WOUND HEALING PROPERTIES OF *ACROSTICHUM AUREUM* AND *A. SPECIOSUM* ON NIH/3T3 FIBROBLASTS CELL LINE AND EXCISIONAL WOUND ON RABBITS

BY

HENDY PUTRA BIN HERMAN

A thesis submitted in fulfillment of the requirement for the degree of Master of Science (Biosciences)

Kulliyyah of Science
International Islamic University Malaysia

JUNE 2014

ABSTRACT

Acrostichum aureum or Piai raya has been known for its medicinal value for wound healing treatment among Malays in Malaysia. In this study, the traditional use of this plant together with *A. speciosum* (Piai lasa) was proven for the first time *in vitro* and *in vivo*. Sampling of both plants have been done in Matang mangroves, Taiping, Perak on February 2011. They were dried, ground into fine powder, extracted with water and ethanol to produce extracts and they were fractionated by Vacuum Liquid Chromatography (VLC) to produce fractions. For the first part of study, screening was done on the extracts to identify their phytochemicals content. From the present study, it was reported that both plants have various potential compounds such as tannins, saponins, alkaloids, and flavonoids. Later, antimicrobial study showed that only *Staphylococcus aureus* was susceptible to the extract treatment and this led to the second part of study which was *in vitro* wound scratch assay. From MTT assay, the best concentration for the proliferative effect of fibroblasts was 200 µg/mL and hence it was used for the wound scratch assay. There were eights extracts and fractions that showed better mean migration rate but only four were selected to be tested on rabbits. For *in vivo* study, three groups of four rabbits each were tested using four different extracts with two different concentrations (5 % and 10 %). The excisional wounds were inflicted at the back of the rabbits and treatments were applied once daily until complete healing. Based on wound contraction, epithelization period and histopathological study, it was found that aqueous extract of rhizomes (10 %) and leaves *A. aureum* (5 %) showed the best wound healing properties with more collagens and fibroblasts proliferation and complete epithelized cells. Therefore, this study has added some novel findings in exploring the use of *A. aureum* and *A. speciosum* as wound healing treatement. In conclusion, this study has successfully justified the medicinal use of rhizomes *A. aureum* in wound healing treatment. In addition to that value, *A. speciosum* which was not well-studied and other parts of the plant, stems and leaves, also have been reported to possess similar medicinal properties for the first time.

