ANTIPROLIFERATIVE AGENTS FROM MUSHROOM *Phellinus* sp.

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A Thesis Submitted in Partial Fulfillment of the Requirements

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สารยับยั้งการเจริญของเซลล์มะเร็งจากเห็ดกระถินพิมาน *Phellinus* sp.

น.ส.กุลรัตน์ พิชยภิญโญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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กุลรัตน์ พิชยภิญโญ : สารยับยั้งการเจริญของเซลล์มะเร็งจากเห็ดกระถิน พิมาน *Phellinus* sp.. (ANTIPROLIFERATIVE AGENTS FROM MUSHROOM *Phellinus* sp.) อ.ที่ปรึกษาหลัก : รศ. ดร.สุรชัย พรภคกุล, อ.ที่ปรึกษาร่วม : ผศ. ดร.จิตรตรา เพียภูเขียว

ในการศึกษานี้ผงเห็ด *Phellinus* (600 กรัม) ถูกสกัดด้วยเมทานอลและน้ำที่กำจัดไอออนแล้ว นำส่วนสารสกัดเมทานอลของเห็ดที่ได้มาแยกส่วนสกัดโดยใช้ตัวทำละลายเฮกเซน ไดคลอโรมีเทน เอธิลอะซิเตต และเมทานอล ตามลำดับ สารสกัดหยาบทั้งหมดได้รับการประเมินฤทธิ์ยับยั้งการเพิ่มจำนวนของเซลล์มะเร็งปอด (A549) และมะเร็งเนื้อเยื่อประสาท (SH-SY5Y) โดยการทดสอบความเป็นพิษต่อเซลล์ด้วยวิธีการวัดสี MTT พบว่าสารสกัดไดคลอโรมีเทน (CDE) แสดงฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งที่ดีที่สุดโดยมีค่าการยับยั้งที่ร้อยละ 50 (IC_{50}) ที่ความเข้มข้น 132.4 และ 106.87 ไมโครกรัมต่อมิลลิลิตร ต่อเซลล์มะเร็งปอดและมะเร็งเนื้อเยื่อประสาท ส่วนสารสกัดเอธิลอะซิเตต (CEE) แสดงฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งที่ระดับปานกลางโดยมีค่า IC_{50} ที่ความเข้มข้น 138.64 และที่ความเข้มข้นมากกว่า 250 ไมโครกรัมต่อมิลลิลิตร ต่อเซลล์ SH-SY5Y และ A549 สารสกัดเฮกเซน สารสกัดเมทานอล และสารสกัดส่วนน้ำจากการสกัดแบบไหลย้อนกลับ และการต้มเคี่ยวแสดงฤทธิ์ยับยั้งเซลล์มะเร็งที่ระดับต่ำต่อเซลล์มะเร็งทั้งสองชนิด ที่ค่า IC_{50} ที่ความเข้มข้นมากกว่า 250 ไมโครกรัมต่อมิลลิลิตร ทำการแยกสารด้วยเทคนิคคอลัมน์โครมาโทกราฟีซ้ำ ตามด้วยการพิสูจน์เอกลักษณ์ของสารสกัดด้วยเทคนิค NMR ได้สาร ergosta-7,22-dien-3-one, 7-methoxyindole-3-carboxylic acid methyl ester, 7-methoxyindole-3-carboxaldehyde และ protocatechualdehyde จากสารสกัดเฮกเซน ไดคลอโรมีเทน และเอธิลอะซิเตต ตามลำดับ

สาขาวิชา เทคโนโลยีชีวภาพ
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Kulrut Pichayaphinyo : ANTIPROLIFERATIVE AGENTS FROM MUSHROOM *Phellinus* sp.. Advisor: Assoc. Prof. SURACHAI PORNPAKAKUL, Ph.D. Co-advisor: Asst. Prof. Jittra Piapukiew, Ph.D.

In this study, *Phellinus* powdered (600 g) was extracted with methanol and DI water. The methanolic crude extract of the mushroom was partitioned with hexane, dichloromethane, ethyl acetate and methanol respectively. All crude extracts were evaluated antiproliferative activity against lung cancer (A549) and neuroblastoma (SH-SY5Y) cell lines by MTT assay. It was found that the dichloromethane extract (CDE) exhibited the strongest antiproliferative activity with half maximal inhibitory concentration (IC_{50}) values of 132.4 and 106.87 µg/mL against human lung cancer and neuroblastoma cell lines, respectively. The ethyl acetate extract (CEE) exhibited moderate antiproliferative activity with IC_{50} values of 138.64 and >250 µg/mL against SH-SY5Y and A549 cell lines, respectively. The hexane extract, methanol extract and aqueous extracts from reflux extraction and decoction exhibited weak antiproliferative activity against both cancer cell lines with IC_{50} >250 µg/mL. Isolation by repeated column chromatographic, followed by NMR characterization, ergosta-7,22-dien-3-one and 7-methoxyindole-3-carboxylic acid methyl ester, 7-methoxyindole-3-carboxaldehyde and protocatechualdehyde from hexane extract, dichloromethane extract and ethyl acetate extract, respectively.

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Student's Signature .....
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Kulrut Pichayaphinyo

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LIST OF ABBREVIATIONS

δ	chemical shift
δ_C	chemical shift of carbon (NMR)
δ_H	chemical shift of proton (NMR)
°C	degree Celsius
μg	microgram
μL	microliter
μm	micrometer
μM	micromolar
%	percentage
>	greater than
:	ratio
br	broad signal (for NMR spectra)
A_0	absorbance of the control
A_1	absorbance of the test sample
A549	lung cancer cell line
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
cm	centimeter
^{13}C NMR	carbon nuclear magnetic resonance spectroscopy
CDE	crude dichloromethane partitioned extract
CEE	crude ethyl acetate partitioned extract
CFD	combine fraction dichloromethane
CFE	combine fraction ethyl acetate
CFH	combine fraction hexane
CHE	crude hexane partitioned extract
CME	crude methanol partitioned extract
COSY	correlated spectroscopy

d	doublet (for NMR spectra)
dd	doublet of doublet (for NMR spectra)
DCM	dichloromethane
DI water	deionized water
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium F12
DNA	deoxyribonucleic acid
et al.	*et alii*
EtOAc	ethyl acetate
EtOH	ethanol
F_{254}	fluorescent indicator 254 nm
g	gram
h	hours
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
HMBC	heteronuclear multiple bong correlation
HPLC	high performance liquid chromatography
HRESIMS	high-resolution electrospray ionization mass spectrometry
HSQC	heteronuclear single quantum correlation
Hz	hertz
IC_{50}	half maximal inhibitory concentration
IR	infrared spectroscopy
J	coupling constant
Kg	kilogram
L	liter
L929	mouse subcutaneous connective tissue
m	multiplet (for NMR spectra)
MeOH	methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyl-tetrazolium bromide

nm	nanometer
NMR	nuclear magnetic resonance spectroscopy
RPM	round per minute
SH-SY5Y	neuroblastoma cell line
SEM	Scanning electron microscope
TLC	thin layer chromatography
UV	ultraviolet
v/v	volume by volume
w/w	weight by weight

CHAPTER I
INTRODUCTION

Cancer is a major public health problem in countries around the world, including Thailand [1]. Cancer is one of the leading causes of death worldwide and an important barrier to increasing life expectancy in every country of the world. World Health Organization (WHO) in 2020, the most common cancer is breast (2.26 million cases), lung (2.21 million cases), colon and rectum (1.93 million cases), prostate (1.41 million cases), skin (non-melanoma) (1.20 million cases) and stomach (1.09 million cases). Lung cancer is the most common as a leading cause of death in 2020 has been reported around 1.80 million deaths [2]. In Thailand, the most common types of cancer in men are liver and bile duct, which these cancers are caused by damage from birth defects, hereditary hemochromatosis disease, liver cirrhosis, alcohol abuse or hepatitis [3]. The most common cancer treatments include surgery, chemotherapy and radiation therapy. Other treatments include targeted therapy, immunotherapy, hormonal therapy and drug therapy [4]. Which chemotherapy is one of the most common of cancer treatment. While chemotherapy drugs disrupt the cancer cells but also side effect harm healthy cells in your body. This side effects depends on overall health, the stage of cancer and the type/amount of drug received. However, the side effects disappear after treatment ends [5].

Nowadays, natural drugs have attracted extensive attention in disease treatments. Natural products cause fewer side effects probably due to their similarity with chemical entities found in the human diet [6]. A natural product is a natural compound or substance produced by a living organism such as plants, animals or microorganism [7]. Secondary metabolites produced from mushroom played an important role of biological activity [8].

Genus *Phellinus* is one of large genera in family Hymenochaetaceae, with over 220 species, which are widely distributed around the world in frigid, temperate, and tropical zones worldwide, with approximately 70 species in China [9]. Which in eastern Asian countries such as China, Japan and Korean, some species of the genus *Phellinus*

were used for therapeutical [8], including in Thailand *Phellinus* mushroom was used by traditional medicinal for the treatment of various diseases including cancer, diabetes, hypertension, infected wound, abscess, melasma, and fever. In addition, the powder of the mushroom mixed with water or honey is useful for skin therapy [10]. Phytochemical compound of genus *Phellinus* has been reported to be rich sources of biological activities, reported that polysaccharides, flavones, coumarins, terpenes, steroids and styrylpyranones [8]. These compounds exhibit different bioactivities, such as anticancer [11-26], anti-inflammatory [27-30], antidiabetic [31], antioxidant [10, 11, 18, 23, 32-35], antimicrobial [36-43] and hepatoprotective [11, 37].

Although many research of phytochemical, bioactivity test and isolation compounds on *Phellinus* mushroom, but only a few reports on bioactivity of the extract of *Phellinus* mushroom in Thailand. Therefore, in this study focused on antiproliferative activity of the extract of *Phellinus* mushroom against two human cancer, lung cancer and human neuroblastoma. According to the objective of this study, *Phellinus* mushroom were extracted and screened for anticancer agents against two human cancer cell lines, A549 (lung cancer) and SH-SY5Y (human neuroblastoma cells) and identification of the active compounds by column chromatography.

CHAPTER II
THEORETICAL

2.1 *Phellinus* sp.

2.1.1 Classification

The genus *Phellinus* is one of the largest genera in Hymenochaetaceae family. The classification is shown as:

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Hymenochaetales

Famliy: Hymenochaetaceae

Genus: *Phellinus* [8]

2.1.2 Description of genus *Phellinus*

Genus *Phellinus* is one of the largest in the family Hymenochaetaceae, which contains around 220 species presently recognized in the world [8]. This mushroom, also known as Sanghuang in Chaina, Meshimakobu in Japan, Sanghwang in Korea and Kratinphiman in Thailand, which are widely distributed in frigid, temperate and tropical zones worldwide. In East Asia countries were found in China, Korea, Japan and Thailand [10]. Genus *Phellinus*, which are found growing on wood. The mushroom forms perennial fruiting bodies as woody-hard, hoof, horseshoes-shapes or disc-shapes, imbricate. Hymenophores showed difference color including yellowish to rusty brown to grey to black. Hymenium is brown with round to angular pores. This mushroom is difference with the other mushroom, it has a woody-hard and building a new surface layer each year and accumulation to the layers [44].

2.2 *Phellinus* sp. in Thailand

In Thailand, Kratinphiman mushroom is found on trees in Dipterocarpaceae, Fabaceae and Moraceae family [10] and more than 100 species of genus *Phellinus* sp. have been found in the northeastern part of country (from the database of the museum of medicinal mushrooms Mahasarakham University). The species of Kratinphiman mushroom in Thailand in Figure 1 has been reported as *P. linteus, P. igniarius*, *P. rimosus, P. gilvus, P. pomaceus, P. tuberculosis* [10], *P. contiguous, P. hippophaeicola, P. pini, P. torulosus, P. trivalis, P. tremulea* [32], *P. conchatus, P. everhatri, P. nigricans,* and *P. noxius* [10].

Figure 1 *Phellinus* sp. was found in Thailand.

2.2.1 Medicinal properties of *Phellinus* sp. in Thailand

In Thailand, *Phellinus* mushrooms are called "Phiman mushroom" which are called Phiman mushrooms because of the books in Thailand of the Royal Institute of Thailand.

There was reported about mushrooms in the genus *Phellinus*. given the Thai name is Phiman/Kratinphiman. *Phellinus* mushroom has been recognition as medicines in Thailand. Medicine book from Bai Lan. Mahachai temple was reported that the *Phellinus* mushroom has medical property as fever, as a cold medicine, stomachache, sore throat, and abscess. Folk medicine doctor in the Northeastern, Thailand that uses this mushroom as diabetic, antihypertensive, erysipelas, herpes zoster, and facial freckle as well as discolored skin patches [10].

2.3 Biological activities and chemical constituents of *Phellinus* sp.

The *Phellinus* mushroom plays a significant role in medicinal properties. In the previous report, polysaccharides, flavones, terpenoids, steroids, and styrylpyranones were found as the main bioactive compounds observed in this genus. The various biological activities of this mushroom have been reported as anticancer, anti-diabetes, anti-inflammation, anti-oxidation, anti-microbial and hepatoprotective. [45]

2.3.1 Anticancer activity

Cancer is one of the leading causes of death worldwide. Common Types of cancer treatment includes surgery, chemotherapy, radiation therapy, hormone therapy, immunotherapy and drug therapy [2], which chemotherapy can cause side effects. Therefore, drug cancer development is needed.

The anticancer properties of *Phellinus*, it was found that the crude ethanolic extract exhibited antitumor (lymphoma and ascites carcinoma) [46] and antiproliferative gastric cancer, cervical cancer, liver cancer, lung cancer [22], colon cancer and prostate cancer [19]. The aqueous extracts exhibited against hepatoma carcinoma and melanoma [26]. Furthermore, the pure compounds were isolation and identification from ethanolic extract of *Phellinus* mushroom, which has reported the anticancer is lung cancer, gastric cancer, breast cancer, liver cancer, kidney cancer, colon cancer. The results showed that the pure compounds are meshimakobnol A, meshimakobnol B, phelligridin C, phelligridin D, phelligridins E, phelligridins F, inoscavin A, hispidin, hispolon and 4-(3,4-dihydroxyphenyl)but-3-en-2-one [20]. phelligridin G was showed active against colon

cancer and human ovary cancer [23], hispolon against epidermoid carcinoma [13] and phelligridin J exhibited against lung cancer, liver cancer, colon cancer and human ovary cancer [24]. The pure compounds were isolation from methanolic extracts were meshimakobnol A, meshimakobnol B, phellifuropyranone A exhibited against lung cancer [18], atactylenoline I exhibited against colon cancer [17], igniarine exhibited against liver cancer and lung cancer [21], ergosterol peroxide exhibited against liver cancer and ergosterol exhibited against liver cancer and breast cancer [21]. In addition, the ethyl acetate extract of *Phellinus* mushroom was isolated to obtain hispolon, which exhibited against leukemia cancer [14], phellinignin A, phellinignin D exhibited against leukemia cancer, hepatocellular carcinoma and colorectal cancer [25], phellinignin B exhibited against hepatocellular carcinoma, conocenol A exhibited against leukemia cancer and conocenal B exhibited against colorectal cancer [25] and the pure compounds were isolation from acetone extract, hispolon, phelligniarin B, phellibaumin A, phellibaumin D exhibited against colon cancer and 3,4-dihydroxybenzalacetone, phelligridinF showed against colon cancer and cervical cancer [12]. According to the previous studies, the pure compounds separated from *Phellinus* mushrooms extracts showed anticancer properties showed in Table 1.

Table 1 Summary of anticancer activity from *Phellinus* mushroom

Species	Part	Solvent	Chemical constituents	Cell lines	Biological activity (IC_{50})	Reference
Phellinus linteus	Fruiting bodies	Ethyl acetate	hispolon **(1)**	Leukemia cancer (NB4)	0.98 µg/mL	[14]
		Ethanol		Epidermoid carcinoma (Kb)	4.62 µg/mL	[13]
		Ethanol		Hepatocarcinoma (Hep G2)	87.6 µM	
				(JS)	54.5 µM	
				(Hep 3B)	35.9 µM	
		Methanol	meshimakobnol A **(2)**	Lung cancer (A549)	22.6 µM	[18]
		Methanol	meshimakobnol B **(3)**	Lung cancer (A549)	15 µM	[18]
		Methanol	phellifuropyranone A **(4)**	Lung cancer (A549)	31.3 µM	[18]
		Methanol	atraclyenolide I **(5)**	Colon cancer (HT-29)	Inhibition rate 20%	[17]
		Ethanol	Crude extracts	Colorectal cancer (HCT116)	Inhibition rate 42.8%	[19]
				Gastric cancer (AGS)	Inhibition rate 40.3%	[19]
				Prostate cancer (DU145)	Inhibition rate 44.8%	[19]
				Cervical cancer (HeLa)	Inhibition rate 11.9%	[19]

Table 1 Summary of anticancer activity from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Cell lines	Biological activity (IC_{50})	Reference
Phellinus igniarius	Fruiting bodies	Ethanol	phelligridin C **(6)**	Lung cancer (A549)	0.016 μM	[20]
				Gastric cancer (A2780)	>0.131 μM	[20]
				Breast cancer (MCF-7)	0.037 μM	[20]
				Liver cancer (Bel-7402)	0.008 μM	[20]
				Kidney cancer (Ketr3)	0.090 μM	[20]
				Colon cancer (HCT-8)	0.099 μM	[20]
Phellinus igniarius	Fruiting bodies	Ethanol	phelligridin D **(7)**	Lung cancer (A549)	0.012 μM	[20]
				Gastric cancer (A2780)	>0.137 μM	[20]
				Breast cancer (MCF-7)	0.072 μM	[20]
				Liver cancer (Bel-7402)	0.010 μM	[20]
				Kidney cancer (Ketr3)	0.094 μM	[20]
				Colon cancer (HCT-8)	0.126 μM	[20]
			phelligridin E **(8)**	Lung cancer (A549)	0.079 μM	[20]
				Gastric cancer (A2780)	0.096 μM	[20]
				Breast cancer (MCF-7)	0.070 μM	[20]
				Liver cancer (Bel-7402)	0.055 μM	[20]

Table 1 Summary of anticancer activity from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Cell lines	Biological activity (IC_{50})	Reference
				Kidney cancer (Ketr3)	>0.105 μM	[20]
				Colon cancer (HCT-8)	>0.105 μM	[20]
			phelligridin F **(9)**	Lung cancer (A549)	0.084 μM	[20]
				Gastric cancer (A2780)	0.092 μM	[20]
				Breast cancer (MCF-7)	0.085 μM	[20]
				Liver cancer (Bel-7402)	0.046 μM	[20]
				Kidney cancer (Ketr3)	>0.104 μM	[20]
Phellinus igniarius	Fruiting bodies	Ethanol		Colon cancer (HCT-8)	>0.104 μM	[20]
			inoscavin A **(10)**	Lung cancer (A549)	>0.108 μM	[20]
				Gastric cancer (A2780)	>0.108 μM	[20]
				Breast cancer (MCF-7)	>0.108 μM	[20]
				Liver cancer (Bel-7402)	0.088 μM	[20]
				Kidney cancer (Ketr3)	>0.108 μM	[20]
				Colon cancer (HCT-8)	>0.108 μM	[20]

Table 1 Summary of anticancer activity from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Cell lines	Biological activity (IC_{50})	Reference
Phellinus igniarius	Fruiting bodies	Ethanol	hispidin **(11)**	Lung cancer (A549)	>0.164 μM	[20]
				Gastric cancer (A2780)	0.146 μM	[20]
				Breast cancer (MCF-7)	0.143 μM	[20]
				Liver cancer (Bel-7402)	0.050 μM	[20]
				Kidney cancer (Ketr3)	0.144 μM	[20]
				Colon cancer (HCT-8)	0.139 μM	[20]
			hispolon **(1)**	Lung cancer (A549)	0.183 μM	[20]
				Gastric cancer (A2780)	0.205 μM	[20]
				Breast cancer (MCF-7)	0.025 μM	[20]
				Liver cancer (Bel-7402)	0.038 μM	[20]
				Kidney cancer (Ketr3)	0.206 μM	[20]
				Colon cancer (HCT-8)	0.199 μM	[20]

