ROLE OF POLYSACCHARIDES FROM *PLEUROTUS SAJOR-CAJU* IN THE IMMUNOMODULATION OF THP-1 HUMAN MACROPHAGE CELLS

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOL AND ABBREVIATIONS

%	Percentage
<	Less Than
=	Equal To
>	More Than
°C	Celsius
µg	Microgram
µL	Microlitre
µm	Micrometre
AMR	Antimicrobial Resistance
APC	Antigen Presenting Cell
CO_2	Carbon Dioxide
CTL	Cytotoxic T Lymphocytes
DC	Dendritic Cell
dH_2O	Distilled Water
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
g	Gram
h	Hour
H_2SO_4	Sulphuric Acid
iNOS	Inducible Nitric Oxide Synthase
KOH	Potassium Hydroxide
LPS	Lipopolysaccharides
M	Molar
MIC	Minimum Inhibitory Concentration
min	Minute
mL	Millilitre
mm	Millimetre
mRNA	Messenger Ribonucleic Acid
NF-κB	Nuclear Factor Kappa-B
NK	Natural Killer
nm	Nanometre
NO	Nitric Oxide
PBS	Phosphate Buffered Saline
PD-L1	Programmed Death Ligand 1
PMA	Phorbol-12-Myristate-13-Acetate
PRR	Pattern Recognition Receptor
PSC	Polysaccharide *Pleurotus Sajor-Caju*
RT	Room Temperature
TLR	Toll-Like Receptor
TNF-α/β	Tumour Necrosis Factor-Alpha/Beta
v/v	Volume/Volume
w/v	Weight/Volume
x g	G-Force/Revolutions Per Minute (Rpm)
RPMI-1640	Roswell Park Memorial Institute 1640 Medium
IL-6	Interleukin 6
IL-1β	Interleukin 1 Beta
THP-1	Human Acute Monocytic Leukemia cells

PERANAN POLISAKARIDA DARIPADA *PLEUROTUS SAJOR-CAJU* DALAM IMUNOMODULASI PADA SEL MAKROFAJ MANUSIA THP-1

ABSTRAK

Cendawan ubat kaya dengan sifat biologi, yang merupakan sumber yang sangat baik untuk penggunaan farmaseutikal dan nutraseutikal. Cendawan ubat juga mempunyai pelbagai khasiat perubatan yang berharga, termasuk tekanan antitumor, hipokolesterolemik, anti-aterogenik, dan anti-oksidatif. *Pleurotus sajor-caju* adalah salah satu *Pleurotus spp.* tergolong dalam filum Basidiomycota, yang terkenal sebagai cendawan berbentuk tiram (basidiocarps) dan umumnya disebut cendawan tiram (OM). Kajian ini bertujuan untuk mengetahui kemampuan ekstrak polisakarida larut dalam air dari ekstrak *Pleurotus sajor-caju* (PSC) dalam mendorong kesan percambahan, imunostimulasi dan anti-radang pada sel makrofag THP-1. Pertama, jumlah kandungan karbohidrat dan β-glukan ditentukan menggunakan ujian individual. Seterusnya, tindak balas percambahan sel dinilai menggunakan ujian MTS. Untuk memahami kesan imunostimulasi ekstrak PSC pada tahap gen, ekspresi gen sitokin terpilih dalam sel THP-1 dinilai menggunakan PCR masa nyata. Pengeluaran Nitrik Oxida (NO) dan tahap ekspresi iNOS diukur dalam makrofag yang dirangsang dengan LPS. Hasil yang diperoleh menunjukkan bahawa 95% jumlah karbohidrat dan 89% kandungan β-glukan masing-masing dikesan dalam 50 mg ekstrak PSC. Ekstrak polisakarida *Pleurotus sajor-caju* (PSC) didapati boleh meningkatkan peningkatan tindak balas proliferatif pada sel THP-1 makrofag dengan cara yang bergantung pada dos, mulai dari 10 µg/ml hingga 80 µg/ml. Pada peringkat transkripsi, PSC merangsang ekspresi tumor nekrosis factor-alpha (TNF-α), interleukin 1β (IL-1β), dan interleukin 6 (IL-6) secara signifikan ($p < 0.05$) pada pelbagai dos ekstrak PSC. Selain

itu, PSC juga menunjukkan kesan perencatan sitokin ini di persekitaran keradangan yang disebabkan oleh LPS. Walaupun begitu, sel yang dirawat dengan PSC menunjukkan 15% - 45% pengurangan pengeluaran NO, 33% - 83% penurunan regulasi ekspresi gen iNOS, dan penurunan regulasi ekspresi gen PD-L1 yang signifikan pada makrofag THP-1 yang dirangsang LPS. Akhir sekali, apabila peningkatan kepekatan PSC digunakan, makrofaj THP-1 juga meningkat dalam bilangan sel yang menunjukkan perubahan ketara. Sebagai kesimpulan, data ini menunjukkan bahawa ekstrak polisakarida mentah PSC dapat menggunakan peristiwa imunostimulasi dengan potensi yang setara dengan LPS melalui pengaktifan beberapa jalur isyarat. Hasil ini memberikan ekstrak polisakarida mentah PSC pemahaman awal tentang mekanisme tindakan untuk aktiviti imunomodulator dan sebagai ‘pembantu’ (di dalam penggunaan vaksin), menunjukkan bahawa ia dapat memberikan landasan untuk pengembangan ‘pembantu’ untuk peningkatan keberkesanan vaksin.

