

A Preliminary Investigation on the Antiviral Activities of the Philippine Marshmint (*Mentha arvensis*) Leaf Extracts against Dengue Virus Serotype 2 *In Vitro*

ANN FLORENCE B. VICTORIANO-BELVIS^{1*}, RAPHAELLA G. LAO¹,
MARIA KATRINA T. MORATO¹, ELMER CASLEY T. REPOTENTE¹,
SHIRHASERNAMIN A. SACLAUSO¹, SAMUEL ALAN B. INOVEJAS¹
and RONALD R. MATIAS¹

¹Research and Biotechnology, St. Luke's Medical Center, E. Rodriguez Avenue, Quezon City, Philippines 1112

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In this study, we investigated the antiviral activity of lyophilized crude leaf extracts of the Philippine marshmint (*Mentha arvensis* L., commonly called yerba buena) against DENV-2 *in vitro*. The plant specimen was authenticated by DNA barcoding analysis using standard primers for amplification of *rbcL*, *matK*, ITS1, ITS2 and *trnH-psbA*. Aqueous, methanol and ethanol leaf extracts were prepared, and lyophilized prior to testing for its cytotoxicity and antiviral activities. All extracts presented cytotoxic activities against Vero cells in a dose-dependent manner. Half maximal cytotoxicity concentration (CC50) was calculated at 2,889.60 µg/mL for the aqueous extract, 1,928.62 µg/mL for the methanol extract, and 3,380.30 µg/mL for the ethanol extract. Antiviral activities assessed by plaque reduction assay revealed reduced DENV-2 viral infectivity, with the ethanol extract observed to have the strongest activity decreasing plaque numbers by 62% relative to the control. The methanol extract was observed to be most effective when added before infection causing 72% reduction in plaque numbers, whereas none of the extracts inhibited plaque formation by more than 40% when added after infection. DENV-2 NS1 antigen production was significantly reduced by the methanol extract, while viral RNA levels were also decreased as determined by real time RT-PCR. Phytochemical analysis revealed the presence of flavonoids, phenolics, tannins, proteins, reducing sugars and saponins.

Our preliminary results are promising, however, it should be interpreted with caution as further studies are needed to establish its potential therapeutic application against dengue infection.

INTRODUCTION

Dengue is an important arboviral disease globally affecting both human health and economy. Over the last two decades, the World Health Organization reports an 8-fold increase of dengue cases from 505,430 in 2000, to over 2.4 million in 2010, and to 4.2 million in 2019. However, actual incidence remains to be under-reported due to majority of cases are asymptomatic or manifests as mild infection, whereas others are misdiagnosed for other febrile illness. A risk of infection among 3.9 billion people spread over 129 countries exists, with 70% of the actual burden is in Asia (1, 2).

In the Philippines, dengue is endemic in all regions of the country with outbreaks occurring mostly during the wet season (June-February) (3). Cases are increasing in the past years, but from January to June 2021, it has been observed to be 44% lower compared to the same period in 2020. It is predicted, however, that cases will increase again in year 2022 based on its trend in the country (4).

Dengue infection can be caused by any of the four dengue virus serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) belonging to the *Flaviviridae* family. Human transmission occurs through the bites of infective female *Aedes* mosquitoes, namely *Ae. aegypti* and *Ae. albopictus*. Infection from one virus serotype does not confer immunity against the other serotypes. In fact, secondary infection could develop into a potentially lethal complication called severe dengue which is accountable for many deaths in Asian and Latin American countries (5).

Dengue fever is usually a mild and self-limiting illness with symptoms similar to other febrile conditions. Currently, there is no specific antiviral treatment for both mild/severe dengue. Due to this plight and the challenges in availing dengue treatment regimens, most people living in tropical and subtropical countries resort to folk/traditional medicines such as the use of medicinal herbs.

Mentha arvensis L., commonly known as yerba buena to Filipinos, is an aromatic plant that is traditionally used as tea and herbal medicine for pain. Yerba buena (YB) is one of the ten herbs that has been endorsed by the

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Philippine Department of Health to relieve pain and body aches. It is also reported to have analgesic, antiseptic, anti-cancer, anti-helminthic, anti-emetic, antibacterial and antiviral properties (6-11).

Medicinal plants and herbs have been recognized to be a rich source of compounds with broad therapeutic potency and limited side effects. Based on ethnobotanical reports, several compounds with anti-dengue potential activity include baicalen (12), 7-O-methyl-glabranine (13), catanospasmine (14), quercetin and fisetin (15, 16), and N-acetyl-D-Glucosamine-specific lectin (17). In this regard, this *in vitro* study is initially carried out to investigate the antiviral potential of yerba buena against DENV-2, a predominant circulating serotype in the country for many years (18-20).

MATERIALS AND METHODS

Cell line

Vero cells were cultured and maintained in Dulbecco's Minimum Essential Medium (DMEM) with 10% Fetal Bovine Serum (FBS) (GIBCO) in 25 cm² tissue culture flasks (Corning, USA) at 37°C with 5% CO₂. For cytotoxicity and antiviral assays, at 80-100% confluency, cells were harvested and plated at indicated cell density (16, 17, 21-23).

Virus strain

Dengue virus serotype 2 (Retentate 00St-022) was obtained from St. Luke's Medical Center, Research and Biotechnology – Center for Basic Science Research. Confirmation of serotype was done by sequencing and comparing with the library of sequences using Basic Local Alignment Search Tool (BLAST) (23).

Plant material and extraction

Yerba buena was purchased from Leonie Agri Corporation in Nueva Ecija, Philippines. The identity of sample was authenticated by the Institute of Biology, University of the Philippines. Plant leaves were washed with 70% alcohol, freeze-dried at -80°C for 3 days and pulverized using mortar and pestle. Powdered leaves were soaked in different solvents (hot water, methanol and ethanol) to produce different extracts that were referred as YBL-A, YBL-M and YBL-E, respectively. Each of the extracts were evaporated by vacuum evaporator or lyophilized (24-26). Working solutions of the extracts were prepared either in DMEM or to a final concentration of 0.1-0.5% DMSO (26, 27).

