ISOLATION, HEMISYNTHESIS AND CHARACTERISATION OF PENTACYCLIC TRITERPENOIDS FROM *Diospyros foxworthyi* BAKH. (EBENACEAE) AND THEIR BIOLOGICAL ACTIVITIES

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UNIVERSITI SAINS MALAYSIA

2024

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by

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Thesis submitted in fulfilment of the requirements for the degree of Master of Science

February 2024

ACKNOWLEDGEMENT

All praise, glory and thanks give to Allah S.W.T. for His amazing grace and generosity that helped me throughout the whole process of completing this Master of Science (MSc.) research work. I would like to convey my utmost, sincere gratitude and heartfelt thanks to my supervisor, Dr Mohamad Nurul Azmi bin Mohamad Taib, for providing me with valuable guidance, time, support, coaching and inspiration throughout the duration of this project. His extensive knowledge and care gave me the motivation to keep going even in difficult situations. I would like to express my sincere appreciation and gratitude to my co-supervisor, Dr Ezatul Ezleen binti Kamarulzaman, for her support, guidance, advice and equipment, especially in the anti-dengue bioassay studies. I am very grateful to her for the valuable time and space, even during the tough trials.

Furthermore, I would like to acknowledge the Universiti Sains Malaysia (USM) Research Grant with codes of FRGS/1/2021/SKK0/USM/02/40 and 1001/PKIMIA/8011072 for funding this research. I would like to thank the Majlis Amanah Rakyat (MARA) for the financial support under the Graduate Excellence Programme (GrEP), which covered my study and living expenses. I would also like to thank the Institute of Postgraduate Studies (IPS), USM, School of Chemical Sciences (SCS) and Malaysian Institute of Pharmaceuticals and Nutraceuticals (IPharm) of NIBM for all the facilities and support during my studies. I would also like to thank the science officers and assistants in the SCS, especially Ms. Arlita, Ms. Alia, Ms. Ainina, Mr. Nazeef, Mr. Fahmy, Ms. Wan Zulilawati, Ms. Asma, Mr. Megat, Mr. Azhar and Mak Cik Fatimah for their help and guidance, especially with the spectroscopies, lab management, cleanliness and glass-blowing facility.

It is my pleasure to thank Prof. Dr. Unang Supratman from Universitas Padjadjaran, Indonesia for HRMS analysis, Dr. Iffah Izzati Zakaria, Dr. Hanim Salin and Ms. Sarah Dyana Khalili from NIBM and IPharm for anti-dengue assessment, Dr. Arba Pramundita Ramadani from Universitas Islam Indonesia for the anti-malarial studies, Dr. Norhafiza binti Mohd Arshad and Mr. Nurhisyam bin Zakaria from Universiti Malaya, Malaysia for cytotoxicity assessment as well as Dr. Mohammad Tasyriq bin Che Omar from School of Distance Education, USM for molecular docking knowledge sharing. Special thanks to Ms. Roziana of SCS for the postgraduate administrative support, help and guidance that led to graduation process. I would also like to thank Fahmy Fared and Ain Latifhaa who assisted me with the chemistry part of this research as part of their final year project (FYP).

Furthermore, my greatest gratitude and love to my mother, Madam Nor Askiah binti Mohd Yusof, and my siblings: Fadli, Fadzilah, Husna, Hidayah, Faiez and Amalina for their countless prayers, inspiration, motivation and blessings to keep me going on my journey. Thanks to your unfailing support and encouragement, the spirit, dedication and memories of Arwah Abah remain alive, even though he is no longer here. I don't want to forget to thank my dear beloved friends, especially Norhadi Mohamad, Zulhelmi Nazri, Amirah Saad, Wan Nur Huda, Nurin Asyiqin, Siti Nurshahira, Ain Syazwani, Lacksany Phongphane, Sarah Dyana, Syifa Amir, Axzel Tan and Akrimi Najihah, who supported and motivated me throughout the project, listened to problems, gave solutions, and created unforgettable moments on the master's journey. Special thanks also go to the other members of the NPSO family: Idris, Ibrahim, Tahir, Mahdi, Mahmoud, Adnan, Tan Cheong Siong, Jun Ming and Selvakumar for their invaluable help, kind words, continuous support and precious shared memories. Thank you everyone!

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LIST OF SYMBOLS

%	Percentage
π	Pi
μg	Microgram
μg mL^{-1}	Microgram per milliliter
±	Plus-minus
°C	Degree Celsius
μL	Microliter
μM	Micromolar
μmol	Micromole
Å	Angstrom
cm	Centimeter
cm^{-1}	Per centimeter
d	Doublet
dd	Doublet of doublet
g	Gram
h	Hour
J	Coupling constant
kcal mol^{-1}	Kilocalorie per mol
kg	Kilogram
L	Liter
m	Multiplet
m/z	Mass-to-charge ratio
mg	Milligram
mg mL^{-1}	Milligram per milliliter
MHz	Mega Hertz
mL	Milliliter
mm	Millimeter
mM	Millimole
nm	Nanometer

ppm	Parts per million
rpm	Rotation per minute
s	Singlet
ν_{max}	Absorption maxima
δ	Chemical shift
δ_C	Carbon chemical shift
δ_H	Proton chemical shift
λ	Wavelength
λ_{em}	Emission wavelength
λ_{ex}	Excitation wavelength
λ_{max}	Wavelength maxima

LIST OF ABBREVIATIONS

^{13}C-NMR	13 Carbon Nuclear Magnetic Resonance
1D-NMR	One Dimensional Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
2D	Two dimensions
2D-NMR	Two Dimensional Nuclear Magnetic Resonance
3D	Three dimensions
ACS	American Cancer Society
ACT	Artemisinin-based combination therapy
AIDS	Acquired immunodeficiency syndrome
AJCC	American Joint Committee on Cancer
AL	Artemether-lumefantrine
AMC	7-amino-4-methylcoumarin
ANOVA	Analysis of variance
AR	Analytical reagents
AS+AQ	Artesunate-amodiaquine
ASMQ	Artesunate-mefloquine
ATR	Attenuated Total Reflection
BAX	Bcl-2-associated X
BE	Barium enema
BOC-Gly-Arg-Arg-MCA	*tert*-Butyloxycarbonylglycyl-L-arginyl-L-arginine-4-methylcoumaryl-7-amide
Bz-Nle-Lys-Arg-Arg-H	Peptidic inhibitor
C=O	Carbonyl
CADD	Computational-aided drug design
CBC	Chair-boat-chair
CC	Column chromatography
CCC	Chair-chair-chair
CCCCB	Chair-chair-chair-chair-boat
CCDC	Cambridge Crystallographic Data Centre
CCSB	The Center for Computational Biology

CDC	Centers for Disease Control and Prevention
$CDCl_3$	Deuterated chloroform
CDs	Communicable diseases
CEA	Carcinoembryonic antigen
CH	Carbon-hydrogen bond
CH_2	Methylene
CH_3	Methyl
CNS	Central nervous system
COSY	Homonuclear correlation spectroscopy
COVID	Coronavirus disease
CPU	Central processing unit
CRC	Colorectal cancer
CT	Computed tomographic
D.	*Diospyros*
D. foxworthyi	*Diospyros foxworthyi*
DCM	Dichloromethane
DENV	Dengue virus
DEPT	Distortionless Enhancement by Polarization Transfer
DF	Dengue fever
DHF	Dengue haemorrhagic fever
Diospyros sp.	*Diospyros* species
DMAP	4-dimethylaminopyridine
DMAPP	Dimethylallyl diphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DRE	Digital rectal exam
DXP	1-deoxy-D-xylulose 5-phosphate
EAC	Electrostatic: Attractive charge
EDG	Electron donating group
ELISA	Enzyme-linked immunosorbent assay
EPC	Electrostatic: Pi-cation
ERUS	Endorectal ultrasound

et al.	And others
Et_3N	Triethylamine
EtOAc	Ethyl acetate
EWG	Electron withdrawing group
FDG PET-CT	^{18}F-fluorodeoxyglucose positron emission tomography-computed tomographic
FEB	Free energy of binding
FIT	Faecal immunochemical test
FPP	Farnesyl pyrophosphate
FPS	Farnesyl pyrophosphate synthase
FT-IR	Fourier Transform Infrared
G3P	Glyceraldehyde-3-phosphate
gFOBT	Guaiac-based faecal occult blood test
GLOBOCAN	Global Cancer Observatory
GRIN	The Germplasm Resources Information Network
H_2O	Water
H_2SO_4	Sulphuric acid
HA	Hydrophobic: Alkyl
HIV	Human immunodeficiency virus
HMBC	Heteronuclear Multiple Bond Correlation
HPA	Hydrophobic: Pi-alkyl
HPIA	Haem polymerisation inhibition activity
HPPS	Hydrophobic: Pi-pi stacked
HPPTS	Hydrophobic: Pi-pi T-shaped
HPS	Hydrophobic: Pi-sigma
HRMS	High Resolution Mass Spectroscopy
HSQC	Heteronuclear Single Quantum Correlation
IC_{50}	Half-maximal inhibitory concentration
IF	Immunofluorescence test
IPharm	Malaysian Institute of Pharmaceuticals and Nutraceuticals
IPP	Isopentenyl pyrophosphate
IR	Infrared
IV	Intravenous

