UNIVERSITI PUTRA MALAYSIA

PHYTOCHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITY OF *EUPHORBIA HIRTA* AND *PHYLLANTHUS WATSONII*

MOHD NAZRUL HISHAM BIN DAUD

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By

MOHD NAZRUL HISHAM BIN DAUD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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DEDICATION

My sisters

&

Friends

My grateful thanks for everything …….

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman: **Associate Professor Khozirah Shaari, PhD**

Institute: **Bioscience**

Preliminery screening for anti-inflammatory activity using 5-lipoxygenase assay has been carried out on 31 species of plant. From the results obtained, *Euphorbia hirta* and *Phyllanthus watsonii* two species of the Euphorbiaceae family showed high inhibition against enzyme 5-lipoxygenase. Based on this results, the phytochemical studies on both plants were carried out. Separation, isolation and purification of compounds from both species were done using solvent-solvent partitioning and chromatography techniques such as normal phase, gel filtration on sephadex LH-20 and reverse phase column chromatography. Two flavonoid *O*-gylcosides known as quercetin 3-*O*-rhamnoside **[34]** and myricetin 3-*O*-rhamnoside **[35]** were isolated from the ethyl acetate fraction of *Euphorbia hirta*. The compounds 26-nor-D:A-friedoolean-14-en-3*β*-ol **[30]** and glochidonol **[36]** have been isolated from *Phyllanthus watsonii*. All the compounds were tested for their inhibitory effect against 5-lipoxygenase enzyme and antioxidants activity

using the ferric thiocyanate (FTC), thiobarbituric acid (TBA), free radical scavenging activity (DPPH) methods. Although the methanolic crude extracts of both plants showed potent inhibition of more than 65% on the 5-lipoxygenase assay, all the compounds isolated showed inhibition below this value. Quercetin 3-*O*-rhamnoside and myricetin 3-*O*-rhamnoside gave 19.0% and 16.0% inhibition against 5-lipoxygenase at the test concentration of 2.5mM. The compounds 26-nor-D:A-friedoolean-14-en-3*β*-ol **[30]** and glochidonol **[36]** showed 17.2% and 17.1% inhibition at the test concentrations of 0.24 and 0.22mM, respectively. Meanwhile, quercetin 3-*O*-rhamnoside **[34]** and glochidonol **[36]** showed strong antioxidant activity, giving 97.7% and 95.5% inhibition in the ferric thiocyanate (FTC) method. In the thiobarbituric acid (TBA) experiment both compounds also showed strong antioxidant activity, giving 92.8% and 92.5% inhibition. Finally the DPPH free radical scavenging activity experiment, quercetin 3-*O*-rhamnoside **[34]** gave 85.8 % inhibition at the test concentration of 100μM. The IC_{50} was calculated to 23.40μM.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan unuk ijazah Master Sains

PENGAJIAN ANALISIS KIMIA TUMBUHAN DAN AKTIVITI BIOLOGI DARIPADA *EUPHORBIA HIRTA* DAN *PHYLLANTHUS WATSONII*

Oleh

MOHD NAZRUL HISHAM BIN DAUD

Januari 2006

Pengerusi: **Profesor Madya Khozirah Shaari, PhD**

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Penyaringan awal aktiviti anti-radang menggunakan cerakin 5-lipoksigenis ke atas 31 spesies tumbuhan mendapati, *Euphorbia hirta* dan *Phyllanthus watsonii* dari famili Euphorbiaceae telah menunujukkan perencatan tinggi terhadap aktiviti enzim 5-lipoksigenis. Berdasarkan keputusan ini, kajian fitokimia keatas kedua-dua tumbuhan telah dijalankan. Pengasingan dan penulenan sebatian kimia daripada kedua-dua spesies telah dilakukan menggunakan kaedah pemisahan pelarut-pelarut dan teknik kromatografi seperti kromatografi fasa biasa, penapisan gel sephadex LH-20 dan kromatografi fasa terbalik. Dua flavonoid *O*-glikosida yang dikenal sebagai kuarsetin 3-*O*-ramnosida **[34]** dan mirisetin 3-*O*-ramnosida **[35]** telah berjaya dipencilkan daripada fraksi etil asetat spesies *Euphorbia hirta*. Sebatian 26-nor-D:A-fiedoolen-14-en-3*β*-ol **[30]** dan glokidonol **[36]** telah berjaya dipencilkan daripada *Phyllanthus watsonii*. Sebatian kimia