YB DNA barcoding

DNA extraction

Approximately 20 mg of plant pulverized leaf samples was weighed in a micro centrifuge tube. Thereafter, 400 µL of lysis buffer containing cetrimonium bromide was added and mixed using a vortex mixer. DNA was extracted using a NucleoSpin Plant II extraction kit according to the manufacturer's instruction.

PCR Amplification

Five sets of primers were used to amplify the 4 target gene regions, *rbcl*, ITS2, *trnH-psbA* and *matK*. There were 2 sets of primers used for the *matK* gene. The primer sequences were obtained from Fazekas, *et al.* (28) as shown in Table I.

Table I. Primer sequences used for DNA barcoding.

Primer name	Primer sequence	Expected size (bp)	Specificity
<i>rbcl</i> A-F	5' - ATGTCACCACAAACAGAGACTAAAGC - 3'	599	<i>rbcl</i>
<i>rbcl</i> A-R	5' - GTAAAATCAAGTCCACCRCG - 3'		
ITS-S2F	5' - ATGCGATACTTGGTGTGAAT - 3'	381	ITS2
ITS4	5' - TCCTCCGCTTATTGATATGC - 3'		
<i>psbA</i> F	5' - GTTATGCATGAACGTAATGCTC - 3'	variable (266/474 bp)	<i>trnH-psbA</i>
<i>trnH</i> 2	5' - CGCGCATGGTGGATTCACAATCC - 3'		
<i>matK</i> -KIM3F	5' - CGTACAGTACTTTTGTGTTTACGAG - 3'	892	<i>matK</i>
<i>matK</i> -KIM1R	5' - ACCCAGTCCATCTGGAAATCTTGGTTC - 3'		
<i>matK</i> -390f	5' - CGATCTATTCATTCAATATTTTC - 3'	939	<i>matK</i>
<i>matK</i> -1326r	5' - TCTAGCACACGAAAGTCGAAGT - 3'		

The PCR master mix for all 5 primer sets were composed of 1x PCR buffer, 2 mM dNTP mix, 2.5 mM MgCl₂, 0.04 U/µL Platinum Taq DNA polymerase and 0.3 µM each of the primers. To enhance the PCR

amplification, trehalose was added to the PCR master mix and a small amount of template was used. The PCR profile used were 94°C for 2 min initial denaturation followed by 30 cycles of 94°C for 10 seconds, 50°C for 30 seconds, 72°C for 30 seconds with a final elongation step of 72°C for 2 min. For the 2 primer sets used for *matK* however, the cycle elongation was increased to 1 min since the expected product size is nearly 1 kb.

PCR products were visualized in a 2% agarose gel using 0.5X TAE buffer. Separation was achieved at constant voltage of 100V for about 30 min. The gel was stained with 3x Gel Red staining solution for at least 30 min and was viewed under UV using a photo documentation apparatus.

Phytochemical Screening of YB extracts

Total Flavonoid content

The total flavonoid content was estimated using the aluminum chloride colorimetric procedure with quercetin as the standard. The calibration curve was prepared by diluting quercetin in methanol (0-100 µg/mL). Briefly, 0.1 mL of the extract solution containing ~0.2 mg of extract or standard quercetin solution (0-0.5 mg) was added to 0.3 mL of 5% sodium nitrite. After 5 min, 0.3 mL of 10% aluminum chloride was added. After 6 min, 2 mL of 1 M sodium hydroxide was added and then the total volume was made up to 10 mL with distilled water and mixed carefully. The absorbance was measured at 510 nm using a spectrophotometer. The total flavonoid content was expressed as milligrams of quercetin equivalent/grams of extract (29-31).

Total Phenolic content

The amount of phenolic content in the extracts was determined as previously described using Folin-Ciocalteu reagent (31, 32). 20 µL of the extract was made up to 1 mL with distilled water. 0.5 mL of Folin-Ciocalteu phenol reagent and 2.5 mL of 20% sodium carbonate were added respectively to the extract solution. The mixture was then allowed to stand at room temperature for 40 min. The absorbance of the mixture was measured at 725 nm using a spectrophotometer. A calibration curve for gallic acid (as standard) in the 20–80 µg/mL range was prepared in the same manner. Amount of phenolic content was expressed as mg gallic acid equivalent (GAE) per gram plant tissue.

Carbohydrates and Sugars

5 g of extracts was dissolved in 5 mL of distilled water and filtered. The filtrate was then subjected to tests for carbohydrates and reducing sugars (31, 32).

Molisch's test: The filtrate was treated with 2-3 drops of 1% alcoholic naphthol and 2 mL of concentrated sulphuric acid was added to the test mixture. Violet color formation indicates the presence of carbohydrates.

Fehling's test: The filtrate was treated with 1 mL of Fehling's solution and heated in a boiling water bath. A reddish orange precipitate was formed and indicates presence of reducing sugar.

Proteins and Amino Acids

Each extract was dissolved in a few mL of water and subjected to the Bradford assay. Briefly, Bradford reagent (900 µL) was mixed with the extract (100 µL) and allowed to react for at least 5 minutes. Absorbance was read at 595 nm. Bovine serum albumin was used as the standard (31).

Tannins

100 µL of the extract was made up to 7 mL with distilled water. 8 mM potassium ferric cyanide and 20 mM ferric chloride prepared in 0.1 M hydrochloric acid were added respectively. The solution was mixed by slowly swirling and absorbance was taken at 700 nm. Tannic acid was used as standard. Tannin content was expressed as mg of tannin per gram of plant tissue (mg TA/g) (31).

Saponins

Froth test

0.5 mL solution of each extract was diluted with distilled water to 5 mL and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins (31).

Cytotoxicity assay

Cells were treated with or without YB extracts at different concentrations for 24 h. Cell viability was evaluated using the resazurin reduction assay according to the manufacturer's instructions. Briefly, cells (5×10^3 cells/well) were seeded into 96-well tissue culture plate and incubated at 37°C with 5% CO₂ overnight. Cells were then treated with increasing YB extract concentrations in triplicates and further incubated at indicated time points followed by the addition of resazurin reagent. Absorbance was measured with an excitation wavelength of

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560 nm and a detection wavelength of 590 nm (22, 23). The half maximal cytotoxic concentration (CC50) was defined as the concentration that reduces cell viability of treated cells to 50% with respect to untreated cells.

