CHARACTERIZATION OF THE ANTI-ANGIOGENESIS ACTIVITY OF *LABISIA PUMILA* AND IDENTIFICATION OF ITS MOLECULAR CONSTITUENTS THAT CONTRIBUTE TO ITS BIOLOGICAL ACTIVITY

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JULY 2010

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Thesis submitted in fulfillment of the

requirements for the degree of

Master of Science

July 2010

ACKNOWLEDGEMENTS

So many good people have helped me along this journey. It is impossible to mention all of them, but let me thank a few which applies equally well to others.

My supervisor, Dr Gurjeet Kaur who accepted me as her student thus opened a door for me in this research work. I'm indebted to her for the wise supervision. I also wish to thank my co-supervisors Dr Amin Malik Shah and Dr Tan Mei Lan for their advice, guidance and assistance throughout.

To my colleagues Hayder, Abed, Zena, Norshirin, Syima, Marina and Zeiad, thank you for being there for me when I need it most.

Last but not least my uttermost gratitude to my family and my husband, Mazrizal Ahamad for their support and patience throughout my study. Their love has no boundary and kept me going at the roughest of times. Thanks for giving me the confidence to seek new challenges and achieve higher goals in life.

This work was funded under the Research University grant, number 1001/PPSK/8120243.

DECLARATION

I hereby declare that the work done in this thesis is my own except for quotations and summaries which have been duly acknowledged

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NOZLENA ABDUL SAMAD

ABSTRACT

Angiogenesis is a process of new blood vessel formation. Inhibition of angiogenesis is considered one of the most promising strategies in treating variety of illnesses including cancer. *Labisia Pumila* var. alata (Myrsiaceae) or locally known as 'kacip fatimah', a lowland plant, is used widely by the Malays in Peninsular Malaysia to treat many health problems and ailments. A literature search did not reveal any reports of its anti-angiogenesis activity. To date this is the first study on anti-angiogenesis activity of *Labisia pumila*. The aim of this study was to investigate anti-angiogenesis activity, anti-oxidant properties, total phenolic content and chemical constituents of *Labisia pumila* leaves extracts and fractions. The plant was pulverized and extracted successively with petroleum ether (PE), chloroform (CE), methanol (ME) and water (WE). The anti-angiogenesis activity of different extracts (PE, CE, ME and WE) of *Labisia pumila* leaves was studied using rat aortic ring assay. The study showed that *Labisia pumila* leaves extracts significantly inhibited microvessels outgrowth of extracts. The methanolic extract gave the most significant anti-angiogenesis activity ($P<0.05$). This extract was further fractionated into three fractions, ethyl acetate fraction (EAF), n-hexane fraction (NF) and water fraction (WF). WF had the highest anti-angiogenesis level, which was lower than the level of the methanol extract (ME). These two samples were found to be non-cytotoxic in the MTT assay against human umbilical vein endothelial cells (HUVEC). Both samples showed no significant cytotoxic activity towards selected cancer cell lines. ME and WF were also found to inhibit the endothelial tube formation in the HUVEC tube formation assay. *Labisia pumila* methanol extract and water fraction demonstrated the inhibition of VEGF protein expression level. Owing

to the importance of anti-oxidants in angiogenesis, both extract and fraction were analysed for their free radical scavenging activity that revealed their potent anti-oxidative properties. The anti-oxidant property was analyzed using DPPH (1, 1–diphenyl-2-picrylhydrazyl) assay and the total phenolic assay was analyzed by Folin-Ciocalteau method. The results demonstrated that methanol extract and water fraction of this plant had potent anti-oxidant activity as observed in the DPPH assays with IC_{50} of the extract and fraction determined to be 0.4021µg/ml and 0.4060µg/ml respectively. The total phenolic content of 1mg of the plant leaves extract and fraction is equivalent to 18µg gallic acid. The chemical constituents of this plant extract and fraction were determined using FTIR, GCMS and LCMS-TOF techniques. LCMS-TOF analysis on ME and WF showed the presence of vitamin D3 derivatives with ME having significant amounts of 4-phatlimidoglutaramic acid, which were absent in the WF fraction. In conclusion, the current study suggests that *Labisia pumila* methanol extract and water fraction may potentially act as potent angiogenesis inhibitor. This activity may be due to the presence of the two compounds previously mentioned.

