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Evaluation for Antiviral Potential of *Ficus deltoidea* against Dengue Virus Type-2

(Penilaian Potensi Antiviral *Ficus deltoidea* terhadap Virus Denggi Jenis-2)

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ABSTRACT

Dengue is one of the most widespread arthropod-borne viral diseases that cause negative impact globally. Presently, no effective drug is available to safeguard people against dengue. *Ficus deltoidea* is Malaysia›s famous traditional herb belonging to Moraceae family due to its pharmacological potential. *F. deltoidea* leaves (FDL) were extracted with n-hexane, ethyl acetate and methanol. Cell cytotoxicity study using MTT assay measuring the formazan absorbance was conducted to determine maximum non-toxic concentration on Vero cells. The antiviral activities of various concentrations of FDL extracts were assessed using virus reduction neutralisation tests against dengue virus type 2. The CC_{20} value of n-hexane, ethyl acetate and methanol FDL extracts were 2.99 ± 0.31, 22.15 ± 2.39, and 25.22 ± 0.42 µg/mL, respectively. Methanol FDL extract at maximum non-toxic concentration exerted strongest direct extracellular virucidal effect against DENV-2. In cell protection assay, ethyl acetate FDL extract achieved highest reduction in viral infectivity (98.17%), whereas n-hexane FDL extract showed strongest inhibition in DENV-2 viral replication in post-infection assay. Methanol FDL extract showed highest selectivity index value in direct virus inhibition, cell protection and post-infection assay. Conclusively, FDL extracts, especially methanol FDL showed potential antiviral activity against DENV-2, thus considered as promising anti-dengue agent.

Keywords: Antiviral; cytotoxicity; dengue virus type 2; *Ficus deltoidei;* MTT assay

ABSTRAK

Denggi ialah salah satu penyakit virus bawaan artropod yang paling meluas yang menyebabkan kesan negatif di seluruh dunia. Pada masa ini, tiada ubat yang berkesan tersedia untuk melindungi orang ramai daripada denggi. *Ficus deltoidea* ialah herba tradisi Malaysia yang terkenal kepunyaan famili Moraceae kerana potensi farmakologinya. Daun *F. deltoidea* (FDL) diekstrak dengan n-heksana, etil asetat dan metanol. Kajian sitotoksisiti sel menggunakan ujian MTT yang mengukur penyerapan formazan telah dijalankan untuk menentukan kepekatan tidak toksik maksimum pada sel Vero. Aktiviti antivirus pelbagai kepekatan ekstrak FDL dinilai menggunakan ujian peneutralan pengurangan virus terhadap virus denggi jenis 2. Nilai CC_{20} bagi ekstrak FDL n-heksana, etil asetat dan metanol masing-masing ialah 2.99 ± 0.31, 22.15 ± 2.39 dan 25.22 ± 0.42 µg/mL. Ekstrak metanol FDL pada kepekatan tidak toksik maksimum memberikan kesan virusidal ekstrasel langsung yang paling kuat terhadap DENV-2. Dalam ujian perlindungan sel, ekstrak FDL etil asetat mencapai pengurangan tertinggi dalam kejangkitan virus (98.17%), manakala ekstrak n- heksana FDL menunjukkan perencatan paling kuat dalam replikasi virus DENV-2 dalam ujian pasca jangkitan. Ekstrak metanol FDL menunjukkan nilai indeks selektiviti tertinggi dalam perencatan virus langsung, perlindungan sel dan ujian pasca jangkitan. Secara kesimpulannya, ekstrak FDL, terutamanya metanol FDL menunjukkan potensi aktiviti antivirus terhadap DENV-2, oleh itu dianggap sebagai agen anti-denggi yang berpotensi.

Kata kunci: Antivirus; *Ficus deltoidei;* sitotoksisiti; ujian MTT; virus denggi jenis 2

Introduction

Dengue infection remains a root cause of our misery, a disease with no efficacious antiviral agents available to safeguard the public against dengue. Supportive care or symptom management is the only therapeutic approach to treat infected patients. Dengue is a mosquito-borne viral disease caused by the four serotypes of dengue virus (DENV) known as DENV-1, DENV-2, DENV-3, and DENV-4 that belongs to the family of *Flaviviridae* (Low et al. 2021). With the escalated geographic spreading of dengue vectors, the incidence of dengue has aroused people's public health concerns and placed a significant economic burden on public health systems in endemic regions in the last few decades (Panda et al. 2021). Nowadays, an extensive variety of either infectious or non-infectious diseases have been treated with plant extracts and phytochemicals obtained from different parts of plant materials. Phytochemicals are numerous chemical compounds derived chiefly from natural origins helps in the discovery and development of novel drugs. Due to the features of readily available, low cost, and rarely reported severe side effects, phytochemical constituents and extracts obtained from plant origins are widely explored as alternative approaches to treat vast and versatile diseases for human use (Bhuiyan et al. 2020; Saleh & Kamisah 2020). *Ficus deltoidea* is one of the most widely used traditional herbs in Malaysia. Extensive published studies have established that *F. deltoidea* is reported in numerous traditional medicine systems with the potential to combat different diseases and infections due to each part of this plant being considered as rich sources of phytochemical constituents with various beneficial therapeutic activities such as antibacterial, anticancer, antidiabetic, anti-inflammatory, antihypertensive, antinociceptive, antiulcerogenic, antioxidative antiviral and uterotonic activity (Abu et al. 2018; Azizan et al. 2017; Khan et al. 2016; Ooi et al. 2021). In addition, previous studies also reported this plant material possesses a vast array of phytochemical constituents such as alkaloids, organic acids, saponin, polyphenols, terpenoids, and their derivatives (Rosnah, Khandaker & Boyce 2015). Considering the skyrocketing global emergence and spread of dengue cases in 129 countries globally (WHO 2023), there is an urgent need for researchers to develop a comprehensive strategy to address this alarming problem since no specific treatment or vaccine is available to prevent and control it. There is considerable scientific and commercial interest in the continuing discovery of novel antiviral products of plant origin, it is essential to develop an insight into the exploration of the antiviral efficiency of *F. deltoidea* leaf (FDL). Despite this plant species having proved to have potential source that contributes extensive important therapeutic applications traditionally, none of the researchers conducted a study to specifically examine the antiviral effects of FDL extracts on DENV-2. To date, there is no published research assessing the anti-dengue potential of FDL extracts but its antiviral properties against *Chikungunya virus* (CHIKV) have been reported in previous studies (Chan, Khoo & Sit 2016; Chan et al. 2021). Based on the information given, we hypothesize that FDL extracts consist of phytochemical constituents with numerous therapeutic benefits, including its antiviral potential against DENV-2 without showing any significant cytotoxic effects. Therefore, the aim/objective of this project was to investigate the *in vitro* antiviral potentials of FDL extracts against DENV-2. We also evaluate the half-maximal cytotoxic concentration, CC_{50} of FDL on the Vero cell lines and half-maximal inhibitory concentration, IC_{50} of FDL extracts against DENV-2 on the Vero cell line via determining the selectivity index (SI) value of FDL extracts against DENV-2.