Plaque reduction assay

Determining the direct virucidal effect of YB extracts on Dengue virus

The method was previously described with slight modifications (15, 16, 22). Briefly, virus suspension was pre-incubated with equal volume of YB extracts at 37°C with 5% CO₂ for 30 min. The mixture was then used to infect monolayer Vero cells cultured in 12-well microplates for 2 h with occasional swirling every 30 min. Inoculum was removed and cells were washed with 1X PBS to remove the unadsorbed virus. An overlay of 1.5 mL consisting of 2% FBS-DMEM-1.25% Avicel was added onto each well and incubated further at 37°C with 5% CO₂ for 3 days. Cells were fixed with 10% formalin, stained with 0.5% crystal violet, and the number of plaques were counted. Untreated uninfected cells and untreated infected cells were used as controls. The percentage of plaque yields was calculated by comparing the treated cells with the untreated infected cells. The half maximal inhibitory concentration (IC50) was defined as the concentration at which the plaque number is 50% of the untreated infected control.

Determining the effect of YB extract on viral entry into cells

2.0×10^5 Vero cells were seeded into 12-well plates and cultured at 37°C with 5% CO₂ overnight. YB extracts were then added into wells except for the control wells and further incubated for 1 h. Cells were washed with serum-free-DMEM and infected with 100 µL of DENV-2 inoculum for 2 h with occasional swirling every 30 min. Inoculum was removed and cells were washed twice with 1X PBS. An overlay of 1.5 mL consisting of 2% FBS-DMEM-1.25% Avicel was added onto each well and the 12-well microplates were incubated further at 37°C with 5% CO₂ for 3 days. Cells were fixed with 10% formalin, stained with 0.5% crystal violet, and the number of plaques were counted (13, 15). Untreated uninfected cells and untreated infected cells were used as controls. The percentage of plaque yields was calculated by comparing the treated cells with the untreated infected cells. The half maximal inhibitory concentration was defined as the concentration at which the plaque number is 50% of the untreated infected control.

Determining the effect of YB extract on virus replication

2.0×10^5 Vero cells were seeded into 12-well plates and cultured at 37°C with 5% CO₂ overnight. Cells were then infected with 100 µL of DENV-2 inoculum and allowed to adsorb for 2 h. Cells were washed twice with 1X PBS to remove unadsorbed virus and then treated with YB extracts for 1 h. An overlay of 1.5 mL consisting of 2% FBS-DMEM-1.25% Avicel was added onto each well and the 12-well microplates were incubated further at 37°C with 5% CO₂ for 3 days (13, 15, 29). Cells were fixed with 10% formalin, stained with 0.5% crystal violet, and the number of plaques were counted. Untreated uninfected cells and untreated infected cells were used as controls. The percentage of plaque yields was calculated by comparing the treated cells with the untreated infected cells. The half maximal inhibitory concentration was defined as the concentration at which the plaque number is 50% of the untreated infected control.

Cell culture for the detection of DENV-2 by real time RT-PCR and DENV-2 NS1 antigen by Enzyme-linked Immunoassay (ELISA)

2.0×10^4 Vero cells were seeded into 96-well plates at 37°C with 5% CO₂ for 24 h. Cells were washed twice and then infected with DENV-2 for 2 h. At the end of incubation, inoculum was removed and cells were washed with sterile PBS twice. Extracts were added to respective wells and were left in contact with cells for 1 h. Subsequently, cells were washed, replenished with complete medium and cultured for an additional 48 h. Supernatants were harvested and stored at -80°C until processed for ELISA and real-time RT-PCR assays. Replicate experiments, each concentration in triplicates, were performed (13, 23). Additional control experiments, run in parallel, include cells only and untreated infected cells.

Dengue virus detection

RNA extraction

Viral RNA was extracted using RNA extraction kit QIAamp Viral RNA mini kit and RNeasy mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The RNA was eluted and stored at -80°C until use.

Reverse Transcription Real Time-PCR

First strand cDNA synthesis was carried out in a 20 µL reaction mixture containing 10 µL of RNA template, 50 ng/µL random primers, and 200 U/µL Superscript III (Thermofisher Scientific, USA).

Dengue virus amplification was performed using Rotor-Gene Q (Qiagen, USA) according to the protocol by Alm *et al.*, (2014) with some modifications. Reaction mixture contained a 25 µL total volume consisting of 1 µL cDNA template, 24 µL Taqman GTXpress Mastermix, 1 µM primers and 0.05 µM probe. Amplification at Ct values less than 35 (Ct < 35) was considered positive while a Ct value greater than 35 cycles (Ct > 35) was considered as negative or not determined. Positive and no template controls were included in each run (13, 33, 34).

Detection of DENV-2 NS1 antigen by Enzyme-Linked Immunoassay (ELISA)

Cell supernatants, collected post-infection were analyzed for non-structural protein 1 (NS1) by using the commercially available enzyme linked immunoassay-based Platelia Dengue NS1 Ag kit (Bio-Rad, Hercules, CA, USA). Briefly, 50 µL of positive and negative controls from the kit, as well as the supernatants from each cell culture condition were tested. Optical density was measured at 450/620 nm using BioTek™ Synergy™ H4 Hybrid Microplate Reader (BioTek, Winooski, Vermont, USA). NS1 concentration in each sample was calculated from an NS1 standard curve, following the Bio-Rad technical insert (35, 36).

Statistical analysis

Means and standard deviations (SDs) were calculated with Microsoft Excel 2011 and GraphPad Prism 7.0 software. All statistical analyses were performed using one-way analysis of variance (ANOVA) and Tukey post-hoc analysis was used for multiple comparisons test by STATA version 14 software; *p* < 0.05 was considered statistically significant.

RESULTS

DNA barcoding

Five DNA barcode fragments were queried against a reference sequence from NCBI to determine the percent similarity using Unipro UGENE (Table II). Results revealed sequence identification for *matK*-KIM, *matK* b, *rbcLa*, *trnH-psbA* and ITS with 99%, 98%, 99%, 98% and 92% similarity to the *Mentha arvensis* consensus sequence, respectively.

