การแยกและการพิสูจน์เอกลักษณ์ของแอนโทไซยานินจากแกลบข้าวพันธุ์ชัยนาท1

Oryza sativa L. cv. Chainat1

นางสาวกาญจมาศ อธิคม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SEPARATION AND IDENTIFICATION OF ANTHOCYANIN FROM RICE HUSK OF *Oryza sativa* L. cv. Chainat1.

Miss Kanchamas Atikom

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biotechnology
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Thesis Title	Separation and identification of anthocyanin from rice husk of *Oryza sativa* L. cv. Chainat1
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Field of Study	Biotechnology
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กาญจมาศ อธิคม : การแยกและการพิสูจน์เอกลักษณ์ของแอนโทไซยานินจากแกลบข้าวพันธุ์ชัยนาท1 *Oryza sativa* L. cv. Chainat1 (SEPARATION AND IDENTIFICATION OF ANTHOCYANIN FROM RICE HUSK OF *Oryza sativa* L. cv. Chainat1) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.ศุภศร วนิชเวชารุ่งเรือง, 44 หน้า.

ในงานวิจัยนี้ได้ทำการแยกและพิสูจน์เอกลักษณ์ของแอนโทไซยานินจากแกลบข้าวพันธุ์ชัยนาท1 โดยนำส่วนสกัดไดคลอโรมีเทนและเอทิลอะซิเตทมาทำการแยกด้วยเทคนิคโครมาโทกราฟี การพิสูจน์เอกลักษณ์ของสารแอนโทไซยานินที่ได้โดยใช้วิธีทางสเปกโตรสโกปี พบว่า คือ มีสารแอนโทไซยานินประเภท malvidin-4-rutinoside เป็นสารหลักที่ให้สีชมพู (คิดเป็น 0.92% โดยน้ำหนัก) และสันนิษฐานว่า compound 2 น่าจะเป็นสารแอนโทไซยานินชนิดใดชนิดหนึ่ง และจากการทดสอบฤทธิ์ต้านอนุมูลอิสระ พบว่า malvidin-3-rutinoside มีค่า IC_{50} เท่ากับ 145 ± 30 ไมโครกรัมต่อมิลลิลิตร ในขณะที่ BHT ให้ค่า IC_{50} เป็น 11.6 ± 2.6 ไมโครกรัมต่อมิลลิลิตร

ภาควิชา....เทคโนโลยีชีวภาพ..... ลายมือชื่อนิสิต.....กาญจมาศ อธิคม.....
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In this work, separation and identification of anthocyanin from rice husk of *Oryza sativa* L. cv. Chainat1 was carried out. The combination of dichloromethane and ethyl acetate crude extracts was isolated by various chromatographic techniques. The isolation and the structure determination of these pink compound was performed as described previously. Compound 2 was indicated that it is one type of anthocyanin. Malvidin-3-rutinoside is a major pigment that give pink color in this compound. Malvidin-3-rutinoside and BHT showed the IC_{50} value of 145 ± 30 and 11.6 ± 2.6 μg/ml, respectively.

Department :.....Biotechnology..... Student's Signature....Kanchamas Atikom.....
Academic Year :.....2009..... Advisor's Signature.....

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%	percentage
cm	centimeter
mm	millimeter
ROS	reactive oxygen species
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
TBHQ	tertiary butylhydroquinone
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
mg	milligram
g	gram
IR	infrared
ESI-MS	electrospray ionization-mass spectrometry
^{1}H NMR	proton nuclear magnetic resonance
^{13}C NMR	carbon nuclear magnetic resonance
HPLC	high-performance liquid chromatography
TLC	thin layer chromatography
γ	gamma
p	para
MHz	megahertz
ppm	part per million

□	chemical shift
Kg	kilogram
mL	milliliter
μL	microlitre
mM	millimolar
nm	nanometer
w/w	weight by weight
R_f	retardation factor
HSQC	heteronuclear single quantum coherence
HMBC	heteronuclear multiple bond correlation
COSY	correlation spectroscopy
λ_{max}	maximum wavelength
M	molar
ε	molar absorptivity
IC_{50}	half maximal inhibitory
J	coupling constant
s	singlet
d	doublet
t	triplet
MW	molecular weight
m/z	mass-to-charge ratio

CHAPTER I
INTRODUCTION

Rice (*Oryza sativa*)

Rice is the principal cereal food in Asia, and the staple food for nearly half of the world's population. The top two rice exporters as of 2009 are Thailand (30.5%) and Vietnam (17.6%). There are two species of rice in the Poaceae (true grass) family, *Oryza sativa* and *Oryza glaberrima*. These plants can be found in native to tropical and subtropical southern and southeastern Asia and Africa. Rice provides more than one fifth of the calories consumed by humans in their global diets[1].

Rice is a monocarpic annual plant, growing to 1-1.8 m tall depending on the variety and soil fertility. The grass has long, slender leaves (50-100 cm long and 2-2.5 cm broad). The small wind-pollinated flowers are produced in a branched arching to pendulous inflorescence 30-50 cm long. The seed is a grain with 5-12 mm long and 2-3 mm thick. The structure of rice grain is illustrated in Figure 1.1

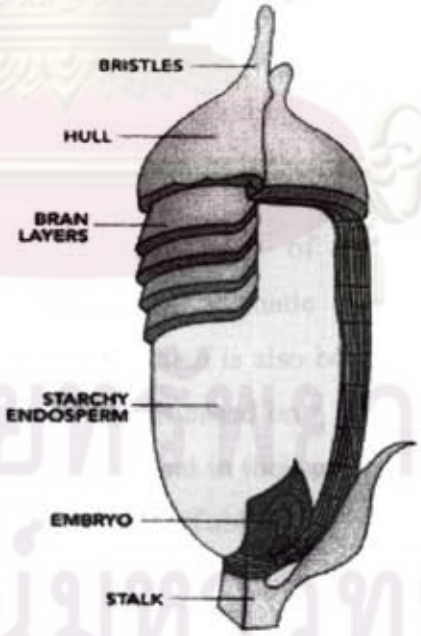


Figure 1.1 The structure of rice grain

Rice husk or rice hull is the hard cover of rice grain which protect the grain from various threats. Although very important in nature, it is one of the major minimal value by-product of the rice industry. Recently, utilization of agricultural residues has been in focused either in an energy aspect or an extraction technologies aspect, both with the aim to make an extra economically value to the residues. Because rice is the Thailand's most important crop, a lot of agricultural residue from rice, including rice husk and rice straw are abundant. However, rice husks are an agricultural byproduct that possessed a very serious problem for the global environment. Attempts to use rice husk as a raw material for extraction of value chemicals has been triggered from a recent supports which indicated that rice husks contain antioxidant substance that protect rice seed from oxidative stress [2]. Also, food applications of rice hull antioxidants are being explored. In the cosmetic industry, attempts are being made to use rice husk antioxidants in the formulation of body lotions end creams used mainly for the protection of the skin against the adverse effects of aging [3].

Anthocyanins

Anthocyanins are the most important pigments of the vascular plants, they are harmless and has been used, as natural water-soluble colorants. These pigments are responsible of the shiny orange, pink, red, violet and blue colours in the flowers and fruits. Another significant property of anthocyanins is their antioxidant activity [4].

Anthocyanidins are the basic structures of the anthocyanins. The anthocyanidins (or aglycons) consist of an aromatic ring [A] bonded to an heterocyclic ring [C] that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring [B]. They are all based on a single basic core structure (Figure 1.2). When the anthocyanidins are found in their glycoside form (bonded to a sugar moiety) they are known as anthocyanins. (Table 1.1)

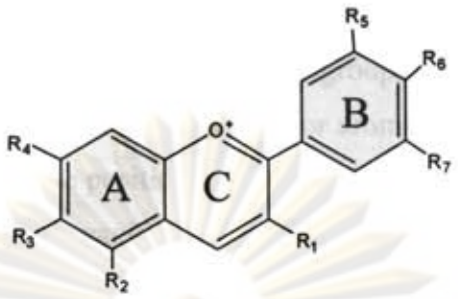


Figure 1.2 The aglycons, the basic structure of anthocyanins

Name	Substitution pattern						
	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Apigeninidin	H	OH	H	OH	H	OH	H
Arrabidin	H	H	OH	OH	H	OH	OMe
Aurantinidin	OH	OH	OH	OH	H	OH	H
Capensinidin	OH	OMe	H	OH	OMe	OH	OMe
Carajurin	H	H	OH	OH	H	OMe	OMe
Cyanidin	OH	OH	H	OH	OH	OH	H
Delphinidin	OH	OH	H	OH	OH	OH	OH
Europinidin	OH	OMe	H	OH	OMe	OH	OH
Hirsutidin	OH	OH	H	OMe	OMe	OH	OMe
3′-Hydroxyarrabidin	H	H	OH	OH	OH	OH	OMe
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	OH
6-Hydroxydelphinidin	OH	OH	OH	OH	OH	OH	OH
6-Hydroxypelargonidin	OH	OH	H	OH	H	OH	H
Peonidin	OH	OH	H	OH	OMe	OH	H
Petunidin	OH	OH	H	OH	H	OH	OH
Pulchellidin	OH	OMe	H	OH	OH	OH	OH
Riccionidin A	OH	H	OH	OH	H	OH	H
Rosinidin	OH	OH	H	OMe	OMe	OH	H
Tricetinidin	H	OH	H	OH	OH	OH	OH

Table 1.1 Structural identification of anthocyanidins (aglycons) [5].

There is a huge variety of anthocyanins spread in nature. The main differences between them are the number of hydroxylated groups, the nature and the number of bonded sugars to their structure, the aliphatic or aromatic carboxylates bonded to the sugar in the molecule and the position of these bonds. The glycoside derivatives of the three non-methylated anthocyanidins (Cyanidin, Delphinidin and Pelargonidin) are the most common in nature, being found in 80% of pigmented leaves, 69% in fruits and 50% in flowers. The distribution of the six more common anthocyanidins in fruits and vegetables is: Cyanidin 50%, Delphinidin 12%, Pelargonidin 12%, Peonidin 12%, Petunidin 7% and Malvidin 7%. The following four classes of anthocyanidin glycosides are common: 3-monosides, 3-biosides, 3,5-diglycosides and 3,7-diglycosides. 3-Glycosides account for about two and half times of 3,5-diglycosides. So, the most wide-spread anthocyanin is cyanidin 3-glucoside. In relation to the stability, anthocyanins are compounds that may suffer reactions that alter their structures through the action of different agents due to the electronic deficiency of their flavylium nuclei. Anthocyanin stability increases with the number of methoxyl groups in the B ring and decreases as hydroxyl groups increase. Thus, among the most common anthocyaninidins, the most stable one is malvidin, followed by peonidin, petunidin, cyanidin and delphinidin. In general, anthocyanins are more stable in an acidic pH. Glycosylation and acylation of the sugars also help increasing the stability and, therefore, the diglycosides are more stable than their corresponding monoglycosides.

Anthocyanins possess known pharmacological properties and are used by humans for therapeutic purposes. The blue, red and purple varieties of some cereals are drawing the attention of scientists and the food industry, since they are potential sources for anthocyanins. These compounds are found in some cereals, such as in purple corn, in such quantities as to make commercialisation of the extracts viable, moreover, the fact that they are frequently located in external tissues of the plant greatly facilitates their production [6, 7].

The phenolic structure of anthocyanins is responsible for their antioxidant activity; i.e., ability to scavenge reactive oxygen species (ROS) such as superoxide, peroxide and hydroxyl radical [8]. They have been shown to suppress angiogenesis

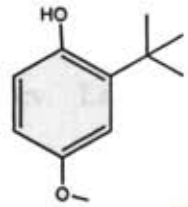
through several mechanisms, and exhibited anti-inflammatory effects in multiple cell types in vitro [9, 10].

Anthocyanins are also found in husk of red rice and black rice grain. Coloured rice has been consumed traditionally in Asian countries and their constituted anthocyanin pigments are used as food colorants in the elaboration of alcoholic beverages [11]. The principal anthocyanin in rice is cyanidin-3-glucoside, followed in minor proportion by peonidin-3-glucoside [12, 13].

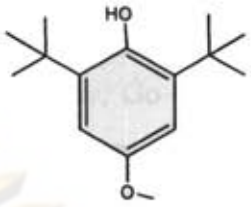
Antioxidant activity

The word "free radicals" and "antioxidants" have become well known for health-conscious consumers. Free radical and reactive oxygen species, collectively known as ROS are generated continuously via normal physiological processes, more so in pathological conditions. Reactive oxygen intermediates are partially reduced forms of atmospheric oxygen. Reactive free radicals have been postulated to contribute to the causes of chronic inflammatory proliferative diseases, especially arteriosclerosis and cancer, through oxidative damage of essential enzymes, cells, and tissues [14].

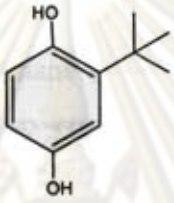
The type of antioxidant are classified into two categories; synthetic and natural antioxidant. In general, the more popular synthetic antioxidants (Figure 1.3) used are phenolic compounds which are always substituted by alkyl to improve their solubility in fats and oils, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butylhydroquinone (TBHQ). However, recently popularily towards natural antioxidant has been increased. Natural antioxidants are found in almost all plants, microorganism, fungi and even in animal tissue. The natural antioxidants are mortly phenolic compounds (tocopherol, flavonoids and phenolic acid), nitrogen containing compounds (alkaloids, chlorophyll derivatives, amino acids and amines) or catotenoids as well as ascorbic acid.



butylated hydroxyanisole (BHA)



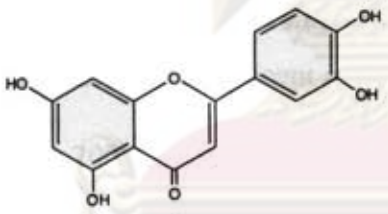
butylated hydroxytoluene (BHT)



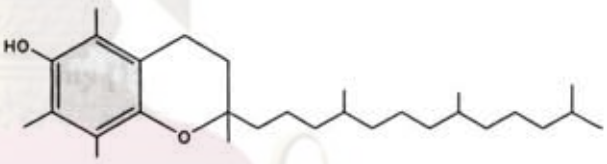
tertiary butylhydroquinone (TBHQ)

Figure 1.3 The synthetic antioxidants

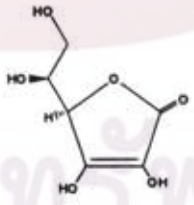
The antioxidant constituents in plants compose of revealed various types of phytochemicals such as luteolin, tocopherol and ascorbic acid (Figure 1.4).



Luteolin



Tocopherol



Ascorbic acid

Figure 1.4 The natural antioxidants

From previous work, rice husk from five strains of Thai rice cultivar, *Oryza sativa* L. cv. Chai-nat1 (CN), *Oryza sativa* L. cv. Look Dang Pattani (LD), *Oryza sativa* L. cv. Leb Nok Pattani (LN), *Oryza sativa* L. cv. Go Kol (GK) and *Oryza sativa* L. cv. Jasmine (JM), were extracted with hexane, dichloromethane, ethyl acetate, methanol and screened for free radical scavenging activity. CN rice husk gave the most potent activity with the highest extraction yield in their dichloromethane and ethyl acetate crude extracts, thus thesethe dichloromethane and ethyl acetate crude extracts of CN were subjected for further isolation and purification. Both the dichloromethane and ethyl acetate crude extracts of CN gave similar TLC pattern hence these two crude extract were combined before subjected to further fractionation. In this study, the isolation and identification for anthocyanin extraction from rice husk of *Oryza sativa* L. cv. Chai-nat1 has been investigated.

