เรียนรู้เพื่อรับใช้สังคม

ANTITYROSINASE AND ANTIOXIDANT ACTIVITIES OF CINNAMIC ACID DERIVATIVES

CHANUTTHA SAK-AIEM

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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ABSTRACT

In this research, three ester derivatives of cinnamic acid and benzenediol were synthesized via esterification reaction between cinnamic acid with excess oxalyl chloride and benzenediol. The derivatives were purified by column chromatography and elucidated by FT-IR, NMR and mass spectroscopy. *In vitro* antioxidant activity and tyrosinase inhibitory were measured. Compound 5c exhibited the highest DPPH scavenging activity with the IC_{50} value of 56.35 µg/ml, followed by compound 5a (26.10 mg/ml) and compound 5b (47.90 mg/ml). Compound 5c also showed the highest inhibitory activity against tyrosinase enzyme with the IC_{50} value of 24.01 µg/ml compared to compound 5b (153.70 µg/ml), and compound 5a (179.62 µg/ml), respectively. The CC_{50} values of compound 5a, 5b, and 5c were higher than 100 µg/ml and lower than that of hydroquinone in the REMA assay against the human skin fibroblast cells.

Keywords: Cinnamic acid, Benzenediol, Cinnamic acid derivatives, Esterification, Antioxidant, Tyrosinase inhibitor

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CHAPTER 1
INTRODUCTION

1.1 Rationale and background

Having white skin is one of the standards for female beauty which is deeply embedded within East Asian culture. Most of people, especially women want to be white and always whiten or protect their skin from the sun. Skin whitening and lightening products account for a large share of the cosmetics market in East Asia region, and will be forecast to be driven by powerful demand with dramatic growth as expected in the future. In human skin melanogenesis, both eumelanin and pheomelanin are synthesized through a series of oxidative reactions involving the amino acid L-tyrosine in the presence of the enzyme tyrosinase. Melanin in skin is the main determining factor of skin color. Tyrosinase is a copper-containing enzyme that catalyzes the melanin synthesis from tyrosine by oxidation. Since tyrosinase is known as the important and rate-limiting enzyme in the synthesis of melanin, tyrosinase inhibition is the important and popular method in decreasing melanin production. Skin-whitening agents are substances that can decrease melanin production. Antioxidant is another interesting choice for whitening ingredient. Antioxidants are substances that inhibit or protect the oxidation of reactive molecules.

Benzenediols, also known as dihydroxybenzenes, are phenolic compounds having the antioxidant activity. Their chemical structures are consisted of two substituted hydroxyl groups onto a benzene ring. There are three isomers of dihydroxybenzene: catechol; the ortho isomer (1,2-benzenediol), resorcinol; the meta isomer (1,3-benzenediol), and hydroquinone; the para isomer (1,4-benzenediol). Hydroquinone shows the highest redox potential of three benzenediols and possesses a strong inhibitory activity of melanin synthesis in melanocytes.

Hydroquinone is the para isomer of benzenediol. It is frequently used as a topical ingredient in both medicinal and cosmetic skin products to whiten the skin

color or reduce the hyperpigmentation. However, this use is banned in some countries because there are some evidences in animal test, showing that hydroquinone may have a carcinogenic effect. The lower cytotoxicity hydroquinone derivatives are used instead of hydroquinone and some become popular and widely used in several whitening cosmetic products.

Cinnamic acid and its derivatives are known ingredients commonly used in cosmetics as fragrance ingredients and sunscreen agents. Cinnamic acid is a widely distributed phenylpropanoid organic compound. It is mainly found in *Cinnamomum cassia* BLUME and *Panax ginseng* and also occurs naturally in a number of plants. Cinnamic acid derivatives possess a wide range of biological activities, such as anticancer, antituberculosis, antifungal, UV rays absorbent activities, etc. Additionally, cinnamic acid was recently reported to have a tyrosinase inhibitory effect. Because of inhibition of tyrosinase activity within melanocytes can cause the reduction of melanin production and whiten the skin color, cinnamic acid might act as a skin whitening agent. [(1)] The effects of cinnamic acid derivatives on the mushroom tyrosinase inhibition activity have been studied and the results show that some derivatives such as 4-hydroxycinnamic acid and 4-methoxycinnamic acid strongly inhibit the tyrosinase activity. [(2)] Furthermore, the antioxidant activities of the cinnamic acid derivatives are also studied. The antioxidant capacity relative to trolox represented that the cinnamic acid derivatives have more antioxidant ability compared to benzoic acid in the same substituted functional group. [(3)]

The objective of this research focuses on synthesizing a series of cinnamic acid ester derivatives, bearing hydroxybenzene moiety as potential skin whitening and antioxidant activities.

1.2 Research objectives

Overall objectives of this research are the following:

1) To synthesize cinnamic acid derivatives

2) To evaluate tyrosinase inhibition activity of cinnamic acid derivatives

3) To evaluate the antioxidant activity of cinnamic acid derivatives

4) To evaluate the cytotoxic activity of cinnamic acid derivatives

1.3 Research hypothesis

1) Cinnamic acid derivatives possess tyrosinase inhibition activity

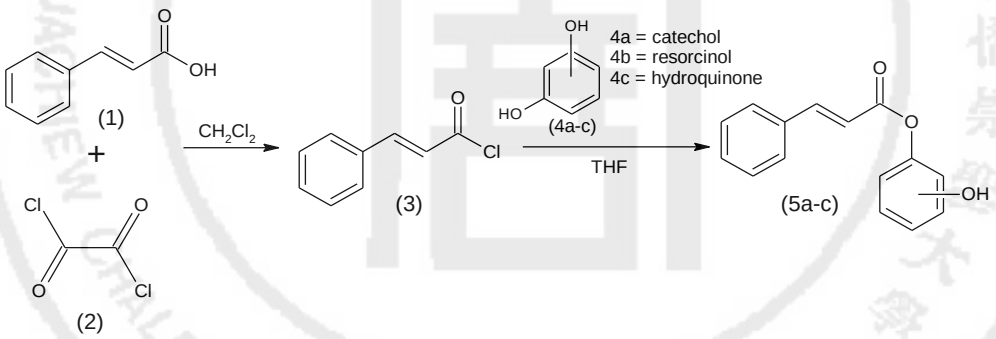
2) Cinnamic acid derivatives show the antioxidant capacity

3) Cinnamic acid derivatives have lower cytotoxicity than hydroquinone

1.4 Scope and limitations of the study

1) Synthesis of cinnamic acid ester derivatives (5a-c), bearing benzenediol moiety is shown in **Scheme 1**

Scheme 1 Synthesis of cinnamic acid ester derivatives bearing benzenediol moiety



2) Purification and chemical structure elucidation of the target compounds

3) Tyrosinase inhibition evaluation

4) Antioxidant activity evaluation

5) Cytotoxicity evaluation

1.5 Expected results

The synthesized compounds possess a tyrosinase inhibition and antioxidant activities with lower cytotoxic activity than hydroquinone.

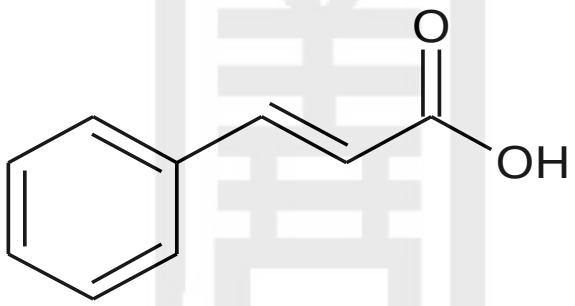
CHAPTER 2

LITERATURE REVIEW

2.1 Cinnamic acid derivatives

Cinnamic acid is an organic compound composed of a phenyl ring substituted with an acrylic acid group as shown in **Figure 1**.

Figure 1 Chemical structure of cinnamic acid (3-phenylacrylic acid)



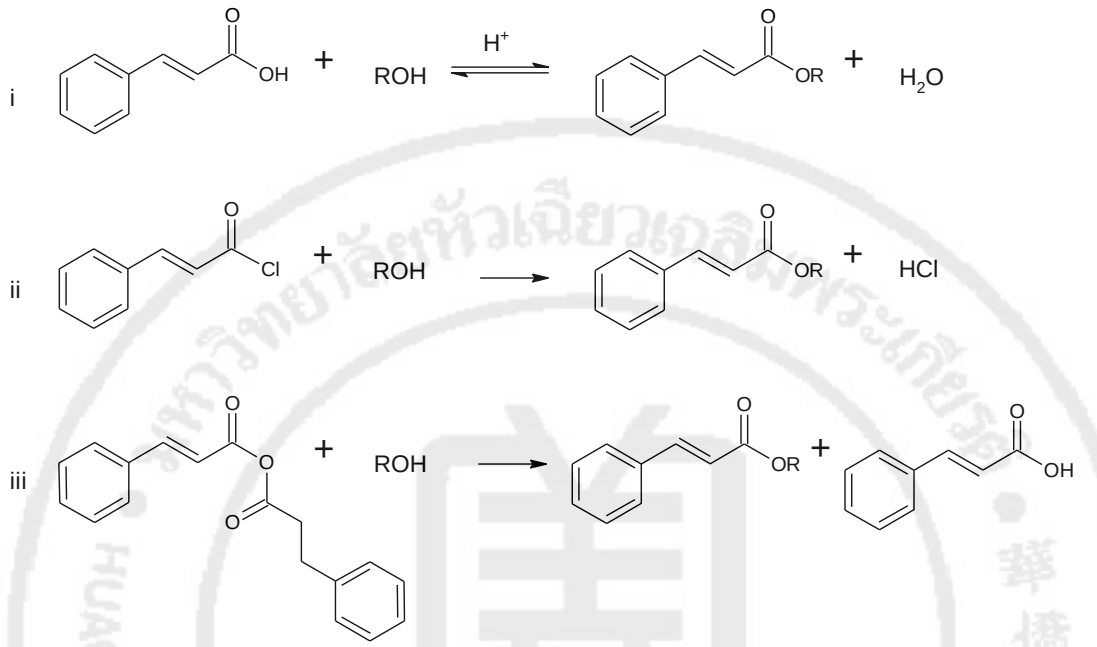
It is a white crystalline and soluble in both polar and non-polar solvent. It can be found naturally in oil of cinnamon, resin and balsam or synthesized by Perkin reaction using benzaldehyde in acetic anhydride and anhydrous sodium acetate. [4] Cinnamic acid esters are widely used as flavor materials in food, perfumery, cosmetic and pharmaceutical industries. [5], [6]

Many cinnamic acid derivatives are studied, some of which are found naturally, and chemically synthesized. In the last decade, both plant - isolated cinnamoyl moiety and chemically synthesized received much attention in medicinal research due to their broad spectrum of biological activities, particularly low toxicity. A series of new substituted cinnamic acids with the aryl ring and the double bond have been synthesized. According to the results of *in vitro* assays, most of these

synthesized acids with different substitution patterns dedicated a significant anticancer activity. [7] The effects on tuberculosis of cinnamic acid derivatives as drug candidates are studied. Modification of cinnamoyl moiety to isoniazid did not notably affect the trend of biological activity or the mode of action. These observations represent that the anti-TB activity depends not only on the α,β-unsaturation but also on the functionalization of the carbonyl part of the cinnamoyl derivatives. [8] The substituted 4-propoxycinnamic acid residues also show an efficiency of antimalarial activity. [9] The antifungal activity has been described in previous paper that some of cinnamic, *p*-coumaric and ferulic acid derivatives have a strong ability of antifungal against *Pythium* spp. and *Corticium rolfsii.* [10], [11] Esters, amides and substituted derivatives of cinnamic acid were synthesized and the antimicrobial activity was determined. Many of the tested cinnamic acid derivatives showed *in vitro* antimicrobial activity against Gram positive *Staphylococcus aureus, Bacillus subtilis*, Gram negative *Escherichia coli* and also showed an antifungal activity against fungi *Candida albicans* and *Aspergillus niger.* [12]

There are several methods for the synthesis of cinnamic acid ester derivatives, for example, cinnamic acid could be reacted directly with an alcohol and small amount of sulphuric acid as shown in **Scheme 2**(i). In addition, cinnamic acid ester derivatives could be synthesized *via* the reaction of cinnamoyl chloride or cinnamoyl anhydride and alcohol as shown in **Scheme 2**(ii, iii).

Scheme 2 Cinnamic acid derivatives synthesis

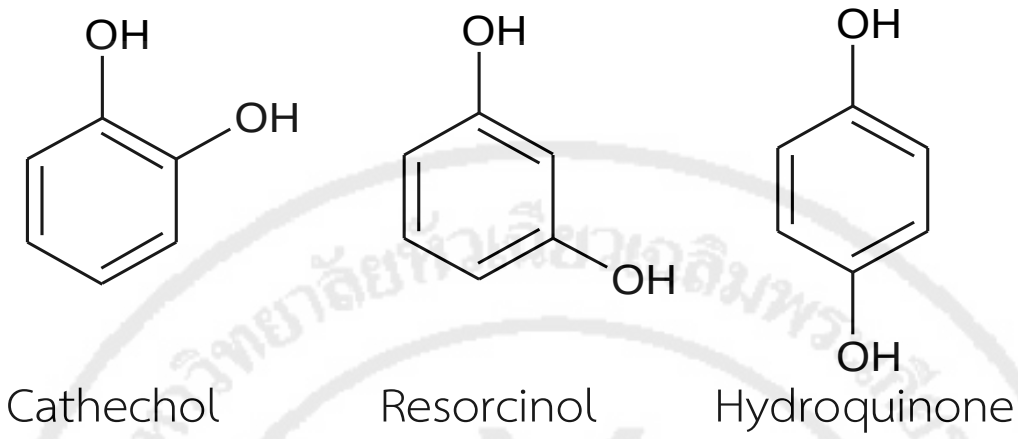


2.2 Benzenediols

Benzenediols are interesting phenolic compounds. Phenols are a class of chemical compounds, consisting of a hydroxyl group bonded directly to an aromatic hydrocarbon group. Although their structures are similar to alcohol, the properties of phenols are unique and different compared to alcohol. Two hydroxyls group on the aromatic ring are weakly acidic. Moreover, a proton from one of the hydroxyls can be deprotonated to form a phenolate ion.

There are three isomers of benzenediol: catechol, resorcinol and hydroquinone. All three isomers have the same chemical formula $C_6H_6O_2$. Two hydroxyl groups attached to a benzene ring in ortho, meta and para position, respectively as shown in **Figure 2**. The chemical formula of benzenediol is $C_6H_6O_2$ and its molecular weight is 110.1106 g/mol. The appearances of the three benzenediols are odorless white crystalline solid that could be darkening after exposure to air or the light. They can be both chemically synthesized and occurred naturally in some plants or animals.

Figure 2 Three isomers of benzenediols



1) Hydroquinone

In cosmetic term, hydroquinone is one of well-known whitening agent that exhibits a strong antioxidant activity. Hydroquinone is an odorless white crystalline solid with two hydroxyl groups bonded to a benzene ring in para position. The melting point of hydroquinone is 172 ºC and the boiling point is 287 ºC. It is very soluble in some polar solvents such as ethanol, methanol, and water, but insoluble in hexane. Hydroquinone was first found as a natural product in non-volatile extracts of coffee beans. [13] Hydroquinone was also found in red wine, wheat cereal and broccoli. Hydroquinone can be originated from many processes in nature. There are reports about the naturally occurring of hydroquinone, such as a direct hydroxylation of phenol to form hydroquinone by cytochrome P-450-enriched extracts of *Streptomyces griseus*, [14] the biodegradation of substituted phenols to form hydroquinone. [15], [16] Hydrolytic p-hydroxylation initiates the formation of substituted hydroquinones from the degradation of many polychlorinated phenolic compounds by *Rhodococcus chlorophenolicus.* [17]

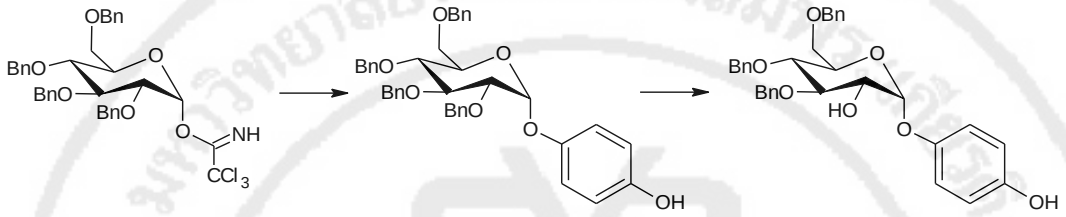
Moreover, hydroquinone can be synthesized and manufactured industrially by several processes. In 1820 hydroquinone was first synthesized by the French chemists Pelletier and Caventou. It was synthesized by pyrolysis of quinic acid and its structure was confirmed in 1844. [18] The most widely used route to produce

hydroquinone is hydroperoxidation of 1,4-diisopropylbenzene. Benzene (or recycled cumene) is alkylated with propylene in the presence of a ZSM-12 catalyst to yield intermediate product: 1,4-diisopropylbenzene. The 1,4-diisopropylbenzene product is then purified and oxidized to a dihydroperoxide. Sulfuric acid is finally added to split the intermediate into hydroquinone and acetone.[19] Hydroquinone can also be manufactured by hydroxylation of phenol with hydrogen peroxide. Hydrogen peroxide is used as a hydroxylation agent. Phenol has been oxidized with aqueous hydrogen peroxide in the presence of strong mineral acids or ferrous or cobaltous salts as catalysts. In the aniline oxidation process, aniline is oxidized by manganese dioxide and sulfuric acid to formed 1,4-benzoquinone. The 1,4-benzoquinone product is reduced by an aqueous solution of iron or by catalytic hydrogenation to hydroquinone. Furthermore, hydroquinone can be synthesized by Reppe's synthesis. Hydroquinone is formed by carbonylation of acetylene and iron pentacarbonyl as a catalyst in the presence of free carbon monoxide gas under pressure. Finally, hydroquinone is obtained from the oxidizing a phenol having a 1-cycloalkenyl or α-methylene-(alkyl or aralkyl) group in the paraposition. The oxidation is carried out using 30% aqueous hydrogen peroxide in an inert solvent. A strong acid which is not oxidized by the peroxide is used as a catalyst to formed p-isopropenylphenol. Then p-isopropenylphenol is degraded by alkaline hydrolysis to give a mixture of hydroquinone, phenol, and acetone.

Furthermore, it can also be found in a variety of forms in many plants and animals. Hydroquinone is used to be a very popular ingredient in skin lightening products because of the strong oxidation ability. Hydroquinone works by inhibiting the activity of tyrosinase, the necessary enzyme that converts tyrosine to DOPA. Thus, an important step in the melanin biosynthesis is blocked. There were some evidences of carcinogenicity of hydroquinone for female F344/N rats and female B6C3F1 mice [20] with an absence of any human counterpart. [21] Because of its carcinogenicity, hydroquinone was banned in Europe. Several hydroquinone structural modifications have been developed. Many substituted hydroquinone compounds were synthesized and tested. Arbutin, a glucoside derivative of hydroquinone is found in the leaves of blueberry, cowberry cranberry and bearberry

plants.[22] α-arbutin is a natural hydroquinone glycoside possessing the strong antioxidant and tyrosinase inhibition activities with less toxic than hydroquinone. In 2006, α-arbutin was successfully prepared by a direct acid-catalyzed glycosylation technique as shown in **Scheme 3**.

Scheme 3 α-arbutin synthesis



2) Resorcinol

Resorcinol is a meta-isomer of benzenediol with the formula $C_6H_6O_2$. It is a white crystal that found naturally in argan oil. It is colorless needles-shaped crystals that can be turned to pink after exposure to air, light, and iron. It is readily soluble in several polar solvent such as water, alcohol, and ether, but insoluble in chloroform and carbon disulfide. Resorcinol is topical used 5 to 10% in ointments as the antiseptic and disinfectant to breaking down rough or hardened skin. It is able to treat the pain itching, skin irritation such as poison, sunburn or skin disorder such as eczema, psoriasis, hidradenitis suppurativa, acne or calluses. On the other hand, in large doses, it is a poison, causing giddiness, deafness, salivation, sweating, and convulsions. Furthermore, in chemical synthesis, resorcinol is known as a chemical intermediate for the synthesis of many organic compounds especially pharmaceuticals. It is also used in the diazo dyes and plasticizers production.

Resorcinol is commercially used in some cosmetics. It is used in many hair products such as dyes, an anti-dandruff agent in shampoos, hair lotions and used in sunscreen product as a UV absorber. A developing use of resorcinol is as a template molecule in supramolecular chemistry. In addition, Resorcinol is also used in Seliwanoff's test as an analytical reagent of the qualitative determination of ketoses. Resorcinol is the starting material for resorcinarene molecules synthesis by condensing of resorcinol and an aldehyde and is the initiating explosive lead

styphnate.[23] Resorcinol can be used as the starting material in the aerogel production. The preparation is carried out by emulsion polymerization of resorcinol and formaldehyde in a slightly basic aqueous solution, followed by supercritical drying with CO_2.

3) Catechol

Catechol or pyrocatechol, a para-isomer of benzenediol with the formula $C_6H_6O_2$ which is feathery white to brown crystal that is very rapidly soluble in water. Catechol was first isolated in 1839 by Edgar Hugo Emil Reinsch (1809 - 1884) by destructive distillation from the solid tannic preparation catechin. Catechin is the residue of catechu that is boiled or concentrated juice from Mimosa catechu (*Acacia catechu* L.f).[24] Catechol can be produced industrially by the hydroxylation of phenol using hydrogen peroxide. Furthermore, catechol occurs naturally in fruits and vegetables with small amount. It is one of the main natural products in argan oil.[25] Catechol is found in *Agaricus bisporus.*[26] It is also a component of castoreum, a chemical compound from the castor sacs of the mature North American Beaver (*Castor canadensis*) and the European Beaver (*Castor fiber*), used as a tincture in some perfumery. Catechol is mainly obtained by chemical synthesis. It is used widely in the production of pesticides and a precursor to fine chemicals such as perfumes and pharmaceuticals.[27]

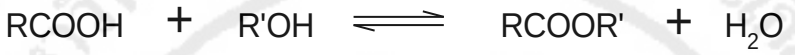
Catechol is used as a black-and-white photographic developer, a developer for fur dyes, as an intermediate for antioxidants in rubber and lubricating oil. It is also used as the precursor of permanent hair dyes, colors, and tints production. Skin contact with catechol causes eczematous dermatitis in humans. Large doses of catechol can cause depression of the central nervous system (CNS) and a prolonged rise of blood pressure in animals. It is not clear whether these health effects were observed following acute (short-term) or chronic (long-term) exposure.

2.3 Chemical synthesis of ester derivatives of cinnamic acid

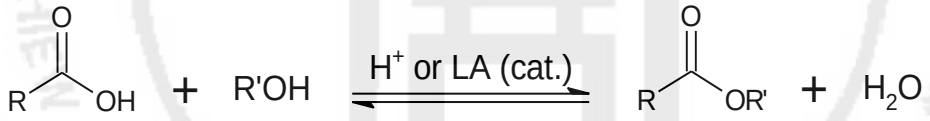
Esterification is the reaction the reaction between two reactants (typically an alcohol and a carboxylic acid) to yield an ester. The most common methods are described as following:

2.3.1 Reaction of acids and alcohols

The alcohol moiety without hydroxyl group is combined together with the dehydrogenated carboxylic acid.

