Tropical Biomedicine 31(2): 286–296 (2014)

Screening of antiviral activities in medicinal plants extracts against dengue virus using dengue NS2B-NS3 protease assay

Rothan, H.A.[1*], Zulqarnain, M.[2], Ammar, Y.A.[1], Tan, E.C.[1], Rahman, N.A.[3] and Yusof, R.[1]
[1]Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]Department of Genetics, Institute of Biological Science, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[3]Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[*]Corresponding author email: rothan@um.edu.my
Received 24 August 2013; received in revised form 20 December 2013; accepted 23 December 2013

Abstract. Dengue virus infects millions of people worldwide and there is no vaccine or anti-dengue therapeutic available. Screening large numbers of medicinal plants for anti-dengue activities is an alternative strategy in order to find the potent therapeutic compounds. Therefore, this study was designed to identify anti-dengue activities in nineteen medicinal plant extracts that are used in traditional medicine. Local medicinal plants *Vernonia cinerea*, *Hemigraphis reptans*, *Hedyotis auricularia*, *Laurentia longiflora*, *Tridax procumbers and Senna angustifolia* were used in this study. The highest inhibitory activates against dengue NS2B-NS3pro was observed in ethanolic extract of *S. angustifolia* leaves, methanolic extract of *V. cinerea* leaves and ethanol extract of *T. procumbens* stems. These findings were further verified by *in vitro* viral inhibition assay. Methanolic extract of *V. cinerea* leaves, ethanol extract of *T. procumbens* stems and at less extent ethanolic extract of *S. angustifolia* leaves were able to maintain the normal morphology of DENV2-infected Vero cells without causing much cytopathic effects (CPE). The percentage of viral inhibition of *V. cinerea* and *T. procumbens* extracts were significantly higher than *S. angustifolia* extract as measured by plaque formation assay and RT-qPCR. In conclusion, The outcome of this study showed that the methanolic extract of *V. cinerea* leaves and ethanol extract of *T. procumbens* stems possessed high inhibitory activates against dengue virus that worth more investigation.

INTRODUCTION

Multiple dengue serotypes (DENV1-DENV4) are members of *Flaviviridae* family that are considered as arthropod-borne human pathogens transmitted by its primary mosquito vector Aedes *aegypti*, and secondary vector *Aedes albopictus* (Rigau-P´erez *et al.*, 1998). Dengue virus infects 50-100 million people each year worldwide and causes various clinical symptoms such as dengue fever (DF) that may later develop to severe dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (Monath *et al.*, 1994; Gubler & Clark, 1995; Botting & Kuhan, 2012). Annually, there are approximately 0.5 million cases of DHF and DSS that lead to more than 20,000 deaths worldwide (Gubler, 2002).

Dengue virus is a small, enveloped virus that contains positive single stranded RNA genome with long open reading frame lacking the poly A tail. The virus uses host cell ribosomes to translate its genomic RNA to full length precursor polyprotein. The post-translational proteolytic cleavage of the precursor protein results in the formation of three structural proteins (namely the capsid, membrane and envelope proteins) and seven non-structural proteins NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Chanprapaph *et al.*, 2005). Protolytic cleavage is achieved by

the combined action of signalase and furin from the host cell, and NS2B-NS3 serine protease from the virus (Stadler *et al*., 1997).

Dengue NS3 protein possesses a trypsin-like serine protease activity in its 180 amino acid N-terminal residues (Chambers *et al*., 1990; Falgout *et al*., 1991) while the C-terminal region is responsible for RNA-helicase and RNA-stimulated NTPase activities (Gorbalenya *et al*., 1989; Wengler, 1993). The protease catalytic activity of NS3 protein depends on the interaction with its cofactor NS2B to form NS2B-NS3 protease (NS2B-NS3pro) (Yusof *et al*., 2000). With the exception of C-prM which is a structural protein, NS2B-NS3pro functions by cleaving viral non-structural proteins at the protein junctions of NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 (Teo, 1997; Stocks & Lobigs, 1998). The disruption of NS2B-NS3pro function is lethal to viral replication (Geiss *et al*., 2009). As such, this protein has been largely considered as an effective target for antiviral drugs (Tomlinson *et al*., 2009).

Drugs that possess potent inhibition of dengue virus are urgently needed to combat the increase of dengue infection worldwide. In this study, local medicinal plants *Vernonia cinerea*, *Hemigraphis reptans*, *Hedyotis auricularia*, *Laurentia longiflora*, *Tridax procumbers* and *Senna angustifolia* were screened for dengue virus inhibition based on the dengue NS2B-NS3pro assay. These medicinal plants are commonly used for the alternative and traditional treatment of various microbial infections. Extracts from plants species that show high inhibitory activities against NS2B-NS3pro were selected for further analysis and subjected to *in vitro* viral inhibition assay.

MATERIALS AND METHODS

Preparation of Plant Extracts

Vernonia cinerea, *Hemigraphis reptans*, *Hedyotis auricularia*, *Laurentia longiflora*, *Tridax procumbers* and *Senna angustifolia* plants were collected from various sites in Kuala Lumpur and Selangor, Malaysia. The botanical identities of each plant were determined and confirmed by a taxonomist from the Institute of Biological Sciences, University of Malaya. Ethanolic and methanolic extracts were prepared based on the availability and relative ease of obtaining the plant samples. Plant leaves, stems and roots were chopped into small pieces and air-dried at room temperature until consistent residual weight has been achieved. The dried pieces were then ground using an electric blender. After grinding, 100 grams of the resultant powders were soaked in 500 ml absolute ethanol or methanol and kept in the dark for five days. The solution was then filtered using Whatman filter paper (20-25 µm) and evaporated at 50°C to yield approximately 7.5 to 9.5 gm of each extract. Stock solution of each extract was prepared by dissolving 100 mg plant extract in 10 ml DMSO, and stored in 4°C until further use.

