ISOLATION OF INDOLE ALKALOIDS FROM THE ROOTS OF *Kopsia singapurensis* RIDL. (APOCYNACEAE) AND ITS CYTOTOXIC ACTIVITY

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ABSTRACT

The objective of this study is to extract, isolate, and elucidate the indole alkaloids from the roots of *Kopsia singapurensis* Ridl. collected from Kluang, Johor. The extraction process of the plant was carried out using hexane followed by dichloromethane (DCM) to yield hexane and DCM crude extracts. The isolation and purification process were performed by various chromatographic techniques namely column chromatography (CC), thin layer chromatography (TLC) and preparative thin layer chromatography (PTLC). The structures of isolated compounds were elucidated by using spectroscopic methods such as 1D-NMR (^{1}H, ^{13}C, and DEPT 135^{o}) and 2D-NMR (COSY, HMQC, and HMBC), ultraviolet (UV), infrared (IR) and mass spectrometry (MS), and also by comparison with previous works. This study has successfully isolated eight indole alkaloids from the roots of *K. singapurensis* Ridl., tetrahydroalstonine, melodinine E, kopsifine, rhazinicine, kopsamine *N*(4)-oxide, aspidodasycarpine, kopsamine, and akuammidine. These isolated indole alkaloids were then screened for cytotoxic activity against human cervical cancer (HeLa) cell, human promyelocytic leukemia (HL-60) cell, and the normal mouse fibroblast (NIH/3T3) cell lines by using MTT assay. The kopsifine showed very strong activity, while kopsamine exhibited a moderate cytotoxic activity against HL-60 cells with Cytotoxic Dose (CD_{50}) values of 0.9 μg/mL and 6.9 μg/mL, respectively. Akuammidine and rhazinicine exhibited significant cytotoxicity effects against HeLa cells with the CD_{50} values 2.8 μg/mL and 2.9 μg/mL, respectively. However, aspidodasycarpine showed a moderate cytotoxicity effects against HeLa cells with the CD_{50} value of 7.5 μg/mL. Rhazinicine, aspidodasycarpine, and kopsifine showed high cytotoxic effects against all tested cancer cells and normal cell with CD_{50} values of 20.8 μg/mL, 6.4 μg/mL, and 20.7 μg/mL, respectively. As a conclusion, these three alkaloids cannot be used as a cancer treatment drugs. The implication of the study showed that there are two indole alkaloids namely kopsamine and akuammidine which can be used as drugs to treat cancer.

Pemencilan Alkaloid Indola dari Akar *Kopsia singapurensis* Ridl. (Apocynaceae) dan Aktiviti Sitotoksik

ABSTRAK

Kajian ini bertujuan mengekstrak, memencil dan mengenalpasti alkaloid indola daripada akar *Kopsia singapurensis* Ridl. yang diperolehi dari Kluang, Johor. Proses pengekstrakan tumbuhan telah dijalankan dengan menggunakan heksana diikuti dengan diklorometana (DCM) untuk menghasilkan ekstrak mentah heksana dan DCM. Proses pemencilan dan penulenan dijalankan dengan pelbagai teknik kromatografi, iaitu kromatografi turus (CC), kromatografi lapisan nipis (TLC) dan kromatografi lapisan nipis preparatif (PTLC). Struktur sebatian yang dipencil dikenalpasti dengan menggunakan kaedah spektroskopi, iaitu 1D-NMR (^{1}H, ^{13}C, dan DEPT 135^{o}) dan 2D-NMR (COSY, HMQC dan HMBC), ultralembayung (UV), inframerah (IR) dan spektrometri jisim (MS), dan juga perbandingan dengan data daripada kajian lepas. Kajian mendapati lapan alkaloid indola telah berjaya dipencilkan daripada akar *K. singapurensis* Ridl. iaitu tetrahidroalstonina, melodinina E, kopsifina, rhazinicina, kopsamina *N*(4)-oksida, aspidodasycarpina, kopsamina, dan akuammidina. Alkaloid indola ini kemudian disaring untuk aktiviti sitotoksik terhadap sel kanser pangkal rahim manusia (HeLa), sel *promyelocytic leukemia* (HL-60), dan sel normal *fibroblast* tikus (NIH/3T3) dengan menggunakan asai MTT. Kopsifina menunjukkan kesan aktiviti sangat kuat, manakala kopsamina memiliki kesan sederhana terhadap aktiviti sitotoksik sel HL-60 dengan nilai *Cytotoxic Dose* (CD_{50}) masing-masing adalah 0.9 µg/mL dan 6.9 µg/mL. Akuammidina dan rhazinicina menunjukkan kesan sitotoksik yang signifikan terhadap sel HeLa dengan nilai CD_{50} masing-masing 2.8 µg/mL dan 2.9 µg/mL. Walau bagaimanapun, aspidodasycarpina menunjukkan kesan sitotoksik sederhana terhadap sel HeLa dengan nilai CD_{50} 7.5 µg/mL. Rhazinicina, aspidodasycarpina, dan kopsifina menunjukkan kesan sitotoksik yang tinggi terhadap semua sel kanser dan sel normal yang diuji dengan nilai CD_{50} masing-masing 20.8 µg/mL, 6.4 µg/mL, dan 20.7 µg/mL. Kesimpulannya, ketiga-tiga alkaloid tersebut tidak boleh digunakan sebagai ubatan rawatan kanser. Implikasi daripada kajian ini menunjukan bahwa terdapat 2 alkaloid indola iaitu kopsamina dan akuammidina dapat digunakan sebagai ubatan bagi merawat kanser.

