ISOLATION, CHARACTERISATION AND ANTI MALARIAL ACTIVITY OF CHEMICAL CONSTITUENTS FROM THE BARK OF *Diospyros adenophora* AND *Diospyros lanciefolia*, AND HEMISYNTHESIS OF LUPEOL AND BETULIN

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by

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LIST OF SYMBOLS

β	Beta
%	Percentage
mg	Microgram
μg mL^{-1}	Microgram per milliliter
±	Plus-minus
°C	Degree Celsius
μL	Microliter
μM	Micromolar
Å	Angstrom
cm	Centimetre
cm^{-1}	Per centimetre
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 millilitre
MHz	Mega Hertz
mL	Millilitre
mm	Millimetre
nm	Nanometre
mM	Millimole
α	Alpha
H	Hydrogen
δ	Delta
λ	lambda
π	Pi
^{1}H	Proton NMR
^{13}C	^{13}Carbon NMR
J	Coupling constants

LIST OF ABBREVIATIONS

δC	Carbon chemical shift
δH	Proton chemical shift
1D	One dimensional
2D	Two dimensional
3D	Three dimensional
^{1}H-NMR	Proton nuclear magnetic resonance
^{13}C-NMR	Carbon 13 nuclear magnetic resonance
IC_{50}	Inhibition concentration at 50 % activity
AACT	Acetoacetyl-CoA thiolase
ATR	Attenuated Total Reflection
ACTs	Artemisinin combination therapies
AL	Artemether-lumefantrine
AS+AQ	Artesunate-amodiaquine
ASMQ	artesunate-mefloquine
AIDS	Acquired immunodeficiency syndrome
BFE	Binding free energy
CC	Column chromatography
C=O	Carbonyl
CH	Methine
CH_2	Methylene
CH_3	Methyl
COSY	Homonuclear correlation spectroscopy
CFR	Case fatality rate

CCDC	Cambridge Crystallographic Data Centre
COVID	Coronavirus disease
$CDCl_3$	Deuterated chloroform
CDC	Centers for Disease Control and Prevention
CFR	case fatality rate (CFR)
CRT	chloroquine resistance transporter
D.	*Diospyros*
d	Doublet
dd	Doublet of doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DNA	Deoxyribonucleic acid
DCM	Dichloromethane
DMAP	Dimethylamine pyridine
D. adenophora	*Diospyros Adenophora*
D. lanciefolia	*Diospyros lanciefolia*
DHA	Dihydroartemisinin
EDG	Electron-donating groups
EWG	Electron-withdrawing groups
EtOAc	Ethyl acetate
Et3N	Triethylamine
et al.	And others
ELISA	Enzyme-linked immunosorbent assay
FT-IR	Fourier Transform Infrared
FMIPA	Faculty of Mathematics and Natural Sciences

HRESIMS	Higher resolution electron spray ionization mass spectroscopy
HPIA	Haem polymerization inhibition activity
HSQC	Heteronuclear Single Quantum Correlation
H_2O	Water
H_2SO_4	Sulphuric acid
HIV	Human immunodeficiency virus
HPIA	Haem polymerization inhibition assay
IR	Infrared
IPS	Institute of postgraduate school
IF	Immunofluorescence test
m	Multiplet
MD	Molecular docking
MM2	Molecular mechanics 2
MeOH	Methanol
MIC	Minimal inhibitory concentration
MS	Mass spectroscopy
NaOH	Sodium hydroxide
NMP	National Malaria Program
NMR	Nuclear Magnetic Resonance
NPs	Natural products
NAA	Nucleic acid amplification
OH	Hydroxyl
P. falciparum	*Plasmodial falciparum*
PDB	Protein Data Bank

PCR	polymerase chain reaction
POWO	*Plants of the World Online*
QSAR	Quantitative structure-activity relationship
RFU	Relative fluorescence units
RPMI	Roswell Park Memorial Institute
RBC	Red Blood Cell
RF	Retention factor
RDT	Rapid Diagnostic Tests
s	Singlet
SD	Standard deviation
t	Triplet
TLC	Thin layer chromatographic
TMS	Tetramethyl silane
UII	Universitas Islam Indonesia
UCSF	University of California, San Francisco
USM	Universiti Sains Malaysia
WFO	World Flora Online
WHO	World Health Organization

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PEMENCILAN, PENCIRIAN DAN AKTIVITI ANTI MALARIA SEBATIAN KIMIA DARIPADA KULIT KAYU *Diospyros adenophora* DAN *Diospyros lanciefolia* DAN HEMISINTESIS LUPEOL DAN BETULIN

ABSTRAK

Malaria merupakan penyakit berjangkit bawaan darah dan terus menimbulkan ancaman besar kepada golongan kanak-kanak serta dewasa, terutamanya di kawasan tropika dan subtropika di dunia. Dalam kajian ini, analisis fitokimia bagi ekstrak etil asetat (EtOAc) daripada kulit kayu dua tumbuhan genus *Diospyros* (*D.*) termasuklah *D. adenophora* dan *D. lanceifolia* telah dijalankan. Sebanyak sembilan (9) sebatian telah berjaya diasingkan daripada kedua-dua tumbuhan dengan menggunakan kaedah kromatografi turus (CC) dan kromatografi lapisan nipis (TLC). Sebatian-sebatian ini telah dikenalpasti sebagai triterpena pentasiklik (5), steroid (1), asid lemak (2) dan sebatian fenolik (1). Tiga sebatian telah diasingkan daripada ekstrak EtOAc kulit kayu *D. adenophora*, termasuklah lupenon (**50**), lupeol (**46**) dan betulina (**33**). Selain itu, pengasingan kromatografi ekstrak EtOAc kulit kayu *D. lanceifolia* telah membawa kepada pemencilan lapan sebatian seperti lupeol (**46**), betulina (**33)**, *β*-sitosterol (**47**), asid oleik (**58**), *α*-amirin asetat (**59**) gliseril trilinoleat (**60**) *β*-amirin (**61**) dan shinanolon (**62**). Oleh yang demikian, lupeol (**46)** dan betulin (**33**) yang merupakan sebatian terpencil utama daripada kedua-dua ekstrak tumbuhan ini telah digabungkan untuk hemisintesis melalui pengasilan, tindak balas hibrid dan tindak balas pengoksidaan, yang membawa kepada sintesis dua puluh tiga (23) sebatian terbitan. Sebatian terbitan lupeol (**46**) merangkumi **64a**-**64j** dan **67a**-**67b**, manakala sebatian terbitan betulin (**33**) terdiri daripada **65a**-**65j** dan **68**, masing-masing. Kesemua struktur sebatian-sebatian terpencil dan sebatian-sebatian terbitan telah dijelaskan