JEV	Japanese encephalitis virus
K_i	Inhibitor constant
KKM	Kementerian Kesihatan Malaysia
Lit. data	Literature data
LDH	Lactate dehydrogenase
MeOH	Methanol
MEP	Methylerythritol phosphate
MIC	Minimal inhibitory concentration
MIZ	Minimal inhibitory zone
MRI	Magnetic resonance imaging
MS	Mass spectroscopy
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MVA	Mevalonic acid / Mevalonate
NAA	Nucleic acid amplification
NaOH	Sodium hydroxide
NCDs	Non-communicable diseases
NH_4Cl	Ammonium chloride
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
NPs	Natural products
NS	Non-structural
OH	Hydroxyl
OSC	Oxidosqualene cyclase
P.	*Plasmodium*
P. falciparum	*Plasmodium falciparum*
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
PDB	Protein Data Bank
POWO	Plants of the World Online
RdRp	RNA-dependent RNA polymerase
RDT	Rapid diagnostic test
R_F	Retention factor

RFU	Relative Fluorescence Unit
RMSD	Root mean square deviation
RNA	Ribonucleic acid
RT	Radiotherapy
SAR	Structure-activity relationship
SCFAs	Short-chain fatty acids
SD	Standard deviation
SI	Selectivity index
sp.	Species
sp^2, sp^3	Hybrid orbitals
SQE	Squalene epoxidase
SQS	Squalene synthase
Sy.x	Standard error of estimate
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNM	Tumour, nodes, metastasis
Tris-HCl	Trisaminomethane hydrochloride
UCSF	The University of California, San Francisco
USDA	United States Department of Agriculture
UTR	Untranslated regions
UV	Ultraviolet
VDW	van der Waals
WFO	World Flora Online
WHO	World Health Organisation
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

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PEMENCILAN, HEMISINTESIS DAN PENCIRIAN TRITERPENOID PENTASIKLIK DARIPADA *Diospyros foxworthyi* BAKH. (EBENACEAE) DAN AKTIVITI BIOLOGINYA

ABSTRAK

Diospyros foxworthyi ialah sejenis spesies tumbuhan tropika daripada famili Ebenaceae yang berasal dari Malaysia. Genus ini didapati kaya dengan triterpenoid pentasiklik dalam bentuk kerangka berasaskan lupana. Kajian awal ke atas genus ini menunjukkan perencatan protease denggi yang baik, seterusnya mencetuskan lanjutan kajian fitokimia terhadapnya. Kajian ini bertujuan untuk menyiasat komposisi kimia daripada *D. foxworthyi*, mensintensis sebatian terbitan triterpenoid dan menilai aktiviti biologinya. Ekstrak etil asetat (EtOAc) daripada kulit tumbuhan ini dikaji melalui proses pemencilan, penulenan dan penentuan struktur menghasilkan penemuan tiga sebatian diketahui dan menarik iaitu betulina (**93**), lupeol (**58**) dan lupenon (**108**). Seterusnya, betulina dan lupeol yang telah dipencilkan ini melalui hemisintesis lanjut secara pengasilan atau pengasetilan yang membawa kepada sintesis dua belas sebatian terbitan betulina **188a-188f** dan sebatian terbitan lupeol **189a-189f** yang menarik. Struktur kimia bagi kesemua sebatian ini dijelas dan dicirikan melalui kaedah spektroskopi terdiri daripada resonans magnet nuklear (RMN)-1D dan 2D bersama dengan analisis spektroskopi inframerah-penjelmaan Fourier (IM-PF) dan spektrometri jisim resolusi tinggi (SJRT), seterusnya dibanding dan disahkan dengan literatur. Triterpenoid **93**, **58** dan **108** yang dipencilkan beserta dengan sebatian hemisintesis **188a-188f** dan **189a-189f** telah dikaji secara *in vitro* dan *in siliko* untuk aktiviti rencatan protease DENV-2 NS2B/NS3 dalam penilaian anti-denggi manakala aktiviti rencatan pempolimeran

haem pula untuk kajian anti-malaria. Selain itu, triterpenoid **93**, **58** dan **108** yang dipencilkan ini juga diuji sitotoksitinya menggunakan asai 3-(4,5-dimetil-2-thiazolil)-2,5-difenil-2H-tetrazolium bromida (MTT) melawan garis sel adenokarsinoma kolorektal manusia HT-29. Antara sebatian yang diuji, **188a**, **108** dan **188b** menunjukkan keberkesanan yang sederhana untuk aktiviti perencatan protease DENV-2 NS2B/NS3 secara *in vitro* dengan nilai IC_{50} masing-masing 155.4 ± 5.16, 169.0 ± 4.61 dan 169.9 ± 5.89 µM. Tambahan pula, tenaga pengikat bebas (TPB) untuk pengedokan molekul *in siliko* dalam kajian anti-denggi bagi **188a**, **108** dan **188b** adalah berjulat antara -7.9 hingga -8.7 kcal mol^{-1}. Dalam aktiviti rencatan pempolimeran haem, sebatian **188d** mempamerkan keberkesanan yang baik dengan nilai IC_{50} sebanyak 6.66 ± 1.36 µM serta TPB bernilai -8.4 kcal mol^{-1}. Bagi kajian sitotoksiti, hasil pemerhatian menunjukkan sebatian **93** dan **85** telah mencetuskan kesan sitotoksik terhadap sel kanser HT-29 dalam cara bersandarkan dos dengan nilai IC_{50} masing-masing iaitu 13.49 ± 0.53 dan 37.57 ± 4.11 µM. Ini ialah laporan pertama yang menerangkan dapatan fitokimia daripada kulit *D. foxworthyi* bersama dengan sebatian terbitan serta aktiviti-aktiviti biologinya yang menunjukkan potensi sebatian-sebatian ini sebagai sumber agen terapeutik dalam menguruskan penyakit denggi, malaria dan kanser.

ISOLATION, HEMISYNTHESIS AND CHARACTERISATION OF PENTACYCLIC TRITERPENOIDS FROM *Diospyros foxworthyi* BAKH. (EBENACEAE) AND THEIR BIOLOGICAL ACTIVITIES

ABSTRACT

Diospyros foxworthyi is a tropical plant species of Ebenaceae family that is native to Malaysia. This genus is rich with pentacyclic triterpenoids in the form of lupane-based skeleton. The preliminary study of this genus showed good inhibition of dengue protease, which subsequently intrigue to further its phytochemical findings. The present study aimed to investigate the chemical composition of *D. foxworthyi*, synthesis of the triterpenoids derivatives and evaluate their biological activities. The ethyl acetate (EtOAc) extract from the bark of the plant was studied through the isolation, purification and structural elucidation processes resulting in the discovery of three known yet interesting compounds, namely betulin (**93**), lupeol (**58**) and lupenone (**108**). Subsequently, these isolated betulin and lupeol were further undergone hemisynthesis by acylation or acetylation which led to the synthesis of twelve interesting betulin derivatives **188a-188f** and lupeol derivatives **189a-189f**. The chemical structures of these compounds were elucidated and characterised by using spectroscopic methods consisting of 1D and 2D-nuclear magnetic resonance (NMR) in combination with Fourier transform-infrared (FT-IR) and high resolution mass spectrometry (HRMS) analysis, which later being compared and confirmed with the literature. The isolated triterpenoids **93**, **58** and **108** together with hemisynthesised derivatives **188a-188f** and **189a-189f** were examined for *in vitro* and *in silico* DENV-2 NS2B/NS3 protease inhibitory activities for anti-dengue evaluation and haem polymerisation inhibition activities for anti-malarial studies.

