Studies on *Quercus lusitanica* Extracts on DENV-2 Replication

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Abstract

This study aimed to search for compounds with potential inhibitory activities towards DENV-2 replication. *In vitro* inhibitory activities of plant extracts towards DENV-2 replication were studied and the proteomics profile of cells infected with dengue virus, followed by treatment with plant extracts, were mapped out. Methanol crude and fractionated extracts of *Quercus lusitanica* were tested. The cytotoxicity of these plant extracts was evaluated by determining the maximum non-toxic dose (MNTD) on C6/36 cells. Antiviral activity was estimated by the reduction of the cytopathic effect (CPE) of DENV-2 in C6/36 cells and by the reduction of virus titre.

The crude methanol extracts of *Q. lusitanica* at the concentration of 180 µg/ml was found to completely inhibit the dengue virus infection at $TCID_{50}$ of 1-1000 by the absence of CPE. Protease inhibition assay of the crude and fractionated methanol extracts indicated more than 90% inhibition at the concentration of 0.2 mg/ml of the extracts.

Methyl gallate purified from fractionated crude extracts of *Q. lusitanica* at the MNTD of 100 µg/mL showed a 96% inhibition at $TCID_{50}$ of 1000. DENV-2 virus protease inhibition assay of methyl gallate showed more than 98% inhibition at 0.3 mg/mL.

Two-dimensional electrophoresis gels of normal, infected and treated cells showed that the treatment with crude methanol extracts as well as methyl gallate purified from the extract down-regulated the expression of the NS1 protein.

Keywords: *Quercus lusitanica,* DENV-2 replication, maximum non-toxic dose, protease inhibition assay.

Introduction

Dengue viruses, mosquito-borne members of the *Flaviviridae* family, are the causative agents of dengue fever and its associated complications, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS).[1] More than 2.5 billion people in over 100 countries are at risk of infection, and there are at least 20 million infections per year[2]. There is currently no treatment or vaccine available for dengue infection.[3]

There have been many reports of higher plant extracts possessing relatively good potential to inhibit viruses.[4] *Quercus lusitanica*,

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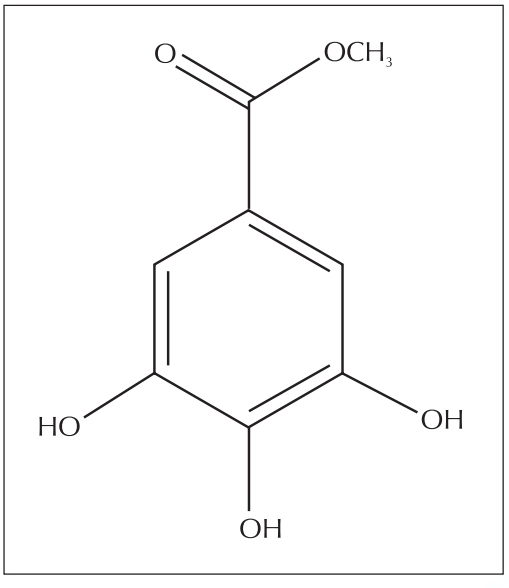
also known as *Quercus infectoria*, is a small tree or a shrub belonging to the *Fagaceae* (*Quercaceae*) family. They are found in the Mediterranean area, mainly in Greece, Asia Minor, Syria and Iran. The galls of *Q. lusitanica* have been shown to have many medicinal properties such as astringent, anti-diabetic, anti-pyretic and anti-Parkinsonian activities.[5] The chemical constituents of the galls have been reported to comprise a large amount of tannins, gallic acid, syringic acid, ellagic acid, ß-sitosterol, methyl betulate and methyl oleanate.[6] Methyl-3, 4, 5-trihydroxybenzoate or commonly-called methyl gallate is one of the compounds isolated from the *Q. lusitanica* methanol extracts in our lab. It is a polyphenol with three hydroxyl (-OH) and ester (R-COO-R) as shown in Figure 1.

