INVESTIGATION OF BIOLOGICAL ACTIVITIES OF *Mallotus paniculatus* LEAF EXTRACT

BY

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ABSTRACT

In this study, *Mallotus paniculatus* (Balik Angin), a plant that was easily found in both rural and urban areas was screened against several biological activities. Traditionally, *M. paniculatus* is used in curing various diseases such as a remedy after child's birth, wound healing and fever treatment. In the present study, four biological properties of the plant were screened, which include antibacterial, antifungal, anticancer and antioxidant activities. Today, synthetic drugs are extensively used in curing most of the human diseases. However, they are expensive, difficult to access for remote areas as well as have limited application for specific treatment compared to the natural products from plants which may be effective against several diseases. Hence, for screening purpose, potential biological compounds were extracted from the plant leaves by sonication with different type of solvents namely hexane, ethyl acetate, and ethanol. The extracted compounds were tested for antibacterial and antifungal properties using disc diffusion agar and broth dilution assay, antioxidant using DPPH scavenging assay and anticancer using MTT assay. From the screening results, *M. paniculatus* ethanolic extract was shown to possess antibacterial, antioxidant and anticancer but not antifungal property. While, ethyl acetate and hexane extracts showed weak or no contribution to any of these activities. The ethanolic extract was also shown to have outstanding antioxidant activity (IC_{50} 30 µg/ml) that is comparable with positive control, butylated hydroxytoluene (BHT) (IC_{50} 30 µg/ml) compared to other biological properties. Due to this, the present study focused on optimizing the sonication extraction process condition for extract with antioxidant activity. Using Face Centered Central Design (FCCCD) to design optimization experiments, the data obtained from the experiments were fit to polynomial equation to determine the best extraction condition. The optimum condition obtain from this study was at 30ºC for 30 min where it gave IC_{50} value of antioxidant activity, 27 µg/ml. Partial purification was carried out to identify which types of compound responsible for the antioxidant activity in the ethanolic extract by fractionation (partitioning) method followed by TLC analysis. It was observed that the extract from partitioning possibly consist of polar compounds which may be responsible for the antioxidant property. In conclusion, *M. paniculatus* possesses multiple medical properties in which its antioxidant property is worth to be further investigated using optimized extraction condition determined in this study.

خلاصه البحث

في هذه الدراسة، (Balik Angin) ***Mallotus paniculatus*** وهو نبات تم العثور عليه بسهولة في كل من المناطق الريفية والحضرية ضد العديد من الأنشطة الطبية. تقليديًا، يستخدم ***M. paniculatus*** في علاج الأمراض المختلفة مثل العلاج بعد ولادة الطفل وعلاج الجروح وعلاج الحمى. في هذه الدراسة، تم فحص أربعة خصائص طبية للنبات، والتي تشمل أنشطة مضادة للجراثيم، مضاد للفطريات، مضادات الأكسدة. اليوم، تستخدم الأدوية الاصطناعية على نطاق واسع في علاج معظم الأمراض التي تصيب الإنسان. ومع ذلك، فهي غالية الثمن، ويصعب الوصول إليها في المناطق النائية بالإضافة إلى أنها محدودة التطبيق لعلاج محدد مقارنة بالمنتجات الطبيعية من النباتات التي قد تكون فعالة ضد العديد من الأمراض. MTT. من نتائج الفحص، تبين أن مستخلص الإيثانول ***M. paniculatus*** يمتلك خاصية مضادة للبكتيريا، مضادات الأكسدة ومضاد للسرطان ولكن ليس خاصية مضادة للفطريات. بينما أظهرت خلاصات إيثيل وخلات الهكسان مساهمة ضعيفة أو معدومة في أي من هذه الأنشطة. كما تبين أن مستخلص الإيثانول له نشاط مضاد للأكسدة متميز (IC50 30 ميكروغرام / مل) يمكن مقارنته بالتحكم الإيجابي، هيدروكسيتولوين بوتيل (IC50 30) (BHT ميكروغرام / مل) مقارنة بالخصائص الطبية الأخرى. بسبب هذا، ركزت الدراسة الحالية على تحسين حالة عملية استخراج صوتنة للاستخراج مع النشاط المضادة للأكسدة. باستخدام التصميم المركزي للوجه (FCCCD) لتصميم تجارب التحسين، كانت البيانات التي تم الحصول عليها من التجارب مناسبة لمعادلة متعددة الحدود لتحديد أفضل حالة استخراج. كانت الحالة المثلى التي حصلت عليها من هذه الدراسة عند 30 درجة مئوية لمدة 30 دقيقة حيث أعطت قيمة IC50 لنشاط مضاد للأكسدة، 27 ميكروغرام / مل. تم إجراء تنقية جزئية لتحديد أنواع المركبات المسؤولة عن نشاط مضادات الأكسدة في طريقة استخراج الإيثانول عن طريق تجزئة (التقسيم) متبوعة بتحليل TLC. وقد لوحظ أن المستخلص من التقسيم ربما يتكون من مركبات قطبية قد تكون مسؤولة عن خاصية مضادات الأكسدة. في الختام، يمتلك ***M. paniculatus*** خواص طبية متعددة والتي تستحق خاصية مضادات الأكسدة الخاصة بها إجراء مزيد من الدراسة باستخدام حالة الاستخراج المثلى المحددة في هذه الدراسة.

