CHARACTERIZATION OF POLYSACCHARIDE EXTRACTED FROM *LENTINUS VELUTINUS* STRAIN WCR1104 AGAINST *IN VITRO* ANTICANCER ACTIVITY

By
MR. Wascharin UDCHUMPISAI

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy MICROBIOLOGY
Department of MICROBIOLOGY
Graduate School, Silpakorn University
Academic Year 2017

การแยกบริสุทธิ์ และการศึกษาสมบัติของพอลิแซคคาร์ไรด์ที่สกัดจากเห็ด *Lentinus velutinus* strain WCR1104 ในการต้านการเจริญของเซลล์มะเร็ง

โดย
นายวัชรินทร์ อุดชุมพิสัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปรัชญาดุษฎีบัณฑิต
สาขาวิชาจุลชีววิทยา แบบ 2.2 ปรัชญาดุษฎีบัณฑิต
ภาควิชาจุลชีววิทยา
บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร
ปีการศึกษา 2560

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Field of Study	MICROBIOLOGY
Advisor	Eakaphun Bangyeekhun

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54313802 : Major MICROBIOLOGY
Keyword : Lentinus spp., cytotoxicity assay, anticancer, polysaccharides, chemical structure

MR. WASCHARIN UDCHUMPISAI : CHARACTERIZATION OF POLYSACCHARIDE EXTRACTED FROM *LENTINUS VELUTINUS* STRAIN WCR1104 AGAINST *IN VITRO* ANTICANCER ACTIVITY THESIS ADVISOR : ASSISTANT PROFESSOR EAKAPHUN BANGYEEKHUN, Ph.D.

The aim of this study was to evaluate some chemical properties and the anticancer activity of polysaccharides extracted from *Lentinus* spp. Several studies were reported about the pharmacological properties of *L. edodes*, especially anticancer properties. While the study of bioactive substance of other species was not much. In this work, four *Lentinus* were collected from Nakhon Pathom, Ratchaburi, and Kanchanaburi province. The morphology, and ITS sequence analysis revealed they were *L. sajor-caju* strain EB1001, *L. swartzii* strain EB1101, *L. squarrosulus* strain WCR1201, and *L. velutinus* strain WCR1104. Crude extracts of dried fruiting bodies and mycelia from five *Lentinus* spp. were extracted using two solvents, hot water and 95% ethanol, and evaluated for their total carbohydrates, proteins, reducing sugar, phenol contents, and cytotoxicity. The yield of crude extracts was 3.36 – 20.53% (w/w). Cytotoxicity was determined with 10 mg/mL of crude aqueous and 1 mg/mL of crude ethanolic extracts by using the [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) method. All extracts showed non-cytotoxicity against the normal cell lines, LLC-MK2 and L929 cells. The aqueous extracted from fruiting bodies of *Lentinus velutinus* (LVFB-Aq) displayed the highest anticancer activity against the HeLa and the HepG2 cells with 49.83% and 48.59% inhibition, respectively. The LVFB-Aq was further separated by re-precipitation method into 3 fractions. The main fraction (Fraction E4) could decrease the viability of both cancer cell lines, HeLa and HepG2, at concentration 5 mg/mL. Fraction E4 was fractionated by anion exchange chromatography into 6 fractions, which fraction E4N5 has the highest anticancer efficacy by significantly inhibiting the growth of HeLa cell line about 17% and 26.65% at concentration 1 and 2 mg/mL, respectively. The fraction E4N5 was purified by size exclusion chromatography into 3 substances e.g. LV1, LV2, and LV3. The average molecular weight of the polysaccharide LV2 was estimated to be ~336 kDa. It slightly inhibited the growth of cancer cell lines, which IC50 of HeLa and HepG2 at 48 h was 2,000 and 1,935 µg/mL, respectively and showed a lower antioxidant activity of approximately 40%SA at 2,000 µg/mL. These studies suggested that the extraction and purification method must be improved for obtaining the high effective anticancer substance from *L. velutinus*.

ACKNOWLEDGEMENTS

First, I am extremely thankful to my advisor Asst. Prof. Eakaphun Bangyeekhun who giving me a golden opportunity to my education. His enthusiasm, encouragement, and faith in me throughout have been extremely helpful. He always offered helpful guidance and supports to me over the years.

I want to acknowledge the thesis chairman, Assoc. Prof. Thanit Pewnim, Ph.D., and thesis committee, Assoc. Prof. Rathapon Asasutjarit, Ph.D. and Waranyou Pulsawat, Ph.D., for comments and recommends.

I am very grateful to Asst. Prof. Thongchai Taechowisan, Ph.D, Asst. Prof. Thanaporn Chuen-Im, Ph.D. (Department of Microbiology, Faculty of Science, Silpakorn university), Mr. Winyou Puckdee (Department of Biology, Faculty of Science and Technology, Rambhai Barni Rajabhat university) and Miss Watcharee Waratchareeyakul, Ph.D. (Department of Chemistry, Faculty of Science and Technology, Rambhai Barni Rajabhat university) for their valuable suggestions in the accomplishment of my thesis.

I would like to thank the Faculty of Science, Silpakorn university through the research grant (SRF – JRG – 2559 – 05) for financial support of this research, as well as the Department of Microbiology, Faculty of Science, Silpakorn university for support and facilities.

Moreover, I wish to thank the GlycaNova company (Norway) for supporting the Lentinex (LentinanXP), the well-known anticancer polysaccharide from Lentinus edodes, that was used as a positive control in this work. I also thank Miss Korapan Sawetsuwannakul, teachers, my friends and lab mates for helping me to complete this thesis successfully. Finally, I would like to express my deepest gratitude to my family who always provide love, care and encouragement.

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ได้รับทุนอุดหนุนการทำวิทยานิพนธ์จากเงินงบประมาณแผ่นดิน

(หมวดเงินอุดหนุนทั่วไป) ของบัณฑิตวิทยาลัย

ประจำปีงบประมาณ 2559 (ครั้งที่ 2)

บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

CHAPTER I

INTRODUCTION

Cancer is a major cause of death in people of various ages and racial background. There are many research efforts and clinical studies in the fight against the disease (Bhanot, Sharma, & Noolvi, 2011; Daba & Ezeronye, 2003; Patel & Goyal, 2012; Zong, Cao, & Wang, 2012). Nowadays, several options are used for cancer treatments, such as surgery, radiotherapy, chemotherapy and hormonal therapy. These methods of cancer treatment and the current anticancer drugs available on the market are pose several side effects for patients (Bhanot et al., 2011; Patel & Goyal, 2012; Zong et al., 2012). Thus, novel effective and less-toxic anticancer agents for cancer therapy are needed.

One of the alternatives for treating cancer is the use of natural products that can be obtained from plants, animals, marine organisms, and microorganisms. They have received increasing attention in recent years because they show lower adverse effects than chemical drugs. They can activate the body's immune system to kill or repress the growth of cancer cells (Bhanot et al., 2011; Mantovani et al., 2008).

Mushrooms are a good source of natural compounds for cancer treatments. Many edible mushrooms and their products have been noted as dietary supplements and sources of medicinal compounds with potential anticancer properties against several types of cancer in human (Ferreira, Vaz, Vasconcelos, & Martins, 2010; Yukawa, Ishikawa, Kawanishi, Tamesada, & Tomi, 2012). *Lentinus edodes* is one of the medicinal mushrooms which was studied and showed numerous pharmacological properties, such as antimicrobial, antiviral, antioxidant, anti-inflammatory, antiatherogenic, hypoglycemic, hepatoprotective, anticancer, and immunomodulating properties (Ferreira et al., 2010; Rai, Tidke, & Wasser, 2005; Zaidman, Yassin, Mahajna, & Wasser, 2005). Several compounds extracted from *L. edodes* such as Lentinan (Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970; M. Zhang, S., Cheung, & Wang, 2007), the polysaccharide L-II (Zheng, Jie, Hanchuan, & Moucheng, 2005), water extracts of fruiting bodies (Israilides et al., 2008), L·E·M (Shen et al., 2009), and LEP (Yukawa et al., 2012), show immunomodulatory and anticancer activity.

Most *Lentinus* species are edible and cultivable. They provide locals with seasonal food, medicine and alternative income (Karunarathna et al., 2011). Many species of *Lentinus* found in Thailand were studies on cultivation and their nutritional value. However, little information is available concerning their bioactivity. Thus, we hypothesized that other species of *Lentinus* can show the pharmacological properties as presence in *L. edodes*, especially anticancer properties. In the current study, the biological extracts from *Lentinus* spp. collected in Thailand were studied. The objectives of this work are to obtain the anticancer polysaccharide extracted from *Lentinus* spp. and to know its structure.

CHAPTER II

REVIEW LITERATURE

1. Cancer and treatments

Cancer is a generic term for a large group of diseases characterized by the uncontrolled proliferation cells which tend to invade adjoining parts of the body and spread to other organs. On the other hand, they are malignant tumors and neoplasm. Cancer is the result of chromosomal DNA mutation of normal cell which can be both activated by external and internal factors. The external factors can be divided into three categories; (1) Physical factors, such as ultraviolet and ionizing radiation; (2) Chemical factors e.g. tobacco use, alcohol use, aflatoxin and so forth; (3) Biological factors, for example viruses, bacteria or parasites infections. Whereas, the internal factors are hormones, aging, immune condition, inherited mutation and mutation occurring in metabolism. Cancer continues to be a major cause of death worldwide, accounting for 14 million new cancer cases in 2012 and about 8.8 million people in 2015. An approximately 70% of cancer deaths occur in low- and middle-income countries. The most causes of cancer death in each year are cancer of lung (1.69 million deaths), liver (788,000 deaths), colorectal (774,000 deaths), stomach (754,000 deaths) and breast (571,000 deaths; World Health Organization, 2017).

Options for the cancer treatment rely on the stage of cancer, such as size of tumor and degree of spread of a cancer from its initial site to another, the metastasis. There are many choices for cancer treatments as followed.

1.1 Surgery

This choice is the oldest and the most effective treatment for the localized primary cancer, in which 100% of excised cells are removed. The surgery offers the greatest chance of cure for many types of cancer.

1.2 Radiotherapy

The medical use of ionizing radiation such as X-rays or gamma rays to control or kill malignant cancer cell. It is one of the most cost-effective cancer therapies. Radiation is regarded to be a local treatment because only cells in the area being treated are affected. However, the radiotherapy can destroy both the cancer cells and cause some effects on some of the surrounding normal cells.

1.3 Chemotherapy

Chemotherapy is the use of drugs to kill a rapidly reproducing cell. However, the chemotherapeutic drugs are not specific to cancer cells and it also affect to normal cells because there is not difference of reproduction between cancer cells and normal cells.

1.4 Hormonal (endocrine) therapy

It is the treatment with drugs that interfere with hormone production or action to kill or slow cancer cells growth. Hormonal therapies have been related with less side effects than cytotoxic chemotherapies which makes them desirable as a

preventative treatment (Urruticoechea et al., 2010).

1.5 Biological therapy (immunotherapy)

All the above cancer treatments and the current anticancer drugs available in the market generally pose several side-effects to patients (Patel & Goyal, 2012). Thus, novel effective and less-toxic anticancer agents for cancer therapy are needed. Biological therapy (immunotherapy) is an alternative cancer treatment options based on promote or support the body's immune system response, or use the immune system components as a basis in order to kill or repress the growth of cancer cells. There are numerous agents whether chemical or biological substances which can stimulate the immune systems and led to the kill the cancer cells.

Nowadays, natural products are an attractive source of new cancer drugs because they show lower adverse effects than chemical drugs (Bhanot et al., 2011; Mantovani et al., 2008; Rocha, Lopes, & Schwartsmann, 2001). There are vast chemical diversity and over 60% of currently used chemotherapeutic agents are derived from natural sources including several species of plant, animals, marine organisms and microorganism (Bhanot et al., 2011; Rocha et al., 2001).

Plants are the major sources of the medicinal compounds. There are numerous anticancer substances isolated from plants, for examples, the vinblastine (VLB) and vincristine (VCR) from the Madagascar periwinkle *Catharanthus roseus*, Podophyllotoxin and Epipodophyllotoxin are derived from the roots of *Podophyllum* species, paclitaxel (Taxol®) is obtained from the bark of the Pacific Yew Taxus brevifolia, camptothecin (CPT) and their derivatives, irinotecan (CPT-11), 9-aminocamptothecin (9-AC), lurtotecan, rubitecan and topotecan, obtained from the bark and stem of Nyssacea *Camptotheca accuminata*, flavopiridol was isolated from the leaves and stems of Amoora rohituka and Dysoxylum binectariferum (Maliaceae), homoharringtonine is isolated from the Chinese tree *Cephalotaxus harringtonia* (Cephalotaxacea) and the others (Bhanot et al., 2011; Nirmala, Samundeeswari, & Sankar, 2011; Rocha et al., 2001).

Natural products obtained from marine organisms are the great potential source of novel chemicals in various classes including polyketides, terpenes, steroids and peptides. They possess cytotoxicity activity against multiple cancer types (Bhanot et al., 2011; Rocha et al., 2001; Simmons, Andrianasolo, McPhail, Flatt, & Gerwick, 2005). The first anticancer product which was derived from the tunicate *Tridemnum solidum*, namely didemnin B, a cyclic depsipeptide, shows a partial activity against non-Hodgkin's lymphoma by inhibiting the protein synthesis and arresting the cell-cycle at G1 phase. Several ecteinascidins have been obtained from murine tunicate *Ecteinascidia turbinate*. Ecteinascidins (ET-743) is the one that is toxic to the most cancer cell lines by selective alkylation of guanine residues in the DNA minor groove. Furthermore, there are several compounds derived from murine organisms that showed the anticancer activity such as bryostatins from *Bugula neritina*, and other marine *bryozoan, halichondrin B* from diverse sponges and psammaplins from verongid sponges (Bhanot et al., 2011; Rocha et al., 2001; Simmons et al., 2005).

Many agents extracted from microorganisms can be used in cancer therapy, e.g. rapamycin from *Streptomyces hygroscopicus* showed a potent

immunosuppressive activity by blocking the cell cycle progression at middle-to-late G1 phase in T cells and B cells, and inhibiting the signaling pathways required for T-cell activation and proliferation, wortmannin, the compound from the fungus *Talaromyces wortmanni*, displays the inhibition of the signal transduction pathway by forming a covalent complex with an active-site residue of phosphoinositide 3 kinase (PI3K). Moreover, there are many anticancer compounds isolated from *Streptomyces* species such as actinomycin, bleomycin, daunomycin, doxorubicin, epirubicin, idarubicin, mitomycin C and geldanamycin (Bhanot et al., 2011; Rocha et al., 2001).

Some effective anticancer compounds can be isolated from yeast and fungal. MOOJ4 and MPPJ5, the polysaccharide fractions isolated from *Penicillium jiangxiense*, as reported by Xiao *et. al.* (2008) showed the slight cytotoxicity effect against the gastric adenocarcinoma SGC-7901 cell proliferation and significantly induced the cell apoptosis and cell cycle arrest at the S phase. *Silfoethyl glucan (SEG)*, a novel derivertive prepared from the yeast β-glucan isolated from *Saccharomyces cerevisiae*, was demonstrated to have DNA-protective and cytotoxic effects against mouse leukemia cells (Zong et al., 2012).

Mushrooms are ones of the attractive sources for anticancer drugs. Several current anticancer drugs were derived from mushrooms such as lentinan, shizophyllan, polysaccharide-K (PSK) and others. They show the strong effective cytotoxicity against many cancer cells while the cytotoxicity against normal cells and the side effects to patients are too low. these are discussed in next section.

2. Bioactive compounds from mushrooms.

'Mushroom' is a macrofungus with a distinctive fruiting body, which can be either hypogenous (growing on a lower/under surface) or epigenous (developing or growing on an upper surface), large enough to be seen with the naked eyes and can be picked by hand (Lindequist, Niedermeyer, & Jülich, 2005; Rai et al., 2005). The number of different mushroom species on the earth is estimated to be 140,000, of which may be only 10% are already known. Assuming that only 5%, or 7000 species are the useful mushrooms among the undiscovered and unexamined mushrooms (Barranco et al., 2010; Ferreira et al., 2010; Lindequist et al., 2005). The higher Basidiomycetes are classified to 80 families, 550 genera and approximately 10,000 species. However, only 2,000 species are safe for human consumption, and about 700 species of these have been found to possess significant medicinal activities (Barranco et al., 2010; Ferreira et al., 2010; Rai et al., 2005).

Medicinal mushrooms have an established history of use as natural products for traditional oriental therapies in most Asian countries such as Japan, China, Korea, and India, whereas the use of medical mushrooms in the Western hemisphere has been lightly increasing only since the last few decades (Lindequist et al., 2005; Zaidman et al., 2005; Zong et al., 2012). They represent a huge source of effective new products with pharmacological properties including antimicrobial, antiviral, antioxidant, anti-inflammatory, immunosuppressive, antiallergic, antiatherogenic, hypoglycemic, hepatoprotective, anticancer, and immunomodulating properties (Ferreira et al., 2010; Rai et al., 2005; Zaidman et al., 2005). Several traditionally used mushrooms, e.g. genera *Ganoderma, Grifola, Lentinus (Lentinula),*

Pleurotus, Schizophylum, Inonotus, Cordyceps, Auricularia, Agaricus, Hericium, Flammulina, and *Tremella*, have been evaluated for their medicinal effects (Barranco et al., 2010; Rai et al., 2005; Silva, Rapior, F., Bahkali, & Hyde, 2012; Zaidman et al., 2005).

Mushrooms are served as dietary and the source of compounds with potential anticancer properties for many years ((Ferreira et al., 2010; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Zaidman et al., 2005). These compounds were isolated from fruiting bodies, mycelia, spores, and culture broth of mushrooms (Moradali et al., 2007). There are approximately 650 species of higher Basidiomycetes that showed the anticancer activity. The first demonstration of the anticancer activity of higher Basidiomycetes was reported by Lucas *et. al.* (1957), who extract the fruiting bodies of *Boletus edulis* Bull. Fr. and other Homobasidiomycetes in tests against the Sarcoma 180 cell line in mice (Zaidman et al., 2005). The compounds from mushrooms with potential anticancer and immunostimulating properties were classified as the followings.

2.1 Low molecular weight compounds (LMW)

Mushrooms contain a variety of complex compounds with various chemical compositions derived from secondary metabolisms such as phenolic compounds, polyketides, triterpenoids, and steroids, which are specific to mushroom species. The secondary metabolites with LMW and anticancer properties in mushrooms including quinones, cerebrosides, isoflavones, catechols, amines, triacylglycerols, sesquiterpenes, steroids, organic germanium, and selenium (Ferreira et al., 2010; Silva et al., 2012).

Several low molecular weight compounds isolated from mushrooms showed the direct effects on cancer development by various mechanisms, such as modulate the cellular signal transduction pathways (NF-κB pathway, MAPK pathway) and support the inhibitory effects on cell differentiation, angiogenesis, carcinogenesis and metastasis (Silva et al., 2012).

Cordycepin (3’-deoxyadenosine), a derivative of the nucleoside adenosine, was initially isolated from an aqueous extract of *Cordyceps sinensis* and a major component of the butanol fraction of *Cordyceps militaris*. It can induce an apoptosis of human leukemia cells in a concentration-dependent manner through a signaling cascade involving a ROS-mediated caspase pathway. Moreover, ~~at a~~ low dose of cordycepin interferes with the mRNA production and protein assembly, resulting in the uncontrolled growth and division of the cells are inhibited, whereas at high doses it can stop cells sticking together by direct impact on the production of proteins (Patel & Goyal, 2012; Silva et al., 2012).

Grifolin is a potential anticancer agent isolated from the fruiting bodies of *Albatrellus confluens*. It induces an apoptosis of some cancer cell lines *in vitro* by activating the caspase-8, -9, and -3, decreasing the Bcl-2 level, and increasing the Bax level. Grifolin can inhibits the ERK ½ and the ERK5 pathway which involved in the inhibition and significantly cause cell-cycle arrest in G1 phase and up-regulation of death-associated protein kinase 1 (DAPK1) via the p53-DAPK1 pathway. Furthermore, grifolin possess many other pharmacological effects e.g. antimicrobial activity, antioxidant activity, and cholesterol lowering activity (Patel & Goyal, 2012; Silva et al., 2012).

Ergosterol (ergosta-5,7,22-trien-3β-ol), the pro-vitamin D_2, is plenty in several mushrooms such as *Agaricus subrufescens, Agaricus brasiliensis, Grifola frondosa, Ganoderma lucidum, Lentinus edodes,* and *Cordyceps sinensis.* Mushroom ergosterol can be converted to vitamin D_2, tachysterol, and lumi-sterol when exposed to ultraviolet (UV) irradiation. It can inhibit the neovascularization, resulting in the growth of solid tumors to slowdown (Patel & Goyal, 2012; Silva et al., 2012). In addition, ergosterol derivative e.g. ergosterol perioxide (5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3β-ol), and (22*E*)-ergosta-7,22-dien-5α-hydroxy-3,6-dione, can be reduce the growth of prostate cancer cells by triggering an apoptotic process (Silva et al., 2012).

Besides, numerous low molecular weight compounds obtained from *Ganoderma lucidum* showed an anticancer activity against some cancer cell lines such as the ganoderic alcohols *lucidumol A ((24S)-24, 25-dihydroxylanost-8-ene-3, 7-dione)* and *lucidumol B (β, (24S)-lanosta-7, 9(11)-diene-3β, 24, 25-triol), ganodermanondiol, ganoderiol F, ganodermanontriol, ganoderic acids A, H, T, W, X* and *Y, lanostane-type triterpenes,* and *germanium* (Ferreira et al., 2010; Silva et al., 2012).

2.2 High molecular weight compounds (HMW)

Several mushrooms contain biologically active compounds in their fruit bodies, mycelium, or culture broth. Most of the high molecular weight compounds are mainly polysaccharides (homo- and heteroglucans, and glycans), polysaccharide-protein complex (glycoproteins, glycopeptides and proteoglycans), proteins, and RNA-protein complex. These are generated through primary metabolism which essential for their growth (Ferreira et al., 2010; Silva et al., 2012).

Polysaccharides and polysaccharides conjugates are the major compounds that have been investigated for anticancer and immunomodulating properties. Some of these are accepted for the clinical treatment of cancer patients in some countries e.g. the β-glucans, *'Lentinan'* from the fruiting bodies of Shiitake (*Lentinus edodes*), and *'Schizophyllan' (Sonifilan, SPG)* from the culture fluid of Suehirotake (*Schizophyllum commune*), and the protein bound polysaccharide *'Krestin' (PSK)* from the cultured mycelium of Kawaratake (*Trametes versicolor*). They showed significant anticancer efficacy against several human cancers (Ferreira et al., 2010; Silva et al., 2012; Zong et al., 2012).

Glycoproteins compose of a proteins core that is covalently attached to glucan chains through O- or N-glycosylation. Several glycoproteins showed an immunostimulatory activity involving with anticancer properties such as α-(1→4)-Glucan-β-(1→6)-glucan-protein complex derived from *Agaricus subrufescens* which inhibited the cancer growth through the host-mediated mechanism, ATOM and the mannan-protein complex (AB-FP) from *Agaricus blazei* exhibited significant anticancer activity in animal studied. Polysaccharide-protein complex (PSPC) isolated from the culture filtrates of *Tricholoma lobayense* could restore and increase phagocytic function of macrophages of the tumor-bearing mice (Ferreira et al., 2010; Silva et al., 2012).

Like glycoproteins, glycopeptides are protein-bound polysaccharides but with a small chain of amino acids such as Krestin (PSK) and PSP which can be prepared from *Trametes versicolor* strains Cov-1 and CM-101. They showed the

cytotoxicity and immunostimulating properties against several cancer cells. Other compound like peptide-polysaccharide complex from *Lentinus edodes* ~~is~~ also showed anticancer properties (Ferreira et al., 2010; Silva et al., 2012; Zong et al., 2012).

Proteoglycans are the compounds that consist of a core protein with one or more covalently attached glycosaminoglycan chain(s) and heavily glycosylated. For examples, GLIS from *Ganoderma lucidum* functioned as a B-cell stimulating factor and acid proteoglycans (PL) from *Phellinus linteus* could stimulate hormonal and cell-mediated immune functions, suppressing cancer growth, and metastasis, and also directly inhibited the adhesion of cancer cell (Ferreira et al., 2010; Silva et al., 2012).

Besides polysaccharides, several proteins isolated from mushrooms showed the anticancer activity. Lectins were found in many species of mushrooms, including *Agaricus bisporus, Pleurotus ostreatus, Tricholoma mongolicum, agaricus subrufescens, Russula lepida, Pholiota adiposa,* and *Grifola frondosa.* They showed anti-proliferative and anticancer activities against several cancer cell lines (Silva et al., 2012). The fungal immunomodulatory protein namely *Ling Zhi-8 (LZ-8),* isolated from mycelia of *Ganoderma lucidum* could activates T lymphocytes via cytokine regulation of intregrin expression and murine macrophages (Ferreira et al., 2010; Silva et al., 2012).

Mushrooms ~~is~~ are increasingly becoming good sources of biologically and physiologically active compounds, especially anticancer substances. There is increasing studies about natural active compounds from numerous medicinal mushrooms. Thus, it may be possible for therapeutic use of medicinal mushrooms as well as use as a diet for supporting good health.

3. Mushroom polysaccharides as the anticancer agents.

Polysaccharides are the most powerful mushroom-derived substances with anticancer and immunostimulating activities (M. Zhang et al., 2007). Mushroom polysaccharides are localized in the intermediate layer of cell wall, adjacent to plasma membrane. There are two major types of polysaccharides of fungal cell wall, (1) fibrillar of chitin or cellulose with the function of to the maintain the rigidity and shape of the cell, and (2) a matrix-like β-glucan, α-glucan and glycoproteins (Mantovani et al., 2008; M. Zhang et al., 2007). Polysaccharides are polymers of monosaccharides linked to each other by glycosidic bond (Daba & Ezeronye, 2003). The natural anticancer polysaccharides include acidic and neutral polysaccharides with different types of glycosidic linkages, while some are very complex molecules because of the monosaccharide units in polysaccharides can interconnect at several points to form a linear or branched structure, or bound to protein, or peptide residues (Daba & Ezeronye, 2003; M. Zhang et al., 2007). This structural variability gives the flexibility to the exact regulatory mechanisms of various cell-cell interactions in higher organisms (Daba & Ezeronye, 2003).

The mushroom polysaccharides with anticancer activity are generally known as glucans which composed of glucose subunits linked by different glycosidic bonds, such as β-(1-3), (1-6) and α-(1-3) glycosidic linkage (Daba & Ezeronye, 2003; Silva et al., 2012). β-glucans, a polymer of D-glucose that linked together with β-glycosidic bonds, are the strong anticancer polysaccharides (Mantovani et al., 2008;

Silva et al., 2012). They showed an effective anticancer and biological activities the with degree of branching is around 0.2 – 0.33, the molecular weight is about 100 – 200 kDa forming a triple-helix structure (Mantovani et al., 2008). Their conformational complexity can be changed in different aqueous solution thus resulting in changes their immune functions (Silva et al., 2012).

The differences in anticancer or immunostimulating activity of mushroom polysaccharides can be correlated with ability to solubilize in water, monosaccharide composition, molecular weight (MW), degree of branching (DB), configuration, position of glycosidic linkages, sequence of monosaccharides, chain conformation, the number and location of appended non-carbohydrate groups, as well as the distribution and length of side chains which provide for stabilization of complex tertiary structures (Daba & Ezeronye, 2003). The high degree of structural complexity is associated with high anticancer and immunomodulatory effects such as polysaccharides with β-(1-3) glucan backbone with additional β-(1-6)-branched showed better anticancer activity than β-glucans with only (1-6) glycosidic linkages. Polysaccharides bound to proteins or peptides display the greater anticancer effects than the free glucans. The high molecular weight glucans displayed more effect against cancers than low molecular weight glucans (Daba & Ezeronye, 2003). However, most polysaccharides are classified as nonspecific bioactive identities because their certain mode of actions were unclear (M. Zhang et al., 2007).

Mushroom-derived polysaccharides have been used for supporting good health for centuries as well as in studies as potential anticancer agents. Many edible mushrooms have been reported to have anticancer properties based on their polysaccharides contents (Table 1).

Table 1 Polysaccharides with anticancer activity from mushroom.

Source	Polysaccharides	Cancer models	Effects	Reference
Auricularia auricular-judae	Water soluble β-D-glucan (AAG)	Sacroma 180 cancer cell line Sacroma 180-bearing mice	Inhibitory effects on cancer cell growth. Induce the cancer cell apoptosis by up-regulation of Bax and down-regulation of Bcl-2 expression.	Ma *et. al.* (2010)
Cordyceps sphecocephala	Polysaccharide-peptide complexes (PPC) named as Fr-1, Fr-II, Fr-III.	HepG2 and SK-N-SH cancer cell lines.	Inhibitory effects on cancer cells by induced apoptosis cells *via* caspase-3 activation and modulation of Bcl-2 and Bax protein.	Young *et. al.* (2008)
Elfvingia applanata	Hot water soluble fraction from fruiting bodies (Fr. HW).	Sacroma 180-bearing mice	Increase the life span of in mice (45.2 %) when treated with Fr. HW previously inoculated with cancer cells	Shim *et. al.* (2012)
Pleurotus tuber-regium	Non-starch polysaccharides (NSPs)	HL-60 cell lines	Anti-proliferative effects and induced apoptosis on HL-60 cells.	Wong *et. al.* (2007)
	water-soluble carboxymethylated β-glucan (CMPTR)	MCF-7 cancer cell line	Inhibitory effect on proliferation of MFC-7 by arresting the G_1 phase and apoptosis induction.	Zhang *et. al.* (2006)

Table 1 Polysaccharides with anticancer activity from mushroom (Cont.).

Source	Polysaccharides	Cancer models	Effects	Reference
Lentinus edodes	Polysaccharide L-II	Sacroma 180-bearing mice	Restore the immunity of mice which was depressed by cancer cells.	Zheng *et. al.* (2005)
			Significant increase of relative thymus and spleen, and phagocytosis ability of macrophage.	
	Water extracts from fruiting bodies	MCF-7 cancer cell lines	Inhibitory effect against MCF-7 cells proliferation on dose-dependent manner.	Israilides *et. al.* (2008)
	L·E·M	Human colon and breast cancer cells implanted in nude mice.	Inhibitory effect on cancer proliferation *via* a reduction in NK cytotoxicity through the suppression of splenic-SNA.	Shen *et. al.* (2009)
	LT1	Solid tumor Sacroma 180 implanted in BALB/c mice	Inhibitory effect against Sacroma 180 solid tumor grown in BALB/c mice	Yu *et. al.* (2010)

Table 1 Polysaccharides with anticancer activity from mushroom (Cont.).

Source	Polysaccharides	Cancer models	Effects	Reference
Lentinus edodes	LEM	Liver cancer-bearing mice	Inhibitory effect on cancer cell and enhance spleen and thymus index.	Fu *et. al.* (2011)
			Inhibitory effect on expression of caspase-3.	
	LEP	HepG2 cancer cell lines	Directly killed the HepG2 cells by direct induce apoptosis via caspase-3 and -8 death receptor pathways.	Yukawa *et. al.* (2012)
	WPLE-N-1, WPLE-N-2 and WPLE-N-3	HCT-116, HT-29 and Sacroma 180 cell lines.	Inhibitory effects against the proliferation of cancer cells in a dose-dependent manner.	Jeff *et. al.* (2013)
Lentinus polychrorus Lév		MCF-7 cell line	Cytotoxic effects against cancer cell line.	Thetsrimuang *et.al.* (2011)

Currently, there are several important anticancer polysaccharide agents isolated from medicinal mushrooms which are described below.