Methyl gallate has been suggested to interact with herpes simplex virus proteins and alter the adsorption and penetration of the virion.[7] In addition, methyl gallate has been shown to have anti-tumour[8] and antibacterial activities.[9]

Figure 1: ***The structure of methyl gallate***



In this study, the *in vitro* inhibitory effects of extracts from *Q. lusitanica* were evaluated against DENV-2 virus replication. Results of these studies are incorporated in the present communication.

Materials and methods

Methanol extract of *Q. lusitanica*

The *Q. lusitanica* galls were air-dried and pulverized. Samples (100 g) were then soaked in methanol (800 mL) at ambient temperature overnight and filtered. The residue was washed with additional methanol and re-extracted. The combined filtrate was concentrated in vacuo and the concentrated methanol extract was used for assay.

Isolation of methyl gallate from the methanol extracts of *Q. lusitanica*

Q. lusitanica methanol extract were first eluted through silica with hexane. The polarity of the eluant was gradually increased (10% v/v-step ladder) in the order of hexane-toluene mixtures, toluene, toluene-diethyl ether, diethyl ether, diethyl ether-ethyl acetate, ethyl acetate, ethyl acetate-acetone, acetone, acetone-methanol and methanol. The flow rate on the fraction collector was set at 2 mL/min, and fractions were collected every 15–20 mL using glass vials.

Preparation of the cell culture

Experiments were carried out on C6/36-cloned cell line derived from larvae of *Aedes albopictus*. The C6/36 cell line was obtained from the Medical Microbiology Department, University of Malaya, and maintained in our laboratory by regular subculturing in RPMI

1640, supplemented with 2mM L-glutamine, 10% fetal bovine serum (FBS) and sodium bicarbonate. Cultivation of cells was at an atmosphere of 28 °C in the absence of 5% CO_2. Confluent monolayers of C6/36 were used for performing *in vitro* antiviral efficacy of plant extracts.

Determination of maximum non-toxic dose (MNTD)

Prior to screening of the plant crude extracts for their inhibitory potential, a control experiment by using methanol alone was carried out in C6/36 cells to rule out the direct effect of methanol toxicity upon cell-virus experiments. Following this, the plant crude extracts were subjected to cytotoxicity studies in order to determine the maximum non-toxic doses which could be non-toxic to C6/36 cells *in vitro*. Basically, the stocks were diluted by using part per million (ppm) calculations and added to the monolayer C6/36 cells in a 25 cm^2 falcon flask.

Virus stocks

Dengue virus (DENV-2), New Guinea C strain, obtained from the Department of Medical Microbiology, University of Malaya, was adapted in C6/36 cell lines. The presence of virus was confirmed by indirect enzyme-linked immunosorbent assay (ELISA) and by RT-PCR as well as nested PCR employing DENV group-specific and type-specific primers. Viral stocks were obtained by inoculating monolayer of C6/36 cells in a 25cm^2 tissue culture flasks with virus diluted 1:5 in 1 ml of maintenance medium containing 2% FBS. After an hour, 4 ml of maintenance medium was added and the cells were cultured for 5 days. Cells and supernatant were then harvested by gentle pipetting. Cell debris was removed by centrifugation at 3000 rpm for 10 minutes, and the viral supernatant was aliquoted in 20% FBS and stored at –80 °C.

Determination of the viral titration

The DENV-2 strain viral stock was removed from –80 °C freezers and immediately thawed in a 37 °C waterbath. Then, the viral stock was diluted by 10-fold serial dilutions (10^{-1}–10^{-8}) in maintenance medium. Four monolayer cultures were infected with 1 ml of each 10-fold virus dilution. Subsequently, the infected cells were incubated at 28 °C in the absence of 5% CO_2. Ninety-six hours after incubation, cells from the flasks were examined under microscope for the presence of cytopathic effects (CPE). The $TCID_{50}$ (tissue culture infective dose 50%) was calculated according to the methods of Reed and Muench.[10]