Materials and Methods

Chemical and reagents

Chemical and reagents used in the extraction and phytochemical screening were kindly provided by the Department of Medicinal Chemistry, Faculty of Pharmacy, MAHSA University. For cytotoxicity assay, MTT powder and sodium dodecyl sulfate (SDS) powder was purchased from Cayman Chemical (Macklin, USA) and Bio-Rad Laboratories (Bio-Rad, US), respectively. Chemical and reagents used in antiviral assay including Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), Foetal Bovine Serum (FBS) (Gibco, USA), penicillin-streptomycin antibiotics (PSA) (Gibco, USA), trypsin (Nacalai Tesque, Japan), phosphate-buffered saline (PBS) (Gibco, USA), carboxymethyl cellulose (CMC) (Sigma, Japan), acetone (Merck, Germany), methanol (Merck, Germany), skim milk (Thermo Fisher, UK), KPL TrueBlue peroxidase substrate (Seracare, USA), DMSO (ATCC, USA), primary antibody (4G2), harvested from HB112 hybridoma cells (ATCC, USA), HRP-conjugated secondary antibody (Invitrogen, USA) were provided by the Centre for Virus and Vaccine Research, School of Medical and Life Sciences, Sunway University.

PREPARATION OF LEAF EXTRACTS

The dried leaves of *F. deltoidea* were purchased from a local supplier, Herbagus Sdn, Bhd, Penang, Malaysia. The plant material was identified and authenticated by a staff from Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia (UPM). Dried leaves samples were blended into powdered form and kept inside a desiccator at room temperature prior to extraction. To have bioactive compounds with increasing polarity, particularly for fractionation purpose, the usual practice is to choose two solvents with low polarity, two with medium polarity, and one with higher polarity. The selection criteria are based on selectivity, safety, cost, reactivity, recovery, viscosity, boiling temperature, and availability. In general, the common polar solvents for polar compound extraction are water, methanol, and ethanol, while nonpolar solvents are hexane dichloromethane. Although water is the most polar solvent, however, it promotes microorganism growth and higher heat is required to concentrate the extract (Abubakar & Haque 2020). For hexane, it can significantly enhance the oil production and have excellent solubilizing ability, but it can react with environmental pollutants to form ozone and photo chemicals (Jeevan Kumar et al. 2017). Thus, in this study, the powdered leaves were extracted sequentially using the Soxhlet extraction method with solvents of increasing polarities that are available in the laboratory, consisting of n-hexane, ethyl acetate, and methanol. Firstly, 400 mL of solvent was transferred to a round bottom flask, and a small amount of clean boiling chip was added to the extraction solvent. The flask containing the solvent was attached to a Soxhlet extractor and condenser on a heating mantle. The thimble was filled with approximately 200 g of powdered plant sample and followed by transferring the thimble inside the Soxhlet extractor. The amount of each solvent added for wetting the plant sample and filling the round flask bottom was at a quantitative relation of around 1:4, provided four solvent parts were added to one part of the plant sample. The thin-layer chromatography (TLC) method was employed to ensure the completion of the extraction process by putting each fraction of the plant sample on a 10 × 10 cm aluminium paper coated with silica gel with 0.2 mm thickness as described by Redfern et al. (2014).

VIRUS AND CELL CULTURE

Vero (African green monkey kidney, ATCC® CCL-81) cell was first cultured in 10% FBS and 1% PSA supplemented DMEM at pH 7 and maintained at 37 °C in a humified 5% CO_2 incubator. DENV-2 (New Guinea C) was propagated in Vero cells for 4 to 5 days. The cells were harvested once the cytopathic effects of DENV-infected Vero cells were achieved at least 80%, followed by three round freeze-thawing processes. The harvested DENV-2 viral supernatant was clarified by centrifugation at 4,000 × g to pellet cell debris, aliquoted into separate 1.5 mL tubes supplemented with 20% FBS. Virus stocks were stored at -80 °C until use. Quantifying of viral titres was performed using foci forming assay. The viral titre of DENV-2 was calculated using the following formula and presented as a foci-forming unit per millimetre (FFU/mL).

$$\text{Viral titre } \left(\frac{\text{FFU}}{\text{mL}}\right) = \frac{\text{Number of foci per well}}{\text{Dilution factor} \times \text{Infection volume (mL)}}$$

CYTOTOXICITY ASSAY

The cytotoxic effect of FDL extracts in Vero cells was assessed by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) using the procedures described by Kumar, Nagarajan and Uchil (2018), and Lalani, Anasir and Poh (2020).