Moreover, the isolated triterpenoids **93**, **58** and **108** were also tested for their cytotoxicity using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay against HT-29 human colorectal adenocarcinoma cell line. Among the tested compounds, **188a**, **108** and **188b** showed moderate potency for *in vitro* DENV-2 NS2B/NS3 protease inhibitory activities with IC_{50} values of 155.4 ± 5.16, 169.0 ± 4.61 and 169.9 ± 5.89 μM, respectively. In addition, the free energy of binding (FEB) of the *in silico* molecular dockings for the anti-dengue study of **188a**, **108** and **188b** were ranged between -7.9 to -8.7 kcal mol^{-1}. In haem polymerisation inhibitory activity, compound **188d** exhibited good potency with IC_{50} value of 6.66 ± 1.36 μM and the FEB of -8.4 kcal mol^{-1}. For cytotoxicity study, the observed result showed that compounds **93** and **85** did induce cytotoxic effect to the HT-29 cancerous cells in a dose-dependent manner with IC_{50} of 13.49 ± 0.53 and 37.57 ± 4.11 μM, respectively. This is the first report describing the phytochemicals finding from the bark of *D. foxworthyi* together with their derivatives and biological activities, suggesting their potential as sources of therapeutic agents for managing dengue, malaria and cancer diseases.

CHAPTER 1

INTRODUCTION

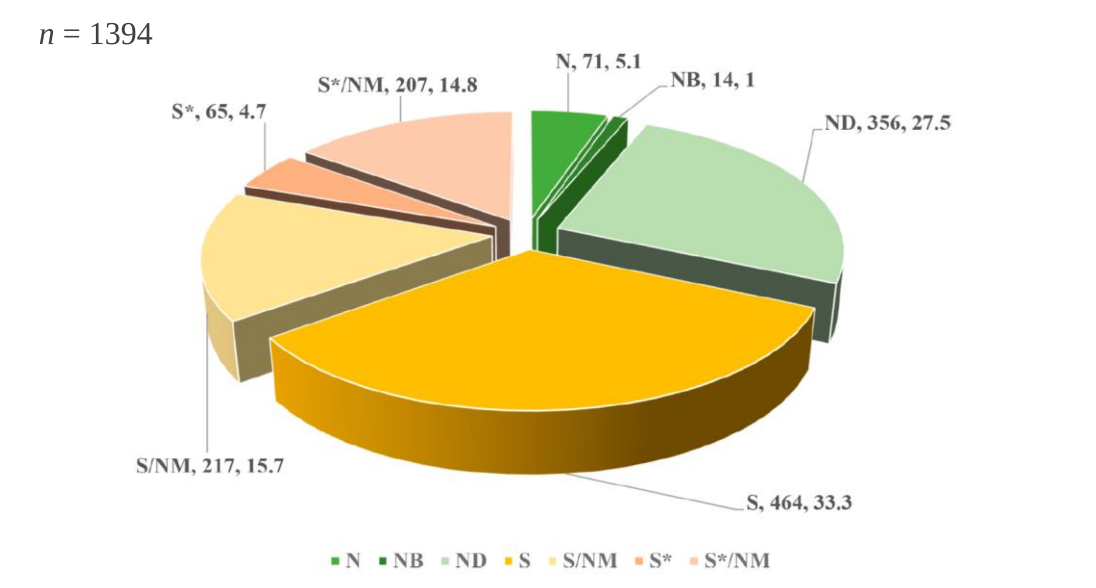
1.1 General introduction

"I consider nature a vast chemical laboratory in which all kinds of composition and decompositions are formed."

Antoine Lavoisier

Nature is an ecosystem with a biotic and an abiotic factor that are symphonically interdependent and form one life (Mader & Andrew, 2010). Nature is the ultimate source of our life and is the greatest contributor to the chemicals of life, namely natural products (NPs). NPs are defined as chemical compounds or substances produced by a living organism. They are synthesised by various biosynthetic pathways and their structures can be relatively simple or highly complex. NPs are required by living organisms in order to survive, develop and reproduce (Rollinger *et al*., 2006; Sorokina *et al*., 2020; Amy, 2022).

NPs have been used primarily to treat human diseases for thousands of years and are becoming increasingly important in drug discovery and research (Harvey *et al*., 2015). NPs or NPs-derived compounds are one of the most important bases for lead candidates in drug development. According to Newman and Cragg (2020), from 1981 to 2019, about 441 of the total 1394 small molecules approved as drugs (32%) were NPs or directly derived from NPs, citing the vitals of NPs as sources of new drugs, as charted in Figure 1.1 (Newman & Cragg, 2020). NPs may derive from both terrestrial and marine organisms, from sources such as plants, fungi, bacteria, protozoa, insects and other animals, as well as from humans (Rollinger *et al*., 2006).

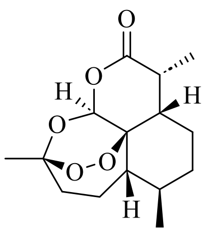


Indicator:
N – natural product; **NB** – natural product botanical; **ND** – natural products derivatives; **S** – totally synthetic drug; **NM** – natural product mimic; **S* –** made by total synthesis but pharmacophore from natural product

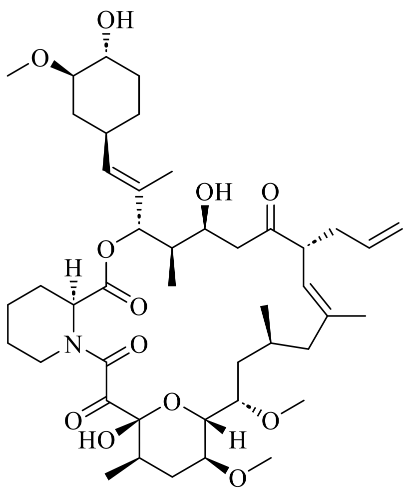
Figure 1.1 Chart showing the distribution of all small molecules approved drugs from 1st January 1981 to 30th September 2019 (Newman & Cragg, 2020).

There are two major classifications of NPs, specifically primary metabolites and secondary metabolites (Elshafie *et al.*, 2023). Primary metabolites are the molecules that are required for the survival of the organism, while secondary metabolites are not required for the normal growth and development of organisms (Elshafie *et al.*, 2023). Secondary metabolites comprise of three main classes that were grouped on the basis of structural similarity and biosynthetic pathways, namely terpenoids, alkaloids and phenolics. These classes of compounds form the backbone of the chemistry of NPs (Teoh, 2015; Hussein & El-Anssary, 2019).

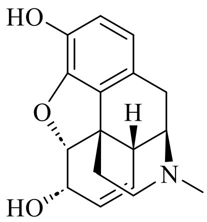
In addition, NPs are distinguished by their structural diversity and complexity, more sp^3 carbon atoms with fewer nitrogen or halogen elements and also the existence of chiral centres and stereochemistry (Guo, 2017). For example, the anti-malarial drug artemisinin (**1**) consists of a diverse and complex fused trioxene system with peroxy, lactone, cyclic acetal and ketal moieties. Moreover, the immunological regulator tacrolimus (**2**) contains many saturated sp^3 carbons compared to sp^2 carbons, which make connections between the tetrahedral carbons to create flexible chains or cyclic structures. The modification or simplification of stereochemistry and chirality led to the derivatisation of the old, highly chiral analgesic morphine (**3**) into simpler, non-chiral synthetic analgesics called fentanyl (**4**).



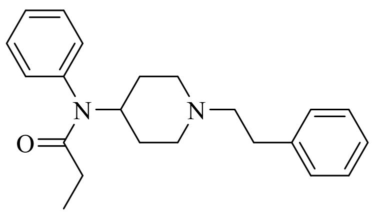
Artemisinin (**1**)



Tacrolimus (**2**)



Morphine (**3**)



Fentanyl (**4**)

Figure 1.2 The structure of chemical compounds with their distinguish structural diversity and complexity.

Numerous pharmaceutical products currently rely on the blueprint of NPs by performing partial or total synthesis in such to produce effective and potent drugs for the targeted diseases (Atanasov *et al*., 2015). Indeed, most anticancer and anti-infective agents are derived from nature (Salah & Bakibaev, 2017). Therefore, the analysis and study of chemical constituents in plant extracts is crucial as the demand for the development and modification of natural products has skyrocketed. As a result of the need for the exploration and derivatisation of NPs, the study of phytochemicals from diverse plant species is required.

