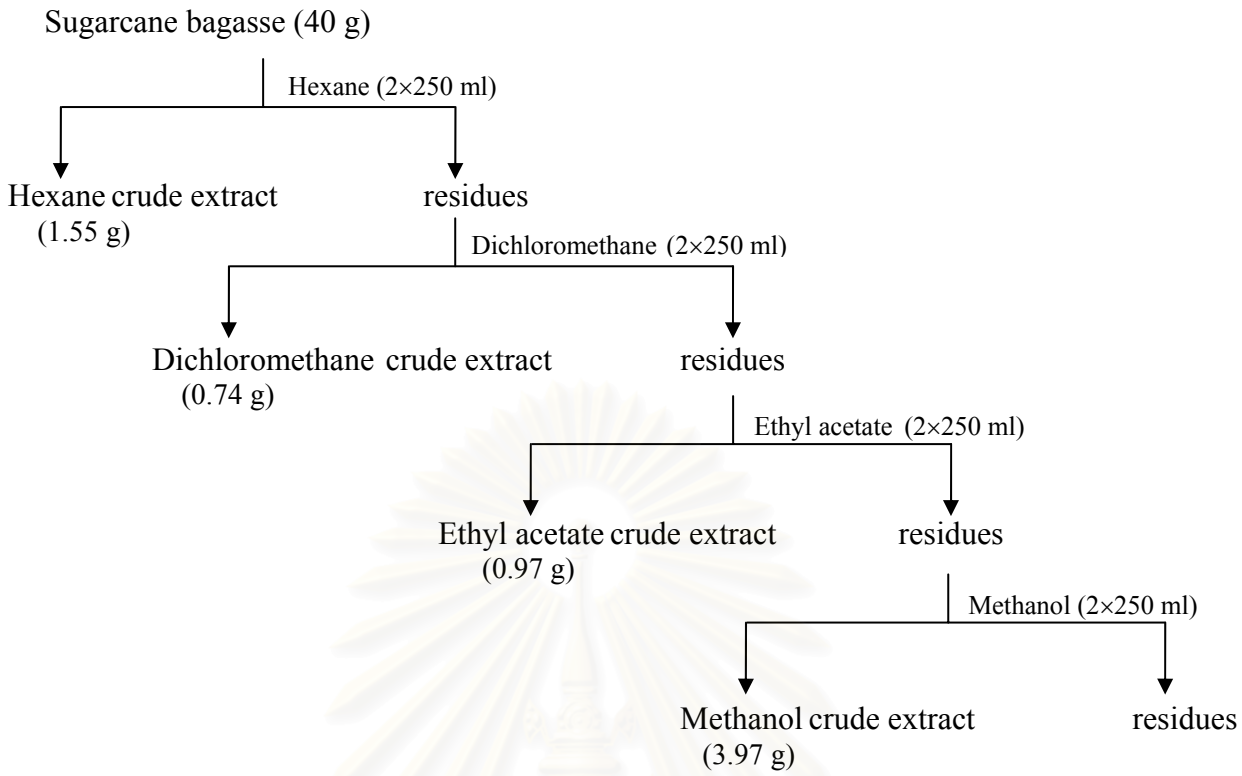
2.4.1 *Preliminary screening*

Fourty grams of grounded sugarcane bagasse was extracted by 250 ml methanol. The extract was filtered and evaporated under reduced pressure. The obtained MeOH extract was tested for antioxidant activity, tyrosinase inhibition activity and UV-sreening activity.

2.4.2 *Extraction*

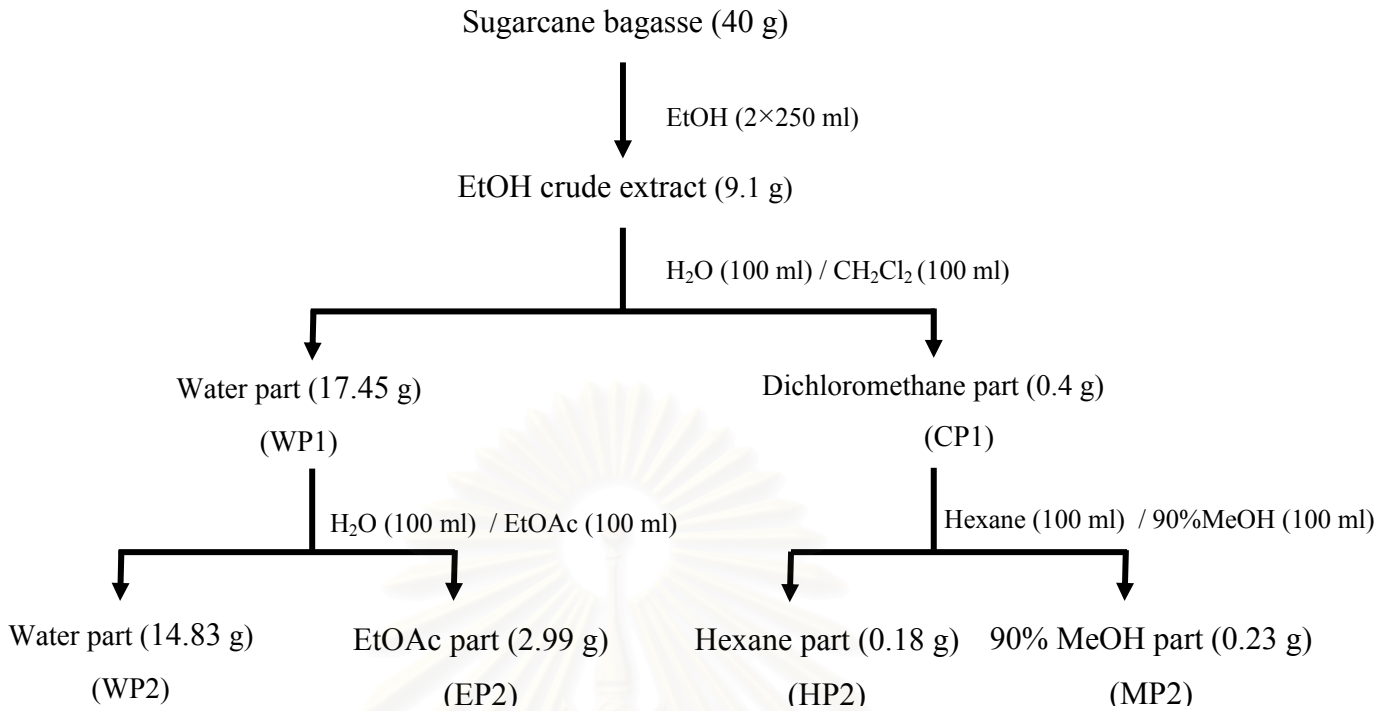
Sequential solvent extraction

Sugarcane bagasse was grounded. The grounded bagasse (40 g) was macerated with hexane (2×250 ml) for 7 days and filtered through a Whatman GF/A filter paper, after which the procedure was repeated with a fresh portion of dichloromethane, ethyl acetate and methanol, respectively. Each extract was evaporated under reduced pressure. The dry extracts were weighted and tested for total phenolic content, antioxidant activity, tyrosinase inhibition activity and UV-screening property.



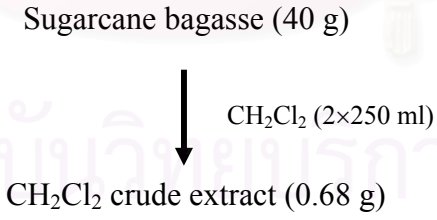
Partition extraction

Fourty grams of ground sugarcane bagasse were macerated in EtOH (2×250 ml) for 7 days at room temperature. The extract was filtered and evaporated to dryness under reduced pressure. The dry extract was partitioned with water and dichloromethane (1:1). The water part was repartitioned with water and EtOAc (1:1) to obtain water extract (WP2) and EtOAc extract (EP2). The dichloromethane part was repartitioned with hexane and 90%MeOH (1:1) to obtain hexane extract (HP2) and 90%MeOH extract (MP2). Each extract was filtered and evaporated to dryness under reduced pressure. Each extract was tested for antioxidant, tyrosinase inhibition and UV-screening activities.



Single dichloromethane extraction

Fourty grams of grounded sugarcane bagasse were macerated in CH_2Cl_2 (2×250 ml) at room temperature. Extracts were filtered and evaporated to dryness under reduced pressure and tested for antioxidant, tyrosinase inhibition and UV-screening activities.



Since the sequential solvent extraction procedure gave extracts with highest antioxidant and tyrosinase inhibition activities, this procedure was used in the following study. Both hexane and dichloromethane extracts were prepared as follows: Three kilograms of sugarcane bagasse (*Saccharum officinarum*) were sun dried for a week. The dry bagasse was grounded into powder and exhaustively extracted by maceration at room temperature with hexane (2×2500 ml) for 7 days followed by dichloromethane (2×2500 ml) for 7 days. Each extract was filtered and evaporated to dryness under reduced pressure.

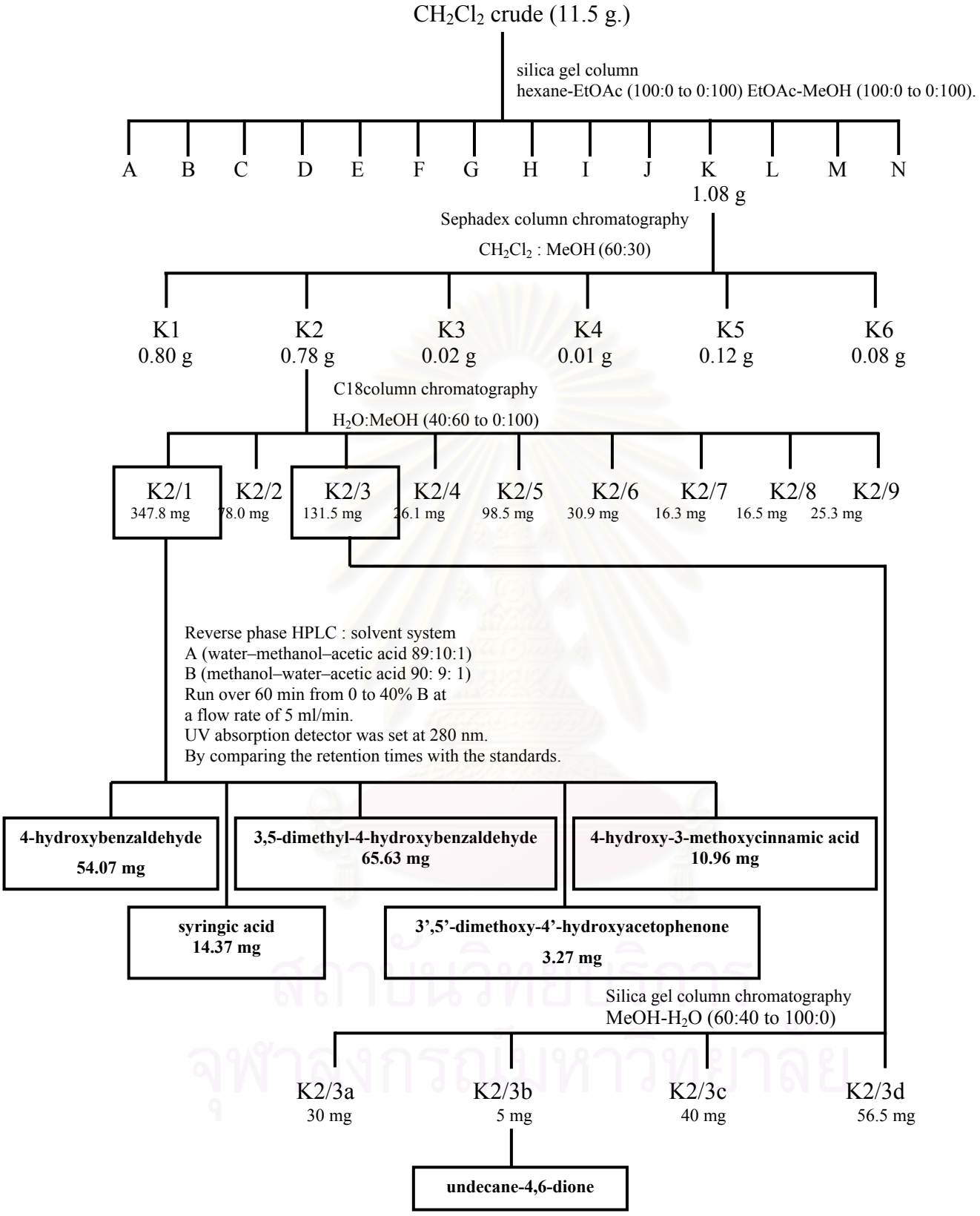
2.4.3 *Isolation of active compounds*

The dichloromethane crude extract (11.5 g) was chromatographed over silica gel column using gradient elution of hexane-EtOAc (100:0 to 0:100) and EtOAc-MeOH (100:0 to 0:100). Fractions were collected and tested for biological activities. Fraction with good activity (K fraction) was further isolated using chromatographic techniques.