خلاصة البحث

عُرف نبات الـ (*Acrostichum aureum*) أو ما يُعرف محلياً عند الماليزين بـ (Piai raya) أنه ذو قيمة طبية وعلاجية لتطبيب الجروح ومعالجتها. في هذه الدراسة ولأول مرة تم استكشاف الفعالية الطبية لهذا النبات بالإضافة إلى نبات الـ(*A. speciosum*) أو الـ(Piai lasa) في هذه الدراسة تم الكشف عن الفعالية الطبية لهذان النباتان على أنسجة الأرانب مباشرة بالإضافية إلى تجربتها على الخلايا النامية على الأطباق المخبرية. جُمعت العينات النباتية خلال شهر شباط 2011 من منطقة متانج وتاينج وبيراك في ماليزيا حيث انتشار الأشجار الإستوائية المستهدفة في هذه الدراسة. تم تجفيف النبات وطحنه للحصول على مسحوق دقيق الحجم ، ومن ثم تم استخلاصة بإستخدام الكحول الإيثيلي والماء، وبعد ذلك تم تجزأة المستخلص بإستخدام تقنية فصل السوائل بالتفريغ (VLC) للحصول على مستخلصات مجزأة من المستخلص الإساسي. في الجزء الأول من الدراسة كُشف عن المكونات الكيميائية النباتية الفعالة المتوفرة في المستخلصات ، وتم التعرف واثبات وجود تنوع من المركبات الحيوية الهامة مثل أنواع التانين ، وأنواع السابونين ، وأنواع الألكلويد ، وأنواع الفلافينويد ، وبينت الدراسة أن وجود أنواع التانين ظهر في مستخلصات الكحول الإيثيلي أكثر من وجوده في المستخلصات المائية. تحليل واختبار الـ (MTT) كشف أن 200 ميكروجرام من المستخلص هي أفضل تركيز لزيادة عملية تراكم وتكون الفيبروبلاست وهو التركيز الذي تم الإعتماد عليه واستخدامه لتجارب الخدش فيما بعد. خلال الدراسة تبين أن هناك ثمانية مستخلصات وأجزاء أظهرت معدل هجرة للخلايا بشكل فعال ، في المقابل فقط تم استخدام أربعة منها في التجارب على الأرانب وذلك لمحدودية الكميات المتوفرة من باقي المستخلصات (أقل من 100 ملجرام). قُسمت الأرانب إلى ثلاث مجموعات كل مجموعة تحتوي على أربعة أرانب وكل مجموعة تم تعريضها لأربع مستخلصات مختلفة بتركيزين مختلفين (5% و 10 %) بالإضافة لإستخدام مادة الـ (Solcoseryl jelly) كمُحكم إيجابي بالإضافة لإستخدام كريم (Aqua) مُحكم سلبي. الجُروح اصطُنعت في ظهر الأرانب وتم تعريضها للعلاج يومياً حتي تم تطبيبها تماماً واختفاء الجروح ، وطبقاً لتركيز الجروح والمدة الزمنية لتكون النسيج الإبثيلي والدراسة النسيجية وُجد أن المستخلص المائي للسيقان الجذعية (10%) وأوراق نبات الـ (*A. aureum*) (5%) أظهروا نتائج وخصائص أفضل من غيرهما في معالجة الجروح ، حيث كان هناك زيادة في تكون الكلاجين والفيبروبلاست والخلايا الإبثيلية. وبناءاً على ما سبق فإننا نستطيع القول بأن هذه الدراسة أضافت معلومات جديدة ومشجعة للإهتمام بهذان النباتان المستهدفين في هذه الدراسة لإستكشاف أهميتهما واستخدامها في معالجة الجُروح وتطبيبها. وأخيراً نستخلص أن هذه الدراسة بيُّنت أهمية استخدام السيقان الجذعية ونبات الـ (*A. aureum*) في علاج الجروح. بالإضافة لما سبق كشفت الدراسة عن القيمة الطبية لنبات الـ (*A. speciosum*) وأهمية دراسة باقي أجزاءه من سيقان وأوراق في علاج الجروح حيث أنهم هذا النبات لم يلقى المزيد من الإهتمام ولم يُدرس بشكل مُكثف لهذا الهدف.

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 (Biosciences).

.....
Deny Susanti
Supervisor

.....
Muhammad Taher
Co-Supervisor

.....
Shahbudin Saad
Co-Supervisor

I certify that I have 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 (Biosciences).

.....
Suzanah Abdul Rahman
Internal Examiner

.....
Mahmood Ameen Abdulla
External Examiner

This thesis was submitted to the Department of Biotechnology and is accepted as a fulfilment of the requirements for the degree of Master of Science (Biosciences).

.....
Noor Faizul Hadry Nordin
Head, Department of Biotechnology

This thesis was submitted to the Kulliyah of Science and is accepted as a fulfilment of the requirements for the degree of Master of Science (Biosciences).

.....
Kamaruzzaman Yunus
Dean, Kulliyyah of Science

DECLARATION PAGE

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 as a whole for any other degrees at IIUM or other institutions.

Hendy Putra Bin Herman

Signature …… Date ……..

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

WOUND HEALING PROPERTIES OF *ACROSTICHUM AUREUM* AND *A. SPECIOSUM* ON NIH/3T3 FIBROBLASTS CELL LINE AND EXCISIONAL WOUND ON RABBITS

Affirmed by Hendy Putra Bin Herman

…….. ……..

