2.1 Materials

In this project, the aqueous extract of *Quercus Infectoria* was preliminary tested on DENV-2 NS2B/NS3 protease complex. The *Quercus Infectoria* plant used in this project was obtained from local market.

2.2 Chemical and Biological Agents

All chemicals used in this project were of the analytical grade or of the highest (HPLC) grade were purchased from Merck, Sigma-Aldrich and JT Baker.

The common solvents used included methanol, ethanol, ethyl acetate, hexane, Chloroform, diethyl ether, toluene, and chemicals included glacial acetic acid, glycerol, hydrochloric acid, potassium choloride, sodium-chloride, sodium-hydroxide, phosphoric acid, and zinc sulfate heptahydrate were purchased from Merck, Sigma-Aldrich and JT Baker. Tris base, yeast extract, tryptone casein peptone, agarose, ampicillin anhydrous and dimethylsulfoxide (DMSO) were purchased from Amresco. HPLC standards (caffeic acid, ferulic acid, chorogenic acid, *p*-coumaric acid, gallic acid and ellagic acid) were purchased from Sigma-Aldrich. Bovine serum albumin, bromophenol blue, brilliant blue R-250, bis-acylamide, N,N,N',N'-tetramethylene-diamine (TEMED), disodium ethylene diamino-tetracetic acid and sodium carbonate were purchased from Sigma-Aldrich. Silica gel for column chromatography 60 F 254 (mesh size 230-400; particle size 0.040-0.063 mm) was purchased from Merck (Germany). Barford reagent has been used for protein estimation from Bio-Rad.

NS2B/NS3 was purified using Isopropy1-B-D-thiogalactopyranoside (IPTG) purchased from Fermentas. Hepes Imidazole was purchased from Sigma-Aldrich; Iysozyme from Roche; Ni-NTA agarose from Qiagen; Ammonium sulfate from Merck;

prestained SDS-PAGE standards (Low range) from Fermentas. Protease assays using fluorogenic peptides substrate: Boc-Gly-Arg-Arg-MCA was purchased from the peptide institute, INC (Japan).

2.3 Escherichia coli

Competent Escherichia coli (E.coli) strain XL1-Blue MRF cultures were transformed with pQE-NS2B (H)-NS3 (185amino acid) plasmid was constructed in our laboratory.

2.4 Media for Bacterial Cell Growth

i. Luria-Bertani (LB) agar

Yeast extracts	0.5g
Tryptone	1.0g
NaCl	1.0g
Agar	1.5g
Distilled H_2O	100ml

(For preparation of four LB agar plates)

The ingredients were dissolved in 75 ml distilled water. The solution was adjusted to pH 7.0 and brought to final volume of 100 ml. Next, the medium was sterilized by autoclaving for 30 minute at 121^{o}C (Tomy autoclave SS-325). Then 25 ml media was poured into LB plates and supplemented with ampicillin. The plates were dried, wrapped and stored at 4^{0}C.

ii. Lucia-Bertani (LB) medium

Yeast extracts	5g
Tryptone	10g
NaCl	10g
Distilled H_2O	1000ml

The ingredients were dissolved in 900 ml distilled water. The solution was Adjusted to pH 7.0 and brought to final volume 1 liter. Next, the medium was Sterilized by autoclaving for 30 minutes at 121^{o}C (Tomy autoclave SS-325). The media was put at room temperature until it is cool and supplemented with ampicillin. The plates were dried, wrapped and stored at 4^{0}C.

2.5 Antibiotic and Solution for Bacterial Culture

i. Ampicllin

A stock 100 mg/ml ampicillin was prepared. Antibiotic solutions were filtered, sterilized, dispensed into aliquots of 1 ml and stored at 4^{o}C. Working concentration used was 100µg/µl.

ii. Isopropy1-B-D-thiogalactopyranoside (IPTG) was prepared at 0.5 M as stock concentration.

1.192 g IPTG was dissolved in 10 ml distilled water and swirled properly. The solution was then filtered through a syringe filter (Sartorius) with 0.22 um pore size cellulose acetate membrane. Finally, it was aliquoted into 1.5 ml microcentrifuge tube in volume size 1.0 ml and stored at 80^{0}C. Working concentration=0.5 mM.

iii. A stock of 5 M Immidazole was prepared, and working concentration of Imidazole is 500 mM and stored at 4^{o}C.

