BIOASSAY-GUIDED PHYTOCHEMISTRY OF *Zingiber zerumbet* (L.) Smith RHIZOME EXTRACT AGAINST DENGUE VIRUS SEROTYPE-2 NS2B/NS3 PROTEASE AND ITS ANTI-PYRETIC ACTIVITIES

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FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ORIGINAL LITERARY WORK DECLARATION

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ABSTRACT

The bioassay-guided study was conducted on *Zingiber zerumbet* (L.) Smith (shampoo ginger) to investigate its anti-dengue potential through the inhibition of DENV2 NS2B/ NS3 protease. In this study, the rhizomes of *Z. zerumbet* were extracted with different polarities of solvents and tested for DENV2 NS2B/NS3 protease inhibition, acute toxicity and anti-pyretic studies. The results showed that the ethyl acetate (EA) extract of *Z. zerumbet* exhibited potent inhibitory activity against NS2B/NS3 protease with IC_{50} value of 2.39 ± 0.86 µg/mL. Acute toxicity test indicated that the EA extract is not toxic up to dosage of 5000 mg/kg. In addition, comparison of anti-pyretic activity between the EA extract (125 mg/kg body weight) and aspirin (**121**) (100 mg/kg body weight) have shown a difference in reduction of the rectal temperature of rats. The EA extract (125 mg/kg body weight) demonstrated faster temperature reduction than aspirin (**121**) within the first two hours after administration. However, the temperature rose after the 2nd hour of the experiment. On the other hand, aspirin (**121**) had slower temperature reduction but the temperature reduction was continued until the 4th hour of the experiment. Therefore, the EA extract (125 mg/kg body weight) showed rapid onset of action compared to aspirin (**121**) but aspirin (**121**) has longer lasting anti-pyretic effect. Furthermore, two compounds were isolated from the potent EA fraction of *Z. zerumbet*, namely zerumbone (**3**) and zerumbone epoxide (**56**). Zerumbone epoxide (**56**) exhibited more potent inhibition against DENV2 NS2B/NS3 protease (72.04%) than zerumbone (**3**) (54.33%). Both compounds were subjected to a molecular docking study. Two hydrogen bonding were observed between the epoxide group of zerumbone epoxide (**56**) and the hydrogen from His51 and Ser135. In addition, five π-alkyl interactions of zerumbone epoxide (**56**) with Tyr150, Tyr161, and His51 were also observed.

Meanwhile, zerumbone (**3**) formed two hydrogen bonds with His51 and Ser135, a π – σ interaction with Tyr161, and three π-alkyl interactions with Pro132, Val155, and Tyr161. The binding energy of zerumbone epoxide (**56**) (-5.60 kcal/mol) was slightly lower than zerumbone (**3**) (-5.58 kcal/mol). Thus, the molecular docking results are consistent with the anti-protease studies of both compounds. In conclusion, the EA of *Z. zerumbet* could be a promising candidate for the development of anti-pyretic and anti-dengue agents.

Keywords: *Zingiber zerumbet*, anti-pyretic, DENV2 NS2B/NS3 protease, toxicity, molecular docking.

PENYELIDIKAN FITOKIMIA BERPANDUKAN BIOASAI KE ATAS EKSTRAK RIZOM *Zingiber zerumbet* (L.) Smith TERHADAP PROTEASE NS2B/NS3 VIRUS DENGGI SEROTAIP-2 DAN AKTIVITI ANTI-PIRETIKNYA

ABSTRAK

Kajian berpandukan bioasai telah dijalankan ke atas *Zingiber zerumbet* (L.) Smith (syampu halia) untuk mengkaji potensi anti-denggi melalui perencatan protease DENV2 NS2B/NS3. Rizom *Z. zerumbet* telah diekstrak dengan kepolaran pelarut yang berbeza dan diuji untuk perencatan protease DENV2 NS2B/NS3, ketoksikan akut dan kajian anti-piretik. Keputusan menunjukkan bahawa ekstrak etil asetat (EA) mempamerkan aktiviti perencatan yang kuat terhadap protease NS2B/NS3 dengan nilai IC_{50} 2.39 ± 0.86 µg/mL. Ujian ketoksikan akut menunjukkan bahawa ekstrak EA rizom *Z. zerumbet* tidak toksik sehingga dos 5000 mg/kg. Di samping itu, perbandingan aktiviti anti-piretik antara ekstrak EA *Z. zerumbet* (125 mg/kg berat badan) dan aspirin (**121**) (100 mg/kg berat badan) telah menunjukkan perbezaan profil mereka dalam mengurangkan suhu rektum tikus. EA ekstrak *Z. zerumbet* (125 mg/kg berat badan) menunjukkan kesan penurunan suhu yang lebih cepat daripada aspirin (**121**) dalam tempoh dua jam pertama selepas ekstrak dimasukkan secara oral ke dalam tikus. Walau bagaimanapun, suhu rektum tikus meningkat selepas jam ke-2 eksperimen. Sebaliknya, kesan penurunan suhu aspirin (**121**) adalah lebih perlahan dan berterusan sehingga jam ke-4 eksperimen. Oleh itu, EA ekstrak *Z. zerumbet* (125 mg/kg berat badan) menunjukkan kesan tindakan yang cepat berbanding aspirin (**121**) tetapi aspirin (**121**) mempunyai kesan anti-piretik yang lebih lama. Tambahan pula, dua sebatian telah dipisahkan daripada bahagian EA *Z. zerumbet* yang aktif, iaitu zerumbon (**3**) dan zerumbon epoksida (**56**). Zerumbon epoksida (**56**) mempamerkan perencatan yang lebih kuat terhadap DENV2 NS2B/NS3 protease (72.04%) berbanding zerumbon (**3**) (54.33%). Kedua-dua sebatian tersebut telah dilanjutkan kepada penyelidikan molekul dok. Keputusan molekul dok menunjukkan kehadiran dua ikatan hidrogen di antara kumpulan epoksida daripada sebatian zerumbon

epoksida (**56**) dan hidrogen daripada His51 dan Ser135. Di samping itu, terdapat juga lima interaksi π-alkil di antara zerumbon epoksida (**56**) dengan Tyr150, Tyr161, dan His51. Sementara itu, zerumbon (**3**) membentuk dua ikatan hidrogen dengan His51 dan Ser135, interaksi π - σ dengan Tyr161 dan tiga interaksi π-alkil dengan Pro132, Val155, dan Tyr161. Tenaga pengikatan zerumbon epoksida (**56**) (-5.60 kcal/mol) adalah lebih rendah berbanding zerumbon (**3**) (-5.58 kcal/mol). Oleh itu, keputusan dok molekul adalah konsisten dengan kajian anti-protease bagi kedua-dua sebatian tersebut. Kesimpulannya, ekstrak EA *Z. zerumbet* berpotensi sebagai calon yang sesuai dalam pembangunan untuk menghasilkan agen anti-piretik dan anti-denggi.

Katakunci: *Zingiber zerumbet*, anti-piretik, DENV2 NS2B/NS3 protease, ketoksikan, penyelidikan molekul dok.

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%	:	Percentage
µg	:	Microgram
µL	:	Microliter
µM	:	Micromolar
µg/mL		Microgram Per Mililitre
^{13}C	:	Carbon with Number Atom 13
^{1}H	:	Hydrogen with Number Atom 1
cm^{-1}	:	Per Centimeter
g	:	Gram
IC_{50}	:	Inhibitory Concentration at 50%
LD_{50}	:	Lethal Dose at 50%
kg	:	Kilogram
L	:	Litre
m	:	Meter
mg	:	Milligram
mL	:	Millilitre
mM	:	Millimolar
min	:	Minutes
nm	:	Nanometer
ppm	:	Part Per Million
prM	:	Pre-membrane
°C	:	Degree Celcius
spp.	:	More Than One Species
w/v	:	Weight Per Volume
α	:	Alpha
β	:	Beta
d	:	Doublet
dd	:	Doublet of Doublets
ddd		Doublet of Doublet of Doublets
δ	:	Chemical Shift
J	:	Coupling Constant
γ	:	Gamma

m/z	:	Mass to Charge Ratio
m	:	Multiplicity
λ	:	Maximum Wavelength
s	:	Singlet
t	:	Triplet
td	:	Triplet of doublets
b.w	:	Body weight
CC	:	Column chromatography
CD_3OD	:	Deuterated methanol
$CDCl_3$	:	Deuterated chloroform
CO_2	:	Carbon dioxide
DENV	:	Dengue Virus
DENV2	:	Dengue Virus Type 2
DMSO	:	Dimethyl Sulfoxide
E	:	Envelope
EA	:	Ethyl Acetate
GC/MS	:	Gas Chromatography/ Mass Spectrometry
GC-FID	:	Gas Chromatography-Flame Ionization Detector
HPLC	:	High-Performance Liquid Chromatography
Hz	:	Hertz
IR	:	Infrared
MHz	:	Mega Hertz
NMR	:	Nuclear Magnetic Resonance
NS2B	:	Nonstructural Protein 2B (Essential Cofactor)
NS3	:	Nonstructural Protein 3
NS3pro	:	Serine Protease Domain of the Nonstructural Protein 3
PTLC	:	Preparative Thin-Layer Chromatography
SD	:	Standard Deviation
TLC	:	Thin-Layer Chromatography
UV-vis	:	Ultraviolet Visible
WHO	:	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 General

Over the decade, many drugs and vaccines have been developed and produced to treat diseases and infections which were caused by microorganisms such as bacteria, fungi and viruses. For example, the diseases caused by bacteria are tuberculosis, ulcer, leptospirosis and typhoid fever, while the diseases that are caused by viruses are HIV, influenza, hepatitis and dengue. Virus-caused diseases and infections are the most difficult to treat because many at times the virus can mutate faster than the immune system's ability to defend the infected body.

Dengue, the world's most important vector-borne disease is caused by flavivirus which can be transmitted through the saliva of the female mosquito of *Aedes aegypti* and *Aedes albopictus*. Dengue is endemic in more than 100 countries, existing mainly in tropical and subtropical regions (Figure 1.1) (CDC, 2021). The World Health Organization (WHO) reported an increase of more than 2 fold of dengue cases from the year of 2010 (2.2 million) to 2019 (5.2 million) (Organization, 2022). According to the World Health Organization's report, the number of dengue infections worldwide was estimated between 100 and 400 million cases per year, creating the entire world's population at risk with Asia country accounting for more than 70% of the actual burden is in Asia (Organization, 2022).

The first recorded epidemics of dengue occurred globally in the 1780s in Asia, Africa and North America, shortly after the disease was reported and named in 1779 (Gubler, 1998). In the 1950s, a pandemic started in Southeast Asia, and by 1975 the dengue haemorrhagic fever (DHF) had become the leading cause of death among children in the country. The first case of DHF was recorded in Manila between 1953 and 1954 (Gubler, 1998).

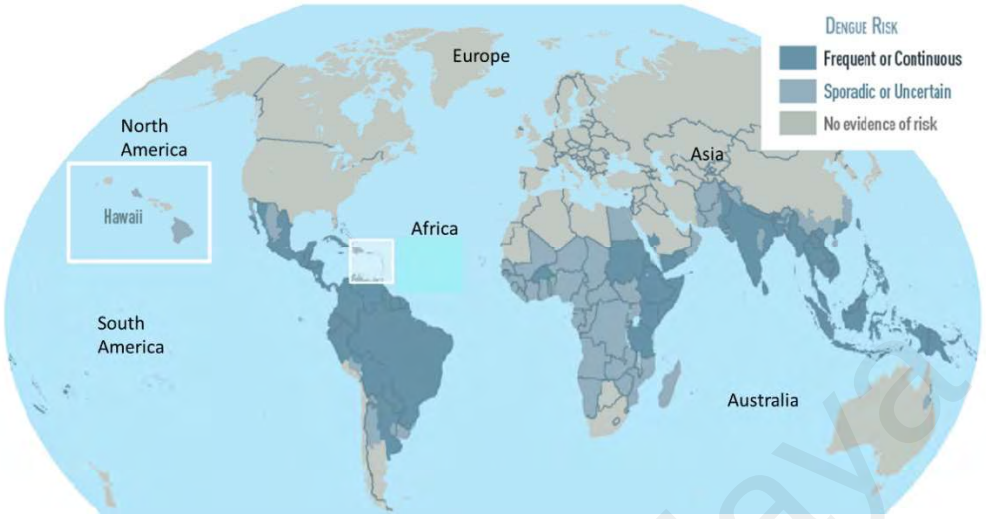


Figure 1.1 : Global map of country infected by dengue adapted from Centers for Disease Control and Prevention (CDC).

(Source : Picture was adopted from Centers for Disease Control and Prevention (CDC) website (CDC, 2021) accessed on 5th September 2021).

In Malaysia, the first outbreak was recognized by Skae in 1902 and then became endemic in 1960 (Abubakar & Shafee, 2002; Skae, 1902), followed by the first dengue haemorrhagic fever in Penang in November 1962 (Abubakar & Shafee, 2002; Lam, 1994). The first major dengue outbreaks were re ported in 1973, and subsequent outbreaks occurred in 1978, 1982, and 1990. (Cheah et al., 2014; Lam, 1994). The number of cases is increasing every year. About 130,101 cases were reported in 2019 compared to 80,615 cases during the same period in 2018 which indicate an increment of 61 percent and found to be the highest cases ever in Malaysia (Fong, 2020; Organization, 2019b). The history of dengue outbreak was depicted in Figure 1.2.

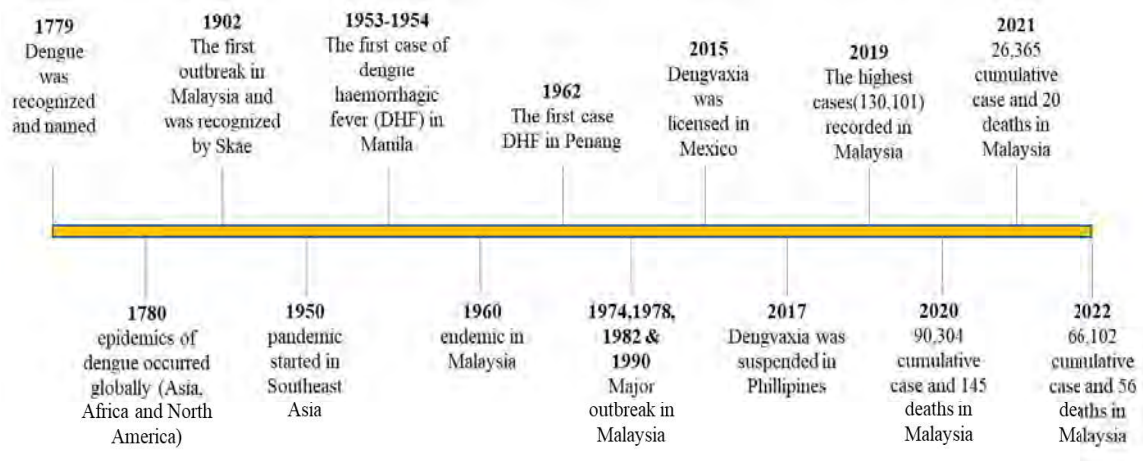


Figure 1.2 : History of dengue outbreak.

The WHO Dengue Situation Update reported that in year 2020, Vietnam presented the highest dengue cases among the Southeast Asia countries (Desk, 2021; Guardian, 2019; Organization, 2018, 2019a, 2020; Thaiger, 2018). Despite COVID-19 pandemic, Singapore showed 2-fold increasing of the dengue cases in 2020 compared to 2019 while the rest of Southeast Asia countries showed decreasing in number of dengue cases as shown in Figure 1.3 (Alam et al., 2021; Desk, 2018; Nurhayati-Wolff, 2021).

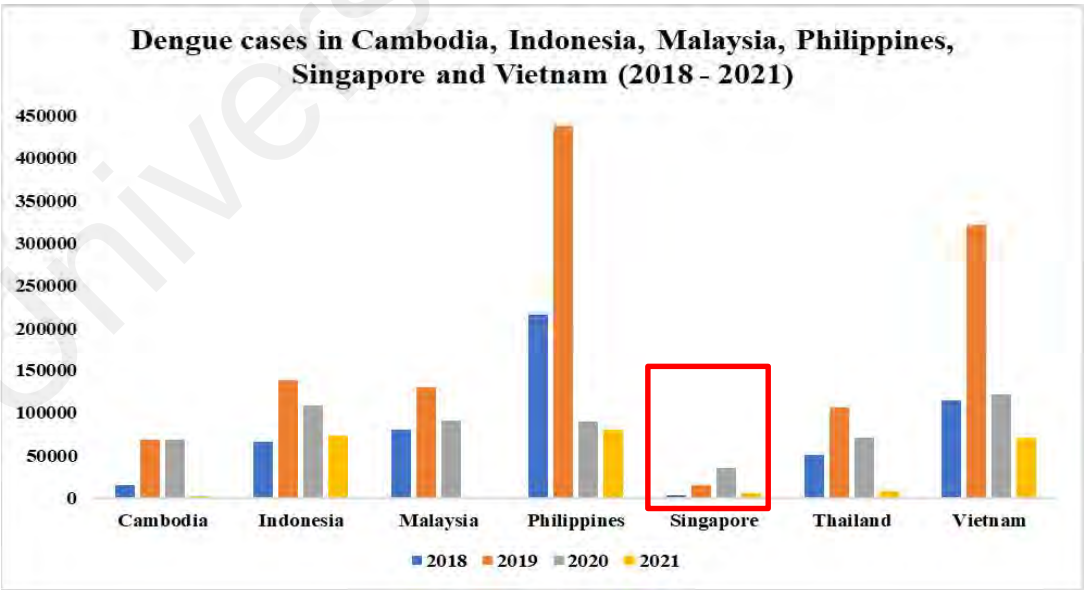


Figure 1.3 : Dengue cases in Cambodia, Indonesia, Malaysia, Philippines, and Vietnam from 2018 to 2021.

Researchers have discovered that inter-annual and seasonal climate variability play a significant role in the transmission of dengue fever (Salim et al., 2021). As discovered by Yang et al. (2009), temperature provides the optimal conditions for the survival of adult mosquitoes, as well as for larvae, pupae, and eggs (in the aquatic phase) (Yang et al., 2009). It has been identified that the rate of dengue transmission increased in warm weather conditions and the humid weather conditions which create an ideal environment for the proliferation of dengue vectors to spread the disease (Masrani et al., 2021; Rahim et al., 2021). As a result, the dengue situation in Malaysia will worsen from early June until September due to the changes in the monsoon season (Razak, 2020).

Dengue virus spread has been aided by a number of other factors, including rapid development, deforestation and population growth, increased migration and globalisation, which lead to recurring outbreaks and high transmission of various serotypes of the virus (Bhatt et al., 2013; Shimu et al., 2022). The total number of cases and deaths decreased in 2021 due to the lockdown implemented to curb COVID-19, but the number of dengue cases and deaths were spiked after the lockdown in 2022. The "iDengue" website from Ministry of Health Malaysia demonstrates an increase in the total number of cases which leads to 66,102 cumulative reported dengue cases and 56 deaths due to dengue in 2022 (Malaysia, 2023) (Figure 1.4). The reported number of dengue cases (66,102 cases) from January to December 2022 increased 150.7% (or 39,737 cases) compared to 26,365 cumulative reported cases last year and the number of deaths increased to 180% (56 deaths) compared to 20 deaths last year (Malaysia, 2023). Therefore, there is an urgent need to look for effective approaches to prevent and cure the dengue disease.

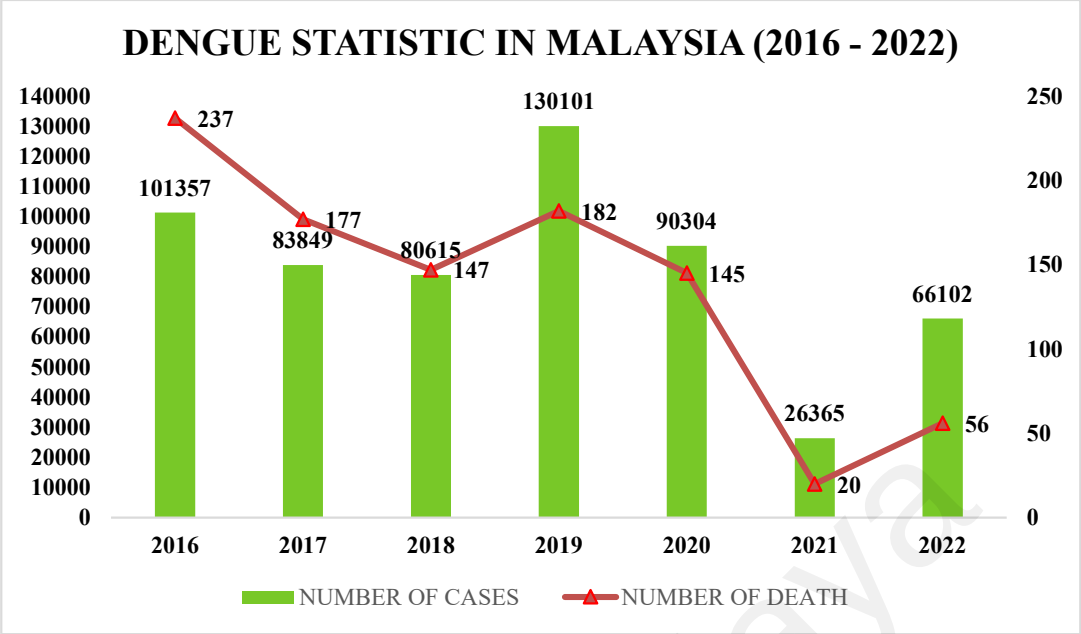


Figure 1.4 : Dengue statistic in Malaysia from 2016 to 2022.

There are several dengue targets which included DENV NS2B/NS3 protease, E-protein, RdRP and O-Mtase. The current study focuses on a specific target site within the non-structural proteins of the dengue virus (DENV), namely the NS2B/NS3. This region includes the NS2B part, consisting of residues 43 to 104, and the NS3 part, consisting of residues 18 to 167 (Purohit et al., 2022).

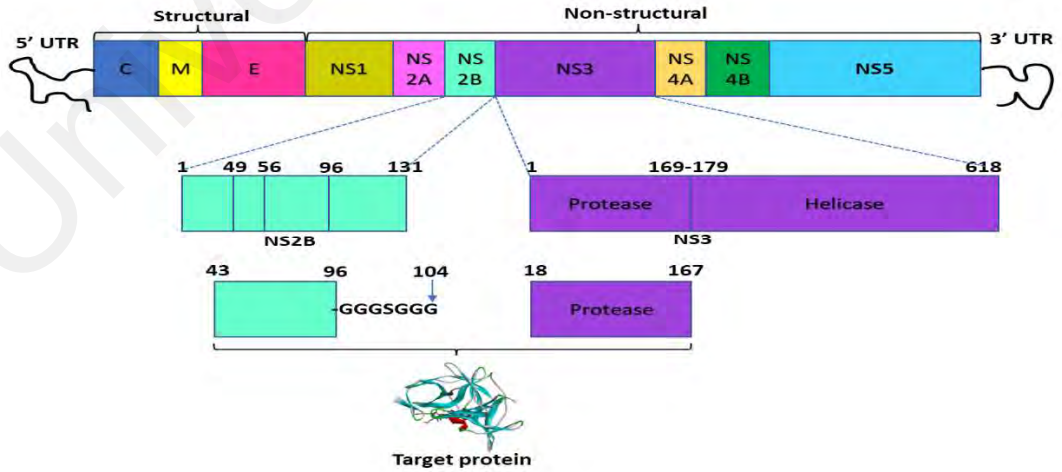


Figure 1.5 : The structure of polyprotein DENV including the target site NS2B/NS3.

A trypsin-like serine protease called DENV protease (NS2B-NS3^{pro}), plays a crucial role in cleaving the dengue polyprotein into individual proteins necessary for viral

replication (Lim et al., 2021). Targeting the DENV NS2B-NS3^{pro} has been a primary focus for developing antiviral treatments against dengue. Suppression of the DENV protease can be achieved at two potential locations which are the active site and the interaction site between the protease (NS3) and its protein cofactor (NS2B). The non-structural NS3 part is essential for post-translation processes in virus growth and maturation (Kronenberger et al., 2021) which consists of a conserved catalytic triad including His51, Asp75, and Ser135 (Noble et al., 2012; Zamri et al., 2019) and the presence of NS2B as a cofactor will enhances the activity of NS3. The hydrophilic domain of NS2B facilitates the initiation of the protease's activity, while the hydrophobic region assists in the cleavage process (Hariono et al., 2019). Structural analysis reveals that the NS2B cofactor forms a β-hairpin structure that surrounds the NS3 protease core, contributing to the dynamic conformation and catalytic activity of the DENV protease (Noble et al., 2012).

The world's first dengue vaccine, Dengvaxia (CYD-TDV), was produced by Sanofi Pasteur and introduced in Mexico in December 2015 (Organization, 2022). Dengvaxia is approved by regulatory authorities in 20 countries for trial use by people ranging from 9–45 years old in a highly endemic area (Guy et al., 2011). Based on the Figure 1.3, Philippines showed the highest number of dengue cases, therefore, it is no doubt that they are eager to approve the use of the Dengvaxia and became the first country to approve the use of the vaccine in 2016. However, the trial outcome showed that the vaccine trial participants in faced a higher risk of more severe dengue fever and hospitalisations compared to unvaccinated participants (Organization, 2022). In Philippine, a more severe circumstances occurred after the vaccination program which led to hospitalisation and death among children (Lo, 2021). In fact, Philippines was the first country to approve the use of the vaccine in 2016, and also the first country to ban the use of it based on safety risks in February, 2019 (Lo, 2021). Therefore, the use of this vaccine is not recommended

for children under 9 years old due to safety concerns (Deng et al., 2020) and the program was terminated by Department of Health, Philippine (DOH). On top of that, Sanofi Pasteur advised the people who have been diagnosed with dengue for the first time are not advised to take the vaccine, because it is only applicable to those who have previously been infected with dengue (Pinheiro-Michelsen et al., 2020).

Furthermore, Dengvaxia has low efficacy against DENV particularly dengue serotype II (39%), which is one of the most prevalent serotypes in Southeast Asian countries (Chew et al., 2017; Deng et al., 2020; Hariono et al., 2019; Suppiah et al., 2018; Zakaria et al., 2019). There are a few others dengue vaccine candidates such as LATV (The National Institute of Allergy and Infectious Diseases, Butantan and Merck), TAK-003 (Takeda), TDEN, DPIV, TVDV by U.S Army Medical Research and Materiel Command as well as V180 by Merck. LATV and TAK-003 are in the third phase of clinical trial and the rest are still under investigations (Pinheiro-Michelsen et al., 2020). There have been numerous procedures and protocols conducted in order to evaluate the vaccines' effectiveness and safety before being released to the public. The lack of adequate animal models for dengue vaccine development, the cost for developing and producing a vaccine, the difficulty of developing a vaccine against all four antigenically distinct DENV serotypes and the need to achieve balanced tetravalent responses that could exhibit significant immunity against all four viruses without the adverse effects of ADE (antibody dependent enhancement) or original antigenic sin are all the major challenges to the development of dengue vaccines (Chew et al., 2017). Dengue vaccination is currently not a viable option due to ineffectiveness and side effects of the vaccine. Instead, currently vector control and surveillance are the primary approaches to dengue prevention (Zaki et al., 2019). Therefore, it is recommended to study the local plants that might lead to the development of the vaccine which are accessible and affordable.

In order to effectively control the dengue borne disease, the program of releasing *Wolbachia*-infected mosquitoes was introduced in Malaysia since July 2019 (Timbuong, 2019). The *Wolbachia*-infected mosquitos were released in Malaysian cities with high populations and dengue cases, such as Kuala Lumpur, Pulau Pinang, Putrajaya and Selangor (Bedi, 2021; Nazni et al., 2019). According to the study, the number of human dengue events has decreased by 5-65% (Nazni et al., 2019). *Wolbachia* bacteria helps to prevent the spread of dengue fever by preventing mosquitos from transmitting the virus to humans (Chrostek et al., 2020; Nazni et al., 2019). However, according to the findings, the large-scale deployment of *Wolbachia*-based control will necessitate monitoring of the growing interaction between mosquito and virus genetic diversity, as well as *Wolbachia*-mediated blocking (Chrostek et al., 2020). Moreover, the long-term prevention by *Wolbachia* is still uncertain as the mechanism of *Wolbachia*-mediated pathogen blocking remains elusive (Chrostek et al., 2020).

Herbal products have experienced consistent growth over the years, and this growth trend is expected to continue through 2023, when the market is expected to reach $ 111 billion. From 2017 to 2023, the market is expected to grow at a compound annual growth rate, or CAGR, of approximately 7.2% (Future, 2018) and it is expected to grow to $5 trillion by 2050 (WHO, 2003). According to the reports by World Health Organization (WHO Traditional Medicine Strategy 2014-2023), the output of Chinese materia medica in 2021 was increased more than 20% as compared to 2011, accounting for approximately US$83.1 billion (Organization, 2013). Besides, United States has spent approximately $14.8 billion on natural product expenditures in 2008 (Organization, 2013). The market growth can be attributed to consumers' rising demand for traditional medicines (Ayurveda, Unani, and Traditional Chinese Medicine) which are less expensive, convenient and have lesser adverse effects compared to synthetic drugs (Ajasa et al.,

2004; Al-Adhroey et al., 2010). Furthermore, increasing significant research investments and funding will support market growth in the future (Muhammad & Awaisu, 2008).

Since herbal formulation has been used traditionally to treat many maladies from the early days from civilization, it is highly recommended that a dengue treatment can be developed from herbal or medicinal plants (Abd Kadir et al., 2013). Several reports have been documented on dengue treatment using traditional or medicinal plants in Asian countries such as Bangladesh, China, India, Philippines and Thailand (de Guzman et al., 2016; Saleh & Kamisah, 2020; Singh & Rawat, 2017). For example, a previous study by Saleh et al. (2020) describes that the leaves extract of papaya (*Carica papaya*) from Caricaceae family and tawa-tawa (*Euphorbia hirta*) from Euphorbiaceae family have been most extensively used to treat dengue infection across the globe (Saleh & Kamisah, 2020). Moreover, evidence of the efficacy of *Carica papaya* in humans against dengue infection has been reported (Singh & Rawat, 2017). The dengue patients consumed 2 tablespoonsful of the *Carica papaya* leaves juices extract in three times a day with six-hour intervals helps in rising the count of blood platelet or thrombocytes within 24 hours of treatment (Kala, 2012; Singh & Rawat, 2017). Meanwhile, the community in Pangasinan (Philippines) used the decoction of roots, stem, and leaves of *Euphorbia hirta* to cure dengue fever (de Guzman et al., 2016; Fiscal, 2017). Interestingly, a clinical study demonstrated that oral administration of an aqueous extract of *Euphorbia hirta* increased platelet and leukocyte counts in dengue fever patients but had no significant effect on haematocrit (Mir et al., 2012). Even though many medicinal plants have been employed for the treatment of dengue fever but the scientific evidence of the biological activity is still lacking.

Thus, we embarked a preliminary screening of medicinal plants for anti-protease activity against DENV2 NS2B/NS3 protease (Salleh et al., 2019). This screening also included *Z. zerumbet*, a traditionally used plant known for its potential in treating fever.

The results showed that *Z. zerumbet* inhibited NS2B/NS3 protease activity with 90% inhibition 200 µg/mL.

As fever is one of the most prominent dengue symptoms therefore, the anti-pyretic activity is required to evaluate the ability of the *Z. zerumbet* extracts in reducing and relieve fever. Additionally, conducting acute oral toxicity tests on the *Z. zerumbet* extracts is necessary to determine their toxicity levels before considering their development as a drug.

Moreover, understanding the relationship between acute toxicity and antipyretic properties is essential for the development of safe and effective drugs. It assists researchers in identifying substances that effectively reduce fever without causing significant adverse effects, leading to improved patient care and treatment outcomes.

Below are the problem statements of the study:

a. To date, there are no specific globally accepted treatments for dengue fever in any medical system since Dengvaxia has a limitation of efficacy and safety.
b. Dengue infections and death still prevails and among the main cause of death in Malaysia.
c. Various herbs or medicinal plants have been discovered to have anti-dengue related pharmacology properties, and they have traditionally been used in the treatment of dengue infection, including *Z. zerumbet*. However, no in-depth study has been done to investigate the potential of *Z. zerumbet*.

1.2 Objectives of the study

This research study is subjected for the bioassay-guided phytochemical study on the rhizomes of *Zingiber zerumbet* (L.) Smith with the following objectives:

1. To extract the rhizomes of *Z. zerumbet* using maceration techniques with different polarity of solvents (hexane, ethyl acetate, ethanol) and using hydrodistillation (essential oils) as well as to evaluate the inhibitory activity of all extracts against dengue virus type 2 (DENV2) via NS2B/NS3 protease,

2. To identify the volatile constituents of the essential oil from the rhizomes of *Z. zerumbet* using GC-FID and GC-MS techniques,

3. To fractionate the most potent extract against DENV2 NS2B/NS3 protease by column chromatography technique and to screen all the fractions for anti-protease activity against DENV2 NS2B/NS3 protease,

4. To isolate and elucidate the structure of compounds from the most potent fraction towards DENV2 NS2B/NS3 protease and to test all the compounds against DENV2 NS2B/NS3 protease inhibition, as well as to conduct molecular docking studies on the isolated compounds in order to determine the possible binding interactions between the compounds and the DENV2 NS2B/NS3 protease,

5. To investigate the acute toxicity and anti-pyretic activity of the most potent extract against DENV2 NS2B/NS3 protease.

CHAPTER 2: LITERATURE REVIEWS

This chapter is divided into three sub-chapters in general. The first sub-chapter discussed about the botanical and chemical aspects of medicinal plants, as well as the chemical constituents found in medicinal plants. The second sub-chapter will give an overview of dengue, acute oral toxicity and anti-pyretic studies. The final sub-chapter explained on molecular docking study especially on DENV2 NS2B/NS3 protease.

2.1 Botanical and chemical aspects

This sub-chapter briefly discussed on botanical and chemical aspects of Zingiberaceae, *Zingiber* sp. and *Zingiber zerumbet*, as well as the chemical constituents found in medicinal plants.

2.1.1 Zingiberaceae

Malaysia was named as one of the world's 17 megadiversity countries. The phrase "megadiverse" country refers to any of a set of countries that are home to many earth's species as well as a large number of endemic species (Pariona, 2021). Malaysia is placed twelfth in the world's biodiversity ranking and third in Asia after Indonesia and China, according to the National Biodiversity Index on the Convention on Biological Diversity in Montreal (2001) (Ng, 2008; T. Y. C. Tan et al., 2020). With only 0.2 percent of the world’s landmass, Malaysia is one of the world's wealthiest countries in terms of biodiversity per unit area due to the profusion of flora and fauna species, which endowed more than 15,000 species of flowering plants and over 151,500 species of invertebrates and vertebrates (Abdullah et al., 2015; Mamat, 2015; Mohamed, 2005).

Medicinal plants are plants that have medicinal abilities or have favourable pharmacological effects on the bodies of humans or animals (Namdeo, 2018). Approximately 10% of the 15,000 flowering plant species have therapeutic

characteristics, including the Apocynaceae, Lauraceae, Rubiceaceae, and Zingiberaceae families (Ibrahim F. H. et al., 2001a; Ibrahim F. H. et al., 2001b). Apart from therapeutic effects, these medicinal plants have a wide range of nutritional properties and possible bioactive chemicals with activity linked to a variety of disorders (Abu Bakar et al., 2018). Several scientific investigations have been conducted to assess the therapeutic characteristics of plants that have historically been used. For example, Zingiberaceae family has been extensively studied due to its traditional uses in meals and flavours, as well as in the treatment of common ailments such as fever, nausea, cold, asthma, and stomachache (Burkill, 1966; Ghosh et al., 2011; Jagadish et al., 2016).

In Malaysia, some plants from Zingiberaceae family are used traditionally. For example, indigenous people using the young shoot and fruits of *Etlingera elatior* as a food condiment and to alleviate ear-ache (Barbosa et al., 2017; Noweg et al., 2003). A decoction of rhizomes of *Zingiber officinale* is believed to relieve gastric and flatulence (Alsarhan et al., 2014), the stems of *Costus speciosus* were pounded and applied topically in healing cuts or wounds (Mohammad et al., 2012) and the juice of *Curcuma xanthorriza* rhizome is applied to the face to cure pimples (Khalid et al., 2011; Ong & Nordiana, 1999). Besides, Al-Adhroey and co-workers (2010) conducted a community-based ethnobotanical study in Peninsular Malaysia and discovered that *Languas galanga* have been used orally and other four plants; *Aeschynanthus sp*., *Alstonia angustiloba*, *Elateriospermum tapos* including *Curcuma domestica*, are used externally in the treatment of malaria (Al-Adhroey et al., 2010).

The genera of Zingiberaceae family namely *Alpinia*, *Costus*, *Curcuma*, *Etlingera* and *Zingiber* were studied for various biological activities such as an anti-inflammatory, anti-fungal, anti-microbial and anti-oxidant properties (Alsarhan et al., 2014; Apisariyakul et al., 1995; Aziz et al., 2013; Chan et al., 2008; Chan et al., 2011; Habsah et al., 2000; Yu

et al., 2009). For example, previous research done by Ibrahim et al. (2003) discovered that essential oils in four out of nine Zingiberaceae species were found to have anti-fungal activity, and these species were *Boesenbergia pandurata*, *Kaempferia galanga*, *Zingiber officinale*, and *Zingiber cassumunar* (Jantan et al., 2003). Furthermore, a study of methanol extracts of the rhizome of *Alpinia galanga* and *Zingiber officinale* revealed the anti-tuberculosis potential of the extracts against *Mycobacterium tuberculosis* with MIC of 1600 μg/mL (Mohamad et al., 2011).

2.1.2 Distribution and habitat

Zingiberaceae, or ginger family, is one of the largest families in the Zingiberales order, with 1500 species and 53 genera spread across tropical Africa, Asia and Americas, with Southeast Asia exhibit the greatest diversity (Saensouk et al., 2016). In Peninsular Malaysia, Zingiberaceae is a component of the herbaceous ground flora of the rainforest. Approximately 160 species of Zingiberaceae (18 genera) have been discovered in Peninsular Malaysia (Holttum, 1950; Khalid et al., 2011).

Zingiberaceae is classified as monocotyledonous plants which naturally grown on the ground flora of the primitive forest of Peninsular Malaysia. Zingiberaceae can be found anywhere in the forest and also usually known as scattered plants, seldom as a thicket. Most of the species are perennial, terrestrial and aromatic herbs found and aromatic herbs found in lowland and mid-mountain forest, with only a few found on high mountain ridges.

Zingiber is one of the largest genera in Zingiberaceae family. It was widely distributed in Southeast Asia and comprised about 150 species, where 51 species had been found in Malaysia according to Mybis database (Chaveerach et al., 2007; Ibrahim H. et al., 2007; *Malaysia Biodiversity Information System (MyBIS)*, 2015). *Zingiber* was distinguished from other genus by its key characteristic features which includes horn-like anther crest,

lateral staminodes that are fused to the labellum, elongated, labellum not connate to the filament, presence of a pulvinus at the base of the petiole and wrapped around the style (Kishor & Leong-Škorničková, 2013). According to Mybis database, 14 species were classified as endangered species in Malaysia. All the species was listed in Table 2.1 according to their distributions in Malaysia. Six species; *Z. chryseum*, *Z. chrysostachys*, *Z. longibracteatum*, *Z. malaysianum*, *Z. montanum*, and *Z. petiolatum* were found in Peninsular Malaysia and five species; *Z. griffithii*, *Z. leptostachyum*, *Z. odoriferum*, *Z. porphyrosphaera*, and *Z. stenostachys* were found in Borneo. Whilst 19 species were found in Sabah whilst 17 species were found in Sarawak (*Malaysia Biodiversity Information System (MyBIS)*, 2015; Milow et al., 2017; *The Plant List*, 2013; Theilade & Mood, 1999; Yen, 2011).

Zingiber zerumbet (L.) Smith, which belongs to the Zingiberaceae family is locally known in Malaysia as lempoyang and Asian ginger, bitter ginger, wild ginger, pinecone ginger and shampoo ginger by other countries (Baby et al., 2009; Ganapathy & Nair, 2017; Padalia et al., 2018). *Z. zerumbet* is mainly distributed in tropical and subtropical areas worldwide and is believed to be native to Southeast Asia. It was typically found in India, Bangladesh, Nepal, Malaysia, and Sri Lanka (Padalia et al., 2018). In Malaysia, *Z. zerumbet* was distributed throughout Peninsular Malaysia, including Sabah and Sarawak. *Z. officinale* was discovered in the states of Negeri Sembilan, Perak, Sabah, and Sarawak. Meanwhile, *Z. ottensii* was found in the islands of Pulau Pinang, Sabah and Sarawak. *Z. montanum* was found in Peninsular Malaysia, Sabah and Sarawak. Other than that, *Z. puberulum* was found in both Johor and Sarawak (*Malaysia Biodiversity Information System (MyBIS)*, 2015; Milow et al., 2017; *The Plant List*, 2013; Theilade & Mood, 1999; Yen, 2011).

Table 2.1 : Distributions of *Zingiber* species in Malaysia and Borneo.

Place of distributions / *Zingiber* species	Peninsular Malaysia	Borneo	Sabah	Sarawak
Z. acuminatum				√
Z. albiflorum				√
Z. argenteum				√
Z. aurantiacum	√			
Z. chlorobracteatum			√	
Z. chryseum	√			
Z. chrysostachys	√			
Z. coloratum			√	
Z. curtisii	√			
Z. eborinum			√	
Z. elatior	√			
Z. flagelliforme			√	
Z. flammeum			√	
Z. fraseri	√			
Z. georgeae				√
Z. gracile	√			
Z. griffithii	√	√		
Z. incomptum		√		√
Z. kelabitianum				√
Z. kelantanese	√			
Z. kunstleri	√			
Z. lambii			√	
Z. latifolium			√	
Z. leptostachyum		√		

‘Table 2.1, continued’

Place of distributions / *Zingiber* species	Peninsular Malaysia	Borneo	Sabah	Sarawak
Z. longibracteatum	√			
Z. longipedunculatum			√	√
Z. martinii				√
Z. mawangense				√
Z. malaysianum	√			
Z. montanum	√		√	√
Z. multibracteatum	√			
Z. odoriferum		√		
Z. officinale	√		√	√
Z. ottensii	√		√	√
Z. pachysiphon			√	√
Z. pendulum			√	
Z. petiolatum	√			
Z. phillippsiae			√	
Z. porphyrosphaera		√		
Z. porphyrosphaerum				√
Z. pseudopungens			√	√
Z. puberulum	√			√
Z. raja	√			
Z. stenostachys		√		
Z. spectabile	√			
Z. sulphureum	√			
Z. velutinum			√	
Z. vinosum			√	
Z. viridiflavum			√	
Z. wrayi	√			
Z. zerumbet	√		√	√

2.1.3 Botanical features and morphology

Zingiberaceae is recognized as herbs perennial, rarely epiphytic, terrestrial, aromatic herbaceous plants with fleshy, tuberous or non-tuberous rhizomes and frequently with tuber-bearing roots. Stems are typically short and replaced by leaf sheath-formed pseudostems. The leaves are distichous and simple with those at the plant's base normally has no blade and reduced to sheaths. Suborbicular or lanceolate to narrowly strap-shaped leaf blades that are rolled longitudinally in bud, hairy or glabrous, with a prominent midvein, numerous lateral veins that are typically pinnate and parallel, and an entire margin. Most of the family members are easily identified by their aromatic leaves and fleshy rhizome when both are crushed, as well as their elliptic-oblong leaves arranged in two ranks on the leaf-shoot.

Zingiber zerumbet (L.) Smith is a vigorous ginger growing up to 3 m tall with short oblong- lanceolate leafy stems that are replaced by the pseudostems, which are approximately 0.6 to 2 m tall (Koga et al., 2016). The rootstocks are aromatic, large and tuberous with pale yellow colour. The leaves are generally 15 to 40 cm long as well as 3 to 8 cm in width and grow on upright thin stems. Apparently, the leaves are numerous, sessile, distichous, short-petiolate or subsessile, the blades are oblong-lanceolate or broadly lanceolate, glabrescent, cuneate at the base and acuminate at the apex, sheaths are green and glabrescent, the lower surface is silky pubescent when young; ligule is 1.5 to 3 cm, entire and membranous. Both the pinecone-like inflorescences and the leaves grow from underground rootstocks of the plant. The flowers are fragrant, three-petaled, usually inconspicuous, creamy-yellow in colour, small, and always exist on the reddish-green cone. Inflorescence green colour when young, peduncle 30 to 70 cm tall, radical, ovate-oblong, spikes are 7 to 12 cm tall, often rounded at tips and formed brighter red colour when mature, bracteoles are 2.5 cm long and linear to lanceolate. The bracts are between 3 to 3.5 cm in length, 2.5 cm in width and it contain an aromatic, clear, slimy

sudsy and creamy fluid, that can be squeezed out and used as a natural hair conditioner and shampoo (Nalawade et al., 2003; Sabu, 2003). This is one of the factors contributing for its popularity and the origin of the name "shampoo ginger".

Figure 2.1 : *Zingiber zerumbet* (L.) Roscoe ex Sm.; (A) Whole plant, (B) Leaves, (C) Rhizomes, (D) Flowers.

The corolla tube is between 2.5 to 3 cm long, lobes are yellowish or white. The labellum is three-lobed, light yellowish creamy colour and the mid-lobe is about 1.8 cm long, suborbicular and bifid. The stamen, which has a longitudinal dehiscence attached

with a horn-like appendage or long-curved beak are distinctive feature that helps to distinguish *Z. zerumbet* from other species. The inner whorl contains one fertile stamen and two staminoids shaped like petals. The ovaries are trilocular and inferior, with an axillary placenta (Yob et al., 2011). The white fruits are about 2.5 cm long, dry or fleshy, glabrous and berry-like in appearance. The seeds are covered with lacerate aril, about 4 mm diameter, oblong, are few too many and black colour (Sabu, 2003). Figure 2.1 represents the whole plant (A), leaves (B), rhizomes (C), and flowers (D) of *Z. zerumbet*, respectively.

2.1.4 Classification of tribes

The earliest classifications of the Zingiberaceae family was suggested by Petersen in 1889 and was refined by other researchers, leading to recognition of four tribes which are Globbeae, Hedychieae, Alpinieae and Zingibereae (Kress et al., 2002). The four tribes are distinguished by morphology features such as the development of staminodia, number of locules and placentation in the ovary, rhizome-shoot-leaf orientation and modifications of the fertile anther (Kress et al., 2002).

The new classification of the Zingiberaceae was identified by Kress et al. in 2002 with the new phylogenetic analyses of DNA sequences of the nuclear internal transcribed spacer (ITS sequence) based together with plastid *matK* coding and uncoding areas. Kress et al. (2002) suggested at least some of these morphological characteristics are homoplasious and three of the tribes are paraphyletic. Consequently, based on the results of these phylogenetic studies, a new classification of the Zingiberaceae is suggested that revealed four tribes and four subfamilies which involves Siphonochiloideae (Siphonochileae), Tamijioideae (Tamijiae), Alpinioideae (Alpinieae, Riedelieae), and Zingiberoideae (Zingibereae, Globbeae) (Kress et al., 2002). The chart of the classification of the subfamilies and tribes of the Zingiberaceae is shown in Table 2.2

(Kress et al., 2002) and the example of the main groups of family Zingiberaceae and the sister family Costaceae as pictured in Figure 2.2 (Kress et al., 2002).

