3.1 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 protein expression and purification profile were monitored at A_{595} nm as shown in figure 3.1 and followed by SDS-PAGE (Figure 3.2).

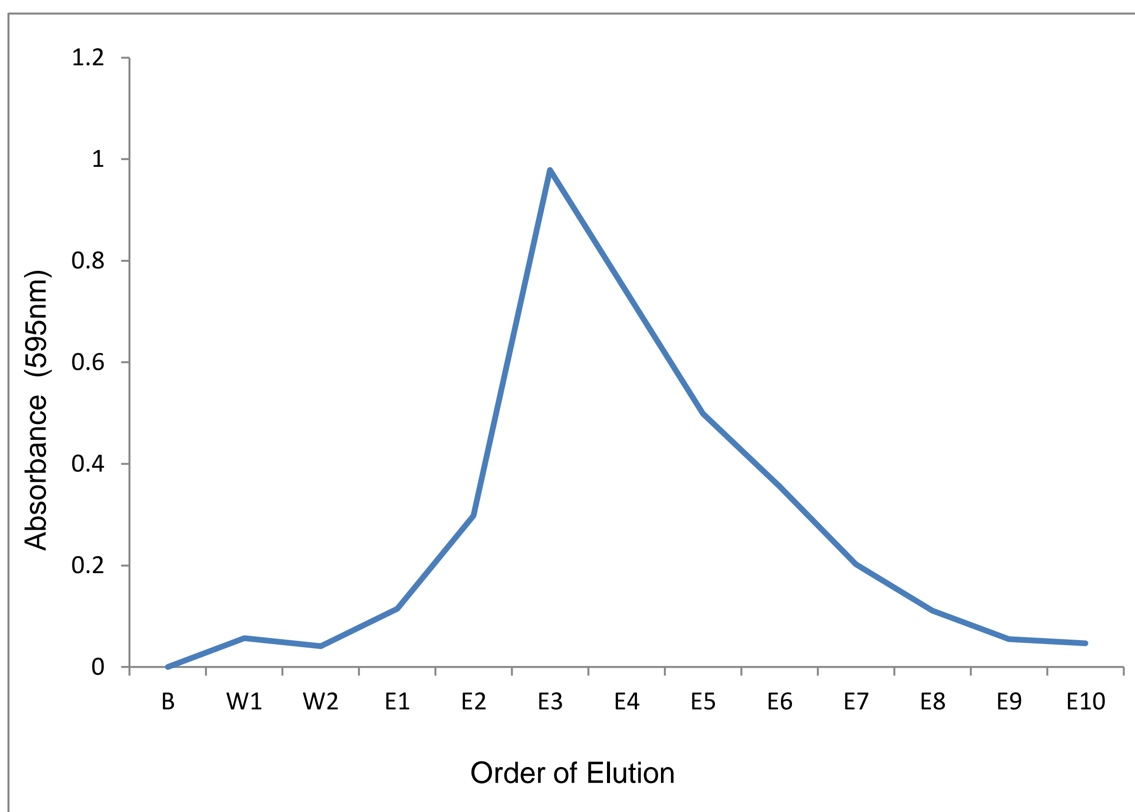

Figure 3.1: Elution profile of protein purification of DENV-2 protease precursor fraction on Nickle-column.

The fractions from Ni^{+2} were collected. W1 and W2 are the wash steps, while E1 to E10 are the elution steps. The peak fractions from tube E2, E3 and E4 were subjected to SDS-PAGE. The absorbance was measured at wavelength of 595 nm.

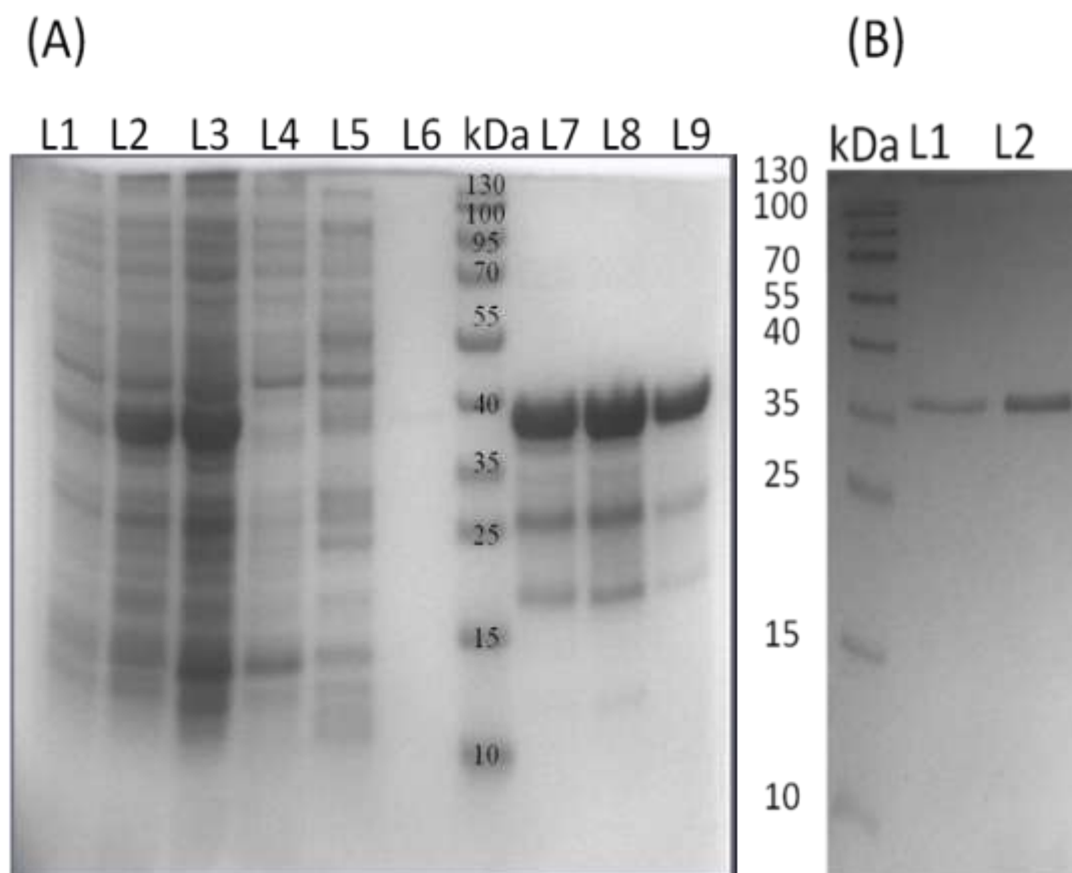

Figure 3.2: SDS-PAGE of NS2B/NS3 before and after purification.

(A) Recombinant NS2B/NS3pro was produced as a single chain in *E coli* (XL1-Blue) and purified using Ni^{+2}NTA affinity chromatography column. L1, the profile of *E. coli* proteins before induction with IPTG; L2, after induction; L3, total protein after cells lyses; L4- L6, washing steps, L7-L9 protein elution steps. (B) Protein purification by gel chromatography, L1-L2, elution steps. kDa protein marker.

3.2 Western Blot

Western blot was used to detect and confirm NS2B/NS3 specific proteins by His-Tag antibody. After SDS-PAGE electrophoresis, the thin layer gel was collected for western blot use as described in (Section 2.17). The Protein bands of interest were detected according to the protein maker.

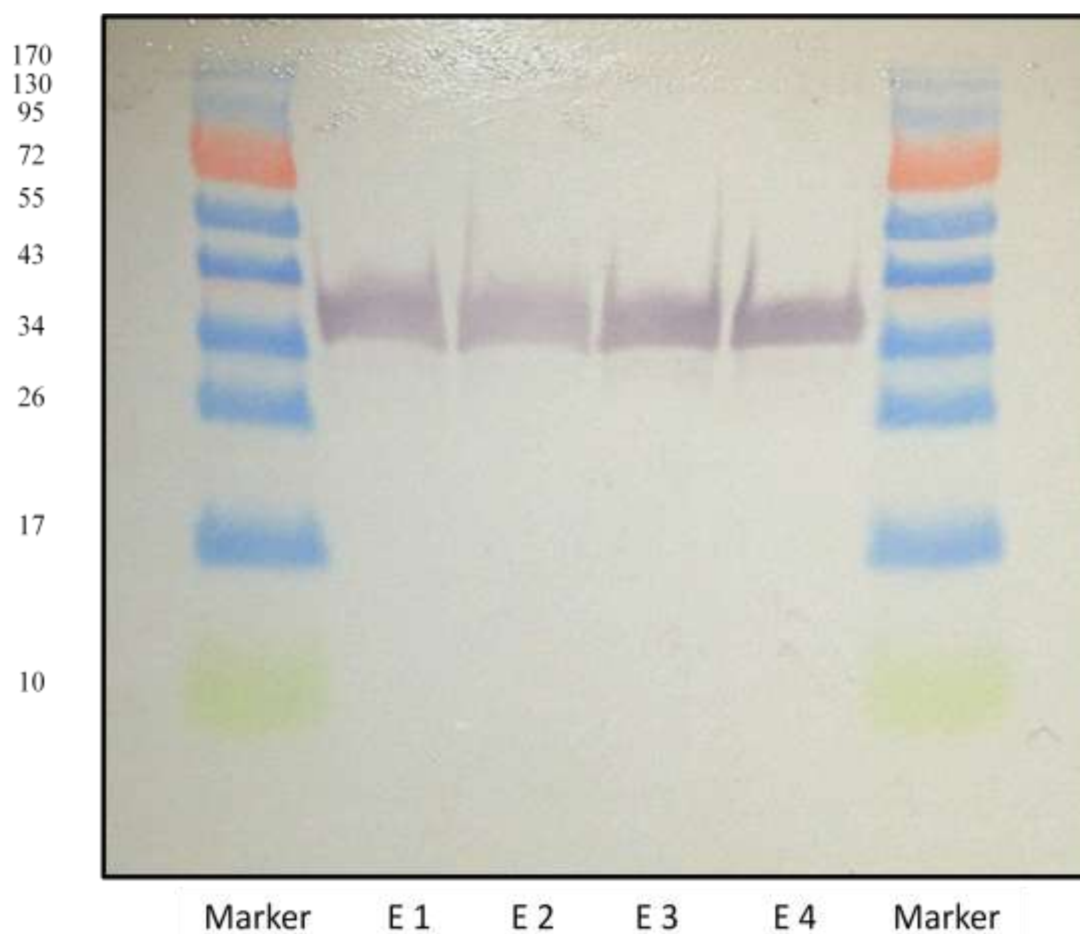

Figure 3.3: Western blot of NS2B/NS3 elution after SDS-PAGE electrophoresis apparatus

The thin layer gel from SDS-PAGE was subjected to western blot. Molecular weight as marker (Fermentas); E1, E2 and E3 showed protein profile after purification Ni^{+2}-NTA column.

3.3 Determination of Enzyme Concentration

Quantitative determination of protein (DENV-2 protease) was based on the Bovine Soluble Albumin (BSA) standard curve which performed using Bradford reagent assay. A series of dilution of protein standard was prepared, which is representative of the protein solution to be tested. The protein solutions were assayed in triplicate. The concentration of the DENV-2 protease was found to be 35.42 μM is based on the linear regression off BSA standard curve (Figure 3.4). The absorbance was measured at a wavelength of 595 nm. The protein was stored at -80°C. This protein was used as an active enzyme for the protease inhibitor assay. A linear equation $y = 0.5235x$, where y is the absorbance at 595 nm, and x is the protein concentration in unit of μg/ml. The linear equation was then used to determine the concentration of DENV-2 NS2B/NS3 purified protease complex.