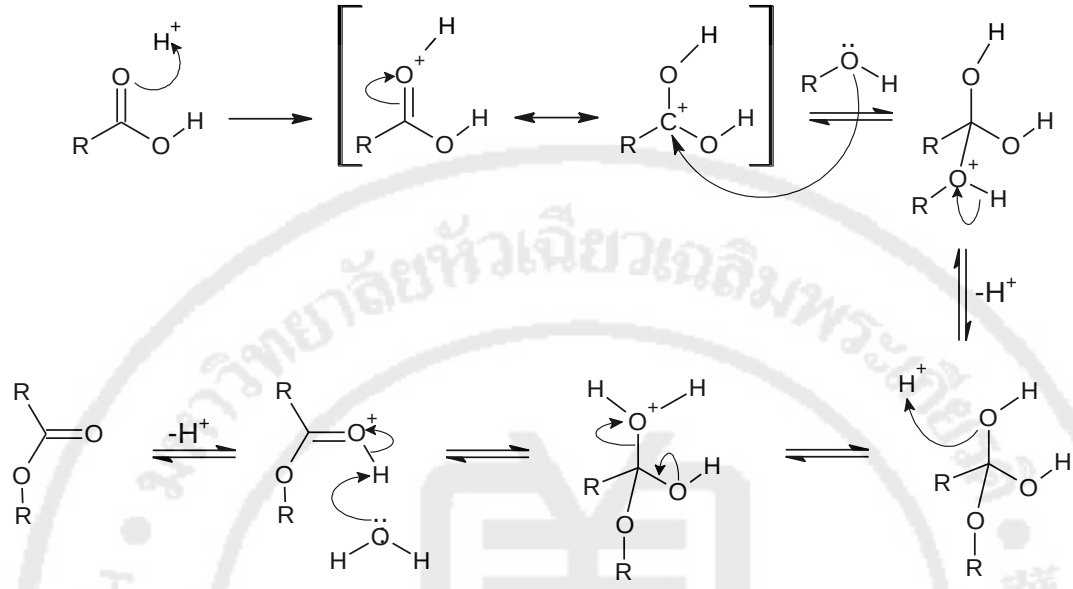


The most commonly reaction is Fischer esterification, which derived from refluxing a carboxylic acid, containing the -COOH group and an alcohol in the presence of an acid catalyst, typically sulfuric acid.



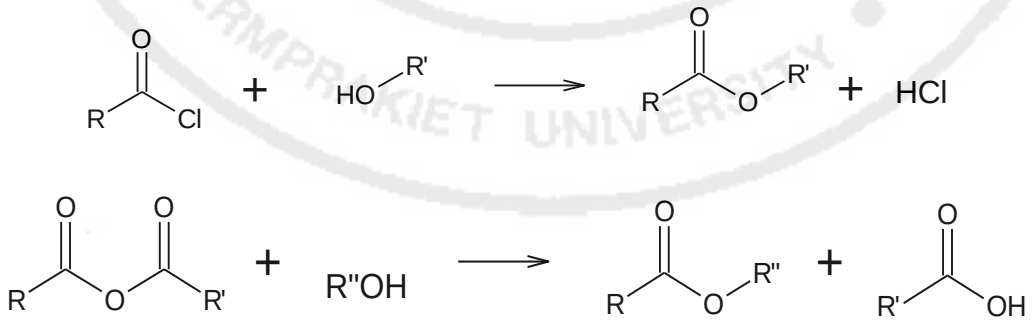
As seen in the **Figure 3**, mechanism of the Fischer Esterification starts by protonation of the carbonyl group in carboxylic acid by *p*-TsOH, H_2SO_4 or a Lewis acid leads to a more reactive electrophile. Then, nucleophile attacks on the activated carbonyl group by 1,2-addition reaction of the alcohol, giving an intermediate with two equivalent hydroxyl groups. After that, a proton is migrated and it leads to be eliminated as water after a proton shift (tautomerism). Finally the molecule is deprotonated to give the ester as a product. [28]

Figure 3 Mechanism of the Fischer Esterification



2.3.2 Reaction of acid chlorides or anhydrides with alcohols

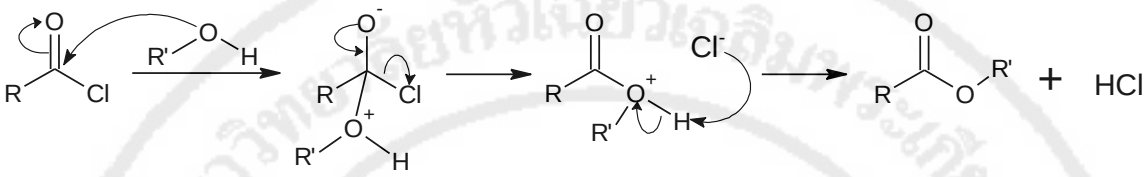
Reaction of acid/acyl chlorides with alcohols can also be called a nucleophilic addition elimination because of their two major stages of the mechanism, an addition and then followed by elimination.



Mechanism of the esterification between acid chlorides or anhydrides with alcohols involves several rearrangements as shown in the **Figure 4**. The nucleophilic alcohol molecule first attacks carbonyl carbon of the acid chloride molecule to generate a reactive intermediate. The chloride ion is eliminated from

the intermediate molecule and then removes a proton from the oxonium ion to provide the ester product. [29), (30), (31)]

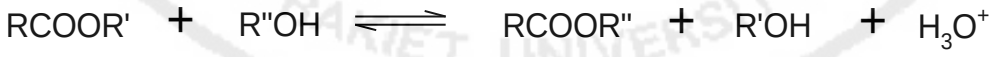
Figure 4 Mechanism of the esterification between acid chlorides with alcohols



To prevent the side reaction, anhydrous condition must be used because acid/ acyl chloride is also reactive with water. Furthermore, some of acyl chloride need to reacts with cold alcohol because of the hydrogen chloride acidic fume generation while the exothermic reaction is occurred. On the other hand, reaction between acid anhydride and alcohol is required to warm during the reaction to speed up the reaction.

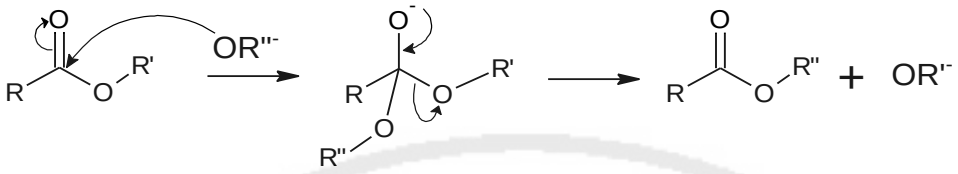
2.3.3 Transesterification (Ester Interchange)

Transesterification, is the reaction changing R' of one ester into another R" group of an alcohol as the equation given below:



Transesterification is catalyzed by acids and bases so there are two mechanisms of the reaction. The mechanism under basic condition starts by a proton removing of the alcohol by a strong base, making that alcohol molecule more nucleophilic (**Figure 5**).

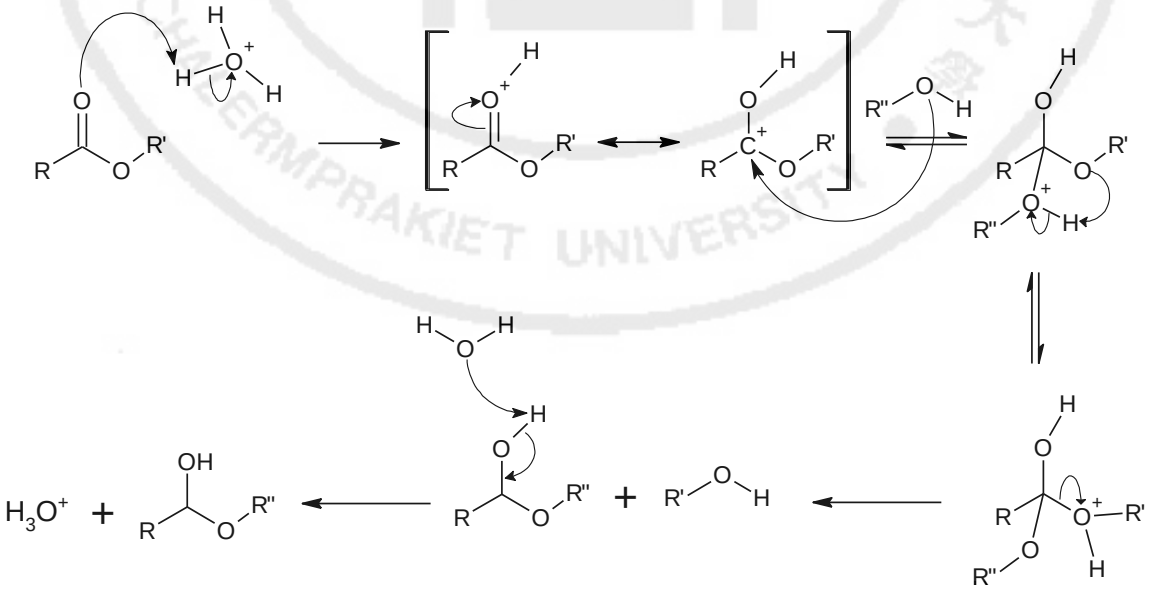
Figure 5 Mechanism of the transesterification in basic condition



The alkoxide ion attacks carbonyl carbon of the starting ester to give a tetrahedral intermediate. The lone pair electron shift to make a C=O bond. Finally, the OR' group is eliminated as a leaving group and the new ester product is completely formed.[32]

On the other hand, in the transesterification mechanism under acidic condition, strong acid donates proton to the carbonyl group of ester to initialize the reaction (**Figure 6**).

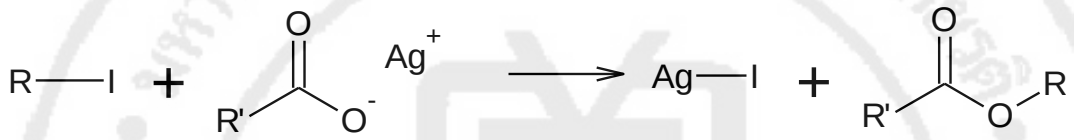
Figure 6 Mechanism of the transesterification in acidic condition



The higher electrophilicity carbon is attacked by the alcohol and gives a tetrahedral intermediate. Then, the proton transfers from the OR'' group to the OR' group. The OR' group is removed as a leaving group. Final step is deprotonation of the molecule and the new ester product is formed.

2.3.4 Reaction of alkyl halide and the salt of and acid

One of the preparations of ester that not widely used is the reaction between alkyl halide and the salt of and acid.



Carboxylate salt anion is generated in situ in the reaction as the strong nucleophile. Alkyl halide is attacked to afford the corresponding esters product. The mechanism can be substitution or elimination reactions depending on the type of alkyl halide (primary, secondary, and tertiary) and the basicity of nucleophile molecules.[33), (34), (35)]

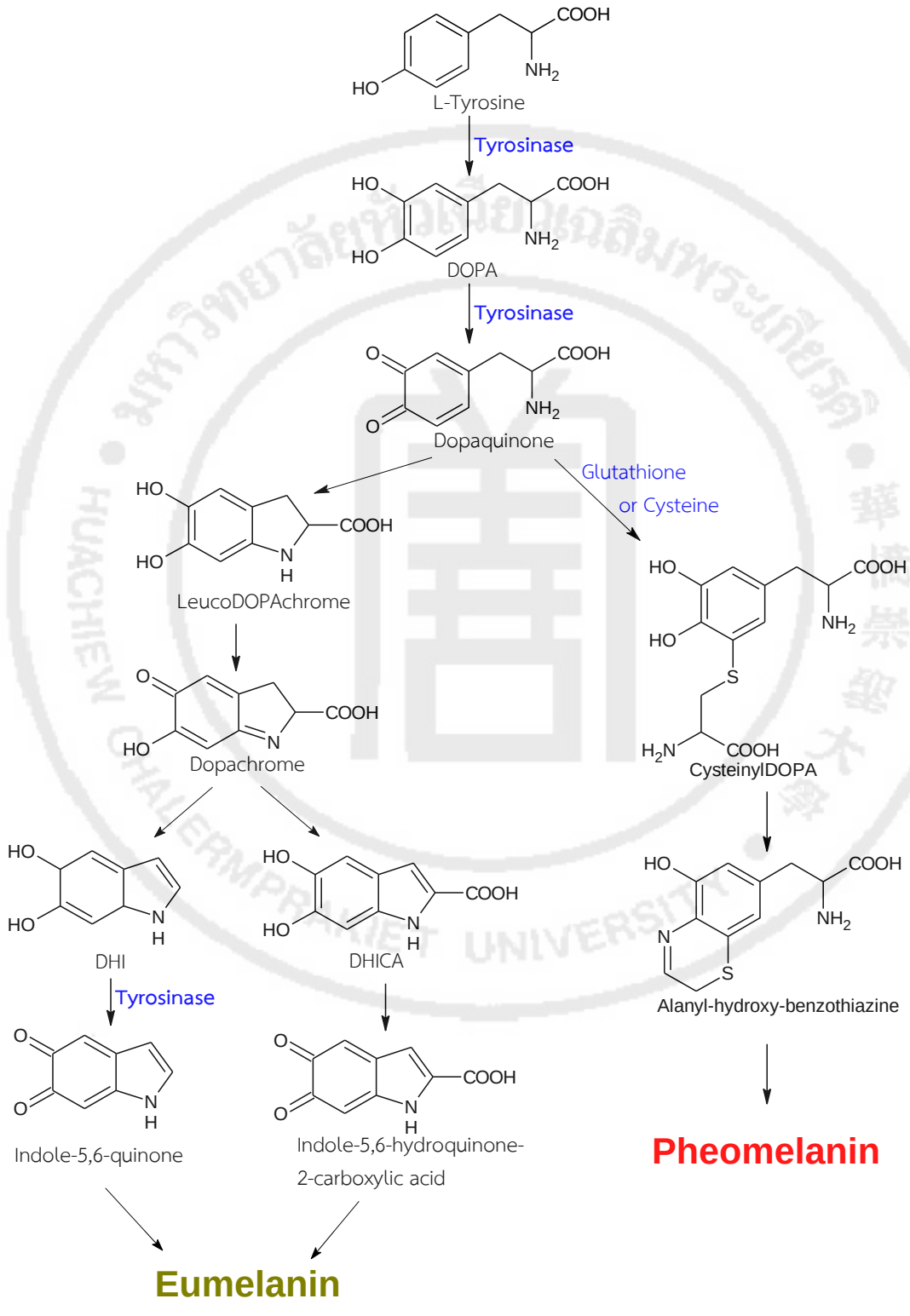
2.3.5 Reaction of an alcohol with amide

This reaction is reversible; it is carried to completion by the use of an acid to take up the ammonia.

2.4 Melanin biosynthesis

Melanin is a pigment derived from an amino acid tyrosine, produced within the skin and mainly regulated by the tyrosinase enzyme. It is occurred inside melanosome. Tyrosianse is an important rate-limiting enzyme, which catalyzes the mammalian melanogenesis, the melanin production as shown in **Scheme 4**. Melanin biosynthesis can be initiated from the hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) in the presence of tyrosinase enzyme. The next step, L-DOPA is oxidized to dopaquinone by tyrosinase again. These two steps are the common step to both pheomelanins and eumelanins production. Dopachrome can be spontaneously converted to 5,6-dihydroxyindole or enzymatically converted to 5,6-dihydroxyindole-2-carboxylic acid via enzymatic conversion by tyrosine-related protein-2 (TRP-2). Finally, the polymerization of 5,6-dihydroxyindole via tyrosine-related protein-1 (TRP-1) and the oxidation of acid leads to form eumelanin. On the other hands, pheomelanogenesis can be formed on the presence of cysteine or glutathione. Dopaquinone can combine with cysteine (or glutathione) to yield cysteinyldopa (or glutathionyldopa). The latter is then converted to benzothazine intermediate that undergo polymerization to form pheomelanin.[36], [37]

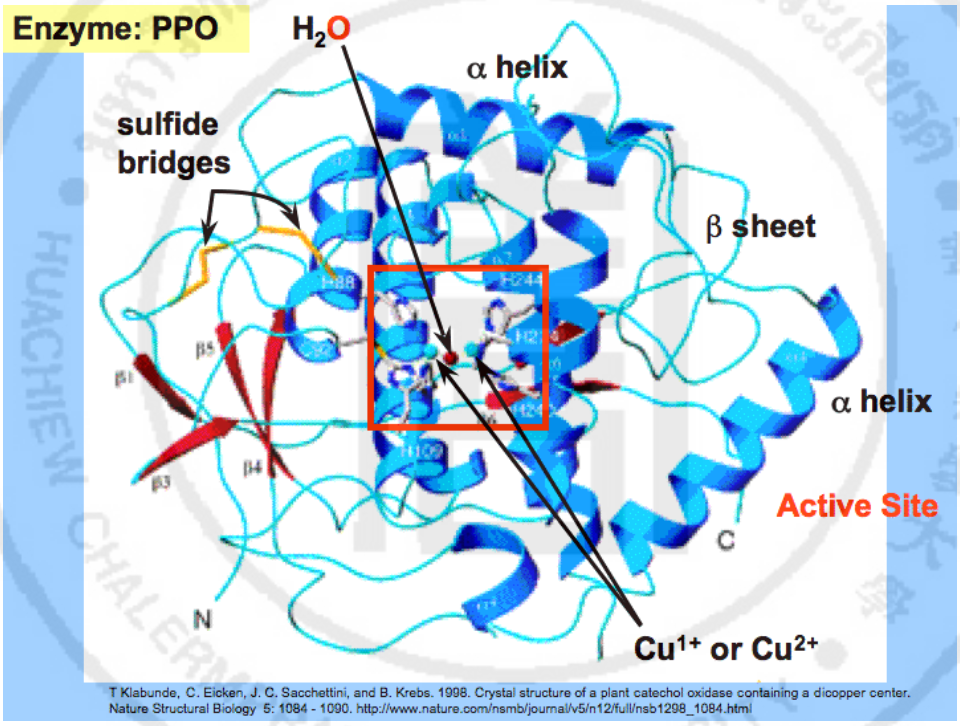
Scheme 4 Melanin synthesis pathway



2.4.1 Tyrosinase enzyme

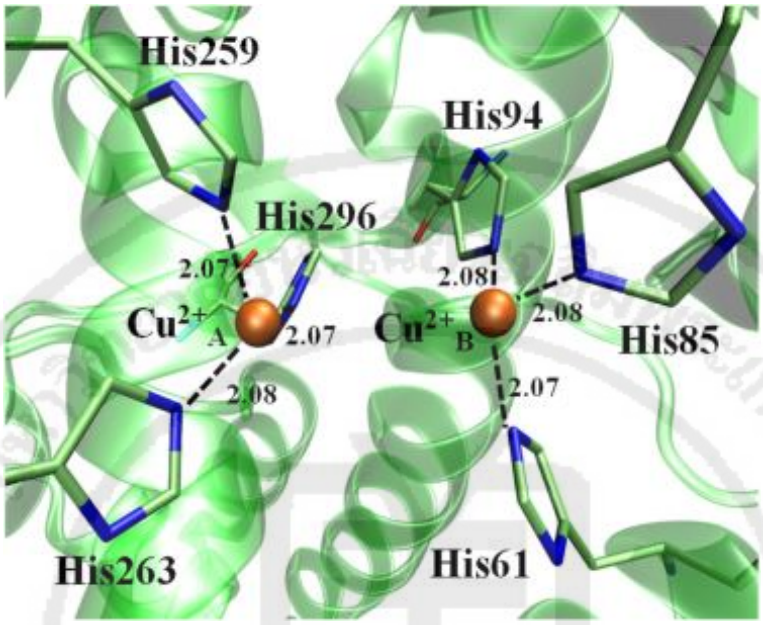
Tyrosinase also known as polyphenol oxidase (PPO), is a copper-containing oxidase as shown in **Figure 7**, which is the rate-limiting enzyme involved in the melanogenesis.

Figure 7 Crystal structure of tyrosinase enzyme [38]



This enzyme is reported to have two binding sites for aromatic substrates and a different binding site for oxygen-copper. [39] The activity of tyrosinase is same as catechol oxidase, a related class of copper oxidase that called polyphenol oxidases. Polyphenol oxidase is a family of dinuclear copper center metalloenzymes that include tyrosinase and catechol oxidase. The two copper atoms active site of tyrosinase enzymes as shown in **Figure 8** are binding with dioxygen to form a highly chemically reactive intermediate.

Figure 8 Active site of tyrosinase enzyme [40]



In plants, both tyrosinase and catechol oxidase can catalyze the oxidation of ortho-diphenols substrates into their corresponding ortho-quinones. The main difference between the tyrosinase and catechol oxidase is that tyrosinase is able to catalyze the hydroxylation of monophenols to diphenols (monophenolase activity) as well as the oxidation of the ortho-diphenol to the ortho-quinone (diphenolase activity) while catechol oxidase represents only diphenolase activity.

2.4.2 Tyrosinase inhibitor

Since tyrosinase is the rate-limiting enzyme that involved in the first two steps of the melanin biosynthesis. Many tyrosinase inhibitors from both natural and synthetic sources are studied and reported. Several tyrosinase inhibitor compounds have been developed and accepted as depigmenting agents or whitening agents in cosmetic products. Inhibitors may broadly be classified as irreversible and reversible.

The irreversible inhibitors attached to target enzymes with covalent bond permanently and then inactivated it. The inhibition cannot be reversed. There are some differences between the tyrosinase irreversible inhibitors and non-

specifically irreversible inactivators such as temperature, and pH which commonly denature all protein structure. The tyrosinase irreversible inhibitors do not inactivate all proteins. Instead, they are specific for the active site of tyrosinase. Moreover, the potential of inhibition ability cannot be measured by IC_{50} value because the irreversible inhibitors play a role in time dependent inhibition. The period of pre-incubation between enzyme and irreversible inhibitor is the important factor of measurement. For example, the antihypertensive drug captopril is the irreversible inactivator of the monophenolase and diphenolase activities of mushroom tyrosinase in a non-competitive and competitive manner, respectively. [41] Captopril only bound the enzyme at its active site by forming copper-captopril complex between cartopril and tyrosinase and disulphide interchange reactions between captopril and cysteine at the active site of the enzyme. [42] Hydrogen peroxide (H_2O_2) is another well-known inactivator of several copper-containing enzymes. It is able to inactivate mushroom tyrosinase in a biphasic manner, which the first phase rate is faster than the later one. The inactivation rate under anaerobic conditions (nitrogen) is also faster than under aerobic conditions (air). [43], [44]

The reversible inhibitors attached to or near the active site of an enzyme and inhibit its activity. Reversible inhibitors bind to the enzymes with non-covalent interactions such as hydrogen bonds, and ionic bonds. They also can be readily removed from the enzyme easily by dilution or dialysis. There are four other categories of reversible inhibitors, i.e., competitive inhibitors, uncompetitive inhibitors, non-competitive inhibitors, and mixed inhibitors.

1) Competitive inhibitors

When the substrate and inhibitor bind to the enzyme in the same way, they have to compete for the active site of the enzyme. This type of inhibitor is called a competitive inhibitor. The competitive inhibitors that bind the active site of the enzyme stronger than the substrate are an effective competitive inhibitor. Competitive inhibitors usually have similar structure to the substrate.

2) Uncompetitive inhibitors

Uncompetitive inhibitors are the inhibitor attached only to the substrate-enzyme intermediate complex at the site distinct from the active site of the enzyme and inhibit the production velocity.

3) Non-competitive inhibitors

Non-competitive inhibitors are the inhibitors that reduce the activity of enzyme by binding at the allosteric sites of enzyme. The substrate and inhibitor can bind the enzyme at the same time. Both the enzyme-inhibitor complex and enzyme-substrate-inhibitor complex are formed equally well but the enzyme-substrate-inhibitor complex cannot form product. The inhibitor binding effect in the complex inhibits the reaction. Then the complex is able to convert to the enzyme-inhibitor complex or eliminate the inhibitor so the reaction can proceed and products chemical reactions may be formed spontaneously.

4) Mixed inhibitors

Mixed inhibitors are the type of inhibitor that mixes between competitive inhibitors and uncompetitive inhibitors. Mixed inhibitors are able to bind both to the free enzyme and to the enzyme-substrate complex. The equilibrium binding constants of the free enzyme and the enzyme-substrate complex are different.

2.5 Free radicals and antioxidants

Free radicals are any atoms, ions or molecules that have unpaired electron. Triphenylmethyl radical was the first organic free radical discovered by Moses Gomberg at the University of Michigan, USA in 1900. They have positive, negative or neutral charge radicals. They are very unstable and highly chemically reactive with other compounds, trying to attack other compounds to gain more stability. Free radicals initiate chain reaction easily. Some free radicals arise normally during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion. Sometimes the body's immune system's cells purposefully create them to attack and kill pathogens. During times of environmental

stress such as exposure of heat, X-rays, ozone, pollutants, cigarette smoke and industrial chemicals can also create free radicals.