The most active fraction, K fraction (brown paste, 1.08g), was fractionized by sephadex LH-20 column using MeOH-CH_2Cl_2 (30:60) as a mobile phase. The most active subfraction, K2 (brown paste, 0.78g), was separated using C_{18} column chromatography with gradient MeOH-H_2O (60:40 to 100:0) elution and subfractions (K2/1-K2/9) were collected of which K2/1 (brown paste, 347.8 mg) showed the highest free radical scavenger activity while K2/3 (brown paste, 131.5 mg) showed the highest tyrosinase inhibition activity **(Scheme 1)**.

Twenty five micrograms (5µl in MeOH) of fraction K2/1 was loaded onto BDS Hypersil C18 reverse phase HPLC column (100 mm long x 4.6 mm i.d., 5 µm, Thermo Electron Corporation, Massachusetts, USA) and elution was carried out using a linear gradient system consisting of solvent A (water–methanol–acetic acid 89:10:1) and solvent B (methanol–water–acetic acid 90:9:1) at 0 to 40% B over 60 min, at a flow rate of 1 ml/min. UV absorption detector was set at 280 nm. By comparing the retention times with those of the standards, 4-hydroxybenzaldehyde, syringic acid, 3,5-dimethyl-4-hydroxybenzal-dehyde, 3',5'-dimethoxy-4'-hydroxyacetophenone and 4-hydroxy-3-methoxycinnamic acid could be identified. Negative ESIMS spectra of all 5 compounds were also obtained.

Fraction K2/3 (brown paste, 131.5 mg) was separated using silica gel column with gradient elution (hexane:EtOAc 100:0 to 0:100). The collected fraction (K2/3b) with best activity was further characterized by spectroscopic technique **(Scheme 1)**.



Scheme 1 Isolation of CH_2Cl_2 crude

2.5 Biological activity assay.

(A) Free radical scavenging activity. Screening of the free radical scavenging activity was performed on collected fractions as previously described [70]. In this assay the exact amount of a sample (0.5-50 µg) was dropped onto a stationary phase using an analytical syringe. After allowing to dry for about 5 min at room temperature, 0.5 mM DPPH solution (in methanol) was sprayed over the entire surface of the stationary phase (0.01 ml/cm^2). Only spots with antioxidant activity appeared white against a purple background.

IC_{50} values for the DPPH scavenging activity were determind as previously described [71]. Briefly, 100 µl of 0.5 mM DPPH solution (in methanol) were added to the 96-well microplate containing 50 µl of sample. The mixture was kept at room temperature for 30 min in the dark. The absorbances of all the sample solutions were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical according to the formula:

% scavenging activity = $[1-A_{sample}/A_{control}] \times 100$

A_{sample} = absorbance at 517 nm. of reaction mixtures containing test compounds.

$A_{control}$ = absorbance at 517 nm. of reaction mixtures without test compounds.

(B) Tyrosinase inhibition activity. Screening of the tyrosinase inhibition activity was performed on fractions as previously described [34]. In this assay the exact amount of a sample (0.5-50 µg) was dropped onto a stationary phase using an analytical syringe. After allowing to dry for about 5 min at room temperature, the enzyme solution (200 units/mL) was sprayed over the entire surface of the stationary phase (28.57 units/cm^2). Immediately after that, L-tyrosine solution was sprayed over the same area (28.57 units/cm^2). After appropriate times (20 min), the plate was then photographed. Only spots with tyrosinase inhibitor(s) appeared white against a brownish-purple background.

IC_{50} values for the tyrosinase inhibition activity were determind as previously described [73]. Briefly, 0.03 ml of tyrosinase solution (333 units/mL), 0.07 ml of 50 mM phosphate buffer solution pH 6.8, were added to the 96-well microplate containing 2 µl of sample. After 5 min incubation at room temperature, 0.1 mL L-tyrosine (2 mM) was added. Absorbances of all the sample solutions were measured

at 492 nm. Tyrosinase inhibition activity was expressed as the inhibition percentage according to the formula:

% inhibition of tyrosinase activity = [[(A-B)-(C-D)]/(A-B)] x 100

A = absorbance of reaction mixtures without test compounds (control)

B = absorbance of blank of control with out enzyme

C = absorbance of reaction mixture containing test compound (sample)

D = absorbance of blank of sample with out enzyme

(C) Total phenolic contents

Total phenolic content was determined by the Folin–Ciocalteu method. Two hundred microliters of diluted sample were added to 1mL of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 μL of saturated sodium carbonate solution (75 g/L) was added. After 2 h of incubation at room temperature, the absorbance at 765nm was measured in triplicate. Gallic acid (0–500 mg/L) was used for calibration of standard curve. The results were expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of sugarcane bagasse.

CHAPTER III
RESULTS AND DISCUSSION

Extraction and Isolation

3.1 Preliminary screening

Sugarcane bagasse methanolic extract was first evaluated for antioxidant, tyrosinase inhibition and UV absorption activities. This was carried out as a preliminary evaluation of biological activities in the bagasse. One hundred grams of dry bagasse gave ~4 g methanolic crude extract. The MeOH crude extract showed both free radical scavenging and tyrosinase inhibition activities. The TLC-DPPH radical-scavenging activity assay of the MeOH extract and the standard BHT gave minimal detectable amount of 5 μg and 0.5 μg, respectively. This indicates that the BHT is approximately 10 times more active than the extract in scavenging free radical. The TLC tyrosinase inhibition activity assay of the MeOH extract and the standard kojic acid gave minimal detectable amount of 5 μg and 0.5 μg, respectively. This indicates that the kojic acid is approximately 10 times more active than the extract. The extract also showed UV absorption band at 320 nm **(Figure 3.1)**. The result encouraged further study of cosmetic actives from the bagasse.

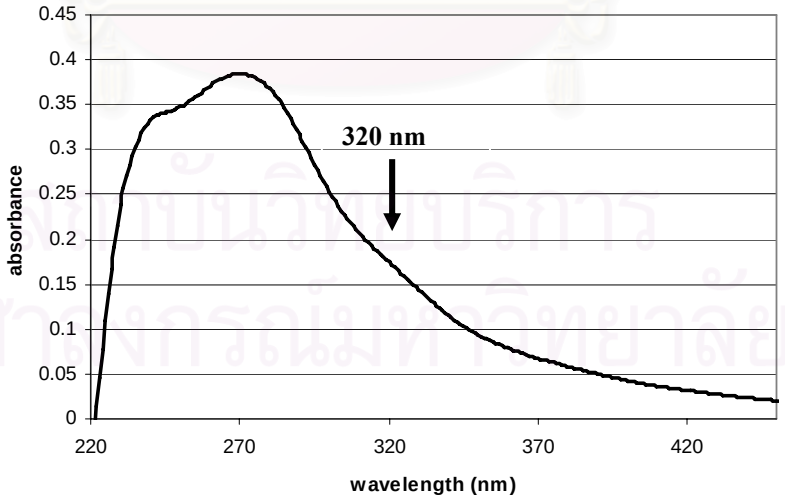


Figure 3.1 UV spectrum of MeOH extract at concentration 50 μg/ml in MeOH.

3.2 Extraction and isolation

Sequential solvent extraction

Sugarcane bagasse (SCB) was sequentially extracted by hexane, dichloromethane (CH_2Cl_2), ethylacetate (EtOAc) and methanol (MeOH). Free radical-scavenging and tyrosinase inhibition activities of each extract were assessed by TLC autographic assay. Although all extracts showed both activities **(Table 3.1)**, the result indicated that the CH_2Cl_2 extract gave the lowest detectable amount in the DPPH-scavenging and tyrosinase inhibition assays **(Table 3.1),** indicating highest activities of this extract. The TLC DPPH radical-scavenging activity assay of the CH_2Cl_2 extract and the standard BHT gave minimal detectable amount of 0.25 μg and 0.25 μg, respectively. This indicates that the extract gave similar activity to the BHT in scavenging free radical. The TLC tyrosinase inhibition activity assay of the CH_2Cl_2 and the standard kojic acid gave minimal detectable amount of 0.05 μg and 0.25 μg, respectively. This indicates that the extract is approximately 5 times more active than the kojic acid in tyrosinase inhibition activity. The CH_2Cl_2 extract showed UV absorption band at 273 nm **(Figure 3.2)**.

Table 3.1 DPPH scavenging activity, tyrosinase inhibition activity and UV absorption activity of hexane, CH_2Cl_2, ethylacetate, and MeOH crude extract comparing with standard antioxidant (BHT) and standard tyrosinase inhibitor (Kojic acid), as estimated by the minimal amount required to produce detectable positive result during the screening.

Crude	%yield (W/W) (as calculated against dry bagasse)	minimal detectable amount in antioxidant assay (μg)	minimal detectable amount in tyrosinase inhibition assay (μg)	UV absorption activity (λ_{max})
Hexane	1.55	0.5	0.25	272
CH_2Cl_2	0.74	0.25	0.05	273
EtOAc	0.97	0.5	0.5	278
MeOH	3.97	0.5	2.5	284
BHT	ND	0.25	ND	ND
Kojic	ND	ND	0.25	ND

ND = Not determined

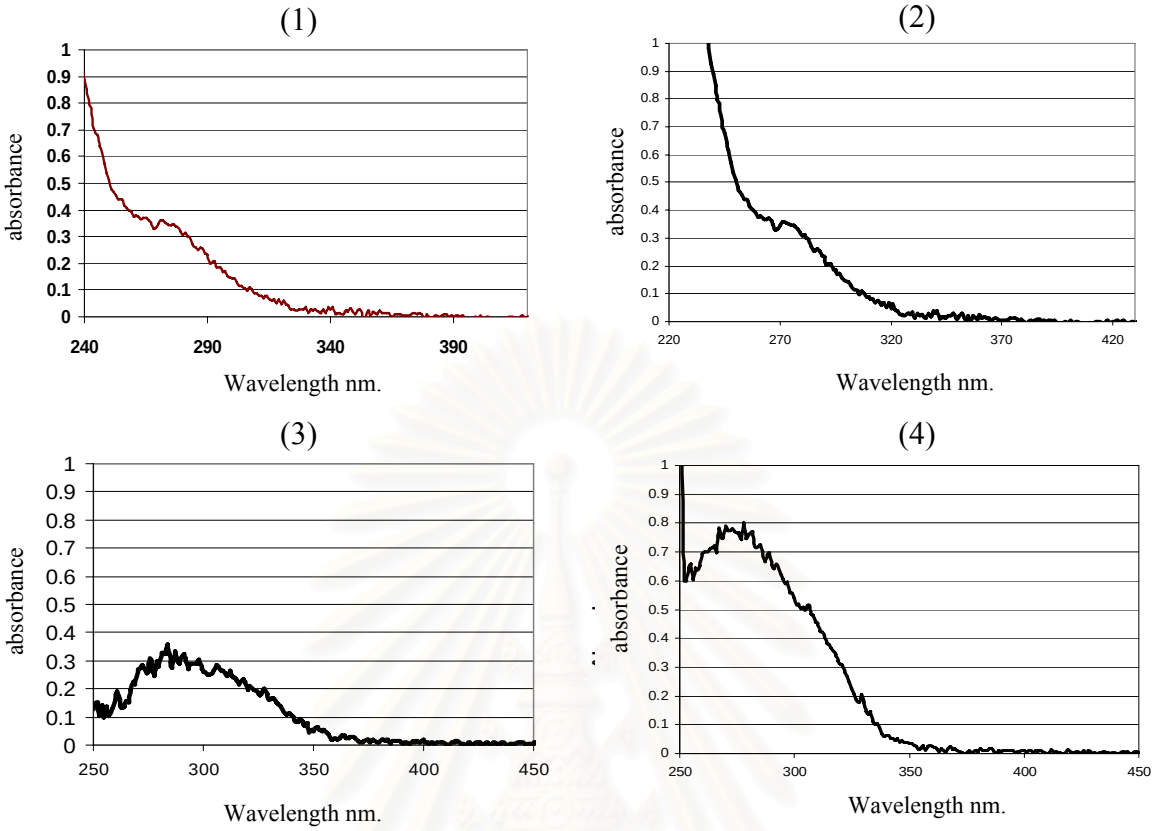


Figure 3.2 UV spectra of (1) hexane, (2) CH_2Cl_2, (3) and (4) MeOH extracts at concentration of 50 µg/ml in MeOH.

Partition extraction

Sugarcane bagasse was macerated in ethanol. The ethanolic extract (EtOH extract) was partitioned with water and dichloromethane (1:1). The water part was repartitioned with water and ethylacetate (1:1) to obtain water extract and ethylacetate extract (EtOAc extract). The dichloromethane part was repartitioned with hexane and 90%methanol (1:1) to obtain hexane extract and 90%methanolic extract (MeOH extract).