Literature reviews

In 1994, Wu and coworker extracted wild rice with methanol, ethanol, and ethyl acetate. The antioxidant activitys of the extracts were measured by thiobarbituric acid reactive substance values in ground beef and by peroxide values in lard. The extracts showed a significant antioxidant activity [15].

In 2002, Oki and coworker extracted hulls from white, black and red rice were prepared by sequential extraction with six different polar solvents, and their radical-scavenging activities were measured by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method. The methanol- and water-extracts exhibit the highest radical scavenging activity. The hull of red rice showed the highest antioxidant activity [16].

In 2004, Awika and coworker analyzed anthocyanins from black sorghums (*Sorghum bicolor* L. Moench) by spectrophotometric and High-Performance Liquid Chromatography techniques (HPLC) methods. Apigeninidin (Figure 1.5A) and luteolinidin (Figure 1.5B) accounted for about 50% of the anthocyanins in the black sorghums. Then the samples were also analyzed for antioxidant activity using the 2,2-azinobis (3-ethylbenzothiaziline-6-sulfonic acid) method. The sorghum grains and their brans possessed high antioxidant activity compared to wheat, barley and oats [17].

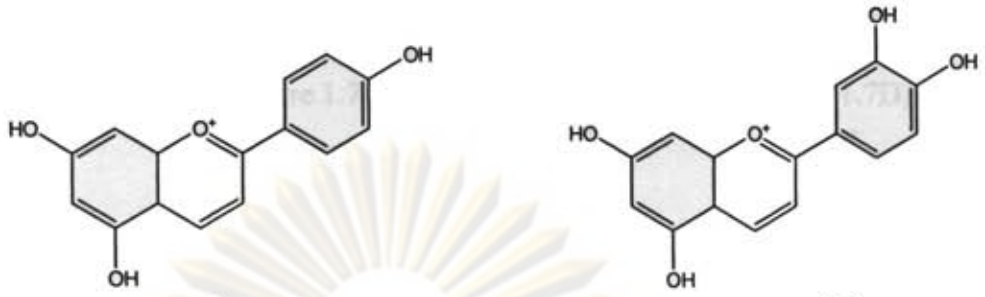


Figure 1.5 Structure of A) apigeninidin, B) luteolinidin

In 2004, Awika and coworker characterized anthocyanins from black, brown (containing tannins), and red sorghums by spectrophotometric and HPLC. The antioxidant activity of the anthocyanins were also determined. Black sorghum bran had the highest anthocyanin content (average of 10.1 mg/g). The brown and red sorghum brans had anthocyanin contents of 2.8-4.3 mg/g. And Only 3-deoxyanthocyanidin (Figure 1.6) was detected in sorghum. Additionally, crude sorghum anthocyanin extracts were more stable than the pure 3-deoxyanthocyanidins [18].

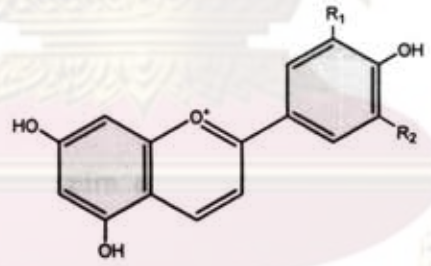


Figure 1.6 Structure of 3-deoxyanthocyanidins

In 2006 Zhang and coworker separated, purified and identified the antioxidative compositions of black rice (*Oryza sativa* L. Indica). The main antioxidative components were separated from the strongest antioxidative fractions by Sephadex LH-20 resin and the structures were analyzed by UV-Visible, IR, ESI-MS, ^{1}H-NMR and FT-NMR spectrums. The spectroscopic analysis indicated that the four active components of the antioxidative extract of black rice were four anthocyanin

compounds of malvidin (Figure 1.7A), pelargonidin-3, 5-diglucoside (Figure 1.7B), cyanidin-3-5-diglucoside (Figure 1.7C) and cyanidin-3-glucoside (Figure 1.7D) [19].

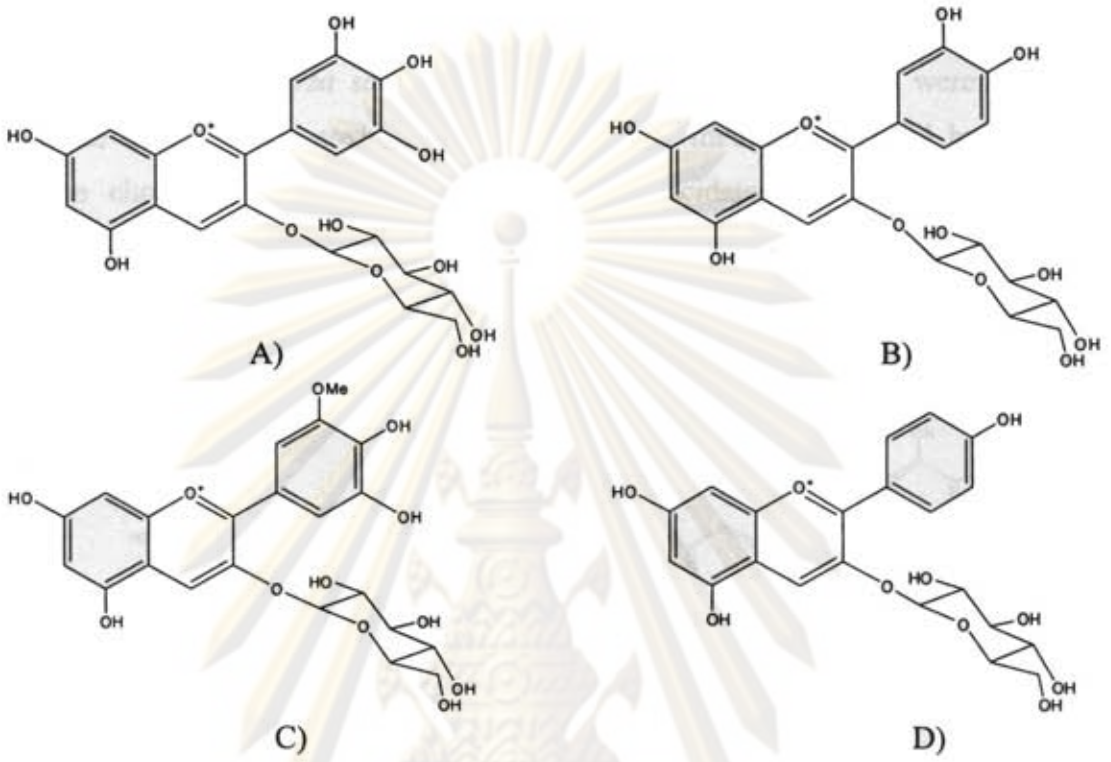


Figure 1.7 Structure of A) malvidin, B) pelargonidin-3,5-diglucoside, C) cyanidin-3,5-diglucoside and D) cyanidin-3-glucoside

In 2007 Prata and Oliveira extracted fresh coffee husks and analyzed the obtained extract by HPLC. Cyanidin-3-rutinoside (Figure 1.8) was characterized as the dominant anthocyanin in fresh coffee husks and the result indicated that the fresh coffee husks was a good source of this pigment [20].

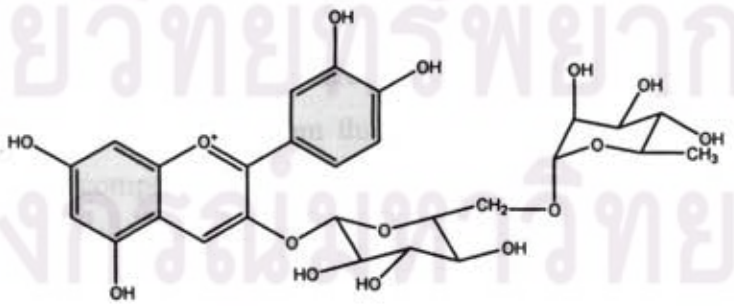


Figure 1.8 Structure of cyanidin-3-rutinoside

In 2008 Hosseinian and coworker isolated anthocyanin, cyanidin-3-glucoside, from purple wheat (*Poaceae Triticum* L.) [21].

In 2009 Lee and coworker isolate and identify the anthocyanins in the black seed-coat soybean (*Oryza sativa* L. cv. Cheongja 3). Anthocyanins were extracted from the coat of black soybeans with 1% TFA in methanol, isolated by RP-C-18 column chromatography, and their structures elucidated by 1D and 2D NMR spectroscopy. The isolated anthocyanins were characterized as delphinidin-3-glucoside (Figure 1.9A), cyanidin-3-glucoside (Figure 1.9B), petunidin-3-glucoside (Figure 1.9C) and pelargonidin-3-glucoside (Figure 1.9D) [22].

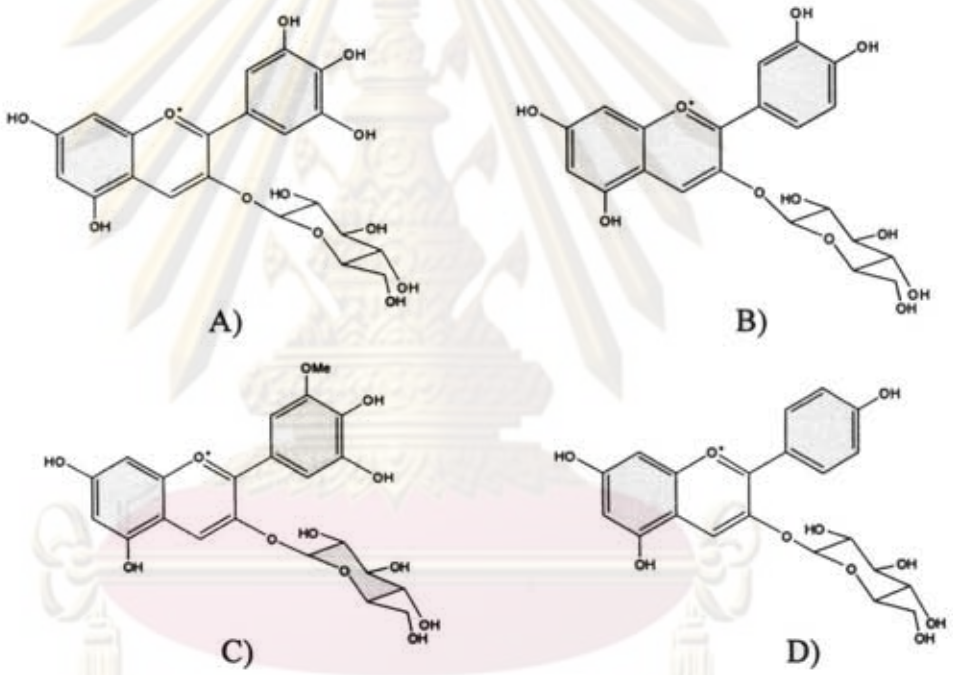


Figure 1.9 Structure of A) delphinidin-3-glucoside, B) cyanidin-3-glucoside, C) petunidin-3-glucoside, D) pelargonidin-3-glucoside

In 2009 Butsat and Siriamornpun collected rice bran, rice husk, brown rice and milled ice of a Thai rice variety from three different growth sites, and determined their phenolic acid composition, γ-oryzanol (Figure 1.10A) and tocopherols (Figure 1.10B). The bran and husk fractions showed higher values of antioxidant activity based on the DPPH assays. In addition, the bran fraction had the highest γ-oryzanol and tocopherols content. On the other hand, the husk fraction showed a greater phenolic acids concentration than the other fractions. Ferulic acid (Figure 1.10C) was

most evident in the bran, whereas vanillic (Figure 1.10D) and *p*-coumaric acids (Figure 1.10E) were mostly found in the husk. This study demonstrates that rice bran and husk as candidates of valuable sources of bioactive components with high antioxidant properties [23].

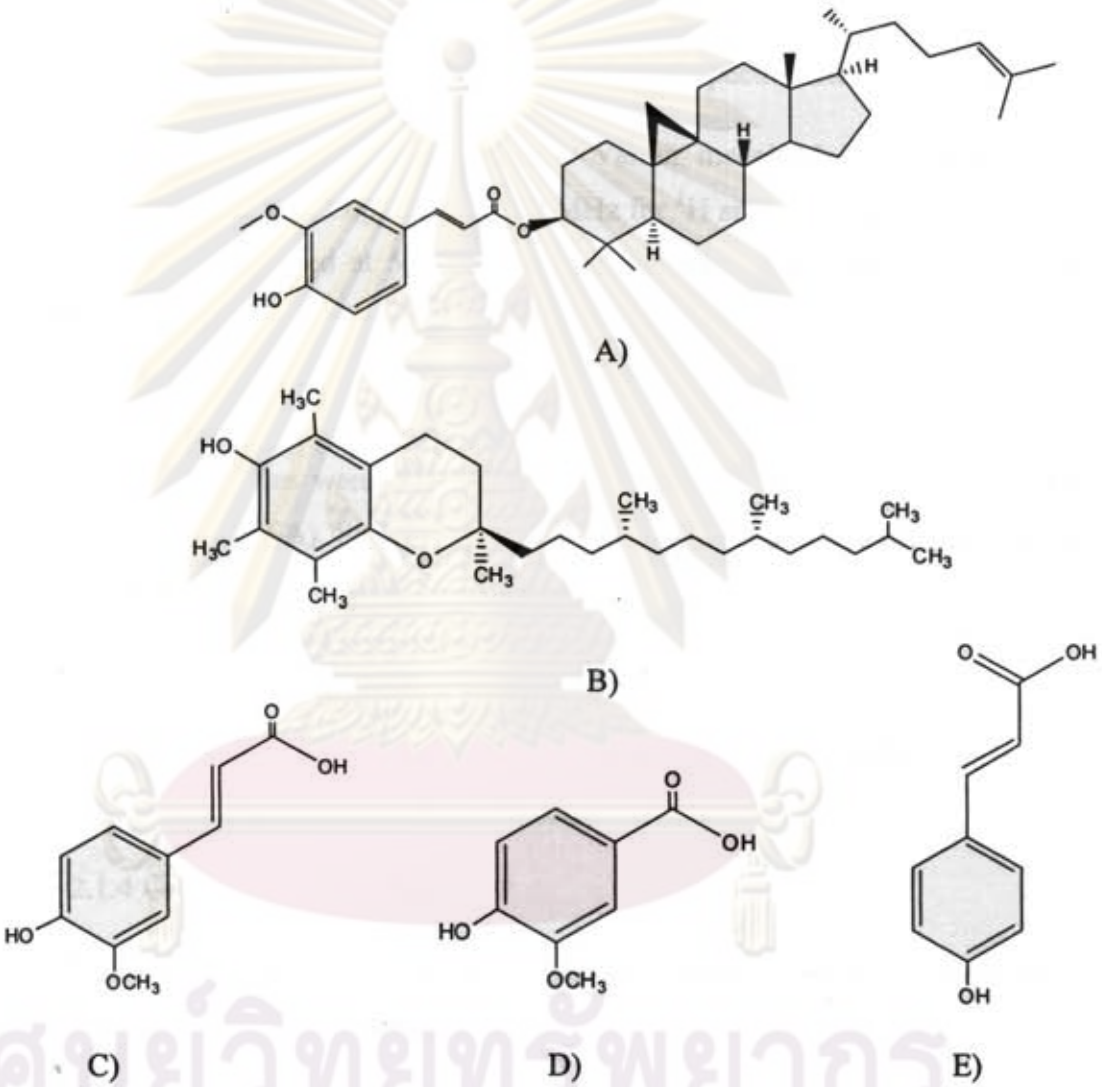


Figure 1.10 Structure of A) γ-oryzanol, B) tocopherol, C) ferulic acid, D) vanillic and E) *p*-coumaric acids

Research's Objective

1. To separate and identify of anthocyanin from rice husk of *Oryza sativa* L. cv. Chainat1.
2. To determine antioxidant activity of the obtained anthocyanin.