Table 1 Summary of anticancer activity from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Cell lines	Biological activity (IC_{50})	Reference
Phellinus igniarius	Fruiting bodies	Ethanol	4-(3,4-dihydroxyphenyl)but-3-en-2-one **(12)**	Lung cancer (A549)	>0.280 μM	[20]
				Gastric cancer (A2780)	0.243 μM	[20]
				Breast cancer (MCF-7)	0.141 μM	[20]
				Liver cancer (Bel-7402)	0.153 μM	[20]
				Kidney cancer (Ketr3)	0.245 μM	[20]
				Colon cancer (HCT-8)	0.227 μM	[20]
			phelligridin G **(13)**	Colon cancer (HCT-8)	30.2 μM	[23]
				Human ovary cancer (A2780)	20.4 μM	[23]
			phelligridin J **(14)**	Lung cancer (A549)	4.2 μM	[24]
				Liver cancer (Bel-7402)	9.2 μM	[24]
				Colon cancer (HCT-8)	8.4 μM	[24]
				Human ovary cancer (A2780)	7.2 μM	[24]
		Methanol	igniarine **(15)**	Liver cancer (Hep G2)	18.4 μM	[21]
				Lung cancer (LU)	29.1 μM	[21]
			meshimakobnol A **(2)**	Liver cancer (Hep G2)	19.2 μM	[21]
				Lung cancer (LU)	35.2 μM	[21]

Table 1 Summary of anticancer activity from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Cell lines	Biological activity (IC_{50})	Reference
Phellinus igniarius	Fruiting bodies	Methanol	meshimakobnol B **(3)**	Liver cancer (Hep G2)	16.7 μM	[21]
				Lung cancer (LU)	21.7 μM	[21]
			ergosterol **(16)**	Liver cancer (Hep G2)	21.5 μM	[21]
				Breast cancer (MCF-7)	43.6 μM	[21]
			ergosterol peroxide **(17)**	Liver cancer (Hep G2)	46.9 μM	[21]
Phellinus igniarius	Culture broth	Ethyl acetate	phellinignin A **(18)**	Leukemia cancer (HL-60)	3.8 μM	[25]
				Hepatocellular carcinoma (SMMC-7721)	12.1 μM	[25]
				Colorectal cancer (SW480)	0.7 μM	[25]
			phellinignin B **(19)**	Hepatocellular carcinoma (SMMC-7721)	17.4 μM	[25]
				Colorectal cancer (SW480)	7.9 μM	[25]

Table 1 Summary of anticancer activity from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Cell lines	Biological activity (IC_{50})	Reference
			phellinignin D **(20)**	Leukemia cancer (HL-60)	21.1 μM	[25]
				Hepatocellular carcinoma (SMMC-7721)	12.3 μM	[25]
				Colorectal cancer (SW480)	13.9 μM	[25]
			conocenol A **(21)**	Leukemia cancer (HL-60)	29.8 μM	[25]
			conocenol B **(22)**	Colorectal cancer (SW480)	16.7 μM	[25]
	Fruiting bodies	Acetone	phelliigniarin B **(23)**	Colon cancer (HT-29)	20 μM	[12]
			phellibaumin A **(24)**		20 μM	[12]
			phellibaumin D **(25)**		20 μM	[12]
			hispolon **(1)**		20 μM	[12]
			3,4-dihydroxbenzal-acetone **(26)**	Cervical cancer (HeLa)	20 μM	[12]
			phelligridin F **(9)**		20 μM	[12]

Table 1 Summary of anticancer activity from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Cell lines	Biological activity (IC_{50})	Reference
Phellinus igniarius	Fruiting bodies	Ethanol	Crude extracts	Gastric cancer (SGC-7901)	110.7 μg/mL	[22]
				Gastric cancer (AGS)	270.5 μg/mL	[22]
				Cervical cancer (HeLa)	314.2 μg/mL	[22]
				Liver cancer (Hep G2)	361.6 μg/mL	[22]
				Lung cancer (A549)	531.7 μg/mL	[22]
		Power with saline	Crude extracts	Hepatoma carcinoma (H22)	Inhibition rate 76.18 %	[26]
				Melanoma (B16)	Inhibition rate 29.71 %	[26]
		Aqueous	Crude extracts	Hepatoma carcinoma (H22)	Inhibition rate 33.71 %	[15]
		Ethanol	Crude extracts	Lymphoma (DLA)	184 mg/mL (antitumor 84 %)	[46]
				Ascites carcinoma (EAC)	92 mg/mL (antitumor 65 %)	[46]
		Methanol	Crude extracts	Lymphoma (DLA)	543 mg/mL (antitumor 96 %)	[46]
				Ascites carcinoma (EAC)	33 mg/mL (antitumor 33 %)	[46]

2.3.2 Antidiabetic activity

The methanolic extract of *Phellinus* mushroom from fruiting body was evaluated rat lens aldose reductase (RLAR) and human recombinant aldose reductase (HRAR). They were identified as phelligridimer A (27), protocatechualdehyde (28), ellagic acid (29), inoscavin A (10), davallialactone (30), methyl davallialactone (31), hispidin (11), caffeic acid (32), interfungin A (33) and hypolomine B (34). Moreover, davallialactone (30) exhibited strong RLAR and HRAR inhibitory activity with IC_{50} values 0.33 and 0.56 μM, respectively [47] showed in Table 2.

2.3.3 Anti-inflammatory activity

The crude aqueous fruiting body extract of *Phellinus* showed anti-inflammation activity (IC_{50}=19.46 μg/mL) on RAW 264.7 cells [48]. Furthermore, the ethanolic extract give 3,4-dihydroxybenzal-acetone (26) test with in acute lung injury (ICR mice) [29], igniaren B (35), igniaren D (36) and ergosta-6,22-die3 β -ol (37) exhibited anti-inflammatory activity test on RAW 264.7 cells with IC_{50} values 5 mg/Kg mouse (*In vivo*), 47.89, 91.74 and 37.57 μg/mL, respectively [27, 28] showed in Table 3.

2.3.4 Antioxidation activity

In the previous studies (Table 4) have found that Phellinus mushroom extract has antioxidant activity. The crude ethyl acetate, ethanol and aqueous fraction of *Phellinus* exhibited against the DPPH radical with IC_{50} values 154.50, 58.10 and 332.85 μg/mL, respectively [49]. The antioxidant activity of 4,(3,4-dihydroxyphenyl)-3-buten-2-one (12), davalliactone (30), inoscavin A (10), protocatechuic acid (38) and protocatechualdehyde (28) were evaluated by using the ABTS assay, which showed IC_{50} values of 8.23, 2.61, 13.3, 2.38 and 5.88 μM, respectively [34]. Inaddition, the antioxidant activity of caffeic acid (32) (0.11, 0.18 μmol/L), 3,14-bihispidinyl (39) (0.90, 055 μmol/L), hypholomine B (34) (0.31, 0.24 μmol/L), hispidin (11) (1.31, 2.27 μmol/L) [35], inotilone (40) (1.55, 2.27/trolox) [33] were evaluated by DPPH and ABTS assay, showed IC_{50} values respectively. phelligridimer A (27) (10.20 μM) [50], phelligridin G

(13) (3.86 μM), phelligridin H (41) (4.80 μM), phelligridin I (42) (3.70 μM), phelligridin J (12) (6.50 μM) and davalliactone (30) (8.20 μM) were evaluated with rat liver microsomal *In vivo* [23, 24].

Table 2 Summary of antidiabetic activity from *Phellinus* mushroom

Species		Solvent	Chemical constituents	Model	Biological activity (IC_{50})	Reference
Phellinus linteus	Fruiting bodies	Methanol	phelligridimer A (27)	Rat lens, Human recombinant	4.26, 7.93 μM	[47]
			protocatechualdehyde (28)		20.52, 35.36 μM	[47]
			ellagic acid (29)		0.63, 1.37 μM	[47]
			inoscavin A (10)		1.06, 1.40 μM	[47]
			davallialactone (30)		0.33, 0.56 μM	[47]
			methyl davallialactone (31)		0.51, 1.15 μM	[47]
			hispidin (11)		0.82, 18.12 μM	[47]
			caffeic acid (32)		>55, >59 μM	[47]
			interfungin A (33)		1.03, 1.82 μM	[47]
			hypolomine B (34)		0.82, 1.82 μM	[47]

Table 3 Summary of anti-inflammation from *Phellinus* mushroom

Species		Solvent	Chemical constituents	Model	Biological activity (IC_{50})	Reference
Phellinus linteus	Fruiting bodies	Ethanol	3,4-dihydroxybenzal-acetone **(26)**	In acute lung injury (ICR mice)	5 mg/kg	[27]
Phellinus igniarius	Fruiting bodies	Ethanol	igniaren B **(35)**	Raw 264.7 cell	47.89 μg/mL	[28]
			igniaren D **(36)**		91.74 μg/mL	[28]
			ergosta-6,22-die3β-ol **(37)**		37.57 μg/mL	[28]
Phellinus gilvus	Fruiting bodies	Aqueous	Crude extracts	Raw 264.7 cell	19.46 μg/mL	[48]
			protocatechualdehyde **(28)**	Sparague Dawley Rats	Inhibition rate 81.1 %	[29]

Table 4 Summary of antioxidation from *Phellinus* mushroom

Species		Solvent	Chemical constituents	Model	Biological activity (IC_{50})	Reference
Phellinus linteus	Fruiting bodies	Methanol	4,(3,4-dihydroxyphenyl)-3-buten-2-one **(12)**	*In vitro*, ABTS	8.23 μM	[34]
			davalliactone **(30)**		2.61 μM	[34]
			inoscavin A **(10)**		13.3 μM	[34]
			protocatechuic acid **(38)**		2.38 μM	[34]
			protocatechualdehyde **(28)**		5.88 μM	[34]
	Mycelial	Ethyl acetate	caffeic acid **(32)**	*In vitro*, DPPH, ABTS	0.11, 0.18 μmol/L	[35]
			3,14-bihispidinyl **(39)**		0.90. 0.55 μmol/L	[35]
			hypholomine B **(34)**		0.31, 0.24 μmol/L	[35]
			hispidin **(11)**		1.31,2.27 μmol/L	[35]
			inotilone **(40)**		1.55, 2.27/Trolox	[33]
			phelligridimer A **(27)**	Rat liver microsomal	10.20 μM	[33]
	Fruiting bodies	Ethanol	phelligridin G **(13)**	Rat liver microsomal	3.86 μM	[23]
			phelligridin H **(41)**		4.80 μM	[24]
			phelligridin I **(42)**		3.70 μM	[24]
			phelligridin J **(12)**		6.50 μM	[24]

Table 4 Summary of antioxidation from *Phellinus* mushroom (continued)

Species		Solvent	Chemical constituents	Model	Biological activity (IC_{50})	Reference
Phellinus linteus	Fruiting bodies	Ethanol	davalliactone **(30)**	Rat liver microsomal	8.20 μM	[24]
Phellinus igniarius	Fruiting bodies	Aqueous	Crude extracts	*In vitro*, DPPH	332.85 μg/mL	[32]
		Ethanol			58.10 μg/mL	[32]
		Ethyl acetate			154.50 μg/mL	[32]
Phellinus rimosus	Fruiting bodies	Ethyl acetate	Crude extracts	Superoxide radical scavenging	2.2 μg/mL	[11]
				Hydroxyl radical scavenging	68 μg/mL	[11]
				Lipid peroxidation inhibition	162 μg/mL	[11]
				Nitric oxide scavenging (male wistar albino rats)	438 μg/mL	[11]

2.3.5 Antimicrobial activity

The antimicrobial activity of *Phellinus* extract was evaluated against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *S. enteritidis*, *Helicobacter pylori* (T96), *H. pylori* (2R), *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Lactobacillus plantarum, Klebsiella pneumonia* [39, 41, 45]. The extract of mycelial was identified as phellinone (43) was evaluated against *B. subtilis* (Minimum inhibitory concentration (MIC) = 10 µg/disc), phellinstatin (44) was evaluated against *S. aureus* (IC_{50}= 6 µg/mL), phellilane I (45), phellidene E (46), **γ**-ionylideneacetic acid (47) and trans-**γ**-monocyclofarnesol (48) were evaluated against *Porphyromonas gingivalis* (MIC= 278, 155, 34.1 and 5.9 µg/mL, respectively [36, 38, 40, 43] showed in Table 5.

2.3.6 Hepatoprotective

The crude ethyl acetate extract of *Phellinus* exhibited potent anti-hepatotoxic activity against carbon tetrachloride induced toxicity in rat liver was obvious from effect on the levels of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT) and serum alkaline phosphatase (ALP) [11]. The ethanolic extract from mycerial of *Phellinus* was identified as phellinulin A (49), phellinulin D (50), phellinulin E (51), phellinulin F (52), phellinulin G (53), phellinulin H (54), phellinulin I (55), phellinulin K (56), phellinulin M (57), phellinulin N (58), phellilin C (59) and **γ**-ionylideneacetic acid (47). The result showed phellinulin K displayed protection against hepatic fibrosis, examined active at rat hepatic stellate cells (HSCs), showed the best inhibition rate at concentration 20 mg/Kg with 67.9 % [37]. (Table 6)

Table 5 Summary of anti-microbial from *Phellinus* mushroom

Species	Part	Solvent	Chemical constituents	Model	Biological activity	Reference
Phellinus linteus	Mycelial	Ethanol	phellinone (43)	*Bacillus subtilis*	10 μg/disc	[43]
			phellinstatin (44)	*Staphylococcus aureus*	6 μg/mL	[36]
			phellilane I (45)	*Porphyromonas gingivalis*	278 μg/mL	[38]
			phellidene E (46)	*P. gingivalis*	155 μg/mL	[40]
			γ-ionylideneacetic acid (47)	*P. gingivalis*	34.1 μg/mL	[40]
			trans-γ-monocyclofarnesol (48)	*P. gingivalis*	5.9 μg/mL	[40]
Phellinus igniarius	Fruiting bodies	Aqueous Ethanol	Crude extracts	*S. aureus*	1.25 mg/mL	[41]
				Escherichia coli	9.33 mm	[42]
				Salmonella typhimurium	1067 mm	[42]
				S. enteritidis	13.00 mm	[42]
				S. aureus	18.33 mm	[42]
				Helicobacter pylori (T96)	17.00 mm	[42]
				H. pylori (2R)	16.83 mm	[42]

Table 5 Summary of anti-microbial from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Model	Biological activity	Reference
Phellinus rimosus	Fruiting bodies	methanol	Crude extracts	*E. coli*	16.30 mm	[39]
				Pseudomonas aeruginosa	11.60 mm	[39]
				S. aureus	12.30 mm	[39]
				S. typhimurium	12.60 mm	[39]
				B. subtilis	18.30 mm	[39]
Phellinus gilvus	Fruiting bodies	Aqueous	Crude extracts	*E. coli*	16.60 mm	[41]
				Lactobacillus plantarum	12.30 mm	[41]
				Klebsiella pneumonia	14.70 mm	[41]
		Ethanol		*S. aureus*	9.67 mm	[42]
Phellinus hippophaeicola	Fruiting bodies	Aqueous		*S. typhimurium*	11.33 mm	[42]
		Ethanol		*E. coli*	9.17 mm	[42]
				S. typhimurium	12.00 mm	[42]
			Crude extracts	*S. enteritidis*	10.67 mm	[42]
				S. aureus	12.67 mm	[42]
				H. pylori (T96)	15.00 mm	[42]
				H. pylori (2R)	21.00 mm	[42]

Table 5 Summary of anti-microbial from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Model	Biological activity	Reference
Phellinus everhrtii	Fruiting bodies	Aqueous	Crude extracts	*S. typhimurium*	11.33 mm	[42]
				S. aureus	12.17 mm	[42]
		Ethanol	Crude extracts	*E. coli*	10.00 mm	[42]
				S. typhimurium	12.00 mm	[42]
				S. enteritidis	11.17 mm	[42]
				S. aureus	12.17 mm	[42]
				H. pylori (T96)	13.33 mm	[42]
				H. pylori (2R)	16.83 mm	[42]
Phellinus noxius	Fruiting bodies	Ethanol	Crude extracts	*S. aureus*	12.50 mm	[42]
				H. pylori (T96)	15.33 mm	[42]
				H. pylori (2R)	16.83 mm	[42]
Phellinus pini	Fruiting bodies	Ethanol	Crude extracts	*S. aureus*	12.50 mm	[42]
				H. pylori (T96)	17.17 mm	[42]
				H. pylori (2R)	18.00 mm	[42]

Table 5 Summary of anti-microbial from *Phellinus* mushroom (continued)

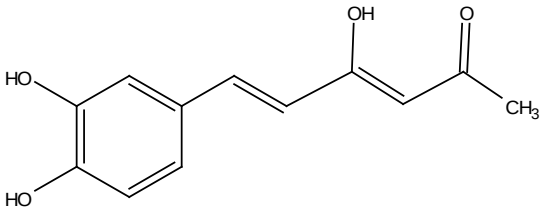
Species	Part	Solvent	Chemical constituents	Model	Biological activity	Reference
Phellinus conchatus	Fruiting bodies	Aqueous		*E. coli*	9.50 mm	[42]
				S. typhimurium	9.17 mm	[42]
		Ethanol	Crude extracts	*S. enteritidis*	9.50 mm	[42]
				S. aureus	10.00 mm	[42]
				H. pylori (T96)	18.00 mm	[42]
				H. pylori (2R)	10.67 mm	[42]
Phellinus nigricans	Fruiting bodies	Aqueous		*S. typhimurium*	9.67 mm	[42]
				S. enteritidis	9.33 mm	[42]
		Ethanol	Crude extracts	*S. aureus*	10.67 mm	[42]
				H. pylori (T96)	15.17 mm	[42]
				H. pylori (2R)	16.00 mm	[42]

Table 6 Summary of hepatoprotective from *Phellinus* mushroom

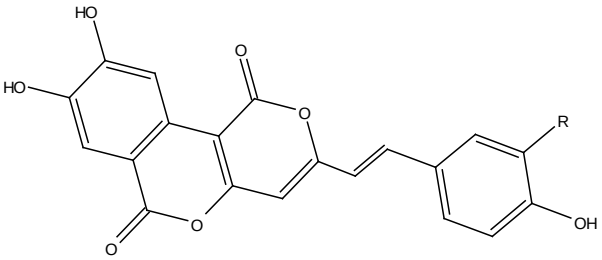
Species	Part	Solvent	Chemical constituents	Model	Biological activity	Reference
Phellinus linteus	Mycelial	Ethanol	phellinulin A **(49)**	Rat hepatic stellate cells (HSCs) 20 mg/Kg	67.2 %	[37]
			phellinulin D **(50)**		4.20 %	[37]
			phellinulin E **(51)**		23.6 %	[37]
			phellinulin F **(52)**		26.7 %	[37]
			phellinulin G **(53)**		15.2 %	[37]
			phellinulin H **(54)**		60.6 %	[37]
			phellinulin I **(55)**		50.9 %	[37]
			phellinulin K **(56)**		67.9 %	[37]
			phellinulin M **(57)**		56.4 %	[37]
			phellinulin N **(58)**		47.2 %	[37]
			phellilin C **(59)**		24.2 %	[37]
			γ-ionylideneacetic acid **(47)**		39.7 %	[37]

Table 6 Summary of hepatoprotective from *Phellinus* mushroom (continued)