Malaysia is one of the world's megadiverse countries. According to the National Biodiversity Index, which is based on estimates of richness and endemicity in four classes of terrestrial vertebrates and plants, Malaysia ranks 12th in the world (Mamat, 2015). Moreover, Malaysia is home to about 15,000 species of vascular plants (Mohamad, 1994), which have always been an attractive geographical target for NPs studies. This diversity of ecosystem offers great opportunities for the discovery of remarkable and potential chemical components from NPs, which is beneficial to pharmaceutical and nutraceutical research for the benefit of human beings. A few studies have shown that many plant species found in Malaysia are suitable for the development of useful drugs, including antiviral and anticancer properties (Jantan, 2004). As Malaysia's forests and lands offer biologically and chemically diverse resources, this phenomenon has led to scientific interest and advances in the study of NPs and medicinal plants. In July 2023, the Ministry of Health Malaysia (MOH) has approved the first registration of NPs with therapeutic claims, namely Fespixon cream. This cream contains *Plectranthus amboinicus* and *Centella asiatica* herbal extracts as the active ingredients for the treatment of diabetic foot ulcers (Ibrahim, 2023), citing the latest developments in NPs from medicinal plants.

In response to the interest on medicinal plants in Malaysia, genus *Diospyros* has been studied for its bioactivity. *Diospyros* has been taxonomically placed in the Ebenaceae family and to date, more than 700 *Diospyros* species have been discovered, making it the largest genus within the Ebenaceae family (Wijedasa *et al*., 2012; POWO, 2023). *Diospyros* is widespread and common in countries with temperate and tropical climates, such as Malaysia. Many recent studies reported the importance of *Diospyros* in pharmacology and chemotaxonomy as researchers have found many potential biochemical compositions from this genus that being used in traditional medicine and showed interesting pharmacological activities (Mallavadhani *et al*., 1998).

According to the Cambridge Dictionary, disease means an illness of living organisms caused by an infection or a failure of health and not by an accident. Diseases are often described as communicable or non-communicable (Ackland *et al*., 2003). Communicable diseases (CDs) are contagious diseases that are transmitted from one person to another through various contacts, carrying agents or vector and through the air. Tuberculosis, measles, dengue, hepatitis, malaria and coronavirus infections are some examples of CDs (Ackland *et al*., 2003; Wong *et al*., 2022). Non-communicable diseases (NCDs), also known as chronic diseases, tend to be of long duration and the result of a combination of genetic, physiological, environmental and behavioural factors. They include cardiovascular diseases, cancers, diabetes and chronic lung disease (Ackland *et al*., 2003; WHO, 2022a). Diseases, whether CDs or NCDs, affect the world through loss of life, impairment of economic and human capital development and reduction in quality of life (Bloom *et al*., 2018; Fan *et al*., 2018).

Among the CDs, dengue and malaria attract the interest in this research. Both of these diseases are arthropod-borne, which the mosquito vector of dengue is *Aedes sp*. while malaria is *Anopheles sp*., causing high morbidity and mortality for many patients around the world (Wiwanitkit, 2011). In general, dengue is a viral infection with dengue virus (DENV) as the causative pathogen (Weiskopf & Sette, 2014) whereas malaria is a protozoan infection with the parasites of *Plasmodium sp*. as the pathogen (Cowell & Winzeler, 2019). There is no doubt that a suitable climate like in a tropical country led to the high prevalence of dengue and malaria infections globally (Wiwanitkit, 2011).

Furthermore, the World Health Organisation (WHO) database shows an 800% increase in reported dengue cases between 2000 and 2019, with a significant jump from 505,430 cases in 2000 to over 2.4 million cases in 2010 and to over 5.2 million cases in 2019. The number of dengue deaths also quadrupled between 2000 and 2015, from 960 to 4032 cases and later increased to 36,055 of deaths in 2019. (Yang *et al*., 2021; WHO, 2023b). In Malaysia, 66,224 cases of dengue fever have been reported cumulatively up to 22 July 2023 (Epidemiological Weeks 29), compared to 29,603 cases in the same period in 2022, representing a huge increase of 123.7%. Unfortunately, 47 casualties due to the complications of dengue fever were reported up to the said period (Kementerian Kesihatan Malaysia, 2023).

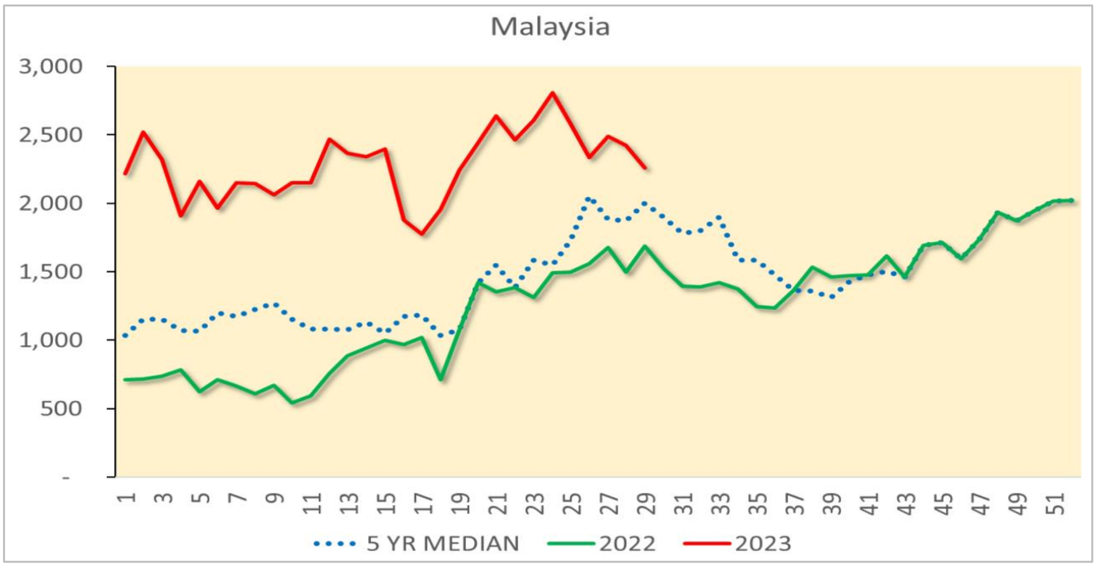


Figure 1.3 The trend comparison for the weekly dengue fever cases in Malaysia for the years of 2023, 2022 and the median of 2018-2022 (Kementerian Kesihatan Malaysia, 2023).

As stated by the latest World Malaria Report, there were 247 million malaria cases in 2021, up from 245 million cases in 2020. The estimated number of malaria deaths was 619,000 in 2021, declined from 625,000 in 2020 (WHO, 2022b). In the two peak years of the pandemic (2020–2021), COVID-related disruptions led to about 13 million more malaria cases and 63,000 more malaria deaths. The WHO African region continues to bear a disproportionate share of the global malaria burden. In 2021, the region accounted for about 95% of all malaria cases and 96% of deaths. Children under five years of age account for about 80% of all malaria deaths in that region. Malaysia had no cases of non-zoonotic malaria (human malaria parasites) for four consecutive years, but for the past five years there has been an increase in the number of zoonotic *Plasmodium knowlesi* malaria cases with a total of 17,125 cases and 48 deaths have been reported (WHO, 2022b).

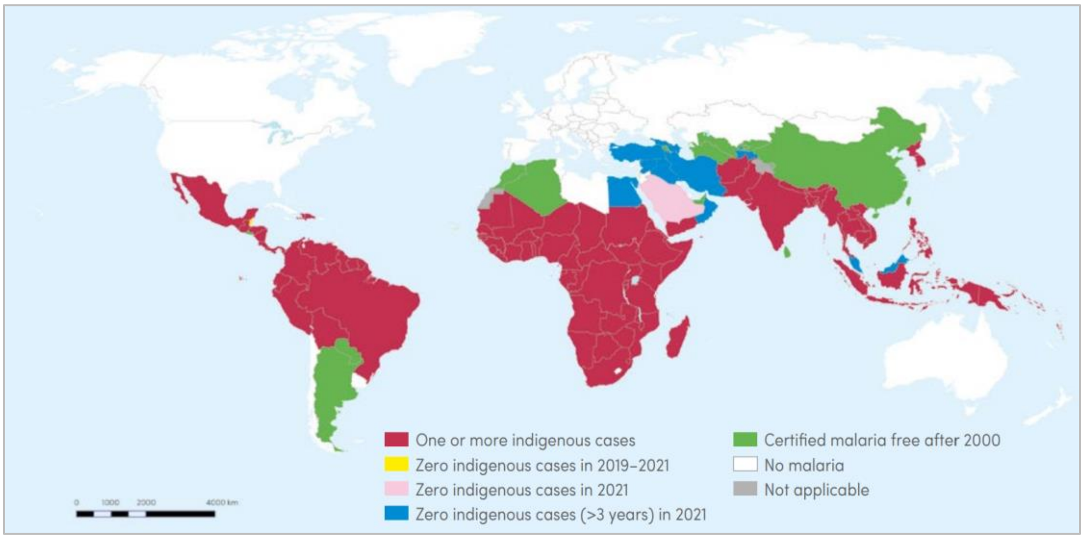


Figure 1.4 Countries with indigenous cases in 2000 and their status by 2021. In 2021, The Islamic Republic of Iran and Malaysia reported zero indigenous cases for the fourth consecutive year (WHO, 2022b).

