4.0 Discussion

Concerning the new evolving viral diseases affecting human health, DF has assumed the utmost significance in urban areas, being mediated through a mosquito vector, *Aedes Aegypti* and *Aedes Albopictus.* According to Duane Gubler from the Division of Vector-Borne Infections Diseases of CDC in Fort Collins, Colorado, dengue is one of the most important emerging tropical diseases in the 21st century (Halstead *et al*., 2005; Guzman, *et al.,* 2010; Allicock *et al.,* 2012). Interestingly, DHF affects most Asian countries and has become a leading cause of hospitalization and death, especially among children (Monath *et al*., 1996; Racloz *et al.,* 2012). It is estimated that there are nearly 100 million yearly reported cases of DF (Halstead, 1992; Monath, 1994; Gubler *et al.,* 1996; Gubler, 1998; McBride *et al.,* 2000; Guzman *et al.,* 2010) with a high mortality rate, especially in children (Gubler 1998; McBride *et al.,* 2000; Marijke *et al.,* 2012). In Malaysia, DENV-2 is the most predominant among the four serotypes (Fong *et al.,* 1998), and the first reported case of DHF in Malaysia was in 1962 (Rudnick *et al*., 1965). As there is no vaccine available, the search for antiviral product is imperative. For this reason, intensive efforts are being directed at research on DENV, such as the development of vaccines and therapeutic agents.

The medicinal value of any plant depends on the presence of active chemical constituent(s). In the selection of plants for scientific study, Slish and co-worker stated that the probability of finding a compound with biological activity from plants which has been used traditionally is higher (Slish *et al*., 1999). To demonstrate this assumption, an experiment was performed to evaluate the effectiveness of traditionally used plants.

In this study *Quercus Infectoria* (known as *manjakani* in Malaysia) has been selected (Sawangjaroen *et al.*, 2004; Kaur, 2004; Wiart *et al.*, 2004; Basri *et al.*, 2005). *Quercus Infectoria* is traditionally used as an astringent; it is used in all cases where astringents are indicated, as in chronic dysentery and diarrhea. It can also be used for children who suffer from chronic diarrhea by boiling the galls with milk (Sawangjaroen *et al.,* 2004). Other prospects of its traditional practices in countries like India, Indonesia, China and Malaysia, is its use after childbirth and to treat vaginal discharge. Numerous researches have demonstrated its other medicinal values. Besides the ability to cure hemorrhoids (Lefkowitz *et al.,* 1999; Bourke *et al.,* 1999; Kaur *et al.,* 2004), there are many other pharmacological uses for this plant such antiparkinsonian (Dar *et al.,* 1976; Dar *et al.,* 1979; Hwang *et al.,* 2000), antibacterial activities (Voravuthikunchai, S. P. *et al.,* 2008), antioxidant activities (Kaur, G. *et al*., 2008), and anti-inflammation (Pithayanukul, P. *et al*., 2009).

Previous studies by Hadinur showed the aqueous-based extract of *Quercus Infectoria*, partially-investigate extract, do inhibit the activity of DENV-2 NS2B/NS3 protease complex (Hadinur *et al.,* 2003). However, he did not identify any specific compound from the extract.

In this study, a second level was done to evaluate the inhibitory potential of the fraction or compound that has shown activity against DENV-2 protease complexes from the aqueous crude plant extract. The aqueous extract of *Quercus Infectoria* was screened in the preliminary testing. The first screening assay of *Quercus Infectoria* crude extract showed strong inhibition towards the DENV-2 protease (greater than

90%). From the aqueous extract of *Quercus Infectoria*, two compounds (ellagic acid and gallic acid) were identified to have activity against NS2B/NS3 protease complex.

Ellagic acid and gallic acid are phenolic compounds, which come from a large and diverse group of compounds that makes up one of the major families of the secondary metabolites in plants that can be classified into soluble and non-soluble compounds (Harbone *et al.,* 2000). Polyphenolic compounds are involved in many metabolic reactions and are widely used as antioxidant food additives (Serrano, A. *et al.*, 1998).