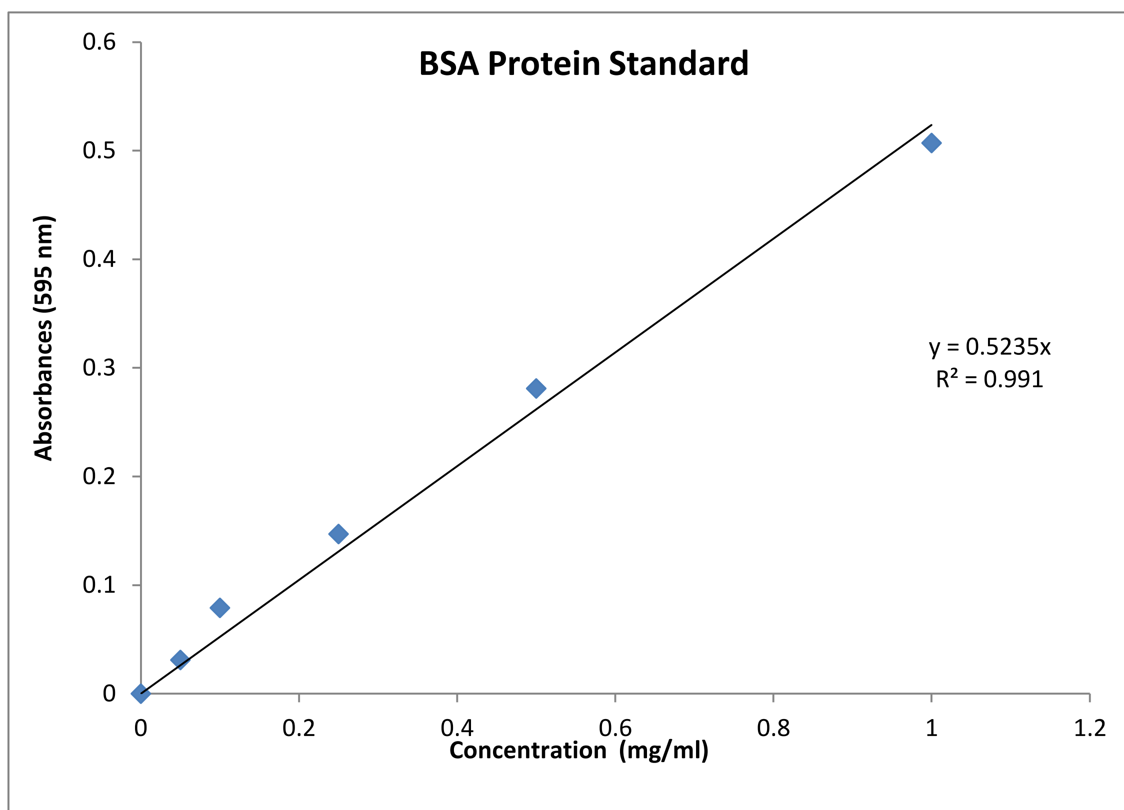

Figure 3.4: Bovine serum Albumin (BSA) standard curve was used to determination of concentration of the protein.

The linear range of the assay for BSA is 0 to 1 mg/ml. the protein solutions were assayed in triplicate. The absorbance was measured at wavelength of 595nm. A linear equation y =0.5235x, where y is the absorbance at 595 nm, and x is the protein concentration in unit of mg/ml. The concentration of the DENV-2 protease complex was found to be 35.42 µM using Bradford reagent for assay and standard BSA curve. The complex was divided into separate 1000 µL aliquots in Eppendorf tubes and stored at -80°C until required.

3.4 Determination of 7-amino-4-methylcoumarin (AMC) Released

Figure 3.5 shows the AMC (fluorogenic moiety, 7-amino-4-methylcoumarin) standard curve which were used for to determine the product of cleavage of the fluorogenic substrate by DENV-2 protease. To determine the amount of AMC particles being released into the assay environment, a standard plot of AMC was prepared. Based on the result, a linear equation $y = 1425.4x$, where y is the intensity and x is the AMC concentration in unit of μM. This AMC standard plot is indicative of AMC being released into the assay solution and thus was used to characterize the NS2B/NS3 protease biochemical and kinetic parameter properties. The standard plot assay was done in triplicate and result was illustrated in Figure 3.5.

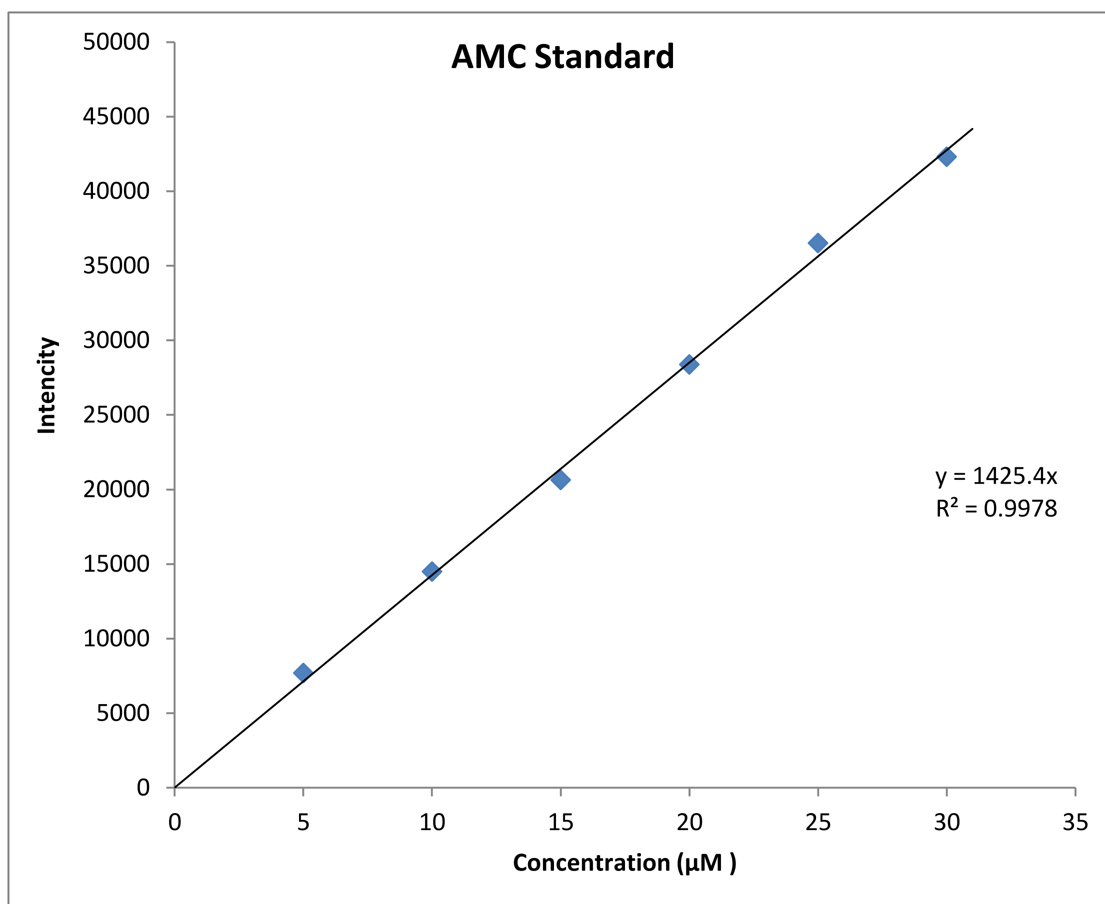

Figure 3.5: 7-amino-4-methylcounarin (AMC) standard curve

The AMC concentrations assayed ranges from 0 to 30 µM. Data are reported as mean standard deviation. A standard plot of AMC was prepared based on result, a linear equation y = 1425.4x, where y is the intensity and x is the AMC in unit of µM. This AMC standard plot directly indicate the amount of AMC being released into the assay solution and thus was used to characterize the NS2B/NS3 protease biochemical and kinetic parameter properties. The standard plot assay was done in triplicate.

3.5 Determination of Optimum Enzyme Concentration for Assay

From the optimum enzymatic studies, the enzymatic concentration for optimum activity found to be 2 μM. Since the concentration of DENV-2 protease obtained earlier was 35.42 μM and was diluted two folds before being used for bioassay. The figure 3.6 maximised at 10 μL enzyme volume. This volume of enzyme was used for all the bioassay. Table 3.1 illustrates the optimum enzyme concentration parameters V_{max} and Km as elucidate from GraphPad Prism 5.0 software.

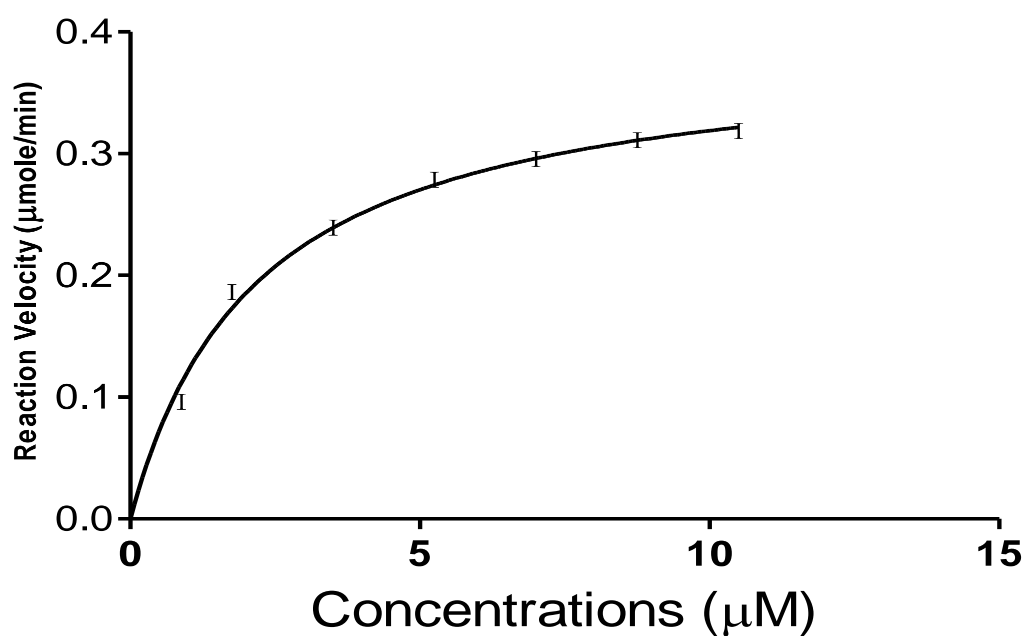

Figure 3.6: Graph of enzyme optimum by GraphPad Prism 5.0 software

This assay was carried out by using enzyme concentration range from 0 to 10.5 μM, 100 μM of substrate and buffered with tris-HCl of 200 mM at pH 8.5.

Table 3.1: The optimum enzyme concentration V_{max} and Km by GraphPad Prism 5.0 software

Parameter	Best-fit value	Standard Error
Vmax	0.3886 μmole/min	± 0.0068
Km	2.189 μM	± 0.1264

3.6 Determination of Optimum Substrate Concentration for Assay

Determination of optimum substrate concentration was needed to find the accurate data. The optimum substrate concentration that is most appropriate to be used for the inhibition assay and characterization of the biochemical and kinetic properties. To determine the optimum substrate concentration, an assay using different substrate concentrations, were varied from 0 to 120 µM. The reaction also contained tris-HCl buffer of 200 mM at pH 8.5 and 2.0 µM of enzyme which was kept constant. The result profile in Figure 3.7 showed that the optimum substrate concentration was 100 µM. Based on this result; further assay was carried out by using 100 µM as the substrate concentration.

3.7 Biochemical and Kinetic Parameters of the Dengue-2 Protease Complex

The enzymatic activity of the DENV-2 NS2B/NS3 protease in cleaving the fluorogenic peptide substrate the Boc-Gly-Arg-Arg-MCA is dependent on time of incubation and the substrate concentration and it followed the Michaels-Menten kinetic. In this experiment the time of incubation was 30 minutes. In this study, the kinetic properties of the purified DENV-2 NS2B/NS3 protease complex were also characterized such as Maximal velocity, V_{max}, and Michaelis-Menten constant, K_m by using GraphPad Prism 5.0 software. This study was carried out using increasing substrate concentration while concentrations of enzyme and inhibitor compound condition were kept constant. This biochemical and kinetic parameters were used for the determination of K_m and V_{max} from DENV-2 NS2B/NS3 protease.

