1.1 Introduction

Dengue viruses (DENVs) belongs to the *Flaviviridae* family and are grouped into four closely related serotypes namely 1, 2, 3 and 4 and transmitted by *Aedes Aegypti* and *Aedes Albopictus* mosquitoes (Westaway *et al.,* 1985; Halstead, 1992; Guha-Sapir *et al.,* 2005; Marijke *et al.,* 2012). (Figure 1.1). DENV is endemic in tropical and sub-tropical regions, mainly in urban and semi-urban areas. Currently, DENV has become a major health threat with regard to its lethal complication particularly the dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF has also become a leading cause of hospitalisation and death especially among children in most Asian countries (Monath *et al.,* 1996; Guzman *et al.,* 2010; Marijke *et al.,* 2012). Duane Gubler, from the Division of Vector-Borne Infections at The Centre for Disease Control (CDC) in Fort Collins, Colorado stated that DENV is one of the most important emerging tropical diseases in the 21st century (Gubler, 1998; Halstead *et al.,* 2005; Allicock *et al.,* 2012). In Malaysia, DHF was firstly reported in 1962 by Rudnick (Rudnick *et al.,* 1965). Furthermore, Fong M. Y. and his co-workers (1998) reported that DENV-2 is the most predominant virus among the four serotypes which can lead to DHF (Fong, M. Y. *et al.,* 1998).

Figure 1.1: Mosquito Vectors of Dengue

Centuries ago natural products were used to cure illnesses by physicians as well as many traditional medical practitioners. The World Health Organization (WHO) stated that approximately 80 percent of the developing world's population relies on traditional medicines (WHO, 2008). This is more prevalent in Arabic countries as well as in Malaysia where various plant extracts have been used to treat different ailments, both as preventive and curative. It is generally accepted that natural products usually have less toxic and low side effects. This has powered many researchers to explore the different potentials of plant extracts for medicinal purposes. Clark stated that natural products are the major source of drugs in modern medicine as they were in ancient times (Clark *et al.,* 1996).

There are numerous plant compounds which have been used for a long time in traditional medicine such as *Cassia Acutifolia* which has been previously used as laxatives and the aqueous extract of *Quercus Infectoria* which is presently used as a potent antimicrobial compound against *Streptococcus Aureus* (Ito *et al*., 2002; Basri *et al.,* 2012). Additionally, many tannin compounds were evaluated as potential inhibitors of Human Deficiency Virus (HIV) replication (Nonaka *et al*., 1990; Lin, *et al.,* 2011) as well as anti-cancer and antiviral activities (Kandil *et al.,* 1998; Huang *et al.,* 2010). A number of tannins and related compounds have been evaluated for their cytotoxicity against human tumor cell lines (Kashiwada *et al*., 1992; Huang *et al.,* 2010). The report by Aswal showed 3800 plants exhibiting efficacy to suppress the growth of several viruses (Aswal *et al*., 1996), with the higher classes of plants possessing relatively higher potential to inhibit viruses as compared to the lower classes (Parida *et al*., 2002; Vijayan *et al.,* 2004). It has been elucidated that extracts from different parts of plants may yield a better source for antiviral compounds rather than synthetic analogues (Monath, 1994; Vijayan *et al.*, 2004).

For many years, DHF and DSS which are caused by DENV were managed only symptomatically, as prevention programs have only achieved minor results. As yet, there has been no one approved vaccine or effective antiviral therapy available against DENV infection, making the development of a new antiviral drug and safe vaccine imperative. For this reason, there is considerable interest in developing a new specific inhibitor and antiviral therapeutic agents to combat this disease. The development of a safe and effective dengue vaccine remains at the experimental stage. This study reports the work carried out in the screening of natural products from a plant for possible leads possessing inhibition activities towards DENV protease.

1.1.1 Objectives of This Study

Being one of the most serious threats to human beings, mosquito-borne viral infections which are dengue fever (DF) and its related DHF compromise a challenge at both tropical and subtropical regions of the world, particularly in Southeast Asia and Malaysia. Mosquito control programs had failed to control the epidemics caused by mosquitoes. This is attributed to the difficult implementation and maintenance of such programs. Attempts to produce a satisfactory DENV vaccine by conventional methods have met with limited success and despite years of research efforts, the development of a safe and effective dengue vaccine remains at the experimental stage.

Based on this information, several research groups have speared into the quest to search and develop an antiviral drug targeting the NS3 protease, the second largest protein required for viral replication either from natural products (Parida *et al.*, 2002) or by chemical synthesis of inhibitors (Leung *et al.*, 2001).

This study reports the work carried out in the screening of natural products from a plant for the possible inhibitor of DENV serine protease. Any identified compound will be instrumental in aiding the drug development process for an anti-DENV drug.

In this research, the main aim of the investigation is to discover an antiviral compound for DENV-2 serine protease from *Quercus Infectoria* and purified natural products. The objectives of this study are as follows:

1. Expression and purification of an active DENV-2 protease complex, NS2B/NS3.
2. Isolation, characterization and identification of the bioactive compounds.
3. Screening of bioactive compounds from *Quercus Infectoria* and purified natural products towards the recombinant DENV-2 protease.
4. Determination of the inhibition constant, Ki for potential compounds.

It is hoped that with the success of this project, a long term locally-based road map for the drug discovery program of DENV-2 could be laid down. This will further enhance the drug discovery program and develop an antiviral drug that is more economical, safe and marketable for DENV-2 infection.

1.2 Dengue Virus (DENV)

DF has been known for over 200 years; the first report of the disease was from Benjamin Rush during an outbreak which occurred in Philadelphia in 1780 (Rigau-Perez *et al.,* 1998). Since then, dengue-like illness have occurred in periodic epidemics at 10 and 30 years intervals in many tropical and subtropical regions like Zanzibar (1823 and 1870), Calcutta (1824,1853,1871,1905), West India (1827), Malaysia and Hong Kong (1901-1902) (Skae, 1902; Rudnick *et al.,* 1965; Gubler, 1994; Henchal *et al.,* 1990; Rozilawati *et al.,* 2007). The first recorded epidemics were recorded in 1977 which occurred almost simultaneously in Cairo, Egypt and Jakarta, Indonesia. Benjamin Rush elucidates DF symptoms as characterized by disseminated body pain, headache, fever, rash, lymphadenopathy and leucopenia (Halstead, 1989; Rigau-Perez *et al*., 1998). However, in the last 5 decades, its worldwide incidence has increased tremendously, placing one-third to nearly half of the world's population at risk of becoming infected. Once, it was considered as mainly an Asian disease but the dengue epidemic has now permeated the tropical areas of the Americas (Phillips, 2008).

