



รายงานวิจัยฉบับสมบูรณ์

โครงการ

สารยับยั้งการเจริญของเชื้อราจากเห็ดหอม (*Lentinus edodes*) :
ผลกระทบต่อการเจริญของเชื้อราและการผลิตสารพิษของเชื้อรา

โดย

ดร. อ้วนวี เพชรคงแก้ว

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ภาควิชาวิทยาศาสตร์และเทคโนโลยีการอาหาร

คณะวิทยาศาสตร์และเทคโนโลยี

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Abstract

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Project Title : Antifungal substance from the Shiitake (*Lentinus edodes*) extract: Effect on growth and mycotoxin production

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Contamination by many mycotoxigenic fungi is often found in foods and feeds. Fungi cause damage of plants, crops, and fruits and their mycotoxins are harmful to human and animal health. Several methods exist for controlling fungal contamination, including physical methods, chemical methods, and biological methods. The biological method is one of the strategies used to prevent infection by fungi. The functional properties of Shiitake mushroom have been previously studied and reported, but research on antifungal activity is rare. In this study, water and chloroform extracts of Shiitake mushroom were investigated for antifungal activity against nine strains of mycotoxigenic fungi: *Aspergillus flavus* 9090, *A. parasiticus* TISTR 3276, *A. ochraceus* TISTR 3557, *Penicillium citrinum* TISTR 3437, *P. expansum* BCC 7541, *Fusarium oxysporum* BCC 4977, *F. graminearum* 1895, *F. moniliforme* TISTR 3175, and *F. verticillioides* 1641. The chloroform extract showed higher antifungal activity than water extract against all tested fungi. The chloroform extract exhibited strong inhibitory activity against *Fusarium* spp. with minimum inhibitory concentration (MIC) ranging from 1.95-3.9 mg/ml. The MIC results for chloroform extract showed reduction in colony radial growth rate (K_r) and specific growth rate of the colony (μ) of four *Fusarium* strains, when compared with the control. The chloroform extract was further evaluated to measure its inhibitory efficiency against mycotoxin production by four *Fusarium* strains. The extract was shown to significantly reduce FB_1 production from *F. verticillioides*. FB_1 production was decreased by about 75% by the end of incubation. Based on these results the biological active compounds from the chloroform extract were purified. The compounds were isolated using chromatographic methods and then

identified by spectroscopy methods. Three purified compounds, ergosterol peroxide (1), ergosterol (2) and linoleic acid (5), were successfully isolated from the extract. It was demonstrated that Shiitake extract may provide an alternative approach to control of the mycotoxigenic fungi, especially fusariotoxin-producing *Fusarium* strains, and inhibition of their mycotoxin production.

Keywords : Shiitake extract, Antifungal activity, Mycotoxigenic fungi, Fumonisin B₁ production

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
v	Volume
i.d.	Inner diameter
Conc.	Concentration
Cont.	Continue
Temp.	Temperature
°C	Degree Celcius
L	Liter
ml	Milliliter
μl	Microliter
M	Molar
mM	Millimolar
h	Hour
min	Minute
s	Second
kg	Kilogram
g	Gram
mg	Milligram
μg	Microgram
cm	Centimeter
mm	Millimeter
eV	Electron volts
rpm	Revolutions per minute
CFU	Colony-Forming Unit
AFB ₁	Aflatoxin B ₁
OTA	Ochratoxin A
FB, FB ₁	Fumonisin, Fumonisin B ₁
DON	Deoxynivalenol
ZEA	Zearalenone

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
CDCl ₃	Chloroform-d1, deuterated chloroform
CH ₂ Cl ₂	Dichloromethane
MeOH	Methanol
EtOAc	Ethyl acetate
DMSO	Dimethyl sulfoxide
EO	Essential oil
IZD	Internal zone diameter
MIC	Minimum inhibitory concentration
MFC	Minimum fungicidal concentration
HPLC	High-Pressure Liquid Chromatography
MS	Mass spectrometry
TLC	Thin-Layer Chromatography
NMR	Nuclear Magnetic Resonance
IR	Infrared
FT-IR	Fourier transform infrared
UV-Vis or UV/Vis	Ultraviolet-Visible
TX	Texas
CA	California
USA	United State
\$	US Dollars

CHAPTER 1

INTRODUCTION

1.1 Rationale and background

Contamination by mycotoxigenic fungi in food and feed is a national problem in many countries (Anukul *et al.*, 2013; Binder *et al.*, 2007). The majority of mycotoxigenic fungi that act as contaminants commodities include *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. Fungal contamination is found in crops and cereals which become contaminated in the food supply chain (pre-harvest, post-harvest, during processing, transportation, and storage).

Mycotoxins are chemical substances produced by secondary metabolism of mycotoxigenic fungi. They affect human and animal health through the food chain. Mycotoxicosis in animals and humans can be acute or chronic depending on the type of mycotoxin and its dose. Important mycotoxins that are often found in agricultural products include aflatoxin B₁ (AFB₁), ochratoxin A (OTA), fumonisin B₁ (FB₁), patulin, deoxynivalenol (DON), and zearalenone (ZEA). Mycotoxins have been reported to cause hepatotoxicity, nephrotoxicity, immunotoxicity, estrogenic effect, haematotoxicity, and carcinogenic effects (Ghitakou *et al.*, 2006; Chulze *et al.*, 2006; Heintz *et al.*, 2010; Briones-Reyes *et al.*, 2007; Zinedine *et al.*, 2007). Mycotoxin contamination becomes a non-tariff trade barrier (NTB) in international trade. This limitation decreases the cost of those products and causes a global economic impact of \$0.5 - \$5 billion per year in the US and Canada.

There are several methods for controlling the growth of mycotoxigenic fungi in food and feed stuffs including chemical, physical, and biological methods. However, the disadvantages of physical and chemical methods are expensive chemicals, environmental pollution, health hazards, and effect on the natural ecological balance. The biological method has been widely studied and developed. Antifungal activity of several plant extracts has been reported, including essential oil from lamiacea (Juárez *et al.*, 2015), savory, thyme, oregano, rose (Stević *et al.*, 2014), and Shiitake mushroom (Hirasawa *et al.*, 1999).

Shiitake mushroom is one of most interesting subjects for study. Shiitake (*Lentinus edodes*) is a well-known edible mushroom, with abundant minerals

and nutrients. It has many biological properties including antifungal activity. The functional properties of Shiitake have been also studied by many researches (Hatvani and Mécs, 2002; Ngai and Ng, 2003 and Kitzberger *et al.*, 2007). The inhibition effect of shiitake extract on several microorganisms has been reported but no studies have investigated the antifungal activity of Shiitake extract on mycotoxigenic fungi. Therefore, this research focused on the effects of Shiitake extract on mycotoxigenic fungi growth inhibition and mycotoxin production. The study also attempted to purify and characterize the biologically active antifungal substances in Shiitake extract.

1.2 Research objectives

- To investigate the antifungal activity of Shiitake (*Lentinus edodes*) extract against mycotoxigenic fungi.
- To study the inhibition effect of Shiitake (*Lentinus edodes*) extract on mycotoxin production.
- To purify and characterize biologically active antifungal compounds from Shiitake (*Lentinus edodes*) extract.

1.3 Research hypotheses

- The Shiitake (*Lentinus edodes*) extract can inhibit growth and mycotoxin production of some mycotoxigenic fungi.
- There are some biologically active compound(s) from Shiitake (*Lentinus edodes*) extract that exhibit the antifungal activity.

1.4 Scope and limitations of the study

- The Shiitake, Donko type was used throughout this study.
- The growth inhibition of Shiitake extract was studied on nine strains of mycotoxigenic fungi including *Aspergillus flavus* 9090, *A. parasiticus* 3276, *A. ochraceus* TISTR 3557, *Penicillium citrinum* TISTR 3437, *P. expansum* BCC 7541, *Fusarium graminearum* 1895,

F. moniliforme TISTR 3175, *F. oxysporum* BCC 4977 and *F. verticillioides* 1641.

- The inhibition effect of chloroform extract on mycotoxin production produced by four *Fusarium* spp. was studied.

1.5 Expected results

- To gain knowledge of the antifungal activity and mycotoxin production of Shiitake (*Lentinus edodes*) extract.
- To obtain the purified compound that exhibit antifungal activity from Shiitake (*Lentinus edodes*) extract and to identify its chemical structure.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

2.1.1 Fungal strains

Nine species of mycotoxigenic fungi were used throughout this study. They were obtained from various culture collections in Thailand. All fungal strains and their collection are presented in Table 2.1. The conidia of each fungal were suspended in 50% glycerol. The suspensions of all stock culture were maintained at -20°C until used.

Table 2.1 Fungal strains and their collection

Fungal strains	Culture collection
<i>Aspergillus flavus</i> 9090	Office of Research and
<i>Fusarium graminearum</i> 1895	Development Crop Protection,
<i>Fusarium verticillioides</i> 1641	Department of Agriculture
<i>Fusarium oxysporum</i> BCC 4977	National Science and Technology
<i>Penicillium expansum</i> BCC 7541	Development Agency (NSTDA)
<i>Aspergillus parasiticus</i> TISTR 3276	Thailand Institute of Scientific and
<i>Aspergillus ochraceus</i> TISTR 3557	Technological Research (TISTR)
<i>Fusarium moniliforme</i> TISTR 3175	
<i>Penicillium citrinum</i> TISTR 3437	

To prepare fungal strains for the experiments, all stock cultures were sub-cultured on Potato Dextrose Agar (PDA; Difco, USA), and incubated at 25°C for 7 days. Then, they were used as inoculum for further experiment. The procedures for culture media preparation are described in Appendix A.

2.1.2 Shiitake mushroom

Dried Shiitake mushroom, Donko type (Figure 2.1), was purchased from a local market in Pathum Thani province, Thailand.



Figure 2.1 Dried Shiitake mushroom, Donko type

2.2 Methods

2.2.1 Extraction of Shiitake mushroom

The dried mushroom was pulverized using a grinder and liquid nitrogen. Two hundred grams of the powder were immersed in 300 ml of water at room temperature for one day. Then, chloroform (800 ml) was added and the mixture left at room temperature for six days. Later, the mixture was filtered and then separated by separatory funnel to a chloroform and a water fraction. The chloroform and water were removed using a rotary evaporator and freeze dryer, respectively. The chloroform extract and water extract were kept in amber bottles at ambient temperature.

The chloroform extract and water extract were evaluated for their antifungal activity against nine strains of mycotoxigenic fungi by the agar diffusion technique as described in section 2.2.2.

2.2.2 Antifungal activity using the agar diffusion technique

2.2.2.1 Antifungal activity of Shiitake extract

For this study, each fungal strain was grown on an appropriate culture medium. *P. citrinum* TISTR 3437, *P. expansum* BCC 7541, *F. moniliforme* TISTR 3175 and *F. verticillioides* 1641 were cultured on PDA. *A. ochraceus* TISTR 3557, *F. oxysporum* BCC 4977 and *F. graminearum* 1895 were cultured on Czapek Dox agar (CZA) (Appendix A). *A. flavus* 9090 and *A. parasiticus* TISTR 3276 were cultured on Yeast Extract Sucrose (YES) agar (Appendix A).

Sterilized plates were prepared with approximately 20 ml of agar medium. The agar medium plate was cut for four wells using a cork borer (0.9 mm) and 250 mg/ml of Shiitake extract (200 µl) were added to each well. For fungal inoculation, all fungal isolates were grown on the PDA for seven day, and cut using a cork borer (0.4 mm), and placed at the center of the same plate. Then, they were incubated at 25°C for seven days. A negative control treatment was performed by incubating the fungal strains without the Shiitake extracts. Experiments were run in triplicate. Fungal growth was recorded by colony diameter measured daily (Kitzberger *et al.*, 2007). The percentage of fungal growth inhibition was calculated by the following equation (1).

$$\% \text{ Fungal growth inhibition} = \frac{(A - B)}{A} \times 100 \text{(1)}$$

where, A = Diameter of fungi without Shiitake extract (cm)

B = Diameter of fungi with Shiitake extract (cm)

2.2.2.2 Minimum inhibitory concentration (MIC) of chloroform extract and growth assessment of fungi

(1) Minimum inhibitory concentration (MIC) of chloroform extract

The minimum inhibitory concentration (MIC) is the bacterial or fungal growth inhibition ability at the lowest concentration of substance (Andrews, 2001). Regarding to the experiment, the concentrations of chloroform extract were obtained by two-fold serial dilution within the range 250-0.31 mg/ml, and antifungal activity was tested using agar diffusion technique.

The nine fungal species, *A. flavus* 9090, *A. parasiticus* TISTR 3276, *A. ochraceus* TISTR 3557, *P. citrinum* TISTR 3437, *P. expansum* BCC 7541, *F. graminearum* 1895, *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, and *F. verticillioides* 1641 were cultured on the appropriate medium as described in section 2.2.2. In this experiment, the chloroform extract was prepared in the range at the concentraion 250-0.31 mg/ml by two-fold dilution method.

The lowest concentration of chloroform extract that demonstrated antifungal activity was assumed to be the MIC. Antifungal activity was estimated by calculating the percentage of fungal growth inhibition using equation (1).

(2) Growth assessment of fungi with chloroform extract

To estimate the specific growth rate of fungi treated with the MIC of chloroform extract, fungal growth was observed on a daily basis for seven days using diameter measurements. Based on the data from section 2.2.2, the growth curve of the fungi was plotted by diameter of colony (cm) versus time (day). The specific growth rate of colony was calculated using equation (2).

$$\text{The specific growth rate of colony} = \frac{K_r}{W} \text{(2)}$$

where, K_r = the colony radial growth rate (cm/day)

W = the width of the peripheral growth zone (cm)

2.2.3 Effect of Shiitake extract on mycotoxin production

2.2.3.1 Conidia preparation

In this study, *Fusarium* spp. was used to determine the effect of the chloroform fraction on inhibition of mycotoxin production. A conidia (macro or micro) suspension of each *Fusarium* strain was used as the stock culture. To induce conidia production, each strain of *Fusarium* spp. was cultured in 250 ml of sterilized Mung bean medium (Appendix A). Next, the culture was incubated at 25°C at 120 rpm for 4 days. After the incubation period, the mycelium was filtered and the conidia were collected by centrifugation. They were counted using Haemocytometer and the stock of conidia suspension was adjusted to 10^8 spores/ml using sterilized water.

2.2.3.2 Mycotoxin production condition of *Fusarium* spp.

Each *Fusarium* strain was cultured in 150 ml of Yeast Malt extract (YM) broth (Appendix A) and incubated at 25°C (for *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, and *F. verticillioides* 1641) and 30°C (for *F. graminearum* 1895) at a speed of 120 rpm. A broth sample was collected at day 7, 10, and 14. Then, Deoxynivalenol (DON), Zeralenone (ZEA), and Fumonisin B₁ (FB₁) were further extracted and analyzed by the method described in section 2.2.3.4.

2.2.3.3 The maximum non-inhibition growth

The concentrations of chloroform fraction were prepared by two-fold serial dilution. The extract (0.48, 0.97, 1.95, 3.9, 7.8 and 15.6 mg/ml) was placed in a sterilized flask and evaporated at 40°C until dry. Then, YM broth was added to the flask. The conidia suspension prepared as in section 2.2.3.1 was inoculated. The medium without extract was used as a control. Samples were incubated at 25°C and 30°C in an incubator shaker at a speed of 120 rpm. The mycelium dry cell weight of each fungus was measured at day seven. To measure the mycelium dry cell weight, the mycelium was filtered and washed twice using sterilized water. Filter paper containing the mycelium was dried in an oven at 105°C until a constant weight was achieved and then left in a desiccator. The filter paper with the mycelium was weighed and the dry weight was calculated according to equation (3) (Hajjaj *et al.*, 2000, Vergopoulou *et al.*, 2001 and Taniwaki *et al.*, 2009).

$$\% \text{Mycelium dry cell weight} = \frac{\text{Mass}_{\text{final}} - \text{Mass}_{\text{initial}}}{\text{Mass}_{\text{initial}}} \times 100 \quad \text{-----}(3)$$

Where, Mass_{initial} = the mass of filter paper without fungal sample

Mass_{final} = the mass of filter paper with dried fungal sample

2.2.3.4 Inhibition of mycotoxin production using Shiitake extract

F. graminearum 1895 and *F. verticillioides* 1641 were used in this experiment because of their mycotoxin production. The chloroform fraction at a concentration of 0.98 mg/ml (for *F. verticillioides* 1641) and 1.95 mg/ml (for *F. graminearum* 1895) was prepared by two-fold serial dilution and 4.5 ml of the extract was added to each sterilized flask. To remove the organic solvent, all flasks were left

at 40°C until dry. Then, 150 ml of YM medium were added into the flask. Besides, the stock of conidia suspension (10^8 spores/ml) was added to give a final concentration of 10^6 spore/ml. Next, the mixtures were incubated at 25°C (for *F. verticillioides* 1641) and 30°C (for *F. graminearum* 1895) in an incubator shaker at a speed of 120 rpm. Samples were collected at day 7, 10, and 14, respectively. The mycotoxins were extracted and analyzed by the methods which were described as following by (1) and (2), respectively.

(1) Mycotoxin extraction

The extraction of mycotoxins was performed following the method as previously published by Sulyok *et al.* (2006). The mycotoxins were extracted by extraction solvent of acetonitrile/water/acetic acid 79+20+1; v/v/v. The sample was centrifuged and the supernatant of each sample was diluted in the ratio 1:1 with a dilution solvent comprising acetonitrile/water/acetic acid 20+79+1 (v/v/v). Then, the samples were analyzed by HPLC-MS/MS.

(2) Mycotoxin detection

Mycotoxin detection was performed with an HPLC-MS/MS system equipped with a Turbo Ion Spray ESI source and a 1100 Series HPLC system (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini® C18 column, 150X4.6 mm i.d., 5 mm particle size, equipped with a C18 4X3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA). Both eluents contained 5 mM ammonium acetate and comprised of methanol/water/acetic acid 10+89+1 (v/v/v; eluent A) or 97+2+1 (eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 100% over 12 min, followed by a hold time of 3 min at 100% B and 4 min column re-equilibration at 100% A. The flow rate was 1 ml/min. The column effluent was transferred via a six-port valve (VICI Valco Instruments, Houston, TX, USA) either to the mass spectrometer (between 2 and 17 min; no flow splitting was used) or to waste (Sulyok *et al.*, 2006).

2.2.4 Purification and characterization of active compounds from

Shiitake extract

2.2.4.1 Purification of active compounds from Shiitake extract

(1) Thin layer chromatography (TLC)

TLC plates (silica gel 60 F254, 0.2 mm; Macherey-Nagel, Germany) were prepared by cutting to a size of 1X5 cm. The sample was spotted on

the TLC plate at an origin line established at 10 mm from the bottom edge. The plate was developed in a chamber containing a mobile phase. After the mobile phase front had moved about 35 mm, the plate was removed from the chamber and dried at ambient temperature. The spots on the TLC plate were then visualized by UV spectrophotometer. Afterward, anisaldehyde staining was applied to the TLC plate and the plate was dried by heating. The retention factor (R_f) revealed the appearance of a spot on the TLC plate. The R_f value was calculated using formula (4) (Martin-Puzon *et al.*, 2015).

$$\text{Retention factor } (R_f) = \frac{\text{Distance travelled by the spot}}{\text{Distance travelled by the solvent}} \quad \text{-----}(4)$$

(2) Column chromatography

The crude extract was placed in a column (3X49 cm) packed with sephadex LH-20 (GE-Healthcare, Sweden) as a stationary phase and was equilibrated with a solvent mixture of methanol:dichloromethane (50:50). The column was washed with the same solvent, and the fraction was then eluted. Each fraction was collected and combined according to TLC results, and the antifungal activity of the combined fractions was tested against four *Fusarium* strains. The details of the method are described in section 2.2.5.