Species	Part	Solvent	Chemical constituents	Model	Biological activity	Reference
Phellinus rimosus			Crude extracts	*In vivo* (male wistar albino rats) -Serum glutamate pyruvate transaminase (SGPT)	175 IU/l	[11]
				-Serum glutamate oxaloacetate transaminase (SGOT)	137 IU/l	[11]
				-Alkaline phosphatase (ALP)	171 IU/l	[11]

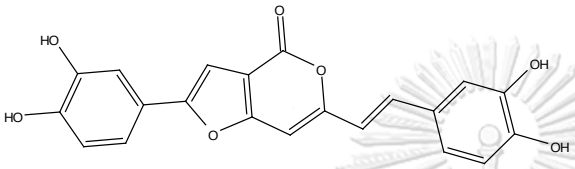


hispolon (1)

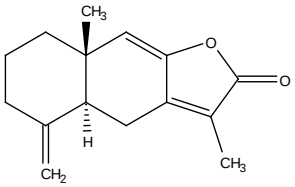


meshimakobnol A (2) R=OH

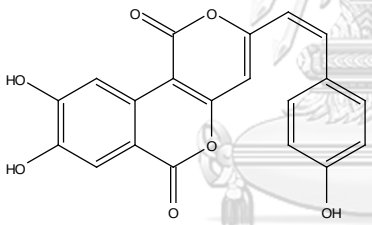
meshimakobnol B (3) R=H



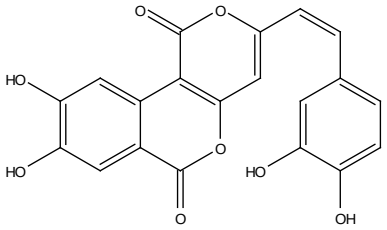
phellifuropyranone (4)



atraclyenoline (5)



phelligridin C (6)



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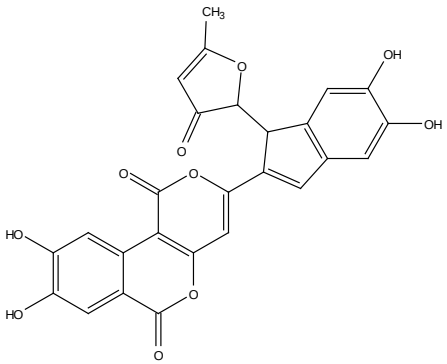

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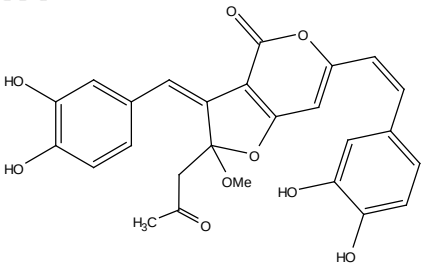
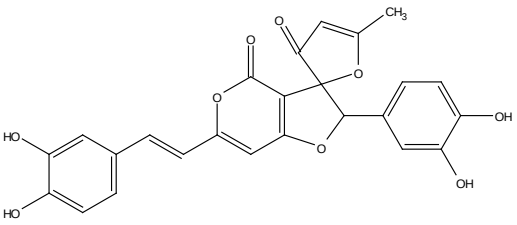
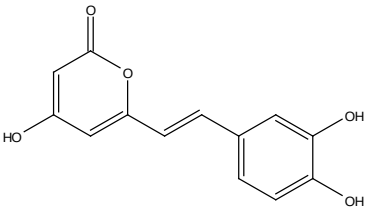


phelligridin F (9)

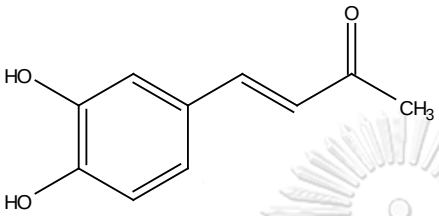
Figure 2 Chemical structure of chemical constituents of *Phellinus* sp.



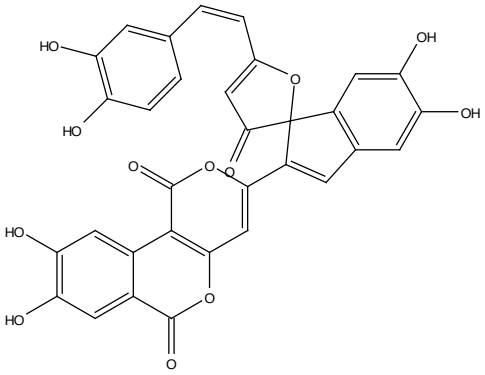
inoscavin A **(10)**



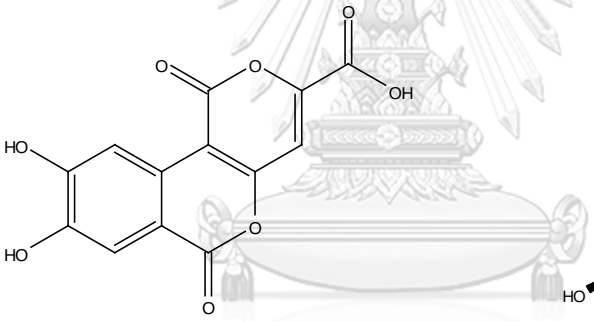
hispidin **(11)**



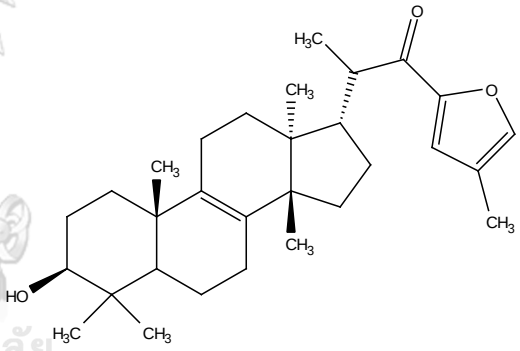
4-(3,4-dihydroxyphenyl)but-3-en-2-one **(12)**



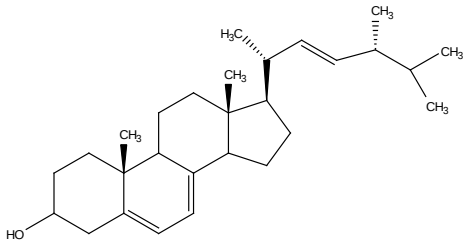
phelligridin G **(13)**



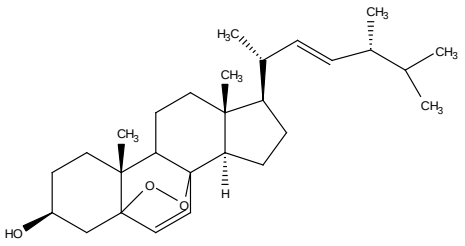
phelligridins J **(14)**



igniarine **(15)**

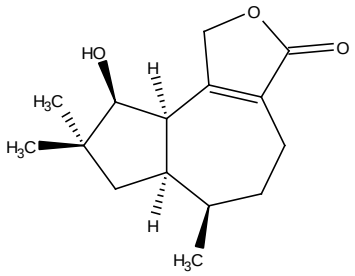


ergosterol **(16)**

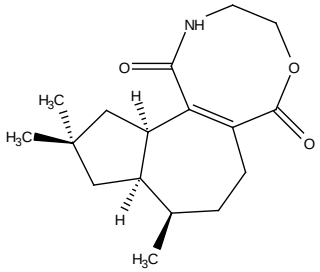


ergosterol peroxide **(17)**

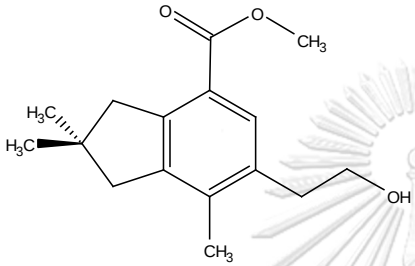
Figure 2 Chemical structure of chemical constituents of *Phellinus* sp. (continued)



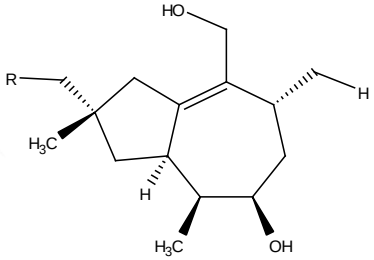
phellinignin A **(18)**



phellinignin B **(19)**

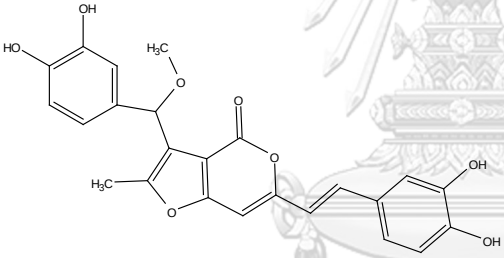


phellinignin D **(20)**

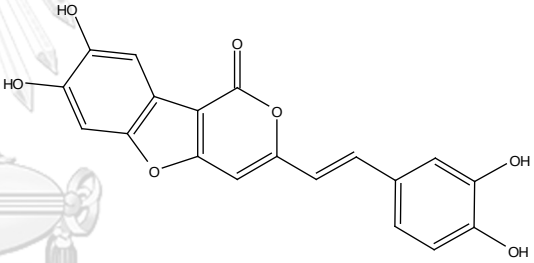


conocenol A **(21)** R=OH

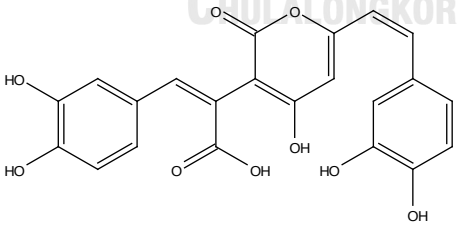
conocenol B **(22)** R= -



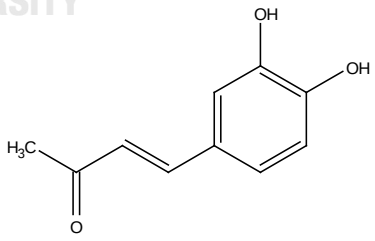
phelliigniarin B **(23)**



phellibaumin A **(24)**

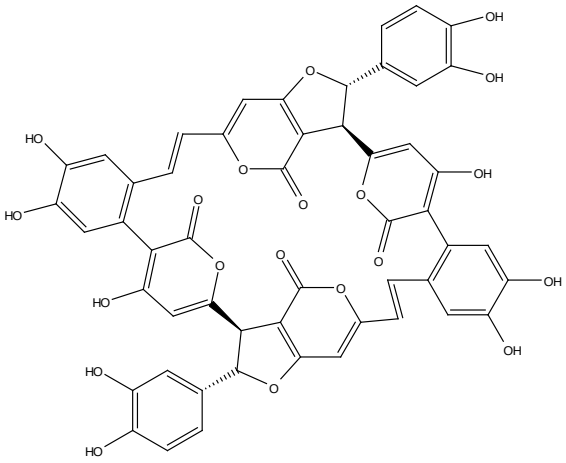


phellibaumin D **(25)**

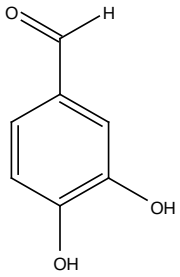


3,4-dihydroxbenzal-acetone **(26)**

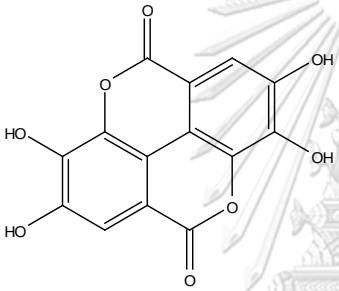
Figure 2 Chemical structure of chemical constituents of *Phellinus* sp. (continued)



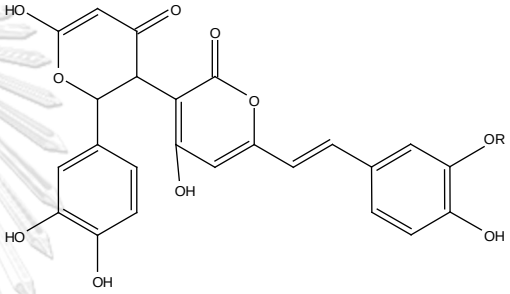
phelligridimer A **(27)**



protocatechualdehyde **(28)**

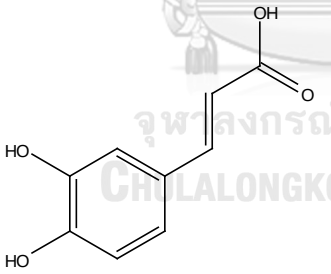


ellagic acid **(29)**

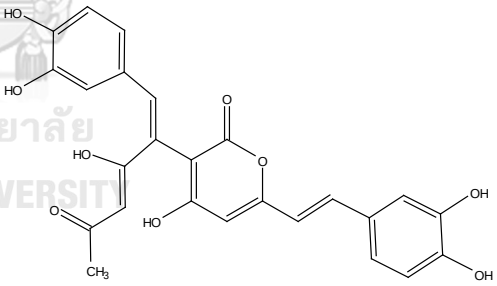


davallialactone **(30)** R=H

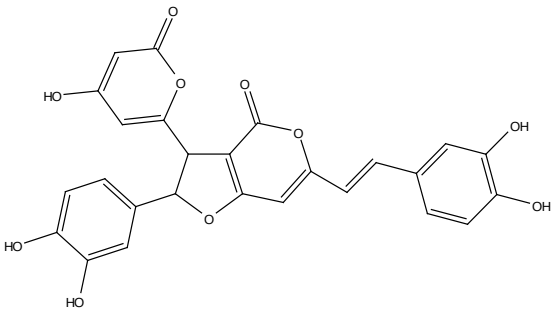
methyldavalliactone **(31)** R=CH_3



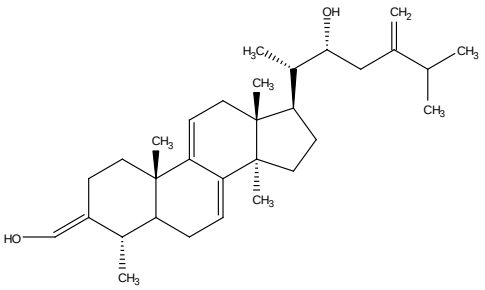
caffeic acid **(32)**



interfungin A **(33)**

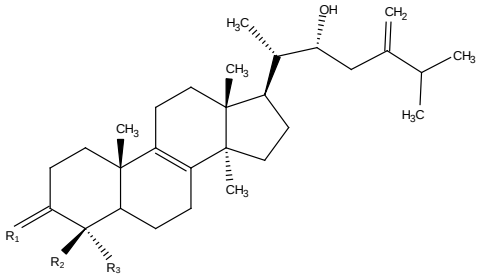


hypolomine B **(34)**



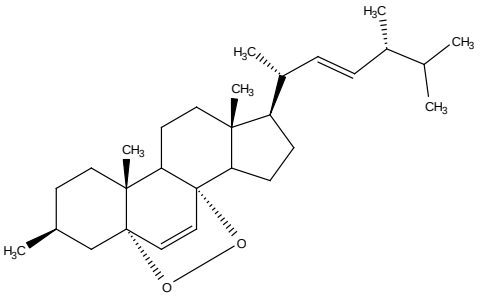
igniaren B **(35)**

Figure 2 Chemical structure of chemical constituents of *Phellinus* sp. (continued)

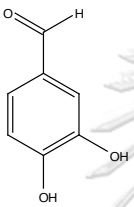


igniaren D (36)

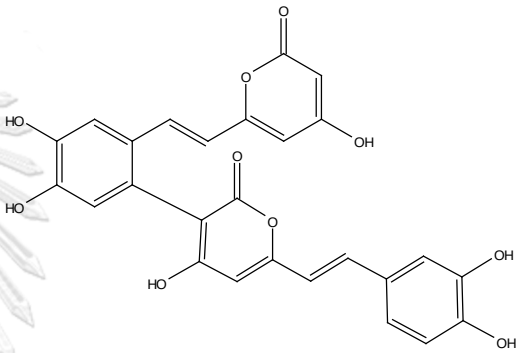
R_1=α-OH, R_2 =β-H, R_3= CH_3



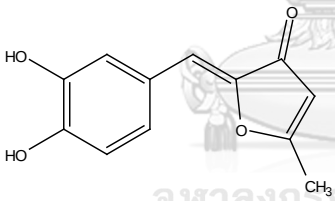
ergosta-6,22-die3β-ol (37)



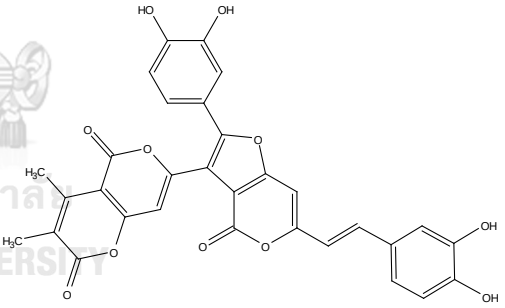
protocatechuic acid (38)



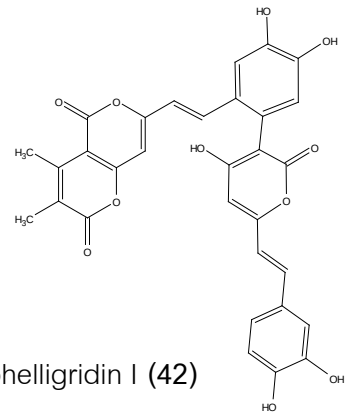
3,14-bihisphidinyl (39)



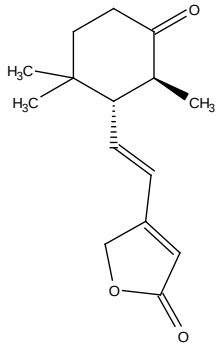
inotilone (40)



phelligridin H (41)

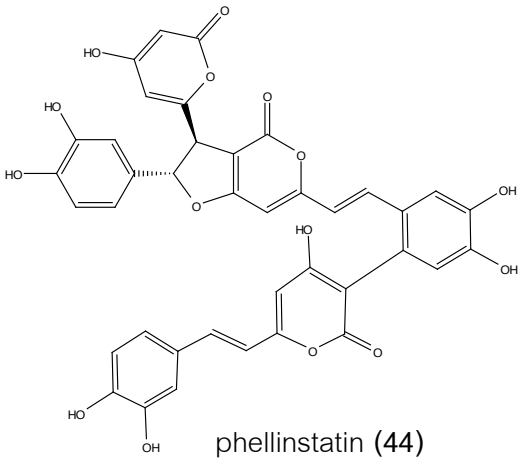


phelligridin I (42)

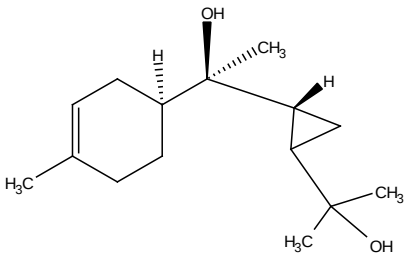


phellinone (43)

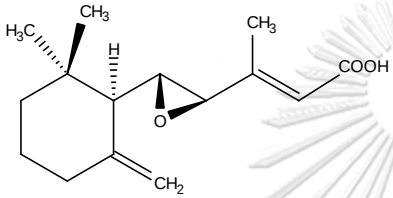
Figure 2 Chemical structure of chemical constituents of *Phellinus* sp. (continued)



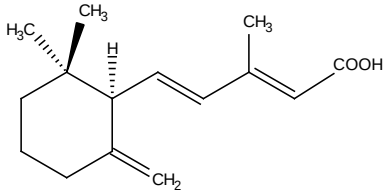
phellinstatin (44)



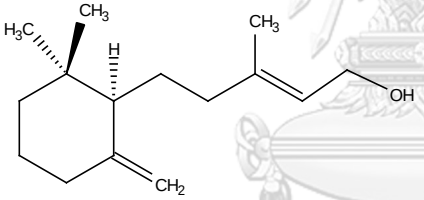
phellilane I (45)