Between the years 1780 and 1940, the disease pattern associated with dengue-like illness was characterized by large epidemics, but relatively infrequent. However, it is likely that DENVs have become endemic in these areas because during inter-epidemic periods when there was no apparent disease transmission, non-immune visitors invariably contracted a dengue-like illness within months of their arrival (Gubler, 1998). Ashburn and colleagues were the first to report that DENV are the causative agent for DF and they identified two serotypes of the virus, namely serotype 1 and serotype 2 (Sabin, 1952), while the other two serotypes called serotype 3 and serotype 4, were only isolated during an epidemic in Manila, Philippines in 1956. This work was conducted by William Mcd Hammon and co-workers utilizing samples

obtained from children contracting the hemorrhagic disease. Since then, all DENVs were fitted antigenically into four serotypes no matter how many viruses have been identified or isolated (Gubler, 1994).

The principal mosquito vector belongs to the genus "Aedes" initially found in tropical and subtropical regions, but is now found in all continents excluding Antarctica. First described and named by Meigen in 1818 (Kettle, 1984), the name comes from the Ancient Greek word *aedes,* meaning "unpleasant" or "odious". Some species of this genus transmit serious diseases, including DF and yellow fever. The first documentation of DENV transmission was by Graham in 1903, and *Aedes Aegypti* was demonstrated to be the principal mosquito vector of DENVs by Ashburn and Craig in 1907 (Nishiura *et al*., 2007). Further studies had shown that *Aedes Albopictus* and *Aedes Polynesiensis* were also efficient secondary vectors for DENVs (Halstead, 1989; Gubler, 1994).

DENV is now believed to be endemic in at least 100 countries in Southeast Asia, the Pacific Islands, the Caribbean, Central America, South America and parts of Africa. It is estimated that there are 100 million cases of DF and half a million cases of DHF annually with a case fatality in Asian countries of 0.5% to 3.5% (Malavige *et al.,* 2004). Malaysia has been one of the endemic countries with DF for decades; however the disease caused by the virus has increased the mortality rate each year. According to the statistics from the Ministry of Health, Malaysia (2009), there is an alarming increase of dengue related clinical cases each year. By the epidemic week 30 (July 26th to August 1st) in the year 2009, a total of 27,542 dengue cases were reported with 66 fatalities. The statistics portrayed an increase in the dengue cases by 7% with 61 deaths reported as compared with the same epidemic week in the year 2008.

1.2.1 Geographic and Seasonal Distribution

Previously, DF was considered a benign, non-fatal disease for visitors to the tropics, with 10-40 years interval between major epidemics (first reported in 1780). This is mainly because at that time the viruses and their mosquito vector could only be transported between population centres by sailing vessels (Gubler, 1994). Nowadays, the viruses are endemic in most urban centres of the tropics with transmission occurring all year round and a peak transmission of DENVS during the periods of higher rainfall. The influencing factors for this seasonal transmission are not well understood. Some are related to the number of mosquitoes in that specific area (which increases during rainy seasons), while others are climate factors such as moderate ambient temperature and higher humidity linked with the rainy season. This kind of weather will increase the survival rate of the vector mosquitoes and hence increase the chances of transmission to secondary hosts.

The hemorrhagic form of DF (DHF) was first recognized in 1950 as a new disease in Philippines, Singapore, Thailand and Vietnam but it soon spread to many countries. The disease is reported in over 100 countries with approximately 2.5 billion people at risk of infection (Guzman *et al.* 2010). (Refer to Figure 1.2). The emergence of DENV related diseases over the years is closely related to population growth, unplanned and uncontrolled urbanization, inadequate waste water management and most importantly, lack of effective mosquito control (Gubler, 2002).

To add to the problem, there are some evidences of microevolution with the existence of more virulent genotypes of DENV which replace the less virulent genotypes (Zhang *et al*, 2003). Although DF and DHF impose a considerable burden on healthcare, public health and international agencies have invested little in dengue control with reasons still unclear (Halstead *et al.,* 2005).

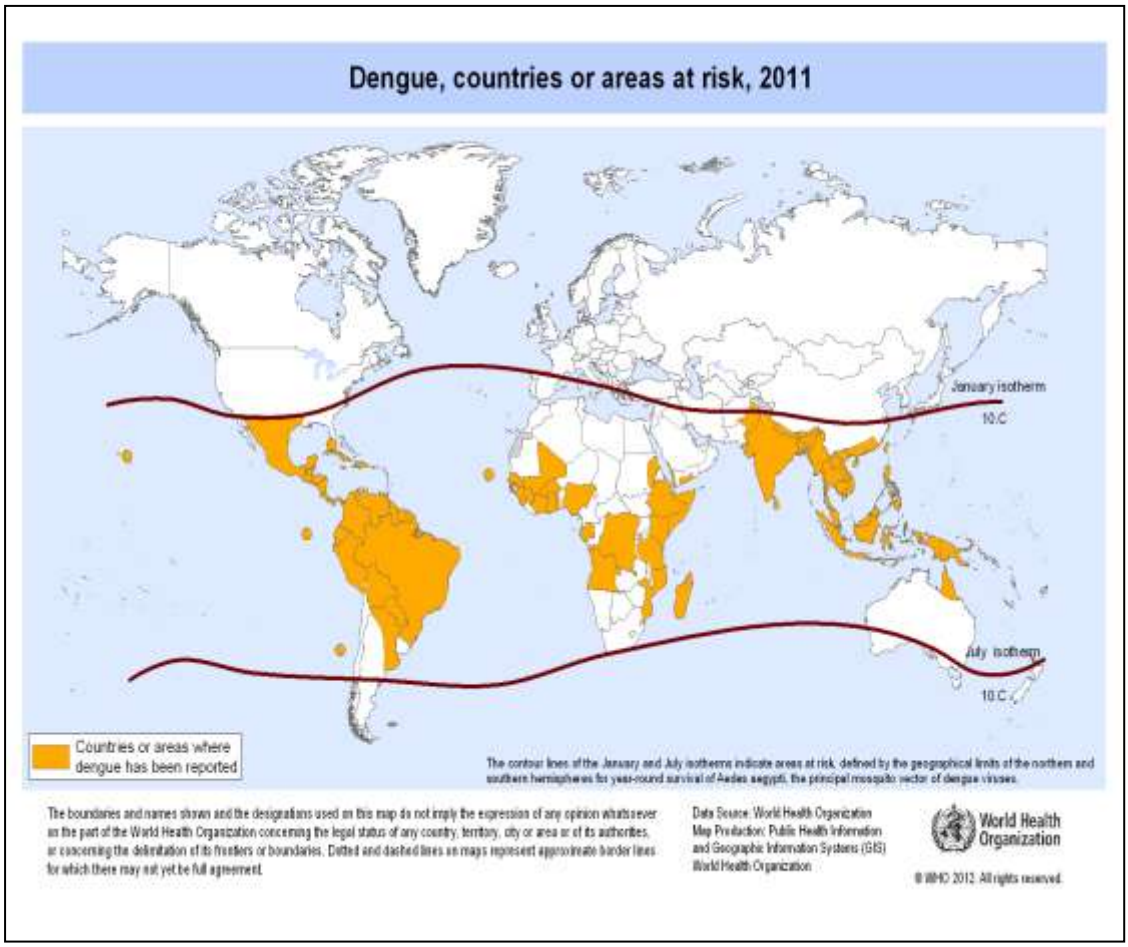

Figure 1.2: The global distribution of the predominant vector for dengue and regions of endemic dengue activity (World Health Organization, 2011).