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ABBREVIATIONS

%	Percent
µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometer
^{13}C	Carbon NMR
1D-NMR	One Dimension Nuclear Magnetic Resonance
^{1}H	Proton NMR
2D-NMR	Two Dimension Nuclear Magnetic Resonance
ATCC	American Type Cell Collection
brd	Broad
C=O	Ketone functional group
CC	Column Chromatography
CD_3OD	Tetradeuteromethanol
CD_{50}	Cytotoxicity Dose of causing 50% cell death
$CDCl_3$	Deuterochloroform
CH_2Cl_2	Dichloromethane
CH_3	Methyl group
$CHCl_3$	Chloroform
cm s^{-1}	Centimeter per Second
cm^{-1}	Per Centimeter
CO_2	Carbon dioxide

COSY	$^{1}H - ^{1}H$ Correlation Spectroscopy
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
dddd	Doublet of doublet of doublet of doublets
DCM	Dichloromethane
DEPT 135^{o}	Distortioness Enhancement by Polarization Transfer at 135^{o}
DMSO	Dimethylsulphoxide
EC_{50}	Effective Concentration of causing 50% cell death
EtOAc	Ethyl acetate
g	Gram
GC-MS	Gas Chromatography-Mass Spectroscopy
H_2O	Water
HCl	Hydrochloric Acid
HeLa	Human cervical cancer cell
HL-60	Human promyelocytic leukemia cell
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HPLC	High Performance Liquid Chromatography
HRESIMS	High Resolution Electrospray Ionisation Mass Spectrometry
Hz	Hertz
IR	Infra Red
IC_{50}	Inhibitory Concentration of of causing 50% cell inhibited
J	Coupling Constant

KB/VJ300	Vincristine-resistant of human oral epidermoid carcinoma cell lines
Kg	Kilogram
M	Molar
m	Meter
m	Multiplet
m/z	Mass per Charge
MeOH	Methanol
MHz	Mega Hertz
min	Minute
mL	Mililitre
mM	Milimolar
mm	Milimeter
m. p.	Melting Point
Na_2CO_3	Sodium carbonate
NC=O	Amide functional group
NH_3	Ammonia
NIH/3T3	Normal mouse fibroblast cell
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
^oC	Degree Celcius
OCH_2O	Methylenedioxy
OCH_3	Methoxyl functional group

ODS	Octa Decyl Silane
OH	Hydroxyl functional group
OR	Optical Rotation ($[\alpha]_D^{25}$, with tungsten lamp at 25°C and wavelength 589 nm (sodium light, D)
PBS	Phosphate buffered saline
pH	Power of Hydrogen
ppm	Part per Million
PTLC	Preprative Thin Layer Chromatography
rf	Radio frequency
R_f	Retention factor
s	Singlet
t	Triplet
td	Triplet of doublets
TLC	Thin Layer Chromatography
UV	Ultra Violet
α	Alpha
β	Beta
δ	Chemical Shift
λ	Maximum Wave Length