Ellagic acid (2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione) is a naturally occurring polyphenolic compound found in different vegetables, nuts and fruits like pomegranate, red raspberry, strawberry, blue berry and walnut in the forms of hydrolysable tannins called ellagitannins (ThulStrup *et al.*, 1999). Ellagic acid has been explored by numerous in vitro (Ashoori *et al.,* 1994; Majid *et al.*, 1991) and in vivo (Sai-Kato *et al.,* 1995; Ashoori *et al.,* 1994) studies over the last few years which provided evidence of its important pharmacological properties, including antioxidant, anti-inflammatory (Hassoun, E. A. *et al.,* 2004; Suzuki, N. *et al.,* 2009), and anticarcinogenic activities (Falsaperla, M. *et al.,* 2005; Glauert, H. P. *et al.,* 2010). Through the effect on p53 and p21 expression, ellagic acid causes G1 arrest and apoptosis in cancer cells in vitro. Other pathways are through the effect on DNA modifying enzymes and polymerase that inhibit the growth of cancerous cells (ThulStrup *et al.,* 1999), and also by breaking hydroxyl group thus terminating the propagation of free radical-mediated reactions working as an antioxidant (Gil *et al.,* 2000). Ellagic acid was also found to be effective in the treatment of hepatitis B viral

infection (Shin *et al.,* 2005). It was shown in experimentally HBeAg-producing transgenic mice (HBeAg-Tg) that host immune tolerance induced by HBeAg during HBV infection, a viral strategy to guarantee HBV infection, can be overcome by ellagic acid; thus it can be used as a therapeutic for HBV-carriers (Kang, E. H. *et al.*, 2006).

Gallic acid (2, 3, 4-trihydroxybenzoic acid) is one of natural phenolic compounds. It can be considered the simplest prototype of vegetable tannins, with a lower molecular weight, widely available in the plant kingdom. Gallic acid is significantly present in human diets and is found in a wide variety of foodstuffs and beverages such as tea and wine (Kahkonen *et al.,* 1999). Its chemical and biochemical properties have been evidenced to be anti-inflammatory, antimutagenic, antibacterial, antiviral and immune-stimulating; the main mechanism proposed for their protective action has been related to their free radical scavenging activity (Cappelli *et al.,* 2005). There is more and more proof indicating that it could be used as drugs for the prevention of pathologies such as cancer due to their antiproliferative and cytotoxic qualities, and strong antimutagenic, anticarcinogenic and antioxidant activities (Wang *et al.,* 2007; Serrano *et al.,* 1998). Gallic acid, as well as other polyphenolic substances, is a compound which is able to inhibit the growth of cells for several types of tumor (Saleem *et al.,* 2002; Zhang *et al.,* 2005).

In a recent study in 2011, the author demonstrated the strong antioxidant effect of gallic acid grafted chitosan in comparison with plain chitosan treated in the same conditions without gallic acid grafting. It was concluded that gallic acid improved the antioxidant capability; furthermore, it also exhibited good cyto-compatibility and

effectively inhibited the formation of intracellular reactive oxygen species (ROS) in time and dose-dependent manner (Cho, Y. S. *et al.*, 2011).

To the best of my knowledge, there have been no studies utilizing these two compounds (ellagic acid and gallic acid) as an antiviral drug against DENV-2 NS2B/NS3 protease complex. For this reason, this study commences to establish and document the antiviral properties that these two compounds may possess.

Purification of virus-specific protein is an essential step for the success of the project. This protein should be isolated from a natural host to ensure proper folding, modification and biochemical activity. For this purpose, precursor NS2B/NS3 serine protease was expressed in competent Escherichia coli strain XL1-Blue MRF. A cofactor (NS2B) for DENV-2 NS3 protein activity is required as shown in previous studies (Chambers *et al.*, 1993; Falgout *et al.*, 1993; Yusof *et al.*, 2000). Both proteins formed NS2B/NS3 protease complex, which recognized a consensus sequence of dibasic amino acids adjacent to the cleavage junction. However, DENV-2 NS2B/NS3 protease purified recombinant shared a similar enzymatic activity.