As mentioned earlier, NCDs also play a major role in the leading causes of death worldwide. NCDs, for example, cancer is the second leading cause of death after heart disease in the US for the period 2015-2020, with 598,932 fatalities in

2020 (Ahmad & Anderson, 2021). The incidence of cancer has increased over time, and efforts to reduce the burden of the disease have been ongoing for decades. According to a 2019 estimate by the WHO, cancer is the second leading cause of death before the age of 70 in 112 countries, including Malaysia (Sung *et al.*, 2021). The National Cancer Registry in Malaysia reported that from 2012-2016, colorectal cancer (CRC) was the second most common cancer in the country as shown in the chart in Figure 1.5. CRC is the most common cancer in men (16.3%) and the second most common in women (10.7%) after breast cancer in Malaysia, and these cases are often detected late (Azizah *et al.*, 2019).

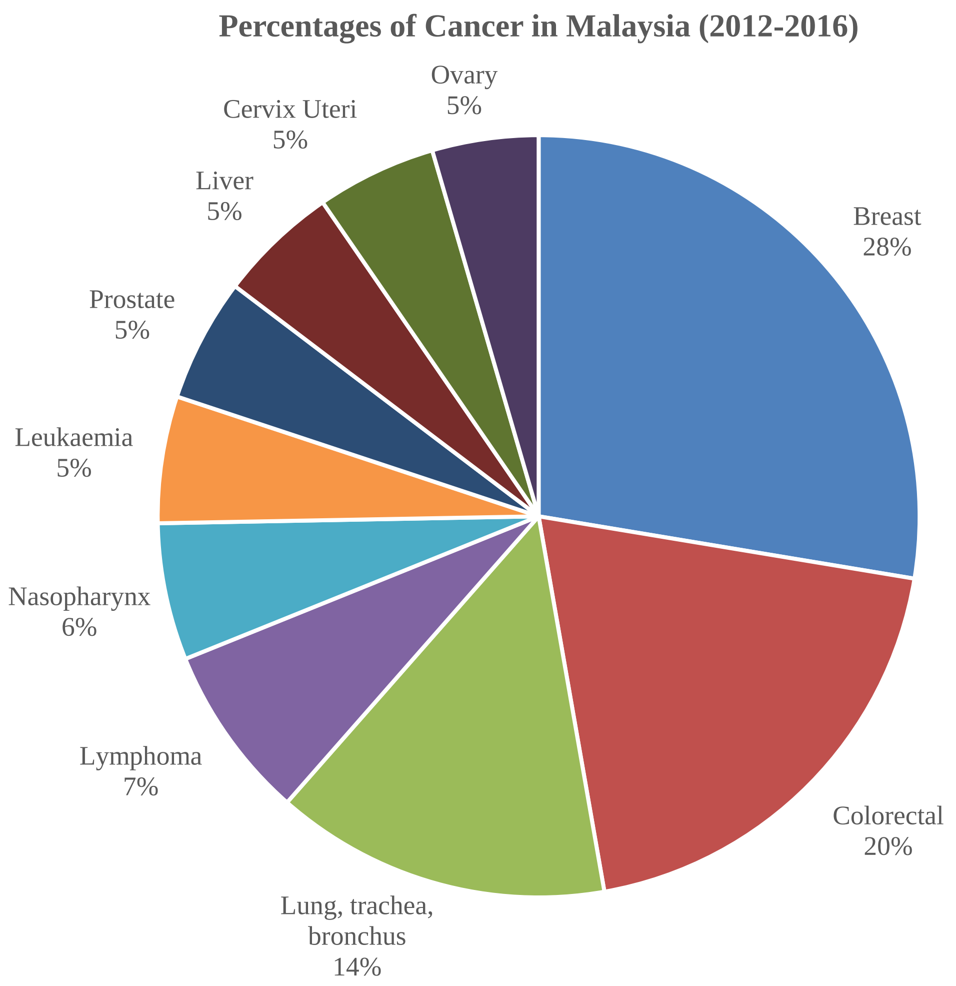


Figure 1.5 Top ten most common cancer in Malaysia from 2012-2016 (Azizah *et al.*, 2019).

Although a genetic inheritance carries a significant risk for CRC, various studies have shown that environmental factors such as smoking, alcohol consumption, sunlight and ionising radiation, harmful chemicals, pathogens, diet and obesity, hormonal therapy, as well as air and water pollution play an important role in the occurrence of CRC (Jeon *et al*., 2018; Witold *et al*., 2018). Current chemotherapies for cancer are non-specific and target not only cancer cells but also healthy cells. Therefore, there is a need to develop chemotherapeutic agents that are more effective, selective and less damaging to healthy cells. Research on cancer drugs from natural resources has shown a positive result in reducing the risk of colon cancer and slowing its progression (Pandey *et al*., 2011). More recently, the use of cancer drugs based on natural products has been highlighted and is currently being developed (Cai *et al*., 2014; Han *et al*., 2017).

To conclude this section, this research focuses on *Diospyros foxworthyi*, a plant native to Malaysia, which is being studied in depth for the first time. Its phytochemical information is collected and analysed for its bioactive components. In addition, using the isolated chemical compounds as starting material, a series of modifications are carried out by hemisynthesis *via* acylation or acetylation. The modifications of these phytochemicals are the key to the potency compared to the precursor compounds in terms of bioactive properties. These isolated and derivatised compounds were assayed for their anti-dengue, anti-malarial and cytotoxic activities against HT-29 human colon cancer cell lines.

1.2 Problem statement

The genus *Diospyros* from family Ebenaceae have been reported to exhibit numerous interesting biological and pharmacological activities. Although many studies highlight different species in genus *Diospyros*, there is one species, *D. foxworthyi* that native to Malaysia, which receives the least attention in terms of research and phytochemical studies. The little-known chemical composition of *D. foxworthyi* remains unexplored as there are very few focused studies on the total active compounds that could be found in this valuable species. The unknown compounds could be used for various purposes and to treat diseases such as dengue, malaria and cancer.

So far, only one study has been conducted on *D. foxworthyi* investigating the antiviral activity of this plant extract against DENV (Peyrat *et al*., 2016). Five Malaysian *Diospyros* plants extracts have shown interesting DENV inhibition ranging from 21–48% at 5 μg/mL dengue replicon. Among them, *D. foxworthyi* showed the highest inhibition of 48%. A preliminary bioassay study by our research group using seven *Diospyros sp.* plants for inhibition of DENV-2 NS2B/NS3 protease indicated the EtOAc crude extract of *D. foxworthyi* bark as a possible protease inhibitor with 68% of inhibition at 0.5 μM of protease concentration. These significant inhibition percentages stimulate further investigation of *D. foxworthyi* for its active chemical diversity.

Moreover, new analogues are generated by modifying the isolated chemical constituents. The effect of these modification strategies is still unknown and will somehow change the characteristics and bioactive properties compared to the original constituents, which needs to be evaluated in this research. These phytochemical findings and structural modifications aim to enhance bioactivities and represents an alternative as new lead compounds for anti-dengue, anti-malarial and cytotoxic chemotherapies. .