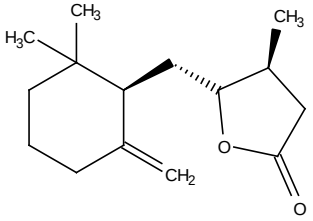
phellidene E (46)



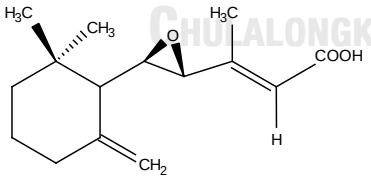
γ-ionylideneacetic acid (47)



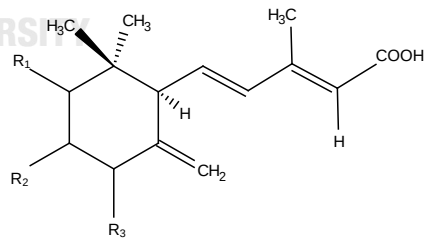
trans-γ-monocyclofarnesol (48)



phellinulin A (49)



phellinulin D (50)



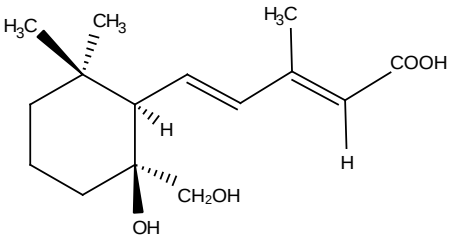
phellinulin E (51) R_1=α-OH, R_2=H, R_3=H

phellinulin F (52) R_1=H, R_2=β-OH, R_3=H

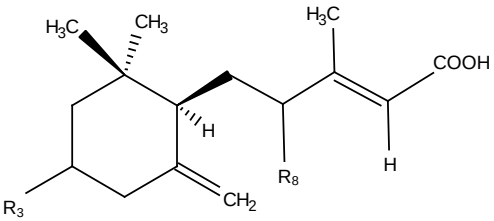
phellinulin G (53) R_1=H, R_2=α-OH, R_3=H

phellinulin H (54) R_1=H, R_2=H, R_3=β-OH

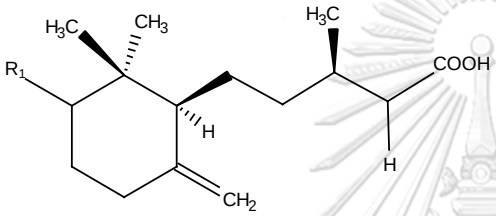
Figure 2 Chemical structure of chemical constituents of *Phellinus* sp. (continued)



phellinulin I (55)

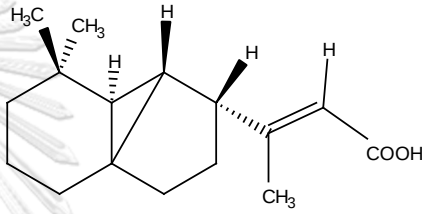


phellinulin K (56)



phellinulin M (57) R_1= H

phellinulin N (58) R_1= β-OH



phellilin C (59)

Figure 2 Chemical structure of chemical constituents of *Phellinus* sp. (continued)

2.3 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is an in vitro test to measure viable cells mitochondrial activity. The mitochondrial activity of live cells is reflected by the conversion of the tetrazolium salt MTT into formazan crystals. This method was evaluated by the colorimetric, involves reducing yellow of dye MTT to formazan crystal (purple) (Figure 3). Thus, number of viable cells can be detected by

measuring absorbance of formazan using a microplate reader [51].

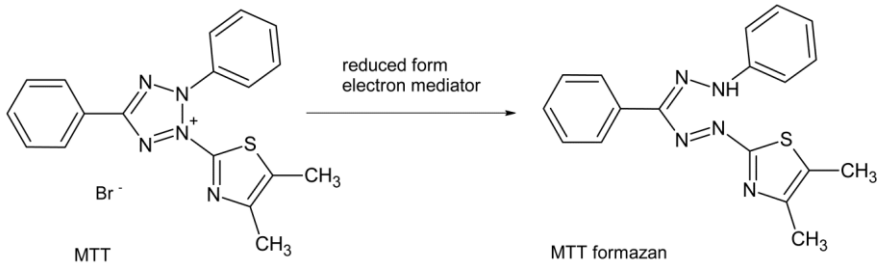


Figure 3 MTT reaction in viable cells by mitochondrial reductase to formazan crystal

CHAPTER III
MATERIALS AND METHODS

3.1 Mushroom materials

Phellinus sp. were collected from Ubon Ratchathani, Thailand in 2020. The mushroom was dried, powdered and stored at -20 °C until use.

3.2 Chemicals and reagents

3.2.1 Commercial grade solvents, *n*-hexane was purchased QREC, Newzealand.

3.2.2 Commercial grade solvents, dichloromethane (DCM) was purchased Lab scan, Supelo.

3.2.3 Commercial grade solvents, ethyl acetate (EtOAc) was purchased Lab scan,
Supelo.

3.2.4 Commercial grade solvents, methanol (MeOH) was purchased Lab scan, Supelo.

3.2.5 Deuterated chloroform and methanol-*d*4 were purchased from Eurisotop.

3.2.6 Deuterium oxide was purchased from Sigma-Aldrich.

3.2.7 Silica gel 60, 70-230 mesh ASTM (0.063-0.200 mm) was purchased from Merk, Germany.

3.2.8 Sephadex LH-20 were purchased from and Pharmacia.

3.2.9 Cetyltrimethylammonium bromide (CTAB) was purchased from Serva.

3.2.10 Isoamyl alcohol was purchased from Carbo Erba.

3.2.11 Dulbecco's Modified Eagle Medium (DMEM) medium was purchased from Thermo Fisher Scientific (U.S.A).

3.2.12 Dulbecco's Modified Eagle Medium F12 (DMEM/F12) was purchased from Thermo Fisher Scientific (U.S.A).

3.2.13 Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (U.S.A).

3.2.14 Penicillin was purchased from Thermo Fisher Scientific (U.S.A)

3.2.15 Sodium pyruvate solution was purchased from Thermo Fisher Scientific (U.S.A).

3.2.16 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Thermo Fisher Scientific (U.S.A)

3.2.17 96-well microplate was purchased from SPL Life Sciences Co., Ltd. (Korea).

3.3 General experimental procedures

3.3.1 Thin layer chromatography (TLC)

TLC analysis using TLC Silica gel coated with 0.2 mm silica gel 60 F_{254}, Merck, Germany. The TLC reverse phase analysis was performed on aluminum sheets coated with silica gel 60 RP-18 F_{254}, Merck, Germany. The test sample was spotted on TLC plate by a capillary tube. The TLC plates were also visualized under UV light at wavelength of 254 and 365 nm and staining with *p*-anisaldehyde and ammonium molybdate followed by heating.

3.3.2 Column chromatography

Column chromatography (CC) was performed using Silica gel 60 F_{254}, Merck, Germany as adsorbent. Size exclusion chromatography was performed by Sephadex LH-20 to separate extracts size exclusion.

3.3.3 Nuclear magnetic resonance spectrometer (NMR)

The ^{1}H and ^{13}C NMR spectra were recorded on Jeol 500 MHz spectrometer. The chemical shifts (δ) were reported in parts per million (ppm) and coupling constants (*J*) in Hertz. Chloroform-*d* ($CDCl_3$), methanol-d_4 (CD_3OD) and deuterium oxide (D_2O) were used as solvent in NMR experiment and chemical shifts were referenced to the residual solvent signals of $CDCl_3$ at δ_H 7.26 ppm, δ_C 77.17 ppm; CD_3OD at δ_H 3.31 ppm, δ_C 49.00

ppm and D_2O at δ_H 4.79 ppm. MestReNova NMR software version 14.1.1 was used to reprocess the spectral data.

3.3.4 Microplate spectrophotometer

Ultraviolet (UV) data of absorbance for cytotoxicity assay was obtained from microplate reader, PowerWave XS2 (Biotek Instruments Inc, USA).

3.4 Identification of mushroom

3.4.1 Morphology observation

The characterization of mushroom was identified on distinguishing characteristics such as shape and color of fruiting body, a tubular hymenophore, color and texture of fruiting body using key in identified of mushroom described by Thomas Læssøe and Jen H. Petersan.

3.4.2 Scanning electron microscopy (SEM)

The dried mushroom fruiting body was cut into 5 x 5 x 1 mm and then coated in vacuum with gold. The sample was analyzed using SEM/EDX microscopy (JSM-IT 100).

3.4.3 Isolation of mycelium from mushroom sample

The mushroom sample was cut into small pieces using a sterile tool in sterile conditions. The small pieces of mushroom were put on Potato Dextrose Agar (PDA) medium and incubated at room temperature.

3.4.4 DNA extraction

DNA was extracted from fruiting body with the solution cetyltrimethylammonium ammonium bromide (CTAB). Fruiting body and bead homogenizer were added into sterile microcentrifuge tube (2 mL) at 20 Hertz for 1 min and then 700 µL of CTAB solution (2%, w/v) was added. The mixture was vortexed and incubated at 65 °C for 1 hour. Then 700

μL of chloroform/isoamyl alcohol (24:1, v/v) was into the mixture, vortexed and centrifuge at 15000 rpm for 8 min at 18 °C. Supernatant was transferred into a new micro centrifuge tube (1.5 mL), mixed with 500 μL of isopropanol solution, stored at -20 °C for 30 min, and centrifuged at 8000 rpm at 4 °C for 10 min. The supernatant was discarded and the pellet was washed with 500 μL of 70% ethanol. After centrifugation at 8000 rpm at 4 °C for 5 min, the supernatant was discarded, and the pellet was dried at room temperature. The dry pellet was dissolved in 20 μL of TE buffer and stored at -20 °C.

3.4.4.1 Amplification and sequencing of ITS region

ITS region of mushroom was amplified with the primer ITS1 (5' CTTGGTCAT TTAGAGGAAGTAA 3') and ITS4 (5'-TCCTCCGCTTATTGATATGC) (White et al., 1990). Amplification was performed in a 30 μL reaction mixture containing each 0.3 mL, 20 μM of primers, 15 mL of Emerald, 3 mL of DNA template and 11.4 mL of sterilized distilled water. The reaction was performed as follows; (1) initial denaturation 94 °C for 5 min, (2) denaturation at 94 °C for 1 min, (3) annealing at 51 °C for 1 min, (4) extension at 72 °C for 1 min, (5) final extension at 72 °C for 5 min and holding 4 °C. The PCR products were analyzed by gel electrophoresis with 1.5% agarose gel (in 0.5% TBE).

3.5 Extraction and isolation

3.5.1 Extraction Kratinpiman mushroom (*Phellinus* sp. fruiting body)

The dried mushroom powders (600 g) were extracted with methanol (1.5 L) (x5 times) by maceration for 24 h at room temperature. After evaporation of the solvents under vacuum with rotary evaporator, crude methanol extract (26.7 g) was obtained. The methanol crude extract was partitioned successively with hexane (200 mL x 10 times), DCM (200 mL x 10 times), ethyl acetate (EtOAc) (200 mL x 10 times) and MeOH (100 mL x 2 times) to give hexane extract (CHE, 2.39 g), DCM extract (CDE, 2.32 g), ethyl acetate extract (CEE, 1.10 g) and methanol extract (CME, 3.16 g), respectively. For aqueous extraction, 10 g of mushroom powder was refluxed with 400 mL of distilled water for 8 h at 75 °C and the mixture was then filtration with Whatman no.1 under vacuum, followed

by lyophilization to give aqueous soluble extract (RE, 10 mg). A 10 g of mushroom powder was decocted with 400 mL of distilled water for 8 h (ten times) at 75 °C and the mixture was then filtration with Whatman no.1 under vacuum. It followed by evaporation water under vacuum by rotary evaporator to give aqueous soluble extract (DE, 10 mg). The extraction procedure is shown in Figure 4. All crude extracts were evaluated their cytotoxic activity against human lung cancer (A549), neuroblastoma (SH-SY5Y) and mouse subcutaneous connective tissue (L929) using the cytotoxicity assay (MTT).

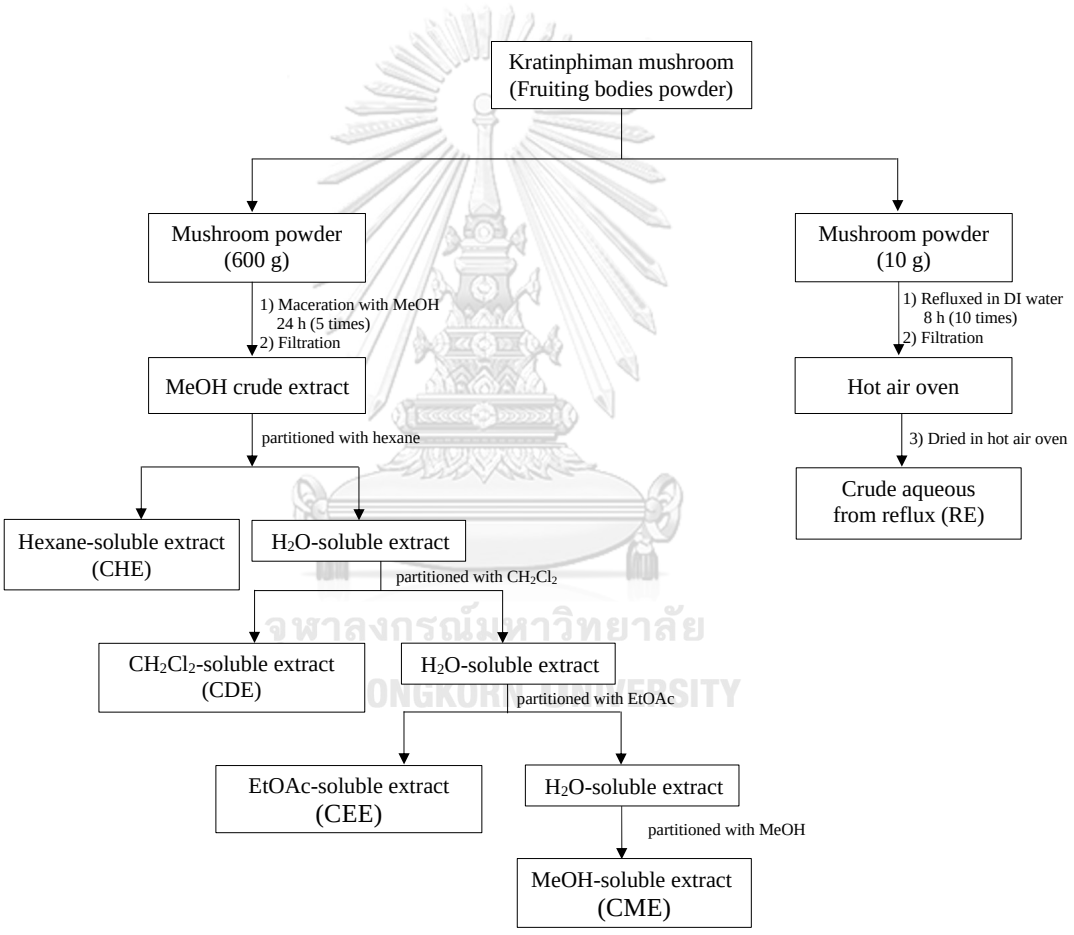


Figure 4 The extraction procedure of Kratinphiman mushroom

3.5.2 Isolation of hexane partitioned extract (CHE) of Kratinpiman mushroom (*Phellinus* sp.)

The CHE extract (CHE, 2.39 g) was separated by column chromatography on Silica gel 60 (300 g). The CHE (2.39 g) was dissolved in hexane, mixed with 10 g of silica gel 60 and evaporation under reduced pressure to give the CHE adsorbed silica. Then it was loaded over silica gel 60 (300 g) in glass column (diameter 10 cm) and then eluted with hexane: EtOAc (9:1, v/v) (2 L), hexane: EtOAc (8:2, v/v) (1.5 L), hexane: EtOAc (6:4, v/v) (2 L), EtOAc (0.5 L), EtOAc: MeOH (6:4, v/v) (1.7 L), EtOAc: MeOH (2:8, v/v) (1.5 L) and MeOH (3 L) , respectively. Eluted fractions (each fraction 30 mL) were collected, and fractions with similar TLC patterns were combined to give eighteen combined fractions (CFH1-CFH18). The combined fractions were evaluated for their antiproliferative activity against with A549 and SH-SY5Y cell lines using the cytotoxicity assay (MTT).

The CFH1 extract (129.6 mg) was separated by silica gel TLC developing by hexane: EtOAC (9:1 v/v, 50 mL) as mobile phase to give eight subfractions (CFH1.1-CFH1.8). Fraction CFH1.4 was further separated by Silica gel TLC using solvent system: hexane: EtOAc (9.5: 0.5 v/v, 50 mL) as mobile phase to give compound 1 (CFH1.4.4) as yellow solid (5.9 mg, 0.24 % w/w of hexane extract).

The fraction CFH 8 (64.7 mg) was separated by column chromatography on silica gel 60 (12 g). The CFH 8 (64.7 mg) was dissolved in hexane, mixed with 0.5 g of silica gel 60 and evaporation under reduced pressure to give the CFH8 adsorbed silica. Then it was loaded over silica gel 60 (12 g) in glass column (diameter 1.5 cm) and then eluted with hexane: EtOAc (8: 2 v/v, 100 mL), hexane: EtOAc (7: 3 v/v, 100 mL), hexane: EtOAc (6: 4 v/v, 100 mL), hexane: EtOAc (5: 5 v/v, 100 mL), hexane: EtOAc (4: 6 v/v, 100 mL), hexane: EtOAc (3: 7 v/v, 100 mL), hexane: EtOAc (2: 8 v/v, 200 mL) and hexane: EtOAc (1: 9 v/v, 200 mL), respectively. Eluted fractions (each fraction 5 mL) were collected, and fractions with similar TLC patterns were combined to give eight combined fractions (CFH 8.1-CFH 8.8) to give compound 2 (CFH 8.4) was obtained as yellow solid (5.2 mg, 0.21 % w/w of hexane extract).

3.5.3 Isolation of DCM partitioned extract (CDE) of Kratinpiman mushroom (*Phellinus* sp.)

The CDE extract (CDE 2.32 g) was separated by column chromatography on Silica gel 60 (300 g). The CDE was dissolved in DCM: MeOH (9:1), mixed with 300 g of silica gel 60 and evaporation under reduced pressure to give the CDE adsorbed silica. Then it was loaded over silica gel 60 (300 g) in glass column (diameter 10 cm) and then eluted with DCM (700 mL), DCM: MeOH (9.75: 0.25, v/v), (1.4 L), DCM: MeOH (9.5: 0.5, v/v), (850 mL), and MeOH (2 L), respectively. Eluted fractions (each fraction 25 mL) were collected, and fractions with similar TLC patterns were combined to give twelve combined fractions (CFD1-CFD12). The combined fractions were evaluated for their antiproliferative activity against with A549 and SH-SY5Y cell lines using the cytotoxicity assay (MTT).

The fraction CFD 3 (20.3 mg) was separated by silica gel TLC reverse phase developed by MeOH: DI (7: 3, 50 mL) as mobile phase to give ten combined fractions (CFD3.1-CFD3.10). Fraction CFD3.4 (compound 2) was obtained as yellow solid (4.6 mg, 0.19% w/w of DCM extract) and Fraction CFD3.3 (compound 3) was obtained as yellow solid (2.5 mg, 0.1% w/w of DCM extract)

3.5.4 Isolation of ethyl acetate partitioned extract (CEE) of Kratinpiman mushroom (*Phellinus* sp.)

The CEE extract (CEE, 1.10 g) was separated by column chromatography on Sephadex LH-20 gel was immersed in absolute MeOH overnight to allow the beads to swell. The CHE (1.10 g) was dissolved in MeOH and then loaded over Sephadex LH-20 gel in glass column (diameter 10 cm) and then eluted with MeOH (4 L). Eluted fractions (each fraction 50 mL) were collected, and fractions with similar TLC patterns were combined fractions (CFE1-CFE16). The combined fractions were evaluated for their antiproliferative activity against with A549 and SH-SY5Y cell lines using the cytotoxicity assay (MTT).