Dengue NS2B-NS3pro assay

This assay was carried out to identify the inhibitory activities of plant extracts against dengue NS2B-NS3pro as previously described (Rothan *et al*., 2012a; Rothan *et al*., 2012b). First, the activity of the recombinant dengue NS2B-NS3pro was evaluated using increased concentrations of protease (1, 2, 3, 4, 5 and 6 µM) with fixed concentration (100 µM) of the fluorescence substrate (Minoh-shi, Osaka, Japan, Lot no: 580907). The end point reaction mixture was performed in black 96-well plates consists of 2 µM recombinant NS2B-NS3pro, 100 µM fluorogenic peptide substrate (Boc-Gly-Arg-Arg-AMC), plant extracts of varying concentrations prepared in DMSO (12.5, 25, 50, 100 and 200 µg/ml), buffered at pH 8.5 with 200 mM Tris-HCl with total volume of 200 µl. The reaction mixture without plant extract, substrate with plant extract, enzyme and different concentrations of plant extract were used as controls. Thereafter, all reaction mixtures were incubated at 37°C for 30 minutes and the substrate was added to the specific reaction mixtures and incubated at the same temperatures for another 30 minutes. Measurements were performed in triplicates using Tecan Infinite M200 Pro fluorescence spectrophotometer (Tecan Group Ltd.,

Switzerland). Substrate cleavage was normalized against buffer only (control) at the emission of 440 nm upon excitation at 350 nm. The IC_{50} values were calculated for each extract from nonlinear regression fitting of signal *vs.* concentration data points to the standard dose–response equation *Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50)))*. In this equation, *X* was the log of compound concentration, *Y* was the response signal, and bottom and top refer to plateaus of the sigmoid response curve. All assays were performed in triplicate and repeated twice.

Maximum non-toxic dose test (MNTD)

The MNTD assay was carried out to determine the cytotoxic concentration of plant extracts. The MNTD test was initiated by seeding Vero cells at $1x10^4$ cells/well in triplicates, at optimal conditions (37ºC, 5% CO_2 in humidified incubator) in 96 well plates with blank control (media only) and cells control (cells only). After overnight incubation, the cells were treated with diluted plant extract stock at the concentrations of 0, 25, 50, 100, 200 and 400 µg/ml with DMEM media supplemented with 2% FBS. The cell culture was analyzed after 72 hours using Non-Radioactive Cell Proliferation assay (Promega, USA) according to the manufacture's protocol. Percentage of cytotoxicity was calculated as follows: (Absorbance of treated cells/Absorbance of untreated cells) x 100. The MNTD was calculated from dose-response curves.

Treatment of DENV2-infected cells with plant extracts

To infect Vero cell lines with DENV2, the cells were cultured in 24-well plates (1.5×10^5 cells/well) for 24 hrs at 37ºC and 5% CO_2. The virus supernatant was added to the cells at MOI of 0.2 followed by incubation for 1 hr with gentle shaking every 15 min for optimal virus to cell contact. The cells were washed twice with fresh serum-free DMEM media after removing the virus supernatant. Then, new complete DMEM media containing 50 µg/ml of plant extract were added and the cultures were incubated for 24, 48 and 72 hrs. Afterwards, cellular supernatants were collected and stored at -80°C for viral load quantification.

Plaque formation assay

To determine the virus yield after treatment with plant extracts, culture supernatants were collected and serially diluted to reduce the effects of extract residues. A 10-fold serial dilution of medium supernatant was added to new Vero cells grown in 24-well plate ($1.5\ x10^5$ cells) and incubated for 1hr at 37ºC. The cells were then overlaid with DMEM medium containing 1.1% methylcellulose. Viral plaques were stained with crystal violet dye after five-day incubation. Virus titers were calculated according to the following formula: Titer (pfu/ml) = number of plaques x volume of diluted virus added to the well x dilution factor of the virus used to infect the well in which plaques were enumerated.

Quantitative Real-time PCR

After treatment with plant extract, the DENV2 RNA copies were quantified using one-step real-time PCR. First, a standard curve was generated by 10-fold serial dilution of known copies of DENV2 RNA. Then, viral RNA was extracted from culture supernatant using QIAmp viral RNA mini kit (QIAGEN, Germany) according to the manufacturer's instructions. Primer pairs were designed to target the 5'UTR region of the virus genome (Forwarded primer 5'-TACGTGGACCGAC AAAGACA-'3 and reverse primer 5'-AAA AACTGTTAGAACTACGTTGAGC-'3). The reactions of qRT-PCR were carried out using SyBr Green Master Kit (Qiagen, Germany) and absolute quantification was performed using ABI7500 machine (Applied Biosystems, Foster City, CA). The thermal cycling profile of this assay consisted of a 30 min reverse transcription step at 50ºC, 15 min of Taq polymerase activation at 95ºC, followed by 35 cycles of PCR at 95ºC of denaturing for 30 sec, 40 sec annealing at 58.0ºC and 50 sec extension at 72.0ºC with a step of a single fluorescence emission data collection followed by 10 min at 72ºC for final extension. The specificity of amplicon was verified by melting curve analysis (72 to 95ºC) with a heating rate 0.5ºC per 5 sec to

check the identity and purity of the amplified products. Results were analyzed using Sequence Detection Software Version 1.3 (Applied Biosystems, Foster City, CA). Percentage of viral inhibition (%) was calculated as follows:

100 – (Viral copy number of treated cells/ viral copy number of untreated cells) x 100.

Statistical analysis

All the assays were done in triplicates and the statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). *P* values <0.05 were considered significant. Error bars are expressed as ± SD.