Signature Date

Dedicated to my family and friends

For outstanding supports and loves

Thank You Allah

ACKNOWLEDGEMENTS

A great journey shall begin with the very first step.

First of all, I would like to praise Allah S. W. T for giving me perseverance and strength to finish my Master study. I would like to extend my utmost appreciation and grateful to my main supervisor Assoc. Prof. Dr. Deny Susanti and two co-supervisors Assoc. Prof. Dr. Shahbdudin Saad and Assoc. Prof. Dr. Muhammad Taher for their constructive guidance and supervision. Not forgetting also Madam Norazsida Ramli for being a cooperative principal researcher. I am so grateful for the opportunity to be a research assistant for this project, thus special thanks to Ministry of Higher Education for providing FRGS grant for this project and providing MyBrain scholarship for me to pursue my Master study.

Million thanks to all staff of Kulliyah of Science and Kulliyyah of Pharmacy for assisting me to carry out the research until the end especially Br. Muzammil, Br. Halim, Br. Zul, Br. Pok Ya, Br. Azizul, Sr. Yanti from Kulliyyah of Science and Sr. Yanti and Sr. Sri from Kulliyyah of Pharmacy. Not forgetting also staff admin office especially Sr. Lin, Sr. Siti, and Sr. Adah.

For my beloved parents (Haji Herman and Hajjah Yusniarti) and six siblings, four sister-in-laws, and 15 nieces and nephews, thank you so much for all your unconditional love and supports during my two years research. I am blessed to have all my loving ones by my side through thick and thin. Special thanks to Dr. Azrul Safuan and my younger sister Haryanti for assisting me in lab work. I am also in debt with Br. Helmi for the Arabic translation of the abstract. May Allah reward you in hereafter. For all my friends, my fellow postgraduates; Jazli, Fuad, Adah, Ija, Mir, Min, Nisah, Rahima, Putri, Sihin, Kak Yan, Kak Husna, Kak Azizah, and Br. Surairi especially my labmates; Hassan, Nissad, and Zaffar. *Jazakallahu khairan kathira*. May Allah bless our effort to disseminate scientific knowledge for the ummah. *Amin*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

Abbreviation	Full name
ATCC	American Type Culture Collection
CAM	Chorioallantoic Membrane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
HIV	Human Immunodeficiency Virus
H & E	Haematoxylin & Eosin
MTT	Methylthiazol Tetrazolium
OH	Hydroxyl Radical
PDGF	Platelet Derived Growth Factor
SOR	Superoxide Radical
VLC	Vacuum Liquid Chromatography
WHO	World Health Organization
m	metre
cm	centimetre
mm	millimetre
g	gram
mg	milligram
µg	microgram
ng	nanogram
L	litre
mL	millilitre
µL	microlitre

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

Commercial drugs are widely available in health industry but in some developing countries, medicinal plants still capture the interest of many researchers. Recently, the interest in medicinal plants is fuelled by the continuous rising costs of prescription drugs in the maintenance of personal health and well-being, and the bioprospecting of new plant-derived drugs (Hoareau and Dasilva, 1999). World Health Organization [WHO], (2002) reported that global market for traditional medicine is around US$ 60 billion. In USA, there are more than 1500-1800 botanical products sold and medicinal herbs is a major segment in their pharmaceutical market (Matthews, Lucier, and Fisher, 1999). The use of herbal medicine was inherited from our ancestors thousand years ago. For example, in old civilization like India, herbal medicine was the key component in their ancient traditional medicine such as Ayurveda and Unani system of medicine used about 700 plant species while Siddha and Amchi used about 600 plant species (Joy, Thomas, Mathew, and Skaria, 1998).