2.6 Reagents and Buffers for SDS-PAGE

i. Tris.HCl/SDS,pH 8.8, 4X

Tris-base	45.5 g (1.5 M tris-Cl)

Tris was dissolved in 200 ml distilled water. The solution was adjusted to pH 8.8 with HCl and made up to 250 ml with dH_2O, filtered and stored at 4^oC.

ii. Tris.HCl / SDS, pH 6.8, 4X

Tris.base	6.05 g (0.5 M tris Cl)
SDS	0.4 g (0.4 % SDS)

The solutions were mixed and pH adjusted to 6.8 with HCl and sterile dH_2O, and was topped up to 100 ml and stored at 4^oC.

iii. 30% acrylamide / 0.8% bisacrylamide solution

Arylamide	30 g
N,N'-bis-methylene acrylamide	0.8 g
Deionized water (dH_2O)	100 ml

The acrylamide was dissolved in 75 ml distilled water. Then, the bisacrylamide was added into the solution and mix until dissolved. The solution was brought to final volume to 100 ml with distilled water, filtered and stored at 4^oC in tightly capped dark bottle.

iv. APS

10 % (w/v) Ammonium persulphate (APS)

APS	0.1 g
Distilled H_2O	1000ul

Balanced APS into a 1.5 ml eppendorf tube mix 1.0 ml distilled H_2O until Dissolve, the solution was prepared fresh prior to usage.

v. N,N,N',N' –tetramethylethylenediamine (TEMED)

vi. 5X Running Buffer / Electrophoresis buffer (Concentrated stock)

Tris-base	7.55 g
Glycine	36.0 g
SDS	2.5 g
Distilled H_2O	500 ml

Tris, glycine and SDS were added in to 300 ml distilled-deionized water stir until dissolved, and brought to final volume 500 ml. This concentrated stock was diluted to 1 X, SDS/ electrophoresis running buffer before use. The solution was stored at 4^oC until further use.

vii. SDS / Sample Loading Buffer 2X

Tris.Cl / SDS pH=6/8 4X	2.5 ml
Glycerol	2.0 ml
SDS	0.4 g
2 ME (Mercapto ethanol)	0.2 ml
Bromophenol blue	0.2 mg
Distilled H_2O	10 ml.

The chemicals were dissolved in distilled, deionized water and brought to final volume 10 ml. The sample dye solution (2 X concentrated stock) was aliquoted into 1 ml volume size and stored in -80^oC. The 2 X concentrated stock was applied to protein samples. Later, it can be further diluted to 1 X stock to be applied on the bacterial pellets.

viii. Coomassie Blue staining solution

Methanol	50%
Coomassie Briliant Blue R250	0.05%
Acetic acid	10%
Distilled H_2O	40%

The Coomassie Briliant Blue R250 was dissolved in methanol before adding acetic acidand water. The solution was stored in open shelves at room temperature for 6 months.

ix. Destaining Solution

Methanol	5%
Acetic acid	7%
Distilled H_2O	88%

The solution was prepared in volume size of 1 liter.

x. Fixing solution

Methanol	50%
Acetic acid	10%
Distilled H_2O	40%

The solution was prepared in volume size of 1 liter and could be stored on open shelves at room temperature for 1 month.

2.7 Buffers and Reagents for Western Blot

I. 10x Transfer Buffer (PVDF membranes)

Tris base	30g
Glycine	144g
Distilled H_2O	1000ml

The chemicals were dissolved in 800 ml distilled water and brought to final volume of 1000 ml. store at 4^0C.

II. 1x working Transfer Buffer

Methanol	200ml
10x Transfer buffer	100ml
Distilled buffer	700ml

III. 10x TBS

Tris base	12.11g
NaCl	87.6g
Distilled H_2O	1000ml

The chemicals were dissolved in 800 ml distilled water. Adjust to pH 8.0 and brought to final volume of 1000 ml with dH_2O.