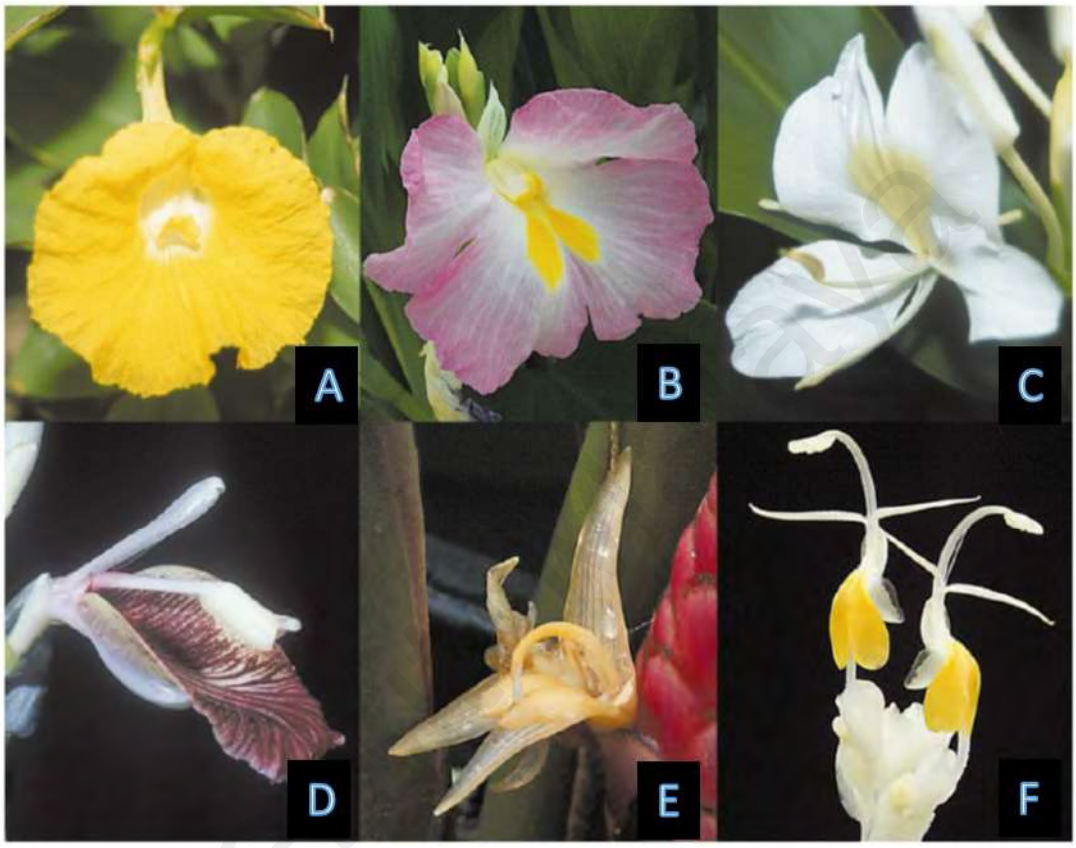


Figure 2.2: The floral of the main groups of family Zingiberaceae and the sister family Costaceae. A) Costaceae (*Monocostus*), B) Hedychieae (*Siphonochilus*), C) Hedychieae (*Hedychium*), D) Alpinieae (*Alpinia*), E) Zingibereae (*Zingiber*) and F) Globbeae (*Mantisia*).

(Source: Picture was adopted from Kress et al., 2002 was accessed on 5th September 2021(Kress et al., 2002)).

Table 2.2 : The new placement of genera in the new classification of the family Zingiberaceae (Kress et al., 2002).

Subfamily	Siphonochiloideae	Tamijioideae	Alpinioideae		Zingiberoideae	
Tribe	**Siphonochileae**	**Tamijieae**	**Alpinieae**	**Riedelieae**	**Zingibereae**	**Globbeae**
Genus	*Siphonochilus*	*Tamijia*	*Aframomum*	*Burbidgea*	*Cornukaempferia*	*Gagnepainia*
			Renealmia	*Pleuranthodium*	*Boesenbergia II*	*Hemiorchis*
			Amomum I	*Riedelia*	*Scaphochlamys*	*Globba*
			Elettariopsis	*Siamanthus*	*Distichochlamys*	*Mantisia*
			Paramomum		*Zingiber*	
			Alpinia I		*Kaempferia*	
			Plagiostachys		*Boesenbergia I*	
			Alpinia III		*Curcumorpha*	
			Alpinia IV		*Haniffia*	
			Alpinia II		*Roscoea*	
			Vanoverberghia		*Cautleya*	
			Etlingera II		*Rhynchanthus*	
			Amomum II		*Pommereschea*	
					Hedychium	
				Incertae Sedis	*Camptandra*	
				Siliquamomum	*Pyrgophyllum*	
					Hitchenia	
					Curcuma I	
					Curcuma II	
					Stahlianthus	
					Curcuma III	
					Smithatris	
					Incertae Sedis	
					Caulokaempferia	

2.1.5 Chemical constituents, ethnomedicinal properties and pharmacological aspects

Zingiber species is among the leading species in the Zingiberaceae family as it has been reported to possess numerous medicinal properties and used traditionally, particularly in food and flavours. In Peninsular Malaysia, the pounded of *Z. ottensii* (Lempoyang hitam) rhizomes have been used by mothers after childbirth as post-partum treatment and sometimes it is also used as appetizer (Malek et al., 2005; Sirat, 1994). Besides that, Samuel et al. (2010) has reported that the juice of *Z. ottensii* rhizomes was employed to cure all types of bacterial infections (Samuel et al., 2010).

Ginger (*Z. officinale*) is one of the world's most widely used natural products in spices, food, dyes, medicines, cosmetics, and fragrance (Jantan et al., 2003; Kumar et al., 2013; Lee & Kim, 2021). Ginger rhizomes are consumed raw or cooked as vegetable and used in the preparation of food as spices and condiments (Djati & Christina, 2019). The most common way to consume ginger is by diluting the juice with water and taken orally as it is a traditional herbal medicine for cough and sore throat (Djati & Christina, 2019). As mentioned in the Malay Tib of MS 174, a decoction of *Z. officinale* rhizomes, onion, garlic, lime and few others herb was drank for three days to treat sinusitis (Fatimah & Yusmaliza, 2019). *Z. officinale* has been studied intensively for its medicinal properties including anti-oxidant, anti-fungal, anti-microbial, anti-cancer, anti-hyperglycemic, anti-inflammatory, and larvicidal activities (Djati & Christina, 2019; Ibrahim H. et al., 2007; Jantan et al., 2003).

Zingiber montanum is also known as bonglai in Peninsular Malaysia, is another interesting *Zingiber* species (Ujang et al., 2015). Many reports have been published regarding the medicinal values of *Z. montanum*. For example, the powdered rhizomes or the rice water paste of the rhizomes is used as an anti-diarrheal medicine (Nair, 2013).

Other than that, the rhizome is used for post-natal treatment, swelling and rheumatism (Ujang et al., 2015). The rhizome extract of *Z. montanum* comprised the highest total curcuminoid content (2.633% w/w) as well as produced the highest amount of essential oil (0.89% v/w) and terpinen-4-ol (**1**) (14.51% v/w) content among the other ten *Zingiber* species (*Z. cornubracteatum*, *Z. ottensii*, *Z. montanum*, *Z. rubens*, Z. *barbatum, Z. bisectum*, *Zingiber* 'Phlai-chompoo', *Z. officinale*, *Z. spectabile*, and *Z. zerumbet*) (Kantayos & Paisooksantivatana, 2012). The main constituents; terpinen-4-ol (**1**) and (*E*)-1(3, 4-dimethylphenyl) butadiene (DMPBD) (**2**), are found to be effective against a variety of pathogenic bacteria and posseses anti-inflammatory property (Jeenapongsa et al., 2003). Furthermore, the methanol extract of *Z. montanum* rhizomes shows strong gastro-protective inhibition for anti-ulcer activity at dosage of 400 mg/kg in mice (Al-Amin et al., 2012).

Zingiber zerumbet (L.) Smith is one of the commonly used wild ginger species in Malay traditional medicine. The fresh rhizomes of *Z. zerumbet* are eaten as an appetizer or used to treat stomachache, fever and worm infestation (Khalid et al., 2011; Ong & Nordiana, 1999; Ruslay et al., 2007). The young stems, rhizomes and inflorescence are also applied topically as a poultice to treat muscle sprain and swollen wounds (Ruslay et al., 2007). The juices of boiled rhizomes are usually taken by postpartum women or post-surgical patients to improve their appetite, enhance recovery or healing as well as to alleviate pain (Grant & Lutz, 2000; Habsah et al., 2000). In Okinawa, Japan, the rhizome is consumed as food since it is believed to have kidney-healing properties (Nakatani et al., 1991). The *Z. zerumbet* has also been mentioned in Malay manuscripts (MSS 1653 and MS 2502), which can be formulated with other herbs such as *Z. offcinale* (ginger) and *Myristica fragrans* (nutmeg) to treat postpartum women; to strengthening reproductive organs, particularly the womb, stomach muscles and pelvic floor muscles (Nawawi,

2017). A summary of the traditional uses and medicinal properties of selected *Zingiber* species is shown in Table 2.3.

Table 2.3 : A summary of the traditional uses and biological properties of selected *Zingiber* species

No.	*Zingiber* species	Traditional medicine	Biological properties	Active compounds	References
1.	*Z. cassumunar*	Inflammation, bruise, sprain and strain, rheumatism, musculoskeletal pain, wound, asthma, cough and respiratory problem, mosquito repellent, carminative, mild laxative as well as anti-dysenteric agent.	Anti-microbial, anti-obesity, anti-inflammatory, analgesic activities, anti-tumor, anti-histamine, anti-fungal, anti-oxidant, anti-cancer, neuroprotective, α-glucosidase inhibitor	Cassumunin A, cassumunin B, sabinene, 26erpinene-4-ol, α-terpinene (*E*)-1-(3',4'-dimethoxyphenyl)but-1-ene, (*E*)-1–(3,4-dimethoxyphenyl)butadiene (DMPBD), (*E*)-1-(3',4'-dimethoxyphenyl)but-1,3-diene, (*E*)-1-(3',4'-dimethoxyphenyl)but-3-en-2-ol, (*E*)-4-(3',4'-dimethoxy-phenyl)but-3-en-2-ol, 1-feruloyloxycinnamic acid, bisdemethoxycurcumin, plain I, plain III, plain VI, neocassumunarins A, neocassumunarins B, (*E*)-4-(2',4',5'-trimethoxyphenyl)but-1,3-diene, (*E*)-4-(2',4',5'-trimethoxyphenyl)but-1-ene, cassumunaquinone 2, curcumin, β-sesquiphellandrene, (*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol, *trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene, *trans*-3-(4'-hydroxy-3'-methoxyphenyl)-4-[(*E*)-3''',4'''-dimethoxystyryl]cyclohex-1-ene, (*E*)-4-(2',4',5'-trimethoxyphenyl)but-1,3-diene, (*E*)-1-(3',4'-dimethoxyphenyl)but-1,3-diene	(Boonyanugomol et al., 2017; Han et al., 2021; Han et al., 2005; Hasimun et al., 2016; Jeenapongsa et al., 2003; Kaewchoothong et al., 2012; Khemawoot et al., 2016; Koysooko et al., 1998; Leelarungrayub et al., 2017; Nagano et al., 1997; Nair, 2013; Panthong et al., 1997; Pithayanukul et al., 2007; Pongprayoon et al., 1997; Sukatta et al., 2009; Vimala et al., 1999)

‘Table 2.3, continued’

No.	*Zingiber* species	Traditional treatment/uses	Biological properties	Chemical constituents	References
2.	*Z. mioga*	To treat cough and rheumatism (China). Leaves: used to wrap and preserve manjyu (bun filled with sweetened red bean paste). Young buds: served as food condiment and tempura.	Anti-cancer, anti-inflammatory, anti-microbial, anti-obesity, anti-oxidant, anti-platelet aggregation effect	Aframodial, miogatrial, miogadial, mioganal, galanal A, galanal B, (*E*)-labda-8(17),12-diene-15,16-dial, 8*β*(17)-epoxy-15-hydroxy-12-(*E*)-labden-16-al	(Abe et al., 2006; Cole & Nürnberger, 2014; Kim et al., 2005; Lee et al., 2016; Park et al., 2020; Shin et al., 2015)
3.	*Z. montanum*	To treat diarrhea and colic. Rhizomes: to reduce fever, vermifuge and for postpartum remedy.	Anti-inflammatory, counter-irritant, and mosquito repellent herb	(*E*)-4-(3’,4’-dimethoxyphenyl)but-3-en-1-ol and (*E*)-1-(3’,4’-dimethylphenyl) butadiene, curcumin	(Mahayothee et al., 2020; M. Sharifi-Rad et al., 2017; Wolff et al., 1999)
4.	*Z. officinale* var. rubrum	Colds, headache, vomiting, cough, flatulence. Rhizomes: stomachache, diarrhea, indigestion problem, fungal infection, chest pain. Water collected by the bracts taken orally (to treat influenza) used topically (to treat conjunctivitis).	Moderate activity of repellent activity against cockroach Larvicidal and repellent activity against the filarial mosquito *Culex quinquefasciatus* Anti-oxidant, anti-fungal, anti-microbial, anti-hyperglycemic, anti-inflammatory, anti-platelet aggregation, anti-ulcer	Zingiberene, ar-curcumene, 6-Shogaol, zingerone, 6-gingerol, 12-gingerol, *β*-sesquiphellandrene, *β*-bisabolene, 6-gingersulfonic acid	(Ahmad et al., 2015; Ghosh et al., 2011; Horie et al., 2004; Mohamad et al., 2011; Ong et al., 2011; Ong & Nordiana, 1999; Rahmani, 2014; M. Sharifi-Rad et al., 2017; Yoshikawa et al., 1994)

‘Table 2.3, continued’

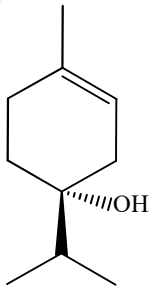
No.	*Zingiber* species	Traditional medicine	Biological properties	Active compounds	References
5.	*Z. ottensii*	Leaves: The poultice was used to treat lumbago and for mother after childbirth. Rhizomes: The juice of the rhizomes is used to cure all types of bacterial infections, gastric, dyspepsia, post-partum medicine, stomacache, fever, cough.	Anti-fungal agent, anti-diabetic, anti-cancer, antiproliferative, *α*-glucosidase inhibitor.	Humulene, humulene epoxide, zerumbone, (*E*)-labda-8(17),12-diene-15,16-dial, zingipain	(Burkill, 1966; Hasimun et al., 2016; Karnchanatat et al., 2011; Panyadee et al., 2019; Samuel et al., 2010; M. Sharifi-Rad et al., 2017; Sinaga et al., 2013; Thitinarongwate et al., 2021)
6.	*Z. purpureum*	Treat fever, headaches, stomach pain, rheumatism, and obesity and serves as an ingredient in post-partum herbal medicine.	Anti-seizure activity, insecticidal activity and larvicidal activity	Terpinen-4-ol, *trans*-banglene and *cis*-banglene	(Kubo et al., 2015; Wang et al., 2015)
7.	*Z. spectabile*	Leaves: food flavour, as a poultice to inflamed eyes and on to the body to reduce swelling and the infusion used for bathing to treat fever, a paste of fresh leaves was patched to heal the open wound, boiled water of leaves was used as facial and nasal wash to treat sinus. Young rhizomes: used to flavour food or as an appetizer.	Anti-oxidant, anti-bacterial, iron chelating agent,	Kaempferol, curcumin, demethoxycurcumin, demethoxycurcumin, spectaflavoside A	(Chee & Kah Hoo, 2010; Hamzah, 1999; H. C. Ong et al., 2012; Hean Chooi Ong et al., 2012; Sadhu et al., 2008; M. Sharifi-Rad et al., 2017; Sivasothy et al., 2012; Sivasothy et al., 2013; Wiart, 2012)

‘Table 2.3, continued’

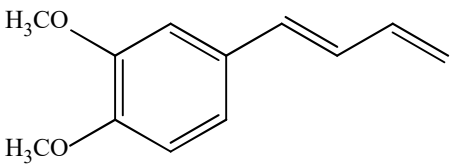
No.	*Zingiber* species	Traditional medicine	Biological properties	Active compounds	References
7.	*Z. spectabile*	Rhizomes: cough, asthma germicide, stimulant, tonic and in the treatment of cancer. Whole plant: the water obtained from boiled of the whole plant was used by women after giving birth for post-natal bath.	Anti-oxidant, anti-bacterial, iron chelating agent,	Kaempferol, curcumin, demethoxycurcumin, demethoxycurcumin, spectaflavoside A	(Chee & Kah Hoo, 2010; Hamzah, 1999; H. C. Ong et al., 2012; Hean Chooi Ong et al., 2012; Sadhu et al., 2008; M. Sharifi-Rad et al., 2017; Sivasothy et al., 2012; Sivasothy et al., 2013; Wiart, 2012)
8.	*Z. wrayi*	Blood circulation, skin and general well-being.	Anti-bacterial	Trans-anethole	(Chairgulprasert et al., 2005)
9.	*Z. zerumbet*	Leaves: Treatment for joint pain and skin diseases. Young shoots and inflorescence: food condiments. Rhizomes: food flavouring, appetizer, cough, cold, fever, swelling, stomachache, ear inflammation, colic pain, diarrhea, tonsillitis, sore throat, peptic ulcer, toothache, worm infestations and skin diseases.	Anti-bacterial, anti-cancer, anti-malaria, anti-oxidant, anti-spasmodic, anti-rheumatic, anti-flatulent and diuretic agents, glucosidase inhibitors, anti-inflammatory and chemopreventive activities. Larvicidal activity against fourth instar larvae of *Cx. quinquefasciatus*	Zerumbone, 6-gingerol, zederone, kaempferol, kaempferol-3-*O*-methylether, 3-*O*-methyl kaempferol, caryophyllene oxide, spathulenol and (*Z*)-*γ*-bisabolene	(Ajish et al., 2014; Chien et al., 2008; Govindarajan et al., 2018; Gyrdymova & Rubtsova, 2021; Jones & German, 1993; Kader et al., 2010; Koga et al., 2016; K. Nascimento et al., 2017; Park et al., 2011; Russo et al., 2018; Schmidt et al., 2010; M. Sharifi-Rad et al., 2017; Silva et al., 2018; Sriphana et al., 2013; Wan Salleh et al., 2015)

The chemical compositions of *Zingiber* species have been studied by many researchers. For example, study has been done by Malek et al. (2005) for the chemical constituents of the essential oil of *Z. ottensii* collected from Johor and Sabah. The study revealed that higher content of zerumbone (**3**) was found in Johor sample compared to Sabah and some of the compounds were not detected in the species from Johor but present in Sabah such as (*E*)-*β*-ocimene (**4**), *trans*-sabinene hydrate (**5**), *cis*-sabinene hydrate (**6**), isoborneol (**7**), bornyl acetate (**8**), *α*-ylangene (**9**), elemol (**10**), (*Z*)-nerolidol (**11**), and caryophyllene oxide (**12**) (Malek et al., 2005). Moreover, three sesquiterpenes and one diterpene were isolated from *Z. ottensii* in 1994, namely humulene (**13**), humulene epoxide (**14**), zerumbone (**3**), and (*E*)-labda-8(17),12-diene-15,16-dial (**15**) (Sirat, 1994).

Other than that, chemical constituents in *Z. officinale* that lead to its anti-oxidant property are *β*-carotene (**16**), terpenoids, alkaloids, and polyphenols (flavonoids, flavones glycosides, and rutin) (Alsarhan et al., 2014; Ghasemzadeh et al., 2010). The *Z. officinale*'s pungency is produced by phenolic chemicals in the rhizomes in which 6-, 8-, and 10-gingerol (**17**)-(**19**) are the most abundant compounds in fresh ginger (Djati & Christina, 2019). The selected compounds found in *Zingiber* species were illustrated in Figure 2.3.



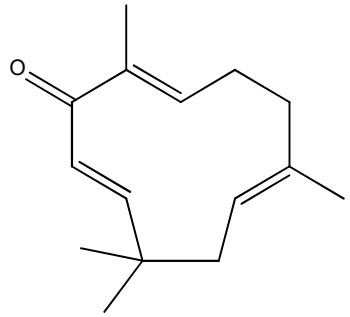
terpinen-4-ol (**1**)



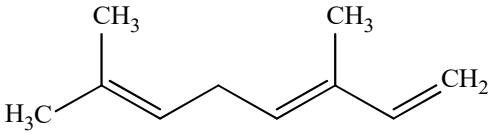
(*E*)-1(3, 4-dimethylphenyl) butadiene (DMPBD) (**2**)

Figure 2.3 : The selected compounds found in *Zingiber* species.

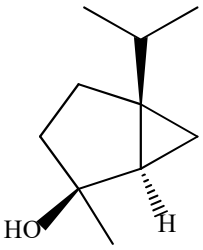
‘Figure 2.3, continued’



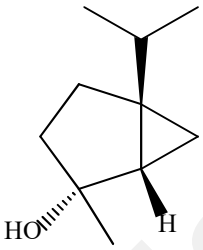
zerumbone (**3**)



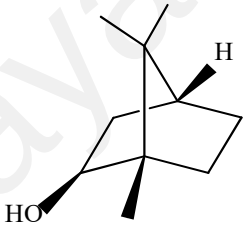
(*E*)-*β*-ocimene (**4**)



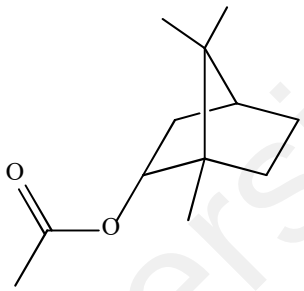
trans-sabinene hydrate (**5**)



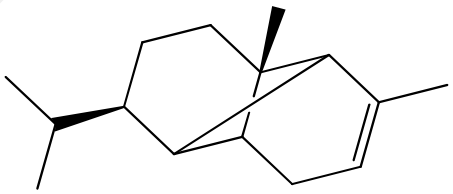
cis-sabinene hydrate (**6**)



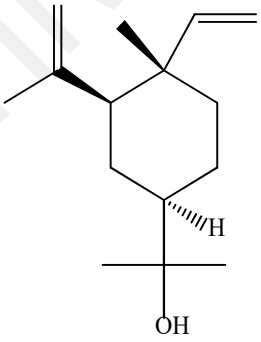
isoborneol (**7**)



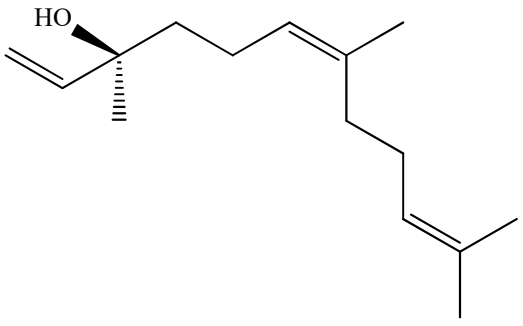
bornyl acetate (**8**)



α-ylangene (**9**)

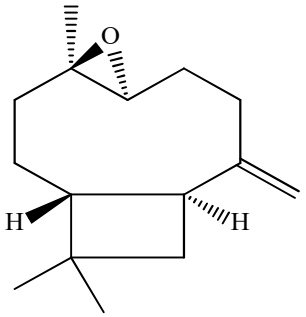


elemol (**10**)

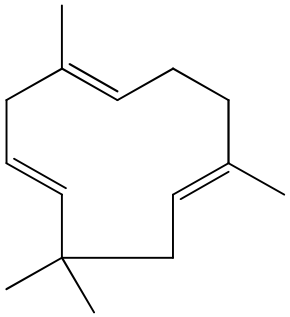


(*Z*)-nerolidol (**11**)

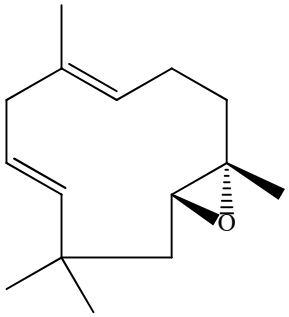
‘Figure 2.3, continued’



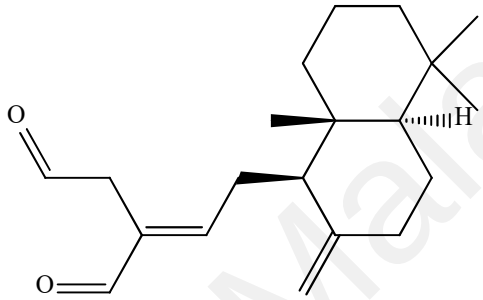
caryophyllene oxide (**12**)



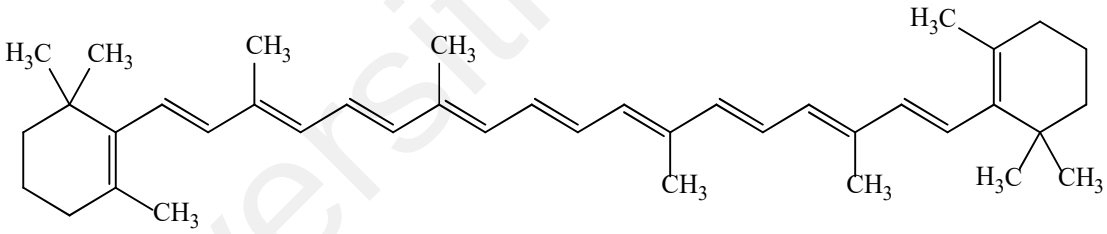
humulene (**13**)



humulene epoxide (**14**)

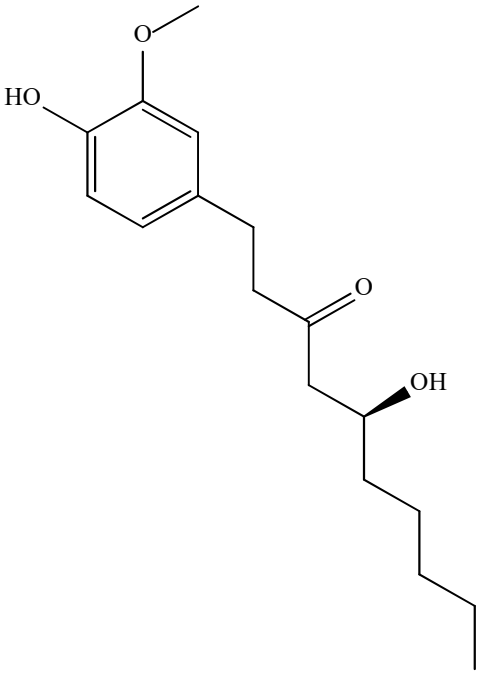


(*E*)-labda-8(17),12-diene-15,16-dial (**15**)

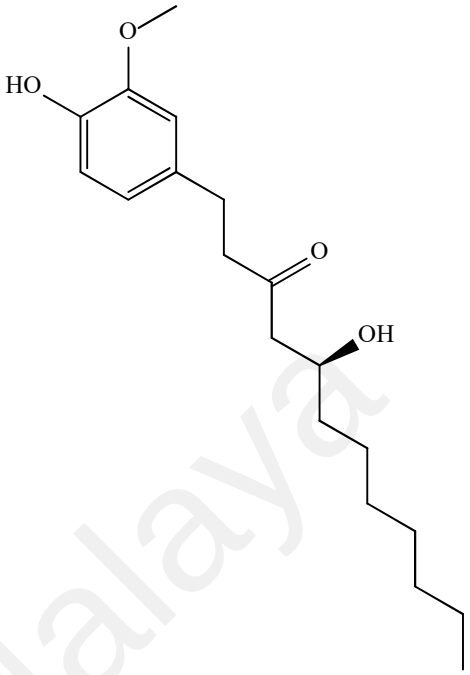


β-carotene (**16**)

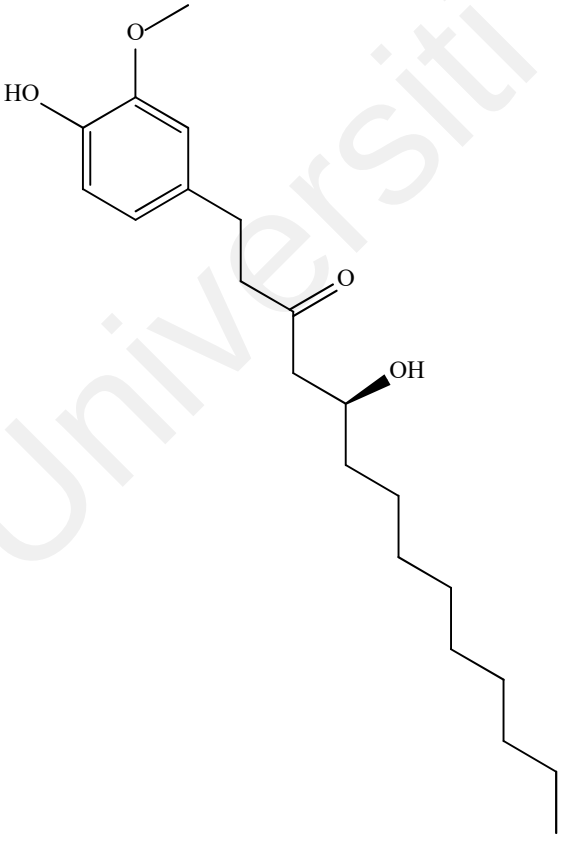
‘Figure 2.3, continued’



6-gingerol (**17**)



8-gingerol (**18**)



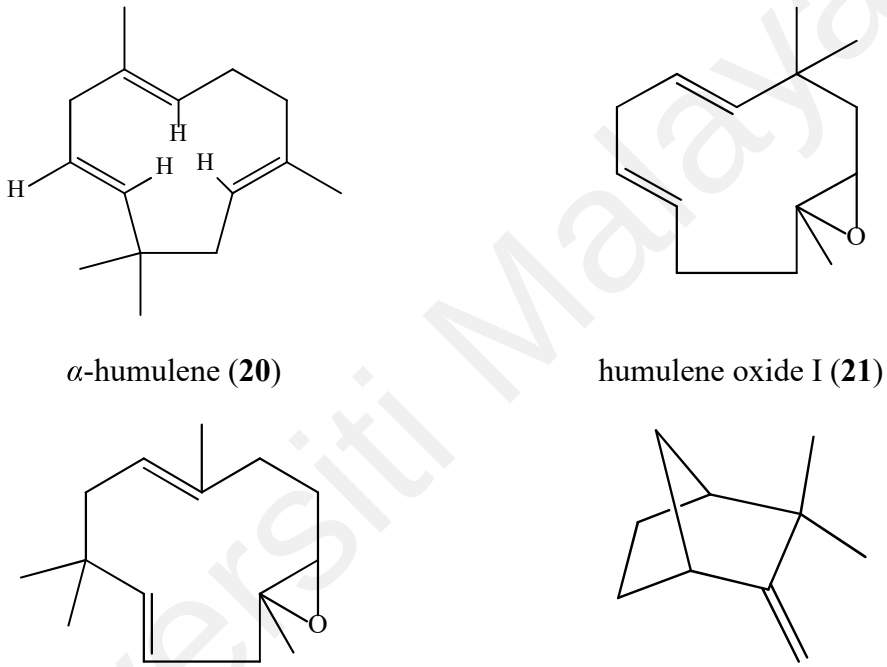
10-gingerol (**19**)

Z. zerumbet is dominated by two major classes of compounds which are terpenes and polyphenols (Hamid et al., 2018; Koga et al., 2016; Matthes et al., 1980; Peng et al., 2022). Varier made the first attempt to isolate and identify chemicals from the rhizomes of *Z. zerumbet* in 1945, he isolated humulene (**13**), a sesquiterpene (Varier, 1945). However, the major compound which is zerumbone (**3**); a monocyclic sesquiterpene, is the most isolated and often used bioactive metabolite. Compound **3** was isolated for the first time from essential oil of *Z. zerumbet* by Dev in 1960 (Dev, 1960). The amount of **3** in the rhizomes of *Z. zerumbet* are dependent on the maturity of the rhizomes (Chien et al., 2008). According to Chien et al. (2008), the rhizome of 5 months old contain **3** at three times higher than young rhizomes and the moisture content in the rhizome decreased significantly after 5 months old (Chien et al., 2008). A study from various geographical locations done by Tian et al. (2020). He reported that **3** is the major compound with ranged from 8.1 to 84.8 % in fresh rhizome essential oil however slightly lower at 1.2 to 35.5 % in the dried rhizome essential oil (Dai et al., 2013; Oliveros & Cantoria, 1982; Sutthanont et al., 2010; Tian et al., 2020). Previous study also mentioned that the oven drying process using oven might be attributed to the loss of **3** in the dried rhizome of *Z. zerumbet* and resulted in the low extraction yield of essential oil (0.39%) compared to fresh rhizome (0.65%). Other studies showed various content of **3** in *Z. zerumbet* from Sabah, Penang (68.9%), and Selangor (36.1%) in which the *Z. zerumbet* from Sabah containing the highest amount of **3** (73.1%) (Baby et al., 2009; Malek et al., 2005; Sulaiman et al., 2010). The content of **3** in *Z. zerumbet* from India was also differed depending on the region (Baby et al., 2009; Dash et al., 2019; Helen et al., 2009; Madegowda et al., 2016; Padalia et al., 2018; Rana et al., 2016; Singh et al., 2014; Srivastava et al., 2000). The most abundance of **3** (84.8%) was identified from the Kallagatta, Kasargode southern Indian state of Kerala while the least amount of **3** (12.6%) was found in Siwan district, Bihar northeastern of India (Baby et al., 2009; Srivastava et al., 2000). Furthermore, a

comparison of extraction technique was conducted in Vietnam to compare the yield of **3** extracted through hydro distillation and steam distillation extraction method. The study revealed that the hydrodistillation of dried rhizomes of *Z. zerumbet* produced a higher yield of **3** (51.3%) than the steam distillation method (31.7%) (Sutthanont et al., 2010; Thi Huong et al., 2020). In addition, the parts of plant also affected the composition of **3** in the essential oil, as studied by Duñg et al. (1993), the highest amount of **3** was found in the stems of *Z. zerumbet* (21.3%) as compared to leaves (2.4%) and flowers of *Z. zerumbet* (3.2%) (Duñg et al., 1993). Therefore, the major compounds and chemical constituents identified varied in accordance with various geographic locations, agroclimatic zone, environment factors, ecological factors, plant age and sample preparation methods involving drying and extraction techniques. The composition of **3** at various geographic regions, differences of extraction methods and parts of plants was compiled in Table 2.4.

Rawat et al. (2023) recently reported that different comminution methods influence the essential oil yield as well as the chemical compositions in *Z. zerumbet* (Rawat et al., 2023). The study employed five different comminution methods were used which are grating, chopping, slicing, using a mixture grinder, and using a pestle mortar. According to the study, the grating techniques (0.97%) produced the highest essential oil yield of *Z. zerumbet*, followed by using a pestle mortar (0.75%), slicing (0.68%), chopping (0.67%), fresh rhizomes (0.44%) and a mixture grinder (0.21%) produced the lowest essential oil yield (Rawat et al., 2023). In addition, thirty-three volatile constituents were identified by GC-FID and GC-MS which accounted for 94.68% (fresh rhizomes), 96.93% (grating), 96.79% (chopping) 96.84% (slicing), 97.42% (mixture grinder) and 98.29% (pestle mortar) of the total essential oils. Furthermore, the study also revealed zerumbone (**3**) (69.66 - 73.90%) was the most abundance in all oil collections followed by α-humulene (**20**) (5.40 - 14.99%), humulene oxide I (**21**) (3.10 - 3.81%), humulene oxide II (**22**) (1.03

- 2.47%) and camphene (**23**) (2.60 - 3.56%) Figure 2.4 (Rawat et al., 2023). Therefore, the study suggested that the extraction of essential oils by crushing the rhizomes in pestle mortar for the higher essential oil yield and produced a better-quality *Z. zerumbet* product due to the total amount of compounds bearing the humulene skeleton (zerumbone (**3**), *α*-humulene (**20**), humulene oxide I (**21**) and humulene oxide II (**22**) were higher in the essential oil of rhizomes crushed using pestle mortar (90.00%) (Rawat et al., 2023).



α-humulene (**20**) humulene oxide I (**21**)

humulene oxide II (**22**) camphene (**23**)

Figure 2.4 : Molecular structures of *α*-humulene (20), humulene oxide I (21), humulene oxide II (22) and camphene (23).

Table 2.4 : A compilation of composition of zerumbone (3) in *Z. zerumbet* from various geographical locations.

Sample collection location	Plant part	Extraction method	Zerumbone (%)	Reference
Bangladesh	Leaves (fresh)	Hydrodistillation	37.0	(Bhuiyan et al., 2008)
	Rhizome (fresh)		46.8	
China	Rhizome (fresh)	Hydrodistillation	75.0	(Tian et al., 2020)
	Rhizome (dried)		41.9	
China	Rhizome (fresh)	Hydrodistillation	40.2	(Wu et al., 2017)
French Polynesia	Rhizome (fresh)	Hydrodistillation	65.3	(Lechat-Vahirua et al., 1993)
India	Rhizome (fresh)	Hydrodistillation	12.6-84.8	(Baby et al., 2009; Dash et al., 2019; Helen et al., 2009; Madegowda et al., 2016; Padalia et al., 2018; Rana et al., 2016; Singh et al., 2014; Srivastava et al., 2000)
Malaysia	Rhizome (fresh)	Hydrodistillation	73.1	(Malek et al., 2005)
Malaysia	Rhizome (fresh)	Hydrodistillation	68.9	(Baby et al., 2009)
Malaysia	Rhizome (fresh)	Hydrodistillation	36.1	(Sulaiman et al., 2010)
Reunion island	Rhizome (fresh)	Steam distillation	37.0	(Chane-Ming et al., 2003)
Thailand	Rhizome (fresh)	Hydrodistillation	8.1	(Suthisut et al., 2011)
Vietnam	Rhizome (fresh)	Steam distillation	72.3	(Duñg et al., 1993)
Vietnam	Stem (fresh)	Steam distillation	21.3	(Dũng et al., 1995)
	Leaves (fresh)		2.4	
Vietnam	Rhizome (dried)	Steam distillation	31.7	(Sutthanont et al., 2010)
Vietnam	Roots (dried)	Hydrodistillation	1.2	(Dai et al., 2013)
Vietnam	Rhizome (dried)	Hydrodistillation	51.3	(Thi Huong et al., 2020)

Humulene monoxide (**24**) and humulene dioxide (**25**) were isolated and identified by Ramaswami and Bhattacharyya from the essential oils of *Z. zerumbet*'s rhizomes (Ramaswami & Bhattacharyya, 1962). Furthermore, Nigam and Levi (1963) reported that twelve compounds were obtained from eight fractions of *Z. zerumbet* essential oil by column chromatography with petroleum ether, benzene, ether, and methanol used as eluants. Those compounds were α-pinene (**26**), *α*-humulene (**20**), α-terpineol (**27**), ar-curcumene (**28**), *β*-caryophyllene (**29**), borneol (**30**), camphor (**31**), humulene monoxide (**24**), humulene dioxide (**25**), limonene (**32**), linalool (**33**), and zerumbone (**3**) (Nigam & Levi, 1963). Six sesquiterpenes were also identified in *Z. zerumbet* essential oil by Damodaran and Dev et al. (1968), which are humulene epoxide I (**34**), humulene epoxide II (**35**), humulene epoxide III (**36**), humulenol I (**37**), humulenol II (**38**) and caryophyllene oxide (**12**) (Damodaran & Dev, 1968).

Besides zerumbone (**3**), three flavonoid compounds were isolated from the methanol extract of the rhizomes of *Z. zerumbet*, which are 3-*O*-methyl kaempferol (**39**), kaempferol-3-*O*-(2,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**40**) and kaempferol-3-*O*-(3,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**41**) (Chien et al., 2008). Other than that, Ruslay et al. (2007) identified other flavonoid compounds from the ethyl acetate fraction of *Z. zerumbet* water extract which are kaempferol-3-*O*-rhamnoside (**42**), kaempferol-3-*O*-(2" or 3"-acetyl)rhamnoside (**43**), kaempferol-3-*O*-(4"-acetyl)rhamnoside (**44**), kaempferol-3-*O*-(2",4"-diacetyl)rhamnoside (**45**) and kaempferol-3-*O*-(3",4"-diacetyl)rhamnoside (**46**) (Ruslay et al., 2007).

The chloroform-soluble part from the rhizomes of *Z. zerumbet* produced eight compounds, which include *p*-hydroxybenzaldehyde (**47**), vanillin (**48**), kaempferol-3-*O*-methylether (**49**), kaempferol-3,4'-*O*-dimethylether (**50**), kaempferol-3,4',7-*O*-trimethylether (**51**), kaempferol-3-*O*-(2,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**40**),

kaempferol-3-*O*-(3,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**41**) and kaempferol-3-*O*-(4-*O*-acetyl- α - L-rhamnopyranoside) (**52**) (Jang et al., 2004). Those acetylated flavanol glycosides ; kaempferol-3-*O*-(4-*O*-acetyl- α -L-rhamnopyranoside) (**52**), kaempferol-3-*O*-(3-*O*-acetyl- α -L-rhamnopyranoside (**53**), kaempferol-3-*O*-(2-*O*-acetyl- α -L-rhamnopyranoside (**54**) and kaempferol-3-*O*-α-L-rhamnopyranoside (**55**) were also obtained from the acetone extracts of the rhizomes of *Z. zerumbet* (Masuda et al., 1991). Nakatani et al. (1991) also reported two acetylated flavanol glycosides ; kaempferol-3-*O*-(2,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**40**) and kaempferol-3-*O*-(3,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**41**), two flavanol; kaempferol-3-*O*-methylether (**49**) and kaempferol-3,4'-*O*-dimethylether (**50**), zerumbone (**3**), zerumbone epoxide (**56**) and curcumin (**57**) from the dichloromethane-soluble part extract of *Z. zerumbet* rhizomes (Nakatani et al., 1991).

Besides that, till date numerous compounds have been identified from various extracts of its rhizomes such as kaempferol (**58**), kaempferol-3-*O*-methylether (**49**), zederone (**59**), diferuloylmethane (**60**), di-*p*-coumaroylmethane (**61**), feruloyl-*p*-coumaroylmethane (**62**) and zerumbone epoxide (**56**).

Ajish et al. (2014) communicated that seven compounds have been isolated from the rhizomes of *Z. zerumbet* which are α-humulene (**20**), zerumbone (**3**), zerumbone epoxide (**56**), zerumbol (**63**), kaempferol (**58**), kaempferol-3-*O*-methylether (**49**), and kaempferol-3-*O*-(3,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**41**) (Ajish et al., 2014). Those compounds have been tested for α-glucosidase, aldose reductase and glycation inhibitory properties for anti-diabetic and its complication treatment. Kaempferol (**58**) and kaempferol-3-*O*-methylether (**49**) are flavonoids (the largest subclass of polyphenol) were found to be potent inhibitors for *α*-glucosidase enzyme, aldose reductase enzyme and glycation reaction (play an important role for the treatment of diabetes) (Ajish et al., 2014).

Zerumbone (**3**) is the major compound in the rhizomes of *Z. zerumbet* which possesses anti-inflammatory, anti-cancer properties and also has the potential to be used in the treatment of Alzheimer's disease (Bustamam et al., 2008; Murakami et al., 2002; Murakami et al., 2004). It also exhibited strong anti-malarial activity against *P. falciparum* with IC_{50} values of 11.8 μM (Sriphana et al., 2013). While, zederone (**59**) showed weak anti-bacterial activity against five strains of *Staphylococcus aureus* with minimum inhibitory concentration ranging between 64-128 μg/mL (Kader et al., 2010). Diferuloylmethane (**60**), di-*p*-coumaroylmethane (**61**), feruloyl-*p*-coumaroylmethane (**62**) were highly toxic against hepatoma tissue culture at 33 μg/mL and zerumbone epoxide (**56**) were not toxic at this dose (Matthes et al., 1980). Other than that, zerumbone (**3**), 3-*O*-methyl kaempferol (**39**), kaempferol-3-*O*-(2,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**40**) and kaempferol-3-*O*-(3,4-di-*O*-acetyl-α-L-rhamnopyranoside) (**41**) were also subjected to anti-inflammatory activity and resulted in zerumbone (**3**) and 3-*O*-methyl kaempferol (**39**) demonstrated potent inhibition of NO production with respective IC_{50} values of 4.37 and 24.35 μM (Chien et al., 2008). All structures of the compounds found in the rhizomes of *Z. zerumbet* were illustrated in Figure 2.5 and the biological activities of selected compounds in *Z. zerumbet* were listed in Table 2.3.

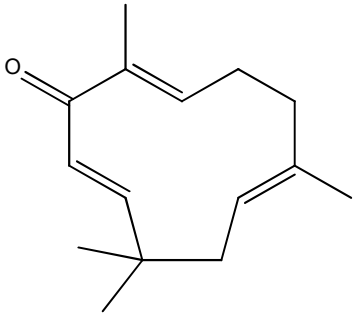
Z. zerumbet has been reported to possess various biological activities such as anti-oxidant (Chang et al., 2014; Jantan et al., 2012; Ramdan et al., 2013; Ruslay et al., 2007), anti-bacterial (Hwang et al., 2000) anti-inflammatory (Chang et al., 2014; Chien et al., 2008; Nurfina et al., 1997), anti-hyperglycaemic (Chang et al., 2012c), anthelmintic (Sahu et al., 2018), anti-cancer (Kirana et al., 2003; Murakami et al., 2004; Ramdan et al., 2013; Sakinah et al., 2007; Songsiang et al., 2010; Xian et al., 2007) and antinociceptive activities (Khalid et al., 2011; Somchit et al., 2005). According to Table 2.5, the aqueous and ethanol extracts were the most studied extracts among the other extracts. The ethanol extract of *Z. zerumbet* was evaluated for anti-hyperglycemic

activity. The results indicated that the rats administered with ethanol extract of *Z. zerumbet* at dosage of 300 mg/kg per day reduced body weight gain, visceral fat-pad weights, and plasma lipid levels in high-fat diet-induced rats (Chang et al., 2012c).

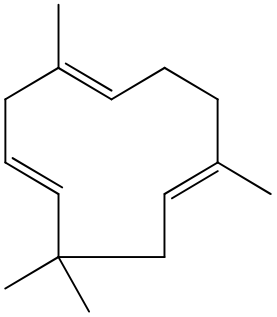
In addition, the hexane crude of *Z. zerumbet* demonstrated the most effective larvacidal activity against *Culex quinquefasciatus* larvae with LC_{50} of 49.28 mg/L and LC_{90} of 83.87 mg/L and *Aedes aegypti* larvae with LC_{50} of 82.05 mg/L and LC_{90} of 121.05 mg/L (Restu Wijaya et al., 2017). Other researchers revealed that the dichloromethane and methanol extracts of *Z. zerumbet* shown the efficient larvicidal activity against *Aedes aegypti* and *Anopheles nuneztovari* larvae (Bucker et al., 2013). Besides, the methanol fraction of *Z. zerumbet* had been reported to exhibit 89% of inhibition against DENV2 virus NS2B/NS3 protease at a concentration of 300 ppm (Tan et al., 2006). The biological activities of various *Z. zerumbet* extracts were summarized in Table 2.5.

Table 2.5 : Summary of biological activities of *Zingiber zerumbet* extracts.