3.1 Lentinan

Lentinan is a well-known anticancer polysaccharide agents isolated from *Lentinus* (*Lentinula*) *edodes*, which is commonly known as the Shiitake mushroom (Mantovani et al., 2008; Silva et al., 2012; Y. Zhang, Li, Wang, Zhang, & Cheung, 2011; Zong et al., 2012). Usually, the extraction of lentinan can be classified into two main types: (1) a solvent extraction method using hot water, alkaline solution and polyethylene glycol; (2) an ultrasonic or ultrahigh pressure extraction (Y. Zhang et al., 2011). Lentinan compose of five β-(1→3)-D-glucan linear residues and two β-(1→6)-D-glucopyranoside side branches (Figure 1), with a triple-helical structure and high molecular weight of 5-15 × 10^5 Da (Mantovani et al., 2008; Silva et al., 2012; Y. Zhang et al., 2011; Zong et al., 2012). The anticancer activity in triple-helical lentinan was close to the reference drug in animal model experiments. But, the activity was decreased when the triple-helical structure transformed to single-helical by alkaline solution, or random coil by DMSO (Silva et al., 2012; Y. Zhang et al., 2011).

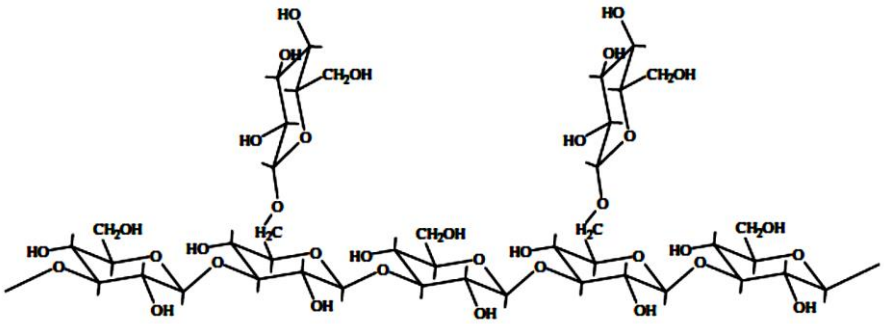

Figure 1 The chemical structure of lentinan (Y. Zhang et al., 2011)

Lentinan was first reported by Chihara *et.al.* (1970) to inhibiting the growth of sarcoma 180 cancer cells which transplanted into CD-1/ICD mice. It could produce over 90% reduction in cancer size (Silva et al., 2012; M. Zhang et al., 2007; Y. Zhang et al., 2011). It also exhibited the immunostimulatory effects against human immunity involving the activation of numerous immune cells and modulating the release of cell signal messengers such as cytokines and chemical messengers. Clinical studies indicated that lentinan could also prolong life in patients with gastric, ovarian, or colorectal cancer (Kidd, 2000; Silva et al., 2012).

The use of lentinan as adjuvants such as S-1/paclitaxel/lentinan, S-1/CDDP/lentinan, S-1/lentinan, lentinan/DCV vaccine, lentinan/TACE/RFA and superfine dispersed lentinan (SLD), showed to prolong the administration period and decrease the incidence rates of adverse chemotherapy effects when use in combination chemotherapies for gastric cancer, pancreatic cancer, colorectal cancer and hepatocellular carcinoma. So, they could prolong the survival time of cancer patients (Zong et al., 2012).

Lentinan also work best in combination with other cancer treatments such as chemotherapy and surgery. It could prevent the cancer recurrence and metastatic after surgical treatment to cancer patients when was combined with IL-2 in pre- and post-operative therapy. When the gastric cancer patients were treated with lentinan and chemotherapy, the survival of patients were significant increased compared with those treated with chemotherapy alone (Y. Zhang et al., 2011)

3.2 Schizophyllan

Schizophyllan, sizofiran or SPG is a mushroom-derived polysaccharide with immunomodulating and anticancer activity. It can be isolated from *Schizophyllum commune*. SPG repeating unit composes of three β-(1→3)-D-glucopyranose with a side branches of a single β-(1→6)-D-glucopyranoside shown in Figure 2. It showed a reversible coiled-helix transition which creating a very strong triple-helical structure in water, with a molecular weight of approximately 4.5 x 10^5 Da in neutral aqoues solutions (Mantovani et al., 2008; Silva et al., 2012; Y. Zhang, Kong, Fang, Nishinari, & Phillips, 2013).

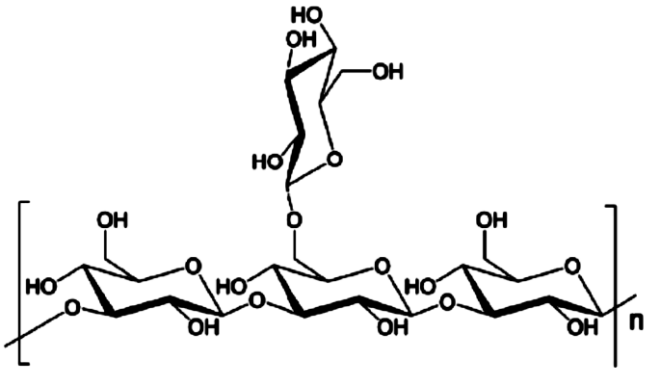

Figure 2 The chemical structure of schizophyllan (Y. Zhang et al., 2013).

Schizophyllan indicated the anticancer activity against several carcinomas and sarcoma cell lines. The first anticancer activity of SPG was reported by Komatsu et al. (1964), an aqueous solution of SPG showed a host-mediated anticancer activity against Sarcoma-180. In addition, it could increase cellular immunity by restoring suppressed killer-cell activity to normal levels in cancer bearing-mice (Y. Zhang et al., 2013). Besides, the folate-conjugated schizophyllan played as a non-cytotoxic cancer targeting antisense carrier, it showed the specific affinity toward folate binding proteins which mediated effective antisense activity in cancer cells (Silva et al., 2012).

Furthermore, when SPG was used in combination with surgery, radiotherapy and chemotherapy (fluorouracil), it prolonged the survival time of patients when compared to patient who did not receive SPG. SPG also significantly prolonged the survival time and time to recurrence in stage II cervical cancer patients when was used in combination with radiotherapy. It showed the increased recovery rates in treatment of patients with head and neck cancer when compared to a control group (Silva et al., 2012; Y. Zhang et al., 2013).

3.3 Krestin (PSK) and PSP

Krestin (PSK) and PSP are the best known commercial protein-bound β-glucan which can be prepared from *Trametes versicolor* (Ferreira et al., 2010; Silva et al., 2012; Zong et al., 2012). PSK is brownish powder derived from the extraction with hot water and precipitation with saturated ammonium sulfate. An average molecular weight of PSK is 9.4 x 10^4 Da. It consists of β-(1→4)-glucan with β-(1→6)- and β-(1→3)-glucopyranosidic side chains (Figure 3), and protein residues were to 25 – 38%; (Tsukagoshi et al., 1984; Zong et al., 2012). The protein portions of PSK consist ~~of~~ predominantly of acidic amino acids such as aspartic acid and glutamic acids, and neutral amino acids such as valine and leucine with basic amino acids including lysine and arginine (Tsukagoshi et al., 1984). Whereas, PSP is a light or dark brown powder extracted with hot water and alcoholic precipitation (Cheng & Leung, 2008). The polysaccharide chains of PSP differ from PSK, consisting of mainly 1-4, 1-2, and 1-3 glucose linkages (Figure 4) and their polypeptides are enriched with aspartic and glutamic acid (Kidd, 2000).

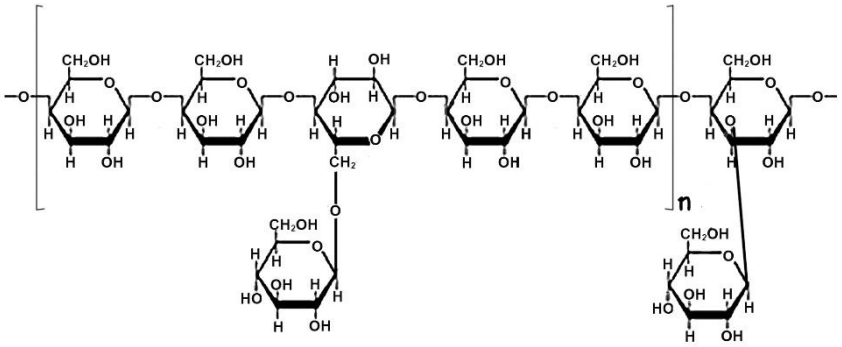

Figure 3 Typical structures of polysaccharide portions of polysaccharide Krestin (PSK; Tsukagoshi et al., 1984).

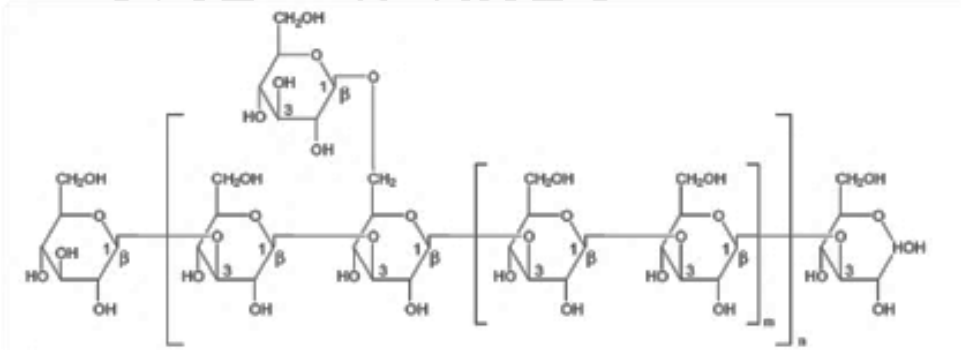

Figure 4 Typical partial structures of polysaccharide portions of the polysaccharide peptide (PSP; Cheng & Leung, 2008).

PSK could inhibit the adhesion, invasion, motility, metastatic and proliferation of cancer cells in various cancer cells *via* the arrest of cell cycle and the apoptosis induction (Silva et al., 2012; Zong et al., 2012). PSK inhibited the cancer

and increased the survival in dimethylbenzanthracene (DMBA)-induces mammary carcinoma inoculated subcutaneously into Fischer rats (Tsukagoshi et al., 1984). PSK also displayed some of the immunoregulation effects such as the prevention of the circulating T cells apoptosis programs and the decrease of the peripheral neuropathy and bone marrow suppression induced by chemotherapy (Zong et al., 2012). PSK was reported in clinical use of cancer patients as non-specific immunostimulant in Japan and used for the treatment of head and neck, upper gastro-intestinal, colorectal, lung and breast cancers (Ferreira et al., 2010; Silva et al., 2012; Zong et al., 2012). In addition, PSK can also improve long-term prognosis, reduce the risk of recurrence, increase the survival rates in cancer patients and enhance the cytotoxicity when uses with chemotherapeutic drugs such as fluoropyrimidines, UFT, S-1, and FOLFOX4 (Zong et al., 2012).

PSP can inhibit the proliferation of cancer cells *via* the apoptosis induction and cell cycle arrest (Zong et al., 2012). It was used in clinical trials by combining with therapeutic drugs such as doxorubicin, etoposide, camptothecin and cyclophosphamide against human cancer cells (Silva et al., 2012; Zong et al., 2012). In addition, it showed a chemopreventive effect on prostate cancer (Zong et al., 2012).

3.4 Maitake D-fraction and MD-fraction

Grifola frondosa or Maitake mushroom was used as a food in Japan for a long time age because ~~of~~ many people believe that it possesses pharmacological properties. The mixed β-D-glucan fraction derived from both the mycelia and fruiting bodies of *Grifola frondosa*. containing both β-(1→6)-D-glucan main chain with β-(1→4)-glucan branches, and β-(1→3)-D-glucan main chains with β-(1→6) branches, with a molecular weight of approximately 1 x 10^6 Da (Silva et al., 2012; Zong et al., 2012). The MD-fraction was derived from further purification of the D-fraction and it showed the anticancer activity like D-fraction (Zong et al., 2012).

The Maitake D-fraction plays a role in mice apoptosis inducer and immune enhancer. In breast cancer cells, this fraction shows apoptotic effect by upregulation of BAK-1 gene activation and the involvement of cytochrome C (Silva et al., 2012; Zong et al., 2012). The anti-cancer activities of D-Fraction relates to its control of the balance between T lymphocyte subsets Th-1 and Th-2 and the activation of helper T-cells, resulting in enhanced cellular immunity (Silva et al., 2012). The MD-fraction showed the synergistic effect which can triggers DNA-PK activation and induces cancer cell arrest at the G1 cell cycle checkpoint when used with IFN-α2b. It can also enhance the anticancer and anti-metastatic activity of cisplatin the chemotherapy drugs and nephrotoxicity in mice (Zong et al., 2012). They have been proposed for phase I/II clinical trials in the United States and Japan. Thus, it can be used as immunotherapeutic agent for cancer patients (Silva et al., 2012; Zong et al., 2012).

4. Hypothesized mechanism of mushroom polysaccharides on cancer cells.

Mushroom polysaccharides play a role in both direct and indirect affect against cancer cells. They can direct anticancer activity by inducing the apoptosis of cancer cells. On the other hand, mushroom polysaccharides are recognized as non-self molecules which indirectly affect against cancer cells by influencing the cancer

cells through the activation of the immune response and exert a nonspecific action of the host organism (Silva et al., 2012; M. Zhang et al., 2007).

The human immunity consists of two major systems, including the innate immune system and the adaptive or acquired immune system. Innate immune system is a non-specific immune system and also known as the first line of defense which does not confer long-lasting or protective immunity to the host. They are capable in responding to many but not all structurally related antigens. Whereas, the adaptive immune system is an immunological memory system to the specific pathogens, leading to a strong and specific immune response. Both immunity systems include humoral immunity components and cell-mediated immunity component (Silva et al., 2012). The mechanism of mushroom polysaccharides against cancer cells is still unclear and there is little evidence from human trials. Nevertheless, these mechanism is composed of a many steps of reaction which involving membrane receptors for modulating the immune systems (Silva et al., 2012).

4.1 Innate immunity system

Innate immunity system, polysaccharides play a role as antigen-presenting cells (APCs) and stimulate macrophage to produce cytokines and activate the other immune system (Moradali et al., 2007). Hong et al. (2004) reported that mushroom polysaccharide like β-glucans can be rapidly enter to small intestinal and were captured by macrophages after oral administration. Then, they were portioned to smaller sized fragments which carried to the bone marrow and endothelial reticular system. These fragments were hypothesized to be released by macrophages and taken up by the circulating granulocytes, monocytes and dendritic cells. The activated macrophages with polysaccharide fragments could attack the dead cells and intracellular pathogens. In addition, these macrophages also create many cytokines, which are cytotoxic to cancer cells (Moradali et al., 2007; Silva et al., 2012).

β-D-glucans are capable of inducing the maturation of bone marrow-derived dendritic cells (DCs) and increasing the membrane molecules in them, resulting in stimulation of cell-mediated immunity and regulation of anticancer effector cells such as NK cells and NK-T cells (Moradali et al., 2007). The NK-cells will secret the chemical substances that kill the cancer cells by bursting cell membrane while the neutrophils will destroy cancer cells by cell mediated phagocytosis (Moradali et al., 2007; Silva et al., 2012). Furthermore, some β-D-glucans (e.g. lentinan) can activate a complement system and inhibit cancer growth *via* alternative pathway (Moradali et al., 2007).

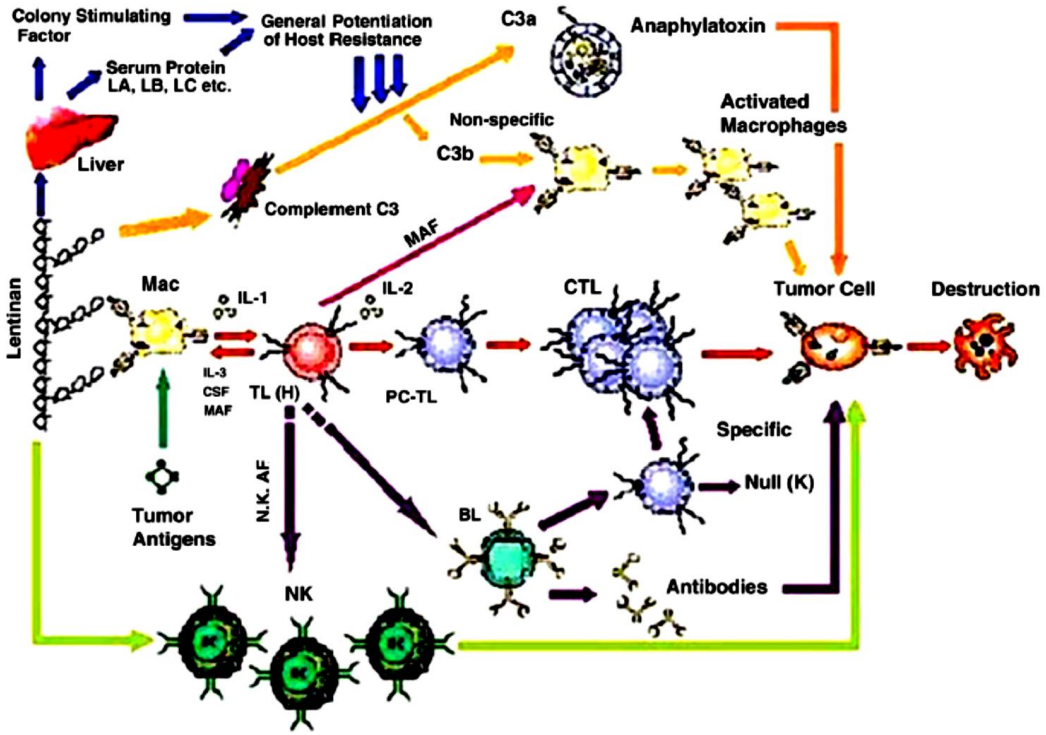

Figure 5 Mechanisms of antitumor activity of lentinan as a β-D-glucan (Moradali et al., 2007). Mac, macrophages; TL(H), T-lymphocyte (helper);NK, natural killer cells; IL-1, -2 and -13, interleukin-1, -2, -13; CSF, colony-stimulating factor; MAF, macrophage-activating factor; PC-TL, precytolytic. T lymphocyte; CTL, cytolytic (cytotoxic) T lymphocyte; BL, B lymphocyte.

4.2 Adaptive immunity system

The mushroom polysaccharides act as pathogen-associated molecular patterns on cell membrane receptors which can detect the strange compounds or non-self structures in the body, and stimulate the immune function. A number of receptors was identified as a β-glucans receptor that mediates the biological response in human such as dectin-1, complement receptor-3 (CR-3), scavenger receptors, lactosylceramide and toll-like receptors (TLR; Silva et al., 2012; Zong et al., 2012).

Toll-like receptors (TLR), a group of transmembrane protein receptors, respond to microbes. There are 11 members of this groups. Most studies indicated that elements from mushroom polysaccharides, such as krestin could be act as ligands binding to toll like receptors 4 and led to induction of TNF-α and interleukin 6 (IL-6) production (Silva et al., 2012).

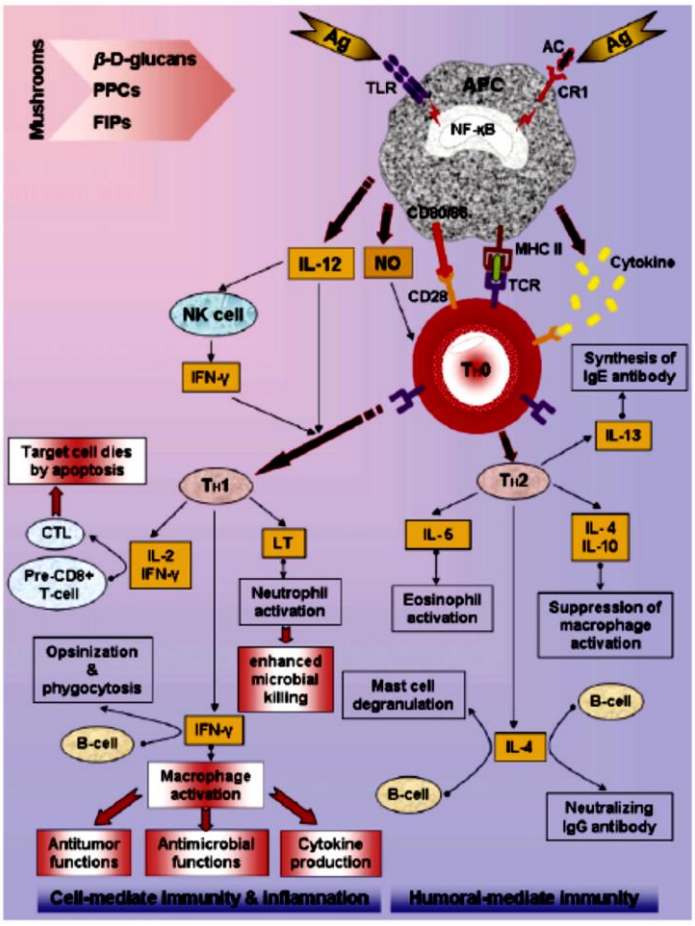

Figure 6 Schematic representation of the affecting of immunomodulatory macrofungi metabolites on the adaptive immune system leading to activation of anticancer pathways (Moradali et al., 2007).

Dectin-1, a type II transmembrane protein receptor, can binds with β-(1→3) and β-(1→6)-glucans and activates several signaling pathways to promote the innate immune responses in macrophages and induce the reactive oxygen species and inflammatory cytokines production (Silva et al., 2012). Dectin-1 in combination with a β-glucan-enriched zymosan derivertive enhances TLR-2-mediated cell activation. This cooperation is necessary for the activation of NF-κB and the induction of TNF-α (Moradali et al., 2007).

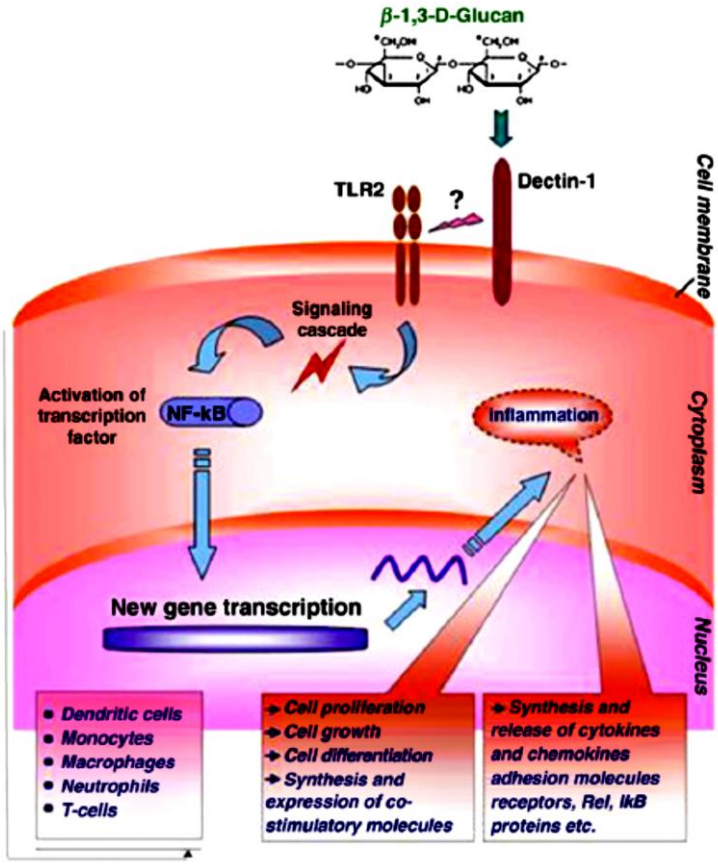

Figure 7 Schematic representation of the β-glucans recognition by certain receptors on the cell surface and activation of NF-κB leading to transcription of many genes in both innate and adaptive immune responses (Moradali et al., 2007).

Apart from inhibition of cancer cells activity, polysaccharides from medicinal mushrooms also prevent the carcinoma in animal study. The mice were treated with medicinal mushroom polysaccharides from *Flammulina velutipes* and *Agaricus blazi* in diet, before they were inoculated with cancer cells. The experiment showed that a number of mice that developed cancers was decreased when compared with the control group (treated with an ordinary diet). Thus, this study displays the cancer-preventive activity of the polysaccharides (M. Zhang et al., 2007).

CHAPTER III

MATERIALS AND METHODS

1. Mushrooms collection and isolation.

Two mushrooms, *Lentinus* sp. strain EB1001 and *Lentinus* sp. strain EB1101, were collected from the Nakhon Pathom province. *Lentinus* sp. strain WCR1104 and *Lentinus* sp. strain WCR1201 were collected from the Chaloem Phrakiat *Thai* Prachan National park, Ratchaburi in 2011 and Tha Maka Operation and Maintenance Project 13, Tha Muang district, Kanchanaburi in 2012, respectively. *Lentinus edodes* AMC#3 was obtained from the Biotechnology Research and Development Office, Department of Agriculture.

The mushroom specimens were isolated at the Department of Microbiology, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand by using the surface sterile technique. Briefly, the outside surface of mushroom fruiting body was cleaned with brush and wiped with 70% ethanol. Then, the fruiting body was cut to small pieces by a new sterile razor blade. The mushroom pieces were treated with 5% Chlorox for 10 seconds and washed with sterile water. After that, the mushroom pieces were treated with 70% ethanol for 10 seconds and washed with sterile water with 50 µg/mL ampicillin and streptomycin. Finally, the mushroom pieces were washed with sterile water, again and dried on sterile filter paper for 10 – 15 minutes. The mushroom pieces were transferred to potatoes dextrose agar (PDA) plate supplement with 50µg/mL ampicillin and streptomycin and incubated at room temperature until the mycelium grown from mushroom pieces. The mycelium was transferred and maintained on PDA at 4°C.

2. Morphological characteristic identification

The identification of the mushrooms was performed using macro- and micro-characteristics of fresh specimens using taxonomic keys according to Pegler (1983). All the mushroom specimens were preserved in FAA reagent.

3. Genomic DNA extraction (Chukeatirote et al., 2012)

The mushroom mycelium was cultured in malt extract broth (MEB) at suitable temperature and appropriate time under shaking (150 rpm). Then, it was collected by filtration and rinsed three times with sterile water. The mycelium was frozen in liquid nitrogen and ground to a fine powder with ceramic mortar and pestle. 50 mg of mycelium powders was transferred to new Eppendorf tube. 500 µL of the extraction buffer was added and mixed by vortex mixer. 300 µL of phenol and 150 µL of chloroform was added and mixed by inverting. Then, the sample was centrifuged at 13,000 rpm for 20 minutes. The supernatant was transferred to a new eppendorf tube. 5 µL of 10 mg/mL RNaseA was added to tube and incubated at 37°C for 10 minutes. DNA was precipitated from aqueous phase by adding 250 µL isopropanol and incubating at -20 °C overnight. The tube was centrifuged at 13,000 rpm for 15 minutes at 4 °C. The supernatant was discarded. A 250 µL of ice-cold 70% ethanol

air-dried for 5-10 minutes. The DNA was dissolved in 50 µL of TE buffer. Then, DNA was stored at -20 °C.

The DNA concentration and purity was determined by absorbance value using a spectrophotometer set at 260 nm and 280 nm. The yield of DNA was calculated by following equation: A_{260} x dilution factor x 50 ng/µL. The DNA purity was calculated by A_{260}/A_{280} ratio. The quality of DNA was verified on 1% (w/v) agarose gel electrophoresis.

4. Amplification of the internal transcribe spacer (ITS) sequence

The ITS DNA fragment was amplified by the PCR technique using ITS1 (5'TCCGTAGGTGAACCTTGCCC3') and ITS4 (5'TCCTCCGCTTATTGATAT GC3') primers (White, Bruns, Lee, & Taylor, 1990)

The PCR reaction was carried out in a total volume of 50 µL containing 100 ng of DNA template, 1X PCR buffer with $(NH_4)_2SO_4$, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 36.5 µL of DEPC water, 2.5 units of *Taq* polymerase, 0.2 µM of ITS1 and ITS4 primer. Amplifications was performed in Esco GeneAmp PCR system programmed for 1 cycle of initial denaturation at 94°C for 5 minutes followed by 35 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for a minute and the final extension at 72 ^{O}C for 20 minutes. The PCR products was then stored at 4 °C. PCR products were analyzed on 1% (w/v) agarose gel electrophoresis.

5. Purification of PCR product by using gel extraction kit (Fermentas, USA)

The expected PCR product in agarose gel was excised using a clean razor blade and weighed in microcentrifuge tube. Three volumes of binding solution (1 g of gel slice equal to 1 mL) was added to the tube with gel slice and incubated at 55°C for 5 minutes or until the gel slice is completely dissolved. The tube was mixed by inversion every few minutes to facilitate the melting process. A volume of 10 µL of the resuspended Silica Powder Suspension was added to the DNA/Binding Buffer mixture and incubated at 55 °C for 5 minutes to allow for binding of the DNA to the silica matrix (the mixture was mixed every few minutes to keep the silica powder in suspension). The silica powder/DNA mixture was spun for 5 seconds to form a pellet. The supernatant solution was carefully removed from the pellet. 500 µL of ice cold washing buffer was added and mixed. Then, the mixture was spun for 5 seconds. The supernatant was discarded. Washing step was repeated three times. The pellet was air-dried for 10-15 min to avoid the presence residual ethanol in the purified DNA solution. The pellet was resuspended with the desired volume of sterile deionized water or TE and incubated the tube at 55°C for 5 minutes. The mixture was spun for 5 seconds and the supernatant was transferred into a new tube.

6. Ligation and transformation (Invitrogen, USA).

The ligation and transformation was performed by using pCR8/GW/TOPO TA cloning kit (Invitrogen, USA) according to manufacture instruction with some

modification. Briefly, 2 µL of fresh PCR product was taken to the 1.5 mL eppendorf tube. A volume of 0.5 µL of salt solution and 0.5 µL of TOPO vector was added. The mixture was mixed gently and incubated at room temperature at room temperature (22 – 23 °C) for 30 minutes. A volume of 25 µL of competent *E. coli* was added to the ligation reaction and mixed gently. The reaction was incubated on ice for 30 minutes. Then, the cells were heat-shocked at 42 ºC for 90 second without shaking and immediately transferred the tube to ice. A volume of 125 µL of room temperature S.O.C medium was added to the reaction. The tube was shaken (200 rpm) at 37 °C for 2 – 3 hours. A volume of 50 – 100 µL of the transformation reaction was spreaded on Luria-Bertani (LB) agar containing 100 µg/mL of spectinomycin and incubated at 37 °C overnight. Colony grown on LB agar plate with spectinomycin was checked for the ITS insert fragment by colony PCR technique.

Colony grown on LB agar plate with spectinomycin was picked with new sterile toothpick and transferred to LB broth containing 100 µg/mL spectinomycin. The transformant was grown at 37 °C under shaking condition (200 rpm) ~~for~~ overnight. The colony PCR reaction was carried out in a total volume of 20 µL containing 1.5 µL of overnight transformant culture broth, 1X PCR buffer with $(NH_4)_2SO_4$, 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 13.5 µL of DEPC water, 2.5 units of *Taq* polymerase, 0.2 µM of ITS1 and ITS4 primer. Amplifications was performed in Esco GeneAmp PCR system programmed for 1 cycle of initial denaturation at 94°C for 5 minutes followed by 35 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for a minute and the final extension at 72 °C for 20 minutes. The PCR products was then stored at 4 °C. PCR products was analyzed on 1% (w/v) agarose gel electrophoresis.

