Phytochemistry, Antioxidant, Anti-inflammation and Anticancer Activities Against Cholangiocarcinoma Cell Lines of Some Species of *Phellinus* Mushroom Extracts

Sonesay Thammavong

A Thesis Submitted in Partial Fulfillment of Requirements for

degree of Doctor of Philosophy in Pharmacy

November 2021

พฤกษเคมี ฤทธิ์ต้านออกซิเดชัน ฤทธิ์ต้านการอักเสบ และ ต้านเซลล์มะเร็งท่อน้ำดีของสารสกัดเห็ดพิมานบางชนิด

วิทยานิพนธ์

ของ

Sonesay Thammavong

เสนอต่อมหาวิทยาลัยมหาสารคาม เพื่อเป็นส่วนหนึ่งของการศึกษาตามหลักสูตร

ปริญญาปรัชญาดุษฎีบัณฑิต สาขาวิชาเภสัชศาสตร์

พฤศจิกายน 2564

Phytochemistry, Antioxidant, Anti-inflammation and Anticancer Activities Against Cholangiocarcinoma Cell Lines of Some Species of *Phellinus* Mushroom Extracts

Sonesay Thammavong

A Thesis Submitted in Partial Fulfillment of Requirements

for Doctor of Philosophy (Pharmacy)

November 2021

The examining committee has unanimously approved this Thesis, submitted by Mr. Sonesay Thammavong , as a partial fulfillment of the requirements for the Doctor of Philosophy Pharmacy at Mahasarakham University

Examining Committee

.... Chairman
(Asst. Prof. Natsajee Nualkaew , Ph.D.)

.... Advisor
(Asst. Prof. Methin Phadungkit , Ph.D.)

.... Co-advisor
(Asst. Prof. Pornpun Laovachirasuwan , Ph.D.)

.... Co-advisor
(Assoc. Prof. Khwanyuruan Naksuwankul , Ph.D.)

.... Committee
(Asst. Prof. Prasoborn Rinthong , Ph.D.)

.... Committee
(Asst. Prof. Bunlue Sungthong , Dr.rer.nat.)

.... Committee
(Asst. Prof. Sakulrat Rattanakiat , Ph.D.)

Mahasarakham University has granted approval to accept this Thesis as a partial fulfillment of the requirements for the Doctor of Philosophy Pharmacy

....
(Asst. Prof. Chanuttha Ploylearmsang , Ph.D.)
Dean of The Faculty of Pharmacy

....
(Assoc. Prof. Krit Chaimoon , Ph.D.)
Dean of Graduate School

TITLE	Phytochemistry, Antioxidant, Anti-inflammation and Anticancer Activities Against Cholangiocarcinoma Cell Lines of Some Species of *Phellinus* Mushroom Extracts		
AUTHOR	Sonesay Thammavong		
ADVISORS	Assistant Professor Methin Phadungkit , Ph.D. Assistant Professor Pornpun Laovachirasuwan , Ph.D. Associate Professor Khwanyuruan Naksuwankul , Ph.D.		
DEGREE	Doctor of Philosophy	**MAJOR**	Pharmacy
UNIVERSITY	Mahasarakham University	**YEAR**	2021

ABSTRACT

Phellinus mushrooms belong to the family Hymenochaetaceae. In Traditional Chinese Medicine, it has been used as an ingredient for the treatment of different types of cancer, ischemia and skin diseases for thousands years. The present study was aimed to evaluate and compare the biological activities of the *Phellinus* mushroom extracts (PE). The methods of this study comprised of different methods including phytochemistry, antioxidant, anti-inflammatory and anticancer against cholangiocarcinoma cells (CCA). The extracts of *Phellinus* mushrooms including *P. igniarius*, *P. linteus*, *P. nigricans* and *P. rimosus* were prepared in two ways: maceration in 95 % ethanol and decoction in distilled water. The antioxidant activity of the sample extracts was evaluated by using the DPPH, ABTS and FRAPS assays. Total phenolic and flavonoid contents were determined by using the colorimetric test. The Nitric oxide assay was used to determine the anti-inflammatory activity. In addition, anticancer against CCA cell lines (KKU-100 & KKUM-213A) were evaluated by using the SRB assay. The results of this study showed that all ethanol extracts of samples demonstrated significantly stronger antioxidant activity when compared to the aqueous extracts ($p < 0.05$). The results also showed that the ethanol extracts contained higher amount of total phenolic and flavonoid contents. The extract of *P. rimosus* presented the highest antioxidant activity, total phenolic and flavonoid contents when compared to *P. igniarius*, *P. linteus* and *P. nigricans* (DPPH: IC_{50}= 9.56 ± 0.47 µg/mL, ABTS: IC_{50} =5.04 ± 0.06 µg/mL, TPC=361.04 ± 5.69 mg GAE/g, TFC=646.55 ± 6.29 mg RE/g). All samples possessed high anti-inflammatory and anticancer activities against CCA cell lines, particularly in the ethanol extracts of *P. rimosus* and *P. linteus*. The anticancer activity was correlated to the phytochemical contents, antioxidant and anti-inflammatory activities of each *Phellinus* mushroom. The water-soluble fraction of the hexane extract and the dichloromethane fraction of the ethanol extract from *P. rimosus* were selected to further investigated. Three chemical compounds were isolated from *P. rimosus* extract including 3-dimethyl-2-hydroxy-2-en-propanoic acid, 4,5-dimethoxy-2,3,5-trihydoxy-2,4 di-en pentanoic acid and 6-deoxydestigloyswietenine acetate. All of the active compounds showed high anti-inflammatory activity with the IC_{50} values of 9.87 ± 0.24, 10.55 ± 1.09 and 8.69 ± 0.08 μg/mL, respectively.

In conclusion, the results of the present study showed that *Phellinus* mushrooms had great potential for further investigation to discover a new anticancer and anti-inflammatory agent from natural products especially from *P. rimosus* extract.

Keyword : Antioxidant, Anti cholangiocarcinoma, Phellinus mushrooms

ACKNOWLEDGEMENTS

The success of this thesis would not be realized without the generosity and assistance of some persons and various institutions to whom I would like to express my deepest gratitude.

I greatly appreciate Asst. Prof. Dr. Methin Phadugnkit, my thesis advisor, for the opportunity to work on this subject and for his comprehensive and stimulating discussions on this thesis and on my other manuscripts, valuable advice, patience, and encouragement throughout this study

I am grateful to Asst. Prof. Dr. Pronpun Laovachirasuwan, my thesis co-advisor, for the kindness, valuable suggestions, and support in ways too numerous to mention.

I am grateful to Assoc. Prof. Kwanruan Naksuwankul, my thesis co-advisor, of the Department of Biology, Faculty of Sciences, Mahasarakham University for her kindness, valuable suggestions, support mushroom and support in ways too numerous to mention.

I would like to acknowledge all the members of my thesis committee for their constructive suggestions and critical review of this thesis.

I especially appreciate Dr. Warapron Saentaweesuk for her kind assistance in dealing with the anticancer activity test and discussion on anticancer activities of the mushroom extract.

I also acknowledge Asst. Prof. Dr. Atit Silsirivanit for his useful discussion and support cells line for this study.

I would like to thank the Fondation Pierre Fabre for the financial support.

I would like to thank the spectroscopic instrument operators of the Scientific Equipment Center, Prince of Songkla University for their helpful assistance in the spectroscopic experiments. Scientific Technological Research Equipment Center Chulalongkorn University and Scientific Equipment Center, Faculty of Science, Khon Kane University

I also thank the Faculty of Pharmacy; Mahasarakham University, Thailand for the place experiment of the mushroom extracts and isolated the pure compounds.

It is a pleasure to thank the students and all staff members of the Faculty of Pharmacy; Mahasarakham University and Faculty of Pharmaceutical Sciences, University of Health Science, Lao PDR for their friendship, kind support, and encouragement throughout the course of my work.

Finally, the most special thanks are due to my beloved family for their encouragement and moral support.

Sonesay Thammavong

TABLE OF CONTENTS

Page

ABSTRACT....D
ACKNOWLEDGEMENTS....F
TABLE OF CONTENTS....H
LIST OF TABLES.... M
LIST OF FIGURES ....N
Chapter I Introduction....1
1. Introduction and Significance....1
1.1 Research Objectives 4
1.2 Research Hypothesis 4
1.3 Scope and Limitation of the Study 4
1.4 Research of Variables....5
1.5 Expected Results 6
1.6 Definition....6
Chapter II Literature Review 8
1. *Phellinus* Mushrooms 8
1.1 Botanical Description 8
1.2 Description of the Study *Phellinus* Mushrooms....8
1.2.1 *Phellinus igniarius* (L.) Quél....8
1.2.2 *Phellinus linteus* (Berk. & M.A. Curtis) Teng 9
1.2.3 *Phellinus nigricans* (Fr.) P. Karst....9
1.2.4 *Phellinus rimosus* (Berk.) Pilát 9
1.3 Chemical Constituents of *Phellinus* Mushrooms 10
1.3.1 Primary Metabolites 10
1.3.2 Secondary Metabolites 11
1.4 Biological Activities of the *Phellinus* Mushrooms 23

1.4.1 Antitumor and Immunomodulation Activity....................................23
1.4.2 Anticancer Activity 24
1.4.3 Anti-inflammatory Activity...25
1.4.4 Antioxidant Activity..26
2. Cholangiocarcinoma (CCA) 26
2.1 Classification of CCA 26
2.2 Causes of CCA 27
2.3 Multiple Risk Factors Revealed a CCA 27
2.4 Chlolangiocarcinoma Cells and Treatment Approaches 28
3. Screening Methods for Development of Anticancer Drugs 30
3.1 Sulforhodamine B (SRB) Cell Cytotoxicity Assay 31
3.2 MTT Assay..32
3.3 Apoptosis Assay 32
4. Free Radicals and Antioxidants 32
4.1 Free Radicals 32
4.2 Oxidative Stress...32
4.3 Antioxidant Activity...33
4.4 Phenolic Compounds..33
4.5 The Correlation between Free Radical and Cancer 35
4.6 Assays for Anti oxidative Capability 36
4.6.1 The DPPH method...36
4.6.2 The ABTS Method 37
4.6.3 The FRAP Method 38
5. Inflammation..39
5.2 Anti-inflammatory Assay..42
5.2.1 Nitric Oxide Assay 42
5.2.2 Cytokine enzyme-linked Immunosorbent Assay (ELISA) 43
5.2.3 COX-1 and COX-2 catalyzed Prostaglandin Biosynthesis Assay ..43
6. Phytochemistry 43

6.1 Extraction 43
6.2 Methods for Drying of Fluid Extracts 46
6.3 Isolation 47
6.4 Identification 49
7. Related Articles 51
7.1 Anticancer Activity 51
7.2 Antioxidant Activity..53
7.3 Anti-inflammatory Activity...53
7.4 Antibacterial Activity 54
1. Contents 56
2. Chemicals and Equipments...56
2.1 Mushrooms and Cell Lines..56
2.2 Chemical Reagents 56
2.3 Types of Equipment 57
3. Phytochemical Study 58
3.1 Extraction of the Mushrooms 58
3.2 Phytochemical Screening 59
3.3 Separation of the Mushroom Extracts 60
3.5 TLC Fingerprint 61
3.6 Isolation of the Pure Compounds 61
3.7 Spectroscopy 62
3.8 Physical Properties 63
3.9 Solvents 63
4. Extraction and Isolation of Compounds from *Phellinus rimosus*......................63
4.1Extraction and Isolation of Compounds from Hexane fraction of *Phellinus rimosus* 63
4.2 Extraction and Isolation of Compounds from Dichloromethane fraction of *Phellinus rimosus* 64
5. Physical and Spectral Data of Isolated Compounds 64
5.1 Compound HPR1 64

5.2 Compound HPR2 65
5.3 Compound DPR 65
6. Evaluation of Biological Activities 65
6.1 Screening of Anticancer, Antioxidant, Anti-inflammatory Activities, ... 65
Total Phenolic and Flavonoid Content Determination 65
6.2. DPPH Scavenging Assay 65
6.3 ABTS Assay 66
6.4 FRAP Assay 67
6.6 Total Flavonoid Content 69
7. Anti-inflammatory Activity 70
7.1 Nitric Oxide Inhibitory Assay 70
7.2 MTT Assay 70
8. Anticancer Activity 71
8.1 Cell lines 71
8.2 Sulforhodamine B (SRB) Assay 73
9. Statistical Analysis 75
Chapter IV Results 76
1. The yield of Mushroom Extracts using Various Solvents 76
2. Preliminary Qualitative Phytochemical Analysis 77
3. TLC fingerprint 78
4. Antioxidant Activity, Total phenolic and Flavonoid content of *Phellinus* Mushroom Extracts 80
5. Anti-inflammatory Activity 83
5.1 Inhibition of nitric oxide production by the *Phellinus* mushrooms extracts 83
5.2 Cell Viability Assessments 85
6. Anticancer Activity 87
6.1 Cholangiocarcinoma Cell Growths Inhibitory Activity of the Ethanolic and Aqueous Extracts of *Phellinus* Mushrooms 87
7. Structure Identification of the Isolated Compounds 90
7.1 Structure Determination of Compound HPR1 90

7.2 Structure Determination of Compound HPR2 93
7.3 Structure Determination of Compound DPR 95
Chapter V Discussion 103
1. Antioxidant, Total Phenolic and Flavonoid Contents.. 103
2. Anti-inflammatory Activity 104
3. Anti-cancer Activity 105
4. Effect of Solvents and Time periods.. 108
Chapter VI Conclusion and Recommendation 110
1. Conclusion 110
2. Recommendation 112
REFERENCES 113
Appendix... 130
BIOGRAPHY 144

LIST OF TABLES

Page

Table 1 Chemical Structure and Part of Mushrooms. 12

Table 2 Anticancer Activity of Pure Compounds Isolated from *Phellinus* Mushrooms. 24

Table 3 Summarizes the Worldwide Epidemiology and Incidence Trends of CCA. . 28

Table 4 Summary of Methods for the Determination of Antioxidant Activities. 38

Table 5 Actions of the Principal mediators of the Inflammatory Response. 42

Table 6 A Brief Summaries of Various Extraction Methods for Natural Products ... 45

Table 7 Concentration of Solution and Volume of Ferrous sulfate. 68

Table 8 Percentage (w/w) yields of mushroom extracts separated by various solvents. 76

Table 9 Preliminary phytochemical screening of ethanolic and aqueous extract of *Phellinus* mushrooms. 78

Table 10 Antioxidant activities, total phenolics and flavonoids contents of *Phellinus* mushrooms extracts. 82

Table 11 Extracts of *Phellinus* species and pure compounds from *P. rimosus* inhibit nitric oxide production in LPS-activated macrophages RAW 264.7. 84

Table 12 Cytotoxicity, safety profile and percentage cell viability of the extracts of the selected *Phellinus* mushrooms on RAW264.7 cells in vitro. 85

Table 13 Effects of the mushroom extracts on the growth of cholangiocarcinoma cell line. 88

Table 14 Effects of the fraction extracts separated from ethanol extract of *P. rimosus* on the growth of cholangiocarcinoma cell lines 89

Table 15 NMR Spectral data of DPR as Compared with 6-O-acetylswietenin acetate. 96

Table 16 Two Dimensional NMR Spectral data of DPR Compound. 97

LIST OF FIGURES

Page

Figure 1 Fruiting body of *Phellinus* Mushrooms..10

Figure 2 Bismuth/Corlette Classification..27

Figure 3 Methods for Investigation on Anticancer Activity..31

Figure 4 Structure of Some Phenolic Compounds Isolated from *Phellinus* Mushrooms 34

Figure 5 Most Active Trihydroxylflavones in Cancer Cell Viability and the Free Radical Scavenging Assay 36

Figure 6 DPPH Free radical Conversion to DPPH by Antioxidant Compound 37

Figure 7 ABTS Chemical Reaction 37

Figure 8 Antioxidant Reaction with Ferric Salt, Fe (III)$(TPTZ)_2Cl_3$ (TPTZ) 2,4,6-Tripyridyls-Triazine..38

Figure 9 Cells and Mediators of the Inflammatory response Molecules derived from Plasma Proteins and Cells in response to Tissue Damage or Pathogens mediate Inflammation by Stimulating Vascular changes Plus Leukocyte Migration and Activation...41

Figure 10 A Soxhlet Extraction Apparatus..45

Figure 11 Extraction of Mushroom by the Decoction Method....................................58

Figure 12 Extraction of Mushroom by the Maceration Method..................................59

Figure 13 Separations of Ethanol Extract from *Phellinus* mushroom 60

Figure 14 Extracts of Ethanol and water from *Phellinus* Mushrooms.........................77

Figure 15 Thin-layer Chromatogram of Ethanol Extract, Hexane, Dichloromethane, Ethyl acetate and Butanol Fractions 79

Figure 16 Thin-layer Chromatogram of Ethanol Extracts from *P. igniarius*, *P. linteus*, *P. nigricans* and *P. rimosus* 80

Figure 17 The Thin-layer Chromatogram of Hexane fraction from *Phellinus rimosus* 90

Figure 18 ^{1}HNMR (400MHz) Spectrum of Compound HPR1 (CDCl3).....................91

Figure 19 ^{13}CNMR (100MHz) Spectrum of Compound HPR1 (CDCl3) 91

Figure 20 FT-IR Spectrum of Compound HPR1 (KBr technique)..............................92
Figure 21 UV-Visible Spectrum of Compound HPR1 ($CDCl_3$)..................................92
Figure 22 HREIMS Mass Spectrum of Compound HPR1 (MeOH)............................92
Figure 23 ^{1}HNMR (400MHz) Spectrum of Compound HPR2 (CDCl3).....................93
Figure 24 ^{13}C NMR (100MHz) Spectrum of Compound HPR2 (CDCl3) 94
Figure 25 FT-IR Spectrum of Compound HPR2 (KBr technique)..............................94
Figure 26 UV-Visible Spectrum of Compound HPR2 ($CDCl_3$)..................................94
Figure 27 HREIMS Mass Spectrum of Compound HPR2 ($CDCl_3$)............................95
Figure 28 Thin-layer chromatogram of dichloromethane fraction from *Phellinus rimosus*..98
Figure 29 ^{1}HNMR (400MHz) Spectrum of Compound DPR (CDCl3).......................99
Figure 30 ^{13}C NMR (400MHz) Spectrum of Compound DPR (CDCl3) 99
Figure 31 FT-IR Spectrum of Compound DPR (KBr technique)................................99
Figure 32 UV-Visible Spectrum of Compound DPR (CDCl3) 100
Figure 33 HREIMS Mass Spectrum of Compound DPR ($CDCl_3$)...........................100
Figure 34 DEPT135 Spectrum of Compound DPR ($CDCl_3$)....................................100
Figure 35 COSY Spectrum of Compound DPR ($CDCl_3$)..101
Figure 36 NEOSY Spectrum of Compound DPR ($CDCl_3$) 101
Figure 37 HSQC Spectrum of compound DPR (CDCl3) 102
Figure 38 HMBC Spectrum of Compound DPR ($CDCl_3$)...102

Chapter I

Introduction

1. Introduction and Significance

Cancer is a major public health problem worldwide and is the second leading cause of death in the United States.[1] Cancer is a leading cause of death worldwide an approximately 60 % of the cancer burden in Thailand, due to five types of cancers such as breast, cervix, colorectal, liver and lung cancers.[2] World Health Organization (WHO)[3, 4] have been reported approximately 23.296 patients per year with Hepatocellular carcinoma (HCC). The cancer is the most common cancer in men with an estimated 16.299 patients per year. In Lao PDR previous study reported that the mortality rate from HCC in male and female is 50.4 % and female 29 % respectively.[3] Recently, HCC and cholangiocarcinoma (CCA) are the common primary liver cancer found in Thailand and Lao PDR. CCA is an important public health problem in several parts of the world including Thailand and Lao PDR. Anatomical classification of CCA was classified into three different types of CCA including intrahepatic cholangiocarcinoma (iCCA), perihilar cholangiocarcinoma (pCCA) and distal cholangiocarcinoma (dCCA).[5,6] The prevalence of CCA in Southeast Asia is much higher than in other areas of the world. The incidence of CCA is relatively low in Korea and Japan whereas the incidence rate of CCA in Thailand is extremely higher. The factor that associated with CCA is *Opisthorchis viverrini* and *Clonorchis sinensis* infection.[7,8,9,10] The cause of increasing in CCA is unknown and is not explained by improvements in diagnosis.[11] The major challenge for CCA control and treatment was the lack of early diagnosis. Nevertheless, the development of multidrug resistance is one of the major challenges to the success of treatment in CCA patients.[12,13] In addition, chemotherapeutics for CCA is largely ineffective and clinical efficacy of the standard treatment with 5-fluorouracil (5-FU) is low. Furthermore, the resistance of this type of cancer to chemotherapy and radiotherapy is a major issue.[14] Even though the clinical response rate is low and the recurrence rate is extremely high. Surgical therapy combined with 5-FU is the alternative treatment for CCA in the present.[11,15,16] The current anti-cancer drugs accessible in the market are not target-specific and pose several side-effects. Complications in the clinical management of various forms of cancer, which highlights the crucial need for novel effective and less toxic therapeutic approaches.[17] The promising therapeutic options in many types of cancers including CCA is the use of combination therapies of standard treatments and in conjugation with alternative therapy with dietary phytochemicals.[18] Discovery and development of effective alternative chemotherapeutics for CCA are therefore the first priority need in focusing.

Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids, and DNA in healthy human cells.[19] Cells are exposed to oxidative stress and thus oxidation free radicals may be important in carcinogenesis at multiple tumor sites.

Inflammation can accelerate the development of cancer. Chronic inflammation is a risk factor for epithelia1 carcinogenesis. Tumor necrosis factor (TNF)-α is an important pro-inflammatory cytokine that is secreted from various cells and exerts many cellular effects. TNF-α has been associated with multiple illness states in humans, including immune and inflammatory diseases, cancer, psychiatric disorders, among others.[20] Prostaglandins generated during the inflammation appear to be important in the pathogenesis of cancer due to their effect on mitogenesis, cellular adhesion, immune surveillance, and apoptosis. Increased production of prostaglandins from arachidonic acid in transformed cells is associated with the up-regulation of COX-2. The enzymes cyclooxygenase-1 and -2 (COX-1 and -2) are the key enzymes involve in recruiting inflammation. Pro-inflammatory cytokines play a crucial role in the initiation and progression of various cancers. Besides the key role of COX in the initiation and progression of inflammation, overexpression of COX has been considered as one of the culprits in the formation of the carcinogenic state in the body.[21] It is this molecular attribute of the COX up-regulation that has made it an attractive target for the design and development of anti-cancer agents also. Nitric oxide (NO) is a signaling molecule that plays a key role in the pathogenesis of inflammation. It gives an anti-inflammatory effect under normal physiological conditions. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to overproduction in abnormal situations. The NO is a potent neurotransmitter at the neuron synapses and contributes to the regulation of apoptosis. NO is involved in the pathogenesis of inflammatory disorders of the joints, gut and lungs. Therefore, NO inhibitors represent an important therapeutic advance in the management of inflammatory diseases.[22] Free radical-induced oxidative stress and its relevance with inflammation and carcinogenesis are well established. The inflammation, free radicals and carcinogenesis are closely related to one another. The drug candidates having anti-inflammatory, free radical scavenging activities are more appreciate as anticancer agents due to lack of effective drugs, cost of chemotherapeutic agents and side effects of drugs. Therefore, efforts are still being made to search for effective natural occurring anti-carcinogens that would prevent, slow or reverse cancer development.[23]

The natural product has played an important role in Western medicine from ancient to modern times. It has an estimated 50 % of the prescription products in Europe and the USA originated from natural products.[24,25] Currently, there is an interested in mushroom which increasing scientific interest in bioactive natural products as chemical leads to a compound for the generation of semi-synthetic derivatives.[26,27] Secondary metabolites produced by mushroom constitute is an important source of novel bioactive substances.[28,29] Mushrooms and herbal remedies offer a valuable alternative treatment in developing countries where traditional therapies are considered cheap and readily available.[30] However, the usage of mushroom and herbal medicine for primary health care needs by people in local communities due to the limited availability and high prices of most pharmaceutical products.[31] In Thailand, mushrooms and medicinal plants are increasingly used by traditional healers for the treatment of various ailments.[32]

The number of mushrooms species on earth was estimated 140,000, which reported that only 10 % are already known. Assuming that the proportion of useful mushrooms among the undiscovered and unexamined mushrooms will be only 5 %, this implies 7,000 yet undiscovered species of possible benefit to mankind. The higher Basidiomycetes include about 10,000 species from 80 families. Furthermore, approximately 700 species of higher Basidiomycetes have been found to possess significant pharmacological activities. The mushroom has been divided into three groups: edible, medicinal and economic purpose, where the properties are less well defined.[29]

Usa Klinhom et al have been reported the number of mushrooms founded in North-Eastern of Thailand. The study showed that mushrooms were 1.147 species.[32] At least 647 edible mushroom species are known to be collected for food and medicine. *Phellinus* is a genus of fungi in the family Hymenochaetaceae. *Phellinus* mushrooms are decay heartwood, causes root and cankers of live standing trees destroy slash and other woody residues. It is parasitic, perthophytic or saprobic causing white root that degrades both lignin and cellulose.[33] *Phellinus* mushrooms are an important fungus, distributed in around the global. It also has about 310 species and 91 plants family's shows infection of *Phellinus* spp. More than 31 species of *Phellinus* mushrooms exist in Thailand and only 2 species have been reported for utilization in medication. The *Phellinus* mushroom is contained important natural substances such as phenolic, flavonoid and terpenoid compounds.[34]

Several studies have distributed with antioxidant activity to find new sources of natural antioxidants to be used in foods, cosmetics, medicine and other purposes. Antioxidant plays an important role in health care to prevent and scavenge free radicals; alleviate chronic diseases and degenerative ailment such as cancer, diabetic and delay the aging process.[35] The experimental evidence suggested that foods containing antioxidant nutrients may be important in disease prevention possibly caused by free radicals.[36,37,38] Moreover, there has some species mushrooms recorded previously described as immunomodulatory,[39,40,41] antitumor,[13,42,43,44,45] anti-cancer,[46,47,48] antigenic and anti-inflammatory.[49,50,51] Beneficial secondary metabolites found in mushrooms include phenolic compounds, sterols, and flavonoids. Thus, the determination of new and safety antioxidants from a natural source has become very important for food and medicinal functions.[35]

The *Phellinus* mushrooms with authenticated anti-cancer properties of their active compounds are of immense interested in the development of anticancer drugs. Many studies and some clinical trials describe the effects of the *Phellinus* mushroom extracts in cancer therapy.[52,53] Their potential uses individually and as ingredients to cancer therapy have occurred. *Phellinus* mushrooms were known to complete chemotherapy and radiation therapy by countering the side-effects of cancer, for example, nausea, bone marrow suppression, and anemia.[54] The *Phellinus* mushrooms are a popular traditional medicine in Thailand for longevity, immune stimulant, and treatment of all types of cancer.[32]

The bioactive compounds isolated from *Phellinus* mushroom include polysaccharides, proteins, fats, glycosides, alkaloids, volatile oils, tocopherols, phenolics, flavonoids, terpenoids, carotenoids, ascorbic acid, enzymes, and organic acids.[55,34] The active components in *Phellinus* mushrooms responsible for conferring anti-cancer potential are hispilin, hispolon, protroglycan, lectin, polysaccharide A and B (HPA and HPB), phenolic compounds, tocopherols, flavonoids, and ascorbic acid.[42,51] In our previous work, the fruiting body extracts of *Phellinus* mushroom exhibited bioactive component and biological activity. Until now, no reports have demonstrated the anti-CCA activity of *Phellinus* mushrooms especially from *P.*

igniarius P. linteus, *P. nigricans*, and *P. rimosus*. Till present time have only few reports about pure compounds isolated from *Phellinus* mushrooms.

Considering the significant importance of cancer chemotherapeutic agents from natural products in chemotherapy, the development of new effective agents with fewer side effects is a compelling urgency. In the present study intensive investigation on the phytochemistry, antioxidant, anti-inflammatory and anticancer activity against CCA cell lines of *Phellinus* mushrooms extracts. The results of this study could be used to confirm the rational basis for the uses of this *Phellinus* mushrooms in traditional medicine against CCA, develop for treatment and prevention of CCA. As mention above, *P. igniarius P. linteus*, *P. nigricans*, and *P. rimosus* were selected for further investigation of their chemical constituents and biological activities. The purposes of this research are as follow:

1.1 Research Objectives

-To screen anticancer activity against cholangiocarcinoma cell lines of *Phellinus* mushroom extracts.

-To isolate and identify pure compound from *Phellinus* mushroom extract was selected

-To evaluate the antioxidant activity using the DPPH, the ABTS and the FRAP assay of *Phellinus* mushroom extracts.

-To evaluate the anti-inflammatory activity using the Nitric oxide inhibitory assay of *Phellinus* mushroom extracts.

-To determine total phenolic compound content using the Folin-Ciocalteu reagent assay of *Phellinus* mushroom extracts.

- To determine total flavonoid compound content using the aluminum chloride colorimetric assay of *Phellinus* mushroom extracts.

1.2 Research Hypothesis

-Anticancer activity of *Phellinus* mushroom extracts will show difference values

-The active compounds isolated from the *Phellinus* extract will be phenolic compounds or triterpene or limonoid

-Antioxidant activity determined by using the DPPH assay, the ABTS and FRAP assays of difference *Phellinus* mushroom extracts will show different values

-Anti-inflammatory activity of *Phellinus* mushroom extracts will show different values.

-Total phenolic contents of difference *Phellinus* mushroom extracts will show different values.

1.3 Scope and Limitation of the Study

-*Phellinus* mushrooms were provided by Natural Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University (MSUT) including *P. igniarius P. linteus, P. nigrican,* and *P. rimosus.*

-*Phellinus* mushrooms were extracted in 95 % ethanol and water.

-Screening cytotoxicity effects of the extracts were analyzed on cholangiocarcinoma cell lines (KKU-100 and KKU-M213A).

-The extracts were investigated for total phenolic and flavonoid content, antioxidant and anti-inflammatory activities.

-Isolation and identification of the isolated compounds were performed.

1.4 Research of Variables

1.4.1 Determination of Anti-cancer Activity Against CCA of *Phellinus* Mushrooms by using the Sulforhodamine B (SRB) assay

-Independent variables

Independent variable composed of 2 types such as crude extracts of mushrooms and control (DMSO).

-Dependent variables

The dependent variable was the half-maximal inhibitory concentration (IC_{50}) value or percentage inhibition of anti-cancer activity against CCA cell lines.

1.4.2 Determination of Antioxidant Activity by Using the DPPH-Scavenging, the ABTS and the FRAP Assay

-Independent variables

Independent variables consist of 2 types such as crude extracts of *Phellinus* mushrooms, standard compounds (ascorbic acid and ferrous sulfate) and method for extracts (95 % ethanol and water).

-Dependent variables

The dependent variable was the half-maximal inhibitory concentration (IC_{50}) antioxidant activity of *Phellinus* mushrooms and standard compounds.

1.4.3 Determination of Anti-inflammatory Activity

-Independent variables

Independent variables consist of 2 types such as crude extracts of *Phellinus* mushrooms, control (DMSO) and method for extracts (95 % ethanol and water).

-Dependent variables

The dependent variable was the half-maximal inhibitory concentration (IC_{50}) anti-inflammatory activity of *Phellinus* mushrooms and control.

1.4.4 Determination of Total Phenolic Compounds of *Phellinus* Mushrooms by Using the Folin-Ciocalteu Reagent Assay

-Independent variables

Independent variable consists of 2 types such as crude extracts of *Phellinus* mushrooms and method for extracts (95 % ethanol and water).

-Dependent variables

The dependent variable was total phenolic contents from a determination by using the Folin-Ciocalteu reagent assay and calculate is mg GAE/g dry mass unit.

1.4.5 Determination of Total Flavonoid Compounds of *Phellinus* Mushrooms by Using the Aluminum Chloride Colorimetric Assay

-Independent variables

Independent variable consists of 2 types such as crude extracts of *Phellinus* mushrooms and method for extracts (95 % ethanol and water).

-Dependent variables

The dependent variable was total flavonoid contents from a determination by using the Aluminum chloride colorimetric assay and calculate is mg GAE/g dry mass unit.

1.5 Expected Results

The anticipated outcomes of this study were:

-The determined phytochemicals, antioxidant, anti-inflammatory and anticancer activity against CCA of *Phellinus* mushroom extracts can be used as basis pharmacological data for consideration of their therapeutic potential use in the future.

-The data can be used to develop CCA cell therapy.

-Comprehending the anti-proliferation of CCA cell lines induced by mushroom extracts the data of anticancer activities can be used in cancer prevention and therapy.

-Enhancing local economics in growing high potential *Phellinus* mushroom.

1.6 Definition

-*Phellinus* mushroom species was meaning fungi contain in family Hymenochaetaceae, this study is considering four mushrooms include *P. nigrican*s, *P. lineteus, P.igniaarius* and *P. rimosus*, deposit at Natural Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University (MSUT) (Thailand).

-Total phenolic compounds were an antioxidant activity which received from the extra body of a human. The structure phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, that compounds are dissolved in water good properties and found in natural products, especially fruit, plant, and herbal medicine.

-Free radical was classified as an atom or molecule capable of an independent existence that contains an unpaired electron in its out orbit. Free radicals are molecules with one or more unpaired electrons, such as superoxide anion (O_2^-) and hydroxyl radical (OH). Free radical-induced biochemical alterations have been documented in ischemic stroke as well as many neurodegenerative diseases. The challenge has been to establish whether these changes initiate cell injury, amplify other pathologic processes, or occur simply as of late markers of cell injury. Free radicals cause damage in four ways: Damage to lipids, Damage to proteins, Damage to genetic material and Lysosome destruction and free radicals are the cause of many diseases such as cancer, hypertension, and diabetic disease.

-Free radical scavenging activity is meaning antioxidant activity or making free radicals become stable molecules were measured by using the DPPH, ABTS and FRAP methods which were reported as IC_{50}.

-IC_{50} Value (50 % Inhibitory Concentration) was meaning the half-maximal inhibitory concentration on a measure of the potency of substance inhibiting a specific biological or biochemical function.

-A cancer cell was referred to cholangiocarcinoma cell lines (CCA), which was used in this study which include KKU 100 and KKUM 213A

-Anti-cancer activity was mean the ability to inhibited cancer cells of crude extracts and semi-extracts, which was report as percentage growth inhibition and the half-maximal inhibitory concentration (IC_{50}).

-Anti-inflammatory activity was mean the ability to inhibited mouse leukemic macrophage cell line (RAW 264.7 cell) of crude extracts and pure compounds, which was report as percentage growth inhibition, cell viability and the half-maximal inhibitory concentration (IC_{50}).

Chapter II
Literature Review

1. *Phellinus* Mushrooms

1.1 Botanical Description

Phellinus is a member of the family Hymenochaetaceae. The members of Hymenochaetaceae form a cosmopolitan group of wood inhibiting fungus, capable of utilizing components of wood cell walls for their growth and reproduction. Wood is composed of the structural polymer cellulose, lignin, and hemicellulose.[39] *Phellinus* is parasitic, perthophytic and/or saprobic causing white rot that degrades both lignin and cellulose, Fruit bodies of *Phellinus* are pileate to resupinate, pileus dark brown to black in species with a crust, hirsute to glabrous, mostly small, tubes usually stratified, context thin and dense. Hyphal system dimitic, generative hyphae usually hyaline, thin-walled and narrow, more rarely wider and pale golden brown, spores of variable shapes, hyaline to rusty brown, thin-walled to thick-walled. All species are on dead wood and Cosmopolitan genus with numerous species, which in many groups can be difficult to separate. There is a problem when it comes to the color of the spores. In some species, they start as pale yellow, but with maturity, they become rustier brown.[33]

Kingdom: Fungi
Phylum: Basidiomycota
Class: Basidiomycetes
Order: Aphylloporal
Family: Hymenochaetaceae
Genus: *Phellinus*
Species: *Phellinus igniarius* (L.) Quél
Phellinus nigricans (Fr.) P. Karst
Phellinus linteus (Berk. & M.A. Curtis) Teng
Phellinus rimosus (Berk.) Pilát

1.2 Description of the Study *Phellinus* Mushrooms

1.2.1 *Phellinus igniarius* (L.) Quél

The Common synonyms of *P. igniarius* include *Boletus igniarius* L, *Polyporus igniarius* (L.) and *Fomes igniarius* (L.). The *P. igniarius* is an inedible, hard and woody, hoof-shaped perennial mushroom growing either solitary or in groups, brown when young, becoming dark-gray and black with age. It is a large mushroom with a diameter and thickness up to 20 cm or sometimes more. The top of an older specimen is dark-gray or black and is often cracked. The bottom surface is cinnamonbrown. Pores are brown. The tube was brown, spores were sub spherical and White color.[56]

P. igniarius has been used in traditional Chinese medicine and in other oriental countries for the treatment of various diseases, include cancer. According to previous studies, *P. igniarius* was employed in Thai traditional medicine to cure a variety of ailments, including lung cancer, liver cancer, prostate cancer, and skin disorders.[32]

1.2.2 *Phellinus linteus* (Berk. & M.A. Curtis) Teng

The common synonyms of *P. linteus* include *Polyporus linteus* Berk, *Tropicoporus linteus* and *Fomes linteus*. The *P. linteus* is a fungus with the growth characteristics of being oppressed, short-downy, homogeneous, adherent, even margins, imprecise, and odorless. It is also woolly and yellowish-orange colonies, with the annual fruiting body and hyphal system, which refers to the appearance of two kinds of hyphae: generative and skeletal. Moreover, the fungus lacks steal hyphae and clamp connections in its hyphae, which is an either thin or thick wall.[57]

P. linteus is widely used in China, Korea, and other Asian countries. According to Chinese clinical empirical practice, as a traditional Chinese medicine with a 2000-year long history, the medicinal applications of *P. linteus* mainly concern treating hemorrhage and diseases related to female menstruation.[34] In Thailand, the previous study reported that *P. linteus* has been used for the treatment of diabetic disease.[58] India and Rusia, this mushroom has been used as an immunomodulator.[59]

1.2.3 *Phellinus nigricans* (Fr.) P. Karst

The common synonyms of *Phellinus nigricans* include *Polyporus nigrican* Fr, *Boletus nigricans* and *Fomes nigricans* Fr. The fruiting body is brow color, pore arranges 5-6 mm, indistinctly laminated tubes, brown hymenium rust to gray-brown, spore around, smooth, hyaline spore with a slightly thickened wall, tetrasporic basidia, not loop, many, subtle, thick-wall, brown, slightly emergent hymenial bristles.[60]

P. nigricans has been traditionally used in the treatment of nervousness, restlessness, pain associated with debility and due to acute inflammation acute meningitis.[61]

1.2.4 *Phellinus rimosus* (Berk.) Pilát

The common synonyms of *P. rimosus* include *P. scaber* (Berk), *Polyporus rimosus* (Berk) and *Phellinus sanjanii* (Lloyd). This is mushroom belongs to family Hymenochaetaceae. It is commonly grown in the northeast of Thailand. The description of this mushroom is basidiocarps pileate, perennial, pileate and solitary. The mostly ungulate to triquetrous with a sloping pileus, semicircular and dimidiate with a contracted base, up to 12 cm wide and long 3-8 cm thick at base, the sharp to rounded, upper surface at first more, except for a narrow marginal zone, fulvous to dark brown, smooth or with a few quit wide sulcate zones, in order specimens, there is often a narrow, more smooth and light-colored zone reflecting new growth; pore surface yellow-brown in actively growing specimens and then pores thin-walled. In older specimens, the pores become more occluded and more round with a thicker wall of 4-5 mm.[62]

P. rimosus has been used as an ingredient in cancer treatment, herpes, earache, and rash. This mushroom has been used to treat a variety of ailments in traditional Chinese and Japanese medicine. Moreover, in India, this mushroom has been used for the treatment of mumps, ischemia, atherosclerosis, and acute hypertension.[63]

A

B

A) *Phellinus igniarius* ((L.) Quél B) *Phellinus linteus* (L.) (Berk & W.Curt) Teng

C

D

C) *Phellinus nigricans* (Fr.) P. Karst D) *Phellinus rimosus* (BerK.) Pilát [64]

Figure 1 Fruiting body of *Phellinus* Mushrooms

1.3 Chemical Constituents of *Phellinus* Mushrooms

According to previous phytochemical studies, compounds from the genus *Phellinus* mushrooms have been isolated. They can be classified as polysaccharide, steroid, terpenoid, glycoside, phenolic, and flavonoid compounds. The distribution of their compounds and the chemical structures were summarized as in Table 1.

1.3.1 Primary Metabolites

Primary metabolites were those metabolites necessary for the growth of an organism, such as proteins and fats. The primary metabolism process was similar among all organisms. Primary metabolites of *Phellinus* are classified as carbohydrate, protein, and lipid.

Polysaccharides are complex bio macromolecules that are made up of chains of monosaccharides. The bonds that form these chains are glycoside bonds. Commonly found monomer units in polysaccharides are glucose, fructose, and mannose, which are simple sugars. The mannose, lactose, glucose, fructose are the main monosaccharide in the fruiting body of mushrooms. Two polysaccharides, namely, PPM (polysaccharide purified from the mycelium) and PPE (polysaccharide purified from the medium extract) were isolated from *P. pini*. The chromatography techniques, namely UV, IR, HPLC, and GC were used identified structure compounds. PPM and PPE compounds were contained mannose, galactose and glucose in the molar ratio of 28.57:1.00:1.47 for PPM; mannose, galactose, and glucose in the molar ratio of 2.99:1.00: 0.34 for PPE.[43] Polysaccharide PL-11 was extracted from *P. linteus*. The polysaccharides were identified by spectroscopic methods and comparison with high-performance liquid chromatography (HPLC).[65]

1.3.2 Secondary Metabolites

The particular characteristics of growth and development of mushrooms in nature result in the accumulation of a variety of secondary metabolites such as terpene, steroid, phenolic, and flavonoid compounds.

1.3.2.1 Phenolic Compounds

Seven phenolic compounds were separated from the ethanol extract of the fruiting body of *P. igniarius*, named 3, 4-hydroxybenzaldehyde, 4-(3,4-dihydroxy phenyl-3-buten-2-one, inonoblin C, phelligridin D, inoscavin C, phelligridin C, interfinger B. These compounds were identified from the ethanol extracted by LCMS and NMR spectoscopy.[66]

1.3.2.2 Flavonoids

Totally 35 Flavonoids, pyranones and furans have been separated from *P. igniarius*. The examples of these flavonoids were four flavones (phelligrins A, B, and meshimakobnol A, B). These compounds were isolated by Chromatography technique (normal phase silica gel and reverse-phase HPLC). The phelligridins A-J were obtained from ethyl acetate fraction of the ethanol extract from *P. igniarius*. The 5, 7, 4′-trihydroxy-6-O-hydroxy benzyl-dihydroxyflavone and 5, 7, 4′-trihydroxy-8-O-hydroxy benzyl dihydroxyflavone were isolated from the fruiting body of P. igniarius. Structures were elucidated by spectroscopic methods including IR, MS, and NMR spectoscopy.[34]

1.3.2.3 Triterpenoids and Steroids

According to some recent reports, some terpenes including sesquiterpenes, diterpenes, and triterpenes were separated from the fruiting body of *P. linteus*. For example, eleven terpenes (gilvsins A-D, igniarens A-D, and phellilins A-C), and three steroids (phellinignincisterol A-C), were characterized as the special ingredients of *P. linteus*. In addition, Shirahata et al have reported the pure compounds from methanol extract of this mushroom, which is consisting of sesquiterpenoids (-)-trans-γ- monocyclofarnesol and γ-ionylidene sesquiterpenoid.[55] Two steroids (3 α,17α, 19, 20-tetrahydroxy-4α-methylpregn-8-ene and 3α, 12α, 17α, 20-tetrahydroxy-4α-methyl pregn-8-ene) and three sesquiterpenoids (12-hydroxy-a-cadinol, 3α,12-dihydroxydi cadinol, and 3α, 6α-dihydroxyspiroax-4-ene) were isolated from cultures of *P. igniarius*.[67] The structures were elucidated by the spectroscopy method (^{1}H & ^{13}C NMR).[55] The distribution of these compounds in *Phellinus* mushrooms and their chemical structure are summarized in Table 1.

Table 1 Chemical Structure and Part of Mushrooms.