1.3 Research objectives

The objectives of this research are as follows:

1. To isolate and purify the chemical constituents from the bark extracts of *D. foxworthyi* using various chromatographic techniques.
2. To hemisynthesise the derivatives of the isolated betulin and lupeol using acylation or acetylation.
3. To elucidate and characterise the isolated and synthesised compounds using various spectroscopic methods such as nuclear magnetic resonance (NMR), Fourier transform-infrared (FT-IR) and high resolution mass spectrometry (HRMS).
4. To evaluate the *in vitro* and *in silico* biological activities of isolated and synthesised compounds as anti-dengue, anti-malarial and cytotoxic agents.

1.4 Significance of study

Through this study, the indigenous *D. foxworthyi*, which is now considered of least concern in terms of species approach, will be adequately highlighted. Likewise, the skeleton or backbone of the interesting components could be utilised in research and development. Hence, this research will explore the hemisynthesis of isolated chemical constituents to produce a new analogue. This derivatisation will be the critical component as the effect of substituting a new functional group will further influence the physical properties and efficacy in terms of biological activities. The mentioned bioactive constituents can be utilised for medicinal benefits such as anti-dengue, anti-malaria and cytotoxic therapeutic agents.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This chapter gives an overview of the Ebenaceae family, the genus *Diopyros*, the terpenoids and a brief overview of dengue, malaria and CRC. First, the distribution, habitat, morphology, classification of tribes and medicinal uses of the Ebenaceae family are described. Then, the botany, morphology and distribution of the genus *Diospyros*, including the studied plant *D. foxworthyi* Bakh. are elaborated for better understanding. The compounds of interest, namely the terpenoid, are also discussed in combination with their biosynthetic pathways. Moreover, the previous studies on chemical compounds and biological activities of the genus *Diospyros* are discussed, summarised and tabulated. Finally, the diseases of dengue, malaria and CRC are presented, including the latest statistics, symptoms and treatments. Overall, this chapter provides a foundation for understanding the botany, chemistry and biology of diseases related to this research topic.

2.2 Ebenaceae family

2.2.1 Distribution and habitat

The Ebenaceae or commonly knowns as the persimmon or ebony family, is classified under the plant order of Ericales (Steven & Luteyn, 2021). Ebenaceae is native in tropics and subtropics which consists of over 800 accepted species of three accepted genera, namely *Diospyros*, *Euclea* and *Lissocarpa* (POWO, 2023). *Diospyros* is pantropical while *Euclea* is restricted to Africa and Arabia whereas *Lissocarpa* is native to South America (World Flora Online, 2023; POWO, 2023). This plant genera are normally discovered in the forest understory as small to medium-sized trees with low population density. Besides, some of these plant species grow along river, swamps, and beach communities while others occur in deciduous forests, dryer vegetation or fire-prone savannahs (Wallnöfer, 2001). The genus *Diospyros* of the tribe is the most significant in the Ebenaceae family, with the highest diversity numerically and economically.

2.2.2 General appearance and morphology

According to the review by Wallnöfer (Wallnöfer, 2001) and GRIN Taxonomy (USDA, 2021), Ebenaceae are distinguished as evergreen dioecious, rarely monoecious, or polygamous trees or shrubs or rarely subshrubs with the bark, roots and heartwood often black. The leaves of Ebenaceae have two-ranked, with flat, dark-coloured glands on the lower surface but no teeth. It is simple, typically alternating, spirally or distichously arranged with most of them petiolate with the stipules absent. The leaf margins are usually whole and extrafloral nectaries are usually present on the abaxial leaf surface. Besides, the flowers of Ebenaceae are commonly unisexual but generally with remnants of the sex present and then articulated at the base with merosity of 3-5(-8). The calyx of the flower is primarily gamosepalous, entire, truncate and deeply lobed. It is spreading or reflexed which is persistent and often accrescent in a fruiting state. Ebenaceae have tricolpate and prolate-spheroidal to prolate pollen grain structure. The pollen is very consistent throughout the family, with the primary differences being in the size and shape of the grains, as well as the apertures. The flowers are reported to be visited by bees, beetles, wasps, and flies as primary pollinators. The bark of tropical species of Ebenaceae is black on the outside while the wood usually is heavy, hard, and fine-textured with pale or dark-coloured. The fruit of *Diospyros* is a multilocular, commonly a berry which not exposed during maturity with 3-8 lobed calyx, which differs from *Euclea*. The seeds of *Euclea* are usually solitary and 3-10 mm in diameter when ripe whereas *Diospyros* contains 1-16 seeds with 8-40 mm long.

2.2.3 Classification of tribes

The family of Ebenaceae can be classified to the botanical taxonomic rank (POWO, 2023; Soepadmo *et al*., 2002) as listed below.

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Ericales
Family	:	Ebenaceae
Genus	:	*Diospyros*, *Euclea* & *Lissocarpa*

2.2.4 Biological properties and medicinal uses of Ebenaceae

Commonly, the utilisation of Ebenaceae plants is heavily focused on as a food source (persimmon) and the needs of timber to a certain socioeconomic region. *Diospyros* plants also have been used in medicine to become a remedy to several ailments such as haemorrhage, incontinence, insomnia, hiccough and diarrhoea (Rauf *et al*., 2017). Some studies reported that important phytochemicals which have medicinal benefits, could be separated and isolated from the *Diospyros* plants, signifying the immense contribution of this genus towards the medicinal progression of society (Rauf *et al*., 2017). Traditionally, ancient Chinese civilisations have opted for the *Diospyros kaki* to produce a cure for ischemia, high blood pressure, atherosclerosis and some infectious diseases (Xie *et al*., 2015). There is also a report claiming the *Diospyros lycioides* is one of the plants used for HIV/AIDS management in Botswana (Thomford *et al*., 2015). In Nigeria, the potent anti-malarial agent that is used frequently is derived from *Diospyros mespiliformis* (Luka *et al*., 2014).

Furthermore, the roots of *Euclea crispa* is used to relieve constipation among Batswana children while its bark and leaves were used for diabetes and rheumatism prevention (Chinsamy *et al*., 2016). In South Africa, the indigenous plant roots of *Euclea natalensis* is applied to the skin lesions of leprosy and also consumed for hookworm infection. Additionally, the crushed and powdered roots of this plant is applied for the treatment of toothache and headache relief (Lall, 2019). For Kenyan people, the chewed fruits of *Euclea divinorum* is believed to detoxicate the bloods and reduced the abdominal upsets (Kigen *et al*., 2017). Table 2.1 have summarised some of the biological properties and medicinal uses of the plants in the Ebenaceae family.

Table 2.1 Biological properties and medicinal uses of plants in the Ebenaceae family.

Plant name	Biological property	Medicinal use	References
D. blancoi	Anti-microbial	Used in the treatment of diarrhoea, dysentery, fever, itchy skin, cough and wounds.	Akter *et al*., 2015
D. celebica	Anti-microbial, anti-inflammatory and anti-thrombosis	Used in wound healing and reduce the endothelial progression.	Chen *et al*., 2018
D. fleuryana	Anti-cancer	Used as inflammation medication and cancer treatment.	Ha *et al*., 2020
D. kaki	Anti-oxidant, anti-inflammatory and anti-hypertensive	Ischemia, high blood pressure and atherosclerosis.	Xie *et al*., 2015; Butt *et al*., 2015; Kim *et al*., 2016

Table 2.1 Continued.

Plant name	Biological property	Medicinal use	References
D. lotus	Anti-microbial, anti-pyretic, antinociceptive, anti-oxidant and anti-proliferative	Used in the treatment of diarrhoea, dry cough and hypertension.	Rauf *et al*., 2014; Loizzo *et al*., 2009
D. lycioides	Anti-viral, anti-inflammatory and anti-microbial	Used in the management of HIV/AIDS diseases. Used as herbal medicine for abdominal pains and wound treatment.	Thomford *et al*., 2015; Maroyi, 2018
D. malabarica	Anti-microbial and anti-oxidant	Used in the microbial infection treatment, wound and ulcer as well as diarrhoea medication.	Moniruzzaman *et al*., 2019
	Anti-diabetic and anti-oxidant	Used in the treatment of diabetes.	Kavatagimath & Jalalpure, 2016
D. melanoxylon	Anti-diabetic and anti-adipogenic	Used traditionally as anti-diabetic medication.	Rathore *et al*., 2014
D. mespiliformis	Anti-bacteria, anti-malaria and anti-inflammatory	Bactericidal, anti-malarial agent and wound healing treatment.	Luka *et al*., 2014; Dangoggo *et al*., 2012; Ebbo *et al*., 2022
D. montana	Anti-cancer	Used traditionally to treat tumours.	Ravishankara *et al*., 2000
D. rhodocalyx	Anti-mycobacteria, anti-malaria and anti-inflammatory	Used for treatments of diarrhoea, bleeding, parasitic infestation abscess and renal disease.	Theerachayanan *et al*., 2007

Table 2.1 Continued.