RESULTS

Inhibitory activities of plant extracts against dengue NS2B-NS3pro

The inhibition profile of nineteen medicinal plant extracts towards dengue NS2B-NS3pro is shown in Figure 1. The apparent IC_{50} for each plant extracts was calculated using increased concentrations form these extracts. Interestingly, ethanolic extract of *S. angustifolia* leaves, methanolic extract of *V. cinerea* leaves and ethanolic extracts of *T. procumbers* stems showed the highest inhibitory activity compared to other extracts (IC_{50} values: 30.1±3.4, 23.7±4.1 and 25.6±3.8 µg/ml respectively). Both methanolic and

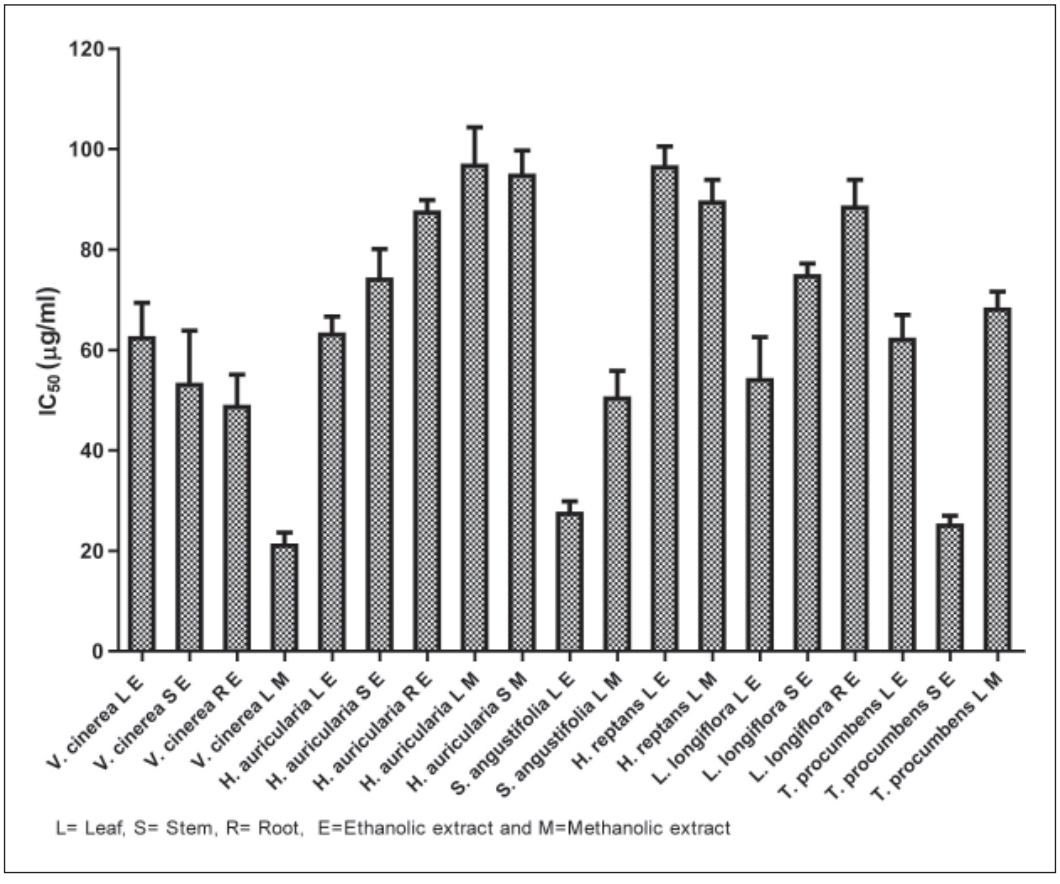

Figure 1. The inhibitory effects of medicinal plant extracts against dengue NS2B-NS3 protease measured by apparent IC_{50} values. The IC_{50} values were calculated from increased concentrations of each plant extract. The total of 5 data points of each extract were used to calculate the apparent IC_{50} value from nonlinear regression fitting of signal *vs.* concentration data points to the standard dose–response equation *Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50)))*. In this equation, *X* was the log of compound concentration, *Y* was the response signal, and bottom and top refer to plateaus of the sigmoid response curve. All assays were performed in triplicate and repeated twice

ethanolic extracts are shown to inhibit NS2B-NS3pro cleavage of the fluorescence substrate. However, the activity of methanolic and ethanolic extracts varied from one plant extract to another. For instance, methanol extracts of *V. cinerea* leaves showed higher inhibitory effect compared to its ethanol extracts. On the other hand, ethanolic extracts of *S. angustifolia* leaves showed higher inhibitory effect compared to its methanolic extracts. A similar trend was also noted for the *T. procumbens* leaf extracts.

Determination of maximum non-toxic dose (MNTD)

In order to evaluate their anti-dengue activity, extracts showing high NS2B-NS3pro-inhibitory activities were first subjected to toxicity test to determine the maximal dose of minimal or non-toxicity to the cells. In this study, the MNTD assay was carried out using serially diluted extracts of each plant followed by further optimisation in order to achieve a specific cytotoxic concentration. The MNTD of each plant obtained through the optimization steps are presented in Figure 2. In general, the selected extracts showed approximately similar toxicity profile. The changes in cell viability were almost insignificant at 50 µg/ml dosage, and this dose was considered in subsequent viral inhibition assays. Cells toxicity was considerably increased at the dose of more than 100 µg/ml of these three extracts.