Then, all active fractions were further separated using a column packed with silica gel (100-200 mesh; RANKEM, India) as the stationary phase. The column was washed with a gradient of ethyl acetate:hexane (0:10, 1:9, 2:8, 3:7, 4:5, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0) and methanol:ethyl acetate (0:10, 1:9, 2:8, 3:7, 4:5, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0) as mobile phase, and the fractions were collected and combined according to TLC results. Purified fractions were identified by nuclear magnetic resonance, mass spectrometry and infrared spectroscopy.

2.2.4.2 Characterization of purified active compounds

(1) Nuclear magnetic resonance (NMR)

The samples were placed under the vacuum condition in order to remove the moisture content. Dried samples were dissolved in CDCl_3 and transferred to an NMR tube. The ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance Ultrashield 400 MHz spectrometer (Switzerland).

(2) Mass spectrometry

The samples were prepared by removing the moisture content and filtered using a 0.22 μm syringe filter. Then, the purified fractions of Shiitake extract were identified by mass spectrometry. The mass spectrum was collected at Electron Impact (EI) mode at 70 eV. Fragmentation was performed with a rate of 1 scan per 0.2 second and a range of mass was from 30 to 1000 m/z.

(3) Infrared spectroscopy

The moisture was removed from the samples by allowing in vacuum. Infrared spectra were obtained on a Fourier Transform Infrared (FT-IR) spectrometer with a detector in a range of 650–4000 cm^{-1} .

(4) Melting point

Dried samples were prepared by removing the moisture content and measured using melting point apparatus.

2.2.5 Antifungal activity of purified compound using microdilution

technique

A microdilution technique was used to determine the fungal growth inhibition ability of the purified compounds. The method was carried out following Espinel-Ingroff *et al.* (1997) with some modification. In this study, four *Fusarium* strains were chosen to evaluate the antifungal activity, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC) of the purified compounds.

The spore suspension of *Fusarium* strains was performed by washing 7-day-old colonies of fungi with 0.85% saline (the details of chemical and spore suspension preparations are described in Appendix A). Then, the suspension was adjusted with sterilized water containing a 2X concentration of resazurin indicator to perform the inoculation suspension of double the desired final concentration. Each tested compound was dissolved in dimethyl sulfoxide (DMSO) and diluted with 2X Roswell Park Memorial Institute 1640 (RPMI 1640) medium to obtain a stock solution of 10 mg/ml (as 2X final concentration). The experiments were performed in 96-well microtiter plates with round bottom wells. The stock solution (100 μl) of tested compound and the inoculation suspension (100 μl) were added to the well of the plate, resulting in a final concentration of 5 mg/ml and a final inoculum of 0.4×10^4 to 5×10^4 CFU/ml, respectively. The microplates were incubated at 25°C for 72 h. In addition, medium without the tested compounds was used as a growth control (negative control).

Drug control (positive control) was performed by using amphotericin B instead of the tested compounds. Growth inhibition was determined according to color change of the indicator. No color change indicated positive fungal growth inhibition, whereas a change from blue to purple indicated a negative result (non-inhibition of growth).

The antifungal activities of the isolated compounds were determined against four *Fusarium* spp. using a microdilution technique. Then, the active compounds that exhibited the activity were further evaluated for their MIC and MFC.

2.2.5.1 Minimum inhibitory concentration (MIC) of isolated compounds

The microdilution technique was used to determine the MIC of the purified compounds. The compounds were dissolved in DMSO, followed by dilution in RPMI 1640. The concentrations of compounds (0.62 to 10 mg/ml) were prepared by two-fold serial dilution. The solution at each concentration (100 µl) and inoculation suspension (100 µl) was then applied to the well, respectively. The final concentration of the tested compounds ranged from 0.31 to 5 mg/ml. The microplates were incubated at 25°C for 72 h. The lowest concentration that inhibited fungal growth was measured as the MIC.

2.2.5.2 Minimum fungicidal concentration (MFC) of purified compounds

The MFC is the lowest concentration that is able to kill 99.9% of the initial inoculum (Zhang *et al.*, 2015). To determine the fungicidal activity of the purified compounds, the concentrations at which no visible growth was observed in the well were further investigated. One hundred microliters from each well were cultured on PDA plates and incubated at 25°C for 72 h. Concentration that showed no visible colonial growth colonies grown on PDA was defined as the MFC.

2.2.6 Statistical analysis

All experiments were conducted in triplicate and the results were determined by One-way Analysis of Variance (ANOVA) with a significance level of 0.05 using Statistic software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Shiitake extracts

In this study, the modified method from Hirasawa *et al.*, (1999) was used. Dried Shiitake mushroom was pulverized with liquid nitrogen, instead of immersing in water and then grinding in a mixer. The appearance of water and chloroform extract was a dark brown liquid, and they were insoluble in each other. The yields of water extract and chloroform extract were 71.47 g (7.147 %yield) and 5.33 g (0.533 %yield), respectively (Table 4.1). Whereas Hirasawa *et al.* (1999) reported obtaining chloroform extract at 0.876 g weight from 200 g Shiitake mushroom (0.438 %yield), the modified extraction method gave a higher %yield.

Table 3.1 Percentage of yield of extracts from dried Shiitake mushroom (*Lentinus edodes*) (1,000 g)

Fraction	Weight of extract (g)	Yield (%)
Chloroform extract	5.33	0.533
Water extract	71.47	7.147

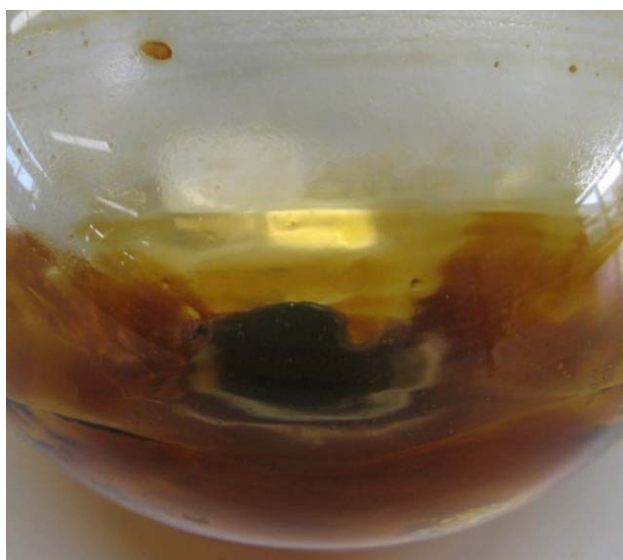


Figure 3.1 Appearance of chloroform extract from Shiitake mushroom

3.2 Antifungal activity of Shiitake extracts

Regarding to this study, chloroform extract and water extract from Shiitake mushroom were investigated for their antifungal activities. Nine strains of mycotoxigenic fungi were used and the experiments were established on agar culture medium. The inhibitory effect of chloroform extract and water extract (250 mg/ml) are shown in Figure 3.2. The percentage of fungal growth inhibition of the chloroform extract was significantly higher than that of the water extract. The water extract showed weak antifungal activity against *A. flavus* 9090, *A. parasiticus* TISTR 3276, and *Fusarium* spp., but no an inhibitory effect on *A. ochraceus* TISTR 3557, *P. citrinum* TISTR 3437, and *P. expansum* BCC 7541. The chloroform extract presented strong antifungal effects against nine strains of fungi, especially *F. graminearum* 1895, *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, and *F. verticillioides* 1641. The percentages of fungal growth inhibition were 64%, 75%, 49%, and 52%, respectively. This may be because the chloroform extract contained more of the active compound than the water extract. In previous research, only one study reported antimicrobial activity of chloroform extract from Shiitake mushroom (Hirasawa *et al.*, 1999). They reported that chloroform extract had the highest activity when compared with ethyl acetate extract and water extract. Rao *et al.* (2009) reported that low polarity compounds in the Shiitake extracts provided the strong antibacterial properties. Moreover, Siddiqui *et al.*

(2013) reported that the chloroform extract from *Mikania scandens* (L.) showed a higher antifungal effect against *F. oxysporum* than ethyl acetate extract. As in the present study, Hearst *et al.* also investigated the antifungal activity of water extract from Shiitake mushroom and reported antifungal activity at a concentration of 1 mg/ μ l (Hearst *et al.*, 2009).

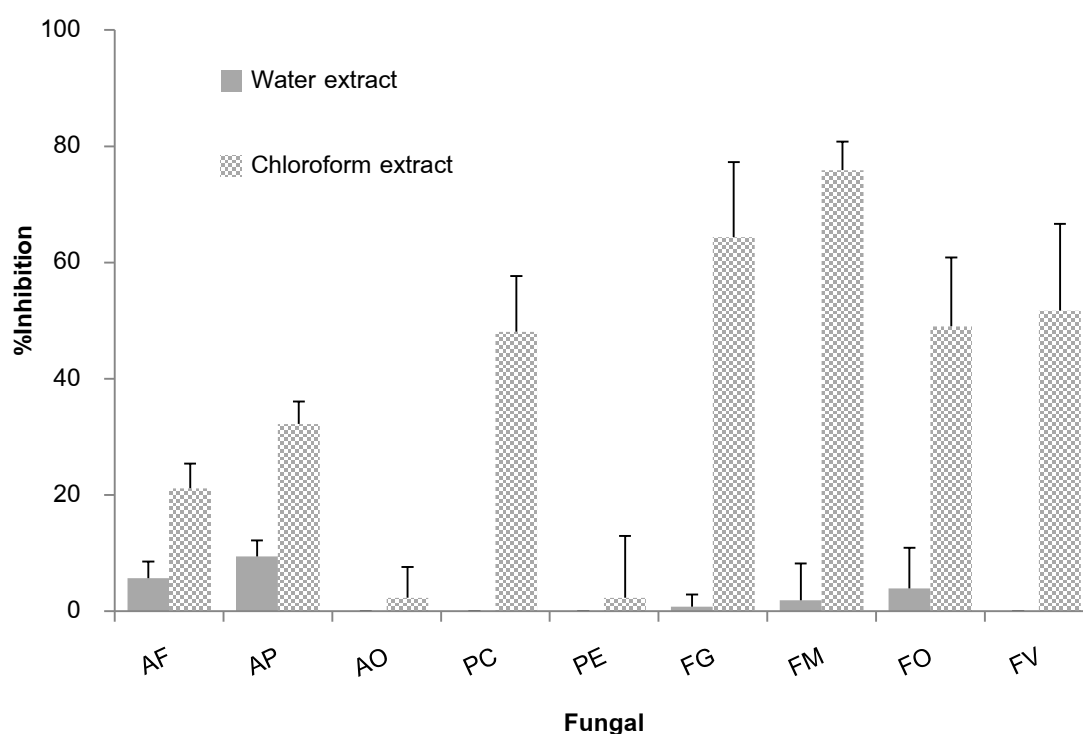


Figure 3.2 Antifungal activity of water extract and chloroform extract against fungal strains. AF; *A. flavus* 9090, AP; *A. parasiticus* TISTR 3276, AO; *A. ochraceus* TISTR 3557, PC; *P. citrinum* TISTR 3437, PE; *P. expansum* BCC 7541, FG; *F. graminearum* 1895, FM; *F. moniliforme* TISTR 3175, FO; *F. oxysporum* BCC 4977, and FV; *F. verticillioides* 1641. Average $\pm$ SD is from a triplicate determination.

3.3 Minimum inhibitory concentration (MIC) of chloroform extract and growth assessment of fungi

As shown in Table 3.2, the chloroform extract displayed potent antifungal activity against *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. The MIC values of chloroform extract on *A. flavus* 9090, *A. parasiticus* TISTR 3276, *A. ochraceus* TISTR 3557, *P. citrinum* TISTR 3437, *P. expansum* BCC 7541, *F. graminearum* 1895, *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, and *F. verticillioides* 1641 were 31.3, 31.3, 250, 62.5, 62.5, 1.95, 1.95, 3.9, and 1.95 mg/ml, respectively. Especially, the chloroform extract exhibited strong inhibitory effect on *F. graminearum* 1895, *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, and *F. verticillioides* 1641. The percentages of fungal growth inhibition that treated with MIC were 6.3%, 11.0%, 2.8%, and 3.2%, respectively (data not shown). To confirm the inhibitory effect of MIC of chloroform extract, the mycelium growth rate of all fungi was investigated. The growth of fungi was tested by comparing the chloroform extract at MIC value and the control (without extract). The results revealed that the colony radial growth rate (K_r) and the specific growth rate of the colony (μ) of all fungi treated with extract was lower than that of the control (Table 3.2). In this study, the chloroform extract exhibited antifungal activity against almost all fungal strains. The most sensitive fungal specie to the chloroform extract was *Fusarium* spp.: *F. graminearum* 1895 ($K_r = 2.350$ cm/day; $\mu = 0.261$ day⁻¹), *F. moniliforme* TISTR 3175 ($K_r = 0.769$ cm/day; $\mu = 0.085$ day⁻¹), *F. oxysporum* BCC 4977 ($K_r = 1.408$ cm/day; $\mu = 0.156$ day⁻¹), and *F. verticillioides* 1641 ($K_r = 1.153$ cm/day; $\mu = 0.128$ day⁻¹). Meanwhile, *Penicillium* spp. was the most resistant one with the extract. For this reason, chloroform extract and four of *Fusarium* spp. were chosen for further studies.

Table 3.2 MIC values and kinetic growth assessment of fungi with and without chloroform extract treatment.

Fungal strain	MIC (mg/ml)	Chloroform extract			
		with extract ^a		without extract ^b	
		K _r (cm/day)	μ (1/day)	K _r (cm/day)	μ (1/day)
<i>A. flavus</i> 9090	31.3	0.703	0.078	0.920	0.102
<i>A. parasiticus</i> TISTR 3276	31.3	0.837	0.093	1.043	0.116
<i>A. ochraceus</i> TISTR 3557	250	0.556	0.062	0.495	0.055
<i>P. citrinum</i> TISTR 3437	62.5	0.268	0.030	0.243	0.027
<i>P. expansum</i> BCC 7541	250	0.280	0.031	0.217	0.024
<i>F. graminearum</i> 1895	1.95	2.350	0.261	2.500	0.278
<i>F. moniliforme</i> TISTR 3175	1.95	0.769	0.085	0.836	0.093
<i>F. oxysporum</i> BCC 4977	3.9	1.408	0.156	1.183	0.131
<i>F. verticillioides</i> 1641	1.95	1.153	0.128	1.073	0.119

K_r; the colony radial growth rate (1/day) and μ; the specific growth rate of the colony (cm/day). ^{a, b} mean values within the same row followed by the different letters are significantly different ($p \leq 0.05$).

In a previous study of antimicrobial activity, Hearst *et al.* (2009) reported that Shiitake mushroom demonstrated antifungal effects. Imtiaj and Lee (2007) also reported that nine Korean wild mushroom types showed antibacterial and antifungal activity. Furthermore, many researchers have studied the antifungal activity of low polarity products such as essential oils and plant extracts. The oils and plant extracts tested include *Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta*, and *Xylothea kraussiana* (Mahlo *et al.*, 2010), *Asparagus* (Rosado-Álvarez *et al.*, 2014), *Trigonella foenum-graecum* (Omezzine *et al.*, 2014), and *Citrullus colocynthis* (Eidi *et al.*, 2015). Farzaneh *et al.* (2015) found that essential oil from three species of savory showed antifungal activity against *A. niger*, *P. digitatum*, *Botrytis cinerea*, and *Rhizopus stolonifer* with MICs in the range 300-600 μl/L. In 2014,

Stević *et al.* reported that 16 essential oils presented antifungal activity against 21 fungi isolated from herbal drugs. The highest potential inhibitory effects in the control of fungi were recorded for thyme, oregano, and rose.

3.4 Effect of Shiitake extract on mycotoxin production

3.4.1 Mycotoxin production condition of *Fusarium* spp.

In this study, mycotoxin production by each fungal strain was studied. The condition for mycotoxin production is summarized in Table 3.3. The results showed that *F. graminearum* 1895 produced Deoxynivalenol (DON) and Fumonisin B₁ (FB₁). The highest production of DON (459.5 ppb) was found using YM broth at 30°C for two weeks. *F. moniliforme* TISTR 3175 can produce zearalenone (ZEA) at 30°C in YM broth, but in very small amounts (data not shown). *F. verticillioides* 1641 produced FB₁ (189 ppb) after incubation for 14 days. Thus, *F. graminearum* 1895 and *F. verticillioides* 1641 were chosen for further study.

Table 3.3 Mycotoxin production condition of *Fusarium* spp.

Fungal strain	Condition		Mycotoxins (ppb)	
	Medium	Temp. (°C)	DON	FB ₁
<i>F. graminearum</i> 1895	YM	30	459.5	4.7
<i>F. moniliforme</i> TISTR 3175		25	-	-
<i>F. oxysporum</i> BCC 4977		25	-	350.0
<i>F. verticillioides</i> 1641		25	-	189.0

- : not produce mycotoxins

YM: Yeast Malt Extract broth, DON: Deoxynivalenol and FB₁: Fumonisin B₁

3.4.2 The maximum non-inhibition growth

To ensure that mycotoxin production was not affected by a decrease in fungal growth, the maximum non-inhibition concentration of chloroform extract was investigated. The concentration at which the extract did not inhibit the growth of fungi is shown in Table 3.4. The result revealed that the concentration of chloroform extract which had no inhibitory effect on the growth of *F. graminearum* 1895 and *F. verticillioides* 1641 were shown to be 1.95 mg/ml and 0.98 mg/ml, respectively. Thus, these concentrations were used in the further experiments.

Table 3.4 Mycelium dry cell weight of *Fusarium* spp. (day 7) at different concentrations of chloroform extract.

Fungal strain	Conc. of chloroform extract (mg/ml)	Mycelium weight (g)
<i>F. graminearum</i> 1895	3.9	0.0754 ^a
	1.95	0.9922 ^b
	control	0.8757 ^b
<i>F. verticillioides</i> 1641	1.95	1.0086 ^a
	0.98	1.0638 ^b
	0.49	1.0712 ^b
	control	1.0719 ^b

^{a, b} mean values within the same column followed by the different letters are significantly different ($p \leq 0.05$), as determined by Duncan's multiple range test.

Although the mycelium dry cell weight of the fungal did not vary, a change in morphology was observed. Bleaching of the *F. verticillioides* mycelium was observed and the appearance of the mycelium also changed. Da Silva Bomfim *et al.* (2015) reported that changes in colony morphology were affected by increase in the conidia size. Reduction in the turgor pressure on the cell wall or cell surface changed the morphology as the osmotic stabilizer attempted to restore the osmotic balance. Increasing the concentration of *Rosmarinus officinalis* L. essential oil (REO) changed the microconidia appearance. This result indicated that antifungal

activity against *F. verticillioidea* take place through the loss of membrane stability and thus the inhibition of cell growth.