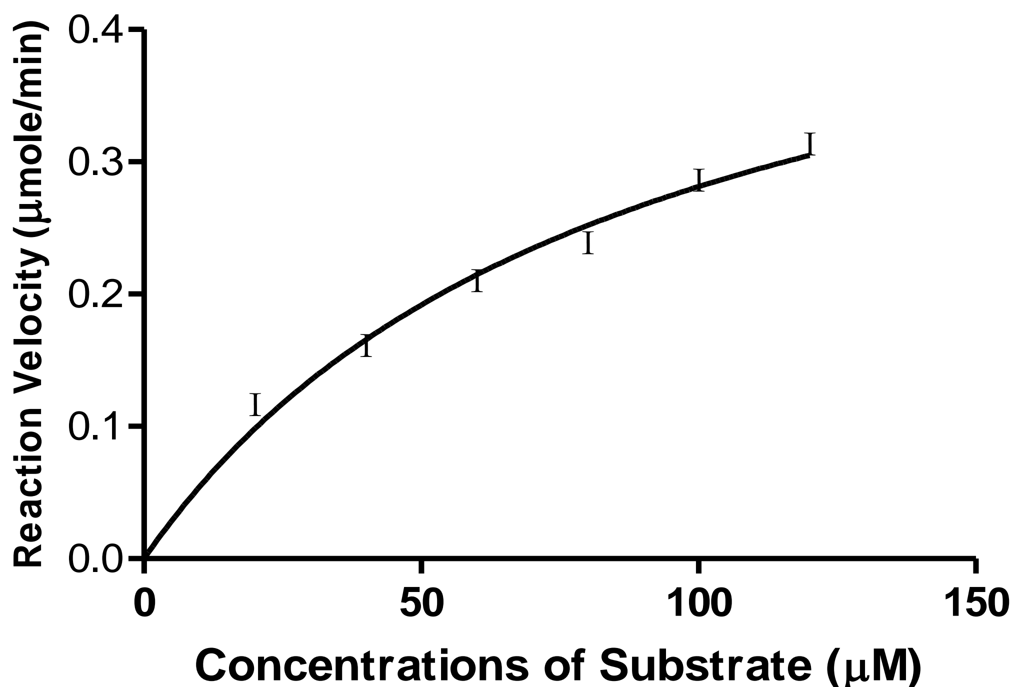

Figure 3.7: Graph of substrate optimum of NS2B/NS3 by Graphpad 5.0 software

This assay carried out by using substrate concentration ranged from 0 to 120 µM, 2.0 µM of NS2B/NS3 protease complex and buffered with tris-HCl of 200 mM at pH 8.5.

Table 3.2: Kinetic properties of NS2B/NS3 protease complex parameter by GraphPad Prism 5.0 software

Parameter	Best-fit value	Standard Error
Vmax	0.5270 µmole/min	± 0.0318
Km	87.382 µM	± 10.8713

3.8 Effect of Solvents on Dengue-2 Protease Complex

The possible effect of solvent on the cleavage of the substrate by the enzyme was separately studied for both the solvents used in dissolving the crude extract, the fractions that obtained from the column chromatography and the purified compounds. This step was taken to evaluate the effect of solvent against NS2B/NS3 and to make sure that solvents have no effect against NS2B/NS3. The solvent concentration from 0 % to 50 % was tested against DENV-2 NS2B/NS3 protease complex. Table 3.3 illustrates the inhibitory effect on the active enzyme and substrate against DMSO. A concentration of 5% (v/v) DMSO caused low effect on enzyme activity at around 11.25 %. Therefore, 10 μL (5 % v/v) DMSO was used in this bioassay and has been fixed as the 0 inhibitor value. Similarly table 3.4 indicates that, at concentration 5 % (v/v) of methanol on enzyme activity found to be around 7.36 % and also used as 0 inhibitor value.

Table 3.3: Determination of inhibitory effect on the active enzyme and substrate against DMSO as solvent at different concentrations range from 0% to 50%.

DMSO Concentration (%)	% Inhibition
0	-
5	11.26
10	20.46
20	30.63
30	45.95
40	51.85
50	55.72

Table 3.4: Determination of inhibitory effect on the active enzyme and substrate against methanol as solvent at different concentrations range from 0% to 50%

Methanol Concentration (%)	% Inhibition
0	-
5	7.36
10	16.88
20	24.78
30	32.78
40	41.90
50	50.44

3.9 Initial Screening of *Quercus Infectoria* Aqueous Extract and Partition Fractions against Dengue-2 NS2B/NS3 Protease Complex

After the preliminary studies of optimum enzyme activity concentration and solvent inhibition limits, the best volume of the stock solution to be used was worked out for each solution. Initial experiments for *Quercus Infectoria* crude extract and 20 fractions were collected from silica gel column chromatography were carried out to screen the activity against NS2B/NS3 protease complex. The concentrations of crude extract used in this experiment were 25ppm, 50ppm, 100ppm, 200ppm, and 400ppm. A fuorogenic substrate, Boc-Gly-Arg-Arg-AMC with concentration 100 µM and 2 µM NS2B/NS3, and Tris buffer (200 mM Tris.HCl/ pH 8.5) were used in this assay. The intensity was red by Tecan Infinite M200 Pro fluorescence spectrophotometer (λ (excitation) = 350 nm, λ (emission) = 440 nm). All measurements were done in triplicate. Of the 25 fractions tested, several have been identified to have inhibition activity against NS2B/NS3 protease complex as shown in (Table 3.5 and Figure 3.8).

In the first screening assay using crude plant extract, the result indicated *Quercus Infectoria* crude extract to demonstrate strong inhibition towards the DENV-2 protease (96.88%). The percentage of inhibition of the crude plant crude extract and fractions on this enzyme increased in a dose dependent manner. All experiments were performed in triplicate.

Table 3.5: Inhibition assay with *Quercus Infectoria* and 5 fractions from column chromatography

No	**Sample tested**	**Mean Percentage Inhibition (%)**				
		25ppm	50ppm	100ppm	200ppm	400ppm
1	Quercus Infectoria	60.97	65.68	73.92	85.06	96.88
2	Fraction 1	60.50	71.03	80.03	89.25	93.75
3	Fraction 2	56.42	69.22	71.92	82.76	88.519
4	Fraction 3	20.77	28.52	40.41	56.76	62.38
5	Fraction 4	15.10	30.34	36.72	53.41	58.88
6	Fraction 5	8.019	20.53	28.23	52.94	55.11

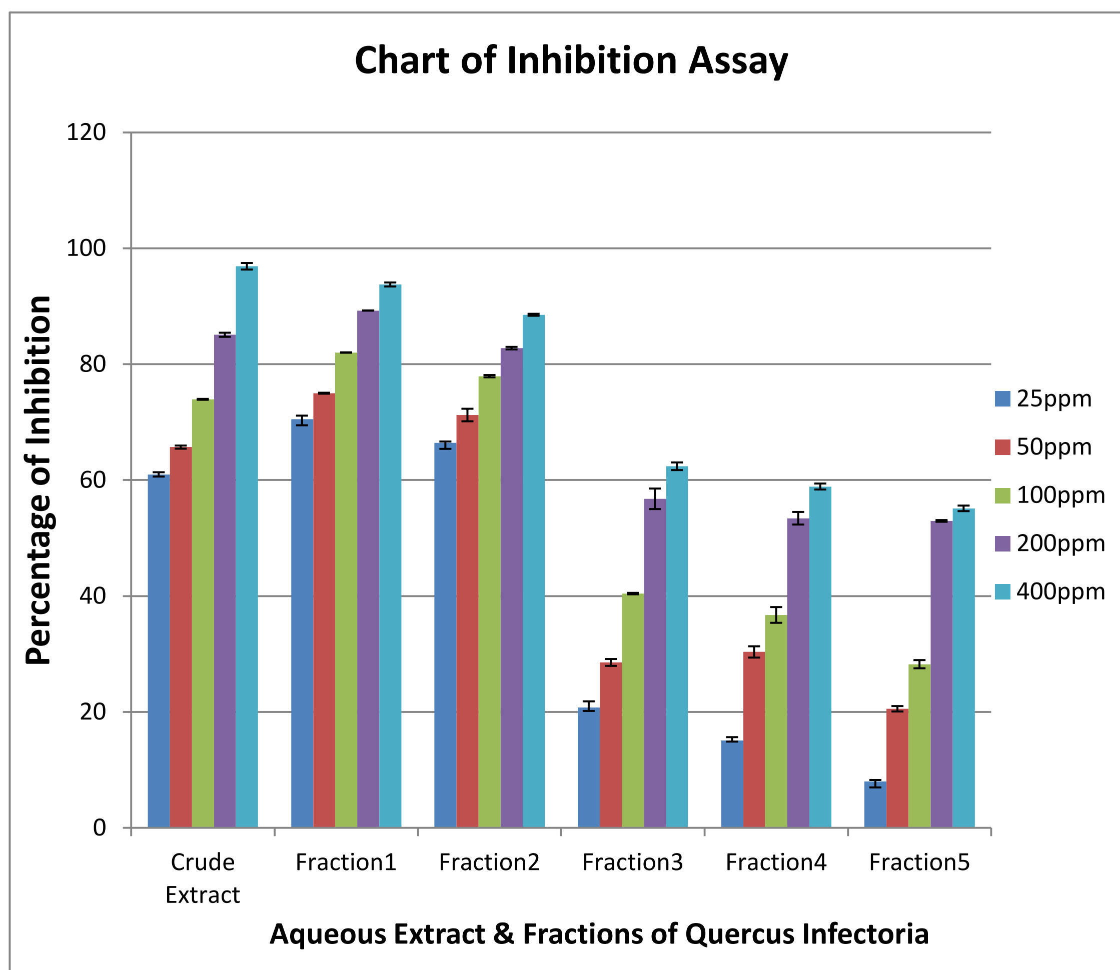

Figure 3.8: Chart of Inhibition assay with *Quercus Infectoria* and 5 fractions from column chromatography towards NS2B/NS3

The concentrations used in this experiment were 25 ppm, 50 ppm, 100 ppm, 200 ppm, 400 ppm. From the 25 fractions tested, only those that showed inhibitory activity against NS2B/NS3 are shown in the chart. The crude extract was dissolved in distilled water while the fractions were dissolved in methanol. In this bioassay 10 µL (5 % v/v) methanol used and have been fixed as the 0 inhibitor value.

3.10 *Quercus Infectoria*

3.10.1 Fractionation and purification of *Quercus Infectoria*

Crude extract was fractionated using the following condition: silica gel (mesh 230-400, particle size 0.040- 0.063 mm), column 3 cm diameter x 50 cm length. The solvent used for elution was: 100% (v/v) 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)). The solvent system was increased in polarity, 10% for each step of each solvent mixture. 15-20 ml elution was collected in glass vials. These fractions were subjected to TLC, with chloroform and ethyl acetate 9:1 (v/v). These spots were visualized under the short wavelength UV as a yellow spot. Staining with ferric chloride-potassium ferricyanide reagent gave the product as green spot. This suggests the compound to be phenolic. Fraction with the same R_f values on the TLC were combined resulting in 5 fractions labeled 1 to 5 (Figure 3.9).

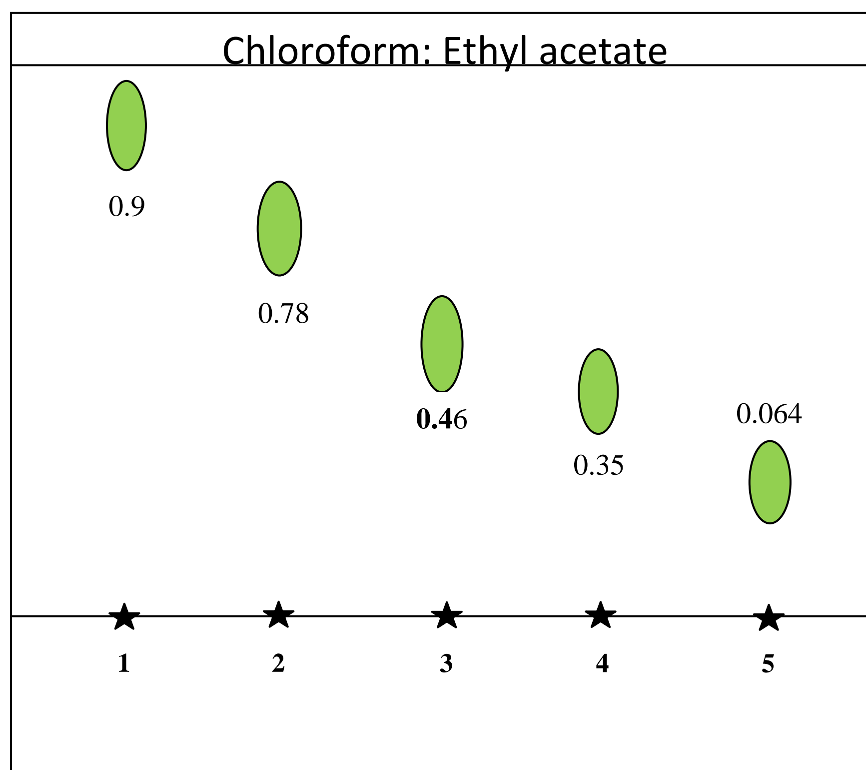

Figure 3.9: Thin layer chromatography of different fractions isolated by column chromatography

Five spots of components separated through preparative plate. They were developed on TLC plate and showed yellow fluorescent under short wave UV light and after staining with ferric chloride-potassium ferricyanide reagent gave the product as green spots.

