THE POTENTIAL ANTIVIRAL ACTIVITY OF MALAYSIA STRAIN SCHIZOPHYLLAN EXTRACT TOWARDS NEWCASTLE DISEASE VIRUS

BY

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ABSTRACT

Antiviral drugs have been one of the major attentions in the world nowadays because of the rise in multiple of illnesses and new diseases. However, many of antiviral drugs were developed synthetically and have high toxicity towards cells. Therefore, it is vital to discover antiviral drugs from natural resources that are effective as well as perceived to be safe. Malaysia has been known for its diversified flora and fauna that have potential benefits to human health. In particular, plants and mushrooms have been long known for their medicinal properties and have been fully implemented in Traditional Chinese Medicine. Many mushrooms such as *Ganoderma lucidum* and *Lentinus edodes* have been widely recognized for its medical treatment and have been commercialized by many pharmaceutical companies. Consequently, this study on antiviral compound from *Schizophyllum commune* (Malaysia strain) is important to determine the properties of *S. commune* isolated locally. *S. commune* is a fungus that excreted an exopolysaccharide known as Schizophyllan and is believed to have antiviral, antimicrobial as well as anticancer activity. The purpose of this study is to determine the effectiveness of locally produced Schizophyllan extract against Newcastle Disease Virus (NDV) F strain and *Mukteswar* 'S' strain. The selected Malaysian strain of *S. commune* mycelia was cultivated by liquid fermentation and Schizophyllan was extracted using isopropanol; then was further analyzed by FTIR. Subsequently, the extract was tested for its cytotoxicity towards normal cell line of DF-1. The extract was tested for its antiviral activity by post-attachment assay where Schizophyllan was introduced towards cells infected by NDV. The antiviral activity was further optimized using Response Surface Method (RSM) to measure the viral inhibition rate. The results showed that the extract prevented the viral replication by inhibiting 83% NDV F strain replication using 931.53 µg/ml of extract and treated 2 times per day towards the infected cells; and inhibiting 40.3% of NDV Mukteswar 'S' strain using 1010.63 µg/ml of extract and treated 2 times per day. In conclusion, Schizophyllan from *S. commune* (Malaysia strain) were highly recommended for further analysis and research towards the advancement of antiviral drug development.