In vitro virus inhibition assay

In vitro inhibitory potential of plant extracts was evaluated in C6/36 cells using virus inhibition assay as described by Premnathan et al.[11] Briefly, plant extracts dilutions were prepared in methanol. Simultaneously, a series of 10-fold dilutions (10^{-1}–10^{-4}) corresponding to 1000 – 1 $TCID_{50}$ of DENV-2 viral stock were prepared separately. Different doses of each plant extracts were mixed with each dilution of the virus in equal proportions and incubated for an hour at 28 °C. After that, each mixture was added into a 25cm^2 falcon flask containing confluent monolayer of C6/36 cells along with suitable cell and virus controls. The flask was then incubated at 28 °C with the absence of 5% CO_2 and observed daily for the presence of cytopathic effects (CPE).

Protease inhibition assay

As described by Clum et al.[12] and Yusof et al.[13], the gene encoding the DENV-2 NS2B/3 protease was cloned and transformed into *Escherichia coli* strain XL1-Blue MRF and was expressed with histidine tag. The expressed NS2B/3 protease enzyme was purified on nickel column followed by Sephadex G-75 gel filtration under denaturing condition. The protease was refolded by dialysis and the activity was determined. All kinetic measurements were performed in 200 mM Tris (pH 8.5) at 37 °C. Fluorescence due to cleavage of the substrate [Boc-Gly-Arg-Arg-MCA (Peptide Institute Inc., Osaka, Japan)] was monitored at 465 nm with excitation at 385 nm.

Protein profiling by proteomic technique: Two-dimensional gel electrophoresis (2-DE)

The 2-DE was performed to determine the protein profile. Briefly, 10 µl (50 µg protein) of infected and treated cell lines was subjected to isoelectric focusing in 13 cm rehydrated pre-cast immobilized dry strips pH 3-10 (Amersham Biosciences, Uppsala, Sweden). For the second dimension, focused samples in the strips were subjected to electrophoresis using the 10% homogenized polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE). The 2-DE gels were then stained with silver staining for further analysis and comparison. All samples were analysed in triplicate.

Results and discussion

In our preliminary studies, extracts from *Q. lusitanica* have been shown to inhibit the activity of the NS2B/3 DENV-2 protease. Hence, in this study, extracts from *Q. lusitinica* as well as purified methyl gallate from fractionated extract of *Q. lusitanica* were subjected to toxicity studies in order to determine the maximum non-toxic dose (MNTD) on the C6/36 mosquito cell lines.

In vitro inhibition assay of methanol crude and fractionated extracts of *Q. lusitanica*

The result from the control experiment using methanol alone showed that methanol was toxic to C6/36 cells at the concentration of 10% (v/v) and above (Figure 2). Based on this result, the concentration of methanol in *Q. lusitanica* crude methanol extracts tested in cell culture used in these experiments did not exceed 10% (v/v).

The inhibitory potential of methanol crude extract (which was concentrated from 100 g powder of *Q. lusitanica* soaked in 800 ml methanol) and fractionated extract of *Q. lusitanica* on DENV-2 virus in *in vitro* systems was then evaluated. The MNTD of these extracts on C6/36 cells is shown in Figure 3. The results indicated the crude extract to be toxic at the concentration of more than 180 µg/mL.