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 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 thesis 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 institution.

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LIST OF ABREVIATIONS

g	gram
mg	milligram
µg	microgram
ml	millilitre
µl	microliter
mm	millimetre
cm^2	Centimetre per square
rpm	revolutions per minute
DPPH	2,2'-Diphenyl-1-picrylhydrazyl radical
IC_{50}	Half maximal inhibition concentration
MIC	Minimum inhibitory concentration
OD	Optical density
w/v	Weight/ volume
CFU	Colony forming unit
UV	Ultraviolet
cm	centimetre
nm	nanometre
et al.	(et alia); and others
etc	(et cetera); and so forth
BHT	Butylated hydroxytoluene
OH	Hydroxyl radical
O^2	Oxygen molecule
CO^2	Carbon dioxide
ROS	reactive oxygen species
EtOH	Ethanol
MeOH	methanol
DNA	deoxyribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMEM	Dulbecco modified eagle medium
min	Minute
Eq	Equation
R_f	Retention factor
TLC	Thin layer chromatography

LIST OF SYMBOLS

°C	Degree Celsius
%	Percentage

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Natural products are very useful and economically essential as it is relatively cost-effective than modern drugs and it has been used for traditional medicine. Undoubtedly, scientific investigation on the traditional plants has greatly helped in natural product commercially and has taken as a secondary role in drug discovery and drug development. The increasing demand for natural products in maintaining health and treating diseases encourages many researchers globally to investigate traditional plants extensively. For instance, in India, neem (*Azadirachta indica*) has been used as treatment and prevention of various diseases (Alzohairy, 2016). While in China, ginseng has been used in traditional Chinese medicine for centuries (Attele et al., 2002; Xie et al., 2005). Whereas in Malaysia, there is many traditional plants like petai (P. speciosa) and pegaga (C. asiatica) were found to possess anti-diabetics, anti-oxidant, as well as anti-hypertensive activities (Bachok et al., 2014).

In the present study, *M. paniculatus* was investigated for its biological properties. The reason to choose this plant as natural product studies is because it is not well known among nowadays people and many potential bioactive compound of this plant are lack of scientific evidence. Mallotus is one of the most diverse genera of the Euphorbiaceae family (Slik and Welzen, 2001) and they are known to have many