3.4.3 Inhibition of mycotoxin production using Shiitake extract

F. graminearum 1895 and *F. verticillioidea* 1641 were cultured with Shiitake chloroform extract at 1.95 g/ml and 0.98 g/ml, respectively. The mycotoxins in the culture medium were analyzed. The effects of chloroform extract on mycotoxin production are illustrated in Figure 3.3. Chloroform extract was able to inhibit FB₁ production from *F. verticillioidea* 1641. Its effect was significantly greater than that of the control (without extract). The quantity of FB₁ decreased about 75% by the end of incubation. On the other hand, the extract had no significant effects with DON production from *F. graminearum* 1895.

A number of studies have investigated the inhibition of FB₁ production by essential oils and plant extracts. In 2013, Pizzolitto *et al.* reported that peanut skin extracts (PSE) demonstrated a mycotoxin inhibitory effect. A yellow fraction (62.5 µg/ml) that was separated from the extract showed effective inhibition of FB₁ production by *F. verticillioidea*. Velluti *et al.* (2003) exhibited that cinnamon, clove, oregano, palmarose, and lemongrass oils significantly inhibited FB₁ production by *F. proliferatum*. Da Silva Bomfim *et al.* (2015) also revealed that essential oil (300-600 ng/ml) from *Rosmarinus officinalis* L. inhibited production of FB₁ and FB₂ by *F. verticillioidea*. The production of fumonisin (both FB₁ and FB₂) was reduced by 97-99%. The inhibition effect of the essential oil against *F. verticillioidea* might be a result of cell wall disruption and loss of the cellular components.

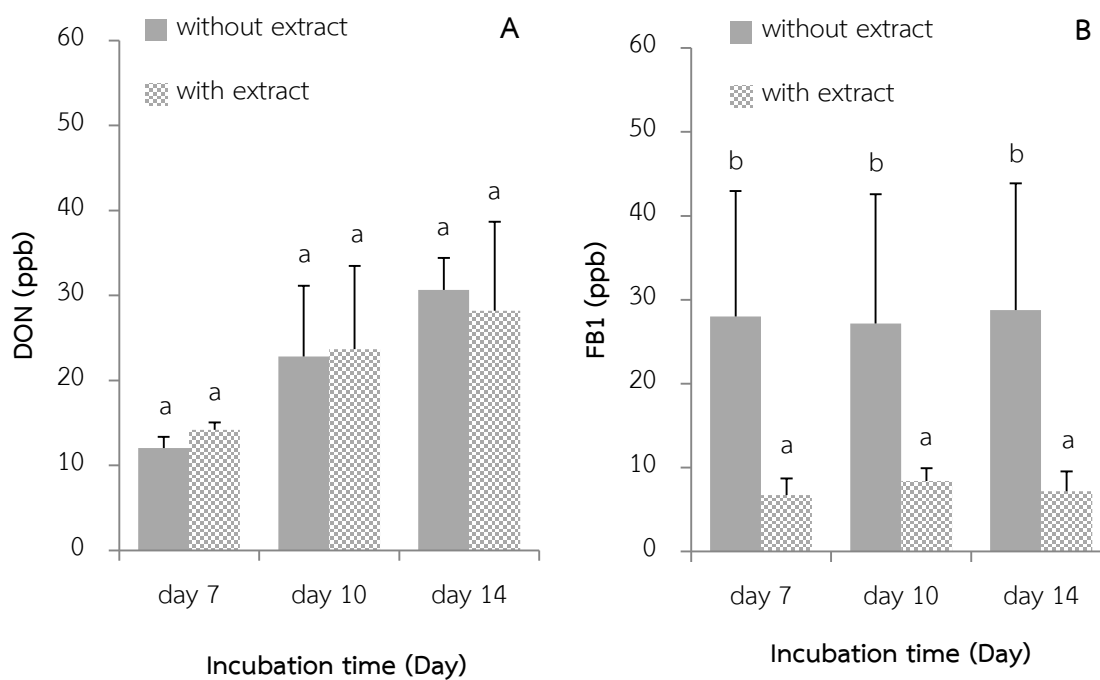


Figure 3.3 Inhibitory effect of chloroform extract at concentrations of 1.95 and 0.98 mg/ml on Deoxynivalenol (DON) production from *F. graminearum* 1895 (A) and Fumonisin B₁ (FB₁) production from *F. verticillioides* 1641 (B). Different letters show a significant difference at $p \leq 0.05$.

3.5 Purification and characterization of active compounds from Shiitake extract

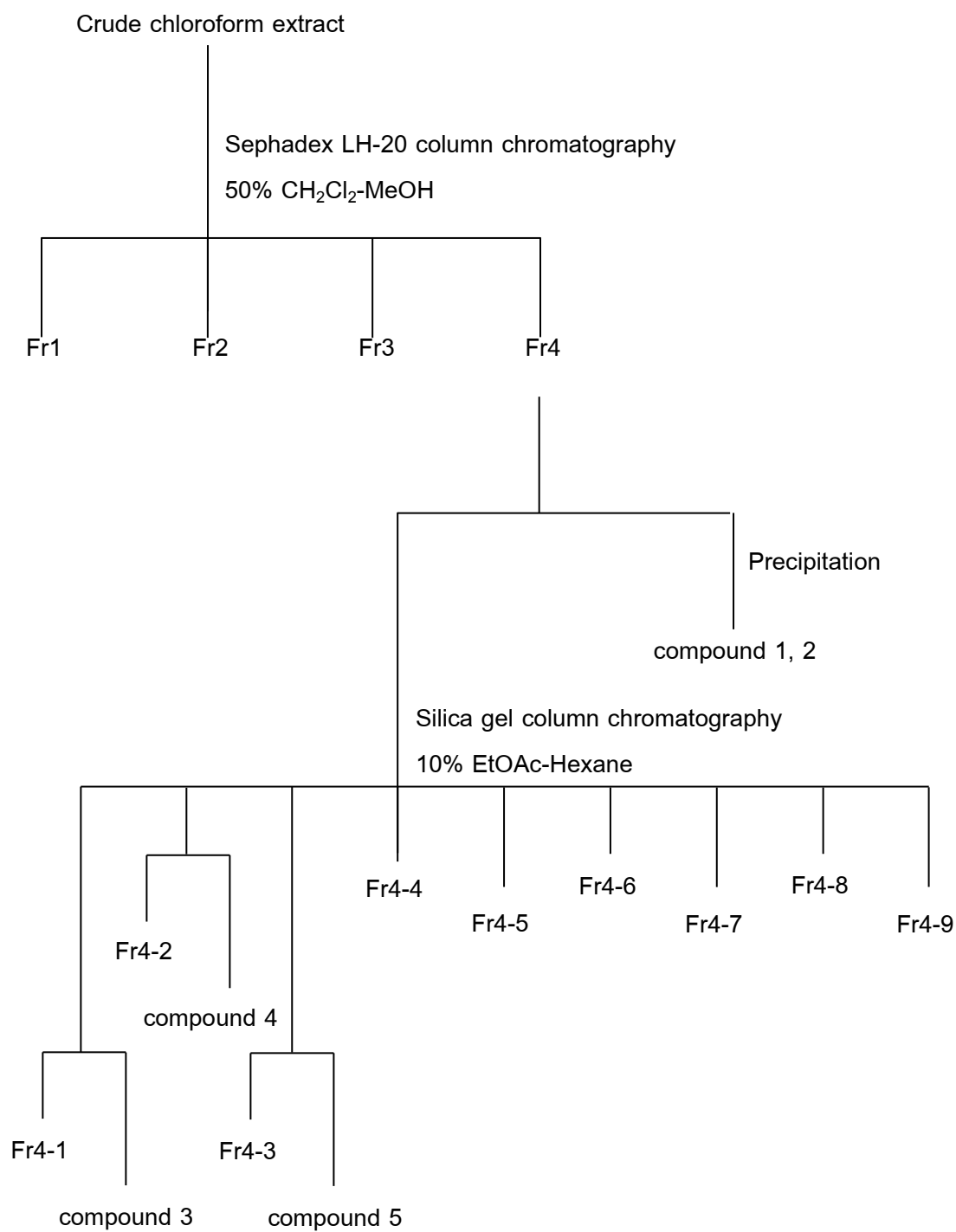


Figure 3.4 Purification of chloroform fraction by column chromatography

3.5.1 Purification of active compounds from chloroform extract

Regarding to the results, water extract and chloroform extract from the Shiitake mushroom were investigated for antifungal activity against nine fungal strains. The chloroform extract displayed stronger inhibitory effects than the water extract. So, the chloroform extract was then purified by chromatography techniques. Figure 3.4 shows the purification steps, the mobile phase was the solvent mixture of methanol:dichloromethane and sephadex was used as the stationary phase. Each eluted fraction was analyzed by TLC and the collections that presented similar spots were combined. Crude chloroform extract was separated by size into Fr1, Fr2, Fr3, and Fr4. Fr4 was redissolved in chloroform and then precipitated with hexane. Two purified compounds from Fr4 were labeled as compound 1 and compound 2.

Table 3.5 Antifungal activity of the fractions isolated from chloroform extract against four *Fusarium* spp.

Fraction	Fungi			
	<i>F. graminearum</i>	<i>F. moniliforme</i>	<i>F. oxysporum</i>	<i>F. verticillioides</i>
Fr1	+	+	+	+
Fr2	+	+	-	-
Fr3	+	-	-	-
Fr4	+	+	+	+
compound 1	+	+	+	+

+ : inhibited

- : non-inhibited

The antifungal activity of Fr1, Fr2, Fr3, Fr4 and compound 1 was evaluated against *Fusarium* spp. (*F. graminearum*, *F. moniliforme*, *F. oxysporum* and *F. verticillioides*) by microdilution technique. The results showed that all fractions exhibited antifungal activity, in which Fr1, Fr4 and compound 1 showing the widest activity (Table 3.5). These fractions were further isolated by chromatography techniques and their isolated fractions were investigated for fungal growth inhibition. However, it proved impractical to isolated sufficient Fr1 to allow full testing of this fraction.

The Fr4 fraction, which showed antifungal activity, was separated again using silica gel as stationary phase and a gradient of ethyl acetate:hexane and

ethyl acetate:methanol as mobile phase. Each fraction eluted from the column was analyzed by TLC and the fractions which showed similar spots were combined. A total of nine fractions (Fr4-1 to Fr4-9) were identified. The antifungal activity of these fractions was tested using microdilution technique. The results revealed that all isolated fractions from Fr4 except Fr4-3 presented an inhibitory effect on the growth of four *Fusarium* spp. The Fr4-3 fraction showed activity against only *F. graminearum* and *F. moniliforme* (Table 3.6).

Table 3.6 Antifungal activity of the fractions isolated from Fr4 against four *Fusarium* spp.

Fraction	Fungi			
	<i>F. graminearum</i>	<i>F. moniliforme</i>	<i>F. oxysporum</i>	<i>F. verticillioides</i>
Fr4-1	+	+	+	+
Fr4-2	+	+	+	+
Fr4-3	+	+	-	-
Fr4-4	+	+	+	+
Fr4-5	+	+	+	+
Fr4-6	+	+	+	+
Fr4-7	+	+	+	+
Fr4-8	+	+	+	+
Fr4-9	+	+	+	+

+ : inhibited

- : non-inhibited

Three isolated compounds were precipitated from Fr4-1, Fr4-2, and Fr4-3 and were labeled as compound 3, compound 4, and compound 5, respectively. The antifungal activity of these fractions was tested using microdilution technique. Compound 3 inhibited growth of all four *Fusarium* spp. while compound 3 and compound 4 showed no inhibitory effect on any fungal strain (Table 3.7).

Table 3.7 Antifungal activity of the compounds isolated from a sub-fraction of Fr4 against four *Fusarium* spp.

Fraction	Fungi			
	<i>F. graminearum</i>	<i>F. moniliforme</i>	<i>F. oxysporum</i>	<i>F. verticillioides</i>
compound 3	-	-	-	-
compound 4	-	-	-	-
compound 5	+	+	+	+

+ : inhibition

- : non-inhibition

According to the results, only compounds 1, 2, and 5 were chosen for identification of chemical structure, because these fractions showed self-precipitation in the receiving flask, demonstrating high purity and making them interesting for identification. The purity of these compounds was verified using nuclear magnetic resonance (NMR) spectroscopy. Compounds 1 and 5 were selected for minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) analysis, because only small amounts of compound 2 were recovered.

The antifungal activity of the crude chloroform extract from Shiitake mushroom was attributed to the presence of biologically active compounds. Since the sensitivity of these compounds was interesting, each purified compound was. The compounds were therefore isolated by chromatography techniques and their structures were elucidated by NMR.

3.5.2 Chemical structure characterization of active compounds from chloroform extract

¹H-NMR spectroscopy is widely used to elucidate the functional group and the type of proton expressed by the form of the chemical shift, signal intensity, and type of signal (singlet, doublet, triplet, quartet, etc.). ¹³C-NMR spectroscopy identifies the number of carbon atoms in the molecule. The number of the chemical shift reflects the number of carbon atoms. In addition, the functional group of each carbon is confirmed by the position of the chemical shift. The number of protons in each carbon atom is determined by Dept135 and Dept90. DEPT-135

presents all types of carbon atom, including CH, CH₂, and CH₃. The signals of CH₂ are negative, while those of CH and CH₃ are positive. DEPT-90 only shows signals of the CH groups. The heteronuclear single quantum coherence (HSQC) is a 2D-NMR technique used to measure the correlation chemical shift of carbon and the directly bonded proton. Each carbon (or other heteroatom) to hydrogen connectivity is determined by a two-dimensional (2D) spectrum with two frequency axes, one axis for carbon and the other for proton. The peak is plotted as a function of two frequencies, usually called F1 and F2. The spectrum shows characteristic peaks of the proton attached to carbon. Cross peaks give the correlation of proton and carbon. Heteronuclear multiple bond correlation (HMBC) is one of the two-dimensional experiments used to determine the long range correlation between carbon and neighboring protons. The correlation is presented by the cross peaks, where the intensity of the cross peaks depends on the coupling constant. The two-dimensional spectrum of the correlation spectroscopy (COSY) experiment is presented with protons along both axes. A diagonal of the signals divides the spectrum in to two equal halves. Peaks that symmetrically present above and below the diagonal are suppressed. Other peaks are used to determine the correlation of proton and proton from neighboring carbon atoms.

Ergosterol peroxide

Appearance: white amorphous powder

Melting point: 179-180°C

R_f: 0.43 (Ethyl acetate:Hexane (5:95))

IR spectra (KBr): $V_{\max}$ 3309, 2924, 2853, 1710, 1457, 1072, 1042, 966 cm⁻¹.

¹H and ¹³C-NMR spectrum: data shown in Table 3.8

Mass spectrum: ion peak m/z 428 [M]⁺

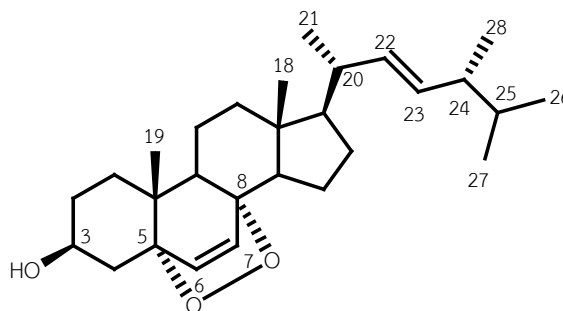


Figure 3.5 Chemical structures of ergosterol peroxide (1) isolated from the chloroform extract

Table 3.8 ^1H -NMR and ^{13}C -NMR data of ergosterol peroxide (1) (in CDCl_3)

Position	Ergosterol peroxide (1)	
	^1H -NMR	^{13}C -NMR
1	1.62	34.69, CH_2
2	1.50	30.10, CH_2
3	4.00	66.49, CH
4	1.82	36.91, CH_2
5	-	79.43, C
6	6.20	135.41, CH
7	6.50	130.75, CH
8	-	82.16, C
9	1.43	51.08, CH
10	1.20	39.34, CH_2
11	0.96	20.63, CH_2
12	1.98	39.74, CH
13	-	44.56, C
14	1.53	51.68, CH
15	1.25	23.40, CH_2
16	1.23	28.65, CH_2
17	1.20	56.20, CH
18	0.80	12.87, CH_3
19	0.86	18.18, CH_3
20	1.23	29.71, CH_2
21	0.98	20.88, CH_3
22	5.12	135.20, CH
23	5.17	132.31, CH
24	1.84	42.78, CH
25	2.30	33.07, CH
26	0.85	19.64, CH_3
27	0.85	19.95, CH_3
28	0.90	17.56, CH_3

The IR spectrum showed the functional group of ergosterol peroxide. The O-H bond showed a distinct characteristic between 3200 and 3500 cm^{-1} . The strong absorptions at 2924 cm^{-1} and 2853 cm^{-1} represented the C-H stretching vibration of the methylene symmetric and asymmetric modes. The stretching vibration band of methylene carbon was observed at 1710 cm^{-1} and the bending vibration band was found at 1457 cm^{-1} . The band identified at 1044 cm^{-1} is typical of the stretching vibrations of C-O.

From the HMBC spectra, the correlation of the carbon and proton within the molecule was described as follows (Figure 3.6). The methylene proton signal at δ 1.62 (H-1) was correlated with the two carbon signals at δ 30.10 (C-2) and 79.43 (C-5). Methylene proton signal at δ 1.50 (H-2) was correlated with the carbon signal at δ 79.43 (C-5). The methylene proton signal at δ 1.82 (H-4) was correlated with two methine carbon signals at δ 66.49 (C-3) and 135.20 (C-6). The methine proton signal at δ 6.50 (H-7) was correlated with the two carbon signals at δ 79.43 (C-5) and 82.16 (C-8) and the two methine carbon signals at δ 135.20 (C-6) and 51.08 (C-9). The methylene proton signal at δ 0.96 (H-11) was correlated with the methylene carbon signal at δ 30.34 (C-10) and the methine carbon signal at δ 39.74 (C-12). The methine proton signal at δ 1.98 (H-12) was correlated with the two methine carbon signals at δ 51.08 (C-9) and 51.68 (C-14). The methine proton signal at δ 1.53 (H-14) was correlated with the methine carbon signal at δ 130.75 (C-7), the carbon signal at δ 44.56 (C-13), and the methylene carbon signal at δ 23.40 (C-15). The carbon signal at δ 44.56 (C-13) was correlated with the methylene proton signal at δ 1.23 (H-16) and the methyl carbon signal at δ 12.87 (C-18) was correlated with the methylene proton signal at δ 1.23 (H-20). The methine proton signal at δ 1.20 (H-17) was correlated with the two methylene carbon signals at δ 28.65 (C-16) and 29.71 (C-20). The methyl proton signal at δ 0.80 (H-18) was correlated with the methylene carbon signal at δ 44.56 (C-13), and the methine carbon signal at δ 56.20 (C-17). The methyl proton signal at δ 0.86 (H-11) was correlated with the two methylene carbon signals at δ 34.69 (C-10) and 39.74 (C-12) and the methine carbon signal at δ 51.08 (C-9). The methyl proton signal at δ 0.98 (H-21) was correlated with the methine carbon signal at δ 135.41 (C-22). The methine proton signal at δ 5.17 (H-23) was correlated with the methine carbon signal at δ 42.78 (C-24) and another methyl carbon signal at δ 17.56 (C-28). The methine proton signal at δ 1.84 (H-24) was correlated with the two methine carbon signals at δ 132.31 (C-23) and 33.07 (C-25). The both methyl proton signals

at δ 0.85 (H-26, H-27) were correlated with the methine carbon signal at δ 42.78 (C-24). The methyl proton signal at δ 0.85 (H-26) was correlated with the methine carbon signal at δ 33.07 (C-25), while the methyl proton signal at δ 0.85 (H-27) was correlated with the methyl carbon signal at δ 19.46 (C-26). The methyl proton signal at δ 0.90 (H-28) was also correlated with the two methine carbon signals at δ 132.31 (C-23), 33.07 (C-25), and the methyl carbon signal at δ 19.46 (C-26). The HMBC spectrum is shown in Figure 3.7. In the ^1H - ^1H COSY spectrum, the signal of the methine proton at δ 6.20 (H-6) coupled with the methine proton signal at δ 6.50 (H-7), as shown in Figure 3.8.