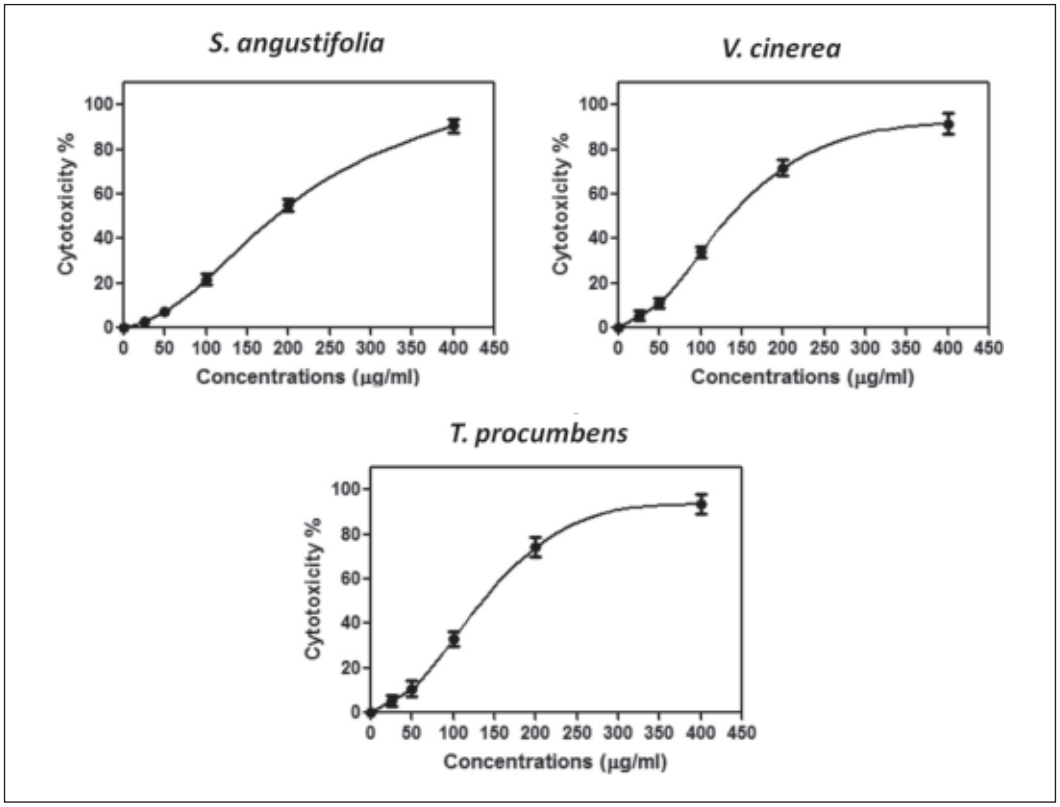

Figure 2. Maximum non-toxic dose test (MNTD). The MNTD assay was carried out to determine the cytotoxic concentration of plant extracts. Vero cells were seeded at $1x10^4$ cells/ well in triplicate at optimal conditions in 96 well plates. After overnight incubation, the cells were treated with diluted plant extract stock at the concentrations 0, 25, 50, 100, 200 and 400 µg/ml. The cell culture was analyzed after 72 hours using Non-Radioactive Cell Proliferation assay. At the dose 50 µg/ml, the changes in cells viability were almost insignificant; therefore, this concentration was used in viral inhibition assay. Cells toxicity was sharply increased at the dose more than 100 µg/ml for both these extracts

***In vitro* antiviral assay**

To further verify their anti-dengue properties, ethanolic extracts of *S. angustifolia* leaves, methanol extract of *V. cinerea* leaves and ethanolic extracts of *T. procumbens* stems that showed the highest inhibition percentage against dengue NS2B-NS3pro were subjected to an *in vitro* antiviral assay. Vero cells were cultured in DMEM media supplemented with 2% FBS and infected with DENV2. The morphology of Vero cells was observed using an inverted light microscope. The uninfected Vero cells were polygonal in shape with a well defined black nuclei in the centre (Fig. 3A). However, the DENV2-infected cells showed various cytopathic effects (CPE) especially after 72 hrs post infection. The main morphological characteristics of CPE observed were random-packed cells pattern, disorganised cell membrane, cells apoptosis and lysis (Fig. 3B). The ethanol extract of *S. angustifolia* leaves showed low ability to reduce CPE in infected cells and this is evidenced by the occurrence of round and spindle shaped cells. Other cell morphology characteristics seen include apoptotic cells, as wells as the failure of treated cells to maintain the monolayer sheet (Fig. 3C and 3D). In contrast however, *V. cinerea* and *T. procumbers* extracts were able to effectively reduce the CPE of the DNEV2-infected cells as observed by maintenance of normal cell morphology and the appearance as a normal monolayer sheet with low amount of apoptotic cells (Fig. 3E and 3F).

Quantitative of viral load by plaque formation assay and Real-time qPCR

In order to confirm the aforementioned observations, plaque formation assay and quantitative real-time PCR was used to determine the viral load in DENV2-infected cells after treatment with plant extracts (Fig. 4). The plaque assay data showed that *S. angustifolia*, *V. cinerea* and *T. procumbers* exhibited significant inhibition against viral replication in infected cells compared to untreated cells ($p<0.0001$). However, both *T. procumbers* and *V. cinerea* extracts showed considerable reduction in plaque formation (80.6% ± 6.1 and 64.0% ± 9.4 respectively) compared to *S. angustifolia* extract (26.3% ± 3.8) ($p<0.0001$) (Fig. 4A and 4B). These data were confirmed by Real-time PCR quantification of viral RNA. The results showed the plant extracts reduced viral RNA in dose dependent manner. The highest percentage of viral inhibition was observed after treating the infected cells with 50 µg/ml of each extract. In parallel with plaque assay data, the highest inhibition of viral load was observed after treating the infected cells with *T. procumbers* and *V. cinerea* extracts (86.3% ± 2.7 and 79.5% 4.3 respectively) compared to *S. angustifolia* (67.2% ± 6.3) ($p<0.0001$) (Fig. 4C).