3.10.2 *Quercus Infectoria* fractions activity towards NS2B/NS3 Protease

After silica gel column chromatography fractionation, the fractions were combined in vials according to their R_f values. The solvent of fractions was allowed to evaporate until dry using solvent evaporation system (automatic environmental speedvac), the yield quantities obtained were 0.25 to 0.42 gram. Five fractions were bioassayed and screened against NS2B/NS3 protease complex to determine the most active fraction. The result of the inhibitor assay showed the fractions 1 and 2, which have R_f value 0.9 and 0.78 to be very effective against NS2B/NS3 protease complex with more than 90 % and 85 % inhibition respectively. While fractions 3, 4 and 5 that have R_f value 0.46, 0.35 and 0.064 showed 62 %, 58 % and 55 % inhibition respectively as shown in figure 3.9 and table 3.5 previously. The fractions were assayed from concentration 25 ppm to 400 ppm and the protease assays were done in triplicate.

3.10.3 High-Performance Liquid Chromatography of Active Fractions

All fractions have been collected from fractions of *Quercus Infectoria* on silica gel column chromatography were subjected to the HPLC. According to TLC plate result, 6 standards stock solutions (caffeic acid, ferulic acid, chorogenic acid, p-coumaric acid, gallic acid and ellagic acid) were prepared as described in (2.13.4).

The chromatogram of fraction 1 with R_f value 0.9 revealed 5 compounds (Figure 3.10). Table 3.7 showed the retention time and the areas of these compounds.

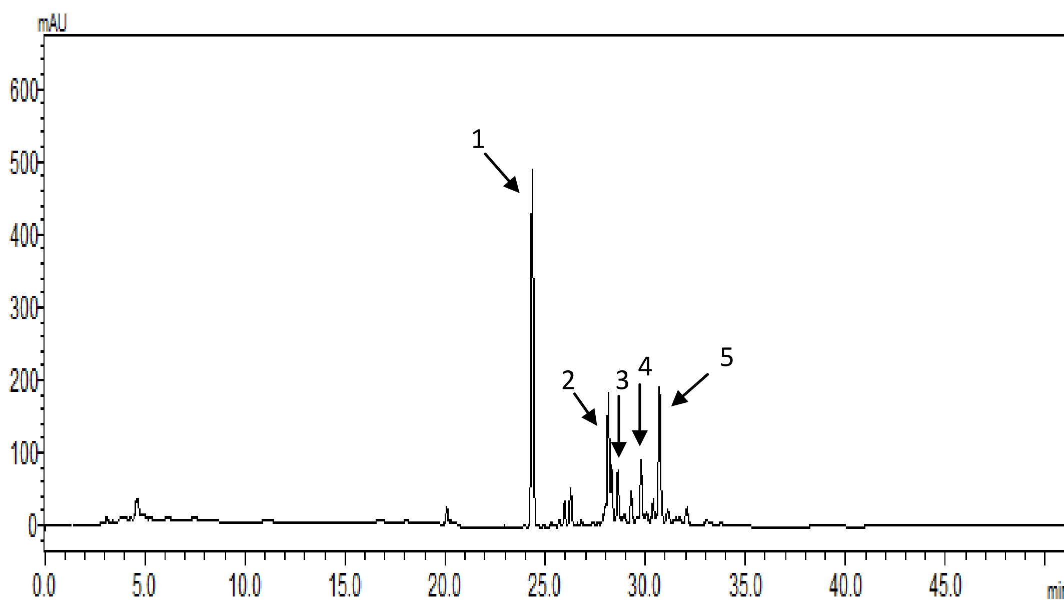

Figure 3.10: The chromatogram of fraction 1 with R_f value 0.90 of *Quercus Infectoria* after extraction using silica gel column chromatography and detected by UV absorption at 290nm revealed 5 compounds.

Table 3.6: Table showing the retention time of the active fraction 1

NO	Retention Time (min)
1	24.21
2	26.50
3	27.29
4	29.35
5	30.40

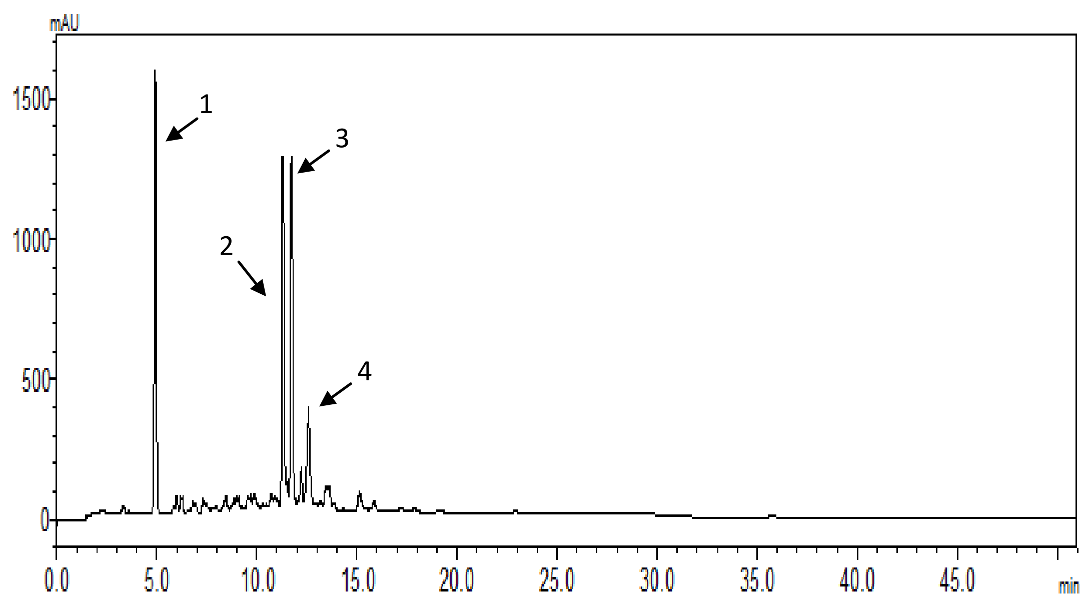

Figure 3.11: The chromatogram of fraction 2 with R_f value 0.78 of *Quercus Infectoria* after extraction using silica gel column chromatography and detected by UV absorption at 290nm revealed 4 compounds.

Table 3.7: The table showed the retention time of the active fraction 2

No	Retention Time (min)
1	4.87
2	11.25
3	11.39
4	12.30

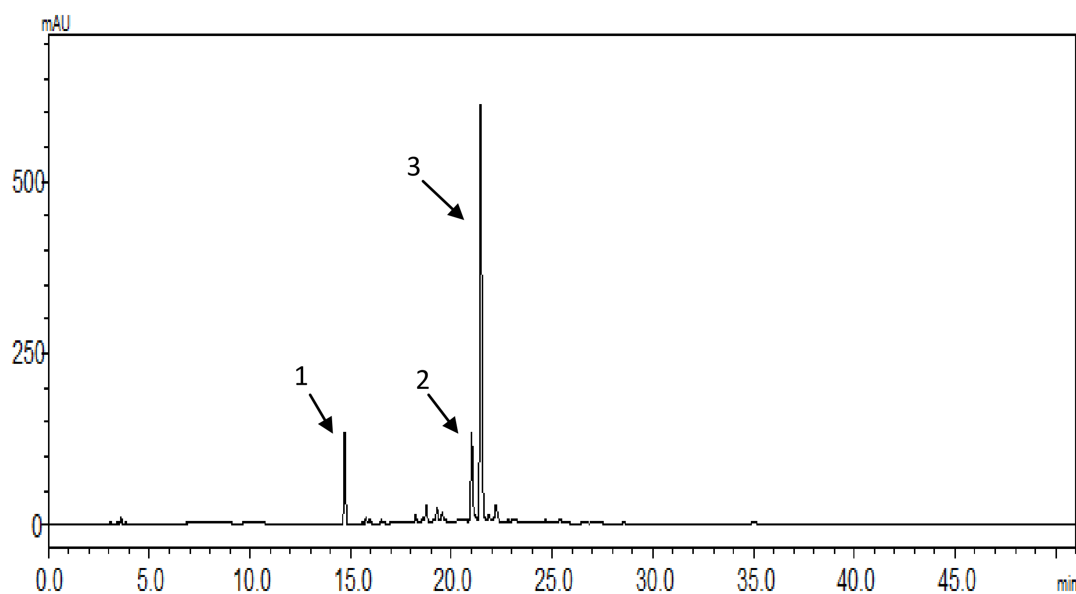

Figure 3.12: The chromatogram of fraction 3 with R_f value 0.46 of *Quercus Infectoria* after extraction using silica gel column chromatography and detected by UV absorption at 290nm revealed 3 compounds

Table 3.8: The table showed the retention time of the active fraction 3

No	Retention Time (min)
1	14.47
2	20.57
3	21.25

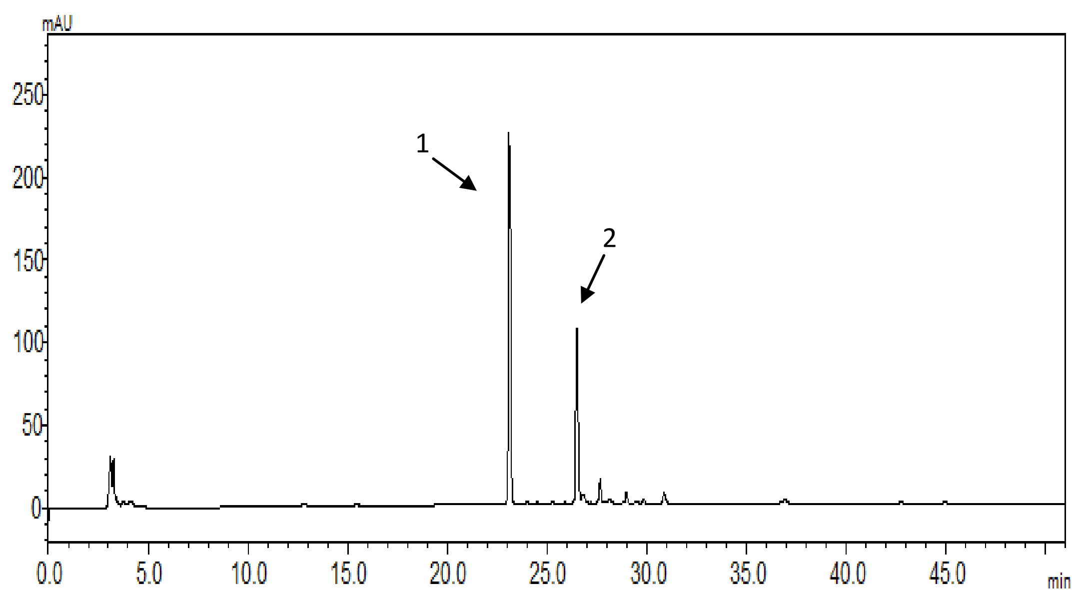

Figure 3.13: The chromatogram of fraction 4 with R_f value 0.35 of *Quercus Infectoria* after extraction using silica gel column chromatography and detected by UV absorption at 290nm revealed 2 compounds.

Table 3.9: The table showed the retention time of the active fraction 4

No	Retention Time (min)
1	22.52
2	22.17

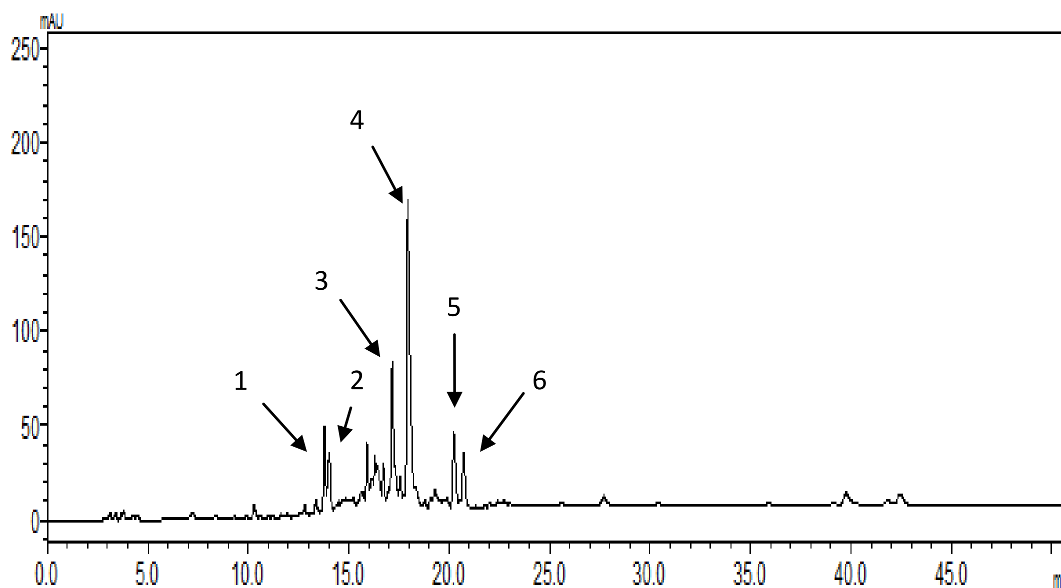

Figure 3.14: The chromatogram of fraction 5 with R_f value 0.064 of *Quercus Infectoria* after extraction using silica gel column chromatography and detected by UV absorption at 290nm revealed 6 compounds.