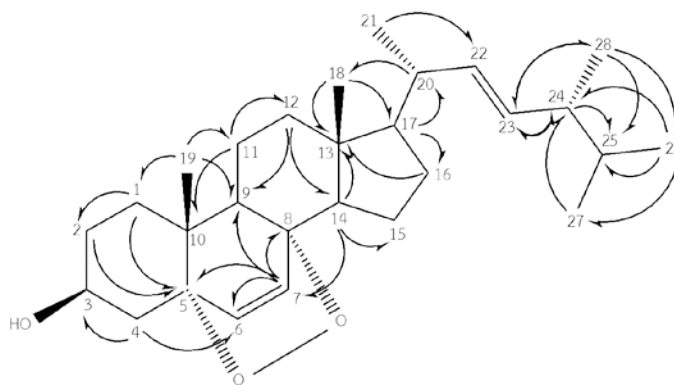


Figure 3.6 Chemical structures of ergosterol peroxide (1) elucidated by HMBC spectrum

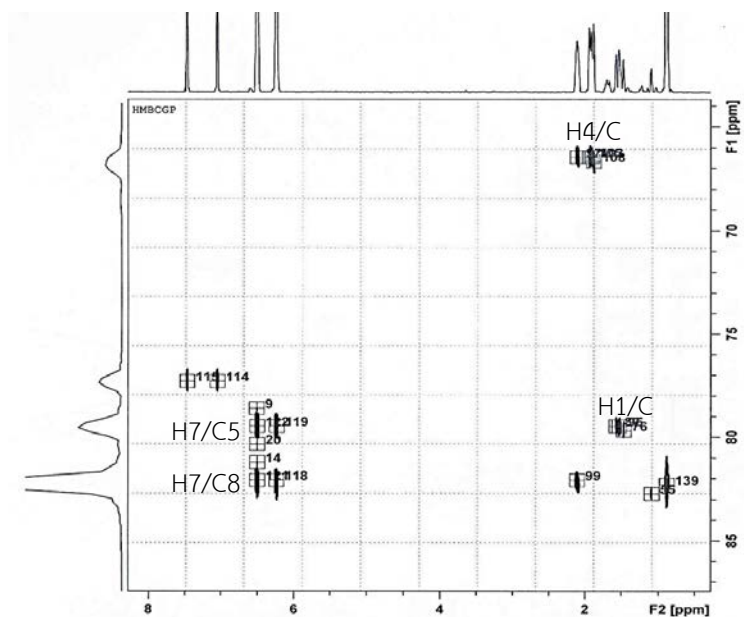


Figure 3.7 HMBC spectrum of ergosterol peroxide (1)

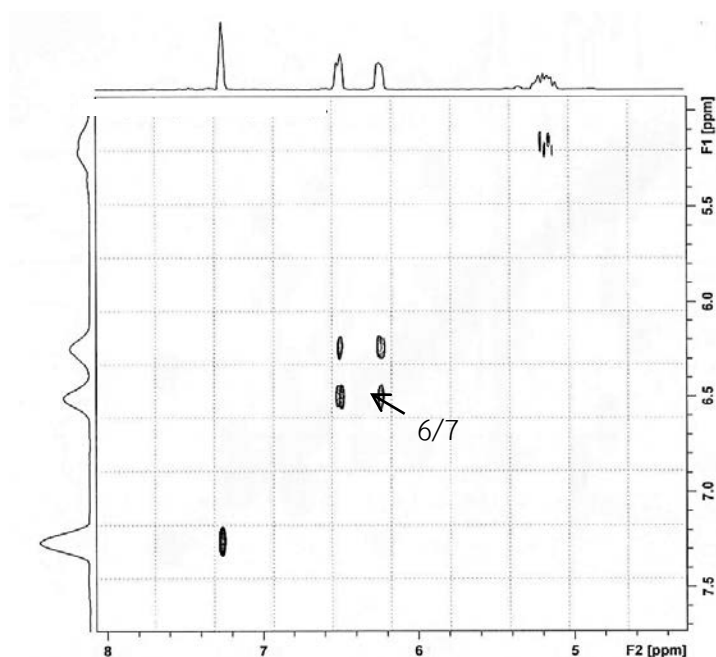


Figure 3.8 COSY spectrum of ergosterol peroxide (1)

Ergosterol

Appearance: white amorphous powder

Melting point: 155-156°C

R_f : 0.63 (Ethyl acetate:Hexane (40:60))

IR spectra (KBr): V_{max} 2955, 2870, 1659, 1458, 1381, 1369, 1069, 1040 cm^{-1} .

1H and ^{13}C -NMR spectrum: data shown in Table 3.9

Mass spectrum: ion peak m/z 396 $[M]^+$

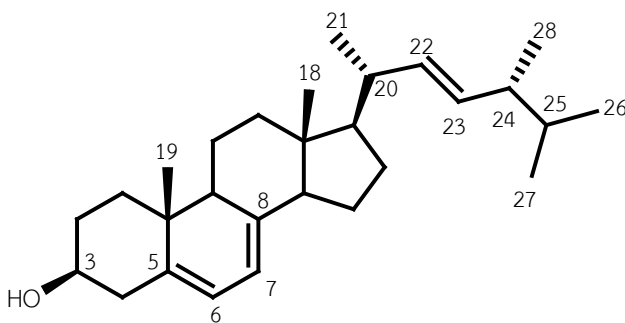


Figure 3.9 Chemical structures of ergosterol (2) isolated from the chloroform fraction

Table 3.9 ^1H -NMR and ^{13}C -NMR data of ergosterol (2) (in CDCl_3)

Position	Ergosterol (2)	
	^1H -NMR	^{13}C -NMR
1	-	38.32
2	-	31.15
3	3.63	69.11, CH
4	-	40.06
5	-	141.18
6	5.57	119.05, CH
7	5.38	117.05, CH
8	-	139.20
9	-	45.97
10	-	36.97
11	-	20.81
12	-	39.35
13	-	42.48
14	-	54.41
15	-	23.19
16	-	28.29
17	-	55.67
18	0.63	12.02, CH_3
19	0.94	15.65, CH_3
20	-	40.97
21	1.03	21.51, CH_3
22	5.24	135.71, CH
23	5.16	131.84, CH
24	-	43.23
25	-	32.85
26	0.91	17.76, CH_3
27	0.82	19.44, CH_3
28	0.83	21.51, CH_3

Linoleic acid

Appearance: brown oil

R_f : 0.37 (Ethyl acetate:Hexane (20:80))

IR spectra (KBr): $V_{\max}$ 2916, 2850, 1721, 1463, 1174, 719 cm^{-1} .

^1H and ^{13}C -NMR spectrum: data shown in Table 3.10

Mass spectrum: ion peak m/z 280 $[\text{M}]^+$

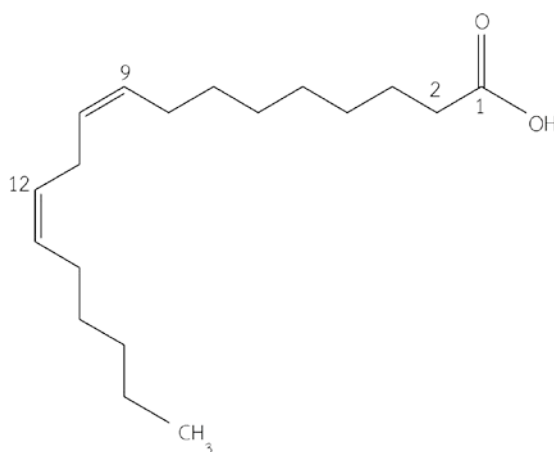


Figure 3.10 Chemical structures of linoleic acid (5) isolated from the chloroform extract

The IR spectrum showed the functional group pattern of linoleic acid. C-H stretching vibrations were observed at 2916 cm^{-1} and 2850 cm^{-1} and the peak C-H bending vibration peak was seen at 1463 cm^{-1} . There also was a strong intensity at 1710 cm^{-1} that was assigned to the C=O stretching vibration of carboxylic acid.

Table 3.10 ^1H -NMR and ^{13}C -NMR data of linoleic acid (5) (in CDCl_3)

Position	Linoleic acid (5)	
	^1H -NMR	^{13}C -NMR
1	-	179.18, C
2	2.37	33.97, CH_2
3	1.70	24.65, CH_2
4	-	29.08, CH_2
5	-	29.03, CH_2
6	-	29.30, CH_2
7	-	29.63, CH_2
8	2.0	27.16, CH_2
9	5.23	129.98, CH
10	5.57	130.19, CH
11	1.3	27.18, CH_2
12	5.38	127.90, CH
13	5.24	128.09, CH
14	-	29.55, CH_2
15	-	25.63, CH_2
16	-	31.52, CH_2
17	-	22.51, CH_2
18	0.9	13.99, CH_3

3.5.3 Antifungal activity of purified compounds using microdilution technique

The minimal inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC) of the compounds purified in this study against four *Fusarium* strains are presented in Table 3.11. The concentration of ergosterol peroxide (1) and linoleic acid (5) were in the range 0.31-5 mg/ml. The ergosterol peroxide (1) showed a strong antifungal activity. At all concentrations, this compound inhibited all four *Fusarium* spp. Both MIC and MFC were lower than 0.31 mg/ml. Linoleic acid (5) showed lower antifungal potential than ergosterol peroxide (1). The MIC and MFC of linoleic acid (5)

against *F. graminearum* 1895, *F. moniliforme* TISTR 3175 and *F. verticilliioides* 1641 were 0.62 mg/ml, while against *F. oxysporum* BCC 4977 they were 0.62 and 1.25 mg/ml.

Table 3.11 MIC values and MFC values of ergosterol peroxide (1) and linoleic acid (5) against *Fusarium* spp.

Fungal strain	Ergosterol peroxide (1)		Linoleic acid (5)	
	MIC	MFC	MIC	MFC
	(mg/ml)	(mg/ml)	(mg/ml)	(mg/ml)
<i>F. graminearum</i> 1895	<0.31	<0.31	0.62	0.62
<i>F. moniliforme</i> TISTR 3175	<0.31	<0.31	0.62	0.62
<i>F. oxysporum</i> BCC 4977	<0.31	<0.31	0.62	1.25
<i>F. verticilliioides</i> 1641	<0.31	<0.31	0.62	0.62

In the previous research, ergosterol peroxide has been reported to have antitumour, anticancer, inflammatory suppression, antioxidative, and immunosuppressive properties (Kobori *et al.*, 2007, Kang *et al.*, 2015). The use of ergosterol peroxide (1) from Shiitake mushroom against mycotoxigenic fungi has not been previously tested. To the best of our knowledge, this is the first study to report antifungal activity of ergosterol peroxide (1) from Shiitake mushroom against mycotoxigenic fungi.

Moreover, linoleic acid (5) showed inhibitory effects on fungal growth. Linoleic acid is an unsaturated fatty acid and the main compound obtained in essential oil from *Perilla frutescens* *linaria*. It has been shown to have strong antifungal activity against eleven strains of phytopathogen with MIC, in the range 0.0069-0.92 µg/ml (Juárez *et al.*, 2015). Walters *et al.* (2004) studied the effect of fatty acids on the growth of plant pathogenic fungi. Linoleic acid exhibited inhibition of the growth of *Rhizoctonia solani*, *Pythium ultimum*, and *Pyrenophora avenae* at a concentration 1,000 µM and *Crinipellis perniciosa* at a concentration 100 µM. In 2008, Liu *et al.* reported that linoleic acid (2,000 µM) inhibited mycelium growth of *F. oxysporum* f. sp. *Cucumerinum*, and *F. oxysporum* f. sp. *lycopersici* by 19% and 16%, respectively. However, while there have been many studies of the antifungal activity of fatty acids

and other compounds from plant extracts, the mechanism remains unclear. A possible mechanism is the disruption or disintegration of the fungal membrane by hydrostatic turgor pressure in cell. The fungal cell membrane can be destroyed by insertion of the antifungal fatty acid. The fatty acid spontaneously attaches to the lipid bi-layer of the fungal membrane and increases causes the membrane fluidity. The increase in fluidity in the membrane produces a disorganization of the cell membrane. This in turn causes conformation changes in the membrane proteins, the release of intracellular components, cytoplasmic disorder, and eventually cell disintegration (Bergsson *et al.*, 2001 and Avis and Bélanger 2001).

CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

In this study, the chloroform and water extracts of Shiitake mushroom were tested for their antifungal activity against nine strains of mycotoxigenic fungi using an agar diffusion technique. The chloroform extract showed a higher level of antifungal activity than the water extract. The chloroform extract (250 mg/ml) exhibited antifungal activity against almost all species of fungi, especially *Fusarium* spp. including *F. graminearum* 1895, *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, and *F. verticillioides* 1641. The percentages of fungal growth inhibition were 64%, 75%, 49%, and 52%, respectively. The MIC values of the chloroform extract for *F. graminearum* 1895, *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, and *F. verticillioides* 1641 were 1.95 mg/ml ($K_r = 2.350$ cm/day; $\mu = 0.261$ day⁻¹), 1.95 mg/ml ($K_r = 0.769$ cm/day; $\mu = 0.085$ day⁻¹), 3.9 mg/ml ($K_r = 1.408$ cm/day; $\mu = 0.156$ day⁻¹), and 1.95 mg/ml ($K_r = 1.153$ cm/day; $\mu = 0.128$ day⁻¹) which K_r and μ values of all fungi treated with the chloroform extract were lower than the control. Chloroform extract (0.98 mg/ml) was able to inhibit approximately 75% of FB₁ production from *F. verticillioides* 1641 at the end of incubation. Then, the chloroform extract was purified and three purified compounds were isolated. They are ergosterol peroxide, ergosterol and linoleic acid, respectively. Ergosterol peroxide exhibited the antifungal effects against four *Fusarium* spp. with MIC and MFC lower than 0.31 mg/ml whereas the MIC and MFC of linoleic acid were 0.62 mg/ml and 0.62-1.25 mg/ml, respectively. Moreover, ergosterol peroxide was firstly reported here for its antifungal activity. This suggests that the Shiitake extract investigated in this study may provide promising compounds for use in agricultural and food processing application.

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APPENDICES

APPENDIX A
CULTURE MEDIA AND REAGENT PREPARATION

APPENDIX A

CULTURE MEDIA AND REAGENT PREPARATION

1. Potato Dextrose Agar (PDA)

PDA powder (Difco, USA)	39 g
Distilled water	1000 mL

Procedure:

Add 39 g of PDA powder in one liter of water. Mix well and sterilize by autoclaving at 121°C, 15 pounds/inch² pressure for 15 minutes. Adjust the pH to 3.5 at 45-50°C.

2. Yeast Extract Sucrose Agar (YES)

Yeast extract	20 g
Agar	20 g
Sucrose	150 g
Distilled water	1000 mL

Procedure:

Weigh the reagents and dissolve in one liter of water. Sterilize by autoclaving at 121°C, 15 pounds/inch² pressure for 15 minutes.

3. Czapek-Dox Agar (CZA)

Sucrose	30 mL
Agar	20 mL
Solution A	50 mL
Solution B	50 g
Solution Metallic	1 g
Distilled water	900 mL

Procedure:

Weigh the sucrose and agar and add 50 ml of solution A, 50 ml of solution B, 1 ml of solution metallic, and 900 ml of water. Sterilize by autoclaving at 121°C, 15 pounds/inch² pressure for 15 minutes.

Solution A (1000 ml)

Sodium nitrite	40 g
Potassium chloride	10 g
Magnesium sulfate	10 g
Ferric sulfate	0.2 g
Distilled water	1000 mL

Solution C (1000 ml)

Dipotassium phosphate	20 g
Distilled water	1000 mL

Solution metallic (100 ml)

Ferric sulfate	1 g
Zinc sulfate	0.5 g
Distilled water	100 mL

4. Roswell Park Memorial Institute (RPMI) 1640 medium

RPMI 1640 powder	10.4 g
Distilled water	1000 mL

Procedure:

Prepare two times (2X) stock solution by weighing out 20.8 g of RPMI 1640 powder, and add 800 ml of water. Then, adjust to pH 7.0 with 0.165 mol/L of MOPS buffer and bring up the volume to one liter by adding water. Sterilize the medium immediately by filtering through a 0.22 µm syringe filter.

5. MOPS buffer (Morpholinepropanesulfonic acid)

MOPS	34.53 g
Distilled water	1000 mL

Procedure:

Prepare 0.165 mol/l of MOPS buffer by weighing out the reagents and dissolving in one liter of water.

6. Alamar blue (Resazurin)

Resazurin	0.01 g
Distilled water	100 mL

Procedure:

Prepare a two times stock solution by weighing out 0.02 g of Resazurin and adding 100 ml of water. Then, mix well and sterilize the solution immediately by filtering through a 0.22 μ m syringe filter.

7. Mung Bean Broth (MB)

Mung bean	20 g
Tap water	1000 mL

Procedure:

Add mung bean to boiling water and boil for 16-20 minutes. Then, filter the mixture using filter paper and collect the filtrate solution. Bring up the volume to one liter by adding water and sterilize by autoclaving at 121°C, 15 pounds/inch² pressure for 15 minutes.

8. Yeast malt extract (YM) broth

Yeast extract	3 g
Malt extract	3 g
Peptone	5 g
Glucose	10 g
Distilled water	1000 mL

Procedure:

Weigh the reagents and dissolve in one liter of water. Sterilize by autoclaving at 121°C, 15 pounds/inch² pressure for 15 minutes.