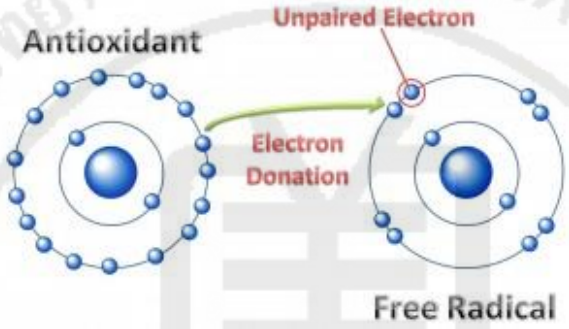
Free radicals react with important macromolecules leading to cell damage and can cause disruption of homeostasis. Furthermore, free radicals can damage the skin leading to inflammation, uneven skin tone and signs of premature skin aging. Once a reactive free radical is generated, it can start a chain reaction. Generally, free radicals attack the nearest stable molecule, steal an electron from that molecule and have now made a new free radical instead. The attacked molecule loses its electron and then becomes a free radical itself, beginning a chain of oxidative reactions. The new free radical goes on to generate yet more free radicals, and so on, like dominoes. Once the process is started, it continues until resulting in the tissue injury of a living cell such as DNA, or the cell membrane. A tremendous chain reaction of free radicals can quickly encounters disaster on all components of the cell. Cells may function abnormally or die if this occurs. Among all cellular components, nucleic acids, proteins, and lipids are the major targets. Oxidative stress, occurring as a result of an imbalance between production and elimination of free radical in the body and results in direct or indirect damage of all kinds of molecular species and has been implicated in carcinogenesis.

Initial damage is mostly indirect and caused by reactive oxygen species (ROS) generated. Reactive Oxygen Species (ROS) such as hydroxyl radical, hydrogen peroxide, superoxide anion radical, oxygen singlet and peroxynitrite radical, are highly reactive oxygen-containing free radicals molecules. ROS are formed as a natural by-product during mitochondrial oxidative phosphorylation and their biological metabolites also play an important role in carcinogenesis. ROS induce DNA damage, as the reaction of free radicals with DNA includes strand break base modification and DNA protein cross-links. Normally, the body can handle free radicals, but if antioxidants are unavailable, or if the free-radical production becomes excessive, damage can occur.

Antioxidants are molecules that inhibit the oxidation of other molecules. They are molecules which can safely interact and neutralize free radicals and terminate the chain reaction as shown in **Figure 9**. Antioxidants relieve oxidative

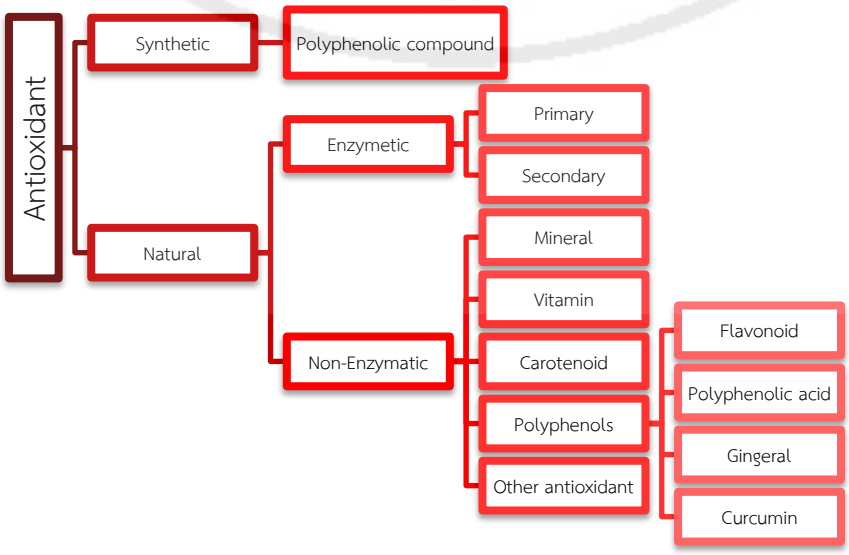
stress induced carcinogenesis with a direct scavenging of ROS by attacking free radicals and stop the chain reaction before molecules are damaged. The antioxidant won't become free radicals after donating an electron to other molecule. They are stable though losing their electrons.

Figure 9 Antioxidant electron donation [45]



Antioxidants have been traditionally classified into two major types based on their source, i.e., natural antioxidants and synthetic antioxidants. Schematic representation of the classification of antioxidants is shown in **figure 10**.

Figure 10 the classification of antioxidants



2.5.1 Natural antioxidants

Natural antioxidants are antioxidants that naturally synthesized in the human body through metabolic process. In addition, they can be found naturally in other sources. Natural antioxidants can be further divided into two categories, i.e., enzymatic antioxidants and non-enzymatic antioxidants.

Enzymatic antioxidants are enzymes that terminate the cellular damage caused by oxidation of free radicals. They can be subdivided into primary antioxidant (superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH)) and secondary antioxidant (glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH)).

Non-enzymatic antioxidants work by interrupting free radical chain reactions. They play an important role in helping the enzymatic antioxidant by neutralization of oxidative stress. Non-enzymatic antioxidants include minerals, polyphenols, vitamins, carotenoids, and other antioxidants such as albumin, ceruloplasmin, hepatoglobin, uric acids, ubiquinol and Coenzyme Q.

2.5.2 Synthetic antioxidants

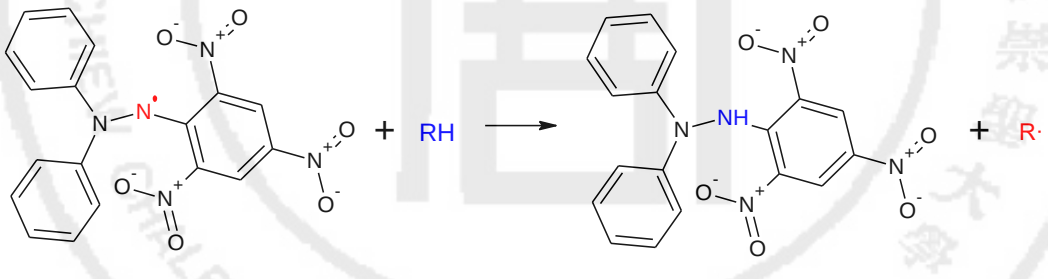
Synthetic antioxidants are polyphenolic compounds that chemically synthesized. They have been mainly used as standard antioxidants in free radical scavenging activity measurement method to compare with natural antioxidants and to be the ingredients into food production manufacturing. Synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), ethylenediaminetetraacetic acid (EDTA), tertiary butylhydroquinone (TBHQ), 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline (ethoxyquin), and propyl gallate (PG). Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are the most widely used synthetic antioxidants.

A huge number of antioxidants are extracted from natural and produced by chemical synthesis. Antioxidant protects the human body from some types of cell damage that can cause many diseases such as cancer, atherosclerosis, and rheumatoid arthritis. Adding an antioxidant to a skin whitening formulation can efficiently decrease the melanin production.

2.5.3 Antioxidant assay

There are various methods for measuring antioxidant activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging is the well-known *in vitro* antioxidant assay [46] because of the simplicity and the short-time analysis. The DPPH radical has a deep violet color in solution, and it becomes colorless or pale yellow when neutralized. Furthermore, the DPPH radical absorbs at 519 nm. This appearance is useful for evaluation the radical scavenging activity by absorbance observation using UV-VIS spectrophotometer. [47], [48] The reaction of DPPH reaction with antioxidant is shown in **Figure 11**.

Figure 11 Reaction of antioxidant with DPPH



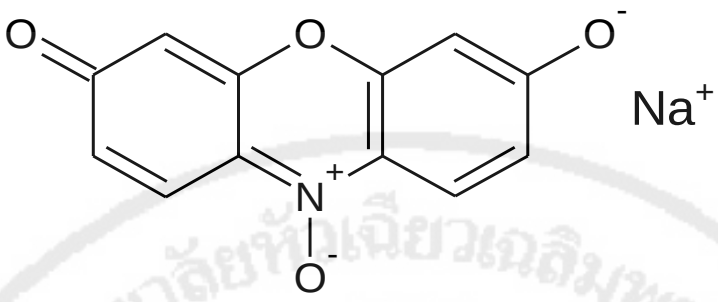
DPPH radical
purple, 519nm

DPPH-H
pale yellow or colorless

2.6 Cytotoxicity

Cytotoxicity refers to the ability of chemicals being toxic to living cells. Cell viability and cytotoxicity assays are the screening test measured the potential toxic effects of the test compound to the living cells. Resazurin Microtiter Assay (REMA) is an *in vitro* fluorometric assay. [49] It is a one of a simple, rapid, accurate and inexpensive colorimetric assay for determining the cell cytotoxicity. [50], [51], [52] Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue non-fluorescent dye that can be reduced to the highly red-fluorescent product resorufin with a 540-550 nm fluorescence excitation wavelengths and 585-595 nm of emission wavelengths. The chemical structure of resazurin sodium is shown in **Figure 12**.

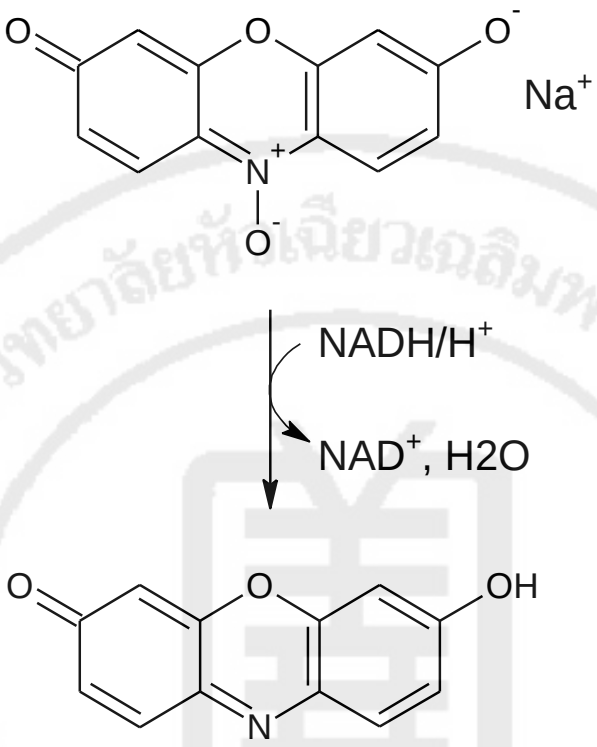
Figure 12 Chemical structure of resazurin



The non-fluorescent resazurin can be used as an oxidation-reduction indicator to monitor and determine the cell viability assays for bacteria, yeast or mammalian cells cultures. [53] Resazurin sodium is soluble in water, aqueous buffers and in organic solvents (e.g. DMSO, DMF), resulting in a deep blue colored solution. It is permeable through cell membranes and non-toxic for the living cell.

The oxidized, non-fluorescent, blue state (resazurin) can be reduced by some enzymes, coenzymes and cofactors in live cell with active metabolism as shown in **Figure 13** and then generates the reduced, fluorescent, pink state (resorufin). Hence, it utilizes for assessing cell cytotoxicity and cell viability assays both aerobic [54] and anaerobic respiration. [55] The number of resorufin produced is relative to the number of viable cells. The changing from resazurin to resorufin can be quantitative measured by colorimetric and fluorometric readings but fluorescence detection is more popular because it is more sensitive and effective than measuring absorbance.

Figure 13 Reduction of resazurin to resorufin in living cells



The advantages of the resazurin reduction assay are much cheaper, easier and safer than the other assays (e.g. formazan-based assays and tritiated thymidine based techniques). It also allows for other measurement such as continuous cell growth monitoring, kinetic studies and can be multiplexed with other methods such as caspase assays and cytokine assays.

CHAPTER 3
METHODOLOGY

3.1 Materials and Equipment

Chemicals

1. Hydroquinone, resorcinol, catechol
2. Cinnamic acid
3. Oxalyl chloride
4. Dimethylformamide (DMF)
5. Sodium bicarbonate ($NaHCO_3$)
6. Sodium carbonate (Na_2CO_3)
7. Deionized water
8. Tetrahyofuran (THF)
9. Dichloromethane (CH_2Cl_2)
10. Hexane
11. Ethyl acetate
12. Silica gel 60 (0.040 – 0.063 mm)
13. Chloroform-*d*
14. 1,1-diphenyl-2-picrylhydrazyl (DPPH)
15. Ethanol
16. Potassium phosphate buffer, pH 6.5
17. L-Tyrosine
18. Tyrosinase enzyme

Equipment

1. Round-bottomed flask and stopper
2. Thin layer chromatography kit
3. Separatory funnel
4. Hot plate and stirrer
5. Rotary evaporator
6. Column

7. Fourier transform Infrared Spectrophotometer (FT-IR) (Spectrum 100; Perkin Elmer, USA)
8. Nuclear Magnetic Resonance (NMR) (Advance Ultrashield spectrometers 400 MHz; Bruker, Germany)
9. Mass spectrometer (MS) (LCQ Advantage; Thermo Finnigan, Germany)
10. Volumetric flask
11. Micropipette
12. 96 well plate
13. Microplate reader

3.2 Method

1) Synthesis of cinnamic acid ester derivatives

1.1) Synthesis method

Cinnamic acid (50 mmol, 7.408 g) was dissolved in CH_2Cl_2 (10 mL) in round-bottomed flask, and stirred gently at room temperature. Oxalyl Chloride (3 mL) were slowly added and a drop of DMF was then added. The reaction flask was stoppered with a glass stopper or a cork (not a rubber stopper) to prevent the solvent from evaporating and then allowed to stir in room temperature for an hour. A piece of blue litmus paper was held over the vapor in the flask to check if the HCl gas was presented in the reaction. Small amount of $NaHCO_3$ was added until a blue litmus paper didn't change to red anymore. Then benzenediol (50 mmol, 5.5055 g) in THF (5 mL) and CH_2Cl_2 (15 mL) were added. The reaction was checked by TLC technique using ethyl acetate (30%): hexane (70%) as the mobile phase system.

The reaction mixture was extracted using separatory funnel. The solution was poured in reaction flask to a separatory funnel and of 10% $NaHCO_3$ (50 mL) solution was added. The separatory funnel was stoppered, shook gently and then vented to release the pressure in the funnel. The separatory funnel was shook and vented until no more gas release. The organic layer was collected and the solvent was distilled by the rotary evaporator to give the pale yellow crude product.

1.2) Purification

Crude product was purified by column chromatography using silica gel 60 (0.040 – 0.063 mm) as the stationary phase and ethyl acetate (10%): hexane (90%) as the mobile phase system. Silica gel was mixed with hexane, made into slurry and poured gently into the column. The silica gel was allowed to set and gently tap the column thus, the stationary phase would be completely packed into the column. Sample was loaded into column by dry technique. The sample was dissolved in ethyl acetate in round-bottomed flask. Dry silica was added to the dissolved sample and stirred until the silica completely absorbs the sample. The solvent was evaporated using rotary evaporator until the silica gel completely dry. All eluated fractions were collected and then dried by evaporation using rotary evaporator. The fractions were determined by TLC technique using ethyl acetate (30%): hexane (70%) as the mobile phase system.

1.3) Chemical structure elucidation

ATR- FTIR Spectroscopy

The sample was prepared by molding the sample using mortar and pestle. The grinded sample was placed on the sample holder and pressed against an ATR crystal. The IR spectrum of each compound was measured.

NMR Spectroscopy

The sample was prepared by dissolving sample in chloroform-d and transferred in NMR tube. The ^{1}H and ^{13}C-NMR spectra of each compound were measured.

Mass Spectroscopy

The purified product fractions from column chromatography were elucidated by mass spectrometry. The sample preparation was started by removing the moisture content by storing in the desiccator equipped with the vacuum pump. The samples were then filtered using 0.22 µm syringe filter. Each sample was injected into the mass spectrometer and the spectrum was recorded at Electron Impact (EI) mode at 70 eV. Rate of fragmentation was 0.2 second/scan and the range of mass was from 30 to 1000 m/z.

2) Bioactivity Test

2.1) Antioxidant capacity assay

The radical-scavenging activity of cinnamic acid derivatives were measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by DPPH assay, as previously reported, with minor modifications (Alam, 2013). [46] The solutions of test compounds were prepared at various concentrations. Hydroquione (50 µg/ml), resorcinol (100 mg/ml), pyrocatechol (100 µg/ml), 5a derivative (250 mg/ml), 5b derivative (250 mg/ml), and 5c derivative (500 µg/ml) were each prepared in DMSO. DPPH solution (1mM) in methanol was prepared by dissolving DPPH (0.0039 g) in methanol and dilute to exactly 10 ml in volumetric flask. The test solution consisted of adding DPPH (1mM, 50 µl) in methanol and samples (10-120 µl). Total volume of solution was adjusted to 270 µl with DMSO in the 96-well microplate (Thermo Fisher Scientific) as shown in

Table 1.

Then test solution was kept in the dark for 30 minutes and the absorbance was measured at 517 nm against a blank. DMSO was served as blank and DPPH in methanol with DMSO in an absence of any samples was served as positive control. Hydroquinone was used as the standard to compare. The percentage of the DPPH radical scavenging was calculated using the equation as given below:

$$\%Inhibition = \frac{(Abs._{Control} - Abs._{Sample})}{Abs._{Control}} \times 100$$

Table 1 Preparation of sample solution in DPPH assay

No	DPPH (µl)	Sample (µl)	DMSO (µl)	Total (µl)
1	50	10	210	270
2	50	20	200	270
3	50	30	190	270
4	50	40	180	270

Table 1 Preparation of sample solution in DPPH assay (continued)

No	DPPH (µl)	Sample (µl)	DMSO (µl)	Total (µl)
5	50	50	170	270
6	50	60	160	270
7	50	70	150	270
8	50	80	140	270
9	50	90	130	270
10	50	100	120	270
11	50	110	110	270
12	50	120	100	270

2.2) Tyrosinase Inhibition assay

Inhibitory effects of cinnamic acid derivatives on mushroom tyrosinase were determined by the method developed by Somvong [56], with minor modifications. In brief, the solution of cinnamic acid derivatives (500 µg/ml) in 10% DMSO in potassium phosphate buffer pH 6.8 solution (20 mM) was prepared. Tyrosinase enzyme solution was also prepared by dissolving 24,988 units of the lyophilized tyrosinase powder from mushroom in potassium phosphate buffer pH 6.8 solution (20 mM). Total volume of solution was adjusted to 1 ml to make tyrosinase enzyme stock solution. Pipette tyrosinase enzyme stock solution (400 µl) into a 10 ml volumetric flask. Then, dilute to exactly 10 ml with potassium phosphate buffer pH 6.8 solution (20 mM) to make tyrosinase enzyme solution (1,000 units/ml, 10 ml). Dissolve L-tyrosine (10 mg) in potassium phosphate buffer pH 6.8 solution (20 mM, 10 ml) to make a L-tyrosine solution (1 mg/ml).

Solutions of cinnamic acid derivatives at various volumes (500 µg/ml, 10 - 120 µl), and L-tyrosine solution (1 mg/ml, 50 µl) were mixed in 96-well plate and incubated at 37 °C for 10 minutes. Then tyrosinase enzyme solution (1,000 units/ml, 50 µl) was added to the tested 96-well plate and then total volume of solution were

adjusted to 260 µl with potassium phosphate buffer pH 6.8 solution (20 mM) as indicated in **Table 2**. Potassium phosphate buffer pH 6.8 solution (20 mM) was served as blank. The mixture between L-tyrosine solution (1 mg/ml) and tyrosinase enzyme solution (1,000 units/ml) in an absence of any samples was served as positive control. Kojic acid was used as the standard.

The test solution was incubated at 37 ºC for another 45 minutes. The absorbance was measured at 475 nm using UV/Vis spectrophotometer and then of %inhibition was calculated using the equation as given below:

$$\%Inhibition = \frac{(Abs._{Control} - Abs._{Sample})}{Abs._{Control}} \times 100$$

Table 2 Preparation of sample solution in tyrosinase assay

No	L-Tyrosine (µl)	Tyrosinase (µl)	Sample (µl)	Buffer (µl)	Total (µl)
1	50	50	10	150	260
2	50	50	20	140	260
3	50	50	30	130	260
4	50	50	40	120	260
5	50	50	50	110	260
6	50	50	60	100	260
7	50	50	70	90	260
8	50	50	80	80	260
9	50	50	90	70	260
10	50	50	100	60	260
11	50	50	110	50	260
12	50	50	120	40	260

2.3) Cytotoxicity assay

This assay was performed in 96-well plate in quadruplicate. First, plates were seeded with cell suspension (200 µl) or blank medium into well, and incubated at 37ºC humidified incubator with 5% CO_2 for 48 hours. Subsequently, culture medium was replaced with fresh medium containing test-compounds (200 µl) or 1% DMSO (200 µl), and plates were further incubated for 24 hours. After incubation period, the plates were added with 50 µl of 125 µg/ml resazurin solution and incubated at 37 ºC humidified incubator with 5% CO_2 for 4 hours. Fluorescence was measured at 530 nm excitation and 590 nm emission wavelengths by using the bottom-reading mode of fluorometer. The signal of test wells was subtracted with that of blank wells before calculations. Percent of cytotoxicity was calculated by the following equation:

$$\%Cytoxicity = [\,1 - (\,FU_T / FU_C\,)\,] \times 100$$

Whereas FU_T and FU_C were the mean fluorescent unit from cells treated with test compound and that treated with 1%DMSO, respectively.

The CC_{50} value was derived from dose-response-curve that was plotted between %cytotoxicity versus the sample concentrations by using SOFTMax Pro software (Molecular Devices, USA).

CHAPTER 4
RESULTS

Cinnamic acid and its derivatives are known ingredients used widely in cosmetics. They were reported to have a tyrosinase inhibitory and antioxidant effect. Benzenediol molecule especially hydroquinone also presents the antioxidant property. In this research, a series of cinnamic acid derivatives were synthesized bearing benzenediol moiety. Furthermore, the antioxidant, tyrosinase inhibitory property and cytotoxicity of the synthesized compounds were evaluated.

4.1 Synthesis of cinnamic acid ester derivatives

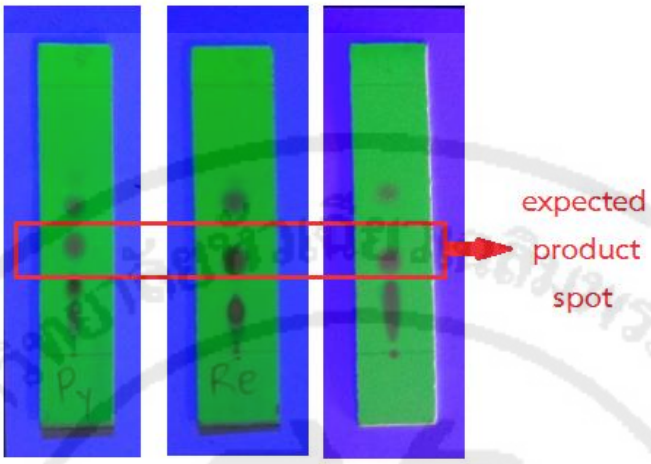
1) The syntheses of Cinnamic Derivatives

The syntheses of cinnamic acid derivatives were accomplished according to **Scheme 1**. The first step, cinnamic acid (1) was reacted with excess oxalyl chloride (2) to give cinnamoyl chloride (3). Then, the cinnamoyl chloride, without isolation was subsequently reacted with benzenediol; catechol (4a), resorcinol (4b), and hydroquinone (4c) to produce the corresponding ester derivative crude products (5a-5c). The Thin Layer Chromatography (TLC) data of crude product of each derivative was shown in **Figure 14**.

