DEVELOPMENT OF STANDARDIZED EXTRACT OF *CARICA PAPAYA* LEAF AS POTENTIAL PHARMACEUTICAL FORMULATION FOR DENGUE

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DEVELOPMENT OF STANDARDIZED EXTRACT OF *CARICA PAPAYA* LEAF AS POTENTIAL PHARMACEUTICAL FORMULATION FOR DENGUE

by

TAN SIEW MEI

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LIST OF SYMBOLS

%	Percentage
% RH	Percentage of relative humidity
% v/v	Percentage in volume per volume
% w/v	Percentage in weight per volume
% w/w	Percentage in weight per weight
%/min	Percent per minute (rate of change as a percentage over time)
/L	Per litre
:	Ratio
<	Less than
=	Equal
>	More than
±	Plus-minus sign
×	Times
≈	Approximately
≤	Less than or equal to
°	Degree (expressed as 2θ angle positions)
µg/g	Microgram per gram
µg/mL	Microgram per millilitre
µL	Microlitre
µmol/L	Micromoles per litre
µm	Micrometre
a.u.	Arbitrary unit
AUC	Area under the curve
℃	Degree Celsius

cm	Centimetre
cm^{-1}	Reciprocal of centimetre
C_e	Critical concentration
C_{max}	Maximum concentration
CPS	Counts per second
df	Degree of freedom
fl	Femtolitres, a unit of volume equal to 10^{-15} litres
g	Grams
g/100g BW	Grams per 100 grams of body weight
g/L	Grams per litre
h	Hours
II	Two
III	Three
kV	Kilovolt
L	Litres
LD_{50}	Lethal Dose, 50% (also known as median lethal dose)
Log *P*	Log of partition coefficient
M	Molarity
mg	Milligrams
mg GAE/g	mg gallic acid equivalents per g of sample extracts
mg RE/g	mg rutin equivalents per g of sample extracts
mg/kg	Milligrams per kilogram
mg/mL	Milligrams per millilitre
min	Minutes
mL	Millilitres
mL/min	Millilitres per minute
mm	Millimetres

mmol/L	Millimoles per litre
n	Number
ng/mL	Nanograms per millilitre
nm	Nanometres
p	p-value
Pa·s	Pascal Second
pg	Picograms, a unit of mass equal to 10^{-12} grams
$P_{o/aq}$	Partition coefficient of n-octanol/aqueous
R^2	Regression coefficient
rpm	Rotation per minute
s	Seconds
S/m	Siemens per metre
$t_{1/2}$	Half-life
T_{max}	Time to peak drug concentration
U/L	Units per litre
w/v	Weight per volume
wt%	Percentage by weight
λ	Wavelength

LIST OF ABBREVIATIONS

2D	Two-Dimensional
3D	Three-Dimensional
3Rs	Replacement, Reduction, and Refinement
AC	Alternating Current
ADE	Antibody-Dependent Enhancement
ADRs	Adverse Drug Reactions
$AlCl_3$	Aluminium Chloride
ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredient
ATR	Attenuated Total Reflectance
ATR-FTIR	Attenuated Total Reflectance-Fourier Transform Infrared
BCS	Biopharmaceutics Classification System
C	Capsid
C. papaya	*Carica papaya*
CO_2	Carbon Dioxide
CP	Freeze-Dried *Carica papaya* Leaf Extract
DENV	Dengue Virus
DHF	Dengue Haemorrhagic Fever
DMAc	N, N-Dimethylacetamide
DMF	N, N-Dimethylformamide
DOX	Doxorubicin
DPX	Dibutylphthalate Polystyrene Xylene
DSS	Dengue Shock Syndrome
E	Envelope
e.g.	exempli gratia (also known as for example)
EDS	Energy-Dispersive
EIP	Extrinsic Incubation Period
EL100	Eudragit L100
EL100-55	Eudragit L100-55
ELISA	Enzyme-Linked Immunosorbent Assays

ES	Electrospun
FDA	Food and Drug Administration
$FeCl_3$	Ferric Chloride
FTIR	Fourier Transform Infrared
GAE	Gallic Acid Equivalents
GCP	Good Clinical Practice
GLP	Good Laboratory Practices
Hb	Haemoglobin
HPLC	High-Performance Liquid Chromatography
HPMC	Hydroxypropyl Methylcellulose
i.e.	id est (also known as that is)
IACUC	Institutional Animal Care and Use Committee
ICH	International Conference on Harmonisation
Ig G	Immunoglobulin G
Ig M	Immunoglobulin M
IMR	Institute for Medical Research
IR	Infrared
KH_2PO_4	Potassium Dihydrogen Phosphate
M	Membrane
MB-HCl	Mebeverine Hydrochloride
MC	Methylene Chloride (also known as dichloromethane)
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Cell Volume
MGV	Mean Gray Value
Na_2HPO_4	Disodium Hydrogen Phosphate
NaCl	Sodium Chloride

$NaNO_2$	Sodium Nitrate
NBF	Neutral Buffered Formalin
NPRA	National Pharmaceutical Regulatory Agency
NSAID	Non-Steroidal Anti-Inflammatory Drug
ODF	Orodispersible Fibre Film
OPD	Optical Path Difference
PCL	Polycaprolactone
PCV	Packed Cell Volume
PDT	Poly(dodecylthiophene)
PEA	Polyethyl Acrylate
PEO	Poly(ethylene oxide)
PIS-CF	Participant Information Sheet and Consent Form
PLGA	Polylactic-co-glycolic acid
PLLA	Poly (L-lactic acid)
PMAA	Poly(methacrylic acid)
PMMA	Polymethyl Methacrylate
PVA	Polyvinyl Alcohol
PVDF	Polyvinylidene Difluoride
PVP	Polyvinylpyrrolidone
PVPK90	Polyvinylpyrrolidone K90
RDW	Red Cell Distribution Width
RE	Rutin Equivalents
RNA	Ribonucleic Acid
ROW	Relative Organ Weight
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SD	Sprague Dawley
SEM	Scanning Electron Microscopy

SFE	Supercritical Fluid Extraction
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
SHS	Simulated Human Saliva
SI	Sink Index
TFC	Total Flavonoid Content
THF	Tetrahydrofuran
Total RBC	Total Red Blood Cell
Total WBC	Total White Blood Cell
TPC	Total Phenolic Content
UAE	Ultrasound-Assisted Extraction
WDS	Wavelength-Dispersive
WHO	World Health Organisation
XRPD	X-ray Powder Diffraction
ZnS	Zinc Selenide