different natural compounds (Nakatsu et al., 1981). It is widely distributed in many countries likely in India, South China, Taiwan, Malaysia and northeastern Australia. In Malaysia, this plant can be easily found in a place nearby to the road, street, river, as well as in rural area. Traditionally, this plant is used in curing various diseases in rural areas such as a remedy after child's birth, wound healing and fever treatment. The plants were found to contain various chemical compounds such as alkaloids, isoflavones, and flavanoids (Rivière et al., 2010) which have been proved in other plants to play a role in protective and preventive properties against a variety of diseases. In other previous studies, there are also have been found to contribute as anti-inflammation, healing skin wounds (Yusof, 2013), antiprotozoal and antileshmanial against L. amazonesis (Lianet et al., 2014; Tistaerta et al., 2012), as well as antioxidant and anticancer from leaves against cancer cervical cell (Riviere et al., 2010). In the present study, we focus on screening *M. paniculatus* medical properties in treating infectious disease and cancer as well as determination of the antioxidant property.

Cancer and infectious diseases are serious health issues in the world causing many people depending on anticancer and antibiotic treatments, respectively. Overuse of antibiotic agents cause a high-risk resistance of antibiotics against bacteria and produce a varied range of side-effects (Kethireddy and Kumar, 2015). The usage of the existing chemotherapy drugs leads to further damage to the human cells whereby they kill fast-growing cells including normal cells that lead to various side effects. An antioxidant agent can be associated with the anticancer treatment through preventing accumulation of reactive free radicals that causes oxidative damage in DNA (Griffiths et al., 2016).

Through many studies conducted previously, natural products that were found to possess anticancer properties include vinblastine, vincristine, etoposide, teniposide, taxol, navelbine, and irinotecan. They had been approved for cancer treatment and available commercially (Pandey, 2009). Till now, many plants have been screened and analysed for their potential to be effective drugs for cancer chemoprevention. Elucidation through mechanisms of cell survival and apoptosis of the plant bioactive compounds that possess anticancer activity were demonstrated. The efficacy of the compounds has been evaluated by modern techniques to treat the cancer.

There were also many plants screened for antimicrobial properties. Bioactive compounds such as alkaloids, flavonoids, glycosides, gums, polysaccharides, phenols, tannins, tenpins and terpenoides from *Aloe vera, Carica papaya* and *Curcuma longa* were demonstrated to be effective antimicrobial drugs (Ross, 2003). These compounds are responsible to inhibit micro-organisms and reduce the risk of fungal infection. In this study, other than screening *M. paniculatus* for anticancer, antimicrobial and antioxidant properties, extraction optimization of compounds responsible for the most effective biological properties were conducted as well. Partial purification of extract was carried out to isolate active compound from the highest biological activity effect.

1.2 PROBLEM STATEMENT AND SIGNIFICANCE OF STUDY

Today, synthetic drugs are losing their effectiveness and have serious threats for human health include tuberculosis, penicillin-resistant pneumonia, and resistant malaria. In addition, synthetic drugs are also expensive where poor people cannot afford and access the treatment and the application of drugs are limited to a specific treatment compared to the natural drugs which may be effective against several

diseases. The prolonged use of antibiotic can cause the emergence of bacterial resistance that difficult to treat. As well as chemotherapy drugs are applied to treat cancer disease, however they may affect both of cancer cells and healthy cells.

The disadvantages of synthetic drugs mentioned above have encouraged many scientists and researchers to investigate alternative treatments from natural products which can reduce both the side effects and bacterial resistance to antibiotics. An investigation of finding a cure based on the natural products provide unlimited potential for new leads of active compound as they have large chemical diversity. Harvey and colleagues (2010) discovered that current strategies for drug discovery through natural products are broad and span a wide range of research approaches and methodologies. *M. paniculatus* is one of the best candidates where it has a promising cure to provide potential sources of novel biologically active agents.

Although, it has been used in folklore treatments such as healing the skin wounds, relieve fever and headache but there are still a limited of scientific evidences on biological properties of this plant. Thus, *M. paniculatus* was screened to investigate four biological properties such as antibacterial, antifungal, antioxidant and anticancer activities and optimize one of the best activity for this plant. Therefore, this study may provide new knowledge as well as increase commercial potential of the plant as biological agent and health supplement due to this plant has limited evidence of pharmacological and phytochemical research.