ABSTRAK

Angiogenesis merupakan suatu proses pembentukan pembuluh darah baru. Penghalangan angiogenesis merupakan strategi yang penting dalam rawatan pelbagai penyakit termasuk kanser. *Labisia pumila* var alata (Myrsiaceae) atau lebih dikenali dengan nama tempatannya sebagai 'kacip fatimah' merupakan tumbuhan di tanah rendah lazimnya digunakan secara meluas oleh penduduk Melayu di Semenanjung Malaysia dalam rawatan pelbagai penyakit di negara ini. Tiada sebarang kajian literasi yang melaporkan aktiviti anti-angiogenesis bagi tumbuhan ini. Oleh yang demikian, kajian ini merupakan kajian pertama bagi melaporkan aktiviti anti-angiogenesis bagi *Labisia pumila.* Tujuan kajian ini adalah untuk mengkaji aktiviti anti-angiogenesis, kandungan anti-oksidan, jumlah kandungan fenol dan juga unsur-unsur bahan kimia yang terdapat dalam *Labisia pumila.* Tumbuhan ini pada mulanya dipotong kecil dan dikisar serta kemudian di ekstrak secara berperingkat dengan eter petroleum (PE), khlorofom (CE), methanol (ME) dan air (WE). Cerakin angiogenesis telah dijalankan dengan menggunakan ekstrak-ekstrak *Labisia pumila* yang berbeza ini (PE, CE, ME dan WE). Keputusan kajian yang diperolehi menunjukkan bahawa pertumbuhan pembuluh darah telah direncatkan dengan rawatan oleh sampel-sampel ini. Ekstrak metanol didapati merupakan ekstrak yang terbanyak bersifat anti-angiogenesis berbanding yang lain dengan kebarangkalian $P<0.05$. Kelompok ini dipecahkan lagi kepada tiga fraksi iaitu fraksi etil asetat (EAF), fraksi n-heksana (NF) dan fraksi air (WF). WF menunjukkan tahap anti-angiogenesis yang tinggi tetapi berada pada tahap yang lebih rendah daripada ekstrak ME. Kedua-dua sampel ini telah didapati tidak sitotoksik dalam cerakinan MTT pada sel-sel endothelium vena umbilicus manusia (human umbilical vein endothelial cells (HUVEC) dan juga

pada sel-sel kanser manusia yang di pilih dalam kajian ini. ME dan WF juga menunjukkan perencatan dalam cerakinan pembentukan tiub HUVEC dan ekspresi protein VEGF. Berdasarkan kepada kepentingan anti-oksidan dalam anti-angiogenesis, aktiviti pencarian radikal bebas bagi kedua-dua sampel telah dianalisa. Kandungan anti-oksidan bagi ME dan WF dikaji dengan menggunakan cerakin 1,1-diphenyl-2-picrylhydrazyl (DPPH). Cerakin jumlah fenol juga dianalisa bagi kedua-dua sampel ini dengan menggunakan kaedah Folin-Ciocalteau. Keputusan yang diperolehi menunjukkan bahawa ekstrak metanol dan fraksi air bagi tumbuhan ini menunjukkan sifat anti-oksidan yang baik sepertimana diperhatikan dalam cerakin DPPH iaitu dengan IC_{50} yang diperolehi bagi ekstrak metanol dan fraksi air masing-masing adalah 0.4021µg/ml dan0.4060µg/ml. Jumlah kandungan fenol bagi 1mg estrak dan fraksi ini adalah bersamaan dengan 18µg asid galik. Unsur-unsur kimia bagi ekstrak dan fraksi tumbuhan ini telah diperolehi dengan menggunakan teknik FTIR, GCMS dan LCMS-TOF. Dalam analisa menggunakan teknik LCMS-TOF pada ME dan WF, kehadiran vitamin D3 dan asid phtalimidoglutaramic telah dikenalpasti. Asid phtalimidoglutaramic hanya terdapat pada ME tetapi tidak pada WF. Kesimpulannya, kajian ini telah mencadangkan bahawa ekstrak metanol dan fraksi air bagi *Labisia pumila* berkemungkinan bertindak sebagai perencat angiogenesis. Aktiviti ini mungkin disebabkan oleh kehadiran dua unsur kimia yang telah dinyatakan.

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LIST OF ABBREVIATIONS

ATCC	American type culture collections
µg/ml	Microgram/ millilitre
3D	Three dimensional
FGF	fibroblast growth factor
CE	Chloroform extract
cm	Centimeter
CO_2	Carbon dioxide
COX-2	Cycloxygenase -2
DMSO	Dimethyl sulfoxide
DPPH	1.1-diphenyl 1-2-picrylhydrozyl
EAF	Ethyl acetate fraction
ECs	Extra cellular cells
ECM	Extra cellular matrix
FTIR	Fourier Transform InfraRed
gm	Gram
GCMS	Gas Chromatography Mass Spectrometry
HCT-116	Human colorectal carcinoma
HIFCS	Heat in activated fetal calf serum
HPLC	High performance liquid chromatography
Hrs	Hours
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Inhibition concentration of 50%
IL	Interleukin