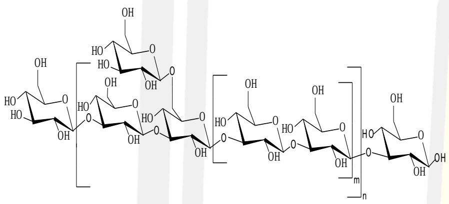
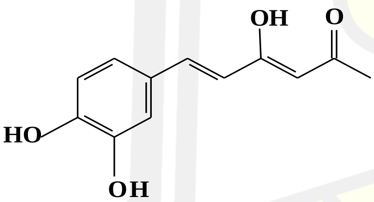
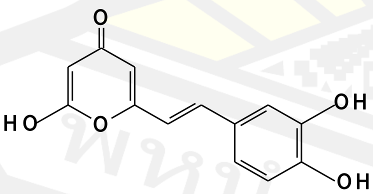
Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References
Polysaccharides,  1) β-glucan		*P. igniarius*	56
2) Polysaccharide PL-11		*P. linteus*	34
Phenolic and Flavonoid compounds  3) Hispolon		*P. linteus* *P. igniarius*	34, 56
 3) Hispidin		*P. linteus* *P. igniarius*	

Table 1 (Continued)

Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References

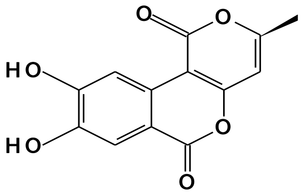
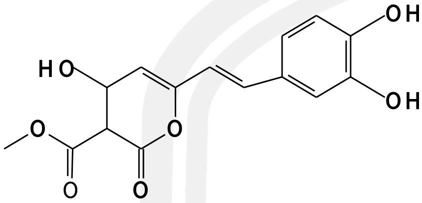
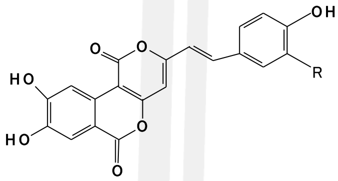
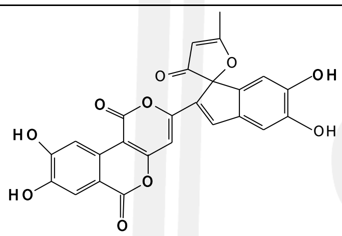
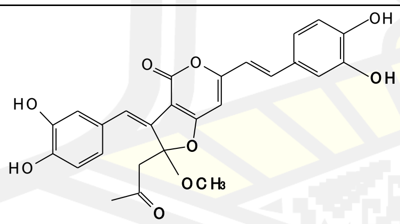
 4) Phelligridin A		*P. linteus* *P. igniarius*	34, 56
 5) Phelligridin B		*P. linteus* *P. igniarius*	34, 56
 6) Phelligridin C	R=H	*P. linteus* *P. igniarius*	34, 56
7) Phelligridin D	R=OH	*P. linteus*	34
 8) Phelligridin E		*P. linteus*	34
 9) Phelligridin F		*P. linteus*	34

Table 1 (Continued)

Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References

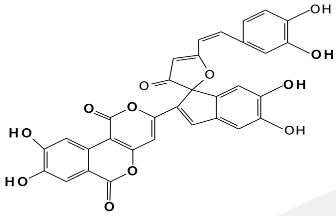
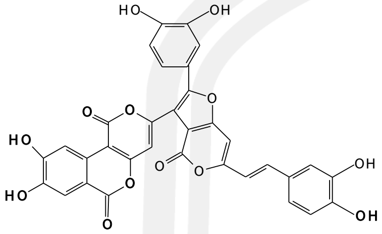
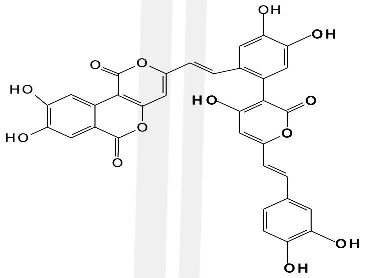
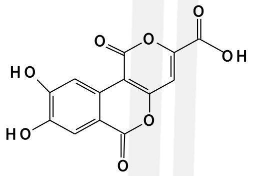
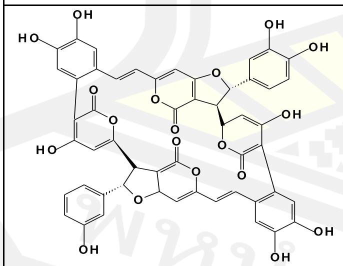
 10) Phelligridin G		*P. linteus*	34
 11) Phelligridin H		*P. linteus*	34
 12) Phelligridin I		*P. linteus*	34
 13) Phelligridin J		*P. linteus*	34
 14) Phelligridimer A (macrolide)		*P. linteus*	34

Table 1 (Continued)

Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References

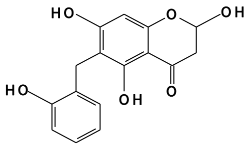
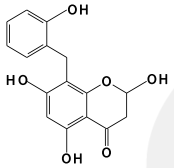
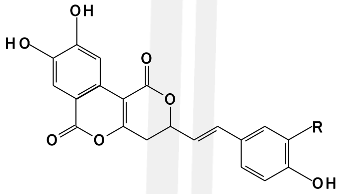
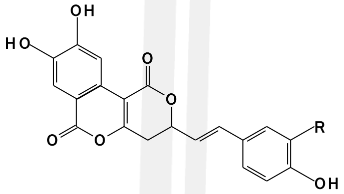
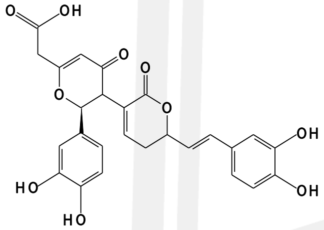
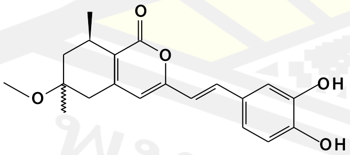
 15) Phelligrins A		*P. linteus*	34
 16) Phelligrins B		*P. linteus*	34
 17) Meshimakobnol A	R=OH	*P. linteus*	34
 18) Meshimakobnol B	R=H	*P. linteus*	34
 19) Baumin		*P. linteus*	34
 20) Phellinin C		*P. linteus*	34

Table 1 (Continued)

Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References

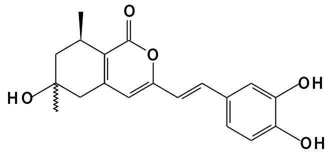
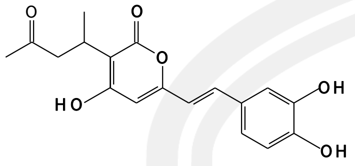
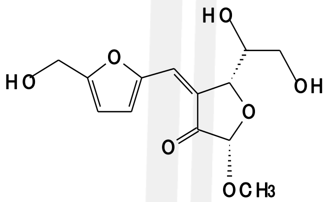
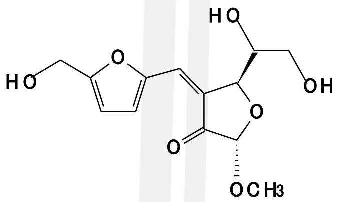
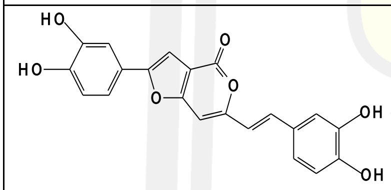
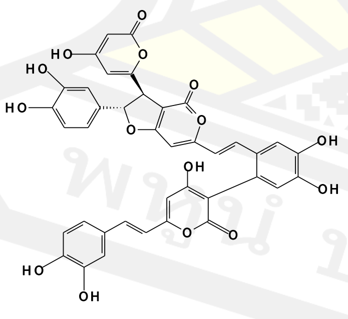
 21) Phellinin B1		*P. linteus*	34
 22) Phellinin B2		*P. linteus*	34
 23) Phellinusfurans A		*P. linteus*	34
 24) Phellinusfurans B		*P. linteus*	34
 25) Phellinusfuropyranone A		*P. linteus*	34
 26) Phellinstatin		*P. linteus*	34

Table 1 (Continued)

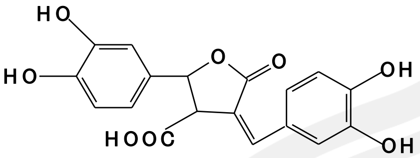
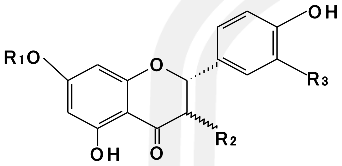
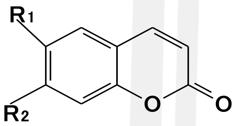
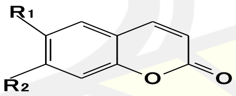
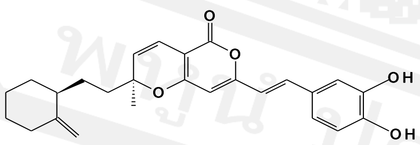
Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References
 27) Phelliusin A		*P. linteus*	34
 28) Naringenin	R_1=R_2=R_3=H	*P. linteus*	34
29) Sakuranetin	R_1=CH_3; R_2=R_3=H	*P. linteus*	34
30) Aromadendrin	R_1=R_3=H; R_2=β-OH	*P. linteus*	34
31) Folerogenin	R_1=CH_3; R_2=α-OH; R_3=H	*P. linteus*	34
32) Eriodictyol	R_1=R_2=H; R_3=OH	*P. linteus*	34
33) Sakuranetin	R_1=CH_3; R_2=R_3=H	*P. linteus*	34
 34) Coumarin	R_1=R_2=H		
 35) Scopoletin	R_1=OCH_3; R_2=OH		34
 36) Phellinin A1		*P. linteus*	34

Table 1 (Continued)

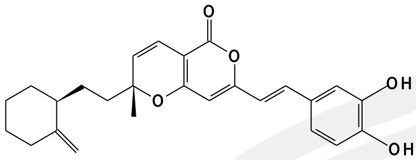
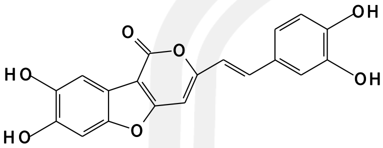
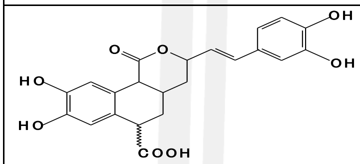
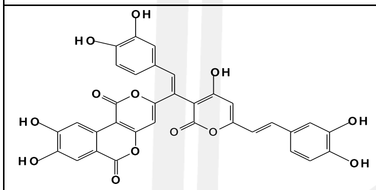
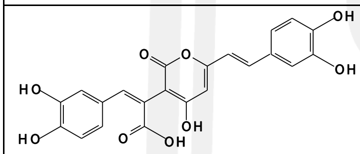
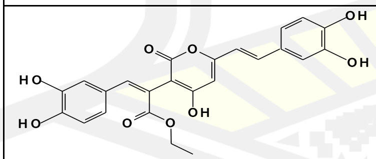
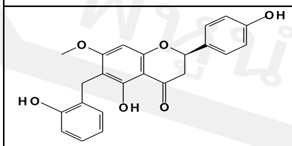
Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References
 37) Phellinin A2		*P. linteus*	34
 38) Phellinbaumin A		*P. linteus*	34
 39) Phellibaumin B		*P. linteus*	34
 40) Phellibaumin C		*P. linteus*	34
 41) Phellibaumin D		*P. linteus*	34
 42) Phellibaumin E		*P. linteus*	34
 43) Methyl phelligrins A		*P. linteus*	34

Table 1 (Continued)

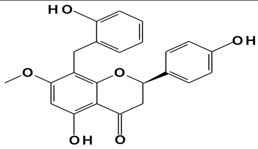
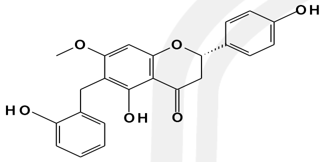
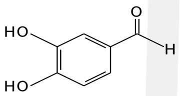
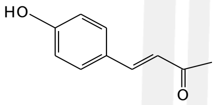
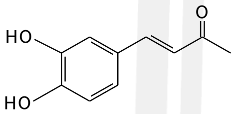
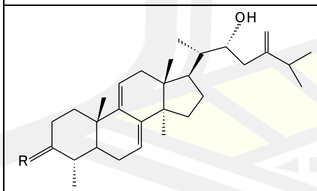
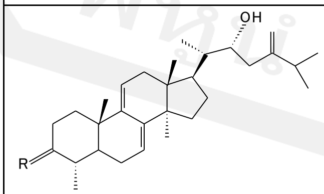
Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References
 44) Methyl phelligrins		*P. linteus*	34
 45) Isomethyl phelligrins A		*P. linteus*	34
 46) 3,4-dihydroxybenzaldehyde		*P. baumii*	68
 47) 4-(4-hydroxyphenyl)-3-buten-2-one		*P. baumii*	68
 48)4-(3,4-hydroxyphenyl)-3-buten-2-one		*P. baumii*	68
Terpenoid and steroids			
 49) Igniarens A	R=O	*P. linteus*	34
 50) Igniarens B	R=α-OH; H	*P. linteus*	34

Table 1 (Continued)

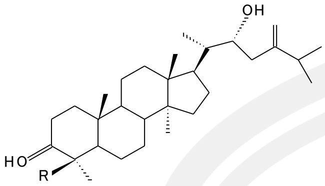
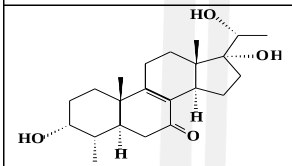
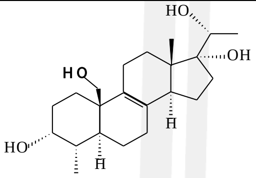
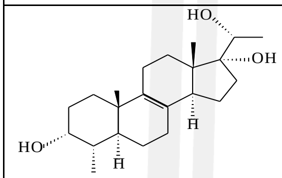
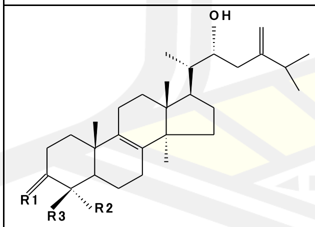
Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References
 51) Igniarens C	R=H	*P. linteus*	34
52) Igniarens D	R=CH_3	*P. linteus*	34
 53) 21-homopregnene derivatives		*P. igniarius*	
 54) 21-homopregnene derivatives		*P. igniarius*	56
 55) 21-homopregnene derivatives		*P. igniarius*	56
 56) Gilvsin A	R_1=O; R_2=R_3=CH_3	The fruiting body of *P. gilvus*	69
57) Gilvsin B	R_1=α-H,β-OH; R_2=COOH; R_3=CH_3	The fruiting body of *P. gilvus*	69
58) Gilvsin C	R_1=O; R_2=CH_3; R_3=H	The fruiting body of *P. gilvus*	69

Table 1 (Continued)

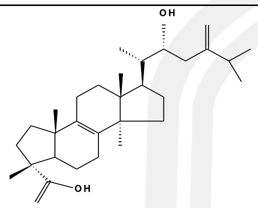
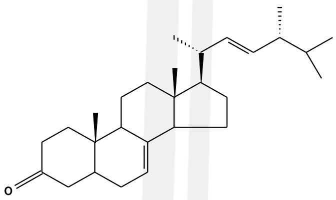
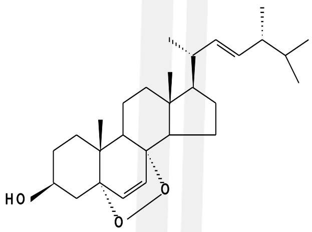
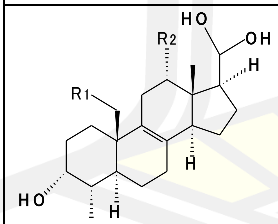
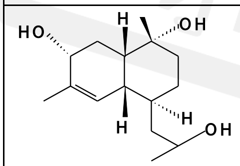
Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References
59) 24-methylenelanost-8-ene-3-β-22-diol	R_1=α-H,β-OH, R_2=R_3=CH_3	The fruiting body of *P. gilvus*	69
 60) Gilvsin D		The fruiting body of *P. gilvus*	69
 61) 5α-egrosta-7,22-diene-3-one		The fruiting body of *P. gilvus*	69
 62) Ergosterol peroxide		The fruiting body of *P. gilvus*	44
 63) 3α,17α,19,20-tetrahydroxy-4α-methylpregn-8-ene	R_1=OH; R_2=H	*P. igniarius*	55
 64) 12-hydroxy-α-cadinol		*P. igniarius*	55

Table 1 (Continued)

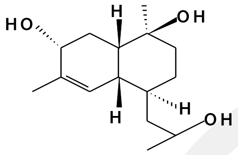
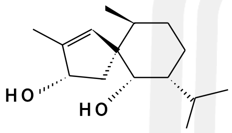
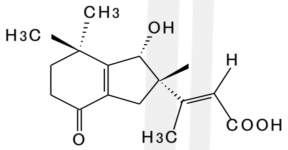
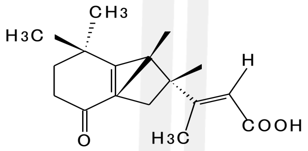
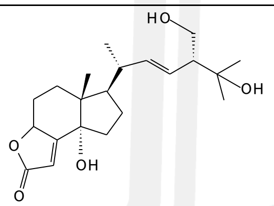
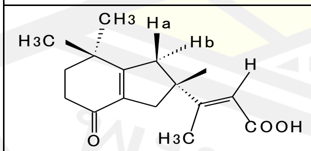
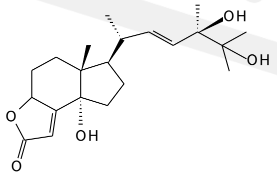
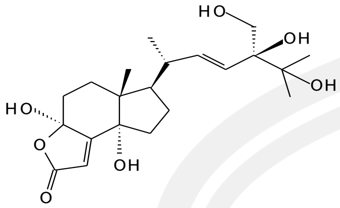
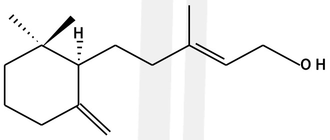
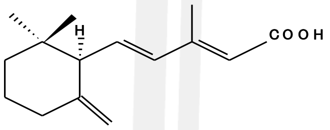
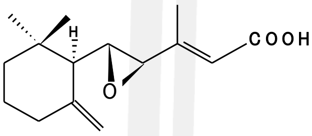
Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References
 65) 3α,12-dihydroxy-d-cadinol		*P. igniarius*	55
 66) 3α,6α-dihydroxyspiroax-4-ene		*P. igniarius*	55
 67) Phellilins B		*P. linteus*	34
 68) Phellilins C		*P. linteus*	34
 69) Phellinignincisterol A		*P. linteus*	34
 70) Phellilins A		*P. linteus*	34
 71) Phellinignincisterol B		*P. linteus*	34

Table 1 (Continued)

Chemical group/structure	Substituted group (R-group)	Mushroom (part)	References
 72) Phellinignincisterol C		*P. linteus*	34
 73) (-)-tran-γ-monocyclofarnesol		Mycelium of *P. linteus*	67
 74) (+)-γ-ionylideneacetic acid		Mycelium of *P. linteus*	67
 75) Phellidene E		Mycelium of *P. linteus*	67

1.4 Biological Activities of the *Phellinus* Mushrooms

Much experimental evidence has revealed that several species of genus *Phellinus* were shown to high cytotoxicity against many different cancer cell lines. [47, 55, 66] The various activities of mushrooms have been studied which includes antitumor,[70-71] immunomodulator,[72] anticancer, antioxidant and antimicrobial activities.[71]

1.4.1 Antitumor and Immunomodulation Activity

In general, antitumor activity was recognized as the most important bioactivity of *Phellinus* mushrooms. Lots of pharmacological experimental studies have verified that *P. igniarius* was an effective anti-cancer medicine. According to Sasaki et al., there have been reported antitumor activity of polysaccharides isolated from *P. linteus*.[73] The present polysaccharides and phenolic compounds were the main components having antitumor effect in *Phellinus* mushrooms.[71] A homogeneous polysaccharide (PPB) was purified from the fruiting body of *P. baumii* and has been investigated on human hepatocellular carcinoma (Hela), human gastric carcinoma cells (SGC-7901) and mouse leukemic macrophage cell line (RAW264.7 cell lines). The results of the study showed that PPB inhibited the proliferation of Hela and SGC-7901 cells significantly. The results implied that PPB was able to induce the cell cycle of Hela arrest at the G0/G1 phase.[70]

1.4.2 Anticancer Activity

Several chemical compounds isolated from *Phellinus* mushrooms demonstrated anticancer properties. For example, Polysaccharides from *P. linteus* and *P. baumii* showed anticancer activity against Hela cells at a concentration of 1.0 mg/mL and 0.025 mg/mL, respectively.[74,42] Furthermore, The pure compound namely hispolon isolate from *P. igniarius* showed anticancer activities against A549 and H661 lung cancer cell lines evaluated by MTT and apoptosis assay. The results showed that hispolon can reduce cell viability in a dose and time-dependent manner. The results also suggested that hispolon enhanced the accumulations of the cells in G0/G1 phase in the cell cycle.

According to the previous studies, the isolated compounds separated from *Phellinus* mushrooms showed anticancer and related activities as shown in Table 2.

Table 2 Anticancer Activity of Pure Compounds Isolated from *Phellinus* Mushrooms.

Chemical/ name	Mushrooms	Molecular Targets	Inhibition (IC_{50})	[Rf]
Hispidin	*P. linteus*	Antitumor (PKC inhibitor)	-	47,75
Proteoglycan	*P. igniarius*	Immunomodulator	-	47
Hispolon	*P. linteus* *P. igniarius*	Anticancer (A549 & H661) Anti-leukemia activity, (Human hepatocellular carcinoma NB4 cells)	8.1 μg/mL & 2.1 μg/mL 0.625 μg/mL	76,47
Hispolon	*P. linteus*	Apoptosis (H661)	40 μM(69%)	47
Proteoglycan	*P. linteus* *P. igniarius* *P. bamii*	Anti-sarcoma activity (Female C57BL/6 mice; MCA-102 tumor cells)	100 mg/kg/day & 100 μg/mL	77
Phelligridimer - A	*P. igniarius*	Rat liver microsomal lipid peroxidation inhibition effect	10.2 μM	78
3α,12-dihydroxy-d-cadinol	*P. gilvus* *P. baumii*	Leukemia HL-60, Hepatocellular carcinoma SMMC-7721, lung cancer A-549 cells, breast cancer MCF-7 and colon cancer SW480 cell lines	<40 μM	34

Table 2 (Continued)

Chemical/ name	Mushrooms	Molecular Targets	Inhibition (IC_{50})	[Rf]
3α,12α,17α,2-tetrahydroxy-4α-methyl-pregn-8-ene	*P. gilvus* *P. baumii*	Leukemia HL-60, Hepatocellular carcinoma SMMC-7721, lung cancer A-549 cells, breast cancer MCF-7 and colon cancer SW480 cell lines	<40 µM	55
12-hydroxy-α-cadinol	*P. gilvus* *p. baumii*	LeukemiaHL-60, Hepatocellular carcinoma SMMC-7721, lung cancer A-549 cells	<40 µM	55
3α,12α,17α,20 tetrahydroxy-4αmethylpregn-8-ene	*P. gilvus,* *P. baumii*	Hepatocellular carcinoma SMMC-7721, A-549 cells, MCF-7 and colon cancer SW480 cell lines	<40 µM	55

1.4.3 Anti-inflammatory Activity

The processes leading to inflammation are usually linked to the activities of the cells involved in the restoration of tissue structure and function. When cells are exposed to immune stimulants, the pro-inflammatory cells, such as macrophages, monocytes, or other host cells, start to produce many molecular mediators that initiate the inflammation process.[79] The ethyl acetate fraction of *P. linteus* exhibited the expression of pro-inflammatory cytokines in IgE/Ag-stimulated RBL-2H3 cells.[80] The BuOH fraction of *P. linteus* showed the highest anti-inflammatory activity in the croton oil-induced ear edema test; moreover, the BuOH fraction indicated the highest inhibitory activity on the chick embryo chorioallantoic membrane angiogenesis in a dose-dependent manner.[81] The inotilone isolated from *P. linteus* had indicated anti-inflammatory activities on RAW264.7 cells and λ-carrageenan (Carr)-induced hind mouse paw edema model. The inotilone can reduce nitric oxide (NO) production and the inducible nitric oxide synthase (iNOS) expression; moreover, the inotilone had inhibited the mitogen-activated protein kinase at 5 mg/kg.[82] According to the study, the analgesic effects of ethanol extract of *P. merrillii* (EPM) were investigated by measuring the acetic acid-induced writhing response and the licking time of hind paws following formalin injection. The results of study showed the anti-inflammatory activities were not statistic significant between EPM (2 g/kg) and indomethacin (10 mg/kg) ($p > 0.001$). EPM (1 and 2 g/kg) was significantly inhibited ($p < 0.001$) the formalin-induced pain in the late phase. EPM may has analgesic and anti-inflammatory activities.[83] The anti-inflammatory activity of the *P. rimosus* extract was evaluated in carrageenan, dextran induced acute, and formalin induced chronic inflammatory models in mice. The extracts showed remarkable anti-inflammatory activity in both models when compared with the standard reference drug (diclofenac). The result was indicated anti-inflammatory activity of methanol extract of *P. rimosus.*[84]

1.4.4 Antioxidant Activity

Several studies have found that *Phellinus* mushroom extract has antioxidative activity.[85, 86,87] The crude ethyl acetate (EtOAc) fraction of *P. pini* showed the highest total phenolic content, with a value of 87.76 ± 1.00 equivalent gallic acid (EGA), while the samples with potent antioxidant activity had a high quantity of total phenolic contents (78.34 ± 0.27 to 51.01 ± 0.38 EGA).[85] The antioxidant activities of davallia-lactone (A) and Interfungins A (B) isolated from *P. linteus* showed the strongest inhibitory effect against the DPPH radical and superoxide anion radical scavenging capacity. In comparison with quercetin (IC_{50}, 44.0 μM), the IC_{50} values of compounds A and B for DPPH radical-scavenging capacity were 19.6 μM and 18.5 μM, respectively.[88] The ethyl acetate extract of *P. rimosus* at a concentration of 0.1 percent showed a high Trolox equivalent antioxidant capacity value of 12.48 μg/mg. That method was evaluated by using the ABTS (2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid) assay.[89] The ethanol extract of *P. rimosus* was evaluated by using the DPPH assay, which showed IC_{50} values of 8.26 ± 1.40 μg/mL.[90]

2. Cholangiocarcinoma (CCA)

HCC (hepatocellular carcinoma) and cholangiocarcinoma (CCA) are two kinds of primary liver cancer with distinct histological characteristics (CCA). HCC is the most common liver cancer which poses a significant disease burden in many parts of the world, especially in Africa and Asia. Chronic infection with the hepatitis B virus (HBV) is the primary cause of HCC in high-incidence areas, although most cases of hepatocellular carcinoma (HCC) and CCA in countries like Japan are linked to cirrhosis caused by chronic hepatitis C virus (HCV) infection. On the other hand, liver cirrhosis associated with chronic alcohol abuse is a major cause of HCC in developed countries.[15] Unlike HCC, CCA has a different geographical distribution, with significant-high peak incidences in Asia especially in the Northeast of Thailand, China, Korea, and Japan.[91]

2.1 Classification of CCA

CCA is classified into three major groups: intrahepatic, perihilar, and distal extra hepatic.[5] Henry Bisthmus had classified into four major groups (Figure 2) according to the anatomic locations: Type 1 - tumor involves hepatic bile duct only; Type 2 - tumor involves bile duct bifurcation; Type 3a - tumor involves bile duct burfication and right hepatic bile duct; Type 3b - tumor involves bile duct burfication and left hepatic bile duct; Type 4 - tumor involves both sides of hepatic bile ducts. This system is used widely in clinical practice.[92]

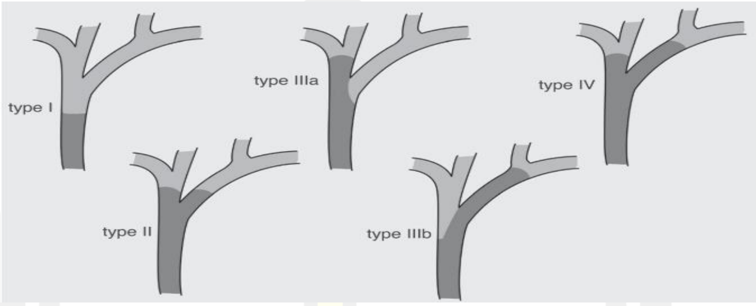


Figure 2 Bismuth/Corlette Classification

2.2 Causes of CCA

Generally, approximately 60-70 percent of CCA occur at the hepatic duct bifurcation, and the piece occurs in the distal common bile duct (20 -30 percent) or inside the liver (5-15 percent).[16] An intrahepatic type of CCA arises from any portion of the intrahepatic bile duct epithelium as well as from the right and left hepatic ducts. CCA is considered an extrahepatic lesion.[93] Intrahepatic CCA is the most common cause of CCA in Thailand and the disease in the area has been definitely related to chronic infection with *Opisthorchis viverrini* (*O. viverrini*). [94,9,95] In other Asian countries like China, Korea, and Japan, *Clonorchis sinensis* (*C. sinensis*) is the main risk factor for CCA.[12] Thailand is still the country with the highest incidence of CCA in the world. The CCA incidence in Thailand is exceedingly high with the age-standardized rate of 33.4 per 100,000 in men, 12.3 per 100,000 in women.[2] *O. viverrini* infections were pathologically associated with a number of hepatobiliary diseases including cholangitis, digestive diseases, hepatomegaly, cholecystitis and choledochal cysts were associated with CCA.[5] The previous studies are experimental and epidemiological evidence indicated that chronic infection with *O. viverrini* liver fluke is the etiology of CCA.[96] The pathological consequences of *O. viverrini* infection (For example; epithelial desquamation, inflammation, epithelial hyperplasia and goblet cell metaplasia) approximately 90 % of the confirmed cases of HCC was intrahepatic CCA, and almost all the CCA cases were found to be related to chronic *O. viverrini* infection in the area of in the previous study.

2.3 Multiple Risk Factors Revealed a CCA

The epidemiological profile of CCA and its subtypes displays enormous geographic differences. Although in most countries CCA is rare cancer, its incidence may reach exceedingly high peaks in North Thailand. In particular, the variation in incidence rates was correlated with the different prevalence of risk factors. In regions of Thailand, for example, it is closely linked to the incidence of liver flukes.[97,5] In previous studies, we can summarize the factors and risk variables related to CCA in the table below.

Table 3 Summarizes the Worldwide Epidemiology and Incidence Trends of CCA.

Risk factor for iCCA	The odds ratio for increased risk	Risk factor for pCCA / dCCA	The odds ratio for increased risk
Bile duct disease & condition		Bile duct disease & condition	
Cholecystitis	8.5	Cholecystitis	5.9
Cholelithiasis	10.23-13.5	Cholelithiasis	2.6-11
Hepatolithiasis	50	Hepatolithiasis	3.09
Chloledochcal cysts	10.7-43	Chloledochcal cysts	47.1
Chlolangistis/PSC	64.2-75.23	Chlolangistis/PSC	45.7
Biliary cirrhosis/PBC	17.08-19.8	Biliary cirrhosis/PBC	11.8
Cholecystectomy	3.6-5.4	Cholecystectomy	5.8-12
Digestive disease		Digestive disease	
Inflammatory bowel disease	1.72-3.95	Inflammatory bowel disease	1.1-1.97
Chronic pancreatitis	5.9	Chronic pancreatitis	9.3
Liver flukes		Liver flukes	
C. sinensis infection	8.6-13.6	*C.s sinensis* infection	6.5
Chronic liver disease	3.1-5.69	Chronic liver disease	4.5

The investigated risk factors could be classified on the basis of the tissue or the cells primarily targeted by diseases or conditions, and are therefore likely to be involved in the carcinogenic process as a cell. In this view, biliary diseases such as cholangitis/PSC, secondary biliary cirrhosis, and liver flukes are pathological conditions that primarily affect large intra or extra hepatic bile ducts. In addition, several toxic and environmental risk factors have been linked to CCA development, including nitrosamine-contaminated food, dioxins, and vinyl chlorides.[98] Moreover, the geographic distribution of the related CCA cases is associated with these risk factors. [5]

2.4 Chlolangiocarcinoma Cells and Treatment Approaches

The limitation of therapeutic options and early detection of CCA are also major problems for controlling CCA. Among the serum tumor markers, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were used as candidate biomarkers for valuation and monitoring after the treatment of several gastrointestinal cancers. According to a previous study, if CCA found an early diagnosis, the surgical method of treatment would improve the 5-year survival rates of patients with iCCA and dCCA at 22-44 percent and 27-37 percent, respectively. The survival of patients depended on local clearance, vascular invasion, and lymph node metastases.[11] Chemotherapy is a treatment with anti-cancer drugs that may be injected into a vein or given by mouth. The standard drugs (5-fluorouracil (5-FU), gemcitabine, and cisplatin) have been indicated to improve local control and prolong the survival rate of patients.[2,99] In 2010, a new standard of care in respective developed with the support of the British Liver Trust and the UK cholangiocarcinoma charity (BTC) was established with the reports of the UK NCRI ABC-01 and ABC-02 trials. ABC-02 was the largest randomized phase III study reported in BTC to date.[100] Four hundred and ten patients with locally advanced or metastatic CCA were randomized to receive 24 weeks of cisplatin plus gemcitabine (CisGem) or gemcitabine (Gem) alone. The median CisGem group and 8.1 months for the Gem group versus 5.0 months for the Gem group was significant. Patients in the CisGem group also had an importantly improved tumor control.[11,100] Other reports also indicated that chemotherapeutic treatment of CCA was largely ineffective, and treatment with 5- FU always produces low in clinical response rates.[15] Moreover, in several stages of CCA, metastatic tumors in the lungs develop by spreading from the liver original through the bloodstream, which was one of the main determined of therapeutic using by gemcitabine and cisplatin. Overall toxicity was similar between the arms, with a slight excess in clinically non-significant hematological toxicities for the CisGem group.[101]

Many studies have been published on the therapy of CCA. For example, in an early prospective open-label trial, 39 patients with unrespectable CCA were randomized to stenting alone and photodynamic therapy (PDT). The PDT group had a significantly higher median survival.[11] Currently, no evidence to support the routine used of radiotherapy postoperatively or for the unrespectable disease.[102] Generally, CCA is considered to be a multidrug and radio resistant tumor that still requires new approaches to treatment. The most common types of treatment for cancer include surgery, chemotherapy, or alternative chemotherapy from dietary phytochemicals.[18] In general, chemotherapy with mushroom-derived compound has emerged as an accessible, alternative, promising approach to cancer control and management in many countries.[103] In such efforts to develop new drugs against CCA from natural products, especially mushrooms. The preclinical study was required and should be conducted to evaluate the safety, efficacy, essential pharmacokinetic aspects of the test substances.

2.4.1 Natural Product Having Anti-cholangiocarcinoma in Cancer Cell Lines

A number of naturally occurring compounds and plant extracts, including phenolic and flavonoid compounds, with anticancer activities against CCA have been reported by Wutka et al. The results of studies showed that many phenolic compounds (capsaicin and myricetin) were indicated active against CCA.[104] Sombaetsri et al reported that atalaphylline, limonophyllines A-C, kaempferol were active against CCA. In addition, medicinal plants and mushrooms showed promising anti-CCA activities through, the in vitro or in vivo assays.[105,106,107,108]

In a search for anti-CCA from Thai medicine plants, the research found that the extracts from seven plant species (*Atractylodes lancea*, *Kaempferia galangal*, *Zingiber* *officinal*, *Piper chaba*, *Mesua ferrea*, *Ligusticum sinense*, *Mimusops elengi*) and one folklore recipe (*Pra-Sa-Prao-Yhai*) exhibited promising activity against the CCA (CL-6) cell line with survival of less than 50 percent at the concentration of 50 µg/mL. Among those, the extracts from the five plants and one recipe showed potential cytotoxic activity with mean IC_{50} values of 24.09, 37.36, 34.26, 40.74, 48.23 and 44.12 µg/mL, respectively.[109]

3. Screening Methods for Development of Anticancer Drugs

To combat the problem of resistance, newer drugs are currently required. The discovery and development projects are now underway throughout the world. It's recognized that most of the methods for anticancer screening and investigation. The principal mechanism of the drug (liberation, absorption, distribution, metabolism, and elimination) has originated from the pharmaceutical research but the same procedure may be applied and optimized for phytochemicals. Several methods including in vitro, in vivo and Cell-Based screening bioassay methods are used for anticancer drug development and evaluation.[110]

There are many institutes interested and conducted research on a new drug for the anticancer. Consequently, previous reports described methods or assays to find a new drug in many types such as incorporate the evaluation of synthetic agents and natural products for antitumor activity. For example, the Southern Research Institute in Alabama has been evaluated and developed programs for anticancer activity. As a result of these efforts, several agents were found with clinical activity, particularly against leukemias and lymphomas. Currently, they provide the battery of available drugs for systemic treatment of cancer (cyclophosphamide, bis (chloroethyl) nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-nitrosourea, antimetabolites (metho- trexate, 5-fluorouracil [5-FU], 6-mercaptopurine), antibiotics (mitomycin C, adriamycin), and hormones (androgens, estrogens, corticoids).[111]

Chemotherapy drugs are sometimes a feared because of a patient's concern about toxic effects. The aim is to decrease the halt of cancer growth and inhibitor the spread of cancer. There are three goals associated with the use of the most commonly used anticancer agents. The goal of the chemotherapy drug was to damage the DNA of the cancer cells. The synthesis inhibited new DNA strands to stop the cell from replicating, which is important for inhibiting what allows the tumor to grow. Chemotherapy's goals for cancer treatment were to stop mitosis or to actually split the original cell into two new cells. Stopping mitosis stops cell division (replication) of cancer and may ultimately halt the progression of cancer.[13]

Mushrooms are known to complement chemotherapy by countering the side effects of cancer, such as nausea, bone marrow suppression, and decreased resistance.

Many experimental pieces of evidence have revealed that several species of the genus *Phellinus* were shown high cytotoxicity against plenty of different cancer cell lines.[55, 70] Furthermore, biological screening worldwide demonstrated that mushroom species of this genus *Phellinus* had presented activities in antitumor[70,71] antioxidant[112,113,72] antimicrobial and immunomodulation activities.[71] Previous studies have reported the methods for anticancer activity evaluation. The common methods include cytotoxicity, anti-proliferative and apoptosis assays as summarized in Figure 3.

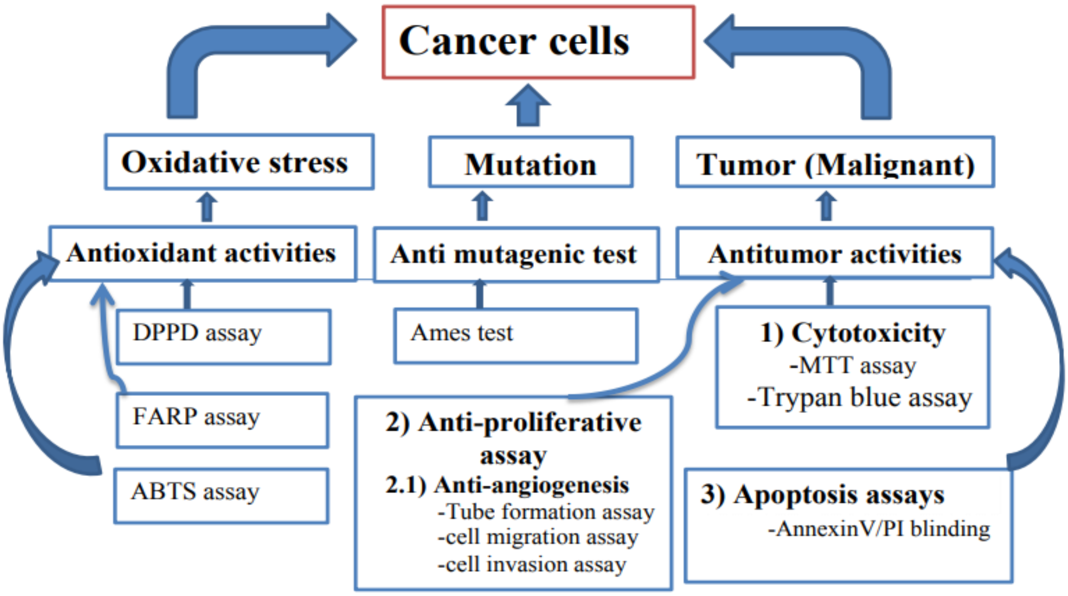


Figure 3 Methods for Investigation on Anticancer Activity

3.1 Sulforhodamine B (SRB) Cell Cytotoxicity Assay

Sulforhodamine B (SRB) cell cytotoxicity assay is one of the widest methods used to detect cell viability or drug cytotoxicity. This assay relies on the ability of SRB to bind cellular protein components and measure the total biomass. SRB is a bright-pink amino xanthene dye that can form an electrostatic complex with basic amino acid residues of proteins in slightly acidic conditions, but it can dissociate under basic conditions. It has been widely used for drug toxicity screening against different types of cancerous and non-cancerous cell lines. In addition, this assay is independent of cell metabolic activity. Since the binding of SRB is stoichiometric, the incorporated dye released from stained cells after washing is directly proportional to the cell biomass and can be measured at 565 nm.114

3.2 MTT Assay

MTT assay is a sensitive and reliable indicator of cellular metabolic activity. The assay relies on the reduction of MTT, a yellow water-soluble tetrazolium dye, primarily by the mitochondrial dehydrogenases to purple-colored formazan crystals. The formazan product is analyzed spectrophotometrically at 550 nm after being dissolved in DMSO. The spectra of nanoparticle-treated and untreated cells give an estimate of the extent of cytotoxicity. The formazan product is analyzed spectrophotometric ally at 550 nm after dissolved in DMSO, the spectra of nanoparticle-treated and untreated cells giving an estimate of the extent of cytotoxicity.[115] The main advantage of MTT assay is the gold standard for cytotoxicity testing while the disadvantages are the conversion to formazan crystals depends on metabolic rate and number of mitochondria resulting in many known interferences.[116]

3.3 Apoptosis Assay

Apoptosis, or programmed cell death, is a normal physiologic process for the removal of unwanted cells. One of the onset events of apoptosis included translocation of membrane phosphatidylserine (PS) from the inner side of the plasma membrane to the surface area. Annexin V, a Ca^{2+} dependent phospholipid-binding protein has a high affinity for PS, and fluorochrome-labeled Annexin V can be used for the detection of exposing PS by using flow cytometry.[27, 117] AnnexinV/PI binding assay is annexin V bind to phosphatidylserine, which migrates to the outer plasma membrane in apoptosis. The analysis is typically by flow cytometer. This method detects the events of apoptosis: if the cells have apoptosis, it detects annexin V & PI staining on cells at the late phase, while the early phase was found to have annexin V only.

4. Free Radicals and Antioxidants

4.1 Free Radicals

A free radical is any atom or molecule that has a single unpaired electron in an outer shell.[118,119] The free radicals, both the reactive oxygen species (ROS) and reactive nitrogen species are derived from both endogenous sources (mitochondria, endoplasmic phagocytic cells) and exogenous sources (pollution, alcohol, tobacco smoke, heavy metals, pesticides, and radiation). [120] Free radicals contribute to various diseases in humans; these include atherosclerosis, arthritis, ischemic central nervous system injury, gastritis, and cancer.[34,121] Free radicals can cause depletion of the immune system. The antioxidant can prevent the changes in gene expression and reduce abnormal proteins. [122]

4.2 Oxidative Stress

Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses. Important free radicals are described and biological sources of origin discussed together with the major antioxidant defense mechanisms. Examples of the possible consequences of free radical damage are provided with special emphasis on lipid peroxidation.[123]

4.3 Antioxidant Activity

Antioxidants are defined as substances that inhibit or delay the oxidation of biologically relevant molecules either by specifically quenching free radicals or by chelation of redox metals.[124] These are radical scavengers and give protection to the human body against chain reactions initiated by free radicals.[125] Due to this property, antioxidants are responsible for delaying the development of chronic diseases that are initiated by free radicals.[126] Antioxidants are compounds known to slow or delay lipid oxidation. Preventative antioxidants can intercept free radical or singlet oxygen before any significant oxidation can occur. However, chain-breaking antioxidants retard or slow the oxidative processes after they begin.[13]

4.3.1 Synthetic Antioxidants

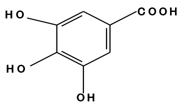
Synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and propyl gallate. Natural and synthetic antioxidants were added into foods to prevent undesirable deterioration. These synthetic antioxidants, however, impose undesirable side effects and recent reports have expressed safety concerns about their usage.[127,128]

4.3.2 Natural Antioxidants

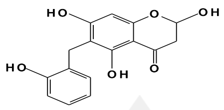
Antioxidants protect against oxidative stress and damage caused by free radicals. Antioxidants such as glutathione, gallic acid, uric acid, and the antioxidant enzymes glutathione peroxidase, superoxide dismutase and catalase, can be generated in the body. However, at times their amount is inadequate when the production of free radicals is increased.[129] Mushrooms possess an almost limitless ability to biosynthesize phytochemicals, which serve as a source for natural antioxidant activities.[130,47,131,50] Compounds with antioxidant properties that found in mushrooms include the vitamins A, E, C and phenolic compounds, including flavonoids, tannins and lignins.[132,133]

4.4 Phenolic Compounds

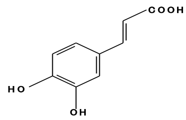
According to the structure of phenolic compounds had divided into several groups, such as simple phenols, hydroxybenzoic, coumarins, stilbenes, anthraquinones, flavonoids and lignins.[134] The chemical structures of the main group of phenolic compounds, phenolic acids and flavonoids including their subclasses, which the present studies are shown as in Figure 4.



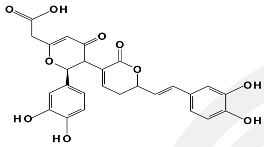
Gallic acid



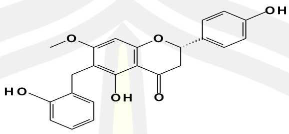
Phelligrin A



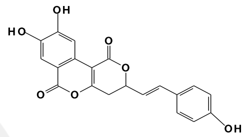
Caffeic acid



Baumin



Isomethyl phelligrins A



Meshimakobnol B

Figure 4 Structure of Some Phenolic Compounds Isolated from *Phellinus* Mushrooms

The phenolic compound is a large class of plant secondary metabolites with a wide range of structures, starting from basic phenolic acids to complex flavonoids. The term 'phenolic' or 'polyphenol' can be defined chemically as a substance that possesses an aromatic ring bearing one or more hydroxyl substituents, including functional derivatives (esters, methyl ethers, glycosides). Most phenolic compounds have two or more hydroxyl groups. The bioactive substances have to occur widely in food plants which are consumed regularly by substantial numbers of people.[49] Phenolic compounds are important for the quality of plant-based foods. They are responsible for the color of red fruits, juices, wines, and the substrate for enzymatic browning. It is also involved in flavor properties. Phenolic compounds have received considerable attention because their dietary intake is related to lower incidence of chronic degenerative diseases, such as cancer, diabetes, cardiovascular diseases.[135]

Naturally occurring phenolic acids contain two distinctive carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures albeit the contents of the latter in plants were less common than that of hydroxycinnamic acids. Again, the number and position of hydroxyl and methoxy substituents in the structure influence anti-oxidative capability.[136] A para hydroxyl group enhances the anti-oxidative capability, which is a hydroxyl group in meta or ortho positions has little or no effect. There were a few free phenolic acids, but the majority of phenolic acids had occurred in conjugated forms, usually linked to cellulose, proteins, lignin, flavonoids, glucose, and terpenes via ester, ether, or acetate bonds.[137,113] The stilbene was phenolic compounds that consist of 1, 2-diphenylethylene, hydroxyl or methoxy group.[58] The potential locations for hydroxyl groups are at the positions of C_3´, C_4´, C_3 and C_5, respectively. It is considered that the OH group at the C_4´ position undergoes oxidation more easily than the other OH groups.[59,138,54]

4.4.1 General Antioxidant Mechanisms of Phenolic Compounds.

The word "phenolic" is used to define substances that possess one or more OH substituents bonded onto an aromatic ring. Compounds that have several or many phenolic hydroxyl substituents were often referred to as polyphenols. Due to their chemical structure, phenolic compounds have the ability to delocalized phenoxide ions.

The phenoxide ion can lose a further electron to form the corresponding radical which can also delocalize. In reference to this property, phenolic compounds have radical scavenging and antioxidant activity.[134,139] Phenolic is large and heterogeneous group of secondary plant metabolites that are distributed throughout the plant kingdom. Phenolics have a wide variety of structures, for example, flavonoids and tannins. Phenolic acids are the main phenolic compounds.[134] These properties were linked to the beneficial health functionality of antioxidants because of their inhibitory effects on the development of many oxidative-stress-related diseases.[126]

Phenolic compounds are able to enactment as antioxidants in a number of models. Phenolic hydroxyl groups are good hydrogen donors*:* Hydrogen-donating antioxidants can react with reactive oxygen and reactive peroxide species.[140,141] The determination reactive break the cycle generation of new radicals. The interaction of the OH of phenolic with the electrons of the benzene ring gives the molecules special properties. The most notably the ability to generate free radicals is stabilized by delocalization. The formation of these relatively long-lived radicals is able to modify radical-mediated oxidation processes.[113,142] The antioxidant capacity of phenolic compounds is also attributed to their ability to chelate metal ions involved in the production of free radicals.[143] However, Phenolics can act as pro-oxidants by chelating metals in a manner that maintains or reduces metals, thus increasing their ability to form free radicals.