Table II. Screening and identification of DNA barcode sequences for yerba buena plant specimen

Species	GenBank accession number				
	<i>matK</i> -KIM	<i>matK</i> b	<i>rbcLa</i>	<i>trnH-psbA</i>	ITS
<i>Mentha arvensis</i>	MK520309.1	KX879078.1	KC571787.1	LC125228.1	KC591661.1

Cytotoxicity of YB extracts

Preceding the screening of the antiviral properties of the YB extract, the effect of the extracts on cell viability was examined in Vero cells for us to determine the concentrations that will be used in our antiviral assays. Results of the resazurin dye assay revealed that treatment of Vero cells with the YB extracts reduced the viability of cells in a dose-dependent manner (Figure 1). Cells remained viable (100%) at 300 µg/mL for all extracts, however, increasing cytotoxicity was demonstrated at the highest concentration tested (3000 µg/mL). No significant change in toxicity was observed for all extracts when exposure time was extended until 72 h (data not shown). Half maximal cytotoxicity concentration (CC50) was calculated at 2,889.60 µg/mL for the aqueous extract (YBL-A), 1,928.62 µg/mL for the methanolic extract (YBL-M), and 3,380.30 µg/mL for the ethanolic extract (YBL-E).

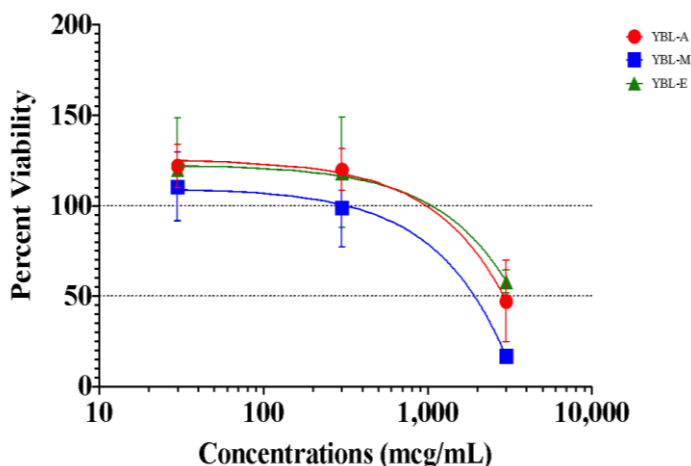
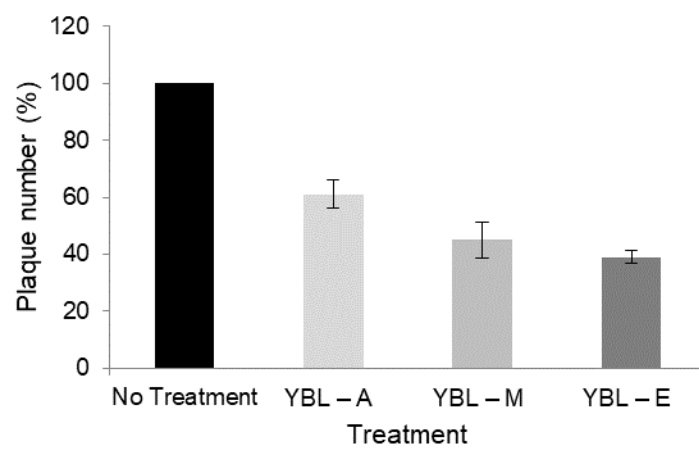
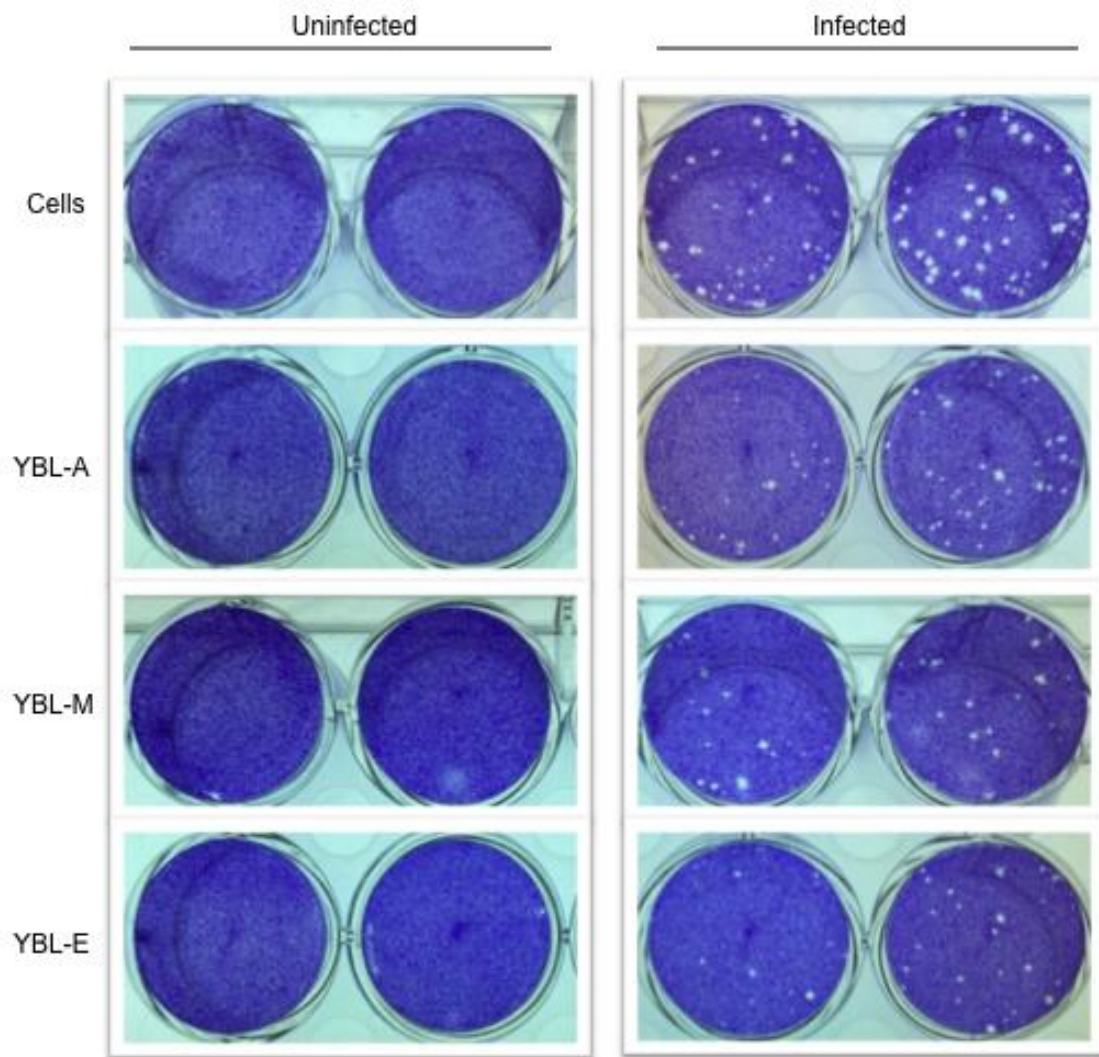