APPENDIX B
ANTIFUNGAL ACTIVITY OF PURIFIED COMPOUNDS
FROM SHIITAKE EXTRACT

APPENDIX B

ANTIFUNGAL ACTIVITY OF PURIFIED COMPOUNDS

FROM SHIITAKE EXTRACT

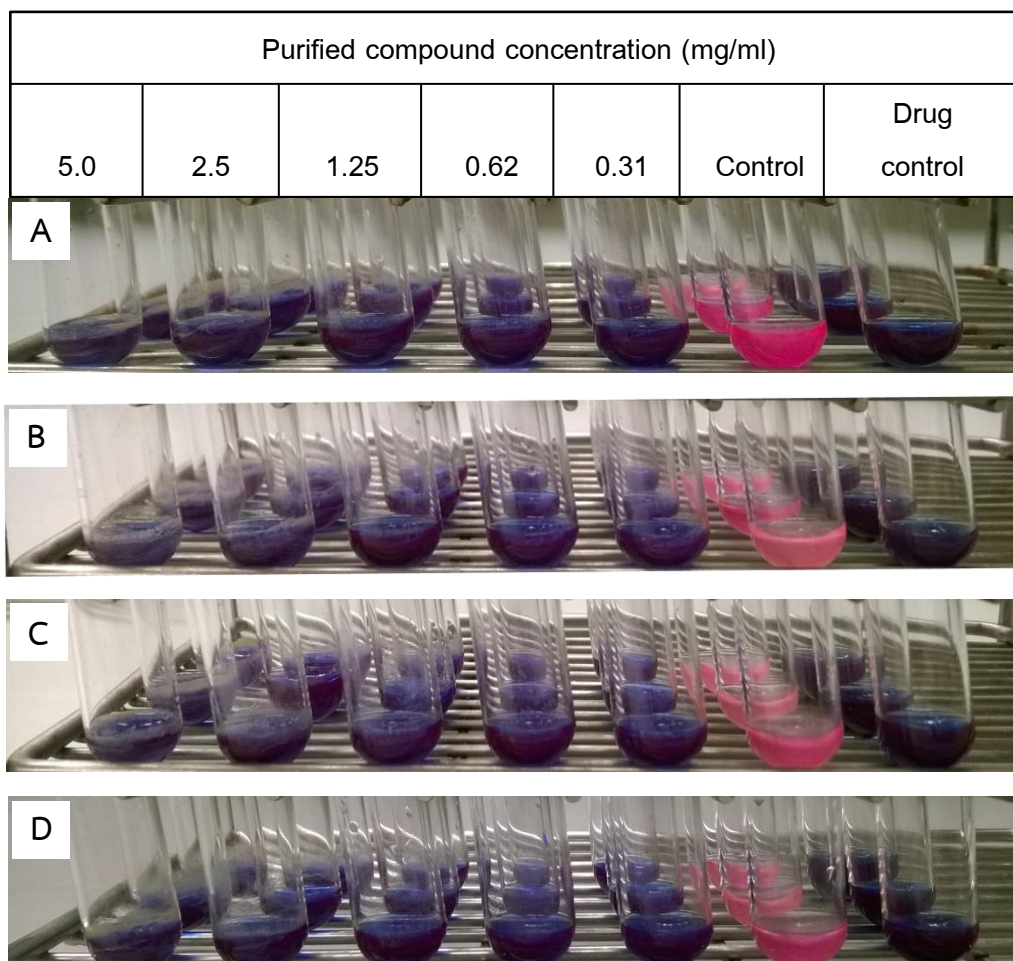


Figure B.1 Minimum inhibitory concentration (MIC) of ergosterol peroxide (1) against *F. graminearum* 1895 (A), *F. moniliforme* TISTR 3175 (B), *F. oxysporum* BCC 4977 (C), *F. verticillioides* 1641 (D)

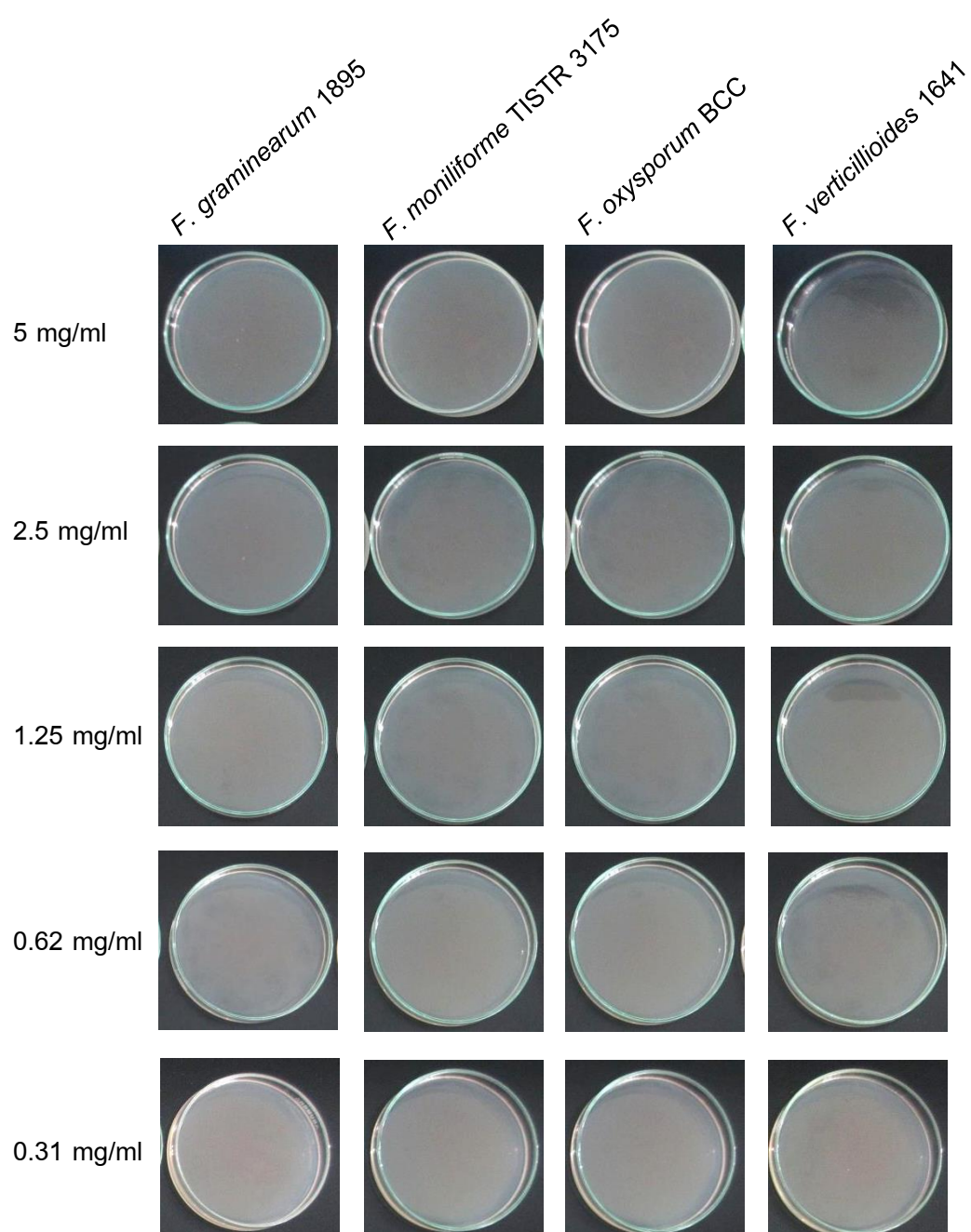


Figure B.2 Minimum fungicidal concentration (MFC) of ergosterol peroxide (1) against *F. graminearum* 1895, *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, *F. verticillioides* 1641

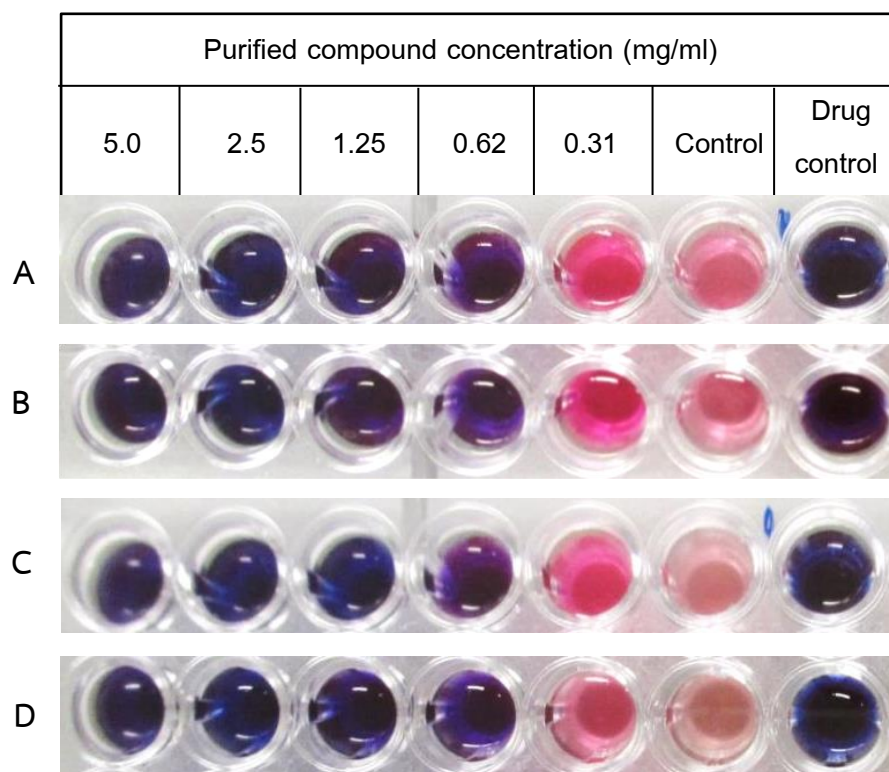


Figure B.3 Minimum inhibitory concentration (MIC) of linoleic acid (5) against *F. graminearum* 1895 (A), *F. moniliforme* TISTR 3175 (B), *F. oxysporum* BCC 4977 (C), *F. verticillioides* 1641 (D)

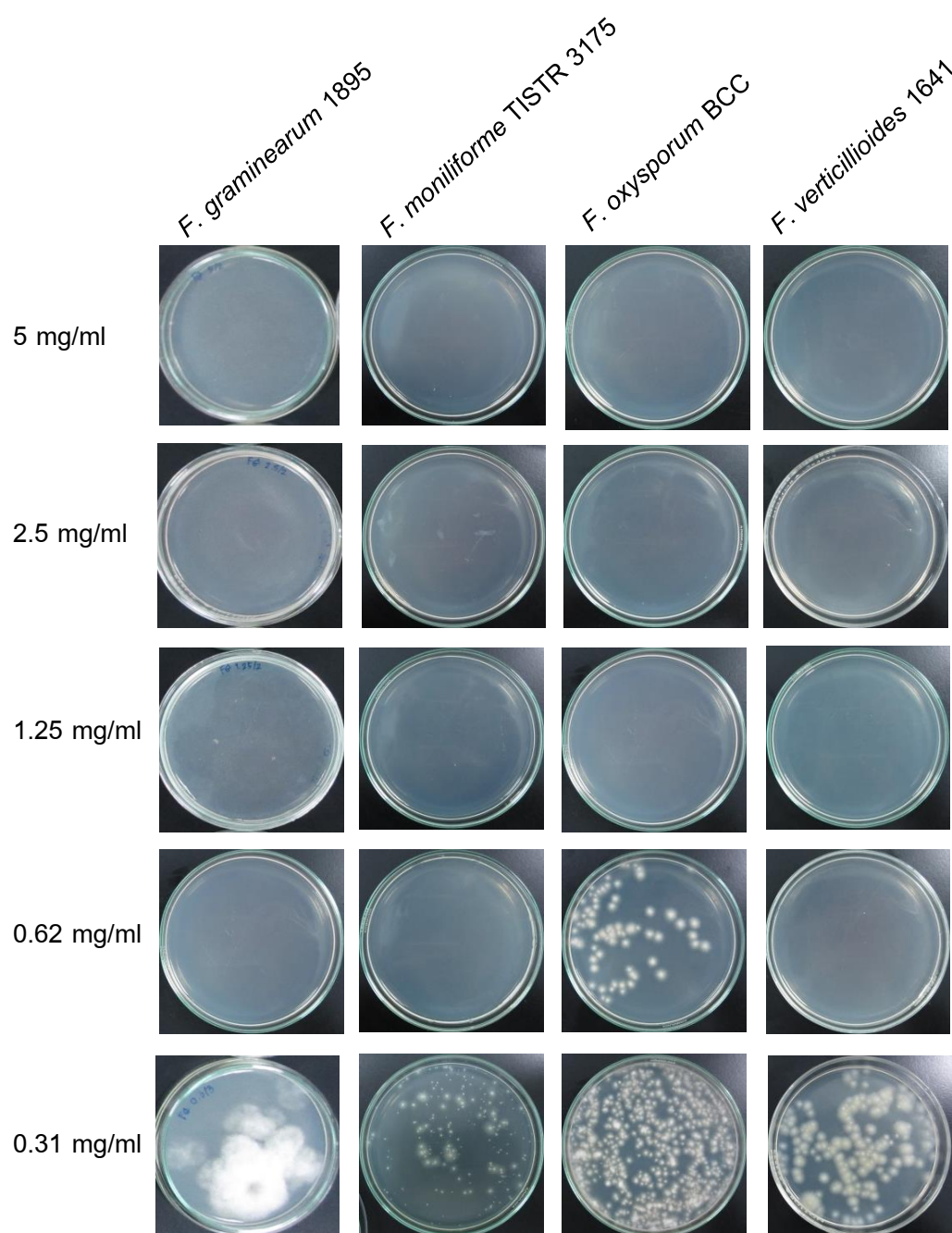


Figure B.4 Minimum fungicidal concentration (MFC) of linoleic acid (5) against *F. graminearum* 1895, *F. moniliforme* TISTR 3175, *F. oxysporum* BCC 4977, *F. verticillioides* 1641

APPENDIX C
NMR SPECTRUM OF PURIFIED ACTIVE COMPOUNDS
FROM SHIITAKE EXTRACT

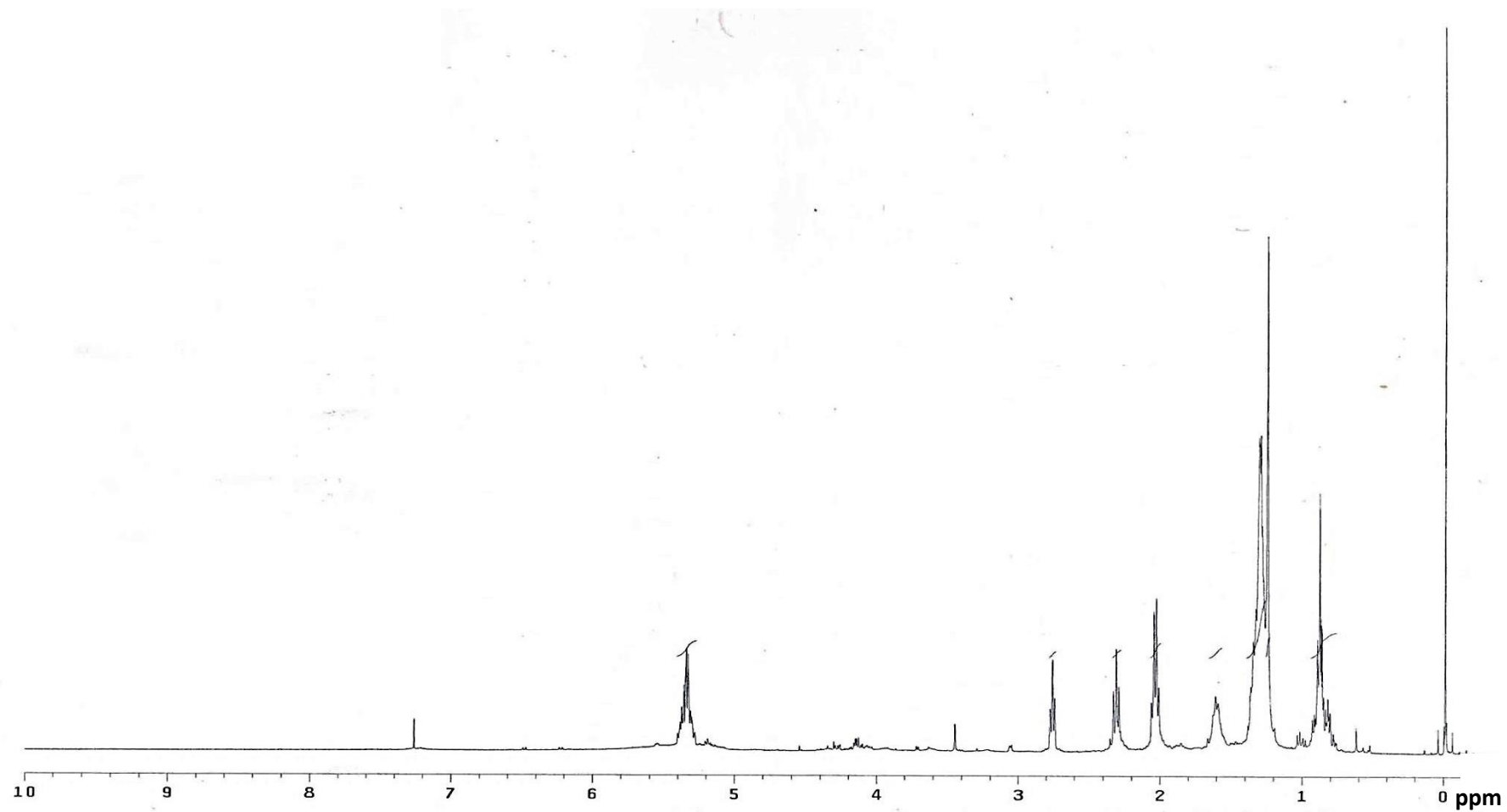


Figure C.1 ^1H -NMR spectrum of crude chloroform extract (in CDCl_3)

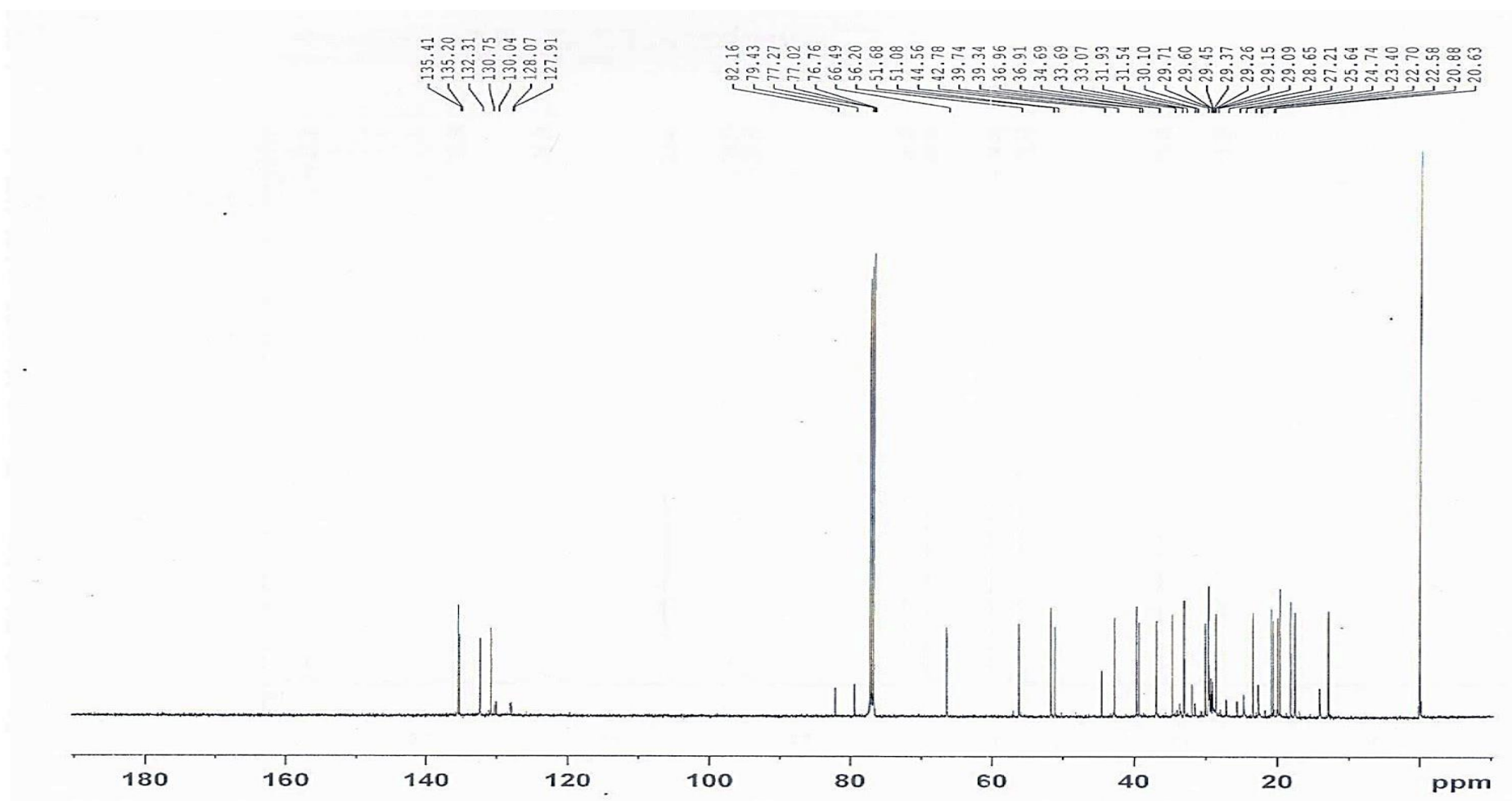


Figure C.3 ¹³C-NMR spectrum of ergosterol peroxide (1) (in CDCl₃)

