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PHYTOCHEMICAL STUDY OF *Phyllanthus amarus* AND ITS ANTICANCER ACTIVITY

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ABSTRACT

This study aimed to isolate and purify compounds from *Phyllanthus amarus* and their potential as anticancer agents. About 2.01 kg of *P. amarus* from Euphorbiaceae family was collected from Melor, Kelantan. The sample was dried at room temperature, grinded and extracted with hexane, ethyl acetate, ethanol and methanol. Compounds were purified using various chromatographic techniques. The structures of the compound were determined by spectroscopic techniques including nuclear magnetic resonance, ultraviolet, infrared, mass spectrometry and comparison with previous study. In addition, compounds were tested by their anticancer effect against HeLa cells and NIH/3T3 cells by MTT assay. The results showed that isolation of hexane and ethanol extract have produced seven compounds which were tanacetene, triglyceride fatty acids, mixtures of stigmasterol and β-sitosterol, hypophyllanthin, niranthin, lintetralin, and nirtetralin. The MTT assay results showed that hypophyllanthin has a strong anticancer effect on HeLa cells compared to niranthin and lintetralin. In conclusion, *P. amarus* consisted of sesquiterpene, glyceride, sterols and lignans. Meanwhile, hypophyllanthin showed a potential as anticancer agent. In implication, the compounds can be used as future reference for secondary metabolites of Malaysian plants and their potential as anticancer agents to be applied in pharmaceutical field.

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KAJIAN FITOKIMIA TERHADAP *Phyllanthus amarus* DAN AKTIVITI ANTIKANSER

ABSTRAK

Kajian ini bertujuan untuk memisahkan dan menulenkan sebatian daripada *Phyllanthus amarus* dan potensinya sebagai agen antikanser. Sebanyak 2.01 kg *P. amarus* daripada keluarga Euphorbiaceae telah dikumpulkan dari Melor, Kelantan. Sampel dikeringkan pada suhu bilik, dikisar dan diekstrak dengan heksana, etil asetat, etanol dan metanol. Sebatian ditulenkan menggunakan pelbagai teknik kromatografi. Struktur sebatian telah ditentukan dengan teknik spektroskopi termasuk resonan nuklear magnetik, ultra lembayung, infra merah, spektrometri jisim dan perbandingan dengan kajian lepas. Sebagai tambahan, sebatian telah diuji dengan kesan antikanser terhadap sel HeLa dan sel NIH/3T3 dengan asai MTT. Hasil kajian menunjukkan penyaringan ekstrak daripada heksana dan etanol telah menghasilkan tujuh sebatian iaitu tanacetena, asid lemak trigliserida, campuran stigmasterol dan β-sitosterol, hipofillantin, nirantin, lintetralin dan nirtetralin. Hasil keputusan dari asai MTT menunjukkan hipofillantin mempunyai kesan antikanser yang kuat ke atas sel HeLa dibandingkan dengan nirantin dan lintetralin. Kesimpulannya, *P. amarus* mengandungi seskuiterpena, gliserida, sterol dan lignan. Sementara itu, hipofillantin menunjukkan potensi sebagai agen antikanser. Implikasinya, sebatian ini boleh digunakan sebagai rujukan masa hadapan untuk metabolit sekunder daripada tumbuhan Malaysia dan potensinya sebagai agen antikanser untuk kegunaan dalam bidang farmaseutikal.

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LIST OF ABBREVIATIONS

^{o}C	Degree celcius
amu	Atomic Mass Unit
cm	Centimeter
cm^{-1}	Per centimeter
mL	Millilitre
mm	Millimetre
ng	Nanogram
nm	Nanometer
d	Doublet
dd	Doublet of doublet
J	Coupling constant (Hz)
m	Multiplet
m/z	Mass per charge
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon Nuclear Magnetic Resonance
1-D NMR	One Dimension Nuclear Magnetic Resonance
2-D NMR	Two Dimension Nuclear Magnetic Resonance
CC	Column Chromatography
$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
CH_3	Methyl group
CH_2Cl_2	Dichloromethane
CO_2	Carbon Dioxide
COSY	^{1}H-^{1}H Correlation Spectroscopy
D	Deuterium Isotope
DEPT	Distortioness Enhancement by Polarization Transfer

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DMSO	Dimethyl sulfoxide
FTIR	Fourier Transform Infrared Spectroscopy
FBS	Fetal Bovine Serum
HeLa	Human Cervical Cancer Cell
H_2SO_4	Sulphuric acid
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
Hz	Hertz
IC_{50}	Inhibition Concentration
IR	Infrared
Kg	Kilogram
LC-MS	Liquid Chromatography-Mass Spectrometry
MeOH	Methanol
MHz	Mega Hertz
MS	Mass Spectrum
NIH/3T3	Normal Mouse Fibroblast Cell
NMR	Nuclear Magnetic Resonance
OCH_3	Methoxyl group
OH	Hydroxy group
PTLC	Preparative Thin Layer Chromatography
Recycling-HPLC	Recycling High Performance Liquid Chromatography
TLC	Thin Layer Chromatography
UV	Ultraviolet