menggunakan pelbagai kaedah spektroskopi: 1D-NMR (^{1}H, ^{13}C dan DEPT-135), 2D-NMR (COSY, HSQC dan HMBC), FT-IR, HRESIMS dan dibandingkan dengan data daripada literatur. Tiga sebatian yang diasingkan daripada *D. adenophora* telah diuji untuk sifat antimalarianya melalui asai perencatan pempolimeran hem *in vitro* dengan menggunakan klorokuin sebagai ubat rujukan. Keputusan *in vitro* mendedahkan bahawa sebatian **46** dan **50** mempamerkan aktiviti tertinggi, dengan IC_{50} masing-masing bernilai 20.2 ± 17 dan 27.5 ± 23 μM, berbanding dengan ubat klorokuin, yang mempunyai IC_{50} sebanyak 37.5 ± 0.6 μM. Tambahan pula, aktiviti antiplasmodial *in vitro* ke atas semua sebatian terpencil, bersama-sama dengan tiga belas (13) sebatian terbitan, mendedahkan bahawa dua belas (12) sebatian mempamerkan aktiviti perencatan yang kuat dengan IC_{50} < 10 μM. Sebatian-sebatian berpotensi ini termasuklah sebatian **50**, **59**, **47**, **60**, **46**, **58**, **64g**, **64j**, **65f**, **67a**, **67b** dan **68** dengan IC_{50} masing-masing bernilai 0.3 ± 0.3, 0.3 ± 0.3, 0.3 ± .2. ± 7.4, 8.4 ± 4.9, 5.7 ± 8.9, 3.3 ± 0.02, 9.9 ± 6.1, 0.03 ± 0.05, 0.7 ± 0.8 dan 5.6 ± 6.4 μM. Secara ketara, aktiviti kuat diperhatikan pada sebatian **50**, **59**, **47**, **67a** dan **67b** berbanding dengan ubat rujukan artemisinin dan klorokuin (0.7 ± 0.3 dan 10.3 ± 2.9 μM). Kajian pengedokan molekul terhadap sebatian-sebatian ini mendedahkan tenaga pengikat yang menggalakkan dengan julat antara 6.0 – 9.8 kcal mol^{-1} dan seterusnya dibandingkan dengan ubat standard artemisinin dan klorokuin. Hasil kajian menunjukkan ikatan hidrogen dan pelbagai interaksi hidrofobik antara sebatian yang dikaji dan asid amino protein PfATP6, seperti ASN A:1039, VAL A: 984, ILE A: 981 dan ILE A: 981. Justeru itu, sebatian-sebatian ini terbukti sebagai struktur utama berpotensi untuk pengesahan lanjut sebagai calon berpotensi dalam membangunkan ubat antimalaria baharu.

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ABSTRACT

Malaria is a blood-borne infectious disease that continues to pose a significant threat to both children and adults, particularly in tropical and subtropical regions of the world. In this research, the phytochemical analysis of the ethyl acetate (EtOAc) bark extract of two *Diospyros* (*D.*) genus plants consisting of *D. adenophora* and *D. lanceifolia* was carried out. A total of nine (9) compounds were successfully isolated from both plants using column chromatography (CC) and thin layer chromatography (TLC) methods. The compounds were identified as five (5) pentacyclic triterpenes, one (1) steroid, two (2) fatty acids and one (1) phenolic compound. Three compounds were isolated from the EtOAc bark extract of *D. adenophora,* including lupenone (**50**), lupeol (**46**) and betulin (**33**). Besides, the chromatographic isolation of the EtOAc bark extract *of D. lanceifolia* has led to the isolation of eight compounds such as lupeol (**46**), betulin (**33**), *β*-sitosterol (**47**), oleic acid (**58**), *α*-amyrin acetate (**59**) glyceryl trilinoleate (**60**), *β*-amyrin (**61**) and shinanolone (**62**). Following this, the major isolated compounds of lupeol (**46**) and betulin (**33**) from the EtOAc extracts of two plants were combined for hemisynthesis via acylation, hybrid reaction and oxidation reaction, which led to the synthesis of twenty-three (23) derivative compounds. The derivatives of lupeol (**46**) encompassed of **64a**-**64j** and **67a**-**67b**, whereas the derivatives of betulin (**33**) consist of **65a**-**65j** and **68**, respectively. The structures of the isolated and derivatised compounds were elucidated using various spectroscopic

methods: 1D-NMR (^{1}H, ^{13}C and DEPT-135), 2D-NMR (COSY, HSQC and HMBC), FT-IR, HRESIMS and by comparison with data from the literature. The three compounds isolated from *D. adenophora* were examined for their antimalarial properties through *in vitro* heme polymerisation inhibition assay by employing chloroquine as a reference drug. The *in vitro* results revealed that compounds **46** and **50** exhibited the highest activity, with IC_{50} values of 20.2 ± 17 and 27.5 ± 23 μM, respectively, in comparison to the chloroquine drug, which had an IC_{50} of 37.5 ± 0.6 μM. Furthermore, the *in vitro* antiplasmodial activity of all the isolated compounds, along with thirteen synthesised derivatives, revealed that twelve (12) compounds exhibited potent inhibitory activity with IC_{50} < 10 μM. These potent compounds include compounds **50**, **59**, **47**, **60**, **46**, **58, 64g, 64j, 65f, 67a, 67b and 68** with IC_{50} values of 0.3 ± 0.3, 0.3 ± 0.3, 0.3 ± 0.3, 1.9 ± 2.2, 4.4 ± 7.4, 8.4 ± 4.9, 5.7 ± 8.9, 3.3 ± 0.02, 9.9 ± 6.1, 0.03 ± 0.05, 0.7 ± 0.8 and 5.6 ± 6.4 μM, respectively. Significantly, strong activity was observed in compounds **50**, **59, 47, 67a** and **67b** compared to reference drugs of artemisinin and chloroquine (0.7 ± 0.3 and 10.3 ± 2.9 μM). The molecular docking studies of these compounds revealed favourable binding energies in the range from 6.0 – 9.8 kcal mol^{-1} and were compared with the standard drugs artemisinin and chloroquine. The results showed hydrogen bonds and various hydrophobic interactions between the studied compounds and the amino acid of the PfATP6 protein, such as ASN A:1039, VAL A: 984, ILE A: 981 and ILE A: 981. Hence, these compounds might be promising lead structures for further validation as potential candidates in developing new antimalarial drugs.

CHAPTER 1

INTRODUCTION

1.1 Natural products

Over the past few decades, researchers have attracted significant interest in natural products (NPs), with a steadily growing interest in their potential applications (Sorokina and Steinbeck, 2020). Their products have proven important for numerous purposes, such as food, dyes, toxins, polymers, adhesives, waxes, fibres, fragrances, and traditional medicine (Zhang *et al.,* 2023). Natural products are chemical compounds produced by living organisms and synthesised by various biosynthetic pathways from which their structure can be relatively simple or very complex (Harvey *et al*., 2015; Sorokina and Steinbeck, 2020). NPs have a distinct chemical diversity that contributes to their various biological effects (Khalifa *et al*., 2019), and they have been used globally for many years as constituents of traditional medicines since before the development of modern chemical pharmacology (Sorokina and Steinbeck, 2020). Generally, NPs are categorised into primary and secondary metabolites (Elshafie *et al.,* 2023). Primary metabolites are essential for the normal functioning of growth, development, and reproduction in organisms (Zaynab *et al.,* 2019). While secondary metabolites do not directly contribute to these processes, they serve ecological functions and are known to have significant functions in plants, microorganisms, and the treatment of human disorders (Nyambe *et al.,* 2021).