خلاصة البحث

إن الأدوية المضادة للفيروسات تعتبر من أحد الاهتمامات الرئيسيةفي العالم في الوقت الحاضر بسبب ارتفاع العديد من الأمراض وظهور الأمراض الجديدة. ومع ذلك، لقد تم تطوير العديد من الأدوية المضادة للفيروسات صناعيا و لكن هذه العقاقير الاصطناعية قد تسبب ارتفاع نسبة السموم عند الخلايا. لذلك، يجب اكتشاف أدوية مضادة للفيروسات فعالة من الموارد الطبيعية التي تعتبر سالمة. و عرفت ماليزيا أيضا بالنباتات المتنوعة وبالحيوانات التي يمكن أن يكون لها فائدة مرجوة على صحة الإنسان. ولقد عرفت الموارد الطبيعية مثل النباتات والفطر لخصائصها الطبية منذ زمن طويل و قد استخدمت بشكل كامل في الطب الصيني التقليدي. هناك اصناف عديدة من الفطر مثل *Ganodermalucidum* و *Lentinusedodes* التي قد تم الاعتراف بها على نطاق واسع للمعالجة الطبية في الوقت الحاضر، وقد تم تسويقها من قبل العديد من شركات الأدوية. و بناءً على ذلك فإن هذه الدراسة على المركّب المضاد للفيروسات من *Schizophyllum commune*(سلالة ماليزيا) ذات اهمية في تحديد خصائص *S.commune* المنفردة في هذا البلد. إنّ *S.commune* من الفطريات التي تفرز عديد السكاريد الخارجي المعروف باسم Schizophyllan و يعتقد بأن يكون له نشاطات مضادة للفيروسات و الميكروبات وكذلك مضادة للسرطان. إن الغرض من هذه الدراسة هو تحديد مدى فعالية مستخلص *Schizophyllan* المنتج محلياً ضد فيروس مرض نيوكاسل (NDV) سلالة 'F' و سلالة 'S' Mukteswar . لقد احضرت السلالة الماليزية المختارة من فطر *S.commune* بطريقة التخمير السائل و تم استخراج Schizophyllan بواسطة الأيزوبروبانول وتم تحليله بالمزيد باستخدام FTIR. بعد ذلك تم فحص المستخلص للسمية الخلوية ضد خط الخلية العادية DF-1 قبل تحليلها لخاصية المضاد للفيروسات. تم فحص المستخلص للنشاط المضاد للفيروسات عن طريق الفحص المرفق حيث قدم Schizophyllan نحو خط خلية NDV المصابة. لقد تمت معالجة مضاد الفيروسات على النحو الأمثل باستخدام منهجية الاستجابة السطحية (RSM) لقياس معدل تثبيط الفيروسية. و أظهرت النتائج أن المستخلص منع العدوى الفيروسية من خلال تثبيط 83٪ NDV والنسخ المتماثل سلالة F باستخدام 931.53 ميكروغرام / مل من المستخلص. وتمت معالجته مرتان في اليوم الواحد نحو الخلايا المصابة، وتثبيط 40.3٪ منNDV Mukteswarسلالة 'S ' باستخدام 1010.63 ميكروغرام / مل من المستخلص ومعالجته مرتان في اليوم الواحد. في الختام، يوصى باستعمال Schizophyllan من *S. commune* (سلالة ماليزيا) للمزيد من التحليل و البحث في تطوير الأدوية المضادة للفيروسات.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology Engineering).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree centigrade
µg	Microgram
µg/ml	Microgram per mililiter
µl	Microliter
cm	Centimeter
CO_2	Carbon dioxide
$C_6H_{12}O_6$	Glucose
CPE	Cytopathic effect
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
IC50	Effective dosage of drug that gives 50% of maximal response
FBS	Fetal bovine serum
FTIR	Fourier Transform Infrared
g	Gram
K_2HPO_4	Potassium phosphate anhydrous
KH_2PO_4	Potassium dihydrogen orthophosphate
L	Late region
M	Molar
mg	Miligram
mg/ml	Milligram per mililiter
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
ml	Mililiter
mm	Millimeter
mw	Molecular weight
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
Na_2CO_3	Natrium carbonate
$MgSO_47H_2O$	Magnesium sulfateheptahydrate
NDV	Newcastle Disease Virus
PBS	Phosphate buffered saline
rpm	Revolution per minute
sp	Species
ß	Beta
UV	Ultraviolet
wt	Weight

CHAPTER ONE

INTRODUCTION

1.1 OVERVIEW

Fungi does not only become an important source of human diet due to its high protein content and lower energy, but also proved to contain medicinal properties such as anticancer, antibacterial, antiviral, immune response-stimulating effects, anti-hypertensive and blood lipid lowering effects (Wasser and Weis, 1999; Khan et al., 2010). Famously known as mushrooms, fungi constitutes of approximately 140 000 species on earth and only 10% have been identified (Hawksworth, 2001). Thus, there is a high potential for a discovery of other useful mushrooms that can benefit humans.

Schizophyllum commune is a widely distributed basidiomycete and also known as *Cendawan Kukur* in Malaysia. This fungal species are also common in many continents around the world due to its endurance towards the habitat hardiness except in the Antarctic (Klaus, 2011). Mushrooms were popular as Traditional Chinese Medicine (TCM) before modern times. Therefore, nowadays many researches on various strains of *S. commune* have been conducted around the globe in order to extract the bioactive compound claimed to have medicinal use.

S. commune claimed to have antitumor, anticancer, antibacterial, antifungal, antiviral, antiproliferative and anti-inflammatory effect (Abraham, 2001; Teoh and Don, 2011; Salvador et al., 2010). This fungus excretes an exopolysaccharide known as Schizophyllan, which is a neutral homoglucan. According to the work of Fukushima (as cited in Ajith and Janardhanan, 2003), Schizophyllan have already established a wide market in China and Japan as anticancer drugs and currently used in cancer treatment. However, current and previous researches of *S. commune* biological

properties were extracted from other strains of this fungi such as*S. commune* Fries, *S. commune* NRCM and *S.commune* ATCC 15205.