IV. 1x TBST

10x TBS	100ml
10% Tween-20	5ml
Distilled H_2O	895ml

V. 10x Sodium Azide solution (Toxic)

Sodium Azide	1g
Distilled H_2O	50ml

VI. 5% Blocking Solution

Non-Fat dry milk	25g
10% Sodium Azide soln	500µl
Distilled H_2O	500ml

2.8 Prestained SDS-PAGE Standards Protein Marker

Prestained Protein Marker: low range (Fermentas)

Prestained marker was stored at -20^{o}C

Approximatery 7 ul of this solution was then loaded into the well

2.9 Bradford Reagent for Protein Estimation (Bio-Rad).

2.10 Buffers for Protein Purification

i. Column Buffer, pH 8.00

50 mM Hepes. pH 8.00/300 mM NaCl

Hepes	11.92 g
NaCl	17.53 g
Distilled H_2O to	1000 ml

The chemicals were dissolved in 800 ml distilled H_2O. The pH adjusted to 8.00 with HCl and made up to 1000 ml with distilled H_2O.

ii. Lysis Buffer

Lysozyme	100 mg
Column Buffer	100 ml
Glycerol	5%

iii. Wash Buffer, pH 8.00

50 mM Hepes pH 8.00/ 300 mM NaCl

Hepes	2.98 g
NaCl	4.38 g
Imidazole	50 mM
Distilled H_2O	250 ml

The chemicals were dissolved in 150 ml distilled H_2O. The pH adjusted to 8.00 with HCl and made up to 250 ml with distilled H_2O.

iv. Elution Buffer

50 mM Hepes pH 8.00 / 300 mM NaCl

Hepes	2.98 g
NaCl	4.38 g
Imidazole	200 mM
Distilled H_2O	250 ml

The chemicals were dissolved in 150 ml distilled H_2O. pH adjusted to 8.00 with HCl and made up to 250 ml with distilled H_2O.

2.11 Buffer for Protease Assay

200mM Tris.HCl pH 8.5

Tris base	12.1 g
Distilled H2O	500 ml

The Tris base was dissolved in 350 ml distilled H_2O. pH adjusted to 8.5 with HCl and made up to 500 ml with distilled H_2O.

2.12 Instruments

The following instruments were used for phytochemical and biological work:

- The NMR spectra were recorded on JEOL FT-NMR 400 MHz, using deuterated methanol-d4 (CD3OD) as solvent with tetramethylsilane (TMS) as the internal reference. The chemical shift values were reported were given in Hz.
- HPLC pump and low gradient system (Shimadzu, Japan), for gradient elution column chromatography.
- Tecan Infinite M200 Pro fluorescence spectrophotometer (Linked to a desktop powered by Intel® i3 CPU 3.20 GHz and Microsoft Windows® 2007) was used to read the intensity in biological assay.
- Savant AES 2000 Automatic Environmental SpeedVac system for solvent evaporation, sample concentration and drying.
- Autoclave—Tommy Sx-700, was used for all sterilizations work.
- pH were measured on Thermo Scientific pH meter instrument.
- Dual-vertical, Mini-Gel unit, Model MGV-201—CBS.Scientific was used for SDS-PAGE.

2.13 PHYTOCHEMICAL ANALYSIS WORK

Since the aqueous extract of *Quercus Infectoria*, showed promising activity as serine protease inhibitor for NS2B/NS3 DENV-2 absorbed in the preliminary inhibitor assay (Section 2.22) when compared to methanol and ethanol extracts, it was chosen for further study.

2.13.1 Crude Plant Extract

Ten kilogram of *Quercus Infectoria* was obtained from local market. Samples collected for extraction were prepared by cleaning several times in water. Fruit part of the plant was chopped into small pieces and air-dried at room temperature until consistent residual weight has been achieved. The dried pieces were then grinded using an electric blender and the resultant powders were soaked in water.

One hundred grams of the resultant powder was soaked in 500 ml water at 60^{o}C overnight. The solution was then filtered using Whatman filter paper. The extract supernatant was concentrated using a freeze-drier and the concentrated extracts was used for preliminary inhibitor assay and subjected to column chromatography.

2.13.2 Fractionation by Column Chromatography

The crude extract was fractionated and purified by silica gel column chromatography. The slurry of silica gel (mesh 230-400, particle size 0.040- 0.063 mm) in 100% (v/v) hexane was run through the column at 2ml/minute to condition the packing of the column. After final packing, 2.5-4 g sample was mixed with silica gel and loaded on to column. The glass column used was 3 cm diameter x 50 cm length.