Figure 2: ***Relative toxicity of methanol in C6/36 cells***

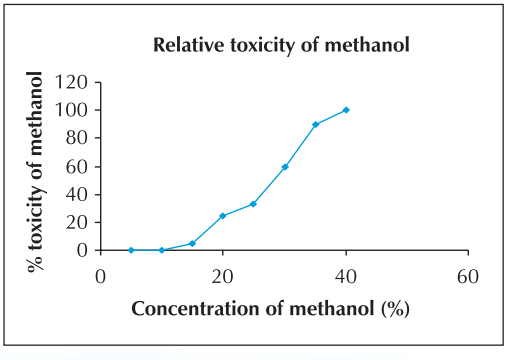
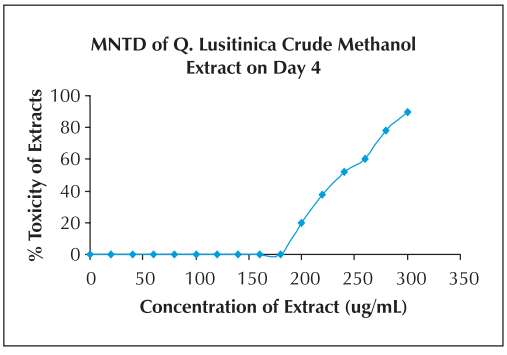


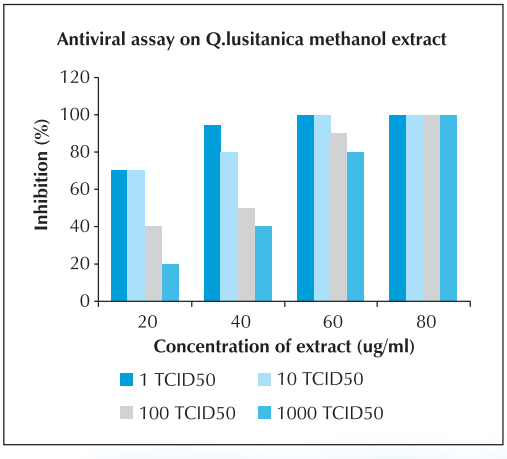
Figure 3: ***Relative toxicity and maximum non-toxic dose (MNTD) of preparations of Q. lusitanica methanol extract in vitro (20 to 300 µg/ml). The MNTD for Q. lusitanica was at 180 µg/ml on C6/36 cells***



The *in vitro* inhibitory potential of *Q. lusitanica* methanol extracts on DENV-2 virus replication in C6/36 cells revealed inhibition of virus replication in dose-dependent manner as depicted in Figure 4.

The extract at its maximum concentration of 80 µg/mL showed 100% inhibition on the replication of the whole range of virus titre used in this study as indicated by the absence of cytopathic effects (CPE). The low dosage of the extract (40 µg/ml) showed 80% inhibition with 10 $TCID_{50}$ of virus but only 50% and 40% inhibition of 100 and 1000 $TCID_{50}$ of virus, respectively (Figure 4).

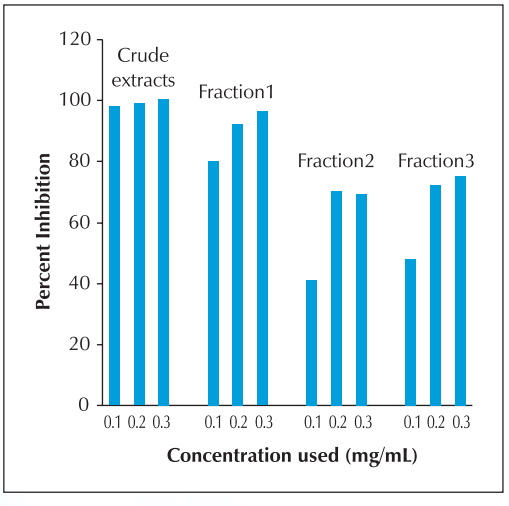
Figure 4: ***Inhibitory potential of various concentrations of Q. lusitanica methanol extracts on different concentrations of DEN-2 virus***



Protease inhibition assay

Protease inhibition assay was performed on the crude and fractionated methanol extracts from *Q. lusitanica* to see the inhibition of NS2B/3 protease complex of DENV-2. The results showed both crude extracts as well as fractionated extracts of *Q. lusitanica* to be active at inhibiting the protease activity (Figure 5). The maximum inhibition observed for the crude extracts was more than 96% at the concentration of 0.20 mg/ml. One of the fractionated compounds also showed high inhibitory activity of the NS2B/3 DENV-2 protease (Figure 5). Further purification of this fraction was carried out in order to identify the possible compounds that may be responsible for the activity.