Table 3.10: The table showed the retention time of the active fraction 5

NO	Retention Time (min)
1	14.31
2	14.34
3	16.49
4	17.42
5	20.08
6	20.36

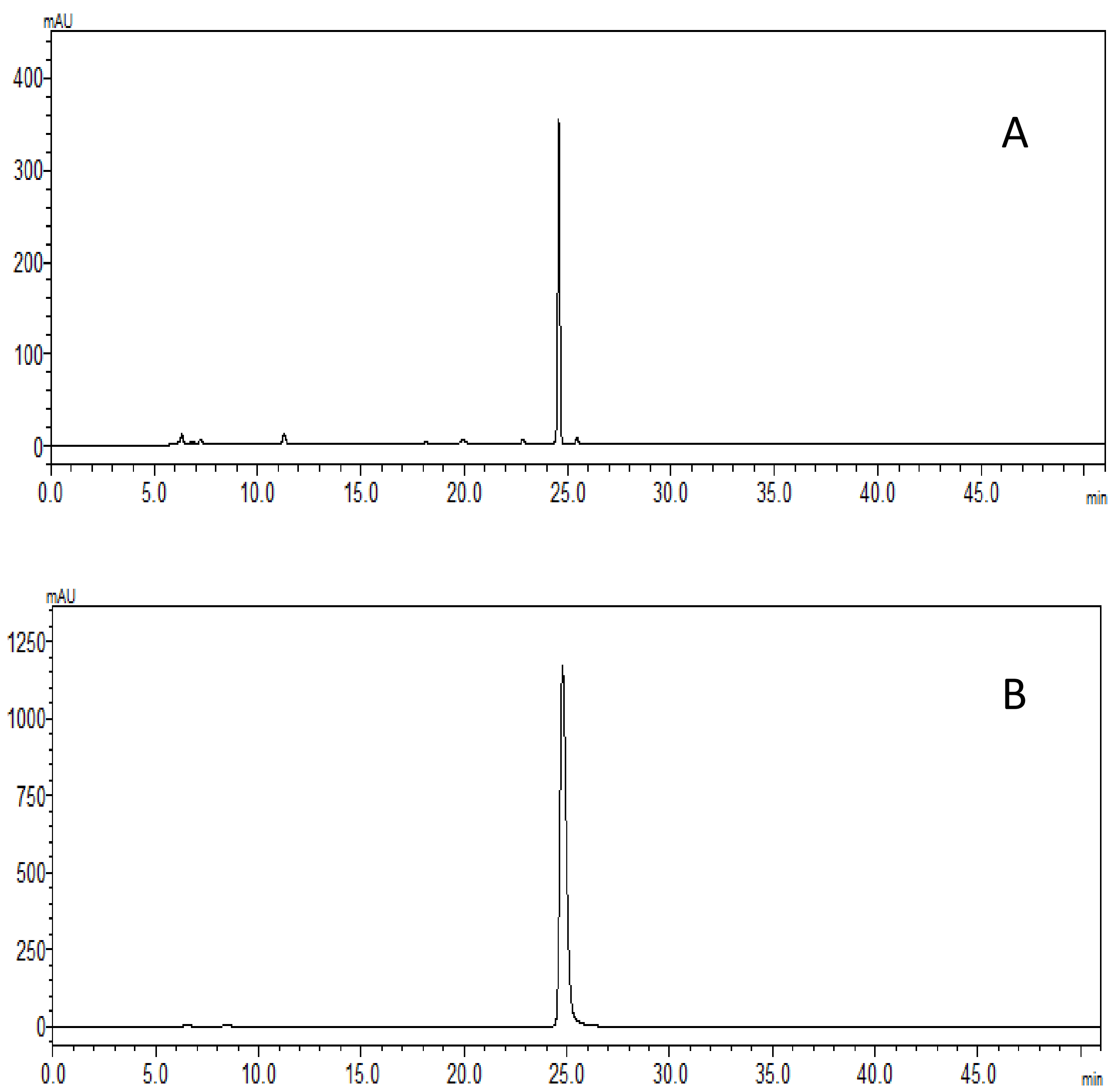

Figure 3.15: Chromatogram of the active compound 1 (ellagic acid)

Where (A) is the active compound 1 (ellagic acid) of aqueous extract of *Quercus Infectoria* after silica gel recolumn chromatography and (B) is ellagic acid standard chromatogram detected by UV absorption at 290nm.

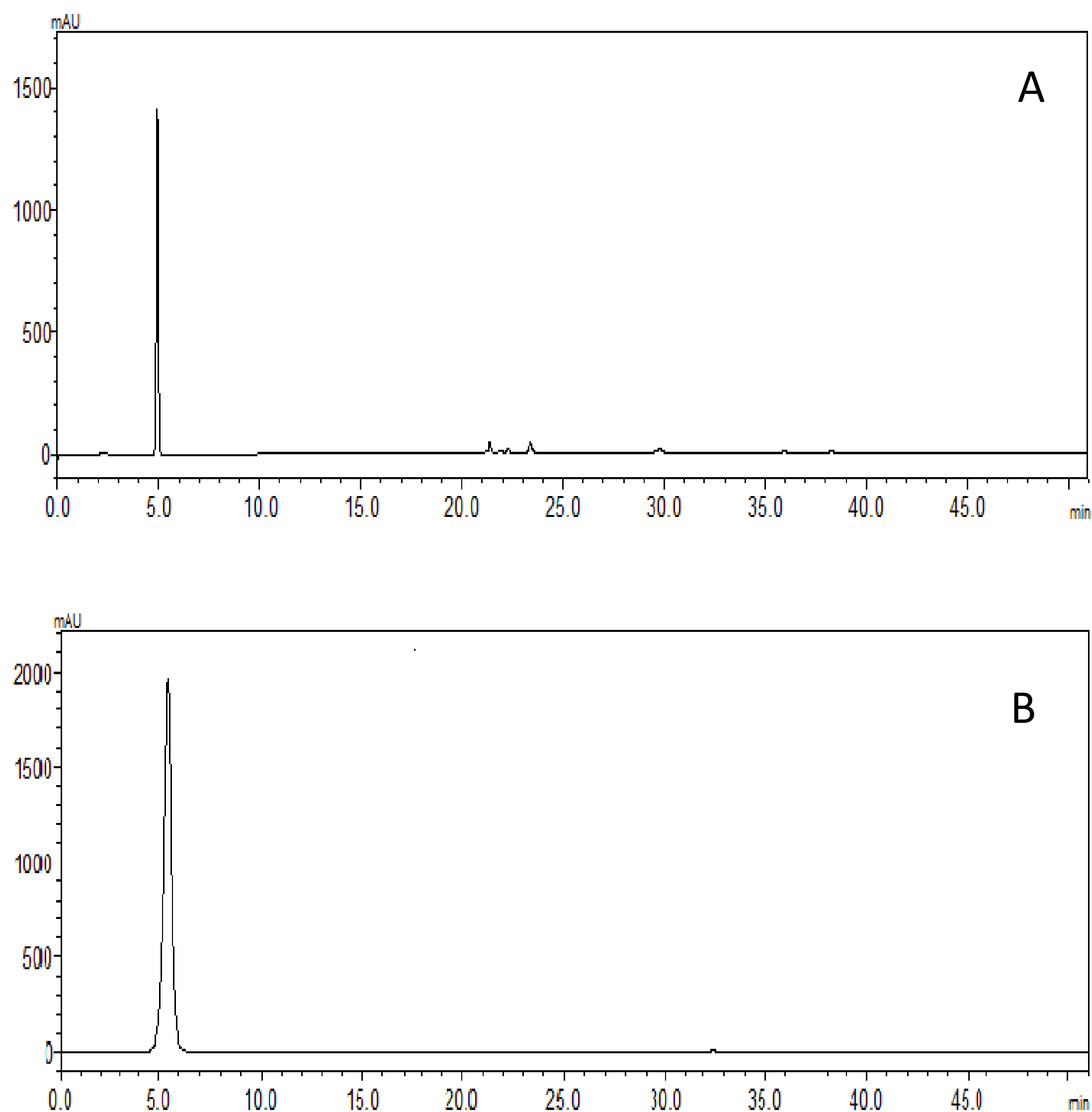

Figure 3.16: Chromatogram of the active compound 2 (gallic acid)

where (A) is for the active compound 2 (gallic acid) of aqueous extract of *Quercus Infectoria* after silica gel recolumn chromatography and B is gallic acid standard chromatogram detected by UV absorption at 290nm.

3.10.4 Nuclear Magnetic Resonance (NMR) Spectra of Purified compounds

Further study carried out to identify the structure for the isolated components were carried out using ^{1}H and ^{13}C NMR spectroscopic methods. The result were recorded on JEOL FT-NMR 400 MHz, using deuterated methanol-d4 (CD3OD) as 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*).

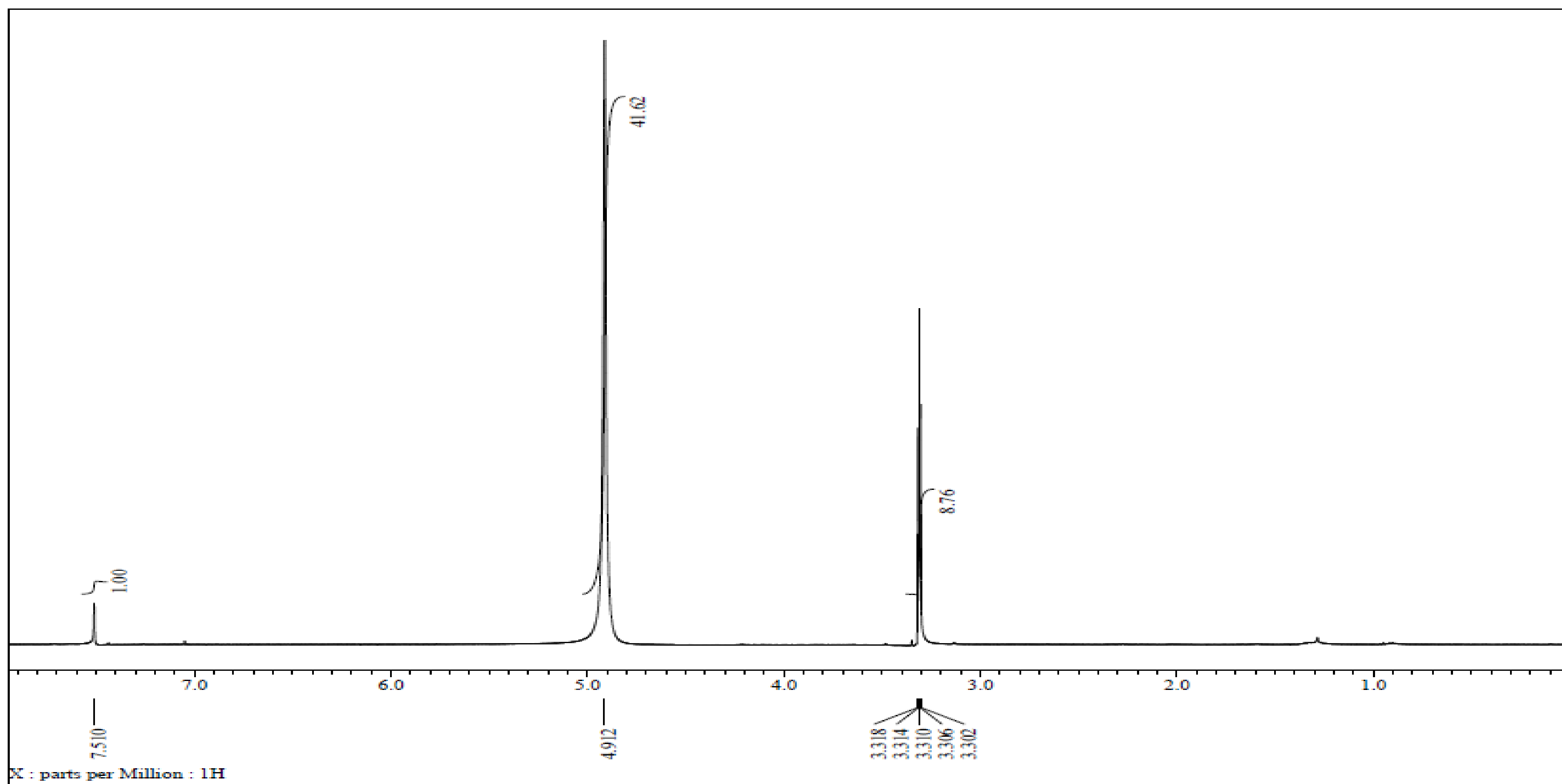

Figure 3.17: ^{1}H NMR Spectrum of the active ellagic acid of aqueous extract of *Quercus Infectoria* after silica gel chromatography