1.3 RESEARCH OBJECTIVES

The objectives of this study are:

1) To screen biological activities such as antibacterial, antifungal, antioxidant and anticancer activities of *M. paniculatus* leaf extracts

2) To determine the best extraction condition based on temperature and time of the most active *M. paniculatus* crude extract by employing Face Centered Composite Design- Response Surface Methodology (FCCCD-RSM)

3) To fractionate the most active *M. paniculatus* extract using optimized extraction condition in order to obtain semi purified fractions

1.4 SCOPE OF RESEARCH

This study involved the investigation of biological properties of *M. paniculatus* crude extract; extraction optimization and semi-purification of the crude extract obtain from optimization experiment. Four biological properties were investigated which include antimicrobial, antifungal, anticancer and antioxidant activities. To investigate these properties, *M. paniculatus* leaves were extracted by sonication with different type of solvents such as hexane, ethyl acetate and ethanol before the following assay/test were conducted.

For antibacterial and antifungal activities, the resulting crude extract were screened using disc diffusion assay and broth dilution method to observe inhibition zone and minimum inhibitory concentration (MIC) as well as half maximal inhibitory concentration (IC_{50}). For antioxidant activity, the extract was tested by DPPH scavenging assay and the effectiveness of crude extract to combat oxidative free

radical was represent by IC_{50} value. For anti-cancer/cytotoxicity activity, MTT assay was used. The cytotoxicity test was carried out against three different cell lines, breast cancer (MCF-7), cervical cancer (HeLa), and colon cancer (HT-29). From MTT assay, graph was plotted between extract concentration and viability of cancer cell to obtain IC_{50} that represent the effectiveness of the extract in inhibiting the growth of cancer cells.

All activities were compared and based on IC_{50} value, the most effective property was selected for optimization extraction experiment. The optimization experiments were designed by Face Central Composite Design (FCCCD) under Response Surface Methodology (RSM) obtained from Design Expert software. The results from the experiments were fitted to polynomial equation to determine the best sonication extraction condition. The extract obtained from the best extraction condition was partially purified using fractionation method and analysed using thin layer chromatography. The extract was not fully purified in this study because it consumes a lot of time where the preliminary result was hugely focused on the screening of biological properties included antibacterial, antifungal, antioxidant and anticancer with various solvents, different type of bacteria and different type of cells. Also, the purification of active compound need more procedures and researches.

1.5 THESIS ORGANIZATION

There are five main parts included in this thesis classified as chapter 1, chapter 2, chapter 3, chapter 4, and chapter 5. In chapter 1, introduction of this research was briefly discussed. Over this chapter, the background of *M. paniculatus* species and the previous studies was discussed. Aside from that, findings of natural product benefits

in Malaysia and over the world were reported as well. For problem statement and significance part, the current health issues in the world was highlighted and discussed briefly. A solution, an alternative modern medicine such as plant can contribute in treating diseases was one of the suggestion to overcome the problems. General method involved in screening biological properties, optimization extraction and semipurification of extracted compound were discussed in the research scope.

In chapter 2, the overview and background of the Mallotus genus and *M. paniculatus* were discussed in this chapter. Collections of journal and book review related to this study were summarized in this chapter.

Chapter 3 describes the routes of experiment involved in details with reference of flow chart for better understanding.

Chapter 4 comprises results of the experiments and extensive discussion on the findings. Previous references were used to support the results obtained from this study and discussion were elaborated on findings that were inconsistent with other studies. Chapter 5 concluded the whole research work and recommended future works that can be continue from this research.

The bibliography section concise all the research works from other investigators that have been reviewed and referred while completing this study. Once all the data was completely compiled, a brief abstract was presented at the beginning of the thesis. Concise information on the problem statement, research methodology and achieved results were stated in the abstract to give readers an overview of the entire thesis.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

A detailed literature review relevant to this research has been elaborated in this chapter. The review focuses mainly in background and fundamental principal of *M. paniculatus*, biological properties and methodology of study as well. Relevant previous study and scientific evidence from collected journals and book reviews are reported and discussed.

