IDENTIFICATION OF PHENOLIC COMPOUNDS IN *PIPER SARMENTOSUM* ROXB. LEAVES METHANOLIC EXTRACT AND ITS CYTOTOXICITY STUDIES AGAINST PATHOGENIC *ACANTHAMOEBA* SPP.

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Biotechnology)

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ABSTRACT

Phenolic compounds are plant secondary metabolites that are beneficial to human health. Previous studies reported that phenolic compounds could lower the risk of heart diseases, occurrence of cancers and many microbial infections. *Piper sarmentosum* or locally known as Kaduk is a tropical herb that has long history in traditional medicines and food. These benefits are significantly dependent on the phenolic content. In this study, the crude methanolic extract of *Piper sarmentosum* leaves were analyzed for phenolic compounds identification and its anti-amoebic properties against two pathogenic *Acanthamoeba* strains namely *Acanthamoeba castelanii* and *Acanthamoeba* sp. of Hospital Kuala Lumpur (HKL) isolate. The total phenolic content in *P. sarmentosum* leaves methanolic extract was first determined by using Folin-Ciocalteu assay. The compounds were further characterized by using gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and reverse phase-high performance liquid chromatography (RP-HPLC) analyses. The cytotoxicity of the plant extract was examined using four assays: determination of IC_{50} by eosin dye method, cell morphological analysis using inverted light and scanning electron microscopies, cell membrane integrity assessment by acridine orange/propidium iodide (AO/PI) staining, and determination of cell death mode by DNA fragmentation assay. The total phenolic content was found to be 142.72 mg[GAE]/g of the extract. A total of 36 phenolic compounds were identified from the three high-resolution chromatography analyses. The IC_{50} values obtained were 74.64 µg/mL for *A. castelanii* while 22.13 µg/mL for *Acanthamoeba* sp. (HKL isolate). Microscopy analyses showed that the extract caused cell encystment indicated by distinctive morphological changes on cell shape and organelles, shortening of acanthopodia and disruption on cell membrane integrity. The extract was also discovered to promote apoptosis in both *Acanthamoeba* species. The result provided the evidence that crude methanolic extract of *P. sarmentosum* leaves contains active phenolic compounds contributing to its anti-acanthamoebic properties.

ملخص البحث

إن المركبات الفينولية هي المستقلبات الثانوية النباتية التي لها فوائد مهمة على صحة الإنسان. ذكرت الدراسات السابقة أن المركبات الفينولية يمكن أن تقلل من خطر أمراض القلب، وحدوث السرطان والعديد من الأمراض الجرثومية. إن عشب *Piper sarmentosum* أو ما يعرف محليا باسم Kaduk هو عشبة استوائية لها تاريخ طويل في الأدوية التقليدية ويستعمل أيضا كغذاء. هذه الفوائد تعتمد بشكل كبير على محتوى الفينول. في هذه الدراسة، تم تحليل المستخلص الميثانولي الخام لأوراق *Piper sarmentosum* لتحديد هوية المركبات الفينولية وخصائصها المضادة للأموبيك ضد سلالتين من نوع *Acanthamoeba* وهما *Acanthamoeba castelanii* و *Acanthamoeba* sp. من مستشفى كوالا لامبور (HKL) . تم تحديد محتوى الفينول الكلي في المستخلص الميثانولي الخام لأوراق *P. sarmentosum* وذلك باستخدام اختبار Folin-Ciocalteuلأول مرة، وتم تمييز المركبات كذلك باستخدام طيف الكتلة الكروماتوغرافي للغاز (GC-MS) ، قياس الطيف الكتلي اللوني السائل (LC-MS) والتحليل الكروماتوجرافي السائل عالي الأداء (RP-HPLC) .تم فحص السمية الخلوية للمستخلص النباتي باستخدام أربع فحوصات: تحديد IC_{50} بطريقة eosin dye ، تحليل شكل الخلية باستخدام الضوء المقلوب ومسح المجهر الإلكتروني ، تقييم سلامة غشاء الخلية بواسطة تلطيخ الأكريدين البرتقالي / البروبيديوم (AO / PI) ، وتحديد وضع موت الخلايا عن طريق فحص تجزئة الحمض النووي. تم العثور على محتوى الفينول الكلي ليكون 142.72 mg[GAE]/g من المستخلص. تم تحديد ما مجموعه 36 مركبات فينول من ثلاثة تحليلات لونية عالية الدقة. كانت قيم IC_{50} التي تم الحصول عليها 74.64 ميكروغرام / مل ل *A. castelanii* بينما 22.13 ميكروغرام / مل من . *Acanthamoeba* sp. (HKL isolate)أظهرت التحاليل المجهرية أن المستخلص قد تسبب في انسحاب الخلية من خلال تغيرات شكلية مميزة على شكل الخلية والعضيات، وتقصيرفيacanthopodi وانقطاع في سلامة الغشاء الخلوي. كما تم اكتشاف أن للمستخلص قدرة لتعزيز الاستموات في كلا النوعين ل *Acanthamoeba* قدمت هذه النتيجة الدليل على أن مستخلص الميثانول الخام من أوراق *P. sarmentosum* يحتوي على مركبات فينولية نشطة تسهم في خصائصه المضادة للالتهاب.