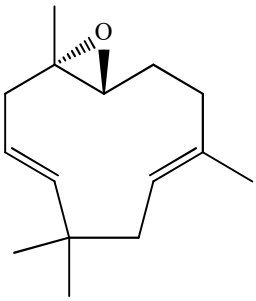
Extract	Biological activities	Reference
Aqueous	Anthelmintic, anti-allergy, anti-fungal, anti-inflammatory, anti-proliferative, anti-pyretic, anti-hypersensisitve	(Chaung et al., 2008; Rashid & Pihie, 2005; Ravi et al., 2017; Sahu et al., 2018; Somchit et al., 2005; Tewtrakul & Subhadhirasakul, 2007)
Benzene	Anti-oxidant	(Ganapathy & Nair, 2017)
Chloroform	Anti-tumor	(Vimala et al., 1999)
Dichloromethane	Larvicidal	(Bucker et al., 2013)
Hexane	Cytotoxicity, larvicidal	(Norfazlina et al., 2013; Restu Wijaya et al., 2017)
Hydro-alcoholic and water (Ethanol (70) : Water (30))	Anthelmintic, anticonvulsant	(Lincy Joseph, 2015; Sahu et al., 2018)
Isopropanol	Anti-oxidant, anti-microbial	(Ganapathy & Nair, 2017; Sreevani et al., 2013)
Methanol	Anti-inflammatory, anti-oxidant, antinociceptive, anti-protease, larvicidal	(Bucker et al., 2013; Jantan et al., 2005; Kiat et al., 2006; Sreevani et al., 2013; Zakaria et al., 2010)
Ethanol	Acute toxicity, analgesic, anthelmintic, anti-allergy, anti-bacterial, anti-fungal, anti-hyperglycemic, anti-hyperlipidaemic, anti-oxidant, anti-pyretic, anti-tumor, anti-retinopathy, cytotoxicity, HIV inhibitory, genotoxicity, phagocytic activity, renoprotective effect, inhibition of sterol regulatory element-binding protein-1c (SREBP-1c) and induction of peroxisome proliferator-activated receptor α (PPARα)	(Akhtar et al., 2019; Chang et al., 2014; Chang et al., 2012a, 2012b; Chang et al., 2012c; Hossain et al., 2012; Kader et al., 2011; Kantayos & Paisooksantivatana, 2012; Nag et al., 2013; Pandey et al., 2011; Raj, 1975; Sinaga et al., 2011; Somchit et al., 2005; Sreevani et al., 2013; Tewtrakul & Subhadhirasakul, 2007; Thummajitsakul et al., 2016; Tzeng et al., 2015; Tzeng et al., 2013)
Ethyl acetate	Anti-oxidant, anti-glycation, hepatoprotective, nephroprotective effects	(Abdul Hamid et al., 2012; Bhavesh et al., 2013; Hamid et al., 2011; Ruslay et al., 2007)



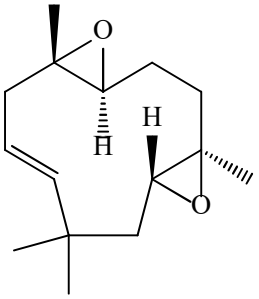
Zerumbone (**3**)



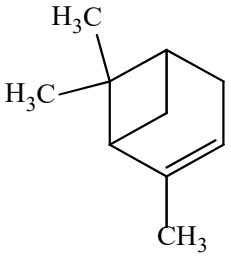
Humulene (**13**)



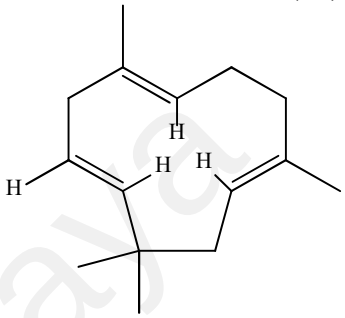
Humulene monoxide (**24**)



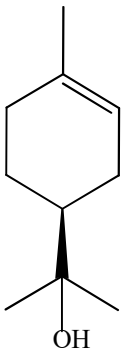
Humulene dioxide (**25**)



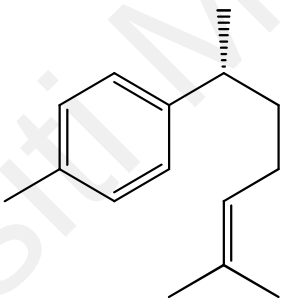
α-pinene (**26**)



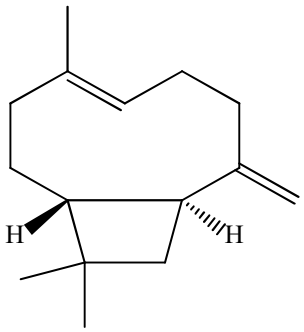
α-humulene (**20**)



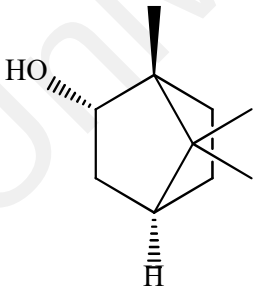
α-terpineol (**27**)



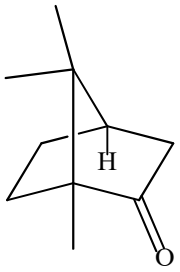
ar-curcumene (**28**)



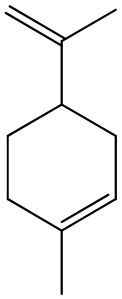
β-caryophyllene (**29**)



`Borneol (**30**)



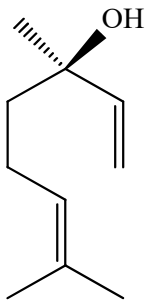
Camphor (**31**)



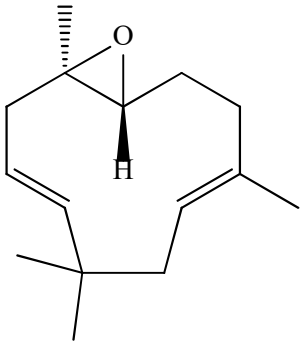
Limonene (**32**)

Figure 2.5 : Structures of selected compounds found in the rhizomes of *Zingiber zerumbet*.

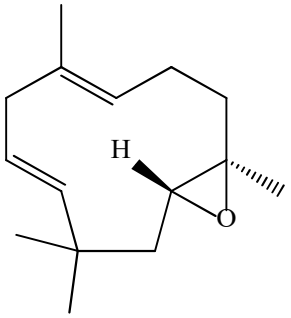
‘Figure 2.5, continued’



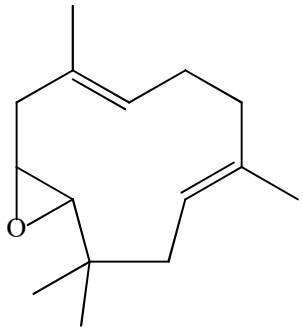
Linalool (**33**)



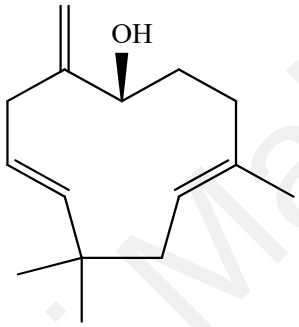
Humulene epoxide I (**34**)



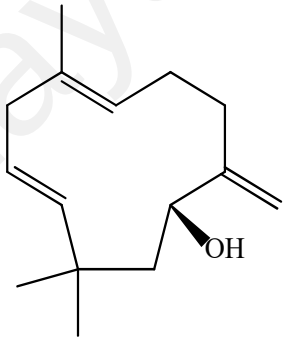
Humulene epoxide II (**35**)



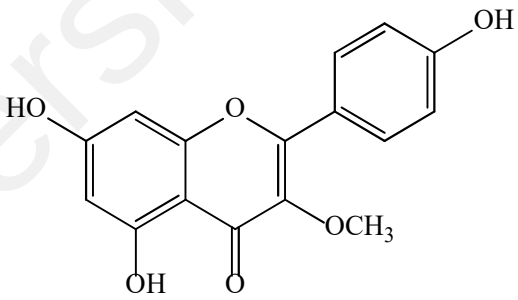
Humulene epoxide III (**36**)



Humulenol I (**37**)

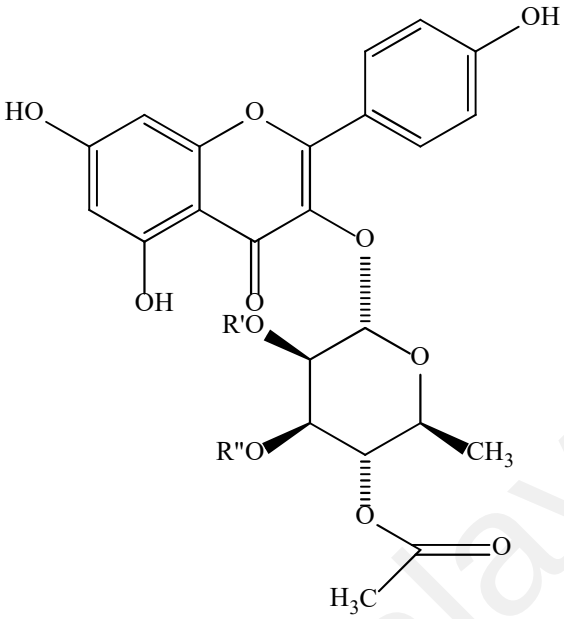


Humulenol II (**38**)



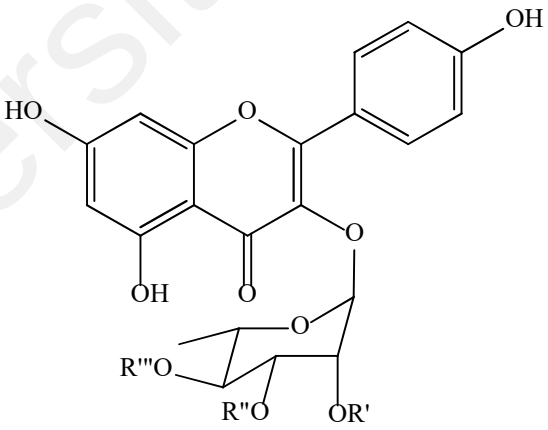
3-*O*-methyl kaempferol (**39**)

‘Figure 2.5, continued’



R’ = $COCH_3$; R” = H Kaempferol-3-*O*-(2,4-di-*O*-acetyl-*α*-L-rhamnopyranoside (**40**)

R’ = $COCH_3$; R” = H Kaempferol-3-*O*-(3,4-di-*O*-acetyl-*α*-L-rhamnopyranoside (**41**)



R’ = R” = R”’ = H Kaempferol-3-*O*-rhamnoside (**42**)

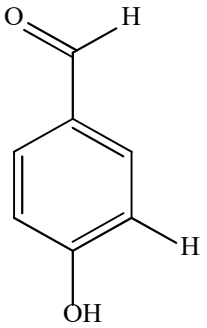
R’ = R”’ = H ; R” = $COCH_3$ Kaempferol-3-*O*-(2” or 3”-acetyl)rhamnoside (**43**)

R’ = R” = H ; R”’ = $COCH_3$ Kaempferol-3-*O*-(4”-acetyl)rhamnoside (**44**)

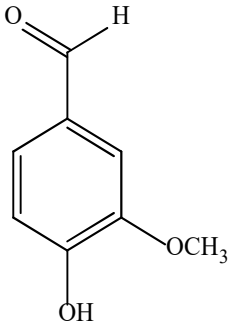
R’ = R”’ = $COCH_3$; R” = H Kaempferol-3-*O*-(2”,4”-diacetyl)rhamnoside (**45**)

R” = R”’ = $COCH_3$; R’ = H Kaempferol-3-*O*-(3”,4”-diacetyl)rhamnoside (**46**)

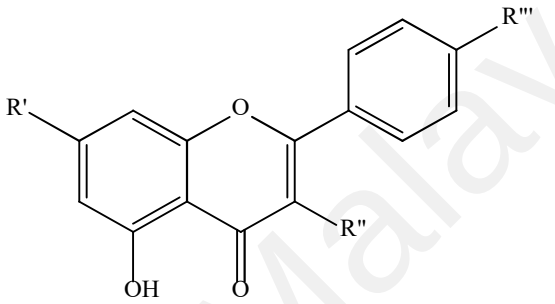
‘Figure 2.5, continued’



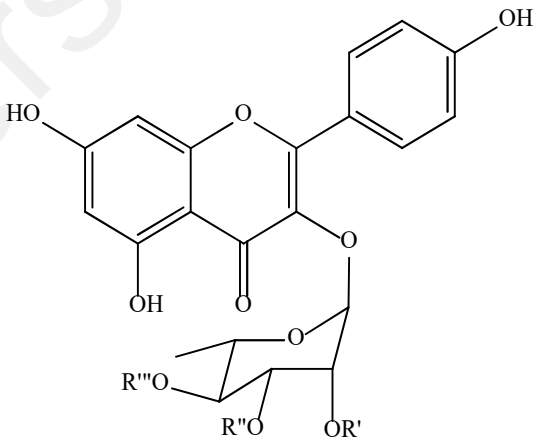
p-hydroxybenzaldehyde (**47**)



Vanillin (**48**)

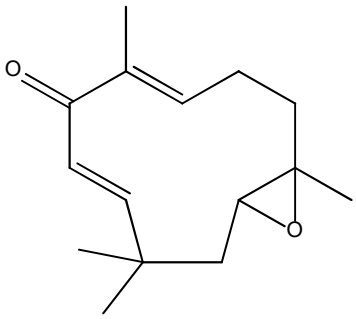


R’ = R”’ = OH ; R” = OCH_3	Kaempferol-3-*O*-methylether (**49**)
R’ = OH ; R” = R”’ = OCH_3	Kaempferol-3,4'-*O*-dimethylether (**50**)
R’ = R” = R”’ = OCH_3	Kaempferol-3,4',7-*O*-trimethylether (**51**)

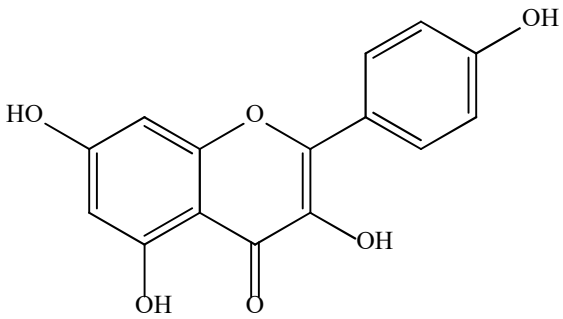


R’ = R” = H ; R”’ = $COCH_3$	Kaempferol-3-*O*-(4-*O*-acetyl-*α*-L-rhamnopyranoside (**52**)
R’ = R”’ = H ; R” = $COCH_3$	Kaempferol-3-*O*-(3-*O*-acetyl-*α*-L-rhamnopyranoside (**53**)
R’ = $COCH_3$; R” = R”’ = H	Kaempferol-3-*O*-(2-*O*-acetyl-*α*-L-rhamnopyranoside (**54**)
R’ = R” = R”’ = H	Kaempferol-3-*O*-*α*-L-rhamnopyranoside (**55**)

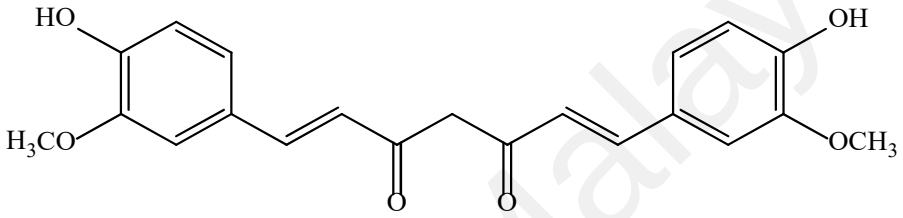
‘Figure 2.5, continued’



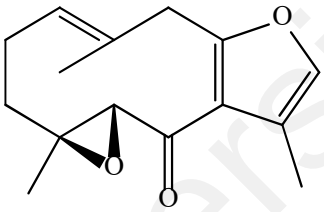
Zerumbone epoxide (**56**)



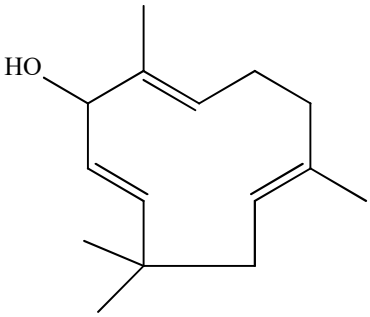
Kaempferol (**58**)



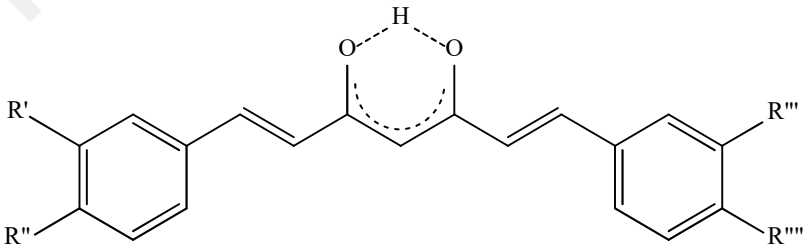
Curcumin (**57**)



Zederone (**59**)



Zerumbol (**63**)



R’ = R”’ = OCH_3 ; R” = R”” = OH	Diferuloylmethane (**60**)
R’ = R”’ = H ; R” = R”” = OH	Di-*p*-coumaroylmethane (**61**)
R’ = OCH_3 ; R”’ = H ; R” = R”” = OH	Feruloyl-*p*-coumaroylmethane (**62**)

2.2 Biological activities: Dengue, acute oral toxicity and anti-pyretic studies

This sub-chapter is divided into three major parts. The first part discussed on dengue disease and virus as well as plants and compounds that have been investigated in dengue related studies. The second part consists of discussions on acute oral toxicity and the last part discussed on anti-pyretic studies.

2.2.1 Dengue

Dengue is an endemic disease in Malaysia, has spread in the community since 1902. Dengue viruses can be spread to human through the bites of infected Aedes mosquitoes. Several factors contribute to the spread of dengue virus, including rapid development, population growth, increased migration and globalisation, as well as the difficulties in implementing effective vector control (Capeding et al., 2014). The infected mosquito injected dengue virus through human skin into the bloodstream. Initially, the virus infected and replicates in the human's skin cells and travel to the lymphatic system, hence the dengue virus travelled and infected all the cells in the body. There are two types of mosquitoes involved, which are *Aedes aegypti* and *Aedes albopictus* (Figure 2.6) and these two mosquitoes are active during dawn and dusk. Dengue virus infections can be classified into three categories, either dengue fever (DF), dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS), which depending on the symptoms and the thrombocytopenia level (Rajapakse, 2011). Dengue fever symptoms may vary from person that can be recognized by high fever and the following signs such as headache, nausea, rashes, myalgias, arthralgias, and low white blood cell count (less than 5,000 cells/mm^3) (Hasan et al., 2016; Kalayanarooj, 2011; Services, 2009; Suppiah et al., 2018). The severe dengue (DHF and DSS) can be identified by bleeding from nose and gums, vomiting blood, abdominal pain, plasma leakage, haemorrhagic manifestation, increasing vascular permeability that may lead to severe complications and death (Hasan et al., 2016; Kalayanarooj, 2011; Services, 2009; Suppiah et al., 2018).

WHO has categorised the patients into three categories; patients with no symptoms, patients with more than one symptoms (fever, rashes) and with coexisting disease (diabetic, renal failure), and patients with severe symptoms (plasma leakage) (Zahoor et al., 2019). The medical teams will diagnose and categorise the patients of dengue fever according to the symptoms and conditions. Most of the patients were admitted to hospital for the treatment of dengue by monitoring the platelet level and they were treated with paracetamol, intravenous (IV) fluid and electrolyte replacement.

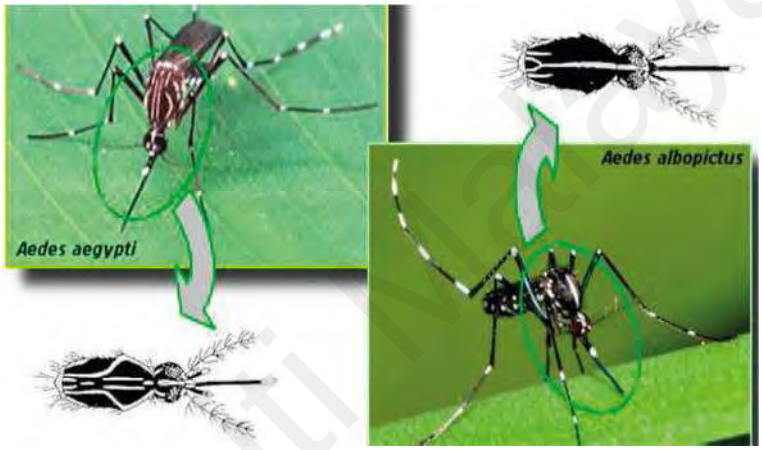


Figure 2.6 : *Aedes aegypti and Aedes albopictus*.

(Source: http://www.mdsaude.com/wp-content/uploads/2012/04/aedes-aegypti-e-aedes-albopictus.jpg accessed on 23rd November 2017).

There are currently no anti-viral drugs available and the only option to relieve symptoms in the early febrile stage is through fluid intake for adequate hydration and anti-pyretic treatment with paracetamol/acetaminophen (**64**) (Hasan et al., 2016). A multitude of efforts have been taken to pacify the disease such as prevalence control, prevention of interaction with infected mosquitoes, public awareness of pest management, insecticide chemical control and others (Zahoor et al., 2019).

2.2.1.1 Dengue virus

Dengue virus comprises a single-stranded RNA of positive polarity, encoding for a single polyprotein precursor of 3,391 amino acids consists of ten proteins involving

capsid (C), membrane (M) and envelope (E) that playing a structural role and seven non-structural (NS) proteins directing DENV replication (5'-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3') (Irie et al., 1989). The structure and genome of dengue virus are shown in Figure 2.7 (Morrison & García-Sastre, 2014). Dengue virus is roughly spherical in shape (50nm diameter). The nucleocapsid, which is composed of the viral genome and C proteins, serves as the centre of the virus. The nucleocapsid is surrounded by the viral envelope which is a lipid bilayer that is taken from the host. The viral envelope contains 180 copies of the E (envelope) and M (membrane) proteins, which span the lipid bilayer and is embedded within the envelope. These proteins form a protective outer layer that controls the entry of the virus into human cells (Mukhopadhyay et al., 2005).

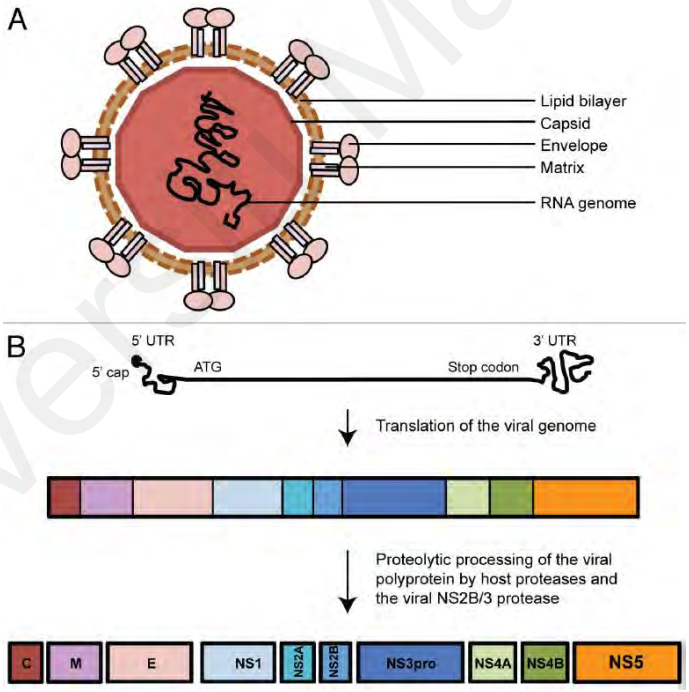


Figure 2.7 : (A) Structure of dengue virus and (B) Genome of dengue virus.

The dengue viral replication process occurs after a dengue virus binds to a human skin cell. The membrane of the skin cell then covers the virus and forming a pouch (endosome) that protect the virus particle. Endosome in normal cell usually ingested the large molecules and particles from outside the cell for nourishment. By exploiting this normal

cell process, the dengue virus is able to invade a host cell. Once a virus has invaded a host cell, it penetrates deeper into the cell while still within the endosome. There are two conditions that allow the virus envelope to fuse with the endosomal membrane, which are the endosome environment is in acidic condition and the endosomal membrane must gain a negative charge. Consequently, this produces the dengue nucleocapsid into the cell cytoplasm. The nucleocapsid opens in the cytoplasm to expose the viral genome and the viral RNA is released into the cytoplasm during this process (Education, 2014).

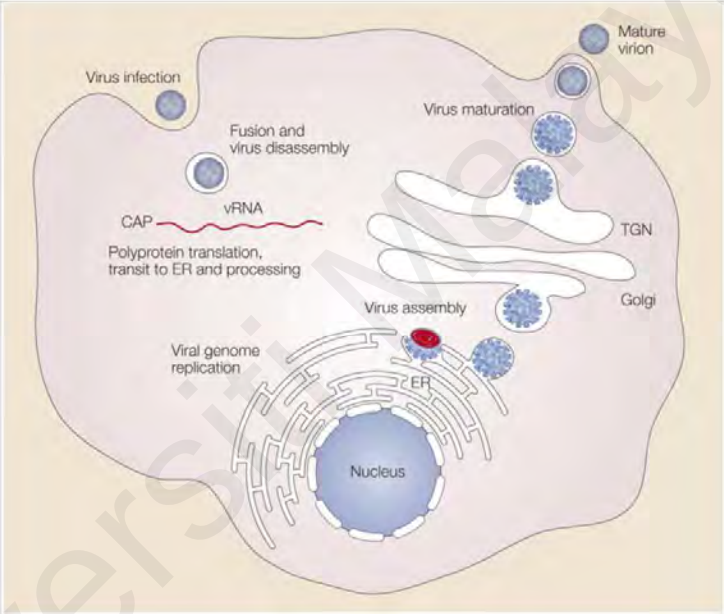


Figure 2.8 : Dengue virus replication (Mukhopadhyay et al., 2005).

(Source:https://www.nature.com/scitable/topicpage/dengue-viruses-22400925/ accessed on 25th August 2022).

Then, in order to replicate itself, the viral RNA takes control of the host cell's machinery. Ribosomes on the host's rough endoplasmic reticulum (ER) are used by the virus to translate viral RNA and generate the viral polypeptide. This polypeptide is then divided into ten dengue proteins. The newly synthesized viral RNA is surrounded by C proteins to form a nucleocapid. The immature virus transport through the Golgi apparatus for the virus to become matured and infectious. Once fully developed, dengue viruses are

then released from the cell and can infect other cells (Education, 2014; Mukhopadhyay et al., 2005). The diagram of the dengue virus replication is shown in Figure 2.8.

Dengue virus replication is dependent on the correct cleavage of this polyprotein and requires both host cell proteases and the virus-encoded, two-component protease, NS2B-NS3 (Falgout et al., 1991; Yusof et al., 2000). Inhibiting the NS2B-NS3 protease will prevent virus replication, thus, it serves as a target in the development of anti-viral drugs for dengue infection disease.

2.2.1.2 Plants and compounds with anti-dengue potentials

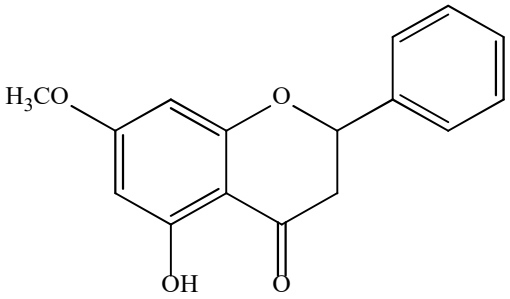
Researchers have turned their attention to nature over the last decades, in searching for natural compounds that can be used as anti-viral agent for dengue. Medicinal plants have richest sources of the bioactive compounds such as alkaloids, flavonoid, terpenoids, phenolics, saponins, and sesquiterpenes which possess therapeutic properties due to scavenging, inhibiting viral entry and viral replication against large spectrum of viruses (da Silva et al., 2019; Idrees et al., 2013; Tahir Ul Qamar et al., 2014). Several studies have reported the use of medicinal plants in ascertaining their inhibitory potentials against dengue virus as listed in Table 2.6.

Zakaria et al. (2019) had screened 150 plants for DENV2 NS2B-NS3 protease assay and only two plants showed the inhibition activity more than 95% with respective IC_{50} values 2 to 5 µg/mL which are *Syzygium campanulatum* and *Syzygium grande* (Zakaria et al., 2019). Both plants showed the IC_{50} values of 2.4397 and 3.7673 µg/mL, respectively (Zakaria et al., 2019).

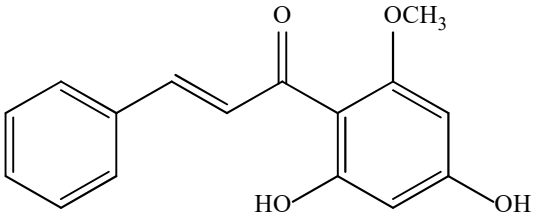
In the absence of an anti-viral drug to treat dengue, various alternative treatments are being investigated such as *Carica papaya* leaf juice. *Carica papaya* leaf juice and extracts have been extensively studied for their beneficial effects on platelet levels in rodents

(Arollado, 2014; Coloma et al., 2015; Norahmad et al., 2019; Patil et al., 2013; Sathasivam et al., 2009). For the past ten years, several clinical trials and clinical case studies on the platelet-increasing property of *Carica papaya* leaf juice or extracts in dengue patients have been published (Ahmad et al., 2011; Gadhwal et al., 2016; Hettige, 2008; Saleh & Kamisah, 2020; Sathyapalan et al., 2020; Yunita et al., 2012). On top of that, chloroform extract of the leaves *Carica papaya* has also been investigated for its *in vitro* anti-DENV2 activity which demonstrated inhibitory activity (EC_{50} = 1 mg/ml) against DENV2 and obtained a selectivity index (SI) value of ± >1 when tested on LLC-MK2 cell line (Adarsh et al., 2017; Sarker et al., 2021) . The water-soluble *Carica papaya* leaf extract inhibited the intracellular replication of DENV-2 on Vero cells with an IC_{50} value of 137.6 μg/mL for 5 hours prior to infection and four days after infection. The CC_{50} and SI value were determined to be 10437 μg/mL and 75.85, respectively (Salim et al., 2018; Sarker et al., 2021).

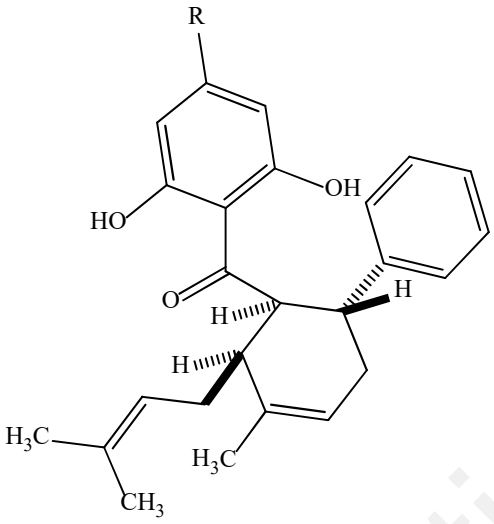
The other study was discovered on the root water extract of *Eurycoma longifolia* Jack which exhibited a dose-dependent inhibition effects against all four DENV serotypes in Vero cells (George et al., 2019). The IC_{50} for four different genotypes of DENV (DENV-1, DENV-2, DENV-3 and DENV-4 was observed at 33.84, 33.55, 58.35, and 119.00 μg/mL, respectively and the lowest of SI value was obtained by DENV-2 at 28.9. The qRT-PCR analysis of dengue virus replication on day 2 of exposure revealed a reduction of 100% for DENV-1, DENV-2, DENV-3, and 80% for DENV-4. On day 6 of the *in vivo* AG129 mouse model, the extract group demonstrated a lower weight loss, higher health score, 30% lower viral load and 13% higher platelet count than the control group. Other medicinal plants such as *Alternanthera photoperiods*, *Andrographis paniculata*, *Ocimum sanctum*, *Catharanthus roseus*, *Cymbopogon citratus*, *Euphorbia hirta*, *Hedyotis auricularia*, *Houttuynia cordata*, *Senna augustifolia*, and *Vernonia cinerea* also exhibited anti-viral properties (Table 2.6).



Pinostrobin (**65**)



Cardamonin (**66**)



R = OCH_3 Panduratin A (**67**)

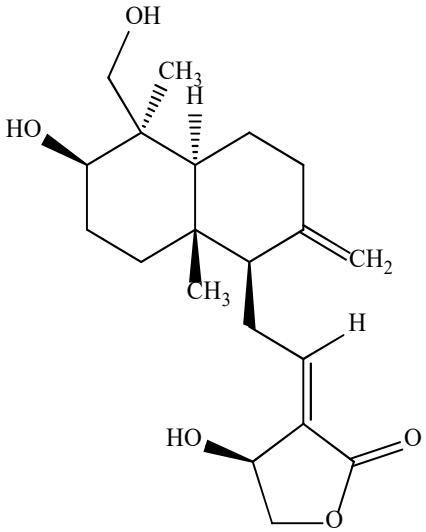
R = OH 4-hydroxypanduratin A (**68**)

Figure 2.9 : Structure of pinostrobin (65), cardamonin (66), panduratin A (67), and 4-hydroxypanduratin A (68).

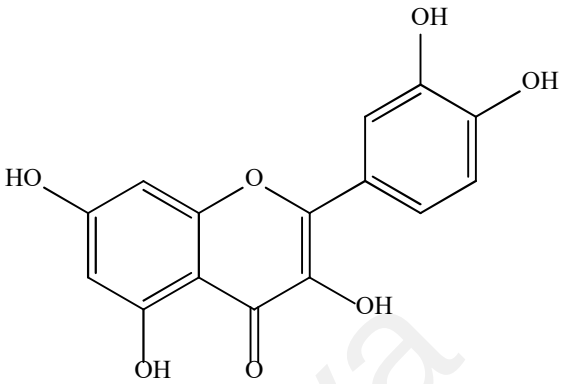
There are many compounds derived from medicinal plants that inhibit the anti-protease activity against NS2B/NS3 such as pinostrobin (**65**), cardamonin (**66**), panduratin A (**67**), and 4-hydroxypanduratin A (**68**) (Figure 2.9) that isolated from *Boesenbergia rotunda* (L.) (Kiat et al., 2006). Kiat el al. (2006) discovered that the pinostrobin (**65**) and cardamonin (**66**) acted as non-competitive inhibitors towards DENV2 protease while panduratin A (**67**) and 4-hydroxypanduratin A (**68**) were determined as competitive inhibitors towards DENV2 protease with K_i values of 25.0 ± 8.0 and 21.0 ± 6.0 μM, respectively. Panduratin A (**67**) and 4-hydroxypanduratin A (**68**) showed promising *in vitro* inhibitory activity againts DENV2 NS2B/NS3 protease due to their low K_i values.

Diterpenoids such as andrographolide (**69**) (Figure 2.10) derived from *Andrographis paniculata* also possesses inhibition on DENV2 in two cell lines (HepG2 and HeLa)

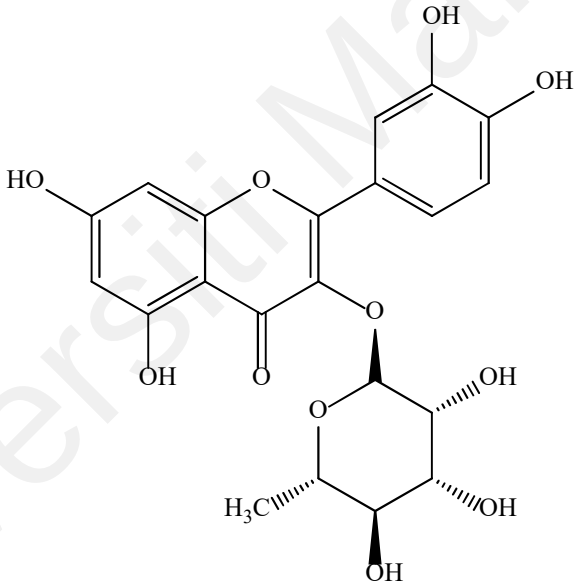
(Panraksa et al., 2017). The EC_{50} value of andrographolide (**69**) against HepG2 cell line is 21.304 μM and for HeLa cell lines is 22.739 μM. Quercetin (**70**) (Figure 2.10) isolated from *Houttuynia cordata*, *Spondias mombin* and *Spondias tuberosa* was found to have anti-viral activity against the DENV2 virus. It possesses IC_{50} value of 176.76 μg/mL and CC_{50} value of 155.38 μg/mL, with SI value of 0.88 (Chiow et al., 2016). Additionally, this compound also was mixed with quercitrin (**71**) (Figure 2.10) (at 1:1 ratio) to determine whether both flavonoid compounds could exert enhanced DENV2 inhibition (Chiow et al., 2016). The results indicated that the mixture performed a synergistic anti-DENV-2 effect and lower cytotoxicity, with improved corresponding values of IC_{50} at 158.21 mg/mL, CC_{50} at 270.00 mg/mL, and SI at 1.71 (Chiow et al., 2016). The selected compounds derived from the medicinal plants which possess anti-viral activity against dengue virus are listed in Table 2.7. Based on all the findings, it is necessary to search and identify the compounds from medicinal plants which have been used as an alternative medicine to treat dengue infections since ancient times.



Andrographolide (**69**)



Quercetin (**70**)



Quercitrin (**71**)

Figure 2.10 : Molecular structure of andrographolide (69), quercetin (70) and quercitrin (71).

Table 2.6 : Medicinal plants with biological properties related to dengue.

Plants	Family	Part used	Type of study	Anti-viral properties	Reference
Alternanthera photoperiods	Amaranthaceae	Whole plant	*In vitro*	Inhibited DENV-2 replication (IC_{50}= 47.43 μg/mL)	(Jiang et al., 2005; Saleh & Kamisah, 2020)
Alternanthera sessillis	Amaranthaceae	Leaves	*In vivo*	Significant platelet increasing activity	(Arollado, 2014; Saleh & Kamisah, 2020)
Acorus calamus	Acoracea	Leaves	*In vitro*	Inhibited DENV-2 replication (96.5% at a dose of 20μg/mL)	(Rosmalena et al., 2019; Saleh & Kamisah, 2020)
Andrographis paniculata	Acanthaceae	Leaves	*In vitro*	Anti-DENV-1 activity in HepG2 (78.3±2.9 PFU/mL)	(Ling et al., 2014; Saleh & Kamisah, 2020)
		Whole plant	*In vitro*	Inhibited DENV-1 replication (75% inhibition)	(Tang et al., 2012)
Annona muricata	Annonaceae	Fruit	*In vitro*	Inhibited DENV-2 replication (99% at a dose of 1.25 mg/mL)	(Saleh & Kamisah, 2020; Wahab, Ibrahim, Kamarudin, Lananan, Juahir, Ghazali, et al., 2018)
Ocimum sanctum	Lamiaceae	Leaves	*In vitro*	Anti-DENV-1 activity in HepG2 (1020.0±271.0 PFU/mL)	(Ling et al., 2014; Saleh & Kamisah, 2020)
Arrabidaea pulchra	Bignoniaceae	Leaves	*In vitro*	Inhibited DENV-2 (EC_{50}= 46.8±1.6 μg/mL)	(Brandão et al., 2013; Saleh & Kamisah, 2020)
Azadirachta indica	Meliaceae	Leaf extract	*In vitro and in vivo*	Inhibited DENV-2 replication in both in vitro and in vivo	(Parida et al., 2002; Saleh & Kamisah, 2020)
Carica papaya	Caricaceae	Leaves	*In vivo*	Increased the platelet counts	(Saleh & Kamisah, 2020; Sathasivam et al., 2009)
		Leaves	*In vivo*	Increased the platelet count and decreased the clotting time in rats	(Patil et al., 2013; Saleh & Kamisah, 2020)

‘Table 2.6, continued’

Plants	Family	Part used	Type of study	Anti-viral properties	Reference
Carica papaya	Caricaceae	Leaf extract	*Human*	The platelet counts, the total white cell counts and the neutrophils had increased	(Ahmad et al., 2011; Hettige, 2008; Saleh & Kamisah, 2020; Sathyapalan et al., 2020; Yunita et al., 2012)
		Leaf extract	*In vivo*	Significant platelet increasing activity	(Arollado, 2014; Saleh & Kamisah, 2020)
		Leaf juice	*Human*	Increased the platelet counts	(Gadhwal et al., 2016; Saleh & Kamisah, 2020; Subenthiran et al., 2013)
		Leaf juice	*In vivo*	Increased the platelet counts	(Coloma et al., 2015; Saleh & Kamisah, 2020)
		Leaf juice	*In vivo*	Increased the plasma CCL2/MCP-1 level	(Norahmad et al., 2019; Saleh & Kamisah, 2020)
Catharanthus roseus	Apocynaceae	Leaves	*In vitro*	Inhibited DENV-2 replication (60% at a dose of 0.078 mg/mL)	(Abd Wahab & Ibrahim, 2020; Saleh & Kamisah, 2020)
Cissampelos pareira	Menispermacea	Aerial part	*Human*	Increased the platelet counts	(Saleh & Kamisah, 2020; Sood et al., 2016)
Cladogynos orientalis	Euphorbiaceae	Whole plant	*In vitro*	Inhibited DENV-2 (34.85% at a dose of 12.5 µg/mL)	(Klawikkan et al., 2011; Saleh & Kamisah, 2020)
Curcuma longa	Zingiberaceae	Not mentioned	*In vitro* and *in vivo*	Anti-DENV activity in Huh-7it-1 cells (IC_{50} =17.91 µg/mL) and reduce viremia period	(Ichsyani et al., 2017; Saleh & Kamisah, 2020)
Cyamopsis tetragonoloba	Fabaceae	Not mentioned	*In vitro*	Inhibited DENV-2 (99.9%)	(Kaushik et al., 2020; Saleh & Kamisah, 2020)

‘Table 2.6, continued’

Plants	Family	Part used	Type of study	Anti-viral properties	Reference
Cymbopogon citratus	Poaceae	Root	*In vivo*	Inhibited DENV-2 replication (98.9% at a dose of 20 µg/mL)	(Rosmalena et al., 2019; Saleh & Kamisah, 2020)
Cynometra cauliflora	Fabaceae	Leaves	*In vitro*	Inhibited DENV-2 replication (78% at a dose of 12.5 mg/mL)	(Abd Wahab, 2018; Saleh & Kamisah, 2020)
Distictella elongata	Bignoniaceae	Leaves, stems, fruits	*In vitro*	Anti-DENV-2 activity (EC_{50} = 9.8 µg/mL)	(Saleh & Kamisah, 2020; Simões et al., 2011)
Dryobalanops aromatica	Dipterocarpaceae	Leaves, barks, fruits	*In vitro*	Inhibited DENV-2 NS2B/NS3 protease (99.70%, 96.52% and 75.00% at 200 µg/mL, respectively)	(Salleh et al., 2019)
Endiandra kingiana	Lauraceae	Bark	*In vitro*	Inhibited DENV-2 (65.05% at 200 µg/mL)	(Sulaiman et al., 2019)
Euphorbia hirta	Euphorbiaceae	Leaves	*In vivo*	Inhibited DENV-2 replication (34.7% at 20 µg/mL dose)	(Perera et al., 2018; Saleh & Kamisah, 2020; Saptawati et al., 2017)
		Whole plant	*In vivo*	Significant platelet increasing activity	(Arollado, 2014; Saleh & Kamisah, 2020)
		Whole plant	*In vitro*	Significantly reduced the plaque forming capacity of the DENV-1-2 (85% and 90% respectively)	(Saleh & Kamisah, 2020; Tayone et al., 2014)
		Whole weed	*Human*	Increased the platelet counts	(Mir et al., 2012; Saleh & Kamisah, 2020)
		Leaf juice	*In vivo*	Increased the platelet counts	(Coloma et al., 2015; Saleh & Kamisah, 2020)

‘Table 2.6, continued’

Plants	Family	Part used	Type of study	Anti-viral properties	Reference
Eurycoma longifolia	Simaroubaceae	Root	*In vitro* and *in vivo*	Inhibited DENV-1, DENV-2, DENV-3 and DENV-4 (IC_{50} = 33.84, 33.55, 58.35 and 119 μg/mL, respectively), 30% lower viral load and 12% higher platelet count	(George et al., 2019; Saleh & Kamisah, 2020)
Flacourtia ramontchi	Salicaceae	Stems barks	*In vitro*	Inhibited DENV-2 NS5 polymerase activity (89% inhibition at 10 μg/mL)	(Bourjot et al., 2012; Saleh & Kamisah, 2020)
Flagellaria indica	Flagellariaceae	Whole plant	*In vitro*	Inhibited DENV-2 (45.52% at a dose of 12.5 μg/mL)	(Klawikkan et al., 2011; Saleh & Kamisah, 2020)
Faramea bahinensis, Faramea hyacinthine and *Faramea truncata*	Rubiaceae	Leaves	*In vitro*	Anti-DENV activity in HepG2 (100% reduction in viral load)	(A. Nascimento et al., 2017; Saleh & Kamisah, 2020)
		Leaves	*In vitro*	Anti-DENV activity in HepG2 (98 and 94% at a dose of 50 μg/mL, respectively)	(Barboza et al., 2017; Saleh & Kamisah, 2020)
		Leaves and stem	*In vitro*	Anti-DENV activity in HepG2 (70 to 93% at a dose of 50 μg/mL)	(Saleh & Kamisah, 2020; Wolff et al., 2019)
Hedyotis auricularia	Rubiaceae	Leaves, Stem and Roots (Ethanol extract)	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 64, 72 and 84 μg/mL, respectively)	(Rothan et al., 2014)
		Leaves and Stem (Methanol extract)	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 99 and 98 μg/mL)	(Rothan et al., 2014)
Hemigraphis reptans	Acanthaceae	Leaves	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 98 μg/mL)	(Rothan et al., 2014)

‘Table 2.6, continued’

Plants	Family	Part used	Type of study	Anti-viral properties	Reference
Hemigraphis reptans	Acanthaceae	Leaves (Methanol extract)	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 88 μg/mL)	(Rothan et al., 2014)
Hippophae rhamnoides	Elaeagnaceae	Leaves	*Human*	Anti-DENV activity in BHK-21 cells (1 PFU/mL at a dose of 50 mg/mL), decreased TNF-*α* and increased IFN-γ	(Jain et al., 2008; Saleh & Kamisah, 2020)
Houttuynia cordata	Saururaceae	Whole plant	*In vitro*	Inhibited DENV-2 replication (35.99% at a dose of 1.56 μg/mL)	(Klawikkan et al., 2011; Saleh & Kamisah, 2020)
		Aerial stem and leaves	*In vitro*	Inhibited the intracellular viral RNA replication (EC_{50} = 0.8 μg/mL)	(Leardkamolkarn et al., 2011; Saleh & Kamisah, 2020)
		Aerial leaves	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 7.50 μg/mL)	(Chiow et al., 2016; Saleh & Kamisah, 2020)
Ipomea batata	Convolvulaceae	Leaves	*In vivo*	Significant platelet increasing activity	(Arollado, 2014; Saleh & Kamisah, 2020)
Justicia adhatoda	Acanthaceae	Leaves	*In vitro*	Inhibited the growth of Vero cells infected with DENV-2 at a dose of 60 μg/mL	(Saleh & Kamisah, 2020; Wilson et al., 2021)
Laurentia longiflora	Campanulaceae	Leaves, Stem and Roots (Ethanol extract)	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 52, 86 and 92 μg/mL, respectively)	(Rothan et al., 2014)
Lawsonia inermis	Lythraceae	Barks	*In vitro*	Inhibited DENV-2 NS2B/NS3 protease (87.99% at 200 μg/mL)	(Salleh et al., 2019)

‘Table 2.6, continued’

Plants	Family	Part used	Type of study	Anti-viral properties	Reference
Momordica charantia	Cucurbitaceae	Fruits and roots	*In vitro*	Inhibited DENV-1 replication (50.00% inhibition)	(Tang et al., 2012)
Moringa oleifera	Moringaceae	Leaf juice	*In vivo*	Increased the platelet counts	(Coloma et al., 2015; Saleh & Kamisah, 2020)
Myristica cinnamomea	Myristicaceae	Fruits	*In vitro*	Inhibited DENV-2 (96.96% at 200 μg/mL)	(Sivasothy et al., 2021)
Myristica fatua	Myristicaceae	Leaves	*In vitro*	Inhibited DENV-2 replication (122.7% at a dose of 20 μg/mL)	(Rosmalena et al., 2019; Saleh & Kamisah, 2020)
Orthosiphon stamineus	Lamiaceae	Leaves	*In vitro*	Inhibited DENV-2 replication (88% at a dose of 0.31 mg/mL)	(Saleh & Kamisah, 2020; Wahab, Ibrahim, Kamarudin, Lananan, Juahir, & Ghazali, 2018)
Piper retrofractum	Piperaceae	Whole plant	*In vitro*	Inhibited DENV-2 replication (53.53% at a dose of 12.5 μg/mL)	(Klawikkan et al., 2011; Saleh & Kamisah, 2020)
Psidium guajava	Myrtaceae	Bark	*In vitro*	Inhibited DENV-2 replication (EC_{50} =7.8 μg/mL)	(Saleh & Kamisah, 2020; Trujillo-Correa et al., 2019)
		Leaves	*In vitro*	Inhibited the growth of Vero cells infected with DENV-2 at a dose of 60 μg/mL	(Saleh & Kamisah, 2020; Wilson et al., 2021)
Phyllanthus spp.	Phyllanthaceae	Whole plant	*In vitro*	Inhibited DENV-2 replication (91.48% at a dose of 250 μg/mL)	(Lee et al., 2013; Saleh & Kamisah, 2020)
Punica granatum	Lythraceae	Leaves and barks	*In vitro*	Inhibited DENV-2 NS2B/NS3 protease (92.23% and 91.84% at 200 μg/mL, respectively)	(Salleh et al., 2019)

‘Table 2.6, continued’

Plants	Family	Part used	Type of study	Anti-viral properties	Reference
Quersus lucitanica	Fagaceae	Seed	*In vitro*	Inhibited DENV-2 (100% at a dose of 0.032 mg/mL)	(Muliawan et al., 2006; Rahman et al., 2006; Saleh & Kamisah, 2020)
Rhizophora apiculata	Rhizophoraceae	Whole plant	*In vitro*	Inhibited DENV-2 replication (56.14% at a dose of 12.5 μg/mL)	(Klawikkan et al., 2011; Saleh & Kamisah, 2020)
Senna angustifolia	Fabaceae	Leaves	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 30 μg/mL)	(Rothan et al., 2014)
		Leaves (Methanol extract)	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 50 μg/mL)	(Rothan et al., 2014)
		Leaves (Ethanol extract)	*In vitro*	Inhibited the growth of Vero cells infected with DENV-2 at a dose of 50 μg/mL (67.2%) and significantly reduced the plaque forming capacity of the DENV-2 (26.3%)	(Rothan et al., 2014)
Spondias mombin and *Spondias tuberosa*	Anacardiaceae	Leaves juice	*In vitro*	Inhibited DENV-2 replication (3.31% and 17.98% at a dose of 500 μg/mL, respectively)	(Saleh & Kamisah, 2020; Silva et al., 2010)
Tridax procumbers	Asteraceae	Leaves	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 64 μg/mL)	(Rothan et al., 2014)
		Leaves (Methanol extract)	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 70 μg/mL)	(Rothan et al., 2014)

'Table 2.6, continued'

Plants	Family	Part used	Type of study	Anti-viral properties	Reference
Tridax procumbers	Asteraceae	Stem (Ethanol extract)	*In vitro*	Inhibited the growth of Vero cells infected with DENV-2 at a dose of 50 μg/mL (86.3%) and significantly reduced the plaque forming capacity of the DENV-2 (80.6%)	(Rothan et al., 2014)
Uncaria tomentosa	Rubiaceae	Stem barks	*In vitro*	Reducing DENV-Ag+ cell rates	(Reis et al., 2008; Saleh & Kamisah, 2020)
Vernonia cinerea	Asteraceae	Leaves, Stem and Roots (Ethanol extract)	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 62, 52 and 48 μg/mL, respectively)	(Rothan et al., 2014)
		Leaves (Methanol extract)	*In vitro*	Inhibited DENV-2 replication (IC_{50} = 24 μg/mL)	(Rothan et al., 2014)
				Inhibited the growth of Vero cells infected with DENV-2 at a dose of 50 μg/mL (79.5%) and significantly reduced the plaque forming capacity of the DENV-2 (64.0%)	(Rothan et al., 2014)
Zingiber zerumbet	Zingiberaceae	Rhizomes	*In vitro*	Inhibited DENV-2 NS2B/NS3 protease (90.32% at 200 μg/mL)	(Salleh et al., 2019)
				Inhibited DENV-2 NS2B/NS3 protease (89.0±1.7% at 300 ppm)	(Tan et al., 2006)
Zizyphus jujuba	Rhamnaceae	Leaves and barks	*In vitro*	Inhibited DENV-2 NS2B/NS3 protease (94.44% and 94.58% at 200 μg/mL, respectively)	(Salleh et al., 2019)

Table 2.7 : Isolated compounds with anti-dengue potential.