PREPARATION OF STOCK SOLUTION

For each type of plant extract, 30 mg/mL of stock solution was first prepared by dissolving 30 mg of plant extract in 1 mL DMSO. Prepared stock solutions were filtered using 0.2 µm pore size filter (Millipore, MA, USA). Filtered stock solutions were aliquoted into 100 µL portions and stored at a temperature of -80 °C until use.

CELL SEEDING AND CELL TREATMENT

Vero cells (2×10^5) were seeded in a 96-well plate and the cells were allowed to grow for 24 h in a 5% CO_2 incubator at 37 °C. A cell confluency of 80-90% was observed after seeding 2×10^5 Vero cells in 96-well plate for 24 h. Different concentrations of each type of FDL extract were prepared using a two-fold serial dilution with 2% FBS supplemented DMEM. Two-fold serial dilution was done in the following manner:

1000 µg/mL > 500 µg/mL > 250 µg/mL > 125 µg/mL > 62.5 µg/mL > 31.25 µg/mL

MTT ASSAY (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)

After 48 h of incubation, 10 µL of MTT reagent (5 mg/mL in sterile PBS) was added to each well and the well plate

was wrapped with aluminium foil. After 4 h of incubation, 100 µL of 10% sodium dodecyl sulphate/0.01 M hydrochloric acid was added to each well for solubilising the insoluble formazan crystals and incubated for 24 h. The cytotoxic potential of plant extracts in the Vero cells was determined using Infinite 200 Pro Multiplate Reader (Tecan, Männdedorf, Switzerland) by measuring the absorbance at 570 nm with a reference wavelength of 630 nm. The percentage of cell viability inhibition was calculated according to the following equation:

$$\text{Percentage of cell viability inhibition (\%)} = 100\% - \left[\frac{(A_t - A_b)}{(A_c)} \times 100\%\right]$$

where A_t represents the absorbance reading of the treated cell; A_br represents the absorbance reading of blank; and A_C represents the absorbance reading of control. GraphPad Prism software was used to construct a curve of cell viability (%) versus log concentration of the tested sample to determine the 20% cytotoxic concentration (CC_{20}) and half-maximal cytotoxic concentration (CC_{50}) by non-linear regression.

FOCI REDUCTION NEUTRALISATION TEST (FRNT)

This assay was carried out to evaluate the antiviral activity of plant extracts by determining the reduction in the number of DENV infectious foci after treatment as mentioned by Low et al. (2021). The antiviral activity of each extract was determined by calculating the percentage of foci reduction (%RF) using the following formula:

$$\text{RF (\%)} = \frac{C-T}{C} \times 100\%$$

where C is the mean number of foci from duplicate treatments without plant extract being added (control); and T is the mean number of foci from duplicate treatments with the respective plant extract.

ANTIVIRAL ASSAY

The following assays were conducted based on a slightly modified procedure described by Lalani, Anasir and Poh (2020) and Low et al. (2021).

DIRECT VIRUS INHIBITION ASSAY

Vero cells (2×10^5 cells/well) were seeded overnight in 24-well plate. 150 µL of DENV-2 was pre-treated with an equal amount of serially diluted FDL extract for 1 h at multiplicity of infection (MOI) of 0.2. 200 µL of pre-treated viral-FDL extract was added to each well and the cells were allowed for incubation at 5% CO_2 incubator at 37 °C for 1 h. After 1 h of adsorption, unbound virus inoculums were removed, the treated Vero cells were washed using PBS (500 µL/well) and the maintenance medium was replaced by 2% FBS supplemented DMEM (200 µL/well). After 96 h of incubation, the supernatants were collected and the antiviral activity of FDL extracts against DENV-2 was determined by FRNT.

CELL PROTECTION ASSAY

First, the Vero cells (2×10^5 cells/well) were seeded in 24-well plate, and the cells were allowed to grow overnight in a 5% CO_2 incubator at 37 °C. After that, Vero cells were treated with two-fold serially diluted FDL extract, and the cells were allowed for incubation at 37 °C. After 1 h of incubation, the leaves extract containing media was discarded and the treated cells were washed with PBS (500 µL/well). Pre-treated cells were infected with DENV-2 at MOI of 0.2 for 1 h (200 µL/well). After 1 h of adsorption, unbound virus inoculums were removed, the treated Vero cells were washed using PBS (500 µL/well) and the maintenance medium will be replaced with 1 mL of 2% FBS supplemented DMEM (200 µL/well). After 96 h of incubation, the supernatants were collected and the antiviral activity of FDL extracts against DENV-2 was determined using FRNT.