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)

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This thesis is dedicated to my family and friends

for outstanding support and love.

Thank you Allah

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LIST OF EQUATIONS

LIST OF ABBREVIATION

AF	Area fraction
AIDS	Acquired Immunodeficiency Syndrome
AK	*Acanthamoeba* keratitis
ANOVA	Analysis of variance
AO	Acridine orange
ARB	Amoeba resistant bacteria
CCAP	Culture Collection of Algae and Protozoa
CCS	Collision cross-section
CNS	Central nervous system
DNA	Deoxyribonucleic acid
ESI	Electron Spray Ionization
FLA	Free-living amoeba
FTA	Forsythoside A
GAE	Gallic acid equivalents
G.A.E	Granulomatous amoebic encephalitis
GC-MS	Gas chromatography mass spectrometry
GNC	Glasshouse and Nursery Complex
HKL	Hospital Kuala Lumpur
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
HSD	Honestly significant difference
IC_{50}	Inhibition concentration for 50% of cell population
IIUM	International Islamic University Malaysia

LC-MS	Liquid chromatography mass spectrometry
MDR	Multi-drug resistance
MRSA	Methicillin resistant *Staphylococcus aureus*
NIST	National Institute of Standards and Technology
OD	Optical density
PBS	Phosphate buffered saline
PHMB	Polyhexamethylene biguanide
PI	Propidium iodide
PSLME	*Piper sarmentosum* leaves methanolic extract
PYG	Peptone-yeast-glucose
QToF	Quadrupole Time-of-Flight
RP-HPLC	Reverse phase-high performance liquid chromatography
rRNA	Ribosomal ribonucleic acid
S.E.M	Standard error of mean
SEM	Scanning electron microscopy
TBE	Tris/Borate/EDTA
TPC	Total phenolic content
UKM	National University of Malaysia
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet

LIST OF SYMBOLS

μm	Micrometre
mm	Milimetre
cm	Centimetre
cm^2	Square centimetre
nm	Nanometre
μL	Microlitre
mL	Mililitre
L	Litre
μg	Microgram
mg	Milligram
g	Gram
%	Percentage
°C	Degree celcius
μM	Micro molar
M	Molar
molar	Molar mass
mbar	Milibar
kPa	Kilopascal
eV	Electronvolt
kV	Kilovolt
m/z	Mass-to-charge ratio
h	Hour
psi	Pound per square inch
rpm	Revolutions per minute
kbp	Kilo basepairs

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Acanthamoeba is a free-living amoeba that could be found ubiquitously in the normal environment especially water, soil and air. As *Acanthamoeba* typically lives as bacterial consumer in natural environment, its distribution is based on the availability of bacterial supply, which are most commonly found in seawater, river, lakes and contaminated water sources (Lorenzo-Morales et al., 2005; Tsvetkova et al., 2004). The reputation of *Acanthamoeba* as an opportunistic human pathogen has been acknowledged in medical history. Primarily, *Acanthamoeba* could cause many diseases primarily by disseminating infections to skin, eyes, lungs, sinuses, and extra-cebral organs. Upon pathogenicity in humans, *Acanthamoeba* can easily enter through lower respiratory tract, or ulcerated and broken skins which then it could produce toxins that can constitute a lethal dose. These infections could progress to chronic diseases such as *Acanthamoeba* keratitis as well as the fatal encephalitis known as granulomatous amoebic encephalitis (G.A.E) although in seldom occurrences. Many reports showed that approximately 85% of all amoebic keratitis cases were associated with contact lens users (Verani et al., 2009). In fact, commercial contact lens solutions were frequently reported to have lack of proper disinfectants against *Acanthamoeba* species. Without appropriate treatment, *Acanthamoeba* keratitis could result in permanent blindness. Meanwhile, G.A.E is a fatal infection of the central nervous system that occurs upon the entrance of *Acanthamoeba* through skin and lungs. The pathogen toxins gradually spread to the brain through the bloodstream causing some