Quercus lusitanica were eluted through silica gel column using 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)) (Increase of 10% v/v- step ladder). The flow rate was set at 2 ml/min, and fraction were collected every about 15-20 ml using glass vials.

Twenty fractions were collected. All fractions were assayed by thin layer chromatography; fraction having the same R_f values were combined and dried by

Savant AES 2000 Automatic Environmental SpeedVac system. Five entire combined fractions were bioassayed against NS2B/NS3 protease. The above procedure was repeated many times to accumulate the quantity for fractionation and biological studies.

2.13.3 Thin-Layer Chromatography (TLC)

Thin layer choromatography (TLC) was carried out on precoated silica gel 60 F254 (Merck). The thickness of the layer was 2.0 mm respectively. The spots were detected by using UV light (254 and 366 nm) and reagent. Quercus lusitanica spot was determined using ferric chloride- potassium ferricynide spray.

2.13.4 High-performance liquid chromatography (HPLC)

Both the pre-hydrolysed and post-hydrolysed samples were analysed using a Shimadzu HPLC system, comprising a Shimadzu dual wavelength absorbance detector (SPD-20A UV-VIS), two Shimadzu pumps (LC 20AC), a manual injector (Rheodyne 7725i) with a 200 µl sample loop and a column oven (CTO-10AS VP). Reverse phase separations were carried out at 30^{o}C using a plus C18 reverse-phase column (*Æ* 4.6 mm x 250 mm) packed with 5 mm diameter particles from California, USA. Standard stock solutions of caffeic acid, ferulic acid, chorogenic acid, *p*-coumaric acid, gallic acid and ellagic acid were prepared in methanol, at concentrations of 1 mg/ml, the standard solutions were filtered through 0.45 mm membrane filter. The standard response curve for each phenolic was a linear regression fitted to triplicate values obtained at each of five concentrations (12.5–200 ppm).

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 dual pumping system, by varying the proportion of solvent A [water-acetic acid (25:1, v/v)] to solvent B (methanol). 10% methanol (B) flowed

through the column isocratically with 90% solvent A for 5 min, then solvent B was increased to 30% in 15 min, to 45% by 25 min, to 60% by 35 min, to 80% by 38 min, to 90% methanol by 43 min, and then followed by isocratic elution with 90% methanol (B) until 45 min. Finally, the gradient was changed to 10% methanol by 48 min, and this composition was held until 50 min at a flow rate of 1.0 ml/min. The elution system was a modified method of (Shahrzad, *et al.*, 1996). The phenolic compounds were detected at both 280 and 360 nm.

2.13.5 Nuclear Magnetic Resonance (NMR)

The ^{1}H and ^{13}C NMR spectra were recorded on JEOL FT-NMR 400 MHz, using deuterated methanol-d4 (CD3OD) as solvent with tetramethylsilane (TMS) as the internal reference. The chemical shift values were reported were given in Hz.

2.14 Biological acticity

As mentioned earlier, the main purpose of this study was to isolate the compound from *Quercus Infectoria* that contributed to inhibitory activities of NS2B/NS3 protease. Below are the procedures of the bioassays carried out:

2.14.1 Transformation of E.coli

A glycerol stock of competent Escherichia coli (E.coli) strain XL1-Blue MRF cultures were transformed with pQE-NS2B (H)-NS3 (185 amino acid) plasmid was constructed in our laboratory.

An inoculum was streaked across one side of a LB agar plate using strerile technique. The inoculating loop was resterilized and streak across a sample from the first streak across a fresh part of the plate. The step was repeated until the plate was covered. The plate was incubated at $37^{0}C$ until colonies appeared.

A volume of 10 ml LB broth was transferred into 5 sterile culture tubes. A single bacterial colony was isolated from overnight LB agar plate and inoculated into the culture tubes. The tubes were loosely capped and grown at 37^0C overnight in a shaker (Innova 4000 incubator shaker, New Brunswick Scientific).

These stocks were used for larger scale culture and storage purposes. For storage purpose, 1.5 ml glycerol was added to 6 ml saturated culture and mixed well. Eventually, the stock was aliquoted into 1.5 ml centrifuge tube in volume size of 1 ml each and stored at -80^0C.