CHAPTER II
EXPERIMENTAL

2.1 Instruments and Equipments

2.1.1 ^{1}H and ^{13}C Nuclear Magnetic Resonance Spectrometer

NMR spectra were recorded with a Varian medel Mecury+ 400 (Varian company, CA, USA) which operated at 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei. Varian Inova 500 operated at 500 MHz for ^{1}H was used to obtain the J-resolved projection spectrum (STREC, Chula, Thailand)

2.1.2 Mass Spectrometer

ESI-MS analyses were performed with Waters Micromass Quattomicro API ESCi (Waters, MA, USA). Sample was dissolved in MeOH and directly injected into the mass spectrometer.

2.1.3 Preparative Thin Layer Chromatography (Prep TLC)

TLC was performed on glass plates precoated with silica gel (Merck Kieselgel 60 GF254) (Merck KgaA, Darmstadi, Germany).

2.1.4 Column chromatography

Column chromatography was performed on silica gel (Merck Kieselgel 60 G) (Merck KgaA, Darmstadi, Germany).

2.1.5 Microtiter plate spectrophotometer

UV-Vis spectrometer, microtiter plate reader, model sunrise (TECAN, Salzburg, Austria) was used in the experiments.

2.1.6 UV-Visible Spectrophotometer

UV-Visible absorption spectra were obtained with the aid of UV 2500 UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan), using a quartz cell with 1 cm path length.

2.1.7 Infrared Spectrometer

The ATR-FTIR spectrum was recorded with Nicolet 6700 FT-IR spectrometer equipped with a mercury-cadmium-telluride (MCT) detector (Thermo Electron Corporation, USA).

2.2 Chemicals

Solvent used in spectroscopic techniques and antioxidant activity assay were reagent or analytical grades purchased from Labscan (Bangkok, Thailand). Solvent used in extraction and column chromatography were purified from commercial grade solvents prior use by distillation. A 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Fluka Chemie (Buchs, Switzerland). 2,6-D-tert-butyl-*p*-hydroxytoluene (BHT) was purchased from Panreac Sintesis (Bacelona, Spain)

2.3 Sample materials

The rice husk of *Oryza sativa* L. cv. Chainat1 was obtained from Rice Research Institute of Patthalung, Thailand.

2.4 Extraction, Separation and Purification

Oryza sativa L. cv. Chainat1 strain rice husk (3.7 kg) was milled and then extracted by maceration at room temperature with hexane (100 mL) for 10 days. The mixture was filtered though a Whatman GF/A filter paper and the liquid hexane extract was collected. The filtered precipitate was macerated with a fresh portion of dichloromethane (CH_2Cl_2) for another 10 days and the CH_2Cl_2 extract was collected. Similar procedures was repeated with ethyl acetate (EtOAc) and methanol (MeOH), to obtain EtOAc and MeOH extracts, respectively. Each extract was filtered and evaporated under reduced pressure to obtain dried crude extract. These extracts were

run on TLC using 7% MeOH in CH_2Cl_2 as a mobile phase. The extracts with similar TLC pattern were combined and named as CNI extract.

The combined dichloromethane and ethyl acetate extract (16.7 g, 0.45% w/w) (CNI) was fractionated over column chromatography using gradient elution with dichloromethane-methanol (100:0 to 60:40) to obtain 8 fractions (CNI-1 to CNI-8) [24].

CNI-7 (5.79 g, 35% w/w) was chromatographed over silica gel column, gave 8 fraction, CNI-7/1 to CNI-7/8. The CNI-7/5 was further purified by silica gel column using gradient elution of ethyl acetate-methanol (100:0 to 50:50) and followed by preparative thin layer chromatography with 50% MeOH in EtOAc. The isolated pink compound was then subjected to spectroscopic analysis (Scheme 2.1).

2.5 Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was selected for determining the antioxidant activity of *Oryza sativa* L. cv. Chainat1 rice husk extract.

DPPH radical scavenging activity

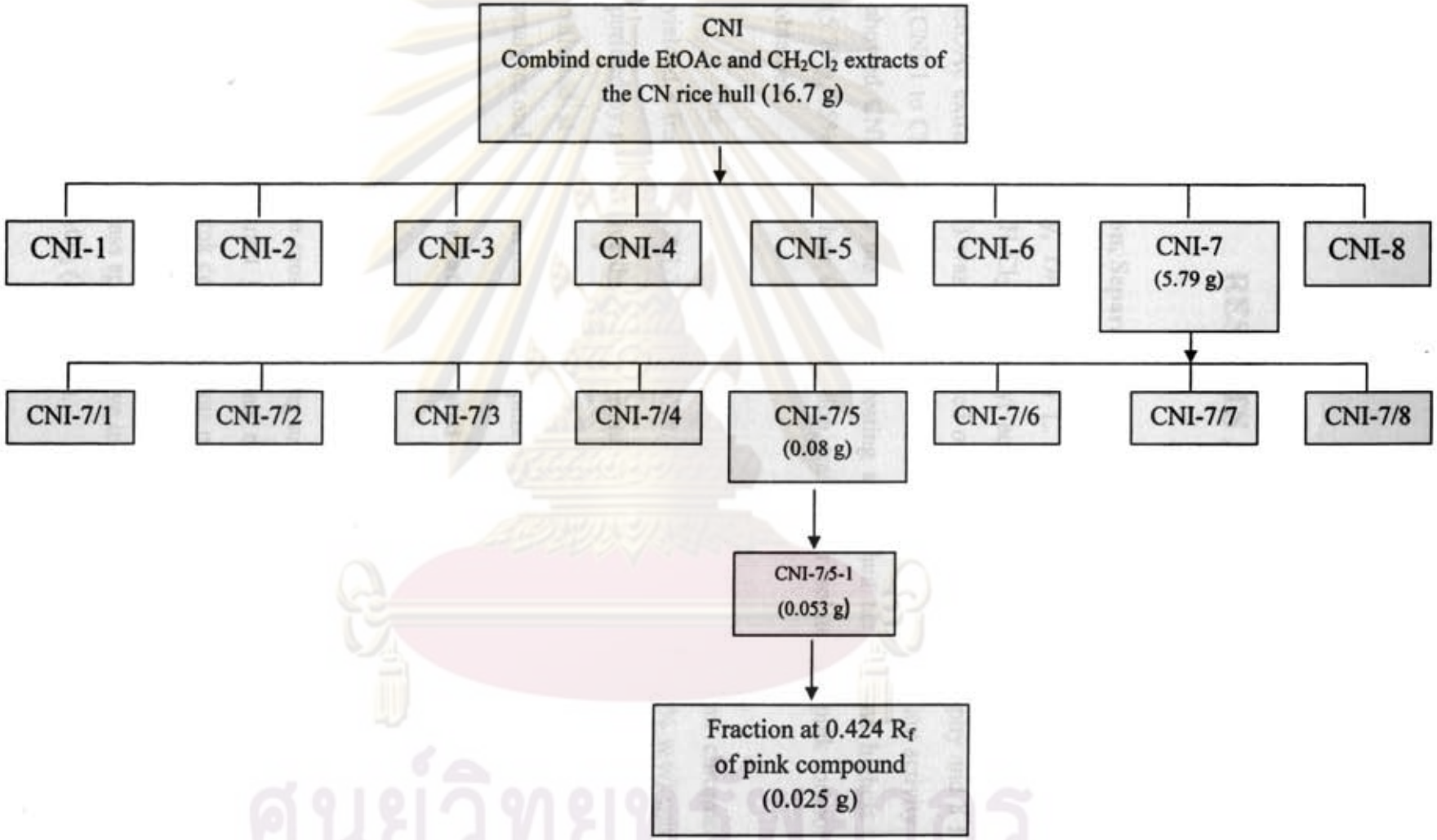
Various concentrations of samples dissolved in methanol (50 μL) were added to methanolic DPPH radical solution (0.25 mM, 100 μL) in the 96-well microplate. After 30 minutes incubation at room temperature in the dark, the absorbance was measured at 517 nm. All tests were run in triplicate. The scavenging activity was evaluated from the decrease in absorbance at 517 nm. The activity is shown as percentage of radical scavenging. BHT was also determined and reported.

$$\text{Radical activity} = [A_1\text{-}A_2 / A_0] \quad [25]$$

A_0 was the absorbance of the control (without extract).

A_1 was absorbance at 517 nm of reaction mixture without test compounds.

A_2 was the absorbance without DPPH.



Scheme 2.1 Isolation of *Oryza sativa* L. cv. Chai-nat1

15

CHAPTER III
RESULTS AND DISCUSSION

3.1 Extraction, Separation and Purification

In this study, *Oryza sativa* L. cv. Chainat1 rice husk was extracted by dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc). The combined CH_2Cl_2 and EtOAc extract (CNI) was fractionated over column chromatography and 8 fractions (CNI-1 to CNI-8). These 8 fractions were tested for their antioxidant activity. Results showed CNI-7 gave the most interesting antioxidant activity and highest % yield (5.79 g, 35% w/w), thus, CNI-7 fraction, was further isolated and pink compound was obtained.

The isolation of CNI-7 performed by silica gel column chromatography yielded 8 fractions, CNI-7/1 to CNI-7/8. The CNI-7/5 (0.08 g, 14% w/w) was further purified by preparative thin layer chromatography (0.025 g, 0.43% w/w). Compound collected at R_f value of 0.424 was subjected to NMR, IR, UV-Visible and MS analyses and the result indicated the pink structure.

3.2 Characterization of isolated compound

Amongst many rice husk extracts, the CH_2Cl_2 and EtOAc crude extracts of *Oryza sativa* L. cv. Chai-nat1 strain possess the most potent free radical scavenging activity and gave the most interesting antioxidant activity. Fractionation and further isolation of the extract yielded pink compound. The isolation and the structure determination of a pink compound was performed as described previously. Malvidin-3-rutinoside (compound 1) is a major pigment that gave pink color in this compound [26,27]. The FAB mass spectrum gave its $[M]^+$ at 639 *m/z*, corresponding to the mass calculated for $C_{29}H_{35}O_{16}$ (Figure 3.1).

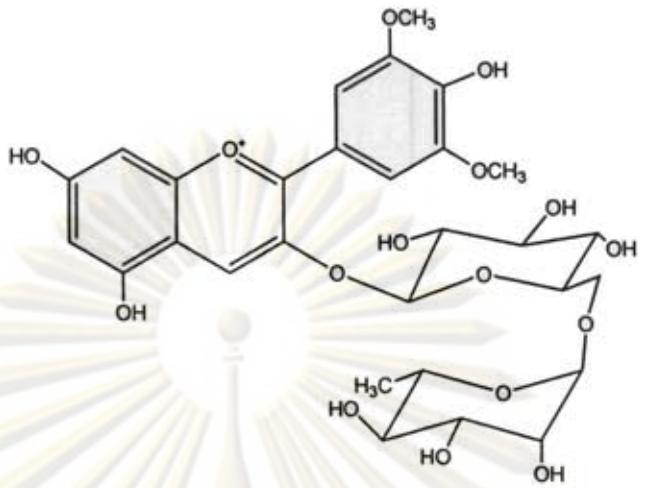


Figure 3.1 Structure of malvidin-3-rutinoside (compound 1).

^{1}H and ^{13}C NMR data and HSQC and HMBC correlation of compound 2 are shown in Table 3.1. The spectroscopic data indicated the presence of one unit of glucose linked to the compound 2 though the ether bond at C-1 of the glucose. IR spectrum showed absorbtion band of O-H stretching at 3365 cm^{-1} , C-H stretching at 2914 cm^{-1} and C=C stretching vibration of aromatic ring at 1582 cm^{-1}(Figure A-1, Appendix A, Page 26).

Table 3.1 ^{1}H and ^{13}C NMR data and HSQC and HMBC correlation for compound 2 (400 MHz, CD_3OD-d_4)

	^{1}H shift (ppm, *m*, *J*)	^{13}C (ppm)	HSQC	HMBC
compound 2				
H-2		168.4		
H-3	7.30 *t*(9.1)	131.8	H-3C	H-4C
H-4	7.01 *dd*(2.3,9.1)	113.5	H-4C	H-3C
H-5	6.79 *d*(9.1)	115.6	H-5C	H-4C

H-6	7.26 *t* (9.1)	128.9	H-6C	H-9C,H-10C
H-7	7.64 *m* (9.1)	129.1	H-7C	H-1'C,H-6'C
H-8	8.11 *dd* (2.3,9.1)	129.7	H-8C	H-6'C
H-1'		114.0		H-2'C,H-6'C
H-2'	6.94 *s*	95.6	H-2'C	H-1'C,H-3'C, H-4'C
H-3'		155.5		H-2'C
H-4'		157.9		H-2'C,H-6'C, H-6"C
H-5'	6.94 *s*	155.5	H-5'C	H-4'C,H-6'C
H-6'		95.6		H-1'C,H-4'C, H-5'C
Glucose				
H-1"	4.89 *s*	94.7	H-1"C	
H-2"		73.4	H-2"C	
H-3"	3.62 *dd* (1.7,7.4)	71.0	H-3"C	
H-4"	3.64 *t* (7.8)	56.9	H-4"C	H-2"C,H-4"C
H-5"	3.60 *t* (7.2)	71.0	H-5"C	
H-6"a	3.68 *m*	45.3	H-6"C	
H-6"b	3.64 *d* (7.0)			H-3"C,H-5"C
	3.66			H-4'C

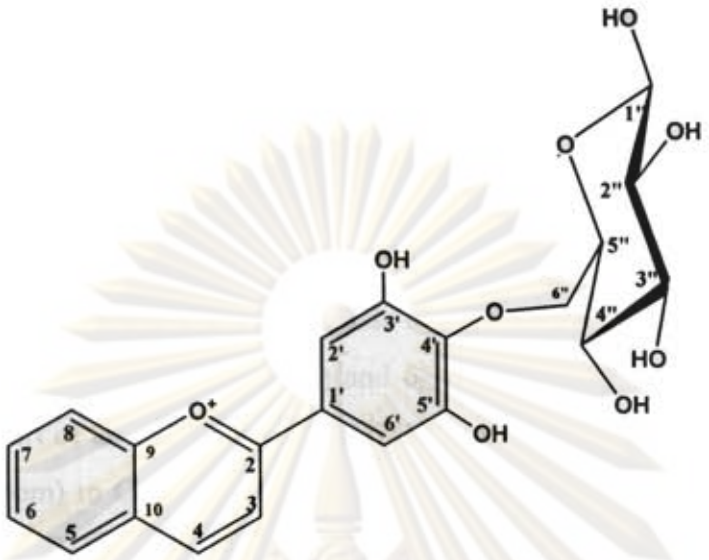


Figure 3.2 Structure of compound 2

The ^{1}H-^{1}H COSY spectrum (Figure B-3to B -5, Appendix B, Page 29-31) revealed the presence of the following connectivity as shown in Figure 3.3