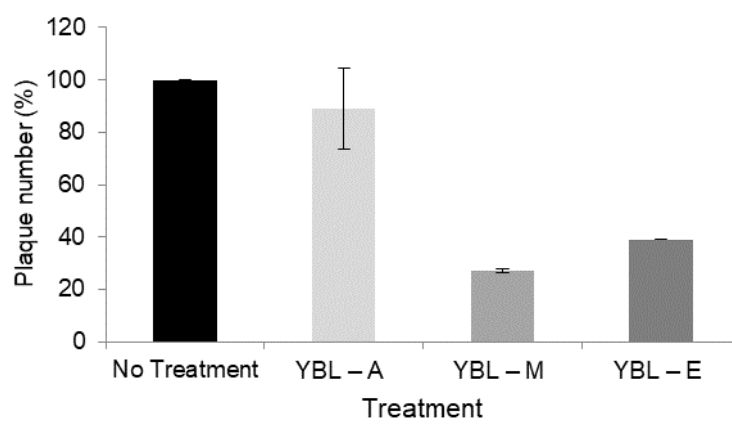
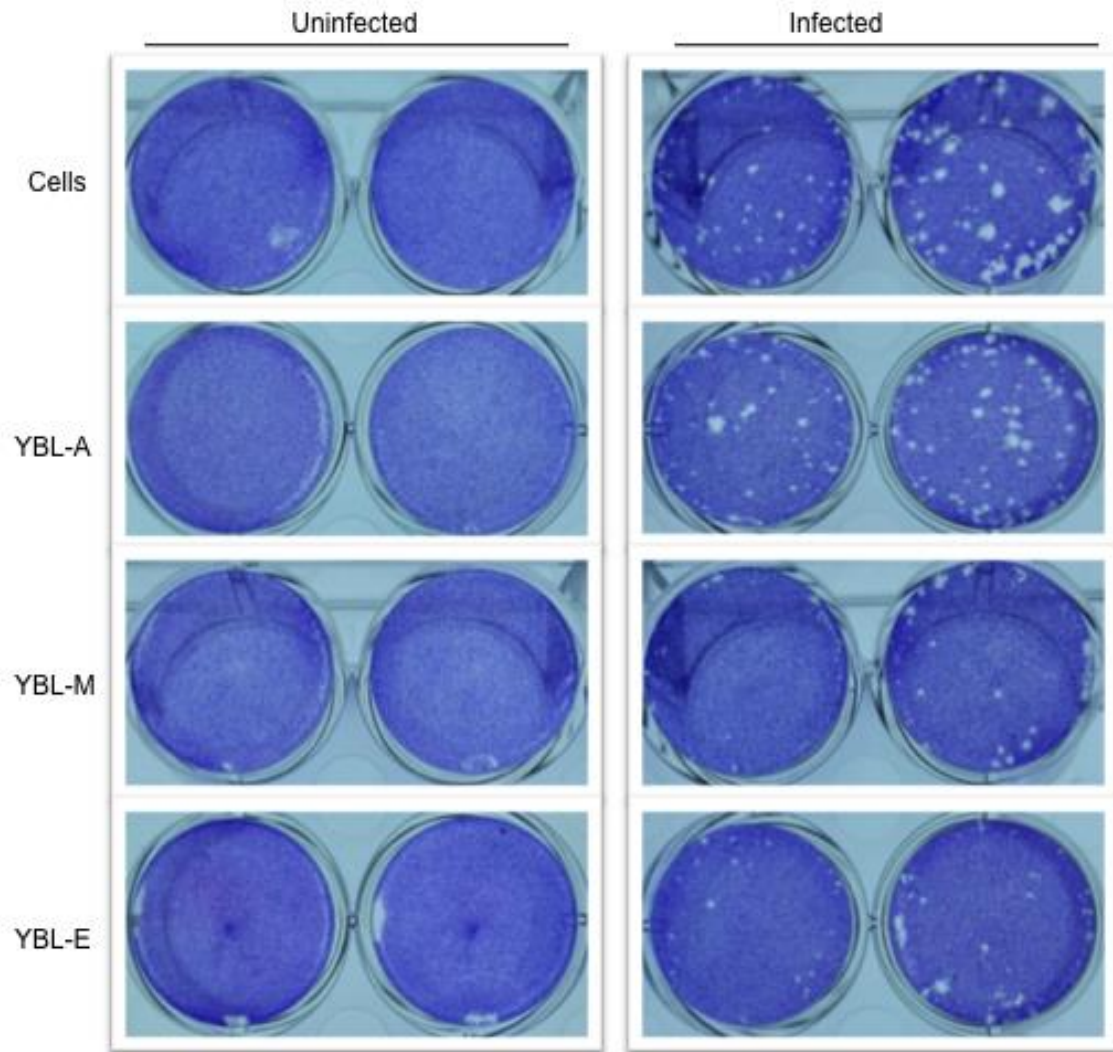
Fig. 1. Cytotoxicity of YB extracts in Vero cells. Vero cells (5×10^3 cells/well) were treated with increasing concentrations of YB extracts at 37°C with 5% CO₂ for 24 h. At the end of the incubation period, cell viability was determined by the resazurin assay. Percent viability was calculated using GraphPrism software by comparing the treated samples with the untreated control. Data represent means ± SD of triplicate wells of a representative experiment.