DENV has grown dramatically around the world in the recent 5 decades. More than 2.5 billion people (over 40% of the world's population) reside in areas in danger of transmission. Dengue infection could be the main reason behind illnesses and deaths within the tropics and subtropics. The World Health Organisation reported that worldwide, as many as one hundred million people are infected yearly (WHO, 2012).

1.2.2 Taxonomy and Classification

Structurally, DENV belongs to the flaviviridae family and genus flavivirus. This family consists of the genera flavivirus, pestivirus, and hepacivirus (Regenmortel *et al.,* 2000; Thurner *et al.,* 2004). The ecological classification of this virus belongs to a larger heterogeneous group of viruses called arbovirus which implies that transmission between vertebrate hosts including humans is dependent upon hematophagous arthropod vectors. Structurally there are four closely related DENVs, which can be distinguished into four serotypes designated as DENV-1, DENV-2, DENV-3 and DENV-4 (Gubler, 1988; Guha-Sapir, 2005) using complement fixation, haemagglutination inhibition and plaque-reduction neutralization tests.

The structure of all flaviviruses including DENV is approximately the same. The main structure of mature virion consists of positive single-stranded RNA genome surrounded by an icosahedral nucleocapsid. The latter is covered by a lipid envelope, which is derived from the host cell membrane from which the virus buds. The complete virion is about 50 nm in diameter. Mature virions contain three structure proteins which are called the nucleocapsid (core protein) (C), membrane-associated protein (M) and the envelope protein (E) (Stoller, 1969) (Figure 1.3). Mainly immature intracellular virus contains a protein known as prM, a precursor of M (Henchal *et al.,* 1990). Antigenically, the four DENVs make up a distinct and unique complex within genus flavivirus. While these four dengue serotypes are antigenically distinct, there is evidence that serologic subcomplexes exist within the group. As an instance, a close genetic relationship between DENV-1 and DENV-4 has been demonstrated using cDNA hybridization probes. Surprisingly, DENV-2 shows a high sequence homology (71%) with the *Edge Hill* virus, an ecologically distinct flavivirus from Australia (Anderson *et al.,* 2005). There are more than 70 other antigenically connected

flavivirus, as well as the yellow fever, *Kunjin*, *Zika*, *Murray Valley Encephalitis*, *Japanese Encephalitis*, *St Louis Encephalitis* and others.

1.2.3 Morphology of Dengue Virus

Morphologically, virion dengue is a small spherical particle surrounded by a nucleocapsid which is 30 nm in diameter. This nucleocapsid is surrounded by a lipid envelope about 10 nm deep (Henchal *et al.,* 1990) with a virion diameter of about 50 nm. DENV contains a single-stranded (ss) positive sense RNA genome of about 10,723 nucleotides (Falgout *et al.,* 1995; Matusan *et al.,* 2001). The dengue genomic RNA is approximately 11 kilobases in length. The 5' end of the RNA is capped but it lacks a poly (A) tail at the 3' end (Rice *et al.,* 1986; Hahn *et al.,* 1988).

The single strand RNA (ss-RNA) genome of DENVs has a sedimentation coefficient of 42 S and a molecular weight of 3.3 x 10^6 with three virion structural polypeptides. (Stodllar *et al.,* 1966). The structural polypeptides of dengue consist of a nucleocapsid protein, a large envelope protein and a small nonglycosylated envelope protein. Molecular weights of the large envelope glycoproteins vary from 57,000 for dengue serotype 4 to 59,000 for dengue serotype 2 (Russel *et al.,* 1980).

The viral RNA has a single open reading frame spanning more than 95% (more than 10 kb) of the genome (Falgout *et al.,* 1991) and encodes a polyprotein precursor (3,391 amino acid residues in DEN-2). The DENVs share approximately 70% amino acid sequence similarity with each other in E gene and approximately 50% similarity with other members of the flavivirus family (Chambers *et al.,* 1990a).