The free radical-scavenging and tyrosinase inhibition activities of each extract was assessed by TLC autographic assay. The result indicated that CH_2Cl_2 and EtOAc extract gave the lowest detectable amount in the DPPH scavenging assay, 0.25 µg of CH_2Cl_2 and EtOAc extracts gave similar activity to 0.25 µg BHT. Only CH_2Cl_2 gave the lowest detectable amount in tyrosinase inhibition assay, 0.25 µg of the CH_2Cl_2 extract gave similar activity to 0.25 µg of kojic acid (**Table 3.2)** and showed neither intense UVB nor UVA absorption (279 nm) **(Figure 3.3)**.

Table 3.2 DPPH scavenging activity, tyrosinase inhibition activity and UV absorption activity of crude extract comparing with standard antioxidant (BHT) and standard tyrosinase inhibitor (Kojic acid), as estimated by the minimal amount required to produce detectable positive result during the screening.

Crude	**%yield (W/W) (as calculated against dry bagasse)**	**minimal detectable amount in antioxidant assay (μg)**	**minimal detectable amount in tyrosinase inhibition assay (μg)**	**UV absorption activity (λ_{max})**
HP1	17.45	-	-	269
CP1	0.4	0.25	0.25	279
HP2	14.83	-	-	273
EP2	2.99	0.25	1.25	272
MP2	0.23	1.25	1.25	275
HP2	0.18	0.5	1.25	270
BHT	ND	0.25	ND	ND
Kojic	ND	ND	0.25	ND

- = No activity, ND = Not determined

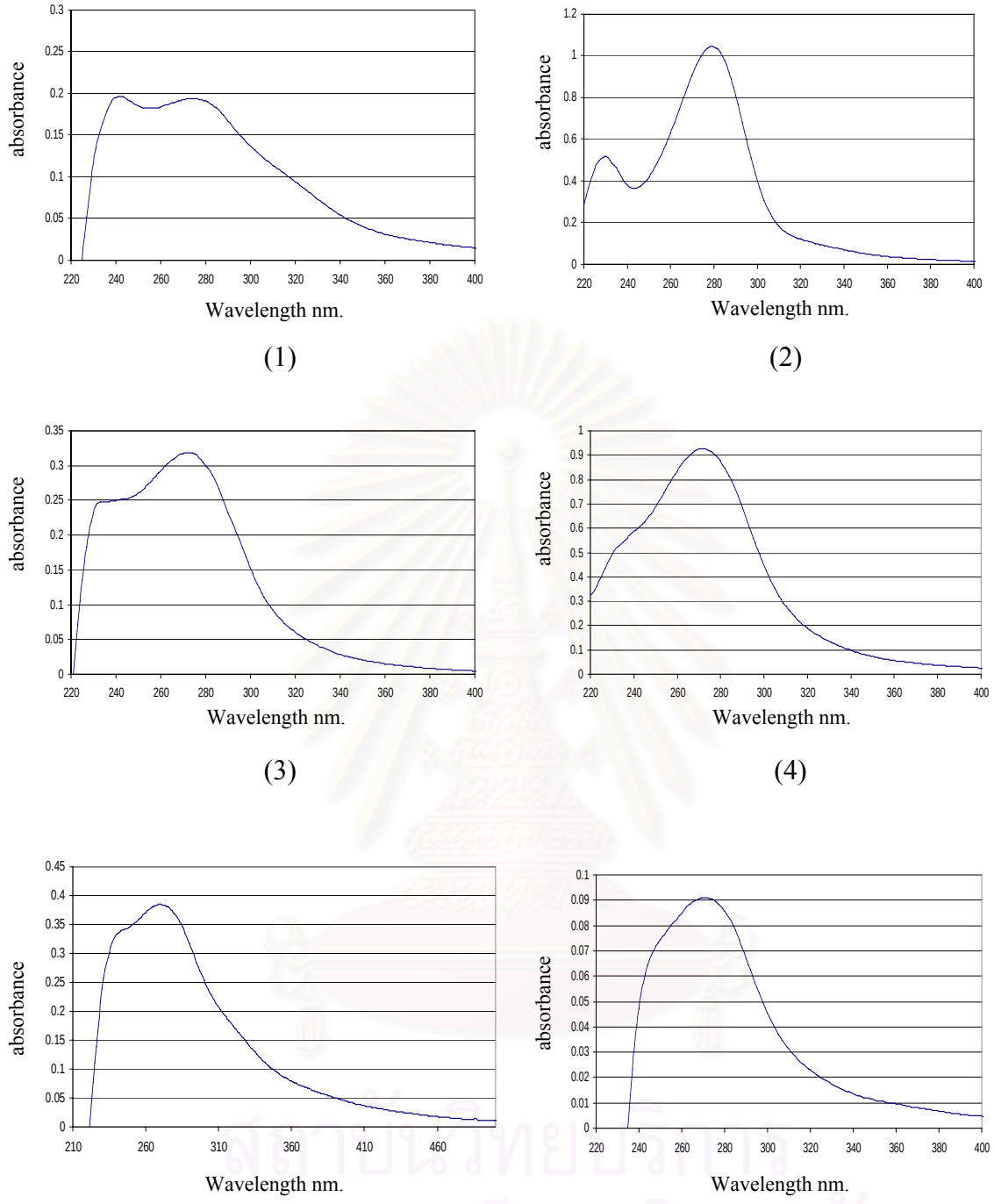


Figure 3.3 UV spectrum of (1) H1, (2) CP1, (3) HP2, (4) EP2, (5) MP2 and (6) HP2 extract at concentration 50 µg/ml in MeOH.

Single dichloromethane extraction

Sugarcane bagasse (SCB) was extracted by dichloromethane (CH_2Cl_2). The process gave 1.69% (W/W) extract as calculated against dry bagasse. The free radical-scavenging and tyrosinase inhibition activities of CH_2Cl_2 extract was assessed by TLC autographic assay.

The result indicated that 0.25 μg of the CH_2Cl_2 extract gave similar activity to 0.25 μg BHT. Similary the potency of tyrosinase inhibition activity of the CH_2Cl_2 extract was equal to that of kojic acid. However, this extract showed neither intense UVB nor UVA absorption **(Figure 3.4)**.

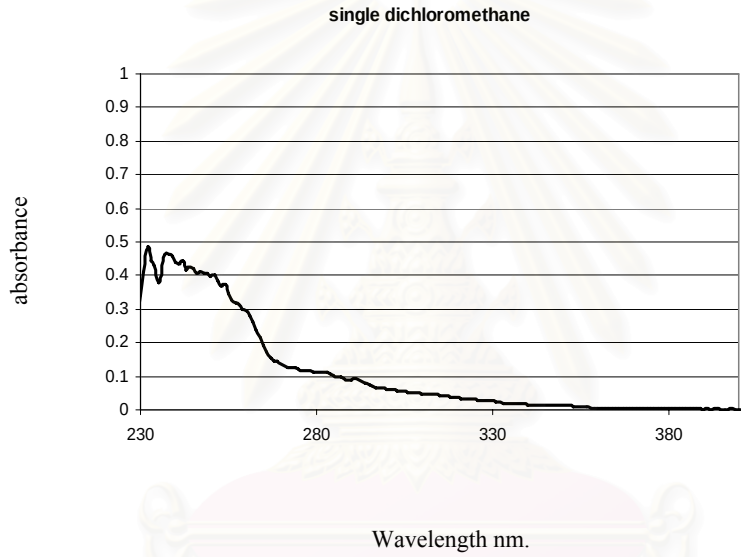


Figure 3.4 UV spectrum of single CH_2Cl_2 extract at concentration 50 μg/ml in MeOH.

Among sequential solvent extraction, partition and single CH_2Cl_2 extraction extraction methods, the CH_2Cl_2 sequential solvent extraction gave the lowest detectable amount for the DPPH-scavenging and tyrosinase inhibition assays. As a result, CH_2Cl_2 sequential solvent extraction was used as a method for the bagasse extraction.

Isolation of active compounds

Powdered sugarcane bagasse (3 kg) was extracted by maceration at room temperature with hexane and CH_2Cl_2. The process gave 0.51% (w/w yield of dry weight sugarcane bagasse).

The CH_2Cl_2 extract was fractionated through silica gel column (Scheme 1) and 14 fractions (A-N) were obtained. Free radical scavenging and tyrosinase inhibition activities of each fraction was assessed by TLC autographic assay.

The result indicated that the K fraction gave the lowest detectable amount in the DPPH-scavenging and tyrosinase inhibition assays **(Table 3.3)**; 0.25 μg of the K fraction gave similar activity to 0.25 of BHT. Similary, the potency of tyrosinase inhibition activity of the K fraction was equal to that of kojic acid. This fraction showed UV absorption band at 282 and 330 nm **(Figure 3.5)**.

Table 3.3 DPPH scavenging activity, tyrosinase inhibition activity and UV absorption activity of crude extract comparing with standard antioxidant (BHT) and standard tyrosinase inhibitor (kojic acid), as estimated by the minimal amount required to produce detectable positive result during the screening.

Crude	%yield (W/W) (as calculated against dry bagasse)	minimal detectable amount in antioxidant assay (μg)	minimal detectable amount in tyrosinase inhibition assay (μg)	UV absorption activity (λ_{max})
A	7.29	-	-	269
B	16.13	5	2.5	272
C	0.27	-	-	274
D	16.25	-	-	270
E	0.39	5	5	268
F	0.37	-	2.5	273
G	4.01	5	-	275
H	0.39	2.5	2.5	279
I	0.68	0.5	-	282 with shoulder 320
J	4.14	0.5	0.5	276 with shoulder 320
K	9.01	0.5	0.25	282 with shoulder 330
L	7.26	5	2.5	274 with shoulder 320
M	1.65	-	2.5	269
N	24.87	5	5	275
BHT	ND	0.25	ND	ND
Kojic	ND	ND	0.25	ND

- = No activity, ND = Not determined

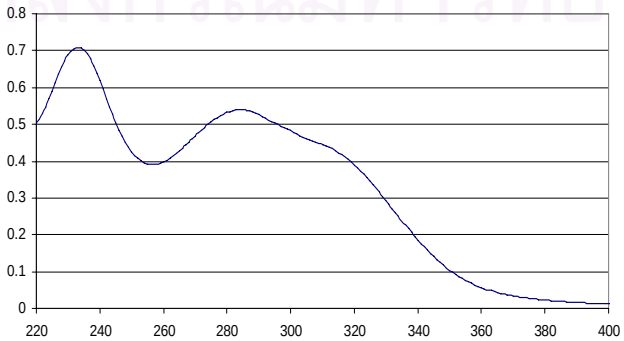


Figure 3.5 UV spectrum of K fraction at concentration 50 μg/ml in MeOH.

The most active fraction, K fraction was fractionized by sephadex LH-20 column using MeOH-CH_2Cl_2 (30:60) as a mobile phase to obtain 6 subfractions (K1-K6). The most active subfraction, K2 (brown paste, 0.78g), was separated using C_{18} column chromatography with gradient MeOH-H_2O (60:40 to 100:0) elution. 9 subfractions (K2/1-K2/9), K2/1 (brown paste, 347.8 mg) showed the highest free radical scavenger activity while K2/3 (brown paste, 131.5 mg) showed the highest tyrosinase inhibition activity.

The K2/1 fraction was fractionated and five constituents, namely, 4-hydroxy benzaldehyde, syringic acid, 3,5-dimethyl-4-hydroxybenzaldehyde, 4-hydroxy-3-methoxycinnamic acid and 3',5'-dimethoxy-4'-hydroxy acetophenone (**Figure 3.8**), could be identified using HPLC method (**Figure 3.6**). Standard compounds were employed in the HPLC identification of 5 constituents (**Figure 3.7**).