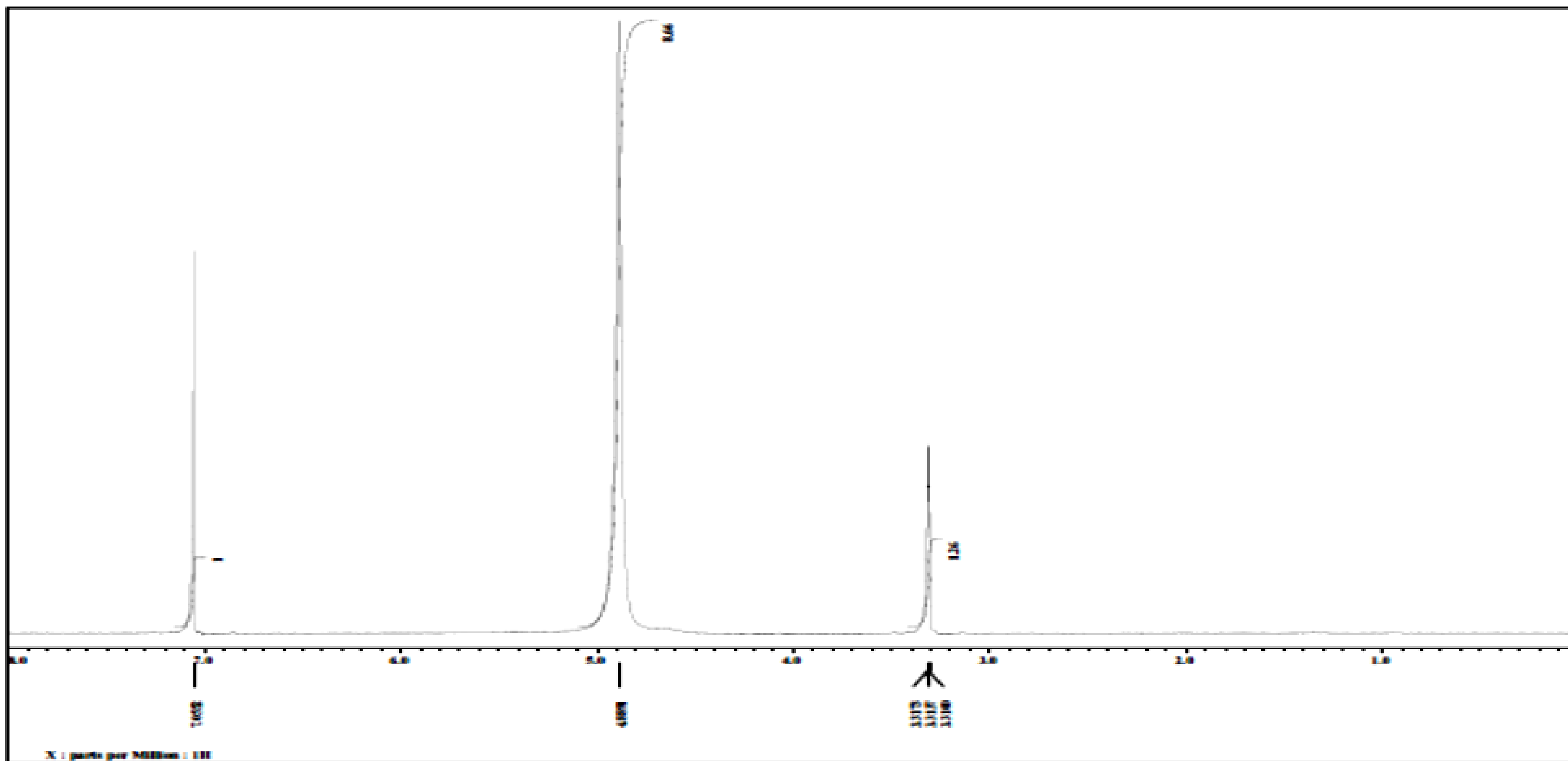

Figure 3.18: ^{1}H NMR Spectrum of the active gallic acid of aqueous extract of *Quercus Infectoria* after silica gel chromatography

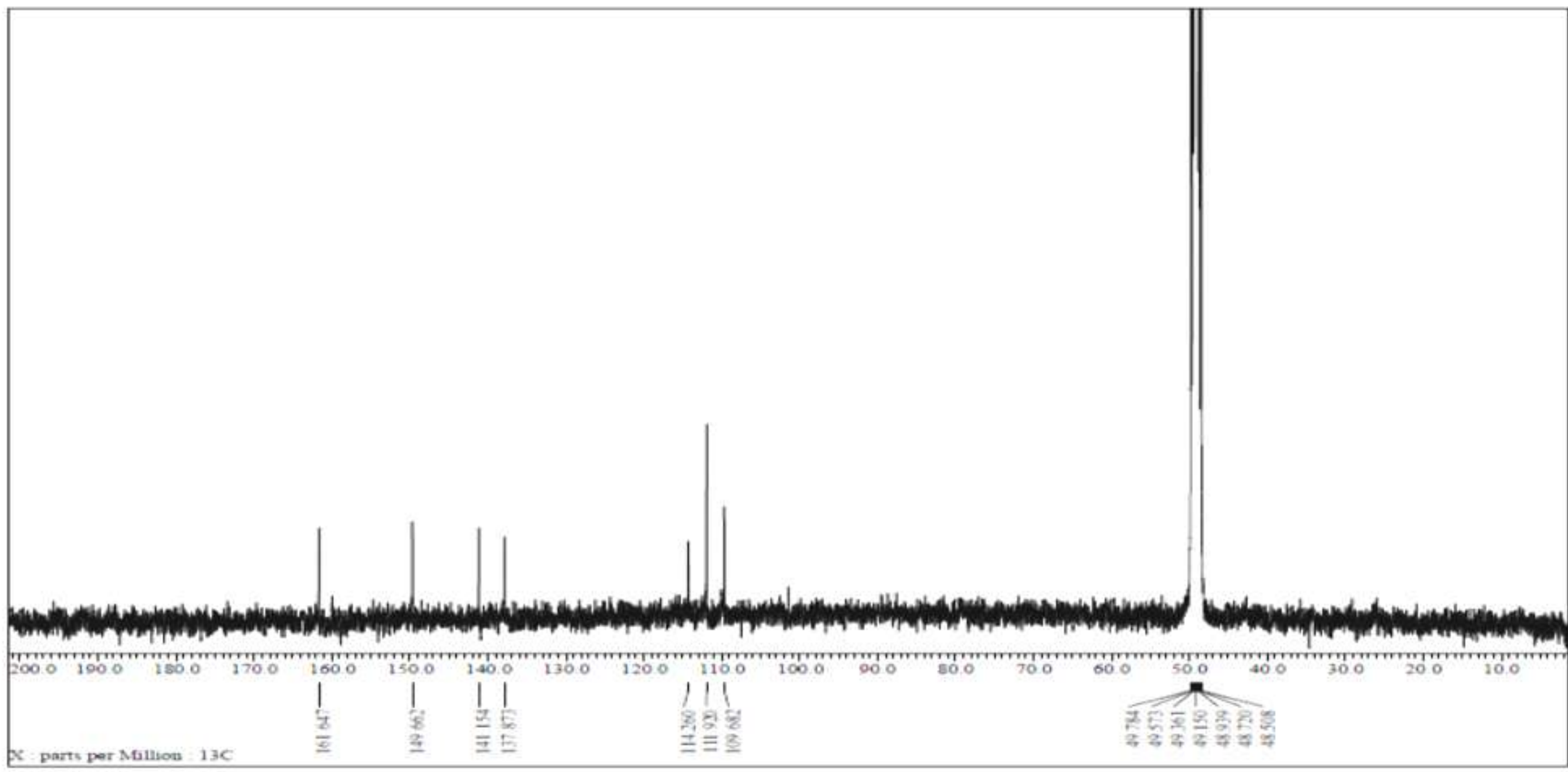

Figure 3.19: ^{13}C NMR Spectrum of the active ellagic acid of aqueous extract of *Quercus Infectoria* after silica gel chromatography.

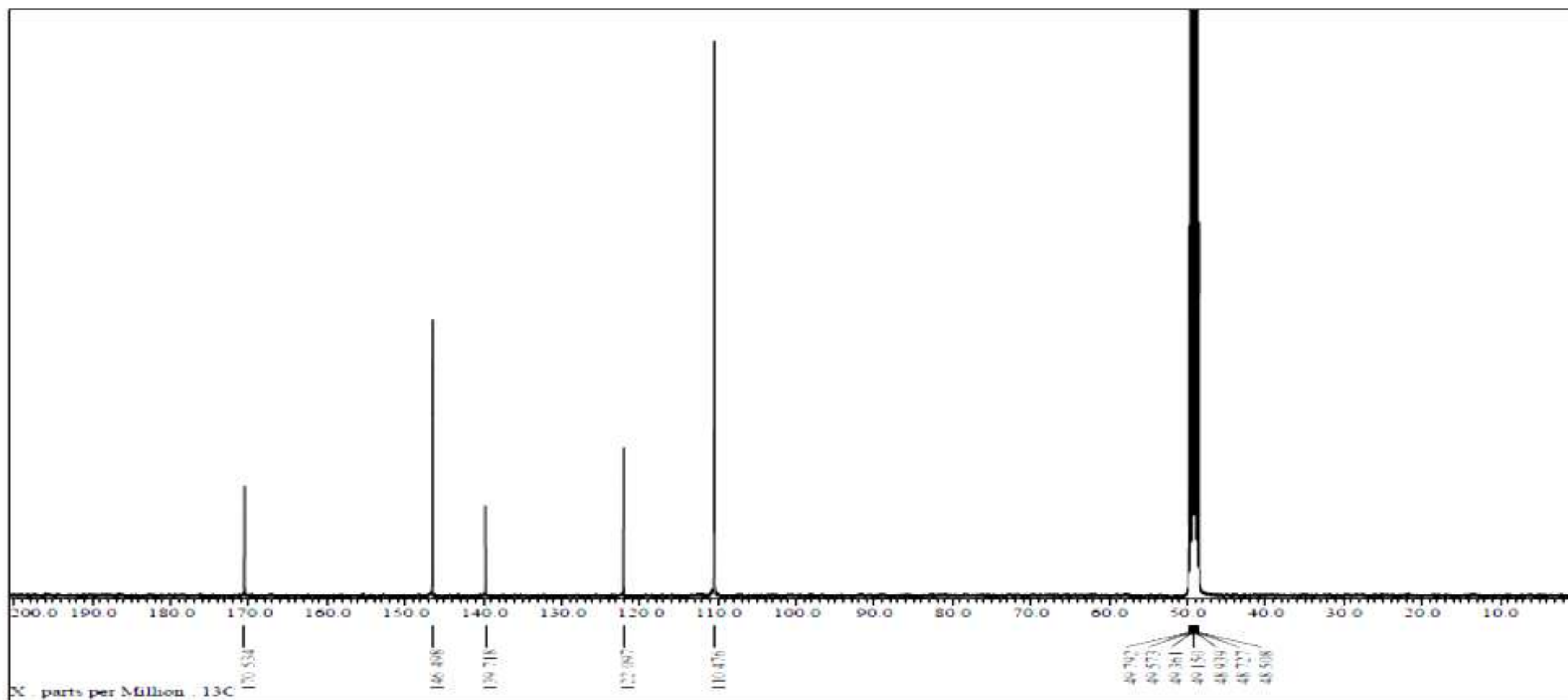

Figure 3.20: ^{13}C NMR Spectrum of the active gallic acid of aqueous extract of *Quercus Infectoria* after silica gel chromatography

Table 3.11: 13C and 1H NMR spectral data of the ellagic acid and gallic acid

Ellagic acid		Gallic acid	
^{1}H NMR	**^{13}C NMR**	**^{1}H NMR**	**^{13}C NMR**
7.51, 2H, (*s*)	109.68	7.05, 2H, (*s*)	110.47
	111.92		122.09
	114.26		139.72
	137.87		146.49
	141.15		170.53
	149.66		
	161.64		

3.10.5 Inhibition Assay of Purified Compounds of *Quercus Infectoria* Aqueous Extract on Dengue-2 NS2B/NS3 Protease Complexes

Another experiment was also carried out to investigate the inhibitory potential of the purified plant extract against NS2B/NS3. The concentrations used in this experiment were also 25 ppm, 50 ppm, 100 ppm, 200 ppm, and 400 ppm as seen in (Table 3.12), which was dissolved in DMSO. A concentration of 5% (v/v) DMSO caused low effect on enzyme activity at around 11.25 %. Therefore, 10 µL (5 % v/v) DMSO was used in this bioassay and has been fixed as the 0 inhibitor value. In this study 2 purified plants extract were identified to have inhibitory activity against NS2B/NS3 protease complex as showed in Table 3.12, and Figure 3.21.