Compounds	Class of compounds	Plants source	Anti-dengue potential	Reference
Andrographolide (**69**)	Diterpenoid	*Andrographis paniculata*	Anti-DENV activity in HepG2 (EC_{50} = 21.304 μM) and HeLa cell lines (EC_{50} = 22.739 μM) for DENV-2	(Panraksa et al., 2017)
			Reduced mean number of *A. aegypti* eggs and increased larvae mortality concentration-dependently (over 90% mortality)	(Edwin et al., 2016)
			Delayed disease onset, reduced mortality and DENV-1, DENV-2, DENV-3 and DENV-4 loads in infected mouse brains	(Panraksa et al., 2017; Tseng et al., 2016)
Apiofuranoside (**72**)	Flavanone glycosides	*Faramea bahiensis*	Controlled viral replication and reduced numbers of infected cells (12%) and RNA copies of DENV-2 (67%) in HepG2 cells	(A. Nascimento et al., 2017)
Baicalein (**73**)	Flavone	*Scutellaria baicalensis*	Inhibited DENV-2 replication (IC_{50} =6.46 μg/mL)	(Zandi et al., 2012)
Betulinic acid 3ß-caffeate (**74**)	Phenyl terpenoid	*Flacourtia ramontchi*	Inhibited DENV replication (IC_{50} = 0.85 μM)	(Bourjot et al., 2012)
(+)-Catechin (**75**)	Flavanoid	*Endiandra kingiana*	Inhibited DENV-2 protease assay (62.02% inhibition at 200 μg/mL)	(Sulaiman et al., 2019)
Caffeoylcalleryanin (**76**)	Phenylethanoid glycosides	*Arrabidaea pulchra*	Anti-DENV-2 activity (IC_{50} = 2.8 μg/mL)	(Brandão et al., 2013)
Cardamonin (**66**)	Phenolic	*Boesenbergia rotunda*	Non-competitive anti-DENV activity (K_i = 377 μM)	(Kiat et al., 2006)

‘Table 2.7, continued’

Compounds	Class of compounds	Plants source	Anti-dengue potential	Reference
Castanospermine (**77**)	Alkaloid	*Castanospermine australe*	Inhibited secretion and infectivity in all DENV serotypes *in vitro* in BHK-21 cells. Only inhibited DENV-2 *in vivo* and prevented mouse mortality and morbidity.	(Whitby et al., 2005)
Chartaceones 1 (**78**)	Dialkylated flavanone	*Crypotocarya chartacea*	Inhibited DENV NS5 polymerase (IC_{50} = 14.8 μM)	(Allard et al., 2011)
Chartaceones 2 (**79**)			Inhibited DENV NS5 polymerase (IC_{50} = 72.5 μM)	(Allard et al., 2011)
Chartaceones 3 (**80**)			Inhibited DENV NS5 polymerase (IC_{50} = 4.2 μM)	(Allard et al., 2011)
Chartaceones 4 (**81**)			Inhibited DENV NS5 polymerase (IC_{50} = 1.8 μM)	(Allard et al., 2011)
Chartaceones 5 (**82**)			Inhibited DENV NS5 polymerase (IC_{50} = 2.9 μM)	(Allard et al., 2011)
Chartaceones 6 (**83**)			Inhibited DENV NS5 polymerase (IC_{50} = 2.4 μM)	(Allard et al., 2011)
Chlorogenic acid (**84**)	Phenolic acid	*Houttuynia cordata*	Synergistic anti-viral effect of combined hyperoside and chlorogenic acid	(Xie et al., 2014)
(−)-Epicatechin (**85**)	Flavanoid	*Endiandra kingiana*	Inhibited DENV-2 protease assay (69.92% inhibition at 200 μg/mL)	(Sulaiman et al., 2019)
Ellagic acid (**86**)	Phenolic acid	*Spondias mombin and Spondias tuberosa*	Inhibited viral replication of DENV-2 at 500 μg/mL by 25.02%	(Silva et al., 2010)
Flacourtoside A (**87**)	Phenolic glycoside	*Flacourtia ramontchi*	Inhibited DENV NS5 polymerase (IC_{50} = 9.3 μM)	(Bourjot et al., 2012)

‘Table 2.7, continued’

Compounds	Class of compounds	Plants source	Anti-dengue potential	Reference
Flacourtoside B (**88**)	Phenolic glycoside	*Flacourtia ramontchi*	Inhibited DENV NS5 polymerase (IC_{50} = 71.1 μM)	(Bourjot et al., 2012)
Flacourtoside C (**89**)			Inhibited DENV NS5 polymerase (IC_{50} = 23.8 μM)	(Bourjot et al., 2012)
Flacourtoside D (**90**)			Inhibited DENV NS5 polymerase (IC_{50} = 35.5 μM)	(Bourjot et al., 2012)
Flacourtoside E (**91**)			Inhibited DENV NS5 polymerase (IC_{50} = 13.4 μM)	(Bourjot et al., 2012)
Flacourtoside F (**92**)			Inhibited DENV NS5 polymerase (IC_{50} = 39.8 μM)	(Bourjot et al., 2012)
Fucoidan (**93**)	Sulfated polysaccharide	*Cladosiphon okamuranus*	Inhibited DENV-2 Reduced infectivity by 20% at 10 μg/mL in BHK-21 cells (IC_{50} = 4.7 μg/mL)	(Hidari et al., 2008)
Galactan (**94**)	Sulfated polysaccharide	*Cryptomenia crenulate*	Inhibited DENV-2 and DENV-3 replication in Vero cells (IC_{50} =1.0 and 14.2 μg/mL, respectively), slight inhibitory effect against DENV-4, inactive against DENV-1	(Talarico et al., 2005)
		Gymnogongrus tolulosus	Inhibited DENV-2 replication in Vero cells (IC_{50} = 0.19–1.7 μg/mL).	(Pujol et al., 2002)
Galactomannan (**95**)	Polysaccharride	*Mimosa scabrella*	Reduced DENV-1 titer 100-fold in C6/36 cells at 347 mg/L	(Ono et al., 2003)
		Leucaena leucocephala	Reduced DENV-1 titer 100-fold in C6/36 cells at 37 mg/L	(Ono et al., 2003)

‘Table 2.7, continued’

Compounds	Class of compounds	Plants source	Anti-dengue potential	Reference
Galactomannan (**95**)	Polysaccharride	*Adenanthera pavonina*	Inhibited DENV-2 replication (77% inhibition at 25 μg/mL)	(Marques et al., 2015)
		Caesalpinia ferrea	Inhibited DENV-2 replication (96% inhibition at 25 μg/mL)	(Marques et al., 2015)
		Dimorphandra gardneriana	Inhibited DENV-2 replication (94% inhibition at 25 μg/mL)	(Marques et al., 2015)
Glabranine (**96**)	Flavanone	*Tephrosia madrensis*	Exhibited 76. % inhibition on DENV in a plaque-forming assay at 25 μM	(Sanchez et al., 2000)
4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3H)-benzofuranone (**97**)	Flavanone	*Endiandra kingiana*	Inhibited DENV-2 protease assay (61.23% inhibition at 200 μg/mL)	(Sulaiman et al., 2019)
4-hydroxypanduratin A (**68**)	Phenolic derivative	*Boesenbergia rotunda*	Competitive inhibitory activity against DENV-2 NS3 protease (K_i = 21 μM)	(Kiat et al., 2006)
Hyperoside (**98**)	Flavonol glycoside	*Houttuynia cordata*	Synergistic anti-viral effect of combination of hyperoside and chlorogenic acid	(Xie et al., 2014)
Itoside H (**99**)	Phenolic glycoside	*Flacourtia ramontchi*	Inhibited DENV replication (IC_{50} = 37.8 μM)	(Bourjot et al., 2012)
κ-Carrageenan (**100**)	Sulfated polysaccharide	*Gymnogongrus griffithsiae*	Selectivity inhibited DENV-2 and DENV-3 replication in Vero cells (IC_{50} = 0.9 and 13.9 μg/mL, resepctively). Inactive against DENV-1 and DENV-4 replication	(Talarico et al., 2005)

‘Table 2.7, continued’

Compounds	Class of compounds	Plants source	Anti-dengue potential	Reference
κ-Carrageenan (**100**)	Sulfated polysaccharide	*Meristiella gelidium*	Inhibited DENV-2 activity in a plaque reduction assay (IC_{50} between 0.14–0.79 μg/mL)	(de S.F-Tischer et al., 2006)
7-*O*-methyl-glabranine (**101**)	Flavanone	*Tephrosia madrensis*	Inhibited DENV in a plaque-forming assay (75% inhibition at 25 μg/mL)	(Sanchez et al., 2000)
Malabaricone C (**102**)	Acylphenol	*Myristica cinnamomea*	Inhibited DENV-2 protease assay (98.54% inhibition at 200 μg/mL, IC_{50} = 27.33 μg/mL)	(Sivasothy et al., 2021)
Malabaricone E (**103**)	Acylphenol	*Myristica cinnamomea*	Inhibited DENV-2 protease assay (99.91% inhibition at 200 μg/mL, IC_{50} = 7.55 μg/mL)	(Sivasothy et al., 2021)
Methyl gallate (**104**)	Phenolic acid	*Quercus lusitanica*	Inhibited DENV-2 protease assay (98% inhibition at 0.3 mg/mL)	(Rahman et al., 2006)
Methyl orsellinate (**105**)	Phenolic	*Endiandra kingiana*	Inhibited DENV-2 protease assay (16.42% inhibition at 200 μg/mL)	(Sulaiman et al., 2019)
Palmatine (**106**)	Isoquinoline alkaloid	*Coptis chinensis*	Inhibited DENV-2 protease assay (IC_{50} = 96 μM)	(Jia et al., 2010)
Panduratin A (**67**)	Phenolic derivative	*Boesenbergia rotunda*	Competitive inhibitory activity against DENV-2 NS3 protease (K_i = 25 μM)	(Kiat et al., 2006)
Pectolinarin (**107**)	Flavone	*Distictella elongate*	Good anti-DENV-2 effect (EC_{50} = 86.4 μg/mL)	(Simões et al., 2011)
Pinostrobin (**65**)	Flavanone	*Boesenbergia rotunda*	Non-competitive DENV replication inhibitory activity (K_i = 345 μM)	(Kiat et al., 2006)
Quercetin (**70**)	Flavonoid	*Houttuynia cordata*	Inhibited DENV-2 replication (IC_{50} = 176.76 μg/mL; CC_{50} = 155.38 μg/mL; Selectivity index = 0.88)	(Chiow et al., 2016)
		Spondias mombin and Spondias tuberosa	Inhibited viral replication of DENV-2 (50% inhibition at 500 μg/mL)	(Silva et al., 2010)

‘Table 2.7, continued’

Compounds	Class of compounds	Plants source	Anti-dengue potential	Reference
Quercitrin (**71**)	Flavonoid glycoside	*Houttuynia cordata*	Inhibited DENV-2 replication (IC_{50} = 467.27 μg/mL). Synergistic DENV-2 effect of quercitrin and quercetin combination by plaque reduction neutralization test (IC_{50} = 158.21 μg/mL)	(Chiow et al., 2016)
Rutin (**108**)	Flavonol glycoside	*Spondias mombin and Spondias tuberosa*	Inhibited viral replication of DENV-2 (68.42% at 500 μg/mL)	(Silva et al., 2010)
Scolochinenoside D (**109**)	Phenolic glycoside	*Flacourtia ramontchi*	Inhibited DENV replication (IC_{50} = 9.5 μM)	(Bourjot et al., 2012)
Trigocherrin A (**110**)	Diterpenoid	*Trigonostemon cherrieri*	Inhibited DENV replication (IC_{50} = 12.7 μM)	(Allard et al., 2012)
Trigocherriolide A (**111**)			Inhibited DENV replication (IC_{50} = 3.1 μM)	(Allard et al., 2012)
Trigocherriolide B (**112**)	Diterpenoid	*Trigonostemon cherrieri*	Inhibited DENV replication (IC_{50} = 16.0 μM)	(Allard et al., 2012)
Ursolic acid (**113**)	Triterpenoid	*Arrabidaea pulchra*	Anti-DENV-2 activity (IC_{50} = 3.2 μg/mL)	(Brandão et al., 2013)
Verbascoside (**114**)	Phenylethanoid glycoside	*Arrabidaea pulchra*	Anti-DENV-2 activity (IC_{50} = 3.4 μg/mL)	(Brandão et al., 2013)
Xylosmin (**115**)	Phenolic glycoside	*Flacourtia ramontchi*	Inhibited DENV replication (IC_{50} = 24.3 μM)	(Bourjot et al., 2012)
Zosteric acid (**116**)	Phenolic acid	*Zostera marina*	Inhibited DENV-2 replication (99% inhibition at 5.0 mM)	(Rees et al., 2008)

2.2.2 Acute oral toxicity

Generally, acute toxicity can be defined as assessment of the side effect resulting from exposure of organisms to single or multiple doses of a test substances (chemicals, compounds, extracts, drugs etc.) through oral, dermal or inhalation within the period of 24 hours (Erhirhie et al., 2018; Saganuwan, 2017). Acute toxicity test findings act as references in the selection of doses for long-term toxicity trials as well as for other studies involving the use of animals and it will lead to a conclusion on the toxicity status of the test substances (Maheshwari & Shaikh, 2016).

There are various alternative methods used in acute toxicity test such as OECD test guidelines 420, OECD test guidelines 423 and OECD test guidelines 425. The OECD guidelines test no. 425 was introduced in 1998, a revised format of OECD test guidelines 420 and OECD test guidelines 423. According to OECD test guidelines 425, animals are treated one at a time. If the previous animal survives, the next animal dose is increased by a factor of 3.2, while the dose is reduced by a factor of 3.2 if the animal dies. If death with these two animals is still observed, further reductions can be made before no death has been observed. It takes 1 or 2 days to observe each animal before the next animal is treated. Animals that survive from the test are then observed for delayed toxicity for 7 days (Chinedu et al., 2013). The behavioural signs of toxicity also should be recorded. However, if no mortality at the starting doses of 2,000 or 5,000 mg/kg is recorded, the lethal dose of a substance described as killing half of the animals in the test group (LD_{50}) of the test substances can be estimated to exceed that limit and therefore to be highly safety (Chinedu et al., 2013; Saganuwan, 2017). Based on Table 2.8, the LD_{50} above than 5000 mg/kg were classified as non-toxic substances (Loomis & Hayes, 1996). The toxicity level based on the dosage range is provided in Table 2.8.

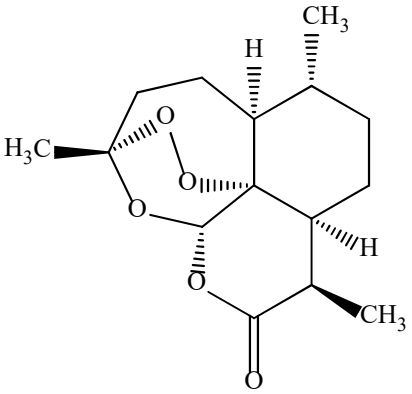
Table 2.8 : Classification of LD_{50} based on dosage range according to Loomis and Hayes, 1996.

LD_{50}	Classification
<5 mg/kg	Extremely toxic
5 – 50 mg/kg	Highly toxic
50 – 500 mg/kg	Moderate toxic
500 – 5,000 mg/kg	Slightly toxic
5000 – 15,000 mg/kg	Practically non-toxic
>15,000 mg/kg	Relatively harmless

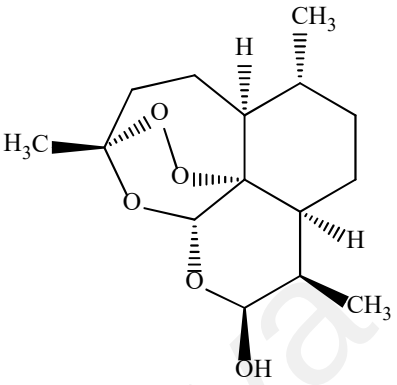
Research on the effectiveness and safety of medicinal plants is the main focus of scientists due to the widespread use of natural herbal remedies (Chen et al., 2006). The medicinal plants should be relatively non-toxic as it has been used for a long-term by humans. However, it has been reported that a number of medicinal plants used in folk medicines have toxic effects (Anywar et al., 2021; Saad et al., 2007). According to a father of toxicology, Paracelsus, who famously said: "All things are poison and nothing (is) without poison; only the dose makes that a thing is no poison". This means that all substances are poisons and there is none which is not a poison, only the proper dosage can distinguish between medicine and poison. Additionally, he also demonstrated that toxins can have both harmful and good effects and that the dose of a drug determines its effects (Hunter, 2008).

Many modern medicines are derived from natural sources (Veeresham, 2012; Yuan et al., 2016). Many of them rely on bioactive compounds found in medicinal plants (Pan et al., 2013; Yuan et al., 2016). For example, artemisinin (**117**) from *Artemisia annua* and its analogues (dihydroartemisinin (**118**), artemether (**119**) and artesunate (**120**)) (Figure 2.11) are currently widely used for the treatment of anti-malaria (Brown, 2010; Davlantes

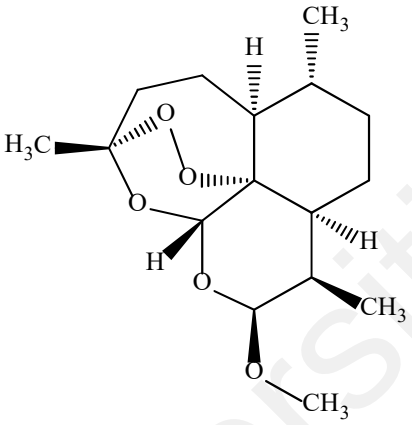
et al., 2018; Pan et al., 2013). Therefore, there is an urgent need to investigate the toxicity of medicinal plants in order to ensure proper dosage and the safety of the medicinal plants.



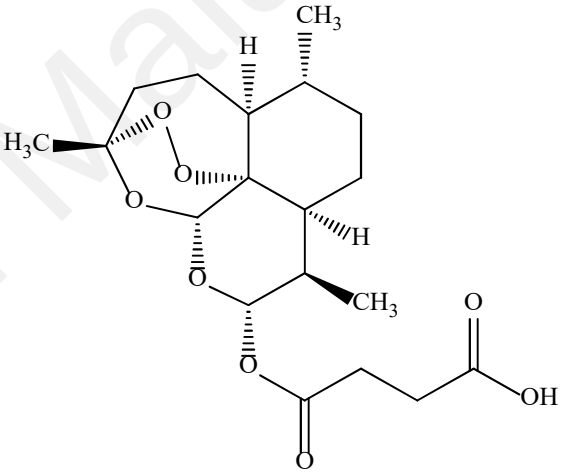
Artemisinin (**117**)



Dihydroartemisinin (**118**)



Artemether (**119**)



Artesunate (**120**)

Figure 2.11 : Molecular structure of artemisinin (117), dihydroartemisinin (118), artemether (119), and artesunate (120).

2.2.3 Anti-pyretic study

Fever or pyrexia has always been the first symptom of illness or infection that can be detected by elevating body temperature beyond the normal range of body temperature, 36.5 - 37.5 °C. Fever can be determined as a natural illness response that are associated with many diseases such as dengue and malaria followed by a variety of symptoms such

as sweating, shivering, failure to focus, depression, anorexia, and insomnia (Hines, 2020; Phumthum & Sadgrove, 2020).

In general, when the body system is in good health, the hypothalamus will set to a normal body temperature. But when the body is infected with the virus, bacteria, or fungal, fever developed. Other than that, fever can also be caused by immunization or medication reactions or long-term chronic inflammatory-associated diseases such as rheumatoid (Publishing, 2018). Fever is a process where our human/body immune system fights with foreign entities as a natural response. Therefore, the virus, bacteria or fungal will be destroyed by our immune system, but at the same time it will also destroy our cells at high temperatures, and the worst thing is it will lead to brain, kidney and liver damage.

The presence of pyrogen will activate the immune system to defend the body against those pathogens (Phumthum & Sadgrove, 2020). There are two types of pyrogens which are exogenous pyrogen and endogenous pyrogen. Exogenous pyrogen is the pyrogens produced from outside the body (external) such as toxins or poison produced by infectious bacteria or viruses (Publishing, 2018). Another one is endogenous pyrogen which is the pyrogens that inside the body (internal). In order to generate cytokines that function as endogenous pyrogens, toxins released from the infected organism and tissue destruction act on the phagocytic cells (monocytes, macrophages and Kupffer cells). The pyrogens are polypeptides and contain interleukin-I (IL-1α and IL-1β) and other cytokines acting on the anterior hypothalamus to increase the production prostaglandin E_2 (PGE_2) (Prajitha et al., 2018). Then, it will send the signals at thermoregulatory center located in the anterior hypothalamus at the central nervous body system to increase the thermostat setting point. The hypothalamus which acts as the body's thermostat was then transfers the signal to other part of organs and system in the body to adapt with the problem with

variety of mechanisms such as sweating, shivering, headache and muscle pain (Publishing, 2020). The illustration of the process of fever caused by infectious diseases as shown in Figure 2.12.

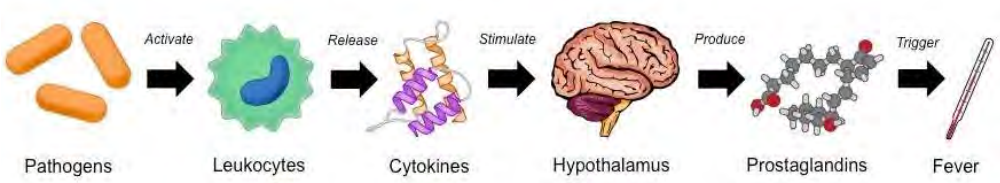


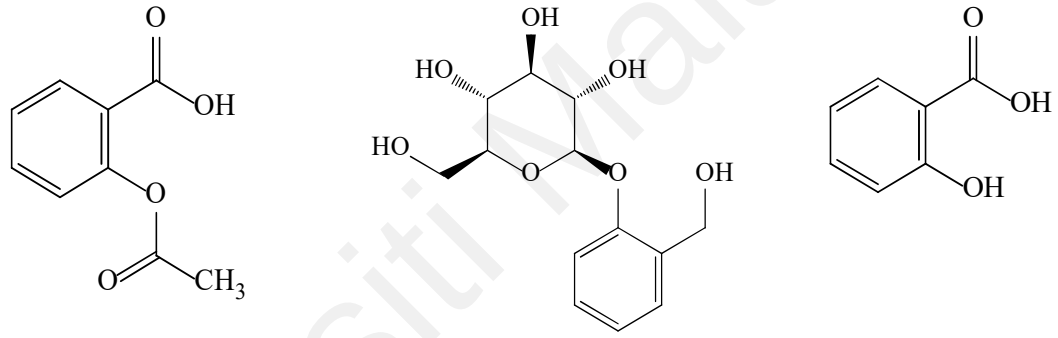
Figure 2.12 : The general mechanism of fever.

(source: https://ib.bioninja.com.au/stan;.dard-level/topic-6-human-physiology/63-defence-against-infectio/inflammation.html accessed on 22nd February 2022)

Pyrexia or fever is the first indicator of our body's defense system by its natural defence mechanism if the body system experienced a secondary impact of infection, tissue injury, inflammation, graft rejection or malignancy (Shilpi & Uddin, 2020). Infected or damaged tissues will promote the formation of cytokines (such as interleukin 1β, α, β and TNF-α), which prone to increase the synthesis of prostaglandin level hence triggeres the hypothalamus to elevate the body temperature (Brunton et al., 2005). Most of the anti-pyretic drugs including aspirin (**121**) will inhibit or suppress cyclooxygenase-2 (COX-2) expression thus inhibit the synthesis of PGE_2 in order to reduce the elevated body temperature. However, these synthetic anti-pyretic drugs are toxic to the hepatic cells, glomeruli, cortex of the brain and heart muscle which tend to cause heart attack and stroke for long term users (Luo et al., 2005). A natural PGE_2 inhibitory anti-pyretic remedy from medicinal plants with minimal toxicity and less side effects are therefore essential.

In ancient times, air or cold water was used to cool down as a means of relieving high fever. Later, physicians discovered herbs such as willow bark and other plants that counteracted fever, although the mode of actions was unclear at the time. Johann Buchner

first discovered the active ingredient in willow bark in 1828 by grinding willow bark into yellow crystals which is salicin (**122**) (Schindler, 1978). The derivatives of salicin (**122**) were developed by other researchers including salicylic acid (**123**), which produced greater and more effective anti-pyretic drugs (Gerhardt, 1853; Leroux, 1830; Piria, 1838). In 1897, Felix Hoffman a medicinal chemist in Bayer successfully produced synthetic anti-pyretic drugs known as aspirin (**121**) (acetylsalicylic acid, ASA (**124**)) by the addition of an acetyl group to salicylic acid (**123**) (Desborough & Keeling, 2017). The molecular structures of acetylsalicylic acid (**124**), salicin (**122**) and salicylic acid (**123**) are shown in Figure 2.13.

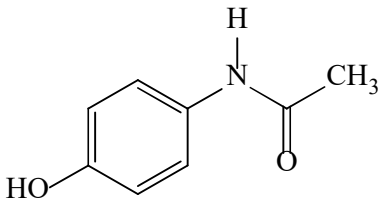


Acetylsalicylic acid (**124**) Salicin (**122**) Salicylic acid (**123**)

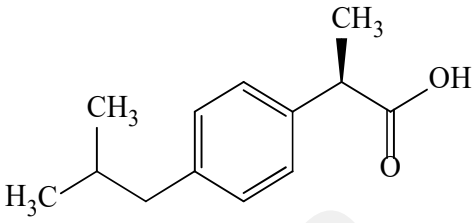
Figure 2.13 : Molecular structures of acetylsalicylic acid (124), salicin (122) and salicylic acid (123).

Nowadays, many synthetic anti-pyretic drugs were developed in treating fever such as acetaminophen (**64**) or paracetamol in 1956 and ibuprofen (**124**) in 1962 (Desborough & Keeling, 2017). The molecular structures of acetaminophen (**64**) and ibuprofen (**124**) are illustrated in Figure 2.14. Aspirin (**121**) and ibuprofen (**124**) are also known as non-steroidal anti-inflammatory drugs (NSAIDs). The uses of anti-pyretic drugs are very efficient in treating fever but the side effect should be considered especially for a long-term consumption or overdosage used. For example, a common side-effect of non-steroidal anti-pyretic drug therapy is gastrointestinal toxicity which can be divided into three categories; mucosal lesions that can be observed by radiographic or endoscopic,

gastrointestinal discomfort (such as dyspepsia, heartburn and nausea) and severe gastrointestinal complications (such as perforated ulcers and gastrointestinal bleeding) (Plaisance, 2000).



Acetaminophen (**64**)



Ibuprofen (**124**)

Figure 2.14 : Molecular structures of acetaminophen (64) and ibuprofen (124).

Other than that, the important adverse effects of NSAIDs, aspirin (**121**) and acetaminophen (**64**) is renal toxicity. It can be classified into four types which are fluid and electrolyte disturbances, acute renal dysfunction, acute interstitial nephritis and analgesic-associated nephropathy (Plaisance, 2000). The FDA is reinforcing the current alert on prescription drug labels and over-the-counter (OTC) drug labels Facts that NSAIDs may increase the risk of heart attack or stroke, either of which may lead to death (Administration, 2015). In 2020, the FDA warns that the use of any sort of pain and fever medications during 20 weeks of pregnancy and beyond, may lead to severe kidney problems in the unborn babies, that resulted in low amniotic fluid and the potential for complications associated with pregnancy (Administration, 2020) .

Considering this, it is an urge to discover an alternative medicine in treating fever which more effective with less adverse effect. Hippocrates, the father of medicines had discovered that 300 medicinal plants classified by physiological action like wormwood and common centaury (*Centaurium umbellatum* Gilib) were applied against fever (Petrovska, 2012). Some of the medicinal plants that were used in treating fever were

Andrographis paniculate, *Centella asiatica*, *Costus speciosus*, *Eurycoma longifolia*, *Helminithotachys zeylanica*, *Hedychium coronarium*, *Hedychium flavescens*, *Kaempferia galanga*, *Curcuma zanthorriza*, *Curcuma zedoaria*, *Curcuma amada* and *Curcuma longa* (Kodjio et al., 2016; Kumar et al., 2013; Mitra et al., 2007; Pitopang et al., 2019). As mentioned in section 2.1.5, *Z. zerumbet* has various medicinal properties including for treating fever (Hamid et al., 2018). Hence, it can be considered as a probable new source of anti-pyretic agent.

2.3 Molecular docking study

In the modern drug discovery era, a lot of technology have been developed in drug discovery. Several modifications and new techniques have been implemented in the development of anti-dengue therapies, including direct-acting anti-virals and host-directed anti-virals (Lim, 2019; Troost & Smit, 2020). The process of drug development and drug discovery is very challenging, expensive, and time-consuming. Approximately 1.8 billion USD have been spent for the conventional *in vitro* research that can take up to a decade to complete the process and many factors have been considered, including the pandemic disease and the country's economic situation. Therefore, *in silico* method is one of the methods that can minimise these costs and accelerate the development of drugs (Macalino et al., 2015; Paul et al., 2010).

In recent years, computer-aided drug design (CADD), also referred to *in silico* screening, has emerged as an effective approach for identifying and developing potential leads in the drug discovery and development process. *In silico* screening is also another option and solutions for the synthesis and screening of selected compounds for better therapeutics. The CADD approach has played a significant role in the search and optimization of potential lead compounds with a considerable gain in time, manpower and cost-effectiveness (Menchaca et al., 2020).

One of the computational-aided techniques used for hit identification and lead optimization is virtual screening. Virtual screening is a more direct and rational drug discovery approach. It also has the advantage of being less expensive and the screening effect is more efficient compared to conventional experimental high-throughput screening (HTS) (Meng et al., 2011). Virtual screening can be divided into two methods which are ligand-based and structure-based method. Ligand-based method is used when the existing active ligand structure is available and little or no structural information of targets or protein is available (Aparoy et al., 2012). The pharmacophore modeling and quantitative structure-activity relationship (QSAR) analysis can be employed for the ligand-based method. On the other hand, the information from the target receptor (protein drug target) is incorporated for the development of its inhibitor in the structure-based method (Aparoy et al., 2012). In most cases, experimental techniques such as nuclear X-ray crystallography and magnetic resonance spectroscopy (NMR) are used to determine the structure of targeted protein. However, if the protein structure is not available, computational methods such as homology modeling and threading can be used to predict it. The most important and widely used tools in structure-based methods are molecular docking strategies. The basic principle and drug design scheme have been laid out in Figure 2.15.

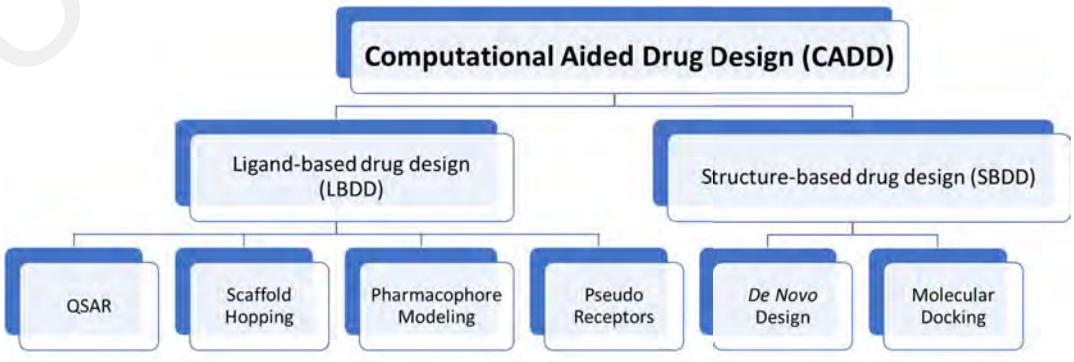


Figure 2.15: Basic principle and drug design scheme.

Molecular docking is a process that involves the prediction of the binding mode of each ligand to the target's binding site to determine the binding affinity of the binding interaction (Aparoy et al., 2012). Docking software such as AUTODOCK, DOCK, ZDOCK, FlexX and GLIDE (Lavecchia & Di Giovanni, 2013) executes the algorithm analysis which evaluate the binding interaction of the docked compound and targeted protein. Then, the compounds are ranked according to their binding scores according to ligand conformation until the lowest energy conformation is obtained which represents the strength of binding interaction. The best fit of docking and ligand conformation are determined by the lowest binding energy. There are several docking programs that use various conformational sampling algorithms and scoring functions commercially or freely available as listed in Table 2.9 (Lavecchia & Di Giovanni, 2013).There are several chemicals and compounds database available in the market such as ChemDB, PubChem and ZINC as recorded in Table 2.10 (Lavecchia & Di Giovanni, 2013). These chemical constituents are known drugs, carbohydrates, synthetic, natural and targeted compounds.

For example, the most popular and free database which is PubChem was developed and managed by National Center for Biotechnology Information (NCBI) that comprises of PubChem Compound (unique structures), PubChem BioAssay (assay results) and PubChem Substance (samples supplied by depositors) (Lavecchia & Di Giovanni, 2013). Additionally, PubChem consists of 30 million chemical compounds focusing on small molecules as system biology probes and potential therapeutic agents.

Table 2.9 : Examples of docking software commercially or freely available.

Docking software	Availability for academia	Website
AUTODOCK	Yes	http://autodock.scripps.edu/
DOCK	Yes	http://dock.compbio.ucsf.edu/
eHiTS	No	http://www.simbiosys.ca/ehits/index.html
FlexX	No	http://www.biosolveit.de/flexx/
FRED	Yes on demand	http://www.eyesopen.com/oedocking
GLIDE	No	http://www.schrodinger.com/
GOLD	No	http://www.ccdc.cam.ac.uk/products/life_sciences/gold/
ICM	No	http://www.molsoft.com/docking.html
LigandFit	No	http://accelrys.com/products/discovery-studio
Ligand-Receptor Docking	No	http://www.chemcomp.com/software-sbd.htm
ROSETTA_DOCK	Yes on demand	http://rosettadock.graylab.jhu.edu/
SLIDE	Yes on demand	http://www.bch.msu.edu/~kuhn/software/slide/index.html
Surflex	No	http://www.tripos.com/index.php
Virtual Docker	No	http://www.molegro.com/mvd-product.php
ZDOCK	Yes	http://zlab.umassmed.edu/zdock/

The other important aspect in molecular docking is the binding site. The binding site is determined by the part of the targeted protein to which the ligand will bind (responsible for the binding of a specific ligand to a targeted protein). As a result, molecular docking

requires three components to be successful: the targeted protein, a ligand or inhibitor, and the targeted protein's binding site.

Table 2.10 : List of chemical compounds databases available.

Name of database	Availability	No. of compounds (million)	Website
Pubchem	Public	30	http://pubchem.ncbi.nlm.nih.gov
ChemSpider	Public	26	http://www.chemspider.com
ZINC	Public	13	http://zinc.docking.org
ChemDB	Public	5	http://cdb.ics.uci.edu
CoCoCo	Public	7	http://cococo.unimore.it/tiki-index.php
ChEMBL	Public	1	http://www.ebi.ac.uk/chembldb/index.php
ChemBank	Public	1.2	http://chembank.broadinstitute.org
eMolecules	Commercial	7	http://www.emolecules.com
ACD	Commercial	3.9	http://accelrys.com/products/databases/sourcing/avaible-chemicalsdirectory.html
Enamine	Commercial	1.7	http://www.enammine.net
ChemDiv	Commercial	1.5	http://www.chemdiv.com

2.3.1 NS2B/NS3 protease

The dengue virus NS2B/NS3 protease is involved in genome replication and viral RNA synthesis by cleaving the viral polyprotein precursor to release individual non-structural macromolecules along with a C-terminal nucleoside triphosphatase (NTPase-dependant) RNA helicase (Dwivedi et al., 2016; Matusan et al., 2001). For this reason,

NS2B/NS3 protease is proposed as a therapeutic target for the development of potential lead compounds in treating the dengue virus infection. In this study, in order to predict the possible binding interaction between the compound (inhibitor) and the enzyme; NS2B/NS3 protease, molecular docking studies were performed by using the DENV2 NS2B/NS3 protease model produced via homology modeling by Wichapong et. al (2010) (Wichapong et al., 2009) as the targeted protein.

According to Wichapong et. al (2009), the molecular docking simulations of DENV2 NS2B/NS3 protease (Figure 2.16) exhibited strong binding modes of the C-terminal domains of NS2B and NS3 that will support the loop regions of the NS3 protease's stability (Wichapong et al., 2009). The protease (host) and NS2B/NS3 (viral protease) are essential for co- and post-translational processing into individual functional protein (Falgout et al., 1991; Wichapong et al., 2009). The enzymatic activity of a classic serine protease catalytic triad of NS3 protease (Asp75, His51 and Ser135) inhibits viral infectivity which suggested that the inhibition of any inhibitors with NS3 may be a promising anti-viral therapeutic (Qamar et al., 2014; Wichapong et al., 2009).

This study observed the interaction between the peptidic inhibitor; Arg-P2 and the residue in the S2 pocket which demonstrated the C-terminal domain of NS2B is significant for the NS3 protease binding interaction as well as involved in the interaction with the P2 residue of the inhibitor (Wichapong et al., 2009). The S1 pocket, on the other hand, only interacts with the inhibitor through the NS3 domain (Asp129, Ser135, Tyr150 and Tyr161). However, both residues from the C-terminal domain of NS2B, together with Asn152 and Asp75 from NS3 that required for preserving the inhibitor's interaction with the P2 residue at the S2 pocket. The main interactions are identified between the backbone of the P3 key residue and NS3 residues (Tyr161 and Gly153) from the S3 pocket whereas only hydrophobic interactions are obtained at the S4 pocket of the NS3 domain.

Therefore, the homology modelling performed by Wichapong et al. (2009) derived the productive conformation of DV NS2B/NS3 model that can be used for structure based drug design in order to discover the potent inhibitors against dengue activity (Wichapong et al., 2009).

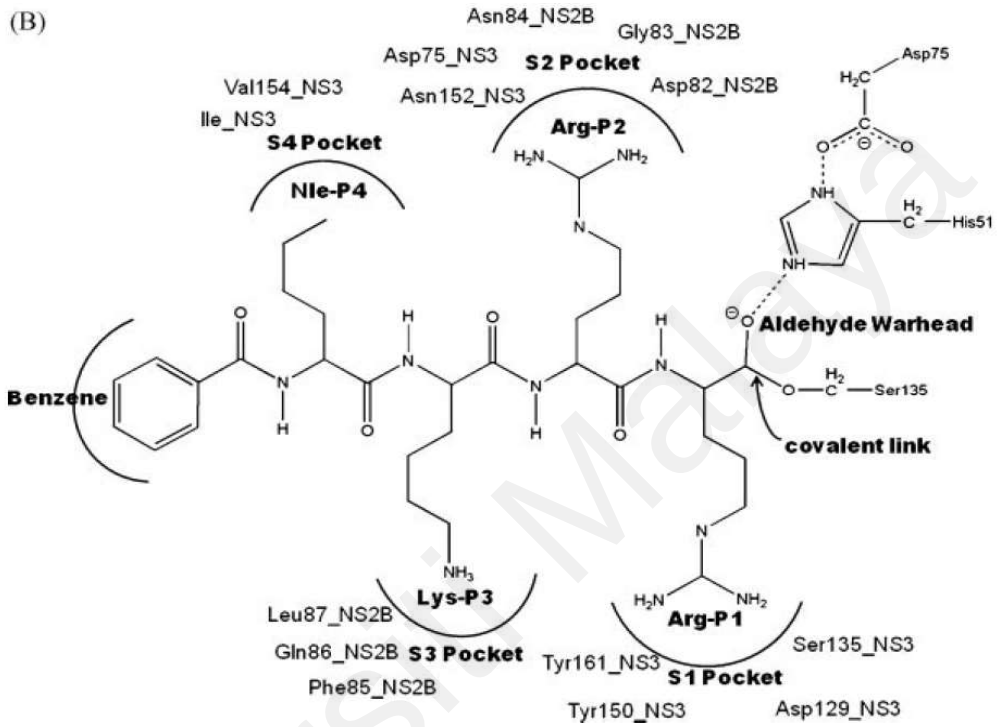


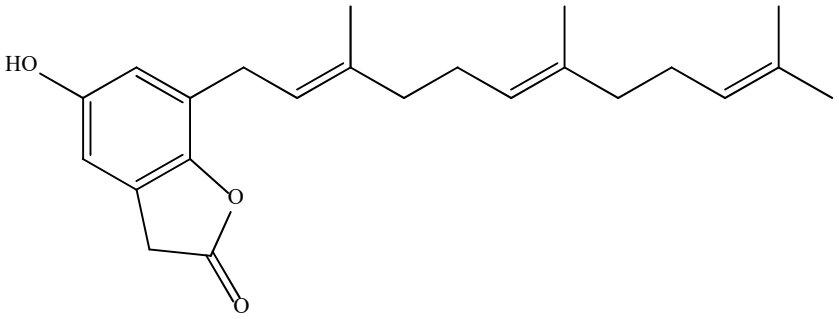
Figure 2.16 : Schematic representation the interaction between the peptidic inhibitors benzoyl-norleucine(P4)-lysine(P3)-arginine(P2)-arginine(P1)-aldehyde (Bz-Nle-Arg-Arg-Arg-H) (2FP7)and the substrate pockets of West Nile Virus DENV2 NS2B/NS3 protease (Wichapong et al., 2009).

2.3.2 Molecular docking study on NS2B/NS3 protease

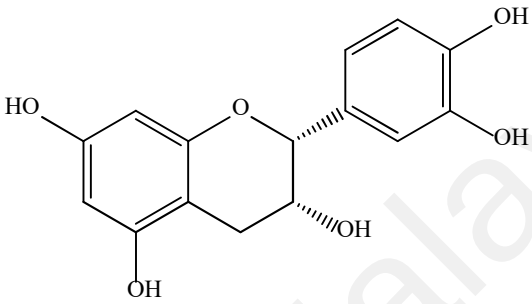
Recently, it was observed that molecular docking has been established in drug development, particularly in the search for dengue fever therapeutics (Neelawala et al., 2019; Rasool et al., 2019; Sulaiman et al., 2019; Yadav et al., 2021). There are a few classes of compounds that have been studied for molecular docking in NS2B/NS3 such as acylphenols, alkaloid, benzofuranone, curcuminoids, flavonoids, phenolic and tetracycline (Lim et al., 2020; Othman et al., 2017; Rahman et al., 2014; Rasool et al., 2019; Sivasothy et al., 2021; Sulaiman et al., 2019).

Sulaiman et al. (2019) has reported a new benzofuranone, 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3H)-benzofuranone (**97**) (Figure 2.17) as an inhibitor against NS2B/NS3 with percentage inhibition of 61.23%. Other compounds including (−)-epicatechin (**85**) and (+)-catechin (**75**) (Figure 2.17) showed moderate inhibition against NS2B/NS3 protease of DENV2 with IC_{50} values of 170.10 ± 5.94 μM and 184.13 ± 2.11 μM, respectively (Sulaiman et al., 2019). The molecular docking study demonstrated that 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3H)-benzofuranone (**97**) forms 2 hydrogen bonds with Ser135 and Asp129at the S1 pocket (Figure 2.18).