early symptoms include skin sores, headache, blurred visions, confusion, seizures and ultimately death if left untreated (Ertabaklar et al., 2006).

Moreover, this microorganism is also capable of being a reservoir for numerous microbial pathogens such as *Campylobacter jejuni, Legionella pneumophila* and the genus *Pseudomonas* spp. (Axelsson-Olsson et al., 2005; Marciano-Cabral & Cabral, 2003). Since the *Acanthamoeba* feed on bacteria, fungi and algae through phagocytosis as their main food sources, some of these microorganisms have successfully evolved and gained resistance to the predation, thus are able to survive, increase the rate of growth and development and easily escape from the host (Marciano-Cabral & Cabral, 2003). Cumulatively, this facilitates for the increased occurrences of microbial infections in humans, animals and plants not only caused by the pathogenicity factors of the amoeba, also by the internalized microorganisms.

Plant secondary metabolites are becoming central players as the basic sources for modern medicines in curing numerous diseases in humans, animals and plants. Phenolic compounds constitute major secondary metabolites in plants as they play important roles for the plant protection against physical pressures such as microbial pathogens, insects and harsh environment condition (Khoddami et al., 2013). Over decades, phenolic compounds are discovered to closely associate with health benefits to humans through consumption of vegetables and fruits. Most frequently, the compounds are found to exhibit strong antioxidant properties, hence are capable of preventing many diseases such as cardiovascular diseases, occurrence of cancers and mutagenesis in human cells, and reducing the risk of diabetes (Khoddami et al., 2013). Apart from that, they are also molecules with wide range of pharmacological properties including anti-inflammatory, anti-allergenic, anti-thrombotic, anti-

artherogenic, and anti-microbial against bacteria, fungi and microalgae (Hussain et al., 2012; Zakaria et al., 2010; Dykes & Rooney, 2007).

The basic structure of phenolic compound is an aromatic or benzene ring bearing one or more hydroxyl groups (Khoddami et al., 2013). Plant phenolics comprise of many groups or subclasses such as simple phenols, coumarins, lignins, lignans, tannins, benzoic acid, cinnamic acid, phenolic acids and flavonoids (Khoddami et al., 2013; Soto-Vaca et al., 2012). Acknowledging the benefits of phenolic compounds to human health, substantial number of studies that focus on the extraction and identification of the compounds from various types of plants has increased over the last 25 years (Khoddami et al., 2013).

Numerous discoveries of plant as a source for diseases management led to execution of many researches that focus on the effectiveness of plant-based antimicrobial agents to combat the harm caused by microbial pathogens on human, plant and animal. *Piper sarmentosum* Roxb. or locally known as "Kaduk" is a terrestrial herb plant that has been used as traditional food and medicines. It is widely distributed in tropical regions including Thailand, Indonesia, Philippines and Malaysia. The plant leaves are commonly consumed as vegetable whilst the whole plant is an effective remedy to treat minor ailments such as coughs, tooth-ache, influenza and feet fungoid dermatitis (Atiax et al., 2011). Previous phytochemical studies of *P. sarmentosum* have led to isolation of several classes of physiologically active compounds especially phenolics such as flavonoids, tannins, lignan and phenylpropanoids.