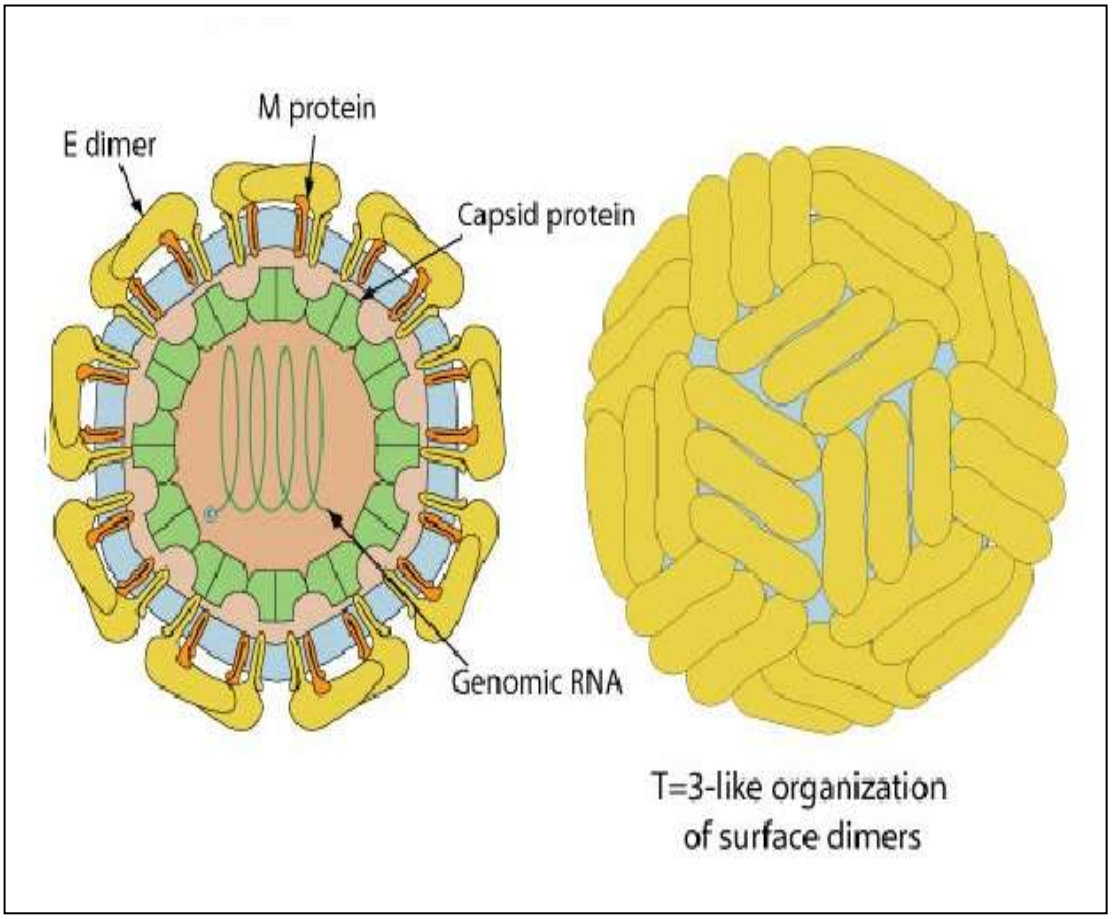

Figure 1.3: Diagram of a Flavivirus Virion.

The flaviviral virion is pictured above showing the main structural parts (Zhang *et al*, 2003).

The viral ss-RNA molecule undergoes translation into the infected cell resulting in the synthesis of a polyprotein precursor which undergoes proteolytic processing to generate 10 virus-specific polypeptides. It has been found that the sequence of the polyprotein precursor from the N to the C terminus would be as follows: NH2-anchC-prM-E-NS1-NS2A-NS2B/NS3-NS4A-NS4B-NS5-COOH (Falgout *et al.,* 1995; Valle *et al.,* 1998; Matusan *et al.,* 2001). The body of sequence information indicates that flaviviruses share the same genomic organization and presumably the same mechanism of gene expression and viral replication (Chambers *et al*., 1990a). The first 3 proteins constitute the structural proteins. They form the virus together with the package RNA molecule called capsid (C, 12-14 kDa), membrane (M, and its precursor prM, 18-22 kDa) and envelope (E, 52-54 kDa), all being encoded in the first quarter of the genome. The remainder of the genome encoded the nonstructural proteins (NS) numbered 1 to 5 in the order of synthesis (Henchal *et al.,* 1990).

1.2.4 Strains of Dengue Virus

It has been shown that DENV proteins contain viral structural and non-structural proteins derived from a large precursor polypeptide, encoded by a long open reading frame ssRNA (Rice, 1990; Chambers *et al*., 1990a). Translation starts at the first AUG codon of ssRNA genome and individual viral proteins are formed by co- and post-translation proteolytic processing of the precursor peptide by host and virus proteases (Rice *et al*., 1985; Hahn *et al*., 1988). It has been proven that the host "signalases" in the lumen of the endoplasmic reticulum is probably responsible for the proteolytic reaction at the N termini of prM, E, NS1 and NS4b (Perlman *et al.,* 1983; Henchal *et al.,* 1990). (Figure 1.4).

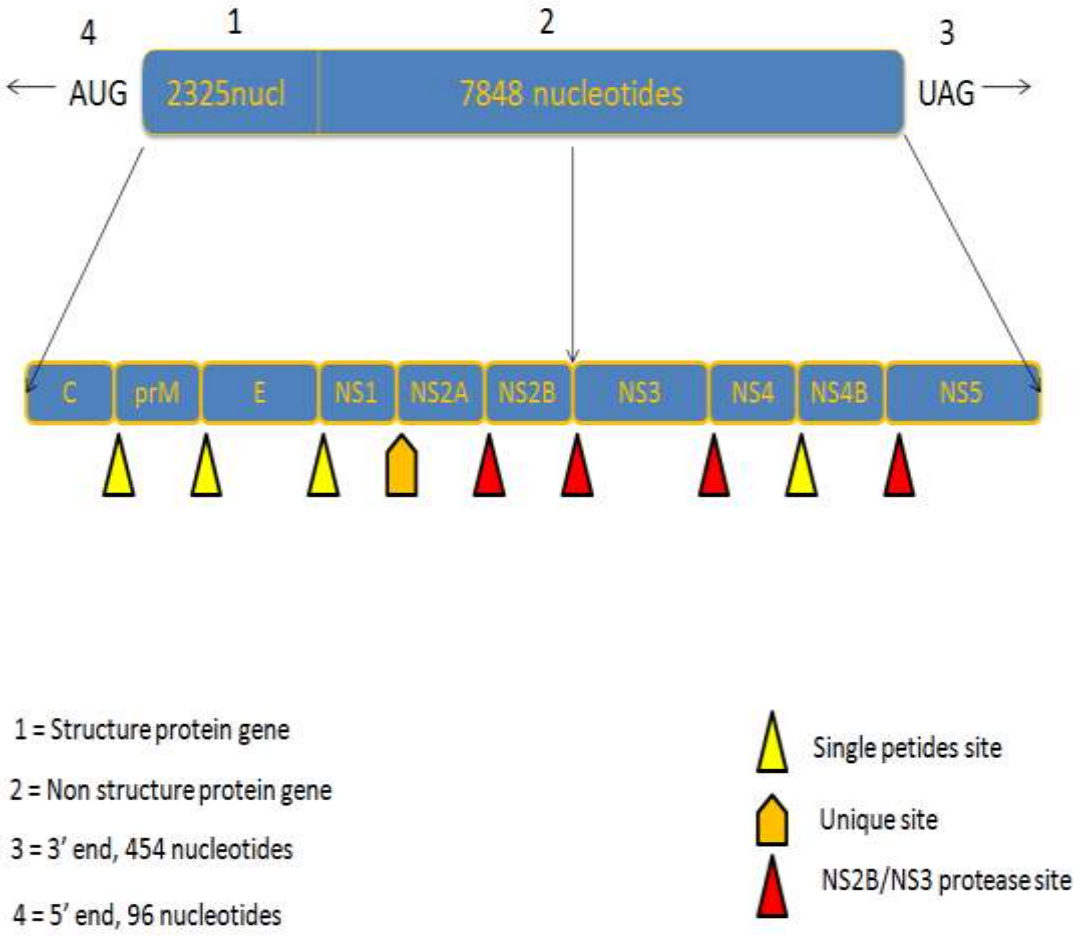

Figure 1.4: Diagram of the Flaviviral Genome.