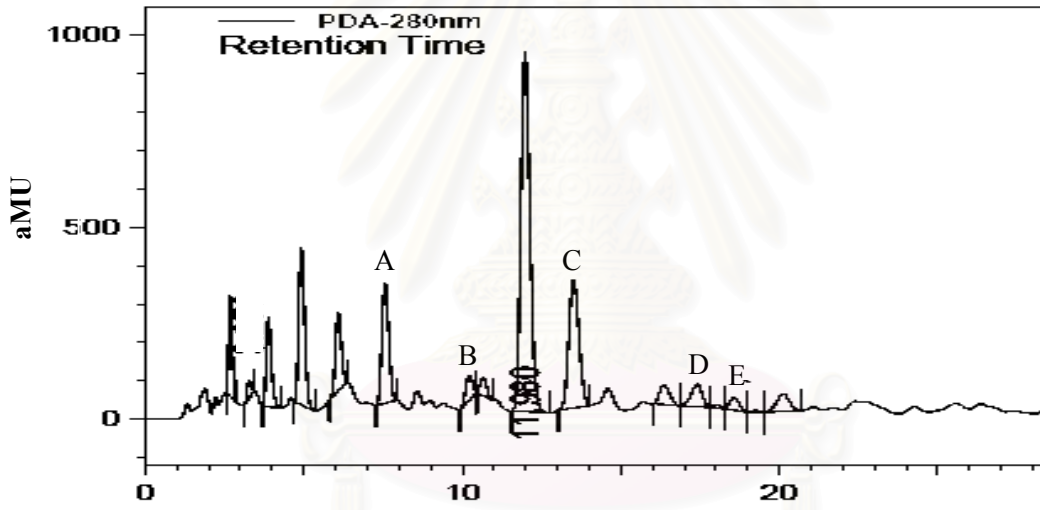


Figure 3.6 Semi-preparative HPLC chromatogram obtained from separation of K2/1 fraction under condition described in the experimental section

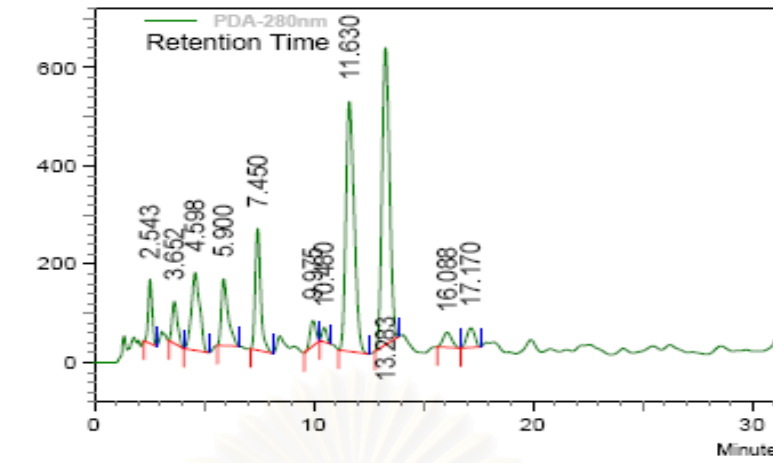
A = 4-hydroxylbenzaldehyde

B = syringic acid

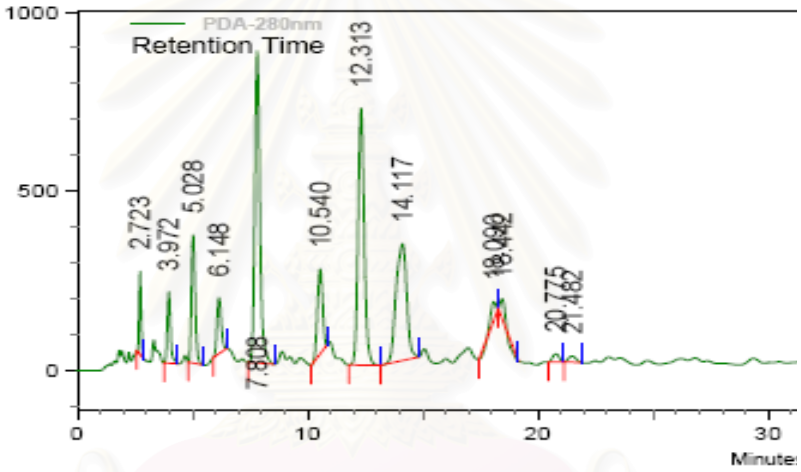
C = 3,5-dimethyl-4-hydroxybenzaldehyde

D = 4-hydroxy-3-methoxycinnamic acid

E = 3',5'- dimethoxy-4'-hydroxyacetophenone



(1)



(2)

Figure 3.7 Semi-preparative HPLC chromatogram

(1) HPLC chromatogram spiked by 3',5'-dimethoxy- 4-hydroxybenzaldehyde.

(2) HPLC chromatogram spiked by 4- hydroxybenzaldehyde, syringic acid, 4-hydroxy-3-methoxycinnamic acid and 3,5-dimethoxy-4-hydroxyacetophenone

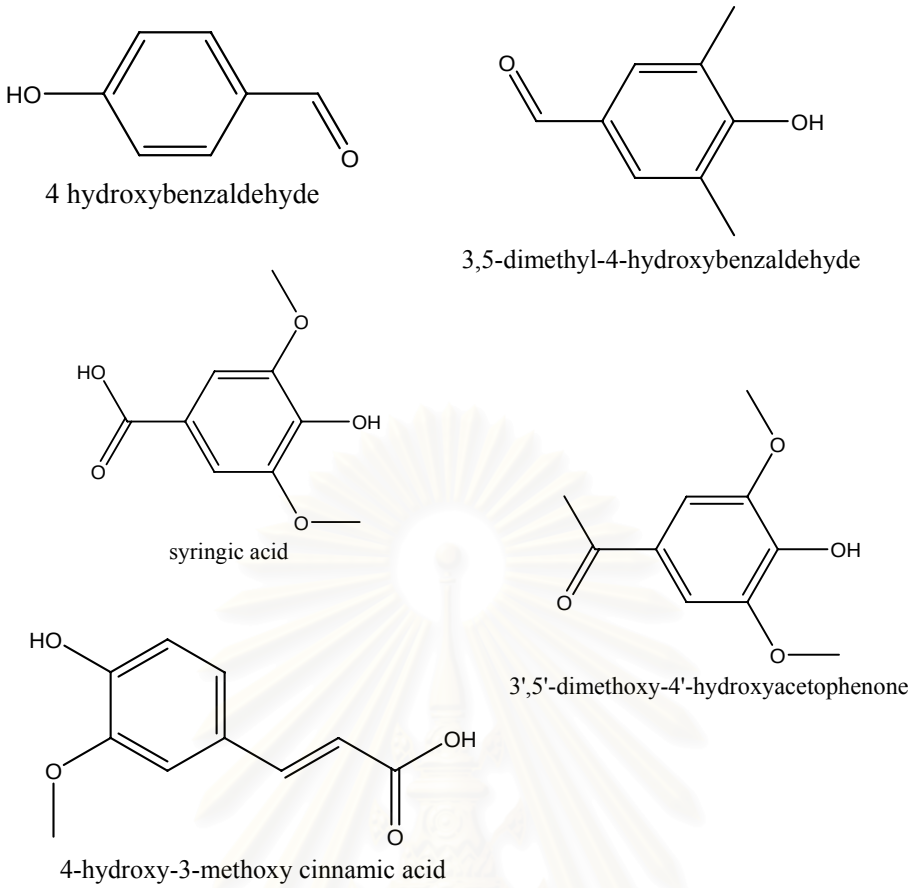


Figure 3.8 Chemical structures 4-hydroxy benzaldehyde, syringic acid, 3,5-dimethyl-4-hydroxybenzaldehyde, 4-hydroxy-3-methoxycinnamic acid, 3',5'-dimethoxy-4'-hydroxy acetophenone

Fraction K2/3 (brown paste, 131.5 mg) was separated using silica gel column with gradient elution (hexane:EtOAc 100:0 to 0:100) to obtain 4 fractions (K2/3a-K2/3d) and undecane-4,6-dione (5 mg) was identified through spectroscopic analysis.

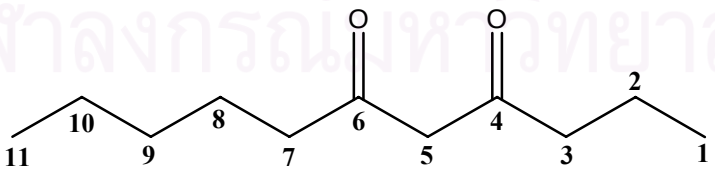


Figure 3.9 Chemical structures of undecane-4,6-dione

Undecane-4,6-dione (**Figure 3.9)**. ESI^- m/z: 367 $[2M-H]^-$. ^1H NMR (400 MHz, $CDCl_3$, δ, ppm): 0.81-0.82 (3H, br, H-1, H-11), 1.20-1.35 (2H, m, H-10), 1.48-1.58 (6H, m, H-2, H-8, H-9), 2.25-2.40 (4H, m, H-3, H-7), 3.60 (2H, s, H-5). ^{13}C

NMR (100 MHz, $CDCl_3$, δ, ppm): 9.0 (C-1), 24.0 (C-2), 33.5 (C-3), 174.0 (C-4), 52.0 (C-5), 178.0 (C-6), 42.0 (C-7), 23.5 (C-8), 23.0 (C-9), 29.0 (C-10), 13.5 (C-11).

Table 3.4 1D and 2D NMR spectroscopic data of undecane-4,6-dione in $CDCl_3$.

position	δ_H (ppm)	δ_C (ppm)
1	0.81 (3H)	9.0
2	1.58 (2H)	24.0
3	2.27 (2H)	33.5
4	-	174.0
5	3.60 (2H)	52.0
6	-	178.0
7	2.30 (2H)	42.0
8	1.55 (2H)	23.5
9	1.48 (2H)	23.0
10	1.25 (2H)	29.0
11	0.82 (3H)	13.5

The HMBC correlation**s (Figure 3.10)** were observed between the correlations of H-2 to C-3, H-3 correlate to C-2 and C-4, H-5 correlate to C-4, H-7 correlate to C-6, C-8 and C-10, H-8, H-9 correlate to C-10, H-10 correlate to C-11 and C-8 and H-11 correlate to C-9.

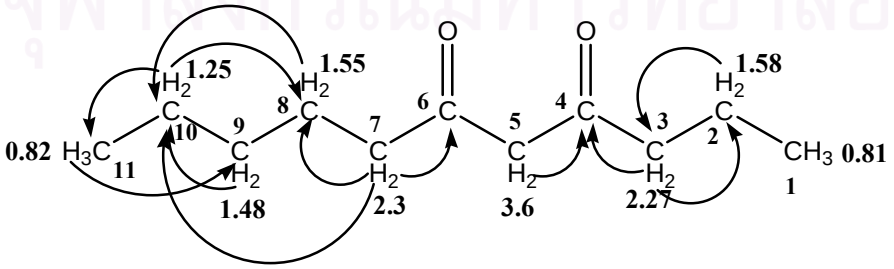


Figure 3.10 HMBC correlation of undecane-4,6-dione.

The ^{1}H-^{1}H COSY spectrum **(Figure 3.11)** revealed the presence of the following connectivity as shown in Figure 3.8

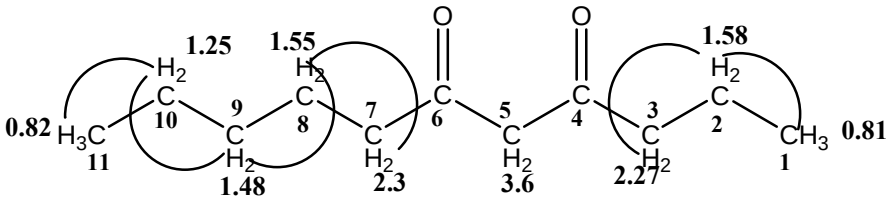


Figure 3.11 The COSY correlation of undecane-4,6-dione.

Among six compounds, 3',5'-dimethoxy-4'-hydroxyacetophenone, 3,5-dimethyl-4-hydroxybenzaldehyde, 4-hydroxy-3-methoxycinnamic acid, 4-hydroxy-benzaldehyde and syringic acid have been identified in sugarcane previously with DPPH scavenging activity also being reported [66,69]. However this was the first time that undecane-4,6-dione was isolated from sugarcane bagasse.

3.3 Biological activity assay

The determination of DPPH-scavenging and tyrosinase inhibition activities of all 6 compounds was carried out as described in chapter II. IC50 values **(Table 3.5)** of each activity could be obtained.

Table 3.5. $[MH]^+$ detected from ESIMS, IC_{50} of DPPH scavenging and tyrosinase inhibition activities and UV absorption property of compounds isolated from sugarcane bagasse as compared to kojic acid and BHT.

Compound	MW. (From ESIMS)	DPPH scavenging activity IC_{50} (μM)	tyrosinase inhibition activity IC_{50} (μM)	UV absorption activity (λ_{max})
4-hydroxybenzaldehyde	121	>50	3.77	282
syringic acid	197	3.18	0.25	264
3,5-dimethyl-4-hydroxybenzaldehyde	181	12.20	-	286
3',5'-dimethoxy-4'-hydroxyacetophenone	195	>50	-	299
4-hydroxy-3-methoxycinnamic acid	193	13.08	>6	311
undecane-4,6-dione	184	-	5.98	275
kojic acid	ND	ND	0.19	ND
BHT	ND	8.81	ND	ND

- = No activity, ND = Not determined

Among the 6 compounds identified, syringic acid gave the highest DPPH scavenging and tyrosinase inhibition activities.

