IDENTIFICATION OF TRITERPENE FROM THE LEAVES AND BARK OF *Kopsia singapurensis* Ridl. (SELADA)

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ABSTRACT

This study was aimed to extract and identify chemical compounds isolated from the leaves and barks of *Kopsia singapurensis*. The isolated compounds were evaluated for cytotoxic (MCF-7), antibacterial (*Bacillus cereus)* and antioxidant activity (DPPH). The isolation process was carried out using chromatographic techniques such as column chromatography and thin layer chromatography. The structures of isolated compounds were elucidated through spectroscopic analysis including 1D-NMR (^{1}H, ^{13}C and DEPT), 2D-NMR (^{1}H-^{1}H COSY, HSQC/HMQC and HMBC), IR, UV, MS (GCMS); also by comparison with previous literature data. Isolation study on the leaves of *K. singapurensis* has yielded lupeol **147**, mixture of stigmasterol **148** and β-sitosterol **149**, lupeol ester **150**, and a mixture of lupeol **147**, α-amyrin **151** and β-amyrin **152**. Compounds β-amyrin **152,** β-amyrenone **153,** β-amyrin acetate **154** and lupeol acetate **155** were successfully isolated from the bark of *K. singapurensis*. These triterpenoids were isolated for the first time from this species. Compounds **147**, **150**, **152**, **153**, **154** and **155** were exhibited cytotoxic effects against MCF-7 in the range of IC_{50} 15.5 - 26.0 μg/mL. All isolated compounds showed weak antioxidant activity (IC_{50} > 500 μg/mL) and were not active against *Bacillus cereus. Kopsia singapurensis* was one of the threatened species however there is no publication on terpene isolated from this species. Hence, study on the chemical constituents of *K. singapurensis* has been embarked to discover new active compound for pharmaceutical industries.

PENGENALPASTIAN TRITERPENA DARIPADA DAUN DAN BATANG *Kopsia singapurensis* RIDL. (SELADA)

ABSTRAK

Kajian ini bertujuan untuk mengekstrak dan mengenal pasti sebatian kimia yang dipencil daripada daun dan batang *Kopsia singapurensis*. Sebatian kimia yang dipencil telah diuji untuk aktiviti sitotoksik (MCF-7), antibakteria (*Bacillus cereus)* dan antioksidan (DPPH). Proses pemencilan telah dijalankan dengan menggunakan teknik kromatografi seperti kromatografi turus dan kromatografi lapisan nipis. Struktur sebatian kimia yang dipencil telah dikenalpasti melalui analisis spektroskopi termasuk 1D-NMR (^{1}H, ^{13}C dan DEPT), 2D-NMR (^{1}H-^{1}H COSY, HSQC / HMQC dan HMBC), IR, UV, MS (GCMS); juga perbandingan dengan data daripada kajian lepas. Pemencilan ke atas daun *K. singapurensis* telah menghasilkan lupeol **147**, campuran stigmasterol **148** dan β-sitosterol **149**, lupeol ester **150** serta campuran lupeol **147**, α-amirin **151** and β-amirin **152**. Sebatian β-amirin **152,** β-amirenone **153,** β-amirin asetat **154** dan lupeol asetat **155** telah berjaya dipencil dari batang *K. singapurensis*. Semua triterpenoid ini merupakan pertama kali dipencil dari spesis ini. Sebatian **147**, **150**, **152**, **153**, **154** dan **155** menunjukkan kesan sitotoksik terhadap MCF-7 dalam julat IC_{50} 15.5 - 26.0 μg/mL. Semua sebatian yang dipencil menunjukkan aktiviti antioksidan yang lemah (IC_{50} > 500 μg/mL) dan tidak aktif terhadap *Bacillus cereus*. *Kopsia singapurensis* merupakan salah satu spesis terancam tetapi tiada penerbitan mengenai terpena yang dipencil daripada spesis ini. Oleh itu, kajian ke atas sebatian kimia daripada *K. singapurensis* telah dilakukan untuk meneroka sebatian aktif yang baharu bagi industri farmaseutikal.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE

05-4506832 pustaka.upsi.edu.my Perpustakaan Tuanku Bainun Kampus Sultan Abdul Jalil Shah PustakaTBainun ptbupsi