IFN	Interferon
LCMS-TOF	Liquid Chromatograph/Mass Spectrometry- Time of Flight
MCF-7	Hormone Dependent Breast Cancer Cells
MDA-MB-231	Metastatic Breast Cancer Cells
ME	Methanol extract
NF	N-hexane fraction
PBS	Phosphate buffer saline
PDGF	Platelet derived growth factor
PE	Petroleum ether
Pen/strep	Penicillin/streptomycin
SD	Standard deviation
TGF- β	Tumor growth factor beta
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor
WE	Water extract
WF	Water fraction

LIST OF SYMBOLS

%	Percent
^{o}C	Degree Celsius
β	Beta
α	Alpha

CHAPTER ONE

INTRODUCTION

1.1 Natural Products of Medicinal Value

Malaysia's biodiversity is rich in natural resources. Today, out of more than 20,000 species of angiosperms (flowering plant) and 600 species of ferns in Malaysia, 1,082 species of angiosperm and 76 species of fern have been documented to have medicinal properties (Noor Rain *et al*., 2007). It has been reported that among 25 best selling medicines in the world, 30% of it are derived from natural products (Kong *et al*., 2003).

In our study, we have chosen to work on *Labisia pumila*, a popular plant in Malaysia that has been used as a herbal remedy to treat various common ailments and to maintain overall good health. Natural products are a preferable choice nowadays. Numbers of known natural products are being developed rapidly because of its potential in discovering new treatment of several illnesses (Alves and Rosa, 2007). Apart from that, medicines derived from natural products or herbal medicines which contain bioactive pytochemical constituents plays a vital role in a physiological action on human body (Krishnaiah *et al*., 2009).

Throughout the whole world, almost 3 billion people depend on herbal remedies as sources of medicines (Mahady, 2001). Figure 1.1 demonstrates the use of natural products as sources of new drugs between years 1981-2002.

The idea of particular plants to be used and the methods of application to cure particular ailments were passed from generation to generation mainly through oral history (Joshi *et al*., 2004).

As the potential of plant based medicines is far from exhausted, biological and phytochemical screenings are rapidly being conducted by researchers to find new drugs to target various illnesses. Table 1.1 shows a list of medicinal agents derived from plant origin.

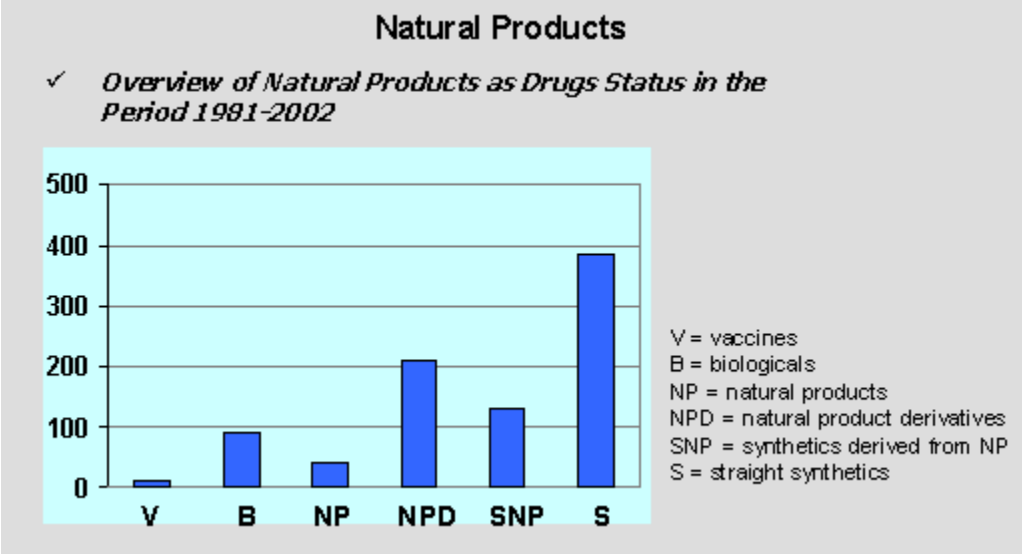

Figure 1.1 The use of natural product as sources of new drugs in 1981-2002 (Newman *et al*., 2003)

Table 1.1 The list of medicinal agents derived from plant origin (Fabricant and Farnsworth, 2001)

Plant	**Medicinal Agent**
Digitalis lanata Ehrh.	Acetyldigoxin
Berberis vulgaris L.	BerberineVincristine and Vinblastine
Curcuma longa L.	CurcuminQuinidine
Digitalis purpurea L.	Digitoxin
Ephedra sinica Stapf	Ephedrine
Hyoscamus niger L.	Hyoscamine
Papaver somniferum L.	Morphine
Ephedra sinica Stapf	Pseudoephedrine
Cinchona ledgeriana Moens ex. Trimen	Quinine
Rhododendron molle G. Don	Rhomitoxin
Salix alba L.	Salicin