2) Purification

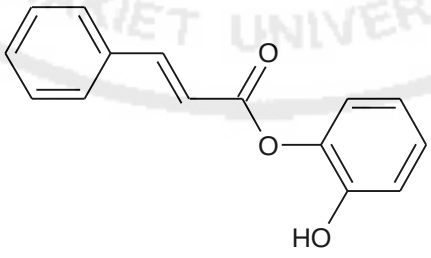
The crude products obtained were extracted three times with 50 mL portions of 10% $NaHCO_3$ solution. The organic layer was collected and removed by the rotary evaporator to give the semi-solid pale yellow crude products. The obtained ester products were finally purified by column chromatography using silica gel as the stationary phase and a gradient elution mixture of ethyl acetate: hexane as the mobile phase system. The eluated fractions were collected and then monitored by TLC technique using 30% ethyl acetate: hexane as the mobile phase.

Figure 14 TLC data of 5a-5c crude product



The TLC spots were visualized under UV light. The ester products were obtained, and characterized by IR, NMR, and mass spectroscopic techniques and the data were indicated as following.

2-hydroxyphenyl cinnamate (5a)



2-hydroxyphenyl (2*E*)-3-phenylprop-2-enoate

Appearance: pale yellow crystal

IR (KBr) (ν, cm^{-1}): 3381 (OH), 1702 ($C=O_{ester}$), 1158 (C=C) (**Figure 1** in Appendix A)

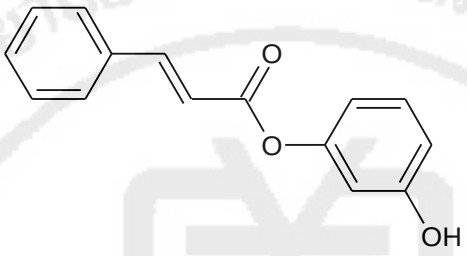
^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.6 (1H, d, J = 16 Hz), 6.9 (1H, d), 7.0 (1H, dd), 7.1 (2H, m), 7.4 (3H, t), 7.6 (1H), 7.8 (1H, d, J = 16 Hz) (**Figure 2** in Appendix A)

^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 116.1, 117.8, 121.3, 122.8, 127.1, 128.2, 128.5, 128.6, 128.7, 130.8, 134.0, 147.3, 147.8, 165.3, 165.4 (**Figure 3** in Appendix A)

Mass spectrum: Calculated mass of $C_{15}H_{12}O_3$ = 240

Found molecular mass = 240 (**Figure 4** in Appendix A)

3- hydroxyphenyl cinnamate (5b)



3-hydroxyphenyl (2*E*)-3-phenylprop-2-enoate

Appearance: colorless crystal

IR (KBr) (ν, cm^{-1}): 3337 (OH), 1702 ($C=O_{ester}$), 1181 (C=C) (**Figure 5** in Appendix A)

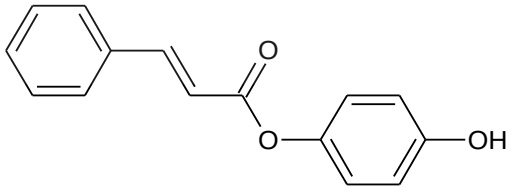
^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.6 (1H, d, *J* = 16 Hz), 6.7 (3H, m), 7.2 (1H, t), 7.4 (3H, t), 7.6 (2H), 7.8 (1H, d, *J* = 16 Hz) (**Figure 6** in Appendix A)

^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 109.4, 113.2, 114.0, 117.3, 128.1, 128.4, 128.5, 128.6, 130.1, 130.8, 134.2, 146.9, 152.6, 157.0, 165.7 (**Figure 7** in Appendix A)

Mass spectrum: Calculated mass of $C_{15}H_{12}O_3$ = 240

Found molecular mass = 240 (**Figure 8** in Appendix A)

4- hydroxyphenyl cinnamate (**5c**)



4-hydroxyphenyl (2*E*)-3-phenylprop-2-enoate

Appearance: white amorphous powder

IR (KBr) (ν, cm^{-1}): 3391 (OH), 1699 ($C=O_{ester}$), 1150 (C=C) (**Figure 9** in Appendix A)

^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.6 (1H, d, *J* = 16 Hz), 6.8 (2H, d, *J* = 7 Hz), 7.0 (2H, d, *J* = 7 Hz), 7.4 (3H, t), 7.6 (2H), 7.8 (1H, d, *J* = 16 Hz) (**Figure 10** in Appendix A)

^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 115.9, 117.6, 122.4, 128.2, 128.3, 128.9, 129.0, 130.6, 134.4, 144.5, 146.3, 153.4, 165.6 (**Figure 11** in Appendix A)

Mass spectrum: Calculated mass of $C_{15}H_{12}O_3$ = 240

Found molecular mass = 240 (**Figure 12** in Appendix A)

4.2 Bioactivity test

The biological activity properties of the target compounds were evaluated, including *in vitro* antioxidant activity and tyrosinase enzyme inhibitory activity.

1) DPPH free radical scavenging test

The synthetic ester series of cinnamic acid derivatives were reacted against DPPH (1,1-diphenyl-2-picrylhydeazyl) free radical. The color changing can be measured by colorimetric technique using UV spectrometer at around 520 nm.

In this study, the antioxidant activities by DPPH assay of 1, 4a, 4b, 4c, 5a, 5b and 5c were determined. The % inhibition of the compounds was plotted against the concentration (g/ml) of compounds. Then, the free radical scavenging activity of each compound was evaluated as the IC_{50} value. All of the test compounds except compound 1 exhibited inhibitory activity. Compound 4c (hydroquinone) showed the highest DPPH scavenging activity with the IC_{50} value of 6.70 µg/ml followed by 4a (pyrocatechol) with the IC_{50} value of 16.99 µg/ml, and 4b (resorcinol) with the IC_{50} value of 105.89 mg/ml, respectively. Among three synthesized isomeric compounds, 5c showed the highest antioxidant activity with the IC_{50} value of 208.71 µg/ml, followed by compound 5a (96.69 mg/ml) and compound 5b (177.42 mg/ml), as shown in **Table 3**.

Table 3 The 50% inhibitory concentration (IC_{50}) value of compound 1, 4a-4c, 5a-5c against DPPH free radical

Compound	IC_{50} (µg/ml)	Equation	R^2 Value
1 (Cinnamic)	-	-	-
4a (Pyrocathechol)	16.99	y = 1.2694x + 28.427	0.9143
4b (Resorcinol)	105.89 mg/ml	y = 0.4004x + 7.6	0.7766
4c (Hydroquinone)	6.70	y = 7.0299x + 2.896	0.9995
5a	96.69 mg/ml	y = 0.284x + 22.539	0.9098
5b	177.42 mg/ml	y = 0.2612x + 3.6574	0.9691
5c	208.71	y = 0.2412x - 0.342	0.9694

2) Tyrosinase inhibitory activity test

In this study, tyrosinase inhibition activity of 1, 4a, 4b, 4c, 5a, 5b and 5c compounds was measured. Kojic acid was used as a positive control. The solutions of cinnamic acid derivatives (500 µg/ml) in DMSO at various volumes from 10 to 120 µl, and L-tyrosine solution (1 mg/ml, 50 µl) were mixed. After that tyrosinase enzyme solution (1,000 units/ml, 50 µl) was added. The test solution was incubated at 37 °C, and then measured at 475 nm using UV/Vis spectrophotometer. The %inhibitions were plotted against the sample concentrations. The result showed that all three synthetic ester derivatives exhibited inhibitory activity. Kojic acid showed the highest tyrosinase an inhibitory activity with the IC_{50} value of 12.17 µg/ml followed by compound 5c with the IC_{50} value of 24.01 µg/ml, compound 5b with the IC_{50} value of 153.70 µg/ml, and 5a with the IC_{50} value of 179.62 µg/ml, respectively.

Table 4 The 50% inhibitory concentration (IC_{50}) value of compound 1, 4a-4c, 5a-5c against tyrosinase enzyme activity

Compound	IC_{50} (µg/ml)	Equation	R^2 Value
Kojic acid	12.17	y = 1.4355x + 32.533	0.9934
1 (Cinnamic)	-	$y = -0.0002x^2 + 0.0668x + 34.604$	0.9642
4a (Pyrocathechol)	-	y = -0.251x + 32.835	-
4b (Resorcinol)	301.09	$y = -0.0009x^2 + 0.264x + 52.103$	0.8595
4c (Hydroquinone)	-	-	-
5a	179.62	y = 0.0572x + 39.726	0.7824
5b	153.70	y = 0.0781x + 37.996	0.6052
5c	24.01	y = 0.0734x + 48.238	0.6534

4.3 Cytotoxicity test

In this research, the series of cinnamic acid ester derivatives as the potential skin whitening agents had been synthesized. The cytotoxicity of those compounds to the human skin fibroblasts had been evaluated through *in vitro* cells *via* the Resazurin Microtiter Assay (REMA).

Compound 4c, 5a, 5b and 5c were selected for the cytotoxic test using the Resazurin Microtiter Assay with the human skin fibroblasts cells. The samples were prepared by dissolving each compound in DMSO with the different various concentrations ranging from from 100, 50, 25, 12.5, 6.2, to 3.13 µg/ml, respectively. The CC_{50} values of the selected compounds were shown in **Table 5**. The CC_{50} values of compound 5a, 5b, and 5c were estimated higher than 100 µg/ml and that of 4c was 20.23 µg/ml.

Table 5 The 50% cytotoxic concentration (CC_{50}) value of compound 5a-5c and hydroquinone

Compound	CC_{50} (µg/ml)
4c (Hydroquinone)	20.23
5a	>100
5b	>100
5c	>100

CHAPTER 5
CONCLUSION AND DISCUSSION

5.1 Discussion

1) Synthesis of cinnamic acid ester derivatives

The cinnamic acid derivatives were synthesized *via* esterification reaction. Several methods of ester syntheses can be used. Here, the reaction of acid halide and alcohol was selected due to the ease, rapid and high yield of reaction. Cinnamic acid was reacted with excess oxalyl chloride to give cinnamoyl chloride (**Figure 15**) which was subsequently reacted with benzenediol without isolation. The obtained ester products were purified by solvent extraction and column chromatography, respectively and then characterized by IR, NMR and mass spectroscopic technique.

The IR spectrum of each ester compound showed strong absorption peaks at 1702, 1702 and 1699 cm^{-1} represented ester carbonyl functionality and also showed hydroxyl absorption peaks at 3381, 3337 and 3391 cm^{-1} as shown in **Figure 16**.

Figure 15 Mechanism of cinnamoyl chloride formation from oxalyl chloride and cinnamic acid

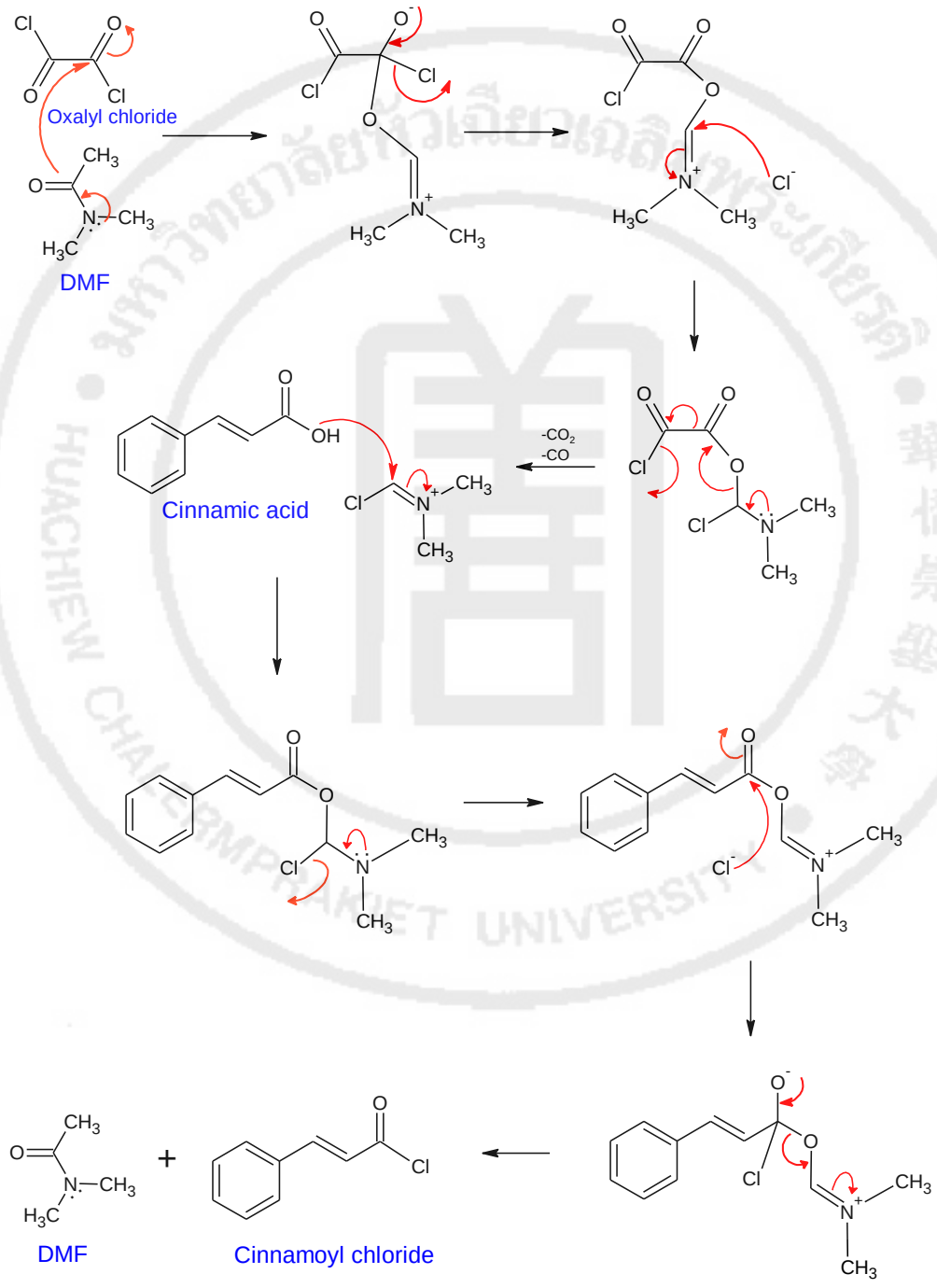
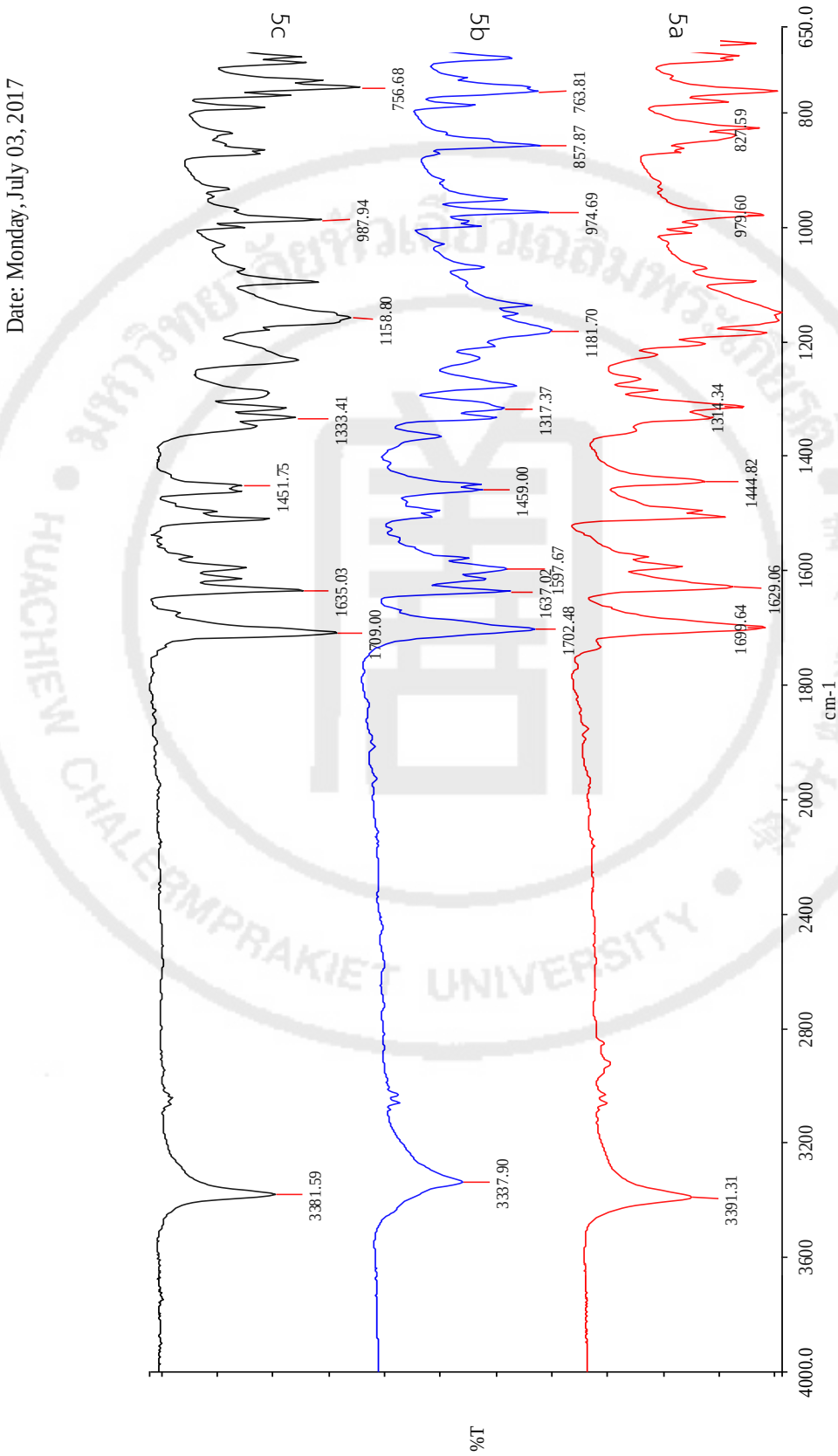
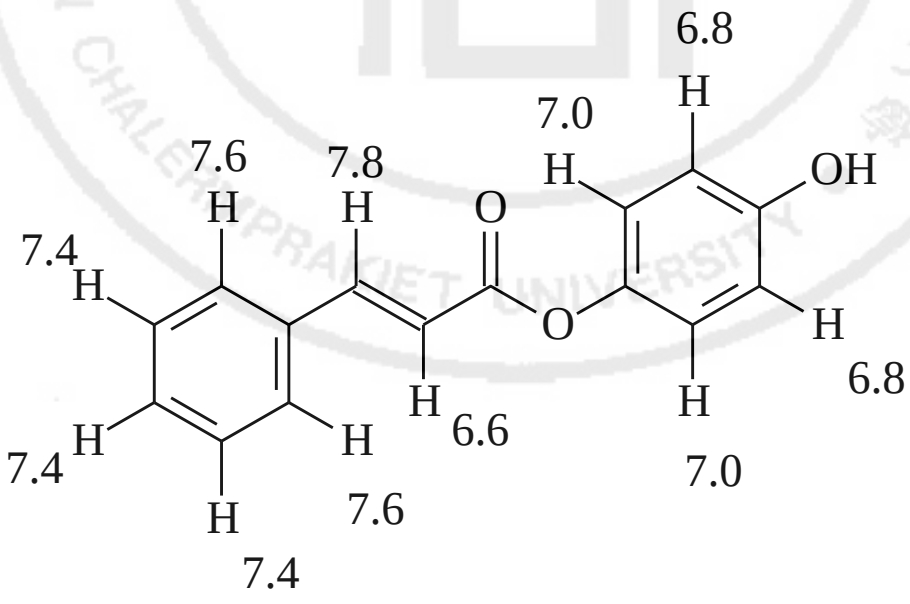


Figure 16 IR Spectrum of compound 5a-5c (Overlay)



The ^{1}H-NMR spectra of compound 5a-5c were shown in **Figure 2**, **6**, **10** in appendix A. The proton signals around δ 0-2.3 ppm of each three spectra might be the proton signals of impurities. However, the chemical structures of compound 5a-5c could be elucidated by ^{1}H-NMR as following. All three ^{1}H-NMR spectra showed characteristic *trans* vinylic protons at δ 6.6 and 7.8 ppm with coupling constant of 16 Hz (*J*=16 Hz) represented alkene moiety of cinnamic acid molecule. Compound 5a-5c showed different peaks around δ 6.6-7.2 ppm. In 5a spectrum, the signal at δ 6.9 (1H), 7.0 (1H), 7.1 (2H) represented chemical shift of protons of catechol moiety. In 5b spectrum, the signal at δ 6.7 (3H) and 7.2 (1H) represented protons of resorcinol moiety. In 5c spectrum, the signal at δ 6.8 (2H), 7.0 (2H) represented proton of hydroquinone moiety and the chemical shift assignment of 5c was indicated in **Figure 17**.

Figure 17 ^{1}H-NMR chemical shift assignment of compound 5c

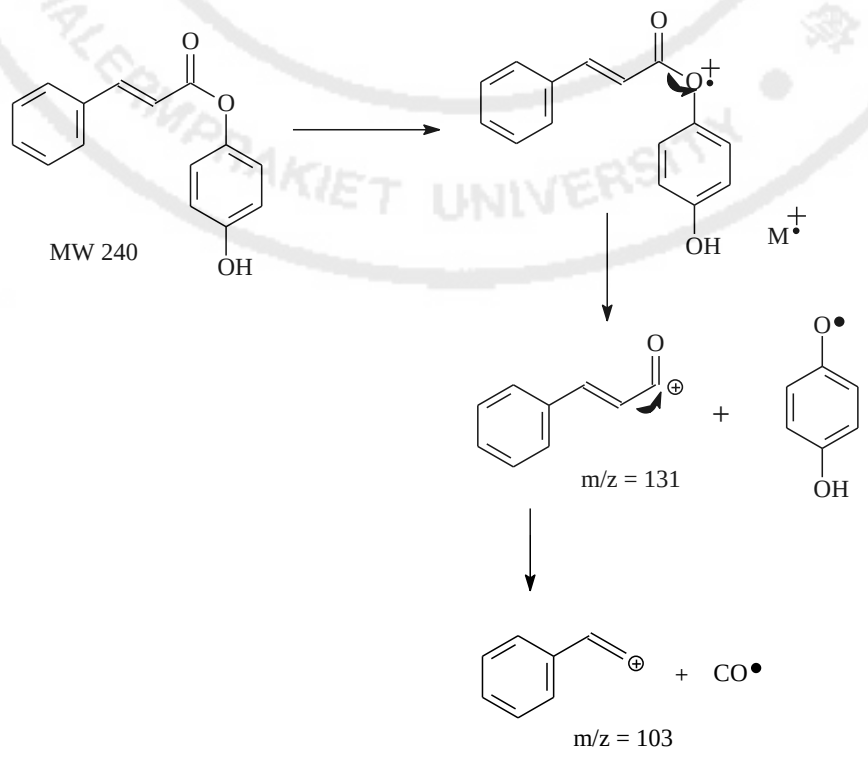


5c

The ^{13}C-NMR spectra of compound 5a-5c were shown in **Figure 3, 7, 11** in appendix A. The signal at δ 165 ppm represented the ester carbonyl carbon. The signals around δ 128 ppm represented the aromatic carbon and the signal at δ 147 ppm and 117 ppm represented the alkene carbon from cinnamic acid.In 5a spectrum, the signal at δ 165.3, 147.3, 127.1, 122.8, 122.3 and 116.1 ppm represented the aromatic carbon of catechol moiety. In 5b spectrum, the signal at δ 157.1, 152.6, 130.8, 114.0, 113.2, and 109.4 represented carbon of resorcinol moiety. In 5c spectrum, the signal at δ 153.4, 144.5, 122.4, and 115.9 represented proton of hydroquinone moiety.

The mass spectra of the three compounds (5a-5c) (**Figure 3, 8, 12** in appendix A) showed the molecular ion peak at m/z 240 could be found in each of the three spectrum. Additionally, the high abundant fragment peaks were observed at m/z 131 and 103 in all three spectra. Those fragment peaks represented the fragment ions occurring as proposed in **Figure 18**.