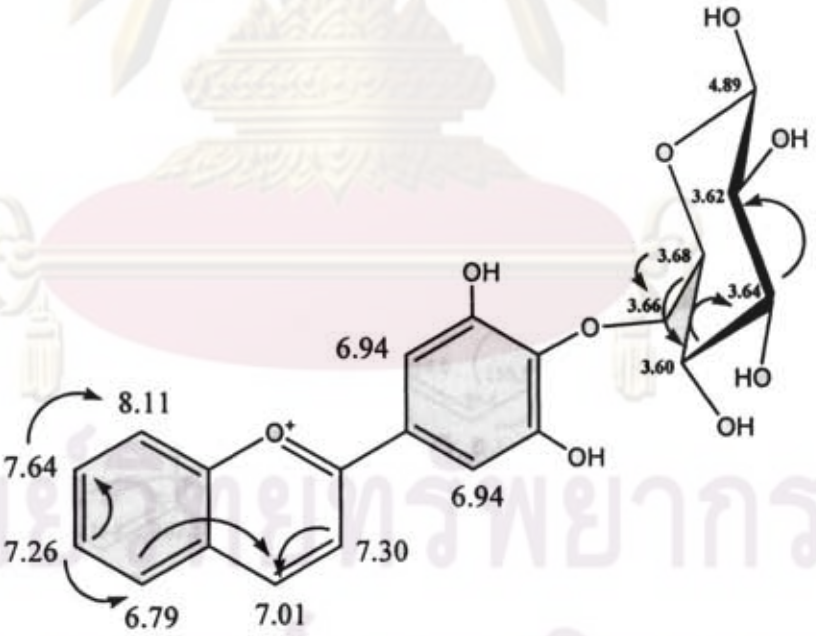


Figure 3.3 The COSY correlation of compound 2

The HMBC correlation (Figure 3.4 and Figure B-6 to B-8, Appendix B, Page 32-34) were observed between the proton signals at δ_H 7.30 (H-3) to carbon signal at δ_C 168.4 ppm (C-2), δ_C 113.5 ppm (C-4) and 162.1 ppm (C-10), between the proton

signals at δ_H 6.79 (H-5) to carbon signal at δ_C 113.5 ppm (C-4), between the proton signals at δ_H 7.26 (H-6) to carbon signal at δ_C 162.1 ppm (C-10), between the proton signals at δ_H 7.64 (H-7) to carbon signal at δ_C 128.9 ppm (C-6), between the proton signals at δ_H 8.11 (H-8) to carbon signal at δ_C 129.1 ppm (C-7), between the proton signals at δ_H 6.94 (H-2') to carbon signal at δ_C 114.0 ppm (C-1'), δ_C 155.5 (C-3') and δ_C 157.9 (C-4'), between the proton signals at δ_H 6.94 (H-6') carbon signal at δ_C 114.0 ppm (C-1'), δ_C 155.5 ppm (C-5') and δ_C 157.9 ppm (C-4') which led to the aromatic ester part of anthocyanidin. The HMBC correlation of the proton signals of H-2" (δ_H 3.54 ppm) to C-4", H-3" (δ_H 3.59 ppm) to C-2" and C-4", H-6" (δ_H 3.65 ppm) to C-2" and C-3" led to the glucose linked to the compound 2 though the ether bond at C-1. The direct connectivity (one bond) of proton and carbon atoms were deduced from a HSQC spectrum (Figure B-9 to B-12, Appendix B, Page 36-39).

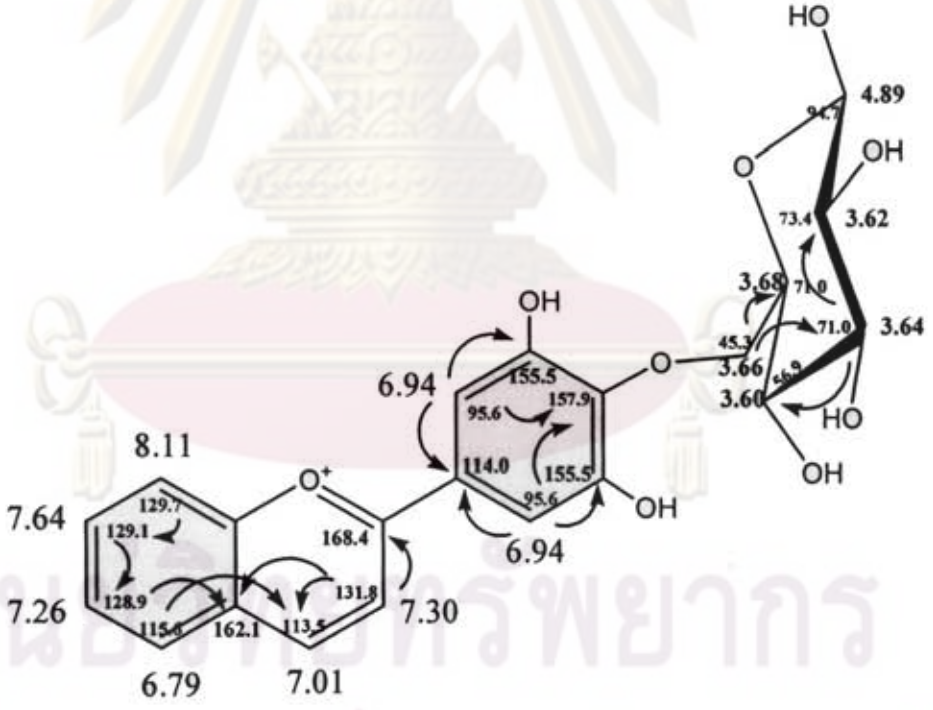


Figure 3.4 Key HMBC correlation of compound 2

The *J*-projection spectrum (Figure 3.5 and Figure B-13, Appendix B, Page 35) clearly indicated H-1" proton signal at δ_H 4.89, H-2" proton at δ_H 3.62, H-3" proton at δ_H 3.64, H-4" proton signal at δ_H 3.60, H-5" proton signal at δ_H 3.68 and H-6"

proton signal at δ_H 3.64 and 3.66. Comparing between the *J*-projection and the ^{1}H-NMR spectrum, splitting pattrun at H-2" was doublet of doublet with the *J* of 1.7 and 7.4. This clearly indicated the β configuration at the C-1" of glucose. This agree well with the resonance proton of H-1" and C-1" at 4.89 and 94.7, respectively.

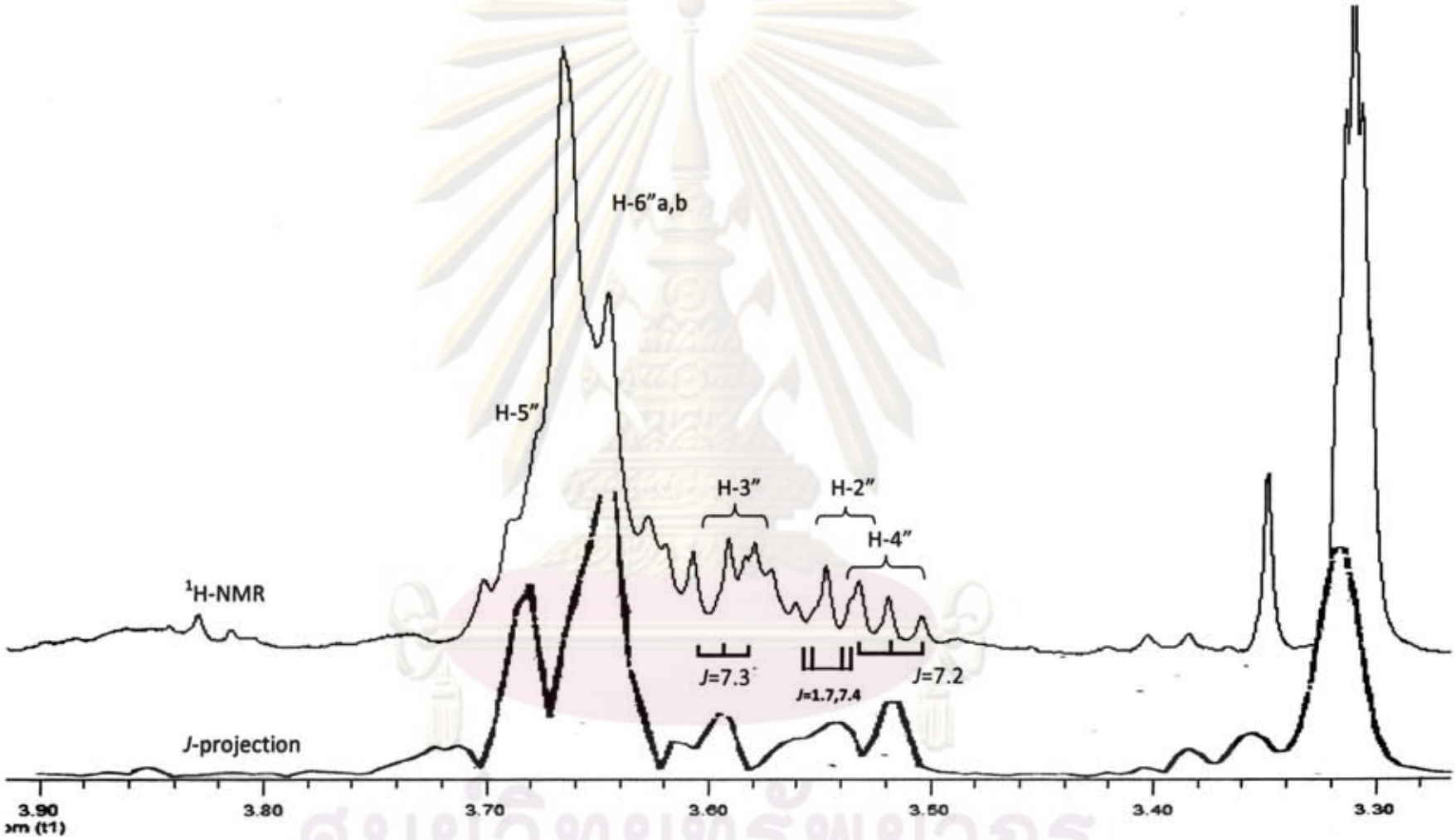


Figure 3.5 The ^{1}H NMR and *J*-projection spectrum (MeOH) of compound 2

22

Analysis of the absorption spectra revealed that compound 2 having a λ_{max} at 542 nm with the molar absorptivity of 7000 M^{-1} cm^{-1} in methanol. (Figure 3.6)

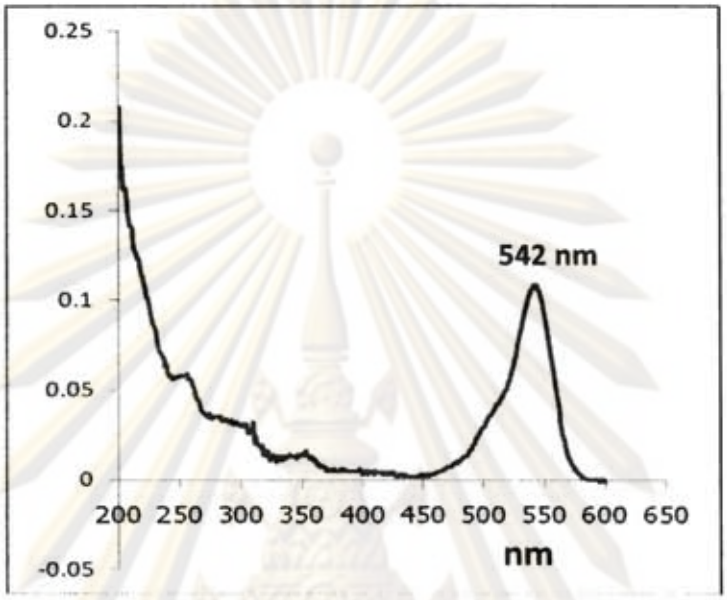


Figure 3.6 UV–visible absorption spectra of compound 2

3.3 Antioxidant activity (DPPH scavenging activity)

Antioxidant activity of compound 2 was tested by TLC autograph method. BHT was used as a control. The test was analyzed by the linear regression analysis. It was found that compound 2 and BHT showed comparable activity. A more specific spectroscopic technique was then carried out and the result indicate the IC_{50} values of 145 ± 30 and 11.6 ± 2.6 μg/ml for compound 2 ($r>0.942$) and BHT ($r>0.981$), respectively (Figure C-1, Appendix C, Page 42).

On one hand, due to conjugated effect, unpaired electrons in oxygen of compound 2 and BHT were not fixed to oxygen atom, but close to benzene ring, hydroxyl bond was weakened as a result, hydrogen activity of hydroxyl group was increased. Both of compound 2 and BHT structure contain a phenolic functionality site on aromatic ring. This phenolic moiety is a well known free radical trap. Its mechanism of action is shown in Figure 3.7

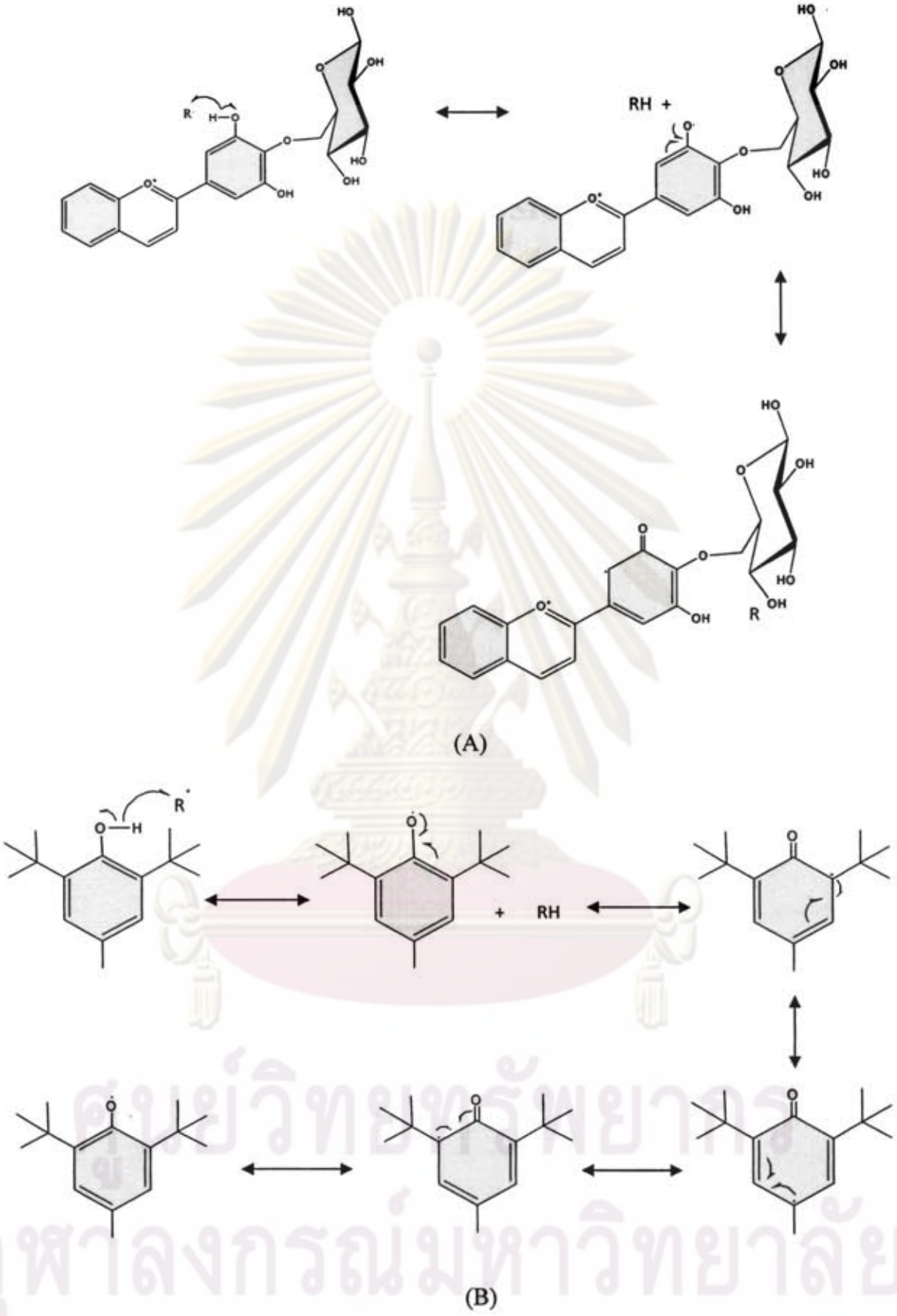


Figure 3.7 Resonance effects of (A) compound 2 and (B) Butylated hydroxytoluene (BHT)

CHAPTER VI

CONCLUSION

Isolation and identification of extract of rice husk (*Oryza sativa* L. cv. Chainat1) gave a pink compound was performed as described previously. Malvidin-3-rutinoside (compound 1) is a major pigment that give pink color in this compound (Figure 4.1). ^{1}H and ^{13}C NMR data and HSQC and HMBC correlation of compound 2 was indicated that it is one type of anthocyanin.