Total phenolics content

Total phenolic contents from various crude extracts were compared (expressed as gallic acid equivalents (GAE)) **(Figure 3.12).**

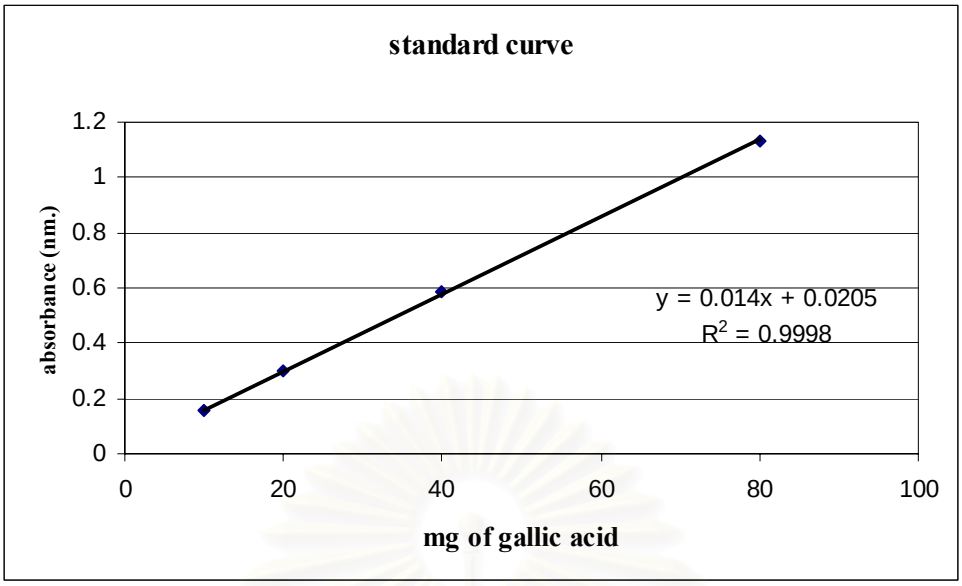


Figure 3.12 Clibration curve of gallic acid

Result from CH_2Cl_2 isolation, the K2/1 fraction had the highest content of total phenolics (243.32 mg GAE /100 g crude extract) **(Table 3.6)**, which was well correlated with the corresponding antioxidant activity and the amount of phenolic compounds.

Table 3.6 Total phenolic contents of CH_2Cl_2 fraction and isolated fractions

Fraction	**Total phenolics content** miligram gallic acid equivalents (GAE) per gram of sugarcane extract.
CH_2Cl_2 sequential solvent extraction	229.20
K	117.21
K2	118.24
K2/1	243.32
K2/3	129.64

Active compounds from sugarcane bagasse extract possess free radicals scavenger and tyrosinase inhibitor. The syringic acid contain highest free radicals scavenging and tyrosinase inhibition activities, 3,5-dimethyl-4-hydroxybenzaldehyde

and 4-hydroxy-3-methoxycinnamic acid are antioxidant synergism and undecane-4,6-dione and 4-hydroxybenzaldehyde are tyrosinase inhibition synergism.

CHAPTER IV
CONCLUSION

The best process to obtain extract with good antioxidant, tyrosinase inhibition activities and UV-absorption property from sugarcane bagasse is CH_2Cl_2 sequential solvent extraction. Separation and purification of sugarcane bagasse (11.5 g) using chromatography gave 6 known biologically active compounds, 3',5'-dimethoxy-4'-hydroxyacetophenone (3.27 mg), 3,5-dimethyl-4-hydroxybenzaldehyde (65.63 mg), 4-hydroxy-3-methoxycinnamic acid (10.96 mg), 4-hydroxybenzaldehyde (54.07 mg), syringic acid (14.37 mg) and undecane-4,6-dione (5.00 mg). The results indicated that free radical scavenging activity of sugarcane extract was partly contributed from the presence of syringic acid, 3,5-dimethyl-4-hydroxybenzaldehyde and 4-hydroxy-3-methoxycinnamic acid. While tyrosinase inhibition activity was partly contributed by the presence of syringic acid, 4-hydroxybenzaldehyde and undecane-4,6-dione. Among the 6 compounds identified, syringic acid gave the highest DPPH scavenging (IC_{50} = 3.18) and tyrosinase inhibition activities (IC_{50} = 0.25).

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APPENDICES

APPENDIX A

A Stock solution preparation

Phosphate buffer pH 6.8 stock solution

Preparation of phosphate buffer stock solution 500 mM 1000 ml with (MW 174.18, 42.5521 g) and KH_2PO_4 (MW 136.09, 34.7982 g) in deionizers water. K_2HPO 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.

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

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

$$0.2 = \log \frac{[H_2PO_4^-]}{[HPO_4^{2-}]}$$

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

$[H_2PO_4^-] = [CA]$
$[CA] = (1.0471/2.0471) \times 0.5$
$[CA] = 0.2557$
KH_2PO_4 0.2557
$[HPO_4^{2-}] = [CB]$
$[CB] = 0.2443$
K_2HPO_4 was used 0.2443 moles, 42.5521 g