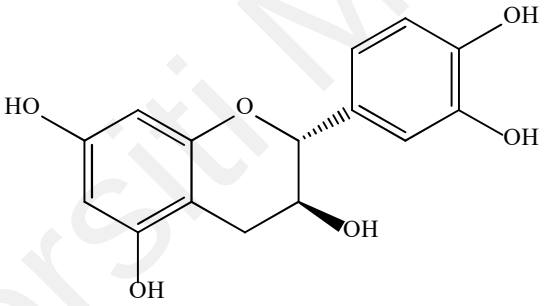
(−)-Epicatechin (**85**) has four hydrogen bonding interactions of hydroxyl (OH) groups with the amino acid residues (Asp 129, Asn 152 and Tyr 161), whereas (+)-catechin (**75**) has three hydrogen bondings with Asn152, Asp 129 and Ser125 residue and one π-π stacking interaction with Tyr161 of DENV2 NS2B/NS3 protease (Figure 2.18). Therefore, the number of hydrogen bonding interaction of hydroxyl groups and the residues may attribute to the anti-protease activity. Besides that, the number of aromatic rings and the position of the hydroxyl groups in the molecule have a significant impact on activity.



(a)

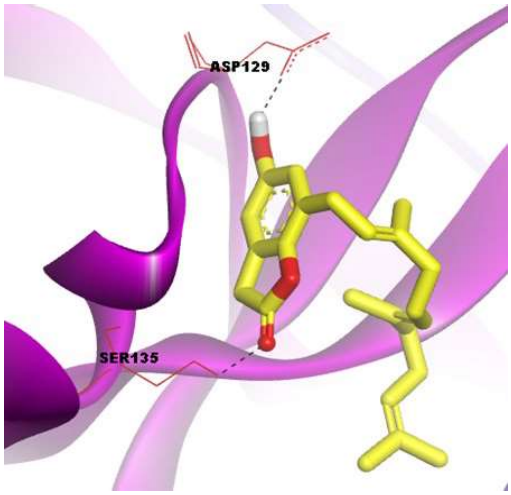


(b)

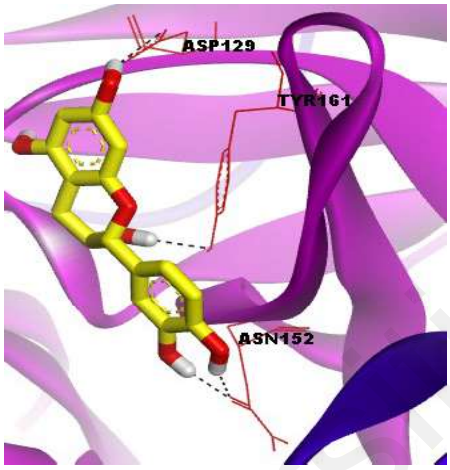


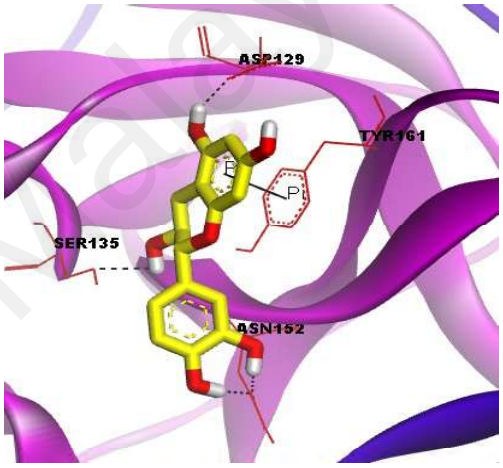
(c)

Figure 2.17 : The molecular structures of (a) 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3H)-benzofuranone (97), (b) (−)-epicatechin (85) and (c) (+)-catechin (75).



(a)



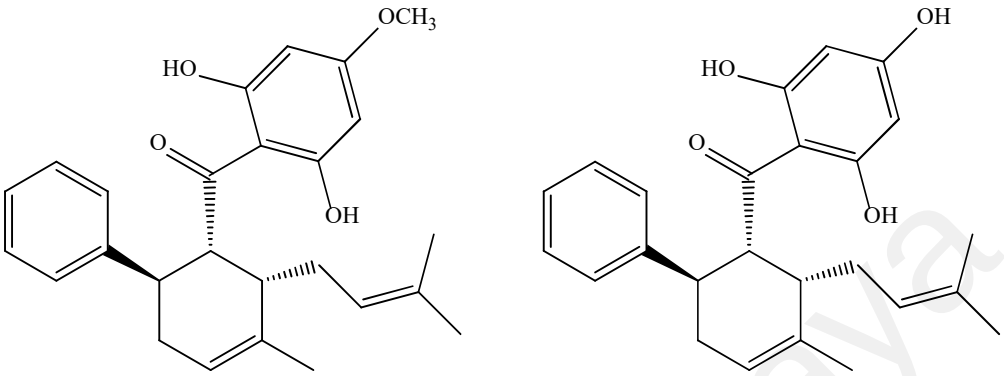


(b) (c)

Figure 2.18: The binding interactions of (a) 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3H)-benzofuranone (97), (b) (−)-epicatechin (85) and (c) (+)-catechin (75) with DENV2 NS2B/NS3 protease (Sulaiman et al., 2019).

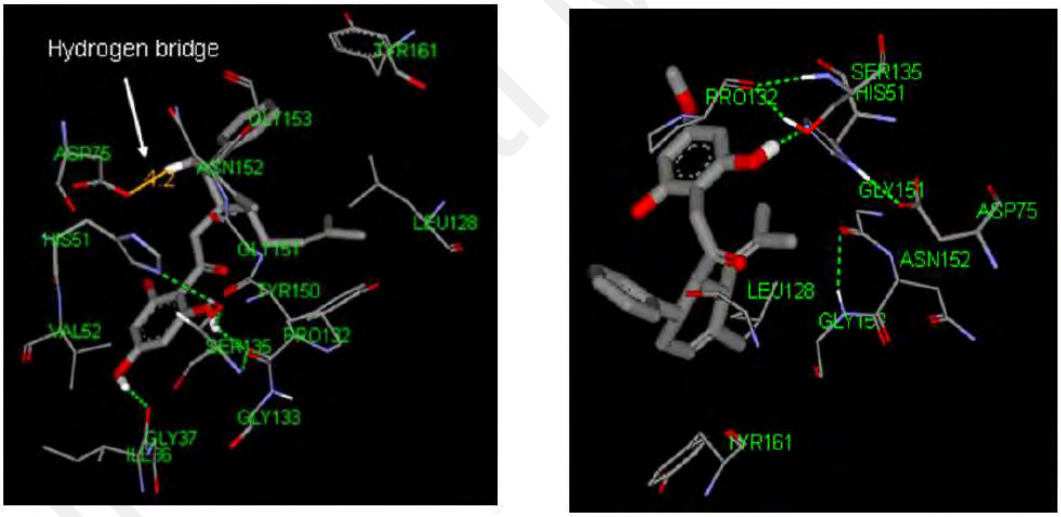
Apart from the aforementioned, panduratin A (**67**) and 4-hydroxypanduratin A (**68**) (Figure 2.19), both natural compounds isolated from *Boesenbergia rotunda* (L.) Mansf. Kulturpfl (BRI), have demonstrated potent inhibition towards NS2B/NS3 with percentage inhibition of 27.1% and 52.0%, respectively at 40 ppm (Kiat et al., 2006). The molecular docking study showed that both compounds having van der Waals interactions with Pro132 and His51, as well as hydrogen interactions with the residues His51 and Gly153 (Figure 2.20) (Frimayanti et al., 2011). Besides, 4-hydroxypanduratin A (**68**) was found to be more potent than panduratin A (**67**) possibly due to shorter hydrogen bridge (the

interaction between a ligand's hydrogen atom and the catalytic triad residues in the binding site which is less than 10 Å) of 4-hydroxypanduratin A (**68**) with the carboxyl group of Asp75 (4.2 Å) compared to panduratin A (**67**) (6.6 Å) (Frimayanti et al., 2011).



(a) (b)

Figure 2.19 : The molecular structure of (a) Panduratin A (67) and (b) 4-hydroxypanduratin A (68).

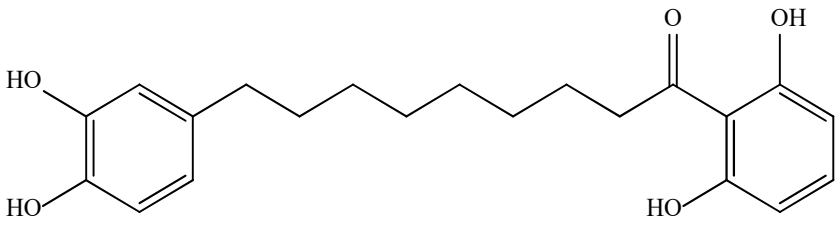


(a) (b)

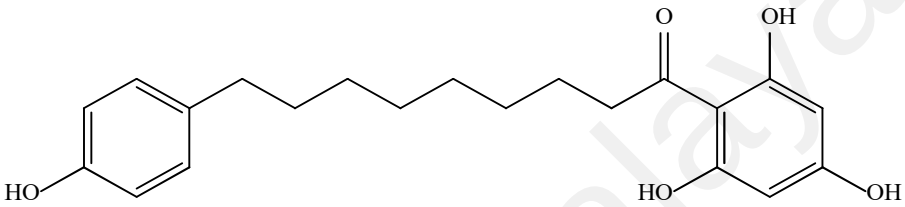
Figure 2.20 : The binding interaction of (a) 4-hydroxypanduratin A (68) with Pro132, His51, Gly153, Asp75 and Ile36 and (b) Panduratin A (67) with Pro132, His51, Gly153 and Asp75 (Frimayanti et al., 2011).

Furthermore, the first report on the DENV2 NS2B/NS3 protease inhibitory activity of acylphenols have been studied by Sivasothy et al. (2021) (Sivasothy et al., 2021). The acylphenols compounds; malabaricones C (**102**) and E (**103**) (Figure 2.21) were isolated

from *Myristica cinnamomea* King, have been found to inhibit DENV2 NS2B/NS3 protease at IC_{50} value of 27.33 ± 5.45 μM and 7.55 ± 1.64 μM, respectively.

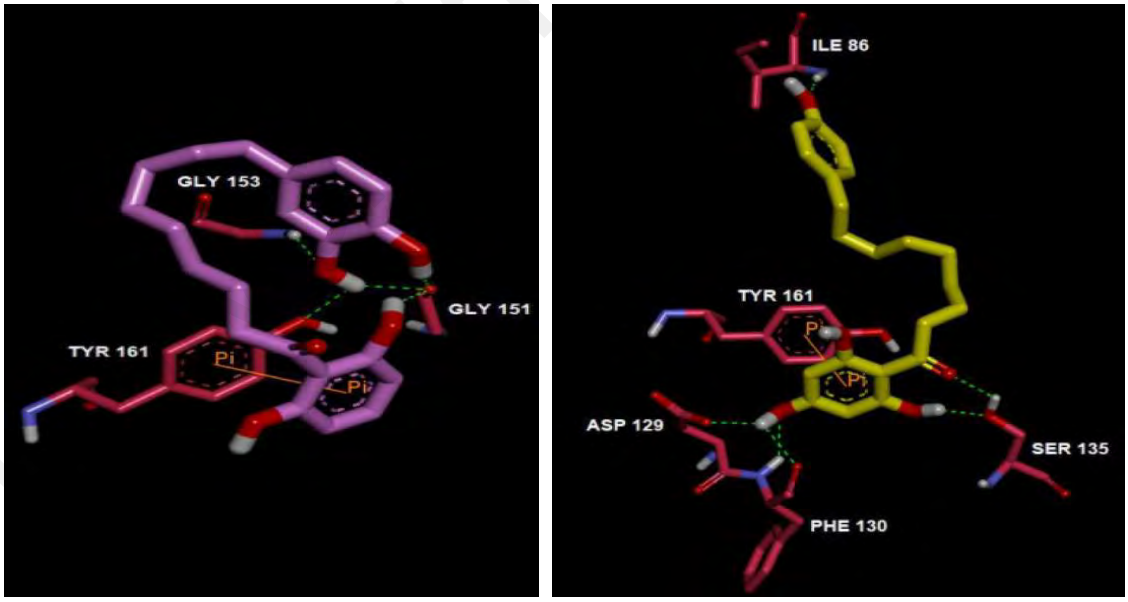


(a)



(b)

Figure 2.21 : The molecular structures of (a) malabaricone C (102) and (b) malabaricone E (103).



(a) (b)

Figure 2.22 : The binding interaction of (a) malabaricone C (102) with Gly151, Gly153 and Tyr161while (b) malabaricone E (103) with Asp129, Ile86, Phe130, Ser135 and Tyr161 (Sivasothy et al., 2021).

Based on the molecular docking studies, both compounds (malabaricones C (**102**) and E (**103**)) have π-π interactions with Tyr161 (Figure 2.22). Malabaricone C (**102**) was demonstrated 3 hydrogen bonding interactions with Gly151, Gly153 and Tyr161, whereas malabaricone E (**103**) formed 4 hydrogen bonds with Ser135, Asp129, Phe130, and Ile86 (Figure 2.22). Interestingly, malabaricone E (**103**) has slightly lower binding energy (-5.89 kcal/mol) in comparison with malabaricone C (**102**) (-5.47 kcal/mol), which indicated that malabaricone E (**103**) is more potent than malabaricone C (**102**) towards inhibition of DENV2 NS2B/NS3 protease.

In the present study, the natural compounds isolated from *Z. Zerumbet*; zerumbone (**3**) and zerumbone epoxide (**56**) together with quercetin (**70**) as a reference inhibitor were selected to investigate the binding interactions involved with protein (DENV2 NS2B/NS3 protease) that are responsible for the compound's anti-viral activity.

CHAPTER 3: EXPERIMENTAL

This chapter covered the experimental procedures that involved in the following activities:

a) the extraction of the rhizomes of *Zingiber zerumbet* (*Z. zerumbet*),
b) anti-protease activity on the crude extracts against DENV2 NS2B/NS3 protease,
c) fractionation of the most potent extract and screening for the anti-protease activity
d) isolation of compounds of active fraction extract based on the anti-protease activity study,
e) anti-protease activity on isolated compounds against DENV2 NS2B/NS3 protease
f) acute oral toxicity and anti-pyretic study on the most active anti-protease crude extract
g) molecular docking of the compounds against DENV2 NS2B/NS3 protease.

Briefly, in this study, the rhizomes of *Z. zerumbet* were extracted with solvent of different polarities. All the extracts were then screened for anti-protease activity against NS2B/NS3 protease. Then, the most potent extract was fractionated and all the fractions were screened for anti-protease activity again. The isolation and purification of compounds were conducted from the most potent fraction. The structure of the isolated compounds was determined through various spectroscopic methods such as 1D-NMR (^{1}H and ^{13}C), 2D-NMR (COSY, HMBC, and HSQC), FTIR, LCMS and UV-vis spectroscopy. The isolated compounds were then tested for the anti-protease activity. In association with dengue fever, the most potent extract in inhibiting DENV2 NS3B/NS3 protease has also

been evaluated for its toxicity test and anti-pyretic activity. The overall research activities are outlined in Figure 3.1.

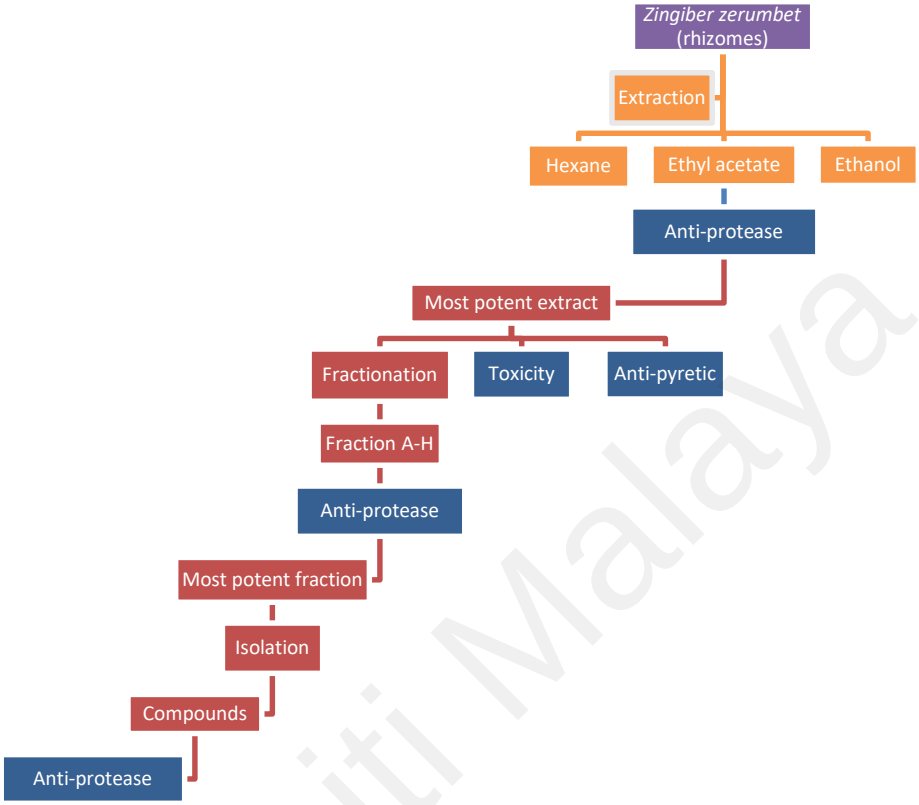


Figure 3.1 : Research Outline.

3.1 Plant material

The plant material, *Z. zerumbet* was purchased from Kiza Herbs in Kg. Baru market. The plant was divided according to its part; leaves and rhizomes. The plant specimen was identified by Mr. Teo Leong Eng (botanist). The voucher specimen (KL 5835) has been deposited at the Herbarium of the Department of Chemistry, Faculty of Science, Universiti Malaya. The voucher specimens of the leaves, stems, roots and flower are presented in Figure 3.2 and Figure 3.3.

Figure 3.2 : The voucher specimen of the leaves, stems and roots.

Figure 3.3 : The voucher specimen of the flower and roots.

3.2 Solvents and chemicals

3.2.1 Phytochemistry

Hexane, ethyl acetate (EA) and ethanol for extraction were distilled prior to use. TLC aluminium sheets, silica gel 60 F_{254} (20 × 20 cm), silica gel 60 (0.040-0.063 mm), celite, vanillin, NMR grade of deuterated chloroform ($CDCl_3$), and deuterated methanol (CD_3OD) were purchased from Merck Sdn. Bhd. Acetonitrile, methanol, and water in LC-MS grade were purchased from Merck Sdn. Bhd. Methanol spectroscopy grade was used for ultra-violet visible spectrometry analysis. The industrial grade solvents (hexane, ethyl acetate and ethanol) were distilled prior to use for extraction.

3.2.2 DENV2 NS2B/NS3 protease inhibition assay

Dimethyl sulfoxide (DMSO) were purchased from Merck Sdn. Bhd. The fluorogenic peptide Boc-Gly-Arg-Arg-MCA (5.2 mg) by Peptide Institute, Inc were purchased from Triway Scientific. Tris-HCl and Tris-base for buffer were purchased from Sigma-Aldrich (M) Sdn. Bhd. The recombinant pET14-NS2B-NS3 was expressed in *Escherichia coli* bacteria BL21 (DE3) according to a previous work by Heh et al. (2013) was obtained by courtesy of Prof. Dr. Habibah binti Abdul Wahab from School of Pharmaceutical Sciences, Universiti Sains Malaysia and Dr. Iffah Izzati binti Zakaria from Natural Product and Drug Discovery Centre, Malaysian Institute of Pharmaceuticals and Nutraceuticals, Ministry of Science, Technology and Innovation, Gelugor, Malaysia (Heh et al., 2013).

3.2.3 Acute toxicity study and anti-pyretic activity

Corn oil (Daisy) was purchased from the supermarket. Aspirin and 0.9% saline solution were purchased from Bio-Diagnostics Sdn Bhd.

3.3 Detecting reagents

Detecting reagent was used to identify the type of skeleton in identification of the isolated compounds. Reagent used were vanillin. The preparation procedure of the reagent were described as below:

3.3.1 Vanillin

1.0 g of vanillin in 10 ml of concentrated H_2SO_4 was added to 90 ml of ethanol before spraying onto the TLC plate. The TLC plate was then heated at 50 °C before complete development of colours had been observed. The appearance of blue, yellow, dark green, gray or brown suggested the presence of terpenes and steroids.

3.4 Phytochemistry study

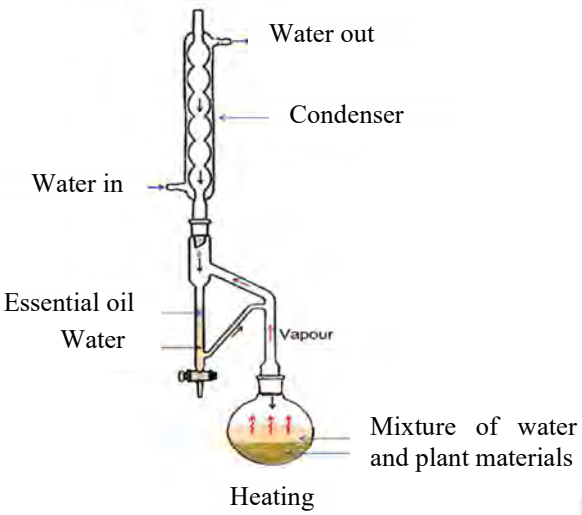
The phytochemistry study is divided into two sections (Part A and Part B):

a) Part A was the analysis of essential oils of *Z. zerumbet*
b) Part B was the extraction, isolation, and structural elucidation of compounds from the potent fraction of *Z. zerumbet*

3.4.1 Essential oil analysis of *Zingiber zerumbet* (Part A)

3.4.1.1 Extraction of essential oil

The dried rhizomes of *Z. zerumbet* (500 mg) were subjected to hydrodistillation on a laboratory scale for 5 hours using Clevenger-type apparatus as illustrated in Figure 3.4 (Sivasothy et al., 2011; Zhang et al., 2021). Pentene was used as the collecting solvent. Oils were separated from the water layer and dried with a drying agent (sodium sulphate, Na_2SO_4). The solvent was then gently removed with a stream of nitrogen gas.



(A)

(B)

Figure 3.4 : (A) The experimental setup for extraction of essential oil (B) Essential oil extraction from *Zingiber zerumbet* using the Clevenger apparatus.

3.4.1.2 Determination of oil yield

The yield of the oils was determined by using the mass of collected oil and calculations were based on the dry weight of *Z. zerumbet* rhizomes. The mean of triplicate percentage oil yield was determined (n = 3) and the percentage of oil yield was calculated by the

following formula (Eq. 3-1). The percentage of yield for three times extraction were shown in Table 3.1.

$$\%\ of\ yield : \frac{Mass\ of\ collection\ oil}{Mass\ of\ dried\ plant\ materials} \times 100 \qquad \textbf{Eq. 3-1}$$

Table 3.1 : The yield of essential oil from *Zingiber zerumbet*

The rhizomes of *Z. zerumbet* (500g of dried rhizome/replicate)	Yield (mg)	Yield (%)	Mean of yield (%)
Replicate 1	1305.4	0.26	0.32
Replicate 2	1888.5	0.38	
Replicate 3	1556.5	0.31	
Total yield (mg)	4750.4		

3.4.1.3 Essential oil analysis

The essential oils of *Z. zerumbet* which collected through the hydrodistillation extraction technique were then subjected to chemical constituents analysis which are GC-FID, GC-MS, and Kovat retention index (KI) analyses.

(a) ***Gas chromatography-flame ionization detector (GC-FID) analysis***

The oils were diluted in hexane (C_5H_{12}) at concentration of 1 mg/mL and injected (injection volume 1.0 µL; splitless) into an Agilent Technologies gas chromatography model 7890A equipped Agilent 7683B series injector flame ionization detector (FID) (Figure 3.5) and HP-5 fused silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The oven temperature was programmed initially at 70 °C and hold time of 5 min, rising from 70 - 160 °C at 2 °C/min and from 160 - 245 °C at 3 °C/min with final hold time of 5 min. Nitrogen gas was used as carrier gas at constant flow rate of 1.0 mL/min.

The standard mixture of homologous series of *n*-alkanes (C_7-C_{30}, Sigma Chemical Co., USA) was used in the GC-FID analysis.

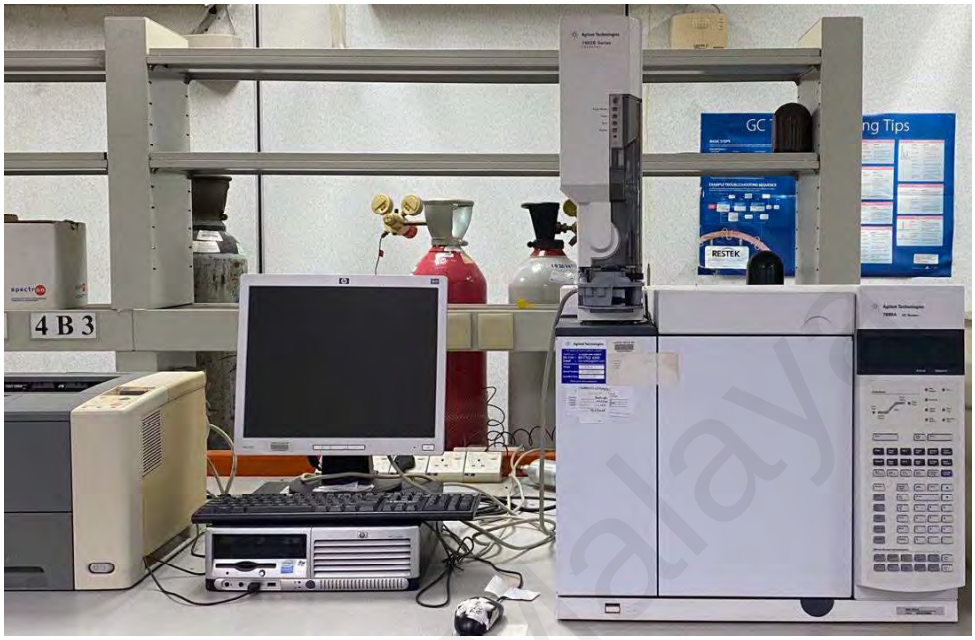


Figure 3.5 : Gas Chromatography-Flame Ionization Detector (GC-FID).

(b) ***Gas chromatography-mass spectrometry (GC-MS) analysis***

An Agilent Technologies gas chromatography 7890A system coupled to a triple quadrupole mass spectrometer 7000B system (Figure 3.6) and fitted with capillary HP-5 MS column (30 m × 250 μm, film thickness 0.25 μm) was used for the GC/MS analysis. The column and temperature parameters were programmed as same conditions as GC-FID as described previously with helium as the carrier gas (1 mL/min). The mass analysis was carried out in electron ionization (EI) mode at 70 eV with the mass scan range of 50-500 a.m.u.

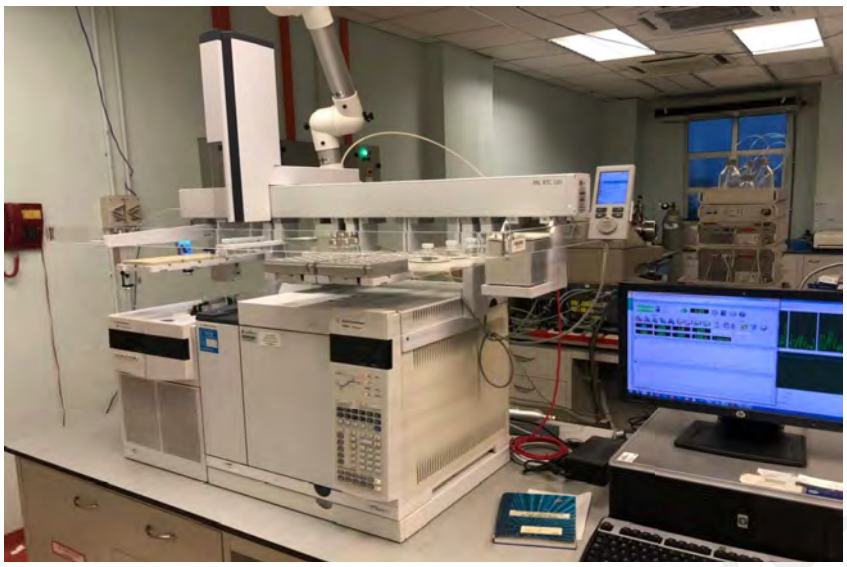


Figure 3.6 : Gas Chromatography-Mass Spectrometry (GC-MS).

(c) ***Kovat retention index (KI)***

The identification of chemical constituents from *Z. zerumbet*'s essential oil was accomplished by comparing Kovat retention index (KI) determined with reference to a homologous series of *n*-alkanes (C_7-C_{30}) under similar experimental parameters as well as by matching their mass spectra with those in NIST Mass Spectral Library (NIST14) and literatures. Relative percentage of the individual compounds of the essential oil were obtained by the GC-FID peak area % reports without FID response correction factor. KI value of each constituent was calculated by the following equation:

$$\textbf{\textit{Kovat Index}} : \frac{100\,(t-t_n)}{(t_{n+1}-t_n)} + 100(n) \qquad \textbf{Eq. 3-2}$$

where:

t = sample component retention time

t_n = retention time of standard hydrocarbon (C_7-C_{30}) elute before retention time of sample component

t_{n+1} = retention time retention time of standard hydrocarbon (C_7-C_{30}) elute after retention time of sample component

n = lowest carbon value

3.4.2 Extraction, isolation and structural elucidation of compounds from the potent ethyl acetate fraction of *Z. zerumbet* (Part B)

3.4.2.1 Extraction from rhizomes of *Z. zerumbet*

The rhizomes of *Z. zerumbet* were washed with water and then were sliced and air-dried until constant mass. Dried rhizomes were ground, and the extraction was proceeded with maceration technique. 2.0 kg of dried rhizomes were soaked in 6 liters of hexane for 3 days. The extract was then filtered and the solvent was removed using rotary evaporator to obtain the hexane extract. The residue was subjected to the same extraction process for two more times to maximize the extraction yield. The residue was then soaked with ethyl acetate and ethanol with the same procedure as mentioned above to obtain the ethyl acetate and ethanol extracts, respectively. The cumulative yields were calculated using the formula:

$$\%\ of\ yield : \frac{mass\ of\ dried\ extract}{mass\ of\ dried\ plant\ materials} \times 100 \qquad \textbf{Eq. 3-3}$$

The yields of each extract obtained from the rhizomes of *Z. zerumbet* are listed in Table 3.2. All extracts were kept in refrigerator until prior used.

Table 3.2 : The yield of extracts from the rhizomes of *Z. zerumbet*

Species	Amount (kg)	Type of extract	Yield (g)	Yield (%)	Description
Z. zerumbet	2.0	Hexane	15.8	0.79	Oily yellowish-brown
		Ethyl acetate	26.1	1.31	Brown colour
		Ethanol	16.9	0.85	Dark brown colour

3.4.2.2 Fractionation of EA

The most potent extract (EA extract) from the results of anti-protease activity against DENV2 NS2B/NS3 protease (refer to Chapter 4.2.1) were fractionated using column chromatography.

An amount 9.0 g of EA was fractionated using column chromatography (CC) technique. Silica gel 60 (0.040-0.063 mm) was used as the stationary phase. The hexane, ethyl acetate and methanol were used as mobile phase. All separations were performed using gradient elution technique. The eluates were collected in test tube (10 mL). The eluates in each test tubes were then checked with thin layer chromatography (TLC) in which the eluates having developed spots with same R_f values on the TLCs were combined. A total of eight fractions (FA-FH) were obtained (Table 3.3). All the fractions were then tested for DENV2 NS2B/NS3 inhibitory assay.

Table 3.3 : Solvent system used for fractionation of EA extract

Fractions	Eluent used for fractionation (ratio)	Yield of fractions (g)	Appearance
FA	Hexane (100)	0.6879	Yellowish oily
FB	Hex : EA (80 : 20)	4.0392	Yellowish solid + white crystal
FC	Hex : EA (60 : 40)	0.5014	Yellowish
FD	Hex : EA (20 : 80)	0.6510	Yellowish sticky
FE	Hex : EA (0 : 100)	1.1418	Yellowish sticky
FF	EA : MeOH (80 : 20)	0.3680	Yellowish sticky
FG	EA : MeOH (60 : 40)	0.5558	Yellowish
FH	MeOH (100)	0.5955	Yellowish

3.4.2.3 Isolation of compounds; zerumbone (3) and zerumbone epoxide (56) from the FB

All fractions were subjected to DENV2 NS2B/NS3 protease activity and the most potent was fraction B (FB) (refer to Chapter 4.2.1). Therefore, FB was further purified to obtain the active compound (Figure 3.7). 0.3324 g of FB was subjected to preparative thin layer chromatography (PTLC) for purification which 20 mg of FB were loaded for each silica gel plate. The PTLC was set up in a covered glass chamber using 90% hexane and 10% EA as the solvent system. Under UV lights, all the spots (separated compounds) were observed and marked. The marked spots were removed and soaked with the desired solvents to extract the compound. The solvent was then removed using rotary evaporator to obtain the compounds. The purification of FB afforded two compounds, zerumbone (**3**) (0.0834 mg) and zerumbone epoxide (**56**) (0.0018 mg) at R_f value 15 cm and 5 cm, respectively. The structural elucidation of the compounds were established by various spectroscopic methods such as nuclear magnetic resonance (NMR), LCMS, IR, and UV-vis.

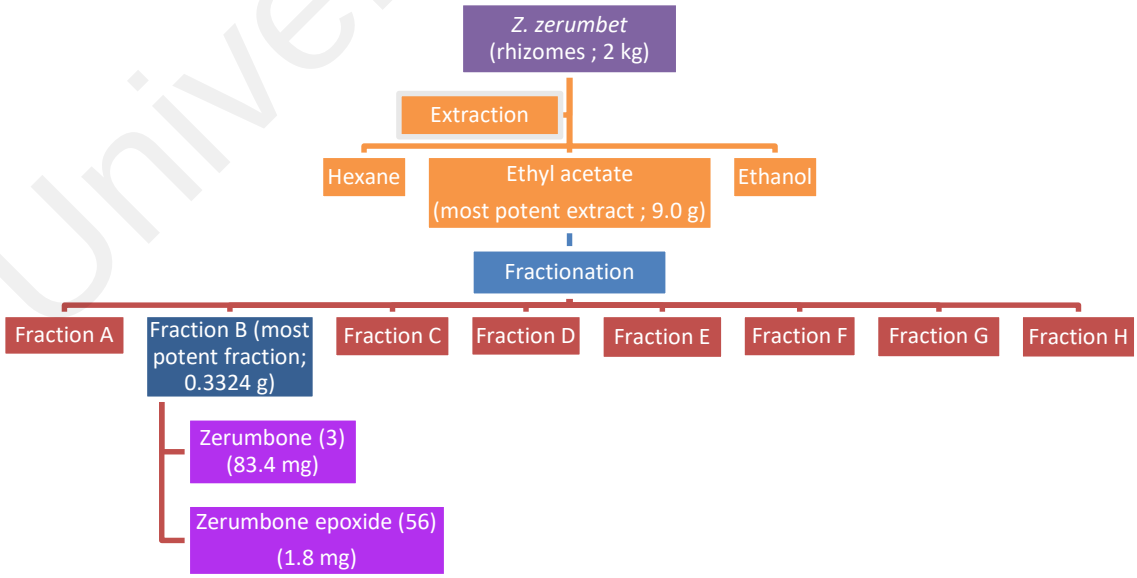


Figure 3.7 : Purification of compounds from the active extracts of *Z. zerumbet* rhizomes.

3.4.2.4 HPLC profiling

HPLC profiling was conducted for the most potent extract based on the anti-protease results which is the EA extract (refer to Chapter 4.2.1).

The profiling of EA extract was performed with high performance liquid chromatography (HPLC) by Shimadzu equipped with Pump LC-20AT, an autosampler SIL-20A HT, DAD detector SPD-M20A and column oven CTO-10AS VP. The isocratic eluents used were acetonitrile (A) and water with 0.1% formic acid (B) with the ratio 60 (A) to 40 (B) at flow rate 1.0 ml/min for 40 minutes. Column used was ZORBAX Eclipse Plus C18 (4.6 mm i.d × 150 mm, 3.5 µm) by Agilent. 1 µL of samples ; EA extract and isolated zerumbone (**3**) were subjected to the HPLC instrument, seperately. The presence of zerumbone (**3**) in EA extract was determined by spiking a small amount of the isolated zerumbone (**3**) (1 mg/mL) to the EA extract and the system was performed using the same method as mentioned above. All HPLC chromatogram were showed in results and discussion (refer to Chapter 4.2.2).

3.4.2.5 LCMS analysis

LCMS analysis was conducted for the isolated compounds from the most potent extract based on the anti-protease results which is the EA extract (refer to Chapter 4.2.1). The procedure of LCMS analysis is as follows:

Zerumbone (**3**) and zerumbone epoxide (**56**) was subjected to liquid chromatography quadruple time of flight (Agilent 1260-6530 Infinity) attached with 1260 DAD detector for profiling. The isocratic eluents used were acetonitrile (A) and water with 0.1% formic acid (B) at ratio of 60 (A) to 40 (B) with flow rate of 1.0 mL/min for 40 minutes. The absorbance was detected at 254 nm. Column used was ZORBAX Eclipse XDB-C18 (2.1 mm i.d × 100 mm, 1.8µm) by Agilent. All samples (zerumbone (**3**) and zerumbone epoxide (**56**)) were filtered through Whatman 13 mm, 0.2 µm nylon membrane syringe

filters before use. 1 µL of sample was injected into the instrument. All LCMS chromatogram with the mass spectrum were showed in results and discussion (refer to Chapter 4.1.2).

3.4.2.6 Infrared (IR) spectroscopy

1.0 mg of compound were subjected to Perkin Elmer Spectrum 1600 FTIR Spectrometer. The IR spectra were analyzed to identify the existence of functional group of the compounds such as hydroxyl, carbonyl, alkyl and alkene groups.

3.4.2.7 Ultra-violet visible spectroscopy

1.0 mg of compound to be tested were diluted with methanol spectroscopy grade to 10 mL volume. The ultra-violet visible (UV-vis) spectra were recorded using Shimadzu UV-250 UV-visible spectrometer.

3.4.2.8 Optical rotation

Optical rotation $[\alpha]_D^{23}$ values were measured using JASCO P-1020 polarimeter instrument with the concentration of compound in g/mL. The compounds were dissolved in chloroform (spectroscopic grade).

3.5 DENV2 NS2B/NS3 protease inhibition assay

In accordance with a previous study by Heh et al., (2013), the recombinant pET14-NS2B-NS3 was expressed in *Escherichia coli* bacteria BL21 (DE3) was obtained through the courtesy of Prof. Dr. Habibah binti Abdul Wahab from School of Pharmaceutical Sciences, Universiti Sains Malaysia and Dr. Iffah Izzati binti Zakaria from Natural Product and Drug Discovery Centre, Malaysian Institute of Pharmaceuticals and Nutraceuticals, Ministry of Science, Technology and Innovation, Gelugor, Malaysia (Heh et al., 2013). *E. coli* BL21 (DE3) containing a pET14-NS2B-NS3 expression vector was inoculated into Luria–Bertani (LB) medium (containing 100 mg/mL ampicillin) and the

mixture was then incubated overnight at 37 °C. 1 litre of LB broth was filled with a 10 mL aliquot of the culture, which was then incubated at 37 °C with 200 rpm of agitation until the optical density (OD_{600}) of the culture reached about 0.8. The cells were induced by addition of 0.5 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG), incubated for an overnight before being harvested by centrifugation (10,000×g, 10 min, 4 °C). The cells were sonicated, ultracentrifuged (10,000×g, 10 min, 4 °C) and the protein suspension was filtered through a membrane filter (0.22 mm). A Ni-NTA (nitrilotriacetic acid) agarose column was used to purified the crude His-tagged fusion protein at 4 °C. The column-retained protein was initially washed with buffer A, Tris–HCl (pH 8.5, 0.5% glycerol, 20 mmol/L imidazole) and followed by buffer B, Tris–HCl (pH 8.5, 0.5% glycerol, 250 mmol/L imidazole). The proteins were then pooled and kept at -80 °C until used.

The bioassay protocol of DENV2 NS2B/NS3 protease activity was employed with minor modification as described by Nawi (Abdul et al., 2016; Nawi, 2015). The protease inhibition activity was carried out using purified DENV2 NS2B/NS3 protease (Abdul et al., 2016; Nawi, 2015) and fluorogenic peptide Boc-Gly-Arg-Arg-MCA as the substrate. In the assay, the enzyme concentration was prepared at 0.5 µM and substrate concentration was diluted to 10 mM in 200 mM Tris-HCl solution (buffer) with pH 8.5. The plant extracts, fractions, isolated compounds (zerumbone (**3**) and zerumbone epoxide (**56**)) and quercetin (**70**) (standard) were dissolved in DMSO and the concentration used in this assay was prepared at 200 µg/mL. All tests were performed in quadruplicate. Firstly, a Tris buffer (pH 8) was pipetted into each wells, followed by 1 µL of sample and 3.1 µL of enzyme. The enzyme and sample were incubated at 37 °C for 10 minutes before adding the substrate. After adding the substrate, the reaction mixtures were incubated at 37 °C for 60 minutes. All reactions were conducted in black 96-well plates with final volume of 100 µL each well and fluorescence intensity of the released AMC was detected after 60 minutes incubation using the Promega Glomax Multi Detection System

microplate reader at 365 nm (excitation) and 410-460 nm (emission) wavelengths, respectively. The IC_{50} evaluation of the samples with more than 80% inhibition against NS2B/NS3 protease at 200 µg/mL were conducted with the same procedure as described above with serial dilutions concentration of sample between 0.78 to 200 µg/mL. The assay conditions were validated by running a positive control (1 µL of 100 mM substrate Boc-Gly-Arg-Arg-MCA and 3.1 µL of 0.5 mM enzyme in 95.9 µL of 200 mM Tris–HCl) and blank control (100 mL of 200 mM Tris–HCl) simultaneously (Zakaria et al., 2019).

3.6 Acute oral toxicity and anti-pyretic studies

3.6.1 Animals

Fifty-two Sprague Dawley rats (190–230 g) were purchased from Animal Experimental Unit of Universiti Malaya. Animals were placed in cages under standard laboratory conditions with access to drinking water and a standard rat diet. A temperature of 22 ± 0.9 °C with a relative humidity of 65 ± 5% and a 12 hours light/dark cycle were maintained. All animal procedures were conducted in accordance with the principles outlined in the guidelines for "Animal Use Protocol" prepared by the University of Malaya Animal Care and Use Committee. Ethic No: 2017-180309/IBS/R/AAB.

3.6.2 Acute oral toxicity

The oral acute toxicity (OAT) test was performed accordance with OECD Test Guideline 425 (tg-425) with minor modifications. Based on early study, a limit test (2000 mg/kg) of EA extract of *Z. zerumbet* was first employed. Animals (4 female rats per dosage group) were fasted for approximately 16 hours prior to dosing and were weighed and given orally an EA extract of *Z. zerumbet* at the doses of 2000, 3000, and 5000 mg/kg body weight. The EA extract was dissolved in corn oil and total volume administered for each rat was 10 mL/kg body weight. A control group was given an equivalent amount of

corn oil. Animals were observed for signs of toxicity or any abnormal behaviour such as sedation, respiratory distress, motor impairment and hyperexcitability during the first 30 min, then 2 hours intervals at least 3 times in the first day, and daily for 16 days after dosing. Food and water were provided *ad libitum*. At the end of the experiment, animals were euthanized by carbon dioxide (CO_2) asphyxiation and were disposed by the UM Registered Bio-disposable contractor.

3.6.3 Induction of pyrexia

Yeast induced pyrexia model was applied to evaluate the anti-pyretic activity of the extract. Pyrexia was induced in both 15 male and 15 female rats according to the method as described previously by Hassan et al. (2015) with minor modifications (Hassan et al., 2015). Prior to the experiment, rectal temperatures of each animal were measured by digital thermometer and rats with constant temperature were selected for the study. Rats were fasted approximately for 16 hours and pyrexia in rats was induced by subcutaneous injection of Brewer's yeast (25% w/v) in 0.9% saline solution at the volume of 20 mL/kg body weight after recording of the initial basal rectal temperature. A group of 6 rats was received an equal amount of 0.9% saline solution and defined as untreated control. After 18 hours of yeast injection, the temperatures of all rats were measured rectally by thermometer and rats with rectal temperature rise at least 0.9 °C were considered pyretic and were selected for anti-pyretic study.

3.6.4 Anti-pyretic activity

Anti-pyretic activity of EA extract (the most potent extract against anti-protease activity) was evaluated in rats following the method as described by Pingsusaen et al. (2015) (Pingsusaen et al., 2015). Untreated rats (3 male and 3 female) with constant rectal temperatures in normal range were defined as control group (Group I). All rats that showed an increament in temperature at least 0.9 °C after the 18 hours of yeast treatment

were divided into 5 subgroups (Group II, III, IV, V and VI) comprising 6 rats in each group. Group II served as negative control. Rats in both Groups I and II were received corn oil at the volume of 10 mL/kg body weight. Rats in Group III served as positive control and were given aspirin (**121**) dissolved in distilled water at the dose 100 mg/kg body weight, while rats in Group IV, V and VI were given EA extract suspended in corn oil at the doses of 125, 250 and 500 mg/kg body weight, respectively. All samples were administered orally. The body temperature of each rats was measured rectally at predetermined time intervals before yeast injection (initial), 18 hours post injection (immediately before extract or aspirin (**121**) administration) and for 4 hours following the treatment. After the evaluation, the animals were euthanized by CO_2 asphyxiation and disposed by the UM Registered Bio-disposable contractor. The difference in rectal temperature was acquired before and after treatment and the percent inhibition of rectal temperature was calculated according to the formula defined by Mworia et al. (2019) as in Eq. 3-4 (Mworia et al., 2019).

$$\%\ \boldsymbol{inhibition\ of\ pyrexia} : \frac{B-Cn}{B} \times\ \mathbf{100} \qquad \textbf{Eq. 3-4}$$

B = Rectal temperature of 18 hours following yeast injection

C_n = Rectal temperature after treatment

3.6.5 Statistical analysis

The results were reported as mean ± standard deviation (M ± SD). Statistical analysis was performed using SPSS (16.0) software. The differences between groups were assessed by analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Statistical significance was defined as $p < 0.05$.

3.7 Molecular docking of isolated compounds with DENV2 NS2B/NS3 protease

The EA extract and the two isolated compounds; zerumbone (**3**) and zerumbone epoxide (**56**) have been found to inhibit the DENV2 NS2B/NS3 protease (Chapter 4.2.1) (Salleh et al., 2019). Hence, molecular docking was performed on the enzyme with these two compounds and the reference drug, quercetin (**70**) in order to understand their binding interactions. The three-dimensional structure of the compounds and the reference drug were retrieved from PubChem and the energy minimization was performed using Hyperchem 8.0 (HyperChem (TM), Profesional 8.0, Hypercube, Inc). In the absence of a 3D structure for the inhibitor-bound form of the DV NS2B/NS3 protease, Wichapong et al. (2009) carried out homology modeling to construct the complex of DV NS2B (2FP7) and NS3 (2FP7) proteases with the inhibitor (Bz-Nle-Lys-Arg-Arg-H) (2FP7). This modelling was based on the available crystal structure of West Nile virus and dengue virus protease. The significant sequence similarity between West Nile virus and dengue virus NS2B/NS3 (56% across both domains) indicates a strong resemblance between these two structures, as reported by Shiryaev et al. in 2007. Hence, the crystal structure of the NS2B/NS3pro West Nile Virus (PDB ID: 2FP7) (Wichapong et al., 2009) was used as the template and residues 28-32 from DENV2 NS3pro (PDB ID: 2FOM) were inserted as described by Hariono et al. (2019) since these particular residues were missed in 2FP7 (Hariono et al., 2019; Wichapong et al., 2009). The docking files for both minimized DENV2 NS2B/NS3 protease and ligands were prepared using AutoDockTools 1.5.6 and the molecular docking was perfomed using AutoDock4.2 software. For the ligands, non-polar hydrogen atoms were merged while the protein was added by polar hydrogen. The Gasteiger partial charges were computed for ligands and the Kollman charges were added to the protein structure. The centre of grid map was set at 21.517(x), 43.428(y) and -1.743(y) coordinates and the grid map was employed at dimension of 60 × 60 × 60 with a grid spacing of 0.375 Å. The dockings were conducted with the Lamarckian genetic

algorithm (LGA) at 100 individual docking runs for ligand conformational search. The output was ranked by increasing order of the free binding energy.