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ROLE OF POLYSACCHARIDES FROM *PLEUROTUS SAJOR-CAJU* IN THE IMMMUNOMODULATION OF THP-1 HUMAN MACROPHAGE CELLS

ABSTRACT

Medicinal mushrooms have a diverse range of biological effects, which are excellent sources for pharmaceutical and nutraceutical use. They possess various valuable medicinal properties, including antitumor, hypocholesterolemic, anti-atherogenic, and anti-oxidative properties. *Pleurotus sajor-caju* is one of the *Pleurotus* spp. belonging to phylum Basidiomycota, well-known as oyster shaped mushroom (basidiocarps) and generally called oyster mushroom (OM). The present study was aimed to determine the ability of the water-soluble polysaccharides extract from *Pleurotus sajor-caju* extracts (PSC) in inducing the proliferation, immunostimulating and anti-inflammatory effects on THP-1 macrophage cells. First, the total carbohydrate and β-glucan contents were determined using the individual assay test. Next, the cell proliferation response was evaluated using the MTS assay. To understand the immunostimulating effect of PSC extract at the gene level, the expressions of selected cytokine genes in THP-1 cells were evaluated using real-time PCR. Nitric Oxide (NO) production and inducible nitric oxide synthase (iNOS) expression level were measured in the LPS-stimulated macrophages. The results obtained showed that 95% of total carbohydrate and 89% of β-glucan contents were detected in 50 mg PSC extracts, respectively. PSC extract was found to induce a marked increase in proliferative response on macrophage THP-1 cell in a dose-dependent manner, ranging from 10 µg/ml up to 80 µg/ml. At transcription level, PSC stimulated the expression of tumor necrosis factor-alpha (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) significantly ($p<0.05$) at various dosages of PSC extract.

Moreover, PSC also showed an inhibitory effect of these cytokines in the inflammatory environment attributed to LPS. Nevertheless, cells treated with PSC showed 15% - 45% inhibition of NO production, 33% - 83% downregulation of iNOS gene expression, and significant downregulation of PD-L1 gene expression on LPS-stimulated THP-1 macrophages respectively. Lastly, when the increasing concentration of PSC was used, the THP-1 macrophages also increased in the number of cells showing significant changes. In conclusion, our data suggest that PSC extract may use its immunostimulating events with comparable potency to LPS via the activation of several signaling pathways. These results provide PSC crude polysaccharide extract a advance understanding into the mechanisms of action for these immunomodulatory and adjuvant activities, suggesting it may provide a foundation for the adjuvant development for the enhancement of vaccine efficacy.

CHAPTER 1

INTRODUCTION

1.1 Background

The Sustainable Development Goals (SDGs) era has commenced. Health is appropriately positioned well within SDGs. It is significant for sustainable development to ensure healthy lives and promote well-being at all ages (SDG 3 : Good health and well-being). The world is currently dealing with a worldwide health crisis unlike any other: COVID-19 is spreading human pain, affecting the global economy, and upending the lives of billions of people all over the world. Specific goal in SDG 3.b mentioning '*Support the research and development of vaccines and medicines for the communicable and non-communicable diseases that primarily affect developing countries, provide access to affordable essential medicines and vaccines…*'(United Nations, 2018) . This goal also briefly explains the affordable medicine which we can provide to all people around the world. To achieve affordable medicine, research and development on medicine using the natural ingredients could be in the best interest of multiple levels of people, from researchers to the pharma company and CEO to stakeholders across sectors are should be embracing the SDGs and leading innovative efforts to build sustainable societies (United Nations Foundation, 2015).

The initiative to discover the natural ingredient to make affordable medicine, and mushroom extracted compounds could be in the best interest because its ongoing supply chain around the world. Mushrooms have been known for hundreds of years ago as edible and medicinal stockpiles specifically around Asian countries China and Japan. These countries are among the earliest countries which has found the benefit of mushroom and use the common name for certain mushroom, for example, lingzhi (*Ganoderma lucidum*), yiner (*Tremella fuciformis*) and shiitake (*Lentinus edodes*).

Mushrooms are grouped among the lower plants in the Divison Thallophyta by Linnaeus. This comes from a very simple and uncomplicated structure (lack of true seeds, true roots, true stems, true leaves, and true flowers). The existence of cell walls in mushrooms contributes to characteristic identification, which is similar to plants rather than animals. The mushrooms have widely been used in varied ways in different countries and at different times (Miles & Chang, 2004).

Mushrooms have a unique flavour which can be considered as gourmet cuisine around the world. It also contains a high nutritional and functional value for the human body, which is acceptable as nutraceutical foods. Moreover, mushrooms are also classified as high in value because of their medicinal properties, organoleptic merit, and economic significance (Miles & Chang, 2004). In Asian countries, it has been a very long tradition to make mushrooms for medicinal use, whereas in the Western hemisphere, they recently started using mushrooms as medicinal therapies. For countries in Asia such as Japan, China, and Korea, they have employed mushrooms as precious traditional therapies and modern clinical practices. Medicinal effects from various species such as *Auricularia spp*., *Flammulina spp*., *Grifola spp*., *Ganoderma spp*., *Pleurotus spp*., and *Tremella spp*. are well documented in the literature (Ooi & Liu, 2000; Wasser, 2002).

Mushrooms can be a useful source for many different miraculous biological properties to be used in pharmaceuticals and nutraceuticals. It is also known as mini-pharmaceutical factories for producing various compounds. It could be another alternative for new antimicrobial compounds such as proteins, quinolones, terpenes, steroids, and oxalic acid. Moreover, mushrooms also can provide various vitamins for human such as Vitamins B1, B2, B12, C, D, and E (Barros et al., 2007a). Numerous studies have described that mushroom-extracted metabolites are suitable to be used as

biological response modifiers (BRM) or as immunomodulators (Mizuno et al., 2014). This study have potentially an overview of the present information about potential compounds from the mushrooms and their health benefits.