Previously, the exclusive pharmaceutical treatments for various diseases comprised crude and semi-purified extracts derived from animals, microorganisms, minerals and plants (Jiménez-González *et al.,* 2023). Plants' secondary metabolites are nitrogen-containing compounds, phenolic compounds, and terpenoids (Anulika *et al.,* 2016). The bioactive constituents present in these secondary metabolites have contributed

to the development of new drugs for different biological activities (Hussein and El-Assary 2019). As a result, it is estimated that more than half of the pharmaceutical drugs currently in use are derived from natural sources, with plants being the models for the development of new compounds either derived directly from plants or through biosynthesis modification of the old ones (Cheuka *et al*., 2017; Najmi *et al.,* 2022). Plants produce various chemical compounds, each characterised by unique properties and serving a specific function (Muhseen *et al.,* 2021; Chaachouay & Zidane, 2024). Some of the pharmaceutical drugs isolated from plants are presented in Table 1.1 and their structures *are* outlined in Figure 1.1.

Table 1.1 List of chemical compounds derived from natural product plants

S/N	Compound	Treatment	Plant
1	Morphine (**1**)	For the relief of pain	*Papaveraceae somniferum*
2	Digitoxin (**2**)	For heart failure and cardiac arrhythmias	*Digitalis lanata*
3	Quinine (**3**)	For malaria treatment	*Cinchona calisa*
4	Artemisinin (**4**)	For malaria treatment	*Artemisia annua*
5	Atropine (**5**)	To reduce saliva and fluid in the respiratory tract	*Atropa belladonna*
6	Pilocarpine (**6**)	To treat dryness of the mouth and throat	*Pilocarpus microphyllus*
7	Galantamine (**7**)	To dementia treatment	*Galanthus nivalis*

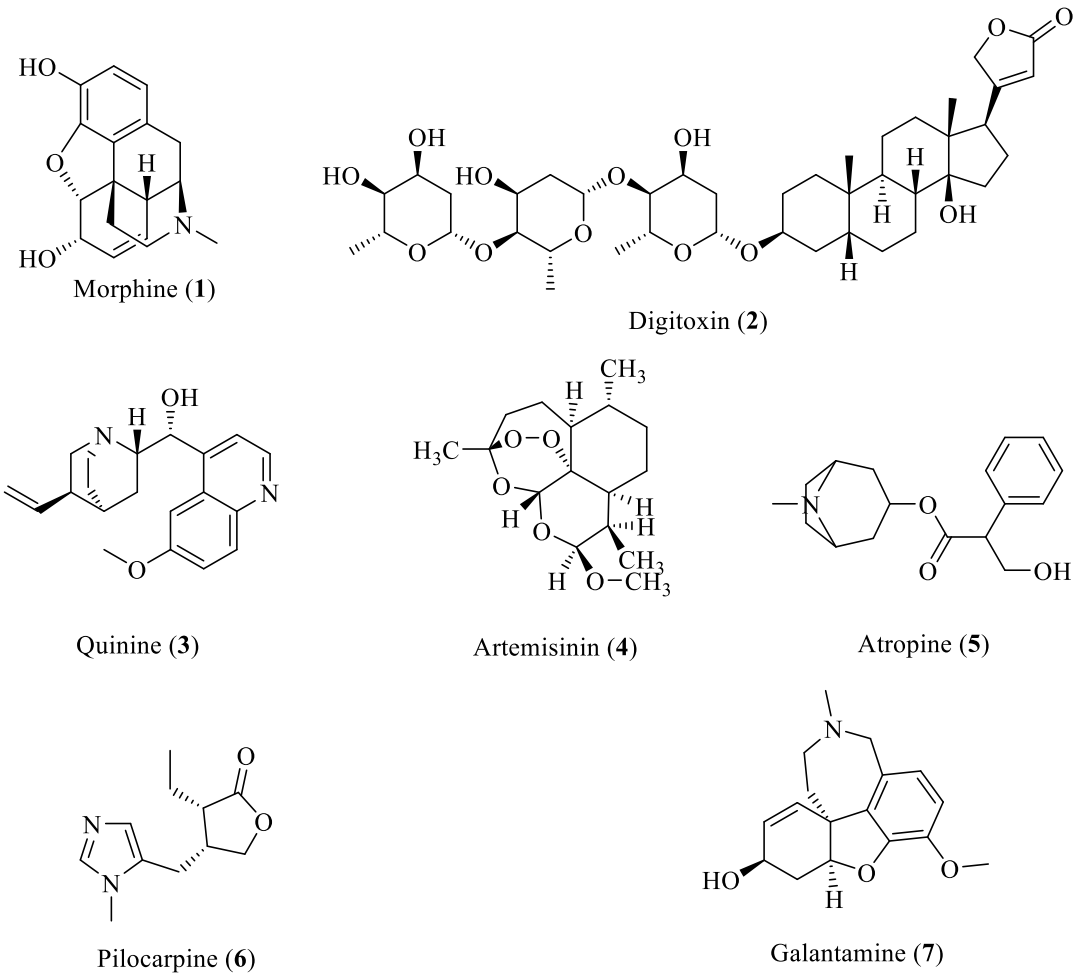


Figure 1.1 Chemical structures of some drugs derived from natural products

The rainforest in Southeast Asia is considered the oldest and most biologically diverse rainforest (Atanasov *et al*., 2015), in which Malaysian species ranks as the second most mega-diverse area globally, boasting a total of 13,739 plant species (Hamidah *et al.*, 2022). The flora of Malaysia is characterised by a tropical climate, which is marked by elevated temperatures, high humidity, and abundant rainfall throughout the year. These environmental conditions are conducive to plant growth, resulting in a rich and diverse thriving flora (Khanalizadeh *et al*., 2023). The rich diversity of Malaysian species is an important resource for the nation's development and could provide opportunities for the discovery of new chemical constituents that might possess useful biological applications

(Ahmad *et al.,* 2015). The first phytochemical study documented in the Malaysian Flora involved the screening of 205 plant species in Sabah. A few years later, the screening of 200 species in Peninsular Malaysia was conducted to identify the presence of alkaloids (Saw & Chung 2015). These initial reports signified the commencement of research on medicinal plants in Malaysia. Following these studies, additional plants were chemically investigated for alkaloids, saponins, triterpenes, and steroids (Ong and Norzalina 1999). Many plants have been explored and researched. However, due to the diversity of this flora, many Malaysian plants have never been the subject of any chemical or biological studies. Therefore, there is an urgent need to conduct research relating to the Malaysian tree flora, particularly the *Diospyros* genus (Ebenaceae family), i.e., *D. adenophora* and *D. lanceifolia* since plants of the Ebenaceae family are known as a rich source of secondary metabolites, including terpenoids (Rauf *et al.,* 2017).