In this research, mycelium of selected *S. commune* strain were grown and cultured in the laboratory for further research. The content of commercial grown mushrooms and wild mushrooms might have slight different nutraceutical properties such as content of sugar, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), tocopherol, ascorbic acid and phenol. However, the quality of bioactive properties does not differbetween commercial and wild mushrooms (Barros et al., 2008).

This research focuses on the antiviral properties of crude extract produce by *S. commune* mycelium. The crude extract was analyzed for its carbohydrate content and existing β-glycosidic bond by Fourier Transform Infrared Spectroscopy (FTIR) method to identify the presence of Schizophyllan as exopolysaccharide. The antiviral effectiveness of *S. commune* extract was tested and optimized towards Newcastle Disease Virus (NDV). Disease from NDV may cause a high potential economic threat to the commercial poultry farming (Azmir et al., 2011). Usually, the disease is controlled by prophylactic vaccination using live and inactivated vaccines. The most virulent strain of NDV might cause a sudden death of the animals while the intermediate virulent strain may cause a decrease in eggs quality and production (Alders and Spradbrow, 2011). Treatment may be given to the infected animal in comparison to the loss of billions of poultry animals causing a higher cost. Therefore,the extract was also tested towards two different strains (NDV S strain and NDV *Mukteswar* S strain) due to the different virulent effects.

1.2 PROBLEM STATEMENTS AND ITS SIGNIFICANCE

Antiviral compounds have a large market potential due to the escalating viral diseases. Poultry animals are more likely prone to infectious viral diseases because of the small and confine farm spaces. Therefore, it will affect the amount of productivity and quality of poultry food source. Many antiviral compounds are developed synthetically; some of the compounds are toxic towards the cell and some are less effective when examined by *in vivo* test. Thus, the discoveries of effective antiviral compounds from natural sources that are less toxic toward the cells are essential.

In addition, most antiviral compounds in Malaysia are either imported from other countries or developed synthetically, subsequently causing a higher import prices on the drugs. Hence, the development of antiviral compound from natural resources in this country will indirectly reduce cost of importing antiviral drugs and transform the pharmaceutical and nutraceutical industries.

Many researches previously focus on plant secreted polysaccharides for antiviral properties. However, fungus would be a more suitable material for cheaper production due to large quantity production, have less growing time and the extraction process of the secondary compound is simpler. Therefore, in this research, *S. commune* is regarded as a suitable raw material to produce an antiviral compound since this mushroom grows abundantly in Malaysia. *S. commune* excreted an exopolysaccharide compound known as Schizophyllan. Schizophyllan excreted from *S. commune* Fries strain have been widely claimed to have antiviral properties. Therefore, it is crucial for strain of *S.commune* from Malaysia to be tested for its antiviral activity as well in order to locally produce such antiviral compound.

1.3 RESEARCH OBJECTIVES

This research aimed to achieve these three objectives:

1. To produce crude extract containing bioactive compounds from local strain of *Schizophyllum commune* using liquid fermentation.
2. To determine the cytotoxicity of the *S. commune* crude extract towards the DF1 cell line that used for Newcastle Disease Virus (NDV) infection.
3. To investigate and optimize the antiviral treatment of the *S. commune* crude extracttowards Newcastle Disease Virus (NDV) strains.

1.4 SCOPE OF RESEARCH

This research is a part of project collaboration with Agro-Biotechnology Institute (ABI), thus the screening of different sample of *S. commune* (Malaysian strains) were collected from seven different places in this country were done by their respective officers. This strain selection is crucial to select the highest production and resistance of these mushrooms towards harsh environment.

The main focus of this research is to investigate the antiviral properties of the crude extract from Malaysian strain of *Schizophyllum commune.* Therefore, this study covered five main parts; the production of the crude extract from the mycelium of *S. commune* (Malaysia strain), the production of DF-1 cell line for virus infection, the cytotoxicity test of *S. commune* extract towards the DF-1 cell line, the infection of Newcastle Disease Virus (NDV) towards the cell line and the antiviral activity test towards NDV.

In the first phase, the production of crude extract from *S. commune* (Malaysia strain) was achieved by liquid culture fermentation. Mycelium was homogenized and separated by centrifugation. Isopropanol was added to separate Schizophyllan from the

supernatant. Centrifuge and filtration methods were used in order to remove the isopropanol and other excess media from the crude extract. In this phase, optimization of media and fermentation of the fungus were not conducted. The parameters of fermentation were according to previous researches obtained from literature reviews while medium formulation is according to an established research of Rau et al. (1999) and Rau (2002). Crude extract were freeze dried and analyze for exopolysaccharide composition using Fourier Transform Infrared (FTIR).