4.5 The Correlation between Free Radical and Cancer

Free radicals are one of the leading causes of death in humans. Free radicals cause different types of chemical changes in DNA, so they could be mutagenic and involved in the etiology of cancer.[144] Cancer cells in particular when compared to normal cells, have higher levels of ROS and more susceptible to mitochondrial dysfunction due to their higher metabolic rate.[145] Cancer cells display elevated levels of oxidative stress due to the activation of oncogenes and loss of tumor suppressors.[146] ROS by altering the growth signals and gene expression cause continuous proliferation of cancer cells.[147] ROS can damage DNA by inducing base modifications, deletions, strand breakage, chromosomal rearrangements and hyper and hypo-methylation of DNA.[148]

Mushrooms have been highlighted not only as chemopreventive, but it also is a potential anticancer substance. Flavones are a subclass of natural flavonoids reported to have anticancer and antioxidant activity. The common active compound was 3’, 4’, 5-trihydroxyflavone, especially against A549 and MCF-7 cell lines. The correlation between antioxidant and anti-cancer activity was only moderate, and it was determined for A549 and U87 cancer cell lines. The most important fragment for those two effects is the ortho-dihydroxy group in ring B.[149]

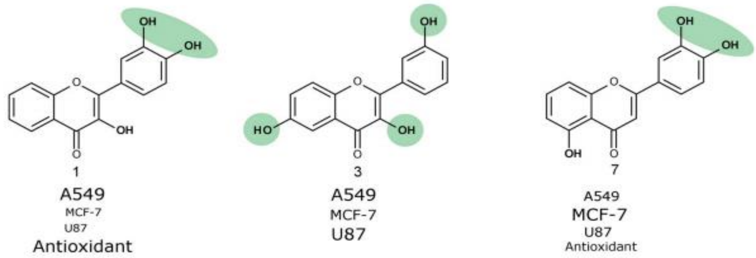


Figure 5 Most Active Trihydroxylflavones in Cancer Cell Viability and the Free Radical Scavenging Assay

4.6 Assays for Anti oxidative Capability

Numbers of studies on the determination of antioxidative capability of natural products have been reported.[150] Antioxidative capability is determined by the amount of given free radical scavenging by sample. Based on the chemical reaction involved, there are two types of assays. In the reaction of the hydrogen atom transfer, the antioxidant and substrate for instance, a biomolecule compete for the reactive species, thus inhibiting is substrate oxidation.[151] The assay measures the ability of an antioxidant to quench free radicals and is described by two parameters: oxygen radical absorbance capacity and total radical-trapping antioxidant parameter.[152] The hydrogen atom transfer assay is preferred in lipid oxidation and other biological systems. The second approach is associated with electron transfer:

Oxidant + *e* (from antioxidant) → reduced probe + oxidized antioxidant

In these assays, the antioxidant interacts with the reactive species, causing a color change of the reaction medium. The degree of color change is considered to be proportional to the antioxidant concentration. The reaction endpoint is reached when the cooler no longer changes. After the substrate is oxidized under standard conditions, either the rate or oxidation is measured. This type of assays includes ferric ion reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) colorimetric assays.[153] The above assays, especially ABTS and DPPH assays have been widely used in the monitoring of anti-oxidative capability because the handling of the above compounds is easier than that of the other reactive oxygen species.[121]

4.6.1 The DPPH method

DPPH• (2,2-diphenyl-1- picrylhydrazyl) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. Thus, DPPH• does not combine with a similar molecule to form a dimer as happens with most free radicals. The occurrence of a purple color is determined by delocalization on the DPPH• molecule, having an absorption band with a maximum around 517 nm. When DPPH•

reacts with a hydrogen donor, the reduced molecule DPPH is generated, accompanied by the disappearance of the violet color. Therefore, the absorbance diminution depends linearly on the antioxidant concentration. Ascorbic acid is used as standard antioxidant. DPPH was applied to antioxidant capacity determination in natural products.[153] The advantages of using a stable free radical like DPPH are its commercial availability. There is no need to generate it with another oxidant, and the exact quantity of the radical required for the study is easy to monitor. DPPH has a strong absorption band at the wavelength of 517 nm in methanol, which does not overlap with the spectra of flavonoids and phenolic acids.[154] The principle of DPPH assay descried in figure 6.

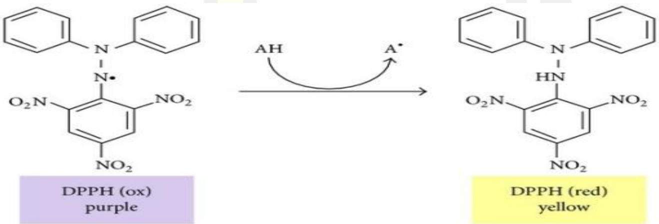


Figure 6 DPPH Free radical Conversion to DPPH by Antioxidant Compound

4.6.2 The ABTS Method

The ABTS cation radical (ABTS•+) which absorbs at 734 nm is formed by the loss of an electron by the nitrogen atom of ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)). In the presence of ascorbic acid, the nitrogen atom quenches the hydrogen atom is yielding the solution decolonization. ABTS can be oxidized by potassium persulphate giving rise to the ABTS cation radical ($ABTS•^{+}$) whose absorbance diminution at 743 nm was monitored in the presence of ascorbic acid chosen as standard antioxidant.[155,156] The principle of ABTS assay described in figure 7.

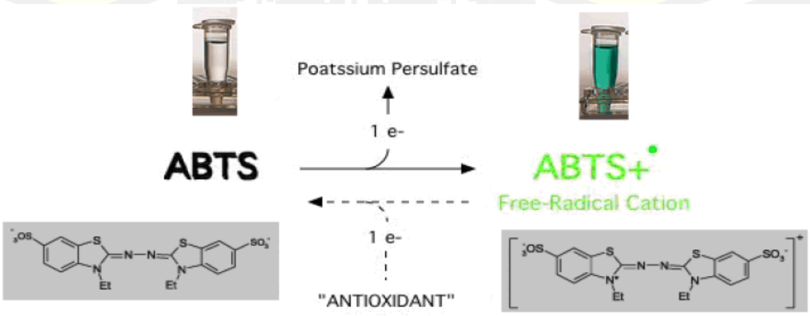


Figure 7 ABTS Chemical Reaction

4.6.3 The FRAP Method

The FRAP method relies on the reduction by the antioxidants of the complex ferric ion-TPTZ (2, 4, 6-tri (2-pyridyl) - 1, 3, 5-triazine). The binding of Fe^{2+} to the ligand creates a very intense navy blue color. The absorbance can be measured to test the amount of iron reduced and can be correlated with the number of antioxidants. Trolox or ascorbic acid or ferrous sulfate were used as references.[157,158] The results were expressed as Trolox or ascorbic acid or ferrous sulfate equivalent antioxidant capacity. The principle of FRAP assay described in figure 8.

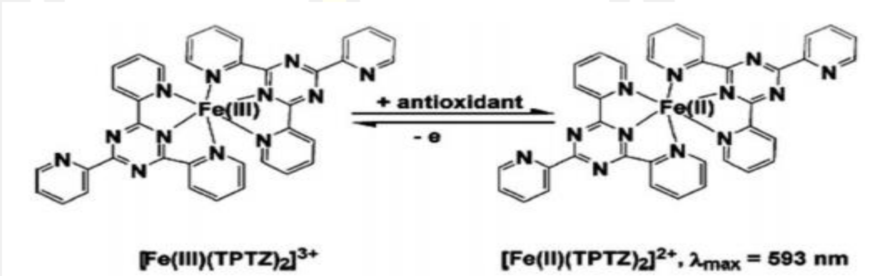


Figure 8 Antioxidant Reaction with Ferric Salt, Fe (III)$(TPTZ)_2Cl_3$ (TPTZ) 2,4,6-Tripyridyls-Triazine

A brief summary of the experimental conditions for the various methods of evaluating the antioxidant capacity is shown in Table 4.[153]

Table 4 Summary of Methods for the Determination of Antioxidant Activities.

Antioxidant capacity assay	Principle of the method	End-product determination
DPPH	Antioxidant reaction with an organic radical	Colorimetry
ABTS	Antioxidant reaction with an organic cation radical	Colorimetry
FRAP	Antioxidant reaction with a Fe(III) complex	Colorimetry
PFRAP	Potassium ferricyanide reduction by antioxidants and subsequent reaction of potassium ferrocyanide with Fe^{3+}	Colorimetry
CUPRAC	Cu (II) reduction to Cu (I) by antioxidants	Colorimetry
ORAC	Antioxidant reaction with peroxyl radicals, induced by AAPH (2,2’-azobis-2-amidino-propane)	Loss of fluorescence of fluorescein

HORAC	Antioxidant capacity to quench OH radicals generated by a Co(II) based Fenton-like system	Loss of fluorescence of fluorescein
Fluorimetry	Emission of light by a substance that has absorbed light or other electromagnetic radiation of a different wavelength	Recording of fluorescence excitation/ emission spectra
Gas chromatography	Separation of the compounds in a mixture is based on the repartition between a liquid stationary phase and a gas mobile phase	Flame ionization or thermal conductivity detection
High performance liquid chromatography	Separation of the compounds in a mixture is based on the repartition between a solid stationary phase and a liquid mobile phase with different polarities, at a high flow rate and pressure of the mobile phase	UV-VIS (e.g. diode array) detection, fluorescence, mass spectrometry or electrochemical detection

5. Inflammation

Inflammation usually occurs when infectious microorganisms such as bacteria, viruses or fungi invade the body reside in particular tissues and/or circulate in the blood. Inflammation is a physiological response to injury, characterized by loss of function and pain, heat, redness and swelling. It is usually associated with the pathogenesis of diseases such as diabetes, arthritis, obesity, metabolic syndrome, cancer, and several cardiovascular diseases.[159] An immune stimulant causes the pro-inflammatory cells such as macrophages and monocytes to start to secrete a number of inflammatory mediators such as interleukins (IL 1β, IL-6, IL-8), tumor necrosis factor (TNF-α), nuclear factor-κB (NF-κB), intercellular adhesion molecule-1 (ICAM- 1), inducible type cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), 5-lipoxygenase (5-LOX), and inducible nitric oxide synthase (iNOS).[20] Uncontrolled products of these inflammatory mediators have been known to cause several cells damage and also initiate the inflammation process.[160] Nitric oxide (NO) is a signaling molecule that plays a key role in the pathogenesis of inflammation. It gives an anti-inflammatory effect under normal physiological conditions. On the other hand, NO is considered as a pro-inflammatory mediator that induces inflammation due to overproduction in abnormal situations. NO is synthesized and released into the endothelial cells with the help of NOSs that convert arginine into citrulline producing NO in the process. Oxygen and

NADPH are necessary co-factors in such conversion. NO is believed to induce vasodilatation in the cardiovascular system and furthermore, it involves immune responses by cytokine-activated macrophages, which release NO in high concentrations. In addition, NO is a potent neurotransmitter at the neuron synapses and contributes to the regulation of apoptosis. NO is involved in the pathogenesis of inflammatory disorders of the joint, gut and lungs. Therefore, NO inhibitors represent an important therapeutic advance in the management of inflammatory diseases.[22]

5.1 Chemical Mediators of Inflammation

The inflammatory response occurs when tissue are injured by stimulation of infection and toxin, chemical factors released upon this stimulation bring about the vascular and cellular changes. The chemicals originate primarily from blood plasma, white blood cells as basophils, neutrophils, monocytes, macrophages and as platelets, mast cells, endothelial cells lining the blood vessels, and damaged tissue cells. Mediators may be produced locally by cells at the site of inflammation, or may be derived from circulating inactive precursors that are activated at the site of inflammation (Table5).[161] Cell-derived mediators are generally stored in intracellular granules and secreted fast upon cellular activation, such as histamine in mast cells, or created de novo in response to a stimulus, such as prostaglandins, nitric oxide, and cytokines produced by leukocytes and other cells. (Figure 9). Most mediators act by binding to specific receptors on different target cells. Mediators may act on only one or a very few cell types, or they may have diverse actions, with differing outcomes depending on which cell type they affect. Other mediator as lysosomal proteases has direct enzymatic and toxic activities that do not require binding to specific receptors. The actions of most mediators are tightly regulated and short-lived. For example, arachidonic acid metabolites are inactivated by enzymes as kininase inactivates bradykinin, eliminated such as antioxidants scavenge toxic oxygen metabolites, or are inhibited such as complement regulatory proteins block complement activation.[162]

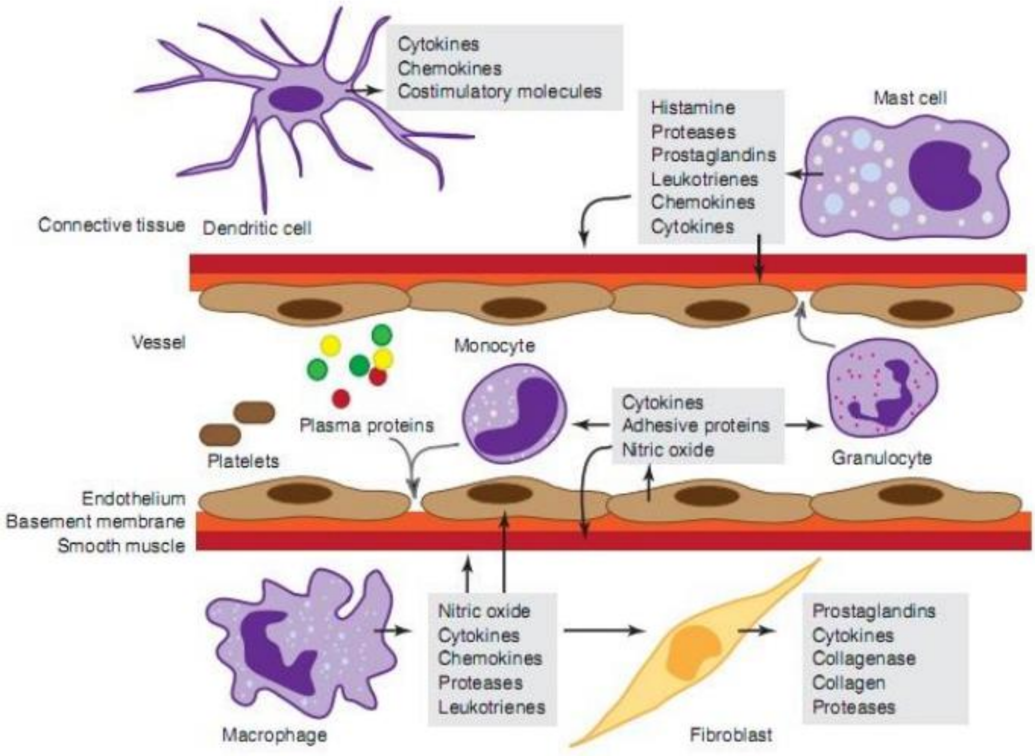


Figure 9 Cells and Mediators of the Inflammatory response Molecules derived from Plasma Proteins and Cells in response to Tissue Damage or Pathogens mediate Inflammation by Stimulating Vascular changes Plus Leukocyte Migration and Activation

Table 5 Actions of the Principal mediators of the Inflammatory Response.

Mediator	Sources	Actions
Histamine	Mast cells, basophils, platelets	Vasodilation, increased vascular permeability, endothelial activation
Serotonin	Platelets	Vasoconstriction
Prostaglandins	Mast cells, leukocytes	Vasodilation, pain, fever
Leukotriene	Mast cells, leukocytes	Increased vascular permeability, chemotaxis, leukocyte adhesion and activation
Platelet activating factor	Mast cells, leukocytes	Vasodilation, increased vascular permeability, leukocyte adhesion, chemotaxis, degranulation oxidative burst
Reactive oxygen species (ROS)	Leukocytes	Killing of microbes, tissue damage
Mediator Sources Actions	Mediator Sources Actions	Mediator Sources Actions
Nitric oxide	Endothelium, macrophages	Vascular smooth muscle relaxation; killing of microbes
Cytokines (TNF, IL-1, IL-6)	Macrophages, endothelium cells, mast cells	Local: endothelial activation (expression of adhesion molecules) Systemic: fever, metabolic, abnormalities, hypotension (shock)
Chemokine	Leukocytes, activated macrophages	Chemtaxis, leukocyte activation

5.2 Anti-inflammatory Assay

5.2.1 Nitric Oxide Assay

The Griess reaction is a simple technique that is widely used for quantification/detection of NO. The basic reaction involves reacting sulphanilamide and N-(1- naphthyl) ethylenediamine (NED) to form a stable compound. The absorbance of this compound at 540 nm is directly proportional to the nitrite concentration in the sample. Several in vitro measurements of NO production in lipopolysaccharide (LPS) stimulated RAW 264.7 cells have been reported by several authors in the past. This is one of the possible ways to screen various extracts and bioactive compounds with potential anti-inflammatory properties. RAW 264.7 cells are seeded in 96-well plates, they are then treated with different concentrations of the sample to be studied followed by stimulation with LPS. The cell culture supernatant is then transferred to a new plate followed by addition of sulphanilamide and NED solutions. The NO produced is determined by measuring the absorbance at 540 nm. This assay is one of the most common and widely used for evaluation of anti-inflammatory activity as reported by different authors.[163]

5.2.2 Cytokine enzyme-linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is used for the detection and quantification of proteins typically secreted or released from cells. This method is usually used for quantification of cytokines and other inflammatory mediators such as interleukin (IL-1β, IL-6, IL-8) and tumor necrosis factor (TNF-α), as reported in a number of publications.[20, 164] RAW 264.7 cells are usually plated in a 24-well plate in the culture medium, and then incubated with the sample to be screened at different concentrations. Cell culture supernatants are finally collected and assayed according to the instructions of the ELISA kit manufacturer to determine the amount of TNF-α and IL-6 released from the cells.

5.2.3 COX-1 and COX-2 catalyzed Prostaglandin Biosynthesis Assay

The cyclooxygenase enzymes have been extensively used to study the anti-inflammatory potential of natural agents. This is not a very common method for anti-inflammatory activity assessment, but it has been reported in some publications.[165] COX activity is usually determined based on the conversion of arachidonic acid to PGE2 and is expressed as a percentage of the control. RAW 264.7 cells are seeded in 96-well plates and incubated, then stimulated with LPS to induce the production of COX-2 and other inflammatory mediators. Induced cells are treated with different concentrations of the samples. Arachidonic acid is added and the cells are further incubated. The amount of PGE2 released in the medium can be determined with PGE2 enzyme immunoassay kit.

6. Phytochemistry

6.1 Extraction

Extraction is the important first step in the analysis of herbal medicinals because it is necessary to extract the desired chemical components from the materials for further separation and characterization. The basic operation included steps such as pre-washing, drying of materials, grinding to obtain a homogenous sample. The contact between sample surfaces with the solvent system is important for analytic extraction. The selection of solvent system largely depends on the specific nature of the bioactive compound is targeted. Different solvent systems are available to extract the bioactive compound from natural products.[166] The extraction of hydrophilic compounds uses polar solvents such as methanol, ethanol or acetone. For extraction of more lipophilic compounds, chloroform, dichloromethane or a mixture of dichloromethane/methanol in the ratio of 1:1 are used. In some sample, extraction with hexane is used to remove chlorophyll.[167] As the target compounds may be non-polar to polar and thermally labile, the suitability of the methods of extraction must be considered. Various methods, such as Sonification, heating under reflux, soxhlet extraction and others are commonly used for the samples extraction. In addition, plant extracts are also prepared by maceration or percolation of fresh green plants or dried powdered plant material in water and/or organic solvent systems.[168] The modern extraction techniques include solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, and solid-phase extraction.

6.1.1 Maceration

In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained. The marc is pressed, and the combined liquids are clarified by filtration or decantation after standing.[169]

6.1.2 Percolation

Percolation is more efficient than maceration because it is a continuous process in which the saturated solvent is constantly being replaced by the fresh solvent.

6.1.3 Reflux Extraction

Reflux extraction is more efficient than percolation or maceration and requires less extraction time and solvent. It cannot be used for the extraction of thermolabile natural products.

6.1.4 Soxhlet Extraction

Soxhlet extraction is a continuous extraction procedure that takes place in a special apparatus. The procedure is a very common technique for extracting organic compounds into a solvent and can be applied to solid and semisolid samples. For extraction, the solid material to be extracted is placed in a thimble made of thick filter paper or in a fritted crucible and introduced in the middle extracting part of the Soxhlet. The thimble is usually made from cellulose and is permeable to the solvent.[170] A consist of a Soxhlet apparatus is showed in figure 10.

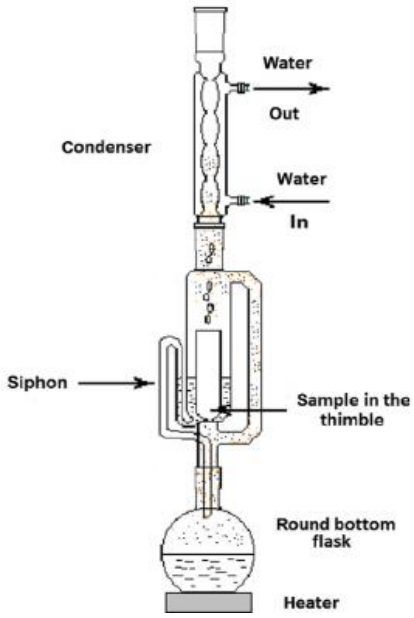


Figure 10 A Soxhlet Extraction Apparatus

A brief summary of the experimental conditions for the various methods of extraction is shown in Table 6.[171]

Table 6 A Brief Summaries of Various Extraction Methods for Natural Products

Method	Solvent	Temperature	Pressure	Time	The volume of organic solvent consumed	The polarity of natural products extracted
Maceration	Water, aqueous (aq) & non-aqueous solvents (nqs)	Room temperature	Atmospheric	Long	Large	Dependent on extracts
Percolation	Water, aq & nqs	Room temperature, occasionally under heat	Atmospheric	Long	Large	Dependent on extracts
Decoction	Water	Under heat	Atmospheric	Moderate	none	Polar compound
Reflux-extraction	aq & nqs	Under heat	Atmospheric	Moderate	Moderate	Dependent on extracts solvent

Table 6 (Continued)

Method	Solvent	Temperature	Pressure	Time	The volume of organic solvent consumed	The polarity of natural products extracted
Soxhlet-extraction	Organic solvents	Under heat	Atmospheric	long	Moderate	Dependent on extracts solvent
Method	Solvent	Temperature	Pressure	Time	The volume of organic solvent consumed	The polarity of natural products extracted
Supercritical fluid-extraction	Supercritical fluid (usually CO_2)	Near room temperature	High	short	None/ small	Nonpolar to moderate Polar compound
Ultrasound-assisted-extraction	Water, aq & nqs	Room temperature/ under heat	Atmospheric	short	Moderate	Dependent on extracts solvent
Microwave-assisted extraction	Water, aq & nqs	Room temperature	Atmospheric	short	none/ Moderate	Dependent on extracts solvent
Hydro-distillation and steam distillation	water	Under heat	Atmospheric	long	none	Essential oil (usually non-polar)

6.2 Methods for Drying of Fluid Extracts

Drying method for extracts include; drying in Vacuum ovens, spray drying and freeze-drying.[172]

6.2.1 Drying in Vacuum Ovens

Vacuum ovens are frequently used in development laboratories for the drying of small samples, especially when the heat stability of the drug or formulation is unsure. The general temperature for drying should be between 60-70 ºC. Lower temperatures up to 50 ºC may be required depending upon the stability of the material.

6.2.2 Spray Drying

The spray drier can be used for drying almost any substance in solution or in suspension. It is most useful for thermolabile materials, especially if handled continuously and in large quantities. The spray drier provides a large surface area for heat and mass transfer by atomizing the liquid to small droplets. The liquids are sprayed into a stream of hot air so that each droplet dries to an individual solid particle. The particles have a characteristic shape in the form of cavernous spheres sometimes with a small hole. This arises from the drying process, as the droplet enters the hot air stream and dries on the outside to form an outer crust with liquid still in the center. This liquid then vaporizes, and the internal vapor escapes by blowing a hole in the sphere.[173]

Advantages of the spray drying process include very short drying times and the temperature of the particles is kept low due to rapid evaporation. The characteristic particle form gives the product a high bulk density and in turn, rapid dissolution. The product formed is also free-flowing with almost spherical particles, and is especially convenient for tablet manufacture as it has excellent flow and compaction properties. Disadvantages of the spray drying process include the bulky and expensive nature of the equipment used and low overall thermal efficiency, as the air must still be hot enough when it leaves the drier to avoid condensation of moisture. Also, large volumes of heated air pass through the chamber without contacting a particle, thus not contributing directly to the drying process.[174]

6.2.3 Freeze Drying

Freeze-drying is a process typically used to remove residual solvent from material to produce a dry powder that can be easily loaded into a cell.[175] Freeze-drying is a process where a product is dried at a low temperature and with a vacuum. The water in the sample is first frozen to a solid and then removed directly by turning the ice into vapor. This is done under vacuum and without having to pass through the liquid phase. The major advantage of freeze-drying practice is that drying takes place at very low temperatures, so that enzyme action is inhibited and chemical decomposition, particularly hydrolysis, is minimized. The two main disadvantages of freeze-drying are first the porosity of the product, which makes it very hygroscopic. Unless products are dried in their final container and sealed in situ, packaging requires special conditions. In addition, the process is very slow and uses a complicated plant, which is very expensive. As a result, it is not a generic method of drying, but rather one that is limited to particular sorts of precious materials that cannot be dried any other way due to their heat sensitivity.[176]

6.3 Isolation

Chromatography is a useful technique for the separation of compounds from a complex mixture such as a mushroom extract. Based on the physical and chemical properties of compound was contained in the sample. Some solid phase materials, a mixture can be separated into its individual compound and solvent system. The chromatography technique was very important for separating complex mixtures into pure compounds. It is necessary to repeat the technique, varying the parameters until compounds of sufficient purity for elucidating the structure. The most common technique employed in the separation and analysis of low molecular weight secondary metabolites is reverse-phase HPLC. Using the same HPLC system with a larger column, semi-preparative or preparative HPLC can be used to isolate and purify individual compounds from a mixture. The larger sized column allows for the introduction of larger samples into the column. Individual peaks can then be collected once they exit the detector.

Isolation of pure metabolites is essential for structural elucidation. Once a pure metabolite has been isolated, mass spectrometry along with 1D and 2D NMR experiments are used to unambiguously elucidate the chemical structure.

6.3.1 Chromatographic Procedures Using Columns

Column chromatography is the oldest form of chromatographic technique. A tube is packed with a solid stationary phase, the sample mixture is applied to the top of the column and the mobile phase is allowed to move down through the column and the samples were eluted by using the mobile phase.

6.3.1.1 Conventional Column Chromatography

Conventionally, the stationary phases in column chromatography were adsorbents of high polarity, e.g. silica gel. However, there are many other forms of stationary phase, e.g. reverse-phase silica, ion-exchange resins, and exclusion medium. The mobile phase passes through the column under the force of gravity and, due to the resistance of the stationary phase packed in the column, the flow rate is not very high. Elution with the mobile phase can be isocratic or gradient. The gradient elution consists of a sequence of different compositions of the mobile phase, most commonly as a gradient of increasing polarity. The gradient is achieved most commonly as a series of steps consisting of aliquots of the mobile phase of fixed composition. More sophisticated systems are now available where a linear gradient is established by means of a mixing pump which uses two separate reservoirs to deliver a constantly increasing concentration of one component in the eluting solvent.

The bands of compounds which eventually eluted from the column cannot be seen unless colored substances are being separated. The usual method of detecting the zones of eluted compounds is to collect the eluted from the column, usually performed automatically using a fraction collector, these fractions can then be analyzed each method.

The most commonly used adsorbents for gravity column chromatography such as silica gel and Sephadex. Silica gel is an acidic adsorbent, being suitable for the chromatography of neutral or acidic components. Meanwhile, the silica gel is also a kind of weak acid cation exchanger, with the silanol group on the surface being able to release weakly acidic hydrogen ions. When meeting a strong alkaline compound, it is capable of absorbing the alkaline compound due to the ion exchange reaction. Silica gel has been widely used as a stationary phase for conventional column chromatographic separations of relatively low polar compounds. The mode of separation is based adsorption into its poly hydroxyl activities sites. The gel itself may be rather acidic or neutral compounds. Silica gel is not recommended for separations of very polar compounds since irreversible adsorption may occur.

Sephadex LH-20 is a liquid chromatography media designed for molecular sizing of natural products such as steroids, terpenoids, lipids, and low molecular weight peptides. Sephadex LH-20 is beaded, cross-linked dextran which has been hydroxypropylated to yield a chromatographic media with both hydrophilic and lipophilic character. Sephadex LH-20 swells in water and a number of organic solvents. Sephadex LH-20 chromatography has been used successfully to separate many height molecules (steroid), particularly the unconjugated biologically active ones. Sephadex LH-20 is commonly employed in the separation of hydrophilic compounds from various plant extracts, chiefly using aqueous methanol, ethanol, and dichloromethane as eluents. Because no material is wasted during separation, Sephadex LH-20 is often used. Sephadex can be re-used many times.

6.4 Identification

6.4.1 Chromatographic Techniques

6.4.1.1 Thin-layer Chromatography (TLC)

TLC is a simple, quick and inexpensive procedure that gives the researcher a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the Ration time factor (R_f) of a compound is compared with the R_f of a known compound. Additional tests involve the spraying of phytochemical screening reagents, which cause color changes according to the phytochemicals existing in a crude extract; or by viewing the plate under the UV light. This has also been used for confirmation of purity and identity of isolated compounds.[168]

6.4.1.2 High Performance Liquid Chromatography (HPLC)

Identification of compounds by HPLC is an important part of an HPLC assay. In order to identify any compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph. The identifying peak should have a reasonable retention time and should be well separated from outside peaks at the detection levels which the assay will be performed. UV detectors are popular among all the detectors because they offer high sensitivity.[177] The high sensitivity of UV detection is a bonus, if a compound of interest is only present in small amounts within the sample. Besides UV, other detection methods are also being employed to detect phytochemicals among which is the diode array detector (DAD) coupled with a mass spectrometer (MS).[178] Liquid chromatography coupled with mass spectrometry (LC/MS) is also a powerful technique for the analysis of complex botanical extracts.[179]

6.4.2 Spectroscopy

6.4.2.1 Mass Spectrometry (MS)

The mass of a pure compound is determined using mass spectrometry (MS). Often a gas or liquid chromatography (GC or LC respectively) system is coupled to a mass spectrometer. Coupling an LC system to electrospray ionization (ESI) MS system allows for the sample to be dissolved in a solvent, introduced into the LC system, separated into its individual components, and then transferred to the gas phase and ionized before entering the mass analyzer.[180] ESI-MS is a useful technique for analyzing high molecular weight biomolecules as well as small non-volatile compounds. It is especially useful when trying to characterize unknown compounds, since it may not be known if the compounds are thermally stable. ESI is a soft ionization technique that, when operated in positive mode, often produces the protonated molecular species ($[M+H]^+$) for a variety of different types of compounds.[181] The mass spectrum analysis can also provide other useful information about the compound being studied. For example, molecules containing chlorine or bromine atoms will display two molecular ion peaks; one for each of its commonly occurring isotopes. This is referred to as the molecular ion cluster.[182] When either chlorine or bromine is present, the heavier isotopes are two mass units heavier than the lighter isotope, resulting in a large M^{+2} peak. The relative intensities of the M^{+2} peaks should be consistent with the relative abundance of the isotope.[180] Mass Spectrometer is a very sensitive technique, critical to the analysis of secondary metabolites.

6.4.2.2 Nuclear Magnetic Resonance (NMR)

In conjunction with the mass of the compound, both ^{13}C and ^{1}H NMR data are necessary to properly characterize a metabolite. Obtaining a 135-DEPT, DEPT 90 spectrum can also be useful since it can help to assign carbons as quaternary, methylene, methyl or methane. To be able to correlate proton signals with their corresponding carbon signals, or to determine whether they belong to an amine or hydroxyl group, hetero nuclear correlation spectroscopy is employed.[182,] Hetero nuclear chemical shift correlation (HETCOR), or hetero nuclear single-quantum correlation (HSQC) reveal the correlation between protons and carbons with the two-dimensional plot. HSQC is a ^{1}H-detected experiment whereas HETCOR is an X-detected experiment (here ^{13}C). HETCOR would be a more useful technique when the carbon spectrum is crowded and better resolution of that parameter is required.[180] Hetero nuclear multiple bond correlation (HMBC) shows two, three and four bond correlations between protons and carbons, suppressing the one bond correlation. This gives much-needed information about the connectivity of carbons. Additional 2D techniques such as COSY and NOESY are often required when analyzing complicated or novel compounds.[183]

6.4.2.3 UV-Visible Spectroscopy (UV-VIS)

Ultraviolet and visible spectroscopy is of limited use in characterizing compounds, however, along with infrared spectroscopy and NMR can provide valuable structural information to support a potential structure. It is possible to correlate some absorption in the UV-VIS wavelengths range with features such as the presence of alkenes, and carbonyls.[180]

6.4.2.4 Infrared Spectroscopy (IR)

Each type of bond in a molecule absorbs a different frequency of energy. Therefore, some characteristic absorption in the IR can give valuable information on the structure of the compound being analyzed. The information from IR used the presence of a broad O-H stretch in the range of 3400-2400 cm^{-1}. A carbonyl stretch between 1730 and 1700 cm^{-1} indicates the presence of a carboxylic acid. If two compounds are identical, it is not possible to use an IR spectrum to identify an unknown metabolite, however, it can be used to confirm the presence of some chemical properties.[180]

7. Related Articles

7.1 Anticancer Activity

Wu et al have reported the anticancer effects of hispolon on lung cancer cells. The method descriptions of cell viability were evaluated by the MTT assay. Cell cycle and apoptosis assays were assessed by flow cytometers. The results of the study showed that hispolon can decrease cell viability in a dose and time-dependent manner. The cell cycle distribution showed that hispolon enhanced the accumulations of the cells in the G0/G1 phase. In addition hispolon induced cell apoptosis through activation of the mitochondrial pathway at least 40 µM.[47]

Li et al studied the antitumor effect of polysaccharides extracted from *P.linteus* on Hep2 cells. The study showed that at the concentration of 200 mg/kg, The volume and weight of solid tumors were significantly decreased when compared with the control mice.[184]

A study was conducted by Song et al to reveal the anti-proliferative and anti-metastatic activity of the ethanol extract from the fruiting body of *P. igniarius* on human hepatocarcinoma SK-Hep-1 cells and rat heart vascular endothelial cells. The results of the study showed that an ethanol extract of *P. igniarius* (at 25 µg/mL) has anti-proliferative and anti-metastatic activities. [185]

Wang et al reported that polysaccharides from *P.linteus* could have a good performance in inhibiting HepG2 human hepatocellular carcinoma cells. The results showed that the polysaccharides with a concentration of 0-1.0 mg/mL markedly suppressed the proliferation of HepG2 cells by 50 percent in a dose and time-dependent manner.[74]

Amuamuta et al investigated the anti-CCA capability and the toxicity of the crude extract of *Kaempferia galangal* Linn (Rhizome) in vitro and in animal models. The results demonstrated that the extract had a stronger cytotoxicity activity with an IC_{50} of 64.2 μg/mL, when compared to the standard reference, 5-FU (IC_{50} = 107.1 μg/mL). Toxicity tests revealed that the animal was well tolerated up to a maximal single oral dose of 5 g/kg body weight and a daily dose of 1 g/kg body weight for 30 days. The extract exhibited promising anti-CCA activity in CL6-xenografted nude mice by inhibiting tumor growth and lung metastasis, as well as prolonging survival time.[12]

Sombatsri et al reported testing the cytotoxicity activity against cholangiocarcinoma and HepG2 cell lines of bioactive compounds. The results showed that buxifoliadine C, N-methylatalaphyllinine, and buxifoliadine E were cytotoxic to the KKU-M156 cell line with IC_{50} values ranging from 3.39 to 4.1 μg/mL, whereas cytotoxicity to the HepG2 cell line had IC_{50} values ranging from 1.43 to 8.4 μg/mL.[106]

Ajith AT et al investigated the cytotoxicity and anticancer properties of *Phellinus rimosus*, which the methods of study were determined by using ascites tumor and solid tumor models. The results of the studies showed that the ethyl acetate and methanol fractions of *P. rimosus* extracts were shown to have cytotoxic activity against cancer cells. The half maximal inhibitory concentration of ethyl acetate extract was 184 ± 3.4 μg/mL and 92 ±10.4 μg/mL for Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines, respectively.[186]

Ajith AT et al reported that they tested the antimutagenic activity of ethyl acetate from *P. rimosus*. The antimutagenic activity was determined using *Salmonella Typhimurium strains* TA 98, TA 100, TA 102 and TA 1535. The methods were determined all antimutagenic activity against mutagens needing activation and antimutagenic assay using direct acting mutagens. The results of this study were that the ethyl acetate extract of *P. rimosus* showed significant antimutagenic activity against both direct acting mutagens and mutagens that require activation. At 2 mg/plate, the extract inhibited sodium azide (NaN_3) induced revertants of *Salmonella* strains TA 100, TA 102, and TA 1535 by 34 %, 50 %, and 66.5 percent, respectively.[187]

Ajith AT et al reported the anticarcinogenic activity of ethyl acetate extract from *P. rimosus.* The study was the determination of antipromotional activity using two-stage carcinogenesis. The result of the study was the topical application of ethyl acetate extract inhibited skin papilloma initiated by 7, 12-dimethyl benzyl [α] anthracene (DMBA) and promoted by croton oil on mouse skin. Group of animals applied with croton oil and DMBA showed 87.5 percent tumor incidence at 15 weeks after DMBA treatment. Application of ethyl acetate extract of *P. rimosus* prior to croton oil reduced the percent of incidence. Topical application of extract at a dose of 1 mg showed a 62.5 percent incidence at 15 weeks and at a dose of 5 mg showed a 37.5 percent incidence at 15 weeks.[188]

7.2 Antioxidant Activity

Yoon et al studied the evaluated antioxidant and antimicrobial activities of methanol (ME) and water extract obtained from the fruiting bodies of *P. gilvus* collected in Korea.The results showed that methanol extracts from *P. gilvus* had 93.65 percent free radical scavenging activity on DPPH at 2 mg/mL, which was equivalent to the positive control like butylated hydroxytoluene (BHT), at the same concentration.[189]

Judprakob et al reported the antioxidant and antimutagenic activities of *P. rimosus*. The objective of the study was to investigate the antioxidant and antimutagenic activities of ethanol, water, and procedures derived from the alkaloid extract of *P. rimosus*. The result of the study was that ethanol extracted, water, and alkaloid showed the best antioxidant activity at IC_{50} values of 8.26 ± 1.4, 20.12 ± 3.65, 94.15 ± 9.08 µg/mL, respectively.[90]

Jeon et al reported the bioactive phytochemicals from the fruiting body of *P. linteus*. The study was investigated using the on-line HPLC-DPPH system. The results of the studies were davalliallactone, interfungins showed the strongest inhibitory effect against the DPPH radical. On comparison with quercetin (IC_{50}, 44.0 µM), the IC_{50} value of davalliallactone, interfungins for DPPH radical-scavenging capacity were 19.6 µM and 18.5 µM, respectively.[88]

Lee et al reported that the hispidin was isolated and screened from the mycelial culture broth of *P. lineteus* to assess its antioxidant effect. The results indicated that 1.0 mM hispidin exerted an antioxidant effect on superoxide anion radical, hydroxyl radical and DPPH radical at the rates of 56.8 percent, 95.3 percent and 85.5 percent, which was similar to the positive control α-tocopherol, but no significant effect on the hydrogen peroxide radical was observed.[190]

Ajith AT et al reported the evaluation of the antioxidant and antihepatotoxic activities of *Phellinus rimosus*. The ethyl acetate extract's superoxide anion scavenging, Fe2+/ascorbate induced lipid peroxidation inhibiting, hydroxyl radical scavenging, and nitric oxide scavenging activities were investigated. The results indicated that ethyl acetate extract of *P. rimosus* exhibited antioxidant activity with IC_{50} values at 22 ± 1, 68 ± 4.1, 162 ± 7 µg/mL for Superoxide, Hydroxyl and Lipid peroxidation inhibiting, respectively. The ethyl acetate extract of *P. rimosus* demonstrated potent antihepatotoxic activity in rat livers when exposed to carbon tetrachloride.[87]

7.3 Anti-inflammatory Activity

Huang et al studied the anti-inflammatory activity of inotilone isolated from *P. lineteus*. The results showed significant inhibition of NO production and iNOS protein expressions concentration-dependently.[191]

Kim et al discovered that the BuOH extract of *P. lineteus* had strong anti-inflammatory action, anti-nociceptive activity, and a good inhibitory effect on chick embryo chorioallantoic membrane angiogenesis, which methods were tested by the croton oil-induced ear edema test. More importantly, the BuOH extract showed the almost equivalent anti-inflammatory effect to indomethacin. In comparison to indomethacin (70.4 %), oral administration of n-BuOH extract of *P. lineteus* (100 mg/kg) reduced the amount of with by 35.9 percent.[81]

Heng et al studied the evaluation of the analgesic and anti-inflammatory effects of ethanol extract of *P. merrillii* (EPM) in the Institute for Cancer Research (ICR) mice. The analgesic effects of EPM were investigated by analyzing the acetic acid-induced writhing response and the licking time of hind paws following formalin injection. The results of the studies showed the treatment of male ICR mice with EPM (2 g/kg) significantly inhibited the number of writhing responses ($p < 0.001$). This inhibition by EPM (2 g/kg) was similar to that produced by positive control indomethacin (10 mg/kg) ($p < 0.001$). EPM (1 and 2g/kg) was significantly inhibited ($p < 0.001$) the formalin-induced pain in the late phase. EPM (1 and 2 g/kg) also inhibited the development of paw edema induced by CARR ($p < 0.05$).[83]

Ajith AT et al investigated the ability of *Phellinus rimosus* to protect rats from carbon tetrachloride-induced chronic hepatotoxicity: antioxidant defense mechanism. Chronic hepatotoxicity was induced by intraperitoneal injection of CCl_4 (1.5 mL=kg body weight.) in paraffin oil. Administration of ethyl acetate extract of *P. rimosus* 25 and 50 mg/kg body weight orally prior to CCl_4 injection significantly and dose-dependently protected the CCl_4-mediated elevation of serum transaminases such as glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT), and of serum alkaline phosphatase (ALP). Previous research has shown that the ethyl acetate extract of *P. rimosus* can protect against CCl_4 induced chronic hepatotoxicity in the rat via restoring the liver's antioxidant status inhibition.[192]

7.4 Antibacterial Activity

Sheena et al studied the antibacterial activity of the methanol extract of three polypore macrofungi including, *Phellinus rimosus*, *Ganoderma lucidum*, and *Navesporus floccosa*. The extracts were evaluated by hole-plate diffusion and microtitre plate dilution methods using *Escherichia coli*, *Pseudomonas aeuroginosa*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Bacillus subtilis*. The methanol extract of *P. rimosus* and *N. floccosa* showed activity against all strains at a concentration of 800 mg/well and 1 mg/well, respectively. The minimum inhibitory concentration (MIC) of *P. rimosus* and *N. flocossa* were found to be 500 µg/well and 1 mg/well, respectively.[193]

Hiralal et al studied the antifungal and antibacterial activity of *Phellinus* samples from the Western Ghats of India. The extracts from six species of *Phellinus* like *P. fastuosus*, *P. merrillii*, *P. aureobruneus*, *P. crocatus*, *P. lloydii*, and *P. sublinteus* were tested against *Acinetobacter calcoaceticus* (NCIB 2886), *Bacillus subtilis* (NCIM 2010) *Candida albicans* (ATCC 2091) virulent strains of bacteria and fungi. The results of the studies were *P. fastuosus* and *P. lloydii* showed the best anti-*Acinetobacter calcoaceticus* and *Candida albicans* activity, respectively.[194]

7.5 Toxicity Study

Ajith AT et al studied the acute and sub-acute toxicity studies of ethyl acetate, methanol and aqueous extracts from *P. rimosus.* The animal used in this study was Male Swiss albino mice weighing 25 ± 2 g used for the study. The results showed that animals administered with ethyl acetate, methanol and aqueous extracts of *P. rimosus* did not produce any external symptoms of toxicity or mortality up to a dose of 2.5 g/kg body weight orally. In sub-acute toxicity studies, treatment of extracts also did not produce any statistically significant change in the hematological or biochemical parameters when compared to the normal group of animals.[195]

Chapter III
Research Methodology

1. Contents

1.1. Chemicals and Equipments
Part 1: Phytochemistry Study
1 Extraction of the Mushrooms
2 Separation of the Mushrooms
3 Isolation and Identification of the Isolated Compounds
Part 2: Chemical Assays
1 Radical Scavenging Activity Assay (DPPH Scavenging Assay)
2 ABTS Assay
3 FRAP Assay
4 Total Phenolic Content Determination
5 Total Flavonoid Content Determination
Part3: Anti-inflammatory Activity Assays
1 Nitric Oxide Assays
2 MTT Assay
Part 4: Anticancer Activity Against Cholangiocarcinoma Cell Lines
1 Anticancer Activity Against Cholangiocarcinoma Cell Lines of the Crude Extracts
2 Anticancer Activity Against Cholangiocarcinoma Cell Lines of The Fractions from *Phellinus rimosus* Extracts

2. Chemicals and Equipments

2.1 Mushrooms and Cell Lines
-*Phellinus igniarius* (L.) Quél (MSUT 2931)
-*Phellinus linteus* (Berk. & M.A. Curtis) Teng (MSUT 2712)
-*Phellinus nigricans* (Fr.) P. Karst. (MSUT 2707)
-*Phellinus rimosus* (Berk.) Pilát (MSUT 2615)
-Cholangiocarcinoma Cell Lines (KKU-100)
-Cholangiocarcinoma Cell Lines (KKU-M213A)
- Mouse Leukemic Macrophage Cell Line (RAW 264.7 cell)

2.2 Chemical Reagents
-Ethanol (Commercial grade, Scitrading. Co. Thailand)
-Methanol (HPLC grade, Merck, Germany)
-Hexane (AR grade, KemAus, Australia)

-Dichloromethane (AR grade, Labscan, ireland)
-Ethyl acetate (AR grade, Merck, Germany)
-Fetal bovine serum (Analytical grade, Gibco, USA)
-Phosphate buffered saline (Analytical grade,Gibco, USA)
-Penicillin and streptomycin (Analytical grade, Gibco, USA)
-Sulforhodamine B (Analytical grade, Gibco, USA)
-Trypsin (Analytical grade,Gibco, USA)
-Dulbecco's Modified Eagle Medium (Analytical grade, Gibco, USA)
-Dimethyl sulfoxide (AR grade, Sigma Chemical, Germany)
-DPPH (1.1-diphenyl-2-picrylhydrazyl) (AR grade, Sigma Chemical, Germany)
-Ascorbic acid (AR grade, Merck, Germany)
-Rutin (AR grade, Sigma Chemical, Germany)
-Ferrous sulfate (Reagent/Food Grade, USA)
-2,2-azino-bis-c 3-ethylbenzene-thiazoline-6-sulfonic acid (AR grad, Sigma Chemical, Germany)
-Potassium persulfate (AR grade, Qrec, Newzealand)
-2,3,5-triphenyl-1,3,4-triaza-2-azoniacyclopenta-1,4-diene chloride (AR grade, Merck, Germany)
-Ferric chloride (AR grade, Qrec, New Zealand)
-Hydrochloric acid (AR grade, Merck, Germany)
-Folin-ciocalteu reagents (AR grade, Sigma Chemical, Germany)
-Sodium carbonate (AR grade, Merck, Germany)

2.3 Types of Equipment

-Balance 4 position (Sartorius LE 2445, Germany)
-Balance 5 position (Sartorius LE 2445, Germany)
-Rotary evaporator (Buchi V700, Switzerland)
-Freeze dryer (Crist Alpha 1-4, Germany)
-Cell culture hood (Thermo Fisher Scientific, Japan)
-Incubator (Thermo Fisher Scientific, Japan)
-Water bath (Memert, Germany)
-Centrifuge (Multifuge X1 Pro Centrifuge Series, Japan)
-Cell counters (Hemocytometer, B4005, India)
-Inverted microscope (Nicolet™ iN™ 5 FTIR Microscope, Japan)
-Autoclave (Remel™ Autoclave Bag, Thermo Scientific™, Japan)
-Refrigerator and freezer (Refrigerated Incubator (EU), 250 L, Powder-coated Stainless Steel, Thermo Scientific™, Japan)
-Thin-layer chromatography (Merck, Germany)
-Column Chromatography (Javener, China)
-Nuclear Magetic Resonannce (BruKer, Model : Ascend-400, Prodigy unit)

-Fourier Transform Infrared Spectrometer,(Perkin Elmer, Spectrum one, Japan)

-UV-visible spectrophotometer (Jasco V530, Japan)

-Mass Spectra (MAT 95 XL Mass Spectrometer, Germany)

3. Phytochemical Study

3.1 Extraction of the Mushrooms

The raw materials were obtained from the Natural Medicinal Mushroom Museum Thailand (MSUT) [*P. igniarius* (MSUT2931) *P. linteus* (MSUT2712), *P. nigricans* (MSUT2707) and *P. rimosus* (MSUT2615)]. A dried, grinded and powdered mushroom material was extracted by decoction with water or maceration (3 days) using 95 % ethanol. For the decoction method, each sample powder of 775 g of the mushroom was mixed with distilled water (3100 mL) and was decocted on hot plate at 100 ^{o}C for 4 hours. After filtration, the water extract was dried by using a freeze dryer at -98 ^{o}C for 26 hours (Figure 11). For the maceration method, a sample power of 1500 g was mixed with 95 % ethanol (6000 mL) in a big glass flask and stored at room temperature for 3 days and repeated 3 times (Figure 12). The liquid of extracts was filtered using gauze and Whatman no. 01 filter paper. The residue was compensated for an additional 72 hours and then filtered. The solvent used in the extraction was evaporated using a rotary evaporator and further concentrated by heating at 60 ^{o}C in a waterbath.[196] The crude extracts obtained were weighed and Percent yields were calculated. The extracts were stored in small glass bottles (covered with aluminum foil) at 4 °C until processed. The crude extracts were evaluated the biological activities and to be isolated to yield the pure compounds and identified by the spectroscopic techniques.