Figure 1.4 shows the structural proteins of Capsid (C), prM, and Envelope (E) with the 7 non-structural proteins. Yellow triangles show possible cleavage sites by signalase enzyme. Red triangles point to possible cleavage by protease which acts after two basic amino acids.

1.2.4.1 Structural Proteins

The mature virion contains 3 structural proteins, specifically the capsid C protein, a non-glycosylated membrane protein M and a glycosylated envelope protein E (Mason *et al.,* 1987). Capsid protein (Molecular weight, 13-16 kDa) is the first viral polypeptide and is synthesized during the translation of virus genome which is rich in lysine and arginine amino acids (about 25%). This highly basic character enables it to interact with the virion ssRNA (Deubel *et al*., 1988; Rice *et al*., 1985). In addition, the C protein contains a carboxyl-terminal of a hydrophobic stretch of amino acid containing 20% of the total C protein amino acid residues (Zhao *et al*., 1986).

The viral NS2B/NS3 protease complex catalyzes cleavage at the COOH (carboxyl) terminus of the C protein on the cytoplasmic side of the endoplasmic reticulum membrane where the C-preM (M) protein cleavage occurs. This is the only site in the structural polyprotein region that is cleaved by this enzyme. Signal peptidase cleavage at the C-prM junction is greatly enhanced in the presence of the viral NS2B/NS3 protease or when prM is expressed using constructs, which do not include the C protein-coding region (Stocks *et al.,* 1998). This cleavage of prM protein results in the formation of 8 kD (Randolph *et al*., 1990).

The E glycoprotein appears as a homotrimer on the surface of mature virions. There are 12 perfectly-conserved cysteine residues that from six disulphide bridges which in turn generate three non-overlapping antigenic structural domains consisting of at least 16 distinct epitopes (Henchal *et al.,* 1990; Volk *et al.,* 2007). The location of these disulphide linkages provides a structural model for the envelope protein (Nowak *et al.,* 1987). The large envelope glycoprotein has a molecular weight of 51-60 kDa (Mason *et al.,* 1987). The exact mechanism of how E protein attached to the cell surface molecules and facilitates DENV entry is still unknown (Abd-Jamil *et al.,* 2008).

1.2.4.2 Non-Structural Proteins

The non-structural proteins consist of seven proteins which are named NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Cahour *et al*., 1992; Falgout *et al.,* 1995) and three highly conserved hydrophobic proteins (NS2A, NS4A and NS4B) (Mackow *et al*., 1987; Chambers *et al*., 1990a). The non-structural protein NS1 is a glycoprotein with a molecular weight of 46 to 50 kDa and is synthesized in the rough endoplasmic reticulum as a hydrophilic, monomeric glycoprotein. The NS1 protein exists as cell-associated, cell surface and extracellular non-virion forms (Schlesinger *et al.,* 1985; Lee *et al.,* 1989; Mason, 1989; Smith and Wright, 1985). The flavivirus NS1 has been recognized as an important immunogenic in infection and has been shown to play a role in protection against diseases (Schlesinger *et al*., 1985; Henchal *et al*., 1988). It has been shown by the anti-soluble complement fixing (SCF) anti-bodies in sera from patients undergoing secondary but not from primary infections (Falker *et al.,* 1973). This supports the findings that the NS1 proteins of DENV are highly immunogenic and can induce humoral immune response after primary DENV infection (Huang *et al*., 2001). This glycoprotein expresses serotype specific, complement-fixing antigenic determinates. The NS1 protein is capable of eliciting a protective immune response in animals (Zhang *et al.,* 1988; Putnak *et al.,* 1991). Therefore, it is hoped to design effective DENV vaccines and the induction of immunity without stimulating the production of virion-reactive anti-bodies.

The NS2 region consists of two hydrophopic proteins, NS2A and NS2B, with a molecular weight of about 14.5 kDa and 20 kDa. Both do not constitute glycoprotein. NS2A and NS2B have been proposed to play a role in modulating proteolytic processing of the nonstructural protein (Preugschat *et al*., 1990; Falgout *et al*., 1991).

It has been known that the NS3 is the second largest viral protein with a molecular weight of 68-70 kDa. It has been shown that the NS3 is a bi-functional, having a protease activity which is needed for the processing of the polyprotein at sites where the cellular proteases will not cleave (Preugshat *et al.,* 1990; Falgout *et al.,* 1991; Yamshchikov and Compans, 1995). It is also needed for nucleoside triphosphatase/helicase activity which is also associated with viral RNA replication (Wengler, 1993). It has recently been reported that NS3 has a serine protease domain (NS3pro) that requires the conserved hydrophilic domain of NS2B for protease activity in the cleavage of the plyoprotein precursor at sites following two basic amino acids (Yusof *et al.,* 2000). The DENV NS3 proteases share with other flavivirus NS3 proteases with trypsin-like character with a classic serine protease catalytic triad (His^{51}, Asp^{75} and Ser^{135}) (Bazan and Fletterick, 1990). The NS2B and NS3 form a stable complex that cleaves NS3 only in mammalian cells but not in insect cells (Arias *et al*., 1993). The cleavage might be part of a regulatory mechanism occurring only during virus replication in mammalian cells. The crystal structures of the protease domain alone and in complex with an inhibitor have been reported (Murthy *et al*., 2000). The NS2B/NS3 protease complex is one of the primary targets for the development of anti-DENV drugs as it is required for viral replication (Tomlinson *et al.,* 2011).

The NS4 consists of proteins NS4A and NS4B; both are hydrophopic with a molecular weight of 16 kDa and 27 kDa. NS4A and NS4B are not well known. It has been suggested that they may serve to anchor the viral replicase to the cellular membranes (Chambers *et al.,* 1989). In addition, they have been shown to contribute to the inhibition of IFN-α/β response of the infected host cell (Munoz-Jordan *et al.,* 2005). Recent studies have shown that NS4A plays a role in the induction of membrane alterations in the infected cells that could serve as a scaffold to anchor the viral replication complex (Miller *et al.,* 2007).

The NS5 is the largest and most highly conserved protein with a predicted molecular weight of 103-104 kDa containing several sequence motifs believed to be common to viral RNA polymerases (Mandl *et al*., 1988; Chambers *et al*., 1990b). NS5 contains the sequence glycine-aspartate-aspartate, which is characteristic of RNA polymerases (Rice *et al.,* 1986) encoded by ss RNA viruses *(*Mandl *et al.,* 1989; O'Reilly and Kao, 1998). The role of NS5 as the viral RNA polymerase and the generation of the N-terminus by cleavage in the cytoplasmic compartment suggest that NS5 is located in the cytoplasm although it is associated with membranes (Speight and Westaway, 1989).