The fraction CFE 5 (56.1 mg) was separated by column chromatography on Sephadex LH-20 was immersed in gradient DCM: MeOH (9:1, v/v) overnight to allow the

beads to swell. The CFE 5 (56.1 mg) was dissolved in MeOH and loaded over Sephadex LH-20 in glass column (diameter 1.5 cm) and then eluted with MeOH (x L). Eluted fractions (each fraction 3 mL) were collected, and fraction with similar TLC patterns were combined to give three fractions (CFE 5.1-CFE5.3). Fraction CFE 5.3 (3.3 mg) was obtained as yellow (3.3 mg, 0.3 % w/w of ethyl acetate extract.

3.6 Cell culture

The human lung cancer (A549, ATCC CCL-185^{TM}) and mouse subcutaneous connective tissue (L929) were obtained from Professor Tanapat Palaga (TP) lab at Chulalongkorn University. Neuroblastoma (SH-SY5Y, ATCC CRL-2266^{TM}) was obtained from James Walker lab at Harvard University. Human lung cancer (A549) and mouse subcutaneous connective tissue (L929) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) and human neuroblastoma (SH-SY5Y) cell line was cultured in Dulbecco's Modified Eagle Medium F12 (DMEM/F12) with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 U/mL penicillin. All cell lines were cultured in an incubator at 37 °C under 5% CO_2 incubator for 24 hours using aseptic technique.

3.7 Cytotoxicity assay

Each stock solution, of dichloromethane (CDE), ethyl acetate (CEE) and methanol (CME) extract was prepared by dissolving 10 mg of each extract in 100 µL of DMSO while a stock of reflux (RE) and decoction (DE) extract were prepared by dissolved 10 mg of the extracts in 100 µL sterilized water. The stock solution (CDE, CEE and CME extracts) was diluted in DMSO to give concentration of 10, 15, 20 and 25 mg/mL and then the solution (10 µL) of each extract was further diluted with 990 µL of the culture medium to result in the final concentration of each extract and DMSO per well of 100, 150, 200 and 250 µg/mL and of 1% v/v, respectively.

Two human cancer cell lines A549 and SH-SY5Y and normal cell lines L929 were seeded in each 96-well plates at 1x10^5 cell/well (100 µL/well) and incubated at 37 °C under 5% CO_2 for 24 hours. Then, the culture medium was removed and 100 µL of the extract with different concentrations were added into each test and incubated at 37 °C

under 5% CO_2 for 24 hours. The culture medium was removed and 100 µL of the MTT solution (0.05 mg/mL) was added. After incubation for 3 hours, the MTT was removed and the formazan remined in each well was dissolved by 100 µL DMSO. Then absorbance (Abs) of formazan was measured by a microplate reader at 570 nm. The cytotoxicity was expressed as IC_{50} values (50% inhibitory concentration) [51].

$$\% \text{ Inhibition} = 100 - \left(\frac{Abs_1 - Abs_{blank}}{\text{Mean } Abs_0 \text{control} - Abs_{blank}} \right) \times 100$$

A_0 is Absorbance of the control (cell lines + culture medium)

A_1 is Absorbance of the test sample (cell lines + culture medium + extract)

A_{blank} is absorbance of blank (culture medium)

CHAPTER VI
RESULT AND DISCUSSION

4.1 Identification of the mushroom

4.1.1 Morphology observation

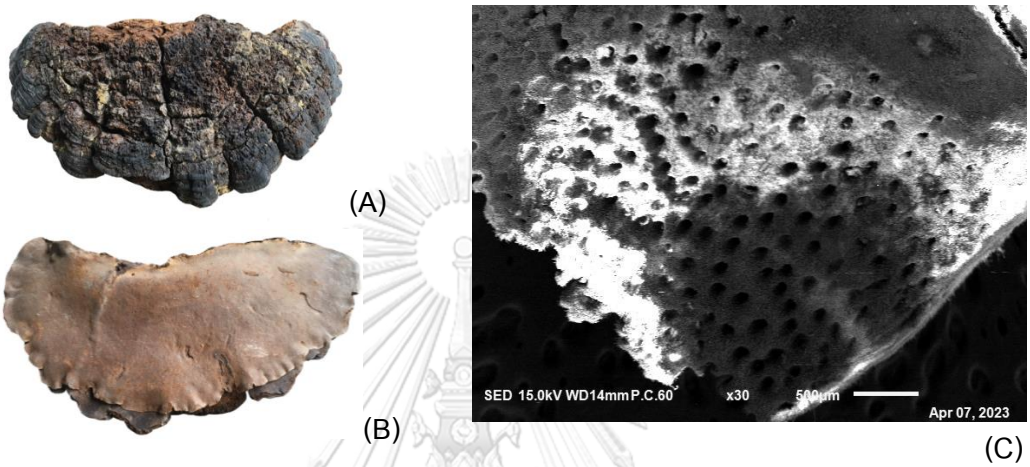


Figure 5 Characteristics of *Phellinus* sp., pileus surface (A), hymenial surface (B) and hymenophore under SEM (C)

4.1.1.2 Key to major groups of mushrooms

The mushroom was identified with the key described by Thomas Læssøe and Jen H. Petersan [52] Form groups of mushrooms were identified with external spore production showed as 18 group such as

1. corticioids (flat or effused-reflexed, smooth, warty, wrinkled or spiny)
2. rosette fungi and the like tongue (shaped or with flattened branches, smooth under/upper side)
3. clavarioids (clavate or coralloid)
4. cyphelloides (with hanging,tiny discs or tubes)
5. dacrymycetals (rubbery, basidia like tunning-forks, spores often septate)
6. jelly fungi (often gelatinous, spores repeating)
7. rusts and smuts (parasitize plants)
8. operculate discomycetes (discoid to clavate apothecia, asci with lids)

9. inoperculate discomycetes (discoid, slit-like to clavate, apothecia, asci without lids)

10. lichens (thalli with enclosed algae and/or cyanobacteria)

11. asexual fungi (asexual)

12. chanterelles (funnel-shaped, hymenophore smooth or veined)

13. agarics (with gills)

14. boletes (with tubes, loosened from the flesh)

15. taphrina (asci on living host tissue, leaves)

16. hydnoids (stem and spines)

17. polypores (tubes firmly attached)

18. fruiting bodies with internal spore production, with or without active spore release

In polypores group can be divided according to character of fruiting bodies and its growths were showed as 5 group such as:

1. clustered polypores (fruitbodies annual, composite, clustered)

2. polypores with a stem (fruitbodies annual, tongue-shaped, with a stem)

3. perennial polypores (fruitbodies perennial, hard or tough)

4. annual, completely resupinate (flat) polypores (fruitbodies annual, tough or soft)

5. annual, capped and adnate polypores (fruitbodies annual, with caps but possibly partly resupinate (reflexed))

The genus in polypores group from the key by Thomas Læssøe and Jen H. Petersan are represented 25 genus such as:

1. *Laricfomes* (high, hoof-shaped flesh very bitter)

2. *Rhodofomes* (pores pink)

3. *Fomitosis* (flesh pale yellow in young parts)

4. *Osmoporus* (strong, sweetish smell)

5. *Gloeophyllum* (pores mostly gill like, stretched)

6. *Fomes* (spore-deposit white, fruitbody with mycelial core)

7. *Ganoderma* (spore-deposit red-brown, on deciduous trees)

8. *Fomitiporia* (mostly with hoof-shaped, cracked caps, on deciduous trees or conifers)

9. *Fuscoporia* (mostly resupinate or with sharp-edged caps, on deciduous trees)
10. *Phellopilus* (with caps or resupinate, with black lines in the flesh, on conifers)
11. *Porodaedalea* (hairy and with large, labyrinthine pores, on conifers)
12. *Phylloporia* (with dark line in the flesh, on Ribes or Euonymus)
13. *Funalia* (deep felt and large pores)
14. *Phellinus* (mostly with hoof-shaped, usually cracked caps, on deciduous trees)
15. *Dichomitus* (darker margin, high on Quercus or Corylus)
16. *Phellinopsis* (with small caps, on Salix)
17. *Daedaleopsis* (reddening, radially stretched pores)
18. *Heterobasidion* (on roots and stumps, on conifers)
19. *Haploporus* (high on Salix or Quercus)
20. *Skeletocutis* (small spores and crystal covered hyphae)
21. *Trametes* (radially stretched pores)
22. *Rigidoporus* (cystidia, flesh mostly pale brown)
23. *Perenniporia* (spores mostly dextrinoid, thick-walled)
24. *Cerrena* (pale grey, labyrinthine pores)
25. *Daedalea* (large labyrinthine pores)

In this study, from the details of mushroom (Figure 5) when comparing with the key of Thomas Læssøe and Jen H. Petersan [52] the results showed that identified a sample is polypores group. Polypores is a form group that is characterized by a tubular hymenophore, the spores are formed on a hymenium underside tubes. The pores of this group have spherical, angular, stretched, labyrinthins or gill-like. Most species in this group have tough or hard fruiting bodies and complicated hyphal structure and in the Figure 5(C) showed pores of mushroom sample have round. In addition, when comparing with character of fruiting bodies and its growths, the result showed as perennial polypores (fruitbodies perennial, hard or tough). From the genus in polypores group from the key [52], the result showed that identified a mushroom sample is genus *Phellinus* which mostly with hoof-shaped, usually cracked caps, on deciduous trees [52].

4.1.2 DNA extraction

4.1.2.1 Isolation of mycelium from mushroom sample

Mycelium of sample was not isolated from fruiting bodies of mushroom sample because the mushroom was dried for prevent contamination of mushroom from other fungi. At high temperatures drying, DNA denaturation and it's not suitable for DNA extraction. The drying method to be using for DNA extraction such as silica gel drying at 22 °C produced the highest yield of dsDNA [53].

4.1.2.2 DNA extraction from fruiting body of mushroom

In this study, genomic DNA was not isolated from fruiting bodies of mushroom sample because of polysaccharides (Figure 26, appendix) and pigment. Similar to the previous report in the literature [54] . In the previous report, polysaccharides, proteins, tannins, alkaloids and polyphenols inhibited DNA polymerase and effected the quality and quantity of DNA, which polysaccharides are the most found contaminants in DNA extraction. Moreover, polysaccharides make DNA pellets slimy[55] .

4.2 Extraction of the *Phellinus* sp. mushroom

The fruiting body of *Phellinus* sp. (600 g) was extracted with methanol and then evaporated by rotary evaporator to obtain methanolic extract as dark brown solid (26.7 g, 4.45 % w/w of mushroom powder). The methanol crude extract was partitioned with four solvents by increasing polarities to obtain for subfractions hexane extract as brown liquid oil (fraction CHE, 2.39 g, 0.39 % w/w of mushroom powder), DCM extract as brown solid (fraction CDE 2.32 g, 0.38% w/w of mushroom powder), ethyl acetate extract as dark brown solid (fraction CEE, 1.10 g, 0.18% w/w of mushroom powder) and methanol extract as dark brown solid (3.16 g, 0.52% w/w of mushroom powder). For aqueous extract, 10 g of mushroom powder was refluxed and decocted with 400 mL of distilled water to give refluxed crude extract as brown solid (RE, 0.01 g, 0.1% w/w of mushroom powder) and decocted crude extract as brown solid (DE, 0.01 g, 0.1% w/w of mushroom powder).

All crude extracts were evaluated their cytotoxic activity against A549, SH-SY5Y, and L929 cell lines. The result in Table 7 showed that DCM extract (CDE) exhibited the

strongest antiproliferative activity with IC_{50} values of 132.4 and 106.87 µg/mL against human lung cancer and neuroblastoma cell lines, respectively. Furthermore, ethyl acetate extract (CEE) exhibited moderate antiproliferative activity against human neuroblastoma and lung cancer cell lines with IC_{50} values of 138.64 µg/mL and at 250 µg/mL showed percentage inhibition as 25.35 %, respectively. Whereas aqueous extract (RE and DE) exhibited weak antiproliferative activity against with both human cancer cells with IC_{50} values of >250 µg/mL showed percentage of inhibition as 24.94 % and 21.05 %. For the damage of normal cell, cytotoxicity of hexane extract (CHE), DCM extract (CDE), ethyl acetate extract (CEE), MeOH extract (CME) and aqueous extract (RE and DE) evaluated cytotoxic activity against mouse subcutaneous connective tissue (L929). The result showed RE and DE extracts were not toxic to L929 cell lines, while CDE extract exhibited toxicity against L929 cell lines exhibited IC_{50} values of 144.86 µg/mL. In addition, the CHE, CEE and CME extracts exhibited toxicity against L929 cell lines at 250 µg/mL with percentage inhibition as 45.66 %, 45.79 % and 5.69 %, respectively. Based on literature, ethanolic extract from fruiting bodies of *Phellinus igniarius* exhibited cytotoxic activity against A549 cell lines with IC_{50} values of 531.7 µg/mL [22], which in this study the dichloromethane extract exhibited cytotoxic activity against A549 cell lines with IC_{50} values 106.87 µg/mL. According to literature, alkaloid are important chemical compounds. Indole alkaloids exhibited cytotoxic activity against various cancer cell lines inducing autophagy, necrosis and apoptosis in the apoptotic pathway [56] . The ^{1}H NMR spectrum (Table16, 17) showed the chemical shift of compound in dichloromethane extract revealed that it contained mainly indole alkaloid. The compounds in the dichloromethane extract were probably active against both lung cancer and neuroblastoma cell lines.

Table *7 In vitro* antiproliferative activity human cancer by MTT assay against two cell lines.

Extracts	IC_{50} (µg/mL), (percentage of inhibition)		
	A549	SH-SY5Y	L929
Hexane	>250, 39.51	>250, 25.31	>250, 45.66
Dichloromethane	132.4	106.87	144.86
Ethyl acetate	>250, 25.35	138.64	>250, 45.79
Methanol	>250, 22.89	>250, 25.33	>250, 5.69
Reflux DI water	>250, 24.94	>250, 23.50	>250, -
Decoction DI water	>250, 21.05	>250, 31.01	>250, -

4.3 Separation of the crude extracts of Kratinpiman mushroom (*Phellinus* sp.)

4.3.1 Separation of fraction hexane extract and antiproliferative activity

The fraction hexane (CHE, 2.39 g) was separated by column chromatography on silica gel (300 g) using hexane: ethyl acetate (9:1, v/v) (2 L), hexane: ethyl acetate (8:2, v/v) (1.5 L), hexane: ethyl acetate (6:4, v/v) (2 L), ethyl acetate (0.5 L), ethyl acetate: methanol (6:4, v/v) (1.7 L), ethyl acetate: methanol (2:8, v/v) (1.5 L) and methanol (3 L) to give eighteen combined fraction (CFH1-CFH18). The result in Table 8 showed that hexane extract (CHE) exhibited antiproliferative against human lung cancer and neuroblastoma cell lines (IC_{50} values >250 µg/mL) with 39.51 and 25.31 percentage of inhibition, respectively. While, the CFH4-6, CFH10-18 exhibited antiproliferative activity against A549 and SH-SY5Y cell lines with percent inhibition at 250 µg/mL.

Table *8 In vitro* antiproliferative activity human cancer by MTT assay against two cell lines of fraction hexane extract of *Phellinus* sp.

Fraction	Weight (mg)	Inhibitory activity (%)	
		A549	SH-SY5Y
CFH 1	129.6	NT	NT
CFH 2	61.6	NT	NT
CFH 3	125.9	NT	NT
CFH 4	80.7	76.17	87.85
CFH 5	65.9	77.98	89.36
CFH 6	62.5	91.78	95.72
CFH 7	138.1	NT	NT
CFH 8	64.7	NT	NT
CFH 9	8.2	NT	NT
CFH 10	162.4	90.49	96.09
CFH 11	252.2	91.26	97.01
CFH 12	64.5	89.17	96.36
CFH 13	93.2	93.10	97.10
CFH 14	252.6	90.25	97.97
CFH 15	70	87.48	96.18
CFH 16	68.6	89.73	93.65
CFH 17	87	43.63	92.77
CFH 18	63.4	32.13	77.03

* NT= not test

The fraction CFH 1 (129.6 mg) was separated by silica gel TLC developed by hexane: EtOAc (9:1 v/v, 50 mL) as mobile phase to give eight subfractions (CFH1.1-CFH1.8). Fraction CFH1.4 was further separated by silica gel TLC using solvent system: hexane: EtOAc (9.5:0.5 v/v, 50 mL) as mobile phase to give compound 1 (CFH1.4.4) as light-yellow solid (5.9 mg, 0.24% w/w of hexane extract) and other fractions as shown in Table 9.

Table 9 Separation of fraction CFH1

Fraction	Weight (mg)	Characteristic of fraction
CFH 1.1	12.8	Yellow
CFH 1.2	28.2	Yellow
CFH 1.3	12.8	White
CFH 1.4 (compound 1)	16.5	Light yellow
CFH 1.5	3.8	Light yellow
CFH 1.6	2.8	Brown
CFH 1.7	3.3	Brown
CFH 1.8	11.3	Brown

The fraction CFH 8 (64.7 mg) was separated by column chromatography on silica gel 60 (12 g). The CFH8 was dissolved in hexane and using hexane: EtOAc (8: 2 v/v, 100 mL), hexane: EtOAc (7: 3 v/v, 100 mL), hexane: EtOAc (6: 4 v/v, 100 mL), hexane: EtOAc (5: 5 v/v, 100 mL), hexane: EtOAc (4: 6 v/v, 100 mL), hexane: EtOAc (3: 7 v/v, 100 mL), hexane: EtOAc (2: 8 v/v, 200 mL) and hexane: EtOAc (1: 9 v/v, 200 mL) as mobile phase, respectively to give eight subfractions (CFH8.1-CFH8.8). Compound 2 (CFH8.4) was obtained as yellow solid (5.2 mg, 0.21% w/w of hexane extract) and other fractions as shown in Table 10.

Table 10 Separation of fraction CFH 8

Fraction	Weight (mg)	Characteristic of fraction
CFH 8.1	3.7	Yellow
CFH 8.2	18.1	Yellow
CFH 8.3	11.4	Yellow
CFH 8.4 (compound 2)	5.2	Yellow
CFH 8.5	1.5	Yellow
CFH 8.6	2.2	Yellow
CFH 8.7	3.1	Yellow
CFH 8.8	4.6	Yellow

4.3.2 Separation of fraction dichloromethane extract and antiproliferative activity

The DCM extract (CDE) exhibited the best antiproliferative activity against A549 and SH-SY5Y cell lines with IC_{50} values of 132.4 and 106.87 µg/mL, respectively. The dichloromethane extract was more potent than the other fraction when compared effective of antiproliferative activity. The extract (CDE, 2.39 g) was separated by silica gel column chromatography eluting with DCM (700 ml), DCM: MeOH (9.75:0.25. v/v), (1.4 L), DCM: MeOH (9.5:0.5. v/v), (850 ml) and MeOH (2 L) to give twelve combined fractions (CFD1–CFD12). Each combined fraction was evaluated for antiproliferative activity at 50 µg/mL. Fraction CFD 6 showed strong activity against SH-SY5Y cell lines with 62.65 percentage of inhibition at concentration 25 µg/mL. While fraction CFD 8 showed strong activity against both cell lines with 51.85 and 91.10 % inhibition at concentration 50 µg/mL, respectively. Moreover, fraction CFD 5, CFD 7 and CFD 9 showed moderate activity against SH-SY5Y cell line with percentage 65.62, 78.12 and 63.71 of inhibition, respectively at concentration 50 µg/mL. The results of separation were shown in Table 11.

Table 11 *In vitro* antiproliferative activity human cancer by MTT assay against two cell lines of fraction dichloromethane extract of *Phellinus* sp.