DISCUSSION

Although several trials are currently underway for vaccine development against DENV (Laughlin *et al*., 2012), there remain real challenges revolving around the practical application of these vaccines, such as the potential of antibody-dependent enhancement (ADE). Throughout a primary infection with dengue virus, the human body produces non-neutralizing antibodies. When an individual is infected with another DENV serotype these antibodies can bind the virus, but the virus remain un-neutralized. Subsequently, the antibody-virus complex binds to the Fc antibody receptors, and the entrance of the virus into host cells are inadvertently facilitated by this process (Vogt *el al*., 2009; Thomas *el al*., 2009).

Among different strategies that have been reported to discover and develop new anti-dengue drugs, inhibition of virus replication with potent inhibitors against dengue protease represents an attractive approach. The present study was designed to determine anti-dengue properties of nineteen plant extracts obtained from local medicinal plants. The identification and subsequent isolation of anti-dengue active compounds in medicinal plant extracts are potentially useful for developing antiviral chemical analogues. The results obtained herein showed that methanolic extract of *V. cinerea* leaves, ethanolic extract of *T. procumbens* stems and ethanol extract of *S. angustifolia*

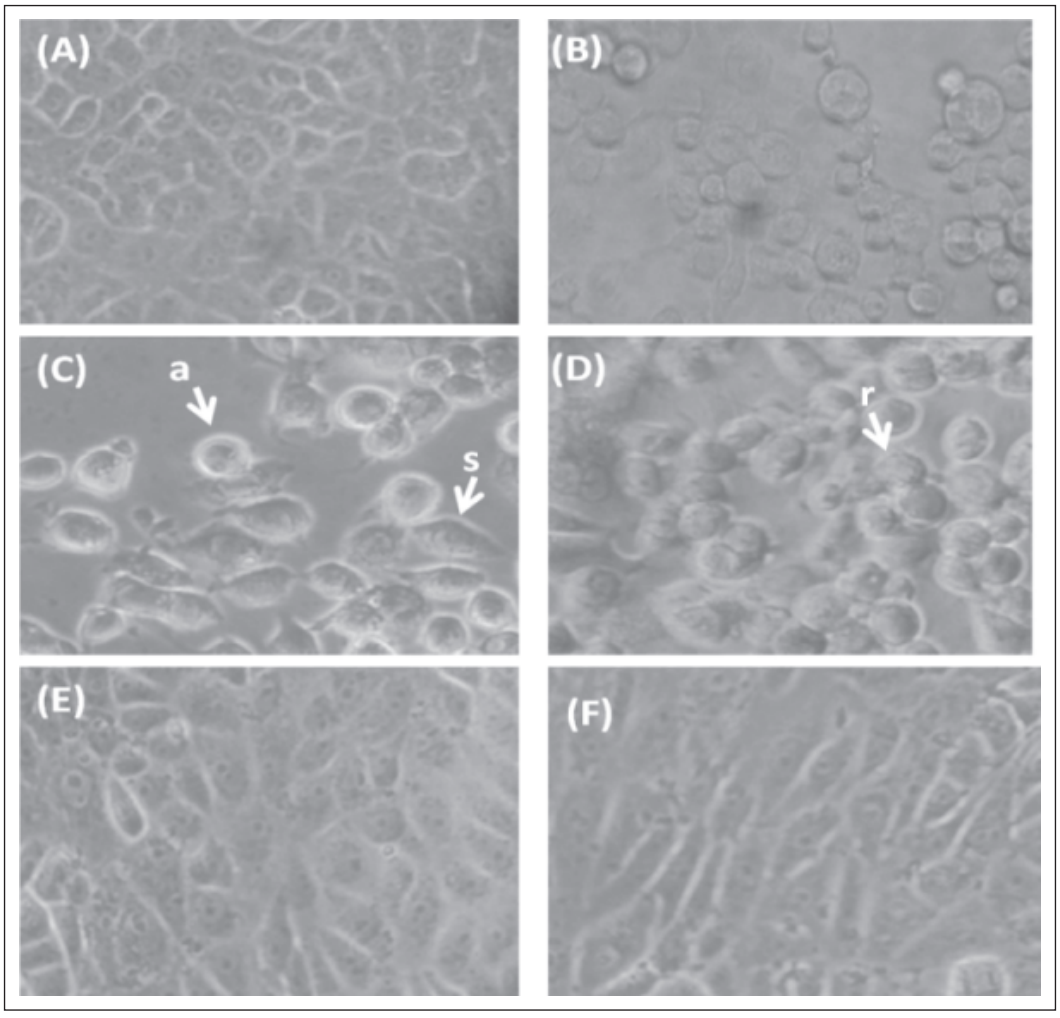

Figure 3. Morphological changes of DENV-2-infected Vero cells treated with plant extracts of three medicinal plants at 72 hrs post infection. (A) Normal cell morphology of Vero cells prior DENV2 infection. (B) Cells Morphology post-infection with various cytopathic effects (CPE). (C and D) Ethanol extract of *S. angustifolia* leaves showed low ability to reduce CPE in infected cells such as the round and spindle shape and the infected cells were not able to maintain the monolayer sheet. (E) Treatment with methanol extract of *V. cinerea* leaves showed approximately normal monolayer sheet without CPE. (F) Infected-Vero cells treated with ethanolic extract of *T. procumbens* stems showed lessen CPE and normal monolayer, similar to that observed in (E). Cell morphology was examined using an inverted microscope at 200× magnification.

a: apoptotic cell, s: spindled shape cell, r: rounded shape cell

leaves possess the highest inhibitory effects against dengue NS2B-NS3pro. These extracts were also able to reduce the CPE to DENV2-infected cells. In parallel, significant reduction in viral load was observed after applying these extract to the DENV2-infected cells. Taken together, results obtained in this study strongly suggest that the NS2B-NS3pro assay is efficient as a means for bulk-screening, especially when large numbers of plant extracts are involved.