The immune system is a host body defence system against infectious organisms and any invaders. Uniquely, this system can differentiate amid self and non-self-antigens for identification, which should remain and should be destroyed to protect the host from infection and invasion. In the human body, the immune system can be classified into innate and adaptive immunity. The innate immunity is the first-line defence in the host, which gives an immediate, broad-spectrum response in encountering a specific antigen and has no memory for foreign substances. It is also known as highly conserving the nature of the response, and even in the simplest animal, it is essential for survival. On the other hand, adaptive immunity is more complex in terms of its ability to identify antigen specifically and intelligence in remembering antigen. Thus, in the adaptive immunity, when the host encounters the same antigen again, the process of eradicating antigens become faster and more efficient whenever the same antigen penetrate the host again (Janeway et al., 1995).

These two systems, the innate and adaptive immune system, cannot work separately. Indeed, their activities work closely together to battle against the external factors by provoking a chain of immune reactions. The cooperation between innate and adaptive immune system are essential in maintaining the regulation of homeostasis of the host. Furthermore, the uptake of antigen becomes the foremost step in the human defense system in the way of fighting any pathogenesis since the adaptive immunity depends remarkably on the mechanism of antigen presentation by antigen-presenting cells (APCs) from the innate immunity (Parkin & Cohen, 2001).

The innate immunity is important because it contains an explanatory role in adaptive immunity due to the capability of molecules and cells from the innate immune system to differentiate between self and non-self-molecules. Thus, it becomes easy to make a specific response by stimulating different particular effectors mechanism for every different pathogen encountered. It is different from the role of lymphocytes in adaptive immunity, which do not have any specific task before activation. Besides from the appearance of antigen by the APCs to the cells of the adaptive immune system, the production of mediators by innate cells acts as a vital role in the stimulation of adaptive immunity. Among various mediators secreted, chemokines and cytokines are playing cohesive roles in regulating several immune responses (Luster, 2002; Jakóbisiak et al., 2003). Moreover, for being a necessity for adaptive immunity, the innate system functions at an early stage of killing and clearance of infectious pathogens and the resolution of the inflammatory response. Hence, the communication between the immune system either internally or externally mediated through small soluble signalling molecules is very important named cytokines. Cytokines are well known as low-molecular-weight proteins to control strength and period of the immune responses by exerting diverse response on countless kinds of immune cells and also play an significant role in the initiation, modulation, and selection of a suitable immune response, while chemokines are small polypeptides which act for chemotaxis and the following regulation of adhesion, activation, and migration of the leukocyte populations (Carolina et al., 2015).

From a therapeutic point of view, immunomodulation is a process of introducing immune cells to a specific agent to modify the host immune system up to the desired level. The immunomodulatory agent can alter the response of the immune system by elevating (immunostimulators) or depleting (immunosuppressive) the

production of serum antibodies (Bascones et al., 2014). Immunostimulator is promoted to enrich the immune response against primary or secondary immunodeficiency, infectious diseases, tumors, and modifications in antibody transfer, among others. Immunosuppressive agents are used to diminishing the immune response against transplanted organ and to treat autoimmune diseases such as allergies, lupus, or pemphigus.

Immunomodulatory agents or drugs act at different levels of the immune system. Thus, the development of these agents has been selectively specified, either intensifying or inhibiting the populations and subpopulations of immune system cells such as cytotoxic T lymphocytes (CTL), neutrophils, natural killer (NK) cells, lymphocytes, and macrophages. Immunomodulator influences the cells to produce soluble mediators such as cytokines (Bascones et al., 2014). Hence, in the immunotherapy concept, the immune system is employed to support the therapeutic of a specific disease. For example, the exogenous antigens produced from the inflammatory process involved in rheumatoid arthritis are taken up and phagocytized by APCs, dendritic cells and/or macrophages. Hence, the stimulation of these several immune cells stimulates the secretion of cytokines as the next step of the body defense process.

An immunosuppressant is an agent or drug that can inhibit the immune response in autoimmune disease and organ transplantation, while immunostimulant is an agent or drug to proliferation the immune response in cancer, immunodeficiency (for example AIDS) and commonly in infection. The term immunomodulation and immunomodulator are the same meaning as a compound or substance that can provoke the measurable modification in immune function. Their action can be specific and non-specific. Immunomodulators can act as a specific-action mechanism to the immune

system following the presence of a specific immunogen or antigen, with selective and specificity for immune response. It becomes selective when the inducement interprets into immunoreaction to one or diverse antigens, as an example in the case of therapeutic vaccines or adjuvants. The immunological adjuvants exaggerate the consequence of vaccines with synthetic antigens, including novel-generation antigens. These agents have also been used in experimental immunization to get monoclonal antibodies and polyclonal antiserums with the purpose of utilization of vaccines. Apart from specific-action immunomodulators, non-specific action immunomodulators are used to trigger and suppress the immune response, without specifying to the activity of stimulated cells to a specific antigen. They have separated into three different types; 1) response to the normal immune system, 2) response to the immunosuppressed immune system and 3) response to the functionally normal and immunosuppressed immune system (Bascones et al., 2014).