CHAPTER I

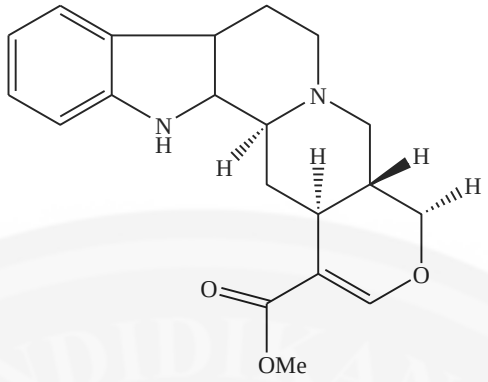
INTRODUCTION

1.1 General

Plant is one of the living organisms that are in the universe. Plants have a long history of providing an innumerable number of molecules with potential for the treatment of many serious diseases, known as medicinal plants which grow naturally around us (Schmidt et al., 2012). The term of medicinal plants include various types of plants used in herbalism and some of these plants have medicinal activities which commonly used as raw materials for extraction of active substances for synthesis of drugs (Rasool Hassan, 2012). For thousands of years natural products have played a very important role in health care and prevention of diseases. Over centuries, cultures around the world have learned how to use plants to fight illness and maintain health (Roberson, 2004). The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural sources for curing various

diseases (Phillipson, 2001). The current review attempts to give an overview on the potential of such plant-derived natural products as antiprotozoal leads and/or drugs in the fight against neglected tropical diseases (Schmidt et al., 2012). Plants contain primary metabolites for growth and thrive, and secondary metabolites for survival and self-protection from environment disturbance. Secondary metabolites are also known as natural products (Dias, Urban, & Roessner, 2012), they are not only found in plants, but also found in animals and microorganisms. The isolation process of them yielded pure compounds such as terpenoids, alkaloids, steroids, etc. (Sarker, Latif, & Gray, 2006), those pure compounds have active chemical structures (Harvey, 2008).

Plants produce many different secondary metabolites which have biological activity on other organisms (Bandi & Lee, 2012; Hussain et al., 2012; Phillipson, 2001). The dominant roles of them are as anticancer (60%) and drugs for infectious diseases (75%) (McChesney, Venkataraman, & Henri, 2007) such as *Catharanthus roseus* G. Don. (Apocynaceae), which contain antihypertensive alkaloid ajmalicine **1** which was also found in *Peganum harmala* L. (Laine, Lood, & Koskinen, 2014) and anticancer alkaloids vincristine **2** and vinblastine **3** (Cragg & Newman, 2005; Cragg, Kingston, & Newman, 2012; Noble, 1990; Rai, Tandon, & Khatoon, 2014). Other anticancer alkaloids are elliptinium **4** (Apocynaceae), camptothecin **5** (Nyssaceae), and paclitaxel (taxol®) **6** (Taxaceae) (Bhanot, Sharma, & Noolvi, 2011; Cragg & Newman, 2005; Malik et al., 2011).



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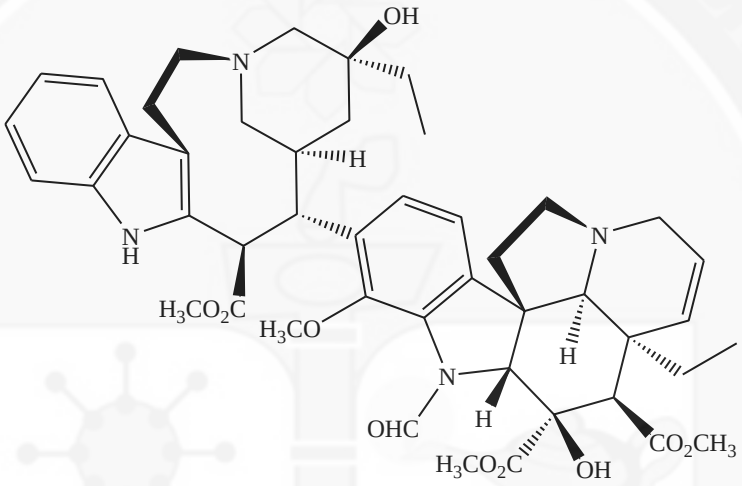