D. adenophora and *D. lanceifolia* are native to Malaysia and are considered less concern in terms of research. These plants are undergoing a comprehensive study for the first time. Its phytochemical information is collected and analysed for its bioactive components. Furthermore, using the isolated chemical compounds as the starting materials, a series of modifications is performed through hemisynthesis via acylation, hybrid and oxidation reactions using Jones reagent. The modifications of these compounds are crucial to their efficacy in terms of their bioactive properties when compared to the precursor compounds. The isolated compounds, along with their synthesised derivatives, were assayed for antimalarial activity against *Plasmodium falciparum*.

1.2 Problem statement

Malaria remains a significant public health issue and exerts a considerable impact on a global scale. This vector-borne disease is caused by a specific species of the unicellular eukaryote known as the *Plasmodium* species (Pandey *et al.,* 2023). Currently, available drugs for the treatment of malaria, such as artemisinin and its derivatives, face limitations as a result of the increasing number of treatment failures in parts of Southeast Asia and it has spread, leaving little or no alternative treatment for malaria. The number of malaria cases in Malaysia requires urgency in exploring *Diospyros* species. (Shibeshi *et al.,* 2020; Chaniad *et al*., 2021; Hanboonkunupakarn & White, 2022). The *Diospyros* genus is known to be a significant source of bioactive compounds. Preliminary studies conducted by our research group with *Diospyros* species available in Malaysia showed significant anti-dengue activity (Abd Ghani *et al.,* 2024). These promising results encourage investigation on *D. adenophora and D. lanceifolia,* respectively, as potential inhibitors of *Plasmodium falciparum*. *D. adenophora* and *D. lanceifolia* received less concern regarding phytochemical analysis, the chemical composition of these plants remains unexplored, so far only two studies have been reported on *D. lanceifolia* investigating the antioxidant activity and antibacterial of this plant (Kalita & Baishya 2016), while no literature reported the phytochemical constituent as well as biological application of *D. adenophora*. Additionally, new analogues were produced through the modification of the isolated chemical constituent. The impact of these modification techniques remains uncertain and is expected to change the characteristics and bioactive properties in comparison to the precursor.

1.3 Significance of the study

Medicinal plants are known to be a significant source of bioactive compounds that would be used as lead candidates in the development of potent drugs (Dzouemo *et al.*, 2022). This study will explore the chemical compositions of *D. adenophora* and *D. lanceifolia* through isolation, purification, and characterisation techniques. Considering the medicinal value of this genus, exploring these plants may be valuable for medicinal purposes, while the nature of the compounds can be explored, and the major compounds will be modified. Therefore, this study focuses on isolating and investigating the chemical constituents of these two Malaysian plants through *in vitro* heme polymerisation inhibition and *in vitro* antiplasmodial activity against *P. falciparum*. These isolated compounds might be an alternative for the treatment of malaria to overcome the artemisinin resistance.

1.4 Research objectives

The general objectives of the study are to enhance knowledge and understanding of the secondary metabolites from the EtOAc bark extract of D. adenophora and D. lanceifolia and explore their potential as antimalarial agents through an in vitro study. This research aims to isolate compounds (terpenes, steroids, and fatty acids) from these two plants and to determine their potential in addressing the issue associated with drug resistance in malaria diseases.

1. To isolate and purify the chemical constituents (terpinene, steroids and fatty acids) from the bark extracts of *D. adenophora* and *D. lanceifolia* using different chromatographic techniques.
2. To elucidate and characterise the chemical structures of the isolated compounds derived from *D. adenophora* and *D. lanceifolia* using various spectroscopic methods.
3. To evaluate the *in vitro* antimalarial activity of the isolated compounds against *beta* hematin polymerisation inhibition and antiplasmodial activity against *Plasmodium falciparum*.
4. To perform chemical modifications on the major compounds isolated from the two plants and subject some of the synthesised derivatives to *in vitro* antiplasmodial activity.
5. To subject the active compounds from the *in vitro* antimalarial assay to molecular docking study using AutoDock Vina.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

This chapter offers a comprehensive study of the Ebenaceae family, particularly emphasising the *Diospyros* genus, terpenoids, phenolic, and fatty acids. It also explores the Ebenaceae family's morphology, habitat, distribution, classification, and traditional medicinal uses. Additionally, it sheds light on two specific plants, *D.* a*denophora* and *D. lanceifolia*. Afterwards, a review of compounds derived from various plants in the *Diospyros* genus that exhibit good antimalarial activity has been tabulated in Table 2.2, while the chemical structures of these compounds are presented in Figure 2.7. Subsequently, the biological activity of some species of this genus is summarised in Table 2.3. Finally, a detailed explanation of malaria diseases and molecular docking is discussed.

2.2 Ebenaceae family

2.2.1 Distributions and habitat

The Ebenaceae family is sometimes called the ebony family, they are a pantropical family that comprises over 800 species (POWO, 2023). The Ebenaceae family is a prominent group of plants that provide a range of economically important products, including timber and fruits and is considered a remarkable forest component of Africa and Asia. Their ebony wood is one of the exotic materials with a long history and is often used in carvings, cabinetry, and ornamental furniture production (Steven & Luteyn, 2021). The family of Ebenaceae is made up of three genera, including *Lassiocarpa*, *Euclea*, and *Diospyros* (Sharma, 2017). *Lissocarpa* is a native American, while *Euclea* species are characterised by their growth as trees or shrubs, reaching heights

of 10 m to 12 m tall (World Flora Online, 2023). Moreover, *Diospyros* is pantropical and is considered the largest genus within the Ebenaceae family, typically ranging from small to medium-sized plants found in the forest understory, although certain species can grow as large as canopy trees (Wijedasa *et al.,* 2012).