yang dipencilkan diuji kesan perencatannya terhadap aktiviti enzim 5-lipoksigenis dan aktiviti biologi lain seperti antioksidan menggunakan kaedah ferik tiosinat (FTC), tiobarbiturik asid (TBA) dan aktiviti pengutipan radikal bebas (DPPH). Walaupun metanol ekstrak kedua-dua tumbuhan mencatatkan perencatan enzim 5-lipoksigenis pada tahap yang berkesan iaitu lebih daripada 65% tetapi sebatian kimia yang dipencilkan mencatatkan perencatan yang lebih rendah daripada itu. Kuarsetin 3-*O*-ramnosida **[34]** dan mirisetin 3-*O*-ramnosida **[35]** masing-masing mencatatkan perencatan sebanyak 19.0% dan 16.0% pada kepekatan 2.5mM. Sebatian kimia 26-nor-D:A-fiedoolen-14-en-3β-ol **[30]** dan glokidonol **[36]** masing-masing mencatatkan perencatat sebanyak 17.2% dan 17.1% pada kepekatan 0.24 dan 0.22mM. Daripada pemerhatian, didapati faktor sinergi mungkin memberi kesan kepada aktiviti perencatan 5-lipoksigenis. Kuarsetin 3-*O*-ramnosida **[34]** dan glokidonol **[36]** menunjukkan kesan antioksidan yang tinggi dengan kadar perencatan masing-masing 97.7% dan 95.5% dalam ujikaji ferik tiosianat (FTC). Manakala dalam ujikaji asid barbiturik (TBA), kedua-dua sebatian kimia mencatatkan perencatan sebanyak 92.8% dan 92.5%. Akhirnya dalam uijikaji pengutipan radikal bebas (DPPH), kuarsetin 3-O-ramnosida **[34]** mencatatkan kadar perencatan sebanyak 85.8% pada kepekatan 100 µM. Nilai IC_{50} bagi sebatian ini ialah 23.40µM.

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I certify that an Examination Committee met on 6th January 2006 to conduct the final examination of Mohd Nazrul Hisham Bin Daud on his Master of Science thesis entitled **"**Phytochemical Analysis And Biological Activity Of *Euphorbia hirta* and *Phyllanthus watsonii''* in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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MOHD NAZRUL HISHAM DAUD

Date:

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LIST OF ABBREVIATIONS

PAF	Platelet Activation Factor
NO	Nitric Oxide
LO	Lipoxygenases
5-LO	5-Lipoxygenase
ROS	Reactive Oxygen Species
UV	Ultraviolet
FT-IR	Fourier Transform Infrared
NMR	Nuclear Magnetic Resonance
LCMS	Liquid Chromatography Mass Spectrometry
EI-MS	Electron Impact Mass Spectrometry
ESI-MS	Electron Spray Impact Spectrometry
GCMS	Gas Chromatography Mass Spectrometry
HSQC	Heteronuclear Single Quantum Coherence
HMBC	Heteronuclear Multiple Bond Correlation
COSY	Correlation Spectroscopy
MeOH	Methanol
$CHCl_3$	Chloroform
EA	Ethyl acetate

CHAPTER 1

INTRODUCTION

1.1 Phytochemicals as a source of drugs

Over 250,000 species of flowering plants exist on this planet and of these, an estimated 155,000 could be found in the tropics (Cordell *et al*., 1991). Malaysia is one of the twelve countries in the world with a rich biodiversity. Apart from fuel, fiber and food, plants have always been known to be a traditional source of medicines since they contain secondary metabolites of high chemical diversity. Chemical diversity is one among several important factors that has given rise to the continuing interests in natural products research. Plants have contributed a lot to modern medicine with the origin of numerous drugs in use today are derived from plants, in one-way or another (Table 1.1). Plants of the tropical rainforests in particular have been said to have very high chemical diversity and thus offers a potential source for the discovery of new and useful natural products for use as medicines.

Natural products are chemical compounds obtained from plants, animals and insects as well as a plethora of other living organisms. The study on natural products encompasses the investigation into their molecular structure, biogenesis, and biological functions in the organism, therapeutic applications and other uses. Studies on natural products have become more and more important with the realization that plants provide a source of useful chemicals that may be used directly or as templates for the development of drugs useful for defense or protection against various diseases.

They are also useful as nutraceutricals and health foods or supplements to promote good health and growth.

The discovery of penicillin from the fungus *Penicillium notatum* by Fleming in 1928 marked a new era in medicine. It promoted the intensive investigation of nature as a source of novel bioactive compounds. Since then plants and microorganisms have together served as a prolific source of structurally diverse and bioactive metabolites, yielding many important products one finds in the pharmaceutical industry today (Cragg *et al*., 1997). This has been the result of systematic investigations carried out on just 5-15% of the total terrestrial flora, mostly of higher plants (Balandrin *et al*., 1993). A larger fraction is virtually untapped and still remains to be investigated. The potential wealth of discovery offered by this biological resource is just enormous. The continuing threat to biodiversity through the destruction of terrestrial and marine ecosystems lends urgency to the need to expand the systematic exploration of this biological resources in the search of new bioactive molecules.