Previous studies have shown that *S. angustifolia* extract has high content of phenolic glycosides, collectively named sennocide A and B (Bala *et al.*, 2001; Srivastava *et al.*, 2006; He *et al.*, 2007; Wu *et*

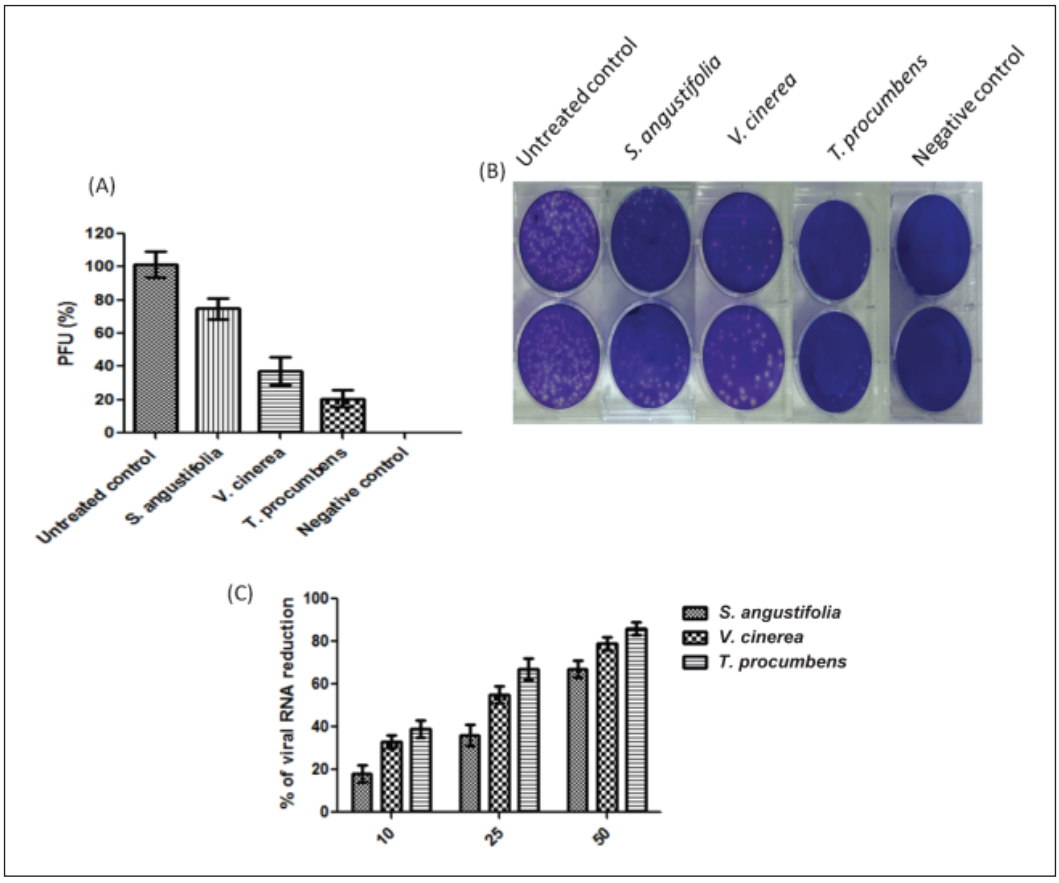

Figure 4. Determining the viral load in DENV2-infected cells after treatment with plant extracts. Vero cells were infected with DENV2 and treated with 50 µg/ml of *S. angustifolia*, *V. cinerea* and *T. procumbens* extract. (A) Histogram shows that plant extracts are able to inhibit infection by DENV2 after normalized to untreated control. (One-way ANOVA with Dunnett's post-test, P< 0.0001), results are expressed as mean ± SD from a representative experiment performed in duplicate. (B) Plaque formation assay shows the reduction of plaque generation. (C) Plant extracts showed significantly reduction in viral RNA in infected cells normalized with untreated control. (Two-way ANOVA with Bonfferoni post test P< 0.0001). Results are expressed as mean ± SD from a representative experiment performed in triplicate.

al., 2008). As the present study showed that the ethanolic extract of *S. angustifolia* has higher inhibitory effect against dengue protease compared to its methanolic extract, we postulate that this could be due to the high content of phenolic glycoside in the ethanolic extracts (Van Hoof *et al.*, 1989). Such antiviral activities of phenolic glycosides have indeed been reported against respiratory syncytial virus, parainfluenza type 3 virus (Ma *et al.*, 2001), HSV-1 and poliovirus virus (De Rodriguez *et al.*, 1990). Conversely however, some glycoside such as ligustroside and neonuezhenide did not show antiviral activity when tested against HSV-1 and Flu A (Ma *et al.*, 2001).

Methanolic extracts of *V. cinerea* and ethanolic extracts of *T. procumbens* stems showed higher inhibitory effect in both NS2B-NS3pro assay and *in vitro* viral inhibition assay compared to *S. angustifolia*. *V. cinerea* extract contains more than 27 phyto-constituents and the predominant phenolic compound is gallic acid (Rajamurugan *et al.*, 2011). This compound showed high antiviral activity against rhinovirus (Choi *et al.*, 2010).

In addition, *V. cinerea* extract contains large number of a sesquiterpene lactone (Zhu *et al*., 2008) that possesses antiviral activity towards *Herpes simplex* type-1, *Para-influenza* [34] and HCV (Hwang *et al.*, 2006).