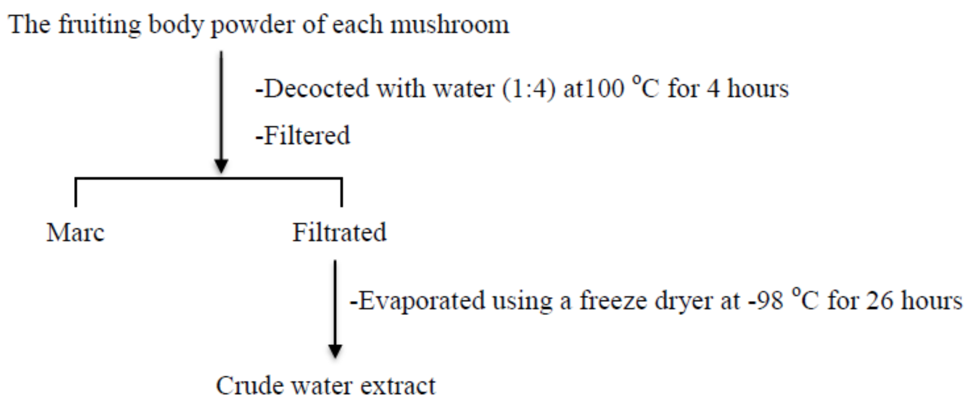


Figure 11 Extraction of Mushroom by the Decoction Method

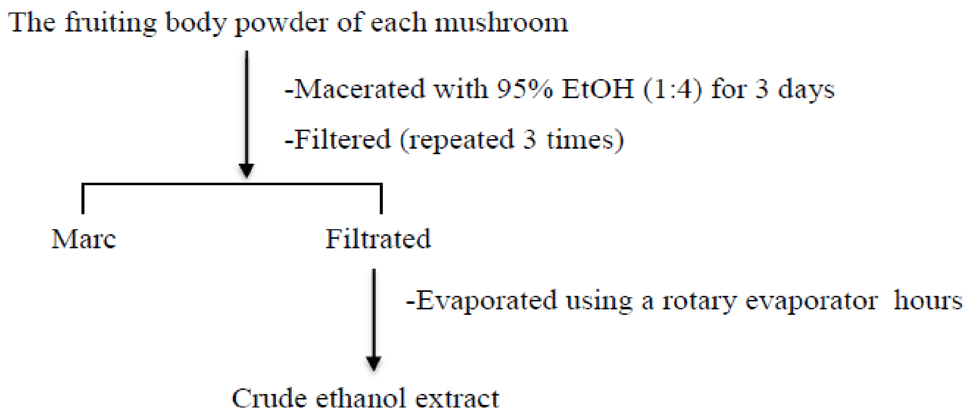


Figure 12 Extraction of Mushroom by the Maceration Method

3.2 Phytochemical Screening

All of the extract was subjected to a phytochemical analysis using standard methods. The qualitative results were expressed as (+) for the presence and (-) for the absence of phytochemicals.

3.2.1 Detection of Alkaloids: Five mg of extract was dissolved individually in 1 mL dilute hydrochloric acid and filtered. Then, the extract was detected using Mayer’s test, Dragendroff’s and Hager's test.

-Mayer’s Test: Filtrate was treated with Mayer's reagent. Formation of a yellow color precipitate indicated the presence of alkaloids.

-Dragendroff’s Test: Filtrate was treated with Dragendroff’s reagent. Formation of a red precipitate indicated the presence of alkaloids.

-Hager’s Test: Filtrate was treated with Hager’s reagent. The presence of alkaloids was confirmed by the formation of a yellow-colored precipitate.

3.2.2 Detection of Phenolic compounds

-Ferric Chloride Test: Extract was treated with 3-4 drops of ferric chloride solution. Formation of bluish-black color indicated the presence of phenolic compounds.

3.2.3 Detection of Flavonoids

-Lead acetate Test: Extract was treated with 3-4 drops of lead acetate solution. Formation of a yellow color precipitate indicated the presence of flavonoids.

-Shinoda Test: the extract was treated with 2-3 fragments of magnesium ribbon and HCl dropwise, pink scarlet, crimson red or occasionally green to blue color appeared after a few minutes.

3.2.4 Detection of Triterpenoids

-Liebermann-Burchard Test: We added 0.5 mL of chloroform and 2 mL of extract dissolved in distilled water. The mixture was added 1.5 mL con. H_2SO_4. A yellow ring that formed at the interphase, which turned reddish-brown after 2 minutes confirms the presence of Terpenoids.[197]

3.3 Separation of the Mushroom Extracts

The crude ethanol and water extracts were assayed for screening anticancer activity against cholangiocarcinoma cell lines (KKU-100 & KKU-M213A) using the SRB assay. Investigations of antioxidant activity and evaluation of the total phenolic compounds were performed using the DPPH scavenging, the ABTS assay, the FRAP assay, and the Folin-Ciocalteu reagent assay, respectively. The criteria for choosing the mushroom extracts for further separation and isolation of the pure compounds were: 1) the extract which possessed the highest activity when tested on antioxidant, anticancer anti-inflammatory activities and total phenolic compound. 2) Economic worthiness as well as the percentage of yield of the extraction. The extract showed fit criteria was subjected to further separation using solvent-solvent extraction with hexane, dichloromethane, ethyl acetate, and n-butanol, respectively.

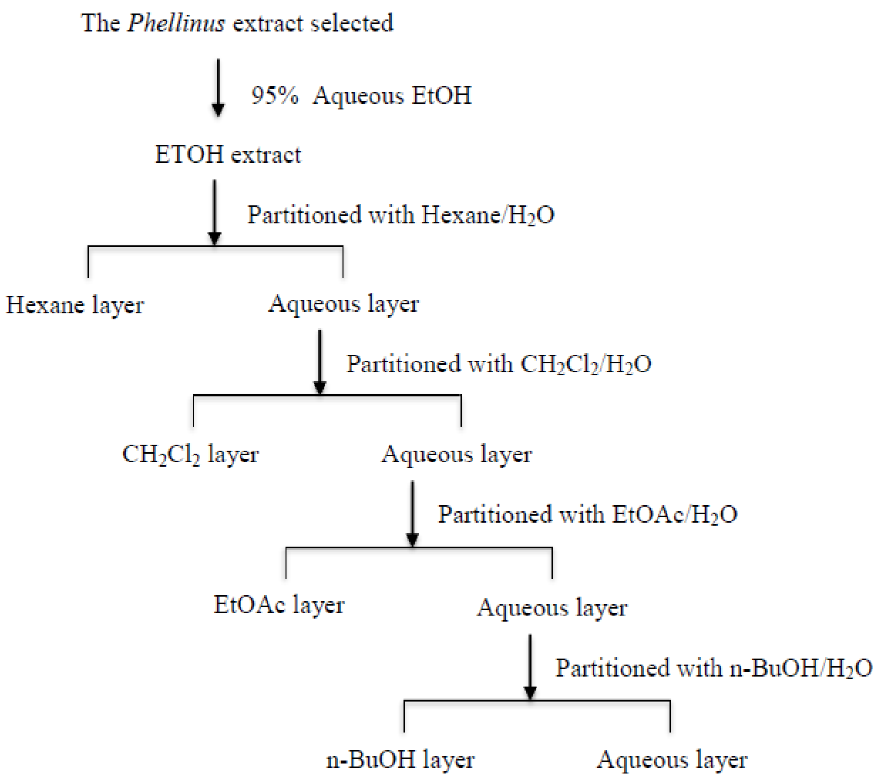


Figure 13 Separations of Ethanol Extract from *Phellinus* mushroom

3.5 TLC Fingerprint

The selected crude extract that fitted the criteria was chosen to separate by using the solvent-solvent separation technique (Figure 13). The TLC fingerprint was then examined using the TLC process. TLC studies of the mushroom fractions were carried out using two different solvent systems. The TLC plate was Aluminum oxide card (GF 254). Using a micropipette about 5 µL of the extract was loaded gradually over the plate and air-dried. The plate was developed in the mobile phase (dichloromethane: ethyl acetate: ethanol: formic acid; 10:1:1:1). Detection was an ultraviolet (254 and 365 nm) and spraying with an anisaldehyde-sulfuric acid solution or ferric chloride after heating at 100 ^{o}C for 5 min. The retention factor (Rf) values of mushroom in two different solvent systems were calculated by using the following formula.

Rf = Distance traveled by the solute (cm)/ Distance traveled by the solvent (cm)

The preparation Anisaldehyde-Sulphuric acid reagent was followed at below: 0.5 ml anisaldehyde was mixed with 10 mL glacial acetic acid, followed by 85 mL ethanol and 5ml concentrated sulphuric acid in that order (The reagent has only limited stability, and is no longer usable when the colour has turned to red violet). The plate was sprayed with anisaldehyde-Sulphuric acid reagent, heated at 100 ^{0}C for 5-10 minutes was observed in visible or in UV 365 for the detection of the terpenoids. The preparation 10 % methanolic ferric chloride reagent was follow by Wagner et al, 1984. The prepare 10g of $FeCl_3$ was dissolved in 100 mL of methanol. Formation of green colour after spraying with this reagent indicates the presence of Phenolic compounds.

3.6 Isolation of the Pure Compounds

The fraction with the highest anti-cancer activity was further isolated by using conventional column chromatography. The isolated compounds were identified by using spectroscopic techniques e.g. UV, IR, Mass, and NMR spectroscopy.

3.6.1 General Techniques

A) Analytical Thin-layer Chromatography (TLC)

Technique	: One dimension, ascending
Adsorbent	: Silica gel 60 F_{254} (20x20 cm, 0.25 mm. Merck) pre-coated plate
Layer thickness	: 0.2 mm
Distance	: 5.0 cm
Temperature	: Laboratory temperature (30-35 ^{o}C)
Detections	:1) Ultraviolet light (254 and 365 nm)
	:2) Spraying with anisaldehyde-sulfuric acid or ferric chloride solution and heating at 100 ^{o}C for 5 min.

B) Column Chromatography (CC)

Liquid column chromatography

Column : Flat bottom glass column (various diameters).

Adsorbent : Silica gel (Merck 60) particle size 70-230 mesh.

Solvent : Various solvent systems depending on materials.

Packing method : Dry and wet packing.

Sample loading : 1) Dry packing: The sample was dissolved in a small amount of suitable organic solvent, mixed with a small quantity of adsorbent triturated, dried and then placed gently on top of the column.

: 2) Wet packing: The sample was dissolved in a small amount of eluent and then applied gently on top of the column.

Detection : Fractions were examined by TLC technique in the same manner as described in section A. Fractions with the similar chromatographic pattern were combined with Gel Filtration Chromatography.

Column size : Glass column, 0.5 cm in diameter.

Gel Filter : Sephadex LH-20 (20-100 µm, Sigma).

Solvent : 100 % MeOH.

Packing method : Gel filter was suspended in the eluent and left standing to well for 24 hours prior to use, then poured into the column and allowed to set tightly.

Sample loading : The sample was dissolved in a small amount of the eluent and then applied gently on top of the column.

Detections : Fractions were examined by TLC technique in the same manner as described in section A.

3.7 Spectroscopy

3.7.1 Ultraviolet (UV) Absorption Spectra

UV absorption spectra were obtained on a UV-JASCO- V-530 spectrophotometer (Faculty of Pharmacy Mahasarakham University).

3.7.2 Infrared (IR) Absorption Spectra

IR absorption spectra (KBr disc and film) were recorded on an EQUINOX 55, Bruker FTIR spectrometer (Scientific Equipment Center, Prince of Songkla University) and Fourier Transform Infrared spectrometer, Perkin Elmer Spectrum One. (Scientific Technological Research Equipment Center Chulalongkorn University).

3.7.3 Mass Spectra (MS)

Electron impact mass spectra (EIMS) and high-resolution electron impact mass spectra (HREIMS) were obtained with MAT 95 XL mass spectrometer (Scientific Equipment Center, Prince of Songkla University).

3.7.4 Nuclear Magnetic Resonance (NMR) spectra

^{1}H (400 MHz) and ^{13}C NMR (100.6 MHz) spectra were obtained on Avance BRUKER 400. (Scientific Equipment Center, Faculty of Science, Khon Kaen University). The solvents for NMR spectra were deuterated chloroform ($CDCl_3$). The chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

3.8 Physical Properties

3.8.1 Melting points

Melting points were obtained on a BUCHI B-540 (Faculty of Pharmacy Mahasarakham University).

3.9 Solvents

Organic solvents used in the extraction were commercial grade. For column chromatography, solvents were redistilled prior to use.

4. Extraction and Isolation of Compounds from *Phellinus rimosus*

4.1Extraction and Isolation of Compounds from Hexane fraction of *Phellinus rimosus*

The *P. rimosus* was chosen for isolation because it had the strongest antioxidant, anticancer, anti-inflammatory, and complete phenolic compound activities. *P. rimosus* had a crude ethanol extract of 51.45 g. (3.43 percent). The ethanol extract was provided as a partition for various solvents, which were arranged in increasing polarity order. The hexane fraction (2.47 g, 12.35 percent yield), dichloromethane fraction (3.75 g, 18.35 percent yield), ethyl acetate fraction (8.7 5 g, 43.51 percent yield), and butanol fraction (3.66 g, 18.02 percent yield) were obtained by pooling the filtrates and drying them under reduced pressure at 40 ^{o}C.

The hexane extract (2.2012 g) was eluted with CH_2Cl_2 in a hexane gradient to give fifty fractions of approximately 25 mL each and then washed down with EtOH using a silica gel (110 g, 1 x 30 cm) as adsorbent. The fractions were then combined on the basis of their TLC profiles, to give seven fractions: fractions H1 (120.5 mg), H2 (125.4 mg), H3 (147.2 mg), H4 (131.3 mg), H5 (129.7 mg), H6 (133.1mg) and H7 (307 mg).

Fraction H2 (125.4 mg) was further chromatograph on a silica gel 60 (28.2 g, 0.5 x 45 cm) column, eluting with 50 % CH_2Cl_2-Hexane gradients to give twelve fractions of approximately 20 mL each and washed down with MeOH. The fractions with similar TLC profiles were then combined to give three fractions: fractions H2.1 (8.5 mg), H2.2 (23.5 mg) and H2.3 (18.4 mg). The fractions H2.2 was dissolved in hexane in chloroform (3:1) and crystallized to yield 15.5 mg of compound HPR1 as colorless.

The fractions H2.3 was dissolved in solvent hexane in chloroform (3:1) ratio and the crystallized in MeOH to yield 10.08 mg of compound HPR2 as colorless neesdles.

4.2 Extraction and Isolation of Compounds from Dichloromethane fraction of *Phellinus rimosus*

The dichloromethane extract (2.5619 g) was subjected to column chromatography by used silica gel (115.28 g, 1 x 40 cm) as adsorbent and eluted with solvent (Toluent: ethyl acetate: methanol: formic acide; 5:5:1:1) gradient to give eleven fractions of approximately 45 mL. Each fraction was washed down with hexane, (fraction 12-20), dichloromethane (fraction 21-29), ethyl acetate (fraction 30-38), acetone (fraction 39-47), ethanol (fraction 48-56) and methanol (fraction 57-62), respectively. The fractions had volume approximately 45 mL of solvent and compounds. The fractions were combined on the basis of their TLC profiles, to give five fractions: fractions D1 (753.9 mg), D2 (1239.9 mg), D3 (191.6 mg), D4 (121.3 mg) and D5 (149.5 mg).

Fraction D1 (753.9 mg) was further chromatograph on a silica gel 60 (34 g, 0.5 x 48 cm) column, eluting with CH_2Cl_2-Hexane (4:1) gradients to give sixty-five fractions of approximately 5 mL. Each fraction was washed down with MeOH. The fractions with similar TLC profiles were then combined to give three fractions: fractions D1.1 (fraction 15-28; 91.6 mg), D1.2 (fraction 29-35; 153 mg) and D1.3 (fractions 50-57; 249.5 mg). The fraction D1.2 (153 mg) was developed with hexane in CH_2Cl_2 (2:3) on TLC plate and detected with ferric chloride. The TLC plate showed one main orange-yellow spot. This fraction was crystallized in EtOH and yielding 131 mg of pale yellow or off-white compound DPR.

5. Physical and Spectral Data of Isolated Compounds

5.1 Compound HPR1

Compound HPR1 was obtained as colorless needles (15.5 mg, 0.03 % yield)

Meting point : 110 -112 ^{o}C

UV : λmax (CDCl3); 291; **Figure 21.**

IR : Vmax cm^{-1}, KB disc; 3435, 2947, 1455, 1368, 1399, 1025, 925, 792, 760; **Figure 20**

^{1}H NMR : δ ppm, 400 MHz, in $CDCl_3$; **Figure 18.**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; **Figure 19.**

HREIMS : m/z 148 g/mol **Figure 22.**

5.2 Compound HPR2

Compound HPR2 was obtained as colorless needles (10.08 mg, 0.02 % yield)

Meting point : 126 -129 ^oC

UV : λmax ($CDCl_3$); 293, 236, 233, 230; **Figure 26.**

IR : Vmax cm^{-1}, KB disc; 3434, 2952, 2920, 1547, 1465, 1394, 1007, 845, 687, **Figure 25.**

^1H NMR : δ ppm, 400 MHz, in $CDCl_3$; **Figure 23.**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; **Figure 24.**

HREIMS : m/z 203 g/mol **Figure 27.**

5.3 Compound DPR

Compound DPR1 was obtained as pale yellow or off-white color. (131 mg, 0.25 % yield)

Meting point : 249-251 ^oC

UV : λmax ($CDCl_3$); 285,217,210; **Figure 32.**

IR : Vmaxcm-1, KB disc; 3458, 3399, 3318, 2561, 2464, 2343, 1599, 1581, 1506, 1440, 1386, 1250, 1207, 1178, 1024; **Figure 31.**

^1H NMR : δ ppm, 400 MHz, in $CDCl_3$; **Figure 29; Table 14.**

^{13}C NMR : δ ppm, 100 MHz, in $CDCl_3$; **Figure 30; Table 14.**

Dept 135 : δ ppm, 100 MHz, in $CDCl_3$; **Figure 34.**

COSY : δ ppm, 400 MHz, in $CDCl_3$; **Figure 35.**

NOESY **:** δ ppm, 400 MHz, in $CDCl_3$; **Figure 36.**

HSQC : δ ppm, 400 MHz, and δ ppm, 125 MHz, in $CDCl_3$; **Figure 37.**

HMBC : δ ppm, 400 MHz, and δ ppm, 125 MHz, in $CDCl_3$; **Figure 38.**

Mass **:** m/z 593.2363 $[M+Na]^+$, **Figure 33.**

6. Evaluation of Biological Activities

6.1 Screening of Anticancer, Antioxidant, Anti-inflammatory Activities, Total Phenolic and Flavonoid Content Determination

The crude ethanol and water extracts were assayed for screening anticancer activity against cholangiocarcinoma cell lines (KKU-100 & KKU-M213A) using the Sulforhodamine B (SRB) assay. The Nitric oxide assay was used to evaluation the anti-inflammatory activity. The MTT assay was used to determine cytotoxicity activity. The investigation of antioxidant activities using the DPPH, the ABTS and the FRAP assays. Total phenolic content was used the Folin-Ciocalteu reagent assay and total flavonoid content was determined by Aluminum chloride colorimetric assay.

6.2. DPPH Scavenging Assay

DPPH radical scavenging assay was carried out according to the method reported earlier, with slight modifications and described by Amic et al (2003).[198, 199]

6.2.1 Reagent Preparation

Test sample: Dissolved the mushroom extracts at the agreed concentration range as 1000 µg/mL stocked in 95 % ethanol and 50 % ethanol for water extractions DPPH radical solution: Dissolved 4 mg of DPPH in100 mL methanol (0.004 % of solution), stored in dark room until used it. Ascorbic acid solution: prepared in different concentrations of 1-100 µg/mL then designed 96 well plate layouts.

6.2.2 Method

-All samples, blank and calibrated were used to add in triplicates.

-In each well of a 96-well plate, add the concentration of each sample from stock solution as 20 µL

-Use the solvent (20 µL) as a blank sample and the calibration curve were used 20 µL of ascorbic acid (10-100 µg/mL) stock solutions.

-Start the reaction by adding 180 µL DPPH radical solutions and start time for 15 minutes from the first addition, then incubated at the room temperature, then on measure the absorbance of each well at 517 nm.

-The radical scavenging activity can be measured in the sample and calibrated as the following equation:

$\%\ \text{inhibition} = 100 \times (A_{blank} - A_{sample})/A_{blank}$

Where A_{blank} = absorbance of blank, A_{sample}= absorbance of the sample.

-From the ascorbic acid calibrate curve, measure the ascorbic acid equivalent antioxidant capacity of the sample.

6.3 ABTS Assay

The ABTS radical scavenging activity was determined using the method described by Payet et al (2005).[200]

6.3.1 Reagent Preparation

Test sample: Dissolve the mushroom extracts in 95% ethanol and 50% ethanol, then prepare was the concentration at 1mg/mL.

6.3.2 Preparation ABTS Reagent

ABTS reagent (2, 2-azino-bis-c 3-ethylbenzene-thiazoline-6-sulfonic acid (diammonium salt) MW = 548.7 g/mol (concentration at 7 mM) is dissolved in DI water and prepared of potassium persulfate (MW = 270.3 g/mol*)* concentration at 2.45 mM for dissolved in DI water after that mixture between ABTS reagent and potassium persulfate in the ratio of 1:0.5 finally, the extracts are stored in a dark room at 25 ^{o}C for 12-16 hours until processed.

6.3.3 Preparation ABTS Working Solution

The ABTS stock solution was diluted with 95 % ethanol. Then the absorbance values were measured at λ max 734 nm, the absorption had been ranged 0.7 ± 0.02.

6.3.4 Preparation Ascorbic Acid

Accurately weigh the standard ascorbic acid 0.001g dissolved in 95 % ethanol 1000 µL after that shake in vortex for 5 mins, then micropipette was added indifference volumes such as 100, 80, 60, 40, 20, 10 and 5 µL to eppendorf is adding 95 % ethanol in the eppendorf until the solution level reached to the neck of the eppendorf at 1000 µL ascorbic acid solution was prepared in different concentrations of 1-100 µg/mL), respectively.

6.3.5 Method

-All samples, blank and calibrated were used to add in triplicates.

-In each well of a 96-well plate, add each sample which concentration from stock solution as 20 µL and the solvent (20 µL) as a blank sample.

-For the calibration curve, use 20 µL of ascorbic acid (1-100 µg/mL) stock solutions

-Start in the reaction by adding 280 µL ABTS working solution, start time for 5 minutes from the first addition, incubate at room temperature.

-Measure the absorbance of each well plate at 734 nm.

-The radical scavenging activity of the sample was measured and calibrated as the following equation: % inhibition = 100 x (A_{blank}-A_{sample})/ A_{blank}

Where A_{blank} =absorbance of blank, A_{sample}=absorbance of sample.

6.4 FRAP Assay

The antioxidant capacity of the mushroom was estimated by applying the spectrophotometric techniques according to the procedure of Benzie et al 1999.

6.4.1 Reagent Preparation

Test sample: Dissolve the mushroom extracts at the agreed concentration range as 1000 µg/ml stock solution in 95 % ethanol and 50 % ethanol for water extractions.

6.4.2 Preparation Working FRAP Reagents

All solution was mixed in between 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride and 10 mM TPTZ solution ratio 10:1:1 mL, respectively.

6.4.3 Preparation 300 mM Acetate Buffer (pH 3.6).

Accurately, weighed 3.1 g of sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) then mix with 16 mL of glacial acetic acid, after that diluted in DI water to 1000 mL, then store in refrigerator at 4^{o}C.

6.4.4 Preparation 20 mM Ferric Chloride Solution

Precisely weigh 0.054 g of ferric chloride ($FeCl_3.6H_2O$) M.W. 270.30, dissolved in DI water, then dilute to 1000 mL.

6.4.5 Preparation of 10 mM TPTZ Solution

10 mM of TPPZ solution was dissolved in 40 mM Hydrochlohydric acid (HCl)

6.4.6 Preparation of 40 mM HCl.

Pipette 1.46 mL of HCl concentration, dissolve in DI water and then dilute to 1000 mL.

6.4.7 Preparation of 10 mM TPTZ Solution

Weigh 0.031 g of TPTZ and dissolve it in 10 mL of 40 mM HCl solution, followed by ultrasonic shaking to ensure that it is well dissolved and homogeneous.

6.4.8 Preparation Standard of 1mM Ferrous Sulfate ($FeSO_4$)

Weigh 0.278 g of ferrous sulfate (FeSO4.7H2O) dissolve in DI water, then dilute to 1000 mL. Ferrous sulfate solution was diluted in DI water to obtained concentration ranged at 0.1-1 mM. Then the following was the steps as shown in table 7.

Table 7 Concentration of Solution and Volume of Ferrous sulfate.

The concentration of a solution (mM)	The volume of $FeSO_4$ solution (μL)	The volume of DI water (μL)
0.1	100	900
0.2	200	800
0.4	400	600
0.6	600	400
0.8	800	200
1.0	1000	0

-Design 96 well plate layouts

6.4.9 Method

-All samples, blank and calibrated were used to add in triplicates.

-In each well plate was adding 20 μL of sample.

-The standard was used the ferrous sulfate (0.1-1mM).

-Start the reaction by adding 150 μL of FRAP reagent and 20 μL of sample.

-Measure the absorbance was well pate at 593 nm at 0 and 4 minutes.

-The absorbance at zero minutes equal the absorbance at 4 minutes was true and selected absorbance of samples.

-The standard cure of ferrous sulfate shown that the correlative between the concentration of ferrous sulfate and absorbance.

-Determination of relative antioxidant activity (FRAP value) from the standard cure of ferrous sulfate. The results were indicated as mg of ferrous sulfate equivalent per g dry weight.[201]

6.5 Total Phenolic Contents

The phenolic content was determined by the Folin-Ciocalteau method (Attar, E 2013).

6.5.1 Preparation 10 % of Folin-ciocalteu Reagents

Pipette 10 mL of Folin-Ciocalteu reagent (100 %) into a volume metric flask (100 mL) and dissolve in 90 mL of DI water.

6.5.2 Preparation 7.5 % of Sodium Carbonate (Na_2CO_3)

Accurately weigh 7.5 g of sodium carbonate (Na_2CO_3) and dissolve in DI water, followed by dilution to 100 mL.

-Design 96 well plate layouts

6.5.3 Method

-All samples, blank and calibrated were used to add in triplicates.

- In each well plate was adding 20 µL of sample.

- The standard was used the gallic acid (10-125 µg/mL).

-Reagent blank use distilled water to prepare.

-Start reagent by adding 200 µL of Folin-Ciocalteu mix 20 µL of the sample, then incubate at 5 minutes, and adding 160 µL of 7.5 % sodium carbonate.

-After incubating for 20 minutes in the room temperature, the absorbance was determined at 630 nm with the UV-Visible Spectrophotometer. The total phenolic content was calculated from the calibration curve and the result was presented as mg of gallic acid equivalent per g dry weight.[202]

6.6 Total Flavonoid Content

The flavonoid content was determined by Aluminum chloride colorimetric assay.

6.6.1 Preparation of 5 % sodium nitrate ($NaNO_2$)

Accurately weigh 5 g of sodium nitrate (Na_2NO_2) and dissolve in DI water, followed by dilution to 100 mL.

6.6.2 Preparation of 10 % Aluminium chloride ($AlCl_3$)

Accurately weigh 10 g of Aluminium chloride ($AlCl_3$) (Na_2CO_3) and dissolve in DI water, followed by dilution to 100 mL.

6.6.3 Preparation of 1M Sodium hydroxide (NaOH)

Accurately weigh 0.4 g of Sodium hydroxide (NaOH) and dissolve in DI water, followed by dilution to 100 mL.

-Design 96 well plate layouts.

6.6.4 Method

-All samples, blank and calibrated were used to add in triplicates.

-In each well plate was adding 50 µL of sample.

-The standard has used the rutin (10-80 mg/mL).

-Reagent blank use distilled water to prepare.

-Start reagent by adding 50 µL of samples mix 20 µL of the 5 % sodium nitrate, then incubate at 5 minutes, added 20 µL of 10 % Aluminium chloride after incubate again 6 minutes at room temperature, then added 1M sodium hydroxide 100 µL.

-The solution was mixed well and the absorbance was measured at 415 nm with the UV-Visible Spectrophotometer. The total flavonoid content was calculated from the calibration curve and the result was presented as mg of rutin equivalent per gram dry weight. [203]

7. Anti-inflammatory Activity

7.1 Nitric Oxide Inhibitory Assay

The Griess reagent was used to measure the accumulation of nitrite, an indication of NO, in the supernatant of RAW 264.7 macrophages after 24 hours of lipopolysaccharide (LPS) treatment with or without the extracts or reference standard (positive control). The method is described below.

-Preparation of RAW 264.7 cells were seed into 96-well plates at the density of 2 x 10^4 cells/well and incubation for 24 hours at 37 °C.

-The cells were activated by incubation in a medium containing 5 µg/mL LPS alone (control).

- Preparation of the mushroom extracts are six concentration (200, 100, 50, 25, 12.5, 6.25 µg/mL)

-The cells were pretreated with the mushroom extracts dissolve in DMSO or water and then incubate at 37 ^{o}C, 5 % CO_2, 24 hours.

-After 24 h of incubation, 100 µL of supernatant from each well of the 96 well-microplates were transferred into new 96-well microplates and adding 100 µL of Griess reagent.

-The absorbance of the mixture was determined at 550 nm on a microplate reader after 10 min of incubation at room temperature.

-The percentage of NO inhibition was calculated based on the ability of each sample to inhibit nitric oxide production by using RAW 264.7 macrophages compared with the control (cells treated with LPS without samples). Subsequently, the cell viability was determined using the MTT assay as described as section 7.2.

-Calculate the percentage NO inhibition using the formula below:

% inhibition = [(O.D.Control − O.D.sample) / O.D.Control] ×100 %

Sample: OD sample (+LPS) - OD sample (-LPS)

Control: Cells treated with LPS without samples.[204]

7.2 MTT Assay

The cytotoxicity assay was performed according to the micro-culture MTT method.[205]

7.2.1 Stock Solution of the MTT

Prepare 12 mM MTT stock solution by adding 1 mL of sterile phosphate-buffered saline (PBS) into a vial (5 mg) of MTT (Component A). The solution was mixed by sonication.

7.2.2 Method

-Plate cells (10^4-10^6 cells) in 100 µL PBS in 96-well (flat bottom).

-Treat cells with test extract and standard drug.

-Add 10 µL of MTT solution (5 mg/mL), mix well.

-Incubate for 24 hr at 37 ^oC in dark room.

-Remove aliquot to analysis; added 100 µL of DMSO and mixed well.

-Incubate additional 1h at 37 ^oC, CO_2.

-Read plate from micro plate reader machine, detect OD at 570 nm wavelength.

-Results were evaluated from three independent experiments (each performed in triplicate).[42]

8. Anticancer Activity

8.1 Cell lines

Human cholangiocarcinoma cell lines KKU-100 and KKU-M213A were kindly provided by Professor Sopit Wongkham and Professor Banchob Sripa, (Khon Kaen University, KhonKaen, Thailand). KKUM-213A, a high-invasive cell line, originated from adenosquamous CCA with well differentiation and KKU-100, a low-invasive cell line was isolated from adenocarcinoma CCA with poor differentiation. The normal cell lines were used as the RAW 264.7 cell line.[206]

8.1.1 Preparation of Reagent

1. Cell lines to be analyzed (KKU100 and KKUM-213A)

2. Standard cell culture medium without fetal calf serum.

3. Phosphate-buffered saline (PBS) solution: Dissolve 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of sodium phosphate (bi-basic), and 0.24 g of potassium phosphate in 800 mL of distilled water. Adjust the pH to 7.2 and then adjust the volume to 1000 mL with distilled water. Dispense into convenient volumes and sterilize by autoclaving. (Store it at room temperature).

4. Ethylenediamine tetra acetic acid (EDTA) solution: Add 0.1 g of ethylenediamine tetra acetic acid disodium salt to 500 mL of PBS. Add sodium hydroxide to adjust the pH to 8.0 and to allow the EDTA to dissolve. Dispense into convenient volumes and sterilize by autoclaving. (Store it at room temperature).

5. Preparation 0.05 % w/v Trypsin or 0.02 % (w/v) EDTA solution.

6. Standard cell culture medium with 10 % v/v heat-inactivated fetal calf serum.

7. Sterile 15 mL centrifuge tubes.

8. Standard cell culture medium with 0.1 % w/v sterile-filtered bovine serum albumin.

9. Hemocytometer

10. Trypan blue solution: 0.25 % w/v trypan blue in PBS, filter sterilized. (Stable at room temperature for several years).

8.1.2 Preparation of the Cells

1. Wash the cell monolayers. Cell culture medium without fetal cal serum gently rocks the flask from side to side so that the entire cell monolayer was covered and discards the spent medium.

2. Obtain a single-cell suspension by first washing the cell monolayer with sterile PBS (5 for 25 cm^2 flask). Pipet this was over the monolayer and then aspirate. Next, incubate the cells with EDTA solution (volumes as PBS) for approximate 10 min in the laminar flow hood. After this time, aspirate the EDTA and pipet on the trypsin-EDTA solution (1 mL for a 25 cm^2 flask).

3. Tighten the cap on the flask and gently swirl the solution over the surface of the cells. Place the flask of cells into the cell culture incubator. After approximate 30 s to 1 min, pipet off any excess solution and monitor the progress of the cells under an inverted micro.

4. Knock the flask sharply to loosen the cells from the bottom. As soon as the majority of the cells were detached from the bottom of the flask stop the action of the trypsin by the addition of cell culture medium with 10 % v/v fetal calf serum (5 mL for a 25 cm^2 flask).

5. Transfer the contents of the flask into a 15 mL sterile centrifuge tube and centrifuge the cells at 2000 rpm for 5 min.

6. Pipet off the medium without disturbing the cell pellet. Gently tap the centrifuge tube against the bench to loosen the cell pellet and resuspend in 5 mL of cell culture medium without fetal calf serum. Repeat twice more.

7. The cell pellet should be suspended in a final volume of 2.5 mL cell culture medium with 0.1 % w/v bovine serum albumin.

8. Count the cell suspension using a hemocytometer.

9. Assess cell viability was used trypan blue. Trypan blue was excluded from living cells, but stains dead/damaged cells blue.

10. Mix 50 mL of trypan blue solution with 50 μL of cell suspension. Transfer the mixture to a hemocytometer and observe under the microscopy. The total number of viable cells can be calculated directly as follows:

$$\frac{\text{Total number of viable cells}}{} \times 2 \times 10^4 = \text{number of viable cells/mL.}$$ [207]

Number of squares counted

8.2 Sulforhodamine B (SRB) Assay

SRB cytotoxicity assay protocol was consisting of 5 steps follows as:

8.2.1 Treatment solution preparation

-Volumes of treatment of choice should be enough for triplicates in 96-well plates and also account for pipetting variation

-Treatment can be prepared in aqueous solution or DMSO, which the final concentration of DMSO was 0.05 percent.

-The sample preparation for the treatment modify by reported of Amuamuta et al (2017), which the sample initially dissolved in 0.5 % DMSO to prepare stock solutions. The working solution was prepared at final concentrations at 500 µg/mL (DMSO 0.05 %) by diluting stock solutions with the medium.[12]

8.2.2. Cell Preparation

-Remove the medium from the cell monolayers and wash the cells once with sterilized PBS.

-Remove phosphate-buffered saline (PBS) and add 1 mL (100 mm plates) 0.25 % (w/v) trypsin to evenly cover the cell growth surface and Incubate at 37 °C for 5 min or until the cells start to dissociate.

-Next, inactivate trypsin with 10 volumes of culture medium containing fetal bovine serum (FBS) and mix up and down to obtain a homogeneous single-cell suspension.

-Transfer the cell suspension to sterile Falcon tube. Determine the cell concentration by counting in a hematocytometer chamber under a microscope using a 1:1 mixture of cell suspension and 0.4 % (w/v) trypan blue solution to determine cell viability prior cell seeding. Optional: before counting, spin down cells in order to wash trypsin and resuspend in the growth medium.

-Adjust the cell concentration with growth medium (10 % FBS) to obtain an appropriate cell seeding density per well in a volume of 100 µL (96-well format).

-Transfer the cell suspension into a sterile reagent reservoir to make it easier to pipette with a multichannel pipette.

8.2.3. Treatment Exposure

-Mix the treatment solutions prepared in step A by pipetting. Dispense 100 µL (96-well format) of solution into each well.

-Mix cell suspension prepared in step (6.2.2) thoroughly and adds 100 µL (96-well format) to each well already containing treatment solutions.

-Set aside three wells in the plate containing the only solvent of choice and cell suspension for an untreated or vehicle control. Also, leave three wells in the plate containing the only medium for background subtraction.

-Incubate the plate at 37 °C in a humidified incubator with 5 % CO_2 until the plate was to be read.

8.2.4. Cell Fixation and Staining

- Add 100 µL (96-well) of cold 10 % (w/v) Trichloroacetic acid (TCA) to each well directly into medium supernatant, and incubate the plates at 4 °C for 30 minutes. Mixing is not required, as this could lead to some cells detaching from the bottom of the well.

- Wash the plates four times (110 µL of DI water) by submerging them in a tub with slow running tap water and removing excess water by gently tapping the plate into a paper towel. Allow the plate to air dry at room temperature after the final wash.

-A SRB solution (100 µL, 0.04 %) was added to each well plate.

-Leave at room temperature for 30 minutes and then quickly rinse the plates four times with 1 % (v/v) acetic acid (110 µL for 96-well) to remove unbound dye.

-Allow for 30 minutes at room temperature before quickly rinsing the plates four times with 1% (v/v) acetic acid (110 µL for 96-well) to remove the unbound dye and allow the plate to air-dry at room temperature.

8.2.5. Absorbance Measurement

- A Tris base solution (10 mM, pH 10.5) of 150 mL was added to each well, and the plate was shaken on an orbital shaker for 15 minutes to solubilize the protein-bound dye.

- The absorbance was measured at 540 nm in a microplate reader.

-Calculations: Correct background by subtracting the O.D. of the control containing only the culture medium (background control well) from all sample readings.

- The formula was used to calculate the percentage of cytotoxicity activity.

% Cytotoxicity = [(O.D.DMSO − O.D.sample) / O.D.DMSO] ×100 %

Where: O.D.DMSO was the O.D. of the DMSO control after background correction (corrected negative control well) O.D.sample is the O.D. of the sample after background correction.[208]

9. Statistical Analysis

All studies had at least five replications (n=5) to determine mean values and standard deviations (SD) of antioxidant, anti-inflammation, and anticancer activity. To replicate measurements, mean values (n=4) for DPPH, ABTS, FRAP, total phenolic, and flavonoid (TPC & TFC) were calculated as indicated in each section. Statistical analysis of the obtained results was performed using one-way analysis of the variance (ANOVA), the differences between samples showed significant variation ($P < 0.05$). Correlation coefficients (R) to determine the relationship between two variables, DPPH, ABTS, FRAP, TPC and TFC tests were calculated using MS Excel 2010 software. The IC_{50} values of sample was calculated by prism graphad program version 8.0

Statistical analysis for detailed biological assays of the sample was performed using the SPSS (Version 15) package (SPSS, Inc., Chicago, IL, USA). Statistical tests were chosen according to the nature of the data analyzed. For the class of solvent extracts, the Student's two-sided t-test was used, and Duncan analysis was used to compare the various mushroom types. Pearson's correlation was used to assess time-response relationships. Statistical significance was described as a P-value of less than 0.05 ($p < 0.05$).

Chapter IV
Results

1. The yield of Mushroom Extracts using Various Solvents

Extraction of antioxidants and other biologically active molecules was requires the use of solvents with varying polarities, Some antioxidants are more soluble in polar solvents like ethanol and water, whilst lipophilic compounds are better isolated by using cyclohexane or dichloromethane. This is investigation, we used the latter strategy extracting by ethanol and water as solvents. From the standpoint of solubility in the employed solvents, it is clear that selected mushrooms are made up of significantly distinct classes of chemicals (Table 8). The yields of total mushroom species was sorted by species represented in Fig 14. Ethanol extract yields ranged from 2.31 percent (*P. igniarus*) to 4.73 percent (*P.nigricans*) depending on the mushroom species, while boiling water yields ranged from 1.05 percent (*P. rimosus*) to 1.91 percent (*P. nigricans*). The yields produced by various solvents are critical aspects in applying the biorefinery concept to biomaterials for successful, waste-free conversion into fractions for various used. The crude extract of *P. rimosus* was performed better than other extracts, hence it was chosen to partition by using a variety of solvents. Hexane, dichloromethane, ethyl acetate, and butanol were used to separate the ethanol extract of *P. rimosus*; the yields of the fractions were 2.4778g (12.35 %), 3.75 g (18.68 %), 8.75 g (43.51 %), and 3.66 g (18.02 %), respectively.

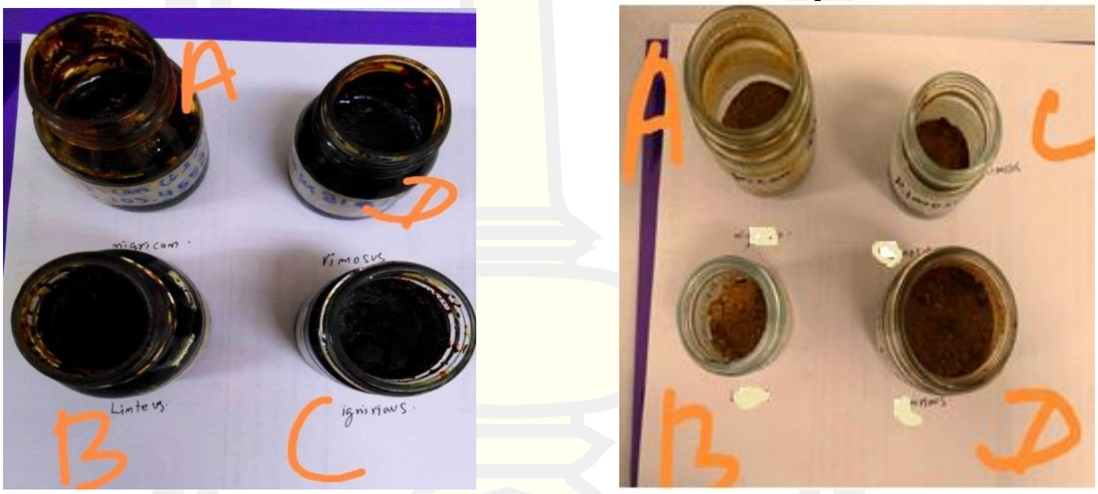
Table 8 Percentage (w/w) yields of mushroom extracts separated by various solvents.

Type of mushrooms	Extract/fraction	weight	% Yield of extracts	Characteristics of extract/fraction
P. igniarius	95 % ethanol extract	34.65	2.31	Dark brownish powder
	Aqueous extract	10.07	1.3	Brownis to yellow
P. lineteus	95 % ethanol extract	45.2	3	dark residue to yellowish substance
	Aqueous extract	9.53	1.23	Dark yellow powder
P. nigricans	95 % ethanol extract	70.95	4.73	Dark yellow residue
	Aqueous extract	14.8	1.91	Brownish to yellow powder

Table 8 (Continued)

Type of mushrooms	Extract/fraction	weight	% Yield of extracts	Characteristics of extract/fraction
P. rimosus	95 % ethanol extract	51.45	3.43	Dark yellow residue
	Aqueous extract	8.13	1.05	Brownish to yellow powder
	Hexane fraction	2.47	12.35	Dark to purple residue
	Dichloromethane fraction	3.75	18.68	Dark to yellow
	Ethyl acetate fraction	8.75	43.51	Dark to purple residue
	Butanol fraction	3.66	18.02	Dark to yellow

Ethanol extracts Aqueous extracts



A) *P. nigricans*, B) *P. linteus*, C) *P. igniarius*, D) *P. rimosus*

Figure 14 Extracts of Ethanol and water from *Phellinus* Mushrooms

2. Preliminary Qualitative Phytochemical Analysis

Phellinus mushroom extracts, both ethanolic and aqueous were found to include phenolics, flavonoids, and triterpenoids. In ethanol extracts, the alkaloids were presented, but not in water extraction (Table 9). The existence of phenolics, flavonoids, alkaloids, and triterpenoids were discovered. The ethanol extract had a higher number of secondary metabolites and a higher degree of precipitation when compared to the water extract. In this study, phenolics, flavonoids, alkaloids, and triterpenoids were shown to be higher in ethanol extract than in water extract, but alkaloids were not discovered in water extract of *Phellinus* mushrooms. Phytochemical analysis of *Phellinus* mushrooms, the results of this study was shown in Table 9

Table 9 Preliminary phytochemical screening of ethanolic and aqueous extract of *Phellinus* mushrooms.

Test	*P. igniarius*		*P. lineteus*		*P. nigricans*		*P. rimosus*	
	EtOH	Aqueous	EtOH	Aqueous	EtOH	Aqueous	EtOH	Aqueous
Alkaloids a) Dragendroff's b) Mayer's test c) Hager's test.	+ + +	- - -	+ + +	- - -	+ + +	- - -	+ + +	- - -
Phenolics	++	++	++	++	+	+	++	++
Flavonoids a) Lead acetate b) Shinoda Test	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++	++ ++
Triterpenoids	++	++	++	++	++	++	++	++

Note: ++ = Present in abundance. + = Present. - = absent

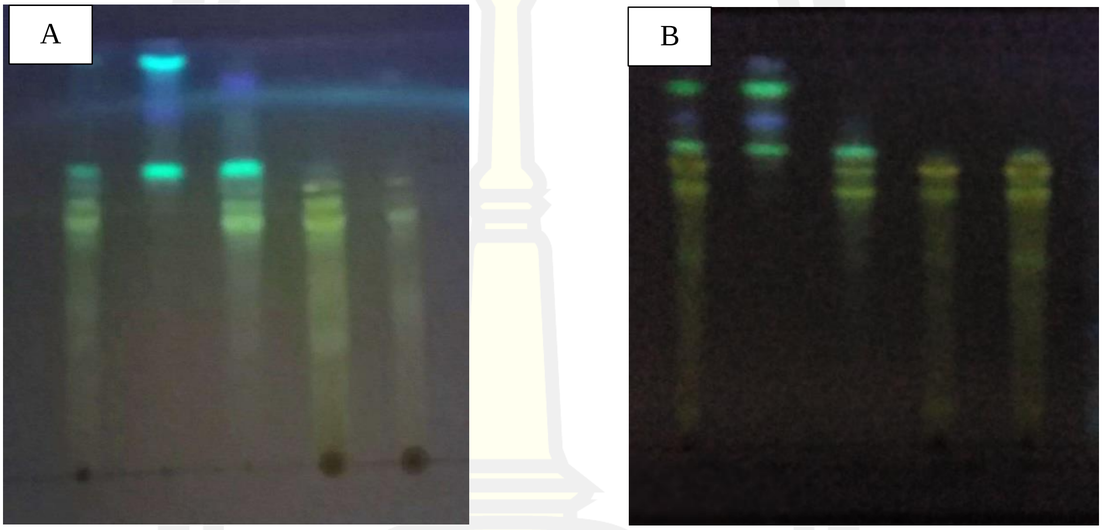
(+)Single plus was used to present amount of substance in the *Phellinus* mushroom extract.