Malaysia as a tropical country is blessed to be a home for some household name of medicinal plants such as Tongkat Ali (*Eurycoma longifolia*), Pegaga (*Centella asiatica*) and Misai Kucing (*Orthosiphon stamineus*) (Rajen, 2004). The herbal industry in Malaysia is blooming annually and it triggers the researchers to develop Malaysian Herbal Pharmacopoeia and Pharmacopoeia Commission in order to assure the quality and reproducibility of the herbal product in the country (Zhari, 2010). This effort is in conformance with one of the strategies by National Policy of

Traditional and Complementary Medicine which is to support efficacy of traditional/ complementary medicinal products by the availability of scientific evidence (Ministry of Health Malaysia, 2001). As a comparison, even though many types of herbal products were consumed by American people but their safety, toxicity, and side effects were yet to be established by scientific research (Bent, 2008).

Historically, Malacca as a renowned port in Southeast Asia played a vital role in herbal trade between foreign traders and Malays. This has resulted in the introduction of foreign herbs into Malay peninsular such as *Mentha arvensis* from Sri Lanka, *Piper sarmentosum* from Indonesia, and *Centella asiatica* from India (Joseph, Sugumaran, and Lee, 2005). Nowadays, many researchers in Malaysia are interested to do various kinds of research on medicinal plants found in its rainforest and mangroves parks since this country is listed as one of twelve megadiversity countries in the world, with 1100 species of ferns and fern allies (Ministry of Science, Environment and Technology, 1998). For that purpose, ethnobotanical studies have provided a very vital information for researchers around the world to explore more on ferns medicinal properties (Singh, Dixit, and Sahu, 2005; Karthik, Raju, Ayyanar, Gowrishankar, and Sekar, 2011; Benniamin, 2011; Singh, S. and Singh, R., 2012).

Pteridaceae is a fern family that consists of 50 genera and 950 species worldwide (Smith, Pryer, Schuettpelz, Korall, Schneider, and Wolf, 2008). One of the prominent characters of this family is the presence of long to short creeping and ascending rhizomes (Smith et al., 2008). *Acrostichum aureum* and *Acrostichum speciosum* are two examples of species under Pteridaceae family that possess medicinal properties in wound healing (Bandaranayake, 1998). These plants are commonly found in mangroves in tropical region especially Malaysia, hence Malays are known to use its rhizome for wound healing (Bandaranayake, 1999). Thus, this

study aimed to test scientifically the effects of the both plant extracts for wound healing treatment. Moreover, to date there is no study ever done on the plant efficacy for wound healing.

Since people in rural area are far away from medicinal facilities, they heavily rely on traditional medicine for wound healing. For example, due to its medicinal value for wound healing treatment, *Aloe vera* is very common herb planted in Malaysia (Joseph et al., 2005). As a comparison, *Acrostichum spp*. could be found in abundance in mangroves area and compared to *A. vera*, these ferns giants need minimum care to grow. Apart from that, since Malays are consuming its young leaves as ulam, therefore they could take the rhizomes to be used as a paste for wound healing treatment. Therefore, in this study, *A. aureum* and *A. speciosum* were chosen to test their wound healing properties due to their abundance in mangroves, lacking of scientific study, and traditional use among Malays in Malaysia.

1.2 OBJECTIVES

1. To identify the phytochemical compounds in *A. aureum* and *A. speciosum*.
2. To test antimicrobial properties of *A. aureum* and *A. speciosum.*
3. To evaluate wound healing properties of ethanol, aqueous extracts and their fractions from leaves, stems, and roots of *A. aureum* and *A. speciosum in vitro* and *in vivo* and to compare their effectiveness as a wound healing agent.

1.3 RESEARCH QUESTIONS

1. Do extracts of *A. aureum* and *A. speciosum* exhibit wound healing properties *in vitro* and *in vivo*?

2. Which extracts (*A. aureum* and *A. speciosum)* exhibit better wound healing properties *in vitro* and *in vivo*?

1.4 HYPOTHESES

Extracts of *A. aureum* and *A. speciosum* exhibit different effectiveness in wound healing properties *in vitro* and *in vivo.*

1.5 OVERVIEW OF THE STUDY

For the first phase of study, phytochemical compounds of both *A. aureum* and *A. speciosum* were screened and total tannin content was done to know their quantitative amount of tannins as a target compound. Next, antimicrobial study was done to assess their properties to enhance wound healing process.