POST-INFECTION ASSAY

Vero cells (2×10^5 cells/well) were seeded in 24-well plate, and the cells were allowed to grow overnight in a 5% CO_2 incubator at 37 °C. Cells were infected with DENV-2 at MOI of 0.2 for 1 h (200 µL/well). After 1 h of infection, unbound virus inoculums were removed, and the treated cells were washed with PBS. Next, virus-infected cells were treated with serially diluted FDL extract prepared in a maintenance medium (200 µL/well) and the cells were incubated at 37 °C for 48 h. After 48 h, the viral supernatants were collected to quantify viral titres. The antiviral activity of FDL extract against DENV-2 was determined using FRNT. The purpose of doing a post-infection assay in anti-dengue activity studies is to analyse the effectiveness of a potential anti-dengue drug after the virus has already infected cells. This type of assay differs from pre-infection assays, which assess the ability of the drug to prevent viral entry or attachment. The post infection assay is the treatment of virus while pre-infection assay is for the prevention against virus.

STATISTICAL ANALYSIS

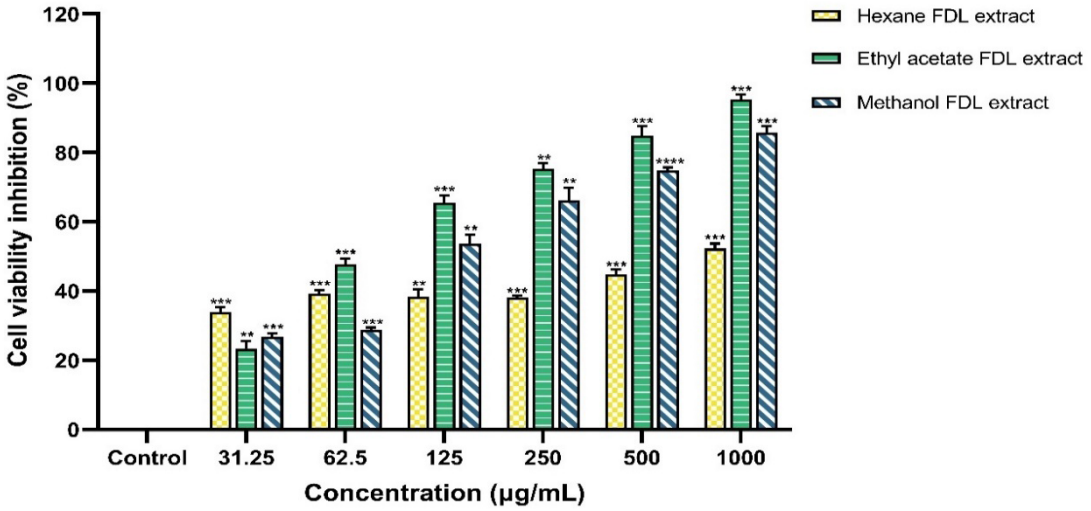
Prism software (GraphPad Prism 8.0, CA, USA) was applied for statistical analysis and graphical illustration. For cytotoxicity assay, CC_{50} and CC_{20} of the tested sample in Vero cells was calculated using Prism software. For antiviral assay, IC_{50} was calculated using Prism software and the calculation of SI was made using the following formula, $SI = CC_{50}/IC_{50}$. The statistical significance of the difference between mean values of the antiviral activity of FDL extract compared to negative control was determined using the student t-test, whereas, the negative control for the study is DMSO, dimethyl sulphoxide, used for dilution of extracts. Differences with $p<0.05$ indicate the result was statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). Data was expressed as mean ± standard deviation of two independent experiments.

RESULTS AND DISCUSSION

CYTOTOXICITY EFFECT OF FDL EXTRACTS ON VERO CELLS

Research on phytochemistry and pharmacology has substantiated the customary use of *F. deltoidea*. The healing qualities of FDL have been known for generations both locally and worldwide, and studies have been done to verify its benefits, especially in the field of medicine. Due to the presence of phenolic and flavonoid bioactive compounds, these results offer strong evidence for the significant and beneficial effects of *F. deltoidea* extract on infectious and chronic diseases involving rates of wound healing, cancer, fever, diabetes, blood pressure, bacterial infection, fungal infection, and many other diseases (Bunawan et al. 2014).

MTT assay was carried to determine the cytotoxicity effects of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract on Vero cells over different concentrations (0, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL, respectively), and the CC_{20} and CC_{50} were tabulated in Figure 1. The CC_{50} value of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract was 21.03 ± 0.63, 69.53 ± 6.92, and 85.13 ± 3.11 µg/mL, respectively, as shown in Table 1. The CC_{20} value of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract was 2.99 ± 0.31, 22.15 ± 2.39, and 25.22 ± 0.42 µg/mL, respectively. Based on the CC_{20} values, 3 µg/mL of n-hexane FDL extract, 25 µg/mL of ethyl acetate FDL extract and 25 µg/mL of methanol FDL extract showed approximately cell viability of 80% and were selected as the highest concentrations to be evaluated in the following antiviral assays. Ethyl acetate FDL extract and methanol FDL extract were found to be well tolerated by Vero cells with CC_{50} values of 69.53±6.92, and 85.13±3.11 µg/mL, respectively. However, n-hexane FDL extract was less tolerated by Vero cells with CC_{50} value of 21.03±0.63 µg/mL.