Figure 18 Proposed fragmentation patterns in the mass spectrum of 5a-5c

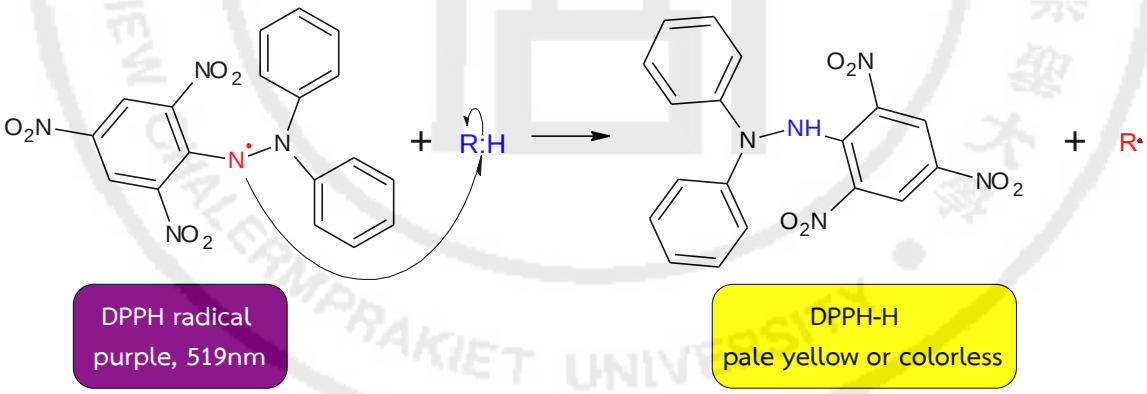


2) Bioactivity test

2.1) Antioxidant activity test

DPPH free radical scavenging test was selected to evaluate the *in vitro* antioxidant activity of the target compounds. It is widely used for preliminary test because it is a rapid, simple, and inexpensive method. The violet color of the DPPH radical accepts an electron from the antioxidant compound; it is reduced and then turns to diphenylpicrylhydrazine radical, a colorless or pale yellow compound. The DPPH assay was believed to involve hydrogen atom transfer reaction as illustrated in **Figure 19**.

Figure 19 Mechanism of DPPH radical scavenging



The antioxidant ability of compounds mainly depends on the active of hydroxyl functional group in their structures. All of the test compounds (1, 4a, 4b, 4c, 5a, 5b and 5c) except compound 1 exhibited an antioxidant activity assessed by DPPH assay. The compound 1 (cinnamic acid) appeared to be non-active via DPPH assay. Compound 4c (hydroquinone) showed the highest DPPH scavenging activity followed by

4b (resorcinol), 4a (pyrocatechol), 5c, 5a and 5b, respectively. It was indicated that the more hydroxyl group in molecule, resulting in the higher scavenging activity of the compound. The position of the hydroxyl groups in molecule also influenced the activity in which the hydroxyl group at para position seemed to be the most active isomer.

2.2) Tyrosinase inhibitory activity assay

Tyrosinase is a copper-containing oxidase enzyme, which is the rate-limiting enzyme involved in the production of the melanin. The skin color tone is determined by the amount and kind of pigment called melanin. The tyrosinase inhibitory activity is the popular properties to study for the developing of functional cosmetics as the skin-whitening product or the hyperpigmentation reducing ingredient.

All compounds (1, 4a, 4b, 4c, 5a, 5b and 5c) displayed various inhibitory activity against tyrosinase enzyme. Among the three synthesized isomeric compounds, 5c showed the highest tyrosinase enzyme inhibitory activity indicating that phenolic –OH at para position was the most active isomer. All derivatives exhibited the tyrosinase enzyme inhibitory activity as measured by tyrosinase inhibition assay although the result gave a slightly low correlation between sample concentration and %inhibition of tyrosinase (R^2 = 0.7824, 0.6052, and 0.6534). It was postulated that enzymes did not seem to display their highest activity or lost some activity due to too high temperature and long period of storage.

2.3) Cytotoxicity test

Compound 4c, 5a, 5b and 5c were selected for the cytotoxic test using the Resazurin Microtiter Assay (REMA) with the human skin fibroblasts cells. The result reported that all of the derivatives are non-toxic. The CC_{50} value of compound 5a, 5b, and 5c were higher than 100 µg/ml while the CC_{50} value of compound 4c (hydroquinone) was 20.23, µg/ml indicating that the synthesized compounds possessed lower cytotoxicity.

5.2 Conclusion

Skin whitening and lightening products account for a large share of the cosmetics market in East Asia region because most of East Asian people, especially

women prefer the white skin color. Cinnamic acid and its derivatives are known ingredients commonly used in cosmetics as fragrance ingredients and sunscreen agents. They possess a wide range of biological activities, including the antioxidant activities. In addition, benzenediols are interesting phenolic compounds with three isomers (ortho, meta, para). The para isomer, hydroquinone; is one of well-known whitening agent that has a strong antioxidant activity and has a potential to inhibit the activity of tyrosinase with the unclear mechanism. Although hydroquinone represents the strong oxidation ability, some evidence shows that hydroquinone can cause the severe effect to the skin. Several hydroquinone structural modifications have been developed.

This present investigation aimed to synthesize a series of cinnamic acid ester derivatives, bearing benzenediol moiety, and then evaluate tyrosinase inhibitory activity and the antioxidant activity. The target compounds could be achieved by one pot synthesis. The chemical structures of all compounds were identified by spectroscopic techniques, mainly IR, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopy.

Study on the antioxidant capacity using DPPH assay revealed that all of the synthetic compounds, including 5a, 5b and 5c exhibited the antioxidant activity. Among the three compounds, compound 5c had the highest activity against DPPH free radical scavenging activity assay, with the IC_{50} value of 56.35 μg/ml, followed by compound 5a (26.10 mg/ml) and compound 5b (47.90 mg/ml).

Moreover, tyrosinase inhibitory activity of 1, 4a, 4b, 4c, 5a, 5b and 5c compounds was also determined. Compound 5c showed the highest tyrosinase inhibitory activity with the IC_{50} value of 24.01 μg/ml. Compound 5b possessed the inhibitory activity with the IC_{50} value of 153.70 μg/ml, and 5a with the IC_{50} value of 179.62 μg/ml, respectively.

Although all of the three synthetic compounds displayed lower DPPH free radical scavenging activity than that of hydroquinone, they possessed lower cytotoxicity than hydroquinone and higher chemical stability. All of the derivatives are non-toxic. The CC_{50} values of compound 5a, 5b, and 5c were higher than 100 μg/ml.

REFERENCES

1. Kong YH, Jo YO, Cho CW, Son D, Park S, Rho J, et al. Inhibitory Effects of Cinnamic Acid on Melanin Biosynthesis in Skin. Biol Pharm Bull. May 2008; 31(5):946-8.
2. Shi Y, Chen QX, Wang Q, Song KK, Qiu L. Inhibitory effects of cinnamic acid and its derivatives on the diphenolase activity of mushroom (*Agaricus bisporus*) tyrosinase. Food Chem. Oct 2005;92(4):707–12.
3. Natella F, Nardini M, Felice MD, Scaccini C. Benzoic and Cinnamic Acid Derivatives as Antioxidants: Structure-Activity Relation. J Agr Food Chem. Apr 1999;47(4):1453-9.
4. Sharma P. Cinnamic acid derivatives: A new chapter of various pharmacological activities. J Chem Pharm Res. 2011;3(2):403-23.
5. Karboune S, Safari M, Lue BM, Yeboah FK, Kermasha S. Lipase-catalyzed biosynthesis of cinnamoylated lipids in a selected organic solvent medium. J Biotechnol. 29 Sep 2005;119(3):281-90.
6. Wang Y, Zhang DH, Chen N, Zhi GY. Synthesis of benzyl cinnamate by enzymatic esterification of cinnamic acid. Bioresour Technol. Dec 2015;198:256-61.
7. Pontiki E, Hadjipavlou-Litina D, Litinas K, Geromichalos G. Novel Cinnamic Acid Derivatives as Antioxidant and Anticancer Agents: Design, Synthesis and Modeling Studies. Molecules. 7 Jul 2014 ;19(7):9655-74.
8. De P, Veau D, Bedos-Belval F, Chassaing S, Baltas M. Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance: Cinnamic Derivatives in Tuberculosis. Shanghai: InTech; 2012. pp. 338-62.
9. Wiesner J, Mitsch A, Wissner P, Jomaa H, Schlitzer M. Structure-activity relationships of novel anti-malarial agents. Part 2: cinnamic acid derivatives. Bioorg Med Chem Lett. 12 Feb 2001;11(3):423-4.
10. Tawata S, Taira S, Kobamoto N, Zhu J, Ishihara M, Toyama S. Synthesis and antifungal activity of cinnamic acid esters. Biosci Biotechnol Biochem. May 1996;60(5):909-10.
11. Sova M. Antioxidant and antimicrobial activities of cinnamic acid derivatives. Mini Rev Med Chem. Jul 2012;12(8):749-67.

REFERENCES (CONTINUED)

12. Narasimhan B, Belsare D, Pharande D, Mourya V, Dhake A. Esters, amides and substituted derivatives of cinnamic acid: synthesis, antimicrobial activity and QSAR investigations. Eur J Med Chem. Oct 2004;39(10):827-34.
13. Högl, O. Some non-volatile extracts of coffee. Mitt Geb Lebensm Hyg. 1958;49:433-41.
14. Trower MK, Sariaslani FS, Kitson FG. Xenobiotic oxidation by cytochrome P-450-enriched extracts of *Streptomyces griseus*. Biochem Bioph Res Co. 1988;157:1417-22.
15. Spain JC, Wyss O, Gibson DT. Enzymatic oxidation of p-nitrophenol. Biochem Biophys Res Commun. 1979;88:634-41.
16. Nyholm N, Jorgensen-Lindgaard P, Hansen N. Biodegradation of 4-nitrophenol in standardized aquatic degradation tests. Ecotoxicol Environ Saf. Oct 1984;8(5):451-70.
17. Häggblom MM, Apajalahti JHA, Salkinoja-Salonen MS. Degradation of chlorinated phenolic compounds occurring in pulp mill effluents. Water Sci Technol. 1988;20:205-8.
18. Wöhler F. Untersuchungen über das Chinon. Justus Liebigs Ann Chem. 1844;51(2):145–63.
19. Koskimis S, Himala S, inventors. Procedure for producing hydroquinone. US Patent 4,801,758. 1989
20. Kari WF. NTP Toxicology and Carcinogenesis Studies of Hydroquinone (CAS No. 123-31-9) in F344/N Rats and B6C3F1 Mice (Gavage Studies). Natl Toxicol Program Tech Rep Ser. Oct 1989;366:1-248.
21. McGregor D. Hydroquinone: an evaluation of the human risks from its carcinogenic and mutagenic properties. Crit Rev Toxicol. 2007;37(10):887-914.
22. Krumenacker L, Costantini M, Pontal P. and Sentenac J. Kirk-Othmer Encyclopedia of Chemical Technology: Hydroquinone, resorcinol, and catechol. Bickford M. 3rd ed. New York: John Wiley & Sons; 2000. pp. 39-69.

REFERENCES (CONTINUED)

23. Department of the Army. Department of the Army Technical Manual, TM 9-1300-214: Military Explosives. Washington, D.C.: University; 1984.
24. Reinsch H. Repertorium für die Pharmacie: Bemerkungen über Catechu. Buchner JA. Nürnberg: Schrag; 1839.
25. Charrouf Z, Guillaume D. Phenols and polyphenols from *Argania spinosa*. AM J Food Technol. 2007;2(7):679-83.
26. Signore AD, Romeo F, Giaccio M. Content of phenolic substances in basidiomycetes. Mycol Res. 1997;101(5):552–6.
27. Fiege H, Voges H-W, Hamamoto T, Umemura S, Iwata T, Miki H, et al. Ullmann's Encyclopedia of Industrial Chemistry: Phenol Derivatives. Elvers B. 7th ed. Weinheim: Wiley-VCH; 2000.
28. Furniss BS, Hannaford AJ, Smith PW, Tatchell AR. Vogel's Textbook of Practical Organic Chemistry. 5th Ed. London: Longman Science & Technical; 1996.
29. Farmer S. Acid chlorides react with alcohols to form esters [database online]. San Francisco: The Chemistry LibreTexts library. c2016 [cited 2017 Jan 18]; Available from: https://chem.libretexts.org/Core/Organic_Chemistry/Acid_Halides/Reactions_of_Acid_Halides/Acid_chlorides_react_with_alcohols_to_form_esters
30. Clark J. Nucleophilic addition/ elimination in the reaction between acyl chlorides and alcohols [database online].Cambridge: Clark J. c2000 [cited 2017 Jan 18]; Available from: http://www.chemguide.co.uk/mechanisms/addelim/alcohol.html
31. Clark J. Esterification [database online]. Cambridge: Clark J. c2000 [cited 2017 Jan 18]; Available from: http://www.chemguide.co.uk/organicprops/alcohols/esterification.html
32. Farmer S. Transesterification [database online]. San Francisco: The Chemistry LibreTexts library. c2014 [cited 2017 Jan 18]; Available from: https://chem.libretexts.org/Core/Organic_Chemistry/Esters/Reactivity_of_Esters/Transesterification

REFERENCES (CONTINUED)

33. James. Synthesis (3) – Reactions of Alkyl Halides [database online]. Ontario: Master Organic Chemistry. c2014 [cited 2017 Jan 18]; Available from: http://www.masterorganicchemistry.com/2014/01/10/synthesis-3-reactions-of-alkyl-halides
34. Hennis HE, EasterlyJr. JP, Collins LR, Thompson LR. Esters from Reactions of Alkyl Halides and Salts of Carboxylic Acids. Reactions of Primary Alkyl Chlorides and Sodium Salts of Carboxylic Acids. Ind Eng Chem Prod Res Dev. 1967; 6(3):193–5.
35. Hennis HE, Thompson LR, Long JP. Esters from the Reactions of Alkyl Halides and Salts of Carboxylic Acids. Comprehensive Study of Amine Catalysis. Ind Eng Chem Prod Res Dev. 1968; 7(2).96-101.
36. Gillbro JM, Olsson MJ. The melanogenesis and mechanisms of skin-lightening agents--existing and new approaches. Int J Cosmet Sci. Jun 2011;33(3):210-21.
37. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol Rev. Oct 2004;84(4):1155-228.
38. Koning RE. Enzyme Basics [database online]. Willimantic: Plant Physiology Information Website. c1994 [cited 2017 Jan 25]; Available from: http://plantphys.info/plant_physiology/enzymebasics.shtml
39. Duckworth HW, Coleman JE. Physicochemical and kinetic properties of mushroom tyrosinase. J Biol Chem. 10 Apr 1970;245(7):1613-25.
40. Lima CR, Silva JA, Cardoso ÉTC, Silva EO, Lameira J, Nascimento JM, et al. Combined Kinetic Studies and Computational Analysis on Kojic Acid Analogs as Tyrosinase Inhibitors. Molecules. 2014:19(7):9591-605
41. Kuo TC, Ho FM. Competitive Inhibition of Mushroom Tyrosinase by Captopril. Res J Biotechnol. 2014;8:26-9.
42. Espín JC, Wichers HJ. Effect of captopril on mushroom tyrosinase activity *in vitro*. Biochim Biophys Acta. 12 Jan 2001;1544(1-2):289-300.
43. Andrawis A, Varda K. Inactivation of mushroom tyrosinase by hydrogen peroxide. Phytochemistry. 1985;24(3):397-405.

REFERENCES (CONTINUED)

44. Karg E, Odh G, Wittbjer A, Rosengren E, Rorsman H. Hydrogen peroxide as an inducer of elevated tyrosinase level in melanoma cells. J Invest Dermatol. Feb 1993;100(2 Suppl):209S-13S.
45. DiSanto B. Antioxidants 101 — The Comprehensive Step-By-Step Guide To Antioxidants [database online]. Paris: Lean It UP. c2012 [cited 2017 Jan 25];Available from: http://www.leanitup.com/antioxidants/
46. Alam MN, Bristi NJ, Rafiquzzaman M. Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. Saudi Pharm J. Apr 2013;21(2):143-52.
47. Carmona-Jiménez Y, García-Moreno MV, Igartuburu JM, Garcia Barroso C. Simplification of the DPPH assay for estimating the antioxidant activity of wine and wine by-products. Food Chem. 15 Dec 2014;165:198-204.
48. Sharma OM, Bhat TK. DPPH antioxidant assay revisited. Food Chem. 2009;113.:1202-5.
49. Page B, Page M, Noel C. A new fluorometric assay for cytotoxicity measurements *in-vitro*. Int J Oncol. Sep 1993;3(3):473-6.
51. Khalifa RA, Nasser MS, Gomaa AA, Osman NM, Salem HM. Resazurin Microtiter Assay Plate method for detection of susceptibility of multidrug resistant Mycobacterium tuberculosis to second-line anti-tuberculous drugs. Egyptian Journal of Chest Disease and Tuberculosis. Apr 2013;62(2):241-7.
52. Kunte S, Karmarkar A, Dharmashale S, Hatolkar S. Resazurin microtitre assay (REMA) plate - A simple, rapid and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. Eur Respir J. 2014;40(Suppl56).
53. O'Brien J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem. Sep 2000 ;267(17):5421-6.
54. González-Pinzón R, Haggerty R, Myrold DD. Measuring aerobic respiration in stream ecosystems using the resazurin-resorufin system. J Geophys Res. Sep 2012;117(G3).

REFERENCES (CONTINUED)

55. Chen JL, Steele TW, Stuckey DC. Modeling and Application of a Rapid Fluorescence-Based Assay for Biotoxicity in Anaerobic Digestion. Environ Sci Technol. 2015;49 (22):13463–71.

56. Somvong K, Prasitpuriprecha C. Antioxidant and Melanogenesis Stimulating activities of Some Thai Traditional Medicinal Plant Extracts for Grey Hair Treatment. In: Proceedings of the 4th Annual Northeast Pharmacy Research Conference of 2012 "Pharmacy Profession in Harmony"; 2012 Feb 11-12; Faculty of Pharmaceutical Sciences Khon Kaen University, Khon Kaen; 2012. pp. 125-34.