LIST OF APPENDICES

PEMBANGUNAN EKSTRAK PIAWAI DAUN *CARICA PAPAYA* SEBAGAI FORMULASI FARMASEUTIKAL BERPOTENSI UNTUK DENGGI

ABSTRAK

Demam denggi kekal sebagai cabaran kesihatan global utama dengan pilihan rawatan yang terhad tersedia. Ekstrak daun betik *Carica* telah digunakan secara tradisional untuk kesan merangsang trombosit dalam pengurusan denggi. Walau bagaimanapun, penggunaan praktikalnya dihalang oleh rasa pahitnya dan ketidakstabilan sebatian bioaktif yang bersifat sensitif haba. Kajian ini bertujuan untuk menangani cabaran-cabaran ini dengan membangunkan ekstrak piawai daun betik *Carica* menggunakan elektrospinning sepaksi untuk menghasilkan filem serat orodispersibel (lapisan) yang berkesan dan rasa sedap. Objektif kajian ini adalah: (1) untuk melakukan pengekstrakan sebatian bioaktif daripada daun betik *Carica* dan menjalankan kajian pra-perumusan ke atas ekstrak piawai yang dikering beku; (2) untuk membangunkan dan mencirikan formulasi ekstrak bioaktif piawai daun betik *Carica* menggunakan pelbagai sistem pembawa polimer yang dihasilkan oleh teknik elektrospinning sepaksi; (3) untuk menilai pelepasan dan kestabilan penyimpanan filem serat orodispersibel (lapisan) yang mengandungi ekstrak bioaktif piawai daun betik *Carica*; dan (4) untuk menyiasat ketoksikan oral akut in vivo dan melakukan penilaian rasa terhadap formulasi filem serat (lapisan) yang mengandungi ekstrak bioaktif piawai daun betik *Carica*. Secara metodologi, kajian ini melibatkan pengekstrakan sebatian bioaktif daripada daun betik *Carica* yang dikering beku, diikuti dengan merumuskan pelbagai filem serat orodispersibel (lapisan) yang mengandungi ekstrak piawai menggunakan teknik elektrospinning sepaksi. Formulasi ini dinilai untuk sifat fizikokimianya, termasuk kandungan lembapan, pelepasan ubat,

dan kestabilan. Selepas itu, keselamatan dan kesedapan formulasi dinilai melalui kajian ketoksikan akut in vivo dan penilaian rasa. Dapatan kajian menunjukkan bahawa teknik elektrospinning sepaksi berjaya mengekalkan rutin sebatian bioaktif sambil menyekat rasa kepahitan ekstrak. Antara rumusan yang dihasilkan, filem ES CP/Gelatin:PVA 1:5 menunjukkan kestabilan yang dipertingkatkan, pelepasan sebatian bioaktif yang terkawal, dan penilaian rasa sedap yang menggalakkan. Selain itu, semua filem serat orodispersibel (lapisan) Gelatin/PVA diterima dengan baik pada dos 2000 mg/kg berat badan pada tikus Sprague Dawley betina semasa ujian ketoksikan yang dijalankan sebelum penilaian rasa. Penyelidikan ini menyumbang kepada pembangunan rawatan botani yang berdaya maju dan mampan untuk denggi dengan mengoptimumkan ekstrak daun betik dalam formulasi orodispersibel yang mesra pengguna. Pendekatan elektrospinning sepaksi bukan sahaja menangani isu kestabilan suhu dan rasa pahit yang dikaitkan dengan penyediaan tradisional, tetapi juga menjanjikan pengkomersilan terapi denggi yang berkesan.

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DEVELOPMENT OF STANDARDIZED EXTRACT OF *CARICA PAPAYA* LEAF AS POTENTIAL PHARMACEUTICAL FORMULATION FOR DENGUE

ABSTRACT

Dengue fever remains a major global health challenge, with limited treatment options available. *Carica papaya* (CP) leaf extract has been traditionally used for its purported platelet-stimulating effects in dengue management. However, its practical use is hindered by its bitter taste and the instability of its thermolabile bioactive compounds. This study aims to address these challenges by developing a standardized CP leaf extract formulation using coaxial electrospinning to produce orodispersible fibre films that are both effective and palatable. The objectives of this study were fourfold: (1) to perform the extraction of bioactive compounds from CP leaf and conduct pre-formulation studies on the standardized freeze-dried extract; (2) to develop and characterise formulations of standardized bioactive CP leaf extract using various polymer carrier systems produced by the coaxial electrospinning technique; (3) to evaluate the release and storage stability of the produced orodispersible fibre film formulations containing standardized bioactive extract of CP leaf; and (4) to investigate the *in vivo* acute oral toxicities and perform human taste evaluation of the fibre film formulations containing standardized bioactive extract of CP leaf. Methodologically, the study involved extracting bioactive compounds from freeze-dried CP leaves, followed by formulating various orodispersible fibre films containing the standardized extract using the coaxial electrospinning technique. These formulations were evaluated for their physicochemical properties, including moisture content, drug release, and stability. Subsequently, the safety and palatability of the

formulations were assessed through *in vivo* acute toxicity studies and taste evaluations. The findings indicated that the coaxial electrospinning technique successfully preserved the bioactive compound rutin while masking the extract's bitterness. Among the formulations, the orodispersible ES CP/Gelatin:PVA 1:5 film showed enhanced stability, controlled release of bioactive compounds, and received favourable taste evaluations. Additionally, all Gelatin/PVA orodispersible fibre films were well-tolerated at a dosage of 2000 mg/kg body weight in female Sprague Dawley rats during toxicity tests conducted prior to taste evaluations. This research contributes to the development of a viable and sustainable botanical treatment for dengue by optimising CP leaf extract in a user-friendly orodispersible formulation. The coaxial electrospinning approach not only addresses the thermostability and taste issues associated with traditional preparations but also holds promise for the commercialisation of effective dengue therapies.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Phytomedicinal compounds are playing a vital role in the attempt to explore potent pharmacological activities and advance both drug discovery and development (Newman & Cragg, 2020). Phytomedicine, often referred to as herbal medicine or phytotherapy, is a branch of traditional and alternative medicine that utilises plant-derived compounds and extracts to prevent, alleviate, or treat various health conditions (Ahmad et al., 2006b). It draws upon the healing properties of various plant parts, including leaves, flowers, seeds, stems, and roots, to produce remedies that have been used for centuries. These remedies are widely employed and consumed as herbal teas, tinctures, capsules, or topical applications. This ancient practice has deep roots in many cultures worldwide and is based on the belief that nature provides a rich source of therapeutic agents within plants.

According to the WHO, more than 80% of the world population employs medicinal plants as a key source of treatment (Bodeker, 2005; Ekor, 2014). It is claimed that among the 460,000 plant species discovered worldwide, at least 6% were recorded for medicinal purposes (Willis, 2017). However, excluding this medicinal plant, less than 16% were cited in medicinal regulatory publications (Allkin, 2017). Due to the limitations of phytochemical stability, consistency, and safety profile (Ahmad et al., 2006a), further studies using cutting-edge technology are still needed to successfully introduce products for clinical trials.