Second phase of the study was *in vitro* study using NIH/3T3 fibroblasts cell line. MTT assay was done to know optimum concentration of the extracts that had proliferative effect on fibroblasts. After that, scratch wound assay was done as a screening of their wound healing potential. Of eight crude extracts and fractions tested that showed better mean migration rate compared to negative control, only four were tested further on rabbits due to their limited amount of fractions.

Third phase of study began with excisional wound inflicment on dorsal part of rabbits. The wounds were treated once daily until complete healing on day 15 with four types of crude extracts with different concentration (5 % and 10 %), Solcoseryl jelly served as a positive control and Aqua cream served as negative control. Next, histopathological study was done on day 15 and the slides were assed individually on three parameters which were collagen, fibroblasts, and epithelization.

CHAPTER TWO

LITERATURE REVIEW

2.1 MEDICINAL PLANTS FOR WOUND HEALING

The treatment of wound healing became human's interest since a long time ago. From east to the west, it became a universal subject when human started to use various kinds of medicinal plants as a wound healing treatment. For example, a bread mold is used in Chinese folk medicine to treat small burns which could be found in written records at least for 2000 years (Brown, 1992). Ibn Sina explained that papyrus is beneficial for haemorrhage to cease and fresh wounds to scar over. This practice is parallel to a hadith narrated by Abu Hazim who had heard Sahl b. Sa'd asked about the treatment for Prophet Muhammad (PBUH)'s wound on the day of Uhud. Fatimah, his daughter burnt a piece of matting to ashes and applied it to the wound. The matting is made from papyrus that contains a strong caustic property that useful to heal wounds (Ibn Qayyim, trans. 2001).

When we trace back the Greek physician practice during Hippocrates (460-370 B. C. E.), the concept of combating and curbing bacterial infection in wound is still a major concern for wound healing treatment until nowadays. Previously, the physicians applied wine into the wound and later it was discovered that polyphenols, malvoside in wine was the compound responsible for bactericidal activity (Eaglstein, 2005). This practice is similar with the use of *Calendula* succus containing fresh juice of *Calendula officinalis* after surgery for enhancing wound healing process. This preparation is included in The Complete German Commission E Monographs for wound healing treatment (MacKay and Miller, 2003). Therefore, various plants have

been studied for wound healing properties worldwide including Malaysia. Various medicinal plants in Malaysia have been proven beneficial in accelerating wound healing process (Table 2.1). The abundance of fauna in Malaysia has been used by many ethnic groups for traditional medicine. Thus, it is a big interest to search for the other potential Malaysian plants and *Acrostichum spp.* is selected in this study due to its traditional Malay practice in wound healing.

Table 2.1 Details of plants have been tested for wound healing study in Malaysia (2006-2012).

	Plant name	Extract used	Model of study	Author
1.	*Plantago major*	Leaves aqueous extract	Excision/ Sprague Dawley male rats	Mahmood and Phipps (2006)
2.	*Allium sativum*	Garlic bulbs aqueous extract	Excision/ Sprague Dawley male rats	Sidik et al. (2006)
3.	*Rafflesia hasseltii*	Flowers methanolic extract	Excision/ Sprague Dawley male rats	Mahmood et al. (2009)
4.	*Ficus deltoidea*	Whole plants aqueous extract	Excision/ Sprague Dawley male rats	Mahmood et al. (2010)
5.	*Elaeis guineensis*	Leaves methanolic extract	Excision/ Sprague Dawley male rats	Sreenivasan et al. (2010)
6.	*Tamarindus indica*	Seed Phosphate Buffered Saline (PBS), aqueous, ethanolic, methanolic extract	Excision/ ICR female mice	Mohd Yusof et al. (2011)
7.	*Phyllanthus niruri*	Leaves aqueous extract	Excision/ Sprague Dawley male rats	Khaled et al. (2012)
8.	*Terminalia coriacea*	Stem bark aqueous and methanolic extract	Excision/ Wistar albino male rats	Mohammed Safwan et al. (2012)