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Malaysia is known as one of the 12 mega diverse countries in the world with a large number of endemic animals, fungi and plants. About 12,500 species of flowering plants and more than 1,100 species of ferns and fern allies are estimated to contain in Malaysia (Ministry of Science, Technology and Environment, 1998a). In Peninsular Malaysia, there are over 26% of endemic tree species. The endemic species are distributed in localized places such as few valleys or mountain tops (Ministry of Science, Technology and Environment, 1998b). About 200 species of medicinal plants from 2,000 species in Peninsular Malaysia have being used by different ethnic groups in all around the country (Siti Fatimah Sabran, Maryati Mohamed, & Mohd Fadzelly Abu Bakar, 2016).

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Remedies from medicinal plants have been traditionally used in many years for human diseases because of its therapeutic benefits. Traditional medicine was using worldwide because of its diversity, flexibility, easy accessibility, broad continuing acceptance in developing countries and increasing popularity in developed countries, relatively low cost, low levels of technological input, relatively low side effect and growing economic importance (Ojo, Esumeh, Osanyinlusi, & Jeje, 2017).

There are common substances that are produced by all living organisms which are often called natural products (Anulika, Ignatius, Raymond, Osasere, & Abiola, 2016). Some natural products have been isolated by humans for treating illnesses, ailments, and infections. The richness of biodiversity, especially as a source of new drugs and carry out systematic collection of samples for chemical analysis and testing were the characteristics seeing by pharmaceutical companies and research institutes for using the natural product as treatment for curing illnesses (Firn, 2010a).

In pharmaceutical industry, some natural products are often used as antibiotics and anticancer drugs. The uses of penicillin **1** as antibiotics make strong belief to scientists that microbes can defend human against other microbes. The era of anticancer drugs also comes from natural products. One of the valuable treatments of several forms of leukemia is the alkaloid in *Catharanthus roseus* called vinblastine **2**. Vinblastine **2** is now still one of the most useful chemotherapeutic agents that used in cancer chemotherapy (Firn, 2010b).

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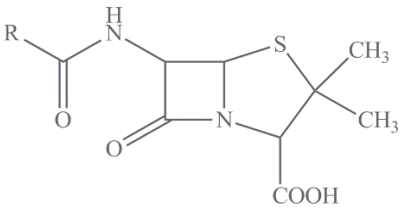

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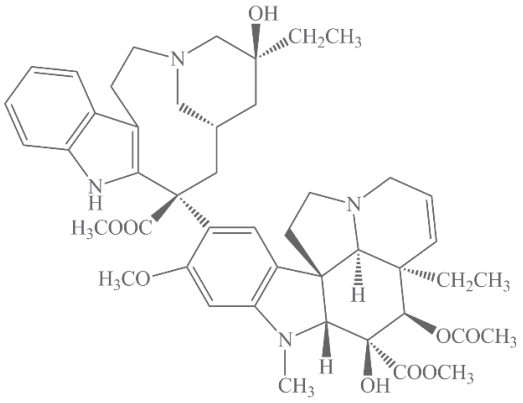

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1.2 Objectives of Study

The research objectives are focusing on:

(i) To extract, isolate and purify chemical constituents from *Phyllanthus amarus*.

(ii) To elucidate and identify the chemical structure of the isolated compounds by using various spectroscopic techniques.

(iii) To determine the anticancer activity of the isolated compounds.

1.3 Significance of Study

A major leading of death in worldwide was caused by cancer. The number of cancer survival was expanding as well as the number of new cancer cases. About 75% to 95% of the populations from the developing countries used traditional medicines from the plant extract products or its active chemical constituents for treatment in human diseases including cancer (Satya et al., 2016).

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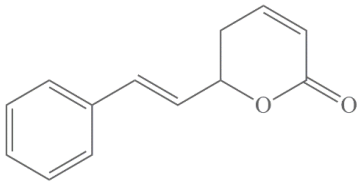
Medicinal plants from Malaysia were rich in many biological activities such as antioxidant, cytotoxic, antiviral, anti-diabetic and anti-inflammatory activities (Mohd Raznan Ramli, Milow, & Chooi, 2015). Several natural product compounds from the medicinal plants such as goniothalamin **3** were successfully isolated from *Goniothalamus* species. This compound was cytotoxic toward leukemic cells and also induced apoptotic cell death (Tajudin et al., 2012).