Figure 5: ***Protease inhibition assay of the crude and fractionated methanol extracts of Q. lusitanica***



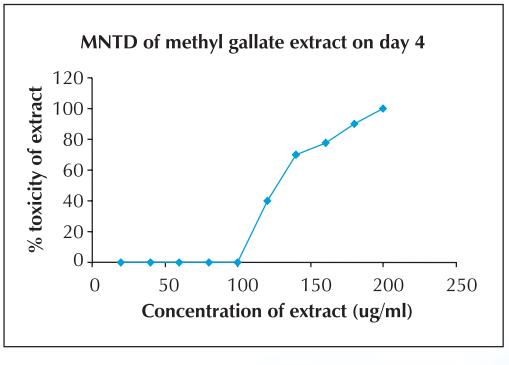
Isolation of methyl gallate from active fractionated methanol extracts of *Q. lusitanica*

Fractions with the R_f value of 0.59 (chloroform-ethyl acetate 9:1 v/v) were collected and the solvent were removed in vacuo to give white crystals as the product. ^{1}H and ^{13}C NMR spectra indicated the white crystals to be methyl gallate, as identified by comparison with an authentic sample. ^{1}H NMR (δ, $CDCl_3$, ppm) 8.07 (3H, brs, O<u>H</u>), 7.03 (2 H, s, aromatic H), 3.76 (3H, s, O<u>C</u>H_3), ^{13}C NMR (δ, $CDCl_3$, ppm) 120.59 (<u>C</u>=O), 166.93 (Ph<u>C</u>), 144.62 (Ph<u>C</u>), 137.24 (Ph<u>C</u>), 109.15 (Ph<u>C</u>), 51.46 (<u>C</u>H_3) and MS (EI, 70 eV) 184 ($M^{\pm}$, 45%), 153 (M-OCH_3, 100%) and 125 (M-CH_3OC=O, 25%).

Inhibitory effect of methyl gallate of *Q. lusitanica* on DENV-2 replication

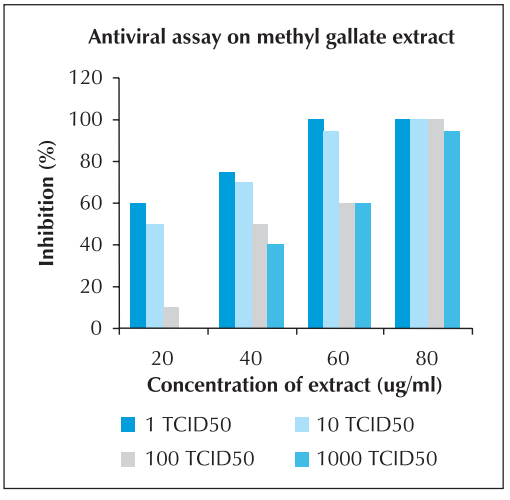
Cytotoxicity studies were performed on the methyl gallate isolated from the fractionated methanol extracts of *Q. lusitanica* on C6/36 cells. The MNTD of methyl gallate was shown to be 100 μg/ml (Figure 6). This is relatively more toxic as compared to that observed with the crude *Q. lusitanica* methanol extract (180 μg/ml).

Figure 6: ***Relative toxicity and maximum non-toxic dose (MNTD) of preparations methyl gallate in vitro (20 to 200 μg/ml). The MNTD for methyl gallate was at 100 μg/ml on C6/36 cells***



Inhibitory assay against DENV-2 replication by methyl gallate at the maximum concentration of 80 μg/ml showed 96% inhibition at the viral titre of 1000 $TCID_{50}$. However, methyl gallate showed a 100% inhibition at the viral titre from 1–100 $TCID_{50}$ (Figure 7).