Over the years, scientific research has provided insights into the bioactive compounds found in plants, helping to better understand their modes of action and potential

therapeutic uses. Moreover, there has been a growing interest in the scientific validation of phytomedicine, leading to the development of standardised herbal preparations and evidence-based herbal medicine. One important aspect of phytomedicine is its link with thermolabile compounds. Thermolabile compounds are substances that are sensitive to heat and easily degrade when exposed to high temperatures. In the context of phytomedicine, many bioactive compounds found in plants are thermolabile, such as enzymes, vitamins, essential oils, and even certain phytochemicals comprising flavonoids. Improper herbal preparation involving high temperatures, such as overheating, can lead to the loss of medicinal efficacy. Thus, it is crucial to use suitable manufacturing methods to preserve the therapeutic properties of these heat-sensitive compounds.

The primary interest of the current study is developing pharmaceutical formulations by employing a heat-free manufacturing method for solving the problem of herbal leaf extracts containing thermolabile compounds. The strategies for producing formulations involving thermolabile compounds require a combination of scientific knowledge and process optimisation to ensure the stability and efficacy of the final product. In this research, coaxial electrospinning technology is employed that enables the encapsulation of thermolabile papaya leaf extract within the core. These aspects will be discussed in the following sections of this chapter.

1.2 Dengue

Dengue is an arboviral disease that has rapidly spread with cases detected in 128 countries worldwide (Brady et al., 2012). In current decades, the global incidence of dengue has grown drastically and almost half of the world citizens are at risk of infection. Every year, an approximately 390 million infections are reported universally (Bhatt et al., 2013). Dengue virus is transmitted via female mosquitoes predominantly of *Aedes aegypti* species, and fairly by *Aedes albopictus*. Figure 1.1 displays the primary infection pathway of dengue virus. Dengue is prevalent in tropical and sub-tropical countries, primarily in urban and semi-urban areas.

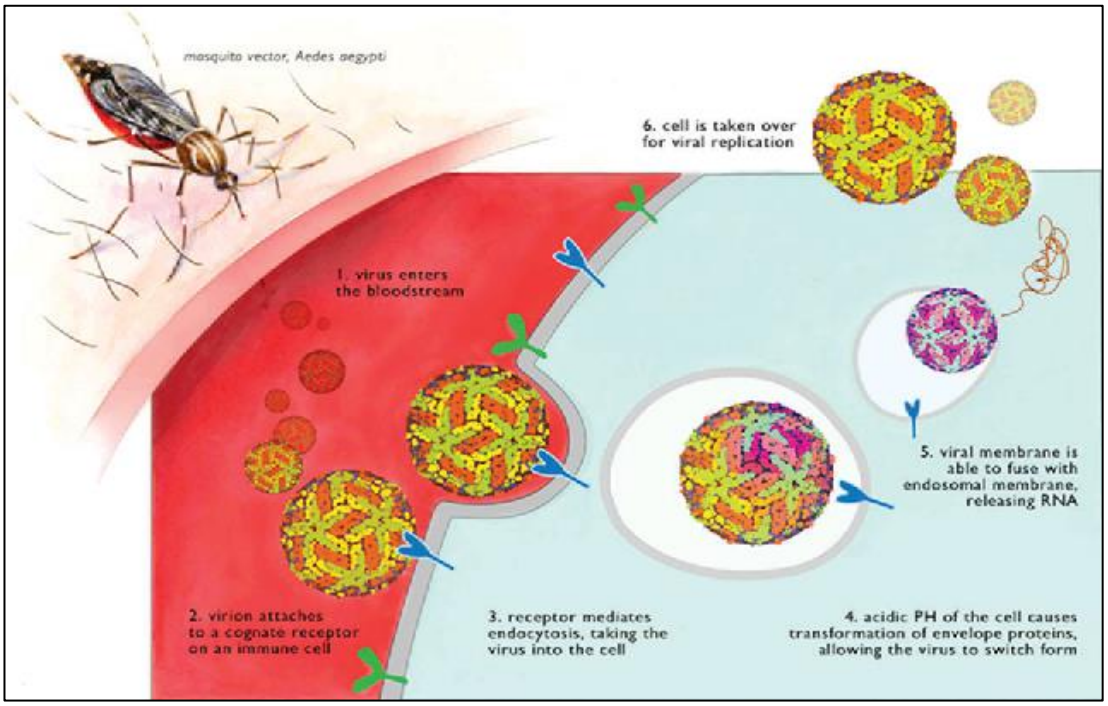


Figure 1.1 Dengue virus pathway. This figure is adapted from (Kumar, 2016b).

The mosquito usually breeds in human habitations, it prefers standing water compared to flowing water. It normally lays its eggs in clear water and its eggs have strong resistant to unfavourable environment which can maintain their vitality after storage in dry environment for several months. Typically, the eggs hatch in 3 days and turn

into adult mosquitoes 8-15 days later. Its average lifespan under normal conditions is most likely not more than 6 weeks and it cannot survive when the surrounding temperature falls below 15℃ (Siler et al., 1926).

This viral infection is categorised into 4 levels based on their severity. It ranges from dengue fever, dengue infection with warning signs to the more severe forms, i.e. dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). The hallmark symptom of dengue infections is thrombocytopenia. As of now, there is no specific anti-viral treatment or vaccine available for dengue infection.

The term thrombocytopenia refers to the condition denoted by abnormal low levels of platelet in blood. Platelets play a crucial role in maintaining vascular integrity and support the vascular endothelium. Platelets secrete and produce a vast amount of substances that are important mediators of coagulation, inflammation, thrombosis and atherosclerosis (Coppinger et al., 2004). Platelets also are called thrombocytes. A normal human platelet count varies from $150-450\times10^9$/L of blood. A platelet count of $<150\times10^9$/L is lower than normal and leads to thrombocytopenia (NHLBI, 2015). Mild thrombocytopenia develops when platelet counts of patients range from $70-150\times10^9$/L while severe thrombocytopenia occurs when platelet counts of patients are $<20\times10^9$/L (Gauer & Braun, 2012).

Thrombocytopenia could be caused by drug interactions (e.g. heparin), immunosuppressants (e.g. cyclophosphamide), infectious diseases and most commonly a crucial symptom of dengue fever. Research proposed that dengue virus was detected in megakaryocyte progenitors and circulating platelets (Noisakran et al., 2009; Saito et al., 2004). This indicates that megakaryocytes and platelets are affected by the dengue virus through direct interactions in the induction of thrombocytopenia.

Another research also found that these virus effect circulating platelet counts independent of the virus attachment or entry into platelets (Hottz et al., 2011). Thus, two mechanisms are involved in dengue induced thrombocytopenia, i.e. impaired thrombopoiesis and peripheral platelet destruction.