The literature demonstrates the advantages and bright future of natural products, including mushrooms for recognizing the intervention of new drugs or agents of immunomodulators. United States has approved 520 new drugs between 1983 until 1994 and 157 drugs were derived and extracted from natural product plus more than 60% contains anticancer and antibacterial (Newman & Cragg, 2016). In current years, there has been a cumulative interest in herbal medicines all around the world. Researchers have attracted large-scale attention towards herbal medicines for making as drug development and discovery (Abdulridha et al., 2020; Zhou et al., 2020).

1.2 Problem statement

Oyster mushroom (*Pleurotus sajor-caju*) is one of the extensively cultivated edible mushrooms (Sarangi et al., 2006) globally. *Pleurotus sajor-caju* is one of the *Pleurotus* spp. belonging to phylum Basidiomycota, well-known as oyster shaped mushroom (basidiocarps) and generally called oyster mushroom (OM). This species is generally seen in white and could be variously coloured, stalked or sessile, underground, and even epiphytic but rarely parasitic. This mushroom is widely distributed from the temperate to the tropical region and grow in the way of saprophytically at a temperature range of 12-32°C. The diverse range of its properties has been associated with this mushroom, including antitumor (Gu & Leonard, 2006), hypocholesterolemic, antiatherogenic, and antioxidative activities (Bobek, 1999). Oyster mushroom was found to trigger immune activity in animals (Paulík et al., 1996). Interestingly, the potential of adjuvanticity of mushroom-derived polysaccharides has widely been discussed in animal and human diseases (Wei et al., 2008; Badalyan et al., 2019). Large numbers of the strain of *Pleurotus* spp. have been studied on the effect of immunomodulation in the normal cells, but there is no evidence showing the immunomodulatory effect of *Pleurotus sajor-caju* extracts on human macrophage cell line THP-1. In this study, the transcriptional profile of cytokines and molecular mechanisms of immune response responsible for the immunomodulating effects by *Pleurotus sajor-caju* on the macrophages may shed light on the potential of adjuvanticity of polysaccharides isolated from the mushroom.

1.3 Research objectives

i. To determine the polysaccharides and beta-glucan contents of *Pleurotus sajor-caju* crude extracts.

ii. To determine the cytotoxicity and cell proliferative effects of PSC crude extract on THP-1 human macrophage cells.

iii. To determine the gene expression levels of cytokines in THP-1 human macrophage cells treated with PSC crude extracts.

iv. To evaluate the nitric oxide (NO) production and gene expression level of inducible nitric oxide synthase (iNOS) in THP-1 human macrophage cells treated with to PSC crude extracts.

v. To observe the morphological changes of THP-1 macrophage cells treated with different concentrations of PSC crude extracts.

1.4 Hypothesis study

Mushroom-derived polysaccharides are known for their nutritional and medicinal values. These active components potentially contribute to the immunomodulation of the immune system in the host. The polysaccharides derived from *Pleurotus sajor-caju* extract is non-toxic and has immunomodulatory effects on THP-1 human macrophages.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Mushroom

For decades, mushrooms have been consumed for their nutritional and medicinal values. The multifunctionality of mushrooms as anti-tumor, anti-cancer, immunomodulatory, and dietary intake has drawn the attention of the health practitioners and researchers around the world for treating various diseases. Mushrooms are well-known ingredients of gourmet cuisine across the world because of their unique savor (Valverde et al., 2015). Mushrooms have been growing an extended tradition in Eastern and Asian countries, especially in China, which is classified as the most prominent producers for mushrooms when compared to worldwide production. In 2007, the Food and Agriculture Organization of the United Nations (2009) found that China has produced more than 1.5 million metric tons, resulting in an increment of about 65% within 10 years. The production rank was followed by the United States and Canada growth in consumer consciousness on the health benefits of mushrooms has resulted in the high consumer demand, which becomes a factor of the production mushrooms continuously increase over time.

Mushroom is described as ‘a macro fungus with a distinctive fruiting body, large enough to be seen with the naked eye and to be picked by hand. There are an estimated 140,000 species of mushrooms distributed over the globe (Minato, 2010). Mushrooms are known for their beneficial source of many different miraculous biological properties to be used in pharmaceuticals and nutraceuticals. It is also known as mini-pharmaceutical factories for producing various biopharmaceutical compounds. They could be other alternatives for new antimicrobial compounds, including proteins, quinolones, terpenes, steroids, and oxalic acid. Moreover,

mushrooms also can provide varied vitamins for human, such as Vitamins B1, B2, B12, C, D, and E (Valverde et al., 2015). There are thousands of studies that discovered mushroom-extracted metabolites are famously used as biological response modifiers (BRM) or as immunomodulators (Mizuno et al., 2014).

An extensive group of fungi, known as Basidiomycetes, have a typical life cycle that starts with the sprout germination by means of a suitable medium of the substratum and becomes a monocaryotic mycelium with genetically identical (n) nuclei that can grow indefinitely. During hyphal fusion or plasmogamy, two compatible monocaryotic mycelia may produce fertile dikaryon. This dicaryon has a clamp and binucleate attachment in each hyphal compartment that contains two genetically distinct nuclei across the mycelium. The effect of sufficient environmental factors (light, relative humidity, and temperature), the dikaryotic mycelium, differentiates into fruit bodies with unique structures known as basidia. Karyogamy (fusion of the paired nuclei; 2n) and meiotic (recombination and separation) occurring in those club-shaped binucleate cells which have developed into lamellae (hymenium) of each fruity body. The four haploid nuclei resulting in this transfer to the basidium sterigmates and form four new basidiospores. When the fruit bodies are mature, basidiospores are released, restarting the sexual life cycle (Ford et al., 2016).