APPENDIX B

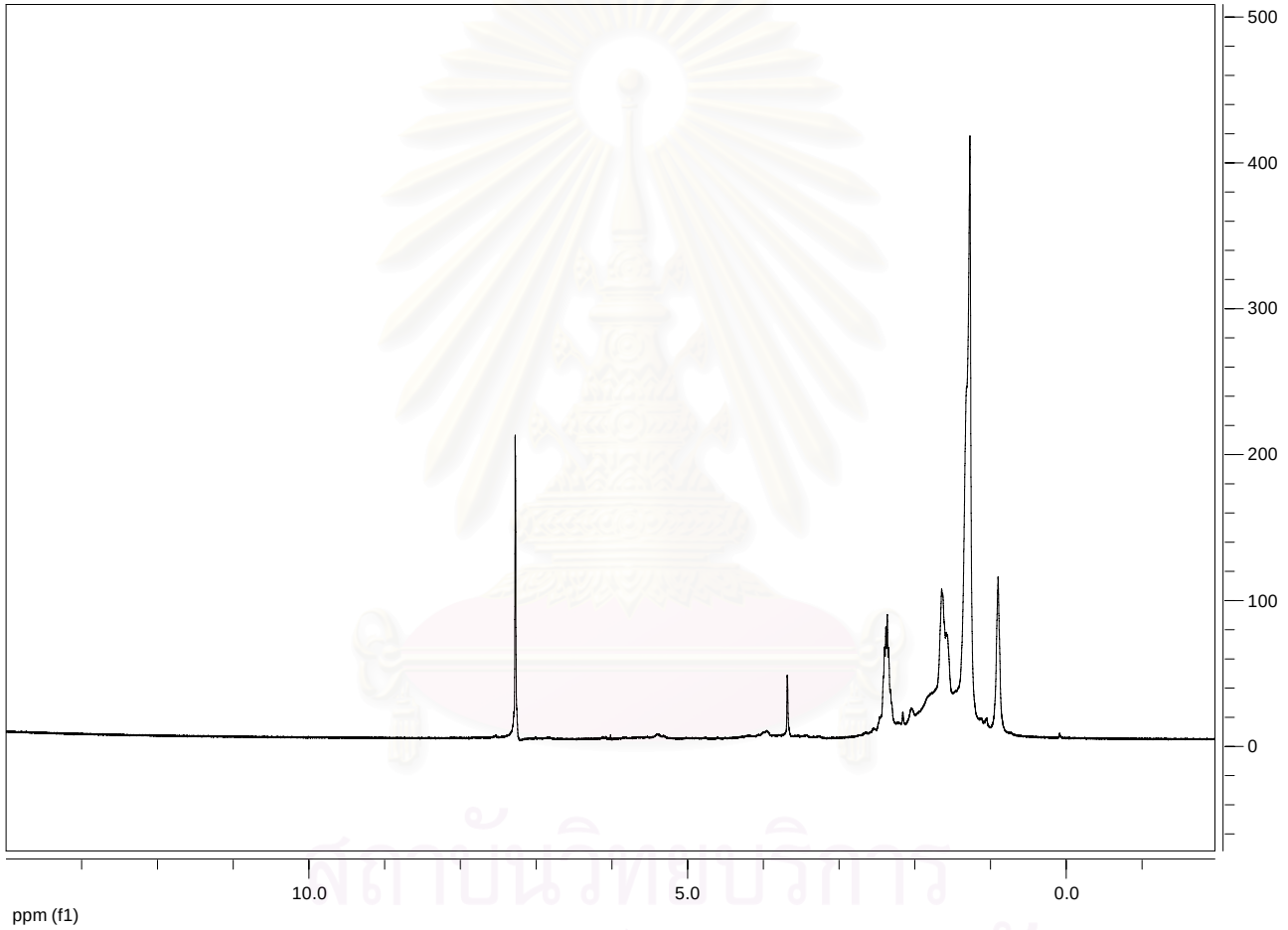


Figure B-1 NMR spectrum of undecane-4,6-dione

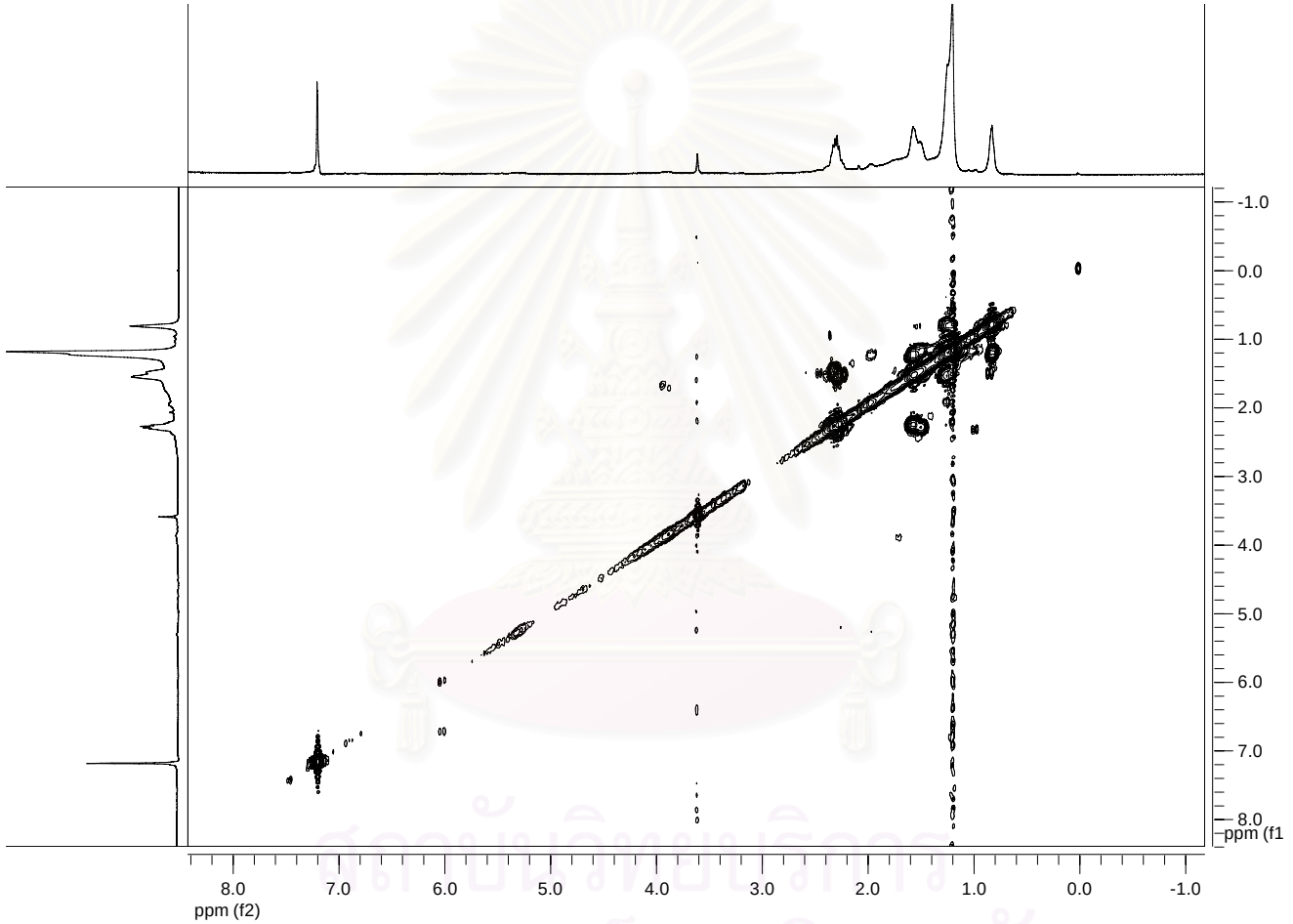


Figure B-2 The COSY spectrum of undecane-4,6-dione

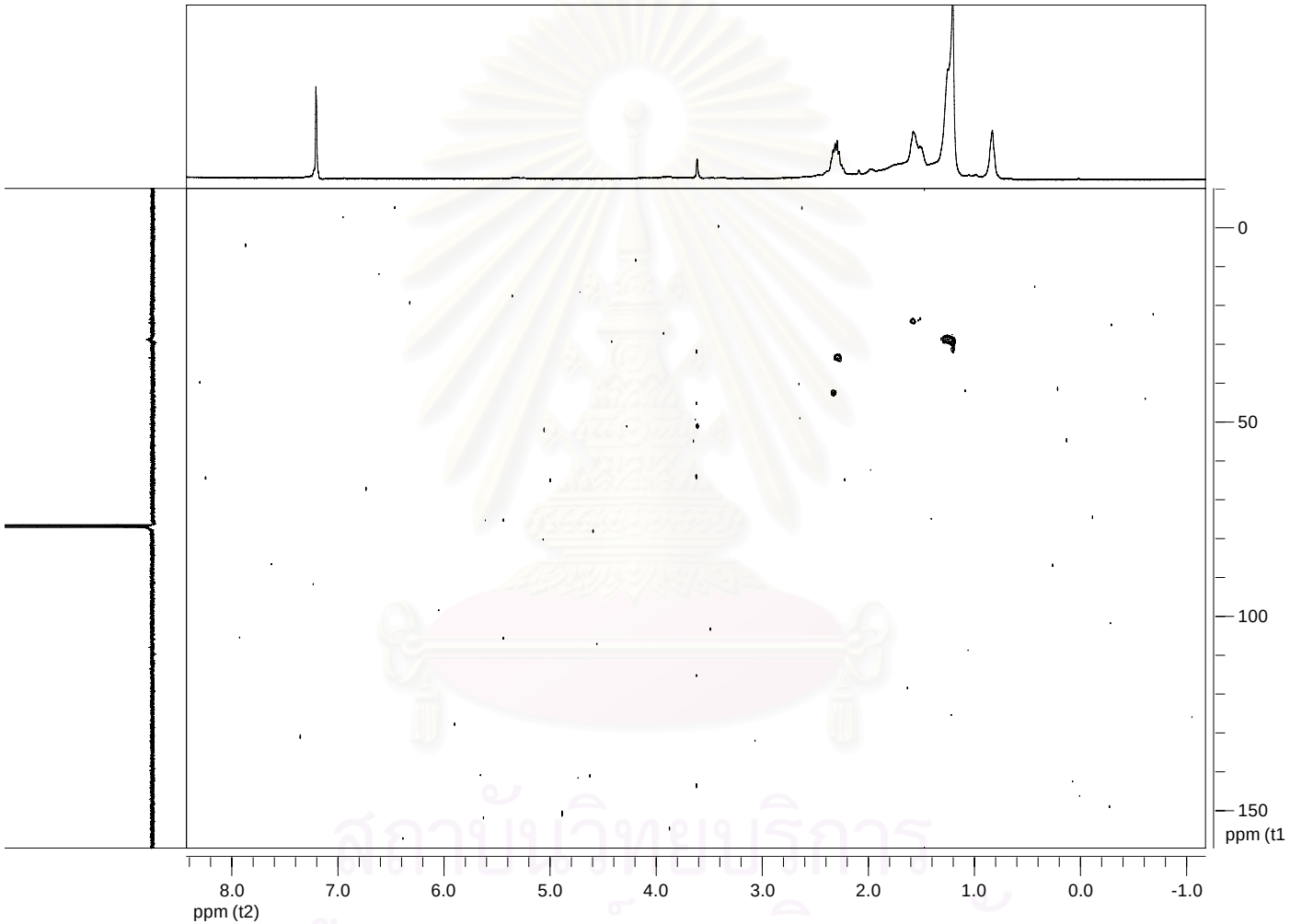


Figure B-3 The HMQC spectrum of undecane-4,6-dione

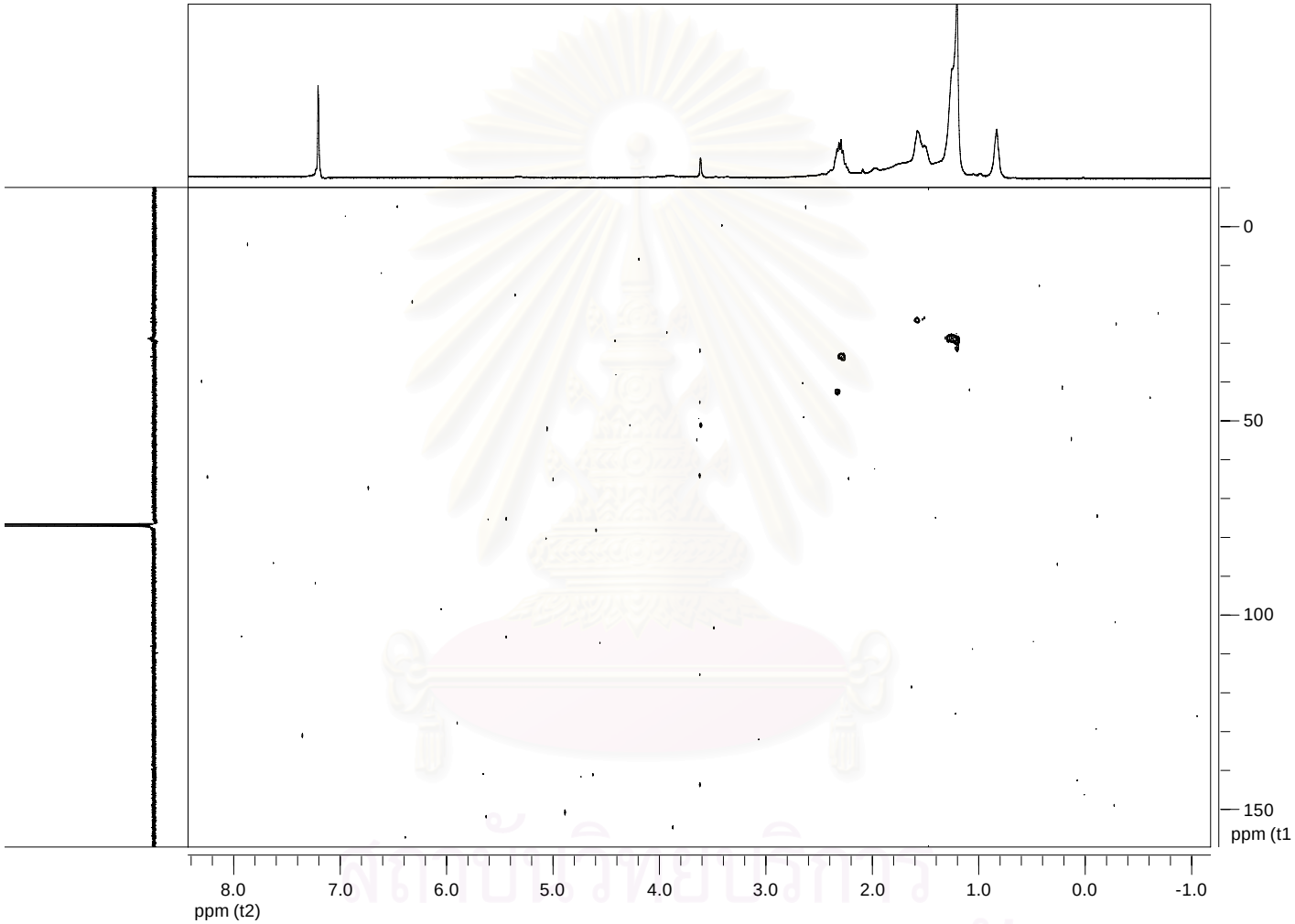


Figure B-4 The HMBC spectrum of undecane-4,6-dione

APPENDIX C

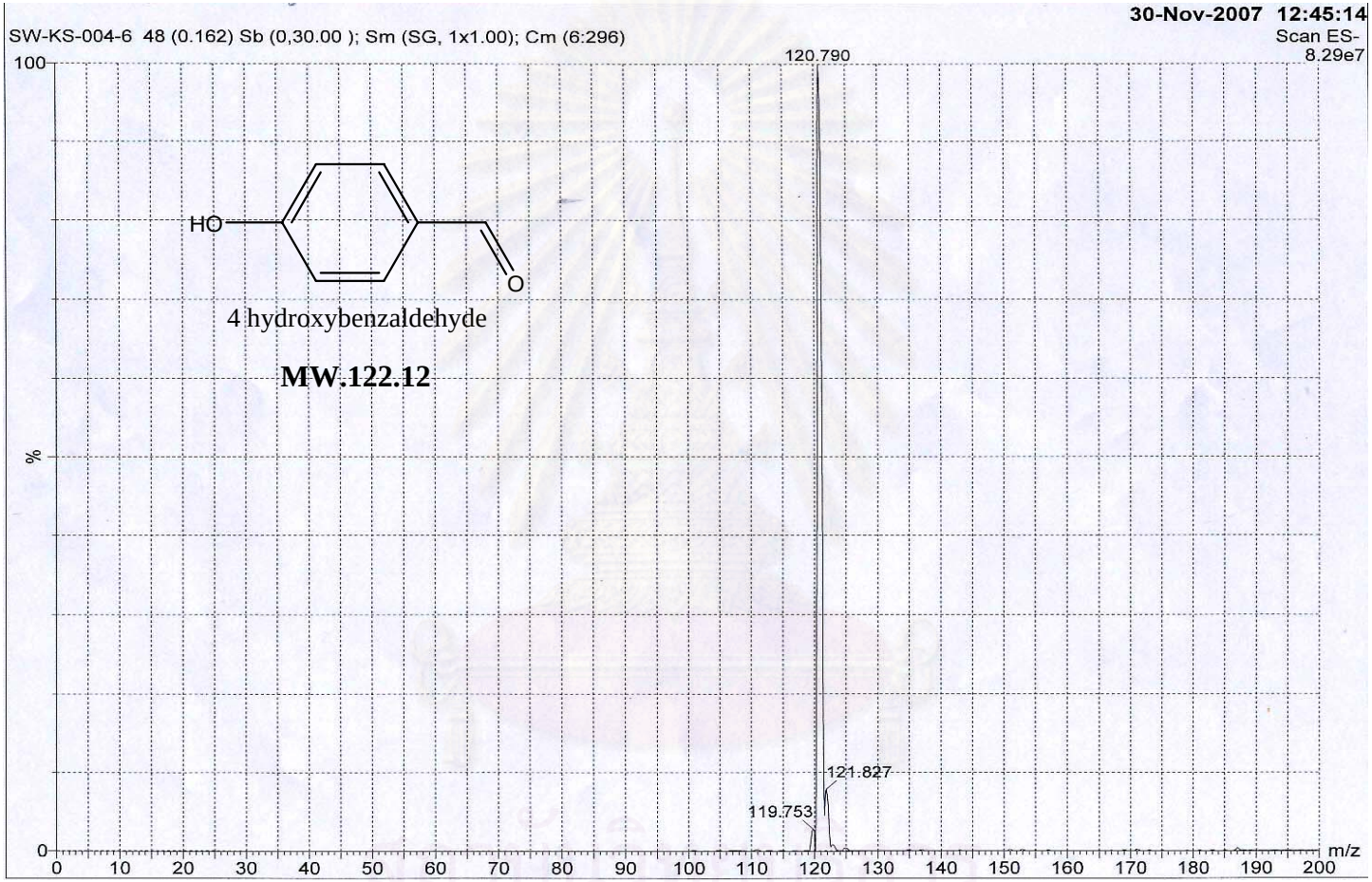