Table 3.12: Result of inhibition assay of purified plant extracts on DENV-2 NS2B/NS3 protease complex

Purified plant Extracts	Solvent	Mean Percentage Inhibition (%)				
		25ppm	50ppm	100ppm	200ppm	400ppm
Ellagic acid	DMSO	48.33	65.14	83.56	92.80	97.83
Gallic acid	DMSO	45.39	59.52	77.04	86.49	92.91

The purified organic compound ellagic acid demonstrated strong inhibition towards the DENV-2 protease (greater than 90%) while gallic acid showed (greater than 85%) inhibition as shown in (Table 3.12) (The percentage of inhibition of the crude plant extract on this enzyme was increased in a dose dependent manner. All experiments were performed in triplicate.

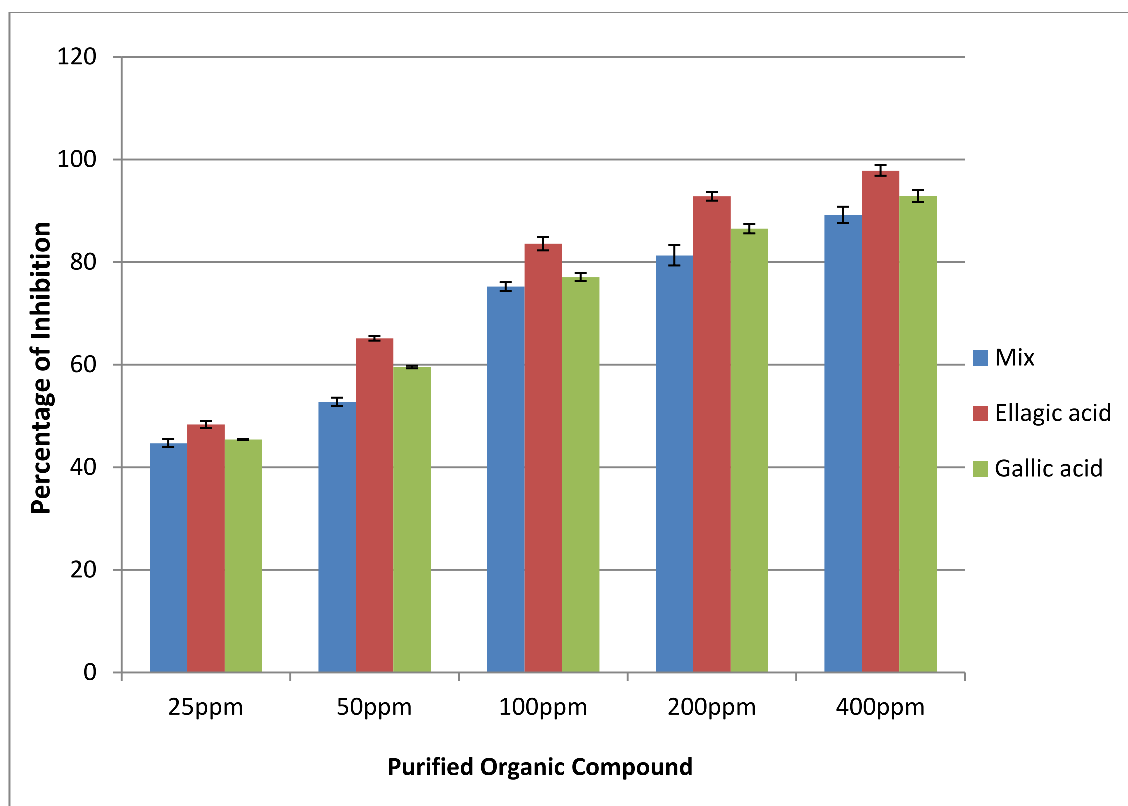

Figure 3.21: Chart of inhibition assay purified compounds on DENV-2 NS2B/NS3 protease complex.

The concentrations used in this experiment were 25ppm, 50ppm, 100ppm, 200ppm, and 400ppm. The blue column (Mix) is mix (v/v) gallic acid and ellagic acid. The reaction contains 2.0 µM enzyme and 100 µM fluorogenic substrate, Boc-Gly-Arg-Arg-AMC. The extract concentrations were varied from 25 ppm to 400 ppm. Substances with potential activity was then screened for their effect on this protease activity by measuring intensity using Tecan Infinite M200 Pro fluorescence spectrophotometer (λ (excitation) = 350 nm, λ (emission) = 440 nm).

3.11 Kinetic Analysis to Determine Type of Inhibition

In this experiment, activity of enzyme activity were also distinguished on the basis of whether the inhibition is or is not relieved by increasing the substrate concentrations. Many inhibitors do not exhibit the ideal properties of pure competitive or noncompetitive inhibition an alternative way to classify inhibitors is by their site of action. Some bind to the enzyme at the same site as does the substrate (the catalytic site) while others bind at some other site (an allosteric site) remove from the catalytic site. For this, Km and Vmax were identified using non-linear regression mixed model inhibition and linear regression model (Copeland, 2002) method in GraphPad Prism 5.0 software. The results showed that ellagic acid (fraction 1) and gallic acid (fraction 2) demonstrated a non competitive inhibition mechanism.

3.12 Purified compounds of *Quercus Infectoria*

Since the results indicated ellagic and gallic acid to have good activity against the NS2B/NS3 protease complex, it is of interest to investigate their potential as lead compound as therapeutic agent against DENV-2. Further works include characterization and determination of the inhibition constant, Ki.

This assay was carried out using increasing concentration of substrate while all other components kept constant. Figures 3.22 and 3.23 showed the kinetic assay plot of the ellagic acid and gallic acid. The inhibition constant, Ki was assayed using fluorogenic substrate at various concentrations ranging from 25 to 400 µM. The compounds were screened at 5 concentrations ranging from 0 to 800 µM. The data was analysed by linear regression model in GraphPad Prism 5 software.

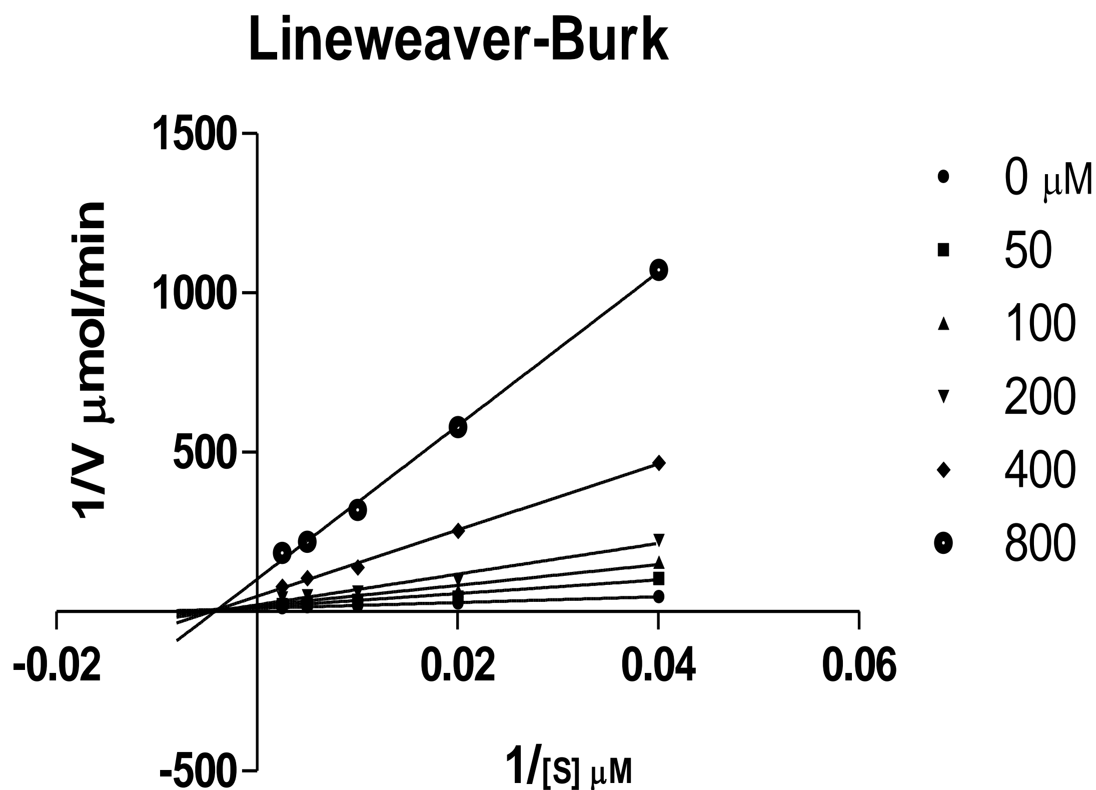

Figure 3.22: Lineweaver-Burk plot of ellagic acid from *Quercus Infectoria*

Lineweaver-Burk plot was carried out using increasing concentration of substrate ranged from 25 μM to 400 μM while all other conditions are kept constant. The data was analysed by linear regression model in GraphPad Prism 5 software.

Table 3.13: The values of Ki, Alpha, V_{max} and Km by GraphPad Prism 5.0 software

Parameter	Best-fit value	Standard Error
Vmax	0.0937µmole/min	± 0.0014
Alpha	1.312	± 0.157
Ki	58.64µM	± 4.638
Km	84.21µM	± 3.435
IC50	64.59µM	± 6.337

Vmax: Maximum enzyme velocity (µmol/min).

Km: Michaelis-Menten constant (µM).

Ki: Inhibition constant (µM)

Alpha: Constant that determines mechanism. If Alpha=1, this is the same as noncompetitive. 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.