2.1.1 *Pleurotus sajor-caju*

2.1.1(a) Background

Instead of the formal name of *Lentinus sajor-caju*, it is also known as *Pleurotus sajor-caju*. The genus of *Lentinus* has constitutes approximately about 120 species and belongs to the kingdom of Fungi, the family of Polyporaceae as stated in Table 2.1.

Polyporaceae are a family of poroid fungi which form fruiting bodies with pores or tube on the underside as shown in Figure 2.1 (Bolhassan et al., 2012; Justo et al., 2017).

Table 2.1: Taxonomy hierarchy of *Lentinus sajor-caju*

Kingdom	**: Fungi**
Phylum	: Basidiomycota
Class	: Agaricomycetes
Order	: Polyporales
Family	: Polyporales
Genus	: *Lentinus*
Species	: *L. sajor-caju*

2.1.1(b) Habitat

Pleurotus sajor-caju breeds extensively in forests having sub-tropical and temperate climates. It is classified as saprotrophic species and play a role in decomposing the deciduous tree specifically the Beech tree. This species of mushroom is also found to be carnivorous. It feeds on nematode, an animal that is itself parasitic. It also be a reason to get the its nitrogen from nematode (Bolhassan et al., 2012).

2.1.1(c) Local Names

The local name *Pleurotus sajor-caju* is a grey oyster mushroom, in Malaysia is ‘cendawan tiram,’ the name given by referring to the same taste of the oyster (tiram) (Bolhassan et al., 2012).

Figure 2.1: Example of oyster mushroom in Malaysia with a characteristic of typically looking like umbrellas that have a stem (stipe) and a cap (pileus).

2.2 Polysaccharides

Polysaccharides extracted from mushrooms are found to possess important roles as antitumor and immunomodulating agents (Zhang et al., 2007; Badalyan et al., 2019). Polysaccharide mushrooms are one of the important components of the fungal cell wall. The mushroom cell wall consists of 2 main polysaccharide groups: cellulose, the other matrix-like β-glucan, α-glucan, and glycoprotein. The mushroom polysaccharide displays linear and branched glucans of different forms on the glycosidic association (1→3), (1→6)-β-glucans, and (1→3)-α-glucans. Even though there are a lot of differences in the chemical components of mushrooms polysaccharides, the group of β-glucans acts as the most significant contributor (Wasser, 2002). Polysaccharides extracted from mushrooms have been proved to be very helpful in the search for efficient, non-toxic constituents with free radical scavenging activity, for example, Lentinus polychrous Lév-extracted polysaccharides which contain scavenging effects on superoxide and hydroxyl radical (Thetsrimuang et al., 2011). In addition to this, immunomodulation and antitumor activity of polysaccharides, which are involved in the processing of biological response modifiers, in immune effector cells such as macrophages, dendrite cells, lymphocytes,

natural killer (NK) cells and hematopoietic stem cells, are involved in innate and adaptive immunity. Polysaccharides may also strengthen the basic role of leukocytes, macrophages and natural killer cells and enhance immune response for the host by activating the complement system (Moradali et al., 2007).

The benefits of polysaccharides act as vaccine adjuvants for bacterial and viral antigens have been proved in animal models in a way to increase the resistance to a variety of bacterial, viral, protozoan, and fungal infections. Recently, most of the pharmacological studies using polysaccharides extracted from some edible mushrooms have been shown to aid in sugar level reduction, serum lipids, antibiotic activity, antiviral properties, antitumor, and immuno-stimulating properties (Khamlue et al., 2012). Polysaccharides isolated from Grifola frondosa GF9801 cultured mycelia expressed potential as anti-proliferative activity in MCF-7 cells (Cui et al., 2007). *Pleurotus* spp. known as oyster mushrooms, are refreshing as a source of biologically active glucan and are known as food supplements due to their immunosuppressive activity (Synytsya et al., 2009). In Traditional Chinese Medicine, *Prunella vulgaris* (PV) is already famous as tumour therapy for centuries. PV extracted polysaccharides are shown to have anti-lung adenocarcinoma activity and have potential in increasing the thymus index and the spleen index as immunomodulation effects in C57BL/6 mouse-Lewis lung carcinoma (LLC) model (Feng et al., 2010).

The present outbreak that affected worldwide related to the acute respiratory disease triggered by SARS-CoV-2 virus named as novel coronavirus disease COVID-19 has been dispersed less than three months rapidly and poses a huge warning to public health. Currently, there are practically no precise drugs targeted for coronavirus treatment. Due to the nature of the virus that can alter and progress the resistance rapidly to these therapies, there is almost no hope for researchers to find the specific

mechanism to prevent the growth of the virus (Liu et al., 2020). During this urgency and desperate period of time, the international medical community needs to step out to develop drugs or vaccines that must be very low toxic, high-efficiency, and specifically targeted to the property and structure of the coronavirus. Polysaccharides from natural products have wide advantages based on their good biocompatibility, low toxicity, and impressive immune regulation (Chen & Huang, 2018; Muralidharan et al., 2019). Polysaccharides possess the unique antiviral mechanisms based on several pathways, 1) directly interacting with the virus, 2) inhibition the virus adsorption and invasion, 3) inhibition the viral transcription and replication, 3) through the activation of the host antiviral immunomodulatory system (Chen et al., 2020). Mushrooms have been used widely as food and drugs for a very long time in the China and Orient centuries. One of the notable species in Asian countries named as *Lentinus edodes* is popularly consumed as health foods. Polysaccharides extracted from this mushroom are most intensively explored with countless immune progressions, which are generally explained as biological response modifiers (Ren et al., 2015). In addition, polysaccharide from cultured Lentinula edodes mycelia has been widely explained as an immunostimulant in animals and human cells affected by many viruses such as hepatitis B virus (HBV), influenza virus and human papillomavirus (HPV) and human immunodeficiency virus (HIV) (Pierro & Bertuccioli, 2020). Due to the mechanism of its action, polysaccharides may be an impressive potential in stimulating a defending response to a broad range of viral infections, including coronavirus.