Drug	Plant source	Therapeutic application
Morphine	*Papaver somniferum*	Pain relief
Quinine	*Cinchona succirubra*	Anti malarial
Taxol	*Taxus brevifolia*	Anti cancer
Lysergic acid diethylamide	*Claviceps purpurea*	Migraine and headaches
Vincristine	*Catharanthus roseus*	Anti leukemic

Table 1.1 Drugs obtained or derived from natural products

1.2 Strategies in natural products research

There have been several strategies used in the past to expedite natural products research. Traditional medicine is a major indicator of activity and the isolation of active compounds in traditional medicines for which the efficacy has been proven, might result in interesting products, although it cannot be excluded that the activity could be due to the combination effects of certain compounds. There is also a degree of predictability in the distribution of natural products in nature. Chemotaxonomy offers certain advantages in that it may be used to search for new and richer sources of known compounds or related structures. It can also be used as 'negative indicators', for example, if a cytotoxic compound with no value as lead has been found in several related species, then other related species may be omitted from the screening. Understanding of the important role of secondary metabolites to plants especially in terms of resistance to pests and diseases, if not completely understood, have improved over the years and tapping a plant natural defense could also be a fruitful approach especially in the search for natural antibiotics.

In the search for bioactive compounds from plants, the choice and availability of plants for evaluation are important and criteria such as traditional uses or ethno pharmacology, chemotaxonomy and plant ecological observations may be used either alone or complementary to each other. Nevertheless preliminary screening of a random collection of plants can also yield fruitful results. In this approach, the usual strategy involves an initial step of putting the plant extracts through a selected biological assay or panel of assays. The bioassay will be decided upon various factors such as therapeutic interest, simplicity, costs and speed. Extracts satisfying a defined

criterion of bioactivity will then be selected for further work. The plants may undergo bioassay-guided isolation of active constituents or may go through a straightforward phytochemical work up to isolate as many compounds as possible. This is then followed by the usual structural elucidation by spectroscopic methods and biological testing of the isolated compounds.

1.3 Aims of the present study

The present study focuses on two plants of the Euphorbiaceae, *Euphorbia hirta* and *Phyllanthus watsonii*. *Euphorbia hirta* is a medicinal plant used for treating asthma, chronic bronchial disorders, acute nasal catarrh, hay fever and emphysema (De Padua *et al*., 1999). There was no record of medicinal uses for *Phyllanthus watsonii* in the literature but the plant is still of interest since it belongs to a genera of plants possessing many medicinal properties. The interest in the two plants arose from the results of a preliminary biological screening exercise on 31 species of medicinal plants and several plants collected at random from a field excursion in the Endau Rompin Forest Reserve. The plants were found to be active in the 5-lipoxygenase inhibition assay used in the preliminary bioactivity screening exercise, indicating that the plants could be useful sources of anti-inflammatory compounds. Thus, the plants were selected for further work with the following specific objectives:

1. To isolate and elucidate the structures of phytochemical constituents from *Euphorbia hirta* (linneus) and *Phyllanthus watsonii* (Airy Shaw)
2. To evaluate the anti-inflammatory and other possible biological activities of the isolated compounds

CHAPTER 2

LITERATURE REVIEW

2.1 The plant family Euphorbiaceae

The Euphorbiaceae is mostly monoecious herbs, shrubs, and trees, sometimes succulent and cactus-like. It comprises about 300 genera and 5,000 species, distributed in the temperate, sub-tropical and tropical regions of the world. The Euphorbiaceae is characterized by the frequent occurrence of milky sap and in many members of the family, including the genus *Euphorbia*, the milky latex or sap is poisonous and skin contact may cause inflammation and a blistering rash.

In Malaysia, common members of the Euphorbiaceae family include *Acalypha*, *Phyllanthus, Euphorbia, Aleurites, Glochidion* and *Breynia*. Phytochemical constituents of this family include terpenoids, alkaloids (most common), anthraquinones (*Clutia spp.*), polyacetates, proanthocyanidins (rare), cyanidin and delphinidin (in one *Phyllanthus* species), flavonols, ellagic acids and saponins/sapogenins (rare) (Watson and Dallwitz, 1985).

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2.1.1 The genus *Euphorbia* (Linneus)

One of the largest genus within the Euphorbiaceae is *Euphorbia* with approximately 2,000 species. They all have latex and a unique flower structure. In Malaysia, the most

common species include *E. antiquorum, E. atoto, E. heterophylla, E. hirta, E. hypericifolia, E. synadenium, E. thymifolia* and *E. tirucali.* (Turner, 1995).