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
λ	Maximum wave length
δ	Chemical shift
g	Gram
kg	Kilogram
µg	microgram
M	Molar
mM	Milimolar
ml	Mililitre
m	Meter
nm	nanometer
MHz	Mega Hertz
Hz	Hertz
UV	Ultraviolet
IR	Infrared
ppm	Part per million
eV	Electron Volt
MeOH	Methanol
$CHCl_3$	Chloroform
CH_2Cl_2	Dichloromethane
DMSO	Dimethylsulphoxide
EA	Ethyl Acetate

pH	Power of Hydrogen
HCl	Hydrogen chloride
TLC	Thin Layer Chromatography
PTLC	Preparative Thin Layer Chromatography
CC	Column Chromatography
NMR	Nuclear Magnetic Resonance
cm^{-1}	Per centimeter
J	Coupling constant
d	Doublet
dd	Doublet of doublet
t	Triplet
dt	Doublet of triplet
s	Singlet
m	Multiplet
dbh	Diameter at Breast Height
℃	Degree Celsius
1D-NMR	One Dimension Nuclear Magnetic Resonance
2D-NMR	Two Dimension Nuclear Magnetic Resonance
^{1}H	Proton NMR
^{13}C	13-Carbon NMR
COSY	^{1}H-^{1}H Correlation Spectroscopy
DEPT	Distortioness Enhancement by Polarization Transfer
HMQC	Heteronuclear Multiple Quantum Correlation
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation

GC-MS	Gas Chromatography Mass Spectrometry
MS	Mass Spectrometry
m/z	Mass per charge
$CDCl_3$	Deuterated chloroform
DPPH	2, 2-diphenyl-1-picrylhydrazyl
MTT	Microculturetetrazolium
FPP	Farnesyldiphosphate
GGPP	Geranylgeranyldiphosphate
GPP	Geranyldiphosphate
GFPP	Geranylfarnesyldiphosphate

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CHAPTER 1

INTRODUCTION

1.1 Introduction

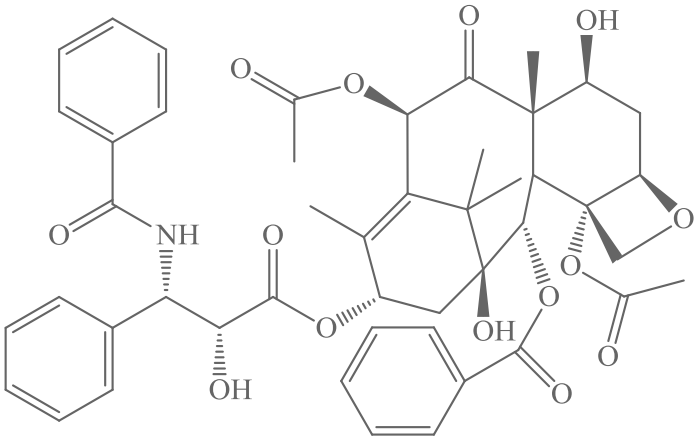
Recently, natural products have been the primary source of commercial medicines and drug leads (Rouhi, 2003). Natural product medicines have come from various sources of materials such as terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates (Newman et al., 2000). A recent survey revealed that 61% of the 877 drugs introduced worldwide can be traced to or were inspired by natural products. This highlights the significance of natural product chemistry. An analysis state that there are 52% of all new chemical entities (NCEs) suggests that natural products are important sources of new drugs and also good lead compound suitable for further modification during drug development (Newman et al., 2000; 2003). Since secondary metabolites from natural product sources have been detailed within living systems, it have been shown that drug-likeness and biological

friendliness is better than totally synthetic molecules, thus making them to have good prospects for further drug development (Balunas & Kinghorn, 2005; Drahl et al., 2005).

Current drug discovery from terrestrial plants has mainly relied on bioactivity-guided isolation methods such as paclitaxel **1** from *Taxus brevifolia* and camptothecin **2** from *Camptotheca acuminate* (Li et al., 2010; Kinghorn, 1994). Several well-known species, including licorice (*Glycyrrhiza glabra*), myrrh (*Commiphora species*), and poppy capsule latex (*Papaver somniferum*), were referred to the treatment of various diseases as ingredients of official drugs or herbal preparations used in systems of traditional medicine. Furthermore, morphine **3**, codeine **4**, noscapine (narcotine) **5**, and papaverine **6** isolated from *Papaver somniferum* were developed as single chemical drugs and are still clinically used (Newman et al., 2000).