7. Extraction of plasmid (Thermo scientific, USA).

The extraction of plasmid was performed by using GeneJET Plasmid miniprep kit (Thermo scientific, USA) according to manufacture instruction. Briefly, the transformant cells was harvested by centrifugation at 6,000 rpm in eppendorf tube for 2 minutes at room temperature. The supernatant was removed, 250 µL of resuspension solution was added to the pellet cells and resuspended by vortex. 250 µL of lysis solution was added and mixed by inverting the tube 4-6 times. Then, 350 µL of neutralization solution was added and the tube was inverted 4-6 times. The mixture was centrifuged at 13,000 rpm for 5 minutes. The supernatant was transferred to the Thermo Scientific GeneJET Spin Column. After that, the column was centrifuged at 13,000 rpm for 1 minute and the flow-through was discard. 500 µL of washing solution was added to column and centrifuge at 13,000 rpm for 30 – 60 seconds. Flow-through was discarded and washed the column again. The empty column was centrifuged at 13,000 rpm for 1 minute. The column was placed to the new tube. Then, 50 µL of elution buffer was added to the column and incubated for 2 minutes. The column was centrifuged at 13,000 rpm for 2 minutes. The flow-through was collected and transferred to new tube.

8. DNA sequencing, comparison of Internal transcribe spacer (ITS) and phylogenetic tree analysis.

The ITS sequences were sequenced by First Base Laboratory, Malaysia. Sequencing result was analyzed by Sequence scanner version 1.0 and CAP3 Sequence Assembly Program. The taxa information and Genbank accession numbers in this work are listed in Table 2. Sequences for each strain were aligned using Clustal X (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997), and phylogenetic analysis of the aligned sequence was performed with neighbor-joining using the MEGA program version 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Gaps were presented as missing data. The strength of the internal branches of the resulting trees was assessed by bootstrap analysis with 1,000 replicates. Phylogenetic trees were figured in Treeview.

9. Mushrooms cultivation

Mushroom cultivation was performed in sterilized sawdust bag (90% sawdust, 3% wheat bran, 1% corn meal, 1% cassava powder, 3% gypsum, 1% cane sugar, 0.05% $(NH_4)_2SO_4$ and 0.05% $CaCO_3$ with 60% Rh). The sawdust bags were incubated at 33±2ºC, except for *L. edodes*, which was incubated at 25ºC. After the completion of mycelium running throughout the sawdust bag, the fruiting bodies of *L. sajor-caju, L. swartzii, L. squarrosulus*, and *L. velutinus* were induced by the removal of plastic bags, and the mushroom substrate was covered with casing soil. The humidity was controlled at 60-70% by watering every day. While the sawdust bags of *L. edodes* were removed from plastic bags and immersed in cooled water at 10ºC overnight, after that the substrates were kept at 60-70% Rh at 20 ºC.

10. Mycelial cultivation

The fungal mycelium was cultured on a Potato Dextrose Agar (PDA) plate at 33±2ºC for 7-15 days, except for *L. edodes*, which was incubated at 25 ºC for 20 days. Then, plugs of active growing mycelium (diameter 0.5 cm) were inoculated to flat-bottoms containing 25 mL of slightly modified Mushroom Complete Medium (Osman, Hassan, Khattab, Ahmed, & El-Henawy, 2009), composed of 1mg Thiamine-HCl; 10mg $FeSO_4.7H_2O$; 1.6mg $MnSO_4.H_2O$; 1mg $CuSO_4$; 1g KH_2PO_4; 0.5g $CaCl_2.2H_2O$; 0.5g $MgSO_4.7H_2O$; 2g $NaNO_3$; 5g yeast extract; 15g fructose; 40g soluble starch and 1L of distilled water, and cultured at 25-30 ºC for 10-30 days.

Table 2 Taxon information and GenBank accession numbers in molecular work.

Taxa	GenBank accession numbers (ITS)	Reference
Lentinus tigrinus strain VKGJ04	JQ428822	Unpublished
Lentinus tigrinus strain VKMK04	GQ849476	Unpublished
Lentinus squarrosulus strain VKGJ05	JQ428823	Unpublished
Lentinus squarrosulus strain 7-4-2	GU001951	Unpublished
Lentinus squarrosulus strain C2-7	JQ717334	Unpublished
Lentinus sajor-caju isolate TFB11739	GU207308	Grand, Hughes and Petersen (2011)
Lentinus sajor-caju isolate TFB11736	GU207309	Grand, Hughes and Petersen (2011)
Lentinus swartzii isolate TFB5206	GU207276	Grand, Hughes and Petersen (2011)
Lentinus swartzii isolate Tage Roland MO166	GU207278	Grand, Hughes and Petersen (2011)
Lentinus swartzii isolate Tage Roland MO194	GU207277	Grand, Hughes and Petersen (2011)
Lentinus velutinus	GQ849478	Unpublished
Lentinus edodes	JX205093	Unpublished
Lentinus edodes strain LEB1	HM561975	Unpublished
Lentinus edodes strain FFBS1 (LEB1)	JN234840	Avin, Bhassu, Shin and Sabaratnam (2012)
Lentinus squarrosulus strain WCR1201	KT956127	In this study
Lentinus sajor-caju strain EB1001	KT956122	In this study
Lentinus swartzii strain EB1101	KT956124	In this study
Lentinus sp. strain WCR1104	KT956126	In this study
Volvariella volvacae strain V11	KC894924	Unpublished

11. Crude polysaccharide extraction

The fruiting bodies and mycelium were taken and cut into small pieces and oven-dried at 60 ºC. Then, they were blended with blender into fine particles.

For aqueous extraction, the fine particles of mushroom were de-fatted with 95% ethanol at room temperature overnight under shaking condition at 180 revolutions per minute (rpm). Then, the ethanol part was discarded. The mushroom biomass was boiled with 20 volumes of water for 3 h (three times). After centrifugation at 8,000 rpm for 30 min, the supernatants were concentrated with boiling and deproteinized with 1 volume of Sevag's reagent (5:1 $CHCl_3$:n-BuOH) for 3 times. Then the aqueous solution was extracted for crude polysaccharides by adding cold ethanol (final concentration was to 80%EtOH) and kept at 4 ºC overnight. The supernatants were discarded after centrifugation at 8,000 rpm for 30 min. The

precipitate was washed with cold absolute ethanol, centrifuged, and washed again. The pellet was collected and air-dried to give the crude aqueous extracts. The crude aqueous extracts were stored at -20 ºC for further use.

For ethanol extraction, they were soaked in 10 volumes of 95% ethanol solution. The sample was extracted by stirring at 180 rpm at room temperature overnight. The mixture was filtered through Whatman's filter paper no 2. The residue was then extracted with two additional 10 volumes of ethanol as described above. The ethanolic extracts were combined and concentrated in a rotary evaporator at 50 °C. The extract was collected, air-dried and stored at -20 °C for further use.

12. Polysaccharide purification

12.1 Re-dissolve and re-precipitation

Crude aqueous extract was re-dissolved in sterile water. After that, the equal volume of cold 95% ethanol was added to aqueous solution and kept at 4 °C ~~for~~ overnight. The precipitate was separated by centrifugation at 8,000 rpm for 30 minutes at 4 °C and washed with cold absolute ethanol. Then, the mixture was centrifuged at 8,000 rpm for 30 minutes at 4 °C. The precipitate was dried which gave a *'Fraction E1'*. After removal of Fraction E1, the aqueous solution was further added 3 volumes of cold 95% ethanol and kept for overnight at 4 °C. The second precipitate was obtained and collected by centrifugation at 8,000 rpm for 30 minutes at 4 °C followed by washing step with cold absolute ethanol. The second precipitate namely *'Fraction E4'* was dried. The aqueous solution was further dried by a rotary evaporator at 40 – 50 °C to give *'Fraction R'*. All fractions were tested for cytotoxicity and anticancer activity.

12.2 Purification of crude polysaccharide by anion exchange chromatography.

The fraction with anticancer activity was further purified on anion exchange chromatography by using DEAE FF column (5 mL). Briefly, the polysaccharide extract was re-dissolved in sterile water at concentration 5 mg/mL and filtered through 0.20 µm cellulose acetate membrane filter. 150 mg of extract was applied to DEAE FF column (5 mL) equilibrated with water by using P-50 pump. The extract was step eluted with water, 0.05M NaCl, 0.1M NaCl, 0.2M NaCl and 0.5M NaCl solution at flow rate 1 mL/min. The fractions (2 mL each) was collected and monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay. A fraction with the same peak was collected and dialyzed through dialysis bag (MWCO: 6-8,000) against distilled water for 5 days to remove salt and small molecules. The material retained inside the dialysis bag was then precipitated with 4 volumes of cold 95% ethanol at 4 °C for overnight. The fraction precipitates were collected by centrifugation at 8,000 rpm for 30 minutes at 4 °C and washed with cold absolute ethanol. The precipitates were dried at 60 °C for overnight. Each fraction was tested for cytotoxicity and anticancer activity.

12.3 Purification of crude polysaccharide by size exclusion chromatography

The fraction with the anticancer activity was further purified on size exclusion chromatography by using Sephadex G100 column (50 cm). Briefly, the polysaccharide extract was re-dissolved in sterile water at concentration 20 mg/mL and filtered through 0.20 µm cellulose acetate membrane filter. 1 mL of extract solution was applied to Sephadex G100 column equilibrated with 50 mM Na-phosphate buffer, pH 7.0 (with 0.15 M NaCl). The extract was eluted with 50 mM Na-phosphate buffer, pH 7.0 (with 0.15 M NaCl) at flow rate 0.1 mL/min. The fractions (5 mL each) was collected and monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay. A fraction with the same peak was collected and dialyze with water for 5 days. The obtained solution was freeze-dried. Each fraction was tested for cytotoxicity and anticancer activity.

13. *In vitro* anticancer activity and cytotoxicity assay

The L929 murine aneuploid fibrosarcoma cell line and LLC-MK2 monkey rhesus kidney cell line were used for the cytotoxicity test and the HeLa human epitheloid cervix carcinoma cell line and HepG2 human hepatocellular liver carcinoma cell line were used for *in vitro* anticancer activities test. The cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) and 50 µg/mL gentamycin. All cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. *In vitro* anticancer activity and cytotoxicity were evaluated using the MTT assay.

Briefly, $1.2x10^4$ cells were seeded in each well of 96-well plates and incubated at 37°C in a humidified atmosphere of 5% CO_2. After 24 h, fresh medium (100 µL) containing test sample was replaced to each well, followed by further incubation for 24 h. Then, the wells were replaced and incubated with fresh culture media containing MTT (0.5 mg/mL) for 4 h at 37 °C. Finally, the media were removed and DMSO was added to the wells (100 µL/well), and absorbance was measured at 540 nm in a microtiter plate reader. The number of viable cells was determined from the absorbance. Assays were performed in triplet wells. Data were expressed as percent viability compared with control (mean±SD).

14. Anitioxidant activity assay (Prieto, 2012)

The antioxidant activity of polysaccharides was evaluated by DPPH radical scavenging assay according to Prieto (2012) with some modification. An aliquot of each 30 µL of sample and 0.2 mM DPPH radical (in methanol) were added to each well in 96-well plate and mixed. Ascorbic acid (1 – 200 µg) was used as standard. Then, the plate was kept in the dark for 30 minutes at room temperature. The absorbance (515 nm) was read with nanodrop. The percentage scavenging activity (%SA) on DPPH radicals was calculated by following expression:

$$\%SA = 100 - \left[100 \times \frac{(Abs\ \text{sample} + \text{DPPH}) - (Abs\ \text{sample blank})}{(Abs\ \text{DPPH})}\right]$$

15. Chemical properties of polysaccharide

15.1 Determination of total carbohydrate content

The carbohydrate content was determined with a phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1951). Briefly, 0.5 mL of crude extract solution was mixed with 0.5 mL of 5% phenol, followed immediately with 2.5 mL of concentrated sulphuric acid and shake well. After 10 min of shaking the contents in tubes, the reaction mixture was placed at 25-30°C for 20 min. The absorbance of the mixture was measured at 490 nm. The total carbohydrate content was calculated with D-glucose as standard (0-100µg/mL).

15.2 Determination of reducing sugar

The reducing sugar was determined by the DNS method (Miller, 1959). Briefly, 2 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added to a sample solution (1 mL). Then, the mixture was heated in boiling water for 15 min and cooled immediately. 1 mL of 40% (w/v) potassium sodium tartrate was added to the mixture and mixed. The volume adjusted to 10 mL with distilled water. The absorbance was measured at 550 nm, and the total reducing sugar was calculated with D-glucose as a standard (0-1,000 µg/mL).

15.3 Determination of total phenol content

The total phenol content of the crude extract was measured by the Folin–Ciocalteu colorimetric method, based on the procedure described by Singleton & Rossi (1965) and Thetsrimuang et al. (2011) with some modifications. Briefly, a sample (0.5 mL) was mixed with 0.5 mL of 1N Folin–Ciocalteu reagent. Three minutes later, 0.5 mL of 7% (w/v) Na_2CO_3 was added, and kept in the dark for 2 h; the absorbance of the mixture was read at 725 nm. The quantification was based on a standard curve of gallic acid (0-32 µg/mL). The total amount of phenol contents was expressed as gallic acid equivalent (mgGAE/g sample).

15.4 Determination of protein content

The total protein content was determined using the procedure described by Lowry et al. (1956) with some modification. Briefly, 6 mL of fresh alkaline copper reagent was immediately mixed with an aliquot of sample solution (1.2 mL). After 10 min, 0.3 mL of 1N Folin-Ciocalteu reagent was added and mixed well. The reaction was incubated for 30 min at room temperature, the absorbance was measured at 500 nm, and the protein content was calculated with bovine serum albumin (BSA) as standard protein (0-1,000 µg/mL).

16. Chemical structure elucidation

16.1 Determination of molecular weight

The molecular weight of polysaccharide was determined by gel permeationn chromatography. Standard dextrans cover a molecular weight range from 342 – 393,000 Dalton and the polysaccharide extract (4 mg/mL) were passed through a OHpak SB-804 HQ column (8.0 mm × 300 mm; Shodex, USA) with a water as mobile phase at flow rate 0.5 mL/min, 40 °C by using Shimadzu LC-

10ADvp HPLC pump (Shimadzu, Japan). The peak of polysaccharide was monitored spectrophotometerically with refractive index (RI) detector. The elution volumes of standard dextrans were plotted against the logarithms of respective their molecular weights, and the average molecular weight of polysaccharide was determined using the standard curve.

16.2 FT-IR

The polysaccharide extract (1 mg) was grounded to fine powder and analyzed with Spectrum 100 FT-IR Spectrometer (PerkinElmer, USA) for detecting functional groups.

16.3 Determination of monosaccharide

The monosaccharide contents were screened by using thin layer chromatography (TLC) technique according to Robyt (2000) with some modification. Briefly, the polysaccharide LV2 (4 mg) was hydrolyzed separately with 4 mL of 2M trifluoroacetic acid (TFA) in a sealed glass tube at 100 °C for 18 h. The excess acid was completely removed by co-distilled with methanol at 60 °C, and then the hydrolyzed products were dried. The hydrolysate polysaccharide and standard were re-dissolved in water, and spotted on TLC plate. The TLC plate was irrigated with 2 solvent systems such as n-butanol-acetone-water (4:3:1, v/v/v), and n-butanol-acetic acid-isopropanol-water (8:4:7:3) at 25 °C, respectively. The solvent was allowed to ascend to the top of the plate, and the plate was removed and placed to dryness between each solvent system. The monosaccharides were detected by dipping the TLC plate into a methanolic solution of 0.3%(w/v) *N*-(1-napthyl)ethylenediamine and 5%(v/v) sulfuric acid, followed by heating at 120 °C for 10 min.

CHAPTER IV

RESULTS

1. Mushroom identification

The morphology of four *Lentinus* spp. were studied and then they were identified according to Pegler (1938) as *L. sajor-caju* strain EB1001 (Fig. 8), *L. swartzii* strain EB1101 (Fig. 9), *L. squarrosulus* strain WCR1201 (Fig. 10), and *L. velutinus* strain WCR1104 (Fig. 11). The morphology of these mushrooms was descripted below:

Lentinus sajor-caju strain EB1001

Pileus 3 – 8(-15) cm diam., convex when young and becoming funnel-shape to depressed when old; surface dry, smooth and hard, sometime with a small brown squamules especially the center; variable in color: whitish at first and then cream to grayish-brown; margin inrolled at first and lobed when old; KOH color change to yellow when drop on the surface. *Lamellae* crowded, 0.5–4 mm broad, decurrent and whitish. *Stipe* central, excentric or lateral, short 0.8–1 × 1-4 cm, cylindric, solid, dry, firm and thick. *Annulus* present, skirtlike, attached toward stipe. *Spore print* white to cream; spores 3-4 × 6-7 µm, oblong to elliptical, hyaline, smooth and not amyloid. *Mycelium* fluffy like a cotton, whitish, very tough when old; 3-5 µm in widths, long, branched, thin cell walls with clamp connection and hyaline under microscope.

Collection site THAILAND, Nakhon Pathom Province, Muang District, Faculty of Science, Silpakorn university. It grows on hardwood logs.

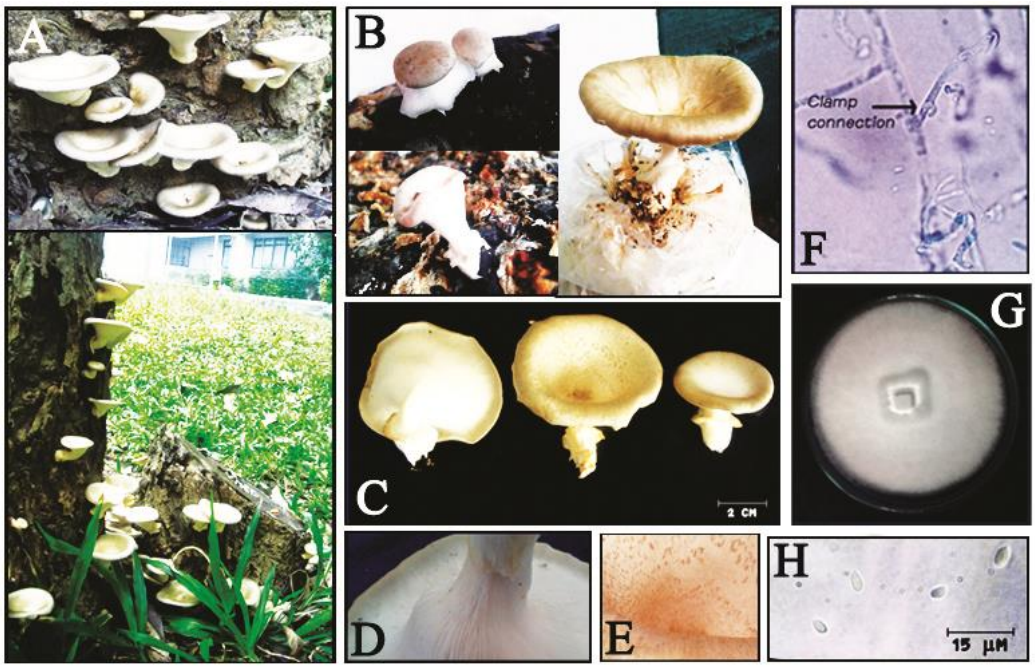

Figure 8 *Lentinus sajor-caju* strain EB1001; **(A)** habitat (on log), **(B)** fruiting bodies on sawdust bags at pinhead stage (top left), young stage (bottom left), and mature stage (right), **(C)** fruiting bodies, **(D)** decurrent gills, **(E)** cap with dark brown squamules, **(F)** clamp connection, **(G)** mycelium on PDA, and **(H)** spores.

Lentinus swartzii strain EB1101

Pileus 1.5 – 7 cm diam., convex to umbilicate or depressed, pale brown; surface covered with tawny brown fibrillous; margin decurved to inrolled; KOH color change to yellow when drop on the surface. *Lamellae* crowded, 1–3 mm broad, short decurrent and cream to pale brown. *Stipe* central, 0.5–1.2 × 1-7 cm, cylindric, solid, thick and covered with brown squamules; white flesh and firm. *Spore print* white; spores 1.5-2 × 4-7 µm, oblong-elliptical to sub-fusiform, hyaline, smooth, thin-walled and not amyloid. *Mycelium* flatted like scurfy, whitish when young and becoming dark with brown exudate when old; 1-3 µm in widths, long, branched, thin cell walls with clamp connection and hyaline under microscope.

Collection site THAILAND, Nakhon Pathom Province, Muang District, Mab-kae subdistrict. They grow on wood log.

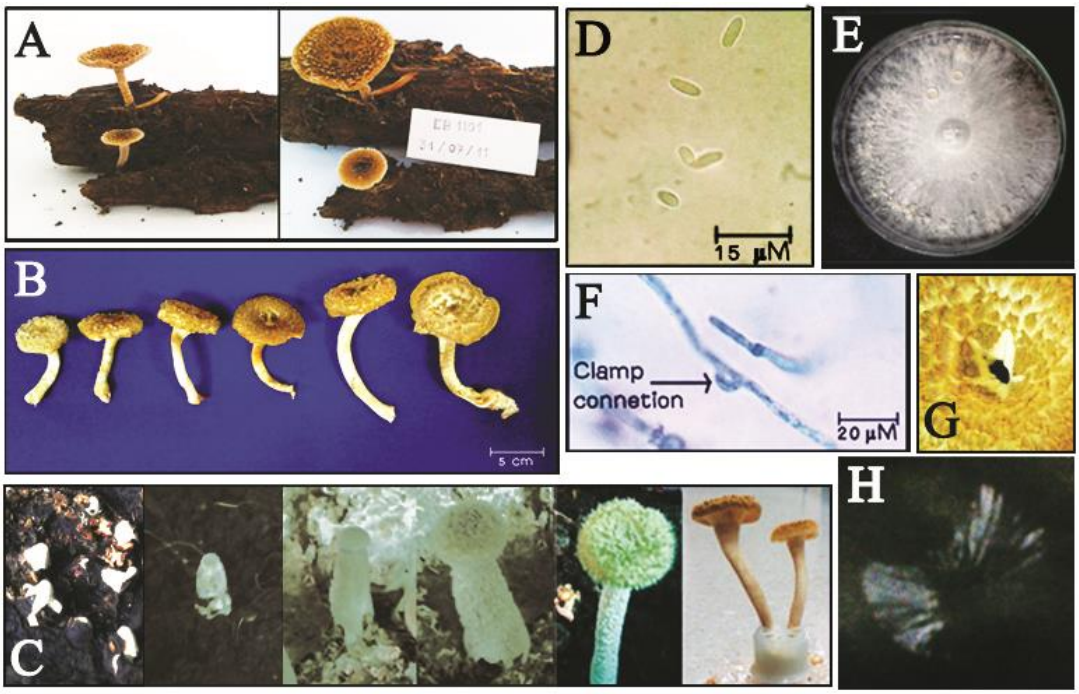

Figure 9 *Lentinus swartzii* strain EB1101; **(A)** habitat (on log), **(B)** fruiting bodies, **(C)** fruiting bodies on sawdust bags at pinhead stage, young stage, and mature stage (left to right), **(D)** spore, **(E)** mycelium on PDA, **(F)** clamp connection, **(G)** brown hairs on cap, and **(H)** spore print.

Lentinus squarrosulus strain WCR1201

Pileus 1.5 – 7(-12) cm diam, at first convex and depressed to umbilicate when old; surface dry and smooth with white to cream squamules; surface whitish to cream and then light brown; margin plane to deeply decurved; KOH color change to yellow when drop on the surface. *Lamellae* crowded, 2–4 mm broad, decurrent and whitish. *Stipe* central or off-center, 0.3–1 × 1-10 cm, whitish to light brown, cylindric, solid and thick. *Spore print* white; spores 2-3 × 3-5 µm, subglobose to oblong-elliptical, hyaline, thin-walled and not amyloid. *Mycelium* fluffy like a cotton, whitish, very tough when old; 3-4 µm in widths, long, branched, thin-walled with clamp connection and hyaline under microscope.

Collection site THAILAND, Kanchanaburi Province, Tha Muang District, Tha Maka irrigation project. They grow on dead wood.

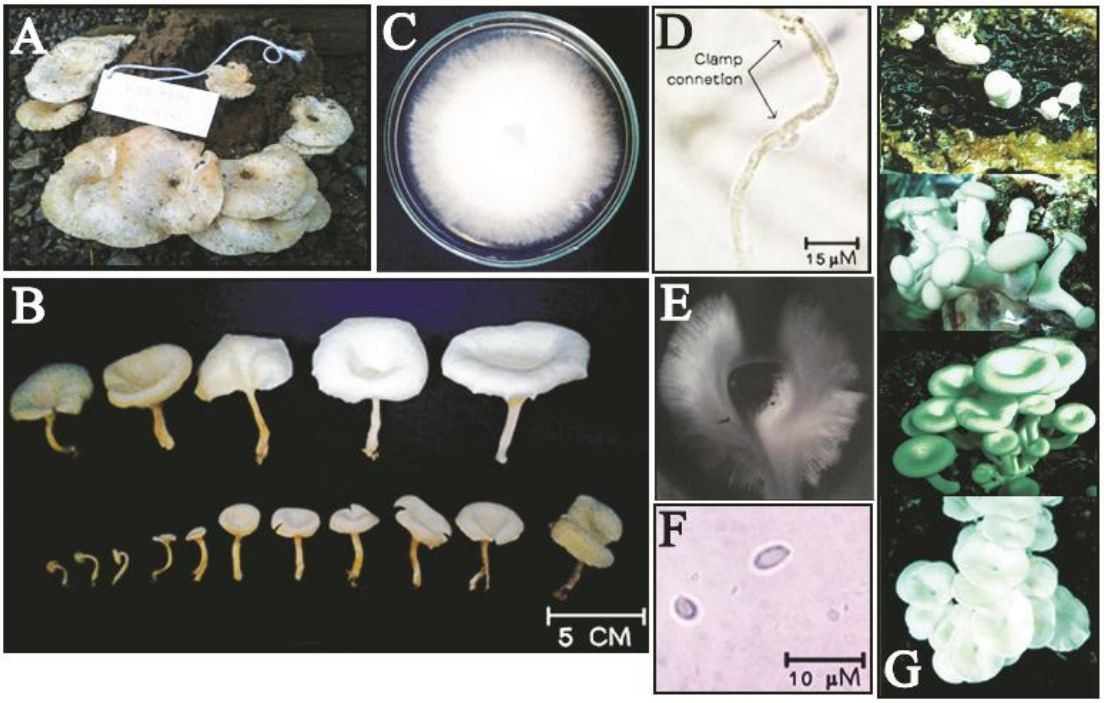

Figure 10 *Lentinus squarrosulus* strain WCR1201; **(A)** habitat (on log), **(B)** fruiting bodies, **(C)** mycelium on PDA, **(D)** clamp connection, **(E)** spore print, **(F)** spores, and **(G)** fruiting bodies on sawdust bags at pinhead stage, young stage, and mature stage (top to bottom).

Lentinus velutinus Strain WCR1104

Pileus 2 – 10(-15) cm diam., funnel-shape when young and becoming depressed to plane when old; surface dry with dark brown hairs; color variable: purplish brown when young and then changing to dark brown; margin incurved to upturned; flesh 0.1-0.3 cm thickness and pale yellow to cream; KOH color change to yellow when drop on the surface. *Lamellae* closed, 1–5 mm broad, decurrent, almond brown and dark brown at margin. *Stipe* central, 0.5–2 × 2.7-10 cm, cylindric to tapering upward, solid, dry, tough and scaly brown surface with brown hairs. *Mycelium* floccose, whitish at first and brown when old with brown exudate.

Pleurocystidia (Fig. 12A) present on the faces of lamella, 4-9 × 23-41 µm, lance-shaped or flask shaped, thickened wall (1-2 µm), hyaline, scarcely projecting beyond the basidioles. *Cheilocystidia* (Fig. 12B) found on the edges of lamella, 2-9 × 27-35 µm, obpyriform shaped, wall 1-3 µm in thickness, hyaline. *Generative hyphae* (Fig. 12C) 2-4 µm diameter, hyaline, thin-walled, frequently branched, with clamp connections. *Hairs on the pileus* (Fig. 12D) dense, 1.5-2.5 µm in diameter, long (80-170 µm), light brown with a thin wall (≈ 1 µm).

Collection site THAILAND, Ratchaburi Province, Pak Tho District, Chaleom Phrakiat Thai Prachan National Park. They grow on died hardwood root.

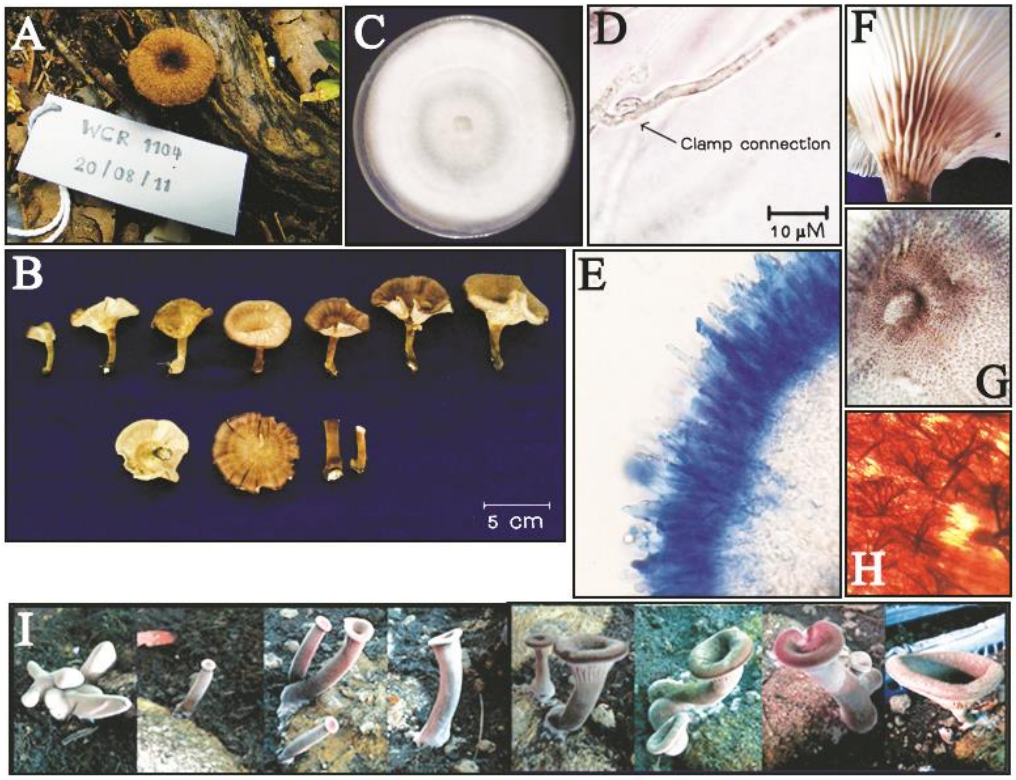

Figure 11 *Lentinus velutinus* strain WCR1104; **(A)** habitat (on log), **(B)** fruiting bodies, **(C)** mycelium on PDA, **(D)** clamp connection, **(E)** cystidia, **(F)** decurrent gills, **(G)** cap with dark brown hairs, **(H)** hairs on cap under microscope (400X), and **(I)** fruiting bodies on sawdust bags at pinhead stage, young stage, and mature stage (left to right).