Data presented as mean±SD. Error bars represent the range of values obtained from two independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 represents a significant difference compared to the negative control analysed using the student t-test

FIGURE 1. Cell viability inhibition of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract in Vero cells

TABLE 1. CC_{20} and CC_{50} of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract determined from MTT assay. Data were obtained from two independent experiments and presented as mean±standard deviation (SD)

Types of extract	CC_{20} (µg/mL)	CC_{50} (µg/mL)
Hexane FDL	2.99 ± 0.31	21.03 ± 0.63
Ethyl acetate FDL	22.15 ± 2.39	69.53 ± 6.92
Methanol FDL	25.22 ± 0.42	85.13 ± 3.11

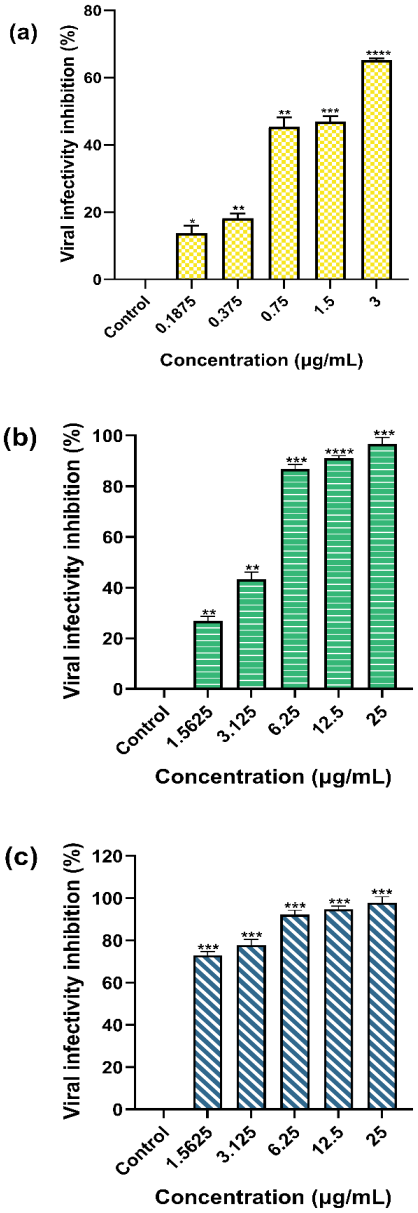
ANTIVIRAL ACTIVITY OF FDL EXTRACTS AGAINST DENV-2

Direct virus inhibition activity of FDL extracts against DENV-2

The direct veridical assay was carried out to investigate the virus-inactivating potential of FDL extract against DENV-2. According to the findings of direct veridical assays, all the tested extracts demonstrated direct extracellular veridical effects on DENV-2 in a dose-dependent manner. The IC_{50} values of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract determined by FRNT was 0.94 ± 0.08, 3.03 ± 0.02, and 1.48 ± 0.02 µg/mL, respectively. The highest inhibition of viral infectivity of DENV-2 by n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract was 65.15% (Figure 2(a)), 96.29% (Figure 2(b)) and 97.78% (Figure 2(c)), respectively, when DENV-2 was treated with 3 µg/mL of n-hexane FDL extract, 25 µg/mL ethyl acetate FDL extract, and 25 µg/mL methanol FDL extract, respectively. In the antiviral assays, there is no baseline or cut-off point showing the degrees of viral inhibition as no positive control/standard anti-dengue agent in the market. Thus, the percentage of viral inhibition was compared to the control without FDL extract treatment. Based on the IC_{50} value, it can be considered that ethyl acetate FDL is the least potent extract as its IC_{50} value is the highest among the tested extracts. In contrast, n-hexane FDL exhibited the lowest IC_{50} value, indicating that it is the most potent extract compared to the remaining two extracts. The SI value for n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract from direct virus inhibition assays was 22.41, 22.96 and 57.23, respectively (Table 2). The SI value of all three tested extracts showed that they are safe and efficacious to develop as antivirals. As methanol FDL extract showed the highest SI value compared to n-hexane FDL extract and ethyl acetate FDL extract, it could be concluded that methanol FDL extract is most effective among the three studied FDL extracts in exerting virus inhibition effect against DENV-2.

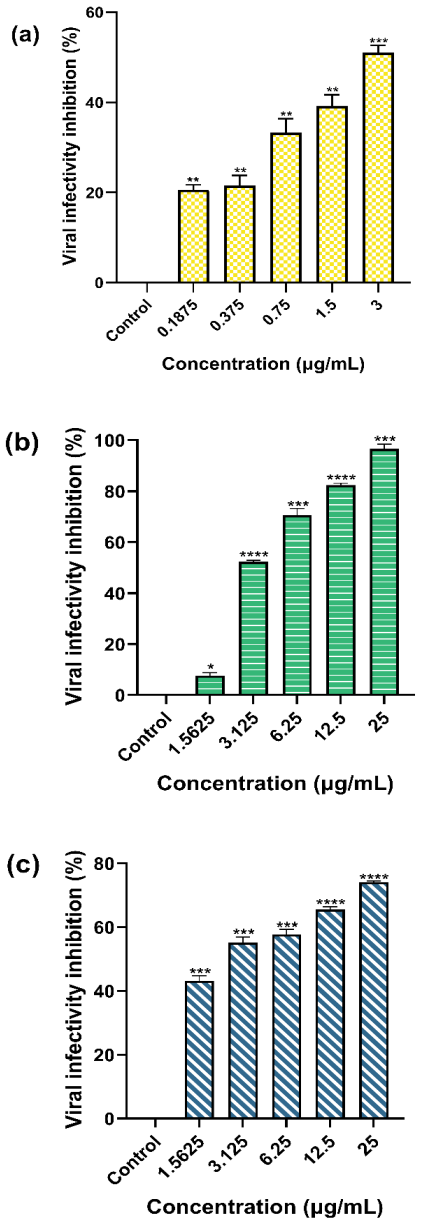
Cell protection activity of FDL extracts against DENV-2