Figure C-1 The negative ESI-MS spectrum of 4-hydroxybenzaldehyde

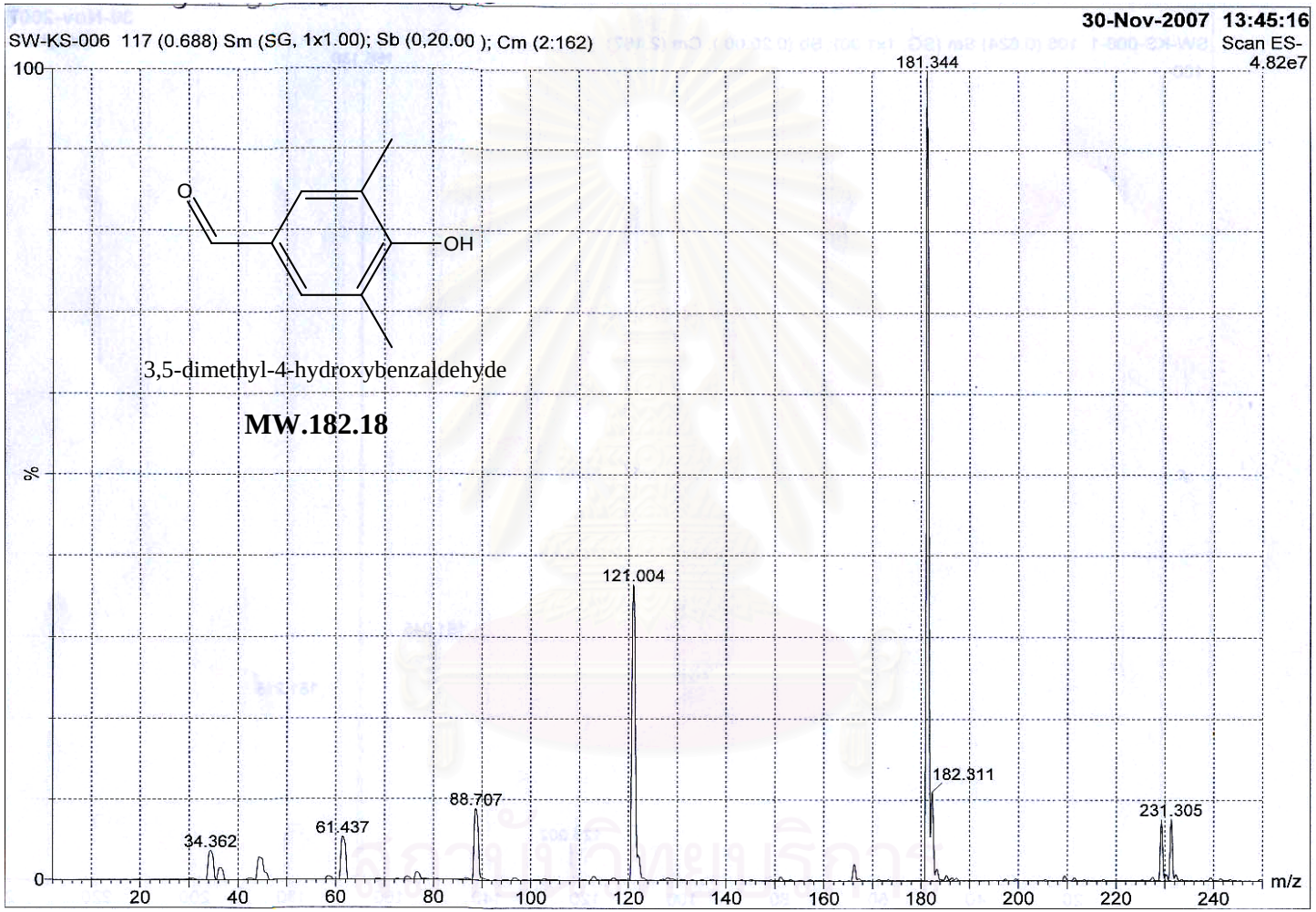


Figure C-2 The negative ESI-MS spectrum of 3,5-dimethyl-4-hydroxybenzaldehyde

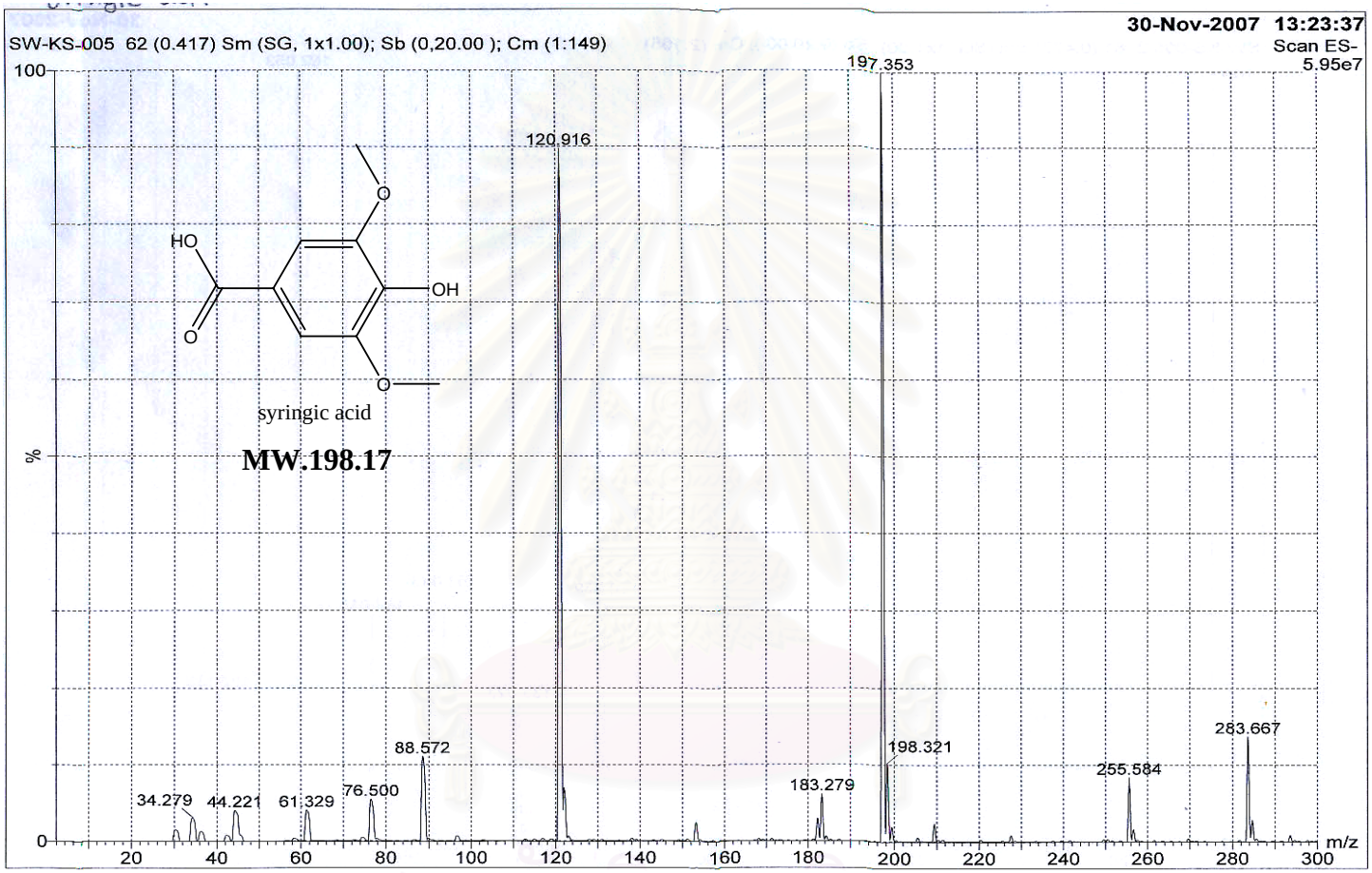


Figure C-3 The negative ESI-MS spectrum of syringic acid

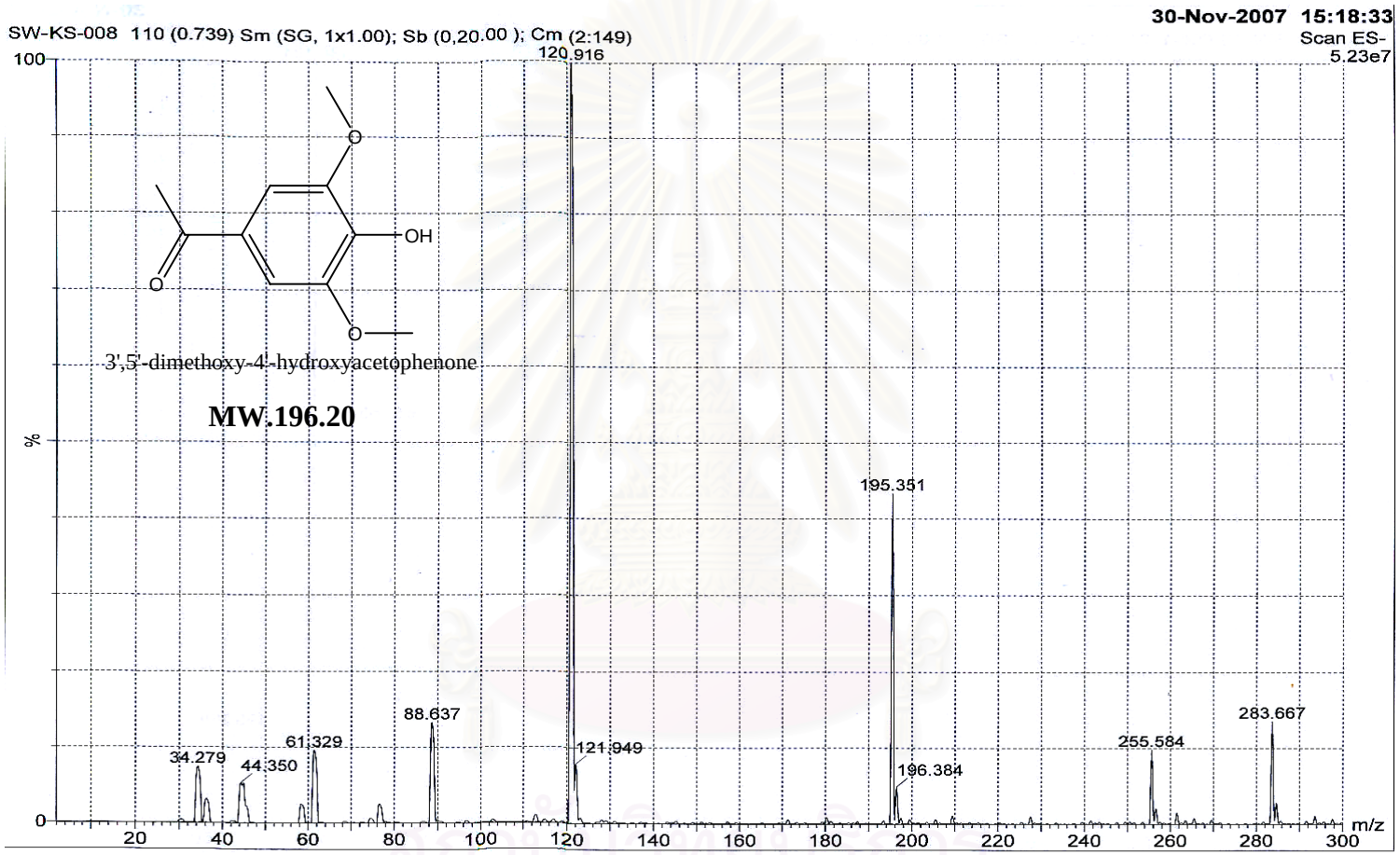


Figure C-4 The negative ESI-MS spectrum of 3’,5’-dimethoxy-4’-hydroxyacetophenone