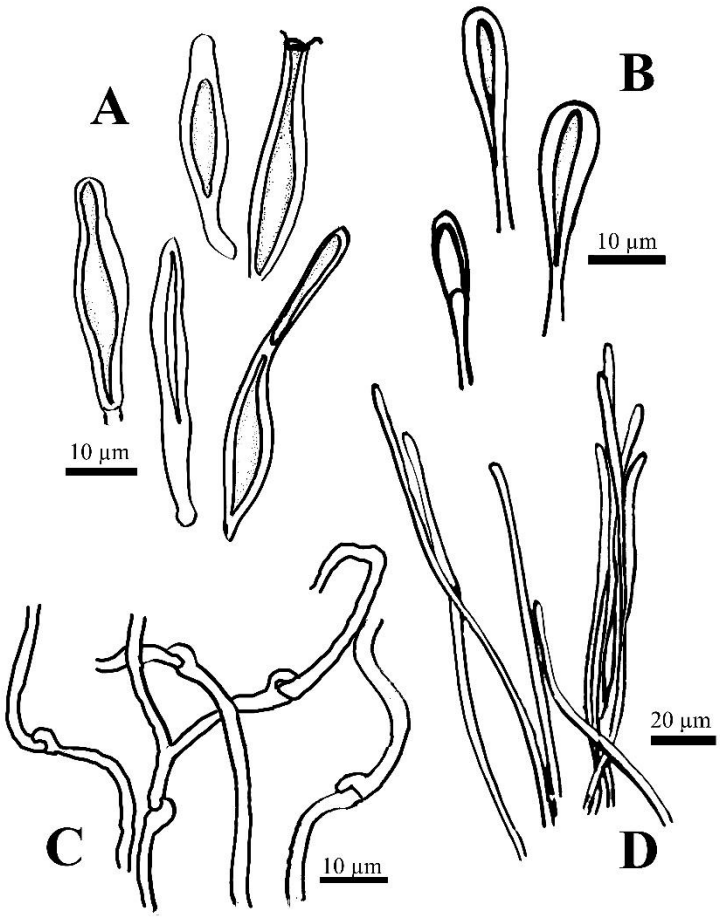

Figure 12 *Lentinus velutinus* strain WCR1104; **(A)** Pleurocystidia, **(B)** Cheilocystidia, **(C)** Generative hyphae, and **(D)** Hairs on the pileus (1000X).

To confirm morphology-based identification, the genomic DNA of mushroom samples was extracted, and the ITS of mushroom samples was amplified and separated on 1% agarose gel electrophoresis. The results showed single bands with an approximate size of 700 bp long, excepted the PCR product of *L. edodes* was about 800 bp long (Fig. 13). Then, the ITS was sequenced and compared to database. A total of 14 ITS sequences of *Lentinus* spp., classified in section Tigrini, Rigidi, Lentinus, and Velutini, were obtained from GenBank. The ITS sequence of *Volvariella volvacae* referred to as the outgroup, was used for sequence analysis. The results indicated that phylogenetic study of the ITS sequences supported the morphological identification (Fig. 14).

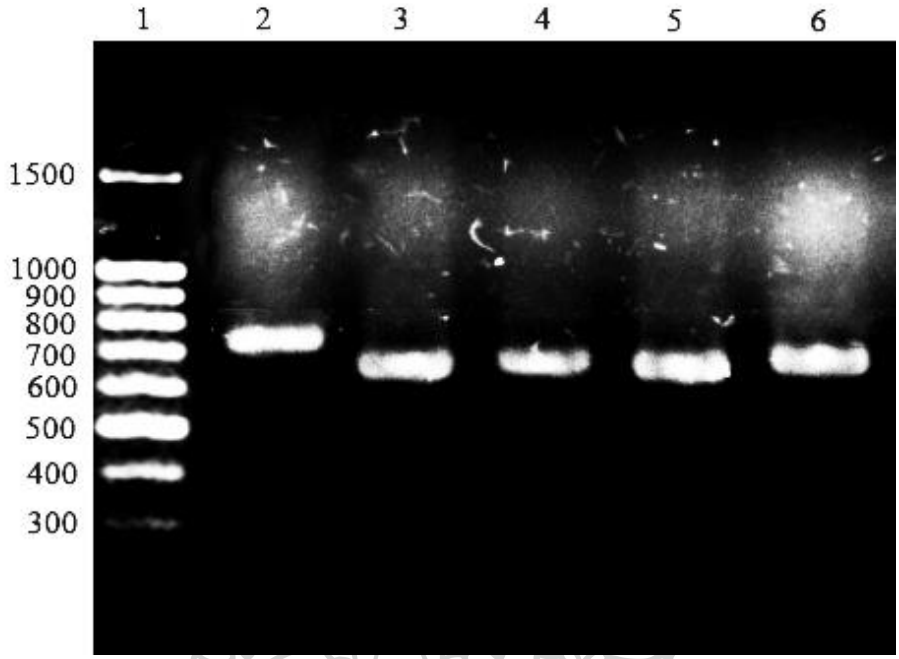

Figure 13 The gel electrophoresis of ITS amplified by PCR technique using ITS1 and ITS4 primer. Lane **(1)** 100 bp marker, **(2)** *L. edodes* #AMC3, **(3)** *L. sajor-caju* strain EB1001, **(4)** *L. swartzii* strain EB1101, **(5)** *L. squarrosulus* strain WCR1201, and **(6)** *L. velutinus* strain WCR1104.

2. Crude polysaccharide yield

A number of studies revealed the polysaccharide from hot water and alcohol extracts of mushrooms exhibited cytotoxicity against cancer cell line and anticancer activity. Hence, hot water and ethanol were chosen as the solvents for extraction of the polysaccharides from mushroom fruiting bodies and mycelia. The yield of crude aqueous polysaccharides from mycelia (13.15% - 18.16%) was higher than that of fruit bodies (3.99% - 6.86%). While the yield of crude ethanolic extracts varied from 3.36% - 52.58% (Table 3). The yield of crude ethanolic extracts was higher than that of crude aqueous extracts because of their textures. The crude ethanolic extracts were light to dark brown viscous liquid with a pungent odor (Fig. 15), while the aqueous extraction yielded dark brown crystals (Fig. 16). The difference in yields might depend on the strains, species and method of extraction.

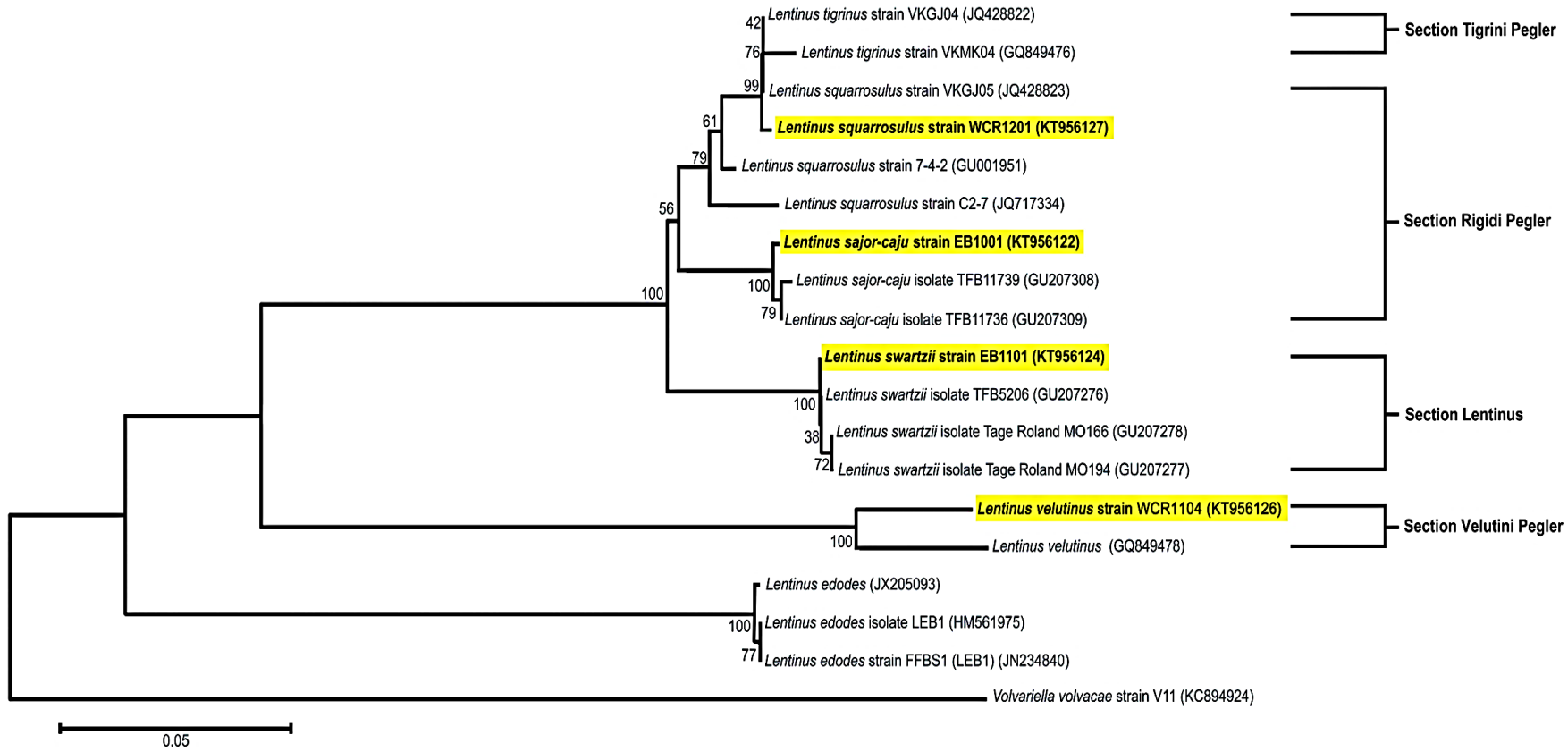

Figure 14 Phylogenetic relationships among four *Lentinus* species, *L. sajor-caju* strain EB1001, *L. swartzii* strain EB1101, *L. squarrosulus* strain WCR1201 and *L. velutinus* strain WCR1104 with some selected *Lentinus* based on ITS sequences. Data were aligned with ClustalW and gaps were treated as missing data. Values above the branches are parsimony bootstrap (≥ 50%). The tree is rooted with *Volvariella volvacae* (KC894924) as outgroup.

Table 3 The yield of crude polysaccharides, amount of proteins, total polysaccharides, reducing sugar, and total phenol content of crude aqoues and ethanolic extracts (n=3).

Crude polysaccharides	Yields[a] (%)	Total carbohydrates[b] (mg/g)	Reducing sugar[b] (mg/g)	Total proteins[b] (mg/g)	Total phenol contents[b] (mgGAE/g)
LEFB-Aq[c]	6.86	411.98 ± 35.95hi[d]	59.16 ± 4.74d	275.42 ± 9.67h	16.77 ± 0.15fgh
LEM-Aq	14.78	368.91 ± 23.41gh	113.83 ± 3.48f	285.65 ± 6.29hi	20.87 ± 0.68ij
LSaFB-Aq	5.91	246.34 ± 12.78de	49.16 ± 3.13bc	284.28 ± 4.11hi	15.56 ± 0.35f
LSaM-Aq	18.16	326.95 ± 34.87fg	114.79 ± 2.37f	305.04 ± 9.60ij	18.39 ± 0.43ghi
LSwFB-Aq	5.47	207.15 ± 34.13cd	40.85 ± 3.10b	453.81 ± 14.57k	23.43 ± 0.65j
LSwM-Aq	16.16	392.01 ± 47.25hi	58.25 ± 2.17cd	241.20 ± 10.97fg	16.37 ± 0.66fg
LSqFB-Aq	6.16	271.06 ± 6.39ef	44.07 ± 2.09b	216.91 ± 4.91f	14.57 ± 0.09f
LSqM-Aq	13.15	405.71 ± 15.86hi	70.49 ± 1.11e	177.26 ± 4.76e	14.13 ± 0.29f
LVFB-Aq	3.99	200.44 ± 9.09cd	69.89 ± 4.03e	260.52 ± 5.81gh	15.58 ±3.33f
LVM-Aq	14.20	443.17 ± 10.40i	73.82 ± 1.21e	312.73 ± 8.49j	18.94 ± 0.48ghi

[a] Yield per hundred grams dry weight of sample.
[b] milligram per gram dry weight of crude extract.
[c] Extracts name; the letter referring to each Lentinus spp. (LE = *L. edodes*, LSa = *L. sajor-caju*, LSw = *L. swartzii*, LSq = *L. squarrosulus* and LV = *L. velutinus*), FB and M referring to fruit bodies and mycelium, and Aq and Et referring to aqueous and ethanolic extract.
[d] Means with different letters within a column are significantly different ($P<0.05$).

Table 3 The yield of crude polysaccharides, amount of proteins, total polysaccharides, reducing sugar, and total phenol content of crude aqoues and ethanolic extracts (n=3; cont.).

Crude polysaccharides	Yields (%)	Total carbohydrates (mg/g)	Reducing sugar (mg/g)	Total proteins (mg/g)	Total phenol contents (mgGAE/g)
LEFB-Et	21.73	83.02 ± 4.37a	20.98 ± 3.61a	77.14 ± 9.05ab	3.29 ± 0.04a
LEM-Et	17.58	191.72 ± 14.29cd	111.46 ± 5.68f	99.26 ± 5.76b	6.42 ± 0.48bc
LSaFB-Et	16.74	172.90 ± 12.85bc	UD[e]	97.31 ± 6.23ab	9.11 ± 0.21de
LSaM-Et	9.84	196.75 ± 8.48cd	56.25 ± 6.57cd	87.90 ± 1.79ab	6.52 ± 0.35cde
LSwFB-Et	3.36	251.70 ± 23.21de	UD	129.12 ± 2.25c	9.74 ± 0.90e
LSwM-Et	20.530	221.72 ± 8.30cde	125.40 ± 1.46g	72.73 ± 1.99a	5.60 ± 0.39abc
LSqFB-Et	32.58	246.14 ± 5.22de	23.76 ± 1.19a	87.31 ± 4.71ab	4.45 ± 0.29ab
LSqM-Et	52.58	123.20 ± 6.44ab	UD	151.28 ± 6.17cd	9.96 ± 0.31e
LVFB-Et	9.69	171.88 ± 18.08bc	126.13 ± 4.66g	162.65 ± 18.69de	19.08 ± 0.21hi
LVM-Et	9.22	175.02 ± 3.78bc	24.31 ± 1.82a	95.78 ± 4.47ab	7.37 ± 0.41cde

[e] UD stand for undetected.

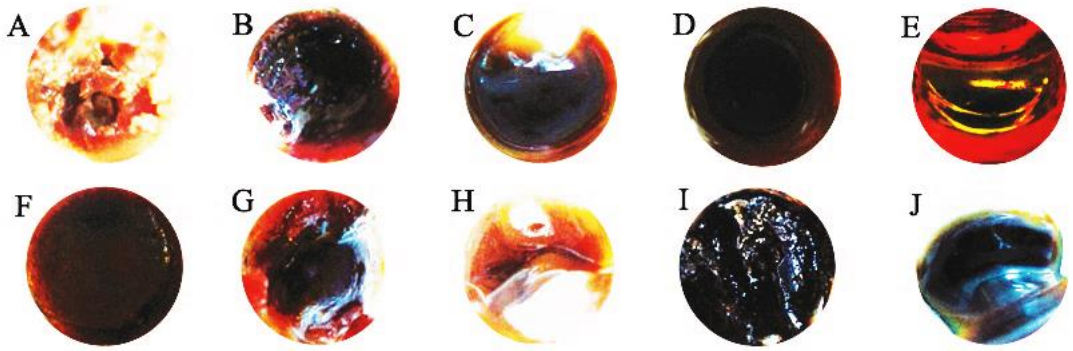

Figure 15 Crude ethanolic extracts; **(A)** LEFB-Et, **(B)** LSaFB-Et, **(C)** LSwFB-Et, **(D)** LSqFB-Et, **(E)** LVFB-Et, **(F)** LEM-Et, **(G)** LSaM-Et, **(H)** LSwM-Et, **(I)** LSqM-Et, and **(J)** LVM-Et.

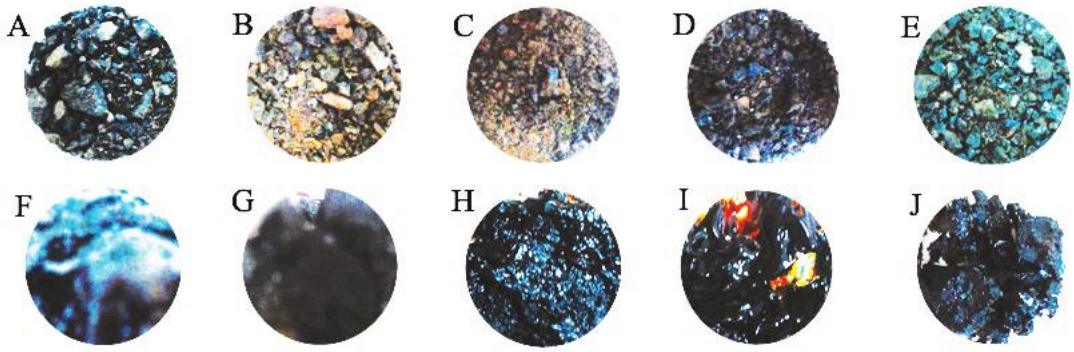

Figure 16 Crude aqueous extracts; **(A)** LEFB-Aq, **(B)** LSaFB-Aq, **(C)** LSwFB-Aq, **(D)** LSqFB-Aq, **(E)** LVFB-Aq, **(F)** LEM-Aq, **(G)** LSaM-Aq, **(H)** LSwM-Aq, **(I)** LSqM-Aq, and **(J)** LVM-Aq.

3. Properties of crude polysaccharides.

Crude aqueous polysaccharides showed a higher content of total carbohydrates, which ranged from 200.44 – 433.17 mg/g dwt (20.04 – 43.32%), than crude ethanolic polysaccharides (8.30% – 25.17%). The percentage of reducing sugar from hot water and ethanol extracts ranged from 0% - 12.61% (Table 3). The total protein contents of crude aqueous and crude ethanolic extracts were between 17.7-45.3% and 7.2-16.2%, respectively. The ratio of polysaccharide to protein from crude aqueous and ethanolic extracts were 1.35 and 1.84 on average, respectively.

The total phenol contents of crude aqueous extracts and crude ethanolic extracts were 14.13 - 23.43 and 3.29 - 19.08 mgGAE/g crude polysaccharides, respectively (Table 3).

4. *In vitro* anticancer activity and cytotoxicity of crude polysaccharides

In preliminary work, the anticancer property and cytotoxicity of crude aqueous extracts was evaluated at concentration 5 mg/mL and 20 mg/mL for 24 h with the cancer cell lines, HeLa and HepG2, and the normal cell lines, LLC-MK2 and L929. The cancer cells were not inhibited by any extracts at 5 mg/mL, while cell lines were completely killed when exposed to the extracts at 20 mg/mL for 24 h. Thus, in this study, the crude aqueous extracts were tested at 10 mg/mL for 24 h. The results indicated that the crude extract from fruiting bodies of *Lentinus velutinus* (LVFB-Aq) showed the highest inhibitory effect against both the HeLa and the HepG2 cell lines, with 49.83% and 48.51% inhibition, respectively. The crude extract from *L. edodes* fruiting bodies (LEFB-Aq) and mycelium (LEM-Aq) and *L. velutinus* mycelium (LVM-Aq) showed the anticancer effects against only the HepG2; the cell viability after treatment with these crude extracts declined to 36.58%, 62.53%, and 55.71%, respectively. While other aqueous extracts had no effects against cancer cell lines (Fig. 17A). The ethanolic extracts from fruiting bodies of *L. velutinus* (LVFB-Et), *L. squarrosulus* (LSqFB-Et), *L. edodes* (LEFB-Et), and *L. sajor-caju* (LSaFB-Et) displayed a toxicity effect against only the HeLa cells after treatment for 24 h; the inhibitory effect was 35.95%, 31.17%, 26.33%, and 15.88%, respectively (Fig 17B). The others had no cytotoxicity toward either the HepG2 and the HeLa.

The cytotoxicity of crude polysaccharides on the normal cell lines were lower than that against cancer cell lines (Fig. 18). Due to these results, the LVFB-Aq was further purified to further characterize this anticancer polysaccharide.

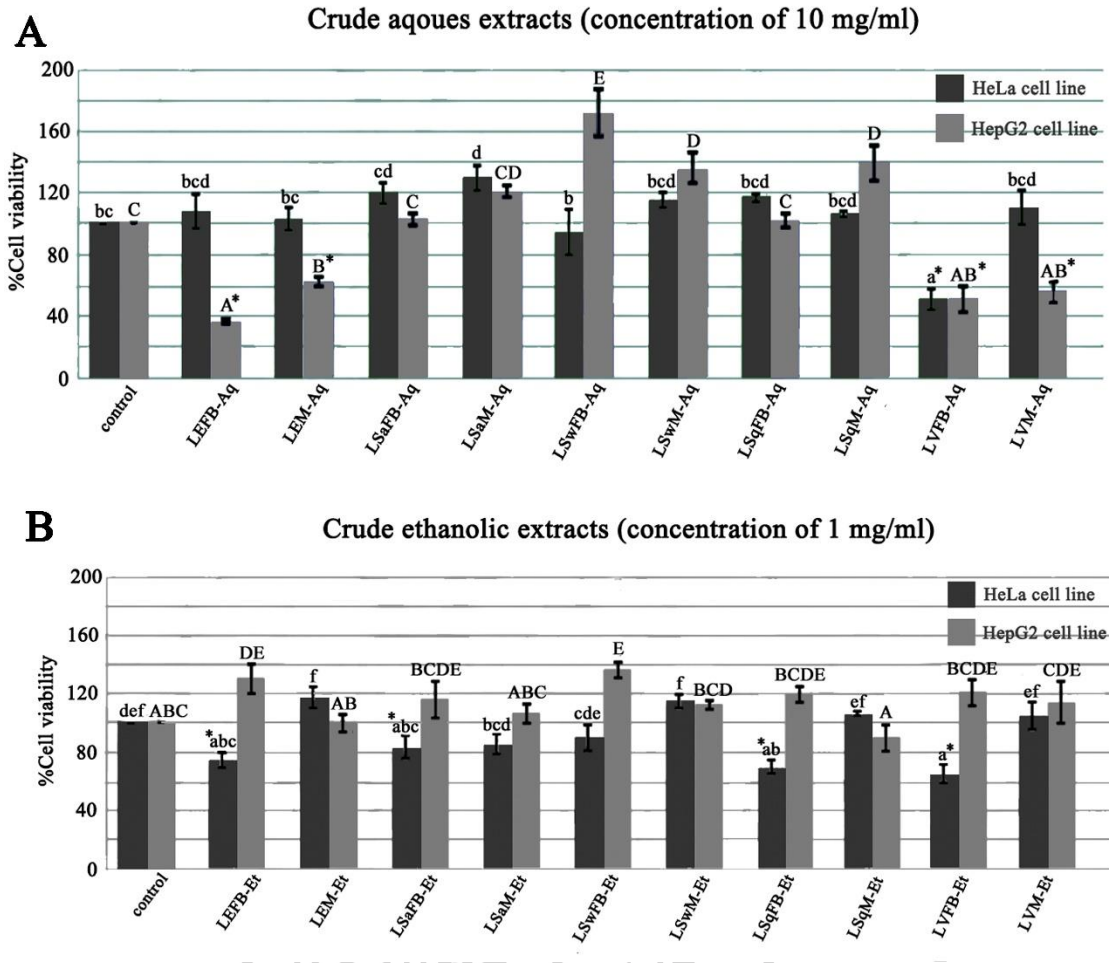

Figure 17 Cell viability (%) of human epitheloid cervix carcinoma cell line (HeLa) and human hepatocellular liver carcinoma cell line (HepG2) after treatment with crude aqueous extracts **(A)** and crude ethanolic extracts (B) of Lentinus spp. for 24 h. Error bars indicate means ± standard deviation (n = 3) and the letters indicated the statistic groups.

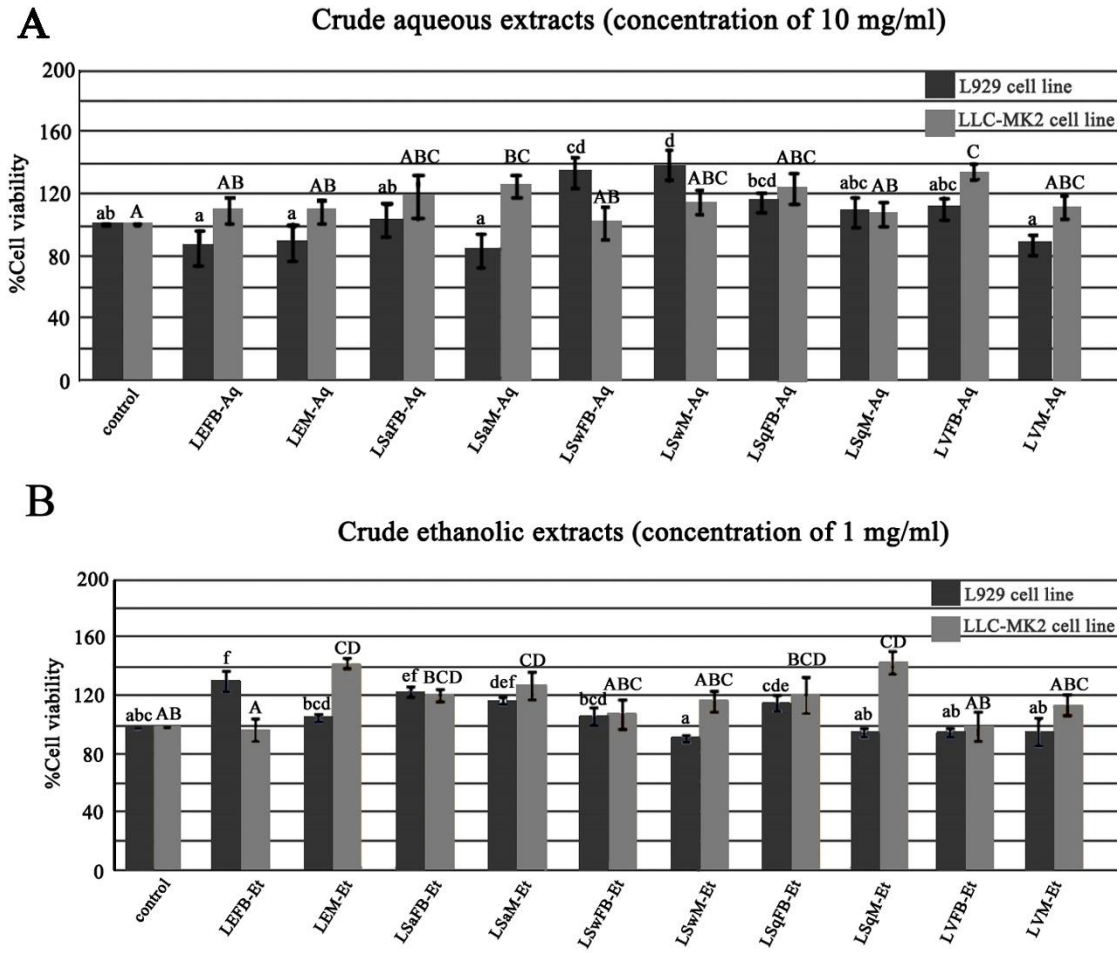

Figure 18 Cell viability (%) of rhesus monkey kidney epithelial cell line (LLC-MK2) and mouse fibroblast cell line (L929) after treatment with crude aqueous extracts (A) and crude ethanolic extracts (B) of *Lentinus* spp. for 24 h. Error bars indicate means ± standard deviation (n = 3) and the letters indicated the statistic groups.

5. The separation of crude aqueous extract, LVFB-Aq, by re-precipitation with ethanol and their cytotoxicity

Crude aqueous extract, LVFB-Aq (5.278 g) was purified by re-dissolving in sterile water and precipitating with cold 95% ethanol. It was separated into three fractions e.g. fraction E1, fraction E4, and fraction R (Fig. 19), which were yields to 1.472, 2.279, and 1.526 g, respectively. The characteristic of fraction E1 and E4 are solid dark brown crystal. While fraction R is sticky substance. In addition, this purification step also demonstrated the high level of total carbohydrate contents up to 99.72% recovery (Table 4). Fraction E1 and fraction E4 included the higher percentage of carbohydrate contents (70.91%, and 59.69%, respectively) than LVFB-Aq (51.72%), whereas fraction R shows only 16.49% (Table 4).

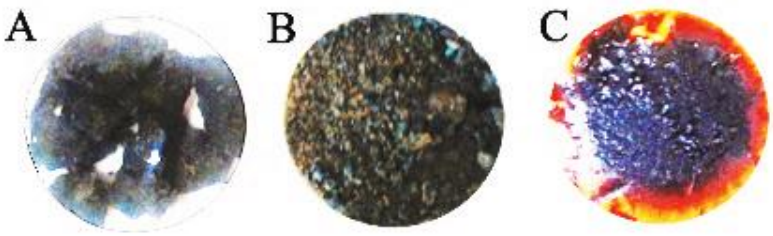

Figure 19 Three fractions including **(A)** fraction E1, **(B)** fraction E4, and **(C)** fraction R, were separated from crude aqueous extract LVFB-Aq by re-dissolving in sterile water and re-precipitating with 95% ethanol.

Table 4 The polysaccharide separation from crude aqueous extract LVFB-Aq by re-precipitation step.

Extract	Yield (mg)	CHO* contents (mg)	CHO contents (%)	Total CHO contents (mg)	% CHO recovery
LVFB-Aq	5,278 (100%)	2,729.94 ± 45.50	51.72	2,729.94	
Fraction E1	1,602 (30.35%)	1,135.99 ± 41.97	70.91		
Fraction E4	2,279 (43.18%)	1,360.33 ± 35.27	59.69	2,722.21	99.72
Fraction R	1,370 (25.96%)	225.89 ± 24.62	16.49		

*CHO refers to carbohydrate.

These fractions were evaluated for anticancer activity and cytotoxicity against HeLa, HepG2, LLC-MK2, and L929 cell lines at concentration 5 and 10 mg/mL for 24 h (Fig. 20). The data indicate that fraction E4 could decreased the viability of both cancer cell lines, HeLa and HepG2, in a concentration-dependent manner. While fraction E1 and R could not inhibit the cancer cells growth. Moreover, fraction R also cytotoxic to L929 cell greater than those cancer cells. At 10 mg/mL, fraction E4 showed better anticancer capability than LVFB-Aq, significantly. Thus, fraction E4 was further applied to anion exchange chromatography for separating the anticancer polysaccharides.

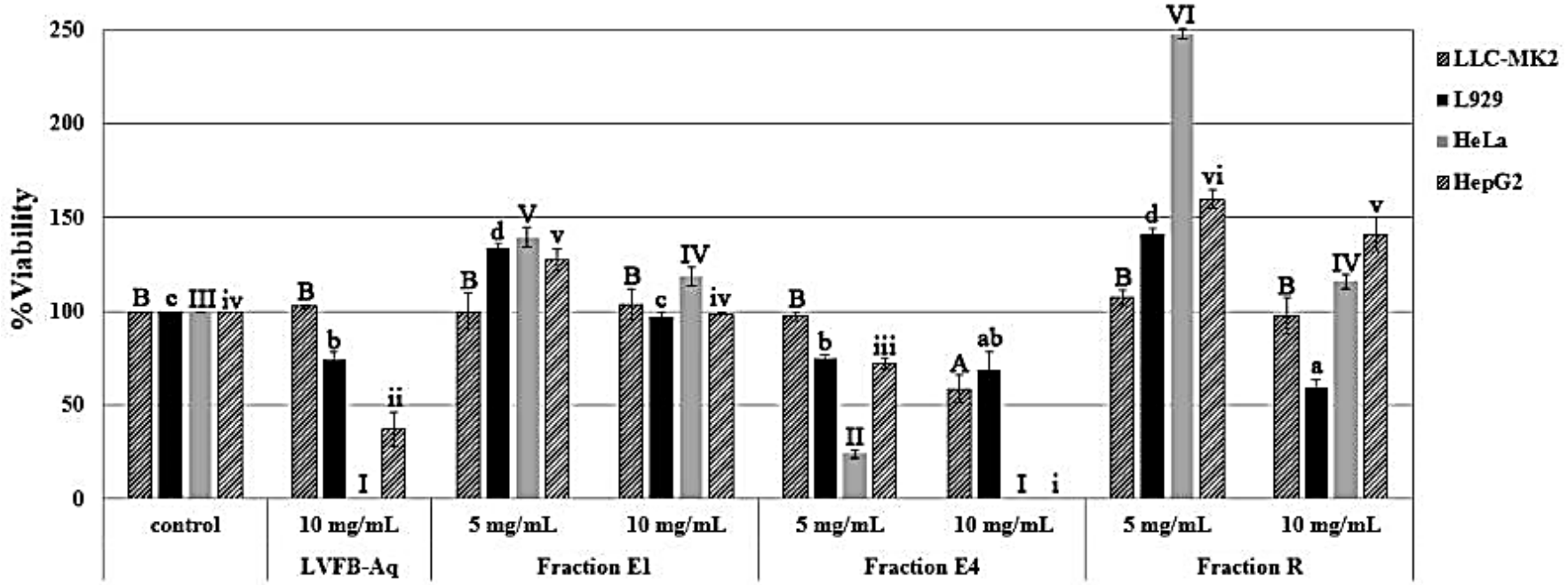

Figure 20 Cell viability (%) of rhesus monkey kidney epithelial cell line (LLC-MK2), mouse fibroblast cell line (L929), human epitheloid cervix carcinoma cell line (HeLa) and human hepatocellular liver carcinoma cell line (HepG2) after treatment with fraction E1, E4, and R at concentration 5, and 10 mg/mL for 24 h. Error bars indicate means ± standard deviation (n = 3) and the letters indicated the statistic groups.