Compound 2 and BHT showed the IC_{50} values of 145 ± 30 and 11.6 ± 2.6 μg/ml, respectively. In addition, compound 2 showed the maximum absorption at 542 nm with the molar absorptivity (ε) of 7000 M^{-1} cm^{-1}.

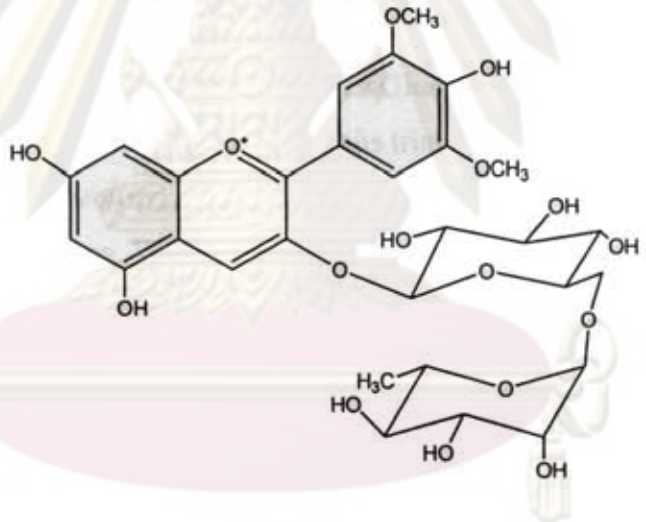


Figure 4.1 Structure of Malvidin-3-rutinoside (compound 1)