Figure 7: ***Inhibitory potential of various concentrations of methyl gallate on different concentrations of DENV-2 virus***



Protease inhibition assay of methyl gallate

Protease inhibition assay was performed on methyl gallate purified from the fractionated extract of *Q. lusitanica* in order to determine the inhibition of NS2B/3 protease complex of DENV-2. The results demonstrated greater than 90% inhibition on DENV-2 protease by methyl gallate (Table).

Kinetic analysis was then carried out to determine the type of inhibition by methyl gallate on the NS2B/3 DENV-2 protease

Table: *Percentage of inhibition by methyl gallate on NS2B/3 DENV-2 protease complex*

Concentration of methyl gallate (mg/ml)	% Inhibition
0.1	76
0.2	86
0.3	98

complex. A Lineweaver-Burk plot was used to determine the K_i by increasing the concentration of the fluorogenic substrate, BOC-Gly-Gly-Arg-MCA, ranging from 50 to 150 μM, while all other conditions were kept constant. Figure 8 shows a Lineweaver-Burk plot of methyl gallate purified from *Q. lusitanica*. Methyl gallate were screened at a concentration of 0.05 mg/ml, 0.20 mg/ml and 0.30 mg/ml. The result from the graph indicated methyl gallate to be a non-competitive inhibitor.

The inhibition constant, K_i value, was determined using the graph of $1/V_{max}$ inhibitor versus concentration inhibitor as shown in Figure 9 and the inhibition constant (K_i) was found to be 0.341 mM.

Two-dimensional gel electrophoresis analysis

Based on the *in vitro* inhibition assay, protein profilings of normal, infected and treated with crude *Q. lusitanica* and methyl gallate C6/36 cell lines were carried out. A comparative analysis made between normal and infected C6/36 cells showed four proteins at the molecular weight of about 50 kDa in the infected cells (Figures 10 a and b). However, this is not detected in the normal cells (Figure 10a). The origins of these proteins were determined through immunoblotting using

Figure 8: ***A Lineweaver-Burk plot of active compound 2 from Q. lusitanica***

Lineweaver-Burk plot was carried out using increasing concentration of substrate (S) while all other condition was kept constant. The reaction was done in the plate.

Line 1 (control): 1.0 μM enzyme and 50-150 μM substrate without inhibitor.

Line 2: 1.0 μM enzyme, 50-150 μM substrate and contained 0.05 mg/ml compound 2.

Line 3: 1.0 μM enzyme and 50-150 μM substrate and contained 0.20 mg/ml compound 2.

Line 4: 1.0 μM enzyme and 50-150 μM substrate and contained 0.30 mg/ml compound 2.

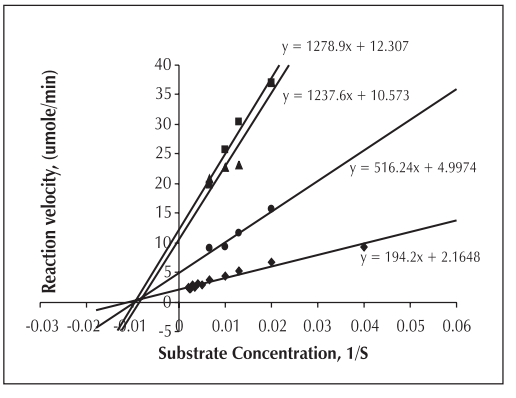
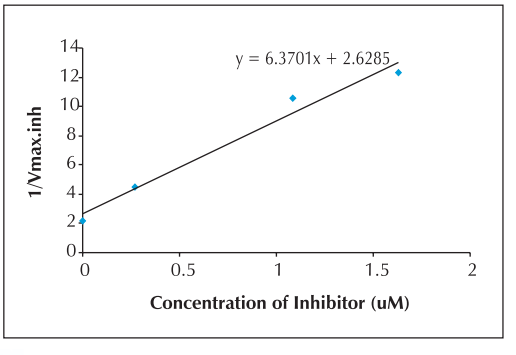


Figure 9: ***Reciprocal maximum reaction velocity of inhibitor ($1/V_{max.inh}$) versus concentration of methyl gallate***