2.1.1.1 *Euphorbia hirta* (Linneus)

Euphorbia hirta (**Figure 2.1**) or *E. pilulifera* is commonly known by many vernacular names such as cats hair, asthma weed, basri dudhi, chara, malnommee, pill bearing spurge, patikan kerbau, patikan kebo, fei yang cao, gelang susu and ara tanah. This hairy plant grows up to 30 cm tall and has numerous small flowers clustered together with opposite oblong leaves (Turner, 1995). The young yellow fruit is a small hairy capsule with three reddish-brown seeds. The plant flowers and fruits all year long.

Figure 2.1. *Euphorbia hirta* (Linneus)

2.1.2 The genus *Phyllanthus* (Linneus)

Phyllanthus comprises over 600 species of shrubs, trees, and annual or biennial herbs. It is well distributed throughout the tropical and subtropical regions of both hemispheres. Most of the plants in this genus are usually in the form of small, erect, annual herb that grows up to 30 to 40 cm in height. It is indigenous to the rainforests

of the Amazon and other tropical areas throughout the world, including the Bahamas, Southern India, and China. There are 20 species of *Phyllanthus* commonly found throughout Malaysia. These are *Phyllanthus albidiscus, P. amarus, P. chamaepeuce, P. columnaris, P. debilis, P. elegans, P. emblica, P. filicifolius, P. gomphocarpus, P. gracilipes, P. oxyphyllus, P. pachyphyllus, P. pulcher, P. reticulates, P. ridleyanus, P. roseus, P. sikkimensis, P. urinaria, P. virgatus and P. watsonii* (Turner, 1995).

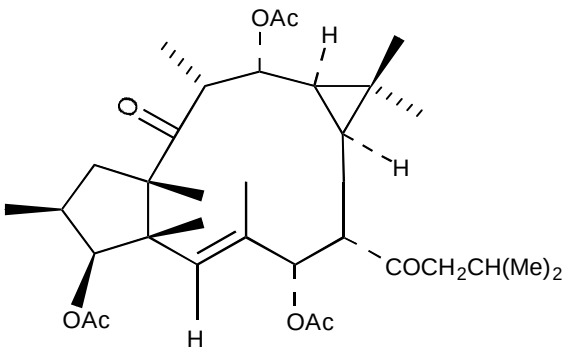
2.1.2.1 ***Phyllanthus watsonii*** (**Airy Shaw**)

Phyllanthus watsonii (**Figure 2.2**) is a small shrub, growing to about 15 cm height, and usually found near fast-flowing rivers. The flowers and fruits for this species usually have thread-like stalks, at least 5 mm long. The flowers are in clusters, on slender racemes to 15 cm borne in groups on main branches behind the leafy twigs (Turner, 1995). The species is found to be endemic to the Endau Rompin area.

Figure 2.2. *Phyllanthus watsonii* (Airy Shaw)

2.2 Chemical constituents and biological properties of the genus *Euphorbia*

The plants of this genus *Euphorbia* contain many kinds of secondary metabolites which may be grouped into three main classes of compounds, namely terpenes (diterpenes, triterpenes and sesquiterpenes), phenolic derivatives (flavonoids, coumarins, acetophenones, lignans) and some alkaloids. More than 120 species of the genus have been studied for their chemical constituents, many of which have yielded several new classes of diterpenoid and triterpenoid skeleton. Some of the unusual diterpenoids are exemplified by 3,7,12-tri-*O*-acetyl-8-isovalerylingol **[1]** isolated from *E. tirucalli* (Abdul Qasim and Abdul Malik, 1990), enukokurin **[2]** isolated from *E. lateriflora* (Connolly *et al*., 1989), euphornin **[3]** isolated from *E. maddeni* (Sahai *et al*., 1980), kansuiphorin A **[4]** and B **[5]** isolated from *E. kansui* (Wu *et al*., 1991), 3,12-diacetyl-8-benzoylingol **[6]** and 3,12-*O*-diacetyl-7-O-angeloyl-8-methoxyingol **[7]**, isolated from *E. nivulia* (Ravikanth *et al*., 2001), caudicifolin **[8],** isolated from *E. caudicifolia* (Saboor *et al*., 1977), and ent-2-hydroxy-1,16(17)-dien-3,14-dione **[9],** isolated from *E. characias* (Appendino *et al*., 1999)



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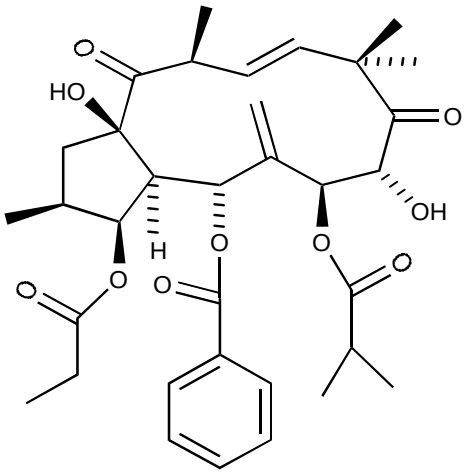

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