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APPENDICES

APPENDIX A

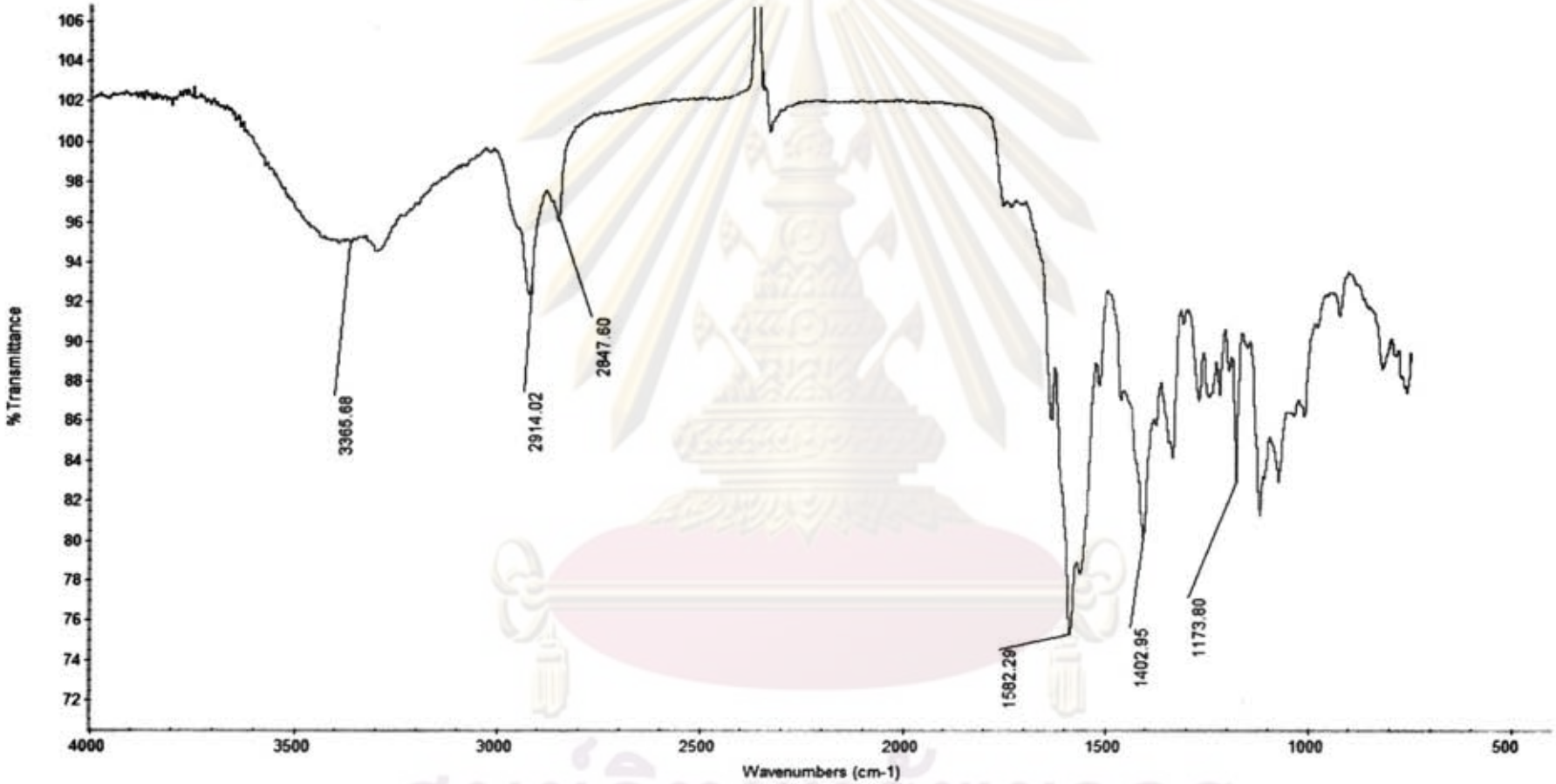


Figure A-1 The IR spectrum of compound 2

APPENDIX B

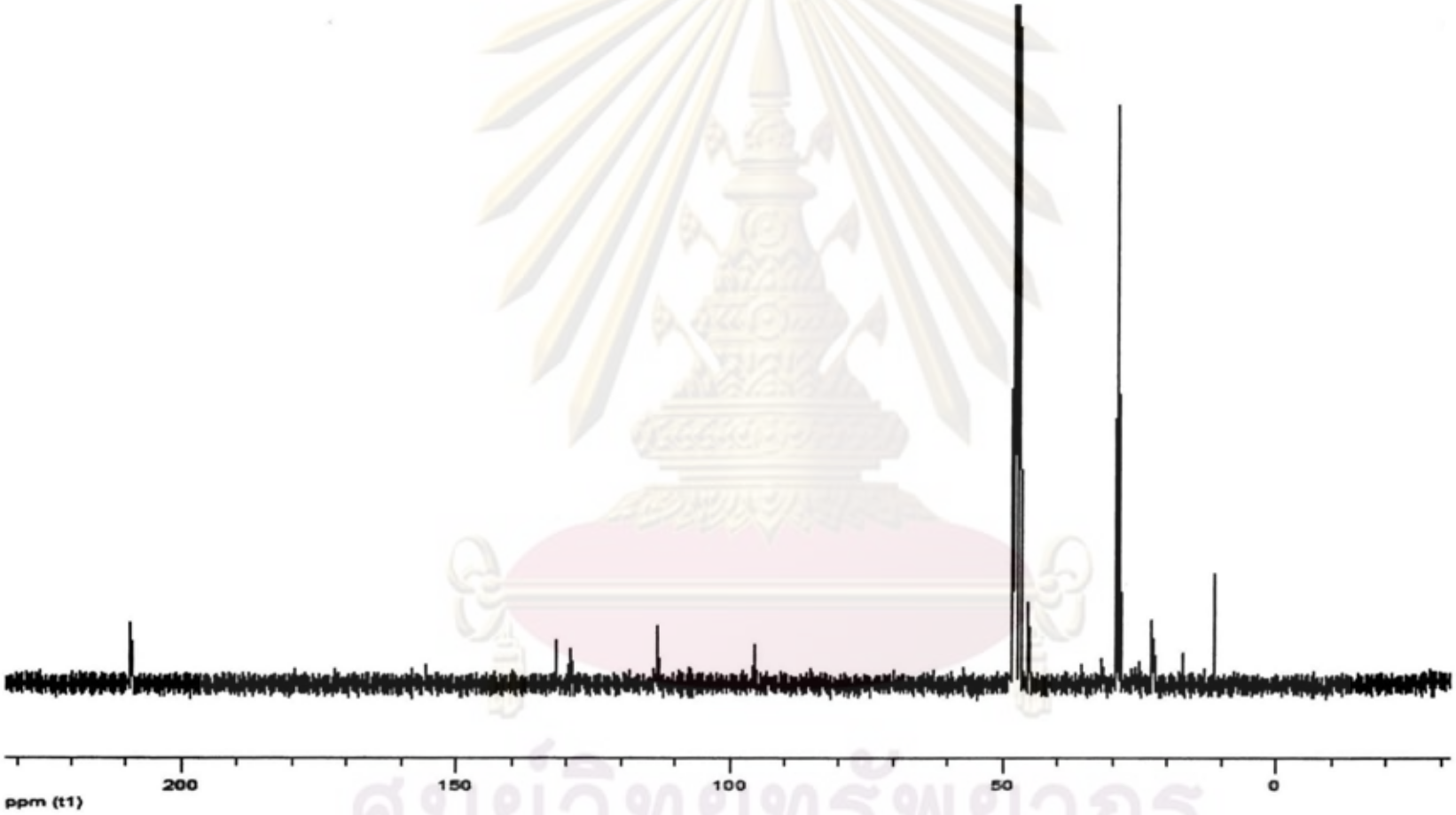


Figure B-1 The ^{13}C NMR spectrum (MeOH) of compound 2

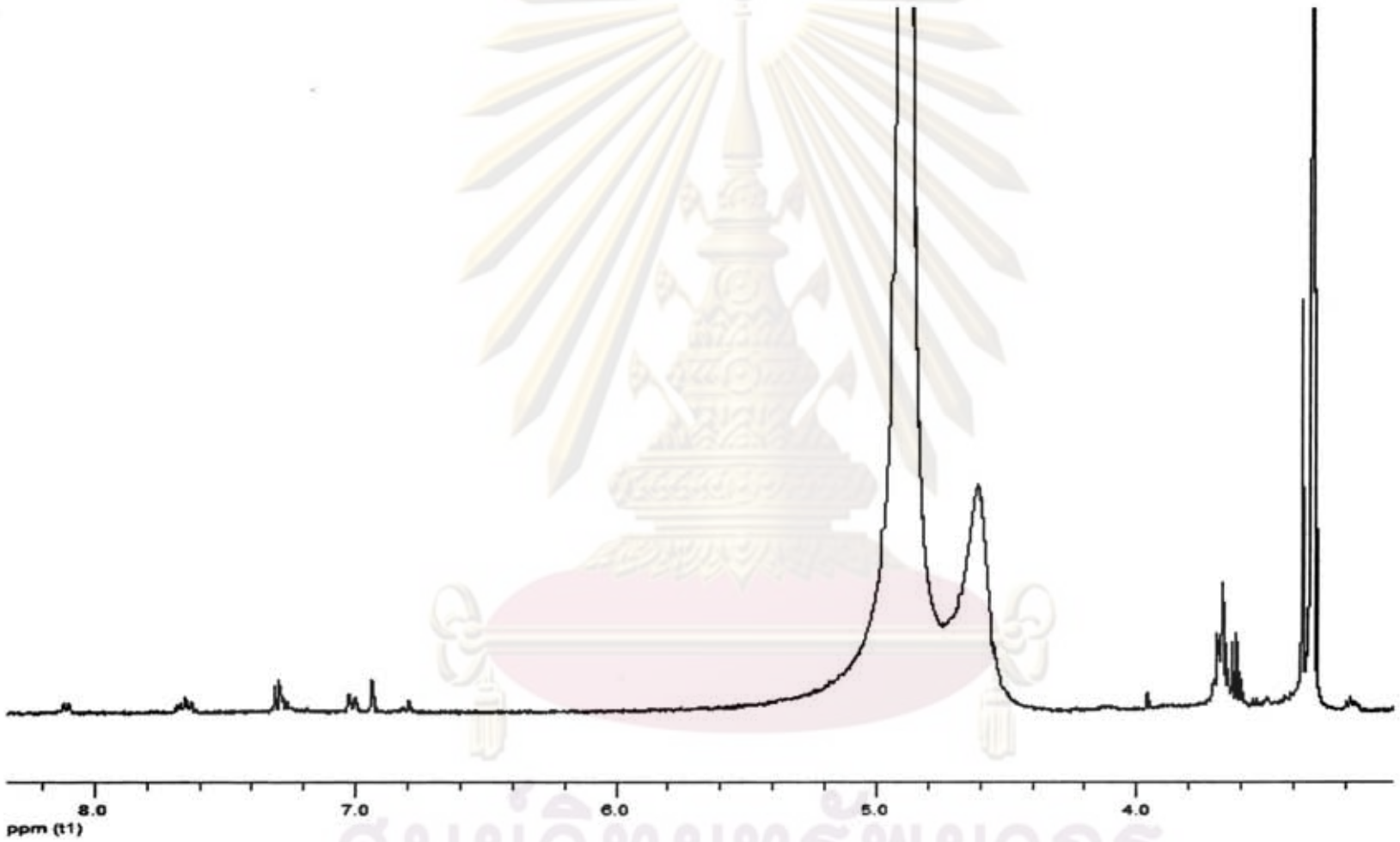


Figure B-2 The ^{1}H NMR spectrum (MeOH) of compound 2

31

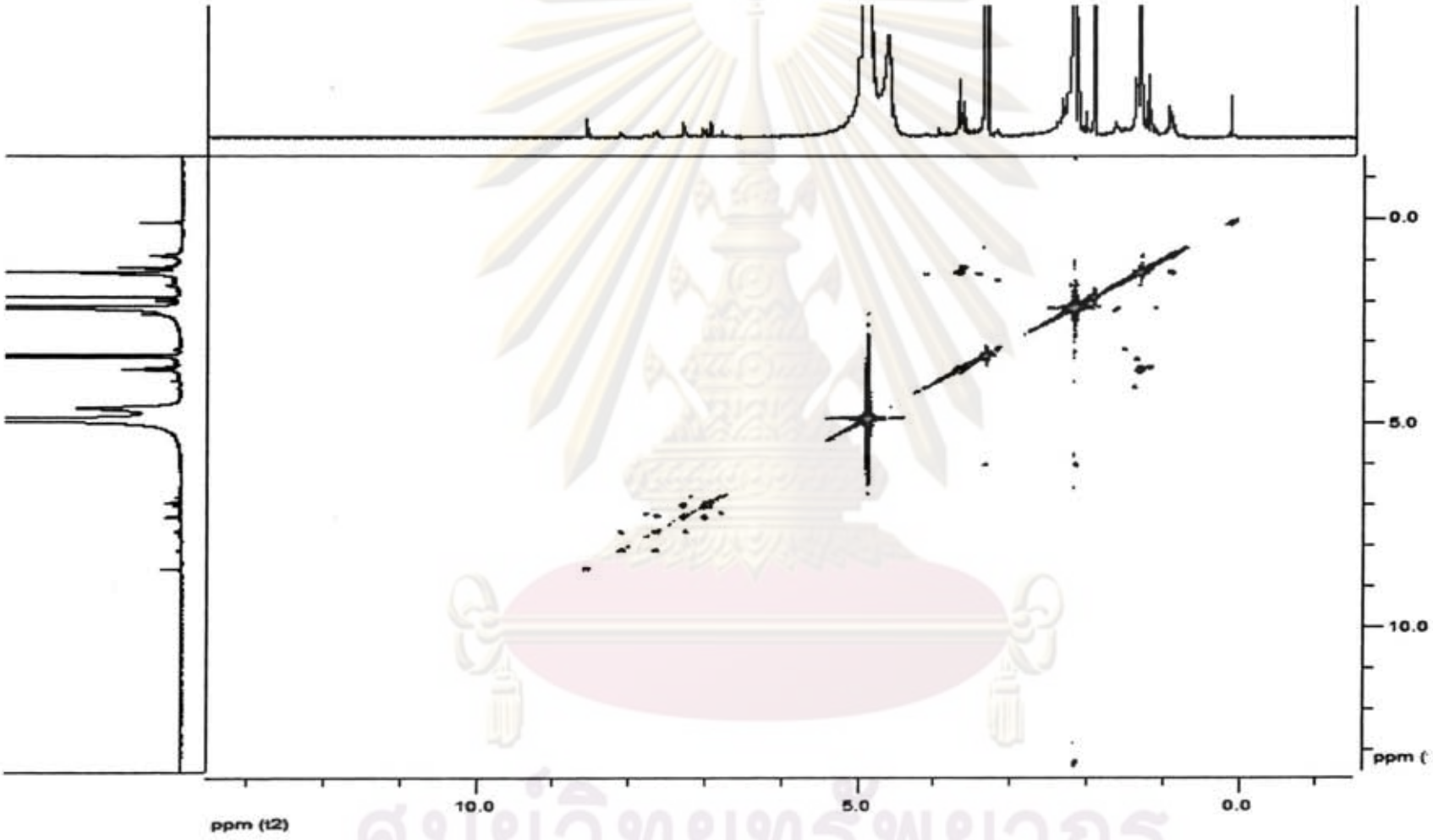


Figure B-3 The COSY spectrum (MeOH) of compound 2

32

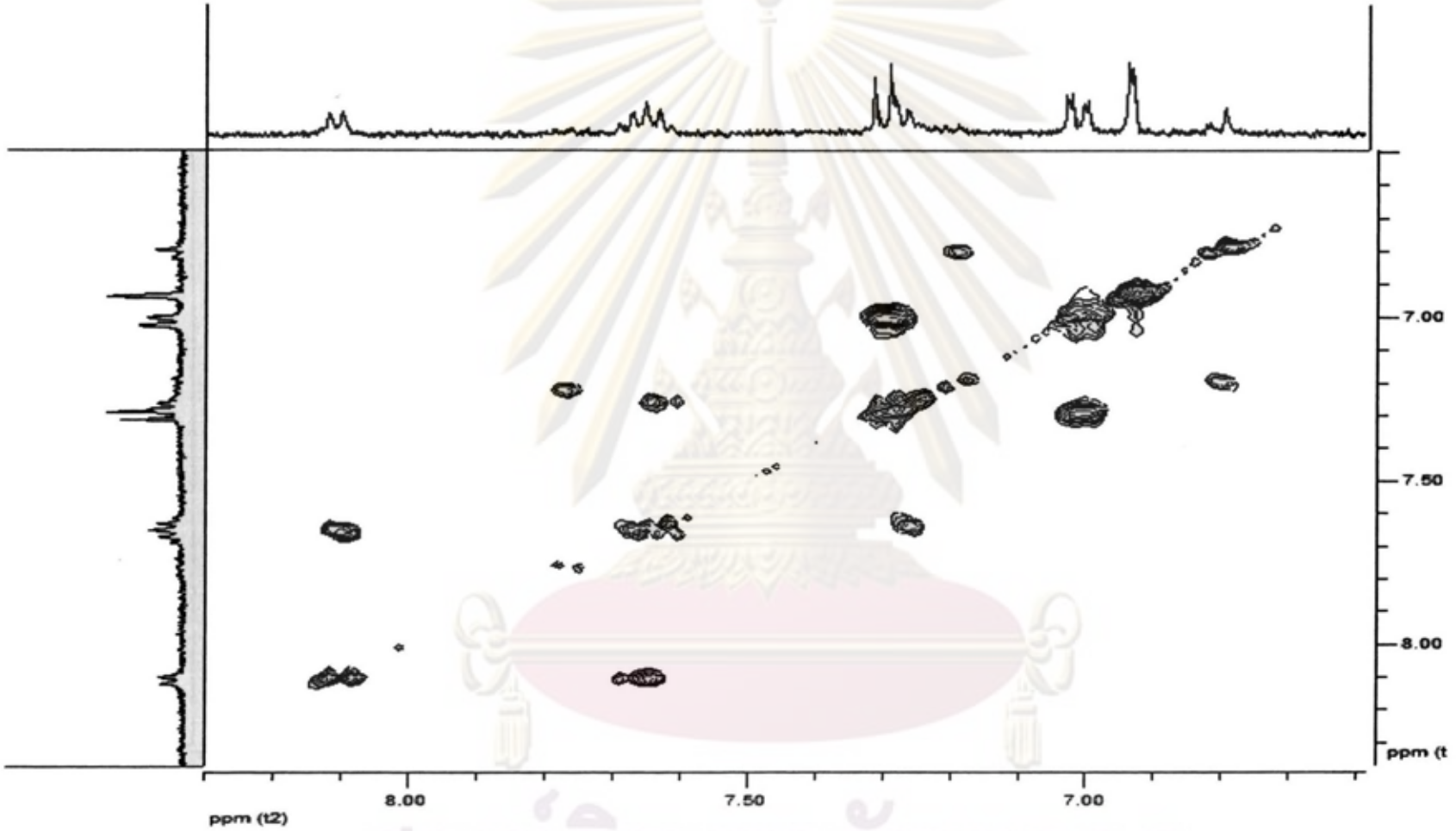


Figure B-4 The COSY spectrum (MeOH) of compound 2

33