Traditional medicines, including herbal medicines have been continuously used in every country around the world in some capacity. In the developing world, 70–95% of the population relied on these traditional medicines for primary care (Robinson & Zhang, 2011; World Health Organization (WHO), 2008; Bauer, 2003; Newman et al., 2000). People around the world use between 50,000 to 80,000 flowering plants for medicinal purposes (Marinelli, 2005). However, the Center for Biological Diversity reports that about 15,000 medicinal plants across the globe are at risk from habitat destruction, overharvesting, and big business (Hamilton, 2008). Based on current extinction rates, the planet may be losing at least one potential major drug every two years (Groombridge & Jenkins, 2002). Loss of medicinal plant means the potential loss of cures for cancer, AIDs and others diseases.

Malaysia's rainforest is considered as the oldest in the world and is rich in biological diversity. It harbors some 185,000 species of fauna and more than 15,000 species of flowering plants, where 2500 species are trees, 3000 species of orchids, 500 species of ferns, 60 species of grasses and bamboos. Malaysia is also richly endowed with aromatic plants. There are more than 600 species of aromatic plants in Malaysia, belonging to at least 21 different families. The largest groups are from the families Annonaceae, Dipterocarpaceae, Lauraceae, Meliaceae and Zingiberaceae (Shukor et al., 2007). In Malaysia, 2,000 species from 14,500 flowering plants have been reported to contain medicinal properties and many have been scientifically proven (Jaganath & Ng, 2000). Many plants from the forest which can produce a large variety of phytochemicals or secondary metabolites are widely used in traditional medicine in Malaysia such as alkaloids that isolated from Gambir (*Uncaria sp.*, Rubiaceae) showed cardio-vascular effects in rats (Shukor et al., 2007). The richness of Malaysian flora provides opportunities for the discovery of many novel compounds, some of which might possess useful bioactivities. Malaysia is wealthy in her plant diversity and potential antiviral drugs are still waiting to be explored. Therefore, a study on the chemical constituents of *Kopsia singapurensis* (Apocynaceae) was embarked.



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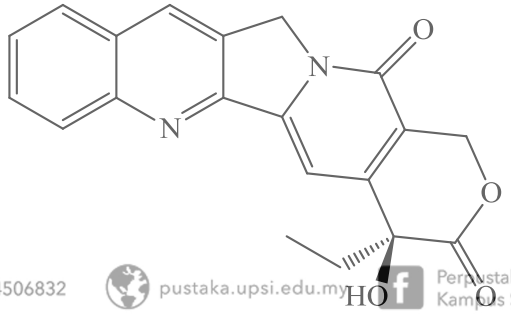

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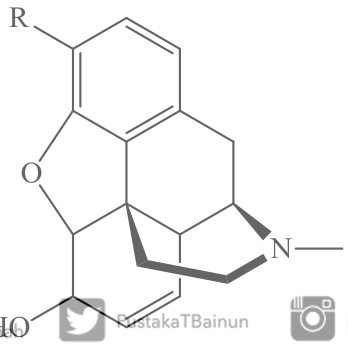
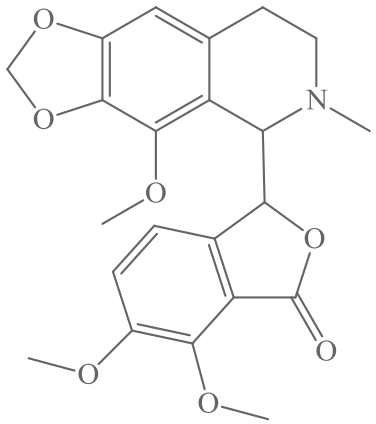


3: R= OH
4: R= OCH_3



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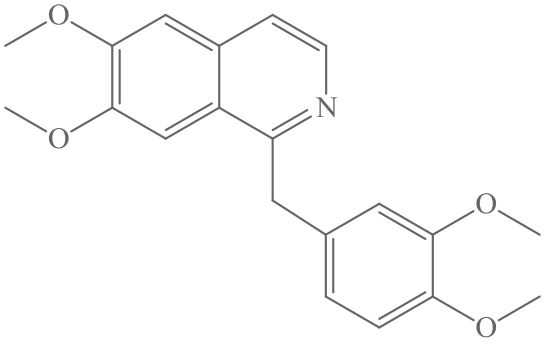


6

1.2 Objectives

The objectives of the study are as follows:

1. To extract, isolate and purify chemical constituents from the leaves and bark of *Kopsia singapurensis.*
2. To elucidate and identify the structures of the isolated compounds using modern spectroscopic methods such as NMR, UV, IR and MS.
3. To determine the bioactivities (cytotoxic, antibacterial and antioxidant) of the isolated compounds.