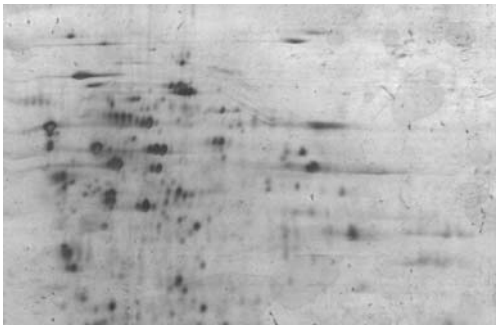


hyper-immune anti-rabbit serum against DENV-2 virus. The result revealed these proteins to be of viral origin as determined by the recognition of the antibody raised against the DENV-2 virus. N-terminal sequencing analysis performed to identify these proteins revealed a sequence of D-S-G-C-V-V-S-W-K-N-K, which was then extrapolated from the Swissprot database to belong to the DEN-2 non-structural protein 1 (NS1).[14•3] Further investigations of the inhibitory potential of plant extracts and compound using 2-DE were carried out. Treatment of the infected cells with low concentration of 40μg/ml of the *Q. lusitanica* extracts caused the NS1 proteins expression to be reduced (data not shown). Increasing the concentration to 120 μg/ml resulted in the disappearance of the NS1 spots completely as shown in Figure 10(c).

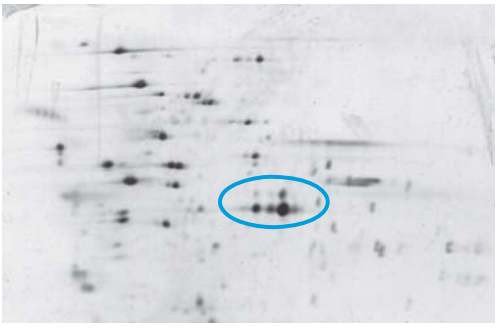
Similarly, cells treated with methyl gallate caused the spots to be reduced in dose-dependent manner as shown in Figure 10(d). This study has shown that the extracts of *Q. lusitanica* as well as methyl gallate have the ability to down-regulate the NS1 protein expression. This down-regulation of the NS1 protein expression could be related to a reduction or absence of CPE on infected C6/36 cells.

Figure 10: ***Protein profile of C6/36 cells infected with DENV-2 virus followed by treatment with crude extracts and purified methyl gallate from Q. lusitanica***

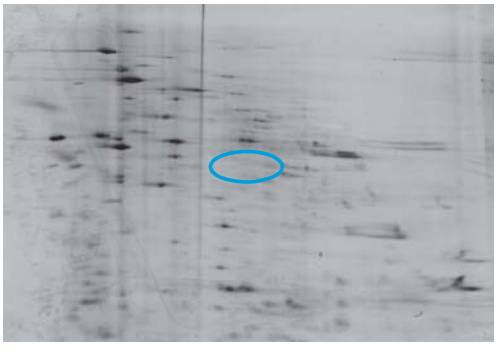
10(a): Uninfected C6/36 cells



10(b): Infected C6/36 cells



10(c): Infected cells treated with 120 μg/ml crude extracts of *Q. lusitanica*



10(d): Infected cells treated with 80 μg/ml of methyl gallate

In summary, methyl gallate and extracts from *Q. lusitanica* are promising antiviral agents against DENV-2 replication as can be observed from the *in vitro* inhibition assay and two-dimensional electrophoresis.

Conclusion

The *in vitro* inhibition assays of the crude extracts of *Q. lusitanica* as well as methyl gallate purified from extracts of *Q. lusitanica* exhibited complete inhibition of the DENV-2 virus replication in inoculated C6/36 cells. 2-DE studies indicated that treatment with crude *Q. lusitanica* and methyl gallate have the ability to down-regulate the NS1 protein expression, which could be related to a reduction or absence of CPE on infected C6/36 cells.

Acknowledgement

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