Research disclosed that dengue virus infects the bone marrow, directly causing thrombocytopenia. Marrow suppression is evident within 2-4 days of infection. The viral ribonucleic acid (RNA) isolates were identified in bone marrow samples of dengue-infected individuals, suggesting that the virus targets the marrow and hematopoietic system (de Araújo et al., 2009). Autoimmune-induced dengue virus proliferation is considered as the significant characterization in dengue disease manifestation. Hypothesis postulates that the host-initiated anti-DENV immune antibodies cross-react with platelets and facilitate clearance. In comparison to typical dengue fever, the serum collected from severe dengue infection patients have higher levels of Ig M and platelet binding (Lin et al., 2006).

1.2.1 Dengue Virus (DENV)

The virus that leads to dengue infection is dengue virus (DENV), also known as *Flavivirus* genus. It belongs to the *Flaviviridae* family, and it has four distinct serotypes, namely, DENV-1, DENV-2, DENV-3 and DENV-4. The fifth variant DENV-5 has been isolated and discovered in October 2013, this serotype obeys the sylvatic cycle that is different from the other four serotypes which follow the human cycle (Mustafa et al., 2015). Dengue patients who have recovered from infection is believed to have lifelong immunity against that particular serotype only. Cross-immunity to other serotypes after recuperation is partial and temporary, which implies that a person is likely to be infected only up to four times in their entire life.

DENV is comprised of basically a nucleocapsid. It is enclosed by glycoproteins and surrounded by a lipid bilayer. It is one of the Class IV viruses and contains positive sense single stranded ribonucleic acid (RNA). The RNA encodes three structural proteins, i.e. capsid (C), membrane (M) and envelope (E) glycoproteins; and seven non-structural proteins, i.e. NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. These structural and non-structural proteins, known as precursor, can be cleaved by host and viral proteases. The schematic diagram of DENV is illustrated in Figure 1.2.

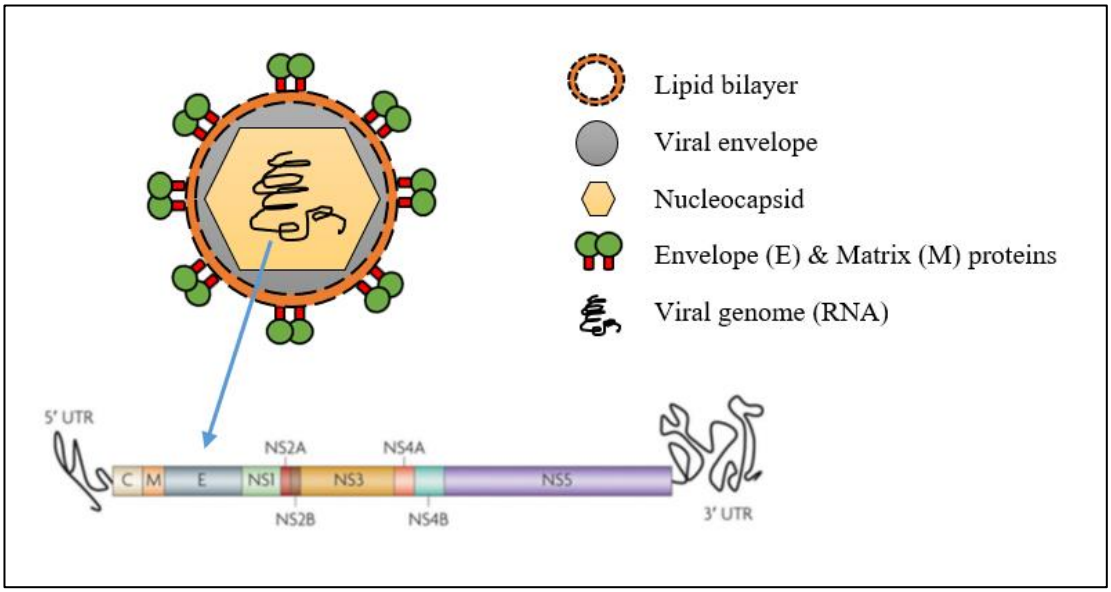


Figure 1.2 Structure and genome of dengue virus. This figure is adapted and modified from (Guzman et al., 2010).

The three structural proteins are incorporated into developed infective virion, while the non-structural proteins work in the replication and assembly of virus only. Researchers have extracted DENV from the macrophages, dendritic cells and polymorphonuclear leukocytes etc. The virus has also been identified in platelets and megakaryocyte progenitors. These discoveries revealed that DENV has the ability to react directly with platelets and megakaryocytes, which may cause thrombocytopenia (Hottz et al., 2011).

1.2.2 Transmission of Dengue Virus

1.2.2(a) Mosquito to Human Transmission

Dengue virus is transmitted to humans through the bites of infected female mosquitoes, typically the *Aedes aegypti* species. Other species of *Aedes* genus such as *Aedes albopictus* can act as vectors, but to a lesser extent as compared to *Aedes aegypti*. The mosquito can transmit virus throughout its lifetime once it is infectious.

The virus, DENV, replicates in the mosquito midgut before it spreads to the secondary tissues, which include the salivary glands. The extrinsic incubation period (EIP), which is the time taken from ingesting the virus to real transmission to a new host spans about 8−12 days at ambient temperature of 25−28℃ (Tjaden et al., 2013; Watts et al., 1987). The differences in EIP are affected by several factors such as initial viral concentration, genotype of virus and daily temperature variations (Anderson & Rico-Hesse, 2006; Lambrechts et al., 2011; Ye et al., 2015).

1.2.2(b) Human to Mosquito Transmission

Mosquitoes may become infected after feeding on a DENV-infected person who is viremic with the specific virus. The DENV-infected person can be pre-symptomatic or asymptomatic. Pre-symptomatic include those who have symptoms of dengue infection or yet to have symptomatic dengue infection, while asymptomatic include people who have no signs of sickness with no detectable symptoms (Duong et al., 2015).

High fever and high viremia in infected humans can easily transmit dengue virus to mosquitoes and increase the risk of mosquito infection. Indeed, high levels of DENV-specific antibodies will lower the risk of mosquito infection. The majority are viremic for 4-5 days; however, viremia can last for up to 12 days (Gubler et al., 1981).

1.2.2(c) Other Modes of Transmission

Although mosquito vectors are the main mode of transmission of dengue virus, there is a likelihood of maternal transmission also. When a mother is DENV-infected during pregnancy, her baby may experience foetal distress, low birthweight or pre-term birth (Basurko et al., 2009). Nevertheless, the risk of vertical transmission (mother-to-child transmission) is low, associated with the timing of dengue infection at the time of pregnancy (Basurko et al., 2018).

1.2.3 Clinical Manifestations

After an incubation period of 3-7 days, the symptoms start to appear. It follows three main phases –febrile phase, critical phase and recovery phase. Figure 1.3 below shows the schematic outlines of the three main phases of dengue infection.