1.2.5 Immune Response

The primary infection of DENV can be characterized by the appearance of immunologybulin M (IgM) and IgG in 5-7 days after the illness onset (Henchal *et al,* 1987; Henchal and Putnak, 1990; Guzman *et al*., 2010). Interestingly, the highest titers of IgM antibody are produced not only in primary dengue infections but in secondary and tertiary infections (Innis, 1989; PAHO, 1994; TDR and WHO, 2009; Guzman *et al*., 2010). In primary dengue infections and after shorter periods in secondary infections, the IgM antibody is transient and disappears in 30-90 days after the illness onset. However, IgG antibody persists for at least 50 years and probably throughout the life of the patient. In addition, the peaks of IgG titers reach the maximum values at days 14 to 21 after the onset of illness especially in the first dengue infection and seldom exceed 1:640 to 1:2180 (Guzman, *et al*., 2010). However, there is an immediate anamnestic IgG immune response to dengue complex or flavivirus-specific antigenic determinants in secondary infections. It has been shown that both IgM and IgG antibodies neutralize DENVs and infection provides life-long immunity to that specific DENV serotype.

Interestingly, both IgM and IgG dengue antibodies can be cross-reacted with other flavivirus antigens, including yellow fever and encephalitis viruses. However, crossing with viruses in dengue complex is more extensive than with other flaviviruses, thus making interpretation of serologic result very difficult.

In geographical areas where there is an endemic of several flaviviruses, definitive laboratory diagnosis can only be made by virus isolation and plaque reduction neutralization for the patients with primary infections. Normally, a combination of clinical, epidemiologic and serologic data is used to diagnose dengue and other flavivirus infections. Although, IgG antibody persists for several years, its presence in a single serum sample is not diagnostic unless it occurs at high titer ($\geq$ 1: 1280 by hemagglutination inhibition or $\geq$ 1: 256 by complement fixation), which is considered presumptive evidence of a recent infection. It has been known that the lower IgG titers simply indicate a previous infection at some time in the past. Paired serum samples are then required to demonstrate a fourfold or greater rise in specific IgG antibody. The presence of detectable IgG antibody in a single serum sample, however, is considered to be diagnostic because it does not persist for long periods. However, since IgM antibody does persist for up to 90 or more days, the diagnosis is considered presumptive. There is some evidence that cell-mediated immunity may play a role in terminating dengue infection T-cell clones that killed DENV infected cells as this has been identified in at least one immune person.

1.2.6 Prevention and Control of Dengue

Currently, the solution to prevent dengue infection is either to control the vector that transmits the virus or through the use of vaccine; however, no dengue vaccines are available. This has also lead researchers to develop new genetically engineered vaccines; but that will be many years before an effective, safe and economical vaccine is commercially available. Therefore the only available way to prevent dengue infection is to control the mosquito vector that transmits the virus.

Unfortunately, controlling *Aedes Aegypti*, the DENV vector, like aiming to kill the adult mosquito or to prevent the larvae stages, is not as easy as it seems. The recommended method for the past 20 years was the use of ultra low volume spraying (ULV) insecticides to kill adult mosquitoes (Gratz, 1991). It was recently shown in field trials in Puerto Rico, Jamaica and Venezuela that this method is not effective in significantly reducing natural mosquito population for any length of time. At present, fogging is another method of controlling but this has also been shown to be ineffective.

The only effective method left for controlling *Aedes Aegypti* is a citizen-based source reduction, that is to eliminate larva habitats occurring in the domestic environment or more importantly, terminate the adult mosquitoes. Mosquito control programs, therefore, must be a community-based integrated intervention. People living in *Aedes Aegypti* infested communities must be educated to accept responsibility for their own health destiny by aiding government agencies to control the vector mosquitoes and thus prevent an epidemic of DF/DHF/DSS.

On the other hand, early recognition and proper management is the key to keeping DHF/DSS fatality rates low. Data from countries such as Thailand showed that

prevention of excess mortality associated with DHF/DSS can be achieved by educating physicians in endemic areas on clinical diagnosis and management of DHF/DSS.

1.2.7 Dengue Virus Challenges

DENV infection is a worldwide health problem affecting tens of millions of people. The presence of four DENV serotypes had led to increased DF reported cases (9100 million), with an associated increased incidence of DHF (500,000 cases reported), with 25,000 deaths and 2.5 billion people at risk of DF (Halstead, 1992; Monath, 1994; Gubler and Clark, 1996; Gubler, 1998; McBride and Othman, 2000; Guzman *et al.* 2010) with a high mortality rate in children (Gubler, 1998; McBride and Ohmann, 2000; Guzman *et al.* 2010).

Many contributing factors are responsible for the dramatic increased incidence and geographical expansion of DHF/DSS. The most important factors are: continued urbanization of the tropics, changing life-styles, increased air travel and lack of effective mosquito control. These trends are anticipated to continue to become a major cause of hospitalization and deaths among children unless something is done to reverse it. More effective and integrated prevention and control strategies must be developed and implemented worldwide in the tropical regions. As a vector-borne illness, millions of individuals are at risk of acquiring a DENV infection. Hence, dengue vaccine and drug antiviral are urgently needed. Efforts towards the development of an economical, safe and effective treatment against dengue must be accelerated. There are many challenges facing the world at this moment with regard to DF control. Most importantly, there is no effective mosquito-control program and if so, it is either too expensive or too difficult to implement and maintain. Secondly, an effective, safe and economical vaccine is not

commercially available at the present time. However, efforts in combating dengue should be continued until such safe, effective and cheap vaccine is available. Another important consideration for the vaccine development is that the envelope (E) protein is different between the different serotypes, which are particularly important for vaccine development as the body produces neutralizing and protective antibodies (Feighny *et al.,* 1992; Lin *et al.,* 1994). It is also the major haemagglutinin of the virus (Della-Porta and Westaway, 1977). Antibody to one serotype does not neutralize the other serotypes. Therefore, a broadly effective dengue vaccine must be produced against all four serotypes to prevent DENV infection. Finally, efforts are needed for the development of safe and effective antiviral medication which can help millions of people around the world.

1.3 Plant under Study

Historically, people have been using plants for different purposes like food resources, domestic tools, housing and fires. One other use was for medicinal purposes - in the fresh form as taken orally or in the processed form as ointment or liniments this is well-known today as a "traditional medicine". These traditional medicines have their roots in Egypt, China and India. Traditional medicine imparts the use of a mixture of various plant parts and has been a popular method of treating diseases which is still widely practised in this region of the world.