A substrate (Boc-Gly-Arg-Arg-MCA) has been tagged with fluorescence particle, AMC, at the cleavage junction. When the purified recombinant DENV-2 NS2B/NS3 protease complex react, AMC particles will be released from the cleavage junction and the protease activity or the enzymatic velocity can be detected by fluorescence spectrum. Hence this approach will provide a sensitive, rapid and quantitative assay that can determine the biochemical and kinetic properties of the protease.

A previous study performed at University of Malaya for the inhibitory potential of the water-base extracts from tropical plants on DENV type 2 protease activities was published in 2003. In this study, 12 different crude extracts were tested for their inhibitory potentials; amongst these 12 extracts, *Quercus Infectoria* showed a strong inhibition towards the DENV-2 protease (greater than 90%) (Hadinur *et al.*, 2003). In the study by Hadinur, methanol was used to extract the bioactive compound from *Quercus Infectoria* crude extract and the compound isolated was found to be methyl gallate. No further studies were carried out to isolate and characterize other bioactive compounds from the aqueous crude extract for this plant against DENV-2 NS2B/NS3 protease complex.

Our crude extract showed good solubility in water and its inhibition towards the DENV-2 NS2B/NS3 protease complex was more than 90%, which is comparable to Hadinur. We prefer to use the water-based extract due to the fact that water is a remarkable molecule whose properties are central to life. Furthermore, it can solubilize and modify the properties of biomolecules through hydrogen bonds and has a low side effect on humans.

To start with, the water-based crude extract from *Quercus Infectoria* was subjected to chromatographic purification to gain its active compound. The components of *Quercus Infectoria* where eluted using a modified solvent system from previously published techniques. Our system using silica gel column was eluted with hexane and, gradually increasing solvent polarity in the order of hexane-diethyl ether mixtures, diethyl ether 100% (v/v), diethyl ether-ethyl acetate mixtures, ethyl acetate 100% (v/v), ethyl acetate-acetonitrile mixtures, acetonitrile 100% (v/v), acetonitrile-methanol

mixtures and finally with pure methanol (100%(v/v), with an increase of 10% v/v- step ladder. The flow rate was set at 2 ml/min, and fractions were collected every 15-20 ml using glass vials.

At the end of the test, 5 fractions were obtained (as shown in Figure 3.9). Each of these fractions was then evaluated using the bioassay technique against DENV-2 NS2B/NS3 protease complex with different concentrations starting with 25ppm, 50ppm, 100ppm, 200ppm and 400ppm to determine the most active fraction. The result of inhibition assay showed fraction 1 and 2 to be the most active fractions against the protease complex with an inhibition of 80.03% and 71.92% at a concentration of 100ppm respectively.

Fraction 1 and 2 were then investigated with further purification in order to identify the active substance(s). As explained in the results, two fractions were found to be the most active among the others. These fractions were visualized under the short wavelength UV as a yellow spot; staining with ferric chloride-potassium ferric cyanide reagent gave the product a green spot. This suggests that these fractions have to be phenolic compounds. Further characterization of these compounds was carried out using High-performance liquid chromatography (HPLC).

According to the results obtained from TLC plate, 6 phenolic standard compounds, namely caffeic acid, ferulic acid, chorogenic acid, *p*-coumaric acid, gallic acid and ellagic acid were prepared. In the beginning, various proportions of acetonitrile-water system were used as mobile phases; however, the separation was not satisfactory. The presence of acid in a mobile phase system gave a much better

separation. The gradient elution of solvent A [water-acetic acid (25:1 v/v)] and solvent B (methanol) had a significant effect on the resolution of compounds. As a result, solvent gradients were formed using the dual pumping system, by varying the proportion of solvent A [water-acetic acid (25:1, v/v)] to solvent B (methanol). Solvent B was increased to 10% methanol (B) through the column isocratically with 90% solvent A for 5 min; then solvent B was increased to 30% methanol after 15 min, to 45% after 25 min, to 60% after 35 min, to 80% after 38 min, to 90% after 43 min, and then followed by isocratic elution with 90% methanol (B) after 45 min. Finally, the gradient was changed to 10% methanol after 48 min; this composition was held until 50 min at a flow rate of 1.0 ml/min.