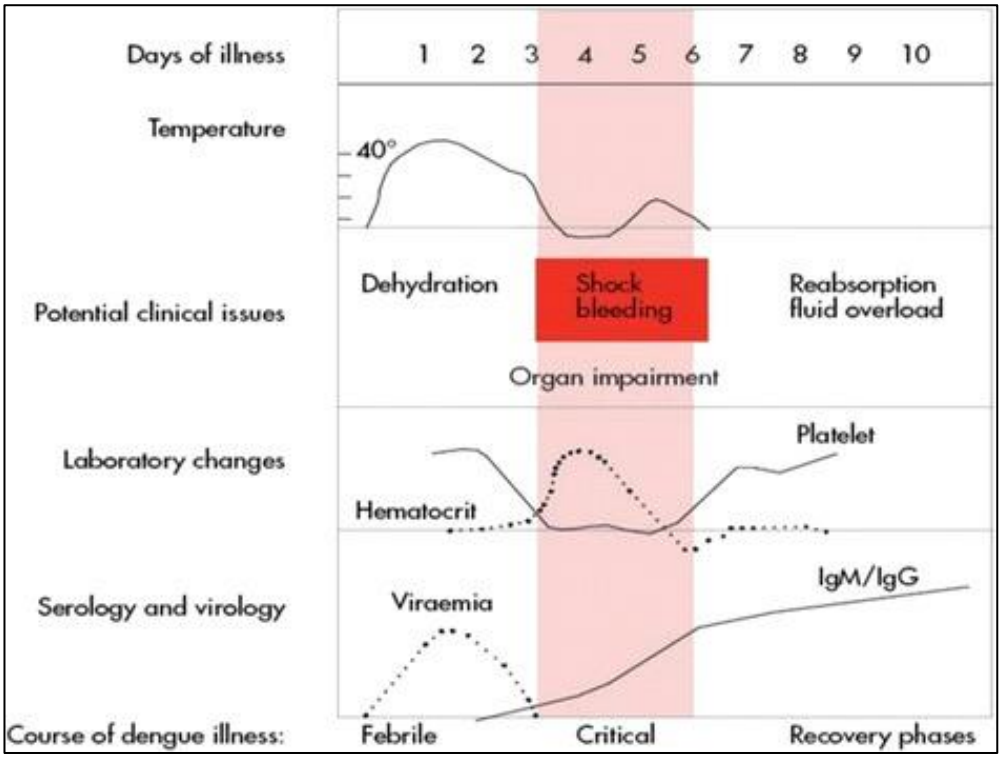


Figure 1.3 Three phases of dengue virus infection. This figure is adapted from (Yacoub & Wills, 2014).

1.2.3(a) Febrile Phase

The initial febrile phase is commonly characterized by high temperature, usually ≥38.5℃. The other symptoms include headache, vomiting, muscle pain (myalgia), joint pain (arthralgia), skin erythema/ transient maculopapular rash and facial flushing. During febrile phase, children also have high fever but typically less symptomatic than adults. Mild hemorrhagic indications such as bruising and petechiae, generally at venipuncture sites and a palpable liver are frequently observed. Clinical findings are mild to moderate thrombocytopenia and leukopenia, associated with moderate increase of hepatic aminotransferase level. Febrile phase lasts for 3-7 days and normally the patients recover without complications (Simmons & Farrar, 2012).

1.2.3(b) Critical Phase

The critical phase happens around the time of defervescence, where the bodily temperature of patients decreases to 37.5−38.0℃ or less. The period of clinically significant plasma leakage generally lasts 24−48 hours, and the fluid management of patient is very critical during that time. Most patients clinically recover during this phase, yet there are people who have significant plasma leakage wherein they may encounter severe dengue within a few hours due to substantial increase in vascular permeability. The clinical observations for those with severe plasma leakage include increasing hemoconcentration, hypoproteinemia, ascites and pleural effusions (Wichmann et al., 2004).

First, physiological compensatory mechanisms are upregulated to maintain sufficient circulation to critical organs, which narrows pulse pressure when loss of plasma volume becomes notable. Diastolic blood pressure increases at this stage. If the pulse pressure narrows to ≤20 mm Hg, associated with signs of peripheral vascular collapse,

dengue shock syndrome is detected, and resuscitation is carried out promptly. Patients may look well during early stages of shock, but once hypotension develops, systolic blood pressure decreases rapidly, where irreversible shock and death may subsequently happen despite resuscitation (Simmons & Farrar, 2012).

In the course of transition from the febrile to critical phase (between days 4 and 7 of infection), it is important for the medical providers to be alert of warning signs such as severe abdominal pain, continuous vomiting, increasing hematocrit level with a rapid drop of platelet count, painful hepatomegaly, mucosal bleeding, serous effusions, and lethargy or restlessness, in which vascular leakage may appear in the patient (CDC, 2015).

During the critical phase, patients may have hemorrhagic manifestations. Typically, in children, bleeding seldom occurs and is accompanied by intense and prolonged shock. On the other hand, hematemesis, critical skin bleeding and/or mucosal bleeding (vaginal or gastrointestinal) may take place in adults, in association with minor plasma leakage and non-apparent precipitating factors. Other rare manifestations are hepatitis/liver failure, myocarditis, pancreatitis, and encephalitis, together with insignificant plasma leakage. Moderate to severe thrombocytopenia with low platelet count $<20\times10^9$/L is usually detected in patients during the critical phase. A temporary increase in activated partial thromboplastin time and a drop in fibrinogen level are commonly observed, but the fundamental mechanisms remain ambiguous (Mairuhu et al., 2003).

1.2.3(c) Recovery Phase

The short-term altered vascular permeability will be returned to a normal level spontaneously after 48−72 hours and associated with rapid improvement in patient's symptoms. The patients start to recover, and their physical condition begins to improve during the recovery phase of dengue. Hence, proper monitoring of symptoms should be carried out. Generally, the appetite of patients will become normal, platelet count increases, blood pressure stabilises and gastrointestinal bleeding reduces. However, a second rash may appear, ranging from a mild maculopapular rash to severe itchy lesions caused by leukocytoclastic vasculitis, soon after recovery, accompanied by skin peeling or shedding of the outermost skin layer for one to two weeks. After recovery, adults may have profound fatigue over a period of several weeks (Simmons & Farrar, 2012).

1.2.4 Diagnostics

There are various methods for diagnosis of dengue virus infection, such as virological tests and serological tests. Virological test is a method that directly detects virus elements while serological test detects human-derived immune constituents that are produced with regards to the virus. The application of distinct diagnostic methods depends on the time of patient presentation, which may be moderately appropriate. Patient specimens collected during the first week of infection should be diagnosed by both virological and serological tests. Figure 1.4 shows the schematic diagrams of dengue virus, antigen and antibody responses used in diagnosis. Among the methods available for dengue infection diagnosis, virus isolation gives the most specific test result. The detection of viral genome or viral antigens is the identification of dengue

infection. Another way of confirming a dengue infection is the seroconversion of IgM or IgG antibodies.