2.2.2 General appearance and morphology

The Ebenaceae family generally consists of tree-like shrubs, with certain species exhibiting evergreen characteristics, while others are classified as deciduous. The bark of these plants is frequently black, fragile, and resembles charcoal, although some species possess hardwood that appears black. The leaves of certain species commonly exhibit dorsiventral characteristics, whereas others display isobilateral traits. Most of these species are dioecious, with monoecious and polygamous occurrences less common (Ugbogu *et al.,* 2016). In addition, the species are also rich in flowering when bearing male flowers and uniflorous when bearing female flowers. The flower of most species is unisexual, very rarely and functionally, most especially in *Diospyros* species. Typically, male and female flowers are actinomorphic, articulated at the base, and have three to five numerous structures (Ugbogu *et al.,* 2016). In *Diospyros,* typically the calyx is accrescent on fruits, the inflorescence is cymose or fasciculate (although female flowers are usually solitary); fruits are typically multi-seeded, rarely one-seeded (Rauf *et al.,* 2017). The seeds are elongated and typically flattened laterally, less commonly circular in cross-section, or somewhat irregular in shape, usually with a persistent, distinctly raised (rarely inconspicuous), straight, and occasionally branched vascular strand that wraps around the fruit longitudinally in a loop; the embryo is straight or slightly curved in its plane (Bati *et al.* 2024). In species with long-lived cotyledons, each cotyledon typically has two, and in species with short-lived cotyledons, *Diospyros* has a continuous cylinder of xylem that is only broken up by thin rays, while in *Royena* and *Euclea*, ray cells have single,

spherical silica grains (Bati *et al.* 2024). The leaves have complete margins, usually alternating, very rarely opposite to sub-opposite in all-tropical. While in *Euclea* calyx species such as *Euclea coriacea, Euclea divinorum*, *Euclea natalensis, Euclea racemosa* and *Euclea undulate,* both subspecies make dense, evergreen, leafy shrubs or small trees that are slender and robust up to 9 m tall with a single trunk up to 30 cm in diameter (Maroyi, 2018).

2.2.3 Classification of tribes

The family of Ebenaceae can be classified into the botanical taxonomic rank (POWO, 2023) as listed below

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

2.2.4 Traditional uses of Ebenaceae

The Ebenaceae family is widely distributed throughout the tropics and subtropics regions with occasional distribution into temperate regions (Rauf *et al.,* 2017; Samuel *et al.,* 2019). Various species from this family are commercially important either for their leaves, edible fruits, or their timber (Rauf *et al.,* 2017). Species of this family are known to have local therapeutic use, like menstruation disorders, leprosy, asthma, dysentery, and abdominal pain (Sharma, 2017). For example, in Nigeria, *D. mespiliformis* is used to treat malaria (Luka *et al.,* 2014). Bark and root are used for the traditional treatment of fever, headaches, diarrhoea, cough, constipation and skin problems (Das *et al*., 2024).

Moreover, the people of Kenya chewed the fruits of *Euclea divinorum* to detoxicate the blood and reduce abdominal pain (Kigen *et al.,* 2017). Also, the root of *Euclea cripsa* relieves constipation among children in Botswana, while its leaves and bark are used to manage rheumatism and diabetes (Chinsamy *et al.,* 2016). Besides, in South Africa, the root of indigenous *Euclea* is used for skin as lotion and the crushed root of this species is used for the treatment of toothache and relief of pain and headache (Lall, 2019). Some species treat diseases like bloody faeces, dysentery, chewing stick, antioxidant, dermatological problems (Sulub-Tun *et al.,* 2020; Sharma *et al*., 2020; Zreen *et al*., 2022; Ribeiro *et al.,* 2023).

2.3 *Diospyros* genus

Diospyros species are evergreen shrubs and trees distributed in the tropical and subtropical regions of the world, like China, India, Indonesia, Malaysia, Korea, India, Pakistan, the Middle East, and some regions of Africa (Rauf *et al.,* 2017; Sharma *et al.*, 2020). *Diospyro*s species have been used in traditional medicine to treat various ailments, particularly infectious diseases. The plants of this genus are known to be a rich source of secondary metabolites, such as naphthoquinones, long-chain fatty acids, triterpenoids, and tannins (Ribeiro *et al.,* 2023). Various chemical constituents have been isolated from the species of this genus, and the compounds isolated from the species of this genus have demonstrated interesting biological applications supported by *in vitro* biological, *in vivo* pharmacological, and clinical tests (Peyrat *et al.*, 2016; Rauf *et al.*, 2017; Ribeiro *et al.*, 2023).

2.3.1 Botany and distribution

Diospyros is a large genus in the family of Ebenaceae, comprising more than 500 species (Jahanbanifard *et al.*, 2020). The trees and bushes of the *Diospyro*s genus come in both deciduous and evergreen varieties. Most leaves are coriaceous, alternating, and simple, flowers are actinomorphic, the inflorescence is a cyme, and the persistent, gamosepalous calyx (Akagi *et al.*, 2014). The fruits of most species are globose berries; some are multiloculate and indehiscent. seeds occur in 1-16 numbers per fruit, which are pendulous and large (Turner *et al.*, 2013). The main physical characteristics of plants of the *Diospyros* genus are their hard and solid dark timber, which is important to the socioeconomic development of the region. Heartwood, or the inner part of a mature trunk of a typical ebony tree, is highly appreciated for its durability and colour, ranging from jet black to dark brown (Jahanbanifard *et al.*, 2020). Another important characteristic possessed by this genus is recognised by the sexuality of plants that could be identified as male and female based on the flowers they produce (Akagi *et al.*, 2014; Nyambe *et al.*, 2021).

2.4 *Diospyros adenophora* (*D. adenophora*)

2.4.1 Botanical description

Diospyros adenophora, commonly known as Kayu ayang, is a plant indigenous to Malaysia that is being revised in the Flora of Peninsular Malaysia and the Flora of Singapore. In Peninsular Malaysia, a total of 65 *Diospyros* species have been identified, with 13 classified as endemic (De Kok and Puglisi, 2021). *Diospyros adenophora* is a confirmed species of the *Diospyros* genus in the family Ebenaceae, found in lowland and limestone hill forests, growing up to 10 m tall and is endemic to Peninsular Malaysia (Hanum *et al.*, 2001). 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 (POWO, 2023). The fruits are velvety and spherical when young, glabrous, and elongated when ripe. When ripe and dry, they tend to shrivel into as many longitudinal furrows as 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, and recurved sides and is slightly expanded. This species occurs in mixed dipterocarp lowland forests in Peninsular Malaysia (Perak, Kelantan and Terengganu) and Borneo (Sabah, Sarawak and Kalimantan) (De Kok and Puglisi, 2021).

Diospyros adenophora

Taxonomy

Kingdom	:	Plantae
Division	:	Magnoliophyte
Class	:	Magnoliopsida
Order		Ericales
Family	:	Ebenaceae
Genus	:	*Diospyros*
Species	:	*Adenophora* Bakh
Vernacular	:	Kayu ayang (Malay)
Residential		Native to Malaysia

A B

Figure 2.1 *Diospyros adenophora* leaves (A) and flowers (B)
(Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur**)**

2.5 *Diospyros lanceifolia* (*D. lanceifolia*)

2.5.1 Botanical description

D. lanceifolia Roxb is one of the species of the *Diospyros* genus from the Ebenaceae family. The name came from Latin, meaning "lance-shaped leaves. The trees of this plant are very big and can grow up to 27 m in height. The twigs of this plant are reddish brown when it is young, and they turn black or dark brown when they are mature. The stem bears cluster flowers up to ten meters while the fruits are spherical and have a diameter of up to 2.5 cm (Kalita & Baishya, 2016). The species of this plant can be found in the lowland and hill rainforests in the Philippines, Indonesia, and India (Kichu *et al.*, 2015). Flowers are monoecious, where the male and female flowers are produced on separate trees, found along the axils. Male flowers are 4-petalled, held in clusters of 3 to many flowers, they are bell-shaped, hairy outside and smooth inside with a slight purple

tinge. The female flowers are 4 to 5-petalled, borne singly, sometimes in clusters of 3; they are unshapen, yellowish, and hairy on both sides.