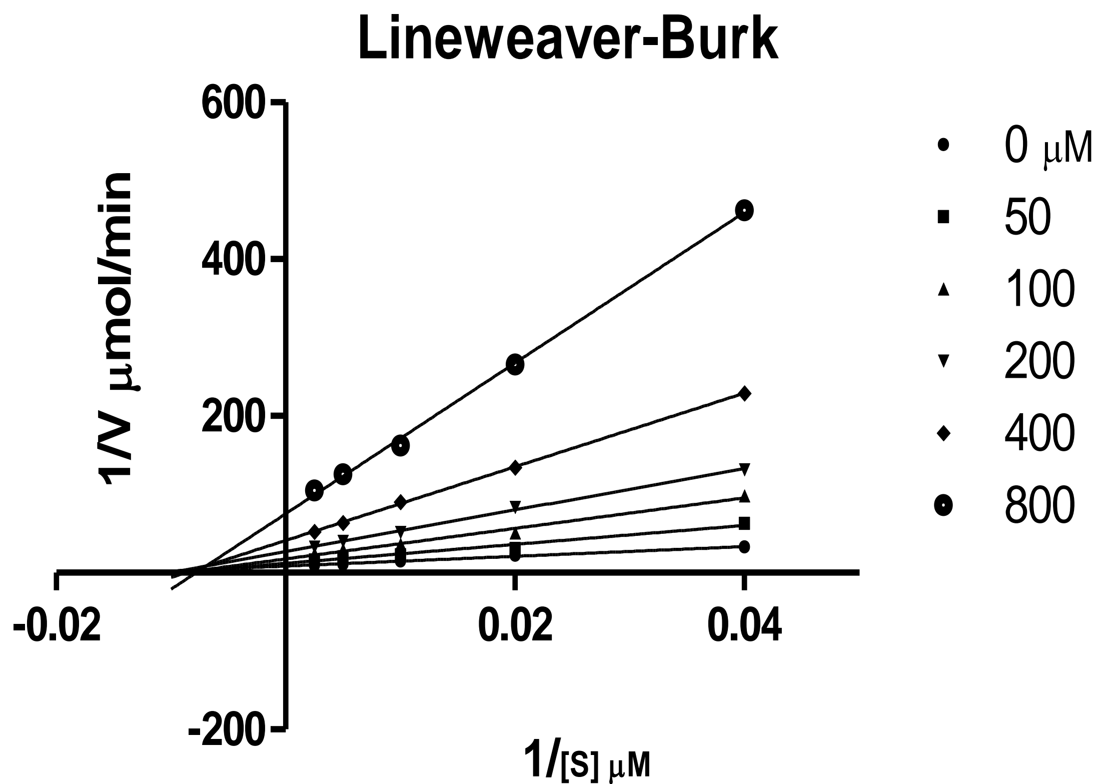

Figure 3.23: Lineweaver-Burk plot of gallic acid from *Quercus Infectoria*

Lineweaver-Burk plot was carried out using increasing concentration of substrate ranged from 25 µM to 400 µM while all other conditions are kept constant. The data was analysed by Michaelis-Menten model under non-linear regression curve fit in GraphPad Prism 5 software.

Table 3.14: The values of Ki, Alpha, V_{max} and Km by GraphPad Prism 5.0 software

Parameter	Best-fit value	Standard Error
Vmax	0.123µmole/min	± 0.0018
Alpha	1.124	± 0.122
Ki	72.62µM	± 6.143
Km	79.22µM	± 3.147
IC50	80.99µM	± 9.341

Vmax: Maximum enzyme velocity (µmol/min).

Km: Michaelis-Menten constant (µM).

Ki: Inhibition constant (µM)

Alpha: Constant that determines mechanism. If Alpha=1, this is the same as noncompetitive. 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.

3.13 Cytotoxicity assay

To evaluate the cytotoxic effect of the crud extract and purified ellagic acid and gallic acid, the MK2 cell lines were incubated individually with increasing concentrations of the crude extract and purified ellagic acid and gallic acid for 24, 48 and 72 hrs. The purified acids obviously showed cytotoxic effects towards MK2 cell lines in concentration dependent manner. The results showed that the IC_{50} of the crude extract was 300µg/ml, ellagic acid 250µg/ml whereas the IC_{50} of gallic acid was 200µg/ml. From the results we can suggest that ellagic acid has less cytotoxicity compared with gallic acid. The data was analysed by Log (inhibitor) vs. normalized response in GraphPad Prism 5 software.

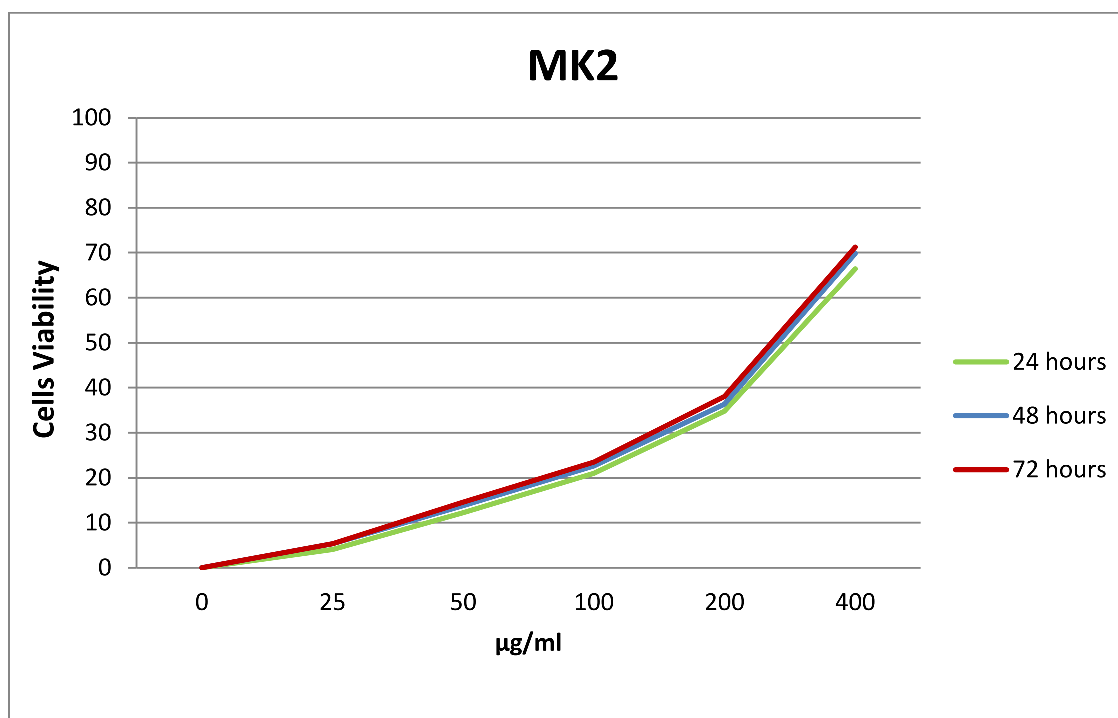

Figure 3.24 The cytotoxicity effect of *Quercus Infectoria* crude extract on MK2 cells.

The MK2 cell lines were seeded at $1x10^4$ cells per well in triplicate at optimal conditions (37˚C, 5% CO_2 in humidified incubator) in 96 well plates. The crude extract was diluted to serial concentration of 0, 25, 50, 100, 200 and 400 µg/ml with DMEM media supplemented with 2% FBS. The cell culture was analyzed at 24, 48 and 72 hrs using Non-Radioactive Cell Proliferation assay (Promega, USA) according to the manufacture protocol. The IC_{50} value of the crude extract was determinate to be 300µg/ml.

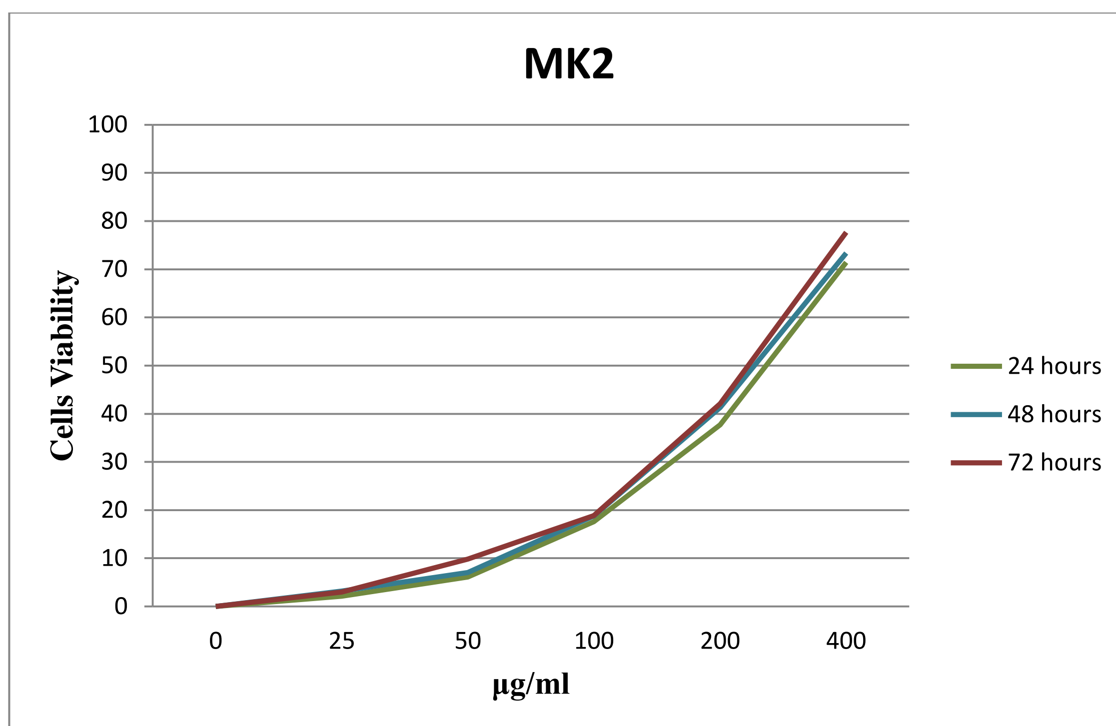

Figure 3.25: The cytotoxicity effect of purified ellagic acid on MK2 cells.

The MK2 cell lines were seeded at $1x10^4$ cells per well in triplicate at optimal conditions (37˚C, 5% CO_2 in humidified incubator) in 96 well plates. Purified acids was diluted to serial concentration of 0, 25, 50, 100, 200 and 400 µg/ml with DMEM media supplemented with 2% FBS. The cell culture was analyzed at 24, 48 and 72 hrs using Non-Radioactive Cell Proliferation assay (Promega, USA) according to the manufacture protocol. The IC_{50} value of ellagic acid was determinate to be 250µg/ml.

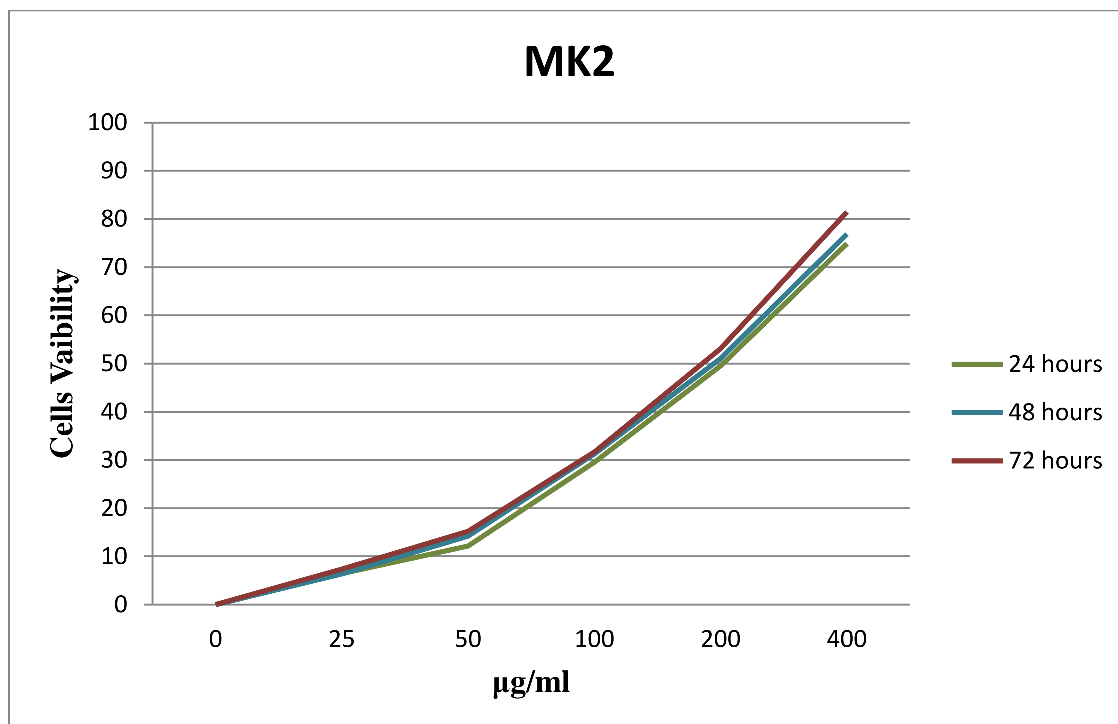

Figure 3.27: The cytotoxicity effect of purified gallic acid on MK2 cells.

The MK2 cell lines were seeded at $1x10^4$ cells per well in triplicate at optimal conditions (37˚C, 5% CO_2 in humidified incubator) in 96 well plates. Purified acids was diluted to serial concentration of 0, 25, 50, 100, 200 and 400 µg/ml with DMEM media supplemented with 2% FBS. The cell culture was analyzed at 24, 48 and 72 hrs using Non-Radioactive Cell Proliferation assay (Promega, USA) according to the manufacture protocol. The IC_{50} value of gallic acid was determinate to be 200µg/ml.