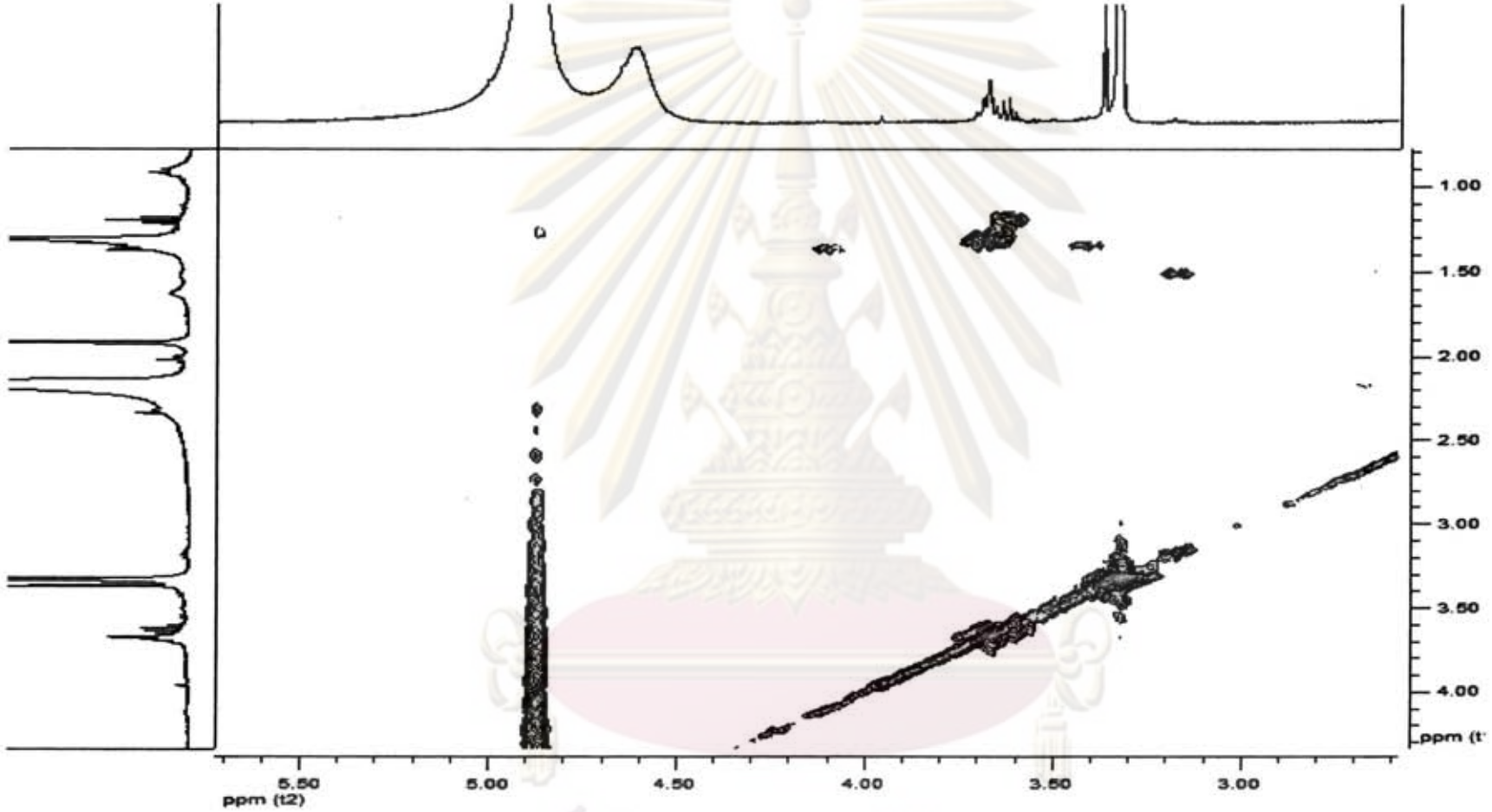


Figure B-5 The COSY spectrum (MeOH) of compound 2

34

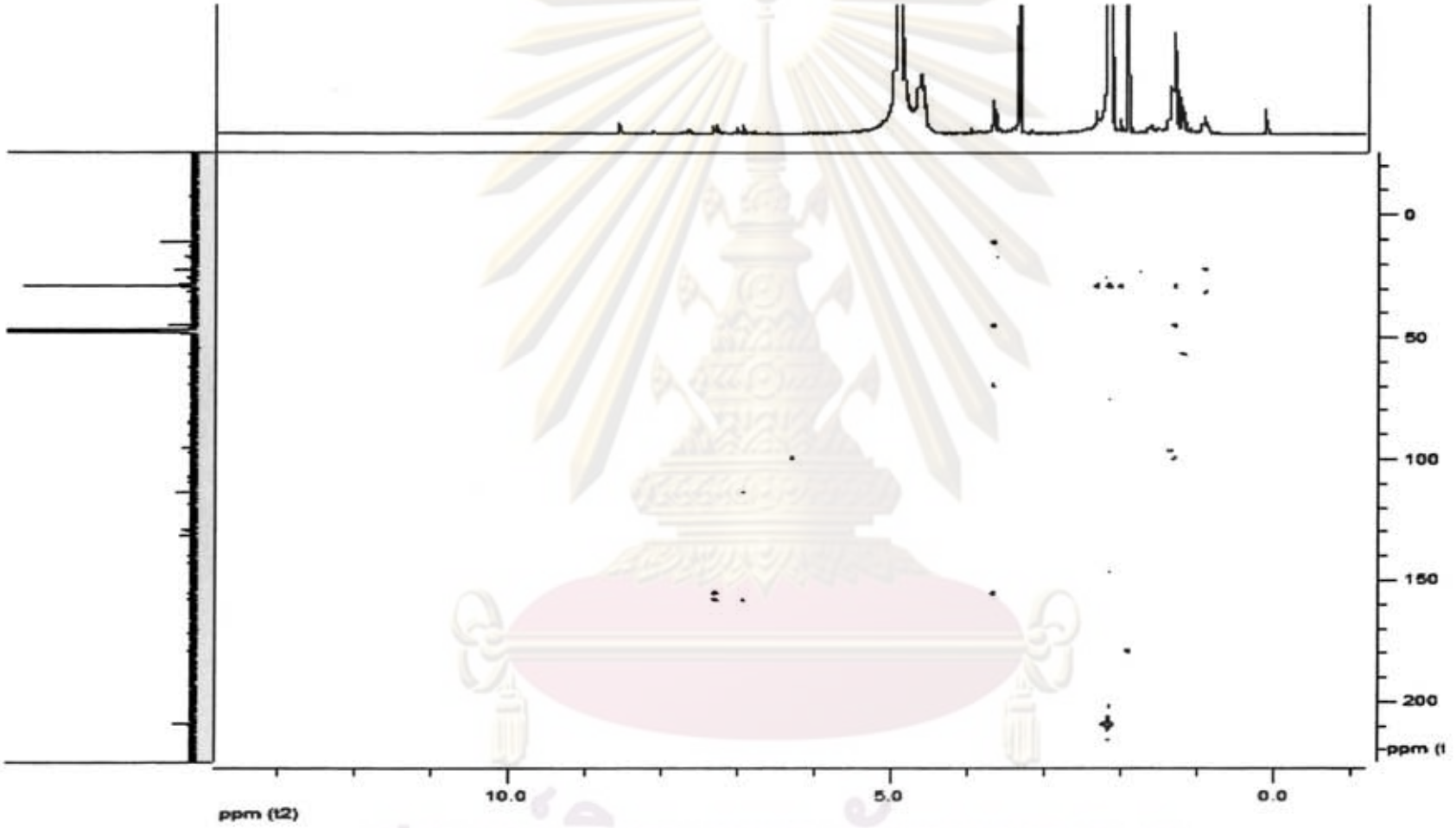


Figure B-6 The HMBC spectrum (MeOH) of compound 2

35

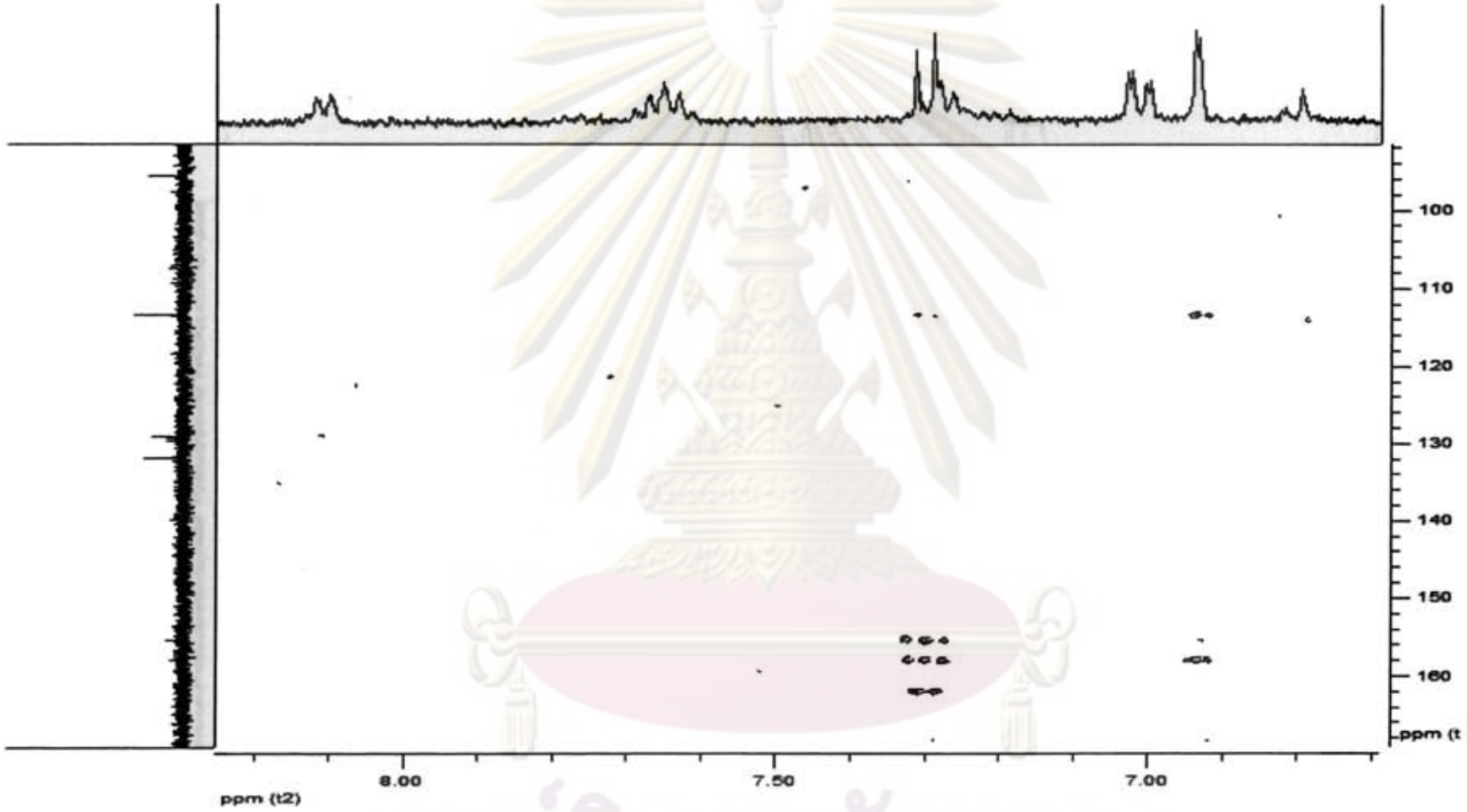


Figure B-7 The HMBC spectrum (MeOH) of compound 2

36

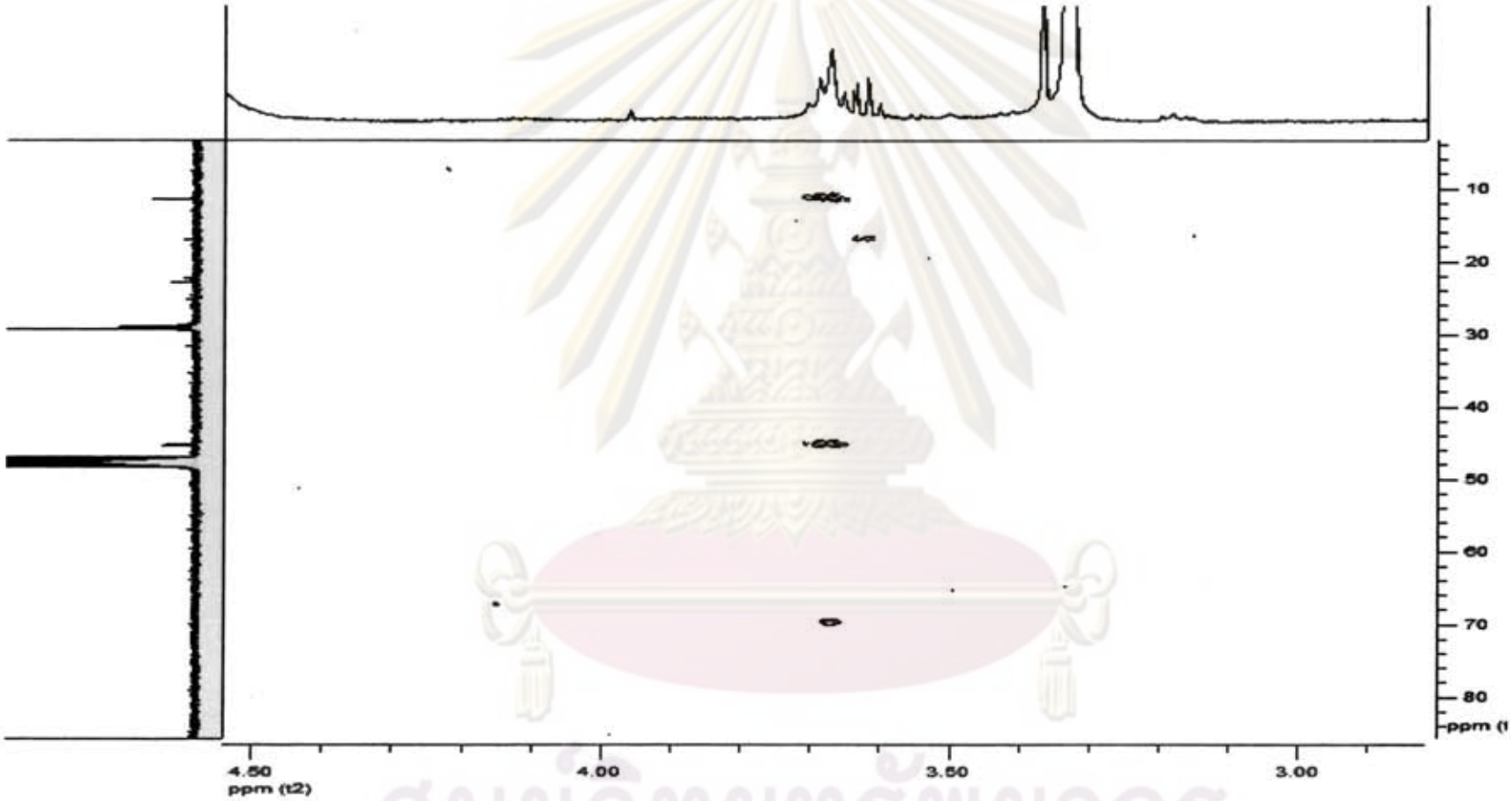


Figure B-8 The HMBC spectrum (MeOH) of compound 2

37

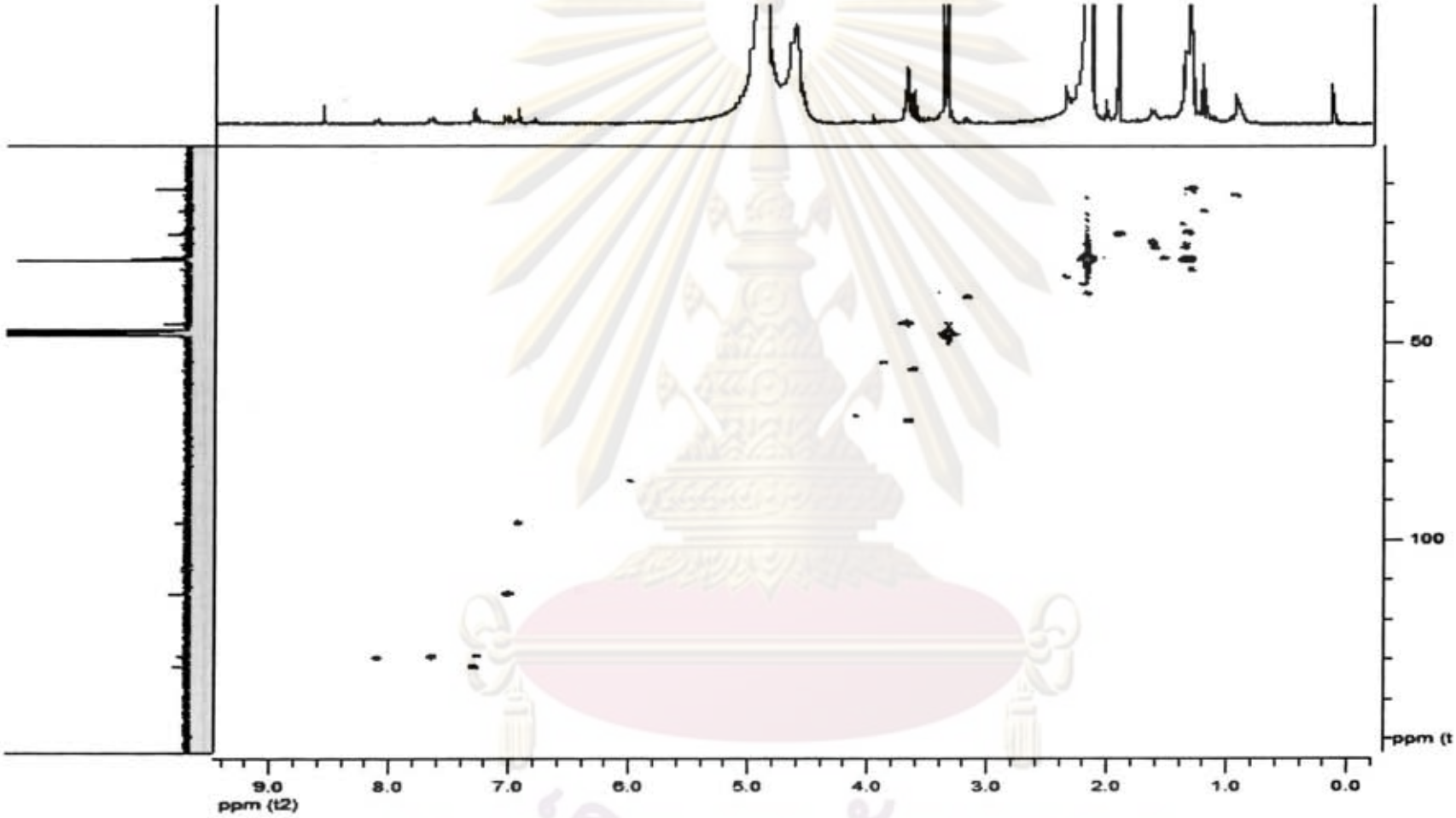


Figure B-9 The HSQC spectrum (MeOH) of compound 2

38

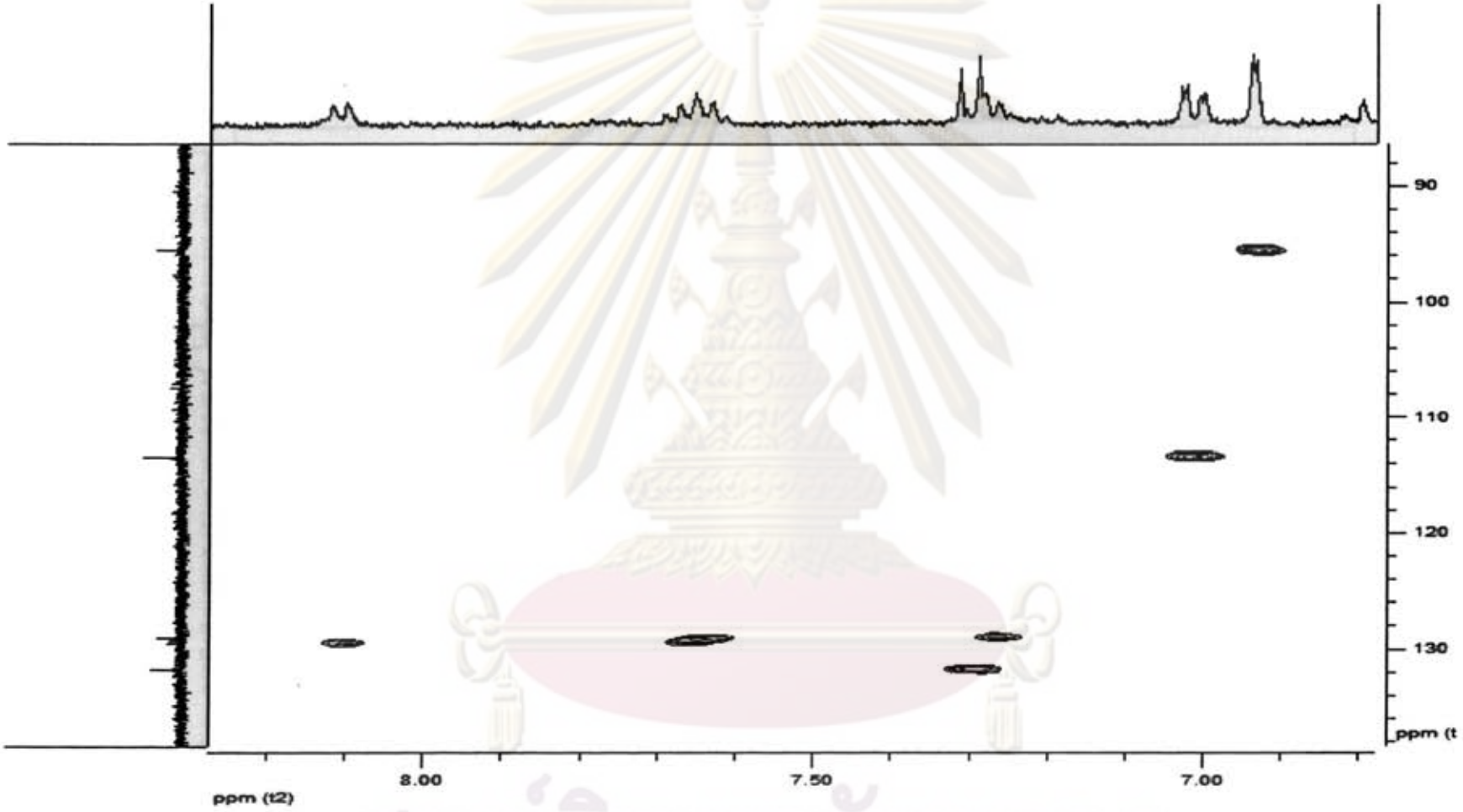


Figure B-10 The HSQC spectrum (MeOH) of compound 2