Diospyros lanceifolia

Taxonomy

Kingdom	:	Plantae
Division	:	Magnoliophyte
Class	:	Magnoliopsida
Order		Ericales
Family	:	Ebenaceae
Genus	:	*Diospyros*
Species	:	*Lanceifolia*
Vernacular	:	Kayu Malam (Malay)
Residential		Native to Malaysia

A B

Figure 2.2 *Diospyros lanceifolia* leaves, fruit (A) and tree (B)
(Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur**)**

2.6 Chemical constituent

In light of the relationship between humankind and plants, it is essential to acknowledge that plants have fulfilled numerous human needs, including medicines. Plant extracts and products play a significant role in our needs, especially as pharmaceutical and biological agents. This section highlights some molecules and secondary metabolites that were previously found in the Diospyros genus.

2.6.1 Terpenoids

Terpenes are naturally occurring compounds produced in the plant kingdom as secondary metabolites and are known to possess different biological applications (Noushahi *et al*., 2022). The name terpenes originated from the word turpentine, a product of coniferous oleoresins. They are a large class of hydrocarbons composed of five-carbon isoprene units typically linked together in a head-to-tail fusion with different degrees of oxidation, unsaturation, functional groups, and ring closures, resulting in a rich diversity of structural classes found in them (Mabou and Yossa 2021). The joining of head-to-tail units is the most common in triterpenoids and carotenoids, while some compounds share by joining the head to the middle, for example, irregular monoterpenoids (Ludwiczuk *et al.*, 2017). The terpene-like compounds are classified according to the number of isoprene units that exist in the parent nucleus, ranging from one to many, and are considered the richest secondary metabolites, with more than 20,000 molecules (Mabou & Yossa 2021). Terpenoids originate from fatty acids but differ from fatty acids due to cyclised and extensive branching in their structure (Noushahi *et al*., 2022). Generally, terpenes are classified according to the number of isoprene units (n) in the molecule, as tabulated in Table 2.1.

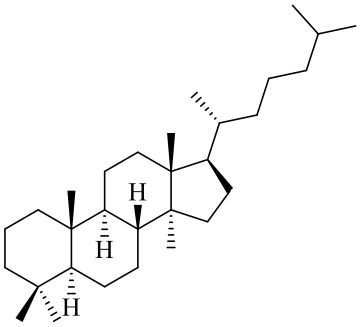
Table 2.1 Classification of terpenes

Number of carbon atoms	Value of n	Class
5	1	Hemiterpenes C_5H_8)
10	2	Monoterpenes ($C_{10}H_{16}$)
15	3	Sesquiterpenes ($C_{15}H_{24}$)
20	4	Diterpenes ($C_{20}H_{32}$)
25	5	Sesterpenes ($C_{30}H_{48}$)
30	6	Triterpenes ($C_{30}H_{48}$),
40	8	Tetraterpenes ($C_{40}H_{64}$)
>40	>8	Polyterpenes $(C_{40}H_{64})_n$

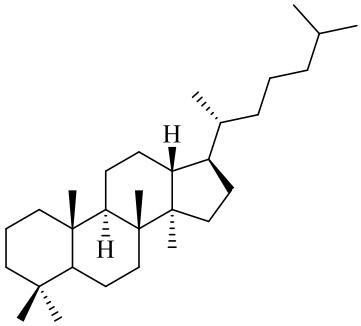
Terpenoids are modified terpenes where oxidised methyl groups are moved or removed at certain positions or additional functional groups are added (Yang *et al.,* 2020). The modified compounds of this class are biologically more active and widely used as medicine to treat different medical conditions. Many terpenoid compounds have intriguing and various therapeutic potentials, making them useful as biological tools or lead compounds for drug discovery research (Mabou & Yossa, 2021).

2.6.2 Triterpenoids

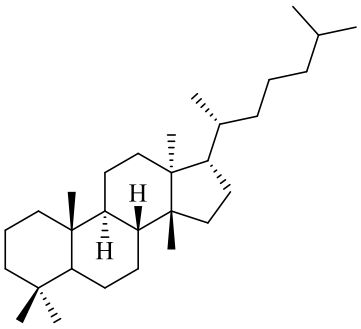
Triterpenoids are molecules with a carbon skeleton composed of six isoprene units; most of them are carboxylic acids, alcohols, or aldehydes with comparatively complex cyclic structures (Ludwiczuk *et al.,* 2017). The active compounds from this class have a high degree of structural homogeneity, with the main difference being their configuration, which differs according to the initial conformation that the squalene epoxide adopted (Cseke *et al.,* 2016). Triterpenoid structures are of different types; the most well-known triterpenoid skeletons are those from tetracyclic and pentacyclic derivatives. The tetracyclic derivatives include lanostane (**8**), dammarane (**9**), euphane (**10**), and cucurbitacin (**11**) and are presented in Figure 2.3.



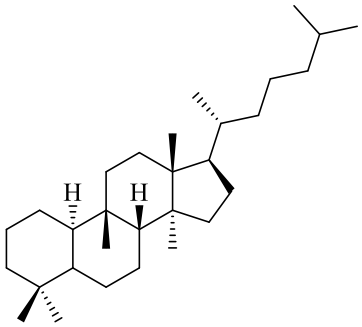
Lanostane (**8**)



Dammarane (**9**)



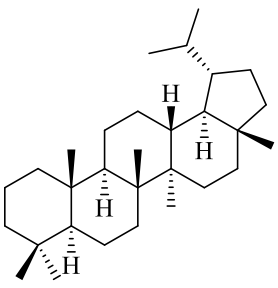
Euphane (**10**)



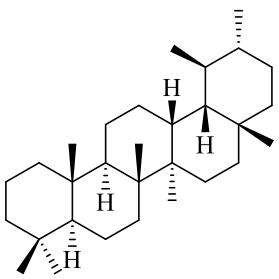
Cucurbitacin (**11**)

Figure 2.3 Example of tetracyclic derivatives

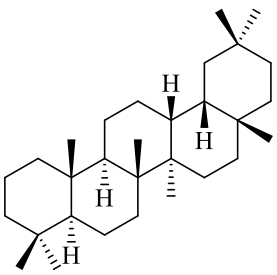
Pentacyclic triterpenes are secondary metabolites produced by the cyclisation of squalene. These compounds can be obtained in various plant parts, such as bark, cork, and the waxy layers on leaves or peels (Laszczyk, 2009). In general, they are widely distributed across different plant species. The derivatives of this group, including lupane (**12**), ursane (**13**) and oleanane (**14**), are illustrated in Figure 2.4.