To investigate the prophylactic effects of FDL extract against DENV-2 in Vero cells, the cell protection assay was performed by incubating Vero cells with different concentrations of three FDL extracts for 1 h at 37 °C. The IC_{50} value of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract determined by FRNT was 0.68 ± 0.08, 3.61 ± 0.28, and 1.93 ± 0.06 µg/mL, respectively. The highest inhibition of DENV-2 by n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract was 50.98% (Figure 3(a)), 98.17% (Figure 3(b)) and 74.14% (Figure 3(c)), respectively, when DENV-2 was treated with 3 µg/mL n-hexane FDL extract, 25 µg/mL ethyl acetate FDL extract and 25 µg/mL methanol FDL extract, respectively. Based on the IC_{50} value, it can be considered that n-hexane FDL extract is the most potent extract as a prophylactic agent because its IC_{50} value is the lowest among these tested extracts. On the contrary, ethyl acetate FDL extract displayed the highest IC_{50} value, implying that it is the least potent extract as a prophylactic agent compared to the other two extracts. Apart from that, the SI value for n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract determined from cell protection assays was 30.95, 19.24 and 44.06, respectively (Table 2). The SI value of all three studied extracts suggested that they are safe and effective for developing as a prophylactic agent against DENV-2. As methanol FDL extract showed the highest SI value compared to n-hexane FDL extract and ethyl acetate FDL extract, it can be suggested that methanol FDL extract is the most effective among the three studied FDL extracts in demonstrating prophylactic effect against DENV-2.



Data presented as mean±SD. Error bars indicate the range of values obtained from two independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 indicates a significant difference compared to the negative control analysed by the student t-test

FIGURE 2. Viral infectivity inhibition (%) of n-hexane FDL extract (a), ethyl acetate FDL extract (b) and methanol FDL extract (c) in direct virus inhibition assay



Data presented as mean±SD. Error bars indicate the range of values obtained from two independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 indicates a significant difference compared to the negative control analysed by the student t-test

FIGURE 3. Viral infectivity inhibition (%) of n-hexane FDL extract (a), ethyl acetate FDL extract (b) and methanol FDL extract (c) in cell protection assay

Post-infection activity of FDL extracts against DENV-2

The post-infection assay was performed to study the antiviral properties of FDL extract against intracellular replication of DENV-2. Based on the findings, the IC_{50} value of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract determined by FRNT were 0.45 ± 0.01, 1.79 ± 0.15, and 1.48 ± 0.01 µg/mL, respectively. The highest inhibition of DENV-2 infectivity by n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract were 98.98% (Figure 4(a)), 96.67% (Figure 4(b))

and 78.29% (Figure 4(c)), respectively, when DENV-2 was treated with 3 µg/mL n-hexane FDL extract, 25 µg/mL ethyl acetate FDL extract and 25 µg/mL methanol FDL extract, respectively. Based on the IC_{50} value, it can be considered that n-hexane FDL extract is the most potent extract to inhibit DENV-2 viral replication because its IC_{50} value is the lowest among these tested extracts. However, ethyl acetate FDL extract demonstrated the highest IC_{50} value, suggesting that it is the least potent extract for inhibiting DENV-2 viral replication compared to the remaining two extracts.

The SI value for n-hexane FDL extract, ethyl acetate FDL extract, and methanol FDL extract determined from the post-infection assays was 47.06, 38.87 and 57.37, respectively (Table 2). As all three FDL, extracts showed SI values greater than 10, hence they are safe and effective to be developed as antiviral. SI value of 10 indicates a promising sample that can be further examined, whereas a SI value of one or less than 1 indicates that the sample may be toxic and cannot be developed as a drug (Indrayanto, Putra & Suhud 2021). As methanol FDL extract showed the highest SI value compared to n-hexane FDL extract and ethyl acetate FDL extract, it can be concluded that methanol FDL extract is the most effective among three studied FDL extracts in inhibiting the replication of DENV-2.

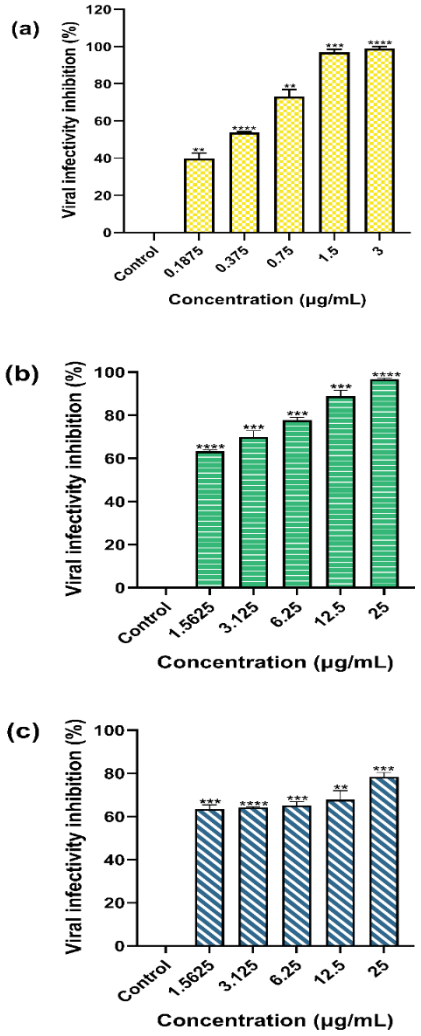
TABLE 2. CC_{50} and SI of n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract determined from MTT assay, direct virus inhibition, cell protection and post-infection assays. Data were obtained from two independent experiments and presented as mean±standard deviation (SD)