A series of the standard mixture solutions of these 6 phenolic compounds were tested to determine the linearity between the standard mixture concentrations and peak areas. The standard response curve for each phenolic was a linear regression fitted to triplicate values obtained at each of the five concentrations (12.5-100 ppm, for each standard). The linearity relationship between peak areas and concentrations was good and the correlation coefficients (*r2*) were greater than 0.9974 for all curves.

During HPLC, the two compounds were identified to be closely related to ellagic acid and gallic acid. This was evident by comparing the standards' retention times with the compounds' retention times of fraction 1 and 2 (as shown in Figure 3.15 and 3.16). To confirm that another HPLC test was run by mixing the 6 standards with each fraction, cumulative effects were observed in two tests while using the standards, ellagic acid and gallic acid, which were presented by increasing the height of the peak.

Another confirmatory test was carried out to identify the structure of the isolated compounds; the isolated compounds were elucidated using ^{1}H and ^{13}C NMR spectra. The results were recorded on JEOL FT-NMR 400 MHz, using deuterated methanol-d4 (CD3OD) as a solvent with tetramethylsilane (TMS) as the internal reference. Ellagic acid was obtained: ^{1}H-NMR (400 MHz, CD3OD) δH: 7.51 (*s*); ^{13}C-NMR (400 MHz, CD3OD) δC: 109.6 (*s*); 111.9 (*s*), 114.2 (*s*), 137.6 (*s*), 141.1 (*s*), 149.6 (*s*), 161.6 (*s*); gallic acid; ^{1}H-NMR (400 MHz, CD3OD) δH: 7.05 (*s*); ^{13}C-NMR (400 MHz, CD3OD) δC: 110.4 (*s*); 122.09 (*s*), 139.7 (*s*), 146.4 (*s*), 170.5 (*s*). The chemical shift values for each compound (ellagic and gallic acids) were identified by comparing the results to earlier reported spectra data, EA (Li *et al.,* 1999; Kwak *et al.,* 2005; Thitilertdecha *et al.,* 2010), GA (Gohar *et al.,* 2003; Anastasiadi *et al.,* 2009).

The substrate concentration that produces half–maximal velocity, termed the Michaelis-Menten constant (Km) value, was determined using GraphPad Prism 5 software. When (S) is approximately equal to the Km, V is very responsive to changes in (S), and the enzyme is working at half-maximal velocity (Wood *et al.,* 1996), (S) is approximately equal to the Km, NS2B/NS3 is shown to possess Km values which are approximately the physiologic concentration of their substrate (Wood *et al.,* 1996). The substrate used is 100 µM, Km value is found to be 86.53 µM and V_{max} = 0.5255µmol/min; these values were obtained by using GraphPad Prism 5 under the Michaelis-Menten model.

The inhibitors were reported to be either competitive or non-competitive in their activities. An alternative way to classify inhibitors is by their site of action. For competitive inhibition, the inhibitor binds to the enzyme at the same site as the substrate

(the catalytic site); others which bind at some site (an allosteric site) removed from the catalytic site are classified as non-competitive inhibitors. The inhibitors of NS2B/NS3 protease activity are classified on the basis of whether there is an increase in inhibition by increasing the substrate concentration. In this study it was found that ellagic and gallic acids have the characteristic of non-competitive inhibitors. The data was analyzed using the Michaelis-Menten model under non-linear regression curve fit in GraphPad Prism 5 software and the type of inhibitors were classified according to *Alpha* value. ***"Alpha: Constant that determines mechanism. If Alpha=1, this is the same as non-competitive. If Alpha is very large, then the model approaches a competitive model. If alpha is very small (but greater than zero), the model approaches an uncompetitive model"***. The Ki value for ellagic acid was found to be 58.64 µM (Table 3.13), and the Ki value for gallic acid was 72.62 µM (Table 3.14). According to *Alpha value,* both inhibitors were determined to be non-competitive (Table 3.13 and 3.14).