2.2 MANGROVES ECOSYSTEM

Mangroves are salt tolerant forest ecosystems found mainly in the tropical and subtropical intertidal regions of the world largely confined to the region between 30° north and south of the equator (Bandaranayake, 2002). In total, there are 84 mangrove plant species in the world, out of which 70 species are true mangroves and 14 species are semi-mangroves tides (Jun, Qiang, Xing, Min, Jian, and Mei-hua, 2008). There are 55 mangrove species from 22 genera and 18 families could be found along Indian Ocean region. This showed that the region has a great diversity of mangrove species with Indonesia has 45 species, followed by Malaysia (40 spp.), India (39 spp.), Thailand (34 spp.) and Singapore (31 spp.) (Kathiresan and Rajendran, 2005).

Basically, mangrove plants are categorized into two groups, true mangrove and semi-mangrove plants. The true mangrove plants are restricted to the typical intertidal mangrove habitats whereas semi-mangrove plants grow on the landward fringe mangrove habitat or in terrestrial marginal zones subjected to irregular high tides. On the other hand, there is a third group called mangrove associated plants which are salt tolerant terrestrial plants occasionally found in landward edge of mangrove habitat and are irregularly flushed by high tide (Jun et al., 2008). There was a controversy regarding *Acrostichum spp.* in which some scientists classified it as true mangroves and others had classified it as associate mangroves. However, a recent study conducted by Wang, Ruan, Cai, Luo, Xu, and Wu (2010) indicated that both *A. aureum* and *A. speciosum* are associate mangroves due to higher specific leaf area (SLA), lower leaf succulence, lower sodium content and chlorine concentration.

2.2.1 Use of Mangroves as a Medicinal Plant

Mangrove plants have been used in folklore medicine to treat various diseases for centuries. They are a great resource for a new pharmaceutical discovery based on the traditional practice (Ashriq, 2013). Bandaranayake (1998) has listed a list of medicinal use of mangrove plants. For instance, they are used as aphrodisiacs (*Acanthus ilicifolius, Xylocarpus moluccensis*), antitumor (*Avicennia africana, Bruguiera parviflora*) and diabetes treatment (*Bruguiera rumphii*, *Nypa fruiticans*). Meanwhile, associate mangrove like *Acrostichum spp*. is used traditionally to treat boils and wounds (rhizome) and rheumatism (leaves) (Polunin, 1988; Bandaranayake, 1999).

The use of medicinal mangrove plants has triggered the interest of scientist worldwide to investigate them scientifically. Many mangrove species have been proven to possess medicinal value. For example, Ravikumar, Gnanadesigan, Suganthi, and Ramalakshmi (2010) reported that ethanolic extract of *Rhizophora mucronata* and *Avicenna marina* plant parts (hypocotyls, collar, bark and flower) had shown antibacterial activity against isolated urinary tract infectious bacterial pathogens. Besides, Premanathan, Chandra, Bajpai, and Kathiresan (1992) demonstrated that five extracts of mangrove plants (bark of *R. mucronata* and leaves of *Excoecaria agallocha*, *Ceriops deeandra*, *Rhizophora apiculata*, *Rhizophora lamarckii*) completely inhibited the Human Immunodeficiency Virus (HIV) adsorption to the cells. Meanwhile, Kumar, Ammani, and Siddhardha (2011) had concluded that leaves extracts of *Derris trifoliata* and *Brugiera gymnorrhiza* were significantly active against some pathogenic bacteria and fungi. Sivaperumal, Ramasamy, Inbaneson, and Ravikumar (2010) had successfully tested bioactive compounds from *Exoecaria agallocha* against several antibiotic resistant bacterial pathogens.