		Direct virus inhibition		Cell protection		Post infection	
Type of extract	CC_{50} (µg/mL)	IC_{50} (µg/mL)	SI	IC_{50} (µg/mL)	SI	IC_{50} (µg/mL)	SI
Hexane FDL	21.03 ± 0.63	0.94 ± 0.08	22.41	0.68 ± 0.08	30.95	0.45 ± 0.01	47.06
Ethyl acetate FDL	69.53 ± 6.92	3.03 ± 0.02	22.96	3.61 ± 0.28	19.24	1.79 ± 0.15	38.87
Methanol FDL	85.13 ± 3.11	1.48 ± 0.02	57.23	1.93 ± 0.06	44.06	1.48 ± 0.01	57.37

This study hypothesized *in vitro* antiviral potentials of FDL extracts against DENV-2, thus, evaluate the CC_{50} and IC_{50} of FDL extracts against DENV-2 on the Vero cell line via determining the SI value of FDL extracts. Among the three FDL extracts investigated, methanol FDL extract has the greatest potential to be a candidate to develop against DENV-2 as anti-dengue agent. The rising inclination of people toward natural products, especially in developing countries, has resulted in the introduction and enhancement of phytochemicals to combat numerous diseases, notably infectious forms. Phytochemicals are a class of biochemicals derived mainly from natural sources, primarily plants, as secondary metabolites. Several plants include *Curcuma longa*, *Urtica dioica*, *Euphorbia hirta*, *Pavetta tomentosa Roxb. ex Sm*, *Amaranthus dubius,* and *Bauhinia holophylla (Bong.) Steud.* have been scientifically proven to exert antiviral activity against DENV (Chang et al. 2020; Dos et al. 2021; Flores-Ocelotl et al. 2018; Pratheeba et al. 2019). Furthermore, active constituents isolated from the plants such as lupeol, voacangine, curcumin, epigallocatechin-3-gallate, luteolin, gallic acid, quercetin, catechin, and silymarin have been found to be possessed anti-dengue activity (Mounce et al. 2017; Raekiansyah et al. 2018; Trujillo-Correa et al. 2019). Although the dengue infection is recognised as an alarming public health concern, no effective and safe antiviral agent is available, and treatment remains supportive. Therefore, discovering an antiviral agent helpful in preventing and managing dengue infection is still an urgent need (Low et al. 2021). Until recently, extensive research has been conducted to identify anti-dengue substances, including small molecules (Byrd et al. 2013), nucleoside analogues (Zandi et al. 2019), peptides (Zandi et al. 2011), and natural plant extracts (Rosmalena et al. 2019). The application of phytochemicals derived from medicinal plants for treating various viral infections has recently gained growing interest (Chan et al. 2021). Several steps are necessary

for obtaining bioactive compounds from medicinal plants, including grinding, milling, homogenisation, and extraction (Do et al. 2014). Among the procedures mentioned earlier, extraction is essential for isolating bioactive chemicals from natural sources. Numerous factors have been found to affect extraction efficiency, including the extraction approach, temperature, duration, solvent system, and bioactive components of natural sources (Ngo et al. 2017). As the condition for performing the extraction process involving three solvent systems with different polarities was constant, it can be considered that the solvent system employed for extraction plays the most significant role in affecting the extraction yield. In our findings, among the three tested solvent systems, methanol resulted in the highest extraction yield (18.79%), followed by ethyl acetate (3.61%) and n-hexane (1.69%), suggesting that the extraction efficiency favours the more polar solvents. The current study's result agrees with the findings of other studies, which showed that the extraction efficiency of medicinal plants increases with the polarity of the solvent system (Kuppusamy et al. 2015; Truong et al. 2019). This can be explained by the fact that therapeutic plants possess more abundant polar bioactive components such as terpenoids, phenolic acids, alkaloids, saponins, and flavonoids. that are more soluble in a polar solvent and claimed to exhibit anti-dengue activities (Saleh & Kamisah 2020). In addition, it is interesting to note that ethyl acetate FDL extract and methanol FDL contained relatively more phytochemical groups than n-hexane extract, which further comprehend the impact of the solvent system on the extraction yield. Previous study supported the fact that ethanol and methanol FDL extracts displayed moderate cytopathic effect inhibitory activity against CHIKV in Vero cells in which ethanol extract of FD inhibited CHIKV cellular entry (Chan, Khoo & Sit 2016). FDL can be considered as one of the promising candidates for treating dengue infection because CHIKV and DENV infections cause similar clinical symptoms (Chan et al. 2021). Nevertheless, not much is known regarding the antiviral activity of FDL against DENV-2. To address this, the *in vitro* inhibitory activities of FDL extract against DENV-2 in Vero cells were studied. In order to determine the concentration limits to be employed in the subsequent antiviral assays, it is necessary to conduct cytotoxicity tests prior to antiviral evaluations, following the precept that an effective and safe compound should exhibit minimal toxicity towards the host cell (Chiamenti et al. 2019). Since the crude extracts from natural sources possess a wide range of bioactive constituents that may exert a cytotoxic effect on normal cells, the maximum non-toxic dose for the plant extract should be determined before performing the antiviral assays (Chan et al. 2021). According to the ISO 10993-5 (2009), the substance with more than 80% cell viability at treated concentration was considered non-cytotoxic at that particular concentration. Therefore, the concentration at which the FDL extract showed cell viability of 80% (CC_{20}) determined from the MTT assay was selected as the highest concentration evaluated in the subsequent antiviral assays. In the field of ethno-pharmacology, the half-maximal inhibitory concentration (IC_{50}) is the most extensively used and informative measure of a drug's potency. In this study, the IC_{50} indicates the concentration at which the tested FDL extract inhibits an infection parameter (e.g., percentage of viral infectivity inhibition) to 50% of its value in an untreated infection. The antiviral activity of each FDL extract was determined by measuring the reduction in the number of DENV-2 infectious foci after treating Vero cells with the FDL extract. SI is the ratio of a sample's cytotoxic concentration to its effective bioactive concentration. In the current study, the SI values for each tested extract were determined as the CC_{50} value divided by the IC_{50} value. An ideal antiviral agent should establish a relatively high SI value since, theoretically, the greater the SI ratio, the more effective and safer a therapy would be in treating a specific viral infection *in vivo*. Evaluation of the SI value for any research on a natural or isolated compound is vital for establishing whether additional studies may be conducted. A SI value of 10 indicates a promising sample that can be further examined, whereas a SI value of one or less than 1 indicates that the sample may be toxic and cannot be developed as a drug (Indrayanto, Putra & Suhud 2021). In this study, the Vero cell line was selected as the normal cell line to evaluate the cytotoxic activity of FDL extracts as the Vero cell is ideal for performing FRNT to evaluate the antiviral activity against DENV (Medina et al. 2012). *In vitro* antiviral assays rely on the ability of a virus to be infected and replicated in specific cell lines, as the cell culture system provides a faster and more reliable way for growing viruses to higher titres (Kohn et al. 2015). The present investigation evaluated the antiviral potential of n-hexane FDL extract, ethyl acetate FDL and methanol FDL using pre- (cell protection assay), co- (direct virus inhibition assay), and post-treatment (post-infection assay) conditions in the permissive Vero cell line. Subsequently, FRNT was performed to determine the antiviral efficacy of plant extracts against DENV-2 by measuring the reduction in the number of DENV infectious foci following treatment. A direct virus inhibition assay was carried out to identify the potential of FDL extracts to exert inactivating effect against DENV-2 virion particles. The significant reduction in DENV-2 infectivity observed in the direct viral inhibition assay for all tested FDL extracts could be explained by the direct effect of FDL extract on the DENV-2 envelope or