The best docked ligand models were chosen based on their ability to bind with the lowest binding energy that involved the largest conformational clusters in each binding (Othman et al., 2017). The lower the binding energy by means of the more negative value resulted in a better binding energy and better binding affinity of the ligands towards the targeted receptor (Nguyen et al., 2021). The binding interactions between the ligands and the NS2B/NS3 protease complex from the docking experiments were analyzed using Discovery Studio 3.5 (www.accelrys.com).

CHAPTER 4: RESULTS AND DISCUSSION

In this chapter, the results and discussion are divided into three parts (Part A, B and C) as follows and the summary of the research pathway is illustrated in Figure 4.1.

Part A (sub-chapter 4.1): Phytochemistry study on the essential oils and the ethyl acetate (EA) extract of *Zingiber zerumbet*.

Part B (sub-chapter 4.2): Anti-protease activity of *Z. zerumbet* extracts, fractions and the isolated compounds followed by the evaluation of acute toxicity and anti-pyretic study of the EA extract.

Part C (sub-chapter 4.3): Molecular docking study on the isolated compounds.

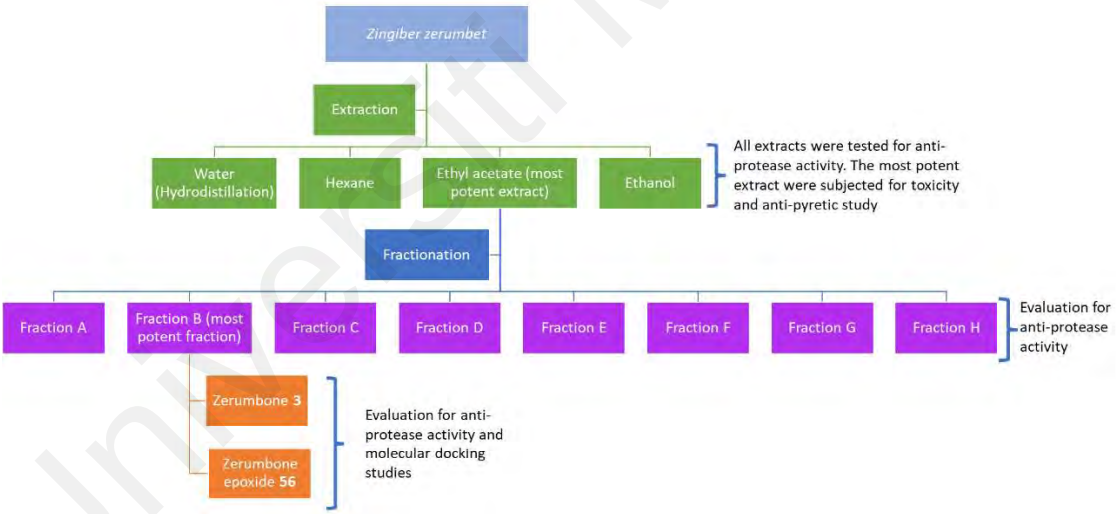


Figure 4.1 : Flow chart of the research study on *Z. zerumbet*.

4.1 Phytochemistry study of the rhizomes of *Zingiber zerumbet* (PART A)

The rhizomes of *Z. zerumbet* were extracted with two extraction methods which are hydrodistillation and organic solvent extraction techniques. The hydrodistillation produced the essential oil of *Z. zerumbet* whereas the solvent extraction yielded three

extracts according to the type of solvents used (hexane, ethyl acetate, and ethanol). The chemical constituents from the essential oils and the most potent extract are discussed in the following sub-chapters.

4.1.1 Essential oil of *Zingiber zerumbet*

The essential oil of the rhizomes of *Z. zerumbet* was obtained by hydrodistillation extraction technique. The essential oil obtained was a mixture of pale-yellow oil and crystal with ginger-like odour (Figure 4.2). Colourless crystals were clearly observed above the liquid portion of the oil during hydrodistillation in the Clevenger-type apparatus.

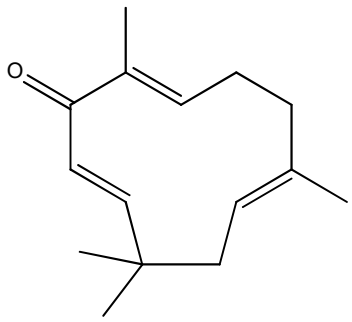
The analysis of the essential oil was performed using gas chromatography–flame ionization detector (GC-FID) and gas chromatography–mass spectroscopy (GC-MS) equipped with HP-5 fused silica capillary column. The parameter used is programmed as described in chapter 3. The essential oil's constituents were identified by comparison of the Kovat retention index (KI) and the mass spectra with the NIST14 library and the reported study by other researchers (Adams, 2001; Dai et al., 2013; Padalia et al., 2018; Rana et al., 2016; Thi Huong et al., 2020; Tian et al., 2020; Wu et al., 2017).

Figure 4.2 : The essential oils from the rhizomes of *Z. zerumbet*.

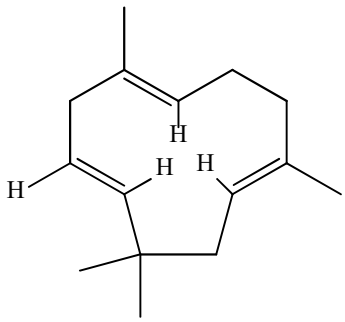
A total of twenty-three compounds were identified from the essential oil of *Z. zerumbet*, comprising 96.94% of the total oil (Table 4.1 and Figure 4.4). The essential oil was characterized by the presence of high concentration of oxygenated sesquiterpenes

which accounted for more than 70% of the oil (composed of 13 compounds) followed by 24.52% of sesquiterpene hydrocarbons (nine compounds) and 0.18% monoterpene hydrocarbon (one compound). Zerumbone (**3**) (sesquiterpene) was identified as the most abundant compound accounted for 43.8% of the essential oil's total composition which is in corroboration with the findings of other reseachers (Malek et al., 2005; Padalia et al., 2018; Rana et al., 2016; Sulaiman et al., 2010; Thi Huong et al., 2020; Tian et al., 2020; Wu et al., 2017). Moreover, the other major compounds were (*Z*)-γ-bisabolene (**125**) (12.1%), humulene epoxide II (**35**) (8.0%), elemol (**10**) (7.5%), (*Z*)-nerolidol (**11**) (5.5%) and α-bergamotene (**126**) (5.1%). The chemical constituents identified in the essential oil of *Z. zerumbet* were listed in Table 4.1 and the GC-FID chromatogram of the essential oil of *Z. zerumbet* rhizomes were presented in Figure 4.4. Chemical structure of the 23 constituents were illustrated in Figure 4.5.

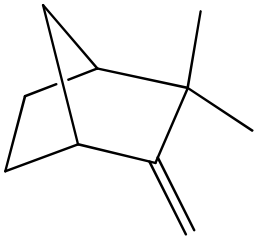
According to previous study, the main constituents of *Z. zerumbet* essential oil from Penang, Malaysia were zerumbone (**3**) (68.9%), α-humulene (**20**) (10.1%), linalool (**33**) (3.6%), 1,8-cineole (**127**) (3.2%) and camphene (**23**) (2.8%) (Figure 4.3). Meanwhile the essential oil obtained from the sample that purchased from Chow Kit's wet market, Kuala Lumpur, Malaysia contained zerumbone (**3**) (36.1%), camphene (**23**) (14.3%), humulene (**13**) (10.0%), borneol (**30**) (4.8%) and camphor (**31**) (4.2%) (Baby et al., 2009; Sulaiman et al., 2010). Moreover, the sample collected from Sabah, Malaysia possessed zerumbone (**3**) (73.1%), α-humulene (**20**) (5.9%), camphene (**23**) (2.8%), caryophyllene oxide (**12**) (2.7%) and camphor (**31**) (1.3%) (Malek et al., 2005).



Zerumbone (3)

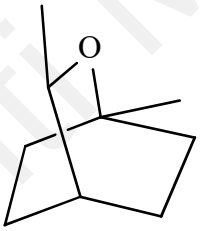


α-humulene (**20**)



Camphene (**23**)

Linalool (**33**)



1,8-cineole (**127**)

Figure 4.3 : Molecular structures of zerumbone (3), α-humulene (20), camphene (23), linalool (33), and 1,8-cineole (127).

Results from our study, revealed that the proportion of these constituents were inconsistent with the findings of the previous studies possibly because some of the compounds were lost during sample preparation, including the drying process (Tian et al., 2020). Other than that, the identification of zerumbone (**3**) and chemical constituents was found to differ depending on several factors as described in Chapter 2.1.5. These factors include agroclimatic zone, geographical location, environmental conditions, ecological factors, plant age, comminution techniques such as grating, chopping, slicing,

using a mixture grinder and using a pestle mortar, as well as the methods used for sample preparation involving drying and extraction techniques.

Table 4.1 : List of chemical constituents in essential oil of *Z. zerumbet* rhizomes

No.	Compounds	Retention time, min	KI[a]	KI[b]	Content (%)[j]
1	Camphor (**31**)	17.68	1141	1146 [c,d,e,g,h,i]	0.18
2	*β*-Caryophyllene (**29**)	34.38	1412	1408 [c,d,g,h,i]	0.19
3	*α*-Bergamotene (**126**)	36.51	1448	1435 [c,e,g]	5.10
4	*α*-Humulene (**20**)	36.85	1454	1455 [c,e,i]	1.39
5	Dehydro-Aromadendrene (**129**)	37.60	1465	1463 [c,f]	0.26
6	Germacrene D (**130**)	37.88	1471	1485 [c,g]	0.48
7	*β*-Selinene (**132**)	38.86	1488	1490 [c,f,h]	0.25
8	(*Z*)-*γ*-Bisabolene (**125**)	40.56	1517	1515 [c,f]	12.14
9	*β*-Sesquiphellandrene (**131**)	40.80	1521	1523 [c,f]	1.07
10	(*Z*)-Nerolidol (**11**)	41.31	1530	1533 [c,f,i]	5.53
11	*β*-Elemenone (**134**)	41.55	1534	1533 [c,d]	0.33
12	*α*-Cadinene (**133**)	41.93	1541	1539 [c,e]	3.64
13	Elemol (**10**)	42.58	1553	1550 [c,f]	7.52
14	Hedycaryol (**135**)	42.85	1557	1560 [c,e]	0.23
15	(*E*)-Nerolidol (**138**)	43.28	1565	1563 [c,f,g]	0.40
16	Spathulenol (**128**)	43.89	1576	1578 [c,d]	0.58
17	*β*-copaen-4*α*-ol (**136**)	44.94	1594	1591 [c,d]	2.20
18	Caryophyllene oxide (**12**)	45.46	1603	1583 [c,e,h]	1.38
19	Humulene epoxide I (**34**)	46.37	1620	1614 [c,h]	0.55
20	Humulene epoxide II (**35**)	47.45	1640	1608 [c,h]	8.02
21	Humulene epoxide III (**36**)	48.15	1653	1651 [c,h]	0.89
22	*β*-Eudesmol (**137**)	49.05	1670	1663 [c,e,h]	0.81
23	Zerumbone (**3**)	52.54	1747	1734 [c,d,e,f,g,h,i]	43.80
				Oxygenated monoterpenes	0.18 %
				Oxygenated sesquiterpenes	72.24 %
				Sesquiterpene hydrocarbons	24.52 %
				Total	**96.94 %**

[a] Kovat indices relative to *n*-alkanes C_7-C_{40} on non-polar HP-5 capillary column
[b] Kovat indices by comparisons with literature reviews ; [c]: (Adams, 2001), [d]: (Rana et al., 2016), [e]: (Tian, 2020), [f]: (Dai et al., 2012), [g]: (Wu et al., 2017), [h]: (Huong et al., 2020), [i]: (Padalia et al., 2018)
[j] Percentage calculated by GC-FID on non-polar HP-5 capillary column

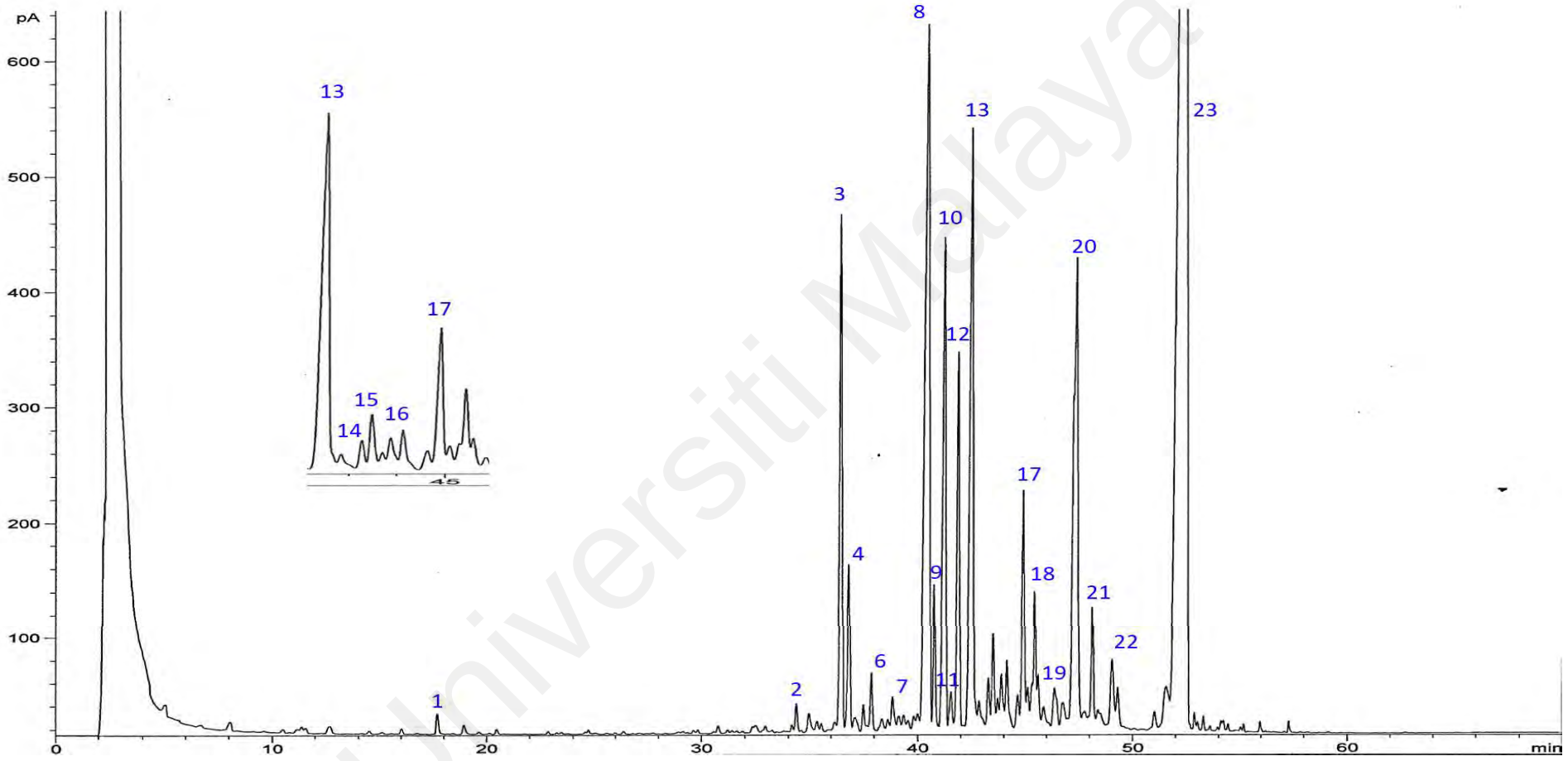


Figure 4.4 : GC-FID chromatogram of essential oil *Z. zerumbet* rhizomes.

Based on our finding, three groups of constituents which were oxygenated monoterpenes, oxygenated sesquiterpenes and sesquiterpenes hydrocarbons were present in the essential oils of *Z. zerumbet*'s rhizome. Zerumbone (**3**) (43.8%), an oxygenated sesquiterpenes, was the most abundance compounds in the essential oils of *Z. zerumbet* followed by (*Z*)-*γ*-bisabolene (**125**) (12.1%) which is sesquiterpene hydrocarbon (Table 4.1).

Generally, essential oils consist of a mixture of hydrocarbons (monoterpene and sesquiterpene hydrocarbons) and their oxygenated derivatives (oxygenated monoterpenes and oxygenated sesquiterpenes) arising from two different isoprenoid pathways (Diass et al., 2021; J. Sharifi-Rad et al., 2017). The monoterpene ($C_{10}H_{16}$) molecules formed by isoprene compounds which could be either linear or cyclic molecules and contribute to organoleptic properties in plants. These molecules have high potential in treating or preventing a variety of diseases due to their anti-microbial, anti-allergic, anti-inflammatory, immunomodulatory, and anti-oxidant properties (de Cássia da Silveira e Sá et al., 2013; Diass et al., 2021; Rocha Caldas et al., 2015; Santos & Rao, 1998). The sesquiterpene ($C_{15}H_{24}$) compounds formed from 3 isoprene units (C_5H_8) commonly presented in cyclic or ring structure. The research and development of drug discovery have led to the discovery of a numerous sesquiterpenes with promising anti-inflammatory, anti-oxidant, anti-parasitic and anti-carcinogenic properties (Bartikova et al., 2014).

(*Z*)-*γ*-bisabolene (**125**) ($C_{15}H_{24}$) is one of the sesquiterpene hydrocarbon that acquired several biological activities including the larvicidal activity (Govindarajan et al., 2018). (*Z*)-*γ*-bisabolene (**125**) was tested for the larvicidal and oviposition deterrent action on 6 mosquito species (*An. stephensi*, *Ae. aegypti*, *Cx. quinquefasciatus*, *An. subpictus*, *Ae. albopictus* and *Cx. tritaeniorhynchus*). The larvicidal activity of (*Z*)-*γ*-bisabolene (**125**)

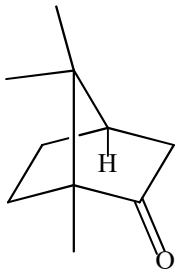
showed the highest acute toxicity on *An. stephensi* (LC_{50} = 2.04 μg/mL) and lowest acute toxicity on *Cx. tritaeniorhynchus* (LC_{50} = 4.87 μg/mL) larvae (Govindarajan et al., 2018). In addition, oviposition deterrent properties of (*Z*)-γ-bisabolene (**125**) were investigated in six mosquito vectors and demonstrating that 25 μg/mL of (*Z*)-γ-bisabolene (**125**) affected to an Oviposition Activity Index lower of - 0.79 in all tested mosquito vectors. Therefore, the study suggested that (*Z*)-γ-bisabolene (**125**) can be used as a highly effective larvicidal and oviposition deterrent against mosquitoes.

Zerumbone (**3**) ($C_{15}H_{24}O$) is oxygenated sesquiterpenes commonly present in essential oil of Zingiberaceae plant families. According to previous research studies, zerumbone (**3**) in the essential oil of *Z. zerumbet* possesses an anti-allergic and immune modulation properties by suppressing Th2-related cytokines (IL-4, IL-5, IL-10, and IL-13) secretion and subsequently decreasing the production of IgE by B cells on allergic asthmatic mice model (Shieh et al., 2015; Tan et al., 2018). Other studies have demonstrated that inhaling *Z. zerumbet* essential oils and zerumbone (**3**) increases body weight by decreasing sympathetic nerve activity and also increases food intake (Batubara et al., 2013). Zerumbone (**3**) also determined to exhibit anti-HIV cytoprotective activity with an EC_{50} value of 0.04 μg/mL, as well as direct cytotoxicity to the host cells at IC_{50} 0.14 μg/mL (Dai et al., 1997).

Other oxygenated sesquiterpenes such as caryophyllene oxide (**12**) and spathulenol (**128**) were known to exhibit various pharmacological activities such as anti-oxidant, anti-bacterial and anti-inflammatory activity (K. Nascimento et al., 2017; Schmidt et al., 2010; Silva et al., 2018; Wan Salleh et al., 2015). For example, caryophyllene oxide (**12**) demonstrates potent anti-cancer activity which exhibited dose-dependent anti-proliferative properties against prostate and human breast cancer (Park et al., 2011) as well as against human osteosarcoma MG-63 cancer cells (Gyrdymova & Rubtsova, 2021;

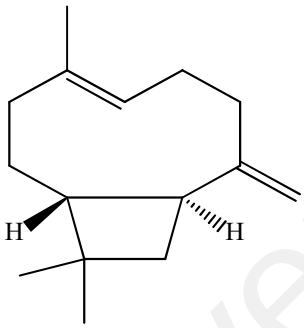
Russo et al., 2018). Therefore, due to the ethnopharmacological used of *Z. zerumbet* and the biological properties of the compounds in the essential oils of *Z. zerumbet*, the essential oil of *Z. zerumbet* was assessed for anti-protease activity against DENV2 NS2B/NS3 protease.

Oxygenated monoterpenes

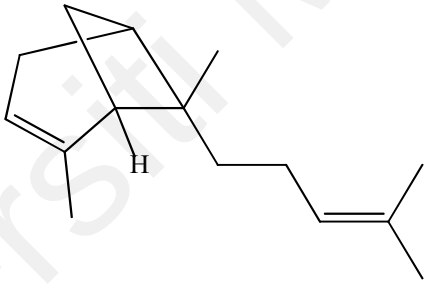


Camphor (**31**)

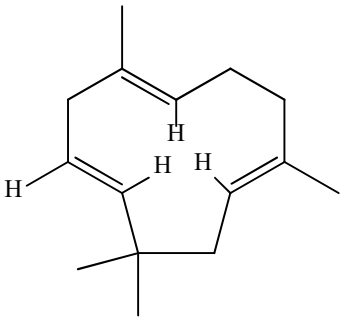
Sesquiterpene hydrocarbons



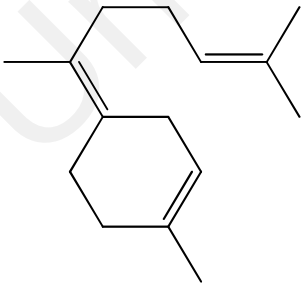
β-Caryophyllene (**29**)



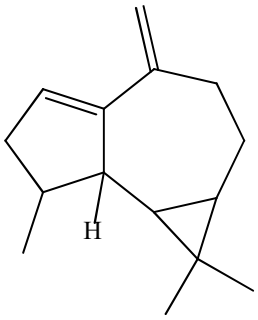
α-Bergamotene (**126**)



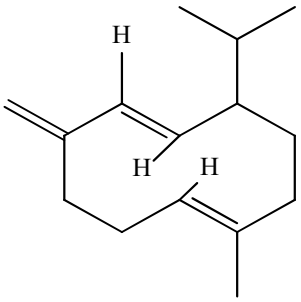
α-Humulene (**20**)



(*Z*)-*γ*-Bisabolene (**125**)



Dehydro-Aromadendrene (**129**)

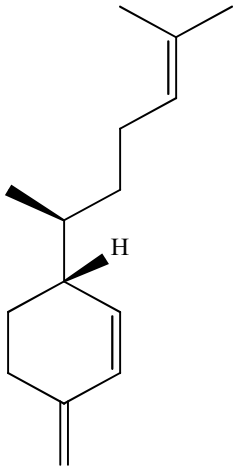


Germacrene D (**130**)

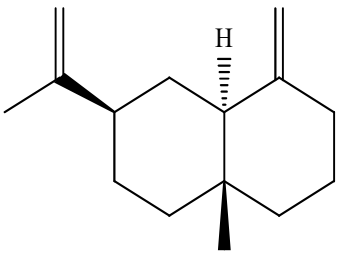
Figure 4.5 : The chemical structures of constituents in the rhizome essential oils of *Z. zerumbet*.

‘Figure 4.5, continued’

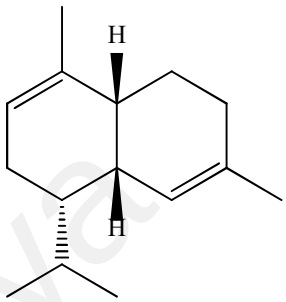
Sesquiterpene hydrocarbons



β-Sesquiphellandrene (**131**)

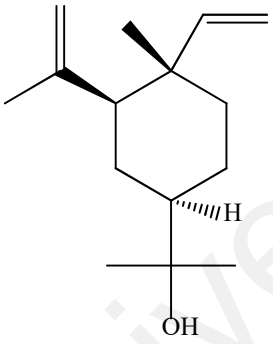


β-Selinene (**132**)

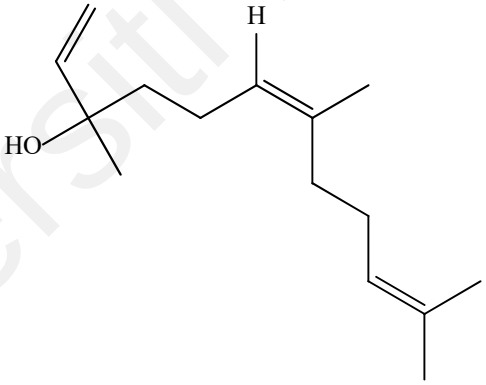


α-Cadinene (**133**)

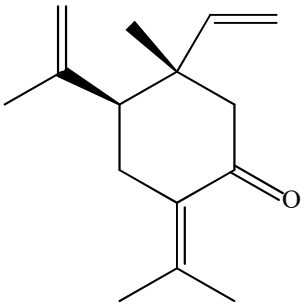
Oxygenated sesquiterpenes



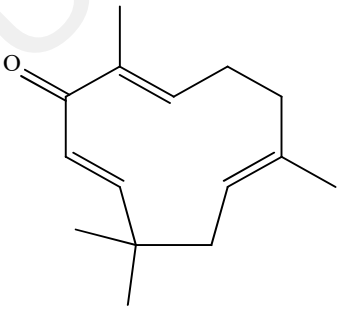
Elemol (**10**)



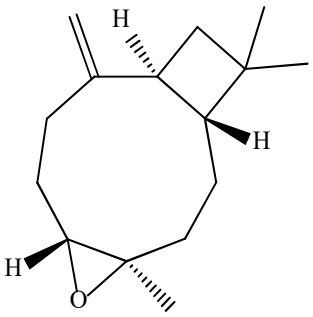
(*Z*)-Nerolidol (**11**)



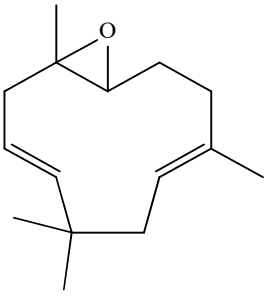
β-Elemenone (**134**)



Zerumbone (**3**)



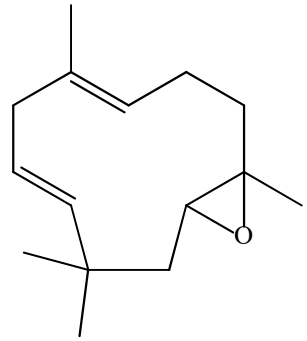
Caryophyllene oxide (**12**)



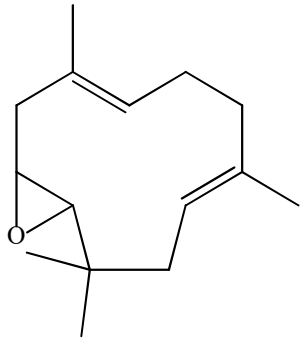
Humulene epoxide I (**34**)

'Figure 4.5, continued'

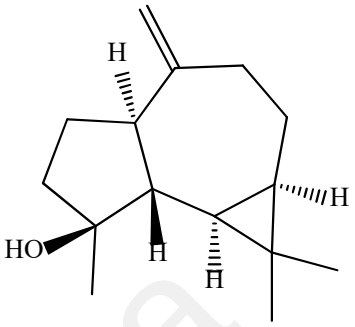
Oxygenated sesquiterpenes



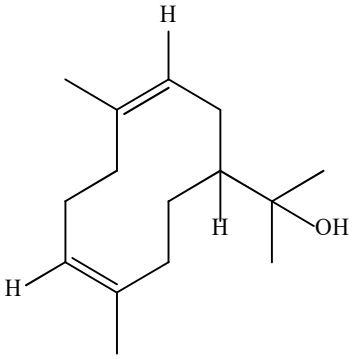
Humulene epoxide II (**35**)



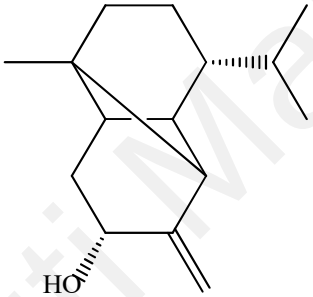
Humulene epoxide III (**36**)



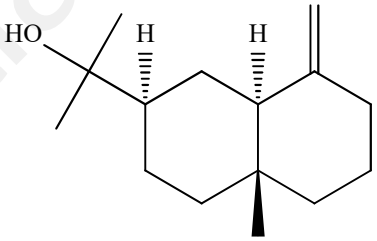
Spathulenol (**128**)



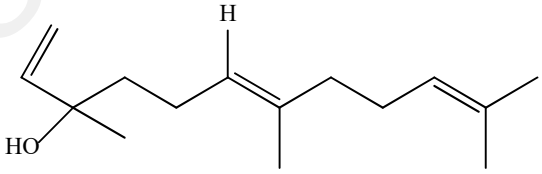
Hedycaryol (**135**)



β-copaen-4*α*-ol (**136**)



β-Eudesmol (**137**)



(*E*)-Nerolidol (**138**)

4.1.2 Bioassay guided and phytochemical studies of EA

Based on bioassay-guided study of anti-protease activity against NS2B/NS3 on *Z. zerumbet* (refer to Chapter 4.2.1, Table 4.4), EA extract was identified as the most potent extract and fraction B (FB) of the EA extract was the most potent fraction. Hence, FB was further purified using preparative thin layer chromatography. Two compounds which

are zerumbone (**3**) and zerumbone epoxide (**56**) were successfully isolated and the structures were elucidated with various spectroscopic methods such as nuclear magnetic resonance, liquid chromatography mass spectrometry, ultra-violet and infra-red spectrometry. The following sub-chapter provided a description on the structural elucidation of both compounds.

4.1.2.1 Zerumbone (3)

Zerumbone (**3**) (0.0834 mg) (Figure 4.6) was isolated as a white needle crystal. It has a molecular formula of $C_{15}H_{22}O$ (calculated 218.1671) as deduced from the LCMS which displayed a pseudomolecular ion peak at *m/z* $[M+H]^+$ 219.2364 (Figure 4.7- Figure 4.8). The UV spectrum of zerumbone (**3**) showed strong absorption at 248 nm. The infrared spectra showed a strong absorption peak at 1652 cm^{-1} indicating the presence of *α,β* unsaturated carbonyl groups (Figure 4.10) (Oliveros & Cantoria, 1982). The methylene carbon and hydrogen stretching was detected at 2858, 2921 and 2962 cm^{-1} while the geminal dimethyl group (C-14 and C-15) absorption peaks were observed at 1385 and 1364 cm^{-1}. Besides, absorption peak at 964 cm^{-1} was assigned for out of plane of trans-sym disubstituted ethylenic linkage while absorption peak of 827 cm^{-1} was due to the presence of out of plane of tri-substituted ethylenic linkage (Baby et al., 2009; Oliveros & Cantoria, 1982).

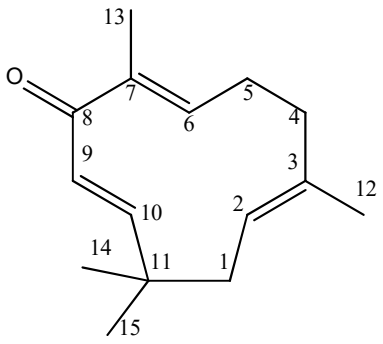


Figure 4.6 : Molecular structure of zerumbone (3).

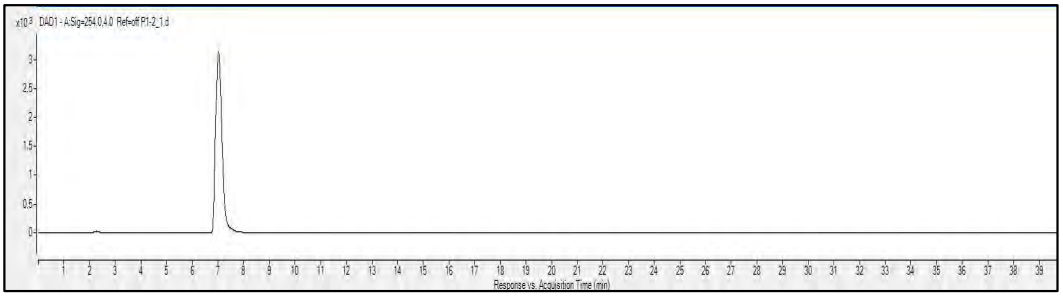


Figure 4.7 : LCMS chromatogram of zerumbone (3).

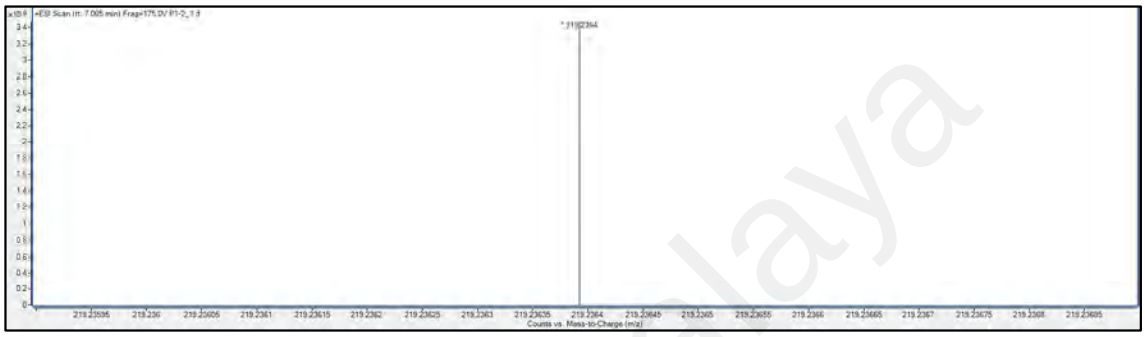


Figure 4.8 : Mass chromatogram peak of zerumbone (3) at 7.005min (254 nm).

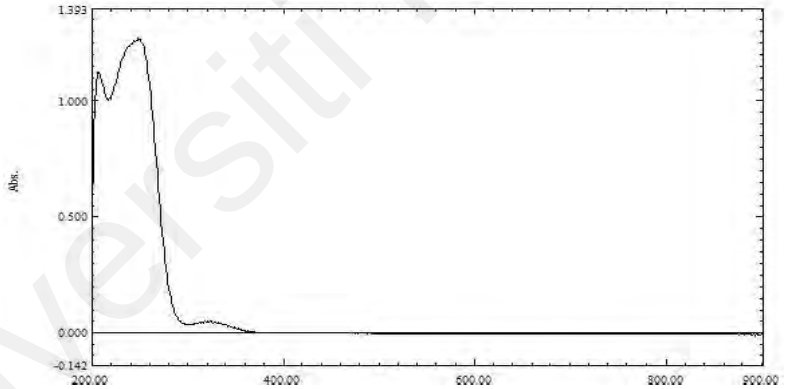


Figure 4.9 : UV spectra of zerumbone (3).

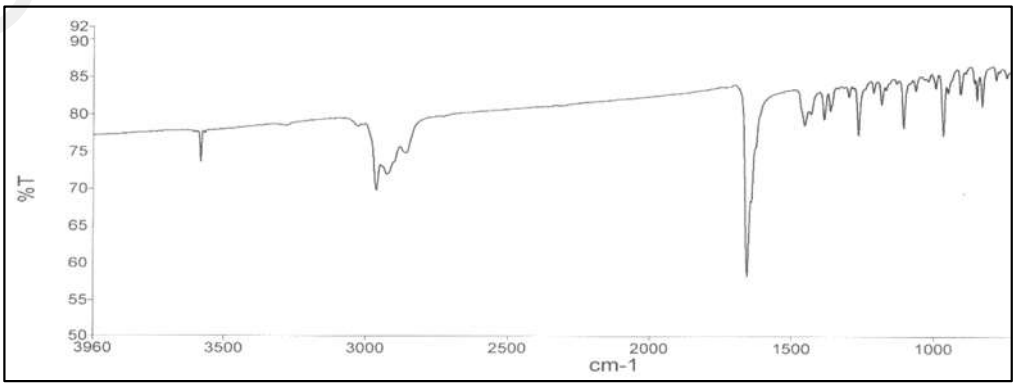


Figure 4.10 : Infrared spectra of zerumbone (3).

Based on ^{1}H NMR (Table 4.2 and Figure 4.12), four methyl singlets of H_3-12, H_3-13, H_3-14 and H_3-15 resonated in the shielded regions at 1.53, 1.79, 1.19, and 1.06 ppm, respectively. Three methylene protons signals were observed at δ_H 1.90 (1H, *d*, *J* = 12.0 Hz, H-1a), 2.34 (1H, *d*, *J* = 12.0 Hz, H-1b), 2.11 (1H, *dd*, *J* = 12.0, 4.0 Hz, H-4a), 2.34 (1H, *d*, *J* = 12.0 Hz, H-4b), 2.23 (1H, *dd*, *J* = 12.0, 4.0, H-5a) and 2.44 (1H, *dd*, *J* = 12.0, 4.0 Hz, H-5b). In addition, four methine protons signals were observed in the deshielded region at δ_H 5.24 (1H, *dd*, *J* = 12.0, 4.0 Hz, H-2), 6.02 (1H, *d*, *J* =12.0 Hz, H-6), 5.99 (1H, *d*, *J* = 16.4 Hz, H-9) and 5.87 (1-H, *d*, *J* = 16.4 Hz, H-10) which correlated with the carbon signals at δ_C 124.9 ppm (C-2), δ_C 148.8 ppm (C-6), δ_C 127.2 ppm (C-9) and δ_C 160.7 ppm (C-10), respectively in the HSQC spectrum (Figure 4.15).

The presence of fifteen carbons were confirmed by the ^{13}C NMR and DEPT-135 spectra (Table 4.2 and Figure 4.13). Four methyl carbons signals of C-12, C-13, C-14, and C-15 were observed at δ_C 15.2, 11.8, 24.2, and 29.4 ppm, respectively. The carbon signals at δ_C 42.4 ppm, 39.4 ppm, and 24.4 ppm were assigned to three methylene carbons C-1, C-4, and C-5, respectively. Furthermore, four methine carbon signals were observed at δ_C 124.9 ppm (C-2), 148.8 ppm (C-6), 127.2 ppm (C-9), and 160.7 ppm (C-10). Additionally, four quaternary carbon signals were observed at δ_C 37.8 ppm (C-11), δ_C 136.2 ppm (C-3), 137.9 ppm (C-7), and 204.4 ppm (C-8) while one carbonyl carbon (C=O) appeared in the downfield region at 204.4 ppm (C-8). From the COSY spectrum (Figure 4.14), a methylene proton, H_2-1 showed a correlation with a methine proton, H-2. The COSY spectrum also demonstrated a correlation of a methine proton, H-6 with a pair of geminal methylene protons, H_2-5. These methylene protons are also coupled with another pair of geminal methylene protons, H_2-4. Furthermore, the correlation between two protons signals of H-9 and H-10 which appeared as doublets can be observed in the COSY spectrum.

The HMBC spectrum demonstrated the correlation of H_3-12 with C-3 and H_3-13 with C-7 indicating the connectivity of the methyl protons with their respective quaternary carbon. Besides, the connectivity of another two methyl protons, H_3-14 and H_3-15 with quaternary carbon, C-11 was shown in the HMBC spectrum. All the COSY and HMBC correlations indicated the presence of the humulane skeleton of the compound (Figure 4.11). The COSY, HSQC, and HMBC spectra were presented in Figure 4.14 – Figure 4.16. With all the aforementioned NMR spectroscopic data analysis and comparison to published data, the structure of zerumbone (**3**) (2,6,9-humulatrien-8-one or 2,6,9,9-tetramethyl-(2*E*,6*E*,10*E*) cycloundeca-2,6,10-trien-1-one) was confirmed (Baby et al., 2009; Riyanto, 2007).

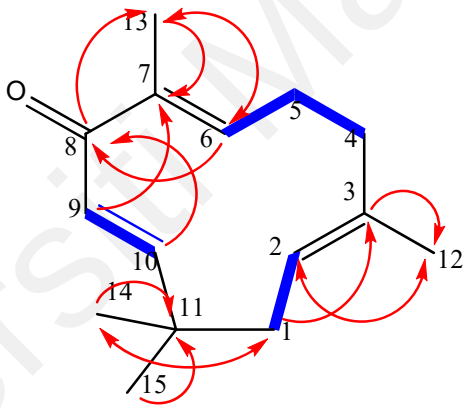


Figure 4.11 : ^{1}H-^{1}H COSY ▬ and selected ^{1}H-^{13}C HMBC ↷ correlations of zerumbone (3).

Table 4.2 : ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of zerumbone (3) (δ in ppm) in $CDCl_3$.

Position	Experimental		Literature	
	Zerumbone		Zerumbone (Riyanto, 2007)	
	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C
1a	1.90 (*d*, *J* = 12.0)	42.4	1.90 (*d*, *J* =13.2)	42.4
1b	2.30-2.37 (*m*)		2.35 (*d*, *J* =13.2)	
2	5.24 (*dd*, *J* =12.0, 4)	124.9	5.25 (*dd*, *J* =16.4, 4)	125.0
3	-	136.2	-	136.3
4a	2.18-2.24 (*m*)	39.4	2.19-2.36 (*m*)	39.5
4b	2.30-2.37 (*m*)			
5a	2.18-2.24 (*m*)	24.4	2.26 (*m*)	24.4
5b	2.44 (*dd*, *J* =12.0, 4)			
6	6.02 (*d*, *J* =12.0)	148.8	6.02 (*t*, *J* =13.2)	148.8
7	-	137.9	-	137.9
8	-	204.4	-	204.4
9	5.99 (*d*, *J* =16.4)	127.2	5.97 (*d*, *J* =16.4)	127.2
10	5.87 (*d*, *J* =16.4)	160.7	5.86 (*d*, *J* =16.4)	160.8
11	-	37.8	-	37.9
12	1.53 (*s*)	15.2	1.54 (*s*)	15.2
13	1.79 (*s*)	11.8	1.80 (*s*)	11.8
14	1.19 (*s*)	24.2	1.20 (*s*)	24.2
15	1.06 (*s*)	29.4	1.07 (*s*)	29.4

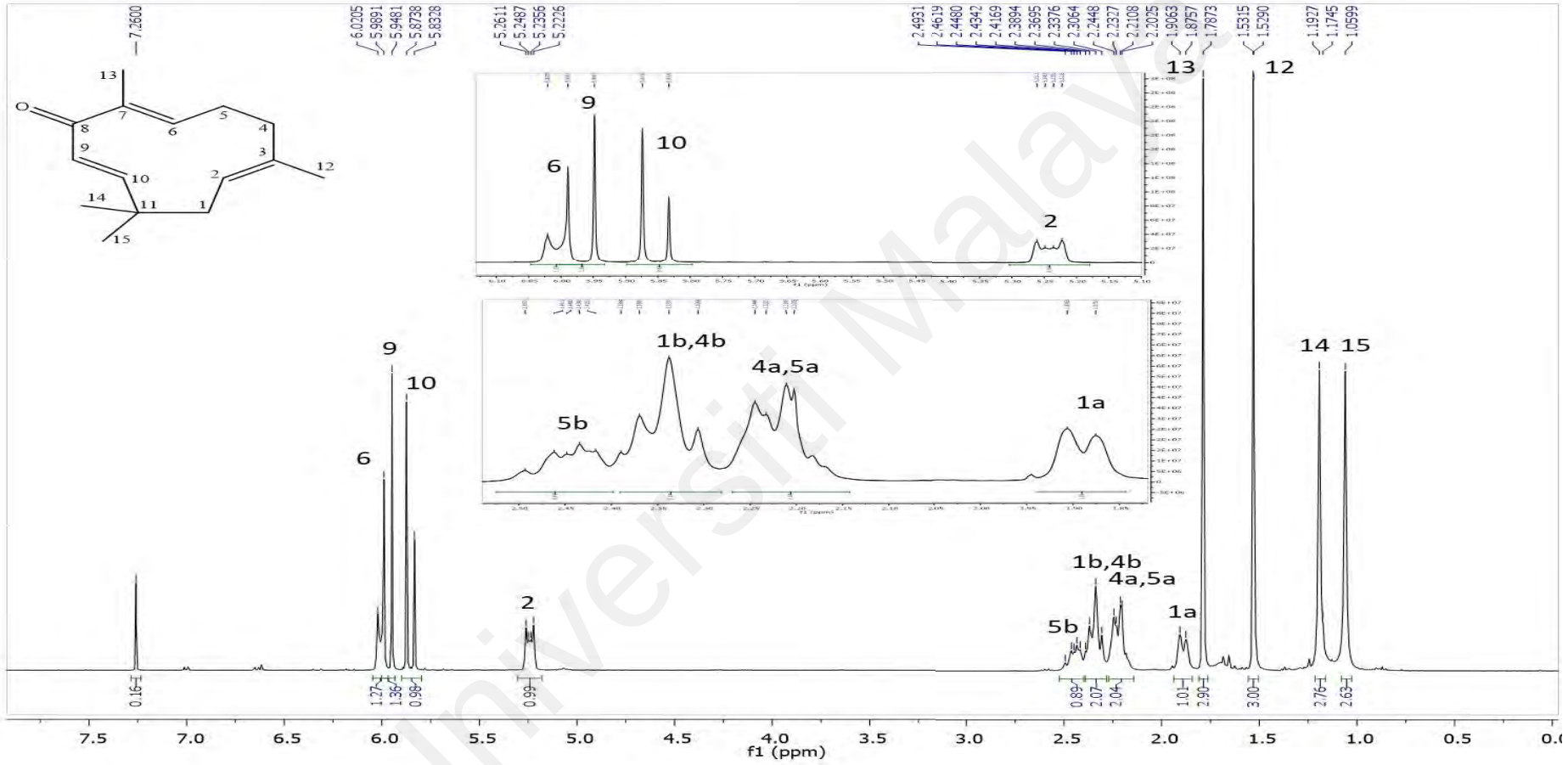


Figure 4.12 : ^{1}H NMR spectrum of zerumbone (3).