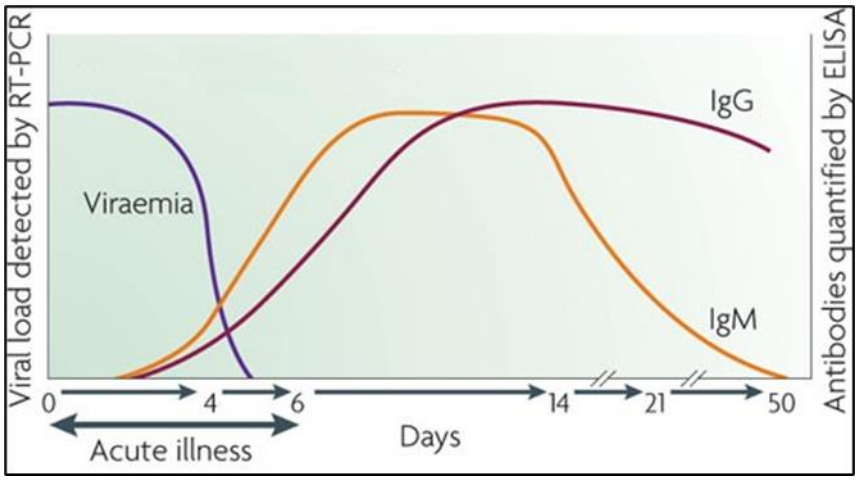


Figure 1.4 Clinical course of dengue virus infection. This figure is adapted from (Yacoub & Wills, 2014).

1.2.4(a) Virological Methods

During the first few days of illness, the dengue virus may be isolated from the blood of patients. Several reverse transcriptase-polymerase chain reaction (RT-PCR) methods are available to test the virus elements. Basically, RT-PCR assays are quite sensitive, but require specialised equipment and experienced technical staff to conduct the test. Hence, this method is uncommon in medical facilities. The RT-PCR products from clinical specimen can be used for virus genotyping, which enable comparisons of virus specimen from various geographical origins (Raengsakulrach et al., 2002).

The virus may also be diagnosed by detecting a virus-produced protein, known as NS1 antigen. There are various rapid diagnostic tests called NS1 antigen detection kits available in the market, as it takes around 20 minutes only to analyze the result and does not require specialized equipment or techniques (Shu et al., 2002; Xu et al., 2006; Young et al., 2000).

1.2.4(b) Serological Methods

Enzyme-linked immunosorbent assay (ELISA) is one of the serological methods used to detect anti-dengue antibodies, including Immunoglobulin M and Immunoglobulin G (Ig M and IgG). The IgM antibodies are identified approximately one week after infection or as early as 4 days after the onset of fever, acting as the first antibodies to fight against a new infection and achieving their highest peak about 2 to 4 weeks after the outbreak of illness. The presence of IgM antibodies indicates that there is a recent dengue virus infection. These antibodies remain detectable for roughly 3 months (Hunsperger et al., 2009). Another antibody, IgG is indicative of a past infection as they take time to form after an infection/immunisation. Normally, IgG antibody levels require longer time to develop compared to IgM. However, IgG antibodies remain in the body for years (Chanama et al., 2004; Falconar et al., 2006).

1.2.5 Management of Dengue

Current treatment regimens are symptom based, so far there is no effective vaccine or specific antiviral treatment available for dengue infection. According to WHO guidelines, three steps are involved in dengue management, which include overall recognition of the disease, diagnosing the phase in terms of severity, and ultimately, focus on the treatment of dengue.

Supportive care with analgesics, fluid replacement and bed rest are usually sufficient. Pain killers and fever reducers can be consumed to reduce symptoms which accompany by dengue infection such as fever, muscle pains and aches. Treatment of common mild dengue infection is only supportive, which include ample amount of oral fluids during febrile phase and paracetamol or acetaminophen (TDR/WHO, 2009). Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen

should be avoided as they will cause blood thinning, which worsen the condition in a disease with risk of hemorrhage (bleeding or abnormal flow of blood).

The presence of any warning sign suggests the necessity for hospitalisation and clinical observation. Administration of intravenous fluids is required to maintain hematocrit levels in severe cases of dengue hemorrhagic fever (DHF) where plasma leakage commonly occurs. If the condition exacerbates to dengue shock syndrome (DSS), rapid fluid resuscitation to rejuvenate plasma volume is vital, followed by continuing fluid therapy to restore circulation to a level that can maintain critical organ perfusion. Isotonic crystalloid solutions should be given to the patients, whereas isotonic colloid solutions should be used for those with severe shock or who do not respond to initial crystalloid treatment (Wills et al., 2005). Intravenous fluid therapy should be kept to minimum to avoid fluid overload and maintain cardiovascular stability until vascular permeability returns to normal levels.

In cases where there is severe bleeding that affects cardiovascular function or plasma leakage reaches very low levels, blood transfusion is the only treatment. However, it should be handled with care due to the risk of fluid overload. Fresh-frozen plasma, platelet concentrates, and cryoprecipitate are required depending on the coagulation profile of patients. As of now, platelet transfusions are ineffective and unworkable for patients who do not have clinically profound bleeding, despite severe thrombocytopenia (Lye et al., 2009; Thomas et al., 2009). For severe dengue infection, adjuvant treatment such as vasopressor and inotropic therapies, renal replacement therapy and other organ impairment treatment are needed.

1.2.6 Challenges and Outlook

The development of vaccine for dengue has been ongoing for several decades. Nevertheless, progress of dengue vaccine has faced a lot of challenges due to the complex pathology of the disease, the requirement to balance neutralising antibody responses to all four virus serotypes and insufficient funds (Hombach, 2007).

Moreover, clinical monitoring discloses that DHF or DSS corresponds to dengue virus secondary infection, which gives rise to exceptional challenge in development of dengue vaccine that such vaccines should develop robust immune response against four DENV serotypes in both the susceptible and previously immune individuals. The lack of understanding on the mechanisms involved in generating immunity against dengue infection exhibits supplementary challenges (Hombach et al., 2007).

Animal models are only partially effective in evaluation of vaccine. Clinical trials are significant for vaccine evaluation to study the immune responses and reactogenicity of vaccine delivered. To indicate the absence of any severe disease or antibody-dependent enhancement (ADE), the prolonged observations of vaccinated populations should be carried out. Therefore, the role of the cellular immune response calls for further study, even though several studies demonstrate neutralising antibodies as the crucial contributors to immunity against dengue infection (Beltramello et al., 2010; Dejnirattisai et al., 2010). There is a broad revolution in using herbal plants for pharmacological applications, however further investigations on the effectiveness of plant extracts to combat dengue are required.