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(A)



(B)



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(C)

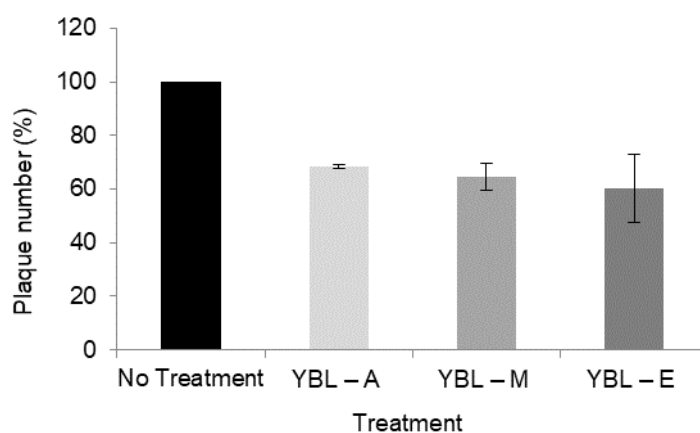
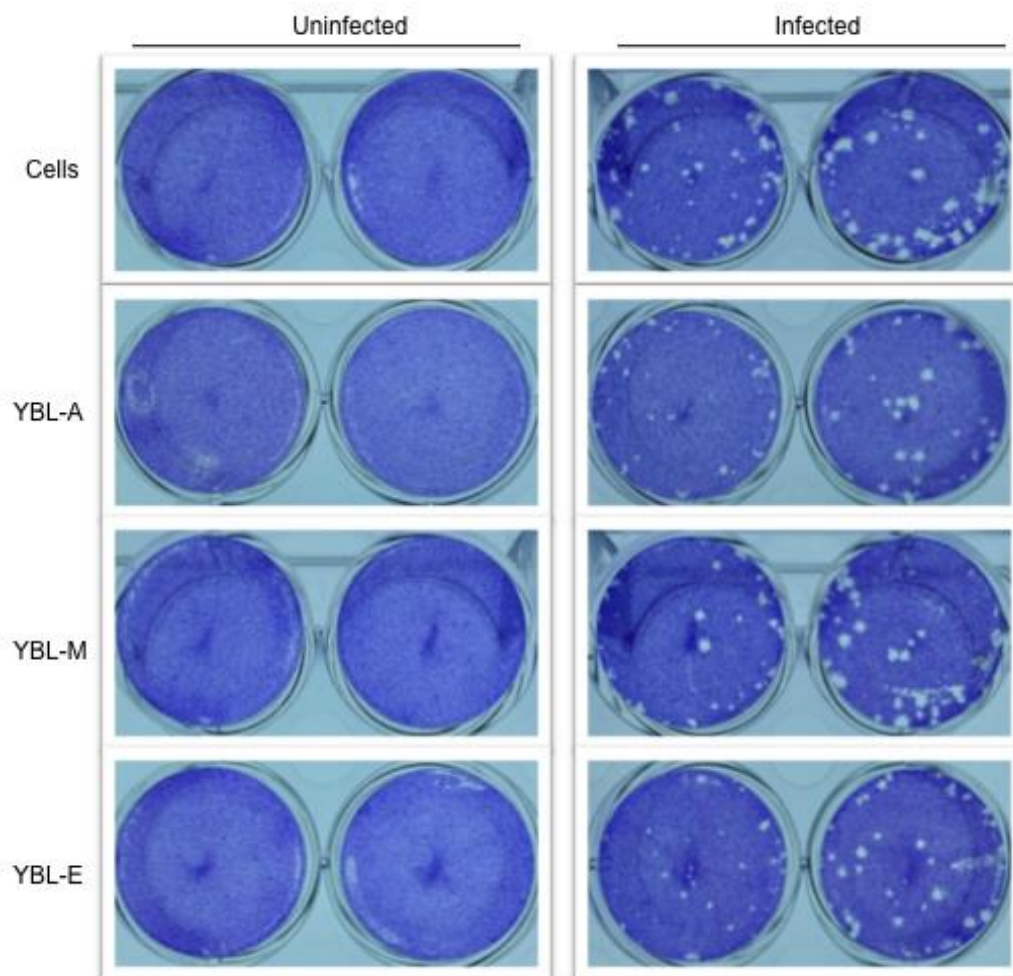


Fig. 2. DENV-2 plaque formation in the presence or absence of YB extracts.

YB extracts (1,464 $\mu\text{g}/\text{mL}$ of YBL-A; 641 $\mu\text{g}/\text{mL}$ of YBL-M, and 1,521 $\mu\text{g}/\text{mL}$ of YBL-E) were evaluated in Vero cells (2.0×10^5) for inhibitory effects in different stages of the DENV-2 life cycle (A) direct virucidal effect, (B) viral entry or adsorption, and (C) post-adsorption. At the end of treatment/infection times, Avicel was added onto each well and the 12-well microplates were incubated further at 37°C with 5% CO_2 for 3 days. Cells were fixed with 10% formalin, stained with 0.5% crystal violet, and the number of plaques were counted. Untreated uninfected cells and untreated infected cells were used as controls. The percentage of plaque yields was calculated by comparing the treated cells with the untreated infected cells. Data are representative images of three independent experiments.

Anti-dengue activities of YB extracts

To investigate the antiviral effects of our extracts, three key steps of DENV-2 viral replication life cycle were investigated; (a) virucidal effect, (b) viral entry, and (c) virus replication or postadsorption events. Firstly, time- and dose-course studies of the YB extracts and inoculum were performed to establish optimized experimental conditions for plaque development (data not shown). Under the optimal condition favorable for plaque formation, the following concentrations (CC10) were used in all antiviral experiments (1,464 $\mu\text{g/mL}$ of YBL-A; 641 $\mu\text{g/mL}$ of YBL-M, and 1,521 $\mu\text{g/mL}$ of YBL-E). The formation of plaques represents the amount of infectious virus unaffected by the extracts, hence, still capable of infecting and replicating in the cells. When DENV-2 was mixed with the extracts prior to infection, residual infectivity was markedly inhibited as indicated by the decrease in plaque numbers (Figure 2A). YBL-E (1,521 $\mu\text{g/mL}$) treatment showed substantially lower plaque numbers (38%) relative to the untreated control (100%), indicating a 62% plaque reduction rate. Meanwhile, exposure to YBL-M (641 $\mu\text{g/mL}$) and YBL-A (1,464 $\mu\text{g/mL}$) led to 50% and 58% plaque appearance, respectively. This finding demonstrated that YB extracts reduced the infectivity of DENV-2, suggesting that YB can directly inactivate the virus.