Lupane (**12**)



Ursane (**13**)



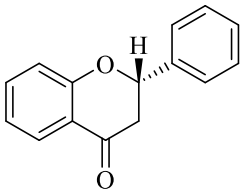
Oleanane (**14**)

Figure 2.4 Example pentacyclic derivatives

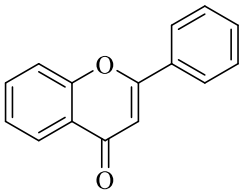
2.7 Phenolics

Phenolic compounds represent a category of secondary metabolites predominantly found in various plant species, displaying significant structural diversity. They may be present in the form of glycosides or aglycones, as well as in matrix or free-bound states, and are primarily composed of either polymerised or monomeric structures (Alara *et al.,* 2021). Furthermore, the distribution of these compounds within plants is not uniform, and their stability can vary considerably**.** Phenolics are known for their role in creating variations in the flavour and colour of various wine brands. In the plant kingdom, phenolics encompass lignans, tannins, phenolic acids, stilbenes, and flavonoids (Fallah *et al.,* 2020)**.**

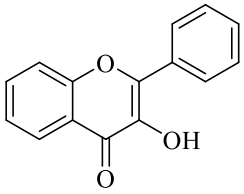
Flavonoids are the primary polyphenolic compounds found in human nutrition. They are characterised by a flavan backbone consisting of 15 carbon atoms arranged into three rings, designated as C6–C3–C6, referred to as A, B, and C, and they are categorised into subgroups, including flavones (**15**), flavanones (**16**), flavanols (**17**), isoflavones (**18**) (Fu *et al.,* 2021). This classification is based on the oxidation state of the central carbon ring within the flavonoid structure. Differences in the structure of each subgroup can be partially explained by the specific patterns and levels of hydroxylation, prenylation, glycosylation, or methoxylation (Alara *et al.,* 2021). The structures of the mentioned flavonoid compounds are given in Figure 2.5.



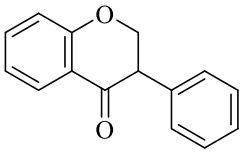
Flavanone (**15**)



Flavone (**16**)



Flavanol (**17**)



Isoflavones (**18**)

2.5 Some classes of flavonoids

2.8 Fatty acid

Fatty acids are essential compounds in complex metabolic pathways, which play a major role in biological processes. The type of fat consumed from various dietary sources directly impacts overall health (De Carvalho & Caramujo, 2018). Fatty acids can be classified as either saturated or unsaturated carboxylic acids, characterised by carbon chains that range from 2 to 36 carbon atoms in length (Tvrzicka *et al.,* 2011). The compounds found in them are involved in plant defence and interspecies communication, and they may serve as extracellular signals or intracellular mediators (De Carvalho & Caramujo, 2018). Generally, the common number of carbons in fatty acid compounds that occur in plant membranes includes 16 or 18 carbons with 0, 1, 2, or 3 double bonds in their structure. The length of the chain, the number of methyl branches, positions, and configuration of unsaturation for example (double and triple bonds), oxidations (hydroxy, keto, epoxy), cyclic structures (cyclopropane, cyclopentene, furan, phenyl), fluoro, cyano, are what diversified the fatty acid structure (Ohlrogge *et al.,* 2018). Remarkable fatty acid compounds are reported with significant biological applications, including

antibacterial activity (Casillas-Vargas *et al.*, 2021). Examples of fatty acid compounds include linoleic acid (**19**), oleic acid (**20**), lauric acid (**21**), capric acid (**22**), stearic acid (**23**), docosahexaenoic acid (**24**) and eicosatetraenoic acid (**25**). The chemical structure of the fatty acid compound is presented in Figure 2.6.