The second phase includes the DF-1 cell culture that will be used in the propagation of the Newcastle Disease Virus (NDV). Third phase comprise the toxicity test of *S. commune* extract towards the DF-1 cell line in order to measure the cell viability and fatality towards the bioactive compound.

The fourth phase consist of replicating the Newcastle Disease Virus in the DF-1 cell line. The virus was replicated to increase the concentration of the viruses for stocking and for the usage during final phase. The final phase includes the assessment of antiviral activity of the *S. commune* extract towards Newcastle Disease Virus (NDV) and the antiviral activity was optimized for its highest efficiency. Antiviral activity optimization consists of different concentration of *S. commune* extract and quantity of treatment given towards the cells.

1.5 THESIS ORGANIZATION

There are five main bodies presented in this research work classified as Chapter 1, Chapter 2, Chapter 3, Chapter 4 and Chapter 5. Chapter 1 discussed the significant and the importance to carry out this study. Suggestions, ideas and the aims of developing antiviral drugs from natural products were further detailed in the problem statement and research objectives. Subsequently, the overview of the raw materials and the background of the methods used in this research were further reviewed in chapter 2; where collections of journal and book review related to this study were summarized in details.

Chapter 3 explained in details the flow and methods involved in this research with reference of a flow chart for better understanding. In this chapter, the extraction of Schizophyllan from *S. commune from* Rau (2002) was described further. Furthermore, thecytotoxicity of extract towards the cell line, the antiviral activity method and the optimization factors of antiviral activity used in this study were explained in details.

Chapter 4 comprised the extensive discussions of obtained results that relate to the methods used in this research. Moreover, different source of references were used to support the results of this study. Chapter 5 concluded the entire research work and recommend future works for this study; whereas the bibliography section listed the research papers and journals that had been used as reference to support this study.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

Currently, about fifty percent of pharmaceutical drugs originated from natural elements due to the urgent therapeutic necessity and, the safety and effectiveness of the products (Khalid, 2000). Viral therapies using prosthetic materials or chemotherapeutic agents are mostly toxic towards human cells and may cause a severe immune suppression towards patients (John and Wiley, 1996). Nevertheless, there are other factors contributing to the demand of drugs derived from natural products such as the wide range of chemical structures and biological activity of the secondary compound, the competency for the biochemical and molecular probes, and also the development of detection, isolation and characterization techniques of natural products (Clark, 1996).

2.2 MEDICINAL MUSHROOMS

According to Chang and Miles (2004), mushroom is a macrofungus with a distinct fruiting body, which can be either hypogeous or not, large enough to be seen by naked eye or picked by hand. According to taxonomy, most of the mushrooms belong to basidiomycetes and some to ascomycetes. The number of mushrooms species on earth were estimated around 140 000 species and 10% have been identified, while only 5% of the identified mushrooms were studied for their medical benefits (Hawksworth, 2011).

In the past, mushrooms are not just supplied nutritional foods for society but also an important source for traditional medicine (Breene, 1990). These resulted into large number of researches on potential benefits of medicinal mushrooms especially in Japan, Korea and China over the past three decades (Veena and Pandey, 2012). Nowadays, medicinal mushrooms have been used widely for pharmaceutical and nutraceutical purposes (Mau et al., 2002). At least 270 species of mushrooms are known to have various therapeutic effects (Ying et al.,1987). Thus, the study of mushrooms' biological activities for medicinal benefit has turned into a very significant research area.

Although the use of natural products in pharmaceuticals are common in Asian countries since decades ago, research from the western region only shows an increasing number starting from few years back. This was proven by the increased of scientific publication in peer-reviewed journals, books and journal reviews that classified biologically active compounds from fungi and its medicinal efficacy (Wasser and Weis, 1999; Ooi and Liu, 2000; Lindequist et al., 2005). Scientists nowadays focused on the medicinal study from mushrooms correspondingly because of the market demand from the world consumers on natural products for its nontoxic properties compared to chemical products. Therefore, study showed an increase number of pharmaceuticals developed antibiotic, antiviral, anti-inflammatory and anticancer drugs from natural products (Asfors and Ley, 1993).