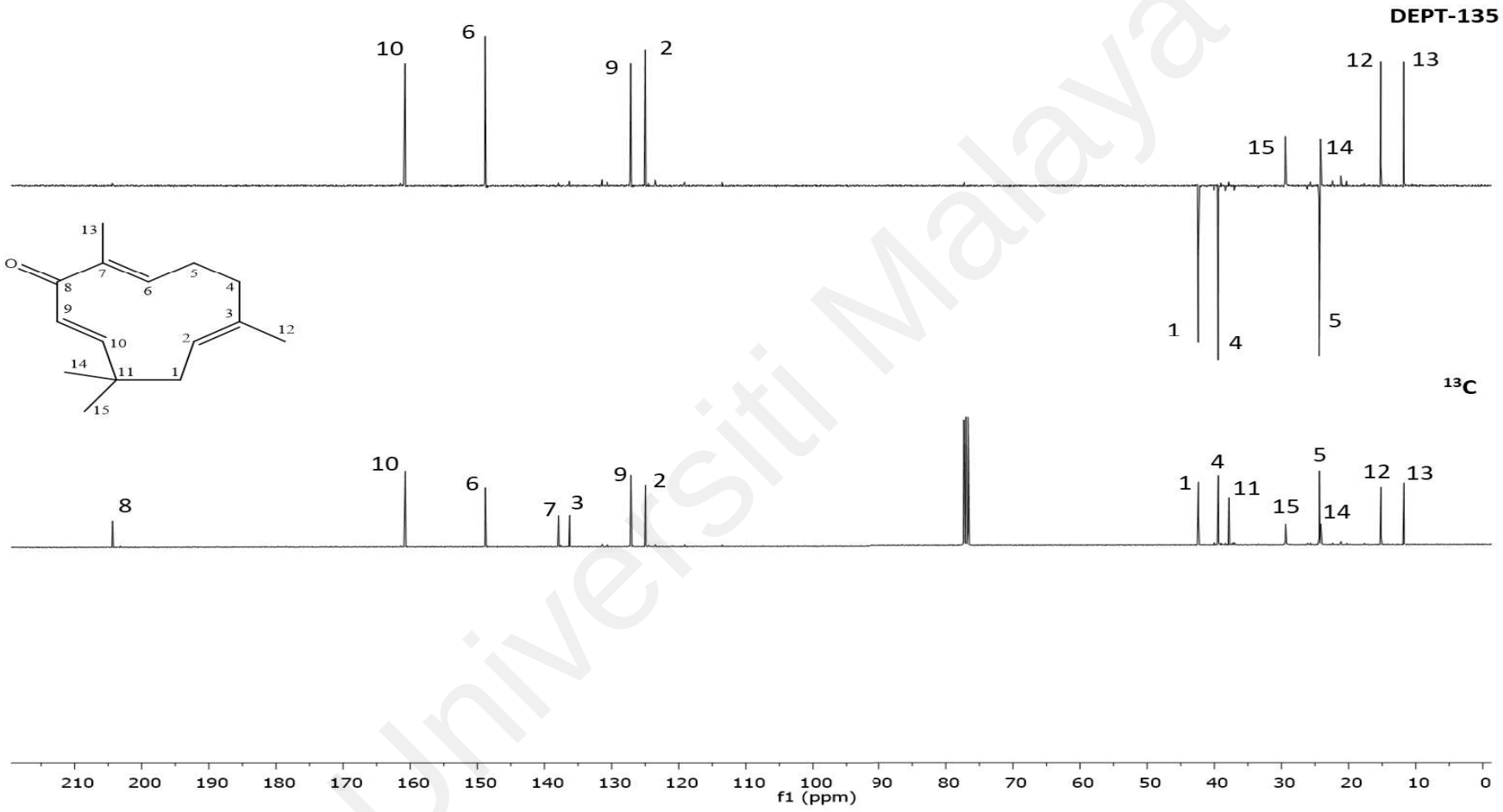


Figure 4.13 : ^{13}C and DEPT-135 NMR spectra of zerumbone (3).

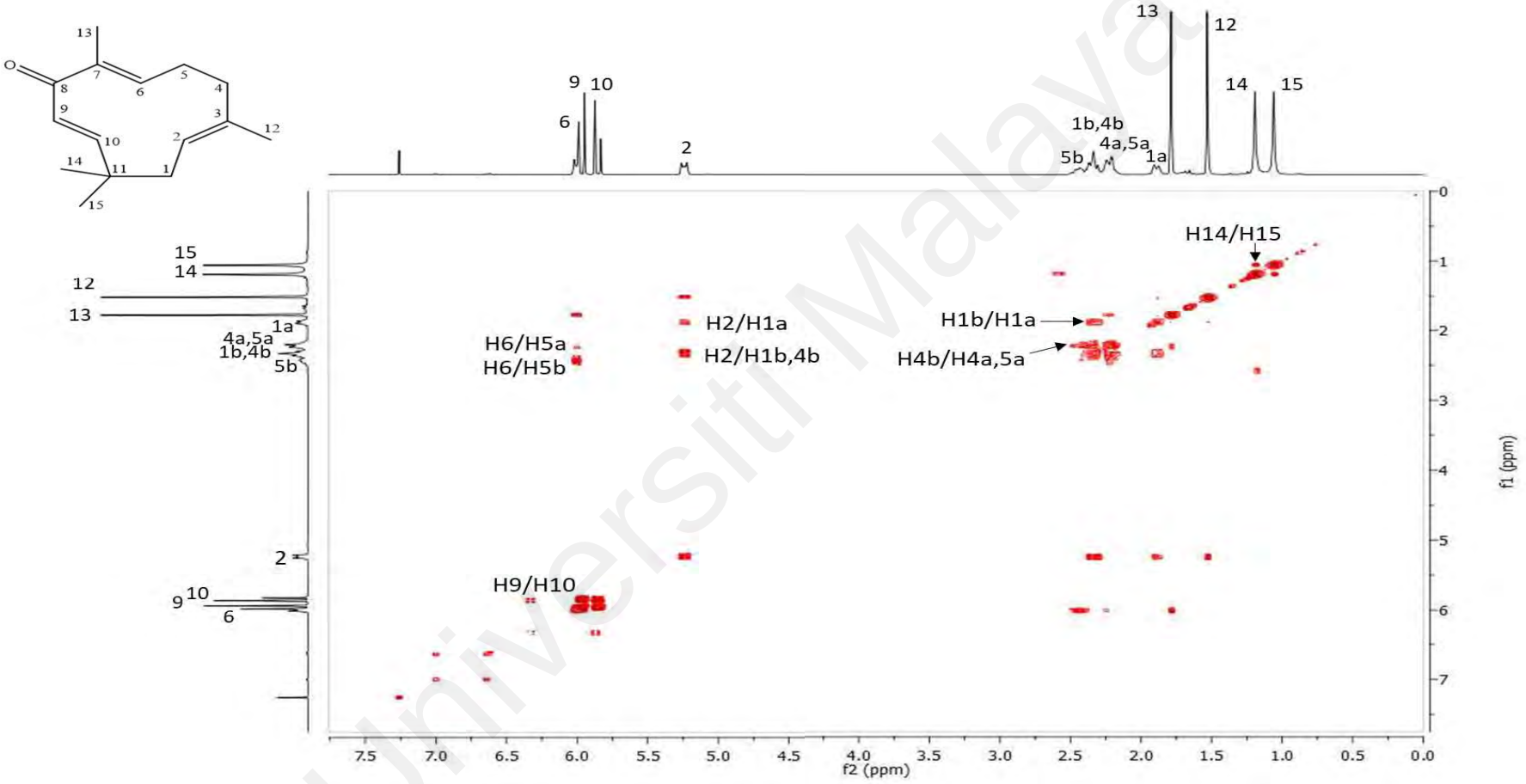


Figure 4.14 : COSY NMR spectrum of zerumbone (3).

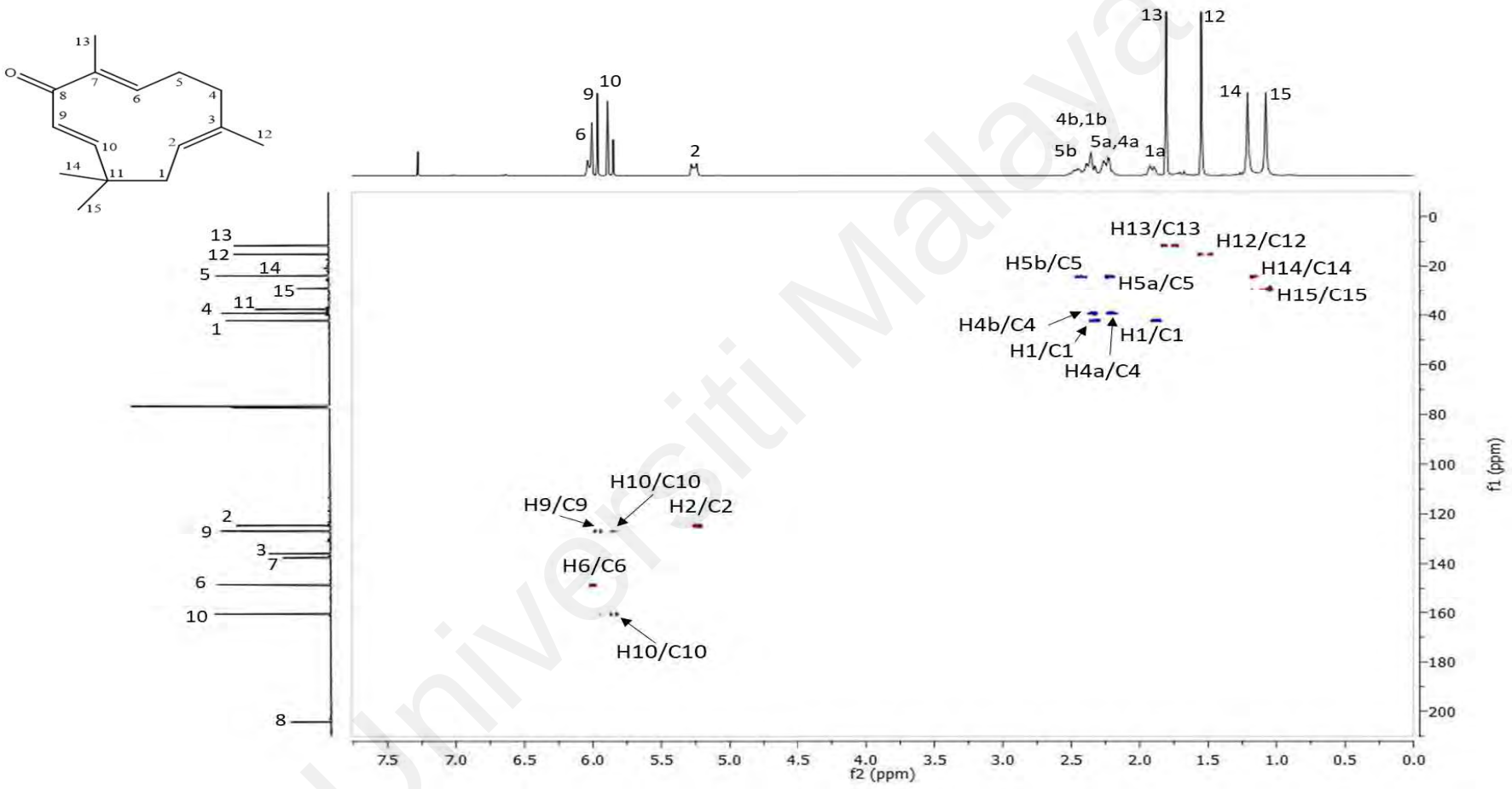


Figure 4.15 : HSQC NMR spectrum of zerumbone (3).

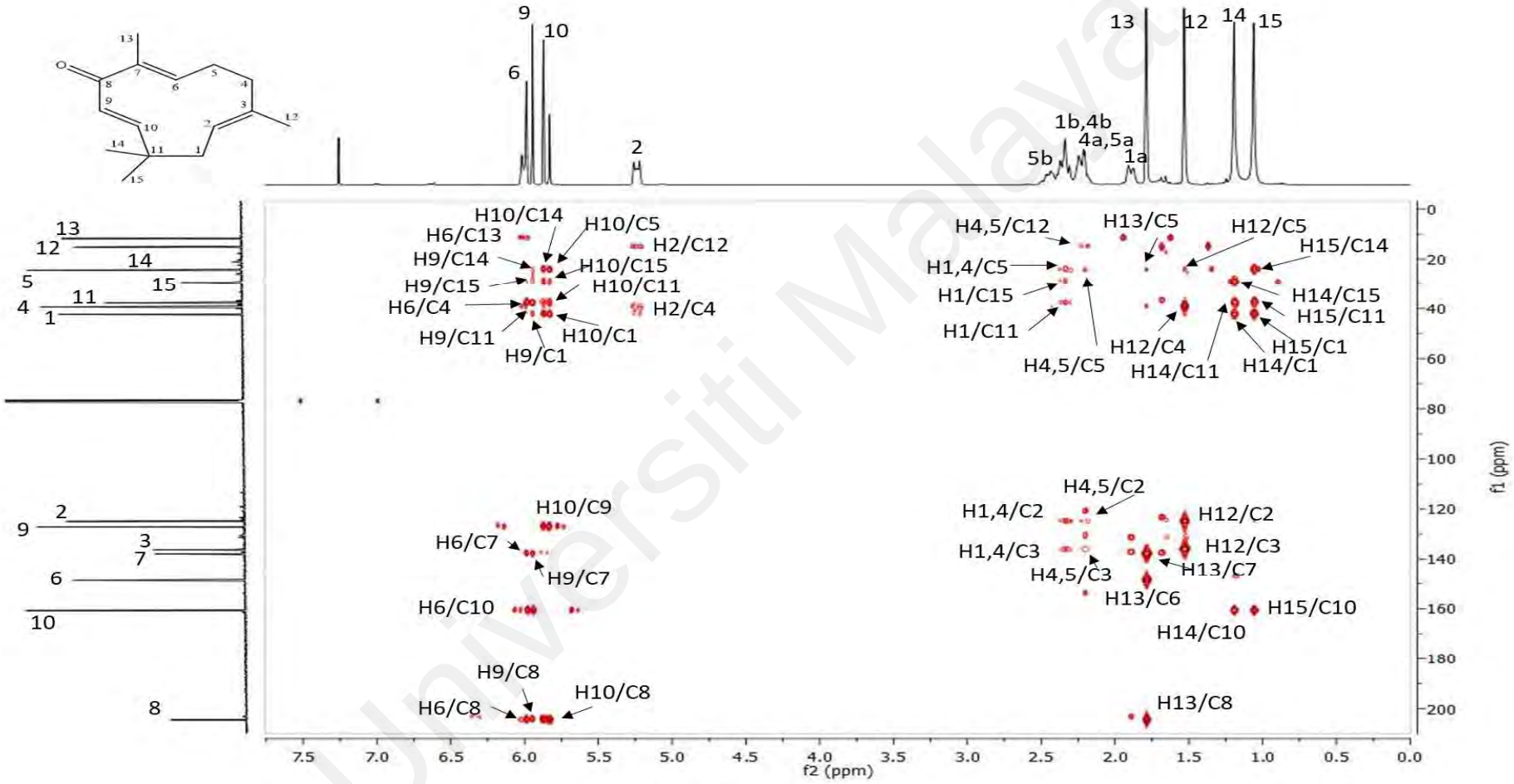


Figure 4.16 : HMBC NMR spectrum of zerumbone (3).

4.1.2.2 Zerumbone epoxide (56)

Zerumbone epoxide (**56**) (0.0018 g) (Figure 4.17) was isolated as pale-yellow compound. The LCMS analysis revealed a pseudomolecular ion peak $[M+H]^+$ at *m/z* 235.2315 which corresponds to the molecular formula $C_{15}H_{22}O_2$ (calculated 234.1620) (Figure 4.18 - Figure 4.19). The optical rotation value of zerumbone epoxide (**56**) is $[\alpha\ ]_D^{23}$ +3.33 (*c* 0.06°, $CHCl_3$). The UV spectrum of zerumbone epoxide (**56**) showed strong absorption at 236 nm. The infra-red spectrum of zerumbone epoxide (**56**) (Figure 4.21) showed a strong absorption of carbonyl stretching at 1658 cm^{-1} which indicates the *α*,*β*-unsaturated ketone (Takashi et al., 2001). A methylene group, C-H stretching was observed at 2854, 2959 and 2925 cm^{-1} (Figure 4.21). Besides, an absorption peak was appeared at 3583 cm^{-1} in the IR spectrum indicating the presence of an epoxide group of zerumbone epoxide (**56**).

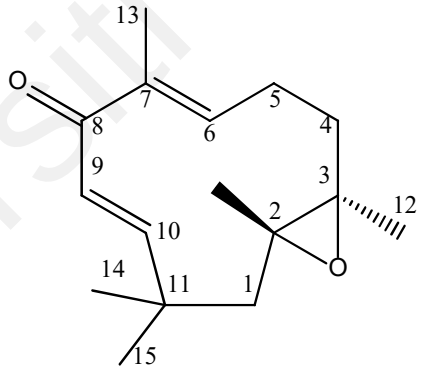


Figure 4.17 : Molecular structure of zerumbone epoxide (56).

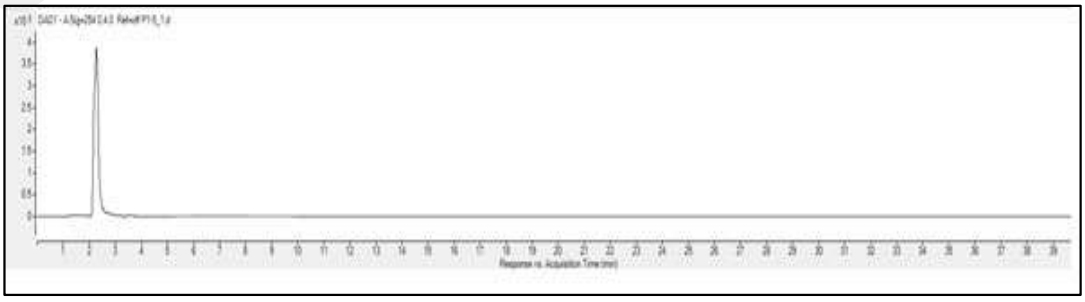


Figure 4.18 : LCMS chromatogram of zerumbone epoxide (56).

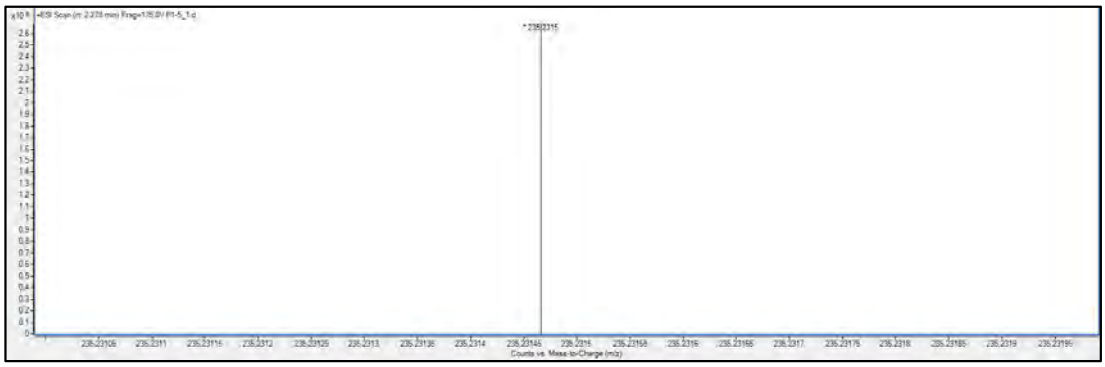


Figure 4.19 : Mass chromatogram of zerumbone epoxide (56) at 2.278 min (254 nm).

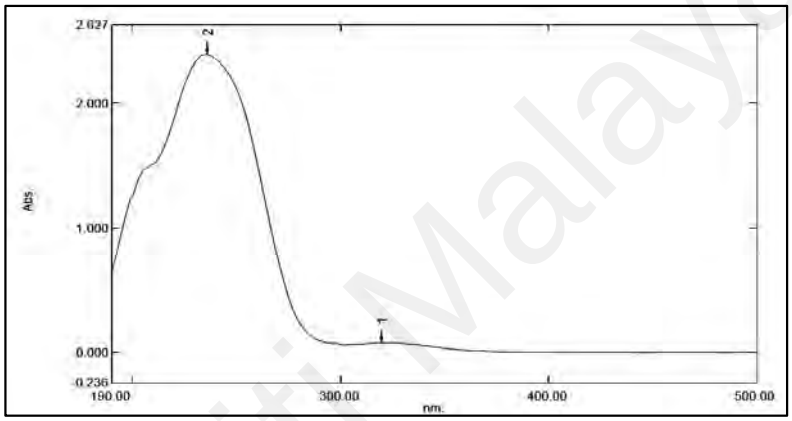


Figure 4.20 : UV spectra of zerumbone epoxide (56).

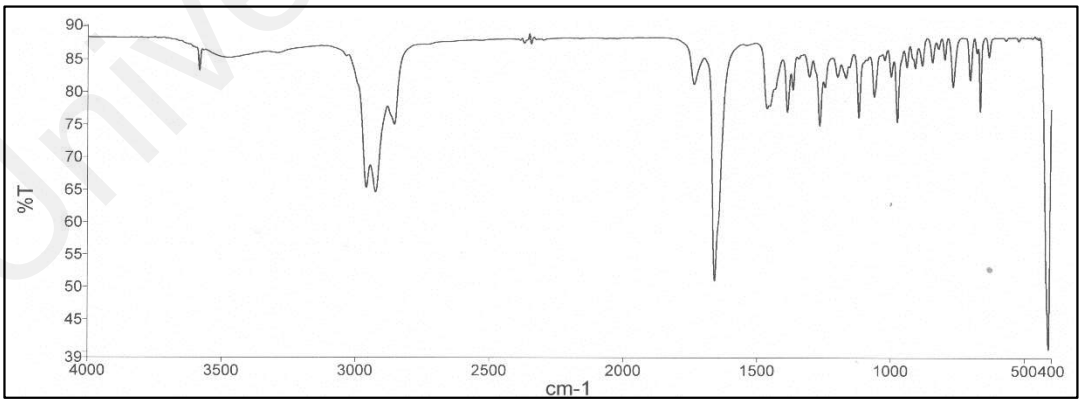


Figure 4.21 : Infrared spectra of zerumbone epoxide (56).

The ^{1}H NMR (Table 4.3 and Figure 4.23) and ^{13}C NMR (Table 4.3 and Figure 4.24) spectra of this compound showed a very similar pattern with that of zerumbone (**3**) which exhibited four distinct methyl singlet proton signals at upfield region δ_H 1.22 (H_3-12), δ_H

1.85 (H_3-13), δ_H 1.29 (H_3-14) and δ_H 1.08 (H_3-15), three methine protons overlapped signals at downfield region, δ_H 6.11 (H-6, H-9, and H-10) and three methylene signals at δ_H 1.45 (H-1a), δ_H 1.93 (H-1b), δ_H 1.34 (H-4a), δ_H 2.29 (H-4b) and δ_H 2.42 (H-5). The only differences observed were the proton of H-2 resonated at δ_H 2.74 (H-2) as well as the carbons of C-2 and C-3 at δ_C 63.0 and δ_C 61.6, respectively. In addition, three methine protons (H-6, H-9, and H-10) overlapping signals were observed in the deshielded region at δ_H 6.11which correlated with the carbon signals at δ_C 147.9 ppm (C-6), δ_C 128.4 ppm (C-9) and δ_C 159.6 ppm (C-10), respectively in the HSQC spectrum (Figure 4.26).

The ^{13}C and DEPT-135 spectra of zerumbone epoxide (**56**) (Table 4.3 and Figure 4.24) revealed a total of 15 carbons corresponding to four sp^3 methyls, three sp^3 methylenes, one sp^3 epoxide, three sp^2 methines, three quaternary carbons, and one carbonyl carbon. The prominent carbon signals of epoxide were observed at δ_C 63.0 (C-2) and δ_C 61.6 (C-3), while the one carbonyl carbon signal, C=O (C-8) appeared at δ_C 203.1.

The ^{1}H-^{1}H COSY correlation of zerumbone epoxide (**56**) (Figure 4.25) was observed for adjacent proton between H-1 and H-2, H-4 and H-5 as well as H-5 and H-6. Two chiral centre were observed in the molecular structure of zerumbone epoxide which is at position carbon 2 and carbon 3. The proton at carbon 2 showed correlation with proton (H-1a) at carbon 1 with a coupling constant 11.2 Hz indicated that it was in cis position. The relative configuration of zerumbone epoxide (**56**) was analyzed by optical rotation and the results were confirmed based on comparison of relative configuration data with previous report (Y. P. Tan et al., 2020). In the HMBC spectrum, the correlations of H-6, H-9, H-10, and H-13 to C-8 were important as it substantiated the presence of *α,β*-unsaturated carbonyl system (Figure 4.27). Correlations of methyl protons, H-12 with epoxy carbon, C-3, and H-13 with quarternary carbon, C-7 were also demonstrated in the HMBC spectrum. Besides, both H-14 and H-15 showed a similar correlation with C-11.

Additionally, long-range coupling of H-15 with C-2 was also revealed in the HMBC spectrum. Therefore, all the COSY and HMBC correlations (Figure 4.25 and Figure 4.27) supported the presence of humulene skeleton in zerumbone epoxide (**56**) (Figure 4.22). From the analysis of the NMR spectral data observed for zerumbone epoxide (**56**) associated with the literature data values, the identity of zerumbone epoxide (**56**) **(**(6E,9E)-zerumbone-2,3-epoxide) was established (Ajish et al., 2014; Matthes et al., 1980; Omer Abdalla, 2015; Sadhu et al., 2008; Y. P. Tan et al., 2020).

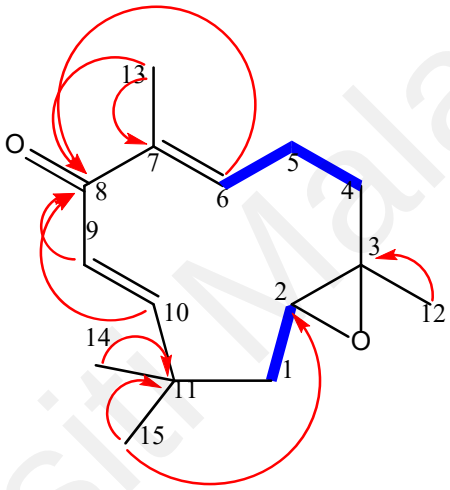


Figure 4.22 : ^{1}H-^{1}H COSY ——— and selected ^{1}H-^{13}C HMBC ⌒ correlation of zerumbone epoxide (56).

Table 4.3 : ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of zerumbone epoxide (56) (δ in ppm) in $CDCl_3$.

Position	Experimental		Literature	
	Zerumbone epoxide		Zerumbone epoxide (Sadhu et al., 2008)	
	δ_H (multiplicity, *J* in Hz)	δ_C	δ_H (multiplicity, *J* in Hz)	δ_C
1a	1.45 (*dd*, *J* = 11.2, 14.0)	42.8	1.39 (*dd*, *J* = 11.0, 14.0)	42.6
1b	1.93 (*d*, *J* =14.0)		1.89 (*d*, *J* = 14.0)	
2	2.74 (*dd*, *J* =11.2, 1.5)	63.0	2.68 (*dd*, *J* = 11.0, 1.5)	62.8
3	-	61.6	-	61.3
4a	1.34 (*m*);		1.27 (*td*, *J* = 13.0, 6.4)	
4b	2.29 (*ddd*, *J* = 13.6, 4.0, 2.8)	38.4	2.22 (*ddd*, *J* = 13.0, 4.0 , 2.5)	38.1
5	2.42 (*m*)	24.8	2.36 (*m*)	24.6
6	6.11 (*m*)	147.9	6.05 (*m*)	147.7
7	-	139.6	-	139.4
8	-	203.1	-	202.9
9	6.11 (*m*)	128.4	6.05 (*m*)	128.2
10	6.11 (*m*)	159.6	6.05 (*m*)	159.4
11	-	36.1	-	35.9
12	1.22 (*s*)	15.8	1.16 (*s*)	15.6
13	1.85 (*s*)	12.2	1.79 (*s*)	12.0
14	1.29 (*s*)	24.2	1.23 (*s*)	23.9
15	1.08 (*s*)	29.9	1.02 (*s*)	29.7

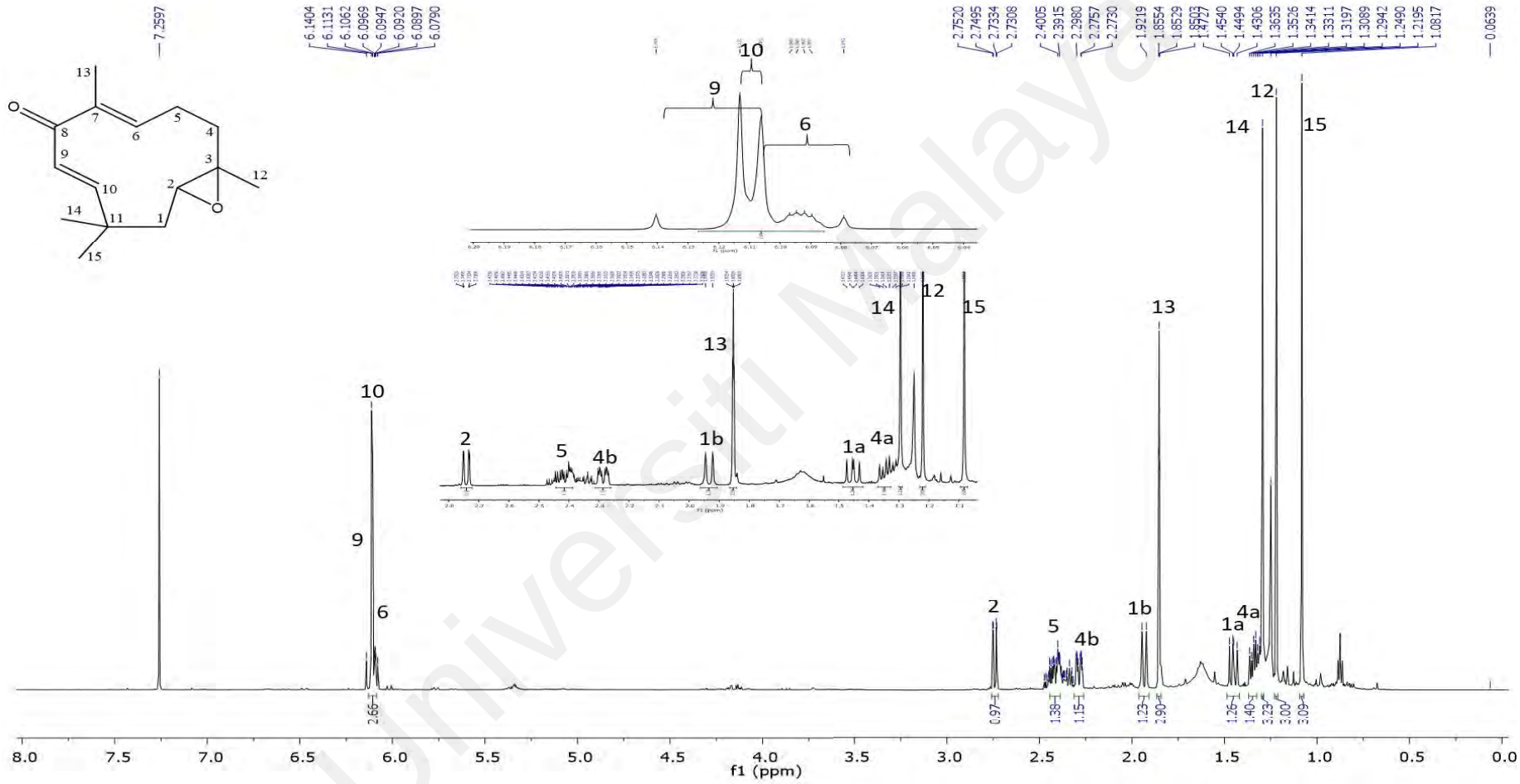


Figure 4.23 : ^{1}H NMR of zerumbone epoxide (56).

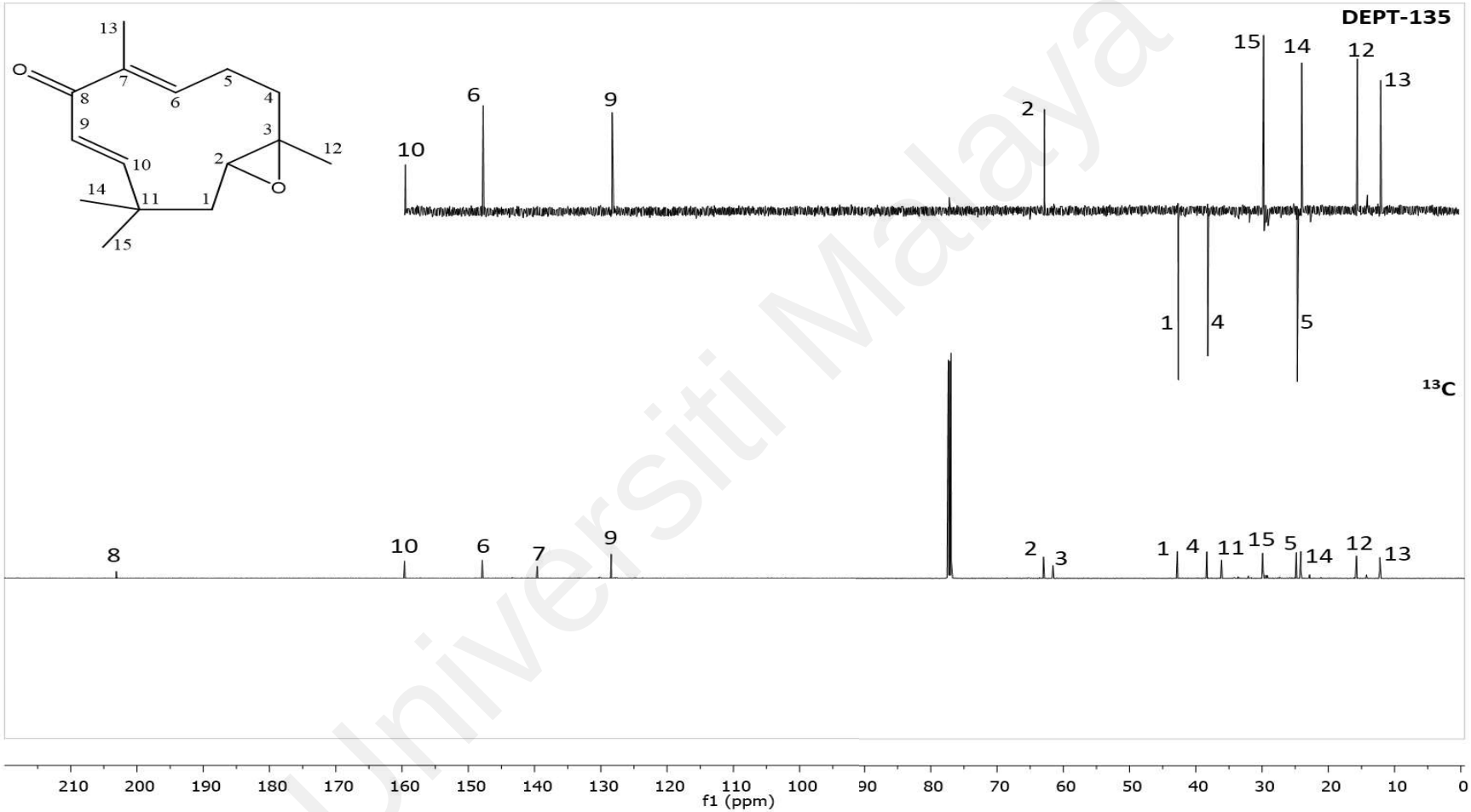


Figure 4.24 : ^{13}C and DEPT NMR spectra of zerumbone epoxide (56).

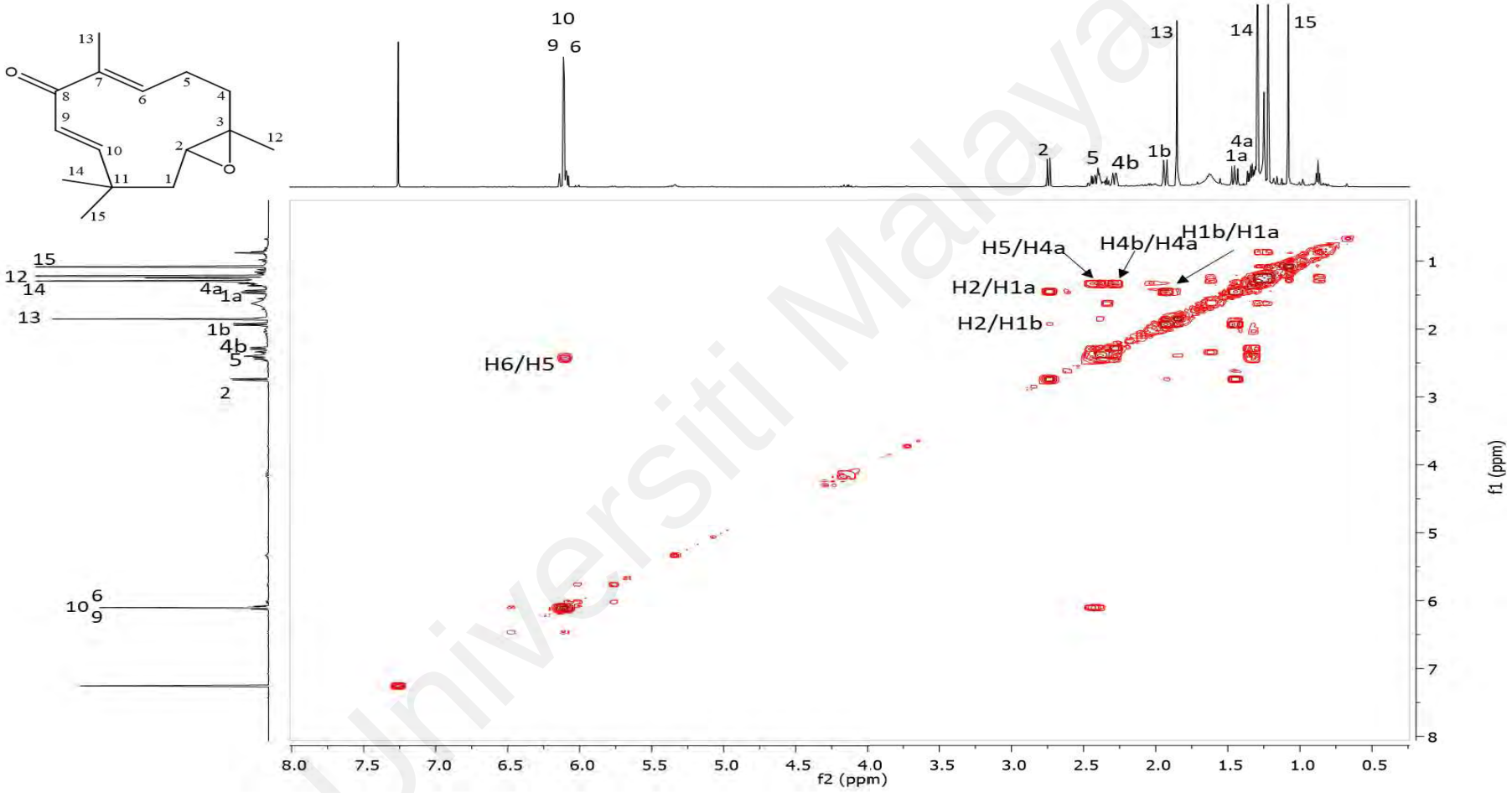


Figure 4.25 : COSY NMR spectrum of zerumbone epoxide (56).

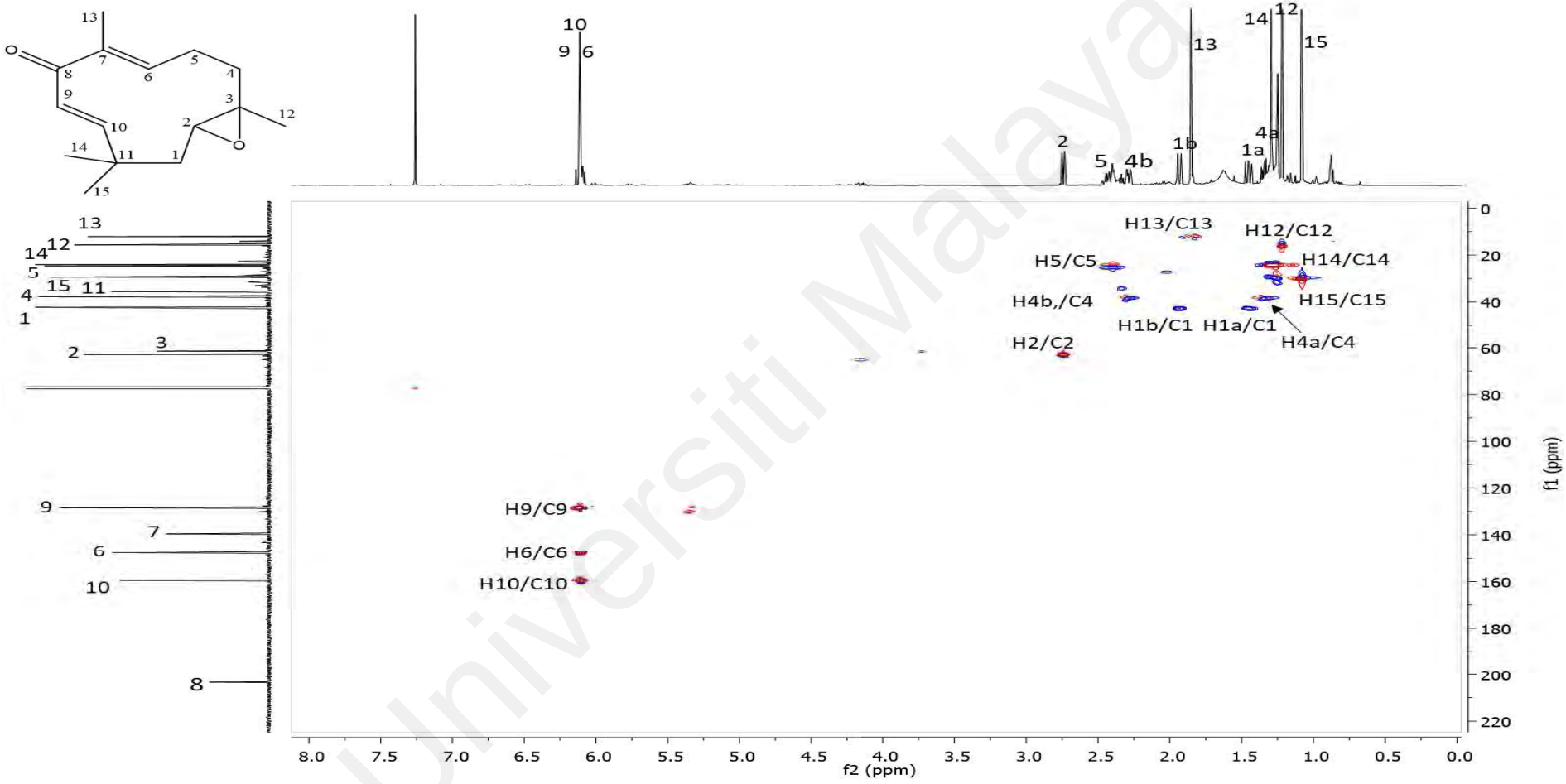


Figure 4.26 : HSQC NMR spectrum of zerumbone epoxide (56).

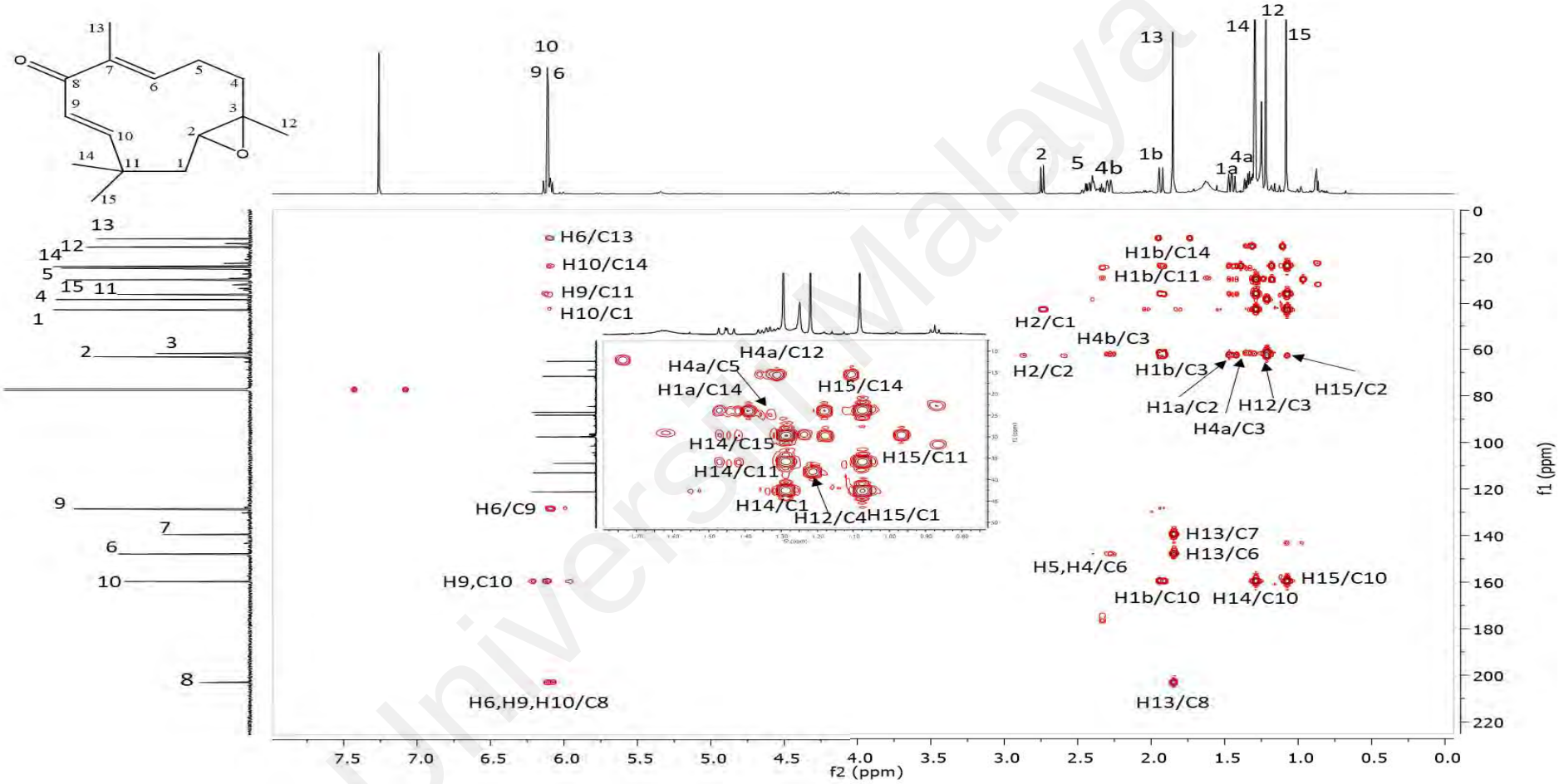


Figure 4.27 : HMBC NMR spectrum zerumbone epoxide (56).

4.2 Biological activity (PART B)

4.2.1 DENV2 NS2B/NS3 protease activity

All extracts were screened for the anti-protease activity against NS2B/NS3 protease. The extracts which exhibited more than 80% of inhibition at 200 µg/mL were subjected to IC_{50} studies. Quercetin (**70**) was used as a standard in this study. The percentages of inhibition of all the extracts against NS2B/NS3 protease were shown in Table 4.4. Ethyl acetate extract showed 90% of inhibition at 200 µg/mL. It exhibited the highest inhibition against NS2B/NS3 protease with IC_{50} value of 2.39 µg/mL. The hexane extract showed moderate anti-protease activity with 63% of inhibition at 200 µg/mL and ethanol extract only possessed 32% of inhibition at 200 µg/mL. The study conducted by Tan et al. (2006) demonstrated that the methanol fractions of *Z. zerumbet* exhibited the strongest inhibitory of DENV2 virus protease activity (89.0 ± 1.7%, 300 ppm) compared to methanol crude extracts (69.3 ± 2.4%, 300 ppm) and hexane fraction (46.6 ± 3.1%, 300 ppm) (Tan et al., 2006). Other Zingiberaceae plants (*Boesenbergia rotunda* (L.)) also showed inhibition against NS2B/NS3 protease in which 4-hydroxypanduratin A (**68**) and panduratin A (**67**) showed K_i values of 21 and 25 µM, respectively corresponded to non-competitive inhibitors (Kiat et al., 2006).