When Vero cells were pre-treated with YB extracts for 1 h prior to DENV-2 infection, the methanol extract showed the strongest antiviral activity with only 28% plaques produced after infection with respect to the untreated infected control (100%) (Figure 2B). The ethanol extract yielded 40% plaques while minimal inhibitory effect was seen with YBL-A. This suggests that YB may have caused cellular structural changes or altered the expression of certain cellular receptors, resulting in DENV-2 entry failure. Meanwhile, when extracts were added after virus infection (Figure 2C), none of the extracts decreased more than 40% in the number of plaques. Therefore, it appears that YB inhibits DENV-2 virus replication.

YB extracts inhibit DENV-2 virus replication.

To further confirm the antiviral activity of YB extracts, its effect on the production of infectious progeny particles was monitored by measuring the amount of NS1 and viral RNA present in the supernatants of infected cells. As shown in Figure 3, there was a statistically significant inhibition of virus production when DENV-2 infected cells were exposed to YB extracts. A significantly decreased NS1 antigen production was observed with YBL-M (48.3%) relative to the untreated infected cells (100%) ($p < 0.005$). On the other hand, a slight impairment was induced by YBL-E (93.9%) while there was no observable activity for YBL-A. In parallel, viral RNA was also extracted from cell culture supernatants to analyze viral copy numbers (Figure 4). At the end of 48 h infection, cells treated with YB extracts showed a significant but small degree of suppression in the amount of virus yield when compared to the control ($p < 0.0001$). This could be attributed to the short 1 h exposure time to the extracts. All extracts were found to have similar extent of antiviral activities, which corresponds with our plaque reduction test result. Collectively, these results suggest that YB extracts inhibit the production of infectious progeny virus.

Looking at all antiviral test results, YBL-M displayed the strongest inhibitory activity, particularly on DENV-2 viral entry, among all extracts tested.

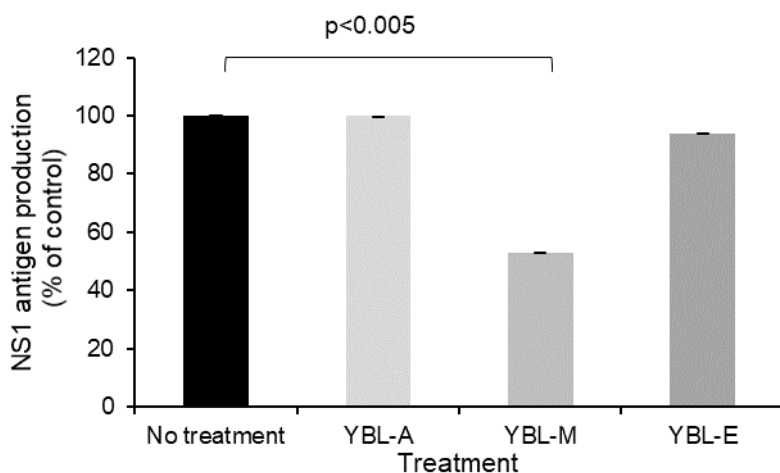


Fig. 3. Effect of YB extracts on DENV-2 production.

Monolayer of Vero cells were infected with DENV-2 (450 PFU/mL) for 2 h at 37°C, 5% CO₂. Virus inoculum was removed, cells were washed and then treated with extracts (1,464 $\mu\text{g/mL}$ of YBL-A; 641 $\mu\text{g/mL}$ of YBL-M, and 1,521 $\mu\text{g/mL}$ of YBL-E) for 1 h. Thereafter, cells were washed, replenished with complete culture medium and cultured for 2 days. Cell culture supernatants were collected and processed for NS1 antigen determination by ELISA. Percentage of antigen expression was calculated based on the amount of NS1 antigen in treated samples compared with untreated control. Data are means \pm SD of triplicate wells from three independent experiments.

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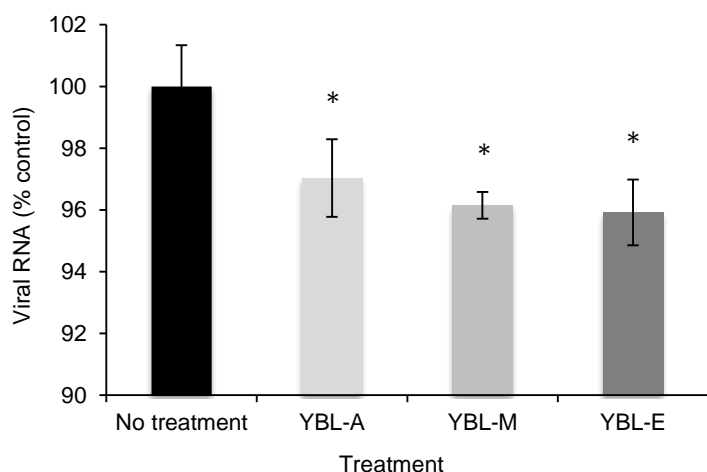


Fig. 4. Quantitation of DENV-2 viral RNA in the presence or absence of YB extracts. Monolayer of Vero cells were infected with DENV-2 (450 PFU/mL) for 2 h at 37°C, 5% CO₂. Virus inoculum was removed, cells were washed and then treated with extracts (1,464 µg/mL of YBL-A; 641 µg/mL of YBL-M, and 1,521 µg/mL of YBL-E) for 1 h. Thereafter, cells were washed, replenished with complete culture medium and cultured for 2 days. Cell culture supernatants were collected and respective DENV RNA production was quantified by real time RT-PCR. Percentage of viral RNA expression was calculated based on the viral RNA (Ct value) in treated samples compared with untreated control. Data are means ± SD of duplicate wells from four independent experiments. *p < 0.0001

Phytochemical analysis

We screened selected compounds in our YB extracts by qualitative and quantitative phytochemical assays. As shown in Table III, our extracts contained high amounts of proteins, phenols, flavonoids and tannins, with YBL-A showing the highest amount (Table IIIA). Qualitatively, we also found reducing sugars while saponin was not detected in YBL-M (Table IIIB).