The extract of *T. procumbens* has been reported as a source of large numbers of flavonoids with known antimicrobial activity such as Apigenin, quercetin, kaempferol (Jindal & Kumar, 2012), falcarinol, selinene, limonene and zerumbone (Joshi & Badakar, 2012), as well as pentahydroxyflavone 7-O-beta-D-glucopyranoside (Ali *et al*., 2001). As observed in the present study the ethanolic extract of this plant showed higher inhibitory effect towards dengue protease compared to its methanolic extract. Interestingly, the antimicrobial activity of *T. procumbens* has also been observed in its ethanolic extract but not in other extracts (Appiah-Opong *et al*., 2011). It is therefore tempting to speculate that the high content of flavonoids in ethanol extract could form the basis of marked differences in inhibitory effects seen in this study. Collectively, flavonoids and other polyphenols have potential inhibition activities against dengue NS2B-NS3pro (Tan *et al*., 2006) and subsequently dengue replication. Therefore, further studies are warranted to identify the active flavonoids in *V. cinerea* and *T. procumbens* extracts that showed high anti-dengue properties in the present study. The main limitation of this study is whether or not the antiviral activity of *S. angustifolia*, *V. Cinerea* and *T. Procumbens* extracts is depending on dengue protease inhibition or these extracts may have other inhibitory capabilities against cellular or viral enzymes or interfering with certain parts of the viral life cycle.

In conclusion, the outcome of present study underlines the need to carry out further investigation in the effort to identify the anti-dengue active compounds in the *V. cinerea* and *T. procumbens* plant extracts. This will subsequently facilitate downstream design of chemical derivatives for anti-dengue drug discovery and production. In addition, screening large number of plant extracts using whole virus assay is quite laborious and expensive. As an alternative, dengue NS2B-NS3pro assay used in this study is efficient for screening plant extracts in a time- and cost-effective manner.

Acknowledgments. This project was funded by the University of Malaya and Ministry of Science, Technology and Innovation (ERGS grant ER016-2013A).

Abbreviations

(DENV2) dengue virus serotype 2, (NS2B) NS2B cofactor amino acids sequence 49-95 in DENV-2 NS2B and 1394-1440 in DENV2 polyprotein; (NS3pro) NS3 protease amino acids sequence 1-185 in NS3 protein and 1476-1660 in DENV2 polyprotein; (NS2B-NS3pro) NS2B fused to NS3pro via 9 amino acids (G4-T-G4); (AMC) fluorogenic peptide substrate (Boc-Gly-Arg-Arg-AMC).

REFERENCES

Ali, M., Ravinder, E. & Ramachandram, R. (2001). A new flavonoid from the aerial parts of Tridax procumbens. *Fitoterapia* **72**: 313-315.

Appiah-Opong, R., Nyarko, A.K., Dodoo, D., Gyang, F.N., Koram, K.A., Ayisi, N.K. *et al*. (2011). Antiplasmodial activity of extracts of Tridax procumbens and Phyllanthus amarus in *in vitro* Plasmodium falciparum culture systems. *Ghana Medical Journal* **45**: 143-150.

Bala, S., Uniyal, G.C. Dubey, T. & Singh, S.P. (2001). An improved method for the analysis of sennosides in Cassia angustifolia by high-performance liquid chromatography," *Phytochem Analysis* **12**: 277-280.

Botting, C. & Kuhn, R.J. (2012). Novel approaches to flavivirus drug discovery. *Expert Opinion on Drug Discovery* **7**: 417-428.

Chambers, T.J., Hahn, C.S. Galler, R. & Rice, C.M. (1990). Flavivirus genome organization, expression and replication," *Annual Review Microbiology* **44**: 649-688.

Chanprapaph, S., Saparpakorn, P., Sangma, C., Niyomrattanakit, P., Hannongbua, S., Angsuthanasombat, C. *et al*. (2005). Competitive inhibition of the dengue virus NS3 serine protease by synthetic peptides representing polyprotein cleavage site. *Biochemical and Biophysical Research Communications* **330**: 1237-1246.

Choi, H.J., Song, J.H., Bhatt, L.R. & Baek, S.H. (2010). Anti-human rhinovirus activity of gallic acid possessing antioxidant capacity. *Phytotherapy Reseasrch* **24**: 1292-1296.

De Rodriguez, D.J., Chulia, J., Simoes, C.M., Amoros, M., Mariotte, A.M., Girre, L. *et al*. (1990). Antiviral activity of a new isoflavonic glycoside from Ulex europaeus. *Planta Medica* **56**: 59-62.

Falgout, B., Pethel, M., Zhang, Y.M. & Lai, C.J. (1991). Both non structural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *Journal of Virology* **65**: 2467-2475.

Geiss, B.J., Stahla, H., Hannah, A.M., Gari, H.H. & Keenan, S.M. (2009). Focus on flaviviruses: current and future drug targets. *Future Medicinal Chemistry* **1**: 327-344.

Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. & Blinov, V.M. (1989). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes *Nucleic Acids Research* **17**: 4713-4730.

Gubler, D.J. & Clark, G.G. (1995). Dengue/ dengue hemorrhagic fever: the emergence of a global health problem. *Emerging Infectious Diseases* **1**: 55-57.

Gubler, D.J. (2002). Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends Microbiology* **10**: 100-103.

He, W.F., Lu, J.C., Yu, X.M. & Ding, Y.M. (2007). Studies on chemical constituents of leaves of Cassia angustifolia. *Zhong Yao Cai* **30**: 1082-1084.

Hwang, D.R., Wu, Y. S., Chang, C.W., Lien, T.W., Chen, W.C., Tan, U.K. *et al*. (2006). Synthesis and anti-viral activity of a series of sesquiterpene lactones and analogues in the subgenomic HCV replicon system. *Bioorganic & Medicinal Chemistry* **14**: 83-91.