1.3 *Carica papaya* Leaf Extract as Herbal Supplement for Dengue Treatment

Literature has reported many platelet-boosting natural products that could facilitate the recovery process of dengue infection. One of the popular plant remedies for this viral infection is *Carica papaya*, commonly known as papaya. It is a sustainable phytochemical resource in Malaysian folk medicine (O'Hare & Williams, 2013).

1.3.1 Therapeutic Benefit

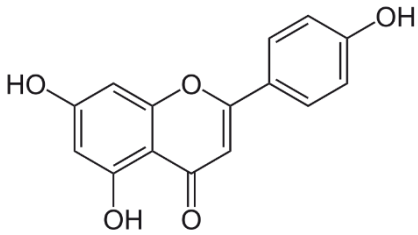
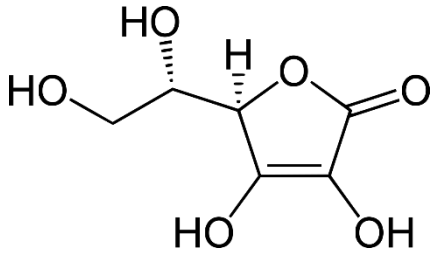
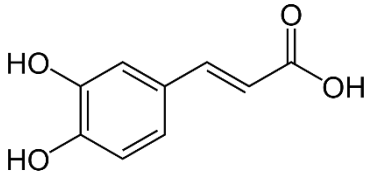
Papaya leaf extract has been a traditional remedy in some cultures, especially in regions where dengue fever is prevalent. *C. papaya* leaf extracts as well as pure compounds derived from the leaves such as caffeic acid, carpaine, chlorogenic acid, cinnamic acid, citropten, kaempferol, nicotiflorin, myricetin, quercetin, rutin, etc. were reported to possess a wide variety of ethnopharmacological activities (Kong et al., 2021; Mohd Abd Razak et al., 2018; Nandini et al., 2021; Razak et al., 2021). A list of these phytochemical compounds found in C. *papaya* leaf along with their chemical structures is provided in Table 1.1. Despite these findings, additional research is warranted to establish its efficacy and safety conclusively.

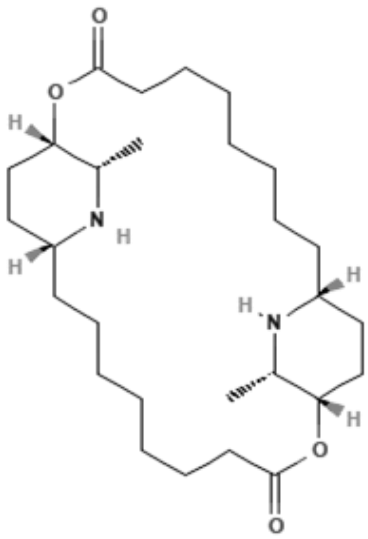
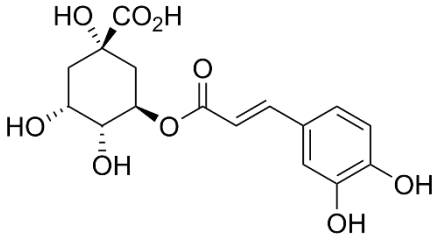
Some studies and anecdotal reports have suggested the potential therapeutic benefits of papaya leaf extract for dengue treatment. One of *Carica papaya* leaf extract's reported mechanisms is to increase platelet counts and reduce clotting time in an *in vivo* model. This suggests that these natural resources could potentially be used to alleviate symptoms of various diseases, including dengue fever or other conditions that lead to thrombocytopenia (Patil et al., 2013). The suggested phytochemicals behind the pharmacological activities of the papaya leaves were of the flavonoid class, including quercetin 3-rutinoside (rutin) and kaempferol 3-rutinoside (nicotiflorin)

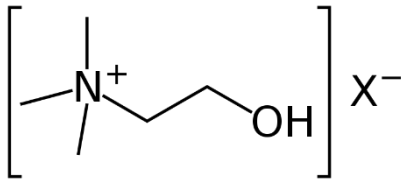
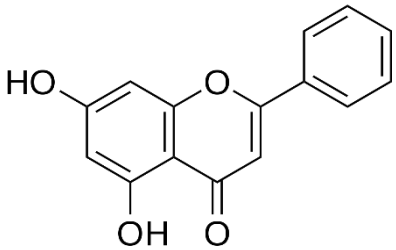
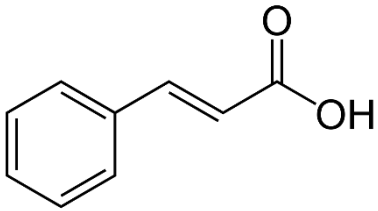
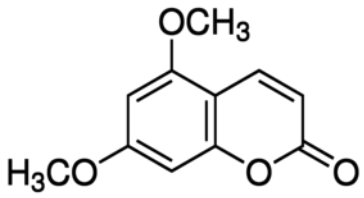
(Nugroho et al., 2017; Zhang et al., 2019). However, such phytochemical classes were reported to be sensitive to the manufacturing process, particularly regarding thermolability and stability issues that are directly influencing their activity (Kumar, 2016b).

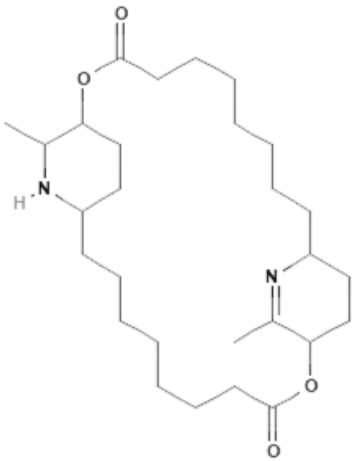
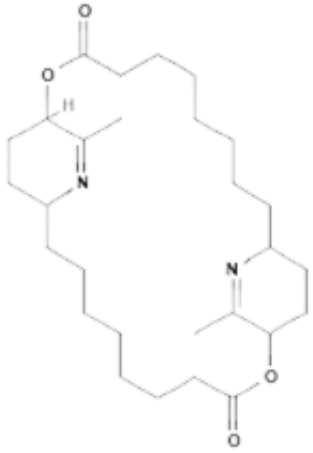
Dengue fever can induce inflammation in the body, leading to symptoms such as fever and joint pain. In this context, *C. papaya* leaf extract possesses anti-inflammatory properties, potentially helping to alleviate inflammatory responses associated with dengue infection (Sarker et al., 2021). Moreover, papaya leaf extract exhibits immunomodulatory properties by affecting the level of cytokine production, which could modulate the body's immune response to the dengue viral infection (Norahmad et al., 2019).