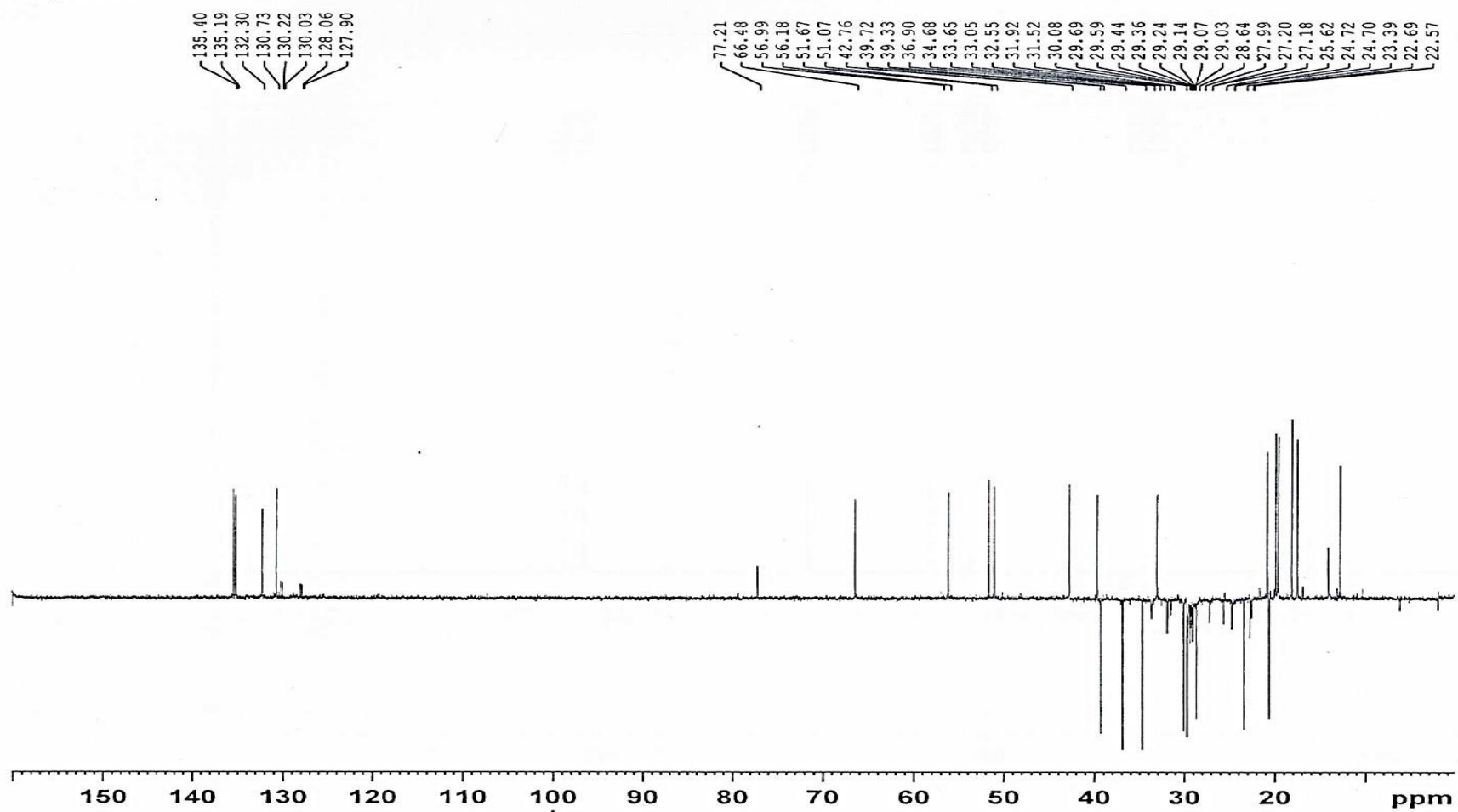


Figure C.4 Dept-135 spectrum of ergosterol peroxide (1) (in CDCl₃)

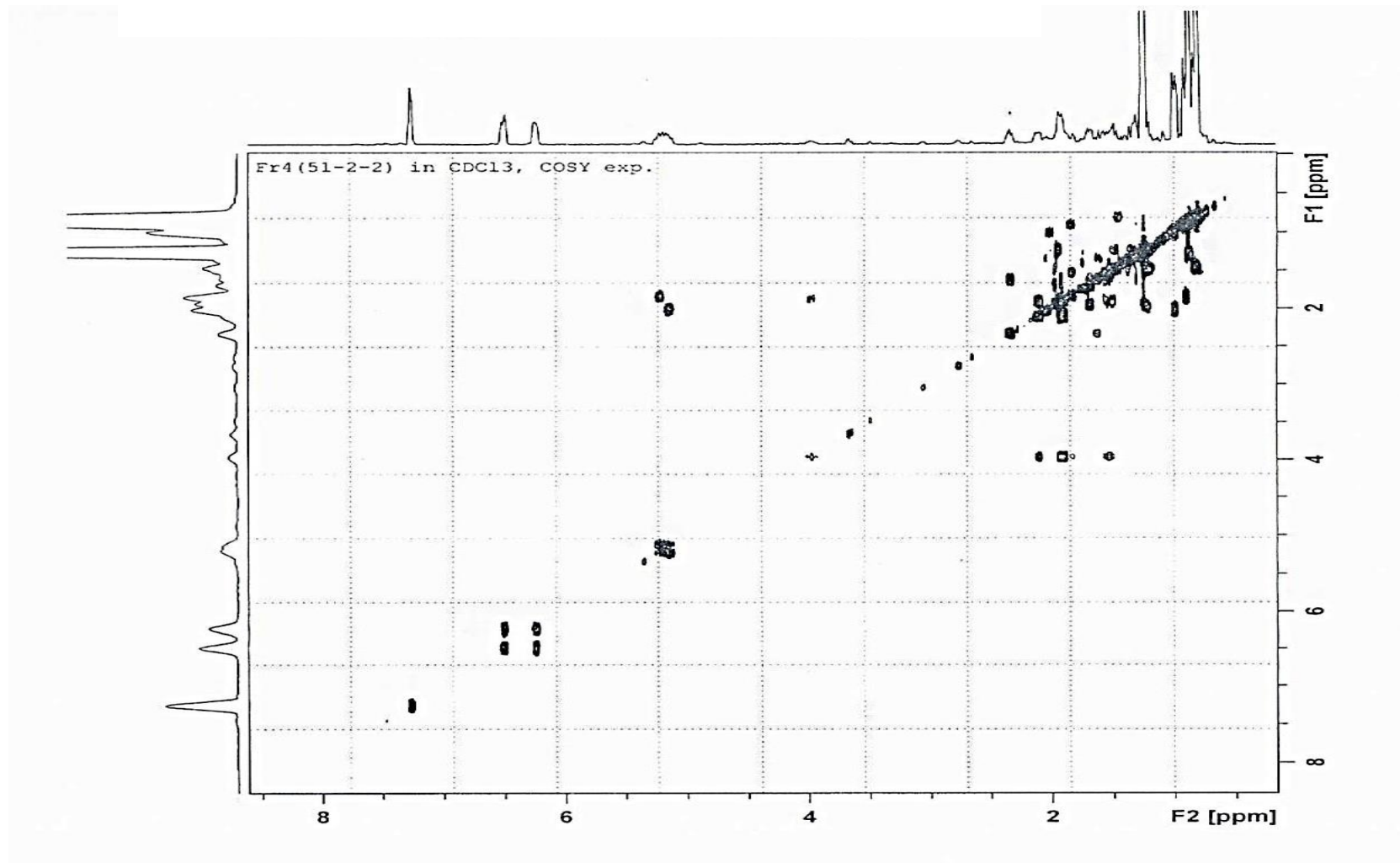


Figure C.5 COSY spectrum of ergosterol peroxide (1) (in CDCl₃)

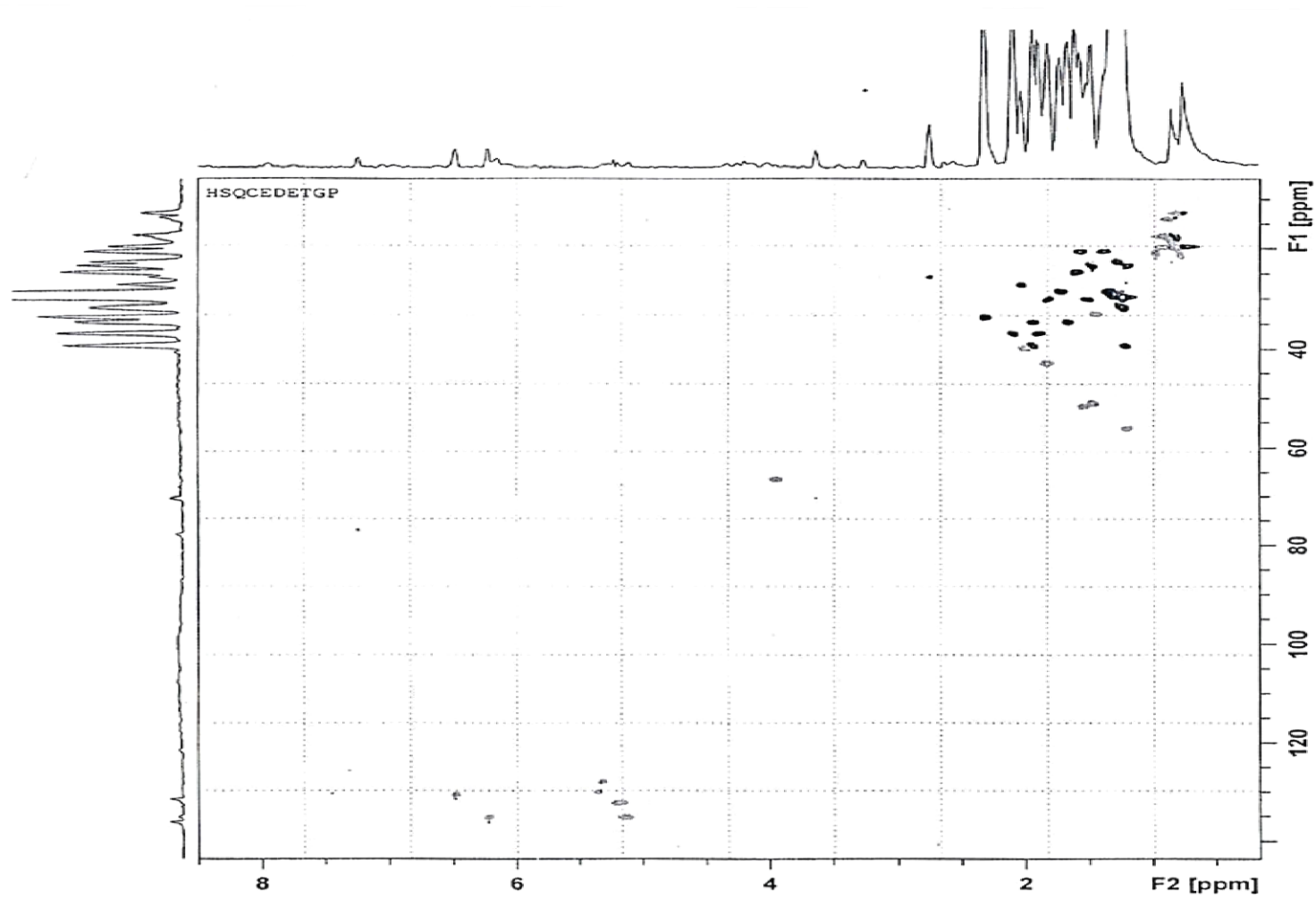


Figure C.6 HSQC spectrum of ergosterol peroxide (1) (in CDCl_3)

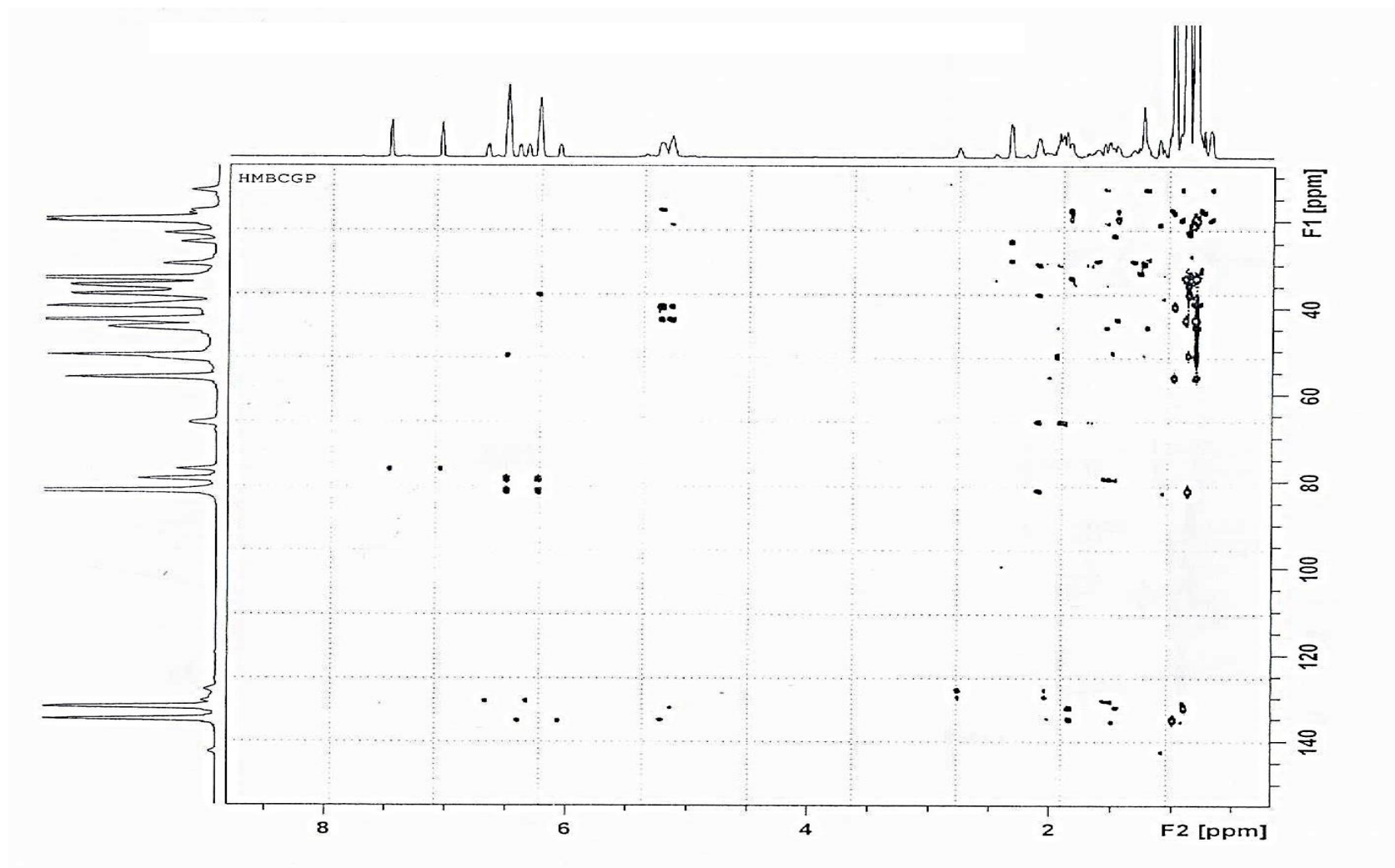


Figure C.7 HMBC spectrum of ergosterol peroxide (1) (in CDCl_3)

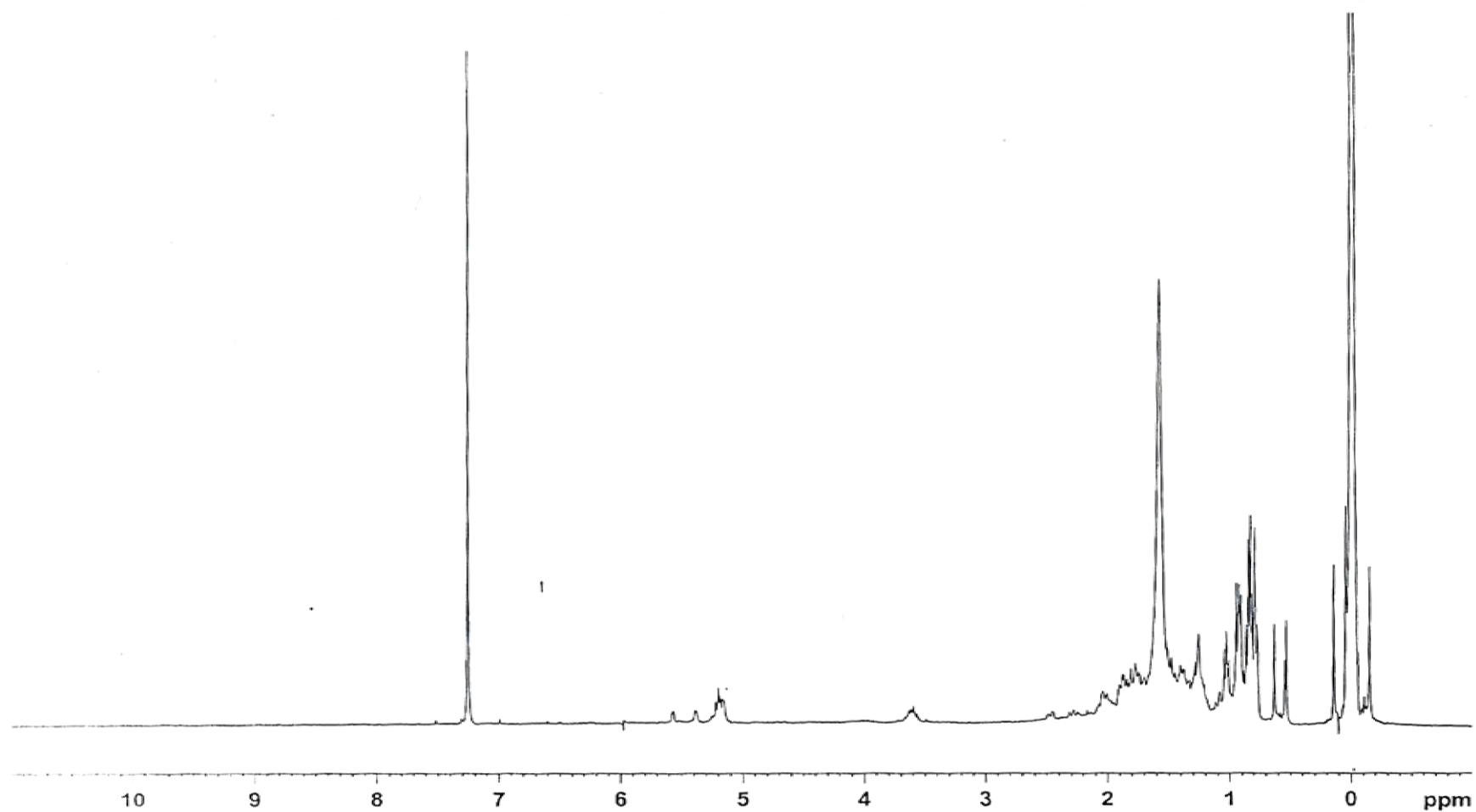


Figure C.8 ^1H -NMR spectrum of ergosterol (2) (in CDCl_3)