Recent researches proved that some solvents-based extracts of the plant showed antimicrobial properties against wide ranges of microorganisms especially bacteria, microalgae, protozoa and fungi (Rahman et al., 2014a; Rukachaisirikul et al.,

2004). These findings reported that the demonstrated antimicrobial properties of the plant associate with high amount of phenolic content. However, the specific potential of phenolic compounds from *P. sarmentosum* against *Acanthamoeba* species has not yet been investigated. This research aimed to identify the phenolic substances present in the *P. sarmentosum* leaves crude methanolic extract (PSLME) and to evaluate the cytotoxicity effects of the extracted phenolic compounds on pathogenic *Acanthamoeba.*

In this study, the phenolic compounds were characterized by using gas chromatography mass spectrometry (GC-MS), reverse phase-high performance liquid chromatography (RP-HPLC) and liquid chromatography mass spectrometry (LC-MS) analyses. Meanwhile, the cytotoxicity studies consisted of determination of half-maximal inhibition concentration (IC_{50}) values, microscopic morphological analyses, assessment of cells' membrane integrity and determination of mode of cell death.

1.2 PROBLEM STATEMENTS

The vision threatening keratitis in human involving *Acanthamoeba* has becoming a serious problem worldwide including in Malaysia. This disease is closely related to contact lens prescribing since few decades ago. Worse still, people with normal eye health are also at the risk of *Acanthamoeba* keratitis especially those who are exposed to contaminated water sources. Current administrations for amoebic eye infections using chlorhexidine and propamidine or polyhexamethylene biguanide (PHMB) were shown as ineffective and adverse side effects were reported in patients (Clarke et al., 2012; Khan, 2008). The prescriptions are monotonous and repetitive, as the patients are required to apply the relevant drugs within interval hours per treatment. Although this topical treatment may progressively reduce the symptoms, complete recovery is

scarcely achieved (Aqeel et al., 2017). Apart from that, in the case of the central nervous system disease of G.A.E caused by *Acanthamoeba*, most of the infected people die as early as 7 to 120 days after symptoms begin (McNeil & Singh, 2012). According to Parija et al. (2015), there is no single drug is effective in treating G.A.E, hence, combination of drugs and antibiotics targeting various proteins are required. More insidiously, successful treatments often require combination of surgical and interventions from multiple medical specialists to initiate appropriate management for G.A.E cases.

It is becoming a mainstream in modern clinical researches that highlight the value of botanical medicine in treating and preventing many diseases, often without significant side effects. Therefore, there is always necessity to discover new candidate materials based on phytochemicals properties, which could be employed as new alternatives for effective drugs in preventing and treating these acanthamoebic diseases.

1.3 RESEARCH OBJECTIVES

The research focused on the *P. sarmentosum* leaves crude methanolic extract potential as anti-amoeba agent against pathogenic *Acanthamoeba* spp. The objectives of this study are listed as below:

1. To identify the phenolic compounds in crude methanolic extracts of *P. sarmentosum* leaves.

2. To evaluate the cytotoxicity of *P. sarmentosum* leaves methanolic extracts on pathogenic *Acanthamoeba* spp.

1.4 SIGNIFICANCE OF STUDY

Although *P. sarmentosum* leaves extracts had shown effective antimicrobial properties against wide range of microorganisms primarily bacteria, fungus as well as protozoa, in our best of knowledge, this is the first study that discovers the potential of *P. sarmentosum* as anti-amoeba agent against *Acanthamoeba.* The aim of identifying the phenolics composition in the plant extract could facilitate in understanding the relationship between phenolic compounds with the exhibited anti-acanthamoebic properties.

Moreover, this fundamental study could provide the preliminary data needed to formulate and further develop improvised version of commercial contact lens disinfectant solution as well as medicines to treat *Acanthamoeba* infections primarily *Acanthamoeba* keratitis and G.A.E. The formulation of using phenolic compounds from *P. sarmentosum* as the foundation ingredient would further promote the incorporation of the herb in pharmaceutical industry although this requires more comprehensive subsequent studies. From this, national consumption of contact lens solution and medicines for *Acanthamoeba* infections will be less dependence on the imported products. This ingredient could also be marketed to global pharmaceutical companies to improve the efficiency of medicines for *Acanthamoeba* infections, in which potentially will avoid the occurrence of *Acanthamoeba* keratitis and disseminated infections caused by the pathogenic amoeba nationwide.

1.5 RESEARCH HYPOTHESIS

Piper sarmentosum leaves methanolic extract contains phenolic compounds that are active to inhbit the proliferation of pathogenic *Acanthamoeba* spp.