Plant name	Biological property	Medicinal use	References
D. villosa	Anti-bacterial, analgesic and anti-cancer	Used traditionally to treat gastrointestinal complaints, worms and flatulence. Used in the treatment of dysmenorrhea and pain management.	Adu *et al*., 2023
D. virginiana	Anti-fungal and anti-pyretic	Used to treat thrush, sore throats and bloody stools. Used to reduce fever symptoms.	Wang *et al*., 2011; Priya & Nethaji, 2015
Euclea racemosa	Anti-diabetic	Used as traditional medicine to treat diabetes.	Keter & Muttiso, 2012
Euclea crispa	Laxatives, anti-diabetic and anti-inflammatory	Used to treat constipation, stomachache and diabetes as well as to prevent rheumatism.	Chinsamy *et al*., 2016
Euclea divinorum	Anti-oxidant, anti-inflammatory and anti-fungal	Used traditionally as blood cleanser. Used to treat abdominal upsets and skin disorders.	Kigen *et al*., 2017
	Anti-bacterial and anti-viral	Treatment for gonorrhoea, genital herpes and HIV/AIDS management.	Chinsembu, 2016
Euclea natalensis	Anti-mycobacterial, anti-parasite, anti-inflammatory and analgesic	Used in the treatment of skin lesion in Hansen's disease, hookworm infection, toothache and headache.	Oosthuizen *et al*., 2020
Euclea undulata	Anti-inflammatory and analgesic	Toothache relief treatment.	Maroyi, 2017

2.3 The genus *Diospyros*

2.3.1 Botany, morphology and distribution

The general name of *Diospyros* is founded on the Greek words, *dios* which means “god or divine” and *puros* meaning “pear, fruit or wheat”, alluding to the good flavour of edible persimmon fruit and their resemblance to a pear. *Diospyros* is the largest genus of the Ebenaceae family with the reported yet accepted number of 779 species. It is documented to be native to the tropical climate region while some of the species even extended to the temperate climate region (POWO, 2023; USDA, 2021). The main physical characteristics of the plants within this genus would be recognised for its hard and solid-dark timber that has a multitude of uses that are important to the socioeconomic of that particular region (USDA, 2021). Another distinct trait that is possessed by the *Diospyros* which it could be identified as the gynodioecious type, where the sexuality of the plants could be differentiated into male and female according to the flower they produce. In general, only the plant with the female flowers would bear the fruits (Akagi *et al*., 2014).

Another main characteristic of *Diospyros* plants are the evergreen leaves and the dark-coloured stem (Britannica, 2019). The main fruit producing within the *Diospyros* genus would be the persimmon fruits which can be further identified into two different species. They are *Diospyros virginiana*, a plant that originated from North America, and the *Diospyros kaki*, which is widely known as the Chinese or Japanese persimmon plant (Britannica, 2019). The fruit is well rounded and voluptuous, between 1-16 seeds per fruit (Turner *et al*., 2013). The *Diospyros* plants could grow in the low-altitude forest, mixed forest, deciduous forest and lowland dry forest (Turner *et al*., 2013). In Table 2.2, the species and distribution of some *Diospyros* species have been listed.

Table 2.2 Some of the *Diospyros sp.* and their distribution.

Species	Distribution	References
D. abyssinica	Native to Africa	Nafiu *et al.*, 2013
D. blancoi	Endemic to Philippines	Ragasa *et al.*, 2009
D. celebica	Endemic to Sulawesi, Indonesia	Alfaizin, 2020
D. decandra	Commonly found in Thailand and Vietnam	Kubola *et al.*, 2011
D. digyna	Originated from Mexico	Jiménez-González *et al.*, 2021
D. fasciculosa	Commonly found in Australia, Fiji and Southeast Asia	Floyd, 2008
D. ferox	Native to Borneo	Soepadmo *et al.*, 2002
D. ferruginescens	Endemic to Borneo	Soepadmo *et al.*, 2002
D. foxworthyi	Found in Peninsular Malaysia and Borneo	Soepadmo *et al.*, 2002
D. kaki	Native to China and Japan	Mallavadhani *et al.*, 1998
D. lotus	Native to China and Asia	Rauf *et al.*, 2016
D. lycioides	Originated from Africa	Maroyi, 2018
D. malabarica	Found in Indian subcontinent	Moniruzzaman *et al.*, 2019
D. melanoxylon	Native to India and Sri Lanka	Sahu *et al.*, 2020
D. mespiliformis	Native to Africa	Mashile *et al.*, 2019
D. mollis	Commonly found in Thailand	Suwama *et al.*, 2018
D. sandwicensis	Endemic to Hawaii	Little, 1989
D. sericea	Found in Brazil and Colombia	Ramaldes *et al.*, 2022
D. singaporensis	Found in Peninsular Malaysia and Borneo	Soepadmo *et al.*, 2002
D. virginiana	Native to certain part of North America	Little, 1989

2.3.2 *Diospyros foxworthyi* Bakh.

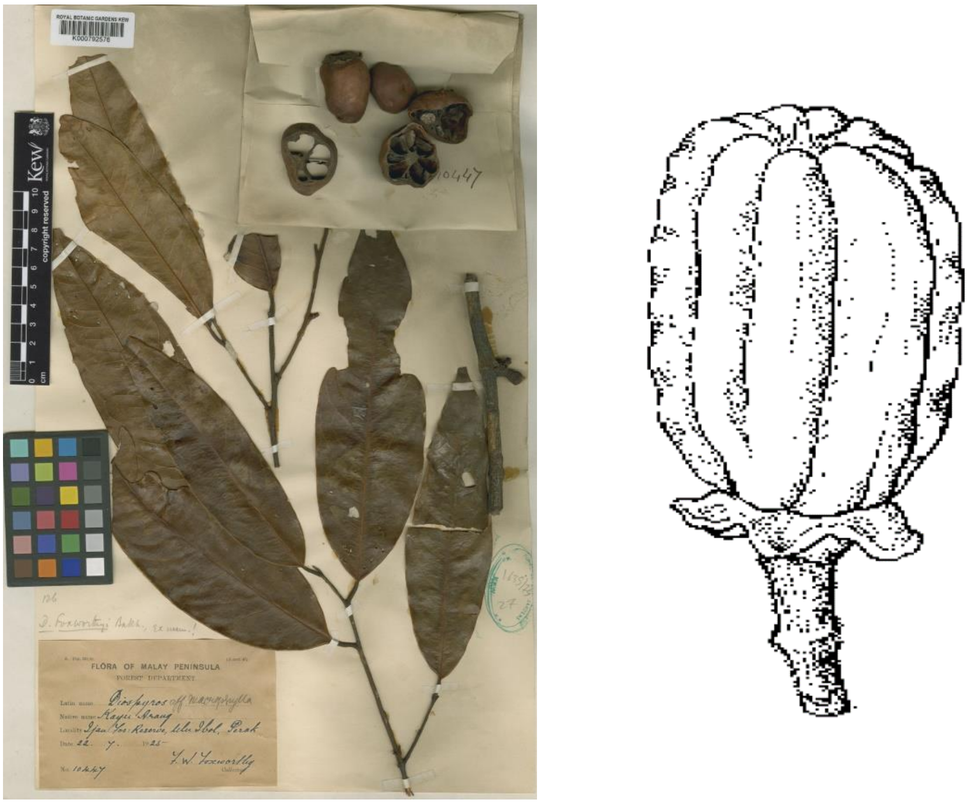
Diospyros foxworthyi Bakh., a plant native to Malaysia (Figures 2.1 & 2.2) is named after the American botanist Frederick William Foxworthy, a forest research officer at Forest Department of Malay Peninsula, Kepong, Malaya (now, Malaysia). This plant is classified as a ‘least concern’ species (Loc, 1998), which prevailed further research of it. This plant botanical classification and description are further explained below (Soepadmo *et al*., 2002; Kodoh *et al*., 2021).