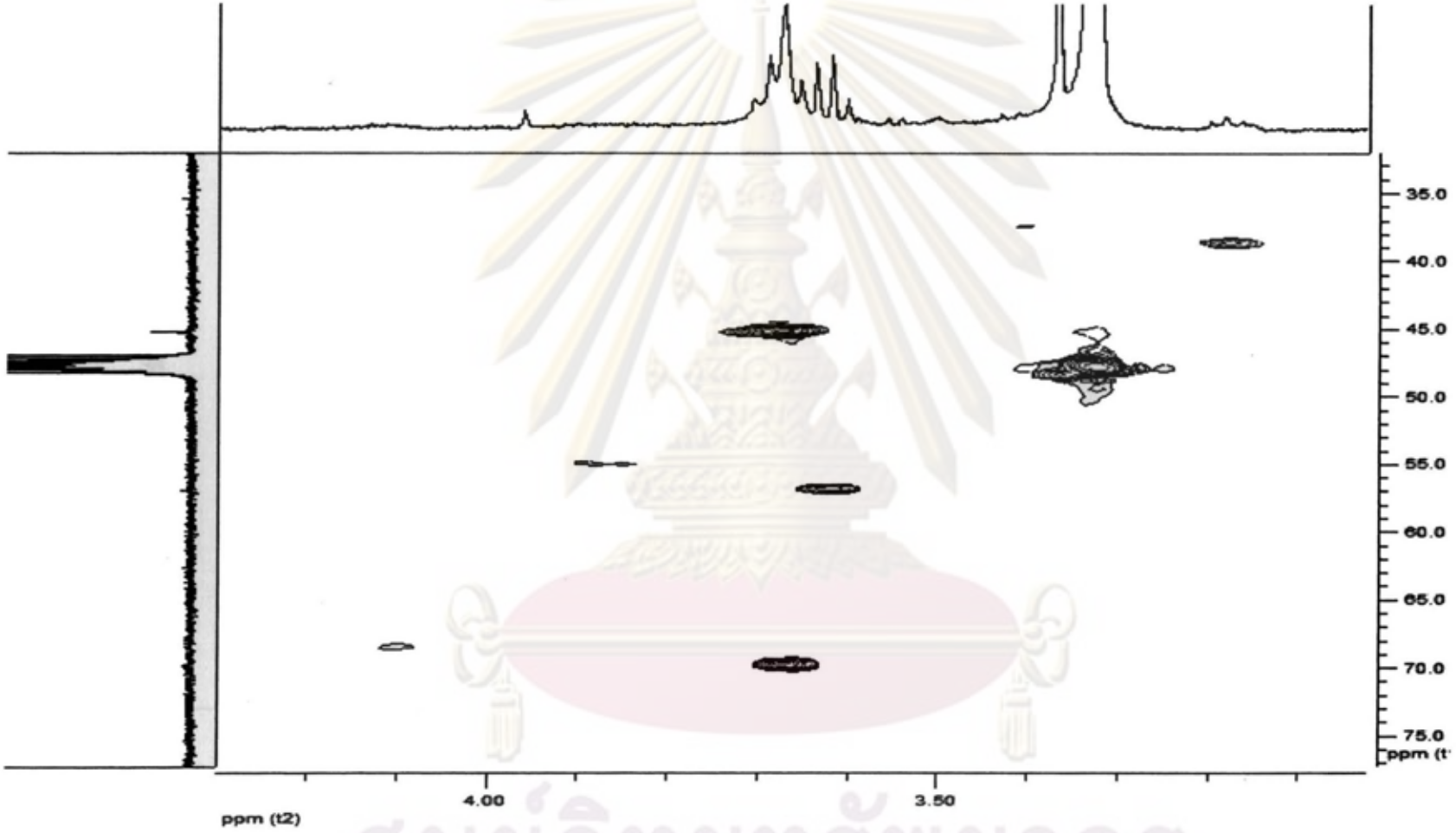


Figure B-11 The HSQC spectrum (MeOH) of compound 2

40

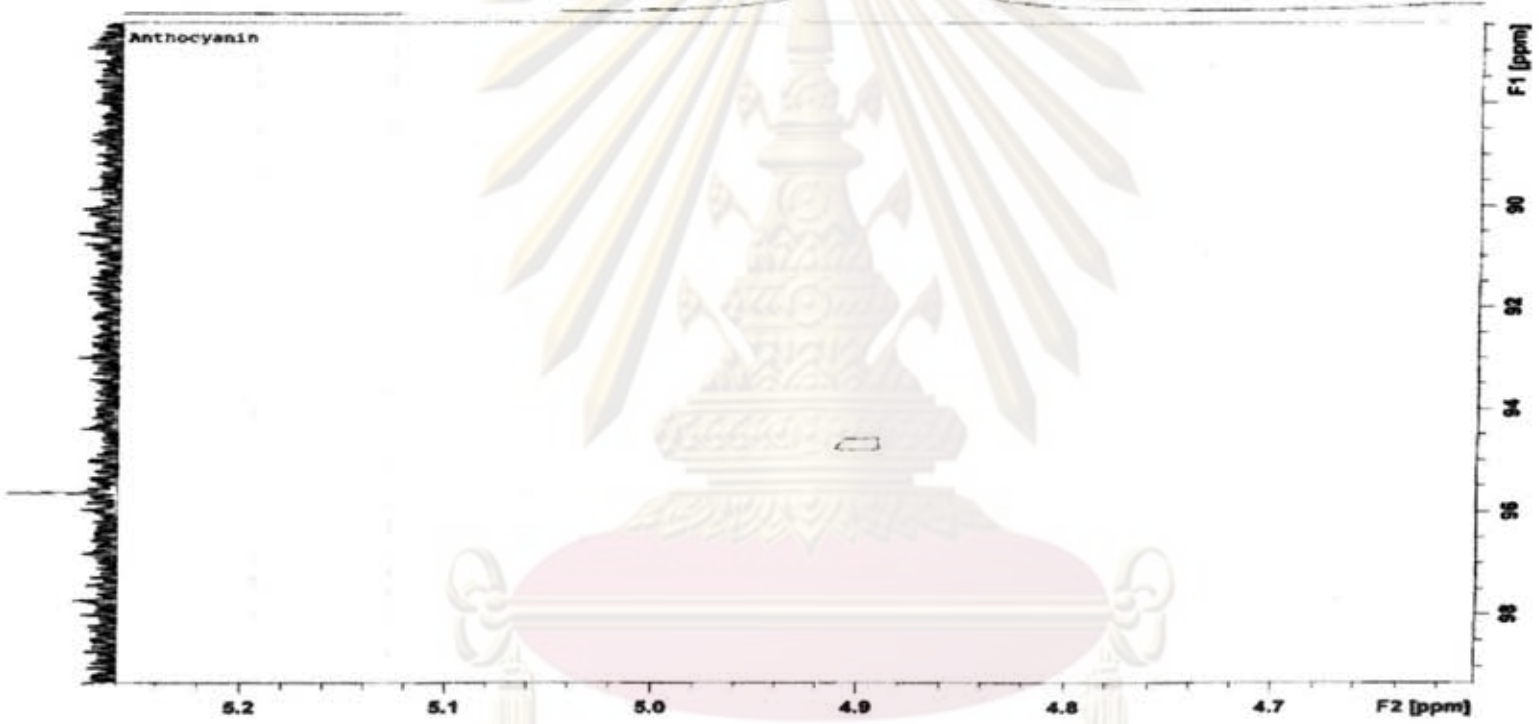


Figure B-12 The HSQC spectrum (MeOH) of compound 2

41

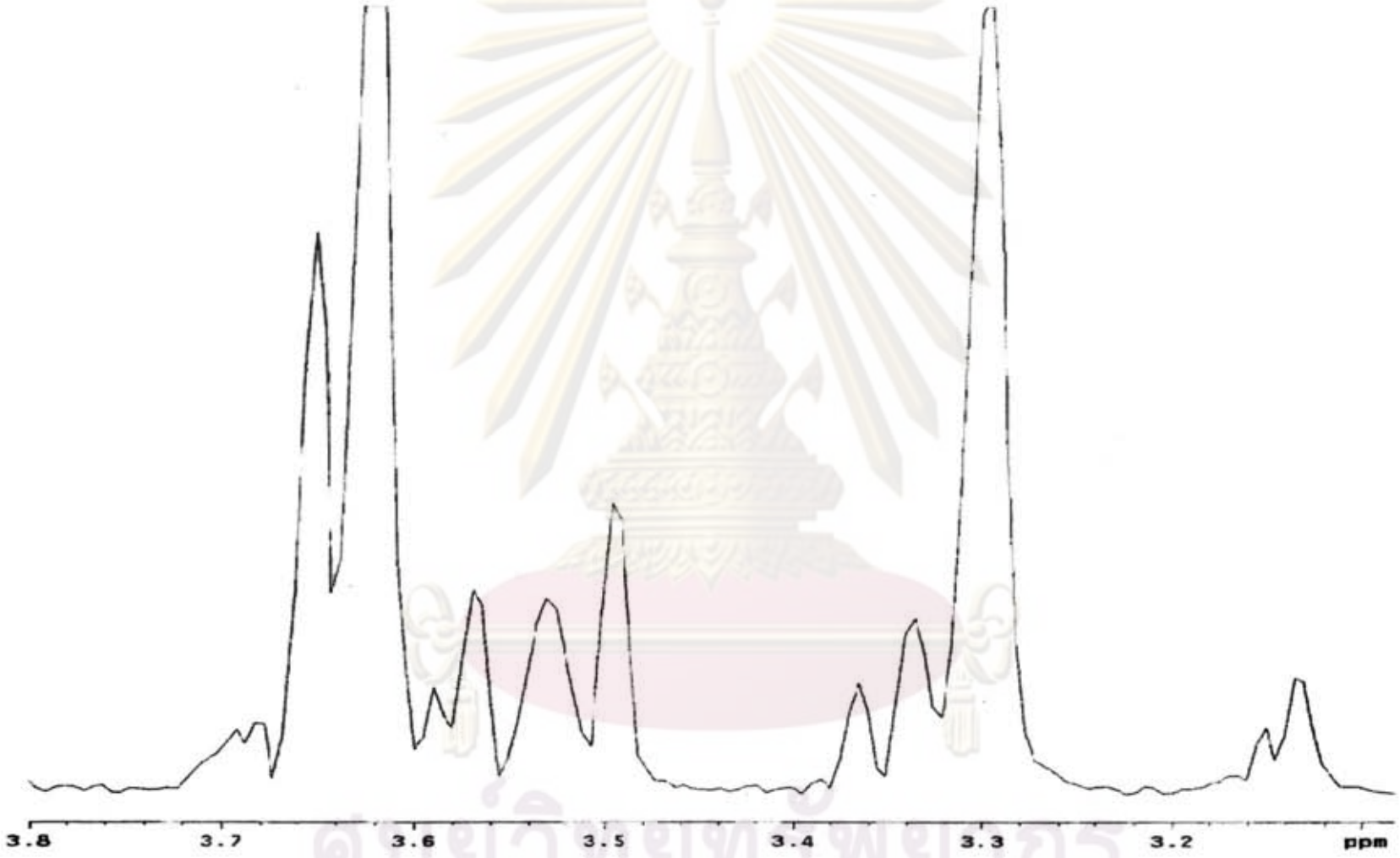
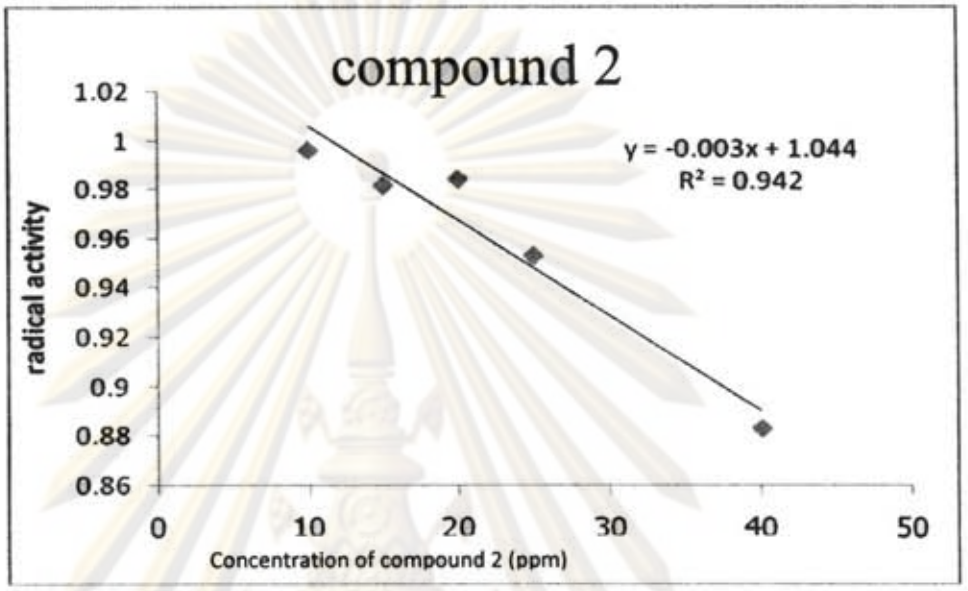


Figure B-13 The *J*-projection spectrum (MeOH) of compound 2

42

APPENDIX C



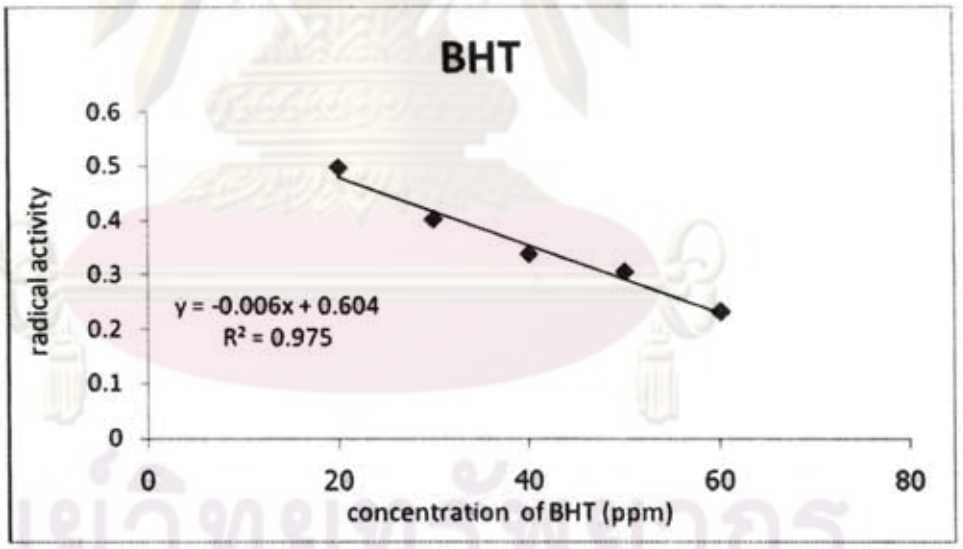


Figure C-1 Free radical activity of (A) compound 2and (B) BHT as determined by the DPPH method.

VITA

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