2.14.2 Expression and Purification of Recombinant Protein

The expression and purification of the recombinant protein were carried out by modifying the method that has been reported by Yusof et al. (2000). Competent Escherichia coli strain XL1-Blue MRF transformed in LB medium in the presence of 100 ug/ml ampicillin at 37^0C with vigorous shaking overnight (200 rpm). When the OD at 600 nm of culture was approximately 0.6, isopropyl-B-D-thiogalactopyranose (IPTG 0.5 mM) was added and consequently incubated for another 2-3 hours. The bacterial cells were collected by centrifugation and the pellet was re-suspended in column buffer (50 mM Hepes, pH 8.00, 300 mM NaC1, 5% Glycerol) and lysed using lysozyme (1 mg/ml) for 30 minutes on ice. The lysed product was sonificated on ice (9 x 10s bursts). The lysate was kept on ice for one hour before denatured lysate was clarified by centrifugation at 8,000 g for 5 minutes at 4^oC.

The supernatant solution was incubated overnight in 2 ml of pre-equilibrated Ni^{2+}-NTA resin in column buffer. The Ni^{2+}-NTA (Nickel (II) nitrilotriacetate, Qiagen) affinity column bound this 6 x His NS2B/NS3 precursor protein making possible its subsequent separation from other proteins. The column was washed with two portions of 10 ml washing buffer and the washings were collected. The histidine-riched protein

NS2B/NS3 was eluted with solution consisting of a mixture of 9:1 (v/v) column buffer (50 mM Hepes, pH 8.00, 300 mM NaC1) and 200 mM imidazole at 4^{o}C (Elution Buffer). The elution profile was monitored by using the SDS PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis) and the peak fractions were pooled.

2.14.3 Protein Estimation

Protein concentration was estimated through Bradford assay.

2.14.3.1 Bovine serum albumin (BSA) standard curve

A stock of bovine serum albumin (BSA) of concentration 1 mg/ml was Prepared in distillation water. A typical standard curve, which was constructed based on standard solutions of bovine serum albumin (BSA) with concentration ranging from 0 to 1 mg. 10 ul of each protein sample, was mixed with 190 ul Bradford solution in 96 wells plate. The mixed solution was incubated at room temperature for 5 minutes. Absorbance was taken at 595 nm using a spectrophotometer. The absorbance of protein after purification was read and estimated based on BSA concentration curve standard.

2.14.3.2 Protein Estimation Using Bradford Reagent

Protein concentration was determined according to method of Bradford, with bovine serum albumin as standard. 10 ul of protein samples after purification was mixed with 190 ul Bradford reagent solution in 96 wells plate. The mixed solution was incubated at room temperature for 5 minutes. Absorbance was taken at 595 nm using a spectrophotometer. The concentration of protein after purification was estimated based on reading absorbance which compared on BSA concentration curve standard.

2.15 7-Amino-4-methylcoumarin (AMC) Standard plot for protein assay

A 7-Amino-4-methylcoumarin (AMC) standard plot was used in determination of the NS2B/NS3 protease complex activity. A working stock of AMC with concentration of 10 uM was prepared. The stock was used to carry out standard assays, ranged from 0-50 uM. The blank and standard assays were triplicate.

2.16 One Dimension Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE analysis was conducted according to the method of Ausubel et al, 1992). First of all, 2 sets of glass-plate sandwich slab were assembled acoording to procedures that have been described in Ausubel et al (1992). Twelve percent polyacrylamide gel and five percent starking gel was prepared using the following solutions as shown table 2.1 (List of solution is in Section 2.1.7).

After mixing, the separating gel was immediately poured into the casting apparatus and allowed to polymerize. The stacking gel solution was then prepared and poured on the top of the polymerized separating solution, and a 12 teeth comb was inserted into the starking gel solution layer to form the sample loading wells.

Table 2.1: Preparation of 12% (w/v) polyacrylamide gel and 5% (w/v) stacking gel

Gel for SDS-PAGE	Sol.i	Sol.ii	Sol.iii	Sol.iv	Sol.v	H_2O
Separating gel (12%w/v)	6.00ml	3.75ml	--	50µl	10µl	5.25ml
Stacking gel (5%w/v)	0.65ml	--	1.25ml	25µl	5µl	3.05ml

The sample for SDS PAGE electrophoresis was mixed in SDS sample buffer loading 2X (1: 1), were boiled at 100^{o}C for 5 minutes and centrifuged briefly. Gels were electrophoresed (Dual vertical mini gel unit, model MGV201, CBS Scientific Co.) at 70 volts (Bio-rad Powerpac 300) until the dye front reached 1 cm from the bottom of the gel edge.