(++) Double plus was used to represent extremely high chemical compounds in the *Phellinus* mushroom extract.

3. TLC fingerprint

P. rimosus had the best effectiveness against cholangiocarcinoma cell lines (KKU-100 & KKUM-213A) as well as antioxidant activities in this assay. Therefore, *P. rimosus* was selected for further studies (separation, isolation, identification and biological activities tested). This study was carried out to investigation the TLC of *P. rimosus* using two the solvent systems. The TLC plate was aluminum oxide card (GF 254). The plates were developed for the first time in dichloromethane: ethyl acetate: ethanol: formic acid (10:1:1:1) ratio (Figure A). The plate was again loaded with simple and developed in toluen: ethyl acetate: methanol: formic acid (5:5:1:1) (Figure B). Various solvent fractions from an ethanol extract of *P. rimosus* (n-hexane, dichloromethane, ethyl acetate, and n-butanol) showed varying spots with different Rf values (Retention factor) in this investigation.

The chromatograms were observed under visible light and photograph. TLC profiling of all mushroom extracts and fractions in the solvent system had indicated the presence of multiple strong biomolecules in these mushrooms. The TLC of *Phellinus* mushroom extract was indicated spots of yellow color, when be detected under blacklight (366 nm) and dark spots under UV light (254 nm), while sprayed with 10 % ferric chloride was shown blue spots that result was represented phenolic compounds. The TLC of *Phellinus* mushroom extract was sprayed with 10 % ethanol potassium hydroxide shown red or brown color spots after spay that result was represented Quinone.



1 2 3 4 5 1 2 3 4 5

Figure 15 Thin-layer Chromatogram of Ethanol Extract, Hexane, Dichloromethane, Ethyl acetate and Butanol Fractions

Adsorbent: Silica gelGF$_{254}$

1= Ethanol extract

2= Hexane fraction

3= Dichloromethane fraction

4= Ethyl acetate fraction

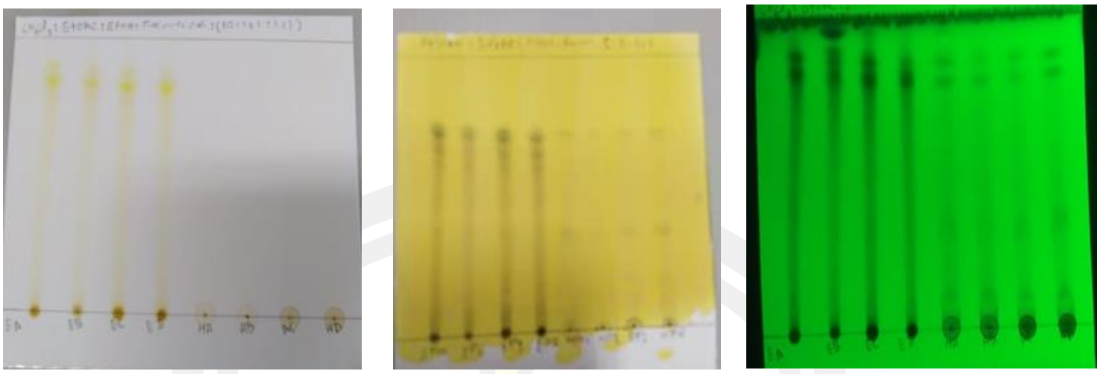
5= Butanol fraction

Solvent system: A) dichloromethane: ethyl acetate: ethanol: formic acid (10:1:1:1)

B) Toluen: ethyl acetate: methanol: formic acid (5:5:1:1).

Detection:

A and B- UV 366 nm



1 2 3 4 5 6 7 8   1 2 3 4 5 6 7 8   1 2 3 4 5 6 7 8

A   B   C

Figure 16 Thin-layer Chromatogram of Ethanol Extracts from *P. igniarius*, *P. linteus*, *P. nigricans* and *P. rimosus*

Adsorbent: Silica gel GF_{254}

1= Ethanol extract of *P. igniarius*	5= Aqueous extract of *P. igniarius*
2= Ethanol extract of *P. linteus*	6= Aqueous extract of *P. linteus*
3= Ethanol extract of *P. nigricans*	7= Aqueous extract of *P. nigricans*
4= Ethanol extract of *P. rimosus*	8= Aqueous extract of *P. rimosus*

Solvent system: 1) dichloromethane: ethyl acetate: ethanol: formic acid (10:1:1:1).

Detection:

1) Visible light (observation)
2) 10 % Ferric chloride in ethanol
3) UV-254 nm

4. Antioxidant Activity, Total phenolic and Flavonoid content of *Phellinus* Mushroom Extracts

According to the findings of this investigation, the ethanol extract of *P. rimosus* had the highest activity compared to other extracts ($p < 0.05$). The IC_{50} value of *P. rimosus* extract in free radical scavenging assays was 9.56 ± 0.47 µg/mL. In this study, all PE demonstrated antioxidant activity in the DPPH experiment, with *P. linteus* inhibiting with IC_{50} values of 28.85 ± 0.56 µg/mL and *P. nigricans* & *P. igniarius* displaying antioxidant activity ($p > 0.05$, IC_{50}=32.46 ± 0.30 and 32.33 ± 0.54 µg/mL, respectively). In the DPPH experiment, PE aqueous extract showed moderate activity, which *P. rimosus*, *P. nigricans*, *P. igniarius* and *P. linteus* were shown to be free radicals, with IC_{50} values of 37.81 ± 0.15, 81.90 ± 0.25, 84.62 ± 1.13 and 176.68 ± 0.77 µg/mL; respectively.

In the ABTS assay, all ethanol extracts of PE had stronger antioxidant activity, the *P. rimosus* was better than other extracts, which each sample inhibited free radical was different a significant ($p < 0.05$). When compared to ascorbic acid, *P. rimosus* had

IC_{50} values of 5.40 ± 0.06 µg/mL, which were not significantly different ($p > 0.05$). The second type of antioxidant activity was found in the ethanol extracts of *P. linteus* and *P. nigricans*, with IC_{50} values of 14.06 ± 0.08 µg/mL and 15.41 ± 0.40 µg/mL, respectively. The ethanol extract of *P. igniarius* showed significant antioxidant activity, with IC_{50} values of 18.44 ± 0.48 µg/mL. According to the findings, aqueous extracts suppressed free radicals ABTS with IC_{50} values ranging from 14.41 ± 0.13 to 29.99 ± 1.62 µg/mL, with a significant difference ($p < 0.05$) in this test when compared between each PE.

The antioxidant activity measured by FRAP method was observed in all extracts. The highest values were 22.70 ± 5.04 to 52.39 ± 0.25 mM $FeSO_4$/100 mg in ethanol extracts of samples, while ethanol extract of *P. rimosus* was the best for the results of this study. The ferric reducing antioxidant capacity of PE aqueous extract ranged from 11.19 ± 0.79 to 12.53 ± 0.42 mM $FeSO_4$/100 mg of samples. The study's findings, which included data on ferric reducing antioxidant capacity, revealed that the extract's reductive capability was not significantly different between PE ($p > 0.05$). The total phenolic content (TPC) of PE showed a difference between sample extracts. The amount of TPC depends on the extraction solvent and the type of sample. When compared to other extracts, the ethanol extract of *P. rimosus* had the highest TPC (361.04 ± 5.69 mg GAE/g of sample) in the current investigation ($p < 0.05$). The quantity of TPC in the ethanol extract of *P. linteus* was second, while the amount of TPC in the ethanol extracts of *P. nigricans* and *P. igniarius* was non-significant ($p > 0.05$, 148.86 ± 3.50 and 145.53 ± 2.10 mg GAE/g of sample). Aqueous extracts of PE exhibited a lower TPC ($p < 0.05$) than ethanol extracts, with *P. rimosus* aqueous extract containing 117.14 ± 6.11 mg GAE/g of sample, whereas *P. nigricans*, *P. igniarius*, and *P. linteus* aqueous extract contained 58.70 ± 2.76, 54.48 ± 2.97, and 50.43 ± 1.65 mg GAE/g of sample, respectively. The results of study, the TPC in each mushroom extract were descripted in table10.

The total flavonoid contents (TFC) of PE were founded dependent on each extract, which ethanol extract of *P. rimosus* showed the highest TFC when compared with other extracts ($p < 0.05$). *P. rimosus* was founded at 646.55 ± 6.29 mg RE/g of sample, while *P. igniarius* showed the second amount of TFC (353.30 ± 1.87 mg RE/g of sample). The present results of study, TFC found in ethanol extract of *P. linteus* and *P. nigricans* showed at 223.67 ± 6.91 and 183.55 ± 5.93 mg RE/g of sample, respectively. The ethanol extract of *P. nigricans* contains the least amount of TFC ($p < 0.05$) when compared to the other extracts. The aqueous extract of *P. rimosus* contained 129.11 ± 5.29 mg RE/g of sample, which was much higher than TFC in PE aqueous extracts. *P. igniarius* exhibited the second highest amount of TFC when compared to other aqueous extracts, while *P. nigricans* and *P. linteus* had no statistically significant difference in TFC levels ($p > 0.05$). The TFC of the aqueous extract of *P. nigricans* was found to be 75.46 ± 1.15 mg RE/g of sample, whereas the TFC of the aqueous extract of *P. linteus* was found to be 74.81 ± 3.52 mg RE/g of sample, as shown in table 10.

Table 10 Antioxidant activities, total phenolics and flavonoids contents of *Phellinus* mushrooms extracts.

Samples	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP (mM Fe^{2+}/100 mg)	Total phenolics (mg GAE/g of sample)	Total flavonoids (mg RE/g of sample)
Ethanol extracts					
P. igniarius	32.33 $\pm0.54^{D}$	18.44 $\pm0.48^{C}$	37.57 $\pm 6.21^{b}$	145.53 $\pm2.10^{c}$	353.30 $\pm1.87^{b}$
P. linteus	28.85 $\pm0.56^{C}$	14.06 $\pm0.08^{B}$	36.90 $\pm0.97^{b}$	184.86 $\pm5.54^{b}$	223.67 $\pm6.92^{c}$
P. nigricans	32.46 $\pm0.30^{D}$	15.41 $\pm0.40^{B}$	22.70 $\pm5.04^{c}$	148.86 $\pm3.50^{c}$	183.55 $\pm5.93^{d}$
P. rimosus	9.56 $\pm0.47^{B}$	5.04 $\pm0.06^{A}$	52.39 $\pm0.74^{a}$	361.04 $\pm5.69^{a}$	646.55 $\pm6.29^{a}$
Aqueous extracts					
P. igniarius	84.62 $\pm1.33^{G}$	25.21 $\pm1.13^{D}$	12.23 $\pm1.08^{d}$	54.48 $\pm2.97^{e}$	97.76 $\pm4.29^{e}$
P. linteus	176.68 $\pm0.77^{H}$	29.99 $\pm1.62^{E}$	12.39 $\pm0.61^{d}$	50.43 $\pm1.65^{e}$	74.81 $\pm3.52^{f}$
P. nigricans	81.90 $\pm0.25^{F}$	24.59 $\pm1.03^{D}$	11.19 $\pm0.79^{d}$	58.38 $\pm2.76^{e}$	75.46 $\pm1.15^{f}$
P. rimosus	37.81 $\pm0.15^{E}$	14.41 $\pm0.13^{B}$	12.53 $\pm0.42^{d}$	117.14 $\pm6.11^{d}$	129.11 $\pm5.29^{c}$
Ascorbic acid	4.53 $\pm0.33^{A}$	5.34 $\pm0.37^{A}$	38.37 $\pm1.55^{b}$	-	-

Statistically significant difference when compared to ascorbic acid (within column), $p < 0.05$; A,B,C,D,E,F,G,H was presented statistically significant difference within columns (uppercase letters, $p < 0.05$; lowercase letters, $p < 0.001$); mM: millimole equivalent; GAE: gallic acid equivalent; RE: rutin equivalent.

5. Anti-inflammatory Activity

5.1 Inhibition of nitric oxide production by the *Phellinus* mushrooms extracts

Nitric oxide is a chemical mediator produced by endothelial cells, macrophages, and neurons that are involved in the development of nitrite in a linear time-dependent manner. The nitric oxide scavenging ability of extracts and compounds were investigated in this study by using a decreased in absorbance at 550 nm caused by a decreased in nitric oxide output. In this research, we found that *Phellinus* mushrooms (ethanol extracts, aqueous extracts, and pure compounds) had anti-inflammatory properties and can inhibited nitric oxide development in LPS-induced Mouse Leukemic macrophage cell line (RAW 264.7 cells). Table 11 indicated the extracts' capacity can be decreased nitric oxide production in LPS-induced RAW 264.7 cells.

Ethanol extracts of *P. rimosus* and *P. linteus* mushrooms had the greatest inhibitory effect on nitric oxide synthesis. At 200 and 100 µg/mL, they had nitric oxide inhibitory activities of 97.95 ± 2.29, 92.08 ± 4.60 percent and 98.83 ± 1.59, 83.13 ±1.58 percent, respectively. *P. nigricans* had second-highest nitric oxide inhibitory activities in RAW 264.7 cells at 93.65 ± 1.87 and 69.26 ± 4.58 percent (concentration at 200 & 100 µg/mL), respectively, while the ethanol extract of *P. igniarius* had least inhibition activity in this study, with statistically significant difference ($p < 0.05$) as compared to other mushroom extracts. The percentage of inhibition of the *P. igniarius* ethanol extract was 72.94 ± 4.75 percent at 200 µg/mL and 21.83 ± 2.45 percent at 100 µg/mL (Table 11). According to the findings of this analysis, ethanol extracts has the greatest potential to inhibited nitric oxide development in RAW 264.7 cells induced by LPS, whereas aqueous extracts had least or no inhibition effect. The pure compounds isolated from *P. rimosus* was indicated a percentage of inhibition ranging from 3.06 ± 2.18 to 100.06 ± 0.97 at 6.25 to 50 µg/mL with IC_{50} values of different (HPR1 IC_{50} = 9.87 ± 0.24 µg/mL, HPR2 IC_{50} = 10.55 ± 1.09 µg/mL and DPR IC_{50} = 8.69 ± 0.08 µg/mL). The result of ethanol extract and all pure compounds, with the exception of the aqueous extract was significantly reduced RAW264.7 cells nitric oxide production. Tables 11 and 12 summarize the findings of reports, including the anti-inflammatory effects of *Phellinus* mushrooms.

Table 11 Extracts of *Phellinus* species and pure compounds from *P. rimosus* inhibit nitric oxide production in LPS-activated macrophages RAW 264.7.

species	Extracts (µg/mL)	IC_{50} (µg/mL)	Percentage inhibition of nitric oxide (NO)	P-values
Ethanol				
P. igniarius	200 100	ND	72.94 ± 4.75* 21.83 ± 2.45^{z}	$p < 0.05$
P. linteus	200 100	ND	98.83 ± 1.59^{a*} 83.13 ± 1.58^{x}	
P. nigricans	200 100	ND	93.65 ± 1.87* 69.26 ± 4.58^{y}	
P. rimosus	200 100	ND	97.95 ± 2.29^{a*} 92.08 ± 4.60^{b}	
Aqueous				
P. igniarius	200 100	ND	-16.87 ± 6.99 -46.09 ± 6.27	-
P. linteus	200 100	ND	-37.42 ± 2.27 -65.15 ± 6.07	
P. nigricans	200 100	ND	7.73 ± 6.86 -12.52 ± 8.37	
P. rimosus	200 100	ND	-9.21 ± 3.70 -20.98 ± 4.30	
HPR1	50 25 12.5 6.25	9.87 ± 0.24**	100.06 ± 0.97 100.68 ± 1.58 74.29 ± 2.97 3.06 ± 2.18	$p < 0.01$
HPR2	50 25 12.5 6.25	10.55 ± 1.09**	97.47 ± 0.91 97.44 ± 0.11 73.48 ± 1.26 13.54 ± 8.53	
DPR	50 25 12.5 6.25	8.69 ± 0.08**	99.63 ± 0.53 97.99 ± 0.36 76.21 ± 0.43 30.01 ± 0.71	
Control media	-	-	0	

Abbreviations: ND: Not done; NO: Nitric oxide; LPS: Lipopolysaccharide.
Star (*) indicated inhibition of NO production in LPS-activated macrophages RAW 264.7 cells a significant at $p < 0.05$ when compared each ethanol extracts of *Phellinus* mushrooms.
Star (**) indicated a significantly difference between pure compounds isolated from *P. rimosus* in LPS-activated macrophages RAW 264.7 cells ($p < 0.01$).
^{a,b,x,y,z} indicate statistically significant difference among ethanol extracts of *Phellinus* mushrooms in LPS-activated macrophages RAW 264.7 cells ($p < 0.01$).

5.2 Cell Viability Assessments

The MTT methods were used to determine cytotoxicity in a mouse leukemic macrophage cell line (RAW 264.7 cells). The nitric oxide inhibitory activity of the *Phellinus* mushroom extracts was promising, as was the cytotoxicity evaluation at 200 µg/mL and 100 µg/mL with different cell viabilities. Table 12 was indicated the percentage cell viability of *Phellinus* mushroom extracts and pure compounds. In this analysis, there was no statistically significant difference in cell viability between RAW 264.7 cells activated by LPS (+ LPS 5 g/mL) and RAW 264.7 cells that were not activated ($p > 0.05$). Extracts were tested in vitro against RAW 264.7 cells to see if they were cytotoxic. In both testion cell lines, the ethanol and aqueous extracts had no cytotoxic effects at concentrations ranging from 100 to 200 µg/mL. The pure compounds had promising nitric oxide inhibitory activity, but the cytotoxicity evaluated at 50 µg/mL with cell viability ranged from 6.79 ± 0.81 to 9.72 ± 0.31 percent for RAW 264.7 cell activated, while percentage cell viabilities ranged from 6.69 ± 0.57 to 30.26 ± 8.1 percent for RAW 264.7 cells none activated, respectively. In fact that the pure compounds showed nitric oxide inhibitory activity indicates that the observed activity was due to a cytotoxic effect, according to the study's findings (Table 12). The ethanol and aqueous extracts of *P. rimosus*, on the other hand, had no cytotoxicity activity (>200 µg/mL), while the pure compounds had cytotoxicity with cell viability at 50 µg/mL. The pure compounds presented percentage of inhibition ranging from 3.06 $\pm$ 2.18 to 100.06 ± 0.97 percent at concentrations ranging from 6.25 to 50 µg/mL with IC_{50} of different, such as HPR1 with IC_{50} = 9.87 0.24 µg/mL, HPR2 $IC_{50} = 10.55 \pm 1.09$ µg/mL & DPR $IC_{50} = 8.69 \pm 0.08$ µg/mL. In comparison, none of the pure compounds showed cytotoxic effect on RAW 264.7 cells at the tested concentration (≤ 25 µg/mL). The compounds DPR had a cell viability of more than 81.66 ± 0.24 percent. Therefore; the results of this study was summarized the findings of this report, including the anti-inflammatory effects of *Phellinus* mushrooms for cytotoxicity, protection profile, and percentage cell viability (Table 12).

Table 12 Cytotoxicity, safety profile and percentage cell viability of the extracts of the selected *Phellinus* mushrooms on RAW264.7 cells in vitro.

species	Extracts	IC_{50}	Percentage cell viability		p-values
Ethanol	(µg/mL)	(µg/mL)	(-LPS)	(+LPS 5µg/mL)	
P. igniarius	200	ND	97.72 ± 5.45^{b}	92.78 ± 5.71^{A}	$p > 0.05$
	100		90.53 ± 2.99^{a}	100.83 ± 3.30^{b}	
P. linteus	200	ND	102.78 ± 3.31^{b}	112.91 ± 3.65^{B}	
	100		98.68 ± 5.96^{a}	108.74 ± 5.67^{b}	
P. nigricans	200	ND	98.71 ± 6.41^{b}	112.20 ± 5.22^{B}	
	100		102.16 ± 4.37^{a}	99.83 ± 3.83^{b}	
P. rimosus	200	ND	90.29 ± 5.03^{b}	96.47 ± 2.29^{A}	
	100		91.84 ± 3.64^{a}	99.22 ± 4.77^{b}	

Table 12 (Continued)

species	Extracts (µg/mL)	IC_{50} (µg/mL)	Percentage cell viability (-LPS)	Percentage cell viability (+LPS 5µg/mL)	p-values
Ethanol					
Aqueous					
P. igniarius	200 100	ND	117.79 ± 3.51 119.19 ± 4.48	119.23 ± 6.03 118.56 ± 5.60	P > 0.05
P. linteus	200 100	ND	124.59 ± 7.27 116.31 ± 6.47	127.72 ± 5.99 116.99 ± 2.99	
P. nigricans	200 100	ND	131.73 ± 8.69 124.73 ± 6.08	125.41 ± 5.28 124.73 ± 6.08	
P. rimosus	200 100	ND	120.37 ± 4.13 117.79 ± 4.77	127.54 ± 4.70 117.51 ± 6.04	
HPR1	50 25 12.5 6.25	9.87 ± 0.24*	30.26 ± 8.19 67.87 ± 3.10 135.30 ± 4.72 130.42 ± 3.88	9.72 ± 0.31 116.68 ± 0.59 109.19 ± 0.74 110.95 ± 2.72	p < 0.01
HPR2	50 25 12.5 6.25	10.55 ± 1.09*	26.81 ± 4.78 62.88 ± 2.37 124.82 ± 6.45 133.67 ± 1.55	50.33 ± 1.83 119.21 ± 6.97 129.01 ± 8.60 116.39 ± 1.28	
DPR	50 25 12.5 6.25	8.69 ± 0.08*	6.69 ± 0.57 74.40 ± 2.40 88.43 ± 4.17 109.11 ± 0.37	6.79 ± 0.81 84.14 ± 1.20 81.66 ± 0.24 94.59 ± 4.88	
Control media	0	-	100 ± 0.72	100 ± 2.15	

Abbreviations: ND, Not done; NO, Nitric oxide; LPS, Lipopolysaccharide.

[A,B] indicate statistically significant difference among ethanol extracts of *Phellinus* mushrooms ($p < 0.05$; within column); difference lowercase presented statistically not significant difference among ethanol extracts of *Phellinus* mushrooms ($p < 0.01$; within column).

Star (*) indicate statistically significant difference at $p < 0.01$ compared between pure compounds isolated from *P. rimosus* on LPS-activated macrophages RAW 264.7.

6. Anticancer Activity

6.1 Cholangiocarcinoma Cell Growths Inhibitory Activity of the Ethanolic and Aqueous Extracts of *Phellinus* Mushrooms

The effect of the extracts on the growth of cholangiocarcinoma cell lines (CCA) was evaluated, according to the procedure adopted in the US National Cancer Institute (NCI) in vitro anticancer drug screening, which uses sulforhodamine B (SRB) assay to assess cell growth inhibition. This was done with eight different *Phellinus* mushroom extracts (PE). The study's findings are provided as a percentage of growth inhibition and an IC_{50} value (concentrations of extract that cause 50 percent cell growth inhibition). These experiments were carried out in CCA (KKU-100 & KKUM-213A), which were chosen as KKU-100 and KKUM-213A representative cells. The ethanol extract of *P. linteus* had a greatest anti-CCA activities in both KKU-100 and KKUM-213 cells when compared to other extracts at all incubation periods (24,48 &72 h) ($p < 0.05$). After treating with *P. linteus* (500 µg/mL), the percentage cell inhibition of KKU 100 ranged from 74.22 ± 2.08 to 95.52 ± 0.14 and 91.42 ± 1.22 to 98.92 ± 0.22 for KKUM-213A cells. This is the first report of PE on anticancer against CCA cell lines. In the present study, *P. rimosus* ethanol extract showed the second manner in anticancer activity in both cells and in all incubation periods with percent cell inhibition ranged from 47.87 ± 1.44 to 91.84 ± 0.36 for KKU-100 and 45.83 ± 1.30 to 95.86 ± 2.61 for KKUM-213A cells. *P. igniarius* inhibited CCA growth by a percentage ranging from 49.72 ± 2.12 to 83.46 ± 0.50 in KKU-100 cells and 75.53 ± 3.08 to 91.83 ± 0.79 in KKUM-213A cells; whereas *P. nigricans* had the least anticancer activity in this study ($p < 0.05$). Inhibition percentages of *P. nigricans* in KKU-100 cells varied from 49.22 ± 1.98 to 51.13 ± 1.06, whereas in KKUM-213A cells they varied from 52.20 ± 0.86 to 79.98 ± 0.50. However, At all incubated periods (24,48 &72 h), the aqueous extracts of PE had the least anticancer efficacy, with sample concentrations of 500 µg/mL indicating less than 50 % CCA growth inhibition in both KKU-100 and KKUM-213A cells. The results of study, anticancer activities of PE were described in table 14. Results were expressed as the average (µg/mL) of a minimum of five independent experiments.

Table 13 Effects of the mushroom extracts on the growth of cholangiocarcinoma cell line.

Species	Crude extracts	Cholangiocarcinoma cell line (% inhibition at 500 µg/mL)					
		KKU-100			KKUM-213A		
	Time	24h	48h	72h	24h	48h	72h
P. igniarius	Ethanolic	49.72 ±2.12^{A}	46.61± 2.16^{D}	83.46 ±0.5^{*c}	75.53 ±3.08*	78.23 ±3.22*	91.83 ±0.79^{*c}
	Aqueous	8.59 ±2.33	23.68 ±1.76	45.69 ±0.71	13.57 ±1.27	11.80 ±1.12	44.82 ±1.68
P. linteus	Ethanolic	74.22 ±2.08*	96.68 ±0.35*	95.52 ±0.14^{*d}	91.42 ±1.22^{b*}	96.74 ±0.46*	98.92 ±0.22*
	Aqueous	-	8.99 ±1.33	13.91 ±2.08	-	9.53 ±1.22	34.51 ±1.99
P. nigricans	Ethanolic	49.22 ±1.98^{B}	47.46 ±3.94^{E}	51.13 ±1.06^{*a}	52.20 ±0.86^{a*}	74.04 ±0.91^{a}	79.98 ±0.50^{*a}
	Aqueous	-	7.85 ±3.27	40.42 ±1.16	-	1.6 ±1.75	17.72 ±1.75
P. rimosus	Ethanolic	47.87 ±1.44^{C}	76.68 ±1.74^{b*}	91.84 ±0.34^{*b}	45.83 ±1.29^{F}	80.72 ±1.03^{d}	95.86 ±2.61^{*d}
	Aqueous	4.05 ±2.91	22.45 ±3.18	34.57 ±3.94	13.45 ±1.91	7.39 ±1.45	48.78^{G} ±1.60

Star (*) indicate statistically significant difference among mushrooms, $p < 0.05$; different lowercase letters (a,b,c,d) indicate statistically significant difference among time points, $p < 0.01$; Different uppercase letters (A,B,C,D,E,F,G) indicate statistically not significant difference among mushrooms and time points, $p < 0.05$, At point 72 hours *P. rimosus* and *P. linteus* for KKUM-213A indicate statistically not significant difference among mushrooms.

When considering the antioxidant activities of the ethanol extract of *P. rimosus* to the total phenolic and total flavonoid contents (TPC and TFC), the ethanol extract of *P. rimosus* showed excellent antioxidant activities, as well as good anticancer activities in both KKU-100 and KKUM-213A cells. As a result, we chose to partition and test anticancer activities of the fractions using an ethanol extract of *P. rimosus*. Hexane, dichloromethane, ethyl acetate, and butanol fractions of *P. rimosus* were chosen for further investigation to test anti-CCA activity. The percentage cell inhibition of each fraction was present in table 15. Dichloromethane, butanol, hexane, and ethyl acetate fractions were found to have cell growth inhibitory effects against at least part of the studied CCA cell line. In the present study, the effects of fraction extract from *P. rimosus* at a concentration ranged of 15.62-250 µg/mL against CCA cells lines (KU-100 & KKUM-213A) were determined by SRB assay. The fraction extract from *P.*

rimosus was shown to be capable of suppressing 50 % of the development of all the CCA lines investigated when used in levels less than 125 µg/mL incubation for 48 hours. The dichloromethane fraction was more potent than other fraction inhibiting the growth of the CCA cell line, when compared effective of anticancer activity ($p < 0.05$). Dichloromethane fraction was inhibited cell viability from CCA at lowest IC_{50} values, which indicated at 35.28 ± 0.80 µg/mL for KKU-100 and 45.15 ± 3.14 µg/mL for KKUM-213A cells. In both KKU-100 and KKUM-213A, the butanol fraction displayed anticancer activity against CCA, with IC_{50} values of 55.06 ± 3.35 µg/mL and 40.97 ± 1.15 µg/mL, respectively ($p < 0.05$). The ethyl acetate fraction showed moderate viability in response to CCA cell lines, with IC_{50} values of 148.05 ± 3.37 µg/mL and 104.27 ± 2.84 µg/mL, respectively, whereas the hexane fraction showed high levels of anticancer against KKUM-213A cell line, but low levels of anticancer against KKU-100 cell lines (IC_{50} 83.04 ± 1.45 & 131.88 ± 3.26 µg/mL). In both cancer cell lines, for example, strong and dose-dependent anticancer activity was reported. Table 15 shows the study's findings, including all fraction extracts.

Table 14 Effects of the fraction extracts separated from ethanol extract of *P. rimosus* on the growth of cholangiocarcinoma cell lines

Samples	Inhibition on the growth of CCA (IC_{50} µg/mL)		p-values
	KKU-100	KKUM-213A	
Ethanol extract	123.95 ± 3.27^{x}	112.11± 3.52^{a}	p <0.05
Hexane fraction	131.88±3.32^{y}	83.04 ± 1.45^{b}	
Dichloromethane fraction	35.28 ± 0.80^{z}	45.15 ± 3.14^{c}	
Ethyl acetate fraction	148.05 ±3.37^{m}	104.27± 2.84^{d}	
Butanol fraction	55.06 ± 3.35^{n}	40.97±1.15^{e}	

Star (**x,y,z,m,n**) and (**a,b,c,d,e**) indicated a significant difference between fraction separated from *P. rimosus* ($p < 0.05$) effects to chlolangiocarcinoma cell line for KKU-100 and KKUM-213A, respectively.

7. Structure Identification of the Isolated Compounds

Chromatographic separation of the hexane fraction from the ethanol extract of *P. rimosus* yielded two compounds including HPR1 and HPR2. Extensive column chromatography was used to extract the dichloromethane fraction of *P. rimosus*, yielding one product, DPR. The structures of these compounds were identified on the basis of their spectroscopic data as well as comparison with previously reported data.

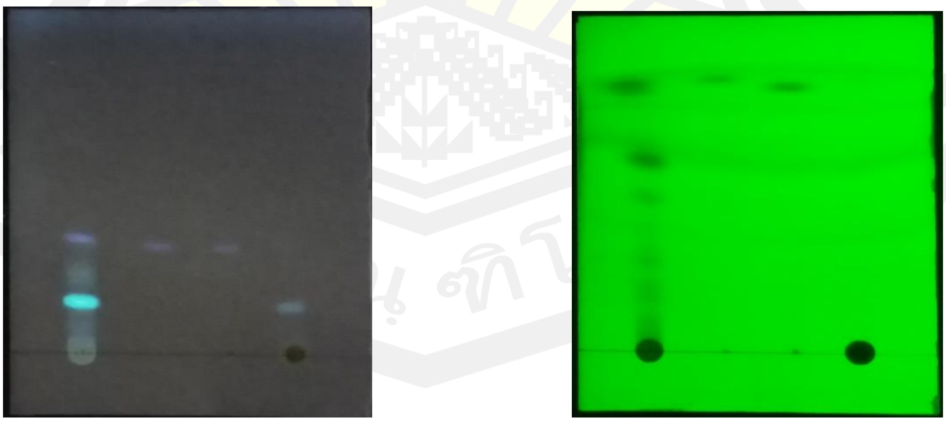
7.1 Structure Determination of Compound HPR1

Compound HPR1 was obtained as colorless needles. A formula $C_5H_8O_5$ was deduced from its $[M^+]$ ion peak at m/z 149 (calcd for $C_5H_8O_5$ 148 + $[H^+]$) in HREIMS (Figure 22). The FT-IR spectrum showed absorption bands for hydroxyl (3435.05 cm^{-1}), the aliphatic stretching (2947.01 cm^{-1}), the double bond stretching (1630.43cm^{-1}), conjugate carbonyl (1445.41 and 1339.55 cm^{-1}), The C-O stretching vibration of alcohols appears in the range 1025.67 cm^{-1}. [209] The UV absorption (Figure 20) at 291.5 nm was indicative of a double bond in the structure. The ^1H and ^{13}C NMR spectrum (Figure 18-19) showed characteristic set of signal at δ_H 3.85 (6H, singlet) was signal of methyl (CH_3) in skeleton.

The ^1H NMR (Figure 18) showed correlations with the ^{13}C NMR signals at δ_C 61.27 (2x) was assigned to methoxyl in skeleton. The ^{13}C NMR signals at δ_C149.84 (C-1) was signal of carbonyl, δ_C 128.17 (C-2) and δ_C 127.52 (C-3) were assigned alkene carbon which carbon at 2 and 3 position in skeleton.[44]

7.1.1 Thin layer chromatography

The thin-layer chromatography of hexane fraction, compound HPR1, HPR2 and ethanol extract from *P. rimosus*. These are compounds indicated Rf values as 1.25 and 1.28 respectively.



1 2 3 4   1 2 3 4

A   B

Figure 17 The Thin-layer Chromatogram of Hexane fraction from *Phellinus rimosus*

1= Hexane fraction

2= HPR1 compound (3-dimethyl-2-hydroxy-2-en-propanoic acid)

3= HPR2 compound (4,5-dimethoxy-2,3,5-trihydoxy-2,4 di-en-pentanoic acid)

4= Ethanol extract

Adsorbent: Silica gelGF$_{254}$

Solvent system: A) Hexane: Dichloromethane (7:3)

Detection:

UV 366 nm

UV 254 nm

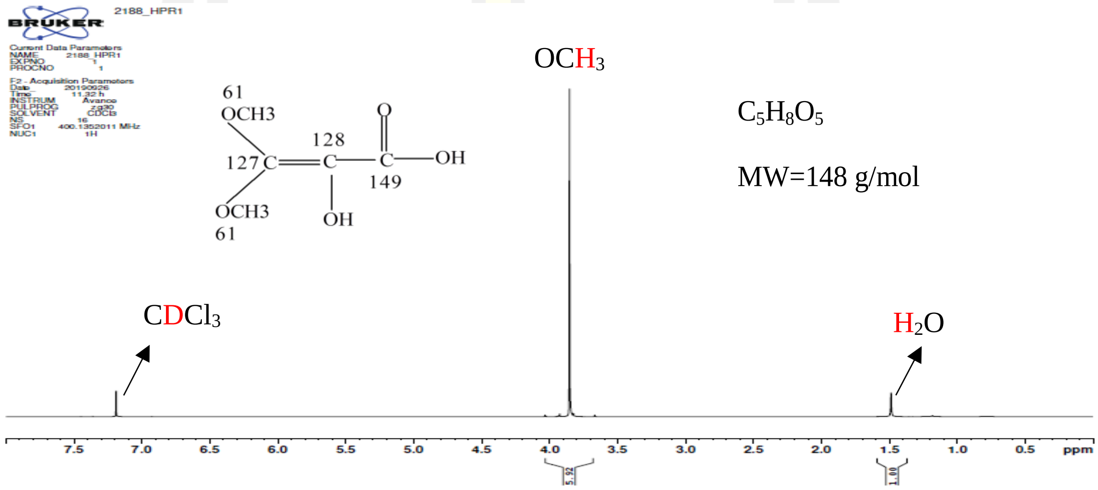


Figure 18 ^{1}HNMR (400MHz) Spectrum of Compound HPR1 (CDCl3)

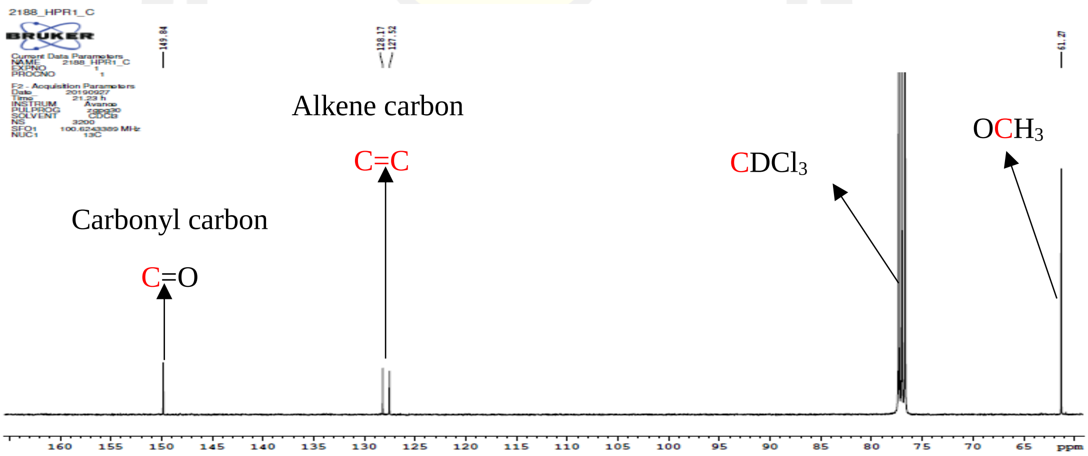


Figure 19 ^{13}CNMR (100MHz) Spectrum of Compound HPR1 (CDCl3)

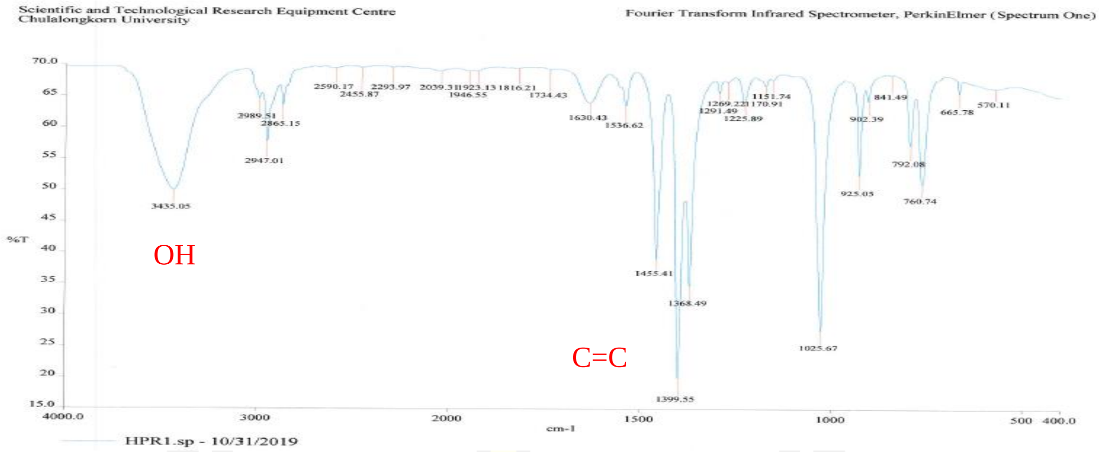


Figure 20 FT-IR Spectrum of Compound HPR1 (KBr technique)

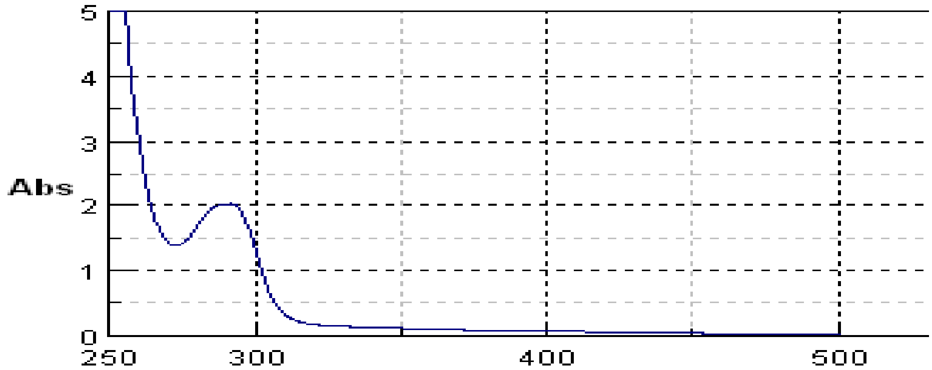


Figure 21 UV-Visible Spectrum of Compound HPR1 ($CDCl_3$)

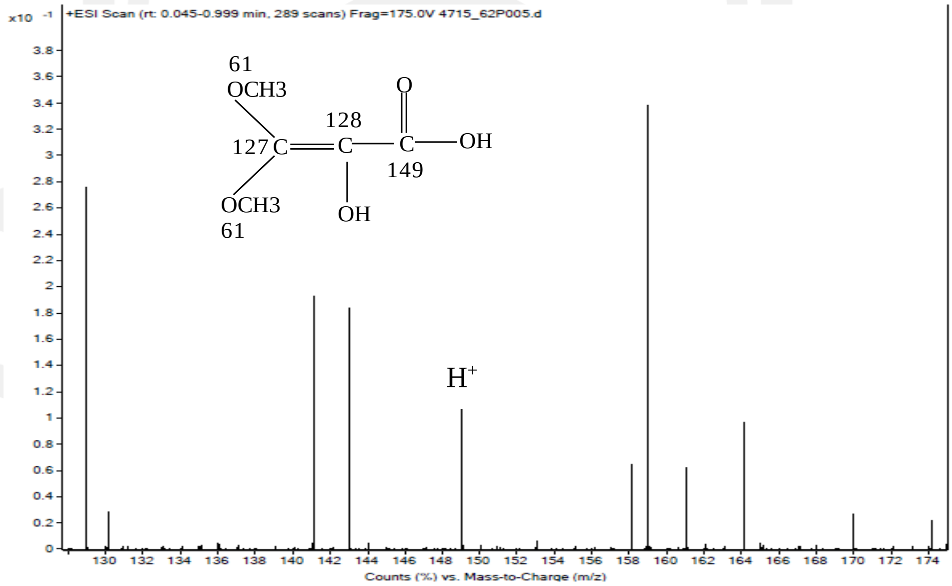


Figure 22 HREIMS Mass Spectrum of Compound HPR1 (MeOH)

7.2 Structure Determination of Compound HPR2

Compound HPR2 was obtained as cream solid. A formula $C_7H_{10}O_7$ was deduced from its $[M^+]$ ion peak at m/z 229.0524 (calcd for $C_7H_{10}O_7$ 206 + $[Na^+]$) in HREIMS (Figure 27). The FT-IR spectrum showed absorption bands for hydroxyl (3434.53 cm^{-1}), the aliphatic stretching (2920.16 cm^{-1}), the double bond stretching (1625.45cm^{-1} to 2705.3cm^{-1}), conjugate carbonyl (1547.51cm^{-1} and 1394.45cm^{-1}). The C-O stretching vibration of alcohols appears in the range 1007.35 cm^{-1}.[209,210] The UV absorptions (Figure 26) at 293 and 236 nm was indicative of double bond in structure.