2.3 General Immune System

The immune system is the body's fundamental defence against cancer growth, tumour, and infectious diseases. It is a network of cells, tissues, organs and containing blood cells, chemicals, and protein. It also plays an important role in defending against

millions of microbes, bacteria, viruses, toxins, and parasites that may invade the body as well. This complex system plays the main role in recognizing, attacking, and destroying any harmful foreign particles to the body (Nicholson, 2016).

There are several factors that may affect the immune response such as age, gender, stress, environment, and health condition. Immunity can be determined by two elements, which are specificity and speed of the reaction. There are two different basic types of responses for eliminating pathogens. These are known as adaptive and innate responses. Based on these two responses, innate immunity provides immediate response, although it is less specific in response. Meanwhile, adaptive immunity response is activated by innate immunity, which produces stronger and highly specific responses against pathogens and able to develop memory in terms of recalling the specific intruders in later challenges (Miller & Segerstrom, 2006).

The innate immunity also known as a natural response and can be activated immediately to defend the human body from an infectious agent and pathogen attacks. Basic principle of innate immune system is to prevent parasites, viruses, bacteria and other foreign particles from the attacking and spreading throughout the human body. Therefore, innate immune defence system contains general defence involving several components for example 1) physical barrier such as eyelashes, skin, cilia and gastrointestinal tract, 2) defence mechanism such as sweat, saliva, tears, and gastric acid, 3) general immune response such as non-specific cellular response, inflammation, and complement. In the innate immune system, there are many forms of cells that function broadly and non-specifically, such as natural killer cells, phagocytic cells (macrophages, monocytes, and neutrophils), and cells that release inflammatory mediators (mast cells, basophils, and eosinophils). The example of molecular mechanisms of innate responses which get involved include cytokines and

complement protein. These innate immune system components take place in attacking pathogens before the active infection is activated. In certain cases, the innate immune response is inadequate in eradicating all pathogens from the human body. Therefore, an innate and adaptive immune system must work together to reduce the severity of the infection and along with eliminating any pathogens or invader microorganisms (Paul, 2011; Parkin & Cohen, 2017).

Adaptive immunity is the hallmark of the immune system of higher species. Adaptive is also known as an acquired reaction, which strengthens with repeated exposure to infection. Adaptive immune response is triggered when exposed to the pathogen and immunological memory is used to improve the immune response that is distinct from the innate immune system, which attacks only on the basis of self-identification and non-self-antigen. In the meantime the adaptive immune response is much slower but precise to respond to the infection, while the inherent immune response is fast but lacks precision. The capacity of adaptive immune response is that from the first episode of exposure, it has immunological memory to recall particular specific antigens, so that subsequent exposure provides a quicker and more vigorous response (Parkin & Cohen, 2001). Contrasting with innate immune system, the adaptive immune system only has two types of cells working in elimination of pathogen, which is B cells and T cells. Antigen-specific B and T cells are stimulated and proliferate once the receptors of these cells bind to the antigen.

Acquired responses consist of the proliferation of antigen-specific B and T cells, which happens when receptors of these cells bind to the antigen, and specialized cells (APCs) often show the antigen to lymphocytes and cooperate with them in response to the antigen. In response, B cells begin to secrete immunoglobulins, also known as antigen-specific antibodies for the removal mechanism of extracellular

microorganisms. T cells also enable B cells to develop different antibodies and destroy pathogens by initiating macrophages and killing virally infected cells. In conclusion, innate and acquired responses normally work together to remove pathogens (Parkin & Cohen, 2001).

2.3.1 Macrophage

A macrophage is the primary cell of innate immunity, which contributes to the supervision of tissues and engulfing and degrading the infected cells and microbes. The macrophage is also an efficient phagocytic cell that can leave the circulatory system by passing through the walls of capillary vessels. This ability makes macrophage important in eliminating pathogens with fewer limits. Activated macrophage excretes a wide range of cytokines to activate, give a signal, and recruit other cells to commence an adaptive immune response. Upon stimulation by lipopolysaccharides (LPS), macrophages in bloodstream produce and release proinflammatory mediators, including tumor necrosis factor (TNF), as an early reaction of cytokine and interleukin-6 (IL-6), which is responding to secreted at the redundant time frame. TNF is a multifunctional cytokine which also can rapidly synthesise and primary release to recruit and activate other cells to the place of invasion and infection. TNF secreted by macrophages has various vital tasks in sepsis. While its production is essential to the immune response, extreme production of TNF may lead to an acute and chronic inflammatory disease which can give a significant clinical problem (Yan, 2007; Ferenbach & Hughes, 2008; Stow et al., 2009).