All eight fractions from the ethyl acetate extract of *Z. zerumbet* were tested for anti-protease activity against NS2B/NS3 protease. Among the eight fractions, fraction B presented the most potent anti-protease activity with IC_{50} value of 0.09 ± 0.62 µg/mL. Fractions A and C also exhibited good inhibition towards NS2B/NS3 protease with IC_{50} values of 9.04 and 2.23 µg/mL, respectively. Since fraction B was the most potent fraction, isolation and purification of the potential compounds were conducted on this fraction.

Table 4.4 : The percentage inhibition of the *Zingiber zerumbet* extracts, fractions, and compounds against NS2B/NS3 serine protease

Extracts	Fractions	Compounds	Percentage inhibition at 200 µg/mL (%)	IC_{50} ± Standard deviation (µg/mL)
Hexane			63.25	NT
Ethyl acetate			90.32	2.39 ± 0.86
Ethanol			32.29	NT
Essential oils			49.21	NT
	Fraction A		57.05	9.04 ± 2.36
	Fraction B		73.55	0.09 ± 0.62
	Fraction C		85.08	2.23 ± 0.41
	Fraction D		82.06	16.83 ± 0.73
	Fraction E		83.32	20.97 ± 8.81
	Fraction F		90.25	12.22 ± 4.22
	Fraction G		56.40	23.07 ± 6.79
	Fraction H		25.10	NT
		Zerumbone	54.33	NT
		Zerumbone epoxide	72.04	NT
Standard		Quercetin	90.90	2.86 ± 2.75

*NT = not tested

Zerumbone (**3**) and zerumbone epoxide (**56**) were successfully isolated from fraction B and both compounds have been tested for anti-protease activity against NS2B/NS3 protease. Zerumbone epoxide (**56**) exhibit the most potent anti-protease with 72.04% of inhibition at 200 ppm while zerumbone (**3**) showed moderate activity in inhibiting DENV2 NS2B/NS3 protease with 54.33% of inhibition at 200 ppm. These results revealed that the sesquiterpenes type of compound may possess good inhibitory activity against DENV2 NS2B/NS3 protease. Besides, Zakaria et al. (2019) has reported sesquiterpenes compounds could be attributed to the inhibitory effect on the DENV2 NS2B/NS3 protease (Zakaria et al., 2019).

4.2.2 Acute toxicity and anti-pyretic potential of ethyl acetate extract of *Zingiber zerumbet*

4.2.2.1 Acute oral toxicity

In this study, EA extract was subjected to an acute oral toxicity study using Sprague Dawley rats as the EA extract exhibited the most potent DENV2 NS2B/NS3 inhibitory activity (refer to Chapter 4.2.1). In the HPLC profiling of the EA extract of *Z. zerumbet*, the peak of zerumbone (**3**) appeared at 10.219 min. The HPLC of isolated zerumbone (**3**) was also subjected to HPLC profiling and it appeared at 10.207 min. The presence of zerumbone (**3**) was confirmed by the HPLC profiling of spiked zerumbone (**3**) in EA extract of *Z. zerumbet*, which showed the increased intensity of peak at 10.165 min. The HPLC-DAD chromatogram of EA extract of *Z. zerumbet*, the spiked zerumbone (**3**) and zerumbone (**3**) in EA extract of *Z. zerumbet* were showed in Figure 4.28.

For an acute toxicity study, 16 Sprague Dawley female rats were divided equally into four groups; control group (Group I), 2000 mg/kg (Group II), 3000 mg/kg (Group III), and 5000 mg/kg (Group IV) of EA extract, respectively. All rats were acclimated to light, food, and water, and provided *ad libitum* for 7 days to adapt to the environment before undergoing the experimental procedures. The body weight of the rats was recorded on day one and day sixteen of the experiment.

The acute toxicity results for 2000 mg/kg (Group II), 3000 mg/kg (Group III), and 5000 mg/kg (Group IV) dosages have been observed till day 16. The observation of the animal behaviour such as the physical movement, eye colour, bleeding, and any mortality have been monitored every hour for 4 hours after the administration of 2000 mg/kg (Group II) extract and aspirin (**121**) (Group I). There was no mortality observed and the rats' behaviour was normal. Hence, the dosage has been increased to 3000 mg/kg (Group

III) and 5000 mg/kg (Group IV) for the respective group. The illustration of the experiment was depicted in Figure 4.29.

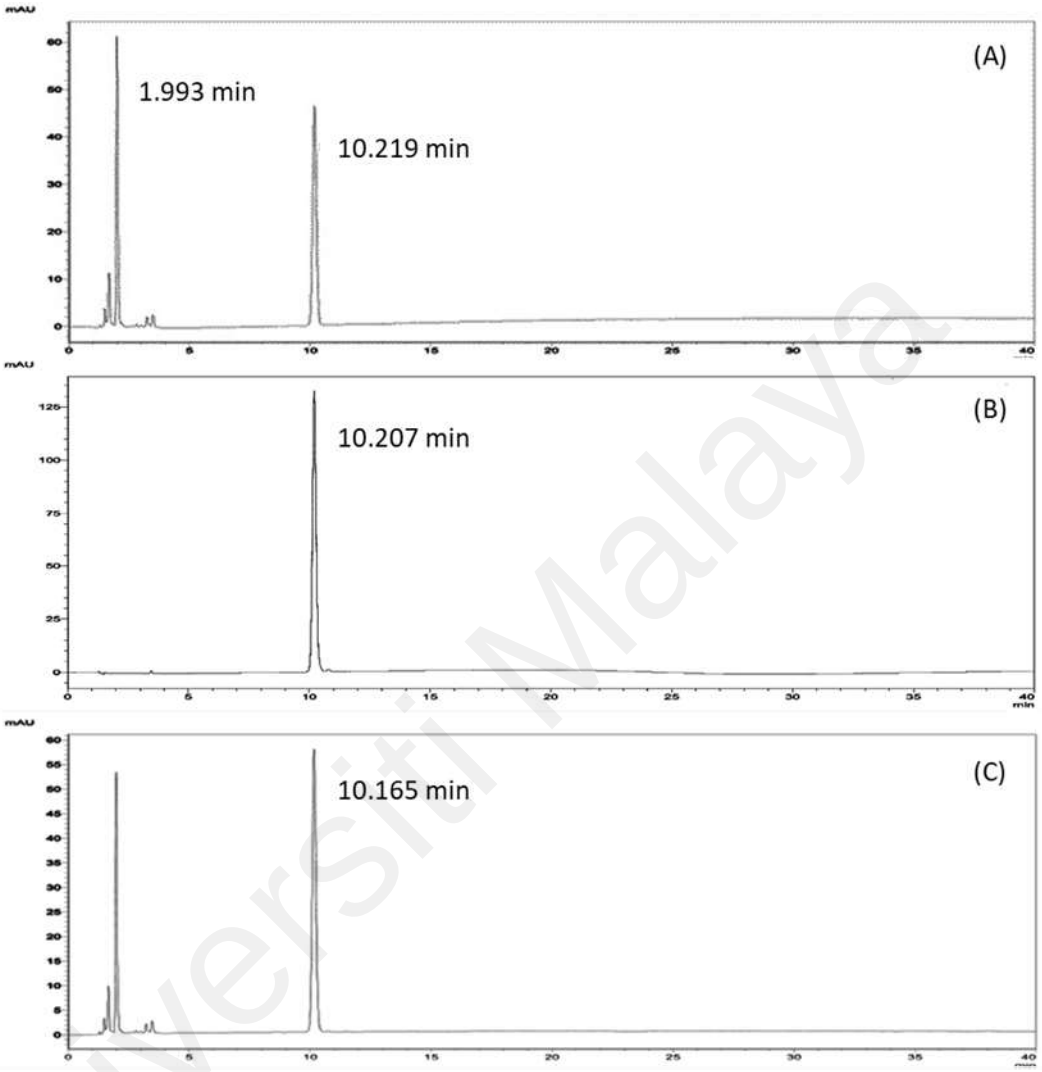


Figure 4.28 : HPLC-DAD chromatogram of (A) ethyl acetate extract of *Z. zerumbet* (B) zerumbone (3) (C) spiked with zerumbone (3) in ethyl acetate extract of *Z. zerumbet*.

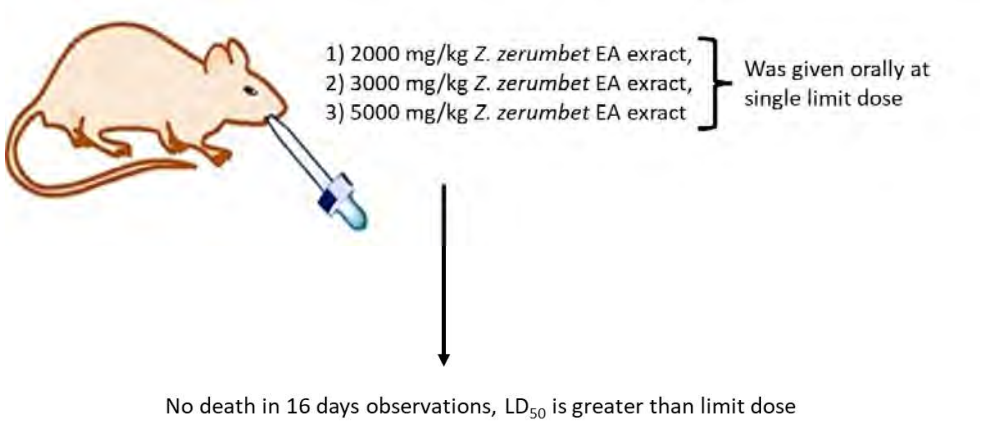


Figure 4.29 : The illustration of the acute toxicity study.

The results showed an increase in body weight from day one to day sixteen for all groups of animals, especially with the dosage of 3000 mg/kg (Group III). The EA extract of *Z. zerumbet* at a limit oral dose of 5000 mg/kg (Group IV) revealed normal behaviour and exhibited no mortality or lethargy or any clinical signs of toxicity in the experimental rats up to 16 days of acute oral toxicity study. The LD_{50} of the ethyl acetate extract of *Z. zerumbet* was greater than 5000 mg/kg, orally. A similar absence of toxic effect was observed in the previously reported case of ethanol extract from *Z. zerumbet* doses of 1000, 2000, or 3000 mg/kg (Chang et al., 2012b). Since the major compound in EA extract of *Z. zerumbet* is zerumbone (**3**), a single injected dose of zerumbone (**3**) at 100 - 200 mg/kg was also reported not to show any ill effect on female Sprague Dawley rats' kidneys and liver tissues (Ibrahim et al., 2010). Therefore, the study suggested that the EA extract of *Z. zerumbet* is safe and non-toxic. Thus, for evaluating the anti-pyretic effect of EA extract of *Z. zerumbet* in rats, the maximum safe starting dose of 500 mg/kg body weight was chosen based on acute toxicity highest dose (1/10th of 5000 mg/kg). Hence, doses of 125 and 250 mg/kg body weight were selected as a comparison to the maximum dose, respectively. The graph represents the average changes in body weight of the rats according to dosage group and control group was shown in Figure 4.30.

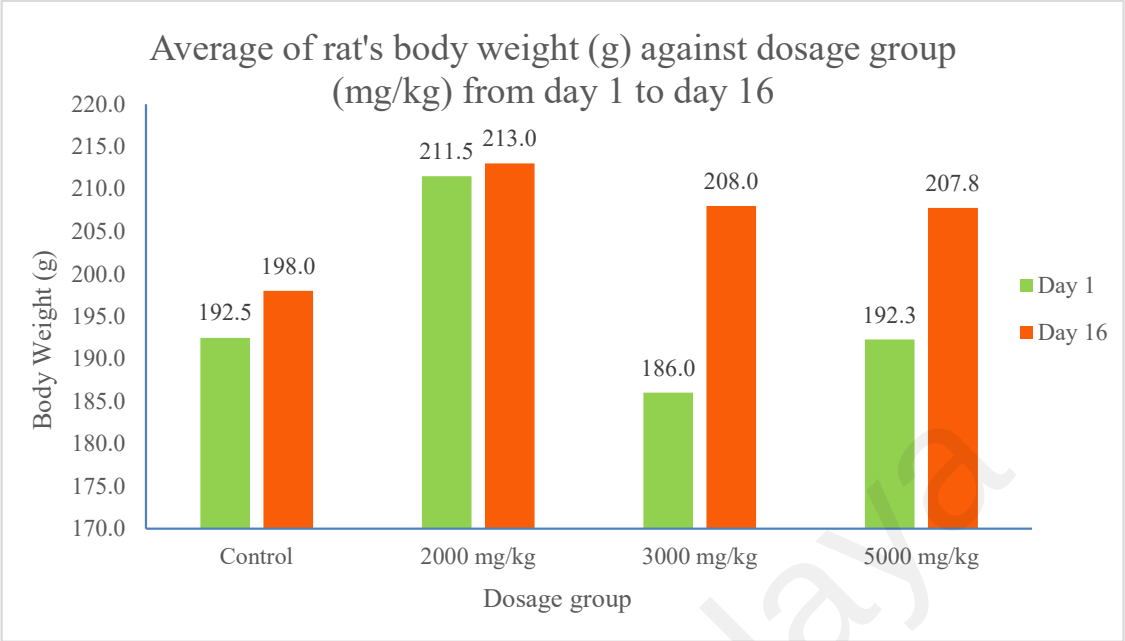


Figure 4.30 : The average of rat's body weight for four rats group against dosage group from day 1 to day 16 of the experiment.

4.2.2.2 Anti-pyretic activity

The anti-pyretic activity of the ethyl acetate extract of *Z. zerumbet* was evaluated using yeast-induced pyrexia in a rat model. The normal body temperatures of rats in this study were 36.15 ± 0.32 °C. The pyrexia model in rats was successfully developed by the significant (p<0.0001) results of increasing rectal temperature after 18 hours of subcutaneous injection yeast in comparison with the normal control group (Group I) as presented in Table 4.5.

The ethyl acetate extract of *Z. zerumbet* at a dose level of 125 mg/kg body weight (Group IV) caused the highest anti-pyretic activity, which significantly (p<0.05) reduced pyrexia by 1.97% after one-hour treatment compared to 0 hours. In addition, a dose level of 250 mg/kg body weight (Group V) demonstrated a significant (p<0.05) decrease in rectal temperature by 1.91%. For the rats group with 500 mg/kg body weight (Group VI) and the rats' group treated with standard drug aspirin (**121**) (Group III), the rectal

temperatures decreased by 1.82% and 1.29%, respectively and both groups showed no significant differences (p<0.05) as compared to 0 hour in respective groups (Figure 4.31).

In the 2nd hours post-treatment, the rats group treated with the ethyl acetate extract of *Z. zerumbet* at the dosage of 125 mg/kg (Group IV) remained significantly (p<0.05) highest decreasing in rectal temperature which is 0.85 °C (2.23%) comparison with 0 hours. Whereas the rats group treated with the ethyl acetate extract of *Z. zerumbet* at dosages of 250 mg/kg (Group V) and 500 mg/kg (Group VI) showed no significant (p<0.05) reduction in pyrexia levels where the rectal temperature was lowered by 1.35% and 0.94%, respectively. Similarly, at this hour, the rats group treated with the standard drug (aspirin (**121**)) at a dosage of 100 mg/kg body weight (Group III) also revealed no significant difference (p<0.05) in fever reduction (1.47%).

In the 3rd and 4th hours post-treatment, Group III which corresponded with the reference drug aspirin (**121**) showed the highest anti-pyretic activity where it exhibited a significant change (p<0.005) in reducing pyrexia by 2.30% and 1.96%, respectively as compared to 0 hours. In contrast, the rat groups that received the ethyl acetate extract of *Z. zerumbet* at 125 mg/kg, 250 mg/kg, and 500 mg/kg body weight dosage levels (Group IV, V, and VI), indicated no significant difference (p<0.005) in anti-pyretic effect in the 3rd hour post-treatment as compared to 0 hours. All three groups of rats (Group IV, V, and VI) demonstrated a slight decrease of rectal temperature by 1.21%, 0.32%, and 0.21%, respectively in the 3rd hour post-treatment and the rectal temperature of all these three groups of rats were not reduced in the 4th hours post-treatment. The finding indicated that the EA extract of *Z. zerumbet* at dosages of 125, 250, and 500 mg/kg body weight was successful in decreasing pyrexia in rats for up to 3 hours, but it was unabled to decrease pyrexia levels at the next hour (in the 4th hour).

The lowest dosage of EA extract of *Z. zerumbet* which is 125 mg/kg body weight (Group IV) demonstrated the most potent and significant (p<0.05) anti-pyretic effect in 1st and 2nd hour of treatment, whereas medium dosage of the EA extract of *Z. zerumbet* (250 mg/kg body weight, Group V) showed significantly (p<0.05) anti-pyretic potential only at the 1st hour of treatment. In contrast, aspirin (**121**) (the standard drug) at a dosage 100 mg/kg (Group III) body weight had no significant (p>0.05) anti-pyretic activity during 1st and 2nd hour of experiment, but it exhibited significant (p<0.05) anti-pyretic activity in the 3rd and 4th hour after administration. Interestingly, the EA extract of *Z. zerumbet* at lower dose (125 mg/kg, Group IV) exhibited more potent anti-pyretic activity at the early stage of the experiment (1st and 2nd hour post-treatment) compared to the standard drug, aspirin (**121**) (Group III).

On the other hand, this study revealed that the EA extract of *Z. zerumbet* was less effective at higher doses (500 mg/kg body weight, Group VI) than at lower (125 mg/kg body weight, Group IV) and medium (250 mg/kg body weight, Group V) levels, indicating its effectiveness declined dose-dependently. These results suggested that the malfunction of the ethyl acetate extract of *Z. zerumbet* at higher doses may be attributed to the high concentration of phytochemicals in the extract which adversely affect the anti-pyretic activity.

According to Bachert et al. (2005), single doses of aspirin (**121**) (500 and 1000 mg) are more efficient at treating fevers for up to 6 hours, and the safety and tolerability of the various treatments were comparable (Bachert et al., 2005). The time-dependent anti-pyretic activity observed for the EA extract of *Z. zerumbet* at 125 mg/kg probably indicated that the active constituents in the extract have a short duration of action than aspirin (**121**). Time-dependent anti-pyretic activity was also observed in the EA extract of *Z. zerumbet* at higher dosage (250 and 500 mg/kg, Group V and VI) where the

effectiveness decreased with time. All dose levels of EA extract of *Z. zerumbet* exhibited anti-pyretic activity immediately after administration, indicating that extracts possess rapid onset of action. Therefore, this finding suggested that the EA extract of *Z. zerumbet* has a significant anti-pyretic effect at a dosage of 125 mg/kg (Group IV) which reduces the pyrexia level within the first 2 hours of administration and could be a potential anti-pyretic agent as a supplement in the treatment of pyrexia. Furthermore, fever is always accompanied by dengue infection. Our study also revealed that the ethyl acetate extract of *Z. zerumbet* had potent anti-protease activity (Salleh et al., 2019) thus, this finding suggests that *Z. zerumbet* could be a promising candidate to be developed as a dengue fever treater.

Body temperature regulation and pain are two significant indicators of the body's resistance to inflammation (Meli et al., 2001). In general, most of the anti-inflammatory drug will exhibit anti-pyretic and analgesic properties as well (Perianayagam et al., 2004). For example, the standard drug employed in this study, aspirin (**121**) has been used among the most commonly prescribed drugs as the non-steroidal anti-inflammatory drug (NSAID), which also possesses anti-pyretic and analgesic properties. The mechanisms of action of aspirin (**121**) that are responsible for all those effects are believed to be involved with both central and peripheral mechanisms by inhibiting the activity of the cyclooxygenase (COX) enzyme to reduce the production of prostaglandin (Aronoff & Neilson, 2001). Previous studies have described that *Z. zerumbet* extract possesses anti-inflammatory and analgesic properties (Chang et al., 2014; Chien et al., 2008; Nurfina et al., 1997; Somchit & Mohamad Hazir, 2003; Somchit et al., 2005). As a result, we believe that the anti-inflammatory effect of the ethyl acetate extract of *Z. zerumbet* may contribute to the mechanism of the anti-pyretic activity.

As reported by many researchers, the main constituent of *Z. zerumbet* is zerumbone (**3**) (Baby et al., 2009; Rout et al., 2009; Sulaiman et al., 2010; Yu et al., 2008) and the present study showed that zerumbone (**3**) is one of the bioactive sesquiterpenes found in *Z. zerumbet*. Somchit et al. (2012) had demonstrated zerumbone (**3**) has inhibitory activities on PGE_2 and λ–Carrageenan which induced inflammation (Somchit et al., 2012). Other researchers have reported that the zerumbone's mechanism of action can be associated with lipoxygenase inhibition and/or cyclooxygenase in peripheral tissues, thus reducing the prostaglandin (PGE_2) synthesis (Sulaiman et al., 2009). Interestingly, this finding was concurrent with the findings made by Murakami and Ohigashi (2007) (Murakami & Ohigashi, 2007). As a result, the anti-pyretic effect shown in this study could be attributed to the high concentration of zerumbone (**3**) in the ethyl acetate extract of *Z. zerumbet*. Consequently, it may be inferred that the anti-pyretic activity of *Z. zerumbet* extract containing zerumbone (**3**) could have a similar mode of action as aspirin (**121**) by suppressing the cyclooxygenase-2 (COX-2) expression and decreasing the production of PGE_2, thereby lowered the body temperature.

As a conclusion, the ethyl acetate extract of *Z. zerumbet* extract and the phytochemicals present may have contributed to the anti-pyretic activity, as established in this study and also validate the use of *Z. zerumbet* for the treatment of fever in traditional Malay medicine.

Table 4.5: Effect of treatment with *Z. zerumbet* extract in a dose of 125, 250, and 500 mg/kg and aspirin in a dose of 100 mg/kg on Brewer's yeast-induced pyrexia in rats.

Groups	Body weight (g)	Rectal temperature (°C) ± SD (percentage temperature change)					
		initial temperature before yeast injection	after 18 hours of yeast injection	Time after drug administration (hour)			
				1	2	3	4
normal control (Group I)	216	36.12 ± 0.29	36.07 ± 0.29	36.35 ± 0.14 (-0.78%)	36.28 ± 0.29 (-0.58%)	36.22 ± 0.20 (-0.42%)	36.20 ± 0.20 (-0.36%)
Yeast (Group II)	238	36.25 ± 0.27	38.30 ± 0.46	38.22 ± 0.35$^{\#}$ (0.21%)	37.87 ± 0.34$^{\#}$ (1.12%)	37.68 ± 0.53$^{\#}$ (1.62%)	37.57 ± 0.61$^{\#}$ (1.91%)
Yeast + ASP (Group III)	226	36.38 ± 0.23	37.33 ± 0.35	36.85 ± 0.51 (1.29%)	36.78 ± 0.48 (1.47%)	36.47 ± 0.56* (2.30%)	36.60 ± 0.36* (1.96%)
Yeast + ZZEA 125 (Group IV)	220	35.98 ± 0.30	38.08 ± 0.60	37.33 ± 0.50* (1.97%)	37.23 ± 0.51* (2.23%)	37.62 ± 0.39 (1.21%)	37.90 ± 0.46 (0.47%)
Yeast + ZZEA 250 (Group V)	209	36.10 ± 0.23	37.71 ± 0.38	36.99 ± 0.74* (1.91%)	37.20 ± 0.36 (1.35%)	37.59 ± 0.29 (0.32%)	37.96 ± 0.41 (-0.66%)
Yeast + ZZEA 500 (Group VI)	238	36.02 ± 0.59	37.35 ± 0.25	36.67 ± 0.28 (1.82%)	37.00 ± 0.37 (0.94%)	37.27 ± 0.55 (0.21%)	37.60 ± 0.32 (-0.67%)

ZZEA: *Zingiber zerumbet* ethyl acetate extract. ASA: aspirin. Yeast + ZZEA 125: Yeast-induced pyrexia rats treated with ZZEA 125 mg/kg body weight; Yeast + ZZEA 250: Yeast-induced pyrexia rats treated with ZZEA 250 mg/kg b.w.; Yeast + ZZEA 500: Yeast-induced pyrexia rats treated with ZZEA 500 mg/kg body weight. The results represent the mean ± SE for 6 rats in each group. $^{\#}p<0.0001$ compared to normal control group, $^{*}p<0.05$ compared to 0 hour in within the group.

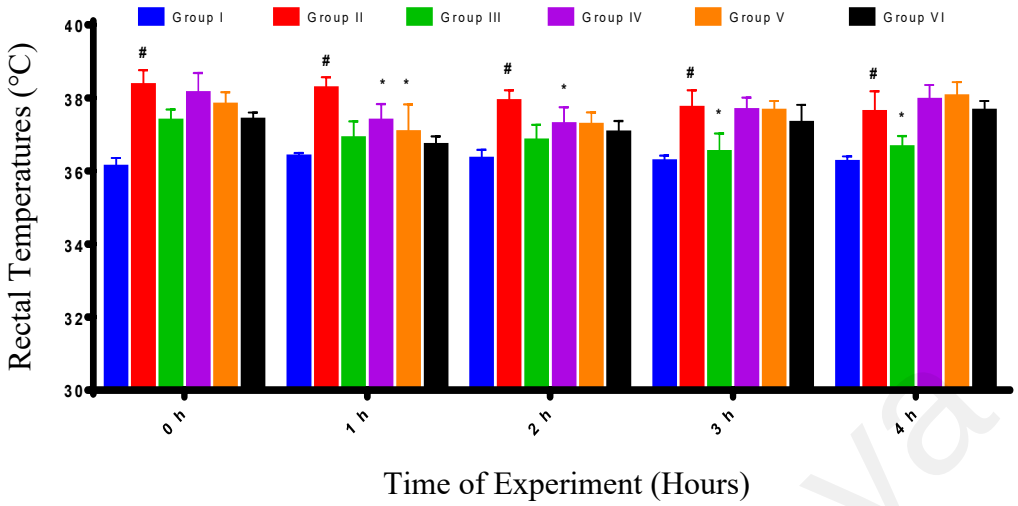


Figure 4.31 : Anti-pyretic effect of the ethyl acetate extract of *Z. zerumbet* in yeast-induced pyrexia.

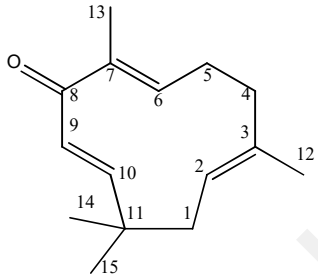
Group I: normal control, Group II: Yeast, Group III: yeast + aspirin (ASA) 100 mg/kg body weight, Group IV: yeast + ZZEA 125 mg/kg body weight, Group V: yeast + ZZEA 250 mg/kg body weight, Group VI: yeast + ZZEA 500 mg/kg body weight. #p<0.0001 compared to normal control group, *p<0.05 compared to 0 hour in within the group.

4.3 Molecular docking study of zerumbone (3) and zerumbone epoxide (56) (PART C)

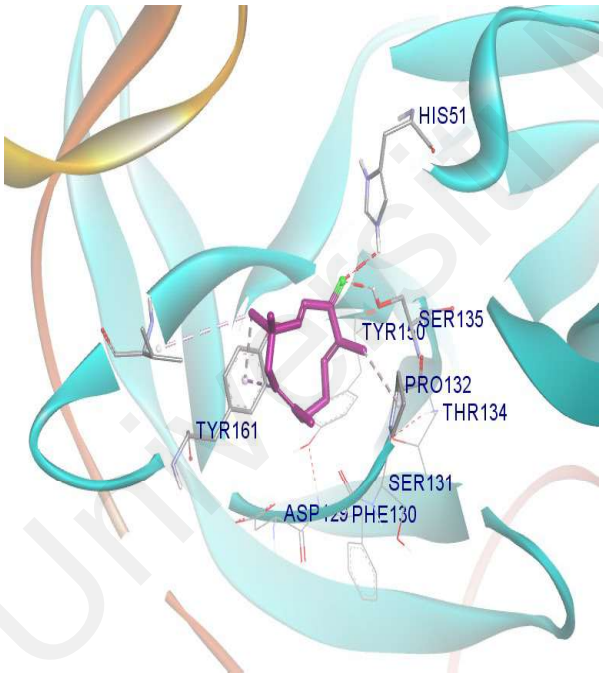
The molecular docking study of the isolated compounds (zerumbone (**3**) and zerumbone epoxide (**56**)) from the most potent fraction of anti-protease activity (refer to Chapter 4.2.1) and the reference inhibitor (quercetin (**70**)) was performed using AutoDock4.2 software. The inhibitor/ligand was docked with the targeted protein NS2B/NS3 protease developed by Wichapong et al. (2009) (Wichapong et al., 2009). The control docking of the DENV2 NS2B/NS3 protease demonstrated the binding energy at -7.60 kcal/mol.

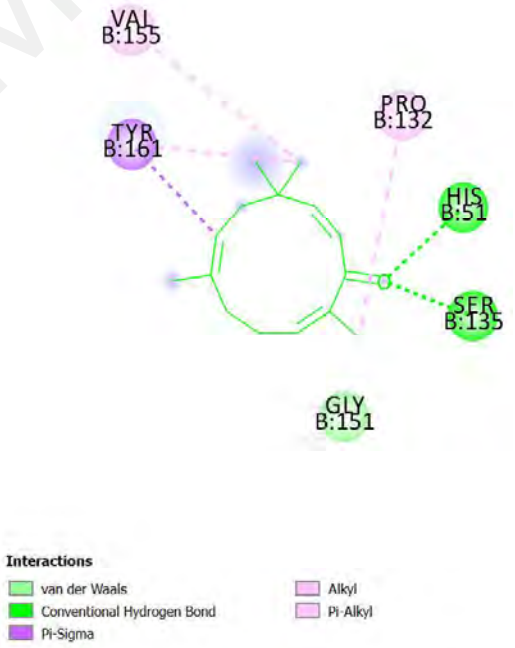
The molecular docking study of zerumbone (**3**) revealed two hydrogen bonding between the carbonyl group of zerumbone (**3**) with amino acid residues of His51 and

Ser135 of DENV2 NS2B/NS3 protease (Figure 4.32(b)). Other interactions involved were π-σ and π-alkyl bonds between the phenyl ring of Tyr161 and the hydrogen of C-2 and C-14 of zerumbone (**3**), respectively. In addition, two π-alkyl interactions were noticed at C-13 and C-14 of zerumbone (**3**) with amino acid residues of Pro132 and Val155, respectively as presented in Figure 4.32(c).



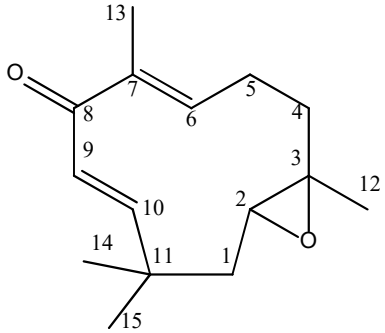
(a)



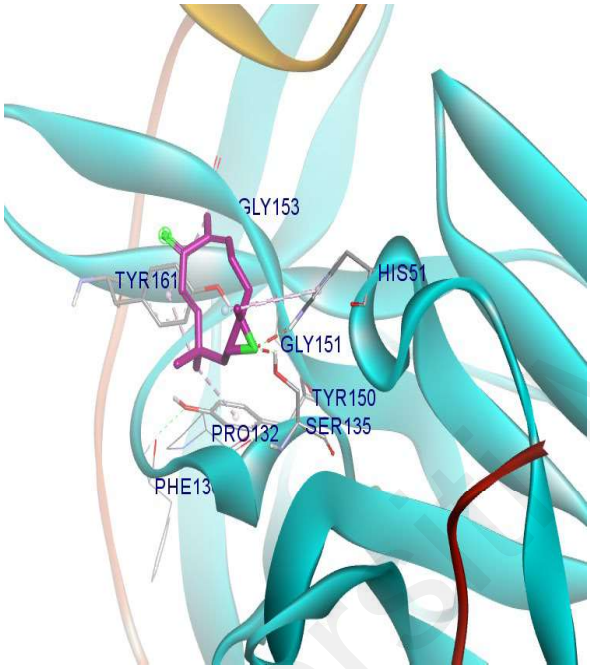


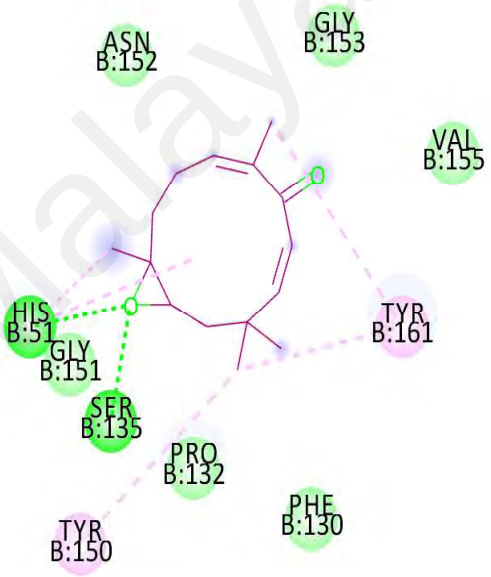
(b) (c)

Figure 4.32 : (a) Molecular structure of zerumbone (3), (b) Binding interactions of zerumbone (3) (purple) with amino acid residues of DENV2 NS2B/NS3 protease (ribbon cyan and brown), (c) 2D diagram representation of the binding interaction involved.



(a)



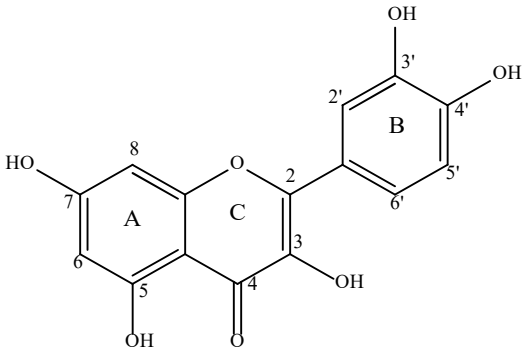


(b) (c)

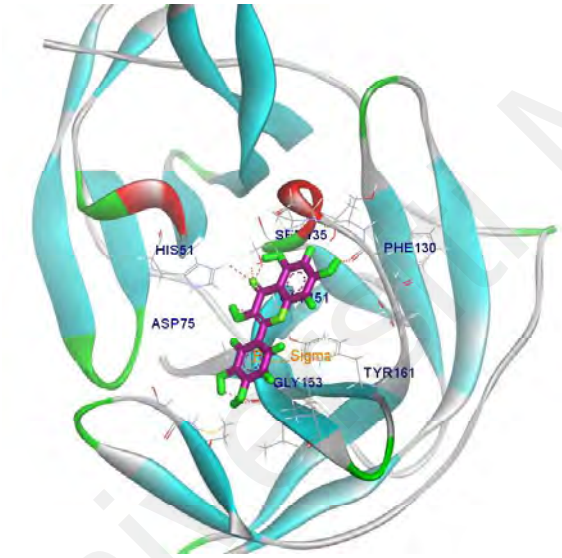
Figure 4.33 : (a) Molecular structure of zerumbone epoxide (56), (b) Visualisation of binding interactions of zerumbone epoxide (56) (purple) with amino acid residues of DENV2 NS2B/NS3 protease represented by solid ribbon cyan and brown, (c) 2D diagram of binding interaction involved between zerumbone epoxide (56) and the amino acid residues where hydrogen bonds were indicated by green dotted lines.

A total of two hydrogen bonds and five π-alkyl bonds interactions were formed between the zerumbone epoxide (**56**) and the key residues involved as labelled in Figure 4.33 (b) and (c). The oxygen from epoxide group of zerumbone epoxide (**56**) formed hydrogen bonds with His51 and Ser135 at the distance of 2.065 Å and 1.831 Å, respectively. A π alkyl interaction was also recorded for alkyl group at C-15 of zerumbone epoxide (**56**) with amino acid residues of Tyr150. In addition, the key residue Tyr 161

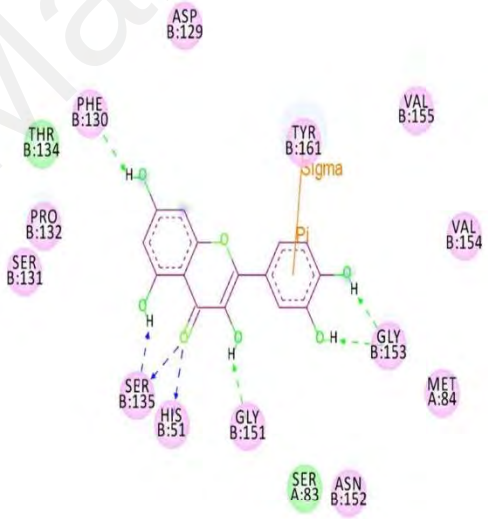
formed π-alkyl interactions with C-13 and C-15 while His51 interacted with C-12 and cyclic of zerumbone epoxide (**56**).



(a)



(b)



(c)

Figure 4.34 : (a) Molecular structure of quercetin (70), (b) Binding interactions of quercetin (70) (purple) with amino acid residues of DENV2 NS2B/NS3 protease (ribbon cyan and brown), (c) 2D diagram of binding interaction involved between quercetin (70) and the amino acid residues where hydrogen bond was indicated by green and blue dotted lines.

For quercetin (**70**) (standard), its five hydroxyl groups formed hydrogen bonds with Gly153, Gly151, Phe130, and Ser135 (Figure 4.36 a and b). In addition, the carbonyl group of ring C interacted with His51 and Ser135 by forming a hydrogen bond. Besides,

Gly153 forms π hydrogen bonding with phenol ring B of quercetin (**70**) while a π alkyl bond is formed between ring A of quercetin (**70**) with Pro132.

The binding energies of zerumbone (**3**), zerumbone epoxide (**56**), and quercetin (**70**) (reference inhibitor) with DENV2 NS2B/NS3 protease were observed as -5.58, -5.60, and -5.65 kcal/mol, respectively. The low binding energy indicates that the interactions between ligands (inhibitors) and the protein were preferable (Qamar et al., 2014). As discussed previously (in Chapter 4.2.1), although the zerumbone epoxide (**56**) demonstrated the most potent activity among the two isolated compounds, the quercetin (**70**) (standard) showed the strongest inhibition against DENV2 NS2B/NS3 protease. This may be due to the hydrogen bonding interaction of carbonyl group at C-4 of the quercetin (**70**) with His51 and Ser 135 (aldehyde warhead) of DENV2 NS2B/NS3 protease (Figure 4.34). Asp75, His51, and Ser135 were the three amino acids known as a catalytic triad that can be found in the active site of DENV2 NS2B/NS3 protease (Abduraman et al., 2018). Any interaction with one of the catalytic triad will contribute to a significant binding interaction between the ligand and the enzyme and leads to effective inhibition of the DENV2 NS2B/NS3 protease activity (Yin et al., 2006). From the results, the interaction of the inhibitors (zerumbone (**3**) and zerumbone epoxide (**56**)) with one of the catalytic triads (His 51, Asp75, and Ser135) may disrupt the transfer of electrons between the carboxyl group of Asp75 and the nitrogen atom on the imidazole group of His51, reducing the ability of nucleophilic attack on the hydroxyl group (*β*-OH) of residue Ser135, which is required to initiate proteolytic activity (Bharadwaj et al., 2019; Dwivedi et al., 2016). While the interaction of inhibitors with other conserved amino acid residues of NS3 such as Asp129, Phe130, Tyr150, Asn152, and Gly153 form a small part of the *β*-sheet that is significant in substrate binding (Bharadwaj et al., 2019; Dwivedi et al., 2016).

Apart from binding energy and the type of interaction involved, the distance between the protein and ligands at active sites within 3.0 Å region also plays an important role in the strong interaction (Nesfu et al., 2019). Summary of binding energies as well as distances of the protein and ligands (zerumbone (**3**), zerumbone epoxide (**56**), and quercetin (**70**)) were tabulated in Table 4.10. At the active site, two amino acid residues of the catalytic triad (His51 and Ser135) interacted with zerumbone (**3**) and zerumbone epoxide (**56**), but the ligand-protein bond of zerumbone epoxide (**56**) was shorter than zerumbone (**3**). The shorter the bond, the stronger the bond, and thus more stable the ligand-protein conformation (Nesfu et al., 2019). Therefore, the zerumbone epoxide (**56**) exhibited more potent activity against DENV2 NS2B/NS3 protease compared to zerumbone (**3**) may also due to this reason.

According to the discussion, apparently the types of binding interaction, the distance of the ligand-protein bond and the binding energy of the conformation were the key factors in contributing to the potency and effectiveness of the inhibitors towards the DENV2 NS2B/NS3 protease. Besides, the hydrogen bonds between the compounds (zerumbone (**3**) and zerumbone epoxide (**56**)) and one of the catalytic triads (His 51, Asp75, and Ser135) may alter the functional of DENV2 NS2B/NS3 protease by reorientate their conformation (Dwivedi et al., 2016). Moreover, the binding interaction between the inhibitors with the amino acid residues (His51, Asp75, and Ser135) at the active site plays a vital role in the inhibition of the enzyme activity, hence inhibiting the virus propagation (Velmurugan et al., 2014).

To the best of our knowledge, there is no report on the molecular docking study of sesquiterpenes compound with DENV2 NS2B/NS3 protease. Therefore, these compounds can be investigated further in order to understand the mechanism of action toward dengue virus inhibition.

Table 4.6 : Summary of binding interactions of the compounds towards DENV2 NS2B/NS3 serine protease.

Ligand/compound	Binding energy (kcal/mol)	Interacting site	Residue	Type of interaction	Distance (Å)	Ligand Interacting
Zerumbone (**3**)	-5.58	Aldehyde warhead	His 51	Hydrogen bond	2.3135	Carbonyl group at C-8
		S1 pocket	Ser 135	Hydrogen bond	1.9646	Carbonyl group at C-8
		S1 pocket	Tyr 161	Pi-sigma		Alkyl group at C-2
			Val 155	Pi-alkyl		Alkyl group at C-14
			Pro 132	Pi-alkyl		Alkyl group at C-7
		S1 pocket	Tyr 161	Pi-alkyl		Alkyl group at C-14
Zerumbone epoxide (**56**)	-5.60	Aldehyde warhead	His 51	Hydrogen bond	2.0645	Epoxide group at C-3 and C-4
		S1 pocket	Ser 135	Hydrogen bond	1.8307	Epoxide group at C-3 and C-4
		Aldehyde warhead	His 51	Pi-alkyl		Alkyl group
		Aldehyde warhead	His 51	Pi-alkyl		Alkyl group at C-12
		S1 pocket	Tyr 150	Pi-alkyl		Alkyl group at C-15
		S1 pocket	Tyr 161	Pi-alkyl		Alkyl group at C-15
		S1 pocket	Tyr 161	Pi-alkyl		Alkyl group at C-13
Quercetin (**70**)	-5.65	Aldehyde warhead	His 51	Hydrogen bond	2.3149	Carbonyl at C-4
		S1 pocket	Ser 135	Hydrogen bond	1.6569	Carbonyl at C-4
		S3 pocket	Gly 153	Hydrogen bond	2.3113	Hydroxyl group at C-4’
		S3 pocket	Gly 153	Hydrogen bond	2.2376	Hydroxyl group at C-3’
			Gly 151	Hydrogen bond	2.1869	Hydroxyl group at C-3
		S1 pocket	Phe 130	Hydrogen bond	1.7626	Hydroxyl group at C-7
		S1 pocket	Ser 135	Hydrogen bond	2.1229	Hydroxyl group at C-5
		S3 pocket	Gly 153	Pi- sigma		Aromatic ring B

CHAPTER 5: CONCLUSION

In this study, the EA extract exhibited the anti-protease activity against DENV2 NS2B/NS3 protease with 90.32% inhibition at 200 μg/mL. All eight fractions were subjected to an anti-protease study and it showed fraction B is the most active fraction towards DENV2 NS2B/NS3 protease with IC_{50} at 0.09±0.62 μg/mL and 73.55% inhibition at 200 μg/mL. Thus, the fraction was purified and two compounds were obtained; zerumbone (**3**) and zerumbone epoxide (**56**). Both compounds were identified and confirmed by various spectroscopic methods including nuclear magnetic resonance (1D NMR and 2D NMR), liquid chromatography mass spectrometry, infrared, and ultra violet visible techniques. Zerumbone epoxide (**56**) exhibited the most potent activity against DENV2 NS2B/NS3 protease with 72.04% of inhibition at the concentration of 200 μg/mL.

Twenty-three compounds from the essential oil of *Z. zerumbet* rhizomes were identified by GCMS, which indicated that oxygenated sesquiterpenes, zerumbone (**3**) (43.80%) as the most abundant constituent in the rhizomes oil of *Z. zerumbet*. The other chemical constituents include *α*-bergamotene (**126)**, *α*-humulene (**20**), (*Z*)-γ-bisabolene (**125**), caryophyllene oxide (**12**), elemol (**10**) and humulene epoxide II (**35**) were also obtained through a hydrodistillation procedure of rhizomes *Z. zerumbet*.

Acute toxicity study of the EA extract *Z. zerumbet* demonstrated no mortality and toxicity signs in the experimental rats for 16 days extract administration up to the dosage of 5000 mg/kg. The investigation on the anti-pyretic property revealed that the extract showed rapid onset of action through a significant reduction of yeast-induced pyrexia in rats (125 mg/kg) for the first two hours of administration. In comparison with aspirin (**121**) the studied extract; EA of *Z. zerumbet* showed faster onset anti-pyretic. However,

the aspirin (**121**) showed longer lasting anti-pyretic effect up to 4 hours. Thus, our findings support the traditional use *of Z. zerumbet* in the treatment of fever.

The molecular docking study showed that the binding energies of zerumbone (**3**), zerumbone epoxide (**56**), and quercetin (**70**) (standard) with DENV2 NS2B/NS3 were -5.58, -5.60, and -5.65 kcal/mol, respectively. The best conformation is indicated by the lowest binding energy. The results showed zerumbone epoxide (**56**) possesses two hydrogen bonds between the epoxide group with two of the catalytic triad (His 51 and Ser 135) and five π alkyl bonds interaction between the key residues (His 51, Tyr 150, and Tyr 161). While the zerumbone (**3**) possesses two hydrogen bonding between the ketone group of zerumbone (**3**) with His51 and Ser135 of DENV2 NS2B/NS3 and three π-alkyl interactions were noticed at C-13 and C-14 of zerumbone (**3**) with Pro132, Tyr 150, and Val155. In addition, an interaction π-σ bonds were noticed between the phenyl ring of Tyr161 and the hydrogen of C-2. The hydrogen bonding and the hydrophobic interaction with one of the catalytic triads (Asp75, His51, and Ser135) amino acid residues of NS3 protease can alter the conformation of the DENV2 NS2B/NS3 protein, thereby modifying its functional characteristic (Dwivedi et al., 2016).

In conclusion, the ethyl acetate extract of *Z. zerumbet* was non-toxic and exhibited rapid temperature reduction in pyrexia. This finding supports the claim of its use in traditional medicine practice to treat fever. Due to *in vitro* and *in silico* evaluations of the isolated compounds, zerumbone epoxide (**56**) can be suggested as a functional sesquiterpene that could be used in the development of natural product-derived drugs against dengue. Thus, these findings indicated that *Z. zerumbet* has potent anti-protease and anti-pyretic properties, and could be a promising candidate in the prevention and/or treatment of dengue infection-related fever.

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