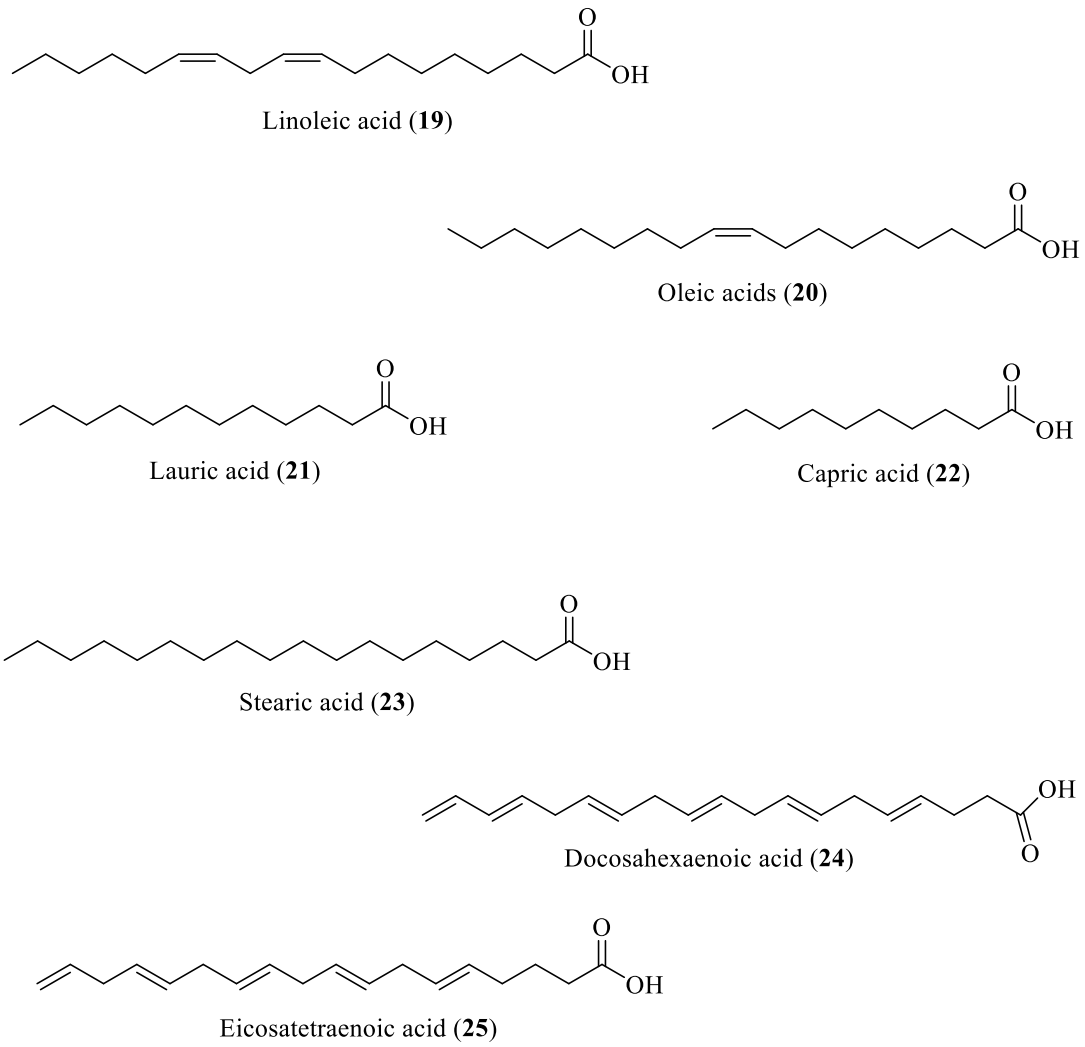


Figure 2.6 Structure of some fatty acids from the *Diospyros* genus

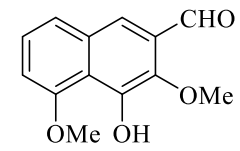
2.9 Previous chemical constituents isolated from the *Diospyros* genus

The genus *Diospyros* (family Ebenaceae) encompasses many species, which have been studied for their chemical constituents and their potential medicinal properties. Previously, various plants of the *Diospyros* genus have undergone phytochemical investigation leading to the isolation of several compounds, including naphthoquinones, triterpenoids, steroids and fatty acid compounds (Rauf *et al*., 2017; Rauf *et al.,* 2020). Table 2.2 lists of compounds isolated from *Diospyros* species that showed antimalarial activity, while Figure 2.7 presents the structure of the listed compounds.

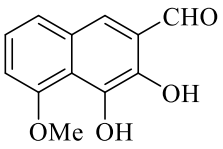
Table 2.2 Plant parts and their previously isolated compounds from *Diospyro*s species that showed antimalarial activity

Plants and their parts	**Chemical compound**	**Reference**
D. assimilis (*Root)*	4-hydroxy-3,5-dimethoxy-2-naphthaldehyde (**26**) 4-hydroxy-5-methoxy-2-naphthaldehye (**27**) 5-hydroxy-4-methoxy-2-naphthaldehyde (**28**) Diospyrin (**29**) 8-hydroxyisodiospyrin (**30**)	Ganapaty *et al*., 2006
D. canaliculata (Stem bark)	Plumbagin (**31**) Canaliculatin (**32**) Betulin (**33**) Ismailin (**34**) Gerberinol (**35**) Betulinic acid (**36**)	Lenta *et al*., 2015
D. filipendula (Root)	Betulonaldehyde (**37**) 3-O-*trans-p*-coumaroylalphatolic acid (**38**)	Wisetsai *et al.*, 2021
D. glans (Bark)	Usnic acid (**39**) 11-oxo-acetyl ursolic acid (**40**) 13,28-epoxy urs-11-ene-3,28-dione (**41**) Betulinic acid (**36**)	Peyrat *et al.*, 2016

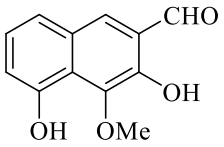
Plants and their parts	**Chemical compound**	**Reference**
	(3*β*)-3,23-dihydroxylup-20(29)-en-28-oic acid (**42**) (3*β*)-3-(acetyloxy)-urs-12-en-28-oic acid (**43**) Betulinic aldehyde (**44**) (3*β*)-3,23-dihydroxylup-12,20(29)-dien-28-oic acid (**45**)	
D. glandulosa and rhodocalyx (Woods)	Lupeol (**46**) *β*-sitosterol (**47**) Stigmasterol (**48**) Diospyrin (**29**)	Theerachayanan *et al*., 2007
D. quaesita (leaves, twigs, and branches)	Betulinic acid 3-caffeate (**49**)	Ma *et al*., 2008
D. rubra (bark)	Lupeol (**46**) Lupenone (**50**) Betulin (**33**) Lupeol acetate (**51**)	Prachayasittikul *et al*., 2010.



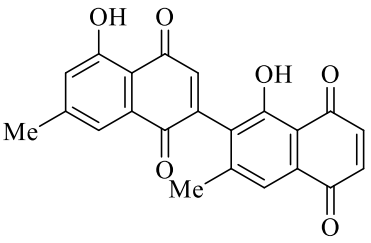
4-hydroxy-3,5-dimethoxy
-2-naphthaldehyde (**26**)



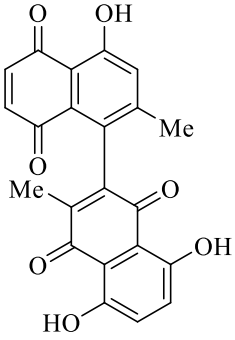
4-hydroxy-5-methoxy
-2-naphthaldehye (**27**)



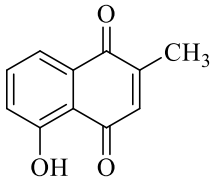
5-hydroxy-4-methoxy-2-
naphthaldehyde (**28**)



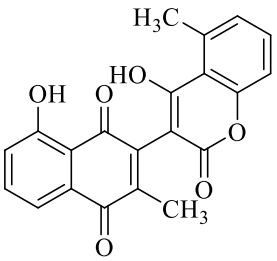
Diospyrin (**29**)



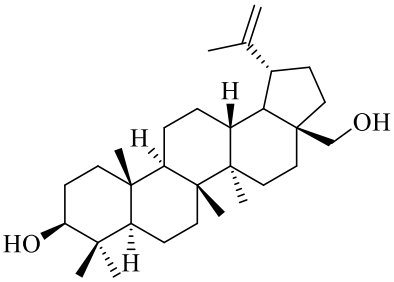
8-hydroxyisodiospyrin (**30**)



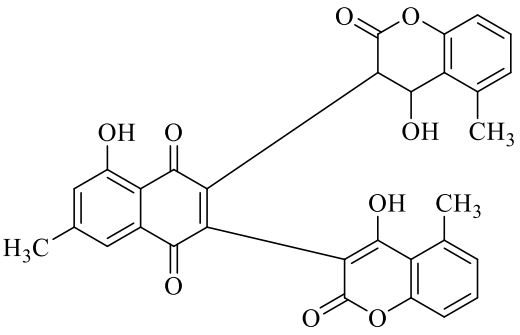
Plumbagin (**31**)



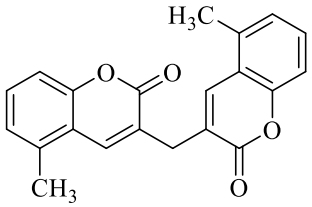
Canaliculatin (**32**)



Betulin (**33**)



Ismailin (**34**)



Gerberinol (**35**)

Figure 2.7 Chemical structures of isolated compounds from *Diospyros* species that shows antimalarial activity