APPENDIX

Appendix A

Spectrum of compound 5a-5c

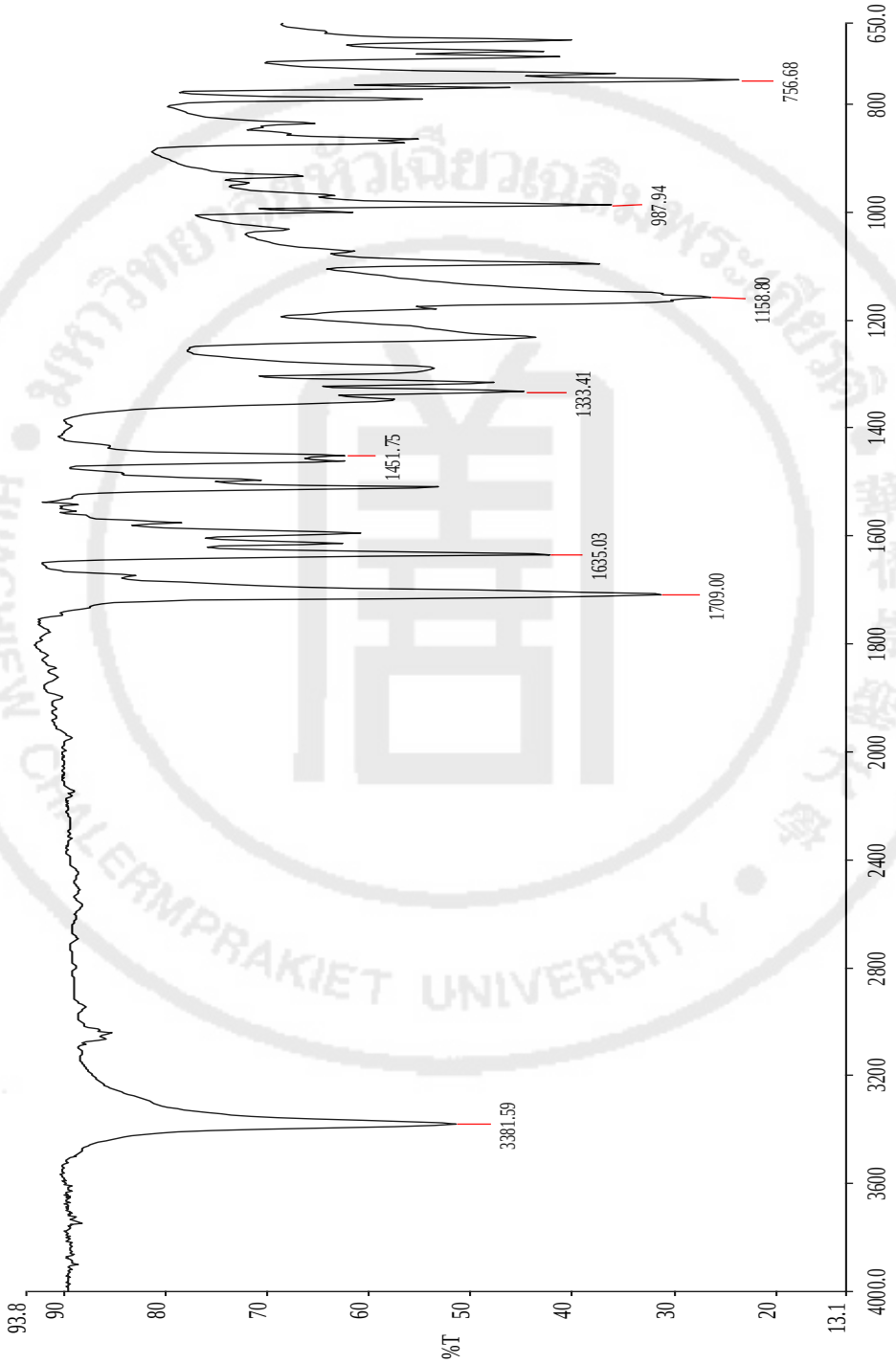


Figure 1 The infrared spectrum of compound 5a

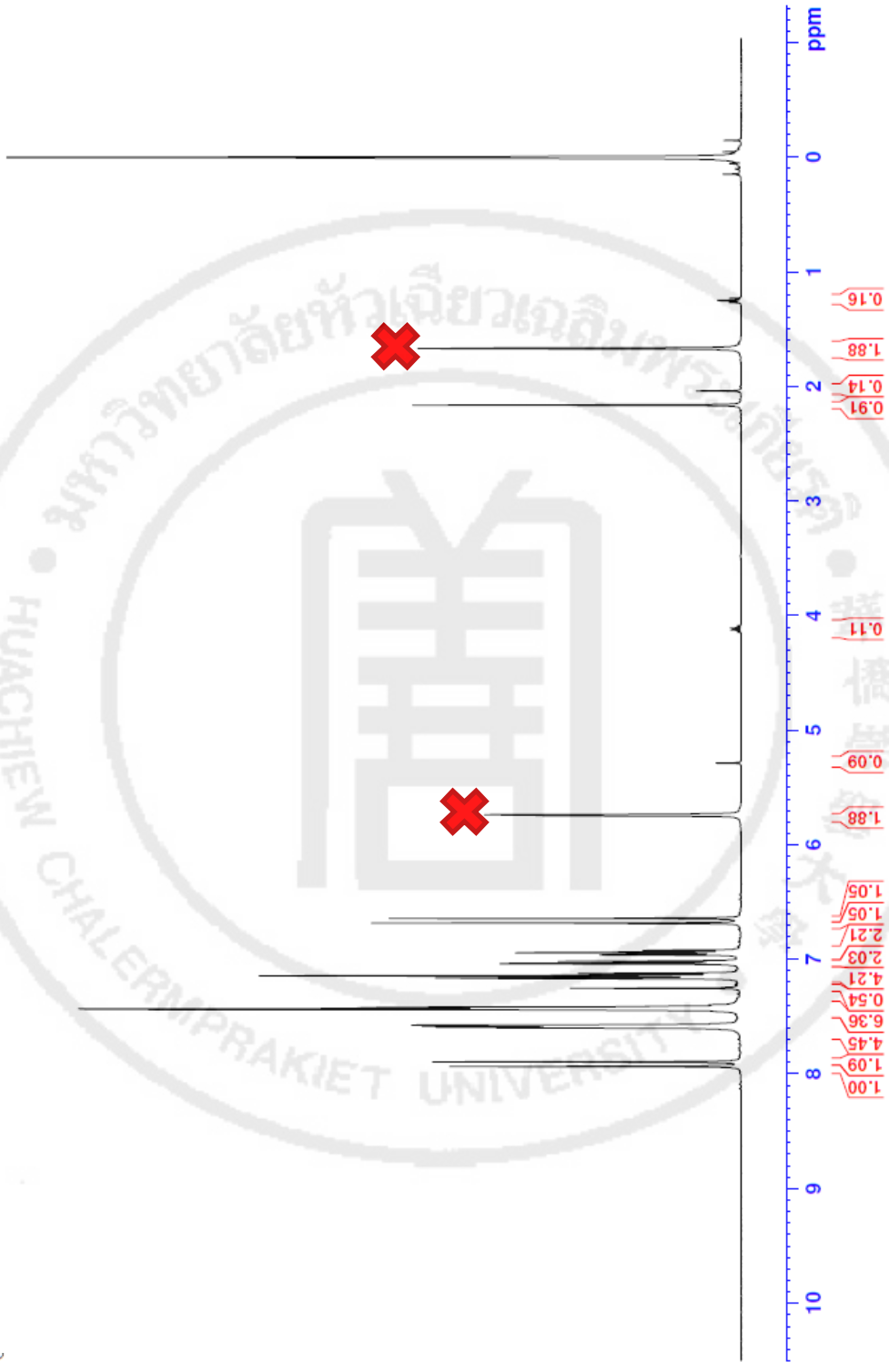


Figure 2 The 400 MHz ^{1}H-NMR spectrum of compound 5a

Figure 3 The 100 MHz ^{13}C-NMR spectrum of compound 5a

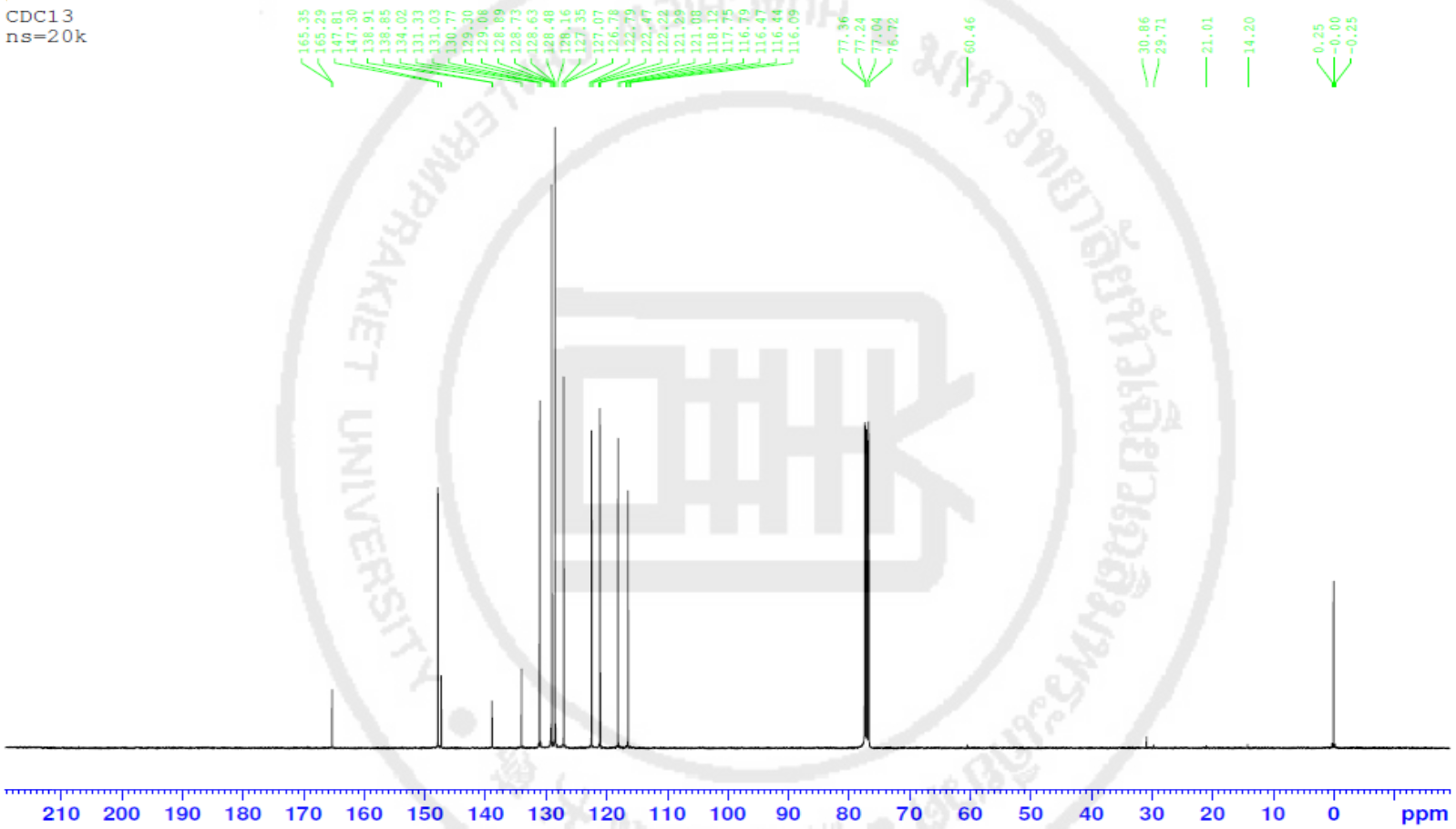
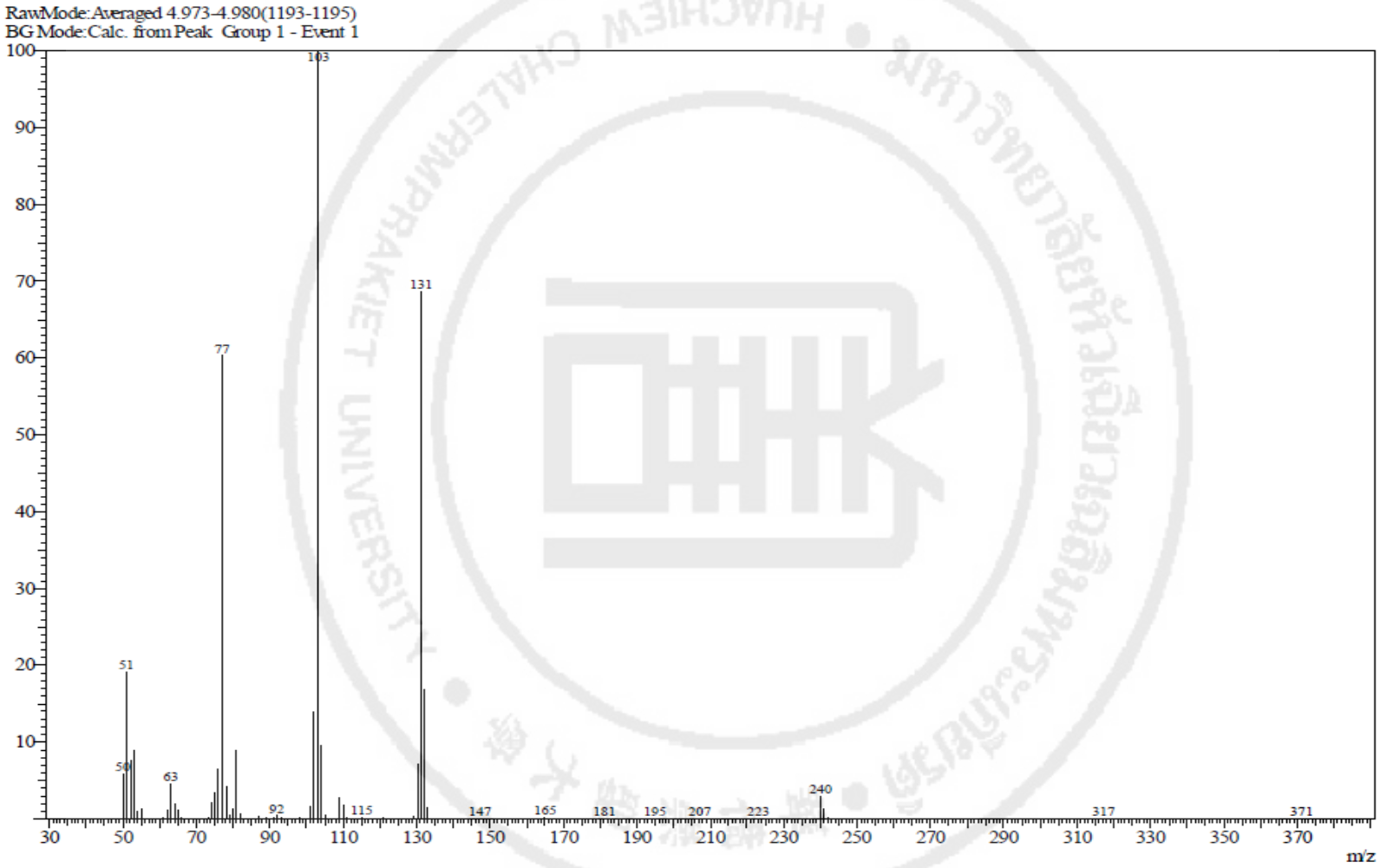