A large portion of the world's rainforest is within the Southeast Asia region. Malaysia alone is home to some 15,000 species of higher plants. Goh, in 1993 (Goh *et al.,* 1993) reported that only more than one thousand of these species have been subjected to chemical screenings (mostly partially) and very few out of these have been

exposed to detailed chemical or pharmacological studies (Goh, 1999; Kong *et al.*, 2003). When applying these figures, one can conclude that only a small proportion of the plant kingdom has been investigated thoroughly. Some studies demonstrated the increasing number of publications in the scientific literature on plant-derived chemicals which reflect the increased trend towards this area (Qin and Xu, 1998; WHO, 2009).

The World Health Organization estimates that approximately 70–95% (WHO, 2011) of the world's inhabitants (1600 million) rely mainly on traditional medicines for their primary health care. Analyzed data on prescriptions dispensed from community pharmacies in the USA from 1959 to 1990 show that out of 119 drugs, 74% was the result of chemical studies directed at isolating the active substances from plants used in traditional medicine while 25% contained plant extracts or active principles derived from higher plants. Research and development into nature's many gifts in jungles to find new drugs and other related products is intensive.

1.3.1 Quercus Infectoria

1.3.1.1 Distribution and Description

Also known as *manjakani* in Malaysia and oak galls in America, *Quercus Infectoria* belongs to the *Fagaceae* family. *Quercus Infectoria* contains large amounts of ellagic acid, gallic acid, methyl oleanate, β-sitosterol, amentoflavone, hexamethyl ether and syringic acid (Dar *et al*., 1976; Ikram and Nowshad, 1977; Hwang *et al*., 2000). The other name for *Quercus Infectoria* is *Quercus Lusitanica Linn*. The *Fagaceae* family has 8 genera and about 900 species, *Fagus* 10 spp, *Costanea* 12 spp, *Quercus* 450 spp (Henrietta, 2009). The following species are used medicinally: in Asia Minor and Malaysia – *Quercus Infectoria*; in China – *Quercus Buangeana Forbes*; in

Europe – *Quercus ilex Linn, Quercus Robur Linn, Quercus Dentata Thunb, Quercus Glauac Thunb*; in North America – *Quercus Alba Linn, Quercus Discolor Ait, Quercus Falcate Mich, Quercus Quercus Velutina Lam, Quercus Virginiana Mill*; in Austria, Hungary and Russia – the bark of *Quercus Pedunculata Ehrhardt*; in France, Germany, Sweden and Switzerland – *Quercus Robur Linn*; in Germany – *Quercus Sessiliflora Salisbury*; in Sweden – *Quercus Sessilis Ehrhardt*. (Kirtikar and Basu, 1980).

Quercus Infectoria is a small shrub of 4 to 6 feet in height. The stems of *Quercus Infectoria* are crooked, the leaves borne on short petioles, 1 to 1½ inches long (Samuelson, 1992; Henrietta, 2009). The leaves are oblong with a few coarse mucronate teeth on each side. It has a bright-green color and is shiny on the upper side. The fruits or acorns are solitary, long and obtuse while the cup is scaly and hemispherical. Galls are subglobular, 1 or 2 cm. (2/5 to 4/5 inch) in diameter, smooth, heavy and hard. It is often with a circular hole near the middle, communicating with the central cavity containing either the partly developed insect or pulverulent remains left by it, is nearly inodorous and taste strongly astringent (Figure 1.5).

Figure 1.5: *Quercus Infectoria* (*manjakani*)

1.3.1.2 History

Dyer's oak or gall oak trees are indigenous to Greece, Asia Minor, Syria and Iran (Kirtikar and Basu, 1980; Basri and Fan, 2005; Umachigi, 2008). These galls are produced by the puncture of the foliaceous or cortical parts of the tree by an insect known as *Cynips Gallae Tinctoriae, Olivier* (*Cynips Quercusfolii*, of *Linnaeus*, or *Diplolepsis Gallae* T*inctoriae*, of *Geoffroy*) (stinging wasps). After the female has made a puncture, she deposits her eggs. This will lead to a spontaneous chemical reaction caused by the penetration which produces a roundish hard ball called an oak gall (Zakaria *et al.,* 1994). Due to irritation, an excrescence is soon formed that quickly leads to the formation of a small tumor of hypertrophied tissue enclosing the eggs what are called galls. Meanwhile, the larva of the insect develops, changing first into the pupa and then into the imago and finally the insect. Then the young insect starts to perforate its prison and escapes. Hence two commercially galls are available; those which are gathered just before the escape of the insect (bluish-black, heavy, not yet perforated and constitute the commercially black, blue or green galls), and those galls from which the insect escaped (commonly larger, lighter colored, perforated and less astringent white galls which commercially command a lesser price) (Zakaria *et al.,* 1994; Hawang, 2000).

1.3.1.3 Chemical Composition

Water is the best solvent for the extraction of galls and proof-spirit for pure alcohol or ether acts more feebly upon them. The chemical reaction of galls in decoction or tincture is similar to those named for tannic acid or tannin (gallotannic acid) as this substance exists in galls in large proportions. The content of tannins varied from 24 per

cent in European galls (German, English and Italian) to 61 per cent in Aleppo galls and 69 per cent or more in Chinese galls. Tannic galic (60-70%), ellagic acids, starch, essential oils and anthocyanins have been isolated from *Quercus Infectoria* (Redwane *et al.,* 1998). Vitamins A and C, calcium, iron, tannic and gallic acid, fiber, protein, carbohydrates and hexagalloyglucose (3-O-digalloyl-1, 2, 4, 6-tetra-O-galloyl-B-D-glucose) have also been isolated (Hwang *et al,* 2000). In general, galls comprise a large amount of tannins, gallic acid, syringic acid, ellagic acid, sitosterol, amentoflavone, hexamethyl ether, isocryptomerin, methyl betulate, methyl oleanate and hexagalloyl glucose (Hwang *et al.,* 2000; Dar *et al.,* 1976; Ikram *et al.,* 1977).