The ^{1}H and ^{13}C NMR spectrum (Figure 23-24) showed characteristic set of signal at δ_H 3.85 (3H, d, *J*=0.08 Hz H-1) and δ_H 3.89 (3H, d, *J*=0.04 Hz H-2) was signal of methyl (CH_3) in skeleton. The ^{1}H NMR (Figure 23) signal at δ_H 3.85 ppm showed correlations with the ^{13}C NMR signal at δ_C 61.26 was assigned to methoxyl in alkene carbon on skeleton at C-4 position, while signal at δ_H 3.90 ppm demonstrated correlation with ^{13}C NMR signal at δ_C 63.15 ppm. The ^{13}C NMR signal at δ_C150.34 ppm (C-1) was signal of carbonyl and δ_C 145.7 (C-2), δ_C 131.91(C-3), δ_C 128.22 ppm (C-5) and δ_C 119.47 ppm (C-4) were assigned alkene carbons in skeleton.[211]

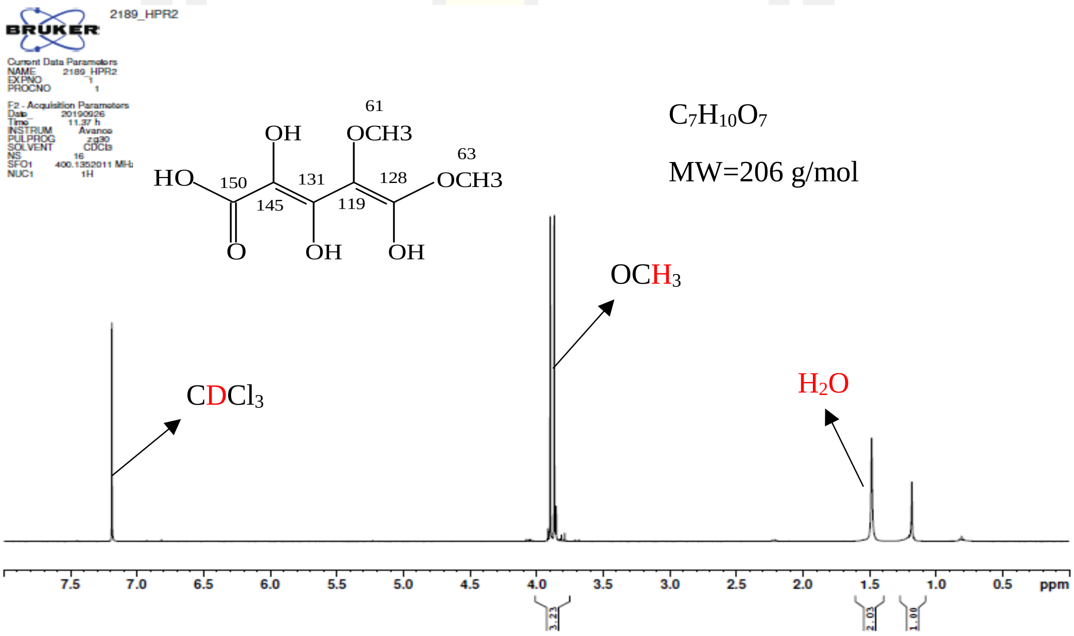


Figure 23 ^{1}HNMR (400MHz) Spectrum of Compound HPR2 (CDCl3)

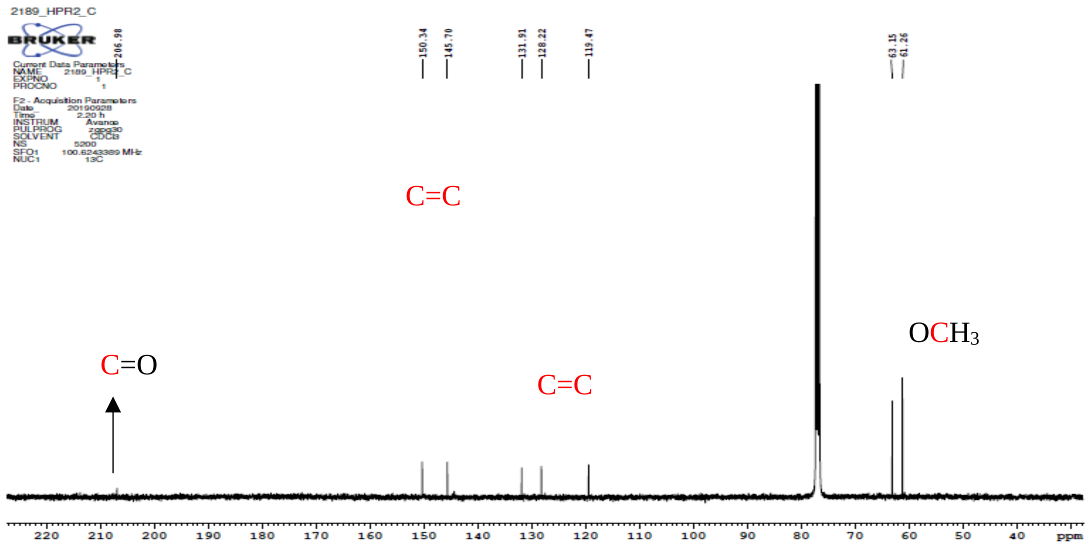


Figure 24 ^{13}C NMR (100MHz) Spectrum of Compound HPR2 (CDCl3)

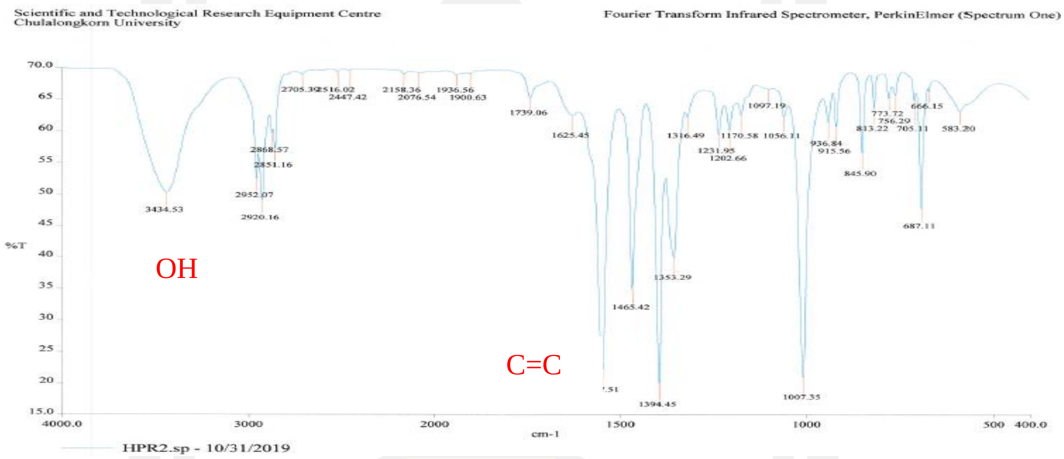


Figure 25 FT-IR Spectrum of Compound HPR2 (KBr technique)

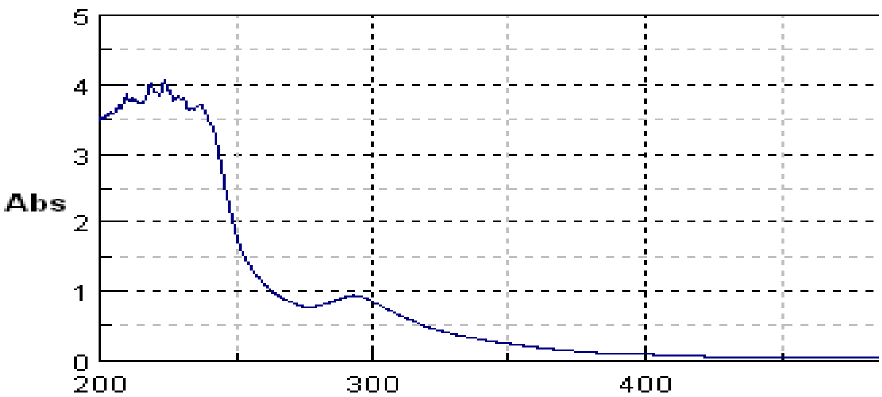


Figure 26 UV-Visible Spectrum of Compound HPR2 ($CDCl_3$)

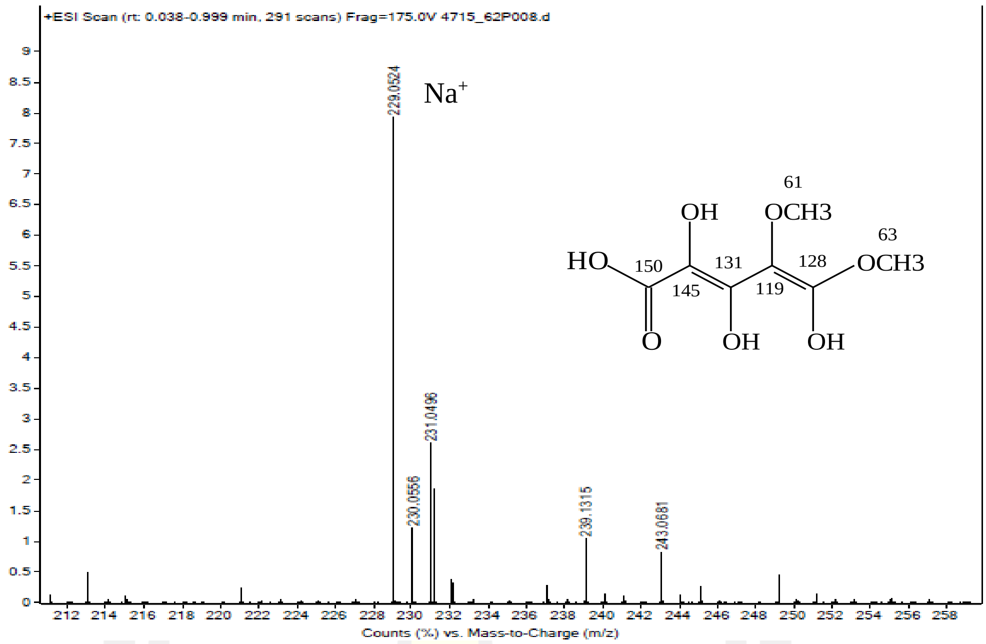


Figure 27 HREIMS Mass Spectrum of Compound HPR2 ($CDCl_3$)

7.3 Structure Determination of Compound DPR

Compound DPR was obtained as cream solid, compound DPR gave $[M + Na]^+$ ion at m/z 593.2357 in HRESIMS, corresponding to the molecular formula of $C_{31}H_{38}O_{10}$. Analysis of the ^1H and ^{13}C NMR spectrum data (table 16) indicate swiestenine, illustrating a β-substituted furan (δ_H 7.67, δ_H 7.41 and δ_H 6.42), four tetiary methyl's (δ_H 1.15, δ_H 1.06, δ_H 1.02 and δ_H 0.91) and acetyl (δ_H 2.16, δ_c169.7 and δ_c 77.3), an ester carbonyl (δ_c 171.1) and a methoxyl (δc 215.8). these evidences are corresponding to mexicanolide-type limonoids based on the above mentioned spectroscopic data and comparison of its NMR spectral data (Table 16-17) with those previously reported Ma et al [212] and Adesida et al[213] the structural compound DPR identify is 6-deoxydestigloyswietenine acetate. The structure was confirmed by HMBC correlations (Figure 38) between H-3 (δ_H 4.61) and C-4, C-5, C-29, C-30, Carbonyl (C=O) and 3-OAc, and between H-6 (δ_H 5.54) to C-4, C-5, C-7, C-10 and Carbonyl (C=O) as well as the COSY and NOESY experiments (figure 35-36).

The structures of compound was identified on the basis and advance of the spectroscopic data as well as comparison with previously reported data (Ma et al [212] and Adesida et al.[213]) Therefore, the structure of DPR was identified at 6-deoxydestigloyswietenine acetate.

Table 15 NMR Spectral data of DPR as Compared with 6-O-acetylswietenin acetate.

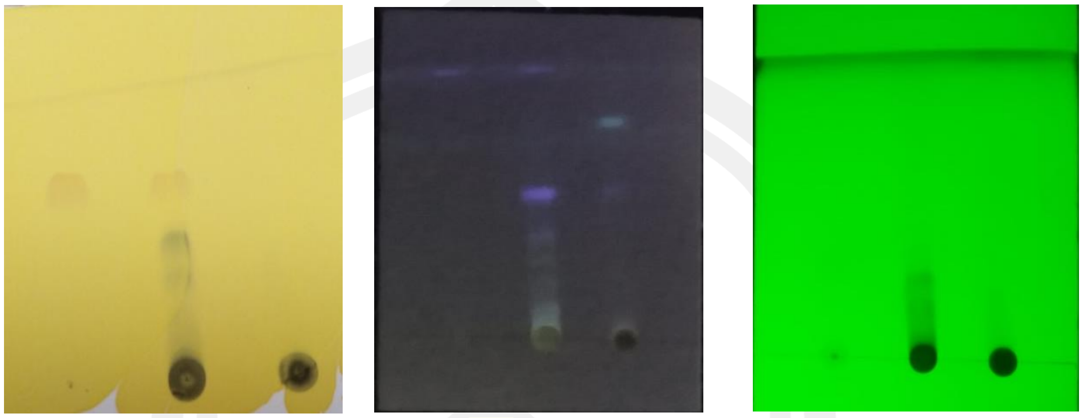
^{1}H NMR spectral data ($CDCl_3$, 400 MHz, δ, mult, J in Hz)			^{13}C NMR spectral data (δ, mult)		
No.	Chemical shift	Previous study (500 MHz)	No.	Chemical shift	Previous study
1	-	-	1	215.8	215.8
2	3.47 m	3.51 dd (9.5, 7.3)	2	48.3	48.5
3	4.61 d (9.5)	4.65 d (9.5)	3	78.2	78.0
4	-	-	4	38.6	38.7
5	3.55 s	3.60 s	5	44.8	44.7
6	5.54 s	5.56 s	6	72.7	72.7
7	-	-	7	171.1	171.1
8	-	-	8	138.8	138.3
9	2.27 m	2.28 dd (13.0, 4.4)	9	57.1	57.2
10	-	-	10	50.1	50.2
11a	1.77-1.80 m	1.79-1.83 m	11	20.8	20.9
11b	2.16-2.22 m	2.14-2.23 m			
12a	1.42 ddd (13.7,13.7, 4.8)	1.45 ddd (14.3, 11.7, 4.4)	12	34.3	34.4
12b	1.73 m	1.75 dt (14.3, 3.3)			
13	-	-	13	36.6	36.6
14	2.23 m	2.25 brd (7.0)	14	45.1	45.2
15a	2.79 dd (18.4, 1.4)	2.81 brd (18.5)	15	29.9	29.9
15b	2.88 dd (18.5, 6.0)	2.90 d (18.5, 7.0)			
16	-	-	16	169.4	169.4
17	5.63 s	5.63 s	17	77.3	77.3
18	1.02 s, 3H	1.05 s, 3H	18	21.8	21.8
19	1.15 s, 3H	1.19 s, 3H	19	15.6	15.7
20	-	-	20	120.8	120.8
21	7.67 t (0.7)	7.69 t (1.0)	21	141.3	141.3
22	6.42 dd (1.8, 0.7)	6.44 dd (1.7, 1.0)	22	109.5	109.5
23	7.41 t (1.7)	7.44 t (1.7)	23	143.0	143.1
28	0.91 s, 3H	1.10 s, 3H	28	22.6	22.6
29	1.06 s, 3H	0.94 s, 3H	29	22.9	23.0
30	5.34 dt (7.4, 2.3)	5.36 dt (7.3, 2.2)	30	122.9	123.0
7-OMe	3.71 s, 3H	3.72 s, 3H	7-OMe	53.2	53.2
2’(3-OAc)	2.05 s, 3H	2.39 q (7.5), 2H	3-OAc	170.7	173.9
			3-OAc (CH_3)	20.3	27.1
3’	-	1.12 t (7.5), 3H	3’	-	8.8
6-OAc	2.16 s, 3H	2.19 s, 3H	6-OAc	169.7	169.7
			6-OAc (CH_3)	21.0	21.0

Table 16 Two Dimensional NMR Spectral data of DPR Compound.

Proton	HSQC	HMBC	COSY	NOESY
7.67 (H-21)	141.3 (C-21)	C-20, C-22, C-23	H-22, H-23	H-17
7.41 (H-23)	143.0 (C-23)	C-20, C-21, C-22,	H-21, H-22	H-22
6.42 (H-22)	109.5 (C-22)	C-20, C-21, C-23	H-21, H-23	H-17, H-23, CH_3-18
5.62 (H-17)	77.3 (C-17)	C-12, C-13, C-14, C-18, C-20, C-21, C-22	-	H-5, H-21, H-22
5.54 (H-6)	72.7 (C-6)	C-4, C-5, 6-OAc (C=O), C-7, C-10	H-5	H-5, CH_3-19
5.34 (H-30)	122.9 (C-30)	C-1, C-9, C-14,	H-2, H-14	H-2, H-15A, H-15B
4.61 (H-3)	78.2 (C-3)	3-OAc (C=O), C-4, C-5, C-29, C-30	H-2	H-2, CH_3-28, CH_3-29
3.71(7-OMe)	53.2 (7-OMe, CH_3)	C-5, C-7	-	CH_3-28
3.55 (H-5)	44.8 (C-5)	C-3, C-4, C-6, C-7, C-9, C-10, C-19, C-29	H-6	H-6, H-11B, H-17, CH_3-28
3.47 (H-2)	48.3 (C-2)	C-1, C-3, C-8, C-10, C-30	H-3, H-30	H-3, H-30
2.88 (H-15b)	29.9 (C-15)	C-8, C-13, C-14, C-16	H-14	H-14, CH_3-18, H-30
2.79 (H-15a)		C-8, C-13, C-14, C-16	H-14	H-14, CH_3-18, H-30
2.27 (H-9)	57.1 (C-9)	C-5,C-8,C-11,C-30	-	-
2.23 (H-14)	45.1 (C-14)	C-8, C-9, C-13, C-15, C-17, C-30	H-15a, H-15b, H-30	H-12a, H-12b, H-15a, H-15b
2.16-2.22 (H-11b)	20.8 (C-11)	C-9, C-12, C-14,	H-12a, H-12b	H-5, H-12a, H-12b
2.16 (6-OAc, CH_3)	21.0 (6-OAc, CH_3)	6-OAc (C=O), C-6	-	-
2.05 (3-OAc, CH_3)	20.3 (3-OAc, CH_3)	3-OAc (C=O), C-3	-	-
1.77-1.80 (H-11a)	20.8 (C-11)	C-9, C-12, C-14	H-12a, H-12b	H-12a, H-12b, CH_3-19
1.73 (H-12b)	34.3 (C-12)	C-11, C-13, C-17	H-11a, H-11b	H-14, CH_3-18
1.42 (H-12a)		C-11, C-13, C-17	H-11a, H-11b	H-14, CH_3-18
1.15 (CH_3-19)	15.6 (C-19)	C-1, C-5, C-9, C-10	-	H-6, H-11a
1.06 (CH_3-29)	22.9 (C-29)	C-3, C-4,C-5, C-28	-	H-3
1.02 (CH_3-18)	21.8 (C-18)	C-12, C-13, C-14, C-17	-	H-12a, H-12b, H-15a, H-15b, H-22
0.91 (CH_3-28)	22.6 (C-28)	C-3, C-4, C-5, C-29	-	H-3, H-5, 7-OMe

7.3.1Thin-layer Chromatography

Thin-layer chromatogram of dichloromethane fraction from *Phellinus rimosus*.



1 2 3 1 2 3 1 2 3

A B C

Figure 28 Thin-layer chromatogram of dichloromethane fraction from *Phellinus rimosus*

1= DPR compounds (6-deoxydestigloyswietenine acetate)

2= Dichloromethane fraction

3= Ethanol extract

Adsorbent: Silica gelGF$_{254}$

Solvent system: A) Dichloromethane: Hexane (8:2)

Detection:

A) 10 % ferric chloride in ethanol

B) UV 366 nm

C) UV 254 nm

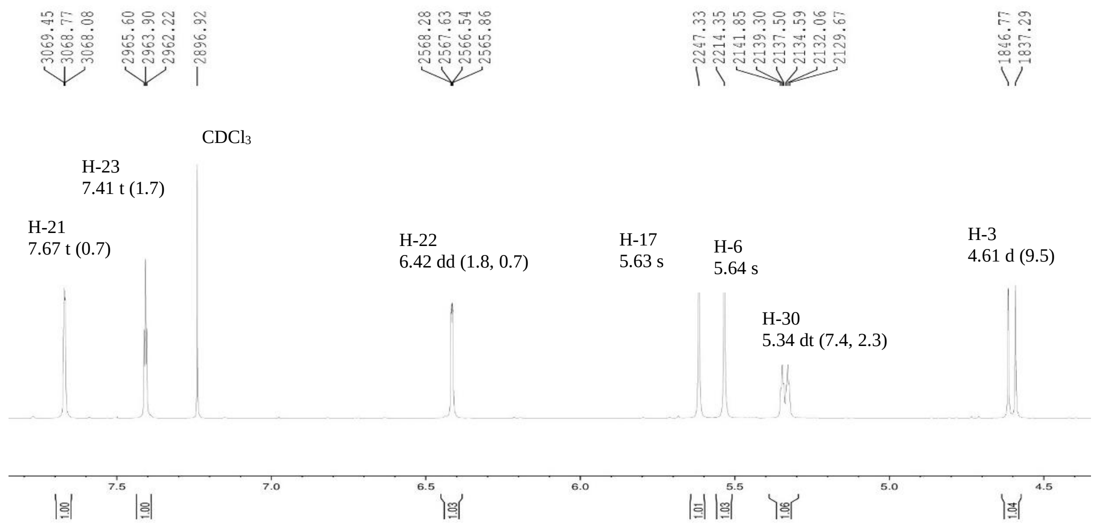


Figure 29 ¹HNMR (400MHz) Spectrum of Compound DPR (CDCl3)

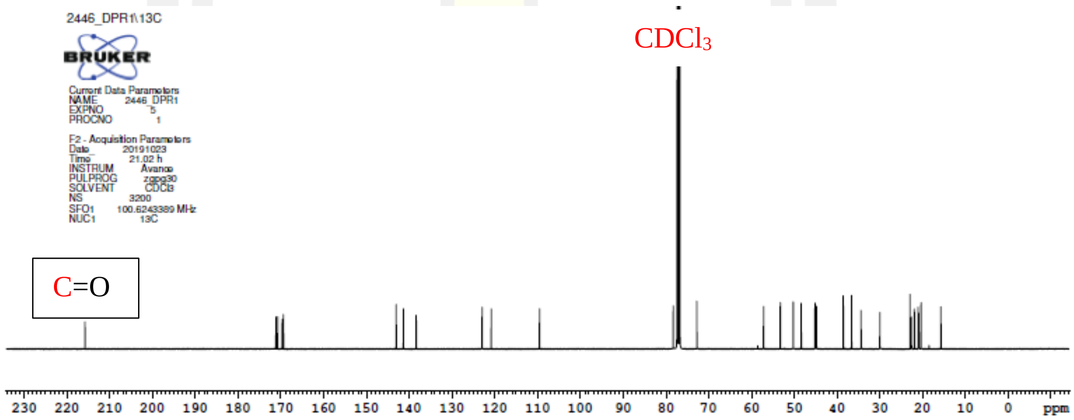


Figure 30 ¹³C NMR (400MHz) Spectrum of Compound DPR (CDCl3)

Scientific Equipment Center , PSU.

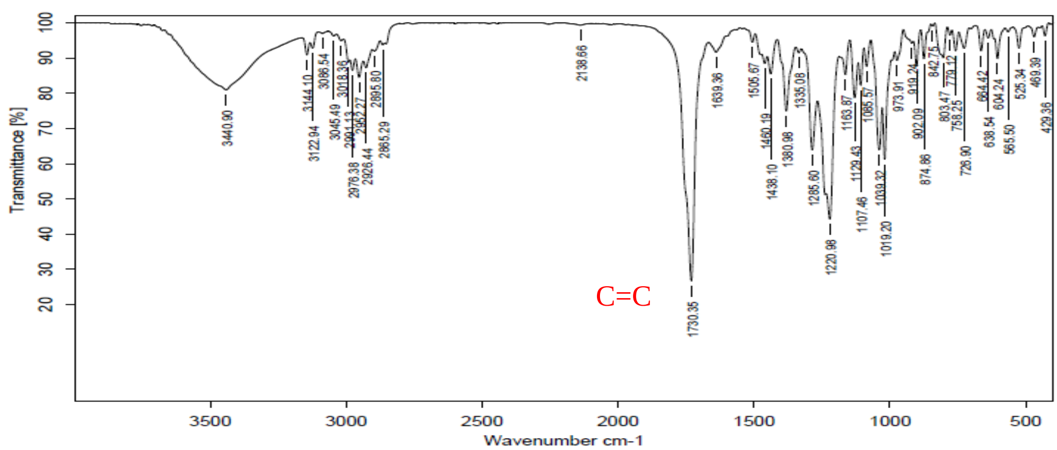


Figure 31 FT-IR Spectrum of Compound DPR (KBr technique)

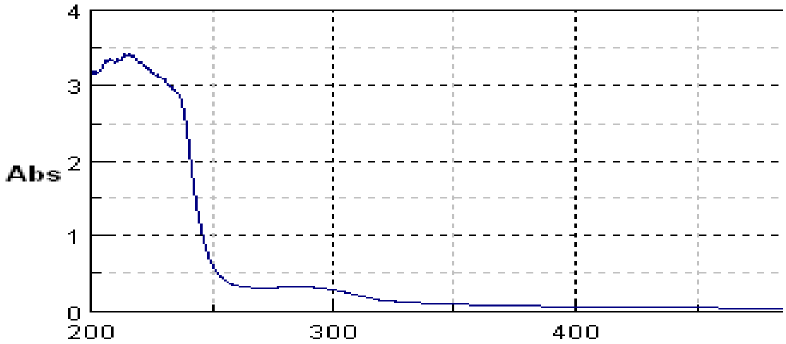


Figure 32 UV-Visible Spectrum of Compound DPR (CDCl3)

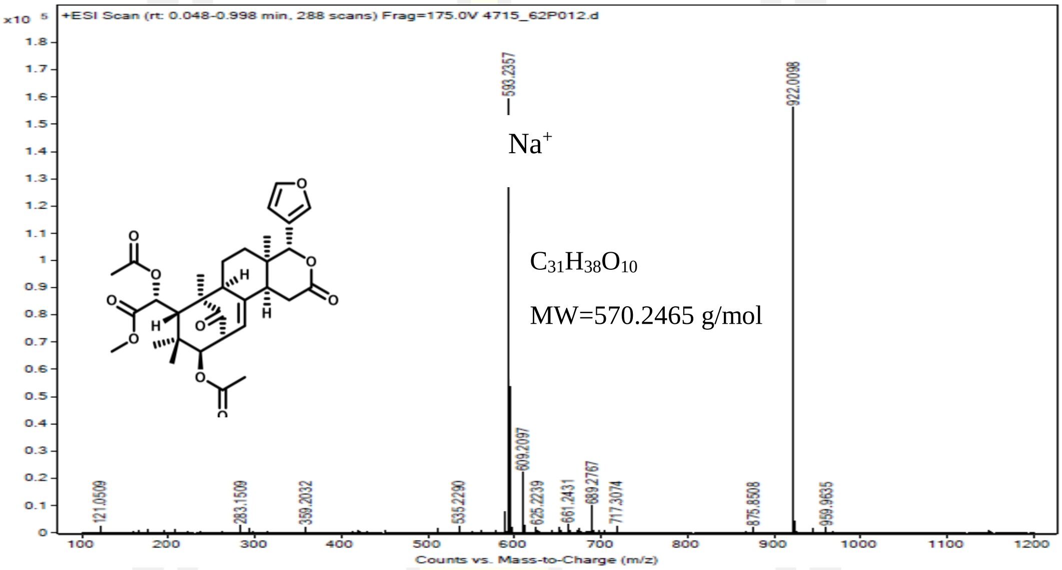


Figure 33 HREIMS Mass Spectrum of Compound DPR (CDCl$_3$)

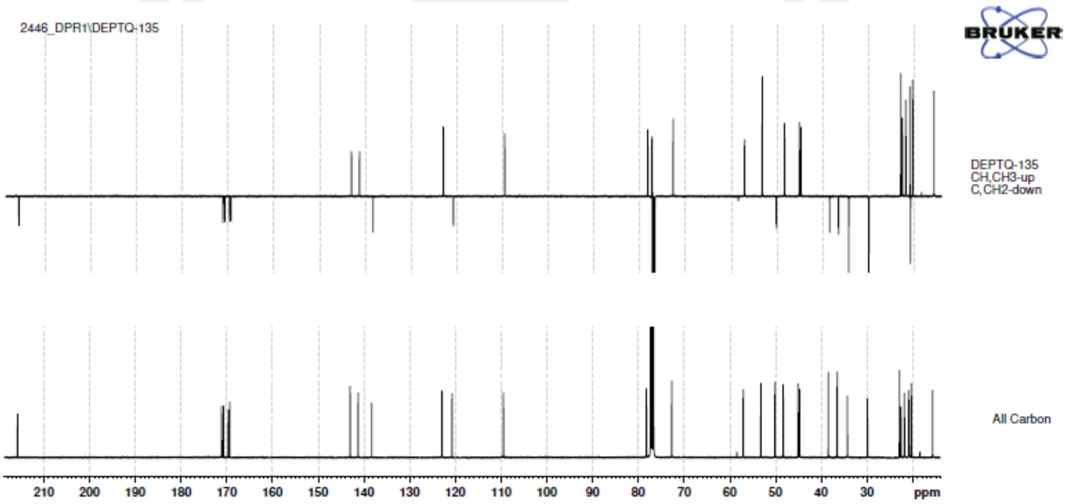


Figure 34 DEPT135 Spectrum of Compound DPR (CDCl$_3$)

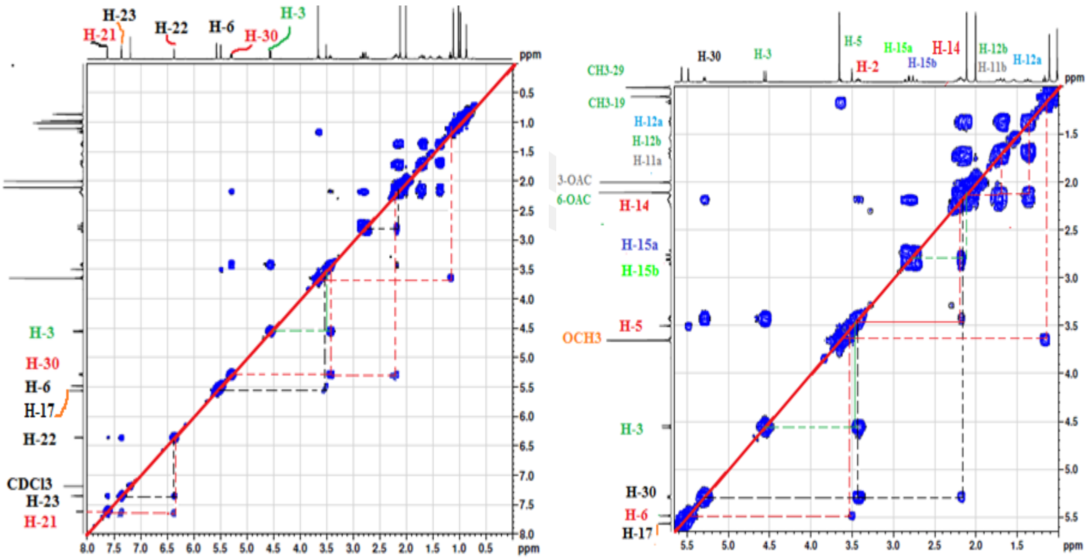


Figure 35 COSY Spectrum of Compound DPR ($CDCl_3$)

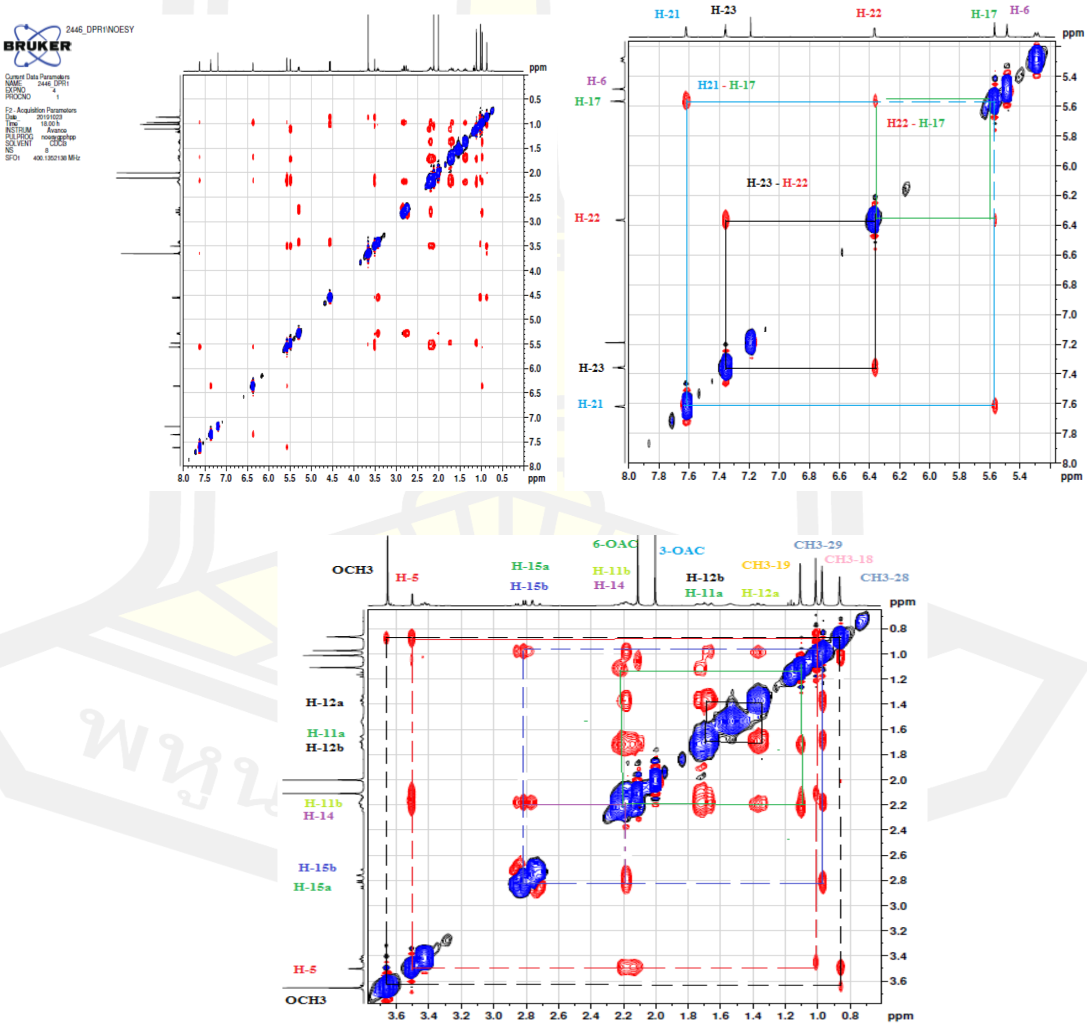


Figure 36 NEOSY Spectrum of Compound DPR ($CDCl_3$)

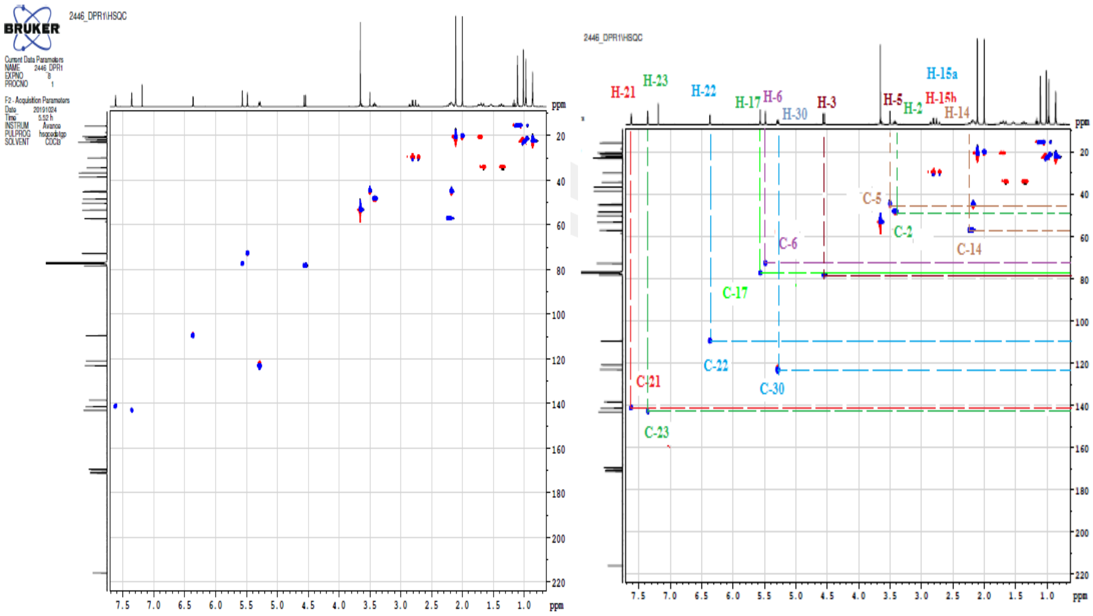


Figure 37 HSQC Spectrum of compound DPR (CDCl3)

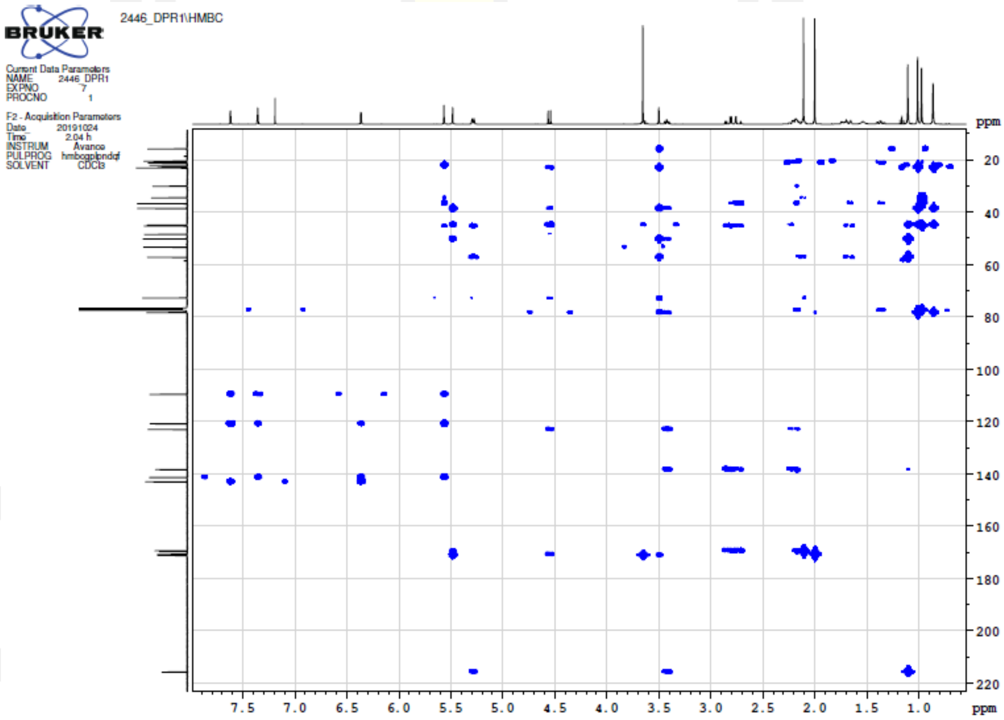


Figure 38 HMBC Spectrum of Compound DPR ($CDCl_3$)

Chapter V
Discussion

1. Antioxidant, Total Phenolic and Flavonoid Contents

The antioxidant capacity differences between extracts were mostly due to the polar solvents utilized to extract antioxidants from materials with polyphenols as the primary anti oxidative active components. However, some fungi-derived materials may contain lipophilic compounds such as carotenoids, terpenoids, and the use of various polarity solvents may provide more comprehensive information on their antioxidant potential, particularly for less studied mushroom species. Our findings discovered that various solvents separated the distribution of antioxidative active components in the extract, which was mostly dependent on mushroom species (Table 10). Our findings suggest that after using polar organic solvents to extract antioxidants from some mushroom species, ethanol and water may be helpful solvents. In terms of toxicity and availability, water was the preferred solvent; however, it is not always sufficient for the isolation of mushroom bioactive components. In DPPH, ABTS, FRAP, TPC, and TFC assays, for example, ethanol extract of *P. rimosus* was the greatest antioxidant activity. The results were confirmed that phenolic compounds and flavonoids presented important contributors to the antioxidant properties of these extracts. In general, ABTS, DPPH• scavenging, and FRAP yields were much higher with ethanol extracts of most species than with other solvents; numerous previous studies have indicated that polar solvents extract more antioxidants from botanicals than lower polarity solvents.[214] The acquired disparities between the applied tests could be explained by a number of factors. Although the use of radical scavenging or reduction tests were based on single-electron transfer and/or hydrogen atom transfer, the reaction processes in each test were unique; they can be influenced by reaction media, pH, the structure of antioxidative chemicals in the extracts, their interactions, and other factors.[215] The values measured with Folin-Ciocalteu reagent and expressed in gallic acid or other phenolic compounds were generally accepted as representing the total phenolic content (TPC). Although it is not fully correct. The Folin-Ciocalteu reagent reacts not only with phenolic, but also with other chemicals in the reaction system that has reducing capacity.[215] As a result, the word TPC can be used conditionally; however, we were utilized it in our study for convenience. The *P. rimosus* had the maximum phenolic content (361.04 ± 5.69 mg GAE/g) in the ethanol extract, while *P. lineteus* and *P. igniarius* had 184.86 ± 5.54 and 145.53 ± 2.10 mg GAE/g, respectively. The TPC in this investigation had corresponded to that published by Seephonkai et al, who found that the TPC in ethanol extracts of *P. lineteus* and *P. igniarius* were 159.60 ± 0.23, 25.8 ± 0.31 mg GAE/g of extract, respectively; however, the findings of our studies were not equivalent. It is impossible process because the extraction method, mushroom source, and other factors were all different.[85] Therefor, the results of TPC for investigation in the present study were different based on reports of Seephonkai et al.[216] The total flavonoid content (TFC) values measured with aluminum colorimetric assay was expressed in rutin or other flavonoid compounds. The result of this study presented TFC was explained in Table 10, which the results were different from the of reported Wang et al, which showed TFC of 40.4 ± 0.1 mg/g for extracts from the dry fruiting body and 52.1 ± 0.09 mg/g for the extract from submerged culture.[217] This result was different because of the solvent for extraction. The reason might be due to the former study used 60 % ethanol while the present study used 95 % ethanol for extraction.

The antioxidant capability of the mushrooms analyzed was found to be somewhat variable: there were variances between the examined species as well as the extraction solvents. In assessing the antioxidant effects of mushroom extracts, the assay method was equally crucial. In almost all of the experiments, ethanol extracts were the most potential of antioxidants. The quantity of TPC, TFC, and antioxidant activity of mushroom extracts were found to be highly correlated in the current study.

2. Anti-inflammatory Activity

Numerous mushroom extracts and single compounds isolated from the genus *Phellinus* have been stated to have pharmacological activities, such as anti-inflammatory and anti-bacterial activity.[218] However, The biological activities of the pure compounds isolated from *P. rimosus* have not been studied, despite its widespread use in folk medicine. For the first time, the authors showed that a compound isolated from *P. rimosus* can exert anti-inflammatory effect in LPS-activated RAW 264.7 macrophages, which may be linked, at least in part, to the inhibitory action of pro-inflammatory mediators like NO development. Inflammation was one of the most significant biological defensive responses to tissue injury, and it may also cause cell injury, resulting in the release of pro-inflammatory mediators.[219] Inflammatory factors were considered fundamental elements in the chronic inflammation associated with many diseases, such as atherosclerosis, obesity, diabetes diseases and cancer. Steroidal and non-steroidal anti-inflammatory drugs were currently used to treat acute inflammation. However, these drugs were not entirely successful in curing chronic inflammatory disorders, and often have side effects. Therefore, the identification of new, safer and more effective anti-inflammatory compound was necessary. In general, the total phenolic content of the mushroom extract was highly correlated with their free radical scavenging activities.[133] Therefore, The crude extract was first evaluated for total phenolic content and free radical scavenging capacities, which will be a convenient way to find a potent inhibitory compound against NO production. In this study, *P. rimosus* exhibited a higher quantity of total phenolics when compared with crude extract and other compounds ($p < 0.05$). The best inhibitory activity on NO synthesis was indicated by *P. rimosus* and *P. linteus* extracts. NO inhibitory activities of DPR compound isolated from *P. rimosus* extracts were 99.63 0.53 percent at 50 µg/mL and 30.01 ± 0.71 percent at 6.25 µg/mL, with an IC_{50} of 8.69 ± 0.08 µg/mL.

The antioxidant and anti-cancer properties of *Phellinus* species has been confirmed by these findings. The existence of various chemical groups such as alkaloids, terpenoids, and flavonoids associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals demonstrated by phenolic compound and flavonoid. The NO scavenging activity of phenolic compounds and flavonoid has been reported previously.[220] This study's findings were found to be strongly inhibitory activity on LPS-stimulated NO production. In the LPS-stimulated macrophages, NO is generated by the process of single enzymes: an inducible isoform of cyclooxygenase-2 (COX-2), and iNOS.[221] Hence, suppression of the iNOS and COX-2 protein expression in LPS-stimulated RAW 264.7 cells are important criteria to inhibit NO production. Further studies are still needed in order to clarify the exact role of the isolated compound 6-deoxydestigloyswietenine acetate, 3-dimethyl-2-hydroxy-2-en-propanoic acid, 4,5-dimethoxy-2,3,5-trihydroxy-2,4 di-en-pentanoic acid on inhibits cytokines such as IL-1β, IL-6, TNF-α and Cox-2 expression.

3. Anti-cancer Activity

The growth of CCA cell lines (KKU-100 & KKUM-213A) was evaluated by using the sulforhodamine B (SRB) assay adopted by the US National Cancer Institute (NCI). Because of their strong antioxidant activity and high total phenolic and flavonoid content, the ethanol extracts of *Phellinus* mushrooms were chosen for further investigation in the current study to test anti-CCA activity. The result of anticancer activity of extracts from *P. linteus* against CCA both KKU-100 and KKUM-213A shown the percentage of growth inhibition ranged from 74.22 ± 2.08 to 95.52 ± 0.14 for KKU-100 and 91.42 ± 1.22 to 98.92 ± 0.22 for KKUM-213A cells ($p<0.05$). This is the first reported of PE on anticancer against CCA cell lines. In the study of Park HJ & et al. revealed that the combination of the ethanol extract of *P. linteus* and a monoclonal antibody, cetuximab increased the sensitivity of KRAS (v-ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutated colon cancer cells to cetuximab. This finding indicated the potential of *Phellinus* mushroom extracts as a medical supplement against colon cancer.[222] *P. linteus* was a common mushroom that has been used in traditional medicine. There are many reported about *P. linteus* such as antioxidative & immunomodulating activities,[41] anticancer property and anti-inflammatory activity. Anticancer capability may be linked to anti-oxidant capability; hence it's possible that phenolic and flavonoid concentration in *P. linteus* extracts had anticancer action against the CCA cell line. In the previous study, the pure compound isolated from *P. linteus* has effective against cancer cells such as hispidin. [34] Many pure chemicals isolated from *P. linteus* have been reported, including phelligridimer A, phelligridins A-J, baumin, phellinin C, phellinin B1 and B2, and phellinusfrans A & B, phellifuropyranone A, and phelliusin A, all of which have biological activity. In this investigation, *P. igniarius* ethanol extract showed anticancer efficacy in both cells, with percentage cell growth inhibition ranging from 46.61 ± 2.16 to 83.46 ± 0.50 for KKU-100 and 75.53 ± 3.08 to 91.83 ± 0.79 for KKUM-213A cells. Result of this study was correlated with Song TY & et al and reported that the ethanolic extract of *P. igniarius* displayed antiproliferative and antimetastatic effects in human hepatocarcinoma (SK-Hep-1) and rat heart vascular endothelial (RHE) cells.[185] The hispolon isolated from *P. igniarius* showed good apoptosis with lung cancer A549 and H661 cells. The half maximal inhibitory concentration (IC_{50}) of hispolon on A549 cells was about 35.9 ± 6.9, 28.8 ± 3.1 and 8.1 ± 2.3 µM for 24, 48 and 72 h, respectively.[47] In the previous the study, *P. igniarius* had indicated many biological activities against cancer cell line; However, the current reported anticancer activity of CCA of *Phellinus* mushrooms for the first time. Hisspolon, 21-homopregnene derivatives, 12-hydroxy-cadinol, naringenin, phelligridimer A, phelligridins G & H were discovered to reduce rat liver microsomal lipid peroxidation and demonstrate cytotoxic effects against human cancer cell lines in the pure substance obtained from *P. igniarius*.[56] When compared to other PE, *P. nigricans* had the least anticancer efficacy in this investigation. The percentage cell inhibition ranged from 49.22 ± 1.98 to 51.13 ± 1.06 for KKU-100 cells and 52.20 ± 0.86 to 79.98 ± 0.50 for KKUM-213A cells. The findings of this investigation revealed that this mushroom was effective; nevertheless, a previous study discovered that proteoglycans extracted from the mycelium of *P. nigricans* has anticancer and immunomodulatory properties.[223] These findings showed that polysaccharides from *P. nigricans* mycelia could be used in functional foods or pharmaceuticals as antioxidants and immunostimulants. [61] Many biological activities of *P. nigricans* have been documented, but none has reported for anticancer activities against CCA cell lines, hence this was the first report on anticancer activity against CCA cell lines. *P. rimosus* inhibited of growth cell viability ranged from 47.87 ± 1.44 to 91.84 ± 0.34 for KKU-100 and 45.83 ± 1.29 to 95.86 ± 2.61 for KKUM-213A cells. In the present study, *P. rimosus* ethanol extracts showed the second manner in anticancer activities against CCA cell line. Ajith. TA. (2004) reported biological activities of *P. rimosus*, including antioxidant, anti-inflammatory, antimutagenic, anticancer, anticarcinogenic activities, and toxicity research. Ajith. TA (2004) found that *P. rimosus* was rich in biologically active chemicals with medicinal potential.[63] The *P. rimosus* was good mushroom for traditional medicine and it is interesting to find a bioactive compound for the treatment of many diseases, particularly CCA.[224] According to the findings of this study, *P. rimosus* possessed great anticancer property against CCA, high antioxidant property, and high amount of TPC and TFC. *P. rimosus* extract also showed the highest biological activities when compared to other PEs in this study. Therefore, *P. rimosus* was selected to further investigation (separation and isolation of the pure components) due to its property that fit the criteria for selection. As the result, *P. rimosus* extract was chosen for separation and isolation of pure compounds.