Figure 4 The mass spectrum of compound 5a



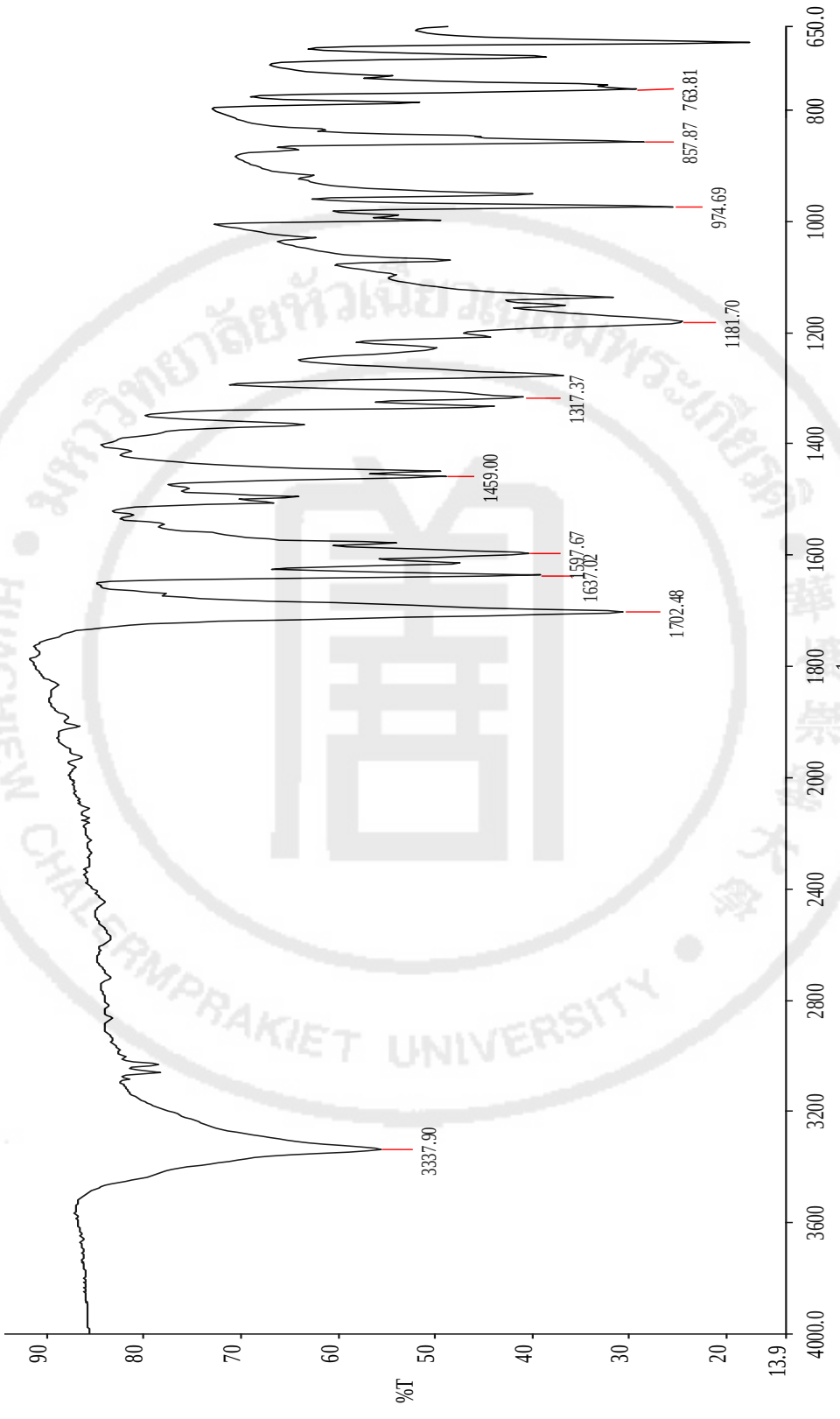


Figure 5 The infrared spectrum of compound 5b

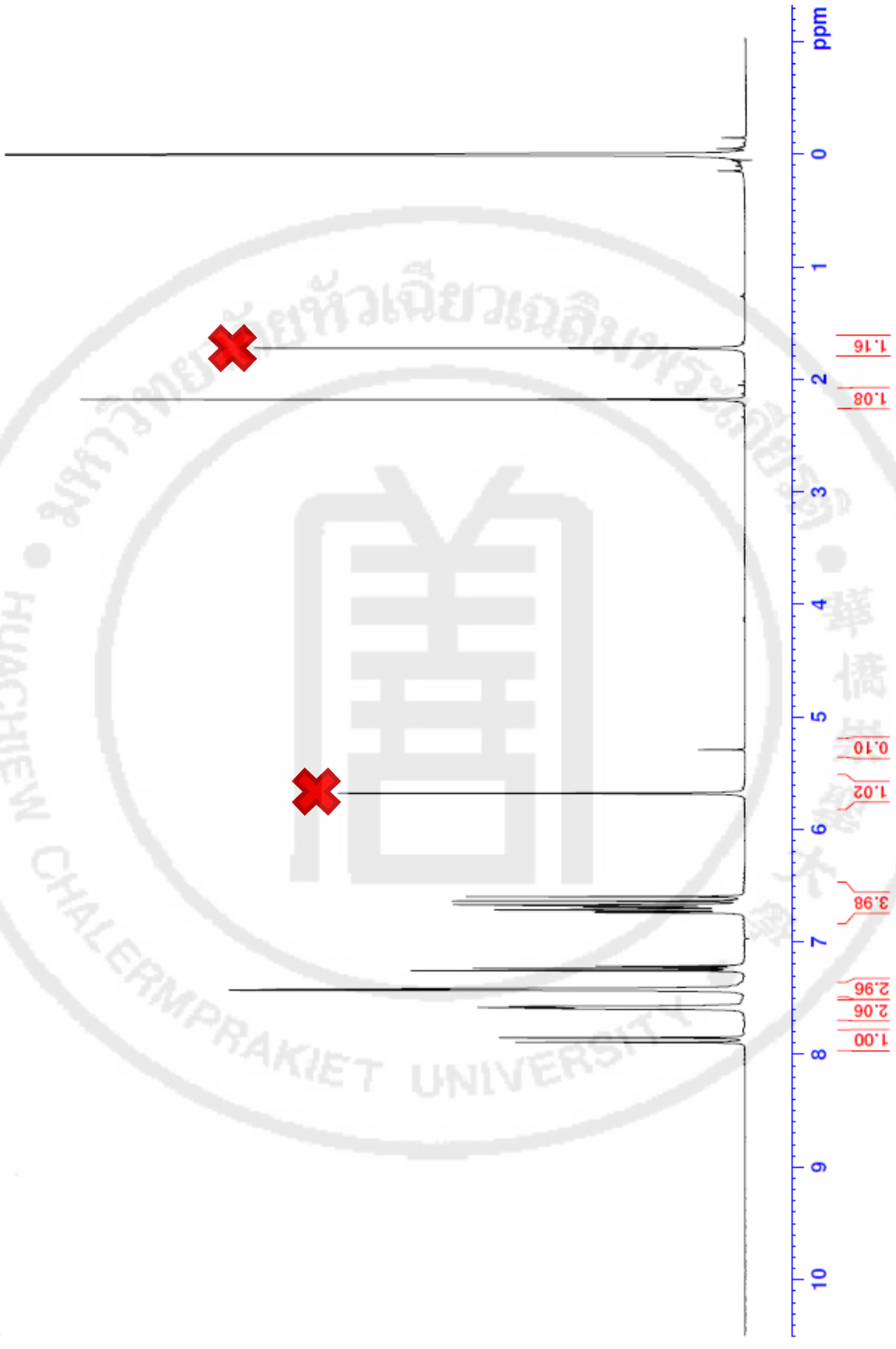


Figure 6 The 400 MHz ^{1}H-NMR spectrum of compound 5b

Figure 7 The 100 MHz ^{13}C-NMR spectrum of compound 5b

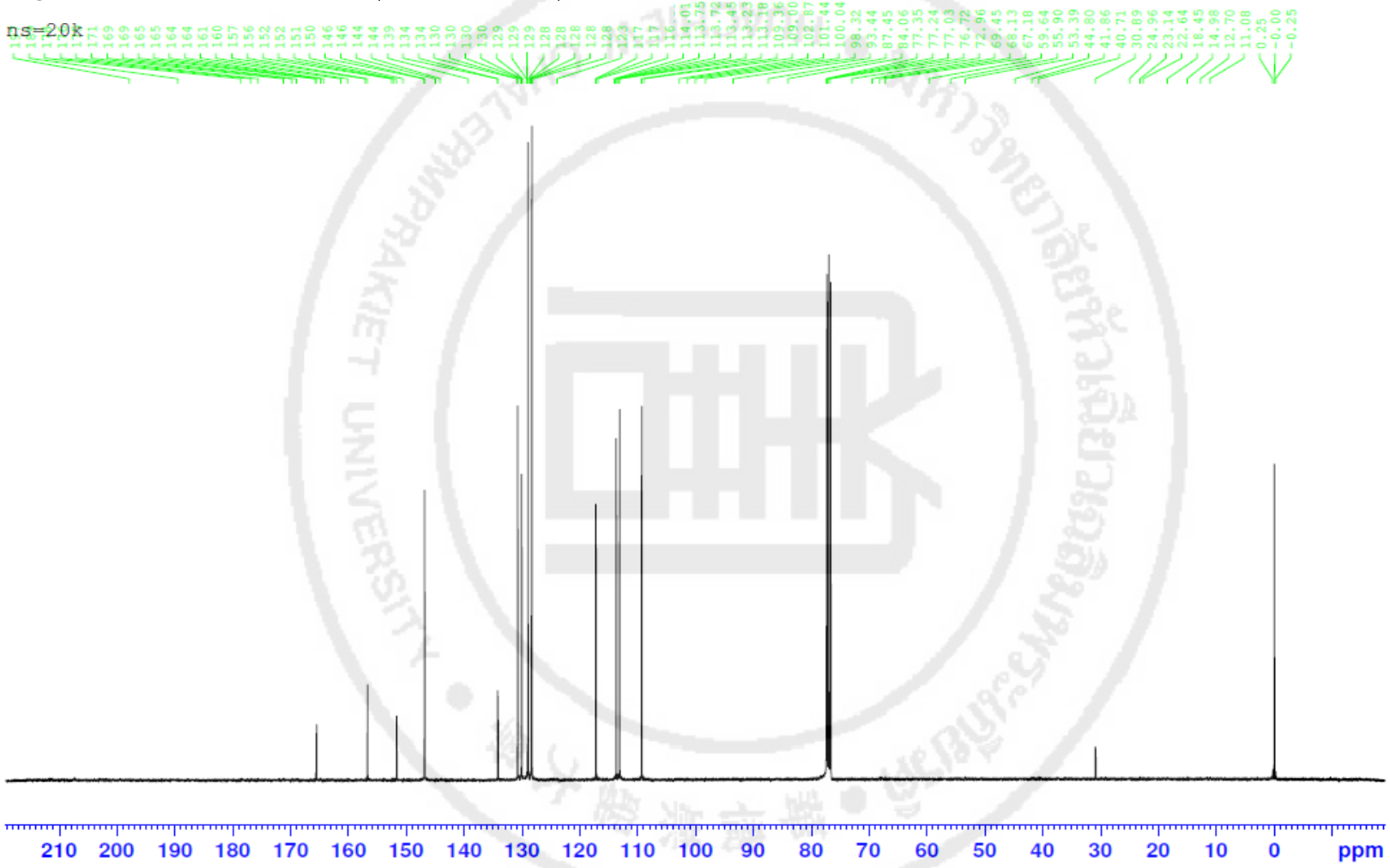


Figure 8 The mass spectrum of compound 5b

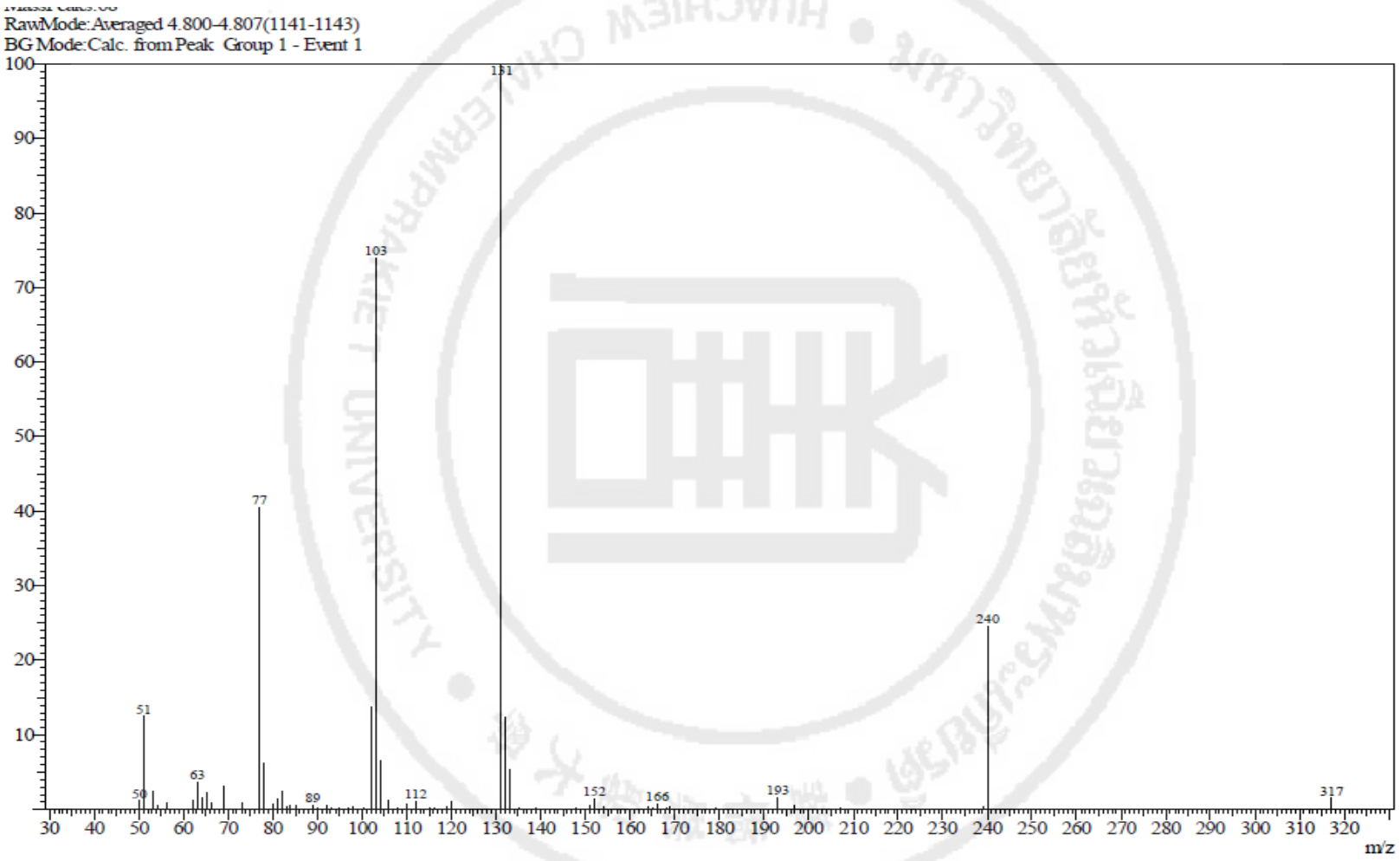
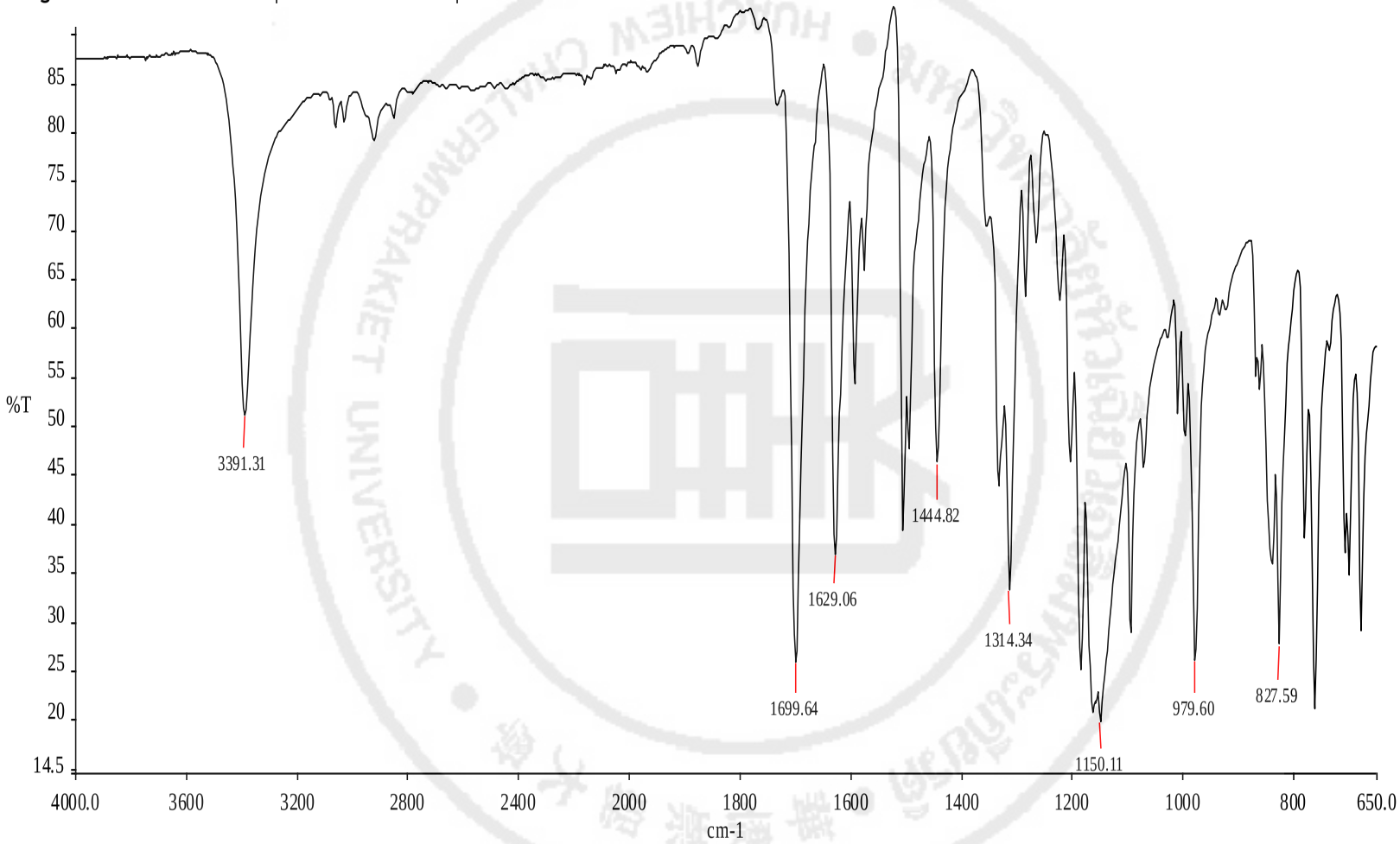


Figure 9 The infrared spectrum of compound 5c



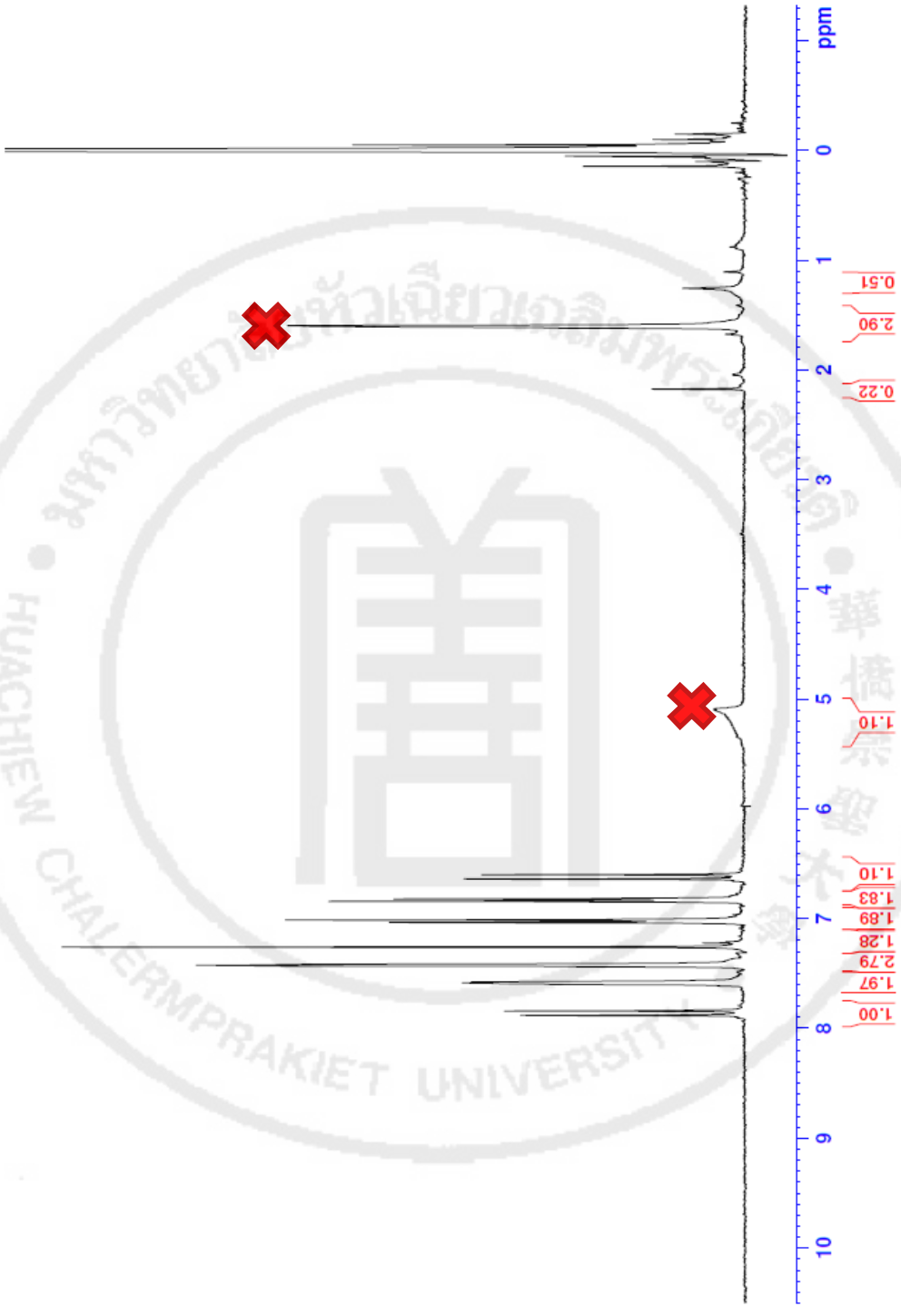


Figure 10 The 400 MHz ^{1}H-NMR spectrum of compound 5c

Figure 11 The 100 MHz ^{13}C-NMR spectrum of compound 5c

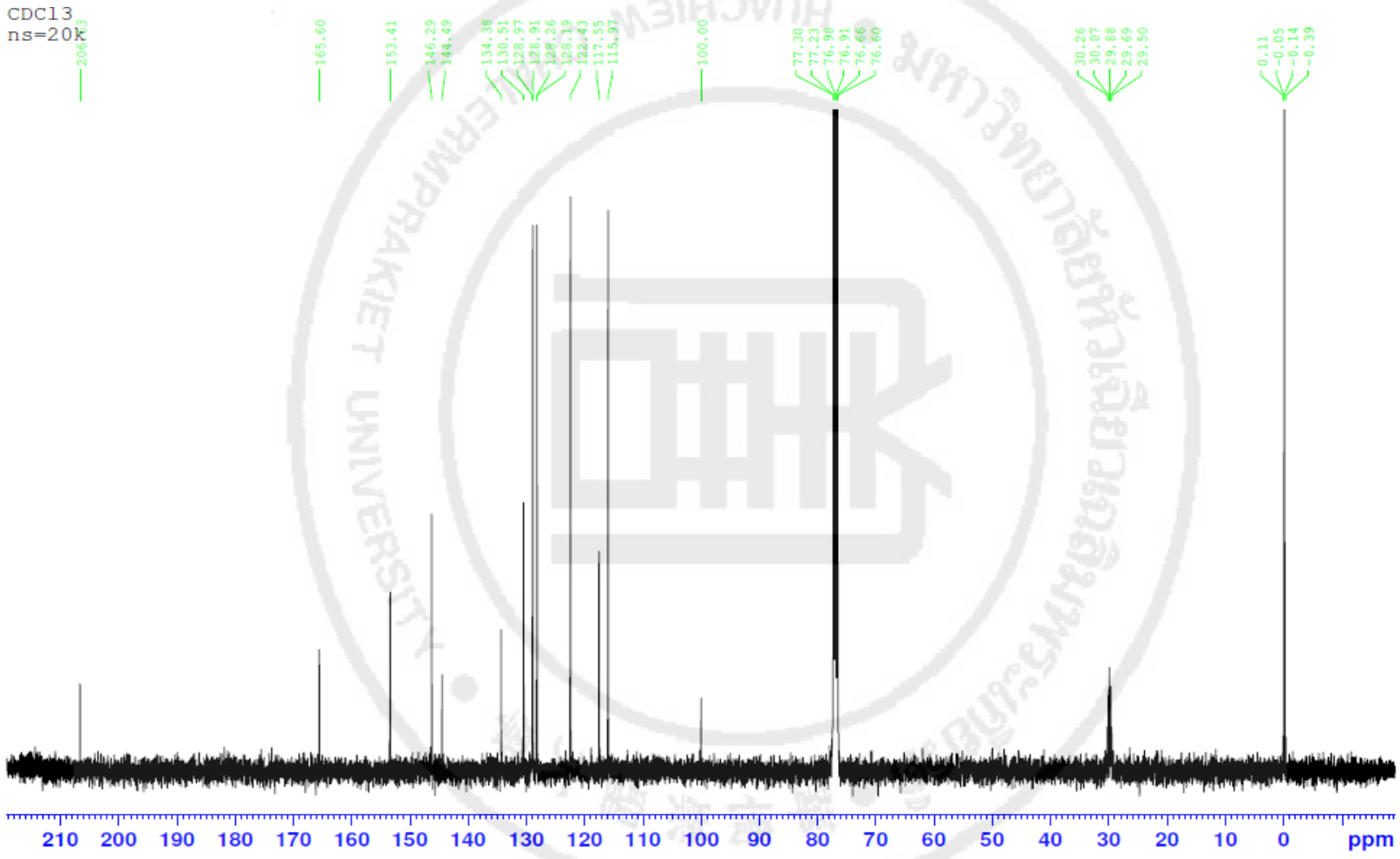
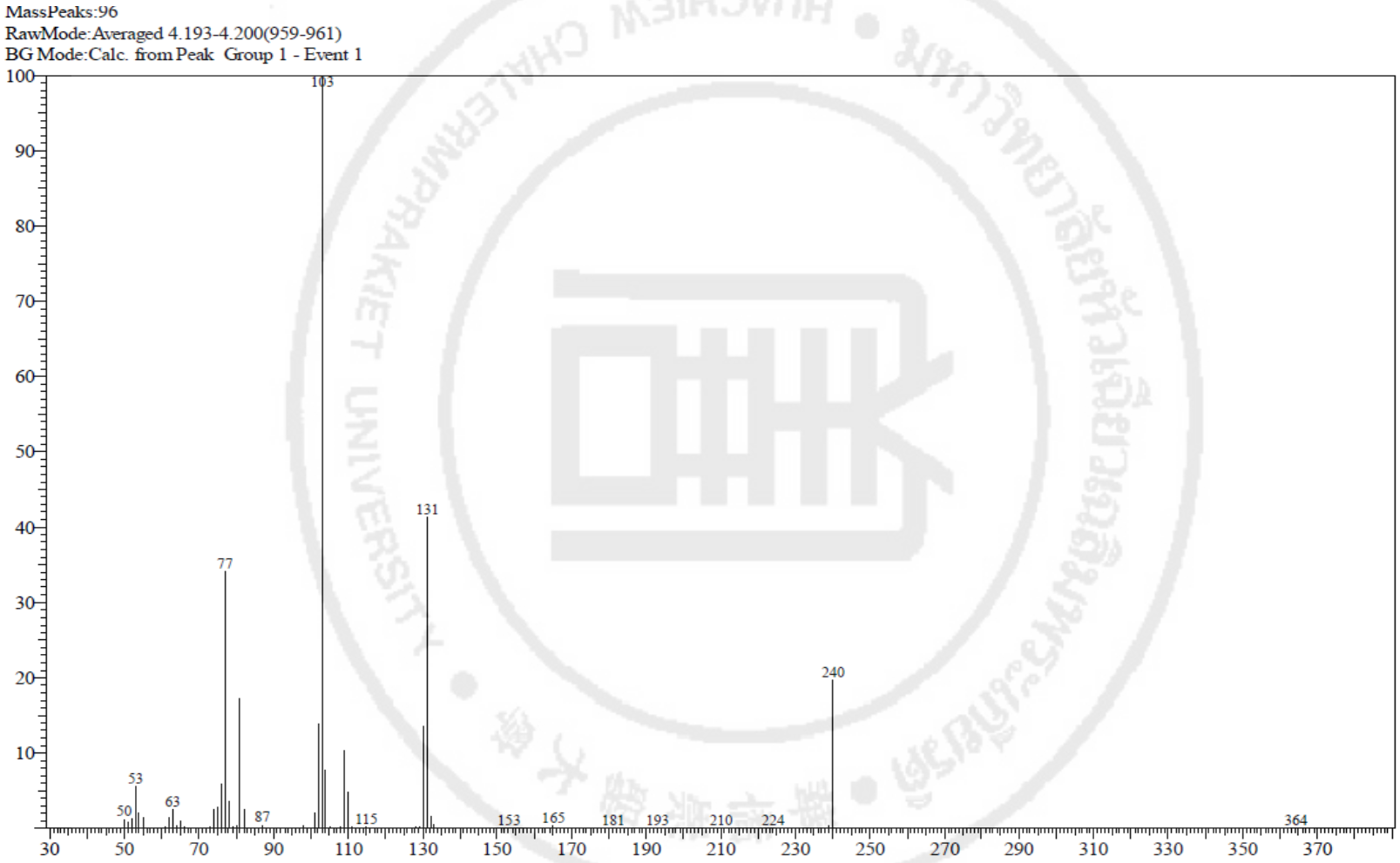