2.2 MALLOTUS

Mallotus is a genus belongs to Malphighiales order and Euphorbiaceae family. Mallotus is one of the most diverse and richest genera of the Euphorbiaceae family, where there is more than 150 Mallotus species. Mallotus is recognised as shrubs to small trees, monoecious or dioecious; branches glabrescent. The large numbers of Mallotus species are classified by subgeneric classifications known as Axenfeldia, Hancea, Mallotus, Rottleropsis, and Stylanthus (Slik and Welzen, 2001). The genus is widely distributed throughout countries including Asia and Africa (Sierra and Welzen, 2005).

Mallotus is known to contain many different natural compounds. In many Mallotus species commonly found triterpenoids, flavonoids, benzopyrans (Nakatsu et

al., 1981). A few compounds have been shown to possess biological properties including antioxidant, antiviral, antimicrobial, anti- inflammatory or cytotoxic (Van Chao et al., 2005).

In Vietnam and China, the roots, stembarks, leaves, and fruits of Mallotus species are used in chronic hepatitis and enteritis treatment for centuries. Certain Mallotus species such as *M. apelta, M. barbatus, M. paniculatus, M. philippinensis* and *M. poilanei* are mostly found in Asian countries used for the various ailments treatment. This study mainly addresses to screening *M. paniculatus* biological properties such as antioxidant, antibacterial, antifungal and anticancer.

2.2.1 Antibacterial Activities of Mallotus sp.

Generally, antibacterial is an agent that inhibits bacterial growth and suppresses their ability to reproduce. The agent includes chemicals that contain antibacterial properties such as chlorine and antibiotic drugs and heat with undesirable temperature such as high temperature could lead to kill bacteria. Commonly, chemical substances are used to kill microorganisms without injuring the host. The most frequent used in biological practices includes aspirin, penicillin and streptomycin. These antibacterial agents help to protect human body from infections of bacteria and pathogens. However, it cannot reduce the risk of viral infectious diseases and cause various side effects due to drug resistances.

In recent years, there is accelerating number of discoveries of antibacterial drugs derived from plants where many researchers including ethnopharmacologists, microbiologists, and natural-products chemists developed the phytochemicals for

treatment of infectious diseases. This is because plants consist of major groups of antimicrobial compounds whereby more than 12,000 secondary metabolites components have been isolated such as alkaloids, flavonoids, glycosides, gums, polysaccharides, phenols, tannins, tenpins and terpenoides (Florkin and Stotz, 2014).

Phenols contribute important role as plant defence mechanisms against predation or pathogen as it consists of single substituted phenolic ring. Terpenenes or terpenoids, one type of phenolic compounds has been shown to be active against bacteria, fungi, viruses, and protozoa in previous study (Harish et al., 2017). Although, it had been demonstrated that the leaves of *M. paniculatus* contain terpenoides, there is yet any investigation carried out to determine the antibacterial activity of *M. paniculatus.*

However, in other mallotus species was found to be possessed as antibacterial activity such as *Mallotus oppositifolius* against *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 12600), *Micrococcus luteus* (ATCC 4698), the gram positive organisms but no antibacterial result for the gram negative organisms, *Klebsiella pneumonae* (ATCC 13883) and *Escherichia coli* (ATCC 11775) (Chukwujekwu et al., 2006).

Figure 2.3 shows the membrane structure of Gram-positive and Gram-negative bacteria which the cell walls are differentiated. Based on their cell wall properties, the Gram-positive bacteria have a thicker layer of peptidoglycan as cell membrane. Meanwhile, Gram negative bacteria have a double phospholipid bilayer (Punopas, 2002). Moreover, the outer membrane of Gram-negative bacteria is an act as extra protection of molecules entering the cell wall and perisplasm. The outer membrane of Gram-negative bacteria has an important component called lipopolysaccharide (LPS).