Four fraction separated from ethanol extract of *P. rimosus* demonstrated percentage of inhibition of growth cell viability with IC_{50} values ranged from 35.28 ± 080 µg/mL to 148.05 ± 3.37 µg/mL for KKU -100 cell line and 40.97 ± 1.15 µg/mL to 104.27 ± 2.84 µg/mL for KKUM-213A cell line. According to the current research, the polarity of a chemical has an effect on cell survival in this mushroom. The KKU-100 cell line was sensitive to a dichloromethane fraction, which has a medium to low polarity, but the KKUM-213A cell line was sensitive to a butanol fraction, which has a high polarity. Preliminary chemical evaluation of the dichloromethane fraction revealed several spots on TLC fingerprint, implying that the main marker components were triterpenoid, flavonoid, and phenolic compounds, but the butanol fraction was suggested to have polyphenol of quinone. Ajith.TA discovered polyphenols and flavonoids in the ethyl acetate and methanol fractions of *P. rimosus* in preliminary research. A number of these chemicals have been shown to have anticancer property.[186] Although the mechanism of anticancer inhibiting activity of flavonoids is not clear, but the differences in compound properties have an effect on cell viability, and cell properties were important for this study's experiment. KKU-100 and KKUM-213A cells have significantly different properties and sources. KKUM-213A is a high-invasive cell line derived from adenocarcinoma CCA with good differentiation, whereas KKU-100 is a low-invasive cell line derived from adenocarcinoma CCA with poor differentiation.[206]

The experimental results indicated that the CCA activities of ethanolic and aqueous extracts of *P. rimosus* might be due to two different classes of substances. Immunomodulating polysaccharides might be responsible for the antitumor activity of aqueous extract. The activity of ethanolic extract could be due to substances other than polysaccharides, possibly polyphenols and flavonoids. The major constituent of the aqueous extract of *P. rimosus* was found to be polysaccharides. Polysaccharides are the most important for modern medicine and *β*-glucan is the best known and the most versatile metabolite with a wide spectrum of biological activity.[224] The aqueous extracts of *Phellinus* mushroom had the least anticancer efficacy against KKU-100 and KKUM-213A, which could be due to mechanisms pathways, and the experimental method was not suitable or specific against CCA cells. The aqueous extracts of *Phellinus* mushroom revealed anticancer and immunomodulatory properties in a previous study.[39, 42]

Previous research has found a link between the consumption of mushroom foods, which are high in antioxidants, and a lower risk of diseases caused by reactive oxygen species.[89] The studies have also suggested that mushroom secondary metabolites acted as excellent anti-inflammatory agents and played an important role in oxidative stress and inflammation.[225] Because of the anti-inflammatory, antioxidant, and anticancer properties of the ethanol extract of *P. rimosus*, as well as the extremely total phenolic and flavonoid contents, the information can be used as a basis pharmacological data for consideration of their therapeutic potential and prevention of illness, particularly against oxidative stress. In this context, mushrooms have a long history of use in the oriental medicine to prevent and fight numerous diseases. Nowadays, mushroom extracts are commercialized as dietary supplements for their properties, mainly for the enhancement of immune function and antitumor activity. To develop natural product as dietary supplements from *P. rimosus*, the traditional healers can prepare dosage form in term of infusions, capsules, powders and macerate with alcohol. Ajith AT has reported the study on acute and sub-acute toxicity of ethyl acetate, methanol, and aqueous extracts from *P. rimosus*, with the concentration at 250 mg/kg had no cytotoxicity (no change in the hematological or biochemical parameters between group sample for treatment and control group).[195] The cytotoxicity study information of mushrooms have a long history of use by traditional healers. Therefore, the evidence is important to confirm the safety of the *P .rimosus* for use in longevity and immune stimulant.

4. Effect of Solvents and Time periods

The solvents used for extraction of PE exhibited resistance to cancer cells, as evidenced by the current study's findings. The difference in anticancer activity between the ethanol extract and the water extract of PE was substantial. The percent yield of PE from ethanol was higher than that of water, indicating that the qualities of the solvents for extraction were important. In previous studies, the solvents had effects on yield and properties of samples.[129, 226]

The amount of time spent experimenting with anticancer activities was an important element to consider. According to the findings of the study, the time point for incubation in the experiment had an effect on survival in both cells (KKU-100 & KKUM-213A), which was consistent with Wu et al (2016). The reports about A549 or H661 cells were treated with various concentrations of hispolon for various time periods to determine the effect of hispolon on the cell viability of non-small cell lung cancer cells.[47] According to the findings, the most effective extracts for antioxidant activity has an IC_{50} value of 14.06 ± 0.08-32.46 ± 0.30 µg/mL, making them the best targeting option for turning natural product into commercial products. The results showed that ethanolic extracts from *P. linteus*, *P. igniarius*, and *P. nigricans* had growth inhibition percentages against CCA of 98.9 ± 0.09, 91.83 ± 1.3, and 79.98 ± 0.85, respectively, which were suitable aspirants for further bioactivity-guided in the search for new active anti-CCA compounds in the future. The *P. rimosus* was demonstrated excellent antioxidant and anti-CCA properties.

At the concentration of 500 µg/mL, *P. linteus* was shown to be the most effective anticancer against CCA cell line, with percentage cell viability inhibition of 96.68 ± 0.35, 95.52 ± 0.14 for KKU-100 and 96.74 ± 0.46, 98.92 ± 0.22 for KKUM-

213A at 48 and 72 hours. At 48 and 72 hours, the *P. rimosus* showed the second active anti-CCA cell line (76.68 1.74, 91.84 ± 0.34 for KKU-100 and 80.72 ± 1.03, 95.86 ± 2.61 for KKUM-213A). The anti-inflammatory activities of *P. linteus* and *P. rimosus* were not statistically significant in inhibiting nitric production on LPS-activated macrophages RAW 264.7, but *P. rimosus* had the best antioxidant activities, high total phenolic and flavonoid contents, and high yield of crude extracts. Considering the properties of all samples and the findings of the previous study, we chose an ethanol extract of *P. rimosus* for further isolation, identification and anti-inflammatory activity study of pure compounds.

Chapter VI
Conclusion and Recommendation

1. Conclusion

Phellinus is a group of medicinal mushroom belonging to the family Hymenochaetaceae. It is found in South East Asia including Thailand. Although Thailand has over 31 species of *Phellinus* mushrooms, only two have been recognized for use in medicine. For thousands of years in Asian countries, *Phellinus* has been used as a component in Traditional Chinese Medicine to treat cancers, herpes, earaches, rash, ischemia, and skin diseases. The fruiting body of this mushroom is used in Thai traditional medicine as a remedy for lung cancer, liver, prostate cancer, and skin diseases. According to a previous study the *Phellinus* mushroom extracts were found to have antitumor, anticancer, antibacterial, anti-inflammatory, and antioxidant properties.

The samples of *Phellinus* mushrooms including *P. igniarius*, *P. linteus, P. nigricans* and *P. rimosus* were prepared in two ways: macerated in 95 % ethanol and decocted in distilled water. The solvents used in the extraction were evaporated until they were completely dry. The ethanol extract was dried in a rotary evaporator, while the aqueous extracts were dried in a freeze dryer at -98 °C for 26 hours.

The phytochemical screening of the sample revealed a number of chemical groups, including phenolic, flavonoid, triterpenoid, and alkaloid, which were detected in higher concentrations in the ethanol extract than in the aqueous extract. DPPH, ABTS, and FRAP tests were used to determine the antioxidant activity of the extracts. Total phenolics and flavonoids content were determined using colorimetric tests. Anti-inflammatory activity was determined using the nitric oxide method, while anticancer activity was determined using the SRB assay against the CCA cell line.

The results showed that the ethanol extracts had the maximum yield and were active against the CCA cell lines, as well as having anti-inflammatory and antioxidant properties. Furthermore, the ethanol extract had the highest amount of total phenolic and flavonoid content. The ethanol extracts from *P. igniarius, P. linteus* and *P. nigricans* inhibit radical scavenging DPPH at IC_{50} values ranging from 28.85 ± 0.56 to 32.46 ± 0.30 μg/mL, ABTS at IC_{50} values ranging 14.06 ± 0.08 to 18.44 ± 0.48 μg/mL, and antioxidant activity measured by FRAP method revealed the highest values of 22.70 ± 5.04 to 52.39 ± 0.25 mM de $FeSO_4$/100 mg.

P. rimosus had the best antioxidant, anticancer, anti-inflammatory, total phenolic and flavonoid contents, (DPPH, IC_{50}=9.56 ± 0.47, ABTS, IC_{50}=5.04 ± 0.06, and FRAP method showed 52.39 ± 0.74 de $FeSO_4$/100 mg). Previously, no pure compound isolated from *P. rimosus* had been reported. As a result, *P. rimosus* ethanol extract was chosen for further research. The ethanol extract of *P. rimosus* was separated with various solvents, isolation and indentification. In the anti-inflammatory study, the ethanol extracts of *P. rimosus* and *P. linteus* mushroom exhibited the best efficacy. The ethanol extracts of *P. rimosus* and *P. linteus* at the concentration of 200 µg/mL indicated percentage inhibition of nitric oxide production on LPS-activated macrophages RAW 264.7 cell at 97.95 ± 2.29 and 98.83 ± 1.59 %, respectively. The pure compounds isolated from *P. rimosus* showed active against anti-inflammatory at IC_{50} values ranging from 8.69 ± 0.08 - 10.55 ± 1.09 µg/mL (HPR1 IC_{50} = 9.87 ± 0.24 µg/mL, HPR2 IC_{50} = 10.55 ± 1.09 µg/mL and DPR IC_{50} = 8.69 ± 0.08 µg/mL). The cytotoxicity of sample was evaluated by using MTT assay. The crude ethanol extracts of *P. rimosus* showed no cytotoxicity at IC_{50} values of 411.25 ± 1.52 µg/mL, whereas the pure compound showed IC_{50} values of less than 25 µg/mL, according to the findings of this investigation.

The fractions separated from *P. rimosus* including hexane, dichloromethane, ethyl acetate and butanol extracts were active against CCA both KKU-100 & KKUM-213A. The dichloromethane fraction was the most effective against KKU-100, with IC_{50} values of 35.28 ± 080 µg/mL, and the butanol fraction was the most sensitive against KKUM-213A, with IC_{50} values of 40.97 ± 1.15 µg/mL. In addition, the ethanol extract revealed IC_{50} values of 123.95 ± 3.27 µg/mL for KKU-100 and112.11 ± 3.52 µg/mL for KKUM-213A, respectively.

The isolation and structural identification of the hexane soluble fraction from ethanol extracts of *P. rimosus* gave 3-dimethyl-2-hydroxy-2-en-propanoic acid and 4,5-dimethoxy-2,3,5-trihydroxy-2,4-di-en-pentanoic acid, while 6-deoxydestigloyswietenine acetate was isolated from dichloromethane fraction. The isolated compounds were tested for anti-inflammatory activity using Nitric oxide assay. Their IC_{50} values of 6-deoxydestigloyswietenine acetate shown inhibit nitric oxide production on LPS-activated macrophages RAW 264.7 cell with IC_{50} values of 8.69 ± 0.08 µg/mL.

The current study reported the efficacy of *P. igniarius*, *P. linteus*, *P. nigrican* and *P. rimosus* extracts on anticancer activity against the CCA cell line for the first time. In addition, the pure compounds isolated from *P. rimosus* showing high efficacy in anti-inflammatory and anticancer assays and was reported for the first time in the current study.

2. Recommendation

Due to the Covid-19 pandemic, A study could not be conducted during the Covid-19 pandemic, which had an impact on the findings of this study. The result of study has limitations in terms of anticancer activity against CCA cells. As previously stated, future research should focus on the anticancer properties of pure substances and molecular studies against CCA cells. The isolated compounds should evaluate apoptosis against CCA cells and determination anti-inflammatory activity (The evaluate COX-2 and IL-1β protein expression in Raw 264.7 cells). This study's findings could be utilized to support the rationale for using this mushroom species in traditional medicine. Furthermore, we should advocate for improving local economics in the cultivation of high-potential *Phellinus* mushrooms, particularly in *P. rimosus*, *P. linteus* and *P. igniarius.*

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin*. 2018;68(1):7-30.
2. Virani S, Bilheem S, Chansaard W, Chitapanarux I, Daoprasert K, Khuanchana S, et al. National and subnational population-based incidence of cancer in Thailand: Assessing cancers with the highest burdens. *Cancers*. 2017;9(8):1-27.
3. World Health Organization - Cancer country profiles "Keya " Cancer mortality profile". *Cancer country profile*. (2019-2020):181-183.
4. Freddie B, Jacques F, Isabelle S, Rebecca L, Siegel L, Torre A, Ahmedin J. Global cancer statistics 2018: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(1):394-422.
5. Bragazzi MC, Ridola L, Safarikia S, Matteo SD, Costantini D, Nevi L, et al. New insights into cholangiocarcinoma: multiple stems and related cell lineages of origin. *Ann Gastroenterol.* 2018;31(1):42-55.
6. Macias RIR. Cholangiocarcinoma: Biology, clinical management, and pharmacological perspectives. *ISRN hepatology.* 2014;16(2):1-13.
7. Kamsa S, Kamsa S, Luvira V, Suwanrungruang K, Vatanasapt P, Wiangnon S. Risk factors for cholangiocarcinoma in Thailand: A Systematic review and meta-analysis. *APJCP*. 2018;19(3):605-614.
8. AbouAlfa GK, Andersen JB, Chapman W, Choti M, Forbes SJ, Gores GJ, et al. Advances in cholangiocarcinoma research: report from the third cholangiocarcinoma foundation annual conference. *J Gastrointest Oncol*. 2016;7(6):819-827.
9. Sripa B, Pairojkul C. Cholangiocarcinoma: lessons from Thailand. *Curr opin Gastroenterol.* 2008;24(3):349-356.
10. Lim MK, Ju YH, Franceschi S, Oh JK, Kong HJ, Hwang SS, et al. *Clonorchis sinensis* infection and increasing risk of cholangiocarcinoma in the Republic of Korea. *Am J Trop Med Hyg*. 2006;75(1):93-96.
11. Khan SA, Davidson BR, Goldin RD, Heaton N, Karani J, Pereira SP, et al. Guidelines for the diagnosis and treatment of cholangiocarcinoma: an update. *Gut*. 2012;61(12):1657-1669.
12. Amuamuta A, Plengsuriyakarn T, NaBangchang K. Anticholangiocarcinoma activity and toxicity of the *Kaempferia galanga* Linn. Rhizome ethanolic extract. *BMC Compl Alternative Med.* 2017;17(1):1-11.
13. Yan JK, Pei JJ, Ma HL, Wang ZB, Liu YS. Advances in antitumor polysaccharides from *Phellinus sensu* lato: Production, isolation, structure, antitumor activity, and mechanisms. *Crit Rev Food Sci Nutr.* 2017;57(6):1256-1269.

14. Plengsuriyakarn T, Viyanant V, Eursitthichai V, Picha P, Kupradinun P, Itharat A, et al. Anticancer activities against cholangiocarcinoma, toxicity and pharmacological activities of Thai medicinal plants in animal models. *BMC Compl Alternative Med*. 2012;12(3):1-7.
15. Lee MA, Woo IS, Kang JH, Hong YS, Lee KS. Epirubicin, cisplatin, and protracted infusion of 5-FU (ECF) in advanced intrahepatic cholangiocarcinoma. *J. Cancer Res Clin Oncol.* 2004;130(6):346-350.
16. Adeson DC, Prinson WC, Berlin J, Chari R. Diagnosis and Treatment of Cholangiocarcinoma. *Oncologist.* 2004;9(5):43-57.
17. Patel S, Goyal A. Recent developments in mushrooms as anti-cancer therapeutics: a review. *Biotech.* 2012;2(1):1-15.
18. Vapiwala N, Mick R, Hampshire MK, Metz JM, DeNittis AS. Patient initiation of complementary and alternative medical therapies (CAM) following cancer diagnosis. *Cancer* 2006;12(6):467-474.
19. Braca A, Sortino C, Politi M, Morelli I, Mendez J. Antioxidant activity of flavonoids from Licania licaniaeflora. *J. Ethnopharmacol.* 2002;79(3):379-381.
20. Azab A, Nassar A, Azab AN. Anti-Inflammatory activity of natural products. *Molecules*. 2016;21(10):1-19.
21. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol.* 2011;31(5):986-1000.
22. Sharma JN, Al-Omran A, Parvathy SS. Role of nitric oxide in inflammatory diseases. *Inflammopharmacology*. 2007;15(6):252-259.
23. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radic Biol Med.* 2010;49(11):1603-1616.
24. Krause J, Tobi G. Discovery, Development, and Regulation of Natural Products. Kulka M, editor. Department of Biomedical Sciences, University of Prince Edward Island, Charlottetown, PE, Canada; (2013).
25. Hadi W. Anticancer and Antimicrobial Potential of Plant-Derived Natural Products. Rasooli I, editor. Department of Clinical Pharmacy and Therapeutics, University of Applied Science, Jordan: Publisher InTech; (2011).
26. Yuan H, Ma Q, Ye L, Piao G. The Traditional medicine and modern medicine from natural products. *Molecules.* 2016;21(5):1-18.
27. Dias DA, Urban S, Roessner U. A historical overview of natural products in drug discovery. *Metabolites*. 2012;2(2):303-336.
28. Rahi DK, Malik D. Diversity of Mushrooms and Their Metabolites of Nutraceutical and Therapeutic Significance. *J. Mycol.* 2016; Article ID 7654123.
29. Lindequist U, Niedermeyer THJ, Julich W. The pharmacological potential of mushrooms. *J Evid Based Complementary Altern Med.* 2005;2(3):285-299.

30. Hollenberg D, Zakus D, Cook T, Wei Xu X. Re-positioning the role of traditional, complementary and alternative medicine as essential health knowledge in global health: Do they still have a role to play. *World health* Popul. 2009;10(4):62-75.
31. Xue CC. Traditional, complementary and alternative medicine: policy and public health perspectives. *Bull. World Health Organ.* 2008;86(1):77-78.
32. กระทรวงสาธารณสุข. เห็ดเพื่อสุขภาพสำหรับอหารเป็นยาและเพื่อเศรษฐกิจตามภูมิปัญญาของหมอพื้นบ้าน. กรุงเทพมหานคร:ชุมชนสหกรณ์การเกษตรแห่งประเทศไทย; (2556).
33. Ranadive K, Jagtap, N, Vaidya, J. Host diversity of *Phellinus* from world. *Elixir Appl Botany* 2012;52(11):11402-8.
34. Chen H, Tian T, Miao H, Zhao YY. Traditional uses, fermentation, phytochemistry and pharmacology of *Phellinus linteus*: A review. *Fitoterapia* (2016);113(9):6-26.
35. Minh TN, Khang do T, Tuyen PT, Minh LT, Anh LH, Quan NV, et al. Phenolic compounds and antioxidant activity of *Phalaenopsis Orchid* Hybrids. *Antioxidants (Basel)*. 2016;5(3):1-12.
36. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact.* 2006;160(1):1-40.
37. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev.* 2010;4(8):118-126.
38. Hajhashemi V, Vaseghi G, Pourfarzam M, Abdollahi A. Are antioxidants helpful for disease prevention. *Res Pharm Sci*. 2010;5(1):1-8.
39. Gao W, Wang W, Sun W, Wang M, Zhang N, Yu S. Antitumor and immunomodulating activities of six *Phellinus igniarius* polysaccharides of different origins. *Exp Ther Med.* 2017;14(5):4627-4632.
40. Saiki P, Nishimura K, Toida T, Van Griensven L. Structural characterization and immunomodulatory effects of polysaccharides from *Phellinus linteus* and *Phellinus igniarius* on the IL-6/IL-10 cytokine balance of the mouse macrophage cell line RAW 264.7. *J Funct Foods.* 2015;6(8):2834-2844.
41. Kozarski M, Klaus A, Niksic M, Jakovljevic D, Helsper J, Van Griensven L. Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*. *Food Chem.* 2011;129(4):1667-1675.
42. Liu M, Zeng P, Li X, Shi L. Antitumor and immunomodulation activities of polysaccharide from *Phellinus baumii*. *Int. J Biol Macromol.* 2016;91(10):1199-1205.

43. Jiang P, Yuan L, Cai D, Jiao L, Zhang L. Characterization and antioxidant activities of the polysaccharides from mycelium of *Phellinus pini* and culture medium. *Carbohydr Polym.* 2015;117(3):600-604.
44. Hsieh P, Wu J, Wu Y. Chemistry and biology of *Phellinus linteus*. *Bio Medicine*. 2013;3(3):106-113.
45. Liu AJ, Sun HC, Chen Y, Wang WH, Liu CH, Wang YM. Preparation and Anti-tumor activity of polysaccharides from *Phellinus pini* mycelia. *Adv Mat Res*. 2014;936(6):728-733.
46. Konno S, Chu K, Feuer N, Phillips J, Choudhury M. Potent anticancer effects of bioactive mushroom extracts (*Phellinus linteus*) on a variety of human cancer cells. *J Clin Med Res*. 2015;7(2):76-82.
47. Wu Q, Kang Y, Zhang H, Wang H, Liu Y, Wang J. The anticancer effects of hispolon on lung cancer cells. *Biochem Biophys Res Commun.* 2016;453(3):385-391.
48. Chang M K, Dai HH, Ho K H, Choi SH, Lee WJ. Anticancer effect of *Phellinus linteus;* potential clinical application in treating pancreatic ductal adenocarcinoma. *J Carcinog Mutagen.* 2013;1(9):1-8.
49. Park HJ. Anti-allergic and anti-inflammatory activity of *Phellinus linteus* grown on *Panax ginseng*. *Food Sci Biotechnol.* 2017;26(2):467-472.
50. Lee YS, Kim YH, Shin EK, Kim DH, Lim SS, Lee JY, et al. Anti-angiogenic activity of methanol extract of *Phellinus linteus* and its fractions. *J. Ethnopharmacol.* 2010;131(1):56-62.
51. Song YS, Kim S, Sa H, Jin C, Lim C, Park E. Anti-angiogenic, antioxidant and xanthine oxidase inhibition activities of the mushroom *Phellinus linteus*. *J Ethnopharmacol*. 2003;88(1):113-116.
52. Torkelson CJ, Sweet E, Martzen MR, Sasagawa M, Wenner CA, Gay J, et al. Phase 1 Clinical trial of trametes versicolor in women with breast cancer. *ISRN oncology.* 2012; Article ID 251632.
53. Ferreira IC, Vaz JA, Vasconcelos MH, Martins A. Compounds from wild mushrooms with antitumor potential. *Anticancer Agents Med Chem.* 2010;10(5):424-436.
54. Sullivan R, Smith JE, Rowan NJ. Medicinal mushrooms and cancer therapy: translating a traditional practice into Western medicine. *Perspect Biol Med* 2006;49(2):159-170.
55. Yin R, Zhao Z, Ji X, Dong Z, Li Z, Feng T, et al. Steroids and sesquiterpenes from cultures of the fungus *Phellinus igniarius*. *Nat Prod Bio Prospect.* 2015;5(1):17-22.

56. Zapora E, Wołkowycki M, Bakier S, Zjawiony J. *Phellinus igniarius*: A Pharmacologically active polypore mushroom. *Nat Prod Commun.* 2016;11(7):1043-1046.

57. Tian X, Yu H, Zhou L, Decock C, Vlasak J, Dai Y. Phylogeny and taxonomy of the *Inonotus linteus* complex. *Fungal Diver.* 2013;58(1):159-169.

58. Chong J, Poutaraud A, Hugueney P. Metabolism and roles of stilbenes in plants. *Plant Sci*. 2009;177(3):143-155.

59. Rzepecka SA, Stojko J, Kurek GA, Gorecki M, Kabała DA, Kubina R, et al. Polyphenols from *Bee Pollen*: Structure, absorption, metabolism and biological activity. *Molecules.* 2015;20(12):21732-21749.

60. *Phellinus nigricans* (Fr.) P. Karst., *Polyporus nigricans* Fr., 1821 Syst. Mycol. 1:375,page 134: http://www.mycobank.org/BioloMICS.aspx?TableKey=14682616000000063&Rec=44542&Fields=All; 1989 [Finl. Basidsv].

61. Wang Z, Wang C, Quan Y. Extraction of polysaccharides from *Phellinus nigricans* mycelia and their antioxidant activities in vitro. *Carbohydr Polym.* 2014;99(1):110-115.

62. Larsen MJ. Re-examination of the nomenclatural types of *Polyporus rimosus* berk. and *P. Badius* berk. *Mycotexon.* 1990;37(4):353-361.

63. Ajith AT, Janardhanan K. Indian medicinal mushrooms as a source of antioxidant and antitumor agents. *J Clin Biochem Nutr.* 2007;40:157-162.

64. *Phellinus igniarius* https://www.google.com/2018

65. Lee SW, Song JG, Hwang BS, Kim DW, Lee YJ, Woo EE, et al. Lipoxygenase inhibitory activity of Korean indigenous mushroom extracts and isolation of an active compound from *Phellinus baumi*. *Mycobiology*. 2014;42(2):185-188.

66. Suabjakyong P, Nishimura K, Toida T, Van Griensven LJ. Structural characterization and immunomodulatory effects of polysaccharides from *Phellinus linteus* and *Phellinus igniarius* on the IL-6/IL-10 cytokine balance of the mouse macrophage cell lines (RAW 264.7). *Food Funct*. 2015;6(8):2834-2844.

67. Shirahata T, Ino C, Mizuno F, Asada Y, Hirotani M, Petersson GA, et al. Corrigendum: gamma-Ionylidene-type sesquiterpenoids possessing antimicrobial activity against Porphyromonas gingivalis from *Phellinus linteus* and their absolute structure determination. *J Antibiot*. 2017;70(9):695-698.

68. Lee S, Lee D, Jang TS, Kang KS, Nam JW, Lee HJ, et al. Anti-Inflammatory Phenolic Metabolites from the Edible Fungus *Phellinus baumi* in LPS-Stimulated RAW264.7 Cells. *Molecules*. 2017;22(9):1-10.

69. Liu H, Tsai T, Chang T, Chou C, Lin L. Lanostane-triterpenoids from the fungus *Phellinus gilvus*. *Phytochemistry*. 2009;70(4):558-563.

70. Liu MM, Zeng P, Li XT, Shi LG. Antitumor and immunomodulation activities of polysaccharide from *Phellinus baumi*. *Int J Biol Macromol.* 2016;91:1199-1205. 71. Gao W, Wang W, Sun W, Wang M, Zhang N, Yu S. Antitumor and immunomodulating activities of six *Phellinus igniarius* polysaccharides of different origins. *Exp Ther Med.* 2017;14(5):4627-4632.

72. Yan JK, Wang YY, Wang ZB, Ma HL, Pei JJ, Wu JY. Structure and antioxidative property of a polysaccharide from an ammonium oxalate extract of *Phellinus linteus*. *Int J Biol Macromol*. 2016;91(9):92-99.

73. Sasaki T, Arai Y, Ikekawa T, Chihara G, Fukuoka F. Antitumor Polysaccharides from Some Polyporaceae, Ganoderma applanatum (PERS.) PAT and *Phellinus linteus* (BERK. et CURT) Aoshima. *Chem Pharm Bull.* 1971;19(4):821-826.

74. Wang G, Dong L, Zhang Y, Ji Y, Xiang W, Zhao M. Polysaccharides from *Phellinus linteus* inhibit cell growth and invasion and induce apoptosis in HepG2 human hepatocellular carcinoma cells. *Biologia.* 2012;67(1):247-254.

75. Zaidman BZ, Yassin M, Mahajna J, Wasser SP. Medicinal mushroom modulators of molecular targets as cancer therapeutics. *Appl Microbiol Biotechnol*. 2005;67(4):453-468.

76. Chen YC, Chang HY, Deng JS, Chen JJ, Huang SS, Lin IH, et al. Hispolon from *Phellinus linteus* induces G0/G1 cell cycle arrest and apoptosis in NB4 human leukaemia cells. *Am J Chin Med.* 2013;41(6):1439-1457.

77. Kim GY, Oh WK, Shin BC, Shin YI, Park YC, Ahn SC, et al. Proteoglycan isolated from *Phellinus linteus* inhibits tumor growth through mechanisms leading to an activation of CD11c+CD8+ DC and type I helper T cell-dominant immune state. *FEBS letters*. 2004;576(3):391-400.

78. Wang Y, Wang SJ, Mo SY, Li S, Yang YC, Shi JG. Phelligridimer A, a Highly oxygenated and unsaturated 26-Membered macrocyclic metabolite with antioxidant activity from the fungus *Phellinus igniarius*. *Org Lett*. 2005;7(21):4733-6.

79. Elsayed EA, El Enshasy H, Wadaan MA, Aziz R. Mushrooms: a potential natural source of anti-inflammatory compounds for medical applications. *Mediators Inflamm*. 2014; Article ID 805841.

80. Park HJ. Anti-allergic and anti-inflammatory activity of *Phellinus linteus* grown on *Panax ginseng. Food Sci Biotechnol.* 2017;26(2):467-472.

81. Kim SH, Song YS, Kim SK, Kim BC, Lim CJ, Park EH. Anti-inflammatory and related pharmacological activities of the n-BuOH subfraction of mushroom *Phellinus linteus*. *J Ethnopharmacol.* 2004;93(1):141-146.

82. Huang GJ, Huang SS, Deng JS. Anti-Inflammatory Activities of Inotilone from *Phellinus linteus* through the Inhibition of MMP-9, NF-κB, and MAPK Activation In Vitro and In Vivo. *Plos One.* 2012;7(5):1-12.

83. Chang HY, Peng WH, Sheu MJ, Huang GJ, Leu ZC, Tseng MC, et al. Analgesic and Anti-inflammatory Activities of *Phellinus merrillii*. *Mid Taiwan J Med.* 2007;12(2):76-82.

84. Ajith TA, Janardhanan KK. Antioxidant and anti-inflammatory activities of methanol extract of *Phellinus rimosus* (Berk) Pilat. *Indian J Exp Biol*. 2001;39(11):1166-1169.

85. Seephonkai P, Samchai S, Thongsom A, Sunaart S, Kiemsanmuang B, Chakuton K. DPPH Radical scavenging activity and total phenolics of *Phellinus* mushroom extracts collected from Northeast of Thailand. *Chin J Nat Med* 2011;9(6):441-445.

86. Yang Y, Wang Q, Liu Y, Wang W, Feng N, Wu D. Antioxidant activities of extracts from the genus *Phellinus* species. Proceedings of the 7th International conference onmushroom biology and mushroom products (ICMBMP7) (2011):232-241.

87. Ajith TA, Janardhanan KK. Antioxidant and antihepatotoxic activities of *Phellinus rimosus* (Berk) Pilat. *J Ethnopharmacol.* 2002;81(3):387-391.

88. Jeon YE, Lee YS, Lim SS, Kim SJ, Jung SH, Bae YS, et al. Evaluation of the antioxidant activity of the fruiting body of *Phellinus linteus* using the on-line HPLC-DPPH method. *J Korean Soc Appl Bi.* 2009;52(5):472-479.

89. Lakshmi B, Tilak JC, Adhikari S, Devasagayam TPA, Janardhanan KK. Evaluation of antioxidant activity of selected Indian mushrooms. *Pharm Biol.* 2004;42(3):179-185.

90. Judprakob C, Laovachirasuwan,P, Phadugkit, M. Antioxidant and Antimutagenic activities of *Phellinus rimosus*. The 5th annual Northeast Pharmacy Research Conference of 2013 " Pharmacy Profession Moving Forward to ASEAN Hamonization" *Faculty of Pharmaceutical Science, KKU, Thailand*. 2013;2(16):175-179.

91. McGlynn KA, London WT. The global epidemiology of hepatocellular carcinoma: present and future. *Clin Liver Dis.* 2011;15(2):223-243.

92. Luo Y. A New Clinical Classification of Hilar Cholangiocarcinoma (Klatskin Tumor). *Int J Surg.* 2017;2(4):1-5.

93. Nakanuma Y, Sato Y, Harada K, Sasaki M, Xu J, Ikeda H. Pathological classification of intrahepatic cholangiocarcinoma based on a new concept. *World J Gastroenterol Hepatol Endosc.* 2010;2(12):419-427.

94. Kurathong S, Lerdverasirikul P, Wongpaitoon V, Pramoolsinsap C, Kanjanapitak A, Varavithya W, et al. Opisthorchis viverrini infection and cholangiocarcinoma. A prospective, case-controlled study;. *Gastroenterology*. 1985;89(1):151-156.

95. Watanapa P, Watanapa WB. Liver fluke-associated cholangiocarcinoma. *Br J Surg* . 2002;89(8):962-970.
96. Suabjakyong P, Saiki R, Van Griensven LJ, Higashi K, Nishimura K, Igarashi K, et al. Polyphenol extract from *Phellinus igniarius* protects against acrolein toxicity in vitro and provides protection in a mouse stroke model. *Plos One*. 2015;10(3):1-14.
97. Sripa B, Kaewkes S, Sithithaworn P, Mairiang E, Laha T, Smout M, et al. Liver fluke induces cholangiocarcinoma. *Plos Med.* 2007;4(7):1148-1155.
98. Patel T. Cholangiocarcinoma-controversies and challenges. *Nat Rev Gastroenterol Hepatol*. 2011;8(4):189-200.
99. Chen J, Chen L, Yu J, Xu Y, Wang X, Zeng Z, et al. Metaanalysis of current chemotherapy regimens in advanced pancreatic cancer to prolong survival and reduce treatmentassociated toxicities. *Mol Med Rep*. 2019;19(1):477-489.
100. Valle JW, Wasan HS, Palmer DD, Cunningham D, Anthoney DA, Maraveyas A, et al. Gemcitabine with or without cisplatin in patients (pts) with advanced or metastatic biliary tract cancer (ABC): Results of a multicenter, randomized phase III trial (the UK ABC-02 trial). *J Clin Oncol.* 2009;27(15):4503-4514.
101. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med*. 2010;362(14):1273-8121.
102. Shinohara ET, Mitra N, Guo M, Metz JM. Radiation therapy is associated with improved survival in the adjuvant and definitive treatment of intrahepatic cholangiocarcinoma. *Int. J Radiat Oncol Biol Phys.* 2008;72(5):1495-1501.
103. Figueiredo L, Regis WCB. Medicinal mushrooms in adjuvant cancer therapies: an approach to anticancer effects and presumed mechanisms of action. *Nutrire*. 2017;42(1):1-10.
104. Wutka A, Palagani V, Barat S, Chen X, El Khatib M, Gotze J, et al. Capsaicin treatment attenuates cholangiocarcinoma carcinogenesis. *Plos One*. 2014;9(4):1-10.
105. Qin Y, Cui W, Yang X, Tong B. Kaempferol inhibits the growth and metastasis of cholangiocarcinoma in vitro and in vivo. *Acta Biochim Biophys Sin*. 2016;48(3):238-245.
106. Sombatsri A, Thummanant Y, Sribuhom T, Boonmak J, Youngme S, Phusrisom S, et al. New limonophyllines A-C from the stem of *Atalantia monophylla* and cytotoxicity against cholangiocarcinoma and HepG2 cell lines. *Arch Pharm Res*. 2018;41(4):431-437.
107. Sun Y, Sun T, Wang F, Zhang J, Li C, Chen X, et al. A polysaccharide from the fungi of Huaier exhibits anti-tumor potential and immunomodulatory effects. *Carbohydr Polym.* 2013;92(1):577-582.

108. Tuponchai P, Kukongviriyapan V, Prawan A, Kongpetch S, Senggunprai L. Myricetin ameliorates cytokine-induced migration and invasion of cholangiocarcinoma cells via suppression of STAT3 pathway. *J Cancer Res Ther*. 2018;15(1):157-163.

109. Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, NaBangchang K. Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro. *BMC Complement Altern Med.* 2010;10(55):1-8.

110. CarbonellCapella JM, Buniowska M, Barba FJ, Esteve MJ, Frigola A. Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: A Review. *Compr Rev Food Sci Food Saf.* 2014;13(2):155-171.

111. Center for Cancer Research National Cancer Institute Besthesda MD and Wasington University school of Medicine S, Louis MO. Handbook of Anticancer Pharmacokinetics and Pharmacodynamics. Rudek MA, Chau, C.H., Figg, W., McLeod, H.L., editor. Spinger science media new york: Humana Press Inc; (2014).

112. Kozarski M, Klaus A, Jakovljevic D, Todorovic N, Vunduk J, Petrovic P, et al. Antioxidants of edible mushrooms. *Molecules*. 2015;20(10):19489-19525.

113. Pereira D, Valentao P, Pereira J, Andrade P. Phenolics: fromc chemistry to biology. *Molecules*. 2009;14(6):2202-11.

114. Ranadive DK, Tarini J, Harshavardhan K, Neeta J, Paramjit J, Vijay R, et al. Host distribution of *Phellinu*s from India. *Indian J For*. 2012;35(1):67-72.

115. Van TA, Joubert AM, Cromarty AD. Limitations of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay when compared to three commonly used cell enumeration assays. *BMC Res Notes*. 2015;8(2):1-10.

116. Kuete V, Karaosmanoglu O, Sivas H. Chapter 10 - Anticancer activities of African medicinal spices and vegetables. In: Kuete V, editor. Medicinal Spices and Vegetables from Africa: Academic Press; (2017).

117. Blanco A, Blanco G. Chapter 32 - Apoptosis. In: Blanco A, Blanco G, editors. Medical Biochemistry: Academic Press; (2017).

118. Martinez A, Reina M. Copper or free radical scavenger. *Comput Theor Chem* 2017;1104(3):1-11.

119. Burini RC, Moreto F, Yu Y. Chapter 15 - HIV-Positive Patients Respond to Dietary Supplementation with Cysteine or Glutamine. In: Watson RR, editor. Health of HIV Infected People. Boston: Academic Press; (2015).

120. Phaniendra A, Jestadi DB, Periyasamy L. Free radicals: properties, sources, targets, and their implication in various diseases. *Indian J Clin Biochem.* 2015;30(1):11-26.

121. Stanojevic L, Stankovic M, Nikolic V, Nikolic L, Ristic D, Canadanovic-Brunet J, et al. Antioxidant activity and total phenolic and flavonoid contents of *Hieracium pilosella* L. extracts. *Sensors.* 2009;9(7):5702-5714.

122. Nithiyanantham S, Selvakumar S, Siddhuraju P. Total phenolic content and antioxidant activity of two different solvent extracts from raw and processed legumes, *Cicer arietinum* L. and *Pisum sativum* L. *J Food Compos Anal.* 2012;27(1):52-60.

123. Betteridge DJ. What is oxidative stress? *Metabolism.* 2000;49(2):3-8.

124. Flora G, Mittal M, Flora SJS. 26-Medical Countermeasures-Chelation Therapy. Flora SJS, editor. Oxford: Academic Press; (2015).

125. Rasool N, Rizwan K, Zubair M, Naveed K, Imran I, Ahmed V. Antioxidant potential of different extracts and fractions of Catharanthus roseus shoots. *Int J Phytomedicine.* 2011;3(1):108-114.

126. Chun S, Vattem DA, Lin Y, Shetty K. Phenolic antioxidants from clonal oregano (Origanum vulgare) with antimicrobial activity against *Helicobacter pylori*. *Process Biochem.* 2005;40(2):809-816.

127. Augustyniak A, Bartosz G, Cipak A, Duburs G, Horakova L, Łuczaj W, et al. Natural and synthetic antioxidants: An updated overview. *Free Radic Res*. 2010;44(10):1216-1262.

128. Kahl R. Synthetic antioxidants: Biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology*. 1984;33(3):185-228.

129. Razali N, MatJunit S, AbdulMuthalib AF, Subramaniam S, AbdulAziz A. Effects of various solvents on the extraction of antioxidant phenolics from the leaves, seeds, veins and skins of *Tamarindus indica* L. *Food Chem*. 2012;131(2):441-448.

130. Boonsong S, Klaypradit W, Wilaipun P. Antioxidant activities of extracts from five edible mushrooms using different extractants. *Agric Nat Resour*. 2016;50(2):89-97.

131. Mujic I, Zekovic Z, Lepojevic z, Vidovic S, Zivkovic J. Antioxidant properties of selected edible mushroom species an antioxidative osobin odabranih jestivih gljva. *J Cent Eur Agric.* 2010;11(4):387-392.

132. Ferreira I, Barros L, Abreu R. Antioxidants in wild mushrooms. *Med Chem*. 2009;16(12):1543-1560.

133. Palacios I, Lozano M, Moro C, D'Arrigo M, Rostagno M, Martínez JA, et al. Antioxidant properties of phenolic compounds occurring in edible mushroom. *Food Chem*. 2011;128(3):674-678.

134. Phenolic Compounds: Introduction. Lattanzio V, editor. Department of Sciences of Agriculture, Food and EnvironmentUniversity of FoggiaFoggiaItaly:

Springer, Berlin, Heidelberg. https://doi.org/10.1007/ 978-3-642-22144-6_57 (2013).

135. Cheynier V. Phenolic compounds: from plants to foods. *Phytochem Rev*. 2012;11(2):153-177.

136. Yao LH, Jiang YM, Shi J, Tomas-Barberan FA, Datta N, Singanusong R, et al. Flavonoids in food and their health benefits. *Plant Foods Hum Nute.* 2004;59(3):113-122.

137. Harnly JM, Bhagwat S, Lin LZ. Profiling methods for the determination of phenolic compounds in foods and dietary supplements. *Anal Bioanal Chem*. 2007;389(1):47-61.

138. Shang YJ, Qian YP, Liu XD, Dai F, Shang XL, Jia WQ, et al. Radical-scavenging activity and mechanism of resveratrol-oriented analogues: influence of the solvent, radical, and substitution. *J Org Chem.* 2009;74(14):5025-5031.

139. Tsao R. Chemistry and biochemistry of dietary polyphenols. *Nutrhu* 2010;2(12):1231-1246.

140. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J Nutr Biochem.* 2002;13(10):572-584.

141. Valentao P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Hydroxyl radical and hypochlorous acid scavenging activity of small centaury (Centaurium erythraea) infusion. A comparative study with green tea (Camellia sinensis). *Phytomedicine.* 2003;10(6-7):517-522.

142. Yang CS, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr.* 2001;21(7):381-406.

143. Huyut Z, Beydemir S, Gulcin I. Antioxidant and Antiradical Properties of Selected Flavonoids and Phenolic Compounds. *Biochem Res Int.* 2017:Article ID 7616791.

144. Dreher D, Junod AF. Role of oxygen free radicals in cancer development. *Eur J Cancer*. 1996;32(1):30-38.

145. Acuna UM, Wittwer J, Ayers S, Pearce CJ, Oberlies NH, EJ DEB. Effects of (5Z)-7-oxozeaenol on the oxidative pathway of cancer cells. Anticancer Res. 2012;32(7):2665-2671.

146. Cairns RA, Harris I, McCracken S, Mak TW. Cancer cell metabolism. *Cold Spring Harb Symp Quant Biol.* 2011;76(12):299-311.

147. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol.* 2007;39(1):44-84.

148. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem.* 2004;266(1-2):37-56.

149. Grigalius I, Petrikaite V. Relationship between Antioxidant and Anticancer Activity of Trihydroxyflavones. *Molecules*. 2017;22(12):1-12.
150. Chang ST, Wu JH, Wang SY, Kang PL, Yang NS, Shyur LF. Antioxidant activity of extracts from Acacia confusa bark and heartwood. *J Agric Food Chem*. 2001;49(7):3420-3424.
151. Antolovich M, Prenzler PD, Patsalides E, McDonald S, Robards K. Methods for testing antioxidant activity. *Analyst.* 2002;127(1):183-198.
152. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem*. 2005;53(10):4290-4302.
153. Pisoschi AM, Negulescu GP. Methods for Total Antioxidant Activity Determination: A Review. Biochemistry & Analytical Biochemistry. 2012;1(1):1-10.
154. Amorati R, Valgimigli L. Advantages and limitations of common testing methods for antioxidants. *Free Radic Res.* 2015;49(5):633-649.
155. Marc F, Davin A, Deglene-Benbrahim L, Ferrand C, Baccaunaud M, Fritsch P. Studies of several analytical methods for antioxidant potential evaluation in food. *J Res Med Sci.* 2004;20(4):458-463.
156. Boligon AA. Technical evaluation of antioxidant activity. *Med Chem*. 2014;4(7):517-522.
157. Benzie IFF, Strain JJ. The Ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. *Anal. Biochem*. 1996;239(1):70-76.
158. Shanab EA, Sa SM. Antioxidant compounds, assays of determination and mode of action. *Afr J Pharm Pharmacol.* 2013;7(10):528-539.
159. Bellik Y, Boukraa L, Alzahrani HA, Bakhotmah BA, Abdellah F, Hammoudi SM, et al. Molecular mechanism underlying anti-inflammatory and anti-allergic activities of phytochemicals: an update. *Molecules*. 2012;18(1):322-353.
160. Kanwar JR, Kanwar RK, Burrow H, Baratchi S. Recent advances on the roles of NO in cancer and chronic inflammatory disorders. C*urr Medicinal Chem*. 2009;16(19):2373-2394.
161. Abdulkhaleq LA, Assi MA, Abdullah R, Zamri-Saad M, Taufiq-Yap YH, Hezmee MNM. The crucial roles of inflammatory mediators in inflammation: A review. *Vet World.* 2018;11(5):627-635.
162. Newton K, Dixit VM. Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol*. 2012;4(3):1-20.
163. Taofiq O, Calhelha RC, Heleno S, Barros L, Martins A, Santos-Buelga C, et al. The contribution of phenolic acids to the anti-inflammatory activity of mushrooms: Screening in phenolic extracts, individual parent molecules and

synthesized glucuronated and methylated derivatives. *Int. Food Res. J.* 2015;76(3):821-827.

164. Gunawardena D, Bennett L, Shanmugam K, King K, Williams R, Zabaras D, et al. Anti-inflammatory effects of five commercially available mushroom species determined in lipopolysaccharide and interferon-gamma activated murine macrophages. Food Chem. 2014;148(4):92-96.

165. Yoshikawa K, Inoue M, Matsumoto Y, Sakakibara C, Miyataka H, Matsumoto H, et al. Lanostane triterpenoids and triterpene glycosides from the fruit body of *Fomitopsis pinicola* and their inhibitory activity against COX-1 and COX-2. *J Nat Prod.* 2005;68(1):69-73.

166. Thouri A, Chahdoura H, El Arem A, Omri Hichri A, Ben Hassin R, Achour L. Effect of solvents extraction on phytochemical components and biological activities of Tunisian date seeds (var. Korkobbi and Arechti). *BMC Complement Altern Med.* 2017;17(1):1-10

167. Cos P, Vlietinck AJ, Berghe DV, Maes L. Anti-infective potential of natural products: How to develop a stronger in vitro 'proof-of-concept'. *J Ethnopharmacol*. 2006;106(3):290-302.

168. Sasidharan YC, D. Saravanan, K.M. Sundram, L. Yoga Latha. Eaxtraction, isolation and characterization of bioactive compounds from plants eaxtracts. *Afr J Tradit Complement AlternMed.* 2011;8(1):1-10.

169. Majekodunmi OS. Review of extraction of medicinal plants for pharmaceutical research. *J Med Med* Sci. 2015;3(11): 521-527.

170. Moldoveanu S, David V. Chapter 6. Solvent eExtraction. Modern Sample Preparation for Chromatography. DOI: 10.1016/B978-0-444-54319-6.00006-2. Elsevier B.V. (2015).

171. Zhang QW, Lin LG, Ye WC. Techniques for extraction and isolation of natural products: a comprehensive review. *Chin Med.* 2018;13(4):1-26.

172. Santos ilva LS, Brumano L, Stringheta P, Aparecida O M, DLO. M, et al. Preparation of dry extract of Mikania glomerata Sprengel (Guaco) and determination of its coumarin levels by spectrophotometry and HPLC-UV. *Molecules*. 2012;17(9):10344-10354.

173. Spray Drying: An Overview. Daniel Santos, Ana Colette Maurício, Vitor Sencadas, José Domingos Santos, Fernandes MH, Gomes PS, editors. DOI: 10.5772/intechopen.72247.(2017).

174. Aulton M. Pharmaceutics: The Science of Dosage Form Design, 2nd Edition. Edinburg: Churchill Livingstone. (2002).

175. Ghalia MA, Dahman Y. Chapter 6 - Advanced nanobiomaterials in tissue engineering: Synthesis, properties, and applications. In: Grumezescu AM, editor.

Nanobiomaterials in Soft Tissue Engineering: William Andrew Publishing; (2016).

176. Duroudier, JeanPaul. 17 - Freeze-drying. In: Duroudier J, editor. Heat Transfer in the Chemical, Food and Pharmaceutical Industries: Elsevier; (2016).