Figure 12 The mass spectrum of compound 5c



Appendix B

Graph of concentration v/s %inhibition of test compounds by DPPH assay

Figure 13 Antioxidant activity of cinnamic acid

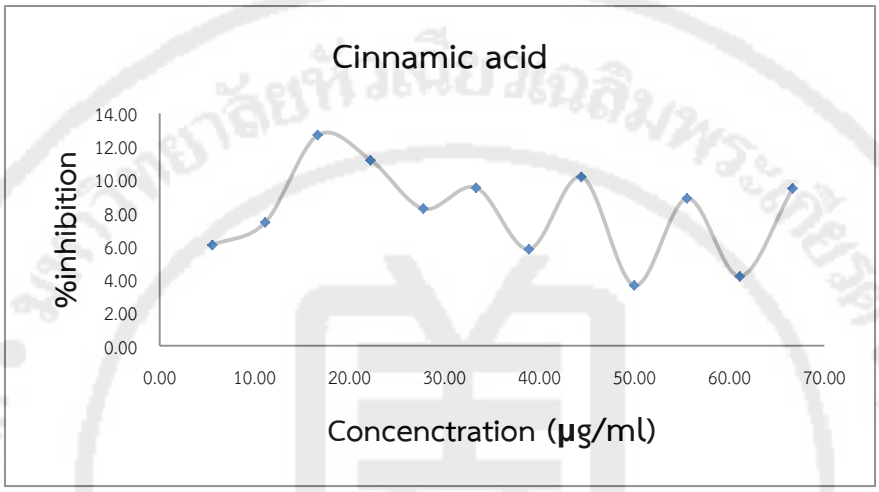


Figure 14 Antioxidant activity of pyrocatechol

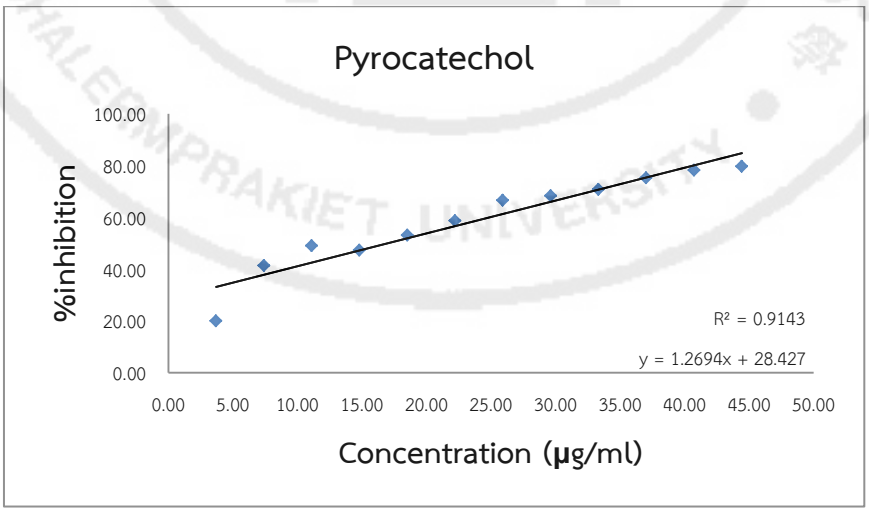


Figure 15 Antioxidant activity of resorcinol

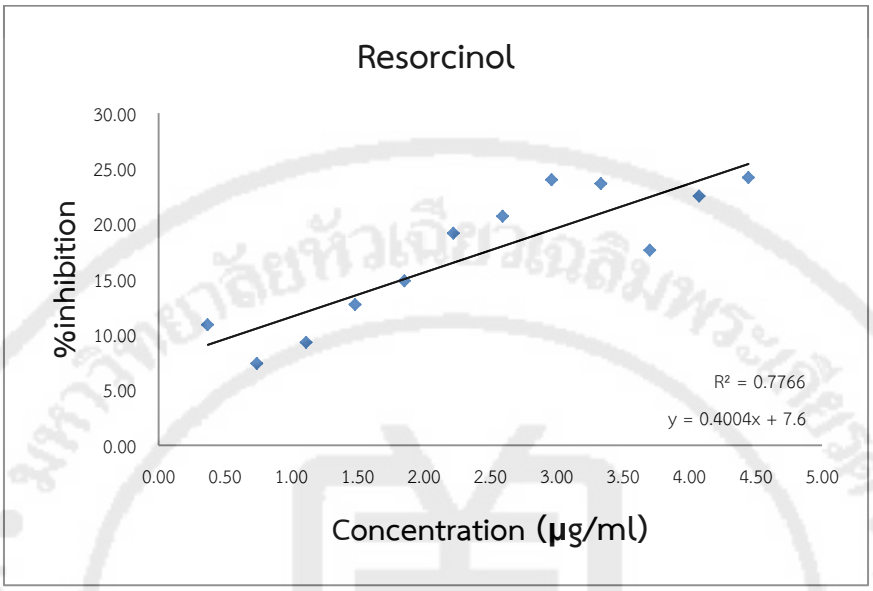


Figure 16 Antioxidant activity of hydroquinone

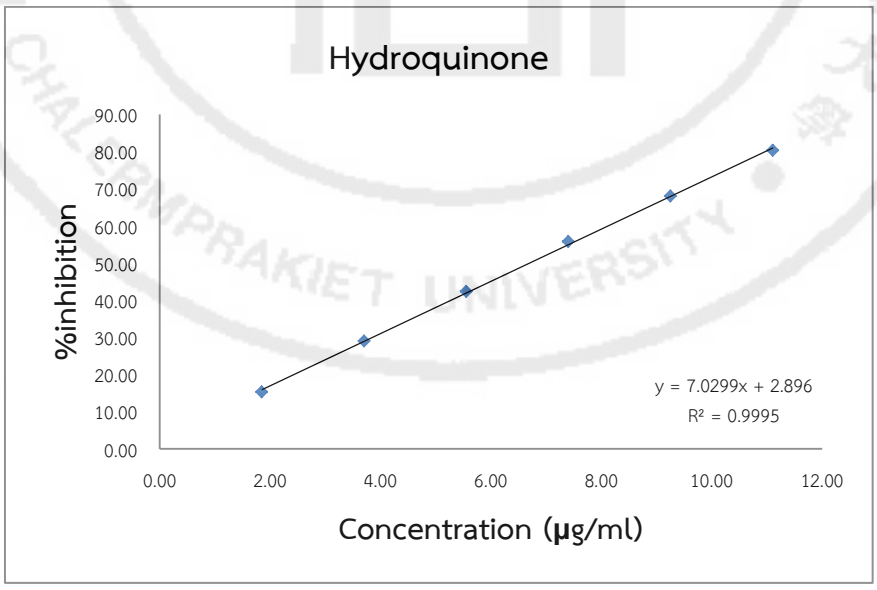


Figure 17 Antioxidant activity of compound 5a

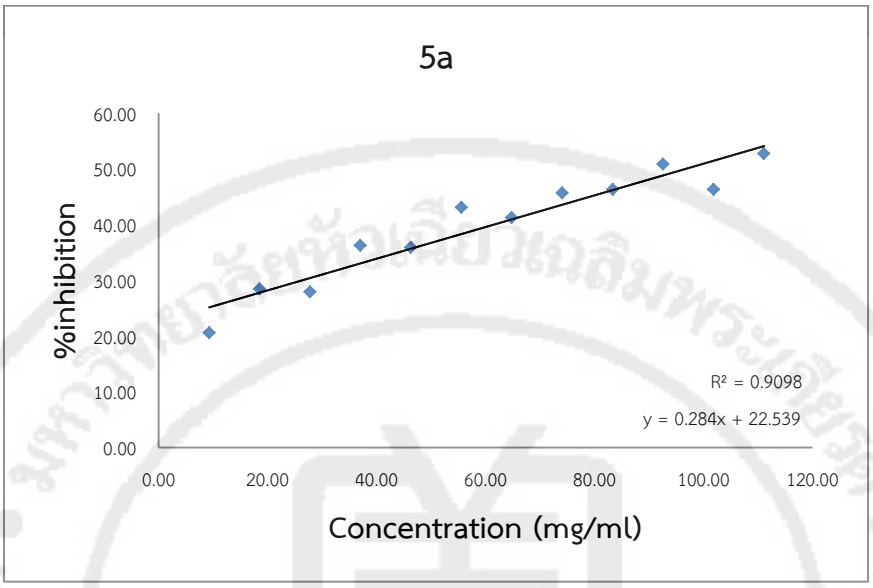


Figure 18 Antioxidant activity of compound 5b

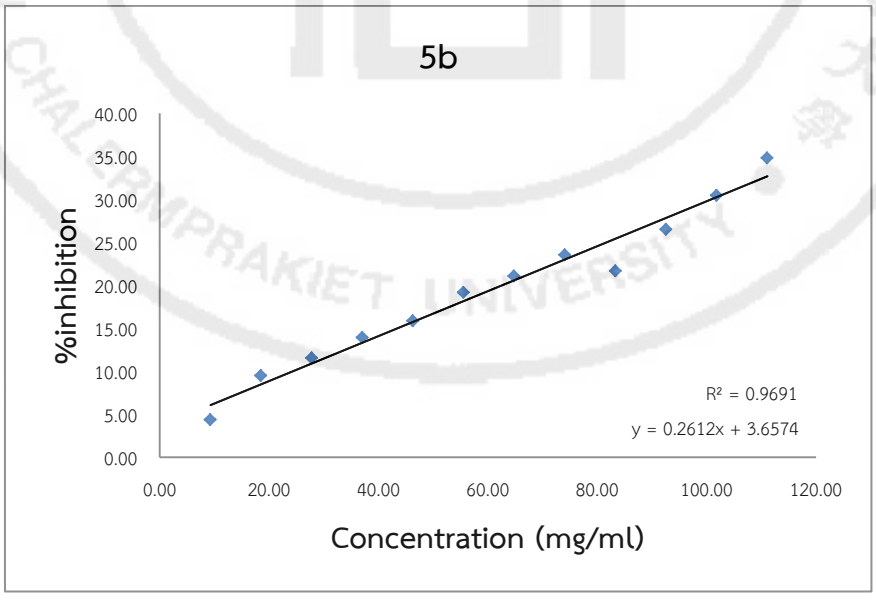
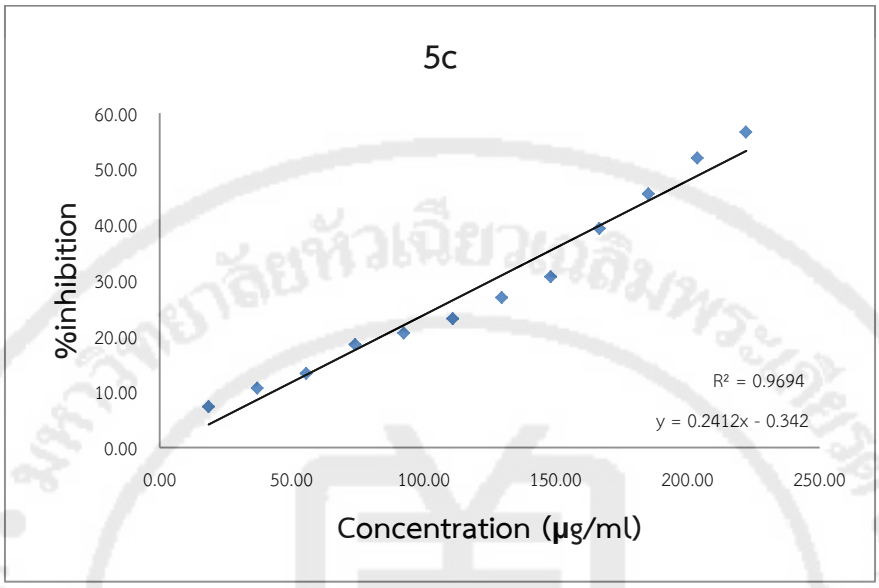


Figure 19 Antioxidant activity of compound 5c



Appendix C

Graph of concentration v/s %inhibition of test compounds by Tyrosinase assay

Figure 20 Tyrosinase inhibitory activity of kojic acid

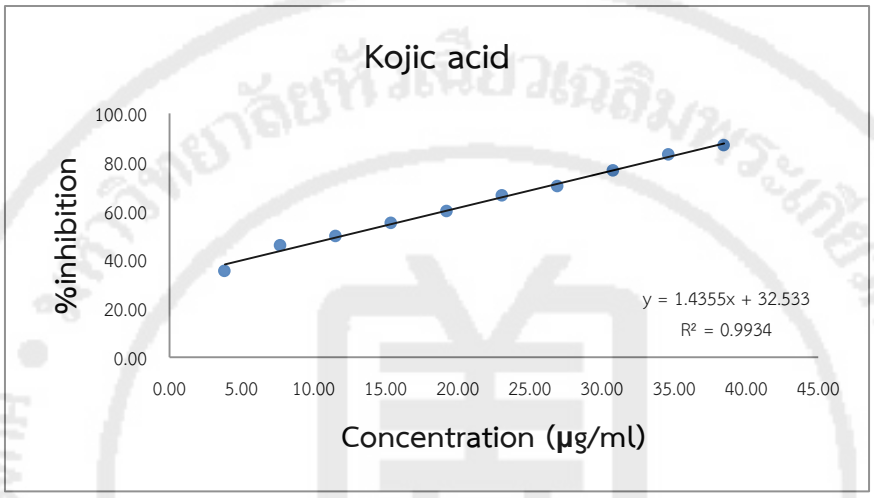


Figure 21 Tyrosinase inhibitory activity of cinnamic acid

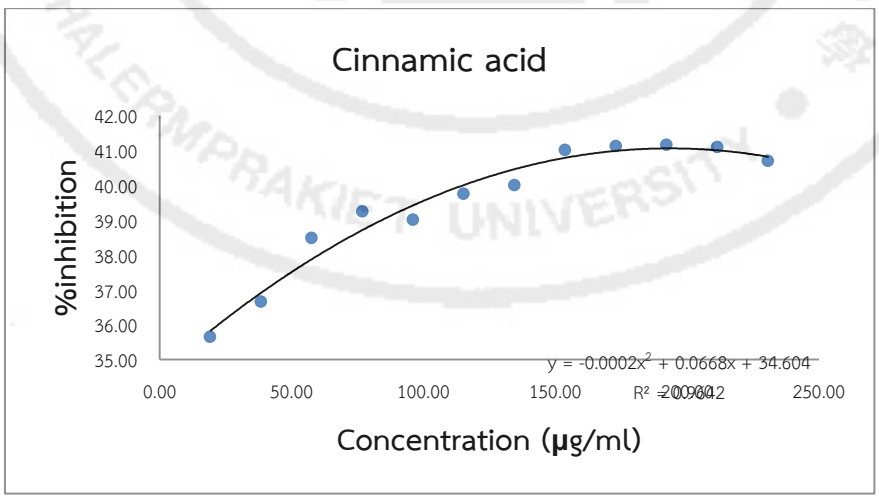


Figure 22 Tyrosinase inhibitory activity of pyrocatechol

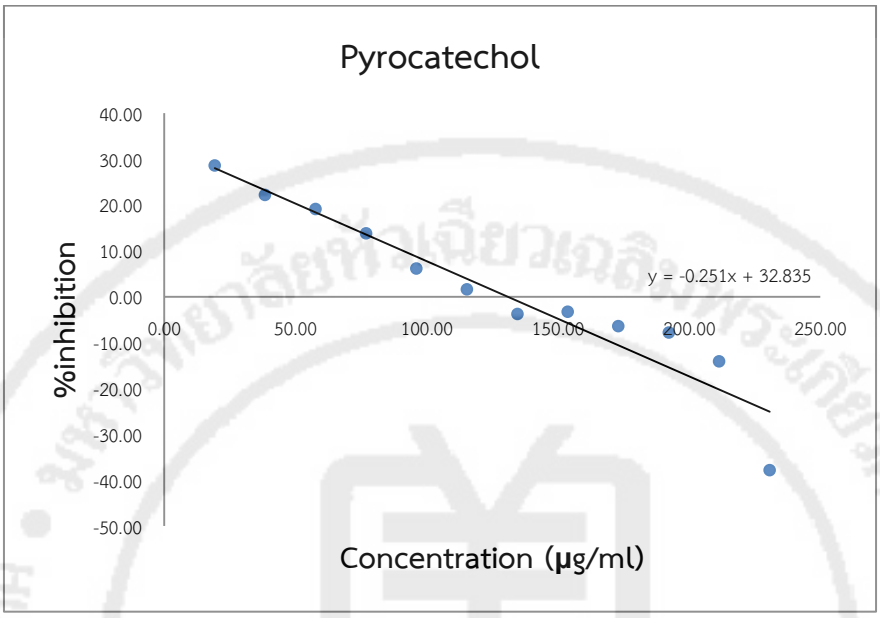


Figure 23 Tyrosinase inhibitory activity of resorcinol

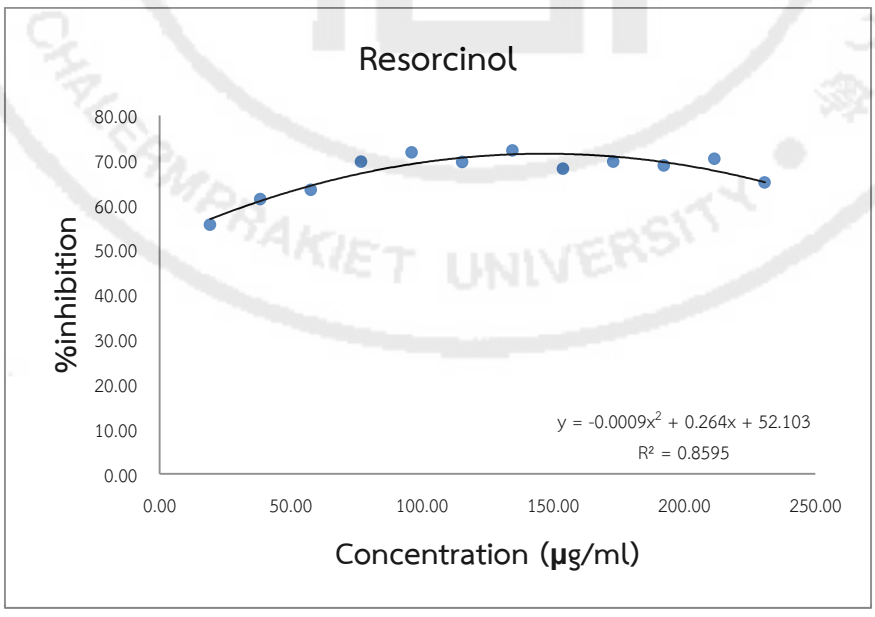


Figure 24 Tyrosinase inhibitory activity of hydroquinone

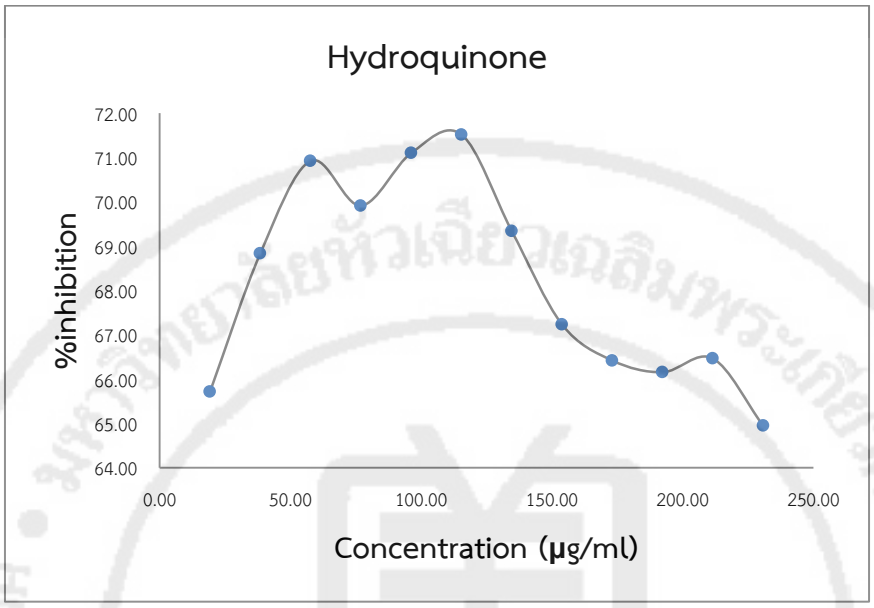


Figure 25 Tyrosinase inhibitory activity of compound 5a

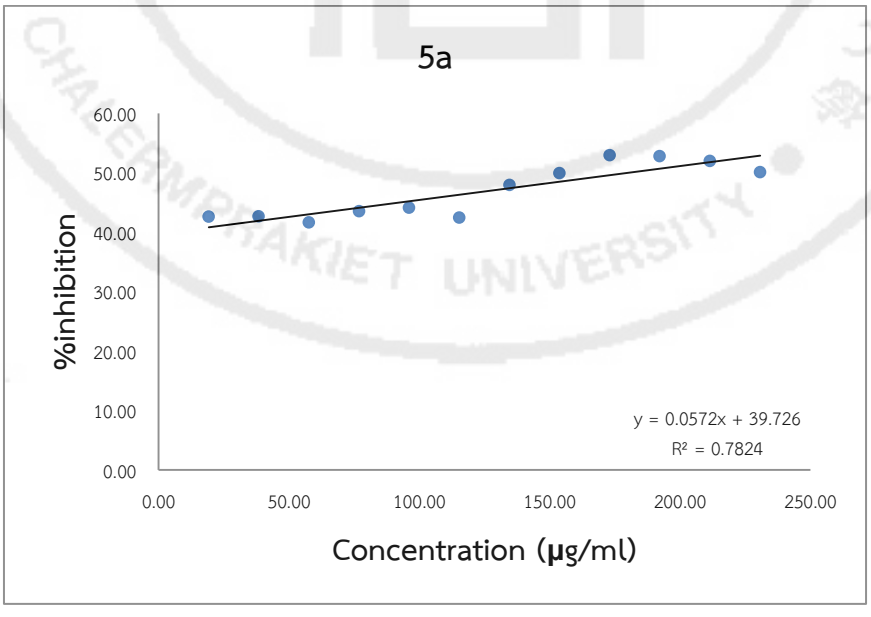


Figure 26 Tyrosinase inhibitory activity of compound 5b

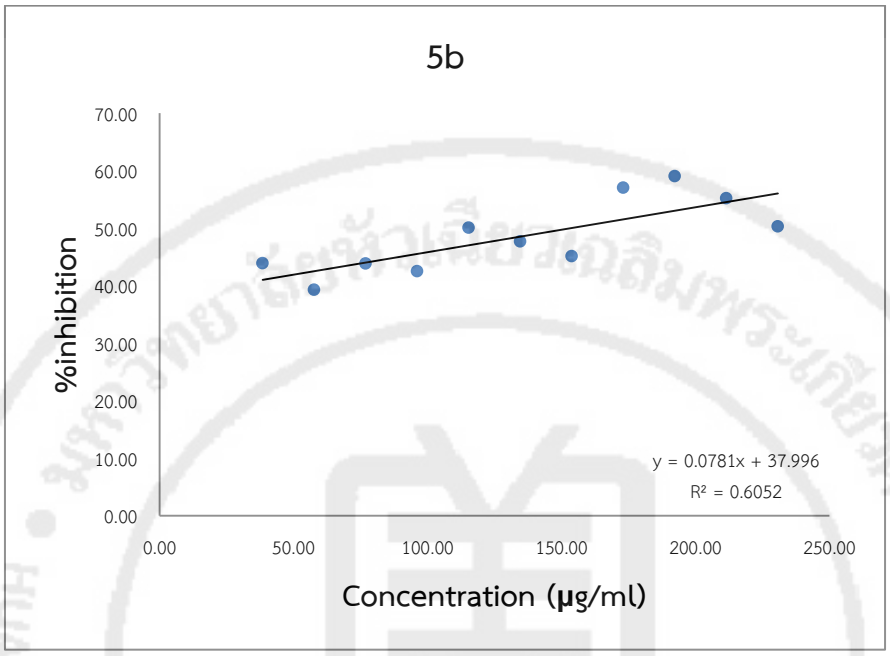
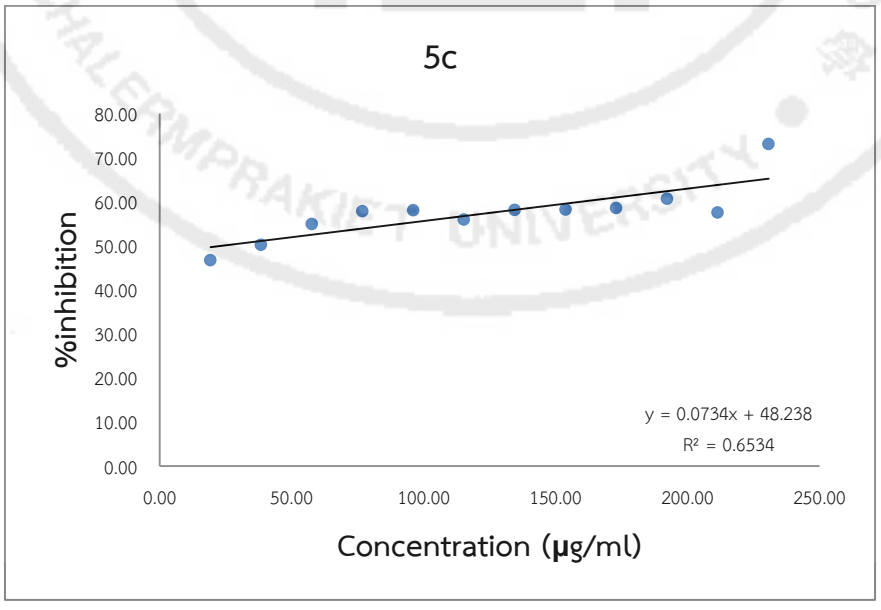


Figure 27 Tyrosinase inhibitory activity of compound 5c



Appendix D

DPPH assay result

Table 1 %inhibition of cinnamic acid against DPPH free radical

Compound	Initial concentration (µg/ml)	Volume (µl)	Final concentration (µg/ml)	%inhibition
Cinnamic	150 µg/ml	10	5.56	6.08
acid		20	11.11	7.44
		30	16.67	12.70
		40	22.22	11.19
		50	27.78	8.26
		60	33.33	9.52
		70	38.89	5.83
		80	44.44	10.17
		90	50.00	3.65
		100	55.56	8.88
		110	61.11	4.19
		120	66.67	9.47

Table 2 %inhibition of pyrocatechol against DPPH free radical

Compound	Initial concentration (µg/ml)	Volume (µl)	Final concentration (µg/ml)	%inhibition
Pyrocathechol	100 µg/ml	10	3.70	19.91
		20	7.41	41.28
		30	11.11	48.98
		40	14.81	47.23
		50	18.52	53.07
		60	22.22	58.66
		70	25.93	66.52
		80	29.63	68.33
		90	33.33	70.74
		100	37.04	75.24
		110	40.74	78.25
		120	44.44	79.63

Table 3 %inhibition of resorcinol against DPPH free radical

Compound	Initial concentration (µg/ml)	Volume (µl)	Final concentration (µg/ml)	%inhibition
Resorcinol	100 mg/ml	10	3.70	10.89
		20	7.41	7.38
		30	11.11	9.29
		40	14.81	12.71
		50	18.52	14.85
		60	22.22	19.16
		70	25.93	20.69
		80	29.63	23.98
		90	33.33	23.64
		100	37.04	17.62
		110	40.74	22.49
		120	44.44	24.18

Table 4 %inhibition of hydroquinone against DPPH free radical

Compound	Initial concentration (µg/ml)	Volume (µl)	Final concentration (µg/ml)	%inhibition
Hydroquinone	50 µg/ml	10	1.85	15.28
		20	3.70	29.03
		30	5.56	42.36
		40	7.41	55.75
		50	9.26	67.98
		60	11.11	80.36

Table 5 %inhibition of compound 5a against DPPH free radical

Compound	Initial concentration (mg/ml)	Volume (µl)	Final concentration (mg/ml)	%inhibition
5a	250 mg/ml	10	9.26	20.61
		20	18.52	28.48
		30	27.78	27.94
		40	37.04	36.30
		50	46.30	35.93
		60	55.56	43.10
		70	64.81	41.28
		80	74.07	45.71
		90	83.33	46.34
		100	92.59	50.84
		110	101.85	46.30
		120	111.11	52.77

Table 6 %inhibition of compound 5b against DPPH free radical

Compound	Initial concentration (mg/ml)	Volume (µl)	Final concentration (mg/ml)	%inhibition
5b	250 mg/ml	10	9.26	4.37
		20	18.52	9.52
		30	27.78	11.56
		40	37.04	13.94
		50	46.30	15.87
		60	55.56	19.18
		70	64.81	21.08
		80	74.07	23.55
		90	83.33	21.67
		100	92.59	26.54
		110	101.85	30.48
		120	111.11	34.81

Table 7 %inhibition of compound 5c against DPPH free radical

Compound	Initial concentration (µg/ml)	Volume (µl)	Final concentration (µg/ml)	%inhibition
5c	500 µg/ml	10	18.52	7.32
		20	37.04	10.64
		30	55.56	13.32
		40	74.07	18.48
		50	92.59	20.54
		60	111.11	23.12
		70	129.63	26.88
		80	148.15	30.65
		90	166.67	39.31
		100	185.19	45.54
		110	203.70	51.93
		120	222.22	56.60

Appendix E

Tyrosinase assay result

Table 8 %inhibition of kojic acid against tyrosinase from mushroom

Compound	Concentration final (µg/ml) (µg/ml)	%inhibition
Kojic acid	3.85	35.32
	7.69	45.91
	11.54	49.67
	15.38	55.12
	19.23	59.89
	23.08	66.31
	26.92	70.15
	30.77	76.55
	34.62	83.14
	38.46	86.92

Table 9 %inhibition of compound 5a-5c, cinnamic acid, pyrocatechol, resorcinol and hydroquinone against tyrosinase from mushroom

Compound / Concentration (µg/ml)	%inhibition						
	5a	5b	5c	Cinna-mic acid	Pyro-catecol	Resor-cinol	Hydro-quinone
19.23	42.59	-	46.62	35.65	28.57	55.52	65.71
38.46	42.64	43.89	50.11	36.66	22.13	61.27	68.84
57.69	41.63	39.23	54.81	38.48	19.08	63.36	70.92
76.92	43.49	43.82	57.76	39.25	13.74	69.60	69.91
96.15	44.09	42.48	58.00	39.01	6.04	71.71	71.10
115.38	42.42	50.04	55.85	39.76	1.54	69.56	71.52
134.62	47.93	47.65	58.04	40.00	-3.85	72.11	69.34
153.85	49.93	45.08	58.13	41.01	-3.36	68.07	67.23
173.08	52.95	57.01	58.48	41.12	-6.48	69.65	66.42
192.31	52.77	58.99	60.55	41.16	-7.89	68.75	66.15
211.54	51.93	55.19	57.49	41.10	-14.13	70.24	66.46
230.77	50.09	50.26	73.03	40.70	-37.85	64.97	64.95

Appendix F

Cytotoxicity assay result

Table 10 %cytotoxicity of compound 5a-5c and hydroquinone

Compound / Concentration (µg/ml)	%cytotoxicity			
	5a	5b	5c	Hydro-quinone
100.00	33.37	-4.80	12.51	98.44
50.00	28.22	-9.03	-1.71	96.41
25.00	21.36	-3.44	-2.19	66.19
12.50	13.39	-7.50	1.04	26.07
6.25	-1.05	-7.82	-5.01	19.15
3.13	-5.58	-0.78	-6.63	16.32

BIOGRAPHY

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2010	Faculty of Science and Technology, Thammasat Univresity
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