6. The separation of fraction E4 by anion exchange chromatography and their cytotoxicity

The fraction E4 (2,179 mg) was further fractionated by applying to DEAE FF anion chromatography column (5 mL), eluted with step concentration of NaCl (0, 0.05, 0.1, 0.2, and 0.5M) at flow rate 1 mL/min, and dialyzed through dialysis bag (MWCO: 6-8,000) against distilled water for 5 days. It was separated into 6 polysaccharide fractions, namely fraction E4W (fraction no. 5-29), E4N1 (fraction no. 48-52), E4N2 (fraction no. 54-67), E4N3 (fraction no. 69-104), E4N4 (fraction no. 108-127), and E4N5 (fraction no. 142-156; Fig. 21). The fraction E4W was a fine light brown powder, while fraction E4N1, N2, and N3 were a light brown thin sheet. Fraction E4N4 and E4N5 were crystalline black brown (Fig. 22).

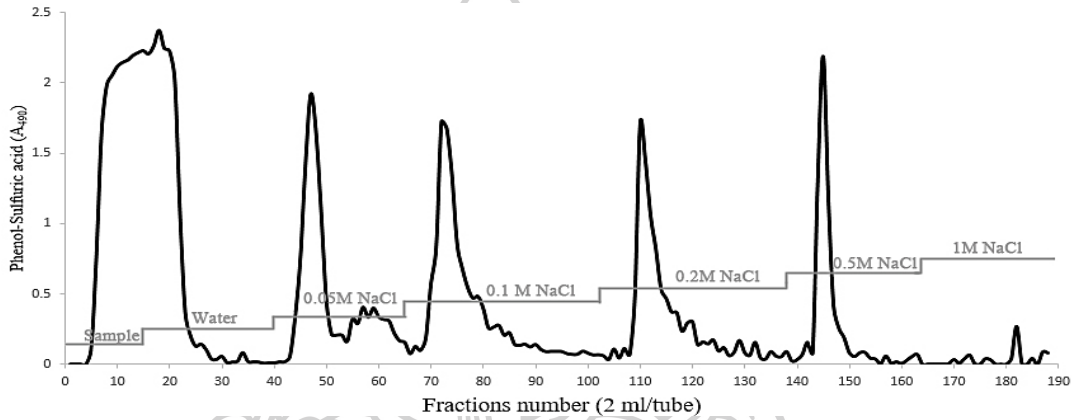

Figure 21 The fractionation of fraction E4 by using DEAE FF anion chromatography column (5 mL), eluted with step concentration of NaCl (0, 0.05, 0.1, 0.2, and 0.5M) at flow rate 1 mL/min into 6 polysaccharide fractions, namely fraction E4W (fraction no. 5-29), E4N1 (fraction no. 48-52), E4N2 (fraction no. 54-67), E4N3 (fraction no. 69-104), E4N4 (fraction no. 108-127), and E4N5 (fraction no. 142-156). The gray line indicated the step elution.

Fraction E4W, E4N1, E4N2, E4N3, E4N4, and E4N5 were yield 210, 77, 64, 83, 105, and 92 mg, respectively, which total yields was only 35.06%(w/w) of fraction E4. While total carbohydrate contents recovery was 38.23% (Table 5). After dialysis and precipitation with ethanol, the carbohydrate contents of fraction E4W, E4N1, E4N2, and E4N3 decreased to approximately 40%, and fraction E4N4 and E4N5 reduced to about 50% and 20%, respectively. Fraction E4W showed the highest yield (210 mg) and percentage of carbohydrate content (91.78%), whereas fraction E4N2 was the lowest (64 mg and 19.5%, respectively; Table 5).

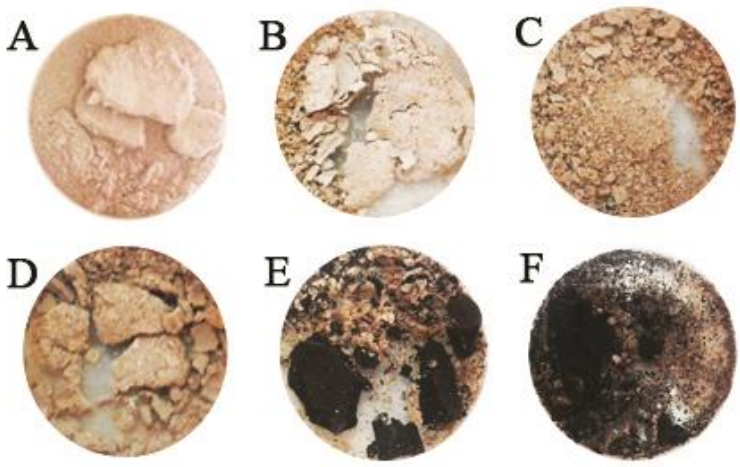

Figure 22 Six fractions including **(A)** fraction E4W, **(B)** fraction E4N1, **(C)** fraction E4N2, **(D)** fraction E4N3, **(E)** fraction E4N4, and **(F)** fraction E4N5 were separated from fraction E4 by using DEAE FF anion chromatography column (5 mL), eluted with step concentration of NaCl (0, 0.05, 0.1, 0.2, and 0.5M).

The anticancer activity and cytotoxicity of the anion fractions were estimated by MTT assay at concentration 1 and 2 mg/mL. The results demonstrated that fraction E4N5 has the greatest anticancer efficacy by significantly inhibiting the growth of HeLa cell line by 17% and 26.65% at concentrations of 1 and 2 mg/mL, respectively. Fraction E4N4 also significantly inhibited the HeLa growth at concentrations of 1 and 2 mg/mL which were 12.52% and 28.59%, respectively (Fig. 23). Furthermore, fraction E4N3 showed merely the inhibition of HeLa cell growth at the concentration of 2 mg/mL which was to 16.03%, significantly. While, fraction E4W and E4N1 could not inhibit any cell lines at the concentration of 2 mg/mL (Fig. 23). All fraction presented the non-cytotoxicity against normal cell line, LLC-MK2 and L929, at the concentration of 2 mg/mL. Moreover, the data showed that fraction E4N5 could also slightly inhibit the HepG2 cell (13.59%) at the concentration of 2 mg/mL, insignificantly as the others could not (Fig. 23). So, fraction E4N5 was chosen for the next separation step.

Table 5 The polysaccharide separation from fraction N4 by anion exchange chromatography.

Extract	Yield (mg)	CHO* contents (mg)	CHO contents (%)	Total CHO contents (mg)	% CHO recovery
Fraction E4	1,800 (100%)	930.96 ± 12.62	51.72	930.96	
BEFORE DIALYSIS					
Fraction E4W		338.52			
Fraction E4N1		50.60			
Fraction E4N2		14.69			
Fraction E4N3		53.07		597.03	64.13
Fraction E4N4		73.04			
Fraction E4N5		67.11			
AFTER DIALYSIS					
Fraction E4W	210 (11.67%)	192.73	91.78		
Fraction E4N1	77 (4.28%)	27.02	35.09		
Fraction E4N2	64 (3.56%)	12.48	19.5		
Fraction E4N3	83 (4.61%)	30.75	37.05	355.86	38.23
Fraction E4N4	105 (5.83%)	37.02	35.26		
Fraction E4N5	92 (5.11%)	55.86	60.72		

*CHO refers to carbohydrate.

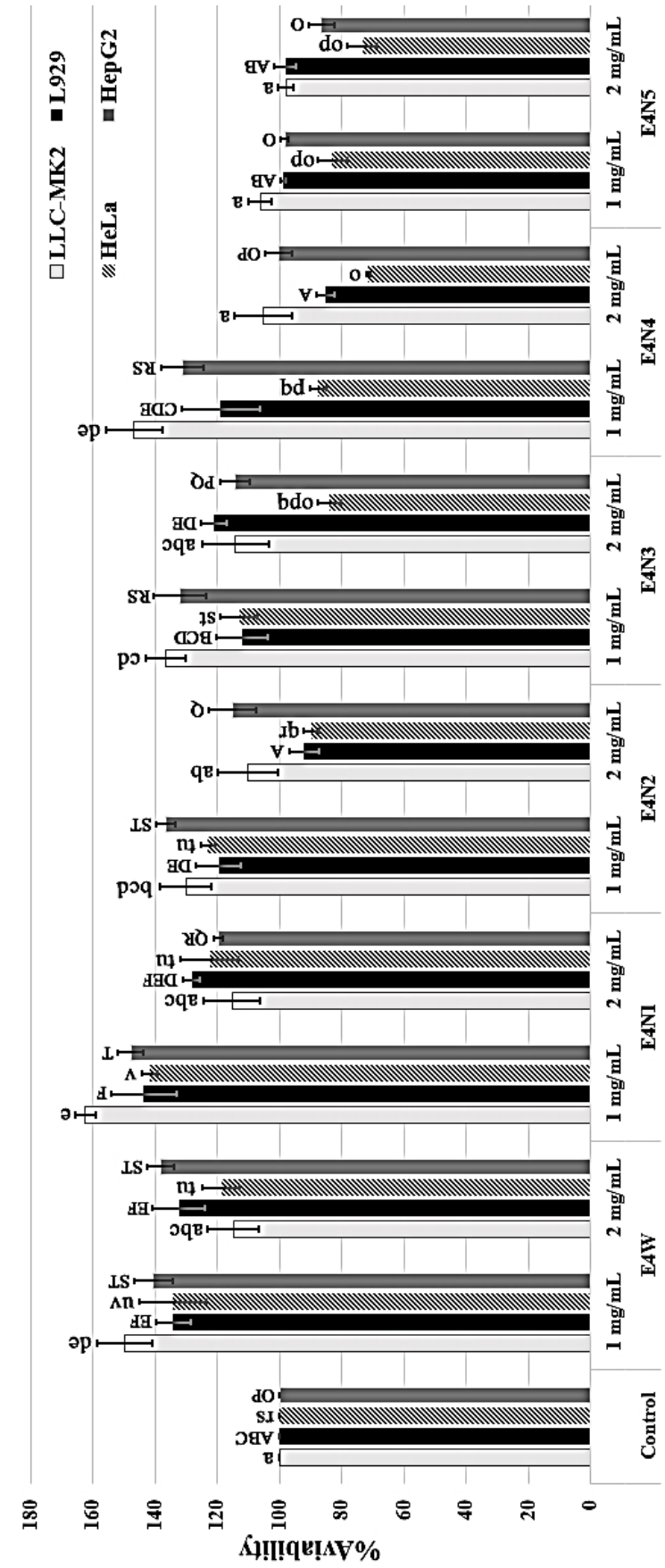

Figure 23 Cell viability (%) of rhesus monkey kidney epithelial cell line (LLC-MK2), mouse fibroblast cell line (L929), human epitheloid cervix carcinoma cell line (HeLa), and human hepatocellular liver carcinoma cell line (HepG2) after treatment with fraction E4W, E4N1, E4N2, E4N3, E4N4, and E4N5 at concentration 1, and 2 mg/mL for 24 h. Error bars indicate means ± standard deviation (n = 3) and the letters indicated the statistic groups.

7. The separation of fraction E4N5 by size exclusion chromatography, and their cytotoxicity and antioxidant activity.

The fraction E4N5 was further separated by using Sephadex G-100 (50 cm) column, which the extracts were separated based on molecular size. The size exclusion chromatography could be separate the fraction E4N5 into 3 substances, namely LV1 (fraction no. 1 – 4), LV2 (fraction no. 6 – 13), and LV3 (fraction no. 16 – 21; Fig. 24). The characteristic of LV1 and LV3 were a light brown plate-like powder, whereas LV2 was a dark brown crystal (Fig. 25). LV2 showed the highest yield and percentage of carbohydrate content, which was 38.4 mg and 99.04%, respectively. While, LV1 and LV3 yield were 3.7 mg and 8.9 mg, respectively (Table 6).

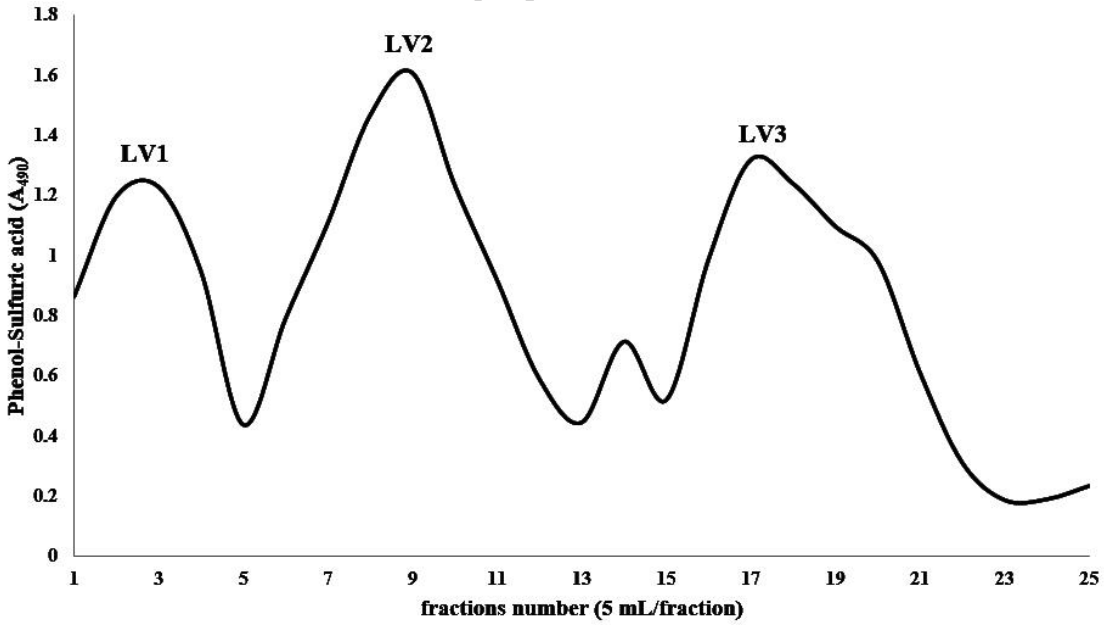

Figure 24 The fractionation of fraction E4N5 by using Sephadex G-100 column (50 cm), eluted with 50 mM sodium-phosphate buffer, pH 7.0 supplemented with 0.15M NaCl at flow rate 0.1 mL/min into 3 polysaccharides, namely LV1 (fraction no. 1-4), LV2 (fraction no. 6-13), and LV3 (fraction no. 16-21).

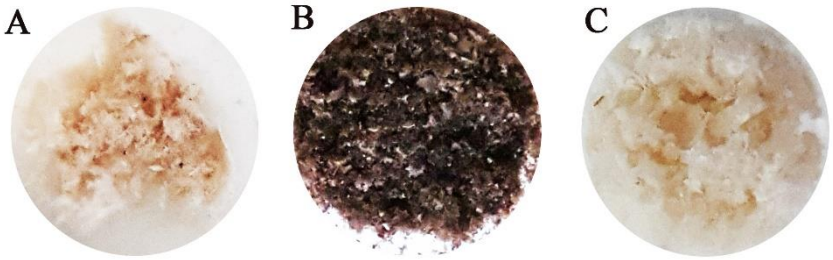

Figure 25 Three polysaccharides including **(A)** LV1, **(B)** LV2, and **(C)** LV3 were separated from fraction E4N5 by using Sephadex G-100 column (50 cm), eluted with 50 mM sodium-phosphate buffer, pH 7.0 supplemented with 0.15M NaCl.

Table 6 The polysaccharide separation from fraction E4N5 by size exclusion chromatography.

Extract	Yield (mg)	CHO* contents (mg)	CHO contents (%)	Total CHO contents (mg)	% CHO recovery
Fraction E4N5	85 (100%)	51.62 ± 0.49	60.72	51.62	
LV1	3.7 (4.35%)	3.35 ± 0.04	90.51	49.92	96.71
LV2	38.4 (45.18%)	38.03 ± 0.24	99.04		
LV3	8.9 (10.47%)	8.54 ± 0.08	95.96		

*CHO refers to carbohydrate.

The anticancer activity was tested with the polysaccharides LV1, LV2 and LV3 at various concentration (10, 50, 100, 250, 500, 1000, 1500, and 2000 µg/mL). In this study, the Lentinan (Lentinex or LentinanXP, Glyconova, Norway) the commercial ß-glucan extracted from *L. edodes,* was used as a benchmark for this test. Cancer cell lines, HeLa and HepG2, decreased after 24 and 48 h when exposure to LV2 and Lentinan in a concentration-dependent and time-dependent manner (Fig 26B, 26D, 27B, and 27D). However, LV1 and LV3 did not have any inhibitory effects against both cancer cell lines (Fig. 26A and 26C). Lentinan indicated the greatest anticancer capability against HeLa and HepG2 cells at both time tests, which IC50 at 24 h was 322.73 and 659.09 µg/mL, and at 48 h was 273.21 and 319.64 µg/mL for HeLa and HepG2, respectively. LV2 slightly inhibited the growth of cancer cell lines, which IC50 of HeLa and HepG2 at 48 h was 2,000 and 1,935 µg/mL, respectively. The cytotoxicity against normal cell lines was tested with LLC-MK2 and L929 cell line (Fig. 26 and 27). The results displayed that all polysaccharides were friendly to LLC-MK2 cell lines at tested concentration. Whereas, the evaluation of L929 cell line found that LV2 and Lentinan were toxic to cell, which IC50 of LV2 and Lentinan at 48 h was 1490 and 550 µg/mL, respectively (Fig 27B and 27C).

After the cancer cells were treated with LV2 and Lentinan, the cell morphology of HeLa and HepG2 cell liness were observed. At low concentration of LV2 and Lentinan, both cancer cells displayed no detectable morphological alterations, they could exhibit normal adherent, and elogation. While, at the higher concentration (>1000 µg/mL of LV2, and >250 µg/mL of Lentinan), the cell morphology of both cancer cells were induced. Cells and their nuclei were swelling and round, the cell density and adherent capacity decreased. Furthermore, the bar- and hexagonal-shaped crystals were detected when the cells were tested with the higher concentration of Lentinan (Fig 28 and 29). These results showed the same way as the tested with MTT.

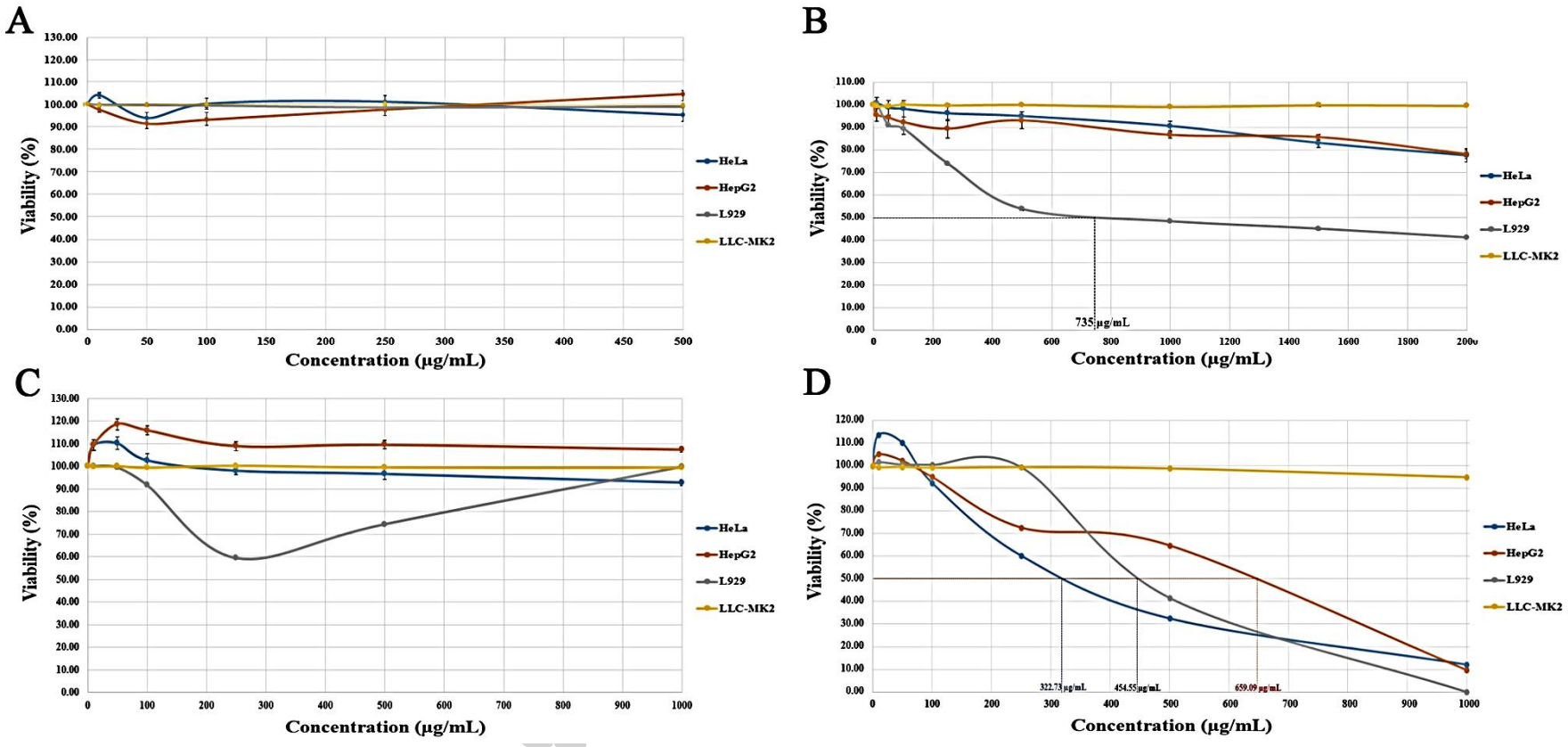

Figure 26 Cell viability (%) of rhesus monkey kidney epithelial cell line (LLC-MK2), mouse fibroblast cell line (L929), human epitheloid cervix carcinoma cell line (HeLa), and human hepatocellular liver carcinoma cell line (HepG2) after treatment with LV1 (A), LV2 (B), LV3 (C), and Lentinan (D) at various concentration (0, 10, 50, 100, 250, 500, 1000, 1500, and 2000 µg/mL) for 24 h. Error bars indicate means ± standard deviation (n = 3) and the line and numbers indicated the IC50 values.

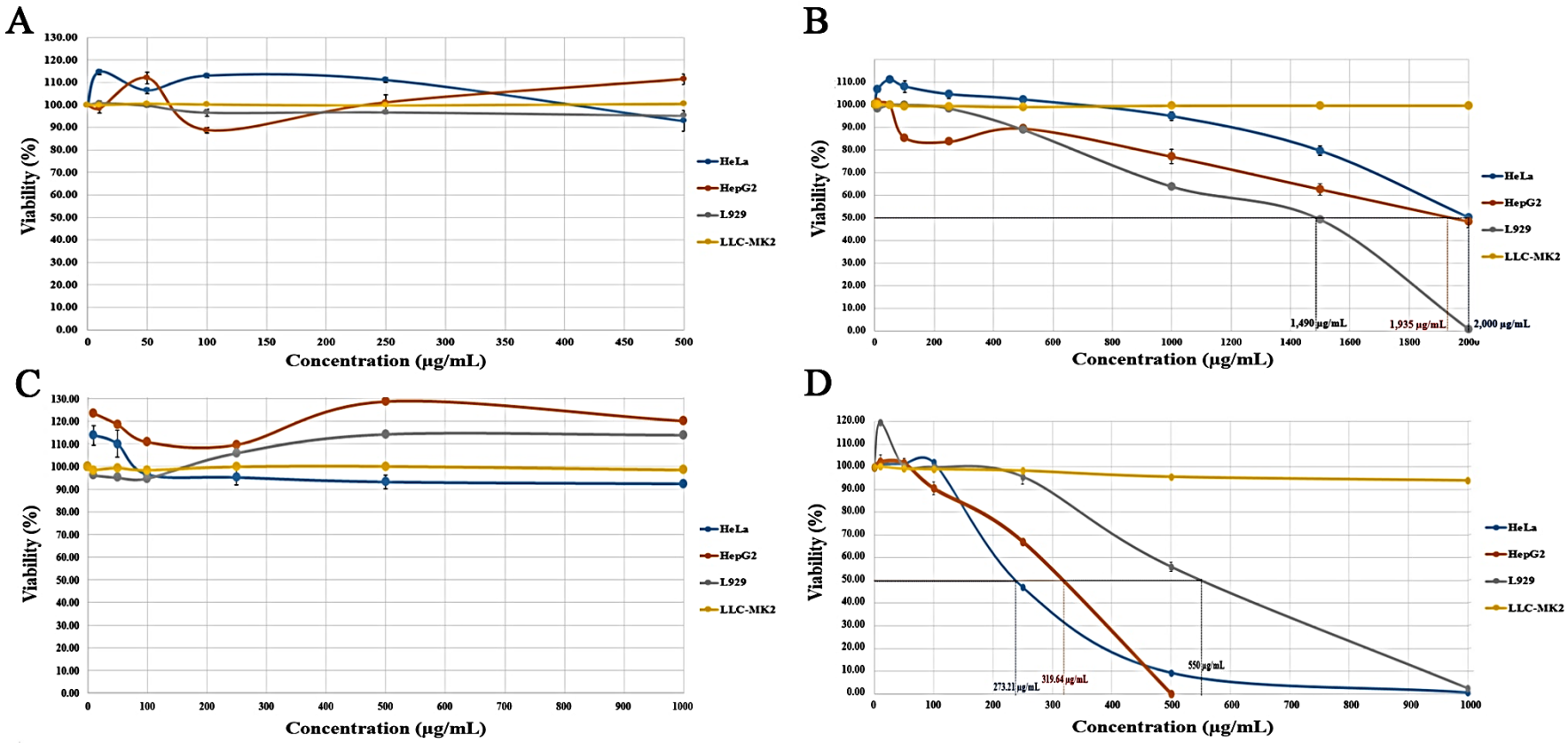

Figure 27 Cell viability (%) of rhesus monkey kidney epithelial cell line (LLC-MK2), mouse fibroblast cell line (L929), human epitheloid cervix carcinoma cell line (HeLa), and human hepatocellular liver carcinoma cell line (HepG2) after treatment with LV1 (A), LV2 (B), LV3 (C), and Lentinan (D) at various concentration (0, 10, 50, 100, 250, 500, 1000, 1500, and 2000 µg/mL) for 48 h. Error bars indicate means ± standard deviation (n = 3) and the line and numbers indicated the IC50 values

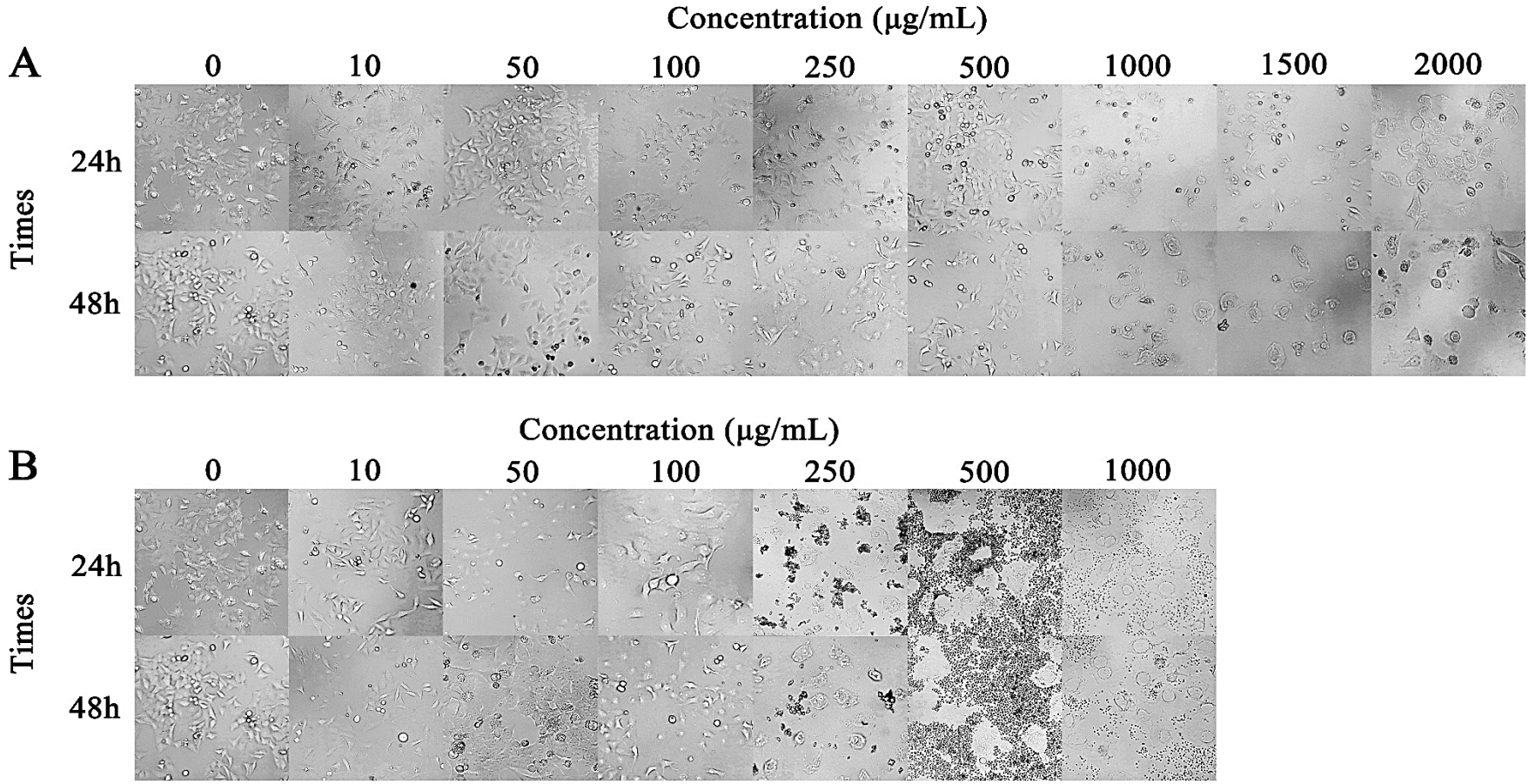

Figure 28 Cell morphology of human epitheloid cervix carcinoma cell line (HeLa) after treatment with (A) LV2 and (B) Lentinan at various concentration (0, 10, 50, 100, 250, 500, 1000, 1500, and 2000 μg/mL) for 24 and 48 h.