Fraction	Weight (mg)	Inhibitory activity (%)	
		A549	SH-SY5Y
CFD 1	6.5	6.79	19.72
CFD 2	27.5	9.42	25.26
CFD 3	20.3	3.12	54.07
CFD 4	96.7	12.89	55.43
CFD 5	45.8	24.30	65.62
CFD 6	12.7	29.50**	62.65**
CFD 7	22.5	28.54	78.12
CFD 8	54.2	51.85	91.10
CFD 9	90.9	46.98	63.71
CFD 10	88.6	43.91	58.36
CFD 11	51.1	30.51	55.90
CFD 12	64.5	15.96	57.07

The fraction CFD 3 (20.3 mg) was separated by silica reverse phase TLC developed twice by the mobile phase MeOH: DI (7:3 v/v) to give ten subfractions (CFD3.1-CFD3.10). The subfraction CFD3.3 (compound 3) was obtained as yellow (2.5 mg, 0.1% of DCM extract). Moreover, CFD3.4 (compound 2) was obtained as yellow (4.6 mg, 0.19 % w/w of DCM extract) and its NMR data were the same as fraction CFH8.4 (compound 2) obtained from the hexane extract as above described. The results of separation were shown in Table 12.

Table 12 Separation of fraction CFD 3

Fraction	Weight (mg)	Characteristic of fraction
CFD 3.1	0.1	White
CFD 3.2	1.4	Yellow
CFD 3.3 (compound 3)	2.5	Yellow
CFD 3.4 (compound 2)	4.6	Yellow
CFD 3.5	3.5	Yellow
CFD 3.6	3.1	Yellow
CFD 3.7	1.1	White
CFD 3.8	1.4	Brown
CFD 3.9	0.8	Light yellow
CFD 3.10	1.3	Brown

4.3.3 Separation of fraction ethyl acetate extract and antiproliferative activity

The ethyl acetate extract (CEE) exhibited the best antiproliferative activity against SH-SY5Y cell line with IC_{50} values of 138.64 μg/mL and exhibited weak activity against A549 cell line. The fraction ethyl acetate (CEE, 1.10 g) was separated by Sephadex LH-20 column chromatography eluting with methanol to give sixteen subfraction (CFE1-CFE16) as shown in Table 13. Fraction CFE 5 showed the best cytotoxic activity against A549 cell line with 53.84 percentage of inhibition at concentration 250 μg/mL. While fraction CFE4, CFE10 and CFE12 exhibited strong activity against SH-SY5Y cell line with 93.27, 91.92 and 91.30 inhibition, respectively.

Table 13 *In vitro* antiproliferative activity human cancer by MTT assay against two cell lines of ethyl acetate extract of *Phellinus* sp.

Fraction	Weight (mg)	Inhibitory activity (%)	
		A549	SH-SY5Y
CFE 1	57.4	12.53	22.63
CFE 2	131.9	21.78	20.52
CFE 3	62.9	14.06	6.56
CFE 4	32	31.11	93.27
CFE 5	56.1	53.84	42.15
CFE 6	38.2	48.48	68.93
CFE 7	38.4	5.60	81.93
CFE 8	3.8	39.50	37.24
CFE 9	36.6	32.66	85.18
CFE 10	41.1	39.68	91.92
CFE 11	123.3	44.05	89.33
CFE 12	40.8	40.87	91.30
CFE 13	21.3	5.76	26.63
CFE 14	23.9	5.29	8.81
CFE 15	64.9	12.10	9.36
CFE 16	58.7	19.49	25.98

The fraction CFE 5 (56.1 mg) showed medium activity and it was separated by column chromatography over Sephadex LH-20 eluted with MeOH to give three subfractions CFE 5.1-CFE5.3 as shown in Table 13 CFE5.3 (compound 4) was obtained as yellow solid (3.3 mg, 0.3 % w/w of ethyl acetate extract).

4.4 Identification of compound 1

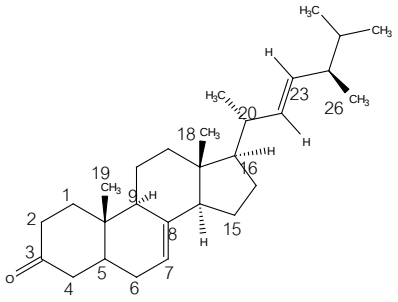


Figure 6 Structure of chemical compound **1**

Compound **1** was identified by ^{1}H, ^{13}C and 2D NMR. ^{1}H NMR spectrum (Figure 6) of compound **1** showed six characteristic ergostane-type steroidal methyl signal at δ_H 1.01 (d, *J*=7.2, H-21), 1.0 (s, H-19), 0.90 (d, *J*=6.9, H-28). 0.82 (d, *J*=6.9, H-26), 0.81 (d, *J*=6.9, H-27) and 0.56 (s, H-18). ^{13}C NMR spectrum of compound 1 (Table 14) displayed 28 carbons, including carbonyl carbon signal at δ_C 212.2 (C-3) and four olefin carbon signals at δ_C 139.61 (C-8), 117.09 (C-7). 135.67 (C-22) and 132.06 (C-23). In comparison NMR data with the literature [54] , compound 1 was identified as ergosta-7,22-dien-3-one (see Table 15). This compound was isolated from *Fomes fomentarius* [57] , *Marthasterias glacialis L* [58] , *Calvatia liacina* [59], *Ganoderma adsperum* [60]. It exhibited antitumor activities against human non-small cell lung cancer (NCI-H 460) cell line, anticancer against human breast cancer (MCF-7), human neuroblastoma (SH-SY5Y) cell lines, colorectal adenocarcinoma (Caco-2) cell line, breast, mammary gland cancer (MDA-MB-231) cell line and antimicrobial activity against a fungus *Cryptococcus neoformans*, respectively. In addition, this compound was isolated from *Ganoderma atum* extract [61] exhibited the potential prevention and treatment of neurodegenerative diseases.

Table 14 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound 1

Position	Compound 1		ergosta-7,22-dien-3-one	
	δ_H (ppm), mult, *J* in Hz	δ_c (ppm)	δ_H (ppm), mult, *J* in Hz	δ_c (ppm)
1		38.91		39.0
2		38.28		38.4
3		212.24		212.2
4		44.39		44.5
5		43.00		43.5
6		30.19		30.3
7		117.14		117.0
8		139.66		139.7
9		48.97		49.2
10		34.54		34.5
11		21.83		21.9
12		39.45		39.6
13		43.40		43.5
14		55.14		55.2
15		23.05		23.1
16		28.24		28.3
17		56.05		56.2
18	0.56 (s)	12.28	0.58 (s)	12.4
19	1.0 (s)	12.61	1.2 (s)	12.7
20		40.64		40.6
21	1.02 (d, *J*= 7.2)	21.26	1.03 (d, *J*=6.2)	21.3
22		135.72		135.8
23		132.11		132.3
24		42.94		43.0
25		33.22		33.3
26	0.83 (d, *J*= 6.9)	20.10	0.83 (d, *J*= 6.4)	20.2
27	0.82 (d, *J*= 6.9)	19.79	0.83 (d, *J*= 6.4)	19.9
28	0.91 (d, *J*=6.9)	17.74	0.93 (d, *J*=6.8)	17.7

4.5 Identification of compound 2

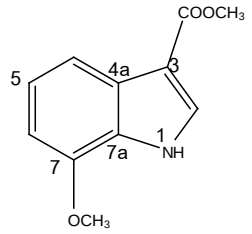


Figure 7 Structure of chemical compound **2**

Compound **2** was identified by ^{1}H, ^{13}C and 2D NMR. ^{1}H NMR spectrum of (Figure 9) compound **2** showed signals of one exchangeable proton and δ_H 8.81 (NH), four aromatic protons at δ_H 6.71 (d, *J*= 7.1 Hz; H-6), 7.18 (t, *J*= 7.9 Hz; H-5), 7.75 (d, *J*= 8.0 Hz; H-4) and 7.88 (d, *J*= 3.0 Hz; H-2) and two methoxy groups at 3.96 (7-OCH_3) and 3.92 (3-$COOCH_3$) (Table 15). ^{13}C NMR of compound **2** showed of eleven carbons, four aromatic methine (δ_c 103.7, C-6; 122.69, C-5; 114.08, C-4 and 130.41, C-2), two methoxy (δ_c 51.24, 3-$COOCH_3$ and 55.53, 7-OCH_3), one oxyquaternary (δ_c 146.21, C-7), three quaternary (δ_c 127.26, C-7a; 126.77, C-4a and 109.39, C-3) and one carbonyl (δ_c 165.86, 3-$COOCH_3$) (Table 16). In comparison NMR data with the literature [62], compound 2 was identified as 7-methoxyindole-3-carboxylic acid methyl ester (see Table 15). It was firstly isolated from crude dichloromethane of *Phellinus linteus*.

Table 15 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data of compound **2**

Position	Compound **2**			7-methoxyindole-3-carboxylic acid methyl ester [62]	
	δ_H (ppm), mult, *J* in Hz	δ_c (ppm)	HMBC	δ_H (ppm), mult, *J* in Hz	δ_c (ppm)
2	7.88 (d, *J*=3.0)	130.41	C-3, C-4, C-7a	7.88 (d, *J*=2.9)	130.2
3		109.39			109.3
4	7.75 (d, *J*=8.1)	114.08	C-4a, C-6	7.75 (d, *J*=8.0)	114.0
5	7.18 (t, *J*=7.9)	122.69	C-7, C-7a	7.19 (dd, *J*=8.0, 7.7)	122.6
6	6.71 (d, *J*=7.1)	103.70	C-4, C-4a, C-7	6.71 (d, *J*=7.7)	103.0
7		146.21			146.1
4a		126.77			126.7
7a		127.26			127.2
1-CH_3					
3-CHO					
3-COO$\underline{C}H_3$	3.92 (s)	51.24	C-3	3.92 (s)	51.0
3-$\underline{C}OOCH_3$		165.86			165.7
7-OCH_3	3.96 (s)	55.53	C-7	3.96 (s)	55.4
NH	8.81 (br s)			8.83 (br s)	

4.6 Identification of compound 3

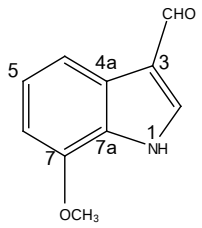


Figure 8 Structure of chemical compound **3**

Compound **3** was identified by ^{1}H, ^{13}C and 2D NMR. ^{1}H NMR spectrum (Figure 12) of compound **3** showed signals of one exchangeable proton and δ_H 9.48 (NH), four aromatic protons at δ_H 6.74 (d, *J*= 7.9 Hz; H-6), 7.20 (t, *J*= 7.9 Hz; H-5), 7.78 (d, *J*= 3.2 Hz; H-2) and 7.83 (d, *J*= 8.1 Hz; H-4), one methoxy groups at 3.94 (7-OCH_3) and carboxaldehyde 9.97 (s, 3-CHO) (Table 16). ^{13}C NMR of compound **2** showed of eleven carbons, four aromatics methine (δ_C 104.3, C-6; 123.7, C-5; 114.2, C-4 and 135.4, C-2), one methoxy (δ_C 55.6, 7-OCH_3), one oxyquaternary (δ_C 146.21, C-7), three quaternary (δ_C 127.26, C-7a; 126.77, C-4a and 109.39, C-3) and one carboxaldehyde (δ_C 185.66, 3-CHO) (Table 16). In comparison NMR data with the literature [63], compound 3 was identified as 7-methoxyindole-3-carboxaldehyde (see Table 17). This compound was found in the methanolic extract of fruiting bodies of wild Sanghuang mushroom *Tropicoporus linteus*.

Table 16 ^{1}H NMR (500 MHz) data of compound **3**

Position	Compound **3**			7-methoxyindole-3-carboxaldehyde	
	δ_H (ppm), mult, *J* in Hz	δ_c (ppm)	HMBC	δ_H (ppm), mult, *J* in Hz	δ_c (ppm)
2	7.78 (d, 3.2)	135.4	C-3, C-4, C-7a	7.79 (d, 3.0)	134.3
3		119.7			120.1
4	7.83 (d, 8.1)	114.2	C-4a, C-6	7.86 (d, 7.9)	114.2
5	7.20 (t, 7.9)	123.7	C-7, C-7a	7.21 (t, 7.9)	123.7
6	6.74 (d, 7.9)	104.3	C-4, C-4a, C-7	6.75 (d, 7.9)	104.3
7		146.2			145.9
4a		125.9			125.7
7a		127.2			127.1
3-CHO	9.97 (s)	185.7	C-3	10.04 (s)	185.3
7-OCH_3	3.94 (s)	55.6	C-7	3.95 (s)	55.5
NH	9.48 (br s)			8.97 (br s)	

4.6 Identification of compound 4

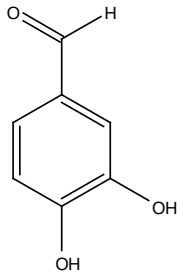


Figure 9 Chemical structure of compound **4**

Compound 4 was identified by comparison ^{1}H NMR data with the literature. ^{1}H NMR spectrum (Figure 9) of compound 4 showed signals as δ_H 9.80 (s, CHO), 7.61 (dd, *J* =8.3, 1.8, H-6), 7.57 (d, *J*= 1.9, H-2), 7.01 (d, *J* =8.0, H-5) (Table 17). In comparison NMR data with the literature , compound 4 was identified as protocatechualdehyde (see Table 18).This compound was isolated form *Phellinus glivus* and *P. linteus* exhibited anticancer against melanoma cells (B16-F10 cell lines) and human colorectal carcinoma cells (HT-29) [64], antioxidant, anti-inflammatory activities [65], anti-diabetes [47].

Table 17 ^{1}H NMR (500 MHz) data of compound 4

Position	δ_H (ppm), mult, *J* in Hz	
	Compound 4	protocatechualdehyde [66]
1		
2	7.57 (d, 1.9)	7.29 (d, 2.0)
3		
4		
5	7.01 (d, 8.0)	6.90 (d, 8.0)
6	7.61 (dd, 8.3, 1.8)	7.30 (dd, 8.0, 2.0)
C<u>H</u>O	9.80 s	9.68 s

CHAPTER IV
CONCLUSION

In conclusion, major group and genus of Kratinpiman mushroom was identified by key for mushroom identification as *Polypores* group and genus *Phellinus*. Due to unsuccess in isolation of DNA and culture of the mushroom, it could be identified by its morphology as *Phellinus* sp. All crude extracts were evaluated for their cytotoxicity. The dichloromethane extract exhibited the best antiproliferative activity with IC_{50} values 132.4 μg/mL. against human lung cancer (A549) and neuroblastoma (SH-SY5Y) cell lines, respectively. The ethyl acetate extract exhibited antiproliferative activity against A549 and SH-SY5Y with IC_{50} values 138.64 and >250 μg/mL (25.35 percentage of inhibition at 250 μg/mL), respectively. Hexane extract, methanol extract and aqueous extracts exhibited weak antiproliferative activity with both cancer cell lines and a normal (L929) cell line while CDE extract exhibited cytotoxicity with L929 cell line exhibited IC_{50} values of 144.86 μg/mL. Isolation of *Phellinus* sp. extracts afforded ergosta-7,22-dien-3-ol (compound 1) and 7-methoxyindole-3-carboxylic acid methyl ester (compound 2) from hexane extract, 7-methoxyindole-3-carboxaldehyde (compound 3) from DCM extract and protocatechualdehyde (compound 4) from ethyl acetate extract. This study demonstrated that metabolites of Kratinpiman (*Phellinus* sp.) showed potential antiproliferative activity against cancer A594 and SH-SY5Y cell lines.

APPENDIX

APPENDIX A

1. Preparing of *ρ*-anisaldehyde

To 135 mL of absolute ethanol add 5 mL of concentrate sulfuric acid and 1.5 mL of glacial acetic acid. Allow the solution to cool to room temperature. Add 3.7 mL of *ρ*anisaldehyde. Stir the solution vigorously to ensure homogeneity. Store refrigerated.

2. Preparing of Cerium ammonium molybdate

Dissolve 0.5 g of ceric ammonium sulfate (Ce $(NH_4)_6(SO_4)_4 \cdot 2H_2O$) and 12 g of ammonium molybdate $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$) in 235 mL of DI water after that, add 15 mL of concentrate sulfuric acid. Wrap the jar with aluminum foil as the stain may be somewhat photo sensitive.

3. Preparing of cell culture medium

Add powders medium DMEM, DMEM/F12 to room temperature autoclaved water, rinse inside of package to remove all traces of powder and filtration with sterile filter. After that, 10% fetal bovine serum (FBS), 100 µg/mL of streptomycin and 100 U/mL of penicillin were added into the medium. This protocol was used aseptic technique working in class II safety hood. The culture media used depend on cell type used, which A549 and L929 were use DMEM, SY-SH5Y was use DMEM/F12 for culture.

4. Preparing of MTT solution for cell viability assay

A 5 mg/mL of MTT solution was prepared by dissolving 0.005 g of MTT powder in sterilized water (1 mL) and a clear yellow solution of the MTT was then stored in -20 °C until use. The stock MTT solution (10 µL) was diluted in 990 µL of culture medium to give the final concentration of MTT solution is 0.05 mg/mL.

5. Tris-buffer (1 M, pH 8.0)

Tris base 121 g

DI water 800 g

Tris base was dissolve in distilled water, adjust pH with hydrochloric acid (HCl) to pH 8.0, then add distilled water until the final volume is 1 L. After that, Tris base was sterile by auto clave at 121 °C, 15 pounds per square inch pressure, 15 min and stored at 4 °C.

6. Washing buffer

Polyvinylpyrrolidone (PVP) 2 g

Ascorbic acid 1.76 g

Tris-buffer 1 M, pH 8.0 20 mL

2-mercaptoethanol 4 mL

Then add sterile distilled water until the final volume is 200 mL. The mixture stored at 4 °C.

7. 2X-Cetyl Trimethyl Ammonium Bromide (CTAB) lysis buffer

Cetyl Trimethyl Ammonium Bromide 4 g

Tris-buffer 1 M, pH 8.0 20 mL

Ethylene Diamine Tetra-acetic acid 1 M, pH 8.0 8 mL

Sodium chloride (NaCl) 16.36 g

2-mercaptoethanol 4 mL

Distilled water until the final volume is 200 mL, stored at room temperature.

8. Ethylenediamine tetra acetic acid 0.5 M

EDTA 186.1 g

Distilled water 800 mL

The mixture was adjusted pH with sodium hydroxide (NaOH) to pH 8.0. Distilled water was added, the final volume is 1 L. After that, EDTA was sterile by autoclave and stored at 4 °C.

9. 10X Tris-boric acid EDTA (10X TBE)

Tris (Hydroxymethyl) amino methane	54 g
EDTA	4.64 g
Boric acid	27.50 g

Add distilled sterile water until the final volume is 500 mL. The mixture was stored at room temperature.

10. Potato dextrose agar (PDA)

Potato	200 g
Glucose	20 g
Agar	15 g
Distilled water	1000 mL

Potatoes were boiled in distilled water for 10-15 min and filtration. Distilled water 1000 mL was added. PDA medium was sterile by autoclave.