The small herbs, *Phyllanthus amarus from* Euphorbiaceae family was used as anticancer medicines due to its toxic chemical compounds such as diterpenoids, flavonoids, lignans and alkaloids. This small herb usually found surrounding area home. In Malaysia, old peoples have been used this herb as the folk medicines to treat diseases such as disturbances of kidneys and urinary bladder, intestinal infections and diabetes (Ramasamy, Norhanom Abdul Wahab, Nurhayati Zainal Abidin, & Manickam, 2011).

Intense research was needed to obtain new anticancer agent as there is no effective treatment for cancer. Therefore, this present study was to extract, isolate and purify the chemical constituents from *P. amarus*. Then, this study will determine whether the pure isolated compounds show any cytotoxic effect on anticancer activity.



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1.4 Family of Euphorbiaceae

The Euphorbiaceae is a large family of flowering plants which comprising 300 genera and around 7 700 species (Satya et al., 2016). Most of the families are herbs, shrubs or trees and some of them are succulent and resemble cacti. They were mainly occurred in tropics and majority of the species are distributed in the Indo-Malayan region and tropical America. The leaves of this family species are alternate with stipules. The flowers are radially symmetrical flowers and unisexual with the male and the female flowers usually occurring on the same plant. They can be monoecious or dioecious (Mahbubur Rahman & Akter, 2013).

Previous phytochemical study revealed that this family contain glycoproteins, lectins, tannins, gcyanogenic glycosides, alkaloids, esters, terpenes, saponins and flavonoids (de Oliveira et al., 2013).

1.5 The Genus of *Phyllanthus*

One of the largest genus in kingdom Plantae is *Phyllanthus* genus (Krithika, Verma, Shrivastav & Suguna, 2011). This genus have more than 600 species which are widely distributed throughout the subtropical and tropical regions (Abhyankar, Rao & Reddy, 2013). There are many researches showed that some species from the genus *Phyllanthus* are widely used in many countries as traditional remedies for the treatment of many illness such as flu, dropsy, diabetes, jaundice and bladder calculus

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(Kassuya, Silvestre, Rehder & Calixto, 2003; Shakil, Pankaj, Kumar, Pandey & Saxena, 2008).

1.5.1 General Characters of Genus *Phyllanthus*

The *Phyllanthus* genus is shrubs or herbs with small, alternate, distichous, stipules narrow leaves and the branchlets resembling pinnate leaves. The capsule of the fruit is crustaceous or thin with 2 valved cocci while the seed is trigonous and rounded at the back. The plants usually have very small flowers that are monoecious, axillary clusters or solitary. The male flowers are in disk of small glands while female flowers are in glands or annular. The male flowers can be varied from 1 to 10 or more while the female flowers are hypogynous which means with big ovaries (Mahbubur Rahman & Akter, 2013).

1.6 *Phyllanthus amarus* Species

Kingdom : Plantae

Division : Angiospermae

Class : Dicotyledoneae

Order : Tubiflorae

Family : Euphorbiaceae

Genus : *Phyllanthus*

Species : *amarus* Schum. & Thonn.

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Phyllanthus amarus is a perennial herb which spreading widely in almost all tropical countries and regions of the world from Africa to Asia including Malaysia, India, America and Nigeria (Fernand, 2003; Maciel, Cunha, & Dantas, 2007; Ajala et al., 2011). In Ayurveda, *Phyllanthus amarus* has been described by Sanskrit name called Bhoomyaamalakee, Taamalakee and Bhoodhatree. Besides, *P. amarus* is also known as Bahupatra and Bhuiamla in Central and Southern India respectively which grows mostly on uncultivated land (Adeneye, 2012). This *P. amarus* also commonly known as 'stonebreaker', 'windbreaker', 'gulf leaf flower' or 'gala of wind'(Ajala et al., 2011).

Phyllanthus amarus are erect annual herbs with characteristics of 10-60 cm tall and have simple or branched stems with scabridulous or smooth in younger parts (Patel, Tripathi, Sharma, Chauhan & Dixit, 2011). The stem has spirally arranged cataphylls each with a deltoid stipule. It has deciduous branches with alternate leaves and each branch has 12 to 32 leaves. The leaves are elliptic, oblong to ovate with five sepals of axillary flowers and apical acute anther (Adeneye & Benebo, 2008; Dhongade & Chandewar, 2013; Khatoon, Rai, Rawat & Mehrotra, 2006). This herb is most commonly used in the medicine of Indian Ayurvedic system for the problems of stomach, genitourinary system, liver, kidney and spleen (Patel et al., 2011).

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Figure 1.1. Branches with Leaves and Fruits of *Phyllanthus amarus*

Figure 1.2. Fruits and Flowers of *Phyllanthus amarus*

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