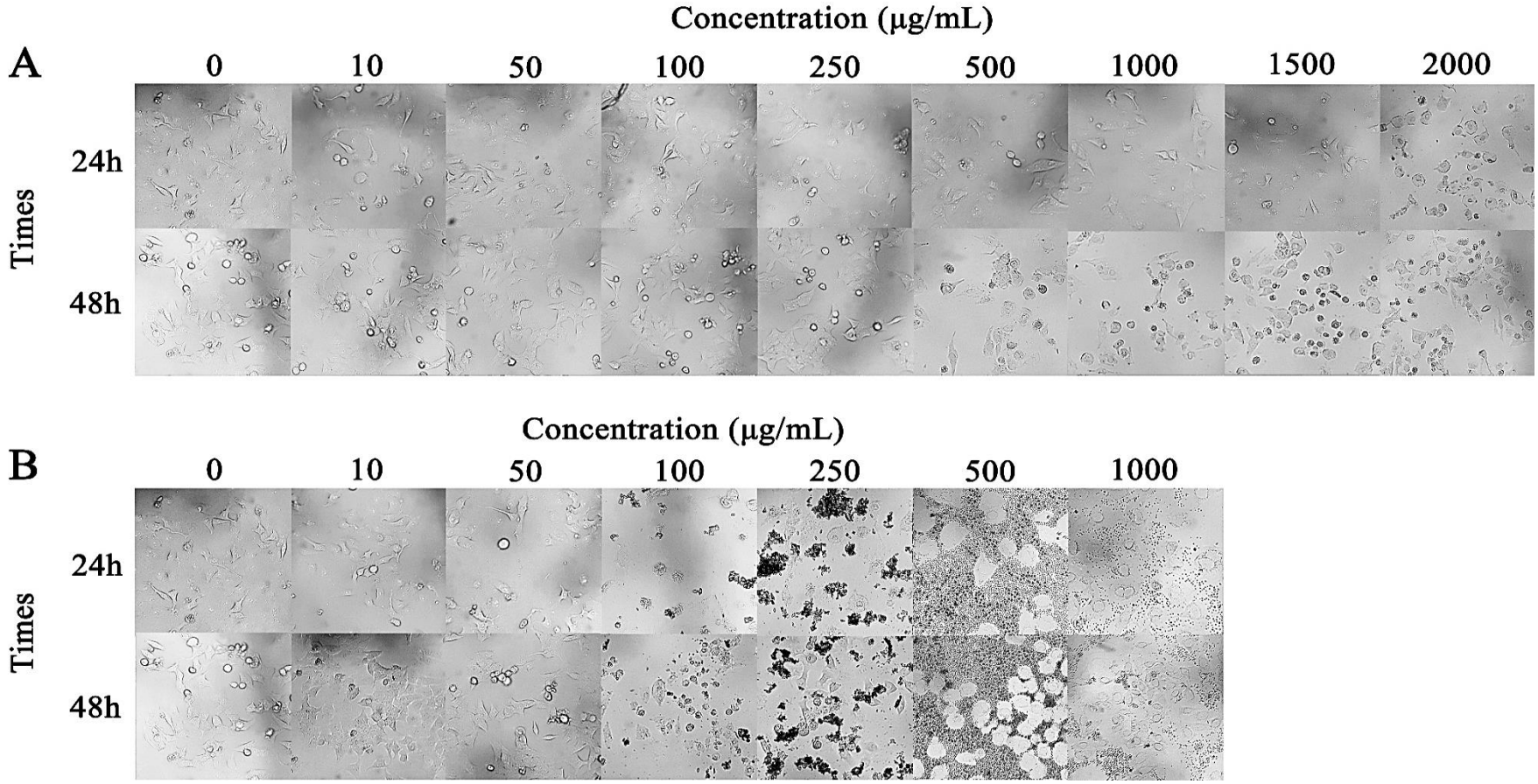

Figure 29 Cell morphology of human hepatocellular liver carcinoma cell line (HepG2) after treatment with (A) LV2 and (B) Lentinan at various concentration (0, 10, 50, 100, 250, 500, 1000, 1500, and 2000 μg/mL) for 24 and 48 h.

Then, the polysaccharides were evaluated for antioxidant activities by scavenging the DPPH radical, compare with ascorbic acid, an antioxidant standard. The decrease in the concentration of DPPH was observed when tested with LV2 and Lentinan in a concentration-dependent manner (Fig. 30). Lentinan showed 50% radicle scavenging activity (IC50) at concentration 341.18 µg/mL (Fig. 30) whereas IC50 value of ascorbic acid was only 37.78 µg/mL (Fig. 31). LV2 had a lower antioxidant activity of approximately 40% at 2,000 µg/mL. In contrast, LV1 and LV2 demonstrated no antioxidant activity (Fig. 30).

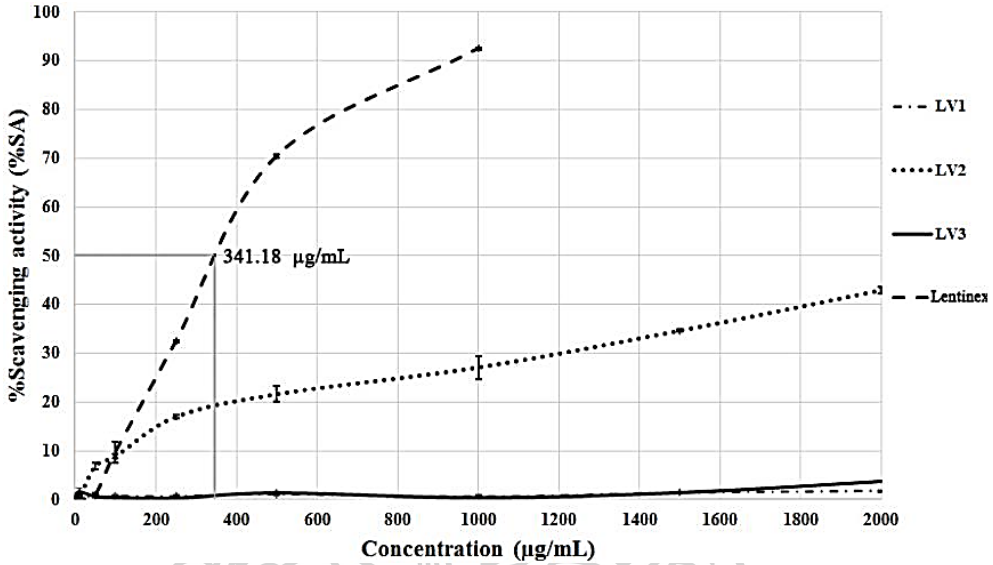

Figure 30 Free radical scavenging activity of LV1, LV2, LV3, and Lentinan on DPPH at various concentration (0, 10, 50, 100, 250, 500, 1000, 1500, and 2000 µg/mL). Error bars indicate means ± standard deviation (n = 3) and the line and numbers indicated the IC50 values.

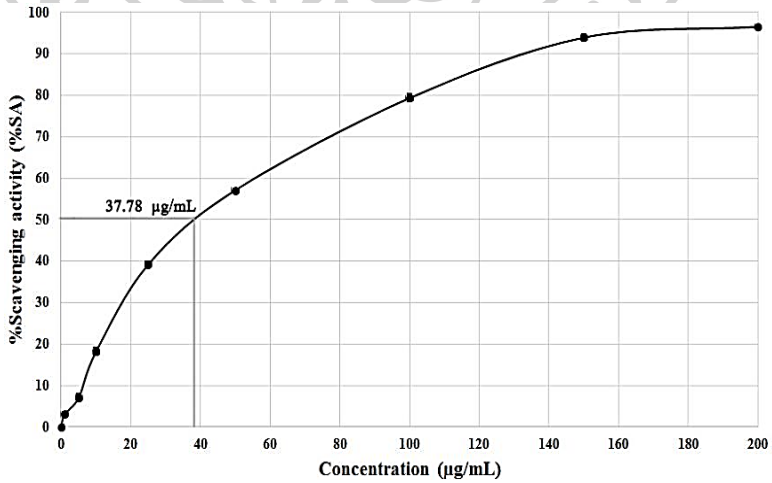

Figure 31 Free radical scavenging activity of ascorbic acid on DPPH at various concentration (0, 1, 5, 10, 25, 50, 100, 150, and 200 µg/mL). Error bars indicate means ± standard deviation (n = 3) and the line and numbers indicated the IC50 values.

The structure characterization of LV2

The chemical structure of polysaccharide LV2 was elucidated by GPC, and FTIR. The total polysaccharide content was 99.01% (Table 6). The absorbance at 260 and 280 nm were not detected, indicating that this polysaccharide does not contain neither protein nor nucleic acid.

The GPC was used for determining the average molecular size. Results showed 2 symmetric peaks with retention times of 14.051 and 18.654 min with average molecular weight of 335,970 and 5,654 Da, respectively (Fig. 32). The polydispersity index (DPI, Mw/Mn) of both peaks were calculated which were 1.56 and 1.84, respectively. This DPI indicates a broader distribution of the molecular weight of polysaccharide LV2.

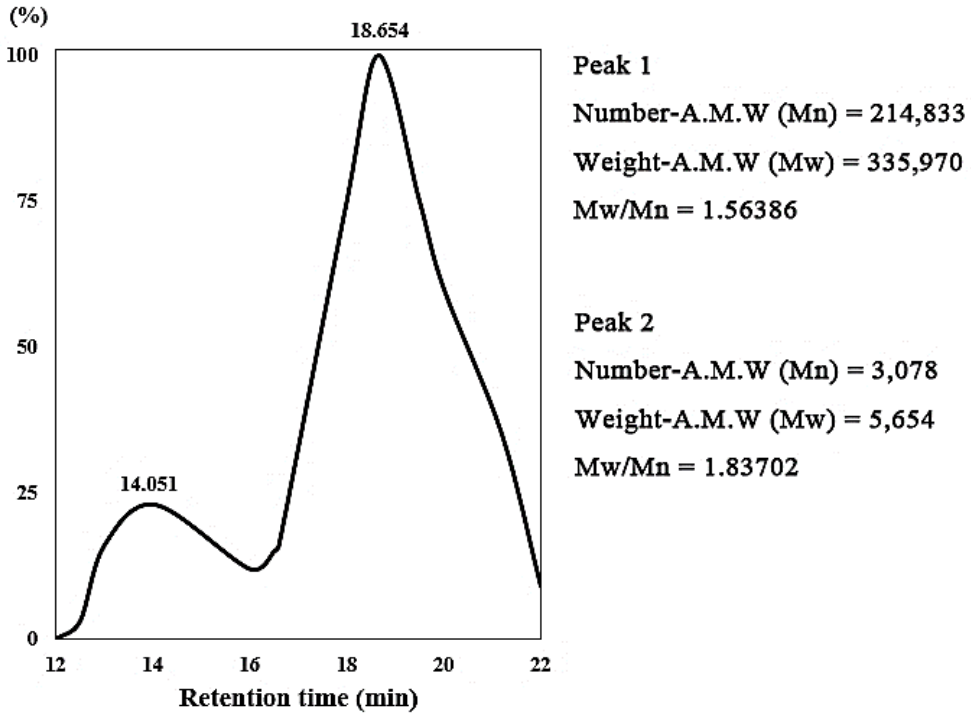

Figure 32 The molecular weight of the polysaccharide LV2 was determined by gel permeation chromatography. The average molecular weights of the polysaccharide LV2 were 3.35×10^5 Da (left), and 5.65×10^3 Da (right).

In the FT-IR spectrum of the polysaccharide LV2 indicated absorption at 3300, 2929, 1640, 1460, 1263, 1106, and 915 cm^{-1} (Fig. 33). The O-H stretching as a broad peak at 3200-3400 cm^{-1}. The bands in the region of 2800 - 3000 cm^{-1} are due to C-H stretching. The band of associated water is occurred around 1600 cm^{-1}. Peak at 1460 cm^{-1} was from CH_2 stretching vibration. The absorptions at about 1200 cm^{-1}, and between 1000 – 1200 cm^{-1} are assigned to be the stretching vibrations of C-O in sugar ring, and C-O-C, respectively. The peaks in the range of 750 – 950 cm^{-1} are anomeric regions. These results showed that polysaccharide LV2 was a polysaccharide with sugar ring structures. Furthermore, there was no absorption peaks between 1700 – 1750 cm^{-1}, indicating the absence of uronic acid (Fig. 33).

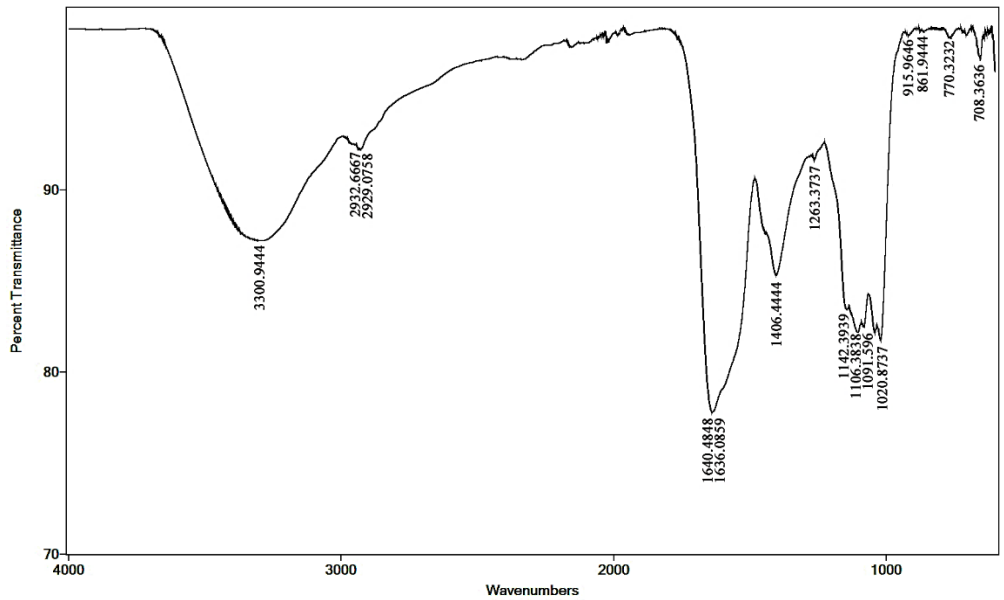

Figure 33 IR spectrum of the polysaccharide LV2 (600 – 4000 cm^{-1}). The polysaccharide LV2 was analyzed the function groups by using ATR-FTIR.

The monosaccharide composition analysis of the polysaccharide LV2 was preliminary determined by TLC technique. Result revealed that the hydrolysate LV2 showed only one green spot with the retention factor (R_f) was 0.51 (Fig. 34) which share the same color and R_f of the standard glucose spot. Thus, the polysaccharide LV2 was composed only glucose as a sugar subunit.

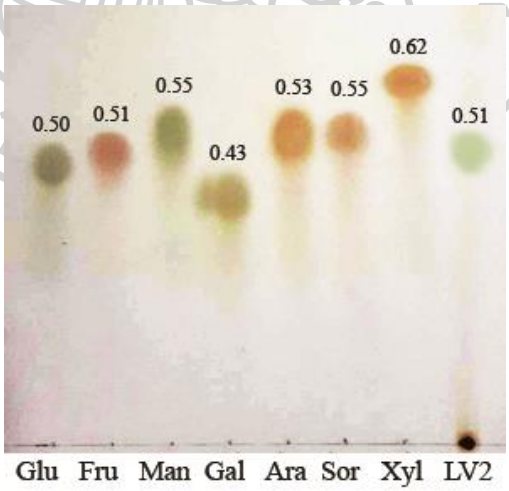

Figure 34 Chromatographic spots from thin layer chromatography (TLC) for monosaccharide of the polysaccharide LV2 which was hydrolyzed with 2M TFA for 18 h. The standard used were glucose (Glu), fructose (Fru), mannose (Man), galactose (Gal), arabinose (Ara), sorbitol (Sor), and xylose (Xyl). The numbers indicated as retention factor (R_f) values.

CHAPTER V
DISCUSSION

Natural products from the edible mushrooms are the one attractive choices for treating the cancer disease which show the less-toxic and lower adverse effects to patients than available chemical drugs. They have been also reported as dietary supplements and sources of medicinal compounds (Ferrari *et al.*, 2012; Yukawa *et al.*, 2012, Bhanot, *et al.*, 2011; Mantovani *et al.*, 2008).

L. edodes is the medicinal mushrooms which was studied and showed numerous pharmacological properties, such as antimicrobial, antiviral, antioxidant, anti-inflammatory, antiatherogenic, hypoglycemic, hepatoprotective, anticancer, and immunomodulating properties (Ferreira *et al.*, 2010; Rai *et al.*, 2005; Zaidman *et al.*, 2005). *L. edodes* is the one of the mushroom species which have been studied on pharmalogical properties, especially immunomodulating and anticancer. Lentinan is the best well-known ß-glucan extracted form *L. edodes*, which shows the great immunomodulatory and anticancer activity (Chihara *et al.*, 1970; Zhang *et al.*, 2007). The most *Lentinus* species are edible and cultivable. They provide locals with seasonal food, medicine and alternative income (Karunarathna *et al.*, 2011). Nevertheless, many species of *Lentinus* found in Thailand were little studies on their bioactivity.

In this study, four *Lentinus* spp. were collected and isolated. They were studied about their morphology and identified according to Pegler (1938) as *L. sajor-caju* strain EB1001, *L. swartzii* strain EB1101, *L. squarrosulus* strain WCR1201, and *L. velutinus* strain WCR1104. To confirm morphology-based identification, the genomic DNA of mushroom samples was extracted, and the ITS of mushroom samples was amplified, sequenced and compared to database. The results indicated that phylogenetic study of the ITS sequences supported the morphological identification.

The crude polysaccharides were extracted by using two different solvents, hot water and ethanol. Due to several studies reported that the mushroom polysaccharides obtained from hot water and alcohol extraction revealed the anticancer activity. The result indicated that the yield of crude ethanolic extracts showed a higher than that of crude aqueous extracts, probably due to their oily textures. However, the total carbohydrates contents of crude aqueous polysaccharides (20.04 – 43.32%) was higher than crude ethanolic polysaccharides (8.30% – 25.17%). The total protein contents of crude aqueous and crude ethanolic extracts were between 17.7-45.3% and 7.2-16.2%, respectively. The ratio of polysaccharide to protein from crude aqueous and ethanolic extracts were 1.35 and 1.84 on average, respectively. Our results agree with Dong and Yao (2008), who reported the ratios of polysaccharide to proteins of crude polysaccharides from fruiting bodies (1.0 ratio) and cultured mycelia (1.9 ratio) of *Cordyceps sinensis*. During extraction, free proteins were eliminated by using Sevag's reagent. However, some protein was presented in crude extracts. Thus, it is possible that the obtained crude extracts may be polysaccharide-protein complex substances; some mushroom extracts were reported as polysaccharide-protein complexes, such as PPC-P11 from

Phaeogyroporus portentosus (Karnchanatat *et al.*, 2013). Nevertheless, this speculation should be examined with respect to the purified fractions.

Next, the crude polysaccharides were evaluated the anticancer activity against HeLa and HepG2 cell lines, the results uncovered that the crude extract from fruiting bodies of *Lentinus velutinus* (LVFB-Aq) showed the highest anticancer activity against both the HeLa and the HepG2 cell lines, which reached 49.83% and 48.51% inhibition, respectively. While, the ethanolic extracts from fruiting bodies of *L. velutinus* (LVFB-Et), and *L. squarrosulus* (LSqFB-Et) showed the most toxicity effect against only the HeLa cells after treatment for 24 h, which was 35.95, and 31.17%inhibition, respectively. Although anticancer capability of the ethanolic extracts were lower than the aqueous extracts which probably due to the tested concentration, but I could not test the ethanolic extracts at the higher concentration because the extracts were precipitated when dissolved with 1%DMSO (in water). Except the concentration, these differences of anticancer activity between aqueous and ethanolic extracts probably was due to the extraction solvents, or the type of obtained substances. Ethanol can extract both polar and nonpolar compounds such as fatty acids, sterols, terpenoids, polypeptides and amino acids, while carbohydrates and some proteins can be soluble in water (Beattie *et al.*, 2011). Most anticancer substances extracted from mushrooms have been reported as polysaccharides, proteins, and polysaccharide-protein complexes. Moreover, the different of polysaccharide structures probably effected to the different properties.

The crude polysaccharide LVFB-Aq was chosen for further separated due to it showed the anticancer activity against both cancer cell lines. The polysaccharide was separated by re-dissolving in water, and re-precipitation with ethanol into three fractions e.g. fraction E1, E4, and R. Fraction E4 was a main fraction which yield 2,279 mg (43.18%). However, the carbohydrate contents of fraction E1 was the highest (70.91%). Among these fractions, the fraction E4 had the greatest anticancer activity. They could decline the viability of both cell lines at concentration 5 which was 24.32% and 72.18% for the HeLa and the HepG2, respectively, and the both cell line were completely killed at concentration 10 mg/mL. While, fraction E1 and R could not decrease the growth of cells. These difference in anticancer activity may be due to the molecular size and structure of polysaccharides. In this step, the ethanol played as antisolvent which can precipitate a various molecular size of polysaccharides in concentration-dependent manner. The higher molecular weight polysaccharides were precipitated at the low concentration of ethanol solution, whereas the high concentration of ethanol favored precipitation of the smaller molecular size of polysaccharides (Zou *et. al.*, 2013; Xue *et. al.*, 2012). Moreover, Xu *et. al.* (2014) reported that not only the molecular size of polysaccharide, but also structural features can be significantly response to ethanol concentration, too.

Next, Fraction E4 was fractionated by anion exchange chromatography using DEAE FF column (5 mL), eluted with step concentration of NaCl (0, 0.05, 0.1, 0.2, and 0.5M) at flow rate 1 mL/min. It was separated into 6 fractions, namely fraction E4W, E4N1, E4N2, E4N3, E4N4, and E4N5. Fraction E4W showed the highest yield (210 mg) and percentage of carbohydrate content (91.78%), whereas fraction E4N2 was the lowest (64 mg and 15.67%, respectively). However, fraction E4N5 indicated the highest inhibition effect against the HeLa which was 26.65% inhibition at concentration 2 mg/mL, and also insignificantly inhibited the HepG2 at

concentration 2 mg/mL (13.59%). The anticancer capability probably due to the negative charges presented on polysaccharides. The DEAE FF column containing the positive charges resins as a stationary phase which can be retained the negatively charged polysaccharides. Polysaccharides could be 'weak' polyelectrolytes called pseudo-polyelectrolytes. Since the neutral polysaccharides typically dissolve in polar solvents e.g. water, they can gain charge by association of proton or small anion with their ionizable units such as electronegative oxygen. After that the polysaccharides were eluted in order of their respective negative charges and replaced by chloride ions once again, the one with weaker negative charge being replaced first. Thus, the negative charged polysaccharide could be sorted as followed: fraction E4N1 < E4N2 < E4N3 < E4N4 < E4N5. These related to the anticancer results, which higher negative charged polysaccharides showed higher inhibition effects against HeLa and HepG2 cells.

Final purification step, the fraction E4N5 was applied to sephadex G-100 column (50 cm), which separated into 3 polysaccharides e.g. LV1, LV2, and LV3. The main polysaccharide was the polysaccharide LV2 (38.4 mg) with 99.01% carbohydrate contents. These polysaccharides were tested the anticancer and antioxidant activity comparing to Lentinan. The data showed that LV2 had a better anticancer against HeLa and HepG2 than LV1, and LV3 in concentration-dependent manner which was IC50 at concentration 2,000 and 1,935 µg/mL when treated cells for 48 h, respectively. Nevertheless, these were still lower than those of Lentinan (IC50 = 273.21 and 319.64 µg/mL for HeLa and HepG2 at 48 h, respectively). Moreover, the both polysaccharides, LV2 and Lentinan, also cytotoxic against L929 cells which the IC50 at 48 h was 1490 and 550 µg/mL, respectively. A number of studies have indicated that polysaccharide extract form agarics inhibited the proliferation of cancer cell line. For instance, LEP, an ethanol precipitate of a dried powder extracted from *Lentinus edodes* mycelium, showed direct cytotoxicity to HepG2. The morphology of HepG2 cells treated with LEP were shrunk, rounded, and floated. The viability of HepG2 treated with LEP at 200 µg/mL for 24 h was 59.9% (Yukawa *et al.*, 2012). Li *et al.* (2012) evaluated the effect of acid, water, and alkaline extract of crude polysaccharide from eight Chinese mushrooms on HeLa and HepG2 cell proliferation. The viability of the cancer cell lines treated with extracts at 600 µg/mL for 48 h was 0-67.9%. The most polysaccharide derived from mushrooms are the non-digestive molecules due to human enzyme cannot break the beta bond, which present in the polysaccharide such as glucan, glycan etc (Bhakta & Kumar, 2013). Thus, these molecules can be absorped and activated the immune system on various mechanism. Innate immunity system, polysaacharides play a role as antigen-presenting cells (APCs). After oral administration, they enter to small intestinal and were captured by macrophage. The activated macrophage with polysaccharide fragment could attack the dead cells. Moreover these macrophages also create mytokines, which are cytotoxic to cancer cells (Moradali et al., 2007; Silva et al., 2012). In addition, the mushroom polysaccharides act as pathogen in adaptive immunity system which can associated with several receptors on cell membrane such as dectin-1, complement receptor-3 (CR-3), scavenger receptor, lactosylceramide, and toll-like receptors (TLR) and activates the signaling pathways to promote the innate immunity responses, and to induce the reactive oxygen species and inflammatory cytokines production (Moradali et al., 2007; Silva et al., 2012; Zong et al., 2012).

The antioxidant evaluation revealed the results as well as the anticancer activity. Lentinan was a good antioxidant followed by the polysaccharide LV2. While LV1 and LV3 were not any effects. The antioxidant effects could be supporting the anticancer therapy, in other words cancer cells have a higher oxidative stress level when compared to normal cells, which related with the rise production of ROS. The ROS may alter the signaling pathway resulting to contribute the malignant cells. The antioxidants could be scavenging the free radical and prevent the oxidative damage which associated with cancer development. As a result, the development of cancer cells was slow down and may be lead cells to apoptosis (Mut-Salud *et. al.*, 2015).

The average molecular weight of the polysaccharide LV2 was estimated as ~336 kDa. However, the second peak presented at retention time 18.654 min which the average molecular weight was about 5,600 Da. This low molecular weight probably was a polysaccharide derived from the degradation of the polysaccharide LV2 during storage. Because the polysaccharide was dialyzed with dialysis bag molecular weight cut-off 6-8,000 Da during purification steps, which larger than the molecular size of appeared polysaccharide. Besides, the polysaccharide LV2 also went through several processes in the purification such as heating, drying, thawing, and freezing, including being kept for a long period. These might be the factors causes the degradation. Szymańska and Winnicka (2015) reported that the rate of chitosan decomposition during heating was accelerate with increase temperatures and heating times. From this experiment, it was possible that the anticancer activity of the polysaccharide LV2 was not good as an expectation, this might due to the lower molecular size polysaccharide interfered the action of effective molecules, causes this effective molecule could not fully functional.

The IR spectra of polysaccharide LV2 displayed the typical signal pattern expected for a carbohydrate moiety, and several bands in the anomeric region (750 – 950 cm^{-1}). These IR spectra was compared to those found in the other works for supporting the results. The infrared spectra of the polysaccharide WPLE (Jeff *et. al.*, 2012) indicated the characteristic absorption at the regions of 1650, 1400, and 1250 cm^{-1} (Fig. 34). The FT-IR spectra of water-soluble polysaccharides from *G. tsugae* mycelium (Fig. 35; Peng *et. al.*, 2005) also exhibited the same pattern and absorption regions as the spectra of LV2. The monosaccharide analysis by TLC method revealed that the polysaccharide LV2 contains only glucose unit which can be assigned to the glucan.

Among the results indicated that the polysaccharide from *L. velutinus* might be an anticancer agent. Nevertheless, the extraction and separation process must be improved and developed for extracting the highest effective polysaccharides.

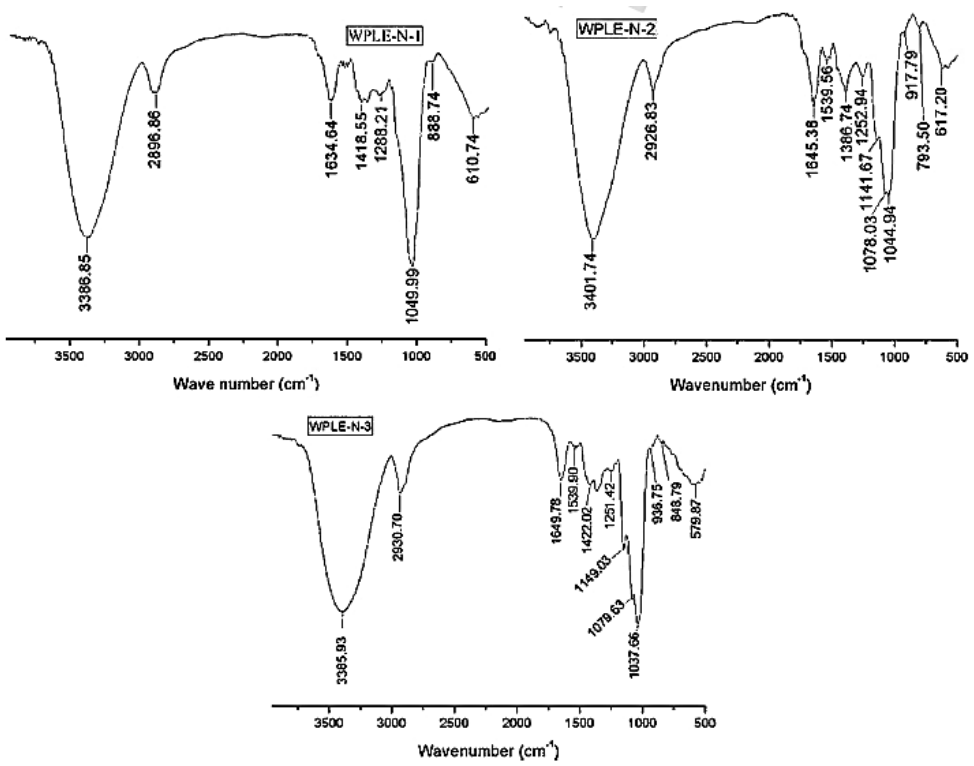

Figure 35 The infrared spectra of WPLE-N-1, WPLE-N-2, and WPLE-N-3 (Jeff *et. al*., 2012)

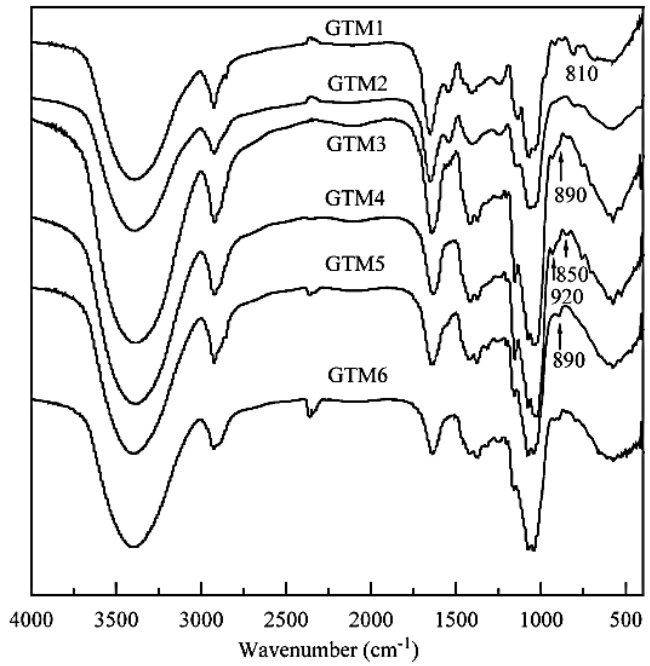

Figure 36 The infrared spectra of water-soluble polysaccharidesfrom *G. tsugae* mycelium (Peng *et. al.*, 2005).