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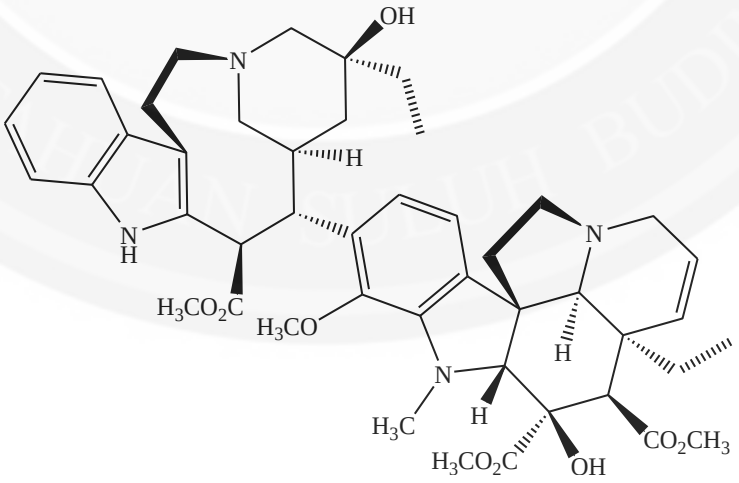

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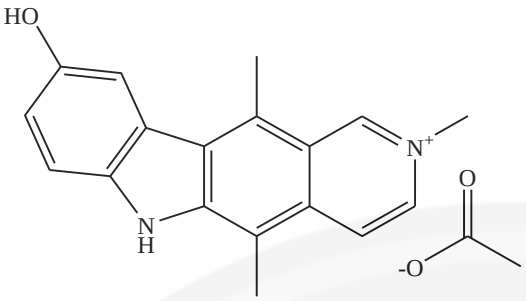

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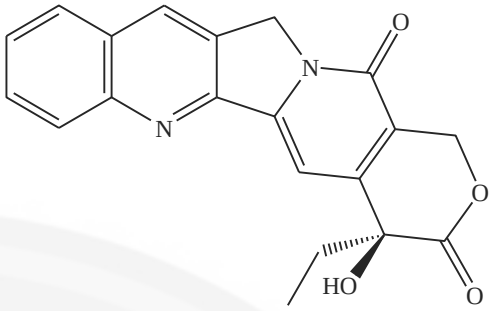

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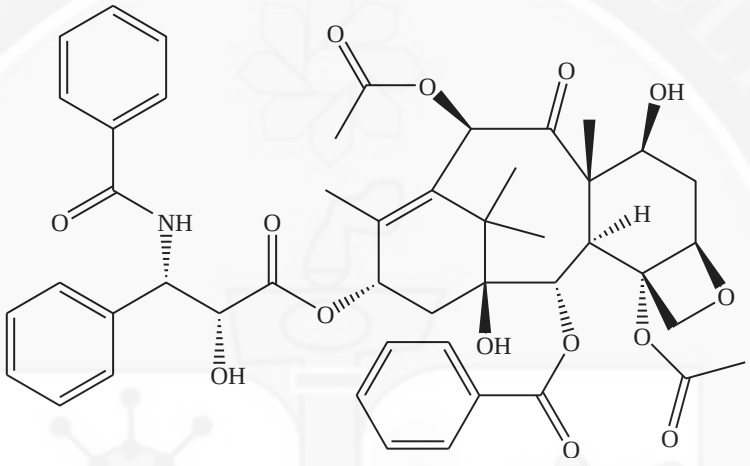

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Secondary metabolites are seen as a potential sources of new natural drugs, insecticides, antibiotics, and herbicides. Besides, they also can use as fibres, glues, waxes, perfumes, etc. (Crozier, Clifford, & Ashihara, 2006). Commonly, the plants which contain the bioactive compounds traditionally used for medicinal plants (Porto, Henriques, & Fett-Neto, 2009) such as the root of some plants of the genus *Kopsia* have the medicinal uses to treat poulticing ulcerated noses in tertiary syphlis, they are *K. fructicosa* (Reanmongkol et al., 2005), *K. Singapurensis* Ridl., *K. Pauciflora* Hook f., *K. Macrophylla* Hook f., and *K. Larutensis* King & Gamble, (Awang et al., 2008; Kam, Tan, & Chuah, 1992). Besides, the stem of *K. Macrophylla* Hook f. also used for treat fever and toxicemia (Reanmongkol et al., 2005). In China, *K. Officinalis*