1.3 Significant of research

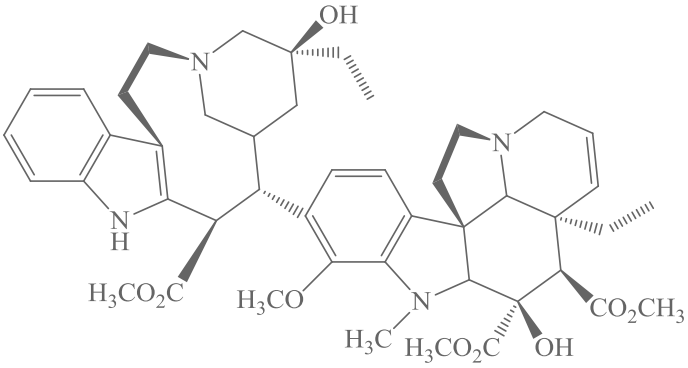
In this study, the researcher is going to isolate and purify chemical constituents from the leaves and bark of *Kopsia singapurensis*. According to Singapore Red Data Book (2008) and IUCN Red List of Threatened Species (Chua, 1998), *Kopsia singapurensis* was threatened by habitat loss. In Singapore, this tree can be found and was protected in the Nee Soon Swamp Forest of the Central Catchment's Nature Reserve. *K. singapurensis* was known as a medicinal plant, where the root of this plant has been used as traditional medicine to treat ulcerated noses in tertiary syphilis (Perry & Metzger, 1980). Thus, it is important to isolate and purify the chemical constituents of *Kopsia singapurensis* to discover potential new drugs before the extinction of this species.

1.4 Botanical aspect of Apocynaceae plants

Apocynaceae, or dogbane family is a large tropical and subtropical family with 424 genera, 1500 species, of trees, shrubs, herbs and climbers, including large woody lianas (Endress & Bruyns, 2000; Wee, 2006; Ng 1991). In Malaysia, nearly 120 species of this family were found and classified into 32 genera which are distributed over lowlands and mountains, which 10 of the species are trees (Corner, 1952; Ng, 1991). Species of Apocynaceae grow in various habitats, from tropical rain forests to semi arid regions. They occur from sea level to mountain tops level, mainly on dry soils, but also on rocks or in flooded areas, and sometimes river margins, such as *Matelea pedalis* (Smith et al., 2004).

Plants in this family usually have simple, opposite leaves, but they may be alternate (*Thevenia*) or whorled (*Alstonia, Rouwolfia*). Some are stem succulents with vestigial or no leaves. Flowers of this family are bisexual. Their calyx and corolla are usually five-lobed with distinct stamens and anthers facing inwards and often adherent to the surface of the stigma (Hodgkiss, 2011). Fruits typically occur in pairs, because they originate from paired ovaries in the flower, but sometimes one member of a pair is aborted. The fruits may be follicles, rarely fused (*Mandevilla spp.*), capsules (*Allamanda*), berries (*Couma*), or drupes (*Thevetia, Vallesia*) and they may contain one to many seeds, usually comose (*Nerium*), less often winged (*Allamanda, Plumeria, Aspidosperma*) or arillate (*Tabernaemontana*) (Ng, 1991; Smith et al., 2004).