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Ericales
Family	:	Ebenaceae
Genus	:	*Diospyros L.*
Species	:	*foxworthyi*
Botanical name	:	*Diospyros foxworthyi* Bakh.
Common name	:	Kayu arang, kayu malam, sungkang seribu

D. foxworthyi is a medium-sized tree up to 20 m tall and 35 cm in diameter. The bark is generally black and hard, while the inner bark is white to cream-coloured. The leaves are alternate, simple, smooth, elongated-elliptical and of chartaceous to coriaceous texture. They are long-pointed to pointed at the tip and wedge-shaped to rounded at the base and 10 – 35 × 3.5 – 12 cm in size. The veins are recessed or flush on the upper side, while they are prominent on the underside of the leaves. The petiole is 0.8 – 1.5 cm long. The male flowers have a calyx that is divided in half into 4 triangular lobes, with a 0.6 cm long salver-shaped corolla. In addition, the male inflorescences are slender, about 1 – 4.5 cm long and bear 3 – 15 flowers each. The female flowers have a calyx divided into 4 triangular lobes with a 0.5 × 0.6 cm corolla. The female inflorescences are 1 – 3.5 cm long and bear single to several flowers each. This plant bears fruits in clusters of 1 – 5, hanging from 1 – 3.5 cm long stems. The fruits are velvety and spherical when young, while they are glabrous and elongated when ripe. When ripe and drying, they tend to shrivel into as many longitudinal furrows as there are seeds. The fruit calyx is slightly enlarged and then forms a flat, woody, 4-pronged plate of 1.5 – 3 cm in diameter. The calyx also has lobes with erect tips, recurved sides and is slightly expanded. This species occurs in mixed dipterocarpic lowland forests in Peninsular Malaysia (Perak, Kelantan and Terengganu) and Borneo (Sabah, Sarawak and Kalimantan) (Soepadmo *et al*., 2002).

A **B**

Figure 2.1 *D. foxworthyi* stem (A) and fruit (B).
(Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur).



A B

Figure 2.2 *D. foxworthyi* herbarium catalogue specimens (A) (POWO, 2023) and the illustrations of its fruit (B) (Soepadmo *et al*., 2002).

2.4 Terpenoids

2.4.1 General

Terpenoids or known as isoprenoids are a substantial and diverse class of natural compounds made up from five carbon isoprene units (C_5H_8) as the building block. Up till now, about 80,000 naturally occurring terpenoid compounds, which serve various functional and structural roles as secondary metabolites (Pemberton *et al.*, 2017). Terpenoids are categorised according to the number and structural organisation of carbons formed by the linear arrangement of isoprene units followed by cyclisation and rearrangements of the carbon backbone with an empirical feature named isoprene rule (Ludwiczuk *et al.,* 2017). The classification of terpenoids are tabulated below in Table 2.3.

Table 2.3 Terpenoids and its classifications.

Classification	Number of isoprene unit(s)	Number of carbon atoms
Hemiterpenoids	1	5
Monoterpenoids	2	10
Sesquiterpenoids	3	15
Diterpenoids	4	20
Sesterterpenoids	5	25
Triterpenoids	6	30
Tetraterpenoids	8	40

2.4.2 Triterpenoids

Triterpenoids are classified under the family of secondary metabolites that typically contain 30 carbon atoms consisting of 6 isoprene units. In fact, about 20,000 different triterpenoids have been identified, making it one of the largest classes of plant natural products (Happi *et al.*, 2022). These metabolites are synthesised from isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP) through the 30-carbon intermediate squalene. Next, 2,3-oxidosqualene is folded into chair-chair-chair conformation (CCC) before cyclisation into multiple assortments of diverse skeletal types of triterpenoids (Thimmappa *et al.*, 2014).

Moreover, triterpenoids have relatively complicated cyclic structures consisting of alcohols, aldehydes and carboxylic acids making them complex and physiologically distinct (Ludwiczuk *et al.,* 2017). There are several important triterpenoids including squalene (**5**), *β*-amyrin (**6**), ursolic acid (**7**), oleanolic acid (**8**), betulinic acid (**9**), stigmasterol (**10**) and campesterol (**11**) which are derived from plants (Figure 2.3). In addition, triterpenoids contain many active sites for glycosylation, leading to the synthesis of steroidal glycoalkaloids name saponins (Mugford & Osbourn, 2012).

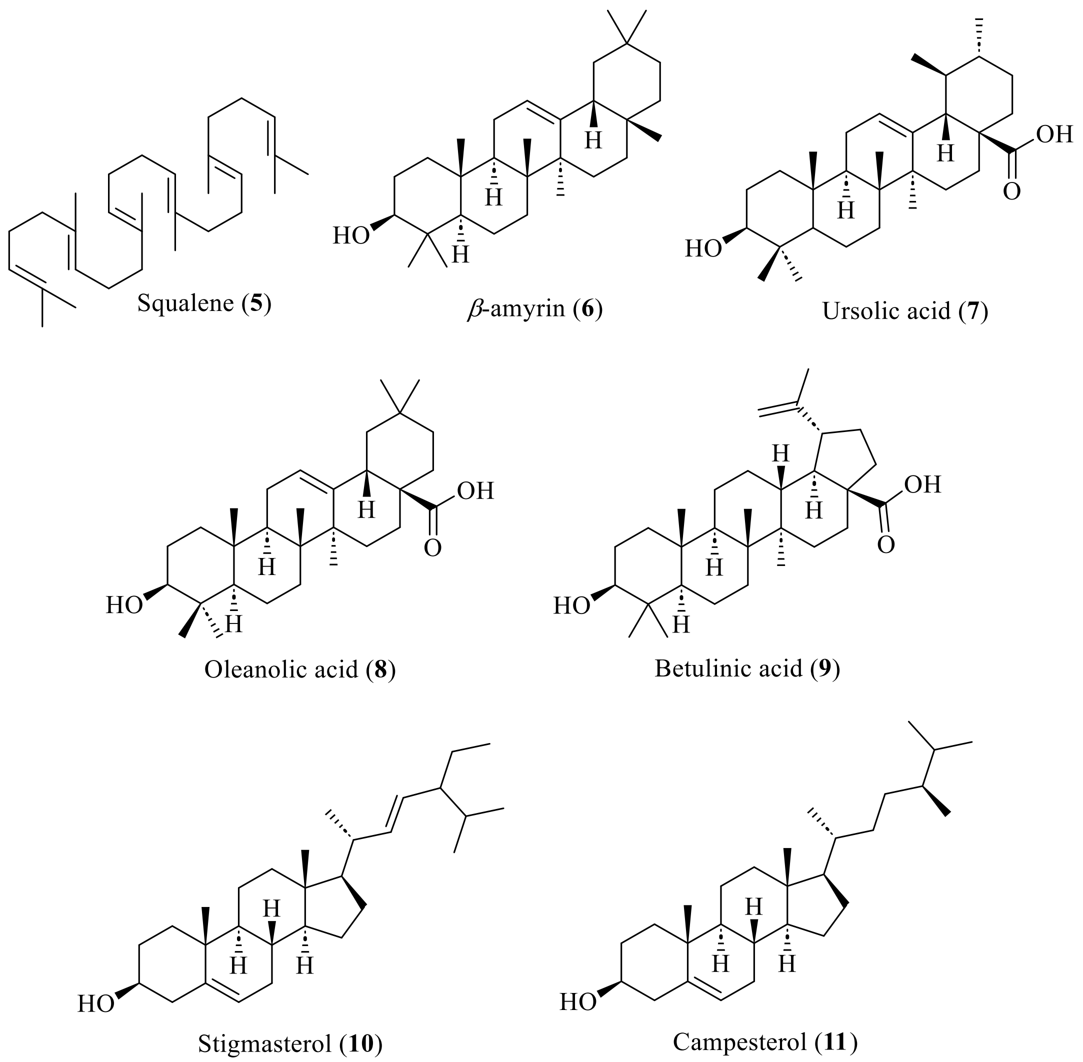


Figure 2.3 Example of important triterpenoids derived from plants.

Triterpenes and triterpenoids are subdivided into acyclic, monocyclic, bicyclic, tricyclic, tetracyclic or pentacyclic compounds based on their structural properties (Nguyen *et al.*, 2015). The main groups of triterpenes are the tetracyclic derivatives (Figure 2.4) including dammarane (**12**), lanostane (**13**), euphane (**14**), cucurbitane (**15**) and cycloartane (**16**), whereas for the pentacyclic derivatives (Figure 2.5) of baccharenyl cation-type compounds incorporating lupane (**17**), ursane (**18**), oleanane (**19**), friedelane (**20**) and taraxerane (**21**) (Noushahi *et al.*, 2022).