To visualize the total protein samples that have been electrophoresed on the 12% SDS PAGE, the gel was stained overnight using Coomassie Brilliant Blue staining solution on a rocking plantform (Biometra, GmBH). It was then destained with destaining solution for a suitable length of time until most of the background is clear. Finally, the gel was soaked in fixing solution to preserve the result profile.

2.17 Western blot

The thin layer gel which was collected from the SDS-PAGE electrophoresis apparatus was used for western blot. The following materials were arranged respectively on the black side of the cassette holder, the first fiber pad was laid, the filter paper on top, the gel on top of the filter paper, the nitrocellulose membrane (Amersham Biosciences, USA), the filter paper and the next fiber pad. It was very important to remove any bubbles between the gel layer and nitrocellulose paper. The cassette holder was fixed in the western blot apparatus (Bio-Rad, USA) with ice block and the transferring buffer was added to the top of the tank. The apparatus was connected with electricity (100 V) for one hour. The nitrocellulose membrane was incubated overnight at 4°C with blocking solution. Then, the membrane was transferred into probing solution with monoclonal anti-human adiponectin antibody as the NS2B/NS3 tag and incubated at room temperature for 60min with slow shaking. After the first antibody incubation, the membrane was rinsed with TBS solution and washed three times (15min each) with TBST solution then rinsed again with TBS solution. The second antibody (mouse IGg antibody, Sigma, USA) was added after covering the membrane with probing solution. The membrane was incubated for one hour and the same step of washing with TBS and TBST solutions was repeated. Finally, the membrane was covered with Western Blue®stabilized substrate (Promega, USA) to catalyse by the alkaline phosphatase which was conjugated with the second antibody. Protein bands of interest were detected according to the protein maker.

2.18 Preparation of Fluorogenic Peptide Substrate

Vials of fluorogenic substrate, BOC-Gly-Arg-Arg-AMC, were purchased from Peptide Institute Inc. (Japan). Each vial contained 5 mg of the fluorogenic substrate. Fluorogenic substrate was dissolved in 800 µl DMSO to yield a concentrated stock of 10 mM. This stock was diluted to 1 mM stock. All these stocks were stored at -80^0C to prolong the substrate stability.

2.19 Expression and Purification of Dengue-2 complex

The purpose of this study is to investigate the action of antiviral compounds against DENV-2 NS2B/NS3 protease. The protein precursor consisting of N-terminal hexahistidine tag fused sequentially to 40-residue NS2B cofactor, a linker of 10 residues and the first 185 amino acids of NS3 was expressed using transformed competent *Escherichia coli* strain XL1-Blue MRF; then harvested and purified by modifying the method that has been reported by Yusof *et al.*(2000). The precursor NS2B/NS3 serine protease was expressed in competent *Escherichia coli* (*E.coli*) strain XL1-Blue MRF cultures grown in Luria Bertani Broth medium as described in section 2.1.5, and was cultured in Luria Bertani Broth medium in the presence of 100 µg/ml of ampicillin at 37ºC with vigorous shaking overnight (200 rpm). Bacterial cells were induced for protein expression by addition of isopropyl-β-D-thiogalactopyranosea (IPTG), when the OD at 600 nm of culture was approximately 0.6 for 3 hours. The bacterial cells were collected by centrifugation at 8900 g for 10 minutes, at 4ºC.

For protein purification, cells were resuspended in column buffer (50 mM Hepes. pH 8.00/300 mM NaCl) as described in (Section 2.10.i) and lysed using lysozyme (1 mg/ml) for 30 minutes in ice to make the bacterial cell wall permeable. The lysed product was centrifuged at 8900 g for 60 minutes at 4ºC and pellet fraction was resuspended in column buffer (50 mM Hepes. pH 8.00/300 mM NaCl) followed by

sonification on ice (9X10s burst). The lysate was kept in ice for another 60 minutes to allow more protease to be release into the suspension and denatured lysate was clarified by centrifugation at 8900 g for 60 minutes at 4ºC.