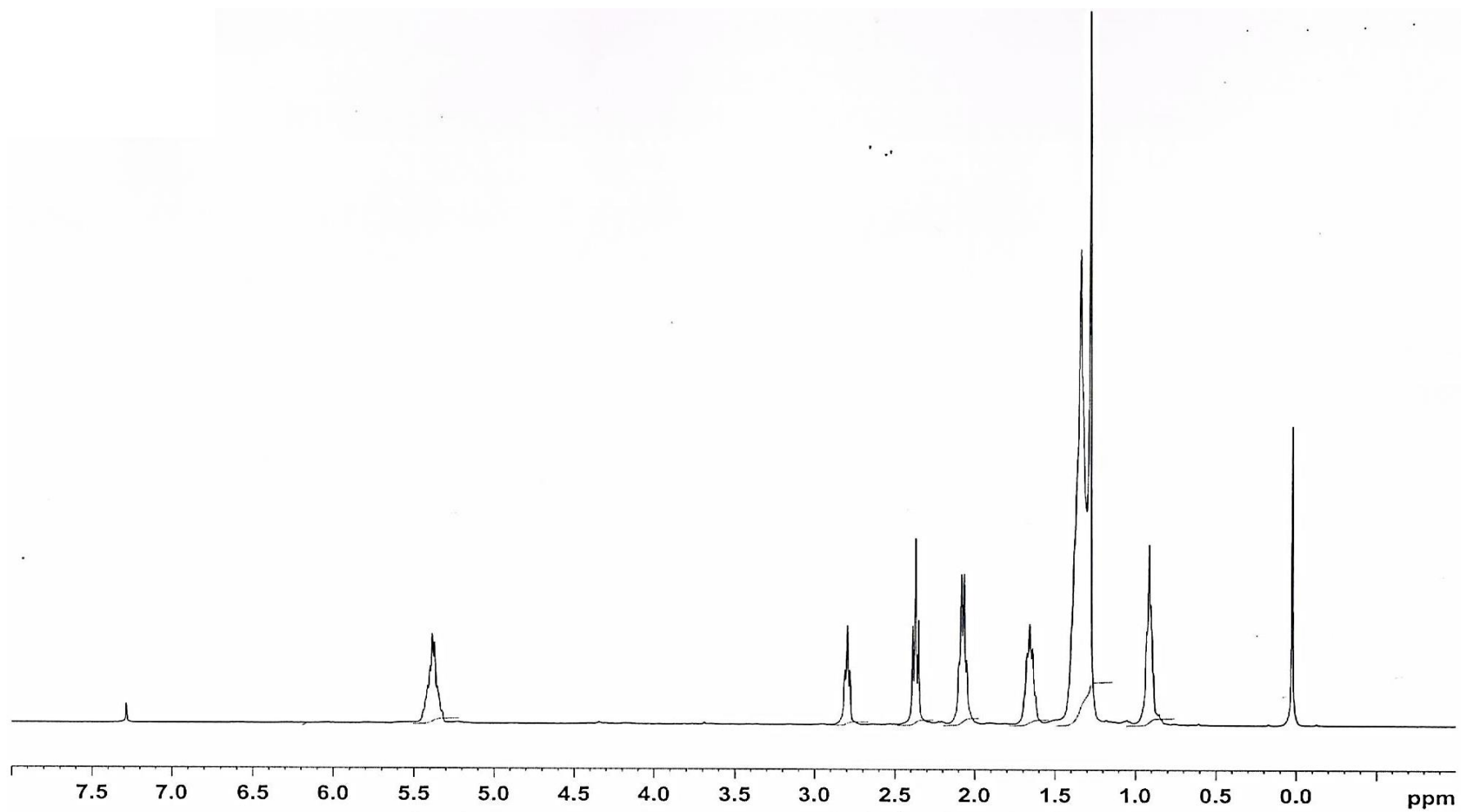


Figure C.9 ^1H -NMR spectrum of linoleic acid (5) (in CDCl_3)

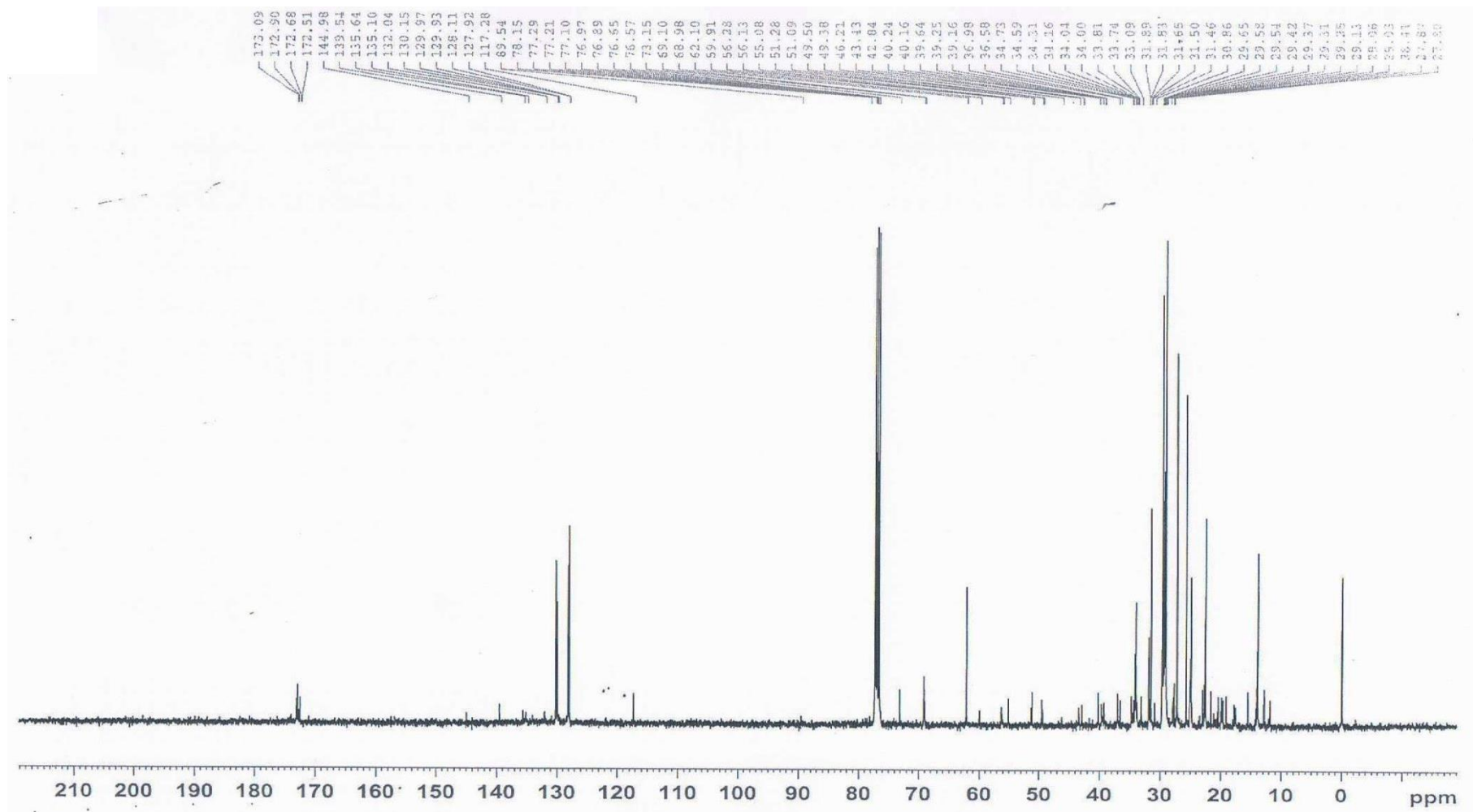


Figure C.10 ^{13}C -NMR spectrum of linoleic acid (5) (in CDCl_3)

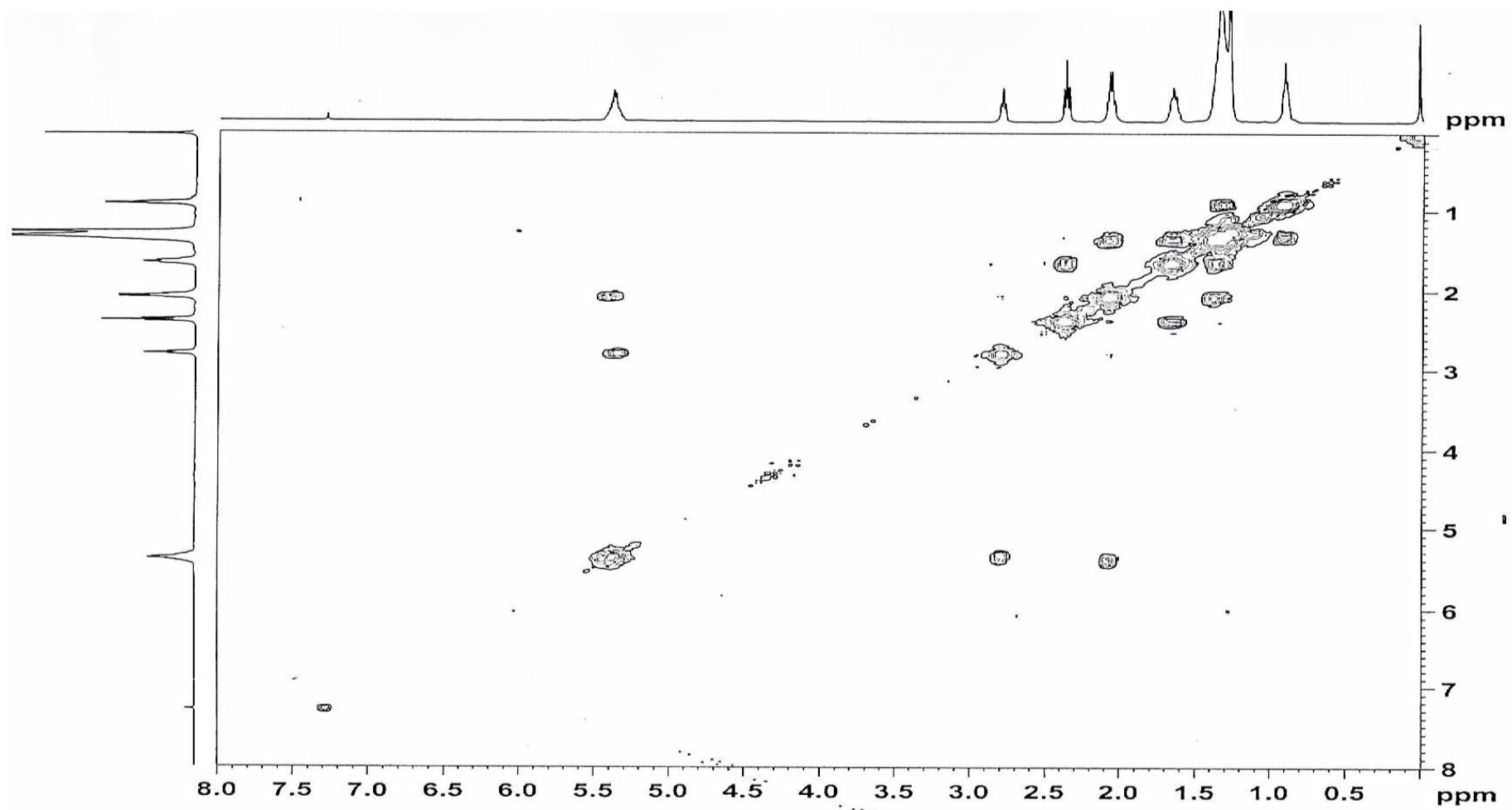


Figure C.11 COSY spectrum of linoleic acid (5) (in CDCl_3)

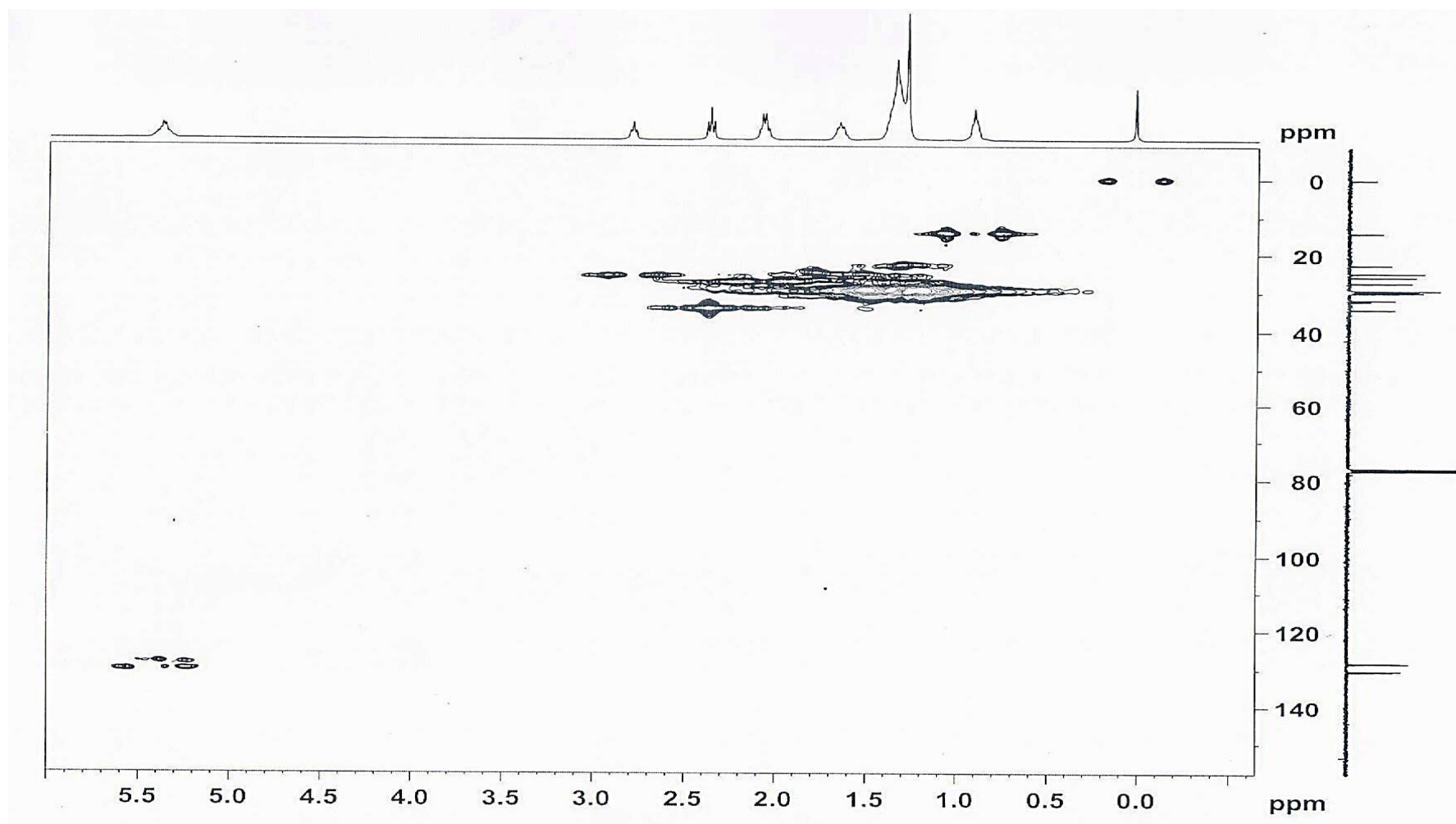


Figure C.12 HMQC spectrum of linoleic acid (5) (in CDCl₃)

APPENDIX D
TLC RESULTS OF PURIFIED ACTIVE COMPOUNDS
FROM SHIITAKE EXTRACT

Table D.1 TLC results of the purified active compounds from Shiitake extract

Compound	TLC plate	R _f	Mobile phase system
Ergosterol peroxide (1)		0.43	Ethyl acetate:Hexane (5:95)
Ergosterol (2)		0.63	Ethyl acetate:Hexane (40:60)
Linoleic acid (5)		0.37	Ethyl acetate:Hexane (20:80)

APPENDIX E
IR SPECTRUM OF PURIFIED ACTIVE COMPOUNDS
FROM SHIITAKE EXTRACT

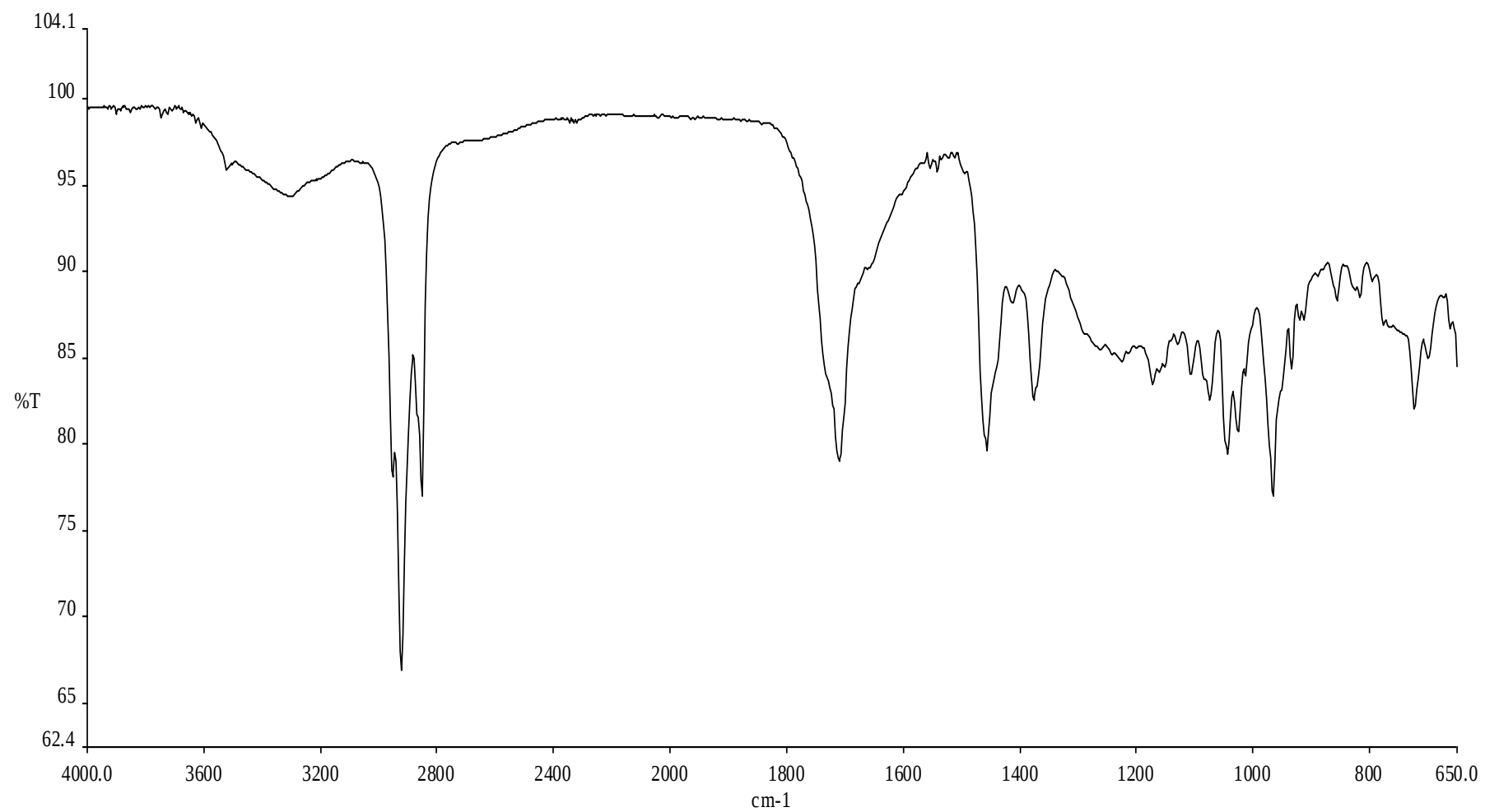


Figure E.1 IR spectrum of ergosterol peroxide (1)