glycoproteins, which prevents DENV-2 virion particles from adsorbing and entering the cell following exposure to FDL extracts (Panda et al. 2021). Besides, significant reduction in viral infectivity determined from cell protection assay indicates that FDL extracts may have the ability to modulate specific host components such as receptors to hinder virus entry or replication. The cell protection assay showed the potential of FDL extracts to be developed as a prophylactic agent through the possible mechanism of inhibiting DENV-2 adsorption and penetration to the host cell. As the cell treatment was given after DENV-2 adsorption, a post-infection assay was conducted to assess the impact of FDL extracts in blocking the viral replication cycle downstream of the DENV-2 entry process. The significant reduction of *in vitro* DENV-2 infectivity by three tested FDL extracts post-infection suggests that FDL extracts could interfere with the functioning of various non-structural proteins, including NS5 methyltransferase, NS5 RdRp and NS2B-NS3 protease, which may result in a decrease in the DENV-2 replication process (Bhatnagar et al. 2021). The findings also proposed that FDL extracts could exert their inhibitory effect at multiple stages of the viral replication cycle (Panda et al. 2021). However, further research is required to determine the exact mechanism of action of anti-dengue effect of the FDL extracts. Additionally, SI index equal to or greater than 10 indicates that the tested compound exhibits good antiviral properties and can kill the virus specifically (Berezin et al. 2019). In the present study, the SI determined from direct virus inhibition, cell protection and post-infection assays showed that n-hexane FDL extract, ethyl acetate FDL extract and methanol FDL extract possess high potential to be developed as antiviral agents against DENV-2. Amongst the tested extracts of FDL, methanol FDL extract was the most selective on DENV-2.



Data presented as mean±SD. Error bars indicate the range of values obtained from two independent experiments. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 indicates a significant difference compared to the negative control analysed by the student t-test

FIGURE 4. Viral infectivity inhibition (%) of n-hexane FDL extract (a), ethyl acetate FDL extract (b) and methanol FDL extract (c) in post-infection assay

Conclusions

This study showed that n-hexane; ethyl acetate and methanol FDL extracts have the potential to be studied as an accessible source of therapeutic drugs for the treatment of DENV-2 infections via direct virus inhibition, cell protection and post-infection testing. After exposure to the extracts, infected cells exhibited a high percentage of suppression of viral infectivity in all assays performed. Among the three FDL extracts investigated, methanol FDL extract has the greatest potential to be a candidate to develop against DENV-2 as anti-dengue agents thus could be proposed for clinical testing following molecular assays and *in-vivo* evaluations. We also suggest the following future standpoints to be undertaken to ensure this study data findings as best as possible to be accomplished. 1). Regulate the FDL extracts' selectivity index against distinct DENV serotypes, including DENV-1, DENV-3 and DENV-4. 2). Determination of molecular mechanism or pathways inhibiting the DENV replication, using *in silico* molecular docking to predict the binding affinity of reported phytochemicals with the dengue viral or host cell targets, and 3). *In-vivo* studies to have a better representative anti-dengue value, toxicity and pharmacokinetic data.

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