The supernatant solution was incubated overnight in 2 ml of pre-equilibrated Ni^{+2}-NTA resin in column buffer, supernatant solution was purified by passage through a Ni^{+2} affinity column and the unbound fraction was removed. The column was washed extensively with washing buffer (Section 2.10.ii) and the washings were collected. The histidine-rich protein NS2B/NS3 was eluted with elution buffer (50 mM Hepes. pH 8.00/300 mM NaCl/ 200 mM imidazole) at 4ºC. The elution profile were monitored at A_{595} nm as shown in figure 3.1 and followed by SDS-PAGE (Figure 3.3).

2.20 Determination of Optimum Activity of the Dengue-2 NS2B/NS3 Protease and Substrate

Studies were executed prior to bioassay to ascertain optimum protease and substrate concentration, and solvent effect. To determine the optimum protease for the enzyme activity, the concentration of enzyme were varied between 0 to 10 μM. The standard reaction mixtures (200 μl) contained Tris buffer, several Enzyme concentration and substrate concentration were kept constant.

The concentration of the substrate was optimized by using different concentrations, from 0 to 120 μM. The reaction contained Tris buffer, enzyme at fix concentration and substrate.

The activity and kinetic parameter of NS2B/NS3 protease complex were characterized by using a fluorogenic peptide substrate BOC-Gly-Arg-Arg-AMC. The fluorogenic moiety (7-amino-4-methyle coumarin, AMC) was release from that substrate and was detected with varian Tecan Infinite M200 Pro fluorescence spectrophotometer λ (excitation) = 350.00 nm, λ (emission) = 440.00 nm.

2.21 Determination of Solvent Effect on Enzyme Activity (Methanol and DMSO)

The possible effect of solvent on the cleavage of the substrate by enzyme was separately studied for both the solvents used to dissolve the plant extract and the compounds. Effect of solvent (DMSO and Methanol) were tested by varying the concentration of solvent from 0% to 50% (v/v). The standard reaction mixtures (200 µl) contained Tris-HCl buffer of 200 mM at pH 8.5, 100 µM fluorogenic peptide substrate, 1.0 µM of enzyme and 0% to 50% (v/v) of solvent (without crude extract of plant). The solvent and buffer were pre-incubated at 37^{o}C for about 10 min before the substrate was added and re-incubated for 30 min, thereafter incubated further for 30 minute at 37^{o}C.

2.22 Inhibition Assays Using Fluorogenic Peptide

The studies of the inhibition assay were executed using Cary Tecan Infinite M200 Pro fluorescence spectrophotometer (λ (excitation) = 350 nm, λ (emission) = 440 nm).

In this experiment, DENV-2 NS2B/NS3 protease complex was screened with plant extract dissolved in methanol and 20 fractions from Quercus Iusitanica obtained from silica gel column chromatography dissolved in DMSO.

2.23 Inhibition Assay by Using Tecan Infinite M200 Pro fluorescence spectrophotometer

The bioassay used in this study was modified from the method published by Kiat and co-workers (Kiat et al., 2006). Reaction mixtures, with total volume of 200 µL, were prepared. These reaction mixtures consisted of 100µM fluorogenic peptide substrate (Boc-Gly-Arg-Arg-MCA), 1 µM DENV-2 NS2B/NS3 protease complex, and with or without inhibitors of varying concentrations, buffered at pH 8.5 by 200 mM Tris-HCl. The inhibitors were initially prepared in Tris-HCl buffer and assayed at

different concentrations, i.e. 12.5-400 µM.The reaction mixtures without fluorogenic peptide substrate were firstly incubated at 37^0C for 30 minutes. Subsequently, the substrate was added and the mixture was further incubated at 37 ^{0}C for 30 mintues. Triplicates were performed for all measurements and the readings were taken using Tecan Infinite M200 Pro fluorescence spectrophotometer. Substrate cleavage was optimized at the emission at 440 nm upon excitation at 350 nm. All measurements were done in triplicate. The readings were then being used for calculating K_i values (in µM) of potential inhibitors and to verify the type of inhibitors by using nonlinear regression mixed model inhibition (Copeland, 2002) method in GraphPad Prism 5.0 software.