In non-competitive inhibition, no competition occurs between substrate (S) and inhibitor (I) and the inhibitor usually bears little or no structural resemblance to the substrate. Hence, it may be assumed to bind to a different domain on the enzyme.

Many inhibitors have been isolated from plant resources and have been assessed against dengue viral infection. Sánchez and his co-workers analyzed the possible antiviral effect on DENVs of different flavonoids extracted from the Mexican plants, *Tephrosia Madrensis*, *Tephrosia Virediflora* and *Tephrosia Crassifolia*. Among the four tested flavonoids, glabranine and 7-O-methyl-glabranine showed an inhibition of 70% on the DENV at a concentration of 25 µM in a dose-dependent manner (Sánchez *et al.*, 2000). In another study reported by Zandi (Zandi *et al.*, 2011) where four types of

bioflavonones (quercetin, naringin, daidzein and hesperetin) were investigated against DENV-2 in Vero cells and $C_{6/36}$ cell lines, anti-DENV activity of these compounds was determined at different of stages of DENV-2 infection and replication cycle measured for Foci Forming Unit Reduction Assay (FFURA) and quantitative RT-PCR. The result from this study suggested that only quercetin significantly demonstrated anti DENV-2 inhibitory activity. However, the mechanism of inhibition of DENV replication was not explored.

Whitby, in his work on *Castanospermine*, a natural alkaloid derived from the black bean tree (*Castanospermum Australae*), reported that infection and viral spread of all four serotypes of the DENV can be inhibited with *Castanospermine* (Whitby *et al.*, 2005). In another work, two cyclohexenyl chalcon derivatives, 4-hydroxypanduratin A and paduratin A, isolated from *Boesenbergia Rotunda* exhibited competitive inhibitory activities towards DENV-2 NS2B/NS3 protease complex. In addition, two other compounds isolated from the same plant, namely pinostrobin and cardamonin, were observed to exhibit non-competitive inhibitory activities (Tan *et al.*, 2006). Talarico and his co-workers studied the antiviral properties of two sulfated polysaccharides obtained from red seaweeds against the four serotypes of DENV. The study found that there were variations in antiviral activity of the compounds depending on the viral serotype and the host cell, and this may be ascribed to differences in the virus-cell interaction leading to virus entry (Talarico L. B. *et al.*, 2005).

In our study, the results with regard to ellagic acid and gallic acid are as follows; ellagic acid and gallic acid showed an inhibition of 83.56 at concentration 100 ppm and 77.04 at concentration 100 ppm, respectively. This concentration is considered low

when compared to other studies using different plant extracts. When Parida evaluated the antiviral activity action of neem (*Azadirachta Indica Juss*) leaves aqueous extract, the isolated *Azadirachtin* was tested against DENV-2 infected $C_{6/36}$ cells; this extract showed an inhibitory effect against DENV-2 at a high concentration (1.897 mg/ml) when evaluated by cytopathic effect inhibition (Parida *et al.,* 2002). To maintain its strong inhibitory effect, using a compound with a lower concentration is a safer option when compared to a compound with a higher concentration. This can be observed from our results.

As mentioned previously, ellagic and gallic acids have been investigated for many other uses; however, only one study reported ellagic acid as an antiviral compound against DENV-2. Silva (2011) evaluated the antiviral action of phenolic components of two Spondias species against DENV-2 infected $C_{6/36}$ cells. The evaluation of antiviral activity against DENV-2 in C6/36 cells suggests that rutin and quercetin have potential for the development of an anti-DENV agent; however the author found that ellagic acid has a low activity against DENV-2 (Silva *et al.,* 2011). The findings in our study were dissimilar with the previous study by Silva which described the low potential of ellagic acid against DENV-2. Thus, our results have evidently confirmed the inhibition of DENV-2 replication by these natural compounds.