Table III. Phytochemical content of YB leaf extracts. The composition of YB leaf extracts were subjected to (A) quantitative and (B) qualitative phytochemical screening.

(A)

Extract	Quantitative Phytochemical Tests (µg/mg)			
	Protein Concentration (BSA Equivalent)	Phenolic Content (Catechin Equivalent)	Flavonoid Content (Quercetin Equivalent)	Tannin Content (Tannic Acid Equivalent)
YBL - A	11.79	22.75	15.21	39.12
YBL - M	4.05	8.18	15.80	18.96
YBL - E	3.57	6.94	20.96	17.04

(B)

Extract	Qualitative Phytochemical Tests			
	Molisch's Test	Fehling's Test	Salkowski's Test	Froth Test
YBL - A	-	+	-	+
YBL - M	-	+	-	-
YBL - E	-	+	-	+

DISCUSSION

Here, we unraveled the antiviral potential of our lyophilized crude yerba buena leaf extracts. Our data demonstrated different activities per solvent used in the extraction at various stages of the virus life cycle.

When the virus was pre-incubated with the extracts before adsorption, viral inactivation was observed as evidenced by the significant reduction in viral plaque numbers three days after cellular virus infection. It has been known that viral entry is mediated by the viral envelope glycoprotein E which contains structural and functional elements (37). The decrease in the plaque number yield may be due to the alteration of viral E protein arrangement or induction of structural abnormalities through modification of viral membrane protein functions, as previously reported to obstruct the binding and entry of virus into cells (38). Another plausible explanation could be that certain constituents in the extract have competed with the virus for binding into the cells or affected the process of virus internalization (39-42). Interestingly, the flavone baicalen extracted from *Scutellaria baicalensis*, a co-member of yerba buena in the Lamiaceae (formerly Labiatae) family, has been shown to exhibit virucidal activity against dengue and whose action is attributed to the interference in binding or inactivation of the structural or non-structural proteins of DENV-2 (35). Anti-adsorption and direct inhibition of DENV-2 were also found in other bioflavonoids like naringin and naringenin (15). As these studies suggest,

flavonoids exert inhibitory activities against dengue. All extracts were found to be enriched with flavonoids, hence the flavonoids, alone or in combination with phenolics and/or other compounds present in the extracts, could be responsible for the observed antiviral activity of YB.

When cells were treated with the extracts prior to DENV-2 infection, viral entry was affected remarkably by the methanol extract. It is plausible that early events in virus replication are restricted and this could include attachment of virus into cells, fusion with cellular membrane or penetration of the virus. Accumulated evidence has highlighted the key role of virus-receptor interaction in cellular invasion. In the cell model system used in this study, it has been reported that heparan sulfate (HS) moieties of proteoglycans are embedded on the surface of Vero cells and act as receptors for successful entry (43). Moreso, functions as a scaffold for viruses to access coreceptors that help in cell penetration has also been cited for PrV, HSV-1, and HIV-1 (44, 45). The treatment of cells with YB extracts could have possibly triggered cellular structural changes or have affected the expression of certain cellular receptors resulting to the failed entry by DENV-2, as has been described by others (46).

When extracts were added after cells were infected, to simulate a natural active DENV infection, all extracts reduced plaque formation at almost similar levels, suggesting a disruption at the intermediate steps in the virus replication cycle. This antiviral activity was substantiated by the reduced dengue NS1 protein levels, a viral biomarker which is correlated with viral replication efficiency (47, 48). Virus multiplication is regulated by a number of viral and host factors which include the viral RNA, viral proteins such as NS2B/NS3 serine protease and cellular RNA polymerases, whose functions can be impaired by flavonoids as reported by others (49-54). This group of compound also interacts with viral enzymes involved in the ARN synthesis or maturation of polyprotein (15). We only observed a minimal reduction in the viral RNA after 48 h post-treatment compared to the untreated infected cells. This subtle effect is most likely attributed to the short time of exposure to the extracts which was only 1 hour after infection. Thus, extending exposure times would resolve this conundrum because it will provide more time for the extract's action to take effect. Interestingly, the flavonoid luteolin which restricts the enzymatic activity of furin producing virions with low infectious capacity is richly found in *Mentha* plant species, which could account for the current observation (55).

Other phenolic compounds like quercetin and fisetin are also reported to inhibit DENV-2 viral replicative cycle through several mechanisms (53, 55) aside from modifying the host innate response. Epigallocatechin from green tea has broad inhibitory activity on DNA and RNA viruses (57, 58). Phenolics together with flavonoids possess platelet increasing potential, a property that can also help address the low platelet count in dengue fever (59, 60). The lyophilized leaf decoction of *E. hirta*, another widely used medicinal plant in the country for dengue, has been demonstrated to augment platelet count in thrombocytopenic rats (59, 61). Purification of its ethyl acetate extract yielded three flavonoid-type of compounds. These studies further support our earlier assumption that the anti-dengue activity could be most likely brought by the flavonoids in our extracts.

Since we are working on crude extracts, different mechanisms could possibly explain the above findings. We also take into account that other bioactive compounds such as menthol, phenolic esters, anthocyanins, diglycerides, phosphatidylcholine, monoterpenes, proteins, coumarins and alkaloids (62-64), previously shown to possess antiviral activities yet still unexplored against dengue, could also be extracted from the leaves and may have played a role. As such, further studies are needed to identify and characterize the bioactive constituent in our active extracts. Moreover, although our extracts seem to work against DENV-2, it remains to be seen whether it will inhibit different serotypes or other viruses.

This study demonstrates the inhibitory activity of yerba buena against DENV-2 *in vitro*. The ethanol extract has been observed to be most effective when mixed directly with the virus before infection while the methanol extract exhibits its activity best when added to cells prior to viral entry. Thus, yerba buena can be considered a lead candidate in the search for novel anti-dengue drugs. Our preliminary results as shown here are promising, however, it should be interpreted with caution as further studies are needed to investigate its potential therapeutic application for dengue infection.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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