Jindal, A. & Kumar, P. (2012). Antimicrobial flavonoids from Tridax procumbens. *Natural Product Research* **26**: 2072-2077.

Joshi, R.K. & Badakar, V. (2012). Chemical composition and in vitro ntimicrobial activity of the essential oil of the flowers of Tridax procumbens. *Natural Product Communication* **7**: 941-942.

Laughlin, C.A., Morens, D.M., Cassetti, M.C., Costero-Saint Denis, A., San Martin, J.L., Whitehead, S.S. *et al*. (2012). Dengue Research Opportunities in the Americas. *Journal of Infectious Diseases* **2012**: doi: 10.1093/infdis/jis351.

Ma, S.C. He, Z.D., Deng, X.L., But, P.P., Ooi, V.E., Xu, H.X. *et al*. (2001). *In vitro* evaluation of secoiridoid glucosides from the fruits of Ligustrum lucidum as antiviral agents. *Chemical and Pharmaceutical Bulletin* **49**: 1471-1473.

Monath, T.P. (1994). Dengue: the risk to developed and developing countries. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 2395-2400.

Rajamurugan, R., Selvaganabathy, N., Kumaravel, S., Ramamurthy, C.H., Sujatha, V., Suresh Kumar, M. *et al*. (2011). Identification, quantification of bioactive constituents, evaluation of antioxidant and *in vivo* acute toxicity property from the methanol extract of Vernonia cinerea leaf extract. *Pharmaceutical Biology* **49**: 1311-1320.

Rigau-P´erez, J.G., Clark, G.G., Gubler, D.J., Reiter, P., Sanders, E.J. & Vorndam, A.V. (1998). Dengue and dengue haemorrhagic fever. *The Lancet* **352**: 9132, 971-977.

Rothan, H.A., Abdulrahman, A.Y., Sasikumer, P.G., Othman, S., Rahman, N.A. & Yusof, R. (2012a). Protegrin-1 inhibits dengue NS2B-NS3 serine protease and viral replication in MK2 cells. *Journal of Biomedicine & Biotechnology* **2012**: 251482.

Rothan, H.A., Han, H.C., Ramasamy, T.S., Othman, S., Rahman, N.A. & Yusof, R. (2012b). Inhibition of dengue NS2B-NS3 protease and viral replication in Vero cells by recombinant retrocyclin-1. *BMC Infectious Diseases* **12**, 314, doi:10.1186/1471-2334-12-314

Srivastava, A., Pandey, R., Verma, R.K. & Gupta, M.M. (2006). Liquid chromatographic determination of sennosides in Cassia angustifolia leaves. *Journal of AOAC International* **89**: 937-941.

Stadler, K., Allison, S.L., Schalich, J. & Heinz, F.X. (1997). Proteolytic activation of tick-borne encephalitis virus by furin. *Journal of Virology* **71**: 8475-8481.

Stocks, C.E., Lobigs, M. (1998). Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. *Journal of Virology* **72**: 2141-2149.

Tan, S.K., Pippen, R., Yusof, R., Ibrahim, H., Khalid, N. & Rahman, N.A. (2006). Inhibitory Activity of Cylohexenyl Chalcone Derivatives and Favanoids of Fingerroot, Boesenbergia rotunda (L), Towards Dengue-2 Virus NS3 Protease. *Bioorganic & Medicinal Chemistry Letters* **16**: 3337-3340.

Teo, K.F. (1997). Wright, P.J. Internal proteolysis of the NS3 protein specified by dengue virus 2. *Journal of General Virology* **78**: 337-341.

Thomas, S.J., Nisalak, A., Anderson, K.B., Libraty, D.H., Kalayanarooj, S., Vaughn, D.W. *et al*. (2009). Dengue plaque reduction neutralization test (PRNT) in primary and secondary dengue virus infections: how alterations in assay conditions impact performance. *American Journal of Tropical Medicine and Hygiene* **81**: 825-833.

Tomlinson, S.M., Malmstrom, R.D. & Watowich, S.J. (2009). New approaches to structure-based discovery of Dengue protease inhibitors.*Infectious Disorders Drug Targets* **9**: 327-343.

Van Hoof, L., Totte, J., Corthout, J., Pieters, L.A., Mertens, F., Vanden Berghe, D.A. *et al*. (1989). Plant Antiviral Agents, VI. Isolation of Antiviral Phenolic Glucosides from Populus Cultivar Beaupre by Droplet Counter-Current Chromatography. *Journal of Natural Products* **52**: 875-878.

Vogt, M., Moesker, R., Goudsmit, B.J., Jongeneelen, M., Austin, S.K., Oliphant, T. *et al*. (2009). Human monoclonal antibodies against West Nile virus induced by natural infection neutralize at a post attachment step *Journal of Virology* **83**: 6494-6507.

Wengler, G. (1993). The NS3 nonstructural protein offlavivirus contains an RNA triphosphatase activity. *Virology* **197**: 265-273.

Wu, Q.P., Wang, Z.J., Fu, M.H., Tang, L.Y., He, Y., Fang, J. *et al*. (2008). Determination of five primary chemical constituents in Cassia angustifolia by HPLC. *Zhongguo Zhong Yao Za Zhi* **33**: 363-365.

Yusof, R., Clum, S., Wetzel, M., Murthy, H.M.K. & Padmanabhan, R. (2000). Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids *in vitro*. *Journal of Biological Chemistry* **275**: 9963-9969.

Zhu, H.X., Tang, Y.P., Pan, L.M. & Min, Z.D. (2008). Studies on bioactive constituents of whole herbs of Vernonia cinerea. *Zhongguo Zhong Yao Za Zhi* **33**: 1986-1988.