2.3.2 Toll-like receptor 4 (TLR4)

To date, 10 and 12 functional toll-like receptors (TLRs) have been identified in human bodies and mice (Uematsu & Akira, 2008). Any microorganism that invades the vertebrate host membrane is initially acquainted with the mechanism called the innate immune system through germline-coded pattern recognition receptors (PRRs). There are a variety of forms of PRRs that identify distinct microbial components, cytoplasm mouse receptors, and directly stimulate immune cells, such as toll-like receptors(TLRs). Exposure of immune cells to the ligands of these receptors activates intracellular signalling cascades that rapidly induce the expression of a number of overlapping and unique genes involved in the inflammatory and immune responses. The beliefs about innate immunity are varying the way we approach our thought about mode of pathogenesis and treatment of infectious, allergy and autoimmunity (Uematsu & Akira, 2008; Kawai & Akira, 2010).

Toll-like receptor 4 recognizes the diverse type of ligands which have a different kind of structures such as heat-shock proteins, the fusion protein of respiratory syncytial virus (RSV), fibronectin, diterpene paclitaxel of the plant and lipopolysaccharides. The flexibility of TLRs expression that can be modulated instantly due to response of pathogens, cytokines, and environmental stresses, it makes TLRs present in a different type of immune cells, such as B cells, specific types of T cells, dendritic cells (DCs), macrophages and also on nonimmune cells such as epithelial cells and fibroblasts (Uematsu & Akira, 2008). The study has shown that polysaccharides extracted from *Cordyceps militaris* proved to stimulate the DCs maturation with the underlying mechanism through TLR4 signalling (Sook et al., 2010). Moreover, the novel polysaccharides isolated culture broth of *Cordyceps militaris* activates macrophages of RAW264.7 under the mechanism of NF-κB and

MAPKs signaling pathways via TLR4 (Lee et al., 2014). The studies above using lipopolysaccharides (LPS) as a control to see the underlying mechanism. LPS is known as the most potent immunostimulant, which contains a lipid portion responsible for the pathogenic phenomena such as endotoxin shock. When LPS is present in the bloodstream, it will bind to the CD14, which is a glycosylphosphatidylinositol (GPI) linked protein expressed on the cell surface phagocytes. After that, LPS then relocates to MD-2, which associates with the extracellular portion of TLR4 before the process of oligomerization as a significant molecule of LPS signaling (Poltorak et al., 1998; Shimazu et al., 1999).

2.3.3 Cytokine

Cytokine is small protein-peptide synthesized and secreted by cells of both innate and adaptive immune system during an immune response and play as the main role in communication and regulate diverse functions within the immune system (Stow et al., 2009). There is thousands type of cytokines in the human body. Cytokines are secreted by diverse different cell types and frequently show overlying activities regulating differentiation or proliferation, depending on the type and development state of the target cells involved. Functional redundancy and pleiotropy characteristics trademark of cytokines, which include interferons, interleukins, growth factors and colony-stimulating factors. The cytokines interleukin 6 (IL-6), interleukin 1 beta (IL-1β), and TNF are increased in most and have been perceived as targets of therapeutic agents intervention. The releasing of cytokines as characteristic of soluble messengers is necessary event for cell-cell communication and regulation within the immune system. Granulocytes and macrophages secrete cytokines to recruit and trigger other cells during inflammation, or as direct destruction (Holgate, 2000). Cytokines also link

cells of the immune system to those in surrounding tissues. During the cell generation, in tumour development and after injury episodes, cytokines can convey the lethal or reparative signals to other cells (Yan & Hansson, 2007). This is an important and complex task for many cells, including macrophages, to secrete variety cytokines, and it is necessary to occur together with other cellular functions as a part of a cell's immune response.

Cytokines are small molecular weight messengers formed by a specific cell to modify the reaction and actions of itself or another cell. By binding to unique cell-surface receptors, cytokines distribute intracellular signals. Most of them are soluble, but some are membrane-bound cytokines. A wide range of cells contain cytokines and have a broad variety of functions. The biological reaction depends on the type of cytokine and the cell involved, but typically, these molecules can influence movement, cell activation, cell generation, division, or apoptosis. Cytokines formed by leukocytes and having reactions primarily to white blood cells are referred to as interleukins. Cytokines with chemoattractant agents are called chemokines. Those that cause the spread and differentiation of stem cells are called colony-stimulating factors. Interferons are those that interfere with viral replication (Parkin & Cohen, 2017).

2.3.4 Tumour Necrosis Factor (TNF)

The word "tumour necrosis factor" originally was taken from two molecules, the factor-alpha (TNF-α) tumour necrosis factor, the factor-beta (TNF-β) tumour necrosis factor, and the factor of lymphocyte-derived tumour necrosis factor. In the 1970s, tumour necrosis factor-alpha (TNF-α) was found as a serum factor caused by endotoxin which is responsible for *in vivo* and *in vitro* necrosis of certain tumours. It participates in the inflammation reaction of the innate immune system, including

cytokine production induction, adhesion molecules activation or expression and growth stimulation. It encourages the proliferation and cytostatic activity of normal cells against tumour, antiviral, immuno-regulatory and inflammatory response. It stimulates the proliferation of normal cells. TNF-α also has demonstrated many additional roles associated with endothelial development, coagulation, metabolism of lipids, and resistance to insulin. It is one of the most common and pleiotropic cytokines that mediate inflammatory and immune responses (Turner et al., 2014).

2.3.5 Interleukin 1 Beta (IL-1β)

The interleukin 1 beta (IL-1β) is also a cytokine that acts as a mediator of the inflammatory activity. Significant for resistance to the pathogen and the host-response, it also can worsen acute tissue injury and chronic disease. Therefore, there is a huge range of interest on how unique this protein is secreted and released from cells. However, the mechanism of IL-1β release has proven to be elusive (Lopez-castejon & Brough, 2011). IL-1β is a dominant pro-inflammatory cytokine, central to host defence responses to infection and injury.