Tsiang and Li used to treat dropsy, rheumatoid, tonsillitis, and arthritis (Awang et al., 2008; Kam, Yoganathan, & Wei, 1996; Wu, Kitajima, Kogure, Zhang, & Takayama, 2008), and *K. hainanensis* used as a treatment of rheumatoid arthritis, tonsilitis, dropsy, pharingytis, and tonsillitis (Chen, Chen, Yao, & Gao, 2011), cytotoxic and anti-multidrug resistant (Tan et al., 2011).

1.2 Problem Statement

Malaysia is one of the diversity flora countries of the world. There are 2,000 species from almost 15,000 flowering plants have been reported to contain medicinal efficacy and many have been scientifically proven (Rizwana et al., 2010). Cancer is a major public health burden in both developed and developing countries (Shoeb, 2006). The treatment of cancer includes surgery, radiation therapy and chemotherapy. Most of the available anticancer drugs are expensive, do not cure cancer and have serious adverse effects. Therefore, majority of cancer patients (up to 80%) look towards alternate and complementary medicine as a primary or adjuvant therapy. Phytotherapy is an alternate modality in the treatment of cancer. Since, in most cases plant-derived products are readily available, are relatively less expensive, less likely to cause dependency, and have low potential for serious side effects (Lamchouri et al., 2013).

The richness of Malaysian flora provides opportunities for the discovery of many novel compounds. Many reviews reported that plants are containing secondary metabolites which have biologically active compounds (Awang et al., 2008; Lamchouri et al., 2013; Lee et al., 2014). Most of them are from alkaloid group. The

major source of alkaloids is in the flowering plants such as Apocynaceae (Fattorusso et al., 2008). One of plants of family Apocynaceae is *Kopsia singapurensis* Ridl. Ahmad et al. (2013) have reported the isolated alkaloids from the roots of *K. singapurensis* Ridl., but less reports about their biological activities. Based on those informations, the investigation of isolated compounds from the roots of *K. singapurensis* Ridl. was carried out and performed the cytotoxic activity against the human cervical cancer (HeLa), the human promyelocytic leukemia (HL-60) and the normal mouse fibroblast (NIH/3T3) cell lines.

1.3 Objectives of Study

Based on the above literatures, the author was interested to start the study of indole alkaloids of the roots of *K. singapurensis* Ridl. (Apocynaceae). The objectives of the study are as follows:

1. Phytochemical analysis on chemical constituents of *K. singapurensis* Ridl.
    - To extract, isolate, and purify indole alkaloids by using various chromatography methodology, such as CC, TLC and preparative TLC.
    - To elucidate and identify the structure of the isolated compounds using various spectroscopic methods such as NMR, UV, IR, MS, and OR.
2. To evaluate in vitro cytotoxic activity on all isolated compounds against HeLa, HL-60, and NIH/3T3 cell lines via MTT assay.

1.4 Significance of Study

This study is significance to the research development that expands information and knowledge bases about the benefits of chemical compounds in the plants, especially in the roots of *Kopsia singapurensis* Ridl. Based on previous work reports, a large number of biologically active compounds in this plant have been reported (Subramaniam et al., 2007; Subramaniam et al., 2008, Awang et al., 2008; Lee et al., 2014), but the chemical compounds in its roots have not been widely reported. Ahmad et al. (2013) have reported the isolated indole alkaloids from the roots of *K. singapurensis* using Sephadex LH-20 column and ODS HPLC, a reverse phase method (the extracting and isolation from polar to non polar). While, this study was carried out on the roots of *K. singapurensis* Ridl. using normal phase method (the extraction and isolation from non polar to polar) to compared the content of indole alkaloids from the root part of this plant by the different methods. The results showed that difference of amount and kind of isolated compounds have yielded. Therefore, this work was performed to develop the research in this area and may lead to serve as part of the chemical characterization for future reference. In addition, in view of the growing need for effective bioactive agents, the study to discover natural products from plant source to serve as safe and effective bioactive agents has become significantly important.