Therapeutic area is particularly influenced by the biologically active compound of mushrooms. Mushrooms are rich sources of nutraceuticals (Çaglarirmak, 2007; Elmastas et al., 2007; Ribeiro et al., 2007) responsible for their anti- oxidant (Mau et al., 2002; Lo and Cheung, 2005; Barros et al., 2007a), antitumor (Wasser and Weis, 1999), and antimicrobial properties (Smânia et al., 1995; Hirasawa et al., 1999;

Hatvani, 2001; Barros et al., 2007b; Turkoglu et al., 2007). Besides their pharmacological features (Lindequist et al., 2005), wild mushrooms are becoming more important in our diet due to their nutritional value, related to the high protein and low fat/energy contents (Diéz and Alvarez, 2001; Agahar-Murugkar and Subbulakshmi, 2005; Barros et al., 2007c). Some famous mushrooms that were commercialized for their potential medicinal benefits are *Lentinus edodes* which reported to possess anti-tumor, antihypertensive, hypocholesterolemic and antibacterial activities (Hirasawa, 1999) and *Ganoderma lucidum* which is proven to have anti-microbial and anti-HIV effects (Yoon et al., 1994; El Mekkawy, 1998).

The first and famous natural product discovered for human drugs is Penicillin antibiotic. Penicillin antibiotic is derived from the extract of *Penicillium notatum* or now known as *Penicillium chrysogenum* (Samson et al., 1977). *P. chrysogenum* is a common fungus in subtropical temperature and nontoxic to human (Anderson et al., 2011). The extracellular bioactive compound of *P. chrysogenum* known as Penicillin was produced by liquid fermentation broth and acts specifically towards gram-positive bacteria (Garrod, 1960).

Cordyceps species are ascomycete fungus that can be classified as endoparasitoids mainly on insects and arthropods. *Cordyceps* mushrooms are used to treat respiration and pulmonary diseases; renal, liver, and cardiovascular diseases; hyposexuality and hyperlipidemia, immune disorders and cancer therapies (Holiday et al., 2008). Commonly, *Cordyceps sinensis* and *Cordyceps militaris* are used in the research and pharmaceuticals drugs (Khan et al., 2010). The water extract and ethanol extract of this mushroom has been proven to have positive reaction in the prevention of tumor metastasis (Nakamura et al., 2003; Shin et al., 2003).

Ganoderma is one highly valued traditional medicine and functional food for

its therapeutic effects; anti-tumor, anti-inflammatory, antiviral, antibacterial, anti-parasitic, blood pressure regulation, cardiovascular disorders, immunomodulating, kidney tonic, hepatoprotective, nerve tonic, sexual potentiator and chronic bronchitis (Wasser and Weis, 1999). *Ganoderma lucidum* is the first medicinal mushroom to gain attention in India where the mushrooms were cultivated on wheat straw and wheat bran for nutritional and pharmaceutical purpose (Mishra and Singh, 2006; Rai, 2003; Veena and Pandey, 2004). It also have been used in Chinese medication for 4000 years and known as 'elixir of life' between Chinese societies. Recently, it has been commercialized both in Asian and Western countries. Other famous medicinal mushroom is *Lentinus edodes* or shitake mushroom extract of oxalic acid is one agent responsible for the antimicrobial against *S. aureus* and other bacteria (Bender et al., 2003).

2.2.1 Common Medicinal Properties of Fungal Extracts

2.2.1.1 Antibacterial Agent

Many antibiotics originated from fungi (Hardman et al.,2001) such as penicillin, streptomycin, chloramphenicol and vancomycin (Griffin, 1994). Antibiotic can be defined as a compound produced by microorganisms that have the capacity to inhibit the growth of bacteria in dilute solution (Black, 2002).

Naturally, fungus excreted antibacterial compound to survive the harsh environment of their habitat. Thus, antibacterial compounds could be isolated from many fungi by manipulating their growth condition and benefited human health (Lindequist et al., 2005). Antimicrobials mechanisms includes inhibition of cell wall synthesis, disruption of cell membrane function, inhibition of protein synthesis, inhibition of nucleic acid synthesis and action as anti- metabolites.