APPENDIX B

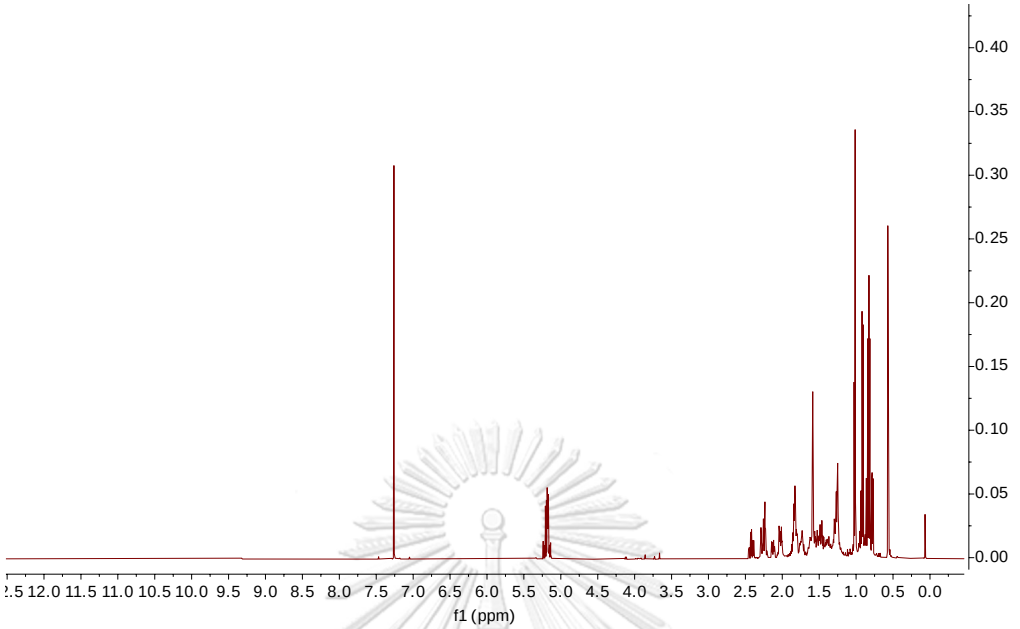


Figure 9 1H NMR spectrum of compound 1

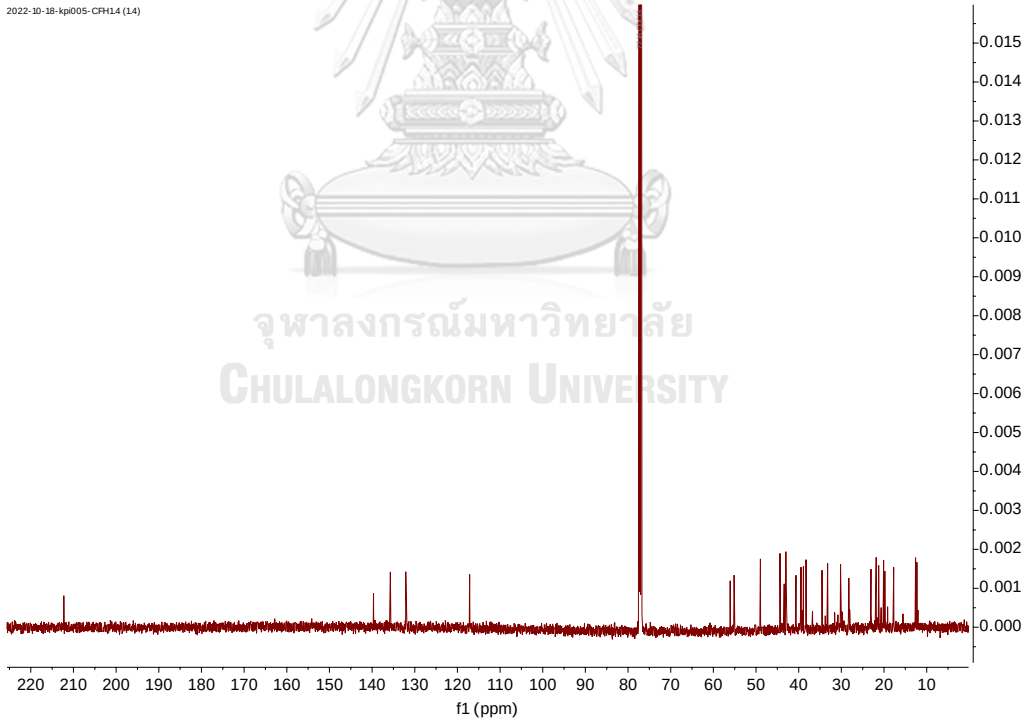


Figure 10 ^{13}C NMR spectrum of compound 1

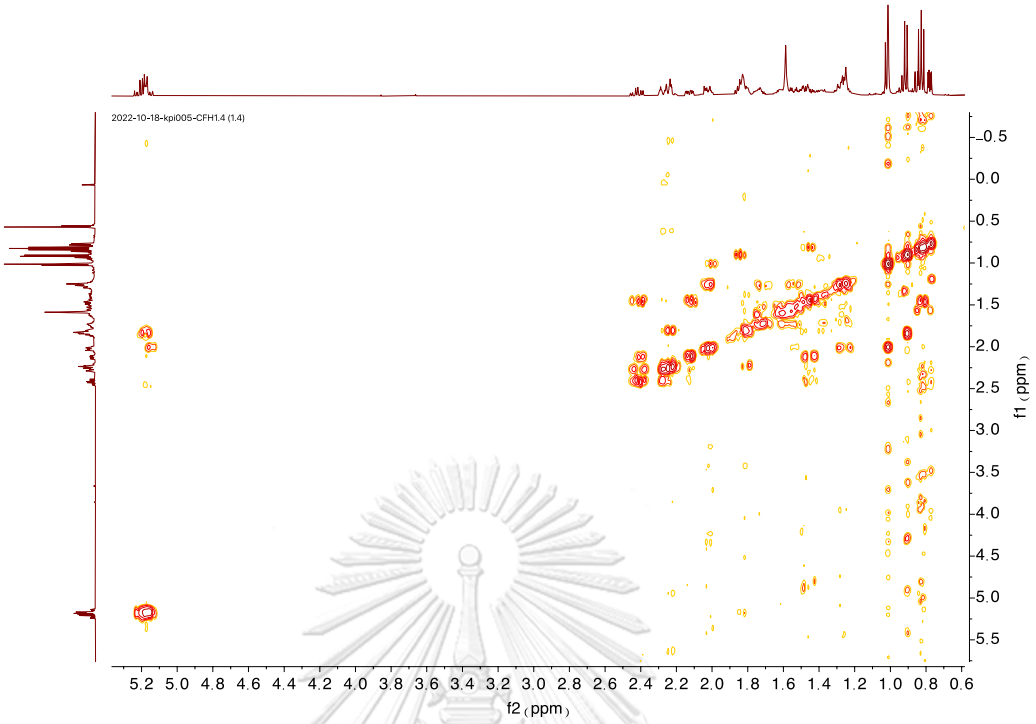


Figure 11 ^{1}H-^{1}H COSY spectrum of compound 1

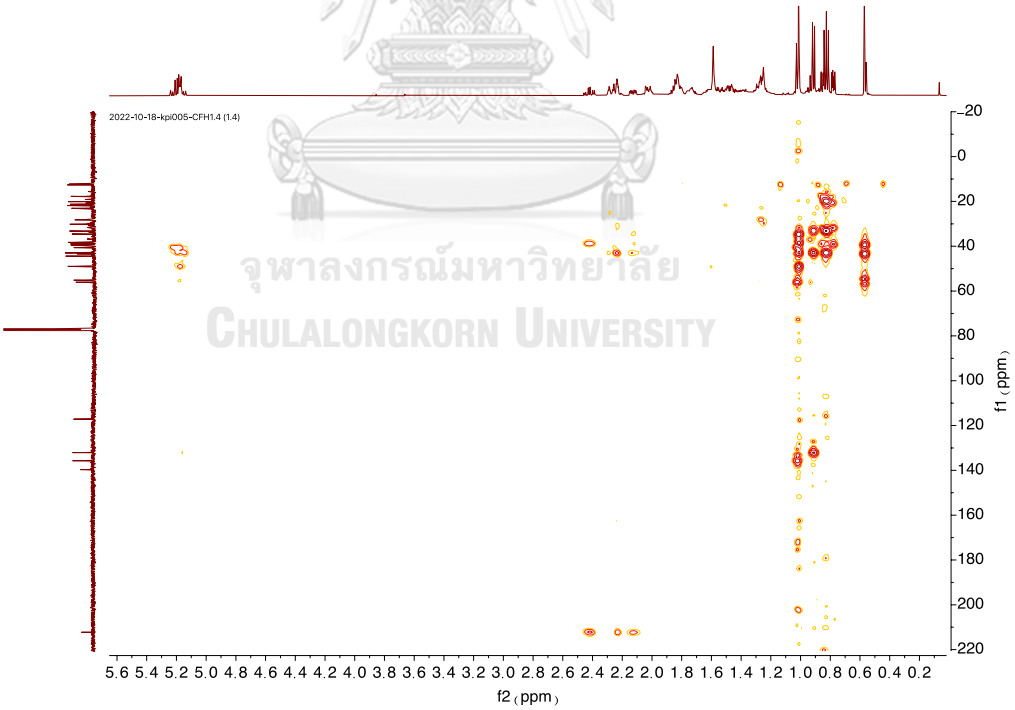


Figure 12 HMBC spectrum of compound 1

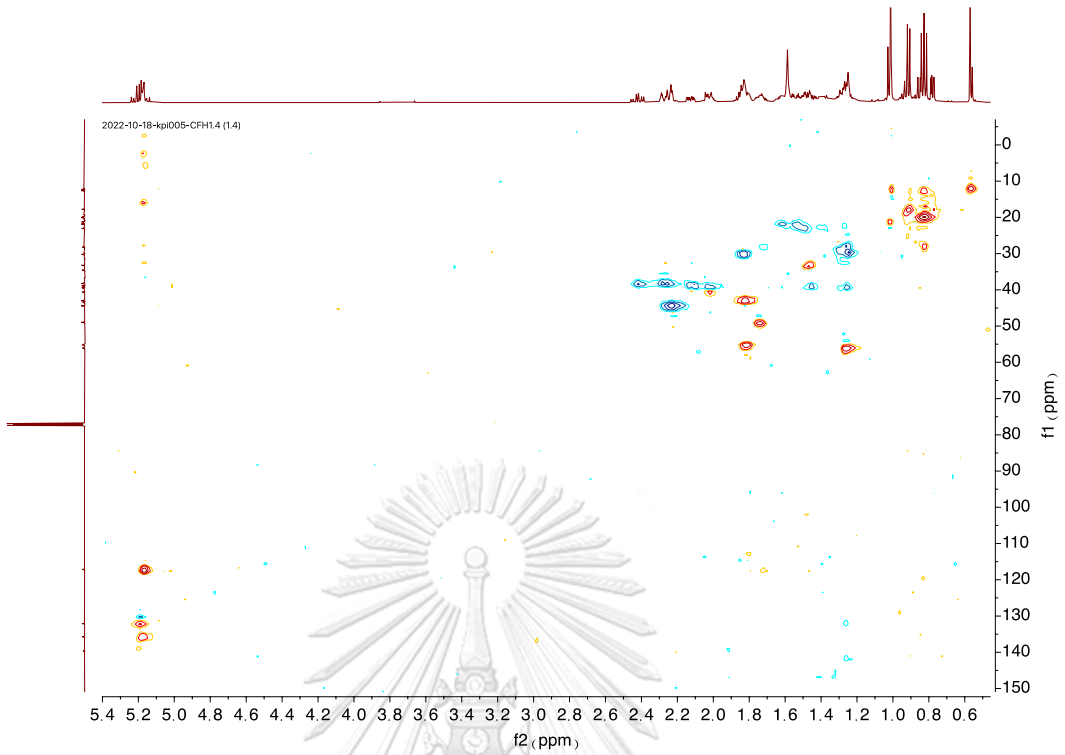


Figure 13 HSQC spectrum of compound 1

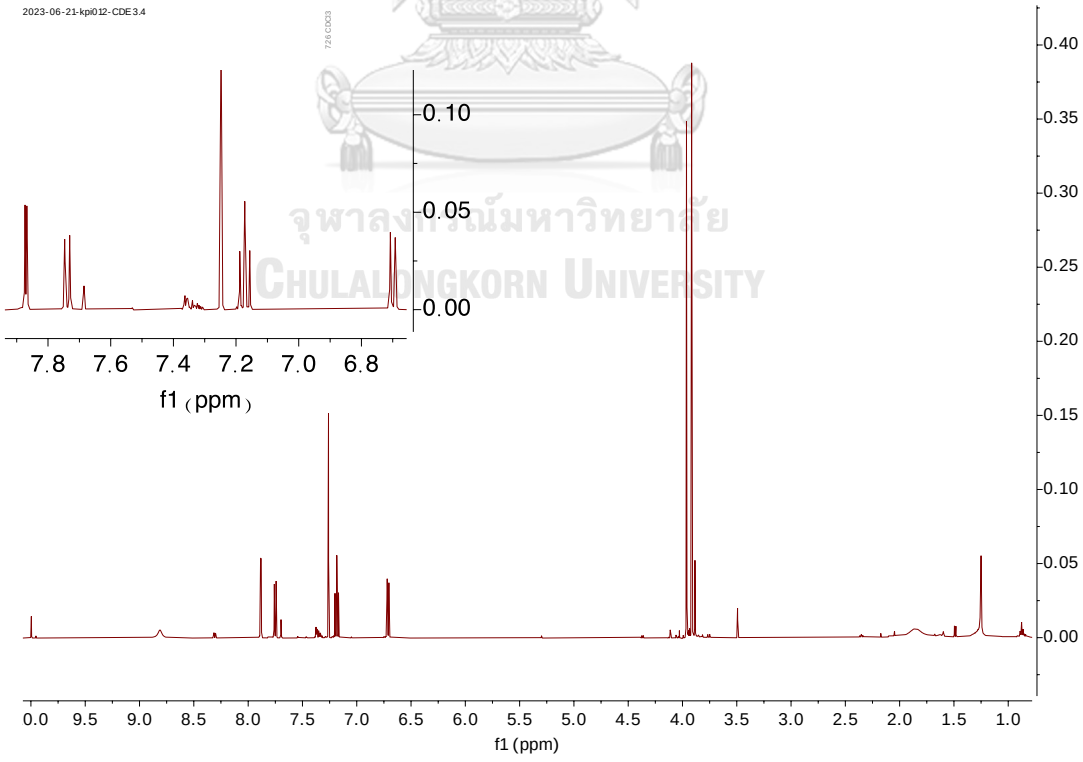


Figure 14 ^{1}H NMR spectrum of compound 2

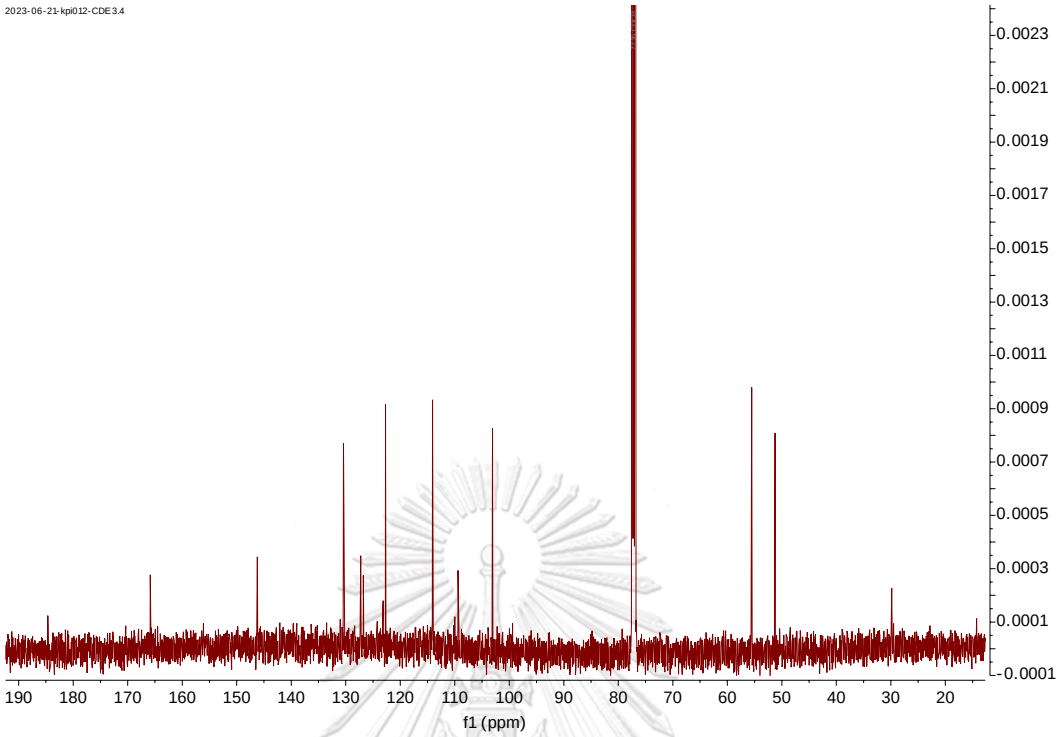


Figure 15 ^{13}C NMR spectrum of compound 2

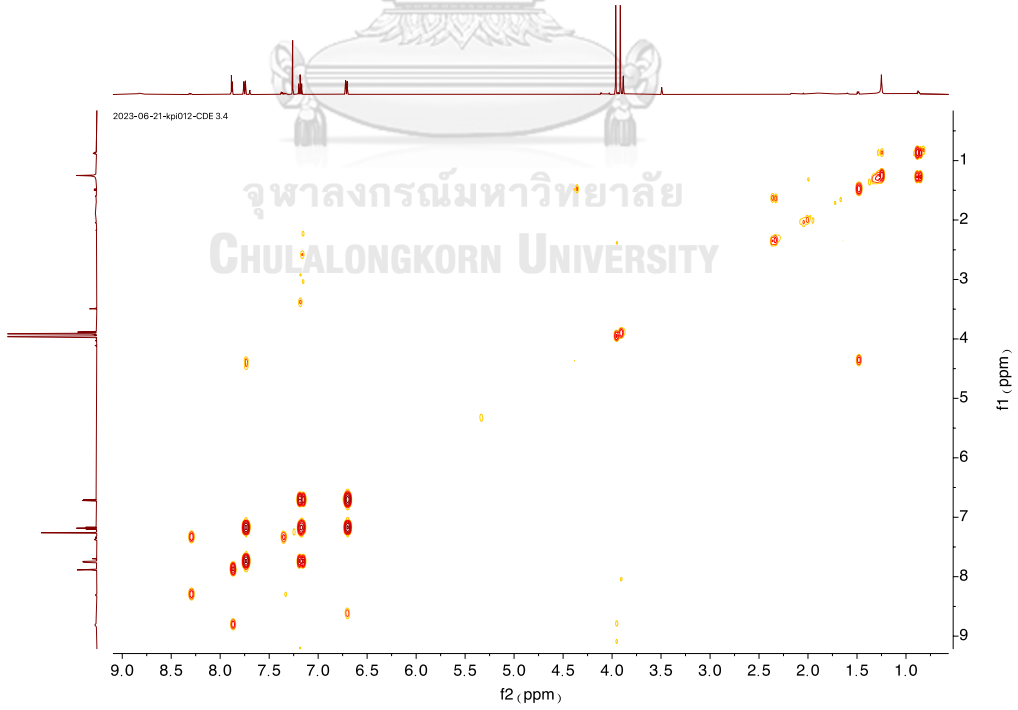


Figure 16 ^{1}H-^{1}H COSY spectrum of compound 2

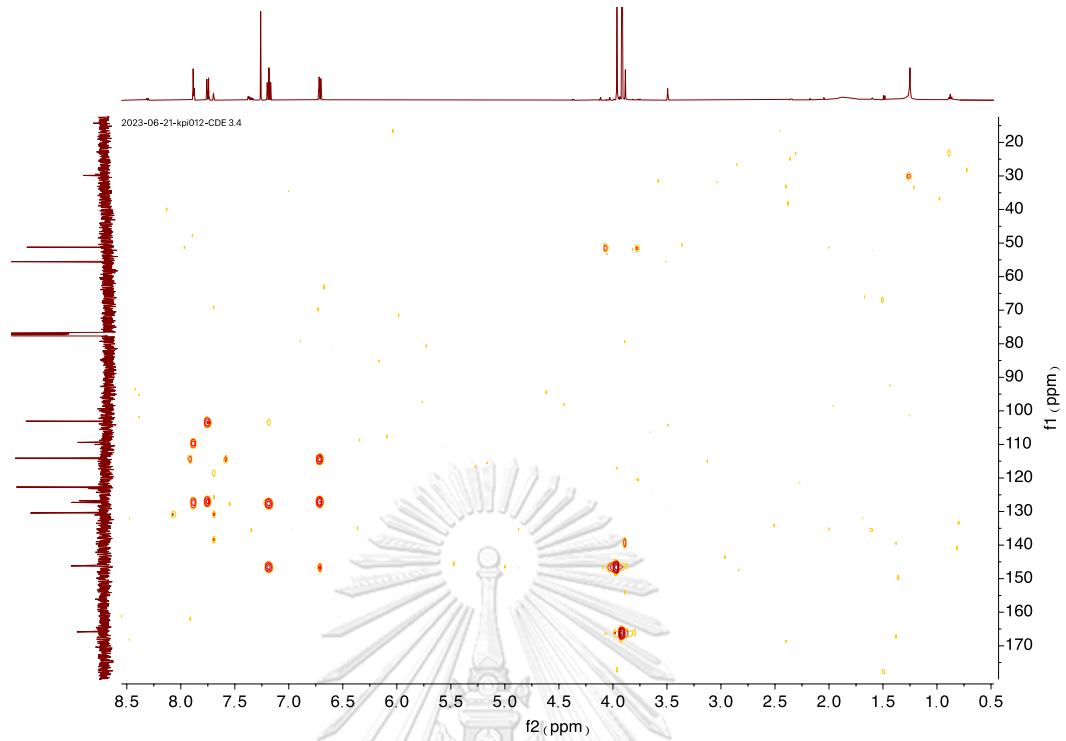


Figure 17 HMBC spectrum of compound 2