177. Wang J, Liu M, Yang C, Wu X, Wang E, Liu P. HPLC method development, validation, and impurity characterization of a potent antitumor indenoisoquinoline, LMP776 (NSC 725776). *J Pharm Biomed Anal*. 2016;124(5):267-273.

178. Tsao R, Deng Z. Separation procedures for naturally occurring antioxidant phytochemicals. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2004;812(2):85-99.

179. De Vos RC, Schipper B, Hall R D. High-performance liquid chromatography-mass spectrometry analysis of plant metabolites in brassicaceae. *Methods Mol Biol.* 2012;860(1):112-128.

180. Electrospray and MALDI Mass Spectrometry: Fundamentals, Instrumentation, Practicalities, and Biological Applications, 2nd Edition. Cole RB, editor. John Wiley & Sons, Inc. All rights reserved.: DOI:10.1002/9780470588901; (2010).

181. Smedsgaard J, Nielsen J. Metabolite profiling of fungi and yeast: from phenotype to metabolome by MS and informatics. *J Exp Bot.* 2005;56(410):273-286.

182. Isolation and Characterization of Bioactive Metabolites from Endophytic Fungi of *Pinus strobus*. Richardson SN, editor. Master of Science in Chemistry with a Specialization in Chemical and Environmental Toxicology.(2014).

183. Balci, M. (2005). Two-Dimensional (2D) NMR Spectroscopy. Basic ^{1}H- and ^{13}C-NMR Spectroscopy, (379-406). doi:10.1016/b978-044451811-8.50016-4

184. Li YG, Ji DF, Zhong S, Liu PG, Lv ZQ, Zhu JX, et al. Polysaccharide from *Phellinus linteus* induces S-phase arrest in HepG2 cells by decreasing calreticulin expression and activating the P27kip1–cyclin A/D1/E–CDK2 pathway. *J Ethnopharmacol*. 2013;150(1):187-195.

185. Song TY, Lin HC, Yang NC, Hu ML. Antiproliferative and antimetastatic effects of the ethanolic extract of *Phellinus igniarius* (Linnearus: Fries) Quelet. *J Ethnopharmacol*. 2008;115(1):50-56.

186. Ajith TA. Cytotoxicity and antitumor activities of *Phellinus rimosus*."Thesis". Amala Cancer Research Centre , University of Calicut.(2004).

187. Ajith TA. Antimutagenic activity of ethyl acetate from *Phellinus rimosus*. " Thesis". Amala Cancer Research Centre , University of Calicut. (2004).

188. Ajith TA. Anticarcinogenic activity of *Phellinus rimosus.*"Thesis". Amala Cancer Research Centre , University of Calicut. (2004).

189. Yoon KN, Jang HS. Antioxidant and Antimicrobial Activities of Fruiting Bodies of *Phellinus gilvus* collected in Korea. *Korean J Clin Lab Sci.* 2016;48(4):355-364.
190. Lee IK, Yun BS. Highly oxygenated and unsaturated metabolites providing a diversity of hispidin class antioxidants in the medicinal mushrooms *Inonotus* and *Phellinus*. *Bioorg Med Chem.* 2007;15(10):3309-3314.
191. Huang GJ, Huang SS, Deng JS. Anti-inflammatory activities of inotilone from *Phellinus linteus* through the inhibition of MMP-9, NF-kappaB, and MAPK activation in vitro and in vivo. Plos One. 2012;7(5):1-12.
192. Ajith TA, Sheenan KK. J. *Phellinus rimosus* Protects Carbon Tetrachloride Induced Chronic Hepatotoxicity In Rats Antioxidant defense mechanism. *Pharm Biol.* 2006;44(6):467-674.
193. Sheena N, AjithTA, Thomas M A, Janardhanan KK. Antibacterial activity of three macrofungi *Ganoderma lucidum Navesporus floccosa* and *Phellinus rimosus* occurring in South India. *Pharm Biol.* 2003;41(8):564-567.
194. Hiralal BS, Asghar SH, Shekhar RB, Sandhya G, GauriB. Antifungal and antibacterial activity of *Phellinus* Samples from Western Ghats of India. *J Pharm Res* 2012;5(12):5339-5342.
195. Ajith TA. Toxicity studies of *Phellinus mushroom*." Thesis". Amala Cancer Research Centre University of Calicut. (2004).
196. Shon M, Kim T, Sung N. Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus* of Hymenochaetaceae) extracts. *Food Chem.* 2003;82(4):593-597.
197. Roghini, R and Vijayalakshmi K. Phytochemical screening, quantitative analysis of flavonoids and minerals in ethanolic extract of *Citrus paradisi*. *Int J Pharm Sci Res.*2018;9(11):4859-4864.
198. Amic D, Amic DD, Beslo D, Trinajsti N. Structure-Radical scavenging sctivity relationships of flavonoids. *Croatica Chemica Acta.* 2003;76(1):55-61.
199. Nara K, Miyoshi T, Honma T, Koga H. Antioxidative activity of bound-form phenolics in potato peel. *Biosci Biotechnol Biochem.* 2006;70(6):1489-1491.
200. Payet B, Shum S, Alain, Smadja J. Assessment of Antioxidant activity of *Cane Brown Sugars* by ABTS and DPPH Radical scavenging assays: determination of their polyphenolic and volatile constituents. *J Agric Food Chem.* 2005;53(26):10074-10079.
201. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Meth Enzymol*. 1999;299:15-27.

202. Attard E. A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. *Cent EurJ Biol*. 2013;8(1):48-53.

203. Marinova FR, MA. Total phenolics and total flavonoids in bulgarian fruits and vegetables. *J Univ Chem Technol Metallurgy*. 2005;40(3):255-260.

204. Mfotie N, Munvera A, Mkounga P, Nkengfack A, McGaw L. Phytochemical analysis with free radical scavenging, nitric oxide inhibition and antiproliferative activity of Sarcocephalus pobeguinii extracts. *BMC Complement Altern Med*. 2017;17(1):1-9.

205. Karakas D, Ari F, Ulukaya E. The MTT viability assay yields strikingly false-positive viabilities although the cells are killed by some plant extracts. T*urk J Biol.* 2017;41(6):919-925.

206. Wasuworawong K, Roytrakul S, Paemanee A, Jindapornprasert K, Komyod W. Comparative proteomic analysis of human Cholangiocarcinoma cell lines: S100A2 as a potential candidate protein inducer of invasion. *Dis Markers.* 2015(4):1-6.

207. Metastasis Research Protocols, Analysis of Cell Behivor Invitro and Invivo. Susan A, Schumacher B, editors. Research school of Biological and Molecular Sciences, Oford Books University, Oford, UK and Udo schumacher Instituent for Anatomy, Department of Neuroanatomy, University Hotpital Hamburg-Eppendrof, Hamburg, Germany.(2001).

208. Orellana EA, Kasinski AL. Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. *Bio Protoc.* 2016;6(21):1-9.

209. Salvatore BA. Chapter 11. NMR Spectroscopy. Organic Chemistry. 2013;109(10):361-417.

210. Promlee B. Fourier transform infrared spectroscopy. Thermogravimetry. (Master's degree) Forensic Science. Thesis. (2013);http://www.sure.su.ac.th/xmlui/handle/123456789/11925.

211. Nuclear magnetic resonance spectroscopy (NMR). A Guide to ^{1}H and ^{13}C NMR chemical shife values From: www.compound chemcom. (2015).

212. Ma YQ, Kun J, Yi Deng LG, YiQun W, Chang H. Mexicanolide-type limonoids from the seeds of *Swietenia macrophylla*. *J Asian Nat Prod Res.* 2017;20(4):1-7.

213. Adesida GA, Adesogana DA.H, Taylo EK. Extractives from *Khayu senegulensis A*. Juss. *Chem Comm* 1967;1(1):790-791.

214. Brahmi F, Mechri B, Dabbou S, Dhibi M, Hammami M. The efficacy of phenolics compounds with different polarities as antioxidants from olive leaves depending on seasonal variations. *Ind Crops Prod.* 2012;38(7):146-152.

215. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. *J Agric Food Chem.* 2005;53(6):1841-1856.

216. Seephonkai P, somchai S, Thongsom A, Sunaart S, Kiemsanmuang B, Chakuton K. DPPH Radical scavenging activity and total phenolics of *Phellinus* mushroom extracts collected from Northeast of Thailand. *Chin J Nat Med.* . 2011;9(6):441-445.

217. Wang Y, Yu J, Zhang, Chen l, Li P, Zhao Y, et al. Influence of flavonoids from *Phellinus igniarius* on sturgeon caviar: Antioxidant effects and sensory characteristics. *Food Chem*. 2012;131(1):206-210.

218. Chang QZ, Ebru Ge, Lee P, Hee H M, Kim C, Heng H, et al. In Vitro antioxidant and anti-Inflammatory activities of protocatechualdehyde isolated from *Phellinus gilvus*. *J Nutr Sci Vitaminol*. 2011;57(1):118-122.

219. Serhan CN, Brain SD, Buckley CD, Gilroy DW, Haslett C, Neill LA, et al. Resolution of inflammation: state of the art, definitions and terms. *FASEB J.* 2007;21(2):325-332.

220. Lakhanpal P, Rai DK. Quercetin: A Versatile Flavonoid. *Internet J. Medical* 2007;2(2):20-35.

221. Joo T, Sowndhararajan K, Hong S, Lee J, Park SY, Kim S, et al. Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells by stem bark of *Ulmus pumila L.* Saudi J Biol sci. 2014;21(5):427-35.

222. Park HJ, Park JB, Lee SJ, Song M. Phellinus linteus Grown on Germinated Brown Rice Increases Cetuximab Sensitivity of KRAS-Mutated Colon Cancer. *Int J Mol Sci.* 2017;18(8):1-12.

223. Li X, Jiao LL, Zhang X, Tian WM, Chen S, Zhang LP. Anti-tumor and immunomodulating activities of proteoglycans from mycelium of *Phellinus nigricans* and culture medium. *Int Immunopharmacol*. 2008;8(6):909-15.

224. AJth,TA. Sheenan KK. Cytotoxic and antitumor activities of a polypore macrofungus, *Phellinus rimosus* (Berk) Pilat. *Ethnopharmacol*. 2003;84(2-3):157-62.

225. Elena Valverde Mara PTHaLP. Edible Mushrooms: Improving Human Health and Promoting Quality Life. *Int J Microbiol*. 2015;2015(ID 376387).

226. Ngo TV, Scarlett CJ, Bowyer MC, Ngo PD, Vuong QV. Impact of different extraction solvents on bioactive compounds and antioxidant capacity from the root of *Salacia chinensis* L. *J Food Qua.* 2017;11(1):1-8.

Appendix

Certificate Ethical for research

คณะกรรมการความปลอดภัยทางชีวภาพระดับสถาบัน มหาวิทยาลัยมหาสารคาม

เอกสารรับรองโครงการวิจัย

เลขที่การรับรอง : IBC12-13/2563

ชื่อโครงการวิจัย

ภาษาไทย : พฤกษเคมี ฤทธิ์ต้านออกซิเดชัน ฤทธิ์ต้านการอักเสบ และต้านเซลล์มะเร็งท่อน้ำดีของสารสกัดเห็ดพิมานบางชนิด

ภาษาอังกฤษ : Phytochemistry, Antioxidant, Anti-inflammation and Anticancer Activities Against Cholangiocarcinoma Cell Lines of Some Species of *Phellinus* Mushroom Extract.

ผู้วิจัย : Mr. Sonesay Thammavong

หน่วยงานที่รับผิดชอบ : คณะเภสัชศาสตร์ มหาวิทยาลัยมหาสารคาม

สถานที่ทำการวิจัย : คณะเภสัชศาสตร์ มหาวิทยาลัยมหาสารคาม

รับรองประเภทที่ 2

วันที่รับรอง : 17 กันยายน 2563 **วันหมดอายุ : 16 กันยายน 2564**

ข้อเสนอการวิจัยนี้ ได้รับการพิจารณาและให้ความเห็นชอบจากคณะกรรมการความปลอดภัยทางชีวภาพระดับสถาบัน มหาวิทยาลัยมหาสารคามแล้ว และอนุมัติในด้านความปลอดภัยทางชีวภาพประเภทที่ 2 ให้ดำเนินการศึกษาวิจัยเรื่องข้างต้นได้ บนพื้นฐานของโครงร่างงานวิจัยที่คณะกรรมการฯ ได้รับและพิจารณา หากมีการเปลี่ยนแปลงใด ๆ ในโครงการวิจัย ผู้วิจัยจักต้องยื่นขอรับการพิจารณาใหม่

....

(ศาสตราจารย์อนงค์ฤทธิ์ แข็งแรง)

รองอธิการบดีฝ่ายพัฒนาโครงสร้างพื้นฐาน วิจัยและนวัตกรรม

ประธานคณะกรรมการความปลอดภัยทางชีวภาพ

Research Journal of Pharmacognosy (RJP) 8(1), 2021: 91-99
Received: 3 Oct 2020
Accepted: 17 Dec 2020
Published online: 21 Dec 2020
DOI: 10.22127/rjp.2020.251089.1632

Original article

Antioxidant and Cytotoxic Activity of *Phellinus* Mushrooms from Northeast Thailand

Sonesay Thammavong[1*] iD, Methin Phadungkit[1], Pornpun Laovachirasuwan[1], Khwanyuruan Naksuwankul[2], Waraporn Saentaweesuk[1], Atit Silsirivanit[3,4], Sopit Wongkham[3,4]

[1]Department of Pharmaceutical Sciences, Faculty of Pharmacy, Mahasarakham University, Kantharawichai District, Maha Sarakham 44150, Thailand.
[2]Department of Biology, Faculty of Science, Mahasarakham University, Kantharawichai District, Maha Sarakham 44150, Thailand.
[3]Department of Biochemistry, Center for Translational Medicine, Faculty of Medicine, Khon Kaen University, Muang District, Khon Kaen 40002, Thailand.
[4]Cholangiocarcinoma Research Institute, Khon Kaen University, Muang District, Khon Kaen 40002, Thailand.

Abstract

Background and objectives: *Phellinus* belongs to the family of Hymenochaetaceae. In Traditional Chinese Medicine, it has been used as an ingredient for the treatment of different types of cancer, ischemia and skin diseases for thousands of years. The present study was aimed to evaluate and compare the mushroom constituents (total phenolic and flavonoid contents) and antioxidant and cytotoxic effect against cholangiocarcinoma cells. **Methods:** The samples of *Phellinus* mushrooms including *P. igniarius*, *P.lineteus* and *P.nigricans* were prepared in two ways: macerated in 95% ethanol and decocted in distilled water. The antioxidant activity of the six extracts were evaluated using the DPPH, ABTS and FRAP assays. Total phenolics and flavonoids were determined using colorimetric tests. In addition, cytotoxic activities against cholangiocarcinoma cell lines (KKU-100 & KKU213A) were assessed by the SRB assay. **Results:** All ethanol extracts of samples showed significantly stronger antioxidant activity compared to aqueous extracts ($p<0.05$), while the ethanol extracts contained higher total phenolic and flavonoid contents. *Phellinus linteus* showed the highest antioxidant activity and total phenolic content when compared to *P. igniarius* and *P.*

nigricans. All samples showed high cytotoxicity against cholangiocarcinoma cell lines, particularly the ethanol extract of *P. linteus.* The cytotoxicity was correlated to the phytochemical contents and antioxidant activity of each *Phellinus* mushroom. **Conclusions:** The cytotoxicity and antioxidant activity are in proportion to the phenolic and flavonoid contents. Therefore, the antioxidant capacity of the mushroom extracts may advocate anti-cancer effects.

Keywords: antioxidant; cytotoxicity; *Phellinus* mushrooms

Citation: Thammavong S, Phadungkit M, Laovachirasuwan P, Naksuwankul K, Saentaweesuk W, Silsirivanit A, Wongkham S. Antioxidant and cytotoxic activity of *Phellinus* mushrooms from northeast Thailand. Res J Pharmacogn. 2010; 8(1): 91-99.

Introduction

Phellinus is a group of medicinal mushroom belonging to the family Hymenochaetaceae [1]. The species decays heartwood, causes root cankers in live standing trees and destroy slash and other woody residues [2]. The pilear surface is light to dark brown or black in color and the hymenial surface is poroid and, light to dark brown in color. Generative hyphae are subhyaline and pale yellow with, simple septate, thin to thick-walled, and branched. Basidiospores are subhyalineand golden yellow to golden brown and are thin-walled to thick-walled [3]. There are 479 species of *Phellinus* worldwide according to the Index Fungorum (2019) [4]. More than 31 species of *Phellinus* mushrooms exist in Thailand, and only two species (*Phellinus linteus* and *Phellinus igniarius*) have been reported for utilization as medication [5]. Traditional Chinese Medicine has used *Phellinus* as an ingredient for the treatment of cancers, herpes, earache, rash, ischemia and skin diseases for thousands of years in Asian countries [6].

Previous studies have shown that some species possess antioxidant [7,8], anti-inflammatory [9,10] and anticancer properties in the fruiting body of mushrooms belonging to the genus *Phellinus* [11,12]. The study of phytochemical constituents of *Phellinus* species have indicated that polysaccharides [4], triterpenoids [13], phenolics and flavonoids [6] were found. For example, scientific investigations demonstrated that hispolon isolated from *Phellinus igniarius* induced apoptosis of lung cancer by increasing apoptosis-related protein expressions, such as the cleavage form of caspase 3, caspase 8 and polymerase [14,15]. The polysaccharide isolated from *P. linteus* effectively inhibited proliferation and colony formation of hepatocellular carcinoma cells (HepG-2) via S-phase cell cycle arrest [16]. In addition, phenolic compounds and polysaccharides prevented cancer formation by their antioxidant effect [17] and immunomodulatory activity [18].

Antioxidants play an important role in neutralizing free radical reactions in the human body [19]. Free radicals have a potential oxidative stress to damage cells. The resultant cell damage contributes to human diseases such as cancer, diabetes mellitus and also inflammation [20]. Therefore, the discovery and research for potent bioactive substances with notable antioxidant activity and low cytotoxicity are important for the development of natural products. Phytochemicals that possess free radical scavenging activity can be used in the prevention and treatment of many inflammation involved diseases including cancer.

Cancer is a major public health problem worldwide and is the second leading cause of death in the United States [21]. Cholangiocarcinoma, a cancer of bile duct, is a significant public health burden in the world, especially in developing countries such as Thailand. The lack of early diagnosis, resistance to chemo and radiotherapies are some of the difficulties and challenges encountered during the treatment [22]. At present, anti-cancer drugs have displayed several side-effects and complications when compared to natural anticancer materials. The natural products are effective and less-toxic agents which are keys in the development of new drugs for the treatment of cancers [23]. Moreover, phytochemicals are gaining popularity in order to be used as prevention and treatment of cancers [24]. A number of bioactive substances show potential to prevent cancer by molecular mechanisms and prove effect against various stages in the neoplastic process [25]. The study of bioactive compounds and development of new drugs for the treatment of cholangiocarcinoma is prioritized; therefore, the objectives of the present study were to evaluate total phenolic and flavonoid contents, antioxidant and cytotoxic activities of *Phellinus* mushroom extracts collected from northeast, Thailand against cholangiocarcinoma.

Materials and Methods

Ethical considerations

The research proposal was approved by the Institutional Biosafety Committee of Mahasarakham University, Mahasarakham District, Thailand (Code; IBC12-13/2563, Date approved; September 17, 2020).

Chemicals and reagents

Cell culture medium including Dulbecco's modified Eagle's medium (DMEM), antibiotic-antimycotic, trypsin, phosphate buffer saline (PBS), and fetal bovine serum (FBS) were purchased from Gibco (USA); dimethyl sulfoxide (DMSO), 2,4,6-tri-(2-pyridyl)-s-triazine(A0382300), Folin-Ciocalteu reagent (LM0821611), methanol (HPLC grade), Rutin (A0257221), ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid, BCBV9734), DPPH (1.1-diphenyl-2-picrylhydrazyl),STBB0828V) were purchased from Sigma Chemicals (Germany).

Mushroom samples and cell lines

Thes amples including *P. igniarius* (MSUT2931), *P. linteus* (MSUT2712), and *P. nigricans* (MSUT2707), were obtained from the Natural Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University, Thailand (MSUT) in June 2019. Cell lines (KKU-100 & KKUM-213A) were obtained from Cholangiocarcinoma Research Institute, KhonKaen University, KhonKaen Mahasarakham District, Thailand.

Extraction

Aqueous extracts

The powder of dried fruit bodies of each sample (77 g) was boiled in distilled water (310 mL) for 4h. After filtration, the water extracts were dried using a freeze dryer at -98 °C for 26 h. The dried extracts were then kept in a refrigerator at 4 ^{o}C for further studies.

Ethanol extract

The powder of each samples (150 g) were extracted sequentially by maceration at room temperature with 95% ethanol (600 mL) in a big glass flask for 3 days (150 × 600 mL, 3 days each). The solution was filtered using gauze and Whatman No. 01 filter paper. The solvents used in the extraction were evaporated by a rotary evaporator and further concentrated by heating at 60 ^{o}C in a water bath, then kept in a refrigerator at 4 ^{o}C [26].

Bioassays

Antioxidant activity

Antioxidant activity of samples were determined using the DPPH, ABTS, and FRAP assays.

The DPPH free radical scavenging activity

The DPPH assay was used to assign free radical scavenging activity, which was described previously by Amid et al. [27]. The samples were prepared at concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL, respectively. Twenty µL of samples and 180 µL of DPPH (300 µM) were added to a 96-well plate, incubated at 37 ^{o}C for 30 min in a dark place. The absorbance was further measured at 517 nm. The negative control was 95% ethanol and ascorbic acid was used as the positive control. Ethanol was replaced instead of the DPPH solution as a blank. The radical scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = [100 \times (A_{blank} - A_{sample})/A_{blank}]$$

Where A_{blank} = absorbance of blank, A_{sample}= absorbance of the sample

ABTS radical scavenging activity

ABTS was produced through the chemical oxidation reaction with potassium persulfate as mentioned by Payet et al. [28]. The blue-green ABTS solution was adjusted with water to obtain an absorbance of 0.7±0.02 at 734 nm. The samples were prepared by mixing 20 µL of the samples (250 µg/mL) with 280 µL ABTS dissolved in water and incubated for 5 min at room temperature. The absorbance was measured at 734 nm using a microplate reader. Ascorbic acid was dissolved in distilled water and used as the positive control. The inhibition percentage of the radical scavenging activity was calculated by using the following equation:

$$\% \text{ inhibition} = [100 \times (A_{blank} - A_{sample})/A_{blank}]$$

Where A_{blank} = absorbance of blank, A_{sample}= absorbance of the sample

FRAP determination

The FRAP assay (ferric reducing antioxidant power) was used to measure the antioxidant power of mushroom extracts. The reduction of ferric tripyridyltriazine [Fe(III)-TPTZ] complex to ferrous tripyridyltriazine [Fe(II)-TPTZ] at low pH is indicated by blue color. The absorbance of the Fe (II)-TPTZ complex was recorded at 593 nm and ferrous sulfate was used as the reference standard [29].

Determination of total phenolics content

The Folin-Ciocalteu colorimetric method was used to investigate the total phenolic content in mushroom extracts [30]. Two hundred µL of Folin-Ciocalteu reagent mixture (1:10 diluted with distilled water) was mixed with 20 µL of a sample (1000 µg/mL) and incubated for 5 min at room temperature; 160 µL of sodium bicarbonate (Na_2CO_3) solution (75g/L) was added to the mixture and incubated at 25 ^{o}C for 30 min. Finally, the absorbance of the solutions was recorded at 630 nm. Gallic acid solution was used to obtain the standard calibration curve (10-125 µg/mL) and total phenolic results were presented as milligrams of gallic acid equivalents (GAE) per gram of dried extract.

Determination of total flavonoids content

Total flavonoids content in the mushroom extracts were determined using the aluminum chloride colorimetric assay [31]. Extracts (100 µL) or standard solution of rutin (10, 20, 40, 60, 80 mg/mL) were added to 30 µL of 5% $NaNO_2$ and 30 µL of 10 % $AlCl_3$. In the 6th min, the total volume was made up to 1 mL by 200 µL of 1M NaOH and distilled water. The absorbance was measured against the prepared blank reagent at 415 nm. The total flavonoids content of the sample was expressed as milligrams of rutin equivalents (RE) per gram of dried extract.

Cell culture

Cholangiocarcinoma cell lines, KKU-100 and KKUM-213A [32,33], were cultured in DMEM containing 10% FBS and 1% antibiotic-antimycotic(100 IU/mL of penicillin-streptomycin solution) in a humidified atmosphere of 5% CO_2 at 37 ^{o}C for 72 h before experiments.

Determination of cytotoxicity against cholangiocarcinoma cells

Cholangiocarcinoma cells were plated to 96-well plates (2×10^3 cells/well) and incubated. After 24 h, the cells were treated with samples (100 µL, 500 µg/mL) and incubated at periods of 24, 48, 72 h. The concentration of DMSO in a vehicle control was 0.5% in DMEM. SRB assay was used to determine the cancer cell viability after treatment of mushroom extracts.

Ten percent of trichloroacetic acid was used to fix the cells in the refrigerator at 4 °C for 30 min. The plate was then washed with distilled water, dried and stained with SRB solution for 30 min and further washed with 1% acetic acid to remove the unbound dye. The bound protein stain in the plate was solubilized using the Tris base. The absorbance was measured at 540 nm [34,35]. The percentage of inhibition was measured as [1-(optical density of test/optical density of vehicle control)] × 100

Statistical analysis

The experiments of the study were performed in triplicates. The results have been shown as mean ± SD. The differences between the groups were considered significant when $p < 0.05$. The Student's t-test was used to define statistical significance among two groups and one-way analysis of variance (ANOVA) followed by Duncan post hoc test was compared for multiple groups.

Results and Discussion

The antioxidant activity, total phenolic and total flavonoid contents of the *Phellinus* mushroom extracts are shown in Table 1. In this study, the ethanol extracts demonstrated significantly higher activities than the water extracts ($p<0.05$) in all assays. In the DPPH assay, the ethanol extract of *P. linteus* showed the highest antioxidant activity compared to other extracts ($p<0.05$, IC_{50} value 28.85 ± 0.56 µg/mL). The ethanol extracts from *P. linteus* and *P. nigricans* exhibited the highest antioxidant activity in the ABTS assay radical, with IC_{50} values of 14.06 ± 0.08 µg/mL and 15.41 ± 0.40 µg/mL, respectively; whereas the ethanol extracts from *P. igniarius* displayed moderate antioxidant activity in response to ABTS radicals with IC_{50} values of 18.44±0.48 µg/mL. The total flavonoids content was found to be higher in *P. igniarius* whereas it was less than other extracts (353.30 ± 1.87 µg/mL; 145.53 ± 2.10 µg/mL). In the FRAP assay, the ethanol extract from *P. linteus* and *P. igniarius* exhibited the highest antioxidant activity ($p<0.05$) while *Phellinus* mushroom extracts from both mushrooms demonstrated the highest total phenolics and flavanoids contents, respectively. The greatest total phenolics content was observed in ethanol extract of *P. linteus* (184.80 ± 5.54 mg GAE/g) while the ethanol extract from *P. igniarius* showed the highest amount of total flavonoid content (353.30 ± 1.87 mg RE/g) ($p <0.05$).

The present study suggested that the ethanol extract of *P. linteus* demonstrated the highest capacity of antioxidant activity in all assays. The reports by Samchai et al.[36] which revealed that the ethanol extract of *P. linteus* showed high free radical scavenging capacity with IC_{50} value of 29.18 ± 0.20 µg/mL, while Seephonkai et al.[37] reported lower free radical scavenging capacity (IC_{50} = 59.24 ± 0.31 µg/mL). The results of the present study also suggested that there was a direct correlation between total phenolics content and antioxidant activity which was observed in the ethanol extract of *P. linteus.*

Table 1. Antioxidant activity, total phenolics and total flavonoids contents of *Phellinus* mushroom extracts

Samples	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	FRAP (mM Fe^{2+}/ 100 mg)	Total phenolics content (mg GAE/g of sample)	Total flavonoids content (mg RE/g of sample)
Ethanol extracts					
P. nigricans	$32.46 \pm 0.30^{B*}$	$15.41 \pm 0.40^{A*}$	$22.70 \pm 5.04^{*b}$	148.86 ± 3.50^{b}	183.55 ± 5.93^{c}
P. linteus	$28.85 \pm 0.56^{A*}$	$14.06 \pm 0.08^{A*}$	36.90 ± 0.97^{a}	184.86 ± 5.54^{a}	223.67 ± 6.91^{b}
P. igniarius	$32.33 \pm 0.54^{B*}$	$18.44 \pm 0.48^{B*}$	37.57 ± 6.21^{a}	145.53 ± 2.10^{b}	353.30 ± 1.87^{a}
Aqueous extracts					
P. nigricans	$81.90 \pm 0. 25^{C*}$	$24.59 \pm 1.03^{C*}$	$11.19 \pm 0.79^{*c}$	58.70 ± 2.76^{c}	75.46 ± 1.15^{e}
P. linteus	$176.68 \pm 0.77^{E*}$	$29.99 \pm 1.62^{D*}$	$12.39 \pm 0.61^{*c}$	50.43 ± 1.65^{c}	74.81 ± 3.52^{e}
P. igniarius	$84.62 \pm 1.13^{D*}$	$25.21 \pm 1.13^{C*}$	$12.23 \pm 1.08^{*c}$	54.48 ± 2.97^{c}	96.76 ± 4.29^{d}
Ascorbic acid	4.53 ± 0.33	5.34 ± 0.37	38.37 ± 1.55	-	-

*Statistically significant difference when compared to ascorbic acid (within column), $p<0.05$; different letters indicate statistically significant difference within columns (uppercase letters, $p<0.05$; lowercase letters, $p<0.001$); mM: millimole equivalent; GAE: gallic acid equivalent; RE: rutin equivalent.

The previous study of Laovachirasuwan et al.[38] suggested that the ethanol extracts of *Phellinus* mushroom possessed higher antioxidant activity than the water extracts which was similar to those observed in the present study. Maingam et al.[39] reported antioxidant activity of crude hot boiling-water extract from cultured mycelia of *P. linteus*, which inhibited DPPH radical scavenging with the IC_{50} value of 243.25 ± 30.82 µg/mL.The growth of cholangiocarcinoma cell lines (KKU-100 & KKU-213A) was evaluated using the sulforhodamine B (SRB) assay modified from the US National Cancer Institute (NCI) [32,35]. In the current study, three ethanol extracts of *Phellinus* mushrooms which presented as the highest antioxidant activity, total phenolics content and total flavonoids content were selected for cytotoxicity against. Percent cell inhibition of each extract is presented in Table 2.

As presented in Table 2, the ethanol extract of *P. linteus* had the greatest cytotoxicity against in both KKU-100 and KKU-213A cells when compared to other extracts at all incubation periods (24, 48, and 72 h) ($p<0.05$). After treating with *P. linteus*, percent cell inhibition of KKU-100 ranged from 74.22 ± 2.08 to 95.52 ± 0.14 and 91.42 ± 1.22 to 98.92 ± 0.22 for KKU-213A cells. This is the first report of *Phellinus* mushroom extracts on cytotoxicity against cholangiocarcinoma cell lines.

The study of Park et al.[40] revealed that the combination of the ethanol extract of *P. linteus* and a monoclonal antibody, cetuximab, increased the sensitivity of KRAS mutated colon cancer cells to cetuximab which indicated the potential of *Phellinus* mushroom extracts as a medical supplement against colon cancer.

The ethanol extract of *P. igniarius* showed the second highest cytotoxicity in both cells and in all incubation periods with percent cell inhibition ranging from 46.61 ± 2.16 to 83.46 ± 0.5 for KKU-100 and 75.53 ± 3.08 to 91.83 ± 0.79 for KKU-213A cells. Song et al.[41] reported that the ethanol extract of *P. igniarius* displayed antiproliferative and antimetastatic effects in human hepatocellular carcinoma (SK-Hep-1) and rat heart vascular endothelial (RHE) cells. This exhibited the potential of *P. igniarius* extract as an adjuvant for cancer chemotherapy.

In the present study, *P. nigricans* was reported to have the least cytotoxic activity in this study when compared to other *Phellinus* mushroom extracts, the percent cell inhibition of *P. nigricans* ranged from 49.22 ± 1.98 to 51.13 ± 1.06 for KKU-100 and 52.20 ± 0.86 to 79.98 ± 0.5 for KKU-213A cells. Li et al.[42] reported that proteoglycans isolated from the mycelium of *P. nigricans* displayed antitumor, and immunomodulating activities. The cytotoxic activity of *Phellinus* mushroom extracts depends on various factors e.g. bioactive compounds in the extracts, incubation times and type of cell lines. A previous study reported that KKU-213A cell line was more sensitive to cancer chemotherapeutic agents than KKU-100,[43] similar result was observed in the present study. For example *P. igniarius* and *P. nigricans* showed weak cytotoxic activity at 24 and 48h when compared with *P. linteus*. The difference of bioactive compounds of *Phellinus* mushroom is an important factor involved in cytotoxicity. In a previous study, hispolon isolated from fruiting bodies of *P. igniarius* exhibited cytotoxicity on lung cancer cells (A549 & H661) and decreased cell viability in a concentration-and times-dependent manner [15].

Table 2. Effects of the *Phellinus* mushroom extracts on growth of cholangiocarcinoma cell lines at different times

Samples/ time point	**CCA cell line (% inhibition at 500 µg/mL)**					
	KKU-100			**KKU-213A**		
Time (h)	24	48	72	24	48	72
P. nigricans	49.22 ± 1.98^{B}	47.46 ± 3.94^{C}	51.13 ± 1.06^{a*}	52.20 ± 0.86^{a*}	74.04 ± 0.91^{a}	79.98 ± 0.50^{a*}
P. linteus	74.22 ± 2.08^{*}	96.68 ± 0.35^{*}	95.52 ± 0.14^{b*}	91.42 ± 1.22^{b*}	96.74 ± 0.46^{*}	98.92 ± 0.22^{*}
P. igniarius	49.72 ± 2.12^{A}	46.61 ± 2.16^{D}	83.46 ± 0.50^{c*}	75.53 ± 3.08^{*}	78.23 ± 3.22^{*}	91.83 ± 0.79^{c*}

Star (*) indicate statistically significant difference among mushrooms, $p<0.05$; different lowercase letters indicate statistically significant difference among time points, $p<0.001$; Different uppercase letters (A,B,C) indicate statistically not significant difference among mushrooms and time points, $p<0.05$

Sarfraz et al. reported that hispolon from *P. linteus* induced apoptosis and sensitized human cancer cells to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through upregulation of death receptors [44]. *Phellinus* mushroom extracts is rich in biologically active compounds with therapeutic potential. Polysaccharide, especially β-glucan, steroid, flavonoid, and phenolic are believed to be responsible for the biological activities as observed in the *Phellinus* mushroom [4] The results on antioxidant and growth inhibition effect of ethanol extracts from *P. linetus*, *P. igniarius*, *P. nigricans* showed their potentials for further bioactivity investigation and may be candidates for development of natural commercial products in the further

In conclusion, the present study demonstrated that all samples showed antioxidant activity and cytotoxicity against cholangiocarcinoma cells. Both biological activities have relation to phytochemical of *Phellinus* mushroom extracts such as total phenolics content and total flavonoids content. Therefore, the mechanisms of antioxidant capacities may advocate anti-cancer effects; *Phellinus* mushroom may be candidates for discovery of new drugs in the future.

Acknowledgments

We convey our appreciation to the support received from the Foundation Pierre Fabre and Mahasarakham University (Grant year 2020). Also, we would like to express our gratitude to the Cholangiocarcinoma Research Institute, Khon Kaen University and Faculty of Pharmacy, Mahasarakham University, Thailand. Finally, we would like to acknowledge Miss. Alice Padmini Albert for proof reading of the manuscript.

Author contributions

Mushroom sample preparation, extraction, biological tests, chemical reaction testing and drafting of the manuscript were carried out by Sonesay Thammavong. Methin Phadungkit and Pornpun Laovachirasuwan were responsible for plan setting and recommendation of biological tests, they were responsible for manuscript modification and editing. Khwanyuruan Naksuwankul was responsible for designing the study, providing mushroom samples, identifying samples and manuscript editing. Waraporn Saentaweesuk assisted with some biological tests. Atit Silsirivanit and Sopit Wongkham advised and guided some experiments.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

References

[1] Ranadive DK, Joshi T, Khare H, Jajtap N, Ranade V, Vaidya J. Host distribution of *phellinus* from india. *Indian J Forestry*. 2012; 35(1): 67-72.

[2] Ranadive DK, Vaidya J. Host diversity of genus *Phellinus* from world. *Elixir Appl Botany*. 2012; 52(2012): 11402-11408.

[3] Azeem U, Dhingra G Shri R. Some additions to the diversity of genus *Phellinus* Quel. from woodrotting fungal flora of district dehradun (uttarakhand), india. *Res J life Sci Bioinform Chem Sci*. 2017; 3(4): 177-189.

[4] Zapora E, Wołkowycki M, Bakier S, Zjawiony J. *Phellinus igniarius*: a pharmacologically active polypore mushroom. *Nat Prod Commun*. 2016; 11(7): 1043-1046.
[5] Thai-Korea Natural *Phellinus* Mushroom Research Center. *Phellinus* Research. [Accessed 2019]. Available from: https://www.phellinus-research.com/
[6] Chen H, Tian T, Miao H, Zhao YY. Traditional uses, fermentation, phytochemistry and pharmacology of *Phellinus linteus*: a review. *Fitoterapia*. 2016; 113(9): 6-26.
[7] Wang Y, Wang SJ, Mo SY, Li S, Yang YC, Shi JG. Phelligridimer a, a highly oxygenated and unsaturated 26-membered macrocyclic metabolite with antioxidant activity from the fungus *Phellinus igniarius*. *Org Lett.* 2005; 7(21): 4733-4736.
[8] Ajith AT, Janardhanan KK. Indian medicinal mushrooms as a source of antioxidant and antitumor agents. *J Clin Biochem Nutr*. 2007; 40(3): 157-162.
[9] Elsayed EA, Enshasy H, Wadaan MA, Aziz R. Mushrooms: a potential natural source of anti-inflammatory compounds for medical applications. *Mediators Inflamm*. 2014; Article ID 805841.
[10] Kim SH, Song YS, Kim SK, Kim BC, Lim CJ, Park EH. Anti-inflammatory and related pharmacological activities of the n-butanol subfraction of mushroom *Phellinus linteus*. *J Ethnopharmacol.* 2004; 93(1): 141-146.
[11] Konno S, Chu K, Feuer N, Phillips J, Choudhury M. Potent anticancer effects of bioactive mushroom extracts (*Phellinus linteus*) on a variety of human cancer cells. *J Clin Med Res*. 2015; 7(2): 76-82.
[12] Moo KC, Dai HH, Ho KH, Sung HC, Woo JL. Anticancer effect of *Phellinus linteus*; potential clinical application in treating pancreatic ductal adenocarcinoma. *J Carcino Mutagen*. 2013; 1(9): 1-8.
[13] Yin RH, Zhao ZZ, Ji X, Dong ZJ, Li ZH, Feng T, Lui KJ. Steroids and sesquiterpenes from cultures of the fungus *Phellinus igniarius. Nat Prod Bioprospect.* 2015; 5(1): 17-22.
[14] Chen YC, Chang HY, Deng JS, Chen JJ, Huang SS, Lin IH, Wan LK, Chao W, Huang GJ. Hispolon from *Phellinus linteus* induces G0/G1 cell cycle arrest and apoptosis in Nb4 human leukaemia cells. *Am J Chin Med.* 2013; 41(6): 1439-1457.
[15] Wu Q, Kang Y, Zhang H, Wang H, Liu Y, Wang J. The anticancer effects of hispolon on lung cancer cells. *Biochem Biophy Res Commun*. 2014; 453(3): 385-391.
[16] Wang G, Dong L, Zhang Y, Ji Y, Xiang W, Zhao M. Polysaccharides from *Phellinus linteus* inhibit cell growth and invasion and induce apoptosis in HepG-human hepatocellular carcinoma cells. *Biologia.* 2012; 67(1): 247-254.
[17] Song W, Helsper JV, Griensven L. Phenolic compounds present in medicinal mushroom extracts generate reactive oxygen species in human cells in vitro. *Int J Med Mushrooms.* 2008; 10(1): 1-13.

[18] Kozarski M, Klaus A, Niksic M, Jakovljevic D, Helsper PFG, Griensven L. Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus. Food Chem.* 2011; 129(4): 1667-1675.
[19] Bagchi D, Bagchi M, Stohs SJ, Das DK, Ray SD, Kuszynski CA, Joshi SS, Pruess HG. Free radicals and grape seed proanthocyanidin extract: Importance in human

health and disease prevention. *Toxicology*. 2000; 148(2-3): 187-197.
[20] Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants, and functional foods: Impact on human health. *Pharmacogn Rev.* 2010; 4(8): 118-126.
[21] Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *Ca Cancer J Clin.* 2018; 68(1): 7-30.
[22] Limeneh A, Plengsuriyakarn T, Nabangchang K. Anticholangiocarcinoma activity and toxicity of the *Kaempferia galanga* Linn. rhizome ethanolic extract. *BMC Complement Altern Med.* 2017; 17: 1-11.
[23] Song FQ, Liu Y, Kong XS, Chang W, Song G. Progress on understanding the anticancer mechanisms of medicinal mushroom: *Inonotus obliquus*. *Asian Pac J Cancer Prev.* 2013; 14(3): 1571-1578.
[24] Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Metha RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science*. 1997; 275(5297): 218-220.
[25] Nosrati N, Bakovic M, Paliyath G. Molecular mechanisms and pathways as targets for cancer prevention and progression with dietary compounds. *Int J Mol Sci*. 2017; 18(10): 1-22.
[26] Shon MY, Kim TH, Sung NJ. Antioxidants and free radical scavenging activity of *Phellinus baumii* (*Phellinus* of hymenochaetaceae) extracts. *Food Chem*. 2003; 82(4): 593-597.
[27] Amic D, Beslo D, Trinajstic N. Structureradical scavenging activity relationships of flavonoids. *Croat Chem Acta.* 2003; 76(1): 55-61.
[28] Payet B, Cheong Sing AS, Smadja J. Assessment of antioxidant activity of cane brown sugars by ABTS and DPPH radical scavenging assays: determination of their polyphenolic and volatile constituents. *J Agric Food Chem.* 2005; 53(26): 10074-10079.
[29] Benzie IFF, Strain JJ. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* 1999; 299: 15-27.
[30] Attard E. A rapid microtitre plate folin-ciocalteu method for the assessment of polyphenols. *Cent Eur Biol.* 2013; 8(1): 48-53.
[31] Marinova D, Ribarova F, Atanassova M. Total phenolics and total flavonoids in bulgarian fruits and vegetables. *J Chem Techno Metall*. 2005; 40(3): 255-260.
[32] Sripa B, Seubwai W, Vaeteewoottacharn K, Sawanyawisuth K, Silsirivanit A, Kaewkong W, Muisuk K, Dana P, Phoomak C, Lertitthiporn W, Luvira V, Pairojkul C, Teh BT, Wongkham S, Chamgramol Y. Functional and genetic characterization of three cell lines derived from a single tumor of an opisthorchis viverrini-associated cholangiocarcinoma patient. *Human Cell.* 2020; Article ID 32207095.
[33] Sripa B, Leungwattanawanit S, Nitta T, Wongkham C, Bhudhisawasdi V, Puapairoj A, Sripa C, Miwa M. Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). *World J Gastroenterol*. 2005; 11(22): 3392-3397.
[34] Indramanee S, Sawanyawisuth K, Silsirivanit A, Dana P, Phoomak C, Kariya R, Kilnhom N, Sorin S, Wongkham C, Okada S, Wongkham S. Terminal fucose mediates progression of human cholangiocarcinoma through EGF/EGFR activation

and the Akt/Erk signaling pathway. *Sci Rep*. 2019; 9: 1-13.
[35] Vichai V, Kirtikara K. Sulforhodamin B colorimetric assay for cytotoxicity screenig. *Nat Protoc*. 2006; 1(3): 1112-1116.
[36] Samchai S, Seephonkai P, Sangdee A, Puntuchai A, Klinhom U. Antioxidant, cytotoxic and antimalarial activities from crude extracts of mushroom *Phellinus linteus*. *J Biol Sci*. 2009; 9(7): 778-783.
[37] Seephonkai P, Samchai S, Thongsom A, Sunaart S, Kiemsanmuang B, Chakuton K. DPPH radical scavenging activity and total phenolics of *Phellinus* mushroom extracts collected from northeast of Thailand. *Chin J Nat Med.* 2011; 9(6): 414-445.
[38] Laovachirasuwan P, Judprakob C, Sinaphet B, Phadungkit M. In vitro antioxidant and antimutagenic activities of different solvent extracts of *Phellinus* spp. *Int Food Res J*. 2016; 23(6): 2608-2615.
[39] Maingam C, Shiponkai P, Chutiman N, Loutchanwoot P, Srivilai P. Total contents of polysaccharide, phenol and flavonoid, and antioxidant activity of crude hot boiling-water extract from cultured mycelia of *Phellinus linteus*. *J Sci Technol MSU*. 2017; 36(6): 793-805.
[40] Park HJ, Park JB, Lee SJ, Song M. *Phellinus linteus* grown on germinated brown rice increases cetuximab sensitivity of kras-mutated colon cancer. *Int J Mol Sci.* 2017; 18(8): 1-12.
[41] Song TY, Lin HC, Yang NC, Hu ML. Antiproliferative and antimetastatic effects of the ethanolic extract of *Phellinus igniarius* (linnearus: fries) Quelet. *J Ethnopharmacol*. 2008; 115(1): 50-56.
[42] Li X, Jiao LL, Zhang X, Tian WM, Chen S, Zhang LP. Anti-tumor and immunomodulating activities of proteoglycans from mycelium of *Phellinus nigricans* and culture medium. *Int Immunopharmacol*. 2008; 8(6): 909-915.
[43] Wasuworawong K, Roytrakul S, Paemanee A, Jindapornprasert K, Komyod W. Comparative Proteomic analysis of human cholangiocarcinoma cell lines: S100A2 as a potential candidate protein inducer of invasion. *Dis Markers.* 2015; Article ID 629367.
[44] Sarfraz A, Rasul A, Iqra Sarfraz I, Hussain G, Shafiq N, Masood M, Adem S, Sarker SD, Li X. Hispolon: a natural polyphenol and emerging cancer killer by multiple cellular signaling pathways. *Environment Res.*2020; 190(11): 1-9.

Abbreviations

PE: *Phellinus* mushroom extract; CCA: cholangiocarcinoma; HepG-2: hepatocellular carcinoma cells; DMEM: Dulbecco’s modified eagle medium; PBS: phosphate buffer solution; FBS: fetal bovine serum; DMSO: dimethyl sulfoxide; ABTS: 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); DPPH: 1.1-diphenyl-2-picrylhydrazyl; FRAP: ferric reducing antioxidant power; TPC: total phenolic content; TFC: total flavonoid content, RE: rutin equivalent.

BIOGRAPHY

NAME	Mr.Sonesay THAMMAVONG
DATE OF BIRTH	10 November 1987
PLACE OF BIRTH	Borikhamxay
ADDRESS	House number 445, Unit 46, Sokyai village, Saysetha District, Vientiane, Lao PDR
POSITION	Assistant Lecturer
PLACE OF WORK	Department of Pharmaceutical Science, Faculty of Pharmacy, University of Health Sciences, Vientiane Lao PDR
EDUCATION	2021 Ph.D. degree in Pharmaceutical Sciences, Faculty of Pharmacy, Mahasarakham University, Thailand 2013 Master's Degree in Thailand, Laboratory of phytochemistry. Prince of Songkla University, Faculty of Thai Traditional Medicine. Fellowship: Prince of Songkla University 2010 Bachelor in Pharmaceutical Sciences, UHS, Vientiane, Lao PDR