2.3.6 Interleukin-6 (IL-6)

Cytokine interleukin-6 (IL-6) not only includes responses to infection and inflammation, but also controls metabolic, regenerative and neural processes. IL-6 activates target cells in traditional signalling through a membrane-bound interleukin-6 receptor that associates the signalling receptor protein gp130 with a ligand binding (Scheller et al., 2011). IL-6 is expressed by a variety of cells, including mononuclear phagocytes, T cells, B cells, fibroblasts, endothelial cells, keratinocytes, and

hepatocytes. IL-6 is involved in hematopoiesis that was important in ending the maturation of B cells into antibody-producing plasma cells, activating T-cells, differentiating and controlling Th2 and Treg phenotypes (Turner et al., 2014).

2.3.7 Nuclear Factor kappa-B (NF-κB)

Nuclear factor kappa-B (NF-κB) is a collection of proteins which comprises of various structurally-related eukaryotic transcription factor including NF-κB1 (p65), NF-κB2, RelA (p50), RelB and c-Rel, controls the transcription of DNA. An NF-κB signalling pathway is one of the possible pathways that has regulated the immunomodulatory function of opioids in inflammation and is important for the pathogenesis of a number of chronic inflammatory diseases. In the normal condition, inactive, NF-κB is bound to its inhibitor proteins called IκBs. When the IκB kinase (IKK) complexes are activated by the LPS, it stimulates the phosphorylation of IκB and proteasome degradation cause of releasing the active NF-κB complexes in the cytoplasm. After the NF-κB activation, this protein is then translocated into the nucleus where it binds to a specific sequence of DNA, resulting in transcription of DNA into mRNA which can be translated into protein, which leads to expression of various inflammatory mediators such as cytokines, chemokines and endothelial cell adhesion molecule (Naliboff et al., 1995). Activation of the NF-κB signalling pathways creates a constructive self-regulatory loop to synchronise and sustain immune response to infection or painful stimuli. The innate immune system activation of NF-κB also activates the release of pro-inflammatory cytokines, and these mediators further facilitate the infiltration and migration of immunocytes into the adaptive immune system to organise the inflammatory response to the pain signals (Rahiman et al., 2016). The binding of polysaccharides at the TLR4 has been reported able to initiate

this NF-κB signalling pathway to produce several cytokines protein as the immune response to pathophysiology and physiology of the human body reaction.

2.3.8 Nitric oxide (NO) and inducible nitric oxide synthase (iNOS)

Nitric oxide (NO) molecule shows an important role in host defence response against several types of pathogens such as bacteria, viruses, fungi, and parasites. Under normal physiological conditions, NO plays a key role in the control of many pathophysiological mechanisms such as vasodilatation, neuronal interaction and neurotoxicity. However, overproduction of NO causes acute and chronic inflammation-related tissue damage. NO is intercellular mediator formed by three forms, namely, endothelium NO synthase (eNOS), neural NO synthase (nNOS) and inductible NO synthase (iNOS) in various mammalian cells. NO is the intercelled mediator produced by three forms of nitric oxide synthases (NOS). In a broad variety of cells and tissues, such as chondrocytes, Kupffer cells, hepatocytes, pulmonary epithelium, neutrophils, colonic epithelium, vasculature, macrophages, and various neoplastic diseases, iNOS involvement has been demonstrated. In response to a wide range of stimuli, iNOS produces NO, most obviously endotoxin and endogenous pro-inflammatory mediators. Regulation of NO output via iNOS necessarily occurs during transcription and translation, iNOS synthesises large amounts of NO before depletion of the substrate. The expression of the iNOS gene and the following mRNA translation are regulated by an increasing amount of agonists, especially pro-inflammatory mediators (Joo et al., 2014; Lechner et al., 2005).

2.3.9 Programmed cell death-ligand 1 (PD-L1)

Costimulatory programmed death ligand 1 (PD-L1) is a constituent membrane protein, and is upregulated on hematopoietic cells such as dendritic, bone-marrow, derived mast calls, B-cells, T-cells, macrophages and non-hematopoietic cells as endothelial, epithelial and muscle cell stimulating effects. The interaction between PD-1 and PD-L1 causes T cell suppression and energy and thus terminates, or prevents efficient T cell response and generally considered a molecule of immunological inhibition. Consequently, the expression of these molecules on antigen presenting cells and T-cells is assisted by many pathogens which both cause acute and chronic infection (Vashishta et al., 2015). Poor prognosis has been associated with a rising level of PD-L1 in many cancers, including melanoma, nasopharyngeal cancer, ovarian cancer, lung cancer, and renal cell cancer. Inhibition of PD-L1 with antibodies in patients suffering from this cancer improved overall survival rates. Many recent studies have shown that inhibition of PD-L1 contributes to therapeutic remedies such as 52% in metastatic bladder cancer, 66% in relapsed follicular lymphoma, and 87% in relapsed or refractory Hodgkin's lymphoma (Zuo et al., 2016).

2.4 Mushrooms as antimicrobial

Nowadays, the prevalence of infectious disease is increasing and presents as a global issue. The antimicrobial resistance (AMR) problem urges improved effort to be developed to discover more efficient antimicrobial agents against the pathogenic microorganisms resistant to current treatment. Hence, the pursuit of the latest products with the antimicrobial properties is very high ranking in research. Many studies focused on use the natural resources approach for that propose. According to the previous study about the effect of mushrooms extract on antimicrobial activity, they using several bacteria for the screening of antibacterial activities such as gram-positive