CHAPTER VI

CONCLUSIONS

Four *Lentinus* spp. collected from Nakhon Pathom, Ratchaburi, and Kanchanaburi province were identified according to Pegler (1938) as *L. sajor-caju* strain EB1001, *L. swartzii* strain EB1101, *L. squarrosulus* strain WCR1201, and *L. velutinus* strain WCR1104.

Hot water and ethanolic extraction of fruit bodies and mycelia of *L. edodes*, and four *Lentinus* species, i.e. *L. sajor-caju, L. swartzii. L. squarrosulus* and *L. velutinus,* showed amounts of crude polysaccharides ranging from 33.6 to 205.3 mg/g dry weight of sample.

The crude aqueous extracts from fruiting bodies of *L. velutinus* (LVFB-Aq) showed the highest anticancer activity against HeLa and HepG2 cell lines which reached 49.83% and 48.51% inhibition, respectively. It had also no any toxic against normal cell lines.

Crude aqueous extract, LVFB-Aq (5.278 g) was separated by re-dissolving in sterile water and precipitating with cold 95% ethanol, into three fractions e.g. fraction E1 (1.472 g), fraction E4 (2.279 g), and fraction R (1.526 g).

Fraction E4 could decreased the viability of both cancer cell lines, HeLa and HepG2, at concentration 5 mg/mL. It had also no any toxic against normal cell lines.

The fraction E4 (2,179 mg) was fractionated by anion exchange chromatography into 6 fractions, namely fraction E4W (210 mg), E4N1 (77 mg), E4N2 (64 mg), E4N3 (83 mg), E4N4 (105 mg), and E4N5 (92 mg).

Fraction E4N5 has the greatest anticancer efficacy by significantly inhibiting the growth of HeLa cell line about 17% and 26.65% at concentration 1 and 2 mg/mL, respectively. It had also no any toxic against normal cell lines.

The fraction E4N5 (85 mg) was separated by size exclusion chromatography into 3 substances, namely LV1 (3.7 mg), LV2 (38.4 mg), and LV3 (8.9 mg). Lentinex indicated the highest anticancer capability against HeLa and HepG2 cells, which IC50 at 24 h was 322.73 and 659.09 µg/mL, and at 48 h was 273.21 and 319.64 µg/mL for HeLa and HepG2, respectively. While, the polysaccharide LV2 slightly inhibited the growth of cancer cell lines, which IC50 of HeLa and HepG2 at 48 h was 2,000 and 1,935 µg/mL, respectively.

The polysaccharide LV2 and Lentinex were toxic to L929 cell line, which IC50 of LV2 and Lentinex at 48 h was 1490 and 550 µg/mL, respectively.

The CPE of HeLa and HepG2 cells were induced at the higher concentration (>1000 µg/mL of LV2, and >250 µg/mL of Lentinex), cells and their nuclei were swelling and round, the cell density and adherent capacity decreased.

The polysaccharide LV2 had a lower antioxidant activity of approximately 40%SA at 2,000 µg/mL. Lentinex showed 50% radicle scavenging activity (IC50) at concentration 341.18 µg/mL, whereas IC50 value of ascorbic acid was only 37.78 µg/mL.

The total polysaccharide content of the polysaccharide LV2 was 99.01%. The protein contents and nucleic acid contamination had no detected.

The weight average molecular weight of the polysaccharide LV2 was ~336 kDa. The polydispersity index (DPI, Mw/Mn) was 1.56.

From IR spectra indicated that the polysaccharide LV2 was a polysaccharide with sugar ring structures and absence of uronic acid.

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APPENDIXES

Appendix A: Culture medium preparation

1. Potato dextrose agar (PDA)

Medium composition:

PDA Powder (Criterion, USA)	39.0	g
Distilled water adjusts to 1 liter		

The PDA powder is dissolved in distilled water and sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes. The cooled medium at 50^{O}C should be poured into Petri dishes.

2. Luria-Bertani (LB) medium

Medium composition:

Tryptone (Biomark, India)	10.0	g
Yeast extract (Biomark, India)	5.0	g
NaCl (Chemex, USA)	10.0	g
Distilled water adjusts to 1 liter		

Tryptone, yeast extract and NaCl are dissolved in 950 mL of distilled water. The pH should be adjusted to 7.0. Then, the volume is adjusted to 1 liter with distilled water and sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

3. Luria-Bertani (LB) agar

Medium composition:

Tryptone (Biomark, India)	10.0	g
Yeast extract (Biomark, India)	5.0	g
NaCl (Chemex, USA)	10.0	g
Agar (Mermaid, Thailand)	15.0	g
Distilled water adjusts to 1 liter		

Tryptone, yeast extract, NaCl, and agar are dissolved in 950 mL of distilled water and heat until the agar was completely dissolved. The pH should be adjusted to 7.0. Then, the volume is adjusted to 1 liter with distilled water and sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes. The cooled medium at 50^{O}C should be poured into Petri dishes.

4. Malt extract broth (MEB)

Medium composition:

Malt extract (Biomark, India)	17.0	g
Peptone (Biomark, India)	3.0	g
Distilled water adjusts to 1 liter		

Malt extract and peptone are dissolved in 950 mL of distilled water. The pH should be adjusted to 7.0. Then, the volume is adjusted to 1 liter with distilled water and sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

5. Super Optimal Broth (SOB) medium

Medium composition:

Yeast extract (Biomark, India)	5.0	g
Tryptone (Biomark, India)	20.0	g
NaCl (Chemex, USA)	0.5	g
Distilled water adjusts to 1 liter		

Yeast extract, tryptone, and NaCl are dissolved in 950 mL of distilled water. The pH should be adjusted to 7.0. Then, the volume is adjusted to 1 liter with distilled water and sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

6. Super Optimal broth with Catabolite repression (SOC) medium

Medium composition:

SOB medium	100.0	mL
2M Glucose solution	1.0	mL

SOB medium and glucose solution were mixed, and heat at 42^{O}C for 10 minutes.

7. Mushroom complete medium (MCM)

Medium composition:

Thiamine-HCl (Biomark, India)	1.0	mg
$FeSO_4 \cdot 7H_2O$ (Biomark, India)	10.0	mg
$MnSO_4 \cdot H_2O$ (Chemex, USA)	1.6	mg
$CuSO_4$	1.0	mg
KH_2PO_4	1.0	g
$CaCl_2 \cdot 2H_2O$	0.5	g
$MgSO_4 \cdot 7H_2O$	0.5	g
$NaNO_3$	2.0	g
Fructose	15.0	g
Starch soluble	40.0	g
Distilled water adjusts to 1 liter		

All chemicals are dissolved in 950 mL of distilled water and boiled until starch soluble is completely dissolving. The pH should be adjusted to 7.0. Then, the volume is adjusted to 1 liter with distilled water and sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

Appendix B: Chemicals preparation

1. 1M Tris-HCl pH 8.5

Chemical composition:

Tris-HCl (Fisher chemicals, UK)	12.11	g
Distilled water adjusts to 100 mL		

Tris-HCl powder is dissolved in 80 mL of distilled water and the pH is adjusted to 8.5. The volume is adjusted to 100 mL. The solution is sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

2. 0.5M EDTA pH 8.0

Chemical composition:

EDTA (VWR International, UK)	14.61	g
Distilled water adjusts to 100 mL		

EDTA powder is dissolved in 80 mL of distilled water and the pH is adjusted to 8.0. The volume is adjusted to 100 mL. The solution is sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

3. 25%(w/v) SDS solution

Chemical composition:

SDS (Fisher chemicals, UK)	25.0	g
Distilled water adjusts to 100 mL		

SDS powder is dissolved in 80 mL of distilled water and heat at 60^{O}C until the powder is completely dissolved. The volume is adjusted to 100 mL.

4. 1M NaCl

Chemical composition:

NaCl (Chemex, USA)	5.84	g
Distilled water adjusts to 100 mL		

NaCl is dissolved in distilled water and sterilized by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

5. Extraction buffer

Chemical composition:

1M Tris-HCl [pH 8.5]	20.00	mL
1M NaCl solution	25.00	mL
0.5M EDTA [pH 8.0]	5.00	mL
25%(w/v) SDS	2.00	mL
Distilled water adjusts to 100 mL		

All solutions are sterile and mixed.

6. TE buffer

Chemical composition:

1M Tris-HCl [pH 8.5]	1.00	mL
0.5M EDTA [pH 8.0]	0.20	mL
Distilled water adjusts to 100 mL		

All solutions are sterile and mixed.

7. Alkaline copper reagent

Reagent A

Chemical composition:

$NaCO_3$ (BDH, UK)	1.00	g
NaOH (Univar, Australia)	0.20	g
Distilled water adjusts to 100 mL		

Reagent B

Chemical composition:

$CuSO_4 \cdot 5H_2O$ (QReC, New Zealand)	0.156	g
$KNaC_4H_4O_6$ (QReC, New Zealand)	0.237	g
Distilled water adjusts to 20 mL		

Alkaline copper reagent is fresh preparation by mixing 100 mL of reagent A and 2 mL of reagent B.

8. DNSA reagent

Chemical composition:

NaOH (Univar, Australia)	10.00	g
3,5 dinitrosalicylic acid (Univar, Australia)	10.00	g
Na_2SO_3 (BHD, UK)	0.5	g
Phenol (Merck, Germany)	2.00	g
Distilled water adjusts to 1 L		

Dissolve the NaOH pellet in 900 mL of distilled water, and then 3,5 dinitrosalicylic acid is added and mixed. Add the Na_2SO_3 and mixed until it is completely dissolve. Followed by adding the phenol pellet. The volume is adjusted to 1 L.

9. 40%(w/v) Potassium sodium tartate

Chemical composition:

$KNaC_4H_4O_6$ (QReC, New Zealand)	40.00	g
Distilled water adjusts to 100 mL		

Dissolve the potassium sodium tartate with 100 mL of distilled water. The solution is sterile by autoclave at 15 psi of pressure, $121^{O}C$ for 15 minutes.

10. 7%(w/v) Sodium carbonate

Chemical composition:

Na_2CO_3 (BHD, UK)	7.00	g
Distilled water adjusts to 100 mL		

Dissolve the sodium carbonate with 100 mL of distilled water. The solution is sterile by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

11. 50 mM Sodium-phosphate buffer [pH 7.0] with 0.15 M NaCl

Chemical composition:

$Na_2HPO_4 \cdot 7H_2O$ (Unilab, Australia)	8.039	g
$NaH_2PO_4 \cdot 2H_2O$ (BHD, UK)	2.496	g
NaCl (Chemex, USA)	8.766	g
Distilled water adjusts to 1 L		

Dissolve the chemical with 1 L of distilled water. The solution is sterile by autoclave at 15 psi of pressure, 121^{O}C for 15 minutes.

12. Sevag's reagent

Chemical composition:

$CHCl_3$ (Merck, Germany)	750	mL
n-Butanol (Merk, Germany)	150	mL

These chemicals are mixed together in bottle amber. The reagent is stored at room temperature.

13. 0.2 mM DPPH solution

Chemical composition:

DPPH (Sigma, USA)	39.4	mg
Metanol (BHD, UK)	500	mL

Dissolve the DPPH powder with absolute methanol. The reagent is stored in bottle amber at 4°C.

14. FAA reagent

Chemical composition:

Formaldehyde (Merck, Germany)	10.00	mL
95%(v/v) Ethanol (Merck, Germany)	70.00	mL
Acetic acid (Merck, Germany)	5.00	mL
Water	15.00	mL

Mix all the chemicals. The reagent is stored in bottle at room temperature.

15. 0.5 mg/mL MTT solution

Chemical composition:

MTT (Sigma, USA)	50.00	mg
RPMI-1640 medium (Gibco, USA)	100	mL

Dissolve the MTT powder with RPMI-1640 medium and sterile by filtration with 0.45 µM filter. The reagent is stored at -20°C until us

Appendix C: Standard curve.

1. Total carbohydrate standard curve by phenol-sulfuric assay

Table 7 The phenol-sufuric assay glucose standard at various concentration by monitoring spectrophotometrically at 490 nm

	Glucose concentration (µg/mL)					
	0	20	40	60	80	100
A490	0	0.191	0.424	0.615	0.838	0.997
	0	0.233	0.419	0.573	0.852	0.988
	0	0.239	0.43	0.642	0.875	0.989
Mean	**0**	**0.221**	**0.424**	**0.61**	**0.855**	**0.991**
SD	0	0.026	0.006	0.035	0.019	0.005

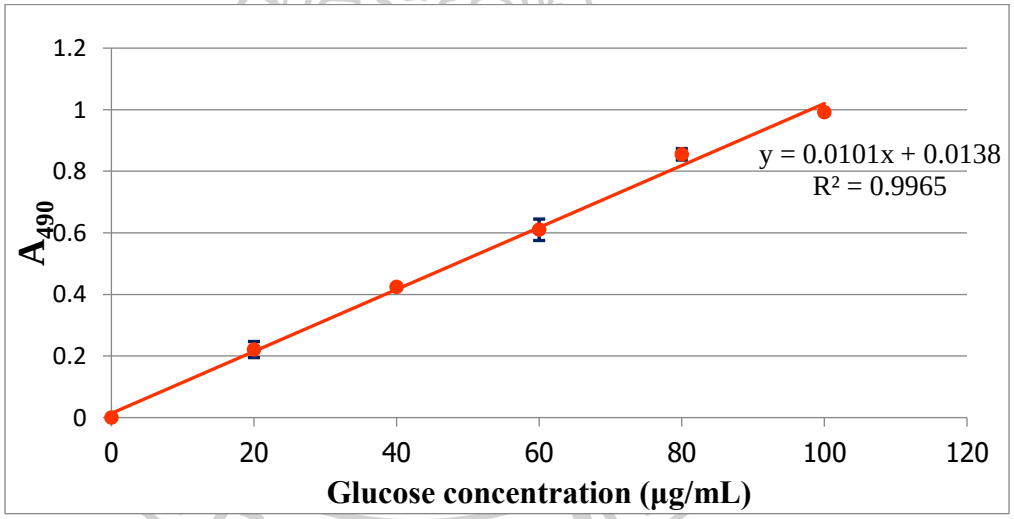

Figure 37 The total carbohydrate standard curve by phenol-sulfuric assay.

2. Total reducing sugar standard curve by DNS assay

Table 8 The DNS assay of glucose standard at various concentration by monitoring spectrophotometrically at 550 nm.

	Glucose concentration (µg/mL)					
	0	20	40	60	80	100
A550	0.000	0.162	0.415	0.625	0.846	1.034
	0.000	0.161	0.396	0.605	0.828	1.062
	0.000	0.170	0.425	0.634	0.842	1.032
Mean	**0.000**	**0.164**	**0.412**	**0.621**	**0.839**	**1.043**
SD	0.000	0.005	0.015	0.015	0.009	0.017

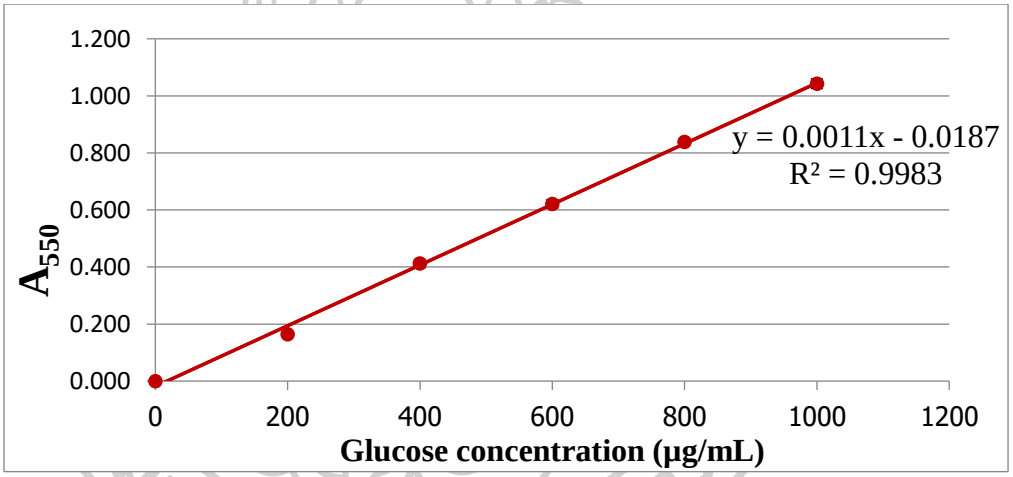

Figure 38 The total reducing sugar standard curve by DNS assay.

3. Total protein standard curve by Lowry's method.

Table 9 The Lowry's method of BSA at various concentration by monitored spectrophotometrically at 500 nm.

	BSA concentration (µg/mL)					
	0	200	400	600	800	1000
A500	0.000	0.115	0.208	0.272	0.322	0.378
	0.000	0.121	0.201	0.272	0.333	0.369
	0.000	0.121	0.190	0.275	0.335	0.366
Mean	**0.000**	**0.119**	**0.200**	**0.273**	**0.330**	**0.371**
SD	0.000	0.003	0.009	0.002	0.007	0.006

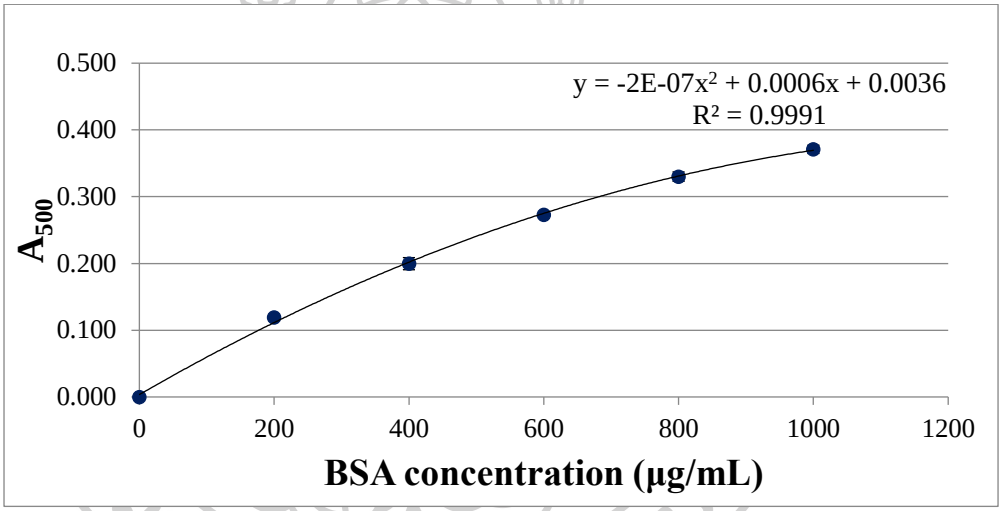

Figure 39 The total protein standard curve by Lowry's method.

4. Total phenol content standard curve by Folin-Ciocalteu reagent method.

Table 10 The Folin-Ciocalteu reagent method of gallic acid at various concentration by monitored spectrophotometrically at 725 nm.

	Gallic acid concentration (µg/mL)					
	0	2	4	8	16	32
A725	0.000	0.071	0.158	0.307	0.657	1.210
	0.000	0.078	0.150	0.324	0.654	1.286
	0.000	0.081	0.159	0.328	0.662	1.308
Mean	**0.000**	**0.077**	**0.156**	**0.320**	**0.658**	**1.268**
SD	0.000	0.005	0.005	0.011	0.004	0.051

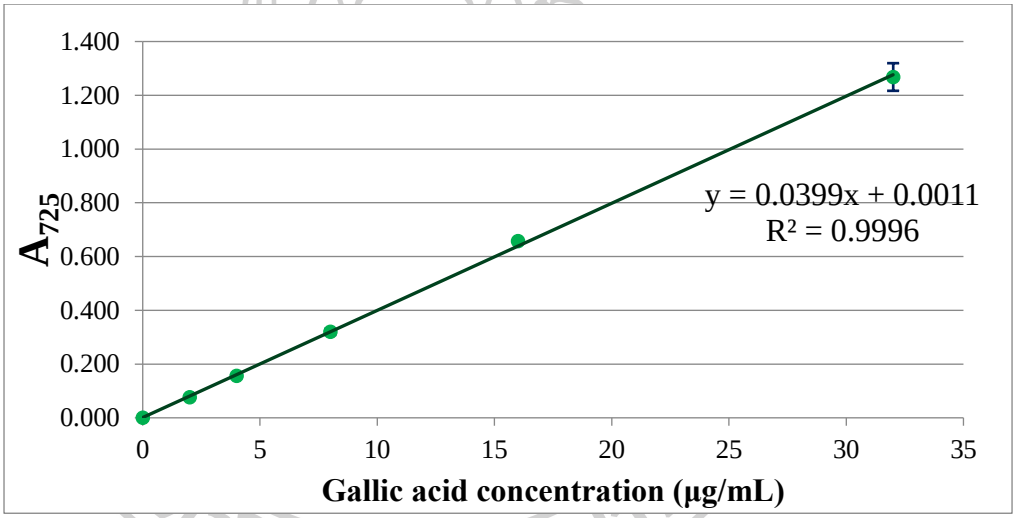

Figure 40 The total phenol content standard curve by Folin-Ciocalteu reagent method.

Appendix D: ITS sequence

1. *Lentinus sajor-caju* strain EB1001 (KT956122)

GAATTCGCCCTTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGT
TATTGAAACGGGTTGTAGCTGGCCTTACGAGGCATGTGCACGCCCTGC
TCATCCACTCTACACCTGTGCACTTACTGTGGGTTTCAGGAGCTTCGAA
AGCGGAGGGCCTTTGTGGGCTTTTCGTTATTAGTTGTGACTGGGCTCAT
GTCCACTACAAACTCTTATAAAGTAACAGAATGTGTATTGCGATGTAA
CGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAG
GAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTGACGGGTTCTTA
ACGGAGCTTGGTTCAGGCTTGGACTTGGAGGCTTGTCGGCTTGCTTTGT
CGAGTCGGCTCCTCTCAAATGCATTAGCTTGGTTCTTTGCGGATCGGCT
CACGGTGTGATAATTGTCTACGCCGCGACCGTTGAAGCGTTTGAATGG
GCCAGCTTATAGTCGTCTCCATCGCGAGACAACATTTCATCGAACTCT
GACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAA
GCGGAGGAAAGGGCGAATTC

2. *Lentinus swartzii* strain EB1101 (KT956124)

TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTTTGAAACGGGT
TGTAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTTCATCCACTCTAC
ACCTGTGCACTTACTGTAGGCTTTCGGGAGCTTCGAAAGCAAAGGTTG
AGGTTCGCGCCTCGCTTTTGCCGTAGTTGTTACCGGGGCTTACGTTCAC
TACAAACCATTACAAGTATCAGAATGTGTATTGCGATGTAACGCATCT
ATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATG
CCTGTTTGAGTGTCATGAAATTCTCAACCTAACGGGTTCTTAACTGGAC
CTGCTTATGGCTTGGACTTGGAGGCTTGTCGGCTCGTTAGTTCGAGGTC
GGCTCCTCTCAAATGCATTAGCTTGGTTCCTCTGCGGATCGGCTTCACG
GTGTGATAATTGTCTACGCCGCGAACTGTTGAAGCGTTTTATAGGCCA
GCTTCTAATCGTCTCCTTGCGAGACAAGCTTTCATCGAACTCTGACCTC
AAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAA
GGA

3. *Lentinus velutinus* strain WCR1104 (KT956126)

TCCGTAGGTGAACCTGCGGAAGGATCATTACTGAATTTATGACAAGGT
TGTAGCTGGCCCTATCCGGGCATGTGCACGCCTTGCTCATTCCAATTCT
ACACCTCTGTGCACTTAACATGGGTTTGGTCGTGGCCTGGTTGCCCGCT
TGGGTGACTGAGCTTTTGACCCTGCCTGTGGTTCTCTACAAACACATCT
ATAGTATCAGAATGTAAACAGCGTATTATAACGCATCTTATACAACTT
TCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTT
GAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGT
GTCATGGTATTCTCAATTCTCTAAATCTTTGCGGATTTGGATGGATTGG
ATGTGGAGGTGATTGCTGGCATCCATGTTAATGGTGTCCCGGCTCCTCT
GAAATACATTAGCAGGAATGTTGCTTTGTCAACCTCAGTGTGATAATT
GTCTACGCTGTTGTTGCATTGCAAAAACTTTCATGTTTCTGCTCCAAAT
CGTCTTCGGACAATTTCTTGACATCTGACCTCAAATCAGGTAGGACTAC
CCGCTGAACTTAAGCATATCAATAAGCGGAGGA

4. *Lentinus squarrosulus* strain WCR1201 (KT956127)

TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTTTGAAACGGGT
TGTAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACA
CCTGTGCACTTACTGTGGGTTTCAGGAGCTTCGAAAGCGAGAAAGGGG
CCTTCACGGGCTTTTTTCTTGCCTAGTTGTTACTGGGCCTACGTTTCACT
ACAAACACTTATAAAGTATCAGAATGTGTATTGCGATGTAACGCATCT
ATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATG
CCTGTTTGAGTGTCATGAAATTCTCAACCTAACGGGTTCTTAACGGGAC
TTGCTTTAGGCTTGGACTTGGAGGTTCTTGTCGGCTTGCTTCAATGTCA
AGTCGGCTCCTCTTAAATGCATTAGCTTGGTTCCTGTGCGGATCGGCTC
ACGGTGTGATAATTGTCTACGCCGCGACCGTTGAAGCGTTTTTATAGG
CCAGCTTCTAGTCGTCTCTTTACGAGACAATAATCACCGAACTCTGACC
TCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGG
AGGA

Appendix E: Chemical contents of crude aqueous and crude ethanolic extracts.

1. Total carbohydrate contents.

Table 11 The total carbohydrate content of crude aqueous and ethanolic extracts.

Crude	A550			Sample concentration (mg/mL)	carbohydrate (µg/mL)			carbohydrate (mg/g crude)			Mean	SD	%Total carbohydrate
LEFB-Aq	0.89	0.86	0.788	0.1	433.76	418.91	383.27	433.76	418.91	383.27	411.98	25.95	41.20
LEM-Aq	0.715	0.809	0.753	0.1	347.13	393.66	365.94	347.13	393.66	365.94	368.91	23.41	36.89
LSaFB-Aq	0.951	1.026	1.05	0.2	231.98	250.54	256.49	231.98	250.54	256.49	246.34	12.78	24.63
LSaM-Aq	1.496	1.236	1.272	0.2	366.88	302.52	311.44	366.88	302.52	311.44	326.95	72.51	32.69
LSwFB-Aq	0.994	0.719	0.839	0.2	242.62	174.55	204.26	242.62	174.55	204.26	207.15	34.13	20.71
LSwM-Aq	0.851	0.696	0.87	0.1	414.46	337.72	423.86	414.46	337.72	423.86	392.01	47.25	39.20
LVFB-Aq	0.425	0.398	0.433	0.1	203.56	190.20	207.52	203.56	190.20	207.56	200.44	9.09	20.04
LVFM-Aq	0.909	0.888	0.93	0.1	443.17	432.77	453.56	443.17	432.77	453.56	443.17	10.40	44.32
LSqFB-Aq	0.565	0.572	0.547	0.1	272.87	276.34	263.96	272.87	276.34	263.96	271.06	6.38	27.11
LSqM-Aq	0.869	0.807	0.824	0.1	423.37	392.67	401.09	423.37	392.67	401.09	405.71	15.86	40.57
LEFB-Et	0.866	0.888	0.803	0.5	84.38	86.55	78.14	84.38	86.55	78.14	83.02	4.37	8.30
LEM-Et	0.754	0.855	0.756	0.2	183.22	208.22	183.71	183.22	208.22	183.71	191.72	14.29	19.17
LSaFB-Et	0.76	0.72	0.657	0.2	184.70	174.80	159.21	184.70	174.80	159.21	172.90	12.85	17.29
LSaM-Et	0.781	0.798	0.847	0.2	189.90	194.11	206.24	189.90	194.11	206.24	196.75	8.48	19.67
LSwFB-Et	1.068	1.1	0.924	0.2	260.94	268.86	225.30	260.94	268.86	225.30	251.70	23.21	25.17
LSwM-Et	0.451	0.453	0.481	0.1	216.44	217.43	231.29	216.44	217.43	231.29	221.72	8.30	22.17

Table 11 The total carbohydrate content of crude aqueous and ethanolic extracts (cont.).

Crude	A550			Sample concentration (mg/mL)	carbohydrate (µg/mL)			carbohydrate (mg/g crude)			Mean	SD	%Total carbohydrate
LVFB-Et	0.36	0.398	0.325	0.1	171.39	190.20	154.06	171.39	190.20	154.06	171.88	18.07	17.19
LVM-Et	0.359	0.374	0.369	0.1	170.89	178.32	175.84	170.89	178.32	175.84	175.02	3.78	17.50
LSqFB-Et	0.522	0.51	0.501	0.1	251.58	245.64	241.19	251.58	245.64	241.19	246.14	5.22	24.61
LSqFB-Et	0.276	0.262	0.25	0.1	129.80	122.87	116.93	129.80	122.87	116.93	123.20	6.44	12.32

2. Total reducing sugar contents.

Table 12 The total reducing sugar content of crude aqueous and ethanolic extracts.

Crude	A550			Sample concentration (mg/mL)	Reducing sugar (µg/mL)			Reducing sugar (mg/g crude)			Mean	SD	%Total reducing sugar
LEFB-Aq	0.277	0.317	0.326	5	53.76	61.04	62.67	53.76	61.04	62.67	59.16	4.74	5.92
LEM-Aq	0.586	0.623	0.613	5	109.95	116.67	114.85	109.95	116.67	114.85	113.82	3.48	11.38
LSaFB-Aq	0.233	0.267	0.255	5	45.76	51.95	49.76	45.76	51.95	49.76	49.16	3.14	4.92
LSaM-Aq	0.617	0.623	0.598	5	115.58	116.67	112.13	115.58	116.67	112.13	114.79	2.37	11.48
LSwFB-Aq	0.192	0.225	0.201	5	38.31	44.31	39.95	38.31	44.31	39.95	40.85	3.10	4.09
LSM-Aq	0.292	0.315	0.298	5	56.49	60.67	57.58	56.49	60.67	57.58	58.25	2.17	5.82
LVFB-Aq	0.342	0.386	0.369	5	65.58	73.58	70.49	65.58	73.58	70.49	69.88	4.03	6.99
LVM-Aq	0.383	0.395	0.384	5	73.04	75.22	73.22	73.04	75.22	73.22	73.82	1.21	7.38

Table 12 The total reducing sugar content of crude aqueous and ethanolic extracts (cont.).