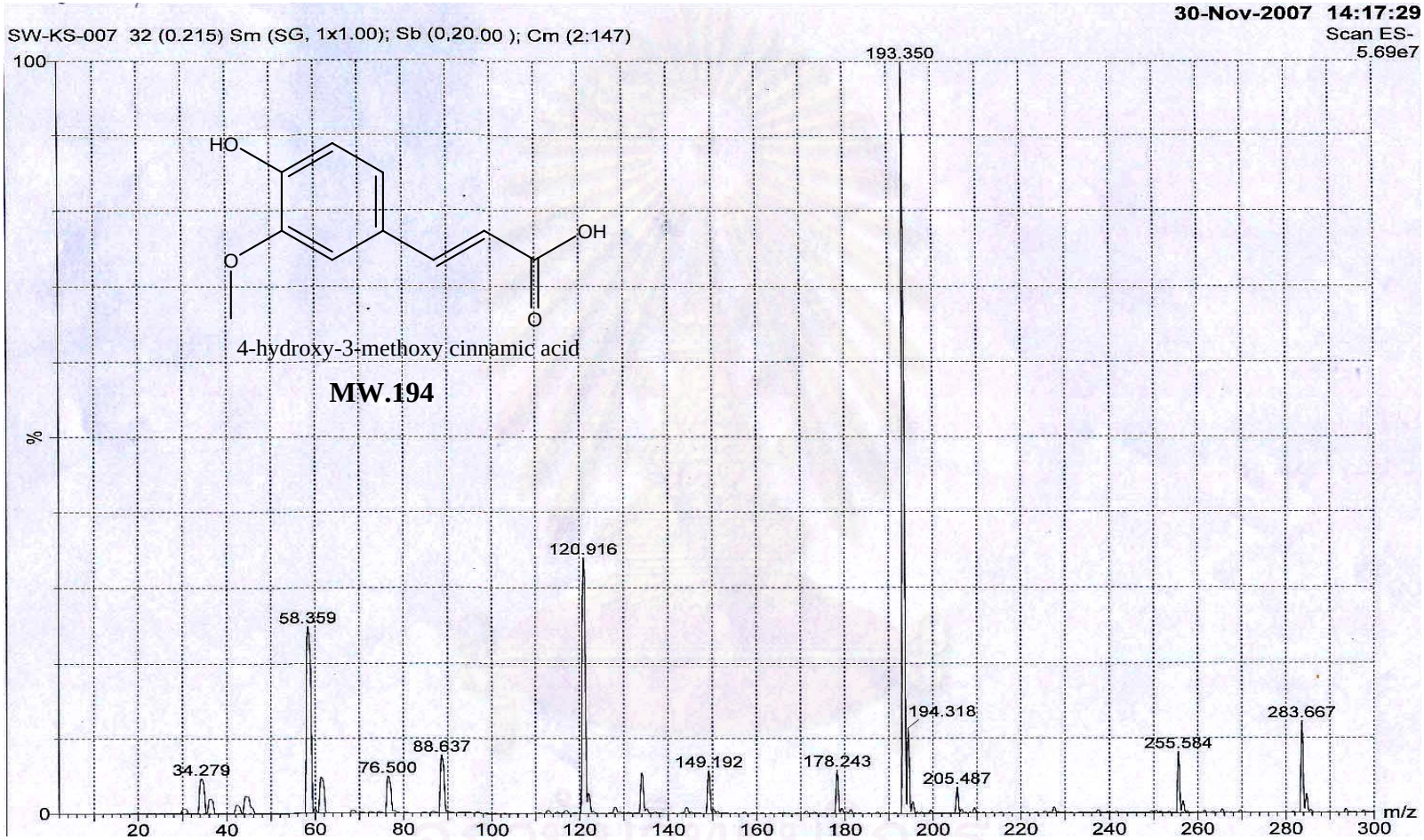
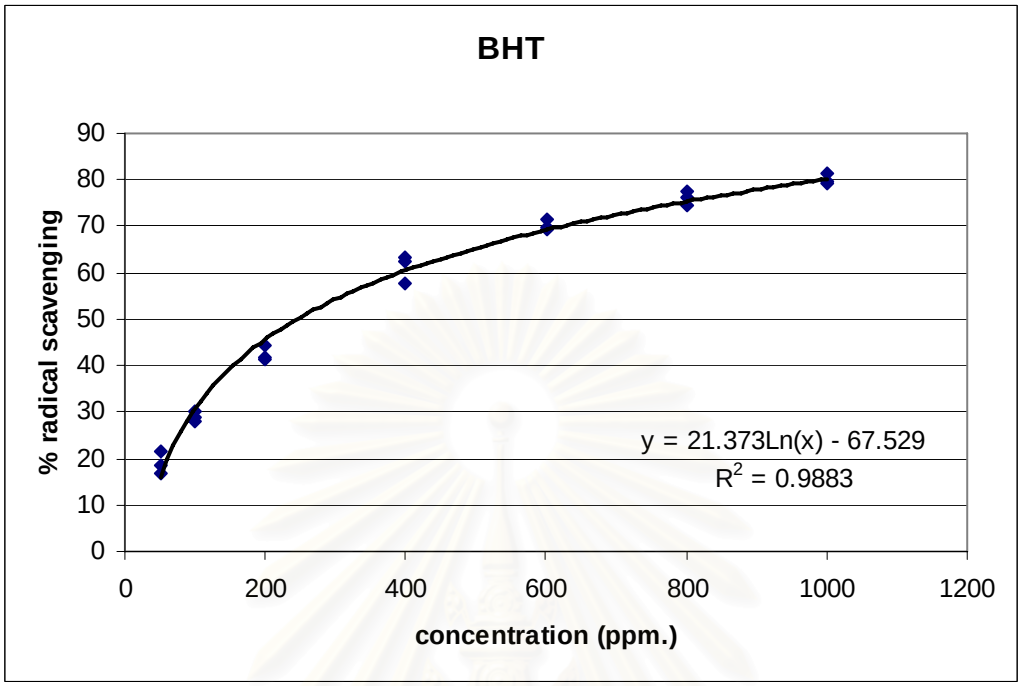
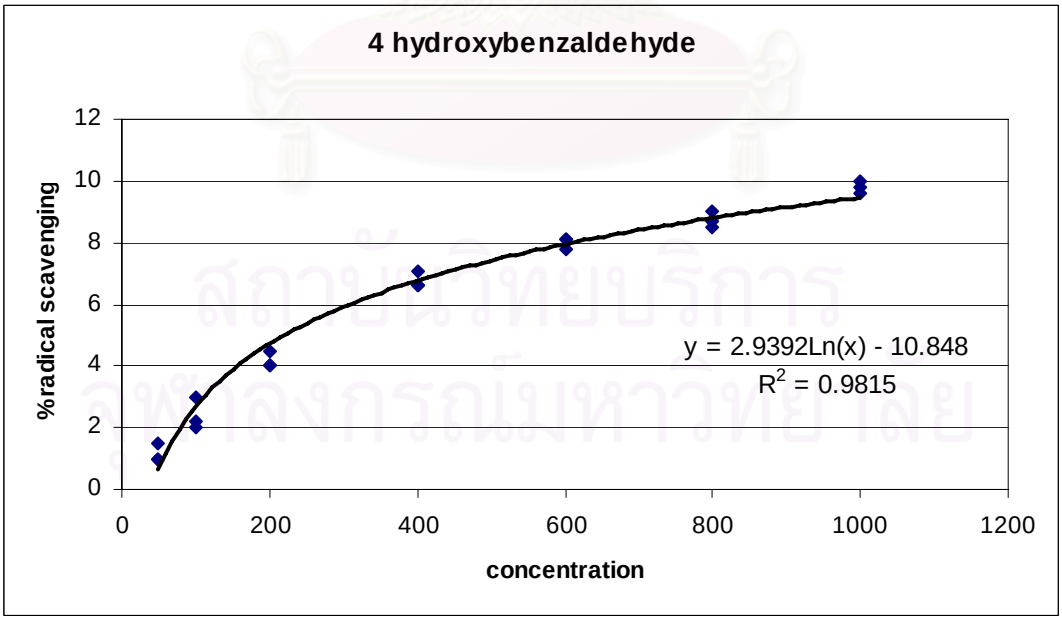


Figure C-5 The negative ESI-MS spectrum of 4-hydroxy-3-methoxycinnamic acid

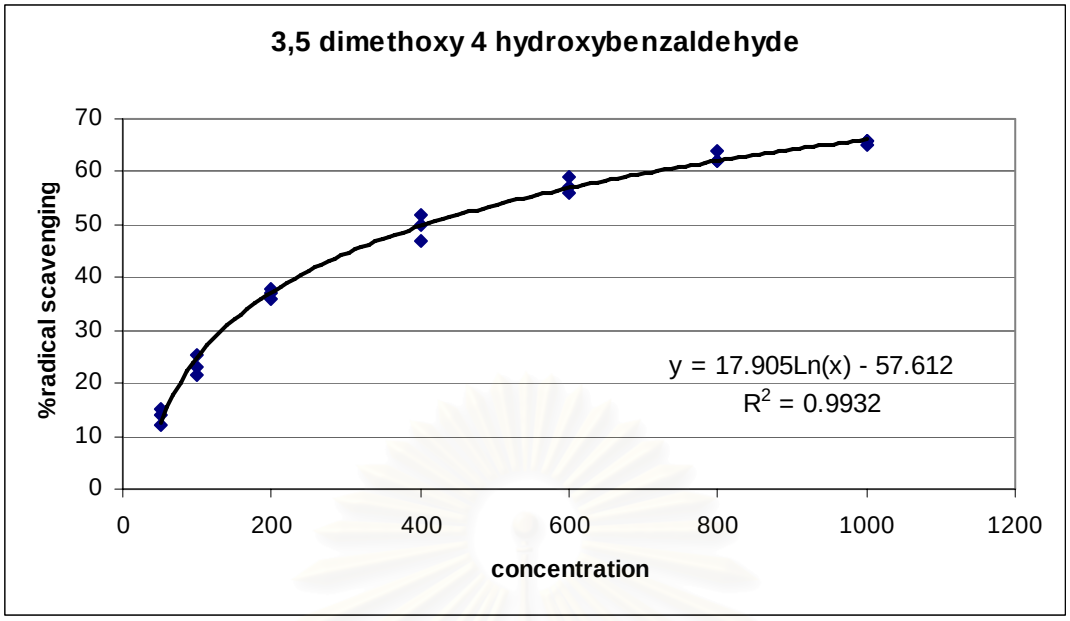
APPENDIX D



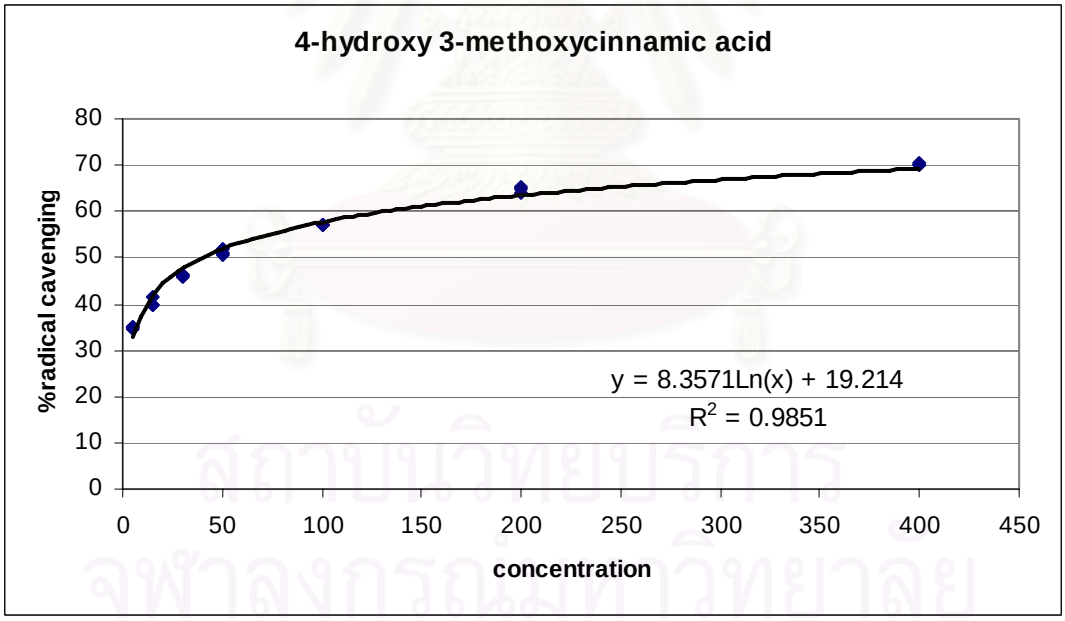
(A)



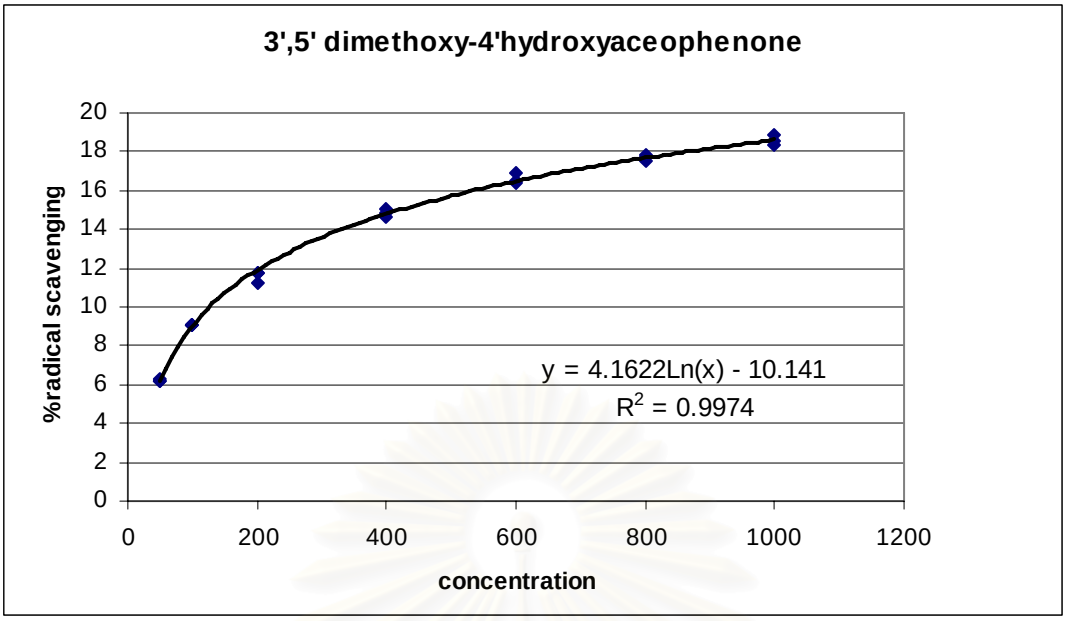
(B)


3,5 dimethoxy 4 hydroxybenzaldehyde
%radical scavenging
concentration
y = 17.905Ln(x) - 57.612
R2 = 0.9932
0
10
20
30
40
50
60
70
0
200
400
600
800
1000
1200

(C)


4-hydroxy 3-methoxycinnamic acid
%radical cavenging
concentration
y = 8.3571Ln(x) + 19.214
R2 = 0.9851
0
10
20
30
40
50
60
70
80
0
50
100
150
200
250
300
350
400
450

(D)



(E)

Figure D-1 IC_{50} of DPPH scavenging activity of compounds isolated from sugarcane bagasse as compared to BHT. (A) BHT, (B) 4-hydroxybenzaldehyde, (C) 3,5-dimethyl-4-hydroxy benzaldehyde, (D) 4-hydroxy-3-methoxycinnamic acid, (E) 3',5'-dimethoxy-4'-hydroxyacetophenone.

VITA

Miss Panpilai Chanchaisak was born on 17th February, 1982 in Bangkok. She got a Bachelor of Science Degree in Biology from Kasetsart University in 2004. After that, Miss Chanchaisak has been gradate student working for Master Degree in Biotechnology program at Chulalongkorn University, During her Master of Science study, she was also awarded a research grant (Graduate School Thesis Grant) from Chulalongkorn University.

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