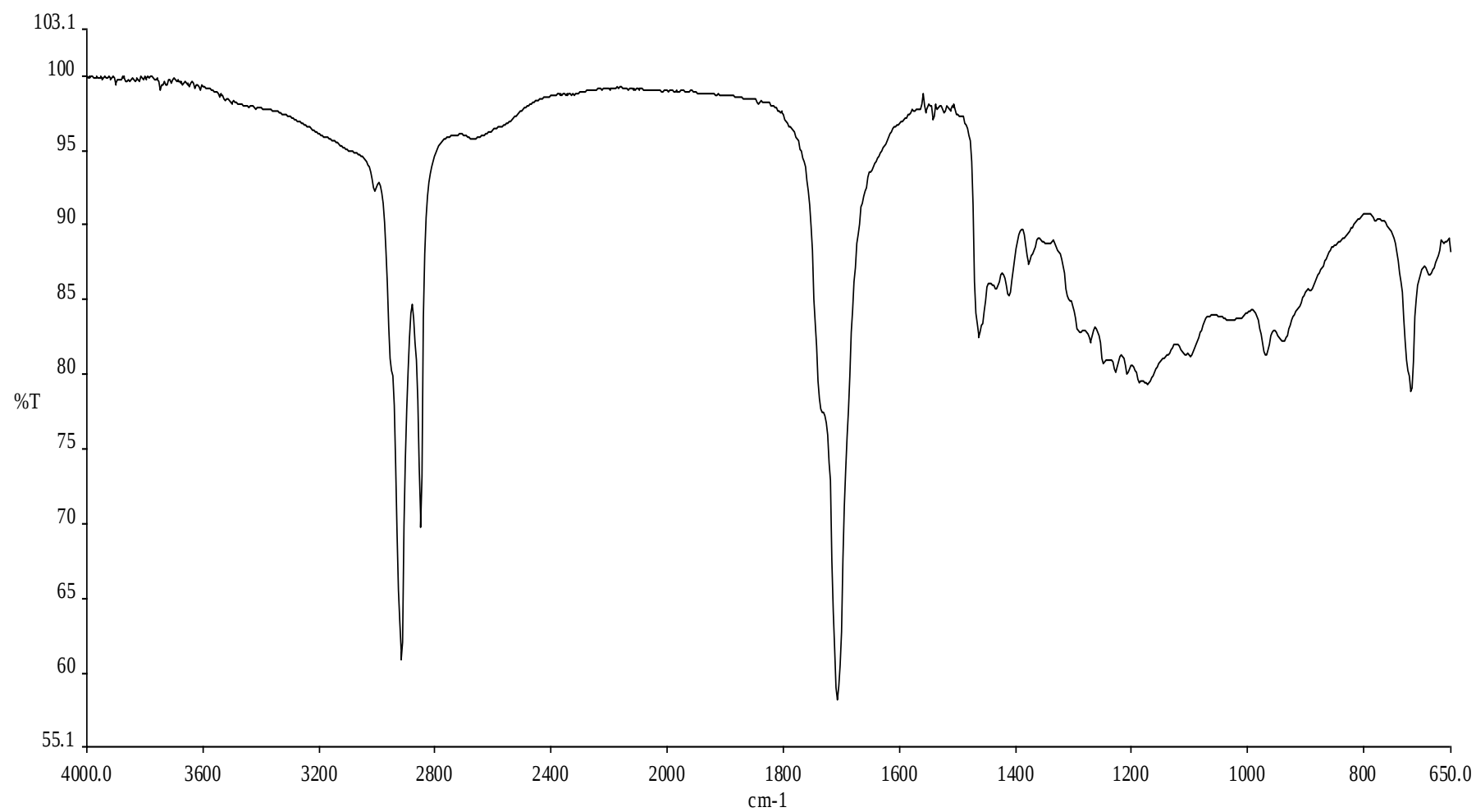
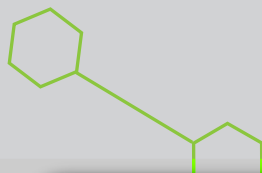
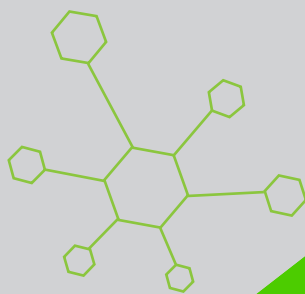


Figure E.2 IR spectrum of linoleic acid (5)



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Shiitake Extract: an Attractive Alternative Control Approach for
***Fusarium* spp.**

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Abstract

Fusarium spp. is mycotoxigenic fungi which can produce fusariotoxins (fumonisin, deoxynivalenol and zearalenone) and lead to mycotoxicoses in farm animal. Shiitake mushroom is a source of many nutrients and it also exhibits several functional properties; for example, anti-oxidant activity, anti-tumor activity, anti-viral activity and anti-microbial activity. Unfortunately, anti-fungal activity especially on mycotoxigenic fungi of Shiitake mushroom have been rarely studied. Therefore, this study was carried out to investigate their extracts upon the growth inhibition of a variety of fusariotoxin producing fungi. Briefly, dried shiitake mushroom, Donko type, were grinded with liquid nitrogen. Next, the Shiitake powder was immersed in water and, further, extracted using chloroform solvent. Afterwards, the mixture was separated to water and chloroform fraction. The water fraction was freeze dried and the chloroform fraction was evaporated using rotary evaporator until dryness. The extract was evaluated for its anti-fungal activity against four fusariotoxins-producing *Fusarium* strains, which are *Fusarium graminearum* 1895, *Fusarium verticillioides* 1641, *Fusarium oxysporum* BCC 4977 and *Fusarium moniliforme* TISTR 3175, using Minimum Inhibition Concentration (MIC) method. The results indicated that all concentration (250, 125 and 62.5 mg/ml) of Shiitake extract exhibited antifungal activity. Shiitake extract; chloroform fraction, showed the antifungal activity higher than Shiitake extract, water fraction. The water fraction of Shiitake displayed an antifungal activity against *F. graminearum* 1895, *F. oxysporum* BCC 4977 and *F. moniliforme* TISTR 3175 with MIC values of 62.5 mg/ml and against *F. verticillioides* 1641 with MIC values of 125 mg/ml whereas the chloroform fraction of Shiitake extract displayed an antifungal activity against all 4 *Fusarium* strains tested with MIC values of 62.5 mg/ml. The Shiitake extract; chloroform fraction, at the lowest concentration of 62.5 mg/ml, showed the growth inhibition of *F. graminearum* 1895, *F. verticillioides* 1641, *F. oxysporum* BCC 4977, *F. moniliforme* TISTR 3175 of with the percentage of inhibition 23.5%, 29.3%, 36.6% and 52.9%, respectively. As a result, Shiitake extract (chloroform fraction) exhibited an attractive anti-fungal activity against *Fusarium* spp. and may be used as an alternative for the control of fusariotoxins-producing *Fusarium* strains.

Keywords: *Shiitake extract, Fusarium* spp., *Anti-fungal activity*



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Introduction

Shiitake (*Lentinus edodes*) is a well known edible mushroom which is abundant with several minerals and nutrients such as protein, iron, ascorbic acid and fibre. The functional properties of Shiitake were also studied and shown on many research as antioxidant, anti-tumor, anti-viral, antimicrobial and antifungal activity (Hatvani and Mécs 2002, Ngai and Ng 2003 and Kitzberger and other 2007).

Fusarium spp. is phytopathogenic fungus that is well known for important agricultural problem across the world. Its infection, especially in cereal and maize, results in a significant disease in plant which is called Fusarium Head Blight (FHB). Infection of *Fusarium* strains not only results in reduce yield and quality of cereals but also cause mycotoxins contamination (Edwards 2004 and Jurado and other 2006). Many species of *Fusarium* produce mycotoxins, which are called fusariotoxins, by secondary metabolism; such as fumonisins (FB₁ and FB₂), trichothecenes (T-2 and HT-2 toxins, deoxynivalenol and nivalenol) and zearalenone (ZEA). However, the most frequently found fusariotoxins in crops are trichothecenes and fumonisins (Glenn 2007 and Jurado and other 2006). These mycotoxins cause mycotoxicosis, both chronic and acute diseases, in animal and human. Briefly, toxicity of FB₁ causes leukoencephalomalacia in horses (Marasas and other 1988), hepatocarcinogen in rats (Gelderblom and other 1991 and Lemmer and other 1999), pulmonary edema in pigs (Harrison and other 1990 and Smith and other 1996) and also esophageal in humans (Marasas and other 2004). Beside, toxicity of DON is acute and toxic to animals such as genotoxicity, immunotoxicity, and teratogenicity. Moreover, ZEA is mycoestrogen, because of its estrogenic-like properties in pigs, cattle and sheep (Zinedine and other 2007). Owing to their toxicity, fusariotoxins contamination is harmful to animal and human health, affecting the trade of agricultural products and exhibits impact to the economic worldwide. Consequently, reduction or inhibition of fungal growth is probable alternative to avoid these problems. Nevertheless, research about antifungal activity of Shiitake extract is quite limited. Therefore, this research focus to study the effects of Shiitake extract on fungal growth inhibition. The objective of this research was to study the effect of Shiitake (*Lentinus edodes*) extract on the growth inhibition of a variety of fusariotoxin producing fungi.



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Materials and Methods

Fungal strains

Four species of *Fusarium*, capable to produce mycotoxins were used through this experiment. For the experimental, each of fungal strains were grown using appropriate culture medium. *Fusarium moniliforme* TISTR 3175 (FM 3175) and *Fusarium verticillioides* 1641 (FV 1641) were cultured on Potato Dextrose Agar (PDA) whereas *Fusarium oxysporum* BCC 4977 (FO 4977) and *Fusarium graminearum* 1895 (FG 1895) were cultured on Czapek Dox Agar (CZA).

Media preparation

Potato Dextrose Agar (PDA) and Czapek Dox agar (CZA) were used as nutrients source for fungal growth. PDA (Difco, USA) was prepared for culture by dissolving 39 g of PDA powder in one liter of distilled water. CZA was prepared by dissolving 6 g of sucrose, 0.4 g of sodium nitrite, 0.1 g of potassium chloride, 0.1 g of magnesium sulfate, 0.002 g of ferric sulfate, 0.2 g of dipotassium phosphate, 0.002 g of zinc sulfate, 0.001 g of copper sulfate and 3 g of agar in 200 ml of distilled water, then, all culture medium were autoclaved at 121°C for 15 min.

Preparation of Shiitake extraction

Dried shiitake mushroom, Donko type, was purchased from local market in Thailand. Firstly, it was powdered using liquid nitrogen. Two hundred grams of shiitake powder were subsequently immersed in 300 ml of water and allowed to stand at room temperature for one day. Then, 800 ml of chloroform was added to wet shiitake paste and left at room temperature for 6 days. Afterwards, the mixture was filtered and the fraction of chloroform and water was separated by separatory funnel. The chloroform was removed using rotary evaporator while the water fraction was freeze dried. The desired concentrations of the Shiitake extract (water fraction and chloroform fraction) were adjusted by two-fold dilution method. Shiitake extract; water fraction and chloroform fraction were dissolved in sterile distilled water and chloroform, respectively.

Effect of Shiitake extract on fungal growth inhibition

Minimum Inhibitory Concentration (MIC) is the determination of bacterial or fungal growth inhibition ability using lowest concentration of substance (Wu, 2008). Briefly,



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approximate of 30 ml of sterilized medium was added into 90 mm Petri dishes, then, the medium was cut using cork borer (0.9 mm) to prepare the wells. Afterwards, different concentrations of Shiitake extract, which were 250, 125 and 62.5 mg/ml, were added to each wells. Then, all Petri dishes were left in the laminar cabinet until dryness in order to eliminate the fungicidal property of chloroform. Later, fungi that cultured on media for 7 days were cut using cork borer (0.4 mm) and placed on the center of Petri dishes before incubated at 25°C for 7 days. Diameter of fungal growth was measured after incubated for 7 days (Kitzberger *et al*, 2007). Further, the percentages of inhibition were calculated by the following equation.■

$$\% \text{ Growth inhibition} = \frac{(A - B)}{B} \times 100$$

Where A = Diameter of fungi without Shiitake extract (cm)

B = Diameter of fungi treated with Shiitake extract (cm)

Results and Discussions

Effect of Shiitake extract on fungal growth inhibition

An antifungal activity of Shiitake extract was investigated *via* percentage of radial growth inhibition of fungi. The Shiitake extract; water and chloroform fraction, exhibited an antifungal activity against all *Fusarium* strains tested as shown in Table 1. At the lowest concentration, the Shiitake extract; water fraction showed a lower antifungal activity, comparing to the chloroform fraction, against *F. graminearum* 1895 (12.3%), *F. oxysporum* BCC 4977 (5.0%), and *F. moniliforme* TISTR 3175 (6.2%) except *F. verticillioides* 1641. The water fraction of Shiitake extract, at the concentration of 62.5 mg/ml, did not inhibit the growth of *F. verticillioides* 1641. The Shiitake extract; chloroform fraction, at the lowest concentration of 62.5 mg/ml, showed a potent inhibitory effect on the growth of *F. graminearum* 1895 (23.5%), *F. oxysporum* BCC 4977 (36.6%), *F. moniliforme* TISTR 3175 (52.9%) and *F. verticillioides* 1641 (29.3%).



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Table 1 Percentage of radial growth inhibition and Minimum Inhibitory Concentration of Shiitake extracts; water fraction and chloroform fraction.

Fungal strains	Percentage of radial growth inhibition (%)							
	Shiitake extract concentration (mg/ml)							
	Water fraction				CHCl ₃ fraction			
	250	125	62.5	0.0	250	125	62.5	0.0
<i>F. graminearum</i> 1895	16.97	16.54	12.33	0.00	67.50	49.86	23.47	0.00
<i>F. oxysporum</i> BCC 4977	20.42	9.72	5.00	0.00	53.82	48.47	36.64	0.00
<i>F. moniliforme</i> TISTR 3175	25.25	14.73	6.17	0.00	75.40	68.45	52.94	0.00
<i>F. verticillioides</i> 1641	10.69	4.66	0.00	0.00	46.70	36.79	29.25	0.00

Both fractions of Shiitake extract were investigated for the antifungal activity using Minimum Inhibitory Concentration (MIC) method. The result of MIC values was presented in Table 1. Shiitake extract; water fraction, displayed an antifungal activity against *F. graminearum* 1895, *F. oxysporum* BCC 4977 and *F. moniliforme* TISTR 3175 with MIC values of 62.5 mg/ml and against *F. verticillioides* 1641 with MIC values of 125 mg/ml whereas the chloroform fraction of Shiitake extract displayed an antifungal activity against all 4 *Fusarium* strains tested with MIC values of 62.5 mg/ml. Comparing to water fraction of Shiitake extract, the chloroform fraction of Shiitake extract was found to be more effective against *F. graminearum* 1895, *F. oxysporum* BCC 4977, *F. moniliforme* TISTR 3175 and *F. verticillioides* 1641.

Because of the *Fusarium* contamination problem in crops, many researchers try to find the alternative ways to eliminate the contamination or inhibit the growth of *Fusarium* mould, and bioactive compounds are one of the methods using for fungal growth control. In this study, dried Shiitake (*Lentinus edodes*), Donko type, was extracted by water and chloroform. Subsequently, both fractions of Shiitake extracts were examined for antifungal activity on the growth of *Fusarium* spp. Regarding to the result, chloroform fraction of Shiitake extract exhibited the highest ability to inhibit the growth of *Fusarium* fungi used in this study. This inhibition mechanism may be due to some bioactive compounds presenting in



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such chloroform fraction of Shiitake extract. There are many studies reported about the bioactive compounds of Shiitake mushroom. Ngai and Ng (2003) reported that Lentinan, a β -1,3-D-glucan polysaccharides which was found as a component of cell wall of mushroom, was able to bind carbohydrates and proteins that have effects on biological and physiological of cell and inhibit growth of fungal. Moreover, the components of the Shiitake extracts were depending on extraction method. Kitzberger and other (2007) reported that the components of Shiitake mushroom demonstrated antioxidant and antimicrobial properties. Shiitake mushroom were extracted using different extraction method according to organic solvents and supercritical fluids. The supercritical fluid extracts had effect on *Micrococcus luteus* and *Bacillus cereus*. Also, the extracts from Shiitake mushroom using supercritical fluid extraction with ethanol as co-solvent had shown the antimicrobial activity. Furthermore, the less polar compounds from Shiitake mushroom exhibited the high level of antibacterial properties and the activity was arisen from disulphides, lenthionine compounds in the organic extracts (Rao and other 2009).

Conclusions

Shiitake (*Lentinus edodes*) is a well-known edible mushroom that was used in herbals remedies since ancient time. Antifungal activity of Shiitake extract; water and chloroform fraction, was investigated on *Fusarium* mould; *F. graminearum* 1895, *F. oxysporum* BCC 4977 and *F. moniliforme* TISTR 3175 using MIC value and percentage of radial growth inhibition. Shiitake extract; chloroform fraction, exhibited antifungal activity higher than the water fraction of Shiitake extract. In this study, the lowest concentration of Shiitake extract; chloroform fraction, could inhibit the growth of *F. graminearum* 1895, *F. verticilioides* 1641, *F. oxysporum* BCC 4977, *F. moniliforme* TISTR 3175. The percentages of radial growth inhibition were 23.5%, 29.3%, 36.6% and 52.9%, respectively, and their MIC value was 62.5 mg/ml. Therefore, the new antifungal agent using to control mycotoxigenic fungi was found in this study. However, the active compound in this chloroform fraction should be identified in further study.



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