Crude	A550			Sample concentration (mg/mL)	Reducing sugar (µg/mL)			Reducing sugar (mg/g crude)			Mean	SD	%Total reducing sugar
LSqFB-Aq	0.212	0.224	0.235	5	41.95	44.13	46.13	41.95	44.13	46.13	44.07	2.09	4.41
LSqM-Aq	0.373	0.372	0.362	5	71.22	71.04	69.22	71.22	71.04	69.22	70.49	1.11	7.05
LEFB-Et	0.119	0.081	0.09	5	25.04	18.13	19.76	25.04	18.13	19.76	20.98	3.61	2.10
LEM-Et	0.599	0.561	0.623	5	112.31	105.40	116.67	112.31	105.40	116.67	111.46	5.68	11.15
LSaFB-Et	0.049	0.043	0.044	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSaM-Et	0.332	0.265	0.275	5	63.76	51.58	53.40	63.76	51.58	53.40	56.25	6.57	5.62
LSwFB-Et	0.071	0.057	0.075	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LSwM-Et	0.671	0.679	0.663	5	125.40	126.85	123.95	125.40	126.85	123.95	125.40	1.45	12.54
LVFB-Et	0.702	0.672	0.651	5	131.04	125.58	121.76	131.04	125.58	121.76	126.13	4.66	12.61
LVM-Et	0.125	0.115	0.105	5	26.13	24.31	22.49	26.13	24.31	22.49	24.31	1.82	2.43
LSqFB-Et	0.105	0.118	0.113	5	22.49	24.85	23.95	22.49	24.85	23.95	23.76	1.19	2.38
LSqM-Et	0.069	0.074	0.071	5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

3. Total protein contents.

Table 13 The total protein content of crude aqueous and ethanolic extracts.

Crude	A550			Sample concentration (mg/mL)	Protein (µg/mL)			Protein (mg/g crude)			Mean	SD	%Total protein
LEFB-Aq	0.15	0.152	0.159	1	267.93	271.99	286.33	267.93	271.99	286.33	275.42	9.67	27.54
LEM-Aq	0.162	0.156	0.158	1	292.52	280.16	284.27	292.52	280.16	284.27	285.65	6.29	28.57
LSaFB-Aq	0.158	0.16	0.156	1	284.27	288.39	280.16	284.27	288.39	280.16	284.27	4.11	28.43
LSaM-Aq	0.173	0.167	0.164	1	315.52	302.92	296.67	315.52	302.92	296.67	305.04	9.60	30.50
LSwFB-Aq	0.24	0.228	0.236	1	466.56	437.93	456.93	466.56	437.93	456.93	453.80	14.57	45.38
LSM-Aq	0.143	0.133	0.134	1	253.81	233.90	235.88	253.81	233.90	235.88	241.20	10.96	24.12
LVFB-Aq	0.143	0.148	0.148	1	253.81	263.88	263.88	253.81	263.88	263.88	260.52	5.81	26.05
LVM-Aq	0.167	0.174	0.174	1	302.92	317.63	317.63	302.92	317.63	317.63	312.73	8.49	31.27
LSqFB-Aq	0.127	0.122	0.124	1	222.11	212.37	216.26	222.11	212.37	216.26	216.91	4.91	21.69
LSqM-Aq	0.104	0.106	0.101	1	177.88	181.67	172.22	177.88	181.67	172.22	177.26	4.75	17.73
LEFB-Et	0.228	0.193	0.194	5	87.59	71.70	72.14	87.59	71.70	72.14	77.14	9.05	7.71
LEM-Et	0.264	0.251	0.241	5	105.27	98.70	93.80	105.27	98.70	93.80	99.26	5.76	9.93
LSaFB-Et	0.171	0.153	0.161	3	103.77	91.34	96.82	103.77	91.34	96.82	97.31	6.23	9.73
LSaM-Et	0.227	0.226	0.233	5	87.12	86.65	89.95	87.12	86.65	89.95	87.90	1.79	8.79
LSwFB-Et	0.206	0.203	0.209	3	129.12	126.88	131.37	129.12	126.88	131.37	129.12	2.25	12.91
LSwM-Et	0.2	0.195	0.191	5	74.79	72.58	70.83	74.79	72.58	70.83	72.73	1.99	7.27
LVFB-Et	0.243	0.273	0.229	3	157.95	183.25	146.76	157.95	183.25	146.76	162.65	18.69	16.27
LVM-Et	0.247	0.253	0.235	5	96.73	99.70	90.91	96.73	99.70	90.91	95.78	4.47	9.58

Table 13 The total protein content of crude aqueous and ethanolic extracts.

Crude	A550			Sample concentration (mg/mL)	Protein (µg/mL)			Protein (mg/g crude)			Mean	SD	%Total protein
LSqFB-Et	0.237	0.217	0.228	5	91.87	82.47	87.59	91.87	82.47	87.59	87.31	4.70	8.73
LSqM-Et	0.241	0.237	0.226	3	156.33	153.11	144.41	156.33	153.11	144.41	151.28	6.17	15.13

4. Total phenol contents.

Table 14 The total phenol content of crude aqueous and ethanolic extracts.

Crude	A725			Sample concentration (mg/mL)	Phenol content (µgGAE/mL)			Phenol content (mgGAE/g crude)			Mean	SD	%Total Phenol
LEFB-Aq	0.665	0.669	0.677	1	16.64	16.74	16.94	16.64	16.74	16.94	16.77	0.15	1.68
LEM-Aq	0.817	0.819	0.865	1	20.45	20.50	21.65	20.45	20.50	21.65	20.87	0.68	2.09
LSaFB-Aq	0.608	0.636	0.622	1	15.21	15.91	15.56	15.21	15.91	15.56	15.56	0.35	1.56
LSaM-Aq	0.736	0.717	0.751	1	18.42	17.94	18.79	18.42	17.94	18.79	18.39	0.43	1.84
LSwFB-Aq	0.934	0.911	0.963	1	23.38	22.80	24.11	23.38	22.80	24.11	23.43	0.65	2.34
LSM-Aq	0.634	0.645	0.684	1	15.86	16.14	17.12	15.86	16.14	17.12	16.37	0.66	1.64
LVFB-Aq	0.776	0.545	0.547	1	19.42	13.63	13.68	19.42	13.63	13.68	15.58	3.33	1.56
LVM-Aq	0.737	0.758	0.775	1	18.44	18.97	19.40	18.44	18.97	19.40	18.94	0.48	1.89
LSqFB-Aq	0.582	0.586	0.579	1	14.56	14.66	14.48	14.56	14.66	14.48	14.57	0.09	1.46
LSqM-Aq	0.554	0.577	0.56	1	13.86	14.43	14.01	13.86	14.43	14.01	14.10	0.30	1.41

Table 14 The total phenol content of crude aqueous and ethanolic extracts (cont.).

Crude	A725			Sample concentration (mg/mL)	Phenol content (µgGAE/mL)			Phenol content (mgGAE/g crude)			Mean	SD	%Total Phenol
LEFB-Et	0.131	0.134	0.132	1	3.26	3.33	3.28	3.26	3.33	3.28	3.29	0.04	0.33
LEM-Et	0.237	0.26	0.275	1	5.91	6.49	6.86	5.91	6.49	6.86	6.42	0.48	0.64
LSaFB-Et	0.355	0.368	0.371	1	8.87	9.20	9.27	8.87	9.20	9.27	9.11	0.21	0.91
LSaM-Et	0.269	0.27	0.245	1	6.71	6.74	6.11	6.71	6.74	6.11	6.52	0.35	0.65
LSwFB-Et	0.431	0.37	0.368	1	10.77	9.25	9.20	10.77	9.25	9.20	9.74	0.90	0.97
LSwM-Et	0.207	0.231	0.236	1	5.16	5.76	5.89	5.16	5.76	5.89	5.60	0.39	0.56
LVFB-Et	0.762	0.754	0.771	1	19.07	18.87	19.30	19.07	18.87	19.30	19.08	0.21	1.91
LVM-Et	0.277	0.301	0.308	1	6.91	7.52	7.69	6.91	7.52	7.69	7.37	0.41	0.74
LSqFB-Et	0.179	0.167	0.19	1	4.46	4.16	4.73	4.46	4.16	4.73	4.45	0.29	0.45
LSqM-Et	0.385	0.409	0.402	1	9.62	10.22	10.05	9.62	10.22	10.05	9.96	0.31	1.00

Appendix F: Polysaccharide purification steps.

1. Anion exchange chromatography.

Table 15 The separation of fraction E4 by anion exchange chromatography. Each fraction was monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay.

Fraction number	A490			Mean	SD
1	0	0	0	0.000	0.000
2	0	0	0	0.000	0.000
3	0	0	0	0.000	0.000
4	0	0	0	0.000	0.000
5	0.102	0.131	0.077	0.103	0.027
6	0.789	0.817	0.678	0.761	0.074
7	1.65	1.674	1.612	1.645	0.031
8	1.982	1.966	1.994	1.981	0.014
9	2.015	2.08	2.055	2.050	0.033
10	2.043	2.17	2.125	2.113	0.064
11	2.095	2.175	2.155	2.142	0.042
12	2.1	2.185	2.19	2.158	0.051
13	2.13	2.23	2.215	2.192	0.054
14	2.155	2.255	2.225	2.212	0.051
15	2.155	2.24	2.28	2.225	0.064
16	2.08	2.31	2.22	2.203	0.116
17	2.145	2.3	2.34	2.262	0.103
18	2.37	2.37	2.37	2.370	0.000
19	2.155	2.325	2.245	2.242	0.085
20	2.125	2.26	2.285	2.223	0.086
21	2.035	2.055	1.958	2.016	0.051
22	0.832	1.25	1.134	1.072	0.216
23	0.354	0.372	0.372	0.366	0.010
24	0.228	0.205	0.177	0.203	0.026
25	0.12	0.168	0.097	0.128	0.036
26	0.081	0.298	0.05	0.143	0.135
27	0.075	0.223	0.013	0.104	0.108
28	0.042	0.048	0.023	0.038	0.013
29	0.052	0.051	0	0.034	0.030
30	0.014	0.137	0.018	0.056	0.070
31	0.007	0.027	0	0.011	0.014
32	0.026	0.022	0	0.016	0.014
33	0.034	0.03	0	0.021	0.019
34	0.092	0.153	0	0.082	0.077
35	0.039	0.019	0	0.019	0.020

Table 15 The separation of fraction E4 by anion exchange chromatography. Each fraction was monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay (cont.).

Fraction number	A490			Mean	SD
36	0.028	0.022	0	0.017	0.015
37	0.033	0.015	0	0.016	0.017
38	0.01	0.011	0	0.007	0.006
39	0	0.027	0	0.009	0.016
40	0.008	0.018	0	0.009	0.009
41	0.012	0.039	0	0.017	0.020
42	0	0.052	0	0.017	0.030
43	0.057	0.097	0	0.051	0.049
44	0.931	0.059	0	0.330	0.521
45	2.14	0.074	0.003	0.739	1.214
46	1.994	1.366	0.89	1.417	0.554
47	1.21	2.3	2.235	1.915	0.611
48	0.511	2.235	2.21	1.652	0.988
49	0.256	1.4	1.58	1.079	0.718
50	0.203	0.4	0.739	0.447	0.271
51	0.161	0.286	0.188	0.212	0.066
52	0.22	0.23	0.172	0.207	0.031
53	0.199	0.235	0.182	0.205	0.027
54	0.225	0.263	0	0.163	0.142
55	0.25	0.284	0.436	0.323	0.099
56	0.263	0.311	0.292	0.289	0.024
57	0.536	0.357	0.321	0.405	0.115
58	0.291	0.359	0.35	0.333	0.037
59	0.334	0.529	0.332	0.398	0.113
60	0.195	0.471	0.342	0.336	0.138
61	0.227	0.514	0.211	0.317	0.171
62	0.154	0.513	0.252	0.306	0.186
63	0.179	0.3	0.175	0.218	0.071
64	0.056	0.308	0.134	0.166	0.129
65	0.083	0.252	0.129	0.155	0.087
66	0	0.099	0.127	0.075	0.067
67	0.066	0.139	0.172	0.126	0.054
68	0	0.125	0.171	0.099	0.088
69	0.275	0.123	0.147	0.182	0.082
70	1.466	0.114	0.141	0.574	0.773

Table 15 The separation of fraction E4 by anion exchange chromatography. Each fraction was monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay (cont.).

Fraction number	A490			Mean	SD
71	1.648	0.392	0.451	0.830	0.709
72	1.574	1.766	1.82	1.720	0.129
73	0.963	2.055	2.005	1.674	0.617
74	0.916	1.694	1.43	1.347	0.396
75	0.419	0.953	1.19	0.854	0.395
76	0.346	0.756	0.933	0.678	0.301
77	0.297	0.663	0.68	0.547	0.216
78	0.315	0.502	0.592	0.470	0.141
79	0.391	0.53	0.522	0.481	0.078
80	0.248	0.46	0.479	0.396	0.128
81	0.06	0.362	0.348	0.257	0.170
82	0.154	0.32	0.318	0.264	0.095
83	0.235	0.308	0.285	0.276	0.037
84	0.069	0.301	0.25	0.207	0.122
85	0.185	0.237	0.248	0.223	0.034
86	0	0.212	0.211	0.141	0.122
87	0.005	0.179	0.22	0.135	0.114
88	0.039	0.186	0.2	0.142	0.089
89	0	0.183	0.184	0.122	0.106
90	0.101	0.167	0.152	0.140	0.035
91	0	0.156	0.16	0.105	0.091
92	0	0.147	0.121	0.089	0.078
93	0	0.144	0.132	0.092	0.080
94	0	0.162	0.113	0.092	0.083
95	0	0.125	0.146	0.090	0.079
96	0	0.116	0.11	0.075	0.065
97	0	0.11	0.1	0.070	0.061
98	0	0.104	0.107	0.070	0.061
99	0	0.104	0.174	0.093	0.088
100	0	0.11	0.127	0.079	0.069
101	0	0.1	0.098	0.066	0.057
102	0	0.097	0.106	0.068	0.059
103	0	0.094	0.086	0.060	0.052
104	0	0.046	0.056	0.034	0.030
105	0.194	0.058	0.071	0.108	0.075

Table 15 The separation of fraction E4 by anion exchange chromatography. Each fraction was monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay (cont.).

Fraction number	A490			Mean	SD
106	0.021	0.05	0.062	0.044	0.021
107	0.228	0.051	0.06	0.113	0.100
108	0.158	0.046	0	0.068	0.081
109	0.498	0.465	0.501	0.488	0.020
110	1.454	1.772	1.894	1.707	0.227
111	1.394	1.59	1.36	1.448	0.124
112	0.89	1.21	1.07	1.057	0.160
113	0.7	0.94	0.82	0.820	0.120
114	0.432	0.54	0.627	0.533	0.098
115	0.42	0.43	0.547	0.466	0.071
116	0.287	0.384	0.443	0.371	0.079
117	0.405	0.358	0.336	0.366	0.035
118	0.212	0.254	0.239	0.235	0.021
119	0.284	0.28	0.306	0.290	0.014
120	0.329	0.251	0.325	0.302	0.044
121	0.136	0.107	0.177	0.140	0.035
122	0.134	0.2	0.143	0.159	0.036
123	0.178	0.105	0.151	0.145	0.037
124	0.274	0.097	0.144	0.172	0.092
125	0.039	0.097	0.146	0.094	0.054
126	0.166	0.065	0.123	0.118	0.051
127	0.104	0.074	0.005	0.061	0.051
128	0.073	0.076	0.111	0.087	0.021
129	0.368	0.04	0.099	0.169	0.175
130	0.077	0.087	0.082	0.082	0.005
131	0.053	0.087	0.052	0.064	0.020
132	0.316	0.101	0.049	0.155	0.142
133	0.026	0.049	0.063	0.046	0.019
134	0	0.064	0.048	0.037	0.033
135	0.151	0.067	0.05	0.089	0.054
136	0.127	0.018	0.026	0.057	0.061
137	0.021	0.02	0.12	0.054	0.057
138	0.176	0.033	0.059	0.089	0.076
139	0	0.049	0.021	0.023	0.025
140	0	0.064	0.032	0.032	0.032

Table 15 The separation of fraction E4 by anion exchange chromatography. Each fraction was monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay (cont.).

Fraction number	A490			Mean	SD
141	0.13	0.051	0.024	0.068	0.055
142	0.395	0.05	0.021	0.155	0.208
143	0.159	0.06	0.025	0.081	0.070
144	1.248	1.6	1.172	1.340	0.228
145	2.295	2.285	1.974	2.185	0.183
146	1.374	1.124	1.03	1.176	0.178
147	0.36	0.454	0.426	0.413	0.048
148	0.363	0.21	0.198	0.257	0.092
149	0.339	0.119	0.107	0.188	0.131
150	0.1	0.064	0.084	0.083	0.018
151	0.096	0.059	0.009	0.055	0.044
152	0.191	0.039	0.016	0.082	0.095
153	0.205	0.034	0.012	0.084	0.106
154	0.045	0.046	0.038	0.043	0.004
155	0.099	0.013	0	0.037	0.054
156	0	0.012	0	0.004	0.007
157	0.158	0.017	0	0.058	0.087
158	0.003	0.022	0	0.008	0.012
159	0.044	0.008	0	0.017	0.023
160	0	0.018	0	0.006	0.010
161	0.073	0.002	0.009	0.028	0.039
162	0.165	0	0	0.055	0.095
163	0.07	0.139	0	0.070	0.070
164	0	0	0	0.000	0.000
165	0	0	0	0.000	0.000
166	0	0	0	0.000	0.000
167	0	0	0	0.000	0.000
168	0	0	0	0.000	0.000
169	0	0	0.01	0.003	0.006
170	0	0.1	0	0.033	0.058
171	0	0	0.022	0.007	0.013
172	0	0	0.113	0.038	0.065
173	0.186	0	0.009	0.065	0.105
174	0	0	0.016	0.005	0.009
175	0	0	0	0.000	0.000

Table 15 The separation of fraction E4 by anion exchange chromatography. Each fraction was monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay (cont.).

Fraction number	A490			Mean	SD
176	0	0.115	0	0.038	0.066
177	0	0.095	0	0.032	0.055
178	0	0	0	0.000	0.000
179	0	0	0	0.000	0.000
180	0	0	0	0.000	0.000
181	0.12	0	0	0.040	0.069
182	0.735	0	0.07	0.268	0.406
183	0	0.02	0	0.007	0.012
184	0	0	0	0.000	0.000
185	0.107	0	0.018	0.042	0.057
186	0	0	0	0.000	0.000
187	0.203	0	0.059	0.087	0.104
188	0.058	0.182	0	0.080	0.093

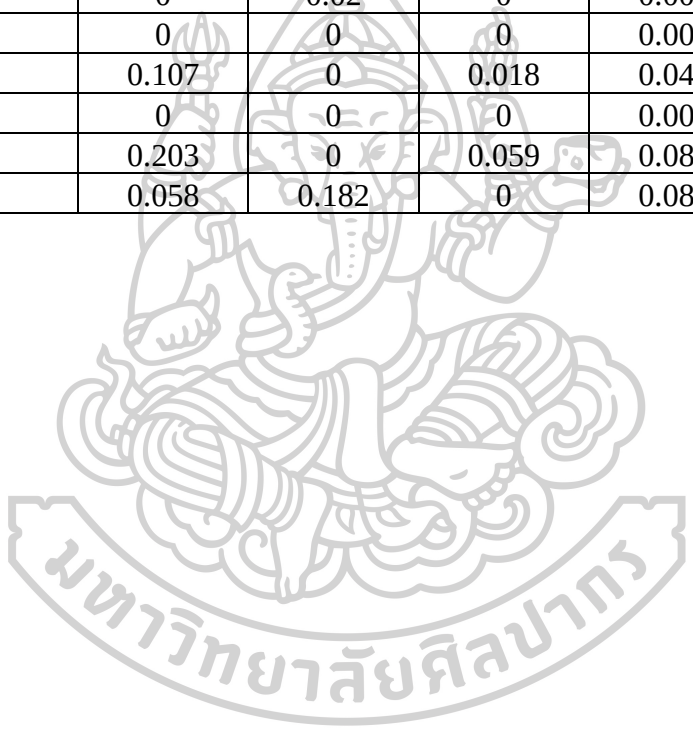

Table 16 The separation of fraction E4N5 by size exclusion chromatography. Each fraction was monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay.

Fraction number	A490								Mean	SD
	Rep 1		Rep 2		Rep 3		Rep 4			
1	0.96	0.956	0.796	0.782	0.823	0.831	0.881	0.877	0.863	0.068
2	1.187	1.233	1.456	1.331	1.086	1.091	1.107	1.091	1.198	0.136
3	1.268	1.277	1.408	1.38	1.218	1.226	1.021	1.038	1.230	0.141
4	1.004	1.054	0.894	0.731	1.09	1.082	0.881	0.854	0.949	0.129
5	0.481	0.476	0.39	0.384	0.398	0.384	0.49	0.496	0.437	0.052
6	0.682	0.692	0.84	0.95	0.634	0.643	0.894	0.99	0.791	0.144
7	1.371	1.377	0.982	0.974	1.098	1.036	0.961	1.077	1.110	0.170
8	1.629	1.584	1.459	1.398	1.419	1.395	1.381	1.48	1.468	0.092
9	1.726	1.642	1.702	1.743	1.383	1.27	1.692	1.714	1.609	0.179
10	0.918	1.147	1.573	1.613	0.862	0.906	1.408	1.453	1.235	0.314
11	0.862	0.97	1.089	1.085	0.752	0.814	0.865	0.914	0.919	0.122
12	0.5	0.591	0.524	0.472	0.657	0.599	0.655	0.719	0.590	0.086
13	0.495	0.486	0.375	0.301	0.55	0.532	0.404	0.417	0.445	0.085
14	0.767	0.783	0.909	0.872	0.52	0.66	0.61	0.593	0.714	0.140
15	0.703	0.708	0.631	0.628	0.321	0.342	0.411	0.414	0.520	0.164
16	1.109	1.066	0.862	0.843	1.092	0.942	0.982	1.012	0.989	0.101
17	1.361	1.372	1.372	1.331	1.33	1.275	1.244	1.276	1.320	0.049
18	1.001	0.966	1.527	1.535	1.292	1.267	1.102	1.228	1.240	0.215
19	0.962	0.918	1.419	1.342	1.062	1.067	0.994	1.016	1.098	0.183
20	0.714	0.723	1.223	1.21	1.043	0.963	0.825	1.146	0.981	0.209

Table 16 The separation of fraction E4N5 by size exclusion chromatography. Each fraction was monitored spectrophotometerically at 490 nm with phenol-sulfuric acid assay (cont.).

Fraction number	A490								Mean	SD
	Rep 1		Rep 2		Rep 3		Rep 4			
21	0.584	0.594	0.437	0.407	0.75	0.628	0.731	0.745	0.610	0.134
22	0.463	0.472	0.207	0.171	0.201	0.211	0.379	0.375	0.310	0.125
23	0.27	0.256	0.178	0.131	0.209	0.223	0.108	0.114	0.186	0.063
24	0.194	0.144	0.159	0.144	0.163	0.179	0.289	0.242	0.189	0.051
25	0.231	0.248	0.226	0.228	0.247	0.123	0.291	0.274	0.234	0.050

Appendix G: Total polysaccharide of fractions

Table 17 Total polysaccharides of each fraction was separated by re-dissolving in water and re-precipitating with ethanol. Total polysaccharides were measured by phenol-sulfuric acid assay at 490 nm.

Crude	A490	Sample dilution	carbohydrate (ug/ml)	Crude weight (g)	carbohydrate (mg)	Mean	SD	%Total carbohydrate
Fraction E1	0.75	1:10	728.91	1.47	1071.50	1042.39	38.51	70.91
	0.7		679.41		998.73			
	0.74		719.01		1056.94			
Fraction E4	0.619	1:10	599.21	2.28	1366.19	1360.93	35.29	59.69
	0.631		611.09		1393.28			
	0.6		580.40		1323.30			
Fraction R	0.167	1:10	151.68	1.37	207.81	225.89	24.62	16.49
	0.173		157.62		215.94			
	0.201		185.35		253.92			

Table 18 Total polysaccharides of each fraction before dialysis was separated from fraction E4 by anion exchange chromatography. Total polysaccharides were measured by phenol-sulfuric acid assay at 490 nm.

Crude	A490	Sample dilution	carbohydrate (ug/ml)	Fraction volume (mL)	carbohydrate (mg)	Mean	SD	Total carbohydrate (mg)
Fraction E4W	0.952	1:50	4644.55	23.40	108.68	110.03	1.95	338.52
	0.983		4798.02		112.27			
	0.956		4664.36		109.15			
	1.35	1:50	6614.85	20.00	132.30	132.50	0.71	
	1.346		6595.05		131.90			
	1.36		6664.36		133.29			
	0.706	1:50	3426.73	26.75	91.67	95.99	7.04	
	0.8		3892.08		104.11			
	0.71		3446.53		92.19			
Fraction E4N1	0.61	3:50	983.83	17.20	16.92	18.18	1.92	50.60
	0.732		1185.15		20.38			
	0.621		1001.98		17.23			
	0.805	3:50	1305.61	14.95	19.52	18.90	0.57	
	0.759		1229.70		18.38			
	0.776		1257.76		18.80			
	0.337	3:50	533.33	24.76	13.21	13.52	0.51	
	0.338		534.98		13.25			
	0.359		569.64		14.10			

Table 18 Total polysaccharides of each fraction before dialysis was separated from fraction E4 by anion exchange chromatography. Total polysaccharides were measured by phenol-sulfuric acid assay at 490 nm (cont.).

Crude	A490	Sample dilution	carbohydrate (ug/ml)	Fraction volume (mL)	carbohydrate (mg)	Mean	SD	Total carbohydrate (mg)
Fraction E4N2	0.305	1:10	288.32	17.70	5.10	5.30	0.17	14.69
	0.321		304.16		5.38			
	0.322		305.15		5.40			
	0.651	1:10	630.89	10.00	6.31	6.56	0.28	
	0.67		649.70		6.50			
	0.707		686.34		6.86			
	0.128	3:50	188.45	15	2.83	2.83	0.14	
	0.123		180.20		2.70			
	0.134		198.35		2.98			
Fraction E4N3	0.423	1:50	2025.74	10.50	21.27	21.96	1.77	53.07
	0.475		2283.17		23.97			
	0.411		1966.34		20.65			
	0.996	1:10	972.48	15.00	14.59	14.70	0.70	
	0.961		937.82		14.07			
	1.054		1029.90		15.45			
	0.551	3:50	886.47	19.1	16.93	16.41	0.94	
	0.5		802.31		15.32			
	0.552		888.12		16.96			

Table 18 Total polysaccharides of each fraction before dialysis was separated from fraction E4 by anion exchange chromatography. Total polysaccharides were measured by phenol-sulfuric acid assay at 490 nm (cont.).

Crude	A490	Sample dilution	carbohydrate (ug/ml)	Fraction volume (mL)	carbohydrate (mg)	Mean	SD	Total carbohydrate (mg)
Fraction E4N4	0.299	3:50	470.63	41.00	19.30	17.87	1.33	73.04
	0.275		431.02		17.67			
	0.26		406.27		16.66			
	0.886	1:10	863.56	33.60	29.02	30.07	1.10	
	0.952		928.91		31.21			
	0.915		892.28		29.98			
	0.704	1:10	683.37	36.25	24.77	25.10	0.29	
	0.72		699.21		25.35			
	0.715		694.26		25.17			
Fraction E4N5	0.11	1:10	95.25	85.46	8.14	11.86	3.24	67.11
	0.18		164.55		14.06			
	0.172		156.63		13.39			
	0.427	1:10	409.11	58.83	24.07	25.70	1.47	
	0.462		443.76		26.11			
	0.476		457.62		26.92			
	0.536	1:10	517.03	57.62	29.79	29.54	3.57	
	0.592		572.48		32.99			
	0.467		448.71		25.85			

Table 19 Total polysaccharides of each fraction after dialysis was separated from fraction E4 by anion exchange chromatography. Total polysaccharides were measured by phenol-sulfuric acid assay at 490 nm.

Crude	A490	Sample dilution	carbohydrate (ug/ml)	Fraction volume (mL)	carbohydrate (mg)	Mean	SD	Total carbohydrate (mg)
Fraction E4W	0.976	3:50	1587.79	29.64	47.06	53.55	6.61	192.73
	1.104		1799.01		53.32			
	1.246		2033.33		60.27			
	0.422	1;100	4041.58	18.85	76.18	77.49	1.22	
	0.435		4170.30		78.61			
	0.43		4120.79		77.68			
	0.42	1:50	2010.89	26.75	53.79	61.69	7.11	
	0.495		2382.18		63.72			
	0.524		2525.74		67.56			
Fraction E4N1	0.354	3:50	561.39	15.96	8.96	8.35	0.53	27.02
	0.322		508.58		8.12			
	0.317		500.33		7.99			
	0.383	1:10	365.54	24.76	9.05	8.72	0.46	
	0.348		330.89		8.19			
	0.377		359.60		8.90			
	0.37	3:50	587.79	20.42	12.00	9.95	1.83	
	0.266		416.17		8.50			
	0.291		457.43		9.34			

Table 19 Total polysaccharides of each fraction after dialysis was separated from fraction E4 by anion exchange chromatography. Total polysaccharides were measured by phenol-sulfuric acid assay at 490 nm (cont.).

Crude	A490	Sample dilution	carbohydrate (ug/ml)	Fraction volume (mL)	carbohydrate (mg)	Mean	SD	Total carbohydrate (mg)
Fraction E4N2	0.27	1:10	253.66	20.00	5.07	3.09	1.72	12.48
	0.117		102.18		2.04			
	0.122		107.13		2.14			
	0.38	1:10	362.57	11.00	3.99	4.11	0.15	
	0.388		370.50		4.08			
	0.406		388.32		4.27			
	0.383	1:10	365.54	15	5.48	5.28	0.28	
	0.348		330.89		4.96			
	0.377		359.60		5.39			
Fraction E4N3	0.436	1:05	209.01	22.74	4.75	4.65	0.64	30.75
	0.478		229.80		5.23			
	0.366		174.36		3.96			
	0.631	1:10	611.09	18.78	11.48	11.20	1.87	
	0.509		490.30		9.21			
	0.708		687.33		12.91			
	0.49	3:50	785.81	19.1	15.01	14.90	0.82	
	0.459		734.65		14.03			
	0.511		820.46		15.67			

Table 19 Total polysaccharides of each fraction after dialysis was separated from fraction E4 by anion exchange chromatography. Total polysaccharides were measured by phenol-sulfuric acid assay at 490 nm (cont.).

Crude	A490	Sample dilution	carbohydrate (ug/ml)	Fraction volume (mL)	carbohydrate (mg)	Mean	SD	Total carbohydrate (mg)
Fraction E4N4	0.286	1:10	269.50	32.97	8.89	8.99	0.25	37.02
	0.298		281.39		9.28			
	0.284		267.52		8.82			
	0.179	3:50	272.61	32.51	8.86	10.22	1.74	
	0.241		374.92		12.19			
	0.193		295.71		9.61			
	0.549	1:10	529.90	35.83	18.99	17.80	1.02	
	0.5		481.39		17.25			
	0.498		479.41		17.18			
Fraction E4N5	0.167	1:10	151.68	61.91	9.39	10.56	1.86	55.86
	0.17		154.65		9.57			
	0.221		205.15		12.70			
	0.135	3:50	200.00	61.78	12.36	12.42	0.61	
	0.142		211.55		13.07			
	0.13		191.75		11.85			
	0.565	1:10	545.74	61.29	33.45	32.88	1.03	
	0.566		546.73		33.51			
	0.536		517.03		31.69			

Table 20 Total polysaccharides of each fraction was separated from fraction E4N5 by size exclusion chromatography. Total polysaccharides were measured by phenol-sulfuric acid assay at 490 nm.

Crude	A490	Sample concentration (mg/mL)	carbohydrate (ug/ml)	Crude weight (mg)	carbohydrate (mg)	Mean	SD	%Total carbohydrate
	0.92		897.23		3.32			
LV1	0.923	0.1	900.20	3.70	3.33	3.35	0.04	90.51
	0.941		918.02		3.40			
	1.021		997.23		38.29			
LV2	1.012	0.1	988.32	38.40	37.95	38.03	0.24	99.04
	1.009		985.35		37.84			
	0.984		960.59		8.55			
LV3	0.997	0.1	973.47	8.9	8.66	8.64	0.08	95.96
	1.001		977.43		8.70			

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