2.24 Determination of NS2B/NS3 Protease Biochemical and Kinetic Properties

2.24.1 Analysis of Kinetics Parameter, K_m and V_{max}

A fluorogenic substrate stock, Boc-Gly-Arg-Arg-MCA with working concentration of 1 mM was prepared (Section 2.3.6) and NS2B/NS3 1 mM, Tris buffer (200 mM Tris.HCl/ pH 8.5) were used in this assay. Substrate concentration was tested from 0-500 µM. The intensity was read by using Tecan Infinite M200 Pro fluorescence spectrophotometer. The control protease assays were done in triplicate. For this, Michaelis-menten constant (K_m), and maximum reaction velocity (V_{max}), by using GraphPad Prism 5 under Michaelis-Menten model.

2.24.2 Determination of the Inhibition Constant, K_i

In this experiment, inhibitions of enzyme activity were distinguished on the basis of whether the inhibition was or were not relieved by increasing the substrate concentration. The reaction velocity was measured at a variety of substrate concentration and a fixed enzyme concentration and a fixed inhibitor concentration. This was repeated at a different inhibitor concentration. A graph of double reciprocal of velocity against substrate concentration was plotted. In this section, the buffer, enzyme and inhibitor, namely gallic acid and ellagic acid, used were similar as described in section 2.3.8.1.

To determine the inhibition constant, Ki with fluorogenic substrate as substrate in the assays, the concentration of the substrate was varied between 25-400 µM but concentration inhibitor were fixed at five different concentrations, 0.1 µM, 0.2 µM, 0.4 µM, 0.8 µM and 1.6 µM for gallic acid and ellagic acid. Substrate used was fluorogenic substrate, Boc-Gly-Arg-Arg-MCA. All measurements were done in triplicate. The readings were then being used for calculating K_i values (in µM) of potential inhibitors and to verify the type of inhibitors by using nonlinear regression mixed model inhibition and linear regression model (Copeland, 2002) method in GraphPad Prism 5.0 software.

2.25 Calculation Methods

2.25.1 Calculation of Retention flow (R_f) Values

As each solute distributes itself between the stationary and the mobile phase, the distance a solute moves is always the same fraction of the distance moved by the solvent.

$$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

2.25.2 Calculation of Inhibition Activity

Measurement of the intensity was carried out after incubation for 30 min and the result were expressed as percentage of control (non-inhibitor compound, only substrate, enzyme and buffer), which was always taken as 100%. All experiments were performed in triplicate. The results were expressed as mean values ± SD, (the corresponding error bars being displayed in the graphical plots).

In the calculation good enzyme activity is needed and other intensity influence should be omitted i.e. colour intensity from extract and solvent. So, in the calculation time is truly intensity was needed; the means of truly intensity of enzyme activity is the intensity caused by the cleavage substrate by the enzyme. It does not have effect solvent on enzyme and solvent does not alter the intensity of extract. The intensity of enzyme activity was decreased in the presence of extract or compound antiviral and however the extract appears to alter intensity of enzyme activity.

$$\% \text{ Inhibition} = \frac{\text{Intensity of Enzyme activity} - \text{Intensity left after Inhibition}}{\text{Intensity of Enzyme activity}} \text{ X } 100$$

Intensity left after inhibition is intensity brought about by cleavage of substrate by NS2B/NS3 protease in the presence of extract was decreased intensity of substrate and extract in the absence of NS2B/NS3 protease.

2.26 Cytotoxicity assay

MK2 cells were seeded at $1x10^4$ cells per well in triplicate in 96 well plates and propagated at optimal conditions (37˚C, 5% CO_2 in humidified incubator). The crude extract and purified ellagic acid and gallic acid were diluted to serial concentrations (0, 25, 50, 100, 200, 400 µg/ml) with DMEM media supplemented with 2% FBS. Each dilution was tested in triplicate. Two control wells were included in each experiment: culture medium without the crude extract or compounds and culture medium with different concentrations of the crude extract and purified compounds in the absence of cells to subtract the background value of the crude extract and compounds in the culture medium. The cell cultures were analyzed at 24, 48 and 72 hrs using Non-Radioactive Cell Proliferation assay (Promega, USA) according to the manufacturer's instructions.