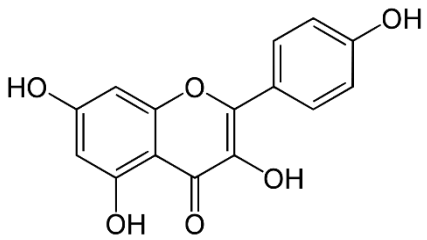
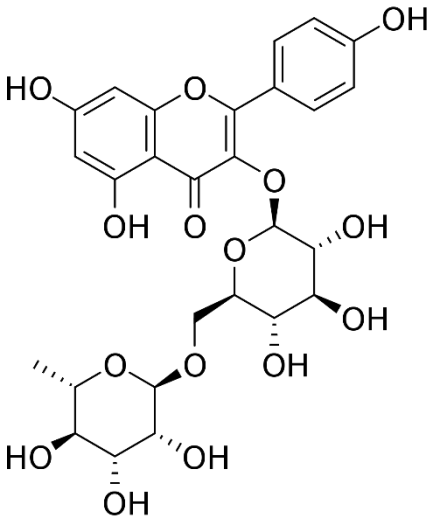
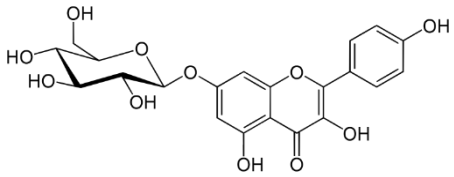
Table 1.1 Phytochemical analysis of *Carica papaya* leaf

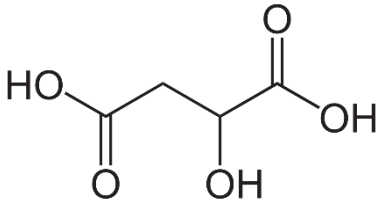
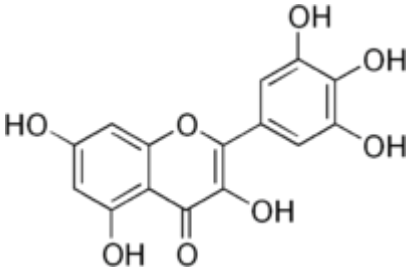
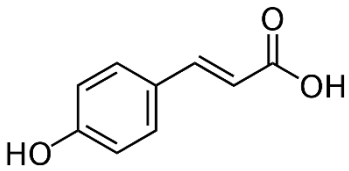
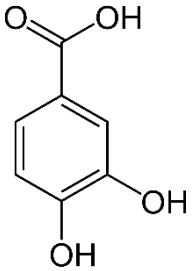
IUPAC Name	Common Name	Chemical Structure	Compound	Reference
5,7-Dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	Apigenin		flavonoid	(Iordănescu et al., 2021)
(2R)-2-[(1S)-1,2-dihydroxyethyl]-3,4-dihydroxy-2H-furan-5-one	Ascorbic acid (Vitamin C)		vitamin	(Al-Seadi et al., 2021)
(E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid	Caffeic acid		phenolics	(Anjum et al., 2017; Kong et al., 2021; Nandini et al., 2021)

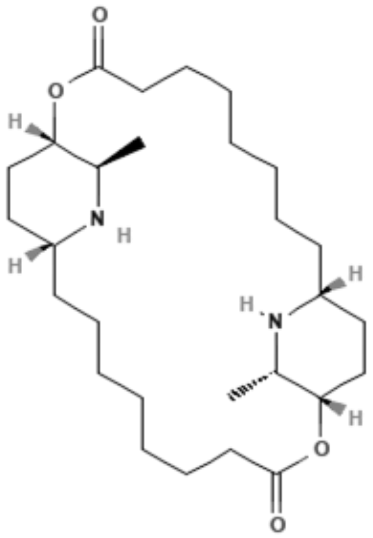
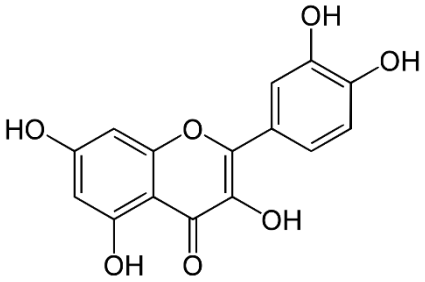
(1S,11R,13S,14S,24R,26S)-13,26-dimethyl-2,15-dioxa-12,25-diazatricyclo[22.2.2.2^{11,14}]triacontane-3,16-dione	Carpaine		alkaloids	(Nandini et al., 2021; Razak et al., 2021)
(1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid	Chlorogenic acid		phenolics	(Mohd Abd Razak et al., 2018)

2-hydroxyethyl(trimethyl)azanium	Choline		alkaloids	(Al-Seadi et al., 2021)
5,7-Dihydroxy-2-phenyl-4H-1-benzopyran-4-one	Chrysin		flavonoids	(Imaga et al., 2009; Nwankwo et al., 2021)
(E)-3-phenylprop-2-enoic acid	Cinnamic acid		styrenes	(Anjum et al., 2017; Nandini et al., 2021)
5,7-dimethoxychromen-2-one	Citropten / 5,7-dimethoxycoumarin		phenolics	(Mohd Abd Razak et al., 2018)

13,26-dimethyl-2,15-dioxa-12,25-diazatricyclo[22.2.2.2^{11,14}]triacont-12-ene-3,16-dione	Dehydrocarpaine I		alkaloids	(Mohd Abd Razak et al., 2018; Tang, 1979)
13,26-dimethyl-2,15-dioxa-12,25-diazatricyclo[22.2.2.2^{11,14}]triaconta-12,25-diene-3,16-dione	Dehydrocarpaine II		alkaloids	(Mohd Abd Razak et al., 2018; Tang, 1979)

3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one	Kaempferol		phenolics	(Anjum et al., 2017; Kong et al., 2021; Mohd Abd Razak et al., 2018)
5,7-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-3-yl 6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside	Kaempferol 3-O-rutinoside / Nicotiflorin		flavonol glycoside	(Norahmad et al., 2019; Razak et al., 2021)
3,5-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yl β-D-glucopyranoside	Kaempferol 7-O-glucoside / Kaempferol 7-O-β-D-glucopyranoside		flavonol glucoside	(Nguyen et al., 2015; Nugroho et al., 2017)

2-hydroxybutanedioic acid	Malic acid		carboxylic acid	(Mohd Abd Razak et al., 2018)
3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4H-1-benzopyran-4-one	Myricetin		flavonoids	(Anjum et al., 2017; Kong et al., 2021)
3-(4-hydroxyphenyl)-2-propenoic acid	p-Coumaric acid		phenolics	(Mohd Abd Razak et al., 2018)
3,4-dihydroxybenzoic acid	Protocatechuic acid		phenolics	(Mohd Abd Razak et al., 2018; Nandini et al., 2021)

(1S,11R,13S,14S,24R,26R)-13,26-dimethyl-2,15-dioxa-12,25-diazatricyclo[22.2.2.2^{11,14}]triacontane-3,16-dione	Pseudocarpaine		alkaloids	(Shrivastava et al., 2022)
2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one	Quercetin		flavonoid	(Kong et al., 2021)