This family is characterized by having copious amounts of white latex in most parts of the plant, usually whorled leaves and twinned seedpods (Corner, 1952). The latex from the sap of a few species has been used as a source of rubber. The latex of jelutong (*Dyera costulata*) can be used to make chewing gum while that of pulai (*Alstonia scholaris*) is used medicinally (Wee, 2006). Nearly all members of this family are poisonous, and many species are used medicinally due to the presence of cardiac glycosides and various alkaloids, especially in the seeds and latex. Some species are valuable sources of medicine, insecticides, fibers, and rubber (Tao, Leeuwenberg & Middleton, 1995). A good example is the Madagascar periwinkle (*Catherantus roseus*). This herb is the source of two important alkaloids, vinblastine **7** and vincristine **8**. The former is used against Hodgkin's disease while the latter to control acute lymphocytic leukemia in children (Wee, 2006). Dried root of *Rauwolfia serpentine* are used medicinally to treat high blood pressure, epilepsy, insanity and cardiac disease. The root and stem of *Hollarrhena antidysantrica* used in the treatment of dysentery. The bark of *Astonia scholaris* is used in treatment of diarrhea. The wood of *Wrightia tinctoria* and *A.scholaris* is soft and used for wood carvings. The fruits of *Carissa caranda L.* are sour and edible. This family also includes many of the most well-known tropical ornamental plants (*Oleander, Frangipani, Allamanda, Mandevilla*) (Bhattacharyya & Johri, 1998; Smith et al., 2004; Singh & Abbas, 2005).



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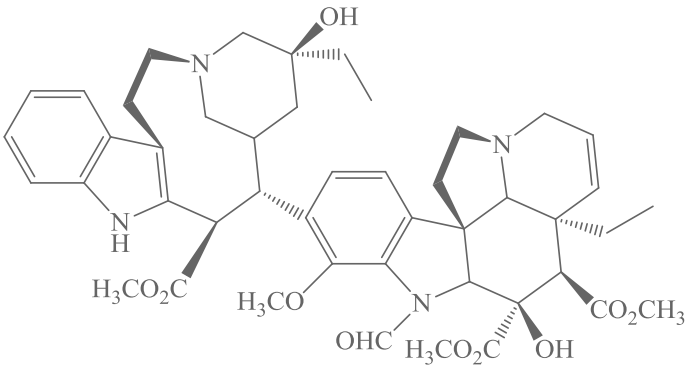

8

1.5 Classification of Apocynaceae plants

Apocynaceae is a natural taxon family and belongs to the order Gentianales (Bhattacharyya & Johri, 1998). This family was traditionally divided into two: Apocynaceae *sensu stricto* and Asclepiadaceae *sense lato*. Apocynaceae *sensu stricto* included two subfamilies: Rauvolfioideae (Plumerioideae) and Apocynoideae (Echitoideae), while Asclepiadaceae *sense lato* included another two subfamilies: Periplocoideae and Asclepiadoideae. However, cladistic studies based on morphological and molecular data have not supported maintaining Apocynaceae and Asclepiadaceae as separate families. Therefore, the Apocynaceae *sensu stricto* and Asclepiadaceae, and the Periplocoideae are broadly defined Apocynaceae (Smith et al., 2004). Recently, five subfamilies in Apocynaceae have been considered by some

authors: Rauvolfioideae (Plumerioideae), Apocynoideae, Periplocoideae, Secamonoideae and Asclepiadoideae (Sennblad & Bremer, 2002; Endress, 2004; Middleton, 2009). Subfamily then further divided into few tribes (Figure 1.1). Each tribe contains several genera. The genus *Kopsia* is placed in the tribe Vinceae, subfamily Rauvolfioideae (Endress & Bruyns, 2000; Sennblad & Bremer, 2002; Middleton, 2009).