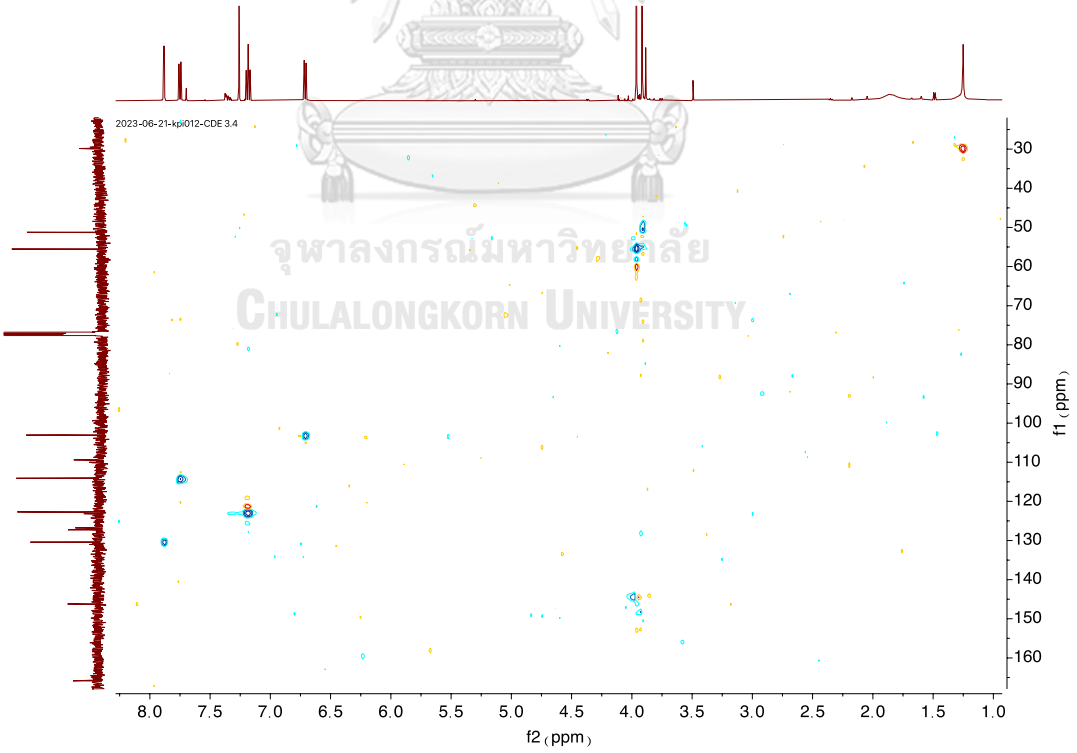


Figure 18 HSQC spectrum of compound 2

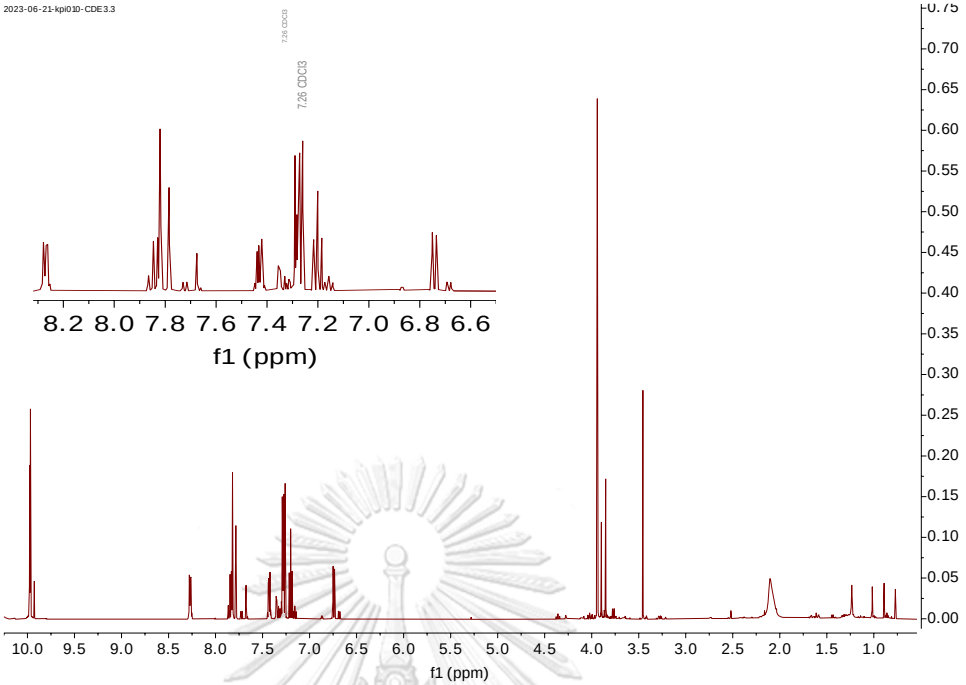


Figure 19 ^{1}H NMR spectrum of compound 3

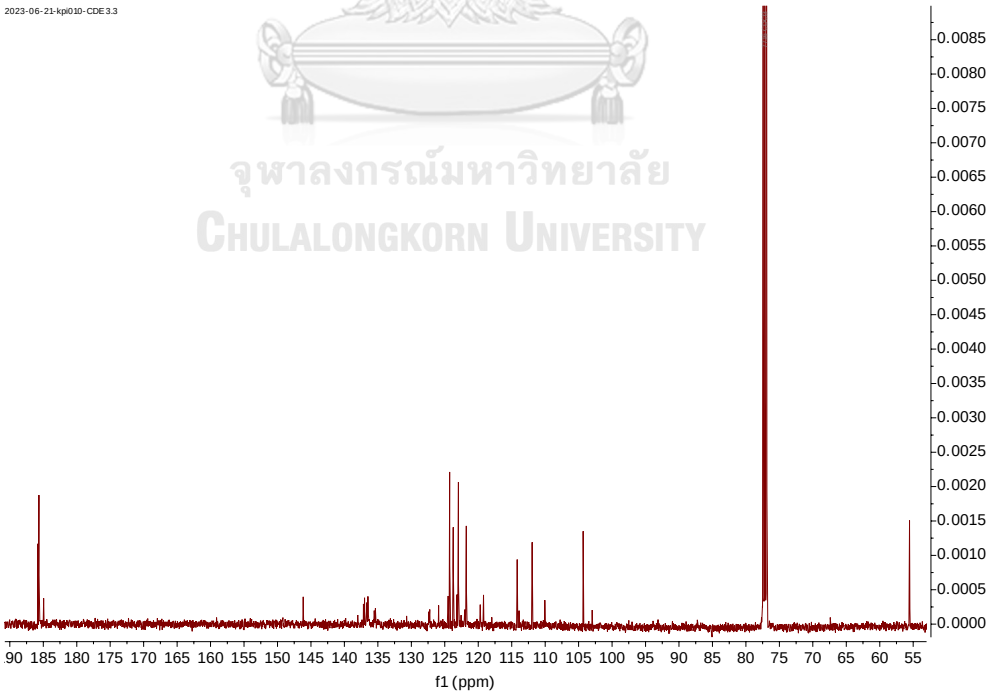


Figure 20 ^{13}C NMR spectrum of compound 3

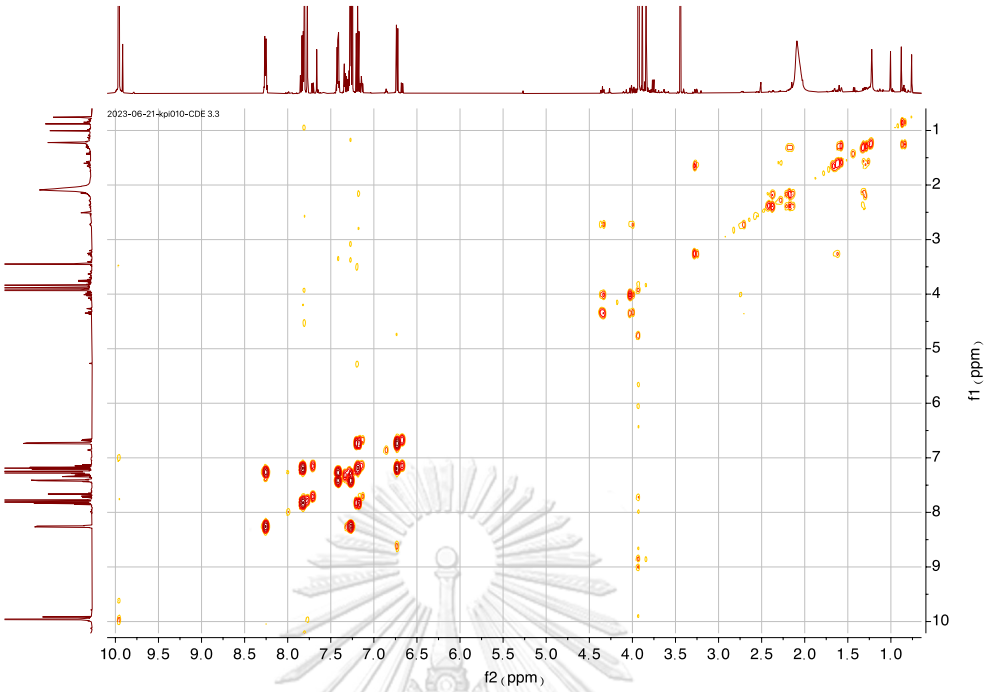


Figure 21 ^{1}H-^{1}H COSY spectrum of compound 3

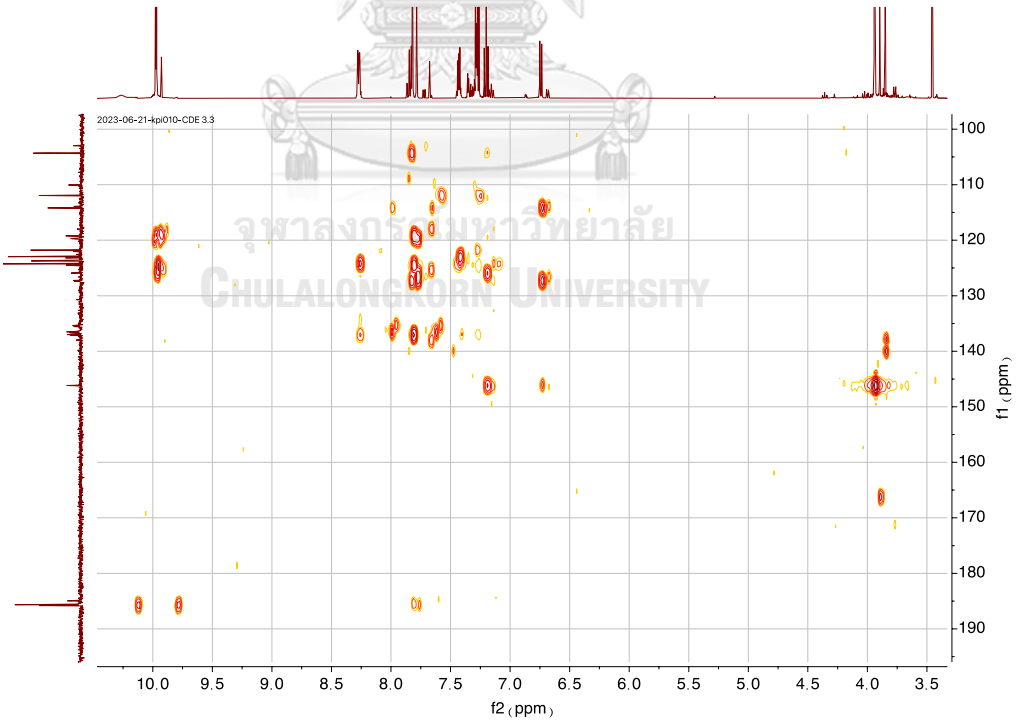


Figure 22 HMBC spectrum of compound 3

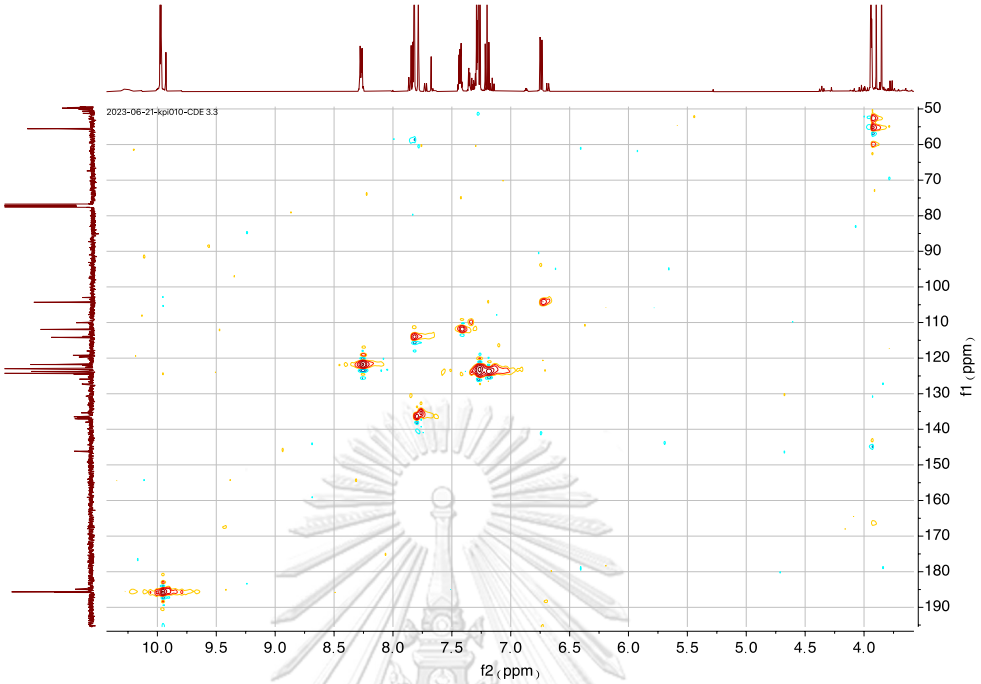


Figure 23 HSQC spectrum of compound 3

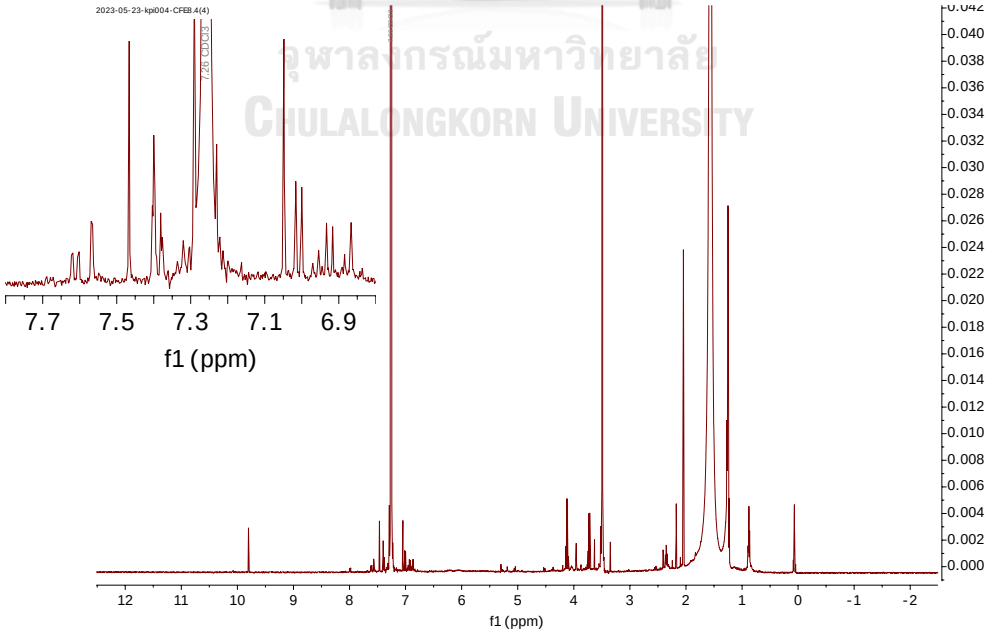


Figure 24 ^{1}H NMR spectrum of compound 4

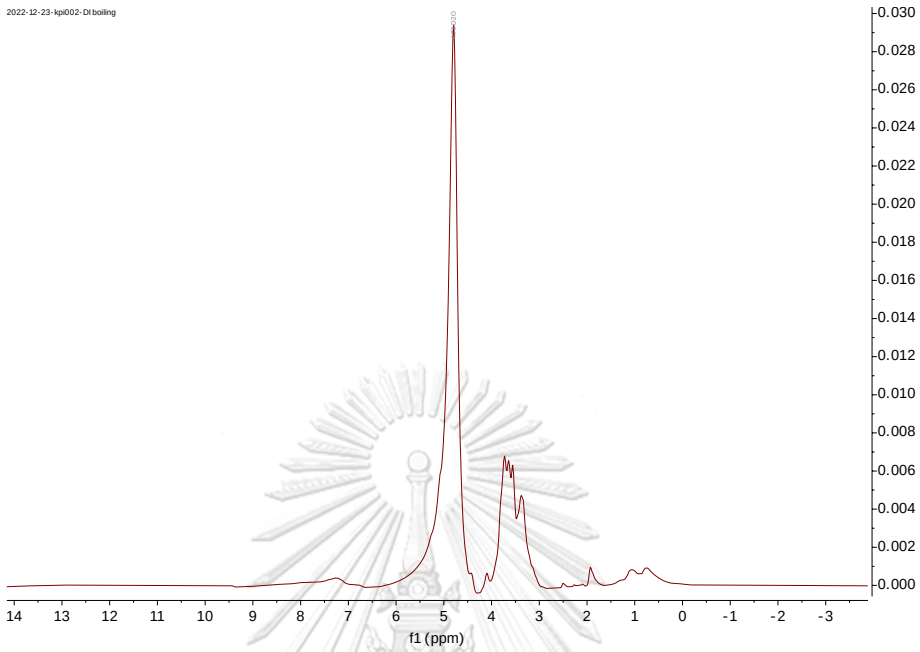


Figure 25 ^{1}H NMR spectrum of distilled water extract

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