1.3.1.4 Action and Medicinal Uses

Quercus Infectoria is an effective medicinal plant distributed widely in the Middle East and Asia. It has been used in Asian countries for centuries as oriental traditional medicine for treating inflammatory diseases (Anonymous, 1995). The galls of *Quercus Infectoria* have many medicinal values, with its astringent being considered to be one of the commonest. It is used in all cases where astringents are indicated, such as in chronic dysentery and diarrhea. It is also used for children who suffer from chronic diarrhea by boiling the galls with milk (Sawangjaroen *et al.,* 2004). In countries such as India, Indonesia, China and Malaysia, it is also used after childbirth and to treat vaginal discharge. Other pharmacological uses are to cure hemorrhoids (Lefkowitz *et al.,* 1999; Bourke and Moynagh, 1999; Kaur, *et al.,* 2004) and also as antidiabetic, antitremorine, local antipyretic and antiparkinsonian anaesthetic (Dar *et al.,* 1976; Dar *et al.,* 1979; Hwang *et al.,* 2000). Accumulated experimental evidence indicates that the galls of *Quercus Infectoria* contain antibacterial components against varied bacterial strains such as *Staphylococcus Aureus, Streptococcus Mutans, Streptococcus Salivarius,*

Porphyromonas Gingivalis and *Fusobacterium Nucleatum* (Voravuthikunchai *et al.,* 2008; Chusri *et al.,* 2009; Basri *et al.,* 2012). Other pleiotropic therapeutic activities of *Quercus Infectoria* extract have been shown, particularly as an inflammatory (Pithayanukul *et al*., 2009; Kaur *et al.,* 2004), antioxidant activity (Kaur *et al*., 2008) and hepatoprotective agent against liver injuries (Pithayanukul *et al.,* 2009). Interestingly, further investigations on *Quercus Infectoria* gall extracts have shown the larvicidal activity against Anopheles Larva (Aivazi *et al.,* 2009) and urban nuisance mosquito (Redwane *et al.,* 2002), giving an efficacy for the development of potential mosquito larvicide.

1.4 Natural Products Evolution

Traditional medicine encompasses systems knowledge developed over generations molded with various cultures before the era of modern medicine. The World Health Organization defines traditional medicine as the health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses or maintain well-being (WHO, 2008).

Traditional medicine utilized many plants. Of these, some have been explored in modern pharmacological and medical applications such as flavonoids and tannins which have been isolated from different plants and used for the study and design of new pharmaceuticals (Da Silva *et al.,* 2008). In most cases both traditional and modern medicines originate from similar raw materials. These may be dried herbs or parts of extracts which are used in treatment. In traditional medicine, these ingredients are used

in the raw form while in modern medicine, the raw extract is processed to obtain the active compounds.

Previous studies by Hadinur et al (Hadinur *et al.*, 2003) have shown that the aqueous extract of *Quercus Infectoria*, has activity against DENV-2 NS2B/NS3 protease complex. However, no specific compound from the extract was reported.

In this study, we isolated pure compounds from the aqueous extract of *Quercus Infectoria* and screened against NS2B/NS3 DENV protease. The compounds isolated and tested are ellagic acid and gallic acid.

1.4.1 Ellagic Acid

Ellagic acid (Figure 1.6) is a natural phenol antioxidant. It has the IUPAC name 2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione and has a molecular weight of 302.197g/mol. Ellagic acid has been detected in many studies with nuts, berries and fruitsin where total ellagic acid was measured by analyzing the ellagic acid content of extracts after acid hydrolysis (Daniel *et al.,* 1989; Häkkinnen *et al.,* 2000). Ellagic acid is of particular interest from a dietary viewpoint as it has been reported to have antiviral (Corthout *et al.,* 1991) and antioxidant activity (Kalt *et al.,* 1999) and provide protection against cancers of the colon (Rao *et al.,* 1991), lungs and oesophagus (Stoner and Morse, 1997).

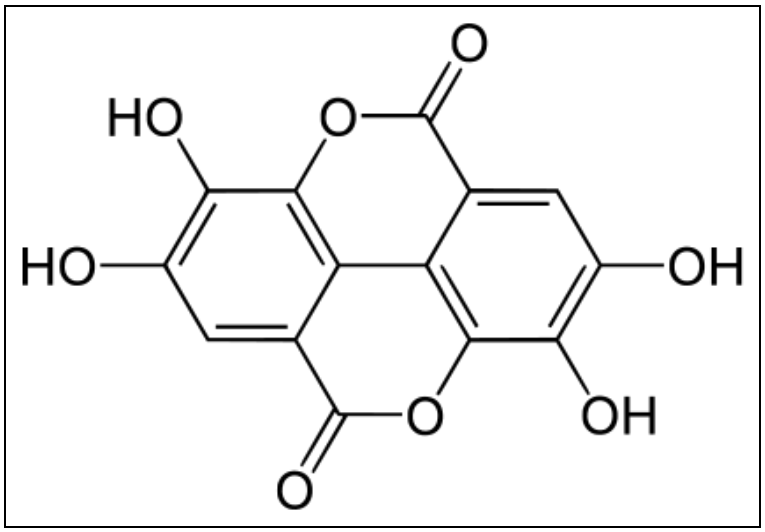

Figure 1.6: Structure of Ellagic Acid

1.4.2 Gallic Acid

Gallic acid (Figure 1.7) is one of the natural phenolic compounds with a lower molecular weight of 170.12g/mol and the IUPAC name known as 2,3,4-trihydroxybenzoic acid. It is widely found in the plant kingdom like green tea, tea leaves, strawberries, barks, apple-peels, bananas, gallnuts, grapes, lemons and pineapples (Madlener, S. *et al.,* 2007). Gallic acid is a strong natural antioxidant (Madsen *et al.,* 1995; Nakatani, 1992; Gali *et al.,* 1992) and is pharmacologically active as an antimutagenic, anti-inflammatory, antiallergic and anticarcinogenic agent (Wang *et al.,* 2007; Gali *et al.,* 1991).

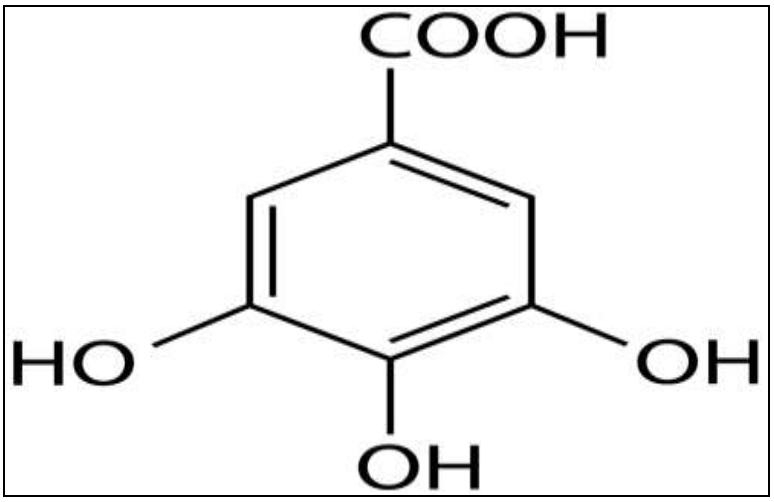

Figure 1.7: Structure of Gallic Acid