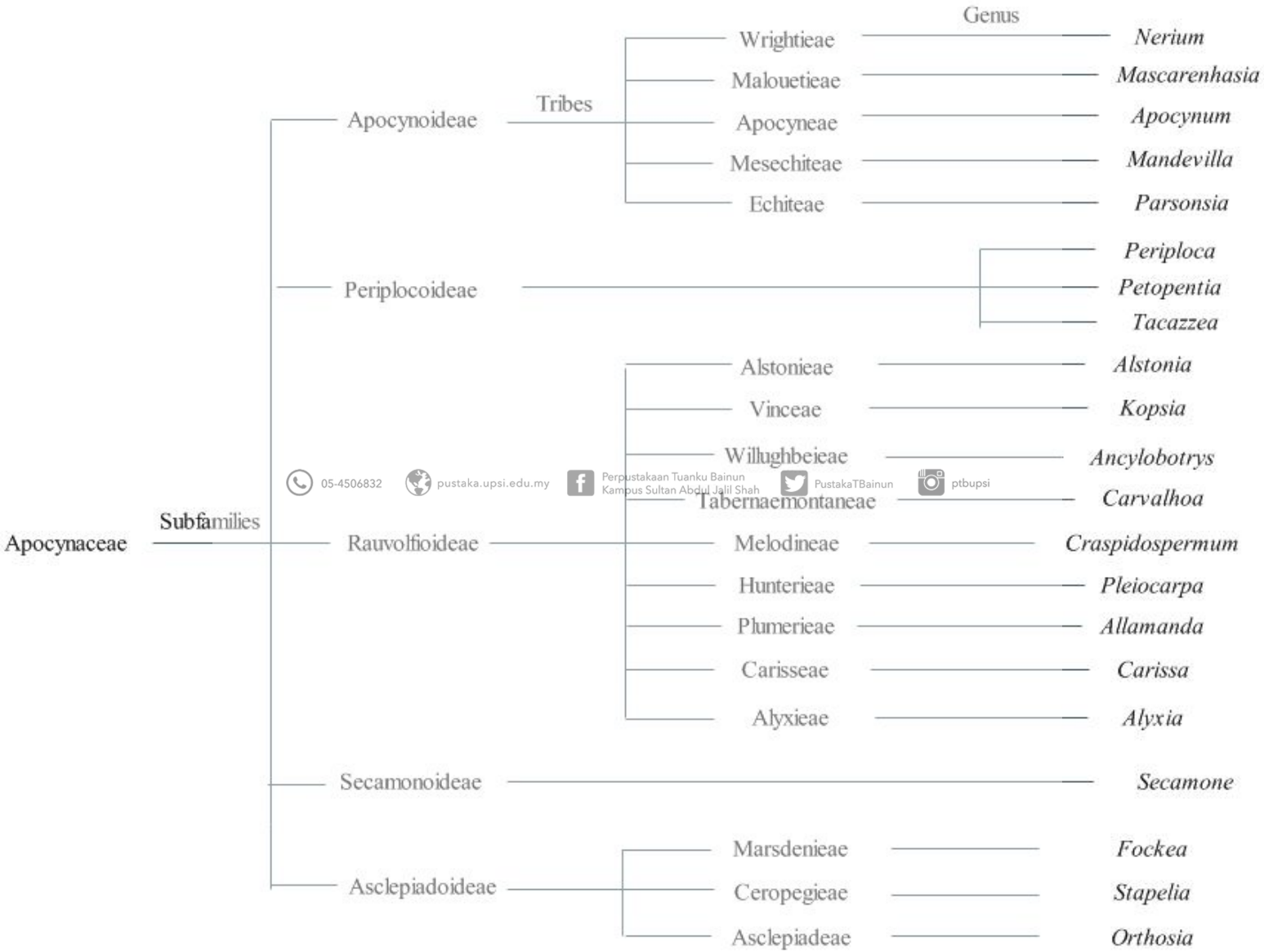

Figure 1.1. Classification of the Apocynaceae according to Endress and Bruyns (2000).

1.6 Botanical aspect of *Kopsia* genus

Plants of the genus *Kopsia* is classified as member of the tribe Vinceae in subfamily Rauvolfioideae. This genus comprises about 30 species, distributed mainly over Southeast Asia, China and India (Awang et al., 2008; Kam et al., 1996). There are 18 species of the genus *Kopsia* are found in Malaysia (Awang et al., 2008; Kam et al., 1997). This genus is most diverse in the Peninsular Malaysia and Sarawak (Malaysian Borneo), also widely distributed in Southeast Asia and is rich in indole alkaloids. Plants of this genus also have been proven to be prodigious sources of indole alkaloids with unusual structures as well as have useful biological activities (Kam & Subramaniam, 2004; Lim & Kam, 2006; Subramaniam et al., 2008a). Besides, the genus contains the most attractive species of any of the Asian genera of Apocynaceae, and a few of them have become widely cultivated. According to Whitmore (1973), most of the species are found mainly in lowland areas and a few species grow on limestone. Almost all species of *Kopsia* are shrubs or trees with white latex, opposite leaves, inflorescence cymose, sometimes raceme-like in appearance.

Most species of *Kopsia* have young branchlets that are somewhat angled and some species the angles become wing-like, such as *K. lancifolia* (Middleton, 2004). Flowers of *Kopsia* are five merous, sepals erect, without collators inside the axils and their corolla lobes are overlapping to the right in bud while the mature corolla are salverform with a narrow tube.

Stamens inserted around the middle of the tube to near the tube throat, very rarely near base, not exerted from throat; filaments are straight, short, and thin;